Studies on the mechanism of organic solvent tolerance of yeast *Saccharomyces cerevisiae* triggered by a transcription factor Pdr1p

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Contents

Abbreviations

4NQO	4-Nitroquinoline 1-oxide
ABC	ATP-binding cassette
CHX	Cyclohexmide
CWI	Cell wall integrity
ChIP	Chromatin immunoprecipitation
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EGFP	Enhanced green fluorescence protein
ER	Endoplasmic reticulum
GAPDH	Glyceraldehyde-3-phosphate-dehydrogenase
IP	Immunoprecipitation
IgG	Immunoglobulin G
KET	Ketoconazole
LB	Luria-Bertani
MAP kinase	Mitogen-activated protein kinase
MDR	Multidrug resistance
NBD	Nucleotide-binding domains
OD	Optical density
PCR	Polymerase chain reaction
PDR	Pleiotropic drug resistance
PDRE	Pleiotropic drug response element
PGAP	GAPDH promoter
SD	Synthetic dextrose
YPD	Yeast extract- peptone-dextrose

Introduction

All living things maintain their internal environments distinct from the external environment for biological activities. However, when the extracellular environment suddenly changes, their internal environments may be perturbed, causing stress to living cells. Even in such severe environments, they recover the internal environment in order to survive and flexibly adapt themselves to the external surroundings. Some cells in the community occasionally have gained mutations that would enhance the stress response. The mutations make them advantaged to adapt to the new environment, resulting in a gain of resistance.

Stress resistance also attracts attention in industries that utilize bioconversions. Currently, some microorganisms are used as whole cell biocatalysts in place of traditional inorganic catalysts. As conditions for bioproduction are often hard for cells to live, cells with stress resistance is expected to promote bioconversion reactions more efficiently.

On the other hand, stress resistance poses problems under certain conditions. One serious problem is that of widespread multidrug resistance (MDR) in pathogenic microbes and cancer cells. Pathogens with MDR pose a serious challenge for therapeutic development. In addition, MDR in pests in agricultures, such as harmful insects and weeds, raises problems.

As above, stress resistance is a kind of double-edged sword. Revealing the mechanisms of stress resistance is important not only to understand the essential mechanisms for living organisms to adapt themselves to environmental challenges, but also in the application for efficient bioproduction, treatments of multidrug pathogens, and extermination of pests in agriculture.

Cellular stresses from the environment

The environment in which organisms live is never maintained the same, and fluctuations in temperature, osmotic levels, and amount of nutrients impose stress to living cells. The living organism may also be exposed to substances toxic to cells, such as oxidants and antibiotics. Therefore, all living cells have several strategies to deal with stress posed by the environment. When they detect stress, they first adjust the transcription levels of stress-related genes, which are followed by reorganized metabolism, strengthened cellular

structures, and protected proteins, among others in order to deal with the stress. However, they cannot live when the fluctuation is above the range that cells can deal with, such as extreme elevation of temperature. Some mutations occasionally boost the stress response, which expands the limit of stress that the cell can manage.

Some organisms called extremophiles can live in harsh environments in which most organisms cannot grow, such as hot springs and deep-sea hydrothermal vent, with extremely high temperature, high or low pH, and high pressure. These extremophiles have specially adapted enzymes, cell structures, or metabolites to live in such environments, e.g., thermotolerant enzymes in hyperthermophilic microorganisms.

The fermentation conditions produce one of the severe environments for microorganisms. In addition to stresses from uncomfortable environment in fermentation process for preventing contamination of other undesirable microorganisms, fermentative microorganisms are exposed to stresses from high concentration of starting materials, solvents, and accumulating products. Therefore, microorganisms utilized for fermentation should gain resistance to those stresses. Yeasts have been long used for the fermentation process in brewing alcohols for several thousand years. As yeasts have high tolerance to alcohols, they are expected to produce ethanol fermentation as biofuels these days. In addition, as the safety of yeasts to humans is certified during the year, yeasts have been expected to produce more useful substances that are difficult to produce in existing chemical reactions such as amino acids and drug precursors.

To apply yeast fermentation to multiple products in purpose, it is necessary for yeasts to elucidate the mechanism of stress response and develop resistance to substances in the reaction systems. In the study of the stress response, yeasts also have great benefits. In molecular biology, yeast is a unicellular eukaryotes considered as a model for studies of eukaryotic cells. The simplicity of single cells, accumulation of many experimental results, and availability of genome sequences make them advantageous to study the mechanisms of stress resistance. So far, several stress response pathways have been investigated in yeast, such as ethanol tolerance, elevated or decreased temperature, osmotic stress, low or high pH, ion concentrations, energy depletion, free radicals, and antibiotics. Previous studies have revealed that the cell wall, which animal cells do not have, contributes substantially to keep cellular shape against osmotic stresses, to protect cells from external stimuli, and to prevent

the invasion of toxic substances.

Cell wall stress in yeast

The cell wall is located in the outermost layer of the yeast cells, and it provides the first barrier to the environment; it is also the first component to gain information directly from the environment. In addition, yeast cell wall has functions such as stabilization of internal osmotic conditions, protection against physical stress, maintenance of cell shape, and scaffolding for proteins (Klis et al., 2006). The cell wall of *S. cerevisiae* consists of mainly β-1,3-linked and β-1,6-linked glucan chains, chitin, and mannoproteins (Klis et al., 2006). Though the cell wall has a rigid structure, it is dynamically remodeled during normal cellular growth and upon extracellular stresses (Jendretzki et al., 2011). As the cell wall functions in fundamental aspects of sustainable life in yeast, damage or stress is monitored by the cell wall integrity pathway (CWI) (Fig. 1).

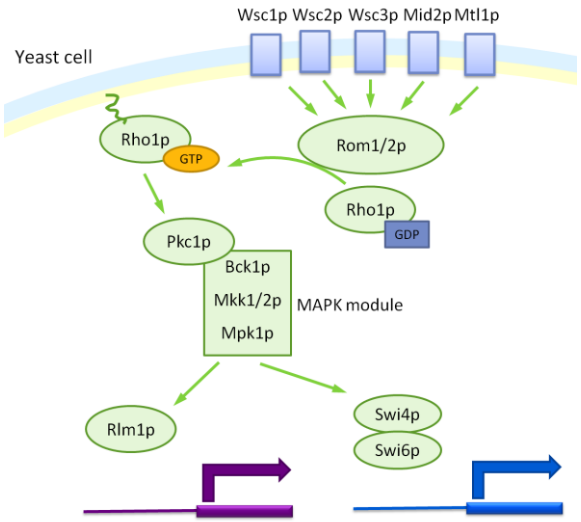

Fig.1 Cell wall integrity pathway

The CWI signal transduction cascade is activated by stress on the cell wall posed by that of heat, hypo-osmotic shock, pheromone-induced morphogenesis, cell wall perturbing reagents such as calcofluor white, actin cytoskeleton depolarization, endoplasmic reticulum (ER) stress, turgor pressure, and plasma membrane stretch, and among others (Levin, 2011). Two types of cell surface sensors, the Wsc-type sensors, (Wsc1p, Wsc2p, Wsc3p), and the other type (Mid2p and Mtl1p) have been identified (Jendretzki et al., 2011). Each cell wall sensor contains a single transmembrane site with a small C terminus in the cytoplasm and a large extracellular region with highly *O*-mannosylated serine- and threonine- rich regions

(Jendretzki et al., 2011). Current working models suggest that cell surface sensors work as mechanosensors to detect stress or strain either on the cell wall or on the plasma membrane. Detection of the stress triggers interaction with the GEFs Rom1/2p, which activates Rho1p and the downstream MAP kinase cascade (Bck1p, Mkk1/2p, and Mpk1p) (Jendretzki et al., 2011). Consequently, Mpk1p activates transcription factors Rlm1p by phosphorylation (Jung et al., 2002), and Swi4/6p complex without phosphorylation (Kim et al., 2008).

Xenobiotic efflux system

Xenobiotic resistance is also important for living cells to protect themselves from toxic substances. Yeast cells should exclude toxic compounds, such as plant secondary products in the environment. Otherwise, they become damaged by the accumulation of such toxins. The xenobiotic efflux system is common among a wide variety of species. As it is necessary for living organisms to exclude xenobiotic substances, they have been developed during evolution. To gain resistance to xenobiotics, cells have several strategies such as increased efflux out of cells, reduced uptake by modifying cell surfaces, sequestration into vacuoles, and by inactivation of xenobiotics by modifying chemicals or target proteins (Fig. 2) (Ernst et al., 2005). Among them, the overproduction of multi-substrate efflux pumps contributes a lot to the gain of xenobiotic resistance. There are mainly two protein families, the ATP-binding cassette (ABC) transporters and the major facilitator superfamily (MFS), in yeast cells.

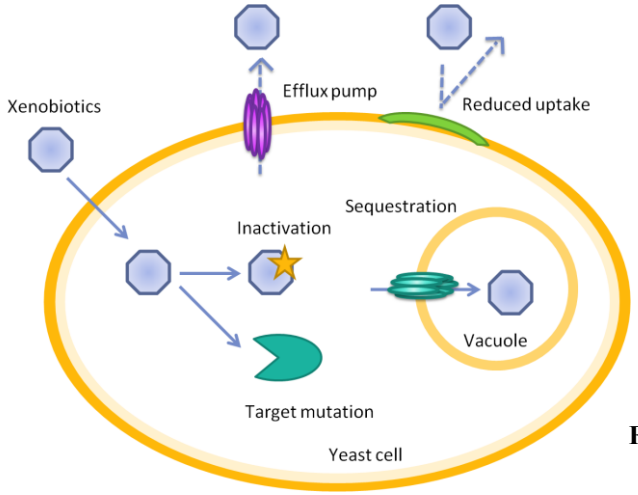

Fig.2 Xenobiotic resistance methods

The ABC transporters catalyze the ATP-dependent transport of diverse compounds across cellular membranes (Paumi et al., 2009). The ABC transporter family is a large

protein group conserved in all living cells from bacteria to humans, and also in yeasts (Bauer et al., 1999). Members of the ABC superfamily share a conserved overall architecture. The ABC core domain consists of two homologous halves, each containing a membrane spanning domain with multiple transmembrane spans and a nucleotide-binding domain, which couples nucleotide hydrolysis to substrate transport (Paumi et al., 2009). In yeast, 22 ABC transporters have been identified. By phylogenetic analysis, yeast ABC transporters have been grouped into six groups, from ABCB to ABCG (Paumi et al., 2009). ABC transporters for xenobiotic transport localize on the plasma membrane and export cytotoxic drugs out of the cell. In addition, some transporters in the ABCC group localize on vacuole to sequester toxins into it.

Another family of transporters of xenobiotics, namely the MFS transporters, is energized by the electrochemical proton-motive force. Yeast drug:H^+ antiport families were identified in Sa-Correia et al. (2009). In yeast, at least 23 putative MDR-H^+-antiporters were identified by yeast genome sequencing, but the role of MFS is less understood as compared to the ABC transporter group.

Xenobiotic efflux system in pathogens: multidrug resistance

This mechanism is also applied to medical drugs, and this causes problems in medical treatment in several ways. When the pathogens, such as pathogenic bacteria, opportunistic microorganisms, and cancer cells, gain strong drug-transport systems, they obtain resistance against several drugs at the same time by amplification of the gene encoding prototypical drug efflux pump. The MDR opportunistic microbes are selected by the use of antibiotics, which kills many but not all microbes (i.e., naturally-occurring drug resistant microbes). The remaining survivors then increase in population, and, as a result, drug-resistant residential flora increases their number. Even if drugs with other mechanisms of function were used, the process to gain resistance would be repeated.

As the development of new functional drugs is time- and cost-consuming, we have fewer options to beat these pathogens as pathogens gain resistance to drugs. To make a breakthrough in this situation, a comprehensive understanding of the MDR mechanism is desired to develop new strategies to block such multidrug resistance.

Applications of xenobiotic efflux systems to industries

Meanwhile, resistance to xenobiotics has advantages for applying cells to industries. These days, microorganisms are used as whole cell biocatalysts in place of traditional inorganic catalysts to produce compounds that cannot be produced efficiently via chemical synthesis, as these types of reactions are highly specific and occur even in mild conditions. As conditions in bioproduction are often severe for cells to live, toxicity leads to cell death, which severely impacts the efficiency of whole cell biocatalysis. Using cells with stress resistance is expected to promote bioconversion reaction efficiently.

Organic solvents, which are used to dissolve hydrophobic compounds, are toxic to living cells because of their hydrophobicity. The accumulation of lipophilic compounds in the membrane lipid bilayer causes loss of membrane integrity and increases in permeability to protons and other ions (Sikkema et al., 1995). They also disrupt functions of membrane-associated proteins, and lead to cellular death (Sikkema et al., 1995). Since organic solvent tolerance is an attractive property for bioconversion in hydrophobic compounds, some bacteria with organic-solvent tolerance have been reported from *Escherichia coli* (Aono et al., 1991), the genus *Pseudomonas* (Inoue and Horikoshi, 1989), and other bacteria (Sardessai and Bhosle, 2002, 2003; Na et al., 2005; Segura et al., 2008; Gao et al., 2011). According to these previous reports, organic solvent-tolerant prokaryotes use several mechanisms to cope with stress from organic solvents as follows. The primary mechanism utilized by prokaryotes is the upregulation of transporter genes, which promote the efflux of toxic organic solvents (Ramos et al., 1998; Rojas et al., 2001). In addition, prokaryotesinduce alterations of the cellular membrane composition in order to decrease solvent permeability (Pinkart et al., 1996), assimilation, solvent biotransformation to a less toxic product (Vangnai et al., 2002), or modifications in the overall morphology of cells (Shi and Xia, 2003; Neumann et al., 2005; Torres et al., 2011).

On the other hand, there are a few reports of organic solvent tolerance in eukaryotic cells such as yeast (Fukumaki et al., 1994). The molecular mechanisms and specific genes responsible for this tolerance have not been determined.

Yeast cells with organic solvent tolerance

The organic solvent-tolerant *S. cerevisiae* diploid strain KK-211 was first isolated

through serial culture in a medium containing isooctane (Kanda et al., 1998). The KK-211 strain can survive and grow well in the presence of isooctane or *n*-nonane. Previous studies used DNA microarray analysis to identify the genes that were upregulated in the organic solvent-tolerant KK-211 strain (Table 1) (Matsui et al., 2006). In the KK-211 strain, four genes encoding ABC transporters, *PDR10*, *SNQ2*, *YOR1*, and *PDR15*, ands6 genes encoding cell wall proteins, *TIP1*, *WSC3*, *PRY3*, *CIS3*, *PIR1*, and *YNL190W* were upregulated (Matsui et al., 2006). Interestingly, the upstream regions of the four transporter-encoding genes harbor the pleiotropic drug-response element (PDRE), which is the Pdr1p-binding site. Further research revealed that the organic solvent tolerance of the KK-211 strain is due to the R821S mutation in a transcription factor Pdr1p (Matsui et al., 2008).

Pdr10p was reported to mediate pleiotropic drug resistance (Kolaczkowska et al., 2002), whereas Snq2p is involved in resistance to cercosporin and other singlet oxygen-generating photosensitizers (Ververidis et al., 2001). Yor1p is associated with oligomycin resistance and rhodamine B transport in *S. cerevisiae* (Katzmann et al., 1995; Decottignies et al., 1998; Grigoras et al., 2008). Pdr15p is the closest homolog of the multidrug efflux transporter Pdr5p, which mediates pleiotropic drug resistance to hundreds of unrelated compounds (Wolfger et al., 2004).

Table 1 Genes upregulated in the KK-211 strain (Matsui et al., 2006, modified)

Systematic name	Standard name	Induction fold	Description
YLR099C	*ICT1*	8.09	Lysophosphatidic acid acyltransferase
YGR281W	*YOR1*	3.52	ABC transporter
YDR406W	*PDR15*	3.04	ABC transporter
YOL151W	*GRE2*	2.41	3-methylbutanal reductase / NADPH-dependent methylglyoxal reductase
YDR011W	*SNQ2*	2.29	ABC transporter
YOR328W	*PDR10*	1.46	ABC transporter
YJL078C	*PRY3*	1.81	Cell wall protein with a role in mating efficiency
YOL105C	*WSC3*	1.77	Sensor-transducer, involved in maintenance of cell wall integrity
YKL164C	*PIR1*	1.35	O-glycosylated protein required for cell wall stability
YBR067C	*TIP1*	1.26	Major cell wall mannoprotein
YJL158C	*CIS3*	1.21	Mannose-containing glycoprotein constituent of the cell wall
YNL190W		1.07	Hydrophilin, cell wall protein

Tip1p has β-1,6 glucose-containing side chains and is a temperature shock-inducible protein (Fujii et al., 1999) (Kondo and Inouye, 1991). Wsc3p is involved in the stress-activated PKC1-MPK1 signaling pathway for the maintenance of cell wall integrity (Verna et al., 1997). Pry3p is a cell wall protein that plays a role in determining mating efficiency (Colman-Lerner et al., 2001). In addition, Cis3p is a mannose-containing glycoprotein constituent of the cell wall and a member of the PIR family (Manning et al., 1997; Mrsa et al., 1997; Moukadiri et al., 1999), and Pir1p is involved in maintaining cell wall stability, and is regulated by the cell integrity pathway (Mrsa and Tanner, 1999; Vongsamphanh et al., 2001). Finally, Ynl190wp is a cell wall protein, and is a member of hydrophilin with unknown function (Hamada et al., 1999; Garay-Arroyo et al., 2000). Though genes encoding several ABC transporters and cell wall proteins were upregulated in the organic solvent tolerant KK-211 strain, it is not clear whether all of the genes contribute to organic solvent tolerance.

Multidrug resistance in yeast

Pdr1p is commonly known as the master transcription regulator MDR in yeast. When cells are exposed to drugs, Pdr1p upregulates transcription levels of genes involved in MDR, such as drug efflux pumps, together with its paralog, Pdr3p (Delaveau et al., 1994); these components constitute the PDR-related pathway. Pdr1p forms homodimers or heterodimers with Pdr3p and recognizes pleiotropic drug resistance elements (PDREs) present in the promoters of target genes (Katzmann et al., 1996; Mamnun et al., 2002). Among the genes upregulated in the KK-211 strain, ABC transporters responsible for MDR harbor PDREs in their promoters, while cell wall proteins do not.

Pdr1p and Pdr3p constantly bind to PDRE in conditions with or without drugs. In response to drug exposure, Pdr1p and Pdr3p are thought to directly interact with various drugs, resulting in recruitment of a mediator complex to the promoters and upregulation of downstream genes (Thakur et al., 2008) (Fig. 3). Some Pdr1p and Pdr3p mutants consistently increase transcription levels of downstream genes and show strong MDR. For example, F815S a point mutation in Pdr1p (*pdr1-3*), and the K257E and G834S single amino-acid mutations in Pdr3p (*pdr3-8* and *pdr3-18*, respectively) have been reported to have such effects (Carvajal et al., 1997; Nourani et al., 1997). Even Pdr3p was shown to have

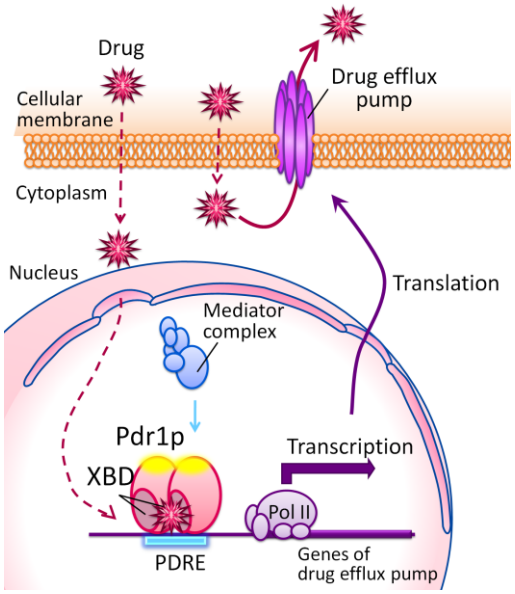

Fig. 3 Pleiotropic drug resistance (PDR) pathway in multidrug resistance in yeast

redundant roles with Pdr1p in MDR (Delaveau et al., 1994; Moye-Rowley, 2003; Jungwirth and Kuchler, 2006; MacPherson et al., 2006), but Pdr3p possesses some different properties from Pdr1p. For example, Pdr3p but not Pdr1p is autoregulated (Delahodde et al., 1995), activated by dysfunctional mitochondria (Hallstrom and Moye-Rowley, 2000; Devaux et al., 2002), and negatively regulated by the Hsp70 chaperone Ssa1p (Shahi et al., 2007).

Of the downstream genes in the PDR-related pathway, Pdr5p has been the most intensively studied drug efflux pump. Pdr5p is a plasma membrane ABC transporter that structurally and functionally eliminates various drugs. It contains four PDREs in its promoter region, which are recognized during transcription regulation by Pdr1p and Pdr3p (Balzi et al., 1994; Katzmann et al., 1994; Katzmann et al., 1996). Other ABC transporters, Pdr10p, Pdr15p, Snq2p, and Yor1p, which were upregulated in organic solvent-tolerant KK-211 strain, have been shown to contribute to MDR, and to be regulated by Pdr1p and Pdr3p via the PDR-related pathway (Servos et al., 1993; Decottignies et al., 1995; Wolfger et al., 1997; Decottignies et al., 1998).

Transcription regulation mechanism of Pdr1p

Pdr1p and Pdr3p are members of zinc cluster transcription factors that recognize PDREs. Members of the zinc cluster protein family are exclusively fungal and possess the well-conserved motif Zn_2C_6 as a DNA binding domain. Pdr1p and Pdr3p have a zinc cluster DNA binding domain at the N terminus and a transcription activation site at the C terminus

(MacPherson et al., 2006). Pdr1p forms homodimers or heterodimers with Pdr3p (Mamnun et al., 2002) and another member of the zinc cluster transcription factors, Stb5p (Akache and Turcotte, 2002). DNA binding domains of Pdr1p and Stb5p are sufficient for heterodimerization. (Akache et al., 2004). However, Stb5p does not seem to form homodimers or heterodimers with Pdr3p. The consensus sequence of PDRE is -TCCGCGGA-. (Katzmann et al., 1994; Delahodde et al., 1995; DeRisi et al., 2000), which contains two CCG triplets. Pdr1p and Pdr3p (Hellauer et al., 1996) recognize PDRE.

In addition, several factors have been individually reported to be involved in transcription regulation in MDR. Yrr1p (Keeven et al., 2002) and Yrm1p (Lucau-Danila et al., 2003) are regulators of PDR genes (Zhang et al., 2001). It has also been reported that many targets of Pdr1p, Pdr3p, and Yrr1p overlap (MacPherson et al., 2006). Yrr1p tend to form homodimers to regulate PDR-related genes (Akache et al., 2004). Yrr1p positively regulates genes through YRREs containing the consensus sequence closely related to PDREs (Le Crom et al., 2002). Pdr8p, a paralog of Yrr1p, also recognizes sequences similar to PDRE (Hikkel et al., 2003). Its target genes overlap with targets of Pdr1p/Pdr3p and Yrr1p (Hikkel et al., 2003). The C terminus of Zuo1p and the N terminus of Ssz1p, with which Zuo1p forms a heterodimer, can independently activate Pdr1p (Hallstrom et al., 1998; Eisenman and Craig, 2004). One model suggests that activation of Pdr1p by direct interaction with the unfolded C-terminuscontaining 13 residues of Zuo1p or Ssz1p would result in upregulation of transporters such as Pdr5p and Yor1p to export signaling molecules involved in quorum sensing (Prunuske et al., 2011; Ducett et al., 2012). A genetic screen revealed that a truncated mutation of *ELM1* encoding a serine/threonine protein kinase suppressed the multidrug resistance of *pdr1-3*. The results suggest that *ELM1* function upstream of *PDR5* in regulation of cycloheximide resistance (Souid et al., 2006). The N terminus of Ngg1p interacted with the C terminus of Pdr1p and Pdr3p to inhibit transcription activation (Martens et al., 1996). Ngg1p forms coactivator/repressor complex (ADA complex) with Ada1p and at least two other yeast proteins, Ada2p and Gcn5p.

The roles of transcription factors in transcription regulation

In spite of the growing importance of epigenetic regulation in transcription regulation such as histone modifications and DNA methylations, transcription factors still play

fundamental roles in transcription regulation to determine which genes to regulate. Transcription factors recognize consensus sequences in the promoters of target genes. Most consensus sequences for transcription factors have motif variants. Some reports have shown binding site motif variants have differential gene expression (Hollenhorst et al., 2009; Rest et al., 2012). In these cases, motif variants result in condition specific differential expression due to either affinity differences between variants, or differential interactions of transcription factors bound to these variants with cofactors; also, there may be differential presence of cofactors across environments (Rest et al., 2012).

PDREs have five sequence variations, in which functional differences are not known. In the genome of *S. cerevisiae*, there are more than 200 genes with PDREs including variants in the promoter regions, but only a part of the genes are actually regulated by Pdr1p and Pdr3p. In addition, there are wide varieties in transcription levels among the genes harboring PDRE(s) in their promoter regions. For selection and regulation of the target genes, the current mechanism of transcription regulation of Pdr1p and Pdr3p is insufficient, in which Pdr1p and Pdr3p constantly bind to PDREs. It is possible that PDRE variants are involved in such differences in transcription regulation.

The purpose of this study was to elucidate the molecular mechanism of organic solvent tolerance of yeast by comparing mechanisms of MDR. In Chapter I, the components that contribute to organic solvent tolerance were identified. In Chapter II, the signaling pathway in response to organic solvent tolerance was investigated. In Chapter III, the difference between homologous transcription factors, Pdr1p and Pdr3p, in organic solvent tolerance was described.

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Chapter I

Identification of factors involved in organic solvent tolerance

The substrates, products, and organic solvents used in the chemical reactions for the production of hydrophobic compounds are usually highly toxic to the catalytic carrier organism. Therefore, organic solvent tolerance is necessary for effective bioproduction. The organic solvent-tolerant *S. cerevisiae* diploid strain KK-211 was first isolated through serial culture in a medium containing isooctane (Kanda et al., 1998) Previous studies used DNA microarray analysis to identify the genes that were upregulated in the organic solvent-tolerant KK-211 strain (Matsui et al., 2006). In the KK-211 strain, 4 genes encoding ABC transporters, *PDR10*, *SNQ2*, *YOR1*, and *PDR15*, and 6 genes encoding cell wall proteins, *TIP1*, *WSC3*, *PRY3*, *CIS3*, *PIR1*, and *YNL190W* were upregulated (Matsui et al., 2006). It was also revealed that the organic solvent tolerance of the KK-211 strain is due to the R821S mutation in the Pdr1p transcription factor (Matsui et al., 2008). However, the molecular mechanisms and specific genes responsible for this tolerance have not been determined.

In this chapter, to identify the genes involved in the organic solvent tolerance, we focused on the upregulated 4 ABC transporters and 6 cell wall proteins in KK-211. We first showed that the MT8-1 PDR1 R821S mutant, which tolerated hydrophobic organic solvents (Matsui et al., 2008), also tolerates the hydrophilic organic solvent dimethyl sulfoxide (DMSO). Then, we tested the contribution to organic solvent tolerance of the 4 ABC transporters and 6 cell wall proteins that were upregulated in the KK-211 strain. The genes were discriminated whether they were responsible for the tolerance to hydrophobic organic solvents, such as *n*-decane and *n*-undecane, and the hydrophilic organic solvent DMSO. Through this study, we identified the genes involved in organic solvent tolerance in yeast, as well as classified the genes depending on solvent specificity.

Materials and methods

Strains and media

The *E. coli* DH5α strain (*F*$^-$, Φ80d*lacZΔ*M15, *Δ*[*lac*ZYA-*arg*F]U169, *deo*R, *rec*A1, *end*A1, *hsd*R17[r_K^-, m_K^+], *phoA, sup*E44, λ^-, *thi*-1, *gyr*A96, *rel*A1) (Toyobo, Osaka, Japan) was used as a host for recombinant DNA manipulation. The *S. cerevisiae* MT8-1 (*MAT***a**, *ade*, *his3*, *leu2*, *trp1*, *ura3*) (Tajima et al., 1985) strain was used as a host for overproduction of transporters and cell wall proteins. The MT8-1 *PDR1* R821S mutant, which is the MT8-1 haploid strain with the *PDR1* R821S point mutation, has been previously constructed (Matsui et al., 2008). *E.coli* was grown in Luria-Bertani (LB) medium (1% [w/v] tryptone, 0.5% yeast extract, and 0.5% sodium chloride) containing 100 μg/mL ampicillin at 37°C. The MT8-1 transformants were grown at 30°C in SDC+AHLU or SDC+AHLW media (0.67% yeast nitrogen base without amino acids, 2% glucose, 0.5% casamino acids, 0.002% adenine, 0.002% L-histidine, 0.003% L-leucine, and 0.002% uracil or 0.002% L-tryptophan). The agar plates for the spotting assay in the organic solvent tolerance test were prepared by adding 2% (w/v) agar to the media. For the DMSO-containing plates, DMSO was added to the media after they were autoclaved. The agar plates were prepared in glass petri dishes to avoid any organic compounds being eluted from plastic containers by the organic solvents.

Construction of the plasmids and strains for the overproduction of the ABC transporters

For the overproduction of transporters, pKMG1 was constructed as a multicopy plasmid that was fused at the C-terminus with enhanced green fluorescence protein (EGFP) under the control of a constitutive glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) promoter (*PGAP*). The primers used for plasmid construction are listed in Table 1. An EGFP-encoding DNA fragment was amplified from pEGFP (Clontech, CA, USA) by polymerase chain reaction (PCR) by using the primers MCS-EGFP-F and MCS-EGFP-R. The primer MCS-EGFP-F includes *Sac*I, *Not*I, *Kpn*I, *Sal*I, and *Bam*HI recognition sites in addition to sequence for EFGP cloning. After digestion with *Sac*I and *Xho*I, the fragment was inserted into the *Sac*I-*Sal*I section of pWGP3 (Takahashi et al., 2001), which included PGAP. The resulting plasmid was named pKMG1 (Fig. 2a). The plasmid pKMG1 has *Sac*I, *Not*I, *Kpn*I,

*Sal*I, and *Bam*HI recognition sequences for the multicloning site.

The open reading frame (ORF) regions of the genes encoding the ABC transporters (*PDR10*, *SNQ2*, *YOR1*, and *PDR15*) and cell wall proteins (*TIP1*, *WSC3*, *PRY3*, *CIS3*, *PIR1*, and *YNL190W*) were amplified from the MT8-1 genome by PCR by using the primers listed in Table 1. The primers were designed by referring to the gene sequences in the *Saccharomyces* genome database (http://www.yeastgenome.org/). The amplified DNA fragments of the ABC transporters were inserted into pKMG1 by using *Sac*I and *Sal*I for *PDR10*, *Not*I and *Bam*HI for *SNQ2* and *PDR15*, and *Not*I and *Sal*I for *YOR1*. The sequences of the inserted DNA fragments were confirmed via DNA sequencing. The plasmids for overexpressing *PDR10*, *SNQ2*, *YOR1*, and *PDR15* were named pKMG1-PDR10, pKMG1-SNQ2, pKMG1-YOR1, and pKMG1-PDR15, respectively. The amplified DNA fragments of the genes encoding the cell wall proteins were inserted into pULI1, a multicopy vector that includes PGAP as the promoter (Miura et al., 2012), by using *Bam*HI and *Sal*I for *TIP1*, *WSC3*, *PIR1*, and *YNL190W*, *Sac*I and *Bam*HI for *CIS3*, and *Sal*I for *PRY3*. The sequences of the inserted DNA fragments were confirmed via DNA sequencing, and the resulting plasmids were named pULI1-TIP1, pULI1-WSC3, pULI1-PIR1, pULI1-YNL190W, pULI1-CIS3, and pULI1-PRY3, respectively. The constructed plasmids were introduced into the laboratory haploid strain MT8-1 by using the lithium acetate method (Ito et al., 1983).

Confirmation of overproduction and subcellular localization of the ABC transporters

The overproduction and subcellular localization of each ABC transporter in the MT8-1 strain were confirmed by observing the green fluorescence emitted from EGFP fused to the ABC transporter. The MT8-1 strains harboring an ABC transporter-producing plasmid or pWGP3 (control) were cultured in 10 mL of SDC+AHLU for 24 h. The cells were harvested and washed with phosphate-buffered saline (PBS; pH 7.4; 137 mM sodium chloride, 8.1 mM disodium hydrogen phosphate, 2.68 mM potassium chloride, and 1.47 mM potassium dihydrogen phosphate). The washed cells were then resuspended in PBS (pH 7.4) and observed using the IX71 fluorescence microscope (Olympus Optical Co., Tokyo, Japan). The green fluorescence from EGFP was detected using a U-MNIBA2 mirror unit with a BP470-490 excitation filter, a DM505 dichroic mirror, and a BA510-550 emission filter (all from Olympus Optical Co.).

Table 1 Primers used in this study

Primers	Sequence[a]
MCS-EGFP-F	5'-ATGCCGAGCTCGCGGCCGCTGGTACCGTCGACGGTGGATCCGGCGGTATGGTGAGCAAGGGCGAGGAG-3' (*Sac*I)
MCS-EGFP-R	5'-TCGCGCTCGAGTTACTTGTACAGCTCGTCCATGCCGAG-3' (*Xho*I)
PDR10-EGFP-F	5'-AKATAGCGGCCGCTATGTTGCAAGCGCCCTCAAG-3' (*Not*I)
PDR10-EGFP-R	5'-ATATTGTCGACTTTCTTTAATTTTTTGCTTTTCTTTGGAACCCGCAC-3' (*Sac*II)
SNQ2-EGFP-F	5'-GTAAAGCGGCCGCTATGAGCAATATCAAAAGCACGCAAG -3' (*Not*I)
SNQ2-EGFP-R	5'-ATCTGGGATCCACCCTGCTTCTTTTTCCTTATGTTTTTAATTTTATTGAG-3' (*Bam*HI)
YOR1-EGFP-F	5'-TGCTAGCGGCCGCTATGACGATTACCGTGGGGGATG-3' (*Not*I)
YOR1-EGFP-R	5'-AATATGTCGACACTTCTGTTCTCGAAATCATTTTCCACAATAC-3' (*Sal*I)
PDR15-EGFP-F	5'-TATCGCGGCCGCATGTCATCAGATATCAGAGACGTAG-3' (*Not*I)
PDR15-EGFP-R	5'-AACGGGATCCCTTCTTGGGTTTTTCGGAAATCTTAC-3' (*Bam*HI)
TIP1-F	5'-CCCGGGGATCCATGTCCGTTTCCAAGATTGCTTTC-3' (*Bam*HI)
TIP1-R	5'-ACCAAGTCGACTTATAACAATAAAGCAGCTGCACCTG-3' (*Sal*I)
WSC3-F	5'-CCCGGGGATCCATGGAAAGAGTATGGTTTGCAAAATTAACAAATAAAG-3' (*Bam*HI)
WSC3-R	5'-ACCAAGTCGACTCAGGCTCGATTATGAGATACG-3' (*Sal*I)
PRY3-F	5'-CTAGAGTCGACATGCTGGAGTTTCCAATATCAGTTC-3' (*Sal*I)
PRY3-R	5'-ACCAAGTCGACCTAGAAGGCGAACAGAACAGC-3' (*Sal*I)
CIS3-F	5'-CCCGGGAGCTCATGCAATTCAAAAACGTCGCCCTA-3' (*Sac*I)
CIS3-R	5'-CTAGAGGATCCTTAACAGTCGACCAAAGAAACAGC-3' (*Bam*HI)
PIR1-F	5'-CCCGGGGATCCATGCAATACAAAAAATCATTAGTTGCCTCC-3' (*Bam*HI)
PIR1-R	5'-ACCAAGTCGACTTAACAGTTGAGCAAATCGATAGCTTG-3' (*Sal*I)
YNL190W-F	5'-CCCGGGGATCCATGAAGTTCTCTTCTGTTACTGCTATTAC-3' (*Bam*HI)
YNL190W-R	5'-ACCAAGTCGACTTATAATAGTAATAAGGCACCGGCTAC-3' (*Sal*I)
Primers for quantitative real-time RT-PCR analysis	
ACT1-Frt	5'-TCGTTCCAATTTACGCTGGTT-3'
ACT1-Rrt	5'-ACCGGCCAAATCGATTCTC-3'
TIP1-Frt	5'-TCCAATGCCGGTCAAAGAG-3'
TIP1-Rrt	5'-GCAACAACAGCACCGAAAGA-3'
WSC3-Frt	5'-GGAAATCGCACGACCAATTAG-3'
WSC3-Rrt	5'-ACCGAACGAATAGGGCTGATAA-3'
PRY3-Frt	5'-GCAGAACTACGCCGACCAAT-3'
PRY3-Rrt	5'-TGGGCCATCGGAATGC-3'
CIS3-Frt	5'-CCCAACCAGCTCCGAAAAG-3'
CIS3-Rrt	5'-AAGAAGATGATGTGGCATTAGTAGATGT-3'
PIR1-Frt	5'-AACTTCTGCAACCATTATACCATCTC-3'
PIR1-Rrt	5'-GCCACTGCTTTTACATGTTTCAG-3'
YNL190W-Frt	5'-CGCCCCTGGTCCATCTAAT-3'
YNL190W-Rrt	5'-GCAGCACTACCAGCGGTAACA-3'

[a] Underlined sequences indicate the restriction sites for enzymes shown in parentheses.

Confirmation of overexpression of the genes encoding cell wall proteins

The overproduction of each cell wall protein in the MT8-1 strain was confirmed by transcription level of its gene using quantitative real-time reverse transcription (RT)-PCR. Total RNAs were isolated using an RNeasy mini kit (QIAGEN, Tokyo, Japan) from cells grown to the mid-log phase, which showed optical densities of 2–3 at 600 nm (OD_{600}). Synthesis of cDNA was performed using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems by Life Technologies, CA, USA) with 2 μg of the total RNA as a template. The reaction was carried out according to the manufacturer's protocol. For the quantitative PCR, the *ACT1* gene was used as an endogenous control to normalize the expression data for each gene. The primers, which are listed in Table 1, were designed using Primer Express® software (Applied Biosystems). Amplification was carried out using Power SYBR® Green PCR Master Mix (Applied Biosystems) in the 7500 Real-Time PCR System (Applied Biosystems). The reporter signals were analyzed using the 7500 Real-Time PCR System.

Cell growth in organic solvent-containing media

The growth of the MT8-1 strains overproducing each ABC transporter was measured in SDC+AHLU medium with or without an organic solvent such as *n*-decane or DMSO. The MT8-1 strain harboring pWGP3 was used as a control. The strains were precultivated in 10 mL of SDC+AHLU medium for 36 h. To measure growth in the presence of *n*-decane, the hydrophobic organic solvent, the preculture was transferred into 100 mL of fresh SDC+AHLU medium or 80 mL of fresh SDC+AHLU medium plus 20 mL of organic solvent, resulting in an initial OD_{600} of 0.4. For growth in the presence of DMSO, the preculture was transferred into 100 mL of fresh SDC+AHLU medium or 93.5 mL of fresh SDC+AHLU medium plus 6.5 mL of DMSO resulting in an initial OD_{600} of 0.1. The growth of MT8-1 *PDR1* R821S was measured in YPD medium with or without DMSO and the MT8-1 wild-type strain was used as a control. These strains were precultivated in 10 mL of YPD medium for 36 h, and transferred into 100 mL of fresh YPD medium or 91 mL of fresh YPD medium plus 9 mL of DMSO resulting in an initial OD_{600} of 0.1. The yeast cells were cultivated by shaking (233 rpm; Bioshaker, BR-3000LFS; TAITEC) at 30˚C, and yeast cell growth was measured by monitoring the OD_{600}.

Spotting assay for evaluation of organic solvent tolerance

The cells were cultivated to the stationary phase in 10 ml SDC media with appropriate amino acids by shaking (250 rpm; Bioshaker, BR-43FL; TAITEC, Saitama, Japan) at 30°C for 36 h. The cells were collected by centrifugation (13040 × g, 10 s), and resuspended in sterilized water. Then the suspensions were serially diluted to OD_{600} of 10×2^{0}, 10×2^{-1}, 10×2^{-2}, 10×2^{-3}, 10×2^{-4}, 10×2^{-5}, 10×2^{-6}, and 10×2^{-7}, and then spotted (5 μl each) onto the prepared agar plates. To examine the tolerance to hydrophobic organic solvents, 5 ml of *n*-undecane, or *n*-decane was overlaid to ensure the surfaces of the plates were entirely covered by the organic solvent. The tolerance to hydrophilic organic solvents was examined by spotting the cells onto agar plates containing DMSO and subsequently sealing the plates with vinyl plastic tape to prevent evaporation of the organic solvents. All the organic solvents were purchased from Wako Pure Chemical Industries (Osaka, Japan). All the plates were incubated at 30°C.

Results

Validation of spotting assay for organic solvent tolerance

To evaluate the organic solvent tolerance, we performed the spotting assay in the presence of organic solvents. We first confirmed whether the spotting assay was able to detect organic solvent tolerance by MT8-1 *PDR1* R821S as a positive control. We had good correlations between the liquid culture experiments (Matsui et al., 2008) and the spotting assay experiments (Fig. 1). Thus, we concluded the spotting assay is appropriate to evaluate organic solvent tolerance.

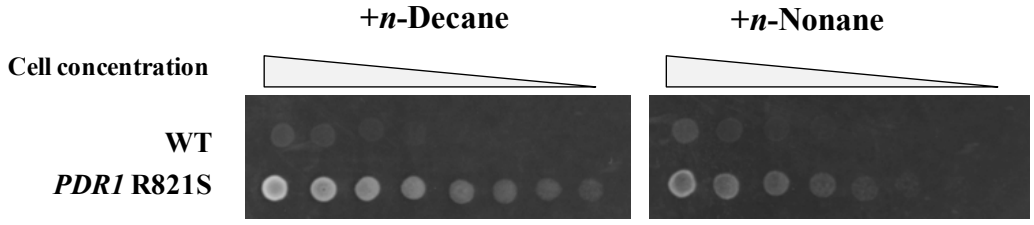

Fig. 1 Validation of spotting assay for organic solvent tolerance. Spotting assay of MT8-1 *PDR1* R821S. MT8-1 and the MT8-1 PDR1 R821S mutant were spotted onto agar plates. 5 ml *n*-decane or *n*-nonane was overlaid on the agar plate prepared in glass petri dishes.

Hydrophilic organic solvent tolerance of the PDR1 R821S mutant

The R821S point mutation in *PDR1* was reported to be responsible for the tolerance to hydrophobic organic solvents, such as isooctane and *n*-nonane (Matsui et al., 2008). However, whether the *PDR1* R821S mutant also shows tolerance to hydrophilic organic solvents such as DMSO is yet to be determined. DMSO is polar aprotic solvent that can dissolve most organic and inorganic compounds. The tolerance of the MT8-1 *PDR1* R821S mutant to the hydrophilic solvent DMSO was evaluated by comparing the growth of the

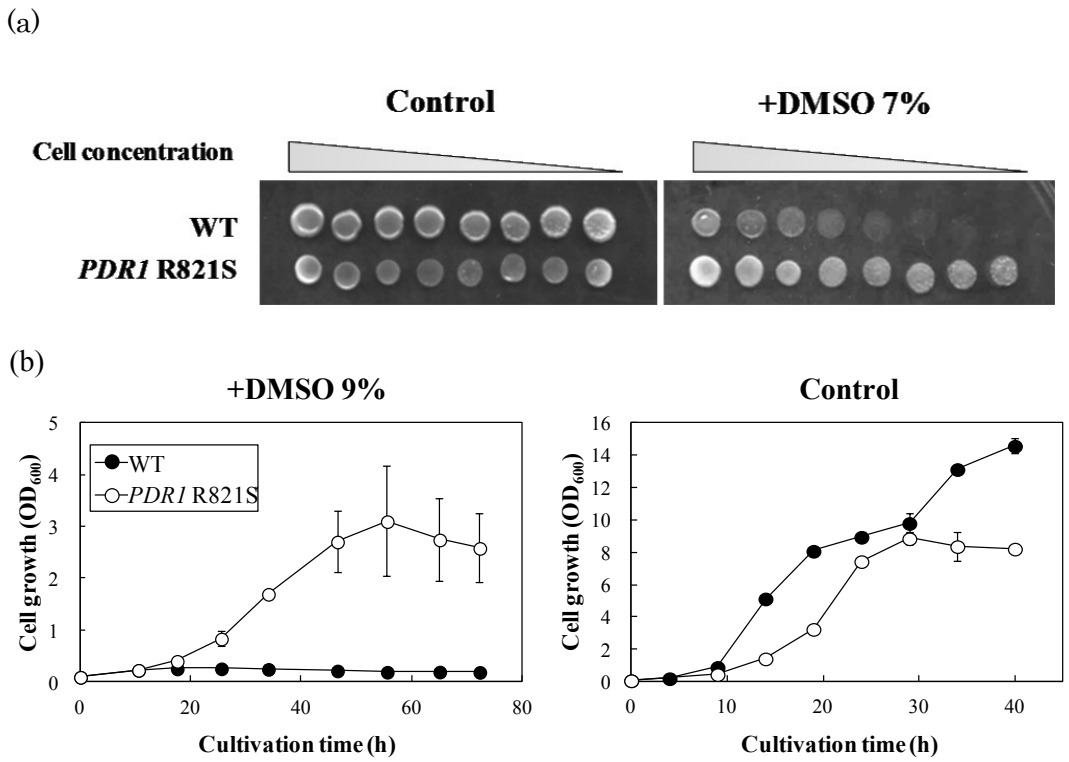

Fig. 2 Hydrophilic organic solvent tolerance of the MT8-1 PDR1 R821S mutant.

(a) MT8-1 and the MT8-1 PDR1 R821S mutant were spotted onto agar plates without or with 7% (v/v) DMSO in glass petri dishes. The cells in the stationary phase were serially diluted by 2-fold at each step starting from OD_{600} = 10, and spotted (5 μl). All the plates were incubated at 30°C for 3 days for the control plates, and 4.5 days for the DMSO-containing plates. (b) Growth curve in DMSO-containing culture of MT8-1 *PDR1* R821S mutant. (Left) Growth curves of the strains cultivated in media containing 9% (v/v) DMSO. MT8-1 *PDR1* R821S (*open circles*) and MT8-1 (WT) (*closed circles*). (Right) Growth curves of the strains cultivated in media without DMSO. The data are mean values of at least 3 independent experiments. The error bars indicate standard deviation.

mutant cells with that of the MT8-1 wild-type strain in the presence of DMSO. In the medium without DMSO, the wild-type strain grew better than the *PDR1* R821S mutant (Fig. 2a). The MT8-1 *PDR1* R821S mutant was able to grow on agar plates containing 7% (v/v) DMSO, while the MT8-1 wild-type strain was not (Fig. 2a). This result showed that the MT8-1 *PDR1* R821S mutant could tolerate hydrophilic organic solvent as well as hydrophobic organic solvents. We also evaluated the tolerance of MT8-1 *PDR1* R821S to DMSO by growth curve in liquid culture, and had the consistent results (Fig. 2b). We, therefore, suggested that one of the Pdr1p-regulated downstream genes was also involved in tolerance to DMSO.

Construction of yeast strains overproducing the ABC transporters

We constructed the MT8-1 laboratory haploid strains overproducing the ABC transporters that were upregulated in the organic solvent-tolerant KK-211 strain

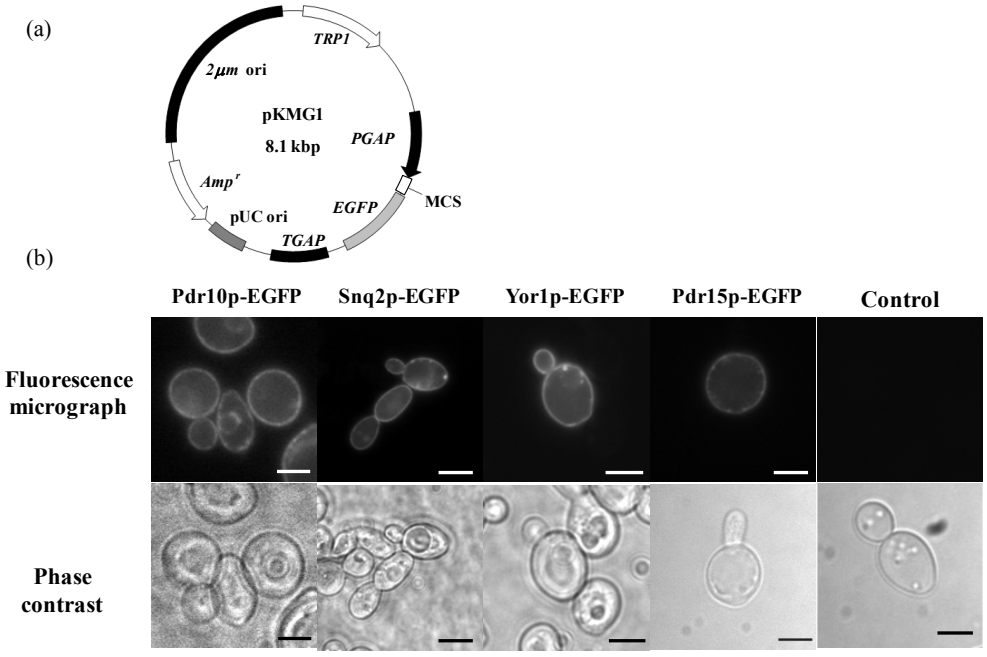

Fig. 3 Overproduction of the yeast ABC transporters fused with EGFP

(a) The pKMG1 vector was used for the overexpression of the ABC transporter genes fused with *EGFP*. (b) Fluorescence observations of the MT8-1 transformants overproducing the EGFP-fused ABC transporters, Pdr10p, Snq2p, Yor1p., and Pdr15p. The transformants were grown in SDC+AHLU to the late log phase. Overproduction and localization of the ABC transporters were observed using a fluorescence microscope. The scale bar is 5 μm.

(Matsui et al., 2006). The pKMG1 plasmid was created for the overproduction of the target transporters fused with *EGFP* at the C-terminus (Fig. 3a). The genes encoding the ABC transporters, namely, *PDR10*, *SNQ2*, *YOR1*, and *PDR15*, were inserted into pKMG1. Then, the production and subcellular localization of Pdr10p, Snq2p, Yor1p, and Pdr15p were observed by monitoring the fluorescence emitted by the fused EGFP (Fig. 3b). Green ring-shaped fluorescence was detected in strains containing the pKMG1 plasmid encoding each ABC transporter. The results indicated successful overproduction and localization of Pdr10p, Snq2p, Yor1p, and Pdr15p at the cellular membrane.

Involvement of the ABC transporters in the tolerance to hydrophobic and hydrophilic organic solvents

To examine whether the ABC transporters upregulated in KK-211 are responsible for organic solvent tolerance, the MT8-1 strains overproducing the ABC transporters were grown on agar plates in the presence of organic solvents. The hydrophobic organic solvent *n*-decane has the lowest log Pow (6.0) value in which the MT8-1 wild-type strain could survive (Kawamoto et al., 2001). The log Pow value is determined by a common logarithm of the *n*-octanol-water partition coefficient of the substance, and is the most general indicator of organic-solvent polarity (Rekker and Kort, 1979). The tolerance assay was performed using *n*-decane and *n*-undecane (log Pow = 6.3). The MT8-1 strains overproducing Pdr10p, Snq2p, Yor1p, and Pdr15p were spotted onto the agar plate, overlaid with *n*-decane or *n*-undecane, and incubated at 30°C (Fig. 4a). Although production of EGFP itself by pKMG1 did not improve organic solvent tolerance (Fig. 4b), the MT8-1 strains overproducing Pdr10p and Snq2p grew better than MT8-1 containing pWGP3 (vector only). Interestingly, the strain overproducing Yor1p grew less than the control strain. All strains showed similar growth patterns in the absence of any organic solvent. We also evaluated the tolerance to *n*-decane of strains overproducing ABC transporters by growth curve in liquid culture, and had the consistent results (data not shown).The results indicated that Pdr10p and Snq2p enhanced the hydrophobic organic solvent tolerance of MT8-1. In contrast, Yor1p was not effective, and even decreased the hydrophobic organic solvent tolerance of the strain.

Furthermore, the results shown in Fig. 2 imply that the ABC transporters upregulated in KK-211 could also be responsible for the DMSO tolerance. To examine the involvement of

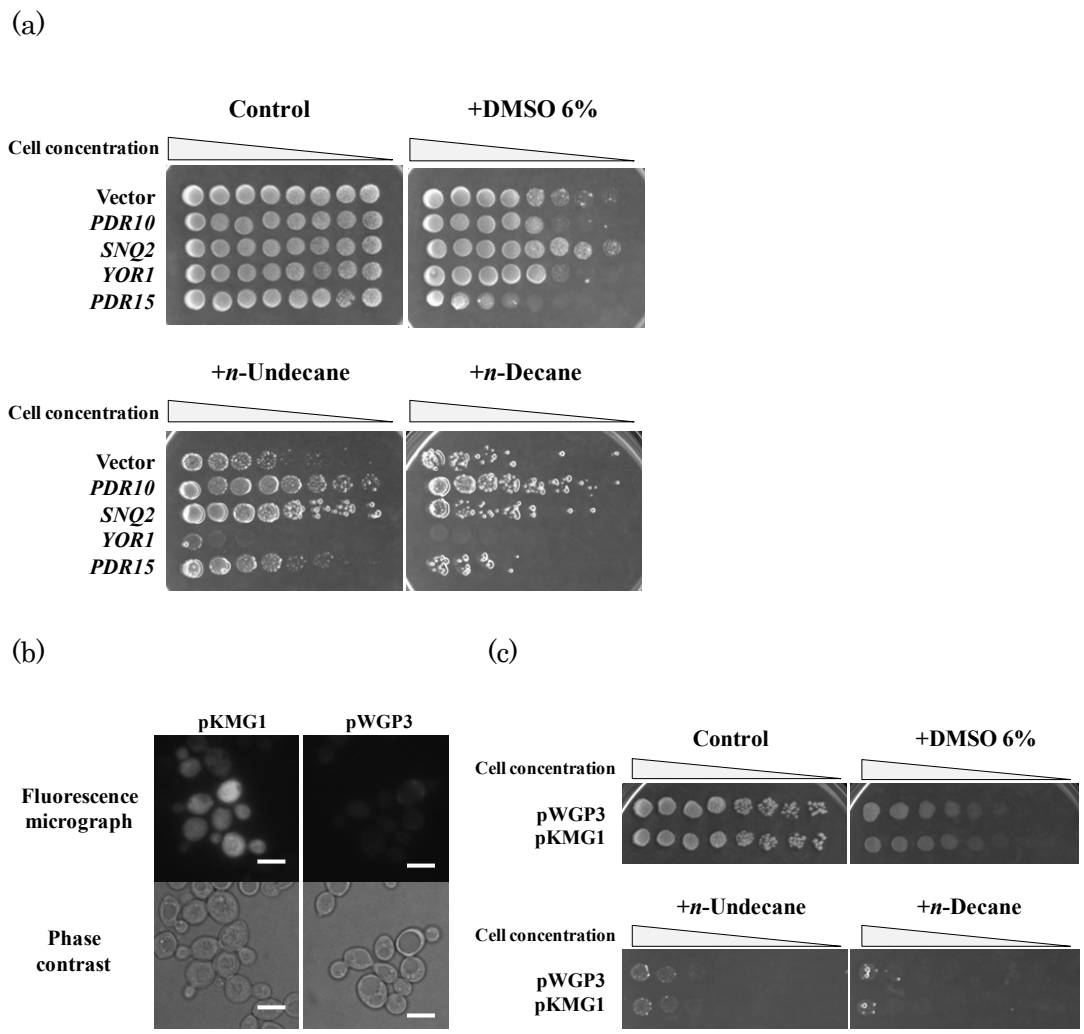

Fig. 4 (a) Spotting assay of the MT8-1 overproducing the ABC transporters (Pdr10p, Snq2p, Yor1p, and Pdr15p) on agar plates in the presence of the organic solvents. For the hydrophobic organic solvent tolerance test, 5 ml *n*-undecane or *n*-decane was overlaid on the agar plate. For the hydrophilic organic solvent tolerance test, the cell suspensions were spotted onto DMSO-containing agar plates prepared in glass petri dishes. All the plates were incubated at 30°C for 3 days for the control plates and *n*-undecane-overlaid plates, and for 4.5 days for the *n*-decane-overlaid plates and DMSO-containing plates.

(b) Overproduction of the yeast ABC transporters fused with EGFP. The scale bar is 5 μm. (c) Spotting assay of the MT8-1 haploid harboring pKMG1 (containing EGFP) or pWGP3 (without EGFP) on agar plates were carried out in the presence of the organic solvents.

the ABC transporters in DMSO tolerance, the growth of the MT8-1 strains overproducing the ABC transporters was measured on agar plates containing 6% (v/v) DMSO (Fig. 3), and

in liquid culture containing 6.5% (v/v) DMSO (data not shown). All strains survived under these conditions, but the yeast strains overproducing Snq2p and Yor1p grew better than the control strain. Therefore, cellular tolerance to DMSO was enhanced by the overproduction of Snq2p and Yor1p, thereby suggesting that these transporters played a role in DMSO tolerance.

Construction of yeast strains overproducing the cell wall proteins

In the MT8-1 strain, the 6 genes encoding cell wall proteins (*TIP1*, *WSC3*, *PRY3*, *CIS3*, *PIR1*, and *YNL190W*) that were upregulated in KK-211 were overexpressed using pULI1 (Miura et al., 2012). We introduced these genes without any fluorescent proteins or tags

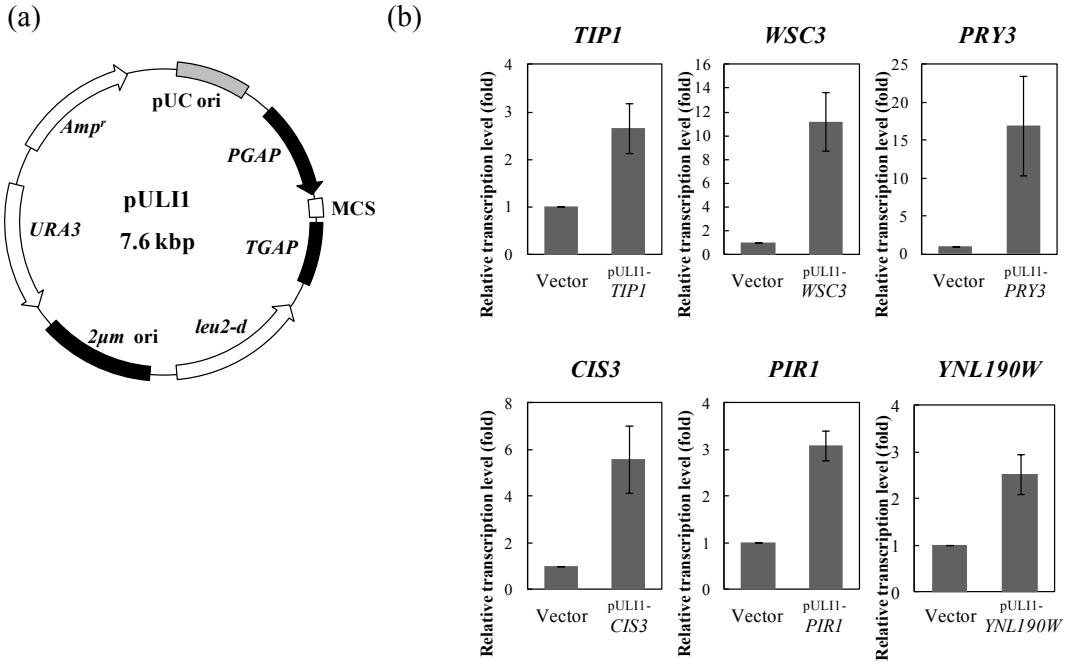

Fig. 5 Overexpression of genes encoding the cell wall proteins in the MT8-1 strain. (a) The pULI1 vector (Miura et al., 2012) was used for the overexpression of the genes encoding the cell wall proteins. (b) Confirmation of overexpression in the MT8-1 transformants harboring plasmids of pULI1-TIP1, pULI1-WSC3, pULI1-PRY3, pULI1-CIS3, pULI1-PIR1, and pULI1-YNL190W. The transformants were grown in SDC+AHLW to the mid-log phase at OD_{600} = 2–3. Overexpression of genes encoding cell wall proteins was confirmed by quantitative real-time PCR. The transcription levels were normalized by using the transcription levels of *ACT1*. The error bars indicate the standard deviation of 3 independent experiments.

because *TIP1* and *YNL190W* were assumed to have signal sequences at both the C and N termini. Overexpression of the genes encoding the cell wall proteins was evaluated by quantitative real-time RT-PCR (Fig. 5b). All genes were confirmed to be upregulated in the strains that contained the overexpression plasmids compared to the genes in the control strain, which harbored the pULI1 vector, although the level of expression varied among the genes. These differences could be due to differences in the basal transcription levels of the genes.

Involvement of the cell wall proteins in the tolerance to hydrophobic and hydrophilic organic solvent

To examine whether the cell wall proteins upregulated in KK-211 are responsible for organic solvent tolerance, the MT8-1 strains overproducing the cell wall proteins were tested for their ability to grow on agar plates in the presence of hydrophobic organic solvents (Fig.

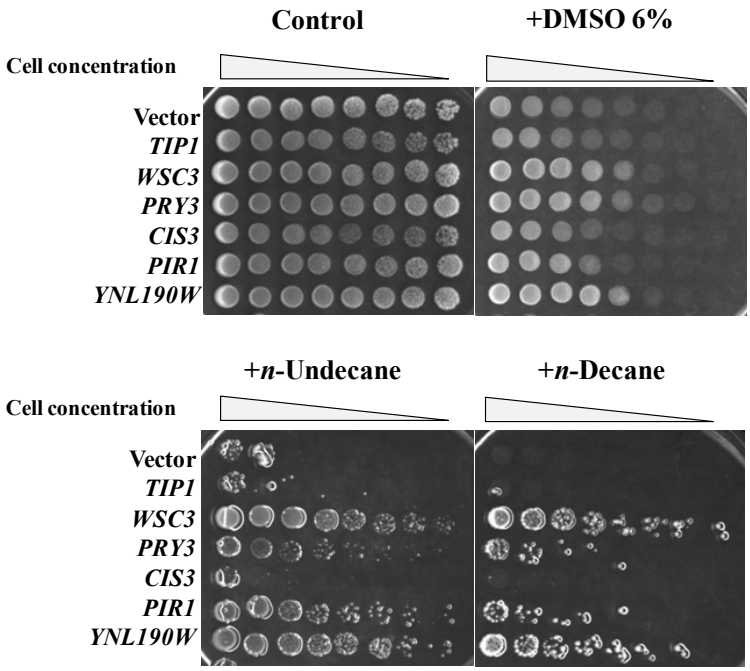

Fig. 6 Spotting assay of MT8-1 overproducing the cell wall proteins (Tip1p, Wsc3p, Pry3p, Cis3p, Pir1p, and Ynl190wp) on agar plates in the presence of organic solvents. For the hydrophobic organic solvent tolerance test, 5 ml of *n*-undecane or *n*-decane was overlaid. For the hydrophilic organic solvent tolerance test, the cell suspensions were spotted onto DMSO-containing agar plates in glass petri dishes. All the plates were incubated for 3 days for the control plates and *n*-undecane-overlaid plates, and for 4.5 days for the *n*-decane-overlaid plates and DMSO-containing plates.

6). The strain harboring pULI1 was used as the control. The strains that overproduced the cell wall proteins were cultivated to the stationary phase and were serially diluted and spotted onto agar plates. The cells were then exposed to hydrophobic organic solvents, or plated onto agar plates containing 6% (v/v) DMSO. Following exposure to the hydrophobic organic solvents, the MT8-1 strains overexpressing *WSC3*, *PRY3*, *PIR1*, and *YNL190W* grew better than the MT8-1 strain containing pUL1 (vector). In the presence of DMSO, the MT8-1 strains overexpressing *WSC3*, *PRY3*, and *YNL190W* grew better than the MT8-1 strain containing pUL1 (vector) (Fig. 6). In the absence of any organic solvents, no significant difference was observed in the growth rates of MT8-1 overproducing the cell wall proteins and MT8-1 containing pULI1 (Fig. 6). These results suggested that *WSC3*, *PRY3*, *PIR1*, and *YNL190W* are involved in hydrophobic organic solvent tolerance, and that *WSC3*, *PRY3*, and *YNL190W* enhance the hydrophilic organic solvent tolerance. In particular, *WSC3* and *YNL190W* seemed to enhance both hydrophobic and hydrophilic organic solvent tolerance.

Discussion

In our previous study (Matsui et al., 2008), *PDR1* was revealed as a regulatory gene related to hydrophobic organic solvent tolerance in the yeast *S. cerevisiae*. In addition, we showed that the MT8-1 *PDR1* R821S mutant, which is a laboratory strain containing a *PDR1* R821S mutation, possessed tolerance to the hydrophobic organic solvents, isooctane and *n*-nonane. Our study revealed that the MT8-1 *PDR1* R821S mutant could also tolerate the hydrophilic organic solvent DMSO as well as hydrophobic organic solvents (Fig. 2). This result indicated that the *PDR1* R821S mutation enabled tolerance of yeast cells to both hydrophobic and hydrophilic organic solvents. In this study, from the transcriptional changes in the hydrophobic organic solvent-tolerant KK-211 strain, we identified and classified some of the genes encoding proteins capable of enhancing hydrophobic and hydrophilic organic solvent tolerance.

Our study showed that ABC transporters in yeast played a role in organic solvent tolerance. Among the 4 ABC transporters that were upregulated in the organic solvent-tolerant KK-211 strain, namely Pdr10p and Snq2p, contributed to the hydrophobic organic solvent tolerance (Fig. 4). Surprisingly, the Yor1p-overproducing strain showed reduced tolerance to hydrophobic organic solvents. Thus, in spite that Yor1p seemed to have

the reverse effect on hydrophobic organic solvent tolerance, although Yor1p was overproduced in the hydrophobic organic solvent-tolerant KK-211 strain. Although it remained unclear and further investigation should be necessary, the activated Pdr1p in KK-211 seemed to upregulate all of the downstream genes including *YOR1* and *PDR15*. In the case of hydrophilic organic solvent tolerance, while all the analyzed strains were able to grow on the agar plates with 6% DMSO, the Snq2p-overproducing and Yor1p-overproducing MT8-1 strains grew better than the control strain (Fig. 4). This result suggested that Snq2p and Yor1p enhance DMSO tolerance in the *PDR1* R821S mutant. Therefore, overproduction of Yor1p was effective only in hydrophilic organic solvent tolerance, and weakened tolerance to *n*-decane and *n*-undecane, (Fig. 4). In addition, overproduction of Snq2p led to increased tolerance to both hydrophobic and hydrophilic organic solvents.

In bacteria, efflux pumps have been involved in organic solvent tolerance (Ramos et al., 1998; Rojas et al., 2001). In *E. coli*, the energy-dependent AcrAB-TolC efflux pump, which is known as multidrug pump (Nikaido, 1996), likely extrudes solvents (Tsukagoshi and Aono, 2000). ABC transporters tested in this study are known as NTP-dependent drug efflux pumps of yeast. Therefore, it is likely that overproduced ABC transporters export organic solvents from inside of the cell, leading to increased tolerance.

In this study, we identified and classified the transporters involved in tolerance to hydrophobic organic solvents, namely, Pdr10p and Snq2p, and the transporters involved in hydrophilic organic solvent tolerance, namely, Snq2p and Yor1p. The sequence similarities within their nucleotide-binding domains (NBDs) dictates that Pdr10p, Snq2p, and Pdr15p belong to the ABC subfamily G (ABCG), and Yor1p belongs to the ABC subfamily C (ABCC) (Paumi et al., 2009). Transporters within the same subfamily have similar functions. ABCG transporters discharge various hydrophobic compounds, including cholesterol, steroids, phospholipids, and many structurally unrelated xenobiotics (Taglicht and Michaelis, 1998; Schmitz et al., 2001; Velamakanni et al., 2007). ABCC transporters predominantly transport xenobiotic compounds or toxic metabolites that are conjugated to glutathione, glucuronide, or sulfur (Homolya et al., 2003; Paumi et al., 2009). However, there was no obvious relationship between the groups to which these transporters belonged and the involvement in hydrophobic or hydrophilic organic solvent tolerance.

We also showed that cell wall proteins are functional in organic solvent tolerance. So far,

studies on organic solvent-tolerant *E. coli* have suggested that the outermost cell surface was involved in the organic solvent tolerance (Aono and Kobayashi, 1997). Our previous study on organic solvent-tolerant KK-211 also indicated that the cell surface affinity of the KK-211 strain to the hydrophobic organic solvent isooctane was reduced (Miura et al., 2000). However, the exact cell surface components that are important for organic solvent tolerance have not been determined. In this study, we first identified and discriminated cell wall proteins that were involved in tolerance to hydrophobic or hydrophilic organic solvents. Among the cell wall proteins upregulated in the organic solvent-tolerant KK-211 strain, overexpression of *WSC3* and *YNL190W* increased tolerance to both hydrophobic and hydrophilic organic solvents. Wsc3p is a sensor in the stress-activating PKC1-MPK1 signaling pathway, which is involved in cell wall maintenance, and is involved in the synthesis of β-1,3-glucan and the stress response (Verna et al., 1997; Zu et al., 2001; Sekiya-Kawasaki et al., 2002; Wojda et al., 2003). Overproduction of Wsc3p possibly boosts the cell wall integrity pathway, and strengthens the cell wall structure. Ynl190wp is a hydrophilin; hydrophillins are proteins that are essential in the desiccation-rehydration process and are characterized by a Gly content >6% and a hydrophilicity index >1.0 (Garay-Arroyo et al., 2000). Therefore, increased levels of Ynl190wp may result in the cell wall becoming more hydrophilic, thereby creating a barrier to protect the inner regions of the cell wall.

Determining which ABC transporters and cell wall proteins were responsible for tolerance to which type of solvent provides useful information to improve the tolerance of yeast to desired solvents. Specific overexpression of the genes involved in tolerance to specific solvents would lead to improved tolerance, compared to expression of whole genes regulated by Pdr1p, which include effective and non-related, even counterproductive-effect genes. The combined expression of effective genes will be also helpful to improve tolerance than expression of a single gene. Importantly, improving the cellular tolerance to particular solvents will facilitate for the use of whole-cell biocatalysts in industrial reactions.

Summary

First, we showed that the *PDR1* R821S mutant, which has been previously shown to be tolerant to hydrophobic organic solvents, also showed tolerance to the hydrophilic organic

solvent DMSO. Among the upregulated genes in the hydrophobic organic solvent-tolerant KK-211 strain, we identified and discriminated genes encoding ABC transporters and cell wall proteins that were involved in hydrophobic and hydrophilic organic solvent tolerance in yeast with overexpression of each target genes. Specific overexpression of the identified genes would improve the tolerance to particular organic solvents for practical use.

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Chapter II

Activation of signaling pathways related to cell wall integrity and multidrug resistance by organic solvents

Organic solvents are toxic to living cells. In eukaryotes, cells with organic solvent tolerance have only been found in *S cerevisiae* (Matsui et al., 2006). In yeast, the R821S mutation in a transcription factor in multidrug resistance, Pdr1p, was identified to confer organic solvent tolerance (Matsui et al., 2008). In the previous chapter, we found that the ABC transporters Pdr10p and Snq2p, and the cell wall-related proteins, particularly Wsc3p and Ynl190wp, contributed to hydrophobic organic solvent tolerance. Overproduction of these proteins led increase of organic solvent tolerance.

Even though it has been possible to both isolate and engineer yeast cells with organic solvent tolerance, the signaling pathways that wild-type cells activate in response to organic solvent stress are not known. Therefore, to understand how yeasts naturally respond to organic solvents, we examined whether the PDR pathway and the CWI pathway are involved in the response to organic solvent stress.

Material and methods

Yeast strains and media

E. coli DH5α strain (Toyobo) was used as a host for plasmid construction as described in Chapter I. *S. cerevisiae* strains used in this study are listed in Table 1. *S. cerevisiae* strains isogenic to BY4741 (*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*) were used in the study. All the single gene deletion strains based on *S. cerevisiae* BY4741 were purchased from Open Biosystems byThermo Fisher Scientific (Waltham, MA, USA). Yeast cells were grown at 30°C in yeast extract- peptone-dextrose (YPD) or SD medium (0.67% yeast nitrogen base without amino acids, 2% glucose) containing appropriate auxotrophic supplements such as 0.002% L-histidine (H), 0.002% uracil (U), 0.003% L-leucine (L), and 0.003% L-methionine (M). To prepare SDC medium, 0.5% casamino acids were added to SD medium. Agar plates

for the spotting assay were prepared by adding 2% (w/v) agar to the media. Drugs were added to autoclaved media using stock solutions of 1 μg/μL cycloheximide (CHX) in aqueous solution; 20 μg/μL ketoconazole (KET) in DMSO or 10 μg/μL 4-nitroquinoline 1-oxide (4NQO) in DMSO. For selection of cells containing pAUR135, a stock solution of 1 μg/μL Aureobasidin A (Takara Bio, Shiga, Japan) in ethanol was added at a final concentration of 0.5 μg/mL. Yeast transformation was performed using the Frozen-EZ Yeast Transformation II Kit (Zymo Research, Irvine, CA, USA).

Table 1 Strains used in this study

Strain	Genotype	Reference
BY4741	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*	EUROSCARF
BY4741 *pdr1Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *pdr1Δ::kanMX4*	Open Biosystems
BY4741 *pdr3Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *pdr3Δ::kanMX4*	Open Biosystems
BY4741 *pdr1Δpdr3Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *pdr1Δ::kanMX4 pdr3Δ0*	This study
BY4741 *wsc3Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *wsc3Δ::kanMX4*	Open Biosystems
BY4741 *ynl190wΔ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *ynl190wΔ::kanMX4*	Open Biosystems
BY4741 *wsc1Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *wsc1Δ::kanMX4*	Open Biosystems
BY4741 *wsc2Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *wsc2Δ::kanMX4*	Open Biosystems
BY4741 *mid2Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *mid2Δ::kanMX4*	Open Biosystems
BY4741 *mtl1Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *mtl1Δ::kanMX4*	Open Biosystems
BY4741 *rom2Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *rom2Δ::kanMX4*	Open Biosystems
BY4741 *mpk1Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *mpk1Δ::kanMX4*	Open Biosystems
BY4741 *rlm1Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *rlm1Δ::kanMX4*	Open Biosystems
BY4741 *swi4Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *swi4Δ::kanMX4*	Open Biosystems
BY4741 *swi6Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *swi6Δ::kanMX4*	Open Biosystems

Plasmid construction

All primers used in this study are listed in Table 2. The vectors pRS415 and pRS416 (Sikorski and Hieter 1989), which are centromeric plasmids with Amp^r, and carrying the *LEU2* and *URA3* genes, respectively, for selection, were used to express *PDR1* and *PDR3*. *PDR1* and *PDR3* were amplified from the *S. cerevisiae* BY4741 genome using the primers

Table 2 Primers used in this study

Primers	Sequence[a]
PDR1-proC-F	5'-TAGTGGGGCCCCCATGAAGAAATCATATAG -3' (*Apa*I)
PDR1-termC-R	5'-CTGGTGAGCTCCATAAGAAATAC -3' (*Sac*I)
PDR3-proC-F	5'-TAGTGGGTACCTCTCAGCTATTCCTTG-3' (KpnI)
PDR3-termC-R	5'-CTGGTGAGCTCTATGCGCAATGTCTTTC -3' (*Sac*I)
PDR1-R821S-F	5'-GACAATCGTCACT**a**GTATCATGCTTTTG-3'
PDR1-R821S-R	5'-CAAAAGCATGATAC**t**AGTGACGATTGTC-3'
PDR1-F815S-F	5'-GAGCTTTTTCTT**c**CCTGACAATCGTC-3'
PDR1-F815S-R	5'-GACGATTGTCAGG**g**AAGAAAAAGCTC-3'
pdr3-8-F	5'-GGATATTTAATT**g**AAAAAAATATCG-3'
pdr3-8-R	5'-CGATATTTTTTT**c**AATTAAATATCC-3'
pdr3-18-F	5'-CGTTGAAAAATCA**a**GCTTTCATTTA-3'
pdr3-18-R	5'-TAAATGAAAGC**t**TGATTTTTCAACG-3'
PDR5-F	5'-TTTAGGAGCTCATGCCCGAGGCCAAGCTTA -3' (*Sac*I)
PDR5-R	5'-TCAAAACTAGTTTATTTCTTGGAGAGTTTACCGTTC-3' (*Spe*I)
PDR5dTAA-R	5'-TCAAAACTAGTTTCTTGGAGAGTTTACCGTTC-3' (*Spe*I)
PDR5-S558Y-F	5'- CTATTTCCGTGGTT**a**TGCTATG -3'
PDR5-S558Y-R	5'- GAATTGCAAAAAACATAGCA**t**AACCACG -3'
CAGT-MCS-Dsred-F	5'-CAGTGAATTCGAGCTCCTCGAGCGGGGATCCTCTAGAGGTGGATCTGGTGGCGAC-3' (MCS; *Eco*RI, *Sac*I, *Xho*I, *Bam*HI, *Xba*I)
CAGT-SalI-stop-Dsred-R	5'-CAGTGTCGACTTACTGGGAGCCGGAGTGG -3' (*Sal*I)
Primers for quantitative real-time RT-PCR analysis	
ACT1-Frt	5'-TCGTTCCAATTTACGCTGGTT-3'
ACT1-Rrt	5'-ACCGGCCAAATCGATTCTC-3'
PDR5-Frt	5'-AGGGCCGTTCAATCTGAATTAG-3'
PDR5-Rrt	5'-GCAGTTATCGAACCTTTCTTTGG-3'
WSC3-Frt	5'-GGAAATCGCACGACCAATTAG-3'
WSC3-Rrt	5'-ACCGAACGAATAGGGCTGATAA-3'
YNL190W-Frt	5'-CGCCCCTGGTCCATCTAAT-3'
YNL190W-Rrt	5'-GCAGCACTACCAGCGGTAACA-3'
PDR1-Frt	5'-GCCCGAATACAGCACACACA-3'
PDR1-Rrt	5'-AAGGGCTGCGGTAAGTGATTT-3'
PDR3-Frt	5'-GCCGCCTTGTGATGACAC-3'
PDR3-Rrt	5'-CAACTGCGACTTCGTTTTATCAA-3'
TFC1-Frt	5'-TGCCACTTAATGTCTCCACAAAA-3'
TFC1-Rrt	5'-CGATGCCCCCACACATTT-3'

[a] Underlines indicate the restriction sites for enzymes shown in parentheses.
Lower case with bold type indicates the mutagenesis sites.

PDR1-proC-F and PDR1-termC-R, and PDR3-proC-F and PDR3-termC-R, respectively, with their own promoter and terminator. Single-amino acid mutations were introduced into Pdr1p or Pdr3p using mutagenesis primer sets (PDR1-R821S-F, PDR1-R821S-R), (PDR1-F815S-F, PDR1-F815S-R), (*pdr3-8*-F, *pdr3-8*-R), and (*pdr3-18*-F, *pdr3-18*-R) by polymerase chain reaction (PCR). The PCR fragments for *PDR1* and *PDR3* were inserted between the *Apa*I and *Sac*I sites of pRS415, and the *Kpn*I and *Sac*I sites of pRS416, respectively. The *PDR1* F815S R821S mutant was constructed by introducing the F815S mutation into the *PDR1* R821S mutant.

The pULI1-Ds vector was constructed to overproduce proteins with the DsRed monomer fused at the C-terminus under the control of the *PGAP* promoter. A DNA fragment with a multicloning site containing *Eco*RI, *Sac*I, *Xho*I, *Bam*HI, and *Xba*I at the 5′ end and a DsRed monomer-encoding sequence with a stop codon at the 3′ end was amplified using pKRD4 (Kuroda et al., 2009) as a template and the primers CAGT-MCS-DsRed-F and CAGT-*Sal*I-stop-DsRed-R. The fragment was then inserted between the *Eco*RI and *Sal*I sites of the multicloning vector pULI1 (Miura et al., 2012). DNA fragments encoding *PDR5* with/without a stop codon were amplified using the primers PDR5-F and PDR5-R/PDR5-dTAA-R from the genomic extract from wild-type *S. cerevisiae* BY4741. *PDR5*-encoding fragments were digested with *Sac*I and *Spe*I and inserted into the *Sac*I and *Xba*I sites of pULI1-Ds to construct pULI1-*PDR5* and pULI1-*PDR5*-Ds. The *PDR5* S558Y mutant, which suppresses resistance to CHX (Sauna et al., 2008), was introduced into pULI-*PDR5*-Ds as described below. DNA fragments encoding *PDR5* S558Y were generated by PCR using the primers PDR5-S558Y-F and PDR5-S558Y-R. PCR fragments were digested with *Bgl*II to replace the *Bgl*II-*Bgl*II section of pULI1-*PDR5*-Ds. The resulting plasmid is referred to as pULI1-*PDR5*-S558Y-Ds.

The pAUR135-*pdr3Δ* plasmid for deletion of the *PDR3* open reading frame (ORF) was constructed as follows. The upstream sequence of the *PDR3* ORF (1200 bp) and terminator sequence (494 bp) were linked via an *Apa*I sequence and cloned into the *Kpn*I and *Sac*I sites of pAUR135 (Takara Bio).

Construction of the S. cerevisiae BY4741 pdr1Δ pdr3Δ double deletion strain

The *S. cerevisiae* BY4741 *pdr1Δ pdr3Δ* double deletion strain was constructed by

deleting the ORF of *PDR3* from the *S. cerevisiae* BY4741 *pdr1Δ* strain using the pAUR135-*pdr3Δ* construct described above. pAUR135-*pdr3Δ* was linearized with *Nae*I, and then introduced into *S. cerevisiae* BY4741 *pdr1Δ*. Transformants were selected based on Aureobasidin A resistance, followed by selection via galactose induction to remove the vector region. The *PDR3* deletion was confirmed by PCR, DNA sequencing, and drug resistance assays.

Spotting assay

Yeast cells were pre-incubated in 10 mL of SD or SDC medium with appropriate amino acids for 36 h until they reached the stationary phase. Cells were collected from 1 mL of culture by centrifugation and resuspended in 1 mL of sterile water. For the spotting assay for drug resistance, the cell suspensions were serially diluted 10-fold from an optical density at 600 nm (OD_{600}) of 10^{0} to 10^{-5}. Next, 10 μL of each cell suspension was spotted on agar plates made either with or without drugs. The spotting assay for organic solvent tolerance tests has been described in Chapter I. Briefly, cell suspensions were adjusted to an OD_{600} of 10, and then serially diluted to OD_{600} of 10×2^{0}, 10×2^{-1}, 10×2^{-2}, 10×2^{-3}, 10×2^{-4}, 10×2^{-5}, 10×2^{-6}, and 10×2^{-7}. Each cell suspension (5 μl) was spotted onto agar plates prepared in glass petri dishes to avoid possible eluted compounds from plastic containers. Organic solvents were overlaid, ensuring that the surfaces of the plates were entirely covered. Plates were incubated at 30°C for 2–4.5 days, depending on the drugs or organic solvents used in the experiment.

RNA extraction for transcription analysis of Pdr1p and Pdr3p mutants

For total RNA isolation of Pdr1p and Pdr3p mutants, cells were pre-cultured in 10 mL of the appropriate media for 36 h. Next, the cells were inoculated into 10 mL of fresh medium, adjusting the concentration to an OD_{600} of 0.1. Cells were cultured for 15–19 h until the mid-log phase, when Pdr1p mutants were at an OD_{600} of 2–3 and Pdr3p mutants were at an OD_{600} of 4.5–5.5. Cells were collected from 10 mL of culture and disrupted using glass beads, and total RNAs were extracted using the RNeasy® Mini kit (QIAGEN, Hilden, Germany) with DNase I digestion according to the manufacturer's protocol. Three independent experiments were performed.

RNA extraction from cells exposed to drugs or organic solvents

Wild-type *S. cerevisiae* BY4741 and isogenic *pdr1Δpdr3Δ* cells were pre-cultured in 10 mL of SDC+HLMU for 36 h until the stationary phase. The cells were inoculated into 100 mL of SDC+HLMU at an initial OD_{600} of 0.1 and incubated for 12 h until the late exponential phase. Each culture was divided into three flasks containing 30 mL each, and exposed to 0.2 μg/mL of CHX, 20 mL of isooctane, or no chemicals (as the negative control), and incubated with vigorous shaking. After 15 min and 60 min, 10 mL of the cell culture was collected for analysis. Total RNAs were extracted using the ISOGEN-LS (Nippongene, Tokyo, Japan) reagent, which contains phenol and guanidine thiocyanate. After ISOGEN-LS was added, cells were disrupted using glass beads. RNAs were extracted with chloroform, precipitated with isopropanol, and washed with 70% (v/v) ethanol. The collected RNAs were dissolved in RNase-free water.

Quantitative real-time RT-PCR analysis

Reverse transcription of total RNA samples and real-time PCR reactions were performed as described in Chapter I. For quantitative PCR analysis, the *ACT1* gene was used as an endogenous control to normalize the expression data for each gene except for *mpk1Δ*; because transcription levels of *ACT1* fluctuated in *mpk1Δ*, *TFC1* was used for normalization in *mpk1Δ* instead. The primers, which are listed in Table 2, were designed using Primer Express® software (Applied Biosystems).

Confirmation of overproduction and subcellular localization of Pdr5p by fluorescence microscopy

Overproduction and subcellular localization of Pdr5p were confirmed by observing the red fluorescence emitted from the DsRed monomer fused to Pdr5p. Cells harboring each Pdr5p-producing plasmid or pULI1-Ds (negative control) were cultured in 10 mL of SDC+HLM for 13 h until the exponential phase. Cells were then harvested and washed with PBS. The washed cells were then resuspended in PBS and observed under an Olympus IX71 fluorescence microscope (Olympus Optical Co., Tokyo, Japan), with a 100× objective lens. Red fluorescence was detected with a mirror unit using XF1077 as an excitation filter and XF3090 as an emission filter (Omega Optical, Inc., VT, USA).

Results

The PDR pathway is involved in the response to organic solvent stress

Among the factors found to confer organic solvent tolerance, the transcription factor Pdr1p (Matsui et al., 2008) and the ABC transporters Pdr10p and Snq2p (identified in Chapter I) are all related to multidrug resistance mediated by the PDR pathway (Decottignies et al., 1995; Wolfger et al., 1997). Thus, we assumed that the PDR pathway would be involved in response to organic solvent stresses.

The R821S mutation in Pdr1p results in organic solvent tolerance (Matsui et al., 2008). It was reported in the previous study that the Pdr1p F815S mutant exhibited multidrug resistance via constitutive activation of the PDR pathway (Carvajal et al., 1997). We hypothesized that the two Pdr1p mutants would operate in the same way, that is, the Pdr1p R821S mutation would confer organic solvent tolerance by constitutively activating the PDR pathway in the same way as F815S. Therefore, the organic solvent tolerance of the Pdr1p F815S mutant and the multidrug resistance of the Pdr1p R821S mutant were evaluated. In fact, as shown in Fig. 1, the Pdr1p F815S mutant exhibited tolerance to the organic solvents *n*-undecane, *n*-decane, and *n*-nonane. Likewise, the Pdr1p R821S mutant conferred resistance to the functionally different drugs CHX (protein synthesis inhibitor), KET (sterol synthesis inhibitor), and 4NQO (a DNA-damaging agent). The levels of drug resistance and organic solvent tolerance of the Pdr1p mutants (F815S, R821S, and F815S-R821S) were identical (Fig. 1), suggesting that the F815S and R821S mutations had the same effect on the transcription regulation byPdr1p. These results suggest that both Pdr1p mutants activate the PDR pathway to cause both organic solvent tolerance and multidrug resistance. The fact that the Pdr1p R821S mutant activated the PDR pathway to cause organic solvent tolerance suggested that wild-type Pdr1p might respond to organic solvent stress by inducing downstream genes in the PDR pathway to improve organic solvent tolerance.

The major transcription factor in the PDR pathway, Pdr3p, in organic solvent tolerance

Pdr3p is a functionally homologous transcription factor of Pdr1p that is involved in thePDR pathway to induce multidrug resistance (Delaveau et al., 1994; Moye-Rowley, 2003;

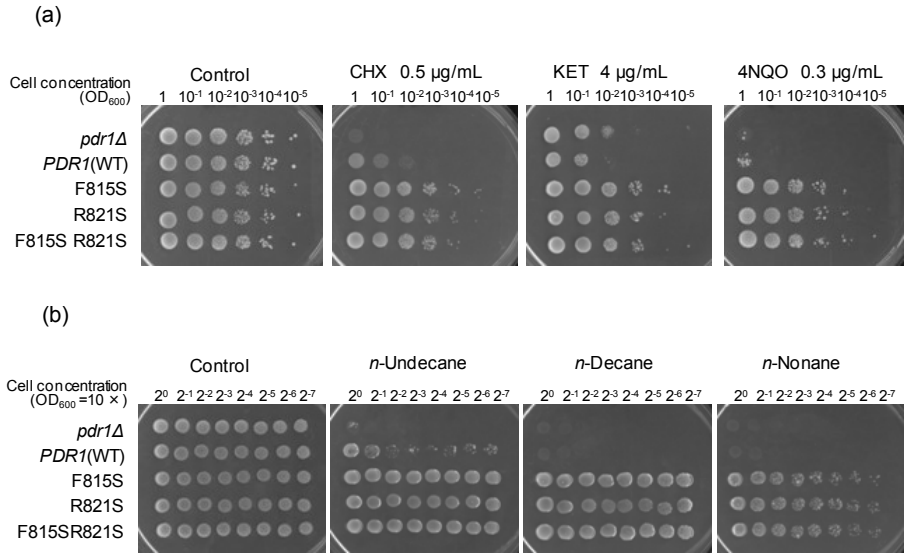

Fig. 1 Multidrug resistance and organic solvent tolerance tests of the *PDR1* F815S mutant and *PDR1* R821S mutant. *S. cerevisiae* BY4741 *pdr1Δ* strains harboring a centromeric vector lacking (*pdr1Δ*) or containing *PDR1* (WT), and *PDR1* with mutations, either multidrug resistance (F815S) or organic solvent tolerance (R821S), or both (F815S R821S) were tested. (a) Spotting assay for evaluation of multidrug resistance. CHX: cycloheximide, KET: ketoconazole, 4NQO: 4-nitroquinoline. Plates were incubated at 30°C for 3 days for the control, KET-, and 4NQO-containing plates, and for 4.5 days for CHX-containing plates. (b) Spotting assay for evaluation of organic solvent tolerance. Plates were incubated for 2 days for the control and *n*-undecane-overlaid plates, 3 days for *n*-decane-overlaid plates, and for 4.5 days for *n*-nonane-overlaid plates.

Jungwirth and Kuchler, 2006; MacPherson et al., 2006). Pdr3p forms homodimers or heterodimers with Pdr1p and recognizes PDREs present in the promoters of target genes (Katzmann et al., 1996; Mamnun et al., 2002). However, Pdr3p possesses some properties that are different from Pdr1p. For example, Pdr3p, but not Pdr1p, is autoregulated (Delahodde et al., 1995), activated by dysfunctional mitochondria (Hallstrom and Moye-Rowley, 2000; Devaux et al., 2002), and negatively regulated by the Hsp70 chaperone Ssa1p (Shahi et al., 2007). To examine whether Pdr3p was also involved in organic solvent tolerance, the organic solvent tolerance of cells with the multidrug resistant Pdr3p mutations *pdr3-8* (K257E) and *pdr3-18* (G834S) (Nourani et al., 1997) was examined. The *pdr3-8* and *pdr3-18* genes expressed under the native *PDR3* promoter were introduced into the BY4741 *pdr3Δ* strain using the centromeric plasmid pRS416. Drug resistance of the constructed *pdr3-8* and *pdr3-18* mutants was confirmed by evaluating CHX resistance with the spotting

assay (Fig. 2a). Next, tolerance of the *pdr3-8* and *pdr3-18* mutants to organic solvents was assessed by the spotting assay on organic solvent-layered agar plates. The *pdr3-8* and *pdr3-18* mutants were able to grow on plates overlaid with organic solvents, whereas the *PDR3* (WT) and vector control strains were not (Fig. 2b). Therefore, the *pdr3-8* and *pdr3-18* mutants showed both organic solvent tolerance and multidrug resistance, indicating that Pdr3p as well as Pdr1p could be involved in the response to organic solvent stress in wild-type cells.

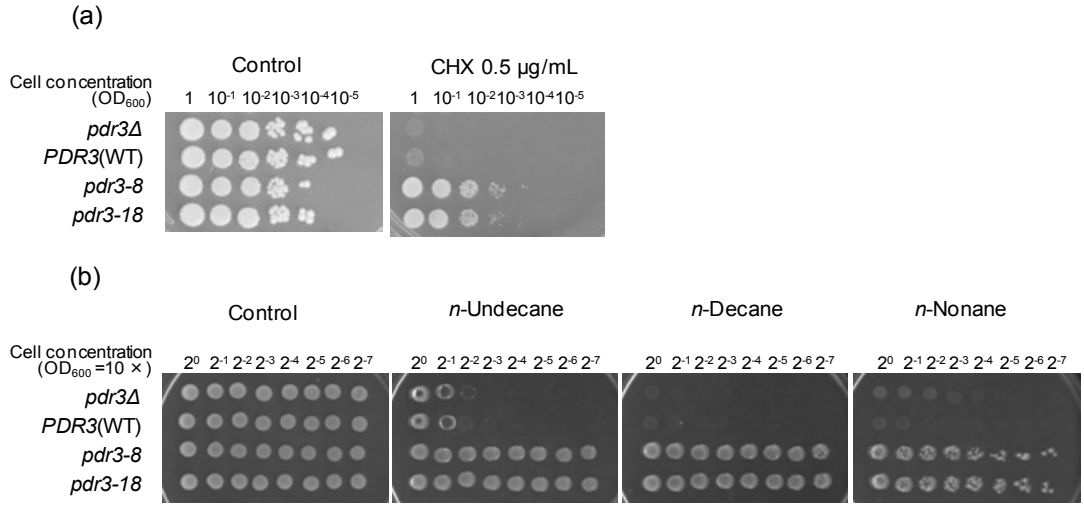

Fig. 2 Organic solvent tolerance test of multidrug-resistant Pdr3p mutants. *S. cerevisiae* BY4741 *pdr3Δ* strains harboring the centromeric vector containing *PDR3* (WT) and the multidrug-resistant mutants *pdr3-8* and *pdr3-18*, and the empty vector (control) were tested for organic solvent tolerance. (a) Spotting assay for the confirmation of multidrug resistance. Incubated for 3 days for control plates, 4 days for CHX-containing plates. (b) Spotting assay for the evaluation of organic solvent tolerance. After cell suspensions were spotted onto agar plates in glass dishes, the plates were overlaid with the indicated organic solvents. Plates were incubated at 30 °C for 2 days for the control and undecane-overlaid plates, 3 days for the decane-overlaid plates, and 4.5 days for the nonane-overlaid plates.

Induction of multidrug transporter Pdr5p by organic solvent stress

To examine whether the PDR pathway is activated by organic solvent stress, the transcriptional response of *PDR5*, which encodes a major transporter in the PDR pathway, was analyzed. Pdr5p is the most widely studied multidrug transporter in the PDR pathway.

Pdr5p is able to recognize and export hundreds of functionally and structurally various drugs out of the cell using energy of ATP hydrolysis (Kolaczkowski et al., 1998). It has been shown that the transcription level of *PDR5* is upregulated by exposure to drugs by the PDR pathway via Pdr1p and Pdr3p (Meyers et al., 1992; Nourani et al., 1997; DeRisi et al., 2000; Simonics et al., 2000). Thus, we hypothesized that Pdr5p might also be upregulated by Pdr1p and Pdr3p in the PDR pathway in response to organic solvents, although the organic solvent tolerant KK-211 strain did not increase transcription levels of *PDR5*.

We first tested whether *PDR5* was induced in response to exposure to organic solvents. Cells grown to exponential phase were exposed to CHX for drug stress (as the positive control), or isooctane for organic solvent stress. Isooctane, which was used for isolating the organic solvent tolerant yeast KK-211 strain (Matsui et al., 2006), was selected for this experiment because it was expected to pose severe stress to cells owing to its high toxicity (Kawamoto et al., 2001). At 15 min and 60 min after the addition of CHX or isooctane, cells were collected and subjected to transcriptional analysis. *PDR5* was upregulated in response to CHX, as previously reported (Gao et al., 2004) (Fig. 3a). *PDR5* was also upregulated after exposure to isooctane (Fig. 3a). These results indicate that *PDR5* is induced by exposure to organic solvents to improve organic solvent tolerance in wild-type cells.

We next examined whether overproduction of Pdr5p would contribute to the induction of organic solvent tolerance. A strain overproducing Pdr5p with the DsRed monomer fused to its C-terminus was constructed by introducing the plasmid pULI1-*PDR5*-Ds. A strain overproducing Pdr5p without a DsRed monomer and a strain harboring the empty pULI1-Ds vector were also constructed as controls. Overproduction and localization of Pdr5p at the cell surface were confirmed by the observation of red fluorescence of the DsRed monomer under a fluorescence microscope (Fig. 3b). The transporter activities of the overproduced Pdr5p with and without the DsRed monomer were verified by their increased resistance to CHX (Fig. 3c). Next, the organic solvent tolerance of Pdr5p-overproducing strains was evaluated using the spotting assay on an *n*-undecane-overlaid plate. Strains overproducing Pdr5p either with or without the DsRed monomer showed better growth on plates overlaid with *n*-undecane than the strain with pULI1-Ds (control plasmid) (Fig. 3d). This result indicates that Pdr5p contributes to organic solvent tolerance as well as to multidrug resistance. The

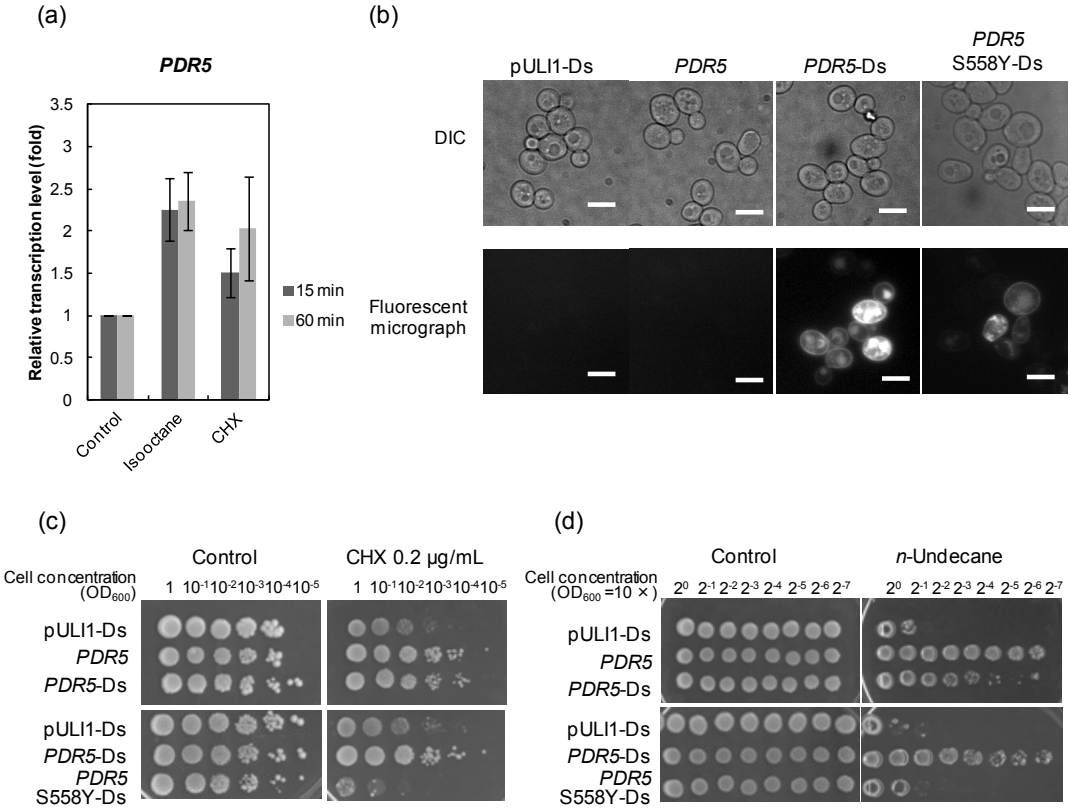

Fig. 3 Induction of *PDR5* encoding significant multidrug transporter by exposure to organic solvent. (a) Transcription analysis after exposure to an organic solvent and a drug. *S. cerevisiae* BY4741 (WT) was grown to the exponential phase and treated with isooctane or 0.2 μg/mL CHX. Transcription levels were normalized to that of *ACT1*. Error bars represent the standard deviation from three biological independent experiments. (b) Overproduction and localization of Pdr5p fused with the DsRed monomer. *S. cerevisiae* BY4741 (WT) carrying the empty vector (pULI1-Ds), plasmids containing *PDR5* fused with the DsRed monomer (*PDR5*-Ds), *PDR5* S558Y mutant fused to the DsRed monomer (*PDR5* S558Y-Ds), or *PDR5* without the DsRed monomer (*PDR5*) were observed under a fluorescence microscope. Scale bar: 5 μm DIC: differential interference contrast. (c) Drug resistance of Pdr5p-overproducing strains. Plates were incubated for 3 days for the control plates and for 4 days for CHX-containing plates. (d) Organic solvent tolerance of Pdr5p-overproducing strains. Cell suspensions were spotted onto plates in glass dishes. The plates were overlaid with undecane as an organic solvent. Plates were incubated for 3 days for both the control and the undecane-overlaid plates.

strain overproducing Pdr5p tagged with the DsRed monomer showed slightly weaker tolerance compared to the strain overproducing Pdr5p without the DsRed monomer. This

result indicated that the DsRed monomer at the C-terminus partially inhibited transporter activity, but did not completely block the activity. This result indicates that, when exposed to organic solvents, yeast cells activate the transcription factors Pdr1p and Pdr3p and upregulate the transcription levels of transporters effective against organic solvents, including Pdr5p, to elevate organic solvent tolerance.

Although overproduction of Pdr5p increased organic solvent tolerance, it possibly altered membrane integrity, which would indirectly improve organic solvent tolerance. To determine whether the transporter activity of Pdr5p contributes to organic solvent tolerance, we performed an organic solvent tolerance test on the *PDR5* S558Y mutant (Sauna et al., 2008). The Pdr5p S558Y mutant retains ATPase activity and drug-binding capability but does not have CHX resistance, supposedly because ATPase activity and transport are uncoupled (Sauna et al., 2008). DsRed monomer-fused Pdr5p S558Y was overproduced in the *S. cerevisiae* BY4741 wild-type strain by introducing pULI1-*PDR5*-S558Y-Ds (Fig. 3b), and organic solvent tolerance was compared to the strain overproducing Pdr5p (wild-type) tagged with the DsRed monomer and the strain containing pULI1-Ds (the control strain). The Pdr5p S558Y mutant lost not only resistance to CHX, but also tolerance to *n*-undecane. This result suggested that organic solvents possibly were transported by Pdr5p in an energy-dependent manner (Fig. 3c, d).

Upregulation of the genes encoding cell wall-related proteins by organic solvent stress

In Chapter I, it was shown that overproduction of the cell wall-related proteins Wsc3p and Ynl190wp improved tolerance to both hydrophobic and hydrophilic organic solvents. Therefore, we examined whether genes encoding Wsc3p and Ynl190wp, that are involved in organic solvent tolerance, were induced in response to exposure to organic solvents. Cells grown to exponential phase were exposed to isooctane as an organic solvent stress. After incubation for 15 min and 60 min, cells were collected and subjected to transcriptional analysis. *WSC3* and *YNL190W* were upregulated by exposure to isooctane (Fig. 4a).

We then attempted to identify the signaling pathway by which *WSC3* and *YNL190W* were induced in response to organic solvents. First, because the PDR pathway was shown to contribute to organic solvent tolerance, we investigated whether *WSC3* and *YNL190W* were regulated via the PDR pathway. The transcription levels of *WSC3* and *YNL190W* were

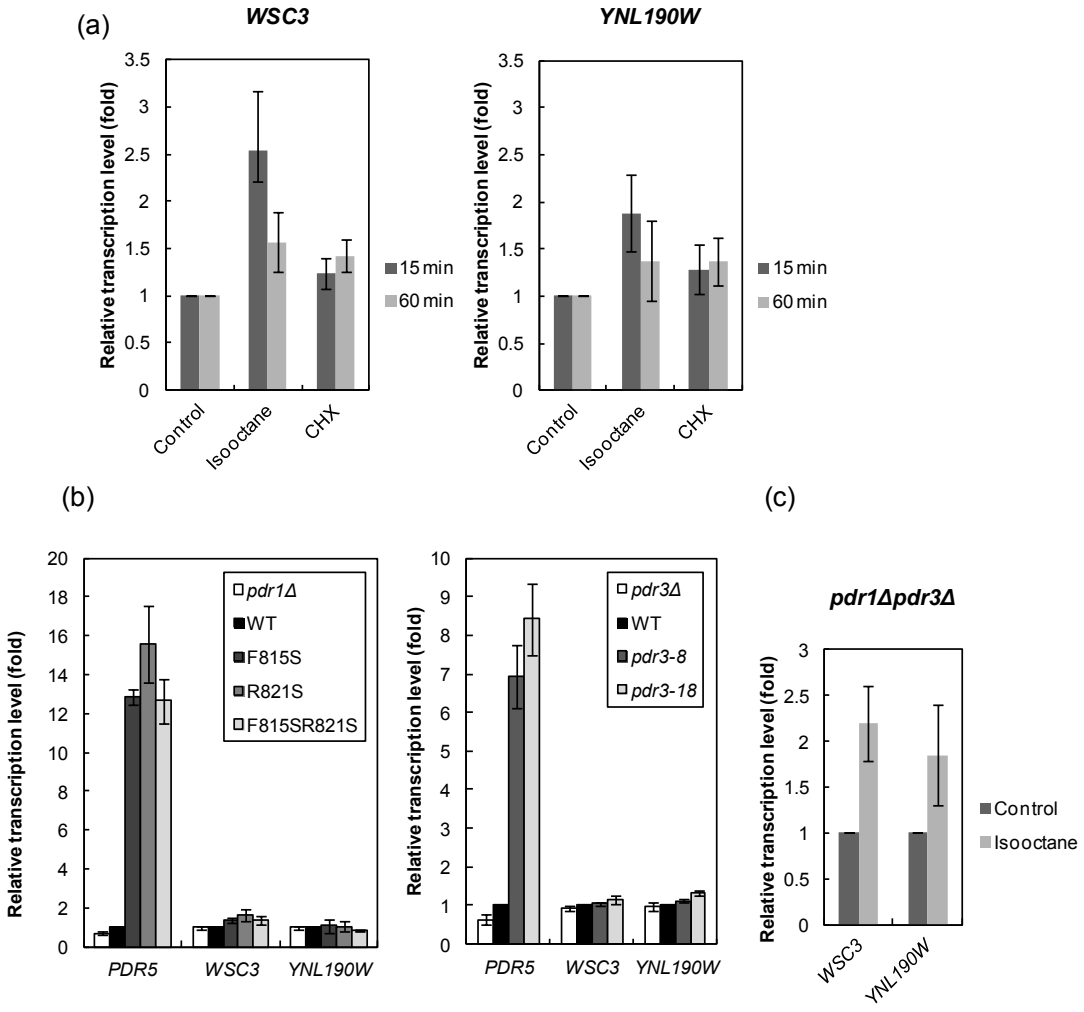

Fig. 4 Transcription levels of genes encoding organic solvent tolerance-related cell wall-related proteins under organic solvent stress or the activated PDR pathway.

Transcription levels of *WSC3* and *YNL190W* in (a) wild-type cells after exposure to an organic solvent (isooctane) or a drug (CHX), (b) Pdr1p mutants (left) and Pdr3p mutants (right), and (c) *pdr1Δpdr3Δ* cells after exposure to organic solvent (isooctane). Total RNAs were extracted from cells grown to the exponential phase. Transcription levels were analyzed using quantitative real-time RT-PCR. The transcription levels were normalized to that of *ACT1*. Error bars represent the standard deviation from three independent experiments.

determined in mutants of two representative transcription factors in the PDR pathway, *PDR1* multidrug resistant mutants (*PDR1* F815S, *PDR1* R821S, or *PDR1* F815S-R821S) and *PDR3* multidrug resistant mutants (*pdr3-8*, *pdr3-18*). *PDR5* transcription levels were also evaluated as a positive control. Transcription levels were analyzed by quantitative real-time RT-PCR. As previously reported (Meyers et al., 1992; Nourani et al., 1997; DeRisi et al.,

2000; Simonics et al., 2000), *PDR5* transcription levels were upregulated in both Pdr1p and Pdr3p mutants (Fig. 4b). In contrast, transcription levels of *WSC3* and *YNL190W* in Pdr1p or Pdr3p mutants remained unchanged from the levels seen in the wild-type strain (Fig. 4b). Based on these results, *WSC3* and *YNL190W* seem not to be induced through the PDR pathway under organic solvent stress, although *WSC3* and *YNL190W* were involved in organic solvent tolerance. This conclusion is also supported by the observation that the *S. cerevisiae* BY4741 *pdr1Δ pdr3Δ* double deletion strain was still able to induce *WSC3* and *YNL190W* in response to organic solvent stress (Fig. 4c). The genes encoding cell wall-related proteins, *WSC3* and *YNL190W*, were suggested to be induced by another signaling pathway independent of the PDR pathway.

Drug-independent regulation of cell wall-related proteins

We also evaluated the transcription levels of *WSC3* and *YNL190W* after exposure to CHX to observe the involvement of drug stress in the transcription regulation of *WSC3* and *YNL190W*. However, *WSC3* and *YNL190W* did not show significant induction in response to CHX (Fig. 4a). These results show that *WSC3* and *YNL190W* are not upregulated by drug stress. In addition, we tested whether Wsc3p and Ynl190wp improved drug resistance as well as organic solvent tolerance. The deletion strains *wsc3Δ* and *ynl190wΔ*, isogenic to *S. cerevisiae* BY4741, were used for the spotting assay to evaluate the contribution of these genes to organic solvent tolerance or multidrug resistance. Consistent with the results in Chapter I, *wsc3Δ* and *ynl190wΔ* showed weaker organic solvent tolerance than wild-type strains (Fig. 5a). In the multidrug resistance test, both deletion mutants showed weaker resistance to CHX, but showed no difference in resistance to KET and 4NQO compared to the wild-type strain (Fig. 5b). These results suggest that while Wsc3p and Ynl190wp participate in organic solvent tolerance, they only improve resistance to specific drugs such as CHX, and do not improve multidrug resistance.

The CWI pathway involved in induction of WSC3 and YNL190W

We next identified the pathway by which *WSC3* and *YNL190W* were activated as a result of exposure to organic solvents. As Wsc3p is one of the sensor proteins in the CWI pathway (Levin 2011), we hypothesized that the CWI pathway might be involved in the

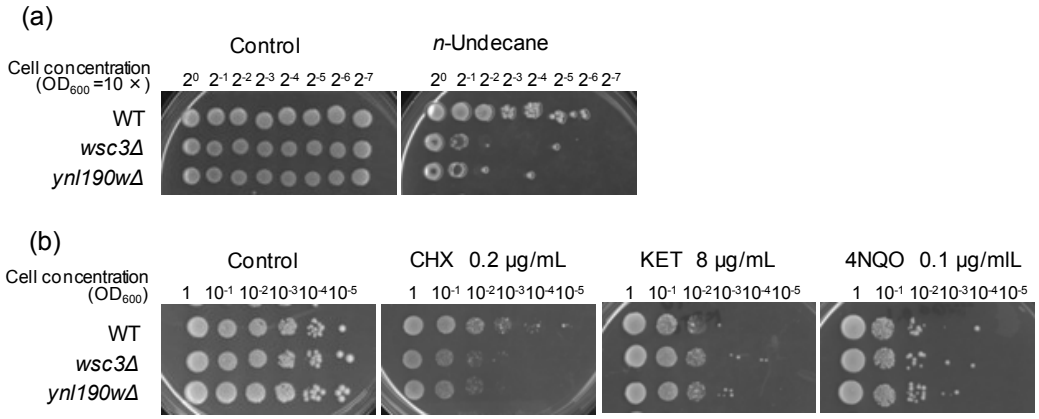

Fig. 5 Multidrug resistance of deletion strains of genes encoding organic solvent tolerance-related cell wall-related proteins, *WSC3* and *YNL190W*

S. cerevisiae BY4741 (WT) cells or isogenic *wsc3Δ* or *ynl190wΔ* strains were tested on (a) organic solvent tolerance and (b) multidrug resistance.

induction of *WSC3* and *YNL190W*. We analyzed the transcription levels of *WSC3* and *YNL190W* after being exposed to isooctane for 60 min in each single deletion strain for components involved in the CWI pathway, such as the five cell surface sensor proteins (Wsc1p, Wsc2p, Wsc3p, Mid2p, and Mtl1p), a GDP/GTP exchange factor (Rom2p), a MAPK component (Mpk1p), and transcription factors (Rlm1p, Swi4p, and Swi6p). Among the strains in which the genes encoding cell surface sensor proteins had been deleted, *wsc3Δ* and *mid2Δ* showed lower induction of both *WSC3* and *YNL190W* (Fig. 6). This result suggests not only that the CWI pathway is involved in the induction of *WSC3* and *YNL190W*, but also that Wsc3p and Mid2p are important for sensing cell wall stresses posed by organic solvents. In addition, deletion of the transcription factors Swi4p and Swi6p resulted in no induction of these genes by isooctane, whereas deletion of the transcription factor Rlm1p did not affect the induction of these genes by isooctane (Fig. 6). These results indicate that *WSC3* and *YNL190W* are regulated by Swi4p and Swi6p, but not by Rlm1p. Deletion of Mpk1p also inhibited the upregulation of these genes by isooctane (Fig. 6), providing further support for the involvement of the CWI pathway in the response to organic solvents. The *YNL190W* transcription level was somewhat upregulated in the *mpk1Δ* strain in the absence of organic solvents compared to wild-type cells, however, the reason for this is not known.

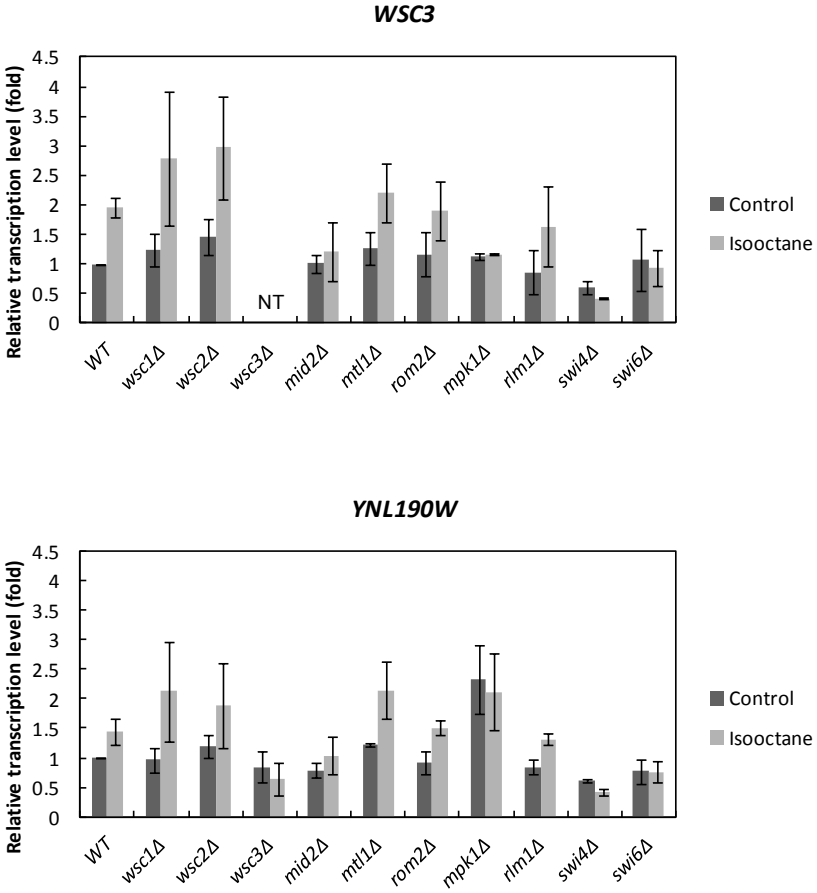

Fig. 6 Transcription levels of genes encoding cell wall-related proteins in deletion strains of components of the CWI pathway under organic solvent stress. *S. cerevisiae* BY4741 (WT) and deletion strains of components of the CWI pathway were grown to the exponential phase and treated with isooctane. Total RNAs were extracted, and transcription levels were analyzed using quantitative real-time RT-PCR. Transcription levels after isooctane exposure were normalized to that of *ACT1* (except for *mpk1Δ*) or *TFC1* (*mpk1Δ*). Error bars represent the standard deviation from three independent experiments.

Discussion

In this study, we showed that exposure to organic solvents induced two pathways, the PDR pathway and the CWI pathway (Fig. 7). Although some of the proteins that are involved in organic solvent tolerance in eukaryotes had been identified in Chapter I and our previous studies, the specific signaling pathways activated to deal with organic solvent stress

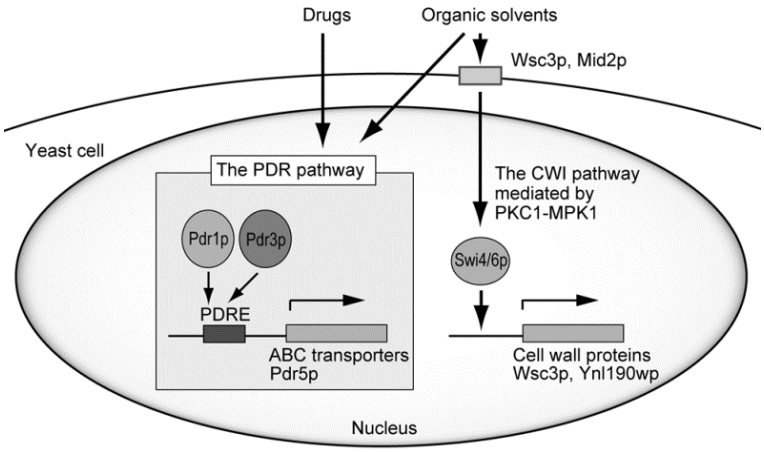

Fig. 7 A model depicting the signaling pathways in response to organic solvents. Following exposure to organic solvent, the PDR-related pathway, which is involved in the response to drugs, is activated to induce ABC transporters such as *PDR5*; in addition, the CWI signaling pathway mediated by PKC1-MPK1 is also activated and upregulates the transcription levels of the genes encoding cell wall-related proteins such as *WSC3* and *YNL190W*.

in wild-type cells were not known. The results of the present study demonstrated that wild-type yeast responded to organic solvents by inducing the PDR pathway and the CWI pathway to alter transcription levels to protect themselves from the stress. The stress posed by organic solvents is thought to be complicated and including membrane damage, protein denaturation, and cell wall damage (Sikkema et al., 1995; Segura et al., 2012). Thus, it is plausible that multiple pathways are simultaneously activated to deal with organic solvent stress.

In our previous studies of organic solvent tolerant yeast, multiple proteins in the multidrug resistance-related PDR pathway were implicated: the transcription factor Pdr1p (Matsui et al., 2008) and the ABC transporters Pdr10p and Snq2p (Chapter I). In this study, we found that the response to organic solvent stress shared the same PDR pathway involved in multidrug resistance. Our observation that the PDR pathway was capable of inducing organic solvent tolerance enhanced the importance of the PDR pathway. In addition to structurally and functionally various drugs, our results added organic solvents to the list of substrates that the PDR pathway can combat. The drug efflux pump Pdr5p seems to recognize at least *n*-undecane as a substrate and to be possible to efflux out of the cell using

energy from ATP hydrolysis (Fig. 3). In the PDR pathway, several drugs interact directly with Pdr1p and Pdr3p to upregulate transcription levels of downstream genes (Thakur et al., 2008). Whether organic solvents interact with Pdr1p and Pdr3p directly in the same manner as drugs to activate the transcription of downstream genes should be further investigated.

In Chapter I, we showed that the cell wall-related proteins Wsc3p and Ynl190wp were involved in organic solvent tolerance. In this study, we showed that *WSC3* and *YNL190W* were also upregulated by organic solvent stress (Fig. 4a). Although organic solvent tolerance and multidrug resistance appeared to share the PDR pathway, we showed that regulation of the cell wall-related proteins is independent of Pdr1p/Pdr3p, the transcription factors in the PDR pathway (Figs. 4b, c). This result is consistent with the fact that there is no known PDRE, the sequence recognized by Pdr1p and Pdr3p, in the promoter regions of both *WSC3* and *YNL190W*. Moreover, according to the previous report of microarray analyses of the multidrug resistance mutants *pdr1-3* and *pdr3-7* (DeRisi et al., 2000), although several genes encoding cell wall-related proteins were upregulated, *WSC3* and *YNL190W* were not. The fact that *wsc3Δ* and *ynl190wΔ* did not affect drug resistance except in the case of CHX (Fig. 5) also supported this result. Therefore, Wsc3p and Ynl190wp may only function in CHX resistance. The difference may be because CHX, KET, and 4NQO have different structural and functional properties. Since the all tested agents do not act directly on cell walls, the differences in the physical and chemical properties of individual drugs may have led to the different resistances of the cell wall deletion strains. However, the results shown in Fig. 4a demonstrated that exposure to CHX was not able to activate the signaling pathway that would induce the cell wall-related proteins. Thus, the cell wall-related proteins are not utilized in response to CHX in wild-type cells even though the cell wall-related proteins themselves have some effect on CHX resistance.

Instead, our study suggests that *WSC3* and *YNL190W* are induced by the CWI pathway (Fig. 6). The CWI pathway, mediated by the PKC1-MPK1 signaling pathway, is induced by several stresses, such as cell wall damage, heat and osmotic stresses, and some chemicals that affect membranes, such as tea tree oil (Straede et al., 2007). It is reasonable that *WSC3* and *YNL190W* are induced by the CWI signaling pathway, since the outermost region of the cell is the first point to make contact with organic solvents. The fact that the CWI pathway responded to organic solvents but not drugs serves to differentiate organic solvent tolerance

from multidrug resistance.

Of the five cell surface sensors, Wsc3p and Mid2p seem to be the ones that detect organic solvents (Fig. 6). There are two groups of cell surface sensors: Wsc-type sensors (Wsc1p, Wsc2p, and Wsc3p) and the others (Mid2p and Mtl1p) (Jendretzki et al., 2011). We hypothesized that organic solvents pose various stresses on the cell wall to activate multiple cell surface sensors, as the sensors from different groups were involved. As cell surface sensors respond to chemicals that damage both the cell wall and the cell membrane, the question as to whether these sensors detect damage to the cell wall or to the cell membrane needs further investigation.

Of the sensors, Wsc1p and Mid2p are thought to be the most important under the conditions tested to date (Levin 2011). Previous studies on deletion strains showed that Wsc1p is the most important for resistance to elevated temperature and to compounds interfering with the cell wall (Calcofluour white, Congo red, or glucan synthase inhibitory echinocandins) and with the plasma membrane, whereas Wsc2p and Wsc3p are thought to be less important (Gray et al., 1997; Verna et al., 1997; Zu et al., 2001). Recently, Wsc2p has been suggested to be as important as Wsc1p (Wilk et al., 2010). In this study, however, we found that Wsc3p was the most important for the response to organic solvent stress, and that Wsc1p and Wsc2p were not necessary (Fig. 6). This result shows that Wsc3p is not a redundant protein version of Wsc1p as was previously thought, but instead has a unique function in organic solvent stress response. In contrast to Wsc1p, Mid2p is suggested to be important for responding to pheromones (Rajavel et al., 1999) and for triggering chitin synthase activity (Levin, 2011). It is possible that organic solvents also perturb chitin structures in the yeast cell wall.

At the downstream end of the CWI pathway, *WSC3* and *YNL190W* were likely to be mainly regulated by the transcription factors Swi4p and Swi6p, but not by Rlm1p (Fig. 6). Swi4p and Swi6p together form the Swi4p/Swi6p-dependent cell cycle box (SCB)-binding factor (SBF) complex to regulate transcription in response to cell wall stress. Swi4p recognizes the SCB sequence, and Swi6 allows Swi4p to bind to DNA by relieving the autoinhibition of Swi4p (Levin 2011). Although there are no SCB sequences in the promoter regions of *WSC3* and *YNL190W*, SBF complexes may regulate *WSC3* and *YNL190W* directly or indirectly.

We suggested that when yeasts came into contact with organic solvents, the cells activated not only the PDR pathway but also the CWI pathway to deal with the stresses induced by organic solvents. This study provided a model how yeast cells overcome the multiple stresses induced by organic solvents.

Summary

We demonstrated that the pleiotropic drug resistance (PDR) pathway contributed to response to organic solvent stress. Activation of the PDR pathway by mutations in the transcription factors Pdr1p and Pdr3p led to organic solvent tolerance. Exposure to organic solvent also induced transcription levels of *PDR5*, which encodes a major drug efflux pump. Overproduction of Pdr5p improved organic solvent tolerance, presumably by exporting organic solvents out of the cell. In addition, we showed that the cell wall integrity (CWI) pathway was induced in response to organic solvents to upregulate genes encoding the cell wall-related proteins Wsc3p and Ynl190wp. *WSC3* and *YNL190W* were upregulated independently of the PDR pathway. Among the components of the CWI pathway, the cell surface sensors Wsc3p and Mid2p and the transcription factors Swi4p and Swi6p appeared to be particularly involved in the response to organic solvents. Our findings indicate that *S. cerevisiae* activates two different signaling pathways, the PDR pathway and the CWI pathway, to cope with stresses from organic solvents.

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Chapter III

Differential roles of the multidrug resistant transcription factors Pdr1p and Pdr3p in organic solvent tolerance in yeast

In *S. cerevisiae*, both transcription factors, Pdr1p and Pdr3p, play important roles in multidrug resistance (MDR). Although they were shown to have redundant roles in MDR (Delaveau et al., 1994; Moye-Rowley, 2003; Jungwirth and Kuchler, 2006; MacPherson et al., 2006), Pdr3p possesses some different properties from Pdr1p. For example, Pdr3p but not Pdr1p is autoregulated (Delahodde et al., 1995), activated by dysfunctional mitochondria (Hallstrom and Moye-Rowley, 2000; Devaux et al., 2002), and negatively regulated by the Hsp70p chaperone Ssa1p (Shahi et al., 2007).

In Chapter I and Chapter II, we showed that the PDR pathway as well as the CWI pathway were induced in response to organic solvents. In this chapter, we analyzed whether Pdr1p and Pdr3p function in the same way in response to organic solvents and we discovered the differential contributions between Pdr1p and Pdr3p.

Materials and methods

Yeast strains and media

S. cerevisiae strains isogenic to BY4741 were used in this study. All yeast strains used are listed in Table 1. Yeasts were grown in SDC+HLMU medium.

RNA extraction from cells exposed to organic solvents or drugs

Total RNAs were extracted from cells after exposure to organic solvents as previously described in Chapter II. Cells grown to the late exponential phase were treated with isooctane or 0.2 μg/ml CHX for 60 min. Total RNAs were extracted using the ISOGEN-LS (Nippongene, Tokyo, Japan) reagent. For reverse transcription of total RNA, a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used.

Table 1 Strains used in this study

Strain	Genotype	Reference
BY4741	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*	EUROSCARF
BY4741 *pdr1Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *pdr1Δ::kanMX4*	Open Biosystems
BY4741 *pdr3Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *pdr3Δ::kanMX4*	Open Biosystems
BY4741 *pdr8Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *pdr8Δ::kanMX4*	Open Biosystems
BY4741 *stb5Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *stb5Δ::kanMX4*	Open Biosystems
BY4741 *yrr1Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *yrr1Δ::kanMX4*	Open Biosystems
BY4741 *rdr1Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *rdr1Δ::kanMX4*	Open Biosystems
BY4741 *rom2Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *rom2Δ::kanMX4*	Open Biosystems
BY4741 *rpd3Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *rpd3Δ::kanMX4*	Open Biosystems
BY4741 *ssa1Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *ssa1Δ::kanMX4*	Open Biosystems
BY4741 *psd1Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *psd1Δ::kanMX4*	Open Biosystems
BY4741 *lge1Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *lge1Δ::kanMX4*	Open Biosystems
BY4741 *med12Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *med12Δ::kanMX4*	Open Biosystems
BY4741 *rtg1Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *rtg1Δ::kanMX4*	Open Biosystems
BY4741 *rtg2Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *rtg2Δ::kanMX4*	Open Biosystems
BY4741 *rtg3Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* *rtg3Δ::kanMX4*	Open Biosystems

Quantitative real-time RT-PCR analysis

Real-time PCR reactions were performed as described in Chapter I. For quantitative PCR analysis, the *ACT1* gene was used as an endogenous control to normalize the expression data for each gene. The primers, which are listed in Table 2, were designed using Primer Express® software (Applied Biosystems).

Table 2 Primers used in this study

Primers	Sequence
ACT1-Frt	5'-TCGTTCCAATTTACGCTGGTT-3'
ACT1-Rrt	5'-ACCGGCCAAATCGATTCTC-3'
PDR5-Frt	5'-AGGGCCGTTCAATCTGAATTAG-3'
PDR5-Rrt	5'-GCAGTTATCGAACCTTTCTTTGG-3'

Results

Differential response to organic solvents between the deletion strains of Pdr1p and Pdr3p

We first examined the transcription levels of *PDR5*, encoding a major multidrug transporter, after exposure to isooctane as an organic solvent. The transcription level after addition of CHX as a drug stress was also measured as a control. The addition of CHX upregulated *PDR5* transcription levels to about two fold in both *pdr1Δ* and *pdr3Δ* strains. This result supports that in MDR both Pdr1p and Pdr3p have overlapping functions.

In response to exposure to isooctane, the transcription level of *PDR5* was elevated to ~two-fold the wild type strain (Fig. 1, also described in Chapter II). Surprisingly, in *pdr1Δ*, transcription levels of *PDR5* were further upregulated to ~seven-fold after exposure to isooctane, while in *pdr3Δ*, upregulation of *PDR5* had almost disappeared (Fig. 1). This result suggested that Pdr3p, but not Pdr1p, was important in the stress response to organic solvents, though Pdr1p and Pdr3p have overlapping functions in MDR. Pdr1p seemed to have a rather inhibitory function in the response to organic solvent. This result underlined the difference between response to organic solvents and that to drugs even in the PDR pathway.

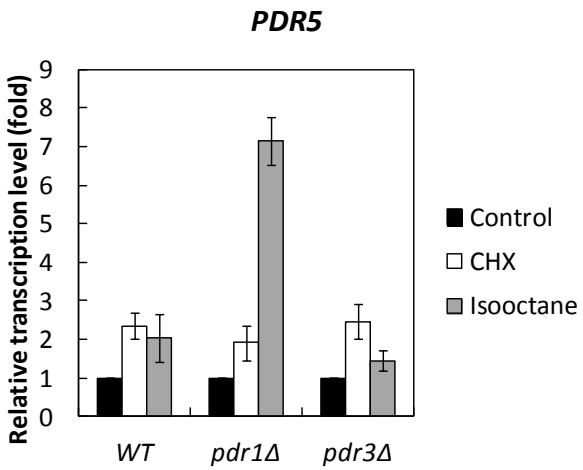

Fig. 1 Expression levels of *PDR5* in *pdr1Δ* and *pdr3Δ* cells under stress from drugs or organic solvents. Transcription levels of *PDR5* after exposure to CHX or isooctane for 60 min were evaluated in BY4741 *pdr1Δ* and *pdr3Δ* cells.

Effect of other transcription factors in PDR5 upregulation by organic solvents

There are other transcription factors that are related to transcription regulation in MDR, such as Stb5p (Akache and Turcotte, 2002), Yrr1p (Cui et al., 1998; Akache et al., 2004), Rdr1p (Hellauer et al., 2002), and Pdr8p (Hikkel et al., 2003). Stb5p was thought to interact

with Pdr1p, but do not interact with Pdr3p (Akache et al., 2004). Rdr1p was reported to have inhibitory effects in MDR via PDREs (Hellauer et al., 2002). Pdr8p seemed to recognize similar sequences to PDREs (Hikkel et al., 2003). Yrr1p has been shown to form homodimers that regulate the expression of PDR-related genes (Cui et al., 1998; Lucau-Danila et al., 2003; Akache et al., 2004).

In order to assess if these transcription factors were cooperatively involved in the response to organic solvents, we analyzed the transcription induction levels of *PDR5* in transcription factor deletion strains.

Deletion of other transcription factors related to PDREs did not show any impact on transcription levels on *PDR5* (Fig. 2). The result indicates that transcription induction of *PDR5* in response to organic solvents was mainly driven by Pdr1p and Pdr3p.

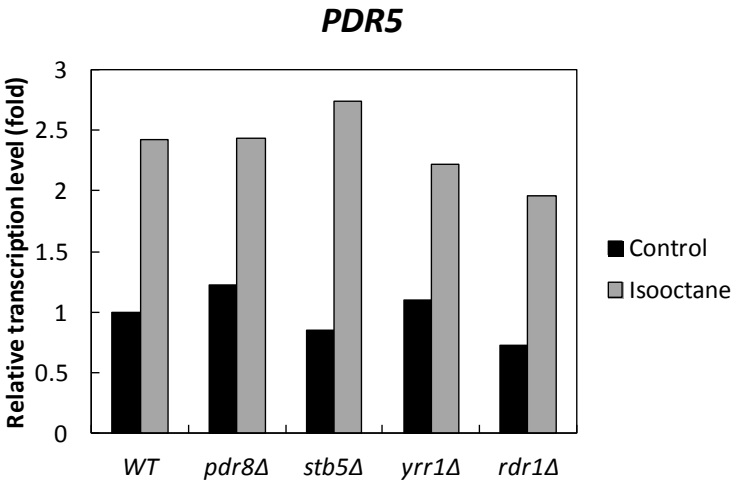

Fig. 2 Expression of *PDR5* in deletion strains of transcription factors under the stress of isooctane. Transcription levels of PDR5 after exposure to isooctane for 60 min was evaluated.

Mitochondrial signaling pathways in response to organic solvents

We then examined which factors are involved in signaling transduction of organic solvent stress, specifically related to Pdr3p, but not to Pdr1p. In the previous study, there were several reports of proteins that acted on Pdr3p, but not on Pdr1p, such as Ssa1p (Shahi et al., 2007). One crucial difference is the involvement of Pdr3p in retrograde signaling pathways from the mitochondria to the nucleus (Hallstrom and Moye-Rowley, 2000; Devaux et al., 2002). Cells that lack their mitochondrial DNA (ρ^0 cells) have been shown to induce

MDR by upregulation of *PDR5* (Hallstrom and Moye-Rowley, 2000). In the retrograde pathway, Lge1p is required for mitochondrial signaling to *PDR5* (Zhang et al., 2005). In addition, Psd1p is also involved in the common retrograde signaling pathway from the mitochondria (Delahodde et al., 2001; Mamnun et al., 2002; Gulshan et al., 2008). We assumed that the retrograde signaling pathway may be involved in the signaling pathway of organic solvent stress, as an inhibitory function of Pdr1p was also reported in the retrograde signaling pathway. In mitochondria-derived upregulation of *PDR5*, deletion of Pdr1p further increases Pdr3p-dependent up-regulation of *PDR5* in ρ^0 cells, indicating that the Pdr1p regulator may also exert inhibitory functions (Zhang and Moye-Rowley, 2001). To determine the proteins involved in signal transduction of organic solvent stress to Pdr3p, transcription levels of *PDR5* in deletion strains of the candidate proteins were evaluated. Transcription levels were normalized by that of *ACT1*.

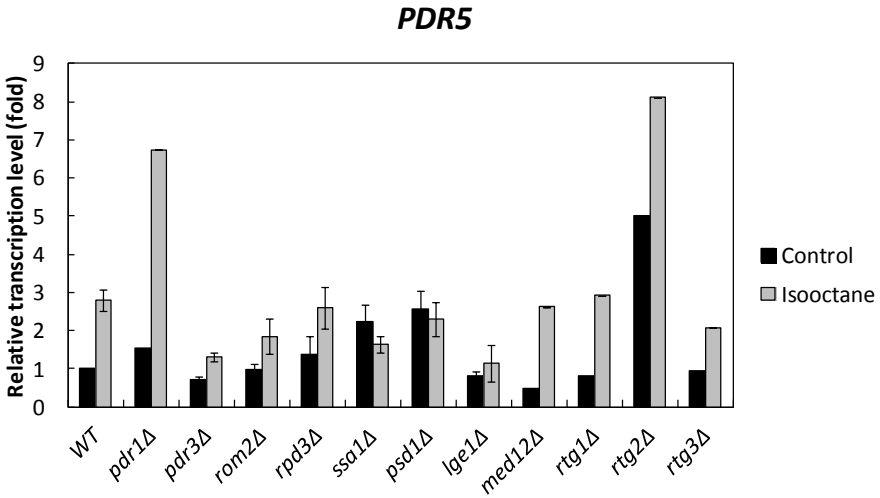

Fig. 3 Expression of *PDR5* in deletion strains of proteins related to Pdr3p under the stress of isooctane. Transcription levels of PDR5 after exposure to isooctane for 60 min was evaluated.

The deletion strain of *lge1Δ* had a lesser upregulation to that of *pdr3Δ*. *psd1Δ* and *ssa1Δ* demonstrated upregulation before isooctane stress, and showed no additional induction by isooctane (Fig. 3). As Psd1p and Lge1p are involved in retrograde pathway from mitochondrial dysfunction, it is possible that the mitochondrial pathway would be involved in organic solvent tolerance. Deletion of Rtg1p and Rtg3p did not affect expression levels of *PDR5*, though Rtg1p and Rtg3p are transcription factors in the retrograde signaling pathways.

This result was consistent with the previous report that the loss of *RTG1* only slightly diminished *PDR5* activation in ρ^0 cells. The previous study indicated that the retrograde pathway connecting MDR to the mitochondria is distinct from that linking *CIT2* expression to mitochondrial status (Moye-Rowley, 2005).

Expression level of Pdr3p in the absence of Pdr1p

Pdr1p and Pdr3p recognize the same sequence, PDREs, in the transcriptional regulation of downstream genes, including *PDR5*. It was possible that production of Pdr3p was increased to compensate for the amount of Pdr1p in the *pdr1Δ* strain, which led to increased induction in *pdr1Δ* by organic solvent stress. To test this hypothesis, transcription levels of *PDR3* were quantified in wild-type and *pdr1Δ* with or without organic solvent stress.

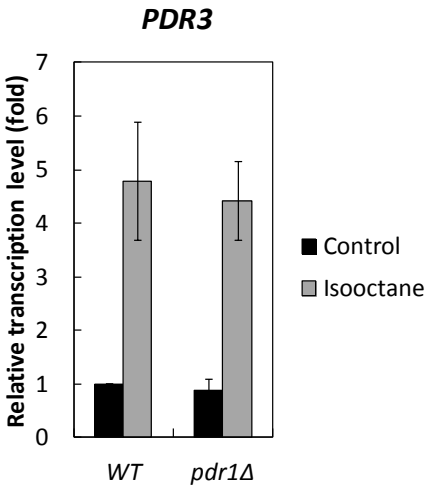

Fig. 4 Transcription levels of *PDR3* in *pdr1Δ* with or without isooctane. Transcription levels were evaluated after 60 min from addition of isooctane.

While expression of *PDR3* was increased after exposure to isooctane in both wild-type and *pdr1Δ* cells, the transcription level of *PDR3* was almost identical in wild-type and *pdr1Δ* strains in both before and after exposure to isooctane (Fig. 4). This result indicated that transcription of *PDR3* was not induced in order to compensate for the absence of Pdr1p. Therefore, the transcription activation of *PDR5* in *pdr1Δ* was not due to the upregulation of Pdr3p.

Discussion

In this study, we found distinct functions of Pdr1p and Pdr3p in response to organic solvent stress. The organic solvent seemed to utilize the mitochondrial signaling pathways

which only transmit signals to Pdr3p but not Pdr1p to induce downstream genes. It should be analyzed whether organic solvents damage mitochondria to induce the retrograde signaling pathway, or whether only utilization of proteins involved in the retrograde signaling pathway. As Psd1p and Lge1p were not reported to directly interact with Pdr3p, the components that directly distinguish Pdr1p from Pdr3p remain unknown.

In organic solvent tolerant yeast strain, KK-211, a point mutation in Pdr1p was identified as key for such tolerance (Matsui et al., 2008). However, this study revealed that Pdr1p itself was not important in response to organic solvent stress. As the downstream genes of Pdr1p and Pdr3p are overlapping, constant activation of downstream genes by the Pdr1p R821S mutation would result in a gain of organic solvent tolerance in the KK-211 strain.

In the *pdr1Δ* strain, induction levels of *PDR5* in response to organic solvents were elevated as compared to the wild-type strain. Fig. 4 shows that this induction was not due to the increase of Pdr3p in the cell. As the amount of Pdr3p was ~nine fold lower than that of Pdr1p (Ghaemmaghami et al., 2003), the amount of PDRE should be seriously deprived in the *pdr1Δ* strain. However, transcription levels of *PDR5* were not decreased in *pdr1Δ*. Therefore, the current transcription model that Pdr3p always binds to PDREs even without stress seemed implausible.

Summary

In response to organic solvent stress, only Pdr3p contributed to the regulation of downstream genes, while Pdr1p had a rather inhibitory role in transcription induction, though Pdr1p and Pdr3p play overlapping roles in MDR. The signal of organic solvent stress was transmitted by proteins involved in the mitochondrial retrograde signaling pathway to Pdr3p. The result discerned the difference between the organic solvent response and MDR. The result also suggested that the organic solvent-tolerant KK-211 strain gained such tolerance by upregulation of the overlapping genes, Pdr1p and Pdr3p by a gain-of-function in Pdr1p.

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Conclusions

This study aimed to describe the yeast response to organic solvent stress. Although the organic solvent tolerant yeast was isolated, the stress response mechanism was not determined so far. This study revealed that yeast cells apply existing signaling pathways to deal with organic solvent stress, which yeast cells have rarely encountered.

In Chapter I, we identified effector proteins directly involved in organic solvent tolerance. Among the genes upregulated in the organic solvent tolerant KK-211 strain, ABC transporters (Pdr10p, Snq2p, and Pdr15p) and cell wall proteins (Wsc3p and Ynl190wp) enhanced organic solvent tolerance. The specificity of ABC transporters to hydrophobic and hydrophilic solvents was also determined.

In Chapter II, we showed that at least two pathways were activated in response to organic solvents in yeast cells. That is, the PDR pathway inducing MDR, and the CWI pathway, which induces cell wall proteins. The result indicated that organic solvents pose various stresses to cells, resulting in induction of multiple signaling pathways. In addition, we underlined the importance of the PDR pathway which is also involved in organic solvent tolerance. At the same time, we dissected MDR and organic solvent tolerance at the point of involvement of the CWI pathway.

In Chapter III, we showed the difference between homologous transcription factors, Pdr1p and Pdr3p, in response to organic solvents. Pdr3p was involved in the response, but Pdr1p seemed to have inhibitory functions in response. Organic solvent stress seemed to be transmitted via components in the mitochondrial retrograde signaling pathway. The Pdr1p R821S point mutation in the organic solvent-tolerant KK-211 strain seemed to attribute organic solvent tolerance by upregulating the downstream genes that are overlapping targets of Pdr3p.

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Publications

Chapter I

Nishida, N., Ozato, N., Matsui, K., Kuroda, K., Ueda, M. ABC transporters and cell wall proteins involved in organic solvent tolerance in *Saccharomyces cerevisiae*. *J. Biotechnol.* **165**, 145-152 (2013).

Chapter II

Nishida, N., Jing, D., Kuroda, K., Ueda, M. Activation of signaling pathways related to cell wall integrity and MDR by organic solvent in *Saccharomyces cerevisiae. Curr. Genet.* in press.

Chapter III

Nishida, N., Mori, H., Kuroda, K., Ueda, M. Differential roles of the multidrug resistant transcription factors Pdr1p and Pdr3p in organic solvent tolerance in yeast. submitted.

Other publication

Nishida, N., Noguchi, M., K., Kuroda, K., Ueda, M. A system designed for controlling apoptosis in genetically modified *Saccharomyces cerevisiae. Biosci. Biotechnol. Biochem.* in press.