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Gamma-glutamylcysteine Synthetase Gene Homolog (*gshA*) is Important in Glutathione Homeostasis in *Aspergillus oryzae*

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Abstract

Aspergillus oryzae is a filamentous fungus used for the manufacture of medicines and fermented foods. Glutathione is a tripeptide contained in variety of organisms and is involved in tolerance to various stresses. *A. oryzae* has a putative γ-glutamylcysteine synthetase gene (*gshA*) in its genome. To investigate the effect of the gene expression level of *gshA* on the amount of glutathione in *A. oryzae*, we generated a transformant where *gshA* expression was regulated by the thiamine-repressible *thiA* promoter. When the transformant was cultured on CzapekDox (CD) agar with added thiamine, the intracellular level of glutathione in the transformant decreased in a dose-dependent manner. Results of this study discovered that in oxidative stress conditions, repression of *gshA* gene expression with thiamine caused inhibition of growth of the transformant. Also, it was found that the *gshA* gene plays an important role in glutathione homeostasis in *A. oryzae*, and this gene may also be necessary for oxidative stress response.

Discipline: Biotechnology
Additional key words: glutathione synthesis, growth, oxidative stress

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 *Aspergilli*, glutathione is important for redox homeostasis (Thön 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 synthase. 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. cerevisiaegsh1* 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 Δ*ligD* Δ*pyrG* (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] $NaNO_3$, 0.15%[w/v] KH_2PO_4, 0.03%[w/v] KCl, 2%[w/v] agar, 0.2%[v/v] 1 M $MgSO_4$ solution, and 0.1%[v/v] trace element solution [0.88%(w/v) $ZnSO_4 \cdot 5H_2O$, 0.1%(w/v) $FeSO_4 \cdot 7H_2O$, 0.04%(w/v) $CuSO_4 \cdot 5H_2O$, and 0.01%(w/v) $Na_2B_4O_7 \cdot 10H_2O$], pH6.0) was used for cultivation. Conidia (2×10^5 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 H_2O_2 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* Δ*ligD* Δ*pyrG* 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

Table 1. PCR primers used in this work

Primer	Sequence (5′ to 3′)
gsh1-5F	TGGCTTGTCAAATCCAGCTAC
gsh1-5R	CAGCGGCCATAAACGTGTTGATAGCTGTTGGGTTGTTGTAGTCTC
gsh1-F	ATGGGTTTGCTGTACGTCG
gsh1-R	TTACTTCTTGCCTCTAAGAAGCTCC
thiA-F	CCTCAGATGCAAAGACAAGGCCAG-TCACTATTGAAAAGGTTCCAGTC
thiA-R	CGACGTACAGCAAACCCATGTTTCAAGTTGCAATGACTATCA
pyrG-F	TTTGTCTGATAGACACTCAGCC
pyrG-R	ATCGCAACGTTTAGCACTCAAA
gsh1-5Fi	ACCTTCTCTTGGGCGTAAAG
gsh1-Ri	GCCTCTAAGAAGCTCCCATC

protoplast-PEG method of Gomi (1987). Insertion of the cassette into transformants was confirmed by PCR with primer pair gsh1-5Fi/gsh1-Ri, using genomic DNA extracted from transformants as the template.

Results and Discussion

We focused on a putative γ-glutamylcysteine 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 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* promotor 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 gsh1-5Fi/gsh1-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 Δ*ligD* Δ*pyrG*, 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

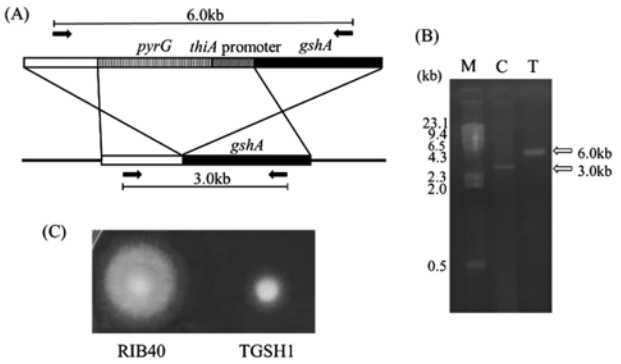

Fig. 1. Construction of *gshA* conditional expression strain of *A. oryzae* by homologous recombination.
(A) *gshA* expression under the control of the *thiA* promoter. Right arrow: gsh1-5Fi. Left arrow: gsh1-Ri. (B) Insertion of the expression cassette into strain TGSH1 was confirmed by PCR with primer pair gsh1-5Fi/gsh1-Ri. Lane M: lambda *Hin*dIII; 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_2O_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_2O_2 was added to the media, compared with the addition of H_2O_2without 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.

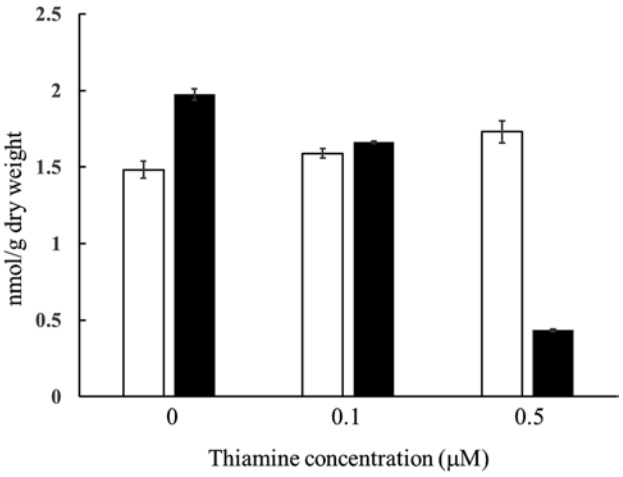

Fig. 2. Effects of thiamine on intracellular glutathione levels.
Strains RIB40 (□) and TGSH1 (■) were cultured on a membrane surface overlaid on solid medium containing 0, 0.1, or 0.5 μM thiamine. The values are means of three replicates with standard deviation.

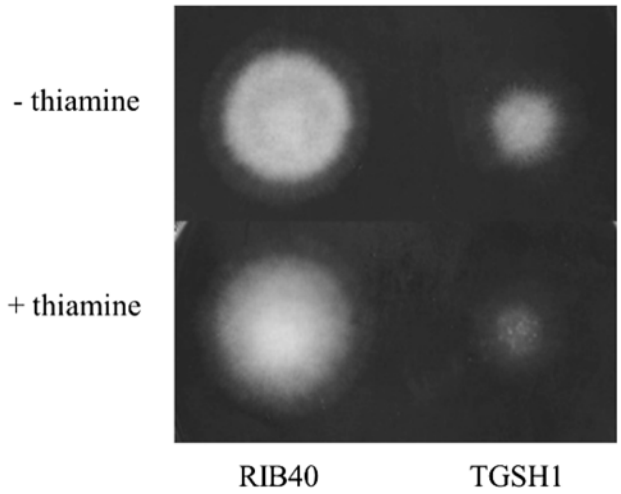

Fig. 3. Reduced resistance to oxidative stress by thiamine addition.
Strains RIB40 and TGSH1 were cultured on Czapek-Dox medium containing 6 mM H_2O_2 with thiamine at 0 or 0.5 μM for three days.

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