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Current Topics in Molecular Biotechnology of a Koji Mold, *Aspergillus oryzae*

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Summary

Aspergillus oryzae is an important filamentous fungus in Japanese fermentation industries and produces a large amount of starch-degrading enzymes, whose gene expressions are regulated by a transcription activator AmyR. We have recently found a gene cluster highly homologous to the yeast maltose-utilizing cluster and showed that expression of the cluster genes is independent on AmyR but is involved in assimilating maltose, a product by starch degradation. In this article are described structural features and expression profiles of the maltose-utilizing cluster genes in *A. oryzae*. On the other hand, *A. oryzae* has been expected as a favorable host microorganism for heterologous protein production. In order to understand the bottlenecks for hyperproduction of heterologous proteins in *A. oryzae*, we have investigated the cellular responses using the mutated α-mannosidase of *Aspergillus saitoi* as a model protein. Interestingly, the mutated α-mannosidase was secreted as a hyperglycosylated form when overexpressed.

I. Structure and expression mechanism of maltose-utilizing gene cluster in *Aspergillus oryzae*

A. oryzae has been widely used in food production in Japan, such as sake, soy sauce and soybean paste (miso) manufacturing. *A. oryzae* produces a copious amount of various hydrolytic enzymes. Among them, amylolytic enzymes such as α-amylase, glucoamylase, and α-glucosidase are most important in sake making. Production of these enzymes is induced in the presence of starch or malto-oligosaccharides. A transcriptional activator gene, *amyR*, involved in the amylolytic gene expression has been cloned[1,2]. The *amyR* encodes 604 amino acid residues of a putative DNA-binding protein carrying a zinc binuclear cluster motif ($Zn(II)_2Cys_6$). The *amyR* gene disruptants showed a significantly poor growth on starch medium and produced little amylolytic enzymes including α-amylase and glucoamylase compared with a non-disruptant, indicating that

amyR is a transcriptional activator gene involved in starch/maltose-induced efficient expression of the amylolytic genes in *A. oryzae*[1]. In contrast to the restricted growth on starch medium, the *amyR* disruptants showed normal growth on maltose medium, indicating the existence of another maltose-utilizing system, whose expression might not be controlled by the *amyR*.

We could find an EST clone homologous to the yeast *Saccharomyces cerevisiae* maltase gene (*MAL62*) in the *Aspergillus* EST database (http://www.nrib.go.jp/ken/EST2/index.html). Since this cDNA clone could have whole coding region according to the 5′-sequencing analysis, we have determined the sequence of the isolated cDNA. The cDNA has an ORF capable of encoding a protein of 574 amino acids. The deduced amino acid sequence of the cDNA showed high homology (46%) to that of the yeast *MAL62* gene[3], and thus the protein encoded by the isolated cDNA seems to be maltase or α-glucosidase, which is probably involved in maltose utilization in *A. oryzae*. In *S. cerevisiae* maltase gene (*MAL62*) constitutes a gene cluster with maltose permease (*MAL61*) and a transcriptional activator (*MAL63*) genes[4]. Therefore, we supposed the possibility of the existence of a maltose-utilizing gene cluster in *A. oryzae*, as in *S. cerevisiae*. Approximately 10,000 clones of *A. oryzae* genomic library based on EMBL3 were screened with the EST clone as a probe, 10 positive clones were isolated. Restriction mapping of the clones revealed the isolation of two different genomic clones. Southern hybridization analysis of the isolated clones indicated that one contains *MAL62* homologue itself and another may have a gene with relatively low homology to the probe. We have sequenced over 10-kb DNA fragment inserted in a representative phage clone containing *MAL62* homologue, and have found three genes which are linked together to form a maltose utilization gene cluster as shown in Fig. 1.

These three genes have high homology to genes in the yeast *MAL* locus: (a) *malP* gene highly homologous to the *MAL61* encoding maltose permease; (b) *malT* which is *MAL62* homologue; (c) *malR* which has homology to the *MAL63*

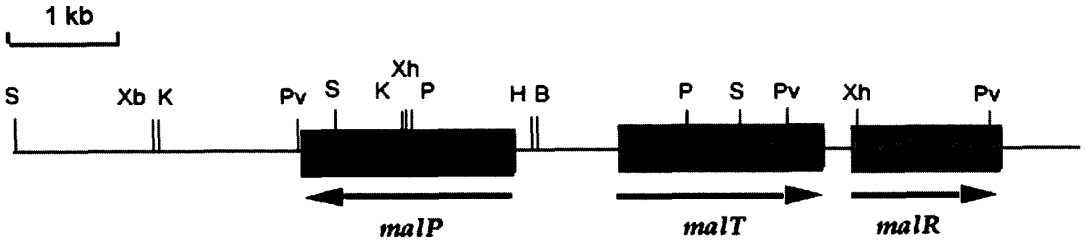

FIG. 1. Partial restriction map of the maltose utilization gene cluster in *Aspergillus oryzae*.
The direction of transcription of the genes is shown by an arrow. Restriction sites are abbreviated as B, *Bam*HI; H, *Hind*III; K, *KpnI*; P, *Psi*A; Pv, *Pvi*II; S, *Sal*II; Xb, *Xba*I; Xh, *Xho*I.

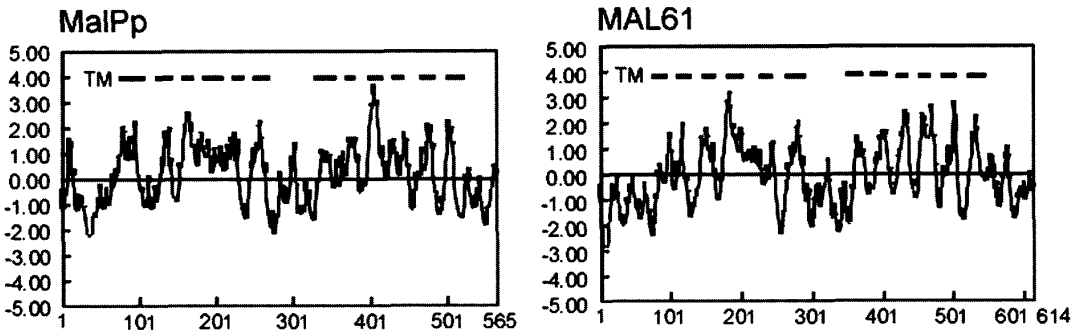

FIG. 2. Hydropathy profiles of the *A. oryzae* and *S. cerevisiae* maltose permeases. MalPp, *A. oryzae* maltose permease: MAL61, *S. cerevisiae* maltose permease TM, transmembrane segment

encoding a transcriptional factor. Gene organization of the cluster resembles well to the yeast *MAL* locus, although the regulatory gene (*malR*) gene is located downstream of the maltase gene (*malT*) but not upstream of the permease gene (*malP*). The *malP* and *malT* genes are transcribed divergently with an intergenic region of 1155 bp. Additionally, all three genes have no intron sequence. The gene cluster identified in this study was designated *MAL* cluster in *A. oryzae*.

The *malP* gene encodes a transmembrane protein of 539 amino acid residues. Comparison of deduced amino acid sequences of the sugar transporters showed that the MalPp has high homologies (40–44% identity and 60–63% similarity) to the yeast maltose permeases and α-glucoside permease. The hydropathy plot of the MalPp showed that there exist 12 putative membrane-spanning segments with a high degree of hydrophobicity in the protein[5] (Fig. 2). These observations indicated that the MalPp could transport maltose or relevant α-glucoside molecules across a membrane.

The hydropathy plot of the MalTp revealed the absence of the putative signal peptide sequence, indicating that the MalTp would be intracellular α-glucosidase or maltase. The BLAST search showed the MalTp has the highest homology (55% identity and 73% similarity) to yeast *Candida albicans* maltase[6], and high homology (43-49% identity and 63-66% similarity) to *Hansenula* (AF261762), *Saccharomyces*[3], and *Kluyveromyces* (AJ007636) α-glucosidases. When the *malT* gene was expressed under control of the high-level expression promoter[7] in *A. oryzae*, high α-glucosidase activity was observed in the cell-free extract of the transformant. In addition, a distinct band corresponding to the molecular size of the MalTp was detected on SDS-PAGE. These results suggest that the *malT* actually encodes functional α-glucosidase responsible for liberating glucose from maltose.

The *malR* gene is located immediately after the *malT* gene with only a 231-bp spacer from the *malT* stop codon to the *malR* translation start codon. The *malR* gene has an ORF capable of encoding a DNA-binding protein of 465

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AomalR   13 CDACGRRKVRCNGQQRCQQCEHMGLVC 39   A. oryzae
AoamyR   28 CDNCRRRKIKCSRELPCDKCQRLLLSC 54   A. oryzae
ScmalR   13 CDCCRIRRVKCDGKRPCSSCLQNSLDC 39   S. cerevisiae
AosugR    7 CDGCSLRKTRCSGGQPCQPCVQSGFEC 33   A. oryzae
ApsugR    7 CDGCSLRKTRCSGGQPCQPCAQSGFEC 33   A. parasiticus
AnxlnR   55 CDQCNQLRTKCDGQHPCAHCIEFGLTC 81   A. niger

Consensus: CD C  rk r/C    PC  C       C
                    k
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FIG. 3. Multiple sequence alignment of the zinc binuclear motifs of fungal transactivators for hydrolytic gene expression

amino acid residues. Deduced amino acid sequence of MalRp showed the presence of a zinc binuclear DNA-binding motif located close to the N-terminus in the region from amino acid residue 13 to 39. This motif has the similarity to the zinc finger of the fungal transcriptional activator proteins containing the consensus sequence, Cys-X_2-Cys-X_6-Cys-X_{5-6}-Cys-X_2-Cys-X_6-Cys[8]. Search for possible homologies in the DNA database revealed that the zinc finger motif of the MalRp has significant homologies to the fungal hydrolytic regulatory proteins (Fig. 3). Amino acid sequence of MalRp zinc finger motif showed 40% identity and 66% similarity to that of AmyRp in *A. oryzae*[1,2]; 37% identity and 55% similarity to that of Mal63p in *S. cerevisiae*[9]; 51% identity and 55% similarity to the *sugR* gene product in *A. parasiticus*[10]. However, the MalRp overall showed relatively low homologies (20–22% identity and 36–40% similarity) with transcriptional activator proteins.

The expression of the genes in *MAL* cluster was examined by Northern blot analysis. The transcripts of the *malP* and *malT* genes were detected in the presence of maltose but not in the absence of maltose, while that of the *malR* gene was observed irrespective of carbon sources. In addition, in the presence of maltose three genes were expressed also in the *amyR* gene disruptant. These indicated that the expression of the *malP* and *malT* genes is induced by maltose, but that of the *malR* gene is constitutive, and furthermore these three genes are not regulated by AmyR. In conclusion, the genes in *MAL* cluster could be responsible for the assimilation of maltose in *A. oryzae*.

II. Cellular response to the overexpression of mutated or exogenous secretory proteins in *Aspergillus oryzae*

Filamentous fungi have been used as hosts that produce useful proteins because of their ability to secrete proteins at a very high level. The protein expression systems have been developed in several filamentous fungi and had a great success in secreting the recombinant proteins derived from eukaryotic

microbes. However, the efficiency of secretion of higher eukaryotic proteins was quite poor in many cases. One of the reasons for a decreased production could be the instability of exogenously expressed proteins in the secretory pathway. In eukaryotic cells, it is known that aberrant secretory proteins are subjected to a protein quality control and degraded intracellularly to maintain their cellular homeostasis. Therefore, the protein production, a situation where exogenous proteins are overexpressed, might put stresses on host cells. In this study, *Aspergillus oryzae* was used as a host strain to analyze the cellular response to the overexpression of a mutated secretory protein.

Tatara *et al.*[12] showed that the cystein residue-443 of 1,2-α-mannosidase from *Aspergillus saitoi* (msdS) was important to maintain its conformation and the substitution of Cys-443 to Phe (C443F) resulted in a marked decrease in its secretion from *A. oryzae* cells, suggesting that the mutant enzyme was subjected to a protein quality control in the host cells. Therefore, the mutant version of msdS was expressed in *A. oryzae* to analyze the cellular response to aberrant proteins loaded into the secretary pathway. Since the expression level is an important consideration to analyze the response to the expression of aberrant proteins, two different promoters were used in this study, i.e. one was the strong No.8142 promoter, and the other was a more moderate *enoA* promoter. Using the resultant transformants, production of the wild-type and C443F msdS proteins was examined with SDS-PAGE and immunoblotting. The wild-type msdS proteins were efficiently secreted to the medium with both promoters, although amount of msdS protein was more than tenfold higher from the No.8142 promoter than the *enoA* promoter. On the other hand, the mutant msdS protein was not detected in the medium by Coomassie Brilliant Blue staining and thus we tried to detect the mutant protein by immunoblotting. The mutant enzyme was still undetectable even by immunoblot analysis when expressed from the *enoA* promoter. In contrast, when overexpressed, the mutant enzymes were found in the medium and extensively subjected to the hyperglycosylation. To test the possibility that the mutant enzyme was accumulated inside the cells, the total cell extract was subjected to immunoblot analysis. At the moderate expression level, only a faint band of the C443F enzyme was detected, although a significant amount of the wild-type enzyme was observed, suggesting that the mutant enzyme was degraded rather than accumulated inside the cells. No obvious difference in amounts of enzymes was seen when overexpressed. Interestingly, the mutant enzyme observed in the cell extract was not hyperglycosylated, suggesting that the mutant enzymes with the hyperglycosylation were efficiently secreted to the medium.

Next, we examined the cellular response to the overexpression of the mutant msdS proteins at the transcription level. Using cDNA microarrays comprising about 5,000 non-redundant EST clones from *A. oryzae*[13], the transcriptions were compared between the strains expressing wild-type and mutant enzymes. The

transcriptions of *bipA* and *pdiA*, which might be involved in protein folding in ER, were increased in the cells expressing the mutant enzyme, suggesting that the overexpression of the mutant msdS induced the unfolded protein response (UPR). Northern analysis confirmed that the overexpression of the mutant enzyme induced the transcriptions of ER chaperons, while the moderate expression did not.

Only one amino acid substitution (Cys-443 to Phe) caused instability of 1, 2-α-mannosidase in *A. oryzae*. At the moderate expression level, the mutant protein might be degraded through the secretory pathway, although the mechanism of how it is degraded is unclear. The hyperglycosylation of secretory proteins occurs at the Golgi apparatus in yeast. Therefore, we reasoned that the degradation might occur at ER because no hyperglycosylated mutant protein was detected in the cell lysate. When overproduced, the mutant proteins could bypass the ER-associated degradation and transport to Golgi apparatus where the hyperglycosylation occurs. The hyperglycosylated mutant proteins were efficiently secreted to the medium. The mutant protein detected in the cell lysate might be localized at ER because they were not hyperglycosylated, and the accumulated aberrant proteins could induce the UPR.

Acknowledgments

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