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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

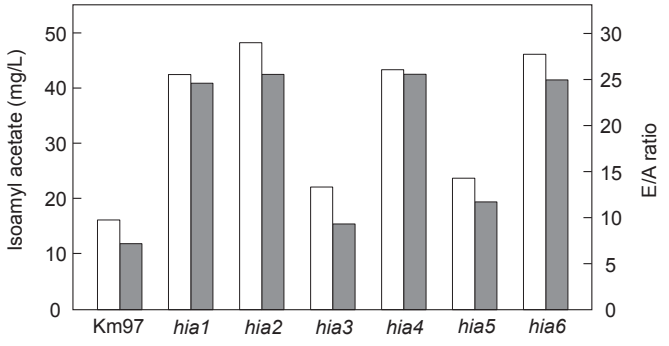

FIG. 1. Comparison of the isoamyl acetate contents and E/A ratios (ratio of isoamyl acetate to isoamyl alcohol) in sake brewed with the Km97 and *hia* mutant strains. Laboratory-scale sake brewing was carried out using 200 g of α-rice and rice-*koji* with a polishing ratio of 70%. The temperature of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

strain Km97 (Fig. 1). Because these mutants were isolated from independent EMS treatments, the mutants are not identical. Surprisingly, the E/A ratios, which are known to correlate highly with the sensory evaluation of *ginjo-shu* (18), of sakes brewed with these mutants were above 24.7 (Fig. 1), higher than any E/A ratio reported previously. These results indicate that the *hia1*, *hia2*, *hia4*, and *hia6* mutants can be used for the production of high levels of isoamyl acetate in the brewing of sake using polished rice with a high polishing ratio. The growth rate and fermentation rate of the *hia1*, *hia2*, *hia4*, and *hia6* mutants were slightly lower than those of strain Km97. However, these strains can be used for sake brewing at the industrial level without any adverse effects. We selected the *hia1* mutant for further study on the basis of the sensory evaluation of its sake.

AATase activity and expression of genes associated with the biosynthesis of isoamyl acetate In order to investigate whether the high isoamyl acetate production of the *hia1* mutant was caused by the activation of AATase, we measured AATase activity using a cell-free extract of yeast cells recovered from fermented sake mash. The AATase activity of the *hia1* mutant was over 4 times higher than that of strain Km97 (Fig. 2A). We also measured the expression levels of genes involved in the biosynthesis of isoamyl acetate through RT-qPCR of yeast cells recovered from the fermented sake mash. Expression of *ATF1* was significantly higher in the *hia1* mutant than in strain Km97, while there was virtually no difference between the two strains in the expression of *ATF2* or *IAH1*, which encodes an isoamyl acetate-hydrolyzing esterase (22) (Fig. 2B). These results indicate that the increase of AATase activity exhibited by the *hia1* mutant is likely due to elevated *ATF1* expression.

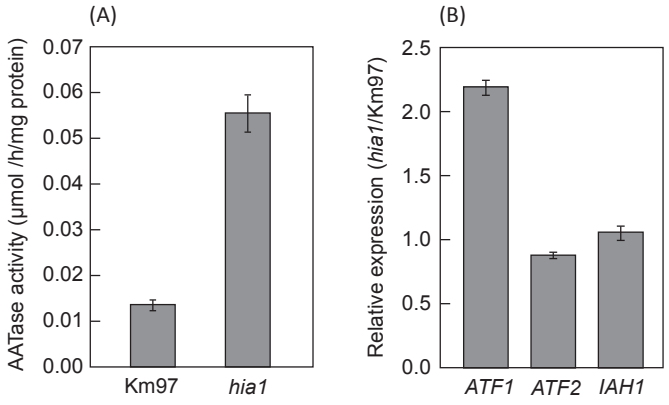

FIG. 2. AATase activity (A) and the expression of genes (*ATF1*, *ATF2*, and *IAH1*) associated with isoamyl acetate biosynthesis (B) during sake brewing in the Km97 and *hia1* mutant strains. Yeast cells were recovered from sake mash prepared using 200 g of α-rice and rice-*koji* with a polishing ratio of 70%, and fermented at 15°C for 6 d. (A) Alcohol acetyltransferase (AATase) activity was measured with cell-free extracts. (B) Quantitative real-time polymerase chain reaction (RT-qPCR) experiments with strain Km97 and *hia1* mutant cells. The relative expression is represented as the ratio of the expression levels obtained for the *hia1* mutant to those obtained for strain Km97. Values shown are the means from three independent experiments, and the error bars indicate standard deviations.

Effect of unsaturated fatty acids on *ATF1* expression The high isoamyl acetate production by the *hia1* mutant may indicate that the repression of *ATF1* expression by unsaturated fatty acids is being inhibited. Therefore, we investigated whether the AATase activity and *ATF1* expression of the *hia1* mutant could be repressed by the addition of unsaturated fatty acids to the growth medium. Cell-free extracts and total RNA were prepared from yeast cells grown in SD10 medium with or without 1 mM linoleic acid. As in the laboratory-scale sake brewing test, the AATase activity of the *hia1* mutant was significantly higher than that of strain Km97 in the absence of linoleic acid (Fig. 3A). In contrast to strain Km97, the AATase activity of *hia1* mutant was not inhibited by the addition of linoleic acid (Fig. 3A). Similarly, *ATF1* expression in the *hia1* mutant was higher than that in strain Km97, and the expression was not inhibited by the presence of linoleic acid (Fig. 3B), indicating that *ATF1* is expressed constitutively and that its repression by unsaturated fatty acids is eliminated in the *hia1* mutant.

The expressions of *ATF1* and *OLE1*, encoding a Δ-9 fatty acid desaturase, are regulated in a coordinated fashion in response to cell membrane fluidity; that is, the expression of both genes is repressed by unsaturated fatty acids (23,24). Therefore, we examined *OLE1* expression in the *hia1* mutant and found the same expression patterns as for *ATF1* (Fig. 3B, C). This indicates that the transcription factor responsible for the regulation of both *ATF1* and *OLE1* may be altered in the *hia1* mutant.

Whole-genome sequence analysis of the *hia1* mutant In order to explore the mechanism underlying the high isoamyl acetate production in the *hia1* mutant, we performed whole-genome sequence analyses on strain Km97 and the *hia1* mutant by using Illumina next-generation sequencing. The obtained sequences were aligned to the reference sake yeast genome sequence (Kyoukai no. 7, K7), and single-nucleotide polymorphisms (SNPs) between strain Km97 and the *hia1* mutant were identified

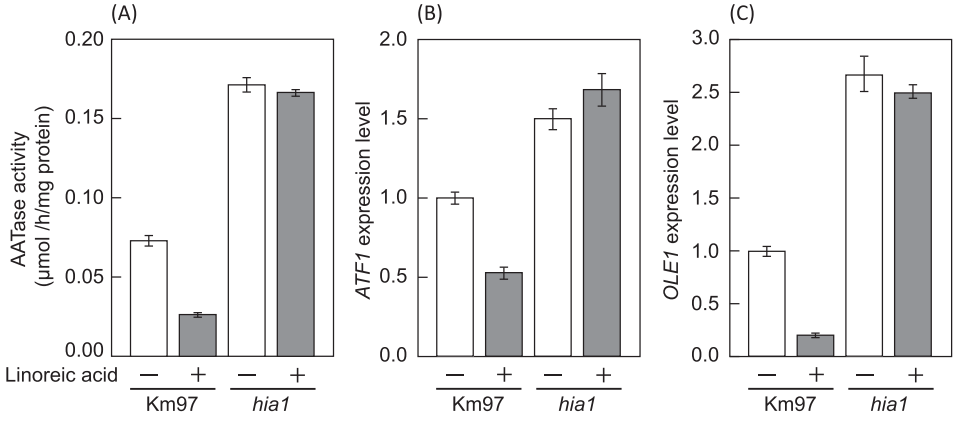

FIG. 3. AATase activity (A) and expression of *ATF1* (B) and *OLE1* (C) in Km97 and the *hia1* mutant strains in the presence and absence of linoleic acid. Values shown are the means of three independent experiments, and the error bars indicate standard deviations. Cell-free extracts and total RNA were prepared from yeast cells grown in SD10 medium without or with 1 mM linoleic acid at 30°C for 20 h. (A) AATase activity was measured with the cell-free extracts. *ATF1* (B) and *OLE1* (C) expression was measured by RT-qPCR. The relative expression levels are presented as fold-changes relative to the expression level obtained for strain Km97 without linoleic acid.

TABLE 1. Mutations discovered in the whole-genome sequence analysis of the *hia1* mutants.

Zygosity	Missense mutations		Nonsense mutations	
	Gene	Amino acid change	Gene	Amino acid change
Heterozygous	207 amino acid changes in 200 genes, listed in Table S2		*YBR168w PEX32*	Trp320*
			YGL124c/MON1	Tyr137*
			YGR060w/ERG25	Trp85*
			YGR157w/CHO2	Trp536*
			YGR184c/UBR1	Gln262*
			YIL146c/ATG32	Trp390*
			YJL108c/PRM10	Trp203*
			YJL107c	Trp590*
			YJL058c/BIT61	Gly281*
			YML128c/MSC1	Trp112*
			YOL145c/CTR9	Gln958*
Homozygous	*YCR014c/POL4*	Leu500Val	*YIR033W/MGA2*	Ser706*
	YCL016c/DCC1	Gln36Lys		
	YCL025c/AGP1	Ala530Ser		
	YCL073c/GEX1	Ala602Thr		
	YLR042c	Thr112Met		
	YLR143w/DPH6	Val254Ile		
	YLR153c/ACS2	Ala101Val		
	YLR207w/HRD3	Pro43Ser		
	YLR357w/RSC2	Arg817Ser		
	YLR358c	His90Gln		
	YLR431c/ATG23	Leu241Met		
	YNL264c/PDR17	Glu17Lys		
	YNL307c/MCK1	Gln220Lys		

(Table 1). We identified 200 genes with a total of 207 heterozygous missense mutations and 13 genes with homozygous missense mutations. In addition, we found 11 genes with heterozygous nonsense mutations and one gene with a homozygous nonsense mutation. Included among the mutations were genes related to AbA resistance in *AUR1*, an inositol phosphorylceramide synthase (Table S2) (25). The *AUR1* sequence of the *hia1* mutant included heterozygous C > T mutations at positions 377 and 469, resulting in amino acid replacements at positions 126 (threonine to isoleucine, Thr126Ile) and 157 (histidine to tyrosine, His157Tyr), respectively (Table S2). A previous study (26) has reported that the His157Tyr missense mutation conferred AbA resistance. However, no significant mutation (deletion or insertion) was detected when the *hia1* mutant was compared with strain Km97.

Because AATase activity is elevated in the *hia1* mutant, we first examined the sequences of the open reading frame (ORF) and promoter region of *ATF1*; however, these sequences were identical in the two strains. This indicated that the high isoamyl acetate production of the *hia1* mutant was not due to a mutation in the amino acid sequence or promoter of Atf1p but perhaps due to upregulation of *ATF1* expression by changes to an *ATF1* transcription factor. The homozygous nonsense mutation we had identified was in *MGA2*, an endoplasmic reticulum (ER) membrane protein involved in the regulation of *ATF1* expression (27). Although position 2117 in this gene was heterozygous (C/A) in strain Km97, it was

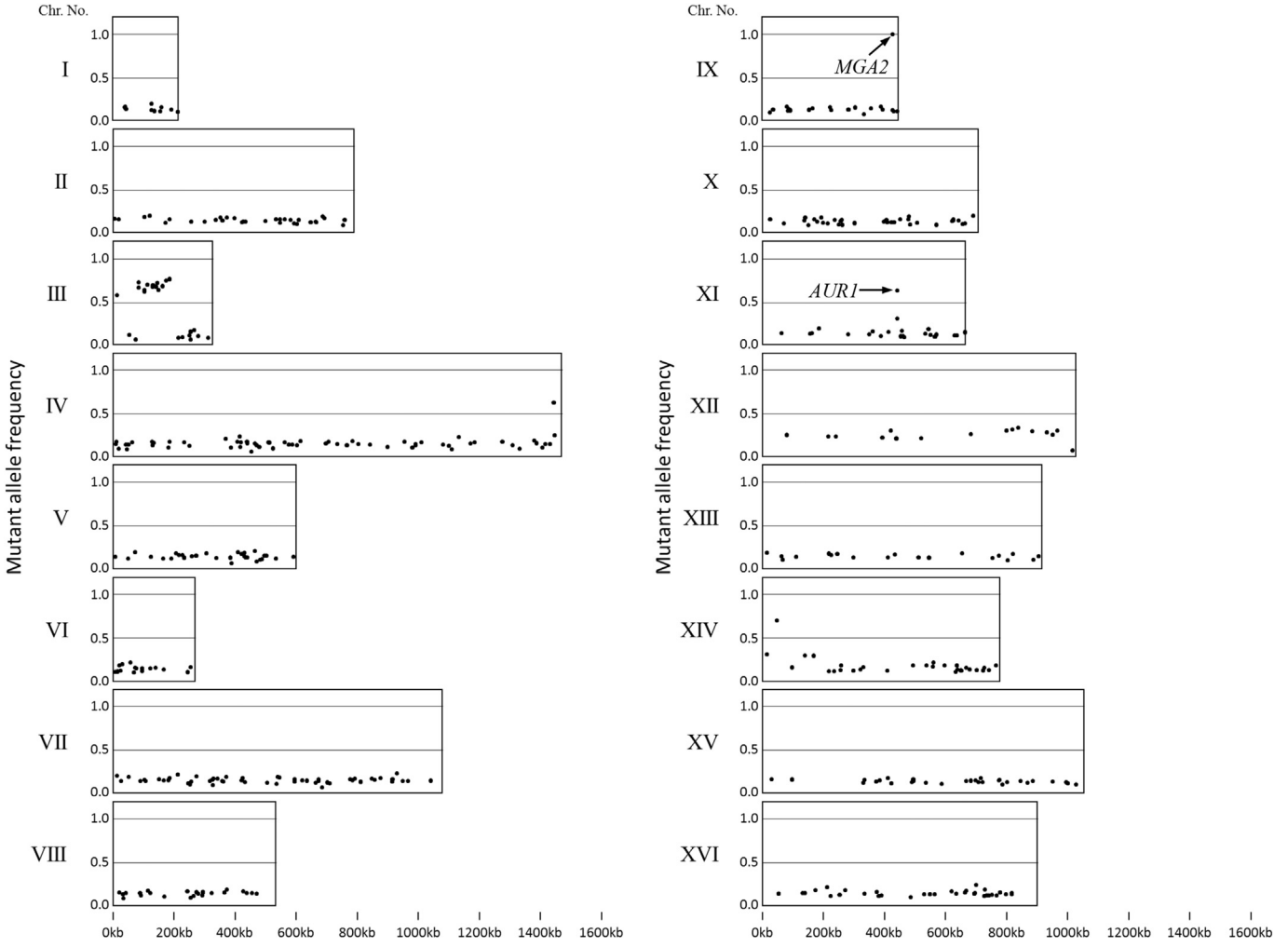

FIG. 4. Genome-wide distribution of mutant allele frequencies of SNPs from pooled *hia* mutant sequences. A genomic DNA pool of the *hia* mutants was constructed by combining equal amounts of genomic DNA extracted from the *hia1*, *hia2*, *hia4* and *hia6* mutants. The pool was then sequenced by using Illumina HiSeq 2000. The mutant allele frequency was calculated as the ratio of the number of reads containing the mutant allele to that of the wild-type allele.

TABLE 2. Mutations discovered in the whole-genome sequence analysis of the pooled *hia* mutant sample.

Gene	Amino acid change	Number of reads	Mutant allele frequency	Mutation in *hia1*	
				Zygosity	Mutation type
YIR033w/MGA2	Ser706*	142	1.000	Homo	Nonsense
YCL025c/AGP1	Ala 530 Ser	221	0.724	Homo	Missense
YCR014c/POL4	Leu 500 Val	214	0.715	Homo	Missense
YNL307c/MCK1	Gln 220 Lys	134	0.694	Homo	Missense
YCR011c/ADP1	Leu 742 Leu	202	0.673	Homo	Synonymous
YCR011c/ADP1	Gly 753 Gly	234	0.667	Homo	Synonymous
YCL025c/AGP1	Ser 445 Ser	187	0.663	Homo	Synonymous
YCL016c/DCC1	Gln 36 Lys	121	0.636	Homo	Missense
YKL004w/AUR1	His 157 Tyr	149	0.624	Hetero	Missense
YCL016c/DCC1	Asp 26 Asp	108	0.611	Homo	Synonymous
YCL073c/GEX1	Ala 602 Thr	116	0.578	Homo	Missense
YLR382c/NAM2	Thr 846 Met	132	0.333	Hetero	Missense
YLR371w/ROM2	His 37 His	129	0.310	Homo	Synonymous
YKL004w/AUR1	Thr 126 Ile	142	0.303	Hetero	Missense
YNL327w/EGT2	Ser 367 Ser	126	0.302	Homo	Synonymous
YLR143w/DPH6	Val 254 Ile	137	0.299	Homo	Missense
YLR440c/SEC39	Ile 344 Ile	151	0.298	Homo	Synonymous
YLR357w/RSC2	Arg 817 Ser	172	0.297	Homo	Missense
YLR358c	His 90 Gln	172	0.297	Homo	Missense
YNL264c/PDR17	Glu 17 Lys	128	0.289	Homo	Missense
YLR299w/ECM38	Gly 199 Gly	158	0.253	Homo	Synonymous
YLR431c/ATG23	Leu 241 Met	153	0.248	Homo	Missense
YPR095c/SYT1	Asp 450 Asn	121	0.231	Hetero	Synonymous
YLR042c	Thr 112 Met	149	0.228	Homo	Missense
YLR057w/MNL2	Leu 27 Leu	172	0.227	Homo	Synonymous
YDR358w/GGA1	Ser 508 Asn	131	0.221	Hetero	Missense
YGR227w/DIE2	Pro 48 Leu	122	0.221	Hetero	Missense
YLR129w/DIP2	Val 509 Met	143	0.217	Hetero	Missense
YFL037w/TUB2	Val 333 Val	141	0.213	Hetero	Synonymous
YLR207w/HRD3	Pro 43 Ser	142	0.211	Homo	Missense
YNL022c/RCM1	Lys 223 Lys	138	0.210	Hetero	Synonymous
YLR153c/ACS2	Ala 101 Val	137	0.204	Homo	Missense
YPL169c/MEX67	Gly 522 Asp	113	0.204	Hetero	Missense
YER100w/UBC6	Pro 76 Pro	125	0.200	Hetero	Synonymous

Homo, homozygous; hetero, heterozygous.

homozygous (A/A) in the *hia1* mutant, resulting in two copies of *MGA2* with stop codons instead of a serine at residue 706 (Ser706*).

Whole-genome sequence analysis of pooled *hia* mutants Following the discovery of the homozygous nonsense mutation (C2117A) in *MGA2* in the *hia1* mutant, we amplified and sequenced the *MGA2* sequences in the *hia2*, *hia4*, and *hia6* mutants. Because all mutants were isolated from independent mutant screenings, it is not a given that they would all carry the same mutation in *MGA2*. Surprisingly, we found that the homozygous C2117A mutation in *MGA2* was indeed present in all the *hia* mutants (data not shown).

We then analyzed the whole-genome sequence of the pooled sample of *hia* mutants to identify any other common mutations among the mutants. The pooled *hia* sequences were aligned to the reference K7 genome sequence, and the mutant allele frequencies of the pooled *hia* mutants were determined at each physical position along each chromosome (Fig. 4). The mutant allele frequency was calculated as the ratio of the number of reads containing the mutant allele to the total number of reads. Hence, the mutant allele frequency should theoretically be 0.5 or 1.0 in the case of common heterozygous or homozygous SNPs, respectively. Genes with mutant allele frequencies greater than 0.2 are listed in Table 2. On chromosome III, the mutant allele frequencies of the region from 75,166 to 185,144 ranged from 0.58 to 0.76, comparatively higher than the rest of the genome. A previous study demonstrated that chromosome III of strain K7 has accumulated a heterozygosity region (28). Therefore, it is more likely that the homozygous mutations were caused by the loss of heterozygosity. The mutant allele frequency of the missense mutation (Gln 220 Lys) in *YNL307c*/*MCK1* on chromosome XIV was also quite high (0.694; Table 2). As the heterozygous missense mutation was detected in this gene in strain Km97, this mutation was also assumed to be caused by the loss of heterozygosity in the two or three *hia* mutants containing the *hia1* mutation.

Surprisingly, the only SNP with a mutant allele frequency of 1.0, indicating a common homozygous mutation, was the nonsense mutation (Ser706*) in *MGA2* on chromosome IX (Fig. 4, Table 2). In contrast, the mutant allele frequency of the missense mutation (His157Tyr) in *AUR1* on chromosome XI was 0.624, a value that indicates a common heterozygous mutation in the four *hia* mutants. These results indicate that the common phenotype of high isoamyl acetate production is likely attributed to the homozygous nonsense mutation (Ser706*) in *MGA2*.

Effect of the *MGA2* nonsense mutation on *ATF1* expression To confirm that the nonsense mutation (Ser706*) in *MGA2* enhances *ATF1* expression and inhibits the repression of *ATF1* by unsaturated fatty acids, we examined the functionality of Mga2p using a BY4743 Δ*mga2* strain complemented with either functional, wild-type *MGA2* (w*MGA2*) or *MGA2* containing the nonsense mutation (m*MGA2*; Fig. 5A). BY4743 Δ*mga2* was transformed with pRS416-wMGA2, pRS416-mMGA2, or pRS416 as a control. These cells were then grown in SC10-URA medium with or without linoleic acid, and RT-qPCR was performed. Treatment with m*MGA2* more than doubled *ATF1* expression compared with w*MGA2* treatment (Fig. 5B). However, unlike in the *hia1* mutant, *ATF1* expression was partially inhibited by the addition of linoleic acid in m*MGA2*-complemented cells, although the extent of suppression was not as much as in w*MGA2*-complemented cells (Fig. 5B).

A previous study (29) indicated that N-terminal fragments of Mga2p lacking the membrane-spanning region (Δ*tm*) acted as powerful transcriptional activators of *OLE1*, although this activity was strongly repressed by unsaturated fatty acids. Therefore, we examined the effect of the nonsense mutation (Ser706*) in *MGA2* on the repression of *OLE1* by unsaturated fatty acids using the same BY4743 Δ*mga2* protocol. As with *ATF1* expression, treatment with m*MGA2* led to an upregulation of *OLE1* expression, which was partially inhibited by the addition of linoleic acid (Fig. 5C).

Finally, we examined whether m*MGA2* conferred AbA resistance. As can be seen in Fig. 5D, complementation of BY4743 Δ*mga2* with m*MGA2* conferred AbA resistance.

DISCUSSION

In this study, we screened 475 yeast mutants that were resistant to AbA, and identified four *hia* mutants with elevated isoamyl acetate productivity during sake brewing with polished rice that has a high polishing ratio. This relatively high frequency of screened mutants (approximately 1%) may seem surprising until we consider that AbA resistance is conferred not only by the known heterozygous missense mutation His157Tyr, but also by the Ser706* nonsense mutation in *MGA2* (Fig. 5D). Our results indicate that the acquisition of the *MGA2* mutation may have been promoted by another factor mediating the involvement of *MGA2* in AbA resistance. Further work is necessary to elucidate the relationship between AbA resistance and the molecular mechanism underlying *MGA2* function.

It is well known that the expressions of *ATF1* and *OLE1* are regulated coordinately in response to cell membrane fluidity and that the expression of both genes is repressed by unsaturated fatty acids (24). However, our results revealed that *ATF1* is expressed constitutively and that its expression is not inhibited by unsaturated fatty acids in the *hia1* mutant (Fig. 3B). A previous study had reported derepression of *OLE1* in a *FAA1*/*FAA4* double disruptant in

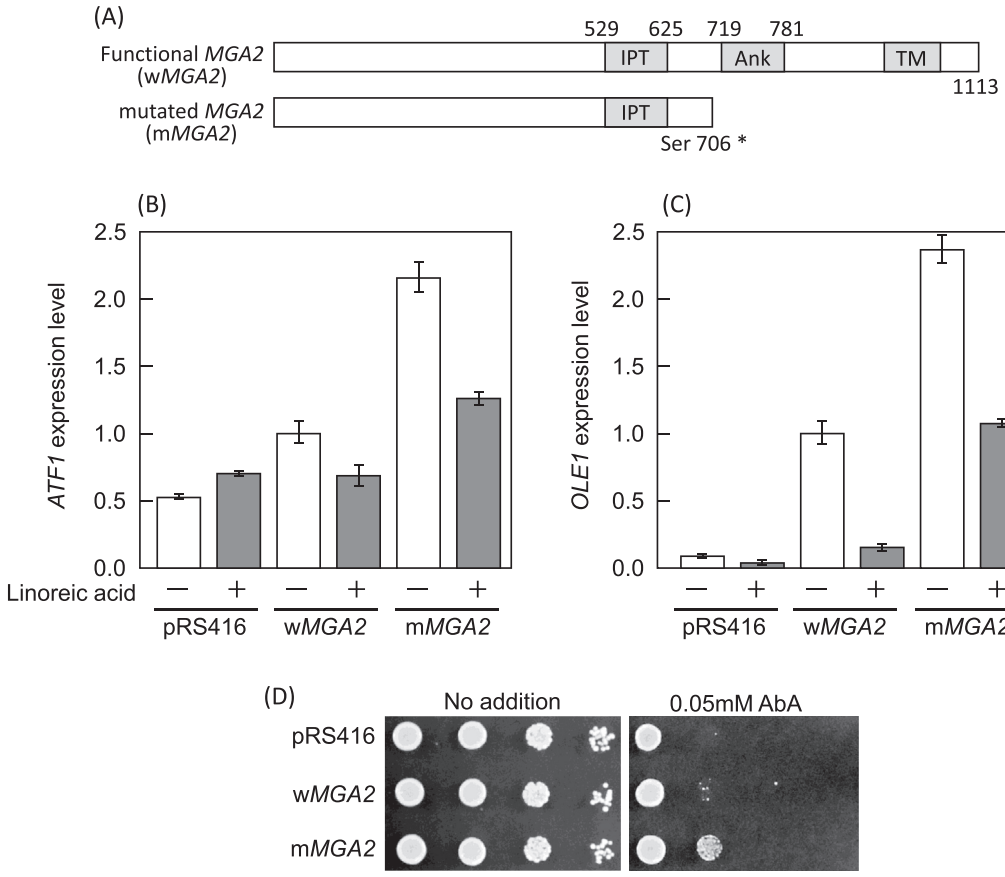

FIG. 5. Evaluation of Mga2p function using the BY4743 *Δmga2* strain complemented with either wild-type *MGA2* (w*MGA2*) or mutant *MGA2* (m*MGA2*). (A) Schematic depiction of wild-type and mutant Mga2p; the latter lacks the ankyrin repeat and transmembrane domains because of a nonsense mutation (Ser706*). The positions of IPT, ankyrin repeat (Ank), and transmembrane (TM) domains are indicated. (B, C) Total RNA was prepared from yeast cells grown in SC10-URA medium without or with 1 mM linoleic acid at 30°C for 9 h. *ATF1* and *OLE1* expression in the presence and absence of linoleic acid in BY4743 *Δmga2* complemented with w*MGA2*, m*MGA2*, or control plasmid (pRS416). Expression levels are shown as fold-changes relative to that of w*MGA2* without linoleic acid. (D) Aureobasidin A (AbA) resistance of BY4743 Δ*mga2* complemented with w*MGA2*, mMGA2, or control plasmid (pRS416). Left panel depicts growth on SC-URA agar medium in the absence of AbA, while right panel depicts growth in the presence of 0.05 mM AbA. Yeast cells were grown in SC-URA at 30°C for 24 h and diluted with distilled water to an OD_{660} value of 1.0. Five-microliter aliquots of serial dilutions (left to right) of cell suspension were placed on SC-URA agar medium containing 0 mM or 0.05 mM AbA, and incubated at 30°C for 2 d.

the presence of unsaturated fatty acids (30). The *FAA1*/*FAA4* genes encode the long-chain fatty acyl-CoA synthase involved in sphingolipid-to-glycerolipid metabolism (31). However, whole-genome sequence analysis revealed that *FAA1* and *FAA4* were not altered in the *hia1* mutant. Instead, the homozygous nonsense mutation Ser706* in *MGA2* was identified as a candidate for conferring elevated *ATF1* expression and high isoamyl acetate production (Table 1). Mga2p consists of 1137 amino acids and localizes to the ER membrane, where it undergoes homodimerization mediated by its central IPT (Ig-like, plexins, transcription factors) domain. Subsequently, it is partially cleaved at the C-terminus, which contains ankyrin repeat and transmembrane domains, in a ubiquitin/proteasome-dependent process. The N-terminal transcription factor domain then translocates into the nucleus to activate the expression of target genes such as *ATF1* and *OLE1* (32,33). Despite the fact that the *hia1*, *hia2*, *hia4*, and *hia6* mutants were derived from independent mutagenesis screens, whole-genome sequence analysis of the pooled *hia* mutant sample revealed that the homozygous *MGA2* nonsense mutation was the only common mutation among all four mutant lines. Furthermore, this mutant version of *MGA2* increased both *ATF1* and *OLE1* expression in a Δ*mga2* strain (Fig. 5B, C), indicating that the lack of the ankyrin repeat and transmembrane domains constitutively increases Mga2p transcriptional activity. We hypothesize that, with these domains eliminated, Mga2p is translated as the active form, consisting only of the N-terminal transcription factor domain (Fig. 5A). A similar mechanism has been reported for Mga2p and NFκB, which is related to Mga2p, where deletion of the ankyrin repeat domain enhanced their transcriptional activity (34,35). This mechanism would explain the high constitutive *ATF1* expression exhibited by *hia* mutants with the homozygous Ser706* nonsense mutation. However, although *ATF1* expression was maintained following the addition of linoleic acid in the *hia1* mutant, expression was partially inhibited by linoleic acid in a Δ*mga2* strain complemented with the mutant version of *MGA2*. On the other hand, in the *hia3* and *hia5* mutants, the homozygous Ser706* nonsense mutation was not detected. These results indicate that another factor without mediating *MGA2* may be involved in the derepression of *ATF1* expression in the presence of unsaturated fatty acids.

In conclusion, we showed in this study that a homozygous nonsense mutation (Ser706*) in *MGA2* enables the brewing of sake with high levels of isoamyl acetate, using polished rice with a high polishing ratio. In addition, we demonstrated that this mutation constitutively activates *ATF1* expression and inhibits its repression by unsaturated fatty acids. Therefore, this mutant may be considered for large-scale application in the fermentation industry to improve the taste and flavor of sake. To develop a suitable method for breeding the yeast strains with enhanced isoamyl acetate productivity, further research is needed to clarify all the regulatory mechanisms underlying isoamyl acetate production.

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