博士論文

Characterization of the traditional sake yeast Hiroshima no. 6
and its application to sake yeast cross-breeding.

（清酒酵母広島 6 号の特性解析及び新規清酒酵母の交配育種への活用）

山 﨑　梨 沙

広島大学大学院先端物質科学研究科

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主論文

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INTRODUCTION

Sake is a traditional Japanese alcoholic beverage made from steamed rice. In sake brewing, the saccharification from rice starch to glucose by the enzymes produced from the mold *Aspergillus oryzae* and the ethanol fermentation achieved with the budding yeast *Saccharomyces cerevisiae* occur simultaneously. This method of producing sake is called "double parallel fermentation". Factors that influence the diversity of sake quality include the brewing method, the variety and quality of the rice, and the microorganisms involved in brewing, but the brewing microorganism is the factor that contributes most to diversity of sake characteristics. In particular, the effect of yeast for sake taste and flavor is remarkable, thus the diversification of sake quality can be achieved by improving the yeast strain.

The Brewing Society of Japan distributes sake yeast strains to brewers. The breeding of sake yeast has a long history. Historically, when sake yeast strains were grown, they were isolated from sake mush (*moromi*). For example, Kyokai no.1 (K1) to Kyokai no.5 (K5) are strains isolated from the breweries of the Kinki district or Hiroshima Prefecture in the early 1900s (Akao, 2014; Ohya *et al.,* 2019). Since then, yeast strains have been isolated from various areas, modern sake yeast Kyokai no.7 (K7) group are the sake yeast strains commonly used today.

The K7 group strains possess many advantageous features (high ethanol production, fermentation at low temperature, non-requirement of biotin for cell growth, and more) (Shimoi *et al.,* 2002; Wu *et al.,* 2005) that differ from those of laboratory yeast strains. Genome sequence analysis of the representative sake yeast, K7, in 2011 enabled comparative genomics between K7 and the laboratory strain S288c (Akao *et al.,* 2011). The sake yeast strains that are most commonly used today are mainly members of the K7 group, including K6, K7, K9, and K10 strains, which are genetically closely related (Azumi and Goto-Yamamoto, 2011).

Characteristics of the K7 group strains include the ability to produce both high concentration of ethanol and the flavor component, ester, at low temperatures. Due to its

valuable characteristics, the K7 group is often used as a parent strain for breeding new sake yeast. However, the genetic crossing of K7 group strains is considered to be difficult, because they commonly possess defects in sporulation and meiosis. In these strains, the low transcriptional level of *IME1*, a master regulator of sporulation, causes low sporulation frequency (Nakazawa *et al.,* 1992). Accordingly, improvement of sake yeast strains relies on mutagenesis breeding to produce desirable properties.

In Japan's Hiroshima Prefecture, unique sake yeast strains that are suitable for the regional environmental conditions have been isolated and used for sake brewing. Hiroshima's sake yeast strain, no.6 (H6), is the oldest among the stock strains whose history has been reported in Hiroshima. H6 was first isolated at Kikubotan Brewery in Hiroshima in 1926 (Shimizu, 1928; Tsutsumi, 1980) and seems to have been used for sake brewing for many years since then; however, it is unclear how long it has been used (Tsutsumi, 1980) and why it has not been used recently. H6 is a very interesting strain as a unique resource.

Recently, the drinking of sake has become more popular worldwide with the spread of Japanese cuisine. As a result, the need for flavor variety has also grown. Brewers want to add a variety of tastes and flavors to their sake. Accordingly, old-type sake strains (K1–K5) and yeast strains that have been stored in sake breweries and naturally occur in *moromi* have been reevaluated. As sake exports increase, new technologies are needed to prevent deterioration during transportation.

Therefore, this study aimed at analyzing the characteristics of the traditional sake yeast strain H6 and utilizing it for sake brewing. Chapter I describes comprehensive characteristic analyses of the fermentation and sporulation abilities of H6. Then, the excellent properties of H6 for sake brewing are reported, and the potential of H6 as a partner strain for the breeding of sake yeast by genetic crossing is described. In chapter II, crossbreeding between H6 and K7 group strains to produce diploid cells with the capacity for sexual reproduction is reported. Sporulation of such crossbred diploid cells should produce a series of haploid cells. These haploid series would be available for

breeding new sake yeast strains with diverse brewing characteristics. Additionally, the relationships between specific genotypes and brewing phenotypes are examined by tetrad analysis.

CHAPTER I

Characteristic analysis of the fermentation and sporulation properties of the traditional sake yeast strain Hiroshima no.6.

I .1 ABSTRACT

General sake yeasts (*e.g.,* Kyokai no.7, K7) show high fermentation ability and low sporulation frequency. The former is related to stress-response defects due to the loss-of-function of *MSN4* and *RIM15*. Later is mainly caused by low *IME1* expression, leading to difficulty in breeding and genetic analysis. Sake yeast Hiroshima no.6 (H6), which had been applied for sake fermentation, has sporulation ability. However, its detailed properties have not been unveiled. In this chapter, I present that the fermentation ability of H6 is suitable for sake brewing, and the precursor of dimethyl trisulfide in sake from H6 is low. *MSN4* but not *RIM15* of H6 has the same mutation as K7. My phylogenetic analysis indicates that H6 is closely related to the K7 group. Unlike K7, H6 shows normal sporulation frequency in a partially *RIM15*-dependent manner, and *IME1* in H6 was expressed. H6 possesses excellent properties as a partner strain for breeding by crossing.

I .2 INTRODUCTION

Sake yeast strains possess many features (high ethanol production, fermentation at low temperature, non-requirement of biotin for cell growth, and more) (Shimoi *et al.,* 2002; Wu *et al.,* 2005) that differ from those of the laboratory yeast strains. The genome sequence analysis of the representative sake yeast Kyokai no.7 (K7) in 2011 enabled comparative genomics between K7 and the laboratory strain S288c (Akao *et al.,* 2011). The sake yeasts that are most commonly used today are mainly members of the K7 group including K6, K7, K9, and K10 strains, which are genetically closely related (Azumi *et al.,* 2011). Characteristics of the K7 group strains include a high ability to produce both alcohol and an aroma component ester at low temperature, and low sporulation ability, *i.e.*, forming only a few spores under sporulation-inducing conditions (Nakazawa *et al.,* 1992).

It was recently reported that the loss-of-function of the *MSN4* and *RIM15* genes involved in the stress response of yeast cause high ethanol fermentation (Watanabe *et al.,* 2011; Watanabe *et al.,* 2012). In budding yeast, the transcription factors Msn2p and Msn4p (hereafter, 'Msn2p/4p') play an important role in the response and adaptation to various stressors such as heat shock and ethanol through the transcriptional activation of target genes, and a genetic analysis indicated that the Rim15p protein kinase is an upstream activator of Msn2p/4p (Cameroni *et al.,* 2004). A genome sequence analysis of K7 revealed that K7 has single-nucleotide polymorphisms (SNPs) causing reduced functions of *MSN4* and *RIM15* genes (Watanabe *et al.,* 2011). The *MSN4* gene of K7 has loss-of-function due to two mutations; one is the single nucleotide substitution at the start codon (from aTg to aCg) and the other is the nonsense mutation at the 1,540th cytosine (codon conversion from Caa to Taa) in the middle of the reading frame. As a result, the expression of the target genes of Msn2p/4p is reduced, and the response to heat and acute ethanol stress is reduced (Watanabe *et al.,* 2011).

Further, on the *RIM15* gene of K7, a single adenine insertion at the 5,055th nucleotide ($rim15^{5055insA}$) results in the deletion of the C-terminal 75 amino acids, the

putative receiver domain, which is a homologous region of the molecules involved in histidine kinase phospho-relay signaling (Wuichet *et al.,* 2010). In budding yeast, under physiological osmotic conditions, the histidine kinase Sln1p is autophosphorylated, and the phosphorylation of the conserved aspartate residue (D554) on the receiver domain of Ssk1p increases. Under high-osmolarity conditions, the Sln1p and D554 of Ssk1p are dephosphorylated, leading to the activation of the mitogen-activated protein kinase (MAPK) Hog1p and the induction of target genes for the osmotic stress response (Kaserer *et al.,* 2009; Posas *et al.,* 1996). However, Rim15p does not have this conserved aspartic acid corresponding to D554 in Ssk1p.

Although the regulatory mechanism of Rim15p *via* its receiver domain is not yet clear, this mutation (*rim15*^{5055insA}) of the *RIM15* gene in a laboratory strain greatly reduced the cell viability under acute ethanol and heat shock stress conditions, and it improved the alcohol fermentation ability in sake brewing (Watanabe *et al.,* 2011). In addition, the *RIM15*-deletion in the laboratory yeast strain showed the same phenotype as this mutation. These results indicated that the *rim15*^{5055insA} mutation caused the defect in the function of Rim15p and improved the sake fermentation ability (Watanabe *et al.,* 2011). Thus, in sake yeasts of the K7 group, both loss-of-function of Rim15p and the decrease in Msn2p/4p markedly reduced the stress response during sake fermentation, leading to the increases in the fermentation rate and ethanol production (Watanabe *et al.,* 2012). However, the expression of the functional *RIM15* gene in K7 did not inhibit the alcohol fermentation ability, indicating that this mutation of *RIM15* gene is not the sole cause of the improved sake fermentation of K7 (Watanabe *et al.,* 2012).

In budding yeast, Rim15p activity is regulated by several nutrient-sensing signaling protein kinases: protein kinase A (PKA), phosphate-sensitive cyclin and cyclin-dependent protein kinase (CDK) complexes Pho80p-Pho85p, and rapamycin target protein kinase complex 1 (TORC1) (Pedruzzi *et al.,* 2003; Wanke *et al.,* 2005). It was recently reported that TORC1 and the PP2A regulatory B subunit Cdc55 (PP2A$^{B55\delta}$) work upstream and downstream of the *RIM15* gene, respectively, and are involved in high

fermentation of sake yeast (Watanabe *et al.,* 2019a). Cdc55p is involved not only in high fermentation but also in the spindle-assembly checkpoint (SAC) in sake yeast (Goshima *et al.,* 2016).

In budding yeast, signals controlling sporulation have been well studied (Piekarska *et al.,* 2010). K7-group sake yeasts have low sporulation ability, leading to difficulties in breeding yeast strains by genetic crossing and genetic analysis of the genes related to brewing properties of sake yeast. It was reported that the transcription of *IME1* is not normally induced in K7, suggesting that one of the causes of low sporulation in K7 is a defect in the expression of *IME1* (Nakazawa *et al.,* 1992), which encodes a transcriptional activator Ime1p playing an important role as the master inducer of sporulation in the initiation of meiosis by both nutrient and cell type signals (Piekarska *et al.,* 2010). However, overexpression of *IME1* in K7 recovered its low sporulation frequency by only a slight degree (Nakazawa *et al.,* 1992). It was also indicated that the K7-group strains' low spore germination ability is due to a defect in chromosomal recombination in meiosis, leading to a loss of chromosomes during meiosis (Shimoi *et al.,* 2019). Although the cause of the low sporulation ability of K7 is due at least in part to a defect in the expression of *IME1*, there are unknown complex causes of this characteristic.

Hiroshima sake yeast strain no.6 (H6) is the oldest among the stock strains whose history has been reported in Hiroshima. H6 seems to have been used for sake brewing for many years after then, but it is unclear how long it has been used (Tsutsumi, 1980) and why it has not been used recently. Previously, Shimizu (Shimizu, 1028) reported several abilities of H6 (spore formation on the sterilized plasterboard and sake fermentation by one-step mashing process at room temperature) but not the detail of sporulation frequency in sporulation medium and the analysis components of sake made from H6. Kawamura (Kawamura, 1996) reported the cell growth and fermentation ability at low temperature (at 5, 10, and 15°C in YPD medium) of three Hiroshima sake yeast strains (H2, H5, and H6) including the haploid cells isolated by random spore method. Farther Hiroshima sake yeast strain no.2 (H2) was constructed by genetically crossing H6 and a yeast strain

isolated from Saikai Brewery in Kumamoto (Shimizu, 1928), and the results indicated that H6 has sporulation ability. However, there is no report or detail analysis of the fermentation and sporulation abilities of H6.

Therefore, Chapter I aimed at analyzing the characteristics of the traditional sake yeast strain H6 and utilizing it for sake brewing. Chapter I describes comprehensive characteristic analyses of the fermentation and sporulation abilities of H6. Then, the excellent properties of H6 for sake brewing are reported, and the potential of H6 as a partner strain for the breeding of sake yeast by genetic crossing is described.

I .3 MATERIALS AND METHODS

I .3. 1 Yeast strains and media

The yeast strains used in this study are listed in Table 1. Disruption of the genes (*RIM15*, *URA3*, and *CAN1*) in H6 was performed by a PCR-based method. The marker cassettes (pFA6-*kanMX*4 and pAG25-*natMX*) were synthesized by fusion PCR using the primers listed in Table 2 Yeast cells were cultured aerobically in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) at 30°C with shaking. Yeast transformation was achieved by the lithium acetate method. For sporulation, I used the sporulation medium (2% potassium acetate). The solid medium was prepared by adding 2% agar to each medium component.

I .3. 2 Small-scale sake fermentation test

I performed a small-scale sake brewing test to examine the fermentation properties of sake yeast strains (Ohnuki *et al.,* 2019). The yeast cells were pre-cultured in 5 mL of YPD medium at 30°C for 24 h. Then, 1 mL (approximately 1×10^8 cells/mL) of the pre-cultured cells was added to 50 mL of YPD medium (approximately 2×10^6 cells/mL) and incubated at 30°C for 24 h with shaking. The cells were harvested and added to sake mash at a final concentration resulting in an optical density at 660 nm of 1.0. The sake mash was prepared from 72.8 g of gelatinized rice (equivalent to 80 g of rare polished rice; Tokushima Seikiku Co., Ltd., Osaka, Japan) at a 60% polishing ratio, 19.2 g of dried rice *koji* (equivalent to 20 g of rare polished rice and a 20% *koji* ratio; Tokushima Seikiku Co., Ltd.) at a 60% polishing ratio, 136 μL of 90% lactic acid, and 174 mL of water (equivalent to a water-to-rice ratio of 130%) containing the yeast cells. The temperature of the sake mash was maintained at 15°C. After 20 days, the sake mash was centrifuged, and the resulted supernatant was collected as the sake samples.

The general composition of the sake samples was analyzed using the methods authorized by the National Tax Agency of Japan (Brewing Society of Japan, 2017). The

organic acid contents in the sake sample were analyzed using a high-performance liquid chromatography system (SCL-10 ASP, equipped with a Shim-pack SPR-H column; Shimadzu, Kyoto, Japan). 1,2-dihydroxy-5-(methyl sulfinyl) pentane-3-one (DMTS-P1) and dimethyl trisulfide producing potential (DMTS-pp), which is defined as the amount of DMTS produced in sake during the storage at 70°C for 7 days, were analyzed as described previously. (Isogai *et al.,* 2010; Wakabayashi *et al.,* 2013).

I .3. 3 Analysis of the strains' sporulation ability and tetrad isolation

The sporulation conditions were as follows. The yeast cells were grown overnight on YPD plates at 30°C, and then the cells were transferred on sporulation media (2% potassium acetate, 2% agar) plates and incubated at 25°C for 3 days. The quantitation of asci was carried out by a light-microscopy examination. At least 300 cells in each strain were counted for the measurement of sporulation frequency and the number of asci with spore(s). Spores to be used for the measurement of germination frequency were isolated with the use of a micromanipulator. Twenty-five tetrads were placed on YPD plates and incubated at 25°C for 4–5 days, and then the germinated spores were counted.

I .3. 4 Measurement of the strains' colony-forming ability

The yeast cells were grown in YPD medium at 30°C overnight. The cells were then diluted to the concentration OD_{660}=0.15 in fresh YPD or SC medium (0.67% yeast nitrogen base without amino acids, 2% glucose, and 0.079% complete supplemental mixture) and incubated at 30°C with shaking at 130 rpm or standing. The cells were serially diluted to the appropriate concentration. The colony-forming ability (CFA) was measured by counting the number of developed colonies or observing viable cells by serially diluted spot-assay.

I. 3. 5 DNA extraction and the DNA sequence analysis

Yeast cells were grown in YPD medium at 30°C overnight. Genomic DNA was

prepared using the DNA Extraction kit for Yeast (Takara Bio, Shiga, Japan). All primers used in this study were designed based on the genome sequence of *S. cerevisiae* found in the *Saccharomyces* genome database (SGD) (http://www.yeastgenome.org/) and are listed in Table 2. A polymerase chain reaction (PCR) was performed using KOD Plus Neo polymerase (Toyobo, Osaka, Japan). The PCR amplification product was purified with an Immu Prep DOUBLE Pure kit (Analytik Jena, Jena, Germany). Sequencing was performed at Macrogen japan Co (Kyoto, Japan) using an Applied Biosystems 3730xl DNA analyzer (Thermo Fisher, San Jose, CA).

I .3. 6 Phylogenetic analysis of the industrial *S. cerevisiae* strains based on five genes

The DNA sequences of the yeast strains, except for H6, were obtained from the DDBJ/EMBL/GenBank. The DNA sequencing of H6 was carried out as described above. The phylogenetic tree was constructed using the sequence of five genes *ZAP1, THI7, PXL1, YRR1*, and *GLG1* of 29 yeasts by Genetyx ver. 13 (GENETYX, Tokyo) as described previously (Futagami *et al.,* 2017).

I .3. 7 Total RNA extraction from the yeast cells under sporulation conditions

For the total RNA extraction from the yeast cells under sporulation conditions, yeast cells were spread widely on YPD plates and grown overnight at 30°C; the cells were then collected and placed on sporulation medium (2% potassium acetate, 2% agar) plates and incubated at 30°C. Cells were then sampled at 5, 7, 11, and 24 h washed with water and frozen at -80°C. Frozen cells were mechanically disrupted with 0.5-mm glass beads by a Mixer Mill MM300 (Retch, Haan, Germany). Total RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany).

To eliminate genomic DNA contamination, I performed an additional DNase treatment according to the RNeasy kit instruction with the RNase-free DNase set (Qiagen). Purified RNA was quantitated using a Nano-Drop 1000 spectrophotometer (Thermo Fisher), and then the total RNA was reverse-transcribed into cDNA in a 20-μL reaction

mixture using the Rever Tra Ace qPCR RT kit (Toyobo, Osaka, Japan).

I .3. 8 Quantitative RT-PCR and data analysis

A quantitative qRT-PCR was performed using a Light Cycler Nano (Nippon Genetics, Tokyo) and a Fast start Essential DNA Green Master kit (Roche, Mannheim, Germany). The final concentration of each primer was 0.5 μM. The thermal cycling conditions were 95°C for 10 min followed by 40 cycles at 95°C for 10 sec, 60°C for 10 sec, 72°C for 15 sec, hold at 95°C for 30 sec, melting at 60°C for 20 sec, 95°C for 20 sec, and hold at 40°C for 30 sec. The data were analyzed using Light cycler software. Delta cycle threshold (ΔC_T) values were calculated by subtracting the C_T of the *ACT1* gene from the C_{Ts} of the *IME1* gene. ΔC_T values were calculated by subtracting the ΔC_T of the X2180 and H6 samples from the ΔC_T of the K7 sample for each hour. Fold changes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak *et al.,* 2001).

I .4 RESULTS AND DISCUSSION

I .4. 1 The fermentation properties of yeast strain H6

To investigate the fermentation properties of H6, I conducted a small-scale sake fermentation test at 15°C using three sake yeast strains, *i.e.*, H6, Sake yeast Kyokai no.7 (K7), and Sake yeast Kyokai no.901 (K901). Since K7 and K901 are common strains that are often used for sake brewing, I used them as controls for H6. As shown in Figure 1A, in the early and late stages of sake mash, the CO_2 production of H6 was significantly lower than those of K7 and K901. The alcohol production of H6 was lower than that of the other strains, although H6 produced 16% alcohol at a low temperature, i.e., 15°C (Table 3). This alcohol production ability of H6 is sufficient for industrial sake brewing.

The acidity in sake from strain H6 was lower than that in sake from the other strains, while the amino acid content in sake from H6 was higher than that in sake from the other strains. The preliminary fermentation test showed that the amino acid content in the sake made from H6 was also higher than that in the sake made from K7 (data not shown), suggesting the possibility that the cell viability of H6 in sake mash is lower than that of K7. To investigate this possibility, I examined the colony-forming ability (CFA) of H6 in sake mash, and observed that this was indeed the case; over the 20 days of the small-scale sake fermentation test, the CFA of H6 was significantly lower than those of K7 and K901 (Figure1B).

I .4. 2 The off-flavor producing ability of H6

Dimethyl trisulfide (DMTS), one of the off-flavors of sake, is generated during storage of products. Isogai *et al.* reported that DMTS-P1 is one of the precursors of DMTS (Isogai *et al.,* 2010). DMTS is a component that occurs during the long-term storage of sake, and the DMTS content of sake tends to increase due to cell death of yeast strains in sake mash (Sasaki *et al.,* 2014; Nishibori *et al.,* 2014). To investigate whether the low CFA of H6 in sake mash (Figure 1B) and the high amino acid content in sake from H6

(Table 3) are derived from the increase in cell death of the H6 strain in sake mash, I measured the contents of DMTS-P1 and the DMTS-producing potential (DMTS-pp), which is defined as the amount of DMTS produced in sake during the storage at 70°C for 7 days, in sake produced from H6. However, the measurements revealed that the contents of both DMTS-P1 and DMTS-pp in sake from H6 were significantly lower than those from the other strains (Figure 1C, D).

Wakabayashi *et al*. investigated the relationship between DMTS-P1 production and the methionine salvage pathway (MTA cycle) using the gene-deleted strains of this pathway and found that DMTS-P1 and DMTS-pp of sake were mainly reduced by the gene-deletion of either *MRI1*, *MDE1*, or *SPE2* (Wakabayashi *et al.,* 2013). I presume the low DMTS-P1 content and DMTS-pp level in sake from H6 might have been caused by defects in the methionine salvage pathway of H6, and examined SNPs in the 14 genes of the methionine salvage pathway of H6. Among the 14 genes, only one SNP (codon conversion from Gaa to Caa, amino acid substitution; from the 54th glutamic acid to glutamine) on the *ADI1* gene (acireductone dioxygenase) was identified (data not shown). Wakabayashi *et al*. showed that the DMTS-P1 content in sake from *ADI1*-deleted cells was slightly reduced compared to wild-type cells (Wakabayashi *et al.,* 2013). To determine whether this mutation (Glu54Gln) of Adi1p is related to the low contents of DMTS-P1 and DMTS-pp in sake from H6, I isolated H6-*ADI1* (Glu54Gln) and K7-*ADI1* (wild-type 54Glu) genes and introduced each separately into an *ADI1*-disrupted H6 strain (H6*Δadi1*), and then constructed two *ADI1*-disrupted H6 strains having H6-*ADI1* (Glu54Gln) or K7-*ADI1* gene. Subsequently, I performed a small-scale sake brewing test using these two strains and measured the DMTS-P1 content and DMTS-pp level in the sake samples. The results revealed no significant difference in the measurement values between the sakes from the two strains (data not shown), indicating that the low DMTS-P1 content and DMTS-pp value in sake from H6 were not caused by a mutation of *ADI1* gene related to the methionine salvage pathway. Further analyses are needed to clarify this feature of H6.

The presence of 4-vinyl guaiacol (4VG) in sake causes smoke-like or phenolic off-flavor, which affects sensory characteristics (Mukai *et al.,* 2014). 4VG is generated from ferulic acid by yeast enzymatic reactions in sake mash, but sake yeast strains do not produce 4VG generally (Mukai *et al.,* 2014). However, a previous report indicated that yeast strains isolated from natural environment produced 4VG in sake mash (Nakagawa *et al.,* 2019). To test 4VG producing ability of H6, I performed the small-scale sake brewing test, and could not detected 4VG off-flavor in the resulted sake sample by sensory evaluation test (data not shown). Further, I confirmed the loss-of-function of two genes essential for 4VG production in H6: *PAD1* (phenylacrylic acid decarboxylase) and *FDC1* (ferulic acid decarboxylase) by a DNA sequence analysis. *FDC1* of H6 contains the nonsense mutation at the 160th adenine (codon conversion from Aaa to Taa) as the case of K7-*PAD1* of H6 contains the same SNPs of K7 (data not shown).

I .4. 3 The sporulation properties of H6

General sake yeasts have a defect in sporulation ability. Previously, Shimizu (Shimizu, 1928) reported that spore formation of H6 on the sterilized plaster board but not the detail of sporulation frequency in sporulation medium, and Kawamura (Kawamura, 1996) isolated haploid cells from H6 by random spore method. I examined the detail of sporulation ability of H6, and first observed the cells in sporulation medium (Figure 2A). I identified asci with three or four spores in H6 but not in K7 (Nakazawa *et al.,* 1992), and this number of spores was also observed as in the laboratory strain X2180. Interestingly, the sporulation frequency of H6 was higher than those of X2180 and K7 (Figure 2B). K7 was reported to have a defect in meiotic recombination (Shimoi *et al.,* 2019), and thus to investigate the meiotic recombination of H6, I followed a method described by Shimoi *et al.* and constructed the H6 strain (*can1:NatMX*, *ura3*:*KanMX*) replacing two genes on chromosome V, *CAN1* and *URA3*, with two genetically selectable cassettes, *NatMX* and *KanMX,* respectively. By observing the nourseothricin (clonNat) or G418 resistance of the spores from a tetrad from this strain, I confirmed that chromosome

recombination in H6 occurred normally (Table 4).

One of the causes of the low sporulation ability of K7 is the low expression of *IME1* under the sporulation conditions. To investigate the expression level of *IME1* in H6, I examined the *IME1* expression in H6 under the sporulation-inducing conditions, and observed that the *IME1* expression in H6 was twofold that in K7 and half that in X2180 (Figure 2C). The *IME1* expression level in H6 might be enough for initiation of sporulation. Taken together, these results indicated that H6 has normal sporulation ability, similar to that of X2180.

I .4. 4 The colony-forming ability (CFA) of H6

In the small-scale sake fermentation test, the CFA of yeast strain H6 was significantly lower than those of K7 and K901 (Figure 1B). To determine the CFA of H6 under several culture conditions (Figure 3A), I examined the CFA of H6 in both a rich medium YPD (Figure 3B, C) and the minimal synthetic medium SC in a shaking culture (Figure 3D), and in YPD with standing culture (Figure 3E). With the YPD and shaking culture (Figure 3B), the CFA of H6 decreased from 4 days. No decrease in the CFA values of the K7 and BY4743 strains was observed. With the YPD and shaking culture, CFA was examined with spot assay (Figure3C). Similar to the results in Figure 3B, no decrease in CFA was observed in the yeast strains X2180 and K7. However, a decrease in CFA of H6 was observed. With the SC medium of shaking culture (Figure 3D), the CFA of H6 decreased rapidly after 5 days of shaking culture. On the other hand, with YPD medium of standing culture (Figure 3E), no significant decrease in the CFA of H6 was observed as was detected in K7 and X2180.

The CFA of strain H6 decreased under shaking culture conditions. To investigate whether H6 is sensitive to oxidative stress, I examined the sensitivity of H6 cells to hydrogen peroxide, and observed that the H6 cells were slightly more sensitive to oxidative stress compared to the control strains K7 and X2180 (Figure 4).

H6-derived haploid cells showed the CFA-decreasing phenotype as H6 WT, and

the hybrid strain of H6Hap and X2180-1A also showed the same phenotype. Therefore, it is inferred that this phenotype is dominant (Figure 5). When a tetrad analysis was performed from H6XHybrid, this phenotype was roughly 2: 2 separated, so that the causative gene may be able to elucidate by pool sequence analysis to identify the causative gene of H6 CFA reduction.

I .4. 5 The phylogenetic tree analysis of H6 based on five genes

Futagami *et al.* reported phylogenetic analysis conducted for strain classification based on five genes: *ZAP1* (zinc-regulated transcription factor), *THI7* (plasma membrane transporter responsible for the uptake of thiamine), *PXL1* (protein localizes at sites of polarized growth), *YRR1* (Zn2-Cys6 zinc-finger transcription factor), *and GLG1* (glycogenin glucosyltransferase) (Futagami *et al.,* 2017). The resultant phylogram should reflect genome-wide genealogical relationship of the strain. To classify H6 among the industrial yeast strains, I examined the nucleotide sequences of these five genes of H6 and constructed the phylogenetic tree as described (Futagami *et al.,* 2017). H6 was classified into the same cluster of sake yeasts and located in the same branch of the K7 group (Figure 6), indicating genetic closeness of H6 and the K7 group. I discuss genetic relationship between them in the next section.

I .4. 6 Analysis of *RIM15* and *MSN4* in H6

In strain K7, the loss-of-function of the *MSN4* and *RIM15* genes involved in the stress response causes high ethanol fermentation (Watanabe *et al.,* 2011; Watanabe *et al.,* 2012). Because my phylogenetic analysis revealed that H6 is closely related to the K7 group (Figure 6), I next examined the DNA sequences of *MSN4* and *RIM15* of H6. On the *MSN4* gene of H6, I identified two mutations that were the same as those in the K7 group: one is a single nucleotide substitution at the 2nd thymine (codon conversion from aTg to aCg), and the other is a nonsense mutation at the 1540th cytosine (codon conversion from Caa to the nonsense Taa) (Figure 7A), resulting in high fermentation

ability (Watanabe *et al.,* 2011).

Surprisingly, on the *RIM15* gene of H6, there was no the single adenine insertion in the 5055th nucleotide (*rim15*^{5055insA}) as was observed on the *RIM15* gene of the K7 group causing the deletion of the C-terminal 75 amino acids. However, the *RIM15* gene of H6 has three specific SNPs (black circles in Figure 7B): L224M [the 224th leucine (Ctg) replaced with methionine (Atg)], S515P [the 515th serine (Tct) replaced with proline (Cct)], and K1738N [the 1738th lysine (aaA) replaced with asparagine (aaC)]. Rim15p has five functional domains: PAS (a Per-arnt-Sim domain), C_2HC (a CCHC-type zinc finger domain), PK1 (a protein kinase catalytic domain), PK2 (a protein kinase catalytic domain), and REC (a putative receiver domain). One mutation (K1738N) but not others (L224M and S515P) is located in the REC domain. Since the 1,738th lysine (K) is not a conserved amino acid in the REC domain (Figure 7C), the Rim15p of H6 can be expected to be functional. Further analyses are required to clarify the effect of these mutations on the function of Rim15p.

Modern sake yeast strains, the K7 group share the same mutations on both *RIM15* and *MSN4* genes, which are involved in high alcohol fermentation ability in sake yeast strains of the K7 group. However, classical sake yeast strains (K1 to K5, K8, and Yabe Kozai), as well as shochu (a Japanese traditional distilled liquor), wine, beer, and laboratory yeast strains, do not possess these mutations on the two genes, indicating specificity of the mutations among the K7 group (Watanabe *et al.,* 2011; Akao *et al.,* 2018). My phylogenetic analysis revealed genetic closeness of H6 and the K7 group strains (Figure 6). Interestingly, H6 possesses the previously reported loss-of-function mutation on *MSN4* but not on *RIM15* (Watanabe *et al.,* 2011; Watanabe *et al.,* 2012). H6 is the first and unique strain revealed to present such distribution pattern of the two mutations. Although H6 is closely related to the K7 group, H6 should not be included in the K7 group because I consider that strains sharing the mutation on both *RIM15* and *MSN4* would be eligible for member of the K7 group (Figure 7) (Akao *et al.,* 2018). Taken together, phylogenetic and evolutionary relationships between them are very intriguing

question, and further genomic study of H6 would provide some important insights for line of evolution to a common ancestor of the K7 group.

I .4. 7 The effects of *RIM15* deletion on the fermentation properties of H6

In the *RIM15* gene in H6, there was no *rim15*^{5055insA} mutation, which is related to the high fermentation ability of the yeast strains in the K7 group. To investigate whether the *RIM15* gene of H6 is related to the strain's fermentation ability, I examined the effect of *RIM15* deletion on the fermentation ability. I constructed single and double deletions of the *RIM15* gene in an H6 diploid (single deletion: H6*Δrim15*, double deletion: H6*Δ/Δrim15*) (Table 1), and I performed another small-scale sake fermentation test using the three strains H6, H6*Δrim15*, and H6*Δ/Δrim15*.

As a result (Figure 8), at the early stage of the fermentation (4 days), the CO_2 production (g/day) of the H6*Δ/Δrim15* strain was significantly lower than that of H6, but from 12 days onward, conversely, the production of H6*Δ/Δrim15* was higher than that of H6. No significant difference in the sake components fermented from the three yeast strains was observed (Table 5). Recent report described that, in barley shochu yeast, the fermentation ability of the *RIM15*-deleted cells was not different from that of the parental strain (Watanabe *et al.,* 2019b).

The PP2A regulatory B subunit (PP2A$^{B55\delta}$)-encoding *CDC55* gene of several sake strains in K7 group has heterozygous loss-of-function mutations (nonsense or frameshift mutations). The dose-dependent reduction of the normal protein level of Cdc55p from these heterozygous mutations would contribute to the fermentation performance as weak suppressors of *rim15*^{5055insA} mutation (Watanabe *et al.,* 2019a). Further, the R48P mutation of Cdc55 in K1801 (Kyokai sake yeast no.1801) causes a defect in spindle-assembly checkpoint (SAC) (Goshima *et al.,* 2016). Therefore, to investigate whether Cdc55p of strain H6 has normal function in SAC, I examined the sensitivity of TBZ of the H6 strain. As shown in Figure 9, no defect in the SAC of strain H6 was observed, suggesting that the Cdc55p of strain H6 is functional in SAC. Further investigations are

needed to clarify the effect of the Cdc55p of H6 on the strain's fermentation ability.

I .4. 8 The effects of *RIM15* deletion on the sporulation properties of H6

Spore formation of H6 was induced in the sporulation medium, *IME1* was normally expressed, and normal spore germination was observed (Figure 2). Although H6 is genetically closely to the K7 group (Figure 6), H6 does not have the *rim15*^{5055insA} mutation like K7 does. To determine whether the sporulation ability of H6 is dependent on the *RIM15* gene, I examined the sporulation frequency and germination ability of spores from a tetrad of three strains: H6, H6*Δrim15*, and H6*Δ/Δrim15*.

As illustrated in Figure 10, the double *RIM15*-deleted cells reduced the sporulation frequency <5%, consistent with the result reported previously (Reinders *et al.,* 1998). However, the germination ratio of spores from the *RIM15*-deleted H6 cells was almost the same as that of the parental H6 strain. These results indicated that the sporulation ability of H6 was partially dependent on the function of Rim15p.

The K7-group strains have defects in both sporulation and chromosomal recombination in meiosis (Shimoi *et al.,* 2019). However, I observed that the chromosome recombination in H6 occurred normally, and the sporulation ability of H6 was partially dependent on Rim15p. Comparison analyses of H6 and K7 will be important to clarify the causes of the defects in sporulation and recombination in K7.

In conclusion, to develop a partner strain for the breeding of sake yeast by genetic crossing, as the first step I analyzed the fermentation and sporulation properties of the traditional sake yeast strain H6. The fermentation ability of H6 was suitable / sufficient for sake brewing. The DMTS-producing ability of H6 was low, and H6 did not produce 4VG. My phylogenetic analysis based on DNA sequences of five genes indicated that H6 is closely related to the K7 group, but interestingly *RIM15* of H6 does not have the same mutation as K7, although *MSN4* of H6 has the same non-functional mutation as that of K7. H6 showed normal sporulation frequency that was similar to that of a lab strain, and consistently, unlike K7 (Shimoi *et al.,* 2019), in H6 both *IME1* expression and meiotic

chromosomal recombination occurred normally. Interestingly, the normal sporulation frequency but not the fermentation ability of H6 was partially dependent on Rim15p. Strain H6 thus has excellent properties as a partner strain for sake yeast breeding by genetic crossing.

Table 1 Yeast strains used in this study

	Remarks	Genotype	Source
K7	Sake yeast Kyokai no.7	*MAT*a/α	Brewing society of Japan
K901	Sake yeast Kyokai no.901	*MAT*a/α	Brewing society of Japan
H6	sake yeast Hiroshima ∩o.6	*MAT*a/α	Hiroshima prefecture yeast Collection
H6Δ*rim15*	H6 *Δrim15::natMX*	*MAT*a/α	This study
H6Δ/Δ*rim15*	H6 *Δrim15::natMX/Δrim15::kanMX*	*MAT*a/α	This study
H6–*UCH1*	H6 *Δura3::natMX/URA3 Δcan1::kanMX/CAN1*	*MAT*a/α	This study
H6–*UCH2*	H6 *Δura3::natMX/URA3 Δcan1::kanMX/CAN1*	*MAT*a/α	This study
BY4743	Lab strain	*MAT*a/α	EUROSCARF
X2180	Lab strain	*MAT*a/α	American type culture Collection
X2180-1A	Lab strain	*MAT*a	American type culture Collection
H6-Hapα3	sake yeast Hiroshima no.6 Haploid	*MAT*α	This study
H6XHyb	Hybrid strain between H6 and lab strain	*MAT*a/α	This study
H6XHyb Tetrad	Haploid derived from H6XHyb	-	This study

Table 2 Primers used in this study

Primer	Sequence(5'-3')	Reference
Sequence for Phylogenetic tree		
THI7- FW	GATTCATCGCAACATCATTTAAAGG	(Futagami *et al.,* 2017)
THI7- RV	CCAATTGGAGGAACCAAATCTC	
ZAP1- FW	TAGTACCAATCACGACTCTCAC	
ZAP1- RV	CTGATTCTGATGAGTGACTTTGG	
PXL1- FW	GGTGTGTCATCATCGTCAAC	
PXL1- RV	CATTCACGATGCCATTGACC	
GLG1- FW	AATTGTTAGAGGAAGCAGGC	
GLG1- RV	GTTAGGAGCAGGGCTATGATC	
YRR1- FW	ACACTAATTCGTCTGCGAGC	
YRR1- RV	CAATAAGGGTCTACCAACGTTAC	
For sequencing FDC1		
FDC1-FW1	TTCCTCTGAGTTATTCTATTCTTG	(Mukai *et al.,* 2014)
FDC1-FW2	CCAATCACTGTTCCTGTGTCATCT	
FDC1-FW3	TCGGTTCCAGTAGTAAAATGTGAG	
FDC1-FW4	AAGCATTGAAGACAACGCCTGAAG	
FDC1-RV1	GAAAGATGGATAGTGTTAATGGCG	
For sequencing PAD1		
PAD1-FW1	CATAATGCTGCAAATATAGATTGA	(Mukai *et al.,* 2014)
PAD1-FW2	TGGCAACCAAGACATACTCTGTTC	
PAD1-RV1	TTTAGCAAGTAACAAATCAACTCT	
PAD1-RV2	GAACAGGGCACAACAATCATACCA	
For sequencing RIM15		
RIM15-P-FW	CCATATTGCCCTAGGTCTTGTTTA	This study
RIM15-P-RV	CTATTCTTCCAGAGGATGAT	
RIM15-S-FW1	CTGGTGGGTGCTTTTACTGAATTCC	
RIM15-S-FW2	ACGACCTTGGAAGCACGTGG	
RIM15-S-FW3	GATACAGGTACGTTCCAACA	
RIM15-S-FW4	CAATCCTAACTCACCCAATG	
RIM15-S-FW5	TCCTCTTCGTCAAGGCTGGG	
RIM15-S-FW6	CTGTTAAATCTCCAACGCCTAGC	
RIM15-S-FW7	CGGTTTATCAAGAGCTGGTC	
RIM15-S-FW8	ATCGCAGCTACCAATACGAA	
RIM15-S-FW9	GATTTTGGAGACGATATCGA	
RIM15-S-FW10	AACAACCTGGGGTTCACAGA	
RIM15-S-FW11	AGAGCTCGAGCACTTCTGAG	
RIM15-S-RV	TAATGAACGGGGGAAAATCC	
For sequencing MSN4		
MSN4-P-FW	TTGCTATTCTCCGGATAAAC	This study
MSN4-P-RV	CGAATGAAATGACCAACCTACTTG	
MSN4-S-FW1	TTTTCGCAACTGGTCAACAGGC	
MSN4-S-FW2	TACCGCCAGTTTAGCTACCA	
MSN4-S-FW3	GTCGCGACGCAAGAAGATACA	
MSN4-S-FW4	GCTTAGATGCAACCACAATG	
MSN4-S-FW5	GCAGTGAGCACTTGAAAAGG	
MSN4-S-RV	CGTTAAAACCAGAACGTGCAC	
For Deletion of URA3		
URA3-DFW	ACCTGCAGGAAACGAAGATAAATCATGTCGAAAGCTAC ATATAAGGAACGTGCTGCTACTCATC	This study
URA3-DRV	TTTACTTATAATACAGTTTTTTAGTTTTGCTGGCCGCATC TTCTCAAATATGCTTCCCAGCCTG	
URA3-ch-FW	GAACAAACACCAGAGTCAAACGACG	
URA3-ch-RV	CTGCCCTACACGTTCGCTATGCTTC	
For Deletion of CAN1		
CAN1-DFW	CAATCTGTCGTCAATCGAAAGTTTATTTCAGAGTTCTTCA GACTTCCGTACGCTGCAGGTCGAC	This study
CAN1-DRV	TGAGAATGCGAAATGGCGTGGGAATGTGATTAAAGGT AATAAAACATCGATGAATTCGAGCTCG	
CAN1-ch-FW	GAATCAGGGAATCCCTTTTTGC	
CAN1-ch-RV	GTTCGGGTGACGTGAAGATAACG	
For Deletion of RIM15		
RIM15-D-FW	CTCTTGCCTCATTTGATAGAATAGATAAGCCCAGTAGAG GAAGACCGTACGCTGCAGGTCGAC	This study
RIM15-D-RV	TTTTTATTCAGTTATTTTTTTTAATTATCTTTATCTTAAAAT TTAATCGATGAATTCGAGCTCG	
RIM15-ch-FW	AAGTTGTTGTTCGTATCACAGC	
RIM15-ch-RV	CTCTAACAAAGGAGAATATATATACG	
For RT-PCR		
IME1-RT-FW	GCCTCATCTCAGCTGCAGAA	This study
IME1-RT-RV	TGAGTGGAACGTAGATGCGG	

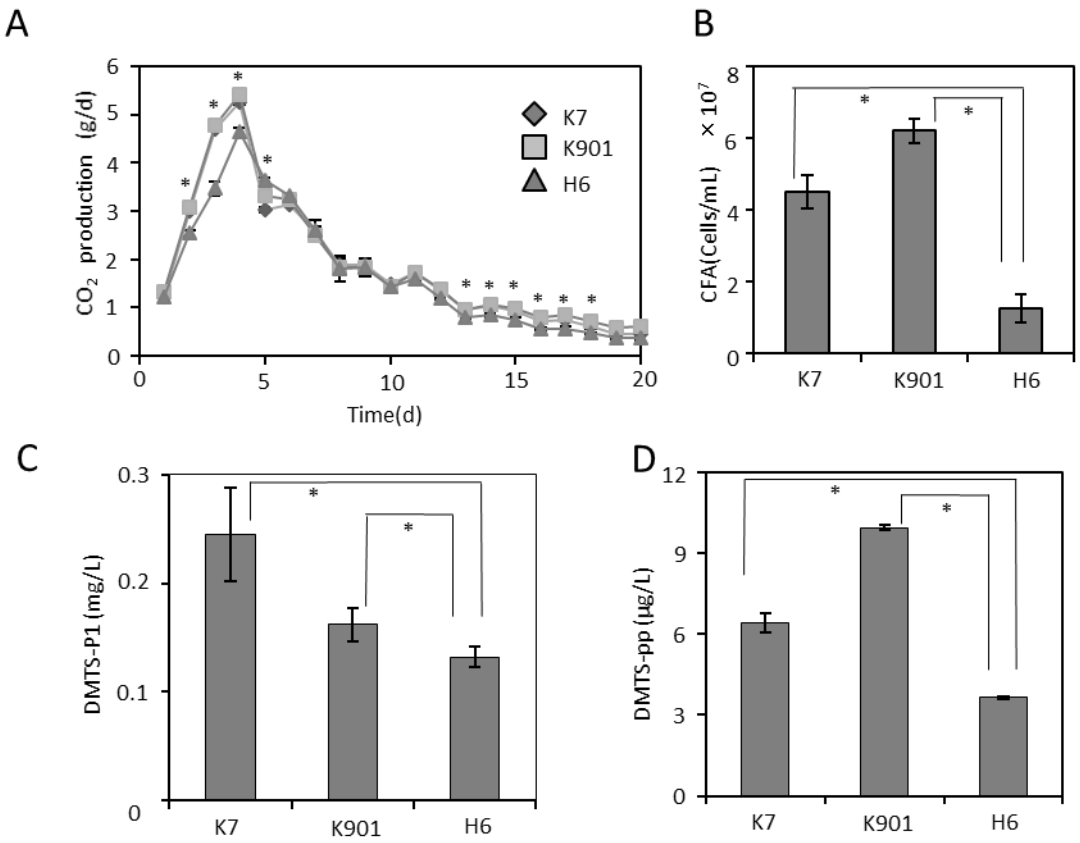


Figure 1 Brewing properties of the sake yeast strain Hiroshima no. 6 (H6).

A: The CO_2 production (g/d) in small-scale sake fermentation tests of strains H6, K7, and K901. The values are the average of three independent experiments. *$p<0.05$ vs. the H6 value (t-test). B: The colony-forming ability (CFA) over the 20-day period in the small-scale sake fermentation tests. *$p<0.05$ vs. the H6 value (t-test). C, D: Contents of dimethyl trisulfide precursor (DMTS-P1) and dimethyl trisulfide producing potential (DMTS-pp). *$p<0.05$ vs. the H6 value (t-test).

Table 3 Values are the averages from assays in at least 3 independent experiments.

	General properties [a]				Organic acids [b] (mg/L)					Flavor component [c] (ppm)			
	ALC (%)	SM	TA (mL)	AA (mL)	Cit	Mal	Suc	Lac	Ace	EtOAc	iAmOAc	iAmOH	EtOCap
K7	17.7	5.1	3.6	2.0	75	171	884	281	606	122	4.2	198	1.4
K901	17.7	6.5	3.7	1.9	80	197	796	433	547	115	3.7	190	1.4
H6	16.1	-8.0	3.1	2.5	84	90	555	357	767	88	2.2	188	0.9

a, c: The general properties (SM, sake meter; Alc, alcohol; TA, total acidity; AA, amino acid content) and flavor components (EtOAc, ethyl acetate; iAmOAc, isoamyl acetate; iAmOAc, isoamyl alcohol; EtOCap, ethyl caproate) of the sake were analyzed by the standard method established by the National Tax Agency of Japan. b: Organic acids (Cit, citric acid; Mal, malic acid; Suc, succinic acid; Lac, lactic acid; Ace, acetic acid) of the sake were analyzed by HPLC.

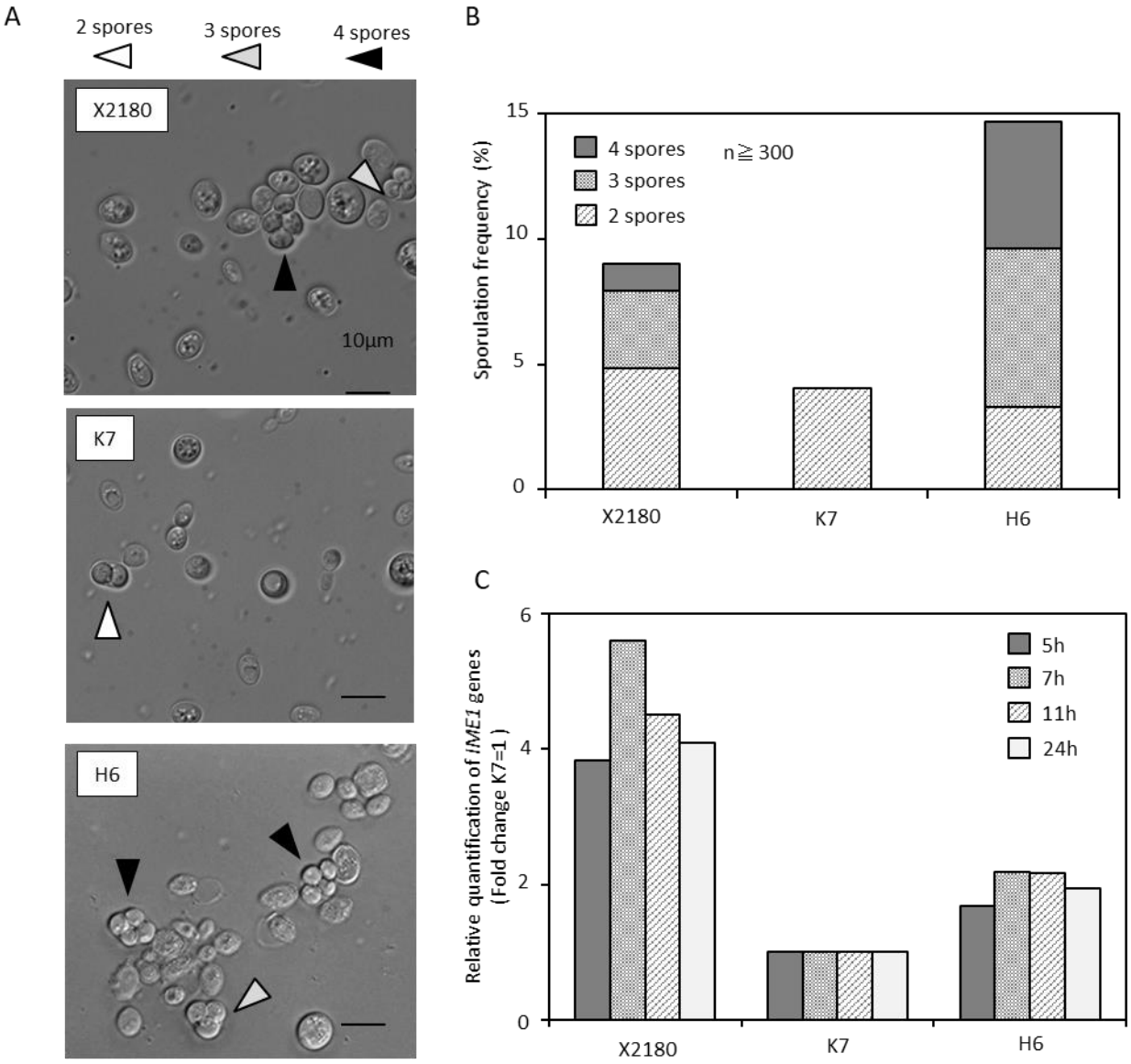


Figure 2 Sporulation properties of H6.

A: Microscope images of strains X2180, K7, and H6 in the sporulation medium. B: Sporulation frequency (%). The numbers of asci with the indicated number of spore(s) in 300 cells of each strain were counted by microscopic observation. C: Expression of *IME1* under the sporulation conditions in H6. The results of qRT-PCR experiments with K7, X2180, and H6 cells isolated from sporulation medium are shown. The relative expression levels are given as fold differences compared to the induction levels obtained for K7 at each time point, using *ACT1* as a reference gene.

Table 4 Genotypes of haploid strains after sporulation of the double heterozygous H6 strain by tetrad analysis.

Parent diploid	Genotypes of haploids			
	CAN1URA3	*can1URA3*	*CAN1ura3*	*can1ura3*
H6−*UCH1*	11	5	5	11
H6−*UCH2*	16	2	2	16

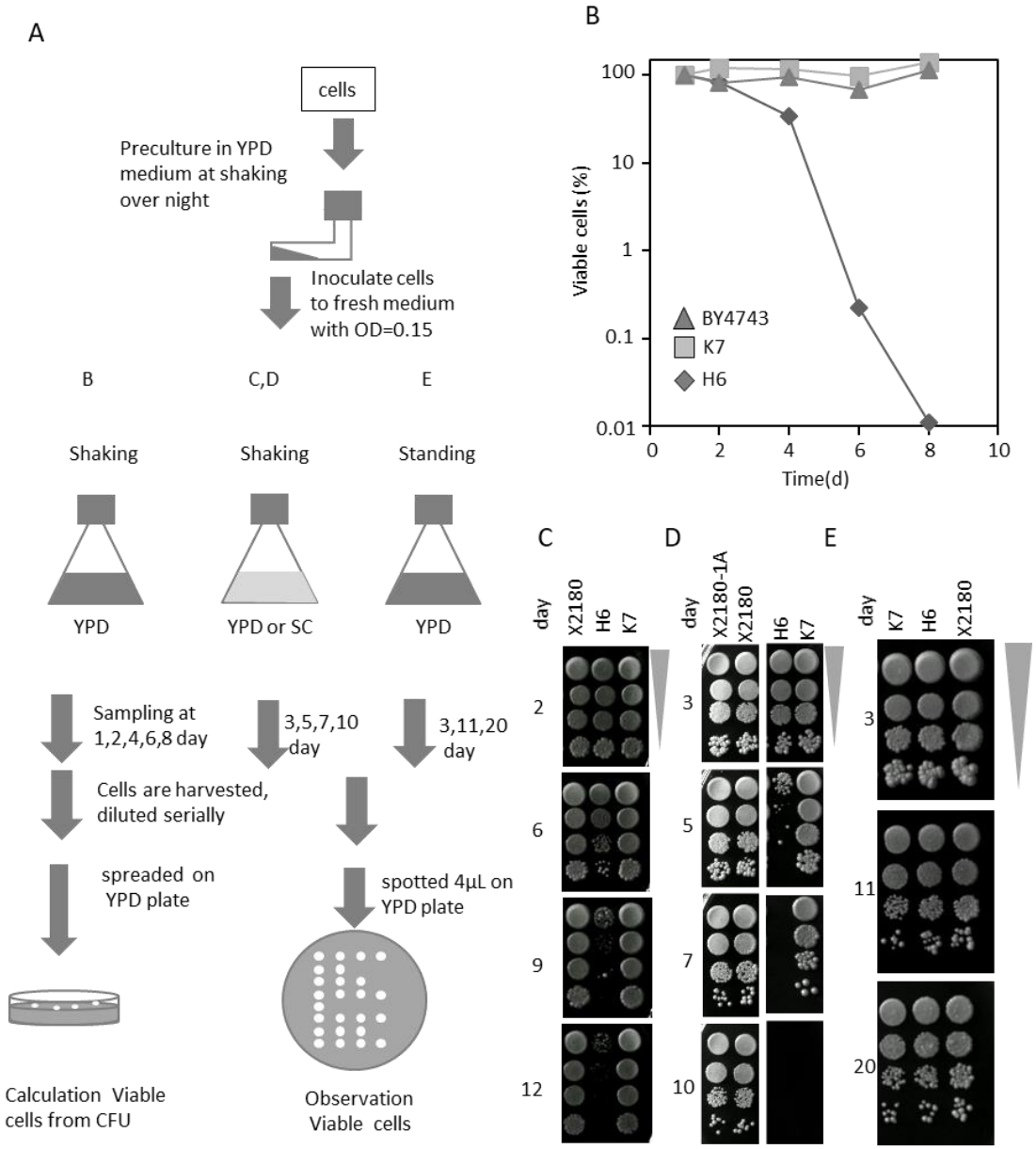


Figure 3 The colony-forming ability (CFA) of sake yeast cells under various culture conditions.

A: Experimental procedure for the investigation of the three sake yeast strains' CFA. B: The CFA of the cells in YPD medium with shaking culture. C: The CFA of the cells in YPD medium with shaking culture. The cells were serially diluted from 1×10^7 cells/mL to 1×10^4 cells/mL. D: The CFA of the cells in SC medium with shaking culture. The cells were serially diluted from 1×10^7 cells/mL to 1×10^4 cells/mL. E: The CFA of the cells in YPD medium with standing culture. The cells were serially diluted as in panel C.

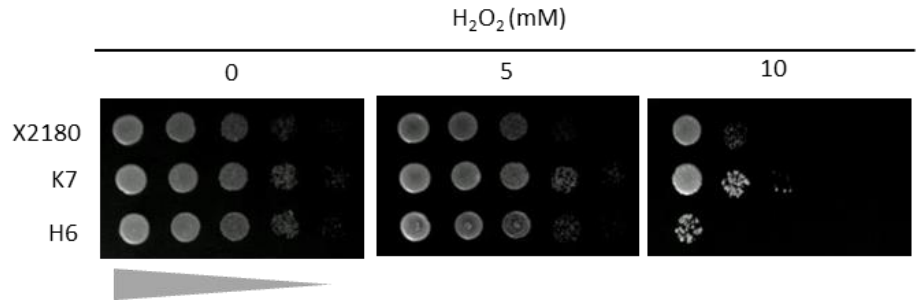


Figure 4 The yeast strains' resistance to oxidative stress.

The yeast strains' resistance to oxidative stress. The strains were grown overnight in YPD medium at 30°C. Cells were diluted to OD660 = 1.0 serially, spotted onto YPD plates with hydrogen peroxide, and incubated at 30°C.

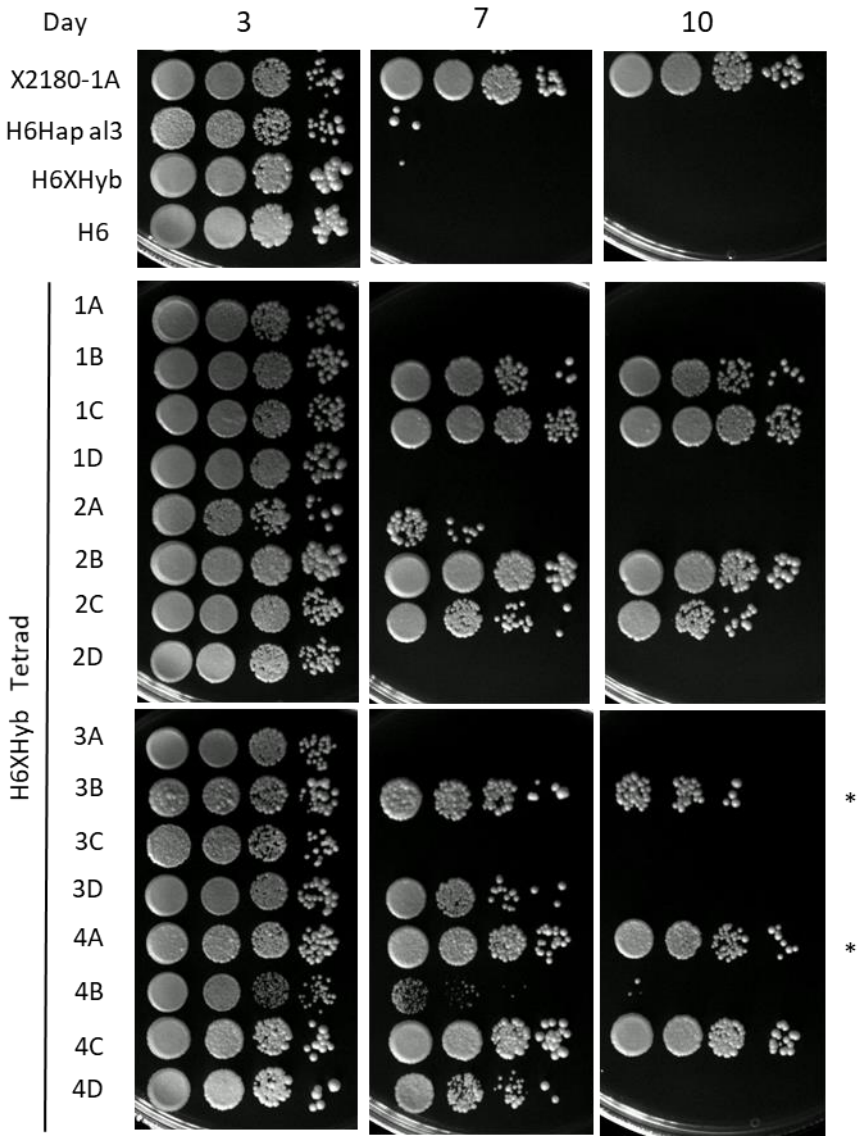


Figure 5 The CFA of the tetrad derived from H6XHyb in SC medium with shaking culture.

The cells were serially diluted from 1×10^7 cells/mL to 1×10^4 cells/mL. * Strains that had strong aggregation during culture.

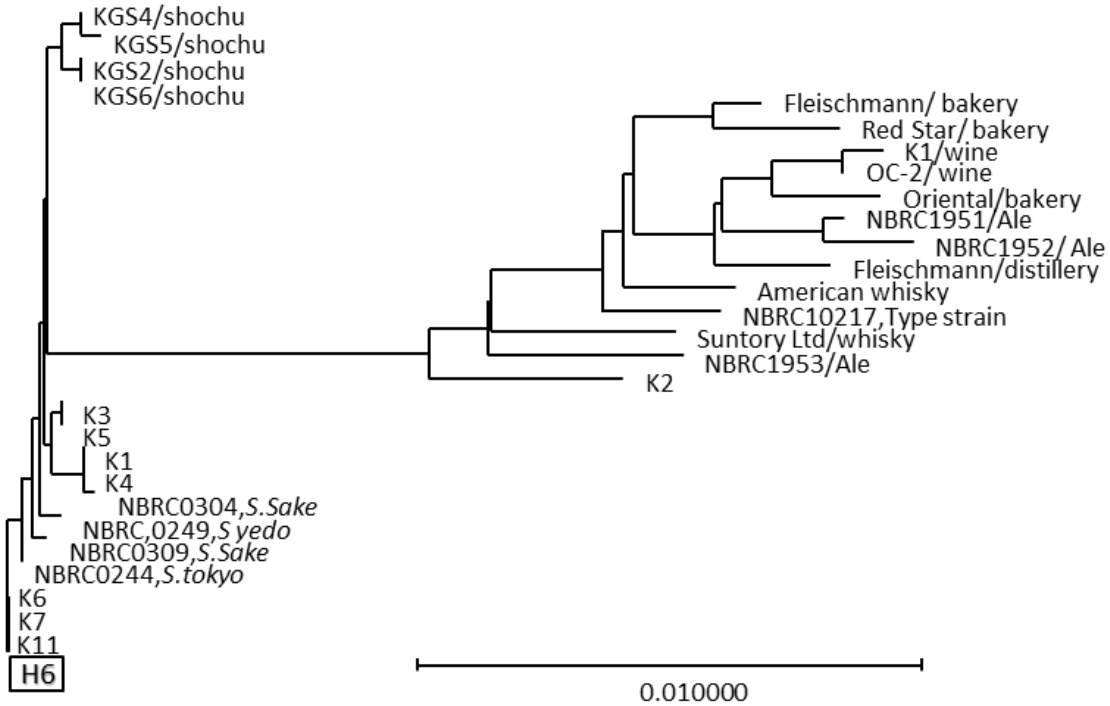


Figure 6 The phylogenetic tree of H6 based on the nucleotide sequence of five genes.

The phylogenetic tree of the industrial *S. cerevisiae* strains based on the DNA sequences of the five genes *ZAP1, THI7, PXL1, YRR1* and *GLG1*. This tree was constructed by the NJ method on Genetyx ver. 13. KGS2/shochu: Kagoshima no.2/shochu yeast, KGS4/shochu: Kagoshima no.4/shochu yeast, KGS5/shochu: Kagoshima no.5/shochu yeast, KGS6/shochu: Kagoshima no.6/shochu yeast, K1: Sake yeast/kyokai no.1, K2: Sake yeast/kyokai no.2, K3: Sake yeast/kyokai no.3, K4: Sake yeast/kyokai no.4, K5: Sake yeast/kyokai no.5, H6: Hiroshima sake yeast/no.6, K6: Sake yeast/kyokai no.6, K7: Sake yeast/kyokai no.7, K-11: Sake yeast/kyokai no.11, Fleischmann/bakery: Fleischmann/bakery yeast, Red Star/bakery: Red Star/bakery yeast, K1/Wine: K1/Wine yeast, OC-2/wine: OC-2/wine yeast, Oriental/bakery: Oriental/bakery yeast, NBRC1951/Ale: NBRC1951/Ale yeast, NBRC1952/Ale: NBRC1952/Ale yeast, NBRC1953/Ale: NBRC1953/Ale yeast, Fleischmann/distillery: Fleischmann/distillery yeast, American whisky: American whisky yeast, Suntory Ltd/whisky: Suntory Ltd/whisky yeast no.1.

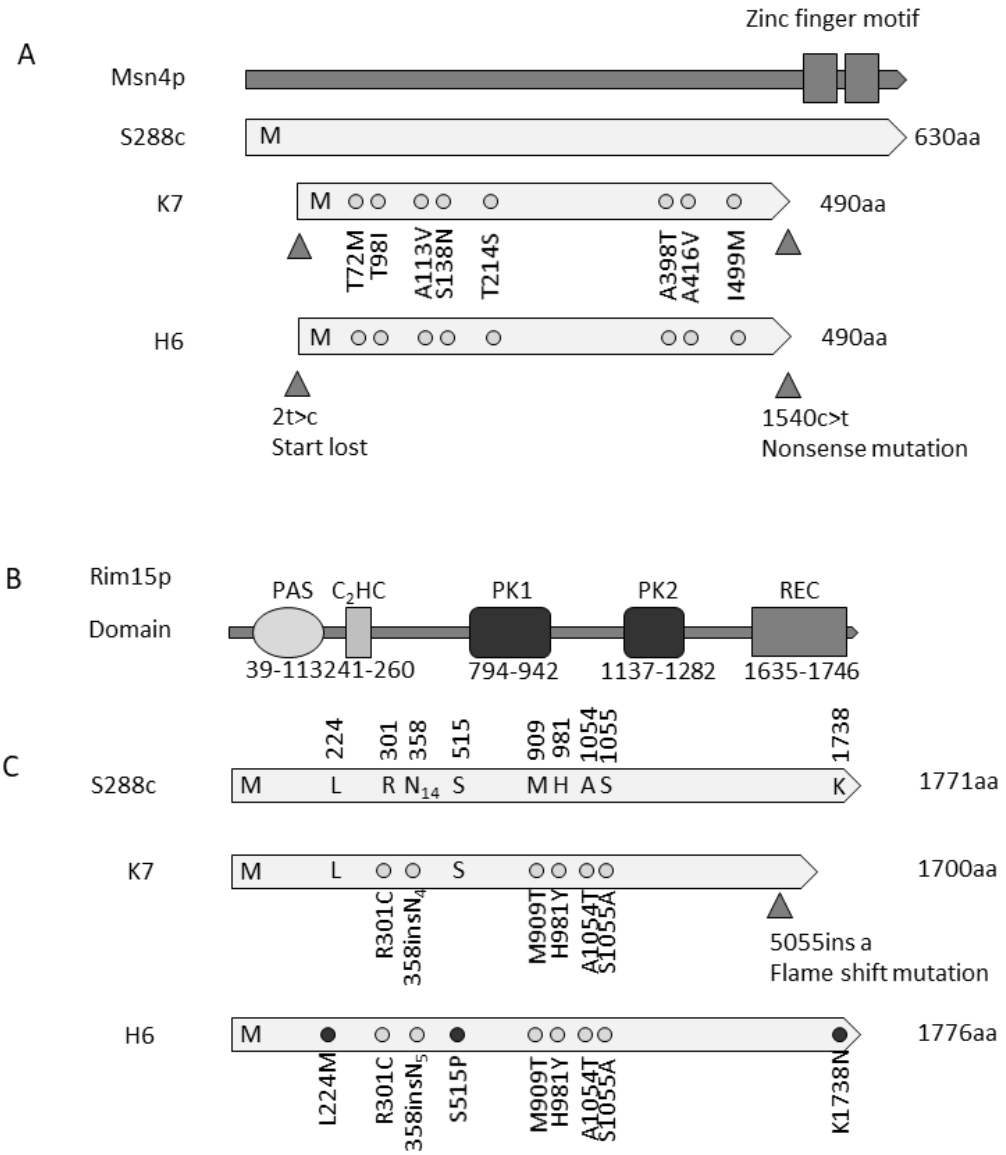


Figure 7 Nucleotide polymorphisms of Msn4p and Rim15p in the sake yeast strains.

A: Mutation sites in the *MSN4* gene of strains H6 and K7. Lowercase letters: nucleotides, uppercase letters: amino acids. Gray circles: Nonsynonymous SNPs of *MSN4*. Triangles: Two common mutation sites of H6-Msn4p and K7-Msn4p; one is the single nucleotide substitution at the 2nd T (codon conversion from aTg to aCg) and the other is the nonsense mutation at the 1540th C (codon conversion from Caa to Taa), leading to the N-terminal- and C-terminal truncations of Msn4p in strains K7 and H6.

B: The functional domain of Rim15p. PAS: the Per-arnt-Sim domain, C2HC: CCHC-type zinc finger domains, PK1: protein kinase catalytic domain, PK2: protein kinase catalytic domain, REC: putative ATP receiver domain. Numbers indicate the amino acids of Rim15p. C: Mutation sites in the *RIM15* gene of strains H6 and K7 Lowercase letters: Substitutions in DNA sequences. Uppercase letters: Substitutions in amino acids. Gray circles: Common SNP of both K7 and H6. Dark circles: Specific SNPs of H6 strains. Triangles: The frame-shift mutation by insertion at the 5055th nucleotide, leading to the C-terminal truncation of Rim15p of K7.

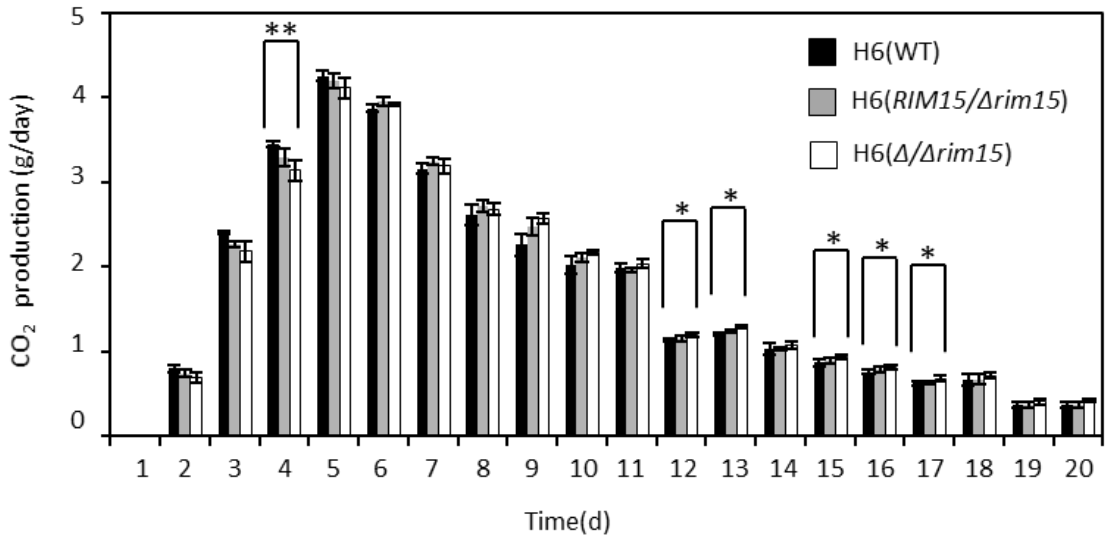


Figure 8 The effect of *RIM15* deletion on the fermentation properties of H6.

The CO_2 production (g/days) in the small-scale sake fermentation tests of the three strains. The values are the average of three independent experiments. **Significantly lower than the H6 (WT) value (*p<0.05, t-test*). *Significantly higher than the H6 (WT) value (*p<0.05, t-test*).

Table 5 The effect of *RIM15* deletion on the fermentation properties of H6.

	General properties [a]				Organic acids [b] (mg/L)					Flavor component [c] (ppm)			
	ALC (%)	SM	TA (mL)	AA (mL)	Cit	Mal	Suc	Lac	Ace	EtOAc	iAmOAc	iAmOH	EtOCap
H6(WT)	17.3	-9.4	3.4	2.3	69	80.2	490	396	502	56	2.0	167	1.0
H6(RIM15/Δrim15)	17.3	-8.3	3.4	2.3	67	77.2	514	385	478	58	2.1	175	1.0
H6(Δ/Δrim15)	17.1	-9.8	3.4	2.2	68	80.4	527	384	482	50	1.7	171	0.8

Values are the averages from assays in at least 3 independent experiments.

a,c: The general properties (SM, sake meter; Alc, alcohol; TA, total acidity; AA, amino acid content) and flavor components (EtOAc, ethyl acetate; iAmOAc, isoamyl acetate; iAmOAc, isoamyl alcohol; EtOCap, ethyl caproate) of the sake were analyzed by the standard method established by the National Tax Agency of Japan.

b: Organic acids (Cit, citric acid; Mal, malic acid; Suc, succinic acid; Lac, lactic acid; Ace, acetic acid) of the sake were analyzed by HPLC.

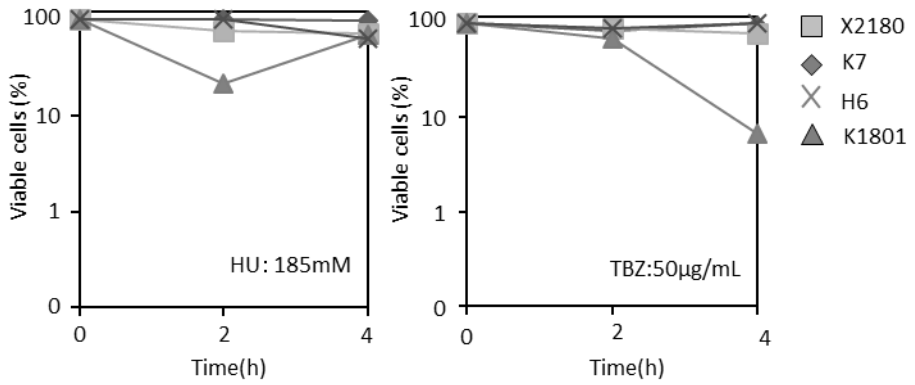


Figure 9 The yeast strains' drug sensitivity.

The strains were grown overnight in YPD medium at 30°C. Cells were inoculated into fresh YPD medium to OD660 = 0.1. After a 4-h incubation, TBZ (50 μg/mL) and HU (185 mM) were added respectively to the culture at the early log phase.

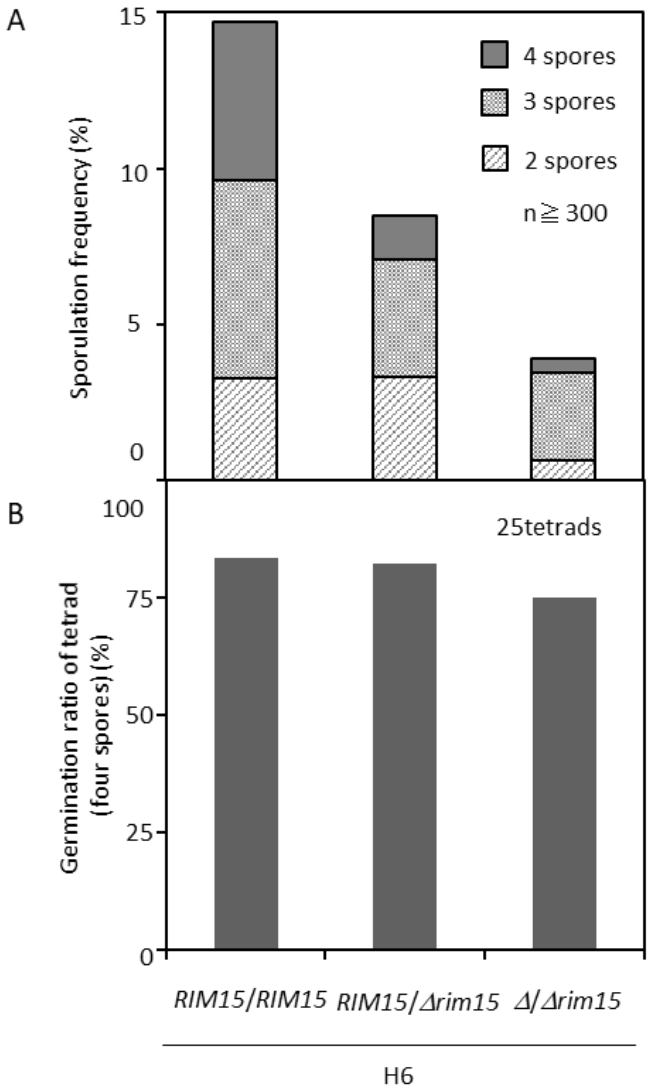


Figure 10 The effect of *RIM15* deletion on the sporulation properties of H6.

A: Sporulation frequency (%). The numbers of asci with indicated number of spore(s) in 300 cells of each strain were counted by microscopic observation. B: The germination frequency of spores from each ascus with four spores. The spores for the germination frequency measurement were isolated by a micromanipulator. Twenty-five tetrads were placed on a YPD plate and incubated at 25°C for 4–5 days, and then the germinated spores were counted.

CHAPTER II

Development of sake yeast haploid set with diverse brewing properties using sake yeast strain Hiroshima no. 6 exhibiting sexual reproduction.

II. 1 ABSTRACT

The long-preserved sake yeast strain Hiroshima no. 6 (H6) is genetically extremely similar to Kyokai no. 7 (K7) and its closely related strains (K7 group). K7 group strains are predominantly used because of their excellent brewing properties but lack normal sporulation ability, including the ability to undergo meiotic chromosomal recombination, which leads to difficulties in crossbreeding. However interestingly H6 exhibited normal sporulation. Thus, we isolated haploid cells from H6 and mated them with previously isolated haploid cells of K7 group strains. The crossbred diploid strains had normal sporulation ability; hence, we performed tetrad analysis. The brewing characteristics of the obtained haploid set were extremely diverse. Principal component analysis based on the volatile and organic acid components measured using small-scale sake brewing tests revealed that the haploid strains derived from each diploid strain displayed a characteristic distribution. Thus, we demonstrated the availability of genetic crossbreeding using H6 with sporulation ability to facilitate both the development of novel sake yeast strains with many desirable characteristics and analyses of the function of sake yeast.

II. 2 INTRODUCTION

Phylogenetically, sake yeast strains belong to the budding yeast *Saccharomyces cerevisiae*, although they possess a number of different characteristics that differ from other *S. cerevisiae* strains, including laboratory strains. The major strains used in industrial sake manufacturing are Kyokai no. 7 (K7) and its closely related strains (K7 group: K6, K7, K9, K10, and their derivatives). These strains produce high concentrations (>20%) of ethanol (Watanabe *et al.,* 2011; Watanabe *et al.,* 2012). The characteristics of sake are also determined by its volatile aroma and the organic acid components produced by yeast, and the breeding of many yeast strains has been performed to produce strains with distinguished organoleptic characteristics.

To date, mutagenesis breeding of sake yeast strains has been employed to produce a number of desired characteristics in a stepwise manner. Regarding breeding *via* mutagenesis, one of the desired/target characteristics for breeding sake yeast is the production of the aroma component ethyl caproate, one of the major favorable aroma components in *ginjo* sake. Previously, it was not easy to produce sake containing large amounts of ethyl caproate. However, this problem was overcome by the development of a new breeding method, namely isolating yeast strains with a specific mutation in *FAS2*, which encodes a subunit of fatty acid synthase, *via* selection using cerulenin resistance as an indicator (Ichikawa *et al.,* 1991). Organic acids are also important components affecting the taste of sake. For example, malic acid gives a fresh sour taste, which is a favorable sake characteristic (Sato *et al.,* 1977). As organic acids are mainly produced by yeast during fermentation, several methods for isolating high malic acid-producing mutants from sake yeast have been developed (Arikawa *et al.,* 1999a; Arikawa *et al.,* 1999b; Enomoto *et al.,* 2002; Motomura *et al.,* 2012; Arikawa *et al.,* 1992; Yoshida *et al.,* 1993; Negoro *et al.,* 2016).

Reducing the health risk of sake products is also an important breeding target for sake yeast. Ethyl carbamate (ECA) is classified as a weak carcinogenic component by the World Health Organization (International Agency for Research on Cancer, 2010), and it

is a major risk component in alcoholic beverages. Because it is chemically generated from urea and ethanol in sake products, preventing urea formation by yeast cells directly results in a decrement of ECA content in sake products (Kitamoto *et al.,* 1991). Arginase, encoded by *CAR1*, converts L-arginine to urea in yeast cells, and selection using medium containing canavanine, arginine, and ornithine (CAO medium) enables the isolation of arginase-deficient cells (Kitamoto *et al.,* 1991). However, mutagenesis breeding has problems including changes in brewing characteristics caused by unexpected mutations, difficulty in obtaining recessive mutants, and low genetic diversity. Thus, the need for genetic crossing is potentially high.

The genetic crossing of K7 group strains has been considered difficult because they commonly possess defects in sporulation and meiosis. In these strains, the low transcriptional level of *IME1*, a master regulator of sporulation, causes low sporulation frequency (Nakazawa *et al.,* 1992). Additionally, aberrant meiotic chromosomal recombination results in uneven chromosomal segregation (Shimoi *et al.,* 2019). Therefore, it is extremely difficult, albeit not completely impossible, to obtain viable spores of K7 group strains. Fortunately, several studies succeeded in the isolation of K7 group haploid cells using the random spore method (Katou *et al.,* 2008) and subsequent crossbreeding (Yoshida, 2006; Goshima *et al.,* 2016; Fujiwara *et al.,* 2017). However, those diploid cells do not spore as well as the wild-type K7 group strains, and thus, backcrossing and genetic analysis as performed in plant breeding are extremely difficult. Furthermore, the range of genetic variation of the segregated haploid cells is almost limited within combinations of 16 chromosomes, due to fixed haplotypes by defective meiotic recombination.

In a previous chapter, I revealed that the long-preserved sake yeast strain Hiroshima no. 6 (H6) possesses sporulation ability. H6 is the oldest stock strain preserved in the Hiroshima Prefecture yeast collection, and it was isolated from the defunct Kikubotan Brewery in Hiroshima in 1926 (Tsutsumi, 1980). In addition, simplified phylogenetic analysis using five genes revealed that H6 is genetically similar to K7 group

strains that are widely used in industrial sake manufacturing. K7 group strains (Watanabe *et al.,* 2012; Akao *et al.,* 2018), but not H6, commonly and specifically possess the *RIM15* mutation (*rim15*^{5055insA}), indicating that H6 does not belong to the K7 group. These genetic similarities and differences imply a unique phylogenetic position of H6 among the sake yeast population. Moreover, I rediscovered that H6 can generate four viable spores in an ascus because of the likelihood of normal sporulation and meiosis.

Based on my observations, it appears to be promising that crossbreeding between H6 and K7 group strains can produce diploid cells with the capacity for sexual reproduction. Sporulation of such crossbred diploid cells should produce a series of haploid cells. These haploid series would be available for breeding new sake yeast strains with diverse brewing characteristics. Accordingly, in this chapter, I isolated H6 haploid cells and selected three types for mating with previously isolated haploid cells from three K7 group strains with specific features. All of the first filial generation (F_1) hybrid cells of the tested haploid combinations exhibited sexual reproduction as expected. In this chapter, I isolated a series of haploid cells from crossbred diploid strains and analyzed their brewing characteristics. Additionally, I examined the relationships of specific genotypes and phenotypes using two functional mutations in *FAS2* and *CAR1* as model traits of targeted crossbreeding.

II. 3 MATERIALS AND METHODS

II. 3. 1 Yeast strains and media

The yeast strains used in this study are listed in Table 6. KArg901-Haploid cells were newly obtained using the random spore method as described previously (Katou *et al.,* 2008). The yeast cells were cultured aerobically in yeast extract-peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% glucose) at 30°C with shaking. For sporulation, I used the sporulation medium (2% potassium acetate). I tested the ability of the cells to grow in the presence of cerulenin by spotting the yeast onto YPD medium containing 2 or 8 mg/L cerulenin. Regarding the growth of yeast cells on arginine-containing medium, I used SD medium plates containing 5 mM arginine or ornithine (Kitamoto *et al.,* 1991).

II. 3. 2 Sporulation and tetrad isolation

The yeast cells were grown overnight on YPD plates at 30°C, and the cells were subsequently transferred to sporulation medium plates and incubated at 25°C for 3 days. Spore generation was confirmed *via* optical microscopy. Spores used to measure germination frequency were isolated using a micromanipulator (SporePlay, Singer Instruments, Somerset, UK). Spores dissected from 25 tetrads were incubated on YPD plates at 25°C for 4–5 days, and then the germinated spores were counted. Separation of tetrads was performed for diploid strains that were selected by fermentation tests and the germination of spores, and only haploid cells isolated *via* tetrad germination were used for subsequent analyses.

Ⅱ. 3. 3 Mating

Yeast cells were cultured in YPD medium at 30°C for 12 h, and then the cells were inoculated into fresh YPD medium at 5.0×10^5 cells/mL and cultured at 30°C until the log phase was reached (1.0×10^6–2.0×10^6 cells/mL). Then, 300 μL of *MAT*α cells and

*MAT*a cells were mixed into 3 mL of fresh YPD medium and incubated at 30°C for 20 h. After confirming zygote formation *via* microscopy, the cells were diluted appropriately and spread on a YPD plate. The colonies that grew well were isolated as diploid strain candidates.

II. 3. 4 Genotyping test

The genotypes of specific polymorphic sites were determined *via* high-resolution melting (HRM) analysis for *RIM15* (Akao *et al.,* 2018; Erali *et al.,* 2008) or mutant allele-specific amplification (MASA) for *FAS2* and *CAR1.* The MASA typing was performed using high single-nucleotide discrimination (HiDi) Taq DNA polymerase (myPOLS Biotec, Konstanz, Germany) (Sakurai *et al.,* 2019). MASA typing primers were designed for each allele so that the single nucleotide polymorphism (SNP) position was 3′. The primers used are shown in Table 7. The mutation sites for each gene were as follows: *rim15*^{m}:5055insA (frameshift mutation), *fas2*^{m}:G3748A (Fas2p:Gly1250Ser), and *car1*^{m}:CTT(848–850) deletion (Car1p:Phe283). The CTT (nucleotide positions 848–850) deletion in KArg901 *car1*^{m} that was presumably responsible for the loss of function was identified *via* sequencing in this study. The genotypes of *MAT* and *RIM15* were examined for all haploid cells.

II. 3. 5 Mating check of diploid strains produced by mating H6 and K7 group haploid cells

The type of *MAT* locus was determined using polymerase chain reaction assays, as described previously (Katou *et al.,* 2008). Additionally, *RIM15* genotyping was performed *via* HRM analysis, which confirmed that the genotype of *RIM15* (*RIM15/rim15*^{5055insA}) displayed heterozygosity in the diploid strain crossbred between H6 and K7 group haploid cells (Watanabe *et al.,* 2012; Akao *et al.,* 2018). The ploidy of the diploid candidates was confirmed using flow cytometry as described previously (Katou *et al.,* 2008).

II. 3. 6 Fermentation test and small-scale sake brewing test

Fermentation tests were performed using *koji* extract solution supplemented with dried *koji* as the fermentation medium to investigate the fermentation properties of H6 haploid strains and diploid strains as described previously (Fujiwara *et al.,* 2017). Additionally, I performed a small-scale sake brewing test to examine the fermentation properties of sake yeast strains (Ohnuki *et al.,* 2019). The yeast cells were pre-cultured in 5 mL of YPD medium at 30°C for 24 h. Then, 1 mL (approximately 1×10^8 cells/mL) of the pre-cultured cells was added to 50 mL of YPD medium (approximately 2×10^6 cells/mL) and incubated at 30°C for 24 h with shaking. The cells were harvested and added to sake mash at a final concentration resulting in an optical density at 660 nm of 1.0. The sake mash was prepared from 72.8 g of gelatinized rice (equivalent to 80 g of rare polished rice; Tokushima Seikiku Co., Ltd., Osaka, Japan) at a 60% polishing ratio, 19.2 g of dried rice *koji* (equivalent to 20 g of rare polished rice and a 20% *koji* ratio; Tokushima Seikiku Co., Ltd.) at a 60% polishing ratio, 136 μL of 90% lactic acid, and 174 mL of water (equivalent to a water-to-rice ratio of 130%) containing the yeast cells. The temperature of the sake mash was maintained at 15°C. After 20 days, the sake mash was centrifuged, and the resulted supernatant was collected as the sake samples.

The general composition of the sake samples was analyzed using the methods authorized by the National Tax Agency of Japan (Brewing Society of Japan,2017). The organic acid contents in the sake sample were analyzed using a high-performance liquid chromatography system (SCL-10 ASP, equipped with a Shim-pack SPR-H column; Shimadzu, Kyoto, Japan). Urea was quantified using an F kit (Roche Diagnostics GmbH, Mannheim, Germany).

II. 3. 7 Statistical analysis of sake components

Statistical significance was assessed using Student's *t*-test. Principal component analysis (PCA) was performed using six volatile parameters and seven organic acid

parameters from the componential analysis of 52 strains (N = 3) using a small-scale sake fermentation test. Clustering analysis was performed using the 19 obtained parameters. All tests were performed in triplicate, and the obtained parameters were averaged for each strain. The general component analysis comprised six parameters, namely sake meter value, ethanol production, acidity, amino acidity, and CO_2 production for 5 and 20 days. Concerning the volatile components, the amounts of ethyl acetate, n-propanol, isobutanol, isoamyl acetate, isoamyl alcohol, and ethyl caproate were used as parameters. Regarding the parameters of organic acids, I used the amounts of phosphoric acid, citric acid, pyruvic acid, malic acid, succinic acid, lactic acid, and acetic acid. PCA, cluster analysis, and discriminant analysis were performed using Microsoft Excel (practical multivariate analysis ver.).

II. 4 RESULTS AND DISCUSSION

II. 4. 1 Isolation and selection of H6 haploid strains

In chapter I, it is indicated that unlike K7 group sake yeast strains, H6 possesses sporulation ability, and its spores germinate well. The acquisition flow of the haploid set derived from H6 and K7 group crossbred diploid cells is summarized in Figure 11. As an initial step, the tetrads of H6 were dissected using a micromanipulator, and then the ploidy and *MAT* type of the haploid cells were confirmed. Haploid cells obtained from 10 asci in which all four spores germinated were judged to have developed from correct meiosis and used for the subsequent analysis. Fermentation tests were conducted under conditions simulating sake mash to select parent candidates for mating. The fermentation tests were performed at 15°C for 2 weeks using *koji* extract solution supplemented with dried *koji* as the medium. K7 was used as a control strain. After the completion of fermentation, the supernatant was obtained *via* centrifugation, and the items of general component analysis (ethanol, total acidity, and amino acids), six volatile components, and six organic acids were analyzed. Then, PCA was performed using all 15 parameters (Figure 11B and 11C). All tests were performed in duplicate, and mean values were used for PCA. In chapter I, I observed lower fermentation ability, higher amino acid contents, and lower ester contents in H6 than in K7 strains. Thus, candidate parent strains for mating were selected to meet these criteria. First, the amount of ethanol produced had to exceed that produced by the H6 strain. Secondly, the amino acid concentration had to be less than that produced by H6, and third, the production of esters, such as ethyl caproate and isoamyl acetate had to be higher. Three strains (H6-Hapα3, H6-Hapα19, and H6-Hapα20) were selected from the strains and used for the subsequent mating. The ethanol production of these three strains was higher than that of the parent strain H6, but other criteria were relatively achieved in the H6 haploid strain population. The results of PCA illustrated that the haploid cluster separated from H6 and the H6 strain were located apart from the K7 position (Figure 11C), indicating that the brewing characteristics of the strains greatly

differ. In particular, there were large differences in the concentrations of isoamyl acetate, ethyl acetate, ethyl caproate, malic acid, and succinic acid, which are explanatory factors in the positive direction of the first principal component (PC1) (Figure 11C).

II. 4. 2 Mating between haploid cells of H6 and K7 group strains

The other parent strains that were used included the haploid 13BY-HapA38 strain derived from Hiroshima Ginjo13BY (HG13BY), which produced a high level of ethyl caproate (Fujiwara *et al.,* 2017; Ohdoi *et al.,* 2004), the haploid K7H868 strain derived from K7, and the haploid KArg901HapA1/A2 strain derived from KArg901, which did not produce urea. By sequencing *FAS2* in the HG13BY genome, it was revealed that G at position 3748 was replaced by A, and the mutation was confirmed at the same site in the previously reported cerulenin-resistant strain (Akada *et al.,* 1999). Therefore, as assumed by the previous study (Fujiwara *et al.,* 2017), it was also confirmed that the HG13BY strain possessed homozygous $fas2^{m}$. Although the mutated site in KArg901 $car1^{m}$ has not been identified, I confirmed homozygous deletion of three bases, namely the CTT sequence at positions 848–850, corresponding to the deletion of phenylalanine at position 283 in the amino acid sequence, which is inferred to be the responsible functional mutation. In a previous sake brewing test, the K7H868 strain was confirmed to produce a relatively higher amount of malic acid, but the genetic reason for the phenotype is unclear (data not shown). Because the organic acid composition of K7H868 is likely to be a quantitative trait, it was used as the third parent strain to investigate the diversity of organic acid composition of haploid strains isolated from the crossbred diploid. I selected the strains for use for mating in line with my expectations of acquiring stocks capable of producing various aroma and taste components (organic acids). Table 8 shows the mating combinations.

I used PCR and HRM methods to confirm that the obtained diploid strain candidates were *MAT* type a/α and that the *RIM15* genotype were $RIM15/rim15^{5055insA}$. Additionally, the ploidy was verified *via* fluorescence-activated cell sorting analysis. The

23 diploid strains obtained using various combinations were subjected to fermentation tests using dried *koji* and *koji* extract together with the parent strain (H6, K7, HG13BY, or KArg901). Of the 18 strains with similar or higher ethanol production than K7, 10 strains with particularly high ethanol production were subjected to sporulation, and four diploid strains with good spore germination were selected (Figure 12). Although the diploid cells inbred within the K7 group strains obtained using the random spore method also exhibited deficient sexual reproduction in my previous examination (data not shown), the crossbred diploid cells between H6 and K7 group haploid cells produced viable spores that probably resulted from normal sporulation and meiosis. These observations indicated that genetic defects of sexual reproduction in the K7 group are recessive. The crossbred strains exhibited significantly higher ethanol production than H6. However, it is unclear whether the result reflected the successful selection of H6 haploid strains.

II.4. 3 Identification of the *FAS2* and *CAR1* genotypes and sake fermentation phenotypes of tetrads dissociated from diploid strains

FAS2 encodes a subunit of fatty acid synthase, and *CAR1* encodes an enzyme that converts arginine to urea. Functional defects induced by mutations in these genes increase ethyl caproate production and block urea synthesis, respectively. I employed these genes as models of specific genetic markers of brewing properties for targeted crossbreeding and tested their effectiveness *via* tetrad analysis. The *FAS2* genotype of tetrads isolated from the diploid H619HG strain determined by SNP typing with MASA (Figure 13) was consistent with cerulenin resistance and separated by 2:2 within the tetrad (Figure 13B). Furthermore, when the amount of ethyl caproate produced in a small-scale sake fermentation test and the *FAS2* genotype were compared, the difference between the two genotypes was statistically significant ($p < 0.01$) (Figure 13C).

The *CAR1* genotype of tetrads isolated from the diploid H620K91 and H620K92 strains determined *via* SNP typing with MASA (Figure 13D) were also consistent with the growth of haploid cells in arginine-containing synthetic complete medium (*i.e.*, the

presence or absence of arginase activity) and separated by 2:2 within the tetrad (Figure 13E). Therefore, it was suggested that the homozygous mutation of KArg901 identified in this study was involved in the loss of Car1p arginase activity. When the amount of urea produced in a small-scale sake fermentation test and the genotype of *CAR1* were compared with those values, the difference between the two genotypes was statistically significant ($p < 0.05$) (Figure 13F). According to these results, SNP typing of marker genes can be used to screen for high ethyl caproate production and low urea production.

In this study, only the germinated tetrads were used for subsequent analysis because they exhibit normal meiosis and gene segregation. A recent report found that K7 is defective in meiotic recombination, which prevents spore germination (Shimoi *et al.,* 2019). Furthermore, these defects found in K7 group strains were complemented by H6 alleles as a result of mating, leading to normal meiosis and normal gene segregation. However, because there were several crossbred diploid strains in which the germination rate of the tetrads was greatly reduced compared with that of H6 (Figure 12), it was necessary to select the diploid strain used for spore germination to solve this problem (Table 6).

II.4. 4 Brewing properties of haploid strains

Small-scale sake fermentation tests were performed using the strain utilized for mating, the crossbred diploid strains, the haploids segregated from the crossbred diploid strains, and the control strains. The brewing properties of these strains are shown in Table 9 and Table 10. PCA was performed using six volatile components as parameters. Figure 14A shows the loading values for each component of PC1 and PC2. The haploid strains derived from each F_1 hybrid strains exhibited a different distribution on the scatter diagram of PC1 and PC2 scores (Figure 14B). The crossbred diploid H603K7H-derived haploid strains were widely distributed between H6 haploid strains and K7H868, which were used for mating. Distribution of the crossbred diploid H619HG-derived haploid strains on the PC2 axis reflected the fact that they harbored one *fas2*^{m}. Interestingly, each

haploid obtained from H603K7H and H619HG were located in a large area of PC1, and these strains produced large amounts of isoamyl acetate (denoted by an asterisk in Figure 14B). Meanwhile, the haploid strains derived from the crossbred diploid H620KA19 and H620KA20 were widely distributed in the PC1 direction, whereas the distribution range was narrow in the PC2 direction.

PCA was also performed using the organic acid composition, which affects the taste of sake, as a parameter. Figure 15A shows the loading values for each component of PC1 and PC2. Similar to the PCA results of the volatile components, concerning PCA of organic acids, the haploid strains derived from each crossbred diploid strain also displayed different distribution patterns from each other (Figure 15B).

Haploid strains derived from H603K7H were widely distributed in both PC1 and PC2. The K7H868 strain displayed a relatively high level of malic acid production (data not shown). Malic acid concentrations in sake produced using haploid strains derived from H603K7H were compared with total acidity values, but no relationship was detected. Based on the segregation of the presumable quantitative traits genes of organic acid composition from the K7H868 strain, haploid strains derived from H603K7H made sake with various organic acid compositions (Figure 15B). Furthermore, correlation analysis between each parameter of sake produced using the H603K7H-derived haploid strains revealed a negative correlation between the amount of malic acid and the amino acid composition (data not shown). On the other hand, the H619HG-derived haploid strains were widely distributed in the PC1 direction, and the H620KA19- and H620KA20-derived haploid strains were widely distributed in the PC2 direction.

In a previous report (Katou *et al.,* 2008), based on the results of PCA of the brewing characteristic of haploid strains isolated from K7 strains using the random spore method, it was described that the parent diploid strain K7 may have many recessive mutations that negatively or positively affect the composition of sake components. In addition, the specific combination of these recessive mutations determines the concentration of volatile aroma components and other sake components, with the

exception of ethyl caproate, in the haploid strains (Katou *et al.,* 2008). This study also suggested that haploid strains with various brewing characteristics resulted from the isolation of the significant SNPs present in the parental strains. The previous report also described that K7 haploid 100 strains displayed a small degree of separation between ethyl caproate and total acidity values (Katou *et al.,* 2008). However, in this study, I used parental strains that produced higher amounts of ethyl caproate or malic acid. Thereby, the diversity of the haploid strains was also obtained in terms of ethyl caproate production and acidity. The aforementioned indicated that the use of H6-derived haploid strains for crossbreeding was effective for obtaining haploid strains with a variety of brewing characteristics after the genetic segregation of crossbred diploid cells.

Cluster analysis was performed using the 19 obtained parameters (Figure 16). The analysis revealed that the Kyokai sake yeast strains (K7, K6, and K11) were located near each other. Notably, K7 and K6 belonged to the same cluster. K7 and K7 H868H were located apart from each other because of the large difference in organic acid composition during brewing. Kyokai no. 1801 (K1801) was reported to be obtained by crossing the haploid cells of K901 and K1601, and it produces a high level of ethyl caproate (Yoshida *et al.,* 2006; Goshima *et al.,* 2016). Because K1801 has excellent sake fermentation characteristics, such as the production of a high ethyl caproate and low isoamyl alcohol levels, it is used preferentially for the production of *ginjo* sake. K1801 formed a cluster with Hiroshima Ginjo 26BY (HG26BY) and diploid H619HG (K1801 cluster). HG26BY was developed *via* inbreeding between haploid cells derived from HG13BY (Fujiwara *et al.,* 2017). Further, there were many haploid strains located close to the diploid strains constructed by mating even though I acquired haploid strains with a wide range of characteristics independent of those of the parent strain. Based on the results of cluster analysis, discriminant analysis was performed between the L (left part of the tree) and R (right part of the tree) groups, which were primarily separated from each other based on isoamyl acetate, isoamyl alcohol, succinic acid, and acetic acid production. K7, K6, and K11 were originally recognized as strains with distinctive brewing properties. However,

in this cluster analysis, they were placed close to each other, and they formed a cluster (K7 cluster). In short, the brewing characteristics of haploid strains derived from the F_1 hybrid exhibited greater diversity than observed for the K7 cluster strains. Interestingly and fortunately, the brewing characteristics of the haploid set were extremely diverse.

A previous study conducted morphological analysis with a large number of *S. cerevisiae* strains including K7 group and other sake yeast strains (Ohnuki *et al.,* 2017). The findings suggested that crossbreeding, even inbreeding, was more effective for generating morphological diversity than mutagenesis, in line with my results.

II.4. 5 The perspective of this method and sake yeast haploid set

I indicated that a set of haploid cells was obtained from crossbred diploid cells between H6 and K7 group haploid cells without experimental difficulty (Figure 11A, Figure 12). Additional effort using various K7 group haploid cells would provide a more extensive haploid library.

The advantages of this haploid set are as follows. 1) H6 and K7 group strains extremely similar genetically, and their hybrid cells harbor less heterozygosity than hybrids between haploid cells of different lineages. Loss of heterozygosity (LOH) is unavoidable in heterozygous diploid cells. Because causal events of LOH such as gene conversion and crossover stochastically occur and result in a relatively large scale of genotypic changes, increase the risk of changes in brewing characteristics, a lower number of heterozygous sites may confer higher genetic robustness to diploid cells. 2) Backcrossing between haploid cells from F_1 hybrid strains and K7 group haploid cells can produce hybrids that are more closely related to the K7 group with sexual reproduction ability. Even repetitive backcrosses may generate such hybrid cells, which would also be available for genetic analysis of sake yeast.

Although most K7 group haploid cells are considered to harbor single or multiple aneuploidy, sporulation of the F_1 hybrid cells may generate euploid haploid cells. The selection of such cells provides a haploid set without the risk of genetic instability caused

by aneuploidy.

I also observed that genotyping of a specific mutation site was effective for the preliminary evaluation of haploid set using *fas2*^{m} and *car1*^{m} as examples. Future genetic mapping studies of industrially important traits involved in brewing characteristics should enable a more precise “genetic diagnosis” of the haploid set, facilitating more targeted crossbreeding.

Table 6 Yeast strains used in this study.

Strain	Remarks	Source
K7	Sake yeast Kyokai no.7	Brewing Society of Japan
K7-H868	Haploid isolated from K7	(Katou *et al.,* 2008)
HG13BY	Hiroshima Ginjo yeast 13BY	Hiroshima Prefecture Yeast Collection (Ohdoi *et al.,* 2004)
13BY-HapA38	Haploid from Hiroshima Ginjyo yeast 13BY	(Fujiwara *et al.,* 2017)
HG26BY	Hiroshima Ginjo yeast 26BY	(Fujiwara *et al.,* 2017)
Karg901	Sake yeast Kyokai no.Arg901	Brewing Society of Japan
Karg901-HapA1	Haploid from sake yeast Kyokai no.Arg901	This study
Karg901-HapA2	Haploid from sake yeast Kyokai no.Arg901	This study
K6	Sake yeast Kyokai no.6	Brewing Society of Japan
K11	Sake yeast Kyokai no.11	Brewing Society of Japan
H6	Sake yeast Hiroshima no.6	Hiroshima Prefecture Yeast Collection
H6-Hap[A1-20], [α1-20]	Haploids isolated from H6	This study
X2180	Laboratory strain	American Type Culture Collection
H603K7H	Diploid(H6-Hapα3*K7-H868)	This study
H619HG	Diploid(H6-Hapα19*13BY-HapA38)	This study
H620KA91	Diploid(H6-Hapα20*KArg901-HapA1)	This study
H620KA92	Diploid(H6-Hapα20*KArg901-HapA2)	This study
H603K7H-Hap[A1-C4]	Haploids isolated from H603K7H	This study
H619HG-Hap[A1-C4]	Haploids isolated from H619HG	This study
H620KA91-Hap[A1-A4]	Haploids isolated from H620KA91	This study
H620KA92-Hap[A1-A4]	Haploids isolated from H620KA92	This study

Table 7 Primers used in this study

Primer	Sequence (5'→3')
MATA specific	ACTCCACTTCAAGTAAGAGTTTG
MATα specific	GCACGGAATATGGGACTACTTCG
MAT specific	AGTCACATCAAGATCGTTTATGG
RIM15-HRM-FW	CGGTGATGAACTAGTTAGCAGAGC
RIM15-HRM-RV	ATTGGTGTTGTCGAATTAGCACCA
RIM15-HRM-PR	CTCAACATCATCAATAACAACCTACp*
FAS2-MASA-FWA	GAGGTTGGTAACTGTTCTGGTTCTG
FAS2-MASA-FWB	GAGGTTGGTAACTGTTCTGGTTCTA
FAS2-MASA-RV	CAAGCGGACATGGTGTTGATAAATG
CAR1-MASA-FWC	GACCTTGAGAGAAGGTCTTTTCT
CAR1-MASA-FWD	GACCTTGAGAGAAGGTCTTTTAG
CAR1-MASA-RV	CAATAAGGTTTCACCCAATGCACA

*The 3' ends of *RIM15*-HRM-PR were phosphorylated not to participate in the elongation steps during PCR.

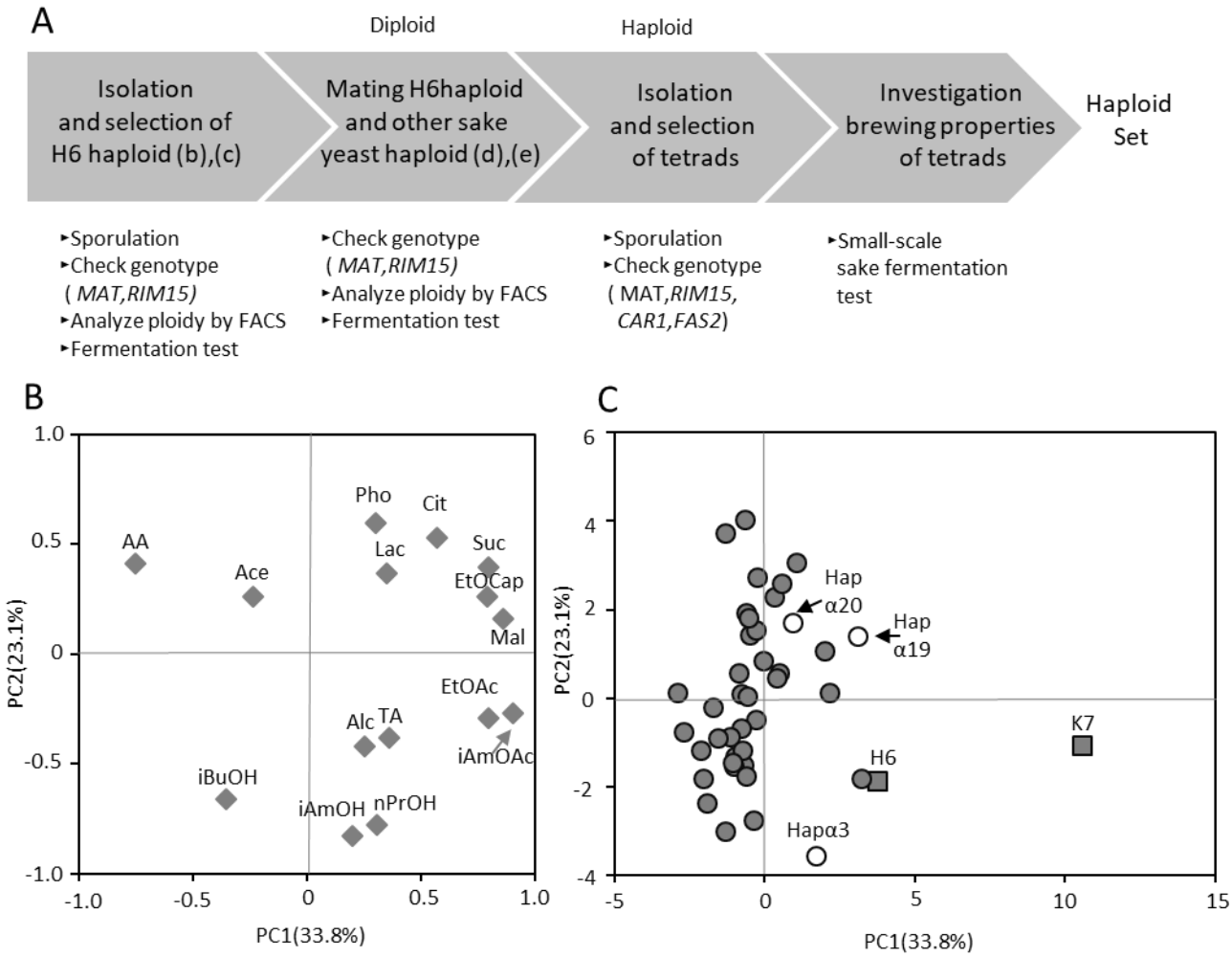


Figure 11 Acquisition flow of the haploid set derived from Hiroshima no. 6 (H6) and Kyokai no. 7 (K7) group crossbred diploids.

A: A haploid set was created *via* diploid strain acquisition, selection, spore acquisition, and sake fermentation tests. First, a haploid fermentation for H6 was conducted. The fermentation test was performed by adding the pre-cultured cells to 30 mL of *koji* extract solution (Brix 10) and 10 g of dried rice *koji* so that the number of cells was 1×10^7, and the cells were incubated at 15°C for 14 days. Next, the H6 haploid cells selected by the fermentation test were crossed with haploid cells obtained from other sake yeast strains. The *MAT* and *RIM15* genotypes and ploidy of the diploid strains were determined using genotyping tests and fluorescence-activated cell sorting analysis. Additionally, fermentation tests of diploid strains were performed (as described previously), and diploid

strains with high ethanol production were selected. The tetrads were dissected using a micromanipulator, and diploid strains with a high rate of spore germination were employed for further analysis. The genotypes of four genes, namely *RIM15*, *MAT*, *CAR1*, and *FAS2*, of the tetrads were investigated, and a small-scale sake fermentation test was conducted. B: Factor loading of *koji* extract fermentation test of H6 haploid strains. The general properties (Alc, alcohol content; TA, total acidity; and AA, amino acid content) and volatile components (EtOAc, ethyl acetate; iAmOH, isoamyl alcohol; iAmOAc, isoamyl acetate; EtOCap, ethyl caproate; iBuOH, isobutanol; and nPrOH, n-propanol) of the fermentation test samples were analyzed using the standard methods established by the National Tax Agency of Japan. Organic acids (Cit, citric acid; Mal, malic acid; Suc, succinic acid; Lac, lactic acid; Ace, acetic acid; and Pho, phosphoric acid) were analyzed using high-performance liquid chromatography. All data are presented as the means of two independent tests. C: PCA analysis of the *koji* extract fermentation test for H6 haploid strains. Squares indicate fermentation samples made from H6 and K7 strains. Gray circles indicate fermentation samples made from H6 haploid strains. Three strains (H6-Hapα3, H6-Hapα19, and H6-Hapα20) selected among the H6 haploid strains and used for subsequent mating are indicated by white circles.

Table 8 Mating combination of haploid cells from H6 and other sake yeast strains.

Mating combination		Haploid from other sake yeast			
		K7H868	13BY HapA38	KArg901 HapA1	KArg901 HapA2
	H6Hapα3	H603K7H			
	H6Hapα19	H619K7H	H619HG	H619KA91	H619KA92
	H6Hapα20	H620K7H	H620HG	H620KA91	H620KA92

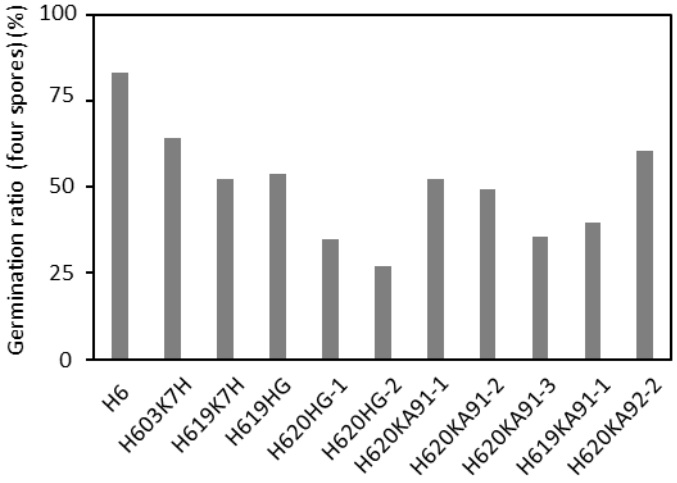


Figure 12 Spore germination rate when tetrads of the diploid strains were isolated using a micromanipulator.

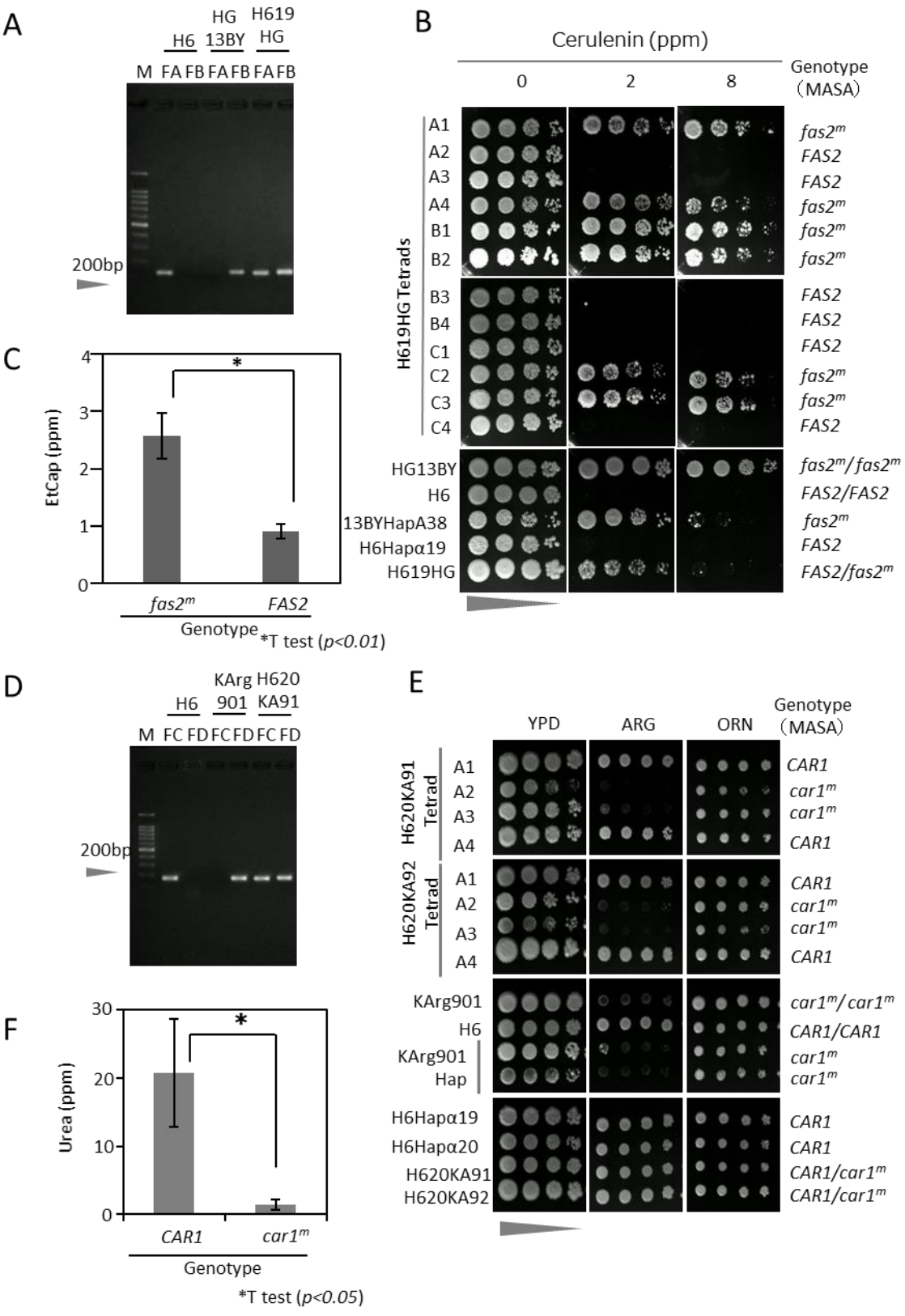

A
H6
HG 13BY
H619 HG
M FA FB FA FB FA FB
200bp
B
Cerulenin (ppm)
0
2
8
Genotype (MASA)
H619HG Tetrads
A1
A2
A3
A4
B1
B2
B3
B4
C1
C2
C3
C4
fas2m
FAS2
FAS2
fas2m
fas2m
fas2m
FAS2
FAS2
FAS2
fas2m
fas2m
FAS2
HG13BY
H6
13BYHapA38
H6Hapα19
H619HG
fas2m/fas2m
FAS2/FAS2
fas2m
FAS2
FAS2/fas2m
C
EtCap (ppm)
4
3
2
1
0
*
fas2m
FAS2
Genotype
*T test (p<0.01)
D
H6
KArg 901
H620 KA91
M FC FD FC FD FC FD
200bp
E
YPD
ARG
ORN
Genotype (MASA)
H620KA91 Tetrad
A1
A2
A3
A4
CAR1
car1m
car1m
CAR1
H620KA92 Tetrad
A1
A2
A3
A4
CAR1
car1m
car1m
CAR1
KArg901
H6
KArg901 Hap
car1m/car1m
CAR1/CAR1
car1m
car1m
H6Hapα19
H6Hapα20
H620KA91
H620KA92
CAR1
CAR1
CAR1/car1m
CAR1/car1m
F
Urea (ppm)
30
20
10
0
*
CAR1
car1m
Genotype
*T test (p<0.05)

Figure 13 Isolation of *FAS2* and *CAR1* genotypes and sake fermentation phenotypes of tetrads dissociated from diploid strains.

A: Polymerase chain reaction (PCR) products were analyzed on a 2.0% agarose gel. FAS2-MASA-FWA (FA) is a primer specific for wild-type *FAS2*, and FAS2-MASA-FWB (FB) is a primer specific for mutant $fas2^{m}$ (Gly1250Ser). B: The resistance of tetrads isolated from H619HG to various concentrations of cerulenin. The cells were cultured overnight at 30°C in yeast extract-peptone-dextrose (YPD) medium, collected by centrifugation, washed with water, resuspended in water to an optical density at 660 nm (OD_{660}) of 0.5, and serially diluted. Then, cells were spotted onto cerulenin plates containing 0, 2, or 8 mg/L cerulenin and incubated at 30°C for 3 days. C: Distribution of ethyl caproate concentrations in small-scale sake fermentation tests of haploid strains with HG13BY or Hiroshima no. 6 (H6) type alleles. D: PCR products were analyzed on a 2.0% agarose gel. CAR1-MASA-FWC (FC) is a primer specific for wild-type *CAR1*, and CAR1-MASA-FWD (FD) is a primer specific for mutant $car1^{m}$. E: Growth of tetrads isolated from H620K91 and H620K92 in synthetic defined (SD) medium containing arginine. The cells were cultured overnight in 30°C YPD medium, collected by centrifugation, washed with water, resuspended in water to an OD_{660} of 0.5, and serially diluted. Then, the cells were spotted on YPD plates and SD plates containing arginine and ornithine and incubated at 30°C for 3 days. F: Distribution of urea concentrations in small-scale sake fermentation tests of haploid strains with KArg901 or H6 type alleles.

Table 9 Genotype and brewing properties of isolated haploid strains, the parental strains, and control strains.

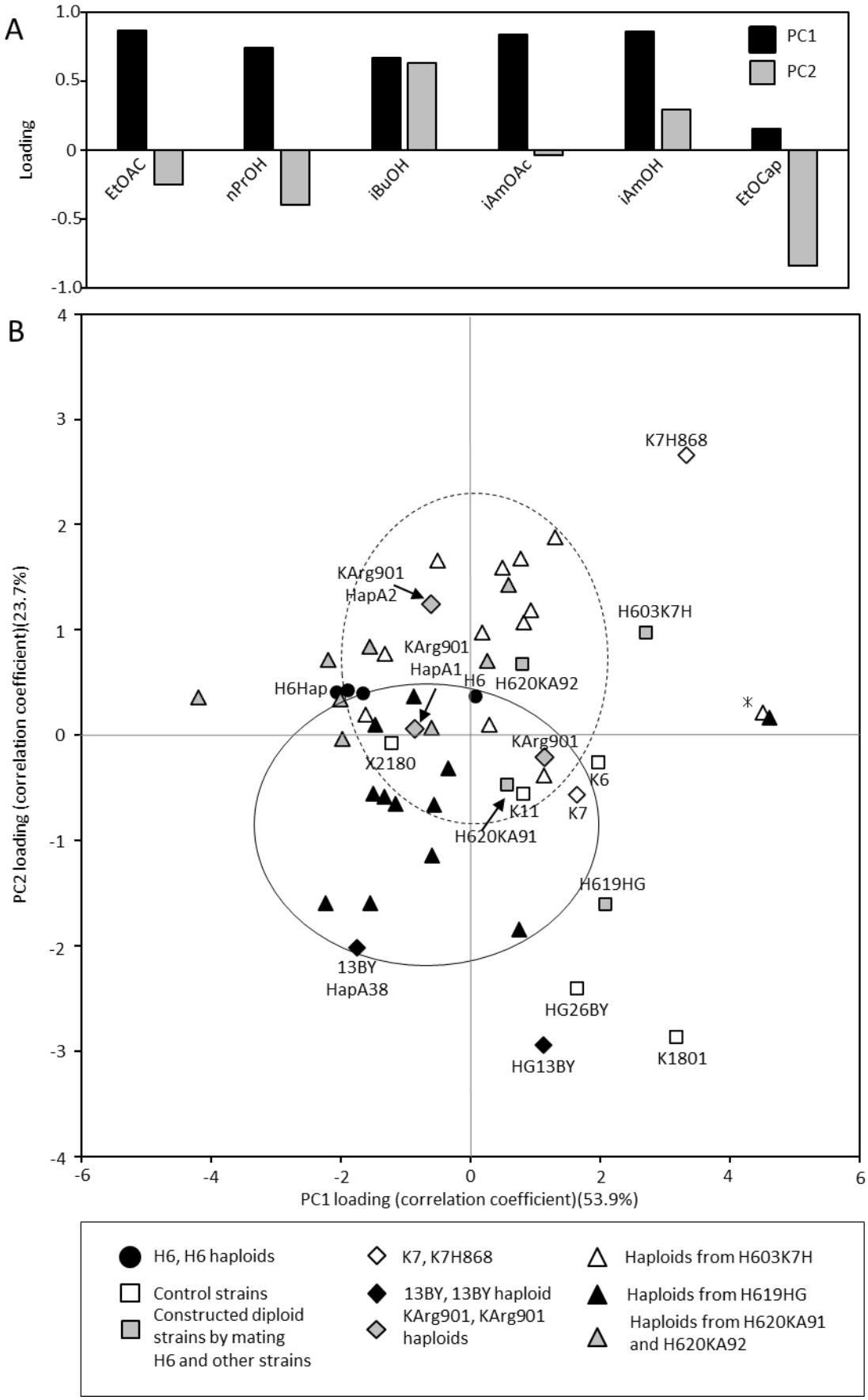
	Genotype				General properties					
	MAT	*RIM15*	*FAS2*	*CAR1*	SM	EtOH	CO_2(5days)	CO_2(Total)	A	AA
H6	a/α	*RIM15/RIM15*	*FAS2/FAS2*	*CAR1/CAR1*	-9.4	17.3	8.2	32.7	3.2	2.0
K7	a/α	*rim15^m/rim15^m*	-	-	1.7	18.7	14.0	36.0	3.7	1.5
13BY	a/α	*rim15^m/rim15^m*	*fas2^m/fas2^m*	-	-1.7	18.4	16.2	35.6	3.1	1.6
K901Arg	a/α	*rim15^m/rim15^m*	-	*car1^m/car1^m*	-9.5	17.6	10.8	33.3	3.8	1.7
K6	a/α	-	-		-1.1	18.5	13.8	35.0	3.8	1.7
K11	a/α	-	-		-0.8	18.5	13.3	35.2	4.3	1.4
K1801	a/α	-	-		4.6	19.1	14.1	36.2	2.8	1.7
26BY	a/α	-	*fas2^m/fas2^m*		-3.2	18.4	14.5	34.8	3.5	1.7
X2180	a/α	*RIM15*	-		-40.7	13.9	11.4	26.1	4.5	1.3
H6-Hapα3	α	*RIM15*	*FAS2*	*CAR1*	-19.6	16.1	7.6	30.7	3.4	2.2
H6Hapα19	α	*RIM15*	*FAS2*	*CAR1*	-21.4	14.5	8.7	30.3	3.2	2.3
H6Hapα20	α	*RIM15*	*FAS2*	*CAR1*	-23.0	14.3	7.2	29.6	3.3	2.5
K7H868A	a	*rim15^m*	-	-	2.2	19.0	16.0	35.5	3.3	1.4
13BYHapA38	a	*rim15^m*	*fas2^m*	-	-19.0	15.0	8.6	30.4	3.7	2.0
K901ArgHapA1	a	*rim15^m*	-	*car1^m*	-16.2	15.6	12.2	32.0	4.1	1.8
K901ArgHapA2	a	*rim15^m*	-	*car1^m*	-33.0	13.2	5.8	26.9	3.5	2.0
H603K7H	a/α	*RIM15/rim15^m*	-	-	5.7	19.3	11.1	36.3	4.0	1.3
H619HG	a/α	*RIM15/rim15^m*	*FAS2/fas2^m*	-	4.1	18.0	11.5	35.8	3.1	1.6
H620KA91	a/α	*RIM15/rim15^m*	-	*CAR1/car1^m*	-0.9	17.5	9.4	34.5	3.3	1.7
H620KA92	a/α	*RIM15/rim15^m*	-	*CAR1/car1^m*	-3.6	17.0	10.7	34.3	3.3	1.8
H603K7H-HapA1	α	*rim15^m*	-	-	-62.3	10.4	4.7	19.5	2.7	2.0
H603K7H-HapA2	α	*RIM15*	-	-	-6.7	18.9	10.3	34.4	3.5	1.7
H603K7H-HapA3	a	*rim15^m*	-	-	-9.0	18.5	7.6	32.4	3.9	1.9
H603K7H-HapA4	a	*RIM15*	-	-	-11.2	18.4	9.2	32.9	5.0	1.9
H603K7H-HapB1	α	*RIM15*	-	-	-14.8	17.3	5.6	30.0	3.4	2.0
H603K7H-HapB2	α	*RIM15*	-	-	-13.9	18.0	12.5	32.2	3.2	2.2
H603K7H-HapB3	a	*rim15^m*	-	-	-22.6	17.1	8.8	30.6	3.6	2.1
H603K7H-HapB4	a	*rim15^m*	-	-	-4.6	19.6	9.5	34.8	3.7	1.9
H603K7H-HapC1	α	*RIM15*	-	-	-20.9	17.4	10.5	29.9	3.3	1.5
H603K7H-HapC2	α	*RIM15*	-	-	-10.9	18.9	8.9	32.8	3.8	1.6
H603K7H-HapC3	a	*rim15^m*	-	-	-45.1	13.4	5.0	23.3	3.2	2.2
H603K7H-HapC4	a	*rim15^m*	-	-	-12.3	18.5	8.5	32.7	3.5	1.9
H619HG-HapA1	a	*rim15^m*	*fas2^m*	-	-21.4	15.4	5.3	29.7	3.0	2.4
H619HG-HapA2	α	*rim15^m*	*FAS2*	-	-13.5	16.6	7.6	31.7	3.4	2.0
H619HG-HapA3	a	*RIM15*	*FAS2*	-	-14.8	16.9	11.3	32.0	4.1	1.7
H619HG-HapA4	α	*RIM15*	*fas2^m*	-	-21.1	15.1	7.2	28.5	2.2	2.2
H619HG-HapB1	α	*RIM15*	*fas2^m*	-	-16.1	15.9	7.6	30.4	3.0	2.1
H619HG-HapB2	a	*RIM15*	*fas2^m*	-	-13.4	16.9	11.8	31.9	4.0	1.8
H619HG-HapB3	α	*rim15^m*	*FAS2*	-	-27.4	14.6	6.6	27.9	2.9	2.3
H619HG-HapB4	a	*rim15^m*	*FAS2*	-	-7.7	17.2	7.7	33.0	3.3	1.9
H619HG-HapC1	α	*rim15^m*	*FAS2*	-	-29.6	14.6	8.4	28.0	3.8	2.2
H619HG-HapC2	a	*rim15^m*	*fas2^m*	-	-8.4	17.3	7.6	33.1	3.1	2.0
H619HG-HapC3	a	*RIM15*	*fas2^m*	-	-10.6	17.3	9.0	32.4	3.6	1.9
H619HG-HapC4	α	*RIM15*	*FAS2*	-	-25.6	14.7	6.5	27.8	2.8	2.3
H620KA91-HapA1	α	-	-	*CAR1*	-22.1	14.8	8.5	30.1	3.2	2.3
H620KA91-HapA2	a	-	-	*car1^m*	-108.2	4.5	3.1	10.7	2.5	2.8
H620KA91-HapA3	a	-	-	*car1^m*	-14.8	15.8	7.0	32.0	4.4	1.8
H620KA91-HapA4	α	-	-	*CAR1*	-47.0	11.4	5.1	23.8	3.0	2.8
H620KA92-HapA1	α	-	-	*CAR1*	-44.7	11.4	4.2	23.6	3.0	2.4
H620KA92-HapA2	a	-	-	*car1^m*	-31.1	13.6	4.3	26.5	3.3	2.1
H620KA92-HapA3	a	-	-	*car1^m*	-42.8	11.6	4.1	24.1	2.8	2.3
H620KA92-HapA4	α	-	-	*CAR1*	-27.7	14.1	7.8	28.6	3.6	2.3

Genotype was determined by HRM or MASA. Mutation sites for each gene are as follows, *rim15^m*:5055insA(Flame shift mutation), *fas2^m*:G3748A (Fas2p:Gly1250thSer), *car1^m*:CTT(848-850)Deletion(Car1p:Phe283thDeletion) General properties (CO_2 (5days), CO_2 production (g /5days); CO_2 (total), CO_2 production (g /20days) ;Alc, alcohol; SM, sake meter; TA, acidity; AA, amino acid content).

Table 10 Brewing properties of isolated haploid strains, the parental strains, and control strains.

	volatile content (ppm)							Organic acid content (mg/L)						
	EtOAC	nPrOH	iBuOH	iAmOAc	iAmOH	EtOCap	E/A ratio	Pho	Cit	Pyr	Mal	Suc	Lac	Ace
H6	56.1	90.6	83.8	1.6	150.8	0.8	1.07	314	81	0	88	639	476	565
K7	71.6	126.2	72.9	3.5	169.6	1.0	2.04	379	71	0	170	849	410	543
13BY	51.3	127.2	56.0	3.4	172.6	4.1	1.95	499	89	0	122	694	426	578
K901Arg	61.1	117.4	79.2	3.3	160.7	1.0	2.04	384	73	0	124	764	480	534
K6	70.1	127.2	82.0	4.1	173.0	0.9	2.39	405	75	0	152	894	387	536
K11	61.0	120.1	62.8	2.9	159.9	0.9	1.80	375	68	0	239	877	464	655
K1801	89.6	136.1	76.4	5.0	182.2	3.8	2.74	565	114	0	187	594	509	109
26BY	56.7	122.3	74.1	3.8	177.8	3.9	2.13	650	114	0	112	734	441	448
X2180	34.4	90.0	49.3	0.4	161.2	0.9	0.27	434	104	173	126	382	676	1028
H6-Hapα3	34.6	66.6	69.0	0.8	111.4	0.7	0.71	322	65	0	83	465	464	906
H6Hapα19	32.4	58.8	58.9	1.0	120.3	0.7	0.82	330	71	0	73	475	466	863
H6Hapα20	36.3	64.3	61.7	1.1	130.0	0.7	0.88	331	80	0	91	557	478	782
K7H868A	60.3	82.7	149.5	3.9	272.0	0.9	1.43	267	69	0	360	518	588	392
13BYHapA38	35.6	62.2	38.9	1.7	126.3	3.3	1.33	380	70	0	95	521	435	846
K901ArgHapA1	49.8	68.2	57.1	2.2	141.7	0.8	1.54	413	72	0	181	800	481	776
K901ArgHapA2	38.3	58.0	94.6	2.5	146.8	0.8	1.72	313	68	0	285	487	549	692
H603K7H	68.9	109.6	113.4	4.1	214.7	0.9	1.91	351	74	0	280	832	424	572
H619HG	72.0	123.9	79.2	3.9	175.9	2.7	2.22	578	126	0	157	706	483	321
H620KA91	75.8	90.1	61.8	2.7	150.6	1.0	1.81	334	80	0	141	667	522	536
H620KA92	57.2	86.6	95.5	2.9	169.3	1.0	1.69	342	92	0	124	730	474	602
H603K7H-HapA1	35.6	44.9	63.2	3.6	108.1	1.1	3.30	79	120	325	155	473	455	122
H603K7H-HapA2	56.4	65.3	94.3	3.9	181.2	0.9	2.13	581	128	0	457	676	488	297
H603K7H-HapA3	48.1	86.8	98.3	1.3	167.2	0.8	0.80	329	62	0	152	621	443	808
H603K7H-HapA4	34.2	68.2	76.9	0.7	144.3	0.8	0.49	400	56	0	199	748	483	1317
H603K7H-HapB1	97.0	88.2	99.8	11.4	195.4	1.1	5.83	269	88	0	221	633	486	405
H603K7H-HapB2	72.5	99.0	66.5	3.7	166.0	1.0	2.21	345	86	0	165	741	484	379
H603K7H-HapB3	30.6	68.8	104.2	1.4	167.7	0.7	0.82	401	65	0	145	592	455	776
H603K7H-HapB4	43.0	69.3	101.4	2.8	210.7	0.7	1.35	631	110	0	270	643	448	681
H603K7H-HapC1	60.2	42.2	79.3	4.3	215.8	0.9	2.01	252	93	0	422	487	554	254
H603K7H-HapC2	64.9	78.1	73.4	3.4	142.2	0.8	2.39	373	76	0	317	694	526	477
H603K7H-HapC3	39.7	66.7	115.1	3.7	167.3	0.9	2.22	218	82	0	255	533	500	407
H603K7H-HapC4	37.8	92.1	123.0	2.9	208.5	0.8	1.39	372	77	0	160	659	390	620
H619HG-HapA1	50.8	60.8	56.4	1.5	132.7	1.8	1.12	354	84	0	134	551	499	519
H619HG-HapA2	49.0	77.9	43.5	1.9	117.2	0.9	1.64	337	72	0	106	629	486	775
H619HG-HapA3	43.4	64.2	64.9	2.3	147.1	0.9	1.56	361	74	0	198	676	501	904
H619HG-HapA4	39.9	74.2	62.1	2.2	155.2	2.8	1.39	245	73	0	126	395	461	309
H619HG-HapB1	34.4	61.7	38.4	1.1	109.5	2.6	0.99	298	68	0	113	508	453	665
H619HG-HapB2	44.1	53.8	46.9	1.7	124.8	3.0	1.38	382	68	0	167	516	455	886
H619HG-HapB3	42.0	65.5	56.0	1.9	123.7	0.8	1.51	288	67	0	96	509	457	713
H619HG-HapB4	94.6	116.3	103.3	8.7	219.4	1.1	3.96	303	115	0	206	644	534	216
H619HG-HapC1	34.8	53.8	49.8	0.9	105.4	0.8	0.88	329	65	0	105	562	440	1140
H619HG-HapC2	68.6	92.8	56.7	3.6	153.0	2.7	2.37	389	84	0	127	524	468	352
H619HG-HapC3	43.8	57.1	68.5	2.9	152.0	2.5	1.88	353	74	0	135	444	465	782
H619HG-HapC4	56.1	83.3	53.8	2.2	150.9	1.0	1.48	276	72	0	164	639	477	327
H620KA91-HapA1	52.8	70.2	65.2	2.9	128.7	0.9	2.26	357	81	0	91	619	480	648
H620KA91-HapA2	12.4	23.8	29.8	0.6	87.7	0.5	0.73	177	55	0	111	258	392	930
H620KA91-HapA3	49.1	70.2	105.9	3.3	170.0	0.7	1.96	393	69	0	270	801	520	737
H620KA91-HapA4	37.4	61.7	48.0	1.6	114.5	0.8	1.40	311	80	0	103	589	474	566
H620KA92-HapA1	37.0	51.9	57.6	1.6	113.9	0.7	1.41	250	70	0	117	546	489	590
H620KA92-HapA2	35.8	51.5	73.8	1.3	136.8	0.8	0.92	255	74	0	183	574	466	658
H620KA92-HapA3	29.2	46.9	67.2	1.0	120.6	0.8	0.87	232	74	0	175	385	532	580
H620KA92-HapA4	46.6	74.3	78.3	3.7	170.9	0.8	2.15	357	92	0	135	858	488	705

Volatile components (EtOAc, ethyl acetate; iAmOAc, isoamyl acetate; iAmOAc, isoamyl alcohol; EtOCap, ethyl caproate), and Organic acids components (Cit, citric acid; Mal, malic acid; Suc, succinic acid; Lac, lactic acid; Ace, acetic acid).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K6
K11
K7
H620KA91
H619HG
13BY
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HG26BY
HG13BY
K1801
H6, H6 haploids
Control strains
Constructed diploid strains by mating H6 and other strains
K7, K7H868
13BY, 13BY haploid
KArg901, KArg901 haploids
Haploids from H603K7H
Haploids from H619HG
Haploids from H620KA91 and H620KA92

Figure 14 Principal component analysis of volatile components in the sake samples from the small-scale sake fermentation tests.

A: Factor loading of six volatile components in small-scale sake fermentation tests. EtOAc, ethyl acetate; iAmOH, isoamyl alcohol; iAmOAc, isoamyl acetate; and EtOCap, ethyl caproate; iBuOH, isobutanol; and nPrOH, n-propanol. B: Score plot of six volatile components from the small-scale sake fermentation tests. The circles on the solid and dotted lines denote sake produced by haploid strains from H619HG and H603K7H, respectively. The asterisks denote haploid strains with high isoamyl acetate production.

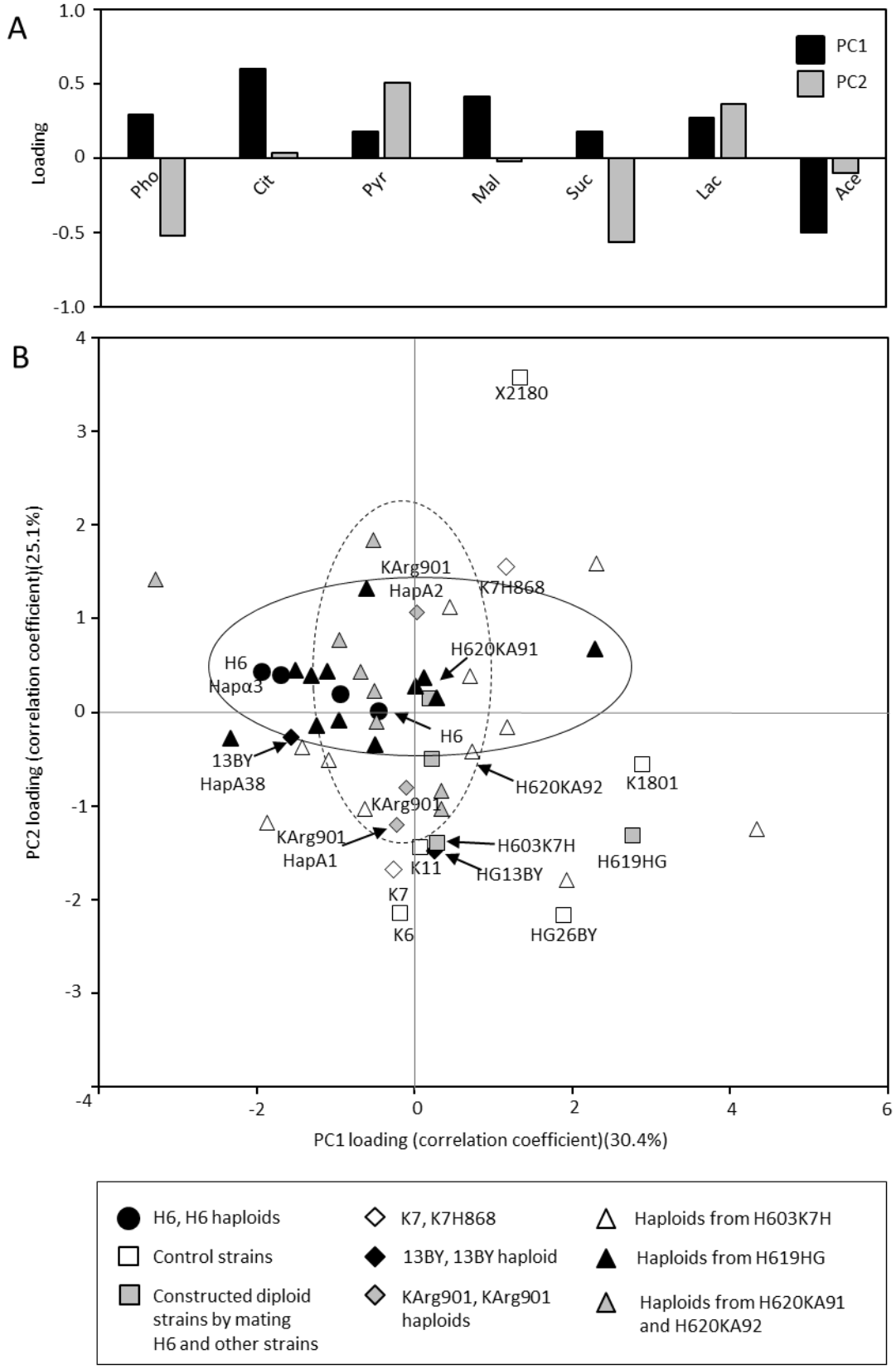
A
1.0
0.5
0
-0.5
-1.0
Loading
PC1
PC2
Pho
Cit
Pyr
Mal
Suc
Lac
Ace
B
X2180
KArg901
HapA2
K7H868
H620KA91
H6
Hapα3
H6
13BY
HapA38
H620KA92
K1801
KArg901
KArg901
HapA1
H603K7H
H619HG
K11
HG13BY
K7
K6
HG26BY
PC2 loading (correlation coefficient)(25.1%)
PC1 loading (correlation coefficient)(30.4%)
H6, H6 haploids
Control strains
Constructed diploid strains by mating H6 and other strains
K7, K7H868
13BY, 13BY haploid
KArg901, KArg901 haploids
Haploids from H603K7H
Haploids from H619HG
Haploids from H620KA91 and H620KA92

Figure 15 Principal component analysis of organic acid components in the sake samples from the small-scale sake fermentation tests.

A: Factor loading of seven organic acid components in small-scale sake fermentation tests. Pho, phosphoric acid; Cit, citric acid; Mal, malic acid; Pyr, pyruvic acid; Suc, succinic acid; Lac, lactic acid; and Ace, acetic acid. B: Score plot of seven organic acid components from the small-scale sake fermentation tests. The solid and dotted lines denote sake produced by haploid strains from H619HG and H620KA91/H620KA92, respectively.

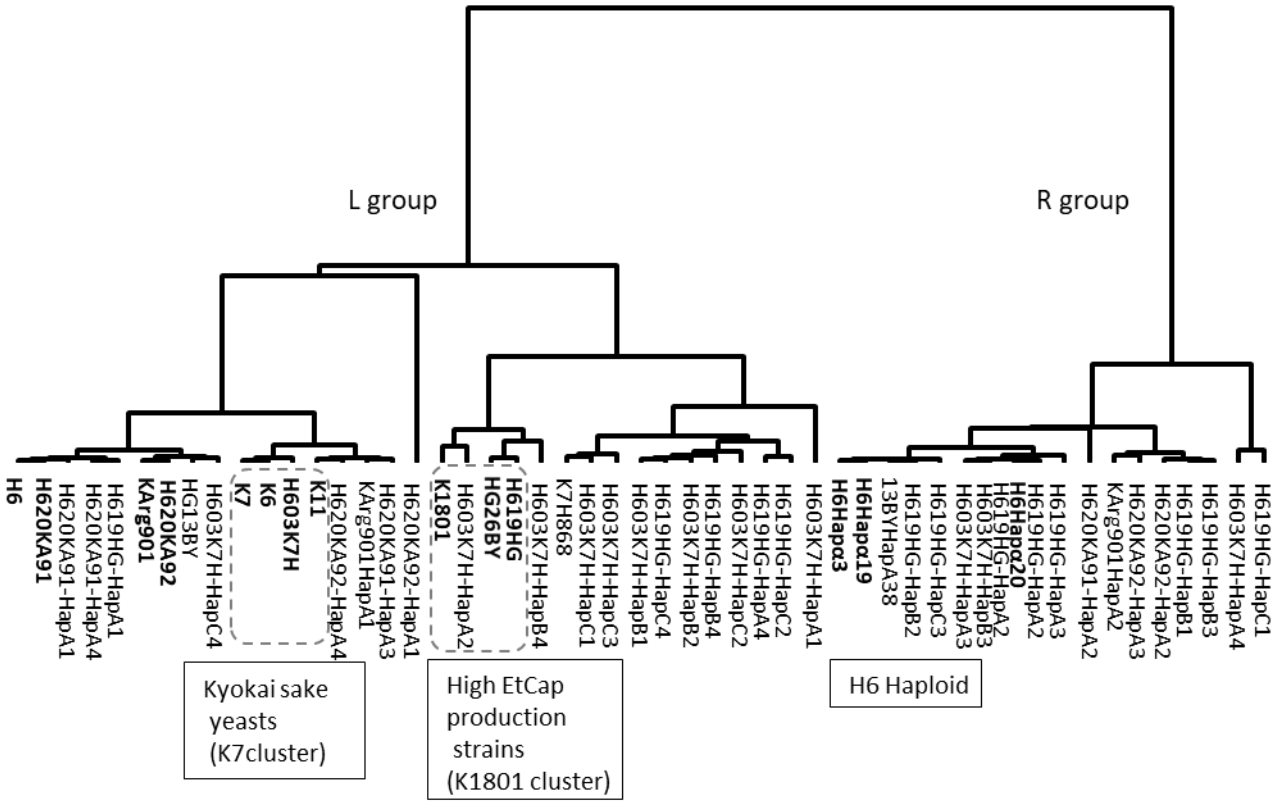

Figure 16 Cluster analysis of the sake components.

The cluster analysis was performed using 19 parameters obtained as follows. Six parameters were obtained *via* general component analysis (sake meter value, ethanol production, acidity, amino acidity, CO_2 production (5days), and CO_2 production (20days)). As the parameters of volatile components, I used the amounts of ethyl acetate, n-propanol, isobutanol, isoamyl acetate, isoamyl alcohol, and ethyl caproate. As the parameters of organic acids, I used the amounts of phosphoric acid, citric acid, pyruvic acid, malic acid, succinic acid, lactic acid, and acetic acid. Ward's method was used for the clustering analysis. Based on the result of cluster analysis, discriminant analysis was performed between the L (left part of the tree) and R (right part of the tree) groups, which were primarily separated from each other based on isoamyl acetate, isoamyl alcohol, succinic acid, and acetic acid production.

CONCLUDING REMARKS

With the advancement of brewing technology and the Westernization of foods, a variety of sake qualities, such as a fruity aroma (*ginjo*) and refreshing acidity, have been required. Sake is becoming increasingly popular around the world, and there is an increasing need not only for traditional sake quality, but also for prevention of deterioration during export.

To develop a partner strain for the breeding of sake yeast by genetic crossing, I focused on the traditional sake yeast strain, H6, and comprehensive characteristic analyses of the fermentation and sporulation abilities of H6 were conducted (Chapter I). The results showed that H6 is genetically closely related to common sake yeast in the K7 group and has normal sporulation ability. In addition, I found that sake made with the H6 strain had excellent features, such as low DMTS precursors and low DMTS-producing potential, so was not easily degraded during long-term storage. If the genes involved in DMTS reduction can be identified, it will be possible to develop sake yeasts with low DMTS production potential and excellent brewing characteristics.

Chapter II shows that crossbreeding between H6 and K7 group strains enable to produce diploid cells with the capacity for sexual reproduction. Sporulation of such crossbred diploid cells should produce a series of haploid cells. These haploid series would be available for breeding new sake yeast strains with diverse brewing characteristics. In this study, H6 haploid cells were isolated and three types were selected for mating with previously isolated haploid cells from three K7 group strains with specific features. All the F_1 hybrid cells of the tested haploid combinations exhibited sexual reproduction as expected. A series of haploid cells from crossbred diploid strains were isolated and their brewing characteristics were analyzed. Interestingly, the brewing characteristics of the haploid set were extremely diverse. Additionally, the relationships between specific genotypes and phenotypes were examined using two functional mutations in *FAS2* and *CAR1* as model traits of targeted crossbreeding.

In the future, as the search for DNA markers linked to brewing characteristics

progresses, the combination of DNA markers and this breeding method will enable efficient selection of strains with desirable brewing characteristics from haploid strains with various phenotypes. Moreover, the spore-forming defect of the K7 group strain may also be better understood by comparative genomic analysis with H6, because H6 is the only strain that sporulates genetically closely related to the K7 group.

There are also movements to use other industrial yeasts, such as wine yeast and shochu yeast, to produce a variety of sake, but those yeast possess characterizations, such as the production of 4-vinyl guaiacol (4VG), the off-flavor of sake and the low fermentability at cold temperature. It is also possible to obtain hybrid strains of H6 and other industrial yeasts and to eliminate those disadvantages by selecting haploid cells. Additional efforts using haploid cells from various industrial yeasts will provide a more extensive haploid library.

I believe that the breeding principle described in this study will be very useful in breeding yeast without relying on mutagenesis or genetic modification technology.

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