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Comparison and characterization of α-amylase inducers in *Aspergillus nidulans* based on nuclear localization of AmyR

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Abstract AmyR, a fungal transcriptional activator responsible for induction of amylolytic genes in *Aspergillus nidulans*, localizes to the nucleus in response to the physiological inducer isomaltose. Maltose, kojibiose, and D-glucose were also found to trigger the nuclear localization of GFP-AmyR. Isomaltose- and kojibiose-triggered nuclear localization was not inhibited by the glucosidase inhibitor, castanospermine, while maltose-triggered localization was inhibited. Thus, maltose itself does not appear to be an direct inducer, but its degraded or transglycosylated product does. Non-metabolizable D-glucose analogues were also able to trigger the nuclear localization, implying that these sugars, except maltose, directly function as the inducers of AmyR nuclear entry. The inducing activity of D-glucose was 4 orders-of-magnitude weaker compared with isomaltose. Although D-glucose has the ability to induce α-amylase production, this activity would generally be masked by CreA-dependent carbon catabolite repression. Significant induction of α-amylase by D-glucose was observed in *creA*-defective *A. nidulans*.

Keywords *Aspergillus nidulans* · Amylase · Inducer · AmyR · Nuclear localization

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Introduction

Filamentous fungi are widely used for industrial production of biomass-degrading enzymes including amylases, xylanases, cellulases, and lipases. These enzymes are generally produced as a mixture of enzymes with a similar function, such as amylolytic enzymes, upon induction by a specific inducing molecule so called as an inducer. Maximal production of the enzymes may be achieved by appropriate control of the inducer concentration. However, it seems that not much attention has been paid in this context, considering that a real inducer, or a physiological inducer in other words, remains to be identified even for production of amylases, the most abundantly produced enzymes in industries.

Amylolytic enzymes, such as α-amylase, glucoamylase, and α-glucosidase, are inducible enzymes in *Aspergillus* species. Previous studies have shown that α-amylase production in *Aspergillus oryzae* and *A. nidulans* is induced by various α-linked glucobioses and glucotrioses including kojibiose, maltose, isomaltose, and panose (Tonomura et al. 1961; Kato et al. 2002a). Isomaltose was proposed to be the physiological inducer in *A. nidulans*, since it has the strongest inducing activity examined to date. It exists in culture supernatants when the organism is grown on either maltose or starch, and does not require enzymatic conversion to exhibit inducing activity (Kato et al. 2002a).

Glucose is generally recognized as a repressing carbon source of amylase production in *Aspergillus* spp. However, it has been reported in *A. oryzae* and *A. niger* that, in addition to acting as a repressing carbon source because of the CreA protein, D-glucose also acts as an inducer of α-amylase

production (Carlsen and Nielsen 2001; Vankuyk et al. 2012). These findings raise the possibility that D-glucose by itself, without any enzymatic conversions, may induce expression of the amylolytic genes.

AmyR is the transcriptional activator responsible for induction of the amylolytic genes in *Aspergillus* species (Petersen et al. 1999; Gomi et al. 2000; Tani et al. 2001b; Nakamura et al. 2006). It has a $Zn(II)_2Cys_6$ DNA binding motif at its N terminus and binds to the $CGGN_8(C/A)GG$ sequence present in the amylase promoters (Tani et al. 2001a; Ito et al. 2004). Transcription of the amylase genes is controlled by regulated nuclear localization of AmyR; the physiological inducer isomaltose triggers nuclear accumulation of AmyR (Makita et al. 2009). In this report, we compared the ability of various sugars to trigger the nuclear localization of AmyR.

Materials and methods

Strains and growth conditions

A. nidulans MGZ5 (*biA1*; *argB2::argB⁺::alcAp-gfp-amyR*; *pyroA4*; *amyR1/malA1*) carrying the GFP-AmyR fusion gene under control of the *alcA* promoter (Makita et al. 2009) was used for sub-cellular localization studies. The strain ABTA16 (*biA1 pyrG89*; *wA3*; *argB2::argB⁺::taaG2*; *pyroA4*) carries a single copy of the *A. oryzae taaG2* gene in the *argB* locus (Kato et al. 2002a). The strain CT3 (*biA1 creA204*; *wA3*; *argB2::argB::taaG2*) was constructed by crossing the strains ABTA16 and CA4 (*creA204 biA1*; *wA3*; *argB2*), the latter strain being progeny of a cross between ABPU1 (*biA1 pyrG89*; *wA3*; *argB2*; *pyroA4*) and MH664 (FGSC A748) (*creA204 biA1*; *niiA4*). *A. nidulans* strains were grown at 37 °C in the standard minimal medium (Rowlands and Turner 1973) supplemented with appropriate requirements, unless otherwise stated.

Fluorescence microscopy

Fluorescence microscopy was performed as described previously (Makita et al. 2009). Briefly, *A. nidulans* spores were inoculated on cover slips dipped in minimal medium with 2% lactose as the sole carbon source and incubated at 37 °C for 14 h. Ethyl methyl ketone was then added at a concentration of 50 mM to trigger the *alcA* promoter-dependent expression of the GFP-AmyR gene. After incubation at 37 °C for 3 h, amylase inducers were added at various concentrations and incubated at 37 °C for 15 min or 60 min. The mycelia on the cover slips were examined by fluorescent microscopy. All microscopy and image preparations were performed with an Olympus BX51TRF microscope equipped with a 40× UPlanApo objective lens set. Olympus filter units, U-MGFPHQ and U-MWU, were used

for detection of GFP and DAPI fluorescence, respectively. Images were captured with a cool CCD camera (Hamamatsu Photonics) controlled by the AQUA-LITE software. Experiments were repeated at least three times for each amylase inducers under the identical conditions.

α-Amylase assay

The α-amylase assay was performed as described previously (Kato et al. 2002a). *A. nidulans* strains were pre-grown for 24 h in the minimal medium with D-glucose replaced by 2% polypeptone (Wako Chemicals Ltd., Japan). After washing the mycelia with the standard minimal medium lacking carbon sources, the mycelia was suspended in a fresh medium of the same composition as used for the pre-culture. Amylase inducers were then added. α-Amylase activity in the culture filtrates was measured after 12 h cultivation as previously described (Kato et al. 2002a).

Chemicals

Maltose and isomaltose were purchased from Sigma (St. Louis, MO, USA) and Seikagakukogyo (Tokyo, Japan), respectively. Kojibiose was kindly provided by Kikkoman Co., Ltd. (Chiba, Japan). Castanospermine was obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA, USA). All other chemicals were of reagent grade.

Results

Amylase inducers trigger nuclear localization of AmyR

α-Glucobioses, such as kojibiose (2-*O*-α-glucosylglucose), maltose (4-*O*-α-glucosylglucose), and isomaltose (6-*O*-α-glucosylglucose), induce amylase synthesis in *A. nidulans* (Kato et al. 2002a). Isomaltose is the strongest inducer, inducing amylase synthesis at 3 μM; kojibiose and maltose are moderate inducers, exhibiting inducing activity at 0.3 mM and above.

We have previously shown that isomaltose triggers rapid nuclear accumulation of GFP-AmyR (Makita et al. 2009). The ability of kojibiose and maltose to trigger the nuclear accumulation of GFP-AmyR was examined to confirm that AmyR localizes to the nuclei in the presence of other amylase inducers (Fig. 1). In the absence of the inducers, GFP fluorescence was distributed in the cytosol of the hyphae. Fifteen minutes after the addition of isomaltose and kojibiose, GFP fluorescence accumulated in the nuclei (bright small dots in Fig. 1). Maltose-triggered nuclear accumulation was not observed at 15 min, but was present at 60 min.

When an α- and β-glucosidase inhibitor, castanospermine, was added, maltose-triggered nuclear localization was inhibited.

Fig. 1 Inducer-dependent nuclear localization of GFP-AmyR. Isomaltose, kojibiose, and maltose were added at a concentration of 3 mM. +CS indicates that castanospermine was added at a concentration of 0.3 mM prior to the addition of the inducers. The samples were examined at 15 and 60 min after the addition of the inducers
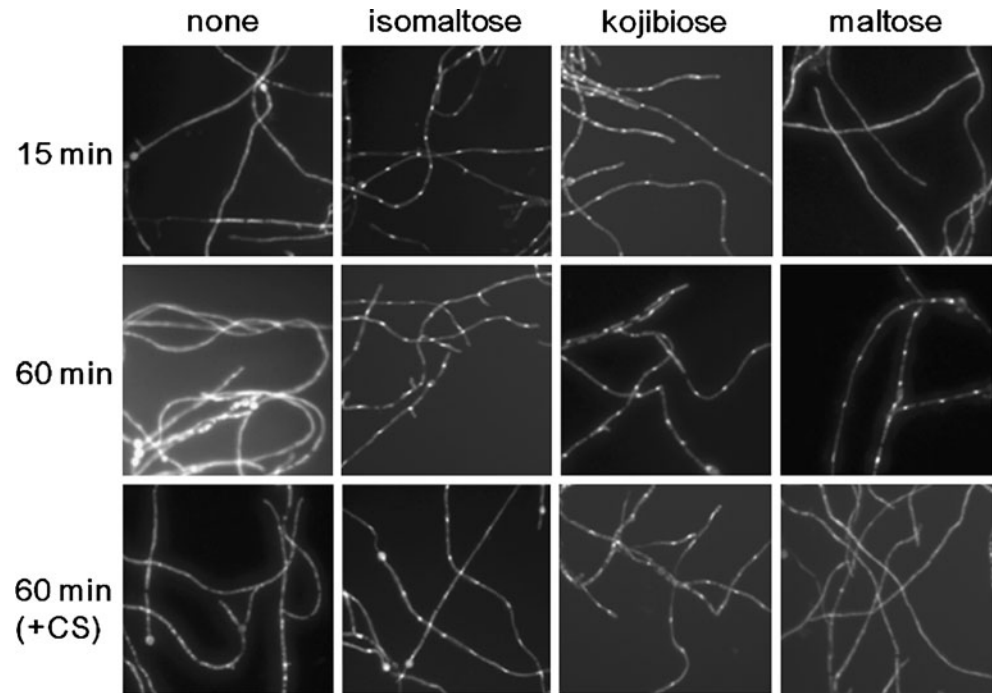
In contrast, isomaltose and kojibiose were still able to trigger the accumulation (Fig. 1). As previously reported, castanospermine inhibits degradation of isomaltose in the culture (Kato et al. 2002a); this is also the case for kojibiose (data not shown). These results indicate that maltose requires a pre-existing α- or β-glucosidase(s) to trigger the nuclear localization of AmyR, while isomaltose and kojibiose do not. This is consistent with our previous findings that maltose induction of α-amylase requires a pre-existing α-glucosidase(s) with isomaltose-producing activity and that isomaltose and kojibiose do not require such an enzyme for this induction (Kato et al. 2002a, b). Based on these findings, we propose that maltose itself is not a direct inducer of α-amylase production at least in *A. nidulans*.

Glucose, a repressing carbon source, triggers nuclear localization

Although D-glucose generally represses the expression of amylolytic genes in a CreA-dependent manner (Felenbok and Kelly 1996), it has also been shown to induce α-amylase in *A. oryzae* (Carlsen and Nielsen 2001). In order to further explore the effects of D-glucose on α-amylase production, sub-cellular localization of GFP-AmyR was examined after 15 min of D-glucose addition and at the final concentration of 3 mM. As shown in Fig. 2a and b, D-glucose triggered nuclear localization of GFP-AmyR, but D-galactose and D-xylose did not. D-Mannose very weakly triggered the nuclear localization; strong GFP fluorescence was observed only in some nuclei at the concentration of 3 mM. While D-glucose induced amylase activity in a *creA* deficient strain as shown below, D-mannose did not (data not shown), suggesting that D-mannose-triggered nuclear accumulation of AmyR is not sufficient to produce detectable amounts of α-amylase. These results indicate that only D-glucose, among the monosaccharides examined, had the ability to trigger substantial nuclear accumulation of AmyR. In addition, rapid nuclear accumulation (within 15 min) in response to D-glucose suggests that D-glucose directly, without any enzymatic conversion, triggers this nuclear accumulation in the same manner as isomaltose and kojibiose.

To confirm this, non-metabolizable D-glucose analogues, 1-deoxyglucose, 2-deoxyglucose, and 6-deoxyglucose, were examined for the ability to trigger the nuclear accumulation. GFP-AmyR accumulated in the nucleus within 15 min of their addition (Fig. 2c). Consequently, D-glucose, without any enzymatic conversion, apparently functions as an inducer of this nuclear accumulation. In addition, these results imply that the hydroxyl groups at C1, C2, and C6 of D-glucose are not essential.

Comparison of effective concentrations of isomaltose, kojibiose, and D-glucose required for triggering the nuclear accumulation revealed the exceptional activity of isomaltose. GFP-AmyR clearly accumulated in the nuclei at 0.3 μM of isomaltose (Fig. 3). In comparison, 30 μM and 3 mM concentrations were required for kojibiose and D-glucose, respectively, to demonstrate an equivalent nuclear accumulation. Thus, kojibiose and D-glucose appeared to be approximately

Fig. 2 Ability of various monosaccharides to trigger the nuclear localization of GFP-AmyR. All monosaccharides were added at a concentration of 3 mM, and microscopic observation was performed at 15 min after their addition. **a** D-Glucose was added and the same mycelia were observed by DAPI (*left*) and GFP (*right*) fluorescence. **b** GFP fluorescence after addition of D-glucose, D-mannose, D-galactose, and D-xylose. **c** GFP fluorescence after addition of 1-deoxyglucose (*1-DOG*), 2-deoxyglucose (*2-DOG*), and 6-deoxyglucose (*6-DOG*), whose structures are shown
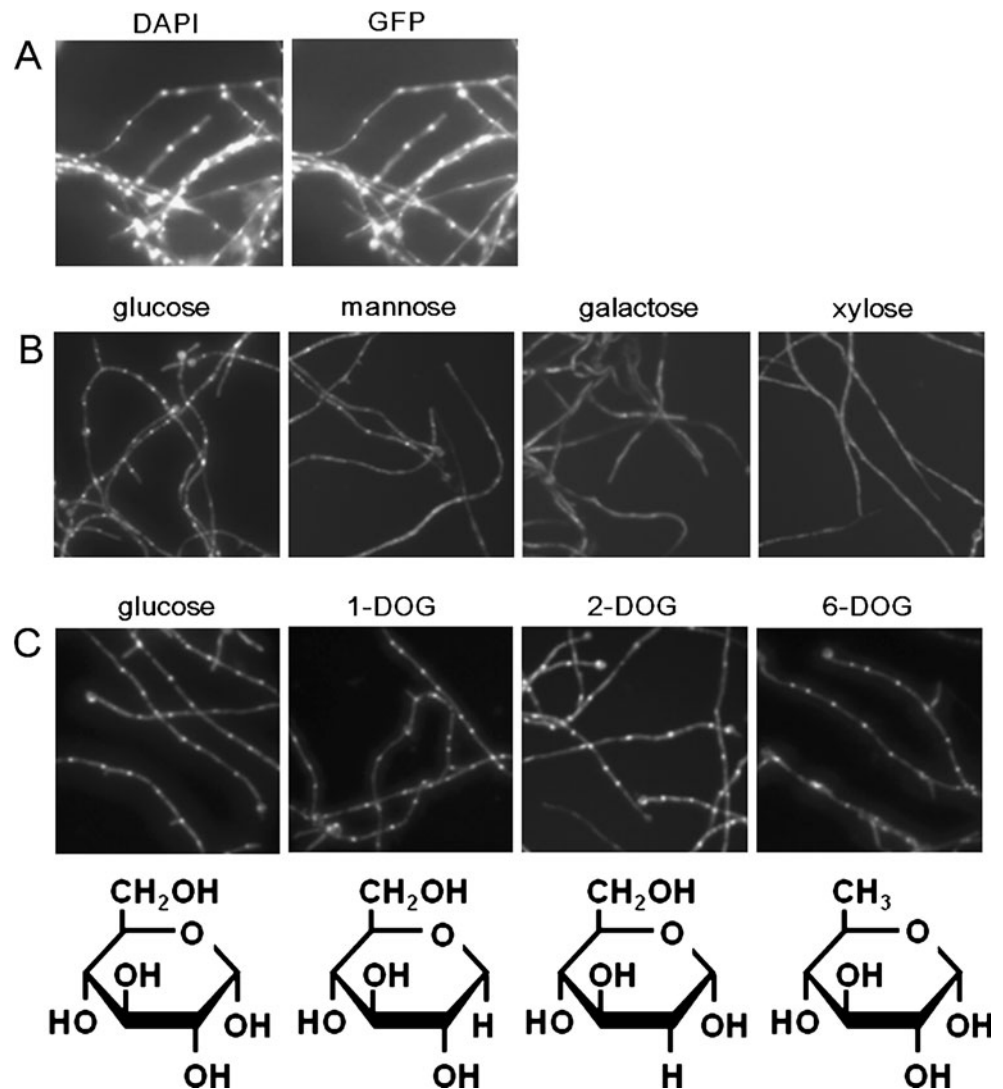
2 and 4 orders-of-magnitude weaker compared with isomaltose when added into the culture.

**Glucose induces α-amylase production in a CreA-deficient strain of *A. nidulans***

To examine whether D-glucose induces α-amylase production, we used *A. nidulans* strains that carry the Taka-amylase A gene (*taaG2*) of *A. oryzae*. The *taaG2* gene is inducibly and abundantly expressed in *A. nidulans* and has been used as a reporter gene for examination of amylase gene regulation in this species (Tani et al. 2001a, b; Ito et al. 2004; Makita et al. 2009).

The strain ABTA16 (*creA$^+$*, *amyR$^+$*) produced approximately 60 times higher α-amylase activity in the presence of 0.1% isomaltose compared with its absence (Fig. 4). When 0.1% D-glucose was used, α-amylase production was decreased approximately eight times compared with that observed in the

presence of isomaltose. In this instance, the observed activity was seven times higher compared with that seen in the non-induced condition. At the higher D-glucose concentration of 2%, α-amylase production decreased to half of that seen with 0.1% D-glucose. α-Amylase induction by D-glucose, and isomaltose, was not detected in the strain ΔamyR103 (*creA$^+$*, *ΔamyR*) (Tani et al. 2001b) (data not shown). This indicates that D-glucose induces α-amylase production in an AmyR-dependent manner, as expected from the results of the sub-cellular localization studies described above.

In the CreA mutant, CT3 (*creA204*, *amyR$^+$*), α-amylase activity was higher at 2% D-glucose compared with 0.1%. In addition, 2-deoxyglucose at 0.1% induced comparable α-amylase activity to that observed with 2% D-glucose. These results clearly indicate that D-glucose induces production of α-amylase, although it represses this production in a CreA-dependent manner.

Fig. 3 Comparison of activity of isomaltose, kojibiose, and glucose to trigger the nuclear localization of GFP-AmyR. The sugars were added at the indicated concentrations and the GFP fluorescence was examined at 15 min after their addition
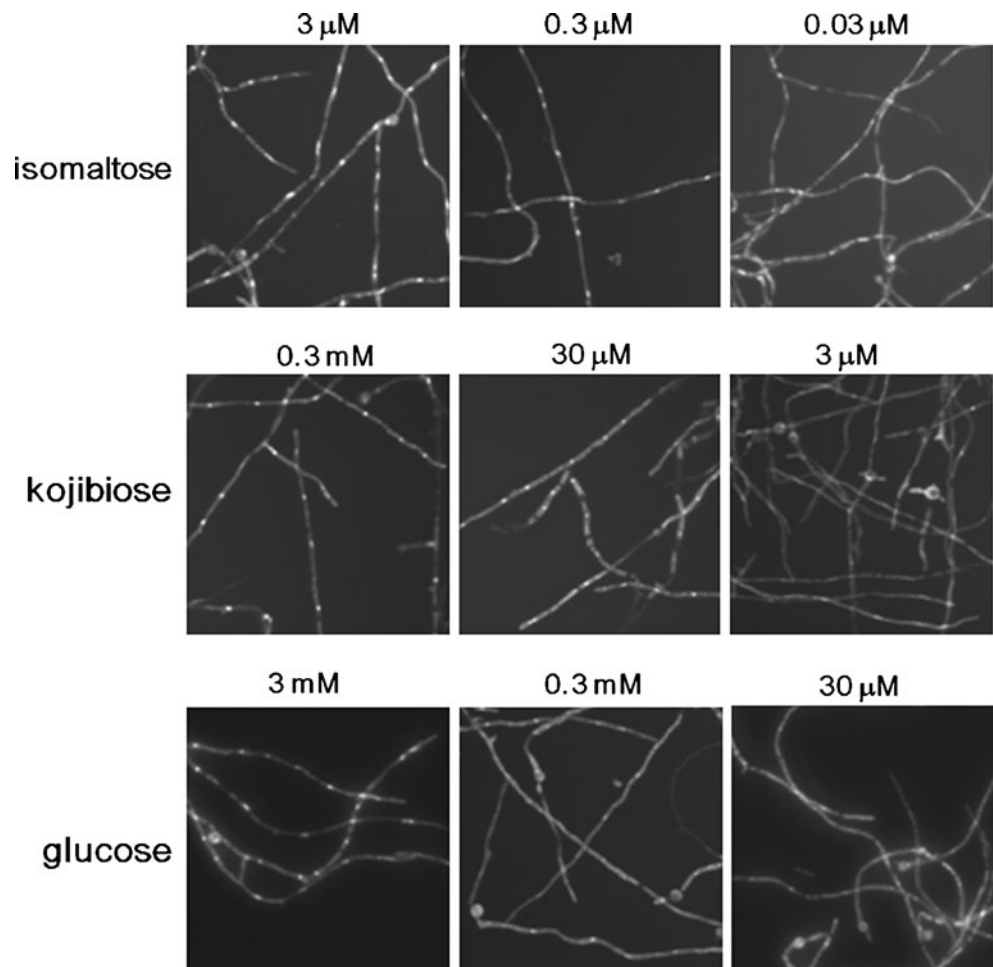
Discussion

Various α-linked glucobioses induce amylase activity in *A. oryzae* and *A. nidulans*, strongest inducer being isomaltose (Tonomura et al. 1961; Kato et al. 2002a). It also appeared to be an extremely effective molecule to cause nuclear localization of AmyR, triggering this within a short period and at very low concentrations (Figs. 1 and 3). Maltose-triggered nuclear localization of AmyR required glucosidase activity, but isomaltose and kojibiose did not. These observations concerning the ability of the glucobioses in triggering the nuclear localization are analogous to those in amylase induction (Kato et al. 2002a), indicating that the activity to trigger the nuclear localization probably determines the strength of the amylase inducers.

Besides acting as a repressing carbon source, D-glucose acts as an amylase inducer in *A. oryzae* (Carlsen and Nielsen 2001). Previous studies had failed to clarify whether the D-glucose directly, without any enzymatic conversions, activates AmyR-mediated expression of the amylolytic genes. Our results, from the studies using non-metabolizable D-glucose analogues, suggest that D-glucose directly triggers nuclear localization of AmyR (Fig. 3). The activity of D-glucose to

trigger nuclear localization is much weaker compared with isomaltose and kojibiose. This weak activity, together with its
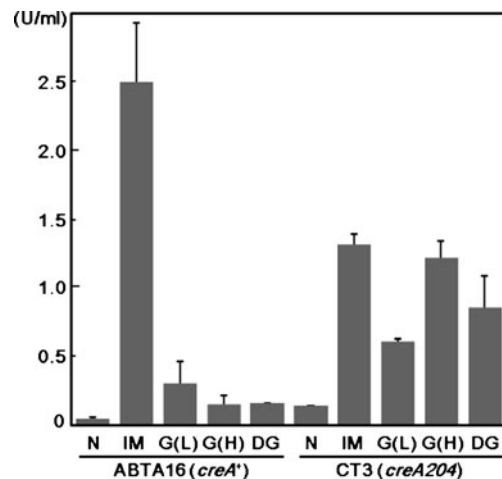

Fig. 4 α-Amylase induction in *A. nidulans* creA[+] and creA204 strains. Isomaltose (*IM*) and 2-deoxyglucose (*DG*) were added at a concentration of 0.1%. The concentrations of D-glucose were 0.1% and 2%, which are indicated as G (L) and G (H), respectively. *N* denotes no inducing carbon sources such as isomaltose, 2-deoxyglucose, and D-glucose

nature to cause strong carbon catabolite repression, meant that sufficient amylase induction by D-glucose could be detected only under a CreA-defective background (Fig. 4).

When starch is present as the sole carbon source, some portion of starch must be first degraded, prior to amylase induction, by α-amylases, glucoamylases and α-glucosidases produced at the basal level, forming maltooligosaccharides, maltose, and D-glucose. At the same time, strong α-1,6-bond-forming transglycosylation activity of some α-glucosidases such as AgdA and AgdB would lead to formation of isomaltose, the strongest inducer reported in *A. nidulans* (Kato et al. 2002a, b). Consequently, two direct inducers, D-glucose and isomaltose, are expected to be present in the starch-grown culture, and this is also the case when maltose is used as the sole carbon source. Approximately the same amounts of D-glucose and isomaltose were detected in the culture broth at 4 h after the induction with 0.1% maltose (data not shown). Considering that D-glucose is a much weaker inducer compared with isomaltose in triggering the nuclear localization of AmyR, and that high levels of D-glucose causes CreA-dependent repression, D-glucose would be expected to contribute little in the induction of α-amylase when grown on maltose or starch. Thus, in laboratory conditions using pure culture, high level of α-amylase production with D-glucose could be achieved only when it is supplied continuously at low concentrations.

However, this situation may be altered in conditions where both starch and high concentrations of D-glucose are present. While CreA represses transcription of amylolytic genes directly and indirectly because of repression of the *amyR* expression (Tani et al. 2001b), AmyR existing at the basal level does accumulate in the nuclei, ready to transactivate its targets. Decrease in the environmental concentration of D-glucose would immediately lead to transcription of amylolytic genes and *amyR* itself, which would be followed by a boost in production of amylolytic enzymes through production of the strongest inducer, isomaltose.

The reason why D-glucose is not the most effective inducer might be speculated from the fact that the major sources of D-glucose in nature are starch and cellulose. If AmyR responded to very low concentrations of D-glucose, high levels of α-amylase would be induced by cellulose. It should also be noted that currently known cellulase inducers are not D-glucose but sophorose in *Trichoderma viride* (Mandels et al. 1962), gentiobiose in *Penicillium purpurogenum* (Kurasawa et al. 1992), and D-xylose in *A. niger* and *A. oryzae* (van Peij et al. 1998; Marui et al. 2002). The glucobioses sophorose and gentiobiose are shown to be produced by the action of β-glucosidase (Kurasawa et al. 1992; Mach et al. 1995), which is analogous to isomaltose formation by α-glucosidases (Kato et al. 2002a, b).

In our previous report, expression of *amyR* was shown to be repressed in a CreA-dependent manner (Tani et al. 2001b). Expression of *amyR* was highest on starch followed by maltose,

and decreased on D-glucose and glycerol in the *creA*⁺ strain of *A. nidulans*. In the *creA204* strain, the expression levels were almost identical on starch, maltose, and D-glucose. The expression level was still low on glycerol, but we could not find a reasonable explanation for this finding. The current results, showing that D-glucose is one of the inducers, suggest the explanation may be that expression of the *amyR* gene is under control of AmyR itself. Further evidence to support this is the presence of an AmyR binding site, CGGN$_8$CGG, at −232 to −245 (referring the translational start site as +1) in the promoter region of the *amyR* gene.

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