Appl Microbiol Biotechnol (1998) 50: 459–467

ORIGINAL PAPER

T. Minetoki · C. Kumagai · K. Gomi · K. Kitamoto
K. Takahashi

Improvement of promoter activity by the introduction of multiple copies of the conserved region III sequence, involved in the efficient expression of *Aspergillus oryzae* amylase-encoding genes

Received: 6 March 1998 / Received revision: 11 May 1998 / Accepted: 14 June 1998

Abstract The role of the conserved sequence region III in the promoter regions of the amylase-encoding genes *amyB*, *glaA* and *agdA* of *Aspergillus oryzae* was examined. Introduction of multiple copies of the region III fragment into the *agdA* promoter resulted in a significant increase in promoter activity at the transcriptional level. This result suggests that the fragment comprising region III consists of one or more *cis*-acting sequence(s). Moreover, expression of the *agdA* gene under the control of the improved *agdA* promoter resulted in efficient overproduction of α-glucosidase, even in the presence of glucose. Thus, overexpression of genes controlled by the improved promoter incorporating region III is possible. Interestingly, expression of the *amyB* and *glaA* genes in the transformant was strongly repressed. This result suggests that the *trans*-acting regulatory protein(s) that interact with region III are common to these amylase genes and that the titration of regulatory protein(s) reduced the expression of the *amyB* and *glaA* genes.

Introduction

Aspergillus species are widely used in the fermentation industry for the production of useful enzymes. In particular, *A. oryzae* is important as a host for heterologous protein production owing to its ability to secrete high levels of protein (Barbesgaard et al. 1992; Christensen et al. 1988). It also produces industrially valuable amylases, such as Taka amylase (TAA), glucoamylase (GLA) and α-glucosidase (AGL). Production of these amylases is known to be induced by starch or oligosaccharides such as maltose, maltotriose and isomaltose. We are interested in the regulation of amylase gene expression, and have cloned and sequenced the *amyB* (Tada et al. 1989), *glaA* (Hata et al. 1991) and *agdA* (Minetoki et al. 1995a) genes encoding TAA, GLA, and AGL respectively. Further analyses have shown that the expression of these three amylase genes is induced at the transcriptional level under inducible growth conditions (Tsuchiya et al. 1992; Hata et al. 1992; Minetoki et al. 1995b).

A comparison of the promoter regions of the *Aspergillus* amylase genes has indicated that there are four highly conserved sequences, designated regions I, II, IIIa, and IIIb. The function of these consensus sequences in the *agdA* promoter (P*agdA*) has been investigated by deletion analysis of a P*agdA*::*uidA* fusion gene integrated at the *niaD* locus (Minetoki et al. 1996). Deletion of region IIIa resulted in a more than 90% reduction in β-glucuronidase activity and also abolished maltose inducibility. This result suggests that region IIIa is a functional element essential for high-level expression and maltose induction. Deletions of region IIIb within the *Aspergillus niger agdA* gene (Nakamura et al. 1997) and region I have suggested that these two elements contain sequences that might be involved in enhancing expression in conjunction with region IIIa. Furthermore, we have observed that TAA activity is markedly reduced in the *glaA* multiple-copy transformants (Hata et al. 1991), and that the expression level of the *amyB* and *glaA* genes in *agdA* multiple-copy transformants is also drastically reduced (Minetoki et al. 1995b). These results imply that the regulation of the three amylase genes is closely correlated at the transcriptional level and that the *A. oryzae* amylase genes may be controlled by a common regulatory mechanism involved in high-level expression and maltose induction.

T. Minetoki (✉) · C. Kumagai
General Research Laboratory, Ozeki Corp., 4-9,
Imazu Dezaike-cho, Nishinomiya-shi, Hyogo 663, Japan
e-mail: ozeki00@po.infosphere.or.jp
Fax: 81-798-34-7475

K. Gomi · K. Takahashi
National Research Institute of Brewing, 3-7-1, Kagamiyama,
Higashi-Hiroshima-shi, Hiroshima 739, Japan

K. Kitamoto
Department of Biotechnology, The University of Tokyo,
1-1-1, Yayoi, Bunkyo-ku, Tokyo 113, Japan

On the other hand, high-level expression signals, such as the *amyB* and *glaA* promoters, are frequently used for the overexpression of homologous and heterologous genes in a variety of filamentous fungi (van den Hondel et al. 1991). If high-level expression of *amyB* and *glaA* is dependent on region III, the insertion of region III into the promoter region could further elevate expression.

In this study, multiple copies of the fragment comprising region III were introduced into P*agdA* to enhance promoter activity and to determine the common regulatory mechanism for the expression of the *A. oryzae* amylase genes. The effect of introducing this fragment on gene expression is discussed. In addition, the merits of a conventional solid-state culture system for protein production are described.

Materials and methods

Strains, plasmids, media and transformation

A. oryzae niaD300, which is a *niaD* mutant derived from the wild-type strain RIB40, was used for transformation experiments and was kindly provided by Dr. J. R. Kinghorn (St. Andrews, UK). *Escherichia coli* JM109 (Yanisch-Perron et al. 1985) was used for DNA manipulation. Plasmid pNAGT4 (Minetoki et al. 1996) was constructed from pUC118 (Vieira and Messing 1987), pBI221 (Jefferson et al. 1987) containing the *E. coli uidA* gene, pSTA14 (Unkles et al. 1989) containing the *A. oryzae niaD* gene and pTGF-1 (Minetoki et al. 1995a) carrying the *A. oryzae agdA* gene. Czapek-Dox (CD) medium, consisting of 0.3% $NaNO_3$, 0.2% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.02% $FeSO_4 \cdot 7H_2O$ and a 2% carbon source adjusted to pH 5.5. In CD-P medium, 1% polypeptone (Wako Pure Chemical, Japan), was substituted for 0.3% $NaNO_3$. Dextrin/peptone (DP) medium, consisting of 2% dextrin, 1% polypeptone, 0.5% KH_2PO_4 and 0.05% $MgSO_4 \cdot 7H_2O$, was used as the complete medium. Transformation of *E. coli* and *A. oryzae* was carried out according to the methods of Hanahan (1983) and Gomi et al. (1987) respectively. *A. oryzae* protoplasts were prepared from mycelia grown at 30 °C for 48 h using Yatalase (Takara Shuzo, Japan).

Construction of the improved *agdA* promoter

The *agdA* promoter d2 (P*agdA*2), designed to generate *Pst*I and *Sal*I sites, was constructed as described previously (Minetoki et al. 1996) and was introduced into pUC118 to give pAGP5-d2. To obtain P*agdA*131, the 340-bp *Eco*RV-*Cla*I region of P*agdA*2 in pAGP5-d2 was removed, and the linearized plasmid was then self-ligated. A 55-bp fragment (−556 to −502) of P*agdA*2, containing region III, was amplified by the polymarase chain reaction (PCR) using primer F and primer R, which had been designed to generate a *Cla*I site at the 5′ end (Fig. 1). For the construction of P*agdA*132 carrying two copies of region III, the 340-bp *Eco*RV-*Cla*I region of P*agdA*2 was replaced by the 55-bp amplified fragment containing the *Eco*RV and *Cla*I sites at the 3′ end. P*agdA*134 and P*agdA*136 were constructed by introducing two or four further copies of the amplified region III fragment into the *Eco*RV and *Cla*I sites of P*agdA*132 respectively. P*agdA*142, carrying 12 copies of tandemly repeated region III, was constructed as follows. The blunted *Cla*I-*Xho*I fragment containing six copies of region III was isolated from P*agdA*136, and cloned between the *Eco*RV and *Cla*I sites of P*agdA*136. The plasmids containing P*agdA*131 to −142 were named pAGP5-d131 to −d142 which were derived from pAGP5-d2. The constructed promoter is shown in Fig. 2.

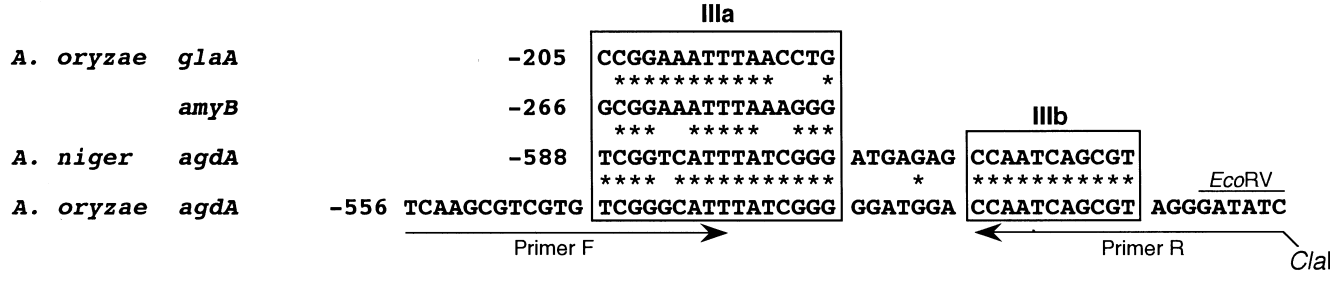


Fig. 1 The conserved nucleotide sequences, including region III, in the upstream regions of *Aspergillus* amylase genes. The nucleotide sequences are based on *A. oryzae glaA* (Hata et al. 1992), *A. oryzae amyB* (Tsuchiya et al. 1992), *A. niger agdA* (Nakamura et al. 1997), and *A. oryzae agdA* (Minetoki et al. 1996). The numbers indicate the nucleotide distance to the translation start point. Regions IIIa and IIIb are boxed. The primers F and R (*arrows*) were used for the polymerase chain reaction amplification of regions IIIa and IIIb

Construction of the expression vector

To obtain plasmids pNAGG1-d131 to −d142, for use as expression vectors for the *uidA* gene under the control of the improved *agdA* promoter, each *Pst*I-*Sal*I fragment carrying the constructed promoter regions pAGP5-d131 to −d142 was introduced into the corresponding sites of plasmid pNAGT4 (Minetoki et al. 1996). Plasmid pNAGL142 (Fig. 5), which was used as the expression vector for the *agdA* gene, was constructed as follows. A 3.8-kb *Cpo*I-*Bam*HI fragment of the *agdA* gene was prepared from plasmid pTGF-1 and introduced into the corresponding sites of pAGP5-d142 to yield pAGL142. A 2.1-kb *Pst*I-*Sal*I fragment containing the *uidA* gene of pNAGT4 was replaced by a 4.8-kb *Pst*I-*Sma*I fragment, which carries the P*agdA*142::*agdA* fusion gene isolated from pAGL142 to yield pNAGL142.

Northern blot analysis

Total RNA was prepared by the method of Cathala et al. (1983) with minor modifications. A 15-μg sample of total RNA was separated on a formaldehyde/agarose gel (Sambrook et al. 1989), then transferred to a Hybond-N membrane (Amersham, UK) and cross-linked under UV light. DNA probes were ^{32}P-labeled with a Megaprime DNA labeling system (Amersham, UK). Hybridization was carried out at 42 °C in 5 × SSPE (0.18 M NaCl, 10 mM sodium phosphate pH 6.7, 1 mM EDTA), 5 × Denhardt's solution, 50% formamide, 0.5% sodium dodecyl sulphate (SDS) and 100 μg/ml denatured salmon sperm DNA.

Enzyme assays

The preparation of a cell-free extract from mycelia was carried out as described previously (Tada et al. 1991). The β-glucuronidase activity of the cell-free extract was measured as described by Jefferson et al. (1986). AGL activity was measured as described previously (Minetoki et al. 1995a). The screening of AGL transformants was carried out by plate assay. Colonies of the overproducing strain produced a strong yellow colour on an agar plate

containing 0.5% 4-nitrophenyl α-D-glucoside. Glucoamylase (GLA) activity was measured by a modification of the method of Iwano et al. (1986). Glucose was assayed with a Glucose-CII test kit (Wako Pure Chemicals, Japan). One unit of activity was defined as the amount that liberated 1 μmol glucose/min. TAA activity was measured as described by Adachi (1954). Culture filtrates dialysed against 20 mM acetate buffer (pH 5.0) were used for the AGL, GLA and TAA assays. Protein concentration was determined with a protein assay kit (BioRad Laboratories, USA) using bovine serum albumin as a standard.

Other methods

Nucleotide sequences were determined by the dideoxynucleotide chain-termination method of Sanger et al. (1977) with a DNA sequencer (Applied Biosystems, model 373A). Preparation of genomic DNA from *A. oryzae* was performed as described previously (Minetoki et al. 1995a). Southern-blot hybridization was carried out by a Gene Images random prime labeling and detection system (Amersham, UK). SDS/polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (1970) using an electrophoresis calibration kit (Pharmacia Biotech, Sweden) as a standard. Proteins were stained with Coomassie brilliant blue R250.

Results

Improvement of promoter activity by the introduction of the fragment comprising region III into the *agdA* promoter

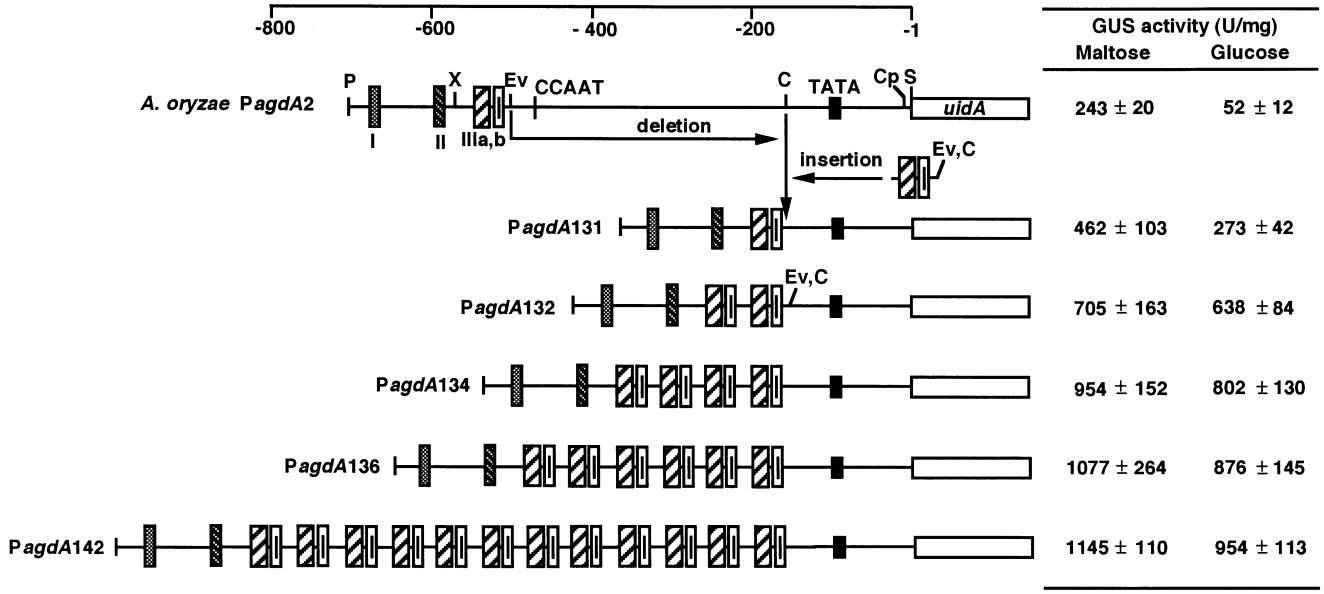
We have previously shown that deletion of the 340-bp *Eco*RV-*Cla*I region in the *agdA* promoter P*agdA* causes a significant increase in promoter activity (Minetoki et al. 1996). To enhance promoter activity further, multiple copies of the fragment comprising region III were inserted into the original promoter (P*agdA*131). Promoter activity was determined using an assay for β-glucuronidase, which was expressed under the control of the improved promoter. As shown in Fig. 2, an increase in β-glucuronidase activity, corresponding to the number of inserted region III copies, was observed with the improved promoter. In P*agdA*142, carrying 12 tandem repeats of region III, the promoter activities were shown to be 2- to 3-fold higher than that of the original promoter (P*agdA*131). P*agdA*142 activity increased 5- and 18-fold in comparison with the intrinsic promoter (P*agdA*2) in the presence of maltose and glucose as a carbon source respectively. This result suggests that the fragment comprising region III consists of one or more *cis*-acting sequence(s).

The dependence of the improved *agdA* promoter on carbon source was analysed further by comparing β-glucuronidase expressed under the control of P*agdA*2 and P*agdA*142 under various growth conditions (Fig. 3A, B). P*agdA*142 activity increased more than 5-fold in comparison to P*agdA*2 activity under all conditions examined, especially in glucose-grown mycelia (more than 20-fold). Thus, although the activity of the intrinsic promoter was repressed by glucose, the improved promoter was capable of constitutive expression in the presence of glucose or other α-glucosyl glucoses.

Transcriptional level of the improved *agdA* promoter

The transcriptional level of *uidA* under the control of the improved promoter and the influence on the expression

Fig. 2 Improvement of promoter activity by the introduction of region III. The location of each conserved element (regions I, II, IIIa and IIIb) within the promoter region is indicated by a different box pattern. The putative CCAAT box and the TATA box are indicated by the vertical line and solid box respectively. The numbers marked at the top indicate the nucleotide distance to the translation start point. Two independently isolated single-copy transformants with each plasmid integrated at the *niaD* locus were grown in 15 ml CD-P medium containing 2% glucose or maltose at 30 °C for 48 h. The β-glucuronidase (*GUS*) activities of cell extracts obtained from two independent experiments are presented as the average with standard errors. *C*, *Cla*I; *Cp*, *Cpo*I; *Ev*, *Eco*RV; *P*, *Pst*I; *S*, *Sal*I; *X*, *Xho*I



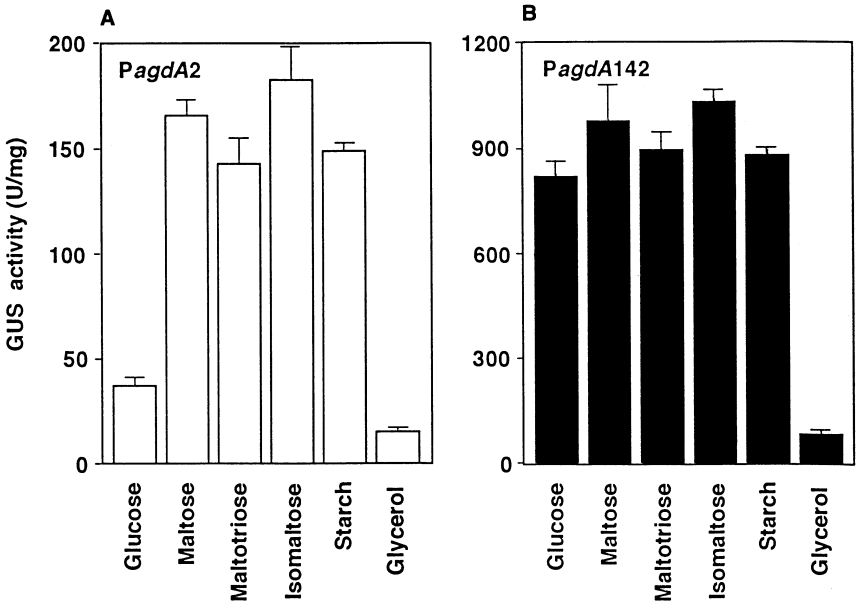


Fig. 3A, B Effect of carbon source on β-glucuronidase (*GUS*) expression under the control of the improved promoter. Two independently isolated single-copy transformants with the *uidA* gene under the control of P*agdA*2 (**A**) and P*agdA*142 (**B**) were grown in 15 ml CD-P medium containing 2% carbon source at 30 °C for 48 h. The β-glucuronidase activities of two independent experiments are plotted as the average. *Bars* standard errors

of *amyB* and *glaA* in the β-glucuronidase transformant were determined by Northern hybridization. Figure 4A shows the transcriptional levels of the *uidA* expressed under both sets of growth conditions. The transcriptional levels were similar to the GUS activities shown in Fig. 2, demonstrating that the *agdA* promoter is improved by deletion of the *Eco*RV-*Cla*I region and insertion of multiple copies of region III.

Since expression of the *amyB* and *glaA* genes was repressed by glucose, these genes had a lower transcription level in the presence of glucose than that observed in the presence of maltose. However, expression of the *amyB* and *glaA* genes in the transformant carrying the P*agdA*142-controlled *uidA* gene was significantly lower in the presence of glucose than that of transformants under the control of other promoters (Fig. 4B, C). A similar decrease was also observed when maltose was used as a carbon source. The TAA and GLA activities of the P*agdA*142 β-glucuronidase transformant grown with maltose also showed an approximately 30% reduction compared to the other transformants (data not shown).

Efficient expression of the *agdA* gene under the control of the improved *agdA* promoter

A pNAGL142 vector (Fig. 5), which carries the P*agdA*142::*agdA* (fusion) gene and the *niaD* gene as a selectable marker, was integrated at the *niaD* locus on the chromosome of an *A. oryzae niaD*$^{-}$ strain (Unkles et al. 1989). AGL142-72 was the most efficient overproducing transformant among the approximately 300 transformants screened. AGL142-72 and 12 transformants selected at random were subjected to Southern analysis (data not shown) to determine the number of *agdA* gene copies. The AGL activities of five transformants with different copy numbers were then determined.

As shown in Table 1, even the AGL activities of transformants carrying a single copy increased to more than 70-fold that of the recipient strain in the presence of glucose, and the transformant with the highest copy number, AGL142-72, showed a 140-fold increase in activity. Although the ratio of the activity in glucose medium to the activity in maltose medium was only 15% in the recipient strain, each transformant had an activity ratio greater than 60%, indicating that the *agdA* gene under the control of the improved promoter was expressed constitutively.

AGL142-95 (single copy) and AGL142-72 (multiple copies) were grown in wheat bran solid-state cultures (Table 2). The AGL activities of the transformant were also significantly higher (e.g. AGL142-72, about a 170-fold increase) than that of the recipient strain. Moreover, the productivity of AGL142-72 was 50-fold higher than that in the submerged culture (see Table 1), indicating that wheat bran solid-state culture is a suitable system for AGL production. On the other hand, the TAA productivity of AGL142-95 and AGL142-72 was reduced 2- and 25-fold, respectively, in comparison with the recipient strain. Further decreases (e.g. less than 1% in AGL142-95) in the TAA productivity of these transformants were observed in solid-state culture containing ammonium sulphate. However, there was no significant effect on AGL productivity. The pH of the crude extract of the solid-state culture containing ammonium sulphate was lower than that the culture without ammonium sulphate. These results suggest that the lower pH of the culture may cause a decrease in TAA production. The reduction in the production of TAA, which is a major secretory protein in *A. oryzae*, resulted in an increase in the specific activity of AGL. A similar result was also observed in the submerged culture containing ammonium sulphate (data not shown). The results of SDS-PAGE analysis (Fig. 6A, B) showed that there was a remarkably high

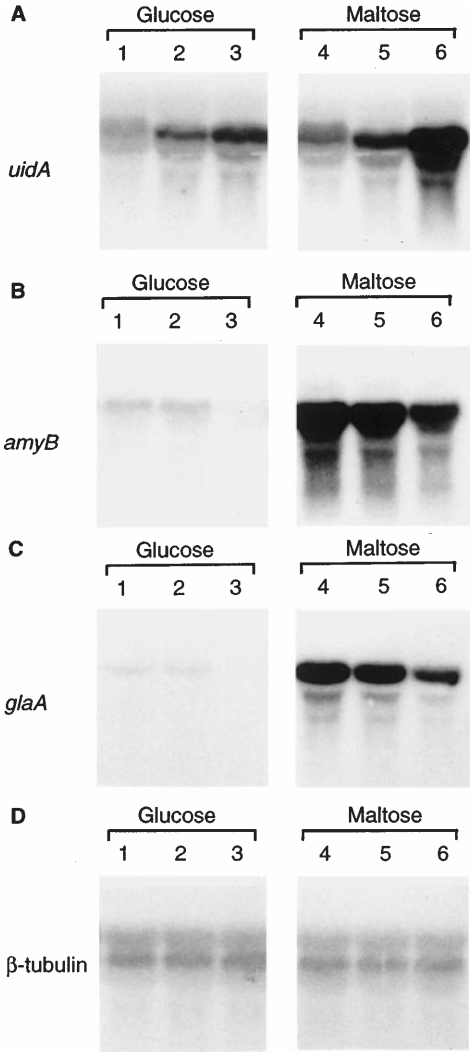


Fig. 4A–D Northern blot analysis of single-copy transformants carrying P*agdA*, and the improved P*agdA* fusion gene with *uidA*. Approximately 1×10^6 conidia were inoculated in 100 ml CD-P medium containing 2% glucose and cultivated at 30 °C. After 72 h, mycelia were transferred to 100 ml CD-P medium containing 2% glucose or maltose. Total RNA was prepared from the mycelia after 12 h cultivation. Four transcripts of the β-glucuronidase transformant under the control of P*agdA*2 (*lanes 1*, *4*), P*agdA*131 (*lanes 2*, *5*), and P*agdA*142 (*lanes 3*, *6*) were determined. The DNA fragments used as a probe were as follows: the 1.9-kb *Xba*I-*Sal*I fragment of the *uidA* gene (**A**), the 3.7-kb *Eco*RI fragment of the *amyB* gene (**B**), the 5.0-kb *Pst*I fragment of the *glaA* gene (**C**), the 4.8-kb *Pst*I fragment of the β-tubulin gene (**D**). The amount of the β-tubulin transcript was used as an internal control

level of AGL production and a significant reduction in TAA.

Transcriptional regulation of the AGL-overproducing transformants

The transcriptional levels of *agdA*, *amyB* and *glaA* were examined by Northern blot analysis to investigate the regulation of the gene expression in the AGL-overproducing transformants. The single-copy transformant of the *agdA* gene, controlled by P*agdA*142, had an extremely high level of *agdA* mRNA, but no further increase in transcription was obtained by the introduction of multiple copies (Fig. 7A). This result suggests that expression is limited by the amount of *trans*-acting regulatory protein(s) interacting with region III. Although the *amyB* and *glaA* genes were highly expressed in the recipient strain, the transformants reduced the transcriptional levels of these genes (Fig. 7B, C). Furthermore, the level of expression of *amyB* and *glaA* in the multiple-copy transformant was significantly lower than that of the single-copy strain. The activities of the three amylases were also measured. As shown in Fig. 7E, increased AGL activity and a concomitant reduction in TAA and GLA activity were observed in the transformants. These results suggest that the *trans*-acting regulatory protein(s) interacting with region III are common to these amylase genes and that the titration of regulatory protein(s) by the introduction of multiple copies of region III leads to a reduction in TAA and GLA production.

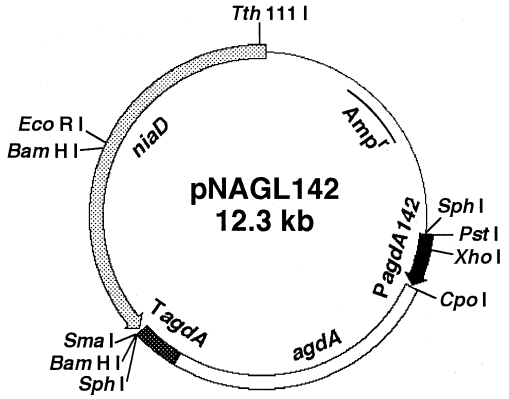


Fig. 5 Structure of the vector used for *agdA* gene expression. The thick lines indicate *Aspergillus oryzae* DNA, and the thin line indicates pUC118. The *niaD* gene was used as a selectable marker gene. P*agdA*142 is the improved promoter region of the *agdA* gene; T*agdA* is the termination region of the *agdA* gene

Discussion

Deletion analysis of P*agdA* indicated that region III is mainly involved in high-level expression and maltose induction of the amylase genes (Minetoki et al. 1996). To enhance promoter activity, we therefore inserted the fragment comprising region III into P*agdA*. As shown in Fig. 2, an increase in promoter activity, which corresponded with the number of copies of region III, was observed. Northern blot analysis showed that there was an increase in promoter activity at the transcriptional level (Fig. 4A). These results clearly demonstrated that region III serves as a target sequence for one or more transcriptional activator(s) in the expression of the *A. oryzae agdA* gene. Punt et al. (1992) have also shown

Table 1 α-Glucosidase activity of the culture broth of the transformants. Approximately 2×10^5 conidia were inoculated into 15 ml CD-P medium containing 2% glucose or maltose, and cultivated at 30 °C for 72 h. The number of *agdA* gene copies was determined by Southern blot analysis. Hybridization signals for the transformants were compared with the endogenous *agdA* gene. The α-glucosidase activity of three independent experiments is presented as the average with standard errors. The value in parentheses indicates the productivity(U/ml culture broth) of the 142–72 transformant grown with maltose

Strain	Copy number	α-Glucosidase activity (U/mg protein)		Glucose Maltose ratio (%)
		Glucose	Maltose	
Recipient	–	0.038 ± 0.005	0.246 ± 0.012	15
142–95	1	2.68 ± 0.10	4.40 ± 0.03	61
142–142	1	2.89 ± 0.32	4.76 ± 0.23	61
142–25	2	3.81 ± 0.24	5.24 ± 0.86	73
142–158	2	4.25 ± 0.25	6.74 ± 0.50	63
142–72	3–4	5.33 ± 0.39	7.37 ± 0.27 (0.076 ± 0.003)	83

that the introduction of a *cis*-acting sequence (the *gpd* box) into the upstream region of an *amdS::lacZ* fusion gene results in a significant increase in gene expression. The insertion of a *cis*-acting sequence into the promoter region is an effective means of increasing promoter activity.

The expression of the *agdA* gene under the control of P*agdA*142 led to the overproduction of AGL, even in the single-copy transformant. However, AGL activity was not proportional to the number of *agdA* copies in the multiple-copy transformant. Northern blot analysis indicated that the amount of AGL production is limited by transcription of the *agdA* gene (Fig. 7A). A similar observation was reported in studies on the introduction of multiple copies of the *glaA* gene into *A. niger* (Verdoes et al. 1993). The multiple-copy transformant (AGL142-72) of the *agdA* gene, controlled by P*agdA*142 comprising 12 copies of region III, carries 36 or 48 copies of region III. In this case, the level of *agdA* mRNA was probably limited by the availability of *trans*-acting regulatory protein(s) interacting with region III. Therefore, further overexpression of the genes controlled by the promoter carrying multiple copies of region III may be accomplished by introducing the gene(s) that encode(s) its regulatory protein(s). To give an example, the expression of a transformant carrying multiple copies of P*alcA* was shown to be limited by the expression level of the *alcR* gene encoding the *trans*-acting regulatory protein in *A. nidulans*. Introduction of multiple copies of the *alcR* gene resulted in a significant increase in the expression level of the gene controlled by P*alcA* (Gwynne et al. 1987; Davis and Hynes 1991).

Interestingly, TAA and GLA activities were strongly reduced in the AGL-overproducing transformants. Northern blot analysis indicated that the expression of the *amyB* and *glaA* genes is repressed at the transcriptional level. As shown in Fig. 1, region III in P*agdA* consists of two subregions, IIIa and IIIb. Our previous study has suggested that IIIa and IIIb are probably different *cis*-acting sequences (Minetoki et al. 1996). Region IIIa is highly conserved in the promoter regions of the *amyB*, *glaA* and *agdA* genes. If the *trans*-regulatory protein(s) interacting with region IIIa is (are) common for these amylase genes, the low transcriptional level of the *amyB* and *glaA* genes would suggest that the introduction of P*agdA*142 carrying multiple copies of region IIIa results in the titration of one or more of the common activator protein(s) involved in the high-level expression of these amylase genes. Additional evidence for such a titration was obtained by the reduction in

Table 2 Amylase activities of the wheat bran solid-state culture of the transformants. A 1-ml sample of conidial suspension (1×10^6/ml) was inoculated onto 5 g wheat bran sterilized by autoclaving with 3 ml water or 0.1 M ammonium sulphate, and then grown at 30 °C for 72 h. The wheat bran was immersed in 50 ml water at 37 °C for 2 h with shaking and centrifuged at 15 000 rpm for 20 min. The supernatant was used as crude extract for the enzyme assay. The pH of the crude extract is shown. The enzyme activities of three independent experiments are presented as the averages with standard errors

Strain	$(NH_4)_2SO_4$ (M)	pH	Enzyme activity		
			α-Glucosidase		α-Amylase Productivity (U/g wheat bran)
			Productivity (U/g wheat bran)	Activity (U/g mg protein)	
Recipient	0	5.2 ± 0.1	0.025 ± 0.001	0.036 ± 0.006	2143 ± 28
142–95	0	5.2 ± 0.1	0.98 ± 0.04	1.76 ± 0.35	1017 ± 7.5
142–72	0	5.2 ± 0.1	3.92 ± 0.13	6.23 ± 0.86	76 ± 5.5
Recipient	0.1	4.1 ± 0.1	0.027 ± 0.006	0.130 ± 0.005	69 ± 15
142–95	0.1	4.2 ± 0.2	0.70 ± 0.10	3.26 ± 0.29	58 ± 9.3
142–72	0.1	4.3 ± 0.2	4.16 ± 0.06	9.50 ± 0.26	12 ± 4.0

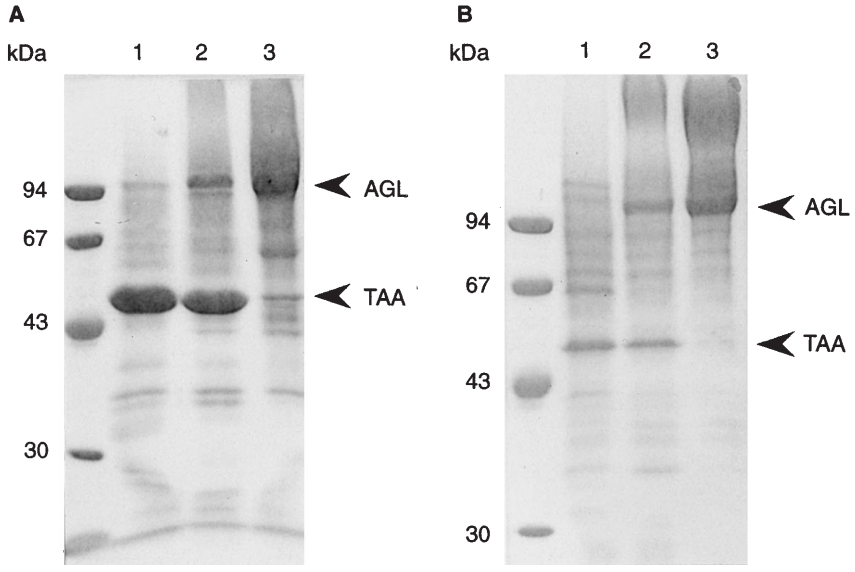
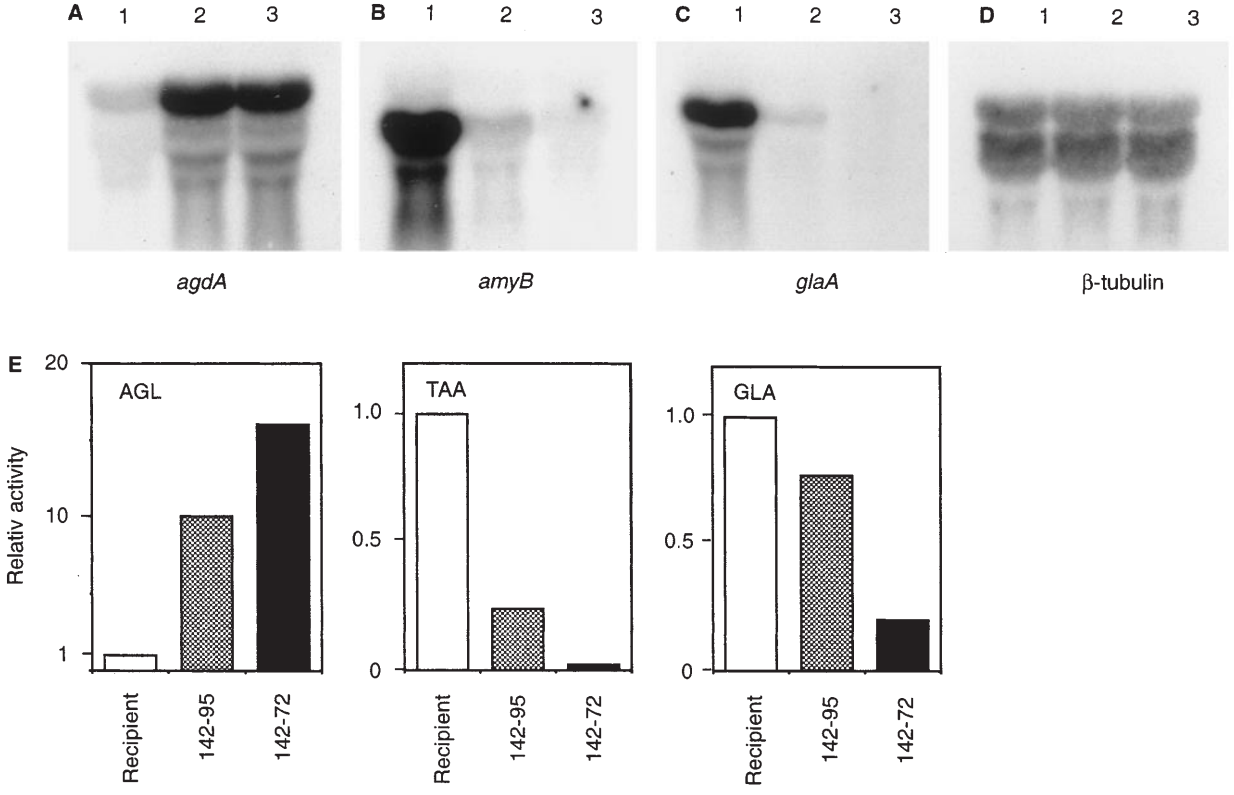


Fig. 6A, B Sodium dodecyl sulphate/polyacrylamide gel electrophoresis analysis of the wheat bran culture extract of the α-glucosidase (*AGL*)-overproducing transformants. The transformants and recipient were grown as described in the legend to Table 1. A 10-μg sample of a crude protein extract of the wheat bran cultivated with no addition (**A**) or in the presence of 0.1 M ammonium sulphate (**B**) was electrophoresed on a 10% polyacrylamide gel. *Arrows* the positions of AGL and α-amylase (*TAA*). Lanes: *1* recipient strain; *2* single-copy transformant (142–95); *3* multiple-copy transformant (142–72)

Fig. 7A–E Northern blot analysis of the AGL-overproducing transformants. Approximately 1×10^6 conidia were inoculated in 100 ml CD-P medium containing 2% glucose and cultivated at 30 °C. After 72 h, the mycelia were transferred to 100 ml DP medium . Total RNA was prepared from the mycelia after 12 h cultivation. Four transcripts, the *agdA* (**A**), *amyB* (**B**), *glaA* (**C**) and β-tubulin (**D**) genes of the recipient strain (*1*), single-copy transformant 142–95 (*2*), and the multiple-copy transformant 142-72 (*3*) were determined. The 5.0-kb *Sca*I fragment of the *agdA* gene was used as a probe. Other probes are described in Fig. 4. The amount of β-tubulin transcript was used as an internal control. The amylase activities of the transformants after 12 h cultivation were also measured (**E**). The value for the amylase activity of the recipient strain was taken as 1.0

expression of the endogenous *amyB* and *glaA* genes in the single-copy transformant carrying the P*agdA*142::*uidA* fusion gene (Fig. 4B, C). Similar titration effects have been observed on the introduction of multiple copies of the *amdS* gene into *A. nidulans* (Kelly and Hynes 1987) or P*glaA* into *A. niger* (Verdoes et al. 1994).

The insertion of region III into P*agdA* increased the promoter activity under all conditions examined, but the promoter activity in glycerol medium was lower than that in the other medium (Fig. 3B). The activity of the intrinsic promoter in glycerol medium was lower than



that in glucose medium. Moreover, similar phenomena were also observed in the expression of the *amyB* (Tada et al. 1991) and *glaA* (Hata et al. 1992) genes. These results suggest that the expression of the amylase genes in the presence of glycerol, which is different from saccharides as a carbon source, is essentially low,and its response to both carbon sources may be different.

The CCAAT sequence is a *cis*-acting element found in the promoter region of a large number of genes in high eukaryotes. Since region IIIb, comprising a CCAAT sequence, is also likely to be a general *cis*-acting element, as shown in our previous study (Minetoki et al. 1996), one of the causes of increased promoter activity under various growth condition is probably the introduction of multiple copies of the region IIIb. Region IIIa is a specific *cis*-acting element that is conserved in the promoter region of the amylase genes. This element is involved in the efficient expression and induction associated with saccharide regulation. Therefore, the insertion of region IIIa into P*agdA* might effect an increase in promoter activity in glucose medium, bringing the promoter activities obtained in glucose and in the other α-glucosyl glucose medium to a similar level. Thus the ratio of the activity in glucose medium to the activity in maltose medium also led to a significant increase in AGL-overproducing transformants. Isolation and analysis of the *trans*-activator proteins are necessary to confirm the role of region IIIa and IIIb regulation in the efficient expression of the improved promoter. Recently the nuclear protein AnCP from *Aspergillus nidulans* has been shown to bind in vitro to a CCAAT sequence in the *amyB*, *amdS* and *gatA* promoters (Nagata et al. 1993; Kato et al. 1997). One of the *trans*-acting regulatory proteins involved in region IIIb may be an *A. oryzae* AnCP-like protein (CCAAT-binding protein). However, there is nothing known about the *trans*-activator protein interacting with region IIIa.

AGL production was more efficient in wheat bran solid-state culture (up to 50-fold) than in the submerged culture (Table 2). Solid-state culture has often been used for the commercial production of enzymes from filamentous fungi. In particular, the addition of ammonium sulphate led to a further reduction in TAA production, resulting in an increase in the specific activity of AGL. Tsuchiya et al. (1994) also reported that solid-state culture with ammonium sulphate resulted in high-level production of recombinant chymosin in *A. oryzae*. The addition of ammonium sulphate is known to lower the pH of the culture and repress protease production (Cohen 1977). However, AGL production is not significantly affected by the lower pH. These results suggest that the pH regulation of the *agdA* and *amyB* genes may be different. Although the pH regulation of the acidic-protease-encoding genes, *pepA* and *pepB*, has been reported (Jarai and Buxton 1994), little is known about that of the amylase genes. Further analysis of the transcriptional level is needed to elucidate the pH regulation.

Introduction of multiple copies of a *cis*-acting sequence that interacts with the regulatory protein(s) can give valuable information about gene regulation (Davis and Hynes 1991). In this study, we have shown the presence in *A. oryzae* of a common regulatory mechanism interacting with region III in the expression of the amylase genes, *amyB*, *glaA* and *agdA*. Moreover, we have demonstrated the advantage of overexpressing genes controlled by the improved promoter using region III. We are now trying to improve other promoters by the introduction of region III, and are investigating their efficiency in the production of extracellular proteins. Furthermore, isolation of the gene(s) encoding the regulatory proteins interacting with region III is also being carried out. The study of these genes will greatly help our understanding of the common regulatory mechanism governing the amylase genes.

Acknowledgement We thank Dr. Gakuzo Tamura for valuable discussion and Dr. Elizabeth Kamei for critical reading of the manuscript.

References

Adachi T (1954) The new method of α-amylase assay of sake-koji (*Aspergillus oryzae*). J Ferment Technol 32: 448–451

Barbesgaard P, Heldt-Hansen HP, Diderichsen B (1992) On the safety of *Aspergillus oryzae*: a review. Appl Microbiol Biotechnol 36: 569–572

Cathala G, Savouret JF, Mandez B, West BL, Karin M, Martial JA, Baxter JD (1983) A method for isolation of intact, translationally active ribonucleic acid. DNA 2: 329–335

Christensen T, Woeldike H, Boel E, Mortensen SB, Hjortshoej K, Thim L, Hansen MT (1988) High-level expression of recombinant genes in *Aspergillus oryzae*. Biotechnology 6: 1419–1422

Cohen BL (1977) In: Smith JE, Pateman JA (eds) Genetic and physiology of *Aspergillus*. Academic Press, New York, pp 281–292

Davis MA, Hynes MJ (1991) Regulatory circuits in *Aspergillus nidulans*. In: Bennett, JW, Lasure LL (eds) More gene manipulations in fungi. Academic Press, New York, pp 151–193

Gomi K, Iimura Y, Hara S (1987) Integrative transformation of *Aspergillus oryzae* with a plasmid containing the *Aspergillus nidulans argB* gene. Agric Biol Chem 51: 2549–2555

Gwynne DI, Buxton FP, Gleeson MA, Davis RW (1987) Genetically engineered secretion of foreign proteins from *Aspergillus species*. In: Burgess RR (ed) Protein purification: micro to macro. A Liss, New York, pp 355–365

Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166: 557–580

Hata Y, Tsuchiya K, Kitamoto K, Gomi K, Tamura G, Hara S (1991) Nucleotide sequence and expression of glucoamylase-encoding gene (*glaA*) from *Aspergillus oryzae*. Gene 108: 145–150

Hata Y, Kitamoto K, Gomi K, Kumagai C, Tamura G (1992) Functional elements of the promoter region of the *Aspergillus oryzae glaA* gene encoding glucoamylase. Curr Genet 22: 85–91

Hondel CAMJJ van den, Punt PJ, Van Gorcom RFM (1991) Heterologous gene expression in filamentous fungi. In: Bennett JW, Lasure LL (eds) More gene manipulation in fungi. Academic Press, San Diego, calif, pp 396–428

Iwano K, Fukuda K, Shiinoki S, Shimada T (1986) The properties of various enzymes of shochu koji (*Aspergillus kawachii*). J Brew Soc Jpn 81: 490–494

Jarai G, Buxton F (1994) Nitrogen, and pH regulation of extracellular acidic proteases of *Aspergillus niger*. Curr Genet 26: 238–244

Jefferson RA, Burgess SM, Hirsh D (1986) β-Glucuronidase from *Escherichia coli* as a gene-fusion maker. Proc Natl Acad Sci USA 83: 8447–8451

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusion: β-glucuronidase as sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901–3907

Kato M, Aoyama A, Naruse F, Kobayashi T, Tsukagoshi N (1997) An *Aspergillus nidulans* nuclear protein, AnCP, involved in enhancement of Taka-amylase A gene expression, binds to the CCAAT-containing *taaG2*, *amdS*, and *gatA* promoters. Mol Gen Genet 254: 119–126

Kelly JM, Hynes MJ (1987) Multiple copies of the *amdS* gene of *Aspergillus nidulans* cause titration of *trans*-acting regulatory proteins. Curr Genet 12: 21–31

Laemmli UK (1970) Cleavaged structural proteins during assembly of the head of bacteriophage T4. Nature 227: 680–685

Minetoki T, Gomi K, Kitamoto K, Kumagai C, Tamura G (1995a) Nucleotide sequense and expression of α-glucosidase-encoding gene (*agdA*) from *Aspergillus oryzae*. Biosci Biotechnol Biochem 59: 1516–1521

Minetoki T, Gomi K, Kitamoto K, Kumagai C, Tamura G (1995b) Characteristic expression of three amylase-encoding genes, *agdA*, *amyB*, and *glaA* in *Aspergillus oryzae* transformants containing multiple copies of the *agdA* gene. Biosci Biotechnol Biochem 59: 2251–2254

Minetoki T, Nunokawa Y, Gomi K, Kitamoto K, Kumagai C, Tamura G (1996) Deletion analysis of promoter elements of the *Aspergillus oryzae agdA* gene encoding α-glucosidase. Curr Genet 30: 432–438

Nagata O, Takashima T, Tanaka M, Tsukagoshi N (1993) *Aspergillus nidulans* nuclear proteins bind to a CCAAT element and the adjacent upstream sequence in the promoter region of the starch-inducible Taka-amylase A gene. Mol Gen Genet 237: 251–260

Nakamura A, Nishimura I, Yokoyama A, Lee DG, Hideka M, Masaki H, Kimura A, Chiba S, Uozumi T (1997) Cloning and sequencing of an alpha-glucosidase gene from *Aspergillus niger* and its expression in *A. nidulans*. J Biotechnol 53: 75–84

Punt PJ, Kramer C, Kuyvenhoven A, Pouwels PH, Hondel CAMJJ vanden (1992) An upstream acting sequence from the *Aspergillus nidulans gpdA* gene. Gene 120: 67–73

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467

Tada S, Iimura Y, Gomi K, Takahashi K, Hara S, Yoshizawa K (1989) Cloning and nucleotide sequence of the genomic Taka-amylase A gene of *Aspergillus oryzae*. Agric Biol Chem 53: 593–599

Tada S, Gomi K, Kitamoto K, Takahashi K, Tamura G, Hara S (1991) Construction of a fusion gene comprising the Taka-amylase A promoter and the *Escherichia coli* β-glucuronidase gene and analysis of its expression in *Aspergillus oryzae*. Mol Gen Genet 229: 301–306

Tsuchiya K, Tada S, Gomi K, Kitamoto K, Kumagai C, Tamura G (1992) Deletion analysis of the Taka-amylase A gene promoter using a homologous transformation system in *Aspergillus oryzae*. Biosci Biotechnol Biochem 56: 1849–1853

Tsuchiya K, Nagashima T, Yamamoto Y, Gomi K, Kitamoto K, Kumagai C, Tamura G (1994) High level secretion of calf chymosin using a glucoamylase-prochymosin fusion gene in *Aspergillus oryzae*. Biosci Biotechnol Biochem 58: 895–899

Unkles SE, Campbell EI, Ruiter-Jacobs YMJT de, Broekhuijsen M, Macro JA, Carrez D, Contreras R, Hondel CAMJJ van den, Kinghorn JR (1989) The development of a homologous transformation system for *Aspergillus oryzae* based on the nitrate assimilation pathway: a convenient and general selection system for filamentous fungal transformation. Mol Gen Genet 218: 99–104

Verdoes JC, Punt PJ, Schrickx JM, van Verseveld HW, Stouthamer AH, Hondel CAMJJ van den (1993) Glucoamylase overexpression in *Aspergillus niger*: molecular genetic analysis of strain containing multiple copies of the *glaA* gene. Transgenic Res 2: 84–82

Verdoes JC, Punt PJ, Stouthamer AH, Hondel CAMJJ van den (1994) The effect of multiple copies of the upstream region on expression of the *Aspergillus niger* glucoamylase-encoding gene. Gene 145: 179–187

Vieira J, Messing J (1987) Production of single-stranded plasmid DNA. Methods Enzymol 153: 3–11

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene 33: 103–119