

Title	Functional analysis of PP2C protein phosphatase genes in response to environmental stresses in Saccharomyces cerevisiae
Author(s)	Sharmin, Dilruba
Citation	大阪大学, 2015, 博士論文
Version Type	VoR
URL	https://doi.org/10.18910/52215
rights	
Note	

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# **Doctoral Dissertation**

# Functional analysis of PP2C protein phosphatase genes in response to environmental stresses in Saccharomyces cerevisiae

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November, 2014

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# Abstract

A key mechanism of signal transduction in eukaryotes is reversible protein phosphorylation mediated through protein kinases and protein phosphatases (PPases). Modulation of signal transduction by this means regulates many biological processes. Saccharomyces cerevisiae has 40 PPases, including seven protein phosphatase 2C (PP2C PPase) genes (*PTC1-PTC7*). However, the precise functions remain poorly understood. To elucidate their cellular functions and to identify those that are redundant, we constructed 127 strains with deletions of all possible combinations of the seven PP2C PPase genes. All 127 disruptants were viable under nutrient rich conditions, demonstrating that none of the combinations induced synthetic lethality under these conditions. However, several combinations exhibited novel phenotypes, e.g., the  $\Delta ptc5\Delta ptc7$  double disruptant and the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  quadruple disruptant exhibited low (13°C) and high (37°C) temperature sensitive growth, respectively. Interestingly, the septuple disruptant  $\Delta ptc1 \Delta ptc2 \Delta ptc3 \Delta ptc4 \Delta ptc5 \Delta ptc6 \Delta ptc7$  showed an essentially normal growth phenotype at 37°C. The  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  quadruple disruptant was sensitive to LiCl (0.4 M). Two double disruptants  $\Delta ptc1 \Delta ptc2$  and  $\Delta ptc1 \Delta ptc4$  displayed slow growth and  $\Delta ptc1 \Delta ptc2 \Delta ptc4$ could not grow on medium containing 1.5 M NaCl. The  $\Delta ptc1\Delta ptc6$  double disruptant showed increased sensitivity to caffeine, congo red (CR) and calcoflour white (CFW) compared to each single deletion. Our observations indicate that S. cerevisiae PP2C PPases have a shared and important role in responses to environmental stresses. These disruptants also provide a means for exploring the molecular mechanisms of redundant PTC gene functions under defined conditions. Here, we sought to determine the cellular role of PTC6 in S. cerevisiae with disruption of PTC genes. We found that cells with  $\Delta ptc6$  disruption were tolerant to the cell wall-damaging agents CR and CFW; however, cells with simultaneous disruption of *PTC1* and *PTC6* were very sensitive to these agents. Thus, simultaneous disruption of *PTC1* and *PTC6* gave a synergistic response to cell wall damaging agents. The level of phosphorylated Slt2 increased significantly after CR treatment in  $\Delta ptc1$  cells and more so in  $\Delta ptc1\Delta ptc6$  cells; therefore, deletion of *PTC6* enhanced Slt2 phosphorylation in the  $\Delta ptc1$  disruptant. The level of transcription of *KDX1* upon exposure to CR increased to a greater extent in the  $\Delta ptc1\Delta ptc6$  double disruptant than the  $\Delta ptc1$  single disruptant. The  $\Delta ptc1\Delta ptc6$  double disruptant than the  $\Delta ptc1$  single disruptant. The  $\Delta ptc1\Delta ptc6$  double disruptant than the  $\Delta ptc1$  single disruptant. The analyses indicate that *S. cerevisiae PTC6* participates in the negative regulation of Slt2 phosphorylation and vacuole morphogenesis under cell wall stress conditions.

# **Chapter 1**

# **General Introduction**

## 1.1 Saccharomyces cerevisiae genome and functional genomics

Saccharomyces cerevisiae, commonly known as budding yeast, has been one of the best characterized organisms for understanding and engineering eukaryotic cell function beside its industrial importance. The genome of yeast *S. cerevisiae* was entirely sequenced with 14 Mb (including ~150 ribosomal DNA units clustered on a single chromosome), elucidating 5,885 protein-encoding genes and made available in public in 1996 (Goffeau *et al.*, 1996). This study showed that more than 50% of the sequenced genes were unknown and uncharacterized (Piskur and Langkjaer, 2004). Yeast became popular model for its easy and precise genetic manipulation, and fundamental eukaryotic biology such as cell cycle control. It is also considered to be a reference eukaryote to discover new genes and for investigating physiological phenomena to characterize those unknown genes. Since decades, scientists have used yeast in research probing the molecular mechanism underlying different biological phenomenon.

Apart from these sequence studies, there have been many works that aimed to unravel the function of numerous unknown genes in yeast genome (Hughes *et al.*, 2000). Different experimental techniques were also first implemented in *S. cerevisiae* to ascertain functional genomics, for example, the first genome-wide cDNA array study was designed for *S. cerevisiae* (DeRisi *et al.*, 1997) which lead to large-scale analysis such as expression profiling (Hughes *et al.*, 2000; Uetz *et al.*, 2000; Ito *et al.*, 2001). The literature based on these approaches enabled us to incorporate available genetic, molecular, and biochemical information for *S. cerevisiae*, which involved significant research effort. Integration of knowledge from genes to protein and further to changes in a genome-scale network will be crucial to understand how the individual components in the system interact and influence fundamental new insights regarding overall cell function.

# **1.2** Yeast response to diverse environmental stresses

Both unicellular and multicellular organisms necessitate specific environments inside the cell for optimal growth and function; however sudden changes in the environments outside cell can pose unique and stressful challenges disrupting normal physiological processes. Therefore, as a result of fluctuations in the external surroundings, cells must equip mechanism(s) to maintain their internal system (Gasch, 2007). Yeast cells have evolved to be exceptionally proficient at persisting sudden and often harsh changes in their external environment. Mechanisms that yeast cells usually practice to protect the internal system from the environmental stresses require maintenance of cellular homeostasis through modification in common gene expression and metabolism (Cheng et al., 2000). This stress response program includes ~900 genes in yeast S. cerevisiae whose expression was stereotypically changed during large variety of environmental stresses including temperature shocks, hydrogen peroxide, hyper- or hypoosmotic shock, amino acid starvation, and nitrogen source depletion (Lucau-Danila et al., 2005). However, the regulation of these expression changes is gene-specific and condition-specific. For example, to initiate signal involving general stress response induced by cell wall damage, a transcriptional alteration in the cell wall integrity is perceived (Garcia et al., 2004) and transcriptional alterations result in erroneous protein synthesis. Finally how a particular protein performs different cellular functions in most of the eukaryotic organisms is regulated by the post-transcriptional modifications.

# 1.3 Reversible protein phosphorylation: Regulation of cellular function

Cells respond to an external molecule that transmit signals to the interior through a process known as signal transduction that mediates the sensing and processing of stimuli during environmental stresses. There are signaling circuits that detect, amplify, and convert a signal from outside the cell to a functional change within the cell, often known as signaling

cascades. Organisms from prokaryote to human develop a number of parallel pathways to avoid unexpected activation or deactivation of the signaling cascades through different mechanisms. Among these mechanisms reversible protein phosphorylation was considered as the main post-transcriptional modification involving protein kinases (PKases) and protein phosphatases (PPases) that phosphorylate and dephosphorylate protein, respectively (Figure 1).



Figure 1: Cellular regulation mediated by reversible protein phosphorylation in S. cerevisae.

We have been interested in cellular regulation by protein phosphorylation and dephosphorylation because this is one of the key regulatory systems in all kinds of organisms from prokaryotes to eukaryotes. Since years, most scientists focused on cellular regulation by PKases and therefore, less research had been done on PPases. Eukaryotic PPases are classified into three major families based on structural and functional diversity: phosphoprotein phosphatases (PPP), metal-dependent protein phosphatases (PPM). The PPP and PPM family include the serine/threonine PPases. Protein tyrosine phosphatases (PTP) family which includes protein tyrosine and dual specificity PPases (Figure 2) (Ariño *et al.*, 2011). In *S. cerevisiae* genome, 129 PKases and 40 PPases genes are found (http://www.yeastgenome.org) and these classes of proteins have their corresponding homologs in both higher plants and humans, although the number is not same (Alonso *et al.*, 2004). The functions of PPase genes in *S. cerevisae* are summarized in Table 1. It is well known that abnormal phosphorylation is implicated as a cause or consequence of human diseases such as cancer, diabetes, rheumatoid arthritis and hypertension (Cohen, 2001; Ariño *et al.*, 2011).



Figure 2: Van diagram showing different classes of PPase genes in *S. cerevisae*.

# 1.4 PP2C subfamily of protein phosphatases

In eukaryotes, most of the phosphorylation (>99%) occurs in Ser and Thr residues of proteins. Therefore, elimination of phosphates from these proteins is mediated by Ser/Thr phosphatases. Protein serine/threonine phosphatases (PPases from PPP and PPM family) are classified into type 1 (PP1) and type 2 (PP2) phosphatases, initially based on their substrate preference, metal cation requirement and sensitivity to inhibitors. PP2 enzymes were further divided into three groups: PP2A, PP2B and PP2C. PP2C is unique in requiring  $Mg^{2+}$  or  $Mn^{2+}$ ions for their activity (Ariño *et al.*, 2011). *S. cerevisiae* genome encodes seven genes in the PP2C subfamily: *PTC1* (Phosphatase two C) to *PTC7* that share a highly conserved aminoterminal domain or carboxyl-terminal domain (Figure 3) (Ariño *et al.*, 2011). *S. cerevisiae* PTC (*PTC1-PTC7*) homologs, PP2C $\alpha$  and PP2C $\beta$ , were identified in mammals (Tamura *et al.*, 1989) and, subsequently several homologs of these genes have been identified in various organisms such as fission yeast (Shiozaki *et al.*, 1995), *Leishmania* (Burns *et al.*, 1993) and *Arabidopsis* (Leung *et al.*, 1994) in the past years. PTCs are known to be involved mainly in the regulation of cell growth and stress signaling in both prokaryotes and eukaryotes (Pereira *et al.*, 2011).



**Figure 3**: Schematic representation of the primary structure and percentage of similarity and identity of overlapping regions of different PP2Cs in comparison with Ptc1in *S. cerevisae*.

Family/	ORF/ gene	Function				
Subfamily						
PPP family						
PP1 subfamily	YER133W/GLC7 YML016C/PPZ1 YDR436W/PPZ2 YPL179W/SAL6	Involved in glycogen metabolism, sporulation and mitosis Involved in salt homeostasis Involved in salt homeostasis Regulation of protein synthesis				
PP2A subfamily	YDL047W/SIT4 YDL134C/PPH21 YDL188C/PPH22 YDR075W/PPH3 YNR032W/PPG1	Cell cycle Cell cycle, cell wall maintenance Highly homologous to Pph21p Cell structure Involved in glycogen accumulation				
PP2B subfamily	YLR433C/CNA1 YML057W/CNA2	Some ion transport, cell polarity, mating response Some ion transport, mating response				
Other	YGR123C/PPT1 YLL010C/PSR1 YLR019W/PSR2 YMR277W/FCP1 YLR361C/DCR2 YHR004C/NEM1 YNL222W/SSU72	Human PP5-related PPase Response to stress Functionally redundant Psrlp homolog Carboxy-terminal domain (CTD) phosphatase Interacts with Sic1P, a inhibitor of mitotic cyclin/cyclin- dependent kinase complexes. Regulates nuclear growth. Transcription/ RNA processing factor.				
<b>PPM Family</b>						
PP2C subfamily	YDL006W/PTC1 YER089C/PTC2 YBL056W/PTC3 YBR125C/PTC4 OR090C/PTC5 YCR079W/PTC6 YHR076W/PTC7	Down-regulate the HOG pathway Cdc28p dephosphorylation, involved in HOG pathway Cdc28p dephosphorylation, involved in HOG pathway Cytoplasmic type 2C PPase Involved in regulation of pyruvate dehydrogenase activity Mitochondria type 2C PPase with similarity to mammalian PPK1s Mitochondrially localized type 2C PPase				
<b>PTP family</b> PTP subfamily	YDL230W/PTP1 YDR208W/PTP2 YER075C/PTP3 YPR073C/LTP1 YNL032W/SIW14 YMR036C/MIH1 YJR110W/YMR1 YNL128W/TEP1	Protein tyrosine phosphatase Down-regulate Hog1P and Fus3p MAPK, sporulation Down-regulate Hog1P and Fus3p MAPK, sporulation Similar to PPase from human placenta Involved in nutritional control of the cell cycle S. pombecdc25p homologue Phosphatidylinositol 3-phosphate phosphatase Similar to human tumor suppressor gene				
DSP subfamily	YER028C/CDC14 YBR276C/PPS1 YIR026C/YVH1 YNL053W/MSG5	Function at the late stage of the cell cycle A role in the DNA synthesis phase of the cell cycle Yeast homologue of Vaccinia virus PTP, VHI Dephosphorylate Fus3p				

# Table 1: Forty protein phosphatase genes in S. cerevisiae

	YILI13W/SDP1 YNL099C/OCA1	Negatively regulates SIt2p MAPK Protein tyrosine/ serine/ threonine phosphatase activity
Other	YNR002C	Weak similarity to PPase

Adopted from http://www.yeastgenome.org and www.proteome.com

# 1.5 Mitogen-activated protein kinase (MAPK) pathways in S. cerevisiae

Signaling pathways are important for cells to sense and respond to their environment. The signaling strategies are conserved from fungi to humans, although their activity and phenotypic significances are extensively variable among individuals within a species (Treusch et al., 2014). In S. cerevisiae, both response and resistance to stressors that activate signaling pathways depend on mitogen-activated protein kinase (MAPK) signaling cascades. MAPK cascades are composed of three sequentially acting kinases which sense an extracellular stimulus (such as the presence of mating pheromones, cell wall damage and high osmolarity) and trigger a cellular response by activating transcription factors and other regulatory proteins (Waltermann and Klipp, 2010; Chen and Thorner, 2007). Adaptation to high osmolarity is controlled by the HOG (high osmolarity glycerol) MAPK pathway while damage to the cell wall is sensed by the cell wall integrity (CWI) pathway (Figure 4). Response to MAPK activating stress conditions is highly variable among different yeast species (Kvitek et al., 2008; Liti et al., 2009; Warringer et al., 2011). However, genes of the core MAPK cascades are highly conserved across species although upstream components (such as stress sensors) and downstream targets (such as transcription factors) are very much diverse (Bahn et al., 2007; Wu et al., 2010). How genetic differences in such elements of the MAPK pathways contribute to the phenotypic differences between species of the same species is still not known.



Figure 4: Mitogen-activated protein kinase (MAPK) pathways in S. cerevisiae.

# 1.6 Transcriptional change in response to cell wall stresses

The cell wall of *S. cerevisiae* consists of four classes of macromolecules such as, cell wall proteins (CWPs),  $\beta$ 1,6-glucan,  $\beta$ 1,3-glucan, and chitin, each of which are interconnected by covalent bonds (Boorsma *et al.*, 2004). Two important proteins that control the cell wall integrity pathway are *PKC1* and the *RHO1* GTPase. Rho1 is a regulatory subunit of the  $\beta$ -1,3 glucan synthase complex that binds to Pkc1 which in turn stimulates the cell wall integrity pathway (Guo *et al.*, 2009).

Activation of Slt2 by means of overexpression of the MAP kinase of this pathway results in changed transcription of 25 genes (20 genes up-regulated; 5 genes down-regulated) and 14 genes among them depend on the transcription factor Rlm1 for their expression after Slt2 activation (Jung and Levin, 1999). The majority of genes that show lowered expression

in  $\Delta rlm1$  disruptant also showed lowered transcription in  $\Delta slt2$  disruptant strain. For example,  $\Delta rlm1$  disruption causes a decrease in the mRNA level of *KDX1* (Becerra *et al.*, 2011).

# **1.7** Objectives of the study

There were so many studies on PKases done before. In contrast, knowledge on PPases is very limited even in *S. cerevisiae*. Therefore, we started functional genome science on PPase several years ago. The ultimate goal of this project is to understand the role of all of these PPases in *S. cerevisiae*. Since genetic screening for mutants is troubled by genetic redundancy in *S. cerevisiae*, we were interested in constructing multiple disruptants at various combinations and analyzing functional redundancy. Towards to this goal, a library of 30 single disruptants (Sakumoto *et al.*, 1999) and 435 double disruptants (Sakumoto *et al.*, 2002) was constructed in all possible combinations of 30 PPase genes known at that time and systematically examined for their phenotypes. This analysis led to discovery of several new phenotypes. From those previous studies, we noted two observations. First is that all of 435 double disruptants showing phenotype was unexpectedly small, that is 4 among 435 double disruptants. We thought that these facts may come from two possibilities. The first possibility is that the number of phenotype that was examined in previous study was not enough and secondly, more than two PPases might be involved in some of the cellular functions.

Based upon these considerations, goal of this study is to examine more phenotype for already constructed single and double disruptants of PTC PPases and also construction of triple and multiple disruptants to discover new phenotypes of PTC PPases. The role of PTC genes seems to be conserved in eukaryotes and also a given PTC gene can exhibit distinctive cellular functions. Despite these facts on PP2C enzymes, numerous vital questions remain unanswered. In this study, we focused on PP2C PPases in *S. cerevisiae*. The specific purpose

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of this study is to elucidate the role of each PTC PPase in cell physiology by construction and systematic phenotypic analysis of multiple disruptants of all possible combinations and to further investigate the molecular mechanism of newly discovered phenotype of PTC disruptants.

In Chapter 1 of this dissertation, I have already summarized the importance of reversible protein phosphorylation and signal transduction in response to environmental stresses. The importance of different MAPK signaling pathways in *S. cerevisiae* has also been discussed. In Chapter 2, construction of a series of multiple disruptants of PTC PPases in all possible combinations and the effects of various combinations of PTC gene deletion on stress responses in *S. cerevisiae* was described. In Chapter 3, I presented evidence indicating that Ptc6 participates in negative regulation of Slt2 phosphorylation and vacuole morphogenesis under cell wall stress conditions. The conclusion and general discussion was described in Chapter 4.

# **Chapter 2**

# Effects of deletion of different PP2C protein phosphatase genes on stress responses in *Saccharomyces cerevisiae*

# 2.1 Introduction

The reversible phosphorylation of proteins is a critical aspect of the control of such fundamental cellular events as metabolism, gene transcription and the cell cycle. This control process involves both protein kinases (PKases) and protein phosphatases (PPases) and is regulated through various signaling pathways. The importance of this regulation by reversible phosphorylation is illustrated by the fact that it affects approximately 30% of the proteome of *Saccharomyces cerevisiae* (Ptacek *et al.*, 2005). In more complex eukaryotes, alterations in the phosphorylation status of proteins are associated with many diseases, such as cancer, diabetes, rheumatoid arthritis and hypertension (Hunter, 1995; Ariño *et al.*, 2011).

Eukaryotic PPases are classified into three major families based on structural and functional diversity: phosphoprotein phosphatases (PPP), metal-dependent protein phosphatases (PPM) and protein tyrosine phosphatases (PTP) (Zolnierowicz and Bollen, 2000). Representative members of the PPP family include protein phosphatase 1 (PP1), PP2A and PP2B subfamilies and other PPases such as *PPT1*. The PPM family comprises  $Mn^{2+}/Mg^{2+}$  ion stimulated PPases, such as the PP2C subfamily. The PTP family consists of protein tyrosine phosphatases (PTPases) and dual-specificity phosphatases (DSPs) in which PTPases dephosphorylate only tyrosine residues; by contrast, DSPs dephosphorylate the serine, threonine and tyrosine residues of proteins (Sakumoto *et al.*, 2002).

The *S. cerevisiae* genome encodes 40 PPase genes, including seven genes in the PP2C subfamily: *PTC1* (Phosphatase two C) to *PTC7* (Table 1). These genes share a conserved PP2C domain that is associated with an amino-terminus in *Ptc5* and *Ptc7* and a carboxyl-terminus in *Ptc2* and *Ptc3* (Ariño *et al.*, 2011). The PP2C subfamily genes do not

show high structural homology except for *PTC2* and *PTC3*, which have 60% identity; however, PPase genes, even from different families and with no structural homology, can show functional redundancy (Evans and Stark, 1997; Sakumoto *et al.*, 2002; Kim *et al.*, 2011). PTCs are involved in the regulation of cell growth and stress signaling in yeast and in higher eukaryotes (Ariño *et al.*, 2011). In yeast, the lethal phenotype of the  $\Delta sln1$  mutant can be rescued by overexpression of *PTC1* through Hog1 hyper-activation. *Ptc1* negatively affects *Pbs2*, the MAPK kinase upstream of Hog1, in the HOG (high-osmolarity glycerol) pathway (Maeda *et al.*, 1994). Several other PTCs (Table 1) also dephosphorylate and inactivate Hog1 MAPK.

In our previous studies, we constructed yeast strains carrying either a single mutation of the 32 PPases (29 strains in total as disruption of *glc7*, *sit4* and *cdc14* proved lethal in HYP100) or two mutations of all the possible combinations (435 strains in total) and performed systematic phenotypic analyses (Sakumoto *et al.*, 1999; 2002). These analyses indicated that none of the 435 double disruptants displayed cell lethality, as might have been expected. Four of the double mutants showed phenotypic changes with regard to temperature-sensitive growth, utilization of carbon sources and sensitivity to cations and drugs, although the effect was relatively small. We interpret these observations to indicate that either the number of phenotypes examined in these earlier studies was insufficient or that more than two PPases have redundant functions.

Here, we investigated the second possibility, focusing on the PP2C subfamily. We constructed yeast strains with multiple deletions of different PTC genes in all possible combinations. In our previous systematic study 111 disruptants among 127 multiple disruptants (including  $\Delta ptc7$ single disruptant and 10 double disruptants involving  $\Delta ptc6$  and  $\Delta ptc7$ ) had not been investigated since only single disruption for *PTC6* gene had been examined and *PTC7* (*YHR076w*) was not identified as PP2C at that time (Sakumoto *et al.*,

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2002). Our specific purpose in this study was to further elucidate the role of each of the seven PP2C PPases in the cellular physiology of *S. cerevisiae*, with special reference to functional redundancy.

# 2.2 Materials and Methods

## 2.2.1 Strains and media

The Saccharomyces cerevisiae and Escherichia coli strains used in this study are listed in Table 2. Disruption of PTC genes was carried out in the wild type parental strain W303 (MATa/MATa ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1-1 his3-11, 15/his3-11, 15 ade2-1/ade2-1 can1-100/can1-100) and its haploid derivatives, W303-1A (MATa) and W303-1B (MATa) (Shirahige et al., 1998). The oligonucleotide primers used for disrupting each of the seven PTC genes are listed in Table 3. Genetic and culture methods for S. cerevisiae and E. coli were performed as described previously (Amberg et al., 2005; Sambrook et al., 2000). The basic media used for the growth of S. cerevisiae were YPDA medium [5% YPD broth (Sigma-Aldrich Co.) and 0.04% adenine (Wako, Tokyo)] or synthetic complete (SC) medium [0.67% yeast nitrogen base without amino acids (Difco) and 2% glucose] supplemented with the required auxotrophic nutrients. Yeast strains were also cultured in YPDA or SC medium containing G418 sulfate (Wako) at a concentration of 200 µg/ml, hygromycin B (Wako) at a concentration of 300 µg/ml, or cerulenin (Wako) at a concentration of 3 µg/ml. E. coli cells were grown in Luria-Bertani (LB) medium containing 2% LB broth (Sigma) and ampicillin (final concentration 50 µg/ml). The minimal sporulation medium (SPM) used here contained 0.5% potassium acetate. When necessary, 2% agar was added to solidify the medium. In all experiments, S. cerevisiae and E. coli strains were cultured at 30°C and 37°C, respectively, unless otherwise specified.

Strain	Genotype and Remarks	Source
S. cerevisiae		
SH4848 (W303-1A)	MAT <b>a</b> ura3-1 leu2-3,112 trp1 his3-11,15 ade2-1	R. Rothstein
SH4849 (W303-1B)	MAT α ura3-1 leu2-3,112 trp1 his3-11,15 ade2-1	R. Rothstein
SH9763	MAT <b>a</b> ura3-1 leu2-3,112 trp1 his3-11,15 ade2-1 ptc1::loxPCgTRP1 ptc2::loxPCgHIS3	Sakumoto et al., 2002
SH682	MATa lys1 trp3 ura1 ura2 pho8-12	Our laboratory
SH683	MATa lys1 trp3 ura1 ura2 pho3 pho8-12	Our laboratory
SH6274	MATa pho3-1 leu1-ELF 52	Our laboratory
SH6275	MATa pho3-1 leu1-SA 54	Our laboratory
	MAT <b>a</b> ura3-1 leu2-3,112 trp1-1 his3-11, 15 ade2-1 TRP1:: pRS304	This study
	MATα ura3-leu2-3,112 trp1-1 his3-1, 15 ade2-1 ptc6:: loxP-HPH-loxP TRP1:: pRS304	This study
E. coli	· · · · ·	
SHB3008	pUG6-CgLEU2	Sugiyama et al., 2005
SHB3009	pUG6- <i>CgHIS3</i>	Sugiyama et al., 2005
SHB3010	pUG6- <i>CgTRP1</i>	Sugiyama et al., 2005
SHB3276	pUG6-∆ <i>loxPURA3</i>	Sugiyama et al., 2008
SHB1463	pUG6	Güldener et al., 1996
	pUG6-YAP1	This study
	pUG6-HPH	This study

 Table 2. Saccharomyces cerevisiae and E. coli strains used in this study.

Table 3. Oligonucleotoide primers used in this study.

Name	Sequence (5'-3')
Disruption	primers
gdPTC1-F	ATCATTTAGGCACTGCATTTATCTTTTAAAAAATCATTATACTTCGTACGCTGCAG
gdPTC1-R	GTCTATGCATAATTTTTGCGCGGTTTATAACGGATCCTTCGCCACTAGTGGATCT
gdPTC2-F	TCCATTGTTGTATAAAATATAGAGAACCAGAAAAAGAAAACTTCGTACGCTGCAG
gdPTC2-R	GTATATAGGTATGTATATATAATGAAGGATGGAAGATCCT GCCACTAGTGGATCT
gdPTC3-F	AGAAGGCCAAGAAGACAAATCGAAGAAGAGAGAGAGAGATAACCTTCGTACGCTGCAG
gdPTC3-R	GACTACTCTTTCGTTGCAAAGTACGGTTCGACAATATTTAGCCACTAGTGGATCT
gdPTC4-F	AGCCATTTTAGAAAGTAACT TCATTTGAAGAAGACAAAACTTCGTACGCTGCAG
gdPTC4-R	GTATGAGAAAAAGGAAGAAGAAAAAATAATT TTTTTTCCGCCACTAGTGGATCT
gdPTC5-F	TTTCAACAGAAGAAGTGCTT TTACTTCTCTCAATCTCTCCCTTCGTACGCTGCAG
gdPTC5-R	ATCCTCTGGTATATACCTACCTCAGCATAAGTTTATATCGGCCACTAGTGGATCT
gdPTC6-F	CTGCAATCGGGGGCAATTAAGCATCAGAAGAGGGGGAATTTGCTTCGTACGCTGCAG
gdPTC6-R	AAGATGATTATCTAGGACTTGTTTCCACCCAGGGGGGGTGTGCCACTAGTGGATCT
gdPTC7-F	ATAAAAGCGGTCCAGAAAACAAACGACAAAGCCACCAAAACTTCGTACGCTGCAG
gdPTC7-R	TTTATTTACACTGCTTTCCAGGAGATTAAAGAGCGGAGTGGCCACTAGTGGATCT

Confirmation primers

cPTC1-F	TTAGTTAAACATTATTATTC
cPTC1-R	GAGATAATGGCGAATTAGAC
cPTC2-F	TTCTGACAGAGCAAATTGAA
cPTC2-R	TGCCCATCCGGGACTGGGGGG
cPTC3-F	AAGTAATATAGCAAGAAACA
cPTC3-R	GGATTCAAAGGTTACCAACA
cPTC4-F	CCCGCGCTGATCTCTTCTTC
cPTC4-R	AATATGTGAATTTATTTCCA
cPTC5-F	AAATTTCCTTATCCCTTGCA
cPTC5-R	GTTCATTTTTTGTGTGAT
cPTC6-F	CTCGCCATAGCCCTTGTAAC
cPTC6-R	GCTTGAAATCAGGGTATACT
cPTC7-F	CACAATAAAATTAGAAATTA
cPTC7-R	TCTTGACTAAAAGCGGTAAG

Primers for plasmid construction

AgTEFp1B	CTCAGATCTGTTTAGCTTGCCTCGTCCCC
AgTEFp2YAP1F	CAGCGACCTCTTGGCGGTAGACACACTCATGGTTGTTTATGTTCGGATGT

#### 2.2.2 Plasmid construction

Plasmid pUG6-HPH was constructed by inserting a 1.8 kb BgIII/SacI\_fragment which has the *hph* gene encoding a hygromycin B phosphotransferase from *Klebsiella pneumoniae* (Goldstein and McCusker, 1999), into the BgIII/SacI sites of pUG6. Plasmid pUG6-*YAP1* was constructed through PCR amplification of about 400 bp of the *Ashbya gossypii TEF* promoter (AgTEFp) using pUG6 as a template and AgTEFp1B and AgTEFp2YAP1F as primers (Table 3). Similarly, an approximately 250 bp fragment of the *A. gossypii TEF* terminator (AgTEFt) was amplified by PCR using pUG6 as a template and AgTEFt1YAP1R and AgTEFt2X as primers. These two fragments were mixed with an approximately 2.0 kb fragment of the *YAP1* gene that was amplified by PCR using the primers YAP1-1 and YAP1-2 and overlap-extension PCR was performed to prepare a fragment consisting of AgTEFp-YAP1-AgTEFt using AgTEFp1B and AgTEFt2X as primers. The resulting AgTEFp-YAP1-AgTEFt fragment was digested with BgIII/XhoI and inserted into the BgIII/XhoI sites of pUG6.

# 2.2.3 Disruption of the *PTC* genes

The seven *PTC* genes were each subjected to a PCR-mediated one step gene disruption strategy by means of homologous recombination by integration of the appropriate gene replacement cassette carrying *CgTRP1*, *CgHIS3*, *CgLEU2*, *ScURA3*, *YAP1*, *KanMX* module or plasmid-borne gene *HPH* (Figure 5A), as described previously (Sakumoto *et al.*, 1999). For gene deletion, ORF-specific primer sets for each *PTC* gene were designed such that 40 bases at the 5' end of the primer were homologous to those at the corresponding

region of the target gene and the 15 bases at the 3' ends were homologous to the pUG6 sequence outside the polylinker region in plasmids. The disruption cassettes were generated by PCR using the corresponding template plasmid and the gene specific primer sets. The amplified PCR products were transformed directly into the yeast parental strains W303-1A and 1B, and Trp<sup>+</sup>, His<sup>+</sup>, Leu<sup>+</sup>, Kan<sup>r</sup>, Cer<sup>r</sup>, Hph<sup>r</sup> and Ura<sup>+</sup> transformants were selected to isolate each gene deletion, i.e.,  $\Delta ptc1$ ,  $\Delta ptc2$ ,  $\Delta ptc3$ ,  $\Delta ptc4$ ,  $\Delta ptc5$ ,  $\Delta ptc6$  and  $\Delta ptc7$ , respectively.

# **A** PCR- mediated one step gene disruption with seven different markers

ptc1 :: loxP-CgTRP1-loxP ptc2 :: loxP-CgHIS3-loxP ptc3 :: loxP-CgLEU2-loxP ptc4 :: loxP-KanMX-loxP ptc5 :: loxP-YAP1-loxP ptc6 :: loxP-HPH-loxP ptc7 :: URA3

# **B** Genetic crossing for multiple gene disruption



C.	List of	127	multiple	disruptants
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Single disruptants (7)	Do	ouble disrupt	ants (21)	Triple disruptants (35)				
$\Delta ptcl$	Δptc1 Δptc2	$\Delta ptc2 \Delta ptc4$	Δptc3 Δptc7	Δptc1 Δptc2 Δptc3	Δptc1 Δptc3 Δptc6	Δptc1 Δptc6 Δptc7	Δptc2 Δptc4 Δptc7	Δptc3 Δptc5 Δptc6
$\Delta ptc2$	$\Delta ptc1 \Delta ptc3$	$\Delta ptc2 \Delta ptc5$	$\Delta ptc4 \Delta ptc5$	$\Delta ptc1 \Delta ptc2 \Delta ptc4$	$\Delta ptc1 \Delta ptc3 \Delta ptc7$	$\Delta ptc2 \Delta ptc3 \Delta ptc4$	$\Delta ptc2 \Delta ptc5 \Delta ptc6$	$\Delta ptc3 \Delta ptc5 \Delta ptc7$
$\Delta ptc3$	$\Delta ptc1 \Delta ptc4$	$\Delta ptc2 \Delta ptc6$	$\Delta ptc4 \Delta ptc6$	$\Delta ptc1 \Delta ptc2 \Delta ptc5$	$\Delta ptc1 \Delta ptc4 \Delta ptc5$	$\Delta ptc2 \Delta ptc3 \Delta ptc5$	$\Delta ptc2 \Delta ptc5 \Delta ptc7$	$\Delta ptc3 \Delta ptc6 \Delta ptc7$
$\Delta ptc4$	$\Delta ptc1 \Delta ptc5$	$\Delta ptc2 \Delta ptc7$	$\Delta ptc4 \Delta ptc7$	$\Delta ptc1 \Delta ptc2 \Delta ptc6$	$\Delta ptc1 \Delta ptc4 \Delta ptc6$	$\Delta ptc2 \Delta ptc3 \Delta ptc6$	$\Delta ptc2 \Delta ptc6 \Delta ptc7$	$\Delta ptc4 \Delta ptc5 \Delta ptc6$
$\Delta ptc5$	$\Delta ptc1 \Delta ptc6$	$\Delta ptc3 \Delta ptc4$	$\Delta ptc5 \Delta ptc6$	$\Delta ptc1 \Delta ptc2 \Delta ptc7$	$\Delta ptc1 \Delta ptc4 \Delta ptc7$	$\Delta ptc2 \Delta ptc3 \Delta ptc7$	$\Delta ptc3 \Delta ptc4 \Delta ptc5$	$\Delta ptc4 \Delta ptc5 \Delta ptc7$
$\Delta ptc6$	$\Delta ptc1 \Delta ptc7$	$\Delta ptc3 \Delta ptc5$	$\Delta ptc5 \Delta ptc7$	$\Delta ptc1 \Delta ptc3 \Delta ptc4$	$\Delta ptc1 \Delta ptc5 \Delta ptc6$	$\Delta ptc2 \Delta ptc4 \Delta ptc5$	$\Delta ptc3 \Delta ptc4 \Delta ptc6$	$\Delta ptc4 \Delta ptc6 \Delta ptc7$
$\Delta ptc7$	$\Delta ptc2 \Delta ptc3$	$\Delta ptc3 \Delta ptc6$	$\Delta ptc6 \Delta ptc7$	$\Delta ptc1 \Delta ptc3 \Delta ptc5$	$\Delta ptc1 \Delta ptc5 \Delta ptc7$	$\Delta ptc2 \Delta ptc4 \Delta ptc6$	$\Delta ptc3 \Delta ptc4 \Delta ptc7$	$\Delta ptc5 \Delta ptc6 \Delta ptc7$
		Quad	ruple disruptants (	35)		· · · · · · · · · · · · · · · · · · ·	Sextuple disru	uptants (7)
Δptc1 Δptc2 Δptc3 Δptc4	Δptc1 Δptc2 Δpt	tc5 Δptc6 Δp	tc1 Δptc3 Δptc5 Δptc7	Δptc2 Δptc3 Δptc4 Δ	ptc6 Δptc2 Δptc4	4 Δptc6 Δptc7	Δptc1 Δptc2 Δptc3 Δj	otc4 Δptc5 Δptc6
$\Delta ptc1 \Delta ptc2 \Delta ptc3 \Delta ptc5$	$\Delta ptc1 \Delta ptc2 \Delta pt$	tc5 Δptc7 Δp	tc1 Δptc3 Δptc6 Δptc7	Δptc2 Δptc3 Δptc4 Δ	ptc7 Δptc2 Δptc.	5 Aptc6 Aptc7	Δptc1 Δptc2 Δptc3 Δμ	$ptc4 \Delta ptc5 \Delta ptc7$
Δptc1 Δptc2 Δptc3 Δptc6	$\Delta ptc1 \Delta ptc2 \Delta pt$	tc6 Δptc7 Δp	tc1 Δptc4 Δptc5 Δptc6	Δptc2 Δptc3 Δptc5 Δ	ptc6 Δptc3 Δptc4	4 Δptc5 Δptc6	Δptc1 Δptc2 Δptc3 Δμ	otc4 Δptc6 Δptc7
$\Delta ptc1 \Delta ptc2 \Delta ptc3 \Delta ptc7$	$\Delta ptc1 \Delta ptc3 \Delta pt$	tc4 Δptc5 Δp	tc1 Δptc4 Δptc5 Δptc7	$\Delta ptc2 \Delta ptc3 \Delta ptc5 \Delta$	ptc7 Δptc3 Δptc4	$4 \Delta ptc5 \Delta ptc7$	$\Delta ptc1 \Delta ptc2 \Delta ptc3 \Delta p$	otc5 Aptc6 Aptc7
$\Delta ptc1 \Delta ptc2 \Delta ptc4 \Delta ptc5$	$\Delta ptc1 \Delta ptc3 \Delta pt$	$tc4 \Delta ptc6 \Delta p$	$tc1 \Delta ptc4 \Delta ptc6 \Delta ptc7$	$\Delta ptc2 \ \Delta ptc3 \ \Delta ptc6 \ \Delta$	$\Delta ptc7  \Delta ptc3  \Delta ptc4$	$4 \Delta ptc6 \Delta ptc7$	$\Delta ptc1 \Delta ptc2 \Delta ptc4 \Delta p$	otc5 Aptc6 Aptc7
$\Delta ptc1 \Delta ptc2 \Delta ptc4 \Delta ptc6$	$\Delta ptc1 \Delta ptc3 \Delta pt$	$tc4 \Delta ptc7 \Delta p$	tc1 Δptc5 Δptc6 Δptc7	$\Delta ptc2 \ \Delta ptc4 \ \Delta ptc5 \ \Delta$	$\Delta ptc6  \Delta ptc3  \Delta ptc.$	5 Δptc6 Δptc7	$\Delta ptc1 \Delta ptc3 \Delta ptc4 \Delta p$	ptc5 Δptc6 Δptc7
Δptc1 Δptc2 Δptc4 Δptc7	Δptc1 Δptc3 Δpt	$tc5 \Delta ptc6 \Delta p$	tc2 Δptc3 Δptc4 Δptc5	Δptc2 Δptc4 Δptc5 Δ	$\Delta ptc7  \Delta ptc4  \Delta ptc.$	5 Δptc6 Δptc7	$\Delta ptc2 \Delta ptc3 \Delta ptc4 \Delta p$	otc5 Δptc6 Δptc7
	Quintuple	e disruptants	(21)		Septu	ple disruptant (1	)	
Δptc1 Δptc2 Δptc3 Δptc4 Δptc	c5 Δptc1 Δptc2	$\Delta ptc4 \Delta ptc5 \Delta p$	tc7 Δptc1 Δptc4 Δptc	5 Δptc6 Δptc7	$\Delta ptc1 \Delta ptc2 \Delta p$	tc3 Δptc4 Δptc5 Δpt	c6 Δptc7	
Δptc1 Δptc2 Δptc3 Δptc4 Δptc	c6 Δptc1 Δptc2	$\Delta ptc4 \Delta ptc6 \Delta p$	tc7 Δptc2 Δptc3 Δptc	$4 \Delta ptc5 \Delta ptc6$				
Δptc1 Δptc2 Δptc3 Δptc4 Δptc	c7 Δptc1 Δptc2	Δρτc5 Δρτc6 Δρ	tc7 Δptc2 Δptc3 Δptc	$4 \Delta ptc5 \Delta ptc7$				
Δptc1 Δptc2 Δptc3 Δptc5 Δptc	c6 Δptc1 Δptc3	$\Delta ptc4 \Delta ptc5 \Delta p$	tc6 Δptc2 Δptc3 Δptc	4 Δptc6 Δptc7				
Δptc1 Δptc2 Δptc3 Δptc5 Δptc	c7 Δptc1 Δptc3	$\Delta ptc4 \Delta ptc5 \Delta p$	tc7 Δptc2 Δptc3 Δptc	$5 \Delta ptc6 \Delta ptc7$				
Δptc1 Δptc2 Δptc3 Δptc6 Δptc	c7 Δptc1 Δptc3	$\Delta ptc4 \Delta ptc6 \Delta p$	tc7 Δptc2 Δptc4 Δptc	$5 \Delta ptc6 \Delta ptc7$				
Δptc1 Δptc2 Δptc4 Δptc5 Δptc	c6 Δptc1 Δptc3	$\Delta ptc5 \Delta ptc6 \Delta p$	tc7 Δptc3 Δptc4 Δptc	5 Δptc6 Δptc7				

**Figure 5.** Strategy for constructing single and multiple gene disruption of *PTC PPases*. (A) Construction of single disruptants of the seven different PTC PPases through PCR-mediated one-step gene deletion strategy by means of homologous recombination through integration of seven different gene replacement cassettes carrying *CgTRP1*, *CgHIS3*, *CgLEU2*, *ScURA3*, *YAP1*, the *KanMX* module and the plasmid-borne gene *HPH*. The deletion cassettes were generated by PCR using the corresponding template plasmid and gene ORF specific primer sets. The amplified PCR products were transformed directly into the yeast parental strain W303-1A (*MATa*) or W303-1B (*MATa*) (B) Construction of 127 multiple gene disruptants by successive genetic crosses. Tetrads were analyzed to screen for Trp<sup>+</sup>, His<sup>+</sup>, Leu<sup>+</sup>, Ura<sup>+</sup>, Kan<sup>r</sup>, Hph<sup>r</sup>, and Cer<sup>r</sup> segregants. (C) List of the 127 *PTC* gene deletion strains (7 single; 21 double; 35 triple; 35 quadruple; 21 quintuple; 7 sextuple and 1 septuple disruptant).

Deletion of the gene was verified by PCR amplification using a forward primer that corresponded to a sequence upstream of the target *PTC* gene and a reverse primer that corresponded to a sequence downstream of the target *PTC* gene.

## 2.2.4 Generation of strains with different deletions of multiple *PTC* genes

Strains carrying different combinations of PTC gene deletions were constructed by genetic crosses. First, heterozygous diploid strains were established by crossing the single disruptants  $\Delta ptc1$ :: loxP-CgTRP1-loxP (W303-1A genetic background) and  $\Delta ptc2$ ::loxP-CgHIS3-loxP (W303-1B genetic background). The diploid cells were induced to sporulate and asci were dissected using a micromanipulator (Sherman and Hicks, 1991). Trp<sup>+</sup> His<sup>+</sup> segregants were selected to obtain the  $\Delta ptc1 \Delta ptc2$  double disruptant. The mating types of the disruptants were determined using two tester strains (MATa: pho3-1 leu1-ELF 52 / MATa: *pho3-1 leu1-SA 54*). We similarly constructed the  $\Delta ptc4\Delta ptc5$  double disruptant from a cross of  $\Delta ptc4::loxP-KanMX-loxP$  and  $\Delta ptc5::loxP-YAP1-loxP$ , and the  $\Delta ptc6\Delta ptc7$  double disruptant from a cross of  $\Delta ptc6::loxP-HPH-loxP$  and  $\Delta ptc7::URA3$ . The MATa  $\Delta ptc1\Delta ptc2$ double disruptant was then crossed with the MATa  $\Delta ptc3$ :: loxP-CgLEU2-loxP single disruptant. The diploid cells were sporulated and the resultant spores from the tetrads were screened. Trp<sup>+</sup> His<sup>+</sup> Leu<sup>+</sup> segregants were selected to obtain the  $\Delta ptc1\Delta ptc2\Delta ptc3$  triple disruptant. Two other double disruptants were also obtained from this cross ( $\Delta ptc1\Delta ptc3$  and  $\Delta ptc2\Delta ptc3$ ). The MATa  $\Delta ptc1\Delta ptc2\Delta ptc3$  triple disruptant was crossed with the  $\Delta ptc4\Delta ptc5$ double disruptant and Trp<sup>+</sup> His<sup>+</sup> Leu<sup>+</sup> Kan<sup>r</sup> Cer<sup>r</sup> segregants were selected to obtain the  $\Delta ptc1 \Delta ptc2 \Delta ptc3 \Delta ptc4 \Delta ptc5$  quintuple disruptant from the tetrads of sporulated diploid cells. From this cross we additionally obtained ten double disruptants, ten triple disruptants, and five quadruple disruptants. The *MAT***a**  $\Delta ptc1 \Delta ptc2 \Delta ptc3 \Delta ptc4 \Delta ptc5$  quintuple disruptant was crossed with the  $\Delta ptc6\Delta ptc7$  double disruptant and Trp<sup>+</sup> His<sup>+</sup> Leu<sup>+</sup> Kan<sup>r</sup> Cer<sup>r</sup> Hph<sup>r</sup> Ura<sup>+</sup> segregants\_were selected from sporulated diploid tetrads. These segregants were selected for the  $\Delta ptc1 \Delta ptc2 \Delta ptc3 \Delta ptc4 \Delta ptc5 \Delta ptc6 \Delta ptc7$  septuple disruptant and all other possible combinations of double, triple, quadruple, quintuple and sextuple disruptants from this cross.

## 2.2.5 Phenotypic test

Cells from each of the 127 PTC gene deletion strains and from two wild-type strains were grown on YPDA plates (3 replicates per strain). SH4848 (W303-1A) and SH4849 (W303-1B) strains were used as the wild-type control strains. The strains were pre-cultured in 5 mL of liquid YPDA medium and then cultured overnight at 30°C on an air-shaker. The next day, the cultures were each inoculated into 5 mL of fresh liquid YPDA and grown to the midlogarithmic phase. From these cultures, cell suspensions ( $1 \times 10^7$  cells) were placed in sterile water  $(OD_{660})$  and aliquots (4 µl) of ten-fold serial dilutions were spotted onto plates with solid medium. Growth of the mutant strains was judged visually by comparison to that of SH4848 and SH4849. Thermo-sensitivity was examined at 13°C, 37°C on YPDA medium and compared to growth at the normal temperature of 30°C. To examine sensitivity to high osmolarity, cell wall stressors, metal ions and genotoxic agents, we used the following compounds and final concentrations: 0.4 M LiCl; 1.5 M NaCl; 1.5 M KCl; 0.3 M CaCl<sub>2</sub>; 5 mM and 10.2 mM caffeine;  $2 \mu g/ml$ ,  $3 \mu g/ml$  and  $4 \mu g/ml$  congo red;  $2 \mu g/ml$ ,  $4 \mu g/ml$  and 5µg/ml calcoflour white; 50 mM hydroxyurea; 0.02% MMS; 0.1 µg/ml rapamycin; and 0.75 M spermine. Growth defects in the mutant strains were confirmed by testing\_phenotypic segregation and co-segregation with the marker in tetrads of the corresponding heterozygous diploid to confirm that disruption of the targeted gene(s) caused the growth defect phenotype.

#### 2.3 Results

# 2.3.1 Construction of the 127 PTC gene deletion strains

In our previous work, the HYP100 parental strain (*MATa ura3-52 leu2-3, 112 trp1* $\Delta$ *his3* $\Delta$  *ade2-101 lys2-801*) (Mukai *et al.*, 1993) was used for construction of strains with single mutations of each PPase (Sakumoto *et al.*, 1999). The HYP100 strain is a meiotic segregant from a diploid strain produced from H4 and YP54, both of which are derivatives of S288C. Since W303 is more generally used in yeast genetics at present, we decided to use this strain and its haploid derivatives in this study. Our first step in constructing strains with disruption of multiple PTC genes in all possible combinations was to generate strains with deletions in the *PTC1* to *PTC7* genes. This step was achieved by transforming either W303-1A (*MATa ura3-1 leu2-3,112 trp1-1his3-11,15 ade2-1*) or W303-1B (*MATa ura3-1 leu2-3,112 trp1-1his3-11,15 ade2-1*) or W303-1B (*MATa ura3-1 leu2-3,112 trp1-1his3-11,15 ade2-1*) with a disruption cassette that corresponded to each PTC gene (Figure 5A). All seven strains with a single *PTC* mutation on a W303 background were viable on YPDA at 30°C, as was previously reported for the HYP100 strain. These seven single *PTC* disruptant strains were used in genetic crosses to construct all 127 different combinations of multiple *PTC* deletions, i.e., 7 single, 21 double, 35 triple, 35 quadruple, 21 quintuple, 7 sextuple and 1 septuple disruptant (Figure 5B). All 127 disruptant strains were viable in nutrient rich medium (YPDA) at 30°C, indicating that no combination of *PTC* PPase gene deletions caused synthetic lethality under this growth condition. The 127 gene deletion strains were used for further phenotypic analyses (Figure 5C).

# 2.3.2 Temperature-sensitive growth in the PTC gene deletion strains

We examined the growth characteristics of the 127 disruptant strains at different temperatures by spotting cells onto YPDA plates and incubating at 13°C, 30°C and 37°C. The  $\Delta ptc1$  single disruptant displayed reduced growth at 37°C and  $\Delta ptc6$  grew slowly at both 13°C and 37°C compared to the wild-type strain (Figure 6A); these results are consistent with previous reports (Sakumoto *et al.*, 2002; Auesukaree *et al.*, 2009). Temperature-sensitive growths in double disruptants of PTC1 - PTC5 were described in the previous study. The  $\Delta ptc1\Delta ptc5$  double disruptant had relatively slower growth than the wild type and single disruptants at 30°C in this study although each of the single disruptants showed normal growth (Figure 6B). Here, we also tested growth at  $13^{\circ}$ C and found that the  $\Delta ptc5\Delta ptc7$  double disruptant also exhibited sensitivity at this temperature (Figure 6C). To confirm that the deletion of these genes was responsible for the temperature-sensitive growth phenotype, we constructed diploids by crossing the  $\Delta ptc5\Delta ptc7$  disruptant (*MATa* ptc5:: loxP-YAP1-loxP ptc7::URA3) and the  $\Delta ptc5$  disruptant (*MATa* ptc5::loxp-YAP1-loxp); the generated diploids were sporulated and dissected for tetrad analysis. We examined 12 asci which yielded a 2 : 2 segregation of uracil auxotrophy and temperature-sensitive growth at 13°C ; both phenotypes have co-segregated in all tetrads tested to date (Figure 6D). To our knowledge, this is the first report that the *PTC5* and *PTC7* genes play a role in growth at low temperature. It should be noted that *PTC7* (*YHR076w*) had not been identified as a PP2C gene and was therefore not included in the previous study (Sakumoto *et al.* (2002).



**Figure 6.** Temperature sensitive growth phenotypes in *PTC* gene deletion strains. (A) Cells of wild-type strain (SH4848),  $\Delta ptc1$  to  $\Delta ptc7$  disruptants were streaked onto two separate YPDA plates (wild-type to  $\Delta ptc5$  in one plate and  $\Delta ptc6$  and  $\Delta ptc7$  in another plate) and incubated at 30°C, 37°C or 13°C. Plates are separated with space in each case. In each plate, wild-type strain was put as a control strain for comparison of the phenotype. (B) Ten-fold serial dilutions of the wild-type,  $\Delta ptc1$ ,  $\Delta ptc5$  single and  $\Delta ptc1\Delta ptc5$  double disruptants were spotted onto YPDA plates and incubated at 30°C. (C) Cells of  $\Delta ptc5$  or  $\Delta ptc7$  single disruptants or of the  $\Delta ptc5\Delta ptc7$  double disruptant were spotted onto YPDA plates, as were those of the wild-type strain; growth was compared at 30°C or 13°C. (D) A diploid heterozygous for the  $\Delta ptc5\Delta ptc7$ :: loxP-YAP1-loxP URA3 double disruptant and the  $\Delta ptc5$ :: loxP-YAP1-loxP single disruptant was sporulated and dissected. Each dissected spore (tetrad), designated A to D, was grown on a YPDA plate at 30°C and cells from each colony that formed were spotted twice on YPDA and SD-Ura plates and incubated at 13°C for 2 days and 30°C overnight.

We also found that the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  quadruple disruptant showed growth sensitivity at 37°C whereas no obvious growth defects occurred in strains with deletion of each individual gene or in double and triple deletion combinations (Figure 6A). The  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  quadruple disruptant also showed a slight growth defect at 30°C although the effect was much weaker that at 37°C (Figure 6B). Although the  $\Delta ptc5\Delta ptc7$ double disruptant exhibited growth sensitivity at 13°C, the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  quadruple disruptant showed normal growth characteristics at this temperature (Figure 6B). These observations suggest that PTC2, PTC3, PTC5 and PTC7 play redundant roles for this growth response. To confirm that these phenotypes were caused by disruption of the PTC genes, diploids were constructed by crossing the quadruple disruptant (MATa ptc2::loxP-CgHIS3loxP ptc3::loxP-CgLEU2-loxP ptc5::loxP-YAP1-loxP ptc7::URA3) with  $\Delta ptc2\Delta ptc3\Delta ptc5$ , or  $\Delta ptc2\Delta ptc5\Delta ptc7$  or  $\Delta ptc3\Delta ptc5\Delta ptc7$  triple disruptants. Tetrad analysis of 12 asci yielded a 2 : 2 segregation for marker auxotrophy and high-temperature sensitive growth; these phenotypes have co-segregated in all tetrads tested to date (Figure 6C). We found that the high-temperature sensitive phenotype of this quadruple disruptant could be partially rescued by the addition of 1.2 M sorbitol to the medium (Figure 6D); this treatment also rescued the swollen shape of the disruptant cells (Figure 7A) suggesting that the growth defect in this quadruple disruptant might have resulted from cell wall instability (Catala et al., 2012).



**Figure 7.** High-temperature growth phenotype of the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  quadruple disruptant. (A) Cells of the wild-type strain, single, double, triple and quadruple disruptants of *PTC2*, *PTC3*, *PTC5* and *PTC7* genes were spotted onto three separate YPDA plates by ten-fold serial dilutions and incubated at 30°C or 37°C. Photos of plates are separated with space in each case. All the strains were compared with wild-type strain in each plate. (B) The growth of the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  quadruple disruptant was compared to that of the wild-type strain on YPDA plates at 30°C, 37°C and 13°C. (C) Heterozygous diploids were

constructed by crossing the quadruple disruptant (*MATa* ptc2::loxP-CgHIS3-loxP ptc3::loxP-CgLEU2-loxP ptc5::loxP-YAP1-loxP ptc7::URA3) with the  $\Delta ptc2\Delta ptc3\Delta ptc3\Delta ptc5$ ,  $\Delta ptc2\Delta ptc5\Delta ptc7$  or  $\Delta ptc3\Delta ptc5\Delta ptc7$  triple disruptants. All the tetrads derived from each cross yielded a 2 : 2 segregation, high-temperature sensitive growth and co-segregation. (D) Cells of the wild-type strain and of the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  quadruple disruptant were spotted onto YPDA plates containing 1.2 M sorbitol and incubated at 37°C overnight.

Interestingly, the  $\Delta ptc1\Delta ptc2\Delta ptc3\Delta ptc4\Delta ptc5\Delta ptc6\Delta ptc7$  septuple disruptant showed a very weak temperature sensitive phenotype compared to the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  quadruple disruptant at 37°C (Figure 7B, C). This observation suggests that  $\Delta ptc1$  and/or  $\Delta ptc4$  and/or  $\Delta ptc6$  might suppress high-temperature sensitivity in the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  disruptant and all the *PTC1*, *PTC4* and *PTC6* genes are required for the temperature sensitivity of the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  quadruple disruptant to occur.

# 2.3.3 Carbon source utilization abilities of PTC gene deletion strains

To examine the relative abilities of the disruptants to use various carbon sources, cells from each of the 127 strains were spotted onto modified YPDA plates with 2% galactose, 2% sucrose and 2% maltose as fermentable carbon sources or 3% glycerol as a non-fermentable carbon source instead of 2% glucose. All 127 strains showed normal growth on both media similar to that of the wild-type strain (data not shown). Even though all the seven *PTC* genes are deleted, the septuple disruptants showed normal growth (Figure 7D). We concluded that *PTC* genes, in any combination, are not indispensable for uptake and assimilation of the tested carbon sources.



Figure 8. Morphology and growth behavior of the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  quadruple disruptant that shows a high-temperature sensitive phenotype. (A) Wild-type (WT) cells and  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  quadruple disruptant cells were grown in liquid YPDA with or without sorbitol at 37°C and their morphologies were observed under the microscope. (B) Cells of the WT, the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$ quadruple disruptant and the  $\Delta ptc1 \Delta ptc2 \Delta ptc3 \Delta ptc4 \Delta ptc5 \Delta ptc6 \Delta ptc7$  septuple disruptant were incubated in liquid YPDA at both 30°C and 37°C overnight. The culture was transferred to a fresh tube at  $OD_{660} = 0.2$ and the ODs were taken every hour until 14 hours. (C) Ten-fold serial dilutions of the wildtype, the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$ quadruple disruptant and the  $\Delta ptc1 \Delta ptc2 \Delta ptc3 \Delta ptc4 \Delta ptc5 \Delta ptc6 \Delta ptc7$  septuple disruptant were spotted on YPDA plates and were incubated at either 30°C or 37°C overnight. (D) Ten-fold serial dilutions of cell
cultures of the wild-type and the  $\Delta ptc1\Delta ptc2\Delta ptc3\Delta ptc4\Delta ptc5\Delta ptc6\Delta ptc7$  septuple disruptant were spotted on modified YPDA medium with 2% galactose, 2% sucrose and 2% maltose as fermentable carbon sources or 3% glycerol instead of 2% glucose) and were incubated at 30°C overnight.

#### 2.3.4 Sensitivity to cations

We examined the sensitivity of the *PTC* gene deletion strains to cations by spotting cells from each of the 127 strains onto YPDA plates containing 1.5 M KCl, 0.3 M CaCl<sub>2</sub>, 1.5 M NaCl or 0.4 M LiCl. None of the 127 disruptants were sensitive to the presence of 1.5 M KCl in the YPDA. However, two double disruptants, namely  $\Delta ptc1\Delta ptc2$  and  $\Delta ptc1\Delta ptc4$ , displayed a growth defect on YPDA medium containing 1.5 M NaCl compared to their growth on YPDA (Figure 8A). Our previous analysis showed that these strains grew normally on medium containing 1.0 M NaCl (Sakumoto *et al.*, 2002); the growth defect was therefore only manifested at the higher salt concentration. We conclude that *PTC1* and *PTC2* and/or *PTC4* has a redundant role for survival when yeast cells are exposed to high levels of Na<sup>+</sup> ions in the medium.

Interestingly, we found that all the double disruptants including  $\Delta ptc1$  disruption exhibited Li<sup>+</sup> sensitive phenotypes except for  $\Delta ptc1\Delta ptc7$  double disruptant (Figure 8A). Thus, disruption of the *PTC7* gene could suppress the Li<sup>+</sup> sensitive phenotype of the  $\Delta ptc1$ deletion and  $\Delta ptc1\Delta ptc7$  double disruptant displayed Li<sup>+</sup> resistance compared to the  $\Delta ptc1$ single disruption. We analyzed 14 tetrads and obtained a 2 : 2 segregation of uracil auxotrophy and LiCl resistant growth; both phenotypes have co-segregated in all the tetrads tested to date (Figure 8B). As expected, the  $\Delta ptc1\Delta ptc2\Delta ptc4$  triple disruptant did not grow on medium containing 1.5 M NaCl (Figure 8C) and a tetrad analysis showed this Li<sup>+</sup> sensitivity co-segregated with geneticin sensitivity (Figure 8D). We also found that the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  quadruple disruptant did



**Figure 9.** Growth phenotype of single *PTC* disruptants and double disruptants combined with  $\Delta ptc1$ . The disruptants listed in (A) were grown on YPDA plates at 30°C and cells were spotted onto various assay plates. Cells of disruptants spotted on the representative plates were incubated at 30°C for 1 day for the control plate (YPDA), and 2 days for 1.5 M NaCl, 0.4 M LiCl and 0.6 M CaCl<sub>2</sub> plates. (B) A diploid heterozygote for the  $\Delta ptc1\Delta ptc7$ :: loxP-CgTRP1-loxP URA3 double disruptant and the  $\Delta ptc1$ :: loxP-CgTRP1-loxP single disruptant was sporulated and dissected. Each dissected spore (tetrad), designated A to D, was grown on a YPDA plate at 30°C and cells from each colony that formed were spotted twice on YPDA and SD-Ura plates and were incubated at 13°C for 2 days or at 30°C overnight. (C) Cells of the wild-type strain, the  $\Delta ptc2\Delta ptc4$  double disruptant and the  $\Delta ptc1\Delta ptc2\Delta ptc4$  triple

disruptant were spotted onto YPDA plates without/with 1.5 M NaCl and incubated at 30°C. (D) A diploid heterozygote for the  $\Delta ptc1\Delta ptc2\Delta ptc4::loxP-CgTRP1-loxP \ loxP-CgHIS3-loxP \ loxP-KanMX-loxP$  triple disruptant and the  $\Delta ptc1\Delta ptc2:: \ loxP-CgTRP1-loxP \ loxP-CgHIS3- \ loxP$  double disruptant was sporulated and dissected. Each dissected spore (tetrad), designated A to D, was grown on a YPDA plate at 30°C and cells from each colony that formed were spotted into YPDA with or without 1.5 M NaCl and YPDA containing geneticin and were incubated at 30°C overnight.

not grow on a YPDA plate containing 0.4 M LiCl (Figure 10A) but did grow on 1.5 M NaCl (Figure 10B); there were no obvious growth defects in single disruptants for each gene, or for the various double and triple deletion combinations (Figure 10A). To confirm that these phenotypes were caused by disruption of these four *PTC* genes, diploids were constructed by crossing the quadruple disruptant (*MATa ptc2::loxP-CgHIS3-loxP ptc3::loxP-CgLEU2-loxP ptc5::loxP-YAP1-loxP ptc7::URA3*) to each of  $\Delta ptc2\Delta ptc3\Delta ptc5$ ,  $\Delta ptc2\Delta ptc5\Delta ptc7$  and  $\Delta ptc3\Delta ptc5\Delta ptc7$  triple disruptants; tetrad analyses were then carried out. We found a 2 : 2 segregation of prototrophy and Li<sup>+</sup> ion-sensitive growth and these two phenotypes co-segregated in the 12 tetrads tested to data (Figure 10C), indicating that *PTC2*, *PTC3*, *PTC5* and *PTC7* are functionally redundant for Li<sup>+</sup> ion sensitive growth.



**Figure 10.** Growth of the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  quadruple disruptant under LiCl stress. (A) Cells of the wild-type strain, single, double, triple and triple disruptants of *PTC2*, *PTC3*, *PTC5* and *PTC7* genes were spotted at ten-fold serial dilutions on three separate YPDA plates with or without 0.4 M LiCl and incubated at 30°C. All the strains were compared with wildtype strain in each plate and photos of the plates are separated with space in both cases. (B) The wild-type strain and the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  quadruple disruptant were spotted onto YPDA plates containing 1.5 M NaCl and incubated at 30°C for 2 days. (C) Heterozygous diploids were constructed by crossing the quadruple disruptant (*MATa* ptc2::loxP-CgHIS3loxP ptc3::loxP-CgLEU2-loxP ptc5::loxP-YAP1-loxP ptc7::URA3) with  $\Delta ptc2\Delta ptc3\Delta ptc5$ ,  $\Delta ptc2\Delta ptc5\Delta ptc7$  or  $\Delta ptc3\Delta ptc5\Delta ptc7$  triple disruptants. All the tetrads derived from each representative cross gave a 2 : 2 segregation, LiCl sensitive growth and co-segregation.

#### 2.3.5 Sensitivity to genotoxic agents

We examined the effects of various genotoxic agents, such as caffeine, congo red, hydroxyurea, methyl methanesulfonate (MMS), spermine and rapamycin, on the 127 deletion strains; these agents are all able to inhibit cell cycle and cell growth. Cells were spotted onto YPDA plates containing various concentrations of these agents (see materials and Methods, Figure 10). We found that two single disruptants,  $\Delta ptc1$  and  $\Delta ptc6$  exhibited caffeine sensitivity and the degree of sensitivity increased in the  $\Delta ptc1\Delta ptc6$  double disruptant (Figure 10A). The  $\Delta ptc1$  and  $\Delta ptc6$  single disruptants were sensitive to 2 µg/ml congo red; the  $\Delta ptc1 \Delta ptc6$  double disruptant showed an increased sensitivity at the higher level of congo red (4  $\mu$ g/ml) (Figure 10B). The  $\Delta ptc1$ ,  $\Delta ptc6$  and  $\Delta ptc1\Delta ptc6$  disruptants showed similar phenotypes when grown on medium with 5 µg/ml calcoflour white (Figure 10B). Analysis of 12 tetrads gave a 2 : 2 segregation for congo red, calcoflour white and caffeine sensitive growth; these phenotypes co-segregated with tryptophan auxotrophy in all tetrads. Our data indicates that the deletion of *PTC6* in combination with the  $\Delta ptc1$  disruptant caused increased sensitivity to caffeine, congo red and calcoflour white (Figure 10C). Taken together, these observations indicate that PTC1 and PTC6 function redundantly in the same metabolic pathways. None of the disruptants showed synthetic sensitivity to hydroxyurea, MMS or spermine (data not shown).



**Figure 11.** Phenotypic analysis of the  $\Delta ptc1$ ,  $\Delta ptc6$  and  $\Delta ptc1\Delta ptc6$  disruptants. (A) Cells of the wild-type,  $\Delta ptc1$  and  $\Delta ptc6$  single disruptants and the  $\Delta ptc1\Delta ptc6$  double disruptants were spotted onto YPDA plates with or without 5 mM or 10.2 mM caffeine and incubated for 2 days at 30°C. (B) Ten-fold serial dilutions of the wild-type,  $\Delta ptc1$  and  $\Delta ptc6$  single disruptants and the  $\Delta ptc1\Delta ptc6$  double disruptants were spotted onto YPDA plates with or without congo red (CR) at a concentration of 2 µg/ml or 4 µg/ml, or calcoflour white (CFW) at a concentration of 2 µg/ml and incubated at 30°C overnight and 2 days respectively. (C) A diploid heterozygote from the  $\Delta ptc1\Delta ptc6$ : loxP-CgTRP1-loxP loxP-*HPH-loxP* double disruptant and the  $\Delta ptc6$ :: loxP-HPH-loxP single disruptant was sporulated at 30°C and cells from each colony that formed were spotted twice into YPDA with or without 4 µg/ml congo red, 5 µg/ml calcoflour white, 5 mM caffeine or SD-Trp and were incubated at 30°C overnight.

#### 2.4 Discussion

One of main purposes of this study was to explore functional redundancy in PTC PPases in *S. cerevisiae*, a model eukaryote. For this purpose, we constructed 127 strains with different numbers and combinations of deletions of the seven PP2C *PPase* genes. Our analyses showed that all of the disruptants were viable in nutrient rich medium, indicating that no combinations of *PTC* gene disruption caused synthetic lethality under the tested culture conditions. Previously, analyses of the then known 32 PPase genes showed that deletion of *glc7*, *sit4* or *cdc14* caused lethality; no double disruptant of the remaining 29 PPases exhibited lethality, indicating that no pair of *PTC1* to *PTC5* genes had a redundant role for normal growth (Sakumoto *et al.*, 1999, 2002). However, when we tested multiple disruptants of *PTC1* to *PTC7* in this study under a range of stressful conditions, such as temperature (high and low), high osmolarity, sensitivity to cell wall-damaging agents, sensitivity to ions and genotoxic agents, we identified several new phenotypes suggesting the occurrence of functional redundancy (summarized in Table 4).

Disruptants	Stress	Condition	Phenotype	
$\Delta ptc1\Delta ptc6$	Congo red	4µg/ml	Sensitive	
	Calcoflour white	5µg/ml	Sensitive	
	Caffeine	10.2 mM	Sensitive	
$\Delta ptc5\Delta ptc7$	Low temperature	13°C	Sensitive	
$\Delta ptc1\Delta ptc2$	NaCl	1.5 M	Sensitive	
$\Delta ptc1\Delta ptc4$	NaCl	1.5 M	Sensitive	
$\Delta ptc1 \Delta ptc2 \Delta ptc4$	NaCl	1.5 M	Sensitive	
$\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$	High temperature	37°C	Sensitive	
	LiCl	0.4 M	Sensitive	

Table 4. Summary of phenotypes discovered in this study.

#### 2.4.1 PTC5 and PTC7 are functionally redundant for cold sensitivity

In this study, we identified the first double disruptant,  $\Delta ptc5\Delta ptc7$ , to show evidence of temperature sensitive growth at 13°C (Figure 6C). Our previous study was limited to examination of strains with single deletions of *PTC1* to *PTC6* or double deletions of *PTC1* to *PTC5* (Sakumoto *et al.*, 2002). Schade *et al.* (2004) identified a set of genes, including *NSR1*, *TIP1*, *TIR1* and *TIR2*, and *OLE1* that are induced in *S. cerevisiae* by low temperatures. The only one PPase known to be induced at low temperature in *S. cerevisiae* is *YVH1*. Transcription of *YVH1* is increased two-fold at 13°C and is also induced by nitrogen starvation (Sakumoto *et al.*, 1999). *PTC7* transcription has been reported to increase in response to osmotic stress through a decrease in Hog1 dephosphorylation (Runner and Brewster, 2003); a significant drop in membrane fluidity induced by low temperature is detected by the Sln1 cell surface cold sensor as the primary signal for HOG pathway activation (Panadero *et al.*, 2006). This suggests the possibility that a decrease in Hog1 dephosphorylation at low temperature might result in increased transcription of *PTC5* as well as of *PTC7*. Therefore, low temperature might induce Hog1 activation by a similar mechanism as for activation of the osmotic stress-regulated HOG pathway.

### 2.4.2 Functional redundancy of *PTC2*, *PTC3*, *PTC5*, and *PTC7* in response to a high temperature stress

The sensitivity of the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  quadruple disruptant to a high temperature was partially suppressed by the addition of 1.2 M sorbitol to the medium (Figure 7D). This high temperature sensitivity phenotype is therefore likely caused by cell lysis due to an osmoregulation defect. It has been reported that, in *Schizosaccharomyces pombe*, aberrant cell morphology and temperature sensitive cell lysis can be suppressed by adding sorbitol to the medium (Shiozaki and Russell, 1995). Osmoregulation of *S. cerevisiae* is mainly controlled through the HOG pathway. *PTC2* and *PTC3* are two negative regulators that directly dephosphorylate Hog1 MAPK through limiting the maximal activation of the HOG pathway (Maeda *et al.*, 1993; Young *et al.*, 2002). The HOG pathway is activated by both osmotic stress and heat stress (Wojda *et al.*, 2003). Two membrane-bound proteins, Sln1p and Sho1, regulate Hog1 activity during osmostress (Wu *et al.*, 2010); in contrast, heat stress activates Hog1 via the membrane protein Sho1 (Winkler *et al.*, 2002). Transcription of *PTC7* in response to osmotic stress is regulated by a feedback mechanism that decreases Hog1 dephosphorylation, although the physiological substrate of Ptc7 is unknown (Runner and Brewster, 2003). Interestingly, the  $\Delta ptc1\Delta ptc2\Delta ptc3\Delta ptc4\Delta ptc5\Delta ptc6\Delta ptc7$  septuple disruptant did not show a temperature sensitive phenotype at 37°C despite the fact that it contains the quadruple deletions of  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$ . This lack of phenotype might be due to the  $\Delta ptc1$  and/or  $\Delta ptc4$  deletions that might act as suppressors of high-temperature sensitivity in the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  disruptant.

#### 2.4.3 Functional redundancy of PTC2, PTC3, PTC5, and PTC7 in the Li<sup>+</sup> sensitivity

It was previously reported that  $\Delta ynr022c$ ,  $\Delta sit4$  and  $\Delta ptc1$  single disruptants and  $\Delta ptc2\Delta msg5$  and  $\Delta ptp2\Delta msg5$  double disruptants exhibit sensitivity to calcium ions (Sakumoto *et al.*, 2002; González *et al.*, 2006; Laviňa *et al.*, 2013). The  $\Delta ptc2\Delta msg5$  double disruptant is also sensitive to 1.0 M NaCl (Sakumoto *et al.*, 2002). Here, we examined the effect of a  $\Delta ptc1$  disruption in combination with one or more deletions of *PTC2* to *PTC7*. The different deletion combinations showed the same degree of Ca<sup>2+</sup> sensitivity (Figure 9A), indicating that disruption of *PTC2* to *PTC7* in combination with the  $\Delta ptc1$  deletion did not suppress or exacerbate calcium sensitivity. *PTC1* has already been recognized to be the major PPase of the Ca<sup>2+</sup> signaling pathway and  $\Delta ptc1$  disruption hyper-activates calcineurin (González *et al.*, 2006). In addition, we obtained the first evidence that Li<sup>+</sup> sensitivity is

increased in the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  quadruple disruptant (Figure 10A). Only the *PTC1* gene has been implicated to date in Li<sup>+</sup> toxicity in *S. cerevisiae* (Ruiz *et al.*, 2006). *PTC2*, *PTC3* and *PTC5* are known to dephosphorylate Cdc28 (Cheng *et al.*, 1999), while the *PKC1* cell integrity pathway is partially dependent on Cdc28 activity (Levin, 2005). We therefore hypothesize that the high Li<sup>+</sup> sensitivity phenotype of the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  quadruple disruptant is caused by osmotic stress due to the high accumulation of Li<sup>+</sup> ions inside the cells. This kind of osmotic stress is known to stimulate Hcs77 (<u>High Copy Suppressor</u>) that activates Rho1 GTPase which in turn triggers a MAP kinase cascade (Martín *et al.*, 2000) to cope with the stress. However, we suggest that the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  quadruple disruptant may be defective in this response. As the *PTC2*, *PTC3*, *PTC5* and *PTC7* genes show redundant roles in growth under conditions of high Li<sup>+</sup> (Figure 10A), we assume that *PTC7* may also dephosphorylate Cdc28 and play a redundant role with *PTC2*, *PTC3* and *PTC5* in the dephosphorylation of Cdc28 under high osmotic conditions. This possibility is currently under investigation.

#### 2.4.4 PTC1, PTC2, and PTC4 are functional redundant PPases for Na<sup>+</sup> sensitivity

The  $\Delta ptc1\Delta ptc2$  and  $\Delta ptc1\Delta ptc4$  double disruptants showed Na<sup>+</sup> sensitivity (Figure 9A). Although  $\Delta ptc2\Delta ptc4$  double disruptant could grow under high Na<sup>+</sup> conditions, the  $\Delta ptc1\Delta ptc2\Delta ptc4$  triple disruptant was very sensitive to 1.5 M NaCl (Figure 9B). All the double disruptants for PPase genes were tested for NaCl sensitivity at a concentration of 1.0 M NaCl which is much lower than the concentration used in this study (Sakumoto *et al.*, 2002). Under the condition of 1.0 M concentration of NaCl,  $\Delta ptc1\Delta ptc2$  and  $\Delta ptc1\Delta ptc4$  double disruptants could grow normally. Various lines of evidence suggest that Ptc1 might negatively regulate the Hal3/Ppz1 and Ppz2 pathways by decreasing expression of the Na<sup>+</sup>-ATPase gene, *ENA1* (de Nadal *et al.*, 1998). Decreased *ENA1* expression has been observed

in cells lacking *PTC1* in a similar manner as cells with  $\Delta hal3\Delta ppz1$  deletions (Ruiz *et al.*, 2006). The mechanism of interaction between *PTC1* and *ENA1* in the Hal3/Ppz system is currently unknown. However, in light of the Na<sup>+</sup> sensitivity shown by  $\Delta ptc1\Delta ptc2$  and  $\Delta ptc1\Delta ptc4$  double disruptants and by the  $\Delta ptc1\Delta ptc2\Delta ptc4$  triple disruptant, we suggest that *PTC2* and *PTC4* might be involved in the Hal3/Ppz system along with *PTC1*.

### 2.4.5 *PTC1* and *PTC6* are functionally redundant in caffeine, congo red and calcoflour white sensitivities

It was reported that the  $\Delta ptcl$  single disruptant did not grow on YPDA plate containing 10 mM of caffeine (Dudley et al., 2005). González et al. (2009) and Hiraski et al. (2011) also reported that the  $\Delta ptc1$  disruptant is caffeine and rapamycin sensitive, suggesting a functional connection of Ptc1with TOR pathway which is not shared by other members of the PP2C sub-family. Sensitivity of the  $\Delta ptc6$  disruptant to 5 mM caffeine (Sakumoto et al., 2002) and 0.1 µg/ml rapamycin (Ruan et al., 2007) was also previously reported. Recently it was reported that additional deletion of *PTC6* recovers the deficient growth caused by the  $\Delta ptc1$  mutation under the stress caused by congo red and calcoflour white (González et al., 2013). By contrast, in this study the lack of Ptc6 increased the sensitivity of the  $\Delta ptc1$  to caffeine, congo red and calcoflour white (Figure 11A, B). The difference between these two observations may come from the different genetic background of BY4741 used by González et al. (2013) from that of our strain W303 since the high temperature sensitive phenotype of the  $\Delta ptc6$  disruptant was also different in HYP100 and W303 genetic background (Sakumoto et al., 2002). Since HYP100 strain is a meiotic segregant of the diploid from H4 and YP54 (both derived from S288C) and BY4741 is the haploid derivative of S288C, whereas, W303 is a diploid derived from a cross of S288C and Sigma1278b strains. These results suggest that

*PTC1* and *PTC6* function redundantly in response to these drugs and the mechanism underlying their involvement in the signaling pathways awaits further analysis.

It is believed that PPases in lower eukaryotes have multiple roles rather than the more specific roles found in higher eukaryotes; certainly, the large number of uncharacterized genes in lower eukaryotes has been presumed to be due to genetic redundancy. The unexpected synthetic phenotypes of the multiple disruptants, such as high temperature sensitivity in the  $\Delta ptc2\Delta ptc3\Delta ptc5\Delta ptc7$  quadruple disruptant revealed in this study, indicates the need to systematically elucidate functional redundancy in more detail and to further investigate the molecular mechanism(s) of functionally redundant genes with respect to their specific roles in particular signaling pathways. Possibly, more extensive phenotypic screens of the 127 PTC gene disruptants constructed in this study will lead to the identification of additional combinations of functionally redundant *PTC* genes.

# **Chapter 3**

### Type 2C protein phosphatase Ptc6 participates in activation of the Slt2mediated cell wall integrity pathway in *Saccharomyces cerevisiae*

#### 3.1 Introduction

Both prokaryotic and eukaryotic cells require a complex network of sensory and signal transduction mechanisms to cope with changes in environmental conditions. Environmental stresses that might reduce cell survival usually elicit modifications in gene expression and metabolic activities that enable growth and proliferation. Particular stresses can cause activity in multiple parallel pathways in the cell in order to lessen the adverse consequences of the improper activation or inactivation of signaling cascades (Laviňa *et al.*, 2013). Cellular regulation is an integral part of signaling cascades, such as involvement of mitogen-activated protein kinase (MAPK), that maintain cellular homeostasis during periods of stress. One of the means that cells employ to mediate signaling is reversible phosphorylation (Laviňa *et al.*, 2014): protein kinases (PKases) can phosphorylate proteins, while protein phosphatases (PPases) can dephosphorylate proteins in response to an environmental stress (Martin *et al.*, 2005).

On average, PKases and PPases comprise 2 - 4% of all the genes in a eukaryotic genome. The importance of these genes is exemplified in Saccharomyces cerevisiae by the PKases fact that the 129 and 40 PPases regulate 30% of the proteome (www.yeastkinome.org). The type 2C Ser/Thr PPases (PP2C) subfamily in S. cerevisiae contains seven members (Ptc1 - Ptc7) Initially, these enzymes were thought to be primarily involved in the regulation of cell growth and in stress signaling in yeast and higher eukaryotes; however, recent studies have suggested that these PPases may have more diverse functions (González et al., 2013). For example, Ptc5 together with Ptc6 dephosphorylates Pda1, the E1α subunit of the pyruvate dehydrogenase (PDH) complex which catalyzes the oxidative decarboxylation of pyruvate to form acetyl coenzyme A (acetyl-CoA) (Gey *et al.*, 2008). Ptc1 is the best characterized member of the subfamily in *S. cerevisiae* and has been shown to share few functions with other members. *S. cerevisiae* cells with a  $\Delta ptc1$  disruption and grown under standard conditions have a different transcriptional profile to that for cells with disruption of *PTC2* to *PTC5* (González *et al.*, 2006); likewise, the transcriptional profile of the  $\Delta ptc6$  disruptant differs from those of  $\Delta ptc1$ -  $\Delta ptc5$  strains (González *et al.*, 2013). The  $\Delta ptc1$  and  $\Delta ptc6$  disruptants show rapamycin and caffeine sensitivities that indicate that these two PPases participate in the TOR signaling pathway (González *et al.*, 2013). Disruption of *PTC1* also results in the activation of the Slt2-mediated cell wall integrity (CWI) pathway (Li *et al.*, 2010). Although an interaction between the Slt2 and HOG pathways is evident, the hypersensitivity of the  $\Delta ptc1$  disruptant to cell wall-damaging agents such as calcofluor white (CFW) is not the result of Hog1 hyperactivation (Bermejo *et al.*, 2008; García *et al.*, 2009). In a previous study, Ptc1 was shown to be important in ensuring proper vacuolar inheritance, and it was suggested that Ptc1-mediated control of vacuolar inheritance does not involve the HOG or CWI pathway s (Jin *et al.*, 2009).

The available evidence suggests that PP2C PPases in yeast and other organisms control a number of processes through the specific interplay of functions that can be overlapping. For example, *Candida albicans* cells that lack both *CaPTC6* and *CaPTC7* show increased tolerance to fluconazole and ketoconazole; by contrast, cells with a single deletion of either *CaPTC6* or *CaPTC7* have a slightly increased sensitivity to these azoles (Zhao *et al.*, 2012). In *S. cerevisiae*,  $\Delta ptc1\Delta slt2$  disruptants are extremely sensitive to cell wall damaging agents, and there is evidence of an additive effect for the two disruptions (González *et al.*, 2006). Cells lacking *PTC1* have a higher level of phosphorylated Slt2 than wild-type cells (Du *et al.*, 2006; González *et al.*, 2006). The decreased tolerance of  $\Delta ptc1\Delta ptc6$  double disruptant to CR and CFW stimulated the present investigation of the functional connection

between *PTC6* and the Slt2 CWI pathway. Our analyses in this chapter indicate that under cell wall stress conditions *PTC6* has distinct role from that of *PTC1* in Slt2 phosphorylation. Cells with both  $\Delta ptc1$  and  $\Delta ptc6$  disruption showed severe defects in their vacuoles under cell wall stress conditions compared to those with only single *PTC* disruption. Therefore, we propose that *PTC6* plays a negative role in the Slt2 pathway as well as in vacuole morphogenesis in *S. cerevisiae*.

#### 3.2 Materials and Methods

#### 3.2.1 Strains, media, growth conditions and general method

The Saccharomyces cerevisiae strains used in this study are summarized in Table 5. The haploid derivatives of W303 (MATa/MATa ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1-1 his3-11, 15/his3-11, 15 ade2-1/ade2-1 can1-100/can1-100), W303-1A (MATa ura3-1 leu2-3,112 trp1-1 his3-11, 15 ade2-1 can1-100) and W303-1B (MATa ura3-1 leu2-3,112 trp1-1 his3-11, 15 ade2-1 can1-100) were used as the parental strain (Shirahige *et al.*, 1998). The cells were grown in YPDA medium or synthetic complete (SC) medium or minimal sporulation medium (SPM) as described previously (Gietz and Schietsl, 2007). Yeast strains were also cultured at 30°C in YPDA or SC medium containing 300 µg/ml hygromycin B (Wako), or 4 µg/ml CR, or 5 µg/ml CFW. The sensitivity of each strain to CR and CFW was evaluated at hourly intervals in liquid cultures; the data are shown as relative growth curves compared to strains grown in YPDA.

#### **3.2.2** Genetic manipulations

Single and double disruptants were constructed using a PCR-mediated one step gene disruption strategy that uses homologous recombination to integrate the appropriate gene replacement cassette carrying the *Candida glabrata TRP1 (CgTRP1)* module or plasmid-

borne *HPH* gene (pUG6-*HPH*) as described previously (Amberg *et al.*, 2005). Disruption of the target gene(s) was verified through by PCR using specific primers. The primers used in this study are listed in Table 2. The double disruptant  $\Delta ptc1\Delta ptc6$  was isolated by tetrad analysis of the diploid strain produced by crossing  $\Delta ptc1$ :: loxP-CgTRP1-loxP (*MAT***a**) and  $\Delta ptc6$ ::loxP-HPH-loxP (*MAT*a) (Sherman and Hicks, 1991). Wild type *TRP1* genes were complemented in W303 (*MAT***a** *ura3-1 leu2-3,112 trp1-1 his3-11*, *15 ade2-1 TRP1*:: pRS304) strain and in  $\Delta ptc6$  (*MAT*a *ura3-1 leu2-3,112 trp1-1 his3-11*, *15 ade2-1 ptc6*:: *loxP-HPH-loxP TRP1*:: pRS304) for tryptophan synthesis. A multicopy vector (YEp) containing *VPS73* gene (YEp51B-*VPS73*) was introduced to the  $\Delta ptc1$  single disruptant and  $\Delta ptc1\Delta ptc6$ double disruptant strains. The YEp51B-*VPS73* plasmid was obtained from National BioResource Project (NBRP) – Yeast.

#### 3.2.3 Phenotypic analysis

Wild-type and deletion strains were assessed for sensitivity to CR or CFW by spot plating on semi-solid media. SH4848 (W303-1A) and SH4849 (W303-1B) strains were used as the wild-type control strains. Ten-fold serial dilutions of the cell suspension ( $10^6$  cells/5 ml) were spotted onto YPDA or YPDA supplemented with 4 µg/ml CR or 5 µg/ml CFW and were incubated at 30°C for 1–2 days. Growth of the disruptant strains was judged visually by comparison to that of SH4848 and SH4849.

#### **3.2.4 Immunoblot analysis**

For immunoblot analysis, protein extracts were prepared using the trichloroacetic acid method: cells were grown until mid-log phase ( $OD_{660} = 1.0$ ) at 30°C in YPD medium with or without 4 µg/ml CR or 5 µg/ml CFW (An *et al.*, 2006) and fractionated on 10% SDS-PAGE. Proteins were transferred to PVDF Immobilon membranes (Millipore Corporation)

and probed overnight at 4°C in the presence of 1% skim milk (Difco<sup>TM</sup>). Antiphospho-p44/42 MAPK (Thr202/Tyr204) (Cell Signaling Technology) or anti-Mpk1 (yC-20):sc-6830 (Santa Cruz Biotechnology, Inc) antibodies at 1:1,000 dilution were used to identify phosphorylated or total Slt2, respectively. These primary antibodies were detected using a 1:10,000 diluted horseradish peroxidase-conjugated anti-goat antibody for total Slt2 and similarly diluted horseradish peroxidase-conjugated anti-rabbit antibody for phosphorylated Slt2 using ECL Western Lightning chemiluminescence Reagent Plus (PerkinElmer LAS, Inc).

#### 3.2.5 RNA isolation

Total RNA was isolated using a Qiagen RNeasy Mini Kit after mechanical disruption of cells grown to mid-log phase ( $OD_{660} = 1.0$ ) at 30°C in 5 ml of YPD medium with or without 4 µg/ml CR. One ml of liquid culture was lysed and homogenized by high-speed agitation in a TissueLyser in the presence of glass beads and buffer. The samples were then applied to the RNeasy Mini spin column. High-quality total RNA bound to the membrane while contaminants were efficiently washed away by the specialized high-salt buffer system; the high-quality RNA was eluted in RNase-free water.

#### **3.2.6** Determination of mRNA levels using real-time PCR (qRT-PCR)

The levels of expression of selected genes were measured by qRT-PCR. Using the total RNA isolated by the Qiagen RNeasy protocol, cDNA was synthesized using a QuantiTect Reverse Transcription Kit that has integrated removal of genomic DNA contamination. The synthesized cDNA was used as the template for two-step real-time PCR in which the reverse transcription reaction step is separated from the real-time PCR assay (Wong and Medrano, 2005). qRT-PCR was performed using 25 µl reaction mixtures and a TAKARA TP800-Thermal Cycler Dice<sup>™</sup> Real Time System with SYBR<sup>®</sup> *Premix Ex Taq* II

(Tli RNaseH Plus). PCR was performed using the following amplification program: initial denaturation for 30 seconds at 95°C, followed by 40 cycles of denaturation for 5 seconds at 95°C, and annealing for 30~60 seconds at 60°C. Triplicate samples of cells were collected at each time point. *ACT1* was used as the reference gene for normalization of mRNA levels. The qRT-PCR primers used in this study were designed using Primer Express version 2.0 and are listed in Table 6.

#### 3.2.7 Statistical analysis

The transcription levels of representative genes were confirmed by a normalized target gene expression analysis  $(2^{-\Delta\Delta C}_{T})$  of the amplification plots derived from real-time RT-PCR (Livak and Schmittgen, 2001). The  $C_{T}$  values were provided from real-time PCR instrumentation. The data were analyzed using Equation, where  $\Delta\Delta C_{T} = C_{T}$  (Target-Actin) -  $C_{T}$  (WT-Actin). The mean  $C_{T}$  values for both the target and internal control genes were determined. The fold change in the target genes were normalized to actin and were calculated for each sample using  $(2^{-\Delta\Delta C}_{T})$  analysis. The mean, SD, and SE were then determined from the triplicate samples at each time.

#### 3.2.8 Vacuolar staining

Vacuoles were stained with FM4-64 as previously described (Hermansyah *et al.*, 2009; Dalmau *et al.*, 2010). Cells grown in YPAD to  $OD_{660} = 1.0$  were concentrated 20-fold in YPAD containing 40 µM FM4-64 (Molecular Probes<sup>TM</sup> Invitrogen) and incubated for 30 min at 4°C with shaking. The cells were harvested at 4°C, resuspended in fresh YPAD at  $OD_{660} = 10$ , and incubated at 30°C with vigorous shaking for 60 min. The cells were centrifuged, resuspended in fresh YPAD and immediately analyzed with a BX61-34-FL-I-D fluorescence microscope (Olympus) using a U-MWIG2 (520–550 nm) filter (Olympus);

images were captured with a CCD-Exi camera (Molecular Devices) and analyzed using MetaMorph version 6.1 software (Molecular Devices).

Strain	Alias	Genotype and Remarks	Source
SH4848	W303-1A	MATa ura3-1 leu2-3,112 trp1 his3-11,15 ade2-1	R. Rothstein
SH4849	W303-1B	MAT α ura3-1 leu2-3,112 trp1 his3-11,15 ade2-1	R. Rothstein
SH8931	$\Delta slt2$	MATα his3 $\Delta$ 1 leu2 $\Delta$ 0 lys2 $\Delta$ 0 ura3 $\Delta$ 0 $\Delta$ slt2::KanMX4	Invitrogen
	W303-1A:: <i>TRP1</i>	MAT <b>a</b> ura3-1 leu2-3,112 trp1:: pRS304-TRP1 his3-11, 15 ade2-1	This study
	$\Delta ptcl$	MATa ura3-1 leu2-3,112 trp1 his3-11,15 ade2-1 ptc1::loxPCgTRP1	Sharmin <i>et al.</i> , 2014
	$\Delta ptc6$	MATa ura3-1 leu2-3,112 trp1:: pRS304-TRP1 his3-11, 15 ade2-1 ptc6:: loxP-HPH-loxP	Sharmin <i>et al.</i> , 2014
	$\Delta ptc1\Delta ptc6$	MATa ura3-1 leu2-3,112 trp1 his3-11,15 ade2-1 ptc1::loxPCgTRP1 ptc6:: loxP-HPH-loxP	Sharmin <i>et al.</i> , 2014

 Table 5. Saccharomyces cerevisiae strains used in this study.

#### Table 6. Oligonucleotide primers used in this study.

Name	Sequence (5'-3')		
Disruption primers			
gdPTC1-F	ATCATTTAGGCACTGCATTTATCTTTTAAAAAATCATTATACTTCGTACGCTGCAG		
gdPTC1-R	GTCTATGCATAATTTTTGCGCGGTTTATAACGGATCCTTCGCCACTAGTGGATCT		
gdPTC6-F	CTGCAATCGGGGCAATTAAGCATCAGAAGAGGGGAATTTGCTTCGTACGCTGCA		
gdPTC6-R	AAGATGATTATCTAGGACTTGTTTCCACCCAGGGGGGGTGTGCCACTAGTGGATCT		
Confirmation primers			
cPTC1-F	TTAGTTAAACATTATTATTC		
cPTC1-R	GAGATAATGGCGAATTAGAC		
cPTC6-F	CTCGCCATAGCCCTTGTAAC		
cPTC6-R	GCTTGAAATCAGGGTATACT		
RT-PCR pr	imers		
RT-KDX1-	F CTCACAGCCTTATTTGTTCTTC		
RT-KDX1-	R CGCGTTTGGTATTTTCTGA		
RT-CRH1-	F GGCTGCCGAAAGTACTGCTA		
RT-CRH1-	R GCGTACAACCTGTAGTTTTTAACG		
RT-SEDI-F	TCCTATTATCTGCCGGTTTAGC		
RT-SEDI-R	AAGTGACATCGGTGGAAGAAG		
RT-ACT1-I	F TGGATTCCGGTGATGGTGTT		
RT-ACT1-I	R TCAAAATGGCGTGAGGTAGAGA		

#### 3.3 Results

### 3.3.1 Deletion of *PTC6* in a $\Delta ptc1$ disruptant causes increased sensitivity to cell wall damaging agents

Exposure of  $\Delta ptc1$  cells to CR or CFW showed that these cells were relatively tolerant to such cell wall-damaging agents; similarly,  $\Delta ptc6$  cells were tolerant to both reagents (Figure 1). By contrast,  $\Delta ptc1$  cells in which *PTC6* was also deleted were completely intolerant of these agents and failed to survive (Figure 12). As both CR and CFW are known to cause activation of the Slt2-CWI pathway and as the Slt2-CWI pathway is also activated in cells lacking *PTC1* (Fuchs and Mylonakis, 2009), we decided to evaluate the potential role of Ptc6 in the Slt2 pathway.



**Figure 12.** Additive genetic effect of  $\Delta ptc6$  disruption in *PTC1* deficient cells. Ten-fold serial dilutions of the wild-type,  $\Delta ptc1$  and  $\Delta ptc6$  single disruptants, the  $\Delta ptc1\Delta ptc6$  double disruptant, and the  $\Delta slt2$  disruptant were spotted onto YPDA plates without or with CR (4 µg/ml) or CFW (5 µg/ml) and incubated at 30°C.

We found that wild-type,  $\Delta ptc1$ ,  $\Delta ptc6$  and  $\Delta ptc1\Delta ptc6$  strains exhibited similar growth patterns under standard conditions (Fig. 2A). However, the  $\Delta ptc1\Delta ptc6$  double disruptant grew very slowly in media containing CR or CFW, whereas the  $\Delta ptc1$  strain was relatively tolerant to both cell wall-damaging agents (Fig. 2B, 2C) and  $\Delta ptc6$  single disruptant displayed similar growth to the wild type (Fig. 2B, 2C). Number of viable cells was low (20%) in *ptc1* single disruptant and further decreased in  $\Delta ptc1\Delta ptc6$  double disruptant (10%) under cell wall stress conditions. It was observed that the  $\Delta ptc1\Delta ptc6$  double disruptant could not reach OD<sub>660</sub> = 1.0 whereas wild type and  $\Delta ptc6$  disruptant were able to reach OD<sub>660</sub> = 1.0 after 9 hours both in the absence and presence of CR and CFW, suggesting that the growth of  $\Delta ptc1\Delta ptc6$  might be decreased due to the decrease in cell density. Thus, sensitivity was increased very considerably in cells lacking both *PTC1* and *PTC6* genes, a response pattern similar to that of the  $\Delta slt2$  disruptant although the growth of the  $\Delta ptc1\Delta ptc6$  double disruptant might be affected by decreased number of viable cells for first three hours but was affected later (Fig. 2A, 2B and 2C). These results suggest that hyper-sensitivity phenotype of the  $\Delta ptc1\Delta ptc6$  double disruptant to cell wall damaging agents involves the Slt2 CWI pathway.

#### 3.3.2 *PTC6* negatively affects Slt2 phosphorylation in the $\Delta ptc1$ disruptant

It was previously demonstrated that a lack of Ptc1 results in the activation of the Slt2 pathway (Li *et al.*, 2010). Our finding here of a synergistic effect between *PTC6* disruption and a  $\Delta ptc1$  background on sensitivity to CR or CFW induced cell wall stress (Figure 13) prompted us to investigate whether there is a functional connection between Ptc6 and Slt2 CWI pathway activation. Cells lacking Ptc1 are known to have higher levels of phosphorylated Slt2 than wild type cells (González *et al.*, 2006; Du *et al.*, 2006). Therefore, we first compared the levels of phosphorylated Slt2 in  $\Delta ptc1\Delta ptc6$  cells with those in wild type and single disruptant cells. Two antibodies were used for this analysis, one that specifically detects the phosphorylated form of Slt2 and the other that detects Slt2 regardless of its phosphorylation status. We found that the level of phosphorylated Slt2 was considerably increased in  $\Delta ptc1\Delta ptc6$  double disruptant cells in the presence of CR (lane 8,

Figure 14A). Quantification of the relative changes in phosphorylation showed an increase in Slt2 phosphorylation and an increase in the total amount of Slt2 protein during growth in the presence of CR in both  $\Delta ptc1$  and  $\Delta ptc1\Delta ptc6$  disruptants compared to wild type cells (Figure 14B). It is noted that a little increase in total Slt2 in  $\Delta ptc6$  single disruptant compared with that in wild type in the presence of CR does not represent an increase in the phosphorylated form of Slt2, as an increase in the amount of total Slt2 did not imply an increment only in the phosphorylated Slt2, rather accumulated large amount of the non-phosphorylated Slt2 (González *et al.*, 2006). Our data indicate that Ptc6 negatively controls the level of Slt2p phosphorylation in  $\Delta ptc1$  cells.



**Figure 13.** Effect of  $\Delta ptc6$  disruption in *PTC1* deficient cells on growth under cell wall stress conditions. (A) Wild-type cells,  $\Delta ptc1$ ,  $\Delta ptc6$  and  $\Delta ptc1\Delta ptc6$  disruptant cells, and *Slt2*-deficient cells were inoculated into liquid YPD medium. Cultures were later transferred to fresh YPDA medium with 4 µg/ml CR (B) or 5 µg/ml CFW (C) at OD<sub>660</sub>= 0.1 and ODs were assessed every hour for 24 hours.



**Figure 14.** Effect of Ptc6 on changes in Slt2 activation. (A) Anti-phospho-Slt2 immunoblot analysis of wild type,  $\Delta ptc1$ ,  $\Delta ptc6$ ,  $\Delta ptc1\Delta ptc6$  and  $\Delta slt2i$  cells. Proteins extracted from cells grown in media with or without 4 µg/ml CR to OD<sub>660</sub> = 1.0 at 30°C were separated on SDS–PAGE gels (lanes 1 - 10), and immunoblotted with anti-antiphospho-p44/42 MAPK (Thr202/Tyr204) antibody. Identical samples were used to detect total Slt2 by immunoblot with anti-Mpk1p/Slt2. (B) Quantification of band intensities was performed by the ImageJ software and shown as Phospho-Slt2/ Slt2 signals ratios.

#### 3.3.3 Deletion of PTC6 causes increased transcription of KDX1

As our immunoblot analysis showed differences in the levels of phosphorylated Slt2 in  $\Delta ptc1$  and  $\Delta ptc1\Delta ptc6$  cells, we next investigated Slt2-mediated transcription of these three genes under cell wall stress conditions. Our analysis showed an increase in *KDX1* transcripts in the presence of CR; this increase was significantly greater in  $\Delta ptc1\Delta ptc6$  double disruptant cells than in wild type,  $\Delta ptc1$ , and  $\Delta ptc6$  cells (Figure 15). The genes *KDX1*, *CRH1* and *SED1* have been shown to be induced under cell wall stress conditions in cells lacking *PTC1* (González *et al.*, 2006). The functions of these genes are described in Table 7. Although *CRH1* and *SED1* are known to be induced in wild type cells following induced cell wall damage (González *et al.*, 2006), they only appeared to be induced here at very low levels in  $\Delta ptc1\Delta ptc6$  cells after exposure to CR (Figure 15). Moreover,  $\Delta ptc1$  and  $\Delta ptc6$  disruptant cells showed no induction of these two genes. These observations suggest that  $\Delta ptc6$  disruption. Our results for *CRH1* and *SED1* transcriptional induction in  $\Delta ptc1$  cells are inconsistent with a previous report (González *et al.*, 2006).



**Figure 15.** Cell wall-related genes are up-regulated in  $\Delta ptc1\Delta ptc6$  double disruptant cells after exposure to CR stress. cDNAs were synthesized from total RNAs isolated from cells grown under standard conditions or in the presence of 4 µg/ml CR. These cDNAs were used as templates for RT-PCR using SYBR Green and specific primers to examine expression of *KDX1*, *CRH1*, and *SED1*. Actin (ACT1) was used as the control. *KDX1*, *CRH1* and *SED1* showed increased expression in  $\Delta ptc1\Delta ptc6$  double disruptant cells following exposure to the CR stress. The relative levels of expression of these three genes were calculated by a normalized target gene expression analysis (2<sup>(- $\Delta\Delta$ Ct)</sup>) of amplification plots derived from real-time RT-PCR. The results are the means of three experiments from three independent preparations.

ORF	Gene	Functions	References		
YDL006w	PTC1	Inactivation of osmosensing MAPK cascade	Warmka et al., 2001		
		through dephosphorylation of Hog1p.			
		Involved in Fus3p activation during	Malleshaiah et al., 2010		
		pheromone-dependent signal transduction.			
		Deletion affects precursor tRNA splicing via	Robinson et al., 1994		
		endonucleolytic cleavage and ligation.			
YCR079w	PTC6	Involved in macroautophagy.	Journo et al., 2009		
		dephosphorylation of the Pda1 subunit of	Gey et al., 2008		
		pyruvate dehydrogenase with Ptc5p.			
YKL161c	KDX1	Implicated in Slt2 mitogen-activated (MAP)	Watanabe et al., 1997		
		kinase signaling pathway in association with			
		Rlm1p.			
		Interacts with numerous components in the	Breitkreutz et al., 2010		
		mating pheromone and CWI MAPK			
		pathways.			
YGR189c	CRH1	Functions in the transfer of chitin to beta(1-	Cabib, 2009		
		6) and beta(1-3) glucans in the cell wall.			
YDR077w	SED1	Mitochondrial genome maintenance.	Phadnis and Ayres Sia,		
		-	2004		
		A major cell wall protein in the stationary	Shimoi et al., 1998		
		phase and is involved in lytic enzyme			
		resistance.			

Table 7. Function of genes discussed in this chapter.

## 3.3.4 Simultaneous deletion of *PTC1* and *PTC6* causes a severe defect in vacuole morphogenesis

In several genomic screens, Ptc1 has been identified as being required for normal vacuolar inheritance. Additionally, there appears to be a linkage between the risk of changes to vacuolar morphology and the hypersensitivity of  $\Delta ptc1$  disruptant cells to caffeine, CR,

zinc and calcium ions, and high pH (Jin et al., 2009). Therefore, we decided to investigate whether vacuolar alterations occurred in  $\Delta ptc6$  and  $\Delta ptc1\Delta ptc6$  cells in the presence of CR and CFW. Microscopic analyses of vacuolar membranes stained with FM4-64 dye showed that vacuolar morphology was normal in  $\Delta ptc1 \Delta ptc6$  double disruptant cells grown in YPDA. However, under CR or CFW stress conditions, approximately 40-50% cells displayed fragmented vacuoles in  $\Delta ptc1\Delta ptc6$  cells (>100 cells per condition were evaluated, depending on the number of vacuoles per cell; Figure 16). The  $\Delta ptc6$  cells displayed normal vacuoles in YPDA and in the presence of 4 µg/ml CR or 5 µg/ml CFW. Previously, it was reported that  $\Delta ptc1$  cells showed fragmented vacuoles even under standard conditions, which caused Slt2 activation (Li et al., 2010). Additionally, it was reported that GRX3 and GRX4 genes played very important role in the defense against oxidative stress. The  $\Delta grx3\Delta grx4$ cells exhibited fragmented vacuole and CR and CFW sensitive phenotype and both of these phenotypes can be rescued by overexpression of Slt2p (Carrion et al., 2013). Therefore, we predicted that the fragmented vacuoles in  $\Delta ptcl$  disruptant cells might be rescued in  $\Delta ptc1\Delta ptc6$  double disruptant cells by the increased expression of Slt2. Contrary to this prediction; we found that fragmented vacuoles were still observed in  $\Delta ptc1\Delta ptc6$  cells in the presence of CR or CFW, although not in YPDA (Figure 16). This observation suggests that although Slt2 overexpression can suppress the occurrence of fragmented vacuoles in  $\Delta ptc1$ disruptant cells under normal growth conditions (Carrion et al., 2013), it cannot do so under the cell wall stress conditions induced by CR and CFW. Therefore, in addition to Ptc1, PTC6 might be required for normal vacuole function under cell wall stress conditions. The introduction of VPS73 gene into  $\Delta ptc 1 \Delta ptc6$  double disruptants cells was unable to rescue the CR and CFW sensitive phenotype of the  $\Delta ptc1 \Delta ptc6$  double disruptant (Figure 17).



**Figure 16.** Vacuolar fragmentation in  $\Delta ptc1 \Delta ptc6$  double disruptant cells. Wild type,  $\Delta ptc6$  and  $\Delta ptc1 \Delta ptc6$  double disruptant cells are stained with FM4-64. White triangles indicate the cellular regions shown in the enlarged images showing fragmented vacuolar membranes. Bar = 5 µm.



**Figure 17.** Effect of overexpression of *VPS73* of the growth defect of the  $\Delta ptc1$  and  $\Delta ptc1 \Delta ptc6$  disruptant strains under cell wall stresses. The  $\Delta ptc1$  and  $\Delta ptc1 \Delta ptc6$  disruptants were transformed with the YEp vectors (without/with *VPS73* gene) and spotted on YPDA plates without or with CR (4 µg/ml) or CFW (5 µg/ml) and incubated at 30°C. Photograph for the growth of transformants was taken after 2 days.

#### 3.4 Discussion

The initial screens of the *S. cerevisiae* genome revealed the presence of a considerable number of genes (more than 50%) that was either previously uncharacterized or of unknown function (Piskur and Langkjaer, 2004). Comparative genomic and phenotypic studies have since provided the opportunity to understand the unexplored biological functions of these genes (Piskur and Langkjaer, 2004; Zhang, 2012). In this work, we focused on the function of two PP2C PPases, namely *PTC1* and *PTC6*, that are responsive to CR and CFW induced cell wall stress. The transcriptional profile of cells with a  $\Delta ptc6$  disruption is similar to that of cells with a  $\Delta ptc1$  disruption (González *et al.*, 2013). However, Ptc1 shares only 13% amino acid similarity with Ptc6 (Figure 6A) and the two proteins localize in different cellular compartments: Ptc1 is present in the nucleus and cytoplasm, while Ptc6 localizes to mitochondria (Figure 6B). Our analyses showed that a lack of *PTC6* in  $\Delta ptc1$  cells caused a significant increase in sensitivity to the cell wall-damaging agents CR and CFW; however, these agents had no effect on *PTC6* deficient cells (Figure 1). These observations suggest that, in addition to its known functions (Table 7), loss of *PTC6* also affects the sensitivity of  $\Delta ptc1$ cells to agents that cause cell wall damage. Although  $\Delta ptc1$  single disruptant used in previous study (González et al., 2013) showed increased sensitivity to CR and CFW compared to  $\Delta ptc1$  single disruptant used here and no sensitive phenotype of  $\Delta ptc6$  single disruptant to CR and CFW is noticeable both in this study and previous study at similar concentrations of these cell wall damaging agents, this might be ascribed to the difference in the genetic background because the genetic background of the wild type strain used in our study (W303) is different from that studied by Gonzalez et al. (González et al., 2013) (BY4741). Hence, it might be possible that the growth of the wild type strain in nutrient rich condition is slower in our study than that of the wild type in the previous study (González et al., 2013). In contrast to the results obtained here, a previous study reported that deletion of PTC6 improved growth in  $\Delta ptc1$  disruptant cells; that study also found that  $\Delta ptc6$  single disruptant cells were tolerant to CR and CFW, as observed here (González et al., 2013). Therefore, it is possible that the mechanism by which PTC6 functions in cell wall stress response is strain specific. We suggest that Ptc6 might have different role from Ptc1 in the negative regulation of Slt2 phosphorylation.

Previous studies suggested that cell wall damage causes activation of the Slt2 mitogenactivated CWI pathway (Levin, 2005) and evidence of a relationship between *PTC1* and the activation of CWI pathway has been reported (Ariño *et al.*, 2011). Thus, for example, cells lacking Ptc1 are sensitive not only to CR and CFW but also to caspofungin, caffeine and alkaline pH, all of which activate the CWI pathway (Markovich *et al.*, 2004; González *et al.*, 2006). It is well known that an increased level of expression of Slt2 is a general response of cells to cope with conditions causing cell wall damage (Garcia *et al.*, 2004). Cells with a  $\Delta ptc1$  disruption have higher levels of active phosphorylated Slt2 (Du *et al.*, 2006). Here, we observed that an elevated level of phosphorylated Slt2 was detectable in the  $\Delta ptc1\Delta ptc6$ 

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double disruptant compared to the  $\Delta ptc1$  single disruptant (Figure 3A, B). There might be a possibility that the expression of Slt2 is affected by the number of viable cells in  $\Delta ptc1\Delta ptc6$  double disruptant. Therefore, we suggest that Ptc6 might negatively regulate Slt2 MAPK under cell wall stress conditions since the additional disruption of *Ptc6* in  $\Delta ptc1$  cells increased the amount of phosphorylated Slt2 in the presence of CR. The detailed mechanism of this suggested function is currently unknown. However, we were unable to detect any changes in the levels of phosphorylation of Slt2 in  $\Delta ptc6$  single disruptant cells even in the presence of CR. Therefore, we suggest that phosphorylation of Slt2 might be triggered by disruption of *PTC1* but not by *PTC6* disruption; however, *Ptc6* disruption might subsequently induce additional phosphorylation of Slt2 in  $\Delta ptc1$  cells.

A previous DNA microarray analysis reported that  $\Delta ptc1$  disruption results in increased expression of genes such as *KDX1*, *CRH1* and *SED1* that are associated with cell wall stress responses and that this effect on expression was dependent on Slt2 MAPK (González *et al.*, 2006). Our results here likewise showed that expression of *KDX1* in wild-type cells was low under normal growth conditions but increased slightly upon exposure to CR; in  $\Delta ptc1\Delta ptc6$ cells exposed to CR, a large increase in *KDX1* mRNA levels was identified (Figure 4). In contrast to Gonzales and colleagues (González *et al.*, 2006), we found only a small increase in *CRH1* expression in  $\Delta ptc1\Delta ptc6$  cells which was lower than in wild type cells, and  $\Delta ptc1$ single disruptant and no increase in the transcription was noticed for *SED1* mRNA (Figure 4). Thus although increased transcription of *CRH1* and *SED1* was previously reported to be induced in  $\Delta ptc1$  single disruptant cells (González *et al.*, 2006), we could not repeat this finding here. Therefore, the role of *PTC1* in the transcription of *CRH1* and *SED1* might be strain specific and, similarly, the effect of  $\Delta ptc6$  disruption on *CRH1* and *SED1* transcription might vary between  $\Delta ptc1$  strains. Our results also suggest that transcription of *KDX1* in  $\Delta ptc6$  disruptant cells was induced by the additional disruption of *PTC1*, irrespective of the

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presence of CR and the regulation of *KDX1* transcription by these two phosphatases might be mediated by distinct mechanisms from those of Slt2 phosphorylation and the vacuolar morphology.

A review of the current works showed that lack of Ptc1 function resulted in numerous phenotypes and many aspects of the phenotype of the  $\Delta ptc1$  disruptant, such as sensitivity to the heavy metals zinc, copper, cesium, or defective sporulation and germination, or altered cell wall structure and function, co-occurred with impaired vacuolar function (González *et al.*, 2013; González *et al.*, 2009). We also report here that  $\Delta ptc1\Delta ptc6$  cells showed fragmented vacuoles in the presence of cell wall-damaging agents (Figure 5) although they displayed a normal vacuole phenotype under standard growth conditions. It is known that overexpression of *VPS73* could rescue the sensitivity of  $\Delta ptc1$  disruptant to cell wall-damaging agents (González *et al.*, 2006). It is known that *VPS73* is a mitochondrial protein (Sickmann *et al.*, 2003). Also *PTC6* is a protein localized in the mitochondria. Since additional  $\Delta ptc1$ disruption drastically increased phosphorylation of Slt2 in  $\Delta ptc6$  disruptant cells in the presence of CR (Figure 14), we propose that the mechanism by which  $\Delta ptc1$  disruption induces vacuolar fragmentation in  $\Delta ptc6$  cells under cell wall stress conditions might involve Slt2 phosphorylation.

	Ptc1	Ptc2	Ptc3	Ptc4	Ptc5	Ptc6	Ptc7	
Α	100	24	29	22	18	13	13	Ptc1
		100	60	25	9	11	9	Ptc2
			100	26	11	4	13	Ptc3
				100	14	14	13	Ptc4
					100	9	10	Ptc5
						100	4	Ptc6
							100	Ptc7

B



**Figure 18.** Amino acid sequence comparison of Ptc proteins (Ptc1 - Ptc7). (A) The level (%) of similarity in the amino acid sequences of the seven PTC PPases. Ptc1 was found to share only 13% similarity with Ptc6. (B) Localization of each PTC PPase in the cell. Localization data were obtained from direct assays collated at <u>http://www.yeastgenome.org</u>.

Previous studies suggested that cell wall damage causes activation of the Slt2 mitogenactivated CWI pathway (Levin, 2005) and evidence of a relationship between *PTC1* and the activation of CWI pathway has been reported (Ariño *et al.*, 2011). Thus, for example, cells lacking Ptc1 are sensitive not only to CR and CFW but also to caspofungin, caffeine and alkaline pH, all of which activate the CWI pathway (Markovich *et al.*, 2004; González *et al.*, 2006). It is well known that an increased level of expression of Slt2 is a general response of cells to cope with conditions causing cell wall damage (Garcia *et al.*, 2004). Cells with a  $\Delta ptc1$  disruption have higher levels of active phosphorylated Slt2 (Du *et al.*, 2006). Here, we observed elevated level of phosphorylated Slt2 in the  $\Delta ptc1\Delta ptc6$  double disruptant compared to the  $\Delta ptc1$  single disruptant (Figure 14A, B). Therefore, we suggest that Ptc6 might negatively regulate Slt2 MAPK pathway under cell wall stress conditions since the additional disruption of *PTC6* in  $\Delta ptc1$  cells increased the amount of phosphorylated Slt2 in the presence of CR. The detailed mechanism of this suggested function is currently unknown. However, we were unable to detect any changes in the levels of phosphorylation of Slt2 in  $\Delta ptc6$  single disruptant cells even in the presence of CR. Therefore, we suggest that phosphorylation of Slt2 might be triggered by disruption of *PTC1* but not by *PTC6* disruption; however, *PTC6* disruption might subsequently induce additional phosphorylation of Slt2 in  $\Delta ptc1$  cells.

A previous DNA microarray analysis reported that  $\Delta ptc1$  disruption results in increased expression of genes such as *KDX1*, *CRH1* and *SED1* that are associated with cell wall stress responses and that this effect on expression was dependent on Slt2 MAPK (González *et al.*, 2006). Our results here likewise showed that expression of *KDX1* in wildtype cells was low under normal growth conditions but increased slightly upon exposure to CR; in  $\Delta ptc1\Delta ptc6$  cells exposed to CR, a large increase in *KDX1* mRNA levels was identified (Figure 15). In contrast to Gonzales and colleague's report (González *et al.*, 2006), we found in this study only a small increase in *CRH1* expression in  $\Delta ptc1\Delta ptc6$  cells which was lower than in wild-type cells, and  $\Delta ptc1$  single disruptant and no increase in the transcription was noticed for *SED1* mRNA (Figure 15). Thus although increased transcription of *CRH1* and *SED1* was previously reported to be induced in  $\Delta ptc1$  single disruptant cells (González *et al.*, 2006), we could not repeat this finding here. Therefore, the role of *PTC1* in the transcription of *CRH1* and *SED1* might be strain specific and, similarly, the effect of  $\Delta ptc6$  disruption on *CRH1* and *SED1* transcription might vary between  $\Delta ptc1$  strains. Our results also revealed that transcription of *KDX1* in  $\Delta ptc6$  disruptant cells was induced by the additional disruption of *PTC1*, irrespective of the presence of CR.

# **Chapter 4**
## General discussion and conclusion

Any conditions that do not favor normal growth are regarded as stress. The response to stresses is an important field of biological science for both industrial and basic research, for diverse organisms from prokaryotic bacteria to plants, and mammals. Several stress conditions such as nutrient limitation, changes in external temperature, osmolarity, humidity, pH, and exposure to toxins have widely been studied for laboratory as well as industrial research. S. cerevisiae retains an inherent ability to manage growth and proliferation during environmental fluctuations by activating diverse parallel signaling pathways that control expression of gene and protein activity. These pathways are regulated by several mechanisms ranging from transcription to post-translational modification in response to environmental stresses. Among these modifications, we have been interested in cellular regulation by reversible protein phosphorylation which is one of key regulatory systems in all kinds of organisms. Since 99% phosphorylation occurs in Ser/ Thr residues of proteins in eukaryotes and PP2C PPases in S. cerevisiae share high osmolarity and identity, in an effort to understand the function of the PP2C subfamily PPases in cell physiology of S. cerevisiae, we constructed 127 multiple disruptants of seven PTC genes in all possible combinations and systematically analyzed various phenotypes. Especially, one of the main purposes of this work was to discover functional redundancy among PTC PPases. With this purpose we discussed here the effects of deletion of different PP2C protein phosphatase genes in response to various environmental stresses.

Common cellular targets are known for specific PP2Cs among different yeasts. For example, Slt2 and Pmk1, MAPKs in *S. cerevisiae* and *S. pombe* cell wall integrity pathways are the targets of Ptc1 (Shiozaki and Russell, 1995). Negative regulation of MAPK pathways by PP2Cs has been also documented for plants and animals. For example, *Arabidopsis* ABI1and ABI2 is two plant PP2Cs involved in abscisic acid signal transduction

(Schweighofer *et al.*, 2004). Mammalian PP2C $\alpha$  and PP2C $\beta$  dephosphorylate p38 MAPK signalling induced by anisomycin and NaCl (Lu and Wang, 2008) and therefore some functions of PP2C PPases seem to be conserved through evolution. However, the same PP2C homologues can reveal reverse cellular roles in different organisms. For instance, *Candida albicans*, *CaPTC1* which shares 52% identity with the *S. cerevisiae PTC1*, contribute to the pathogenicity of *C. albicans* by increasing tolerance to cell wall damaging agent, CR. By contrast,  $\Delta ptc1$  in *S. cerevisiae* is sensitive to cell wall damaging agents like CR and CFW.

My results described in this study revealed that that no disruptants of *PTC* PPases were lethal at least in nutrient rich medium, suggesting that no combinations of *PTC* gene disruption had a redundant role at least under standard growth condition. However, systematic phenotype testing under various stresses i.e., high and low temperatures, high osmolarity, sensitivity to cell wall-damaging agents, and sensitivity to ions and genotoxic agents, led us to discover several new phenotypes (summarized in Table 4) and several combinations of genes were found to be functionally redundant under those conditions for the first time in this study. For example *PTC5* and *PTC7* is such pair of genes having redundant function for cold sensitivity, *PTC1* and *PTC6* are redundant for CR, CFW and caffeine sensitivity. Also more than two PPases were found to be functionally redundant. For example, *PTC1*, *PTC2* and *PTC7* genes were found to be redundant for both high temperature and Li<sup>+</sup> sensitivity and this is also the first report about four genes having redundant function. These findings should be future research subjects at molecular level.

In my study we discovered that simultaneous disruption of *PTC1* and *PTC6* showed increased sensitivity upon exposure to cell wall-damaging agents although these two PPases shared only 13% amino acid sequence similarity. Also these PPases are localized in different cellular compartments; *PTC1* in cytoplasm and nucleus and *PTC6* in mitochondria (Figure

18). Since an elevated amount of phosphorylated Slt2 was observed in the  $\Delta ptc1\Delta ptc6$  double disruptant compared to the  $\Delta ptc1$  and  $\Delta ptc6$  single disruptant cells under cell wall stress (Figure 14A, B), we suggested that *PTC1* and *PTC6* are functionally associated in Slt2 phosphorylation although they have less similarity and different localization. In contrast, a transcriptional increase in cell wall related gene, *KDX1* was significantly induced in  $\Delta ptc1\Delta ptc6$  double disruptant compared to the  $\Delta ptc1$  and  $\Delta ptc6$  single disruptants irrespective of the presence of CR (Figure 15), suggesting regulation of Slt2 pathway by *PTC6* in  $\Delta ptc1$  cells even in the absence of cell wall damaging agents. Therefore, from these observations we suggest that *PTC6* might function in Slt2 CWI pathway in addition to *PTC1* and play a secondary role to enhance after the Slt2 pathway is activated due to the deletion of *PTC1*.

We also found that  $\Delta ptc1\Delta ptc6$  cells showed fragmented vacuoles in the presence of both CR and CFW although a normal vacuolar phenotype was observed in  $\Delta ptc6$  single disruptant both in the absence or presence of cell wall stresses (Figure 16). Previous studies revealed that  $\Delta ptc1$  disruptant was defective in normal vacuole functioning (Jin *et al.*, 2009). In this study we suggest that phenotypes derived from the deletion of other *PTCs* along with *PTC1* deletion such as sensitivity to the heavy metals zinc, copper, cesium, or defective sporulation and germination, or altered cell wall structure and function that co-occurred with impaired vacuolar function are due to the secondary effect of *PTC6* deletion in conjunction with the loss of prime function of *PTC1*. In connection with the increased Slt2 phosphorylatyion in the  $\Delta ptc1\Delta ptc6$  cells, we also suggest the involvement of Ptc6 that functions following  $\Delta ptc1$  disruption supporting the possibility that *PTC6* might function in Slt2 CWI pathway in addition to *PTC1*.

In yeast, signaling pathways have been implicated in the condition-specific regulation of gene expression under environmental stresses. HOG pathway is activated by a

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number of conditions, including hyper-osmotic stress, high temperature and low temperature although in each of these situations the signal that activates the HOG pathway may be due to the loss of balance in intracellular glycerol content (Panadero *et al.*, 2006). The human homolog of the MAPK Hog1, p38 $\alpha$ , not only mediates the response to hyper-osmolarity as well (Waltermann and Klipp., 2010), but also plays key roles in inflammation and cancer (Wagner and Nebreda, 2009.) Although resistance to MAPK-activating stresses is very common among different organisms, genes of the core MAPK cascades exhibit high levels of divergence (Bahn *et al.*, 2007; Wu *et al.*, 2010).

It is still unknown that how differences in the elements of the MAPK pathways contribute to the phenotypic differences in the same species. Our laboratory aims to screen improved strain with stress resistance emphasizing their industrial importance. This study gives us fundamental knowledge and understanding about the mechanism for resistance by seven PP2C PPase genes in *S. cerevisiae* in response to environmental stresses. Therefore, knowledge from this finding about stress sensitive strains how and which genes are responsible for particular function can also be the basis for strain improvement. Finally, it can be said that *PTC* PPases are mostly involved in stress signaling pathways but still they might possess unknown function that enhance or inhibit cellular regulation irrespective of their structural similarity and cellular localization.

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## **Publications**

Sharmin, D., Sasano, Y., Sugiyama, M. and Harashima, S. 2014. Effects of deletion of different PP2C protein phosphatase genes on stress responses in *Saccharomyces cerevisiae*.Yeast. 10: 393-409.

**Sharmin, D.**, Sasano, Y., Sugiyama, M. and Harashima, S. 2014. Type 2C protein phosphatase Ptc6 participates in activation of the Slt2-mediated cell wall integrity pathway in *Saccharomyces cerevisiae*. **Journal of Bioscience and Bioengineering**. (In press).

## Acknowledgement

First and foremost, I would like to thank almighty God for all his blessing in my life, especially with the opportunity to study at Graduate School of Engineering, Osaka University and giving me the strength, courage, and the ability to do my research here for the past five years.

I wish to express my sincere thanks to my supervisor Professor Dr. Satoshi Harashima for giving me an excellent opportunity to study yeast genetics and molecular biology in his laboratory, who has been an invaluable scientific resource and outstanding teacher in various ways. His wide knowledge and logical way of thinking have been of great value for me. He has deeply enriched my knowledge and experience as an independent scientist by offering moral support and providing critical evaluation of my study that often led to exciting new insights into my research. Being unusually busy with many things, Dr. Harashima is always graciously available for problem solving, research discussion, manuscript editing or for personal help even in midnight.

I am especially grateful to Professor Dr. Yoshinobu Kaneko, Associate Professor Dr. Minetaka Sugiyama and Assistant Professor Dr. Yu Sasano who has provided me with numerous helpful scientific discussions and valuable advice over the last five years. Their consultation, advice and strong support unfailingly helped me to quickly overcome difficulties or to fulfill a goal. I also thank my co-supervisors, Professor Dr. Takuya Nihira and Professor Dr. Toshiya Muranaka that have provided me with the insight and direction needed to complete my thesis.

My thankfulness is also extended to all members of the Harashima (molecular genetics) laboratory for the support and sincere thanks to the faculty and staff of the Osaka University for their kind cooperation during my study period. I also appreciate Miss. Ayako Tomoda for her kind help in administrative matters.

I wish to extend my appreciation to the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) for financial support throughout this research period.

Last, but of course not the least, thank to my parents and brother, for their warmhearted encouragement and regular support. I could not have made it through successful without their love and support. I am very lucky to have such a warm and caring family. I hope they know how much I love them and how much I appreciate all their help and support over the past few years.

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