Hiroshi Takagi · Hiroshi Kitagaki *Editors*

Stress Biology of Yeasts and Fungi

Applications for Industrial Brewing and Fermentation

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Preface

In past millennia, humans have had a history of using the power of microorganisms (particularly yeasts and fungi) that possess strong productivities of ethanol, carbon dioxide, taste and flavor compounds, or enzymes during their fermentation processes for making breads and brewing alcoholic beverages. Recently, bioethanol is one of the renewable fuels important for the reduction of the global warming effect and environmental damage caused by the worldwide use of fossil fuels. However, we should recognize that, during fermentation, cells of yeasts and fungi, mostly *Saccharomyces cerevisiae* and *Aspergillus oryzae*, respectively, are exposed to a variety of fermentation stresses, including high concentrations of ethanol, high/low temperature, freezing, desiccation, high osmotic pressure, low pH, hypoxia, nutritional starvation, and redox imbalance. Such stresses induce protein denaturation and reactive oxygen species generation, leading to growth inhibition or cell death. Under severe stress conditions, their fermentation ability and enzyme productivity are rather limited. Therefore, in terms of industrial application, stress tolerance is the key characteristic for yeast and fungus cells.

The focus of this book is on stress response/adaptation mechanisms of yeasts and fungi and their applications for industrial brewing and fermentation. Our purpose is to facilitate the development of fermentation technologies by addressing strategies for stress tolerance of yeast and fungus cells. We believe that readers benefit nicely from novel understandings and methodologies of these industrial microbes.

The book consists of two parts. The first, comprising the first eight chapters, presents advances and mechanisms based on our current understanding of the stress tolerance of yeast used for the production of bread, sake, beer, wine, and bioethanol in the presence of various fermentation stresses such as freeze–thaw, high sucrose, air-drying (so-called baking-associated stresses), nutrient deficiency, high concentrations of ethanol, high hydrostatic pressure, and various inhibitors (glycolaldehyde, furan derivatives, weak organic acids, and phenolic compounds). The second part, comprising the last five chapters, covers mechanisms and approaches based on our recent knowledge of the stress response of fungi, including environmental

changes (hypoxia, nitric oxide, cell wall, and osmotic pressure) and biological processes (cell wall biosynthesis; polarized, multicellular, or hyphal morphogenesis; and conidiation).

This book provides detailed descriptions of stress response/adaptation mechanisms of yeasts and fungi during fermentation processes, suggesting numerous promising strategies for breeding of industrial yeast and fungus strains with improved tolerance to stresses. This publication also introduces the traditional Japanese alcoholic beverage sake, made from steamed rice by multiple parallel fermentation of the fungus *Aspergillus oryzae* (national microbe of Japan, *Kokkin*: 国菌) and the yeast *Saccharomyces cerevisiae* (*Kyokai* sake yeast), which produce saccharification enzymes for making the dried fermentation starter (*koji*) and high concentrations of ethanol (~20 % [vol/vol]) from glucose, respectively. The book is suitable for both academic scientists and graduate-level students involved in applied microbiology and biochemistry and biotechnology and industrial researchers and engineers who are experts with fermentation-based technologies.

Finally, we would like to thank all contributing authors for their excellent work, effort, and dedication in this project, which were indispensable for the production of the book. We believe that the authors can be proud of such an achievement. We are also grateful to Springer Japan for publishing this monograph, and our special thanks are due to Kaoru Hashimoto and Momoko Asawa for their great assistance and support.

Nara, Japan — Hiroshi Takagi
Saga, Japan — Hiroshi Kitagaki

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Part I
Stress Biology of Yeasts

Chapter 1
The Breeding of Bioethanol-Producing Yeast by Detoxification of Glycolaldehyde, a Novel Fermentation Inhibitor

Lahiru N. Jayakody, Nobuyuki Hayashi, and Hiroshi Kitagaki

Abstract The inhibitory effect of lignocellulose hydrolysates poses a significant technological barrier to the industrialization of second-generation bioethanol production. Even though approximately 60 inhibitory compounds have been reported to be present in lignocellulose hydrolysates, we discovered glycolaldehyde as a novel fermentation inhibitor and established a key role for the toxic compound in second-generation bioethanol production. Glycolaldehyde is primarily generated from retro-aldol condensation of monomeric sugars liberated during the lignocellulosic biomass pretreatment process. It substantially inhibits yeast growth and ethanol fermentation at a very low concentration. Moreover, glycolaldehyde is a stronger growth inhibitor than other reported major fermentation inhibitors such as 5-hydroxymethyl furfural (5-HMF) and furfural. Through comprehensive genomic analysis and in-depth analysis of fermentation metabolic consequences in response to redox cofactor perturbation with glycolaldehyde, we discovered the toxic mechanisms and pathways necessary to ultimately engineer a glycolaldehyde-tolerant yeast strain. This chapter provides novel knowledge on glycolaldehyde toxicity and molecular mechanisms for in situ biological detoxification of glycolaldehyde to improve the bioethanol fermentation of *Saccharomyces cerevisiae*.

Keywords *ADH1* • Bioethanol • Glycolaldehyde • *GRE2* • Hot-compressed water • Redox cofactor • Yeast

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1.1 Background

Bioethanol production is a promising strategy to ensure meeting future global transportation fuel demand while mitigating global warming issues. However, first-generation biofuel production is limited by availability of raw material as well as debate over arable land utilization, the so-called fuel versus food issue (Tenenbaum 2008; Naik et al. 2010). Therefore, to substitute for sugar-based or starch-based bioethanol production, technologies for producing second-generation bioethanol from lignocellulosic biomass are rapidly developing worldwide. The United States, Sweden, Canada, and Japan are currently operating lignocellulosic ethanol plants on a pilot scale (Schubert 2006). Lignocellulose is the most abundant renewable resource that can be used in biorefineries to obtain biofuel, chemicals, and polymers, establishing an alternative to the current oil refineries (Landucci et al. 1994; Ragauskas et al. 2006). Lignocellulose feedstock mainly consists of homopolysaccharide cellulose, heteropolysaccharide hemicelluloses, and phenypropane units containing lignin (Wright 1988). Thus, the first step of bioethanol generation using lignocelluloses involves the conversion of these polysaccharides into fermentable sugars, such as glucose and xylose.

The recovery of fermentable sugars for the production of lignocellulosic bioethanol is typically a two-step process, involving pretreatment followed by enzymatic hydrolysis. Biological, physical, and chemical pretreatment technologies have been applied to break β-1,4-linked D-glucopyranose-containing celluloses and β-1,4-linked D-xylopyranose-containing hemicelluloses. Among these pretreatment technologies, hot-compressed water treatment has been identified as an advanced, nonenzymatic sugar-producing biomass pretreatment method (Adschiri et al. 1993; Bonn et al. 1983; Kumagai et al. 2004; Mosier et al 2005; Nakata et al. 2006). Water in a subcritical or supercritical stage or at a temperature above 150 °C with various pressures (5–22 MPa) is defined as hot-compressed water. It breaks down celluloses and hemicelluloses into various compounds through pyrolytic cleavage, swelling, and dissolution reactions (Yu et al. 2007; Lu et al. 2009). The degradation of cellulose with hot-compressed water at temperatures of 270–400 °C mainly yielded glucose, fructose, erythrose, mannose, and cello-oligosaccharides. On the other hand, the hydrolysis of hemicelluloses at temperatures of 230–270 °C yielded xylose, galactose, rhamnose, mannose, arabinose, and xylo-oligosaccharides. Treatment of lignocelluloses with hot-compressed water has several advantages. No hazardous wastes are produced in the process, and fermentable sugar fractions of hemicellulose (primarily pentose) and cellulose (primarily hexose) can be separately recovered through a two-step hydrolysis process (Lu et al. 2009), improving the degradation of crystallized cellulose (Mosier et al. 2005). The reaction rate is quite fast and economically feasible for mass-scale production (Kumar et al. 2009). However, pretreatment of lignocelluloses with hot-compressed water generates a variety of inhibitory compounds by further degrading simple sugars (Jayakody et al. 2013b), and this is recognized as one of the greatest bottlenecks for the success of the industrial application of this advanced technology.

Approximately 60 inhibitory compounds have been reported to be present in a hot-compressed water-treated lignocellulosic hydrolysate (Lu et al. 2009; Palmqvist and Hahn-Hägerdal 2000a, b). However, furfural, 5-hydroxymethyl furfural (5-HMF), methylglyoxal, acetic acid, and newly identified glycolaldehyde are recognized as the main inhibitors present in the hot-compressed water-treated lignocelluloses (Jayakody et al. 2013b). These compounds predominantly exist in the hydrolysate and inhibit the growth and fermentative capacity of the robust and extensively used industrial workhorse *Saccharomyces cerevisiae* at very low concentrations (Jayakody et al. 2011; Liu 2011; Palmqvist and Hahn-Hägerdal 2000a, b). In general, three common methods have been implemented to handle inhibitors: inhibitor formation reduction by process controlling, chemical detoxification, and development of inhibitor-tolerant strains to overcome the toxicity. The third alternative has been widely adopted at the industrial scale for techno-economic concerns. Hence, engineering strains for the biotransformation of inhibitors into less toxic compounds is the primary driving force for developing inhibitor-tolerant *S. cerevisiae*. Comprehensive studies performed during the past two decades based on genome-wide, transcriptome, and metabolome analyses have uncovered the molecular mechanisms of yeast tolerance to these major inhibitors (Jayakody et al. 2013b; Jönsson et al. 2013; Liu 2011). In *S. cerevisiae*, NADPH-dependent oxidoreductase activities primarily are involved in the in situ detoxification of lignocellulose hydrolysate inhibitors. Figure 1.1 shows the major biological conversion

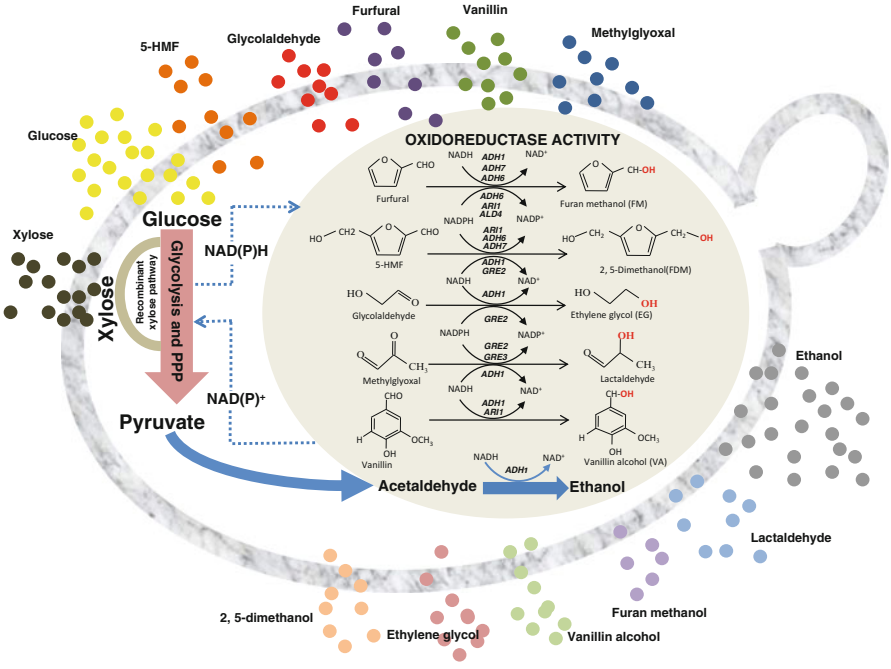

Fig. 1.1 Biological conversion pathways of key inhibitors present in hot-compressed water-treated lignocellulose hydrolysate

pathways of these inhibitors. However, until we discovered glycolaldehyde as a novel key inhibitor of bioethanol fermentation, it was largely unstudied. Therefore, this report provides novel knowledge on the toxicity and detoxification mechanisms of glycolaldehyde, which can be adopted to develop robust yeast strains for lignocellulosic ethanol production.

1.2 The Role of Glycolaldehyde as a Fermentation Inhibitor

1.2.1 Physiochemical Background of Glycolaldehyde Formation

The hydrolytic degradation of hemicelluloses and cellulose requires the catalytic activities of H^+ and OH^-. However, the catalytic activities of these ions cause not only degradation of hemicelluloses and cellulose but also degradation or chemical conversion of single sugar units. The chemical conversion is mainly via dehydration, because water content is far from equilibrium in a concentrated sulfuric acid solution. Therefore, substances such as 5-HMF, which is formed from 6-carbon sugars, and furfural, which is formed from 5-carbon sugars, are generated. Because these substances inhibit yeast fermentation, their effects on bioethanol production have been studied. At the same time, when temperatures are high, retro-aldol condensation of sugar units occurs. Retro-aldol condensation produces glycolaldehyde (Yu et al. 2007; Lu et al. 2009). This reaction depends on the reaction energy, and thus, formation of glycolaldehyde occurs at high temperatures (≥200 °C). Three molecules of glycolaldehyde are formed through the two-step retro-aldol condensation reaction of glucose followed by erythrose. However, when xylose is used, one molecule of glycolaldehyde is produced with the glyceraldehydes (Yu et al. 2007) (Fig. 1.2a). Other than retro-aldol condensation in plant hydrolysates, glycolaldehyde is formed from serine through Strecker degradation (Yaylayan 2003) (Fig. 1.2b). The concentration of glycolaldehyde in the lignocellulosic hydrolysate ranges from 1 to 22 mM, depending on pretreatment conditions and the type of biomass used (Lu et al. 2009; Katsunobu and Shiro 2002).

1.2.2 Toxicity of Glycolaldehyde

Glycolaldehyde is a highly reactive α-hydroxyaldehyde that contains a strongly electrophilic carbon atom. It has a hydroxyl bond next to the aldehyde bond, which discriminates this molecule from other general aldehydes. The Maillard reaction activity of glycolaldehyde is 2,109-fold higher than that of glucose (Hayashi and Namiki 1986). Glycolaldehyde forms aldolamines with proteins, followed by Schiff base formation and Amadori rearrangement (Glomb and Monnier 1995). Furthermore, the keto base of aldolamine attacks the noncovalent

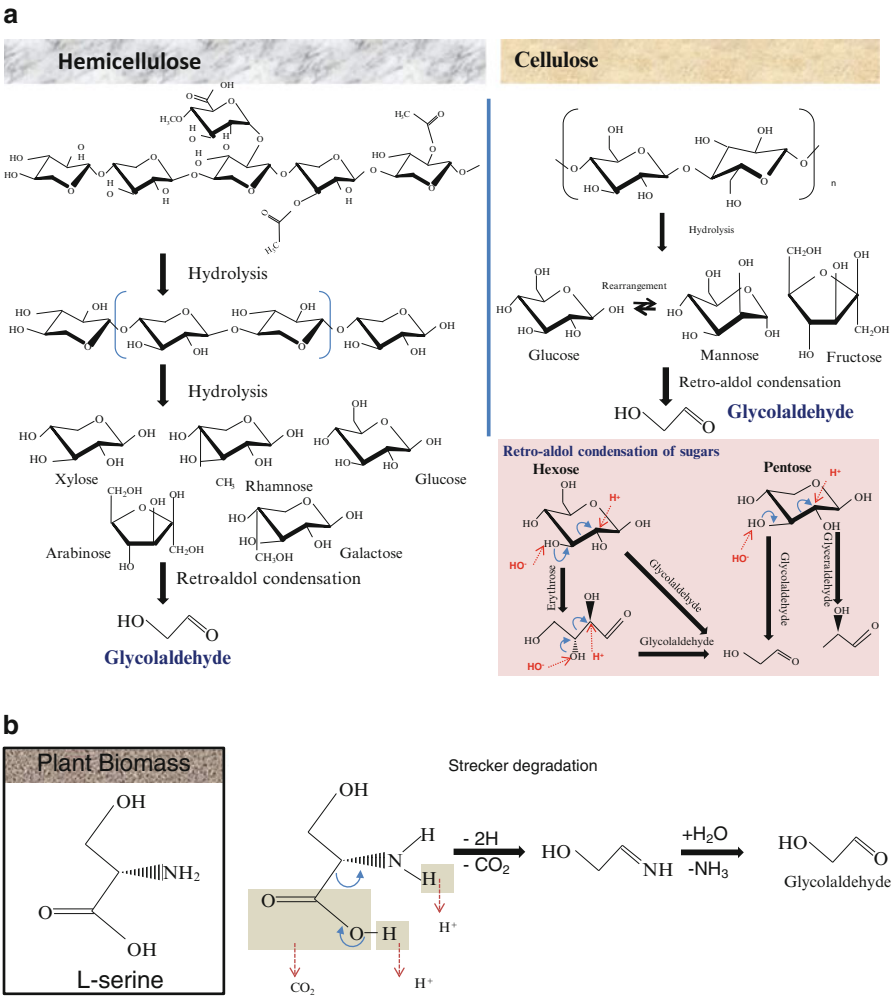

Fig. 1.2 Proposed chemical pathways of generating glycolaldehyde from hot-compressed water treatment of biomass. **a** Hydrolysis followed by retro-aldol condensation. **b** Strecker degradation

electron pair of the nitrogen atom of amino bases of other proteins, thereby crosslinking proteins and eventually forming mellanoidin (Hayashi and Namiki 1986) (Fig. 1.3). In contrast to protein crosslinking, aldolamine leads to the formation of carboxymethyllysine (CML) (Fig. 1.3). In humans, glycolaldehyde is generated from myeloperoxidase activity on L-serine, protein glycation, and oxygen-dependent cleavage of glucose or Schiff bases (Takeuchi and Makita 2001). Its concentration is estimated to be approximately 0.1 to 1 mM. Moreover, glycolaldehyde causes diabetes complications in patients by forming advanced glycation end products (AGEs) (Glomb and Monnier 1995; Matsumoto et al. 2010).

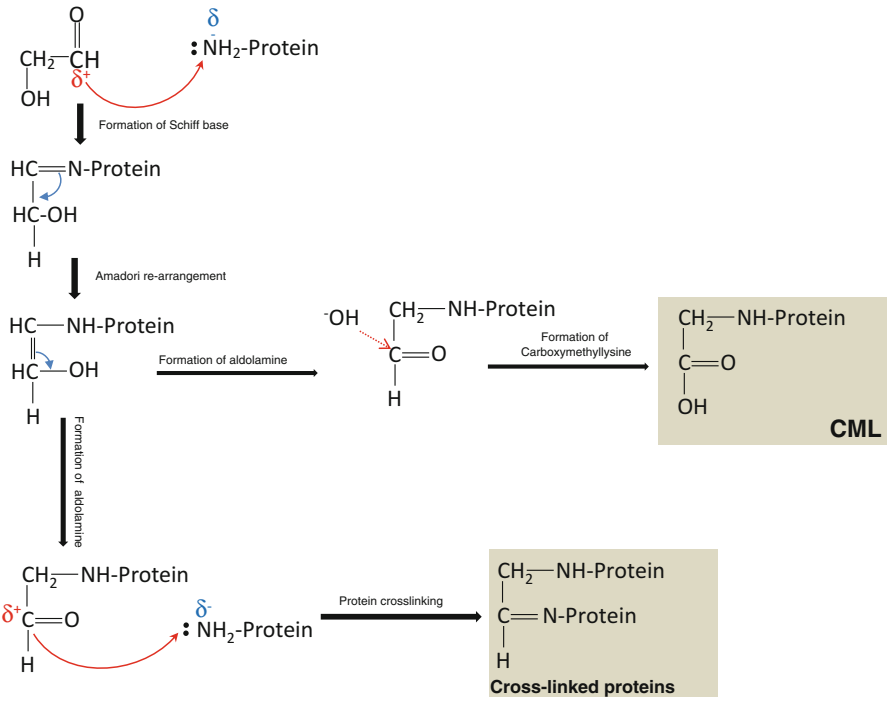

Fig. 1.3 Reaction scheme for the modification of proteins by glycolaldehyde

Although glycolaldehyde has a significant impact on human health, its effects on fermentation inhibition have not been studied. However, the described toxic background of glycolaldehyde has been suggested to impact yeast physiology.

1.2.3 Glycolaldehyde Mediates Yeast Fermentation Inhibition: Effects and Mechanisms

Glycolaldehyde significantly inhibits yeast cell growth at a very low concentration (Fig. 1.4a). The IC_{50} value of glycolaldehyde on *S. cerevisiae* is approximately 10 mM (Jayakody et al. 2011). Hence, the concentration of glycolaldehyde contained in the actual pressurized hot-compressed water-treated lignocellulose hydrolysate is high enough to inhibit yeast growth. Furthermore, growth analysis indicated that glycolaldehyde affects both the cell growth rate (Table 1.1) and lag phase of cell growth (Fig. 1.4a) (Jayakody et al. 2011). Fermentation profile analysis in the presence of glycolaldehyde revealed that glycolaldehyde reduces the ethanol production rate and final ethanol titer as well as the glucose consumption rate (Table 1.2) (Jayakody et al. 2011). To date, furfural and 5-HMF have been recognized as the major fermentation inhibitors in second-generation biofuel production (Liu 2011).

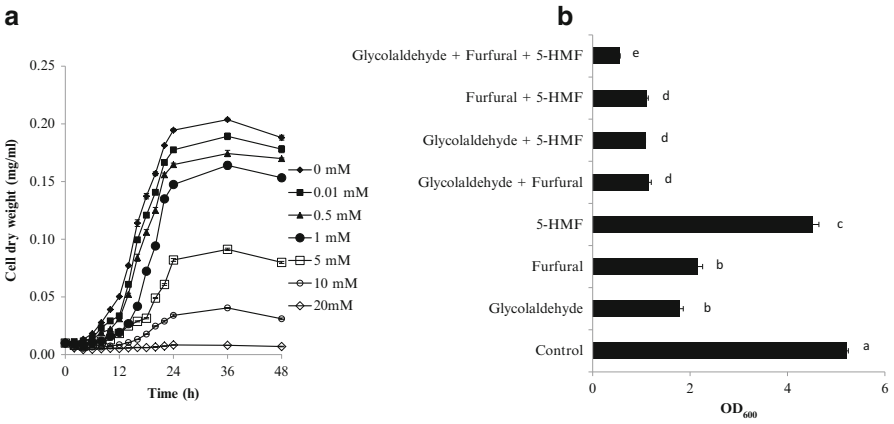

Fig. 1.4 Glycolaldehyde is a key inhibitor of *Saccharomyces cerevisiae*. **a** *S. cerevisiae* BY4743 cells were grown at 30 °C in 96-well plates containing 100 µl SC media supplemented with CSM and different concentrations of glycolaldehyde. Growth was monitored at OD_{600} at different time intervals. Cell dry weights were calculated based on the OD_{600} of 1 equaling 0.45 mg cell dry weight. **b** BY4743 + pRS426 in media containing 2.3 mM glycolaldehyde, 3.3 mM furfural, 3.5 mM 5-HMF, and their combinations. OD_{600} values were measured at 24 h. The results are expressed as the mean ± SEM of independent triplicate experiments from the respective independent starter cultures

Table 1.1 Specific growth rate of strains in the presence of 5 mM glycolaldehyde

	Specific growth rate (g/g/h)	
	Without glycolaldehyde	With glycolaldehyde
BY4743 + pAUR123	0.153 ± 0.001	0.070 ± 0.002
BY4743 + pAUR123-*GRE2*	0.154 ± 0.002	0.072 ± 0.000
BY4743 + pRS426-*ADH1* + pAUR123	0.153 ± 0.002	0.114 ± 0.003*
BY4743 + pRS426-*ADH1* + pAUR123-*GRE2*	0.155 ± 0.001	0.132 ± 0.002*

Asterisks indicate statistically significantly different values ($p < 0.05$, $n = 3$) compared to the control strain

However, as shown in Table 1.3, the growth inhibitory activity of glycolaldehyde is higher than those of furfural and 5-HMF at a 5 mM concentration. Moreover, glycolaldehyde exhibits combinational inhibitory effects with 5-HMF and furfural (Fig. 1.4b), indicating that glycolaldehyde is the key inhibitory substance present in the hot-compressed water-treated lignocellulose hydrolysate.

Genome-wide screening was used to identify the exact molecular targets of glycolaldehyde during yeast growth and ethanol fermentation. As a result, 170 genes were identified to be required for glycolaldehyde tolerance by screening the complete mutant collection of *S. cerevisiae* BY4743, comprising 4,848 homozygous diploid deletion strains, with 0.01 mM glycolaldehyde (Jayakody et al. 2011). Furthermore, Table 1.4 shows the major cellular functional categories that are involved in glycolaldehyde resistance of yeast according to the Gene Ontology

Table 1.2 Ethanol production and glucose consumption rates per biomass of strains in the presence of 5 mM glycolaldehyde

	Ethanol production (mM/g/h)		Glucose consumption (mM/g/h)	
	Without glycolaldehyde	With glycolaldehyde	Without glycolaldehyde	With glycolaldehyde
BY4743 + pAUR123	1.68 ± 0.39	1.12 ± 0.18	1.15 ± 0.01	0.93 ± 0.00
BY4743 + pAUR 123-*GRE2*	1.63 ± 0.04	1.3 ± 0.35	1.14 ± 0.01	1.11 ± 0.02
BY4743 + pRS426-*ADH1* + pAUR123	1.886 ± 0.15	1.438 ± 8.85	1.090 ± 0.001	1.029 ± 0.002
BY4743 + pRS426-*ADH1* + pAUR123-*GRE2*	1.885 ± 0.17	1.614 ± 0.01*	1.090 ± 0.003	1.076 ± 0.007

Asterisks indicate statistically significantly different values ($p < 0.05$, $n = 3$) compared to the control strain

Table 1.3 Specific growth rates of yeast strains treated with fermentation inhibitors

	Control	Glycolaldehyde-treated	5-HMF-treated	Furfural-treated
Specific growth rate (h^{-1})	0.148 ± 0.0019 a	0.0682 ± 0.0018 b	0.114 ± 0.0017 ac	0.00771 ± 0.00158 bc

Specific growth rates were measured in their exponential growth phases ($OD_{600} = 0.2–4.0$) with or without 5 mM inhibitors. The results are mean values ± SEM of triplicate independent experiments. Values labeled with different letters indicate statistical significance

Table 1.4 Functional categories that are overrepresented in the glycolaldehyde-sensitive mutants

Category[a]	*p* Value[b]	Gene name
GO cellular component		
Mitochondrial respiratory chain complex IV	0.00262	*COX9 COX6 COX5B*
Ubiquitin ligase complex	0.00740	*SLX8 BUL2* YNL311c
Polysome	0.00740	*PBP1 CTK1 SSB2*
Elongator holoenzyme complex	0.00821	*ELP2 IKI3*
GO biological process		
Response to acid	0.00148	*BCK1 MID2 RLM1*
Golgi to vacuole transport	0.00327	*VPS54 VPS45 APS3 APL2*
Mitochondrial electron transport, cytochrome *c* to oxygen	0.00334	*COX9 COX6 COX5B*
GO molecular function		
Chromatin DNA binding	0.00556	*GON7 RED1*
Phospholipase activity	0.00821	*PLB1* YOR022c

[a]The gene functions were identified by analyzing the Gene Ontology (GO) database with the FunSpec statistical evaluation program

[b]The probability of the functional set occurring from chance is shown

(GO) yeast databases on the FunSpec web-based clustering tool. Given that glycolaldehyde is involved in the posttranslational modification of proteins by forming CML and crosslinking proteins, these results show that mutants defective in ubiquitin ligase complex and polysomes were significantly more sensitive to glycolaldehyde. The results of genome-wide analysis and the physiochemical characteristics of glycolaldehyde suggested that the positively charged α-carbon of the glycolaldehyde molecule has a key role in the inhibition of yeast because the electrophilic attack of the α-carbon of glycolaldehyde on negatively charged molecules inside the cells is the main cause of toxicity.

1.3 Biological Detoxification of Glycolaldehyde

1.3.1 The Role of Oxidoreductase Activity in Reducing the Functional Group of Glycolaldehyde

Consistent with the uncovered molecular toxicity mechanism of glycolaldehyde, the reduction of the positively charged carbonyl carbon of the glycolaldehyde molecule by nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) was implemented as the principal strategy to develop a resistant strain (Fig. 1.5). Although not detected in the functional categories of glycolaldehyde-resistant genes in GO-based statistical analysis, mutants defective in aldehyde dehydrogenases, such as the NADH-dependent alcohol dehydrogenate gene *ADH1* and the NADPH-dependent methyl glyoxal reductase gene *GRE2*, were obtained as genes that countered toxicity in the glycolaldehyde screen (Jayakody et al. 2011). These results suggest that dehydrogenases confer glycolaldehyde tolerance. Glycolaldehyde functions as an aldehyde within cells, and the enzymes that reduce the glycolaldehyde to ethylene glycol can effectively mitigate the damage. These findings are consistent with a previous study that reported that aldehyde dehydrogenases, such as *ADH6*, *ADH7*, *ALD4*, *ARI1*, and *GRE3*, detoxify the aldehyde functional group of inhibitors, including 5-HMF, furfural, vanillin, and methyl glyoxal (Liu and Moon 2009; Liu 2011; Petersson et al. 2006). Moreover, ethylene glycol was not toxic to yeast cells when it was administered at the same concentration as glycolaldehyde (Jayakody et al. 2012). This result is consistent with the fact that the attached aldehyde functional groups of the furan inhibitors 5-HMF and furfural are toxic to yeast growth or fermentation, but the reduced forms of the furan compounds, furanmethanol and furan 2,5-dimethanol, are not (Liu 2011). Because Adh1 was able to confer tolerance to glycolaldehyde and Adh1 is capable of reducing short-chain aldehydes such as acetaldehyde and formaldehyde by using NADH as a cofactor (Leskovac et al. 2002; Grey et al. 1996), it was selected to biochemically reduce glycolaldehyde into ethylene glycol (Jayakody et al. 2012). This hypothesis was verified by the constructed *ADH1*-expressing strain. Indeed, the constructed strain enhanced ethylene glycol production by 2.5-fold relative to the control strains (Fig. 1.6), suggesting that the *ADH1*-expressing strain is highly

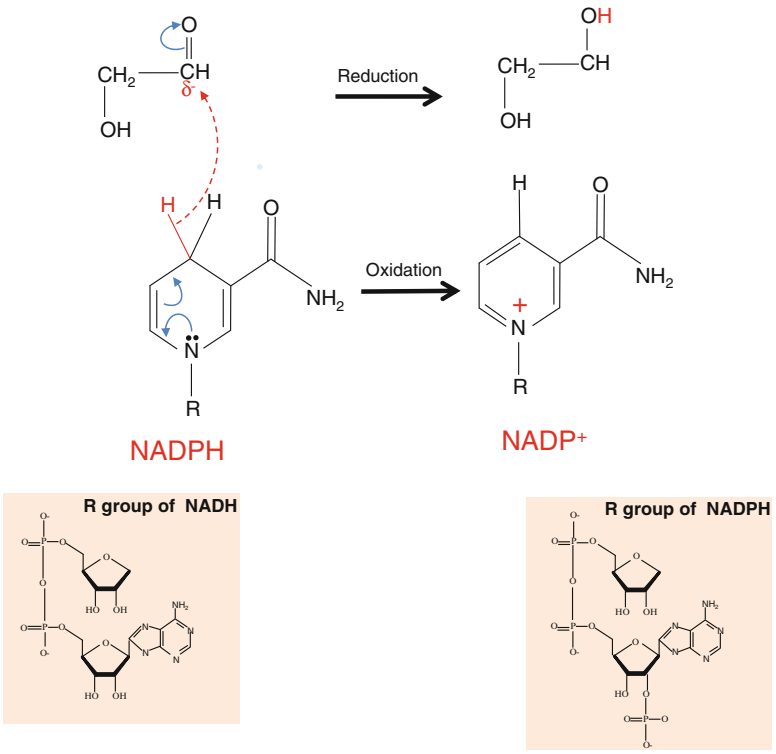

Fig. 1.5 Oxidoreductase-catalyzed conversion pathway of glycolaldehyde into ethylene glycol coupled with NADPH

capable of converting glycolaldehyde into ethylene glycol. Moreover, the *ADH1*-expressing strain exhibited a significantly improved growth (Table 1.1) and fermentation profile in synthetic medium with glycolaldehyde as well as in the actual hot-compressed water-treated lignocellulose hydrolysates (Table 1.2, Jayakody et al. 2012). These results suggest that the reduction of glycolaldehyde into ethylene glycol is a promising strategy and key target to decrease the toxicity of hot-compressed water-treated lignocellulose hydrolysates.

1.3.2 Altering Redox Cofactor Usage to Enhance the Glycolaldehyde Reduction Reaction

1.3.2.1 NADH Perturbation in the *ADH1*-Expressing Strain Limits Glycolaldehyde Reduction

Even though we successfully developed an efficient glycolaldehyde-reducing strain by expressing NADH-dependent *ADH1*, the developed strain was only partially tolerant to high concentrations of glycolaldehyde (>10 mM). The in vitro analysis of

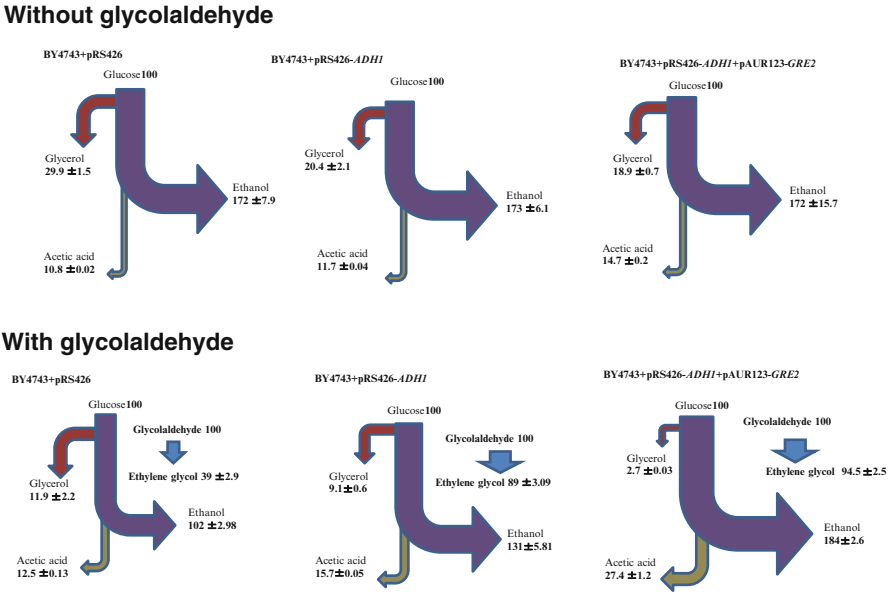

Fig. 1.6 Effect of glycolaldehyde on extracellular fermentation metabolic flux distribution. Extracellular fermentation metabolic flux distributions of strains are represented as moles of products per moles of consumed glucose; the thicknesses of the *arrows* represents the extent of flux. The results are expressed as mean values ± SEM of independent triplicate experiments from respective independent starter cultures

whole-cell lysates for NADH- and NADPH-dependent glycolaldehyde-reducing activities indicated that glycolaldehyde reduction in the *ADH1*-expressing strain mainly occurs through an NADH- and not an NADPH-dependent manner (Jayakody et al. 2013a). Further analysis of the redox profile of glycolaldehyde-treated yeast cells by monitoring intracellular NADH and NADPH levels showed that NADPH perturbation occurs in response to the glycolaldehyde detoxification reaction (Jayakody et al. 2013a). Indeed, the *ADH1*-expressing strain showed dramatically reduced NADH content with high concentration of glycolaldehyde (≥5 mM), suggesting that the *ADH1*-expressing strain has reduced redox capacity in terms of NADH to handle high concentrations of glycolaldehyde. Therefore, restoring the NADPH-dependent glycolaldehyde-reducing pathway could reinforce the glycolaldehyde tolerance capacity of the *ADH1*-expressing strain.

1.3.2.2 Role of Gre2 in NADPH-Dependent Glycolaldehyde Reduction

Based on the results of a genome-wide survey, NADPH-dependent Gre2 was selected to augment the NADPH-dependent glycolaldehyde reduction pathway in yeast (Jayakody et al. 2013a). Although not as effective as that of *ADH1*, expression of *GRE2* (11.5 ± 3.2 μM/g/h) increased ethylene glycol production (Fig. 1.6) as well

as the rate of production relative to the parent strain (5.9 ± 3.2 μM/g/h). Moreover, whole-protein lysates of the *GRE2*-expresing strain exhibit high NADPH-dependent glycolaldehyde-reducing activity relative to both parental and *ADH1*-expressing strains. Interestingly, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *GRE2* expression levels in the presence of glycolaldehyde unveiled that its expression significantly increases at high concentrations of glycolaldehyde (≥5 mM) relative to low concentrations (≤2 mM) (Jayakody et al. 2013a). In addition, intracellular redox cofactor analysis confirmed that the *GRE2*-expressing strain had significantly higher $NADP^+$ levels compared to that of the parental strain in 5 mM but not in 2 mM glycolaldehyde-containing medium. Altogether, these results confirmed that Gre2 catalyzed the glycolaldehyde reduction to ethylene glycol in an NADPH-dependent manner, and its activity increased with high concentrations of glycolaldehyde (≥5 mM).

1.3.2.3 Activation of the Pentose Phosphate Pathway at High Concentrations of Glycolaldehyde

The pentose phosphate pathway (PPP) is the main source of intracellular NADPH. Mutants defective in the pentose phosphate pathway, such as *gdn1*△, *tkl1*△, and *sol1*△, were highly sensitive to glycolaldehyde at high concentrations (≥5 mM), but not at low concentrations (≤2 mM) (Jayakody et al. 2013a). Furthermore, qRT-PCR analysis revealed that key genes in the pentose phosphate pathway such as *ZNF1*, which encodes glucose-6-phosphate dehydrogenase, and *GND1*, which encodes 6-phosphogluconate, were upregulated in response to high concentrations of glycolaldehyde (≥5 mM), but not in response to low concentrations (≤2 mM) (Jayakody et al. 2013a). Both enzymes reduce $NADP^+$ to generate NADPH. These results further established the importance of NADPH-dependent glycolaldehyde tolerance mechanisms and the vital role of the pentose phosphate pathway in yeast resistance, especially at high concentrations of glycolaldehyde.

1.3.2.4 The Shift in Redox Cofactor Preference of Glycolaldehyde

Analysis of intracellular $NADP^+$ levels confirmed that both the *GRE2*-expressing strain and the *ADH1*-expressing strain had an increased level of $NADP^+$ in response to 5 mM glycolaldehyde when compared with 2 mM glycolaldehyde (Jayakody et al. 2013a). This finding suggests that at high concentrations of glycolaldehyde (≥5 mM), the glycolaldehyde reduction reaction predominantly utilizes NADPH as a cofactor, in contrast to NADH as the predominant cofactor at low concentrations of glycolaldehyde (≤2 mM) (Fig. 1.7c). The shift in the cofactor preferences of yeast cells for aldehyde reduction according to their concentrations has been reported for several aldehyde reduction reactions, including those with furfural and 5-HMF (Almeida et al. 2007; Heer et al. 2009; Liu 2011). The reduction of furfural into the less toxic furan methanol at a concentration less than 6 mM in yeast cells

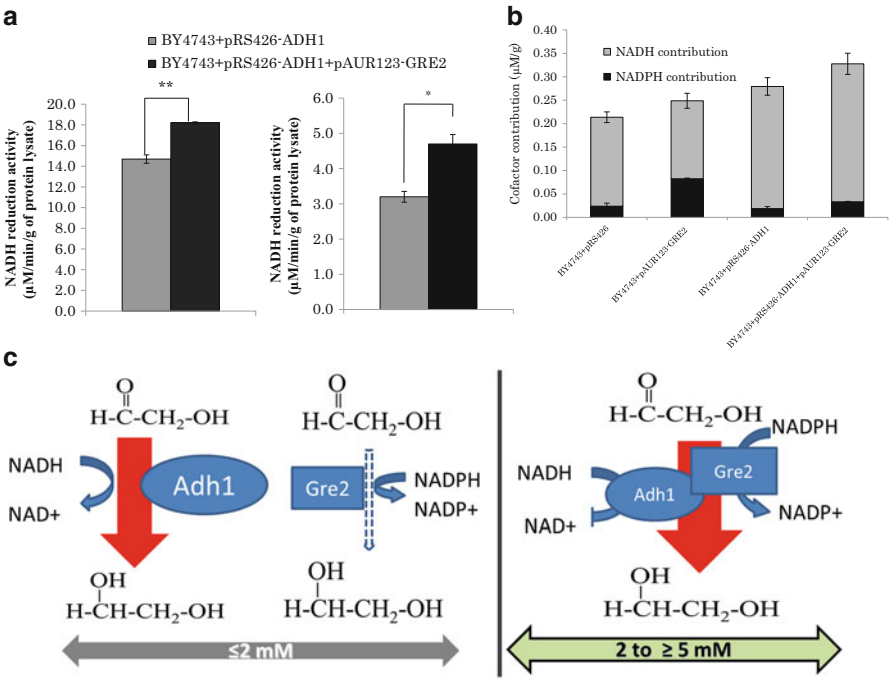

Fig. 1.7 Redox cofactor preference for the glycolaldehyde reduction reaction. **a** In vitro NADH and NADPH-dependent glycolaldehyde-reducing activities of strains both expressing *ADH1* and *GRE2*. **b** Contribution of NADH and NADPH to glycolaldehyde reduction reaction of different yeast strains. **c** Schematic illustration of the concentration-dependent redox cofactor preference of glycolaldehyde reduction reaction

involves the use of NADH as a cofactor (Horvath et al. 2001). By contrast, at higher concentrations (>15 mM) of furfural, the reaction utilizes the cofactor NADPH because of an insufficient supply of NADH (Heer et al. 2009). Celton et al. (2012) demonstrated that a similar shift in cofactor preference occurs in the acetoin-reducing reaction. However, in the case of glycolaldehyde, the shift in cofactor preference occurs at lower concentrations (2–5 mM) compared to the other aldehydes, possibly as a result of its strong toxicity (Jayakody et al. 2012). Hence, redox cofactor regeneration, redox balance, and redox cofactor preference are key targets to enhance *S. cerevisiae* tolerance to aldehyde inhibitors.

1.3.2.5 Coexpression of *ADH1* and *GRE2* in Yeast Cells Creates Better Redox Balance for Glycolaldehyde Reduction Reactions

To maintain better redox balance for the glycolaldehyde-reducing reaction, we augmented the NADPH-dependent glycolaldehyde pathway in the *ADH1*-expressing strain by introducing a *GRE2* expression plasmid. Indeed, the strain expressing both

ADH1 and *GRE2* converted significantly more glycolaldehyde (101.0 ± 11.2 μM/g/h) into ethylene glycol using NADH and NADPH as cofactors compared to *ADH1-expressing* strains (90.3 μM/g/h) (Fig. 1.6). The in vitro analysis of NADPH-dependent glycolaldehyde reduction activity of whole-cell lysates from the strain expressing both *ADH1* and *GRE2* confirmed that the strain has significantly higher NADH- and NADPH-dependent glycolaldehyde-reducing activity relative to other strains (Fig. 1.7a). Furthermore, intracellular redox profile analysis of the novel strain showed notable low NADH and high $NADP^+$ levels in response to 5 mM glycolaldehyde when compared with those of other strains (Jayakody et al. 2013a). Taken together, these results suggest that the strain expressing both *ADH1* and *GRE2* has efficient and enhanced NADPH-dependent glycolaldehyde-reducing capability that is attributed to a better redox balance. Figure 1.8 outlines the relationship between the glycolaldehyde-reducing reactions, redox flux, and principal pathways of central carbon metabolism. In brief, the strain expressing oxidoreductase genes such as *ADH1 and GRE2* remarkably enhanced the NADPH-dependent glycolaldehyde-reducing reaction (Fig. 1.7b), increasing the accumulation of NAD^+ and the $NADP^+$ in cells. Reduction of generated $NADP^+$ in the pentose phosphate pathway by Zwf1p and Gnd1p as well as the action of acetaldehyde oxidation by Ald4p regenerated the NADPH for the glycolaldehyde reduction system where the

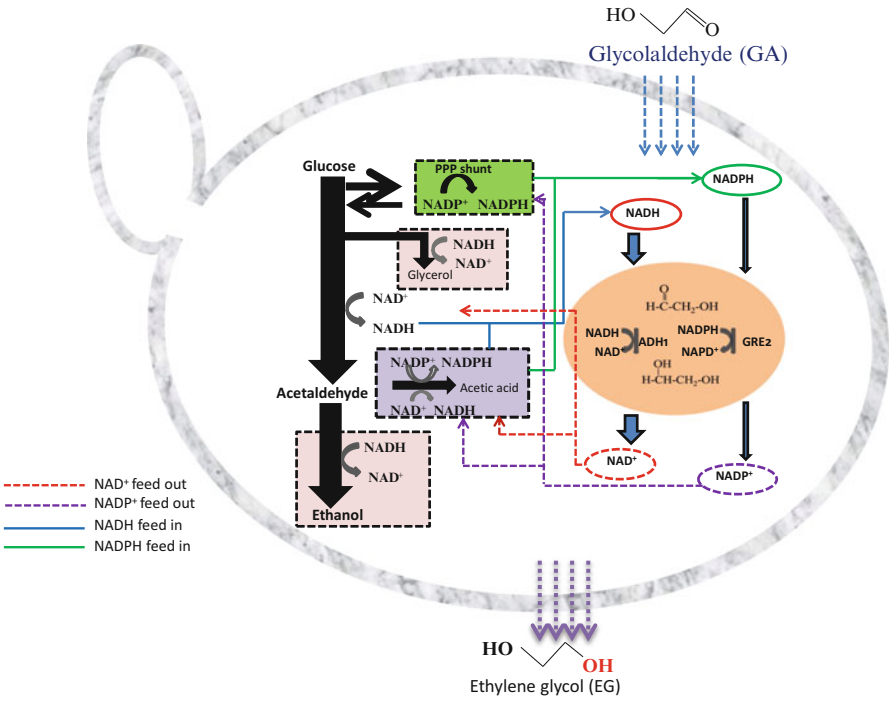

Fig. 1.8 Schematic illustration of the coordination of redox cofactors in *Saccharomyces cerevisiae* and the conversion of glycolaldehyde into ethylene glycol

NADH is supplied from the central glycolysis pathway for the glycolaldehyde reduction reaction. Even though the developed strain exhibited a lag phase of growth, it resumed full growth with 10 mM glycolaldehyde treatment. Moreover, the developed strain showed significantly improved ethanol production in the presence of glycolaldehyde (Table 1.2). In summary, we developed a glycolaldehyde-hypertolerant yeast strain by altering redox cofactor utilization.

1.4 Metabolic Impact of Redox Cofactor Perturbation Resulting from Glycolaldehyde Reduction

Redox cofactors have pivotal roles in driving the at least 307 metabolic reactions in yeast cells (Förster et al. 2003; Bruinenberg et al. 1983). Because the redox carrier NADPH is involved in the glycolaldehyde-reducing reaction, redox cofactor perturbation occurs in the yeast cell. Therefore, it affected the metabolic network and led to an extensive change in metabolic outputs. We rationally exploited the redox system to efficiently increase the reduction of glycolaldehyde by expressing putative oxidoreductase genes (Jayakody et al. 2013a). Interestingly, other than establishing a redox balance, the engineered redox system improved the desired fermentation metabolites in favor of ethanol production.

1.4.1 Alternative NADPH-Regeneration Pathways Activate in Response to Glycolaldehyde Reduction

In glycolaldehyde-treated cells, consumption of excess NADPH during the glycolaldehyde-reducing reaction of Gre2 is primarily balanced by the reduction of $NADP^+$ in the oxidative branch of the pentose phosphate pathway. In addition to the pentose phosphate pathway as a major source of NADPH, the acetate pathway has been reported to produce NADPH in response to the excess NADPH demand to a lesser extent (Grabowska and Chelstowska 2003). Hence, the increase in acetic acid production in glycolaldehyde-containing medium of the *GRE2*-expressing strain and the *ADH1*- and *GRE2*-expressing strain is interconnected with the excess NADPH demand of the cells (Jayakody et al. 2013a). Moreover, the glycerol-dihydroxyacetone cycle (DHA cycle), which has been reported to be activated by saturation of the pentose phosphate pathway and acetate pathway at high levels of NADPH oxidation, converts NADH into NADPH (Celton et al. 2012; Costenoble et al. 2000). The lower glycerol content in the *GRE2*-expressing strain in glycolaldehyde-reducing conditions suggests that the DHA cycle might be activated in *GRE2*-expressing strains as a third source of NADPH generation (Jayakody et al. 2013a). Taken together, analysis of extracellular metabolites revealed that yeast cells manipulate their metabolic flux to generate NADPH to reduce glycolaldehyde.

1.4.2 The Metabolic Costs of Improving Ethanol Yields Through Glycolaldehyde Reduction Reactions Related to Glycerol Formation

In anaerobic conditions, NADH is primarily generated through reduction of NAD^+ in glycolysis and amino acid biosynthesis. Because glycolysis is redox neutral, the redox balance is maintained by reducing acetaldehyde into ethanol. Because *S. cerevisiae* lacks pyridine nucleotide transhydrogenase-like activities (Bruinenberg et al. 1983), glycerol forms to balance the excess NADH generated under anaerobic conditions and acts as a sink for electrons (Bakker et al. 2001). Glycerol is a major by-product of yeast ethanol fermentation and is estimated to use 5 % of the carbon sources in fermentation media (Zaldivar et al. 2001; Oura 1977). In addition, the decrease in glycerol concentration reflects a shortage of NADH within cells. Therefore, decrease in glycerol production in glycolaldehyde-treated cells can be explained by the competition between the dihydroxyacetone phosphate to glycerol-3-phosphate reaction and the glycolaldehyde to ethylene glycol reaction for the reductive potential of NADH. Given that, glycerol production from the reduction of dihydroxyacetone phosphate utilizes NADH, and it competes with the production of ethanol from the reduction of acetaldehyde. Therefore, it has been reported that reducing the rate of glycerol production increases ethanol yield (Pagliardini et al. 2013). Lower glycerol production has been reported to be achieved through ablation of the *GDH1* gene and expression of the *GDH2* gene (Roca et al. 2003), as well as engineering of the phosphoketolase pathway (Sonderegger et al. 2004). The results reported in our studies indicate that, in the presence of glycolaldehyde, the oxidoreductase-expressing strain predominantly oxidizes surplus cytosolic NADH and generates NAD^+, which substantially reduces glycerol formation and increases ethanol formation. Thus, the glycolaldehyde-treated strain expressing *ADH1* and *GRE2* decreased the glycerol yield by 70 % and increased the ethanol yields by approximately 7 % relative to the glycolaldehyde-untreated parental strain. This result is also similar to the decrease of glycerol observed with overexpression of an H_2O-forming NADH oxidase (Vemuri et al. 2007).

1.5 Future Challenges

Even though we succeeded in engineering a glycolaldehyde-hyperresistant strain by efficiently detoxifying glycolaldehyde, the developed strain was unable to overcome the overall toxicity of hot-compressed water-treated lignocellulose hydrolysate, and thus has a long lag phase of growth. It is widely accepted that the complexity of inhibitors in actual hydrolysate elicits complex yeast stress responses, which are distinct from responses to single inhibitors (Skerker et al. 2013). Moreover, we identified that glycolaldehyde has a synergistic inhibitory effect with 5-HMF and furfural (Fig. 1.4b). The mechanism of glycolaldehyde action implies that the

combinational inhibitory effect and tolerance pathways are not well understood. Therefore, the specific biological responses of yeast to complex inhibitory stresses induced by lignocellulosic hydrolysate are yet to be discovered.

1.6 Conclusion

By incorporating physicochemical understanding into the study of fermentation inhibition, we discovered glycolaldehyde as a key novel fermentation inhibitor in second-generation biofuel production. We used a genomic approach to determine the mechanisms of glycolaldehyde toxicity and tolerance pathways in yeast and identified 170 putative glycolaldehyde tolerance genes in *S. cerevisiae*. Based on genomic analysis, we rationally developed a glycolaldehyde-tolerant yeast strain by expressing *ADH1*. The developed strain was capable of converting glycolaldehyde into the less toxic ethylene glycol and alleviated glycolaldehyde toxicity. Moreover, based on dynamic redox cofactor analysis, we further improved the glycolaldehyde tolerance of the *ADH1*-expressing strain by engineering redox cofactors for glycolaldehyde reduction. Furthermore, the developed strain enhanced ethanol fermentation significantly in the presence of glycolaldehyde. This novel knowledge and rational strategy that we have adopted to develop a yeast strain that is tolerant to glycolaldehyde toxicity can be widely used to develop tolerant strains for other inhibitors. However, more detailed analysis of glycolaldehyde toxicity mechanisms and detoxification strategies is needed to develop robust industrial yeast strains suitable to ferment hot-compressed water-treated lignocellulose hydrolysates.

References

Adschiri T, Hirose S, Malaluan R, Arai K (1993) Noncatalytic conversion of cellulose in supercritical and subcritical water. J Chem Eng Jpn 26:676–680

Almeida JRM, Modig T, Petersson A et al (2007) Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J Chem Technol Biotechnol 82:340–349

Bakker BM, Overkamp KM, van Maris AJA et al (2001) Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. FEMS Microbiol Rev 25:15–37

Bonn G, Concin R, Bobleter O (1983) Hydrothermolysis—a new process for the utilization of biomass. Wood Sci Technol 17:195–202

Bruinenberg PM, Van Dijken JP, Scheffers WA (1983) A theoretical analysis of NADPH production and consumption in yeasts. J Gen Microbiol 129:953–964

Celton M, Goelzer A, Camarasa C et al (2012) A constraint-based model analysis of the metabolic consequences of increased NADPH oxidation in *Saccharomyces cerevisiae*. Metab Eng 14: 366–379

Costenoble R, Valadi H, Gustafsson L et al (2000) Micro aerobic glycerol formation in *Saccharomyces cerevisiae*. Yeast 16:483–495

Förster J, Famili I, Fu P et al (2003) Genome-scale reconstruction of the *Saccharomyces cerevisiae* metabolic network. Genome Res 13:244–253

Glomb MA, Monnier VM (1995) Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction. J Biol Chem 270:10017–10026

Grabowska D, Chelstowska A (2003) The *ALD6* gene product is indispensable for providing NADPH in yeast cells lacking glucose-6-phosphate dehydrogenase activity. J Biol Chem 278:13984–13988

Grey M, Schmidt M, Brendel M (1996) Overexpression of *ADH1* confers hyper-resistance to formaldehyde in *Saccharomyces cerevisiae*. Curr Genet 29:437–440

Hayashi T, Namiki M (1986) Role of sugar fragmentation in an early stage browning of amino-carbonyl reaction of sugar with amino acid. Agric Biol Chem 50:1965–1970

Heer D, Heine D, Sauer U (2009) Resistance of *Saccharomyces cerevisiae* to high concentrations of furfural is based on NADPH-dependent reduction by at least two oxireductases. Appl Environ Microbiol 75:7631–7638

Horvath IS, Taherzadeh MJ, Niklasson C, Liden G (2001) Effects of furfural on anaerobic continuous cultivation of *Saccharomyces cerevisiae*. Biotechnol Bioeng 75:540–549

Jayakody LN, Hayashi N, Kitagaki H (2011) Identification of glycolaldehyde as the key inhibitor of bioethanol fermentation by yeast and genome-wide analysis of its toxicity. Biotechnol Lett 33(2):285–292

Jayakody LN, Horie K, Hayashi N, Kitagaki H (2012) Improvement of *Saccharomyces cerevisiae* to hot-compressed water treated cellulose by expression of *ADH1*. Appl Microbiol Biotechnol 94:273–283

Jayakody LN, Horie K, Hayashi N, Kitagaki H (2013a) Engineering redox cofactor utilization for detoxification of glycolaldehyde, a key inhibitor of bioethanol production, in yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 94:273–283

Jayakody LN, Hayashi N, Kitagaki H (2013b) Molecular mechanisms for detoxification of major aldehyde inhibitors for production of bioethanol by *Saccharomyces cerevisiae* from hot-compressed water-treated lignocelluloses. In: Mendez-Vilas A (ed) Material and process for energy: communicating current research and technology development. Formatex Research Center, Badajox, Spain, pp 302–311

Jönsson LJ, Alriksson B, Nilvebrant N (2013) Bioconversion of lignocellulose: inhibitors and detoxification. Biotech Biofuel 6:16

Katsunobu E, Shiro S (2002) A comparative study on chemical conversion of cellulose between the batch-type and flow-type systems in supercritical water. Cellulose 9:301–311

Kumagai S, Hayashi N, Sasaki T et al (2004) Fractionation and saccharification of cellulose and hemicellulose in rice hull by hot-compressed-water treatment with two-step heating. J Jpn Inst Energy 83:776–781

Kumar P, Diane MB, Michael JD, Stroeve P (2009) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind Eng Chem Res 48:3713–3729

Landucci R, Goodman B, Wyman C (1994) Methodology of evaluating the economics of biologically producing chemicals and materials from alternative feedstocks. Appl Biochem Biotechnol 1:677–696

Leskovac V, Trivić S, Pericin D (2002) The three zinc-containing alcohol dehydrogenases from baker's yeast, *Saccharomyces cerevisiae*. FEMS Yeast Res 2:481–494

Liu ZL (2011) Molecular mechanisms of yeast tolerance and in situ detoxification of lignocellulose hydrolysates. Appl Microbiol Biotechnol 90:809–825

Liu ZL, Moon JA (2009) Novel NADPH-dependent aldehyde reductase gene from *Saccharomyces cerevisiae* NRRL Y-12632 involved in the detoxification of aldehyde inhibitors derived from lignocellulosic biomass conversion. Gene 446:1–10

Lu X, Yamauchi K, Phaiiboonsilpa N, Saka S (2009) Two-step hydrolysis of Japanese beech as treated by semi-flow hot-compressed water. J Wood Sci 55:367–375

Matsumoto T, Ozawa Y, Taguchi K et al (2010) Diabetes-associated changes and role of N-epsilon-(carboxymethyl)lysine in big ET-1-induced coronary vasoconstriction. Peptides 31:346–353

Mosier N, Wyman C, Dale B et al (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresourc Technol 96:673–686

Naik SN, Vaibhav VG, Rout PK, Dalai AK (2010) Production of first and second generation biofuels: a comprehensive review. Renew Sust Energ Rev 14:578–597

Nakata T, Miyafuji H, Saka S (2006) Bioethanol from cellulose with supercritical water treatment followed by enzymatic hydrolysis. Appl Biochem Biotechnol 129:476–485

Oura E (1977) Reaction-products of yeast fermentations. Process Biochem 12:19–21

Pagliardini J, Hubmann G, Alfenore S et al (2013) The metabolic costs of improving ethanol yield by reducing glycerol formation capacity under anaerobic conditions in *Saccharomyces cerevisiae*. Microb Cell Fact 12:29

Palmqvist E, Hahn-Hägerdal B (2000a) Fermentation of lignocellulosic hydrolysates. I: inhibitors and mechanisms of inhibition. Bioresourc Technol 74:17–24

Palmqvist E, Hahn-Hägerdal B (2000b) Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresourc Technol 74:25–33

Petersson A, Almeida JRM, Modig T et al (2006) A 5-hydroxymethyl furfural reducing enzyme encoded by the *Saccharomyces cerevisiae ADH6* gene conveys HMF tolerance. Yeast 6:455–464

Ragauskas AJ, Charlotte KW, George B et al (2006) The path forward for biofuel and biomaterials. Science 311:484–489

Roca C, Nielsen J, Olsson L (2003) Metabolic engineering of ammonium assimilation in xylose-fermenting *Saccharomyces cerevisiae* improves ethanol production. Appl Environ Microbiol 69:4732–4736

Schubert C (2006) Can biofuel finally take center stage. Nat Biotechnol 24:777–784

Skerker JM, Leon D, Price MN et al (2013) Dissecting a complex chemical stress: chemogenomic profiling of plant hydrolysates. Mol Syst Bio 9:674

Sonderegger M, Schumperli M, Sauer U (2004) Metabolic engineering of a phosphoketolase pathway for pentose catabolism in *Saccharomyces cerevisiae*. Appl Environ Microbiol 70:2892–2897

Takeuchi M, Makita Z (2001) Alternative routes for the formation of immunochemically distinct advanced glycation end-products in vivo. Curr Mol Med 1:305–315

Tenenbaum DJ (2008) Food vs. fuel: diversion of crops could cause more hunger. Environ Health Perspect 116:254–257

Vemuri GN, Eiteman MA, McEwen JE et al (2007) Increasing NADH oxidation reduces overflow metabolism in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 104:2402–2407

Wright DJ (1988) Ethanol from lignocellulosics: an overview. Energy Prog 84:71–80

Yaylayan VA (2003) Recent advances in the chemistry of Strecker degradation and Amadori rearrangement: implications to aroma and color formation. Food Sci Technol Res 9:1–6

Yu Y, Lou X, Wu H (2007) Some recent advances in hydrolysis of biomass in hot compressed water and its comparison with other hydrolysis methods. Energy Fuel 22:46–60

Zaldivar J, Nielsen J, Olsson L (2001) Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. Appl Microbiol Biotechnol 56:17–34

Chapter 2
Stress Tolerance of Baker's Yeast During Bread-Making Processes

Hiroshi Takagi and Jun Shima

Abstract During the fermentation of dough and the production of baker's yeast, cells of baker's yeast are exposed to numerous and multiple environmental stresses including freeze–thaw, high-sucrose, and air-drying, the so-called baking-associated stresses. In addition, such stress conditions could induce oxidative stress in yeast cells with an increase in reactive oxygen species level because of the denaturation of proteins including antioxidant enzymes and the severe damage to the mitochondrial membrane or respiratory chain. To avoid lethal damage, baker's yeast cells need to acquire a variety of stress-tolerant mechanisms, such as the induction of stress proteins, accumulation of stress protectants or compatible solutes, change of membrane composition, and repression of translation, by regulating the corresponding gene expression via stress-triggered signal transduction pathways. For example, proline and trehalose are important compounds involved in the stress tolerance of baker's yeast. In fact, the engineering of proline and trehalose metabolism is a promising approach for the development of stress-tolerant baker's yeast. Moreover, the multiomics approach such as comprehensive phenomics and functional genomics is promising for the identification of novel genes required for the stress tolerance. To further improve the fermentation ability or the production efficiency of yeasts, however, the detailed mechanisms underlying the stress response, adaptation, and tolerance of yeast cells should be understood. We believe that not only baker's yeast, but also other important industrial yeasts with higher tolerance to various stresses, could contribute to the yeast-based industry for the effective production of bread doughs and alcoholic beverages or a breakthrough in bioethanol production.

Keywords Air-drying stress • Baker's yeast • Baking-associated stress • Bread making • Fermentation • Freeze–thaw stress • High-sucrose stress • Nitric oxide • Omics • Oxidative stress • Proline • *Saccharomyces cerevisiae* • Stress protectant • Stress tolerance • Trehalose

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2.1 Introduction

Baker's yeast (mostly strains of *Saccharomyces cerevisiae*) is an essential ingredient in bakery products produced by fermentation (Linko et al. 1997; Randez-Gil et al. 1999). Around the world, about 2 million tons of baker's yeast are produced per year based on 30 % dry weight (Attfield 1997; Evans 1990). The function of baker's yeast in bread making can be summarized as follows: (1) to increase dough volume by gas generation during fermentation, (2) to develop structure and texture in the dough, and (3) to add a distinctive flavor to the dough (Burrows 1970). Baker's yeast is produced in the form of cream yeast (aqueous suspension containing approximately 20 % of dry weight cells), compressed yeast, or dried yeast. The compressed yeast is manufactured by partial dehydration and contains approximately 30 % of dry weight cells. In Japan, most baker's yeasts are produced as cream or compressed yeasts. However, dried yeast, which contains less than 5 % water, is imported from other countries and used in home baking and bakery shops for reasons of the convenience of its storage and delivery in Japan.

Flavor is an important factor in the quality of bread. Consumers prefer bread with a delicious characteristic flavor. Bread flavor is influenced by various compounds, such as alcohols, diacetyl, esters, organic acids, and carbonyl compounds, produced during fermentation and baking stages in bread making (Pence and Kohler 1961; Wick et al. 1964). Isobutyl alcohol (i-BuOH) and isoamyl alcohol (i-AmOH) would affect the flavor of bakery products and could make a new type of bread. In Japan, mutants producing a large amount of i-BuOH from 4-aza-DL-leucine-resistant mutants derived from baker's yeasts confer a favorable flavor on bread, although using more i-AmOH was evaluated to be unfavorable (Watanabe et al. 1990). The mutants overproducing i-BuOH or i-AmOH were released from inhibition of the key enzymes, acetohydroxy acid synthase and α-isopropylmalate synthase, respectively, in the pathway of branched-chain amino acids synthesis.

Other targets for breeding of baker's yeast are the utilization of melibiose and maltose, which is the disaccharide converted from raffinose in molasses used for the production of baker's yeast and one of the free sugars in flour, respectively (Dequin 2001). Baker's yeast cannot utilize melibiose because of the lack of α-galactosidase (melibiase) responsible for the hydrolysis of melibiose into the fermentable sugars galactose and glucose. When the *MEL1* gene encoding α-galactosidase found in bottom-fermenting brewing yeast was expressed in baker's yeast, all available melibiose was utilized in a beet molasses medium, resulting in higher yeast yields (Liljeström et al. 1991; Liljeström-Suominen et al. 1988). Fermentation continues as a result of the action of amylases present in the dough, which release maltose from starch. Maltose utilization requires maltose permease and maltase, both of which are repressed by glucose (Needleman 1991), causing a lag phase in carbon dioxide production. To avoid this, maltose-utilizing enzymes have been derepressed by replacing the native promoters of the maltase and maltose permease with constitutive promoters (Osinga et al. 1988).

During the bread-making process, baker's yeast cells are exposed to a variety of environmental stresses, such as freeze–thaw, high-sucrose concentrations, and

air-drying. These treatments induce oxidative stress-generating reactive oxygen species (ROS) because of the mitochondrial damage. This chapter focuses on the mechanisms of cellular response, adaptation, and tolerance to baking-associated environmental stresses and the construction of stress-tolerant baker's yeast strains for commercial use.

2.2 Baking-Associated Stresses

The demands of modern society require bread-making technology to make further advances despite its long history. One of the important technologies in the baking industry is developing baker's yeast strains with high fermentation ability and durability in response to various baking methods. For example, dried yeast is widely used because of its longer storage time and lower transport costs than compressed yeast. Sweet dough (high-sugar dough) contains up to 40 % sucrose per weight of flour. Frozen-dough technology has been developed to supply oven-fresh bakery products to consumers. During the fermentation of dough and the production of baker's yeast, yeast cells are exposed to numerous environmental stresses including freeze–thaw, high-sucrose, and air-drying (baking-associated stresses) (Attfield 1997; Shima and Takagi 2009) (Fig. 2.1). In addition, yeast cells encounter such stresses in a multiple and sequential manner (for example, freeze–thaw plus

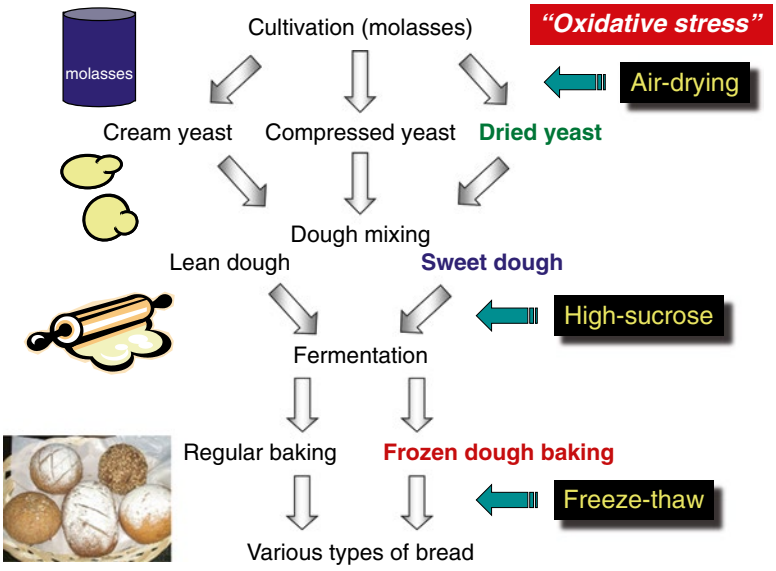

Fig. 2.1 Schematic view of the processes for baker's yeast production and bread making. During these processes, baker's yeast cells are exposed to baking-associated stresses, such as air-drying, high-sucrose, and freeze–thaw. These treatments also induce oxidative stress in yeast cells

high-sucrose) (Attfield 1997). It is believed that by undergoing freeze–thaw, high-sucrose, and air-drying treatments yeast cells are exposed to oxidative stress (Ando et al. 2007; Attfield 1997; Landolfo et al. 2008; Sasano et al. 2010, 2012a; Shima et al. 2008). These treatments induce the generation of ROS by the denaturation of proteins including antioxidant enzymes and severe damage to the mitochondrial membrane or respiratory chain.

In general, microorganisms have some degree of adaptation ability to environmental stress. Yeast cells also need to acquire a variety of stress-adaptation mechanisms, such as induction of stress proteins, accumulation of stress protectants or compatible solutes, change of membrane composition, and repression of translation, by regulating the corresponding gene expression via stress-triggered signal transduction pathways. Under severe stress conditions that induce protein denaturation and ROS generation, leading to growth inhibition or cell death, the fermentation ability of yeast is rather limited. In terms of industrial applications, stress tolerance is the key characteristic for yeast cells. To develop the commercial fermentation and production process of baker's yeast, it is necessary to construct yeast strains with higher tolerance to various stresses.

2.2.1 Freeze–Thaw Stress

Frozen-dough baking not only improves labor conditions by saving working hours in the bakery industry but also allows for the supply of oven-fresh bakery products to consumers. However, freezing and the subsequent thawing treatments cause severe damage to various cellular components, and this damage leads to cell death and low fermentation ability. The processes of freeze–thaw also induce oxidative stress to cells (Park et al. 1997). In particular, free radicals and ROS are generated and cause oxidative damage to cellular components (Park et al. 1998). For this reason, baker's yeast strains that are tolerant of freeze–thaw stress are highly desirable. Freeze–thaw-tolerant yeasts have been isolated from natural sources (Hahn and Kawai 1990; Hino et al. 1987) and culture collections (Oda et al. 1986) and have also been constructed by conventional mutation or hybrids (Nakagawa and Ouchi 1994a). Freeze–thaw damage to cells could be reduced by heat treatment of the fermented doughs before freezing (Nakagawa and Ouchi 1994b).

Saccharomyces yeast available for frozen dough accumulates trehalose in cells compared with non-freezing-tolerant yeasts (Hino et al. 1990; Oda et al. 1986). Yeast cells induce trehalose synthesis under various stress conditions (Kaino and Takagi 2008; Van Dijck et al. 1995) and accumulated trehalose functions as a stress protectant (Hino et al. 1990; Oda et al. 1986). Intracellular levels of trehalose are controlled by metabolic balance between its synthesis and degradation. The neutral trehalase Nth1 is one of the degradation enzymes. The disruption of the *NTH1* gene increased the intracellular trehalose level and conferred freeze–thaw tolerance on baker's yeast (Shima et al. 1999).

In response to osmotic stress, proline is accumulated in many plant and bacterial cells as an osmoprotectant (compatible solute) (Csonka 1981; Verbruggen and Hermans 2008). Under various stress conditions, yeast cells induce glycerol or trehalose synthesis, but do not increase the intracellular proline level (Kaino and Takagi 2008). Extracellular proline also has cryoprotective activity nearly equal to that of glycerol or trehalose in yeast (Takagi et al. 1997). Proline has many functions in vitro, such as protein and membrane stabilization during freezing and dehydration, lowering the T_m of DNA during salinity stress, and hydroxy radical scavenging under oxidative stress (Takagi 2008). Also, elevated proline in plants has been shown to reduce the levels of free radicals in response to osmotic stress (Hong et al. 2000). Probably because of the extremely high water solubility, proline is suggested to inhibit ice crystal formation and dehydration by forming strong hydrogen bonds with intracellular free water. However, the mechanisms of these functions in vivo are poorly understood. Previously, Takagi's laboratory constructed *S. cerevisiae* strains that accumulate proline, and the engineered strains successfully showed enhanced tolerance to many stresses, including freeze–thaw, desiccation, hydrogen peroxide, and ethanol (Matsuura and Takagi 2005; Morita et al. 2002, 2003; Takagi et al. 1997, 2000a, 2005; Terao et al. 2003). In *S. cerevisiae*, γ-glutamyl kinase (the *PRO1* gene product) is the first enzyme of the proline synthetic pathway from glutamate and proline oxidase (the *PUT1* gene product) catalyzes the first step of proline degradation pathway (Fig. 2.2). The activity of Pro1 is subjected to feedback inhibition by proline, indicating that Pro1 is the rate-limiting enzyme that controls

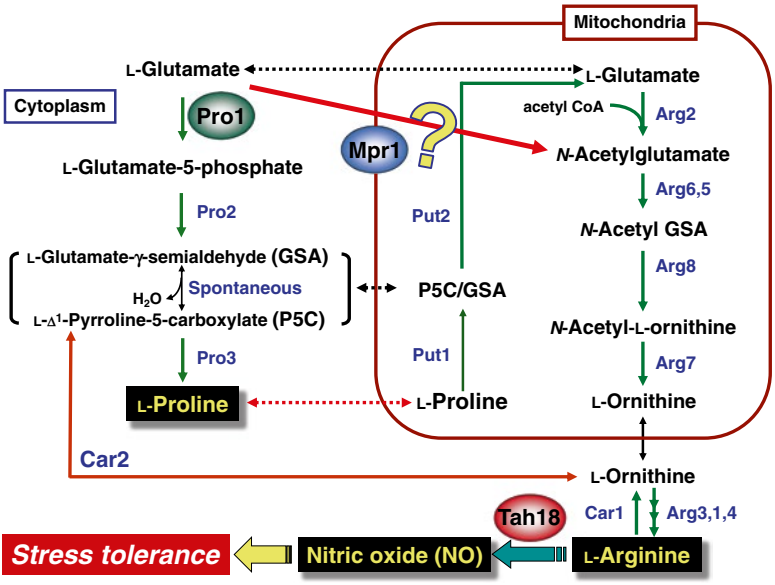

Fig. 2.2 Metabolic pathways of proline and arginine in yeast cells. Normally, both amino acids are synthesized from glutamic acid. In response to oxidative stress, nitric oxide (NO) is produced from the increased arginine through the Mpr1- and Tah18-dependent pathway

intracellular proline level (Sekine et al. 2007). Interestingly, the Asp154Asn and Ile150Thr Pro1 variants were less sensitive to feedback inhibition leading to proline oversynthesis, and yeast cells expressing these Pro1 variants accumulated proline and showed a higher tolerance to freeze–thaw stress (Sekine et al. 2007).

With respect to industrial yeast, Japanese sake yeast accumulated proline by disrupting the *PUT1* gene and carrying a mutant allele of *PRO1*^{D154N}, and it was more tolerant to ethanol stress than was the control strain (Takagi et al. 2005). Furthermore, the fermentation profiles of diploid sake yeast strains that accumulate proline were analyzed during sake brewing (Takagi et al. 2007). For the application of recombinant baker's yeasts for commercial use, self-cloning (SC) yeast, which does not contain any foreign genes or DNA sequences except for yeast DNA, might be more acceptable for consumers than genetically modified (GM) yeasts. Recently, Kaino et al. (2008) constructed SC diploid baker's yeast strains by disrupting the *PUT1* gene and replacing the wild-type *PRO1* gene with the *PRO1*^{D154N} or *PRO1*^{I150T} allele. In commercial frozen-dough processes, prefermentation before freezing is desirable in terms of texture and taste (Teunissen et al. 2002). Yeast cells activated during prefermentation produce the metabolites, such as alcohols and organic acids, that influence the taste and flavor of the bread. The reason for the loss of the gassing power remains unclear; however, it is possible that prolonged prefermentation causes serious damage to the membranes of yeast cells in the dough (Kline and Sugihara 1968). Therefore, the dough was prefermented for 120 min at 30 °C before freezing and was kept frozen for 9 days. The remaining gassing power of wild-type cells was dramatically decreased to 40 % of that before freezing (Fig. 2.3a). It is noteworthy that proline-accumulating cells showed approximately 50 % greater fermentation ability than did wild-type cells (Fig. 2.3a). These results indicate that proline-accumulating baker's yeast has a higher tolerance to freeze–thaw stress and is suitable for frozen-dough baking.

To enhance the freeze–thaw stress tolerance of yeast cells, a diploid baker's yeast strain that simultaneously accumulates proline and trehalose was constructed (Sasano et al. 2012c). It showed greater tolerance to freeze–thaw stress and higher fermentation ability in frozen dough than the single accumulating strains showed separately. It is possible to produce breads with greater swelling after freezing, to reduce the freezing period, and to cut the manufacturing cost using the diploid baker's yeast strain. The simultaneous accumulation of proline and trehalose could be promising for breeding novel yeast strains that are useful for frozen-dough baking.

The transcriptional activator Msn2 induces approximately 180 genes in response to oxidative stress, heat shock, and high concentrations of ethanol (Causton et al. 2001; Estruch 2000). When cells are exposed to such stresses, Msn2, which usually forms a heterodimer with Msn4, is imported into the nucleus and binds to the stress responsive element (STRE), found in the promoter region, and finally activates the transcription of many stress protein genes (Marchler et al. 1993; Martinez-Pastor et al. 1996). Various cellular functions are dependent on genes regulated by Msn2, including the oxidative stress response (*CTT1*, *SOD2*), molecular chaperoning (*HSP12*, *HSP104*), and trehalose synthesis (*TPS1*, *TPS2*) (Boy-Marcotte et al. 1998). Yeast strains that overexpress Msn2 have shown tolerance to oxidative stress,

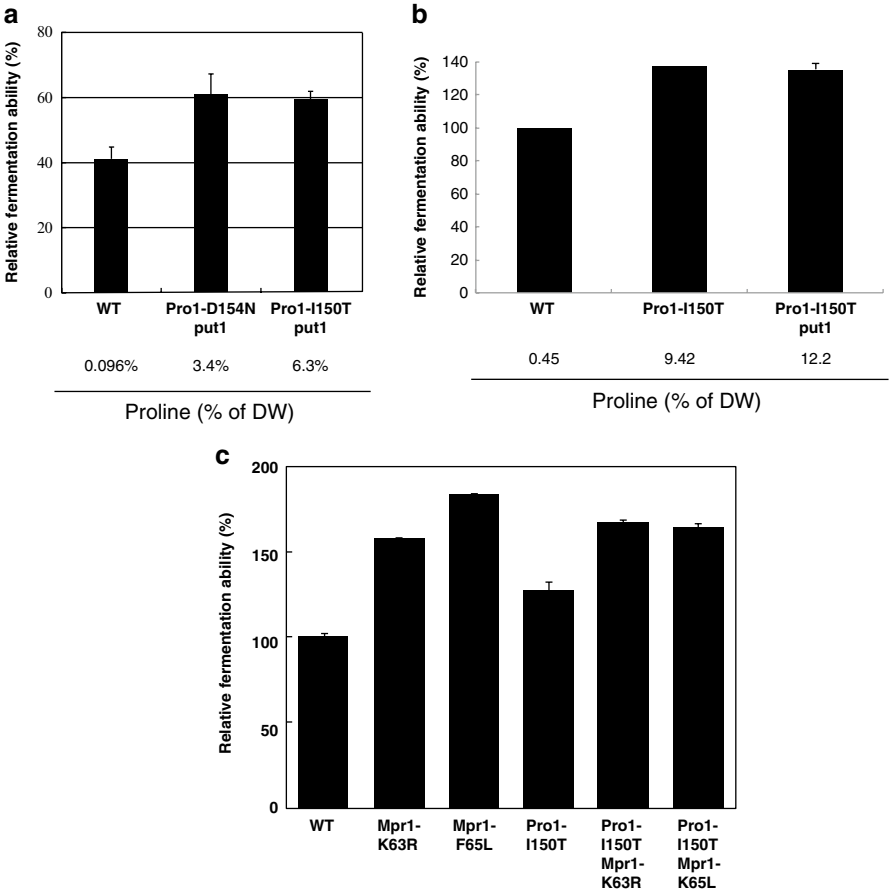

Fig. 2.3 Fermentation abilities of baker's yeast strains under baking-associated stresses. **a** Doughs were prefermented for 120 min at 30 °C and then frozen for 9 days at −20 °C. The frozen dough was thawed for 30 min at 30 °C, and the remaining CO_2 gas production was measured. The gassing power before freezing was defined as 100 % (Kaino et al. 2008). **b** Fermentation ability in sweet dough (30 % sucrose per weight of flour) was monitored by CO_2 gas production. Total amounts of CO_2 production after 2 h were measured. The gassing power of wild-type strain (WT) was defined as 100 % (Sasano et al. 2012d). **c** Compressed yeast was treated with air-drying stress for 4 h at 37 °C. Dough containing the stress-treated yeasts was fermented for 3 h, and the remaining CO_2 gas production was measured. The amount of CO_2 production of WT after air-drying stress treatment was defined as 100 % (Sasano et al. 2010)

mainly from the high-level transcription of antioxidant genes (Cardona et al. 2007; Sasano et al. 2012e; Watanabe et al. 2009; Zuzuarregui and del Olmo 2004). Recently, Sasano et al. (2012b) constructed a SC diploid baker's yeast strain that overexpressed Msn2. It showed higher tolerance to freeze–thaw stress and higher intracellular trehalose level than observed in the wild-type strain. The Msn2-overexpressing strain showed an approximately 2.5-fold increase in fermentation

ability in the frozen dough as compared with the wild-type strain. Hence, Msn2-overexpressing baker's yeast could be useful in frozen-dough baking.

The *POG1* gene, encoding a transcription factor involved in cell-cycle regulation (Leza and Elion 1999), is a multicopy suppressor of *S. cerevisiae* E3 ubiquitin ligase Rsp5 mutant (Demae et al. 2007). The *pog1* mutant is sensitive to various stresses, suggesting that the *POG1* gene is involved in stress tolerance in yeast cells. Interestingly, deletion of the *POG1* gene drastically (55–70 %) increased the fermentation ability in bread dough after freeze–thaw stress, whereas overexpression of the *POG1* gene conferred increased fermentation ability in high-sucrose-containing dough (Sasano et al. 2013). Thus, the engineering of yeast strains to control the *POG1* gene expression level would be a novel method for molecular breeding of baker's yeast.

2.2.2 High-Sucrose Stress

Baler's yeast must adapt to different sucrose concentrations during dough-fermentation processes (Tanaka et al. 2006). Dough can be classified into lean or sweet dough based on the sugar concentrations contained in the dough. Lean dough contains no sugar (non-sugar dough) or small amounts of sugar (less than 5 % per weight of flour). In general, sweet dough (high-sugar dough) contains up to approximately 40 % sucrose per weight of flour. Such high-sucrose concentrations exert severe osmotic stress that seriously damages cellular components (Verstrepen et al. 2004) and inhibits the optimal fermentation ability of yeast. To avoid lethal injury, baker's yeast cells need to acquire osmotolerance, but the development of osmotolerant baker's yeast requires knowledge of the mechanism involved in high-sucrose stress tolerance, for example, by the induction of stress proteins, the accumulation of stress protectants or compatible solutes, and the changes in membrane composition (Shima and Takagi 2009).

When high osmotic pressure is sensed, *S. cerevisiae* cells accumulate glycerol and trehalose (Cronwright et al. 2002; De Virgilio et al. 1994; Hino et al. 1990; Hirasawa et al. 2006; Shima et al. 1999). Microarray analysis and genome-wide screening using a deletion strain collection revealed that the metabolism of glycerol and trehalose, both of which are known as osmoprotectants, is important for high-sucrose stress tolerance (Ando et al. 2006; Tanaka-Tsuno et al. 2007). In response to osmotic stress, proline is accumulated in many bacterial and bacterial cells as an osmoprotectant (Csonka 1981; Verbruggen and Hermans 2008). With regard to high osmotic pressure, the proline oxidase-deficient strain, which had a significantly higher proline level, was clearly more osmotolerant than were other strains in the presence of 1 M NaCl (Takagi et al. 1997). Proline-accumulating baker's yeast was found to retain higher-level fermentation ability in the frozen dough than that of the wild-type strain (Kaino et al. 2008).

Based on these results, it is possible that proline confers tolerance to high-sucrose stress on baker's yeast. Sasano et al. (2012d) constructed SC diploid baker's yeast

strains that accumulate proline. To examine the effect of proline accumulation on high-sucrose stress tolerance, cell viability was measured after inoculation into the high-sucrose-containing liquid fermentation medium. The proline-accumulating strains showed higher cell viability than that of wild-type cells, suggesting that proline accumulation confers tolerance to high-sucrose stress on yeast cells.

Landolfo et al. (2008) reported that ROS accumulation caused oxidative damage to wine yeast cells during fermentation of high-sugar-containing medium, probably because of the denaturation of antioxidant proteins or the dysfunction of mitochondrial membranes. When baker's yeast cells were inoculated into the high-sucrose-containing liquid fermentation medium, the ROS level increased approximately twofold in all the strains tested under the high-sucrose stress condition, indicating that, as in the case of wine yeast, ROS accumulation occurs after exposure to high sugar concentrations in baker's yeast. It appears that proline accumulation confers tolerance to high-sucrose stress on yeast cells by reducing the ROS level. It was also found that the specific activity of superoxide dismutase was significantly higher in cells that accumulate proline than that of wild-type cells (approximately 1.7-fold). Intracellular proline is suggested to protect antioxidant enzymes from high osmotic pressure.

Next, the high-sucrose tolerance of proline-accumulating strains was assayed in sweet dough containing 30 % sucrose per weight of flour. Stationary-phase cells cultivated in cane molasses medium for 48 h were used for sweet dough fermentation. Interestingly, proline-accumulating strains showed an approximately 40 % increase in gassing power compared with wild-type strain, indicating that proline accumulation enhanced the leavening ability in sweet dough (Fig. 2.3b). It was also revealed that an appropriate proline level (approximately 9 %) in yeast cells is important for its stress-protective effect (Fig. 2.3b). These data clearly demonstrate that the proline-accumulating baker's yeast strains are suitable for sweet bread making. It is possible to produce bread with greater swelling to reduce the fermentation period and to cut the manufacturing cost.

2.2.3 Air-Drying Stress

Dried yeast is exposed to air-drying stress during the preparation process. Air-drying stress exerts many harmful influences such as accumulation of misfolded proteins, mitochondrial malfunction, and vacuolar acidification (Nakamura et al. 2008; Shima et al. 2008), leading to decreased fermentation ability. Thus, air-drying stress tolerance is a necessary characteristic of baker's yeast used in dried yeast preparation. During the drying process, the flow of hot air increases the temperature of yeast cells to around 37 °C. Therefore, air-drying stress is considered to be a combination of two stresses, high temperature and dehydration. Both stresses are reported to accumulate intracellular ROS (Franca et al. 2007; Nomura and Takagi 2004). During normal respiratory metabolism in all aerobic organisms including yeast, several ROS, which are produced as by-products, would be scavenged by a

variety of antioxidant enzymes. However, the transient heat shock and loss of water might promote dysfunctions in the enzymes capable of detoxifying ROS. As a result, the increased ROS levels damage cellular components, leading to low fermentation ability or cell death.

The Mpr1 protein was identified as a novel *N*-acetyltransferase that detoxifies the proline analogue L-azetidine-2-carboxylate (AZC) in the *S. cerevisiae* Σ1278b strain (Shichiri et al. 2001; Takagi et al. 2000b; Nasuno et al. 2013). The Σ1278b background strain has two isogenes of the *MPR* gene, *MPR1* on chromosome XIV and *MPR2* on chromosome X (sigma 1278b gene for proline-analogue resistance). These gene products (Mpr1 and Mpr2) have similar roles in AZC resistance (Takagi et al. 2000b). Mpr1 decreases the intracellular ROS levels when yeast cells are exposed to oxidative stresses such as heat-shock, H_2O_2, freeze–thaw, or ethanol treatment (Du and Takagi 2005, 2007; Nomura and Takagi 2004). Recently, two Mpr1 variants with improved enzymatic functions (Lys63Arg and Phe65Leu) were isolated (Iinoya et al. 2009). Overexpression of the K63R variant decreased intracellular ROS levels and increased cell viability under oxidative stress conditions compared with the wild-type Mpr1. In addition, the F65L mutation greatly enhanced the thermal stability.

Interestingly, among industrial yeast strains, Japanese baker's yeast strains possess one copy of the *MPR2* gene on chromosome X (Sasano et al. 2010). To examine the role of *MPR2* in baker's yeast, the cell viability and intracellular ROS level of diploid industrial baker's yeast strains was tested after exposure to air-drying stress (Sasano et al. 2010). Wild-type cells showed a significant increase in ROS level after exposure to air-drying stress. The *Δmpr2* strain was more sensitive to air-drying stress than the wild-type strain. Interestingly, the ROS levels in *Δmpr2* were approximately 40 % higher than those observed in wild-type cells, indicating that the *MPR2* gene protects baker's yeast from air-drying stress by reducing the intracellular ROS levels.

The fermentation ability of *Δmpr2* to air-drying stress was assayed in dough. There were no significant differences in gassing power between the wild-type and *Δmpr2* strains before the air-drying stress treatment. However, the fermentation ability of *Δmpr2* cells treated with air-drying stress fell to approximately 60 % of wild-type cells, indicating that the *MPR2* gene in baker's yeast is involved in the fermentation performance in dough after exposure to air-drying stress. Interestingly, the K63R and F65L Mpr1 variants exhibited increased fermentation ability compared with wild-type Mpr1 after air-drying stress (Fig. 2.3c). In particular, an approximately 1.8-fold increase was observed in F65L compared with the gassing power of wild-type Mpr1, probably from enhanced thermal stability of the F65L variant (Fig. 2.3c).

The effect of proline accumulation on air-drying stress tolerance was also examined: proline accumulation significantly enhanced fermentation ability after air-drying stress in baker's yeast (Fig. 2.3c). Furthermore, the Mpr1 variant-expressing cells showed an approximately 40 % increase in fermentation ability after air-drying treatment as compared with cells expressing the wild-type Mpr1 (Fig. 2.3c). Hence, the antioxidant enzyme Mpr1/2 could be promising for breeding novel yeast strains with higher tolerance to air-drying stress.

2.3 Novel Approach and Mechanism for Baking-Associated Stress Tolerance

2.3.1 Omics Approach to Identify the Genes Required for Stress Tolerance

To determine the uncharacterized genes required for stress tolerance, both comprehensive phenomics analysis and functional genomics analysis were carried out under various stress conditions simulating the commercial baking process. These analyses indicate that many genes are involved in the stress tolerance of baker's yeast.

To clarify the genes required for freeze–thaw tolerance, genome-wide screening was performed using the complete deletion strain collection of diploid *S. cerevisiae* (Ando et al. 2007). The screening identified 58 gene deletions that conferred freeze–thaw sensitivity. These genes were then classified based on their cellular function and on the localization of their products. The results showed that the genes required for freeze–thaw tolerance were frequently involved in vacuole functions and cell wall biogenesis. The highest numbers of gene products were components of vacuolar H^+-ATPase. Next, the cross-sensitivity of the freeze–thaw-sensitive mutants to oxidative stress and to cell wall stress was studied: both are environmental stresses closely related to freeze–thaw stress. Ando et al. (2007) showed that defects in the functions of vacuolar H^+-ATPase conferred sensitivity to oxidative stress and to cell wall stress. In contrast, defects in gene products involved in cell wall assembly conferred sensitivity to cell wall stress but not oxidative stress. These results suggest the presence of at least two different mechanisms of freezing injury: oxidative stress generated during the freeze–thaw process and defects in cell wall assembly.

In the modern baking industry, high-sucrose-tolerant (HS) and maltose-utilizing (LS) yeast were developed using breeding techniques and are now used commercially. Sugar utilization and high-sucrose tolerance differ significantly between HS and LS yeasts. Tanaka-Tsuno et al. (2007) analyzed the gene expression profiles of HS and LS yeasts under different sucrose conditions. Two-way hierarchical clustering was performed to obtain the overall patterns of gene expression. The clustering clearly showed that the gene expression patterns of LS yeast differed from those of HS yeast. Quality threshold clustering was used to identify the gene clusters containing upregulated genes (cluster 1) and downregulated genes (cluster 2) under high-sucrose conditions. Clusters 1 and 2 contained numerous genes involved in carbon and nitrogen metabolism, respectively. The expression level of the genes involved in the metabolism of glycerol and trehalose, which are known to be osmoprotectants, was higher in LS yeast than that in HS yeast under sucrose concentrations of 5–40 %. No clear correlation was found between the expression level of the genes involved in the biosynthesis of the osmoprotectants and the intracellular contents of the osmoprotectants.

Nakamura et al. (2008) analyzed changes in the gene expression of baker's yeast during an air-drying process that simulated dried yeast production. The intracellular

accumulation of trehalose was reported to contribute to dehydration tolerance (Gadd et al. 1987). However, the genes related to trehalose synthesis were not increased during the air-drying process, which could account for the high concentration of intracellular trehalose in the compressed yeast. On the other hand, the genes involved in the molecular chaperone, proteasome, and autophagy were transiently upregulated at an early stage, suggesting that the folding of cellular proteins and removal of denatured proteins may play important roles in the adaptation of cells to air-drying stress. It was found that the genes involved in β-oxidation were constantly upregulated during the air-drying process. It can be speculated that yeast cells may acquire the energy needed to adapt to air-drying conditions by degrading intracellular fatty acids because yeast cells were not able to uptake environmental nutrients during the air-drying process. These expression data may accelerate improvements in dried yeast production and in the breeding of a strain that has higher tolerance to air-drying stress.

From the foregoing analyses, vacuolar H^+-ATPase was suggested to have an important role in yeast cells under baking-associated stress conditions, particularly air-drying stress (Shima et al. 2008). Gene expression and phenotypic analyses suggest that copper ion homeostasis is required for freeze–thaw stress tolerance of yeast cells (Takahashi et al. 2009). Moreover, the ER membrane protein Eos1, which was originally identified as the gene product required for tolerance to oxidative stress, could be involved in zinc homeostasis (Nakamura et al. 2007, 2010).

With a view to a more comprehensive understanding of oxidative stress tolerance, the essential genes required for oxidative stress tolerance using a heterozygous deletion mutant collection were screened (Okada et al. 2014). The classification of the identified essential genes by function and localization suggested that nuclear functions, such as RNA synthesis, were implied in a critical role for the oxidative stress response.

2.3.2 Nitric Oxide-Mediated Stress-Tolerant Mechanism Found in Yeast

Nitric oxide (NO) is a ubiquitous signaling molecule involved in the regulation of a large number of cellular functions. NO confers oxidative stress tolerance by enhancing cellular antioxidative activity in mammals. NO plays an important role in mammalian cells, mainly through the cGMP-mediated signaling pathways by activating soluble guanylate cyclase and through posttranslational activation of proteins via *S*-nitrosylation. In the unicellular eukaryote yeast, low levels of NO may be involved in various stress response pathways including responses to high hydrostatic pressure, copper metabolism, and H_2O_2-induced apoptosis (Almeida et al. 2007; Domitrovic et al. 2003; Shinyashiki et al. 2000). However, the synthetic mechanism and the physiological role of NO in *S. cerevisiae* remain unclear because of the lack of mammalian NO synthase (NOS) orthologues in the genome. Recently, Nishimura et al. (2010, 2013) revealed a novel antioxidative mechanism in a laboratory yeast

strain that is involved in stress-induced NO synthesis-mediated proline and arginine metabolism via Mpr1 (Fig. 2.2).

Previously, the expression of the proline-feedback inhibition-less sensitive variant Pro1-I150T and the thermostable variant Mpr1-F65L resulted in an enhanced fermentation ability of baker's yeast after freeze–thaw stress and air-drying stress, respectively (Kaino et al. 2008; Sasano et al. 2010). Sasano et al. (2012a) constructed a SC diploid baker's yeast strain with enhanced proline and NO synthesis by coexpressing Pro1-I150T and Mpr1-F65L. The engineered strain increased the intracellular NO level in response to air-drying stress, and the strain was tolerant not only to oxidative stress but also to both air-drying and freeze–thaw stresses because of the reduced intracellular ROS level (Fig. 2.4a). The resultant strain was shown to retain higher leavening activity in bread dough after air-drying and freeze–thaw stress than that of the wild-type strain (Fig. 2.4b). This result suggests that NO is

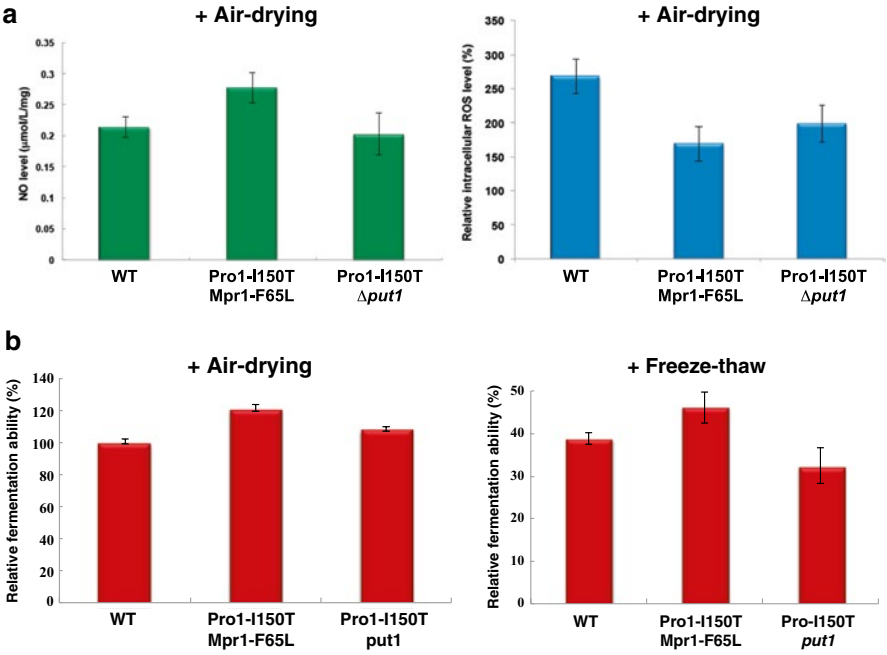

Fig. 2.4 Intracellular NO and reactive oxygen species (ROS) levels and fermentation abilities of baker's yeast strains under baking-associated stresses (Sasano et al. 2012a). **a** Intracellular NO (*left*) and ROS (*right*) levels after air-drying stress (at 42 °C for 90 min). For ROS level, the fluorescence intensity before stress treatment of each strain was defined as 100 %. **b** After exposure to air-drying stress, baker's yeast strains were mixed with dough and fermented. The remaining CO_2 gas production after 2 h was measured. The gassing power of the wild-type strain (WT) is relatively taken as 100 % (*left*). Doughs were prefermented for 2 h at 30 °C and then frozen at −20 °C for 3 weeks. The frozen dough was thawed for 30 min at 30 °C, and remaining CO_2 gas production after 2 h was measured. The gassing power before freezing of each strain was defined as 100 % (*right*)

synthesized in baker's yeast in response to oxidative stresses that induce ROS generation and that increased NO plays an important role in baking-associated stress tolerance. The enhancement of proline and NO synthesis could be promising for breeding novel baker's yeast strains.

Nishimura et al. (2013) showed a novel antioxidative mechanism mediated by NO in yeast cells, but the mechanism underlying the oxidative stress tolerance remained unclear. Takagi's group recently found one of the downstream pathways of NO involved in the stress-tolerance mechanism in yeast (Nasuno et al., unpublished data). Microarray and real-time quantitative PCR analyses revealed that exogenous NO treatment induced the expression of genes responsible for copper metabolism under the control of the transcription factor Mac1, including the *CTR1* gene encoding high-affinity copper transporter. NO was found to produce under high-temperature stress conditions that increased the transcription level of the *CTR1* gene, intracellular copper content, the activity of Cu,Zn-superoxide dismutase Sod1, and cell viability after exposure to high temperature in a manner dependent on Mac1. NO did not affect the expression of the *MAC1* gene, indicating that NO activates Mac1 through its posttranslational modification. Based on these results, a novel NO-mediated antioxidative mechanism through the activation of Mac1 has been proposed.

2.4 Conclusions and Future Perspective

As described in this chapter, baker's yeasts are exposed to multiple environmental stresses including freeze–thaw, high-sucrose, and air-drying stress. Yeast cells possess a wide variety of strategies in response to these environmental changes. If stress levels are higher than those to which yeast cells can adapt, however, the fermentation abilities of the yeast are greatly restricted. In terms of applied aspects, stress "tolerance" is the key characteristic for yeast cells. For example, proline and trehalose are important compounds involved in the stress tolerance of baker's yeast. In fact, the engineering of proline and trehalose metabolism is a promising approach for the development of stress-tolerant baker's yeast. To further improve the fermentation ability or the production efficiency of yeasts, however, the detailed mechanisms underlying the stress response, adaptation, and tolerance of yeast should be understood at the molecular, metabolic, and cellular levels. We believe that not only baker's yeast for making breads but also other important industrial yeasts for brewing alcoholic beverages (beer, wine, sake, *shochu*, etc.) and producing bioethanol with higher tolerance to various stresses could contribute to the yeast-based industry for the effective production of bread doughs and alcoholic beverages or the breakthrough of bioethanol production.

Apart from science and technology, problems that should be resolved still remain for practical use of engineered baker's yeasts. First, it is relatively difficult to breed an industrial baker's yeast strain with a higher stress tolerance than a laboratory strain. The process that involves adding stress protectant or antioxidant externally to

the cell or to the dough remains somewhat laborious for practical application. However, baker's yeast strains that express the Pro1 variant to accumulate proline or express the Mpr1 variant with improved antioxidant activity might overcome this problem.

Second, according to Japanese government guidelines, SC yeast does not need to be treated as a GM yeast, because SC processes are considered to be the same as naturally occurring gene conversions, such as recombination, deletion, and transposition (Hino 2002). However, the baking industry and consumers have not yet accepted such a technology. Instead, we propose an acceptable method for consumers to construct baker's yeasts that have the same stress-tolerant mechanism (Fig. 2.5). The proline toxic analogue AZC is transported into the cells via proline permease. It causes misfolding of the proteins into which it is incorporated competitively with proline and thereby inhibits cell growth. However, by introducing a favorable mutation in the *PRO1* or *MPR1* gene, the cells that accumulate large quantities of proline or acetylate AZC efficiently are tolerant to AZC as a consequence of the incorporation of proline into the proteins. After treatment with the conventional mutagen, we will be able to obtain desirable strains derived from AZC-resistant mutants.

Finally, we propose new sophisticated strategies for constructing baker's yeast strains with desired properties by both rational and reverse engineering, which are based on "omics" sciences and technologies (Fig. 2.6).

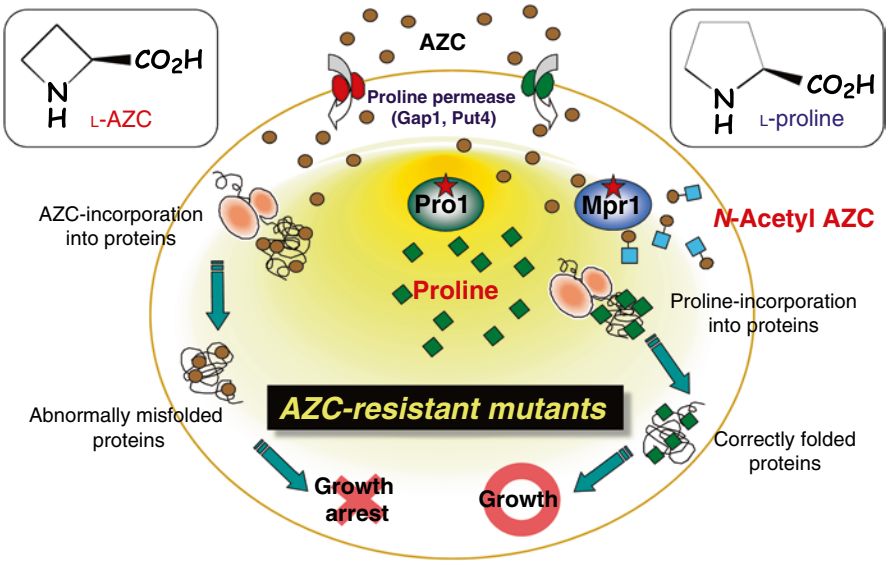

Fig. 2.5 Construction of commercial baker's yeast strains. By introducing a favorable mutation in the *PRO1* or *MPR1* gene, yeast cells that accumulate large quantities of proline or acetylate L-AZETIDINE-2-carboxylate (*AZC*) efficiently are tolerant to AZC, respectively

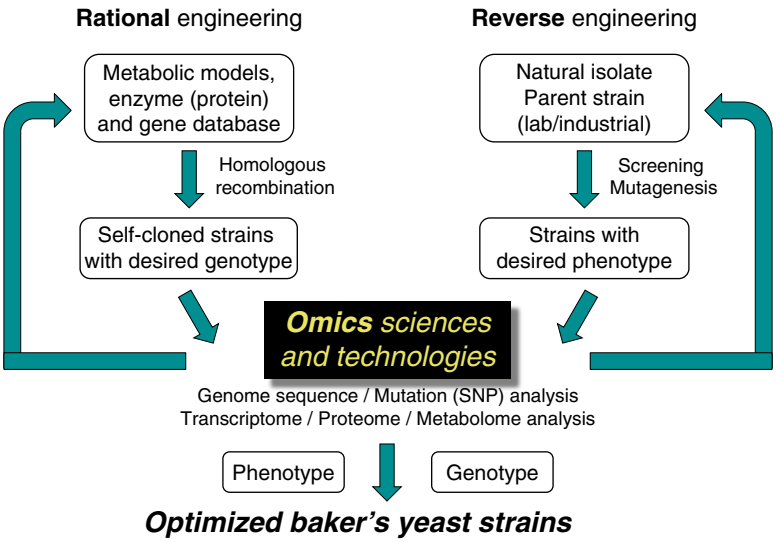

Fig. 2.6 Schematic view of omics-based baker's yeast breeding. *SNP* single-nucleotide polymorphism

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References

Almeida B, Buttner S, Ohlmeier S, Silva A, Mesquita A, Sampaio-Marques B, Osorio NS, Kollau A, Mayer B, Leao C, Laranjinha J, Rodrigues F, Madeo F, Ludovico P (2007) NO-mediated apoptosis in yeast. J Cell Sci 120:3279–3288

Ando A, Tanaka F, Murata Y, Takagi H, Shima J (2006) Identification and classification of genes required for tolerance to high-sucrose stress revealed by genome-wide screening of *Saccharomyces cerevisiae*. FEMS Yeast Res 6:249–267

Ando A, Nakamura T, Murata Y, Takagi H, Shima J (2007) Identification and classification of genes required for tolerance to freeze-thaw stress revealed by genome-wide screening of *Saccharomyces cerevisiae* deletion strains. FEMS Yeast Res 7:244–253

Attfield PV (1997) Stress tolerance: the key to effective strains of industrial baker's yeast. Nat Biotechnol 15:1351–1357

Boy-Marcotte E, Perrot M, Bussereau F, Boucherie H, Jacquet M (1998) Msn2p and Msn4p control a large number of genes induced at the diauxic transition which are repressed by cyclic AMP in *Saccharomyces cerevisiae*. J Bacteriol 180:1044–1052

Burrows S (1970) Baker's yeast. In: Rose AH, Harrison JS (eds) The yeasts, vol 3, Yeast technology. Academic, London, pp 349–420

Cardona F, Carrasco P, Perez-Ortin JE, del Olmo M, Aranda A (2007) A novel approach for the improvement of stress resistance in wine yeasts. Int J Food Microbiol 114:83–91

Causton HC, Ren B, Koh SS, Harbison CT, Kanin E, Jennings EG, Lee TI, True HL, Lander ES, Young RA (2001) Remodeling of yeast genome expression in response to environmental changes. Mol Biol Cell 12:323–337

Cronwright GR, Rohwer JM, Prior BA (2002) Metabolic control analysis of glycerol synthesis in *Saccharomyces cerevisiae*. Appl Environ Microbiol 68:4448–4456

Csonka LN (1981) Proline over-production results in enhanced osmotolerance in *Salmonella typhimurium*. Mol Gen Genet 182:82–86

Demae M, Murata Y, Hisano M, Haitani Y, Shima J. Takagi H (2007) Overexpression of two transcriptional factors, Kin28 and Pog1, suppresses the stress sensitivity caused by the *rsp*5 mutation in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 277:70–78

De Virgilio C, Hottiger T, Dominguez J, Boller T, Wiemken A (1994) The role of trehalose synthesis for the acquisition of thermotolerance in yeast. I. Genetic evidence that trehalose is a thermoprotectant. Eur J Biochem 219:179–186

Dequin S (2001) The potential of genetic engineering for improving brewing, wine-making and bread making. Appl Microbiol Biotechnol 56:577–588

Domitrovic T, Palhano FL, Barja-Fidalgo C, DeFreitas M, Orlando MT, Fernandes PM (2003) Role of nitric oxide in the response of *Saccharomyces cerevisiae* cells to heat shock and high hydrostatic pressure. FEMS Yeast Res 3:341–346

Du X, Takagi H (2005) *N*-Acetyltransferase Mpr1 confers freeze tolerance on *Saccharomyces cerevisiae* by reducing reactive oxygen species. J Biochem 138:391–397

Du X, Takagi H (2007) *N*-Acetyltransferase Mpr1 confers ethanol tolerance on *Saccharomyces cerevisiae* by reducing reactive oxygen species. Appl Microbiol Biotechnol 75:1343–1351

Estruch F (2000) Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. FEMS Microbiol Rev 24:469–486

Evans IH (1990) In: Spencer JFT, Spencer DM (eds) Yeast technology. Springer-Verlag, Berlin, pp 13–45

Franca MB, Panek AD, Eleutherio EC (2007) Oxidative stress and its effects during dehydration. Comp Biochem Physiol A Mol Integr Physiol 146:621–631

Gadd GM, Chalmers K, Reed RH (1987) The role of trehalose in dehydration resistance of *Saccharomyces cerevisiae*. FEMS Microbiol Lett 48:249–254

Hahn YS, Kawai H (1990) Isolation and characterization of freeze-tolerant yeasts from nature available for the frozen-dough method. Agric Biol Chem 54:829–831

Hino A (2002) Safety assessment and public concerns for genetically modified food products: the Japanese experience. Toxicol Pathol 30:126–128

Hino A, Takano H, Tanaka Y (1987) New freeze-tolerant yeast for frozen dough preparations. Cereal Chem 64:269–275

Hino A, Mihara K, Nakashima K, Takano H (1990) Trehalose levels and survival ratio of freeze-tolerant versus freeze-sensitive yeasts. Appl Environ Microbiol 56:1386–1391

Hirasawa T, Nakakura Y, Yoshikawa K, Ashitani K, Nagahisa K, Furusawa C, Katakura Y, Shimizu H, Shioya S (2006) Comparative analysis of transcriptional responses to saline stress in the laboratory and brewing strains of *Saccharomyces cerevisiae* with DNA microarray. Appl Microbiol Biotechnol 70:346–357

Hong Z, Lakkineni K, Zhang Z, Verma DPS (2000) Removal of feedback inhibition of $\Delta^{1\text{-pyrroline}}$-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiol 122:1129–1136

Iinoya K, Kotani T, Sasano Y, Takagi H (2009) Engineering of the yeast antioxidant enzyme Mpr1 for enhanced activity and stability. Biotechnol Bioeng 103:341–352

Kaino T, Takagi H (2008) Gene expression profiles and intracellular contents of stress protectants in *Saccharomyces cerevisiae* under ethanol and sorbitol stresses. Appl Microbiol Biotechnol 79:273–283

Kaino T, Tateiwa T, Mizukami-Murata S, Shima J, Takagi H (2008) Self-cloning baker's yeasts that accumulate proline enhance freeze tolerance in doughs. Appl Environ Microbiol 74: 5845–5849

Kline L, Sugihara TG (1968) Factors affecting the stability of frozen bread doughs. I. Prepared by the straight dough method. Bakers Dig 42:44–69

Landolfo S, Politi H, Angelozzi D, Mannazzu I (2008) ROS accumulation and oxidative damage to cell structures in *Saccharomyces cerevisiae* wine strains during fermentation of high-sugar-containing medium. Biochim Biophys Acta 1780:892–898

Leza MA, Elion EA (1999) *POG1*, a novel yeast gene, promotes recovery from pheromone arrest via the G_1 cyclin *CLN2*. Genetics 151:531–543

Liljeström PL, Tubb, RS, Korhola MP (1991) Construction of new α-galactosidase-producing yeast strains and the industrial application of these strains. U.S. Patent 5,055,401

Liljeström-Suominen PL, Joutsjoki V, Korhola MP (1988) Construction of a stable α-galactosidase producing baker's yeast. Appl Environ Microbiol 54:245–249

Linko Y, Javanainen P, Linko S (1997) Biotechnology of bread baking. Trends Food Sci Technol 8:339–344

Marchler G, Schuller C, Adam G, Ruis H (1993) A *Saccharomyces cerevisiae* UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. EMBO J 12:1997–2003

Martinez-Pastor MT, Marchler G, Schuller C, Marchler-Bauer A, Ruis H, Estruch F (1996) The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress-response element (STRE). EMBO J 15:2227–2235

Matsuura K, Takagi H (2005) Vacuolar functions are involved in stress-protective effect of intracellular proline in *Saccharomyces cerevisiae*. J Biosci Bioeng 100:538–544

Morita Y, Nakamori S, Takagi H (2002) Effect of proline and arginine metabolism on freezing stress of *Saccharomyces cerevisiae*. J Biosci Bioeng 94:390–394

Morita Y, Nakamori S, Takagi H (2003) L-Proline accumulation and freeze tolerance of *Saccharomyces cerevisiae* are caused by a mutation in the *PRO1* gene encoding γ-glutamyl kinase. Appl Environ Microbiol 69:212–219

Nakagawa S, Ouchi K (1994a) Construction from a single parent of baker's yeast strains with high freeze tolerance and fermentative activity in both lean and sweet doughs. Appl Environ Microbiol 60:3499–3502

Nakagawa S, Ouchi K (1994b) Improvement of freeze tolerance of commercial baker's yeasts in dough by heat treatment before freezing. Biosci Biotechnol Biochem 58:2077–2079

Nakamura T, Ando A, Takagi H, Shima J (2007) Eos1, whose deletion confers sensitivity to oxidative stress, is involved in *N*-glycosylation in *Saccharomyces cerevisiae*. Biochem Biophys Res Commun 353:293–298

Nakamura T, Mizukami-Murata S, Ando A, Murata Y, Takagi H, Shima J (2008) Changes in gene expression of commercial baker's yeast during an air-drying process that simulates dried yeast production. J Biosci Bioeng 106:405–408

Nakamura T, Takahashi S, Takagi H, Shima J (2010) Multicopy suppression of oxidant-sensitive *eos1* mutation by *IZH2* in *Saccharomyces cerevisiae* and the involvement of Eos1 in zinc homeostasis. FEMS Yeast Res 10:259–269

Nasuno R, Hirano Y, Itoh T, Hakoshima T, Hibi T, Takagi H (2013) Structural and functional analysis of the yeast *N*-acetyltransferase Mpr1 involved in oxidative stress tolerance via proline metabolism. Proc Natl Acad Sci U S A 110:11821–11826

Needleman R (1991) Control of maltase synthesis in yeast. Mol Microbiol 5:2079–2084

Nishimura A, Kotani T, Sasano Y, Takagi H (2010) An antioxidative mechanism mediated by the yeast *N*-acetyltransferase Mpr1: oxidative stress-induced arginine synthesis and its physiological role. FEMS Yeast Res 10:687–698

Nishimura A, Kawahara N, Takagi H (2013) The flavoprotein Tah18-dependent NO synthesis confers high-temperature stress tolerance on yeast cells. Biochem Biophys Res Commun 430:137–143

Nomura M, Takagi H (2004) Role of the yeast acetyltransferase Mpr1 in oxidative stress: regulation of oxygen reactive species caused by a toxic proline catabolism intermediate. Proc Natl Acad Sci U S A 101:12616–12621

Oda Y, Uno K, Ohta S (1986) Selection of yeasts for breadmaking by the frozen-dough method. Appl Environ Microbiol 52:941–943

Okada N, Ogawa J, Shima J (2014) Comprehensive analysis of genes involved in the oxidative stress tolerance using yeast heterozygous deletion collection. FEMS Yeast Res 14:425–434

Osinga KA, Beudeker RF, Plaat JB van der, Hollander JA de (1988) New yeast strains providing for an enhanced rate of the fermentation of sugars, a process to obtain such strains and the use of these strains. Eur. Patent 0306107A2

Park JI, Grant CM, Attfield PA, Dawes IW (1997) The freeze-thaw stress response of the yeast *Saccharomyces cerevisiae* is growth phase specific and is controlled by nutritional state via the *RAS*-cyclic AMP signal transduction pathway. Appl Environ Microbiol 63:3818–3824

Park JI, Grant CM, Davies MJ, Dawes IW (1998) The cytoplasmic Cu, Zn superoxide dismutase of *Saccharomyces cerevisiae* is required for resistance to freeze-thaw stress. J Biol Chem 273:22921–22928

Pence JW, Kohler GO (1961) Investigations of bread flavor in the U.S. Department of Agriculture. Brot Gebaeck 15:129–134

Randez-Gil F, Sanz P, Pietro JA (1999) Engineering baker's yeast: room for improvement. Trends Biotechnol 17:237–244

Sasano Y, Takahashi S, Shima J, Takagi H (2010) Antioxidant *N*-acetyltransferase Mpr1/2 of industrial baker's yeast enhances fermentation ability after air-drying stress in bread dough. Int J Food Microbiol 138:181–185

Sasano Y, Haitani Y, Hashida K, Ohtsu I, Shima J, Takagi H (2012a) Enhancement of the proline and nitric oxide synthetic pathway improves fermentation ability under multiple baking-associated stress conditions in industrial baker's yeast. Microb Cell Fact 11:40. doi:10.1186/1475-2859-11-40

Sasano Y, Haitani Y, Hashida K, Ohtsu I, Shima J, Takagi H (2012b) Overexpression of the transcription activator Msn2 enhances fermentation ability of industrial baker's yeast in frozen dough. Biosci Biotechnol Biochem 76:624–627

Sasano Y, Haitani Y, Hashida K, Ohtsu I, Shima J, Takagi H (2012c) Simultaneous accumulation of proline and trehalose in industrial baker's yeast enhances fermentation ability in frozen dough. J Biosci Bioeng 113:592–595

Sasano Y, Haitani Y, Ohtsu I, Shima J, Takagi H (2012d) Proline accumulation in baker's yeast enhances high-sucrose stress tolerance and fermentation ability in sweet dough. Int J Food Microbiol 152:40–43

Sasano Y, Watanabe D, Ukibe K, Inai T, Ohtsu I, Shimoi H, Takagi H (2012e) Overexpression of the yeast transcription activator Msn2 confers furfural resistance and increases the initial fermentation rate in ethanol production. J Biosci Bioeng 113:451–455

Sasano Y, Haitani Y, Hashida K, Oshiro S, Shima J, Takagi H (2013) Improvement of fermentation ability under baking-associated stress conditions by altering the POG1 gene expression in baker's yeast. Int J Food Microbiol 165:241–245

Sekine T, Kawaguchi A, Hamano Y, Takagi H (2007) Desensitization of feedback inhibition of the *Saccharomyces cerevisiae* gamma-glutamyl kinase enhances proline accumulation and freezing tolerance. Appl Environ Microbiol 73:4011–4019

Shichiri M, Hoshikawa C, Nakamori S, Takagi H (2001) A novel acetyltransferase found in *Saccharomyces cerevisiae* Sigma1278b that detoxifies a proline analogue, azetidine-2-carboxylic acid. J Biol Chem 276:41998–42002

Shima J, Takagi H (2009) Stress-tolerance of baker's-yeast (*Saccharomyces cerevisiae*) cells: stress-protective molecules and genes involved in stress tolerance. Biotechnol Appl Biochem 53:155–164

Shima J, Hino A, Yamada-Iyo C, Suzuki Y, Nakajima R, Watanabe H, Mori K, Takano H (1999) Stress tolerance in doughs of *Saccharomyces cerevisiae* trehalase mutants derived from commercial Baker's yeast. Appl Environ Microbiol 65:2841–2846

Shima J, Ando A, Takagi H (2008) Possible roles of vacuolar H^+-ATPase and mitochondrial function in tolerance to air-drying stress revealed by genome-wide screening of *Saccharomyces cerevisiae* deletion strains. Yeast 25:179–190

Shinyashiki M, Chiang KT, Switzer CH, Gralla EB, Valentine JS, Thiele DJ, Fukuto JM (2000) The interaction of nitric oxide (NO) with the yeast transcription factor Ace1: a model system for NO-protein thiol interactions with implications to metal metabolism. Proc Natl Acad Sci U S A 97:2491–2496

Takagi H (2008) Proline as a stress protectant in yeast: physiological functions, metabolic regulations, and biotechnological applications. Appl Microbiol Biotechnol 81:211–223

Takagi H, Iwamoto F, Nakamori S (1997) Isolation of freeze-tolerant laboratory strains of *Saccharomyces cerevisiae* from proline-analogue-resistant mutants. Appl Microbiol Biotechnol 47:405–411

Takagi H, Sakai K, Morida K, Nakamori S (2000a) Proline accumulation by mutation or disruption of the proline oxidase gene improves resistance to freezing and desiccation stresses in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 184:103–108

Takagi H, Shichiri M, Takemura M, Mohri M, Nakamori S (2000b) *Saccharomyces cerevisiae* sigma 1278b has novel genes of the *N*-acetyltransferase gene superfamily required for L-PROLINE analogue resistance. J Bacteriol 182:4249–4256

Takagi H, Takaoka M, Kawaguchi A, Kubo Y (2005) Effect of L-proline on sake brewing and ethanol stress in *Saccharomyces cerevisiae*. Appl Environ Microbiol 71:8656–8662

Takagi H, Matsui F, Kawaguchi A, Wu H, Shimoi H, Kubo Y (2007) Construction and analysis of self-cloning sake yeasts that accumulate proline. J Biosci Bioeng 103:377–380

Takahashi S, Ando A, Takagi H, Shima J (2009) Insufficiency of copper ion homeostasis causes freeze-thaw injury of yeast cells revealed by indirect gene expression analysis. Appl Environ Microbiol 75:6706–6711

Tanaka F, Ando A, Nakamura T, Takagi H, Shima J (2006) Functional genomic analysis of commercial baker's yeast during initial stages of model dough-fermentation. Food Microbiol 23:717–728

Tanaka-Tsuno F, Mizukami-Murata S, Murata Y, Nakamura T, Ando A, Takagi H, Shima J (2007) Functional genomics of commercial baker's yeasts that have different abilities for sugar utilization and high-sucrose tolerance under different sugar conditions. Yeast 24:901–911

Terao Y, Nakamori S, Takagi H (2003) Gene dosage effect of L-proline biosynthetic enzymes on L-proline accumulation and freeze tolerance in *Saccharomyces cerevisiae*. Appl Environ Microbiol 69:6527–6532

Teunissen A, Dumortier F, Gorwa MF, Bauer J, Tanghe A, Loïez A, Smet P, Van Dijck P, Thevelein JM (2002) Isolation and characterization of a freeze-tolerant diploid derivative of an industrial baker's yeast strain and its use in frozen doughs. Appl Environ Microbiol 68:4780–4787

Van Dijck P, Colavizza D, Smet P, Thevelein JM (1995) Differential importance of trehalose in stress resistance in fermenting and nonfermenting *Saccharomyces cerevisiae* cells. Appl Environ Microbiol 61:109–115

Verbruggen N, Hermans C (2008) Proline accumulation in plants: a review. Amino Acids 35: 753–759

Verstrepen KJ, Iserentant D, Malcorps P, Derdelinckx G, Van Dijck P, Winderickx J, Pretorius IS, Thevelein JM, Delvaux FR (2004) Glucose and sucrose: hazardous fast-food for industrial yeast? Trends Biotechnol 22:531–537

Watanabe M, Fukuda K, Asano K, Ohta S (1990) Mutants of baker's yeasts producing a large amount of isobutyl alcohol or isoamyl alcohol, flavor components of bread. Appl Microbiol Biotechnol 34:154–159

Watanabe M, Watanabe D, Akao T, Shimoi H (2009) Overexpression of *MSN2* in a sake yeast strain promotes ethanol tolerance and increases ethanol production in sake brewing. J Biosci Bioeng 107:516–518

Wick EL, De Figueiredo M, Wallace DH (1964) The volatile components of white bread prepared by a preferent method. Cereal Chem 41:300–315

Zuzuarregui A, del Olmo ML (2004) Expression of stress response genes in wine strains with different fermentative behavior. FEMS Yeast Res 4:699–710

Chapter 3
Yeast mRNA Flux During Brewing and Under Ethanol Stress Conditions

Shingo Izawa

Abstract Yeast is a eukaryotic cell in which transcription (the nucleus) is separated from translation (the cytoplasm) by the nuclear membrane. The synthesized mRNA of yeast is translated through various steps, such as intranuclear processing and nuclear export. Various cytoplasmic processes, such as mRNA degradation and the formation of mRNP granules, are also involved in the regulation of gene expression. Thus, the expression of genes in yeast should be understood as a series of steps (mRNA flux) that include the processing, transport, translation, and degradation of mRNA, as well as transcriptional regulation. Yeast mRNA undergoes capping, poly (A) tailing, and splicing, and also forms mRNP complexes with mRNA export factors in the nucleus, which are then exported from the nucleus into the cytoplasm. However, under heat-shock conditions (42 °C), the transport of nonessential mRNAs is suppressed whereas various *HSP* mRNAs are preferentially transported in yeast cells (i.e., selective nuclear export of mRNA). Not all mRNAs are translated following their cytoplasmic transport; the expression of some mRNAs is regulated by processes such as translational repression or degradation, and is dependent on the conditions present. Untranslated mRNAs leave ribosomes under various stress conditions and form cytoplasmic mRNP granules (processing bodies and stress granules) with mRNA-degrading enzymes or translation factors. Untranslated mRNAs are sequestered from the translation apparatus in processing bodies and stress granules during stress responsive translational regulation. A recent study demonstrated the unique phenomena of mRNA nuclear export and translational regulation under ethanol stress conditions or during brewing. I herein describe yeast mRNA flux, which was found to be tightly regulated at various steps.

Keywords Ethanol stress • *HSP* mRNAs • mRNA export • Processing bodies • Stress granules

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3.1 mRNA Nuclear Export Mechanism

mRNA should be matured through processing, such as capping and poly(A) tailing. Along with this processing, the nuclear export of mRNA is gradually prepared. The nuclear export of mRNA is achieved by the formation of a complex with Mex67/Mtr2, mRNA export factors. The binding of mRNA to Mex67/Mtr2 requires an adapter, such as Sub2, a homologue of human splicing factor, or Yra1, which belongs to the Ref (RNA and export factor binding proteins) family. Sub2 is a component of the TREX (transcription-export) complex (Strässer et al. 2002). The TREX complex facilitates mRNA transcription and nuclear export. A dysfunction in the TREX complex has been shown to deregulate the elongation, poly (A) tailing, and release of mRNA from a transcription site, which consequently disrupts the nuclear export of mRNA (Rougemaille et al. 2008). A previous study reported that the binding of Yra1 to mRNA required an interaction with Pcf11 (3′-end processing factor) in the cleavage factor 1A (CF1A) (Johnson et al. 2009). These findings suggested that the nuclear export of mRNA is prepared along with its transcriptional elongation and 3′-terminal processing (cotranscriptional recruitment of the mRNA export machinery) (Molina-Navarro et al. 2011; Rodríguez-Navarro and Hurt 2011). Besides Mex67/Mtr2 and Yra1, Npl3, Pab1, Nab2, Hrb1, Gbp2, Sub2, and cap-binding proteins bind to the mRNA exported out of the nucleus to form mRNA–protein complexes (mRNP complexes) (Meinel et al. 2013).

The mRNA that binds to Mex67/Mtr2 is translocated to the nucleus through the nuclear pore, which serves as an exit from the nucleus and an entrance to the cytoplasm. A TREX-2 complex consisting of Sac3-Sus1-Cdc31-Thp1-Sem1 is involved in stable translocation to the nuclear pore (González-Aguilera et al. 2008). The TREX-2 complex was also previously found to be strongly involved in transcription and genome stability, which suggests that the nuclear export of mRNA proceeds with multiple intranuclear events (Faza et al. 2009; García-Oliver et al. 2012).

The nuclear pore complexes (NPC) that form nuclear pores are considered to assess the quality of mRNPs transported as a “barrier” against the nuclear export of mRNA. mRNP binds to the nuclear pore through an interaction between the C-terminal region of Mlp1, which is located on the nuclear side of the NPC and Nab2 in the mRNP complex (Green et al. 2004). Mlp1 and Mlp2 serve as an mRNA-binding point in the NPC and also evaluate the quality of mRNP, that is, serve as a gatekeeper of the barrier (Niepel et al. 2013). mRNP complexes that contain quality defects cannot pass through the nuclear pore, and undergo processing, such as degradation and repair, in the nucleus. Following the mRNP quality check, Yra1 is known to be ubiquitinated by Tom1, an E3 ligase, sequestered from the mRNP, and then enters the nuclear pore (Iglesias et al. 2010).

Nuclear pore complexes are composed of proteins called nucleoporins. Approximately 30 kinds of nucleoporins have been identified to date, and approximately half have FG (PheGly)-repetitive sequences (Wente and Rout 2010). mRNP is transported from the nuclear pore to the cytoplasm while interacting with the FG repeat sequence of nucleoporin. Gle1 and Dbp5, mRNA nuclear export-related factors, exist around the exit of the cytoplasmic side of the nuclear pore and separate

the transport carrier, Mex67/Mtr2, from mRNP transported from the nucleus, thereby releasing mRNA into the cytoplasm and completing the nuclear transport of mRNA (Folkmann et al. 2011; Ledoux and Guthrie 2011). It has been suggested that Dbp5 possesses ATP-dependent helicase activity. Gle1 and its cofactor, IP6 (inositol-6-phosphate) have been shown to activate Dbp5, which disassociates mRNA-binding proteins such as Mex67/Mtr2 and Nab2 (Folkmann et al. 2011; Ledoux and Guthrie 2011; Kaminski et al. 2013; Tieg and Krebber 2013).

3.2 Selective Nuclear Export of mRNA During Stress Responses

The nuclear export of mRNA in yeast is known to be restricted under stress conditions. Transcription patterns in yeast cells are markedly changed under stress conditions by stress-responsive transcription factors, such as Msn2/Msn4 and Hsf1 (Lee et al. 2013). Previous studies examined transcriptional regulation during stress responses in detail. Most of these studies have been conducted at the transcription level. However, eukaryotic cells undergo multiple steps from transcription to translation. Changes in transcription patterns may not necessarily be directly associated with changes in gene expression. If the processing or nuclear export of mRNA is adjusted or negatively influenced by stress, mRNA levels, which are increased by transcriptional activation, may not necessarily be reflected by changes in protein levels. As described next, the expression of genes during stress responses may be misunderstood or misinterpreted if it is only examined based on changes in mRNA levels, because a series of mRNA flux from transcription to translation under stress conditions is in a variable state (Gygi et al. 1999).

The nuclear export of mRNA was previously shown to be completely suppressed under heat-shock conditions, such as at a temperature of 42 °C, and the mRNAs of the *HSP* genes, which encode heat-shock proteins, preferentially underwent nuclear export (Saavedra et al. 1996). Figure 3.1 shows the intracellular localization of mRNA by fluorescence in situ hybridization (FISH), clearly demonstrating the accumulation of bulk poly $(A)^+$ mRNA in the nucleus under heat-shock conditions. No signal was detected for the mRNA of *SSA4*, which encodes Hsp70, because its synthesis is negligible under non-stress conditions; however, it was rapidly transported to the cytoplasm without accumulating in the nucleus under heat-shock conditions. For the proteins required to respond to stress situations, such as heat shock, to be efficiently synthesized, nonessential mRNAs temporarily remain in the nucleus, and nuclear export pathways and translation apparatuses are preferentially used for *HSP* mRNAs to achieve a rapid and efficient response to the stress (Fig. 3.2).

Nucleoporin Nup42 (Rip1) is required for the nuclear export of *HSP* mRNA under heat-shock conditions. The nuclear export of *HSP* mRNA is blocked in the gene-disrupted *nup42*Δ strain (Saavedra et al. 1997; Stutz et al. 1997). Nup42 is localized in the cytoplasm of the NPC and interacts with Gle1; therefore, it is believed to be involved in selective transport regulation in the final step of the

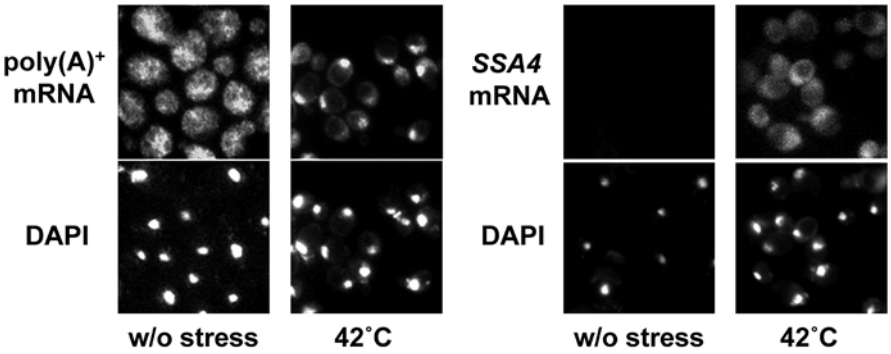

Fig. 3.1 Effects of heat shock on the nuclear export of mRNA in yeast. Cells in exponential phase were treated with heat shock (42°C) for 60 min. *Left:* Intracellular localization of bulk poly(A)$^+$ mRNA was analyzed by in situ hybridization using a DIG-labeled oligo(dT)$_{50}$ probe. *Right:* Intracellular localization of *SSA4* mRNA was analyzed using Cy3-labeled *SSA4* probes. Nuclear DNA was stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). (Quoted from Takemura et al. 2004)

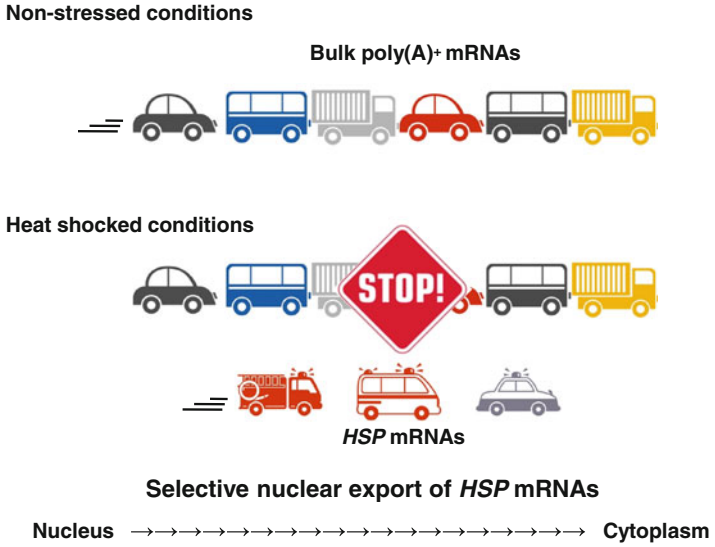

Fig. 3.2 Selective nuclear export of mRNA under heat shock in yeast. Export of nonessential mRNAs is blocked for the rapid response to heat shock, whereas *HSP* mRNAs can be preferentially exported

nuclear export of mRNA (Strahm et al. 1999; Aitchison and Rout 2012). In addition, Gle2, another mRNA nuclear export-related factor, is known to be translocated into the nucleus under heat stress conditions (Izawa et al. 2004). A previous study showed that Nab2 is phosphorylated by Slt2, a kind of MAP kinase (Thr178/Ser180

of Nab2 is phosphorylated), and aggregates with Yra1 and Mlp1 in the nucleoplasm away from the NPC (Carmody et al. 2010). Thus, mRNP, which is formed with Mex67/Mtr2 in bulk poly $(A)^+$ mRNA (non-*HSP* mRNAs), cannot access the NPC, does not undergo nuclear export, and accumulates in the nucleus. No bulk poly $(A)^+$ mRNAs accumulated in the nuclei of the *slt2*Δ strain under heat-shock stress conditions. The altered localization of these mRNA transport-related factors is involved in the selective nuclear export of mRNA under heat-shock conditions. However, the mechanisms responsible, including those that distinguish between *HSP* mRNAs and other mRNAs, remain unclear.

3.3 Translational Repression, P-Bodies, and Stress Granules

mRNAs that are transported into the cytoplasm are normally translated in ribosomes for protein synthesis. Active translation under non-stress conditions induces the binding of multiple ribosomes to a single mRNA molecule, thereby forming a polysome. Polysome analysis of ribosomes identified a fraction with a high sedimentation coefficient, together with small (40 S) and large (60 S) subunits as well as monosome (80 S) fractions (Fig. 3.3). However, stress often suppresses translation, which reduces the formation of polysomes. The polysome fraction rapidly disappeared under glucose depletion and sodium azide (NaN_3) stress conditions, whereas the 80 S monosome fraction increased (Buchan et al. 2011; Iwaki and Izawa 2012) (Fig. 3.3).

Translation is suppressed and polysomes disappear under certain stress conditions, which separates untranslated mRNAs from the ribosomes and forms complexes between mRNAs and RNA-binding proteins in the cytoplasm. This complex is referred to as an mRNP granule. Two kinds of mRNP granules have been identified to date: cytoplasmic processing bodies (P-bodies) and stress granules (SGs) (Balagopal and Parker 2009; Buchan and Parker 2009) (Fig. 3.4). Untranslated mRNAs caused by stress are sequestered in the P-bodies and SGs, and subsequently undergo processing. It is likely that segregation of untranslated mRNAs in P-bodies

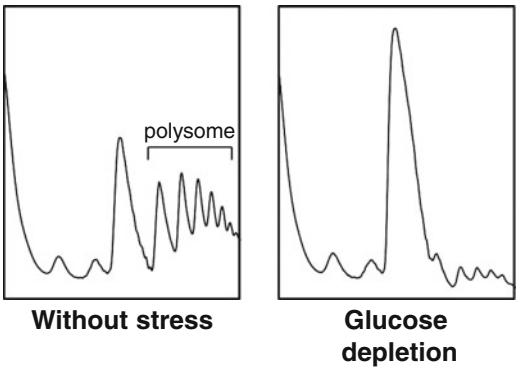

Fig. 3.3 Polysome profiles of cells under glucose-depleted conditions. Glucose depletion caused a significant reduction in the polysome fraction and a concomitant increase in the monosome fraction (80S), indicating a bulk attenuation in translation initiation. (Quoted from Iwaki and Izawa 2012)

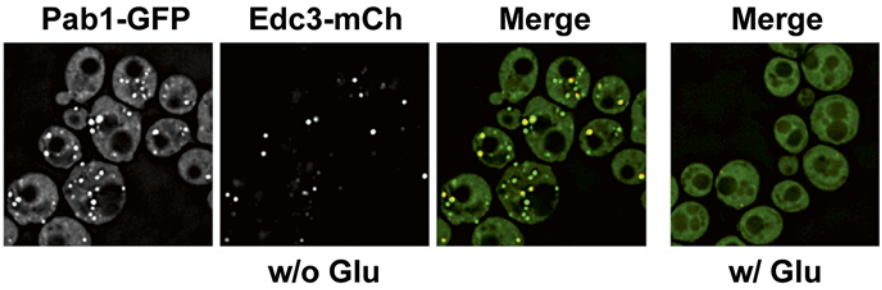

Fig. 3.4 P-bodies and stress granules (SGs) under glucose-depleted conditions. Pab1-GFP was used as a SG marker and Edc3-mCh was used as a P-body marker. *Glu* glucose. (Quoted from Buchan et al. 2008)

and SGs facilitates the preferential translation of mRNAs that are urgently required under stress conditions.

P-bodies and SGs have structures without membranes that are composed of untranslated mRNAs and RNA-binding proteins (Parker and Sheth 2007; Franks and Lykke-Andersen 2008; Balagopal and Parker 2009; Buchan and Parker 2009). Component proteins are basically different, although partially shared. Component factors vary with the kinds of stresses encountered (Hoyle et al. 2007; Buchan et al. 2008, 2011; Grousl et al. 2009; Kato et al. 2011; Yamamoto and Izawa 2013). SGs are composed of untranslated mRNA, the translation initiation factor, eIF, poly (A) binding protein, and 40S ribosomal protein (Balagopal and Parker 2009; Buchan and Parker 2009). P-bodies are composed of proteins involved in mRNA degradation (e.g., exonuclease and decapping enzyme) (Parker and Sheth 2007; Franks and Lykke-Andersen 2008). Thus, P-bodies were initially considered to have important roles in the degradation of mRNA, such as nonsense-mediated mRNA decay (NMD), and were subsequently considered to have important functions in translational regulation under stress conditions, as well as the degradation of mRNA.

Untranslated mRNAs in animal cells are known to be collected in SGs, followed by (1) their return to translation in the ribosomes, (2) degradation in the P-bodies, or (3) sequestration and storage in SGs. Thus, SGs function as a place for triage (prioritizing) to determine the fates of mRNAs (Anderson and Kedersha 2009). In *Saccharomyces cerevisiae*, the formation of P-bodies is first induced in response to stress (Buchan et al. 2008). The formation of yeast SGs is induced by more stressful conditions or the passage of time (Buchan et al. 2008; Kato et al. 2011; Iwaki et al. 2013; Yamamoto and Izawa 2013). The formation of P-bodies in yeast was previously reported to be regulated by Ras/PKA activity and protein kinases, such as Pkh1/Pkh2 and Pkc1 (Luo et al. 2011; Ramachandran et al. 2011). The formation of SGs was found to be efficiently induced when Ste20 directly phosphorylated Dcp2 (Yoon et al. 2010).

3.4 P-Body and Stress Granule Formation Under Glucose Depletion and Heat-Shock Conditions

The formation of P-bodies, followed by that of SGs, has been reported under glucose depletion conditions (Brengues et al. 2005; Buchan et al. 2008). The formation of P-bodies was also shown to be induced by NaN_3, and this was followed by the formation of SGs (Buchan et al. 2011). P-bodies and SGs were formed in cells exposed to severe heat shock at 46 °C and were closely localized within the cells (Grousl et al. 2009). Based on these findings, Parker et al. proposed the possible formation of SGs in yeast using P-bodies as a scaffold (Balagopal and Parker 2009). SG, P-body, and scaffold factors and assembly orders have been shown to differ slightly depending on the kinds of stresses encountered (Buchan et al. 2011), whereas the number of polysomes markedly decreased under both stress conditions. On the other hand, lactic acid can induce the formation of P-bodies but not SGs (Iwaki and Izawa 2012). In contrast to mammalian cells, oxidative stress and osmotic stress have little or no effect on the formation of SGs in *S. cerevisiae* (Buchan et al. 2008; Balagopal and Parker 2009).

Untranslated mRNAs may be transported between P-bodies and SGs (Brengues et al. 2005). The cessation of stress by the readdition of glucose led to the transport of the mRNAs sequestered in the P-bodies and SGs back to the translation apparatus, which rapidly resumed translation (Balagopal and Parker 2009; Arribere et al. 2011). However, only a limited number of mRNAs are known to be recycled from P-bodies for translation (Arribere et al. 2011). Therefore, the kinds of mRNAs that are sequestered, as well as the frequency by which mRNAs are sequestered for recycling, remain unclear.

3.5 mRNA Nuclear Export Under Ethanol Stress Conditions

The selective nuclear export of mRNA is also caused by ethanol stress. Bulk poly $(A)^+$ mRNAs accumulated in the nuclei of yeast cells exposed to more than 6 % (v/v) ethanol stress in the logarithmic growth phase, whereas most mRNAs remained in the nuclei of cells treated with more than 9 % (v/v) ethanol stress, as demonstrated by FISH analysis. The mRNAs that accumulated in the nucleus were rapidly transported out of the nucleus after treatment with less than 6 % ethanol (Takemura et al. 2004).

Changes in the localization and functions of the mRNA export related factors were predicted as suppressors of the entire nuclear export of mRNA. The localization of Gle2, a nuclear export-related factor, under heat-shock or greater than 6 % ethanol stress conditions was shown to differ from that under normal conditions (Izawa et al. 2004). However, heat-shock and ethanol stresses did not suppress the nuclear export of mRNA through the same mechanism. For example, the localization of Dbp5, an important factor in the release of mRNAs from nuclear pores into the cytoplasm (Folkmann et al. 2011; Ledoux and Guthrie 2011), reversibly changed

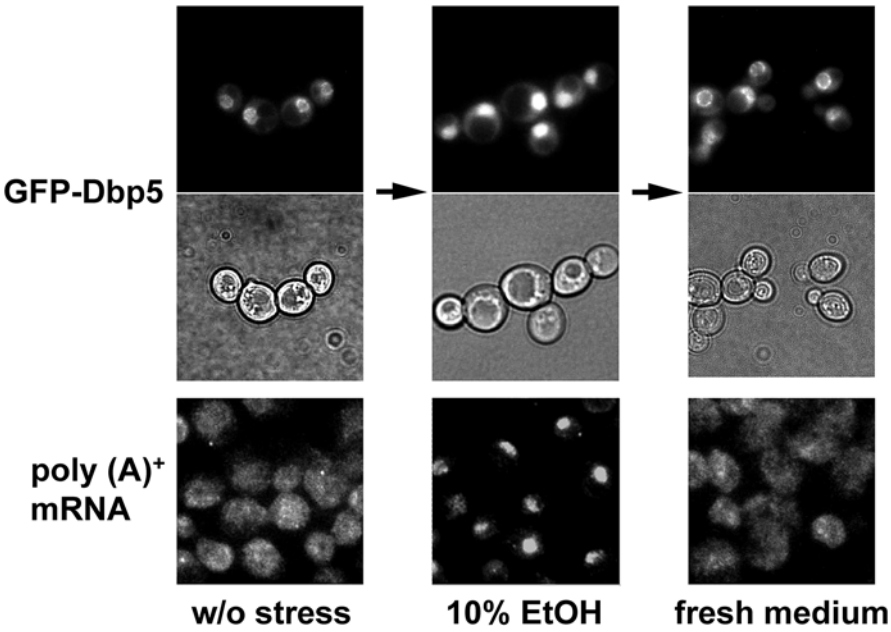

Fig. 3.5 Rapid and reversible change in the intracellular localization of GFP-Dbp5. Cells in exponential phase were treated with 10 % ethanol for 30 min, collected, and transferred to fresh SD medium without ethanol. GFP-Dbp5 fluorescence was visualized before ethanol treatment (w/o stress), after ethanol treatment for 30 min (10 % ethanol) and 5 min after the shift to fresh SD medium. Cells at each stage were fixed for in situ hybridization to detect bulk poly(A)$^+$ RNA using a DIG-labeled oligo(dT)$_{50}$ probe. (Quoted from Takemura et al. 2004)

in an ethanol concentration-dependent manner (Takemura et al. 2004) (Fig. 3.5). Dbp5 gradually accumulated in the nucleus and stopped functioning in the presence of more than 6 % ethanol. However, the localization of Dbp5 did not change under heat-shock conditions (Takemura et al. 2004). Dbp5 did not respond to heat-shock conditions, which suggested the different effects of the heat-shock and ethanol stresses on the mRNA nuclear export pathway. Dbp5, which specifically responds to ethanol, may function as an ethanol-responsive regulator of bulk poly (A)$^+$ mRNA nuclear export.

3.6 Localization of *HSP* mRNAs Under Ethanol Stress

Significant differences have also been observed in *HSP* mRNA transport between heat-shock and ethanol stresses. The general stress-responsive transcription factors Msn2 and Msn4 are activated by ethanol stress and promote the transcription of various *HSP* genes and increase *HSP* mRNA levels (Izawa et al. 2008). However, a previous study demonstrated that *HSP* mRNAs were not preferentially exported

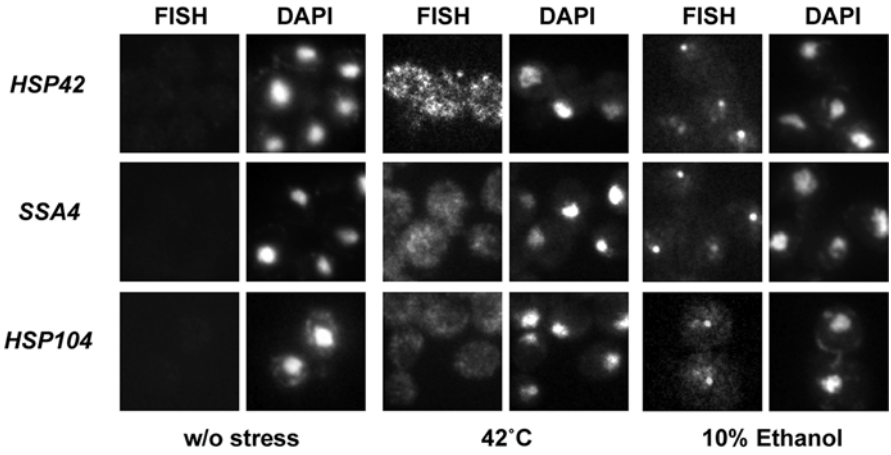

Fig. 3.6 Nuclear retention of *HSP42*, *SSA4*, and *HSP104* mRNAs in ethanol-treated cells. Cells were treated with heat shock (42 °C) or ethanol stress (10 %) for 30 min. The intracellular localization of each *HSP* mRNA was monitored by in situ hybridization. Probes were labeled with Cy3 and nuclear DNA was stained with DAPI. *FISH* fluorescent in situ hybridization. (Quoted from Izawa et al. 2008)

from the nucleus under ethanol stress conditions and remained in the nucleus, similar to bulk poly $(A)^+$ mRNAs (Izawa et al. 2008). FISH analysis (Fig. 3.6) revealed the localization of *HSP* mRNAs within the nuclei. *HSP* mRNAs that remain in the nucleus in spite of activated synthesis appear to be wasteful. However, this can be interpreted as a standby state for rapid nuclear export and translation after an adaptation to ethanol stress or removal of the stress condition. Even if transcriptional activation is wasteful, we speculated that "incorrect stress responses" that cannot be corrected for during transcription could be compensated for during mRNA transport, thereby providing the appropriate regulation of gene expression. The expression of genes is known to be regulated by multiple steps, including transcription, transport, and translation. Therefore, each step is not the sole critical regulatory mechanism; furthermore, multiple regulations can provide rapid and strict responses to changing conditions.

The mechanism by which *HSP* mRNAs remain in the nucleus under ethanol stress conditions has been correlated with hyperadenylation, which generates an excessively long poly (A) tail at the 3′-terminus (Izawa et al. 2008). mRNAs were previously shown to be hyperadenylated by abnormalities in mRNA nuclear export-related factors, including Dbp5 and Nab2 (Hilleren and Parker 2001; Kelly et al. 2010). The poly (A) tail lengths of *HSP* mRNAs reversibly and rapidly change in response to ethanol concentrations. *HSP* mRNAs with unusually long poly (A) tails remain in the nucleus (Izawa et al. 2008). The poly (A) tail lengths of *HSP* mRNAs are normal under heat-shock conditions. These findings suggest the presence of regulatory mechanisms for the processing of mRNA in the nucleus as well as nuclear export during stress responses; however, whether hyperadenylated mRNAs are the result or cause of suppressed nuclear export remains unclear (Soucek et al. 2012).

3.7 mRNA Transport During Brewing

The nuclear export of mRNA differs markedly during the brewing of Japanese sake and white wine. When the ethanol concentration of grape juice exceeds 6 % during white wine brewing, Dbp5 starts to migrate into the nucleus and bulk poly $(A)^+$ mRNAs start to accumulate in the nucleus (Izawa et al. 2005a). When the ethanol concentration exceeds 9 %, Dbp5 mostly accumulates in the nucleus, which lowers the signal of mRNA in the cytoplasm (Izawa et al. 2005a). The nuclear export of mRNA is suppressed and Dbp5 accumulates in the nucleus in response to greater than 6 % ethanol in the same manner during white wine brewing and under laboratory conditions (Takemura et al. 2004).

A small-scale fermentation test of sake revealed that ethanol concentration-dependent inhibition of the nuclear export of mRNA was not induced (Izawa et al. 2005b). Even when the ethanol concentration of sake mash increased to 6–9 %, neither Dbp5 nor bulk poly $(A)^+$ mRNAs accumulated in the nucleus. When the ethanol concentration reached 12–13 %, the nuclear export of mRNA finally began to be suppressed. These phenomena may be specific for a solid–liquid mixture of sake mash regardless of the kind of yeast because wine yeast exhibited the same behaviors as Japanese sake yeast during sake brewing (Izawa et al. 2005b).

3.8 P-Body and Stress Granule Formation Under Ethanol Stress Conditions

Polysome analysis using cells under ethanol stress conditions demonstrated the negligible effects of less than 5 % ethanol on the formation of polysomes, 80 S monosomes, and ribosomal subunits (Yamamoto and Izawa 2013). However, the formation of polysomes was gradually suppressed whereas that of monosomes increased at greater than 6 % ethanol concentration. In addition, polysomes were reduced to a negligible level at ethanol concentrations greater than >9–10 %, which demonstrated the strong suppression of translational activity (Iwaki et al. 2013). However, specific proteins are synthesized even in the presence of 10 % ethanol, which indicated selective translation. Similar to the nuclear export of mRNA, overall translational activity was found to be reduced to suppress the translation of nonessential mRNAs, whereas urgently required proteins were intensively synthesized.

A previous study reported that P-bodies were formed under greater than 6 % ethanol stress conditions (Izawa et al. 2007), and this was dependent on the concentration of ethanol; the number and size of the P-bodies increased as the concentration increased. P-bodies were no longer formed when the release of mRNA from the ribosomes was inhibited using cycloheximide. P-bodies were formed by ethanol stress, and, subsequently, degraded and disappeared soon after the removal of ethanol stress (Izawa et al. 2007).

In a synthetic medium, SGs were formed in yeast cells treated with greater than 10 % ethanol stress, whereas their formation was negligible under 6–9 % ethanol stress (Kato et al. 2011). Both P-bodies and SGs were formed by 10 % ethanol stress. The time required for SGs to form (20 min) is longer than that for P-bodies (approximately 5–10 min) (Kato et al. 2011). In addition, the localization of SGs overlaps with the formation of P-bodies. These findings demonstrated that more severe ethanol stress conditions (i.e., longer time and higher concentration) were required for the formation of SGs than P-bodies. Thus, the hypothesis of Parker et al., "SGs of yeast are formed using P-bodies as a scaffold," was also supported under ethanol stress conditions (Buchan et al. 2008; Balagopal and Parker 2009). In contrast to animal cells, untranslated mRNAs first gather in the P-bodies, which may function as a place for triage.

The roles of yeast SGs under ethanol stress conditions were examined. The formation of SGs by ethanol was suppressed in the knockout mutants, *pbp1*Δ, *pub1*Δ, and *tif4632*Δ, and their sensitivities to 15 % ethanol stress were similar to that of the wild type (Kato et al. 2011). However, these knockout mutants showed a significant difference from the wild type in the recovery process from 15 % ethanol. These mutants were treated with 15 % ethanol for 20 min and then transferred to a new ethanol-free medium to observe their growth. Growth immediately recovered in the wild type, but either did not recover or took a long time to resume in the knockout mutants (Kato et al. 2011). These results suggested that the mRNAs sequestered in SGs quickly returned to the ribosomes to resume translation, thereby allowing rapid growth recovery by the wild strain. A possible interpretation of these results is as follows: under stress situations, the mRNAs required for defense against stress are preferentially used by the translation apparatus, and most nonessential mRNAs are sequestered in the P-bodies and SGs (Brengues et al. 2005). When the stress ceases, mRNAs sequestered in the SGs are immediately sent to the ribosomes to resume translation, and this contributes to rapid recovery and growth resumption. The mRNAs sequestered in the cytoplasmic mRNP granules return to the ribosomes to save the time and energy required to restart transcription, mRNA nuclear export, and translation. The mRNAs of stress-responsive genes are preferentially translated under stress conditions, which suggests that the mRNAs of housekeeping genes that are required for normal growth may be sequestered in the SGs (Arribere et al. 2011). However, the mechanisms involved have yet to be elucidated in detail.

3.9 P-Body and Stress Granule Formation During Brewing

The size and number of P-bodies formed during white wine brewing were shown to increase under laboratory conditions (>6 % ethanol), whereas P-bodies were actively formed when the ethanol concentration of mash reached 13 % during sake brewing (Izawa et al. 2007). The formation of P-bodies reflected translational repression, which suggested that translation was active in sake mash until the late stage of brewing. Bulk poly $(A)^+$ mRNA nuclear export was found to be suppressed

in the presence of approximately 13 % ethanol during sake brewing (Izawa et al. 2005b). SGs were formed when the ethanol concentration reached approximately 10 % during wine brewing. However, the marked formation of SGs has not been reported in sake mash, which suggests that the effects of ethanol stress are weak, at least on mRNA flux in sake mash. The ethanol stress-relieving effects of sake mash (solid–liquid coexisting substance) may be stronger than those of synthetic medium or grape juice.

3.10 Roles of P-Bodies and Stress Granules

Only P-bodies exist at ethanol concentrations of 6–9 %, which suggests that untranslated mRNAs, including the mRNAs of housekeeping genes, may be sequestered in the P-bodies. When SGs exist at high ethanol concentrations, the mRNAs to be degraded may mainly exist in P-bodies, and this suggests that the mRNAs of housekeeping genes, which are retranslated with the removal of the stress condition, may be preferentially stored in SGs. The kinds of mRNAs transported to mRNP granules under severe stress conditions, the kinds of mRNP granules, and the fates of the mRNAs have significant effects on cell fate. Therefore, the adaptation strategy of yeast to ethanol stress should be elucidated from the aspect of mRNA flux. However, the kinds and amounts of mRNAs contained in mRNP granules upon ethanol stress remain unclear. In my laboratory, the mRNAs contained in polysomes, P-bodies, and SGs at various ethanol concentrations are under analysis to reveal specific mRNA flux in the cytoplasm under ethanol stress conditions. We speculate that mRNAs contained in polysomes even under high-concentration ethanol stress must be quite important for the tolerance and adaptation to severe ethanol stress. Identification of the mRNAs might be a crucial clue for the dramatic improvement of ethanol stress tolerance and fermentation capacity of yeast cells.

3.11 Conclusion

As described here, initiation of transcription is followed by the nuclear export, translation, and degradation of mRNA (mRNA flux) in yeast cells, that is, eukaryotic cells. This mRNA flux should be properly regulated to facilitate life activities. The selective transport, translation, and degradation of mRNA are precisely regulated under stress conditions. Thus, the nuclear export, translation, and degradation of mRNA, as well as transcription, should be considered when examining the expression of certain genes in yeast under stress conditions: this may be important for the applications of yeast. The physiology of yeast should be reexamined from the aspect of mRNA flux to facilitate understanding of poorly defined mechanisms.

References

Aitchison JD, Rout MP (2012) The yeast nuclear pore complex and transport through it. Genetics 190:855–883

Anderson P, Kedersha N (2009) RNA granules: post-transcriptional and epigenetic modulators of gene expression. Nat Rev Mol Cell Biol 10:430–436

Arribere JA, Doudna JA, Gilbert WV (2011) Reconsidering movement of eukaryotic mRNAs between polysomes and P bodies. Mol Cell 44:745–758

Balagopal V, Parker R (2009) Polysomes, P bodies and stress granules: states and fates of eukaryotic mRNAs. Curr Opin Cell Biol 21:403–408

Brengues M, Sheth U, Parker R (2005) Movement of eukaryotic mRNAs between polysomes and cytoplasmic processing bodies. Science 310:486–489

Buchan JR, Parker R (2009) Eukaryotic stress granules: the ins and outs of translation. Mol Cell 36:932–940

Buchan JR, Muhlrad D, Parker R (2008) P bodies promote stress granule assembly in *Saccharomyces cerevisiae*. J Cell Biol 183:441–455

Buchan JR, Yoon JH, Parker R (2011) Stress-specific composition, assembly and kinetics of stress granules in *Saccharomyces cerevisiae*. J Cell Sci 124:228–239

Carmody SR, Tran EJ, Apponi LH, Corbett AH, Wente SR (2010) The mitogen-activated protein kinase Slt2 regulates nuclear retention of non-heat shock mRNAs during heat shock-induced stress. Mol Cell Biol 30:5168–5179

Faza M, Kemmler S, Jimeno S, Gonzalez-Aguilera C, Aguilera A, Hurt E, Panse V (2009) Sem1 is a functional component of the nuclear pore complex-associated messenger RNA export machinery. J Cell Biol 184:833–846

Folkmann AW, Noble KN, Cole CN, Wente SR (2011) Dbp5, Gle1-IP6 and Nup159: a working model for mRNP export. Nucleus 2:540–548

Franks TM, Lykke-Andersen J (2008) The control of mRNA decapping and P-body formation. Mol Cell 32:605–615

García-Oliver E, García-Molinero V, Rodríguez-Navarro S (2012) mRNA export and gene expression: the SAGA-TREX-2 connection. Biochim Biophys Acta 1819:555–565

González-Aguilera C, Tous C, Gómez- González B, Huertas P, Luna R, Aguilera A (2008) The THP1-SAC3-SUS1-CDC31 complex works in transcription elongation-mRNA export preventing RNA-mediated genome instability. Mol Biol Cell 19:4310–4318

Green DM, Johnson CP, Hagan H, Corbett AH (2004) The C-terminal domain of myosin-like protein 1 (Mlp1p) is a docking site for heterogeneous nuclear ribonucleoproteins that are required for mRNA export. Proc Natl Acad Sci USA 100:1010–1015

Grousl T, Ivanov P, Frydlova I, Vasicova P, Janda F, Voltova J, Malinska K, Malcova I, Novakova L, Janoskova D, Valasek L, Hasek J (2009) Robust heat shock induces eIF2alpha-phosphorylation-independent assembly of stress granules containing eIF3 and 40S ribosomal subunits in budding yeast, *Saccharomyces cerevisiae*. J Cell Sci 122:2078–2088

Gygi SP, Rochon Y, Franza BR, Aebersold R (1999) Correlation between protein and mRNA abundance in yeast. Mol Cell Biol 19:1720–1730

Hilleren P, Parker R (2001) Defects in the mRNA export factors Rat7p, Gle1p, Mex67p, and Rat8p cause hyperadenylation during 3′-end formation of nascent transcripts. RNA 7:753–764

Hoyle NP, Castelli LM, Campbell SG, Holmes LE, Ashe MP (2007) Stress-dependent relocalization of translationally primed mRNPs to cytoplasmic granules that are kinetically and spatially distinct from P-bodies. J Cell Biol 179:65–74

Iglesias N, Tutucci E, Gwizdek C, Vinciguerra P, Von Dach E, Corbett AH, Dargemont C, Stutz F (2010) Ubiquitin-mediated mRNP dynamics and surveillance prior to budding yeast mRNA export. Genes Dev 24:1927–1938

Iwaki A, Izawa S (2012) Acidic stress induces the formation of P-bodies, but not stress granules, with mild attenuation of bulk translation in *Saccharomyces cerevisiae*. Biochem J 446:225–233

Iwaki A, Kawai T, Yamamoto Y, Izawa S (2013) Biomass conversion inhibitors furfural and 5-hydroxymethylfurfural induce formation of messenger RNP granules and attenuate translation activity in *Saccharomyces cerevisiae*. Appl Environ Microbiol 79:1661–1667

Izawa S, Takemura R, Inoue Y (2004) Gle2p is essential to induce adaptation of the export of bulk poly(A)$^+$ mRNA to heat shock in *Saccharomyces cerevisiae*. J Biol Chem 279:35469–35478

Izawa S, Takemura R, Miki T, Inoue Y (2005a) Characterization of the export of bulk poly (A)$^+$ mRNA in *Saccharomyces cerevisiae* during wine-making process. Appl Environ Microbiol 71:2179–2182

Izawa S, Takemura R, Ikeda K, Fukuda K, Wakai Y, Inoue Y (2005b) Characterization of Rat8 localization and mRNA export in *Saccharomyces cerevisiae* during the brewing of Japanese sake. Appl Microbiol Biotechnol 69:86–91

Izawa S, Kita T, Ikeda K, Miki T, Inoue Y (2007) Formation of the cytoplasmic P-bodies in *sake* yeast during Japanese *sake* brewing and wine making. Biosci Biotechnol Biochem 71:2800–2807

Izawa S, Kita T, Ikeda K, Inoue Y (2008) Heat shock and ethanol stress provoke distinctly different responses in 3′-processing and nuclear export of *HSP* mRNA in *Saccharomyces cerevisiae*. Biochem J 414:111–119

Johnson SA, Cubberley G, Bentley DL (2009) Cotranscriptional recruitment of the mRNA export factor Yra1 by direct interaction with the 3′ end processing factor Pcf11. Mol Cell 32: 215–226

Kaminski T, Siebrasse JP, Kubitscheck U (2013) A single molecule view on Dbp5 and mRNA at the nuclear pore. Nucleus 4:1–8

Kato K, Yamamoto Y, Izawa S (2011) Severe ethanol stress induces assembly of stress granules in *Saccharomyces cerevisiae*. Yeast 28:339–347

Kelly SM, Leung SW, Apponi LH, Bramley AM, Tran EJ, Chekanova JA, Wente SR, Corbett AH (2010) Recognition of polyadenosine RNA by the zinc finger domain of nuclear poly(A) RNA-binding protein 2 (Nab2) is required for correct mRNA 3′-end formation. J Biol Chem 285:26022–26032

Ledoux S, Guthrie C (2011) Regulation of the Dbp5 ATPase cycle in mRNP remodeling at the nuclear pore: a lively new paradigm for DEAD-box proteins. Genes Dev 25:1109–1114

Lee P, Kim MS, Paik SM, Choi SH, Cho BR, Hahn JS (2013) Rim15-dependent activation of Hsf1 and Msn2/4 transcription factors by direct phosphorylation in *Saccharomyces cerevisiae*. FEBS Lett 587:3648–3655

Luo G, Costanzo M, Boone C, Dickson DC (2011) Nutrients and the Pkh1/2 and Pkc1 protein kinases control mRNA decay and P-body assembly in yeast. J Biol Chem 286:8759–8770

Meinel DM, Burkert-Kautzsch C, Kieser A, O'Duibhir E, Siebert M, Mayer A, Cramer P, Söding J, Holstege FCP, Sträßer K (2013) Recruitment of TREX to the transcription machinery by its direct binding to the phospho-CTD of RNA polymerase II. PLoS Genet 9:e1003914. doi:10.1371/journal.pgen.1003914

Molina-Navarro MM, Martinez-Jimenez CP, Rodriguez-Navarro S (2011) Transcriptional elongation and mRNA export are coregulated processes. Genet Res Int 2011:652461. doi:10.4061/2011/652461

Niepel M, Molloy KR, Williams R, Farr JC, Meinema AC, Vecchietti N, Cristea IM, Chait BT, Rout MP, Strambio-De-Castillia C (2013) The nuclear basket proteins Mlp1p and Mlp2p are part of a dynamic interactome including Esc1p and the proteasome. Mol Biol Cell 24: 3920–3938

Parker R, Sheth U (2007) P bodies and the control of mRNA translation and degradation. Mol Cell 25:635–646

Ramachandran V, Shah KH, Herman PK (2011) The cAMP-dependent protein kinase signaling pathway is a key regulator of P body foci formation. Mol Cell 43:973–981

Rodríguez-Navarro S, Hurt E (2011) Linking gene regulation to mRNA production and export. Curr Opin Cell Biol 23:302–309

Rougemaille M, Dieppois G, Kisseleva-Romanova E, Gudipati RK, Lemoine S, Blugeon C, Boulay J, Jensen TH, Stutz F, Devaux F, Libri D (2008) THO/Sub2p functions to coordinate 3′-end processing with gene-nuclear pore association. Cell 135:308–321

Saavedra C, Hammell CM, Heath CV, Cole CN (1997) Yeast heat shock mRNAs are exported through a distinct pathway defined byRip1p. Genes Dev 11:2845–2856

Saavedra C, Tung KS, Amberg DC, Hopper AK, Cole CN (1996) Regulation of mRNA export in response to stress in *Saccharomyces cerevisiae*. Genes Dev 10:1608–1620

Soucek S, Corbett AH, Fasken MB (2012) The long and the short of it: the role of the zinc finger polyadenosine RNA binding protein, Nab2, in control of poly(A) tail length. Biochim Biophys Acta 1819:546–554

Strahm Y, Fahrenkrog B, Zenklusen D, Rychner E, Kantor J, Rosbach M, Stutz F (1999) The RNA export factor Gle1p is located on the cytoplasmic fibrils of the NPC and physically interacts with the FG-nucleoporin Rip1p, the DEAD-box protein Rat8p/Dbp5p and a new protein Ymr 255p. EMBO J 18:5761–5777

Strässer K, Masuda S, Mason P, Pfanstiel J, Oppizzi M, Rodriguez-Navarro S, Rondón AG, Aguilera A, Struhl K, Reed R, Hurt E (2002) TREX is a conserved complex coupling transcription with messenger RNA export. Nature 417:304–308

Stutz F, Kantor J, Zhang D, McCarthy T, Neville M, Rosbash M (1997) The yeast nucleoporin Rip1p contributes to multiple export pathways with no essential role for its FG-repeat region. Genes Dev 11:2857–2868

Takemura R, Inoue Y, Izawa S (2004) Stress response in yeast mRNA export factor: reversible changes in Rat8p localization are caused by ethanol stress but not heat shock. J Cell Sci 117:4189–4197

Tieg B, Krebber H (2013) Dbp5—from nuclear export to translation. Biochim Biophys Acta 1829:791–798

Wente SR, Rout MP (2010) The nuclear pore complex and nuclear transport. Cold Spring Harb Perspect Biol 2:a000562. doi:10.1101/cshperspect.a000562

Yamamoto Y, Izawa S (2013) Adaptive response in stress granule formation and bulk translational repression upon a combined stress of mild heat shock and mild ethanol stress in yeast. Genes Cells 18:974–984

Yoon JH, Choi EJ, Parker R (2010) Dcp2 phosphorylation by Ste20 modulates stress granule assembly and mRNA decay in *Saccharomyces cerevisiae*. J Cell Biol 189:813–827

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.

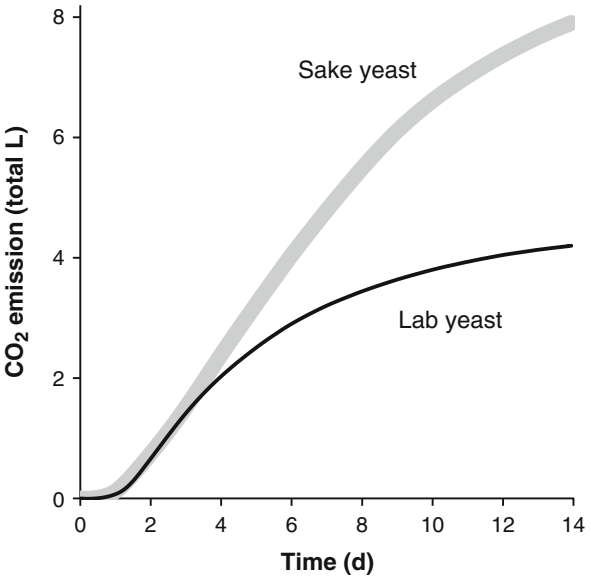

Fig. 4.1 Effects of different yeasts on the sake fermentation profile. Sake fermentation tests were performed using sake yeast (K7) or laboratory yeast (X2180). Fermentation rates were monitored by measuring CO_2 emission from the sake mash

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

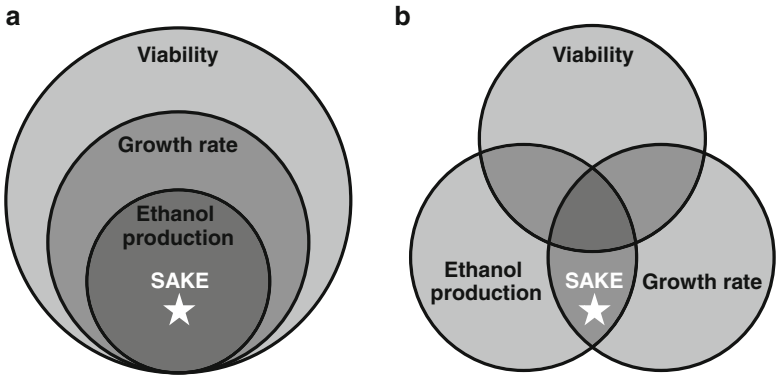

Fig. 4.2 Definitions of ethanol tolerance in yeast. Three *circles* indicate the sets of yeast strains with (i) high cellular viability under ethanol stress, (ii) high growth rates under ethanol stress, and (iii) high productivity of ethanol. Although it has traditionally been considered that these sets 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

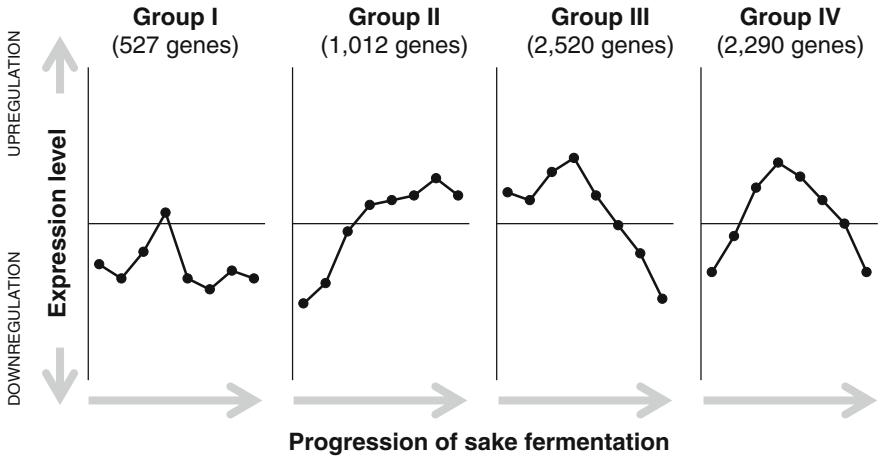

Fig. 4.3 Expression profiles during sake fermentation of the four gene groups established by *k*-means clustering. (Modified from Wu et al. 2006)

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

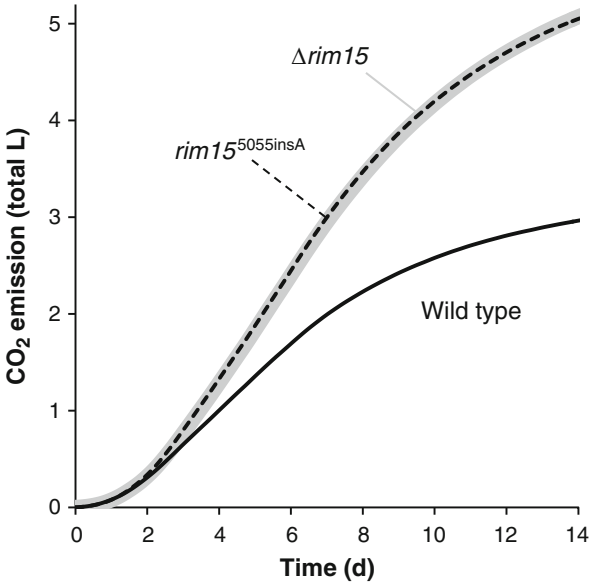

Fig. 4.4 Effects of deletion of the *RIM15* gene or the *rim15*^{5055insA} mutation on the sake fermentation profile. The fermentation rates were monitored by measuring CO_2 emission from the sake mash. (Modified from Watanabe et al. 2012)

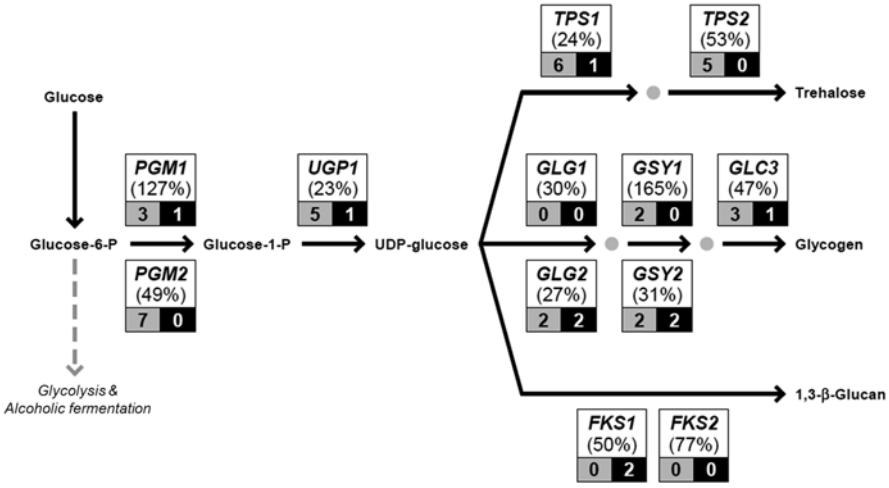

Fig. 4.5 Storage and structural carbohydrate synthesis-related gene expression in sake yeast. Percentages in *parentheses* indicate gene expression level ratios in K701 relative to X2180. Gene expression data during sake fermentation (day 5) were derived from Watanabe et al. (2011c). Values in *gray* 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.

References

Akao T, Yashiro I, Hosoyama A, Kitagaki H, Horikawa H, Watanabe D, Akada R, Ando Y, Harashima S, Inoue T, Inoue Y, Kajiwara S, Kitamoto K, Kitamoto N, Kobayashi O, Kuhara S, Masubuchi T, Mizoguchi H, Nakao Y, Nakazato A, Namise M, Oba T, Ogata T, Ohta A, Sato M, Shibasaki S, Takatsume Y, Tanimoto S, Tsuboi H, Nishimura A, Yoda K, Ishikawa T, Iwashita K, Fujita N, Shimoi H (2011) Whole-genome sequencing of sake yeast *Saccharomyces cerevisiae* Kyokai no. 7. DNA Res 18:423–434

Alper H, Moxley J, Nevoigt E, Fink GR, Stephanopoulos G (2006) Engineering yeast transcription machinery for improved ethanol tolerance and production. Science 314:1565–1568

Amorós M, Estruch F (2001) Hsf1p and Msn2/4p cooperate in the expression of *Saccharomyces cerevisiae* genes *HSP26* and *HSP104* in a gene- and stress type-dependent manner. Mol Microbiol 39:1523–1532

Aranda A, Querol A, del Olmo ML (2002) Correlation between acetaldehyde and ethanol resistance and expression of *HSP* genes in yeast strains isolated during the biological aging of sherry wines. Arch Microbiol 177(4):304–312

Azumi M, Goto-Yamamoto N (2001) AFLP analysis of type strains and laboratory and industrial strains of *Saccharomyces sensu stricto* and its application to phenetic clustering. Yeast 18:1145–1154

Bessonov K, Walkey CJ, Shelp BJ, van Vuuren HJ, Chiu D, van der Merwe G (2013) Functional analyses of *NSF1* in wine yeast using interconnected correlation clustering and molecular analyses. PLoS One 8:e77192

Bleoanca I, Silva AR, Pimentel C, Rodrigues-Pousada C, Menezes Rde A (2013) Relationship between ethanol and oxidative stress in laboratory and brewing yeast strains. J Biosci Bioeng 116:697–705

Blieck L, Toye G, Dumortier F, Verstrepen KJ, Delvaux FR, Thevelein JM, Van Dijck P (2007) Isolation and characterization of brewer's yeast variants with improved fermentation performance under high-gravity conditions. Appl Environ Microbiol 73:815–824

Boorsma A, Foat BC, Vis D, Klis F, Bussemaker HJ (2005) T-profiler: scoring the activity of predefined groups of genes using gene expression data. Nucleic Acids Res 33((Web Server Issue)):W592–W595

Borneman AR, Desany BA, Riches D, Affourtit JP, Forgan AH, Pretorius IS, Egholm M, Chambers PJ (2011) Whole-genome comparison reveals novel genetic elements that characterize the genome of industrial strains of *Saccharomyces cerevisiae*. PLoS Genet 7:e1001287

Cameroni E, Hulo N, Roosen J, Winderickx J, De Virgilio C (2004) The novel yeast PAS kinase Rim15 orchestrates G_0-associated antioxidant defense mechanisms. Cell Cycle 3:462–468

Casey GP, Ingledew WM (1986) Ethanol tolerance in yeasts. Crit Rev Microbiol 13:219–280

Causton HC, Ren B, Koh SS, Harbison CT, Kanin E, Jennings EG, Lee TI, True HL, Lander ES, Young RA (2001) Remodeling of yeast genome expression in response to environmental changes. Mol Biol Cell 12:323–337

D'Amore T, Stewart GG (1987) Ethanol tolerance of yeast. Enzyme Microb Technol 9:322–330

Eastmond DL, Nelson HC (2006) Genome-wide analysis reveals new roles for the activation domains of the *Saccharomyces cerevisiae* heat shock transcription factor (Hsf1) during the transient heat shock response. J Biol Chem 281:32909–32921

Estruch F, Carlson M (1993) Two homologous zinc finger genes identified by multicopy suppression in a *SNF1* protein kinase mutant of *Saccharomyces cerevisiae*. Mol Cell Biol 13:3872–3881

Ferguson SB, Anderson ES, Harshaw RB, Thate T, Craig NL, Nelson HC (2005) Protein kinase A regulates constitutive expression of small heat-shock genes in an Msn2/4p-independent and Hsf1p-dependent manner in *Saccharomyces cerevisiae*. Genetics 169:1203–1214

Furukawa K, Kitano H, Mizoguchi H, Hara S (2004) Effect of cellular inositol content on ethanol tolerance of *Saccharomyces cerevisiae* in sake brewing. J Biosci Bioeng 98:107–113

Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO (2000) Genomic expression programs in the response of yeast cells to environmental changes. Mol Biol Cell 11:4241–4257

Gibson BR, Boulton CA, Box WG, Graham NS, Lawrence SJ, Linforth RS, Smart KA (2008) Carbohydrate utilization and the lager yeast transcriptome during brewery fermentation. Yeast 25:549–562

Görner W, Durchschlag E, Martinez-Pastor MT, Estruch F, Ammerer G, Hamilton B, Ruis H, Schüller C (1998) Nuclear localization of the C_2H_2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. Genes Dev 12:586–597

Hahn JS, Thiele DJ (2004) Activation of the *Saccharomyces cerevisiae* heat shock transcription factor under glucose starvation conditions by Snf1 protein kinase. J Biol Chem 279:5169–5176

Hahn JS, Hu Z, Thiele DJ, Iyer VR (2004) Genome-wide analysis of the biology of stress responses through heat shock transcription factor. Mol Cell Biol 24:5249–5256

Hayashida S, Feng D, Hongo M (1974) Function of the high concentration alcohol-producing factor. Agric Biol Chem 38:2001–2006

Hong ME, Lee KS, Yu BJ, Sung YJ, Park SM, Koo HM, Kweon DH, Park JC, Jin YS (2010) Identification of gene targets eliciting improved alcohol tolerance in *Saccharomyces cerevisiae* through inverse metabolic engineering. J Biotechnol 149:52–59

Huuskonen A, Markkula T, Vidgren V, Lima L, Mulder L, Geurts W, Walsh M, Londesborough J (2010) Selection from industrial lager yeast strains of variants with improved fermentation performance in very-high-gravity worts. Appl Environ Microbiol 76:1563–1573

Imazu H, Sakurai H (2005) *Saccharomyces cerevisiae* heat shock transcription factor regulates cell wall remodeling in response to heat shock. Eukaryot Cell 4:1050–1056

Inai T, Watanabe D, Zhou Y, Fukada R, Akao T, Shima J, Takagi H, Shimoi H (2013) Rim15p-mediated regulation of sucrose utilization during molasses fermentation using *Saccharomyces cerevisiae* strain PE-2. J Biosci Bioeng 116:591–594

Ivorra C, Pérez-Ortín JE, del Olmo M (1999) An inverse correlation between stress resistance and stuck fermentations in wine yeasts. A molecular study. Biotechnol Bioeng 64:698–708

Jakobsen BK, Pelham HR (1988) Constitutive binding of yeast heat shock factor to DNA *in vivo*. Mol Cell Biol 8:5040–5042

Kitagaki H, Shimoi H (2007) Mitochondrial dynamics of yeast during sake brewing. J Biosci Bioeng 104:227–230

Kitagaki H, Takagi H (2014) Mitochondrial metabolism and stress response of yeast: applications in fermentation technologies. J Biosci Bioeng 117:383–393

Lee P, Kim MS, Paik SM, Choi SH, Cho BR, Hahn JS (2013) Rim15-dependent activation of Hsf1 and Msn2/4 transcription factors by direct phosphorylation in *Saccharomyces cerevisiae*. FEBS Lett 587:3648–3655

Liti G, Carter DM, Moses AM, Warringer J, Parts L, James SA, Davey RP, Roberts IN, Burt A, Koufopanou V, Tsai IJ, Bergman CM, Bensasson D, O'Kelly MJ, van Oudenaarden A, Barton DB, Bailes E, Nguyen AN, Jones M, Quail MA, Goodhead I, Sims S, Smith F, Blomberg A, Durbin R, Louis EJ (2009) Population genomics of domestic and wild yeasts. Nature 458:337–341

Liu XD, Thiele DJ (1996) Oxidative stress induced heat shock factor phosphorylation and HSF-dependent activation of yeast metallothionein gene transcription. Genes Dev 10:592–603

Luo X, Talarek N, De Virgilio C (2011) Initiation of the yeast G_0 program requires Igo1 and Igo2, which antagonize activation of decapping of specific nutrient-regulated mRNAs. RNA Biol 8:14–17

Ma M, Liu LZ (2010) Quantitative transcription dynamic analysis reveals candidate genes and key regulators for ethanol tolerance in *Saccharomyces cerevisiae*. BMC Microbiol 10:169

Marks VD, Ho Sui SJ, Erasmus D, van der Merwe GK, Brumm J, Wasserman WW, Bryan J, van Vuuren HJ (2008) Dynamics of the yeast transcriptome during wine fermentation reveals a novel fermentation stress response. FEMS Yeast Res 8:35–52

Martínez-Pastor MT, Marchler G, Schüller C, Marchler-Bauer A, Ruis H, Estruch F (1996) The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). EMBO J 15:2227–2235

Mendes-Ferreira A, del Olmo M, García-Martínez J, Jiménez-Martí E, Mendes-Faia A, Pérez-Ortín JE, Leão C (2007) Transcriptional response of *Saccharomyces cerevisiae* to different nitrogen concentrations during alcoholic fermentation. Appl Environ Microbiol 73:3049–3060

Mortimer RK, Johnston JR (1986) Genealogy of principal strains of the yeast genetic stock center. Genetics 113:35–43

Noguchi C, Watanabe D, Zhou Y, Akao T, Shimoi H (2012) Association of constitutive hyperphosphorylation of Hsf1p with a defective ethanol stress response in *Saccharomyces cerevisiae* sake yeast strains. Appl Environ Microbiol 78:385–392

Nozawa M, Takahashi T, Hara S, Mizoguchi H (2002) A role of *Saccharomyces cerevisiae* fatty acid activation protein 4 in palmitoyl-CoA pool for growth in the presence of ethanol. J Biosci Bioeng 93:288–295

Ogawa Y, Nitta A, Uchiyama H, Imamura T, Shimoi H, Ito K (2000) Tolerance mechanism of the ethanol-tolerant mutant of sake yeast. J Biosci Bioeng 90:313–320

Olesen K, Felding T, Gjermansen C, Hansen J (2002) The dynamics of the *Saccharomyces carlsbergensis* brewing yeast transcriptome during a production-scale lager beer fermentation. FEMS Yeast Res 2:563–573

Pedruzzi I, Dubouloz F, Cameroni E, Wanke V, Roosen J, Winderickx J, De Virgilio C (2003) TOR and PKA signaling pathways converge on the protein kinase Rim15 to control entry into G_0. Mol Cell 12:1607–1613

Pereira FB, Guimarães PM, Gomes DG, Mira NP, Teixeira MC, Sá-Correia I, Domingues L (2011) Identification of candidate genes for yeast engineering to improve bioethanol production in very high gravity and lignocellulosic biomass industrial fermentations. Biotechnol Biofuels 4:57

Rossignol T, Dulau L, Julien A, Blondin B (2003) Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. Yeast 20:1369–1385

Rossouw D, Bauer FF (2009) Comparing the transcriptomes of wine yeast strains: toward understanding the interaction between environment and transcriptome during fermentation. Appl Microbiol Biotechnol 84:937–954

Rossouw D, van den Dool AH, Jacobson D, Bauer FF (2010) Comparative transcriptomic and proteomic profiling of industrial wine yeast strains. Appl Environ Microbiol 76:3911–3923

Santos J, Sousa MJ, Cardoso H, Inácio J, Silva S, Spencer-Martins I, Leão C (2008) Ethanol tolerance of sugar transport, and the rectification of stuck wine fermentations. Microbiology 154:422–430

Sasano Y, Watanabe D, Ukibe K, Inai T, Ohtsu I, Shimoi H, Takagi H (2012) Overexpression of the yeast transcription activator Msn2 confers furfural resistance and increases the initial fermentation rate in ethanol production. J Biosci Bioeng 113:451–455

Schmitt AP, McEntee K (1996) Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 93:5777–5782

Shahsavarani H, Sugiyama M, Kaneko Y, Chuenchit B, Harashima S (2012) Superior thermotolerance of *Saccharomyces cerevisiae* for efficient bioethanol fermentation can be achieved by overexpression of *RSP5* ubiquitin ligase. Biotechnol Adv 30:1289–1300

Shobayashi M, Mitsueda S, Ago M, Fujii T, Iwashita K, Iefuji H (2005) Effects of culture conditions on ergosterol biosynthesis by *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 69:2381–2388

Sorger PK, Pelham HR (1987) Purification and characterization of a heat-shock element binding protein from yeast. EMBO J 6:3035–3041

Sorger PK, Pelham HR (1988) Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. Cell 54:855–864

Takahashi T, Shimoi H, Ito K (2001) Identification of genes required for growth under ethanol stress using transposon mutagenesis in *Saccharomyces cerevisiae*. Mol Genet Genomics 265:1112–1119

Talarek N, Cameroni E, Jaquenoud M, Luo X, Bontron S, Lippman S, Devgan G, Snyder M, Broach JR, De Virgilio C (2010) Initiation of the TORC1-regulated G_0 program requires Igo1/2, which license specific mRNAs to evade degradation via the 5′-3′ mRNA decay pathway. Mol Cell 38:345–355

Tao X, Zheng D, Liu T, Wang P, Zhao W, Zhu M, Jiang X, Zhao Y, Wu X (2012) A novel strategy to construct yeast *Saccharomyces cerevisiae* strains for very high gravity fermentation. PLoS One 7:e31235

Treger JM, Schmitt AP, Simon JR, McEntee K (1998) Transcriptional factor mutations reveal regulatory complexities of heat shock and newly identified stress genes in *Saccharomyces cerevisiae*. J Biol Chem 273:26875–26879

Urbanczyk H, Noguchi C, Wu H, Watanabe D, Akao T, Takagi H, Shimoi H (2011) Sake yeast strains have difficulty in entering a quiescent state after cell growth cessation. J Biosci Bioeng 112:44–48

Varela C, Cárdenas J, Melo F, Agosin E (2005) Quantitative analysis of wine yeast gene expression profiles under winemaking conditions. Yeast 22:369–383

Walkey CJ, Luo Z, Borchers CH, Measday V, van Vuuren HJ (2011) The *Saccharomyces cerevisiae* fermentation stress response protein Igd1p/Yfr017p regulates glycogen levels by inhibiting the glycogen debranching enzyme. FEMS Yeast Res 11:499–508

Walkey CJ, Luo Z, Madilao LL, van Vuuren HJ (2012) The fermentation stress response protein Aaf1p/Yml081Wp regulates acetate production in *Saccharomyces cerevisiae*. PLoS One 7:e51551

Watanabe M, Tamura K, Magbanua JP, Takano K, Kitamoto K, Kitagaki H, Akao T, Shimoi H (2007) Elevated expression of genes under the control of stress response element (STRE) and Msn2p in an ethanol-tolerance sake yeast Kyokai no. 11. J Biosci Bioeng 104:163–170

Watanabe M, Watanabe D, Akao T, Shimoi H (2009) Overexpression of *MSN2* in a sake yeast strain promotes ethanol tolerance and increases ethanol production in sake brewing. J Biosci Bioeng 107:516–518

Watanabe D, Nogami S, Ohya Y, Kanno Y, Zhou Y, Akao T, Shimoi H (2011a) Ethanol fermentation driven by elevated expression of the G_1 cyclin gene *CLN3* in sake yeast. J Biosci Bioeng 112:577–582

Watanabe D, Ota T, Nitta F, Akao T, Shimoi H (2011b) Automatic measurement of sake fermentation kinetics using a multi-channel gas monitor system. J Biosci Bioeng 112:54–57

Watanabe D, Wu H, Noguchi C, Zhou Y, Akao T, Shimoi H (2011c) Enhancement of the initial rate of ethanol fermentation due to dysfunction of yeast stress response components Msn2p and/or Msn4p. Appl Environ Microbiol 77:934–941

Watanabe D, Araki Y, Zhou Y, Maeya N, Akao T, Shimoi H (2012) A loss-of-function mutation in the PAS kinase Rim15p is related to defective quiescence entry and high fermentation rates of *Saccharomyces cerevisiae* sake yeast strains. Appl Environ Microbiol 78:4008–4016

Watanabe D, Hashimoto N, Mizuno M, Zhou Y, Akao T, Shimoi H (2013) Accelerated alcoholic fermentation caused by defective gene expression related to glucose derepression in *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 77:2255–2262

Wiederrecht G, Seto D, Parker CS (1988) Isolation of the gene encoding the *S. cerevisiae* heat shock transcription factor. Cell 54:841–853

Wu H, Zheng X, Araki Y, Sahara H, Takagi H, Shimoi H (2006) Global gene expression analysis of yeast cells during sake brewing. Appl Environ Microbiol 72:7353–7358

Wu H, Watanabe T, Araki Y, Kitagaki H, Akao T, Takagi H, Shimoi H (2009) Disruption of ubiquitin-related genes in laboratory yeast strains enhances ethanol production during sake brewing. J Biosci Bioeng 107:636–640

Xie MW, Jin F, Hwang H, Hwang S, Anand V, Duncan MC, Huang J (2005) Insights into TOR function and rapamycin response: chemical genomic profiling by using a high-density cell array method. Proc Natl Acad Sci U S A 102:7215–7220

Yamada T, Shimoi H, Ito K (2005) High expression of unsaturated fatty acid synthesis gene *OLE1* in sake yeasts. J Biosci Bioeng 99:512–516

Yamaji K, Hara S, Mizoguchi H (2003) Influence of Ras function on ethanol stress response of sake yeast. J Biosci Bioeng 96:474–480

Yang J, Bae JY, Lee YM, Kwon H, Moon HY, Kang HA, Yee SB, Kim W, Choi W (2011) Construction of *Saccharomyces cerevisiae* strains with enhanced ethanol tolerance by mutagenesis of the TATA-binding protein gene and identification of novel genes associated with ethanol tolerance. Biotechnol Bioeng 108:1776–1787

Yoshida S, Imoto J, Minato T, Oouchi R, Sugihara M, Imai T, Ishiguro T, Mizutani S, Tomita M, Soga T, Yoshimoto H (2008) Development of bottom-fermenting *Saccharomyces* strains that produce high SO_2 levels, using integrated metabolome and transcriptome analysis. Appl Environ Microbiol 74:2787–2796

Zuzuarregui A, del Olmo M (2004) Analyses of stress resistance under laboratory conditions constitute a suitable criterion for wine yeast selection. Antonie Van Leeuwenhoek 85:271–280

Chapter 5
Stress Responses of the Yeast *Saccharomyces cerevisiae* Under High Hydrostatic Pressure

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Abstract Effects of high hydrostatic pressure on biological systems have been mainly investigated from three perspectives: (1) structural understanding of macromolecules such as proteins and lipids and kinetic analysis of biochemical reactions, (2) adaptation of microbes such as mesophiles and piezophiles to high pressure, and (3) inactivation of food-spoiling microbes and application of nonthermal food processing. The yeast *Saccharomyces cerevisiae* has been an invaluable organism in establishing the molecular basis of cellular responses to high pressure as well as in applying a basic knowledge of the effects of pressure on industrial processes involving various microbes. In this chapter, the general effects of high pressure on biological systems and recent advances in research on the response of *S. cerevisiae* to high pressure with respect to intracellular pH homeostasis, significance of tryptophan uptake, ubiquitin-dependent degradation of tryptophan permeases, global analyses on transcription and gene functions, and attempts toward industrial applications are reviewed.

Keywords *DAN/TIR* family genes • Fermentation • Global functional analysis • Global transcriptional analysis • High hydrostatic pressure • High-pressure growth mutants • Hsp104 • Intracellular acidification • Rsp5 ubiquitin ligase • *Saccharomyces cerevisiae* • Tryptophan permeases Tat1 and Tat2 • Ubiquitination • Volume changes

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5.1 General Effects of High Hydrostatic Pressure on Biological Systems

The oceans occupy 70 % of the earth and have an average depth of 3,800 m. In terms of volume, the majority of the biosphere is made up of high-pressure environments. Therefore, organisms have to experience a broad range of hydrostatic pressures from 0.1 to 100 MPa (0.1 MPa = 1 bar = 0.9869 atm = 1.0197 kg of force/cm^2; for clarity, "MPa" is used throughout) in combination with varying temperatures. There is a growing interest in deep-sea microorganisms, namely "piezophiles," that prefer high hydrostatic pressure for growth (Yayanos 1995; Abe and Horikoshi 2001; Bartlett 2002; Oger and Jebbar 2010). However, the molecular basis of high-pressure adaptation has not yet been established in piezophiles occurring in natural environments because of the difficulty of cultivation and genetic manipulation (e.g., gene disruption, overexpression, or mutagenesis). Humans also experience high hydrostatic pressure. The knees are exposed to several dozen MPa of pressure during exercise (Muir 1995); the back teeth are also exposed to the same levels of pressure. Nonetheless, the mechanisms underlying the effects of high pressure on living systems are not yet sufficiently understood.

Inhibitory effects of high pressure on biological processes are listed in Table 5.1. In general, high hydrostatic pressures in the range of several dozen MPa do not readily kill microorganisms but exert adverse effects on the activities of organisms that are adapted to atmospheric pressure (Abe et al. 1999; Bartlett 2002). These effects depend not only on the magnitude but also on the duration of the applied pressure, temperature, pH, oxygen supply, and composition of culture media. Accordingly, the effects are very complex, thus making their interpretation difficult. Practically, oxygen supply is one of the limitations for a long-term culture of organisms in a closed hydrostatic chamber. The yeast *Saccharomyces cerevisiae* is a facultative anaerobe and thus a useful model organism in this experimental situation. The yeast genome encodes approximately 6,600 genes, including more than 4,800 nonessential genes. The recent, large-scale phenotypic screening of the *S. cerevisiae*

Table 5.1 Inhibitory effects of high pressure on biological processes

Cellular process/structure	Inhibitory pressure (MPa<)
Motility	10
Cell division	20
Nutrient uptake	15–20
Ethanol fermentation	25–50
Membrane protein function	25–50
Replication	50
Transcription	50–100
Protein synthesis	50
Protein oligomerization	50–100
Soluble enzyme function	100
Viability	100–200
Protein structure (monomer)	200
DNA structure (double helix)	1,000

gene-deletion library revealed numerous unexpected genes and metabolic pathways that are involved in the tolerance of environmental stress.

Application of pressure yields a fundamental physical parameter in any reaction, that is, volume change. The following two equations describe the effect of hydrostatic pressure on the equilibrium $A \leftrightarrow B$ and the reaction $A \rightarrow B$, respectively:

$$\left(\partial \ln K / \partial p\right)_T = -\Delta V / RT \tag{5.1}$$

$$\left(\partial \ln k / \partial p\right)_T = -\Delta V^{\neq} / RT, \tag{5.2}$$

where K is the equilibrium constant; k, rate constant; p, pressure (MPa); T, absolute temperature (K); R, gas constant (ml MPa K^{-1} mol^{-1}); ΔV, difference between the final and initial volumes in the entire system at equilibrium (reaction volume), including the solute and the surrounding solvent; and $\Delta V^{\neq}$, apparent volume change in activation (activation volume), representing the difference in volume between the reactants and the transition state. The direction and degree of pressure effects on any reaction are governed by the sign and magnitude of volume change. When a reaction is accompanied by volume increase, it is inhibited by increasing pressure. When a reaction is accompanied by volume decrease, it is facilitated by increasing pressure. A definite distinction between pressure and temperature is that temperature accelerates reactions, as defined by the Arrhenius equation, whereas pressure accelerates, inhibits, or does not affect reactions depending on the sign and magnitude of ΔV and $\Delta V^{\neq}$ values.

High hydrostatic pressure of approximately 100 MPa generally dissociates oligomeric proteins because hydration of charged groups, and exposure of nonpolar groups to water is usually accompanied by negative volume change (Gross and Jaenicke 1994; Meersman et al. 2006); therefore, high pressure prefers the dissociated forms of proteins in aqueous solution. Ribosome subunits are dissociated at pressures above 60 MPa in *Escherichia coli*, which could be the upper limit of pressure for growth of this bacterium (Gross and Jaenicke 1994). High pressure, above 200–300 MPa, causes unfolding of the protein monomer (Meersman et al. 2006; Fourme et al. 2012; Akasaka et al. 2013). During protein unfolding, water molecules penetrate into cavities within the proteins, and nonpolar groups are exposed to the solvent. Consequently, proteins form aggregates within the cell, which have harmful effects on cell viability.

Phase behavior of lipid bilayers is one of the most pressure-sensitive processes in biological systems. High pressure and low temperature increase the order of fatty acid acyl chains; consequently, the membrane becomes packed and its fluidity is decreased (Winter 2002; Matsuki et al. 2013). In artificial lipid bilayers such as dipalmitoylphosphatidylcholine, the temperature for the transition (T_m) from ripple gel ($P\beta'$) phase to a liquid crystalline ($L\alpha$) phase is 41.6 °C at atmospheric pressure; however, T_m increases to 66 °C at a pressure of 100 MPa (Ichimori et al. 1998). Although biological membranes do not exhibit a clear phase transition, high pressure inevitably stiffens the membranes, thereby affecting membrane proteins to a greater or lesser extent. In the following sections, the occurrence and responses of *S. cerevisiae* cells to high pressure are described (Table 5.2).

Table 5.2 Pressure-induced effects on yeast physiology and survival[a]

Pressure (MPa)	Effect/occurrence	Notes	References
0.1–100 MPa	Arrest of cell growth	Cell-cycle arrest in G_1 phase in Trp^- strains[b] (15–25 MPa and 24 °C)	Abe and Horikoshi (2000); Abe and Iida (2003)
		Trp^{+c} or *HPG* strains,[d] (50 MPa and 24 °C)	
	Metabolic change	Calorimetry	Tamura et al. (1999)
	Inhibition of amino acid uptake	Severity, Trp>Lys>His>Leu	Abe and Horikoshi (2000)
	Stress-inducible gene expression	During growth (25 MPa and 24 °C)	Abe (2007)
	Stress-inducible gene expression	During growth (30 MPa and 25 °C)	Iwahashi et al. (2005)
	Stress-inducible gene expression	After pressure release (40 MPa and 4 °C)	Iwahashi et al. (2003)
	Enhancement of esterase activity	Nonspecific esterases	Abe and Minegishi (2008)
	Inhibition of ethanol fermentation	Internal ATP level is unchanged.	Abe and Horikoshi (1997, 1998)
	Cytoplasmic and vacuolar acidification	Fluorescence analysis; internal pH decreases by 0.3–0.5 units	Abe and Horikoshi (1997, 1998)
	Ubiquitin-dependent degradation	Degradation of tryptophan permeases Tat1 and Tat2 (25 MPa and 25 °C)	Abe and Iida (2003)
	Growth defects	Nonessential gene disruptants (25 MPa and 24 °C)	Abe and Minegishi (2008)
	The role of unsaturated fatty acids	Increased viability, C18:3>C18:2>C18:1>C16:1	de Freitas et al. (2012)
100 MPa–	Reduction in viability	Colony-forming unit measurement	Iwahashi et al. (1991); Hamada et al. (1992); Kobori et al. (1995)
	Disruption of microtubules	Electron microscopy	Kobori et al. (1995); Sato et al. (1996)
	Depolymerization of F-actin	Electron microscopy	Sato et al. (1996, 1999)
	Nuclear membrane perturbation	Electron microscopy	Kobori et al. (1995); Sato et al. (1996, 1999)
	Acquired piezotolerance	Heat shock (42 °C); Hsp104, Hsc70, and trehalose have a role	Iwahashi et al. (1991, 1997, 2000)
	Acquired piezotolerance	Msn2/Msn4 transcription factors	Domitrovic et al. (2006)
	Stress-inducible gene expression	After pressure release (180 MPa and 4 °C)	Iwahashi et al. (2005)

(continued)

Table 5.2 (continued)

Pressure (MPa)	Effect/occurrence	Notes	References
200 MPa–	Acquired piezotolerance	H_2O_2, ethanol, or cold-shock treatment	Palhano et al. (2004)
	Induction of petite mutation	Strain- and growth phase dependent	Rosin and Zimmerman (1977)
	Induction of homozygous diploids	Dye-plate method	Hamada et al. (1992)
	Shrinkage of cells	Direct microscopic observation	Perrier-Cornet et al. (1995)
	Leakage of internal substrates	Amino acids and ions	Shimada et al. (1993)
	Stress-inducible gene expression	After pressure release (200 MPa, 30 min)	Fernandes et al. (2004)

[a]It should be noted that most effects are dependent on time, strain, growth phase, and analytical procedure
[b]*Trp*$^-$ tryptophan auxotrophic
[c]*Trp*$^+$ tryptophan prototrophic
[d]*HPG* high-pressure growth

5.2 High Pressure Induces Intracellular Acidification

High pressure affects chemical reactions of intracellular small molecules. During ethanol fermentation, large amounts of carbon dioxide (CO_2) are produced by yeast cells. At atmospheric pressure, more than 99 % of aqueous CO_2 exists as dissolved gas and less than 1 % exists as carbonic acid H_2CO_3, which partly dissociates to produce H^+, HCO_3^-, and CO_3^{2-}. The reaction volume (ΔV) of the equilibrium $H_2CO_3 \leftrightarrow H^+ + HCO_3^-$ is negative (−26.0 ml/mol), indicating that high pressure operates to shift the equilibrium considerably toward generating protons. The simple equilibrium shift occurs in the yeast cytoplasm during ethanol fermentation. Cytoplasmic pH and vacuolar pH were monitored in a hydrostatic chamber with transparent windows. Two pH-sensitive fluorescence probes, 5- (and 6-) carboxy SNARF-1 and 6-caroboxyfluorescein, were used for staining the cytoplasm and the vacuole, respectively (Abe and Horikoshi 1997, 1998). The labeled cells were placed in the chamber, and fluorescence was emitted through the window under various pressure conditions. High hydrostatic pressure in the range of 40–60 MPa acidified the cytoplasm, with a pH decrease by approximately 0.3 units; this resulted in the concomitant acidification of the vacuole (Abe and Horikoshi 1997). Because the key glycolytic enzyme phosphofructokinase is highly sensitive to acidic pH, high pressure will indirectly diminish ethanol fermentation in yeast. Neutral cytoplasmic pH is primarily maintained through proton extrusion by plasma membrane H^+-ATPase Pma1, whereas the acidic vacuolar pH is maintained by vacuolar H^+-ATPase. The pressure-induced internal acidification is attributed to the enhanced

generation of protons along with the equilibrium shift because it occurs only with fermentable sugars such as glucose or fructose, but not with nonfermentable carbohydrates such as ethanol or glycerol (Abe and Horikoshi 1998). The same levels of hydrostatic pressure activate nonspecific esterase in the cells (Abe 1998). Esterase activity influences the production of isoamyl alcohol and ethyl caproate, which are important flavor compounds of Japanese sake. Yeast cells undergo a very small fluctuation in hydrostatic pressure during circulation in a large fermenting vessel. Therefore, production of such flavor compounds may vary with the depth at which the yeast cells exist. Although the relationship between internal acidification and esterase activation is unclear, the chemical reactions of internal small molecules should be considered for elucidating the physiological effects of high hydrostatic pressure in living yeast cells.

5.3 Tryptophan Availability Is a Limiting Factor for Cell Growth Under High Pressure

Experimental *S. cerevisiae* strains such as YPH499 and W303 carry nutrient auxotrophic markers (e.g., *ade2*, *ura3*, *his3*, *lys3*, *leu2*, and *trp1*) for plasmid selection. Tryptophan auxotrophic strains appear to be highly sensitive to high pressure. Regardless of other auxotrophic markers, Trp^- strains exhibit growth defects at nonlethal hydrostatic pressures. When the cells are exposed to pressures of 15–25 MPa for 5–10 h, the cell cycle is arrested in the G_1 phase without any loss of viability (Abe and Horikoshi 2000). Trp^- strains also exhibit growth defects at low temperature, 10–15 °C. The sensitivity of Trp^- cells to high pressure and low temperature is thought to have originated from the following two specific properties of tryptophan uptake mediated by tryptophan permeases Tat1 and Tat2: (1) Tat1 and Tat2 activity is readily compromised by stiffening the membrane either by high pressure or low temperature, and (2) Tat1 and Tat2 undergo ubiquitination-dependent degradation in response to high pressure (see following). Trp^+ strains, including distillers' yeasts, are capable of growing at 15–25 MPa; however, the growth rate is decreased. Addition of excess amounts of tryptophan in the growth medium or overexpression of *TAT1* or *TAT2* in a multicopy plasmid confers Trp^- strains the ability to grow at 25 MPa (Abe and Horikoshi 2000; Abe and Iida 2003). Accordingly, *S. cerevisiae* cells are capable of growing at pressures up to 25 MPa if tryptophan availability is ensured.

5.4 Ubiquitin-Dependent Degradation of Tryptophan Permeases in Response to High Pressure

Even at nonlethal levels, hydrostatic pressure results in the structural perturbation of biological membranes, thus directly or indirectly affecting membrane proteins. Denatured membrane proteins are likely to undergo ubiquitination-dependent

degradation (Hershko and Ciechanover 1998). Ubiquitination was initially described to promote proteasomal degradation of proteins and has since been shown to regulate other processes, including DNA repair, signaling, endocytosis, or membrane trafficking (Lauwers et al. 2010). The ubiquitin ligase (E3) Rsp5 complex plays an essential role in the ubiquitination and subsequent endocytosis of yeast plasma membrane proteins by vacuole for degradation (Belgareh-Touze et al. 2008; Rotin and Kumar 2009). The Rsp5 complex is also required for the ubiquitination of endosomal proteins. The primary structure of Rsp5, including the N-terminal C2 domain, three central WW domains, and C-terminal HECT (homologous to E6-AP C-terminus) domain, is similar to that of Nedd4 family proteins. Ubiquitination of Tat2 was initially reported as a starvation response of yeast, in which covalent binding of ubiquitin occurs on one or more of the five lysine residues within the N-terminal cytoplasmic domain (Beck et al. 1999). Then, ubiquitin-bound Tat2 undergoes endocytosis, followed by vacuolar degradation. Upon exposure of the cells to high pressure, Tat2 also undergo ubiquitination-dependent degradation.

High-pressure growth (*HPG*) mutants, which are capable of growing at 25 MPa, were isolated from Trp^- strain YPH499 and were classified into four semidominant complementation groups, namely, *HPG1* to *HPG4* (Abe and Iida 2003). *HPG1* mutation sites were found within the catalytic HECT domain of Rsp5. Because of the reduction in Rsp5 activity, Tat2 becomes undegraded, leading to a substantial accumulation in the plasma membrane (Abe and Iida 2003). Given that the accumulated Tat2 proteins retain some tryptophan import activity, *HPG1* cells become endowed with the ability to grow at high pressure. *HPG2* mutation sites were found within the N- and C-terminal cytoplasmic domains of Tat2 (E27F, D563N, or E570K) (Nagayama et al. 2004). All three *HPG2* mutations result in the loss of a negative charge within the cytoplasmic tails. Accordingly, negatively charged residues at certain positions in the cytoplasmic tails might be required for recruiting the Rsp5 complex through ionic interactions. The Rsp5 WW domains are responsible for binding to proteins that have a PPxY motif (Gupta et al. 2007). However, most substrate proteins do not have a canonical PPxY motif. In this situation, several Rsp5 adaptors are characterized, acting on ubiquitination. Bul1 is a PPxY motif-containing protein that was initially identified as an Rsp5-binding protein (Yashiroda et al. 1996). Loss of Bul1 and its close homologue Bul2 results in a considerable accumulation of Tat2. Eventually, the *bul1*Δ*bul2*Δ mutant becomes capable of growing under high pressure (Abe and Iida 2003). The W > G mutation of the WW3 domain also results in Tat2 accumulation (Hiraki and Abe 2010a). Therefore, Bul1 and/or Bul2 are likely to interact with Rsp5 on the WW3 domain to ubiquitinate Tat2. Other PPxY motif-containing proteins Ssh1, Ear1, and Sna3 also contribute to Rsp5-Tat2 interaction because overexpression of one of these three proteins inhibits Tat2 degradation (Hiraki and Abe 2010b). Thus, Ssh1, Ear1, and Sna3 impinge on Tat2 in the direction opposite to Bul1 and Bul2. Although Tat2 is a short-lived protein, the other tryptophan permease Tat1 is a long-lived protein with a half-life of more than 3 h. High pressure of 25 MPa dramatically accelerates Tat1 degradation in an Rsp5-dependent manner (Suzuki et al. 2013). In this process, multiple adaptors, including Bul1/Bul2 and arrestin-like proteins ARTs, have a redundant role in

mediating Rsp5 and Tat1. However, how the denatured states of Tat1 and Tat2 are defined in terms of structural alterations and how denatured proteins are recognized by the Rsp5 complex remains unclear.

5.5 Global Screening of Genes Responsible for Growth Under High Pressure

As already mentioned, tryptophan availability is the primal requirement for yeast to grow under high pressure. However, it has been shown that many genes other than those involved in tryptophan biosynthesis or uptake are responsible for growth under high pressure. Tryptophan prototrophic strain BY4742 is capable of growing at pressures up to 30 MPa. By using BY4742 as a parental strain, systematic gene deletion has been performed using PCR-generated deletion strategy (http://www-sequence.stanford.edu/group/yeast_deletion_project/project_desc.html#intro) (Giaever et al. 2002). Of the 6,600 genes encoded by the yeast genome, single deletions in approximately 4,800 genes do not cause lethality under normal culture conditions. These genes are assumed to be necessary for particular cellular functions such as stress resistance or sporulation, although some of them are functionally redundant. Genetic defects that result in sensitivity to high pressure (25 MPa and 24 °C) or low temperature (0.1 MPa and 15 °C) were elucidated using the deletion library in which 1 of the 4,800 nonessential genes was disrupted: This procedure revealed 80 genes, of which 71 were required for growth under high pressure and 56 were required for growth under low temperature (Abe and Minegishi 2008). Given the significant overlap of 47 genes, a number of shared biological processes support growth under both high pressure and low temperature. The 80 genes are involved in a broad range of cellular functions such as amino acid biosynthesis, mitochondrial function, actin cytoskeleton formation, membrane trafficking, transcription, ribosome biogenesis, chromatin structure, and some unknown functions (Abe and Minegishi 2008). It should be noted that the 80 deletion mutants display a broad range of sensitivities to high pressure and low temperature. Genes involved in the biosynthesis of aromatic amino acids, such as *ARO1*, *ARO2*, *TRP1*, *TRP2*, *TRP4*, and *TRP5*, are highly important for growth under high pressure and low temperature, which is consistent with the defective growth observed in Trp^- strains as previously described. Deletions in *HOM3*, *THR4*, and *SER1* result in auxotrophy for methionine (*hom3*Δ), threonine (*thr4*Δ), and serine (*ser1*Δ); thus, the growth of yeast cells harboring these deletions depends on the corresponding amino acid permeases. Similarly, deletions in two mitochondrial protein genes, *ACO1* and *CAF1*, result in auxotrophy for glutamine or glutamate. Therefore, multiple amino acid permeases are the likely targets of high pressure and low temperature. Genes encoding mitochondrial ribosomal subunits (*MRPL22*, *MRP51*, and *MRPL38*) or a protein involved in the folding of mitochondrially synthesized proteins (*MDJ1*) are also required for growth under the conditions.

Genes that give rise to remarkable growth sensitivity under high pressure and low temperature upon deletion are those encoding for components of the EGO complex (*EGO1*, *EGO3*, *GTR1*, and *GTR2*) (Dubouloz et al. 2005; Abe and Minegishi 2008). The EGO complex is a vacuolar membrane-associated protein complex comprising Ego1 (also known as Meh1 and Gse2), Ego3 (also known as Slm4, Nir1 and Gse1), and Gtr2 (Dubouloz et al. 2005). Cells lacking one of the EGO components normally show growth arrest after the addition of the immunosuppressive drug rapamycin to culture medium and do not revert to the growth phase upon the release of the rapamycin block. The EGO complex interacts with and activates the target of rapamycin complex 1 (TORC1) in an amino acid-sensitive manner (Binda et al. 2009). Taken together, high pressure and low temperature are likely to compromise biological processes that are involved in amino acid signaling in yeast cells. Requirement of TORC1 and EGO complex for growth under high pressure and low temperature could be a common feature in eukaryotes because of the substantial conservation in the primary structures.

Numerous genes involved in membrane trafficking (*VID24*, *VPS34*, *SEC22*, *PEP3*, *CHC1*, *PEP5*, *VPS45*, *ERG24*, *VPS54*, *AKR1*, and *SAC1*) are responsible for growth under high pressure and low temperature (Abe and Minegishi 2008). The products of these genes might facilitate the delivery of newly synthesized proteins to appropriate locations, such as the bud neck, cell surface, or cell wall, under high pressure and low temperature. The appropriate actin network is a prerequisite for bud formation and polarized cell growth in yeast. Several genes involved in these processes (*LTE1*, *HOF1*, *SLM3*, *CLA4*, *CDC50*, and *SLM6*) are required for growth under high pressure and low temperature. Of these, *CDC50* encodes a protein that interacts with Drs2, a P-type ATPase of the aminophospholipid translocase (Kishimoto et al. 2005). Drs2 is required for the organization of the actin cytoskeleton and trafficking of proteins between the Golgi complex and the endosome/vacuole (Chen et al. 2006). The lack of *DRS2* also confers high-pressure and low-temperature sensitivity. Therefore, the Drs2–Cdc50 complex is likely to modulate protein trafficking to establish appropriate actin network formation under high pressure and low temperature. Ergosterol is a major constituent of the plasma membrane and has multiple functions such as maintenance of plasma membrane rigidity and solute impermeability, resistance to alkaline cations, and trafficking of membrane proteins. Deletion mutants for one of the enzymes that catalyze the later steps of ergosterol biosynthesis, such as *ERG24*, *ERG2*, *ERG6*, and *ERG3*, accumulate structurally abnormal sterols in the cells. Such mutants exhibit marked high-pressure and low-temperature sensitivity. Therefore, ergosterol plays a role in maintaining an appropriate membrane property that opposes the stiffening effect of high pressure and low temperature.

Genes involved in transcription and mRNA degradation (*SNF6*, *MOT2*, *POP2*, *SHE3*, *CDC73*, *RPB4*, *HFI1*, *PAF1*, *ELF1*, *SNF1*, *SRB5*, *TAF14*, *CCR4*, and *SAP155*) comprise a major class of essential genes for growth under high pressure and low temperature (Abe and Minegishi 2008). Among them, the loss of a constituent of the Ccr4–Not complex (Pop2, Not4, or Cdc73) results in marked sensitivity. This finding raises the possibility that the Ccr4–Not complex might fail

to assemble in cells under high pressure and low temperature if Pop2, Not4, or Cdc73 is lost. Eventually, several genes under its control will not be transcribed. Twelve genes without any functional annotation were obtained in the same screening. The products of these genes might have an overlapping function with any proteins with functional identification.

5.6 Resistance of Cells to Lethal Levels of High Pressure

High hydrostatic pressure, greater than 100 MPa, is considered to be lethal. This fact is put to practical application for sterilization of foodstuffs without heat treatment (Reineke et al. 2013). A pressure of 100 MPa increases the occurrence of cytoplasmic petite mutants that are characterized by a small colony size and respiration deficiency of cells, reflecting high-pressure sensitivity of the mitochondrial function (Rosin and Zimmerman 1977). Pressures of 100–150 MPa disrupt the spindle pole bodies and microtubules (Kobori et al. 1995), and those of 200–250 MPa induce tetraploids and homozygous diploids, potentially providing industrial uses for strains with increased growth rates (Hamada et al. 1992). It is well known that a mild heat treatment (e.g., 42 °C for 30 min) dramatically increases the viability of cells against a subsequent severe heat shock treatment (e.g., 50 °C for 10 min). Among the numerous heat-shock proteins (Hsps), the molecular chaperone Hsp104 is essential in this acquired heat tolerance by unfolding denatured intracellular proteins in an ATP-dependent manner (Glover and Lindquist 1998). Such a mild heat treatment also allows the cells to survive high pressures of 140–180 MPa, with a 100- to 1,000-fold increase in viability (Iwahashi et al. 1991). After high-pressure treatment at 140 MPa, Hsp104 associates with an insoluble protein fraction, suggesting that Hsp104 unfolds high pressure-induced denatured intracellular proteins (Iwahashi et al. 1997, 2000). Indeed, the *hsp104*Δ mutant is unable to acquire heat-inducible high-pressure tolerance (hereafter, piezotolerance; (Iwahashi et al. 1997). Cold shock and moderate concentration of H_2O_2 or ethanol also increase piezotolerance of the cells (Palhano et al. 2004). Similarly, a pressure treatment at a nonlethal level (50 MPa for 1 h) increases the viability of the cells at 200 MPa (Domitrovic et al. 2006). The acquired piezotolerance by moderate pressure treatment is governed by two transcription factors, Msn2 and Msn4, that are induced by various types of stress. The loss of both *MSN2* and *MSN4* genes results in susceptibility to high pressure (Domitrovic et al. 2006). However, how high pressure regulates Msn2 and Msn4 after the transcription of their downstream genes remains to be clarified. Trehalose is a nonreducing disaccharide known to protect proteins, membranes, and other macromolecules against various stresses. This disaccharide plays a role in the piezotolerance of yeast by preventing the formation of protein aggregates and promoting the refolding of Hsp104. Although yeast mutants defective in the accumulation of trehalose are susceptible to increasing pressure (Iwahashi et al. 1997), a mutant lacking neutral trehalase Nth1, which

catalyzes the hydrolysis of one trehalose molecule to two glucose molecules, shows susceptibility to high pressure (Iwahashi et al. 2000). Neutral trehalase is required for recovery after high-pressure treatment but is not required during the treatment. Accordingly, glucose is likely to be required as an energy source for recovery from pressure-induced damage at atmospheric pressure.

5.7 Transcriptional Regulation Under High Pressure

Global transcriptional analysis under high pressure has been performed using DNA microarray hybridization in several laboratories, but the results vary with strains and experimental conditions. Under a pressure of 30 MPa and 25 °C, which allows the growth of tryptophan prototrophic strains, 366 genes were upregulated by more than twofold and 253 genes were downregulated by more than twofold (Iwahashi et al. 2005). According to the functional categories of the Munich Information Center for Protein Sequences (http://mips.gsf.de/), the highly upregulated genes were categorized to cell cycle and DNA processing, cell rescue, defense and virulence, and metabolism. It is rationalized that heat shock-responsible genes such as *HSP12*, *HSP150*, *HSP26*, *SSE2*, and *HSP104* are induced by high pressure in terms of the acquired piezotolerance (Iwahashi et al. 2005). However, the result does not fully coincide with another report showing that *HSP104*, *HSP10*, *HSP78*, *HSP30*, *HSP42*, and *HSP82* are upregulated by more than twofold but others are not (Abe 2007). This difference is possibly the result of differences in strains (S288C versus BY4742), culture medium (YPD versus SC medium), and culture conditions (30 MPa and 25 °C for 16 h versus 25 MPa and 24 °C for 5 h). In addition, deletion of *HSP104*, *HSP78*, *HSP30*, *HSP42*, *HSP82*, *HSP150*, *HSP12*, or *HSP26* resulted in a fourfold increase in the survival rate at 125 MPa from 1 % to 4 %, suggesting that the cellular defensive system against high pressure could be strengthened upon the loss of *HSP* genes (Miura et al. 2006). The function of Hsp104 is seemingly contradictory, but these observations indicate that cell death at 140–180 MPa is mainly caused by the misfolding or denaturation of cellular proteins whereas cell death at 125 MPa could occur for other reasons.

High pressure and low temperature also upregulate the transcription of *DAN/TIR* family mannoprotein genes, which are optimally expressed under hypoxia (Abramova et al. 2001). In support of the role of mannoproteins in cell wall integrity, cells preexposed to high pressure or low temperature acquire tolerance against treatment with low concentrations of sodium dodecyl sulfate (SDS) and zymolyase or at lethal levels of high pressure (125 MPa for 1 h) (Abe 2007). However, how seemingly unrelated environmental factors induce the *DAN/TIR* family genes remains to be addressed. High pressure and low temperature increase the order of fatty acid acyl chains in the membrane, thus decreasing membrane fluidity. Hypoxia potentially exerts a decrease in membrane fluidity because acyl chain unsaturation, which requires molecular oxygen, could be compromised under hypoxia.

In this sense, membrane sensor(s) likely exist in the membrane and might transduce the changes in membrane fluidity into an intracellular signal that stimulates the transcription of *DAN/TIR* family genes.

Global transcriptional analysis was performed under pressures that did not allow yeast cell growth at 180 MPa and 4 °C or 40 MPa and 4 °C. During recovery after the treatments, transcription of genes required for energy metabolism, cell defense, and protein metabolism was highly upregulated (Iwahashi et al. 2003). According to a hierarchical clustering analysis, transcriptional patterns at growth-nonpermissive pressures are analogous to the treatment of cells with detergents such as SDS or sodium *n*-dodecyl benzene sulfonate (LAS). Hence, membrane perturbation by high hydrostatic pressure or detergents might have a direct effect on gene expression on a genome-wide level (Iwahashi et al. 2003). Fatty acid composition affects cell survival under lethal levels of pressure at 150–200 MPa. A strain lacking Ole1, a membrane-bound Δ9 desaturase, was cultured in a medium supplemented with various fatty acids such as palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2), or linolenic acid (C18:3). Subsequently, the cells were subjected to pressures of 150–200 MPa for 30 min. The order of promoting survival by fatty acids was linolenic acid > linoleic acid > oleic acid > palmitoleic acid, indicating that a higher proportion of unsaturated fatty acids would help maintain a favorable membrane fluidity under high pressure and that reduced membrane fluidity was fatal under high pressure (de Freitas et al. 2012). However, reduced membrane fluidity is not the sole reason for high pressure-induced cell death because yeast cells are still alive at low temperatures of 0–4 °C.

Pretreatment with a nonlethal pressure level increases ethanol production in a yeast strain occurring in Brazilian spirit. After 4 h of fermentation, ethanol concentration reached 0.3 % in cells treated with a pressure of 50 MPa for 30 min whereas cells treated with no pressure produced negligible levels of ethanol (Bravim et al. 2013). After extended time periods, the difference in ethanol production between the two treatments became small. Global transcriptional analysis of this naturally occurring strain to identify the genes responsible for pressure-enhanced ethanol fermentation showed that genes associated with stress response (*CTT1*, *SOD2*, *STF1*, *GAC1*, *HSP12*, *HSP26*, *HSP30*, *HSP104*, *SSE2*, and *SYM1*), methionine biosynthesis and transport (*SAM3*, *MET14*, *MET16*, *MUP3*, *MET2*, and *MXR1*), glutamate transformation (*GAD1*, *UGA1*, and *UGA2*), or glycogen and trehalose metabolism (*TPS1*, *GSY2*, *NTH1*, and *NTH2*) were upregulated during recovery from pressure treatment (Bravim et al. 2013). Among the ten genes responsible for stress response, *SYM1* conferred resistance of 12–15 % and enhanced the production of ethanol upon its overexpression. Sym1 is a mitochondrial matrix protein, and its overexpression might have a general defect in NADH oxidation. To compensate for the defect, NADH could be oxidized by ethanol production in the cytoplasm, leading to enhanced ethanol production. In this way, application of high hydrostatic pressure offers an opportunity to modulate gene expression associated with the fermentation capacity of yeast in industrial production.

5.8 Conclusion

Hydrostatic pressure is a thermodynamic parameter that merely affects equilibria or the rate constant of chemical reactions without introducing any components into the experimental system. The system returns to its original state after the release of pressure. Differing from heat treatment, hydrostatic pressure uniformly transmits through the system in a moment. In this sense, hydrostatic pressure is a unique tool to modulate cellular metabolism or material production with saving energy. However, we have not completely understood the effects of high pressure on living systems or how to handle the complex cellular responses caused by high pressure. Evidently, a mechanistic understanding of the effects of high pressure on proteins, membranes, or intracellular small compounds is necessary to elucidate the complete picture of cellular responses to high pressure. Furthermore, advanced studies at levels of the transcriptome, proteome, and metabolome should converge on the analysis in a unified way of using the same yeast strains, media, temperature, or holding time of pressure in worldwide research.

References

Abe F (1998) Hydrostatic pressure enhances vital staining with carboxyfluorescein or carboxydichlorofluorescein in *Saccharomyces cerevisiae*: efficient detection of labeled yeasts by flow cytometry. Appl Environ Microbiol 64:1139–1142

Abe F (2007) Induction of *DAN/TIR* yeast cell wall mannoprotein genes in response to high hydrostatic pressure and low temperature. FEBS Lett 581:4993–4998

Abe F, Horikoshi K (1997) Vacuolar acidification in *Saccharomyces cerevisiae* induced by elevated hydrostatic pressure is transient and is mediated by vacuolar H^+-ATPase. Extremophiles 1:89–93

Abe F, Horikoshi K (1998) Analysis of intracellular pH in the yeast *Saccharomyces cerevisiae* under elevated hydrostatic pressure: a study in baro-(piezo-)physiology. Extremophiles 2: 223–228

Abe F, Horikoshi K (2000) Tryptophan permease gene *TAT2* confers high-pressure growth in *Saccharomyces cerevisiae*. Mol Cell Biol 20:8093–8102

Abe F, Horikoshi K (2001) The biotechnological potential of piezophiles. Trends Biotechnol 19: 102–108

Abe F, Iida H (2003) Pressure-induced differential regulation of the two tryptophan permeases Tat1 and Tat2 by ubiquitin ligase Rsp5 and its binding proteins, Bul1 and Bul2. Mol Cell Biol 23:7566–7584

Abe F, Minegishi H (2008) Global screening of genes essential for growth in high-pressure and cold environments: searching for basic adaptive strategies using a yeast deletion library. Genetics 178:851–872

Abe F, Kato C, Horikoshi K (1999) Pressure-regulated metabolism in microorganisms. Trends Microbiol 7:447–453

Abramova N, Sertil O, Mehta S, Lowry CV (2001) Reciprocal regulation of anaerobic and aerobic cell wall mannoprotein gene expression in *Saccharomyces cerevisiae*. J Bacteriol 183: 2881–2887

Akasaka K, Kitahara R, Kamatari YO (2013) Exploring the folding energy landscape with pressure. Arch Biochem Biophys 531:110–115

Bartlett DH (2002) Pressure effects on in vivo microbial processes. Biochim Biophys Acta 1595:367–381

Beck T, Schmidt A, Hall MN (1999) Starvation induces vacuolar targeting and degradation of the tryptophan permease in yeast. J Cell Biol 146:1227–1238

Belgareh-Touze N, Leon S, Erpapazoglou Z, Stawiecka-Mirota M, Urban-Grimal D, Haguenauer-Tsapis R (2008) Versatile role of the yeast ubiquitin ligase Rsp5p in intracellular trafficking. Biochem Soc Trans 36:791–796

Binda M, Peli-Gulli MP, Bonfils G, Panchaud N, Urban J, Sturgill TW, Loewith R, De Virgilio C (2009) The Vam6 GEF controls TORC1 by activating the EGO complex. Mol Cell 35: 563–573

Bravim F, Lippman SI, da Silva LF, Souza DT, Fernandes AA, Masuda CA, Broach JR, Fernandes PM (2013) High hydrostatic pressure activates gene expression that leads to ethanol production enhancement in a *Saccharomyces cerevisiae* distillery strain. Appl Microbiol Biotechnol 97:2093–2107

Chen S, Wang J, Muthusamy BP, Liu K, Zare S, Andersen RJ, Graham TR (2006) Roles for the Drs2p-Cdc50p complex in protein transport and phosphatidylserine asymmetry of the yeast plasma membrane. Traffic 7:1503–1517

de Freitas JM, Bravim F, Buss DS, Lemos EM, Fernandes AA, Fernandes PM (2012) Influence of cellular fatty acid composition on the response of *Saccharomyces cerevisiae* to hydrostatic pressure stress. FEMS Yeast Res 12:871–878

Domitrovic T, Fernandes CM, Boy-Marcotte E, Kurtenbach E (2006) High hydrostatic pressure activates gene expression through Msn2/4 stress transcription factors which are involved in the acquired tolerance by mild pressure precondition in *Saccharomyces cerevisiae*. FEBS Lett 580:6033–6038

Dubouloz F, Deloche O, Wanke V, Cameroni E, De Virgilio C (2005) The TOR and EGO protein complexes orchestrate microautophagy in yeast. Mol Cell 19:15–26

Fernandes PM, Domitrovic T, Kao CM, Kurtenbach E (2004) Genomic expression pattern in *Saccharomyces cerevisiae* cells in response to high hydrostatic pressure. FEBS Lett 556: 153–160

Fourme R, Girard E, Akasaka K (2012) High-pressure macromolecular crystallography and NMR: status, achievements and prospects. Curr Opin Struct Biol 22:636–642

Giaever G, Chu AM, Ni L, Connelly C, Riles L, Veronneau S, Dow S, Lucau-Danila A, Anderson K, Andre B, Arkin AP, Astromoff A, El-Bakkoury M, Bangham R, Benito R, Brachat S, Campanaro S, Curtiss M, Davis K, Deutschbauer A, Entian KD, Flaherty P, Foury F, Garfinkel DJ, Gerstein M, Gotte D, Guldener U, Hegemann JH, Hempel S, Herman Z, Jaramillo DF, Kelly DE, Kelly SL, Kotter P, LaBonte D, Lamb DC, Lan N, Liang H, Liao H, Liu L, Luo C, Lussier M, Mao R, Menard P, Ooi SL, Revuelta JL, Roberts CJ, Rose M, Ross-Macdonald P, Scherens B, Schimmack G, Shafer B, Shoemaker DD, Sookhai-Mahadeo S, Storms RK, Strathern JN, Valle G, Voet M, Volckaert G, Wang CY, Ward TR, Wilhelmy J, Winzeler EA, Yang Y, Yen G, Youngman E, Yu K, Bussey H, Boeke JD, Snyder M, Philippsen P, Davis RW, Johnston M (2002) Functional profiling of the *Saccharomyces cerevisiae* genome. Nature (Lond) 418:387–391

Glover JR, Lindquist S (1998) Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. Cell 94:73–82

Gross M, Jaenicke R (1994) Proteins under pressure. The influence of high hydrostatic pressure on structure, function and assembly of proteins and protein complexes. Eur J Biochem 221: 617–630

Gupta R, Kus B, Fladd C, Wasmuth J, Tonikian R, Sidhu S, Krogan NJ, Parkinson J, Rotin D (2007) Ubiquitination screen using protein microarrays for comprehensive identification of Rsp5 substrates in yeast. Mol Syst Biol 3:116

Hamada K, Nakatomi Y, Shimada S (1992) Direct induction of tetraploids or homozygous diploids in the industrial yeast *Saccharomyces cerevisiae* by hydrostatic pressure. Curr Genet 22: 371–376

Hershko A, Ciechanover A (1998) The ubiquitin system. Annu Rev Biochem 67:425–479
Hiraki T, Abe F (2010a) Overexpression of Sna3 stabilizes tryptophan permease Tat2, potentially competing for the WW domain of Rsp5 ubiquitin ligase with its binding protein Bul1. FEBS Lett 584:55–60
Hiraki T, Abe F (2010b) Overexpression of *EAR1* and *SSH4* that encode PPxY proteins in the multivesicular body provides stability of tryptophan permease Tat2 allowing yeast cells to grow under high hydrostatic pressure. High Press Res 30:514–518
Ichimori H, Hata T, Matsuki H, Kaneshina S (1998) Barotropic phase transitions and pressure-induced interdigitation on bilayer membranes of phospholipids with varying acyl chain lengths. Biochim Biophys Acta 1414:165–174
Iwahashi H, Kaul SC, Obuchi K, Komatsu Y (1991) Induction of barotolerance by heat shock treatment in yeast. FEMS Microbiol Lett 64:325–328
Iwahashi H, Obuchi K, Fujii S, Komatsu Y (1997) Effect of temperature on the role of Hsp104 and trehalose in barotolerance of *Saccharomyces cerevisiae*. FEBS Lett 416:1–5
Iwahashi H, Nwaka S, Obuchi K (2000) Evidence for contribution of neutral trehalase in barotolerance of *Saccharomyces cerevisiae*. Appl Environ Microbiol 66:5182–5185
Iwahashi H, Shimizu H, Odani M, Komatsu Y (2003) Piezophysiology of genome wide gene expression levels in the yeast *Saccharomyces cerevisiae*. Extremophiles 7:291–298
Iwahashi H, Odani M, Ishidou E, Kitagawa E (2005) Adaptation of *Saccharomyces cerevisiae* to high hydrostatic pressure causing growth inhibition. FEBS Lett 579:2847–2852
Kishimoto T, Yamamoto T, Tanaka K (2005) Defects in structural integrity of ergosterol and the Cdc50p-Drs2p putative phospholipid translocase cause accumulation of endocytic membranes, onto which actin patches are assembled in yeast. Mol Biol Cell 16:5592–5609
Kobori H, Sato M, Tameike A, Hamada K, Shimada S, Osumi M (1995) Ultrastructural effects of pressure stress to the nucleus in *Saccharomyces cerevisiae*: a study by immunoelectron microscopy using frozen thin sections. FEMS Microbiol Lett 132:253–258
Lauwers E, Erpapazoglou Z, Haguenauer-Tsapis R, Andre B (2010) The ubiquitin code of yeast permease trafficking. Trends Cell Biol 20:196–204
Matsuki H, Goto M, Tada K, Tamai N (2013) Thermotropic and barotropic phase behavior of phosphatidylcholine bilayers. Int J Mol Sci 14:2282–2302
Meersman F, Smeller L, Heremans K (2006) Protein stability and dynamics in the pressure-temperature plane. Biochim Biophys Acta 1764:346–354
Miura T, Minegishi H, Usami R, Abe F (2006) Systematic analysis of *HSP* gene expression and effects on cell growth and survival at high hydrostatic pressure in *Saccharomyces cerevisiae*. Extremophiles 10:279–284
Muir H (1995) The chondrocyte, architect of cartilage. Biomechanics, structure, function and molecular biology of cartilage matrix macromolecules. Bioessays 17:1039–1048
Nagayama A, Kato C, Abe F (2004) The N- and C-terminal mutations in tryptophan permease Tat2 confer cell growth in *Saccharomyces cerevisiae* under high-pressure and low-temperature conditions. Extremophiles 8:143–149
Oger PM, Jebbar M (2010) The many ways of coping with pressure. Res Microbiol 161:799–809
Palhano FL, Orlando MT, Fernandes PM (2004) Induction of baroresistance by hydrogen peroxide, ethanol and cold-shock in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 233:139–145
Perrier-Cornet JM, Marechal PA, Gervais P (1995) A new design intended to relate high pressure treatment to yeast cell mass transfer. J Biotechnol 41:49–58
Reineke K, Mathys A, Heinz V, Knorr D (2013) Mechanisms of endospore inactivation under high pressure. Trends Microbiol 21:296–304
Rosin MP, Zimmerman AM (1977) The induction of cytoplasmic petite mutants of *Saccharomyces cerevisiae* by hydrostatic pressure. J Cell Sci 26:373–385
Rotin D, Kumar S (2009) Physiological functions of the HECT family of ubiquitin ligases. Nat Rev Mol Cell Biol 10:398–409
Sato M, Kobori H, Ishijima SA, Feng ZH, Hamada K, Shimada S, Osumi M (1996) *Schizosaccharomyces pombe* is more sensitive to pressure stress than *Saccharomyces cerevisiae*. Cell Struct Funct 21:167–174

Sato M, Hasegawa K, Shimada S, Osumi M (1999) Effects of pressure stress on the fission yeast *Schizosaccharomyces pombe* cold-sensitive mutant *nda3*. FEMS Microbiol Lett 176:31–38

Shimada S, Andou M, Naito N, Yamada N, Osumi M, Hayashi R (1993) Effects of hydrostatic pressure on the ultrastructure and leakage of internal substances in the yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 40:123–131

Suzuki A, Mochizuki T, Uemura S, Hiraki T, Abe F (2013) Pressure-induced endocytic degradation of the *Saccharomyces cerevisiae* low-affinity tryptophan permease Tat1 is mediated by Rsp5 ubiquitin ligase and functionally redundant PPxY motif proteins. Eukaryot Cell 12: 990–997

Tamura K, Kamiki Y, Miyashita M (1999) Measurement of microbial activities under high pressure by calorimetry. In: Ludwig H (ed) Advances in high pressure bioscience and biotechnology. Springer, Heidelberg, pp 47–50

Winter R (2002) Synchrotron X-ray and neutron small-angle scattering of lyotropic lipid mesophases, model biomembranes and proteins in solution at high pressure. Biochim Biophys Acta 1595:160–184

Yashiroda H, Oguchi T, Yasuda Y, Toh-E A, Kikuchi Y (1996) Bul1, a new protein that binds to the Rsp5 ubiquitin ligase in *Saccharomyces cerevisiae*. Mol Cell Biol 16:3255–3263

Yayanos AA (1995) Microbiology to 10,500 meters in the deep sea. Annu Rev Microbiol 49: 777–805

Chapter 6
Environmental Stresses to Which Yeast Cells Are Exposed During Bioethanol Production from Biomass

Jun Shima and Toshihide Nakamura

Abstract Bioethanol is one of the most important renewable fuels for the reduction of global warming effects and environmental damage caused by the worldwide utilization of fossil fuels. Yeasts such as *Saccharomyces cerevisiae* are frequently used for bioethanol production from mono- or disaccharides derived from biomass, including sugar cane, corn, and lignocellulosic materials. During bioethanol production, yeast cells are exposed to various environmental stresses including chemical, temperature, oxidative, and acid stresses. The development of yeast strains tolerant to such environmental stresses must improve the bioethanol production process. This chapter focuses on the environmental stresses to which yeast cells are exposed during bioethanol production. We also discuss the exploration and breeding of stress-tolerant yeast strains and their application to bioethanol production.

Keywords Bioethanol • Environmental stresses • Fermentation breeding • *Saccharomyces cerevisiae* • Stress tolerance • Yeast

6.1 Yeast Cells Are Exposed to Various Stresses During Bioethanol Production

Bioethanol is one of the most important renewable fuels for the reduction of the global warming effect and environmental damage caused by the worldwide use of fossil fuels. Bioethanol production generally utilizes derivatives from food crops such as corn grain and sugar cane. Yeasts such as *Saccharomyces cerevisiae* are

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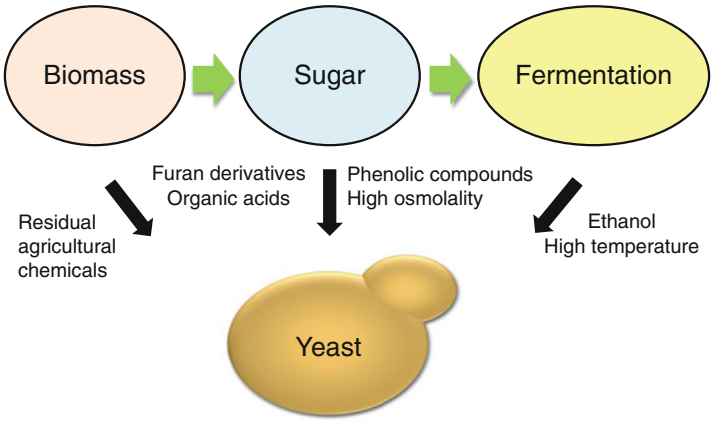

Fig. 6.1 Inhibitory factors in bioethanol production. Pesticides remain in agricultural materials in some cases. After pretreatment of the biomass, furan derivatives, organic acids, and phenolic compounds are generated in sugar solution. Fermentation is inhibited by both the heat of the process and the ethanol produced by yeasts

frequently used for ethanol production from mono- or di-saccharides derived from biomass including sugar cane, corn, and lignocellulosic materials (Balat et al. 2008). As occurs in the production of traditional liquor, such as sake, and with bread fermentation (Shima and Takagi 2009), yeast cells are exposed to numerous environmental stresses including chemical, temperature, acid, and osmolality stresses (Fig. 6.1). In addition, it can be considered that yeast cells encounter such stresses in a multiple and sequential manner. In general, microorganisms show some ability to adapt to environmental stresses. Yeast cells also need to acquire a variety of stress-adaptation mechanisms, such as the induction of stress proteins, the accumulation of stress protectants, changes in membrane composition, and the repression of translation, and by regulating the corresponding gene expression via stress-triggered signal transduction pathways (Shima and Takagi 2009; Attfield 1997). Under severe stress conditions, however, the fermentation ability of yeast is rather limited. To develop a commercial bioethanol fermentation method, it is necessary to breed yeast strains that have higher tolerances to various environmental stresses.

The efficient use of natural resources for bioethanol production has been explored by several research groups (Hasunuma and Kondo 2012; Balat 2011; Binod et al. 2010; Sarkar et al. 2012). At present, most bioethanol is produced from food crops such as corn grain or sugar cane (Kim and Dale 2004; Sanchez and Cardona 2008). However, the use of starch and sugar for the production of bioethanol competes with the use of crops as food supplies. As an alternative, a lignocellulosic biomass product such as corn stover, corn fiber, rice straw, bagasse, or wheat straw could become an indispensable resource for bioethanol production. In Japan, for example, because approximately 75 % of rice straw is not used effectively, it could be an abundant feedstock for bioethanol production.

It is known that the pretreatment methods of biomass employed as feedstock in bioethanol production strongly affect saccharification with hydrolysis enzymes and subsequent fermentation by yeast cells. Many attempts to construct a pretreatment system for lignocellulosic biomass, such as crop residues and wood chips, have been carried out (Binod et al. 2010; Sanchez and Cardona 2008). Because environmental stresses to which yeast cells are exposed depend on a pretreatment system, we considered the design and construction of yeast strains with careful consideration of pretreatment systems. Plant cell walls are composed of three main components: cellulose, hemicellulose, and lignin. To achieve high-efficiency ethanol production, it is desirable to use both the glucose and xylose contained in the cellulose and hemicellulose (Kuhad et al. 2011). However, a few types of yeast, such as *Scheffersomyces stipitis* (formerly known as *Pichia stipitis*), *Scheffersomyces shehatae* (formerly known as *Candida shehatae*), and *Spathaspora passalidarum*, have been found capable of xylose fermentation (Hou 2012; Jeffries et al. 2007; Tanimura et al. 2012); the simultaneous utilization of these sugars has been problematic. The yeast strain most generally used in current bioethanol production processes, *S. cerevisiae*, can ferment glucose derived from cellulose to ethanol; however, it normally lacks the ability to produce ethanol by fermenting the xylose present in hemicellulose (Jeffries and Jin 2004; Hasunuma and Kondo 2012; Kuhad et al. 2011). Thus, it is important to develop xylose-fermenting yeast strains. Toward this end, researchers have attempted to genetically improve yeast strains. The genetic improvement strategies are founded on the metabolism of wild xylose fermentable yeasts, such as *Sch. stipitis*. For example, xylose reductase and xylitol dehydrogenase genes from *Sch. stipitis* have been introduced into *S. cerevisiae* to make yeast with an improved xylose-fermenting ability (Kim et al. 2010; Madhavan et al. 2009).

6.2 Chemical Stresses During Bioethanol Production

Lignocellulosic materials are among the most important potential sources for bioethanol production (Gray et al. 2006; Hahn-Hagerdal et al. 2007). Lignocellulosic plant residue contains up to 70 % carbohydrates (as cellulose and hemicellulose), so it is a prominent substrate for inexpensive bioethanol production (Zaldivar et al. 2001). However, because of the close associations of cellulose and hemicellulose with lignin in the plant cell wall, pretreatment is necessary to make carbohydrates available for enzymatic hydrolysis and subsequent fermentation. For economic reasons, dilute acid hydrolysis is commonly used to prepare lignocelluloses for enzymatic saccharification and fermentation (Balat et al. 2008). Numerous by-products, including furan derivatives, weak organic acids, and phenolic compounds, are generated during pretreatment (Fig. 6.2). It has been suggested that many of these components inhibit the growth and fermentation of yeast (Fig. 6.1) (Olsson and HahnHagerdal 1996; Saha 2003; Palmqvist and Hahn-Hagerdal 2000). Furan derivatives, such as furfural and 5-hydroxymethylfurfural (HMF), which are

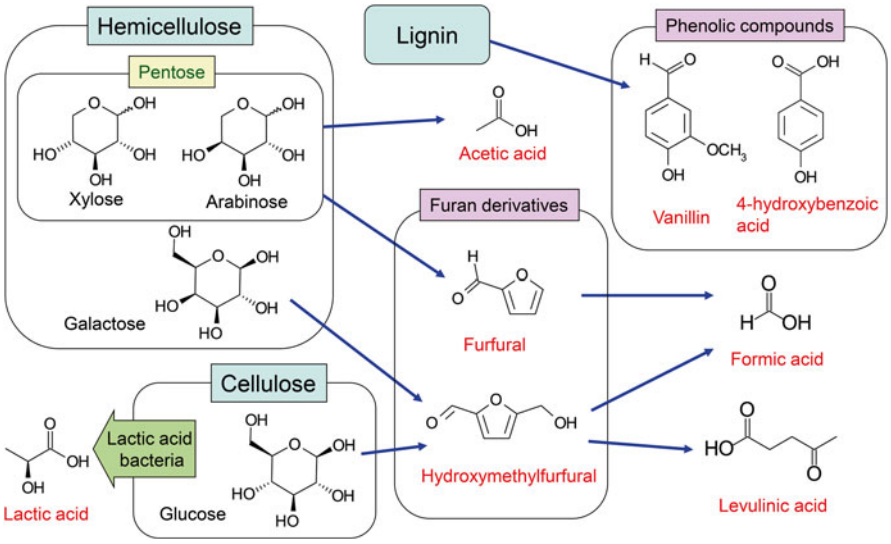

Fig. 6.2 Chemical structures of fermentation inhibitors. During the pretreatment of biomass, lignin and sugars derived from the cell wall are processed by heat or acid, and then fermentation inhibitors are generated. Lactic acid produced by contaminated lactic acid bacteria inhibits the fermentation

generated by the breakdown of sugars, have been reported to be fermentation inhibitors (Klinke et al. 2004). Furfural and HMF have been shown to reduce enzymatic and biological activity as well as the breakdown of DNA (Modig et al. 2002; Khan and Hadi 1994). It is also known that furfural and HMF induce oxidative stress in yeast cells (Kim and Hahn 2013). We discuss oxidative stress tolerance in Sect. 6.3.

Phenolic compounds such as vanillin and 4-hydroxybenzoic acid, generated by lignin degradation, have also been shown to be potent fermentation inhibitors (Klinke et al. 2004). In particular, vanillin has been suggested as a more effective inhibitor of growth and bioethanol fermentation than furan derivatives, weak acids, and other phenolic compounds, because vanillin inhibits fermentation at low concentrations (Klinke et al. 2004; Endo et al. 2008, 2009). In a later section we discuss weak acids as fermentation inhibitors and tools to control contaminated bacteria.

In this section, we employ vanillin as a model fermentation inhibitor, and we discuss the analysis of mechanisms that endow *S. cerevisiae* with tolerance to fermentation inhibitors. Our group showed the genes required for tolerance to vanillin using collections of yeast deletion mutants (Endo et al. 2008). Collections of such mutants can be powerful tools: the functions of particular genes can be clarified by analyzing the phenotypes of mutants lacking genes of interest; this is known as "phenomics" (Giaever et al. 1999; Ando et al. 2006; Okada et al. 2014). We suggested that the genes involved in ergosterol biosynthesis are required for vanillin tolerance, which in turn suggested that ergosterol is a key component of vanillin tolerance (Endo et al. 2008). Ergosterol is a ubiquitous component of cellular mem-

Table 6.1 Cross-sensitivity of ergosterol mutants to various inhibitors

	Sensitivity[a]						
Gene	Enzyme	VA	HB	GU	SY	FU	HMF	AA
ERG24	C-14 sterol reductase	0.19	0.04	0.02	0.10	0.09	0.27	0.01
ERG6	Delta(24)-sterol C-methyltransferase	0.29	0.03	0.03	0.04	0.03	0.08	0.02
ERG2	C-8 sterol isomerase	0.03	0.06	0.03	0.13	0.05	0.57	0.02
ERG3	C-5 sterol desaturase	0.20	0.05	0.06	0.12	0.05	0.26	0.02

[a]Sensitivity values are the growth ratios of mutant to wild-type strain; values <0.3 are shaded
VA vanillin, *HB* 4-hydroxybenzoic acid, *GU* guaiacol, *SY* syringaldehyde, *FU* furfural, *HMF* 5-hydroxymethylfurfural, *AA* acetic acid

branes in yeast and is required for the correct fluidity and functioning of the cellular membrane (Daum et al. 1998). The deletion mutants of the genes for ergosterol biosynthesis also showed cross-sensitivity to furan derivatives, weak acids, and phenolic compounds (Table 6.1). Thus, ergosterol is very important for tolerance to fermentation inhibitors. We screened a vanillin-tolerant strain of *S. cerevisiae* and compared its intracellular ergosterol levels with several laboratory yeast strains to study the potential relationship between ergosterol content and vanillin tolerance (Endo et al. 2009). The vanillin-tolerant strain contained a higher level of ergosterol, suggesting the amount of ergosterol is related to vanillin tolerance. Iwaki et al. showed the cellular processes affected by vanillin using high-content, image-based profiling (Iwaki et al. 2013). Among yeast deletion mutants, the morphology of those defective in the large ribosomal subunit was very similar to that of vanillin-treated cells. The defects in these mutants were clustered in three domains of the ribosome: the mRNA tunnel entrance, exit, and backbone required for small subunit attachment. Processing bodies and stress granules, which are composed of non-translating mRNAs and various proteins, were formed after treatment with vanillin. These results suggest that vanillin represses translation in yeast cells (Iwaki et al. 2013). Nguyen et al. reported that vanillin causes the activation of Yap1, which is an oxidative stress-responsive transcription factor, and mitochondrial fragmentation in *S. cerevisiae* (Nguyen et al. 2014). It can be considered that such molecular analyses may contribute to the design and construction of yeast strains tolerant to chemical stresses.

6.3 Temperature and Oxidative Stresses During Fermentation

Fermentation at higher temperatures is desirable for the reduction of cooling costs, thus requiring the use of yeasts that can produce good yields of ethanol even at elevated temperatures. In separate hydrolysis and fermentation (SHF), enzymatic hydrolysis and ethanolic fermentation are performed separately. To reduce reactor cooling costs, the temperature difference between the two processes must be reduced. During simultaneous saccharification and fermentation (SSF), a high

temperature is required to accelerate enzymatic hydrolysis (Abdel-Banat et al. 2010; Rodrussamee et al. 2011; Fonseca et al. 2008). The fermentation reaction temperature is determined by the optimal fermentation temperature of the yeast used in either the SHF or SSF process, with the majority of yeasts growing well in the range of 25–30 °C. The most widely used yeast in industrial bioethanol production is *S. cerevisiae*, of which the optimal temperature is about 30 °C. Consequently, the operating temperatures in ethanol conversion systems are fixed in this temperature range. Yeast strains tolerant to high temperature would clearly be desirable, as detailed by Banat and Marchant (1995) as well as by Ishchuk et al. (2008). In Sect. 6.5.3, we discuss yeast strains recently found to be tolerant of high temperature.

Most environmental stresses to which yeast cells are exposed induce oxidative stress by the decline in intracellular pH and excessive generation of reactive oxygen species (ROS), leading to cell damage and low fermentation ability (Attfield 1997). During the bioethanol production process, it is obvious that yeast cells are exposed to oxidative stress. Yeast cells possess several response systems to oxidative stress. In *S. cerevisiae*, it is known that transcriptional factors such as Yap1, Msn2/4, and Skn7 are important in the response to oxidative stress (Attfield 1997; Jamieson 1998; Herrero 2005; Morano et al. 2012). These factors are involved in the activation or expression of specific antioxidants and thereby the detoxification of the excess intracellular ROS and normalization of internal pH. Alternatively, *S. cerevisiae* cells possess several groups of enzymes that act directly as ROS detoxifiers: superoxide dismutases (SODs) are involved in the detoxification of superoxide anions; glutathione peroxidases (GPXs), catalases (Cta1 and Ctt1), and thioredoxin peroxidases (TSAs) reduce hydrogen peroxide (H_2O_2); and thioredoxins (TRXs) and thioredoxin reductases (TRRs) are involved in thiol reduction. In addition, the loss of the proton-transporting activities of V-ATPases, which are responsible for maintaining cytosolic pH and ion homeostasis, is known to dampen the tolerance of yeast cells to various stresses including oxidative stress, suggesting that V-ATPases play a role in stress tolerance (Thorpe et al. 2004; Ando et al. 2007; Shima and Takagi 2009). It is possible that oxidation-tolerant yeast cells improve bioethanol production by various bioprocesses from biomass.

Because oxidative stress has a negative impact under various conditions, a comprehensive understanding of the mechanism underlying oxidative stress tolerance would provide useful insights for the further development of molecular breeding of industrial yeasts. Yeast deletion collections are a valuable resource for examining loss-of-function phenotypes of yeast genes (Giaever et al. 1999), enabling genome-wide screening to clarify the relevance of genes in oxidative stress tolerance (Higgins et al. 2002; Ando et al. 2007; Shima and Takagi 2009). However, previous genome-wide screenings for stress response genes have used either haploid or homozygous deletion collections (Giaever et al. 2002; Ando et al. 2007) and thereby have been limited to nonessential genes because of the lethality resulting from the deletion of essential genes. Therefore, although essential genes account for about 20 % of all genes, the relevance of essential genes to oxidative stress tolerance remains obscure. As most heterozygotes show no obvious growth defects, the heterozygous deletion collection is a promising tool for enabling genome-wide screen-

ing including essential genes. Recently we clarified the involvement of essential genes in oxidative stress tolerance using a heterozygote deletion collection in *S. cerevisiae* (Okada et al. 2014).

6.4 Contaminations by Bacteria and Wild Yeast Are Serious Inhibitory Factors

Bacterial contamination of yeast can be a major cause of reduced ethanol yield during ethanol production from feedstock (Nishino et al. 2007; Skinner and Leathers 2004). These bacterial contaminants grow under conditions suitable for the growth of yeast and reduce ethanol yields by consuming sugars such as glucose. Among bacteria that contaminate corn mash and cane juice, lactic acid bacteria (LAB) and *Bacillus* sp. may be the most serious because of their rapid growth (Thomas et al. 2001; Saithong et al. 2009). It is reported that *Lactobacillus* strains, such as *Lb. brevis* and *Lb. fermentum*, are the most abundant isolates from commercial plants among LAB (Skinner and Leathers 2004). Lactate and acetate produced by contaminating bacteria have been reported to strongly inhibit ethanol production by yeast strains (Makanjuola et al. 1992). To avoid the reduction of ethanol yields by bacterial contamination, various agents have been examined. It has been reported that hydrogen peroxide, potassium metabisulfate, 3,4,4′-trichlorocarbanilide, and antibiotics effectively inhibit LAB (Aquarone 1960; Bayrock et al. 2003; Chang et al. 1997; Hynes et al. 1997; Narendranath et al. 1997; Oliva-Neto and Yokoya 1998; William and Carl 1986). In fact, antibiotics such as penicillin and virginiamycin are used in commercial bioethanol production today (Bayrock et al. 2003; Hynes et al. 1997). However, the addition of antibiotics to the broth may not be preferable from an ecological point of view because the waste generated during bioethanol production should be recycled as useful products such as forage or fertilizer. Antibiotics remaining in this waste can lead to the emergence and spread of mutants resistant to antibiotics, which would threaten the safety of food and human health. Therefore, it is important to develop a method to control bacterial contamination during bioethanol production without the use of antibiotics. In Sect. 6.5.2, we show the combined use of organic acid-tolerant strains and organic acids as agents for bacterial contamination.

6.5 Development of Stress-Tolerant Yeast Strains Useful for Bioethanol Production

6.5.1 Screening and Breeding

Molecular genetic engineering techniques for the breeding of yeast are well established. Such techniques could improve a yeast's characteristics, such as fermentation ability or stress tolerance, and could improve the bioethanol production process

(Hasunuma and Kondo 2012; Inaba et al. 2013). Genetic engineering techniques produce two categories of yeasts: genetically modified (GM) yeast, which contains a heterologous DNA segment derived from organisms taxonomically different from their host cells, and self-cloning (SC) yeast, which does not contain any DNA derived from other organisms and does not produce any additional proteins except for proteins originally produced in the yeast (Akada 2002; Kaino et al. 2008; Ando et al. 2005). SC processes are considered the same as naturally occurring gene conversion, such as recombination, deletion, and transposition, and thus SC yeast is not considered a GM organism. For this reason, SC yeast might be more acceptable for industry than GM yeast. However, genetically engineered yeasts, not only GM but also SC, are not frequently used commercially. One reason for hesitation in commercial use of GM or SC strains of yeast is the lack of scientific data on their effects on the natural environment and on human health. It is important to assess the viability of yeasts constructed by GM and SC techniques in natural environments because such yeasts might be inadvertently or intentionally released into environments such as soil and water if such yeasts are not contained. Several researchers consider that recombinant yeasts are impractical for industrial use because they require special containment methods to confine the engineered microorganisms. The use of recombinant yeasts drastically increases initial investment and maintenance costs. Because our group considers that both the expansion of natural yeast strains and molecular breeding are important for future bioethanol production industry, both strategies are employed.

To breed xylose-fermenting yeasts, genetic improvement strategies are founded on the metabolism of wild xylose-fermentable yeasts, such as *Sch. stipitis*. For example, xylose reductase and xylitol dehydrogenase genes from *Sch. stipitis* have been introduced into *S. cerevisiae* to make yeasts with improved xylose-fermenting ability (Kim et al. 2010; Madhavan et al. 2009).

6.5.2 Organic-Acid-Tolerant Yeasts

As described in Sect. 6.4, it is known that organic acids strongly inhibit ethanol fermentation by yeasts and that contaminating bacteria consume sugar. However, if the organic acid levels are elevated, the growth and viability of contaminated bacteria are drastically decreased. We therefore consider that the combined use of yeast strains tolerant to organic acids and organic acids as control reagents for bacterial contaminants may contribute to the construction of effective fermentation systems as a low-cost means to prevent bacterial contamination (Fig. 6.3).

First, we attempted to screen yeast strains tolerant to organic acids. From this, we found lactate-tolerant yeast *Candida glabrata* (Watanabe et al. 2008) and acetate-tolerant *Schizosaccharomyces (Sc.) pombe* (Saithong et al. 2009) and also *S. cerevisiae* ATCC 38555, which have relatively high levels of acetate tolerance compared with other *S. cerevisiae* strains (Haitani et al. 2012). It is known that several strains of *Candida* are not able to sufficiently grow and produce ethanol under anaerobic

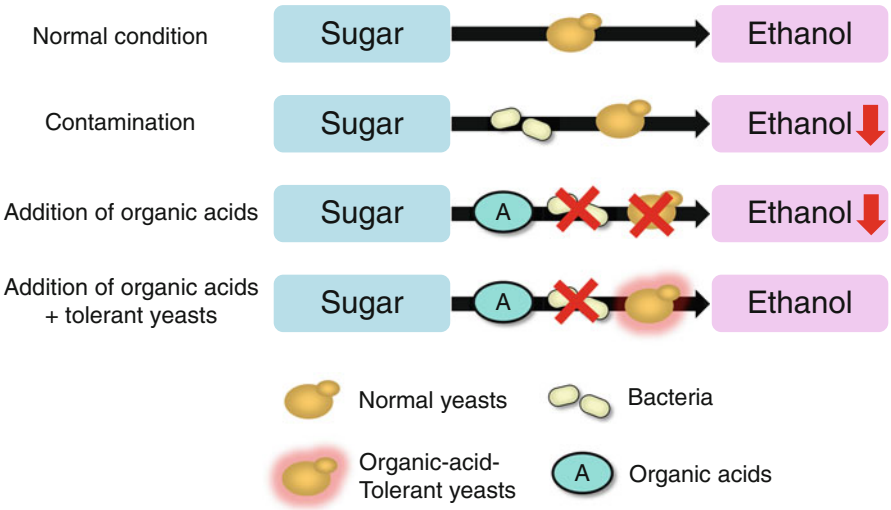

Fig. 6.3 Concepts of improvement in bioethanol fermentation. Ethanol productivity is reduced by bacterial contamination. It is possible to inhibit the growth of bacteria by the addition of organic acids, this also inhibits the fermentation of normal yeasts. The addition of yeasts that can tolerate organic acids makes possible both fermentation and bacterial growth suppression

conditions (Visser et al. 1990). However, *C. glabrata* produces high levels of ethanol and grows well under anaerobic conditions; the characteristics of *C. glabrata* in ethanol production were similar to those of *S. cerevisiae* (Watanabe et al. 2008). It is considered that the conditions for conventional ethanol production using *S. cerevisiae* can be applied to ethanol production using *C. glabrata*. We also show the yeast strain ability to produce ethanol under conditions including artificial infection with LAB (Watanabe et al. 2008). Our study shows the possibility of designing a novel bioethanol production system using lactate-tolerant yeast. We suggested that the combined use of *C. glabrata* and lactate is useful for the construction of fermentation systems to avoid the occurrence of bacterial contaminants. Because *C. glabrata* does not have the ability to assimilate sucrose, the use of *Sc. pombe* for molasses fermentation was attempted. It is suggested that the combined use of *Sc. pombe* and acetate enables bioethanol production even in the presence of bacterial contamination (Saithong et al. 2009).

On the other hand, we used ATCC 38555, an acetate-tolerant strain of *S. cerevisiae*, as a genetic resource for breeding *S. cerevisiae* strains that have higher levels of acetate tolerance. Because *S. cerevisiae* is considered a Generally Recognized as Safe (GRAS) microorganism, recycled products including DDGS produced by a fermentation system using *S. cerevisiae* are valuable as forage. In addition, *S. cerevisiae* is known as a good model system to study acid stress and defense against it in eukaryotes (Piper 2011). We found that *S. cerevisiae* ATCC 38555 is acetate tolerant, with a fermentation profile indicating that it has a high level of acetate

adaptation. Global gene expression analysis using DNA microarray indicated that genes regulated by Haa1, which is known as a transcriptional activator responsible for acetate response, are clearly upregulated in *S. cerevisiae* ATCC 38555 (Haitani et al. 2012). We show that the constitutive *HAA1*-overexpressing strain acquired a higher level of acetic acid tolerance when a laboratory *S. cerevisiae* strain was employed (Tanaka et al. 2012). Under conditions of acetic acid stress, the intracellular level of acetic acid was significantly lower in *HAA1*-overexpressing cells than in the wild-type cells (Tanaka et al. 2012). Furthermore, we constructed an *HAA1-overexpressing* diploid strain (*MAT a*/α, named ER HAA1-OP) derived from the industrial bioethanol strain ethanol red (ER) (Inaba et al. 2013). ER HAA1-OP showed tolerance not only to acetate but also to lactate, and this tolerance was dependent on the increased expression of the *HAA1* gene. The ethanol production ability of ER HAA1-OP was almost equivalent to that of the parent strain during the bioethanol production process from sugarcane molasses in the absence of acetate. The addition of acetate at 0.5 % (w/v, pH 4.5) inhibited the fermentation ability of the parent strain, but no such inhibition was observed in the ethanol production process using ER HAA1-OP (Inaba et al. 2013). This strategy can be considered useful for breeding acetate-tolerant yeast strains and for developing effective fermentation processes to prevent bacterial contamination. Using yeast strains tolerant to organic acids, we are attempting to develop fermentation systems without a sterilization process, because such a process consumes much energy.

6.5.3 High-Temperature-Tolerant Yeasts

Recently, isolated yeast strains having high-temperature tolerance have been studied (Abdel-Banat et al. 2010). Because thermotolerance in yeast might depend on quantitative trait locus (QTL), the development of thermotolerant yeasts was mainly obtained by isolation from natural resources.

It is pointed out that strains belonging to *Kluyveromyces marxianus* have higher tolerance to high temperatures (approximately 49 °C) (Nonklang et al. 2008). Abdel-Banat et al. suggested that a 5 °C increase in the fermentation temperature greatly decreased fuel ethanol production costs (Abdel-Banat et al. 2010).

Our group found that *C. glabrata*, which was described as a lactate-tolerant yeast in Sect. 6.5.2, ferments glucose at 45 °C. To improve fermentation performance for bioethanol production, a respiratory-deficient mutant and a flocculation mutant were obtained (Watanabe et al. 2009, 2010). These mutants showed superior fermentation properties.

We also succeeded in isolating a yeast strain, ATY839, capable of ethanolic fermentation at temperatures above those previously reported for yeasts able to ferment both glucose and xylose (Tanimura et al. 2012). Strain ATY839 produced a substantial amount of ethanol at up to 37 °C from 2 % glucose or 2 % xylose. The results of phylogenetic analysis suggest that strain ATY839 belongs to *Sch. shehatae*. The whole genome sequencing of this strain is ongoing.

6.6 Conclusions

In this chapter, we overviewed environmental stresses to which yeast cells are exposed during bioethanol production. At present, many research groups are attempting to construct effective pretreatment and scarification processes for various biomasses. The environmental stresses to which yeast strains are exposed during fermentation must depend on such factors. It can be considered that the breeding of customized yeast strains for each bioethanol production process forms various biomasses.

For the development of yeast strains for bioethanol production, molecular breeding and the combination of exploration and classical breeding can be used today. It is important to construct systems to develop yeast strains that contribute bioprocesses that are cost-effective and that decrease negative impacts on the natural environment.

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References

Abdel-Banat BMA, Hoshida H, Ano A, Nonklang S, Akada R (2010) High-temperature fermentation: how can processes for ethanol production at high temperatures become superior to the traditional process using mesophilic yeast? Appl Microbiol Biotechnol 85:861–867

Akada R (2002) Genetically modified industrial yeast ready for application. J Biosci Bioeng 94(6):536–544

Ando A, Suzuki C, Shima J (2005) Survival of genetically modified and self-cloned strains of commercial baker's yeast in simulated natural environments: environmental risk assessment. Appl Environ Microbiol 71(11):7075–7082

Ando A, Tanaka F, Murata Y, Takagi H, Shima J (2006) Identification and classification of genes required for tolerance to high-sucrose stress revealed by genome-wide screening of *Saccharomyces cerevisiae*. FEMS Yeast Res 6(2):249–267

Ando A, Nakamura T, Murata Y, Takagi H, Shima J (2007) Identification and classification of genes required for tolerance to freeze-thaw stress revealed by genome-wide screening of *Saccharomyces cerevisiae* deletion strains. FEMS Yeast Res 7(2):244–253

Aquarone E (1960) Penicillin and tetracycline as contamination control agents in alcoholic fermentation of sugar cane molasses. Appl Microbiol 8:263–268

Attfield PV (1997) Stress tolerance: the key to effective strains of industrial baker's yeast. Nat Biotechnol 15(13):1351–1357

Balat M (2011) Production of bioethanol from lignocellulosic materials via the biochemical pathway: a review. Energy Conserv Manag 52:858–875

Balat M, Balat H, Oz C (2008) Progress in bioethanol processing. Prog Energ Combust 34(5):551–573

Banat IM, Marchant R (1995) Characterization and potential industrial applications of 5 novel, thermotolerant, fermentative, yeast strains. World J Microbiol Biotechnol 11:304–306

Bayrock DP, Thomas KC, Ingledew WM (2003) Control of *Lactobacillus* contaminants in continuous fuel ethanol fermentations by constant or pulsed addition of penicillin G. Appl Microbiol Biotechnol 62(5–6):498–502

Binod P, Sindhu R, Singhania RR, Vikram S, Devi L, Nagalakshmi S, Kurien N, Sukumaran RK, Pandey A (2010) Bioethanol production from rice straw: an overview. Bioresour Technol 101:4767–4774

Chang IS, Kim BH, Shin PK (1997) Use of sulfite and hydrogen peroxide to control bacterial contamination in ethanol fermentation. Appl Environ Microbiol 63(1):1–6

Daum G, Lees ND, Bard M, Dickson R (1998) Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*. Yeast 14(16):1471–1510

Endo A, Nakamura T, Ando A, Tokuyasu K, Shima J (2008) Genome-wide screening of the genes required for tolerance to vanillin, which is a potential inhibitor of bioethanol fermentation, in *Saccharomyces cerevisiae*. Biotechnol Biofuels 1(1):3

Endo A, Nakamura T, Shima J (2009) Involvement of ergosterol in tolerance to vanillin, a potential inhibitor of bioethanol fermentation, in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 299(1):95–99

Fonseca GG, Heinzle E, Wittmann C, Gombert AK (2008) The yeast *Kluyveromyces marxianus* and its biotechnological potential. Appl Microbiol Biotechnol 79:339–354

Giaever G, Shoemaker DD, Jones TW, Liang H, Winzeler EA, Astromoff A, Davis RW (1999) Genomic profiling of drug sensitivities via induced haploinsufficiency. Nat Genet 21(3): 278–283

Giaever G, Chu AM, Ni L, Connelly C, Riles L, Veronneau S, Dow S, Lucau-Danila A, Anderson K, Andre B, Arkin AP, Astromoff A, El-Bakkoury M, Bangham R, Benito R, Brachat S, Campanaro S, Curtiss M, Davis K, Deutschbauer A, Entian KD, Flaherty P, Foury F, Garfinkel DJ, Gerstein M, Gotte D, Guldener U, Hegemann JH, Hempel S, Herman Z, Jaramillo DF, Kelly DE, Kelly SL, Kotter P, LaBonte D, Lamb DC, Lan N, Liang H, Liao H, Liu L, Luo C, Lussier M, Mao R, Menard P, Ooi SL, Revuelta JL, Roberts CJ, Rose M, Ross-Macdonald P, Scherens B, Schimmack G, Shafer B, Shoemaker DD, Sookhai-Mahadeo S, Storms RK, Strathern JN, Valle G, Voet M, Volckaert G, Wang CY, Ward TR, Wilhelmy J, Winzeler EA, Yang Y, Yen G, Youngman E, Yu K, Bussey H, Boeke JD, Snyder M, Philippsen P, Davis RW, Johnston M (2002) Functional profiling of the *Saccharomyces cerevisiae* genome. Nature 418(6896):387–991

Gray KA, Zhao L, Emptage M (2006) Bioethanol Curr Opin Chem Biol 10(2):141–146

Hahn-Hagerdal B, Karhumaa K, Fonseca C, Spencer-Martins I, Gorwa-Grauslund MF (2007) Towards industrial pentose-fermenting yeast strains. Appl Microbiol Biotechnol 74(5): 937–953

Haitani Y, Tanaka K, Yamamoto M, Nakamura T, Ando A, Ogawa J, Shima J (2012) Identification of an acetate-tolerant strain of *Saccharomyces cerevisiae* and characterization by gene expression analysis. J Biosci Bioeng 114(6):648–651

Hasunuma T, Kondo A (2012) Development of yeast cell factories for consolidated bioprocessing of lignocellulose to bioethanol through cell surface engineering. Biotechnol Adv 30(6): 1207–1218

Herrero E (2005) Evolutionary relationships between *Saccharomyces cerevisiae* and other fungal species as determined from genome comparisons. Rev Iberoam Micol 22(4):217–222

Higgins VJ, Alic N, Thorpe GW, Breitenbach M, Larsson V, Dawes IW (2002) Phenotypic analysis of gene deletant strains for sensitivity to oxidative stress. Yeast 19(3):203–214

Hou X (2012) Anaerobic xylose fermentation by *Spathaspora passalidarum*. Appl Microbiol Biotechnol 94(1):205–214

Hynes SH, Kjarsgaard DM, Thomas KC, Ingledew WM (1997) Use of virginiamycin to control the growth of lactic acid bacteria during alcohol fermentation. J Ind Microbiol Biotechnol 18(4):284–291

Inaba T, Watanabe D, Yoshiyama Y, Tanaka K, Ogawa J, Takagi H, Shimoi H, Shima J (2013) An organic acid-tolerant *HAA1*-overexpression mutant of an industrial bioethanol strain of *Saccharomyces cerevisiae* and its application to the production of bioethanol from sugarcane molasses. AMB Express 3:74

Ishchuk OP, Voronovsky AY, Stasyk OV, Gayda GZ, Gonchar MV, Abbas CA, Sibirny AA (2008) Overexpression of pyruvate decarboxylase in the yeast *Hansenula polymorpha* results in

increased ethanol yield in high-temperature fermentation of xylose. FEMS Yeast Res 8: 1164–1174
Iwaki A, Ohnuki S, Suga Y, Izawa S, Ohya Y (2013) Vanillin inhibits translation and induces messenger ribonucleoprotein (mRNP) granule formation in *Saccharomyces cerevisiae*: application and validation of high-content, image-based profiling. PLoS One 8(4):e61748
Jamieson DJ (1998) Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. Yeast 14(16):1511–1527
Jeffries TW, Jin YS (2004) Metabolic engineering for improved fermentation of pentoses by yeasts. Appl Microbiol Biotechnol 63:495–509
Jeffries TW, Grigoriev IV, Grimwood J, Laplaza JM, Aerts A, Salamov A, Schmutz J, Lindquist E, Dehal P, Shapiro H, Jin YS, Passoth V, Richardson PM (2007) Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis*. Nat Biotechnol 25(3):319–326
Kaino T, Tateiwa T, Mizukami-Murata S, Shima J, Takagi H (2008) Self-cloning baker's yeasts that accumulate proline enhance freeze tolerance in doughs. Appl Environ Microbiol 74(18): 5845–5849
Khan QA, Hadi SM (1994) Inactivation and repair of bacteriophage lambda by furfural. Biochem Mol Biol Int 32(2):379–385
Kim S, Dale BE (2004) Global potential bioethanol production from wasted crops and crop residues. Biomass Bioenerg 26:361–375
Kim D, Hahn JS (2013) Roles of the Yap1 transcription factor and antioxidants in *Saccharomyces cerevisiae*'s tolerance to furfural and 5-hydroxymethylfurfural, which function as thiol-reactive electrophiles generating oxidative stress. Appl Environ Microbiol 79(16):5069–5077
Kim J-H, Block DE, Mills DA (2010) Simultaneous consumption of pentose and hexose sugars: an optimal microbial phenotype for efficient fermentation of lignocellulosic biomass. Appl Microbiol Biotechnol 88:1077–1085
Klinke HB, Thomsen AB, Ahring BK (2004) Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl Microbiol Biotechnol 66(1):10–26
Kuhad RC, Gupta R, Khasa YP, Singh A, Zhang YHP (2011) Bioethanol production from pentose sugars: current status and future prospects. Renew Sust Energ Rev 15:4950–4962
Madhavan A, Tamalampudi S, Srivastava A, Fukuda H, Bisaria VS, Kondo A (2009) Alcoholic fermentation of xylose and mixed sugars using recombinant *Saccharomyces cerevisiae* engineered for xylose utilization. Appl Microbiol Biotechnol 82:1037–1047
Makanjuola DB, Tymon A, Springham DG (1992) Some effects of lactic acid bacteria on laboratory-scale yeast fermentations. Enzyme Microb Technol 14:350–357
Modig T, Liden G, Taherzadeh MJ (2002) Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. Biochem J 363(Pt 3):769–776
Morano KA, Grant CM, Moye-Rowley WS (2012) The response to heat shock and oxidative stress in *Saccharomyces cerevisiae*. Genetics 190(4):1157–1195
Narendranath NV, Hynes SH, Thomas KC, Ingledew WM (1997) Effects of lactobacilli on yeast-catalyzed ethanol fermentations. Appl Environ Microbiol 63(11):4158–4163
Nguyen TT, Iwaki A, Ohya Y, Izawa S (2014) Vanillin causes the activation of Yap1 and mitochondrial fragmentation in *Saccharomyces cerevisiae*. J Biosci Bioeng 117(1):33–38
Nishino N, Hattori H, Kishida Y (2007) Alcoholic fermentation and its prevention by *Lactobacillus buchneri* in whole crop rice silage. Lett Appl Microbiol 44(5):538–543
Nonklang S, Abdel-Banat BMA, Cha-aim K, Moonjai N, Hoshida H, Limtong S, Yamada M, Akada R (2008) High-temperature ethanol fermentation and transformation with linear DNA in the thermotolerant yeast *Kluyveromyces marxianus* DMKU3-1042. Appl Environ Microbiol 74:7514–7521
Okada N, Ogawa J, Shima J (2014) Comprehensive analysis of genes involved in the oxidative stress tolerance using yeast heterozygous deletion collection. FEMS Yeast Res 14(3):425–434. doi:10.1111/1567-1364.12136

Oliva-Neto PD, Yokoya F (1998) Effect of 3,4,4′-trichlorocarbanilide on growth of lactic acid bacteria contaminants in alcoholic fermentation. Bioresour Technol 63(1):17–21

Olsson L, Hahn-Hagerdal B (1996) Fermentation of lignocellulosic hydrolysates for ethanol production. Enzyme Microb Technol 18(5):312–331

Palmqvist E, Hahn-Hagerdal B (2000) Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of inhibition. Bioresour Technol 74(1):25–33

Piper PW (2011) Resistance of yeasts to weak organic acid food preservatives. Adv Appl Microbiol 77:97–113

Rodrussamee N, Lertwattanasakul N, Hirata K, Suprayogi LS, Kosaka T, Yamada M (2011) Growth and ethanol fermentation ability on hexose and pentose sugars and glucose effect under various conditions in thermotolerant yeast *Kluyveromyces marxianus*. Appl Microbiol Biotechnol 90:1573–1586

Saha BC (2003) Hemicellulose bioconversion. J Ind Microbiol Biotechnol 30(5):279–291

Saithong P, Nakamura T, Shima J (2009) Prevention of bacterial contamination using acetate-tolerant *Schizosaccharomyces pombe* during bioethanol production from molasses. J Biosci Bioeng 108(3):216–219

Sanchez OJ, Cardona CA (2008) Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresour Technol 99(13):5270–5295

Sarkar N, Ghosh SK, Bannerjee S, Aikat K (2012) Bioethanol production from agricultural wastes: an overview. Renew Energy 37:19–27

Shima J, Takagi H (2009) Stress-tolerance of baker's-yeast (*Saccharomyces cerevisiae*) cells: stress-protective molecules and genes involved in stress tolerance. Biotechnol Appl Biochem 53(Pt 3):155–164

Skinner KA, Leathers TD (2004) Bacterial contaminants of fuel ethanol production. J Ind Microbiol Biotechnol 31(9):401–408

Tanaka K, Ishii Y, Ogawa J, Shima J (2012) Enhancement of acetic acid tolerance in *Saccharomyces cerevisiae* by overexpression of the *HAA1* gene, encoding a transcriptional activator. Appl Environ Microbiol 78(22):8161–8163

Tanimura A, Nakamura T, Watanabe I, Ogawa J, Shima J (2012) Isolation of a novel strain of *Candida shehatae* for ethanol production at elevated temperature. Springerplus 1:27

Thomas KC, Hynes SH, Ingledew WM (2001) Effect of lactobacilli on yeast growth, viability and batch and semi-continuous alcoholic fermentation of corn mash. J Appl Microbiol 90(5):819–828

Thorpe GW, Fong CS, Alic N, Higgins VJ, Dawes IW (2004) Cells have distinct mechanisms to maintain protection against different reactive oxygen species: oxidative-stress-response genes. Proc Natl Acad Sci U S A 101(17):6564–6569

Visser W, Scheffers WA, Batenburg-van der Vegte WH, van Dijken JP (1990) Oxygen requirements of yeasts. Appl Environ Microbiol 56(12):3785–3792

Watanabe I, Nakamura T, Shima J (2008) A strategy to prevent the occurrence of *Lactobacillus* strains using lactate-tolerant yeast *Candida glabrata* in bioethanol production. J Ind Microbiol Biotechnol 35(10):1117–1122

Watanabe I, Nakamura T, Shima J (2009) Characterization of a spontaneous flocculation mutant derived from *Candida glabrata*: a useful strain for bioethanol production. J Biosci Bioeng 107(4):379–382

Watanabe I, Nakamura T, Shima J (2010) Strategy for simultaneous saccharification and fermentation using a respiratory-deficient mutant of *Candida glabrata* for bioethanol production. J Biosci Bioeng 110(2):176–179

William RG, Carl AW (1986) Effects of sodium meta bisulfite on diffusion fermentation of fodder beets for fuel ethanol production. Biotechnol Bioeng 30(7):909–916

Zaldivar J, Nielsen J, Olsson L (2001) Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. Appl Microbiol Biotechnol 56(1-2):17–34

Chapter 7
Mechanism of Yeast Adaptation to Weak Organic Acid Stress

Minetaka Sugiyama, Yu Sasano, and Satoshi Harashima

Abstract The budding yeast *Saccharomyces cerevisiae* has great potential to tolerate weak organic acid stress. This trait is becoming important for refining bioproduction systems for chemicals such as lactic acid. In this chapter, we summarize mechanisms of *S. cerevisiae* adaptation to weak organic acids. For example, yeast cells counteract lipophilic organic acids such as benzoic or sorbic acid by enhancing the production of plasma membrane transporters, including Pdr12, which pump the acid anions out of cells. For acetic acid stress, a less lipophilic organic acid, yeast cells increase their resistance through inactivation of the plasma membrane channel Fps1, which facilitates diffusional entry of acetic acid. On the other hand, protons generated by dissociation of weak organic acids are actively pumped out of the cell by the plasma membrane proton transporter Pma1. During the period of adaptation, the transcription activators War1 and Haa1 are important in stimulating transcription-mediated adaptation responses. Haa1 is also essential to activate the adaptation response to lactic acid stress. Our recent analysis suggested that one of the adaptation responses to lactic acid stress is mediated by nuclear localization of Haa1. This nuclear localization is thought to be linked to the extent of Haa1 phosphorylation, which seems to be important for Msn5-mediated nuclear export of Haa1. Studies for improving lactic acid resistance are also discussed from the point of view of efficient lactic acid production by *S. cerevisiae*.

Keywords Fps1 • Haa1 • Lactic acid • Lactic acid production • Pdr12 • *Saccharomyces cerevisiae* • War1 • Weak organic acid resistance

7.1 Introduction

Yeasts, as represented by *Saccharomyces cerevisiae*, have been widely used for making fermented food and alcoholic beverages because these microorganisms possess a strong fermentation ability that produces carbon dioxide or ethanol.

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During the traditional fermentation process, weak organic acids such as acetic or lactic acid are often produced by competing bacteria and inhibit the proliferation of yeasts. In addition, yeasts themselves also intrinsically produce organic acids such as lactic or succinic acid as metabolites, which are important determinants of the flavor of their products, resulting in lowering of the pH of the environment. Although it is unclear whether the struggle against organic acids creates a selection pressure on the evolution of yeasts, it is known that *S. cerevisiae* prefers mild but acidic conditions (pH 5–6) for its growth, and generally shows higher resistance to acid as compared with bacteria such as *Escherichia coli* or even lactic acid bacteria.

Together with sulfite, weak organic acids including benzoic, sorbic and acetic acids are the most widely used preservatives for foods and beverages under low pH conditions (Piper 2011). Because of their higher acid resistance, yeasts such as *S. cerevisiae* or *Zygosaccharomyces bailii* are among the major spoilers of food and beverages, and thus the adaptation responses of yeasts to weak organic acids have been extensively investigated. On the other hand, the higher acid resistance of yeasts is becoming important in the efficient bioproduction of acidic chemicals including lactic acid (Okano et al. 2010). In this chapter, we summarize current knowledge about the adaptation mechanisms of *S. cerevisiae* to weak organic acids and also discuss strategies to improve the lactic acid resistance of this microorganism for the efficient production of lactic acid.

7.2 Mechanism of Adaptation to Weak Organic Acids in *S. cerevisiae*

In solution, weak organic acids such as benzoic, sorbic, acetic, and lactic acids are present in a pH-dependent equilibrium between their undissociated and dissociated forms. These weak organic acids have their own pK_a value (Fig. 7.1), which represents the pH at which half of the acid molecules lose protons; the amount of the undissociated form of the acid increases as the pH decreases from the pK_a value. When a weak organic acid is added to the medium, the undissociated form of the acid preferentially enters yeast cells by passive diffusion because the uncharged acid diffuses across the plasma membrane into the cytoplasm more readily than the charged form. Once the acid invades the cells as the undissociated form, however, it dissociates in the cell as a result of the near-neutral pH of the cytosol to generate a proton and the respective counter-anion.

The protons that are released potentially cause a decrease in the intracellular pH (pHi) of yeast (Guadalupe Cabral et al. 2004; Ullah et al. 2012) and thereby decrease the activities of metabolic functions (Orij et al. 2012; Pearce et al. 2001). In addition, changes in pHi affect signal transduction (Dechant et al. 2010), protein interaction (Young et al. 2010), and cell division rate (Orij et al. 2012). As a result of their charged state, the anions released also accumulate inside the cell where they possibly cause an increase in internal turgor pressure and exert growth inhibitory actions, depending on the nature of the anion (Hazan et al. 2004; Piper 1999; Ullah et al. 2012;

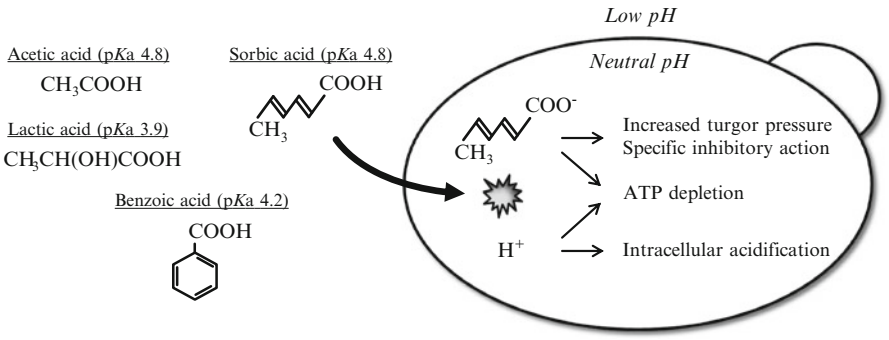

Fig. 7.1 Weak organic acid stress. Diffusional entry and dissociation of sorbic acid in a cell is depicted as an example of weak organic acid stress. At lower pH values, sorbic acid (pK_a 4.8) exists substantially in the undissociated state, resulting in a large diffusional flux of acid into cells that, in turn, causes weak organic acid stress via the dissociation-mediated generation of protons and anions as a result of the near-neutral pH inside the cell

Stratford et al. 2013). Furthermore, the energy demand for pumping protons and anions out of the cell through plasma membrane ATPases, in addition to the inhibition of glycolytic enzymes, is thought to cause depletion of the intracellular ATP pool (Holyoak et al. 1996).

7.2.1 Adaptation Response to Decreased Intracellular pH

The protons released inside the cells are buffered by intracellular materials such as proteins or metabolites. In addition, intracellular protons are also effectively pumped out of the cell by the plasma membrane H^+-ATPase Pma1. Activation of Pma1 is observed during the adaptation period to weak acids (Guadalupe Cabral et al. 2004; Holyoak et al. 1996). For example, posttranslational activation of Pma1 is suggested to be involved in the adaptation response (Carmelo et al. 1997). More recent studies showed that acetic acid and more lipophilic sorbic or benzoic acid affect pHi differently (Stratford et al. 2013; Ullah et al. 2012). At growth inhibitory concentrations, sorbic and benzoic acids do not cause a severe reduction of pHi, indicating that intracellular acidification is not a major cause of growth inhibition by these acids; however, export of the anion is reported to be involved in restoration of pHi (Ullah et al. 2012). By contrast, acetic acid stress causes long-term intracellular acidification leading to growth inhibition; therefore, activity of Pma1 is much important for growth recovery under acetic acid condition (Ullah et al. 2012).

The vacuolar proton pump (V-ATPase) also contributes to maintaining cytosolic pH by translocating protons into the lumen. A genome-wide screen of single-gene deletion mutants revealed that yeast cells lacking a functional vacuole, especially those lacking V-ATPase subunits, display a defect in adaptation to weak organic

acid stresses (Kawahata et al. 2006; Mollapour et al. 2004; Suzuki et al. 2012). Yeast cells are also reported to activate V-ATPase to counteract the deleterious effects of the weak acid 2,4-dichlorophenoxyacetic acid (Fernandes et al. 2003). The wide roles that V-ATPase plays in cytosolic pH homeostasis through not only sequestration of protons into the vacuole but also targeting of Pma1 to the plasma membrane have been discussed by Martinez-Munoz and Kane (2008). In the following sections, we present more specific adaptation responses of *S. cerevisiae* to benzoic, sorbic, acetic, and lactic acids.

7.2.2 *Adaptation Response to Sorbic Acid and Benzoic Acid Stress*

Intracellular accumulation of sorbic and benzoic anions is reported to cause growth inhibition (Stratford et al. 2013; Ullah et al. 2012) because these acids perturb the intracellular membrane dynamics of yeast (Hazan et al. 2004; Stratford and Anslow 1998) because of their higher lipophilicity. Uptake of aromatic amino acids including tryptophan is inhibited in the presence of sorbic acid (Bauer et al. 2003). Sorbic and benzoic acids also increase generation of ROS from the respiratory chain of mitochondria in aerobically cultured *S. cerevisiae* (Piper 1999). Furthermore, a recent study suggested that sorbic acid seems to exert its toxic action primarily through the inhibition of Pma1 (Stratford et al. 2013).

To increase resistance to these hydrophobic acids, yeast cells markedly upregulate expression of the *PDR12* gene (Piper et al. 1998) (Fig. 7.2). Pdr12 is a drug efflux pump localized on the plasma membrane that mediates ATP-dependent extrusion of sorbate and benzoate. Expression of *PDR12* is induced strongly in response to sorbic and benzoic acids, but not to high concentrations of acetic acid, indicating that the carbon chain length or lipophilicity of acids is an important determinant of the transcriptional activation of *PDR12* (Hatzixanthis et al. 2003).

Transcriptional induction of *PDR12* is mainly controlled by War1 protein (Kren et al. 2003). War1 is a $Zn(II)_2Cys_6$ zinc cluster transcription factor that binds to the *cis*-acting element WARE in the promoter region of the *PDR12*. Disruption of *WAR1*, as well as *PDR12*, causes hypersensitivity to sorbic and benzoic acids. Furthermore, overexpression of *PDR12* leads to a substantial recovery of sorbic acid resistance in the Δ*war1* disruptant, indicating that induction of *PDR12* might be the main function of War1 in the War1-mediated sorbic acid adaptation response. War1 can form homodimers and is continuously localized in the nucleus irrespective of the presence or absence of acid stress (Kren et al. 2003). However, strong recruitment of War1 to the *PDR12* promoter, in association with changes in its conformation, occurs in response to sorbic acid stress (Gregori et al. 2008). Similar to other transcriptional activators, therefore, direct binding of anions to War1 is thought to be the mechanism of its activation. The involvement of two other drug:H^+ antiporters, Tpo2 and Tpo3, in export of the benzoate has also been reported (Fernandes et al. 2005).

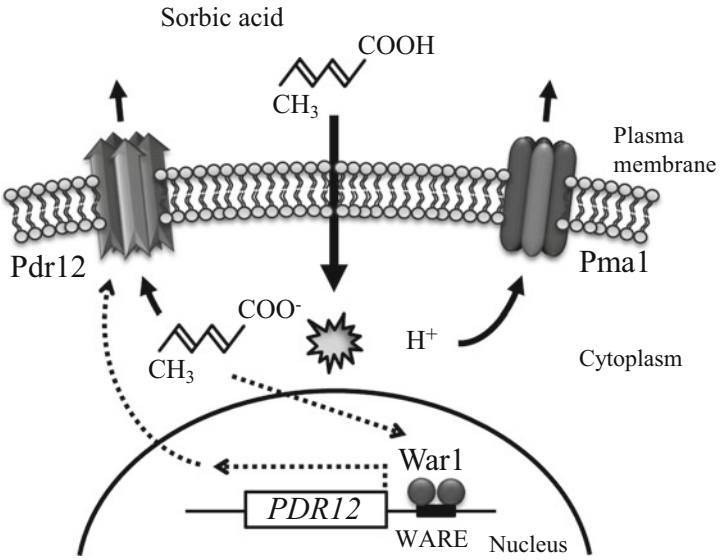

Fig. 7.2 War1-mediated induction of *PDR12* in response to sorbic acid stress. The transcription factor War1 is activated by the presence of sorbate anions and increases its binding affinity to the *cis*-acting element WARE in the promoter of the *PDR12* encoding an ATP-dependent multidrug transporter. Rapid and potent induction of Pdr12 enhances the efflux of sorbate anions at the expense of ATP

Analysis of the transcriptional profile of cells challenged with several weak organic acids revealed that four regulatory pathways controlled by four transcription factors, Msn2/Msn4, War1, Haa1, and Rim101, are involved in the adaptation responses of yeasts to these acids (Mira et al. 2010b). Among these factors, the War1 regulon is thought to be most important for sorbic acid adaptation (Schuller et al. 2004). Expression of *SPI1*, encoding a glycosylphosphatidylinositol (GPI) cell wall protein, is enhanced under benzoic acid conditions in a Msn2-dependent manner (Simoes et al. 2006). This response leads to a decrease in the porosity of the cell wall and thus contributes to prevent diffusional (re)entry of the acid. *HSP30*, which encodes a plasma membrane heat-shock protein, is also upregulated, probably to prevent ATP exhaustion caused by the activation of Pma1 in response to sorbic acid stress (Piper et al. 1997; Schuller et al. 2004).

7.2.3 Adaptation Response to Acetic Acid Stress

Intracellular acidification is proposed to be the main inhibitory effect of acetic acid stress (Stratford et al. 2013; Ullah et al. 2012). In *S. cerevisiae*, acetic acid is incorporated into the cell both by facilitated diffusion via the aquaglyceroporin Fps1

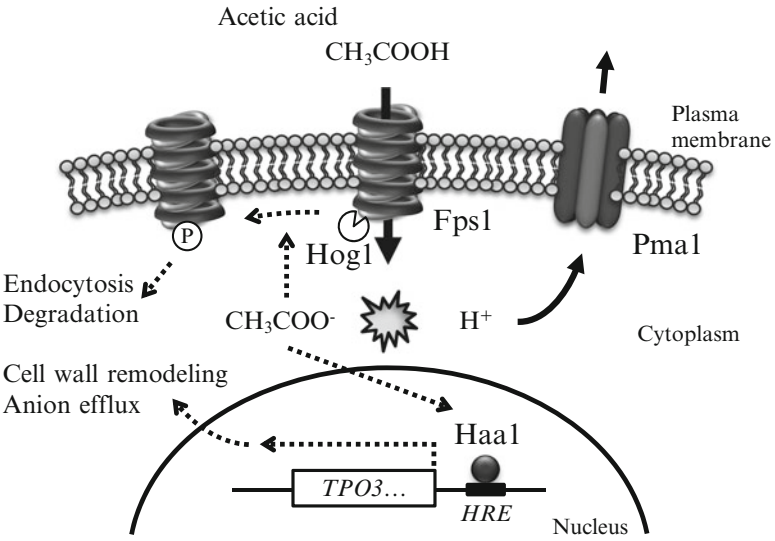

Fig. 7.3 Hog1-mediated degradation of Fps1 in response to acetic acid stress. The aquaglyceroporin Fps1 is involved in diffusional entry of acetic acid. Influx of acetic acid into the cell stimulates phosphorylation of Fps1 by Hog1 MAPK, followed by degradation of Fps1 in the vacuole, resulting in higher resistance to acetic acid stress. The Haa1-mediated transcriptional adaptation response under acetic acid stress is also depicted

(Mollapour and Piper 2007) and by passive diffusion across the plasma membrane. Acetic acid inhibits the uptake of some nutrients such as histidine, leucine, and glucose, which is possibly caused by decreased levels of intracellular ATP and also decreased expression of some nutrient transporter genes such as *HXT1*, *HXT3*, or *BAP2* (Ding et al. 2013). A sublethal concentration of acetic acid also induces programmed cell death (Ludovico et al. 2002).

To cope with acetic acid stress, the yeast cell promotes destabilization of Fps1, a diffusional entry channel for acetic acid, by an endocytosis-mediated internalization and degradation in the vacuole (Mollapour and Piper 2007) (Fig. 7.3). This degradation is mediated by the Hog1 mitogen-activated protein kinase (MAPK). In the absence of acid stress, a subfraction of Hog1 physically associates with the N-terminal cytosolic domain of Fps1. In the presence of acetic acid, however, transient activation of Hog1 occurs, and consequently Fps1 is phosphorylated by the activated Hog1. Phosphorylated Fps1 is then ubiquitinated and endocytosed, and ultimately degraded in the vacuole. Because disruption of *FPS1* confers acetic acid resistance by the decreased accumulation of acetic acid inside the cell, this adaptation response is a rational way for yeast cells to counteract stress from this less lipophilic acid. Acetic acid stress also activates the Slt2 MAPK involved in cell wall integrity, possibly by causing inhibition of glucan synthesis (Mollapour et al. 2009).

In contrast to its pivotal function under more lipophilic sorbic or benzoic acid conditions, Pdr12 does not play an important role in acetic acid stress (Bauer et al. 2003).

Instead, the drug:H^+ antiporters Tpo2 and Tpo3, in addition to Aqr1, have been implicated in reducing the intracellular concentration of acetate in acetic acid-challenged cells (Fernandes et al. 2005; Tenreiro et al. 2002). In fact, expression of *TPO2* and *TPO3* is upregulated in response to acetic acid stress, and this transcriptional upregulation requires the transcription activator Haa1 (Fernandes et al. 2005). Subsequent analysis has further revealed that Haa1 is a key transcription activator for the transcription-mediated adaptation response to acetic acid stress (Mira et al. 2010a). Among the genes regulated by Haa1, *SAP30*, encoding a component of the histone deacetylase Rpd3L complex, and *HRK1*, encoding a protein kinase whose deletion increases intracellular acetate concentrations, have been found to render the strongest protective effect against acetic acid. By identifying the *cis*-acting element HRE to which Haa1 binds in promoters of putative target genes, a transcriptional regulatory network mediated by Haa1 under acetic acid stress has been deduced (Mira et al. 2011). Cell wall proteins encoded by *SPI1* and *YGP1*, which are both in the Haa1 regulon, play a protective role against diffusional (re)entry of the acid (Fernandes et al. 2005; Mira et al. 2011; Simoes et al. 2006). Consistent with the proposed important roles of Haa1, overexpression of *HAA1* confers enhanced resistance to acetic acid (Tanaka et al. 2012; Sugiyama et al. 2014).

7.2.4 Adaptation Response to Lactic Acid Stress

Acetic and lactic acids are thought to have different growth inhibition mechanisms (Narendranath 2001). Activation of Pma1 was observed on exposure of yeast to acetic acid stress, whereas lactic acid stress caused a decrease in Pma1 activity with a reduction in unsaturated fatty acids. Because unsaturated fatty acids affect the membrane fluidity and stability of membrane proteins, their reduction may cause the decrease in activity and stability of Pma1. As a result, lactic acid stress causes a decrease in pHi and inhibits cell growth (Halm et al. 2004). Lactic acid stress also impairs maintenance of intracellular amino acid homeostasis (Suzuki et al. 2012). ROS stress is also induced by lactic acid under aerobic growth conditions (Abbott et al. 2009).

An analysis of the transcriptional responses of *S. cerevisiae* to high concentrations of lactic acid at pH 3, where more undissociated acid is present, indicated that the transcription activator Haa1 regulates the transcriptional response to undissociated lactic acid (Abbott et al. 2008). Moreover, the transcription activators Ace2 and Swi5, which are necessary for cell-cycle progression, were reported to be involved in the transcriptional response at pH 3. However, at pH 5, where lactate is the predominant form, the main response of *S. cerevisiae* was related to iron homeostasis: lactate chelates metal cations, especially iron that is required for yeast growth; as a result, the transcription activator Aft1, which is involved in iron utilization, was found to be translocated to the nucleus upon exposure to lactic acid where it induced transcription of its target genes (Abbott et al. 2008; Kawahata et al. 2006). Genome-wide gene deletion analysis also revealed that a variety of proteins, such as the Hog1

and Slt2 MAPKs, Uba4 involved in protein urmylation, and Nat3 involved in recovery from DNA damage through N-terminal acetylation of proteins, were essential for tolerance to lactic acid stress (Kawahata et al. 2006; Suzuki et al. 2012). However, the adaptation response to lactic acid stress is still poorly understood as compared with those to stresses such as sorbic or acetic acid.

To study the adaptation response to lactic acid and search for a key factor for improving resistance under low pH conditions, a screen for *S. cerevisiae* genes that, via increased copy number, rendered improved lactic acid resistance, was performed (Sugiyama et al. 2014). By this screen, the gene encoding Haa1 was found to confer resistance to high levels of lactic acid at low pH when overexpressed. A similar result was also reported by Inaba et al. (2013).

The transcriptional induction of some genes that are targeted by Haa1 under acetic acid conditions was reported to occur under conditions of lactic acid stress as well (Abbott et al. 2008). When *HAA1* was overexpressed, further enhanced induction of its target genes was observed under lactic acid conditions (Sugiyama et al. 2014), suggesting that Haa1 activity is an important determinant of lactic acid resistance and that improved expression of its target genes confers stronger protective effects on yeast cells so that they can survive when exposed to toxic levels of lactic acid.

Identification of the Haa1 target genes that are necessary for lactic acid adaptation will be beneficial for understanding the mechanism of the adaptation response. However, disruption of some known target genes of Haa1, including *SPI1*, *TPO2*, *TPO3*, and *YGP1*, which are essential for acetic acid resistance, either alone or in combination in an *HAA1* overexpression (Sugiyama et al. 2014) or the wild-type (Abbott et al. 2008) strain, failed to identify genes that are important for lactic acid resistance. In addition, *PDR12*, which is important for conferring resistance to sorbic and benzoic acid, was not essential for lactic acid resistance (Kawahata et al. 2006; Suzuki et al. 2012). Furthermore, disruption of *FPS1* did not render resistance to lactic acid, in contrast with acetic acid (Kawahata et al. 2006; Suzuki et al. 2013), suggesting that mechanisms differing from those regulating adaptation to other acids may operate during lactic acid adaptation.

Analysis of the molecular basis of the improved lactic acid resistance conferred by *HAA1* overexpression suggested that the adaptation response involves translocation of Haa1 to the nucleus to upregulate expression of its target genes, and that this nuclear localization is linked to the extent of phosphorylation of Haa1, which appears to be important for its Msn5-mediated nuclear export (Sugiyama et al. 2014) (Fig. 7.4). In the absence of lactic acid, Haa1 was localized in both cytoplasm and nucleus, and appeared to be phosphorylated. Under conditions of lactic acid stress, however, Haa1 mainly accumulated in the nucleus (although a subfraction is still localized in the cytoplasm), although the degree of phosphorylation decreased. Because Msn5 is reported to interact with Haa1 (Uetz et al. 2000) and is known to preferentially export the phosphorylated form of various transcriptional factors from the nucleus to the cytoplasm (Kaffman et al. 1998), it was suggested that nuclear accumulation of Haa1 might be linked to a reduction in the degree of phosphorylation of Haa1. It was recently reported that Haa1 also accumulated in the

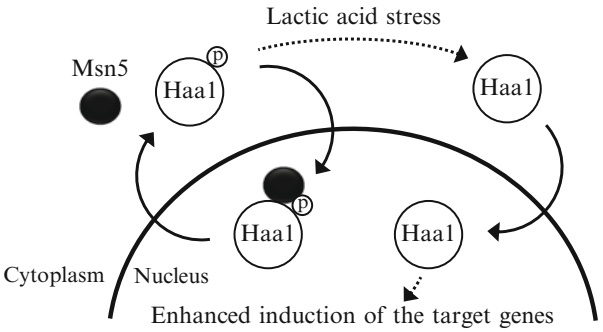

Fig. 7.4 Working hypothesis of the nuclear localization and induction of target genes of Haa1 in response to lactic acid stress. Phosphorylation sites of Haa1 involved in lactic acid adaptation response are currently unclear

nucleus on exposure to hydroxyurea or methyl methanesulfonate (Tkach et al. 2012). Because nuclear accumulation of transcription factor is an efficient strategy by which cells can rapidly execute a transcriptional adaptation response to stressful conditions, an increase in nuclear localization of Haa1 may be one of the adaptation mechanisms in yeast that protects against lactic acid stress.

7.3 Improvement of Lactic Acid Production Through Enhanced Lactic Acid Resistance

Plastics made from chemicals derived from renewable carbon sources such as polylactic acid are receiving increasing attention relative to controlling atmospheric CO_2 emissions. Lactic acid bacteria have been used to produce lactic acid, the building block chemical for polylactic acid. These bacteria, however, are sensitive to low pH and have complex nutritional requirements (Skory 2003); in addition, the lactic acid that they produce often does not have high optical purity (Hofvendahl and Hahn-Hagerdal 2000), which in turn affects the physical properties of polylactic acid, making the bacteria suboptimal for the bulk production of lactic acid for renewable plastics.

S. cerevisiae, which can tolerate more acidic conditions as compared with lactic acid bacteria and thus has the potential to eliminate expensive procedures to neutralize the lactic acid produced and desalinate the resulting lactate, and has fewer nutritional requirements, offers an alternative approach to the bioproduction of lactic acid. Although wild-type *S. cerevisiae* produces very little lactic acid because it mainly produces ethanol, genetically engineered *S. cerevisiae* harboring an exogenously introduced lactate dehydrogenase gene (*LDH*) can produce lactic acid from pyruvic acid (Dequin and Barre 1994) (Fig. 7.5).

In *S. cerevisiae*, two sequential enzymatic reactions carried out by pyruvate decarboxylase (Pdc), followed by alcohol dehydrogenase (Adh and Sfa1), convert pyruvate to ethanol. *PDC1*, *PDC5*, and *PDC6* are responsible for the conversion of

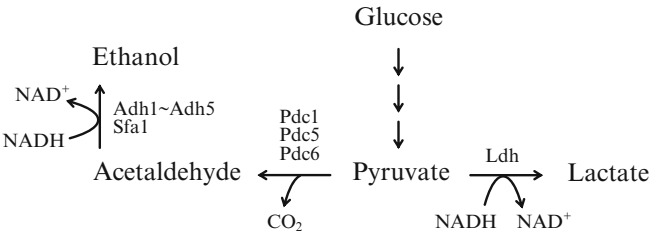

Fig. 7.5 Lactic acid production by heterologous expression of the lactate dehydrogenase gene in *S. cerevisiae*. The *S. cerevisiae* genome encodes three genes for pyruvate decarboxylase activity (Pdc1, Pdc5, and Pdc6) and at least six genes for alcohol dehydrogenase activity (Adh1, Adh2, Adh3, Adh4, Adh5, and Sfa1)

pyruvic acid into acetaldehyde, and Pdc1 is the main Pdc that functions during glucose fermentation. Genetically engineered *S. cerevisiae* strains harboring L-*LDH* still produce ethanol; however, disruption of *PDC1* by replacement with *LDH* has been found to result in the more efficient production of L-lactic acid (Ishida et al. 2005) with extremely high optical purity (Saitoh et al. 2005), even without pH control (Ishida et al. 2005). Further improvement of the production yield was achieved by simultaneous deletions of *PDC1* and *PDC5* (Ishida et al. 2006), or *PDC1*, *ADH1–ADH5*, and *SFA1*, in addition to disruption of the glycerol synthesis genes *GPD1* and *GPD2* (Ida et al. 2013), although growth of the latter disruptants was severely repressed in glucose medium. However, it was also observed that lactic acid production with the engineered yeast strain slowed down as the pH value dropped below 2.8 because of accumulation of the product (Ishida et al. 2005). Therefore, increasing the lactic acid resistance of *S. cerevisiae* would be an efficient approach to improve the production yield of lactic acid under nonneutralized conditions.

Abbott et al. (2009) reported that ROS stress is induced by lactic acid under aerobic conditions, possibly through the lactate–Fe^{3+} complex-mediated conversion of hydrogen peroxide into hydroxy radicals that are highly reactive with cellular components, and this ROS stress is one of the causes of growth inhibition in cells challenged with lactic acid. To alleviate the deleterious effect of hydroxy radicals generated by lactic acid, *CTT1*, which encodes a cytosolic catalase that can reduce intracellular hydrogen peroxide concentration, was overexpressed (Abbott et al. 2009). *CTT1* overexpression improved specific growth rate by 25 % and reduced ROS levels significantly under lactic acid stress in a Δ*cyb2* strain in which lactic acid cannot be metabolized. Because aerobic and microaerobic conditions are thought to be suitable for lactic acid production in the pyruvate decarboxylase-negative *S. cerevisiae* (van Maris et al. 2004), improvement of lactic acid resistance by reducing the level of ROS may contribute to increased lactic acid production in yeast.

Lactic acid stress causes a decrease in pHi, and ability to maintain pHi homeostasis partly contributes to the resistance of yeasts to lactic acid (Halm et al. 2004). During lactic acid fermentation, the pHi of lactic-acid producing yeast is expected to decrease as the yeast produces lactic acid, and this will hamper the production

ability of the microorganism. Valli et al. (2006) analyzed the pHi and lactic acid generation of lactic acid-producing *S. cerevisiae*. They observed a correlation between pHi and L-lactic acid productivity, and showed that a strain with higher pHi can produce much more lactic acid. Cells in which pHi dropped significantly during the fermentation were also found to be dead. Given these results, they used a screening assay to isolate lactic acid-producing *S. cerevisiae* cells showing higher viability and pHi after UV mutagenesis, followed by flow cytometry-assisted sorting of cells stained with a pH-dependent probe. After several rounds of sorting, more than 65 % of the mutants selected showed improved lactic acid production as compared with the parental strain. Although the mechanisms responsible for higher pHi during lactic acid fermentation are currently unclear, improved ability to maintain pHi and viability against lactic acid would lead to cost-effective production of lactic acid in yeast.

Lactic acid resistance can also be improved by multiple accumulations of mutations, each of which confer some resistance (Suzuki et al. 2013). To this end, a genome-wide screening for strains showing resistance to L-lactic acid was performed using the single gene deletion collection of *S. cerevisiae*. The Δ*dse2*, Δ*scw11*, Δ*eaf3*, and Δ*sed1* disruptants exhibited the highest lactic acid resistance of 94 deletion mutations identified; therefore, all 15 possible combinations of deletions for *DSE2* and *SCW11* encoding glucanases, *EAF3* encoding a component of the NuA4 acetyltransferase complex, and/or *SED1* encoding a major stress-induced GPI cell wall glycoprotein were investigated for further improvement of lactic acid resistance. Although the degree of lactic acid resistance differed across the 15 deletion mutants depending on the combination of deleted genes, the quadruple Δ*dse2*Δ*scw11*Δ*eaf3*Δ*sed1* disruptant displayed the highest cell growth with the shortest lag phase in media containing 6 % lactic acid. To evaluate the ability to produce lactic acid, the bovine L-*LDH* gene was introduced into the *PDC1* locus of each of the 15 deletion mutants. The quadruple disruptant showed not only the highest resistance up to 7 % lactic acid stress but also the highest lactic acid productivity. A 27 % increase in lactic acid productivity was achieved in the quadruple disruptant relative to the parental strain carrying the L-*LDH* gene under nonneutralized conditions. This finding suggests that improving resistance by the combinatorial accumulation of disruption mutations individually rendering lactic acid resistance provides a simple way to improve lactic acid production under nonneutralized conditions.

7.4 Concluding Remarks

The yeast *S. cerevisiae* offers an excellent platform as a microbial cell factory owing to its many attributes suitable for industrial applications. One such useful attribute is strong acid resistance. This trait has opened up new opportunities for the production of organic acids including lactic acid without pH control that can then be used as building blocks for the subsequent generation of many high-value chemicals.

To make these production systems more efficient and sustainable, however, intensive studies of adaptation responses to organic acids will be essential to improve the resistance of yeast to organic acid. Knowledge of the organic acid resistance of yeast will also facilitate the bioproduction of a variety of materials that could replace oil-based ones from biomass because organic acids such as acetic, formic, or lactic acids, which are contained in the hydrolysate of lignocellulosic biomass as a substrate for fermentation, inhibit the growth and fermentation of microorganisms and significantly reduce production yields. Furthermore, increasing our understanding of organic acid resistance of yeast will also promote the development of effective strategies for food and beverage preservation. Therefore, we believe that elucidating the mechanisms of acid adaptation should greatly contribute to increasing the biotechnological potential of yeast.

References

Abbott DA, Suir E, van Maris AJ, Pronk JT (2008) Physiological and transcriptional responses to high concentrations of lactic acid in anaerobic chemostat cultures of *Saccharomyces cerevisiae*. Appl Environ Microbiol 74(18):5759–5768. doi:10.1128/AEM.01030-08

Abbott DA, Suir E, Duong GH, de Hulster E, Pronk JT, van Maris AJ (2009) Catalase overexpression reduces lactic acid-induced oxidative stress in *Saccharomyces cerevisiae*. Appl Environ Microbiol 75(8):2320–2325. doi:10.1128/AEM.00009-09

Bauer BE, Rossington D, Mollapour M, Mamnun Y, Kuchler K, Piper PW (2003) Weak organic acid stress inhibits aromatic amino acid uptake by yeast, causing a strong influence of amino acid auxotrophies on the phenotypes of membrane transporter mutants. Eur J Biochem 270(15):3189–3195

Carmelo V, Santos H, Sa-Correia I (1997) Effect of extracellular acidification on the activity of plasma membrane ATPase and on the cytosolic and vacuolar pH of *Saccharomyces cerevisiae*. Biochim Biophys Acta 1325(1):63–70

Dechant R, Binda M, Lee SS, Pelet S, Winderickx J, Peter M (2010) Cytosolic pH is a second messenger for glucose and regulates the PKA pathway through V-ATPase. EMBO J 29(15):2515–2526. doi:10.1038/emboj.2010.138

Dequin S, Barre P (1994) Mixed lactic acid-alcoholic fermentation by *Saccharomyces cerevisiae* expressing the *Lactobacillus casei* L(+)-LDH. Biotechnology 12(2):173–177

Ding J, Bierma J, Smith MR, Poliner E, Wolfe C, Hadduck AN, Zara S, Jirikovic M, van Zee K, Penner MH, Patton-Vogt J, Bakalinsky AT (2013) Acetic acid inhibits nutrient uptake in *Saccharomyces cerevisiae*: auxotrophy confounds the use of yeast deletion libraries for strain improvement. Appl Microbiol Biotechnol 97(16):7405–7416. doi:10.1007/s00253-013-5071-y

Fernandes AR, Durao PJ, Santos PM, Sa-Correia I (2003) Activation and significance of vacuolar H^+-ATPase in *Saccharomyces cerevisiae* adaptation and resistance to the herbicide 2,4-dichlorophenoxyacetic acid. Biochem Biophys Res Commun 312(4):1317–1324

Fernandes AR, Mira NP, Vargas RC, Canelhas I, Sa-Correia I (2005) *Saccharomyces cerevisiae* adaptation to weak acids involves the transcription factor Haa1p and Haa1p-regulated genes. Biochem Biophys Res Commun 337(1):95–103. doi:10.1016/j.bbrc.2005.09.010

Gregori C, Schuller C, Frohner IE, Ammerer G, Kuchler K (2008) Weak organic acids trigger conformational changes of the yeast transcription factor War1 *in vivo* to elicit stress adaptation. J Biol Chem 283(37):25752–25764. doi:10.1074/jbc.M803095200

Guadalupe Cabral M, Sa-Correia I, Viegas CA (2004) Adaptative responses in yeast to the herbicide 2-methyl-4-chlorophenoxyacetic acid at the level of intracellular pH homeostasis. J Appl Microbiol 96(3):603–612

Halm M, Hornbaek T, Arneborg N, Sefa-Dedeh S, Jespersen L (2004) Lactic acid tolerance determined by measurement of intracellular pH of single cells of *Candida krusei* and *Saccharomyces cerevisiae* isolated from fermented maize dough. Int J Food Microbiol 94(1):97–103. doi:10.1016/j.ijfoodmicro.2003.12.019

Hatzixanthis K, Mollapour M, Seymour I, Bauer BE, Krapf G, Schuller C, Kuchler K, Piper PW (2003) Moderately lipophilic carboxylate compounds are the selective inducers of the *Saccharomyces cerevisiae* Pdr12p ATP-binding cassette transporter. Yeast 20(7):575–585. doi:10.1002/yea.981

Hazan R, Levine A, Abeliovich H (2004) Benzoic acid, a weak organic acid food preservative, exerts specific effects on intracellular membrane trafficking pathways in *Saccharomyces cerevisiae*. Appl Environ Microbiol 70(8):4449–4457. doi:10.1128/AEM.70.8.4449-4457.2004

Hofvendahl K, Hahn-Hagerdal B (2000) Factors affecting the fermentative lactic acid production from renewable resources(1). Enzyme Microb Technol 26(2–4):87–107

Holyoak CD, Stratford M, McMullin Z, Cole MB, Crimmins K, Brown AJ, Coote PJ (1996) Activity of the plasma membrane H(+)-ATPase and optimal glycolytic flux are required for rapid adaptation and growth of *Saccharomyces cerevisiae* in the presence of the weak-acid preservative sorbic acid. Appl Environ Microbiol 62(9):3158–3164

Ida Y, Hirasawa T, Furusawa C, Shimizu H (2013) Utilization of *Saccharomyces cerevisiae* recombinant strain incapable of both ethanol and glycerol biosynthesis for anaerobic bioproduction. Appl Microbiol Biotechnol 97(11):4811–4819. doi:10.1007/s00253-013-4760-x

Inaba T, Watanabe D, Yoshiyama Y, Tanaka K, Ogawa J, Takagi H, Shimoi H, Shima J (2013) An organic acid-tolerant *HAA1*-overexpression mutant of an industrial bioethanol strain of *Saccharomyces cerevisiae* and its application to the production of bioethanol from sugarcane molasses. AMB Express 3(1):74. doi:10.1186/2191-0855-3-74

Ishida N, Saitoh S, Tokuhiro K, Nagamori E, Matsuyama T, Kitamoto K, Takahashi H (2005) Efficient production of L-lactic acid by metabolically engineered *Saccharomyces cerevisiae* with a genome-integrated L-lactate dehydrogenase gene. Appl Environ Microbiol 71(4):1964–1970. doi:10.1128/AEM.71.4.1964-1970.2005

Ishida N, Saitoh S, Onishi T, Tokuhiro K, Nagamori E, Kitamoto K, Takahashi H (2006) The effect of pyruvate decarboxylase gene knockout in *Saccharomyces cerevisiae* on L-lactic acid production. Biosci Biotechnol Biochem 70(5):1148–1153

Kaffman A, Rank NM, O'Neill EM, Huang LS, O'Shea EK (1998) The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus. Nature 396(6710):482–486. doi:10.1038/24898

Kawahata M, Masaki K, Fujii T, Iefuji H (2006) Yeast genes involved in response to lactic acid and acetic acid: acidic conditions caused by the organic acids in *Saccharomyces cerevisiae* cultures induce expression of intracellular metal metabolism genes regulated by Aft1p. FEMS Yeast Res 6(6):924–936. doi:10.1111/j.1567-1364.2006.00089.x

Kren A, Mamnun YM, Bauer BE, Schuller C, Wolfger H, Hatzixanthis K, Mollapour M, Gregori C, Piper P, Kuchler K (2003) War1p, a novel transcription factor controlling weak acid stress response in yeast. Mol Cell Biol 23(5):1775–1785

Ludovico P, Rodrigues F, Almeida A, Silva MT, Barrientos A, Corte-Real M (2002) Cytochrome *c* release and mitochondria involvement in programmed cell death induced by acetic acid in *Saccharomyces cerevisiae*. Mol Biol Cell 13(8):2598–2606. doi:10.1091/mbc.E01-12-0161

Martinez-Munoz GA, Kane P (2008) Vacuolar and plasma membrane proton pumps collaborate to achieve cytosolic pH homeostasis in yeast. J Biol Chem 283(29):20309–20319. doi:10.1074/jbc.M710470200

Mira NP, Becker JD, Sa-Correia I (2010a) Genomic expression program involving the Haa1p-regulon in *Saccharomyces cerevisiae* response to acetic acid. OMICS 14(5):587–601. doi:10.1089/omi.2010.0048

Mira NP, Teixeira MC, Sa-Correia I (2010b) Adaptive response and tolerance to weak acids in *Saccharomyces cerevisiae*: a genome-wide view. OMICS 14(5):525–540. doi:10.1089/omi.2010.0072

Mira NP, Henriques SF, Keller G, Teixeira MC, Matos RG, Arraiano CM, Winge DR, Sa-Correia I (2011) Identification of a DNA-binding site for the transcription factor Haa1, required for *Saccharomyces cerevisiae* response to acetic acid stress. Nucleic Acids Res 39(16):6896–6907. doi:10.1093/nar/gkr228

Mollapour M, Piper PW (2007) Hog1 mitogen-activated protein kinase phosphorylation targets the yeast Fps1 aquaglyceroporin for endocytosis, thereby rendering cells resistant to acetic acid. Mol Cell Biol 27(18):6446–6456. doi:10.1128/MCB.02205-06

Mollapour M, Fong D, Balakrishnan K, Harris N, Thompson S, Schuller C, Kuchler K, Piper PW (2004) Screening the yeast deletant mutant collection for hypersensitivity and hyper-resistance to sorbate, a weak organic acid food preservative. Yeast 21(11):927–946. doi:10.1002/yea.1141

Mollapour M, Shepherd A, Piper PW (2009) Presence of the Fps1p aquaglyceroporin channel is essential for Hog1p activation, but suppresses Slt2(Mpk1)p activation, with acetic acid stress of yeast. Microbiology 155(Pt 10):3304–3311. doi:10.1099/mic.0.030502-0

Narendranath NV (2001) Acetic acid and lactic acid inhibition of growth of *Saccharomyces cerevisiae* by different mechanisms. J Am Soc Brew Chem 59(4):187–194

Okano K, Tanaka T, Ogino C, Fukuda H, Kondo A (2010) Biotechnological production of enantiomeric pure lactic acid from renewable resources: recent achievements, perspectives, and limits. Appl Microbiol Biotechnol 85(3):413–423. doi:10.1007/s00253-009-2280-5

Orij R, Urbanus ML, Vizeacoumar FJ, Giaever G, Boone C, Nislow C, Brul S, Smits GJ (2012) Genome-wide analysis of intracellular pH reveals quantitative control of cell division rate by pH(c) in *Saccharomyces cerevisiae*. Genome Biol 13(9):R80. doi:10.1186/gb-2012-13-9-r80

Pearce AK, Booth IR, Brown AJ (2001) Genetic manipulation of 6-phosphofructo-1-kinase and fructose 2,6-bisphosphate levels affects the extent to which benzoic acid inhibits the growth of *Saccharomyces cerevisiae*. Microbiology 147(Pt 2):403–410

Piper PW (1999) Yeast superoxide dismutase mutants reveal a pro-oxidant action of weak organic acid food preservatives. Free Radic Biol Med 27(11-12):1219–1227

Piper PW (2011) Resistance of yeasts to weak organic acid food preservatives. Adv Appl Microbiol 77:97–113. doi:10.1016/B978-0-12-387044-5.00004-2

Piper PW, Ortiz-Calderon C, Holyoak C, Coote P, Cole M (1997) Hsp30, the integral plasma membrane heat shock protein of *Saccharomyces cerevisiae*, is a stress-inducible regulator of plasma membrane H(+)-ATPase. Cell Stress Chaperones 2(1):12–24

Piper P, Mahe Y, Thompson S, Pandjaitan R, Holyoak C, Egner R, Muhlbauer M, Coote P, Kuchler K (1998) The Pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. EMBO J 17(15):4257–4265. doi:10.1093/emboj/17.15.4257

Saitoh S, Ishida N, Onishi T, Tokuhiro K, Nagamori E, Kitamoto K, Takahashi H (2005) Genetically engineered wine yeast produces a high concentration of L-lactic acid of extremely high optical purity. Appl Environ Microbiol 71(5):2789–2792. doi:10.1128/AEM.71.5.2789-2792.2005

Schuller C, Mamnun YM, Mollapour M, Krapf G, Schuster M, Bauer BE, Piper PW, Kuchler K (2004) Global phenotypic analysis and transcriptional profiling defines the weak acid stress response regulon in *Saccharomyces cerevisiae*. Mol Biol Cell 15(2):706–720. doi:10.1091/mbc.E03-05-0322

Simoes T, Mira NP, Fernandes AR, Sa-Correia I (2006) The *SPI1* gene, encoding a glycosylphosphatidylinositol-anchored cell wall protein, plays a prominent role in the development of yeast resistance to lipophilic weak-acid food preservatives. Appl Environ Microbiol 72(11):7168–7175. doi:10.1128/AEM.01476-06

Skory CD (2003) Lactic acid production by *Saccharomyces cerevisiae* expressing a *Rhizopus oryzae* lactate dehydrogenase gene. J Ind Microbiol Biotechnol 30(1):22–27. doi:10.1007/s10295-002-0004-2

Stratford M, Anslow PA (1998) Evidence that sorbic acid does not inhibit yeast as a classic "weak acid preservative". Lett Appl Microbiol 27(4):203–206

Stratford M, Nebe-von-Caron G, Steels H, Novodvorska M, Ueckert J, Archer DB (2013) Weak-acid preservatives: pH and proton movements in the yeast *Saccharomyces cerevisiae*. Int J Food Microbiol 161(3):164–171. doi:10.1016/j.ijfoodmicro.2012.12.013

Sugiyama M, Akase SP, Nakanishi R, Horie H, Kaneko Y, Harashima S (2014) Nuclear localization of Haa1, which is linked to its phosphorylation status, mediates lactic acid tolerance in *Saccharomyces cerevisiae*. Appl Environ Microbiol 80(11):3488–3495. doi:10.1128/AEM.04241-13

Suzuki T, Sugiyama M, Wakazono K, Kaneko Y, Harashima S (2012) Lactic-acid stress causes vacuolar fragmentation and impairs intracellular amino-acid homeostasis in *Saccharomyces cerevisiae*. J Biosci Bioeng 113(4):421–430. doi:10.1016/j.jbiosc.2011.11.010

Suzuki T, Sakamoto T, Sugiyama M, Ishida N, Kambe H, Obata S, Kaneko Y, Takahashi H, Harashima S (2013) Disruption of multiple genes whose deletion causes lactic-acid resistance improves lactic-acid resistance and productivity in *Saccharomyces cerevisiae*. J Biosci Bioeng 115(5):467–474. doi:10.1016/j.jbiosc.2012.11.014

Tanaka K, Ishii Y, Ogawa J, Shima J (2012) Enhancement of acetic acid tolerance in *Saccharomyces cerevisiae* by overexpression of the *HAA1* gene, encoding a transcriptional activator. Appl Environ Microbiol 78(22):8161–8163. doi:10.1128/AEM.02356-12

Tenreiro S, Nunes PA, Viegas CA, Neves MS, Teixeira MC, Cabral MG, Sa-Correia I (2002) *AQR1* gene (ORF YNL065w) encodes a plasma membrane transporter of the major facilitator superfamily that confers resistance to short-chain monocarboxylic acids and quinidine in *Saccharomyces cerevisiae*. Biochem Biophys Res Commun 292(3):741–748. doi:10.1006/bbrc.2002.6703

Tkach JM, Yimit A, Lee AY, Riffle M, Costanzo M, Jaschob D, Hendry JA, Ou J, Moffat J, Boone C, Davis TN, Nislow C, Brown GW (2012) Dissecting DNA damage response pathways by analysing protein localization and abundance changes during DNA replication stress. Nat Cell Biol 14(9):966–976. doi:10.1038/ncb2549

Uetz P, Giot L, Cagney G, Mansfield TA, Judson RS, Knight JR, Lockshon D, Narayan V, Srinivasan M, Pochart P, Qureshi-Emili A, Li Y, Godwin B, Conover D, Kalbfleisch T, Vijayadamodar G, Yang M, Johnston M, Fields S, Rothberg JM (2000) A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. Nature 403(6770):623–627. doi:10.1038/35001009

Ullah A, Orij R, Brul S, Smits GJ (2012) Quantitative analysis of the modes of growth inhibition by weak organic acids in *Saccharomyces cerevisiae*. Appl Environ Microbiol 78(23):8377–8387. doi:10.1128/AEM.02126-12

Valli M, Sauer M, Branduardi P, Borth N, Porro D, Mattanovich D (2006) Improvement of lactic acid production in *Saccharomyces cerevisiae* by cell sorting for high intracellular pH. Appl Environ Microbiol 72(8):5492–5499. doi:10.1128/AEM.00683-06

van Maris AJ, Winkler AA, Porro D, van Dijken JP, Pronk JT (2004) Homofermentative lactate production cannot sustain anaerobic growth of engineered *Saccharomyces cerevisiae*: possible consequence of energy-dependent lactate export. Appl Environ Microbiol 70(5):2898–2905

Young BP, Shin JJ, Orij R, Chao JT, Li SC, Guan XL, Khong A, Jan E, Wenk MR, Prinz WA, Smits GJ, Loewen CJ (2010) Phosphatidic acid is a pH biosensor that links membrane biogenesis to metabolism. Science 329(5995):1085–1088. doi:10.1126/science.1191026

Chapter 8
Nutrient Stress Responses of the Bottom-Fermenting Yeast

Satoshi Yoshida and Hiroyuki Yoshimoto

Abstract Beer is an alcoholic beverage that is made by yeast fermentation of the raw ingredients malt and hops. Since the mid-1990s, varieties of alcoholic beverages such as low-malt beer and no-malt brews made from soybean proteins or peptides instead of malt, and liquid sugar, have been launched. In the process of developing these alcohol beverages, a number of problems that have not previously been encountered in beer production have arisen. To solve these problems, therefore, it is necessary to investigate the physiological state of brewing yeast under various stressed conditions. In this chapter, we describe cellular responses to stress caused by different sugars, by nutrients other than sugars, and by mineral and vitamin deficiency, in addition to other environmental stresses that are predicted to be related to genes specific to bottom-fermenting yeast.

Keywords Beer • Low-malt • Mineral • No-malt • *Saccharomyces pastorianus* • SICD • Stress • Sugar • Vitamin • Yeast

8.1 Introduction

Beer is an alcoholic beverage that is made by yeast fermentation of the raw ingredients malt and hops. In Japan, rice and corn are sometimes used as auxiliary materials to give a mild and fresh taste. In standard beer, malt is milled and mashed to make wort. The sugar in wort is then converted to alcohol by yeast fermentation. Since the mid-1990s, a low-malt beer, happoushu, which is made from wort containing liquid sugars and a smaller amount of malt as raw materials, and no-malt brews, made from soybean proteins or peptides instead of malt and liquid sugars, have been produced. These worts contain sugars and nitrogen sources including amino acids, but their ratio of sugars to nutrients other than sugars (hereafter "non-sugar nutrients") is higher than that of standard beer wort. Therefore, yeast is

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exposed to many stresses other than the high concentration of sugar, high osmolarity, and high alcohol content produced during fermentation, which are known to exert stress on yeast during beer production.

Brewing yeasts for beer are largely divided into two groups: top-fermenting yeast and bottom-fermenting yeast. The former is used to brew top-fermenting beer including ale-type beers, whereas the latter is used to brew bottom-fermenting beer including pilsner-type beer. Almost all top-fermenting yeasts belong to the species *Saccharomyces cerevisiae*. This type of yeast floats to the surface of the tank at the end of the fermentation. In contrast, the bottom-fermenting yeast flocculates and settles at the bottom of the tank when the main fermentation has finished. All bottom-fermenting yeasts belong to the species *Saccharomyces pastorianus*, which is a natural hybrid formed from aneuploidy between *S. cerevisiae* and *Saccharomyces eubayanus* based on their genome sequences (Libkind et al. 2011). Regarding *S. pastorianus*, the whole genome sequence has been determined (Nakao et al. 2009), transcriptomic analysis of the genes has been performed (Yoshida et al. 2007a), and the structure and regulation of the expression of orthologous genes have been investigated (Minato et al. 2009).

Various environmental stress responses of laboratory *S. cerevisiae* yeast have been investigated. Because *S. cerevisiae* is the simplest eukaryote, it has also been used extensively for basic research. On the basis of the cellular response of yeast to antibiotics, the working mechanisms of antibiotics in higher eukaryotes including mammals have become evident and have facilitated the analysis and application of related cellular functions. For example, by analysis of response to the immunosuppressant rapamycin, function of the TOR (*To*lerant to *r*apamycin) signal transduction pathway related to nutrient signal response has been elucidated (Schmelzle and Hall 2000). In addition, analysis of response to the protein kinase C (PKC) inhibitor staurosporine has facilitated elucidation of the PKC-phosphatidylinositol (PI) signal transduction pathway related to regulation of cell growth and cell wall biosynthesis (Yoshida et al. 1992). Similarly, the PI 3-kinase signal transduction pathway has been investigated by analysis of the response to wortmannin (Zewail et al. 2003). On the other hand, during the process of brewing beer, yeast encounters various important environmental stresses, including higher sugar concentration, higher water pressure, and greater alcohol and organic acids content. With respect to osmolarity, yeast has a two-component system related to the HOG (*h*igh *o*smolarity *g*lycerol production) signal transduction pathway, which has been investigated in detail (Saito and Posas 2012). High-gravity brewing has been investigated from the aspect of saving costs and energy. In addition, structure and composition of the plasma membrane, including ergosterol and transcription factors, are known to be involved in tolerance to alcohol (Ding et al. 2009; Alper et al. 2006); in particular, sake yeast *MSN2/4* and *RIM15* have been shown to be related to alcohol tolerance in a comparison of sake yeast with laboratory yeast (Watanabe et al. 2012). With respect to organic acids, a mechanism for tolerance to weak organic acid (Piper et al. 2001) and lactic acid (Suzuki et al. 2012) has been reported. Among these various stress responses, the nutrient stress responses of brewing yeast relative to sugars and minerals are the main focus of this chapter.

8.1.1 Cellular Response to Nonsugar Nutrient Starvation in Bottom-Fermenting Yeast

Laboratory yeast *S. cerevisiae* cells are known to arrest at G_1 phase under conditions of carbon starvation (Thevelein 1992). In addition, under conditions of thiamine starvation, the activity of enzymes requiring thiamine diphosphate as a coenzyme is decreased, resulting in metabolic deficiency (Hohmann and Meacock 1998). When the balance between sugar and nonsugar nutrients is disturbed, for example, the level of nonsugar nutrients is markedly reduced, yeast cells are likely to die (Granot and Snyder 1991). Moreover, the senescence of cells is also involved in the nutrient stress response, providing a link between sugar consumption and cell death.

8.1.1.1 Cell Senescence

With respect to the senescence of yeast cells, the gene *SIR2* (*s*ilent *i*nformation *r*egulator 2) encoding an NAD-dependent histone deacetylase, sirtuin, has been isolated. This gene was initially discovered in yeast, but shortly afterward homologous genes were discovered in mammals, and *SIR2* has been found to be conserved among various organisms. Disruption of *SIR2* was found to result in a 50 % decrease in the lifespan of cells, whereas an increase in *SIR2* gene expression elongated the lifespan by 1.3-fold. In addition, when the concentration of glucose (Glc) in the medium was decreased from 2 to 0.5 %, the lifespan of the yeast cells was increased by 1.3-fold, showing that calorie restriction elongates lifespan in yeast cells as in mammals (Bitterman et al. 2003). In humans, activation of this gene might be used to control aspects of senescence, such as the elimination of reactive oxygen molecules, fat burning, muscle strengthening, control of arteriosclerosis and high blood sugar, prevention of diabetes, and control of cancer. That is, the lifespan of humans might be increased by activation of *SIR2* via moderate calorie restriction, a diet with moderate nutrient balance, and avoidance of overeating. Indeed, it has been reported that resveratrol, which is present at high levels in red wine, is an activator of Sir2p, and there is a relationship between red wine and senescence (Chen and Guarente 2006). It follows that the lifespan of the yeast cells might be elongated via the activation of this gene, for example, by providing growth media with a moderate nutrient balance.

8.1.1.2 Sugar-Induced Cell Death (SICD)

Notably, laboratory yeast cells die immediately when they are transferred to D-Glc solution, but they live when they are transferred to a synthetic minimum medium containing YNB (yeast nitrogen base without amino acid) and D-Glc, L-Glc solution, or water. This phenomenon is called SICD (*s*ugar-*i*nduced *c*ell *d*eath) and is caused by a deficiency of nonsugar nutrients (Granot and Snyder 1991).

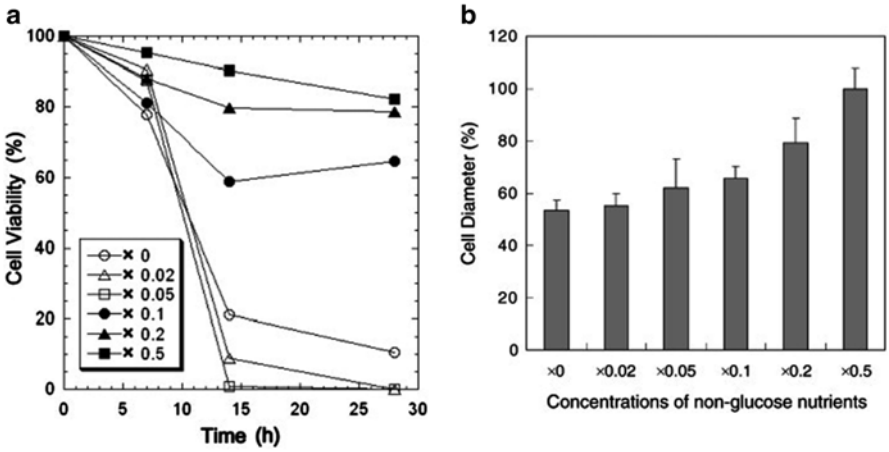

Fig. 8.1 Changes in cell viability and morphology are influenced by the balance between Glc and non-Glc nutrients. Fermentation tests were carried out in synthetic medium containing various concentrations of nonsugar nutrients in the original basal medium containing a fixed amount of sugars. X0, X0.02, X0.05, X0.1, X0.2, and X0.5 indicate 0-, 0.02-, 0.05-, 0.1-, 0.2-, and 0.5-fold concentrations of nonsugar nutrients, respectively. **a** Cell viability was measured after 7, 14, and 28 h of incubation. **b** The diameter of yeast cells was calculated for cells after 28 h of incubation. Data are mean ± SD of at least three determinations

SICD is partially suppressed by the addition of a nitrogen source, suggesting that it is also related to a deficiency of nutrients other than nitrogen. SICD is induced in a solution of fermentable sugars including fructose (Frc) and sucrose in addition to D-Glc. SICD also occurs in nonfermentable sugars such as glycerol, galactose, acetic acid, and ethanol. In addition, laboratory yeast cells that cannot use maltose (Mal) do not undergo SICD in a solution containing 2 % Mal, just as in water.

Collectively, these observations show that yeast cells die when they consume sugars in the absence of nonsugar nutrients (Granot and Snyder 1993). Yeast cells that have died from SICD show DNA and RNA degradation, plasma membrane damage, nuclear fragmentation, and cell shrinkage, all of which are typical of apoptosis. That is, SICD is a type of apoptosis, in which the cells die and release nutrients including nitrogen sources and trace elements such as minerals and vitamins into the medium. These nutrients are then used by surrounding cells to survive. Under the conditions of SICD, reactive oxygen species (ROS) are produced, and addition of the antioxidant agent ascorbate suppresses cell death (Granot et al. 2003).

Yeast cells tend to die in low-malt beer fermentation as compared with normal beer fermentation. Therefore, we investigated whether SICD occurs during the fermentation of bottom-fermenting yeast under anaerobic conditions (Yoshimoto et al. 2009). We found that the viability of bottom-fermenting yeast cells decreased as the ratio of sugars to nonsugar nutrients increased (Fig. 8.1a). At the same time, the diameter of the cells decreased as the ratio of sugars to nonsugar nutrients increased, suggesting that cell-cycle control is destroyed during SICD (Fig. 8.1b). In other words, it is important to regulate nonsugar nutrients for suitable fermentation.

Cell death caused by an imbalance between sugars and nonsugar nutrients should be an important consideration in the fermentation of low-malt beer and no-malt brews. Taking account of the concentration of D-Glc for the lifespan previously described, we suggest that a balance between sugars and nonsugar nutrients is a key factor for life events such as growth, fermentation, and longevity.

We found that the bottom-fermenting yeast died in D-Glc solution under both aerobic and anaerobic conditions (Yoshimoto et al. 2009). Next, we investigated whether respiratory function is required for SICD. We isolated pet⁻ mutants from wild-type bottom-fermenting yeast and showed that these mutant yeast cells died rapidly in D-Glc solution. These results suggest that mitochondrial respiratory function is not required for SICD (Yoshimoto et al. 2009).

8.1.1.3 Pathways Involved in SICD

It is important to determine how yeast cells die in SICD. A microarray analysis was performed on bottom-fermenting yeast cells incubated in 10 % D-Glc solution or water at 20 °C for 24 h. It was found that genes related to the Ras/cAMP signal transduction pathway were more highly induced in cells incubated in D-Glc solution than in those incubated in water. Genes that function upstream of the glycolysis pathway and TCA cycle were also highly induced in D-Glc solution. By contrast, expression of the genes functioning downstream of the glycolysis pathway and those related to protein synthesis was lower in cells incubated in D-Glc solution than in those incubated in water. In addition, the expression of many genes involved in cell-cycle control was affected (Fig. 8.2). On the basis of the transcriptome analysis, in the presence of consumable sugars, the yeast cells took in these sugars and started glycolysis. Although genes functioning upstream of glycolysis and TCA cycle were induced, genes functioning downstream of glycolysis were not induced, resulting in an imbalance in sugar metabolism. Under these conditions, although protein synthesis was decreased, some cell-cycle regulatory genes, including those in the Ras/cAMP signal transduction pathway, were activated and cell division was induced. As a result, the yeast cells would die because of an imbalance at the cell-cycle checkpoint.

Yeast has other degradation systems: namely, the ubiquitin/proteosome and autophagy systems. The ubiqutin/proteosome system is used for protein recycling, as well as protein degradation. Autophagy, which is induced by nitrogen starvation, is used to degrade proteins in the vacuole; the amino acids from the degraded protein are then used for yeast cell growth. Autophagy also includes mitophagy, which is a dynamic degradation system by which organella including mitochondria are degraded in the vacuole. It is possible that autophagy is one of the pathways of SICD. Moreover, formic acid and acetic acid induce apoptosis-like cell death (Du et al. 2008; Guaragnella et al. 2011), and phosphate and succinic acid have been reported to inhibit SICD (Lee et al. 2011), suggesting that there is a relationship between organic acids and SICD.

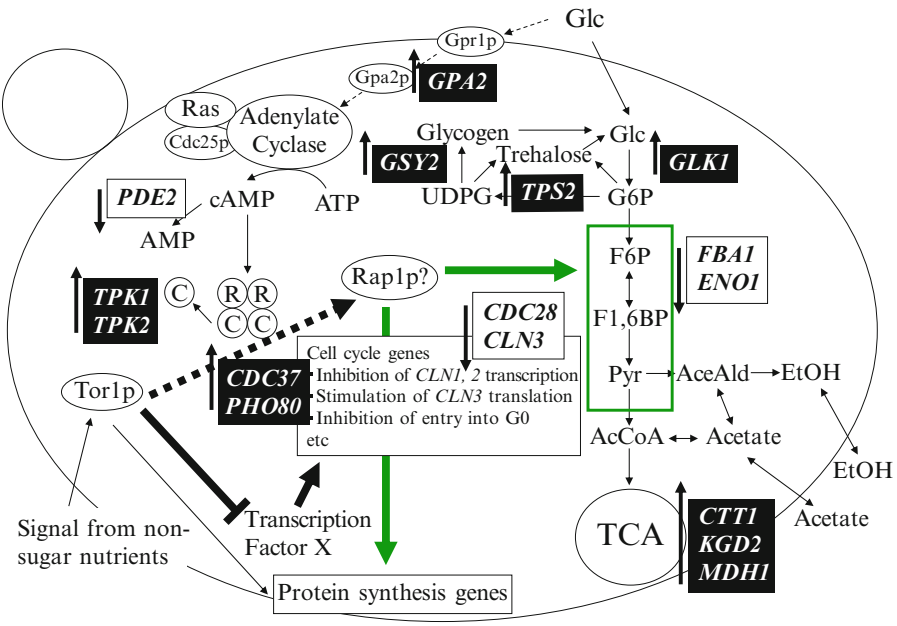

Fig. 8.2 Cartoon of metabolic pathways in yeast summarizing the results from microarray analysis of gene expression in yeast cells incubated in Glc10 and water. Genes described in *italic* and *outlined characters* with *upward arrow* show increased expression in Glc10 as compared with water, and genes described in *italic font* and *parentheses* with *downward arrow* show decreased gene expression

8.1.2 Sugars in the Wort and Fermentative Ability of Bottom-Fermenting Yeast

Beer wort contains various kinds and concentrations of sugars (Table 8.1). In this section, we describe the sugar fermentation ability, genomic structure, and environmental stress responses of the bottom-fermenting yeast *S. pastorianus*. By a comparison of the genome and ESTs between bottom-fermenting yeast and the laboratory yeast S288C, bottom-fermenting yeast was found to possess the *MAL* gene cluster involved in transport and utilization of Mal at subtelomeric regions (Jespersen et al. 1999), in addition to bottom-fermenting yeast-specific genes encoding transporters of Frc (Goncalves et al. 2000) and maltotriose (Dietvorst et al. 2005), which are not present in the genome of S288C. These results are consistent with the fact that beer wort contains abundant Mal and maltotriose as compared with Glc, and bottom-fermenting yeasts that use Mal and maltotriose efficiently to produce beer have been selected until now.

When different kinds of sugars are present in the media, all yeast including bottom-fermenting yeast first utilize monosaccharides such as Glc and sucrose,

Table 8.1 Sugar composition of a typical wort

Sugar	Amount (%)
Hexose	7–9
Sucrose	3
Maltose	43–47
Maltotriose	11–13
Dextorin (low MW)	6–12
Dextorin (high MW)	19–24
Pentosan	3–4
β-Glucan	0.2–0.3

which are easy to ferment. Next, Mal and maltotriose are used; however, oligosaccharides that are more complex than tetrasaccharides are barely fermented. With respect to monosaccharides, wort mainly contains Glc, although some Frc is present. These monosaccharides are transported into the cells by hexose transporters (HXT), and are used to produce ethanol through glycolysis and a large amount of ATP through the TCA cycle and respiratory chain reaction. Frc is also transported into the cells via a transporter encoded by *FSY1* (Goncalves et al. 2000). With respect to sucrose utilization, either invertase is secreted and degrades sucrose to Glc for uptake, or sucrose is transported into the cell by an Agt1p transporter that dose not transport Glc and degraded to Glc intracellularly (Alves et al. 2008). The *MAL* gene cluster that comprises *MALX1*, *MALX2*, and *MALX3* encoding maltase, a Mal repressor, and a Mal transporter, respectively, is related to Mal utilization (Jespersen et al. 1999). In addition, maltotriose is transported by Mtt1p (Dietvorst et al. 2005), and Agt1p (Alves et al. 2008). Mtt1p also transports Mal at low affinity. Bottom-fermenting yeast cannot utilize oligosaccharides, probably for lack of an oligosaccharide transporter rather than the lack of a degradative enzyme (Zastrow et al. 2001).

Notably, *FSY1* is conserved among various *Saccharomyces* species (de Sousa et al. 2004), in addition to *Kluyveromyces* and *Zygosaccharomyces* species (Diezemann and Boles 2003; Pina et al. 2004). Because these yeast species would use smaller amount of sugar as rapidly as possible under severe wild conditions, they have evolved to possess these Frc transporters to survive

8.1.3 The Effect of Differences in Sugar on Ethanol Fermentation of Bottom-Fermenting Yeast

In the production of low-malt alcohol beverages, liquid sugars are usually used to supplement the lack of carbohydrates. Because liquid sugars composed of monosaccharides such as Glc and Frc, and disaccharides including Mal, are used under

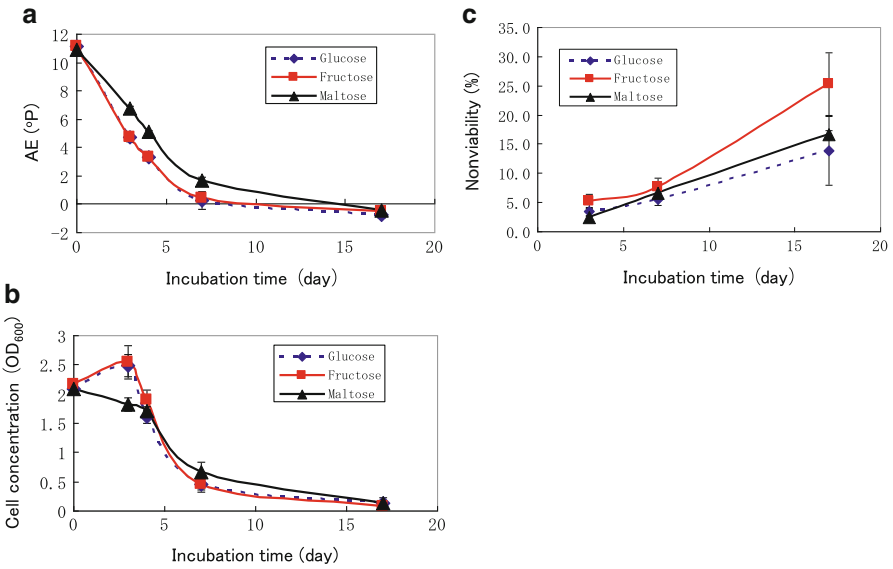

Fig. 8.3 Fermentation of the bottom-fermenting yeast in the YNB medium containing Glc, Frc, or Mal. **a** Apparent extract (AE) of the medium; **b** optimal density at 600 nm yeast cells; **c** nonviability of yeast cells floating in the tube. Yeast cells were incubated in 1 1 0.67 % YNB medium containing 10 % Glc (*diamonds*), Frc (*squares*), or Mal (*triangles*) in the EBC tube at 20 °C. Data are mean ± SD of at least three determinations

some conditions, we investigated the effect of these sugars on the fermentation of bottom-fermenting yeast (Yoshida et al. 2013). The fermentation ability and viability of the yeast cells were investigated in YNB medium containing Glc, Frc, or Mal as the sole carbon source. There was no significant difference in the fermentation ability of the bottom-fermenting yeast between the Glc and the Frc media, but its fermentation ability in the Mal medium was lower than that in Glc medium. By contrast, the viability of the yeast cells was lower in the Frc medium than in the Glc or Mal medium (Fig. 8.3). Furthermore, the bud size of the yeast cells was smaller in the Frc medium. Next, we carried out a microarray analysis of the yeast cells in the Glc and Frc media (Table 8.2). Many genes related to sugar metabolism including glycolysis were upregulated in the Frc medium as compared with the Glc medium. In addition, genes related to ergosterol biosynthesis, ribosome biogenesis, and transcription, many of which are essential genes, were downregulated in the Frc medium (Table 8.2). These results indicate that, as the expression level of these essential genes is lowered and sugar metabolism is activated in the Frc medium, the balance of cell-cycle control is destroyed and the viability of the cells is decreased (Yoshida et al. 2013). On the other hand, because the fermentation rate was lower in the Mal medium than in the Glc medium, the metabolic rate of the cells and the

Table 8.2 Microarray analysis of yeast cells incubated in medium containing various sugars

Gene name	Frc/Glc	Glc/Sor	Function
SUC2	1.71	9.17	Invertase
HXK1	1.68	18.38	Hexokinase
MAL12	1.56	10.03	α-Glucosidase
HXK2	1.49	9.83	Hexokinase
DAK2	1.49	2.40	Dihydroxyacetone kinase
YNR073C	1.48	8.79	Putative mannitol dehydrogenase
IMA2	1.40	3.79	Isomaltase
IMA3	1.40	3.84	Isomaltase
GSY1	1.37	5.49	Glycogen synthase
YFL052W	1.29	3.68	Putative zinc cluster protein
PGM2	1.26	11.69	Phosphoglucomutase
GLC3	1.24	6.59	Glycogen branching enzyme
YPR196W	1.23	3.06	Putative transcription factor
RTC2	1.23	2.66	Restriction of telomere capping
EMI2	1.19	7.03	Early meiotic induction
BDH1	1.19	6.66	Butanediol dehydrogenase
ADH4	1.40	0.43	Alcohol dehydrogenase
AST1	1.12	0.33	ATPase-stabilizing
*ERG1**	0.66	1.96	Ergosterol biosynthesis
ERG5	0.66	2.56	Ergosterol biosynthesis
*ERG25**	0.70	2.48	Ergosterol biosynthesis
CYB2	0.72	18.76	Cytochrome B
ERG6	0.79	2.03	Ergosterol biosynthesis
ARA1	0.86	3.16	Arabinose dehydrogenase
*ERG9**	0.89	3.83	Ergosterol biosynthesis
CMR3	0.77	0.55	Putative zinc finger protein
JID1	0.80	0.65	DnaJ protein
*YEF3**	0.82	0.16	Translation elongation factor 3
YML108W	0.83	0.59	Unknown
*RRS1**	0.84	0.44	Regulator of ribosome synthesis
YEH1	0.85	0.29	Steryl ester hydrolase
NUP84	0.85	0.58	Nuclear pore complex subunit
*NOP53**	0.88	0.35	Nucleolar protein
HEF3	0.90	0.37	Translational elongation factor EF-3
*RIX1**	0.93	0.25	Ribosome export

Frc/Glc indicates the relative gene expression ratio of yeast cells incubated in YPF10 (1 % yeast extract, 2 % polypeptone, and 10 % fructose) and YPD10 (1 % yeast extract, 2 % polypeptone, and 10 % glucose) at 20 °C for 4 days. Glc/Sor indicates the relative gene expression ratio of yeast cells incubated in 10 % glucose and 10 % sorbitol at 20 °C for 24 h
Asterisk indicates that the gene is essential gene for cell growth in YPD medium
Frc fructose, *Glc* glucose, *Sor* sorbitol

viability of the cells were lower in the Mal medium than in the Glc medium. These results indicate that we should select a kind of liquid sugars depending on the sugar composition and cost.

In a study where yeast cells were incubated in Mal medium, the Mal transporters were immediately degraded via endocytosis and the ubiqutin/proteosome system after the addition of Glc (Hatanaka et al. 2009). This observation was used to select a mutant whose Mal transporter was resistant to degradation caused by the addition of Glc. The mutant was found to be resistant to 2-deoxyglucose, which is a glucose analogue (Hatanaka et al. 2009). This mutant should be useful for brewing beer-like alcoholic beverages in wort containing liquid sugars because it can use both Glc and Mal without delaying fermentation. At present, it is necessary to determine the optimal fermentation temperature and the amount of pitching yeast cells for which the Mal transporters will still be intact after Glc is completely consumed.

8.1.4 Effect of Minerals and Vitamins on Alcohol Fermentation

It has been reported that yeast cells alter their metabolism in response to mineral availability. Among the various minerals, cations including zinc, manganese, magnesium, calcium, copper, potassium, and iron are components of enzymatic activity and are essential for cell growth (Eide et al. 2005). Apart from zinc, these cations are usually abundant in beer wort; in low-malt wort, by contrast, yeast cells show inhibition of growth and fermentation because of a deficiency of cations. In particular, with respect to zinc, yeast cells show growth inhibition, delayed fermentation, and an increase of off-flavors including acetaldehyde when the concentration of zinc is less than 0.1 ppm (Jacobsen et al. 1981). Therefore, it is very important to maintain a stable concentration of zinc in wort by altering the way in which wort is made. A deficiency of potassium has also been reported to cause an increase in ROS, outflow of phosphatidylserine, alteration of chromatin structure, and fragmentation of DNA and the nucleus, resulting in cell death (Lauff and Santa-Maria 2010). Moreover, when the concentration of potassium in the medium is decreased, the concentration ratio of intracellular sodium to intracellular potassium increases, and the yeast cells produce more acetic acid as a product of incomplete fermentation (Kawakubo et al. 2012).

With regard to vitamins, most bottom-fermenting yeast strains require biotin for cell growth (Ohsugi and Imanishi 1985). In addition, some top-fermenting yeast strains require thiamine (Hammond 1993). Nearly all vitamins except biotin and thiamine are abundant in normal wort. There are many reports about thiamine in yeast and the enzymes that require thiamine, including enzymes of glycolysis and TCA as a coenzyme (Hohmann and Meacock 1998). If thiamine levels are decreased in brewing, any enzymatic activity requiring thiamine as a coenzyme will be lowered and consequently more pyruvic acid and 2-oxyoglutaric acid will be produced

(Kawakubo et al. 2012). In the process of making of wort, it is most important to maintain the levels of thiamine needed for the fermentation of low-malt beer and beer-like alcoholic beverages.

8.1.5 *Bottom-Fermenting Yeast-Specific Genes and Their Relationship to Environmental Stress Responses*

Comparison of laboratory yeast with bottom-fermenting yeast shows that many genes are specific to bottom-fermenting strains, including *RTM1*, which provides tolerance to molasses (Ness and Aigle 1995). This gene is conserved among industrial yeasts including bottom-fermenting yeast. It is possible that bottom-fermenting yeast might utilize sugars present in the environment, for example, molasses, as efficiently as possible via the function of Rtm1p. The tyrosine transporter *TAT3* is also a bottom-fermenting yeast-specific gene (Omura et al. 2007). Because few amino acids are available in the environment, yeast cells may have evolved to possess *TAT3* to enable them to use amino acids as efficiently as possible.

The *AMI1* gene has also been isolated by EST analysis of the bottom-fermenting yeast (Yoshida et al. 2007a, b). *AMI1* was deduced to encode a protein with amidase-homologous regions, but with unknown function. On the basis of overexpression of the *AMI1* gene in laboratory yeast, Ami1p was found to be involved in morphogenesis of the vacuole. In addition, metabolomic analysis of yeast cells overexpressing *AMI1* showed that Ami1p is related to the metabolism of amino acids. Because basic amino acids such as histidine, arginine, and lysine are stored in the vacuole, it is possible that Ami1p is also related to vacuolar function. Furthermore, nicotinic acid was found to be dramatically decreased in *AMI1*-overexpressing cells; thus, Ami1p has a relationship with the metabolism of NAD, which is important in the regulation of redox balance. Taken together, these observations suggest that, by breeding yeast cells with a focus on *AMI1*, a yeast strain that is tolerant to stresses and changes in redox balance might be obtained.

8.2 Conclusion

In the brewing of low-malt beer and no-malt brews, the lack of nutrients often causes a delay in fermentation and produces an "off-flavor," problems that have rarely occurred in standard beer brewing. Yeast cells respond to environmental nutrients, including carbon and nitrogen sources, by regulating their intracellular metabolism. For yeast cells that are sensitive, such nutrient stresses cause an unbalanced metabolism and lead to the production of intracellular ROS. It is highly important to avoid ROS and to reduce the stress load for yeast cells during the production of alcoholic beverages. Thus, to keep nutrient stresses to a minimum and thereby produce beer, low-malt beer, and no-malt beer with a good taste, the cellular responses of yeast cells should be suitably regulated.

References

Alper H, Moxley J, Nevoigt E et al (2006) Engineering yeast transcription machinery for improved ethanol tolerance and production. Science 314:1564–1568

Alves SL Jr, Herbert RA, Hollatz C et al (2008) Molecular analysis of maltotriose active transport and fermentation by *Saccharomyces cerevisiae* reveals a determinant role for the *AGT1* permease. Appl Environ Microbiol 74:1494–1501. doi:10.1128/AEM.02570-07

Bitterman KJ, Medvedik O, Sinclair DA (2003) Longevity regulation in *Saccharomyces cerevisiae*: linking metabolism, genome stability, and heterochromatin. Microbiol Mol Biol Rev 67:376–399

Chen D, Guarente L (2006) SIR2: a potential target for calorie restriction mimetics. Trends Mol Med 13:64–71. doi:10.1016/j.molmed.2006.12.004

de Sousa HR, Spencer-Martins L, Goncalves P (2004) Differential regulation by glucose and fructose of a gene encoding a specific fructose/H^+ symporter in *Saccharomyces* sense strict yeasts. Yeast 21:519–530. doi:10.1002/yea.1118

Dietvorst J, Londesborough J, Steensma HY (2005) Maltotriose utilization in lager yeast strains: *MTT1* encodes a maltotriose transporter. Yeast 22:775–788. doi:10.1002/yea.1279

Diezemann A, Boles E (2003) Functional characterization of the Frt1 sugar transporter and of fructose uptake in *Kluyveromyces lactis*. Curr Genet 43:281–288. doi:10.1007/s00294-003-0392-5

Ding J, Huang X, Zhang L, Zhao N, Yang D, Zhang K (2009) Tolerance and stress response to ethanol in the yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 85:253–263. doi:10.1007/s00253-009-2223-1

Du L, Su Y, Sun D et al (2008) Formic acid induces Yca1-iindependent apoptosis-like cell death in the yeast *Saccharomyces cerevisiae*. FEMS Yeast Res 8:531–539. doi:10.1111/j.1567-1364.2008.00375.x

Eide DJ, Clark S, Nair TM et al (2005) Characterization of the yeast ionome: a genome-wide analysis of nutrient mineral and trace element homeostasis in *Saccharomyces cerevisiae*. Genome Biol 6:R77. doi:10.1186/gb-2005-6-9-r77

Goncalves P, Rodrigues de Sousa H, Spencer-Martins I (2000) *FSY1*, a novel gene encoding a specific fructose/H^+ symporter in the type strain *Saccharomyces carlsbergensis*. J Bacteriol 182:5628–5630

Granot D, Snyder M (1991) Glucose induces cAMP-independent growth-regulated changes in stationary-phase cells of *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 88:5724–5728

Granot D, Snyder M (1993) Carbon source induces growth of stationary phase yeast cells, independent of carbon source metabolism. Yeast 9:465–479

Granot D, Levine A, Dor-Hefetz E (2003) Sugar-induced apoptosis in yeast cells. FEMS Yeast Res 4:7–13

Guaragnella N, Antonacci L, Passarella S et al (2011) Achievements and perspectives in yeast acetic acid-induced programmed cell death pathways. Biochem Soc Trans 39:1538–1543. doi:10.1042/BST0391538

Hammond JRM (1993) Brewer's yeasts. In: Rose AH, Harrison JS (eds) The yeasts, vol 5, 2nd edn. Academic, London, pp 7–67

Hatanaka H, Omura F, Kodama Y et al (2009) Gly-46 and His-50 of yeast maltose transporter Mal21p are essential fro its resistance against glucose-induced degradation. J Biol Chem 284:15448–15457. doi:10.1074/jbc.M808151200

Hohmann S, Meacock PA (1998) Thiamin metabolism and thiamin diphosphate-dependent enzymes in the yeast *Saccharomyces cerevisiae*: genetic regulation. Biochim Biophys Acta 1385:201–219

Jacobsen T, Lie S, Haga T (1981) Wort quality and the zinc content of malt. In: Proceedings of the 18th Congress European Brewers Convention, Copenhagen, pp 97–104

Jespersen L, Cesar D, Meaden PG et al (1999) Multiple α-glucoside transporter genes in brewer's yeast. Appl Environ Microbiol 62:450–456

Kawakubo T, Iwasaki K, Tanaka M et al (2012) Effects of non-sugar nutrients concentration on fermentation and beer taste. In: Abstracts of the annual meeting of the Japan Society for Bioscience, Biotechnology, and Agrochemistry 2012, Kyoto Women's University, Kyoto, 23–25 March 2012

Lauff DB, Santa-Maria GE (2010) Potassium deprivation is sufficient to induce a cell death program in *Saccharomyces cerevisiae*. FEMS Yeast Res 10:497–507. doi:10.1111/j.1567-1364.2010.00628.x

Lee YJ, Burlet E, Galiano F et al (2011) Phosphate and succinate use different mechanisms to inhibit sugar-induced cell death in yeast. J Biol Chem 286:20267–20274. doi:10.1074/jbc.M110.209379

Libkind D, Hittinger CT, Valerio E et al (2011) Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. Proc Natl Acad Sci U S A 108:14539–14544. doi:10.1073/pnas.1154301108

Minato T, Yoshida S, Ishiguro T et al (2009) Expression profiling of the bottom fermenting yeast *Saccharomyces pastorianus* orthologous genes using oligonucleotide microarrays. Yeast 26:147–165. doi:10.1002/yea1654

Nakao Y, Kanamori T, Itoh T et al (2009) Genome sequence of the lager brewing yeast, an interspecies hybrid. DNA Res 16:115–129

Ness F, Aigle M (1995) *RTM1*: a member of a new family of telomeric repeated genes in yeast. Genetics 140:945–956

Ohsugi M, Imanishi Y (1985) Microbiological activity of biotin-vitamers. J Nutr Vitaminol (Tokyo) 31:563–572

Omura F, Hatanaka H, Nakao Y (2007) Characterization of a novel tyrosine permease of lager brewing yeast shared by *Saccharomyces cerevisiae* strain RM11-1a. FEMS Yeast Res 7:1350–1361. doi:10.1111/j.1567-1364.2007.00310.x

Pina C, Goncalves P, Prista C et al (2004) Ffz1, a new transporter specific for fructose from *Zygosaccharomyces bailii*. Microbiology 150:2429–2433. doi:10.1099/mic.0.26979-0

Piper P, Colderon CO, Hatzixanthis K et al (2001) Weak acid adaptation: the stress response that confers yeast with resistance to organic acid food preservatives. Microbiology 147:2635–2642

Saito H, Posas F (2012) Response to hyperosmotic stress. Genetics 192:289–318. doi:10.1534/genetics.112.140863

Schmelzle T, Hall MN (2000) TOR, a central controller of cell growth. Cell 103:253–262

Suzuki T, Sugiyama M, Wakazono K et al (2012) Lactic-acid stress causes vacuolar fragmentation and impairs intracellular amino-acid homeostasis in *Saccharomyces cerevisiae*. J Biosci Bioeng 113:421–430. doi:10.1016/j.jbiosc.2011.11.010

Thevelein JM (1992) The RAS-adenylate cyclase pathway and cell cycle control in *Saccharomyces cerevisiae*. Antonie Van Leeuwenhoek 62:109–130

Watanabe D, Araki Y, Zhou Y et al (2012) A loss-of-function mutation in the PAS kinase Rim15p is related to defective quiescence entry and high fermentation rates of *Saccharomyces cerevisiae* sake yeast strains. Appl Environ Microbiol 78:4008–4016. doi:10.1128/AEM.00165-12

Yoshida S, Ikeda E, Uno I et al (1992) Characterization of a staurosporine- and temperature-sensitive mutant, stt1, of S*accharomyces cerevisiae*: *STT1* is allelic to *PKC1*. Mol Gen Genet 231:337–344

Yoshida S, Hashimoto K, Shimada E et al (2007a) Identification of bottom-fermenting yeast genes expressed during lager beer fermentation. Yeast 24:599–606. doi:10.1002/yea.1494

Yoshida S, Minato T, Ikado et al (2013) Nutrient stress response of bottom-fermenting yeast. In: Abstracts of the 65th annual meeting of the Society for Biotechnology Japan, International Conference Center Hiroshima, Hiroshima, 18–20 September 2013

Yoshimoto H, Ohuchi R, Ikado K et al (2009) Sugar induced death of the bottom fermenting yeast *Saccharomyces pastorianus*. J Biosci Bioeng 108:60–62. doi:10.1061/j.jbiosc.2008.12.022

Zastrow CR, Hollatz C, de Araujo PS et al (2001) Maltotriose fermentation by *Saccharomyces cerevisiae*. J Ind Microbiol Biotechnol 27:34–38

Zewail A, Xie MW, Xing Y et al (2003) Novel functions of the phosphatidylinositol metabolic pathway discovered by a chemical genomics screen with wortmannin. Proc Natl Acad Sci U S A 100:3345–3350. doi:10.1073/pnas.0530118100

Part II
Stress Biology of Fungi

Chapter 9
Unique Metabolic Responses to Hypoxia and Nitric Oxide by Filamentous Fungi

Shunsuke Masuo and Naoki Takaya

Abstract To understand fungal metabolism under stress is important for the industrial production of organic acids and enzymes from fungi, and also for traditional large-scale fermentation because inadequately controlled cultures impose stress on fungi that reduces performance efficiency. This chapter describes recent advances in studies of the hypoxic regulation of fungal metabolism. Transcriptome and proteome analyses of the model filamentous fungus *Aspergillus nidulans* have identified global metabolic changes in carbon source utilization and energy conservation under hypoxia. Insufficient nitrate reduction under hypoxia results in the generation of nitrite or reactive nitrogen species (RNS) that constitute a prevalent nitrogen source for filamentous fungi such as *Aspergillus*. Fungi have developed novel nitrate reduction mechanisms to survive under hypoxia that are likely to be industrially important because they can generate RNS during fermentation. This chapter also describes recent findings of heme biosynthesis and nitrosothionein that are involved in fungal responses to RNS and detoxification mechanisms.

Keywords Ammonia fermentation • Denitrification • Heme • Hypoxia • Nitric oxide • Nitrosothionein • Proteomics • Transcriptomics

Abbreviations

GABA	γ-Aminobutyric acid
GSNOR	GSNO reductase
iNT	Nitrosothionein
Nar	Nitrate reductase
Nir	Nitrite reductase
Nor	NO reductase
PPP	Pentose phosphate pathway
RNS	Reactive nitrogen species
TCA	Tricarboxylic acid TrxR, thioredoxin reductase

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9.1 Industrial Applications of Filamentous Fungi

The production of East Asian wines that require filamentous fungi to saccharify cereal starch has been established for centuries, and such fungi have since become indispensable to the global production of industrial enzymes, organic materials, and pharmaceuticals. Hydrolyzing enzymes derived from various filamentous fungi are applied to detergent manufacturing, food processing, and the saccharification of starch and biomass cellulose. Some filamentous fungi are sources of antibiotics such as penicillins and cephalosporins, cholesterol-lowering statins, and other drugs. These applications depend on the fermentative properties of filamentous fungi that are supported by their unique metabolic activities. Culture conditions, namely nutrients, temperature, aeration, and pH, must be precisely controlled to maximize fermentation performance because inadequately controlled conditions impose stress on fungi, which in turn threatens the fermentation process and affects fungal survival. Thus, understanding fungal metabolism under stress is important for industrial applications of fungi.

The genus *Aspergillus* contains several industrially valuable species. *Aspergillus oryzae* is used to hydrolyze rice and soy beans during fermentation to manufacture Japanese rice wine (sake), soy sauces, and pastes. *Aspergillus niger* and *Aspergillus terreus* produce citric and itaconic acids that are useful in the food and chemical industries, respectively. All fermentation processes are affected by culture conditions as just described. Aeration is critical because most filamentous fungal species are absolutely aerobic, or require oxygen for growth, cellular metabolism, and hence fermentation efficiency. For example, *A. niger* produces less citric acid under low oxygen (hypoxic) conditions than under normoxic conditions. To understand the metabolic changes induced by hypoxia is therefore important for industrial applications, yet little is known about the biology of filamentous fungi under a hypoxic milieu.

This chapter briefly describes recent advances in studies of the hypoxic regulation of fungal metabolism. Transcriptome and proteome analyses of the model filamentous fungus *A. nidulans* have found that global metabolic changes occur in the utilization of carbon sources and energy conservation under hypoxia. More recent studies have found that hypoxia generates nitrite or reactive nitrogen species (RNS) because of the insufficient reduction of nitrate added to fermentation mixtures as a nitrogen source. This process results in disrupted metabolism and damaged DNA. Fungi have developed novel nitrate reduction mechanisms to survive in a hypoxic milieu. Such mechanisms are likely to be industrially important because they can generate RNS during fungal fermentation that includes nitrate, which is a popular nitrogen source for filamentous fungi such as *Aspergillus*. The following chapter describes recent findings of fungal responses to RNS and detoxification mechanisms.

9.2 Hypoxic Stress Responses of Filamentous Fungi

Most eukaryotes are obligate aerobes that require oxygen not only for the final electron acceptor of respiration but for biosynthesizing sterol, NAD, heme, and other metabolites (Bunn and Poyton 1996). Filamentous fungi as well as other eukaryotes were originally considered to be obligate aerobes, and when faced with a low-oxygen milieu, their growth was limited by oxygen-requiring metabolism. However, some fungi can survive very low (<0.5 ppm) concentrations of oxygen (hypoxic conditions) (Hall and Denning 1994). These findings suggested that fungi cannot be considered obligate aerobes in terms of energy conservation. This chapter describes these fungal hypoxic mechanisms and how filamentous fungi respond to oxygen availability and regulate their metabolic mechanisms at the cellular level.

9.2.1 Nitrogen Metabolism Under Hypoxia

Filamentous fungi can assimilate a diverse range of nitrogen sources. In addition to mechanisms of nitrate assimilation, unique fungal dissimilation mechanisms of nitrate that conserve energy under oxygen-limited conditions have been proposed. A denitrification mechanism was originally identified in *Fusarium oxysporum* during the 1990s (Shoun and Tanimoto 1991). This fungus reduces nitrate or nitrite to gaseous nitrous oxide under oxygen-limiting conditions via the activities of nitrate reductase (Nar), nitrite reductase (Nir), and NO reductase (Nor) (Fig. 9.1). Currently known filamentous fungi lack N_2O reducing activity, and the final product of fungal denitrification is not the N_2 that is generated by bacteria, but N_2O. The denitrifying fungus *F. oxysporum* produces Nar activity in mitochondria and in the cytosol, processes that are dependent on ubiquinol and NADPH, respectively (Fujii and Takaya 2008). Nitrate reduction by ubiquinol-Nar is associated with ADP phosphorylation, indicating the physiological significance of hypoxic denitrification as respiration (nitrate respiration). Genes encoding Nor have been isolated from *F. oxysporum*, *A. oryzae*, and other filamentous fungi, and they are essential for NO reduction to nitrous oxide (Takaya 2009). In contrast to bacterial Nor, which contains cytochrome *bc* as a cofactor, fungal Nor is unique among cytochrome P450 enzymes as it has an NO–heme complex heme in the active center to which NADH is a direct electron donor (Nakahara et al. 1993). The inducible production and ability of fungal Nor to oxidize NADH under hypoxic conditions suggest that it has a physiological role in dissimilating NO as an alternative electron acceptor to oxygen (Shoun and Tanimoto 1991; Fujii and Takaya 2008).

Another unique hypoxic mechanism in filamentous *F. oxysporum* and *A. nidulans* is ammonia fermentation (Zhou et al. 2002; Takasaki et al. 2004). These fungi reduce nitrate to ammonium, which is coupled with the oxidation of ethanol (carbon source) to acetate under hypoxic conditions, and ATP is then generated through

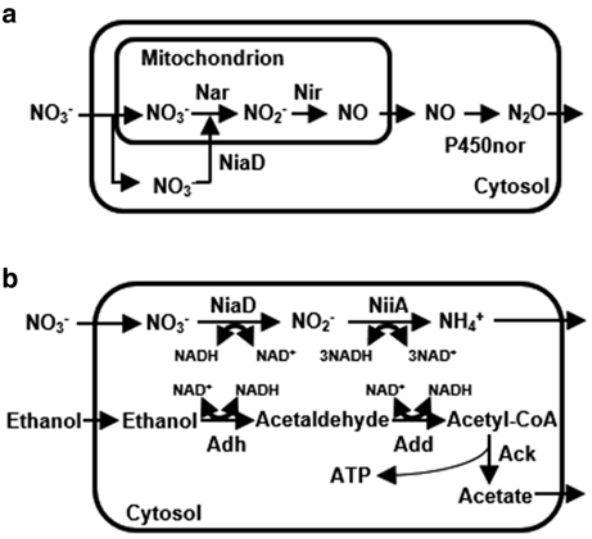

Fig. 9.1 Schematic representations of fungal hypoxic nitrogen metabolism. **a** Denitrification. **b** Ammonia fermentation. *Ack* acetate kinase, *Add* aldehyde dehydrogenase (acylating), *Adh* aldehyde dehydrogenase, *Nar* nitrate reductase, *NiaD and NiiA* Nar and Nir encoded by *niaD* and *niaD*, *Nir* nitrite reductase, *P450nor* cytochrome P450nor

substrate-level phosphorylation (Zhou et al. 2002; Takasaki et al. 2004). The enzymes involved in the oxidation of ethanol to acetate are alcohol dehydrogenase, coenzyme A-acylating aldehyde dehydrogenase, and acetate kinase (Ack). The key enzyme in this process is Ack, which generates acetate from acetyl-coA coupled with AMP and ADP phosphorylation in *A. nidulans* and *F. oxysporum*, respectively (Zhou et al. 2002; Takasaki et al. 2004). The nitrate-reducing reaction comprises nitrate and nitrite reductases (Takasaki et al. 2004), encoded by *niaD* and *niiA*, which are nitrate-assimilating enzymes in *A. nidulans*. These studies indicated that this fungus utilizes the nitrate-reducing mechanism to ammonium for both assimilation and dissimilation, the latter of which is a coping mechanism under low oxygen availability. The findings of denitrification and ammonia fermentation imply that fungal hypoxic mechanisms are far more complex than was previously thought.

9.2.2 Global Metabolic Changes Upon Hypoxia

Despite the potential significance of fungal hypoxic responses as dissimilation mechanisms, global metabolic changes remained obscure until recent genome-wide analysis using transcriptome and proteome approaches in the model fungus *A. nidulans* started to yield some clarification (Masuo et al. 2010; Shimizu et al. 2009). The transcriptional responses of *A. nidulans* in glucose minimal medium after transfer from normoxia to hypoxia were analyzed using DNA microarrays. The resulting transcriptome indicated that 27 % of the total number of genes was up- or downregulated by the altered oxygen availability (Masuo et al. 2010). The expression of genes for glycolysis, the tricarboxylic acid (TCA) cycle, and the

γ-aminobutyrate (GABA) shunt were upregulated upon hypoxia (Masuo et al. 2010). The same study found upregulated enzymes that convert pyruvate to ethanol and lactate under hypoxia, indicating that glucose in the medium is fermented to these compounds through glycolysis, and that this process generates ATP by substrate-level phosphorylation as it does in other microorganisms (ethanol and lactate fermentation) (Masuo et al. 2010). Glycolysis is also upregulated in *A. oryzae*, *A. fumigatus*, and *Saccharomyces cerevisiae* as well as in other filamentous fungi and yeasts (Terabayashi et al. 2012; Vödisch et al. 2011; Barker et al. 2012), implying that this phenomenon is conserved among fungi.

Transcripts associated with the TCA cycle are downregulated under hypoxic conditions in *Candida albicans*, *S. cerevisiae*, and *Trichoderma reesei*, whereas *A. niger* produces more intermediate metabolites of the TCA cycle under hypoxia, suggesting that TCA cycle metabolism is upregulated (Diano et al. 2009). Transcripts involved in the TCA cycle are induced, whereas the expression of respiratory genes is downregulated in *A. nidulans* (Masuo et al. 2010) as well as in *A. oryzae* (Terabayashi et al. 2012). Proteome studies of *A. nidulans* cells utilizing C-2 carbon (ethanol) showed that the production of TCA cycle enzymes does not change under hypoxia (Shimizu et al. 2009). The TCA cycle seems to be differently regulated in *A. fumigatus* when cultured under long-term hypoxia with limited glucose and under short-term hypoxia with sufficient glucose (Vödisch et al. 2011; Barker et al. 2012). These results suggest that culture conditions differentially regulate the TCA cycle among fungal species under hypoxic conditions. Detailed analyses are required to understand the regulation and physiological roles of the TCA cycle under hypoxia.

The GABA shunt requires glutamate dehydrogenase, glutamate decarboxylase, GABA transaminase, and succinic semialdehyde dehydrogenase; it bypasses two steps of the TCA cycle (Fig. 9.2). This pathway, which was discovered in plants around 50 years ago, is in fact conserved in almost all organisms (Bouché and Fromm 2004). The GABA and the GABA shunt are involved in various physiological processes such as neurotransmission, the regulation of carbon:nitrogen flux, and the oxidative stress response in diverse organisms, suggesting the critical importance of this pathway. Both transcripts and enzymes constituting this pathway are upregulated in *A. nidulans* under hypoxic conditions (Masuo et al. 2010; Shimizu et al. 2009). The GABA shunt is also upregulated in *A. oryzae*, *A. fumigatus*, and *F. oxysporum* (Terabayashi et al. 2012; Barker et al. 2012), implying that this bypass pathway is conserved among these filamentous fungi. Oxygen limitation under hypoxic conditions elevates the cellular NADH:NAD^+ ratio in *A. nidulans* (Masuo et al. 2010). The increased NADH level disrupts the cellular redox status, induces severe redox stress, and causes extreme metabolic changes in cells. Because the GABA shunt bypasses the NADH-generating step of TCA cycle, it is considered to be physiologically significant as a mechanism that prevents excessive NADH accumulation.

Hypoxia also causes changes in amino acid metabolism. Intracellular and extracellular alanine and glutamate levels are increased in *A. nidulans* under hypoxia

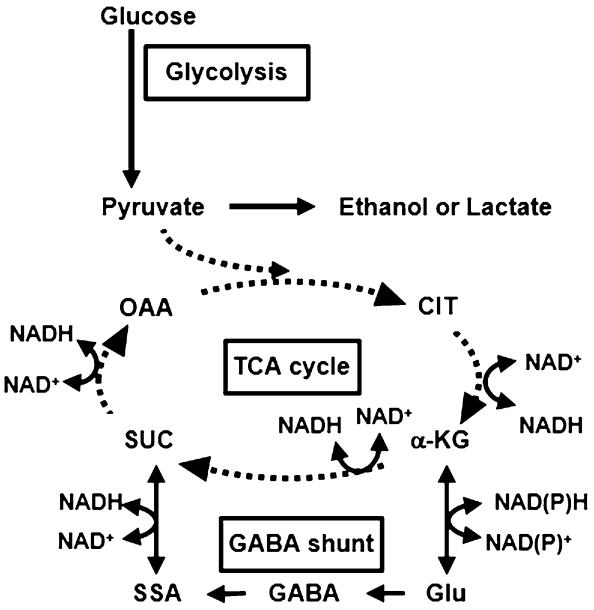

Fig. 9.2 Fungal hypoxic metabolism in glycolysis, tricarboxylic acid (TCA) cycle, and gamma-aminobutyric acid (GABA) shunt. *α-KG* α-ketoglutarate, *CIT* citrate, *GABA* γ-aminobutyrate, *Glu* glutamate, *OAA* oxaloacetate, *SSA* succinic semialdehyde, *SUC* succinate

(Masuo et al. 2010; Shimizu et al. 2010). These amino acids are the products of aminotransferase reactions against pyruvate and 2-oxoglutarate, as well as of upregulated glycolysis and the TCA cycle. Thus, levels of alanine and glutamate should increase under hypoxic conditions. Metabolism involving the GABA shunt is linked to the conversion of ammonium and pyruvate to alanine or glutamate, and it is likely to contribute to this phenomenon. *Aspergillus nidulans* overproduces branched-chain amino acids under hypoxia to regenerate NAD^+, which is referred to as branched-amino-acid fermentation (Shimizu et al. 2010). This process also seems to participate in the production of branched amino acids by hypoxic *A. fumigatus* cells, suggesting that these filamentous fungi maintain the intracellular NADH/NAD^+ balance via various mechanisms under hypoxic stress conditions.

Proteomic analysis has shown that hypoxic *A. nidulans* increases the production of enzymes associated with the pentose phosphate pathway (PPP) (Shimizu et al. 2009). One physiological role of PPP is the generation of NADPH, which serves as a substrate for the NADPH dehydrogenases that are involved in stress responses. Upregulated PPP might contribute to the ability of *A. nidulans* to tolerate redox states disrupted by hypoxia. The other role of PPP is the production of ribose-5-phosphate, which is a nucleotide precursor. *Aspergillus nidulans* cells accumulate more nitrite under hypoxia than in normoxia. Nitrite deaminates DNA purine bases under physiological conditions. Purine nucleotide metabolism is also activated in *A. nidulans* cells under hypoxia, indicating that this fungus repairs DNA damaged by accumulated nitrite under such conditions (Shimizu et al. 2009). Hypoxic organisms produce NO by reducing nitrite in mitochondria (Poyton et al. 2009). Although the mechanism of NO production by hypoxic fungal mitochondria remains

unclear, NO-detoxifying flavohemoglobin is upregulated in hypoxic *A. oryzae* and *A. fumigatus*, indicating a possible pathway (Terabayashi et al. 2012; Vödisch et al. 2011).

9.3 Nitric Oxide Responses of *Aspergillus nidulans*

9.3.1 Stress Imposed by Reactive Nitrogen Species and Responses of Filamentous Fungi

The findings of novel nitrate and RNS metabolic pathways in hypoxic *A. nidulans* cells opened up fungal RNS responses and adaptation as a new field of study. More knowledge of these pathways might be important to deepen understanding of the physiology of fermentative fungi because nitrate is often included in the fermentation media of filamentous fungi. Nitrite is an intermediate of nitrate reduction to ammonium, and it is in equilibrium with the powerful oxidant and mutagen, nitrous acid (HNO_2). Further protonation generates the highly reactive RNS nitrosonium (NO^+) (Poole 2005). Nitrate reductase (NADPH-dependent) in plants and probably isozymes in fungi produce NO as a by-product of nitrate reduction (Desikan et al. 2002). These nitrogenous oxides inhibit fungal activities under both hypoxic and aerobic conditions, and thus the fungal mechanisms that respond to and tolerate RNS stress should be considered to achieve maximal fermentation efficiency under both hypoxic and normoxic (aerobic) conditions.

Fungal mechanisms in response to RNS are best characterized as those to NO (Benhar et al. 2009). Upon exposure to exogenous NO, the yeast *Saccharomyces cerevisiae* induces the production of flavohemoglobin (Fhb) that oxygenates NO to nitrate and sequesters NO. Nitric oxide reacts with cellular thiols such as free cysteine, thiol moieties of antioxidant glutathione (GSH), and cysteine thiolate of proteins, and also generates *S*-nitrosothiols. The reaction with GSH generates *S*-nitrosoglutathione (GSNO), which GSNO reductase (GSNOR) reduces to GSH. The yeast GSH-GSNOR system and Fhb constitute a control mechanism of NO that eliminates stress imposed by RNS (Liu et al. 2001). The recent findings of Fhb in *A. oryzae* (Zhou et al. 2012) and *A. nidulans* (Zhou et al. 2013) indicate that these filamentous fungi have NO-detoxifying functions. However, the functions of the GSH/GSNOR system and other proteins in the responses of filamentous fungi to NO remain unknown.

9.3.2 Heme Biosynthesis

Zhou et al. screened *A. nidulans* for genes that tolerate NO to identify novel fungal mechanisms of RNS tolerance and generated 26,000 transformants from which six RNS-tolerant clones were isolated (Zhou et al. 2012). An *A. nidulans* genomic DNA

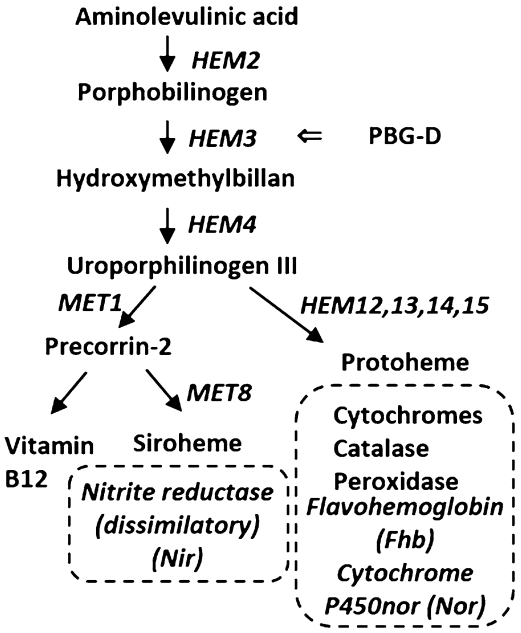

Fig. 9.3 Models of heme biosynthesis involved in reactive nitrogen species (RNS) tolerance. Heme biosynthetic pathway prediction of filamentous fungi. Corresponding *S. cerevisiae* genes are designated using conventional nomenclature

library along with a unique *A. nidulans–E. coli* shuttle vector (Gems et al. 1991) was introduced into *A. nidulans* and RNS-resistant transformants were then screened using acidified nitrite as the NO donor. This vector enabled recovery of the introduced plasmid by the simple transformation of *E. coli* with total DNA of the fungal transformants. As this type of vector cannot be used for other filamentous fungi, thus *A. nidulans* confers a strategic advantage when screening filamentous fungal genes for RNS tolerance.

Heme is an essential cofactor for the respiratory chain of the mitochondria and for some enzymes. The genetic screen for NO-tolerating genes previously described identified an orthologue of the *S. cerevisiae* porphobilinogen deaminase (PBG-D) gene (*HEM3*). This enzyme condenses four porphobilinogen molecules to form hydroxymethylbilane, which is an essential intermediate of the heme biosynthetic pathway (Fig. 9.3). The *A. nidulans* genome encodes a set of heme biosynthetic genes similar to those of yeast, indicating that this fungus synthesizes heme via the same metabolic pathway. A conditional mutant of the *HEM3* orthologue of *A. nidulans* (*hemC*) that expresses *hemC* under control of the inducible gene promoter confers a growth defect under the repressive conditions of *hemC* expression, indicating that this gene is critical for the normal growth of *A. nidulans* (Zhou et al. 2012). The growth of the mutant is more sensitive to acidified nitrite than the wild-type strain under *hemC*-repressive conditions. The cellular content of protoheme, which is found in most intracellular heme cofactors, decreases in *hemC*-repressed strains, indicating that the heme biosynthetic pathway is a novel determinant of cellular NO resistance.

Fungal enzymes that contain heme are involved in sterol synthesis, as well as sulfate and nitrate utilization. Adding sterol or other sulfur and nitrogen sources does not recover growth of the *hemC*-repressed strain that becomes defective in the presence of NO, indicating that the absence of this enzyme activity does not account for the NO-sensitive growth induced by a *hemC* deficiency. In fact, less Fhb-dependent NO dioxygenase activity is produced by the *hemC*-repressed strain than the wild-type strain. Intact cells of the strain consume NO more slowly than the wild type. These findings can be accounted for by *hemC* supplying protoheme to Fhb to upregulate NO detoxification (Fig. 9.3). The study of Zhou et al. was notable because it revealed more complex fungal RNS-detoxifying mechanisms; NADPH-nitrite reductase (NiiA, *niiA* gene product) reduces nitrite to ammonium, but detoxified acidified nitrite when added as the main NO donor under their experimental conditions. A contribution of PBG-D to growth in the presence of acidified nitrite has been suggested by adding an apoenzyme of NiiA with siroheme, which is an essential cofactor for producing enzyme activity.

9.3.3 Nitrosothionein

Another recently identified NO-tolerating gene of *A. nidulans* encodes a 23-amino-acid peptide called inducible nitrosothionein (iNT) (Zhou et al. 2013). This peptide is similar to the N-terminal half of metallothionein (MT) (Fig. 9.4), which is a ubiquitous, cysteine-rich peptide constituting N- and C-terminal β- and α-domains that both contain several cysteine resides (Capdevila and Atrian 2011). The overproduction of iNT or its loss induced by gene disruption respectively increases or decreases fungal growth in the presence of acidified nitrite as the NO donor, indicating that iNT is involved in the NO tolerance mechanism of *A. nidulans*. The gene disruptant of the gene encoding iNT produces less aconitase activity, and the fungus becomes more susceptible to respiratory oxygen consumption than the wild type after exposure to acidified nitrite. These impairments are likely to be involved in the retarded growth of the iNT gene disruptant because these activities are essential cellular metabolic processes.

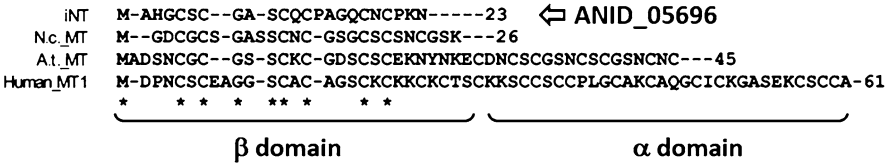

Fig. 9.4 Nitrosothionein and RNS tolerance. Alignment of nitrosothionein (iNT), classical metallothioneins, and fungal iNT-like peptide. Cysteine residues are *highlighted. A.t. Arabidopsis thaliana*, *N.c. Neurospora crassa*

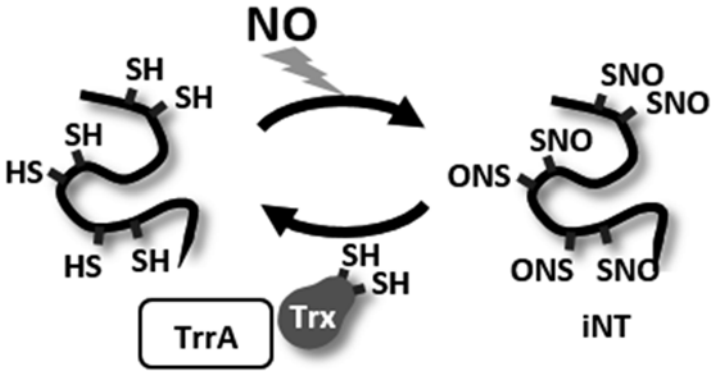

Fig. 9.5 Role of iNT coupled with thioredoxin (*Trx*) system in NO detoxification. *TrrA* thioredoxin reductase encoded by *trrA*

The chemistry of thiols (–SH) and NO is established, and their reactions under physiological conditions generates *S*-nitrosothiols (–SNO). This is also true for the iNT peptide, and thiols of the six cysteine residues are stoichemetrically converted to *S*-nitrosothiols in vitro (Zhou et al. 2013). Studies using the gene disruptant and a strain that produces an excess of iNT found a negative correlation between cellular iNT levels and the amount of *S*-nitrosated proteins, indicating that iNT protects cellular protein thiols from *S*-nitrosation by scavenging NO under RNS stress. The NO scavenging role of iNT seems to be mediated by thioredoxin-dependent catalysis (Zhou et al. 2013). Reconstitution studies in vitro have shown that thioredoxin (TrxA) rapidly reduces *S*-nitrosated iNT (iNT-SNO) to iNT, and the resultant oxidized thioredoxin is reduced by thioredoxin reductase (TrrA) and NADPH (Fig. 9.5). Reverse genetics studies have demonstrated that the NO-tolerance function of iNT requires TrrA, and the evidence described here supports this conclusion.

Metallothionein binds heavy metal cations at both the α- and β-domains, decreases their cellular concentration, and thus induces tolerance against them. Judging from its structural similarity to the metallothionein β-domain, the iNT peptide not only reacts with NO but also efficiently binds copper (I) (Zhou et al. 2013), suggesting that metallothionein and iNT both bind metals. However, disruption of the iNT gene does not affect fungal growth in the presence of various heavy metals. This is evident in the genetic background of this fungus that lacks typical metallothioneins, suggesting that iNT is dispensable for fungal heavy metal tolerance in vivo. This discrepancy between the chemical and physiological functions of iNT in metal tolerance might be explained by the regulation of iNT production. Zhou et al. found that adding acidified nitrite increases iNT gene expression, and that intracellular levels of iNT consequently increase to 5.0 ± 1.0 nmol g protein^{-1}, which is comparable to that of metallothionein in other organisms. This finding indicates that under normal conditions without added NO, the amount of iNT is insufficient to cause heavy metal tolerance, although this remains to be confirmed.

The physiological roles of *S*-nitrosothiols have recently been investigated in detail and iNT has provided a novel example of RNS-tolerant peptides in addition to GSH. In contrast to the metal-binding ability of iNT, thiol apparently imposes *S*-nitrosothiol generation on metallothionein cysteine residues both in vitro and in vivo. Mammalian metallothionein-3 is not involved in heavy metal tolerance (Vašák and Meloni 2011). Although NO-related functions remain unknown, NO induces the gene expression of metallothionein in mesangial cells (Datta and Lianos 2006). These metallothioneins might constitute an NO-detoxifying machinery similar to that of iNT.

9.4 Future Prospects

The metabolic mechanisms of *S. cerevisiae* under hypoxia and NO have been characterized in detail and those of filamentous fungi are just beginning to emerge. Both *S. cerevisiae* and *Aspergillus* are grouped in the phylum Ascomycotina or as imperfect fungi that are closely related to Ascomycotina. Their evolutional relationship indicates that they share some of the same response mechanisms to exogenous stimuli, such as upregulated glycolysis in response to hypoxia and the involvement of Fhb in NO tolerance. However, *S. cerevisiae* is unequivocally different in that it grows under hypoxic (and probably under anoxic) conditions, implying that *Aspergillus* will supersede this yeast under hypoxia as the most important model organism for studying general fungal responses to hypoxia. Furthermore, the fact that filamentous fungi produce proteins related to nitrogen oxides and RNS metabolism that have never been found in *S. cerevisiae* is hardly surprising because filamentous fungi efficiently reduce nitrate for utilization as a nitrogen source and electron acceptor. These proteins include cytochrome P450nor, iNT, and nitrate/nitrite reductases, as described in this chapter. Other proteins are being discovered in filamentous fungi. Genes regulating hypoxic gene expression and RNS regulation in filamentous fungi as well as cues that could explain the overall scheme of responses will be discovered in the near future.

References

Barker BM, Kroll K, Vödisch M et al (2012) Transcriptomic and proteomic analyses of the *Aspergillus fumigatus* hypoxia response using an oxygen-controlled fermenter. BMC Genomics 13:62

Benhar M, Forrester MT, Stamler JS (2009) Protein denitrosylation: enzymatic mechanisms and cellular functions. Nat Rev Mol Cell Biol 10:721–732

Bouché N, Fromm H (2004) GABA in plants: just a metabolite? Trends Plant Sci 9:110–115

Bunn HF, Poyton RO (1996) Oxygen sensing and molecular adaptation to hypoxia. Physiol Rev 76:839–885

Capdevila M, Atrian S (2011) Metallothionein protein evolution: a miniassay. J Biol Inorg Chem 16:977–989

Datta PK, Lianos EA (2006) Nitric oxide induces metallothionein-1 gene expression in mesangial cells. Transl Res 148:180–187

Desikan R, Griffiths R, Hancock J, Neill S (2002) A new role for an old enzyme: nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A 99:16314–16318

Diano A, Peeters J, Dynesen J et al (2009) Physiology of *Aspergillus niger* in oxygen-limited continuous cultures: influence of aeration, carbon source concentration and dilution rate. Biotechnol Bioeng 103:956–965

Fujii T, Takaya N (2008) Denitrification by the fungus *Fusarium oxysporum* involves NADH-nitrate reductase. Biosci Biotechnol Biochem 72:412–420

Gems D, Johnstone IL, Clutterbuck AJ (1991) An autonomously replicating plasmid transforms *Aspergillus nidulans* at high frequency. Gene 98:61–67

Hall LA, Denning DW (1994) Oxygen requirements of *Aspergillus* species. J Med Microbiol 41:311–315

Liu L, Hausladen A, Zeng M et al (2001) A metabolic enzyme for *S*-nitrosothiol conserved from bacteria to humans. Nature 410:490–494

Masuo S, Terabayashi Y, Shimizu M et al (2010) Global gene expression analysis of *Aspergillus nidulans* reveals metabolic shift and transcription suppression under hypoxia. Mol Gen Genet 284:415–424

Nakahara K, Tanimoto T, Hatano K et al (1993) Cytochrome P-450 55A1 (P450dNIR) acts as nitric oxide reductase employing NADH as direct electron donor. J Biol Chem 268:8350–8355

Poole RK (2005) Nitric oxide and nitrosative stress tolerance in bacteria. Biochem Soc Trans 33:176–180

Poyton R, Castello P, Ball K et al (2009) Mitochondria and hypoxic signaling. Ann N Y Acad Sci 1177:48–56

Shimizu M, Fujii T, Masuo S et al (2009) Proteomic analysis of *Aspergillus nidulans* cultured under hypoxic conditions. Proteomics 9:7–19

Shimizu M, Fujii T, Masuo S et al (2010) Mechanism of de novo branched-chain amino acid synthesis as an alternative electron sink in hypoxic *Aspergillus nidulans* cells. Appl Environ Microbiol 76:1507–1515

Shoun H, Tanimoto T (1991) Denitrification by the fungus *Fusarium oxysporum* and involvement of cytochrome P-450 in respiratory nitrate reduction. J Biol Chem 266:11078–11082

Takasaki K, Shoun H, Yamaguchi M et al (2004) Fungal ammonia fermentation, a novel metabolic mechanism that couples the dissimilatory and assimilatory pathways of both nitrate and ethanol. J Biol Chem 279:12414–12420

Takaya N (2009) Response to hypoxia, reduction of electron acceptors, and subsequent survival by filamentous fungi. Biosci Biotechnol Biochem 73:1–8

Terabayashi Y, Shimizu M, Kitazume T et al (2012) Conserved and specific responses to hypoxia in *Aspergillus oryzae* and *Aspergillus nidulans* determined by comparative transcriptomics. Appl Microbiol Biotechnol 93:305–317

Vašák M, Meloni G (2011) Chemistry and biology of mammalian metallothioneins. J Biol Inorg Chem 16:1067–1078

Vödisch M, Scherlach K, Winkler R et al (2011) Analysis of the *Aspergillus fumigatus* proteome reveals metabolic changes and the activation of the pseurotin A biosynthesis gene cluster in response to hypoxia. J Proteome Res 10:2508–2524

Zhou Z, Takaya N, Nakamura A et al (2002) Ammonia fermentation, a novel anoxic metabolism of nitrate by fungi. J Biol Chem 277:1892–1896

Zhou S, Fushinobu S, Kim SW et al (2011) Functional analysis and subcellular location of two flavohemoglobins from *Aspergillus oryzae*. Fungal Genet Biol 48:200–207

Zhou S, Narukami T, Nameki M et al (2012) Heme-biosynthetic porphobilinogen deaminase protects *Aspergillus nidulans* from nitrosative stress. Appl Environ Microbiol 78:103–109

Zhou S, Narukami T, Masuo S et al (2013) NO-inducible nitrosothionein mediates NO removal in tandem with thioredoxin. Nat Chem Biol 9:657–663

Chapter 10
Cell Wall Biosynthesis in Filamentous Fungi

Takuji Oka, Taiki Futagami, and Masatoshi Goto

Abstract The cell wall provides physical strength to cells and defines the morphology of fungi. During hyphal development of filamentous fungi, the apical region and the branching sites of the cell are remodeled to support hyphal extension and formation of a new hypha. The cell wall has contact with the environment and thus is the place of first contact with external stresses originating outside of the cells. The cell wall also acts as a matrix for various extracellular proteins such as enzymes and sensor proteins. Budding yeast (*Saccharomyces cerevisiae*) and filamentous fungi (*Aspergillus* species) are industrially and medically important fungi belonging to the Ascomycota. These fungi share similar composition in the cell wall although they are morphologically different. Fungal cell walls are usually composed of glucose, mannose, *N*-acetyl-D-glucosamine, proteins, and lipids. Some glycans composed of galactofuranose or *N*-acetyl-D-galactosamine are found characteristically in the cell wall of *Aspergillus* species. In this chapter, we present an overview of current knowledge on cell wall biogenesis and wall-stress sensing in fungi, particularly focusing on recent findings in filamentous fungi.

Keywords *Aspergillus* • Cell wall • Cell wall integrity • Fungi • Glucans • Glycosylation • GPI anchor • *Saccharomyces* • Stress response

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10.1 Introduction

The cell wall provides physical strength to cells and defines the morphology of fungi. During hyphal development of filamentous fungi, the apical region and the branching sites of the cell are remodeled to support hyphal extension and formation of a new hypha. The cell wall has contact with the environment and thus is the place of first contact with external stresses originating outside the cells. The cell wall also acts as a matrix for various extracellular proteins such as enzymes and sensor proteins. Budding yeast (*Saccharomyces cerevisiae*) and filamentous fungi (*Aspergillus* species) are industrially and medically important fungi belonging to the Ascomycota. These fungi share similar composition in the cell wall although they are morphologically different. Fungal cell walls usually are composed of glucose, mannose, *N*-acetyl-D-glucosamine, proteins, and lipids. Some glycans composed of galactofuranose or *N*-acetyl-D-galactosamine are found characteristically in the cell wall of *Aspergillus* species. Excellent review articles of the fungal cell wall have been published (Farkaš 1985; De Groot et al. 2005; Osherov and Yarden 2010; Ruiz-Herrera 2012). In this chapter, we present an overview of current knowledge on cell wall biogenesis and wall-stress sensing in fungi, particularly focusing on the recent findings in filamentous fungi.

10.2 Cell Wall Glycans Shared in Fungi

10.2.1 Chitin

Chitin is a skeletal cell wall constituent giving the wall mechanical rigidity in the majority of fungi. Chitin is a β-1,4-linked polymer of *N*-acetyl-D-glucosamine (GlcNAc). The adjacent chains of chitin are mutually linked by hydrogen bonds between the amide groups, so that the whole structure consists of a series of chitin chains. In the fungal wall, chitin occurs in its α-form, in which adjacent chains are organized in the antiparallel direction. In the fungal walls, chitin usually occurs in a complex with β-1,3-glucan, β-1,6-glucan, and cell wall proteins (Fig. 10.1) (Farkaš 1985).

Chitin is synthesized by chitin synthases, which are membrane proteins catalyzing the polymerization of GlcNAc using UDP-GlcNAc as a substrate. *S. cerevisiae* has three chitin synthase-encoding genes, whereas *Aspergillus nidulans* has more than eight chitin synthase-encoding loci in its genome. The function of the eight individual chitin synthases in *A. nidulans* is regulated spatially and temporally, and they have distinct roles in hyphal extension, septum formation, and conidium formation under various environmental conditions (Horiuchi 2009). Among the eight chitin synthases are two unique chitin synthases (CsmA and CsmB) with a myosin motor-like domain. Homologues of these proteins are found only in certain filamentous fungi. Myosins are motor proteins that move along actin filaments.

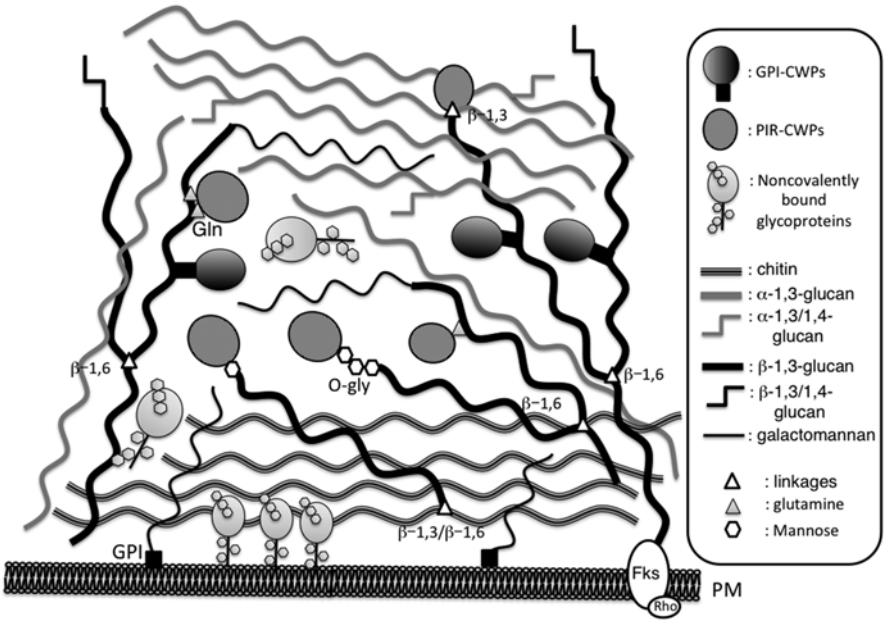

Fig. 10.1 Cell wall structure of *Aspergillus* spp.

The actin cytoskeleton is important to the hyphal polarity. CsmA and CsmB localize near actin structures at hyphal tips and forming septa, where the proteins play a role in polarized growth (Takeshita et al. 2006; Tsuizaki et al. 2013).

10.2.2 β-Glucans

β-1,3-Glucan is a typical skeletal cell wall constituent in most fungi. It is composed of D-glucose residues linked by β-1,3-glucosidic bonds. In fact, β-1,3-glucans branch through β-1,6 linkages from the main β-1,3-glucan chain (Fig. 10.1). The degree of branching by β-1,6-linkage varies in different fungal species and greatly influences the crystalline properties and solubility of β-glucans. Long linear β-1,3-glucan chains show an increased tendency for the formation of intra- and intermolecular hydrogen bonds and thus an increased tendency to crystallize than the highly branched chains. β-1,6-Glucan is found in the yeast *S. cerevisiae*, but is not found in a number of filamentous fungi, including *Aspergillus* species (Osherov and Yarden 2010). β-1,3-Glucan and β-1,6-glucan play important roles in giving the wall mechanical rigidity and cell wall assembly, respectively. In contrast, the cell wall of *Aspergillus fumigatus* contains a linear β-1,3/1,4-glucan that is covalently linked to the nonreducing end of β-1,3-glucan side chains (Fontaine et al. 2000).

β-1,3-Glucan is synthesized mainly by a β-1,3-glucan synthase complex, which is composed of a plasma membrane-localized catalytic subunit (FKS) and a regulatory subunit (Rho GTPase) and uses UDP-glucose as a substrate. The echinocandin class of antifungal drugs, which includes caspofungin and micafungin, inhibits the activity of FKS. *S. cerevisiae* possesses three *FKS* genes, and *A. fumigatus* has a single *fks* gene based on the genome analysis (Latgé et al. 2005). β-1,3-Glucan chains are further elongated and reorganized by β-1,3-glucanosyltransferases, which in filamentous fungi are designated as Gel proteins. Gel proteins cleave β-1,3-glucan molecules internally and transfer the newly generated reducing end to the nonreducing end of another β-1.3-glucan molecule, resulting in the elongation of the β-1.3-glucan chain. Gel proteins are glycosylated and attached to the cell wall through a glycosylphosphatidylinositol (GPI) anchor. *A. fumigatus* has seven *gel* homologues, and three are expressed at each stage of growth (Mouyna et al. 2013).

Synthesis of β-1,6-glucan in *S. cerevisiae* has been the subject of detailed analysis. The most active version of the enzyme catalyzing the polymerization of β-1, 6-glucan is the KRE6 protein. KRE6 is a type II membrane protein with amino acid sequence homology to glycoside hydrolase. KRE6 accumulates at the sites of polarized growth of yeast where and when β-1,6-glucan is synthesized (Kurita et al. 2011). Enzymes involved in the crosslinking of β-1,3-glucan chains via the β-1, 6-linkage have not been identified in fungi. Transglycosylases involved in the connection between both β-1,3-/β-1,6-glucans and chitin include the *S. cerevisiae* CRH1 and CRH2 proteins, both of which are GH16 family proteins, as classified in CAZY (http://www.cazy.org) in Cabib (2009). Five CRH orthologues are present in the genome of *A. fumigatus* (Mouyna et al. 2013).

10.2.3 α-Glucans

α-1,3-Glucan is a major cell wall constituent of most ascomycetous and basidiomycetous fungi. α-1,3-Glucan functions as a polysaccharide of the wall matrix and is located primarily in the outer wall layer, where this glucan forms thick, irregular microfibrils removable by alkaline extraction (Fig. 10.1). During alkaline extraction of *Aspergillus* walls, α-1,3-glucan copurifies with nigeran, an α-glucan with alternating α-1,3- and α-1,4-glucosidic bonds (Farkaš 1985).

α-1,3-Glucan is synthesized by the action of α-1,3-glucan synthase (AGS). AGSs are transmembrane proteins with high molecular masses (>200 kDa); these enzymes catalyze the polymerization of glucose via α-1,3-glucosidic linkages using UDP-glucose as a sugar donor. AGS-encoding loci have been identified in *A. nidulans* (two genes) and in *A. fumigatus* (three genes), but not in *S. cerevisiae* (Beauvais et al. 2013). In *A. nidulans*, disruption of both genes (*agsA* and *agsB*) does not lead to a lethal phenotype, indicating that these genes are dispensable in this fungus. However, AgsB is required for normal growth characteristics under liquid culture conditions (Yoshimi et al. 2013), indicating importance of α-1, 3-glucan for the cell wall.

10.3 Glycans Characteristically Found in Filamentous Fungi

10.3.1 Galactomannan

Fungal-type galactomannan of *Aspergillus* is composed of a linear mannan core with an α-1,2-linked mannotetraose repeating unit attached via an α-1,6-linkage and β-1,5-galactofuranose (Gal_f) oligomers (galactofuran side chain) of up to five residues; the galactofuran side chains are attached to the mannan backbone via β-1,6- or β-1,3-linkages (Fig. 10.2) (Latgé et al. 1994). Galactomannan is covalently linked either to the nonreducing end of a short β-1,3-glucan chain or to a GPI anchor (Fontaine et al. 2000; Costachel et al. 2005). The glycan structure of the GPI anchor, Manα1,2Manα1,2Manα1,6Manα1,4$GlcNH_2$, is α-1,6-linked to an inositol phosphoceramide. The galactomannan structure is thought to be linked to the terminal mannose of the anchor. Galactomannan also is released from the cell wall as a free polysaccharide in *A. fumigatus* cultures.

Several enzymes involved in the synthesis of Gal_f-containing sugar chains have been studied in filamentous fungi (Fig. 10.3). UDP-glucose 4-epimerase (UgeA) is

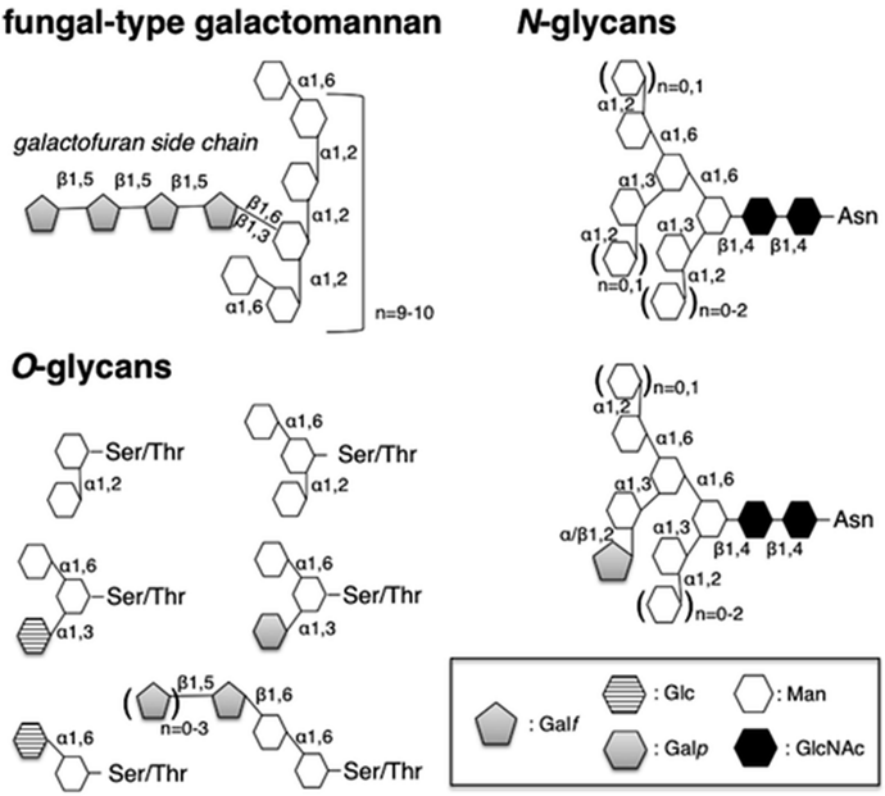

Fig. 10.2 Structure of *N*-glycans and *O*-glycans: galactomannan in *Aspergillus* spp.

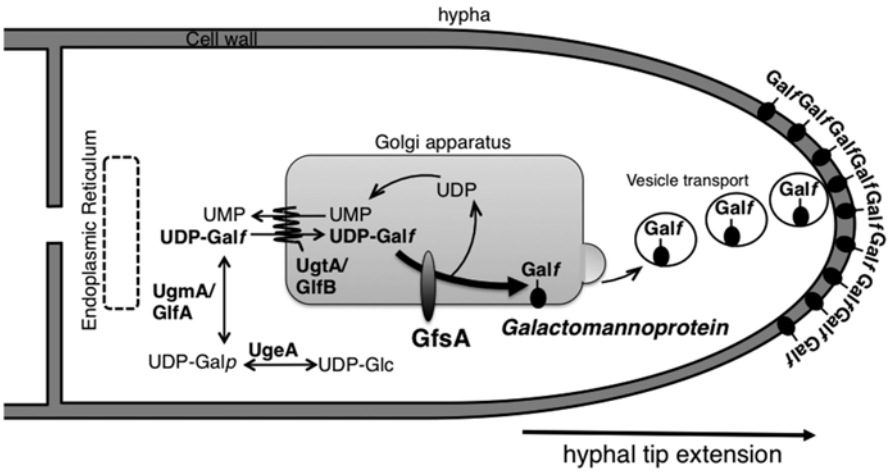

Fig. 10.3 Biosynthesis of Gal*f*-containing glycans in *Aspergillus* spp. *UMP* uridine 5′-monophosphate, *UDP* uridine 5′-diphosphate, *Galf* galactofuranose, *Galp* galactopyranose, *Glc* glucose

responsible for the conversion of UDP-glucose to UDP-galactopyranose (Gal_p) (El-Ganiny et al. 2010), which then is converted to UDP-Gal_f by the cytosolic UDP-Gal*p* mutase (UgmA/GlfA) (Bakker et al. 2005; Damveld et al. 2008; El-Ganiny et al. 2008; Schmalhorst et al. 2008). The synthesized UDP-Gal_f then is transported into the Golgi lumen by the Golgi-localized UDP-Gal_f transporter (UgtA/GlfB) (Engel et al. 2009; Afroz et al. 2011), which is encoded as a unique locus in the *Aspergillus* genome and is required for Gal_f deposition in the cell wall. UDP-Gal_f plays an important role in the Gal_f biosynthetic pathway, where it acts as a sugar donor for galactofuranosyltransferases (Engel et al. 2009; Afroz et al. 2011). Strains mutated for the gene encoding UDP-Gal_p mutase (*A. nidulans* Δ*ugmA* or *A. fumigatus* Δ*glfA*) display abnormal phenotypes that appear to result from the loss of all Gal_f-containing polysaccharides and glycoconjugates, including *O*-glycans, *N*-glycans, galactofuran side chains of galactomannan, and glycosylinositol phosphoceramides (El-Ganiny et al. 2008; Schmalhorst et al. 2008). These results indicate that Gal_f residues play an important role in forming the proper cell wall structure in *Aspergillus* spp. Galactofuranosyl transferase-encoding genes involved in the biosynthesis of galactomannan have not been identified.

10.3.2 Galactosaminogalactan

Galactosaminogalactan is a component of the extracellular matrix that covers the mycelium of *A. fumigatus*. Galactosaminogalactan is composed of α-1,4-linked galactose and α-1,4-linked *N*-acetylgalactosamine residues, with both monosaccharides randomly distributed at per-chain galactose percentages ranging from 15 % to

60 % (Fontaine et al. 2011). In *A. fumigatus*, this polysaccharide promotes fungal development in immunocompetent mice by its immunosuppressive activity, as evidenced by diminished neutrophil infiltration (Fontaine et al. 2011). In *A. fumigatus*, this polysaccharide also functions as the dominant adhesion for host cells and suppresses host inflammatory responses, in part through masking cell wall β-glucans from recognition by dectin-1 (Gravelat et al. 2013).

UDP-glucose 4-epimerase (Uge3 and Uge5) is responsible for the conversion of UDP-glucose to UDP-galactopyranose (Gal_p) (Lee et al. 2014). Uge3 can mediate production of both UDP-Gal_p and UDP-*N*-acetylgalactosamine (GalNAc) and is required for the production of galactosaminogalactan in *A. fumigatus* (Lee et al. 2014). In *A. nidulans*, UgeB possesses both UDP-glucose 4-epimerase and UDP-GalNAc 4-epimerase activities (Oka et al., unpublished data). Glycosyltransferases involved in the biosynthesis of galactosaminogalactan have not been identified.

10.4 Cell Wall Proteins

10.4.1 Covalently and Nocovalently Attached Wall Proteins

Cell wall proteins (CWPs) generally enhance the strength of the cell wall structure by crosslinking glycans and by forming an outer coat that protects wall glycans from hydrolytic degradation (Fig. 10.1). CWPs are either covalently or noncovalently linked to cell wall glycans. Covalently attached CWPs include GPI-anchored CWPs, covalently linked to the β-1,3/1,6-glucans core through a trimmed form of their GPI anchor, which includes a phosphodiester bridge connecting ethanolamine to the third mannosyl residues of the glycan core structure. Many GPI-CWPs are have not been functionally characterized. However, as already mentioned, some GPI-CWPs are involved in cell wall synthesis and remodeling, such as transglycosylases, chitinases, and glycoside hydrolases, indicating that the cell wall itself serves as a working place for these enzymes. Based on genomic analyses of fungi, individual species are predicted to encode 60 to 120 GPI-CWP-encoding genes (Osherov and Yarden 2010).

Another class of covalently attached CWPs are the alkali-sensitive linkage proteins, which include proteins with internal repeats (PIRs) that are directly linked to the β-1,3-glucan network through glutamine residues. The repetitive sequences in PIR-CWPs appear to be important for cell wall anchoring. In contrast to GPI-CWPs, PIR-CWPs with multiple anchoring sites to β-1,3-glucan are uniformly distributed throughout the inner polysaccharide layer of cell wall; upon cell wall damage, the PIR-CWP-encoding genes are strongly upregulated to compensate for weakening of the cell wall (De Groot et al. 2005). The reducing end of a β-1,3-glucan chain may be coupled to an *O*-linked side chain of PIR-CWPs (Fig. 10.1), resulting in the putative polysaccharide–CWP complex: β-1,3-glucan:Man_{1-7}-*O*-CWP (Klis et al. 2001) .

Another type of CWPs, noncovalently bound CWPs, often includes proteins of cytosolic origin and secretory proteins. These CWPs are bound to mannosylated proteins by electrostatic interactions or by the formation of disulfide bonds. The noncovalently bound CWPs are embedded as glycosylated forms in the fungal wall and determine the porosity of the wall (De Groot et al. 2005).

10.4.2 N-*Glycans and* O-*Glycans in Glycoproteins*

Because CWPs are usually secreted proteins that are produced with an N-terminal signal sequence, CWPs often are detected as glycosylated forms in the fungal wall. During the secretion process, the secretory proteins are glycosylated in the endoplasmic reticulum (ER) and Golgi apparatus. Protein glycosylation is believed to play a critical role in various cell activities such as quality control of secretory proteins, cell wall integrity, environmental adaptation, antigenicity, and pathogenicity in some pathogenic fungi. Protein glycosylation appears to modulate various functions, including protein stability and localization. *N*-glycan and *O*-glycan structures attached to the proteins differ among *S. cerevisiae* and *Aspergillus* species (Fig. 10.2) (Goto 2007; Jigami 2008; Deshpande et al. 2008).

Protein *N*-glycosylation is initiated in the ER by an oligosaccharyltransferase (OST) complex using $Glc_3Man_9GlcNAc_2$-PP-dolichol as a sugar donor. After transfer of the $Glc_3Man_9GlcNAc_2$ to the Asn residue of a polypeptide, three glucose residues and one mannose residue are removed by the sequential actions of a Gls2–Gtb1 complex and Mns1, respectively. This process is crucial for the quality control of secretory proteins in ER and ER-associated degradation of misfolded proteins upon recognition by lectins. Glycoproteins transported into the Golgi apparatus are subjected to further elongation of *N*-glycans. Yeast glycoproteins localized at the cell wall and periplasmic space contain a long, branched polymer of approximately 200 mannoses with phosphate in *N*-glycans (Jigami 2008). However, *N*-glycans in *Aspergillus* species are observed as $Man_{5–24}GlcNAc_2$-N, which are shorter in length than those in yeast (Maras et al. 1999; Wallis et al. 2001). The differences in *N*-glycan structures between these fungi are the result of the presence (in yeast) of the long α-1,6-mannan backbone heavily substituted with short α-mannosyl side chains, including some degree of phosphorylation. The long α-1,6-mannan backbone and phosphate residue in *N*-glycans are synthesized by the actions of Och1 and Mnn6, respectively. Given that the *A. fumigatus OCH1* orthologue complements the yeast *OCH1* mutant (Lambou et al. 2010), *A. fumigatus* would appear to have the potential ability to elongate *N*-glycans as seen in yeast. Taking into account the fact that *Aspergillus* species possess equivalents of all the yeast genes involved in the elongation of *N*-glycans, it is likely that *N*-glycans are subjected to partial degradation by α-mannosidases in *Aspergillus* species, yielding shortened *N*-glycans.

As with *N*-glycans, *O*-glycans attached to proteins from *Aspergillus* spp. also are shorter in length than those from yeast; *Aspergillus* spp. *O*-glycans additionally are

characterized by the presence of a branching form of oligosaccharide (Fig. 10.2, Goto 2007). Protein *O*-glycosylation is initiated in the ER by the action of protein *O*-mannosyltransferase (Pmt) using dolichyl phosphate mannose as a sugar donor (Willer et al. 2003). *S. cerevisiae* harbors seven *pmt* genes, which in turn are classified into three protein subfamilies (designated the Pmt1, Pmt2, and Pmt4 subfamilies). Each Pmt4 subfamily protein forms dimer complexes with proteins of the same subfamily, whereas each Pmt1 subfamily member forms dimer complexes with Pmt2 subfamily members. Simultaneous disruption of more than three *pmt* genes gives rise to a lethal phenotype in yeast. On the other hand, filamentous fungi possess only three *pmt* genes, corresponding to one from each Pmt subfamily. The three *Aspergillus pmt* genes have been characterized in both *A. nidulans* and *A. fumigatus*, where the encoded proteins play crucial roles in hyphal development, morphology, and asexual conidiation of these fungi (Oka et al. 2004; Zhou et al. 2007; Goto et al. 2009; Kriangkripipat and Momany 2009; Mouyna et al. 2010). After initial mannosylation by Pmt in the ER, further glycosylation occurs by the action of sugar transferases in the Golgi apparatus during the secretory process. Multiple α-1,2- and α-1,3-mannosyltransferases have been characterized in yeast, but no sugar transferase responsible for this elongation process has been characterized in *Aspergillus* spp.

10.4.3 Gal$_f$*-Containing* N- *and* O*-Glycans*

As described previously (Sect. 10.3.1), galactofuranose residues also are incorporated into the *N*- and *O*-glycans of *Aspergillus* spp. and closely related filamentous fungi (Wallis et al. 2001; Leitao et al. 2003). A single α- or β-1,2-linked Gal$_f$ residue is present at the nonreducing terminus in *N*-glycans (Fig. 10.2). Terminally β-1, 5-linked Gal$_f$ oligosaccharides connected to mannan of *O*-glycans via a β-1,6 or β-1,3 glucosidic bond are present in *O*-glycans. Sugar nucleotide donor UDP-Gal$_f$ biosynthesis is well studied, as already described (Sect. 10.3.1). However, knowledge of galactosyltranferase remains limited. Recently, the enzyme involved in the synthesis of Gal$_f$-containing *O*-glycans was identified as a Golgi-localized β-1, 5-galactofuranosyltransferase (GfsA), which catalyzes the transfer of UDP-Gal$_f$ as a sugar donor to β-1,6-Gal$_f$-attached galactomannoprotein in the presence of Mn^{2+} (Fig. 10.3, Komachi et al. 2013). In both *A. nidulans* and *A. fumigatus*, Δ*gfsA* cells exhibit an abnormal morphology characterized by poor hyphal extension, hyphal curvature, and limited formation of conidia. Genes related to *gfsA* are widely distributed in the Ascomycota subphylum Pezizomycotina, but are not found in the subphyla Saccharomycotina or Taphrinomycotina. The Pezizomycotina subphylum contains fungi pathogenic to humans, animals, and plants. It has been suggested that Gal$_f$-containing *O*-glycans from these fungi are related to virulence and infection (Komachi et al. 2013).

10.5 Stress Response

10.5.1 Cell Wall Integrity Signaling Pathway

In *S. cerevisiae*, cell wall biogenesis is regulated primarily by the cell wall integrity (CWI) signaling pathway (Levin 2011). This pathway is acknowledged to have an important role in adapting to environmental stresses such as hypo-osmosis. The orthologues of components of this pathway are widely conserved in fungal species. To date, the gene disruptants of *wscA*, *wscB*, *mtlA*, *rhoA*, *mpkA*, and *rlmA*, and a conditional mutant of *pkcA* have been characterized in the filamentous fungus *A. nidulans* (see Chaps. 12 and 13). These genes have been demonstrated to play essential roles in defining the appropriate cell morphology, differentiation, and CWI of *A. nidulans*. Interestingly, the stress sensor orthologues are considered to have evolved more rapidly than central signaling transducers among signaling components (Rispail et al. 2009; Nikolaou et al. 2009).

The putative model of the CWI signaling pathway of *A. nidulans* has been outlined (Fig. 10.4a). The upstream Wsc-family (WscA and WscB) and Mid2-family (MtlA) proteins are putative stress sensors and localize to the cell surface (Futagami et al. 2011, 2014; Futagami and Goto 2012). The downstream pathway has been uncovered based on the studies in *Aspergillus* and yeast models (Levin 2011; see Chap. 12 and 13). Specifically, the activated sensor proteins interact with a GDP/GTP exchange factor (Rom2). Rom2 then converts a small G protein, RhoA, from the GDP-bound state to the GTP-bound state. The GTP-bound RhoA in turn activates protein kinase C (PkcA) and the mitogen-activated protein kinase (MAPK) cascade, which consists of MAPKKK (BckA), MAPKK (Mkk1), and MAPK (MpkA), through sequential phosphorylation by GTPase activity. The phosphorylated MpkA moves into the nucleus to activate a transcriptional factor (RlmA) that regulates the expression of cell wall-related genes (Fujioka et al. 2007). MpkA-RlmA signaling has been shown to regulate (at least) the α-1,3-glucan synthase-encoding genes (*agsA* and *agsB*) and a glutamine-fructose-6-phosphate transaminase-encoding gene (*gfaA*) involved in chitin biosynthesis. Interestingly, these MpkA-RlmA regulated genes represent distinct activities from those in the well-studied *S. cerevisiae*. RlmA also has been shown to be involved in asexual development and autolysis in *A. nidulans* (Kovács et al. 2013).

10.5.2 Stress-Sensing Proteins

A. nidulans WscA, WscB, and MtlA show phylogenetic relationships to *S. cerevisiae* Wsc1, Wsc4, and Mid2, respectively (Futagami and Goto 2012). In *S. cerevisiae*, Wsc1 and Mid2 act as the primary stress sensors for CWI signaling (Levin 2011). The domain compositions are well conserved between *A. nidulans* and *S. cerevisiae*

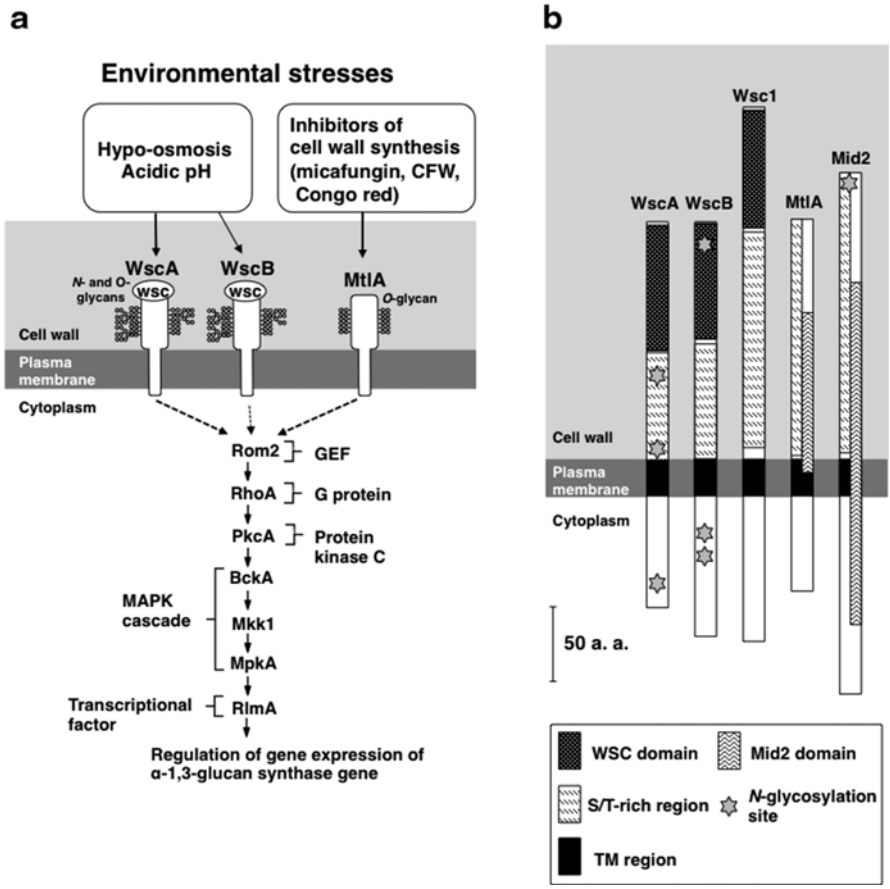

Fig. 10.4 A model of cell wall integrity (CWI) signaling pathways in *Aspergillus nidulans*. **a** Model for CWI signaling pathways in response to environmental stimuli. *Dotted lines* indicate unclear relationships. The contribution of these putative sensors to downstream CWI pathway signaling remains unclear because MpkA-RlmA signaling remains active in *wscA wscB* double disruptants and the *mtlA* single disruptant during exposure to stressors (hypo-osmotic shock and micafungin addition, respectively) (Futagami and Goto 2012; Futagami et al. 2014). The MAP kinase (Pmk1) signaling also has been found to remain active in the *mtl2* and *wsc1* disruptants of the fission yeast *Schizosaccharomyces pombe* (Cruz et al. 2013). Note that the *line* between Rom2 and Rho1 has been characterized solely for *A. fumigatus* within the genus *Aspergillus*; the two proteins were shown to interact with each other by co-immunoprecipitation (Samantaray et al. 2013). *WSC* cell wall integrity and stress response component domain, *CFW* Calcofluor white, *GEF* guanine nucleotide exchange factor, *G protein* guanine nucleotide-binding protein, *MAPK* mitogen-activated protein kinase. **b** Comparison of *A. nidulans* WscA, WscB, and MtlA to *Saccharomyces cerevisiae* Wsc1 and Mid2. Signal sequences are not depicted. *a.a.* amino acids, *S/T* serine/threonine. Note that WscA and WscB are *N*-glycosylated but the site of modification remains undefined (Futagami et al. 2011)

(Fig. 10.4b). The Wsc proteins are characterized by the presence of an N-terminal signal sequence, a WSC (cell wall integrity and stress response component) domain, a serine/threonine-rich region, a transmembrane region, and a highly charged C-terminal cytoplasmic region. The WSC domain contains up to eight conserved cysteine residues that may form S–S bonds; this domain is believed to mediate non-covalent binding with cell wall glucans. The primary difference in Wsc family proteins between *A. nidulans* and *S. cerevisiae* is found in the length of the serine/threonine-rich region and glycosylation profiles. The serine/threonine-region is shorter in *A. nidulans* WscA and WscB than in *S. cerevisiae* Wsc1. The *A. nidulans* WscA and WscB proteins are modified with both *O*- and *N*-glycans, whereas *S. cerevisiae* Wsc1 is modified with *O*-glycan but not with *N*-glycan (Lommel et al. 2004; Goto et al. 2009; Futagami et al. 2011). The *O*-glycan has an important role in stabilizing WscA by preventing proteolysis (Goto et al. 2009). *O*-glycosylation is believed to be required for the rod-like structure of mature Wsc1, providing extension and stiffening of the polypeptide (Lommel et al. 2004). Wsc1 behaves like a nanospring that is capable of resisting a high level of mechanical force and of responding to cell-surface stress in the live cell (Dupres et al. 2009). *O*-glycosylation enhances this protein's spring-like properties.

Next, in the case of Mid2-family proteins, *A. nidulans* MtlA and *S. cerevisiae* Mid2 proteins are characterized by the presence of an N-terminal signal sequence, a Mid2 domain, a transmembrane region, and a C-terminal cytoplasmic tail (Fig. 10.4b). The domain structures of MtlA and Mid2 also are well conserved, but the length of the Mid2 domain of MtlA is shorter than that of Mid2. In contrast to the Wsc proteins, MtlA is highly *O*-glycosylated, but is not *N*-glycosylated, whereas Mid2 is modified by both *O*- and *N*-glycans (Hutzler et al. 2008; Rajavel et al. 1999; Futagami et al. 2014). *O*-glycosylation of Mid2 is required for protein stability (Rajavel et al. 1999), whereas *N*-glycosylation near the N-terminus (N35) is considered to be directly involved in sensory activity (Hutzler et al. 2008). The N35 residue of Mid2 is not conserved in MtlA and its orthologues in Aspergilli, indicating that the sensing mechanism in the filamentous fungus MtlA may be different from that in *S. cerevisiae* Mid2.

WscA, WscB, and MtlA localize to the cell surface (Futagami et al. 2011, 2014). Localization at the cell surface is consistent with predicted function as a sensor protein that senses cell wall perturbations caused by environmental stimuli. MtlA may also localize to the septum, although the role of MtlA at the septum is still unclear (Futagami et al. 2014). On the other hand, in *S. cerevisiae* and *K. lactis*, Wsc1 proteins reside in membrane patches within the plasma membrane (Straede and Heinisch 2007; Rodicio et al. 2008). The WSC domain of *S. cerevisiae* Wsc1 is required for clustering, a phenomenon that is stimulated by stressful conditions (Heinisch et al. 2010). Thus, it has been proposed that the function of Wsc1 is coupled to its local enrichment within membrane patches called the Wsc1 "sensosome."

WscA/WscB and MtlA are considered to have different sensing spectra in *A. nidulans* (Fig. 10.4a). Based on the phenotypes of these gene mutant strains, WscA and WscB are required for tolerance to hypo-osmotic and acidic pH stresses

A. nidulans FGSC A4		*A. fumigatus* Af293	*A. niger* CBS 513.88	*A. oryzae* RIB40	*A. kawachii* IFO 4308
Locus tag/ Protein name	Function	Identities (%)	Identities (%)	Identities (%)	Identities (%)
AN5660/WscA	Stress sensor	53	51	56	56
AN4674/WscB	Stress sensor	54	53	53	47
AN4897/MtlA	Stress sensor	57	63	72	66
AN4719/Rom2	GDP/GTP exchange factor	83	84	82	84
AN5740/RhoA	G protein	94	98	96	98
AN0106/PkcA	Protein kinase C	84	83	84	83
AN4887/BckA	MAP kinase kinase kinase	60	61	60	61
AN4187/Mkk1	MAP kinase kinase	81	81	80	81
AN5666/MpkA	MAP kinase	88	89	90	89
AN2984/RlmA	Transcriptional factor	66	67	61	67
AN3154/Swi4	Transcriptional factor	75	77	78	76
AN6715/Swi6	Transcriptional factor	78	81	77	78

Color for BLASTP Identities (%)

40-60
60-80
80-100

Fig. 10.5 Degree of conservation of orthologues of components of cell wall integrity signaling pathway among *Aspergillus* species. Identities were obtained by BLASTP searches using each *A. nidulans* protein. *GEF* guanine nucleotide exchange factor, *G protein* guanine nucleotide-binding protein

but not to cell wall inhibitors (Futagami et al. 2011). In contrast, MtlA is required for tolerance to cell wall stress associated with exposure to Congo red, Calcofluor white, and micafungin, but not to hypo-osmotic stress (Futagami et al. 2014). The sensing spectrum also is different from those of Wsc1 and Mtl2 of *S. cerevisiae*. For example, *S. cerevisiae* Wsc1 is involved in the response to stress associated with exposure to alkaline conditions (Serrano et al. 2006). In addition, Wsc1, but not Mid2, mediates caspofungin-induced PKC pathway activation in *S. cerevisiae* (Reinoso-Martín et al. 2003). Thus, the functional roles of sensor orthologues apparently differ between *A. nidulans* and *S. cerevisiae*. Moreover, the sensing spectrum of sensor orthologues might be different even among *Aspergillus* species. Three candidate sensors, Wsc1, Wsc3, and MidA, have been characterized in *A. fumigatus* (Dichtl et al. 2012). *A. nidulans* WscA, WscB, and MtlA were counterparts to *A. fumigatus* Wsc1, Wsc3, and MidA, respectively, but the *A. fumigatus wsc1* and *midA* disruptants showed different growth deficiencies than those of the *A. nidulans wscA* and *mtlA* disruptants. MidA is involved in tolerance of elevated temperature, CFW, and Congo red in *A. fumigatus*, but is dispensable in resistance to caspofungin. In *A. fumigatus*, Wsc1 is required for caspofungin tolerance. Thus, the Mid2- and Wsc family proteins have different functions in *A. fumigatus* and *A. nidulans*. These differences might be explained by different evolutionary histories. For example, in contrast to *A. nidulans*, *A. fumigatus* is an opportunistic pathogen that causes invasive aspergillosis. The rapid evolution of sensor orthologues among *Aspergillus* species is also supported by BLASTP searches using *A. nidulans* CWI component orthologues (Fig. 10.5). Although all the components are well conserved, the sensor proteins show lower similarities than do the other components, even among congeneric species, which is consistent with the hypothesis that upstream sensors and transcriptional factors have evolved more rapidly than the central signaling transducers in fugal signaling pathways (Rispail et al. 2009; Nikolaou et al. 2009).

10.5.3 High Osmolarity Glycerol Pathway

The CWI signaling pathway is required for adaptation to hypo-osmotic environments, whereas the HOG (high osmolarity glycerol) MAPK (mitogen-activated protein kinase) pathway is important in adaptation under high osmotic stress (Saito and Posas 2012, see Chap. 13). The HOG pathway also is conserved among fungal species (Rispail et al. 2009; Nikolaou et al. 2009). During activation of the HOG pathway, phosphorylated HogA induces the expression of glycerol biosynthesis genes such as the glycerol-3-phosphate dehydrogenase-encoding *gfdB* locus (Furukawa et al. 2007). From the aspect of cell wall biogenesis, transcriptional factor Skn7 of the HOG pathway is involved in oxidative stress response and also the control of cell wall biogenesis in *S. cerevisiae* (Levin and Bartlett-Heubusch 1992; Alberts et al. 1998). The Skn7 orthologue was characterized in *A. fumigatus* (Lamarre et al. 2007). Similar to the *S. cerevisiae skn7* mutant, growth of the *A. fumigatus skn7* mutant is inhibited in the presence of hydrogen peroxide; in contrast to the yeast mutant, the *A. fumigatus skn7* mutant does not exhibit elevated susceptibility to the yeast cell wall inhibitor Calcofluor white.

10.6 Future Perspectives

Fungi have characteristic cell wall structures that permit the cells to maintain themselves and adapt under various environments. Thus, fungal cell wall components that are absent in mammals become attractive targets for antifungal drugs. Indeed, polyoxins and nikkomycins, inhibitors of chitin synthases, were discovered in cultures of *Streptomyces* spp. (Endo and Misato 1969; Dähn et al. 1976). Echinocandins, including caspofungin and micafungin, specifically inhibit β-1,3-glucan synthesis in fungi and have been the subject of recent development by pharmaceutical companies. Rhodanine-3-acetic acid derivatives and pradimicins inhibit mannosylation in fungal glycoproteins (Walsh and Giri 1997; Orchard et al. 2004). These antifungals were identified based on targeting of the fungal cell wall components generated by well-studied cell wall synthetic processes. However, recent findings regarding crosslinking enzymes, enzymes involved in the synthesis of galactofuranose-containing glycans and galactosaminogalactan, and proteins for cell wall integrity should allow us to discover new classes of antifungals.

Because fungi include both harmful and beneficial microorganisms, fungal cell wall studies contribute to their application not only in the medical field but also in the fermentation industry. *A. oryzae* and *A. kawachii* have been used as koji molds for brewing alcoholic beverages (sake and shochu, respectively) in Japan. Koji is rice or barley that has been polished, steamed, and covered with the hyphal growth of a fungus; the secreted fungal enzymes convert the starch present in the grains to glucose (Akiyama 2010). A large number of filamentous fungal conidia with high germination rates are absolutely required as a seed for making koji. Defects in

fungal cell wall often give rise to reduced conidial formation by filamentous fungi; thus, understanding cell wall biogenesis supports production of the high-quality and mass-quantity conidia needed by the fermentation industry. During shochu-koji making, *Aspergillus kawachii* is subjected to growth at a high temperature of 40 °C to induce the production of amylase and glucoamylase, followed by a shift to about 30 °C to induce the production of citric acid. Under these conditions, *A. kawachii* is subjected to stress caused by the high temperature applied at the earliest stage of incubation. Microarray analysis of the *A. kawachii* genome revealed that various cell wall-related genes (including β-1,3-glucanosyltransferase, chitinases, β-1, 3-gluconases, and uncharacterized glycoside hydrolases) were induced in response to the temperature shift (our unpublished data). Thus, studies of cell wall biogenesis under stress conditions are expected to provide improved culturing conditions and strains for the development of alternative koji-making processes.

References

Afroz S, El-Ganiny AM, Sanders DA, Kaminskyj SG (2011) Roles of the *Aspergillus nidulans* UDP-galactofuranose transporter, UgtA in hyphal morphogenesis, cell wall architecture, conidiation, and drug sensitivity. Fungal Genet Biol 48:896–903

Akiyama H (2010) Sake: the essence of 2000 years of Japanese wisdom gained from brewing alcoholic beverages from rice. Brewing Society of Japan, Tokyo

Alberts AS, Bouquin N, Johnston LH, Treisman R (1998) Analysis of RhoA-binding proteins reveals an interaction domain conserved in heterotrimeric G protein β subunits and the yeast response regulator protein Skn7. J Biol Chem 273:8616–8622

Bakker H, Kleczka B, Gerardy-Schahn R, Routier FH (2005) Identification and partial characterization of two eukaryotic UDP-galactopyranose mutases. Biol Chem 386:657–661

Beauvais A, Bozza S, Kniemeyer O et al (2013) Deletion of the α-(1,3)-glucan synthase genes induces a restructuring of the conidial cell wall responsible for the avirulence of *Aspergillus fumigatus*. PLoS Pathog 9(11):e1003716. doi:10.1371/journal.ppat.1003716

Cabib E (2009) Two novel techniques for determination of polysaccharide cross-links show that Crh1p and Crh2p attach chitin to both β(1-6)- and β(1-3) glucan in the *Saccharomyces cerevisiae* cell wall. Eukaryot Cell 8:1626–1636

Costachel C, Coddeville B, Latgé JP, Fontaine T (2005) Glycosylphosphatidylinositol-anchored fungal polysaccharide in *Aspergillus fumigatus*. J Biol Chem 280:39835–39842

Cruz S, Muñoz S, Manjón E et al (2013) The fission yeast cell wall stress sensor-like proteins Mtl2 and Wsc1 act by turning on the GTPase Rho1p but act independently of the cell wall integrity pathway. Microbiol Open 2:778–794

Dähn U, Hagenmaier H, Höhne H et al. (1976) Stoffwechselprodukte von mikroorganismen. 154. Mitteilung. Nikkomycin, ein neuer hemmstoff der chitinsynthese bei pilzen. Arch Microbiol 107:143–160

Damveld RA, Franken A, Arentshorst M et al (2008) A novel screening method for cell wall mutants in *Aspergillus niger* identifies UDP-galactopyranose mutase as an important protein in fungal cell wall biosynthesis. Genetics 178:873–881

De Groot PWJ, Ram AF, Klis FM (2005) Features and functions of covalently linked proteins in fungal cell walls. Fungal Genet Biol 42:657–675

Deshpande N, Wilkins MR, Packer N et al (2008) Protein glycosylation pathways in filamentous fungi. Glycobiology 8:626–637

Dichtl K, Helmschrott C, Dirr F, Wagener J (2012) Deciphering cell wall integrity signaling in *Aspergillus fumigatus*: identification and functional characterization of cell wall stress sensors and relevant Rho GTPases. Mol Microbiol 83:506–519

Dupres V, Alsteens D, Wilk S et al (2009) The yeast Wsc1 cell surface sensor behaves like a nanospring in vivo. Nat Chem Biol 5:857–862

El-Ganiny AM, Sanders DA, Kaminskyj SG et al (2008) *Aspergillus nidulans* UDP-galactopyranose mutase, encoded by *ugmA* plays key roles in colony growth, hyphal morphogenesis, and conidiation. Fungal Genet Biol 45:1533–1542

El-Ganiny AM, Sheoran I, Sanders DA, Kaminskyj SG (2010) *Aspergillus nidulans* UDP-glucose-4-epimerase UgeA has multiple roles in wall architecture, hyphal morphogenesis, and asexual development. Fungal Genet Biol 47:629–635

Endo A, Misato T (1969) Polyoxin D, a competitive inhibitor of UDP-N-acetylglucosamine: Chitin N-acetylglucosaminyltransferase in Neurospora crassa. Biochem Biophys Res Commun 37:718–722

Engel J, Schmalhorst PS, Dörk-Bousset T et al (2009) A single UDP-galactofuranose transporter is required for galactofuranosylation in *Aspergillus fumigatus*. J Biol Chem 284:33859–33868

Farkaš V (1985) The fungal cell wall. In: Peberdy JF, Ferenczy L (eds) Fungal protoplasts. Dekker, New York, pp 3–29

Fontaine T, Simenel C, Dubreucq G et al (2000) Molecular organization of the alkali-insoluble fraction of *Aspergillus fumigatus* cell wall. J Biol Chem 275:27594–27607

Fontaine T, Delangle A, Simenel C et al (2011) Galactosaminogalactan, a new immunosuppressive polysaccharide of *Aspergillus fumigatus*. PLoS Pathog 7:e1002372

Fujioka T, Mizutani O, Furukawa K et al (2007) MpkA-dependent and -independent cell wall integrity signaling in *Aspergillus nidulans*. Eukaryot Cell 6:1497–1510

Furukawa K, Yoshimi A, Furukawa T et al (2007) Novel reporter gene expression systems for monitoring activation of the *Aspergillus nidulans* HOG pathway. Biosci Biotechnol Biochem 71:1724–1730

Futagami T, Goto M (2012) Putative cell wall integrity sensor proteins in *Aspergillus nidulans*. Commun Integr Biol 5:206–208

Futagami T, Nakao S, Kido Y et al (2011) Putative stress sensors WscA and WscB are involved in hypo-osmotic and acidic pH stress tolerance in *Aspergillus nidulans*. Eukaryot Cell 10:1504–1515

Futagami T, Seto K, Kajiwara Y et al (2014) The putative stress sensor protein MtlA is required for conidia formation, cell wall stress tolerance, and cell wall integrity in *Aspergillus nidulans*. Biosci Biotechnol Biochem. doi:10.1080/09168451.2014.878218

Goto M (2007) Protein *O*-glycosylation in fungi: diverse structures and multiple functions. Biosci Biotechnol Biochem 6:1415–1427

Goto M, Harada Y, Oka T et al (2009) Protein *O*-mannosyltransferases B and C support hyphal development and differentiation in *Aspergillus nidulans*. Eukaryot Cell 10:1465–1474

Gravelat FN, Beauvais A, Liu H et al (2013) *Aspergillus* galactosaminogalactan mediates adherence to host constituents and conceals hyphal β-glucan from the immune system. PLoS Pathog 9:e1003575

Heinisch JJ, Dupres V, Wilk S et al (2010) Single-molecule atomic force microscopy reveals clustering of the yeast plasma-membrane sensor Wsc1. PLoS One 5:e11104

Horiuchi H (2009) Functional diversity of chitin synthases of *Aspergillus nidulans* in hyphal growth, conidiophore development and septum formation. Med Mycol. doi:10.1080/13693780802213332

Hutzler F, Gerstl R, Lommel M, Strahl S (2008) Protein N-glycosylation determines functionality of the *Saccharomyces cerevisiae* cell wall integrity sensor Mid2p. Mol Microbiol 68:1438–1449

Jigami Y (2008) Yeast glycobiology and its application. Biosci Biotechnol Biochem 3:637–648

Klis FM, De Groot P, Hellingwerf K (2001) Molecular organization of the cell wall of *Candida albicans*. Med Mycol 39:1–8

Komachi Y, Hatakeyama S, Motomatsu H et al (2013) GfsA encodes a novel galactofuranosyltransferase involved in biosynthesis of galactofuranose antigen of *O*-glycan in *Aspergillus nidulans* and *Aspergillus fumigatus*. Mol Microbiol 90:1054–1073

Kovács Z, Szarka M, Kovács S et al (2013) Effect of cell wall integrity stress and RlmA transcription factor on asexual development and autolysis in *Aspergillus nidulans*. Fungal Genet Biol 54:1–14

Kriangkripipat T, Momany M (2009) *Aspergillus nidulans* protein *O*-mannosyltransferases play roles in cell wall integrity and developmental patterning. Eukaryot Cell 8:1475–1485

Kurita T, Noda Y, Takagi T et al (2011) Kre6 protein essential for yeast cell wall β-1,6-glucan synthesis accumulates at sites of polarized growth. J Biol Chem 286:7429–7438

Lamarre C, Ibrahim-Granet O, Du C et al (2007) Characterization of the *SKN7* ortholog of *Aspergillus fumigatus*. Fungal Genet Biol 44:682–690

Lambou K, Perkhofer S, Fontaine T et al (2010) Comparative functional analysis of the *OCH1* mannosyltransferase families in *Aspergillus fumigatus* and *Saccharomyces cerevisiae*. Yeast 8:625–636

Latgé JP, Kobayashi H, Debeaupuis JP et al (1994) Chemical and immunological characterization of the extracellular galactomannan of *Aspergillus fumigatus*. Infect Immun 62:5424–5433

Latgé JP, Mouyna I, Tekaia F et al (2005) Specific molecular features in the organization and biosynthesis of the cell wall of *Aspergillus fumigatus*. Med Mycol 43:15–22

Lee MJ, Gravelat FN, Cerone RP et al (2014) Overlapping and distinct roles of *Aspergillus fumigatus* UDP-glucose 4-epimerases in galactose metabolism and the synthesis of galactose-containing cell wall polysaccharides. J Biol Chem 289:1243–1256

Leitao EA, Bittencourt VC, Haido RM et al (2003) β-Galactofuranose-containing *O*-linked oligosaccharides present in the cell wall peptidogalactomannan of *Aspergillus fumigatus* contain immunodominant epitopes. Glycobiology 13:681–692

Levin DE (2011) Regulation of cell wall biogenesis in *Saccharomyces cerevisiae*: the cell wall integrity signaling pathway. Genetics 189:1145–1175

Levin DE, Bartlett-Heubusch E (1992) Mutants in the *S. cerevisiae* PKC1 gene display a cell cycle-specific osmotic stability defect. J Cell Biol 116:1221–1229

Lommel M, Bagnat M, Strahl S (2004) Aberrant processing of the WSC family and Mid2p cell surface sensors results in cell death of *Saccharomyces cerevisiae O*-mannosylation mutants. Mol Cell Biol 24:46–57

Maras M, van Die I, Contreras R et al (1990) Filamentous fungi as production organisms for glycoproteins of bio-medical interest. Glycoconj J 16:99–107

Mouyna I, Kniemeyer O, Jank T et al (2010) Members of protein *O*-mannosyltransferase family in *Aspergillus fumigatus* differentially affect growth, morphogenesis and viability. Mol Microbiol 76:1205–1221

Mouyna I, Hartl L, Latgé JP (2013) β-1,3-Glucan modifying enzymes in *Aspergillus fumigatus*. Front Microbiol 4:81. doi:10.3389/fmicb.2013.00081

Nikolaou E, Agrafioti I, Stumpf M et al (2009) Phylogenetic diversity of stress signalling pathways in fungi. BMC Evol Biol 9:44

Oka T, Hamaguchi T, Sameshima Y et al (2004) Molecular characterization of protein *O*-mannosyltransferase and its involvement in cell-wall synthesis in *Aspergillus nidulans*. Microbiology 6:1973–1982

Orchard MG, Neuss JC, Galley CM, et al. (2004) Rhodanine-3-acetic acid derivatives as inhibitors of fungal protein mannosyl transferase 1 (PMT1). Bioorg Med Chem Lett 14:3975–3978

Osherov N, Yarden O (2010) The cell wall of filamentous fungi. In: Borkovich KA, Ebbole DJ (eds) Cellular and molecular biology of filamentous fungi. ASM, Washington, pp 224–237

Rajavel M, Philip B, Buehrer BM et al (1999) Mid2 is a putative sensor for cell integrity signaling in *Saccharomyces cerevisiae*. Mol Cell Biol 19:3969–3976

Reinoso-Martín C, Schuller C, Schuetzer-Muehlbauer M, Kuchler K (2003) The yeast protein kinase C cell integrity pathway mediates tolerance to the antifungal drug caspofungin through activation of Slt2p mitogen-activated protein kinase signaling. Eukaryot Cell 2:1200–1210. doi:10.1128/EC.2.6.1200-1210.2003

Rispail N, Soanes DM, Ant C et al (2009) Comparative genomics of MAP kinase and calcium-calcineurin signalling components in plant and human pathogenic fungi. Fungal Genet Biol 46:287–298

Rodicio R, Buchwald U, Schmitz HP, Heinisch JJ (2008) Dissecting sensor functions in cell wall integrity signaling in *Kluyveromyces lactis*. Fungal Genet Biol 45:422–435
Ruiz-Herrera J (2012) Fungal cell wall, structure, synthesis, and assembly. CRC, Boca Raton
Saito H, Posas F (2012) Response to hyperosmotic stress. Genetics 192:289–318
Samantaray S, Neubauer M, Helmschrott C, Wagener J (2013) Role of the guanine nucleotide exchange factor Rom2 in cell wall integrity maintenance of *Aspergillus fumigatus*. Eukaryot Cell 12:288–298
Schmalhorst PS, Krappmann S, Vervecken W et al (2008) Contribution of galactofuranose to the virulence of the opportunistic pathogen *Aspergillus fumigatus*. Eukaryot Cell 7:1268–1277
Serrano R, Martin H, Casamayor A, Arino J (2006) Signaling alkaline pH stress in the yeast *Saccharomyces cerevisiae* through the Wsc1 cell surface sensor and the Slt2 MAPK pathway. J Biol Chem 281:39785–39795
Straede A, Heinisch JJ (2007) Functional analyses of the extra- and intracellular domains of the yeast cell wall integrity sensors Mid2 and Wsc1. FEBS Lett 581:4495–4500
Takeshita N, Yamashita S, Ohta A et al (2006) *Aspergillus nidulans* class V and VI chitin synthases CsmA and CsmB, each with a myosin motor-like domain, perform compensatory functions that are essential for hyphal tip growth. Mol Microbiol 59:1380–1394
Tsuizaki M, Ohta A, Horiuchi H (2013) Myosin motor-like domain of class VI chitin synthase CsmB of *Aspergillus nidulans* is not functionally equivalent to that of class V chitin synthase CsmA. Biosci Biotechnol Biochem 77:369–374
Wallis GL, Easton RL, Jolly K et al (2001) Galactofuranoic-oligomannose *N*-linked glycans of alpha-galactosidase A from *Aspergillus niger*. Eur J Biochem 268:4134–4143
Walsh TJ, Giri N (1997) Pradimicins: a novel class of broad-spectrum antifungal compounds. Eur J Clin Microbiol Infect Dis 16:93–97
Willer T, Valero MC, Tanner W et al (2003) *O*-Mannosyl glycans: from yeast to novel associations with human disease. Curr Opin Struct Biol 13:621–630
Yoshimi A, Sano M, Inaba A et al (2013) Functional analysis of the α-1,3-glucan synthase genes agsA and agsB in *Aspergillus nidulans*: agsB is the major α-1,3-glucan synthase in this fungus. PLoS One 8(1):e54893. doi:10.1371/journal.pone.0054893
Zhou H, Hu H, Zhang L et al (2007) *O*-Mannosyltransferase 1 in *Aspergillus fumigatus* (AfPmt1p) is crucial for cell wall integrity and conidium morphology, especially at an elevated temperature. Eukaryot Cell 6:2260–2268

Chapter 11
Stress Responses of *Koji* Mold Cells with Highly Polarized and Multicellular Morphology

Jun-ichi Maruyama and Katsuhiko Kitamoto

Abstract The *koji* mold *Aspergillus oryzae* has long been used in traditional Japanese fermentation for the manufacture of sake, soy sauce, and *miso*, and it is also utilized for the industrial production of enzymes and recombinant proteins. *A. oryzae* grows by extending and compartmentalizing hyphae into individual cells, a morphological feature that increases the possibility that hyphae differentially encounter environmental and physical stresses. Stress responses of *A. oryzae* are conventionally analyzed using whole mycelial samples; however, recent studies conducted at the cellular level have provided new insights into the spatial specificity of stress responses in individual hyphae. In contrast to unicellular yeast, *A. oryzae* has complex morphological features that are characterized by multicellularity and polarized filamentous growth. The intercellular connectivity of hyphae via the septal pore is strictly regulated by the Woronin body, which is a Pezizomycotina-specific organelle that plugs the septal pore upon the physical wounding of cells. AoSO protein, which is required for hyphal fusion, also accumulates at the septal pore in response to stress. Stress granules, which sequester nontranslating mRNAs, form cytoplasmic foci that include a novel component AoSO protein at the hyphal tip, which is the main site of protein secretion. In this review, we present the most recent cell biology-based evidence for the stress response mechanisms in *A. oryzae* and discuss how this knowledge could contribute to the industrial application of this important fungus.

Keywords *Aspergillus oryzae* • Filamentous fungi • Multicellularity • Polarized growth • Septal pore • Stress granules • Woronin body

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11.1 Introduction

The *koji* mold *Aspergillus oryzae* is a filamentous fungus that has long been used in the production of traditional Japanese fermented foods such as sake, soy sauce, and *miso* (Kitamoto 2002). *A. oryzae* is also a suitable host for heterologous protein production because of its ability to secrete large amounts of enzymes into the medium and its Generally Regarded as Safe (GRAS) status based on its extensive use in fermented food production. A number of industrially useful proteins have been produced using *A. oryzae* (Nakajima et al. 2006; Ito et al. 2007; Chen et al. 2010), and hyperproducing mutant strains have also been isolated (Nemoto et al. 2009). Advances in molecular genetic techniques for generating multiple gene disruptions (Maruyama and Kitamoto 2008; Maruyama and Kitamoto 2011) have facilitated the molecular breeding of hyperproducing *A. oryzae* host strains (Yoon et al. 2009; Yoon et al. 2011; Zhu et al. 2013). As recently reported, inhibition of vacuolar degradation machineries, such as vacuolar protein sorting and autophagy, enhanced the ability of heterologous protein production in *A. oryzae* (Yoon et al. 2010; Yoon et al. 2013). This finding indicates that cell biology-based approaches are important for optimizing the potential of *A. oryzae* as a host for industrial protein production.

Compared to unicellular eukaryotic microorganisms such as yeast, *A. oryzae* has complex morphological features that are characterized by multicellularity and highly polarized growth. *A. oryzae* forms straight hyphae via polarized elongation of the hyphal tip. Visualization of fluorescent protein fusions in *A. oryzae* has allowed the subcellular localizations of various organelles and proteins to be determined (Fig. 11.1) (Maruyama et al. 2001). Secretory proteins accumulate at the hyphal tip (Masai et al. 2003; Kimura et al. 2010; Hayakawa et al. 2011), a localization that is consistent with that of the vesicle supply center called the Spitzenkörper

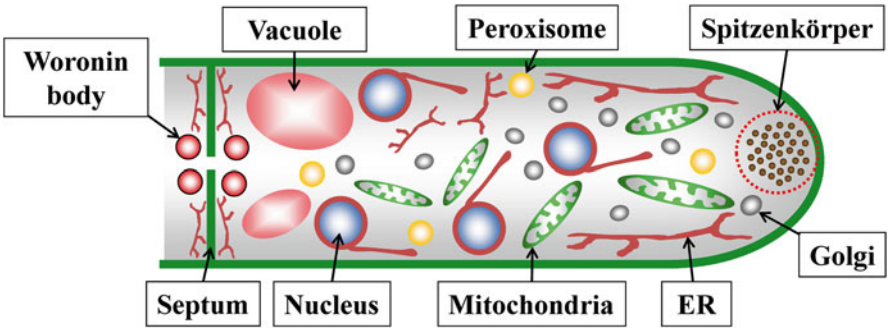

Fig. 11.1 Schematic model showing the morphology and organelles of *Aspergillus oryzae* hyphal cells. *ER* endoplasmic reticulum

(Harris et al. 2005). Organelles involved in protein secretion, such as the endoplasmic reticulum (ER) and the Golgi apparatus, are also distributed in a polarized manner near the hyphal tip (Maruyama et al. 2006; Maruyama and Kitamoto 2007; Kuratsu et al. 2007; Kimura et al. 2010). However, upon exposure to environmental stresses such as heat and cold, a portion of the secretory proteins targeted to the hyphal tip are delivered to the vacuoles (Masai et al. 2003). Such spatial regulation of secretory proteins in response to stress may markedly reduce polarized secretion at the hyphal tip, which has industrial importance for the use of *A. oryzae* as a cell factory for heterologous protein production. In prolonged culture accompanied with nutrient starvation, autophagy delivers misfolded proteins and organelles, such as ER, mitochondria, peroxisomes, and nuclei, into vacuoles for degradation (Shoji et al. 2010; Kimura et al. 2011), thereby limiting secreted protein yields.

A. oryzae forms multicellular networks of hyphae that are compartmentalized into distinct cells by the formation of the septum (Fig. 11.1). The septum functions to divide hyphae into sections with distinct growth states, such as mitotic and non-mitotic cells (Shen et al. 2014). However, the septum does not completely separate hyphae because there is a perforated structure, called the septal pore, that allows the passage of cytoplasm and organelles between adjacent hyphal cells as reported in other filamentous fungi (Lew 2005; Tey et al. 2005; Ng et al. 2009). Such cell-to-cell connectivity resembles that found in higher eukaryotes, such as gap junctions in animal cells and plasmodesmata in plant cells, and suggests that *A. oryzae* possesses an intercellular channel system that modulates responses to environmental stressors. Thus, the septal pore may have an important role in the organized growth of *A. oryzae* and other filamentous fungi. However, the interconnection of mycelia makes them susceptible to catastrophic damage as a result of excessive cytoplasmic loss when individual hyphae are physically wounded.

The morphological characteristics of *A. oryzae* hyphal cells increase their susceptibility to various kinds of stresses. For example, in the process of *koji*-making for sake fermentation (Kitamoto 2002), *A. oryzae* conidia are germinated on streamed rice, producing hyphae that grow rapidly and increase the temperature of the *koji* rice. To reduce the impact of heat stress on mycelia, the *koji* rice is frequently mixed to lower the temperature below 38–42 °C. After 40 to 45 h of cultivation, the *koji* rice is incubated at low temperature to halt fungal growth. During *koji* rice preparation, actively growing cells must adapt to rapidly changing environments, including heat and cold, whereas older cells are frequently exposed to starvation and oxidative stresses. For the study of mycelial samples containing different types of stressed cells, such as those found in *koji* rice, conventional analysis techniques do not allow for accurate insight into the stress physiology of *A. oryzae* hyphal cells. Thus, determining the subcellular dynamics of organelles and proteins of individual hyphae is critical for understanding fungal responses to stress.

In this review, we present the emerging cell biology-based evidence related to stress responses in *A. oryzae* and discuss its importance for the industrial application of this fungus.

11.2 Stress and Multicellularity in *Aspergillus oryzae*

11.2.1 The Woronin Body, a Unique Organelle That Shuts Down Intercellular Connectivity

Solid-state culture is a traditional culturing technique that leads to higher levels of protein production by *A. oryzae* when compared to submerged culture (Tsuchiya et al. 1994). When the protein extraction process of solid-state culture is mimicked by adding water to *A. oryzae* colonies grown on agar medium, hyphal tip bursting is induced (Fig. 11.2a) (Maruyama et al. 2005). Although hyphal damage is associated with the risk of cytoplasmic loss because of the intercellular connectivity via the septal pore, differential interference contrast (DIC) and fluorescence microscopic analyses have shown that approximately 80 % of cells immediately adjacent to the burst hyphal tip cell retain their cytoplasmic constituents (Fig. 11.2b) (Maruyama et al. 2005). The protected cells then initiate hyphal regrowth by producing a new

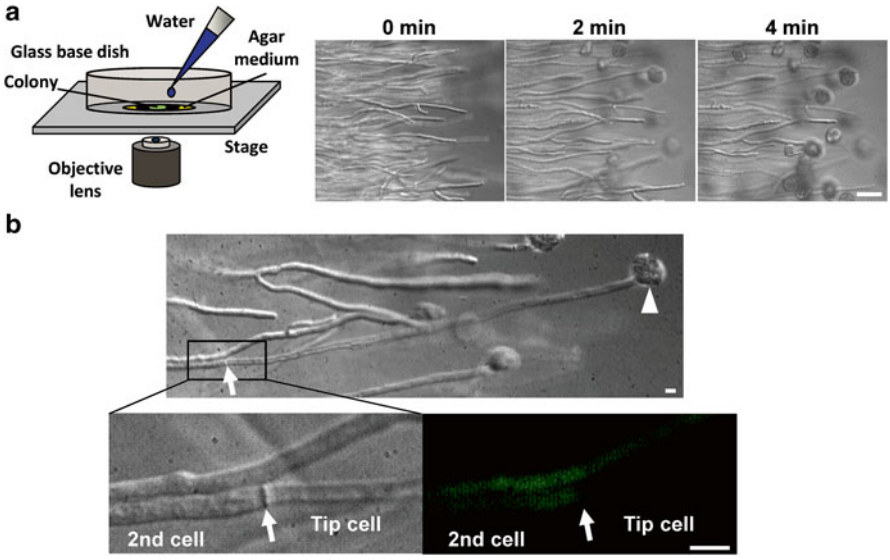

Fig. 11.2 Hyphal tip bursting upon hypotonic shock in *A. oryzae* (Maruyama et al. 2005). **a** Time-lapse observation of hyphal tip bursting upon hypotonic shock. Hyphal tips at the edge of a colony grown on agar medium were observed by differential interference contrast (DIC) microscopy (*right*) before and after flooding hyphae with water (*left*). **b** Excessive loss of cytoplasmic constituents is prevented upon hyphal tip bursting induced by hypotonic shock. The cytoplasm was labeled by expression of EGFP. The *arrowhead* indicates a burst hyphal tip and the *arrows* show a septum adjacent to the burst tip cell. Note that the cell (2nd cell) adjacent to the burst cell (Tip cell) retained its cytoplasmic constituents, as observed by DIC and fluorescence microcopy. *Bars* **a** 50 μm; **b** 10 μm

hyphal tip (Maruyama et al. 2006; Maruyama and Kitamoto 2007). Thus, *A. oryzae* has an inherent defense system that aids survival by preventing the simultaneous loss of cytoplasm from multiple cells upon hyphal wounding.

The Woronin body is an organelle specific to species of Pezizomycotina, including *A. oryzae*, and functions to plug the septal pore upon hyphal wounding (Fig. 11.3a) (Markham and Collinge 1987). Jedd and Chua (2000) first identified Hex1 as a major protein of the Woronin body in the red bread mold

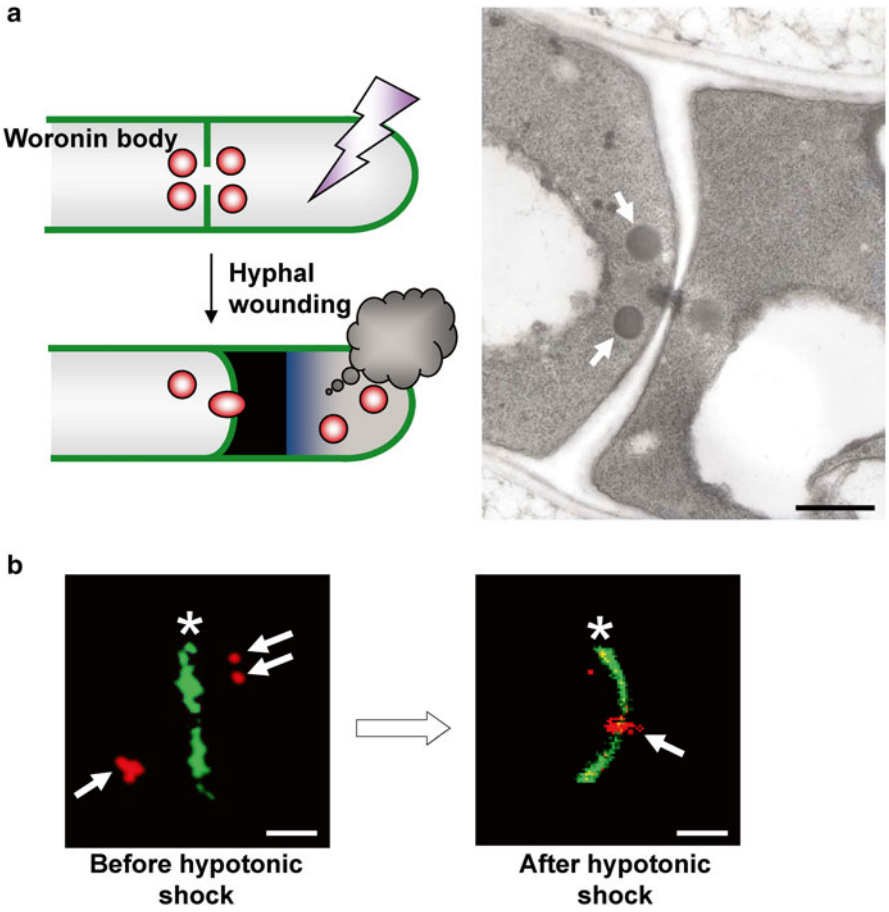

Fig. 11.3 Function and morphology of the Woronin body (Maruyama et al. 2005). **a** Model of Woronin body function (*left*) and transmission electron microscopic image of Woronin bodies (*arrows*) in the vicinity of the septal pore in *A. oryzae* (*right*) (Maruyama et al. 2005). **b** Confocal images of Woronin bodies (*red*, *arrows*) and septa (*green*, *asterisks*) before (*left*) and after (*right*) hyphal tip bursting induced by hypotonic shock. Woronin bodies and septa were fluorescently labeled by expressing DsRed2-AoHex1 and RNase T1-EGFP fusion proteins, respectively. *Bars* **a** 500 nm; **b** 2 μm

Neurospora crassa, and genes encoding the Hex1 protein are conserved in Pezizomycotina species (Jedd and Chua 2000; Asiegbu et al. 2004; Curach et al. 2004; Soundararajan et al. 2004; Maruyama et al. 2005; Beck et al. 2013). In *A. oryzae*, *Aohex1* deletion results in defective Woronin body formation and significantly reduces the ability of hyphae to prevent the excessive loss of cytoplasm upon wounding (Maruyama et al. 2005). Woronin bodies visualized using a fluorescent protein fused to AoHex1 were shown to plug the septal pore adjacent to damaged hyphal cells (Fig. 11.3b) (Maruyama et al. 2005).

As reported for Woronin bodies in *N. crassa*, self-assembled Hex1 proteins form a mechanically solid core that provides resistance to the protoplasmic streaming pressure arising from hyphal lysis (Jedd and Chua 2000; Yuan et al. 2003). Protein kinase C (PKC)-dependent phosphorylation of AoHex1 has a role in the formation of the multimeric core of Woronin bodies (Juvvadi et al. 2007). Consistent with the fact that AoHex1 contains peroxisomal targeting signal sequence 1 (PTS1), in the absence of the peroxisome proliferator protein AoPex11 Woronin bodies fail to differentiate from peroxisomes, and the ability of hyphae to prevent the excessive loss of cytoplasm is reduced by about 30 % (Escaño et al. 2009).

Recently, Bleichrodt et al. (2012) reported that the Woronin body reversibly plugs the septal pore during the normal growth of *A. oryzae*, a property that contrasts the conventionally regarded function of this organelle in wound healing. In addition, Woronin bodies were shown to be required for generating heterogeneous gene expression among hyphae located at the periphery of colonies (Bleichrodt et al. 2012). It was proposed that Woronin bodies generate populations of the leading hyphae with different cellular activities by preventing intercellular connectivity via plugging of the septal pore. Thus, the generation of such colonial heterogeneity under normal growth conditions may protect *A. oryzae* cells against environmental stresses, as evidenced by the sensitivity of the Δ*Aohex1* strain to heat stress (Bleichrodt et al. 2012). Accordingly, the function of Woronin bodies has been expanded from simple plugging behavior upon hyphal wounding to include a gatekeeper role at the septal pore (Jedd and Pieuchot 2012).

11.2.2 AoSO Protein Accumulates at the Septal Pore and Protects Against Hyphal Wounding

With the exception of Woronin bodies, the cellular components involved in septal plugging in filamentous fungi have not been extensively studied. *N. crassa* SO protein exhibits unique localization behavior; it accumulates at the septal pore in injured hyphae (Fleißner and Glass 2007), a characteristic that is similar to the Woronin body. SO and its *Sordaria macrospora* homologue Pro40 were initially identified as important proteins for hyphal fusion (Fleißner et al. 2005; Fleißner and Glass 2007; Fleißner et al. 2009) and sexual reproduction (Engh et al. 2007). SO proteins are specific to Pezizomycotina species, and homologues are not found

in yeasts or basidiomycetes (Fleißner et al. 2005). These proteins are composed of approximately 1,200 amino acids and are predicted to be intrinsically disordered and contain a WW domain (Lai et al. 2012). Intrinsically disordered proteins range from partially to completely unstructured and lack globular folds; however, they are capable of folding upon binding to target molecules (Wright and Dyson 2009). In recent years, the number of reports related to intrinsically disordered proteins has increased significantly, and many biological functions have been revealed (Oldfield and Dunker 2014). In *N. crassa*, several proteins containing disordered domains were recently shown to localize to the septum, where some of these proteins are involved in intercellular connectivity (Lai et al. 2012).

An *A. oryzae* homologue of SO protein (AoSO) has been identified, and disruption of the *Aoso* gene completely impairs hyphal fusion (Tsukasaki et al. 2014). A similar phenotype was also observed by mutation of the *N. crassa so* gene (Fleißner et al. 2005). Upon hyphal tip bursting induced by hypotonic shock, cells of the *Aoso* disruptant strain (Δ*Aoso*) are unable to prevent the excessive loss of cytoplasm (Maruyama et al. 2010), similar to the Δ*Aohex1* strain, which is defective in Woronin body formation. Localization analysis with AoSO-EGFP revealed that this fusion protein is dispersed throughout the cytoplasm under normal growth conditions, but accumulates as a punctuate dot at the septal pore adjacent to wounded cells (Maruyama et al. 2010). Based on these findings, AoSO is thought to prevent the excessive loss of cytoplasm by accumulating at the septal pore, similar to the observed function of Woronin bodies (Maruyama et al. 2005). However, the proportion of Δ*Aoso* hyphae that retained their cytoplasm was higher (approximately 50 %) than that of the Δ*Aohex1* strain (~20 %). In addition, a double-disruptant strain (Δ*Aoso* Δ*Aohex1*) did not exhibit more severe cytoplasmic leakage than the Δ*Aohex1* strain (Maruyama et al. 2010). Thus, AoSO appears to play a supportive role for the function of Woronin bodies.

11.2.3 Stress-Responsive Accumulation of AoSO at the Septal Pore

In response to sudden environmental changes, multicellular organisms appear to regulate cytoplasmic streaming between adjacent cells to maintain cellular homeostasis. Under transmission electron microscopy, the septal pore in *A. oryzae* hyphae is often surrounded by an electron-dense material distinct from Woronin bodies (Fig. 11.3a) (Maruyama et al. 2005). It is hypothesized that the septal pore serves as a cell-to-cell channel for responding to environmental changes; however, the underlying molecular mechanisms have not been elucidated in filamentous fungi. In *N. crassa*, SO protein accumulates at the septal pore in aging hyphae (Fleißner and Glass 2007), which are frequently exposed to various stresses, particularly that of nutrient depletion, which raises the possibility that specific proteins respond to various environmental stresses in filamentous fungi.

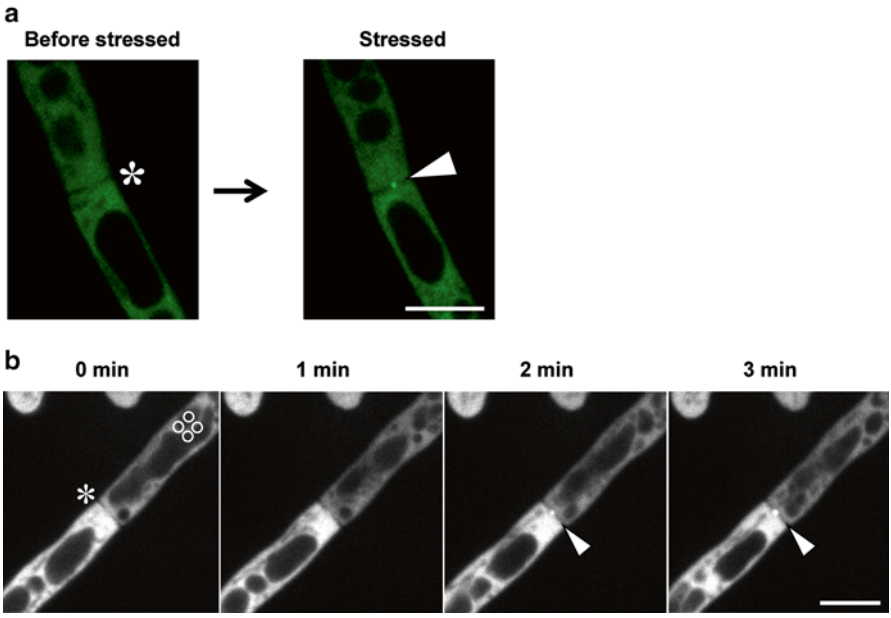

Fig. 11.4 Accumulation of AoSO-EGFP fusion protein at the septal pore in response to stress conditions induced by low pH (**a**) and pulse laser treatment (**b**) (Maruyama et al. 2010). A strain expressing the AoSO-EGFP fusion protein was subjected to low pH (pH 2.0, 24 min) and pulse laser treatment (at 0 min). *Circles* indicate the area where the pulse laser was applied, and *arrowheads* indicate the appearance of a punctate dot at the septal pore upon pulse laser treatment. *Bar*: 5 μm

In *A. oryzae*, the subcellular localization of AoSO was visualized under various stress conditions, including low/high temperature, highly acidic/alkaline conditions, and nitrogen/carbon depletion (Maruyama et al. 2010). In each of these conditions, the AoSO-EGFP fusion protein accumulated as a punctate dot at the septal pore (Fig. 11.4a). In response to pulsed laser treatment, which physically stresses cells without causing hyphal wounding, AoSO rapidly accumulates at the septal pore nearest to the stressed hyphal area (Fig. 11.4b) (Maruyama et al. 2010). These findings demonstrate that AoSO accumulates at the septal pore in response to various stresses, suggesting that this protein might regulate intercellular connectivity via the septal pore in a stress-dependent manner.

The experimental monitoring of cytoplasmic streaming through the septal pore after hyphal injury induced by laser ablation has suggested that stress-responsive changes in intercellular connectivity occur in the basidiomycete *Schizophyllum commune* (van Peer et al. 2009), but not in *A. oryzae* (Bleichrodt et al. 2012). In contrast to *N. crassa* and *Sordaria fimicola*, which exhibit extensive cytoplasmic streaming through septal pores (Lew 2005; Tey et al. 2005; Ng et al. 2009), the movement of cytoplasm across the septum is rarely seen in *A. oryzae* (Maruyama et al., unpublished results). Recently, a sensitive assay for assessing septal pore

permeability in *Aspergillus nidulans* based on fluorescence recovery after photobleaching (FRAP) analysis revealed that mitosis disrupts intercellular connectivity via the septal pore in a manner independent of Woronin bodies and SO protein (Shen et al. 2014). As it remains to be determined if intercellular connectivity is regulated by stress, further studies employing the FRAP-based technique may provide insight into the apparent gatekeeping role of AoSO at the septal pore.

11.3 Stress Granule Formation in *A. oryzae*

11.3.1 Spatially Polarized Formation of Stress Granules

Environmental stress response mechanisms in eukaryotic cells induce global translational inhibition, which attenuates protein synthesis to conserve anabolic energy and reorganizes gene expression profiles for adaptation to stress conditions. It was recently demonstrated that this response involves the remodeling of mRNAs translated from polysomes into stress granules, which are composed of nontranslating messenger ribonucleoproteins (mRNPs) and appear as discrete cytoplasmic foci (Buchan and Parker 2009). However, a subset of mRNAs required for cell survival under stress conditions are not delivered to stress granules, but rather are stabilized and preferentially translated in the cytoplasm (Lu et al. 2006; Powley et al. 2009).

Recently, stress granules have been characterized in *A. oryzae* (Huang et al. 2013) using an EGFP fusion construct of the *A. oryzae* homologue of *S. cerevisiae* Pab1p (poly (A)-binding protein), which serves as a marker of stress granules (Buchan et al. 2008). The *A. oryzae* homologue of Pab1p, AoPab1, exhibits dispersed distribution throughout the cytoplasm under normal growth conditions. However, in response to various stresses, including low/high temperature, glucose deprivation, and osmotic, oxidative, and ER stresses, AoPab1 accumulates as cytoplasmic foci (Fig. 11.5) (Huang et al. 2013).

The processing body (P-body) is another type of mRNP that consists of cellular components involved in mRNA decapping and degradation (Sheth and Parker 2003; Nissan et al. 2010). In *S. cerevisiae*, Dcp2p, a component of P-bodies (Eulalio et al. 2007; Parker and Sheth 2007), is the catalytic subunit of the decapping enzyme that cleaves the 5′-cap of mRNAs (Steiger et al. 2003). An EGFP fusion protein of the *A. oryzae* homologue of *S. cerevisiae* Dcp2p (AoDcp2) forms small punctate dots in the cytoplasm under normal growth conditions (Huang et al. 2013). However, under stress conditions, such as high and low temperatures and carbon deprivation, the number of punctate fluorescent dots markedly increases. Fluorescence microscopy analysis also revealed that most stress granules colocalize or are closely associated with P-bodies, a finding that is consistent with previous reports in *S. cerevisiae* and mammalian cells (Kedersha et al. 2005; Buchan et al. 2008; Grousl et al. 2009).

The afore-described findings indicate that the formation of stress granules is a general phenomenon in response to external stress and suggest that the stress-induced reprogramming of mRNAs to enter stress granules occurs in *A. oryzae*.

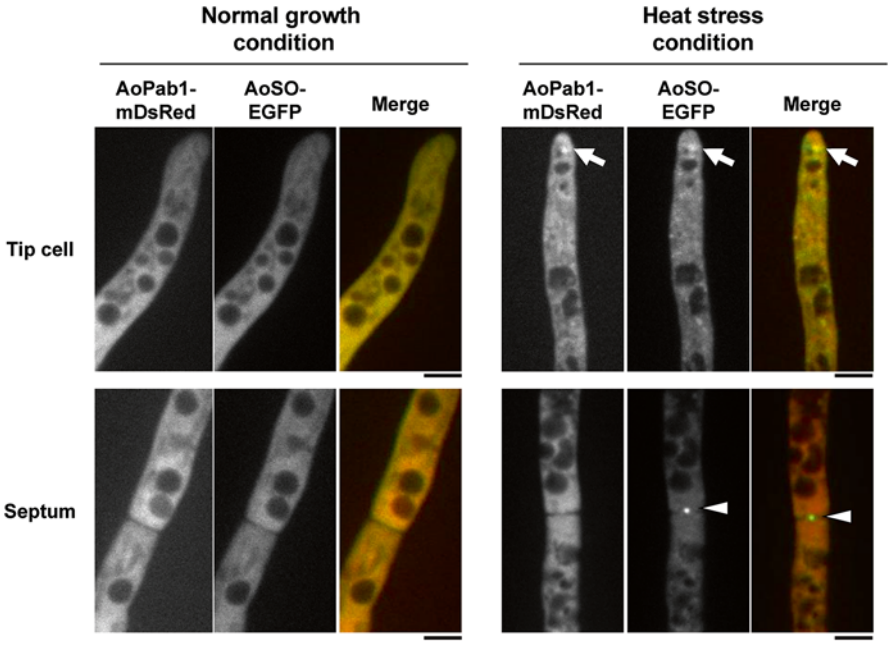

Fig. 11.5 Stress-induced formation of stress granules and colocalization of AoSO protein at the hyphal tip (Huang et al. 2013). A strain coexpressing AoSO-EGFP and AoPab1-mDsRed was observed by fluorescence microscopy. Hyphae were observed in normal growth conditions (*left*) and under heat stress (*right*; 45 °C, 10 min). Colocalization of a stress granule and an AoSO cytoplasmic focus is indicated by the *arrows*. *Arrowheads* show accumulation of AoSO at the septal pore

The polarized localization of stress granules at the hyphal tip suggests that the posttranscriptional regulation of gene expression is spatially specific. *A. oryzae* has a highly polarized cell structure, and secretory proteins and related components are concentrated at the hyphal tip (Masai et al. 2003; Maruyama et al. 2006; Kuratsu et al. 2007; Kimura et al. 2010; Hayakawa et al. 2011). Taken together with the fact that ribosome-related genes are abundantly expressed in the peripheral regions of colonies, which has a high density of hyphal tips (Masai et al. 2006), these observations support the idea that mRNA translation actively occurs in this region.

11.3.2 Identification of a Novel Stress Granule Component AoSO

As AoSO protein accumulates at the septal pore in response to various stresses (Maruyama et al. 2010), further characterization of this protein may shed light on the function and properties of stress granules in *A. oryzae*. In normal growth conditions,

AoSO-EGFP is evenly distributed throughout the cytoplasm, but accumulates as cytoplasmic foci at hyphal tips when cells are exposed to heat stress (Huang et al. 2013). Fluorescence microscopy revealed that AoSO-EGFP colocalizes in the cytoplasm with stress granules labeled with AoPab1-mDsRed (Fig. 11.5) in a manner that is sensitive to cycloheximide treatment, demonstrating that cytoplasmic AoSO foci are typical mRNP granules, as previously reported (Kedersha et al. 2000; Buchan et al. 2008; Grousl et al. 2009). Under any of the examined stress conditions, however, AoPab1-mDsRed did not localize to the septal pore where AoSO protein accumulates in a manner insensitive to cycloheximide treatment (Fig. 11.5). The physical association of AoSO protein with the stress granule component AoPab1 was confirmed by co-immunoprecipitation analysis, which demonstrated that AoSO is a novel component of stress granules.

In the Δ*Aoso* strain, although stress granules are formed in response to stress, the amount of granules is approximately 10 % lower compared to the wild-type strain (Huang et al. 2013). Moreover, the distribution of stress granules in the Δ*Aoso* strain is farther from the hyphal tip (Huang et al. 2013). Taken together, these findings indicate that AoSO affects the formation and localization of stress granules. In addition to the known functions of SO proteins in hyphal fusion, sexual development, and septal plugging (Fleißner et al. 2005; Engh et al. 2007; Maruyama et al. 2010; Tsukasaki et al. 2014), it is also possible that SO proteins might have pleiotropic functions including the regulation of mRNAs.

11.4 Conclusion and Perspectives

The series of findings described here indicate that stress responses in *A. oryzae* hyphal cells are region specific. The plugging of septa with Woronin bodies and septal accumulation of AoSO protein (Fig. 11.6a, b) indicate that intercellular connectivity in *A. oryzae* is tightly regulated in response to physical and environmental stresses. The septal pore is therefore a physiologically important site for maintaining the cellular function and multicellularity of *A. oryzae*, as supported by the septal localization of numerous proteins, including microtubule organizing center components (Zekert et al. 2010), signaling molecules (Juvvadi et al. 2011), and intrinsically disordered proteins (Lai et al. 2012) in other filamentous fungi. The phenomenon of hyphal tip bursting induced by hypotonic shock (Fig. 11.2) was originally observed when mimicking the extraction of proteins produced by *A. oryzae* in solid-state culture (Maruyama et al. 2005). A proportion of secretory proteins are retained intracellularly in response to heat and cold (Masai et al. 2003), suggesting that the excessive loss of intracellular constituents in the absence of Woronin bodies may increase the protein extraction efficiency in solid-state culture. Alternatively, in submerged culture, which is used for large-scale industrial protein production, *A. oryzae* cells form mycelial pellets by gathering and attaching with each other. The use of physical agitation to increase the amount of dissolved oxygen

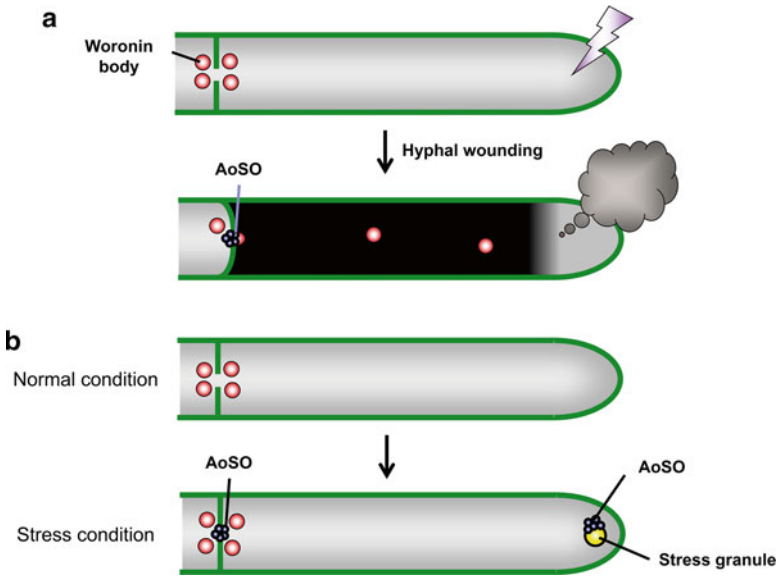

Fig. 11.6 Schematic model of stress responses in *A. oryzae* hyphal tip cells. **a** Upon hyphal wounding, Woronin bodies plug the septal pore and AoSO accumulates at the septal pore. **b** Under stressed conditions, stress granules are formed as cytoplasmic foci together with AoSO at the hyphal tip, but only AoSO accumulates at the septal pore

often breaks mycelia into cell fragments, thereby reducing cell activity. Thus, the rapid response to such damage, such as septal plugging, is required for the efficient reinitiation of growth and would affect the productivity of industrial enzymes and recombinant proteins in *A. oryzae*.

The polarized formation of stress granules with the novel component AoSO at hyphal tips (Fig. 11.6b) (Huang et al. 2013) highlights the spatial specificity of stress responses in *A. oryzae*, which is in contrast to the formation of stress granules throughout the cytoplasm in other eukaryotes. As the hyphal tip is enriched with secretory proteins/machineries (Masai et al. 2003; Kuratsu et al. 2007; Kimura et al. 2010; Hayakawa et al. 2011), mRNAs for secretory proteins/machineries might also accumulate at the hyphal tip. If such mRNAs are sequestered to stress granules to prevent translation, protein production activity in individual hypha may be reduced under stress conditions. Additionally, heterologous protein expression induces the unfolded protein response, indicating the occurrence of ER stress (Ohno et al. 2011). As ER stress induces the formation of stress granules at the hyphal tip (Huang et al. 2013), it is possible that overexpression of heterologous proteins may attenuate their own translational activity and secretion efficiency. Further characterization of stress granules is expected to reveal approaches for further enhancing the potential of *A. oryzae* as a host for heterologous protein production.

It could be said that the *A. oryzae* cells encounter various stresses during the fermentation process. For example, in the course of *koji*-making, rapid and vigorous hyphal growth raises the culture temperature, which may lead to the formation of stress granules and cause a reduction in translational activity. During the mixing process to lower the temperature of *koji* rice, it is possible that physical stress might reduce the activity of *A. oryzae* cells, as suggested by the septal accumulation of AoSO in response to pulse laser treatment (Fig. 11.4b; Maruyama et al. 2010). Furthermore, in *koji*-making for the production of "Dai-Ginjo-Shu," which is the highest grade of sake and is brewed from white rice polished to 50 % or less, *A. oryzae* cells may experience nitrogen starvation stress because of the low nitrogen content of the rice. A recent finding has revealed that the efficiency of hyphal fusion is increased in nitrogen-starved conditions (Tsukasaki et al. 2014). As the stress-responsive protein AoSO is required for hyphal fusion, it is possible that stress response machineries have critical roles not only for acute adaptation to stress but also for specialized stages of growth. Further studies of stress responses at the cellular level will provide new insights into the underlying biology of *koji-making* and help develop innovative ideas for advanced brewing technologies.

References

Asiegbu FO, Choi W, Jeong JS, Dean RA (2004) Cloning, sequencing and functional analysis of *Magnaporthe grisea MVP1* gene, a *hex-1* homolog encoding a putative "Woronin body" protein. FEMS Microbiol Lett 230:85–90

Beck J, Echtenacher B, Ebel F (2013) Woronin bodies, their impact on stress resistance and virulence of the pathogenic mould *Aspergillus fumigatus* and their anchoring at the septal pore of filamentous Ascomycota. Mol Microbiol 89:857–871

Bleichrodt RJ, van Veluw GJ, Recter B, Maruyama J, Kitamoto K, Wösten HA (2012) Hyphal heterogeneity in *Aspergillus oryzae* is the result of dynamic closure of septa by Woronin bodies. Mol Microbiol 86:1334–1344

Buchan JR, Parker R (2009) Eukaryotic stress granules: the ins and outs of translation. Mol Cell 36:932–941

Buchan JR, Muhlrad D, Parker R (2008) P bodies promote stress granule assembly in *Saccharomyces cerevisiae*. J Cell Biol 183:441–455

Chen X, Ishida N, Todaka N, Nakamura R, Maruyama J, Takahashi H, Kitamoto K (2010) Promotion of efficient saccharification with *Aspergillus fumigatus* AfSwo1 towards crystalline cellulose. Appl Environ Microbiol 76:2556–2561

Curach NC, Te'o VS, Gibbs MD, Bergquist PL, Nevalainen KM (2004) Isolation, characterization and expression of the *hex1* gene from *Trichoderma reesei*. Gene 331:133–140

Engh I, Würtz C, Witzel-Schlömp K, Zhang HY, Hoff B, Nowrousian M, Rottensteiner H, Kück U (2007) The WW domain protein PRO40 is required for fungal fertility and associates with Woronin bodies. Eukaryot Cell 6:831–843

Escaño CS, Juvvadi PR, Jin FJ, Takahashi T, Koyama Y, Yamashita S, Maruyama J, Kitamoto K (2009) Disruption of the *Aopex11-1* gene involved in peroxisome proliferation leads to impaired Woronin body formation in *Aspergillus oryzae*. Eukaryot Cell 8:296–305

Eulalio A, Behm-Ansmant I, Izaurralde E (2007) P bodies: at the crossroads of post-transcriptional pathways. Nat Rev Mol Cell Biol 8:9–22

Fleißner A, Glass NL (2007) SO, a protein involved in hyphal fusion in *Neurospora crassa*, localizes to septal plugs. Eukaryot Cell 6:84–94

Fleißner A, Sarkar S, Jacobson DJ, Roca MG, Read ND, Glass NL (2005) The *so* locus is required for vegetative cell fusion and postfertilization events in *Neurospora crassa*. Eukaryot Cell 4:920–930

Fleißner A, Leeder AC, Roca MG, Read ND, Glass NL (2009) Oscillatory recruitment of signaling proteins to cell tips promotes coordinated behavior during cell fusion. Proc Natl Acad Sci U S A 106:19387–19392

Grousl T, Ivanov P, Frýdlová I, Vasicová P, Janda F, Vojtová J, Malínská K, Malcová I, Nováková L, Janosková D, Valásek L, Hasek J (2009) Robust heat shock induces eIF2alpha-phosphorylation-independent assembly of stress granules containing eIF3 and 40S ribosomal subunits in budding yeast, *Saccharomyces cerevisiae*. J Cell Sci 122:2078–2088

Harris SD, Read ND, Roberson RW, Shaw B, Seiler S, Plamann M, Momany M (2005) Polarisome meets spitzenkorper: microscopy, genetics, and genomics converge. Eukaryot Cell 4:225–229

Hayakawa Y, Ishikawa E, Shoji JY, Nakano H, Kitamoto K (2011) Septum-directed secretion in the filamentous fungus *Aspergillus oryzae*. Mol Microbiol 81:40–55

Huang HT, Maruyama J, Kitamoto K (2013) *Aspergillus oryzae* AoSO is a novel component of stress granules upon heat stress in filamentous fungi. PLoS One 8:e72209

Ito K, Asakura T, Morita Y, Nakajima K, Koizumi A, Shimizu-Ibuka A, Masuda K, Ishiguro M, Terada T, Maruyama J, Kitamoto K, Misaka T, Abe K (2007) Microbial production of sensory-active miraculin. Biochem Biophys Res Commun 360:407–411

Jedd G, Chua NH (2000) A new self-assembled peroxisomal vesicle required for efficient resealing of the plasma membrane. Nat Cell Biol 2:226–231

Jedd G, Pieuchot L (2012) Multiple modes for gatekeeping at fungal cell-to-cell channels. Mol Microbiol 86:1291–1294

Juvvadi PR, Maruyama J, Kitamoto K (2007) Phosphorylation of the *Aspergillus oryzae* Woronin body protein, AoHex1, by protein kinase C: evidence for its role in the multimerization and proper localization of the Woronin body protein. Biochem J 405:533–540

Juvvadi PR, Fortwendel JR, Rogg LE, Burns KA, Randell SH, Steinbach WJ (2011) Localization and activity of the calcineurin catalytic and regulatory subunit complex at the septum is essential for hyphal elongation and proper septation in *Aspergillus fumigatus*. Mol Microbiol 82:1235–1259

Kedersha N, Cho MR, Li W, Yacono PW, Chen S, Gilks N, Golan DE, Anderson P (2000) Dynamic shuttling of TIA-1 accompanies the recruitment of mRNA to mammalian stress granules. J Cell Biol 151:1257–1268

Kedersha N, Stoecklin G, Ayodele M, Yacono P, Lykke-Andersen J, Fritzler MJ, Scheuner D, Kaufman RJ, Golan DE, Anderson P (2005) Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. J Cell Biol 169:871–884

Kimura S, Maruyama J, Watanabe T, Ito Y, Arioka M, Kitamoto K (2010) *In vivo* imaging of endoplasmic reticulum and distribution of mutant α-amylase in *Aspergillus oryzae*. Fungal Genet Biol 47:1044–1054

Kimura S, Maruyama J, Kikuma T, Arioka M, Kitamoto K (2011) Autophagy delivers misfolded secretory proteins accumulated in endoplasmic reticulum to vacuoles in the filamentous fungus *Aspergillus oryzae*. Biochem Biophys Res Commun 406:464–470

Kitamoto K (2002) Molecular biology of the *koji* molds. Adv Appl Microbiol 51:129–153

Kuratsu M, Taura A, Shoji JY, Kikuchi S, Arioka M, Kitamoto K (2007) Systematic analysis of SNARE localization in the filamentous fungus *Aspergillus oryzae*. Fungal Genet Biol 44:1310–1323

Lai J, Koh CH, Tjota M, Pieuchot L, Raman V, Chandrababu KB, Yang D, Wong L, Jedd G (2012) Intrinsically disordered proteins aggregate at fungal cell-to-cell channels and regulate intercellular connectivity. Proc Natl Acad Sci U S A 109:15781–15786

Lew RR (2005) Mass flow and pressure-driven hyphal extension in *Neurospra crassa*. Microbiology 151:2685–2692

Lu X, de la Pena L, Barker C, Camphausen K, Tofilon PJ (2006) Radiation induced changes in gene expression involve recruitment of existing messenger RNAs to and away from polysomes. Cancer Res 66:1052–1061

Markham P, Collinge AJ (1987) Woronin bodies of filamentous fungi. FEMS Microbiol Rev 46:1–11

Maruyama J, Kitamoto K (2007) Differential distribution of the endoplasmic reticulum network in filamentous fungi. FEMS Microbiol Lett 272:1–7

Maruyama J, Kitamoto K (2008) Multiple gene disruptions by marker recycling with highly efficient gene-targeting background (Δ*ligD*) in *Aspergillus oryzae*. Biotechnol Lett 30:1811–1817

Maruyama J, Kitamoto K (2011) Targeted gene disruption in koji mold *Aspergillus oryzae*. Methods Mol Biol 765:447–456

Maruyama J, Nakajima H, Kitamoto K (2001) Visualization of nuclei in *Aspergillus oryzae* with EGFP and analysis of the number of nuclei in each conidium by FACS. Biosci Biotechnol Biochem 65:1504–1510

Maruyama J, Juvvadi PR, Ishi K, Kitamoto K (2005) Three-dimensional image analysis of plugging at the septal pore by Woronin body during hypotonic shock inducing hyphal tip bursting in the filamentous fungus *Aspergillus oryzae*. Biochem Biophys Res Commun 331:1081–1088

Maruyama J, Kikuchi S, Kitamoto K (2006) Differential distribution of the endoplasmic reticulum network as visualized by the BipA-EGFP fusion protein in hyphal compartments across the septum of the filamentous fungus, *Aspergillus oryzae*. Fungal Genet Biol 43:642–654

Maruyama J, Escaño CS, Kitamoto K (2010) AoSO protein accumulates at the septal pore in response to various stresses in the filamentous fungus *Aspergillus oryzae*. Biochem Biophys Res Commun 391:868–873

Masai K, Maruyama J, Nakajima H, Kitamoto K (2003) *In vivo* visualization of the distribution of a secretory protein in *Aspergillus oryzae* hyphae using the RntA-EGFP fusion protein. Biosci Biotechnol Biochem 67:455–459

Masai K, Maruyama J, Sakamoto K, Nakajima H, Akita O, Kitamoto K (2006) Square-plate culture method allows detection of differential gene expression and screening of novel, region-specific genes in *Aspergillus oryzae*. Appl Microbiol Biotechnol 71:881–891

Nakajima K, Asakura T, Maruyama J, Morita Y, Oike H, Shimizu-Ibuka A, Misaka T, Sorimachi H, Arai S, Kitamoto K, Abe K (2006) Extracellular production of neoculin, a sweet-tasting heterodimeric protein with taste-modifying activity, by *Aspergillus oryzae*. Appl Environ Microbiol 72:3716–3723

Nemoto T, Watanabe T, Mizogami Y, Maruyama J, Kitamoto K (2009) Isolation of *Aspergillus oryzae* mutants for heterologous protein production from a double proteinase gene disruptant. Appl Microbiol Biotechnol 82:1105–1114

Ng SK, Liu F, Lai J, Low W, Jedd G (2009) A tether for Woronin body inheritance is associated with evolutionary variation in organelle positioning. PLoS Genet 5:e1000521

Nissan T, Rajyaguru P, She M, Song H, Parker R (2010) Decapping activators in *Saccharomyces cerevisiae* act by multiple mechanisms. Mol Cell 39:773–783

Ohno A, Maruyama J, Nemoto T, Arioka M, Kitamoto K (2011) A carrier fusion significantly induces unfolded protein response in heterologous protein production by *Aspergillus oryzae*. Appl Microbiol Biotechnol 92:1197–1206

Oldfield CJ, Dunker AK (2014) Intrinsically disordered proteins and intrinsically disordered protein regions. Annu Rev Biochem 83:553–584

Parker R, Sheth U (2007) P bodies and the control of mRNA translation and degradation. Mol Cell 25:635–646

Powley IR, Kondrashov A, Young LA, Dobbyn HC, Hill K, Cannell IG, Stoneley M, Kong YW, Cotes JA, Smith GC, Wek R, Hayes C, Gant TW, Spriggs KA, Bushell M, Willis AE (2009) Translational reprogramming following UVB irradiation is mediated by DNAPKcs and allows selective recruitment to the polysomes of mRNAs encoding DNA repair enzymes. Genes Dev 23:1207–1220

Shen KF, Osmani AH, Govindaraghavan M, Osmani SA (2014) Mitotic regulation of fungal cell-to-cell connectivity through septal pores involves the NIMA kinase. Mol Biol Cell 25(6): 763–775

Sheth U, Parker R (2003) Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. Science 300:805–808

Shoji J, Kikuma T, Arioka M, Kitamoto K (2010) Macroautophagy-mediated degradation of whole nuclei in the filamentous fungus *Aspergillus oryzae*. PLoS One 5:e15650

Soundararajan S, Jedd G, Li X, Ramos-Pamploña M, Chua NH, Naqvi NI (2004) Woronin body function in *Magnaporthe grisea* is essential for efficient pathogenesis and for survival during nitrogen starvation stress. Plant Cell 6:1564–1574

Steiger M, Carr-Schmid A, Schwartz DC, Kiledjian M, Parker R (2003) Analysis of recombinant yeast decapping enzyme. RNA 9:231–238

Tey WK, North AJ, Reyes JL, Lu YF, Jedd G (2005) Polarized gene expression determines Woronin body formation at the leading edge of the fungal colony. Mol Biol Cell 16: 2651–2659

Tsuchiya K, Nagashima T, Yamamoto Y, Gomi K, Kitamoto K, Kumagai C, Tamura G (1994) High level secretion of calf chymosin using a glucoamylase-prochymosin fusion gene in *Aspergillus oryzae*. Biosci Biotechnol Biochem 58:895–899

Tsukasaki W, Maruyama J, Kitamoto K (2014) Establishment of a new method to quantitatively evaluate hyphal fusion ability in *Aspergillus oryzae*. Biosci Biotechnol Biochem 78(7):1254–1262

van Peer AF, Muller WH, Boekhout T, Lugones LG, Wosten HA (2009) Cytoplasmic continuity revisited: closure of septa of the filamentous fungus *Schizophyllum commune* in response to environmental conditions. PLoS One 4:e5977

Wright PE, Dyson HJ (2009) Linking folding and binding. Curr Opin Struct Biol 19:31–38

Yoon J, Kimura S, Maruyama J, Kitamoto K (2009) Construction of quintuple protease gene disruptant for heterologous protein production in *Aspergillus oryzae*. Appl Microbiol Biotechnol 82:691–701

Yoon J, Aishan T, Maruyama J, Kitamoto K (2010) Enhanced production and secretion of heterologous proteins by the filamentous fungus *Aspergillus oryzae* via disruption of vacuolar protein sorting receptor gene *Aovps10*. Appl Environ Microbiol 76:5718–5727

Yoon J, Maruyama J, Kitamoto K (2011) Disruption of ten protease genes in the filamentous fungus *Aspergillus oryzae* highly improves production of heterologous proteins. Appl Microbiol Biotechnol 89:747–759

Yoon J, Kikuma T, Maruyama J, Kitamoto K (2013) Enhanced production of bovine chymosin by autophagy deficiency in the filamentous fungus *Aspergillus oryzae*. PLoS One 8:e62512

Yuan P, Jedd G, Kumaran D, Swaminathan S, Shio H, Hewitt D, Chua NH, Swaminathan K (2003) A HEX-1 crystal lattice required for Woronin body function in *Neurospora crassa*. Nat Struct Biol 4:264–270

Zekert N, Veith D, Fischer R (2010) Interaction of the *Aspergillus nidulans* microtubule-organizing center (MTOC) component ApsB with gamma-tubulin and evidence for a role of a subclass of peroxisomes in the formation of septal MTOCs. Eukaryot Cell 9:795–805

Zhu L, Maruyama J, Kitamoto K (2013) Further enhanced production of heterologous proteins by double-gene disruption (Δ*AosedD* Δ*Aovps10*) in a hyper-producing mutant of *Aspergillus oryzae*. Appl Microbiol Biotechnol 97:6347–6357

Chapter 12
Protein Kinase C of Filamentous Fungi and Its Roles in the Stresses Affecting Hyphal Morphogenesis and Conidiation

Hiroyuki Horiuchi and Takuya Katayama

Abstract Protein kinase C (PKC) is known to play pivotal roles in the various signal transduction pathways in mammalian cells. Its functions have been extensively explored in mammalian cells, whereas those of the PKC of filamentous fungi remain largely unknown, with the exception that PKC is known to function in the cell wall integrity signaling pathway similar to that in the yeast *Saccharomyces cerevisiae*. Recent advances in the functional analyses of *Aspergillus nidulans* PKC suggest that it has functions in germination, hyphal morphogenesis, and spore formation under heat stress. These functions are suppression of apoptosis induction and the establishment of cell polarity during germination, reestablishment of hyphal polarity after depolarization, and repression of conidiation. In this chapter, we present these functions of PKC and describe them in detail.

Keywords Apoptosis • *Aspergillus* • Cell wall integrity signaling pathway • Conidiation • Morphogenesis • Protein kinase C • Stress response

12.1 Introduction

Fungi grow in nature under many environmental stress conditions. To respond to these stresses, fungi have various signal transduction pathways and change their morphology to adapt to their environment. Protein kinases, such as protein kinase A, G, and C (AGC protein kinases), calmodulin-dependent protein kinase (CAMK),

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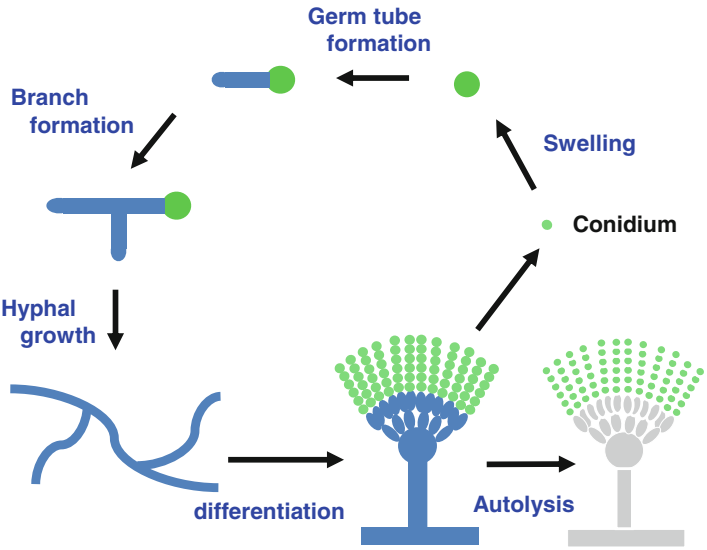

Fig. 12.1 Asexual life cycle of *Aspergillus nidulans*. Details described in text

and kinases involved in the mitogen-activated protein (MAP) kinase cascade, are speculated to have important roles in these processes (De Souza et al. 2013). The functions of these fungal kinases have been investigated primarily using *Saccharomyces cerevisiae*, and little is known about their functions in filamentous fungi.

Filamentous fungi belonging to Ascomycota undergo asexual and sexual life cycles and form asexual spores (conidia) and sexual spores (ascospores). In the asexual life cycle of *Aspergillus nidulans*, conidia grow isotropically at 37 °C for 2 to 3 h and establish cell polarity to form germ tubes. Then, polar growth proceeds with branching followed by hyphae formation. The hyphae differentiate conidiophores, which form conidia after approximately 20 h of cultivation. Finally, autolysis occurs at a late stage of cultivation (Fig. 12.1). These specific structural changes in the life cycle of filamentous fungi are thought to be closely related to both their industrial merits and demerits. Thus, elucidating the mechanisms of conidial germination, hyphal growth, conidiophore, and conidia development at the molecular level is very important for the efficient utilization of filamentous fungi in industry. Fungal cells are surrounded by cell walls, which consist mainly of polysaccharides and proteins and form very rigid structures. The cell wall is crucial for the formation of differentiated structures of filamentous fungi, and the architecture changes during the progression of the life cycle and in response to various cell wall stresses.

Protein kinase C (PKC) is a serine/threonine kinase that is ubiquitous from fungi to mammals. There are more than ten isozymes in mammalian cells that function in various signal transduction pathways in these cells (Steinberg 2008). In contrast to mammalian cells, there are only one or a few PKC-encoding genes in the genomes of filamentous fungi and yeasts. Although PKC is known to have a crucial role in the

signal transduction pathway in *S. cerevisiae* under cell wall stress, its functions in filamentous fungi remain poorly understood.

In this chapter, we mainly focus on the protein kinase C (PKC)-related signal transduction pathway of filamentous fungi.

12.2 PKC Signaling Pathway

Fungal PKCs have a specific domain organization consisting of two HR1 domains, a C2-like domain, a pseudo-substrate sequence, two C1 domains, a protein kinase domain, and a protein kinase C-terminal domain (Fig. 12.2). The organization of these domains is similar to that of the mammalian novel PKC (nPKC) subclass with the exception of the HR1 domain. The HR1 domain is not present in nPKC but is present in the PKC-related kinase, protein kinase N (PKN) (Mukai 2003). In these domains, the HR1 domain is known to bind to the GTPase Rho (Schmitz et al. 2002) and the C2-like domain is a sequence homologous to the C2 Ca^{2+}-binding domain. However, the amino acids required for Ca^{2+} binding are not conserved in the "C2-like" domain. The C1 domain, a region that binds to diacylglycerol (DAG), is followed by a pseudo-substrate sequence located between C2-like and C1 domains. The pseudo-substrate sequence resembles the amino acid sequence of substrate

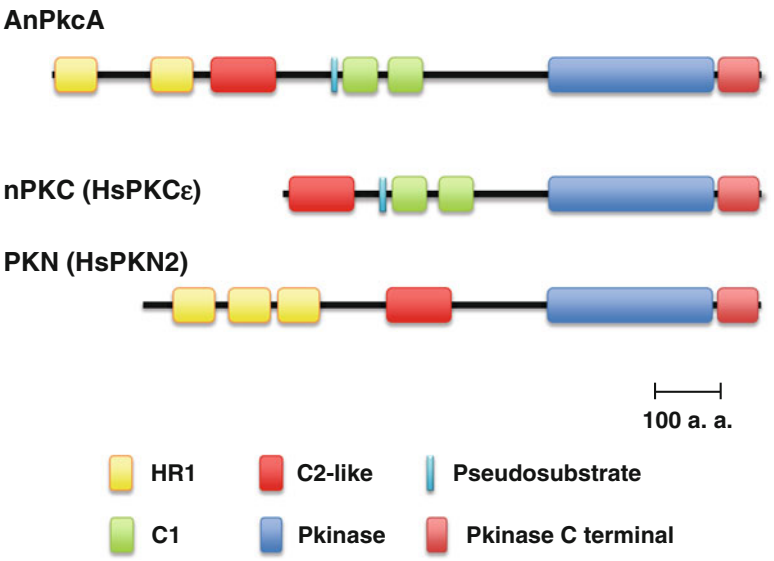

Fig. 12.2 Domain organization of the fungal PKC and human nPKC and PKN. Hs *Homo sapiens*, An *A. nidulans*

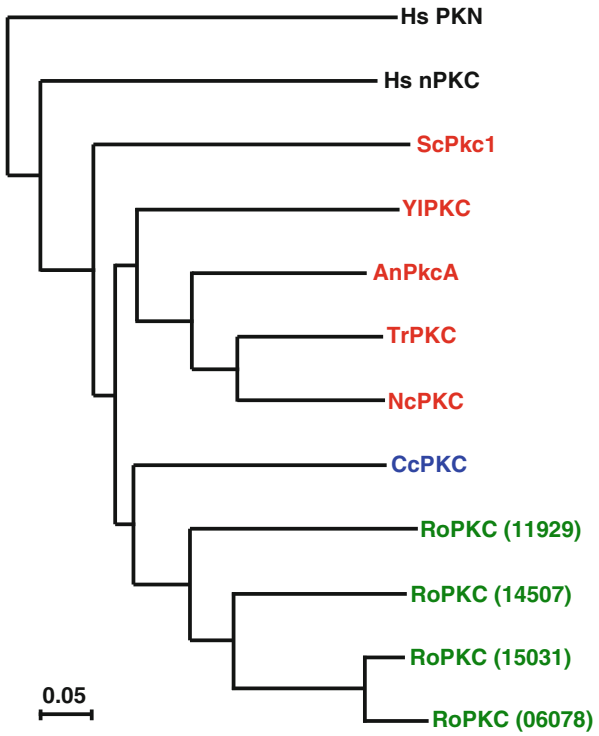

Fig. 12.3 Phylogenetic tree of fungal PKC and human nPKC and PKN. Alignment was performed with ClustalW using the entire amino acid sequence of each PKC and PKN. The tree was drawn using NJprot. Branch length indicates evolutionary distance. PKCs of the fungi belonging to Ascomycota, Basidiomycota, and Mucoromycotina are shown in *red*, *blue*, and *green*, respectively. Hs *Homo sapiens*, Sc *Saccharomyces cerevisiae*, An *A. nidulans*, Tr *Trichoderma reesei*, Nc *N. crassa*, Yl *Yarrowia lipolytica*, Cc *Coprinopsis cinerea*, Ro *Rhizopus delemar*. The numbers in parentheses indicate the gene IDs in the genome database of *R. delemar*

proteins, with the exception of the phosphoacceptor residue, which is replaced by alanine. The pseudo-substrate sequence is thought to bind to the catalytic subunit of PKC and keep the kinase inactive.

The phylogenetic relationships among fungal PKCs and human nPKC and PKN are shown in Fig. 12.3. In general, fungi belonging to Ascomycota or Basidiomycota have one or two PKCs, whereas those belonging to Mucoromycotina (formerly known as Zygomycota) (Hibbett et al. 2007) have three or four PKCs.

Saccharomyces cerevisiae has the only PKC-encoding gene, *PKC1*. The gene product of *PKC1*, Pkc1, has the typical domain organization of fungal PKC (Fig. 12.2), and its activity is not stimulated by Ca^{2+} or DAG (Kamada et al. 1996). Amino acids that are thought to be required for binding to DAG are not conserved in the C1 domain of fungal PKCs (Schmitz and Heinisch 2003). The C1 domain of Pkc1 has also been reported to bind Rho1 in *S. cerevisiae* (Nonaka et al. 1995).

Pkc1 is known to have pivotal function in the cell wall integrity (CWI) signaling pathway. The *pkc1* deletion mutant could not form colonies unless an osmotic stabilizer was added to the medium. It has been suggested that *PKC1* was involved

in various cellular processes, such as the cell wall integrity (CWI) signaling pathway, cell-cycle progression, and phospholipid synthesis (Levin 2005). However, its functions at the molecular level remain poorly understood except for the function in the CWI signaling pathway. In the *Aspergillus nidulans* genome, there is a PKC-encoding gene, *pkcA*. *pkcA* was cloned and its functions have been investigated. Deletion of *pkcA* caused frequent hyphal lysis and its deletion mutant did not form colonies, indicating that *pkcA* is essential for hyphal growth (Ichinomiya et al. 2007). This growth defect was not remedied by the addition of osmotic stabilizers to the medium, which suggests that PkcA has other functions besides the function in the CWI signaling pathway (see following). It was reported that *pkcA* was involved in penicillin biosynthesis and was crucial for the nuclear localization of the transcription factor AnBH1 (Herrmann et al. 2006).

Pkc1 of *S. cerevisiae* localizes to polarized growth sites. However, Denis and Cyert showed that the deletion of the HR1 domain causes the relocalization of Pkc1 to the mitotic spindle. They also determined a nuclear localization signal (NLS) and a nuclear export signal (NES) in Pkc1. These results suggest that the small portion of Pkc1 is shuttled between the nucleus and cytoplasm (Denis and Cyert 2005). Recently, Pkc1 has been reported to disappear at the polarized growth site and to accumulate in damage sites caused by lasers (Kono et al. 2012).

PkcA of *A. nidulans* mainly localizes to polarized growth sites, such as hyphal tips, forming septa, and tips of phialides (Teepe et al. 2007). The PKC of *Neurospora crassa* also localizes at some hyphal tips and subapical membranes in actively growing hyphae and forming septa. The PKC in the cytoplasm accumulates in the plasma membrane after treatment with the phorbol ester, 12-myristate 13-acetate (Khatun and Lakin-Thomas 2011).

12.2.1 Function of PKC in the Cell Wall Integrity Signaling Pathway

Fungal cells sense cell wall stresses and transduce signals inside the cells. The signals are transmitted to transcription factors that induce the expression of certain genes. This signal transduction pathway is called the CWI signaling pathway. In this pathway, transmembrane sensor proteins, Wsc1–3, Mid2, and Mtl1, which localize in the plasma membrane, sense cell wall stresses and transmit signals to the downstream effectors Rom1 and 2. Rom1 and 2 are GDP/GTP exchange factors (GEFs) for the GTPase Rho1, a small G protein that is active when it binds to GTP. The GTP-bound form of Rho1 interacts with Pkc1 and activates it, and Pkc1 phosphorylates MAP kinase kinase kinase Bck1. Signals are transduced to transcription factors Rlm1 and the Swi4/Swi6 SBF complex through the MAP kinase cascade. Because the genes encoding almost all the components involved in the CWI pathway are conserved in the genomes of Ascomycota, Basidiomycota, and Mucoromycotina fungi (Table 12.1), signal transduction pathways are speculated to be present in

Table 12.1 Presence of orthologous genes involved in the cell wall integrity (CWI) pathway in fungi

	Saccharomyces cerevisiae	*Aspergillus nidulans*	*A. oryzae*	*C. cinerea*[a]	*R. delemar*[b]
Sensor	Wsc1–3, Mid2, Mtl1	WscA, WscB, MtlA	AO090009000205, AO090020000482	?	?
GEF	Rom1, Rom2	AN4719	AO090120000205	Rgf2	RO3G_07161, RO3G_00951, RO3G_03084, RO3G_02600, RO3G_10407, RO3G_08775, RO3G_14312, RO3G_16337
GTPase	Rho1	RhoA	AO090003000071	CC1G_03166	RO3G_11556, RO3G_02938
PKC	Pkc1	PkcA	AO090120000316	CC1G_07190	RO3G_14507, RO3G_11929, RO3G_06078, RO3G_15031
MAPKKK	Bck1	BckA	AO090003000662	CC1G_10513	RO3G_13559
MAPKK	Mkk1, Mkk2	MkkA	AO090009000347	CC1G_01983	RO3G_02520
MAPK	Slt2	MpkA	MpkA	CC1G_02007, CC1G_14865	RO3G_05693, RO3G_10828
Transcription factors	Rlm1	RlmA	AO090005001424	CC1G_07160	RO3G_15773, RO3G_02782, RO3G_03857, RO3G_06615
	Swi4/Swi6	AN3154/AN6715	AO090012000763/ AO090005000424	CC1G_01306/CC1G_00846	RO3G_13109, RO3G_10059, RO3G_11837, RO3G_08554, RO3G_06039/RO3G_03281

[a] *Coprinopsis cinerea* (Basidiomycota)
[b] *Rhizopus delemar* (Mucoromycotina)

filamentous fungi. The functions of upstream components in this signaling pathway have been analyzed and are described in Chap. 10. In *A. nidulan*s, MpkA and RlmA, a MAP kinase and a transcription factor in the CWI signaling pathway, respectively, are involved in the regulation of the expression of the genes encoding alpha-glucan synthases (*agsA* and *agsB*) and a glutamine-fructose-6-phosphate amidotransferase (*gfaA*) when the cells are treated with caspofungin, a β-1,3 glucan synthase inhibitor (Fujioka et al. 2007). RlmA of *A. niger* is also involved in the expression of *agsA* and *gfaA* (Damveld et al. 2005).

When *pkcA* expression is reduced in *A. nidulans*, the growth of the *pkcA*-conditional mutant is sensitive to a β-1,3 glucan synthase inhibitor, caspofungin, the chitin-binding dyes Congo red and Calcofluor white, and a PKC inhibitor, staurosporine. Abnormalities in the cell wall organization of this mutant have been observed under the *pkcA*-downregulating conditions (Ronen et al. 2007). The overexpression of *pkcA* has also been observed to induce growth sensitivity to a β-1,3-glucan synthase inhibitor, a chitin-binding dye, and chitin synthase inhibitors (Ichinomiya et al. 2007), suggesting that PkcA has a critical role in the CWI signaling pathway. To investigate the function of PkcA in this pathway, we constructed a strain that produces a constitutively active form of PkcA and analyzed the expression patterns of the genes encoding chitin synthases and alpha- and beta-glucan synthases. The results showed that most of these genes, including *chsB*, *csmA*, and *csmB*, are upregulated. Moreover, this upregulation was completely or partially dependent on RlmA (Katayama et al. 2015). These results suggest that chitin synthesis and glucan synthesis are also regulated by the CWI signaling pathway through PKC and Rlm1 orthologues in filamentous fungi. The gene *chsB* encodes a class III chitin synthase, and its gene product, ChsB, tagged with EGFP (EGFP-ChsB), was observed to localize at hyphal tips and forming septa (Fukuda et al. 2009; Horiuchi 2009). ChsB is crucial in hyphal tip growth (Borgia et al. 1996; Ichinomiya et al. 2002). The genes *csmA* and *csmB* encode chitin synthases with myosin motor-like domains, and they are located in a head-to-head configuration in the genome. There are five putative RlmA-binding sites in the promoter region between them. Their gene products, CsmA and CsmB, colocalize at hyphal tips, forming septa, and have compensatory essential functions for tip growth (Takeshita et al. 2006). CsmA is suggested to be involved in repairing cell wall damage (Yamada et al. 2005), and both CsmA and CsmB have similar characteristics (Takeshita et al. 2005; Tsuizaki et al. 2009). It is reasonable to regulate the expression of *csmA* and *csmB* by the signaling pathway through PkcA and RlmA.

Recently, RlmA has been shown to be involved in the regulation of *brlA* expression (Kovács et al. 2013). Because BrlA is a transcription factor that has a crucial role in asexual development (see following), the CWI signaling pathway and asexual development signaling pathway are suggested to be somewhat related.

12.2.2 Functions of PKC Under Heat Stress

12.2.2.1 Germination

As described in the former section, *pkcA* is essential for the growth of *A. nidulans*. In contrast, *bckA* encoding a MAP kinase kinase kinase of the CWI signaling pathway and *mpkA* (Table 12.1) are not essential for growth at 37 °C, an optimal growth temperature for *A. nidulans* (Katayama et al. 2012), which suggests that the PkcA has other function(s) essential for the growth of *A. nidulans*. To investigate these functions, we constructed and characterized a temperature-sensitive mutant of *pkcA*. The resulting *pkcA*-ts mutant grew as well as the wild-type strain below 30 °C, although it showed a partial growth defect and a severe conidiation defect at 37 °C. The *pkcA*-ts mutant did not form colonies at 42 °C. Furthermore, the conidiation defect at 37 °C were partially remedied by the addition of an osmotic stabilizer in the medium, whereas the growth defect at 42 °C was not remedied even in the presence of an osmotic stabilizer. Although the growth defect of *the S. cerevisiae pkc1*-ts mutant at a restrictive temperature was suppressed when Ca^{2+} was added to the medium, that of the *A. nidulans pkcA*-ts mutant was not suppressed on the addition of Ca^{2+}.

During germination, the conidia of *A. nidulans* swell isotropically for a period of time, then the growth polarity is established and germ tubes form (Fig. 12.1). The conidia of the *pkcA*-ts mutant swelled slightly and stopped growing at 42 °C. Reactive oxygen species (ROS) and DNA-strand breaks, common characteristics that are phenotypes of apoptosis, accumulated in swollen conidia, suggesting that apoptosis was induced in the *pkcA*-ts mutant at 42 °C and that PkcA suppresses the induction of apoptosis under heat stress. In contrast, induction of apoptosis was not observed in the *pkcA*-ts mutant grown at 37 °C. The *bckA* and *mpkA* deletion mutants did not form colonies, and the conidia of these mutants swelled and stopped growing at 42 °C. The accumulation of ROS and DNA-strand breaks were also observed in the swollen conidia of both mutants. These results suggest that suppression of apoptosis induction is required for the function of BckA and MpkA. On the other hand, the *rlmA* deletion mutant grew well at 42 °C, suggesting that RlmA is not required for suppression. Taken together, the suppression of apoptosis induction under heat stress conditions would occur through PKC and the downstream MAP kinase cascade but not through RlmA.

The *bckA* and *mpkA* deletion mutants formed colonies at 42 °C when osmotic stabilizer was added to the medium, although the *pkcA*-ts mutant did not form colonies under the same condition, suggesting that PkcA has another function in the growth of *A. nidulans* under heat stress conditions. To clarify the function of PkcA, we analyzed the terminal phenotype of the *pkcA*-ts mutant in the presence of the osmotic stabilizer at 42 °C. Under this condition, apoptosis was not induced in the *pkcA*-ts mutant. However, the mutant formed extremely swollen conidia and did not form germ tubes 8 h after incubation at 42 °C. The DNA content in the swollen conidia of the mutant increased as well as that of the wild-type strain, and polar localization of actin filaments was not observed in the mutant. These results suggest

that the cell cycle progressed regularly, but polarity was not established in the mutant. In contrast, the *bckA* deletion mutant established cell polarity and formed germ tubes under the same condition (Katayama et al. 2012), suggesting that PkcA is required to establish polarity in the swollen conidia, although BckA and downstream factors of the CWI signaling pathway are not involved.

12.2.2.2 Hyphal Growth

The role of PkcA in hyphal tip growth under heat stress conditions was investigated. When the *pkcA*-ts mutant was cultivated for 16 h to induce hyphal growth at a permissive temperature (30 °C) and shifted to a restrictive temperature (42 °C), the growth of the *pkcA*-ts mutant stopped and its hyphal tips swelled. These observations suggest that the hyphal tips were depolarized. Actin filaments are usually observed at the hyphal tips in *A. nidulans*. When hyphae were exposed to heat stress, actin filaments at the hyphal tips rapidly disappeared in both the *pkcA*-ts mutant and the wild-type strain. In the wild-type strain, actin filaments at the hyphal tips reappeared within 60 min after the shift to 42 °C, whereas they did not reappear 120 min after the sift in the *pkcA*-ts mutant. The reappearance of actin filaments at the tips was observed in the *bckA* deletion mutant as well as the wild-type strain (Katayama et al. 2012), suggesting that the repolarization of hyphae does not depend on the function of BckA. As already mentioned, EGFP-ChsB localizes at the hyphal tips. After the shift to 42 °C, EGFP-ChsB disappeared from hyphal tips in the *pkcA*-ts mutant and the wild-type strain. EGFP-ChsB was observed again at the hyphal tips a few hours after the shift in the wild-type strain, whereas it was not observed at the hyphal tips 5 h after the shift in the *pkcA*-ts strain. The localization of microtubules was not affected in the *pkcA*-ts mutant and wild-type strain when exposed to heat stress (Takai et al., unpublished results). These results suggest that the redistribution of EGFP-ChsB to hyphal tips depends on the actin filament repolarization through the function of PkcA. A model for the PKC functions during germination and hyphal tip growth is shown in Fig. 12.4.

12.2.2.3 Asexual Development

As described in the former section, induction of the expression of *brlA* is an essential step for conidiation in *Aspergillus nidulans. brlA* encodes a C_2H_2 transcription factor and controls the expression of genes involved in regulating the early stages of conidiation. The expression of *brlA* was not induced in the *pkcA*-ts mutant at 40 °C, suggesting that PkcA is required to induce the expression of *brlA* (Katayama and Horiuchi, unpublished results). The expression of *brlA* is induced by the upstream activators FluG, FlbA, FlbB, FlbC, FlbD, and FlbE (Fig. 12.5). FluG is a key activator of the initiation of conidiation and induces conidiation via two independent processes (Etxebeste et al. 2010; Park and Yu 2012). First, FluG inhibits the vegetative growth via the activation of FlbA, which is a key regulator of the G-protein

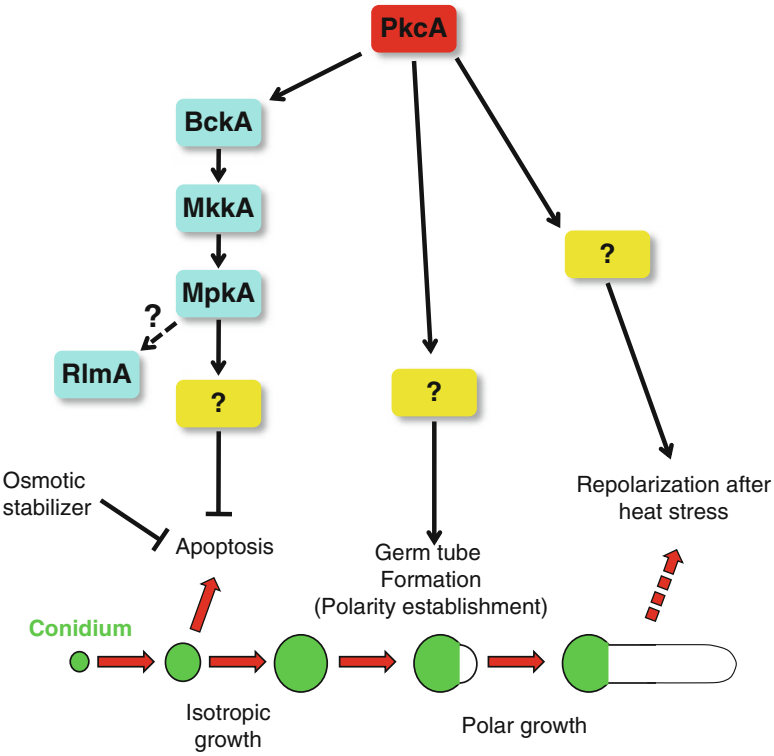

Fig. 12.4 A model for the function of PkcA under heat stress. "?" in *yellow panels* means the possible presence of unknown factors. (Modified from fig. 10 in a previous paper by Katayama et al. 2012

signaling pathway that regulates the balance between hyphal proliferation and conidiophore differentiation. Second, FluG induces the expression of *brlA* by activating the two separate pathways. One pathway contains FlbC, which is a C_2H_2 transcription factor and induces the expression of *brlA* by directly binding to the promoter of *brlA*. Another pathway consists of FlbE, FlbB, and FlbD. In this pathway, FlbB functions as a bZIP-type transcription factor and forms a complex with FlbE. This complex induces the expression of *flbD*, which encodes a cMyc-type transcription factor. FlbE interacts and colocalizes with FlbB at the hyphal tips, suggesting that FlbE ensures the proper localization and function of FlbB (Garzia et al. 2009). Both FlbB and FlbD induce the expression of *brlA* by directly binding to the promoter of *brlA*. The expression levels of *brlA* significantly decrease in mutants of the genes encoding these activators, and the mutants show the fluffy phenotype (Wieser et al. 1994). Negative regulators of conidiation have also been identified. SfgA is a putative transcription factor with a Gal4-type $Zn(II)_2Cys_6$ binuclear cluster DNA-binding domain. The deletion of *sfgA* suppressed the fluffy phenotype of the *fluG* deletion mutant but not the deletion mutants of *flbA*, *flbB*, *flbC*, and *flbD*, indicating that SfgA acts as a negative regulator of conidiation by functioning

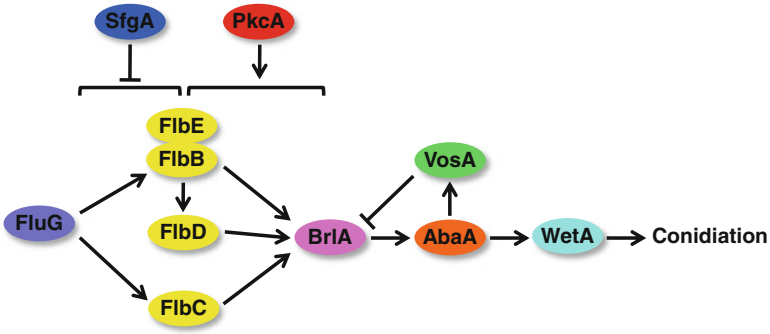

Fig. 12.5 Involvement of PkcA in conidiation. Details discussed in text

downstream of FluG but upstream of FlbA, FlbB, FlbC, and FlbD (Seo et al. 2006). The deletion of *sfgA* did not suppress the decrease in conidiation efficiency of the *pkcA*-ts mutant at 37 °C, suggesting that PkcA regulates the expression of *brlA* downstream of SfgA (Katayama and Horiuchi, unpublished results). The *velvet* protein VosA is suggested to repress conidiation during vegetative growth (Bayram and Braus 2012; Park et al. 2012). In addition, VosA binds to the promoter of *brlA* and represses the expression of *brlA* (Park et al. 2012). The expression of *vosA* is induced by AbaA, which is a TEA/ATTS transcription factor that functions downstream of BrlA, suggesting that VosA plays a role in the negative feedback regulation of *brlA* (Park and Yu 2012). The deletion of *vosA* did not suppress the decrease in the conidiation efficiency of the *pkcA*-ts mutant at 37 °C, suggesting that VosA is not involved in the PkcA regulation of *brlA* expression (Katayama and Horiuchi, unpublished results). These results are summarized in Fig. 12.5.

Because conidiophores of *Aspergillus* have distinctive structural features and those are not conserved in the filamentous fungi other than Ascomycota, there are no orthologues of the genes involved in the formation of conidiophores and conidia in filamentous fungi belonging to Basidiomycota and Mucoromycotina. In Ascomycota, only the orthologous genes of *brlA* are conserved in the genomes belonging to the genus *Aspergillus* or *Penicillium*. Future studies are required for understanding the role of PKC in asexual development of filamentous fungi of other genera.

12.2.3 Other Functions of PKC in Filamentous Fungi

PKCs in filamentous fungi have also been reported to have other functions, as described next.

Unfolded protein response (UPR) is a stress response caused by endoplasmic reticulum stresses. Farnesol induces apoptosis in filamentous fungi, and farnesol treatment of *Aspergillus nidulans* has been reported to induce UPR via the PKC signaling pathway, suggesting that PkcA is involved in the induction of UPR (Colabardini et al. 2010). In *Neurospora crassa*, PKC is known to regulate light

responses via phosphorylating the WC-1 protein, which is a blue light photoreceptor (Franchi et al. 2005). In *Aspergillus oryzae*, the assembly of a structural protein of Woronin body, AoHex1, has been suggested to be regulated by PKC activity (Juvvadi et al. 2007) (see Chap. 11). Because the orthologues of the genes encoding WC-1 and AoHex1 are present in the genome of filamentous fungi belonging to Ascomycota, PKCs in these fungi likely have similar functions.

Recently, transcriptome analysis of *A. niger* was performed under carbon starvation conditions. Carbon starvation induces hyphal morphological changes and asexual development. The expression of a PKC-encoding gene was slightly upregulated under this starvation condition (Nitsche et al. 2012). This observation may suggest a novel function of PKC for morphogenesis in filamentous fungi.

In this chapter, we described the functions of PKC in the morphogenesis of filamentous fungi by focusing on the *A. nidulans* PkcA under cell wall stress or heat stress. The factors that function upstream of PKC are currently unknown under these conditions, except for MtlA of *A. nidulans* (Futagami et al. 2014) (see Chap. 10).

Although PKC signaling pathways in *S. cerevisiae* are activated by many environmental stresses, such as cell wall stress, heat stress, oxidative stress, hypoosmotic stress, and endoplasmic reticulum stress (Levin 2005, 2011), these functions remain largely uncharacterized in filamentous fungi, partly because of the difficulties of handling filamentous fungi at the molecular level. Because filamentous fungi encounter various stresses in the culture conditions used in industry, it is very important to understand their responses to these stresses. This information enables us to improve the culture conditions and how to breed strains at the molecular level. PKC is probably pivotal in many aspects of these conditions. Because molecular biological techniques have much improved recently in filamentous fungi, other functions of PKCs related to the stresses affecting morphogenesis and conidiation will be elucidated in the near future.

References

Bayram O, Braus GH (2012) Coordination of secondary metabolism and development in fungi: the velvet family of regulatory proteins. FEMS Microbiol Rev 36:1–24. doi:10.1111/j.1574-6976.2011.00285.x

Borgia P, Iartchouk N, Riggle P, Winter K, Koltin Y, Bulawa C (1996) The *chsB* gene of *Aspergillus nidulans* is necessary for normal hyphal growth and development. Fungal Genet Biol 20: 193–203. doi:10.1006/fgbi.1996.0035

Colabardini AC, de Castro PA, de Gouvêa PF, Savoldi M, Malavazi I, Goldman MHS, Goldman GH (2010) Involvement of the *Aspergillus nidulans* protein kinase C with farnesol tolerance is related to the unfolded protein response. Mol Microbiol 78:1259–1279. doi:10.1111/j.1365-2958.2010.07403.x

Damveld R, Arentshorst M, Franken A, van Kuyk P, Klis F, van den Hondel C, Ram A (2005) The *Aspergillus niger* MADS-box transcription factor RlmA is required for cell wall reinforcement in response to cell wall stress. Mol Microbiol 58:305–319. doi:10.1111/j.1365-2958.2005.04827.x

De Souza CP, Hashmi SB, Osmani AH, Andrews P, Ringelberg CS, Dunlap JC, Osmani SA (2013) Functional analysis of the *Aspergillus nidulans* kinome. PLoS One 8:e58008. doi:10.1371/journal.pone.0058008

Denis V, Cyert MS (2005) Molecular analysis reveals localization of *Saccharomyces cerevisiae* protein kinase C to sites of polarized growth and Pkc1p targeting to the nucleus and mitotic spindle. Eukaryot Cell 4:36–45. doi:10.1128/EC.4.1.36-45.2005

Etxebeste O, Garzia A, Espeso EA, Ugalde U (2010) *Aspergillus nidulans* asexual development: making the most of cellular modules. Trends Microbiol 18:569–576. doi:10.1016/j.tim.2010.09.007

Franchi L, Fulci V, Macino G (2005) Protein kinase C modulates light responses in *Neurospora* by regulating the blue light photoreceptor WC-1. Mol Microbiol 56:334–345. doi:10.1111/j.1365-2958.2005.04545.x

Fujioka T, Mizutani O, Furukawa K, Sato N, Yoshimi A, Yamagata Y, Nakajima T, Abe K (2007) MpkA-dependent and -independent cell wall integrity signaling in *Aspergillus nidulans*. Eukaryot Cell 6:1497–1510. doi:10.1128/EC.00281-06

Fukuda K, Yamada K, Deoka K, Yamashita S, Ohta A, Horiuchi H (2009) Class III chitin synthase ChsB of *Aspergillus nidulans* localizes at the sites of polarized cell wall synthesis and is required for conidial development. Eukaryot Cell 8:945–956. doi:10.1128/EC.00326-08

Futagami T, Seto K, Kajiwara Y, Takashita H, Omori T, Takegawa K, Goto M (2014) The putative stress sensor protein MtlA is required for conidia formation, cell wall stress tolerance, and cell wall integrity in *Aspergillus nidulans*. Biosci Biotechnol Biochem 78:326–335. doi:10.1080/09168451.2014.878218

Garzia A, Etxebeste O, Herrero-García E, Fischer R, Espeso EA, Ugalde U (2009) *Aspergillus nidulans* FlbE is an upstream developmental activator of conidiation functionally associated with the putative transcription factor FlbB. Mol Microbiol 71:172–184. doi:10.1111/j.1365-2958.2008.06520.x

Herrmann M, Sprote P, Brakhage A (2006) Protein kinase C (PkcA) of *Aspergillus nidulans* is involved in penicillin production. Appl Environ Microbiol 72:2957–2970. doi:10.1128/AEM.72.4.2957-2970.2006

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson OE, Huhndorf S, James T, Kirk PM, Lücking R, Lumbsch HT, Lutzoni F, Matheny PB, Mclaughlin DJ, Powell MJ, Redhead S, Schoch CL, Spatafora JW, Stalpers JA, Vilgalys R, Aime MC, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, Crous PW, Dai Y, Gams W, Geiser DM, Griffith GW, Gueidan C, Hawksworth DL, Hestmark G, Hosaka K, Humber RA, Hyde KD, Ironside JE, Kõljalg U, Kurtzman CP, Larsson K, Lichtwardt R, Longcore J, Miadlikowska J, Miller A, Moncalvo J, Mozley-Standridge S, Oberwinkler F, Parmasto E, Reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio JP, Schüßler A, Sugiyama J, Thorn RG, Tibell L, Untereiner WA, Walker C, Wang Z, Weir A, Weiss M, White MM, Winka K, Yao Y, Zhang N (2007) A higher-level phylogenetic classification of the Fungi. Mycol Res 111:509–547. doi:10.1016/j.mycres.2007.03.004

Horiuchi H (2009) Functional diversity of chitin synthases of *Aspergillus nidulans* in hyphal growth, conidiophore development and septum formation. Med Mycol 47:S47–S52. doi:10.1080/13693780802213332

Ichinomiya M, Motoyama T, Fujiwara M, Takagi M, Horiuchi H, Ohta A (2002) Repression of *chsB* expression reveals the functional importance of class IV chitin synthase gene *chsD* in hyphal growth and conidiation of *Aspergillus nidulans*. Microbiology 148:1335–1347

Ichinomiya M, Uchida H, Koshi Y, Ohta A, Horiuchi H (2007) A protein kinase C-encoding gene, pkcA, is essential to the viability of the filamentous fungus *Aspergillus nidulans*. Biosci Biotechnol Biochem 71:2787–2799

Juvvadi PR, Maruyama J, Kitamoto K (2007) Phosphorylation of the *Aspergillus oryzae* Woronin body protein, AoHex1, by protein kinase C: evidence for its role in the multimerization and proper localization of the Woronin body protein. Biochem J 405:533–540. doi:10.1042/BJ20061678

Kamada Y, Qadota H, Python C, Anraku Y, Ohya Y, Levin D (1996) Activation of yeast protein kinase C by Rho1 GTPase. J Biol Chem 271:9193–9196

Katayama T, Ohta A, Horiuchi H (2015) Protein kinase C regulates the expression of cell wall-related genes in RlmA-dependent and independent manners in *Aspergillus nidulans*. Biosci Biotechnol Biochem in press doi:10.1080/09168451.2014.973365

Katayama T, Uchida H, Ohta A, Horiuchi H (2012) Involvement of protein kinase C in the suppression of apoptosis and in polarity establishment in *Aspergillus nidulans* under conditions of heat stress. PLoS One 7:e50503. doi:10.1371/journal.pone.0050503

Khatun R, Lakin-Thomas P (2011) Activation and localization of protein kinase C in *Neurospora crassa*. Fungal Genet Biol 48:465–473. doi:10.1016/j.fgb.2010.11.002

Kono K, Saeki Y, Yoshida S, Tanaka K, Pellman D (2012) Proteasomal degradation resolves competition between cell polarization and cellular wound healing. Cell 150:151–164. doi:10.1016/j.cell.2012.05.030

Kovács Z, Szarka M, Kovács S, Boczonádi I, Emri T, Abe K, Pócsi I, Pusztahelyi T (2013) Effect of cell wall integrity stress and RlmA transcription factor on asexual development and autolysis in *Aspergillus nidulans*. Fungal Genet Biol 54:1–14. doi:10.1016/j.fgb.2013.02.004

Levin DE (2005) Cell wall integrity signaling in *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 69:262–291. doi:10.1128/MMBR.69.2.262-291.2005

Levin DE (2011) Regulation of cell wall biogenesis in *Saccharomyces cerevisiae*: the cell wall integrity signaling pathway. Genetics 189:1145–1175. doi:10.1534/genetics.111.128264

Mukai H (2003) The structure and function of PKN, a protein kinase having a catalytic domain homologous to that of PKC. J Biochem 133:17–27. doi:10.1093/jb/mvg019

Nitsche BM, Jørgensen TR, Akeroyd M, Meyer V, Ram AFJ (2012) The carbon starvation response of *Aspergillus niger* during submerged cultivation: insights from the transcriptome and secretome. BMC Genomics 13:380. doi:10.1186/1471-2164-13-380

Nonaka H, Tanaka K, Hirano H, Fujiwara T, Kohno H, Umikawa M, Mino A, TakaiI Y (1995) A downstream target of Rho1 small GTP-binding protein is Pkc1, a homolog of protein-kinase-C, which leads to activation of the MAP kinase cascade in *Saccharomyces cerevisiae*. EMBO J 14:5931–5938

Park H, Yu J (2012) Genetic control of asexual sporulation in filamentous fungi. Curr Opin Microbiol 15:669–677

Park H, Ni M, Jeong KC, Kim YH, Yu J (2012) The role, interaction and regulation of the velvet regulator VelB in *Aspergillus nidulans*. PLoS One 7:e45935. doi:10.1371/journal.pone.0045935

Ronen R, Sharon H, Levdansky E, Romano J, Shadkchan Y, Osherov N (2007) The *Aspergillus nidulans pkcA* gene is involved in polarized growth, morphogenesis and maintenance of cell wall integrity. Curr Genet 51:321–329. doi:10.1007/s00294-007-0129-y

Schmitz H, Heinisch J (2003) Evolution, biochemistry and genetics of protein kinase C in fungi. Curr Genet 43:245–254. doi:10.1007/s00294-003-0403-6

Schmitz H, Lorberg A, Heinisch J (2002) Regulation of yeast protein kinase C activity by interaction with the small GTPase Rho1p through its amino-terminal HR1 domain. Mol Microbiol 44:829–840. doi:10.1046/j.1365-2958.2002.02925.x

Seo J, Guan Y, Yu J (2006) FluG-dependent asexual development in *Aspergillus nidulans* occurs via derepression. Genetics 172:1535–1544. doi:10.1534/genetics.105.052258

Steinberg SF (2008) Structural basis of protein kinase C isoform function. Physiol Rev 88:1341–1378. doi:10.1152/physrev.00034.2007

Takeshita N, Ohta A, Horiuchi H (2005) CsmA, a class V chitin synthase with a myosin motor-like domain, is localized through direct interaction with the actin cytoskeleton in *Aspergillus nidulans*. Mol Biol Cell 16:1961–1970

Takeshita N, Yamashita S, Ohta A, Horiuchi H (2006) *Aspergillus nidulans* class V and VI chitin synthases CsmA and CsmB, each with a myosin motor-like domain, perform compensatory functions that are essential for hyphal tip growth. Mol Microbiol 59:1380–1394

Teepe AG, Loprete DM, He Z, Hoggard TA, Hill TW (2007) The protein kinase C orthologue PkcA plays a role in cell wall integrity and polarized growth in *Aspergillus nidulans*. Fungal Genet Biol 44:554–562. doi:10.1016/j.fgb.2006.10.001

Tsuizaki M, Takeshita N, Ohta A, Horiuchi H (2009) Myosin motor-like domain of the class VI chitin synthase, CsmB, is essential for its functions in *Aspergillus nidulans*. Biosci Biotechnol Biochem 73:1163–1167

Wieser J, Lee B, Fondon J, Adams T (1994) Genetic requirements for initiating asexual development in *Aspergillus nidulans*. Curr Genet 27:62–69. doi:10.1007/BF00326580

Yamada E, Ichinomiya M, Ohta A, Horiuchi H (2005) Functions of *csmA* in *chsA chsC* double mutant of *Aspergillus nidulans*. Biosci Biotechnol Biochem 69:87–97

Chapter 13
Response and Adaptation to Cell Wall Stress and Osmotic Stress in *Aspergillus* Species

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Abstract *Aspergillus* species are one of the most important filamentous fungi from the point of view of industry, pathogenesis, and mycotoxin production. Some aspergilli are used to produce traditional Japanese fermented foods such as sake, shoyu (soy sauce), and miso. In the fermentation steps, fungi are cultivated on solid substrates (steamed or baked cereal grains), under which conditions fungi produce and secrete a large amount of hydrolytic enzymes. During this solid-state fermentation, fungi must cope with various abiotic stresses including temperature, pH, osmotic stress, and low oxygen. To understand fungal biology and to make more use of fungi for fermentation and enzyme production, intensive research on stress adaptation mechanisms have been performed in *Aspergillus* species. This review focuses on the responses to cell wall stresses and osmotic stresses to which aspergilli should adapt during solid-state cultivation. In a cell wall integrity signaling pathway, the MpkA mitogen-activated protein kinase (MAPK) cascade plays a central role in the regulation of α-1,3-glucan synthase genes and consequently affects fungal cell wall composition. An osmotic stress signaling pathway is composed of the combination of the upstream two-component phosphorelay signaling and the downstream HogA/SakA MAPK cascade, and the signaling pathway is responsible for adaptation to environmental osmotic changes. Here, we provide recent findings on the two stress

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response signaling pathways in aspergilli. The accumulated knowledge will be useful for understanding and improving the fermentation processes of industrial aspergilli in solid-state cultivation.

Keywords *Aspergillus* species • Cell wall integrity signaling • HogA/SakA MAPK cascade • MpkA MAPK cascade • Osmotic stress signaling • Solid-state culture • Stress response • α-1,3-Glucan

13.1 Introduction

Filamentous fungi are ubiquitous, commonly found on decaying woods and plants as well as in the soil. They produce a large amount of different kinds of hydrolytic enzymes, which degrade biomass in nature and thus contribute to carbon and nitrogen recycling. The saprophytic property is important for ecosystems, but some plant pathogenic fungi spoil farm crops, which is problematic all over the world. Small but significant numbers of filamentous fungi cause serious infection in humans, to which much attention has been paid as the number of immunocompromised people is increasing because of aging populations or immunocompromising diseases including human immunodeficiency virus (HIV) infection. As a beneficial aspect, some fungi produce a variety of enzymes and organic acids that are applicable to the food and pharmaceutical industry, and they are used for manufacturing fermented foods. Aspergilli includes a wide variety of species such as the industrial fungi *Aspergillus oryzae* and *Aspergillus niger*, the human pathogen *Aspergillus fumigatus*, the aflatoxin producers *Aspergillus flavus* and *Aspergillus parasiticus*, and the model fungus *Aspergillus nidulans*. Because of their industrial, medical, and agricultural importance, the genome sequences of aspergilli have been determined (Machida et al. 2005; Nierman et al. 2005; Galagan et al. 2005; Pel et al. 2007), and the understanding of the biology of filamentous fungi has been extensively advanced in the past decade.

Some *Aspergillus* species, such as *A. oryzae*, *A. sojae*, and *Aspergillus luchuensis*, have been used for hundreds of years in traditional Japanese fermentation industries to produce *sake* (rice wine), *shoyu* (soy sauce), *miso* (soybean paste), and *shōchū* (distilled beverage) (Machida et al. 2008; Hong et al. 2013). *Aspergillus oryzae* has been used for the fermentations because of its notable potential for the secretory production of various enzymes such as amylases and proteases in solid-state cultivation. The ability to secrete such hydrolytic enzymes in solid-state culture is further enhanced compared to submerged (liquid) culture (Oda et al. 2006). Because of this advantage, the solid-state cultivation of *A. oryzae* has been applied to the production not only of fermented foods but also of various enzymes and chemicals (Yu et al. 2003). During solid-state cultivation, fungi have to adapt to various abiotic stresses including temperature, pH, and oxidative and osmotic

conditions on the surface of the solid substrates. Low oxygen is an additional condition to which fungi should adapt inside the solid substrates. At present, our understanding of the stress response biology under solid-state culture conditions is limited. Therefore, it is necessary to intensively accumulate knowledge about this state, which would advance technology in the fermented food, enzyme production, and chemical industries.

Because fungi confront a variety of stresses in nature, well-established mechanisms for sensing and responding to environmental stimuli must be inherent. Of these stress adaptation mechanisms, responses to cell wall stress and osmotic stress, which are also important in solid-state cultivation, have been well studied in several *Aspergillus* species. This review focuses on response mechanisms to the stresses and describes the up-to-date findings and current understanding of the response mechanisms.

13.2 Cell Wall Integrity Signaling System of *Aspergillus* Species

13.2.1 Overview of Cell Wall Integrity (CWI) Signaling

The fungal cell wall is an essential structure that defines the shape of the cell and shields it from environmental stress. In solid-state fermentation, fungi are exposed to a variety of environmental stimuli, including changes in osmolality, temperature, and pH. These stresses primarily act on the fungal cell wall, which generates a need for remodeling or repair. Therefore, understanding the mechanism of cell wall biogenesis is important for fermentation industries.

Proper cell wall architecture of filamentous fungi requires several cell wall components that are mainly composed of polysaccharides: α-glucans (α-1,3-glucan and α-1,4-glucan), β-glucans (β-1,6-branched β-1,3-glucan), galactosaminogalactan, galactomannan, and chitin (Fig. 13.1) (Fontaine et al. 2000; Kapteyn et al. 1999; Klis 1994; Latgé 2007, 2010). Cell wall biogenesis and a signal transduction system that monitors the cell wall and promotes cell wall remodeling have been studied in detail in *Saccharomyces cerevisiae* (Heinisch et al. 1999; Levin 2005). Sensing of cell wall perturbations requires surface sensors, which are encoded by the cell wall integrity and stress response component (WSC) genes, upstream of the intracellular signal transduction pathway responsible for maintaining cell wall integrity in yeast. The Wsc1p, Wsc2p, and Wsc3p proteins are highly glycosylated plasma membrane proteins that contain an extracellular domain with a cysteine-rich domain, a Ser/Thr-rich region that carries glycosylation sites, and a highly charged C-terminal cytoplasmic region (Levin 2011; Lodder et al. 1999; Rodicio and Heinisch 2010; Verna et al. 1997). Additional cell wall stress sensors are partially redundant to Mid2p and Mlt1p cell-surface proteins. These proteins act as

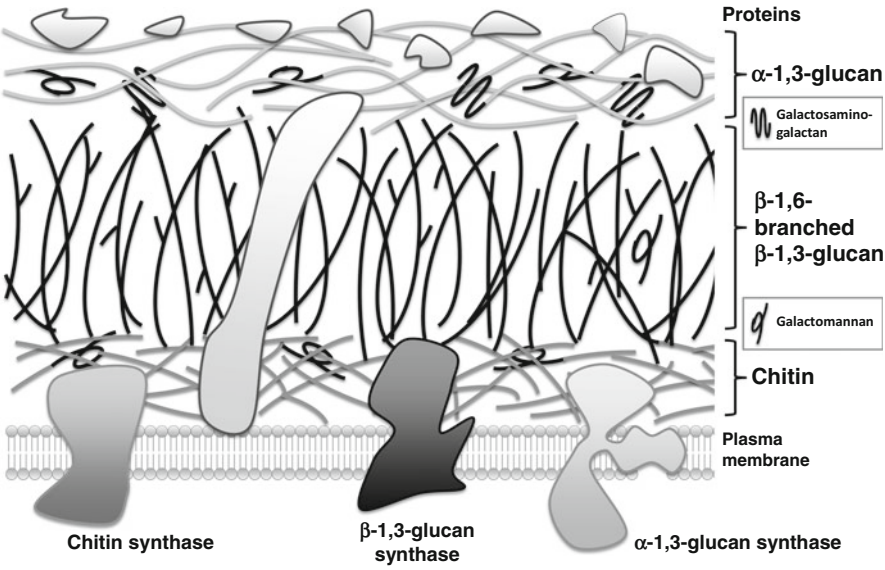

Fig. 13.1 Cell wall organization of *Aspergillus nidulans*. The central core of the cell wall is mainly composed of β-1,6-branched β-1,3-glucan crosslinked to chitin; the amorphous α-1,3-glucan is present in the outer layer of the cell wall. Polysaccharides, such as galactosaminogalactan and galactomannan, and proteins, such as GPI-anchored protein and surface proteins, are also present in the cell wall

mechanosensors and detect cell wall perturbations caused by cell wall stress during growth or pheromone-induced morphogenesis, exposure to high temperature, or changes in osmolality, and they transmit signals to the downstream signaling pathway (Ketela et al. 1999; Rajavel et al. 1999; Verna et al. 1997). The activation of the cell wall integrity (CWI) signaling pathway proceeds through the small G protein Rho1p (Levin et al. 1990; Levin 2011). Rho1p is a small GTPase that is activated by the guanosine nucleotide exchange factors (GEFs) Rom1p, Rom2p, and Tus1p (Levin 2011; Philip and Levin 2001) and is downregulated by the GTPase-activating proteins (GAPs), including Bem2p and Sac7p (Levin 2011; Schmidt et al. 1997). In the active form, Rho1p binds to and activates protein kinase C (Pkc1p) (Kamada et al. 1996), which in turn activates the MAPK (mitogen-activated protein kinase) cascade. The MAPK cascade is a linear pathway that is composed of the MAPK kinase kinase Bck1p (Lee and Levin 1992), a pair of redundant MAPK kinases, Mkk1p and Mkk2p (Irie et al. 1993), and the MAPK Mpk1p/Slt2p (Martin et al. 1993). The Mpk1p phosphorylates and activates the transcription factor Rlm1p, which regulates the transcription of at least 25 genes involved in cell wall biogenesis, including β-1,3-glucan synthase genes and chitin synthase genes (Jung and Levin 1999). In the following sections, recent findings related to CWI signaling in aspergilli are described.

13.2.2 *Cell-Surface Sensors for CWI Signaling in* Aspergillus *Species*

In *Aspergillus* species, the genes for CWI sensor proteins have been characterized (Dichtl et al. 2012; Goto et al. 2009; Futagami et al. 2011; Futagami and Goto 2012). Goto et al. (2009) identified the *A. nidulans* gene for the cell wall stress sensor WscA from the genome, which is a homologue of Wsc1p in *S. cerevisiae* (Goto et al. 2009). WscA contains a Wsc motif that is rich in cysteine residues, a Ser/Thr-rich region that carries three putative *N*-glycosylation sites, a transmembrane region, and a cytoplasmic region at the C-terminus (Goto et al. 2009). They also suggested that *A. nidulans* WscA is *O*-mannosylated at the conserved Ser/Thr-rich region by PmtA and PmtC, but not by PmtB, which are protein *O*-mannosyltransferases in *A. nidulans*, and that the *O*-glycan attachment has a significant impact on the stability of WscA (Goto et al. 2009; Kriangkripipat and Momany 2009). Protein *O*-glycosylation has a critical role, as demonstrated by the observation that the *pmtA* mutant exhibits an abnormal cell morphology and altered cell wall composition (Oka et al. 2004; Goto 2007). Futagami et al. (2011) determined that WscA and WscB were both *N*- and *O*-glycosylated and localized on the cell surface. They also reported that the *wsc* disruptants (*wscA*Δ and *wscB*Δ) showed reduced colony size, the formation of fewer conidia, and a high frequency of swollen hyphae in hypo-osmotic conditions, whereas the defective phenotype was restored by osmotic stabilization with potassium chloride (Futagami et al. 2011). Moreover, the transcription levels of the α-1,3-glucan synthase genes, *agsA* and *agsB*, are markedly altered in the *wsc* disruptants, resulting in an increase in the amount of alkali-soluble cell wall glucan, including soluble α-1,3-glucan (Futagami et al. 2011). On the other hand, exposure to micafungin, a β-1,3-glucan synthase inhibitor, leads to the activation of the *agsB* transcription in both the wild-type and the *wscA*Δ *wscB*Δ strains. This alteration in expression was dependent on MpkA, which is an orthologue of yeast Mpk1p in *A. nidulans*, indicating that WscA and WscB are not essential for MpkA signaling (Fig. 13.2) (Futagami et al. 2011). In addition, it has been reported that the *A. nidulans* genome possesses a Mid2-like protein, which has structural features that are similar to the CWI sensor protein Mid2p of *S. cerevisiae* (Fig. 13.2) (Futagami et al. 2011; Futagami and Goto 2012). However, the involvement of the protein in *A. nidulans* CWI signaling is yet to be investigated.

In *Aspergillus fumigatus* CWI signaling, the potential CWI sensor proteins Wsc1, Wsc2, Wsc3, and MidA, which are homologues of yeast Wsc1p, Wsc2p, Wsc3p, and Mid2p, respectively, and the Rho GTPases Rho1, Rho2, and Rho4 were functionally characterized (Dichtl et al. 2012). On the basis of experimental data obtained from phenotypic analysis in the mutants of the genes encoding CWI sensor proteins and the Rho GTPases, Dichtl et al. (2012) proposed a model for a CWI signaling pathway in *A. fumigatus*. Cell wall stress induced by Congo red (CR), Calcofluor white (CFW), and heat shock is sensed by MidA. MidA and an unknown sensor activate the MpkA pathway, probably via Rho1 and PkcA. Wsc1

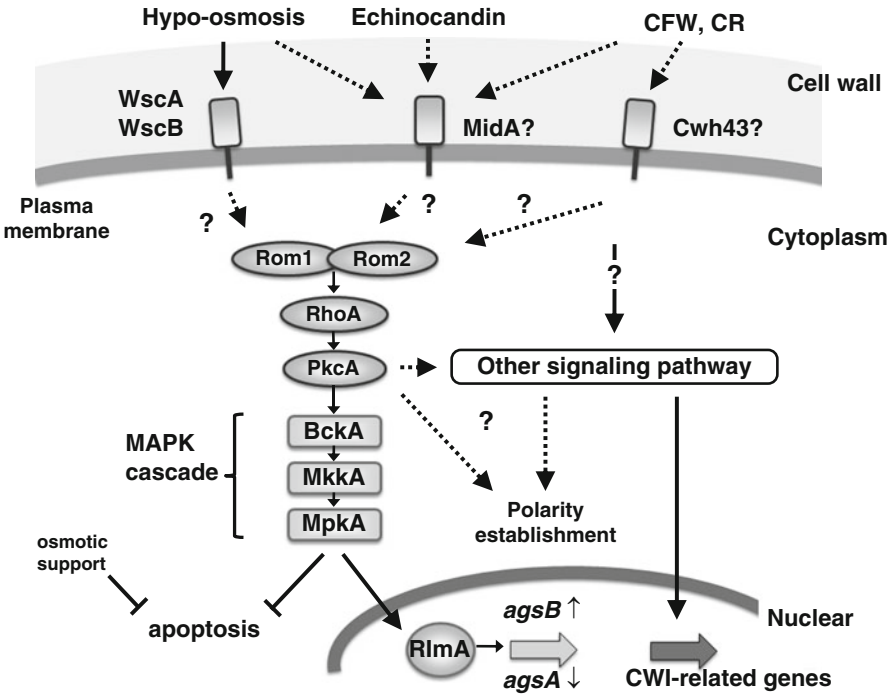

Fig. 13.2 Schematic model of cell wall stress signaling in *A. nidulans*. Based on the study results, we hypothesize that *A. nidulans* has the following cell wall integrity (CWI) signaling system. (1) Putative sensor proteins in the CWI signaling pathway, WscA and WscB, are important in CWI signaling under hypo-osmotic conditions, but WscA and WscB are not essential for MpkA-RlmA signaling. (2) PkcA is involved in the CWI pathway in *A. nidulans*. In addition, PkcA is a factor in the suppression of apoptosis induction via the MpkA pathway, but not in polarity establishment, during hyphal growth independent of the MpkA pathway under heat stress conditions. (3) *AgsA* and *agsB* expression is dependent on MpkA and partly dependent on RlmA. (4) Other CWI-related genes, such as *fksA*, *gelA*, *gelB*, *chsA*, *chsB*, *chsC*, *chsD*, *csmA*, and *csmB*, are independent of the MpkA-RlmA system. The CWI pathway mainly regulates the transcription of α-1,3-glucan biogenesis-related genes. The transcripts of β-1,3-glucan and chitin biogenesis-related genes are mainly regulated by other unknown signals that might be activated by a cell wall stress such as echinocandin (micafungin) treatment

is specifically required to be tolerant of β-1,3-glucan synthase inhibitors, such as caspofungin, which is an echinocandin antifungal drug. Wsc1, Wsc3, and MidA are redundantly required to promote radial growth and conidiation, possibly via the MpkA pathway. Rho2 and Rho4 seem to not directly contribute to MpkA phosphorylation, but both are important for CWI signaling. Moreover, Rho4 is essential for septum formation and contributes to tolerance of β-1,3-glucan synthase inhibitors (Dichtl et al. 2012).

13.2.3 *Protein Kinase C Pathway in* Aspergillus nidulans

In addition to CWI sensor proteins and Rho GTPases, the PKCs have been isolated from several filamentous fungal species. For example, *Neurospora* PKC is suggested to be essential for viability and is involved in a light-signaling pathway (Arpaia et al. 1999; Franchi et al. 2005). With regard to *Aspergillus* species, an *A. nidulans* PKC-encoding gene, *pkcA* (a counterpart of yeast *pkc1*), is suggested to be essential for its viability even under osmotic stabilization, whereas the lethality caused by deletion of yeast *pkc1* is suppressed by osmotic stabilization (Herrmann et al. 2006; Ichinomiya et al. 2007; Ronen et al. 2007; Teepe et al. 2007). Repression of *pkcA* expression led to hypersensitivity to cell wall-defective agents, such as caspofungin and CFW, and defects in the cell wall structure, suggesting that PkcA is involved in the CWI pathway in *A. nidulans* (Fig. 13.2) (Ronen et al. 2007; Teepe et al. 2007). It was shown that PkcA localized to the hyphal apices, forming septa, and tips of phialides (Teepe et al. 2007). In addition, PkcA is suggested to be associated with numerous functions, including conidiation, germination, secondary metabolism, and farnesol-induced cell death (Colabardini et al. 2010; Herrmann et al. 2006; Ichinomiya et al. 2007; Ronen et al. 2007; Teepe et al. 2007). Katayama et al. (2012) constructed and characterized temperature-sensitive mutants of *pkcA* of *A. nidulans*. These mutants exhibited apoptotic phenotypes at 42 °C, a restrictive temperature, although the mutants showed almost normal growth and conidiation at 30 °C. They also suggested that PkcA functions in the suppression of apoptosis induction via the MpkA pathway. However, polarity establishment during hyphal growth under heat stress conditions, which involves PkcA, is independent of the MpkA pathway (Fig. 13.2). Direct deletion mutants of *pkcA* have not yet been constructed in any filamentous fungi, and the evidence collected thus far suggests that *pkcA* is essential in *A. nidulans*.

13.2.4 *MAP Kinase Pathway for CWI Signaling in* Aspergillus *Species*

The genes encoding a counterpart of yeast Mpk1p (Slt2p) have also been characterized in aspergilli (Bussink and Osmani 1999; Fujioka et al. 2007; Jain et al. 2011). Deletion analysis of *A. nidulans mpkA* has suggested that the kinase is involved in conidial germination and in polarized growth (Bussink and Osmani 1999). In *A. fumigatus*, MpkA is involved in the response against reactive oxygen species, siderophore production during iron starvation, and the production of secondary metabolites (Jain et al. 2011). Besides these physiological functions, the involvement of MpkA in CWI signaling has been demonstrated in *Aspergillus* species.

Fujioka et al. (2007) constructed disruptant strains of *A. nidulans, mpkA*, *rlmA*, and *Answi4*/*Answi6* (orthologues of *SWI4*/*SWI6*, which encodes the Mpk1p-activating transcription factor Swi4p–Swi6p complex in *S. cerevisiae*): *mpkAΔ*,

rlmAΔ, *Answi4Δ*, and *Answi6Δ* strains (Fujioka et al. 2007). The transcriptional regulation of cell wall-related genes and *mpkA* via CWI signaling was investigated in the disruptants under cell wall stress induced by micafungin, a β-1,3-glucan synthase inhibitor. The transcription of most cell wall-related genes except two α-1, 3-glucan synthase genes (*agsA* and *agsB*) is transiently upregulated by micafungin treatment, but this action is independent of MpkA, RlmA, and AnSwi4-AnSwi6, suggesting that transcription of the β-1,3-glucan synthase gene *fksA* and several chitin synthase genes (*chsA–chsD*, *csmA*, and *csmB*) is regulated by non-MpkA signaling (Fig. 13.2). Transcription of *agsB*, which encodes a major α-1,3-glucan synthase, depends mainly on MpkA–RlmA signaling (Fig. 13.2) (Fujioka et al. 2007). The *agsA* gene is scarcely transcribed in *A. nidulans* wild-type strains, but its transcription is weakly upregulated in the *mpkAΔ* and *rlmAΔ* strains (Fig. 13.2). Fujioka et al. (2007) further reported that the GUS reporter gene controlled by the *mpkA* promoter was expressed in the wild-type and *rlmAΔ* strains but not in the *mpkAΔ* strain, suggesting that *mpkA* transcription is autoregulated by CWI signaling via MpkA but is independent of RlmA and AnSwi4-AnSwi6. In contrast to the prominent roles of Rlm1p and Swi4p-Swi6p in the maintenance of CWI in *S. cerevisiae*, neither RlmA nor AnSwi4-AnSwi6 in *A. nidulans* is a major transcription factor that controls the expression of *mpkA* or most cell wall-related genes (except the α-1,3-glucan synthase genes *agsA* and *agsB*) as the target of MpkA, and expression of *mpkA* is autoregulated by CWI signaling via an unknown transcription factor that is the target of MpkA. The transcriptional regulation of most genes involved in the biosynthesis of β-1,3-glucan and chitin seems to be regulated by an unknown signaling pathway that is activated by cell wall stresses (e.g., treatment with micafungin) rather than CWI signaling via MpkA in *A. nidulans* (Fujioka et al. 2007).

In *Aspergillus niger*, the genes that encode glutamine:fructose-6-phosphate amidotransferase (*gfaA*) and α-1,3-glucan synthase (*agsA*) are induced in response to stress at the cell wall (Damveld et al. 2005a; Ram et al. 2004). In silico analysis of the promoter region of the two genes revealed the presence of putative DNA-binding sites targeted by the transcription factors RlmA and MsnA that are orthologues of the stress-responsive transcription factors Rlm1p and Msn2p/Msn4p in *S. cerevisiae*. Promoter analysis using a GUS reporter indicated that induction of *agsA* in response to stress at the cell wall depends fully on a single putative RlmA-binding site in its promoter region (Damveld et al. 2005a). Deletion of the *rlmA* gene in *A. niger* eliminates the induction of *agsA* and results in reduced induction of *gfaA* during cell wall stress induced by CFW. The increase in cell wall chitin content in the presence of CFW is also affected in the *rlmA* deletion strain. In addition, the deletion strain is more sensitive to agents that induce cell wall stress. The results indicate that *A. niger* responds to cell wall stress by transcriptional activation of cell wall-reinforcing genes, including *agsA* and *gfaA*, by RlmA.

In *A. oryzae*, understanding of the CWI signaling pathway has been advanced by a functional study of the *kexB* gene encoding a subtilisin-like processing protease KexB that is homologous to *S. cerevisiae* Kex2p (Mizutani et al. 2004). The *kexB* disruptant (Δ*kexB*) forms shrunken colonies with poor generation of conidia on Czapek–Dox (CD) agar plates and hyperbranched mycelia in CD liquid medium.

The phenotypes of the Δ*kexB* strain are restored under high-osmolality conditions in both solid and liquid culture. Gene expression profiles of the Δ*kexB* and wild-type strains were analyzed by using *A. oryzae* cDNA microarrays (Mizutani et al. 2004). Transcription levels of the *mpkA* gene, which encodes a putative MAPK involved in the CWI signaling pathway, is significantly higher in Δ*kexB* cells than in wild-type cells. Constitutively higher levels of phosphorylated MpkA are also observed in Δ*kexB* cells in CD plate culture. High osmotic stress remarkably downregulates the level of *mpkA* transcripts and the phosphorylated form of MpkA in Δ*kexB* cells, concomitantly suppressing the aforementioned morphological defects (Mizutani et al. 2004). The Δ*kexB* cells also contain higher levels of transcripts for cell wall-related genes that encode β-1,3-glucan synthase, β-1, 3-glucanosyltransferases, and chitin synthases. Taken together, these results suggest that KexB is required to maintain normal cell wall structure or integrity, and that the KexB defect induces disordered CWI signaling. To confirm whether the higher levels of transcripts of cell wall-related genes in *A. oryzae* Δ*kexB* cells depend on MpkA or non-MpkA signaling, it is further necessary to construct an *A. oryzae kexB*Δ *mpkA*Δ strain.

13.2.5 Targets of CWI Signaling in Aspergillus *Species*

Because filamentous fungi, including aspergilli, seem to use the MpkA MAPK pathway mainly to regulate the transcription of α-1,3-glucan synthase genes (Fujioka et al. 2007), the biological functions of α-1,3-glucan have been investigated. Originally, the importance of cell wall α-1,3-glucan relative to fungal virulence has been studied in several human pathogenic fungi, such as *Blastomyces dermatitidis*, *Cryptococcus neoformans*, and *Histoplasma capsulatum* (Hogan and Klein 1994; Reese and Doering 2003; Rappleye and Goldman 2006), and the plant pathogenic fungus *Magnaporthe grisea* (Fujikawa et al. 2009). To reveal biological functions of α-1,3-glucan in the *Aspergillus* species, α-1,3-glucan synthase genes have been characterized (Beauvais et al. 2005; Damveld et al. 2005b; Maubon et al. 2006). *A. fumigatus* contains three AGS genes, *ags1* to *ags3* (Fig. 13.3). *A. fumigatus ags1*, which is an orthologue of *A. nidulans agsB* (Fig. 13.3), is involved in the formation of 50 % of the cell wall α-1,3-glucan, whereas disruption of *ags2*, which is an orthologue of *agsA* (Fig. 13.3), had no detectable effect on glucan levels (Beauvais et al. 2005). Disruption of the third gene, *ags3*, which has no orthologue in *A. nidulans* (Fig. 13.3), results in the overexpression of *ags1*, which may serve to compensate for the lost enzyme activity and maintain normal cell wall composition (Maubon et al. 2006). In addition, the disruption of *ags3* in *A. fumigatus* causes hypervirulence, whereas the disruption of *ags1* and *ags2* did not affect virulence (Beauvais et al. 2005; Maubon et al. 2006). Furthermore, a triple-mutant strain of *A. fumigatus* lacking the three α-1,3-glucan synthase genes (*ags1*, *ags2*, and *ags3*) was generated, and the growth of the triple mutant in plate culture was similar to that of the parental strain (Henry et al. 2011). The triple mutant showed slightly

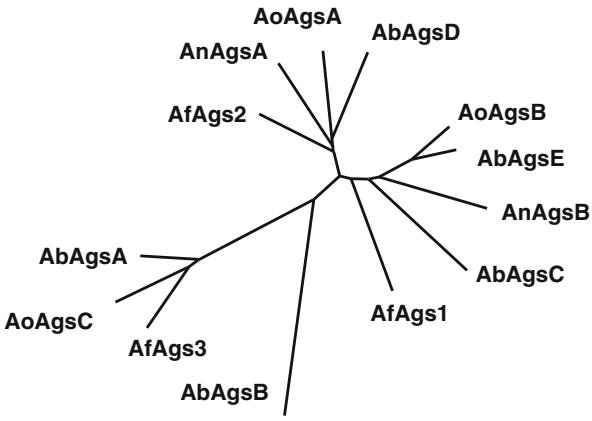

Fig. 13.3 Phylogenetic tree of the **α**-1,3-glucan synthases in *Aspergillus* species. The tree was constructed using the neighbor-joining method based on the alignment of the amino acid sequences. *An A. nidulans*, *Ao A. oryzae*, *Af A. fumigatus*, *Ab A. niger*

decreased conidiogenesis, as did the single *ags1* and *ags2* mutants (Beauvais et al. 2005; Henry et al. 2011), and the lack of cell wall α-1,3-glucan led to an increase in β-1,3-glucan and chitin levels in mycelia of the triple mutant (Henry et al. 2011). *A. niger* has five α-1,3-glucan synthases encoded by *agsA* to *agsE* (Fig. 13.3). The expression of *agsA* (an orthologue of *A. fumigatus ags3*) and *agsE* (an orthologue of *A. fumigatus ags1* and *A. nidulans agsB*) was induced in the presence of cell wall stress-inducing compounds such as CFW, sodium dodecyl sulfate, and caspofungin (Damveld et al. 2005b). In *A. nidulans*, several mutants for the α-1,3-glucan synthase genes *agsA* and *agsB* were constructed (Yoshimi et al. 2013). The *agsA* disruption strains did not show markedly different phenotypes from those of the wild-type strain. The *agsB* disruption strains and the double-disruption strains showed increased sensitivity to CR and lysing enzymes (Yoshimi et al. 2013). In addition, the *agsB* disruption strains formed dispersed hyphal cells under liquid culture conditions regardless of the *agsA* genetic background (Yoshimi et al. 2013). Biochemical analysis of the cell wall polysaccharides revealed that the disruption of *agsB* led to almost complete loss of cell wall α-1,3-glucan, which was mainly composed of linear α-1,3-glucan with a structure that is similar to that of mutan, a biofilm component that is produced by the oral bacterium *Streptococcus mutans* with its glucanosyltransferase reaction (Yoshimi et al. 2013). Recently, He et al. (2014) demonstrated that *agsA* was mainly expressed during conidiation, and the *agsB* disruptant showed increased sensitivity to CFW but not to CR in *A. nidulans*. In contrast to the results of He et al., Yoshimi et al. (2013) reported that the *agsB* disruption strain showed increased sensitivity to CR, but not to CFW, and the amount of CR adsorption to the hyphae of the *agsB* disruptant strain was significantly greater than that of the wild-type strain. Both CR and CFW interact with various polysaccharides, although β-1,3-glucan shows a strong interaction with CR but a weak interaction with CFW. In addition, the amount of CR adsorbed to α-1,3-glucan is significantly less than the amount adsorbed to β-1,3-glucan or chitin (Yoshimi et al. 2013). It is reasonable to hypothesize that the loss of α-1,3-glucan from the cell wall

led to increased exposure of β-1,3-glucan on the cell surface and the resulting increased sensitivity to CR. The differences between the results of the two research groups in relation to the sensitivity to CR and CFW might be related to the fact that the two groups used different parental *A. nidulans* strains. These observations indicate that α-1,3-glucan is involved not only in fungal virulence but also in multiple functions in cell wall biogenesis, such as the maintenance of normal growth characteristics and protection against a certain cell wall stress in *Aspergillus* species.

13.3 Osmotic Stress Signaling in *Aspergillus* Species

13.3.1 Overview of the High Osmolality Glycerol (HOG) Pathway

In solid-state cultivation, which is used in the fermentation industry, *A. oryzae* grows on solid substrates including steamed rice grain, roasted wheat grain, and steamed ground soybean (Abe and Gomi 2007). These conditions make the organism produce a large amount of enzymes that hydrolyze starch and proteins into sugars and peptides/amino acids, respectively. Thus, fungi are thought to be exposed to elevated osmotic stress in a microenvironment during solid-state cultivation.

Some fungi are known to synthesize glycerol as a main osmolyte to adapt to the surrounding hyperosmotic environment. In *S. cerevisiae*, the production of glycerol is initiated by the conversion of dihydroxyacetone phosphate through a two-step reaction of glycerol-3-phophate dehydrogenase (Gpd1p, Gpd2p) and glycerol-3-phosphatase (Gpp1p, Gpp2p) (Albertyn, et al. 1994; Norbeck et al. 1996). The expression of *GPD1* and *GPP2* and subsequent production of glycerol are under control of the Hog1p MAPK cascade that consists of the MAPK kinase kinases Ssk2p/Ssk22p, MAPK kinase Pbs2p, and MAPK Hog1p (Hohmann 2002). The Hog1p MAPK cascade is regulated by two different upstream branches, the Sho1p shunt and a two-component signaling (TCS) system, and is activated via these pathways in response to hyperosmotic stresses (Fig. 13.4) (Maeda et al. 1994, 1995; Posas et al. 1996). The *S. cerevisiae* TCS system has one membrane-anchored histidine kinase, Sln1p, which seems to act as a sensor for environmental osmotic conditions. In hypo-osmotic conditions, Sln1p is phosphorylated and, in turn, a phospho group is relayed from Sln1p to the downstream response regulator (RR), Ssk1p, via an intermediate protein Ypd1p (Fig. 13.4). The phosphorylated form of Ssk1p inactivates the downstream Hog1p MAPK cascade (Posas et al. 1996; Posas and Saito 1998). In hyperosmotic stress conditions, Sln1p shows phosphatase activity and thus deprives the phospho group from Ypd1p-Ssk1p components. The dephosphorylated form of Ssk1p activates the Hog1p MAPK cascade, which in turn facilitates glycerol biosynthesis for osmotic adaptation.

In *Aspergillus* species, counterparts of the Hog1p MAPK cascade and TCS system have been extensively studied. We provide up-to-date findings of the aspergilli HOG pathway in the following sections.

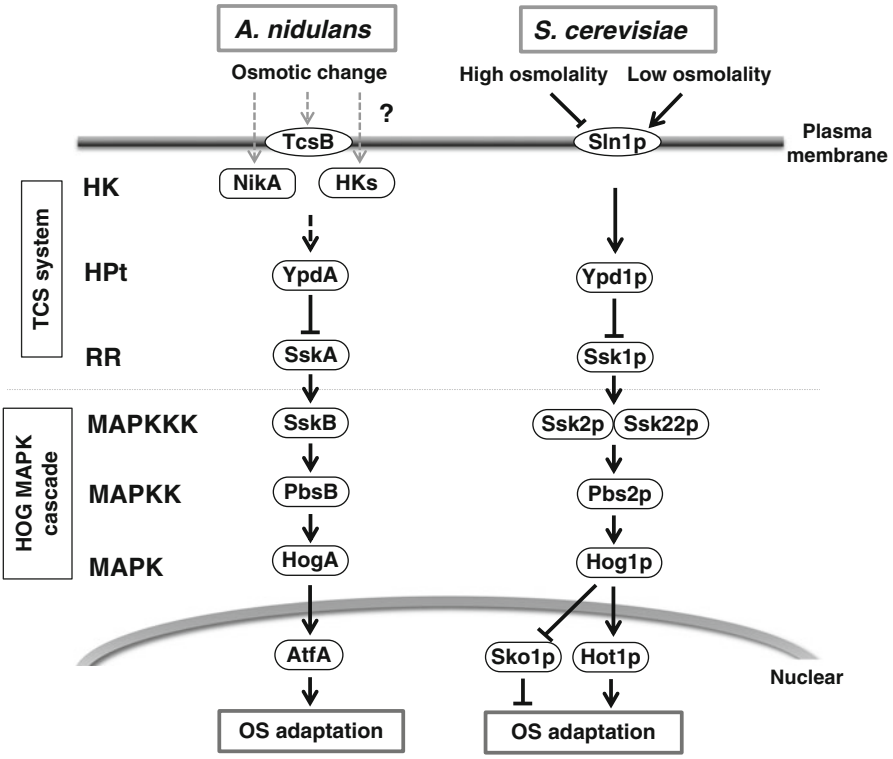

Fig. 13.4 Schematic model of osmotic stress signaling in *A. nidulans*. Osmotic stress signaling [the high-osmolality glycerol (HOG) pathway] involves the two-component system (TCS) and HOG mitogen-activated protein kinase (MAPK) cascade in *A. nidulans*, which corresponds to the well-studied *Saccharomyces cerevisiae* (*S. cerevisiae*) HOG pathway. *OS* osmotic stress

13.3.2 TCS System for Osmotic Stress Signaling in Aspergillus *Species*

The TCS (also known as His-Asp phospho-relay signaling) system, which was first described in bacteria, is a common signal transduction mechanism found in organisms ranging from bacteria to fungi and higher plants, but it is not in animals (Mizuno 1998). The eukaryotic TCS system consists of three types of common signal transducers, a histidine kinase (HK), a histidine-containing phospho transmitter (HPt), and an RR, resulting in a multistep phospho-relay signal. Each component has an invariant amino acid residue, His or Asp, within a conserved motif, and a phospho group is transferred (relayed) from His to Asp or Asp to His in response to external stimuli (Appleby et al. 1996).

BLAST of the genome sequences of *A. nidulans*, *A. oryzae*, and *A. fumigatus* revealed that 13 to 15 HKs, 3 RRs, and 1 HPt were found in their genomes.

Considering that the model yeasts *S. cerevisiae*, *Schizosaccharomyces pombe*, and *Candida albicans* have 1, 3, and 3 HKs, respectively, the larger number of HKs found in the aspergilli genomes is in sharp contrast to the small number of HKs in the yeasts (Kobayashi et al. 2007). This divergence suggests that signaling networks might be more complex in filamentous fungi.

Of the multiple *Aspergillus* HKs, TcsB is an orthologue of *S. cerevisiae* Sln1p and was thought to have a crucial role in the osmotic stress response. In contrast to expectation, the *tcsB* deletion mutant of *A. nidulans* and *A. fumigatus* does not exhibit any detectable phenotypic defects on osmotic stress medium, and *tcsB* is not required for the phosphorylation of the HogA/SakA MAPK, which is a counterpart of Hog1p, in response to osmotic stress (Furukawa et al. 2002, 2005; Du et al. 2006). In a recent report, *A. fumigatus* TcsB is involved in the phosphorylation of the SakA MAPK in response to a cold shock stress, and it is required for growth under high-temperature conditions (Ji et al. 2012). Although the involvement of TcsB in certain stress responses has been reported, a sensor for osmotic conditions, which functions in the HOG pathway, is yet to be identified.

Among filamentous fungi, NikA is a widely conserved HK that has a characteristic motif, a repeated HAMP domain, in its N-terminus. Although the function of the HAMP domain has remained elusive so far, some reports suggested a potential role of the domain in the perception of osmotic conditions (Meena et al. 2010; El-Mowafy et al. 2013). Indeed, in both *A. nidulans* and *A. fumigatus*, disruption of the *nikA* gene results in a growth defect on plate medium containing high osmolality stress (Hagiwara et al. 2009b, 2013). This result raised the hypothesis that, instead of TcsB, NikA protein might regulate the HOG pathway in response and in adaptation to osmotic conditions. However, phosphorylation of the SakA MAPK in response to osmotic stress occurs irrespective of NikA in *A. fumigatus*, whereas the SskA RR is indispensable for the phosphorylation of SakA (Hagiwara et al. 2013). This finding suggests that the other HKs may contribute to the response and adaptation to the osmotic changes through the HOG pathway in the fungus.

13.3.3 HogA/SakA MAPK Cascade in the Osmotic Stress Response in Aspergillus *Species*

With regard to osmotic stress signaling, the HogA/SakA MAPK cascade plays a central role among aspergilli and other filamentous fungi (Bahn 2008). *A. nidulans* possesses the SskB MAPK kinase kinase, the PbsB MAPK kinase, and the HogA MAPK in the HogA MAPK cascade (Fig. 13.4). The *sskB*, *pbsB*, and *hogA* deletion mutants show growth inhibition under high osmolality. In response to osmotic shock, the HogA MAPK is phosphorylated in an SskA RR-, SskB-, and PbsB-dependent manner (Furukawa et al. 2005). Along with *A. nidulans*, the *A. fumigatus* SakA MAPK cascade is composed of the SskB MAPK kinase kinase, the PbsB MAPK kinase, and the SakA MAPK. The *sakA* and *pbsB* deletion mutants show

retarded growth under high osmolality conditions (Hagiwara et al. 2013, unpublished data). SakA is phosphorylated in response to osmotic shock in an SskA RR-dependent manner (Hagiwara et al. 2013). Importantly, these studies on two *Aspergillus* HOG pathways indicated that the activation of the HogA/SakA MAPK cascade is exclusively dependent on the TCS system (at least the SskA RR, although the responsible HK is undetermined). In contrast to *S. cerevisiae*, the ShoA (counterpart of Sho1p) shunt is not responsible for the regulation of the HogA/SakA MAPK cascade in *Aspergillus* species. This view is supported by the fact that *A. nidulans* PbsB protein lacks the Pro-rich motif that is required for binding to the Src-homology 3 domain of Sho1p (Furukawa et al. 2005). Taken together, osmotic stress signaling in the HOG pathway involves the HogA/SakA MAPK cascade following the TCS system in aspergilli.

Transcriptome analysis in response to osmotic shock was conducted in *A. nidulans* using a DNA microarray (Hagiwara et al. 2009a). The study identified 181 and 85 genes as osmotic stress-upregulated (>2 times) genes and osmotic stress-downregulated (<0.5 times) genes, respectively. Of those, 123 genes (68 %) and 22 genes (26 %) were dependent on HogA, respectively, which revealed that the HOG pathway is a central regulator for environmental osmolality changes. In the global expression study, the expression profile in response to a treatment of fludioxonil, a filamentous fungi-specific fungicide widely used in agricultural applications, was also investigated. A mode of action of fludioxonil had been thought to be the HOG pathway from studies on several filamentous fungi. Indeed, transcriptome data indicated that treatment with fludioxonil activates the *Aspergillus* HOG pathway, and a large number of genes were expressed differentially in a HogA-dependent manner. Notably, the genes that were differentially expressed largely overlapped with those that were differentially expressed with fludioxonil treatment or osmotic shock, suggesting that the HOG pathway is shared by the fludioxonil and osmotic stress responses in the fungus.

13.3.4 Targets of the HOG Pathway in Aspergillus *Species*

The *Aspergillus* HOG pathway is activated in osmotic stress and is involved in growth under high osmolality conditions, which at least partly shows how the cells perceive environmental osmolality changes. The next question is the adaptive mechanism of how cells adapt to the extracellular osmotic conditions. As stated, *S. cerevisiae* accumulates glycerol as an osmolyte in the cell when treated with a high concentration of sodium chloride as osmotic stress, which serves as osmotic adaptation. In a similar fashion, *A. fumigatus* accumulates a marked amount of glycerol in hyphae in response to osmotic stress treatment, and the glycerol de novo biosynthesis is dependent on the SakA MAPK cascade (Hagiwara et al., unpublished data). It appears that the HOG pathway functions in osmotic adaptation by glycerol accumulation as an osmolyte in the fungus.

A. nidulans has homologues for the glycerol biosynthesis-related genes GfdA and GfdB (presumed glycerol-3-phophate dehydrogenases) and GppA (a presumed

glycerol-3-phosphatase) (Miskei et al. 2009). Although the inactivation of *gfdA* results in decreased glycerol accumulation during growth under osmotic stress conditions, the mutant does not show an apparent sensitivity to high osmolality stress (Fillinger et al. 2001). Another *A. nidulans* GPD homologue is GfdB, the expression of which is increased in response to osmotic shock in a HogA MAPK cascade-dependent manner, but the involvement of the protein in osmotic adaptation remains to be determined (Furukawa et al. 2007).

Transcription factors that function downstream of the HOG pathway in aspergilli have not been identified except AtfA, which is an orthologue of *S. pombe* Atf1. From the transcriptome comparison analysis already stated here, AtfA was found to function in the *A. nidulans* HOG pathway (Hagiwara et al. 2009a). Notably, the upregulation of *gfdB* and other genes that are under the control of HogA MAPK are largely dependent on AtfA. The deletion mutant of *atfA* shows growth retardation on plates containing high osmolality stress. From these results, the TCS system-HogA MAPK cascade-AtfA transcription factor signaling pathway has a crucial role in the osmotic stress response and adaptation in *A. nidulans* (Fig. 13.4).

The inferred osmotic stress signaling pathway also is crucial in stress tolerance in conidia of *Aspergillus* species. Sakamoto et al. (2009) reported that conidia from an *A. oryzae atfA* deletion mutant showed a decreased germination rate and a higher sensitivity to oxidative stress. Indeed, the expression of a conidia-specific catalase gene, *catA*, and trehalose content were lower in conidia of the *atfA* mutant than the control strain. The decreased conidia viability was also observed in *atfA* mutants of *A. nidulans* and *A. fumigatus* (Hagiwara et al. 2008, unpublished data). Importantly, keeping conidia alive is a key technology enabling the cultivation and distribution of fungi in fermentation industries. Therefore, the osmotic stress signaling pathway appears to contribute to applications of industrially important *Aspergillus* species.

13.4 Concluding Remarks

In this decade, studies of stress responses and adaptation mechanisms in filamentous fungi have been greatly advanced by sequenced genome information and the development of genome-wide analysis tools. In this chapter, we described two major stress response signaling systems that seem to be crucial in solid-state fermentation processes. The CWI and osmotic stress signaling systems of aspergilli are largely similar to those of *S. cerevisiae*, whereas some components are missing or added in the pathways (i.e., the Sho1 shunt is missing in osmotic stress signaling; *ags* genes and the NikA HK are added in the CWI and osmotic stress signaling pathways, respectively). *Aspergillus* fungi evolutionally must have remediated the stress response mechanisms in adapting to their harsh environmental niches. Because fungal hyphae invade the substrates in solid-state fermentation, this condition somehow mimics the process of infection of pathogenic fungi for plants and animals. Therefore, further studies of solid-state cultivation and the fungal infection process may mutually accelerate the understanding of stress biology in filamentous fungi.

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References

Abe K, Gomi K (2007) Food products fermented by *Aspergillus oryzae*. In: Osmani SA, Goldman GH (eds) The Aspergilli: genomics, medical applications, biotechnology, and research methods. CRC, Boca Raton, pp 428–438

Albertyn J, Hohmann S, Thevelein JM et al (1994) *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. Mol Cell Biol 14:4135–4144

Appleby JL, Parkinson JS, Bourret RB (1996) Signal transduction via the multi-step phosphorelay: not necessarily a road less traveled. Cell 86:845–848

Arpaia G, Cerri F, Baima S et al (1999) Involvement of protein kinase C in the response of *Neurospora crassa* to blue light. Mol Gen Genet 262:314–322

Bahn YS (2008) Master and commander in fungal pathogens: the two-component system and the HOG signaling pathway. Eukaryot Cell 7:2017–2036

Beauvais A, Maubon D, Park S et al (2005) Two α(1-3) glucan synthases with different functions in *Aspergillus fumigatus*. Appl Environ Microbiol 71:1531–1538

Bussink HJ, Osmani SA (1999) A mitogen-activated protein kinase (MPKA) is involved in polarized growth in the filamentous fungus, *Aspergillus nidulans*. FEMS Microbiol Lett 173:117–125

Colabardini AC, De Castro PA, De Gouvêa PF et al (2010) Involvement of the *Aspergillus nidulans* protein kinase C with farnesol tolerance is related to the unfolded protein response. Mol Microbiol 78:1259–1279

Damveld RA, Arentshorst M, Franken A et al (2005a) The *Aspergillus niger* MADS-box transcription factor RlmA is required for cell wall reinforcement in response to cell wall stress. Mol Microbiol 58:305–319

Damveld RA, vanKuyk PA, Arentshorst M et al (2005b) Expression of *agsA*, one of five 1,3-α-D-glucan synthase-encoding genes in *Aspergillus niger*, is induced in response to cell wall stress. Fungal Genet Biol 42:165–177

Dichtl K, Helmschrott C, Dirr F et al (2012) Deciphering cell wall integrity signalling in *Aspergillus fumigatus*: identification and functional characterization of cell wall stress sensors and relevant Rho GTPases. Mol Microbiol 83:506–519

Du C, Sarfati J, Latge JP et al (2006) The role of the *sakA* (Hog1) and *tcsB* (sln1) genes in the oxidant adaptation of *Aspergillus fumigatus*. Med Mycol 44:211–218

El-Mowafy M, Bahgat MM, Bilitewski U (2013) Deletion of the HAMP domains from the histidine kinase CaNik1p of *Candida albicans* or treatment with fungicides activates the MAP kinase Hog1p in *S. cerevisiae* transformants. BMC Microbiol 13:209

Fillinger S, Ruijter G, Tamás MJ et al (2001) Molecular and physiological characterization of the NAD-dependent glycerol 3-phosphate dehydrogenase in the filamentous fungus *Aspergillus nidulans*. Mol Microbiol 39:145–157

Fontaine T, Simenel C, Dubreucq G et al (2000) Molecular organization of the alkali-insoluble fraction of *Aspergillus fumigatus* cell wall. J Biol Chem 275:27594–27607

Franchi L, Fulci V, Macino G (2005) Protein kinase C modulates light responses in *Neurospora* by regulating the blue light photoreceptor WC-1. Mol Microbiol 56:334–345

Fujikawa T, Kuga Y, Yano S et al (2009) Dynamics of cell wall components of *Magnaporthe grisea* during infectious structure development. Mol Microbiol 73:553–570

Fujioka T, Mizutani O, Furukawa K et al (2007) MpkA-dependent and -independent cell wall integrity signaling in *Aspergillus nidulans*. Eukaryot Cell 6:1497–1510

Furukawa K, Katsuno Y, Urao T et al (2002) Isolation and functional analysis of a gene, *tcsB*, encoding a transmembrane hybrid-type histidine kinase from *Aspergillus nidulans*. Appl Environ Microbiol 68:5304–5310

Furukawa K, Hoshi Y, Maeda T et al (2005) *Aspergillus nidulans* HOG pathway is activated only by two-component signalling pathway in response to osmotic stress. Mol Microbiol 56:1246–1261

Furukawa K, Yoshimi A, Furukawa T et al (2007) Novel reporter gene expression systems for monitoring activation of the *Aspergillus nidulans* HOG pathway. Biosci Biotechnol Biochem 71:1724–1730

Futagami T, Goto M (2012) Putative cell wall integrity sensor proteins in *Aspergillus nidulans*. Commun Integr Biol 5:206–208

Futagami T, Nakao S, Kido Y et al (2011) Putative stress sensors WscA and WscB are involved in hypo-osmotic and acidic pH stress tolerance in *Aspergillus nidulans*. Eukaryot Cell 10:1504–1515

Galagan JE, Calvo SE, Cuomo C et al (2005) Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. Nature 438:1105–1115

Goto M (2007) Protein *O*-glycosylation in fungi: diverse structures and multiple functions. Biosci Biotechnol Biochem 71:1415–1427

Goto M, Harada Y, Oka T et al (2009) Protein *O*-mannosyltransferases B and C support hyphal development and differentiation in *Aspergillus nidulans*. Eukaryot Cell 8:1465–1474

Hagiwara D, Asano Y, Yamashino T et al (2008) Characterization of bZip-type transcription factor AtfA with reference to stress responses of conidia of *Aspergillus nidulans*. Biosci Biotechnol Biochem 72:2756–2760

Hagiwara D, Asano Y, Marui J et al (2009a) Transcriptional profiling for *Aspergillus nidulans* HogA MAPK signaling pathway in response to fludioxonil and osmotic stress. Fungal Genet Biol 46:868–878

Hagiwara D, Mizuno T, Abe K (2009b) Characterization of NikA histidine kinase and two response regulators with special reference to osmotic adaptation and asexual development in *Aspergillus nidulans*. Biosci Biotechnol Biochem 73:1566–1571

Hagiwara D, Takahashi-Nakaguchi A, Toyotome T et al (2013) NikA/TcsC histidine kinase is involved in conidiation, hyphal morphology, and responses to osmotic stress and antifungal chemicals in *Aspergillus fumigatus*. PLoS One 8:e80881

He X, Li S, Kaminskyj SGW (2014) Characterization of *Aspergillus nidulans* α-glucan synthesis: roles for two synthases and two amylases. Mol Microbiol 91:579–595

Heinisch JJ, Lorberg A, Schmitz HP et al (1999) The protein kinase C-mediated MAP kinase pathway involved in the maintenance of cellular integrity in *Saccharomyces cerevisiae*. Mol Microbiol 32:671–680

Henry C, Latgé JP, Beauvais A (2011) α1,3-Glucans are dispensable in *Aspergillus fumigatus*. Eukaryot Cell 11:26–29

Herrmann M, Sprote P, Brakhage AA (2006) Protein kinase C (PkcA) of *Aspergillus nidulans* is involved in penicillin production. Appl Environ Microbiol 72:2957–2970

Hogan LH, Klein BS (1994) Altered expression of surface α-1,3-glucan in genetically related strains of *Blastomyces dermatitidis* that differ in virulence. Infect Immun 62:3543–3546

Hohmann S (2002) Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev 66:300–372

Hong SB, Lee M, Kim DH et al (2013) *Aspergillus luchuensis*, an industrially important black *Aspergillus* in East Asia. PLoS One 8:e63769

Ichinomiya M, Uchida H, Koshi Y et al (2007) A protein kinase C-encoding gene, pkcA, is essential to the viability of the filamentous fungus *Aspergillus nidulans*. Biosci Biotechnol Biochem 71:2787–2799

Irie K, Takase M, Lee KS et al (1993) MKK1 and MKK2, which encode *Saccharomyces cerevisiae* mitogen-activated protein kinase kinase homologs, function in the pathway mediated by protein kinase C. Mol Cell Biol 13:3076–3083

Jain R, Valiante V, Remme N et al (2011) The MAP kinase MpkA controls cell wall integrity, oxidative stress response, gliotoxin production and iron adaptation in *Aspergillus fumigatus*. Mol Microbiol 82:39–53

Ji Y, Yang F, Ma D et al (2012) HOG-MAPK signaling regulates the adaptive responses of *Aspergillus fumigatus* to thermal stress and other related stress. Mycopathologia 174:273–282

Jung US, Levin DE (1999) Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signalling pathway. Mol Microbiol 34:1049–1057

Kamada Y, Qadota H, Python CP et al (1996) Activation of yeast protein kinase C by Rho1 GTPase. J Biol Chem 271:9193–9196

Kapteyn JC, Van Den Ende H, Klis FM (1999) The contribution of cell wall proteins to the organization of the yeast cell wall. Biochim Biophys Acta 1426:373–383

Katayama T, Uchida H, Ohta A et al (2012) Involvement of protein kinase C in the suppression of apoptosis and in polarity establishment in *Aspergillus nidulans* under conditions of heat stress. PLoS One 7:e50503

Ketela T, Green R, Bussey H (1999) *Saccharomyces cerevisiae* Mid2p is a potential cell wall stress sensor and upstream activator of the PKC1–MPK1 cell integrity pathway. J Bacteriol 181:3330–3340

Klis FM (1994) Review: Cell wall assembly in yeast. Yeast 10:851–869

Kobayashi T, Abe K, Asai K et al (2007) Genomics of *Aspergillus oryzae*. Biosci Biotechnol Biochem 71:646–670

Kriangkripipat T, Momany M (2009) *Aspergillus nidulans* protein *O*-mannosyltransferases play roles in cell wall integrity and developmental patterning. Eukaryot Cell 8:1475–1485

Latgé JP (2007) The cell wall: a carbohydrate armour for the fungal cell. Mol Microbiol 66:279–290

Latgé JP (2010) Tasting the fungal cell wall. Cell Microbiol 12:863–872

Lee KS, Levin DE (1992) Dominant mutations in a gene encoding a putative protein kinase (*BCK1*) bypass the requirement for a *Saccharomyces cerevisiae* protein kinase C homolog. Mol Cell Biol 12:172–182

Levin DE (2005) Cell wall integrity signaling in *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 69:262–291

Levin DE (2011) Regulation of cell wall biogenesis in *Saccharomyces cerevisiae*: the cell wall integrity signaling pathway. Genetics 189:1145–1175

Levin DE, Fields FO, Kunisawa R et al (1990) A candidate protein kinase C gene, PKC1, is required for the *S. cerevisiae* cell cycle. Cell 62:213–224

Lodder AL, Lee TK, Ballester R (1999) Characterization of the Wsc1 protein, a putative receptor in the stress response of *Saccharomyces cerevisiae*. Genetics 152:1487–1499

Machida M, Asai K, Sano M et al (2005) Genome sequencing and analysis of *Aspergillus oryzae*. Nature 438:1157–1161

Machida M, Yamada O, Gomi K (2008) Genomics of *Aspergillus oryzae*: learning from the history of Koji mold and exploration of its future. DNA Res 15:173–183

Maeda T, Wurgler-Murphy SM, Saito H (1994) A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature 369:242–245

Maeda T, Takekawa M, Saito H (1995) Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. Science 269:554–558

Martin H, Arroyo J, Sanchez M et al (1993) Activity of the yeast MAP kinase homologue Slt2 is critically required for cell integrity at 37 degrees C. Mol Gen Genet 241:177–184

Maubon D, Park S, Taugy M et al (2006) *AGS3*, an α(1-3)glucan synthase gene family member of *Aspergillus fumigatus*, modulates mycelium growth in the lung of experimentally infected mice. Fungal Genet Biol 43:366–375

Meena N, Kaur H, Mondal AK (2010) Interactions among HAMP domain repeats act as an osmosensing molecular switch in group III hybrid histidine kinases from fungi. J Biol Chem 285:12121–12132

Miskei M, Karányi Z, Pócsi I (2009) Annotation of stress-response proteins in the aspergilli. Fungal Genet Biol 46(sSuppl 1):S105–S120

Mizuno T (1998) His-Asp phosphotransfer signal transduction. J Biochem 123:555–563

Mizutani O, Nojima A, Yamamoto M et al (2004) Disordered cell integrity signaling caused by disruption of the *kexB* gene in *Aspergillus oryzae*. Eukaryot Cell 3:1036–1048

Nierman WC, Pain A, Anderson MJ et al (2005) Genome sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. Nature 438:1151–1156

Norbeck J, Pahlman AK, Akhtar N et al (1996) Purification and characterization of two isoenzymes of DL-glycerol-3-phosphatase from *Saccharomyces cerevisiae*. Identification of the corresponding GPP1 and GPP2 genes and evidence for osmotic regulation of Gpp2p expression by the osmosensing mitogen-activated protein kinase signal transduction pathway. J Biol Chem 271:13875–13881

Oda K, Kakizono D, Yamada O et al (2006) Proteomic analysis of extracellular proteins from *Aspergillus oryzae* grown under submerged and solid-state culture conditions. Appl Environ Microbiol 72:3448–3457

Oka T, Hamaguchi T, Sameshima Y et al (2004) Molecular characterization of protein *O*-mannosyltransferase and its involvement in cell-wall synthesis in *Aspergillus nidulans*. Microbiology 150:1973–1982

Pel HJ, de Winde JH, Archer DB et al (2007) Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nat Biotechnol 25:221–231

Philip B, Levin DE (2001) Wsc1 and Mid2 are cell surface sensors for cell wall integrity signaling that act through Rom2, a guanine nucleotide exchange factor for Rho1. Mol Cell Biol 21:271–280

Posas F, Saito H (1998) Activation of the yeast SSK2 MAP kinase kinase kinase by the SSK1 two-component response regulator. EMBO J 17:1385–1394

Posas F, Wurgler-Murphy SM, Maeda T et al (1996) Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 "two-component" osmosensor. Cell 86:865–875

Rajavel M, Philip B, Buehrer BM et al (1999) Mid2 is a putative sensor for cell integrity signaling in *Saccharomyces cerevisiae*. Mol Cell Biol 19:3969–3976

Ram AF, Arentshorst M, Damveld RA et al (2004) The cell wall stress response in *Aspergillus niger* involves increased expression of the glutamine: fructose-6-phosphate amidotransferase-encoding gene (*gfaA*) and increased deposition of chitin in the cell wall. Microbiology 150:3315–3326

Rappleye CA, Goldman WE (2006) Defining virulence genes in the dimorphic fungi. Annu Rev Microbiol 60:281–303

Reese AJ, Doering TL (2003) Cell wall alpha-1,3-glucan is required to anchor the *Cryptococcus neoformans* capsule. Mol Microbiol 50:1401–1409

Rodicio R, Heinisch JJ (2010) Together we are strong—cell wall integrity sensors in yeasts. Yeast 27:531–540

Ronen R, Sharon H, Levdansky E et al (2007) The *Aspergillus nidulans pkcA* gene is involved in polarized growth, morphogenesis and maintenance of cell wall integrity. Curr Genet 51:321–329

Sakamoto K, Iwashita K, Yamada O et al (2009) *Aspergillus oryzae atfA* controls conidial germination and stress tolerance. Fungal Genet Biol 46:887–897

Schmidt A, Bickle M, Beck T et al (1997) The yeast phosphatidylinositol kinase homolog TOR2 activates RHO1 and RHO2 via the exchange factor ROM2. Cell 88:531–542

Teepe AG, Loprete DM, He Z et al (2007) The protein kinase C orthologue PkcA plays a role in cell wall integrity and polarized growth in *Aspergillus nidulans*. Fungal Genet Biol 44:554–562

Verna J, Lodder A, Lee K et al (1997) A family of genes required for maintenance of cell wall integrity and for the stress response in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 94:13804–13809

Yoshimi A, Sano M, Inaba A et al (2013) Functional analysis of the α-1,3-glucan synthase genes *agsA* and *agsB* in *Aspergillus nidulans*: AgsB is the major α-1,3-glucan synthase in this fungus. PLoS One 8:e54893

Yu J, Proctor R, Brown D et al (2003) Genomics of economically significant *Aspergillus* and *Fusarium* species. In: Arora DK, Khachatourians GG (eds) Applied mycology and biotechnology: fungal genomics. Elsevier, Amsterdam, pp 249–283