

# Improved $\alpha$ -amylase production by *Aspergillus oryzae* after a double deletion of genes involved in carbon catabolite repression

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**Abstract** In filamentous fungi, the expression of secretory glycoside hydrolase encoding genes, such as those for amylases, cellulases, and xylanases, is generally repressed in the presence of glucose. CreA and CreB have been observed to be regulating factors for carbon catabolite repression. In this study, we generated single and double deletion *creA* and/or *creB* mutants in *Aspergillus oryzae*. The  $\alpha$ -amylase activities of each strain were compared under various culture conditions. For the wild-type strain, mRNA levels of  $\alpha$ -amylase were markedly decreased in the later stage of submerged culture under inducing conditions, whereas this reduced expression was not observed for single *creA* and double *creA/creB* deletion mutants. In addition,  $\alpha$ -amylase activity of the wild-type strain was reduced in submerged culture containing high concentrations of inducing sugars, whereas all constructed mutants showed higher  $\alpha$ -amylase activities. In particular, the  $\alpha$ -amylase activity of the double deletion mutant in a medium containing 5 % starch was >10-fold higher than that of the wild-type strain under the same culture conditions. In solid-state cultures using wheat bran as a substrate, the  $\alpha$ -amylase activities of single *creA* and double deletion mutants were >2-fold higher than that of the wild-type strain. These results suggested that deleting both *creA* and *creB* resulted in dramatic

improvements in the production of secretory glycoside hydrolases in filamentous fungi.

**Keywords** *Aspergillus oryzae* ·  $\alpha$ -Amylase production · Carbon catabolite derepression · CreA transcription repressor · CreB ubiquitin processing protease · Double deletion mutant

## Introduction

The filamentous fungus *Aspergillus oryzae* has been used for producing traditional Japanese fermented foods, such as sake, soy sauce, and miso (soybean paste), for over a thousand years (Machida et al. 2008). *A. oryzae* can produce considerable amounts of amylolytic enzymes in the presence of maltooligosaccharides, such as starch and maltose, and the induction of the corresponding amylolytic genes is regulated by a fungal-specific Zn<sub>2</sub>Cys<sub>6</sub> transcription activator, AmyR (Gomi et al. 2000; Petersen et al. 1999). In the presence of glucose, however, the production of amylolytic enzymes is repressed, even in the presence of inducing sugars (Gomi et al. 2000; Tada et al. 1989; Tonomura et al. 1961). Therefore, the production levels of amylolytic enzymes are limited by glucose, resulting from the enzymatic hydrolysis of starch or maltose used as inducers.

The glucose-induced repression of amylolytic genes is mediated by a well-known carbon catabolite repression (CCR) system. It is well established that CCR is regulated by the Cys<sub>2</sub>His<sub>2</sub> type transcription factor CreA in filamentous fungi, particularly in *Aspergillus nidulans* (Dowzer and Kelly 1989, 1991; Kato et al. 1996). In addition to CreA that functions as a transcription repressor, CreB, CreC, and CreD have also been shown to be involved in CCR by modulating the ubiquitination/deubiquitination of the CreA protein in *A. nidulans*. *creB* and *creC* mutants also show defective CCR, and a *creD* mutation

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suppresses the *creB* and *creC* mutant phenotypes (Hynes and Kelly 1977; Kelly and Hynes 1977).

It has been shown that CreB and CreC form a complex in vivo and that the CreB protein is a ubiquitin processing protease (Lockington and Kelly 2001, 2002). In contrast, CreD belongs to a family of arrestin-like proteins and is presumably involved in recruiting a ubiquitin ligase to a target protein for ubiquitination (Boase and Kelly 2004).

In *Trichoderma reesei*, Rut-C30 and PC3-7 were identified as hyper-producing mutant strains of cellulases and hemicellulases, and these strains had mutations in *cre1*, an ortholog of *creA* (Ilmén et al. 1996; Porciuncula et al. 2013). In addition to these mutants, a *cre1* deletion strain produced considerably high levels of these hydrolytic enzymes (Nakari-Setälä et al. 2009). Moreover, disrupting a *creB* ortholog (*cre2*) in *T. reesei* also resulted in increased cellulase activity under inducing conditions (Denton and Kelly 2011). These findings suggest that carbon catabolite derepression is highly effective for improving glycoside hydrolyase production by filamentous fungi.

In this study, we generated single and double deletion *creA* and *creB* gene mutants in *A. oryzae*. The  $\alpha$ -amylase production capabilities of these deletion mutants under submerged and solid-state cultures were compared with those of the wild-type strain.

## Materials and methods

### Strains and media

An *A. oryzae*  $\Delta ligD::loxP$  *pyrG*-deficient strain ( $\Delta ligD::loxP$ , *niaD*<sup>-</sup>, *sC*<sup>-</sup>, *pyrG*<sup>-</sup>), which was derived from a  $\Delta ligD::loxP$  strain (Mizutani et al. 2012) and provided by Dr. Mizutani at the National Research Institute of Brewing (Higashi-hiroshima, Japan), was used as the recipient strain for gene deletion. The  $\Delta ligD::loxP$  strain was constructed from *A. oryzae* NS4 (*niaD*<sup>-</sup>, *sC*<sup>-</sup>) (Yamada et al. 1997; National Research Institute of Brewing Stock Culture) derived from the wild-type strain, *A. oryzae* RIB40 (National Research Institute of Brewing Stock Culture). *Escherichia coli* DH5 $\alpha$  (*endA1*, *hsdR17*, *supE44*, *recA1*, *gyrA96*, *thi-1*, *relA1*, *deoR*,  $\Phi 80lacZ$   $\Delta$  M15,  $\Delta(lacZYA^{-}argF)$ U169) was used for the construction and propagation of plasmid DNAs. *Saccharomyces cerevisiae* BY4741 (*MATa his3*  $\Delta$  *leu2*  $\Delta$  *met15*  $\Delta$  *ura3*  $\Delta$ ) was used for in vivo plasmid construction for gene deletion. The minimal medium (MM) for *A. oryzae* culture was Czapek–Dox (CD) medium, which contained 0.5 % (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 0.05 % KCl; 0.2 % KH<sub>2</sub>PO<sub>4</sub>; 0.05 % MgSO<sub>4</sub>; trace amounts of FeSO<sub>4</sub>, ZnSO<sub>4</sub>, CuSO<sub>4</sub>, MnSO<sub>4</sub>, Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>, and (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>; and 1 % sugar, and supplemented with 0.0003 % (0.02 mM) methionine. For culture of the  $\Delta ligD::loxP$  *pyrG*<sup>-</sup> strain, 0.2 % uracil was added to the medium. To assess  $\alpha$ -amylase production in liquid medium, YP complete medium containing 0.5 %

yeast extract and 1 % Bacto-peptone supplemented with a 1 or 5 % carbon source was used. For solid-state culture, 5 g of wheat bran (small particles were removed through a 10 standard mesh sieve; opening 1.7 mm) was moistened with 2 ml of distilled water after sterilization.

### Construction of plasmid DNAs

The plasmids for *creA* and *creB* deletions were constructed as follows: To generate a plasmid for *creA* deletion, the 5'- and 3'-flanking regions of *creA* were amplified using PCR with primer sets P1 + P2 and P3 + P4 using the genomic DNA of *A. oryzae* RIB40 as the template. An *A. nidulans* *sC* cassette encoding ATP sulfurylase was prepared from pUSC (Yamada et al. 1997) digested with *Bam*HI and *Pst*I. A yeast vector, pYES2 (Life Technologies, Carlsbad, CA), was digested with *Eco*RI and *Bam*HI. These four DNA fragments were assembled in *S. cerevisiae* using an endogenous homologous recombination system (Colot et al. 2006; Oldenburg et al. 1997), which resulted in  $\Delta creA$ /pYES2.

To generate a plasmid for *creB* deletion using *sC* as a selectable marker, the 3'-flanking region of *creB* was amplified by PCR with primers (designated creBdownsenPstI and creBdownanti) using the genomic DNA of *A. oryzae* RIB40 as the template. This amplified fragment was digested with *Pst*I and inserted into *Pst*I-digested pUSC, which resulted in creBdown/pUSC. The 5'-flanking region of *creB* was also amplified by PCR using the primers creBupsen and creBupantiBamHI and digested with *Kpn*I and *Bam*HI. This fragment was subsequently cloned into *Kpn*I/*Bam*HI-digested creBdown/pUSC, which generated  $\Delta creB$ /pUSC.

To generate a plasmid for *creB* deletion using the orotidine-5'-decarboxylase gene, *pyrG*, as a selectable marker, a *pyrG* fragment was amplified by PCR with *A. nidulans* genomic DNA using the primers AnpyrGsen and AnpyrGantiBgIII. The *pyrG* fragment was digested with *Bgl*II and ligated to *Bam*HI-digested  $\Delta creB$ /pUSC, which resulted in a  $\Delta creB$ /pyrG plasmid.

To construct plasmid DNA for complementation of the *pyrG*-deficient strain, a *pyrG* fragment was amplified by PCR with *A. nidulans* genomic DNA using the primers AnpyrGsen and AnpyrGantiPstI. The fragment obtained was digested with *Pst*I and *Bgl*II and inserted into *Pst*I/*Bam*HI-digested pUC119, which gave pUC/pyrG. A *niaD* fragment encoding nitrate reductase was obtained from a pNGA142 vector (Tamalampudi et al. 2007) by digestion with *Nru*I and inserted into *Sma*I-digested pUC/pyrG, which generated pUC/pyrG/*niaD*. The nucleotide sequences of all primers used for plasmid construction are shown in Supplemental Table S1.

### Fungal transformation

Transformation of *A. oryzae* was performed according to the method of Gomi et al. (1987). To obtain the DNA fragments

for deleting *creA* and *creB*,  $\Delta creA/pYES2$  and  $\Delta creB/pyrG$  plasmids were digested with *NotI/KpnI* and *EcoRI/SphI*, respectively. The pUC/*pyrG/niaD* plasmid was introduced into the recipient *pyrG*-deficient strain and  $\Delta creA$  mutant for complementation of *pyrG* deficiency.

#### Southern blot analysis

*A. oryzae* genomic DNA preparation and Southern blot analysis were performed as previously described (Tanaka et al. 2012).

#### $\alpha$ -Amylase activity

$\alpha$ -Amylase activity was determined using the iodine–starch method (Sato et al. 2011). In a submerged culture of *A. oryzae*, secreted  $\alpha$ -amylase is absorbed onto the fungal cell wall (Sato et al. 2011). Therefore, the mycelia harvested by filtration through Miracloth (EMD Chemicals Inc., San Diego, CA) were incubated in 0.1 M phosphate buffer (pH 7.0) for 60 min to release  $\alpha$ -amylase that had been adsorbed on the fungal cell wall. The  $\alpha$ -amylase activities in the extraction buffer and culture supernatant were summed for total  $\alpha$ -amylase activity. After incubation in phosphate buffer, the mycelia were dried in a vacuum freeze drier (VD-550R; TAITEC, Saitama, Japan), after which the dry mycelia weight was determined. The  $\alpha$ -amylase activity in submerged cultures was expressed as the activity per dry mycelia weight of the respective strains. To determine  $\alpha$ -amylase activity in solid-state culture, wheat bran, on which *A. oryzae* had grown, was suspended in 50 ml of 10-mM acetate buffer (pH 5.0) and incubated for 3 h at room temperature to extract secreted proteins. Subsequently, wheat bran was removed by filtration through Miracloth, and  $\alpha$ -amylase activity in the filtered sample was determined.

#### Quantitative RT-PCR analysis

To prepare total RNA, mycelia grown in liquid cultures were harvested and dried in a vacuum freeze drier. Total RNA was extracted from a freeze-dried mycelia suspension and disrupted in ISOGEN (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. After treating total RNA with DNase I (TaKaRa Bio, Otsu, Japan), first-strand cDNAs were synthesized using M-MLV reverse transcriptase (Life Technologies, Carlsbad, CA) with an oligo(dT) primer, after which synthesized cDNA was treated with RNase H (Life Technologies, Carlsbad, CA). PCR reactions and subsequent analyses were performed using SYBR<sup>®</sup> Green PCR Master Mix and a StepOnePlus<sup>™</sup> Real-Time PCR system (Life Technologies, Carlsbad, CA). The histone H4 gene was used as reference, and relative mRNA expression was calculated by the  $-\Delta\Delta C_T$  method (Livak and Schmittgen 2001). The nucleotide sequences of primers used for quantitative RT-PCR analysis are shown in Supplemental Table S1.

## Results

### Construction of single and double *creA* and *creB* deletion mutants

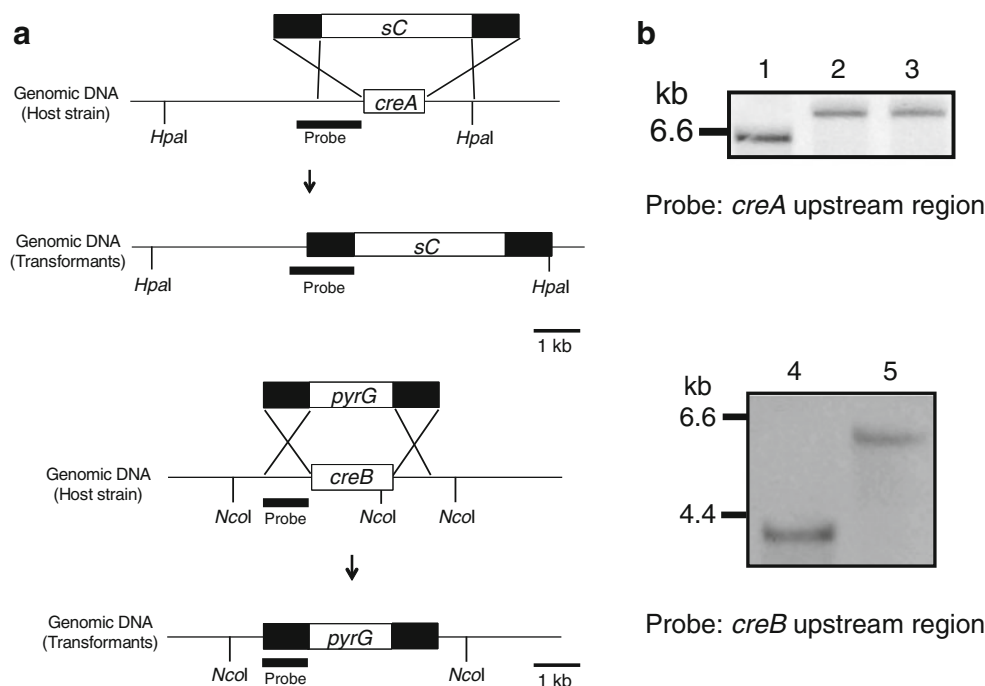
To investigate the effect of carbon catabolite derepression on amylolytic enzyme production, we generated gene deletion mutants for *creA* and *creB* orthologs in *A. oryzae*. The amino acid identities of *A. oryzae* CreA (AO090026000464) and CreB (AO090009000320) to those of *A. nidulans* were 80.6 and 70.5 %, respectively. DNA fragments for deleting *creA* and *creB* using the *A. nidulans* *sC* and *pyrG* genes as selectable markers were individually constructed, and the resulting DNA fragments were introduced into a recipient strain (Fig. 1a). These gene deletions were confirmed by PCR (data not shown) and Southern blot analysis (Fig. 1b). The *creA* deletion cassette was introduced into the  $\Delta creB$  mutant, and a double deletion mutant ( $\Delta creA\Delta creB$ ) was successfully obtained. For complementation of *pyrG* deficiency, pUC/*pyrG/niaD* plasmid DNA was introduced into the recipient and  $\Delta creA$  mutant strains. For this study, the *pyrG*-complemented recipient was defined as the wild-type strain.

Because it is known that deleting *creA* causes severe morphological defects in filamentous fungi, we examined the growth characteristics of these gene deletion mutants. On MM agar plates, the  $\Delta creA$  and  $\Delta creA\Delta creB$  mutants showed distinctive growth defects compared with the wild-type strain (Fig. 2a). In contrast, the  $\Delta creB$  mutant showed no apparent growth abnormalities (Fig. 2a). These phenotypes were identical to those of the *creA* and *creB* disruptants of other filamentous fungal species, such as *A. nidulans*, *Neurospora crassa*, and *T. reesei* (Denton and Kelly 2011; Hynes and Kelly 1977; Nakari-Setälä et al. 2009; Ziv et al. 2008). However, in submerged cultures with YP plus 1 % glucose (YPD) medium, the wild-type and  $\Delta creB$  mutant strains generally grew in compact hyphal pellets, whereas the  $\Delta creA$  and  $\Delta creA\Delta creB$  mutants were more likely to grow in pellets of smaller size or with a pulpy-like morphology.

Mycelia weights of the  $\Delta creA$  and  $\Delta creA\Delta creB$  mutants in 24-h culture were relatively lower than those of the wild-type and  $\Delta creB$  mutant strains, which was consistent with the growth defects observed on agar medium. However, after an additional 48-h culture, the  $\Delta creA$  and  $\Delta creA\Delta creB$  mutants showed rather better growth, which resulted in an increase in their dry mycelia weights compared with that of the wild-type and  $\Delta creB$  mutant strains. This was presumably due to the differences in the growth morphologies of these mutants (Fig. 2b).

### Carbon catabolite derepression by *creA* and *creB* deletions

To investigate the effects of deleting the *creA* and/or *creB* genes on CCR, each of these strains was grown on MM agar



**Fig. 1** Construction of single and double deletion strains. **a** Strategies for *creA* and *creB* deletion by homologous recombination using *A. nidulans* *sC* and *pyrG* genes as selectable markers.  $\Delta ligD::loxP pyrG^-$  was used as the host strain for single deletion strains. To generate a double deletion strain, a DNA fragment for *creA* deletion was introduced into a *creB* deletion mutant. **b** Southern blot analysis of single and double deletion

strains. The genomic DNAs of wild type (lanes 1 and 4),  $\Delta creA$  (lane 2),  $\Delta creA \Delta creB$  (lane 3), and  $\Delta creB$  (lane 5) were digested with *HpaI* or *NcoI*, electrophoresed on a 0.8 % agarose gel, and transferred onto a nylon membrane. The labeled DNA fragments of the *creA* or *creB* upstream region were used as a probe

plates containing 1 % starch, with or without 1 % glucose. Clear zones formed by starch degradation were observed. On both types of agar plate, 0.25 % Triton X-100 was added to limit the colony size so that the clear zone could be clearly seen. The wild-type and all mutant strains showed clear zones around the colonies on the agar plates, containing starch as the sole carbon source (Fig. 3). In comparison, the formation of clear zones around wild-type strain colonies was strongly inhibited by adding glucose. In contrast, all of the deletion mutants formed distinct clear zones on starch medium supplemented with glucose (Fig. 3). This clearly indicated that CCR was relieved by deleting *creB* and *creA*.

#### Comparisons of $\alpha$ -amylase activities in submerged culture

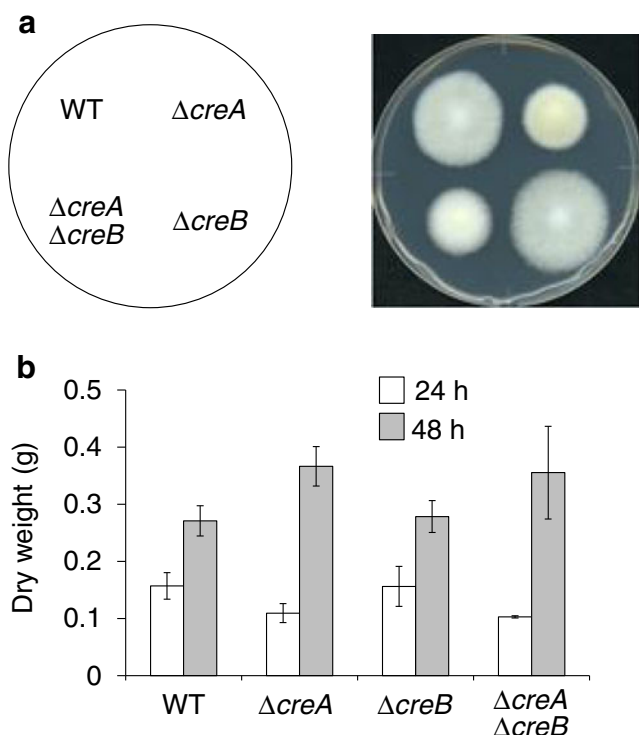
To examine the effects of the *creA* and/or *creB* deletions on  $\alpha$ -amylase production, all transformants were grown in liquid YP medium supplemented with 1 % sugar, after which secreted  $\alpha$ -amylase activity was measured. In YPD medium, the wild-type and  $\Delta creB$  mutant strains produced small amounts of  $\alpha$ -amylase, whereas the  $\Delta creA$  and  $\Delta creA \Delta creB$  mutants showed significantly high  $\alpha$ -amylase activities (Fig. 4a). This indicated that deleting the *creA* gene resulted in inducing  $\alpha$ -amylase production concomitant with CCR relief. This also suggested that glucose could act as an inducer for  $\alpha$ -amylase

production, as reported for *A. nidulans* by Murakoshi et al. (2012). Another study using carbon-limited chemostat cultures also suggested that  $\alpha$ -amylase production by *A. oryzae* was induced by glucose (Carlsen and Nielsen 2001).

In YP plus 1 % starch (YPS) medium after 48 h of culture, the  $\alpha$ -amylase activities of the single  $\Delta creA$  or  $\Delta creB$  and double  $\Delta creA \Delta creB$  mutants were approximately 1.5-, 2-, and 3-fold higher, respectively, compared with that of the wild-type strain. In addition, in YPS medium supplemented with the repressing sugar glucose, the single  $\Delta creA$  and double  $\Delta creA \Delta creB$  mutants retained high  $\alpha$ -amylase activity similar to that in YPS medium without glucose, whereas the wild-type strain showed significantly reduced  $\alpha$ -amylase activity (Fig. 4b). This indicated that the *creA* deletion resulted in carbon catabolite derepression of  $\alpha$ -amylase production in submerged culture, as described above using the plate assay (Fig. 3).

In the presence of glucose, the  $\Delta creB$  mutant showed decreased  $\alpha$ -amylase activity. However, the degree of reduced  $\alpha$ -amylase activity by the  $\Delta creB$  mutant was not comparable with that of the wild-type strain, which suggested that the *creB* deletion partially resulted in carbon catabolite derepression. Together with the results for  $\alpha$ -amylase production in YPD and YPS media, the *creA* and *creB* double deletion was the most effective for improving  $\alpha$ -amylase production in submerged culture.

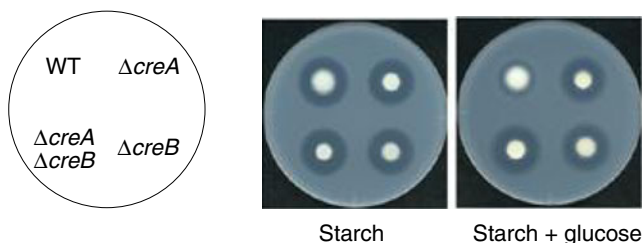




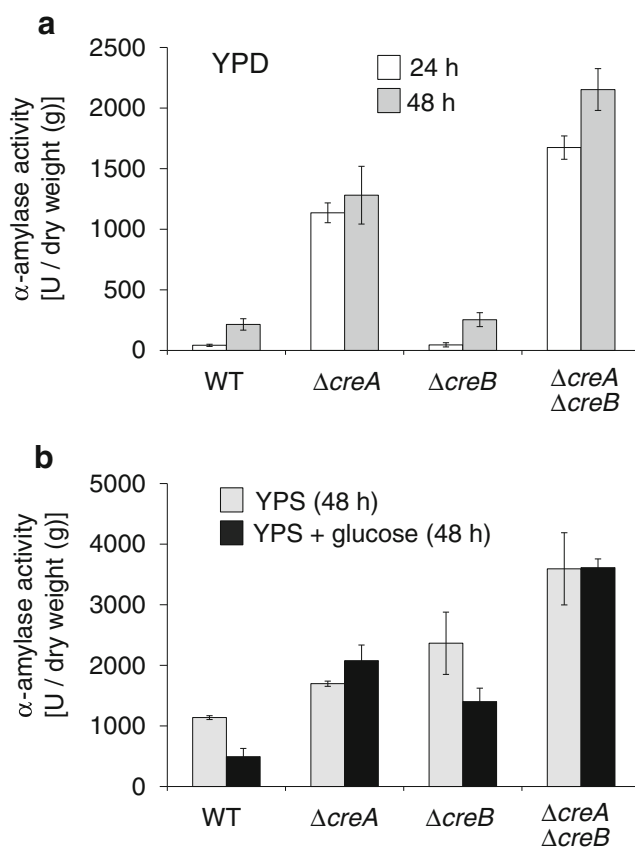
**Fig. 2** Growth of single and double deletion strains. **a** Growth on minimal agar plate. Approximately  $1 \times 10^4$  conidiospores of each strain were grown on a minimal agar plate containing 1 % glucose as the sole carbon source at 30 °C for 3 days. *WT* wild type. **b** Growth in submerged culture. Approximately  $2 \times 10^6$  conidiospores of each strain were grown in YP + 1 % glucose (YPD) medium at 30 °C, after which mycelia dry weights were determined. *Error bars* indicate the standard errors of three independent experiments

Comparisons of  $\alpha$ -amylase activities in submerged cultures containing high concentrations of inducing sugars

We subsequently examined  $\alpha$ -amylase production in liquid medium containing 5 % inducing sugars maltose and starch to determine whether higher production levels could be achieved with these conditions. As shown in Fig. 5, all deletion mutants had significantly higher  $\alpha$ -amylase activity than the wild-type strain. In YP plus 5 % maltose (YP5M) medium in the 48-h culture, both the single  $\Delta creA$  and  $\Delta creB$  mutants had approximately 4-fold higher  $\alpha$ -amylase activity compared with



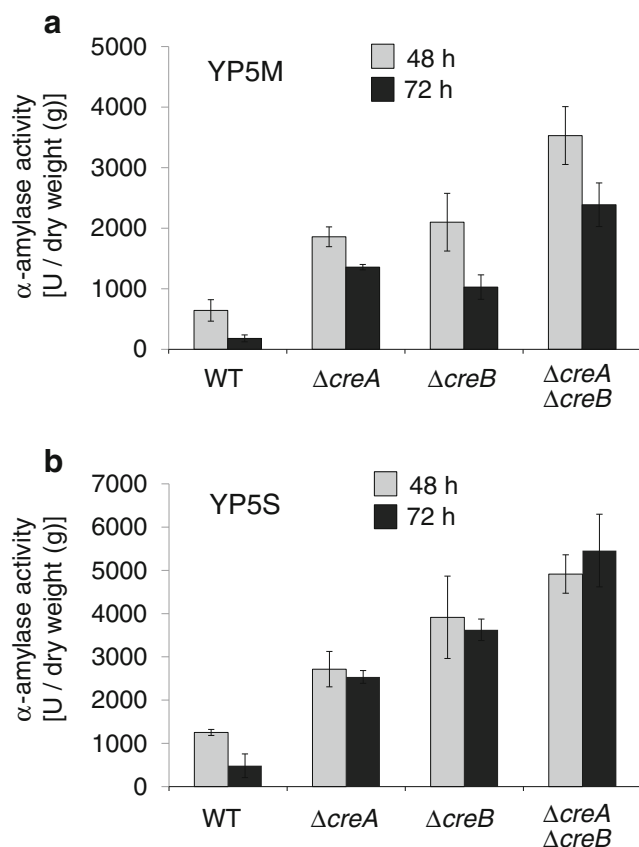
**Fig. 3** Effects of gene deletions on carbon catabolite repression. Approximately  $1 \times 10^4$  conidiospores of each strain were grown on minimal agar medium containing 1 % starch supplemented with or without 1 % glucose at 30 °C for 2 days. In both agar media, 0.25 % Triton X-100 was added to more clearly discriminate the clear zones



**Fig. 4**  $\alpha$ -Amylase activities in submerged culture. Approximately  $2 \times 10^6$  conidiospores of each strain were grown either in **a** YP + 1 % glucose (YPD) medium at 30 °C for 24 and 48 h or in **b** YP + 1 % starch (YPS) and YPS + 1 % glucose media for 48 h at 30 °C. Harvested mycelia were incubated in 100 mM phosphate buffer for 60 min to release  $\alpha$ -amylase bound to the cell wall. The  $\alpha$ -amylase activities in the culture broth and phosphate buffer were measured, and total activity was divided by the mycelia dry weight. *Error bars* indicate the standard errors of three independent experiments

the wild-type strain. Under the same conditions, the double  $\Delta creA \Delta creB$  mutant had an approximately 7-fold higher activity than the wild-type strain (Fig. 5a). Further, culture for 72 h in YP5M resulted in reduced  $\alpha$ -amylase activity in all strains, although the activity of the deletion mutants remained at a higher level compared with the wild-type strain, for which the activity in the medium was markedly reduced (Fig. 5a).

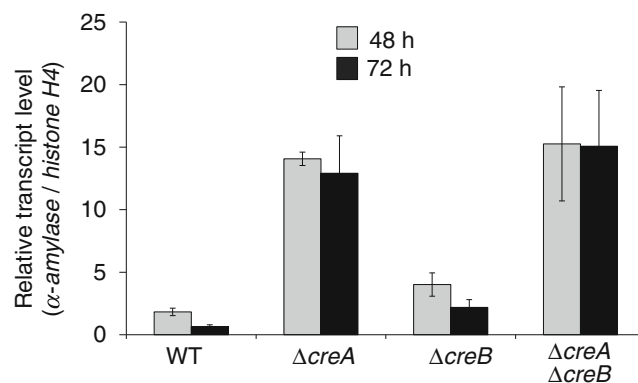
In the presence of a high concentration of maltose, the wild-type and deletion mutant strains continued to grow during the entire culture period, although their  $\alpha$ -amylase production was repressed by CCR induced by glucose resulting from maltose degradation. Thus, the  $\alpha$ -amylase activity per dry mycelia weight of the wild-type strain was significantly reduced compared with activities of the deletion mutants. In comparison, in YP plus 5 % starch (YP5S) medium,  $\alpha$ -amylase production by all of the strains was considerably higher than in YP5M medium for the same culture period (Fig. 5b). In particular, the single  $\Delta creA$ ,  $\Delta creB$ , and double  $\Delta creA \Delta creB$  mutants had approximately 2-, 3-, and 4-fold



**Fig. 5**  $\alpha$ -Amylase activities in liquid YP + 5 % sugar media. Approximately  $2 \times 10^6$  conidiospores of each strain were grown in **a** YP5M or **b** YP5S medium for 48 and 72 h at 30 °C. Total  $\alpha$ -amylase activities were determined as described in Fig. 4

higher  $\alpha$ -amylase activity, respectively, than the wild-type strain. Furthermore, the wild-type strain showed a remarkable decrease in activity in a 72-h culture, similar to that in YP5M medium, whereas  $\alpha$ -amylase production by all of the deletion mutants in the 72-h culture was almost equal to that in the 48-h culture (Fig. 5b). These results clearly indicated that carbon catabolite derepression was considerably effective for improving  $\alpha$ -amylase production in submerged cultures with high concentrations of inducing sugars.

To investigate whether the increased  $\alpha$ -amylase production by the  $\Delta creA \Delta creB$  mutant was due to an increase in transcript levels,  $\alpha$ -amylase mRNA expression in each strain grown in YP5M medium was compared by quantitative RT-PCR (qRT-PCR) analysis (Fig. 6). The recipient strain used in this study was originated from RIB40 and contained three  $\alpha$ -amylase genes (*amyA*, *amyB*, and *amyC*) with almost the same nucleotide sequence in the genome (Tada et al. 1989). Thus, the primers used in qRT-PCR were designed to amplify DNA fragments corresponding to mRNAs transcribed from all three genes. The  $\alpha$ -amylase mRNA levels in the  $\Delta creA$  and  $\Delta creA \Delta creB$  mutants were notably higher than that in the wild-type strain during the entire culture period. The single

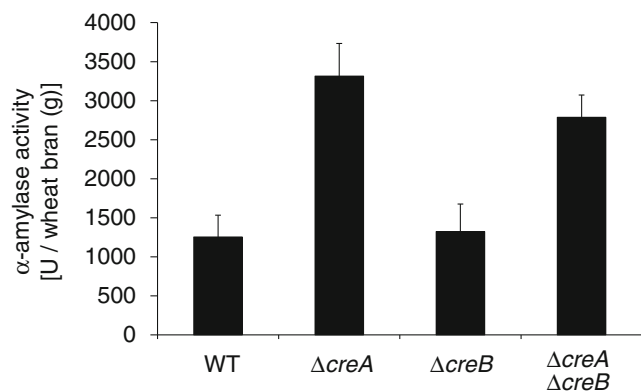


**Fig. 6** Quantitative RT-PCR analysis for  $\alpha$ -amylase mRNA. Approximately  $2 \times 10^6$  conidiospores of each strain were grown in YP5M medium. Total RNA was prepared from harvested mycelia, and the resulting cDNA was subjected to qRT-PCR analysis using specific primers for  $\alpha$ -amylase genes. The relative expression level of  $\alpha$ -amylase genes was normalized to that of histone H4 gene. Error bars indicate the standard errors of three independent experiments

$\Delta creB$  mutant also showed an approximately 2-fold higher transcription level than the wild-type strain, although its transcription level was considerably lower compared with those in the  $\Delta creA$  and  $\Delta creA \Delta creB$  mutants. These results suggested that under culture conditions with high concentrations of inducing sugars, the *creA* deletion was considerably more effective for enhancing the transcription level of the  $\alpha$ -amylase gene and resulted in high-level production of secreted  $\alpha$ -amylase compared with the wild-type strain. The *creA* and *creB* double deletion further increased the  $\alpha$ -amylase mRNA level and resulted in an additional increase in  $\alpha$ -amylase production. However, although it had a higher transcription level, the single *creA* deletion did not result in an increased yield of  $\alpha$ -amylase compared with the single  $\Delta creB$  mutation (Figs. 5a and 6).

#### Comparisons of $\alpha$ -amylase activities in solid-state cultures

It is well known that *A. oryzae* produces larger amounts of proteins when in solid-state culture compared with submerged culture (Machida et al. 2008). Thus, we assessed  $\alpha$ -amylase production by deletion mutants in solid-state culture using wheat bran as the substrate. In solid-state culture, there were no apparent differences in growth between the wild-type and deletion mutant strains (data not shown). After the 48-h culture of wheat bran, secreted proteins were extracted with acetate buffer, and  $\alpha$ -amylase activity was determined. The  $\alpha$ -amylase activity of the  $\Delta creB$  mutant was not high and was comparable with that of the wild-type strain. However, the  $\Delta creA$  and  $\Delta creA \Delta creB$  mutants had approximately 2–2.5-fold higher activity than the wild-type strain (Fig. 7). This suggested that  $\alpha$ -amylase production in solid-state culture improved after the *creA* deletion, although the *creB* deletion had no apparent additive effects.



**Fig. 7**  $\alpha$ -Amylase activities in solid-state culture. Approximately  $1 \times 10^6$  conidiospores of each strain were grown in solid-state culture with wheat bran as a substrate at 30 °C for 48 h. Secreted proteins were extracted with acetate buffer, after which the  $\alpha$ -amylase activity in the acetate buffer was determined. Error bars indicate the standard errors of three independent experiments

## Discussion

In this study, we generated single and double deletion mutants for the genes involved in CCR, *creA* and *creB*, in *A. oryzae*. The  $\alpha$ -amylase activity of these mutant strains was compared under various culture conditions to evaluate the effect of these gene deletions on  $\alpha$ -amylase production.

Deleting *creA* resulted in reduced growth on MM agar medium, consistent with observations of other filamentous fungi in which their orthologous genes had been deleted. The reduced growth of the  $\Delta creA$  mutants was assumed to render them unsuitable for industrial use in enzyme production. However, in submerged cultures using YPD medium, the growth of mutants with a *creA* deletion ultimately exceeded that of the wild-type and  $\Delta creB$  mutant strains in the 48-h culture, even though their growth in the early culture period of 24 h was relatively worse compared with that of the wild-type strain. This was also observed for submerged cultures with high concentrations of inducing sugars using YP5M and YP5S media (data not shown).

For *T. reesei*, a *creI* disruption mutant grew either poorly or failed to grow in liquid glucose or maltose medium, respectively (Denton and Kelly 2011). The media used for *T. reesei* were based on MM, whereas we used YP as the liquid medium in this study. Thus, it is likely that the  $\Delta creA$  mutants can grow normally in media rich in nitrogen and vitamins. In addition, there were no apparent differences in growth among the wild-type strain and deletion mutants under solid-state culture conditions. These results indicated that the *creA* deletion would not be disadvantageous for industrial applications.

The plate assay for  $\alpha$ -amylase activity showed that a *creA* or *creB* deletion resulted in relief from CCR induced by glucose. Similarly, the  $\alpha$ -amylase activities of the deletion mutants in YPS medium supplemented with glucose were considerably higher than that of the wild-type, which also

indicated that a *creA* or *creB* deletion resulted in carbon catabolite derepression. In the presence of glucose, the double deletion mutant showed the highest  $\alpha$ -amylase production and suggested that deleting both *creA* and *creB* was most effective for enhancing  $\alpha$ -amylase production, possibly by completely circumventing glucose repression.

The  $\alpha$ -amylase gene expression level by the *creA* deletion mutants ( $\Delta creA$  and  $\Delta creA \Delta creB$ ) in YP5M liquid medium was highly increased compared with that of the wild-type strain. In particular, a significant reduction in the  $\alpha$ -amylase mRNA level in the 72-h culture was observed for the wild-type but not for the  $\Delta creA$  mutants. These results also indicated that expression of the  $\alpha$ -amylase gene was repressed by glucose derived from maltose and that the *creA* deletion resulted in carbon catabolite derepression. In contrast to the *creA* deletion, the single *creB* deletion mutant showed only a slightly increased  $\alpha$ -amylase gene expression. This suggested that the *creB* deletion caused partial CCR relief at the transcriptional level.

As reported for *A. nidulans*, CCR is directly regulated by CreA, the stability of which is modulated by its ubiquitination and deubiquitination. Under CCR conditions, ubiquitinated CreA is deubiquitinated by a CreB ubiquitin processing protease, which allows it to escape degradation by the proteasome; thus, it can exert its repressor function. In contrast, a defect in CreB results in the ubiquitination and destabilization of CreA, and proteolytic degradation of CreA results in derepression of CCR (Lockington and Kelly 2002). Because CreA is directly involved in CCR, the absence of CreA could result in nearly complete CCR relief.

In contrast, even in the absence of CreB, if a small amount of deubiquitinated CreA may be present, it could repress the expression of genes that are subject to CreA-mediated CCR. This may be a reason why *creB* deletion did not result in an increased transcript level of the  $\alpha$ -amylase gene. However, despite similar expression levels of the  $\alpha$ -amylase gene for the single  $\Delta creA$  and the double  $\Delta creA \Delta creB$  mutants, the double mutant had considerably higher  $\alpha$ -amylase activity than the single  $\Delta creA$  mutant in the submerged cultures examined in this study. Thus, *creB* deletion appeared to have an additive effect on carbon catabolite derepression.

In addition, the  $\Delta creB$  mutant had a higher level of  $\alpha$ -amylase production than the  $\Delta creA$  mutant in YP5M medium, although the  $\alpha$ -amylase mRNA level in the single  $\Delta creB$  mutant was not increased to a level comparable to that in the  $\Delta creA$  mutant. This was also observed when using cultures with YPS and YP5S media. In both cultures, the  $\alpha$ -amylase mRNA levels in the  $\Delta creB$  mutant were notably lower than those in the  $\Delta creA$  mutant in YP5M medium (data not shown). These observations raise the intriguing possibility that *creB* deletion has an effect on  $\alpha$ -amylase production other than CCR relief through the destabilization of ubiquitinated CreA.

Because CreB has been inferred to be a deubiquitinating enzyme, it is likely that there are several target proteins for CreB

other than CreA. An as yet unidentified target protein(s) may be involved in inhibiting  $\alpha$ -amylase production, presumably at the posttranscriptional level. Thus, *creB* deletion may cause an increased level of  $\alpha$ -amylase. In *A. nidulans*, CreB has been proposed to be involved in the expression of genes required for utilizing proline and quinate as carbon or nitrogen sources by regulating either transcription activators or enzymes and permeases (Lockington and Kelly 2002). However, the possible pleiotropic functions of CreB are uncertain. Thus, further analysis of CreB function will be necessary. In this context, because it was reported that CreB functions as a deubiquitinating enzyme with another component CreC (Lockington and Kelly 2002), we are now constructing a *creC* deletion mutant to examine whether a similar phenomenon is observed in the *creC* mutant. Furthermore, we are also planning the microarray analyses of the mutant strains obtained in this study to find the putative target gene(s) or protein(s) of CreA and CreB.

It is known that CCR in filamentous fungi is generally circumvented by solid-state fermentation (Viniestra-González and Favela-Torres 2006). However, the  $\alpha$ -amylase activities of the  $\Delta creA$  and  $\Delta creA \Delta creB$  mutants in solid-state culture using wheat bran were approximately 2–2.5-fold higher than that of the wild-type strain. This suggested that deleting *creA* was also effective for improving the production of enzyme proteins in solid-state culture, whereas no synergistic effect of *creA* and *creB* double deletion was observed. The reason why no effect of *creB* deletion on the  $\alpha$ -amylase production was observed in the single and double deletion mutants remains unclear. However, since the transcription profiles of many genes, such as *gluB* encoding glucoamylase B, *pepA* encoding aspartic protease, and *melB* encoding secretory tyrosinase, are known to be different in between solid-state culture and submerged culture (Hata et al. 1998; Kitano et al. 2002; Obata et al. 2004), there might be a different effect of the *creB* deletion on the protein production under both culture conditions. Because the yield of *A. oryzae*-secreted proteins in solid-state culture was considerably higher than that in submerged culture, an increase in protein production during solid-state fermentation by *creA* deletion would be advantageous for enzyme protein production.

In conclusion, we report that a *creA* and *creB* double deletion is highly effective for improving  $\alpha$ -amylase production by *A. oryzae*. The maximum  $\alpha$ -amylase activity of this double deletion mutant was achieved at 72 h of culture with YP5S medium, and its  $\alpha$ -amylase activity was more than 10-fold higher than that of the wild-type strain. It was recently reported that a *creB* deletion also resulted in carbon catabolite derepression in *A. oryzae*, although no experimental results for single *creA* or double *creA* and *creB* deletions were reported (Hunter et al. 2013). Based on our results, we anticipate that a *creA* and *creB* double deletion would be extremely effective for improving the production of hydrolytic enzymes in filamentous fungi, such as cellulases and xylanases of *T. reesei*. In addition to the production of amylolytic enzymes,

*A. oryzae* has attracted attention as a host organism for recombinant protein production. Because the promoters for amylolytic genes have been widely used as expression promoters for heterologous genes, we also anticipate that a *creA* and *creB* double deletion strain could be useful as a suitable host strain for recombinant protein production.

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