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Studies on gene expression and promoter analyses

for protein production in *Aspergillus oryzae*

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Contents

Abbreviations

BSA	bovine serum albumin
catA	catalase A-encoding gene
catB	catalase B-encoding gene
CBB	Coomassie Brilliant Blue
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic gel mobility shift assay
Endo H	endoglycosidase H
EST	expressed sequence tag
glaB	glucoamylase-encoding gene
GUS	β-glucuronidase
hCG	human chorionic gonadotropin
HSE	heat shock element
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
SDS	sodium dodecyl sulfate
SSC	solid-state culture
sodM	superoxide dismutase-encoding gene
TMB	3,3′,5,5′-tetramethylbenzidine
uidA	β-glucuronidase-encoding gene
VHH	heavy-chain antibody fragment
WCE	whole-cell extracts

Introduction

The filamentous fungus *Aspergillus oryzae* is used widely in traditional Japanese fermentation industries, including sake, *mirin*, *miso* and soy sauce breweries. In sake brewing, *A. oryzae* has been used for making rice *koji,* malted rice, for over 1,000 years. Therefore, *A. oryzae* is called "*koji* mold" and is honorary titled as "national fungus (*kokkin*)" (Machida *et al.* 2008). In the process of *koji*-making in sake brewing, polished and steamed rice is traditionally used as a solid-state culture medium. As the advancement of fermentation technology, the process of sake brewing has been mechanized and regulated by a computer-assisted operation in a plant, but the process of making *koji* still remains as the traditional solid-state culture on steamed rice. It is extremely important to make a high quality *koji* for brewing good quality sake, but the process of making *koji* is still dependent largely on manual craftsmanship procedures of skilled artisans called *Toji* group. A chief sake brewer or *Toji* says that the most important process in sake brewing is in the process of *koji* making, sending words as "1st *koji*, 2nd yeast mash (*moto*) and 3rd alcohol fermentation (*tsukuri*)".

A well-known "simultaneous saccharification and fermentation process" of sake brewing is quite unique. The saccharification process, a highly important step of starch hydrolysis before alcohol fermentation, depends on starch degradation enzymes such as glucoamylase and α-amylase, and it is *koji* that provides these starch degradation enzymes. Therefore, if the manufacturing process of *koji* fails, glucoamylase activity becomes so low as to inadequately degrade starch or maltooligosaccharide to glucose, thereby resulting in failure in subsequent alcohol fermentation to deteriorate the quality of the final product sake. For example, when the water content of steamed rice is too high, the manufacture of high quality *koji* is most likely to fail; this is so-called "submerged *koji* (*nure koji* or *hazeochi*)". Although the

3

phenomenon of submerged *koji* is empirically known, this problem has been resolved only by intuitions and experiences of skilled craftsmanship of sake brewers with complicated process control.

The importance of *koji* is deeply concerned with the expression of saccharifying enzymes such as glucoamylase and α-amylase expressed in a solid-state culture of *A. oryzae*. The glucoamylase-encoding gene (*glaB*) has already been cloned from *A. oryzae* (Hata *et al.* 1998). The *glaB* is expressed excessively on solid-state culture such as *koji*, but is expressed neither in submerged *koji* nor in submerged (liquid) culture (Ishida *et al.* 1998). The difference between solid-state culture and submerged culture lies mainly in water activity, nutrient diffusion and the presence of insoluble material that serves as a scaffold of mold growth (Akao *et al.* 2007). Since these culture conditions and factors significantly affect the growth of *A. oryzae* and its gene expressions, it is highly important to elucidate the mechanism of regulation of gene expression of the *glaB* in a solid-state culture as compared to that in submerged *koji* or liquid culture.

Since the whole genome sequence of *A. oryzae* was determined in 2005 (Machida *et al.*), comprehensive analyses, such as DNA microarray analysis and proteomic analysis, have become available for various culture conditions (Akao *et al.* 2007; Oda *et al.* 2006). These comprehensive analyses have identified several proteins that are produced only in a solid-state culture, but the regulation mechanism of solid-state-specific gene expression, including the *glaB*, is yet to be elucidated. If this mechanism is revealed, it should significantly benefit not only sake brewing, but also *mirin*, *miso* and soy sauce brewing that includes a *koji* making process. Since the identification of the regulation mechanism would make *koji* manufacture much easier, the analysis of regulation of individual gene of culture-condition-specific expression has become increasingly important.

Whereas other *Aspergilli*, including *A. nidulans*, *A. fumigatus*, *A. niger* and *A. awamori*, produce a high level of glucoamylase in submerged cultures, *A. oryzae* produces a limited amount of glucoamylase in submerged culture (5 U/ml) compared with that on solid-state *koji* (200 U/g) (Narahara *et al.* 1982). It seems quite difficult to know the reason why *A. oryzae* produces glucoamylase in a culture-condition-specific manner, but it was proposed recently that *A. oryzae* was the specific man-made mold that has been bred for sake brewing over 500 years so that it produces a higher level of glucoamylase only in a solid-state culture (Kitamoto 2012).

In Chapter I of this study, the author focused catalases as an essential enzyme for detoxification of reactive oxygen species. Since catalase-encoding genes would be strongly expressed and tightly regulated under aerobic conditions, these genes are suitable for gene expression analysis. First, the author cloned two distinct, but apparently catalase-encoding genes (*catA* and *catB*). The overexpression of the *catA* and *catB* indicated that both genes encoded functional catalases. In the next step, the author revealed gene expression analysis that the *catB* gene was expressed under both submerged (liquid) and solid-state culture, and was induced with hydrogen peroxide addition. In contrast, the *catA* gene was found to be expressed only in solid-state culture and was not inducible with hydrogen peroxide.

Since a gene expression is regulated directly by its promoter, the analysis of promoters is a key to understand the regulation mechanism of culture-condition-specific gene expression. Hence, in Chapter II, the author focused on the analysis of the promoters of the genes that express in a culture-condition-specific manner to determine the relationship between culture condition and promoter regulation. Promoters of three types of genes were analyzed to see how the difference in gene regulation affects the difference in gene expression depending on culture conditions: a gene of submerged-specific expression, a gene of non-specific

expression, and a gene of solid-state-specific expression.

In Section 1, the author focused on the promoter of a superoxide dismutase-encoding gene (*sodM*) as a liquid-culture-specific expression gene (Ishida *et al*. 2004). Although the *sodM* gene plays a central role in detoxification of reactive oxygen species in concert with catalase-encoding gene, the *sodM* gene is strongly expressed only in submerged or liquid culture. Deletion analysis and electrophoretic mobility shift assay (EMSA) revealed the region that contains *cis*-elements concerned with strong gene expression. Comparison with the *sodM* ortholog promoters in *Aspergilli* showed that the region was found only in *A. oryzae*. This genetic trait indicates that *A. oryzae* has specifically been bred as a safe fungus for brewing and that is totally different from other *Aspergilli* such as pathogenic *A. fumigatus*.

The catalase-encoding gene (*catB*) is expressed non-selectively depending on culure conditions. Threrefore, the promoter of *catB* was studied as a second topic of promoter analysis (Section 2). Deletion analysis and EMSA identified the region containing *cis*-elements concerned with strong gene expression. The region contains a well-known heat shock element (HSE) that responds to various stresses. Comparison with the *catB* ortholog promoters showed that the region was conserved widely in *Aspergilli*, suggesting that non-specific expression gene promoter is conserved during breeding.

The glucoamylase-encoding gene (*glaB*) and acid protease-encoding gene (*pepA*) of *A. oryzae* are very important in *koji* and are expressed only in solid-state culture (Ishida *et al*. 1998; Kitano *et al*. 2002). Since common *cis*-elements concerned with solid-state-specific gene expression was not known, the *glaB* promoter was analyzed in Section 3. Deletion analysis and EMSA successfully found a region responding to low water activity, a unique property of solid-state culture. Comparison with other *glaB* ortholog promoters showed that the region was conserved in a limited member of *Aspergilli*, *A. oryzae*, *A. clavatus* and *A.*

terreus. These results suggested that *A. oryzae* have been selected and bred intentionally for sake brewing among *Aspergilli*.

A. oryzae is an attractive mold as a host for production of valuable proteins from different origins. In Chapter III, the author describes the production of a llama variable heavy-chain antibody fragments (VHH) fused to glucoamylase in *A. oryzae*. Because the VHH consisting of a single polypeptide is more easily manipulated through protein engineering, this class of antibodies has been the subject of significant research effort, e.g., for improved thermal stability (Hagihara *et al*. 2007; Hussack *et al*. 2011). The llama VHH was previously produced and secreted in submerged culture of *A. awamori* (Joosten *et al*. 2005a, b), but at very low expression levels (7.5 to 30 mg protein/l culture medium). By searching for a leader protein fused with llama VHH for optimal production, the author successfully expressed a functional VHH with antigen binding ability in *A. oryzae* by fusing with glucoamylse. The productivity was over 1.0 g/l culture medium in 5 day.

The results shown in this study reveals an interesting genetic trait of *A. oryzae* as the uique and safe microbe that has been bred specifically over years by skilled brewers for brewing traditional Japanese fermented foods where solid-state culture is a key. The author believes that the present study lays a "solid" platform for efficient production of saccharifying enzymes in submerged or liquid-culture *koji*, a long-cherished goal of brewing industries in Japan.

Chapter I

Expression analysis of two different catalase-encoding genes
under various culture conditions

Many organisms have functionally equivalent genes that are regulated differently. In *A. oryzae*, two genes encoding glucoamylase (*glaA* and *glaB*) were identified (Hata *et al*. 1998). Both genes encode the same class of enzyme, but *glaA* is expressed only in submerged cultures, while *glaB* is expressed only in solid-state cultures (*koji*). Recently, tyrosinase-encoding genes *melO* and *melB* were also found to act in a similar manner (Ishida *et al*. 2001; Obata *et al*. 2004).

The author screened for strong promoters for the overexpression of homo- and hetero-proteins. In the screening process, the author hypothesized that the genes required for detoxification of reactive oxygen species would be constitutively expressed under strong promoters and would be induced easily by increasing oxidative stress. Oxidative stress causes damage to various organs; it was recently reported that reactive oxygen species are involved in Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (Barnham *et al*. 2004). Reactive oxygen is not only the cause of such diseases, but is also used to signaling processes such as apoptosis (Cantara *et al*. 2004), life span determination (Dröge 2003), cell differentiation (Carter *et al*. 2004; Hansberg and Aguirre 1990) and pathogen defense (Paris *et al*. 2003). Hydrogen peroxide, superoxides and hydroxyl radicals are formed inevitably during aerobic metabolism. All aerobic organisms have enzymatic and non-enzymatic detoxification systems to combat these reactive oxygen species. Catalases, superoxide dismutases and peroxidases play a central role in enzymatic detoxification (Muradian *et al*. 2002). Among the detoxification enzymes, catalases are the most important enzymes to degrade hydrogen

peroxide and are classified into three separate families: Mn-catalases, catalase-peroxidases and mono-functional catalases (Igarashi *et al*. 1996; Klotz *et al*. 1997). The mono-functional catalases are the best characterized, and they are homotetrameric and heme-containing enzymes. As catalases are found in organisms from eubacteria to eukaryotes (Fowler *et al*. 2003; Klotz *et al*. 1997; Michán *et al*. 2002; Paris *et al*. 2003; Varnado *et al*. 2004), they are essential, strongly expressed, and tightly regulated.

Here the author describes the cloning of two distinct catalase-encoding genes of *A. oryzae* and the characterization of their physiological roles in *A. oryzae* by promoter analysis.

Materials and methods

Strains, plasmids and growth conditions   The genomic DNA library was constructed from the wild-type *A. oryzae* OSI1013 (deposited with the International Patent Organism Depository [IPOD] as FERM P-16528) (Hata *et al*. 1998). The *A. oryzae* OSI1013 AON-2, a *niaD* mutant (deposited with IPOD as FERM P-17707) derived from *A. oryzae* OSI1013, was used for the gene expression and promoter experiments. Submerged cultures were incubated at 30°C for 72 h in 100 ml of modified Czapek-Dox medium (2% glucose, 0.2% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$) or in 100 ml of DPY medium (2% dextrin, 1% peptone, 0.5% Yeast extract, 0.2% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$). Solid-state cultures were incubated at 30°C for 48 h on DPY-agar (DPY medium, 1.5% agar) or on wheat bran (Sigma-Aldrich, St. Louis, MO; containing 80% $H_2O[w/v]$). The *Escherichia coli* JM109 strain (TaKaRa Bio, Otsu, Japan) was used for DNA manipulations.

The pNia2 plasmid used for the catalase gene expression experiments was generated by

inserting the nitrogen reductase (*niaD*) selectable gene marker into the pUC119 vector. The

melO promoter (-999 to -1) was amplified by polymerase chain reaction (PCR) with the upper

primer MO-1 (5′-AAAA<u>CTGCAG</u>GCATGTCATTGCAGCCAGGT-3′; the synthesized *Pst*I

site underlined), and the lower primer MO-2 (5′-<u>TATTGGCCATTGTGAACCA</u>

<u>A</u>AGTAATCAGA-3′; the underlined sequence being capable of annealing with that in the

CA-1 primer) (Ishida *et al*. 2001). The *catA* open reading frame (ORF) was amplified by PCR

with the upper primer CA-1 (5′-<u>TTGGTTCACAATGGCCAATA</u>TTGTGGCTGG-3′; the

underlined sequence being capable of annealing with that in the MO-2 primer), and the lower

primer CA-2 (5′-AAAA<u>CTCGAG</u>TTAGAATGCAACTGTCGAGG-3′; the synthesized *Xho*I

site underlined). Both PCR products were fused and subcloned into pNia2, and it was named

pMCA (*catA*). Similarly, the *melO* promoter (-999 to -1) was amplified with the upper primer

MO-1, and the lower primer MO-3 (5′-<u>GGGCGCGCATTGTGAACCAA</u>AGTAATCAGA-3′;

the underlined sequence being capable of annealing with that in the CB-1 primer). The *catB*

ORF was amplified with the upper primer CB-1 (5′-<u>TTGGTTCACAATGCGCGCCC</u>TC

TCCCTTGC-3′; the underlined sequence being capable of annealing with that in the MO-3

primer), and the lower primer CB-2 (5′-AAAA<u>GTCGAC</u>TTAATGGTCCAGCTTGAAAC-3′;

the synthesized *Sal*I site underlined). Both PCR products were fused and subcloned into

pNia2, and it was named pMCB (*catB*).

The *A. oryzae* integration vector pNGS1 used for promoter analysis was derived from

pUC119 and harbors the *E. coli uidA* gene, the *A. oryzae glaB* terminator, and *A. oryzae niaD*

as a reporter, terminator, and selectable marker, respectively (Ishida *et al*. 2000). The *catA*

promoter region (-1000 to -1) was amplified by PCR with the forward primer CAp-1

(5′-AAAA<u>CTGCAG</u>TGATGGGATTCAATAATTGA-3′; the synthesized *Pst*I site underlined),

and the reverse primer CAp-2 (5′-AAAA<u>GTCGAC</u>GATTGGGGTCTGATGGATAT-3′; the

synthesized *Sal*I site underlined). Similarly, The *catB* promoter region (-1000 to -1) was amplified with the forward primer CBp-1 (5′-AAAACTGCAGGCCAATATTCTCG AAACGTC-3′; the synthesized *Pst*I site underlined), and the reverse primer CBp-2 (5′-AAAAGTCGACCGTGTTGCTAACTGTCAAGT-3′; the synthesized *Sal*I site underlined). Each promoter fragment was individually subcloned upstream of the reporter gene using these sites, and they were named pCAPG (*catA*) and pCBPG (*catB*).

Transformation of the plasmids into AON-2 was done by the method of Gomi *et al.* (1987). Transformants were selected on plates containing sodium nitrate as the sole source of nitrogen.

Catalase assay   Catalase activity was measured by the method of Beers and Sizer (1952). One unit of catalase activity is defined as the amount of enzyme that decomposes 1 μmol of H_2O_2/min at 25°C (pH 7.0). Consumption of H_2O_2 was measured as the decrease in absorbance at 240 nm. The rate of H_2O_2 consumption was determined over the range of 10.3 mM to 9.2 mM of H_2O_2. Spectrophotometric assays were performed on a UV-2450 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan).

β-Glucuronidase (GUS) assay and determination of protein concentration   Cell-free extracts were prepared by the method of Ishida *et al*. (1998). β-Glucuronidase (GUS) activity was measured according to Jefferson *et al*. (1986). One unit of GUS is defined as the amount of enzyme that produces 1 nmol of *p*-nitrophenol/min at 37°C using a molar extinction coefficient of 14,000 at an absorbance of 430 nm. Protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin (BSA) as the standard.

<center>**Results**</center>

Cloning of two developmentally distinct catalase genes To identify genes expressed under strong and easily induced promoters, the author screened an *A. oryzae* expression sequence tag (EST) library consisting of approximately 80,000 clones. Accordingly, the author identified two catalase-encoding genes that were expressed in developmentally distinct ways. To clone the full-length genes from *A. oryzae*, the author constructed a phage genomic library from the *A. oryzae* OSI1013 strain. Approximately 15,000 plaques of the EMBL3 phage library were screened by hybridization with DNA probes made from the ESTs by PCR. Three positive clones were isolated, and sequence analysis confirmed that each EST sequence was represented by the cloned genes. According to standard nomenclature, the genes were named *catA* and *catB*. Sequence analysis of the *catA* gene revealed an ATG-initiated ORF interrupted by two putative introns that predicted a 747 amino acid polypeptide (Fig. 1-1). This polypeptide is homologous to the *A. fumigatus catA* protein (81%) and the *A. nidulans catA* protein (77%). The *catB* gene consists of an ATG-initiated ORF interrupted by five introns that predicts a putative polypeptide of 725 amino acids (Fig. 1-2). This polypeptide shares homology with the *A. fumigatus catB* protein (82%) and the *A. nidulans catB* protein (75%). However, the newly cloned *A. oryzae* catalase genes share much less homology (41%, Table 1-1).

<center>12</center>

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tgatgggattcaataattgacatagttcagcaatagttacatcggagaatttttctggtcccattactagggtcgtggctatggtagtatccgccgaggac
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ATGGCCAATATTGTGGCTGGGGGCCTCCACAAGGTTCAAGAAGCAGTGCAGGGCGCTGCTTCCAAGGATAAGAAGCTAGTTGACCTAGCACCCGACACCC
 M  A  N  I  V  A  G  G  L  H  K  V  Q  E  A  V  Q  G  A  A  S  K  D  K  K  L  V  D  L  A  P  D  T  H
ATAATGTACAGTCCAGCAAGGAGCCACTGACCACCGACCATGGTGTGCGTATCAGCGATACGGACCACTGGCTGAAGGAGGTGAATGACAACCACACCGG
  N  V  Q  S  S  K  E  P  L  T  T  D  H  G  V  R  I  S  D  T  D  H  W  L  K  E  V  N  D  N  H  T  G
TCCTATGATGCTTGAGGACCAGATTGCACGAGAGAAGgtatgattccccgaatcggtatgggtcagaaccatagattgaacgaatcgcaacccagATTCA
 P  M  M  L  E  D  Q  I  A  R  E  K                                                              I  H
TCGTTTCGATCATGAGCGCATTCCCGAGAGAGTCGTCCATGCGCGTGGCACCGCTGCATTCGGAAACTTCAAGCTCCATGAGAGCGCTGAAGATGTATCC
  R  F  D  H  E  R  I  P  E  R  V  V  H  A  R  G  T  A  A  F  G  N  F  K  L  H  E  S  A  E  D  V  S
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 Y  A  G  I  L  T  D  T  S  R  N  T  P  V  F  L  R  F  S  T  V  Q  G  S  K  G  S  A  D  T  V  R  D  V
TTCGTGGGTTTGCCGTGAAATTCTACACCGACGAAGGAAATTGGGATCTGGTTGGAAACAACATCCCCGTTTTCTTTATCCAAGATGCGATTAAGTTCCC
  R  G  F  A  V  K  F  Y  T  D  E  G  N  W  D  L  V  G  N  N  I  P  V  F  F  I  Q  D  A  I  K  F  P
GGATTTTGgtacgtacctcctcccaactatgagtccaaaactctagagctaacgagtgtagTCCATGCTGTTAAGCCCGAGCCGCACAACGAGGTACCAC
 D  F  V                                                        H  A  V  K  P  E  P  H  N  E  V  P  Q
AGGCCCAAACTGCTCACAACAACTTCTGGGACTTTGTCTATCTTCACCCGGAAGCCACCCATATGTTCATGTGGGCCATGTCTGATCGGGCCATTCCTCG
 A  Q  T  A  H  N  N  F  W  D  F  V  Y  L  H  P  E  A  T  H  M  F  M  W  A  M  S  D  R  A  I  P  R
GTCATACCGTATGATGCAGGGTTTCGGTGTCAACACATTCAGTCTCATCAACAAGGAAGGAAAGCGCCATTTTGTCAAGTTCCATTTCATCCCCCACCTG
 S  Y  R  M  M  Q  G  F  G  V  N  T  F  S  L  I  N  K  E  G  K  R  H  F  V  K  F  H  F  I  P  H  L
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 G  V  H  S  L  V  W  D  E  A  L  K  L  A  G  Q  D  P  D  F  H  R  K  D  L  M  E  A  I  D  N  G  A  Y
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 P  K  W  D  F  A  I  Q  V  I  P  E  E  K  Q  D  D  F  E  F  D  I  F  D  A  T  K  I  W  P  E  E  L
CGTGCCTCTGCGCGTGATCGGCGAACTGGAACTGAACCGCAACGTCGACGAGTTCTTCCCTCAAACCGAGCAAGTCGCCTTCTGCACCAGCCACATCGTC
 V  P  L  R  V  I  G  E  L  E  L  N  R  N  V  D  E  F  F  P  Q  T  E  Q  V  A  F  C  T  S  H  I  V
CCCGGCATTGACTTCAGTGACGACCCGCTTCTCCAGGGCCGTAACTTCTCCTACTTCGACACTCAGATCAGTCGACTGGGCATCAACTGGGAAGAAATCC
 P  G  I  D  F  S  D  D  P  L  L  Q  G  R  N  F  S  Y  F  D  T  Q  I  S  R  L  G  I  N  W  E  E  I  P
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 I  N  R  P  V  C  P  V  L  N  H  N  R  D  G  A  K  R  H  R  I  A  Q  G  T  V  T  T  W  S  N  R  S
CGAGGCCGGACCACCCGCACCAGTAGAACATGGTGGCTTCGCGTCCTACCCTGCGAAACTGAACGGTATCAAGAAGCGCGGCCTGAGCCCCAAGTTCCGC
 E  A  G  P  P  A  P  V  E  H  G  G  F  A  S  Y  P  A  K  L  N  G  I  K  K  R  G  L  S  P  K  F  R
GAGCACCACAACCAGGCTCAACTCTTCTACAACTCTCTCTCCGAGCACGAGAAGGTCCACGTCAAGAAGGCCTTCGGCTTCGAACTGGACCACTGCGACG
 E  H  H  N  Q  A  Q  L  F  Y  N  S  L  S  E  H  E  K  V  H  V  K  K  A  F  G  F  E  L  D  H  C  D  D
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 P  I  V  Y  E  R  L  A  G  H  R  L  A  E  I  D  L  T  L  A  Q  E  V  A  E  L  V  G  A  P  I  P  D
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 K  A  L  R  P  N  H  G  K  R  S  K  H  L  S  Q  T  E  F  P  G  K  Q  P  T  I  A  S  R  R  I  A  I
ATTATCGGCGACGGATACGACCCCGTCGCTTTCAATGGCCTCAAGGGCGCCATCACGGCGGTTGGAGCCTTACCGTTCGTCATTGGCACCAAGCGGTCAC
 I  I  G  D  G  Y  D  P  V  A  F  N  G  L  K  G  A  I  T  A  V  G  A  L  P  F  V  I  G  T  K  R  S  P
CTATCTACGCCGACGGTGAGGACAAATCATCTTCCAAGGGCGTGATCGCCGACCACCAGTATGACGGACAGCGTTCGACGATGTTTGACGCTACCTTCAT
  I  Y  A  D  G  E  D  K  S  S  S  K  G  V  I  A  D  H  Q  Y  D  G  Q  R  S  T  M  F  D  A  T  F  I
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 P  G  G  P  H  V  E  S  L  K  A  N  G  Q  I  R  Y  W  I  I  E  T  F  G  H  L  K  A  L  G  A  T  G
GAAGCGGCGGGCTTTCATCAAGGAAGCCCTGGGCTCCGCGCTTGATGTGAAGGTCGCTACGTCTGATAACCCCAGCCGGTTGAGTGGTATGGTGTTGTCA
 E  A  A  A  F  I  K  E  A  L  G  S  A  L  D  V  K  V  A  T  S  D  N  P  Q  P  V  E  W  Y  G  V  V  T
CGGCTGGAAAGATCCACAAACCTGAGAGCTTCAAGGAGGGTATCCAGATTGTCAAGGATGCGAAGGATTTCATTAGCACCTTCTTCTACCAGATCAGTCA
 A  G  K  I  H  K  P  E  S  F  K  E  G  I  Q  I  V  K  D  A  K  D  F  I  S  T  F  F  Y  Q  I  S  Q
GCATCGGAACTACAAGCGTGAACTGGATGGCCTCGCCTCGACAGTTGCATTCTAAatgctttcgtgattggttgaggacatggaggcttgtgttaacgca
 H  R  N  Y  K  R  E  L  D  G  L  A  S  T  V  A  F  *
aaagtggcattttagttaatgtcatccttgtaatgaattatgtctctaactgtggatggccagaatgtacgctaatatgaatcatgaaaatactctattc
taattgtgaatgtgaaagtgaaacggcgtcgaaaggtagttatcaatgttatcctgaggtatctaatatacaacatctttgatattgtaggaaagaaacg
taagaaaggatcgtacatagtggggtatcataatctggtacagcgtccaaggctcggctt
```

Fig. 1-1 The nucleotide and predicted polypeptide sequence of the *catA* gene. A potential TATA box and consensus splice sites are underlined. DDBJ/EMBL/GenBank accession no. is AB079611.

```
gccaatattctcgaaacgtctctagcatcagatcgggatgaggcccgtcttcgccgacccacgcaaggggcgatcaaacaggaaaaacctcccttgttct
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 M  R  A  L  S  L  A  S  L  I  G  I  A  S  A  A  C  P  Y  M  T  G  E  L  E  R  R  D  T  G  T  D  D  A
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  T  A  A  T  E  E  F  L  S  Q  Y  Y  M  A  D  N  D  T  F  L  T  S  D  V  G  G  P  I  E  D  Q  N  S
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                                          V  P  E  R  A  V  H  A  R  G  V  G  A  H  G  V  F  T  S  Y
CGGTGACTACTCTAACATCACTGCTGCCTCGTTCCTTGGAGCTGAGGGCAAGGAGACTCCGGTTTTCGTGCGGATTCTCGACTGTTGCGGGGTAGCCGTGGT
  G  D  Y  S  N  I  T  A  A  S  F  L  G  A  E  G  K  E  T  P  V  F  V  R  F  S  T  V  A  G  S  R  G
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  S  S  D  L  A  R  D  V  H  G  F  A  T  R  F  Y  T  D  E  G  N  F  D
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                                I  V  G  N  N  I  P  V  F  F  I  Q  D  A  I  L  F  P  D  L  I  H
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  L  W  A  M  S  G  H  G  I  P  R  S  L  R  H  V  D  G  F  G  I  H  T  F  R  F  V  T  D  N  G  D  S
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  Q  D  L  F  E  A  I  E  A  G  R  Y  P  E  W  S                                             L  G
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  K  M  T  L  N  R  N  P  R  N  Y  F  A  E  T  E  Q  V  M
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                    F  Q  P  G  H  I  V  R  G  V  D  F  T  E  D  P  L  L  Q  G  R  L  F  S  Y  L  D
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  T  Q  L  N  R  H  G  G  P  N  F  E  Q  L  P  I  N  Q  P  R  V  P  V  H  N  N  N  R  D  G  A  G
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                                                         Q  M  F  I  P  L  N  P  N  A  Y  S  P  N
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  T  L  N  K  G  S  P  K  Q  A  N  Q  T  V  G  K  G  F  F  T  A  P  G  R  E  S  T  G  R  F  T  R  A
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  N  S  N  V  K  S  S  V  V  R  N  N  V  I  I  Q  L  N  R  V  S  N  D  L  A  R  R  V  A  R  A  I  G
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  V  E  E  P  E  A  D  P  T  Y  Y  H  N  N  K  T  T  D  V  G  T  F  G  Q  K  L  K  K  L  D  G  L  K
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  V  G  F  L  A  S  V  E  T  P  A  S  I  E  A  A  S  E  L  S  K  Q  L  S  E  D  G  V  D  V  V  V  A
CGGAGCGTCTGTCCGATGGCGTTGATCAGACTTACTCCGGATCGGATGCCATCCAGTTCGATGCTGTGATCGTCGCGCCCGGGGCGCTGAGGGTCTTTTCTC
    E  R  L  S  D  G  V  D  Q  T  Y  S  G  S  D  A  I  Q  F  D  A  V  I  V  A  P  G  A  E  G  L  F  S
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  F  V  D  G  I  K  D  G  L  R  T  F  K  F  L  D  R  F  K  L  D  H  *
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gcccaatttcagcttaaaaataggttatctgataa
```

Fig. 1-2 The nucleotide and predicted polypeptide sequence of the *catB* gene. A potential TATA box and consensus splice sites are underlined. DDBJ/EMBL/GenBank accession no. is AB078864.

Table 1-1 Homologies at amino acid level of catalase-encoding genes from *Aspergilli*

	AfCatA	AnCatA	CatR	AoCatB	AfCatB	AnCatB
AoCatA	81	77	43	41	40	39
AfCatA		78	47	41	38	41
AnCatA			45	42	43	40
CatR				61	60	60
AoCatB					82	75
AfCatB						75

AoCatA and AoCatB, *catA* and *catB* from *A. oryzae*; AfCatA and AfCatB, *catA* (DDBJ/EMBL/Genbank accession no. U87630) and *catB* (no. Y07763) from *A. fumigatus*; AnCatA and AnCatB, *catA* and *catB* from *A. nidulans* (Kawasaki *et al*. 1997); CatR, *catR* from *A. niger* (Fowler *et al*. 2003).

Overexpression of the *catA* and *catB* genes under the *melO* promoter   To determine whether the *catA* and *catB* genes encode functional catalases, the author overexpressed each one in DPY medium. The coding regions of *catA* and *catB* were fused downstream of the *melO* promoter and were inserted into the pNia2 plasmid. The *melO* promoter was chosen because it is one of the strongest promoters available (Ishida *et al*. 2001). Both constructs, pMCA (*catA*) and pMCB (*catB*) (Fig. 1-3) were introduced into the host AON-2 strain, and transformants harboring single copies of the fused genes were analyzed. Figure 1-4 shows that the catalase activities of the overexpressed transformants were approximately 2-fold higher than the control transformant. These results demonstrate that the *catA* and *catB* genes encode functional catalases. In Czapek-Dox medium, the similar results were observed.
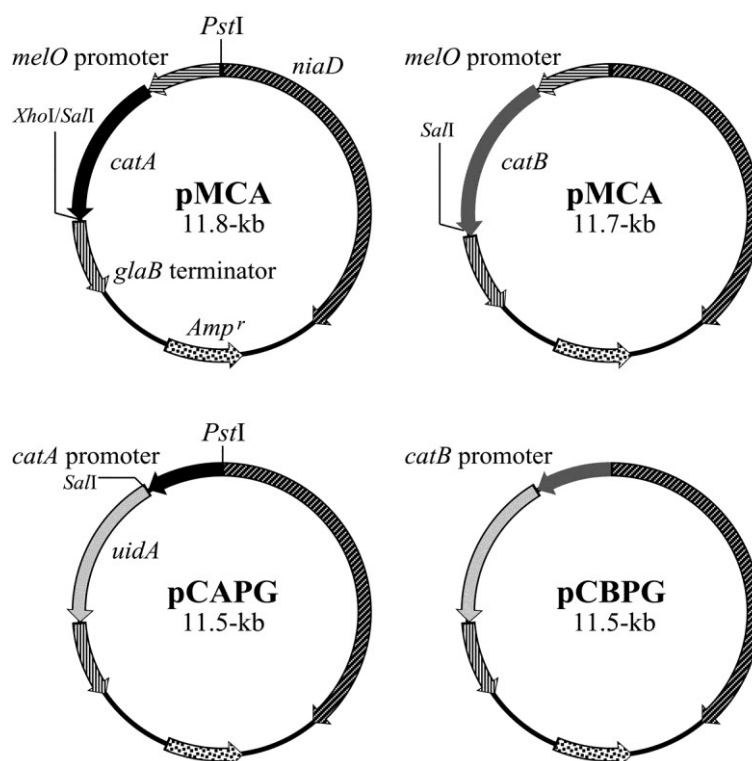
Fig. 1-3 Plasmids used in Chapter I. The constructed plasmids for overexpression of the *catA* and *catB* gene were pMCA and pMCB, respectively. The plasmids used for promoter analysis of the *catA* and *catB* promoters were pCAPG and pCBPG, respectively.
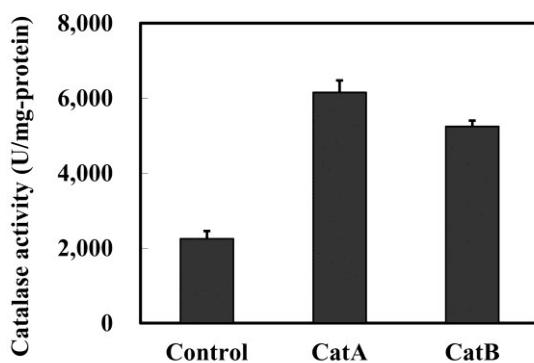
Fig. 1-4 Catalase activities of the overexpressed transformants. The AON-2 was individually transformed with constructed plasmids. Control, transformant with pNia2; CatA, transformant with pMCA (*catA*); CatB, transformant with pMCB (*catB*). Each transformant was cultured in 100 ml DPY medium at 30°C for 8 d. Catalase activities were determined with cell-free extracts (Beers and Sizer 1952; Ishida *et al*. 1998).

Expression analysis of the *catA* and *catB* promoters by GUS reporter assay   To study the regulation of the two catalase genes, the author constructed pNGS1-based plasmids for the β-glucuronidase (GUS) reporter assay that contained the *catA* gene promoter (pCAPG) or the *catB* gene promoter (pCBPG) fused upstream of the β-glucuronidase-encoding gene (*uidA*). Both plasmids were introduced into AON-2, and transformants were cultivated under various conditions. GUS activity from the extracts of transformants was measured to analyze the regulation of the two catalases (Table 1-2). The *catB* gene was expressed under all culture conditions, but was highly induced in submerged (liquid) cultures containing DPY medium and Czapek-Dox medium. The *catA* gene was expressed only in wheat bran (solid-state) cultures in which many spores were formed.

Table 1-2   GUS activity under various culture conditions

Culture	*catA* promoter[a]	*catB* promoter[a]	Sporulation
DPY	295±15	25,400±903	−
Czapek-Dox	306±17	12,391±131	−
DPY-agar	500±21	8,479±321	+
Wheat Bran	2,532±21	2,154±189	+++

[a] GUS activity (U/mg-protein)

The effect of hydrogen peroxide on *catA* and *catB* gene expression   In an *in vivo* study examining the gene expression of two *Saccharomyces cerevisiae* catalases, it was demonstrated that one catalase gene was induced upon hydrogen peroxide exposure (Wieser *et al*. 1991). To determine the effect of hydrogen peroxide on *A. oryzae* catalases, the author examined gene expression under two experimental conditions. Hydrogen peroxide was added either to a Czapek-Dox submerged culture or to a wheat bran solid-state culture (Fig. 1-5). Under both culture conditions, hydrogen peroxide induced the *catB* gene but not the *catA* gene.
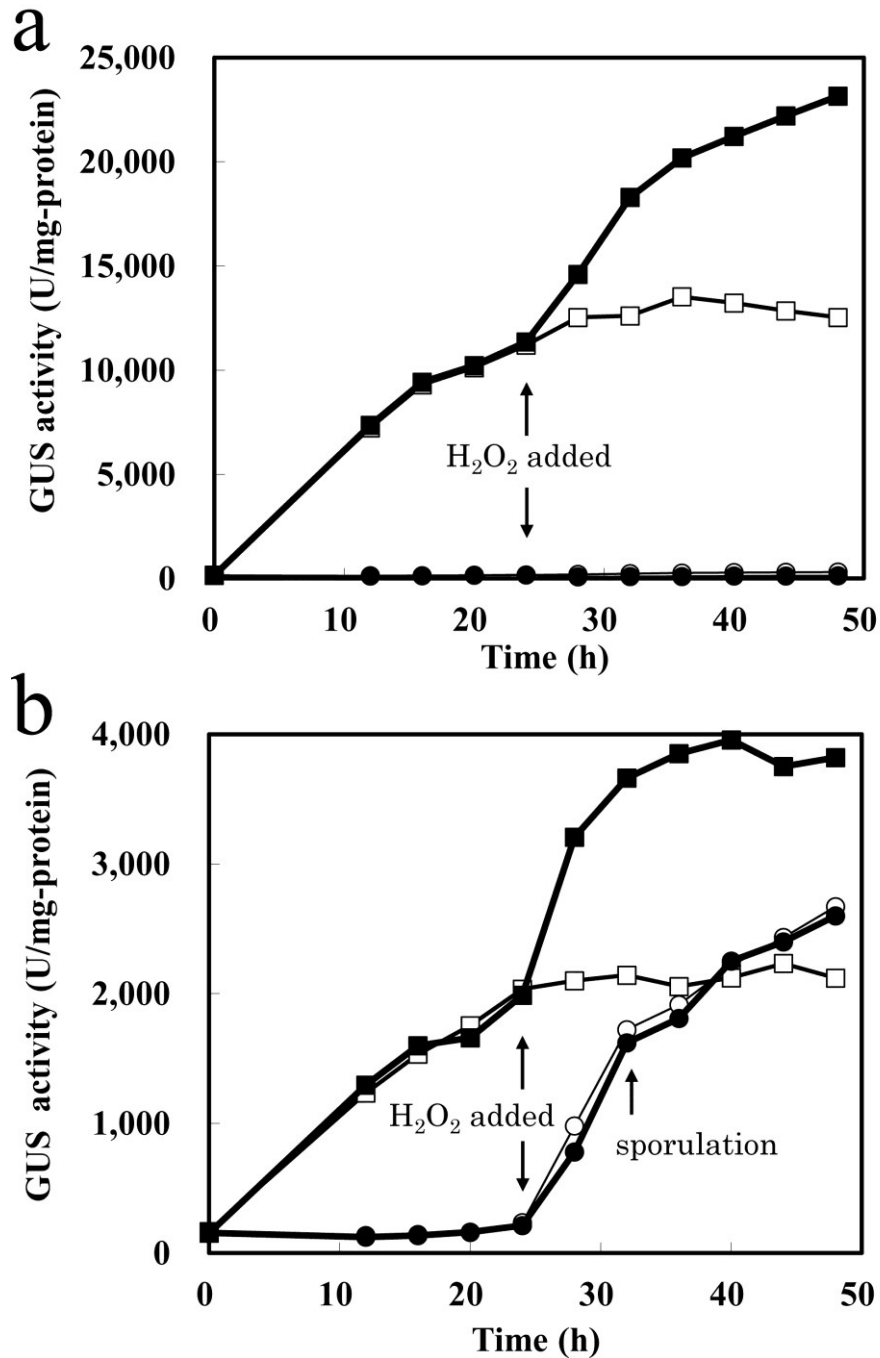
Fig. 1-5 Time-course of *catA* and *catB* expression. GUS activity was measured from cultures grown in Czapek-Dox medium (**a**) and on wheat bran solid-state medium (**b**). H_2O_2 was added to the cultures (final concentration 0.1% v/v in Czapek-Dox medium, or 0.1% v/w on wheat bran solid-state medium mixed well with vigorously shaking) 24 h after the initial inoculation. Symbols: open circles, *catA* promoter; closed circles, *catA* promoter plus H_2O_2; open squares, *catB* promoter; closed squares, *catB* promoter plus H_2O_2.

Discussion

Here the author has cloned and characterized the *A. oryzae catA* and *catB* genes and shown that they encode different catalases. Based on sequence similarity, it is likely that *A. oryzae catA*, *A. fumigatus catA* and *A. nidulans catA* compose one family of genes, while *A. oryzae catB*, *A. fumigatus catB* and *A. nidulans catB* compose another (Table 1-1). The *catA* and *catB* genes show little similarity to one another (41%), further suggesting that they do not belong to the same gene family. Both gene families are conserved in other fungi. For example, the *Neurospora crassa cat1* gene shares 63% homology with the *catA* gene, and its *cat3* gene shares 61% homology with the *catB* gene (Michán *et al*. 2002).

Overexpression of the *catA* and *catB* genes under the strong *melO* promoter produced 2-fold higher activity than the control confirming that both genes encode a functional catalase. Because equal levels of activity were observed with equal amounts of protein from cell-free extracts, these experiments also suggest that there is no difference in *catA* and *catB* catalytic activities.

To elucidate the regulation of the *catA* and *catB* genes, the author employed the promoter-GUS gene fusion system. The transcriptional efficiency of the *catA* and *catB* promoters was measured under various culture conditions. These experiments revealed much about the regulatory mechanisms of both the *catA* and *catB* genes and allowed insight into their roles *in vivo*. The *catB* gene was constitutively expressed under all culture conditions and could be induced with hydrogen peroxide. In comparison to the *catB* promoter, the *melO* promoter was strong in submerged culture, but its overexpression needed more time (7-10 d) (Ishida *et al*. 2001). The *glaA* promoter was only expressed in submerged cultures, on the other hand the *glaB* promoter was specifically expressed on solid-state cultures. Furthermore,

polysaccharides were necessary for expression (Hata *et al*. 1998; Ishida *et al*. 1998). Thus, the *catB* promoter will be very useful for homo- or heterologous protein expression. On the other hand, the *catA* gene was expressed only in solid-cultures undergoing sporulation and was not induced with hydrogen peroxide. These developmental differences have also been noted in other fungi (Kawasaki *et al*. 1997; Navarro *et al*. 1996). The author hypothesizes that the main function of *catB* is to reduce the toxic effects of oxidative stress and that the *catA* gene product is required during the germination stage. Intriguingly, disruption of the *catB* gene but not the *catA* gene in *A. nidulans* rendered spores sensitive to hydrogen peroxide (Kawasaki *et al*. 1997). This piece of evidence suggests that the *catA* gene is required for a role other than detoxification of the spore.

Both catalase genes subcloned in Chapter I belong to large-subunit catalase families (Klotz *et al*. 1997). A motif search of the proteins (GenomeNet, http://www.genome.jp/) showed that *catB* contains a catalytic domain (44-434 amino acid residues) and a catalase-related domain (514-567 amino acid residues). However, *catA* not only contains a catalytic domain (81-469 amino acid residues) and a catalase-related domain (475-529 amino acid residues), but also harbors a DJ-1/PfpI domain (598-745 amino acid residues). The DJ-1/PfpI domain is found in members of the DJ-1/ThiJ/PfpI superfamily, which have both chaperone and protease activities (Bandyopadhyay and Cookson 2004; Wilson *et al*. 2004). This suggests that the function of the *catA* gene is required for proper folding and degradation of proteins in the spore.

Chapter II

Promoter analysis of culture-condition-specific expression genes and comparison of homologous promoters in *Aspergilli*

Section 1

Analysis of superoxide dismutase-encoding gene (*sodM*) promoter

driving strong expression only in submerged cultures

A. oryzae is one of the most important fungi used in the fermentation of traditional Japanese foods such as sake, *miso* and soy sauce. Because *A. oryzae* produces a variety of useful enzymes without concurrent production of harmful secondary metabolites such as aflatoxins, it is considered to be an excellent host for the production of homologous and heterologous proteins (Barbesgaard *et al*. 1992). Economical production of such proteins in *A. oryzae* requires a better understanding of how expression of each gene is regulated.

Previous promoter analysis in *A. oryzae* focused on two gene families. One comprises the saccharolytic enzyme encoding-genes such as *amyB* (Tsuchiya *et al*. 1992; Tsukagoshi *et al*. 1989), *glaA* (Hata *et al*. 1992), *agdA* (Minetoki *et al*. 1996), and *xynF1* (Kitamoto *et al*. 1998). Regulatory regions of these promoters such as Region III (Minetoki *et al*. 1998) and the GGCTAAA-box (Van Peij *et al*. 1998), and regulatory factors such as AmyR (Gomi *et al*. 2000) and AoXlnR (Marui *et al*. 2002) have been described. The other family comprises genes that encode glycolytic enzymes such as *gpdA*, *pgkA*, and *enoA* (Nakajima *et al*. 2000). The consensus regulatory region of these glycolytic promoters has been analyzed using an electrophoretic gel mobility shift assay (EMSA) (Toda *et al*. 2001).

The author previously reported that genes required for detoxification of active oxygen in

A. oryzae were highly expressed. The manganese superoxide dismutase (*sodM*) (DDBJ/EMBL/GenBank accession no. AB078724) promoter is one of the strongest promoters in *A. oryzae*, and has been exploited for protein production (Ishida *et al*. 2004). The *sodM* gene was highly expressed in submerged cultures but less expressed in solid-state cultures. Thus, the present promoter analysis of the *sodM* gene was undertaken to learn the regulatory basis for its high-level expression and to allow rational construction of an even stronger promoter. Although *Aspergillus fumigatus* manganese superoxide dismutase is known to be one of the factors responsible for aspergillosis (Latge 1999), *A. oryzae* is not a causative agent, in spite of having the *sodM* gene. Therefore, the *sodM* promoter from *A. fumigatus* and from *A. oryzae* was compared to determine if sequence divergence could account for this difference in pathogenicity.

Materials and methods

Strains, growth conditions, expression plasmid, and primers   The *A. oryzae* OSI1013 AON-2 was used throughout the transformation experiments for GUS reporter assay. GPY medium (2% glucose, 1% peptone, 0.5% yeast extract, 0.2% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.002% $FeSO_4 \cdot 7H_2O$) was used to grow the *A. oryzae* OSI1013 AON-2 and transformants. *E. coli* JM109 was used for DNA manipulations. The *A. oryzae* integration vector pNGS1 was used for promoter deletion analysis. Primers are listed in Table 2-1.

Transformation experiments   *A. oryzae* AON-2 mycelia were grown in GPY at 30°C for 24 h and transformed as described (Gomi *et al*. 1987). Transformants were selected on plates containing sodium nitrate as sole nitrogen source.

Construction of *sodM* promoter deletion mutants   DNA fragments containing the *sodM* promoter region of various lengths were amplified by PCR using *A. oryzae* genomic DNA and the appropriate primers listed in Table 2-1. To generate the *Pst*I and *Sal*I sites at both ends of all PCR products, additional sequences, (5′-AAAACTGCAG-3′) and (5′-ACGCGTCGAC-3′), respectively, were added to the 5′-ends of each upstream and the common downstream (CDS) primer was shown in Table 2-1. The CDS primer contained the antisense nucleotide sequence between -20 to -1 of the *sodM* promoter. As an example of how the deleted promoters were constructed, the mutant promoter with a site-specific deletion of 30-bp between -209 to -178 was prepared by overlap extension PCR method (Higuchi 1989). The upper fragment (-1,038 to -209) was amplified by PCR using the Del. 1 and Del. 9-u primers, and the lower fragment (-178 to -1) was amplified using the Del. 9-d and CDS primers. To obtain the final site-specific deletion promoter, the two PCRs were combined and subjected to a second PCR using Del. 1 and CDS as primers. All synthesized promoters were digested with *Pst*I and *Sal*I, and individually subcloned upstream of the *uidA* gene using these same sites in pNGS1.

Construction of enhanced *sodM* promoters   A partial *sodM* promoter region (-209 to -1) was amplified by PCR using TD-u and CDS primers, and was ligated to pNGS1 to yield pTD1. The region from -209 to -178 of the *sodM* promoter was then amplified by PCR using TD-u and TD-d primers and was then digested with *Pst*I and *Sal*I, and subcloned into the *Pst*I and *Xho*I sites of pTD1. This method was repeated multiple times to construct pTD5 and pTD9, containing five and nine copies of this region, respectively.

Table 2-1　PCR primers

Primer	Sequence　(5′ to 3′)
Del. 1	AAAACTGCAGTTATGTACTCCGTACTCGGT
Del. 2	AAAACTGCAGTTCTTGAACTATATCTTCTT
Del. 3	AAAACTGCAGATATCAGAGTTCTACAGAGT
Del. 4	AAAACTGCAGAGGCTGTAGTAGGCGGCCTT
Del. 5	AAAACTGCAGTCCCGCCAACATGATTGGTA
Del. 6	AAAACTGCAGTATATTCTCCCTCGCCATAA
Del. 7	AAAACTGCAGAGTCACATCCCGCCAACATG
Del. 8	AAAACTGCAGATGAAGGTACCTTGGGGGTC
Del. 9-u	GTACCTTCATATGAGCGAGTTTGAGGGGAT
Del. 9-d	ACTCGCTCATATGAAGGTACCTTGGGGGTC
TD-u	AAAACTGCAGCTCGAGAGTCACATCCCGCCAACATG
TD-d	ACGCGTCGACTTTTACCAATCATGTTGGCG
CDS	ACGCGTCGACTTTGGGTGGTTTGGTTGGTA

GUS assay　*A. oryzae* transformants were cultured in 100 ml GPY at 30°C for 72 h. Cell-free extracts were prepared by the method of Ishida *et al*. (1998). GUS activity was measured as described (Jefferson *et al*. 1986). Protein was determined (Bradford 1976) using a commercial kit (Bio-Rad Laboratories) with BSA as a standard.

Southern blot analysis　Genomic DNA from *A. oryzae* transformants prepared according to Tsuchiya *et al*. (1992) was digested with *Sal*I before Southern blot analysis. The entire 1.8-kbp *uidA* gene fragment was used as a probe. The DIG-DNA labeling and detection kit (Roche, Basel, Switzerland) was used for signal detection.

Preparation of whole-cell extracts (WCE) and EMSA   Whole-cell extracts (WCE) were prepared from *A. oryzae* OSI1013 mycelia that were cultured in 100 ml GPY at 30°C for 24 h (Machida and Jigami 1994). Oligonucleotides and those labeled with IRDye800 were chemically synthesized and annealed to prepare double-stranded probes. The coding region of the *A. oryzae pgmA* gene (+191 to +240) (DDBJ/EMBL/GenBank accession no. AB032275) was used as a non-specific competitor. DNA fragments that had the same sequences as the probes were used as specific competitors. WCE and probes were incubated for 20 min at room temperature as described by Toda *et al*. (2001), and were then subjected to EMSA performed using a LI-COR 4200L DNA sequencer (LI-COR, Lincoln, NE) (Sano *et al*. 2001).

Results

Deletion analysis of the *sodM* promoter   Plasmids for deletion analysis were constructed by insertion of truncated *sodM* promoter fragments into pNGS1 for fusion with the *uidA* gene. A series of sequential deletions at the 5′-end of the 1,038-bp *sodM* promoter was constructed (Fig. 2-1). These plasmids were introduced into the AON-2 strain, and transformants were selected on the basis of nitrate utilization as sole nitrogen source. Transformants harboring a single copy of the *uidA* gene were confirmed by genomic Southern blot analysis. Functional analysis of the *sodM* promoter was performed on submerged cultures by measuring GUS activity. Deletions, spanning the region from -1,038 to -200 (Del. 1-5 in Fig. 2-1), were found not to affect GUS activity. In contrast, a deletion from -1,038 to -138 (Del. 6), resulting in loss of the sequence upstream of the TATA-box resulted in almost complete loss of activity.
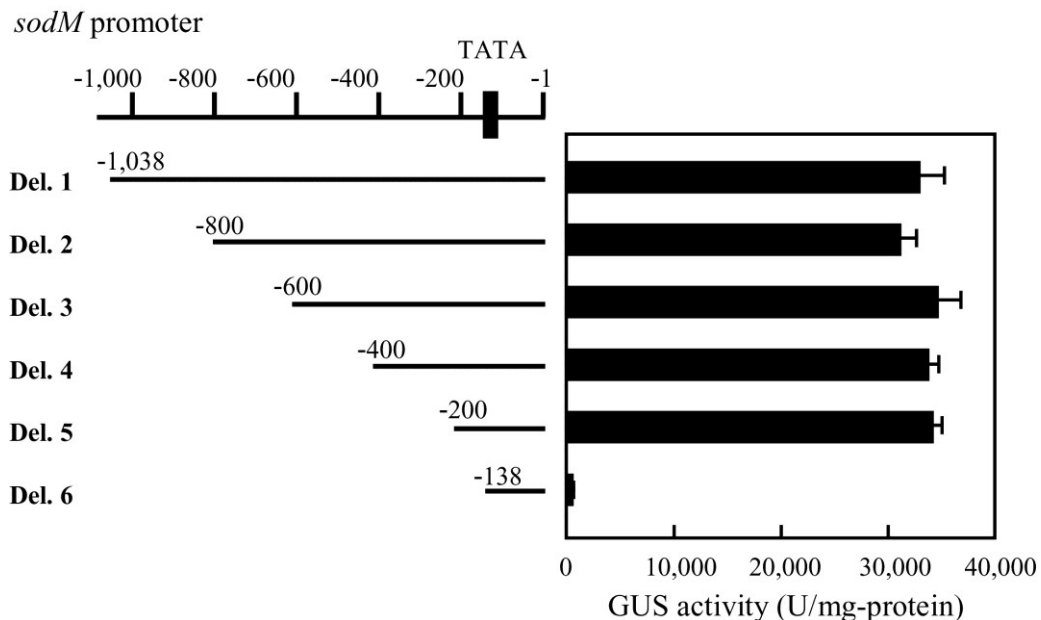
Fig. 2-1   GUS activity of the *sodM* promoters. The numbers noted in each mutant denote the distance from the start codon ATG. GUS activities are the means (filled bars) and standard deviations (error bars) for cell-free extracts from three independent transformants.

26

Determination of protein-binding regions   EMSA was employed to detect specific binding of intracellular factors to the regulatory regions identified by the deletion analysis. The probe for EMSA was designed to include a part of the *sodM* promoter sequence (-209 to -138). The competitors were synthesized so as to contain an entire (C0, -209 to -138) or a part (C1, -169 to -138; C2, -189 to -158; C3, -209 to -178) of the promoter sequence. A strongly shifted band (arrow adjacent to lane 2, Fig. 2-2) was detected in the sample incubated with a WCE. The shifted band was detected in the presence of the non-specific competitor, but disappeared upon the addition of the specific competitor (C0). While the short competitor, C3, inhibited formation of the shifted band as did C0, the competitors C1 and C2 did not. These results clearly indicate that the 30-bp region from -209 to -178 is the target of the sequence-specific binding factor(s) present in the WCE.
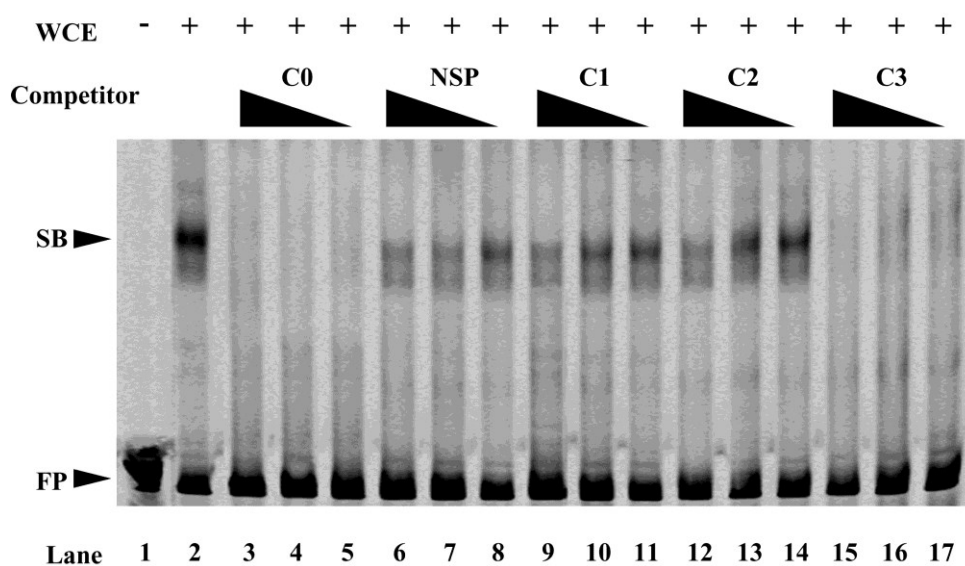
Fig. 2-2   EMSA of the *sodM* promoter. WCE (5 μg) and 200 fmol of DNA probe derived from the *sodM* promoter region (-209 to -178) were mixed and incubated. The specific competitor (C0), partial competitor 1 (C1), partial competitor 2 (C2), and partial competitor 3 (C3) were derived from regions -209 to -178, -169 to -138, and -189 to -158 of the *sodM* promoter, respectively. NS indicates non-specific competitor. Either 10 pmol (lanes 3, 6, 9, 12, and 15), 5 pmol (lanes 4, 7, 10, 13, and 16), or 1 pmol (lanes 5, 8, 11, 14, and 17) of each competitor were added. SB and FP indicate the shifted band and the free probe, respectively.

Detailed promoter deletion analysis of *sodM* promoter   Detailed deletion analysis was undertaken to confirm the EMSA results. Deletion of the *sodM* promoter from -209 to -178 (Del. 8, Fig. 2-3) and the site-specific deletion (Del. 9) led to almost complete loss of GUS activity. Weak activity remained after deletion of the region from -209 to -178 and was not affected by further deletions up to position -138 (Del. 6, 8). These results together with the EMSA strongly suggest that the 30-bp region from -209 to -178 is the *cis*-regulatory element essential for strong expression of the *sodM* promoter.
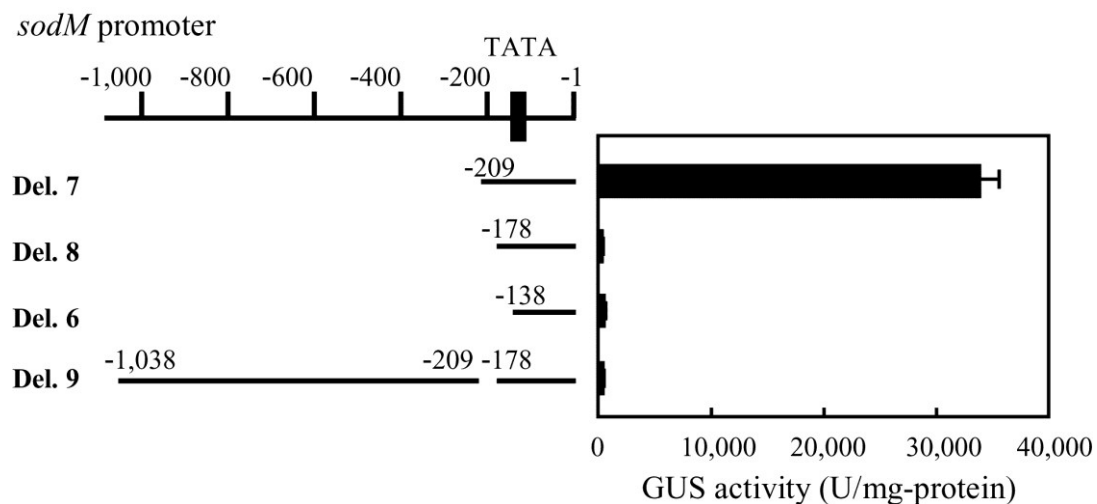
Fig. 2-3   GUS activity of the *sodM* promoter with detailed deletion. The numbers, filled bars, and error bars are as described in the Fig. 2-1 legend.

Construction of an enhanced *sodM* promoter   To confirm the deletion analysis and EMSA results, *sodM* promoters containing multiple insertions of the putative *cis*-acting element (-209 to -178) upstream of the minimum promoter (-209 to -1) were constructed. Five and nine tandem copies of the putative *cis*-element are present upstream of the minimum

promoter (Del. 7 in Fig. 2-3) in pTD5 and pTD9, respectively. These promoters containing the multiple repeats of the putative *cis*-element had about 2-fold higher activity than the minimum *sodM* promoter (Fig. 2-4).
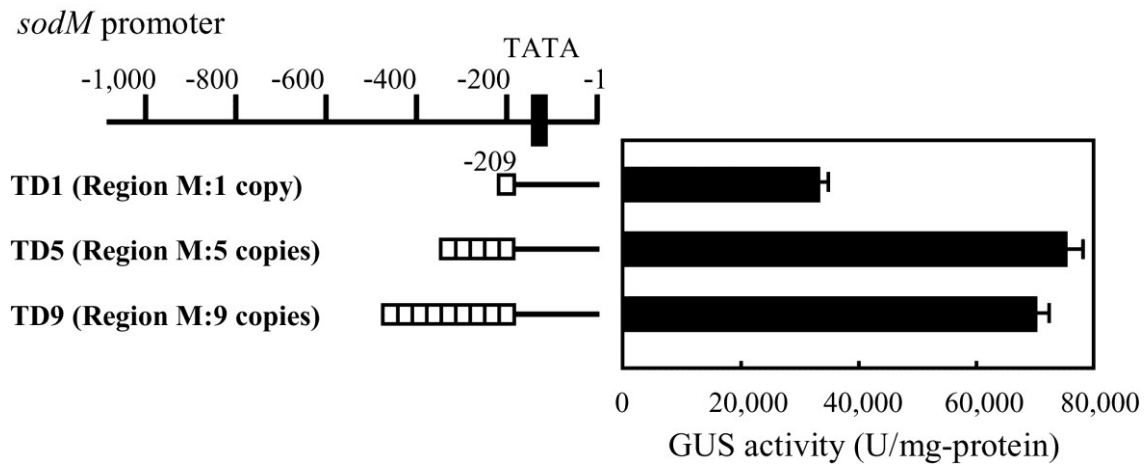
Fig. 2-4 GUS activity of the enhanced *sodM* promoters. The numbers, filled bars, and error bars are as described in the Fig. 2-1 legend. The open box indicates Region M (-209 to -178). TD1 contains only the *sodM* promoter region (-209 to -1) and original single copy of Region M. TD5 and TD9 contain five and nine tandem copies of region M, respectively.

Discussion

The initial deletion analysis of the *sodM* promoter revealed that a 63-bp region (-200 to -138) upstream of the TATA-box was essential for high-level expression of *sodM*. By way of comparison, the *cis*-element(s) needed for effective expression in other promoters such as *amyB* and *glaA* are more distant from the TATA-box, although the basic enhancers such as the CCAAT-box are located close to the TATA-box (Minetoki *et al*. 1996). These basic enhancers, in general, do not have a full promoter activity. However, the short region (-209 to -138) of the *sodM* promoter appeared to have a full promoter activity, judging from similarity to that of the *enoA* enhancer (Toda *et al*. 2001), the strongest promoter reported to date.

The EMSA identified the region from -209 to -178 which contained *cis*-acting element(s), and these results were consistent with the detailed deletion analysis. This region required for high-level expression was named Region M. In Region M, two *cis*-acting elements were found, the CCAAT-box (Tanaka *et al*. 2000) and the GC-box (Ishida *et al*. 2000) (Fig. 2-5a). These motifs have been reported in various promoters and have enhanced activity by changing local chromatin structure (Tsukagoshi *et al*. 2001). The author speculated that the strong enhancing activity of Region M was due to nearby arrangements of both the CCAAT-box and the GC-box. These arrangements are usually found in the promoters of vertebrates (Bernadt and Rizzino 2003), but have not been reported in other promoters of *A. oryzae* that have been analyzed to date.

To confirm that Region M has a strong enhancing activity, the enhanced *sodM* promoters were constructed by connecting five or nine tandem copies of this region. These promoters exhibited 2-3 times higher expression than wild-type (Fig. 2-4), indicating that Region M is a *bona fide cis*-acting enhancer element. The observation that GUS activity was almost the

same, irrespective of the presence of five or nine consecutive elements, suggests exhaustion of the regulatory protein(s) interacting with Region M, which is known as titration previously reported by Minetoki *et al*. (1998). A slight decrease in the expression level when the copy number was increased from five to nine is likely to be within the experimental error, but possibly is due to other uncharacterized effects (e.g., conformational change of DNA unfavorable to the binding of the regulatory factor).

In Section 1, the author showed that Region M was responsible for high-level expression of the *sodM* gene in submerged (liquid) cultures. The *A. fumigatus* manganese superoxide dismutase (MnSOD) gene (DDBJ/EMBL/GenBank accession no. U53561, Crameri *et al*. 1996) is a *sodM* homologue. The product of this homolog is known to be one of the factors responsible for aspergillosis (Latge 1999). The *A. fumigatus* MnSOD gene was reported to be expressed strongly on the surface of human lung under growth conditions resembling solid-state cultures (Crameri *et al*.1996). In Section 1, the *A. oryzae sodM* gene was found to be repressed in solid-state cultures such as growth on the surface of an agar plate, wheat bran, or rice *koji* (GUS activities< 100 unit/mg-protein). The *sodM* homologs from the *A. nidulans* and *A. fumigatus* genomes were searched, and their promoter regions were compared. A highly conserved region (HCR) was found upstream of Region M (Fig. 2-5b), but this region did not affect promoter activity under various culture conditions in *A. oryzae*. Alignment of the regions spanning from the HCR to the translation initiation codon revealed that Region M was found only in the *A. oryzae sodM* promoter, but not in the other two *Aspergilli*, although approximately 50% similarity was observed throughout the promoter regions of the three genes, except in Region M (Fig. 2-5b). This would suggest that Region M evolved in the *sodM* promoter after *A. oryzae* branched off from *A. fumigatus*. Modification of *sodM* expression by insertion of Region M might be one of the mechanisms that prevent human

pathogenicity. The very weak expression of the modified *sodM* promoter lacking Region M (Del. 9 in Fig. 2-3) in solid-state cultures suggests extensive mutations in HCR of the *A. oryzae* promoter. This feature of the promoter could contribute to the safety of *A. oryzae*, as truncation of the transcriptional regulator, AflR, is already known to work as one of the multiple safety locks that prevents aflatoxin production (Kusumoto *et al.* 1998).
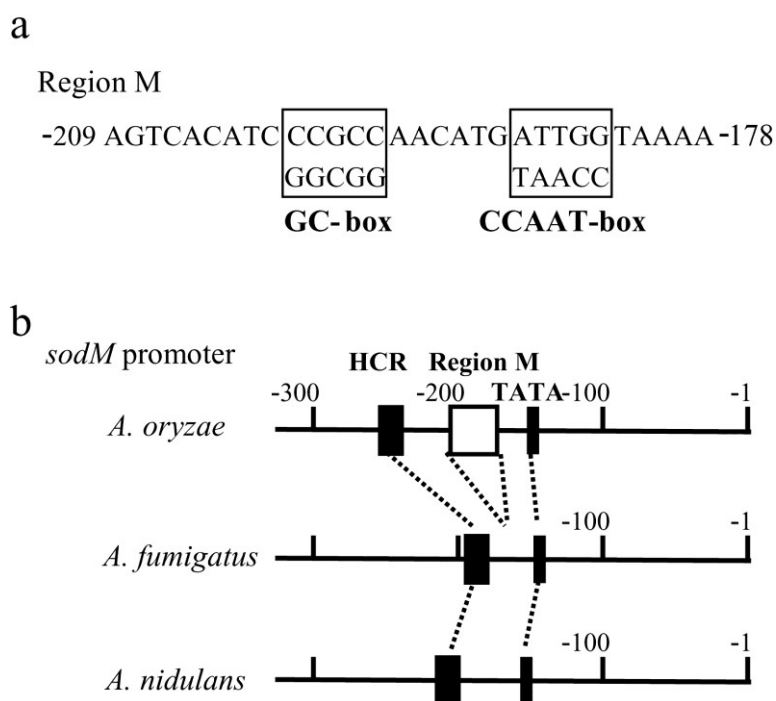

Fig. 2-5a, b Nucleotide sequence of *cis*-acting regions. **a** Nucleotide sequence of Region M. The open boxes indicate the GC-box and the CCAAT-box. **b** Interspecific comparison of regulatory regions in the *sodM* promoter. The solid box, open box, and hatched box indicate the TATA-box, Region M, and the highly conserved region (HCR), respectively.

Section 2

Analysis of catalase-encoding gene (*catB*) promoter expressed constitutively and induced with hydrogen peroxide

Aspergilli are one of the most important fungi for useful enzyme production. In particular, *A. oryzae* is used not only in the manufacture of traditional Japanese foods such as sake and soy sauce, but is also in the production of homo- and heterologous proteins. Recently, the *A. oryzae* genome sequence was identified (Machida *et al*. 2005), and post-genomic analysis such as transcriptome was done (Masai *et al*. 2006). Gene expression analysis is very important in the analysis of transcriptome, but promoter analysis and determination of *cis*-elements have not well been examined. These analyses are laborious and require much time, but the results are necessary not only for basic research but also for applications such as constructing strong promoters (Ishida *et al*. 2006a).

Recently, the authors isolated the catalase-encoding gene (*catB*) from *A. oryzae* and found strong expression in various cultures. In other *Aspergilli, catB* homolog genes are well expressed, but it is difficult to determine their regulatory *cis*-elements *in silico*; a catalase is commonly involved in detoxification of active oxygen, but it has been reported that regulatory *cis*-elements are different in species, such as *S. cerevisiae*, *Schizosaccharomyces pombe*, and *Homo sapiens* (Nakagawa *et al*. 1999; Nenoi *et al*. 2001; O'Rourke *et al*. 2002).

Furthermore, *catB* homolog genes such as the *A. fumigatus cat1* gene are involved in aspergillosis (Shibuya *et al*. 2006), and the elucidation of the *cis*-elements of *catB* promoter should be useful for understanding this disease. Hence in Section 2, the author chose the *A. oryzae catB* promoter for its analysis.

Materials and methods

Strains and growth conditions   The *A. oryzae* OSI1013 AON-2 was used in all the transformation experiments by GUS reporter assay. GPY medium was used to grow the *A. oryzae* AON-2 and transformants. Czapek-Dox medium was used to select transformants. *E. coli* JM109 was used in DNA manipulations.

Transformation experiments   *A. oryzae* AON-2 mycelia were grown in GPY at 30°C for 24 h and transformed as described previously (Gomi *et al.* 1987). Transformants were selected using Czapek-Dox medium containing sodium nitrate as sole nitrogen source.

Construction of *catB* promoter deletion mutants   The *A. oryzae* integration vector pNGS1 used for promoter deletion analysis. Various lengths of DNA fragments of the *catB* promoter region were amplified by PCR using *A. oryzae* genomic DNA as a template. Each upstream primer (pd2-pd10) was tailed with 10-mer oligonucleotide (5′-AAAACTGCAG-3′) to generate a *Pst*I site at the 5′ end (pd2: 5′-AAAACTGCAGTAGCTATCAACGCAAGTAGT-3′, pd3: 5′-AAAACTGCAGGCCAATATTCTCGAAACGTC-3′, pd4: 5′-AAAACTGCAGAGTCAACATTCTCATCTTAT-3′, pd5: 5′-AAAACTGCAGCTCTCATACCAATTATTGGG-3′, pd6: 5′-AAAACTGCAGTAGAGAGCAAGACCCAGTAG-3′, pd7: 5′-AAAACTGCAGGCATATAAGTACCTCGCAAG-3′, pd8: 5′-AAAACTGCAGCATCAGATCGGGATGAGGCC-3′, pd9: 5′-AAAACTGCAGTCGCCGACCCACGCAAGGGG-3′, pd10: 5′-AAAACTGCAGAGGCCAAAATTCTGAATAAG-3′, pd11: 5′-AAAACTGCAGGAGGTATTGATCATCGAAGC-3′). One upstream primer and the common downstream primer (cdp) were tailed with 10-mer oligonucleotide (5′-ACGCGTCGAC-3′) to generate a *Sal*I site at the 5′ end (pd1: 5′-ACGCGTCGACTTGAGTTATTATCGTATC-CT-3′, cdp: 5′-ACGCGTCGACCGTGTTGCTAACTGTCAAGT-3′). The cdp primer contained an antisense nucleotide sequence between -20 to -1 of the *catB* promoter. The mutant promoter with site-specific deletion of 25-bp between -1,000 to -975 was prepared by overlap extension

PCR (Higuchi, 1989). The upper fragment (-1,400 to -1,000) was amplified by PCR using the pd1 and the pd12u primers (5′-CGATCTGATGTGGAACACTTTCTCCTGAAT-3′), and the lower fragment (-975 to -1) was amplified using the pd12d (5′-AAGTGTTCCACATCAGA TCGGGATGAGGCC-3′) and cdp primers. To obtain the site-specific deletion promoter, the two PCR products were combined and subjected to a second PCR using the pd1 and cdp primers. Each promoter fragments were digested with *Pst*I and/or *Sal*I, and were individually introduced upstream of the *uidA* gene using these same sites in pNGS1.

GUS assay   *A. oryzae* transformants were cultured in 200 ml GPY at 30°C for 72 h with shaking. Preparation of cell-free extracts was done as described by Ishida *et al*. (2004). The GUS activity of cell-free extract was measured according to Jefferson *et al*. (1986). Protein concentration was determined using a protein assay kit (Bio-Rad Laboratories) with BSA as a standard.

Southern blot analysis   Preparation of genomic DNA from *A. oryzae* transformants was carried out as described Tsuchiya *et al*. (1992). The entire 1.8-kbp *uidA* gene fragment was used as a probe. A DIG-DNA labeling and detection kit (Roche) was used for signal detection.

Preparation of WCE and EMSA   WCE were prepared from *A. oryzae* OSI1013 mycelia that were cultured in 100 ml GPY at 30°C for 24 h (Machida and Jigami 1994). A double-stranded probe was prepared by PCR with a chemically labeled IRDye800 primer (5′-AATGTTGACTGGTTTCTTGGCAGCATCCCC-3′) and a non-labeled primer (5′-GCCAATATTCTCGAAACGTCTCTAGCATCA-3′). A specific competitor was prepared by the same method as both non-labeled primers. The coding region of the *A. oryzae pgmA* gene (+191 to +240, DDBJ/EMBL/GenBank no. AB032275) was used as a non-specific competitor. WCE and probes were incubated for 20 min at room temperature, as described by Toda *et al*. (2001), and were then subjected to EMSA performed using a LI-COR 4200L DNA sequencer (LI-COR) (Sano *et al*. 2001).

Results

Deletion analysis of the *catB* promoter   Plasmids for promoter deletion analysis were obtained by insertions of the truncated *catB* promoter fragments into the pNGSl to fuse the *uidA* gene. A series of sequential deletions at the 5′-end of the 1400-bp *catB* promoter was constructed. These plasmids were introduced into the AON-2 strain, and the transformants were selected according to their ability to utilize sodium nitrate as sole nitrogen source. Transformants harboring a single copy of *uidA* gene were selected by the genomic Southern blot analysis. Functional analysis of the *catB* promoter was performed on submerged cultures by measuring GUS activity. In the culture condition without hydrogen peroxide, deletion of the region from -1,200 to -1,000 caused a dramatic increase in GUS activity (Del. 2, 3 in Fig. 3-1). Oppositely, deletion of the region from -1,000 to -800 caused marked decreases (Del. 3, 4). A deletion from -800 to -600 (Del. 4, 5) resulted in almost complete loss of activity. Deletions spanning the region from -600 to -200 (Del. 5-7) were found not to affect GUS activity. In the culture condition containing hydrogen peroxide, deletions spanning the region from -1,400 to -1,000 had higher GUS activities than the first condition (Del. 1-3), but deletions spanning the region from -1,000 to -200 (Del. 4-7) were not different in GUS activity.
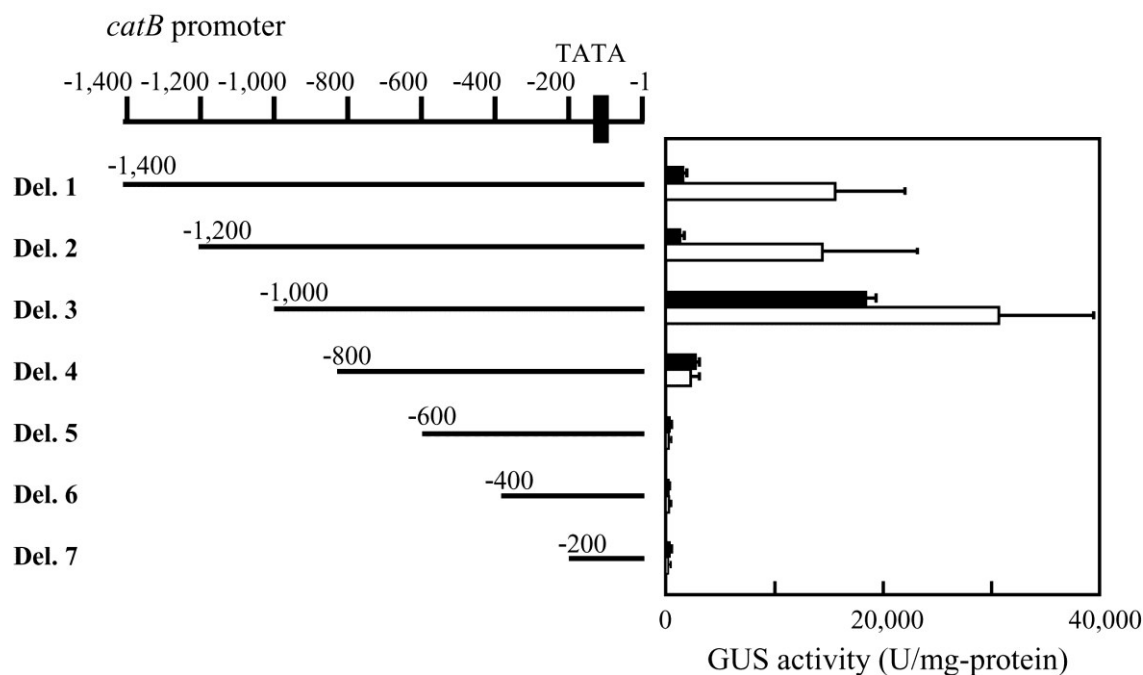
Fig. 3-1 GUS activity of the *catB* promoters. The numbers noted for each mutant denote the distance from the start codon, ATG. GUS activities in the culture condition without hydrogen peroxide (filled bars) and with hydrogen peroxide (open bar) are the means and standard deviations (error bars) for cell-free extracts from three independent transformants. Hydrogen peroxide was added to the cultures (final concentration, 0.1% v/v in culture medium) 24 h after inoculation.

Determination of protein-binding regions with EMSA   EMSA was employed in order to determine the specific binding of intracellular factors to the transcription regulatory regions identified by promoter deletion analysis. The probe and a specific competitor for EMSA were designed to include part of the *catB* promoter sequence (-1,000 to -791). The partial competitors were synthesized so as to contain part (Cl, -1,000 to -956; C2, -965 to -921; C3, -930 to -886; C4, -895 to -851; C5, -860 to -816; and C6, -825 to -791) of the promoter sequence. A strongly shifted band (Fig. 3-2, arrow adjacent to lane 2) was detected in the sample incubated with WCE. The shifted band was detected in the presence of the non-specific competitor, but disappeared upon addition of the specific competitor. While the short competitor, Cl, inhibited formation of the shifted band, as did specific competitor,

competitors C2 to C6 did not. These results clearly indicate that the 45-bp region from -1,000 to -956 was the target of the sequence-specific binding factors present in the WCE. Other probes including the *catB* promoter sequences (-1,200 to -1,000 and -800 to -600) were designed. But no shifted bands were detected when these probes were used in EMSA.
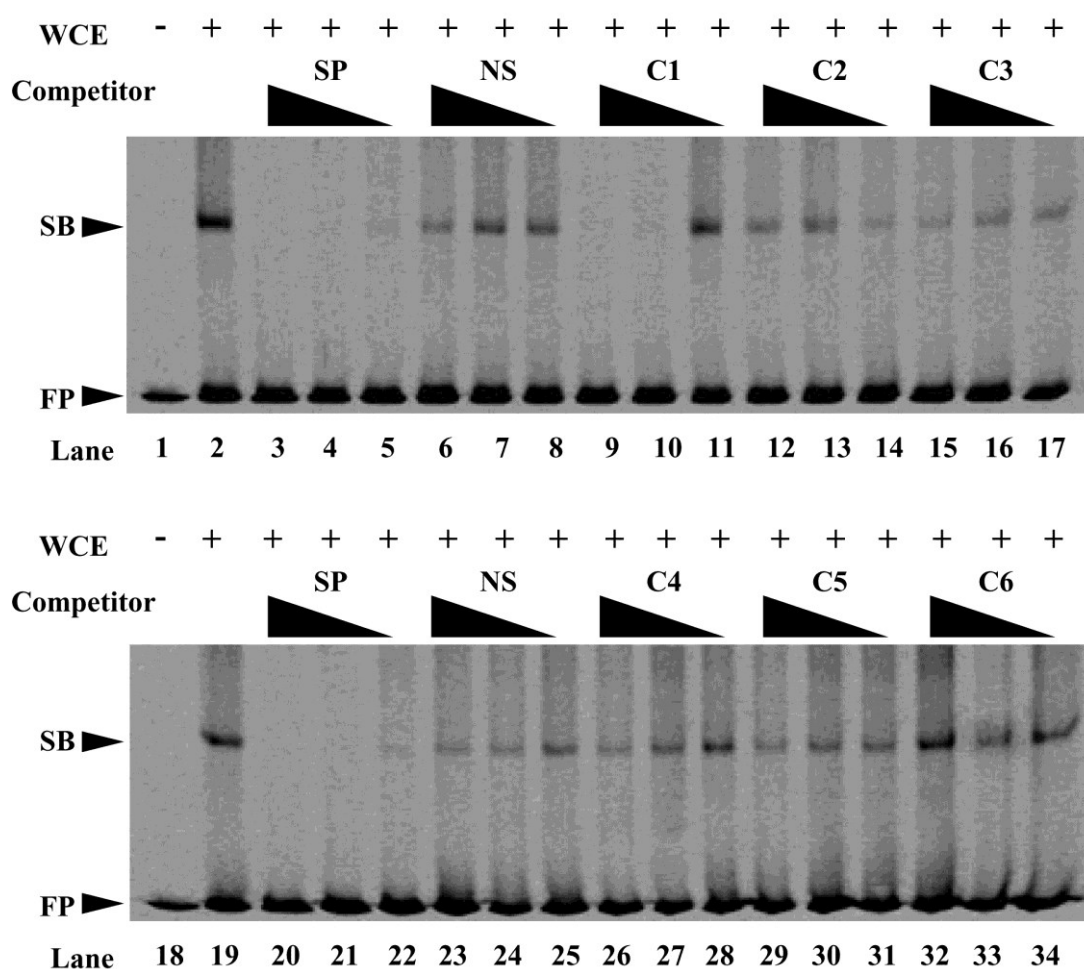
Fig. 3-2 EMSA of the *catB* promoter. WCE (10 μg) and 100 fmol of DNA probe derived from the *catB* promoter region (-1,000 to -791) were mixed and incubated. The specific competitor (SP) and partial competitors (C1-6) were derived from *catB* promoter regions (SP, -1,000 to -791; C1, -1,000 to -956; C2, -965 to -921; C3, -930 to -886; C4, -895 to -851; C5, -860 to -816; and C6, -825 to -791). NS indicates non-specific competitor. Either 10 pmol (lanes 3, 6, 9, 12, 15, 20, 23, 26, 29, and 32), 5 pmol (lanes 4, 7, 10, 13, 16, 21, 24, 27, and 30), or 1 pmol (lanes 5, 8, 11, 14, 17, 22, 25, 28, and 31) of the competitors were added. SB and FP indicate the shifted band and the free probe, respectively.

Detailed promoter deletion analysis of *catB* promoter   Detailed deletion analysis was undertaken to confirm these results. Deletion of the *catB* promoter from -1,000 to -975 (Fig. 3-3, Del. 8), and the site-specific deletion (Del. 12) led to loss of GUS activity in both culture conditions, with and without hydrogen peroxide. Weak activity remained after deletion of the region from -1,000 to -975 and was not affected by further deletions up to -800 (Del. 9-11). Theses results together with those of the EMSA strongly suggest that the 25-bp region from -1,000 to -975 is the *cis*-regulatory element essential for strong expression and induction with hydrogen peroxide of the *catB* promoter.
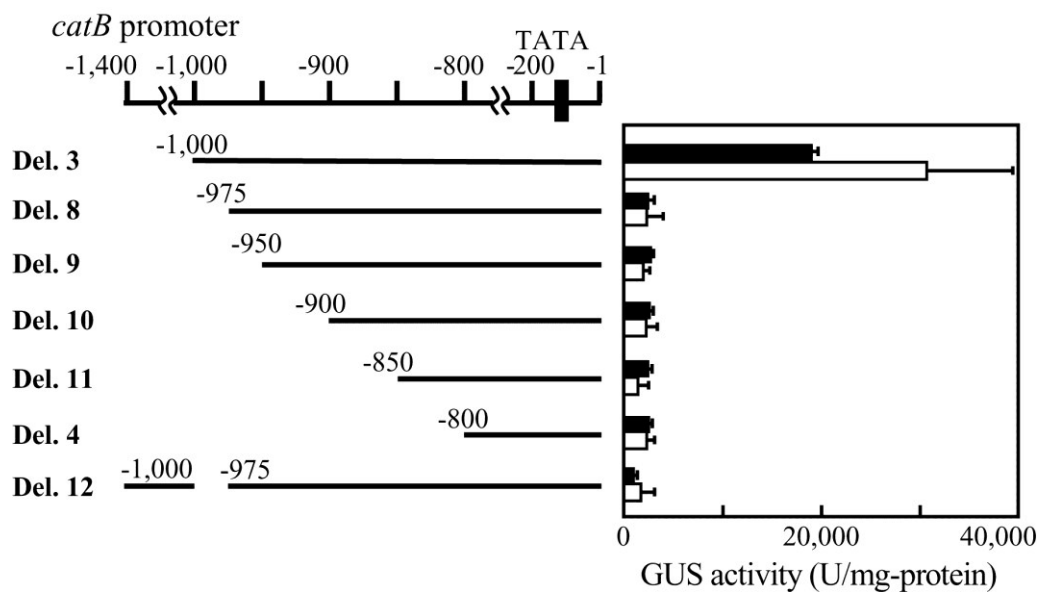
Fig. 3-3   GUS activity of the *catB* promoter with detailed deletion analysis. The numbers, filled bars, open bars, error bars, and culture conditions are as described in the legend to Fig. 3-1.

Discussion

In the initial deletion analysis, the *catB* promoter was deleted by 200-bp systematically, since 200-bp is the maximum limitation length for a probe length in EMSA. This deletion analysis revealed three regulated regions. The first region, from -1,200 to -1,000, was concerned with repression. The second region, from -1,000 to -800, was necessary for strong expression and induction with hydrogen peroxide. The third region, from -800 to -600, contributed to weak expression. Each region concerned with regulation was distant from the TATA-box or the translation start site, such as *agdA* gene promoter (Minetoki *et al*. 1996).

EMSA identified the region from -1,000 to -956 that contained *cis*-acting elements. Furthermore, detailed promoter deletion analysis indicated that a 25-bp element from -1,000 to -975 was more important. In this 25-bp element, a heat shock elements, (HSE, nTTCnnGAAnnTTCn) and a CCAAT-box, were found (Fig. 3-4). It has been reported that the HSE and its binding protein, a heat shock transcription factor, are highly conserved from yeast to humans (Sakurai and Takemori 2007; Yamamoto *et al*. 2005; Xiao *et al*. 1991).

It is well known that heat shock and hydrogen peroxide induces catalase gene expression in *Aspergilli* (Abrashev *et al*. 2005; Calera *et al*. 1997; Kawasaki *et al*. 1997), and other species (Nakagawa *et al*. 1999; Nenoi *et al*. 2001; O'Rourke *et al*. 2002; Wieser *et al*. 1991), and that the promoter of each catalase gene has a regulatory element for stress response. However, the regulatory elements are different in species. There is Msn2 or Msn4 binding site in the *S. cerevisiae* catalase gene *CTT1* promoter (O'Rourke *et al*. 2002), an element A and the Atf1 binding site in the *S. pombe* catalase gene promoter (Nakagawa *et al*. 1999), the multi CCAAT-box, and the Sp1 binding site in the human catalase gene promoter (DDBJ/EMBL/GenBank no. AB034940) (Nenoi *et al*. 2001). Considering these reports, in *A*.

oryzae, HSE and the CCAAT-box have to do with strong expression and induction with hydrogen peroxide and with a different regulation mechanism of *catB* in comparison with other species.

In *Aspergilli*, the *catB* gene and its homologs are highly conserved and are thought of as housekeeping genes. These genes are regulated by the same mechanism as the glyceraldehyde-3-phosphate dehydrogenase gene (Sano *et al*. 2001). When the *catB* gene and its homologous promoters from *A. fumigatus*, *A. nidulans*, *A. niger*, *A. terreus*, and *A. flavus* are compared, two highly conserved motifs were found in addition to HSE (Fig. 3-5). The first motif, a CGGAGT-box, which is consistent with the *S. cerevisiae* transcription factor Adr1p binding motif GG(A/G)G (Cheng *et al*. 1994), was located in the first region, from -1,200 to -1,000, which was concerned with repression. In the *A. oryzae* genome database, an *ADR1* homolog gene was found (AO090023000650, http://www.bio.nite.go.jp/dogan/top). The *ADR1* homolog gene had high similarity to the DNA binding domain, but no similarity to the activation domain. Further, a DNA binding region and other regions were swapped structurally as compared with *ADR1* gene, so it is thought that the *ADR1* homolog gene and its binding CGGAGT-box are concerned with repression of the *catB* gene. In the third region, from -800 to -600, which contributed to weak expression, a second motif, an ATTACCAAG-box, which was not consistent with any already-known motifs, was located. But neither motifs was detected in EMSA, and a method such as detailed promoter deletion analysis is necessary to identify these motif functions. Since HSE and the two putative motifs were conserved and aliened sequentially in *Aspergilli*, the *catB* gene homologs, including *A. fumigatus cat1* gene, were regulated in the same way as *A. oryzae catB* gene described in Section 2.
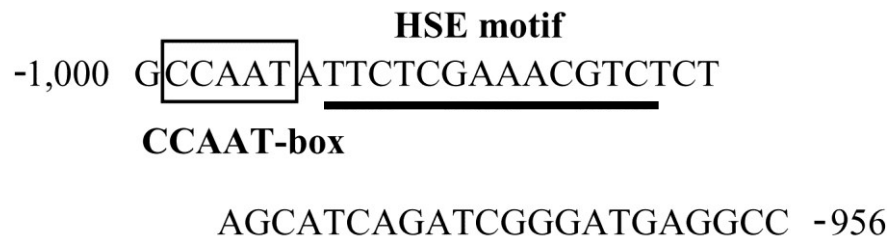
HSE motif

-1,000 GCCAAT ATTCTCGAAACGTCTCT

CCAAT-box

AGCATCAGATCGGGATGAGGCC -956

Fig. 3-4 Nucleotide sequence of *cis*-acting regions. The HSE motif is underlined. The open box indicates the CCAAT-box.
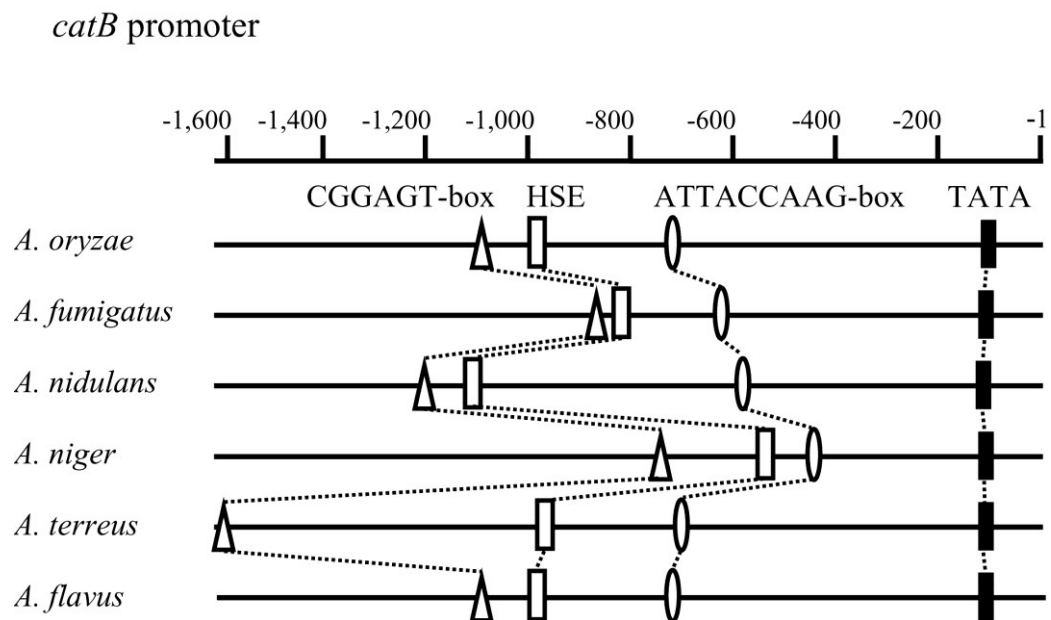
catB promoter

Fig. 3-5 Comparison of *catB* promoters in *Aspergilli*. The numbers denote the distances from the start codon, ATG. The solid box, triangle, open box, and circle indicate the TATA-box, CGGAGT-box, HSE, and ATTACCAAG-box, respectively.

Section 3

Analysis of glucoamylase-encoding gene (*glaB*) promoter expressed only in solid-state cultures and determination of *cis*-elements responding to low water activity

The filamentous fungus, *A. oryzae*, is widely used in traditional Japanese fermentations, including sake, soy sauce, *miso*, and *mirin*. These fermentations are conducted using a solid-state cultivation (SSC) process. Protein secretion is enhanced during SSC relative to submerged cultures. Proteomic analysis of extracellular proteins produced as a function of cultivation process identified one set of proteins produced specifically during SSC, a different set produced in submerged cultures, and a third set produced in both cultivation systems (Oda *et al*. 2006). EST analysis showed that the *A. oryzae* gene expression profile changed in a cultivation process-dependent manner (Akao *et al*. 2007). Because submerged culture fermentations have been widely used relative to SSC fermentations, a number of gene expression analyses have focused on the former, involving promoter deletion analysis and EMSA (Ito *et al*. 2004; Toda *et al*. 2001). In spite of the experimental complications involving insoluble substrates (e.g., polysaccharides), several genes have been identified that were expressed specifically during SSC (Kitano *et al*. 2002; Kobayashi *et al*. 2007; Obata *et al*. 2004).

Significant differences between submerged and solid-state media include water activity, diffusion of nutrients and gasses, continuity of medium distribution, and physical barriers to hyphal extension invading the substrates (Akao *et al*. 2007). With specific reference to SSC-based production of the *koji* mold for sake brewing, control of low water activity and high temperature is of critical importance. One reason is that sake brewing is affected by the glucoamylase activity of the *koji*. The *glaB* gene encoding glucoamylase, one of the most

extensively studied SSC-specific genes, is only expressed during SSC (Ishida *et al*. 2000, 2006). In Section 3, the author used EMSA and promoter deletion analysis to determine important *cis*-elements responsive to low water activity stress during SSC.

Materials and methods

Strains, growth conditions *A. oryzae* OSI1013 AON-2 was used in all the transformation experiments involving GUS reporter assay. GPY medium (1% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, and 2% glucose or 2% soluble starch, pH 6.0) was used in the transformation experiments. Plate culture (GPY medium containing 2% soluble starch, 1.5% agar, and 0.2 μm mixed cellulose ester membrane (ADVANTEC Inc., Tokyo, Japan)) was used for preparing WCE (Ishida *et al*. 1998). Steamed rice polished to 70% of total weight (Ishida *et al*. 2006a) and steamed wheat bran (Kobayashi *et al*. 2007) were used for SSC. Standard growth conditions for *A. oryzae* transformants were 30°C for 48 h.

Preparation of WCE and EMSA WCE were prepared from mycelia of *A. oryzae* OSI1013 grown on plate culture medium containing 50% maltose at 42°C for 48 h (Machida and Jigami 1994). Oligonucleotides and those labeled with IRDye800 were chemically synthesized and annealed to prepare double-strand probes. The coding region of the *A. oryzae pgmA* gene (+191 to +240) (DDBJ/EMBL/GenBank accession no. AB032275) was used as a non-specific competitor. DNA fragments that had the same sequences as the probes were used as specific competitors. WCE and probes were incubated for 20 min at room temperature, and were then subjected to EMSA performed using a LI-COR 4200L DNA sequencer (Toda *et al*. 2001).

Construction of *glaB* promoter deletion mutants   DNA fragments containing various *glaB* promoter regions were amplified by PCR using *A. oryzae* genomic DNA and the appropriate primers listed in Table 4-1. To generate the *Pst*I and *Sal*I sites at both ends of all PCR products, additional sequences, (5′-AAAACTGCAG-3′) and (5′-ACGCGTCGAC-3′), respectively, were added to the 5′-ends of each common upstream (CUS) and common downstream (CDS) primer as shown in Table 4-1. The CUS primer contained the sense nucleotide sequence between -420 and -401 of the *glaB* promoter. The CDS primer contained the antisense nucleotide sequence between -20 and -1 of the *glaB* promoter. As an example of how the deleted promoters were constructed, the mutant promoter with a site-specific 30-bp deletion between -382 and -354 was prepared by overlap extension PCR (Higuchi *et al*. 1988). The upper fragment (-420 to -383) was amplified by PCR using CUS and Del1-u primers, and the lower fragment (-352 to -1) was amplified using the Del1-d and CDS primers. To obtain the final construct containing the site-specific deletion, the two PCRs were combined and subjected to a second PCR using CUS and CDS as primers. All synthesized promoters were digested with *Pst*I and *Sal*I, and individually subcloned upstream of the *uidA* gene using these same sites in pNGS1.

Transformation of *A. oryzae*   Mycelia from *A. oryzae* AON-2 were grown in GPY at 30°C for 24 h and transformed as described (Gomi *et al*. 1987). Transformants were selected on plates containing sodium nitrate as sole nitrogen source.

GUS assay   Cells obtained from a submerged culture and SSC were frozen in liquid nitrogen and disrupted mechanically using a mortar. The cell-free extracts were suspended in 20 mM sodium acetate buffer (pH 5.0), and GUS activities in the supernatants were measured as described (Jefferson *et al*. 1986).

Table 4-1 PCR primers

Primer	Sequence (5′ to 3′)
CUS	AAAACTGCAGAGTCCGCCTTCTAAGAGCAT
CDS	ACGCGTCGACGATGGTGGTGACTTCCAAGA
Del1-u	TAGTTCTCACAGTCCTAGTCGAGTCTTAAT
Del1-d	GACTAGGACTGTGAGAACTAAGAGAATGGC
Del2-u	TGCCCGTGCCTGGTGGAAACCCCATCGGAC
Del2-d	GTTTCCACCAGGCACGGGCAGATGTCGGGA
Del3-u	AAAGTGATCAGCCATTCTCTTAGTTCTCAC
Del3-d	AGAGAATGGCTGATCACTTTTAGTCGCTCC
Del4-u	ATTGAAATCGTCCCGACATCTGCCCGTGCC
Del4-d	GATGTCGGGACGATTTCAATTGGATCCGCC
Del5-u	ATTTCCGGGAATATTGCGTCGGAGCGACTA
Del5-d	GACGCAATATTCCCGGAAATTAATACCGGA
Del6-u	TGAAGCAGATTGCAGCGTGGCGGATCCAAT
Del6-d	CCACGCTGCAATCTGCTTCATGATGGGGCC

Results

Determination of protein-binding regions   In a previous study, it was suggested that the GC-box and a related region of the *glaB* promoter were the essential *cis*-elements involved in the low water activity response (Ishida *et al*. 2000). Here, the author employed EMSA to detect specific binding of intracellular factors. The probe for EMSA was designed to include a part of the *glaB* promoter region (-352 to -313). A shifted band (arrow adjacent to lane 2, Fig. 4-1) was detected in the sample incubated with WCE from SSC. The shifted band was detected in the presence of non-specific competitors, but disappeared upon the addition of a specific competitor. The shifted band was not detected in the presence of submerged culture-derived WCE.

Determination of protein-binding neighboring regions   To determine other protein-binding regions, two probes were designed (-382 to -333 and -332 to -283). A shifted band (arrow adjacent to lane 2, Fig. 4-2a, b) was detected with each probe. Further, neither shifted band was affected by the presence of a non-specific competitor, but both disappeared in the presence of a specific competitor. Using the -382 to -333 probe, the shifted band was not affected by the presence of partial competitor GB1 (-352 to -333), but it disappeared in the presence of partial competitor C1 (-382 to -353) (Fig. 4-2a). These results indicate that the 30-bp region from -382 to -353 is the target of the sequence-specific binding factor(s) present in WCE. Using the -332 to -283 probe, the band which was shifted strongly disappeared in the presence of the partial competitor GB2 (-332 to -313) as observed for the specific competitor (Fig. 4-2b). These results also indicate that the 20-bp region from -332 to -313 is the target of the sequence-specific binding factor present in WCE. Using other probes (-420 to -383 and -282 to -254), no additional shifted bands were detected. Using the -253 to -236 probe, a weak and broad shifted band was detected.
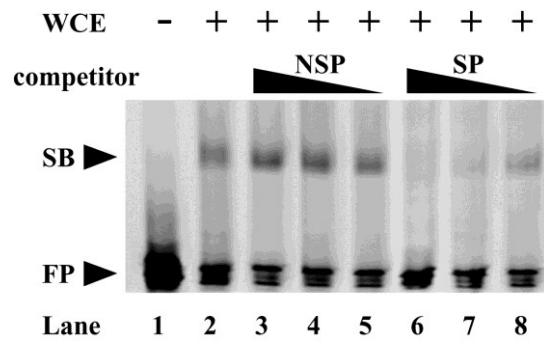
Fig. 4-1 EMSA of GC-box. WCE (5 μg) and 200 fmol of DNA probe derived from the *glaB* promoter region (-352 to -313) were mixed and incubated. SP and NSP indicate specific competitor and non-specific competitor, respectively. Either 10 pmol (lanes 3 and 6), 5 pmol (lanes 4 and 7), or 1 pmol (lanes 5 and 8) of each competitor were added. SB and FP indicate the shifted band and free probe, respectively.
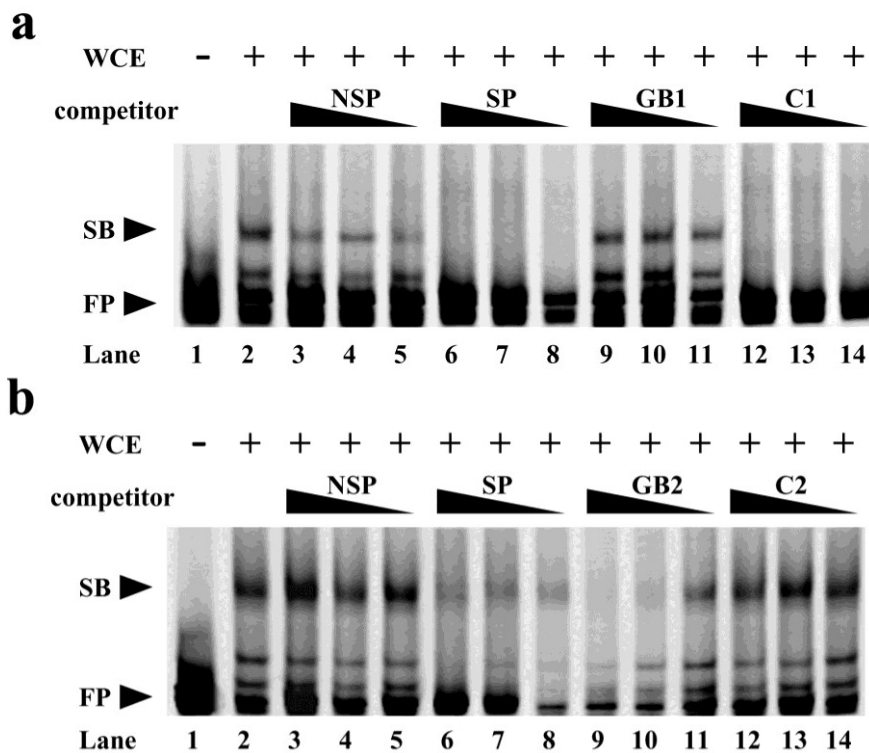

Fig. 4-2 EMSA of neighboring regions. SP, NSP, SB, and FP are described in the Fig. 4-1 legend. Partial competitors (GB1, GB2, C1, and C2) are derived from the *glaB* promoter region (-352 to -333, -332 to -313, -382 to -353, and -312 to -283, respectively). Either 10 pmol (lanes 3, 6, 9, and 12), 5 pmol (lanes 4, 7, 10 and 13), or 1 pmol (lanes 5, 8, 11 and 14) of each competitor were added. **a** Results obtained using a DNA probe from the *glaB* promoter region -382 to -333; **b** Results obtained using a probe from -332 to -283.

48

Deletion analysis of the *glaB* promoter   Deletion analysis was undertaken to confirm the EMSA results. Plasmids for deletion analysis were constructed by insertion of site-specific deleted *glaB* promoter fragments into pNGS1 for fusion with the *uidA* gene. These plasmids were introduced into the AON-2 strain, and the transformants harboring a single copy of the *uidA* gene were selected. Initially, each transformant was cultivated in a submerged culture and on steamed rice—an SSC method. Compared to the wild-type *glaB* promoter (Fig. 4-3, Del. 0), Del. 1, Del. 3, and Del. 6 had less GUS activity. In the submerged culture, Del. 1 and Del. 3 were induced by starch, but Del. 6 was not. Among the deleted promoters, Del. 3 resulted in a drastic reduction, especially during growth on steamed rice. In general, GUS activities obtained from steamed rice culture were higher than those observed in the submerged culture.

Induction by low water activity and high temperature   Based on the deletion analysis, three promoter regions (Del. 1, Del. 3, and Del. 6) were found to play a role in *glaB* expression as a function of culture condition. To determine which regions might be involved in induction by low water activity and high temperature stress, each transformant was cultured on plate and steamed wheat bran, the easily-controlled SSC conditions. The Del. 1, Del. 3, and Del. 6 constructs, which had less GUS activity than Del. 0, also had much lower activity under these two SSC conditions (Fig. 4-4). Under conditions of low water activity (plate culture with 50 % maltose and steamed wheat bran containing 50 % moisture), only the Del. 3 construct was not induced relative to the controls. At high temperature (growth at 42°C), only the Del. 1 construct was not induced. While generally having lower GUS activity, the Del. 6 construct was induced by both low water activity and high temperature stress.

	GUS Activity (U/mg)		
	Submerged culture		Steamed rice culture
	+Glucose	+Starch	
Del. 0	45	330	7,320
Del. 1	35	203	3,330
Del. 2	42	290	6,260
Del. 3	11	85	631
Del. 4	28	301	5,250
Del. 5	30	316	5,980
Del. 6	38	45	2,420

Fig. 4-3   GUS activity of the *glaB* promoters in submerged culture and steamed rice culture. The numbers noted in each mutant denote the distance from the start codon ATG. GUS activity in submerged culture is indicated by +Glucose and +Starch for 2% glucose or 2% starch as sole carbon source.

a GUS Activity (U/mg) plate culture

b GUS Activity (U/mg) wheat bran culture

	a GUS Activity (U/mg) plate culture			b GUS Activity (U/mg) wheat bran culture		
	control	+50% Maltose	42°C	control	50% moisture	42°C
Del. 0	422	2,810	825	1,512	7,838	3,130
Del. 1	323	2,230	385	1,160	5,657	1,335
Del. 2	360	2,722	881	1,383	7,145	2,983
Del. 3	85	65	237	310	323	1,231
Del. 4	378	2,955	637	1,681	6,678	2,859
Del. 5	405	2,691	721	1,379	7,678	2,978
Del. 6	212	1,374	389	792	3,256	1,404

Fig. 4-4   Effect of promoter deletions at low water activity and high temperature. GUS activity of the *glaB* promoters in plate culture and wheat bran culture. **a** The control indicates GUS activity in plate culture at 30°C on standard medium with 2% starch as sole carbon source. "+50% maltose" indicates a low water activity condition with 50% maltose. "42°C" indicates the control medium incubated at 42°C. **b** The control refers to GUS activity during wheat bran plate culture at 30°C with 10 g steamed wheat bran and 10 ml water. "50% moisture" indicates low water activity conditions with 5 ml water. "42°C" indicates the control medium incubated at 42°C.

Discussion

The EMSA analysis around the GC-box identified three regions as the targets of sequence-specific binding factors (-382 to -353, -332 to -313, and -253 to -236, designated regions H, S, and R, respectively). The results of the deletion analysis together with the EMSA data strongly suggest that the three regions are *cis*-regulatory elements responsive to environmental stress.

Because the addition of starch increased GUS activity in submerged cultures, except for the constructs containing region R deletions, the author presumes that region R is involved in starch induction (Fig. 4-3, Del 6). In fact, region R contains a single CGG triplet, a starch-responsive element recognized in the regulatory transfactor AmyR (Ito *et al*. 2004). Recently, it was also reported that *glaB* was not expressed in an *amyR* gene deletion mutant (Watanabe *et al*. 2011). These previous studies strongly support the possibility that region R is a starch-responsive element.

During SSC at high temperature, GUS activity increased except for the construct with a deleted region H (Fig. 4-4, Del. 1), suggesting that region H is involved in induction by high temperature. In region H, HSE like motif (TTCnnnnnGATnnnnnTTC) was found (Matsushita *et al*. 2009). In the previous study, f-HSE (-349 to -345) or b-HSE (-340 to -336) substitution is highly effective in GUS activities about 2-fold lower (plate culture) and 2-fold upper (SSC), respectively), but our EMSA results show that no proteins bind these regions (Ishida *et al*. 2000). Perhaps, these substitutions affect of sequence-specific binding factors on their binding ability to *cis*-regulatory elements.
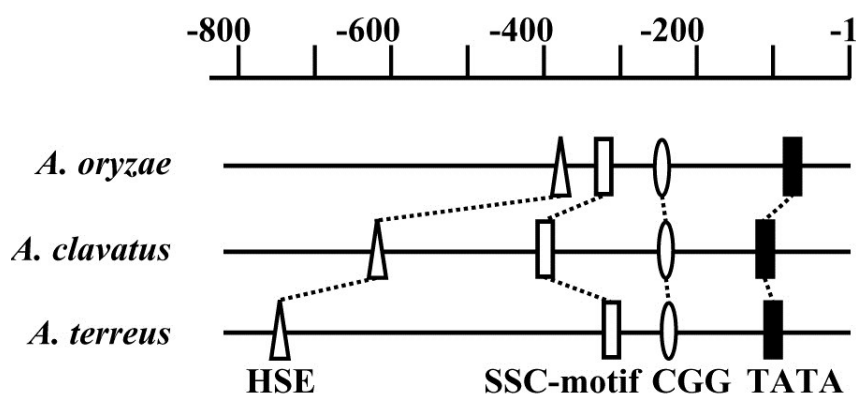
Fig. 4-5 Comparison of *glaB* promoters in *Aspergilli*. The numbers denote the distances from the start codon. The solid box, triangle, open box and circle indicate the TATA-box, HSE, SSC-motif, and single CGG triplet, respectively.

During SSC, deletion of region S caused a dramatic decrease in GUS activity, suggesting that region S is essential for strong *glaB* expression. Although GUS was inducible by starch and high temperature in the region S deletion mutant, it was not affected by low water activity (Fig. 4-4, Del 3). Unlike regions R and H, no known motifs were found in region S. While glucoamylases in *Aspergilli* generally contain catalytic and starch-binding domains, the glucoamylase encoded by *glaB* only contains a catalytic domain. Recent genomic analysis found that a *glaB* ortholog exists in *Aspergilli* including *A. clavatus* (DDBJ/EMBL/GenBank accession no. XM_001272680) and *A. terreus* (XM_001215158). When the *glaB* promoter was compared to those of orthologs from *A. clavatus* and *A. terreus*, a highly conserved motif CGGnnAGnnGTCGGG (SSC-motif) was found (Fig. 4-5). In a previous study, the sequence was identified as CGG-N8-CGG, the AmyR-binding motif (Petersen *et al*. 1999). More recent work identified the functional binding motif as CGGnnnTTTnTCGG (Wang *et al*. 2012). Together with these previous studies, our results strongly suggest that a transfactor(s) other than AmyR binds to this motif in *A. oryzae*. In Section 3, the author identified region S which

contains *cis*-elements responding to low water activity stress. Because the *glaB* gene is found on a syntenic block based on comparative genomics (Machida *et al*. 2008), the author compared the sequences of several adjacent genes. These genes are partially conserved in *A. oryzae*, *A. clavatus*, and *A. terreus* (Fig. 4-6). Although six genes including *glaB* are located in this region, insertion, reversal, and transfer events could have occurred, there is no evidence for insertion or reversal events within the first promoter region between *glaB* and an integral membrane protein-encoding gene (*impB*) in these three species. This holds true for the second promoter region between an ER membrane protein-encoding gene (*pkr1*) and a monosaccharide transporter-encoding gene (*mstB*). When the two regions were compared, a region S-like sequence was found (5′-GGTGGAATGGGCAGATATGGTG-3′) in the second promoter region. Because the length of the promoter regions was 1,339 and 2,066-bp, respectively, it is likely that only one region S sequence was shared by the *glaB* and *impB*. The author speculated that the *pkr1* and *mstB* genes also shared the region S-like promoter sequence. Region S-like sequences were not found in the promoters of the anaphase-promoting complex subunit-encoding gene (*apcB*) or taurine catabolism dioxygenase-encoding gene (*tauD*). These findings suggest that gene insertion, reversal, or transfer events may have resulted in loss of region S-like sequences. Further, because the fungal-specific transcription factor-encoding gene in *A. oryzae* (Fig. 4-6, *T1*, *fstA*) and transcription factor-encoding gene in *A. clavatus* (Fig. 4-6, *T2*, *rfeD*) are found on non-syntenic blocks, the transcription factor-encoding gene subject to region S regulation would be transferred to another syntenic block.

Section 3 is the first to analyze *cis*-elements in the *glaB* promoter that are responsive to SSC-specific conditions (e.g., low water activity) by EMSA and deletion analysis. Further, our combined approach involving WCE preparation, easily-controlled SSC conditions, and

comparative genomics can be applied directly to identify other genes whose promoters are expressed specifically during SSC.
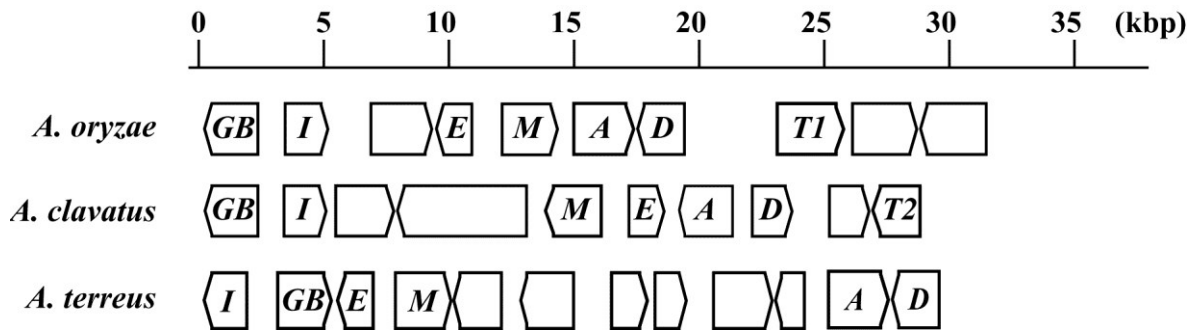

Fig. 4-6 Comparative genomics of the *glaB* gene region in *A. oryzae*, *A. clavatus*, and *A. terreus*. *GB*, *I*, *E*, *M*, *A*, *D*, *T1*, and *T2* indicate the *glaB* gene (DDBJ/EMBL/GenBank accession no. XM_001819466 in *A. oryzae*), integral membrane protein-encoding gene (*impB*, XM_001819467), ER membrane protein-encoding gene (*pkr1*, XM_001819469), monosaccharide transporter-encoding gene (*mstB*, XM_001819470), anaphasepromoting complex subunit-encoding gene (*apcB*, XM_001819471), taurine catabolism dioxygenase-encoding gene (*tauD*, XM_001819472), fungal-specific transcription factor-encoding gene (*fstA*, XM_001819473), and transcription factor-encoding gene (*rfeD*, XM_001272671 in *A. clavatus*), respectively. Box-like arrows indicate gene orientation.

Chapter III

High production of llama variable heavy-chain fused to glucoamylase (GlaB)

in submerged culture

Antibodies and their derivatives are proteins that possess specific and high-affinity binding ability. These abilities have been applied successfully to human therapy in the form of humanized antibodies, e.g., tocilizumab (Nishimoto *et al*. 2003), omalizumab (Spector 2004), and ustekinumab (Nora *et al*. 2010). Antibodies have traditionally and widely been used as reagents for the detection and purification of antigens. In the area of food safety, reliable and cost-effective detection of caffeine (Carvalho *et al*. 2010), aflatoxin (Uchigashima *et al*. 2009), and botulinum toxin (Ponmariappan *et al*. 2011) requires highly stable antibodies that can be produced at low cost.

Hamers-Casterman *et al*. (1993) reported a novel class of two heavy chain immunoglobulins, in addition to conventional four-chain immunoglobulins, in members of the camelid family (including camels and llamas). Because these antibodies are devoid of light chains and do not require a constant heavy chain domain to bind antigens, their heavy-chain variable domain (VHH) has been proposed as a potentially valuable biotechnological tool (Muyldermans 2001). Recently, other two heavy-chain immunoglobulins were found in various vertebrates, including cartilaginous fish (ratfish, shark, skates, and rays), monotreme (platypus), and marsupial (koala) (Flajnik *et al*. 2011). Because an antibody consisting of a single polypeptide is more easily manipulated through protein engineering, this class of antibodies has been the subject of significant research effort, e.g., improved thermal stabilization (Hagihara *et al*. 2007; Hussack *et al*. 2011).

Aspergilli have a long history of use in biotechnology as expression platforms for the

production of various excreted homologous and heterologous proteins (Meyer *et al*. 2011). In a first report, non-fused VHH were produced by *Aspergillus awamori*, resulting in functional VHH excreted into culture medium (Joosten *et al*. 2005a). Following this early study, VHH fusion proteins (e.g., VHH-peroxidase) were produced (Joosten *et al*. 2005b). These studies have shown that VHH and fused VHH fusion proteins could be produced, but at low levels (7.5 and 30 mg protein/l culture medium, respectively), which is inadequate for large-scale industrial application.

A. oryzae is capable of secreting a significant amount of proteins into a culture medium (Archer and Peberdy 1997). Production of a heterologous target protein fused to homologous secreted proteins such as Taka-amylase and glucoamylase A (Hirayama *et al*. 2010; Ohno *et al*. 2011) is one means of overcoming the problem of low productivity. In Chapter III, the author describes the expression of VHH against human chorionic gonadotropin (hCG) in *A. oryzae* by fusing VHH to the C-termini of four highly secreted homologous leader proteins. C-terminal His-tag sequences were added to confirm that full-length VHHs were produced. The VHH proteins were assayed for native binding ability, and evaluated for overproduction.

Materials and methods

Strains and growth conditions   The *A. oryzae* OSI1013 leu-5, a *leuA* mutant (deposited with IPOD as FERM P-20079), derived from wild-type *A. oryzae* OSI1013 was used throughout the transformation experiments for target gene expression and production (Ishida *et al*. 2006b). GPY medium was used for growing the *A. oryzae leuA* mutant and transformants. *E. coli* HST08 strain (TaKaRa Bio) was used for DNA manipulations.

Plasmid construction and transformation of *A. oryzae*   The plasmids constructed and used in Chapter III are summarized in Table 5-1 and Table 5-2, respectively. Plasmid phlACB which contains the *hlyA* promoter and *glaB* terminator (Bando *et al*. 2011) was used as the target gene expression cassette. All target genes and vectors were amplified by PCR using *A. oryzae* genomic DNA, the phlACB plasmid, and a synthetic VHH-encoding gene (Hokkaido System Science, Sapporo, Japan; Fig. 5-1), to construct the plasmids in Chapter III. As an example of how the *amyA-His* (*amyA*, DDBJ/EMBL/GenBank accession no. M33218) expression vector pAA was constructed, the leader protein-encoding gene and common expression vector (CEV) were amplified by PCR separately, after which the protein-encoding DNA fragment was subcloned into CEV. Initially, the CEV was amplified by PCR using vec-u and vec-d primers and phlACB plasmid as a template. Next, the *amyA* gene fragment was amplified by PCR with AA-u and AA-d primers and *A. oryzae* OSI1013 strain genomic DNA as template. The *amyA* gene fragment was then subcloned into the CEV using an In-Fusion™ Advantage PCR cloning kit (TaKaRa Bio) to generate the expression vector pAA plasmid. In the case of VHH expression, e.g., *amyA-VHH-His*, the *amyA* gene was amplified using primer AA-d-VHH instead of AA-d. The common VHH-encoding gene fragment (CV) was amplified using VHH-u and VHH-d primers and the synthetic VHH-encoding gene as template. The *amyA* gene fragment and CV were then subcloned into the CEV using the same cloning kit to generate expression vector pAAV. Other expression vectors were generated similarly by PCR using the appropriate primers.

A. oryzae was transformed as described by Bando *et al*. (2011). DNA used for transformations was generated as described below. Each expression cassette was amplified by PCR using M13-P7 and M13-P8 primers and appropriate constructed plasmids as templates. The isopropylmalate isomerase-encoding gene (*leuA*) from *A. nidulans* was amplified by PCR

using the same primer noted above in Bando *et al.* (2011). The resultant transformants were subcultured and selected on solid Czapek-Dox medium.

Table 5-1   Genes, plasmids and relevant proteins used in Chapter III

Gene	Source	Relevant protein
amyA	M33218[a]	α-amylase (AmyA[b])
glaB	AB007825[a]	glucoamylase (GlaB[b])
celA	D83731[a]	endo-1,4-β-glucanase (CelA[b])
celB	D83732[a]	endo-1,4-β-glucanase (CelB[b])
VHH	1G9E_A[c]	heavy-chain antibody fragment (VHH[b])
His		6xHis-tag (His[b])
Plasmid	Expressed gene	Relevant protein
pAA	*amyA-His*	AmyA-His
pAAV	*amyA-VHH-His*	AmyA-VHH-His
pGB	*glaB-His*	GlaB-His
pGBV	*glaB-VHH-His*	GlaB-VHH-His
pCA	*celA-His*	CelA-His
pCAV	*celA-VHH-His*	CelA-VHH-His
pCB	*celB-His*	CelB-His
pCBV	*celB-VHH-His*	CelB-VHH-His

[a] DDBJ/EMBL/GenBank accession no.   [b] Abbreviation   [c] PDB accession no.

```
CAGGTCCAGCTCCAGGAGTCCGGTGGTGGTCTCGTCCAGGCCGGTGGTTCCCTCCGCCTC    60
TCCTGGGCCGCCTCCGGTCGCACCGGTTCCACCTACGACATGGGTTGGTTCCGCCAGGCC   120
CCCGGTAAGGAGCGCGAGTCCGTCGCCGCCATCAACTGGGACTCCGCCCGCACCTACTAC   180
GCCTCCTCCGTCCGCGGTCGCTTCACCATCTCCCGCGACAACGCCAAGAAGACCGTCTAC   240
CTCCAGATGAACTCCCTCAAGCCCGAGGACACCGCCGTCTACACCGCCGGTGCCGGTGAG   300
GGTGGTACCTGGGACTCCTGGGGTCAGGGTACCCAGGTCACCGTCTCCTCC           351
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Fig. 5-1   Nucleotide sequence of a synthetic VHH-encoding gene (PDB accession no. 1G9E_A).

Table 5-2 PCR primers

Primer	Sequences (5′ to 3′)
vec-u	GGTGTTGTGGTGTGAAGGGTGATTGATGTG
vec-d	CACCATCACCACCACCACTAAATGTACTTTCCAGTGCGTGTAGTCTACTCT
AA-u	TCACACCACAACACCATGATGGTCGCGTGGTGGTCTCTATTTCTG
AA-d	GTGGTGGTGATGGTGCGAGCTACTACAGATCTTGCTACCTGCCAA
GB-u	TCACACCACAACACCATGCGGAACAACCTTCTTTTTTCCCTCAAT
GB-d	GTGGTGGTGATGGTGCCACGACCCAACAGTTGGGGTAGTGTAACT
CA-u	TCACACCACAACACCATGAAGCTCTCATTGGCACTTGCTACGCTC
CA-d	GTGGTGGTGATGGTGGTTGACACTGGCAGTCCAGTTGGGAACTGT
CB-u	TCACACCACAACACCATGATCTGGACACTCGCTCCCTTTGTGGCA
CB-d	GTGGTGGTGATGGTGATGCCTGTAGGTAGATCCAATATCTCCCCA
VHH-u	CAGGTCCAGCTCCAGGAGTCCGGTGGTGGT
VHH-d	GTGGTGGTGATGGTGGGAGGAGACGGTGACCTGGGTACCCTGACC
AA-d-VHH	CTGGAGCTGGACCTGCGAGCTACTACAGATCTTGCTACCTGCCAA
GB-d-VHH	CTGGAGCTGGACCTGCCACGACCCAACAGTTGGGGTAGTGTAACT
CA-d-VHH	CTGGAGCTGGACCTGGTTGACACTGGCAGTCCAGTTGGGAACTGT
CB-d-VHH	CTGGAGCTGGACCTGATGCCTGTAGGTAGATCCAATATCTCCCCA
M13-P7	GGGTAACGCCAGGGTTTTCCCAGTCACGAC
M13-P8	GTGAGCGGATAACAATTTCACACAGGAAAC

Purification of His-tagged proteins   Transformants were grown in 100 ml GPY medium at 30°C for 3 d. To recover culture media, mycelia were removed by filtration using a 0.45-μm filter. The culture medium was adjusted to pH 7.4, and loaded onto a His GraviTrap™ system (GE Healthcare Bio-Sciences, Little Chalfont, United Kingdom) from which the His-tagged proteins were purified as described by the manufacturer. Impurities were first removed by use of a wash buffer (20 mM sodium phosphate, 500 mM NaCl, 10 mM imidazole, pH 7.4). His-tagged proteins were then recovered by elution with 20 mM sodium phosphate, 500 mM NaCl, and 500 mM imidazole, pH 7.4. Desalting and buffer exchange were performed by loading eluates onto PD-10 desalting columns (GE Healthcare Bio-Sciences) as described by the manufacturer.

Deglycosylation of purified proteins with endoglycosidase H (Endo H)   The purified protein was deglycosylated with endoglycosidase H (Endo H from *Streptomyces plicatus*; Merck KGaA, Darmstadt, Germany) using the modified protocol. The protein was diluted to 0.5 mg/ml using denaturing reaction buffer (50 mM sodium phosphate, 0.1% sodium dodecyl sulfate [SDS], and 50 mM β-mercaptoethanol, pH 5.5), and incubated at 80°C for 10 min. The denatured protein sample was then treated with Endo H (final concentration 0.1 U/ml) at 37°C for 3 h.

Binding assay of fused VHH protein against hCG   The purified proteins were immobilized on CNBr-activated sepharose™ 4B (GE Healthcare Bio-Sciences) using the standard protocol supplied by the manufacturer. Each purified protein (40 μg) and the activated sepharose beads, 20 μl, were mixed to immobilize the purified protein. The binding assay using the immobilized sepharose beads and human chorionic gonadotropin (hCG, Wako Pure Chemical, Osaka, Japan) was performed as described (Hagihara *et al*. 2005). The protein-immobilized sepharose beads, 20 μl, and hCG, 10 μg, were incubated for 90 min at

room temperature in binding buffer (20 mM Tris-HCl, 150 mM NaCl, pH 8.0). After the beads were washed with binding buffer three times, they were boiled for 15 min at 95°C with 10 μl of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. The supernatant recovered from the beads was loaded onto an SDS-PAGE gel (Mini-PROTEAN TGX™ Any kD gels, Bio-Rad Laboratories) and stained with Coomassie Brilliant Blue (CBB; GelCode Blue Safe Protein Stain™, TaKaRa Bio).

His-tagged protein staining in polyacrylamide gel His-tagged proteins in polyacrylamide gels were stained using a commercial staining kit (InVision™ His-tag In-gel Stain, Life Technologies, Carlsbad, CA). Stained bands were detected using a UV transilluminator and signal intensity was quantified by Image J software (Abramoff *et al*. 2004).

Detection of hCG-binding activity of fused VHH protein by enzyme-linked immunosorbent assay (ELISA) A microtiter plate (type H 96-well microtiter plate; Sumitomo Bakelite, Tokyo, Japan) was coated overnight at 4°C with 20 μg hCG (20 mM Tris-HCl, pH 8.0) and then washed three times with 20 mM Tris-HCl buffer (pH 8.0). After the plate was blocked with blocking buffer (1% BSA, 20 mM Tris-HCl, pH 8.0) for 3 h at room temperature, it was washed three times with binding buffer. After addition of the purified protein, the plate was incubated for 3 h at room temperature followed by another wash with binding buffer. This was then followed by addition of substrate solution (50 mM sodium citrate, 5 mg/ml maltose, 15 ng/ml 3,3′,5,5′-tetramethylbenzidine [TMB], 10 U/ml glucose oxidase from *A. niger*, 10 U/ml peroxidase from horseradish, pH 5.0). After incubation for 30 min at room temperature, 1 M sulfuric acid solution was added to stop the reaction (Christensen *et al*. 1995).

Results

Overexpression and overproduction of leader proteins and fused VHH proteins

The VHH gene was fused to genes encoding leader proteins amylase (AmyA-His), glucoamylase (GlaB-His), and endo-glucanases (CelA-His, and CelB-His) to generate cassettes for expression in *A. oryzae* (AmyA-VHH-His, GlaB-VHH-His, CelA-VHH-His, and CelB-VHH-His, Table 5-1). Transformants harboring each expression cassette were cultured in GPY medium at 30°C for 3 d, after which culture media were analyzed by SDS-PAGE analysis (Fig. 5-2). Although the CelA-VHH-His protein was produced at a very low level, the other proteins were produced more efficiently, as the apparent molecular weight of fused VHH protein increased. Each VHH fusion protein was about 10 kDa larger than the corresponding leader protein.
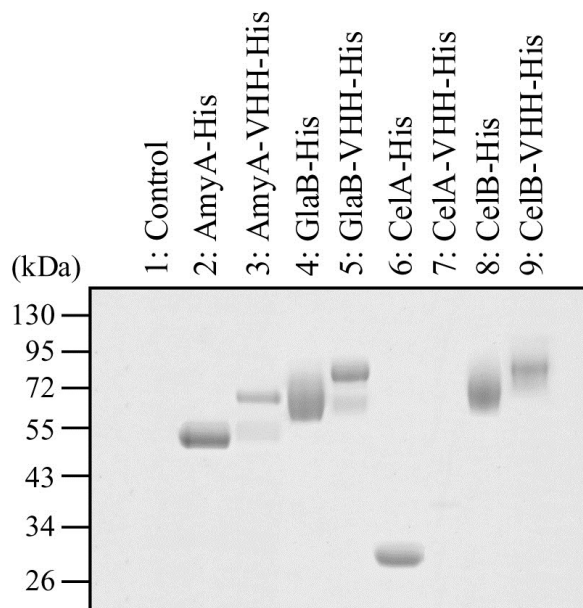
Fig. 5-2 Overproduction of VHH fusion proteins by *A. oryzae* transformants. Supernatants (7.5 μl) from 3-day culture media were analyzed by SDS-PAGE. Control (lane 1), leader proteins AmyB-His (lane 2), GlaB-His (lane 4), CelA-His (lane 6), and CelB-His (lane 8) and VHH-leader fusion proteins AmyB-VHH-His (lane 3), GlaB-VHH-His (lane 5), CelA-His (lane 7), and CelB-VHH-His (lane 9).

Purification of VHH fusion proteins and deglycosylation   VHH fusion proteins were purified using a commercial His-tag column. Concentrations of the purified proteins were determined using BSA as a standard. The GlaB-VHH-His protein was recovered at the highest concentration, about 0.155 mg protein/ml culture medium. AmyA-VHH-His and CelB-VHH-His were recovered at 0.033 and 0.101 mg protein/ml culture medium, respectively. Very little of the CelA-VHH-His protein was detected, less than 0.005 mg protein/ml culture medium.

The apparent molecular weight of the purified proteins was larger than the calculated molecular weight (Fig. 5-3). To determine exact molecular weight of the fused VHH proteins, a deglycosylation treatment was performed. AmyA-VHH-His, GlaB-VHH-His, and CelB-VHH-His (calculated molecular weight of 65.8, 64.0, and 56.0 kDa, respectively) were detected by SDS-PAGE analysis (Fig. 5-3). These results indicate that the full-length fused VHH proteins were produced by *A. oryzae*.
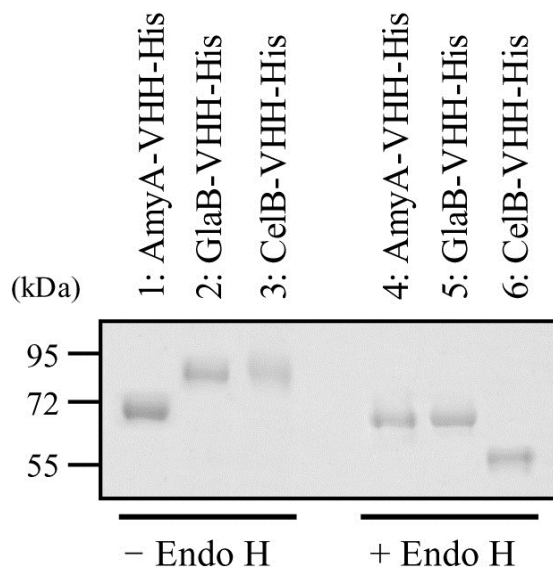
Fig. 5-3   Deglycosylation of VHH fusion proteins by Endo H treatment. Purified VHH fusion proteins incubated with Endo H (lanes 4-6), and without Endo H (lanes 1-3) were analyzed by SDS-PAGE. Lanes 1 and 4, purified AmyA-VHH-His; lanes 2 and 5, purified GlaB-VHH-His; lanes 3 and 6, purified CelB-VHH-His.

Binding assay of VHH fusion proteins with the hCG antigen   The GlaB-His and purified VHH fusion protein GlaB-VHH-His were immobilized on sepharose, and the presumptive immobilized sepharoses were incubated with hCG, washed, and then treated with SDS-PAGE sample buffer to elute the bound hCG. hCG was detected in the eluent from the GlaB-VHH-His immobilized sepharose by SDS-PAGE analysis. hCG was detected in the eluent from the GlaB-VHH-His-immobilized sepharose, but not in those from the GlaB-His-immobilized and non-immobilized (Fig. 5-4). These results indicate that hCG binds specifically with the VHH region of the VHH fusion protein. Similar results were obtained with the other VHH fusion proteins, AmyA-VHH-His and CelB-VHH-His.
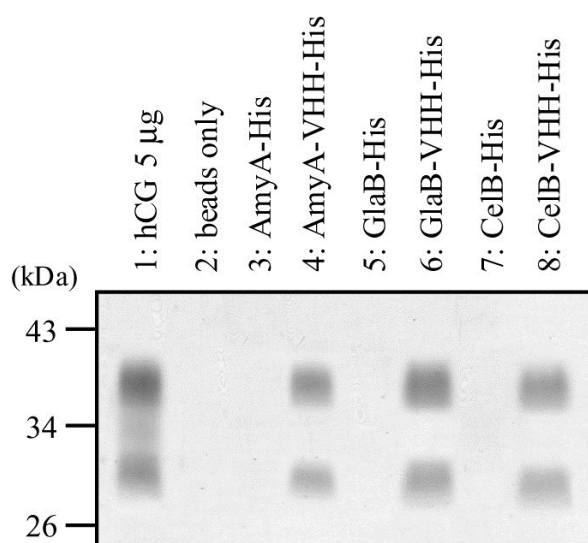
Fig. 5-4   Binding of the hCG antigen to immobilized and purified VHH fusion proteins. Leader proteins AmyB-His (lane 3), GlaB-His (lane 5), and CelB-His (lane 7) and VHH fusion proteins AmyB-VHH-His (lane 4), GlaB-VHH-His (lane 6), and CelB-VHH-His (lane 8) were immobilized on sepharose beads. Beads and hCG were incubated at room temperature for 90 min. After washing and elution, bound hCG was subjected to SDS-PAGE.

Detection of the hCG antigen by ELISA using glucoamylase activity   Because purified GlaB-VHH-His had glucoamylase activity, the author demonstrated the coupled glucoamylase assay involving glucoamylase, glucose oxidase, and peroxidase in an ELISA-based format to confirm binding ability of GlaB-VHH-His. While addition of GlaB-His did not cause color formation in the microtiter plate coated with hCG, addition of GlaB-VHH-His did at least 0.1 μg (Fig. 5-5).
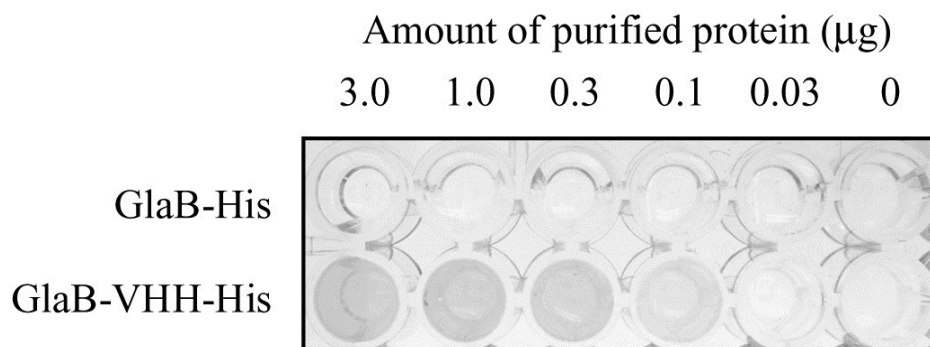
Fig. 5-5   Detection of the hCG antigen with GlaB-VHH-His using glucoamylase activity. The hCG-binding activity of GlaB-VHH-His was tested by ELISA. Microtiter plates were coated with hCG and various amounts of purified GlaB-VHH-His were subsequently loaded onto the plates. Bound GlaB-VHH-His was detected using the coupled enzymatic assay (based on addition of glucose oxidase and peroxidase and substrates maltose and TMB). Purified GlaB-His was used as a control to confirm non-specific binding.

Production of GlaB-VHH-His Because higher amount of GlaB-VHH-His was produced than any of the other fusion proteins, the author analyzed GlaB-VHH-His productivity by the *A. oryzae* transformant. Ammonium sulfate (final concentration, 1% w/v) or potassium phosphate (final concentration 100 mM) was added to the culture medium to prevent proteolysis, or to improve secretion efficiency, respectively (Sato *et al.* 2011). The amount of GlaB-VHH-His was determined by SDS-PAGE analysis, using purified GlaB-VHH-His as a standard. Using CBB staining, low molecular weight signals were detected in addition to the expected band corresponding to the standard. Staining with the His-tag specific reagent generated signals only corresponding to the expected band for GlaB-VHH-His. By CBB staining, 7-day culture media with or without potassium phosphate addition indicated the highest productivity. Using the His-tag-specific stain, the 5-day culture medium with potassium phosphate addition had the highest productivity (1.35 mg protein/ml, Fig. 5-6). Using the His-tag column, GlaB-VHH-His was recovered at 0.610 mg protein/ml above-mentioned culture medium. Addition of ammonium sulfate caused lower productivity than non-addition and addition of potassium phosphate.
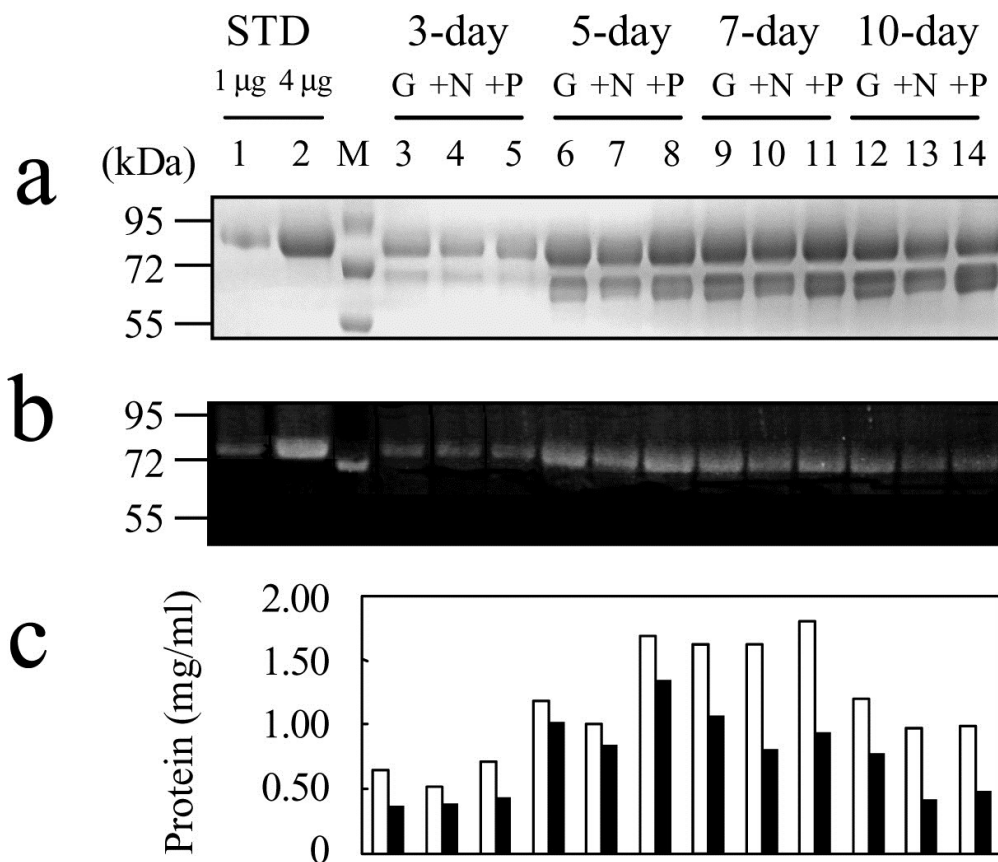
Fig. 5-6 Time course of GlaB-VHH-His production by *A. oryzae* transformant harboring *glaB-VHH-His* expression cassette. Supernatants (2.5 µl) from culture media were analyzed by SDS-PAGE by CBB staining (**a**) and by His-tag-specific staining (**b**). Signal intensity was estimated with imaging software (**c**). Purified GlaB-VHH-His was used as a standard (lane 1, 1 µg; lane 2, 4 µg). 3-day cultivation (lanes 3-5), 5-day cultivation (lanes 6-8), 7-day cultivation (lanes 9-11), and 10-day cultivation (lanes 12-14). Lanes 3, 6, 9, and 12, GPY medium with no addition; lanes 4, 7, 10, and 13, GPY medium with ammonium sulfate addition; lanes 5, 8, 11, and 14, GPY medium with potassium phosphate addition; lane M, protein ladder. In **c**, the empty box indicates the calculated amounts of GlaB-VHH-His from CBB staining while the filled box indicates calculated amounts of GlaB-VHH-His from His-tag-specific staining.

Discussion

In Chapter III, four highly secreted proteins were used as leader proteins fused to VHH in order to achieve high-level production and secretion in *A. oryzae*. The productivity of the fusion proteins was greatly affected by the choice of the leader protein. As the apparent molecular weight of the leader protein increased, productivity improved. The author speculated that the apparent molecular weight of the leader protein might play a role in protection against proteolysis, because the productivity of the fusion proteins was higher than that of VHH alone produced by *A. awamori* as reported in previous studies (Joosten *et al.* 2005a, b).

The ability of the VHH fusion proteins to bind hCG was determined using the same method tested for constructs previously expressed in *E. coli* (Hagihara *et al.* 2005). The author detected similar binding ability for constructs expressed in *A. oryzae*.

To confirm binding ability of the fused VHH proteins in a practical ELISA-based format, a coupled enzymatic assay was developed for the GlaB-VHH-His construct that had glucoamylase activity. By this assay, as little as 0.1 μg per well of the GlaB-VHH-His fusion protein was detectable. An ELISA requires a first antibody to bind its target antigen and a secondary antibody typically conjugated to peroxidase or alkali phosphatase for detection of binding. Although the first antibody was replaced by VHH alone produced by *E. coli* and *A. awamori*, a secondary antibody was found necessary for detection in previous work (Hagihara *et al.* 2005; Joosten *et al.* 2005a). In contrast, our results here show not only that a secondary antibody was not necessary for detection, but also that the GlaB-VHH-His was more suitable for practical use than peroxidase-VHH fusion protein (Joosten *et al.* 2005b).

For large-scale industrial application, more than 1 g of VHH fusion protein/l is required

(Krüger *et al*. 2006). The author examined a high-level production of the GlaB-VHH-His construct. Although 1.35 mg of protein/ml culture medium was obtained for full-length GlaB-VHH-His by His-tag staining, yields of the purified protein using His-tag column were much lower. In *A. niger*, it was reported that a C-terminal His-tag was easily degraded, resulting in low binding ability to His-tag affinity columns. In fact, immunoblot analysis using an anti-His-tag antibody was able to detect non-adsorption of His-tagged proteins in the flow through fractions from the affinity columns (Roth and Dersch 2010), indicating that actual GlaB-VHH-His productivity was close to that estimated using the His-tag-specific stain. An ELISA-based estimate using the glucoamylase activity indicated more than 1.0 mg protein/ml culture medium.

Degradation of GlaB-VHH-His could have occurred over long-term cultivation (Fig. 5-5a, b) as described for a peroxidase-VHH fusion protein in *A. awamori* (Joosten *et al*. 2005b). The host strain used for production of GlaB-VHH-His in Chapter III possesses proteases that might be responsible for degradation of expressed GlaB-VHH-His. Hence the use of a protease-deficient host might increase protein production dramatically (Yoon *et al*. 2011). In Chapter III, the author demonstrated high-level production of a VHH fusion protein, approaching industrially relevant levels. Not all tested leader proteins were found to be effective in terms of yields of the fusion constructs. Incorporation of an enzymatically active leader protein allowed development of a novel ELISA for sensitive detection of the desired protein as an additional advantage of this approach. The author anticipates that the method described here should be promising also for the overproduction of valuable heterologous proteins and peptides.

Conclusions

A final and long-cherished goal of fermentation industries in Japan is to manufacture a high quality of "liquid *koji*" that contains a significant amount of saccharifying enzymes such as glucoamylase and α-amylase for use in modern industrial manufacture of good quality of traditional Japanese fermentation foods such as sake, *mirin, miso* and soy sauce. Due to lack of detailed information about the mechanisms and regulation of expression of saccharifying enzyme-encoding genes in *Aspergillus oryzae*, an essential microbe for *koji* manufacture, a good quality of *koji* has so far been and can only be manufactured in a solid-state culture with a delicate and complicated process by craftsmanship of skilled brewers. Therefore, it is critically important to understand how the gene expression is controlled depending on culture conditions in *A. oryzae,* a specifically bred fungus over years for *koji* manufacture (solid-state culture). The present study describes gene expression and promoter analysis of the genes that are expressed in a culture-condition dependent manner in *A. oryzae*. This study reveled in Chapter I that the expression of two catalase isozyme-encoding genes, *catA* and *catB*, is controlled independently by culture conditions and in response to H_2O_2.

Since gene expression is regulated directly by its promoter, the analysis of promoters is important in understanding the regulation mechanism of culture-condition-specific gene expression. The promoter analysis of submerged (liquid) culture-specific expression gene (*sodM*) showed that the promoter carried unique *cis*-elements that are not found in other *Aspergilli*, suggesting that *A. oryzae* would be bred specifically for use in fermentation as a fungus of high safety. In contrast, the promoter analysis of the gene expressed under various culture conditions (*catB*) showed that the promoter held universal *cis*-elements conserved widely in other *Aspergilli* and that the gene was regulated similarly. Lastly, the analysis of the

promoter of solid-state culture-specific expression gene (*glaB*) revealed that the promoter harbored unique *cis*-elements that are found in a limited number of species of *Aspergilli*, a genetic trait suggesting that *A. oryzae* have been intentionally selected for sake brewing among *Aspergilli*. The results of the promoter analyses of three genes revealed several unique features of *A. oryzae* in *Aspergilli* as a result of breeding over years for sake brewing, and suggested the potential of *A. oryzae* as a benign and useful microbe of high safety and fidelity for liquid *koji* manufacture in the future that is suitable for highly mechanized fermentation industries.

A. oryzae is also an attractive mold as a host for production of valuable proteins from different organisms. A llama variable heavy-chain antibody (VHH) fused with glucoamylase was produced and secreted in submerged culture of *A. oryzae*. The fusion protein was functional and had an antigen binding ability as the original VHH. Furthermore, the glucoamylase activity derived from the fusion leader protein was employed successfully as a marker for single protein ELISA. The expression and secretion of the fusion protein as much as over 1 g/l was achieved in a liquid culture. These results expand the scope of *A. oryzae* as a valuable host for production of heterologous and useful proteins. This might lead to a "functional sake" with an additional health benefit as a medicated beverage by including proteins useful for keeping healthy conditions.

The author believes that the results and expertise shown in this study should give an important step toward transition from traditional solid-state *koji* to more sophisticated submerged (liquid) *koji* for efficient and elegant manufacture of sake and other traditional Japanese fermentation foods.

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Acknowledgments

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