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# Characterization and expression analysis of a maltose-utilizing (*MAL*) cluster in *Aspergillus oryzae*

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ABSTRACT

Starch and maltooligosaccharides such as maltose and maltotriose induce the production of amylolytic enzymes including α-amylase in *Aspergillus oryzae*. A transcriptional activator gene *amyR*, required for maltose induction of amylolytic enzymes, has been cloned and characterized. The *amyR* gene deletion mutant showed significantly poor growth on starch medium but normal growth on maltose medium. This indicated the existence of another maltose-utilizing system, whose expression might not be controlled by *amyR*. We have identified a gene cluster homologous to the *MAL* cluster of *Saccharomyces cerevisiae* in the *A. oryzae* genome. The cluster consists of a *MAL61* homolog (designated *malP*), a *MAL62* homolog (designated *malT*), and a *MAL63* homolog (designated *malR*). Overexpression of *malT* in *A. oryzae* resulted in a significant increase in intracellular α-glucosidase activity, and that of *malP* allowed *S. cerevisiae mal61Δ* to grow on maltose. The expression of both *malP* and *malT* genes was highly up-regulated in the presence of maltose, but *malR* expressed constitutively irrespective of carbon sources. Disruption of *malR* resulted in the loss of *malP* and *malT* expression and thus in restricted growth on maltose medium. In addition, a *malP* disruptant showed a significantly reduced expression of *malT* and *malR* and exhibited a growth defect on maltose similar to the *malR* disruptant. These results suggest that the *MAL* cluster of *A. oryzae* is responsible for the assimilation of maltose in *A. oryzae*.



## 1. Introduction

A filamentous fungus *Aspergillus oryzae* is used in traditional Japanese fermentation industries including sake, soy sauce, and soybean paste (*miso*) manufacturing (Hara et al., 1992; Machida et al., 2008). *A. oryzae* produces copious amounts of amylolytic enzymes such as α-amylase (Taka-amylase A), glucoamylase, and α-glucosidase. Production of these enzymes is induced in the presence of starch or maltooligosaccharides, but not glucose (Gomi et al., 2000; Tada et al., 1989; Tonomura et al., 1961). A transcriptional activator gene *amyR* involved in amylolytic gene expression has been cloned (Gomi et al., 2000; Petersen et al., 1999). The *amyR* gene encodes 604 amino acid residues of a putative DNA-binding protein carrying a zinc binuclear cluster motif ($Zn(II)_2Cys_6$). AmyR binds to the *cis*-element sequence designated region III that occurs in the promoters of amylolytic genes (Petersen et al., 1999). The *amyR* gene disruptants showed a significantly poor growth on starch medium and produced few amylolytic enzymes including α-amylase and glucoamylase compared with a non-disruptant. These results indicated that *amyR* is a transcriptional activator gene involved in starch/maltose-induced efficient expression of the amylolytic genes in *A. oryzae* (Gomi et al., 2000). In contrast to the restricted growth on starch medium, *amyR* disruptants showed normal growth on maltose medium. This observation suggested that a certain maltose-utilizing system whose expression might be regulated independently of *amyR* might exist in *A. oryzae*.

In *Saccharomyces cerevisiae*, utilization of maltose is based on intracellular transport and hydrolysis of this disaccharide by maltose permease and maltase, respectively. These encoding genes are known to be clustered at the *MAL* loci; *MAL1*, *MAL2*, *MAL3*, *MAL4*, and *MAL6* (Charron et al., 1989; Michels and Needleman, 1984; Needleman, 1991; Needleman et al., 1984; Needleman and Michels, 1983). Each locus is comprised of a cluster of at least three different genes encoding maltose permease (*MALx1*), maltase (*MALx2*), and a transcriptional activator for these genes (*MALx3*) ("*x*" representing the number of *MAL* locus). *MALx3* has a zinc binuclear motif that is highly homologous to that of AmyR in *A. oryzae* (Gomi et al., 2000; Petersen et al., 1999). The genes of *S. cerevisiae* have been functionally analyzed, showing that the *MAL1* or *MAL6* gene is essential for maltose utilization (Chang et al., 1988; Charron et al., 1986; Cohen et al., 1984). We therefore speculated on the possibility of the presence of a maltose utilization system in *A. oryzae* similar to that in yeast, and found an EST clone (AoEST1652) homologous to the maltase gene (*MAL62*) in the *A. oryzae* EST database (Akao et al., 2007; http://www.nrib.go.jp/

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ken/EST2/index.html). By screening the phage library of *A. oryzae* using the EST clone as a probe, we isolated a positive clone that contained a gene homologous to yeast maltose permease gene (*MAL61*) located adjacent to the *MAL62* homologous gene (DDBJ Accession No. AB044389). Recently, we completed the whole-genome sequencing analysis of *A. oryzae* (Machida et al., 2005), and found that an additional gene homologous to the regulatory gene for maltose utilization in yeast (*MAL63*) is located immediately downstream of the genes that appear to encode maltose permease and maltase. This gene organization closely resembles the *MAL* cluster in *S. cerevisiae* and thus is thought to play an important role in maltose utilization in *A. oryzae*. In this study, we analyze the function and expression profiles of the genes contained in the cluster designated the *MAL* cluster in *A. oryzae*.

## 2. Materials and methods

### 2.1. Strains and culture conditions

*A. oryzae* RIB40 (Machida et al., 2005; Tada et al., 1989) and NS4 (*niaD*$^{-}$, *sC*$^{-}$) (Yamada et al., 1997) were used as a DNA donor and host strain for transformation, respectively. The functionality of the genes for maltose permease and transcription factor in the *A. oryzae MAL* cluster was examined by complementation of the *S. cerevisiae* strains deficient in maltose permease (CMY1050 (*mal61-Δ::HIS3*)) (Medintz et al., 2000) and regulatory factor (CMY1030 (*mal13Δ::G418*)) (X. Wang and C.A. Michels, personal communication), respectively. Isogenic wild-type strain CMY1001 (*MAT*a *MAL61/HA MAL12 MAL13 GAL leu2 ura3–52 lys2–801 ade2–101 trp1-Δ63 his3–200*) (Medintz et al., 1996) of these mutants was used as a control. CMY1001, CMY1030, and CMY1050 were kindly provided by Prof. C.A. Michels. *Escherichia coli* DH5α (*supF*44, *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1, *lacU*169/Φ80*lacZ*M15) was used to construct and propagate plasmid DNAs.

Czapek–Dox (CD) medium consisting of 0.3% $NaNO_3$, 0.15% KCl, 0.1% $KH_2PO_4$, 0.05% $MgSO_4$, trace amounts of $FeSO_4$, $ZnSO_4$, $CuSO_4$, $MnSO_4$, $(NH_4)_6Mo_7O_{24}$, and 1% sugar supplemented with appropriate nutrients was used as a minimal medium for *A. oryzae*. The sugar used as a carbon source in this study was 1% maltose, glucose, or glycerol for total RNA extraction from submerged cultures, and 0.1% starch, maltose, or glucose for growth characterization on agar plates. Minimal medium (YNB) for yeast contained 0.67% yeast nitrogen base without amino acids (Difco) supplemented with appropriate amino acids and bases. Modified YPD medium containing 0.5% yeast extract, 1% poly peptone, and 1% glucose was used as a complete medium for both yeast and mold. *A. oryzae* and *S. cerevisiae* strains were grown at 30 °C, and *E. coli* was grown at 37 °C.

### 2.2. Transformation experiments

The transformation of *E. coli*, *S. cerevisiae*, and *A. oryzae* was performed following the method of Inoue et al. (1990), Gietz and Schiestl (1995), and Gomi et al. (1987), respectively.

### 2.3. Complementation analysis of malP and malR in yeast deletion mutants

For the yeast complementation experiments, a YCp (yeast centromere plasmid) vector pRA-6 (Nagashima et al., 1994), which contains *URA3* as the selection marker and a promoter of *ADH1* for constitutive high-level expression, was used. Since both *A. oryzae malP* and *malR* have no introns, their ORFs were amplified by PCR with primers, malP FW-EcoRI + malP RV-EcoRI and malR FW-EcoRI + malR RV-EcoRI, respectively (listed in Table 1), digested with the restriction enzyme *Eco*RI, and cloned downstream of the *ADH1* promoter in pRA-6, yielding pYCmalP and pYCmalR, respectively. The resulting plasmids pYCmalP and pYCmalR were used to transform *S. cerevisiae* CMY1050 (*mal61Δ::HIS3*) and CMY1030 (*mal13Δ::G418*), respectively. For reference, the mutant strains and wild-type CMY1001 (29) were transformed with the empty vector pRA-6. The resulting transformants were pre-cultured in YNB liquid medium containing 1% glucose for 24 h, followed by incubation in YNB without a carbon source for 6 h, and were then patched onto the YNB agar medium containing 0.1% maltose or glucose.

### 2.4. Construction of the malT overexpression strain

An *Xba*I–*Eco*RI fragment containing the entire ORF of *malT* was isolated by digestion of the EST clone AoEST1652 (National Research Institute of Brewing, Higashi-Hiroshima) with restriction enzymes, and introduced into *Xba*I/*Eco*RI-digested pBluescript SK(+). The resultant plasmid was digested with *Hin*dIII and *Xba*I, and the fragment of *malT* ORF was inserted between the improved *glaA* promoter and *agdA* terminator of the expression vector pNGA142 (Minetoki et al., 2003), which contains the *niaD* gene as a selectable marker, yielding pNmalT. This plasmid was used to transform *A. oryzae* NS4 to construct the *malT* overexpression strain.

### 2.5. Construction of disruptants of each gene in the MAL cluster

Disruption of three different genes was performed by integrative disruption (Shortle et al., 1982). For disruption of the *malP* gene, a plasmid pDMP800 was constructed by ligating a *Bam*HI–*Eco*RV fragment of the *Aspergillus nidulans sC* gene from pUSC to pmalP800, which carries a truncated *malP* fragment lacking 0.07 kb and 0.75 kb of the ORF from the 5′- and 3′-termini, respectively, and inserted into the *Eco*RV–*Xho*I site of pBluescript KS(−) (TaKaRa). The resulting plasmid was digested with *Sph*I, the site which is unique to the truncated *malP*, and introduced into NS4 by restriction enzyme-mediated integration (REMI) (Sanchez et al., 1998) (Supplementary Fig. 1A).

For disruption of the *malR* gene, a 0.85-kb *Eco*T22I-*Bgl*II fragment containing a truncated *malR* that lacks 0.28- and 0.27-kb fragments of the coding region from the 5′- and 3′-termini, respectively, was cloned into *Pst*I–*Bam*HI digested pBluescript KS(−), resulting in pmalR850. Then, the *Kpn*I–*Xba*I fragment containing the truncated *malR* from pmalR850 was introduced into the *Xba*I–*Kpn*I site of pUSC, yielding pDMR850. The resulting plasmid was digested with *Psh*AI, the site which is unique to the truncated *malR*, and introduced into NS4 by REMI (Supplementary Fig. 1B).

For disruption of the *malT* gene, pDMT840 was constructed by inserting a *Xba*I–*Kpn*I fragment of the *sC* gene from pUSC into pmalT 840, which carries a truncated *malT* that lacks 0.7 kb and 0.2 kb of the coding region from the 5′- and 3′-termini, respectively, cloned into *Cla*I–*Bam*HI site of pBluescript KS(−). The resulting plasmid was digested with *Aat*II, the site which is unique to the truncated *malT*, and introduced into NS4 by REMI (Supplementary Fig. 1C).

All candidate strains for disruption of each cluster gene obtained were verified by Southern blot analysis.

### 2.6. Southern blot analysis

Fungal strains were grown in 50 ml of YPD medium at 30 °C with shaking (120 rpm) for 36 h. Genomic DNA was prepared by pulverization of the mycelium in liquid nitrogen and was purified as described previously (16). Briefly, the mycelium was harvested by filtration through Miracloth (Calbiochem), washed with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), flash-frozen in liquid