Doctoral Dissertation

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

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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 Δ*ptc5*Δ*ptc7* double disruptant and the Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*ptc7* quadruple disruptant exhibited low (13ºC) and high (37ºC) temperature sensitive growth, respectively. Interestingly, the septuple disruptant Δ*ptc1*Δ*ptc2*Δ*ptc3*Δ*ptc4*Δ*ptc5*Δ*ptc6*Δ*ptc7* showed an essentially normal growth phenotype at 37ºC. The Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*ptc7* quadruple disruptant was sensitive to LiCl (0.4 M). Two double disruptants Δ*ptc1*Δ*ptc2* and Δ*ptc1*Δ*ptc4* displayed slow growth and Δ*ptc1*Δ*ptc2*Δ*ptc4* could not grow on medium containing 1.5 M NaCl. The Δ*ptc1*Δ*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 Δ*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 Δ*ptc1* cells and more so in Δ*ptc1*Δ*ptc6* cells; therefore, deletion of *PTC6* enhanced Slt2 phosphorylation in the Δ*ptc1* disruptant. The level of transcription of *KDX1* upon exposure to CR increased to a greater extent in the Δ*ptc1*Δ*ptc6* double disruptant than the Δ*ptc1* single disruptant. The Δ*ptc1*Δ*ptc6* double disruptant cells showed normal vacuole formation under standard growth conditions, but fragmented vacuoles were present in the presence of CR or CFW. Our 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).

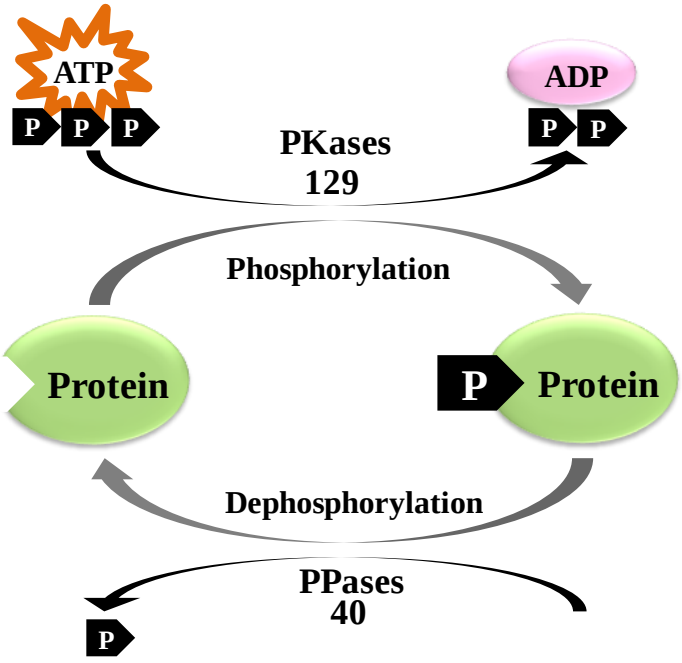


Figure 1: Cellular regulation mediated by reversible protein phosphorylation in *S. cerevisae*.

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).

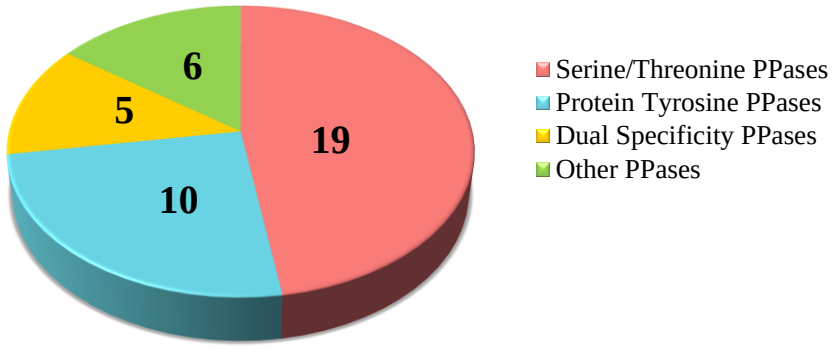


Figure 2: Van diagram showing different classes of PPase genes in *S. cerevisae*.

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 amino-

terminal domain or carboxyl-terminal domain (Figure 3) (Ariño *et al*., 2011). *S. cerevisiae* PTC (*PTC1-PTC7*) homologs, PP2Cα and PP2Cβ, 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).

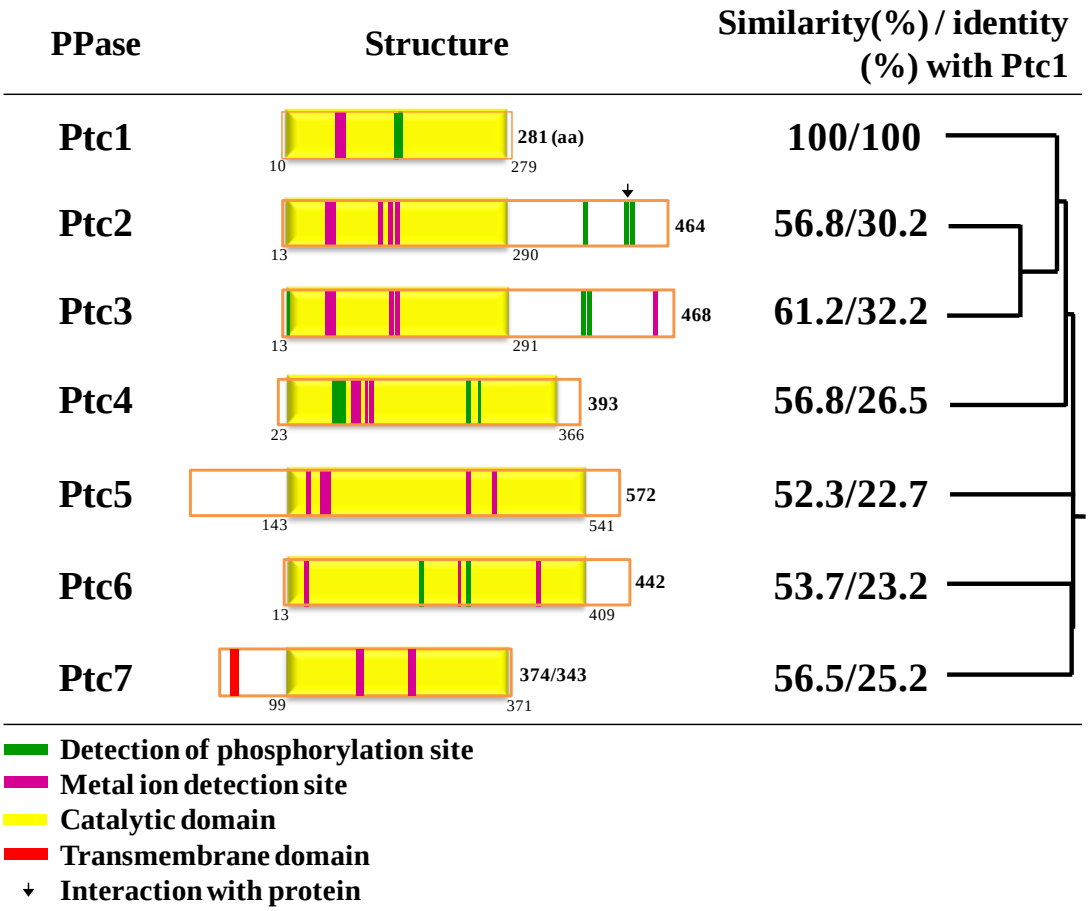


Figure 3: Schematic representation of the primary structure and percentage of similarity and identity of overlapping regions of different PP2Cs in comparison with Ptc1in *S. cerevisae*.

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

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

	YIL113W/SDP1	Negatively regulates Slt2p MAPK
	YNL099C/OCA1	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.

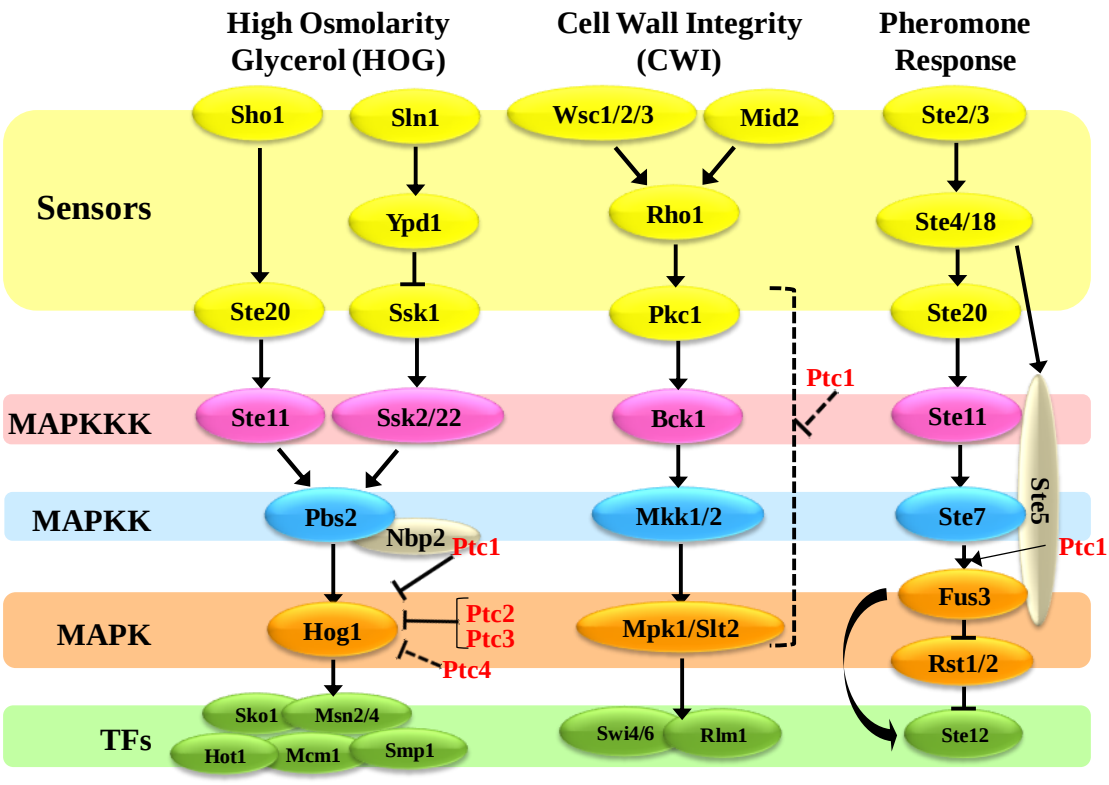


Figure 4: Mitogen-activated protein kinase (MAPK) pathways in *S. cerevisiae*.

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), ß1,6-glucan, ß1,3-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 ß-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 Δ*rlm1* disruptant also showed lowered transcription in Δ*slt2* disruptant strain. For example, Δ*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 never showed synthetic lethality. Secondly, the number of 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

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 Δ*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 Δ*ptc7*single disruptant and 10 double disruptants involving Δ*ptc6* and Δ*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*.,

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 (*MAT***a**/*MATα 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 (*MAT***a**) and W303-1B (*MATα*) (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 30ºC and 37ºC, respectively, unless otherwise specified.

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

Strain	Genotype and Remarks	Source
S. cerevisiae		
SH4848 (W303-1A)	*MAT***a** *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***a** *ura3-1 leu2-3,112 trp1 his3-11,15 ade2-1 ptc1::loxPCgTRP1 ptc2::loxPCgHIS3*	Sakumoto *et al*., 2002
SH682	*MAT***a** *lys1 trp3 ura1 ura2 pho8-12*	Our laboratory
SH683	*MAT***a** *lys1 trp3 ura1 ura2 pho3 pho8-12*	Our laboratory
SH6274	*MAT***a** *pho3-1 leu1-ELF 52*	Our laboratory
SH6275	*MAT***a** *pho3-1 leu1-SA 54*	Our laboratory
	*MAT***a** *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-*l*oxP TRP1*:: pRS304	This study
E. coli		
SHB3008	pUG6-*CgLEU2*	Sugiyama *et al*., 2005
SHB3009	pUG6-*CgHIS3*	Sugiyama *et al*., 2005
SHB3010	pUG6-*CgTRP1*	Sugiyama *et al*., 2005
SHB3276	pUG6-Δ*loxPURA3*	Sugiyama *et al*., 2008
SHB1463	pUG6	Güldener *et al*., 1996
	pUG6-*YAP1*	This study
	pUG6-*HPH*	This study

Table 3. Oligonucleotoide primers used in this study.

Name	Sequence (5´-3´)
Disruption primers	
gdPTC1-F	ATCATTTAGGCACTGCATTTATCTTTTAAAAATCATTATACTTCGTACGCTGCAG
gdPTC1-R	GTCTATGCATAATTTTTGCGCGGTTTATAACGGATCCTTCGCCACTAGTGGATCT
gdPTC2-F	TCCATTGTTGTATAAAATATAGAGAACCAGAAAAAGAAAACTTCGTACGCTGCAG
gdPTC2-R	GTATATAGGTATGTATATATAATGAAGGATGGAAGATCCT GCCACTAGTGGATCT
gdPTC3-F	AGAAGGCCAAGAAGACAAATCGAAGAAAGAGAGAGATAACCTTCGTACGCTGCAG
gdPTC3-R	GACTACTCTTTCGTTGCAAAGTACGGTTCGACAATATTTAGCCACTAGTGGATCT
gdPTC4-F	AGCCATTTTAGAAAGTAACT TCATTTGAAGAAGACAAAACTTCGTACGCTGCAG
gdPTC4-R	GTATGAGAAAAAGGAAGAAGAAAAATAATT TTTTTTCCGCCACTAGTGGATCT
gdPTC5-F	TTTCAACAGAAGAAGTGCTT TTACTTCTCTCAATCTCTCCCTTCGTACGCTGCAG
gdPTC5-R	ATCCTCTGGTATATACCTACCTCAGCATAAGTTTATATCGGCCACTAGTGGATCT
gdPTC6-F	CTGCAATCGGGGCAATTAAGCATCAGAAGAGGGGAATTTGCTTCGTACGCTGCAG
gdPTC6-R	AAGATGATTATCTAGGACTTGTTTCCACCCAGGGGGGTGTGCCACTAGTGGATCT
gdPTC7-F	ATAAAAGCGGTCCAGAAAACAAACGACAAAGCCACCAAAACTTCGTACGCTGCAG
gdPTC7-R	TTTATTTACACTGCTTTCCAGGAGATTAAAGAGCGGAGTGGCCACTAGTGGATCT
Confirmation primers	
cPTC1-F	TTAGTTAAACATTATTATTC
cPTC1-R	GAGATAATGGCGAATTAGAC
cPTC2-F	TTCTGACAGAGCAAATTGAA
cPTC2-R	TGCCCATCCGGGACTGGGGG
cPTC3-F	AAGTAATATAGCAAGAAACA
cPTC3-R	GGATTCAAAGGTTACCAACA
cPTC4-F	CCCGCGCTGATCTCTTCTTC
cPTC4-R	AATATGTGAATTTATTTCCA
cPTC5-F	AAATTTCCTTATCCCTTGCA
cPTC5-R	GTTCATTTTTTTGTGTGAT
cPTC6-F	CTCGCCATAGCCCTTGTAAC
cPTC6-R	GCTTGAAATCAGGGTATACT
cPTC7-F	CACAATAAAATTAGAAATTA
cPTC7-R	TCTTGACTAAAAGCGGTAAG
Primers for plasmid construction	
AgTEFp1B	CTCAGATCTGTTTAGCTTGCCTCGTCCCC
AgTEFp2YAP1F	CAGCGACCTCTTGGCGGTAGACACACTCATGGTTGTTTATGTTCGGATGT

AgTEFt1YAP1R	CAATTAGCTTTGAATAAGCATATGAACTAAGGATCCTCAGTACTGACAATAAAAAG
AgTEFt2X	CTCCTCGAGAGCTCGTTTTCGAC
YAP1-1	ATGAGTGTGTCTACCGCCAA
YAP1-2	TTAGTTCATATGCTTATTCA

2.2.2 Plasmid construction

Plasmid pUG6-HPH was constructed by inserting a 1.8 kb BglII/SacI_fragment which has the *hph* gene encoding a hygromycin B phosphotransferase from *Klebsiella pneumoniae* (Goldstein and McCusker, 1999), into the BglII/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 BglII/XhoI and inserted into the BglII/XhoI sites of pUG6.

2.2.3 Disruption of the *PTC* genes

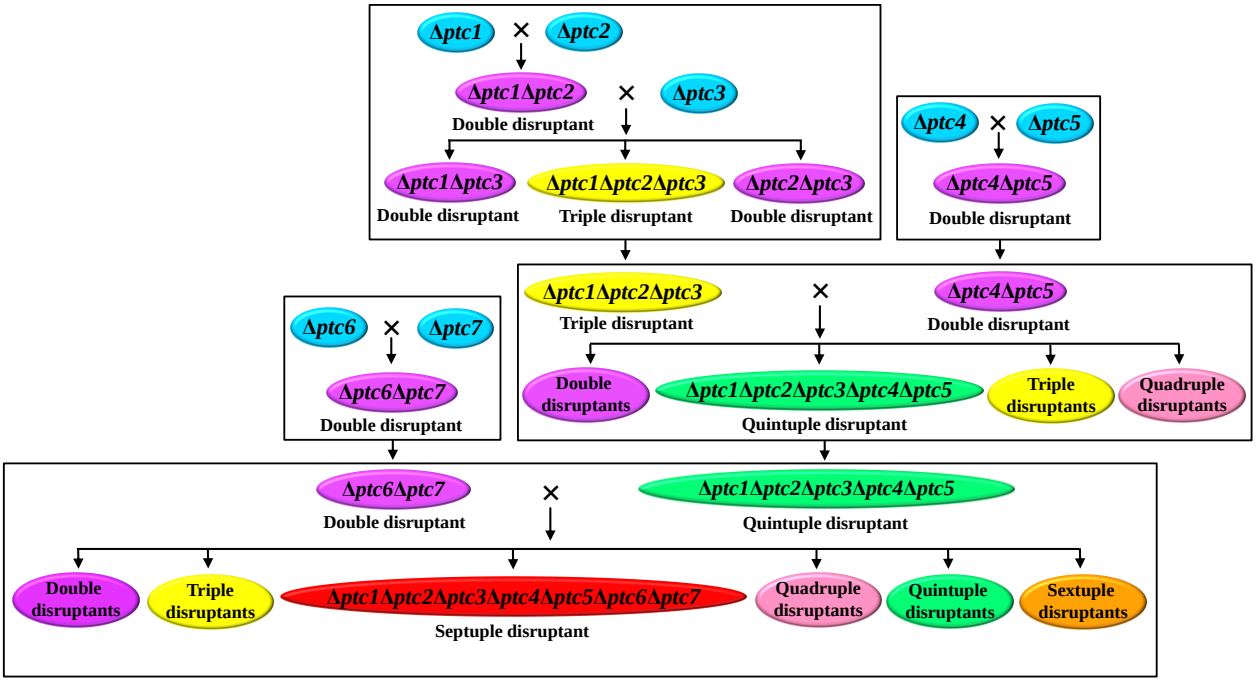
The seven *PTC* genes were each subjected to a PCR-mediated one step gene disruption strategy by means of homologous recombination by integration of the appropriate gene replacement cassette carrying *CgTRP1*, *CgHIS3*, *CgLEU2*, *ScURA3*, *YAP1*, *KanMX* module or plasmid-borne gene *HPH* (Figure 5A), 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^+, His^+, Leu^+, Kan^r, Cer^r, Hph^r and Ura^+ transformants were selected to isolate each gene deletion, i.e., Δ*ptc1*, Δ*ptc2*, Δ*ptc3*, Δ*ptc4*, Δ*ptc5*, Δ*ptc6* and Δ*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

Single disruptants (7)
Δ*ptc1*
Δ*ptc2*
Δ*ptc3*
Δ*ptc4*
Δ*ptc5*
Δ*ptc6*
Δ*ptc7*

Double disruptants (21)		
Δ*ptc1* Δ*ptc2*	Δ*ptc2* Δ*ptc4*	Δ*ptc3* Δ*ptc7*
Δ*ptc1* Δ*ptc3*	Δ*ptc2* Δ*ptc5*	Δ*ptc4* Δ*ptc5*
Δ*ptc1* Δ*ptc4*	Δ*ptc2* Δ*ptc6*	Δ*ptc4* Δ*ptc6*
Δ*ptc1* Δ*ptc5*	Δ*ptc2* Δ*ptc7*	Δ*ptc4* Δ*ptc7*
Δ*ptc1* Δ*ptc6*	Δ*ptc3* Δ*ptc4*	Δ*ptc5* Δ*ptc6*
Δ*ptc1* Δ*ptc7*	Δ*ptc3* Δ*ptc5*	Δ*ptc5* Δ*ptc7*
Δ*ptc2* Δ*ptc3*	Δ*ptc3* Δ*ptc6*	Δ*ptc6* Δ*ptc7*

Triple disruptants (35)				
Δ*ptc1* Δ*ptc2* Δ*ptc3*	Δ*ptc1* Δ*ptc3* Δ*ptc6*	Δ*ptc1* Δ*ptc6* Δ*ptc7*	Δ*ptc2* Δ*ptc4* Δ*ptc7*	Δ*ptc3* Δ*ptc5* Δ*ptc6*
Δ*ptc1* Δ*ptc2* Δ*ptc4*	Δ*ptc1* Δ*ptc3* Δ*ptc7*	Δ*ptc2* Δ*ptc3* Δ*ptc4*	Δ*ptc2* Δ*ptc5* Δ*ptc6*	Δ*ptc3* Δ*ptc5* Δ*ptc7*
Δ*ptc1* Δ*ptc2* Δ*ptc5*	Δ*ptc1* Δ*ptc4* Δ*ptc5*	Δ*ptc2* Δ*ptc3* Δ*ptc5*	Δ*ptc2* Δ*ptc5* Δ*ptc7*	Δ*ptc3* Δ*ptc6* Δ*ptc7*
Δ*ptc1* Δ*ptc2* Δ*ptc6*	Δ*ptc1* Δ*ptc4* Δ*ptc6*	Δ*ptc2* Δ*ptc3* Δ*ptc6*	Δ*ptc2* Δ*ptc6* Δ*ptc7*	Δ*ptc4* Δ*ptc5* Δ*ptc6*
Δ*ptc1* Δ*ptc2* Δ*ptc7*	Δ*ptc1* Δ*ptc4* Δ*ptc7*	Δ*ptc2* Δ*ptc3* Δ*ptc7*	Δ*ptc3* Δ*ptc4* Δ*ptc5*	Δ*ptc4* Δ*ptc5* Δ*ptc7*
Δ*ptc1* Δ*ptc3* Δ*ptc4*	Δ*ptc1* Δ*ptc5* Δ*ptc6*	Δ*ptc2* Δ*ptc4* Δ*ptc5*	Δ*ptc3* Δ*ptc4* Δ*ptc6*	Δ*ptc4* Δ*ptc6* Δ*ptc7*
Δ*ptc1* Δ*ptc3* Δ*ptc5*	Δ*ptc1* Δ*ptc5* Δ*ptc7*	Δ*ptc2* Δ*ptc4* Δ*ptc6*	Δ*ptc3* Δ*ptc4* Δ*ptc7*	Δ*ptc5* Δ*ptc6* Δ*ptc7*

Quadruple disruptants (35)				
Δ*ptc1* Δ*ptc2* Δ*ptc3* Δ*ptc4*	Δ*ptc1* Δ*ptc2* Δ*ptc5* Δ*ptc6*	Δ*ptc1* Δ*ptc3* Δ*ptc5* Δ*ptc7*	Δ*ptc2* Δ*ptc3* Δ*ptc4* Δ*ptc6*	Δ*ptc2* Δ*ptc4* Δ*ptc6* Δ*ptc7*
Δ*ptc1* Δ*ptc2* Δ*ptc3* Δ*ptc5*	Δ*ptc1* Δ*ptc2* Δ*ptc5* Δ*ptc7*	Δ*ptc1* Δ*ptc3* Δ*ptc6* Δ*ptc7*	Δ*ptc2* Δ*ptc3* Δ*ptc4* Δ*ptc7*	Δ*ptc2* Δ*ptc5* Δ*ptc6* Δ*ptc7*
Δ*ptc1* Δ*ptc2* Δ*ptc3* Δ*ptc6*	Δ*ptc1* Δ*ptc2* Δ*ptc6* Δ*ptc7*	Δ*ptc1* Δ*ptc4* Δ*ptc5* Δ*ptc6*	Δ*ptc2* Δ*ptc3* Δ*ptc5* Δ*ptc6*	Δ*ptc3* Δ*ptc4* Δ*ptc5* Δ*ptc6*
Δ*ptc1* Δ*ptc2* Δ*ptc3* Δ*ptc7*	Δ*ptc1* Δ*ptc3* Δ*ptc4* Δ*ptc5*	Δ*ptc1* Δ*ptc4* Δ*ptc5* Δ*ptc7*	Δ*ptc2* Δ*ptc3* Δ*ptc5* Δ*ptc7*	Δ*ptc3* Δ*ptc4* Δ*ptc5* Δ*ptc7*
Δ*ptc1* Δ*ptc2* Δ*ptc4* Δ*ptc5*	Δ*ptc1* Δ*ptc3* Δ*ptc4* Δ*ptc6*	Δ*ptc1* Δ*ptc4* Δ*ptc6* Δ*ptc7*	Δ*ptc2* Δ*ptc3* Δ*ptc6* Δ*ptc7*	Δ*ptc3* Δ*ptc4* Δ*ptc6* Δ*ptc7*
Δ*ptc1* Δ*ptc2* Δ*ptc4* Δ*ptc6*	Δ*ptc1* Δ*ptc3* Δ*ptc4* Δ*ptc7*	Δ*ptc1* Δ*ptc5* Δ*ptc6* Δ*ptc7*	Δ*ptc2* Δ*ptc4* Δ*ptc5* Δ*ptc6*	Δ*ptc3* Δ*ptc5* Δ*ptc6* Δ*ptc7*
Δ*ptc1* Δ*ptc2* Δ*ptc4* Δ*ptc7*	Δ*ptc1* Δ*ptc3* Δ*ptc5* Δ*ptc6*	Δ*ptc2* Δ*ptc3* Δ*ptc4* Δ*ptc5*	Δ*ptc2* Δ*ptc4* Δ*ptc5* Δ*ptc7*	Δ*ptc4* Δ*ptc5* Δ*ptc6* Δ*ptc7*

Sextuple disruptants (7)
Δ*ptc1* Δ*ptc2* Δ*ptc3* Δ*ptc4* Δ*ptc5* Δ*ptc6*
Δ*ptc1* Δ*ptc2* Δ*ptc3* Δ*ptc4* Δ*ptc5* Δ*ptc7*
Δ*ptc1* Δ*ptc2* Δ*ptc3* Δ*ptc4* Δ*ptc6* Δ*ptc7*
Δ*ptc1* Δ*ptc2* Δ*ptc3* Δ*ptc5* Δ*ptc6* Δ*ptc7*
Δ*ptc1* Δ*ptc2* Δ*ptc4* Δ*ptc5* Δ*ptc6* Δ*ptc7*
Δ*ptc1* Δ*ptc3* Δ*ptc4* Δ*ptc5* Δ*ptc6* Δ*ptc7*
Δ*ptc2* Δ*ptc3* Δ*ptc4* Δ*ptc5* Δ*ptc6* Δ*ptc7*

Quintuple disruptants (21)		
Δ*ptc1* Δ*ptc2* Δ*ptc3* Δ*ptc4* Δ*ptc5*	Δ*ptc1* Δ*ptc2* Δ*ptc4* Δ*ptc5* Δ*ptc7*	Δ*ptc1* Δ*ptc4* Δ*ptc5* Δ*ptc6* Δ*ptc7*
Δ*ptc1* Δ*ptc2* Δ*ptc3* Δ*ptc4* Δ*ptc6*	Δ*ptc1* Δ*ptc2* Δ*ptc4* Δ*ptc6* Δ*ptc7*	Δ*ptc2* Δ*ptc3* Δ*ptc4* Δ*ptc5* Δ*ptc6*
Δ*ptc1* Δ*ptc2* Δ*ptc3* Δ*ptc4* Δ*ptc7*	Δ*ptc1* Δ*ptc2* Δ*ptc5* Δ*ptc6* Δ*ptc7*	Δ*ptc2* Δ*ptc3* Δ*ptc4* Δ*ptc5* Δ*ptc7*
Δ*ptc1* Δ*ptc2* Δ*ptc3* Δ*ptc5* Δ*ptc6*	Δ*ptc1* Δ*ptc3* Δ*ptc4* Δ*ptc5* Δ*ptc6*	Δ*ptc2* Δ*ptc3* Δ*ptc4* Δ*ptc6* Δ*ptc7*
Δ*ptc1* Δ*ptc2* Δ*ptc3* Δ*ptc5* Δ*ptc7*	Δ*ptc1* Δ*ptc3* Δ*ptc4* Δ*ptc5* Δ*ptc7*	Δ*ptc2* Δ*ptc3* Δ*ptc5* Δ*ptc6* Δ*ptc7*
Δ*ptc1* Δ*ptc2* Δ*ptc3* Δ*ptc6* Δ*ptc7*	Δ*ptc1* Δ*ptc3* Δ*ptc4* Δ*ptc6* Δ*ptc7*	Δ*ptc2* Δ*ptc4* Δ*ptc5* Δ*ptc6* Δ*ptc7*
Δ*ptc1* Δ*ptc2* Δ*ptc4* Δ*ptc5* Δ*ptc6*	Δ*ptc1* Δ*ptc3* Δ*ptc5* Δ*ptc6* Δ*ptc7*	Δ*ptc3* Δ*ptc4* Δ*ptc5* Δ*ptc6* Δ*ptc7*

Septuple disruptant (1)
Δ*ptc1* Δ*ptc2* Δ*ptc3* Δ*ptc4* Δ*ptc5* Δ*ptc6* Δ*ptc7*

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 (*MAT***a**) or W303-1B (*MAT*α) (B) Construction of 127 multiple gene disruptants by successive genetic crosses. Tetrads were analyzed to screen for Trp^+, His^+, Leu^+, Ura^+, Kan^r, Hph^r, and Cer^r 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 Δ*ptc1*:: *loxP-CgTRP1-loxP* (W303-1A genetic background) and Δ*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^+ His^+ segregants were selected to obtain the Δ*ptc1*Δ*ptc2* double disruptant. The mating types of the disruptants were determined using two tester strains (*MAT***a**: *pho3-1 leu1-ELF 52* / *MAT*α: *pho3-1 leu1-SA 54*). We similarly constructed the Δ*ptc4*Δ*ptc5* double disruptant from a cross of Δ*ptc4*::*loxP-KanMX-loxP* and Δ*ptc5*::*loxP*-YAP1-*loxP*, and the Δ*ptc6*Δ*ptc7* double disruptant from a cross of Δ*ptc6*::*loxP-HPH-loxP* and Δ*ptc7*::*URA3*. The *MAT***a** Δ*ptc1*Δ*ptc2* double disruptant was then crossed with the *MAT*α Δ*ptc3*:: *loxP-CgLEU2-loxP* single disruptant. The diploid cells were sporulated and the resultant spores from the tetrads were screened. Trp^+ His^+ Leu^+ segregants were selected to obtain the Δ*ptc1*Δ*ptc2*Δ*ptc3* triple disruptant. Two other double disruptants were also obtained from this cross (Δ*ptc1*Δ*ptc3* and Δ*ptc2*Δ*ptc3*). The *MAT***a** Δ*ptc1*Δ*ptc2*Δ*ptc3* triple disruptant was crossed with the Δ*ptc4*Δ*ptc5* double disruptant and Trp^+ His^+ Leu^+ Kan^r Cer^r segregants were selected to obtain the Δ*ptc1*Δ*ptc2*Δ*ptc3*Δ*ptc4*Δ*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** Δ*ptc1*Δ*ptc2*Δ*ptc3*Δ*ptc4*Δ*ptc5* quintuple disruptant was crossed with the Δ*ptc6*Δ*ptc7* double disruptant and Trp^+ His^+ Leu^+ Kan^r Cer^r Hph^r Ura^+ segregants were selected from sporulated diploid tetrads. These segregants were selected for the Δ*ptc1*Δ*ptc2*Δ*ptc3*Δ*ptc4*Δ*ptc5*Δ*ptc6*Δ*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 30ºC on an air-shaker. The next day, the cultures were each inoculated into 5 mL of fresh liquid YPDA and grown to the mid-logarithmic phase. From these cultures, cell suspensions (1×10^7 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 13ºC, 37ºC on YPDA medium and compared to growth at the normal temperature of 30ºC. 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 $CaCl_2$; 5 mM and 10.2 mM caffeine; 2 µg/ml, 3 µg/ml and 4 µg/ml congo red; 2 µg/ml, 4 µg/ml and 5 µg/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 (*MAT***a** *ura3-52 leu2-3, 112 trp1*Δ *his3*Δ *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 derivative**s** 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 (*MAT***a** *ura3-1 leu2-3,112 trp1-1his3-11,15 ade2-1*) or W303-1B (*MATα 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 30ºC, 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 30ºC, 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 13ºC, 30ºC and 37ºC. The Δ*ptc1* single disruptant displayed reduced growth at 37ºC and Δ*ptc6* grew slowly at both 13ºC and 37ºC 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 Δ*ptc1*Δ*ptc5* double disruptant had relatively slower growth than the wild type and single disruptants at 30ºC in this study although each of the single disruptants showed normal growth (Figure 6B).

Here, we also tested growth at 13^{o}C and found that the Δ*ptc5*Δ*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 Δ*ptc5*Δ*ptc7* disruptant (*MAT***a** *ptc5*:: *loxP*-YAP1-*loxP ptc7*::*URA3*) and the Δ*ptc5* disruptant (*MAT*α *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 13ºC ; 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).

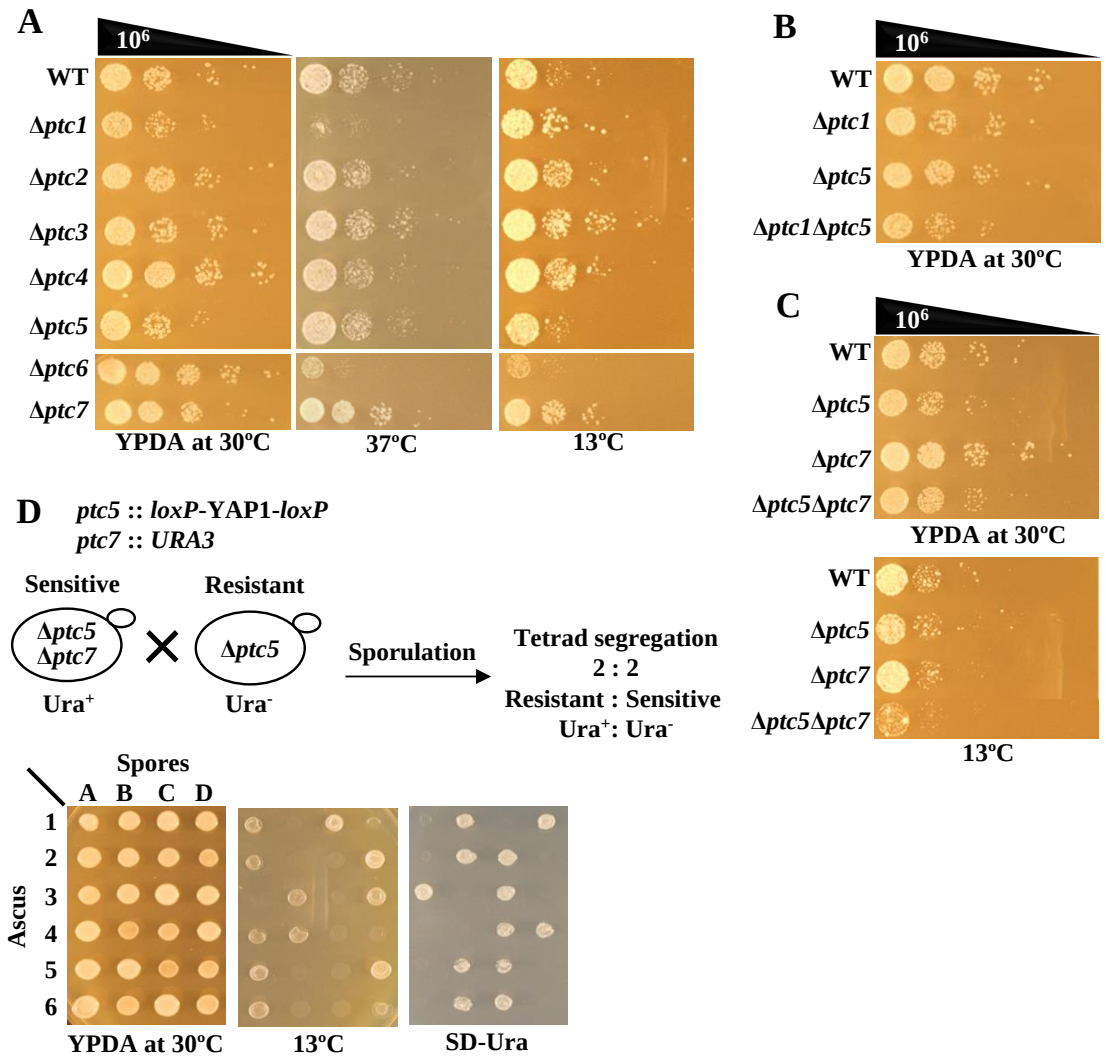


Figure 6. Temperature sensitive growth phenotypes in *PTC* gene deletion strains. (A) Cells of wild-type strain (SH4848), Δ*ptc1* to Δ*ptc7* disruptants were streaked onto two separate YPDA plates (wild-type to Δ*ptc5* in one plate and Δ*ptc6* and Δ*ptc7* in another plate) and incubated at 30ºC, 37ºC or 13ºC. 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, Δ*ptc1*, Δ*ptc5* single and Δ*ptc1*Δ*ptc5* double disruptants were spotted onto YPDA plates and incubated at 30ºC. (C) Cells of Δ*ptc5* or Δ*ptc7* single disruptants or of the Δ*ptc5*Δ*ptc7* double disruptant were spotted onto YPDA plates, as were those of the wild-type strain; growth was compared at 30ºC or 13ºC. (D) A diploid heterozygous for the Δ*ptc5*Δ*ptc7*:: *loxP-YAP1-loxP URA3* double disruptant and the Δ*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 30ºC and cells from each colony that formed were spotted twice on YPDA and SD-Ura plates and incubated at 13ºC for 2 days and 30ºC overnight.

We also found that the Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*ptc7* quadruple disruptant showed growth sensitivity at 37ºC whereas no obvious growth defects occurred in strains with deletion of each individual gene or in double and triple deletion combinations (Figure 6A). The Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*ptc7* quadruple disruptant also showed a slight growth defect at 30ºC although the effect was much weaker that at 37ºC (Figure 6B). Although the Δ*ptc5*Δ*ptc7* double disruptant exhibited growth sensitivity at 13ºC, the Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*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 (*MATα ptc2*::*loxP-CgHIS3-loxP ptc3*::*loxP-CgLEU2-loxP ptc5*::*loxP*-YAP1-*loxP ptc7*::*URA3*) with Δ*ptc2*Δ*ptc3*Δ*ptc5*, or Δ*ptc2*Δ*ptc5*Δ*ptc7* or Δ*ptc3*Δ*ptc5*Δ*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).

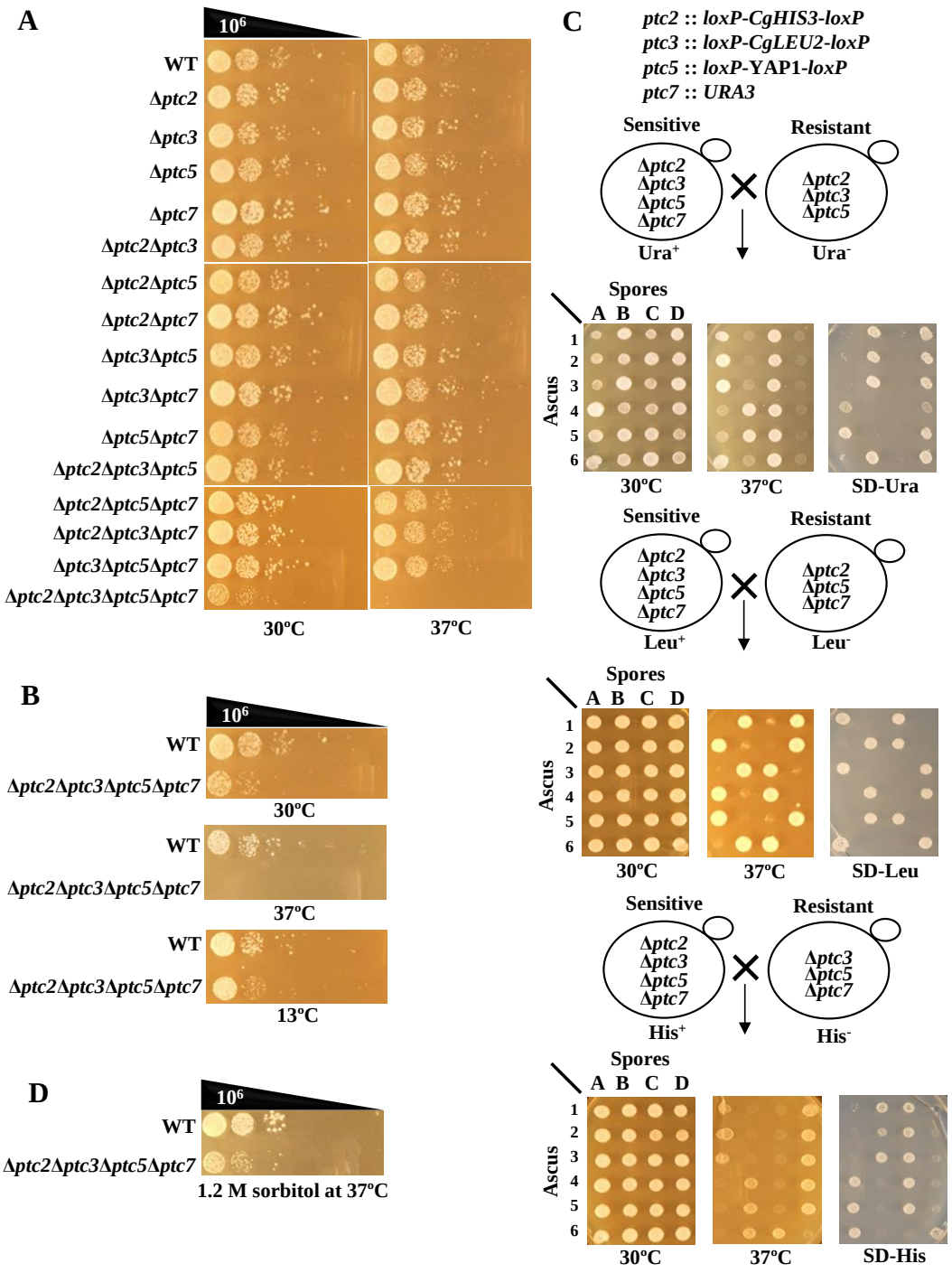


Figure 7. High-temperature growth phenotype of the Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*ptc7* quadruple disruptant. (A) Cells of the wild-type strain, single, double, triple and quadruple disruptants of *PTC2*, *PTC3*, *PTC5* and *PTC7* genes were spotted onto three separate YPDA plates by ten-fold serial dilutions and incubated at 30ºC or 37ºC. 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 Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*ptc7* quadruple disruptant was compared to that of the wild-type strain on YPDA plates at 30ºC, 37ºC and 13ºC. (C) Heterozygous diploids were

constructed by crossing the quadruple disruptant (*MATα ptc2*::*loxP-CgHIS3-loxP ptc3*::*loxP-CgLEU2-loxP ptc5*::*loxP-YAP1-loxP ptc7*::*URA3*) with the Δ*ptc2*Δ*ptc3*Δ*ptc5*, Δ*ptc2*Δ*ptc5*Δ*ptc7* or Δ*ptc3*Δ*ptc5*Δ*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 Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*ptc7*quadruple disruptant were spotted onto YPDA plates containing 1.2 M sorbitol and incubated at 37°C overnight.

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

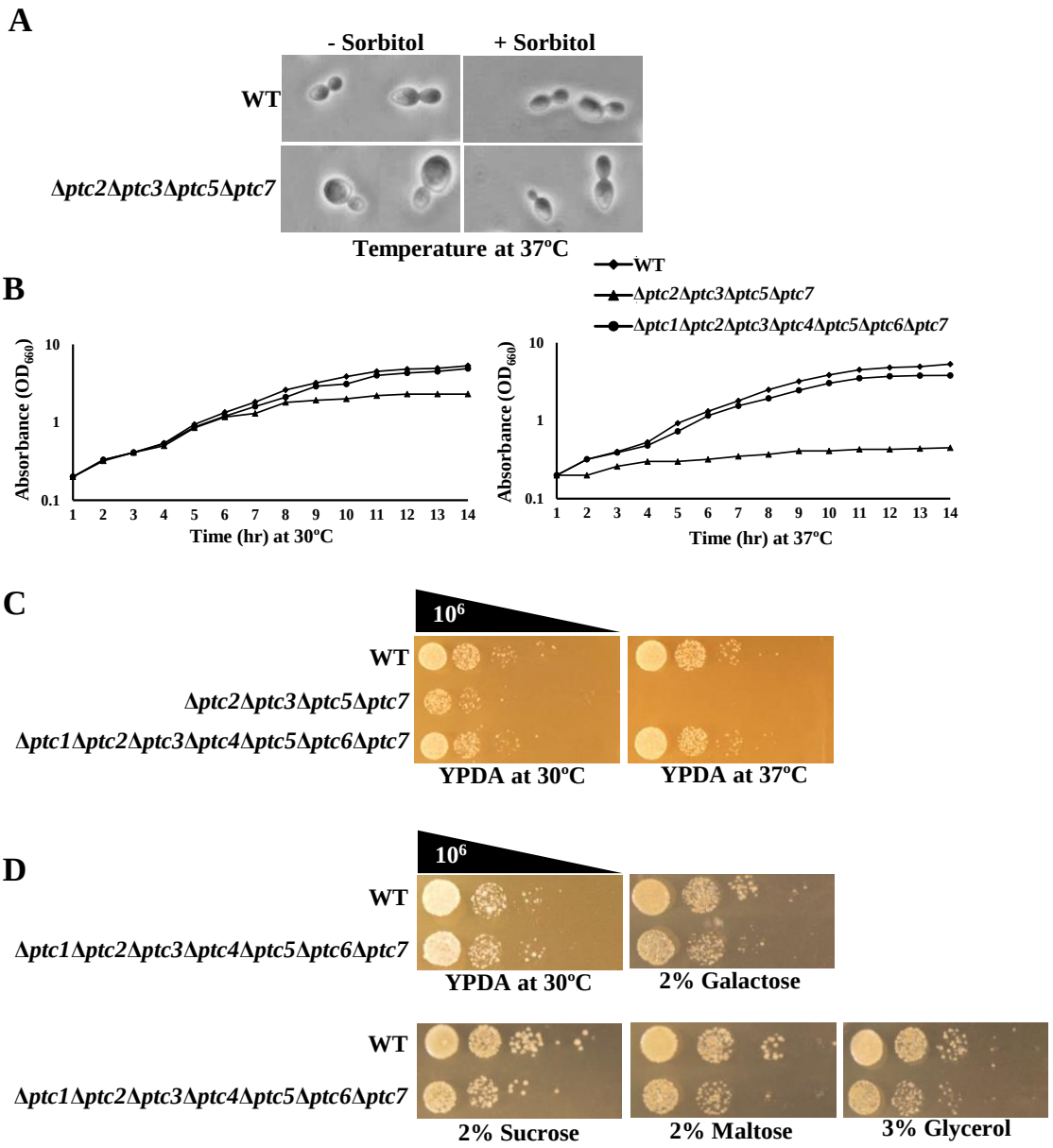


Figure 8. Morphology and growth behavior of the Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*ptc7* quadruple disruptant that shows a high-temperature sensitive phenotype. (A) Wild-type (WT) cells and Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*ptc7* quadruple disruptant cells were grown in liquid YPDA with or without sorbitol at 37ºC and their morphologies were observed under the microscope. (B) Cells of the WT, the Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*ptc7* quadruple disruptant and the Δ*ptc1*Δ*ptc2*Δ*ptc3*Δ*ptc4*Δ*ptc5*Δ*ptc6*Δ*ptc7* septuple disruptant were incubated in liquid YPDA at both 30ºC and 37ºC 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 wild-type, the Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*ptc7* quadruple disruptant and the Δ*ptc1*Δ*ptc2*Δ*ptc3*Δ*ptc4*Δ*ptc5*Δ*ptc6*Δ*ptc7* septuple disruptant were spotted on YPDA plates and were incubated at either 30ºC or 37ºC overnight. (D) Ten-fold serial dilutions of cell

cultures of the wild-type and the Δ*ptc1*Δ*ptc2*Δ*ptc3*Δ*ptc4*Δ*ptc5*Δ*ptc6*Δ*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 30ºC 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 $CaCl_2$, 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 Δ*ptc1*Δ*ptc2* and Δ*ptc1*Δ*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^+ ions in the medium.

Interestingly, we found that all the double disruptants including Δ*ptc1* disruption exhibited Li^+ sensitive phenotypes except for Δ*ptc1*Δ*ptc7* double disruptant (Figure 8A). Thus, disruption of the *PTC7* gene could suppress the Li^+ sensitive phenotype of the Δ*ptc1* deletion and Δ*ptc1*Δ*ptc7* double disruptant displayed Li^+ resistance compared to the Δ*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 Δ*ptc1*Δ*ptc2*Δ*ptc4* triple disruptant did not grow on medium containing 1.5 M NaCl (Figure 8C) and a tetrad analysis showed this Li^+ sensitivity co-segregated with geneticin sensitivity (Figure 8D). We also found that the Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*ptc7* quadruple disruptant did

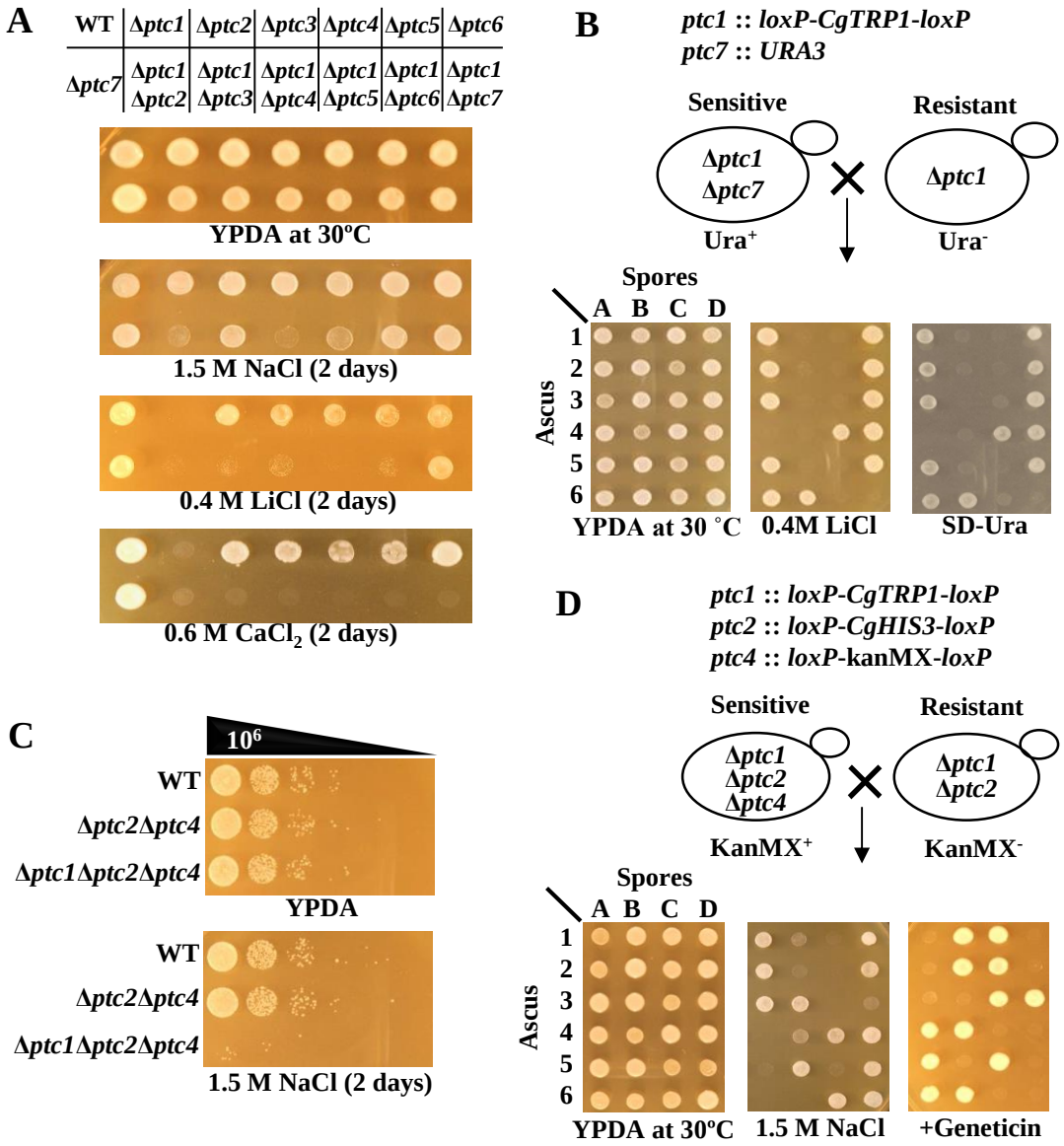


Figure 9. Growth phenotype of single *PTC* disruptants and double disruptants combined with Δ*ptc1*. The disruptants listed in (A) were grown on YPDA plates at 30ºC and cells were spotted onto various assay plates. Cells of disruptants spotted on the representative plates were incubated at 30ºC for 1 day for the control plate (YPDA), and 2 days for 1.5 M NaCl, 0.4 M LiCl and 0.6 M $CaCl_2$ plates. (B) A diploid heterozygote for the Δ*ptc1*Δ*ptc7*:: *loxP-CgTRP1-loxP URA3* double disruptant and the Δ*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 30ºC and cells from each colony that formed were spotted twice on YPDA and SD-Ura plates and were incubated at 13ºC for 2 days or at 30ºC overnight. (C) Cells of the wild-type strain, the Δ*ptc2*Δ*ptc4* double disruptant and the Δ*ptc1*Δ*ptc2*Δ*ptc4* triple

disruptant were spotted onto YPDA plates without/with 1.5 M NaCl and incubated at 30ºC. (D) A diploid heterozygote for the Δ*ptc1*Δ*ptc2*Δ*ptc4*::*loxP-CgTRP1-loxP loxP-CgHIS3-loxP loxP-KanMX-loxP* triple disruptant and the Δ*ptc1*Δ*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 30ºC 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 30ºC 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 (*MATα ptc2*::*loxP-CgHIS3-loxP ptc3*::*loxP-CgLEU2-loxP ptc5*::*loxP-YAP1-loxP ptc7*::*URA3*) to each of Δ*ptc2*Δ*ptc3*Δ*ptc5*, Δ*ptc2*Δ*ptc5*Δ*ptc7* and Δ*ptc3*Δ*ptc5*Δ*ptc7* triple disruptants; tetrad analyses were then carried out. We found a 2 : 2 segregation of prototrophy and Li^+ 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^+ ion sensitive growth.

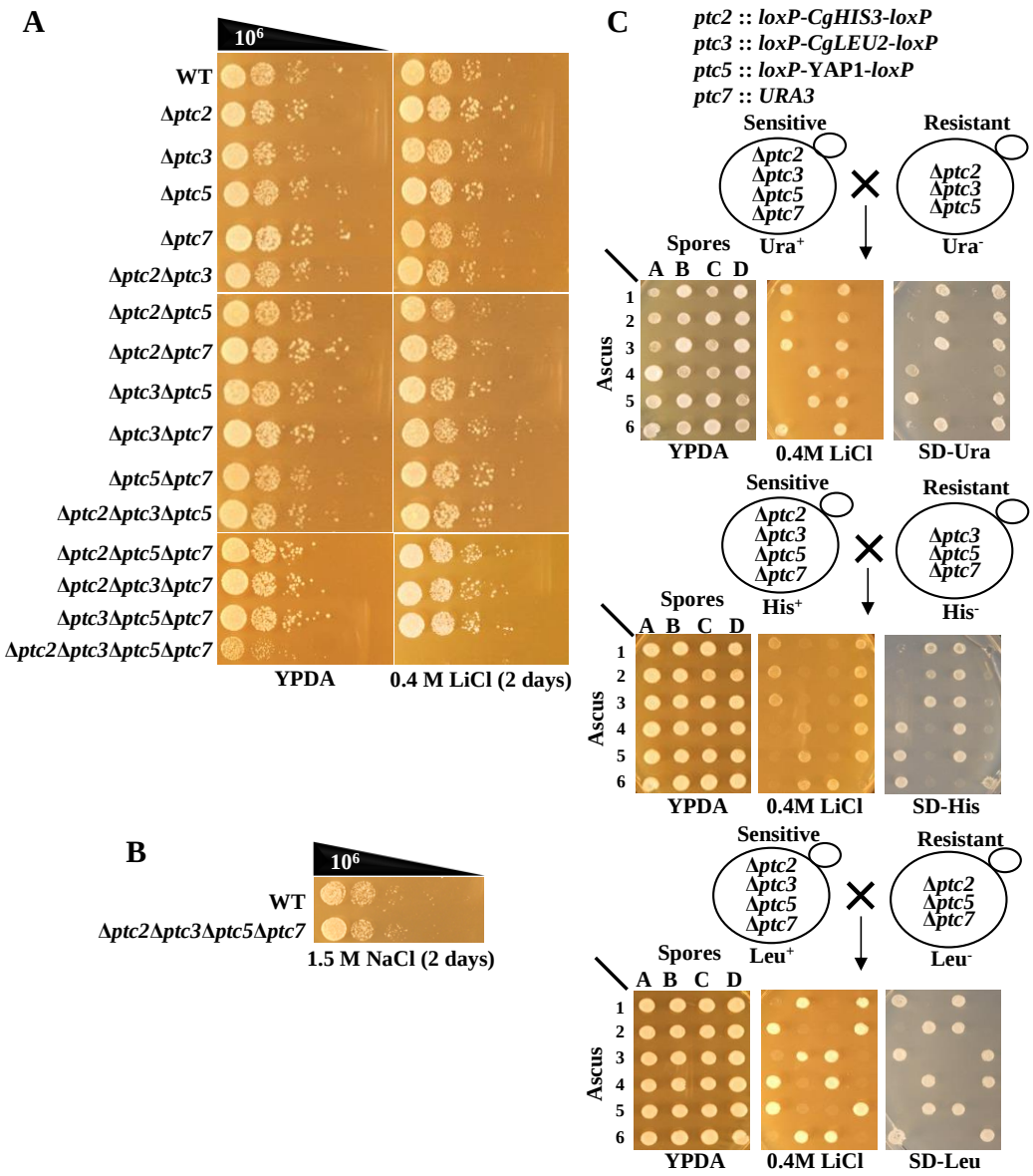


Figure 10. Growth of the Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*ptc7* quadruple disruptant under LiCl stress. (A) Cells of the wild-type strain, single, double, triple and triple disruptants of *PTC2, PTC3, PTC5* and *PTC7* genes were spotted at ten-fold serial dilutions on three separate YPDA plates with or without 0.4 M LiCl and incubated at 30ºC. All the strains were compared with wild-type strain in each plate and photos of the plates are separated with space in both cases. (B) The wild-type strain and the Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*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 (*MATα ptc2*::*loxP-CgHIS3-loxP ptc3*::*loxP-CgLEU2-loxP ptc5*::*loxP-YAP1-loxP ptc7*::*URA3*) with Δ*ptc2*Δ*ptc3*Δ*ptc5*, Δ*ptc2*Δ*ptc5*Δ*ptc7* or Δ*ptc3*Δ*ptc5*Δ*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, Δ*ptc1* and Δ*ptc6* exhibited caffeine sensitivity and the degree of sensitivity increased in the Δ*ptc1*Δ*ptc6* double disruptant (Figure 10A). The Δ*ptc1* and Δ*ptc6* single disruptants were sensitive to 2 µg/ml congo red; the Δ*ptc1*Δ*ptc6* double disruptant showed an increased sensitivity at the higher level of congo red (4 µg/ml) (Figure 10B). The Δ*ptc1*, Δ*ptc6* and Δ*ptc1*Δ*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 Δ*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).

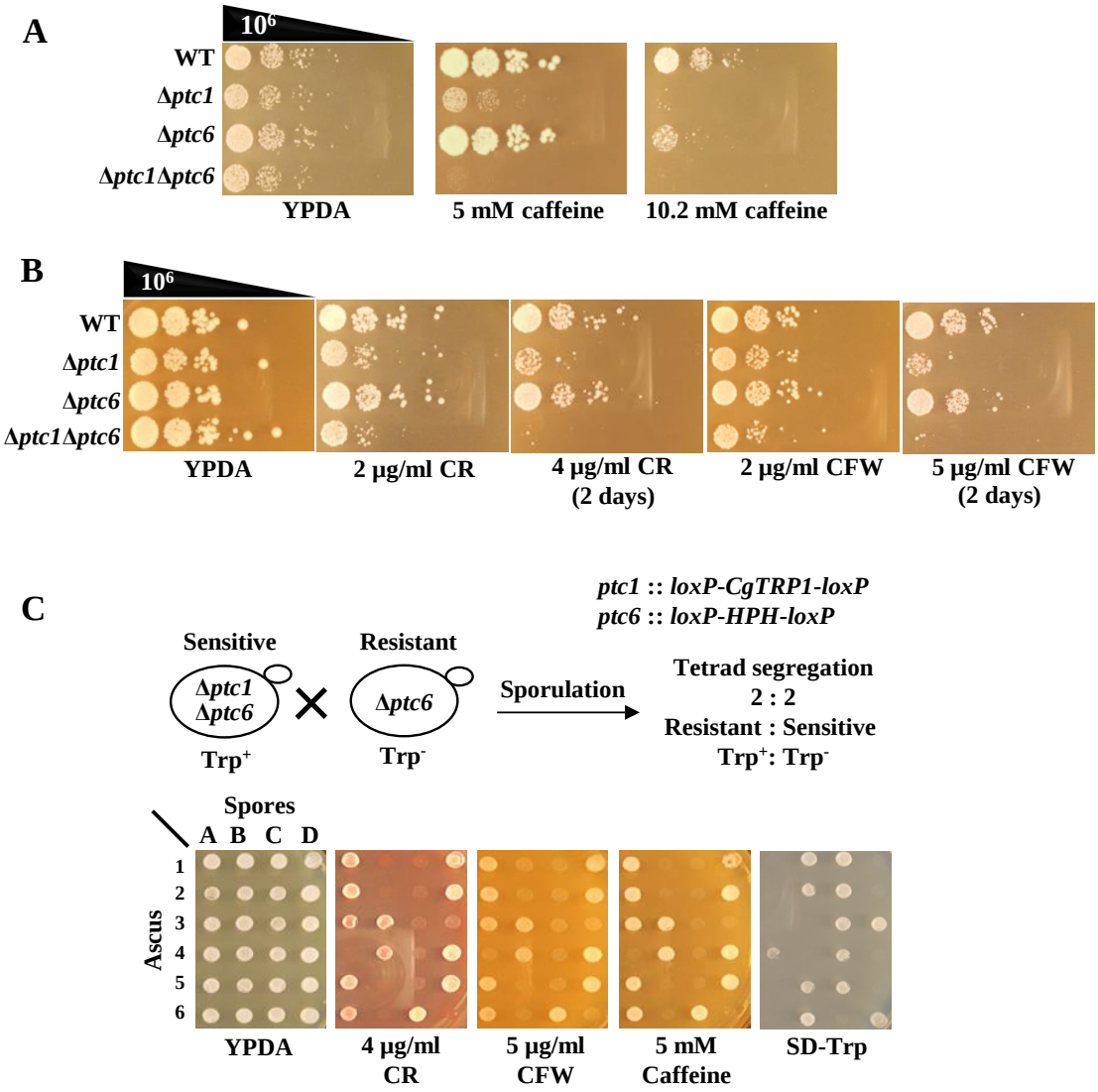


Figure 11. Phenotypic analysis of the Δ*ptc1*, Δ*ptc6* and Δ*ptc1*Δ*ptc6* disruptants. (A) Cells of the wild-type, Δ*ptc1*and Δ*ptc6* single disruptants and the Δ*ptc1*Δ*ptc6* double disruptants were spotted onto YPDA plates with or without 5 mM or 10.2 mM caffeine and incubated for 2 days at 30ºC. (B) Ten-fold serial dilutions of the wild-type, Δ*ptc1* and Δ*ptc6* single disruptants and the Δ*ptc1*Δ*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 or 5 µg/ml and incubated at 30ºC overnight and 2 days respectively. (C) A diploid heterozygote from the Δ*ptc1*Δ*ptc6*:: *loxP-CgTRP1-loxP loxP-HPH-loxP* double disruptant and the Δ*ptc6*:: *loxP-HPH-loxP* single disruptant was sporulated and dissected. Each dissected spore (tetrad), designated A to D, was grown on a YPDA plate at 30ºC 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 30ºC 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).

Table 4. Summary of phenotypes discovered in this study.

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

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

In this study, we identified the first double disruptant, Δ*ptc5*Δ*ptc7*, to show evidence of temperature sensitive growth at 13ºC (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 13ºC 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 Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*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 Δ*ptc1*Δ*ptc2*Δ*ptc3*Δ*ptc4*Δ*ptc5*Δ*ptc6*Δ*ptc7* septuple disruptant did not show a temperature sensitive phenotype at 37ºC despite the fact that it contains the quadruple deletions of Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*ptc7*. This lack of phenotype might be due to the Δ*ptc1* and/or Δ*ptc4* deletions that might act as suppressors of high-temperature sensitivity in the Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*ptc7* disruptant.

2.4.3 Functional redundancy of *PTC2, PTC3, PTC5,* and *PTC7* in the Li^+ sensitivity

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

increased in the Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*ptc7* quadruple disruptant (Figure 10A). Only the *PTC1* gene has been implicated to date in Li^+ 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^+ sensitivity phenotype of the Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*ptc7* quadruple disruptant is caused by osmotic stress due to the high accumulation of Li^+ ions inside the cells. This kind of osmotic stress is known to stimulate Hcs77 (High Copy Suppressor) 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 Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*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^+ (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^+ sensitivity

The Δ*ptc1*Δ*ptc2* and Δ*ptc1*Δ*ptc4* double disruptants showed Na^+ sensitivity (Figure 9A). Although Δ*ptc2*Δ*ptc4* double disruptant could grow under high Na^+ conditions, the Δ*ptc1*Δ*ptc2*Δ*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, Δ*ptc1*Δ*ptc2* and Δ*ptc1*Δ*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^+-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 Δ*hal3*Δ*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^+ sensitivity shown by Δ*ptc1*Δ*ptc2* and Δ*ptc1*Δ*ptc4* double disruptants and by the Δ*ptc1*Δ*ptc2*Δ*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 Δ*ptc1* 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 Δ*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 Δ*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 Δ*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 Δ*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 temperaure sensitive phenotype of the Δ*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 Δ*ptc2*Δ*ptc3*Δ*ptc5*Δ*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 Slt2-mediated 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 fact that the 129 PKases 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 Δ*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 Δ*ptc6* disruptant differs from those of Δ*ptc1*- Δ*ptc5* strains (González *et al*., 2013). The Δ*ptc1* and Δ*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 Δ*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*, Δ*ptc1*Δ*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 Δ*ptc1*Δ*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 Δ*ptc1* and Δ*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 (*MAT***a**/*MATα 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 (*MAT***a** *ura3-1 leu2-3,112 trp1-1 his3-11, 15 ade2-1 can1-100*) and W303-1B (*MATα 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 30ºC 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 Δ*ptc1*Δ*ptc6* was isolated by tetrad analysis of the diploid strain produced by crossing Δ*ptc1*:: *loxP-CgTRP1-loxP* (*MAT***a**) and Δ*ptc6*::*loxP-HPH-loxP* (*MAT*α) (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 Δ*ptc6* (*MAT*α *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 Δ*ptc1* single disruptant and Δ*ptc1*Δ*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 30ºC 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™). 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™ Real Time System with SYBR® *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™ 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).

Table 5. *Saccharomyces cerevisiae* strains used in this study.

Strain	Alias	Genotype and Remarks	Source
SH4848	W303-1A	*MAT***a** *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	Δ*slt2*	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 Δslt2::KanMX4*	Invitrogen
	W303-1A:: *TRP1*	*MAT***a** *ura3-1 leu2-3,112 trp1:: pRS304-TRP1 his3-11, 15 ade2-1*	This study
	Δ*ptc1*	*MAT***a** *ura3-1 leu2-3,112 trp1 his3-11,15 ade2-1 ptc1::loxPCgTRP1*	Sharmin *et al*., 2014
	Δ*ptc6*	*MATα ura3-1 leu2-3,112 trp1:: pRS304-TRP1 his3-11, 15 ade2-1 ptc6:: loxP-HPH-loxP*	Sharmin *et al*., 2014
	Δ*ptc1*Δ*ptc6*	*MAT***a** *ura3-1 leu2-3,112 trp1 his3-11,15 ade2-1 ptc1::loxPCgTRP1 ptc6:: loxP-HPH-loxP*	Sharmin *et al*., 2014

Table 6. Oligonucleotide primers used in this study.

Name	Sequence (5´-3´)
Disruption primers	
gdPTC1-F	ATCATTTAGGCACTGCATTTATCTTTTAAAAATCATTATACTTCGTACGCTGCAG
gdPTC1-R	GTCTATGCATAATTTTTGCGCGGTTTATAACGGATCCTTCGCCACTAGTGGATCT
gdPTC6-F	CTGCAATCGGGGCAATTAAGCATCAGAAGAGGGGAATTTGCTTCGTACGCTGCAG
gdPTC6-R	AAGATGATTATCTAGGACTTGTTTCCACCCAGGGGGGTGTGCCACTAGTGGATCT
Confirmation primers	
cPTC1-F	TTAGTTAAACATTATTATTC
cPTC1-R	GAGATAATGGCGAATTAGAC
cPTC6-F	CTCGCCATAGCCCTTGTAAC
cPTC6-R	GCTTGAAATCAGGGTATACT
RT-PCR primers	
RT-KDX1-F	CTCACACAGCCTTATTTGTTCTTC
RT-KDX1-R	CGCGTTTGGTATTTTTCTGA
RT-CRH1-F	GGCTGCCGAAAGTACTGCTA
RT-CRH1-R	GCGTACAACCTGTAGTTTTTAACG
RT-SEDI-F	TCCTATTATCTGCCGGTTTAGC
RT-SEDI-R	AAGTGACATCGGTGGAAGAAG
RT-ACT1-F	TGGATTCCGGTGATGGTGTT
RT-ACT1-R	TCAAAATGGCGTGAGGTAGAGA

3.3 Results

3.3.1 Deletion of *PTC6* in a Δ*ptc1* disruptant causes increased sensitivity to cell wall damaging agents

Exposure of Δ*ptc1* cells to CR or CFW showed that these cells were relatively tolerant to such cell wall-damaging agents; similarly, Δ*ptc6* cells were tolerant to both reagents (Figure 1). By contrast, Δ*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.

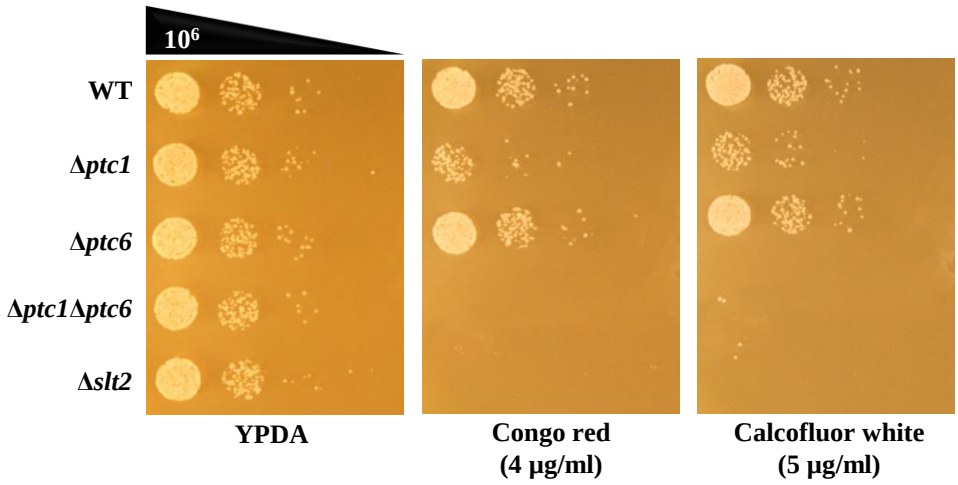


Figure 12. Additive genetic effect of Δ*ptc6* disruption in *PTC1* deficient cells. Ten-fold serial dilutions of the wild-type, Δ*ptc1* and Δ*ptc6* single disruptants, the Δ*ptc1*Δ*ptc6* double disruptant, and the Δ*slt2* disruptant were spotted onto YPDA plates without or with CR (4 µg/ml) or CFW (5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

We found that wild-type, Δ*ptc1*, Δ*ptc6* and Δ*ptc1*Δ*ptc6* strains exhibited similar growth patterns under standard conditions (Fig. 2A). However, the Δ*ptc1*Δ*ptc6* double disruptant grew very slowly in media containing CR or CFW, whereas the Δ*ptc1* strain was relatively tolerant to both cell wall-damaging agents (Fig. 2B, 2C) and Δ*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 Δ*ptc1*Δ*ptc6* double disruptant (10%) under cell wall stress conditions. It was observed that the Δ*ptc1*Δ*ptc6* double disruptant could not reach OD_{660} = 1.0 whereas wild type and Δ*ptc6* disruptant were able to reach OD_{660} = 1.0 after 9 hours both in the absence and presence of CR and CFW, suggesting that the growth of Δ*ptc1*Δ*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 Δ*slt2* disruptant although the growth of the Δ*ptc1*Δ*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 Δ*ptc1*Δ*ptc6* double disruptant to cell wall damaging agents involves the Slt2 CWI pathway.

3.3.2 *PTC6* negatively affects Slt2 phosphorylation in the Δ*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 Δ*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 Δ*ptc1*Δ*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 Δ*ptc1*Δ*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 Δ*ptc1* and Δ*ptc1*Δ*ptc6* disruptants compared to wild type cells (Figure 14B). It is noted that a little increase in total Slt2 in Δ*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 Δ*ptc1* cells.

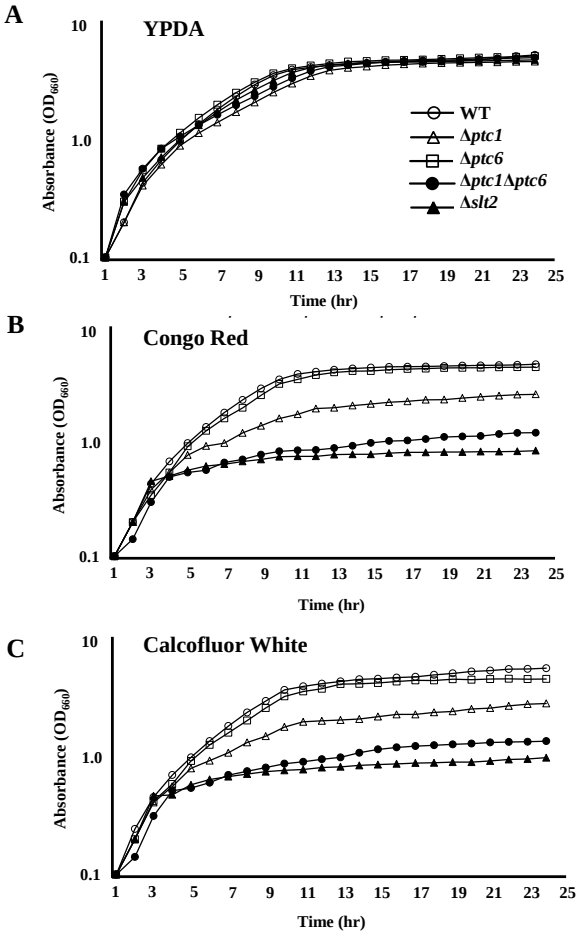


Figure 13. Effect of Δ*ptc6* disruption in *PTC1* deficient cells on growth under cell wall stress conditions. (A) Wild-type cells, Δ*ptc1*, Δ*ptc6* and Δ*ptc1*Δ*ptc6* disruptant cells, and *Slt2*-deficient cells were inoculated into liquid YPD medium. Cultures were later transferred to fresh YPDA medium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

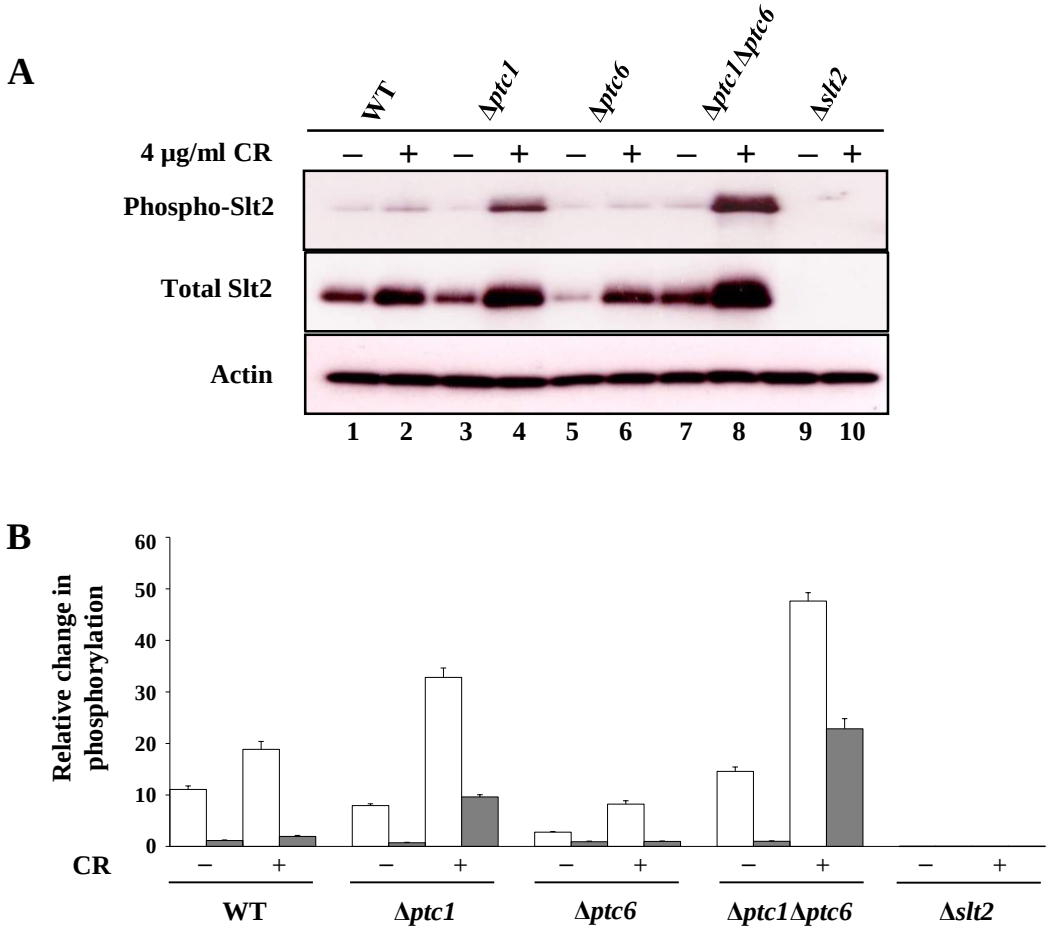


Figure 14. Effect of Ptc6 on changes in Slt2 activation. (A) Anti-phospho-Slt2 immunoblot analysis of wild type, Δ*ptc1*, Δ*ptc6*, Δ*ptc1*Δ*ptc6* and Δ*slt2i* cells. Proteins extracted from cells grown in media with or without 4 µg/ml CR to OD_{660} = 1.0 at 30°C were separated on SDS–PAGE gels (lanes 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 Δ*ptc1* and Δ*ptc1*Δ*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 Δ*ptc1*Δ*ptc6*

double disruptant cells than in wild type, Δ*ptc1*, and Δ*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 Δ*ptc1*Δ*ptc6* cells after exposure to CR (Figure 15). Moreover, Δ*ptc1* and Δ*ptc6* disruptant cells showed no induction of these two genes. These observations suggest that Δ*ptc6* disruption positively increases transcription of *KDX1* but not *CRH1* and *SED1* transcription. Our results for *CRH1* and *SED1* transcriptional induction in Δ*ptc1* cells are inconsistent with a previous report (González *et al*., 2006).

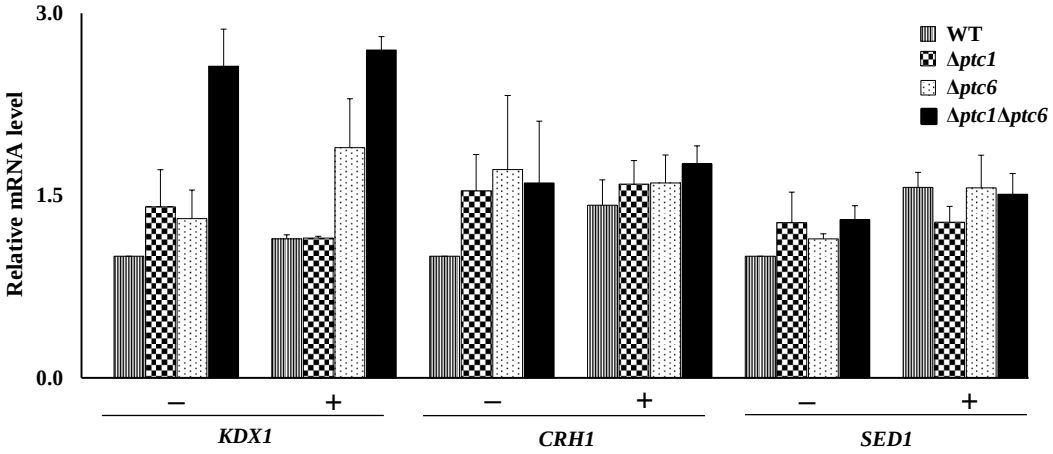


Figure 15. Cell wall-related genes are up-regulated in Δ*ptc1*Δ*ptc6* double disruptant cells after exposure to CR stress. cDNAs were synthesized from total RNAs isolated from cells grown under standard conditions or in the presence of 4 μg/ml CR. These cDNAs were used as templates for RT-PCR using SYBR 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 Δ*ptc1*Δ*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 ($2^{(-\Delta\Delta Ct)}$) of amplification plots derived from real-time RT-PCR. The results are the means of three experiments from three independent preparations.

Table 7. Function of genes discussed in this chapter.

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

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 Δ*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 Δ*ptc6* and Δ*ptc1*Δ*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 Δ*ptc1*Δ*ptc6* double disruptant cells grown in YPDA. However, under CR or CFW stress conditions, approximately 40-50% cells displayed fragmented vacuoles in Δ*ptc1*Δ*ptc6* cells (>100 cells per condition were evaluated, depending on the number of vacuoles per cell; Figure 16). The Δ*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 Δ*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 Δ*grx3*Δ*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 Δ*ptc1* disruptant cells might be rescued in Δ*ptc1*Δ*ptc6* double disruptant cells by the increased expression of Slt2. Contrary to this prediction; we found that fragmented vacuoles were still observed in Δ*ptc1*Δ*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 Δ*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 *Δptc1Δptc6* double disruptants cells was unable to rescue the CR and CFW sensitive phenotype of the *Δptc1Δptc6* double disruptant (Figure 17).

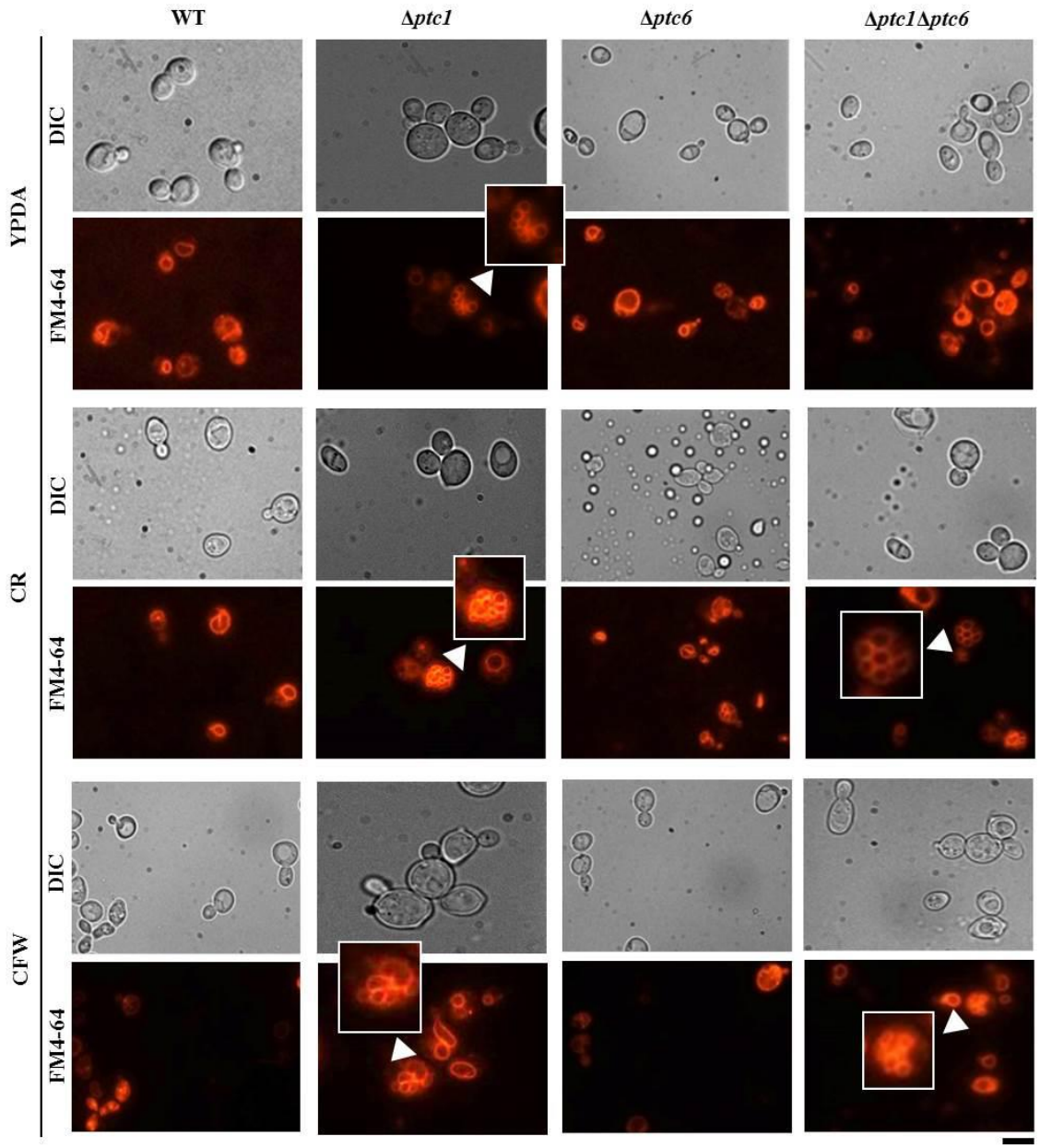


Figure 16. Vacuolar fragmentation in Δ*ptc1*Δ*ptc6* double disruptant cells. Wild type, Δ*ptc6* and Δ*ptc1*Δ*ptc6* double disruptant cells are stained with FM4-64. White triangles indicate the cellular regions shown in the enlarged images showing fragmented vacuolar membranes. Bar = 5 μm.

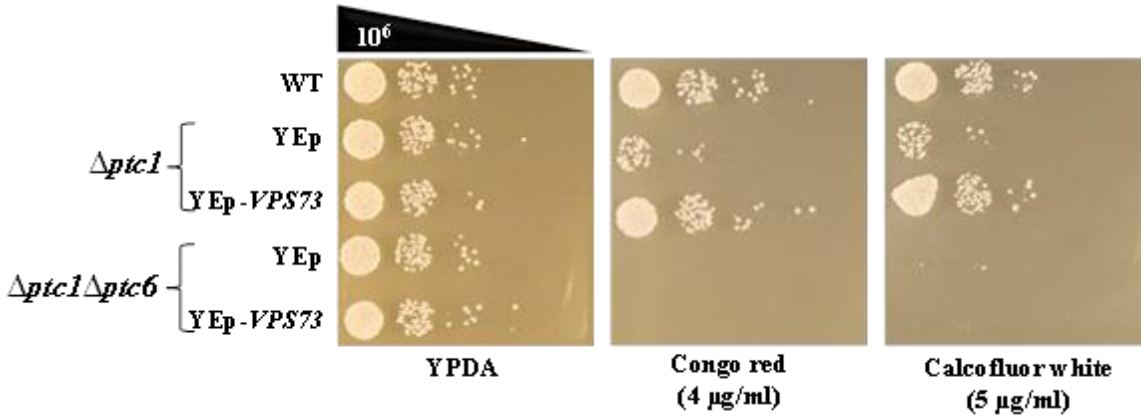


Figure 17. Effect of overexpression of *VPS73* of the growth defect of the Δptc1 and *Δptc1Δptc6* disruptant strains under cell wall stresses. The *Δptc1* and *Δptc1Δptc6* disruptants were transformed with the YEp vectors (without/with *VPS73* gene) and spotted on YPDA plates without or with CR (4 µg/ml) or CFW (5 µg/ml) and incubated at 30ºC. 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 Δ*ptc6* disruption is similar to that of cells with a Δ*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 Δ*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 Δ*ptc1* cells to agents that cause cell wall damage. Although Δ*ptc1* single disruptant used in previous study (González *et al*., 2013) showed increased sensitivity to CR and CFW compared to Δ*ptc1* single disruptant used here and no sensitive phenotype of Δ*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 Δ*ptc1* disruptant cells; that study also found that Δ*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 mitogen-activated 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 Δ*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 Δ*ptc1*Δ*ptc6*

double disruptant compared to the Δ*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 Δ*ptc1*Δ*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 Δ*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 Δ*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 Δ*ptc1* cells.

A previous DNA microarray analysis reported that Δ*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 Δ*ptc1*Δ*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 Δ*ptc1*Δ*ptc6* cells which was lower than in wild type cells, and Δ*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 Δ*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 Δ*ptc6* disruption on *CRH1* and *SED1* transcription might vary between Δ*ptc1* strains. Our results also suggest that transcription of *KDX1* in Δ*ptc6* disruptant cells was induced by the additional disruption of *PTC1*, irrespective of the

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 Δ*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 Δ*ptc1*Δ*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 Δ*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 Δ*ptc1* disruption drastically increased phosphorylation of Slt2 in Δ*ptc6* disruptant cells in the presence of CR (Figure 14), we propose that the mechanism by which Δ*ptc1* disruption induces vacuolar fragmentation in Δ*ptc6* cells under cell wall stress conditions might involve Slt2 phosphorylation.

A

	Ptc1	Ptc2	Ptc3	Ptc4	Ptc5	Ptc6	Ptc7	
	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

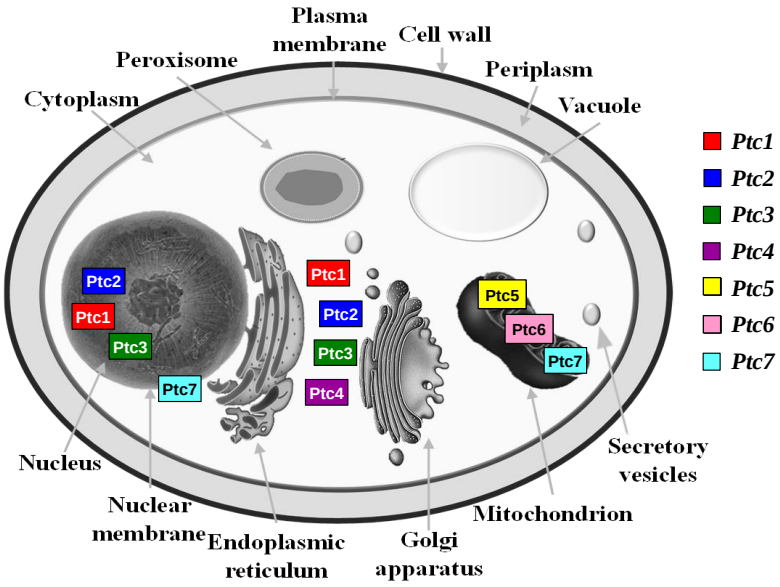


Figure 18. Amino acid sequence comparison of Ptc proteins (Ptc1 - Ptc7). (A) The level (%) of similarity in the amino acid sequences of the seven PTC PPases. Ptc1 was found to share only 13% similarity with Ptc6. (B) Localization of each PTC PPase in the cell. Localization data were obtained from direct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

Previous studies suggested that cell wall damage causes activation of the Slt2 mitogen-activated 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 Δ*ptc1* disruption have higher levels of active phosphorylated Slt2 (Du *et al*., 2006). Here, we observed elevated level of phosphorylated Slt2 in the Δ*ptc1*Δ*ptc6* double disruptant compared to the Δ*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 Δ*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 Δ*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 Δ*ptc1* cells.

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Δ*ptc6* disruption on *CRH1* and *SED1* transcription might vary between Δ*ptc1* strains. Our results also revealed that transcription of *KDX1* in Δ*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 PP2Cα and PP2Cβ 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, Δ*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 *PTC4* were functionally redundant for Na^+ sensitivity. Remarkably, *PTC2*, *PTC3*, *PTC5* and *PTC7* genes were found to be redundant for both high temperature and Li^+ 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 Δ*ptc1*Δ*ptc6* double disruptant compared to the Δ*ptc1* and Δ*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 Δ*ptc1*Δ*ptc6* double disruptant compared to the Δ*ptc1* and Δ*ptc6* single disruptants irrespective of the presence of CR (Figure 15), suggesting regulation of Slt2 pathway by *PTC6* in Δ*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 Δ*ptc1*Δ*ptc6* cells showed fragmented vacuoles in the presence of both CR and CFW although a normal vacuolar phenotype was observed in Δ*ptc6* single disruptant both in the absence or presence of cell wall stresses (Figure 16). Previous studies revealed that Δ*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 Δ*ptc1*Δ*ptc6* cells, we also suggest the involvement of Ptc6 that functions following Δ*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

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, p38α, 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.

References

Alonso A, Sasin J, Bottini N, Friedberg I, Friedberg I, Osterman A, Godzik A, Hunter T, Dixon J, Mustelin T. 2004. Protein tyrosine phosphatases in the human genome. Cell 117: 699–711.

Amberg DC, Burke DJ, Strathern JN. 2005. Methods in yeast genetics, a cold spring harbor laboratory course manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

An X, Zhang Z, Yang K, Huang M. 2006. Cotransport of the heterodimer small subunit of the *Saccharomyces cerevisiae* ribonucleotide reductase between the nucleus and the cytoplasm. Genetics 173: 63–73.

Ariño J, Casamayor A, González A. 2011. Type 2C protein phosphatases in fungi. Eukaryot Cell 10: 21–33.

Auesukaree C, Damnernsawad A, Kruatrachue M, Pokethitiyook P, Boonchird C, Kaneko Y, Harashima S. 2009. Genome-wide identification of genes involved in tolerance to various environmental stresses in *Saccharomyces cerevisiae*. J Appl Genet 50: 301–310.

Bahn YS, Xue C, Idnurm A, Rutherford JC, Heitman J, Cardenas ME. 2007. Sensing the environment: lessons from fungi. Nat Rev Microbiol 5: 57–69.

Becerra M, Lombardía LJ, Lamas-Maceiras M, Canto E, Rodríguez-Belmonte E, Cerdán ME. 2011. Comparative transcriptome analysis of yeast strains carrying slt2, rlm1, and pop2 deletions. Genome 54: 99–109.

Bermejo C, Rodriguez E, Garcia R, Rodríguez-Peña JM, Rodríguez de la Concepción ML, Rivas C, Arias P, Nombela C, Posas F, Arroyo J. 2008. The sequential activation of the yeast HOG and SLT2 pathways is required for cell survival to cell wall stress. Mol Biol Cell 19: 1113–1124.

Boorsma A, de Nobel H, ter Riet B, Bargmann B, Brul S, Hellingwerf KJ, Klis FM. 2004. Characterization of the transcriptional response to cell wall stress in *Saccharomyces cerevisiae*. Yeast 21:413–427.

Breitkreutz A, Choi H, Sharom JR, Boucher L, Neduva V, Larsen B, Lin ZY, Breitkreutz BJ, Stark C, Liu G, Ahn J, Dewar-Darch D, Reguly T, Tang X, Almeida R, Qin ZS, Pawson T, Gingras AC, Nesvizhskii AI, Tyers M. 2010. A global protein kinase and phosphatase interaction network in yeast. Science 328: 1043–1046.

Burns JM, Parsons M, Rosman DE, Reed SG. 1993. Molecular cloning and characterization of a 42-kDa Protein phosphatase of *Leishmania chagasi*. J Biol Chem 268: 17155–17161.

Cabib E. 2009. Two novel techniques for determination of polysaccharide cross-links show that Crh1p and Crh2p attach chitin to both beta (1-6)- and beta(1-3) glucan in the *Saccharomyces cerevisiae* cell wall. Eukaryot Cell 8: 1626–1636.

Carrion NP, Petkova MI, Serrano L, de la Torre-Ruiz MA. 2013. The MAP kinase Slt2 is involved in vacuolar function and actin remodeling in *Saccharomyces cerevisiae* disruptants affected by endogenous oxidative stress. Appl Environ Microbiol 79: 6459–6471.

Catala M, Aksouh L, Elela SA. 2012. RNA-dependent regulation of the cell wall stress response. Nucleic Acids Res 40: 7507–7517.

Chen RE and Thorner J. 2007. Function and regulation in MAPK signaling pathways: lessons learned from the yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta 1773:1311–1340.

Cheng A, Ross KE, Kaldis P, Solomon MJ. 1999. Dephosphorylation of cyclin-dependent kinases by type 2C protein phosphatases. Genes Dev 13: 2946–2957.

Cheng TH, Chang CR, Joy P, Yablok S, Gartenberg MR. 2000. Controlling gene expression in yeast by inducible site-specific recombination. Nucleic Acids Res 28: E108.

Cohen P. 2001. The role of protein phosphorylation in human health and disease. Eur J Biochem 268: 5001–5010.

Dalmau JF, Gonzalez A, Platara M, Navarrete C, Martinez JL, Barreto L, Ramos J, Arino J, Casamayor A. 2010. Ref2, a regulatory subunit of the yeast protein phosphatase 1, is a novel component of cation homoeostasis. J Biol Chem 426: 355–364.

de Nadal E, Clotet J, Posas F, Serrano R, Gomez N, Ariño J. 1998. The yeast halotolerance determinant Hal3p is an inhibitory subunit of the Ppz1p Ser/Thr protein phosphatase. Proc Natl Acad Sci USA 95: 7357–7362.

DeRisi JL, Iyer VR, Brown PO. 1997. Exploring the metabolic and genetic control of gene expression on a genomic scale. Science 278: 680–686.

Du Y, Walker L, Novick P, Novick SF. 2006. Ptc1 regulates cortical ER inheritance via Slt2p. EMBO J 25: 4413–4422.

Dudley AM, Janse DM, Tanay A, Shamir R, Church GM. 2005. A global view of pleiotropy and phenotypically derived gene function in yeast. Mol Syst Biol 1: 2005 0001.

Evans DR and Stark MJ. 1997. Mutations in the *Saccharomyces cerevisiae* type 2A protein phosphatase catalytic subunit reveal roles in cell wall integrity, actin cytoskeleton organization and mitosis. Genetics 145: 227–241.

Fuchs BB and Mylonakis E. 2009. Our paths might cross: the role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways. Eukaryot Cell 8: 1616–1625.

Garcia R, Bermejo C, Grau C, Perez R, Rodriguez-Pena JM, Francois J, Nombela C, Arroyo J. 2004. The global transcriptional response to transient cell wall damage in

Saccharomyces cerevisiae and its regulation by the cell integrity signaling pathway. J Biol Chem 279: 15183–15195.

García R, Rodríguez-Peña JM, Bermejo C, Nombela C, Arroyo J. 2009. The high osmotic response and cell wall integrity pathways cooperate to regulate transcriptional responses to zymolyase-induced cell wall stress in *Saccharomyces cerevisiae*. J Biol Chem 284: 10901–10911.

Gasch AP. 2007. Comparative genomics of the environmental stress response in ascomycete fungi. Yeast 24: 961–976.

Gey U, Czupalla C, Hoflack B, Rödel G, Krause-Buchholz U. 2008. Yeast pyruvate dehydrogenase complex is regulated by a concerted activity of two kinases and two phosphatases. J Biol Chem 283: 9759–9767.

Gietz RD and Schiestl RH. 2007. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat Protoc 2: 31–34.

Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M, Louis EJ, Mewes HW, Murakami Y, Philippsen P, Tettelin H, Oliver SG. 1996. Life with 6000 genes. Science 274: 546–567.

Goldstein AL and McCusker JH. 1999. Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. Yeast 15: 1541–1553.

González A, Casado C, Ariño J, Casamayor A. 2013. Ptc6 is required for proper rapamycin-induced down-regulation of the genes coding for ribosomal and rRNA processing proteins in *S.cerevisiae*. PLoS ONE 8: e64470.

González A, Ruiz A, Casamayor A, Ariño J. 2009. Normal Function of the TOR pathway requires the yeast Type 2C Protein Phosphatase Ptc1. Mol Cell Biol 29: 2876–2888.

González A, Ruiz A, Serrano R, Ariño J, Casamayor A. 2006. Transcriptional profiling of the protein phosphatase 2C family in yeast provides insights into the unique functional roles of Ptc1. J Biol Chem 281: 35057–35069.

Güldener U, Heck S, Fielder T, Beinhauer J, Hegemann JH. 1996. A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res* 24: 2519–2524.

Guo S, Shen X, Yan G, Ma D, Bai X, Li S, Jiang Y. 2009. A MAP kinase dependent feedback mechanism controls Rho1 GTPase and actin distribution in yeast. PLoS One 4: e6089.

Hermansyah, Sugiyama M, Kaneko Y, Harashima S. 2009. Yeast protein phosphatase Ptp2p and Msg5p are involved in G1-S transition, CLN2 transcription, and vacuole morphogenesis. Arch Microbiol 191: 721–733.

Hirasaki M, Horiguchi M, Numamoto M, Sugiyama M, Kaneko Y, Nogi Y, Harashima S. 2011. *Saccharomyces cerevisiae* protein phosphatase Ppz1 and protein kinases Sat4 and Hal5 are involved in the control of subcellular localization of Gln3 by likely regulating its phosphorylation state. J Biosci Bioeng 111: 249–254.

Hughes TR, Marton MJ, Jones AR, Roberts CJ, Stoughton R, Armour CD, Bennett HA, Coffey E, Dai H, He YD, Kidd MJ, King AM, Meyer MR, Slade D, Lum PY, Stepaniants SB, Shoemaker DD, Gachotte D, Chakraburtty K, Simon J, Bard M, Friend SH. 2000. Functional discovery via a compendium of expression profiles. Cell 102: 109–126.

Hunter T. 1995. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. Cell 80: 225–236.

Ito T, Chiba T, Ozawa R, Yoshida M, Hattori M, Sakaki Y. 2001. A comprehensive two-hybrid analysis to explore the yeast protein interactome. Proc Natl Acad Sci 98: 4569–4574.

Jin Y, Taylor EP, Tang F, Weisman LS. 2009. *PTC1* is required for vacuole inheritance and promotes the association of the myosin-V vacuole-specific receptor complex. Mol Biol Cell 20: 1312–1323.

Journo D, Mor A, Abeliovich H. 2009. Aup1-mediated regulation of Rtg3 during mitophagy. J Biol Chem 284: 35885–35895.

Jung US and Levin DE. 1999. Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signalling pathway. Mol Microbiol 34: 1049–1057.

Kim JA, Hicks WM, Li J, Tay SY, Haber JE. 2011. Protein phosphatases pph3, ptc2, and ptc3 play redundant roles in DNA double-strand break repair by homologous recombination. Mol Cell Biol 31: 507–516.

Klumpp S, Thissen MC, Krieglstein J. 2006. Protein phosphatases types 2Calpha and 2Cbeta in apoptosis. Biochem Soc Trans 34: 1370–1375.

Kvitek DJ, Will JL, Gasch AP. 2008.Variations in stress sensitivity and genomic expression in diverse *S. cerevisiae* isolates. PLoS Genetics 4: e1000223.

Laviña WA, Hermansyah, Sugiyama M, Kaneko Y, Harashima S. 2013. Functionally redundant protein phosphatase genes *PTP2* and *MSG5* co-regulate the calcium signaling pathway in *Saccharomyces cerevisiae* upon exposure to high extracellular calcium concentration. J Biosci Bioeng 115: 138–146.

Laviña WA, Shahsavarani H, Saidi A, Sugiyama M, Kaneko Y, Harashima S. 2014. Suppression mechanism of the calcium sensitivity in *Saccharomyces cerevisiae ptp2*Δ*msg5*Δ double disruptant involves in novel HOG-independent function of Ssk2, transcription factor Msn2 and the PKA component Bcy1. J Biosci Bioeng 117: 135–141.

Leung J, Bouvier-Durand M, Morris PC, Guerrier D, Chefdor F, Giraudat J. 1994. Arabidopsis ABA response gene ABI1: features of a calcium-modulated protein phosphatase. Science 264: 1448–1452.

Levin DE. 2005. Cell wall integrity signaling in *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 69: 262–291.

Li X, Du Y, Siegel S, Ferro-Novick S, Novick P. 2010. Activation of the mitogen-activated protein kinase, Slt2p, at bud tips blocks a late stage of endoplasmic reticulum inheritance in *Saccharomyces cerevisiae*. Mol Biol Cell 21: 1772–1782.

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

Livak KJ and Schmittgen TD. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ Method. Methods 25: 402–408.

Lu G and Wang Y. 2008. Functional diversity of mammalian type 2C protein phosphatase isoforms: new tales from an old family. Clin Exp Pharmacol Physiol 35: 107–112.

Lucau-Danila A, Lelandais G, Kozovska Z, Tanty V, Delaveau T, Devaux F, Jacq C. 2005. Early expression of yeast genes affected by chemical stress. Mol Cell Biol 25:1860–1868.

Maeda T, Tsai AYM, Saito H. 1993. Mutations in a protein tyrosine phosphatase gene (PTP2) and a protein serine/threonine phosphatase gene (PTC1) cause a synthetic growth defect in *Saccharomyces cerevisiae*. Mol Cell Biol 13: 5408–5417.

Maeda T, Wurgler-Murphy SM, Saito H. 1994. A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature 369: 242–245.

Malleshaiah MK, Shahrezaei V, Swain PS, Michnick SW. 2010. The scaffold protein Ste5 directly controls a switch-like mating decision in yeast. Nature 465:101–105.

Markovich S, Yekutiel A, Shalit I, Shadkchan Y, Osherov N. 2004. Genomic approach to identification of mutations affecting caspofungin susceptibility in *Saccharomyces cerevisiae*. Antimicrob Agents Chemother 48: 3871–3876.

Martin H, Flandez M, Nombela C, Molina M. 2005. Protein phosphatases in MAPK signaling: we keep learning from yeast. Mol Microbiol 58: 6–16.

Martín H, Pachón JMR, Ruiz C, Nombela C, Molina M. 2000. Regulatory mechanisms for modulation of signaling through the cell integrity Slt2-mediated pathway in *Saccharomyces cerevisiae*. J Biol Chem 275: 1511–1519.

Mukai Y, Harashima S, Oshima Y. 1993. Function of the ste signal transduction pathway for mating pheromones sustains MAT alpha 1 transcription in *Saccharomyces cerevisiae*. Mol Cell Biol 13: 2050–2060.

Panadero J, Pallotti C, Rodríguez-Vargas S, Randez-Gil F, Prieto JA. 2006. A downshift in temperature activates the high osmolarity glycerol (HOG) pathway, which determines freeze tolerance in *Saccharomyces cerevisiae*. J Biol Chem. 281: 4638–4645.

Pereira SFF, Goss L, Dworkin J. 2011. Eukaryote-Like Serine/Threonine Kinases and Phosphatases in Bacteria. Microbiol Mol Biol Rev 75: 192–212.

Phadnis N and Ayres Sia E. 2004. Role of the putative structural protein Sed1p in mitochondrial genome maintenance. J Mol Biol 342: 1115–1129.

Piskur J and Langkjaer RB. 2004. Yeast genome sequencing: the power of comparative genomics. Mol Microbiol 53: 381–389.

Ptacek J, Devgan G, Michaud G, *et al*. 2005. Global analysis of protein phosphorylation in yeast. Nature 438: 679–684.

Robinson MK, van Zyl WH, Phizicky EM, Broach JR. 1994. *TPD1* of *Saccharomyces cerevisiae* encodes a protein phosphatase 2C-like activity implicated in tRNA splicing and cell separation. Mol Cell Biol 14: 3634–3645.

Ruan H, Yan Z, Sun H, Jiang L. 2007. The YCR079w gene confers a rapamycin-resistant function and encodes the sixth type 2C protein phosphatase in *Saccharomyces cerevisiae*. FEMS Yeast Res 7: 209–215.

Ruiz A, González A, Garcia-Salcedo R, Ramos J, Ariño J. 2006. Role of protein phosphatases 2C on tolerance to lithium toxicity in the yeast *Saccharomyces cerevisiae*. Mol Microbiol 62: 263–277.

Runner VM and Brewster JL. 2003. A genetic screen for yeast genes induced by sustained osmotic stress. Yeast 20: 913–920.

Sakumoto N, Matsuoka I, Mukai Y, Ogawa N, Kaneko Y, Harashima S. 2002. A series of double disruptants for protein phosphatase genes in *Saccharomyces cerevisiae* and their phenotypic analysis. Yeast 19: 587–599.

Sakumoto N, Mukai Y, Uchida K, *et al*. 1999. A series of protein phosphatase gene disruptants in *Saccharomyces cerevisiae*. Yeast 15: 1669–1679.

Sambrook J, Fritsch EF, Maniatis T. 2000. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Schade B, Jansen G, Whiteway M, Entian KD, Thomas DY. 2004. Cold adaptation in budding yeast. Mol Biol Cell 15: 5492–5502.

Schweighofer A, Hirt H, Meskiene I. 2004. Plant PP2C phosphatases: emerging functions in stress signaling. Trends Plant Sci 9: 236–243.

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

Sherman F and Hicks J. 1991. Micromanipulation and dissection of asci. Methods Enzymol 194: 21–37.

Shimoi H, Kitagaki H, Ohmori H, Iimura Y, Ito K. 1998. Sed1p is a major cell wall protein of *Saccharomyces cerevisiae* in the stationary phase and is involved in lytic enzyme resistance. J Bacteriol 180: 3381–3387.

Shiozaki K and Russell P. 1995. Counteractive roles of protein phosphatase 2C (PP2C) and a MAP kinase kinase homolog in the osmoregulation of fission yeast. EMBO J 14: 492–502.

Shirahige K, Hori Y, Shiraishi K, Yamashita M, Takahashi K, Obuse C, Tsurimoto T, Yoshikawa H. 1998. Regulation of DNA-replication origins during cell-cycle progression. Nature 395: 618–621.

Sickmann A, Reinders J, Wagner Y, Joppich C, Zahedi R, Meyer HE, Schönfisch B, Perschil I, Chacinska A, Guiard B, and other 3 authors. 2003. The proteome of *Saccharomyces cerevisiae* mitochondria. Proc Natl Acad Sci 100:13207–13212.

Sugiyama M, Ikushima S, Nakazawa T, Kaneko Y, Harashima S. 2005. PCR-mediated repeated chromosome splitting in *Saccharomyces cerevisiae*. Biotechniques 38: 909–914.

Sugiyama M, Nakazawa T, Murakami K, Sumiya T, Nakamura A, Kaneko Y, Nishizawa M, Harashima S. 2008. PCR-mediated one-step deletion of targeted chromosomal regions in haploid *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 80: 545–553.

Tamura S, Lynch KR, Larner J, Fox J, Yasui A, Kikuchi K, Suzuki Y, Tsuiki S. 1989. Molecular cloning of rat type 2C (IA) protein phosphatase mRNA. Proc Natl Acad Sci USA 86: 1796–1800.

Treusch S, Albert FW, Bloom JS, Kotenko IE, Kruglyak L. 2014. genetic dissection of mapk-mediated complex traits across *Saccharomyces cerevisiae*. BioRxiv (In press).

Uetz P, Giot L, Cagney G, Mansfield TA, Judson RS, Knight JR, Lockshon D, Narayan V, Srinivasan M, Pochart P, Qureshi-Emili A, Li Y, Godwin B, Conover D, Kalbfleisch T,

Vijayadamodar G, Yang M, Johnston M, Fields S, Rothberg JM. 2000. A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. Nature 403: 623–631.

Wagner EF and Nebreda AR. 2009. Signal integration by JNK and p38 MAPK pathways in cancer development. Nature Reviews Cancer 9: 537–549.

Waltermann C and Klipp E. 2010. Signal integration in budding yeast. Biochemical Society transactions 38: 1257–1264.

Warmka J, Hanneman J, Lee J, Amin D, Ota I. 2001. Ptc1, type 2C Ser/ Thr phosphatase, inactivates the HOG pathway by dephosphorylating the mitogen-activated protein kinase Hog1. Mol Cell Biol 21: 51–60.

Warringer J, Zörgö E, Cubillos FA, Zia A, Gjuvsland A, Simpson JT, Forsmark A, Durbin R, Omholt SW, Louis EJ, Liti G, Moses A, Blomberg A. 2011. Trait variation in yeast is defined by population history. PLoS Genet 7: e1002111.

Watanabe Y, Takaesu G, Hagiwara M, Irie K, Matsumoto K. 1997. Characterization of a serum response factor-like protein in *Saccharomyces cerevisiae*, Rlm1, which has transcriptional activity regulated by the Mpk1 (Slt2) mitogen-activated protein kinase pathway. Mol Cell Biol 17: 2615–2623.

Winkler A, Arkind C, Mattison CP, Burkholder A, Knoche K, Ota I. 2002. Heat stress activates the yeast High-Osmolarity Glycerol Mitogen-Activated protein kinase pathway and protein tyrosine phosphatases are essential under heat stress. Eukaryot Cell 1: 163–173.

Wojda I, Alonso-Monge R, Bebelman JP, Mager WH, Siderius M. 2003. Response to high osmotic conditions and elevated temperature in *Saccharomyces cerevisiae* is controlled by intracellular glycerol and involves coordinate activity of MAP kinase pathways. Microbiology 149: 1193–1204.

Wong ML and Medrano JF. 2005. Real-time PCR for mRNA quantitation. Biotechniques 39: 75–85.

Wu X, Chi X, Wang P, Zheng D, Ding R, Li Y. 2010. The evolutionary rate variation among genes of HOG-signaling pathway in yeast genomes. Biol Direct 5: 46–56.

Young C, Mapes J, Hanneman J, Al Zarban S, Ota I. 2002. Role of Ptc2 type 2C Ser/Thr phosphatase in yeast high-osmolarity glycerol pathway inactivation. Eukaryot Cell 1: 1032–1040.

Zhang J. 2012. Genetic redundancies and their evolutionary maintenance. Adv Exp Med Biol 751: 279–300.

Zhao Y, Feng J, Li J, Jiang L. 2012. Mitochondrial type 2C protein phosphatases CaPtc5p, CaPtc6p, and CaPtc7p play vital roles in cellular responses to antifungal drugs and cadmium in *Candida albicans*. FEMS Yeast Res 12: 897–906.

Zolnierowicz S and Bollen M. 2000. Protein phosphorylation and protein phosphatases. De Panne, Belgium, September 19-24, 1999. EMBO J 19: 483–484.

Publications

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