Chapter 4
Mechanism of High Alcoholic Fermentation Ability of Sake Yeast

Daisuke Watanabe, Hiroshi Takagi, and Hitoshi Shimoi

Abstract Sake yeast strains belonging to the budding yeast *Saccharomyces cerevisiae* exhibit higher rates of alcoholic fermentation and ethanol yields in the sake mash than the other types of *S. cerevsiae* strains. Although this has traditionally been regarded to be caused by their higher resistance against ethanol and various environmental stresses, recent studies revealed that they are rather defective in stress responses. Our genomic and transcriptomic approaches has led to the identification of the sake yeast-specific loss-of-function mutations in the *MSN4*, *PPT1*, and *RIM15* genes, each of which has important roles in the responses to environmental changes. Surprisingly, each of these mutations contributes to the increase of alcoholic fermentation rate. Thus, we first reported the causal mutations for the high alcoholic fermentation ability of industrial yeast strains. These findings have drastically changed how we understand the relationship between ethanol tolerance and ethanol production ability of yeast cells. In this review, we introduce the history and progression of sake yeast studies, especially focusing on their superior alcoholic fermentation properties.

Keywords Alcoholic fermentation • DNA microarray • Ethanol tolerance • Quiescence • *Saccharomyces cerevisiae* • Sake yeast • Stress response

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4.1 Introduction

Sake, a Japanese traditional alcohol beverage, is produced by saccharification of rice starch by koji (rice molded by the filamentous fungus *Aspergillus oryzae*) and alcoholic fermentation by the budding yeast *Saccharomyces cerevisiae*. One of the most prominent characteristics of sake is its high alcohol concentration; undiluted sake generally contains 18–20 vol.% of ethanol, whereas most beer and wine usually contain 4–6 vol.% and 12–15 vol.%, respectively. It has been proposed that this high ethanol production during a single batch fermentation is achieved by the unique fermentation process of simultaneous saccharification and alcoholic fermentation, which leads to the gradual supply of glucose in the sake mash and prevents severe osmotic stresses to yeast cells. A proteolipid component derived from *A. oryzae* is also reported to be responsible for high alcohol production (Hayashida et al. 1974), although it has not yet been identified from the sake mash. More recent comparative studies have shown that the *S. cerevisiae* strains that are not used for sake brewing (e.g. the laboratory reference strain S288c and its relatives) perform alcoholic fermentation less effectively in the sake mash than sake yeast strains (Urbanczyk et al. 2011; Watanabe et al. 2011a, b; Wu et al. 2009; Yamada et al. 2005), indicating that the fermentation properties of sake yeast at least partly contribute to the high yield of ethanol (Fig. 4.1). Sake yeast strains are taxonomically categorized as *S. cerevisiae* and form a closely related subgroup distinct from others, such as wine, ale, baking, clinical, and laboratory strains (Azumi and Goto-Yamamoto 2001; Borneman et al. 2011; Liti et al. 2009). In this chapter, we focus on their phenotypic and genomic differences from the other subgroups, which are responsible for the high fermentation ability of the sake yeast strains.

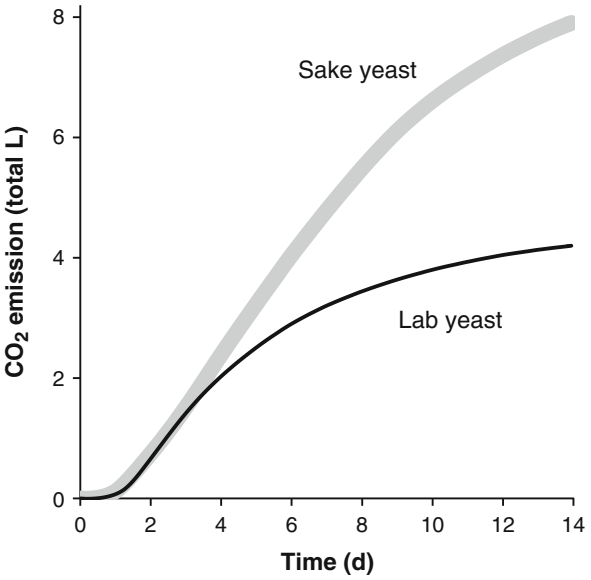

Fig. 4.1 Effects of different yeasts on the sake fermentation profile. Sake fermentation tests were performed using sake yeast (K7) or laboratory yeast (X2180). Fermentation rates were monitored by measuring CO_2 emission from the sake mash

4.2 Ethanol Tolerance of Yeasts

Ethanol has three major inhibitory effects on (1) cell viability, closely related to stress responses and typically tested by colony-forming assays after acute ethanol stress; (2) cell growth, related to cell-cycle progression and tested by OD measurements or spot assays using ethanol-containing media; and (3) ethanol production, related to metabolic control and tested by quantification of ethanol (Casey and Ingledew 1986; D'Amore and Stewart 1987). Therefore, the term ethanol tolerance has been traditionally used for representing these distinct properties or these mixed phenotypes (Fig. 4.2). Thus, it has long been believed that the yeast strains with high cell viability or with healthy growth ability under ethanol stress are able to produce high concentrations of ethanol. This explanation is at least partly true. Especially under severe stresses, such as high concentrations of ethanol and other fermentation-inhibitory compounds, several yeast strains that are mutagenized or genetically modified to increase the viability or the growth rate under ethanol stress are reported to exhibit improved fermentation ability (Alper et al. 2006; Hong et al. 2010; Ma and Liu 2010; Pereira et al. 2011; Sasano et al. 2012; Shahsavarani et al. 2012; Tao et al. 2012; Watanabe et al. 2009; Yang et al. 2011). However, a comparative study of beer and sake strains indicated that ethanol tolerance does not strictly correlate to ethanol production (Casey and Ingledew 1986), indirectly denying that high ethanol tolerance is an essential prerequisite for yeast cells to be equipped with high fermentation properties.

In winemaking, ethanol tolerance of wine yeast strains has been considered as one of their most valuable aspects. As the extracellular ethanol level rises, alcoholic fermentation often slows down or stops before all the available sugar is fully consumed. Although this sluggish or stuck fermentation is closely linked to low

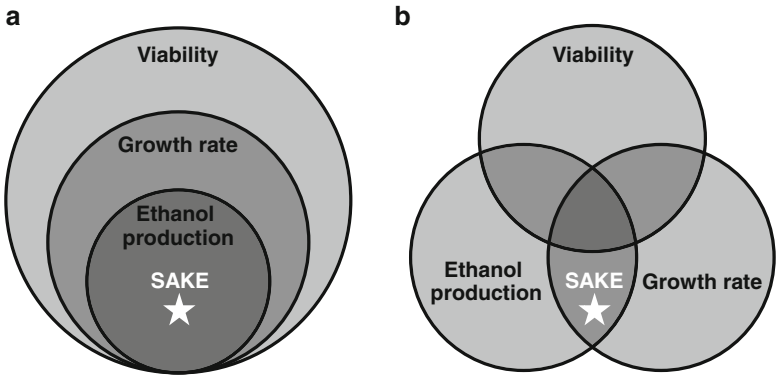

Fig. 4.2 Definitions of ethanol tolerance in yeast. Three *circles* indicate the sets of yeast strains with (i) high cellular viability under ethanol stress, (ii) high growth rates under ethanol stress, and (iii) high productivity of ethanol. Although it has traditionally been considered that these sets are mixed and completely overlapped (**a**), three phenotypes should be separately recognized (**b**). *Asterisks* indicate plausible positions of sake yeast

ethanol tolerance in general (Santos et al. 2008; Zuzuarregui and del Olmo 2004), Ivorra et al. (1999) discovered an inverse correlation between the viability under various stresses including a high concentration of ethanol and stuck fermentations in the wine yeasts examined. Moreover, the fermentative non-*Saccharomyces* yeast *Torulaspora delbrueckii* is more resistant to a combination of ethanol and acetic acid stresses but less effectively consumes fructose in a medium simulating a stuck fermentation than *S. cerevisiae* (Santos et al. 2008). These data raise the possibility that some unknown mechanism(s) contributing to the viability of yeast cells under ethanol stresses might negatively regulate alcoholic fermentation. In addition to main alcoholic fermentation, aging of sherry wine may be associated with ethanol tolerance; the flor yeast strains isolated from the solera aging processes maintain high viability under ethanol and acetaldehyde stresses (Aranda et al. 2002). As for beer, because the final ethanol concentrations are not so high as to cause stuck fermentations, ethanol tolerance is not a major concern in industrial brewing. Because of the successive recycling of lager yeast (*S. carlsbergensis*) cells, however, their viability after completion of alcoholic fermentation is an important indicator for the quality of yeast slurry (Bleoanca et al. 2013; Blieck et al. 2007; Huuskonen et al. 2010).

In sake yeast strains, several factors responsible for the cell viability or the growth rate under ethanol stress have been identified: Cell wall integrity (Takahashi et al. 2001), inositol content (Furukawa et al. 2004), palmitoyl-CoA pool (Nozawa et al. 2002), ergosterol biosynthesis (Shobayashi et al. 2005), and stress-responsive gene expression (Ogawa et al. 2000; Watanabe et al. 2007, 2009; Yamaji et al. 2003). So far, however, it is not easy to correlate them with the alcoholic fermentation ability specifically of the sake yeast strains.

4.3 Gene Expression Profiles During Alcoholic Fermentation

To elucidate the yeast genes responsible for the control of alcoholic fermentation, a series of transcriptomic analyses have been performed. Rossignol et al. (2003) first reported a DNA microarray analysis of a representative wine strain EC1118 during alcoholic fermentation using a synthetic medium MS300. Because many targets of rapamycin (TOR)-pathway genes are induced upon nitrogen starvation, they argued that the inhibition of TOR by nitrogen depletion might be the initial trigger of the stress response. A serial analysis of gene expression (SAGE) of EC1118 cells in MS300 was also performed (Varela et al. 2005). They especially focused on different expression patterns of glycolytic genes and hexose transporter genes. Mendes-Ferreira et al. (2007) investigated the effects of nitrogen limitation on the gene expression profile of a Portuguese wine strain PYCC4072. They found the genes encoding ribosomal proteins and involved in ribosome biogenesis are upregulated in response to nitrogen starvation. Marks et al. (2008) analyzed the gene expression of an industrial wine strain Vin13 fermenting in Riesling grape juice. Among the genes that dramatically induced during fermentation, a group of 223 genes was

identified and designated as fermentation stress response (FSR) genes, which are distinct from well-defined yeast stress-responsive genes, such as environmental stress response (ESR) genes (Gasch et al. 2000) and common environmental response (CER) genes (Causton et al. 2001). The FSR genes contain novel regulators of glycogen debranching (Walkey et al. 2011), acetate production (Walkey et al. 2012), and sulfur metabolism (Bessonov et al. 2013), each of which is expected to have an important role in the control of wine fermentation. More recently, a comparison of the transcriptomes of different industrial wine strains (Rossouw and Bauer 2009) and a comparison between transcriptomic and proteomic profiles (Rossouw et al. 2010) were reported. Although very few transcriptomic analyses of lager brewing yeast have been reported (Gibson et al. 2008; Olesen et al. 2002), an integrated transcriptome and metabolome analysis is worth noting, which has led to breeding of a bottom-fermenting yeast strain that produces a high level of SO_2 to slow the development of oxidation haze and staling of flavors in beer (Yoshida et al. 2008).

In regard to sake yeast, Wu et al. (2006) first reported a DNA microarray analysis of a representative strain, Kyokai no. 701 (K701), in the fermenting sake mash. The genes were classified into four groups by *k*-means clustering analysis of the expression profiles (Fig. 4.3) and categorized in accordance with their biological processes by using the *Saccharomyces* genome database (SGD) gene ontology (GO) slim mapper (http://www.yeastgenome.org/cgi-bin/GO/goSlimMapper.pl). Group I contained the genes whose expression levels were almost constantly low throughout the fermentation process. This group included 527 genes, representing 8.3 % of the total genes. Group II contained the genes whose expression levels increased along with the progression of sake fermentation. This group, which consisted

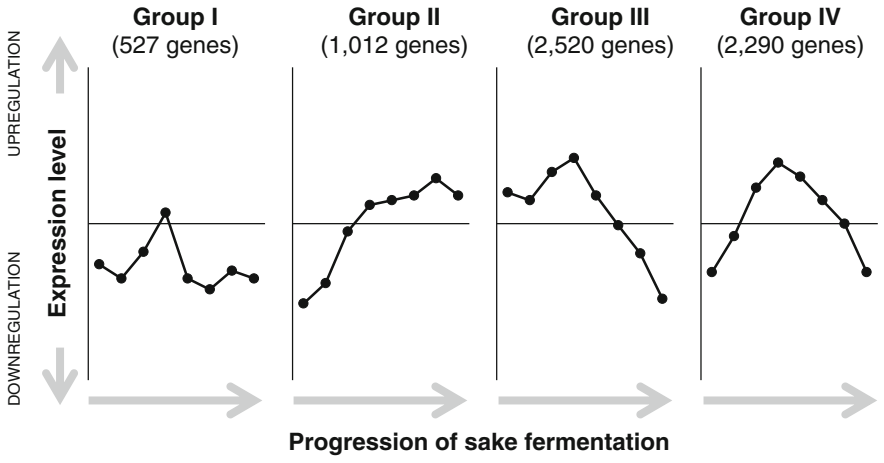

Fig. 4.3 Expression profiles during sake fermentation of the four gene groups established by *k*-means clustering. (Modified from Wu et al. 2006)

of 1,012 genes (15.9 % of the total genes), contained many genes involved in electron transport and generation of precursor metabolites and energy. Recent studies of the roles of mitochondria in alcoholic fermentation (Kitagaki and Shimoi 2007; Kitagaki and Takagi 2014) may help in understanding the significance of the upregulation of these genes. Group III contained the genes whose expression levels were high during the early growth phase but decreased in the late fermentation stage, 2,520 genes, representing 39.7 % of the total genes. The genes belonging in this group are mainly related to cell growth, such as ribosome biogenesis and assembly, protein biosynthesis, and amino acid and derivative metabolism, which is consistent with the fact that cell growth occurs only in the early stage of the sake mash. Group IV contained the genes whose expression levels increased during the early stage, reached maximum levels in the middle stage, and then decreased in the late stage. This group included 2,290 genes, representing 36.1 % of the total genes, and contained many genes involved in protein catabolism, protein modification, signal transduction, and morphogenesis. These genes likely play important roles in the adaptation to the sake fermentation condition. Thus, this study revealed several characteristics of yeast gene expression during the sake fermentation process and provided a scaffold for molecular-level understanding of alcoholic fermentation in the sake mash.

To find sake yeast-specific factors that function during sake fermentation, the gene expression profile of K701, already described, was compared with that of a laboratory diploid strain X2180 that arose through spontaneous self-diploidization of S288c (Mortimer and Johnston 1986), under identical sake fermentation condition (Watanabe et al. 2011c). Analysis of differential DNA microarray data using T-profiler (http://www.t-profiler.org), which is used for scoring changes in the average expression levels of predefined groups of genes (Boorsma et al. 2005), revealed that the expression of genes under the control of several transcription factors that are responsible for stress responses and nutrient signaling, including Msn2p and Msn4p (Msn2/4p), was significantly repressed in K701. It is thus expected that the inactivation of these transcription factors might account for the sake fermentation properties of sake yeast. Hereafter, we introduce the pathways mediated by these transcription factors and their physiological significance in the control of alcoholic fermentation in the sake mash.

4.4 Defective Stress Responses in Sake Yeast Strains

4.4.1 Msn2/4p

Msn2/4p (Estruch and Carlson 1993; Görner et al. 1998; Martínez-Pastor et al. 1996; Schmitt and McEntee 1996) are functionally redundant transcription factors that have been best characterized among the regulators of ESR (Gasch et al. 2000) and CER (Causton et al. 2001). In response to various stresses, Msn2/4p migrate

into the nucleus and bind to stress response elements (STRE) (CCCCT or AGGGG) within the promoters of stress-induced genes. The zinc-finger DNA-binding domains of Msn2/4p located at their carboxyl termini are essential for recognition of STRE sequences. Interaction of Msn2/4p and STRE leads to global transcriptional activation of a large set of stress-responsive genes, including those related to oxidative stress defense, carbohydrate metabolism, and protein folding chaperones. Consistently, a Δ*msn2* Δ*msn4* double mutant displays severe defects in stress-protective gene expression and thus exhibits pleiotropic stress sensitivity.

Based on the data of the DNA microarray experiment already described and a reporter assay using a STRE-pCYC1-*lacZ* fusion gene (Watanabe et al. 2011c), it was demonstrated that sake yeast has severe defects in stress-inducible gene expression mediated by Msn2/4p and STRE specifically during sake fermentation. Taking this into consideration, it is hypothesized that sake yeast cells might exhibit lower stress tolerance than laboratory yeast cells, which is contradictory to the traditional assumption that sake yeast is more tolerant to ethanol stress than other yeasts. To confirm this, we compared the colony-forming ability of the K701 and X2180 cells isolated from the fermenting sake mash and found that K701 cells show significantly lower viability than X2180 cells under heat-shock or ethanol stress (Urbanczyk et al. 2011). Furthermore, the other sake yeast strains tested (Kyokai no. 6, 7, 9, and 10) also exhibited decreased viability under heat or ethanol stress, comparing to X2180. Therefore, the stress-sensitive phenotype, which is consistent with the impaired activity of Msn2/4p, is considered a general feature of the commonly used modern sake yeast strains.

As the whole-genome sequencing of a sake yeast strain Kyokai no. 7 (K7) was completed (Akao et al. 2011), now it is possible to search the mutations in the specific genes of sake yeast in silico. To investigate the cause of the defective stress responses in sake yeast, we focused on nucleotide polymorphisms in the *MSN2* and *MSN4* genes. *MSN2* nucleotide sequences are well conserved between K7 and S288c, except for three nonsynonymous polymorphisms. Previous studies indicated that Msn2p of K7 is functional (Watanabe et al. 2007, 2009). In contrast, *MSN4* has more nonsynonymous polymorphisms. We focused on two point mutations (T2C and C1540T) in sake yeast K7 that result in the truncation of the amino- and carboxyl-termini, respectively, of Msn4p (Watanabe et al. 2007, 2011c). Of these mutations, at least C1540T was proven to be a loss-of-function mutation, as it deletes the carboxyl-terminal zinc-finger DNA-binding motifs of Msn4p. Next, we examined the distribution of the T2C and C1540T polymorphisms among sake, wine, beer, and laboratory yeast strains. This comparison revealed that genetically closely related modern sake yeast strains, including Kyokai no. 6, 9, 10, 11, 12, 13, 14, and 15 (Azumi and Goto-Yamamoto 2001), had both mutations identical to those of K7 and K701. In contrast, nearly all the other yeast strains tested, including the classical sake yeast strains (Kyokai no. 1, 2, 3, 4, 5, and 8 and Yabe Kozai), as well as the wine, beer, and laboratory yeast strains, showed no double truncation of *MSN4* as was observed in X2180. Furthermore, the fungal sequence alignment analysis in SGD (http://www.yeastgenome.org/cgi-bin/FUNGI/showAlign) revealed that both the T2 and C1540 nucleotides are universally conserved among

orthologous genes in *Saccharomyces* sensu stricto. These data suggest that an ancestor of the modern sake strains may have acquired these mutations in the *MSN4* gene during the selection of sake yeast with desirable brewing properties.

We thus hypothesized that the modern sake yeast-specific loss of Msn2/4p functions might be linked to their fermentation properties. The sake fermentation tests using the Δ*msn2*, Δ*msn4*, and Δ*msn2* Δ*msn4* disruptants in a laboratory yeast background revealed that all three disruptant strains displayed significant increases of evolved carbon dioxide gas in the early stage of sake fermentation compared to the wild-type strain, with the double mutant showing the largest increase (Watanabe et al. 2011c). After 20 days of fermentation, the ethanol concentrations were higher in the sake produced using the disruptants, also indicating that the abrogation of Msn2/4p leads to improved alcoholic fermentation.

Yeast stress responses and the resultant stress tolerance are generally considered to be important characteristics for effective alcoholic fermentation; therefore, numerous recent studies have focused on the enhancement of ethanol tolerance to achieve high ethanol productivity. In contrast to this approach, however, our experimental data revealed that Msn2/4p-mediated environmental stress responses act as a physiological "brake" for ethanol production.

4.4.2 Hsf1p

As described here, conserved loss-of-function mutations in *MSN4* partly contribute to the phenotypes of low cell viability under stresses and high alcoholic fermentation rates of modern sake yeast strains. However, they are insufficient to fully account for their phenotypes because Msn2p, a transcription factor redundant with Msn4p, is still functional in the modern sake strains (Watanabe et al. 2007, 2009), and because loss of Msn4p only slightly increases the fermentation rate of laboratory strains (Watanabe et al. 2011c). Therefore, we next focused on the other stress-responsive transcription factors, because most Msn2/4p target genes are redundantly or coordinately regulated by Gis1p, Yap1p, and heat-shock factor protein 1 (Hsf1p) (Amorós and Estruch 2001; Cameroni et al. 2004; Gasch et al. 2000; Treger et al. 1998).

Although Hsf1p was originally isolated as a heat-shock transcription factor (Sorger and Pelham 1987; Wiederrecht et al. 1988), it responds to a variety of stress conditions, including ethanol stress. Hsf1p is constitutively localized in the nucleus, where it is bound to heat-shock elements (HSEs) (Jakobsen and Pelham 1988), and has an essential function in cell proliferation even under normal growth conditions. In response to stress, however, Hsf1p becomes highly active and induces the transcription of hundreds of target genes related to protein folding, detoxification, energy generation, carbohydrate metabolism, and cell wall organization (Eastmond and Nelson 2006; Hahn et al. 2004). Mutants of *hsf1* therefore exhibit pleiotropic phenotypes and temperature sensitivity.

The phosphorylation state of Hsf1p in response to stress provides an important clue for understanding the regulatory mechanism of this transcription factor. In the absence of stress, Hsf1p is subjected to basal low-level phosphorylation, which appears to be negatively controlled by protein kinase A (Ferguson et al. 2005). Further phosphorylation by the AMP-activated kinase Snf1p is required for glucose starvation-induced activation of Hsf1p (Hahn and Thiele 2004). Upon heat shock and oxidative stress, Hsf1p is also extensively phosphorylated, although the responsible kinases are unknown (Liu and Thiele 1996; Sorger and Pelham 1988). Based on these findings, constitutive hypophosphorylation and stress-responsive hyperphosphorylation of Hsf1p seem closely related to its low basal and stress-induced high activities, respectively.

To examine Hsf1p- and HSE-mediated gene expression during sake fermentation, the activity of the HSE-pCYC1-*lacZ* fusion gene product in strains K701 and X2180 was monitored during the sake fermentation process (Noguchi et al. 2012). Although the β-galactosidase activity kept increasing in X2180, no significant upregulation of the activity was observed in K701 throughout the fermentation period. Although ten nonsynonymous mutations were identified in the *HSF1* gene based on the whole-genome sequence of K7 (Akao et al. 2011), none of them significantly affected the gene induction activity of Hsf1p (Noguchi et al. 2012). These data suggest that sake yeast is defective in the Hsf1p- and HSE-mediated stress response, which is not caused by the mutations in the *HSF1* gene, but by sake yeast-specific impairment of the Hsf1p activity. To reveal differences in the regulatory mechanism of Hsf1p between laboratory and sake yeast strains, we next examined the phosphorylation state of Hsf1p in the sake mash or under acute ethanol stress. Upon these stress conditions, X2180 Hsf1p decreased its mobility, as was previously observed under heat shock or oxidative stress (Liu and Thiele 1996; Sorger and Pelham 1988). In contrast, the mobility of K701 Hsf1p was lower than the low-mobility form of X2180 Hsf1p, even under normal growth conditions (Noguchi et al. 2012). Furthermore, differences in the mobility of Hsf1p between the two strains were no longer observed when the lysates were treated with alkaline phosphatase, demonstrating that the mobility changes were the results of variations in the phosphorylation levels. Altogether, it is suggested that hyperphosphorylation of Hsf1p in X2180 is closely related to its activation under ethanol stress conditions, whereas the constitutive hyperphosphorylation of Hsf1p in K701 presumably triggers its dysfunction.

To identify the regulatory factor controlling the phosphorylation state of Hsf1p, we screened nonessential protein phosphatase-encoding gene disruptants (Noguchi et al. 2012). Among known *S. cerevisiae* protein phosphatase genes, 29 disruptants in the laboratory strain BY4743 background were subjected to Western blot analysis with the anti-Hsf1p antiserum. Although most disruptants showed mobilities of Hsf1p similar to that of wild-type cells under both non-stress and stress conditions, Hsf1p in the Δ*ppt1* strain migrated significantly slower, even without stress, as was observed in K701. From this result, Ppt1p is suggested to be a putative phosphatase that constitutively dephosphorylates Hsf1p and is defective in sake yeast. Intriguingly, we found that the *PPT1* gene is completely deleted in K7; a 2.6-kb

region including the *PPT1* gene was replaced by a Ty2 element (Akao et al. 2011). This type of *PPT1*-gene loss occurs specifically in the modern sake yeast strains, as determined by Southern blot and PCR analyses. These results demonstrated that loss of the *PPT1* gene was closely linked to the sake yeast-specific constitutive hyperphosphorylation of Hsf1p.

Furthermore, we investigated the effects of deleting *PPT1* on fermentation properties (Noguchi et al. 2012). Small-scale sake fermentation tests revealed that deletion of the *PPT1* gene leads to significantly higher levels of carbon dioxide emission and ethanol production. Taken together, the foregoing findings demonstrated that the Δ*ppt1* disruptant mimicked every examined phenotypic characteristic of modern sake yeast, including the constitutive hyperphosphorylation of Hsf1p, defective HSE-mediated expression induction under ethanol stress, and superior fermentation ability. This finding, together with the inactivation of Msn2/4p in modern sake yeast (Watanabe et al. 2011c), provides novel insight into yeast stress responses as major impediments of effective ethanol fermentation.

4.4.3 Rim15p

Although our findings help in understanding in vivo regulatory mechanisms of alcoholic fermentation, how to orchestrate these two pathways still needs to be elucidated. Furthermore, the identified sake yeast-specific loss-of-function mutations, *msn4*^{C1540T} and Δ*ppt1*::*Ty2*, still do not appear to be solely responsible for the high alcoholic fermentation ability of sake yeast, because loss of the *MSN4* or *PPT1* gene in the laboratory strains only modestly increases their fermentation rates (Noguchi et al. 2012; Watanabe et al. 2011c). It is thus suggested that another novel factor might enhance both Msn2/4p and Hsf1p activities and contribute more significantly to the fermentation properties.

We focused on the nucleotide sequence of *RIM15* (Watanabe et al. 2012), which encodes the Per-Arnt-Sim kinase (PASK) functioning upstream of Msn2/4p and Hsf1p as a master regulator of initiation of the yeast quiescent program (Cameroni et al. 2004; Imazu and Sakurai 2005; Lee et al. 2013; Pedruzzi et al. 2003; Talarek et al. 2010). Although the length of the open reading frame (ORF) of the *RIM15* gene is 5,313 nucleotides in S288c, the same ORF is composed of 5,100 nucleotides in K7 and contains 34 single nucleotide substitutions, 5 of which are nonsynonymous (R301C, M909T, H981Y, A1054T, and S1055A), an insertion of 4 AAT trinucleotide repeats (358insN_4), and the insertion of a single adenine nucleotide immediately after A5067 (named 5055insA because the inserted site corresponds to A5055 in S288c). This frameshift is predicted to lead to a premature stop codon that shortens the gene product by 75 amino acids in the carboxyl-terminal region. As the insertion of an adenine nucleotide at the same site in laboratory strain BY4743 resulted in a rapamycin-sensitive growth phenotype, similar to that observed in the Δ*rim15* disruptant (Xie et al. 2005), we anticipated that the function of Rim15p might be severely impaired or lost in K7 because of the 5055insA mutation. This

finding is also consistent with the fact that the sake yeast strains displays pleiotropic phenotypes associated with defective entry into quiescence, such as lower cell viability under stresses, decreased levels of intracellular glycogen and trehalose, lower cellular buoyant density in the stationary phase, and less effective G_1 arrest (Urbanczyk et al. 2011; Watanabe et al. 2011a, 2012). It is worth noting that this mutation, as well as *msn4*^{C1540T} and Δ*ppt1*::*Ty2*, is conserved among the modern sake yeast strains and not found in any other kinds of strains. Thus, the 5055insA mutation in the *RIM15* gene represented a novel modern sake yeast-specific loss-of-function mutation.

As we already proved that the stress responses mediated by Msn2/4p and Hsf1p negatively regulate alcoholic fermentation (Noguchi et al. 2012; Watanabe et al. 2011c), it is expected that the loss of Rim15p functions might be associated with the fermentation properties of sake yeast cells. As a result of sake fermentation tests (Watanabe et al. 2012), the disruption of *RIM15* dramatically promoted alcoholic fermentation in the sake mash, compared to loss of Msn4p or Ppt1p function. We also confirmed that the ethanol concentration after 20 days of sake fermentation was markedly higher in the finished sake made from BY4743 Δ*rim15* (~17 vol.%) than that made from wild-type BY4743 (~11 vol.%). Moreover, the *rim15*^{5055insA} mutant also displayed an improved sake fermentation profile and ethanol concentration (~17 vol.%) that were almost identical to those of the Δ*rim15* disruptant (Fig. 4.4). Taken together, these results clearly demonstrated that dysfunction of Rim15p leads to a striking increase in alcoholic fermentation rates, and that the 5055insA mutation likely led to a complete loss of the Rim15p functions related to the control of alcoholic fermentation. Only a single adenine nucleotide insertion in the *RIM15* gene accounts for both the quiescence-related deficient phenotypes and the increased

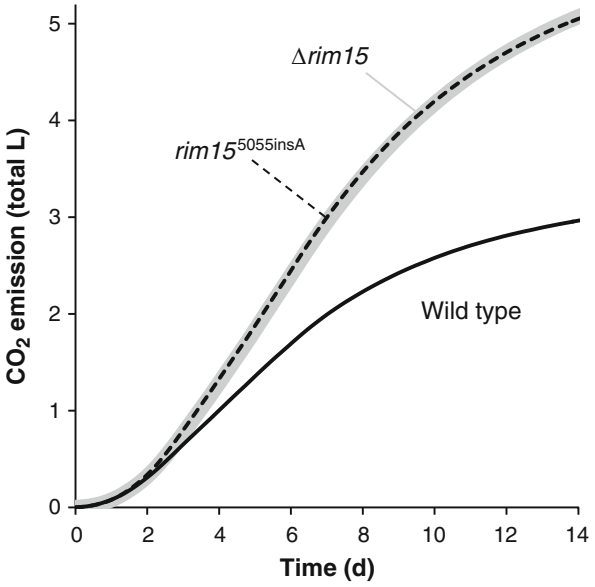

Fig. 4.4 Effects of deletion of the *RIM15* gene or the *rim15*^{5055insA} mutation on the sake fermentation profile. The fermentation rates were monitored by measuring CO_2 emission from the sake mash. (Modified from Watanabe et al. 2012)

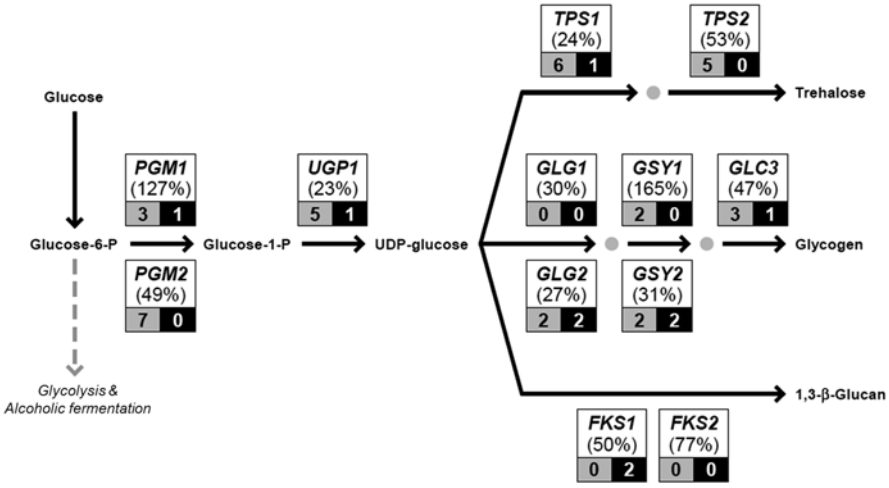

Fig. 4.5 Storage and structural carbohydrate synthesis-related gene expression in sake yeast. Percentages in *parentheses* indicate gene expression level ratios in K701 relative to X2180. Gene expression data during sake fermentation (day 5) were derived from Watanabe et al. (2011c). Values in *gray* and *black boxes* represent the numbers of STREs (CCCCT) and HSEs (NGAANNTTCN or NTTCNNGAAN), respectively, in the 5′-UTR (1,000 bases immediately upstream from the start codon) of each gene, as determined by searches of the YEASTRACT database (http://www.yeastract.com/)

fermentation rates, suggesting that this *rim15*^{5055insA} mutation might have played a pivotal role in establishing the unique core physiological properties of modern sake yeast strains.

How does the dysfunction of Rim15p result in increased ethanol production rates? The most plausible explanation is that the loss of Rim15p activity leads to elevated fermentation rates through inactivation of Msn2/4p and Hsf1p. Inactivation of these transcription factors contributes to the repression of the genes related to stress-responsive carbohydrate metabolism, which diversifies the cellular glucose flux (Fig. 4.5). In agreement with this phenomenon, we showed that the expression of a functional *RIM15* gene in a modern sake yeast strain appears to recover the synthesis of the storage carbohydrates trehalose and glycogen (Watanabe et al. 2012). We also found that Rim15p dysfunction also affects cell-cycle G_1 arrest under rapamycin treatment in the present study (Watanabe et al. 2012). Because G_1 progression is involved in the regulation of fermentation rates (Watanabe et al. 2011a), Rim15p dysfunction might partly contribute to rapid ethanol production through a decrease in G_1 arrest efficiency during fermentation. In addition, we revealed that the only known targets of Rim15p, namely, Igo1p and Igo2p (Luo et al. 2011; Talarek et al. 2010), also inhibit fermentation (Watanabe et al. 2012), albeit by unknown mechanisms. To elucidate the complete regulatory mechanisms underlying Rim15p-mediated quiescence entry and alcoholic fermentation, the downstream effectors of Rim15p and their roles in fermentation control should be investigated comprehensively.

4.5 Closing Remarks

It has long been vaguely understood that yeast strains with good alcoholic fermentation ability are resistant to ethanol stress. However, our studies of sake yeast revealed that this is not necessarily the case. In the past 80 years, a group of genetically closely related modern sake yeast strains that exhibit high fermentation rates in sake mash have been isolated. Based on our genomic (Akao et al. 2011) and transcriptomic (Watanabe et al. 2011c; Wu et al. 2006) analyses, it was discovered that the modern sake strains commonly possess the *msn4*^{C1540T}, Δ*ppt1*::*Ty2*, and *rim15*^{5055insA} mutations, each of which leads to defective stress responses and an increased rate of alcoholic fermentation (Noguchi et al. 2012; Watanabe et al. 2011c, 2012, 2013). Consistently, the modern sake yeast cells exhibit lower viability than the laboratory reference strain (Urbanczyk et al. 2011). It was thus demonstrated that sake yeast acquired high fermentation ability by abrogating the stress-responsive mechanisms. Genetic engineering of these factors represents a potential strategy for improving the ethanol production rates of other industrial yeast strains. For example, we achieved improvement of the molasses fermentation properties of an industrial ethanol-producing strain PE-2 by deleting the *RIM15* gene (Inai et al. 2013). Yeast stress responses may involve metabolic regulation to prevent the cells from the synthesis of toxic compounds, including ethanol. Therefore, sake yeast strains without this protective system might have been highly domesticated under sake fermentation conditions containing much glucose and probably do not survive in adverse natural environments. Elucidating the whole map of yeast stress responses from the genomic, transcriptomic, proteomic, and metabolic aspects will provide the basis for the breeding of yeast strains that effectively produce useful compounds.

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