Deletion Analysis of the Taka-amylose A Gene Promoter Using a Homologous Transformation System in *Aspergillus oryzae*

KOZO TSUCHIYA, SETSUZO TADA, KATSUYA GOMI,† KATSUMHIKO KITAMOTO,* CHIEKO KUMAGAI,* and GAKUZO TAMURA

Research Institute of Brewing Resources Co., Ltd., 1-54-18, Takanogawa, Kita-ku, Tokyo 114, Japan

*National Research Institute of Brewing, 2-6-30, Takanogawa, Kita-ku, Tokyo 114, Japan

Received June 8, 1992

The Taka-amylose A gene (amyB) of *Aspergillus oryzae* is induced by starch or maltose. The molecular mechanism of the induction was investigated using a fusion of the amyB promoter and the *Escherichia coli* uidA gene encoding β-glucuronidase (GUS). To identify the region responsible for high-level expression and regulation within the amyB promoter, a series of deletion promoters was constructed and introduced into the *A. oryzae* met locus by homologous recombination. Deletion of the region between −377 to −290 (the number indicates the distance in base pairs from the translation initiation point (+1) to the deletion end point) significantly reduced the GUS activity, but slight reduction of the GUS activity was observed in deletions up to −377. Northern blot analysis showed that reduction of the GUS activity depended upon the expression level of the GUS gene. The region between −377 to −290 is suggested to include the sequence required directly for high-level expression and regulation of the amyB gene.

*Aspergillus oryzae* produces a copious amount of α-amylose, which is known as “Taka-amylose A (TAA)”. The production of TAA is induced by starch or maltose-oligosaccharides such as maltose, iso-maltose, and panose, but repressed by glucose.†−3 To investigate the induction mechanism of this enzyme would provide us with a model for gene regulation in *Aspergillus*. We showed recently that a 617-bp promoter sequence was sufficient to confer the TAA gene (amyB) regulation using a fusion gene of the amyB promoter and the *E. coli* uidA gene encoding β-glucuronidase (GUS). To locate functional regions responsible for high-level expression and regulation within the upsteam region of the amyB gene, plasmids consisting of a series of deletions within 617-bp of the promoter region and the GUS gene were introduced into *A. oryzae*. A sequence required for high-level expression and regulation of the amyB gene was first found from −377 to −290 (the number indicates the distance in base pairs from the translation initiation point (+1) to the deletion end point) of the amyB promoter region, however, the copy number and the integration position of the fusion gene in the transformants remain to be found.

In fungal transformation, a plasmid integrates at various sites within the genome, and integration is often associated with tandem duplication of the plasmid giving a variety of copy numbers. Such variation of the integration manner complicates the interpretation of the effects of promoter deletion on the reporter gene expression. Therefore, functional analysis of the amyB promoter would be best accomplished using a system in which the integration of a single copy of the transforming plasmid could occur by site-specific homologous recombination. In *A. oryzae*, the met gene, which was isolated from *A. oryzae* to complement a methionine-auxotrophic mutation, could be used as an integration target. A single copy of plasmid integration occurred when the plasmid containing a part of the met sequence was linearized by cutting within this sequence and was introduced into *A. oryzae*. This transformation system would make it possible to detect fine alterations of the levels of the reporter gene product and to facilitate precise analysis of the amyB promoter.

In this paper, we describe the development of the homologous transformation system for *A. oryzae* through the met locus-targeted recombination and analysis of the series of the amyB promoter deletion mutants with this system. The region required for high-level expression and regulation was identified, and its role is also discussed.

**Materials and Methods**

Strains, plasmids, and media. *A. oryzae* M-2-3 (argB⁻) was used as a recipient strain for transformation. Escherichia coli JM109 [recA1, lac-proAB, endA1, gyrA96, thi-1, hsdR17, relA1, supE44, F’traD36, proAB⁺, lacZΔM15] and the plasmid vectors pUC118 and pUC119 were used for DNA manipulation.

Dextrin-peptone (DP) medium consisting of 2% dextrin, 1% Polypepton (Wako Co., Ltd., Japan), 0.5% KH₂PO₄, and 0.05% MgSO₄·7H₂O was used as the complete medium. CD-P medium is Czapek-Dox medium consisting of 0.3% NaNO₃, 0.2% KCl, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.001% FeSO₄·7H₂O, and 2% carbon source supplemented with 1% Polypepton.

Construction of a deleted amyB promoter-GUS fusion gene. Figure 1 shows the nucleotide sequence of the 5’ upstream region of the amyB gene. The original plasmid, pMTAG1 (see Fig. 2), contains an amyB promoter-GUS fusion gene and a part of the *A. oryzae* met gene as an integration target. On the pMTAG1, the promoter region from −617-bp to −10-bp and a followed poly-linker sequence (SalI to HindIII) derived from pUC118 was ligated with a HindIII linker ligated GUS gene. A synthetic oligonucleotide linker, 5'-GAATTCCATGATGCCGATTCGATTCC-3', which includes an EcoT22I site and a BamHI site, was inserted into the EcoR1 site upstream of the amyB promoter to give pMTAG-Δ617. The resulting plasmid was digested with EcoT22I and BamHI and unidirectional deletions were introduced by the method of Henikoff. The deletion length of each plasmid was identified by nucleotide

† To whom correspondence should be addressed.
sequencing.131

Transformation experiment. The transformation of A. oryzae and E. coli were done by the method of Gomi et al.130 and Hanahan,133 respectively.

Southern blot analysis. Transformants were grown in 10 ml of DP medium at 30°C for 3 days with shaking. The mycelia were quickly frozen in liquid nitrogen and were immediately ground with a mortar and pestle. The disrupted mycelia were suspended in 3 ml of TE buffer (pH 8.0) followed by addition of 3 ml of 50 mM EDTA and 0.5% SDS. After repeated phenol extractions and ethanol precipitation, the sample was suspended in TE buffer and incubated with 0.1 mg/ml RNase A at 37°C. Then whole DNA was purified by phenol extraction and ethanol precipitation. Southern hybridization was done by the method of Sambrook et al.108

β-Glucuronidase assay. Approximately 106 conidia of each transformant were inoculated into 10 ml of CD-P medium in duplicate, incubated at 30°C for 3 days with shaking, and then were harvested. Preparation of cell-free extracts of transformants were done as described previously.44 β-Glucuronidase activity was measured by spectrophotometry,59 using p-nitrophenyl glucuronide as a substrate. One unit was defined as the amount of enzyme producing one nanomole of p-nitrophenol per minute at 37°C. The protein concentration was measured by the method of Lowry et al.,144 using bovine serum albumin as a standard.

Preparation of total RNA. Representative transformants were grown in 100 ml CD-P medium containing 2% maltose as a carbon source at 30°C for 3 days with shaking and were harvested through a glass filter. The RNA was isolated by the modified method of Cathala et al.135

Northern blot analysis. Total RNA (about 50 µg) was fractionated by formaldehyde-agarose gel electrophoresis, transferred to a Hybond-N nylon membrane (Amersham Corp. UK), and hybridized with a probe radiolabeled as described.130

Results

Scheme of the homologous integration of plasmid at the met locus

The met locus is on a 3.5-kb BamHI fragment within the genome of A. oryzae.8) The pMTAG1 contains a 2.5-kb BgII fragment of the met gene as an integration target. Figure 2 is a schematic diagram of the possible homologous recombination at the met locus when the linearized plasmid is introduced. The integration of the plasmid through homologous recombination at the resident met locus is expected to shift the size of the 3.5-kb BamHI fragment. When the single copy of the plasmid, pMTAG1, integrates at the resident met locus, the 3.5-kb BamHI fragment is expected to be replaced by 5.6-kb and 8.5-kb BamHI–EcoRI fragments in the transformant (Fig. 2c). Since the 5.6-kb fragment includes the amyl gene, it becomes

![Diagram](image-url)

Fig. 2. The Scheme of the Homologous Integration at the met Locus.

The transformation plasmid pMTAG1 (a) and its derived deletion promoter plasmids were linearized at a unique SmaI site within the part of the met sequence. Dotted line and shadow boxes indicate the sequences originated from the introduced plasmid. b, integration of the linearized plasmid at the met locus. c, single copy integration of the plasmid. d, multiple copy integration of the plasmid. Abbreviations: B, BamHI; Bg, BgII; E, EcoRI; S, SmaI.
smaller according to the deletions. When the tandem repeats of the plasmid integrate into the met locus, an additional 10.6-kb EcoRI fragment is expected to be generated (Fig. 2d).

Homologous transformation of A. oryzae with the amyB promoter deletion plasmid

pMTAG1-Δ617 and its derivative promoter deletion plasmids were linearized by digestion at a unique Smal site within the met gene and then were used for transformation of A. oryzae. The argB+ transformant was selected on CD medium. Approximately ten transformants generated with each promoter deletion plasmid were analyzed by Southern blot analysis (Table I). The transformants in which a single copy of the plasmid integrated at the met locus were obtained with the promoter deletion plasmid, pMTAG1-Δ617, pMTAG1-Δ377, and pMTAG1-Δ290 (the number after the Δ symbol indicates the length of the deletion promoter). No homologous recombinant was obtained with pMTAG1-Δ061 or pMTAG1-Δ019. Since the level of the GUS activity in the Δ061 and Δ019 deletion constructs were very low in a preliminary study,19 these deletion promoters are thought to include no functional regions. The homologous integration event occurred in 14% of transformants examined. Figure 3 shows Southern blot analysis of the representative transformants. Absence of the 3.5-kb BamHI fragment in the transformants indicates that the plasmid integrated at the met locus through homologous recombination. In the transformants Δ617-11, Δ377-1, and Δ290-5, observation of the 5.6-kb (in Δ617-11; it becomes smaller according to the deletion) and the 8.5-kb fragments indicates that a single copy of the plasmid integrated into the met locus. Since transformants Δ501-8, Δ233-6, and Δ161-3 contain an additional 10-kb fragment, these transformants are thought to harbor more than two copies of plasmids integrated at the met locus judging from the intensity. These transformants were used in the measurement of the GUS activity.

Effects of the promoter deletions on GUS activity in A. oryzae

Figure 4 shows the GUS activity of the selected transformants grown in CD-P medium containing maltose as an inducing carbon source or glucose as a non-inducing one. The level of GUS activity of the transformant Δ501-8 increased approximately two-fold compared with that of

| Table I. Integration Manner of the Plasmid in the Transformants |
|-----------------------------|-----------------------------|-----------------------------|
| Plasmid | Homologous | Non-homologous |
| | Singlea | Multi b | |
| pMTAG1-Δ617 | 1 | 0 | 11 |
| pMTAG1-Δ501 | 0 | 3 | 5 |
| pMTAG1-Δ377 | 1 | 1 | 6 |
| pMTAG1-Δ290 | 1 | 1 | 6 |
| pMTAG1-Δ233 | 0 | 3 | 7 |
| pMTAG1-Δ061 | 0 | 2 | 5 |
| pMTAG1-Δ019 | 0 | 0 | 9 |

a Numbers of the transformant in which a single copy of the plasmid integrated at the met locus.

b Numbers of the transformant in which multiple copies of the plasmid integrated at the met locus.

c Numbers of the transformant in which plasmids integrated at various sites within the genome.

Fig. 3. Southern blot analysis of A. oryzae transformants in which the plasmid integrated at the met locus.

Five microgram of whole DNAs prepared from transformants was digested with BamHI and EcoRI and were separated by 0.8% agarose gel electrophoresis. Then DNAs were transferred onto a Hybond-N nylon membrane and hybridized with the radiolabeled 3.5-kb BamHI fragment containing the met gene. Lane 1, Δ617-11; Lane 2, Δ501-8; Lane 3, Δ377-1; Lane 4, Δ290-5; Lane 5, Δ233-6; Lane 6, Δ161-3; Lane 7, Δ amyB M-2-3, a host strain.

<table>
<thead>
<tr>
<th>Strain</th>
<th>GUS activity [U/mg-protein]</th>
<th>Ratio (M/G)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltose</td>
<td>Glucose</td>
<td></td>
</tr>
<tr>
<td>Δ617-11</td>
<td>955</td>
<td>28.9</td>
</tr>
<tr>
<td>Δ501-8</td>
<td>1660</td>
<td>52.2</td>
</tr>
<tr>
<td>Δ377-1</td>
<td>700</td>
<td>17.6</td>
</tr>
<tr>
<td>Δ290-5</td>
<td>30.1</td>
<td>2.18</td>
</tr>
<tr>
<td>Δ233-6</td>
<td>5.52</td>
<td>5.19</td>
</tr>
<tr>
<td>Δ161-3</td>
<td>14.5</td>
<td>10.6</td>
</tr>
</tbody>
</table>

Fig. 4. GUS activities in the amyB promoter deletion mutants.

Numbers for the deletion mutants denote the distances in base pairs from the initiation ATG codon at +1 to the deletion endpoint. A putative TATA box is at −100-bp and two putative CAAT boxes are at −192-bp and −377-bp. Promoter region of the amyB gene is indicated by an open box and E. coli 16S rRNA coding region by a solid box. The transformants harboring deletion promoter plasmids were grown in the CD-P medium containing maltose as an inducing carbon source or glucose as a non-inducing one. The value of the GUS activity represents an average of duplicate cultures. The ratio of maltose/glucose (induction efficiency) is indicated in column M/G.

NII-Electronic Library Service
dosage effect and influence of the integration sites on the GUS level. In general, poor gene targeting methods in the transformation systems for *Aspergillus* has made it difficult to analyze a promoter function. So far, therefore, only a few genes have been subject to functional analysis of the promoter region. Fowler et al.\(^{17}\) reported the deletion analysis of the glucoamylase gene (glaA) promoter of *A. niger*. Although they estimated the copy numbers of the integrated plasmids from the results of Southern blot analysis, the level of glucoamylase activity in the transformant was not always correlated with the copy number of the plasmid. The plasmid probably integrated at various sites within the genome. On the other hand, in *A. nidulans*, functional analysis of *trpC* promoter was done with transformants in which the fusion gene of the promoter and *E. coli lacZ* gene integrated in the resident *argB* locus.\(^{18}\)

A homologous recombination system is necessary for a precise promoter analysis.

In this study we describe *met* locus-targeted homologous transformation with which the *amyB* promoter function can be analyzed in *A. oryzae*. In the Δ377 deletion construct, the level of GUS activity was reduced slightly but induction efficiency (M/G, Fig. 4) was the same as in the Δ617 deletion. Since a significant reduction of the GUS activity was observed in the Δ290 deletion construct, the region from -377 to -290 is likely to be required for high-level expression and induction of the *amyB* gene. The region from -617 to -377 may be concerned with efficient expression because of the slight reduction of the GUS level in the Δ377 deletion. The induction efficiency of the Δ290 deletion construct was not completely lost, suggesting that the Δ290 deletion contains a part of the functional sequence for the *amyB* gene regulation. In the *amyB* promoter, there are several elements reported to be involved in promoter function (see Fig. 1). Two putative CAAT elements\(^{19}\) are located at -375 and -192. A hexamer sequence, GGGCGG, which is the consensus sequence for Sp1 binding,\(^{19}\) is found at -265. Sp1 is a factor concerning the promoter function in a mammalian cell through sequence-specific binding. The region from -377 to -290 contains one of the putative CAAT elements (CAAAT) and an inverted repeat (−342), thus it is of great interest whether these elements are concerned with high-level expression and regulation.

It has been known that several enzymes are also induced with starch or malto-oligosaccharides in *A. oryzae*. The glaA gene of *A. oryzae* has been cloned\(^{20}\) and its promoter function was also analyzed using a fusion gene of the promoter and the GUS gene. By comparing the nucleotide sequences of the glaA promoter with that of the *amyB* promoter, two regions, designated Region I and Region II, were found to be highly conserved.\(^{21}\) The octamer sequence, TCACGGGC, located on the region from -307 to -300 in the *amyB* promoter was identical with the sequence from -344 to -337 in Region I of the glaA promoter. Deletion of this octamer sequence in the glaA promoter resulted in significant reduction of the expression level. This fact strongly suggests that this octamer sequence is required for high-level expression and probably serves as a binding site for transcription factor(s). In Region II, the sequence from -224 to -210 in the glaA promoter is highly conserved in the sequence from -207 to -193 of the *amyB* promoter.

Fig. 5. Transcriptional Level of the GUS Gene Expressed under the Control of the Deletion Promoter of the *amyB* Gene. About 50 μg of total RNA extracted from transformants grown in the CD-P medium containing maltose as a carbon source at 30°C for 3 d with shaking was put on a formaldehyde-agarose gel. Lane 1, Δ617-11; Lane 2, Δ501-8; Lane 3, Δ377-1; Lane 4, Δ290-5; Lane 5, Δ233-6; Lane 6, Δ617-3. a, northern blot analysis using the GUS gene as a probe; b, ethidium bromide staining of total RNA.

Transcriptional level of the GUS gene under the control of the *amyB* deletion promoter

Transformants used to measure the GUS activity were grown in CD-P medium containing 2% maltose as a carbon source at 30°C for 3 d with shaking, and RNAs were extracted from the mycelia. Northern blot analysis using the GUS gene as a probe showed that the GUS levels were regulated at the transcriptional level (Fig. 5). The amount of GUS gene-specific mRNA was correlated with the levels of GUS activity, confirming that alteration of GUS activity reflects the deletion promoter that subsequently caused the reduction of the GUS gene expression.

Discussion

We indicated previously that a 617-bp promoter sequence was sufficient to confer the *amyB* gene regulation.\(^{40}\) To identify the sequence required for high-level expression and regulation of the *amyB* gene, we constructed a series of deletions in the *amyB* promoter fused to the GUS structural gene as a reporter gene. Since the copy number and integration site of the introduced plasmid within the genome were underlined in the transformation system used in our previous study,\(^{61}\) it was impossible to eliminate the gene

---

K. Tsuchiya et al.
This sequence is included in the region required for basal transcription in the amyB gene (unpublished results). Further analyses including site-specific deletions or substitutions must be done to identify the function of these elements on the amyB promoter. Homology between the sequence of the amyB promoter and that of the glaA promoter led us to speculate about a similar mechanism concerning high-level expression and regulation in these genes. This will be tested by further investigation about the factor(s) which interact with these regions.

We developed a homologous transformation system which is applicable to analysis of the promoter function in A. oryzae. In the met locus-targeted transformation system, however, single copy integration was observed in only 4% of the transformants analyzed. Since there are three TAA genes, amyA, amyB, and amyC in A. oryzae, the amyB promoter and terminator on the pMTAG1 might work as an integration target, resulting in the low frequency of integration at the met locus. Homologous transformation was recently reported in A. oryzae using the nitrate reductase gene (niaD) as a selectable marker. This transformation system seems to be suitable for the promoter analysis because of its high frequency of single copy integration.

In transformation systems in which multiple plasmids integrate at random sites within the genome, it is impossible to compare the expression level among the transformants. This met locus-targeted transformation system made it possible to detect a fine alternation of the expression level, and provided us precise information for identification of the cis-acting elements.

Acknowledgement. We thank Mr. Y. Hata for his helpful discussion.

References
2) M. Yabuki, N. Ono, K. Hoshino, and S. Fukui, Appl. Environ.