Introduction

*Aspergillus oryzae* has been used in the production of traditional fermented foods and beverages for over 1,000 years in Japan, for example, soy sauce, soybean paste, and rice wine. *A. oryzae* is recognized as safe and is listed as “generally recognized as safe” by the US Food and Drug Administration (FDA; Taylor et al. 1979, Machida et al. 2008). In the production of koji culture used as a starter culture for traditional fermented foods, *A. oryzae* (also called koji mold) grows on the surface of steamed rice or soybean. This process is important for the quality of fermented food because this growth affects the taste. *A. oryzae* produces various catabolic enzymes (e.g., protease and amylase) and substances that facilitate the growth of yeast and lactic acid bacteria in the koji culture.

*A. oryzae* grows in oxidative stress conditions for around 40 h during the production of koji culture. Oxidative stress damages DNA, proteins, and lipids and causes various disorders (Halliwell et al. 1994). It is suggested that oxidative stress tolerance is required for effective conidial germination of *A. oryzae* (Sakamoto et al. 2009). Oxidative stress tolerance is also important for efficient production of various enzymes, which may lead to reduction of cost in production of fermented food. Glutathione is generally involved in oxidative stress tolerance in microorganisms (Pócsi et al. 2004). In *Aspergillus nidulans*, the model organism representative of *Aspergillus*, glutathione is important for redox homeostasis (Thôn et al. 2007). It is likely that glutathione possesses a key role in oxidative stress tolerance in *A. oryzae*. Moreover, glutathione is related in “kokumi” flavor, which has properties such as continuity, mouthfulness and thickness, and is one of the tastes described by Ueda et al. (1997). Thus, glutathione may be related to the taste of fermented food.

Many enzymes are involved in glutathione metabolism. In most microorganisms, glutathione is synthesized through two consecutive reactions catalyzed by γ-glutamylcysteine synthetase and glutathione synthetase. In *Saccharomyces cerevisiae*, these enzymes are encoded by *GSH1* and *GSH2*, respectively. In the

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genome sequence of *A. oryzae* (Machida et al. 2005), using the genome database DOGAN; http://www.bio.nite.go.jp/dogan/project/view/AO, we found putative γ-glutamylcysteine synthetase genes and glutathione synthase genes, which are homologs of *S. cerevisiae* GSH1 and GSH2; thus, it appears that *A. oryzae* may have a similar glutathione synthesis pathway to *S. cerevisiae*.

A putative γ-glutamylcysteine synthetase gene (AO090012000764) is the focus of a first step toward characterizing the production of glutathione in *A. oryzae*. γ-Glutamylcysteine synthesis is thought to be the rate-limiting step of glutathione synthesis in *S. cerevisiae*. Glutathione accumulation was almost absent in a *S. cerevisiae gsh1* deletion mutant (Gales et al. 2008). The result obtained in this study suggests that this gene is implicated in glutathione synthesis and tolerance of oxidative stress in *A. oryzae*.

Materials and Methods

1. Strains, media, and culture conditions

 *Aspergillus oryzae* strain ΔligD ΔpyrG (Tada et al. unpublished), which is derived from *A. oryzae* strain RIB40, was used as the host strain for generating a mutant in which γ-glutamylcysteine synthetase expression was regulated by the thiA promoter (Shoji et al. 2005). Strain RIB40 was the DNA donor for genome sequencing analysis (Machida et al. 2005). Czapek-Dox (CD) medium (1%[w/v] D-glucose, 0.6%[w/v] NaNO₃, 0.15%[w/v] KH₂PO₄, 0.03%[w/v] KCl, 2%[w/v] agar, 0.2%[w/v] 1 M MgSO₄ solution, and 0.2%[v/v] 1 M MgSO₄ solution, and 0.1%[v/v] trace element solution [0.88%(w/v) ZnSO₄·5H₂O, 0.1%(w/v) FeSO₄·7H₂O, 0.04%(w/v) CuSO₄·5H₂O, and 0.01%(w/v) Na₂B₄O₇·10H₂O], pH6.0) was used for cultivation. Conidia (2 × 10⁵ in a 5 μL spot) were inoculated onto the solidified medium, followed by incubation at 30 °C. For glutathione assay, a spore suspension was spotted onto autoclaved membrane filter (A020A090C: Advantec, Tsukuba, Japan) and cultured on CD medium containing 0, 0.1, or 0.5 μM thiamine. CD medium containing 0 or 0.5 μM thiamine and 6 mM H₂O₂ was used to examine oxidative stress effects.

2. Glutathione assay

On the second day after inoculation, colonies were collected and frozen in liquid nitrogen. These samples were ground and added to 5%(w/v) 5-sulfosalicylic acid. The supernatant was collected by centrifugation at 15,000×g, 4 °C for 10 min and used as the intracellular glutathione extract. An HT Glutathione Assay Kit (Trevigen, Gaithersburg, USA) was employed for glutathione assay.

3. Construction of *gshA* conditional expression strain of *A. oryzae*

To create a transformant in which *gshA* gene expression was under the control of the *A. oryzae thiA* promoter, a DNA fragment containing the *thiA* promoter and *pyrG* was inserted upstream of *gshA* in *A. oryzae* ΔligD ΔpyrG by homologous recombination (Fig. 1A). The gene replacement construct was created by fusion PCR (Szewczyk et al. 2006), using Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific, Waltham, USA). PCR primer sequences are given in Table 1. The 5′- flanking regions of the *gshA* gene and the genes *gshA*, *thiA*, and *pyrG* were amplified from *A. oryzae* RIB40 genomic DNA using primer pairs gsh1-5F/gsh1-5R, gsh1-F/gsh1-R, thiA-F/thiA-R, and pyrG-F/pyrG-R, respectively. A mixture of these PCR products was then used as the template for PCR using primer pair gsh1-5F/gsh1-R. The resulting PCR product was introduced into the host strain by fungal transformation using the

| Primer   | Sequence (5’ to 3’)                  |
|----------|--------------------------------------|
| gsh1-5F  | TGGCTTGCAAAATCCAGCTAC                |
| gsh1-5R  | CAGGGGCCATAAAGCTTGTTAGCTGTTGGTGTAGTCTC |
| gsh1-F   | ATGGGTTGCTGTCAGTCC                    |
| gsh1-R   | TTACCTTCTTGGCCTCTAAAGAGCTCC          |
| thiA-F   | CCTCAGATGCAAAGAAGCCAG-TCACTATTGAAAGGTTCCAGTC |
| thiA-R   | CGACGTACAGCAAACCAATGGTCTTCAAGACTATCA |
| pyrG-F   | TTTGTCTAGATAGACTGACGCC               |
| pyrG-R   | ATCGGAAAGTTTACGACTCAA                |
| gsh1-5F  | ACCCTCTCTTGGGCGTAAG                  |
| gsh1-R   | GCCTCAGAGAAGGCTCC                    |
protoplast-PEG method of Gomi (1987). Insertion of the cassette into transformants was confirmed by PCR with primer pair gshl-5Fi/gshl-1-Ri, using genomic DNA extracted from transformants as the template.

Results and Discussion

We focused on a putative γ-glutamylecysteine synthetase gene (AO090012000764) of A. oryzae, which has the highest homology (38%) to S. cerevisiae GSH1 in amino acid sequence, according to DOGAN. This gene, which is named gshA, codes for a protein of 680 amino acids with predicted molecular mass of 77,256.3 Da. We planned to elucidate the function of gshA in A. oryzae. Initially, construction of a gshA deletion strain of A. oryzae was attempted, but this could not be obtained despite several trials. In transformation experiments involving A. oryzae, enzymatically prepared protoplasts are used for DNA uptake and regenerated on agar medium containing NaCl for osmotic stabilization (Gomi et al. 1987). Salt stress induces damage to mitochondria, which may be linked to oxidative damage (Hamilton & Heckathorn 2001). This result supports the conjecture may be linked to oxidative damage (Hamilton & Heckathorn 2001). That oxidative damage to A. oryzae protoplasts may have occurred during transformation, as has been previously observed in plants. If gshA is important in glutathione synthesis, the transformed protoplast whose gshA has been deleted may not be able to resist oxidative stress during its regeneration because it cannot synthesize adequate glutathione. The gsh1 mutant of S. cerevisiae was unable to grow on glutathione-deficient medium (Grant et al. 1997). However, the A. oryzae gshA disruptant could not be obtained using medium containing glutathione. Therefore, we concluded that gshA is essential in A. oryzae, at least for the cell wall regeneration process, and we decided to change our strategy for elucidation of gshA function.

Next, a conditional gshA expression strain of A. oryzae was constructed. The A. oryzae thiA promoter is known as a tool for molecular biological studies. The concentration of thiamine in medium can control the expression level from the thiA promoter (Shoji et al. 2005). Expression from this thiA promoter is repressed both transcriptionally and, to a greater extent, post- transcriptionally in the presence of thiamine (Shoji et al. 2005). To express the A. oryzae gshA gene under the control of the thiA promoter, a transformant was constructed where the thiA promoter was inserted upstream of gshA. This transformant was designated TGSH1. Figure 1A shows the structure of the modified gshA region of this strain. Insertion of the cassette into TGSH1 was confirmed by PCR with primer pair gshl-5Fi/gshl-Ri, using genomic DNA extracted from the host strain and TGSH1 as templates. The size of the PCR products was 3.0 KB from strain ΔligD ΔpyrG, and 6.0 KB from TGSH1 (Fig. 1B; lanes C and T, respectively). However, the growth of this mutant strain was defective compared with that of RIB40 (Fig. 1C). Repression of gshA gene by addition of thiamine to the medium did not change the phenotype (data not shown). It is possible that the insertion of the thiA promoter and pyrG gene in the upstream region of gshA in strain TGSH1 may affect expression of neighboring genes. There is predicted protein-coding gene (AO090012000763) near the upstream region of the gshA gene. This gene has high homology to the APSES transcription factor, which regulates morphological changes in fungi (Zhao et al. 2015). If the expression of the transcription factor gene changes, the growth of A. oryzae may be defective.

The total glutathione level in TGSH1 on the second day after inoculation when thiamine was added into the medium during the membrane culture of TGSH1 was examined. Membrane filter culture mimics solid-state culture conditions (Tamano et al. 2007) and enables easy collection of fungal bodies. In our experiments, without thiamine, the glutathione level in A. oryzae RIB40 and TGSH1 was 1.48 nmol/g dry weight and 1.97 nmol/g dry weight, respectively (Fig. 2). Addition of thiamine to the medium at 0.1 and 0.5 μM slightly increased the intracellular glutathione level in A. oryzae RIB40 strain, to 1.59 nmol/g dry weight and 1.73 nmol/g dry weight, respectively. However, a lower glutathione level in

![Fig. 1. Construction of gshA conditional expression strain of A. oryzae by homologous recombination.](image)

(A) gshA expression under the control of the thiA promoter. Right arrow: gshl-5Fi. Left arrow: gshl-Ri. (B) Insertion of the expression cassette into strain TGSH1 was confirmed by PCR with primer pair gshl-5Fi/gshl-1-Ri. Lane M: lambda HindIII; lane C: strain ΔligD ΔpyrG; lane T: strainTGSH1. (C) Phenotypes of strains RIB40 and TGSH1. These strains were cultured for three days.
TGSH1 in the presence of thiamine was observed. As Fig. 2 shows, the glutathione content of TGSH1 grown in the presence of 0.5 μM thiamine was decreased to 21.9% of the control level (0.43 nmol/g dry weight); and the glutathione content of TGSH1 grown in the presence of 0.1 μM thiamine was decreased to 86.3% of the level in the control culture (1.66 nmol/g dry weight). In *S. cerevisiae*, GSH1 is needed to accumulate glutathione (Grant et al. 1997). In our experiments, decreasing the expression of *gshA* by adding thiamine to the growth media caused reduction of intracellular glutathione in *A. oryzae* strain TGSH1. This means that *gshA* plays an important role in glutathione synthesis in *A. oryzae*.

Our results show only a 13.7% decrease of the glutathione level when TGSH1 was grown with 0.1 μM thiamine in solid culture at pH 6.0. The *thiA* promoter is highly responsive to thiamine. With 0.1 μM thiamine, gene transcription was almost repressed in liquid culture at pH 5.5 (Shoji et al. 2005). However, Shoji et al. (2005) reported that the *thiA* promoter failed to be regulated appropriately in alkaline conditions. It seems that differences in culture type and pH affect the responsiveness of this promoter to thiamine. The drop of intracellular glutathione level in TGSH1 remained at 21.9% of the control level even at 0.5 μM thiamine.

Next, the effect of intracellular glutathione levels on tolerance to oxidative stress was examined. The growth of *A. oryzae* RIB40 and TGSH1 with or without thiamine in the presence of H$_2$O$_2$ was observed. The addition of thiamine decreased the tolerance of TGSH1 to oxidative stress (Fig. 3). The colony size of TGSH1 reduced when 0.5 μM of thiamine together with H$_2$O$_2$ was added to the media, compared with the addition of H$_2$O$_2$ without thiamine. Considering the results in Fig. 2 and Fig. 3 together, there may not be enough intracellular glutathione in thiamine-treated TGSH1 to tolerate the cellular oxidative damage. These results show that the intracellular glutathione level is important for oxidative stress tolerance in *A. oryzae*, and that *gshA* plays a role in that tolerance.

In this study, it is suggested that *A. oryzae* *gshA* plays an important role in glutathione synthesis and in oxidative stress tolerance. We thought that glutathione synthesis and accumulation in *A. oryzae* are important for its growth in the production of koji culture. However, the function of other homologs involved in these mechanisms for glutathione accumulation remains to be elucidated in *A. oryzae*. Next, we will study glutathione synthetase (*GSH2* homolog) in *A. oryzae*. Furthermore, the γ-glutamyl peptide contributes to the taste of fermented food (Kuroda et al. 2013). Further unraveling of glutathione synthesis may lead to higher glutathione production in *A. oryzae*, which is expected to add value to fermented foods.
gshA is Important in Glutathione Homeostasis in Aspergillus oryzae

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