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Review

Development of high expression system with the improved promoter using the *cis*-acting element in *Aspergillus* species

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Aspergillus species are widely used for the production of useful enzymes. We found that there exist three highly conserved sequences, designated Region I, II and III, in the promoter regions of the genes encoding α-amylase (*amyB*), glucoamylase (*glaA*), and α-glucosidase (*agdA*) of *A. oryzae*. Deletion analyses of the promoter regions indicated that Region III is mainly involved in high level expression and maltose induction of the amylase genes. Introduction of the multiple copies of the fragment comprising Region III into the *agdA* promoter resulted in a significant increase in promoter activity at the transcriptional level. In addition, introduction of the multiple copies of Region III into No.8 promoter that was isolated from *A. niger* also resulted in a remarkable increase. Using the improved promoter, a remarkably high yield of nuclease S1 and 1,2-α-D-mannosidase, of which production levels are low in wild-type strain, could be gained. In such transformants, the production of α-amylase and glucoamylase was significantly reduced, simultaneously. These phenomena could be caused by titration of the common regulatory protein(s) which interact with Region III. In this study, we have shown the presence in *A. oryzae* of a common regulatory mechanism interacted with Region III in the expression of the amylase genes. Moreover, we have demonstrated the advantage of over-expression of genes controlled by the improved promoter using Region III in various *Aspergillus* species.

Introduction

The useful protein (enzyme) produced by gene manipulation technology is used in fields broad now, such as a medicine use and an industrial way. Thus, recombinant protein has brought modern society many benefits. Then, various protein expression systems by the microorganism, the insect and the plant host are developed in order to produce such useful protein more efficiently. The high expression system by filamentous fungi which authors developed is also one in it.

There are mainly two merits which use *Aspergillus* species as a host. In the 1st, *Aspergillus* species which essentially produces various enzymes is that the protein productivity is superior to other hosts. As the 2nd, it is estimated that *A. oryzae* and *A. niger* which are used for manufacture of Japanese fermentation food, such as *sake*, *miso*, and soy sauce, are safe also as a host of protein production[1]. Recently, the report of production of various recombinant proteins by the *Aspergillus* species host is increasing notably[2,3]. And industrial use of some enzyme has already been carried out.

When producing the target protein by genetic engineering, the production efficiency is regulated by various factors, such as promoter

activity, translation efficiency, glycosylation to the expressed protein, the copy number of a target gene, and degradation by fungal proteases. However, the most important factor in it is how to increase the transcriptional level. From this viewpoint, discovery of a strong promoter is important. Recently, also in *Aspergillus oryzae*, there is movement toward extensive acquisition of the promoter using EST (expressed sequence tag) analysis data and acquisition of the intellectual property rights about the promoter. However, since it depends for production of useful enzyme on the selected promoter activity and the expression characteristic in this case, the promoter who uses it has chosen according to the purpose of target enzyme. In this review, it introduces about the high expression system in *Aspergillus* species developed by using the improved promoter containing *cis*-element of the amylase genes[4,5].

Regulatory mechanism for the *A. oryzae* amylase genes

A. oryzae produces industrially valuable amylases, such as Taka-amylase (TAA)[6], glucoamylase (GLA)[7] and α-glucosidase (AGL)[8]. The expression of *amyB*, *glaA* and *agdA* genes encoding TAA, GLA and AGL respectively is induced by starch or maltose and repressed by glucose as a carbon source at the transcriptional level.

A comparison of the promoter regions of the *Aspergillus* amylase genes has indicated that are four highly conserved sequences, designated Region I, II, IIIa and IIIb (Fig. 1)[9]. The function of these consensus sequences has been investigated by deletion analysis in the *agdA* promoter (P*agdA*). This result suggests that Region IIIa is a functional element essential for high-level expression and maltose induction. Deletions of Region IIIb have suggested that this element contains sequences that might be involved in enhancing expression in conjunction with region IIIa. Accordingly, multiple copies of the fragment comprising with Region IIIa, b 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[10]. The improved promoter (P*agdA*142) carrying 12 tandem repeats of Region IIIa,b resulted in a significant increase in promoter activity. Moreover, the transformant carrying the *agdA* gene under the control of the improved *agdA* promoter resulted in efficient overproduction more than 100-fold AGL activities of the recipient strain.

Interestingly, expression of the *amyB* and *glaA* genes in the transformant was strongly repressed. Since this phenomena was observed at the transcriptional level, these results suggest that the fragment comprising Region IIIa,b consist of one or more *cis*-acting sequence(s). Recently, a gene, *amyR* which

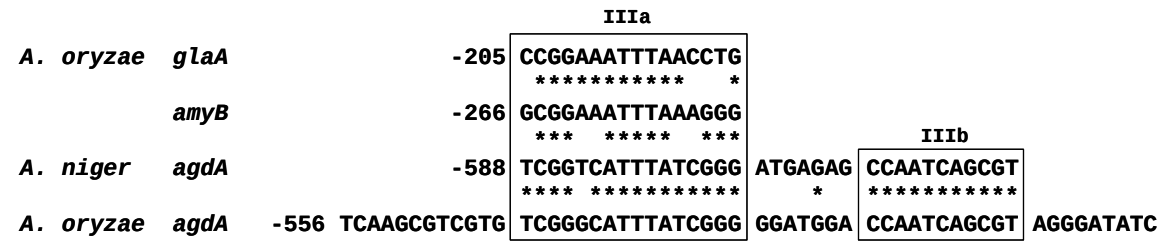

Fig. 1 The conserved nucleotide sequences, including Region III, in the upstream regions of Aspergillus amylase genes. The numbers indicate the nucleotide distance to the translation start point.

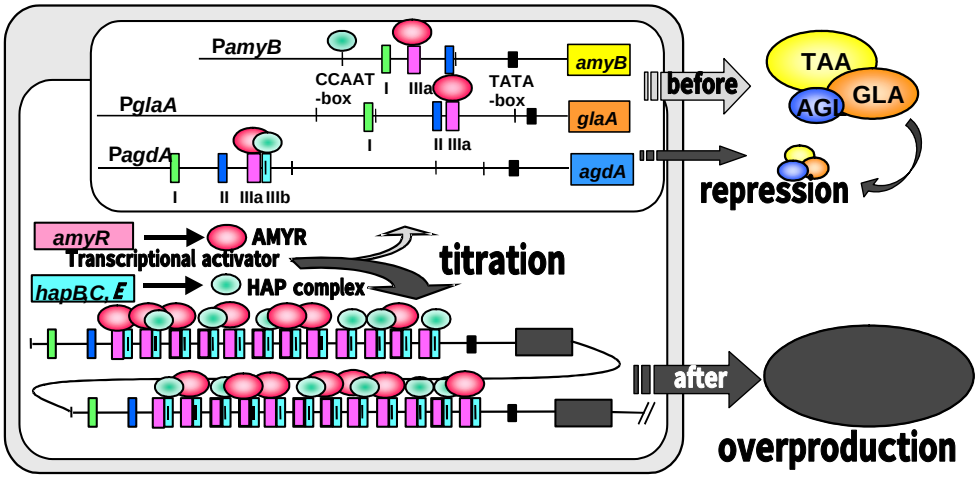

Fig. 2 Schematic diagram of titration event by the introduction of the multiple copies of Region III into the promoter

encodes a transcriptional activator of the amylase genes has been cloned from *A. oryzae*[11,12], *A. niger* and *A. nidulans*[13,14]. And the CCAAT binding protein which has an influence on the overall expression of several fungal genes has been also detected and cloned from *A. nidulans*[15~17] and *A. oryzae*[18]. Now, as shown in Fig. 2, regulatory mechanism model for the *A. oryzae* amylase genes is demonstrated. The *amyR* protein, AMYR, which involved in maltose induction and high-level expression, binds to Region IIIa. The *A. oryzae* CCAAT binding protein, AoCP, consists of the HAP complex (HAPB, HAPC and HAPE)[18], and binds to Region IIIb. In the wild-type strain, the expression of the *A. oryzae* amylase genes is enhanced by two regulatory mechanisms of Region IIIa-AMYR and Region IIIb-AoCP. However, in the AGL-overproducing transformant, the transcriptional activator such as AMYR and AoCP in interacting with Region IIIa,b are common to these amylase genes and that the titration of regulatory proteins by the introduction of multiple copies of Region IIIa,b leads to a reduction in TAA and GLA production.

Development of high expression system with the improved promoter

We demonstrated that Region III comprising two *cis*-elements is important on the regulatory mechanism for *A. oryzae* amylase genes. Furthermore, improvement of promoter activity has been exhibited by the introduction into the *agdA* promoter of multiple copies of the Region III. In this chapter, we describe about the effects of Region III insertion and utilities of the improved promoter in various *Aspergillus* species.

(1) The effects of Region III insertion

High-level expression signals such as the *amyB* and *glaA* promoters are frequently used for the over-expression of homologous and heterologous genes in a variety of filamentous fungi. If the effects of Region III insertion are observed in the other promoters, the application of Region III could extend to the efficient production of enzymes in filamentous fungi. Accordingly, multiple copies of the fragment comprising Region III were introduced into the *glaA* promoter (P*glaA*) and No. 8 promoter (P-No8)[19] that was high-level

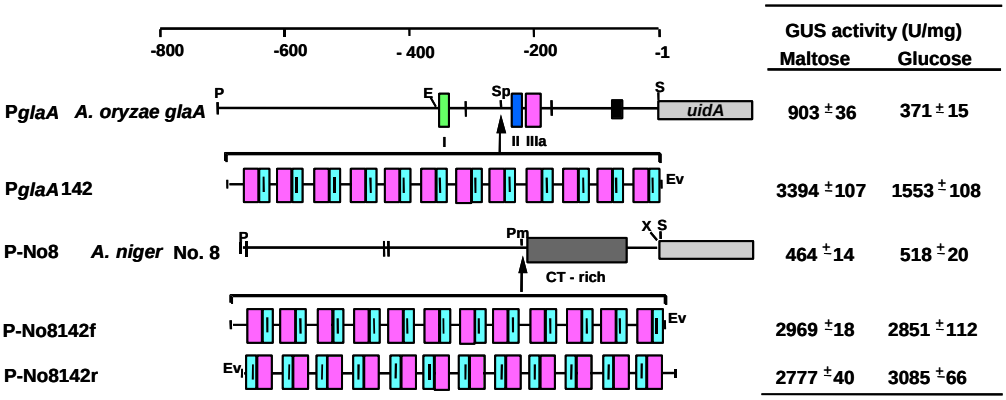

Fig. 3 Improvement (1) of promoter activity by the introduction of Region III. Two independently isolated single-copy transformants with each plasmid integrated at the *niaD* locus were grown in CD-P medium containing 2% glucose or maltose. The ß-glucuronidase (GUS) activities of cell extracts obtained from two independent experiments are presented as the average with standard errors. E, *Eco*RI; Ev, *Eco*RV; P, *Pst*I; Pm, *Pma*CI; S, *Sal*I; Sp, *Spe*I; X, *Xho*I

expression signal isolated from *A. niger*. The promoter activities of the improved *glaA* promoter (P*glaA*142) and the improved No. 8 promoter (P-No8142) increased to be 4-6 folds higher than that of the original promoter (Fig. 3). And these improved promoters resulted in 3-fold higher activities than the improved *agdA* promoter (P*agdA*142)[4, 5].

Moreover, introduction of the multiple copies of Region IIIa,b into the *enoA* promoter (P*enoA*)[20] that was one of the most strongly expressed genes in *A. oryzae* glycolytic genes also resulted in the improved promoter (P*enoA*142) more than 20-fold activities of the native promoter (Table 1)[21]. And the activity of P*enoA*142 increased to be 2.5-fold higher than that of P-No8142. This improved promoter is probably most strong in *A. oryzae.* In addition, introduction of the reverse fragment into P-No8 and P*enoA* also resulted in a significant increase.

These results suggest that Region III not only is useful to improvement of the promoter of amylase genes, but also that of various promoters from *Aspergillus* species. The insertion of a *cis*-acting sequence into the promoter region is an effective means of increasing promoter activity.

Table 1 Improvement (2) of promoter activity by the introduction of Region III.

Promoter [a]	GUS activity [b] (U/mg)
P*enoA*	719
P*enoA*142f-1(forward)	16425
P*enoA*142r-1(reverse)	13750
P-No8142f	6474

a) The fragment of 12 tandem repeats of Region III was introduced into the *sty*I site of *enoA* promoter.
b) GUS activity of transformant was measured as described in Fig. 3.

(2) Utilities of the improved promoter in various *Aspergillus* species

To confirm utilities of the improved promoter in various *Aspergillus* species other than *A. oryzae*, the activity of improved No. 8 promoter (P-No8142) was compared with the intrinsic No. 8 promoter (P-No8) in *A. niger* and *A. usamii*. The P-No8142 activity increased to be 2- to 3-fold higher than that of the P-No8 both hosts. This result suggests that the improved promoter using Region III is useful to

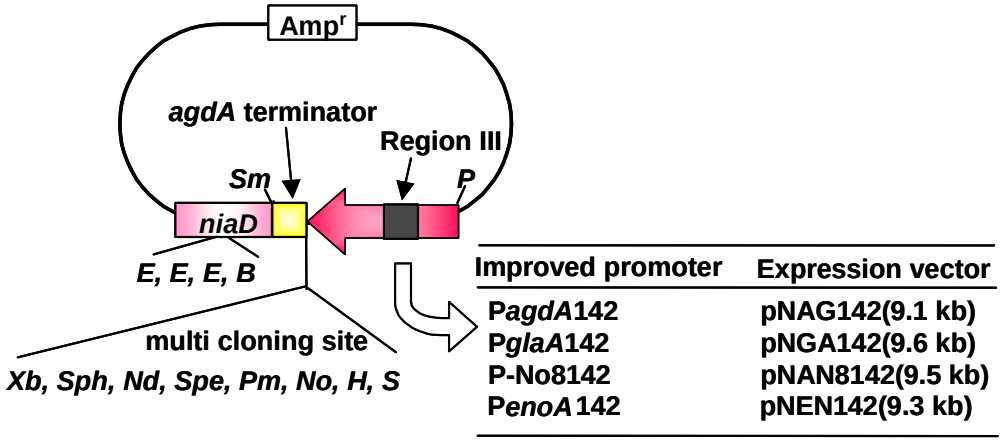

Fig. 4 Construction of the high-level expression vector comprising several improved promoter. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Nd, *Nde*I; No, *Not*I; Pm, *Pma*CI; S, *Sal*I; Spe, *Spe*I; Sph, *Sph*I; Xb, *Xba*I

over-expression of the genes in various *Aspergillus* species as a host. Moreover, the increasing activity of the improved promoter shows the presence of a common regulatory mechanism interacted with Region III in *Aspergillus* species. In fact, the *amyR* gene has been cloned from *A. niger* and *A. nidulans*.

Molecular breeding of useful enzyme overproducing transformant in *Aspergillus* species

It has constructed the expression vector that consist of the improved promoter, the *agdA* terminator, multi cloning site and the *niaD* gene[22] encoding nitrate reductase as a selectable marker (Fig. 4). We have obtained several overproducing transformants of useful enzyme by the transformation system using *Aspergillus* species *niaD* mutant as a host. In this chapter, we describe about the overproducing transformant of nuclease S1 (NUC) and 1, 2-α-D-mannosidase (MSD).

A. oryzae NUC is one of the nuclear degradation enzymes and useful enzyme as a reagent of gene manipulation. However, since the expression level of *nucS* gene[23] encoding NUC is very low in *A. oryzae* wild-type strain, using the improved *agdA* promoter (P*agdA*142), the NUC-overproducing transformants are obtained in *A. oryzae*. In these transformants, the NUC activities increased to be 1000-fold higher than that of *A. oryzae* host strain, and concomitant reduction of the TAA and GLA activities were remarkably observed. The result of SDS-PAGE analysis showed that the amount of NUC is more than 80% yield of the total secreted proteins in the submerged culture. Similar phenomena have been also observed in wheat bran solid-state culture. In addition, NUC production was more efficient in wheat bran solid-state culture (up to 20-fold) than in the submerged culture, resulting that the productivity was more than 1.0g/kg-wheat bran.

In the next place, to compare the ability of NUC production in several host, the P-No8142::*nucS* fusion gene was introduced into *A. oryzae*, *A. usamii*, *A. niger* and *A. saitoi*. Each transformant carrying similar multiple copies showed high-level and same degree production both the solid-state and the submerged culture. This results that the examined four *Aspergillus* species is excellent as a host. SDS-PAGE profile of *A. niger* transformant in the submerged culture showed in Fig. 5A.

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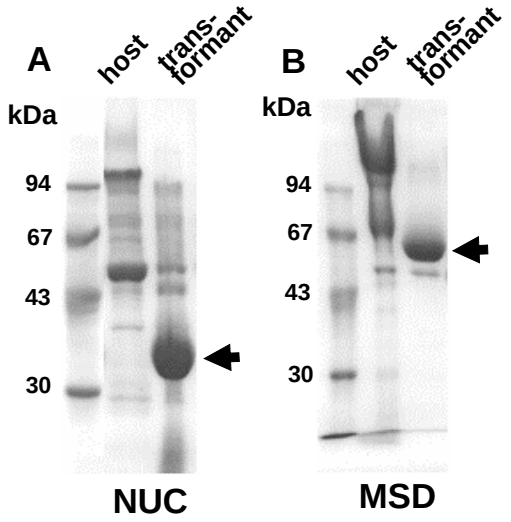

Fig. 5 High-purity overproduction profile; SDS-PAGE of the culture supernatant of the transformants. A, nuclease S1 (NUC) in *A. niger*; B, 1,2-α-D-mannosidase (MSD) in *A. saitoi*

overproducing transformant of *A. saitoi* 1, 2-α-D-mannosidase (MSD) which is intracellular enzyme and useful as a reagent of glycotechnology. The *msdS* gene encoding MSD was cloned by Ichishima et al[24]. The fusion gene which was constructed with the signal sequence of aspergillopepsin I gene, *apnS*, and the *msdS* fragment, was introduced into *A. oryzae* using the improved No. 8 promoter (P-No8142)[25]. The secreted MSD of transformant was remarkably high level (0.5-0.6g/l). We also obtained the MSD-overproducing transformants in *A. saitoi* host. Those transformant showed high-purity and overproduction to more than 2000-fold in comparison with *A. saitoi* wild-type strain (Fig. 5B).

Recently, Kawaguchi et al. have reported that the transformants of *A. aculeatus* ß-mannosidase achieve a remarkable high production (more than 2.5g/l) using the P-No8142[26]. These results demonstrate that the high expression system using the improved promoter is useful to efficiency overproduction of low level enzymes. Moreover, the reduction in the production of amylase, which is a major secretory protein in *Aspergillus* species, resulted in an increase in the specific activity of the target enzyme. Therefore, the purification of target enzyme is easy and price down of production costs is possible.

Future development

Whole genome sequence of various organism such as plant, mammalian and microorganism have been determined in the past several years so that it may be represented by human genome analysis. In *A. oryzae*, it was vigorously promoted in our country, and first, EST analysis was carried out in about two years after 1999, and the partial sequences of about 5000 genes was determined. Then, draft sequence completion of whole genomes was released in January, 2002, and it was shown in *A. oryzae* that about 13000 genes exist[27]. *A. oryzae* strains and their products have been used in food and the food processing industry and have resulted in a so-called GRAS (generally regarded as safe) status[1]. Therefore, *A. oryzae* is safe also as a gene resource and the usefulness is high.

We are continuing research of *Aspergillus* species from ten years or more before, and have succeeded in development of an effective high expression system. Moreover, we have participated also in the project of above-mentioned EST analysis and genome analysis, and can receive also about the newest gene information. That is, we have the core technology about recombinant protein production and gene information on *A. oryzae*, and consider positive business deployment using technology and information. We want to strengthen joint development between companies, and cooperation with industrial, administrative and academic sectors, and to get a business chance for that purpose, more than former. Please tell us, if some interested in our technology and business plan.

Conclusion

Now, gene manipulation technology is used not only in the overproduction of useful protein but in various fields. For example, these technologies are also used in the biomass, environmental preservation and the ecology field, and these fields will develop greatly from now on. Only one being regrettable is that a hurdle is still expensive about the application to food. This cause is not being obtained in sufficient public acceptance about safety. In order to improve this situation, industrial, administrative and academic sectors pull together, and much more honest efforts are required. We want to believe that an understanding of application of gene manipulation technology to food or fermentation industry is obtained in the near future.

In this study, we have shown the presence in *A. oryzae* of a common regulatory mechanism interacted with Region III in the expression of the amylase genes, *amyB*, *glaA*, and *agdA*. Moreover, we have demonstrated the advantage of over-expression of genes controlled by the improved promoter using Region III in various *Aspergillus* species. However, this high expression system by *Aspergillus* species is probably further improvable. For that purpose, it is necessary to analyze a series of regulatory mechanisms, such as transcription, translation, glycosylation, secretion and proteolytic degradation, in detail. We will try the development of a more efficient high expression system based on new finding in the near future. And we want to contribute to development of biotechnological industry using this expression system.

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