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*Aspergillus oryzae*에서의 이종 Promoter들의 발현

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Expression of Heterologous Promoters in *Aspergillus oryzae*

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ABSTRACT

The expression of *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpd*A) and *trp*C promoters in *A. oryzae* were compared using *E. coli lac*Z gene fusions. The specific acitivities of the expressed *E. coli* β-galactosidase in *A. oryzae* transformants containing the *A. nidulans gpd*A promoter were around 2,000 units per ug of protein. The specific activities of transformants containing the *A. nidulans trp*C promoter were very low, ranging from 10.5 to 52.3 units per ug of protein. These results showed that the expression of the *A. nidulans gpd*A promoter in *A. oryzae* was approximately 70 times greater than the *A. nidulans trp*C promoter. In western blot analysis, immunoreactive bands of a similar molecular weight as the *E. coli* β-galactosidase were observed in *A. oryzae* carrying the *gpd*A-*lac*Z fusion and to a lesser intensity in those carrying the *trp*C-*lac*Z fusion. Southern analysis showed that the higher expression of the *gpd*A-*lac*Z fusion as compared to the *trp*C-*lac*Z fusion was not due a greater number of integrated plasmids.

INTRODUCTION

The *E. coli lac*Z gene is well-characterized and has been used to assay the expression of heterologous promoters fused to it. Up to the first 27 amino acids of β-galactosidase can be removed without appreciable loss of activity. The expression of the heterologous promoter and controling sequences can be monitored by assaying β-galactosidase activity (1). The *E. coli lac*Z fusion has been used to characterize the upstream activation sites essential for gene expression in several organisms including bacteria, fungi and mammalian cells (2-5). It was fused to the upstream regulatory sequence of the *A. nidulans trp*C gene, resulting in the production of an active β-galactosidase when transformed into *A. nidulans* (6). The expression of native β-galactosidase in *A. nidulans* is repressed by growth on glucose or sucrose and induced by growth on lactose and galactose (7). Therefore promoters fused to the *E. coli lac*Z can be studied in *A. nidulans* without in-

terference from endogenous β-galactosidase activity by including glucose or sucrose in the medium (6).

The *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (GPD; EC1.2.1.12) gene (*gpd*A) was isolated from an *A. nidulans* gene library using the *S. cerevisiae gpd*A gene as a prove (8). GPD plays a crucial role in glycolysis and gluconeogenesis. In glycolysis, it catalyzes the conversion of glyceraldehyde-3-phosphate into biphosphate glycerate while in gluconeogenesis, it catalyses the reverse reaction. Yelton *et al.* (9) isolated the *A. nidulans trp*C gene from a genomic DNA library by complementation of *trp*C mutation in *E. coli* (10). The *trp*C gene encodes a trifunctional polypeptide, having glutamine amido transferase (GAT), indoleglycerolphosphate synthase (IGPS) and phosphoribosylanthranilate isomerase (PRAI) activities. It formed a complex with the product of the *trp*A gene and this complex was involved in tryptophan biosynthesis (11, 12).

In this study, We compared the expression of β-galactosidase with the *A. nidulans gpd*A- and *trp* C-*lac*Z fusions in *A. oryzae*. Plasmid pAN5-41B and plasmid pAN923-21B were transformed into *A. oryzae* YTH-1. The expression of the *gpd*A and *trp*C promoters were assayed by measuring β-galactosidase activity in cell free extracts and on indicator plates containing the chromogenic substrate X-gal. The transformants were analyzed by using the Southern blot hybridization and the western blot analysis with rabbit antiserum against *E. coli* β-galactosidase.

MATERIALS AND METHODS

Microorganisms, Plasmids and Media

E. coli strains, HB101 and JM83, and *Aspergillus oryzae*, NRRL492 (wild type) and YTH-1 (*arg*B$^-$), were used in this study.

Plasmid pAN5-41B and pAN923-21B (kindly provided by Dr. Punt, TNO, Netherlands) are *lac* Z fusion vectors (4). pAN5-41B contains the promoter and part of the coding region of *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpd*A) gene fused in frame with *lac*Z gene. pAN923-21B carries the *A. nidulans trp*C promoter and part of its coding region fused to *lac*Z gene. Both plasmids contain the *A. nidulans arg*B gene as selectable marker and *A. nidulans trp*C transcriptional termination sequence. Plasmids were isolated as described by Sambrook *et al.* (13).

For *E. coli*, Luria-Bertani medium (LB) and 2YT medium were used as complex media. The composition of LB medium is 1% tryptone, 0.5% yeast extract and 1% NaCl (pH 7.5) and 2YT medium, 1.6% tryptone, 1% yeast extract and 0.5% NaCl. For *A. oryzae*, Czapek-Dox broth (30g of Bacto Saccharose, 3g of $NaNO_3$, 1g of K_2HPO_4, 0.5g of $MgSO_4 \cdot 7H_2O$, 0.5g of KCl and 0.01g of $FeSO_4 \cdot 7H_2O$) as minimal medium and YPD medium (2% peptone, 2% glucose and 1% yeast extract) as complex medium were used.

Preparation of *A. oryzae* Spore Stocks

A single colony of *A. oryzae* was inoculated into 100ml of YPD agar medium in a 500ml Erlenmeyer flask and grown at 30℃ for 7days. The spores were harvested by scrapping the sporulated mycelia in 1% triton X-100 solution. The spore solution was filtered through cheesecloth and centrifuged at 8,000 ×g for 15min. The spore pellet was washed, resuspended in 1% triton X-100 solution and stored at 4℃. The titer of the spore stock was measured by plating serial dilutions on YPD agar plates and counting the colony forming units after incubation for 2days at 30℃.

A. oryzae Transformation

The preparation of *A. oryzae* protoplasts and their transformation were performed as described by Yelton *et al.* (14). The modifications were the use of 5mM 2-mercaptoethanol in the protoplasting mixture (15) and lysing enzyme (Sigma Co.) for the preparation of protoplasts instead of Novozyme 234.

Preparation of Cell Extracts from *A. oryzae*

Approximately $10^7 \sim 10^8$ spores (conidia) were

inoculated into 100ml of minimal medium with 2 % glucose and incubated at 30℃ for 2days with aeration (150rpm). The mycelia were collected on a miracloth and washed twice with 100ml of 0.7% NaCl. The mycelia were frozen with liquid nitrogen and powdered with glass beads in mortar. The powdered mycelia were placed in a 50ml polypropylene contrifuge tube and 3ml of extraction buffer (50mM sodium phosphate buffer, pH 7.0, 1mM EDTA, 20uM PMSF) was added per gm of ground mycelia, mixed and centrifuged at 5,000×g for 15min. The supernatant was divided into 200ul aliquots and stored at −70℃. For SDS PAGE, the cell extract was dialyzed against 0.7% NaCl solution for 2hrs at 4℃. An equal volume of 20% trichloroacetic acid (TCA) was added to the dialyzed extract and placed on ice for 30min. The precipitate was collected by centrifugation at 10,000×g for 15min. The pellet was washed with 500 ul of ice-cold acetone, dried under vacuum, and resuspended 15 ul of 1X sample buffer.

Protein Assay

Protein was quantified by the Bio-Rad protein assay. A standard curve was prepared using bovine serum albumin (BSA) as a standard. A 0.1ml of sample was placed in a 10ml test tube and 5ml of the dye reagent diluted 1:4 with distilled water was added. The mixture was incubated at room temperature for 5min and the OD_{595} was measured. The concentration of protein was calculated from the standard curve obtained from the bovine serum albumin. For samples containing less than 1ug of protein, 0.8ml of sample and 0.2ml of dye reagent concentrate were mixed and incubated at room temperature for 5min. The OD_{595} was then measured and compared to a standard curve.

β-galactosidase Activity Assay

β-galactosidase activity was assayed in extracts of *A. oryzae* as described by Miller (16). A 100 ul aliquot of cell extract was diluted to 1ml with Z buffer (16.1g of $Na_2HPO_4 \cdot 7H_2O$, 5.5g of $NaH_2PO_4 \cdot H_2O$, 0.75g of KCl, 0.246g of $MgSO_4 \cdot 7H_2O$ and 2.7ml of 2-mercaptoethanol per liter; pH adjusted to 7.0) and incubated at 37℃ for 5min. To start the reaction, 0.2ml of o-nitrophenyl-galactoside (ONPG, 4mg/ml in water) was added. After sufficient yellow color developed, the reaction was stopped by addition of 0.5ml of 1M Na_2CO_3 and the reaction time was recorded. The OD_{420} was measured and the activity expressed as umole of ONP (o-nitro-phenyl) released/ug of protein/min based upon a standard curve determined by using o-nitro-phenol.

SDS PAGE and Western Blotting

Proteins were separated using denaturing PAGE as described by Laemmli (17). For the western blotting, the SDS PAGE gel after electrophoresis was soaked in three changes of transfer buffer (25mM Tris-HCl, pH 8.6, 190mM glycine, 20% methanol) for 20min. The proteins were electroblotted onto a 0.45um nitrocellulose membrane overnight at 0.1amp. The filter was blocked in NET buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 3mM EDTA, 0.25% gelatin, 0.05% Triton X-100) at room temperature for 1hr. The filter was incubated with an 1:2,000 dilution of the rabbit antiserum against β-galactosidase for 30min. The filter was washed four time with NET buffer for a total of 1hr. A 1:2,000 dilution of goat-rabbit antibody conjugated with horse radish peroxidase (HRP) was added and incubated for 2hrs. The filter was washed with 50ml of transfer buffer for 15min. The HRP conjugate was detected using chloro-1-napthol (2mg/ml) in 17% methanol, 10mM Tris-HCl, pH 7.4 and 0.02% H_2O_2. The reaction was stopped by washing the filter in water.

Southern Blot Hybridization

A. oryzae chromosmal DNA isolated by using a modification of the method as described by Davis *et al.* (18) and southern blot hybridization was performed as described by Sambrook *et al.* (13). Plasmid pILJ16(15) carrying the *A. nidulans arg* B gene was used as probe DNA which was labeled by nick translation with ^{32}P.

RESULTS

β-galactosidase Activities of Transformants

The plasmids pAN5-41B and pAN923-21B were transformed into *A. oryzae* YTH-1 selecting for complementation of the *arg*B$^-$ marker. The expressions of *gpd*A-*lac*Z and *trp*C-*lac*Z fusion in *A. oryzae* transformants were evaluated by incubating the transformants on minimal medium containing 2% glucose and 0.4% X-gal. After 3days incubation at 30℃, blue colonies were observed only in *A. oryzae* transformants carrying the *gpd*A-*lac*Z fusion but not the *trp*C-*lac*Z fusion (Table. 1).

β-galactosidase activities were assayed in cell extracts of 2-day-old transformants cultures (Table 2). The specific enzyme activities in *A. oryzae* transformants containing the *gpd*A-*lac*Z vector were around 2,000 units per ug of protein. The specific activities of transformants containing the *trp*C-*lac*Z vector were very low, ranging from 10.5 to 52.3 units per ug of protein. No activity was detected in the control *A. oryzae* YTH-1. The expression of the *A. nidulans gpd*A promoter in *A. oryzae* was approximately 70 times greater than the *A. nidulans trp*C promoter. In *A. nidulans*, the level of β-galactosidase expression from the *gpd*A promoter was approximately 10 times greater than the *trp*C promoter when only a single copy was integrated into the *arg*B locus of the genome (4). When the *gpd*A-*lac*Z fusion vector was integrated into the *gpd*A locus of genome, a similar level of β-galactosidase expression was also observed.

Table 1. Appearence of *A. oryzae* YTH-1 carrying the *gpd*A-*lac*Z or *trp*C-*lac*Z fusions on minimal medium agar plates containing X-gal. Colonies were incubated for 3 days at 30℃.

Strains	Plasmid	Promoter	Colony coloration[1]
YTH-1	–	–	white
Transformant-1	pAN5-41B	*A. nidulans gpd*A	blue
Transformant-2	pAN5-41B	*A. nidulans gpd*A	blue
Transformant-3	pAN923-21B	*A. nidulans trp*C	white
Transformant-4	pAN923-21B	*A. nidulans trp*C	white
Transformant-5	pAN923-21B	*A. nidulans trp*C	white
Transformant-6	pAN923-21B	*A. nidulans trp*C	white

[1] Scored after 3days growth on minimal medium containing 2% glucose and 0.4% X-gal.

Table 2. β-galactosidase specfic activity in cell extract of *A. oryzae* transformants carrying either the *gpd-lac*Z or *trp-lac*Z fusion. Cultures were grown in minimal medium containing 2% glucose for 2days at 30℃. Activity is measured by the hydrolysis of ONPG in Z buffer at 37℃.

Strains	Plasmid	Promoter	Specific activity[1]
YTH-1	–	–	–
Transformant-1	pAN5-41B	*A. nidulans gpd*A	2,289.8
Transformant-2	pAN5-41B	*A. nidulans gpd*A	1,954.0
Transformant-3	pAN923-21B	*A. nidulans trp*C	52.3
Transformant-4	pAN923-21B	*A. nidulans trp*C	21.3
Transformant-5	pAN923-21B	*A. nidulans trp*C	38.8
Transformant-6	pAN923-21B	*A. nidulans trp*C	10.5

[1] μmoles o-nitro-phenol released per min per mg of protein.

SDS PAGE and Western Blot Analysis of *A. oryzae* Transformants

Cell extracts from *A. oryzae* [pAN5-41B] grown for 2 days in minimal medium with 2% glucose were separated on a 12.5% SDS PAGE gel and stained with coomassie blue G-250. An intense band with a similar molecular weight to the *E. coli* β-galactosidase was observed in the cell extract (Fig. 1).

The level of β-galactosidase activity in cell extracts of *A. oryzae* transformant was compared with the amount of immunoreactive proteins observed in western blotting using rabbit antiserum against the *E. coli* β-galactosidase. Immunoreactive bands of a similar molecular weight as the *E. coli* β-galactosidase were observed in *A. oryzae* carrying the *gpd*A-*lac*Z fusion and to a lesser intensity in those carrying the *trp*C-*lac*Z fusion. No

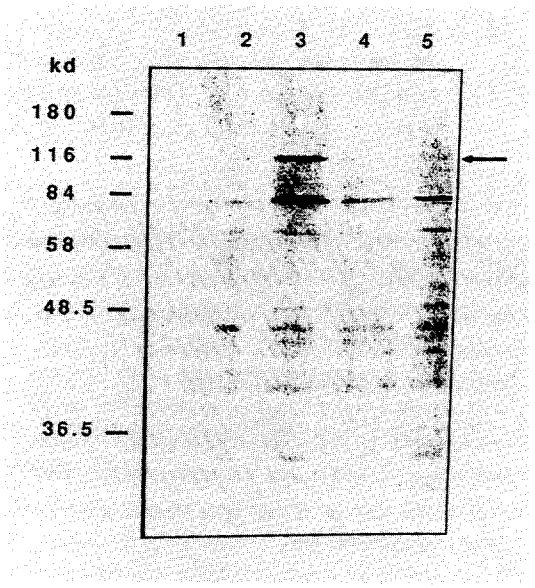

Fig. 1. SDS PAGE analysis of cell extracts from 2-day-old cultures grown in minimal media with 2% glucose at 30℃. Lane 1, purified *E. coli* β-galactosidase; lane 2, cell extract from *A. oryzae* YTH-1; lane 3, cell extract from transformant with *gpd*A-*lacZ* vector (Transformant-1); lane 4, cell extract from transformant with *trp*C-*lacZ* (Transformant-3); lane 5, cell extract from transformant with *trp*C-*lacZ* (Transformant-4). A arrow indicates the position of *E. coli* β-galactosidase. Molecular markers are shown in left.

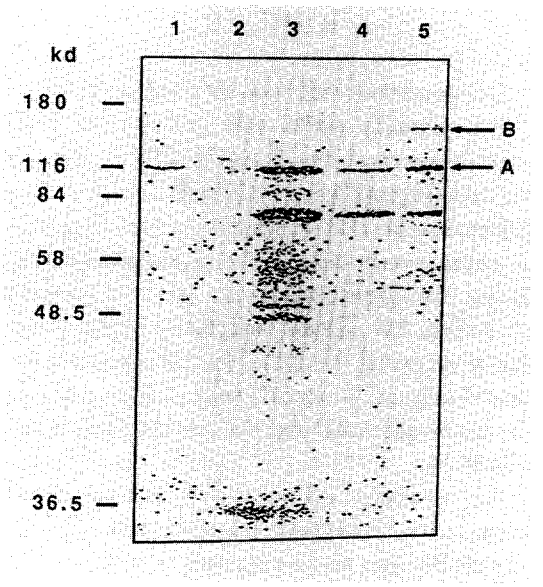

Fig. 2. Western blot analysis of cell extracts from 2-day-old cultures using rabbit antisera against *E. coli* β-galactosidase. Lane 1, purified *E. coli* β-galactosidase; lane 2, cell extract from *A. oryzae* YTH-1; lane 3, cell extract from transformant with *gpd*A-*lacZ* vector (Transformant-1); lane 4, cell extract from transformant with *trp*C-*lacZ* (Transformant-4). Arrow A indicates the position of *E. coli* β-galactosidase and arrow B, denotes an apparent *trp*C-*lacZ* fusion protein. Molecular markers are shown in left.

bands were visible in the cell extract from control *A. oryzae* YTH-1 (Fig. 2).

An immunoreactive 160kDa protein was observed in *A. oryzae* [pAN923-21B] which might be a fusion protein of *trp*C-*lac*Z fusion. Immunoreactive proteins with molecular weights less than 116kDa which are probably the degraded products were observed in the *A. oryzae* transformant carrying the *gpd*A-*lac*Z fusion. These results show that fused proteins were recognized as foreign and rapidly degraded by endogenous proteinase(s) of *A. oryzae*.

Southern Analysis of *A. oryzae* Transformed with *gpd*A- and *trp*C-*lac*Z Fusions

Undigested chromosomal DNA from *A. oryzae* YTH-1, one transformant carrying the *gpd*A-*lac* Z and two carrying the *trp*C-*lac*Z were hybridized to ^{32}P-labeled pILJ16 (Fig. 3). Bands hybridizing with the probe migrated with the bulk chromosomal DNA in the DNA isolated from transformants carrying either plasmids. This indicates that transforming plasmid DNA was integrated into host chromosomal DNA. Chromosomal DNA from these transformants and from YTH-1 were digested with *Xho*I, separated on an 0.8% agarose gel along with *Xho*I-digested pAN5-41B and pAN923-21B. The DNA fragments were transferred to a nytran filter paper and probed with ^{32}P-labeled pILJ16 (Fig. 4). The hybridizing bands found in transformant DNA migrated similarly to the corresponding linearized plasmids

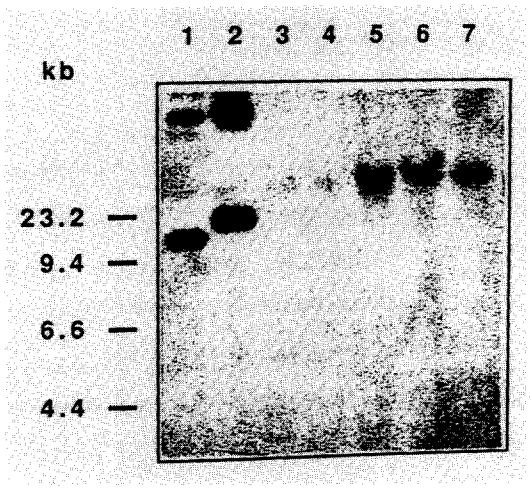

Fig. 3. Autoradiogram of Southern gel run on total DNA isolated from *A. oryzae* YTH-1 transformed with pAN5-41B and pAN923-21B and probed with ^{32}P-labeled pILJ-16 carrying *A. nidulans argB* gene. Lane 1, uncut plasmid pAN5-41B; lane 2, uncut plasmid pAN923-21B; lane 3, *A. oryzae* NRRL 492, uncut chromosomal DNA; lane 4, *A. oryzae* YTH-1, uncut chromosomal DNA; lane 5, *A. oryzae* YTH-1 transformed with pAN923-21B (Transformant-3), uncut chromosomal DNA and lane 7, *A. oryzae* YTH-1 transformed with pAN923-21B (Transformant-4), uncut chromosomal DNA.

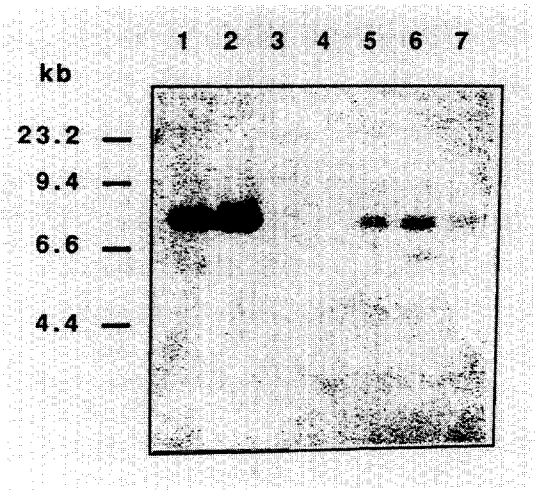

Fig. 4. Autoradiogram of Southern gel run on total DNA isolated from *A. oryzae* YTH-1 transformed with pAN5-41B and pAN923-21B and probed with ^{32}P-labeled pILJ-16 carrying *A. nidulans argB* gene. Plasmids and chromosomal DNA were digested with *Xho*I. Lane 1, plasmid pAN-41B; lane 2, plasmid pAN923-21B; lane 3, *A. oryzae* NRRL 492, chromosomal DNA; lane 4, *A. oryzae* YTH-1, chromosomal DNA; lane 5, *A. oryzae* YTH-1 transformed with pAN5-41B (Transformant-1), chromosomal DNA; lane 6, *A. oryzae* YTH-1 transformed with pAN923-21B (Transformant-3), chromosomal DNA and lane 7, *A. oryzae* YTH-1 transformed with pAN923-21B (Transformant-4), chromosomal DNA.

(Fig. 3 and 4).

The observed differences in the expression of *lacZ* fusions might be due to the copy number of the integrated plasmids or the site of integration in the genome. Although the intensity of the band in the transformant carrying *trpC-lacZ* vector (lane 6, Fig. 4) was greater than the transformant carrying the *gpdA-lacZ* (lane 5, Fig. 4), the expression of β-galactosidase in the former was much lower. The lower level of expression from *trpC-lacZ* fusion as compared to the *gpdA-lacZ* fusion in *A. oryzae* was therefore probably not due to a lower relative copy number of the integrated plasmids. Since there is a lack of genetic markers mapped in *A. oryzae*, it is difficult to ascertain whether the sequences flanking the site of insertion affects the expression of β-galactosidase. However, the poor expression of *E. coli* β-galactosidase in all transformants carrying the *trpC-lacZ* fusion in *A. nidulans* (4) relative to the *gpdA* promoter or *trpC* gene is expressed in later stage when the nutrients become deficient.

DISCUSSION

The level of *gpd* gene expression in both *S. cerevisiae* (19) and rat muscle tissue (20) is high, consisting of up to 5% of the total cellular proteins. In this study, an intense 116 kDa band was observed in a coomassie blue stained SDS-PAGE gel run on the cell extract of a *A. oryzae* transformant carrying the *gpdA-lacZ* fusion. It also re-

acted with rabbit antiserum raised against *E. coli* β-galactosidase. The relatively greater intensity of this band in the SDS PAGE, compared to other cell proteins, suggests that it is a significant proportion of the total cellular proteins.

Yelton *et al.* (9) reported that the level of *trp*C mRNA in *A. nidulans* was regulated by both the medium composition and the developmental stage of the fungus. mRNA levels were highest in cultures grown in minimal medium containing nitrate as the sole nitrogen source and were 3- to 4-fold lower in minimal medium with ammonium as the sole nitrogen source. In a medium containing yeast extract, the lowest level of expression was obtained. *A. nidulans* cultures induced to conidiate on minimal medium containing yeast extract had levels of *trp*C mRNA equivalent to those found in hyphae grown on minimal medium with nitrate as the sole nitrogen source. The developmental regulation of *trp*C mRNA might be the result of a greater requirement for tryptophan or a compound derived from tryptophan during conidiation. If the *A. nidulans trp*C gene is developmentally regulated, a lower level of β-galactosidase expression in *A. oryzae* transformants carrying the *trp*C-*lac*Z fusion can be expected in cell extracts form 2-day-old culture since they were not conidiated.

Finally, since the plasmid was integrated into the chromosome at random sites with multiple tandem copies, the expression of β-galactosidase might not truely reflect the actual promoter activity of each gene. In *A. nidulans* (4), the transforming vector carrying *A. nidulans arg*B gene integrated with a high percentage at the *arg* B locus on the chromosome. It minimized the effect of chromosomal environment for the quantitative analysis of the expression and regulation signals. Thus, the integration of a single copy of the *trp*C or *gpd*A fusions at the same site in the chromosome is required to precisely quantitate their expression in *A. oryzae*.

요 약

*Aspergillus oryzae*에서 *A. nidulans*의 glyceraldehyde-3-phosphate dehydrogenase (*gpd*A)와 *trp*C prmoter의 발현 능력을 *E. coli lac*Z gene fusion을 이용하여 비교·분석하였다. *A. oryzae* 내에서 발현된 *E. coli* β-galactosidase의 specific activity를 조사하여 본 결과, *gpd*A promoter를 가지는 transformant들에서는 2,000unit/ug of protein 정도의 activity를 보이는 반면, *trp*C promoter를 가지고 있는 transformant들에서는 10.5~52.3unit/ug of protein 정도의 activity를 보였다. 이 결과로부터 *A. oryzae* 내에서 *A. nidulans*의 *gpd*A promoter가 *trp*C promoter에 비해 70배 정도 더 강한 발현 능력을 보이고 있음을 알 수 있다. Western blot 분석에서도 *gpd*A promoter를 가지고 있는 transformant에서 더 많은 *E. coli* β-galactosidase가 발현된 것을 보여 주고 있다. 또한 southern blot 분석에서는 이러한 강한 발현이 transform된 plasmid의 copy number와 상관 없음을 보여주고 있다.

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