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Note

Characterization of the *gatA* gene from *Aspergillus oryzae*

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We analyzed the role of the gene encoding GABA transaminase (*gatA*) in *Aspergillus oryzae* by gene disruption. The *gatA*-disrupted strain could not grow on minimal medium with GABA as the sole nitrogen source. These results indicate that *gatA* is essential for GABA metabolism in *A. oryzae*.

Keywords: *Aspergillus oryzae*, *gatA*, GABA transaminase, GABA

γ-Aminobutyric acid (GABA) is a ubiquitous non-protein amino acid found in plants, animals, and microorganisms. It acts as a neuronal transmission inhibitor at inhibitory synapses in the brain by binding to a specific transmembrane receptor in the plasma membrane (1). Clinical administration of GABA regulates pain and anxiety and reduces blood pressure (2); furthermore, daily ingestion of GABA, which is approved as a dietary supplement, has a beneficial therapeutic effect for hypertension (3).

GABA is produced from glutamate by the action of glutamate decarboxylase in the GABA shunt pathway or from putrescine degradation; it is converted to succinate semialdehyde (SSA) through

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transamination by GABA transaminase (*gatA*). SSA dehydrogenase converts SSA to succinate (SUCC), which subsequently feeds into the TCA cycle. GABA transaminase activity has been detected in several fungal and yeast strains. Moreover, the GABA shunt pathway is conserved from bacteria to fungi, plants, and vertebrates (4).

GABA is a metabolic by-product of plants and microorganisms. Although it is not found in fresh food, it is produced by microbial fermentation. In Japan, GABA-rich dietary components such as sake and beni-koji (5) are marketed as supplements. Sake contains approximately 20 ppm GABA, and rice-koji contains approximately 200 mg GABA/kg dry koji (1). The filamentous fungus *Aspergillus oryzae* is widely used in the preparation of traditional Japanese fermented foods such as sake, soy sauce, miso, and mirin (6). GABA levels in this fungal strain must be enhanced to improve the health benefits of these foods. Therefore, we produced a *gatA*-disrupted mutant. This mutant could not degrade GABA, so GABA accumulated within *A. oryzae* cells. In this study, we investigated the role of *gatA* in *A. oryzae* by gene disruption.

gatA (XM_001823026) from *A. oryzae* was homologous to *gatA* from other organisms, showing 87%, 98%, and 100% homology to *gatA* from *A. nidulans*, *A. fumigates*, and *A. flavus*, respectively. The high degree of similarity indicated that *gatA* is conserved in *Aspergillus* species (7).

To analyze the expression of *gatA* using real-time PCR, we isolated total RNA and mRNA from *A. oryzae* RIB40. The strain was precultured at 30 °C for 24 h in YPD medium. The main culture was then established for 6 h at 30 °C in minimal medium (2% glucose, 0.3% KH_2PO_4, 0.1% KCl, and 0.1% $MgSO_4$) containing 100 mM GABA or 100 mM $(NH_4)_2SO_4$. Total RNA was extracted using RNAiso Plus (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. mRNA was purified using an *Oligotex-dT30<super>* mRNA Purification Kit (TaKaRa) following the manufacturer's protocol. Reverse transcription and real-time PCR were

performed using the SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen, Carlsbad, CA) (8). Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ technique (ABI User Bulletin 2). The sequences (5′→3′) of the *gatA* primers were GCAACTTCCCCTCTGCTGACT (forward) and GCGGTGAAGACCTGGTTCA (reverse). Histone H1 was used as an endogenous control. *gatA* expression was strongly induced by addition of 100 mM GABA and was 39-fold higher than with addition of 100 mM $(NH_4)_2SO_4$ (Fig. 1). In *Saccharomyces cerevisiae,* GABA transaminase is encoded by *UGA1*. When *S. cerevisiae* was grown with GABA as the sole nitrogen source, the expression of *UGA1* was similar to the expression of *gatA*. Thus, the real-time PCR assays indicated that *gatA* was inducible by GABA (9).

Δ*gatA* was created as described by Tamano *et al*. (10). The *gatA* gene disruption cassette was generated by fusion polymerase chain reaction (PCR) using an Expand High Fidelity PCR

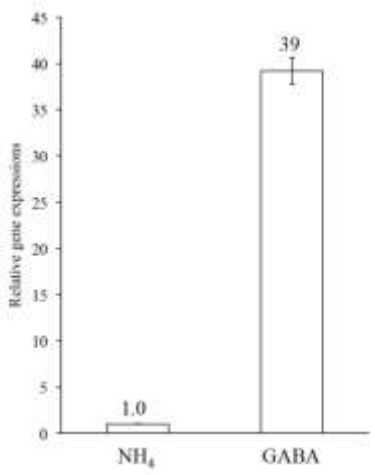

Fig. 1. Relative *gatA* expression analyzed by real-time PCR.

gatA expression in cells grown in minimal medium containing 100 mM $(NH_4)_2SO_4$ was set to 1.0. Error bar indicates standard deviation (n = 3).

System (Roche Diagnostics, Mannheim, Germany). The 5′- and 3′-arms of *gatA* were amplified from genomic DNA with the primers LU/LL (5′-arm) and RU/RL (3′-arm). *pyrG* was amplified with the primer pair PU/PL. Amplified fragments were purified using the Wizard Gel extraction kit (Promega, Madison, WI). The 5′-flanking:pyrG:3′-flanking amplicon (1:3:1 molar ratio) was used as a template. The PCR products were used for a second round of PCR with the primer pair LU/RL to fuse the 5′ and 3′

regions of the target gene at each end of the *pyrG* gene. The primer sequences (5′→3′) were as follows: CCATGATCTCGTAGAGAGG (LU), CATCACAGGGTACGTCTGTTGTGG TGTACAGAGGTGTGG (LL), AATCGCCACACCTCTGTACACCAC AACAGACGTACCCTGTGA (PU), AACCAATGAACGCCGGACTCTGGC AGATACAACTGCACCTC (PL), TCTTCTGAGGTGCAGTTGTATCTGC CAGAGTCCGGCGTTCATT (RU), and AGGTCCGTCGTCGCAATT (RL). The amplified fragment was purified with the Wizard Gel extraction kit and used for transformation.

A. oryzae strain Δ*ligD* (*pyrG*-, *niaD*-) was used as a host for transformation (11). The uridine prototrophic transformants were subjected to two consecutive rounds of single sporing. The control strain for these experiments was transformed with only *pyrG*. The *gatA*-disrupted mutant and control strains were grown on yeast-peptone-dextrose (YPD) medium at 30 °C for 24 h. DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturer's instructions. Disruption of *gatA* was confirmed by Southern blotting. Southern blotting of genomic DNA was performed following digestion with *Hind*III. The DNA fragment containing sequence upstream of *gatA* gene was used as a probe. The DIG-DNA labeling and detection kit (Roche) was used for signal detection. We observed the expected hybridization signals at 1.3 kb and 4.6 kb for digested genomic DNA isolated from the control and disrupted strains, respectively (Fig. 2).

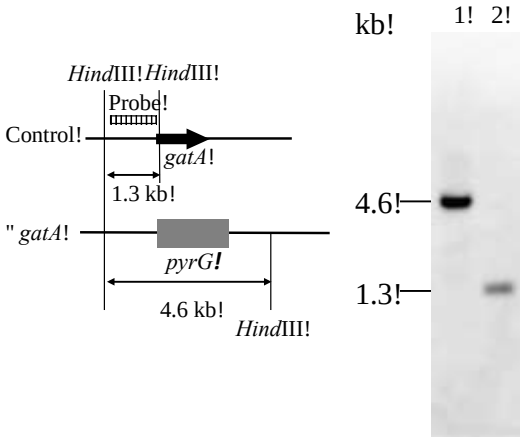

Fig. 2. Southern blot analysis of genomic DNA (20 μg/lane) from *gatA*-disrupted (lane 1) and control (lane 2) strains.

These results indicated successful homologous recombination at the resident *gatA* locus.

We observed that *A. oryzae* could efficiently utilize GABA as a nitrogen source. However, the *gatA*-disrupted strain did not grow on GABA plates (Fig. 3). These data suggest that *gatA* is essential for GABA metabolism in *A. oryzae*. An *S. cerevisiae* mutant strain lacking *UGA1* was incapable of growth on minimal medium with GABA as the sole nitrogen source. However, a *Ustilago maydis* strain with a GABA transaminase gene disruption (*ugatA*) was able to grow with GABA as the sole nitrogen source (12). This result indicated the presence of an alternative pathway for the utilization of GABA in *U. maydis*. These results revealed that the pathway for GABA metabolism is diverse among microorganisms.

In conclusion, we analyzed the function of *gatA* in *A. oryzae*. We found that *gatA* was inducible by GABA and that a *gatA*-disrupted strain could not be grown with GABA as the sole nitrogen source. These results indicate that *gatA*, the gene encoding a putative GABA aminotransferase, is essential for GABA metabolism in *A. oryzae*. Furthermore, the function of *gatA* in *A. oryzae* is similar to that of *UGA1* in *S. cerevisiae* but differs from that of *ugatA* in *U. maydis*.

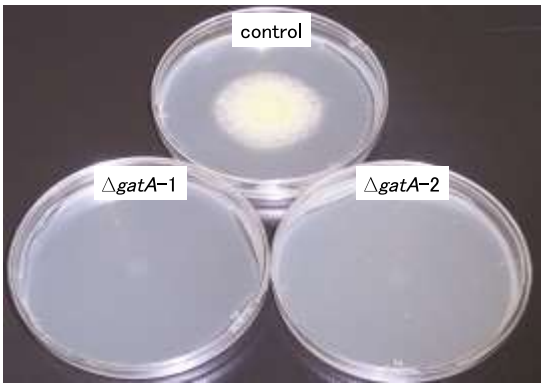

Fig. 3. Growth phenotype of *gatA*-disrupted strains. Control and *gatA*-disrupted strains were grown at 30 °C for 5 days on minimal medium containing 100 mM GABA.

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