Form 3

# Synopsis of Thesis

Title: Multi-level functional redundancy mechanisms of *Saccharomyces cerevisiae* in response to high extracellular calcium stress

(出芽酵母における高濃度カルシウムストレス応答の多層的制御メカニズム)

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## Chapter 1: General Introduction

Upon the completion of the Yeast Genome Project in 1996, *Saccharomyces cerevisiae* became the first eukaryotic genome to be sequenced fully; defining 5,885 potential protein-encoding genes. More importantly, the complete sequence revealed the magnitude of research that was needed to be done as more than 50% of the sequenced genes were unknown and uncharacterized by previously established methods such as mutant hunting. Furthermore, the yeast genome showed a large amount of genes with apparent genetic redundancy. This particular problem necessitated the need to systematically characterize the biological function of the all genes in the yeast genome and to elucidate functional redundancy in more detail.

Regulatory systems such as reversible phosphorylation involving protein kinases (PKases) and phosphatases (PPases) play key roles in mediating stress response and other cellular processes thus, they serve as important research subjects for molecular breeding and strain improvement. In an effort to understand the role of each protein PPase in the cellular physiology of *S. cerevisiae*, Sakumoto and colleagues (2002) constructed a PPase library of single and double disruptants and systematically screened for interesting phenotypes; one of which is that the disruption of two PPase genes, *PTP2* and *MSG5*, causes calcium sensitivity indicating that functional redundancy exists between the two PPases in response to high extracellular calcium. Furthermore, Hermansyah (2010) revealed that additional disruption of PKase genes *BCK1*, *MKK1* and *SLT2* (Cell Wall Integrity SLT2 MAPK pathway), *MCK1* (Chromosome segregation), *YAK1* (Glucose sensing) and *SSK2* (High Osmolarity Glycerol (HOG) MAPK pathway) in the *ptp2Δmsg5Δ* background confers calcium tolerance. In this study, I clarified the redundant role of Ptp2 and Msg5 in calcium-induced signaling and the suppression mechanisms conferred by the PKase disruptions using genetic and transcriptional analyses.

## Chapter 2: Functionally redundant protein phosphatase genes *PTP2* and *MSG5* co-regulate the calcium signaling pathway in *S. cerevisiae* upon exposure to high extracellular calcium concentration

Cell regulation is an integral part of signaling cascades that ensures the maintenance of cellular homeostasis upon changes in the environment. Since over-activation and down-regulation of a particular signal can have damaging effects, cells possess a myriad of ways to combat improper activation or down-regulation of signaling pathways. In this study, I found a redundant function for Ptp2 and Msg5 in the regulation of SLT2 pathway in response to high extracellular calcium. Furthermore, I discovered that inactivation of calcineurin by the disruption of the calcineurin regulatory subunit, *CNB1* or treatment with

a calcineurin inhibitor, FK506, can suppress the calcium sensitive phenotype of the *ptp2Δmsg5Δ* double disruptant. This indicated that the Ptp2 and Msg5-mediated calcium signaling is under the parallel control of SLT2 and calcineurin pathways. In the wake of a calcium-induced, calcineurin-driven signaling pathway activation, the calcium sensitivity of the *ptp2Δmsg5Δ* double disruptant can be suppressed by regulating the SLT2 pathway through the disruption of the major kinases in the SLT2 signal cascade that include *BCK1*, *MKK1* and *SLT2*. I propose that Ptp2 and Msg5 are key regulatory phosphatases that prevent over-activation of the calcium-induced signaling cascade under the parallel control of the SLT2 and calcineurin pathways.

**Chapter 3: Suppression mechanism of the calcium sensitivity in *S. cerevisiae* *ptp2Δ msg5Δ* double disruptant involves a novel HOG-independent function of Ssk2, transcription factor Msn2 and the PKA component Bcy1**

Although the suppressor roles of *BCK1*, *MKK1* and *SLT2* have been characterized in Chapter 2, the mechanism of suppression of the calcium sensitivity by *ssk2Δ* and *yak1Δ* disruption is poorly understood. Through genetic analysis, I found that only *ssk2Δ* disruption could confer calcium tolerance to the *ptp2Δ msg5Δ* disruptant among the HOG components, indicating a HOG-independent suppressor function of Ssk2 in relation to calcium signaling. Using microarray analysis, I identified 19 induced genes in the *ptp2Δ msg5Δ* disruptant that were subsequently repressed in the *ptp2Δ msg5Δ ssk2Δ* disruptant ("rise and fall" pattern of expression), indicating their involvement in the suppression mechanism conferred by *ssk2Δ* disruption. Furthermore, *msn2Δ* disruption was able to suppress the calcium sensitivity of the *ptp2Δmsg5Δ* disruptant, implying a regulatory role in the calcium signaling pathway. The same "rise and fall" patterned expression was observed in *ptp2Δ msg5 Δyak1Δ* and *ptp2Δ msg5Δ bcy1Δ* disruptants thus, linking the PKA-related suppression mechanism with Ssk2 and Msn2. These results suggest that the regulation of the "rise and fall" genes serves as a merging point for the suppression of the calcium sensitive phenotype of the *ptp2Δ msg5Δ* double disruptant mediated by *ssk2Δ*, *yak1Δ*, *msn2Δ* and *bcy1Δ* disruption.

**Chapter 4: General discussion and conclusion**

This work describes functional redundancy in response to high extracellular calcium existing at both the protein and cascade levels. At the protein level, Ptp2 and Msg5 have redundant functions as negative regulators of the SLT2 pathway. This indicates the importance of preventing the hyper-activation of the calcium-induced signaling pathway mediated by the parallel SLT2 and calcineurin pathways. Based on the proposed model, calcineurin is the primary signaling cascade activated upon calcium exposure while SLT2 acts as an alternate, secondary pathway if calcineurin is impaired. Thus, in the normal calcium-exposed setting, SLT2 pathway should be repressed via negative regulation by Ptp2 or Msg5. At the cascade level, functional redundancy between SLT2 and calcineurin pathways in response to high extracellular calcium was revealed. By inactivating either SLT2 or calcineurin pathway, the calcium sensitivity of the *ptp2Δmsg5Δ* double disruptant can be suppressed by preventing over-activation of the calcium-induced signaling cascade under the parallel control of the SLT2 and calcineurin. Another alternative pathway that can suppress the calcium sensitivity of the *ptp2Δ msg5Δ* double disruptant was discovered that involves the PKase suppressors *ssk2Δ*, *msn2Δ*, *bcy1Δ* and *yak1Δ*. The Msn2-mediated regulation of the "rise and fall" genes served as the convergence point between Ssk2 and

PKA (*yak1Δ* and *bcy1Δ*) with regards to the suppression of the calcium sensitivity of the *ptp2Δ msg5Δ* double disruptant. In conclusion, this work presented evidence of multi-level, functionally redundant mechanisms in *S. cerevisiae* that act as safeguards for buffering the lethal effects of a hyper-activated signaling pathway upon exposure to high extracellular calcium.

# 論文審査の結果の要旨及び担当者

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## 論文審査の結果の要旨

Upon the completion of the Yeast Genome Project in 1996, *Saccharomyces cerevisiae* became the first eukaryotic genome to be sequenced fully; defining 5,885 potential protein-encoding genes. More importantly, the complete sequence revealed the magnitude of research that was needed to be done as more than 50% of the sequenced genes were unknown and uncharacterized by previously established methods such as mutant hunting. Furthermore, the yeast genome showed a large amount of genes with apparent genetic redundancy. This particular problem necessitated the need to systematically characterize the biological function of the all genes in the yeast genome and to elucidate functional redundancy in more detail.

In Chapter 1, Mr. Laviña reviewed the previous studies related to protein phosphatases and several signaling pathways that are explored in this study. Regulatory systems such as reversible phosphorylation involving protein kinases (PKases) and phosphatases (PPases) play key roles in mediating stress response and other cellular processes thus, they serve as important research subjects for molecular breeding and strain improvement. In an effort to understand the role of each protein PPase in the cellular physiology of *S. cerevisiae*, Sakumoto and colleagues (2002) in their previous work constructed a PPase library of single and double disruptants and systematically screened for interesting phenotypes; one of which was that the disruption of two PPase genes, *PTP2* and *MSG5*, causes calcium sensitivity, indicating that functional redundancy exists between the two PPases in response to high extracellular calcium. Furthermore, Hermansyah (2010) revealed that additional disruption of PKase genes *BCK1*, *MKK1* and *SLT2* (Cell Wall Integrity SLT2 MAPK pathway), *MCK1* (Chromosome segregation), *YAK1* (Glucose sensing) and *SSK2* (High Osmolarity Glycerol (HOG) MAPK pathway) in the *ptp2Δmsg5Δ* background confers calcium tolerance. Based upon this background, in this study, Mr. Laviña clarified the redundant role of Ptp2 and Msg5 in calcium-induced signaling and the suppression mechanisms conferred by the PKase disruptions using genetic and transcriptional analyses as follows.

Cell regulation is an integral part of signaling cascades that ensures the maintenance of cellular homeostasis upon changes in the environment. Since over-activation and down-regulation of a particular signal can have damaging effects, cells possess a myriad of ways to combat improper activation or down-regulation of signaling pathways. In Chapter 2 of this study, Mr. Laviña found a redundant function for Ptp2 and Msg5 in the regulation of SLT2 pathway in response to high extracellular calcium. Furthermore, Mr. Laviña discovered that

inactivation of calcineurin by the disruption of the calcineurin regulatory subunit, *CNB1* or treatment with a calcineurin inhibitor, FK506, can suppress the calcium sensitive phenotype of the *ptp2Δmsg5Δ* double disruptant. This indicated that the Ptp2 and Msg5-mediated calcium signaling is under the parallel control of SLT2 and calcineurin pathways. In the wake of a calcium-induced, calcineurin-driven signaling pathway activation, the calcium sensitivity of the *ptp2Δmsg5Δ* double disruptant can be suppressed by regulating the SLT2 pathway through the disruption of the major kinases in the SLT2 signal cascade that include *BCK1*, *MKK1* and *SLT2*. Mr. Laviña proposes that Ptp2 and Msg5 are key regulatory phosphatases that prevent over-activation of the calcium-induced signaling cascade under the parallel control of the SLT2 and calcineurin pathways.

Although the suppressor roles of *BCK1*, *MKK1* and *SLT2* have been characterized in Chapter 2, the mechanism of suppression of the calcium sensitivity by *ssk2Δ* and *yak1Δ* disruption is poorly understood. In Chapter 3, through genetic analysis, Mr. Laviña found that only *ssk2Δ* disruption could confer calcium tolerance to the *ptp2Δ msg5Δ* disruptant among the HOG components, indicating a HOG-independent suppressor function of Ssk2 in relation to calcium signaling. Using microarray analysis, Mr. Laviña identified 19 induced genes in the *ptp2Δ msg5Δ* disruptant that were subsequently repressed in the *ptp2Δ msg5Δ ssk2Δ* disruptant ("rise and fall" pattern of expression), indicating their involvement in the suppression mechanism conferred by *ssk2Δ* disruption. Furthermore, *msn2Δ* disruption was able to suppress the calcium sensitivity of the *ptp2Δmsg5Δ* disruptant, implying a regulatory role in the calcium signaling pathway. The same "rise and fall" patterned expression was observed in *ptp2Δ msg5 Δyak1Δ* and *ptp2Δ msg5Δ bcy1Δ* disruptants thus, linking the PKA-related suppression mechanism with Ssk2 and Msn2. These results suggest that the regulation of the "rise and fall" genes serves as a merging point for the suppression of the calcium sensitive phenotype of the *ptp2Δ msg5Δ* double disruptant mediated by *ssk2Δ*, *yak1Δ*, *msn2Δ* and *bcy1Δ* disruption.

In Chapter 4, Mr. Laviña summarized his important findings and proposed several models that illustrate the mechanisms of suppression of the calcium sensitive phenotype of the *ptp2Δmsg5Δ* double disruptant. Mr. Laviña's work describes functional redundancy in response to high extracellular calcium existing at both the protein and cascade levels. At the protein level, Ptp2 and Msg5 have redundant functions as negative regulators of the SLT2 pathway. This indicates the importance of preventing the hyper-activation of the calcium-induced signaling pathway mediated by the parallel SLT2 and calcineurin pathways. Based on the proposed model, he hypothesized that calcineurin is the primary signaling cascade activated upon calcium exposure while SLT2 acts as an alternate, secondary pathway if calcineurin is impaired. Thus, in the normal calcium-exposed setting, SLT2 pathway should be repressed via negative regulation by Ptp2 or Msg5. At the cascade level, functional redundancy between SLT2 and calcineurin pathways in response to high extracellular calcium was revealed. By inactivating either SLT2 or calcineurin pathway, the calcium sensitivity of the *ptp2Δmsg5Δ* double disruptant can be suppressed by preventing over-activation of the calcium-induced signaling cascade under the parallel control of the SLT2 and calcineurin. He also discovered another alternative pathway that can suppress the calcium sensitivity of the *ptp2Δ msg5Δ* double disruptant and involves the PKase suppressors *ssk2Δ*, *msn2Δ*, *bcy1Δ* and *yak1Δ*. He proposed that the Msn2-mediated regulation of the "rise and fall" genes served as the convergence point between Ssk2 and PKA (*yak1Δ* and *bcy1Δ*) with regards to the suppression of the calcium sensitivity of the *ptp2Δ msg5Δ* double disruptant. In conclusion, Mr. Laviña's work presented evidence of multi-level, functionally redundant mechanisms in *S. cerevisiae* that act as safeguards for buffering the lethal effects of a hyper-activated signaling pathway upon exposure to high extracellular calcium. Judging from these achievements, this dissertation deserves the degree of Doctor of Engineering.