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Isolation and characterization of sake yeast mutants with enhanced isoamyl acetate productivity

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Isoamyl acetate is an important flavor compound in sake. However, production of isoamyl acetate by *Saccharomyces cerevisiae* is significantly reduced during sake brewing with rice that has a high polishing ratio, because unsaturated fatty acids derived from the outer layer of rice repress the expression of *ATF1*, which encodes an alcohol acetyl transferase. Yeast mutants capable of relieving this repression would allow the brewing of rice with high polishing ratios, improving the diversity of taste and flavor of sake. Atf1p is also believed to contribute to biological membrane homeostasis. We isolated four yeast mutants (*hia1*, *hia2*, *hia4*, and *hia6*) that have high isoamyl acetate productivity and are resistant to aureobasidin A, an inhibitor of sphingolipid biosynthesis. The isoamyl acetate content of sake brewed with the *hia1* mutant was 2.6 times higher than that of the parental strain. *ATF1* was expressed constitutively in the *hia1* mutant during brewing and remained derepressed upon the addition of unsaturated fatty acids. Whole-genome sequence analysis of the *hia* mutants revealed a homozygous nonsense mutation (Ser706*) in *MGA2* in all four mutants. Mga2p, an endoplasmic reticulum (ER) membrane protein, regulates *ATF1* transcription. The expression of *ATF1* was elevated in BY4743 *Δmga2* cells complemented with *MGA2* (Ser706*), and this was not completely inhibited by the addition of unsaturated fatty acids. These results indicate that a nonsense mutation in *MGA2* induces high levels of isoamyl acetate production in *S. cerevisiae*. This finding has applications for brewing sake with high levels of isoamyl acetate.

[**Key words:** Sake yeast; Isoamyl acetate; Alcohol acetyl transferase; *ATF1*; *MGA2*]

Ginjo-shu is a type of sake that is brewed using highly polished rice with a polishing ratio of 60% or lower, for long periods at low temperatures. *Ginjo-shu* is characterized by a fruity flavor, referred to as *ginjo-ko*, which is mainly derived from isoamyl acetate and ethyl caproate. Isoamyl acetate is synthesized from acetyl-CoA and isoamyl alcohol by alcohol acetyltransferase (AATase, EC 2.3.1.84), which is encoded by *ATF1* or *ATF2* in yeast (1–4). Studies on *ATF1*/*ATF2* deletion mutants have revealed that Atf1p plays a major role in isoamyl acetate production in the brewing processes of beer (5). However, *ATF1* expression is inhibited by unsaturated fatty acids (6,7). Specifically, an 18-bp fragment that encodes the Rap1p-binding domain of the 5′-flanking region of *ATF1*, is crucial for transcriptional regulation of the gene by unsaturated fatty acids (8). In sake brewing using polished rice with a high polishing ratio, isoamyl acetate production by *Saccharomyces cerevisiae* markedly decreases because *ATF1* expression is repressed by unsaturated fatty acids derived from the outer layer of rice.

Several attempts have been made to develop yeast mutants with high isoamyl acetate productivity. Isoamyl acetate is synthesized from β-keto isocaproate, an intermediate in the L-leucine synthesis pathway. The first enzyme in the biosynthesis of leucine in yeast, α-isopropylmalate synthetase (9), is inhibited by L-leucine. In a mutant resistant to 5′,5′,5′-DL-trifluoroleucine, which is an analog for L-leucine, this enzyme is markedly resistant to inhibition by L-leucine. Hence, a mutant that produces large amounts of isoamyl acetate was bred by using resistance to 5′,5′,5′-DL-trifluoroleucine as an indicator (10). To further improve the production of isoamyl acetate, yeast mutants resistant to L-canavanine, 1-farnesylpyridinium, or hygromycin B have been isolated (11–13). Although these mutants exhibit high AATase activity, none is reported to inhibit the unsaturated fatty acid-mediated repression of *ATF1*. Therefore, these mutants have primarily been used for sake brewing using polished rice with a low polishing ratio.

The antibiotic myriocin (also known as ISP-1) inhibits a serine palmitoyltransferase that is the primary enzyme in sphingolipid biosynthesis. The gene *SLI1* exhibits weak similarity to *ATF1* and *ATF2* and encodes an *N*-acetyltransferase, the overexpression of which confers resistance to ISP-1 (14). Overexpressed Sli1p blocks the ISP-1-induced inhibition of sphingolipid biosynthesis (14). These findings indicate that *ATF1* may play an important role in not only esterification but also sphingolipid metabolism. Therefore, we attempted to isolate sake yeast mutants with enhanced isoamyl acetate productivity from mutants resistant to aureobasidin A (AbA), which inhibits inositol phosphorylceramide synthase within the sphingolipid biosynthesis pathway (15).

In this study, we report the brewing characteristics and *ATF1* expression of these mutants with increased isoamyl acetate production. Furthermore, we demonstrate that a nonsense mutation in *MGA2*, an *ATF1* transcription factor, induces constitutive expression of *ATF1* and inhibits the unsaturated fatty acid-mediated repression

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of *ATF1*. Discovery of mutants capable of inhibiting the unsaturated fatty acid-mediated repression of *ATF1* would allow the brewing of polished rice with high polishing ratios, and would help improve the diversity of taste and flavor of sake.

MATERIALS AND METHODS

Strains and media Sake yeast *S. cerevisiae* strain Km97 is an arginase-deficient mutant derived from K901 (a non-forming variant of Kyokai no. 9). BY4743 and BY4743 Δ*mga2* were provided by EUROSCARF (Hamburg, Germany). YPD medium (1% yeast extract, 2% Bacto-peptone, and 2% glucose) was used as a rich medium for yeast growth. SD10 medium (0.67% Bacto-yeast nitrogen base without amino acids and 10% glucose) was used for assays of *ATF1* expression and AATase activity. SC-URA medium (0.67% Bacto-yeast nitrogen base without amino acids, 2% glucose, and 0.077% CSM-URA) was used for the transformation of BY4743 Δ*mga2*. SC10-URA medium (0.67% Bacto-yeast nitrogen base without amino acids, 10% glucose, and 0.077% CSM-URA) supplemented with 1 mM linoleic acid and 1% Brij 58 as an emulsifier was used to assay *ATF1* expression in the BY4743 Δ*mga2* transformant. For all procedures involving *Escherichia coli*, strain DH5α was used. LB medium (1% Bacto-tryptone, 0.5% yeast extract, and 1% NaCl) containing 100 μg/mL ampicillin was used for the growth of *E. coli*.

Plasmids For the construction of pRS416-wMGA2 and pRS416-mMGA2, a DNA fragment containing the *MGA2*-coding region and 5′ and 3′ flanking sequences was amplified using TaKaRa Ex Taq (Takara Bio, Kusatsu, Japan) from the genomic DNA of either strain K901 or the *hia1* mutant by polymerase chain reaction (PCR), using the upstream primer 5′-GCAGCCCGGGGGATCCTTTCGTAGATTAAGACTGAA-3′ and the downstream primer 5′-TAGAACTAGTGGATCCCCCTCACAACCCCATCCC-3′, corresponding to positions −1018 to +3762. The underlined bases indicate sequences that are complementary to the ends of the linearized pRS416, a single-copy yeast vector (16), when treated with *Bam*HI. The resulting fragments were cloned into the *Bam*HI site of pRS416 using an In-Fusion HD Cloning Kit (Clontech, Mountain View, CA, USA).

Isolation of mutants resistant to AbA Strain Km97 cells were mutagenized by treatment with 4% ethyl methanesulfonate (EMS) in 100 mM phosphate buffer (pH 8.0) at 30°C for 1 h. Mutagenized cells were washed twice with 5% sodium thiosulfate and then twice with sterilized water. They were spread on YPD medium containing 1 μg/mL AbA and incubated at 30°C for 4 d. Mutants that grew on the medium were selected as AbA-resistant mutants.

Analysis of flavor compounds Headspace gas chromatography coupled with flame ionization detection (GC-FID) was used for the measurement of flavor components. The GC-FID was calibrated for isoamyl alcohol and isoamyl acetate. Samples were analyzed with a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan) with a Turbo Matrix HS40 headspace sampler (PerkinElmer Life Sciences, Waltham, MA, USA) and a capillary column of DB-WAX (length, 60 m; internal diameter, 0.32 mm; layer thickness, 1.2 μm; J&W Scientific, Folsom, CA, USA). Samples were heated at 50°C for 30 min in the headspace autosampler. The injection block and flame ionization detector temperatures were kept constant at 160°C and 200°C, respectively. The oven temperature was held at 40°C for 5 min and then increased to 100°C at 5°C min^{-1} and to 230°C at 20°C min^{-1} before being held at 230°C for 5 min (17). The content ratio of isoamyl acetate to isoamyl alcohol is represented as the E/A ratio (18).

Fermentation test Rice-saccharified liquid medium was prepared as follows. First, 64 g of α-rice and 36 g of rice-*koji* with a polishing ratio of 70% were mixed with 200 mL of water and incubated at 55°C for 4 h. The saccharified liquid was then diluted 1.5 times with water, and the titratable acidity was adjusted to a value of 4.0 with lactic acid. AbA-resistant mutants were inoculated in the rice-saccharified liquid medium and fermented at 15°C for 11 d. After recovery of the supernatant of the fermented medium by centrifugation, the isoamyl acetate content was analyzed by the headspace gas chromatography method described above.

Sake brewing Laboratory-scale sake brewing was carried out according to the method reported by Namba et al. (19) using 200 g of α-rice and rice-*koji* with a polishing ratio of 70%. The temperature of the sake mash was maintained at 15°C through the entire fermentation period, and the fermentation was monitored by measuring the weight reduction of the sake mash, which represents CO_2 evolution. When CO_2 evolution reached 60 g, the sake mash was centrifuged and the isoamyl acetate content of the supernatant was analyzed by the headspace gas chromatography method described above.

Assay of AATase activity Yeast cell-free extracts were prepared by disrupting yeast cells with glass beads using a multi-bead shocker (Yasui Kikai, Osaka, Japan) in buffer A (25 mM imidazole-HCl at pH 7.5, 0.1 M NaCl, 20% glycerol, 1 mM dithiothreitol, 46 mM isoamyl alcohol, and 0.1% Triton X-100) at 4°C and recovering the supernatant by centrifugation at 15,000 ×*g* for 10 min (4). AATase activity was measured as described by Minetoki et al. (20), with modifications. The reaction mixture consisted of 1 mL of cell-free extracts and 1 mL of buffer A containing 1.6 mM acetyl-coA. After incubation at 25°C for 1 h, the reaction was terminated by the addition of 2.25 mL of saturated NaCl solution. After addition of 0.75 mL of ethanol, the isoamyl acetate content was measured as described above. The protein concentrations of the cell-free extracts were measured with a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

AbA sensitivity test Yeast cells grown in SC-URA with shaking at 30°C for 24 h were harvested, washed with sterile water, and diluted with sterile water to an OD_{660} value of 1.0. Cell suspensions were serially diluted thrice by a factor of 10 each. Aliquots (5 μL) of each dilution were spotted onto SC-URA agar medium with or without 0.05 mM AbA, and incubated at 30°C for 2 d.

Quantitative real-time PCR Total RNA was extracted from yeast cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany). The quality of the total RNA sample was evaluated by measuring the OD_{260}/OD_{280} ratio. cDNA was synthesized from 1 μg of total RNA in a final volume of 20 μL, using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Quantitative real-time PCR (RT-qPCR) primers were designed with Primer3Plus (http://primer3plus.com/), as shown in Table S1 in the supplementary materials. cDNA (2 μL each) was used in 25-μL RT-qPCR mixtures with 10 μM primers and SYBR Premix EX Taq II (Takara Bio). RT-qPCR was performed with a Thermal Cycler Dice Real Time System II (Takara Bio). The thermal cycling conditions were 95°C for 30 s and 40 cycles of 95°C for 5 s and 60°C for 30 s. The ΔΔCt method was used for the relative quantification of gene expression (21). Gene expression levels were normalized to that of the transcription factor class C (*TFC1*) gene, and were expressed as percentages of control levels.

Whole-genome sequence analysis Strain Km97 and all the *hia* mutants were grown individually in YPD medium at 30°C for 1 d with shaking. Yeast cells were then harvested and washed twice with sterile water. Genomic DNA was extracted by using a Dr. GenTLE (from Yeast) High Recovery kit (Takara Bio). Genomic DNAs extracted from strain Km97 and the *hia1* mutant were sequenced individually. In addition, a pooled sample of the genomic DNAs of the *hia1*, *hia2*, *hia4*, and *hia6* mutants was also sequenced. Libraries for sequence analysis were prepared using the TruSeq Nano DNA LT Sample Prep Kit (Illumina, San Diego, CA, USA), and paired-end short reads of ∼100 bp were produced using Illumina HiSeq 2000, following the manufacturer's instructions.

RESULTS

Screening for yeast mutants with elevated isoamyl acetate production After four independent rounds of EMS mutagenesis, we isolated 475 AbA-resistant mutants derived from the parental strain Km97. We then performed a fermentation test with rice-saccharified liquid medium and found six mutants whose fermentation led to elevated levels of isoamyl acetate in the fermented medium compared with that of strain Km97. These were selected as candidates for high isoamyl acetate production (*hia1*–*6*). Finally, to investigate the isoamyl acetate content in sake brewed with *hia* mutants, a laboratory-scale sake brewing test was carried out using 200 g of α-rice and rice-*koji* with a polishing ratio of 70%. The isoamyl acetate contents of sakes brewed with the *hia1*, *hia2*, *hia4*, and *hia6* mutants were found to be 2.6, 3.0, 2.7, and 2.9 times higher, respectively, than that of

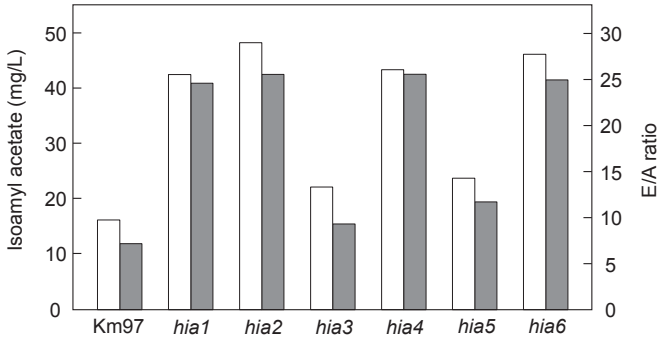

FIG. 1. Comparison of the isoamyl acetate contents and E/A ratios (ratio of isoamyl acetate to isoamyl alcohol) in sake brewed with the Km97 and *hia* mutant strains. Laboratory-scale sake brewing was carried out using 200 g of α-rice and rice-*koji* with a polishing ratio of 70%. The temperature of the sake mash was maintained at 15°C through the entire fermentation period. Open bars, isoamyl acetate contents; closed bars, E/A ratios.