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Functional Characterization of the Phosphorelay Protein MPR1 from *Schizosaccharomyces pombe*

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Functional Characterization of the Phosphorelay Protein MPR1 from *Schizosaccharomyces pombe*

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF CHEMISTRY AND BIOCHEMISTRY

By

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

General Abbreviations

Ax	Absorbance at x nanometers
ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
5FOA	5-Fluoroorotic acid
IPTG	Isopropyl-β-D-thiogalactoside
HPt	Histidine-containing phosphotransfer
HK	Histidine kinase
LiAc	Lithium acetate
MAP	Mitogen-activated protein
ODx	Optical density at x nanometers
PCR	Polymerase chain reaction
SC	Synthetic complete (selection media for yeast)
YPAD	Yeast extract, peptone, adenine sulfate, dextrose (rich media for yeast)
CPRG	Chlorophenol-red-β-D-galactopyranoside
ONPG	O-nitrophenyl-β-D-galactopyranoside
RR	Response regulator

Protein Designations

ATF1	activating transcription factor; *S. pombe*
ArcB	Anoxic redox control B; *E. coli*
HOG1	High osmolarity glycerol response; *S. cerevisiae*
MAK1,2,3	MCS4 associated kinase 1, 2, 3; *S. pombe*
MCS4	Mitosis catastrophy suppressor 4; *S. pombe*
MPR1	Multistep phosphorelay protein; *S. pombe*
PRR1	*S.pombe* response regulator; *S. pombe*
SKN7	Suppressor of Kre9 mutation; *S. cerevisiae*
SLN1	Synthetic lethal of the N-end rule 1; *S. cerevisiae*
SSK1	Suppressor of the sensor kinase 1; *S. cerevisiae*
YPD1	Tyrosine (Y) phosphatase dependent 1; *S. cerevisiae*
MAPK	mitogen-activated protein kinase

Abstract

The yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* utilize not only serine/threonine/tyrosine phosphorylation that is dominant in the eukaryotic kingdom, but also employ histidine/aspartate phosphorylation that is mainly used in prokaryotic cell signal transduction systems. MPR1, the histidine-containing phosphotransfer (HPt) protein from *S. pombe*, has a C-terminal portion (160 residues out of 295 total) that is 42% identical to YPD1, the HPt protein in *S. cerevisiae*. Not only is the histidine phosphorylation site of YPD1 (residues 62-71) completely conserved in MPR1, but also the solved structure of YPD1 is very similar to the predicted structure of the C-terminal domain of MPR1. Yet the additional N-terminal 135 residues of MPR1 have no apparent sequence homology to any known protein. To identify the possible roles of the N-terminal portion of MPR1, studies were conducted to test the phosphotransfer ability *in vivo* and *in vitro* of truncated MPR1 (the C-terminal domain of MPR1, or MPR1ΔN), in comparison to wild type MPR1 to see the effect of deletion of the N-terminal portion of MPR1. Yeast two-hybrid analysis was also used to assay protein-protein interactions between MPR1/MPR1ΔN and the cognate response regulators (RR). The results revealed that the C-terminal domain of MPR1 could function as an HPt protein, while the N-terminal domain might enhance the phospho-receiving and phosphotransfer ability of the C-terminal domain by enhancing the interaction between the HPt protein and the response regulator proteins. The N-terminal domain of MPR1 might help to form a binding surface that would dock the response regulator proteins.

The yeast two-hybrid assay indicates that MPR1 interacts with the response regulator domains of MAK3 (one of the three histidine kinases) and MCS4, but not with MAK1, MAK2 and PRR1. PRR1-RR weakly interacts with MAK1-HK, MAK2-HK and MAK3-HK. *In vitro* phosphorylation assay results showed that MPR1 could receive phosphoryl groups from phospho-MAK2-RR and transfer them to MCS4-R4, but not to PRR1-RR. These results suggest that one branch of the pathway is formed by MAK2/MAK3→MPR1→MCS4, and the other one is MAK1→PRR1 in the His-Asp phosphorelay pathway in *S. pombe*.

HPt proteins may have phosphotransfer specificity toward different response regulators. The phosphotransfer between YPD1 and SSK1-R2 was more rapid and favored than phosphotransfer between YPD1 and SKN7-R3 *in vitro*, while in a heterologous assay MPR1 favors phosphotransfer to SKN7-R3 over SSK1-R2, which is opposite to YPD1. To investigate any structural basis behind this phenomenon, the phosphotransfer specificity of mutated YPD1 and MPR1 was tested *in vitro*. None of the mutants could invert phosphotransfer specificity of YPD1. It is possible that the combined differences between the two proteins are responsible for the difference in phosphotransfer specificity.

1. Introduction

1.1. Two-component signal transduction

Signal transduction systems are abundant in all living cells. Upon sensing stimuli from the extracellular environment, the corresponding signal transduction pathway then transduces the signal from the surface of the cell to the inside of the cell, leading to changes in gene expression and protein activity. Cell responses like these are critical for cells to survive and adapt to drastic changes in their extracellular environment. In bacteria, the predominant signaling mechanism is a phosphotransfer pathway referred to as a "two-component" signaling pathway, which transmits signals by protein phosphorylation (reviewed in Parkinson *et al*., 1992; Stock *et al*., 1995; Mizuno *et al*., 1998; Stock *et al*., 2000; West *et al*., 2001).

The central core of the two-component pathway is a histidine protein kinase (HK) and a response regulator protein (RR) (Stock *et al*., 1995; Stock *et al*., 2000). HKs do not resemble the better-studied serine/threonine and tyrosine kinases, either in sequence or structure (Thomason *et al*., 2000). HKs belong to an ancient enzyme family that utilizes ATP to drive reactions, a family that also includes topoisomerase II and the chaperone Hsp90 (Tanaka *et al*., 1998; Bilwes *et al*., 1999; Stock *et al*., 1999). HKs usually consist of a transmembrane domain that detects extracellular stimuli (sensor domain) and a conserved kinase core (histidine kinase domain) that catalyzes autophosphorylaton at a conserved His residue (Robinson *et al*., 2000). RRs contain a conserved receiver domain that catalyzes phosphotransfer from the phospho-His of the HK to its own conserved Asp residue (Robinson *et al*., 2000). Being multidomain

proteins, RRs also contain a variable domain that elicits the output response (Figure 1-1). The most common response is the regulation of gene expression; other responses in eukaryotes include regulation of the mitogen-activated protein kinase pathway (MAPK pathway) (Maeda *et al*., 1994), control of motility (Falke *et al*., 1997), and modulation of cyclic nucleotide levels (Thomason *et al*., 1998).

1.2. His-Asp phosphorelay signal transduction in lower eukaryotes

The first discoveries of the two-component pathway in eukaryotes were through the finding of the SLN1 gene when studying the N-end rule in protein degradation (Ota *et al*., 1993), and the discovery of the ETR1 gene when isolating ethylene-induced genes in plants (Chang *et al*., 1993). Then genomic projects expanded the list of sensor kinases and response regulators that exist in lower eukaryotes like plants and fungi (Maeda *et al*., 1994; Perraud *et al*., 1999). It seems that a multistep "phosphorelay" instead of the simple two-component system is more popular in these eukaryotes. In these phosphorelays, sensor kinases are hybrid proteins that contain both a histidine kinase domain and a response regulator or "receiver" domain. Furthermore, phosphotransfer to a second response regulator is mediated by a third protein, a histidine-containing phosphotransfer (HPt) protein (Figure 1-1) (Brown *et al*., 1993).

Although no His-Asp phosphorelay pathway has been found in the animal kingdom, histidine kinase-like proteins exist in mammals (reviewed by Besant *et al*., 2005). They either contain sequence motifs similar to histidine kinases, or were found to be able to transfer a phosphoryl group from a histidine residue to another molecule like

histone H4. Possible existence of histidine phosphatases supports this idea (reviewed by Besant *et al.*, 2005).

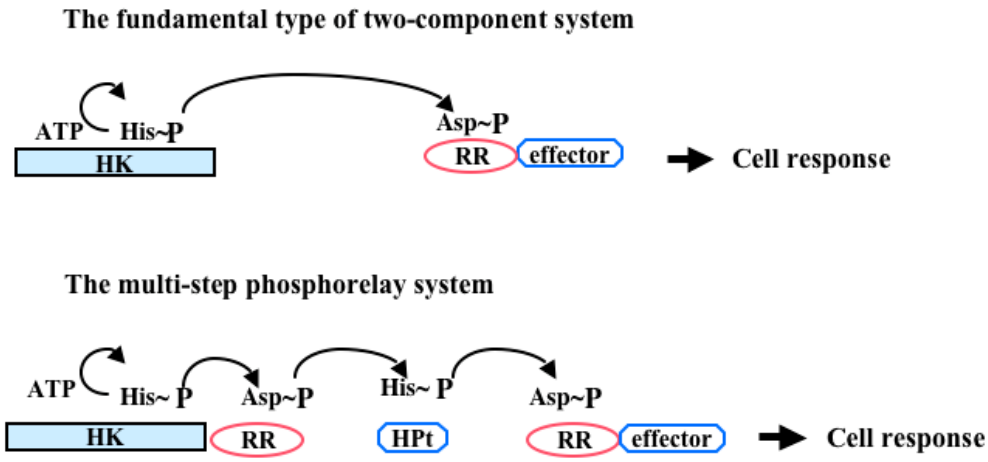

Figure 1-1. Two-component phosphotransfer system. A fundamental two-component phosphotransfer system consists of a transmembrane sensor HK and a cytoplasmic RR. HKs catalyze ATP-dependent autophosphorylation of a conserved residue H (histidine). The phosphoryl group (P) is then transferred to a conserved Asp residue (D) within the conserved regulatory domain of an RR. Phosphorylation of RR activates a downstream effector domain, which elicits a cellular response. The multi-step phosphorelay system has a hybrid HK that has a RR domain. Multiple phosphotransfer reactions occur and a His-containing phosphotransfer (HPt) protein is involved.

1.3. His-Asp phosphorelay pathway in *Saccharomyces cerevisiae*

The His-Asp phosphorelay system in *Saccharomyces cerevisiae* was the first one identified in eukaryotes and is also the best characterized to date. This two-component system is located upstream of the HOG (high osmolarity glycerol) MAPK (mitogen-activated protein kinase) cascade involved in adaptation to osmotic stress (Brewster *et al.*, 1993; Maeda *et al.*, 1994; Posas *et al.*, 1996) (Figure 1-2). The components are: SLN1, a hybrid sensor kinase, with an N-terminal extracellular sensing domain, linked to

a cytoplasmic histidine kinase domain (HK), and a response regulator domain at the C-terminal end (RR); YPD1, the histidine-containing phosphotransfer protein (HPt); SSK1 and SKN7, the response regulators (Maeda *et al*., 1994; Posas *et al*., 1996). Under normal growth conditions, SLN1 autophosphorylates and transfers the phosphoryl group to YPD1 via its receiver domain, and ultimately to SSK1. Phosphorylated SSK1 inhibits activation of the redundant MAPKKK SSK2 and SSK22, which are the components in the downstream HOG pathway. Hyperosmotic stress inhibits SLN1, which in turn leads to dephosphorylation of SSK1 and the activation of the HOG pathway (Posas *et al*., 1996) (Figure 1-2). SKN7 is a transcription factor that reacts to cell wall damage and oxidative stress (Brown *et al*., 1993; Brown *et al*., 1994; Morgan *et al*., 1997; Alberts *et al*., 1998; Ketela *et al*., 1998; Li *et al*., 1998).

1.4. His-Asp phosphorelay pathway in *Schizosaccharomyces pombe*

A His-Asp phosphorelay system is also found in *Schizosaccharomyces pombe* (Cottarel *et al*., 1997; Shieh *et al*., 1997; Ohmiya *et al*., 1999; Aoyama *et al*., 2000; Nguyen *et al*., 2000). The system controls the activity of the WAK1 (also WIK1, WIS4)-WIS1-STY1 (also SPC1 and PHH1) MAPK pathway (Figure 1-2) (Shiozaki *et al*., 1997). Many of the components have been found to be homologous to their counterparts within the HOG pathway of *S. cerevisiae*. STY1 is the MAP kinase in the cascade that reacts to multiple stresses, including osmotic stress, oxidative stress, heat shock and UV light.

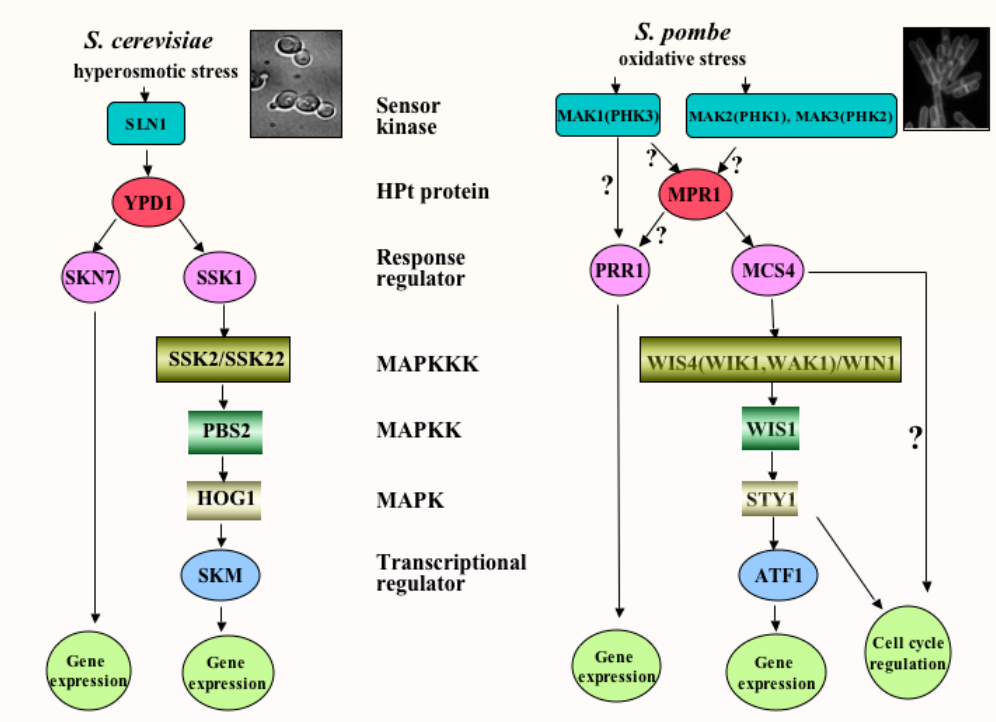

Figure 1-2. Multi-step phosphorelay pathways in *S. cerevisiae* and *S. pombe*. The phosphorelay pathways in the two kinds of yeast react to different stresses. Both of them control a downstream MAPK pathway. The components are homologous to the counterparts in the other yeast.

1.4.1 Three histidine kinases: MAK1, MAK2, MAK3

Three histidine kinases exist in *S. pombe*: MAK1, MAK2 and MAK3 (for MCS4-activating kinase, Buck *et al*., 2000) (also known as PHK3, PHK1 and PHK2 respectively, as pombe histidine kinase, Aoyama *et al*., 2001). All of them have a histidine-kinase domain and a response regulator domain at the C-terminal portion. They also contain several additional domains that are absent in *S. cerevisiae* homologue SLN1 (Buck *et al*., 2000). Both MAK2 and MAK3 contain one PAS/PAC domain, a domain that has been identified in a variety of prokaryotic sensors of oxygen or redox (Crews *et al*., 1999), while MAK1 has two such domains in parallel (Buck *et al*., 2000) (Figure 1-3). Two motifs are found in MAK2 and MAK3, but not in MAK1. The first one is a

GAF domain, which has been identified in a number of phototransducing proteins like phytochromes and plant ethylene receptors (Aravind *et al*., 1997; Buck *et al*., 2000). The GAF domain has a well-defined function, which is cGMP binding (Charbonneau *et al*., 1990). The second is a domain that is homologous to a family of atypical serine/threonine kinases from prokaryotes, such as *Mycobacterium tuberculosis* PknB (Buck *et al*., 2000) (See Figure 1-3).

MAK2 and MAK3 regulate STY1 activity in response to peroxide stress, while deletion of the *MAK1* gene did not affect STY1 phosphorylation. *MAK2* and *MAK3* gene deletions caused reduction of *PYP2* and *GPX1* mRNA transcription in presence of 1 mM hydrogen peroxide, while *MAK1* deletion affected $CTT1^{+}$ mRNA level. It was believed that two phospho-relay systems were required to protect *S. pombe* from peroxide stress, one controlled by MAK1, the other one controlled by MAK2/3 (Buck *et al*., 2000).

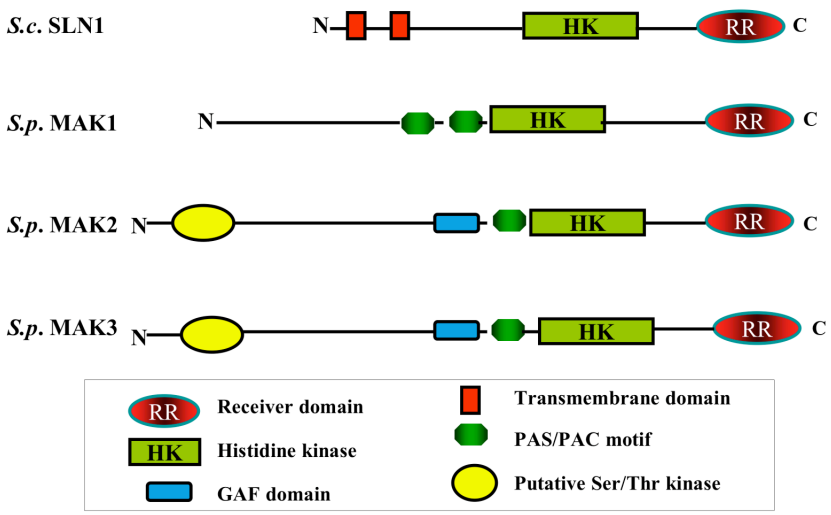

Figure 1-3. The domain structure of sensor kinases in *S. pombe* and *S. cerevisiae*. SLN1 in *S.cerevisiae* has a transmembrane domain. MAK1, MAK2 and MAK3 have domains that do not exist in SLN1, the PAS/PAC domain, GAF domain and a putative Ser/Thr kinase domain. All of the four kinases have a RR and an HK domain at the C-terminal portion.

1.4.2 Two response regulators: MCS4 and PRR1

MCS4 (mitotic catastrophe suppressor) in fission yeast was originally identified as a positive regulator of mitosis, suppressing the lethal hyperactivation of the central mitotic regulator CDC2 (Booher *et al*., 1987; Mloz *et al*., 1989; Shieh *et al*., 1997). The *S. pombe cdc2-3w wee1-50* double mutant displays a temperature-sensitive lethal phenotype termed mitotic catastrophe. Six mitotic catastrophe suppressor (*MCS1-6*) genes were identified and mutations in them suppress the *cdc2-3w wee1-50* temperature-sensitive growth defect (Cottare *et al*., 1997).

MCS4 is structurally and functionally homologous to SSK1 from the budding yeast *S. cerevisiae* (Maeda *et al*., 1994), thus it is thought to be the putative response regulator in the stress pathway in fission yeast (Mloz *et al*., 1989; Cottare *et al*., 1997; Shieh *et al*., 1997; Shiozaki *et al*., 1997). The identity between the two proteins is mostly confined to the C-terminal ends where the conserved response regulator domains reside (Cottarel *et al*., 1997) (Figure 1-4). The MCS4 522 amino acid protein is 55% identical to SSK1 in the carboxy-terminal response regulatory domain, while it is 18% identical on average to a number of bacterial response regulators (Shieh *et al*., 1997) (Figure 1-4). It is believed to be a response regulator monitoring environmental signals and regulating the G_2/M transition partially through the WIK1 (also WAK1, WIS4)-WIS1-STY1 kinase cascade (Shiozaki *et al*., 1997). This conclusion was based on the findings that deleting the *MCS4* gene prevented activation of STY1 in response to a variety of environmental stresses (Shieh *et al*., 1997), and that double deletion of the *WAK1* and *MCS4* genes gave an effect on STY1 activation and PYP2 induction similar to the single deletion of *WAK1*

(Shieh *et al*., 1997). Also, MCS4 was shown to associate with WAK1 *in vitro* (Shieh *et al*., 1997).

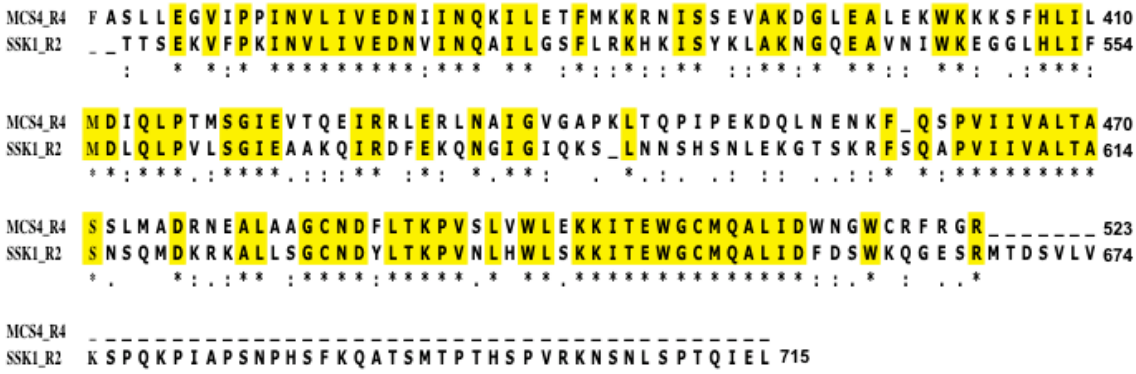

Figure 1-4. Sequence alignment of the response regulator domains of SSK1-R2 and MCS4-R4. The RR domain of SSK1 (named as SSK1-R2) and MCS4 (named as MCS4-R4) are 55% identical. The identical residues are indicated by *. The highly conserved and conserved residues are indicated by : and . respectively.

Besides regulating the STY1 MAP kinase pathway, MCS4 also controls the G_2/M transition in cell division in *S. pombe* by influencing an unknown pathway that is required for the correct timing of CDC2 kinase activation (Shieh *et al*., 1997). This was based on the observation that neither a *WIS1* deletion nor a *STY1* deletion was sufficient to suppress mitotic catastrophe caused by hyperactivation of CDC2, while a *MCS4* deletion mutation could suppress mitotic catastrophe (Shieh *et al*., 1997).

Although deletion of the *MCS4* gene in *S. pombe* was viable to cells, the transition from G2 to M phase in cell division was delayed and cells divided in elongated shape (Aoyama *et al*., 2000). The conserved aspartate residue in the RR domain of MCS4 is D412. A D412N mutation in MCS4 did not affect cell growth rate, but caused cells to divide at a smaller size (11.4±0.4μm) compared to a wild-type cell (14.3±0.2μm)

(Buck *et al*., 2000), suggesting that dephosphorylation of MCS4 partially blocked activation of STY1. The phenotype resembles that of the deletion of *PYP1*, the gene that encodes MAP kinase phosphatase (Buck *et al*., 2000).

Another putative response regulator in *S. pombe* is PRR1, a protein consisting of 539 amino acid residues. It is homologous to the *S. cerevisiae* response regulator SKN7 (622 amino acids), sharing 32.8% identity overall (Ketela *et al*., 1999) (Figure 1-5). Both of them contain a receiver domain at the C-terminal domain, which may serve as a phospho-acceptor in a phosphorelay. D418 in PRR1 is thought to be the phospho-accepting aspartate residue. The N-terminal portion of PRR1 is similar to mammalian heat shock factors (HSFs), with 24.7% identical to mouse HSF-2 (Ketela *et al*., 1999) (Figure 1-6).

Deletion of *PRR1* in *S. pombe* was not lethal to cells, but caused cell sensitivity to cold temperatures (20^{o}C), heavy metal (cadmium) and *t*-BOOH/H_2O_2 treatment, but not to diamide treatment or to osmotic pressure caused by 2.2 M sorbitol (Ketela *et al*., 1999; Li *et al*., 2002). Another phenotype exhibited was failure of *prr1Δ* cells to undergo sexual development (sterility). PRR1 controls not only the expression of *CTT1*$^{+}$ (encodes catalase) and *TRR1*$^{+}$ (encodes thioredoxin), but also the expression of *STEL1*$^{+}$ (the gene that controls sexual development in *S. pombe*) (Ketela *et al*., 1999; Li *et al*., 2002) and *MAM2*$^{+}$ (encodes mating pheromone M-factor), *MEI2*$^{+}$ (encodes a protein that is committed to meiosis) (Nakamichi *et al*., 2003). Although one group found that PRR1 was not responsible for *GPD1*$^{+}$ (encodes glycerol-3-phosphotate dehydrogenase) gene induction in presence of 1 M KCl (Ketela *et al*., 1999), another group revealed that deletion of the *PRR1* gene reduced the mRNA level of *CTA3*$^{+}$, *CTT1*$^{+}$ and *GPX1*$^{+}$

(encodes glutathione peroxidase) in presence of 0.6 M KCl (Greenall *et al*., 2002) (*CTA3*$^{+}$ encodes an intracellular cation transporter, a stress gene whose expression is positively controlled by the STY1 pathway and negatively regulated by TUP repressors). It is believed that PRR1 contributed to the high basal level of *CTA3*$^{+}$ level when *TUP* repressor genes were deleted.

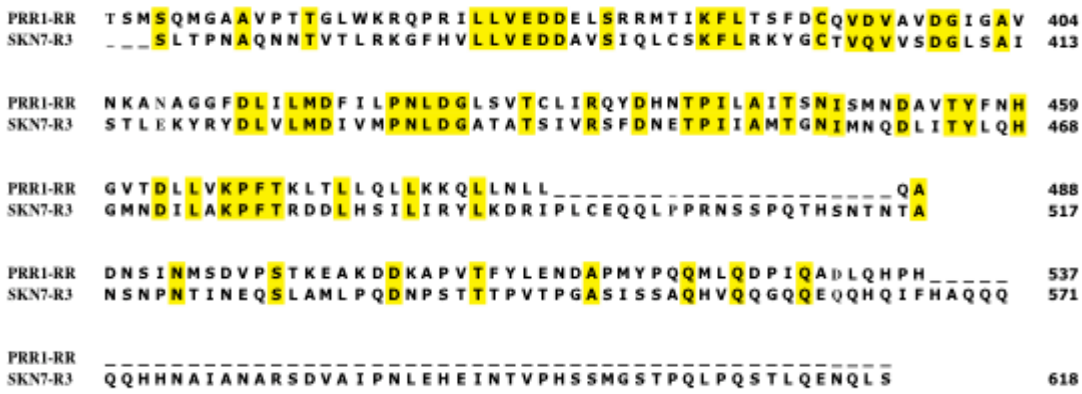

Figure 1-5. Sequence alignment of the response regulator domains of PRR1 and SKN7. The amino acid sequences of response regulator domain of PRR1 (named as PRR1-RR) and that of SKN7 (named as SKN7-R3) were aligned. The identical amino acid residues are highlighted in yellow boxes.

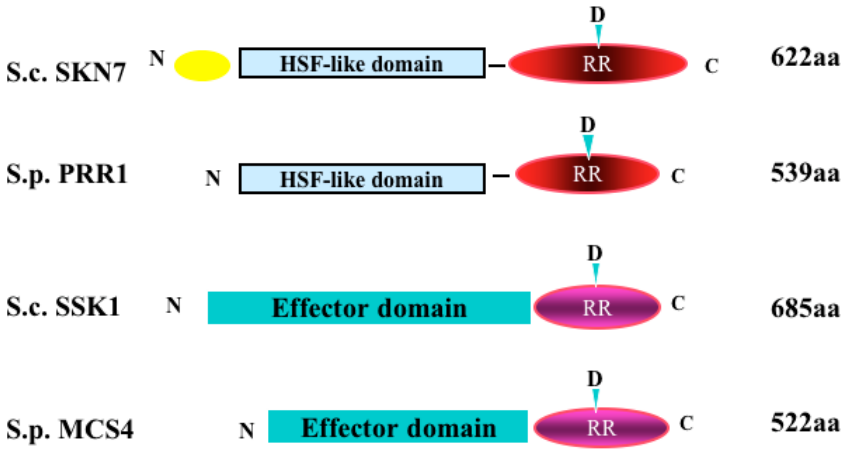

Figure 1-6. The domain structure of the four response regulators in *S. pombe* and in *S. cerevisiae*. RR means response regulatory domain. The arrows point to the active centers that contain the conserved aspartate residue. S.p.: *S.pombe*, S.c.: *S.cerevisiae*. HSF is the abbreviation for heat shock factor.

While the MCS4 response regulator plays a role in mitotic cell cycling, PRR1 seems to control the *S. pombe* meiosis process (Greenall *et al.*, 2002). In *S. pombe*, the decision to exit the mitotic cell cycle and to start mating requires both the pheromone (mating partners) and specific nutrient signals (Sugimoto *et al.*, 1991; Aono *et al.*, 1994; Watanabe *et al.*, 1998). Under starving conditions (when the nitrogen source is limited), there are several cellular changes, including arrest of cell growth at the G1 phase, lowering intracellular cAMP levels, the expression of certain genes (*STE11*$^{+}$, *MAM2*$^{+}$, and *MEI2*$^{+}$), and initiation of meiosis (Nakamichi *et al.*, 2003). While *prr1Δ* cells were sterile in nitrogen-deficient medium, the PRR1D418N mutation resulted in a precocious (or rapid) sexual development. The mutated cells underwent mating even in rich medium and/or anaerobic growth conditions (limited external oxygen). *S. pombe* wild type cells cannot enter into the meiotic cell cycle in a nitrogen-deficient medium if under anaerobic conditions. This suggested that the D418N mutation of PRR1 is a "gain-of-function" and

PRR1 could be negatively regulated by the histidine kinases (Nakamichi *et al*., 2003). The *mak1/2/3Δ* cells and PRR1D418N cells could efficiently enter into meiosis in nitrogen-deficient medium under anaerobic growth conditions, while wild-type cells could not, suggesting that the sexual development of *S. pombe* may also be regulated in response to oxidative stress (Nakamichi *et al*., 2003).

Li *et al*., 2002 cited their own unpublished data that showed truncated PRR1 mutants (truncation of either the N-terminal portion or the C-terminal portion alone) were not able to complement the *prr1Δ* phenotype, while the full-length gene could (Li *et al*., 2002), suggesting that full-length PRR1 is required to accomplish its function.

GFP-PRR1 protein (a GFP-PRR1D418N mutation) (GFP stands for green fluorescent protein) was located mainly in the nucleus, and the localization was not affected by prior treatment of the cells with H_2O_2 (Li *et al*., 2002). This suggests that PRR1 may predominantly be located in the nucleus under normal and oxidative stress conditions (Li *et al*., 2002).

mak1Δ cells have reduced $CTT1^+$ levels like *prr1Δ* cells, suggesting that PRR1 may be under the control of MAK1 (Crews *et al*., 1999), but more direct evidence will be needed to make the conclusion.

1.4.3 One HPt protein: MPR1

MPR1 (multistep phosphorelay protein, Nguyen *et al*., 2000), also known as SPY1 (*S. pombe* YPD1 like protein, Aoyama *et al*., 2000), is highly homologous to the YPD1 protein in budding yeast and is thought to be the HPt protein in fission yeast. This 32.5 kDa protein is 128 residues longer than YPD1. The C-terminal region of 160 residues is 42% identical to the YPD1 sequence. The histidine phosphorylation site of

YPD1 (residues 62-71) is completely conserved in MPR1, whose phosphorylation site is His-221 (Figure 1-7). However, the additional N-terminal 135 residues of MPR1 have no apparent sequence homology to any known proteins (Nguyen *et al*., 2000).

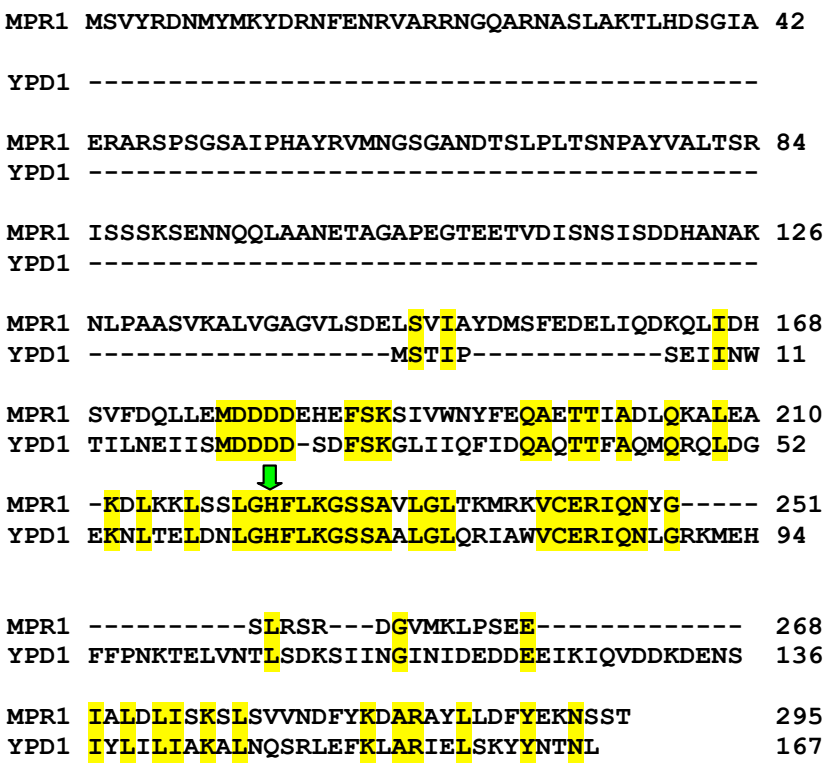

Figure 1-7. Alignment of the amino acid sequence of MPR1 with that of YPD1. YPD1 is 42% identical to the C-terminal portion of MPR1. Identical residues of the two proteins are shaded in yellow boxes. A green arrow marks the phosphorylation site, which is completely conserved between the two proteins.

1.4.4 What is experimentally known about MPR1?

Besides the sequence alignment and homology, some experimental evidence supports the hypothesis that MPR1 acts as a HPt protein in *S. pombe*. The *YPD1* deletion mutation of *S. cerevisiae* is lethal to the cells (Posas *et al*., 1996), however, the expression of the *MPR1* gene can complement the deletion (Aoyama *et al*., 2000).

Although deletion of the *MPR1* gene (*mpr1*Δ) in *S. pombe* does not kill the cells, it causes severe impairment of STY1 activation (Nguyen *et al*., 2000). To test whether His221 of MPR1 (the residue that aligns with His 64 in YPD1, the site of phosphorylation) is the phosphorylation site, His221 was substituted with Gln (MPR1H221Q). This mutated MPR1 was unable to complement the disruption of *YPD1* in *S. cerevisiae* (Aoyama *et al*., 2000). The same mutation impaired STY1 activation upon oxidative stress, showing that His221 to Gln mutation completely abolished the phosphotransfer activity of the protein.

The natural downstream target of MPR1 is thought to be MCS4. Two different *in vivo* studies showed that MPR1 interacts with MCS4. One study was a yeast two-hybrid analysis (Aoyama *et al*., 2000), the other one used a coprecipitation analysis in which MCS4 was copurified with MPR1 (Nguyen *et al*., 2000). Besides regulating STY1 MAPK pathway, MPR1 together with MCS4 may also regulate G_2/M cycle during cell division, because *MPR1*-deficient cells were precocious in entering the M phase (Aoyama *et al*., 2000).

The *MPR1* gene is not essential for cell growth under standard growth conditions since its deletion, unlike the *YPD1* deletion in *S. cerevisiae*, is not lethal to the cell (Aoyama *et al*., 2000). Furthermore, *mpr1Δ* cells grew well on YES agar plates containing 1 M KCl (Nguyen *et al.,* 2000, unpublished data), suggesting that the *MPR1* gene is not essential for controlling high-osmotic stress (Nguyen *et al*., 2000).

1.4.5 Binding specificity of HPt proteins to response regulators

Both *S. pombe* and *S. cerevisiae* cells have two response regulators in their phosphorelay system. SSK1 in *S. cerevisiae* is involved in hyperosmotic stress response, whereas SKN7 is involved in cell wall metabolism (Brown *et al*., 1993, Ketela *et al*., 1999, Li *et al*., 2002), heat shock response (Raitt *et al*., 2000), nitrogen starvation-induced diploid filamentous growth (Lorenz *et al*., 1998), cell cycle control (Morgan *et al*., 1995; Bouquin *et al*., 1999), and oxidative stress response (Charizanis *et al*., 1999; Charizanis *et al*., 1999). To induce the proper cellular reaction in signal transduction, the HPt protein needs to transfer the phosphoryl group to the correct RR in response to a specific situation. Binding specificity may decide the direction of the phosphoryl group flow from the HPt protein to the response regulators. Previous results through the yeast two-hybrid assay (Porter *et al*., 2003; Porter *et al*., 2005) and the YPD1/SLN1-R1 co-crystal structure observation (Xu *et al*., 2003) showed that the hydrophobic patch around the site of phosphorylation on YPD1 is the primary binding surface, while the peripheral residues may be responsible for the specificity of binding to RRs. Although sharing the similar core binding area, SSK1-R2 and SKN7-R3 have specific binding residues on YPD1. Mutation of the specific residues would dramatically affect the binding affinity (Porter *et al*., 2005).

1.5. Similarities and differences between the *S. cerevisiae* and *S. pombe* phosphorelay pathways and MAP kinase pathways

There are other similarities between the *S. cerevisiae* and *S. pombe* phosphorelay pathways beside the sequence homology of their proteins. Like YPD1, MPR1 may also

negatively regulate the MCS4 response regulator, that is, MCS4 is phosphorylated under normal conditions but is dephosphorylated under the appropriate stress condition (Aoyama *et al*., 2000; Nguyen *et al*., 2000).

However, there are also important features that distinguish the *S. pombe* phosphorelay pathway from that of *S. cerevisiae*. Unlike the SLN1-YPD1-SSK1 pathway, which is inactivated by hyperosmotic stress, the putative MAK2, MAK3-MPR1-MCS4 pathway is inactivated by oxidative stress (reviewed by Hohmann, 2002). The MAPK pathway controlled by the SLN1-YPD1-SSK1 pathway is activated only by hyperosmotic stress, while the MAPK pathway that is downstream of the MAK2/MAK3-MPR1-MCS4 pathway (STY1 pathway) responds to oxidative, heat shock stresses, and nutrient limitation (Baunuett, 1998). Beside the differences in type of stress, another striking difference is that the STY1 pathway is involved not only in the regulation of the stress response, but also in mitosis and sexual differentiation (Baunuett, 1998). Furthermore the STY1 pathway controls a transcription factor (ATF1) that is similar to the transcription factor (ATF2) activated in mammalian cells (Baunuett, 1998) (Figure 1-2).

As discussed above, the His-Asp phosphorelay systems in budding yeast and fission yeast have similarities and differences. Differences in sequence homologies may result in functional similarities or differences. MPR1 in fission yeast has an additional 160 residues at its N terminal region compared to the sequence of YPD1 in budding yeast, and that raises an obvious question: what function does the N-terminal portion perform? Does it contribute to the phosphotransfer activity of MPR1, by enhancing or inhibiting the function of the C-terminal portion in phosphotransfer? Is it involved in

binding of response regulators and/or in interacting with the upstream histidine kinases? Or, does it have some other unknown function yet to be determined?

HPt proteins play an important role in His-Asp phosphorelay pathways. Although only a small group of prokaryotes employ a separate HPt protein in a phosphorelay (Freeman *et al*., 1999), HPt proteins are predominantly used in eukaryotic His-Asp pathways. HPt proteins function as a link between RRs. Genomic sequencing programs have revealed that many phosphorelay systems have redundant HKs and/or RRs (Thomason *et al*., 2000). For example, in *Arabidopsis thaliana*, there are at least 11 HKs and 16 RRs that participate in multiple interactions (West *et al*., 2001), but the number of HPt proteins found is lower than that of HKs or RRs in each of the organisms that use a His-Asp phosphorelay. Most of the organisms have only one HPt, while some of them do not have any; only *Arabidopsis thaliana* has 5 HPt proteins (Miyata *et al*., 1998, Suzuki *et al*., 1998). Although more HPt genes may be found with the completion of genomic programs, it is unlikely that their number will exceed that of HKs or RRs (Thomason *et al*., 2000).

In *S. cerevisiae*, six distinct MAPK pathways have been identified, including the osmoregulation pathway (HOG pathway), pheromone pathway, morphological switch pathway, cell wall integrity pathway, cell wall construction pathway, and sporulation pathway (reviewed by Hohmann, 2002). In *S. pombe*, only three MAPK cascades have been defined so far, including the mating pheromone responsive MAPK, the stress activated MAPK (STY1 pathway), and the cell integrity pathway (reviewed by Yang *et al*., 2001).

The HOG pathway in *S. cerevisiae* is stimulated by osmotic shock and controls expression of genes that allow adapting to this stress. The STY1 pathway in *S. pombe* also controls the osmotic stress response in *S. pombe*. The components in the two pathways share relative high identity and similarity (reviewed by Hohmann, 2002). However, differences exist between the two pathways since *S. pombe* is a single cell organism that shares more features with higher eukaryotes than does *S. cerevisiae*. First, in contrast to the HOG pathway in *S. cerevisiae,* which is activated only by hyperosmotic stress, the STY1 pathway in *S. pombe* is activated by hyperosmotic stress, oxidative and heat shock stresses, nutrient limitation and anisomycin (a protein synthesis inhibitor), UV light and carbon starvation (Baunuett 1998). In this respect, the STY1 pathway resembles the p38 and SAPK/JNK pathway of mammalian cells (Baunuett 1998). Second, the STY1 pathway uses a transcription factor (ATF1) that is highly homologous to mammalian transcription factor ATF2. Another feature that distinguishes the *S. pombe* MAP kinase pathway from that in *S. cerevisiae* is that the STY1 pathway not only regulates stress responses but also integrates the responses with two processes essential to all eukaryotes: control of mitosis and initiation of meiosis (Baunuett 1998). The G2-mitosis transition of fission yeast could be delayed if the STY1 pathway was blocked, resulting in delaying of mitosis and causing elongation in cell shape and size. Shorter sized fission yeast cell could be generated if the STY1 pathway was over-activated. The STY1 pathway is also required to initiate the mating of fission yeast cells (Baunuett 1998). Although some observations do link the HOG pathway to cell cycle control in *S. cerevisiae*, such as osmotic shock resulting in a temporary arrest in the G2

phase in mitosis (Alexander *et al*., 2001), more investigations are needed to relate the HOG pathway to the mitosis and meiosis processes in *S. cerevisiae*.

The two-component pathway in *S. cerevisiae* is responsible for activation of the HOG pathway under hyperosmotic stress, while that in *S. pombe* activates the STY1 pathway under oxidative stress. It is not understood yet how osmotic stress signals are transduced by the STY1 pathway, if it is not through the two component system. PRR1 might partially be responsible for activating the STY1 pathway under osmotic stress, since CTA^{3+} transcripts were significantly reduced when the *prr1Δ* strain was exposed to 0.6 M KCl (Greenall *et al*., 2002). Others found that PRR1 may not be involved in the transcriptional response to high salt stress (Ohmiya *et al*., 1999).

1.6. Significance

The fission yeast *Schizosaccharomyces pombe* is a member of the archiascomycetes, believed to belong to an ancestral assembly of the ascomycetes. *S. pombe* has been an excellent alternative fungal model to *S. cerevisiae*. First of all, *S. pombe* is almost as easily cultured and manipulated as budding yeast (*S. cerevisiae*). Secondly, *S. pombe* is well characterized as to molecular genetics, since the genome sequence has been finished recently. More importantly, a phylogenetic analysis using nuclear small subunit rRNAs (GOBASE), suggests that *S. pombe* branches off before the divergence of budding yeasts. This statement is supported by phylogenetic analyses using mitochondrial sequence data (Fungal mitochondrial genome project led by B.F.Lang). Another advantage of using *S. pombe* as a model is that *S. pombe* uses the universal translation code for all ubiquitous mitochondrial genes, which clearly

distinguishes it from other ascomycetes that have one or more codon reassignments (GOBASE). Thus, using *S. pombe* as a model organism will help us to analyze proteins from human and other higher eukaryotes, leading to a better understanding of biological processes. *S. pombe* is only second to *S. cerevisiae* in ease of genetic manipulation within the kingdom of eukaryotic organisms. It has served as an excellent model system in biological research (Wood *et al*., 2002). The genome sequencing was completed by the Wellcome Trust Sanger Institute (www.sanger.ac.uk). The total number of the genes in *S. pombe* is substantially lower than the number found in *S. cerevisiae* (Wood *et al*., 2002). This fact, combined with the fact that *S. pombe* is farther evolved than *S. cerevisiae*, implies that the genes in *S. pombe* are more efficiently used. Yet MPR1, the only HPt protein found in *S. pombe*, contains additional sequence at its N-terminus that is not homologous to any known protein.

Although there is evidence that mammalian histidine kinases exist as a remnant of mammalian evolution from bacterial origins (Besant *et al*., 2005), no such genes that resemble HPts and RRs in His-Asp signal pathways have been found in animals. This fact makes His-Asp phosphorelay systems potential therapeutic targets for drug development.

1.7. Research focus

The two-component signal transduction system in *S. cerevisiae* has been studied quite thoroughly so far, but the one in *S. pombe*, whose host is closer to higher eukaryotes, is less characterized. Many questions remain to be solved. What is the possible function of the N-terminal portion of the HPt protein MPR1 in the

phosphotransfer pathway? How is the phosphoryl group transferred in the pathway, starting from three histidine kinases, ending up with two response regulators? What are the functional differences between MPR1 and YPD1? How would the mutations on the binding surface of YPD1 affect the phosphotransfer to RRs *in vitro*, if the YPD1 is altered to be more like MPR1, and vice versa?

The presence of three histidine kinases (HK) in the His-Asp phosphotransfer pathway in *S. pombe* seems redundant when compared to the number of histidine kinases in *S. cerevisiae*, the less evolved species (there is only one HK). Having more than one HK may help distinguish interaction with RRs in the pathway under different stresses. The yeast-two hybrid method was used to detect interactions between the proteins (or domains) of the phosphorelay pathway in *S. pombe*. The HPt protein in *S. pombe* has an extra N-terminal portion which is not homologous to any known protein. To investigate the possible function of the N-terminal portion, MPR1 was truncated at the N-terminus and the function of the mutants was tested *in vivo* and *in vitro*. To further investigate the binding preference of YPD1 to RRs, some of the residues that are putatively responsible for binding specificity were mutated to the corresponding ones on MPR1. The mutants were expressed and their phosphotransfer activity was tested *in vitro*.

2. Functional Characterization of the Phosphorelay Protein MPR1 from *S. pombe*

2.1. Introduction

The multi-step phosphorelay system in the fission yeast *Schizosaccharomyces pombe* is primarily involved in regulating cellular responses to oxidative stress (Nguyen *et al.*, 2000; Buck *et al.*, 2001), but also is implicated in regulation of the cell cycle (Shieh *et al.*, 1997; Aoyama *et al.*, 2001). Many of these components have homologous counterparts within the HOG1 MAPK pathway of *S. cerevisiae*. In *S. pombe,* however, there are three hybrid histidine kinases (MAK1, MAK2 and MAK3) (Aoyama *et al.*, 2001; Buck *et al.*, 2001), and two response regulator proteins, MCS4 and PRR1, that are homologous to the *S. cerevisiae* SSK1 and SKN7 response regulators, respectively (Cottarel, 1997; Shieh *et al.*, 1997; Shiozaki *et al.*, 1997). MPR1 is highly homologous to the YPD1 protein in budding yeast. Deletion of the *MPR1* gene in *S. pombe* causes severe impairment of STY1 activation (Nguyen *et al.*, 2000). MPR1 together with MCS4 has been implicated as a regulator of the G_2/M cell cycle transition during cell division, because MPR1-deficient cells are precocious in entering M phase (Aoyama *et al.*, 2000). The C-terminal 135 residues of MPR1 is ~42% identical to the full-length YPD1 sequence, including the putative site of phosphorylation (His221) (Nguyen *et al.*,

2000) (Figure 1-7). However, the N-terminal 160 residues of MPR1 have no apparent sequence homology to any other known protein.

Expression of the full-length *MPR1* gene in *S. cerevisiae* can complement a *ypd1*Δ strain and rescue cell lethality (Aoyama *et al.*, 2000). However, unlike the *ypd1*Δ lethal phenotype in *S. cerevisiae,* the *MPR1* gene is not essential for cell viability under standard growth conditions (Aoyama *et al.*, 2000). The downstream target of MPR1 is thought to be the response regulator MCS4. Two kinds of *in vivo* studies suggest that MPR1 interacts with MCS4. One is the yeast two-hybrid analysis (Aoyama *et al.*, 2000), the other is co-immunoprecipitation in which MCS4 was copurified with MPR1 (Nguyen *et al.*, 2000).

HPt proteins (or HPt domains) are generally composed of about 170 amino acids but share weak overall sequence similarity. However, their three-dimensional structures are highly conserved (Madhusudan *et al.*, 1996; Kato *et al.*, 1997; Xu & West, 1999; Mourey *et al.*, 2001; Sugawara *et al.*, 2006), all composed of a four-helix bundle core with the histidine phosphorylation site located in the middle of the helical bundle in a solvent-exposed position.

Among the many HPt genes identified from 20 different fungal genomes, only the *S. pombe MPR1* gene appears to encode a multidomain protein (295 amino acids) (Krantz *et al.*, 2006). The high degree of sequence similarity between YPD1 and the C-terminal region of MPR1 suggested to us that the MPR1 C-terminal domain alone may be able to function as an HPt protein *in vivo* and in an *in vitro* reconstituted phosphorelay assay. We show here that N-terminal truncation mutants of MPR1 (MPR1ΔN) are indeed able to complement an *S. cerevisiae ypd1*Δ strain. An obvious

question arose: what function does the N-terminal domain perform? Here we present evidence that the N-terminal domain of MPR1 enhances protein-protein interactions and consequently phosphotransfer between MPR1 and its upstream and downstream response regulator domains.

2.2. Materials and methods

2.2.1 Materials

All chemicals (including water) used were of ultrapure grade. Gateway cloning kit, restriction enzymes and T4 DNA ligase were purchased from Invitrogen. Pfu, Pfu Turbo and Pfx DNA polymerases were obtained from Stratagene. Culture media for bacterial and yeast growth was bought from Difco. Low-melting agarose was purchased from Cambrex Bio Sciences. Plasmid DNA was purified using QIAprep® spin Miniprep kit purchased from QIAGEN Inc. Plasmid DNA samples were sequenced by Microgen in the University of Oklahoma Health Sciences Center to confirm designed mutations. Chitin beads used to purify protein was from New England Biolabs. Chelating Sepharose™ Fast Flow was from Amersham Pharmacia Biotech. 30% Acrylamide/Bis used in SDS gel was purchased from Bio-Rad. TEMED was from Amersham Pharmacia Biotech. 5-Fluoroorotic acid (5FOA) was obtained from Sigma-Aldrich. IPTG (Isopropyl-β-D-thiogalactoside) was from Gold Biotechnology. [γ-^{32}P]-ATP was from Amersham Pharmacia Biotech. CPRG (Chloro-phenol-red-β-D-galactopyranoside) and ONPG (O-nitrophenyl-β-D-galactopyranoside) was purchased from Roche. Anti-Gal4 activation domain antibody was from Sigma-Aldrich. Fluorescent ECF substrate was purchased from Amersham Pharmacia Biotech. The rabbit anti-MPR1 antibody was

produced by Cocalico Biologicals Inc. All of the *E.coli* expression hosts were from Invitrogen.

2.2.2 Construction of deletion mutants of MPR1

Wild-type *MPR1* and *YPD1* genes were subcloned into the pRS315 low copy shuttle vector (a kind gift from Dr. Jan Fassler, University of Iowa). The resulting plasmid pRS315-*MPR1* (OU plasmid No. 198, abbreviated as OU pl#198) served as a template for making six of the seven N-terminal deletion constructs (ΔN36, ΔN70, ΔN101, ΔN120, ΔN145 and ΔN160) using the QuikChange insertion/deletion mutagenesis strategy (Stratagene). The resulting OU pl# are 253, 254, 255, 256, 259, 257, respectively. The primer pairs used in PCR were two complementary oligonucleotides designed to remove the desired gene segment (listed in Table 2-1). For the PCR reactions, the denaturation temperature was set to 95°C for 50 seconds, the annealing temperature was 50°C for 50 seconds, and the extension temperature was 68°C for 14 minutes. Dpn I (10 units) was used to digest methylated parental DNA while retaining the newly synthesized DNA. After transformation into *E. coli* DH5α cells and overnight culture, plasmid DNA was isolated and subjected to DNA sequencing (University of Oklahoma Health Sciences Center Laboratory for Genomics and Bioinformatics) to confirm the deletion mutations. For the MPR1ΔN167 construct (OU pl#258), the primer pairs were designed to amplify the gene segment encoding residues 168-296 with unique flanking restriction enzyme sites to facilitate subcloning of the amplified fragment into the pRS315 vector.

MPR1 construct	deletion	Oligonucleotide sequence
AW289	ΔN36 fprimer	5'-CACACAATAATGTCTACTCACGATTCTGGCATAGCTGAACG-3'
AW290	ΔN36 rprimer	5'-CGTTCAGCTATGCCAGAATCGTGAGTAGACATTATTGTGTG-3'
AW406	ΔN70 fprimer	5'-CACACAATAATGTCTACTCCACTGACCTCAAATCCTGCTTATG-3'
AW407	ΔN70 rprimer	5'-CATAAGCAGGATTTGAGGTCAGTGGAGTAGACATTATTGTGTG-3'
AW291	ΔN101 fprimer	5'-CACACAATAATGTCTACTGCTGGCGCACCTGAAGGCACGGAGG-3'
AW292	ΔN101 rprimer	5'-CTCCGTGCCTTCAGGTGCGCCAGCAGTAGACATTATTGTGTG-3'
AW408	ΔN120 fprimer	5'-CACACAATAATGTCTACTGACCATGCGAATGCCAAAAATC-3'
AW409	ΔN120 rprimer	5'-GATTTTTGGCATTCGCATGGTCAGTAGACATTATTGTGTG-3'
AW293	ΔN145 fprimer	5'-CACACAATAATGTCTACTCTTTCAGTAATTGCTTACGATATG-3'
AW294	ΔN145 rprimer	5'-CATATCGTAAGCAATTACTGAAAGAGTAGACATTATTGTGTG-3'
AW410	ΔN160 fprimer	5'-CACACAATAATGTCTACTCAAGACAAACAGCTCATTGATC-3'
AW411	ΔN160 rprimer	5'-GATCAATGAGCTGTTTGTCTTGAGTAGACATTATTGTGTG-3'
AW428	ΔN167 fprimer	5'-CAAGACAAACAGATGTCTACTCATTCCGTTTTTGACCAGTTG-3'
AW429	ΔN167 rprimer	5'-CCCCGATATCTTATGTAGAAGAATTTTTTTCATAAAAG-3'

Table 2-1. Oligonucleotide primer pairs used for PCR-mediated deletion mutagenesis. For each primer, an AW number was designated to it. Fprimer means the primer is forward, rprimer means the primer is reverse.

2.2.3 Protein expression and purification

The *MPR1* gene cloned in a pCR2.1 vector was a kind gift from Dr. Kazuhiro Shiozaki (University of California, Davis) (OU pl#94). The *MPR1* gene was then sub-cloned into the pDEST17 expression vector using the Gateway cloning reagents and recommended procedures (Invitrogen). The plasmid pDEST17-*MPR1* (OU pl#115) was transformed into BL21(SI) (Invitrogen) cells (OU strain 243) in order to express the His_6-tagged MPR1 protein in *E. coli*. Protein expression was induced with 0.2 M NaCl at 16°C for 20 hours. A 4 mL Ni^{2+} resin column (Chelating Sepharose Fast Flow, Amersham Pharmacia Biotech) was used to purify the His_6-MPR1 protein generated

from 1 L cell culture. The pDEST17-*MPR1* plasmid was truncated to pDEST17-*MPR1ΔN160* using QuikChange mutagenesis (OU pl#276). Purification of His_6-MPR1ΔN160 in BL21(DE3)plysS cell (Invitrogen) (OU strain 345) was similar to that of His_6-MPR1.

The PCR product encoding the C-terminal RR domain of MCS4 (MCS4-R4, residues Glu345-Arg523) was generated using *S. pombe* genomic DNA as a template. The *MCS4-R4* gene fragment was cloned into a pET16b expression vector with Nde I and Bam HI restriction sites (OU pl#274), and the resultant protein, His_{10}-MCS4-R4, was expressed in BL21(DE3)pLysS cells (Invitrogen) (OU strain 351). Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM to induce expression of His_{10}-MCS4-R4 at 16°C for 6 hours.

The *PRR1-RR* gene fragment was cloned into a pET21a vector (using Nde I and Xho I sites, OU pl#287) in order to express a C-terminal His-tagged protein, PRR1-RR-His_6 (residues Lys365-Lys540). Expression of PRR1-RR-His6 was in BL21(DE3)Star RIL cells (Invitrogen) (OU strain 368) with 0.6 mM IPTG at 16°C for 20 hours. Similarly, the RR domain of MAK2 (residues Ser1695-Arg2022) was cloned into a pET21a vector (using Nhe I and Xho I sites, OU pl#344) and the resultant protein, MAK2-RR-His_6, was expressed in BL21(DE3)Star cells (Invitrogen) (OU strain 435) and induced with 0.4 mM IPTG at 16°C for 22 hours. All His-tagged proteins were purified using Ni^{2+}resin column chromatography. The His_{10}-MCS4-R4 protein purification required an additional gel filtration (Sephadex G50) step. The proteins were buffer exchanged to final buffer (20 mM Tris-HCl, pH8.5 for MAK2-RR-His_6, pH 7.0 for other proteins, 10% Glycerol, 100 mM NaCl, 1 mM βME) to get rid of imidazole.

The *S. cerevisiae* phosphorelay proteins, YPD1, GST-SLN1-HK, SLN1-R1, SSK1-R2, SKN7-R3, were purified following methods described previously in our laboratory (Janiak-Spens & West, 2000).

2.2.4 *In vitro* phosphorylation assay

GST-tagged SLN1-HK was purified bound to glutathione-sepharose 4B resin (Amersham Pharmacia Biotech AB) as described previously (Janiak-Spens & West, 2000). After buffer exchange to a reaction buffer [50 mM Tris pH 8.0, 100 mM KCl, 15 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 20% glycerol], 7 μM [γ-^{32}P]-ATP (Amersham, 8.3 μCi/μL) and 2.5 μM SLN1-R1 (or MAK2-RR-His_6) were added into the 7 μM GST-SLN1-HK solution. After incubating at room temperature for 1 hour, phospho-SLN1-R1 (or phospho-MAK2-RR- His_6) was separated from the GST-SLN1-HK-bound glutathione-sepharose resin by gentle centrifugation. Phospho-SLN1-R1 was then added to tubes containing an equal molar amount of an HPt protein (YPD1, MPR1, or MPR1ΔN160) to react at room temperature for a designated amount of time. Purified RR domains (SSK1-R2, SKN7-R3, His_{10}-MCS4-R4, PRR1-RR-His_6) were then added (0.6 μM). Aliquots were removed at designated time intervals and the reactions quenched by the addition of 4X Stop buffer (250 mM Tris pH6.8, 40% glycerol, 8% SDS, 50 mM EDTA). The samples were then applied to a 15% SDS polyacrylamide gel and electrophoresed at 200 V for 45 minutes. The SDS gels were wrapped in plastic wrap and exposed to a phosphorimager screen. The radioactivity of each band was quantified using a Storm 840 phosphorimager (Molecular Dynamics).

2.2.5 *In vivo* complementation assay

The *S. cerevisiae* strain JF2153 (Matα, can^R, cyh^R, his3Δ200, leu2Δ1, ura3-52, trp1Δ63, lys2Δ202, *ypd1*Δkan, 2μ-*PTP2*(*URA3*$^+$)), a kind gift from Dr. Jan Fassler (University of Iowa), was streaked onto a YPAD plate (1% Bacto-yeast extract, 2% Bacto-peptone, 0.01% adenine sulfate, 2% dextrose, 2% agar**)**. Colonies were picked from the plate and used to inoculate 5 mL synthetic complete (SC)-Ura (uracil) media in a 50 mL flask. After overnight shaking at 30°C, about 1 mL culture was added to 95 mL SC–Ura liquid media in a 500 mL flask at an initial optical density at 600 nm (A_{600}) of ~0.1. After shaking at 30°C for ~6 hours (A_{600} ~0.5), cells were collected by centrifugation and then washed with 40 mL autoclaved distilled water, followed by 10 mL 1X TE/LiAc solution (10 mM Tris-HCl pH7.5, 10 mM EDTA, 100 mM lithium acetate). The cell pellet was resuspended in 300 μL 1X TE/LiAC solution. For each transformation, 50 μL cells, 5 μL freshly boiled carrier DNA (sonicated salmon sperm DNA, 10 mg/ml) and 100 ng each plasmid DNA were combined in an autoclaved 1.5 mL microcentrifuge tube. 300 μL PEG/LiAc solution (1X TE/LiAc, 40% PEG-3350) was added and gently mixed with the solution. The mixtures were incubated for 30 minutes in a 30°C water bath, followed by 15 minutes in a 42°C water bath. Then the reaction mixtures were buffer exchanged with 500 μL autoclaved, distilled water and plated onto the SC-Ura-Leu (leucine) plates. The plates were incubated for 48-72 hours at 30°C. Three colonies were picked from each plate and streaked onto SC–Leu plates to promote loss of the 2μ-*PTP2(URA*$^+$*)* plasmid. Again the plates were incubated at 30°C for 24-48 hours. Overnight culture in SC–Leu liquid media was prepared by picking colonies into liquid media and shaking at 30°C. Equal amount of cells were plated on

SC–Leu+5-FOA (5-fluoroorotic acid) plates (with different concentration of 5-FOA: 0.05%, 0.1%, 0.2% v/v), undiluted or with 1:10 serial dilutions. After incubating at 30°C for 48 hours, digital pictures of the plates were taken. The assay was repeated three times.

2.2.6 Yeast two-hybrid assay

Genes of full-length proteins or individual functional domains were cloned into the two yeast two-hybrid vectors: pDB (Leu^+) that produces DNA-binding domain (BD) fusion proteins, pPC86 (Trp^+) that produces activation domain (AD) fusion proteins (ProQuest Yeast Two-Hybrid System, Invitrogen). The genes were: *MPR1, MPR1ΔN160, MPR1ΔC135, MAK1~3-RR* (RR domains of Mak1~3p), *MCS4, MCS4-R4* (RR domain of MCS4), *PRR1-RR* (RR domain of PRR1). Plasmids (pPC86-MCS4-R4 OU $pl^{\#}308$, pDBleu-MPR1 OU $pl^{\#}309$, pDBleu-MPR1ΔC135 OU $pl^{\#}312$, pDBleu-PRR1-RR OU $pl^{\#}320$, pPC86-MAK1-RR OU $pl^{\#}325$, pPC86- MAK2-RR OU $pl^{\#}326$, pPC86- MAK3-RR OU $pl^{\#}327$, pPC86-PRR1-RR OU $pl^{\#}328$, pDBleu-MPR1ΔN160 OU $pl^{\#}330$, pPC86-MCS4 OU $pl^{\#}331$) (with one gene in pDBleu vector and another gene in pPC86 vector) were co-transformed into the host strain MaV203 (*MAT*α, *leu*2-3, 112, *trp*1-901, *his3*Δ200, *ade*2-101, *gal*4Δ, *gal*80Δ, *SPAL*10::*URA*3, *GAL*1::l*acZ*, $HIS3_{UAS\ GAL1}$::*HIS*3@*LYS*2, $can1^R$, $cyh2^R$), using the PEG-LiAc method described in complementation assay. Controls were one plasmid alone or one plasmid with the other vector. Transformation mixtures were plated onto SC selective medium agar plates, depending on the plasmid(s) transformed. Three cultures were prepared from three separate colonies for each transformation in selective media, either SC-Leu, or SC-Trp,

or SC-Leu,-Trp. The cultures were shaken at 30°C overnight. One mL of such overnight culture was added to 4 mL of YPAD liquid media (1% yeast extract, 2% peptone, 0.01% adenine sulfate, 2% dextrose, pH 6.0), and shaken at 30°C until an optical density at 600 nm (OD_{600}) reached 1.0. Cells were harvested into 1.5 mL Eppendorf tubes with l mL into each tube, 3 tubes per inoculants. Thus each colony was assayed in triplicate. Cells were pelleted in a microcentrifuge, 8000 g x 1 minute and were washed twice with 1.0 ml assay buffer (100 mM HEPES, 150 mM NaCl, 5 mM L-aspartate, 1% bovine serum albumin, 0.05% Tween 20, pH7.0). Dry cell pellets were frozen in a -80°C freezer overnight and were resuspended in 100 μL assay buffer the next day. Cells were lysed by six rounds of immersion in liquid nitrogen and incubation in 37°C water bath. CPRG (2 mM) (chlorophenol-red-β-D-galactopyranoside in 900 μl assay buffer) was added to the cell lysate to start the reaction. The reaction mixture was incubated at room temperature overnight, then quenched by adding 250 μL 6 mM $ZnCl_2$. Cellular debris was removed by centrifugation, 8000 g x 1 minute, and the OD_{574} value of the supernatant was measured. β-galactosidase units were calculated using the equation β-gal unit = 1000 x OD_{574}/ (t x V x OD_{600}), where t is time in minutes and V is the volume of the initial aliquot.

2.2.7 Western blot analysis

Cells were handled in the same way as in the β-galactosidase assay until right after the freeze/thaw cycles. A parallel culture was set aside for Western blot analysis. Glycerol was added to 10% final concentration as a cryopreservative to store the sample at –20°C. Total cell protein concentration was measured using BioRad's Bradford

assay. Gel loading buffer (4X: 250 mM Tris pH6.8, 40% glycerol, 8% SDS) was mixed with the samples. The same amount of protein per lane was loaded on 10% or 15% SDS polyacrylamide gels and electrophoresed at 200 V for 40 minutes. The gel was then electroblotted onto polyvinylidene difluoride (PVDF) membranes in transfer buffer (25mM Tris pH8.3, 192 mM glycine) at 35 V for 30 minutes and then at 25 V for 1 hour. After washing the PVDF membranes with blocking buffer (20 mM Tris, 500mM NaCl, 0.1% Tween 20, 5% dry milk) for 1 hour, GAL4 activation domain (AD) antibody (Sigma, 1:2000 dilution, in 5% milk) or anti-MPR1 antibody (custom generated at Cocalico Biologicals Inc., 1:3000 dilution) was added to probe the membranes at 4°C overnight while gently shaking. An alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Sigma, 1:3000 dilution, in 5% milk) was added to probe the 1st antibody at room temperature for 1 hour. TTBS (20 mM Tris, 500 mM NaCl, 0.1% Tween 20) buffer was used to wash the membranes between the two antibodies. Fluorescent ECF substrate (Amersham Biosciences) was added to the membranes and allowed to react for 5 minutes. After being dried, the membranes were analyzed using a Storm 840 phosphorimager (Molecular Dynamics).

2.3 Results

2.3.1 Genetic complementation of a *ypd1*Δ *S. cerevisiae* strain

The sequence of MPR1 was aligned with other representative HPt proteins, including YPD1 from *S. cerevisiae*, YPD1 from *Candida albicans*, MPR1 from *S. pombe*, ZmHP2 from the plant *Zea mays*, and the C-terminal 125 residues of ArcB from *E. coli* (ArcB^{C}, the HPt domain of ArcB sensor kinase) using the program Clustal X

(Jeanmougin *et al.*, 1998). The secondary structure of the C-terminal domain of MPR1 was predicted using the web-based program 123D (http://123d.ncifcrf.gov) (Alexandrov *et al.*, 1996). This information combined with the known secondary structure of YPD1 from *S. cerevisiae* (Xu & West, 1999), enabled us to produce a structure-based sequence alignment shown in Figure 2-1. The crystal structure of YPD1 from *S. cerevisiae* (Song *et al.*, 1999; Xu & West, 1999) resembles that of ZmHP2 (Sugawara *et al.*, 2006) and $ArcB^C$ (Kato *et al.*, 1997), all having a four-helix bundle as the core of the protein. The secondary structure prediction of MPR1 indicates that the C-terminal region also has a helical rich composition, with each helix aligning very well with those of YPD1 from *S. cerevisiae* (Figure 2-1). While the C-terminal 135 residues of MPR1 show the highest sequence homology to the other two fungal HPt proteins (41 and 42% identity to YPD1 from *S. cerevisiae* and *C. albicans*, respectively), there appears to be a high degree of structural conservation among all five of these HPt proteins from diverse organisms. Several amino acid residues around the active site histidine, His64 in ScYPD1 and His221 in MPR1, are completely conserved (highlighted in red font in Figure 2-1). The most significant difference is that YPD1 has a long linker region between the D and G helices (residues 89-132), whereas neither ZmHP2, $ArcB^C$ nor MPR1 has such a linker region.

The 123D program further predicts both α and β secondary structure in the N-terminal region of MPR1. A nested set of N-terminal truncation mutations of the *MPR1* gene was created based on the sequence alignment of HPt proteins and protein secondary structure prediction of MPR1 as presented in Figure 2-1. The resultant deletion mutants were named according to the number of N-terminal residues removed: ΔN36, ΔN70,

ΔN101, ΔN120, ΔN145, ΔN160, ΔN167. The gene fragments for wild type *MPR1* and the mutant genes were cloned into pRS315, a low copy yeast shuttle vector. As a control, the wild type *YPD1* gene was also subcloned into the same pRS315 vector. The plasmids were then transformed into the *S. cerevisiae* strain JF2153, a *ypd1*Δ strain that contains a *2μ*-plasmid that overexpresses PTP2, a HOG1-specific protein tyrosine phosphatase. Deletion of *YPD1* is lethal to *S. cerevisiae* cells, due to constitutive tyrosine phosphorylation and activation of the HOG1 MAPK and resultant overproduction of intracellular glycerol. Overexpression of the *PTP2* gene in the *ypd1*Δ strain can restore cell viability. In our complementation assay, the 2μ-*PTP2* plasmid will be lost due to negative selection with 5-FOA, and YPD1 function must therefore be compensated for in order for cells to survive.

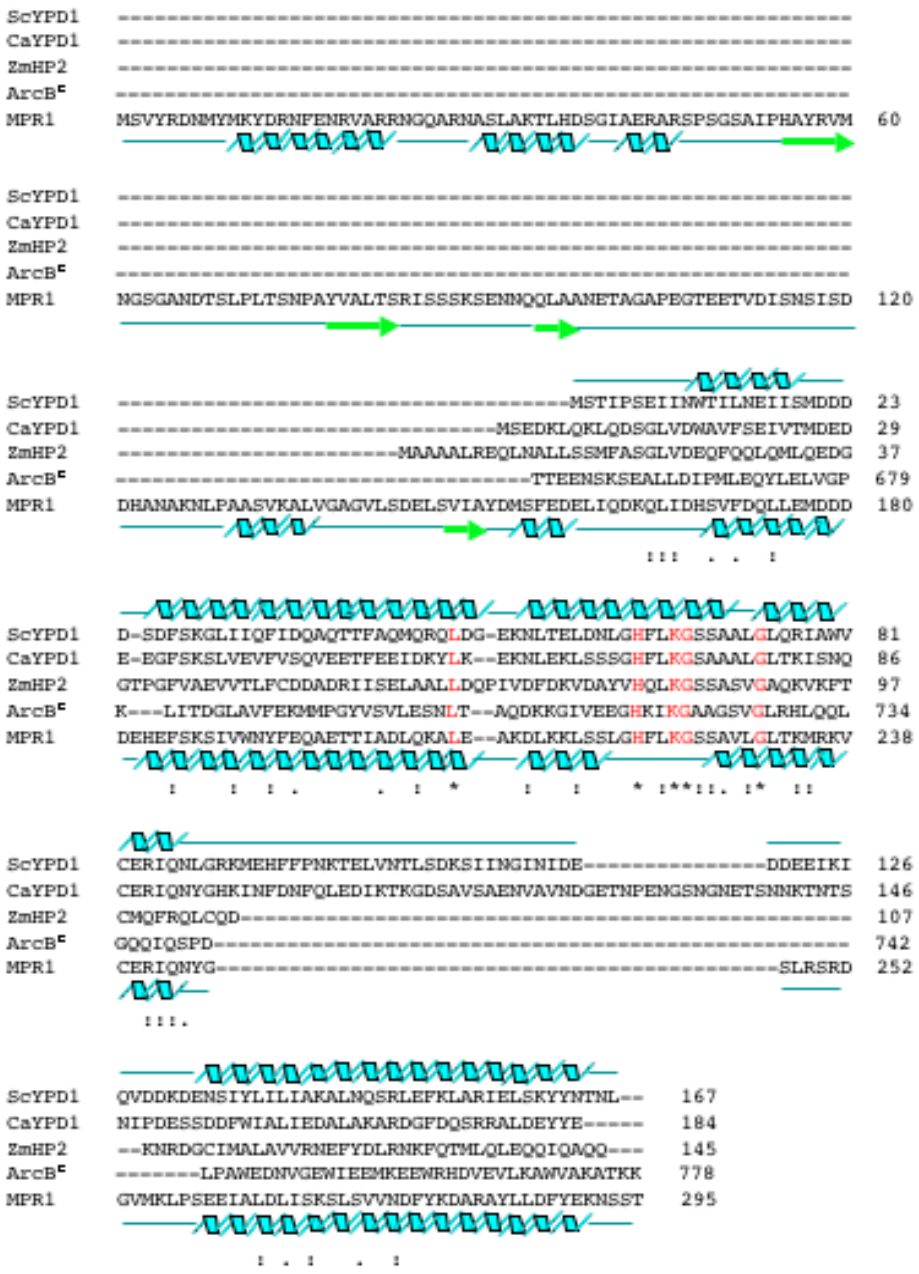

Figure 2-1. Structure-based sequence alignment of HPt proteins. Amino acid sequences of full-length MPR1 from *S. pombe*, YPD1 from *S. cerevisiae* (ScYPD1), YPD1 from *Candida albicans* (CaYPD1), ZmHP2 from *Zea mays*, and the C-terminal domain of the *E. coli* ArcB sensor kinase ($ArcB^C$) were aligned using Clustal X (Jeanmougin et al., 1998). The secondary structure of the known crystal structure of ScYPD1 (above) and the predicted secondary structure of MPR1 (below) are also shown with α-helices in blue and β-strands in green. The completely conserved residues among the five proteins are highlighted in red font and indicated below with an asterisk. Other highly conserved (:) or less conserved (.) residues are also indicated below the sequence alignment.

As shown in Figure 2-2, a pRS315 plasmid that contains either the wild type *YPD1* or *MPR1* gene can rescue *ypd1*Δ *S. cerevisiae* cells, indicating that expression of the full-length *MPR1* gene can compensate for loss of YPD1 *in vivo* (this is consistent with data reported by the Mizuno group (Aoyama *et al.*, 2000)). Most of the *MPR1* N-terminal deletion mutants were also able to complement the *ypd1*Δ strain except for MPR1ΔN70 and MPR1ΔN167. Although cells that express the MPR1ΔN70 mutant partially survived on 0.05% and 0.1% 5FOA media, they were not viable on 0.2% 5FOA plates (Figure 2-3). Western blot analysis showed comparable levels of expression and appropriate molecular sizes for the ΔN36, ΔN101, ΔN120, ΔN145 and ΔN160 expressed mutant proteins (Figure 2-4). However, bands for the ΔN70 and ΔN167 were not visible, indicating that these two truncated proteins may be poorly expressed and/or structurally unstable *in vivo*. Although larger deletions were expressed, ΔN70 may have cut right through a helix or a β-sheet. The damaged protein could be digested by the Ubiquitin-proteasome system inside cells shortly after expression. Cells containing MPR1ΔN101 exhibited lower survival than cells containing larger deletions (e.g. ΔN120 and ΔN145) even though the expressed protein could be detected by Western blotting. This suggests that residues 101-120 of the MPR1 protein are required for full HPt function. MPR1ΔN160 is the largest truncation construct that still retains HPt function according to the complementation assay. Overall, these results demonstrate that the C-terminal 135 amino acids of MPR1 can function as an HPt protein and can rescue a *S. cerevisiae ypd1*Δ strain.

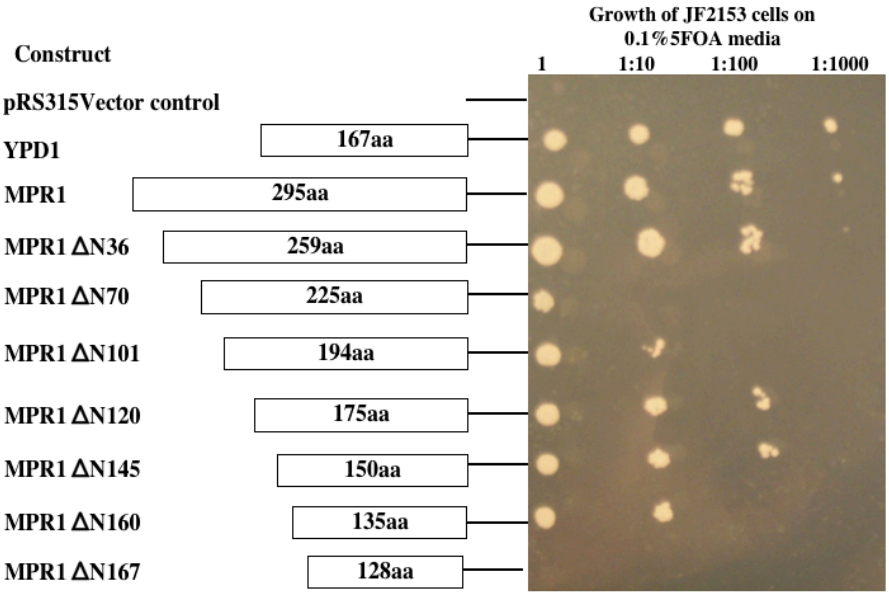

Figure 2-2. *In vivo* complementation assay. *S. cerevisiae ypd1*Δ cells (JF2153) that contain indicated plasmids were plated on selective media (SC –Leu + 0.1% 5FOA). The N-terminal truncations of MPR1 are named based on the number of residues removed (ΔN#) or indicated by the number of C-terminal residues remaining (boxes). Undiluted and serial diluted cells were plated after plasmid transformation. pRS315 vector control is the negative control, while YPD1 is the positive control.

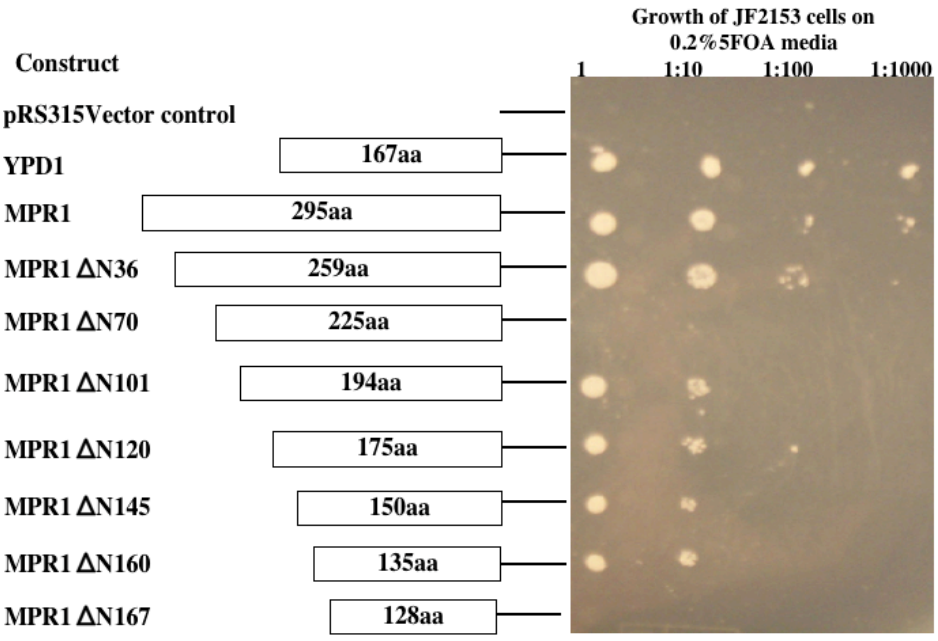

Figure 2-3. *In vivo* complementation assay (continued). The concentration of 5FOA was 0.2% comparing with that in Figure 2-2.

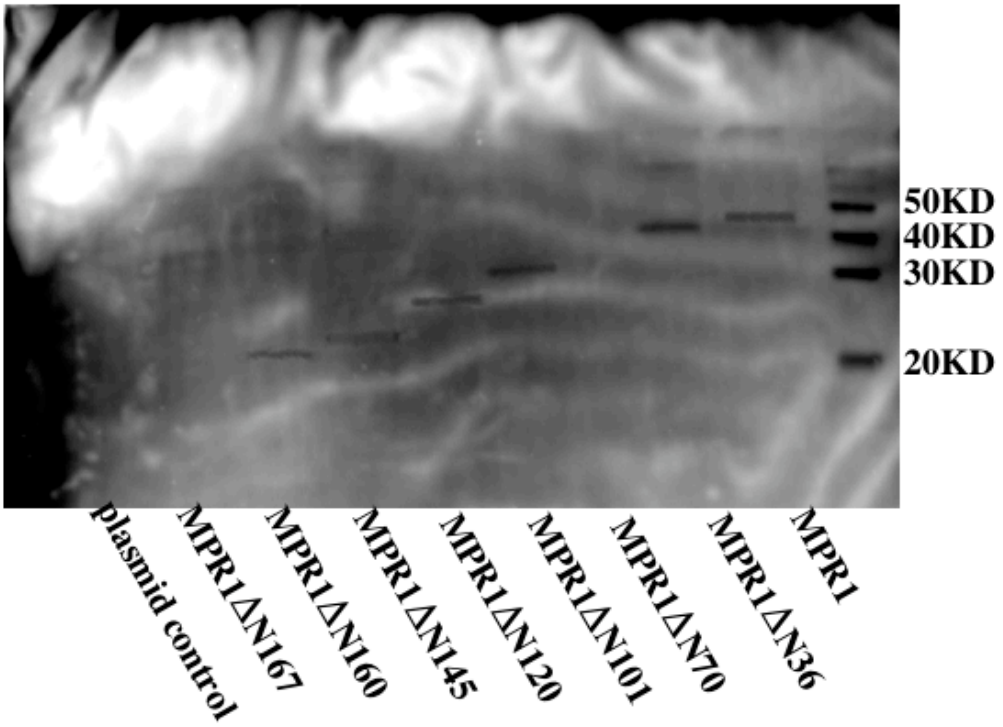

Figure 2-4. Western blot of the samples in complementation assay. Anti-MPR1 antibody from Cocalico Biologicals Inc. was used to detect the expressed MPR1 or MPR1 mutants. There was no band shown in plasmid control sample. Except MPR1ΔN70 and MPR1ΔN167 samples, the other samples had proteins detected by anti-MPR1 antibody, indicating that the two distorted proteins might be digested by proteases.

2.3.2 MPR1 and response regulator phosphotransfer specificity

To avoid cross-talk during signal transduction, HPt proteins need to transfer phosphoryl groups to the appropriate downstream RR in response to a specific stress. In *S. cerevisiae*, YPD1 transfers phosphoryl groups to two distinct RRs, SSK1 and SKN7. In *S. pombe*, there are also two RRs downstream of MPR1, MCS4 and PRR1. However, it is not known whether MPR1 exhibits preferred specificity towards one RR over the other.

Here we show, using an *in vitro* phosphotransfer competition assay in which both RRs are present in equal molar amounts, that YPD1 exhibits a marked preference for

phosphotransfer to SSK1 over SKN7 (Figure 2-5A), consistent with kinetic analysis in which a 100-fold faster rate of phosphotransfer between YPD1 and SSK1 was observed relative to SKN7 (Janiak-Spens *et al.*, 2005). Interestingly, if MPR1 is used in place of YPD1, we observe the opposite specificity, that is, MPR1 favors formation of phospho-SKN7-R3 over phospho-SSK1-R2 (Figure 2-5B). It is also worth noting that although SLN1-R1 can serve as a phosphodonor to MPR1, it does so with far less efficiency than with the cognate HPt protein YPD1. In control experiments lacking YPD1 or MPR1, direct phosphotransfer from one response regulator domain to another was not observed. It is tempting to speculate that the RR specificity exhibited by MPR1 is due to the involvement of SKN7 in oxidative stress in *S. cerevisiae*, which would be consistent with the role of MPR1 in oxidative stress responses in *S. pombe*. It seems more likely that molecular structure and surface characteristics of SKN7 resemble that of MCS4, the cognate RR for MPR1.

The phosphotransfer activity of the truncation mutant, MPR1ΔN160, was also compared with that of full-length MPR1 using the *in vitro* phosphorylation assay with *S. cerevisiae* components. Both full-length MPR1 (Figure 2-6A) and MPR1ΔN160 (Figure 2-6B) can receive phosphoryl groups from the *S. cerevisiae* phosphodonor SLN1-R1 *in vitro*. When the two RR domains SKN7-R3 and SSK1-R2 were included in the reaction, MPR1ΔN160 still favored phosphotransfer to SKN7-R3 over SSK1-R2 (Figure 2-6C).

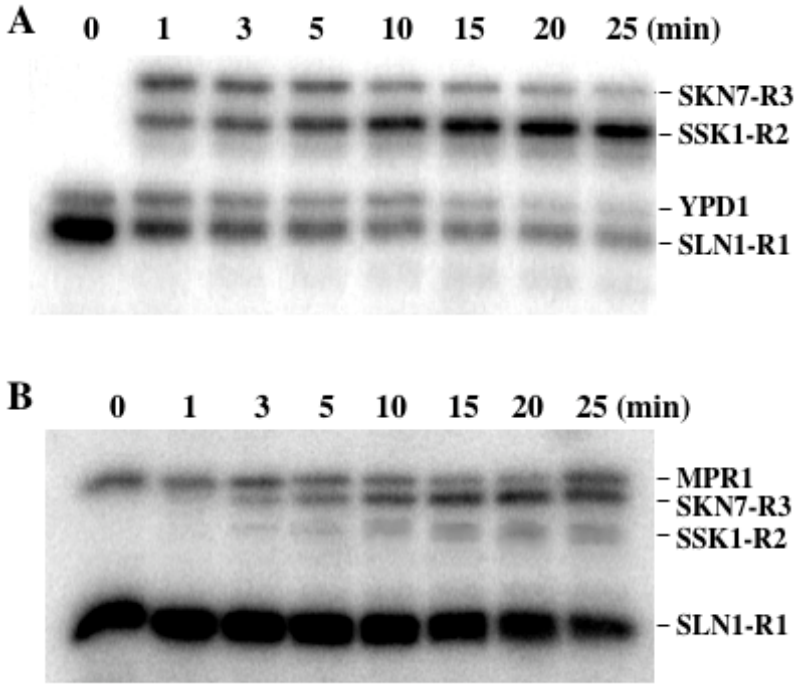

Figure 2-5. Comparison of YPD1 versus MPR1 with respect to response regulator phosphotransfer specificity. Phospho-SLN1-R1 was used as a phosphodonor to YPD1 (panel A) or Mpr1 (panel B). Both SSK1-R2 and SKN7-R3 response regulator domains were included in the reaction mixture in order to test for HPt-to-RR phosphotransfer specificity over the given time course.

Prey protein/domain	Bait protein	
	MPR1	MPR1ΔN160
MAK1-RR	±	–
MAK2-RR	–	–
MAK3-RR	+	±
MCS4	+++	++
MCS4-RR	++	+
PRR1-RR	±	–

Table 2-2. Comparative results of protein-protein interactions using a yeast two-hybrid assay. Control sets of interacting pairs provided with the ProQuest Yeast Two-Hybrid System (Invitrogen) were used to guage relative strengths of interaction as follows: +++ (moderate), ++ (moderate – weak), + (weak), ± (borderline), – (non-detectable). The cDNA for the weak interaction control was the human *RB* and *E2F1* genes, while the cDNA for the moderately strong interaction was the *Drosophila DP* and *E2F* genes.

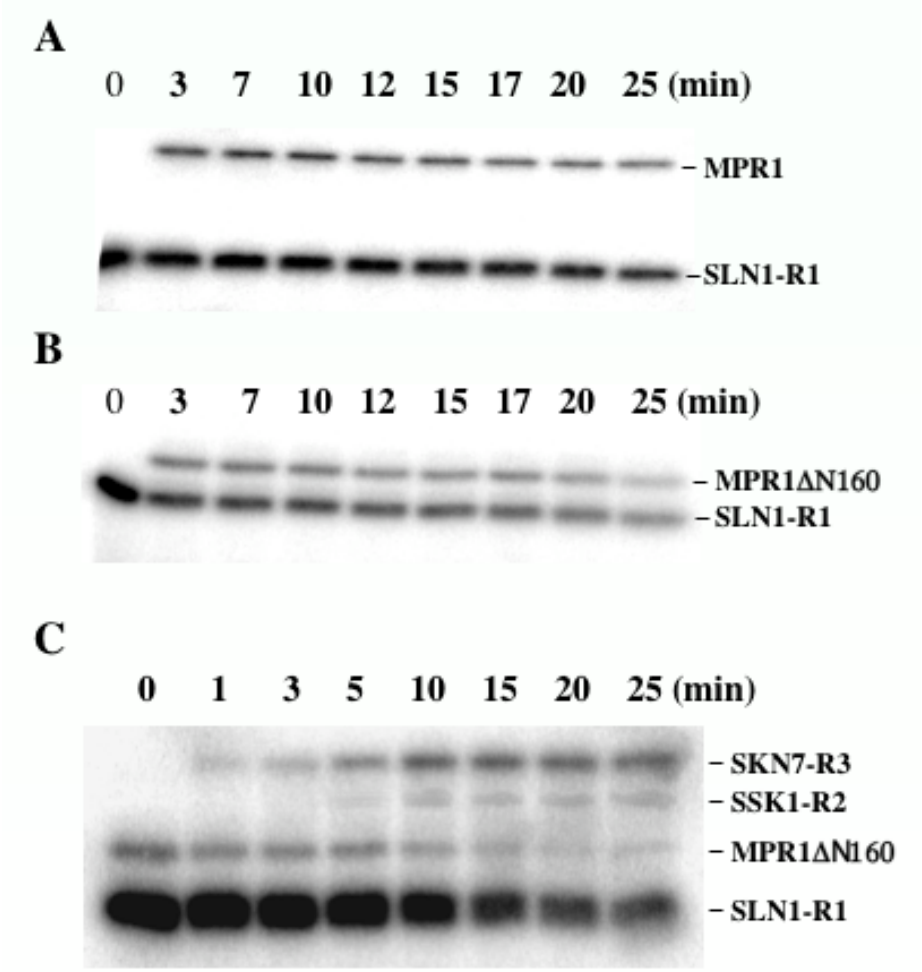

Figure 2-6. *In vitro* phosphotransfer activity of MPR1 versus MPR1ΔN160. The ability of Mpr1 (panel A) or MPR1ΔN160 (panel B) to be phosphorylated by phospho-SLN1-R1 is shown. Response regulator specificity was determined for the MPR1ΔN160 mutant (panel C) using the RR competition assay as shown in Figure 2-5.

2.3.3 The role of the N-terminal domain of MPR1 in protein-protein interactions

In the phosphorelay system in *S. pombe*, there are three hybrid HKs (MAK1, MAK2, MAK3) and two RRs (MCS4, PRR1) that possibly react to oxidative stress, with only one HPt protein (MPR1) functioning between these HKs and RRs. MPR1 has been shown to interact with and transfer phosphoryl groups to MCS4 (Aoyama *et al.*, 2000; Nguyen *et al.*, 2000; Aoyama *et al.*, 2001). Genetic studies also support the existence of

a phosphorelay system from MAK2 or MAK3 to MPR1 to MCS4 and possibly direct phosphotransfer from MAK1 to PRR1, without participation of MPR1 (Buck *et al.*, 2001). We have used a yeast two-hybrid assay to investigate all possible interactions between full-length MPR1 or the Mpr1ΔN160 mutant, the three RR domains from the three HKs, and the two RRs MCS4 and PRR1. Full-length *MPR1* or the gene fragment expressing MPR1ΔN160 were cloned into the pDBleu vector in order to express a "bait" fusion protein with the Gal4p DNA-binding domain, while gene fragments encoding the RR proteins/domains were cloned into the pPC86 vector in order to express a "prey" fusion protein with the Gal4p activation domain. Protein expression levels of both bait and prey fusion proteins were checked by Western blot analysis using antibodies to the Gal4 activation domain or MPR1 (See Figure 3-3, 4, 5 in Chapter 3). β-galactosidase reporter activity was measured and the comparative results are shown in Table 2-2. The strength of interaction was based on comparisons to the control sets of interacting pairs supplied with the ProQuest Yeast Two-Hybrid System (Invitrogen).

MPR1 showed strong interaction with full length MCS4, and a more moderate level of interaction with only the RR domain of MCS4 (MCS4-RR). MPR1 exhibited relatively low interaction with the MAK3-RR domain and barely detectable interaction with PRR1-RR and MAK1-RR. Interaction with MAK2-RR was undetectable. The results of the yeast two-hybrid assay using the truncation mutant MPR1ΔN160 were consistent with that of full-length MPR1 but at lower levels of interaction. An effort was made to delete the C-terminal domain of MPR1 and test the interaction between the cognate proteins (domains) with the N-terminal domain of MPR1. But when fused with the DNA binding domain of GAL4, the C-terminal domain of MPR1 displayed

interaction with the activation domain of GAL4 (i.e. false positive). The approach was not pursued further.

2.3.4 The role of the N-terminal domain of MPR1 in response regulator phosphotransfer specificity

In order to confirm our observations from the yeast two-hybrid interaction assay, we expressed and purified the RR domains from MCS4 and PRR1 and tested whether MPR1 could transfer phosphoryl groups to the RRs *in vitro*. Using the heterologous *in vitro* phosphotransfer assay with SLN1-R1 as the initial phosphodonor, we observe that MPR1 shuttles phosphoryl groups from SLN1-R1 to MCS4 (Figure 2-7A), but not PRR1 (Figure 2-7B). These results are consistent with genetic data that supports a MPR1-MCS4 phosphorelay system but not MPR1-PRR1 phosphotransfer (Buck *et al.*, 2001).

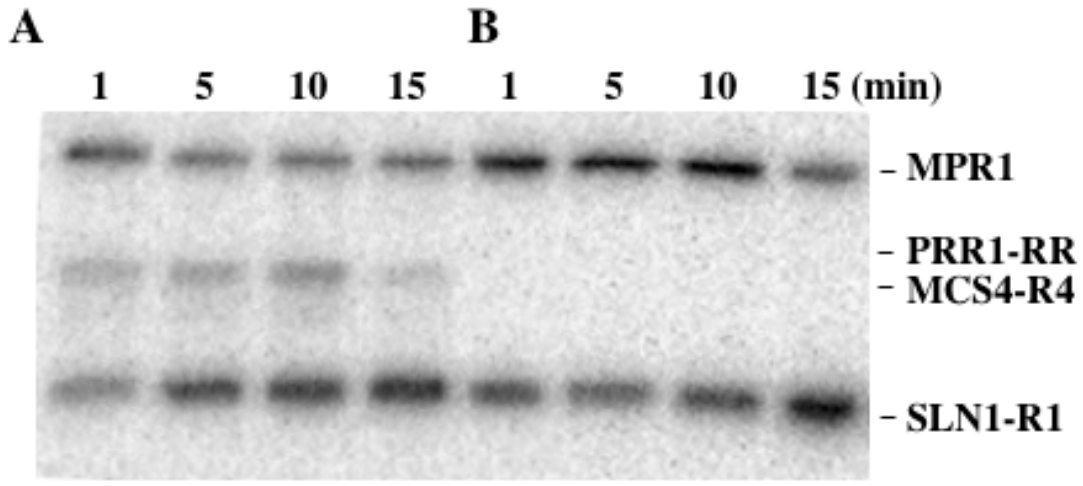

Figure 2-7. *In vitro* phosphotransfer from MPR1 to its *S. pombe* response regulator proteins. MPR1 was phosphorylated using *S. cerevisiae* SLN1-R1 as a phosphodonor and then the ability of MPR1 to shuttle phosphoryl groups to either the *S. pombe* MCS4 RR domain (panel A) or the PRR1 RR domain (panel B) was examined.

The yeast two-hybrid interaction data indicated a lack of interaction between the MAK2-RR domain and MPR1, however, this result would be in conflict with the genetic data of Buck *et al.,* which indicates that both MAK2 and MAK3 function upstream of MPR1 and MCS4 (Buck *et al.*, 2001).

In order to corroborate our yeast two-hybrid results, we used purified protein domains and tested them for phosphotransfer activity *in vitro*. The MAK1-RR, MAK2-RR and MAK3-RR domains were expressed in BL21(DE3)Star cells. GST-SLN1-HK was used to phosphorylate these three RR domains in the presence of [γ-^{32}P]-ATP. However, we only observed phosphorylation of the MAK2-RR (Figure 2-8) under the conditions described in Methods. To test whether the MAK2-RR can serve as an *in vitro* phosphodonor to MPR1, we incubated phospho-MAK2-RR with either full-length MPR1 or MPR1ΔN160. More efficient phosphotransfer is observed with the full-length construct (Figure 2-8A) than with the truncation mutant (Figure 2-8B), suggesting that the N-terminal region of MPR1 also plays a role in phosphotransfer. Thus, we conclude that the hybrid HK MAK2 is part of a multistep phosphorelay system with MPR1 as its immediate downstream phosphorelay partner. It is possible that interaction between MAK2-RR and MPR1 *in vivo* was not observed due to weak binding affinity or steric hinderance due to the two-hybrid fusion construct.

The yeast two-hybrid assay also indicated that MPR1 interacted weakly with the MAK3-RR domain. We were unable to confirm whether this is a productive interaction in terms of *in vitro* phosphotransfer due to difficulties in phosphorylating the MAK3-RR domain.

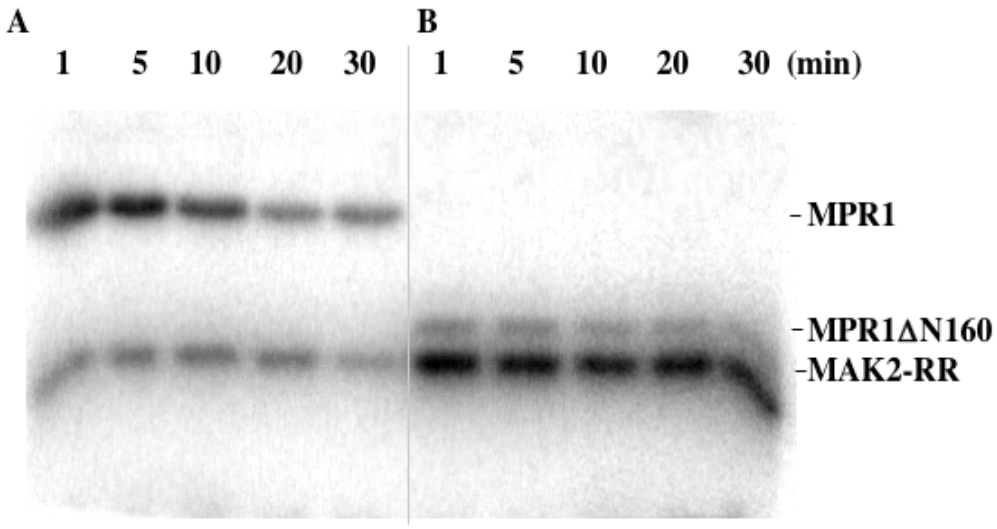

Figure 2-8. *In vitro* phosphotransfer from MAK2-RR to MPR1. The RR domain of the hybrid HK MAK2 was phosphorylated by phospho-SLN1-HK. The ability of full-length MPR1 (panel A) or MPR1ΔN160 (panel B) to receive phosphoryl groups from MAK2-RR was tested.

2.4 Discussion

HPt protein plays an important role in His-Asp phosphorelay pathways. Although only a small minority of prokaryotes employ a separate HPt protein in a phosphorelay (Freeman, *et al.,* 1999), HPt proteins are widely used in eukaryotic His-Asp muitiple phosphorelay pathways. HPt proteins function as a connection between RRs. Genomic sequencing program has revealed that many phosphorelay systems have redundant HKs and/or RRs (Thomason, *et al.,* 1999). For example, in *Arabidopsis thaliana*, there are at least 11 HKs and 16 RRs taking part in multiple interactions (West, *et al.,* 2001). But the number of HPt proteins found is far less than that of HKs or RRs in each organism that uses His-Asp phosphorelay. Most of them have only one HPt, some of them do not have any, only *Arabidopsis thaliana* has 5 HPts (reviewed by West, *et al.,* 2001). Although more HPt genes may be found with the completion of genomic program, it is unlikely

that their number will exceed that of HKs or RRs (Thomason, *et al.,* 1999). The HPt protein MPR1 in *S. pombe* has more than one domain. The C-terminal domain could receive phosphoryl groups from SLN1-R1 and transfer them to SSK1-R2/SKN7-R3 both *in vivo* and *in vitro*, showing that the C-domain of MPR1 could perform HPt protein function without the N-domain. Having a multi-domain HPt protein in the His-asp phosphotransfer pathway in *S. pombe* must have a purpose, which is different in comparison with the one domain HPt protein YPD1 in *S. cerevisiae*. One of the obvious differences between the pathways in *S. pombe* and *S. cerevisiae* is that there are three HKs in *S. pombe* pathway while there is only one HK in *S. cerevisiae* pathway. Our first hypothesis was that the additional domain of MPR1 could have something to do with distinguishing the three HKs upstream. This turned out not to be the case. Yeast two-hybrid results were consistent between MPR1 and MPR1ΔN160, but the level of interaction is weakened when the N-terminal domain is deleted. Deletion of the N-terminal domain also decreased the phosphotransfer from MAK2-RR to MPR1 *in vitro*. This suggests a role for the N-terminal region of MPR1 in enhancing or stabilizing protein-protein interactions. On the other hand, the N-terminal 160 residues of MPR1 may not be essential for RR phosphotransfer specificity, according to the *in vitro* phosphorylation assay results. MPR1 has been shown to react with MCS4 *in vitro* and *in vivo* (Aoyama *et al.,* 2000; Nguyen *et al.,* 2000), which agrees with *in vitro* and *in vivo* results in this research. MPR1 did not interact with PRR1-RR *in vivo*, neither could it transfer phosphoryl groups to PRR1-RR *in vitro*, which is consistent with the genetic deletion result that *MPR1* caused a mitosis change (Aoyama *et al.,* 2000), but *PRR1* caused meiosis disability (Greenall *et al.,* 2002).

By comparing the two-component systems in fungi and plants, we see a trend indicating that the N-terminal domain of MPR1 may have other functions other than discussed above. There is only one HK in *S. cerevisiae*, three HKs in *S. pombe*, and three in *C. albicans*, 8 HKs in *Arabidopsis* that contain all the conserved residues required for kinase activity (Schaller *et al*., 2002). *Arabidopsis* also contains proteins related to HK but lack essential residues for kinase activity (Schaller *et al*., 2002). These so called diverged histidine kinases have serine/threonine kinase activity instead of histidine kinase activity. The HPt protein (YPD1) in *S. cerevisiae* interacts with both of the two RRs downstream, while the HPt protein (MPR1) in *S. pombe* seems react with only one of the two RRs. There are 5 HPt proteins found in *Arabidopsis* that contain the conserved histidine residue, and a pseudo-HPt is also found that does not have the histidine phosphorylation site. Genomic research showed that 22 response regulators may exist in *Arabidopsis* that contain all the conserved residues for enzymatic activity, and 9 pseudo-response regulators may also exist that miss essential residues for activity. Although there are multiple HKs, HPts and RRs, not all of them participate in the two-component system. They may have other biological functions other than histidyl-aspartyl phosphorelay functions. It is possible that two-component signaling elements evolved to shift functions to non-histidine/aspartate-related ones (Schaller *et al*., 2002). From this point of view, we can suspect that the components in the two-component pathway in *S. pombe*, a species that is closer to animals than plants (Schaller *et al*., 2002), may perform other functions beside histidyl-aspartyl phosphorelay. Indeed, MCS4 is required for the activation of STY1 in response to many stresses beside oxidative stress, such as UV light, protein synthesis inhibitor anisomycin stress, and temperature shock, but these

stress-responses do not require phosphotransfer to MCS4 (Thomason *et al*., 2000; Shieh *et al*., 1997). And we cannot exclude the possibility that MPR1 may participate in other reactions that does not involve the C-terminal portion phosphorelay. The trend of His-Asp phosphorelay pathway seems to go this way: dominant in prokaryotes → not so dominant in low eukaryotes → absent in animals. The transition from "not dominant" to "absent in aninal" generated pseudo His-Asp phosphorelay components that are unlikely to function in this pathway. Take example of *Arabidopsis*, it contains pseudo-response regulators that have complete receiver domain but do not have the essential Aspartate residue for phosphorylation (Popov *et al*., 1993; Makino *et al*., 2000; Schaller *et al*., 2000). Similarly, the trend of change of HPt protein from prokaryotes to low eukaryotes to animals seems to be: exist as a domain in hybrid kinase → independent HPt protein with one domain → HPt protein with more than one domain → pseudo HPt → no HPt (no His-Asp phosphorelay). When HPt proteins change to pseudo HPt proteins, they must have obtained new function that no longer rely on phosphorylation of histidine. Then the appearance of multidomain HPt proteins before pseudo type of HPt protein could enable HPt proteins to act in both phosphorelay and nonphosphorelay systems. Or, the domains other than HPt domain of HPt proteins may play a role in interacting with other pathways that regulate similar stress conditions.

Another possibility about the role of the N-terminal domain of MPR1 is that it might be involved with the N-terminal domains of MAK2 and MAK3. The N-terminal domain of MPR1 are predicted to have the following site (Prosite): Casein kinase II phosphorylation site (residue35-38 TlhD; 88-91 SksE; 117-120 SisD; 154-157 SfeD) Protein kinase C phosphorylation site (residue 82 - 84 Tsr; 87-89 SsK; 132-134 SvK).

Both MAK2 and MAK3 have an atypical Serine/Threonine kinase domain at the end of the N-terminal of the proteins (Figure 2-9). This domain has homology to the serine/threonine kinases from prokaryotes like *Mycobacterium tuberculosis* PKNB, and a similar domain also exists in HK1 histidine kinase from Candida albicans. (Buck *et al*., 2000). Actually, the alignment result shows that the serine/threonine kinase domains from PKNB and MAK2 have 23% identity and about 21% highly conserved, while those from PKNB and MAK3 share 18% identity and 20% high similarity (Figure 2-10).

Protein phosphorylation forming a phosphoester bond (on residue serine, threonine or tyrosine) mainly happens in eukaryotes. Bacterial genomic sequence data and the use of antiphosphoprotein antibodies showed that some prokaryotes also utilize serine/threonine kinases (PSTKs) (Potts *et al*., 1993; Zhang *et al*., 1996). PSTKs have been shown to participate in two processes, cell development and pathogenicity. Although PKNB could phosphorylate MBP (myelin basic protein) (Av-Gay *et al*., 1999), a conventional in vitro substrate for serine/threonine kinases, little is known about the physiological substrates. GarA was found to be a putative physiological substrate of PKNB (Villarino *et al*., 2005). Although GarA is a protein of 162 amino acid residues, similar to the size of the N-terminal MPR1, these two proteins do not share obvious similarity. The N-terminal MPR1 may or may not be a substrate of the atypical serine/threonine kinase domains of MAK2 and MAK3.

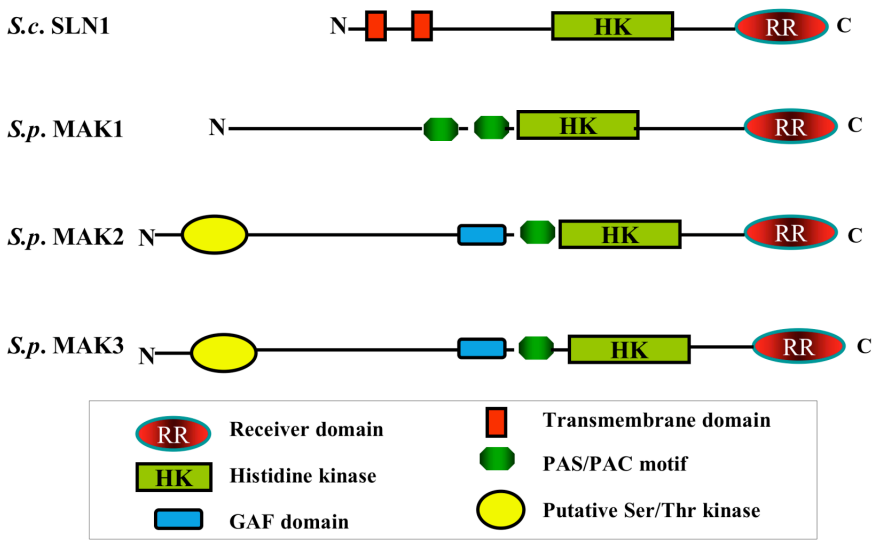

Figure 2-9. Domain structure of the HKs from *S. cerevisiae and S. pombe*. The domain structures of SLN1 from *S. cerevisiae* (*S.c.*) and MAK1-3 from *S. pombe* (*S.p.*) were depicted using colored shapes. Beside an RR domain and an HK domain at the C-terminal end of the four proteins, SLN1 has two transmembrane domains, MAK2 and MAK3 have a putative Ser/Thr kinase domain that does not exist on others.

```
MAK2-s/T   LNETLGALIYDDPGPNILDEWLGNPNPLDLKLFLKFALGVSYVLCFLHEKKIVHGEIRLD
MAK3-S/T   -----------------------------AEFVRFCIAICKCIEYLHSKGMVHGEIRLD
PKNB       -----------------------------KRAIEVIADACQALNFSHQNGIIHRDVKPA
                                         :..    .  : : *.: ::* :::

MAK2-s/T   TFHYDLNAPIHAKLLTIGSSVSPIRFTLSSLNWKRLYQVQNICHKLQFFSPEQIGNVGRP
MAK3-S/T   SFIP-ISSYDNVYMLTVGSGASYFHNCLQAHNWRKYSEDSESMSRILFISPEQTGRTSYS
PKNB       NIMISATNAVKVMDFGIARAIADSGNSVT--------QTAAVIGTAQYLSPEQAR--GDS
           .:    .   :.  : :. . :     :         :         ::****    . .

MAK2-s/T   LDSRSDIYSLGILFYVILTKQYPWGGQSMRIVQSIHMRQFPSVLPRRPD-AFPALDQLIQ
MAK3-S/T   VGYRTDIYSLGVLFFHYLSDCSPYTGSFVQRIRSILTEPLPDISKSCPK-LPHLIFKIIE
PKNB       VDARSDVYSLGCVLYEVLTGEPPFTGDSPVSVAYQHVREDPIPPSARHEGLSADLDAVVL
           :. *:*:**** :::  *:   *: *.    :     .  *       .     :  ::

MAK2-s/T   KMTAKSMNSRISSATDLCYTIV------
MAK3-S/T   KMTRKNPDERYTSCSGIVNDLE------
PKNB       KALAKNPENRYQTAAEMRADLVRVHNGE
               *    *. :.:. :  :
```

Figure 2-10. The alignment of the serine/threonine domains of MAK2, MAK3 from *S. pombe* and PKNB from *Mycobacterium tuberculosis*. The identical residues are indicated by *, while the highly conserved residues by :. The serine/threonine kinase domains from PKNB and MAK2 have 23% identity and ~ 21% highly conservative, while those from PKNB and MAK3 share 18% identity and 20% similarity.

2.5 Summary

In summary, we conclude that the C-terminal domain of MPR1 is a functional HPt domain capable of complementing a *ypd1*Δ strain and substituting for YPD1 in *in vitro* phosphotransfer reactions. Our data further suggests that the N-terminal domain of MPR1 contributes to phosphotransfer efficiency, but not specificity, by enhancing the physical interaction (*e.g.* providing a docking site) between the C-terminal HPt domain and the upstream or downstream RR domains that MPR1 engages. Our data, when combined with data from the current literature, is consistent with the existence of two parallel His-Asp phosphorelay pathways in *S. pombe* (Figure 2-11). One pathway integrates environmental stress signals through the MAK2 and MAK3 sensor histidine kinases to MPR1 and MCS4, while the other pathway responds to environmental signals via MAK1 and PRR1 through a MPR1-independent mechanism. More detailed discussion about the MAK1 to PRR1 branch will be stated in Chapter 3.

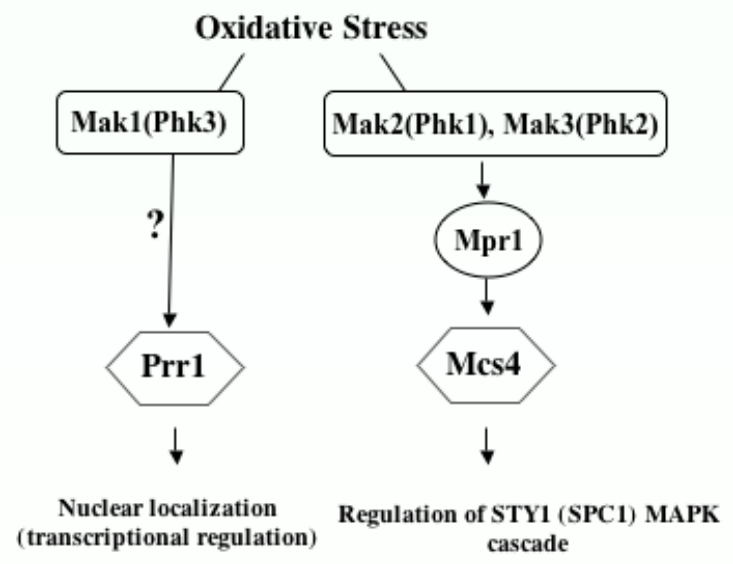

Figure 2-11. Model of His-Asp phosphorelay pathways in *S. pombe*. The current data supports the theory that the hybrid HKs MAK2 and MAK3 lie upstream of MPR1 and MCS4 and form a multi-step His-Asp phosphorelay, while the response regulator PRR1 may be phosphorylated directly by MAK1.

3. Additional studies on protein-protein interactions in the His-Asp phosphotransfer pathway in *S. pombe*

3.1. Introduction

Ever since MCS4 was identified as an upstream response regulator that mediates activation of the STY1 MAP kinase pathway in 1996 (Shieh *et al*., 1997), there unveiled the research in the two-component pathway in *S. pombe*. Originally the discovery of the putative Histidine-Aspartate phosphotransfer pathway was based on amino acid sequence comparisons. Homologous proteins were found in *S. pombe* that were similar to those in the hyperosmotic pathway in *S. cerevisiae*, thus the assumption was made that there was a similar two-component pathway in *S. pombe*. The three histidine kinases in *S. pombe* are homologous to SLN1, the HK in *S. cerevisiae*, in both the histidine kinase domain and the response regulator domain. The C-terminal domain of the HPt protein, MPR1 is similar to YPD1. MCS4 and PRR1 are homologous to SSK1 and SKN7, respectively. After finding the possible components of the pathway, the majority of the published work was focused on genetic deletion studies. The genes that encode components of the pathway were deleted singly, doubly, or triply, and phenotypes were observed and compared with that of wild type cells, or the down stream gene expression level was checked and compared (Ohmiya *et al*., 1999; Aoyama *et al*., 2000; Buck *et al*., 2000; Nguyen *et al*., 2000; Ohmiya *et al*., 2000; Aoyama *et al*., 2001). The *S. pombe* two-component pathway regulates cell mitosis and meiosis processes, thus phenotypic observations included cell size, cell shape and sexual development. The viability of mutated strains was also tested under appropriate stress conditions. Although the

proteins are homologous to their counterparts in *S. cerevisiae* pathway, but there may be at least two distinct phospho-relay pathways in *S. pombe*, regulating oxidative stress responses. One is comprised of the MAK2/MAK3 through MPR1 to MCS4, while the other one begins with MAK1 and ends with PRR1, possibly without participation of MPR1 (Buck *et al*., 2000).

Phosphorylation and interaction have been tested between only MPR1 and MCS4 out of the six in the pathway, showing that MPR1 interacts with and transfers phosphoryl groups to MCS4 *in vitro* (Aoyama *et al*. 2000; Tan *et al*., in press). Phospho-transfer and/or interaction among the other components in the pathway have not yet been studied. In Chapter 2, the yeast two-hybrid assay was used to test protein-protein interactions between MPR1 and the response regulator proteins in the pathway. We showed that MPR1 has no interaction with PRR1-RR in the assay, nor does MPR1 transfer phosphoryl groups to PRR1-RR *in vitro*. In this Chapter, the yeast two-hybrid assay is used to determine which of the histidine kinase(s) PRR1-RR may interact with.

3.2. Materials and methods

3.2.1 Yeast two-hybrid assay

See Materials and methods in Chapter 2.

3.2.2 Western blot analysis

See Materials and methods in Chapter 2.

3.3. Results

The yeast two-hybrid method is a popular and valuable technique to fish out possible protein-protein interactions *in vivo*. This system employs reconstitution of the two domains of a transcription factor GAL4, GAL4 DNA binding domain (BD) and GAL4 activation domain (AD) (Chien *et al*., 1991: White, 1996; Bartel *et al*., 1997; Robinson *et al*., 1998; Serebriiskii *et al*., 2001; Maher, 2002). The AD interacts with RNA polymerase and initiates transcription of a reporter gene in the galactose operon. The two GAL4 domains must be physically linked for the reporter gene to be transcribed. Two hybrid proteins are created by fusing one of the two proteins of interest to the BD domain, while fusing the other to the AD domain. If the two proteins of interest interact, the GAL4 transcription factor is reconstituted, transcription occurs and the reporter gene is expressed. If the proteins do not react, no transcription would occur. The reporter gene here is LacZ gene that expresses β-galactosidase. By measuring β-galactosidase activity, the interaction between the pair of the protein can be quantified. Experiments aimed at comparing *in vitro* data with yeast two-hybrid assay data were successful (Estojak *et al*., 1995). The study indicated that the interaction predicted in the yeast two-hybrid assay correlated well with *in vitro* data and a direct correlation existed between the strength of interaction estimated from both assays. Besides the yeast two-hybrid assay, an *in vitro* phosphorylation study was also carried out to provide further evidence.

Proteins/domains were cloned into the two yeast two-hybrid vectors: pDBleu that gives the BD fusion protein, pPC86 that gives the AD fusion protein. The

proteins/domains are MAK1~3-HK (HK domains of MAK1~3) and PRR1-RR (RR domain of PRR1).

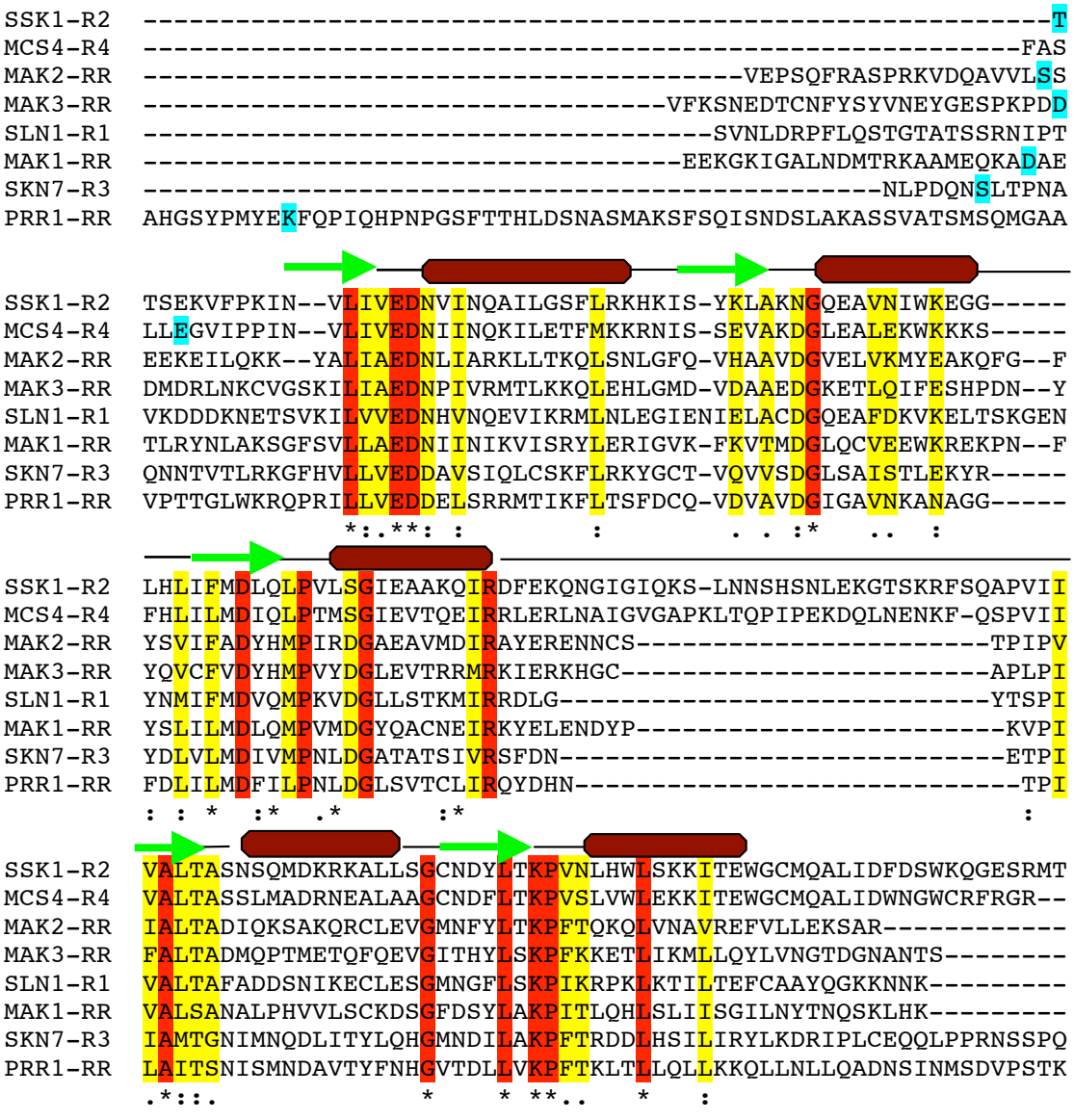

Figure 3-1. Alignment of eight RRs from *S. pombe* and *S. cerevisiae*. The RR domains of SSK1, SKN7, MCS4, PRR1, SLN1, MAK1-3 were aligned. The identical amino acid residues are highlighted in red, conserved ones in yellow. Predicted 2nd structure (unless solved) were also aligned. Green arrows represent β sheets, dark red rectangles represent α helices, black lines are linkers. The first amino acid residue of each RR is highlighted in turquoise. The alignment is accomplished using Clustal X (Jeanmougin *et al*., 1998).

The yeast two-hybrid method has proved effective in assaying protein-protein interactions and there was a direct correlation between the strength of interaction and the level of reporter gene expression (Estojak *et al*., 1995). Thus, yeast two-hybrid data obtained from testing MPR1 and the cognate proteins (domains) will provide useful data in verifying that there are two branches in the His-Asp phosphorelay pathway in *S. pombe.*

With the exception of MCS4, which was full-length, domains were used instead of full length proteins, for two reasons. First reason, MAK2 and MAK3 are large genes that have nearly seven thousand base pairs. It is very difficult to clone them into pDBleu or pPC86, which are the same size as the two genes. Second, both the *PRR1* gene and the *MAK1* genes have introns that are outside RR domains. Effort has been made to generate *S. pombe* mRNA but failed.

The sequences of PRR1, MCS4, MAK1-3 were aligned with the already known RR domains of SLN1, SSK1 and SKN7 from *S. cerevisiae* to determine the RR domains of the proteins in *S. pombe* (Figure 3-1). The HK domains of MAK1-3 were determined by aligning with the HK domain of SLN1 (Figure 3-2). Secondary structures were also predicted for the proteins to ensure the integrity of the RR or HK domains. Predictions for RRs are in Figure 3-1, while those predictions for the HK domains of MAK1, 2, 3 are in Figure 3-3, 3-4, 3-5, respectively.

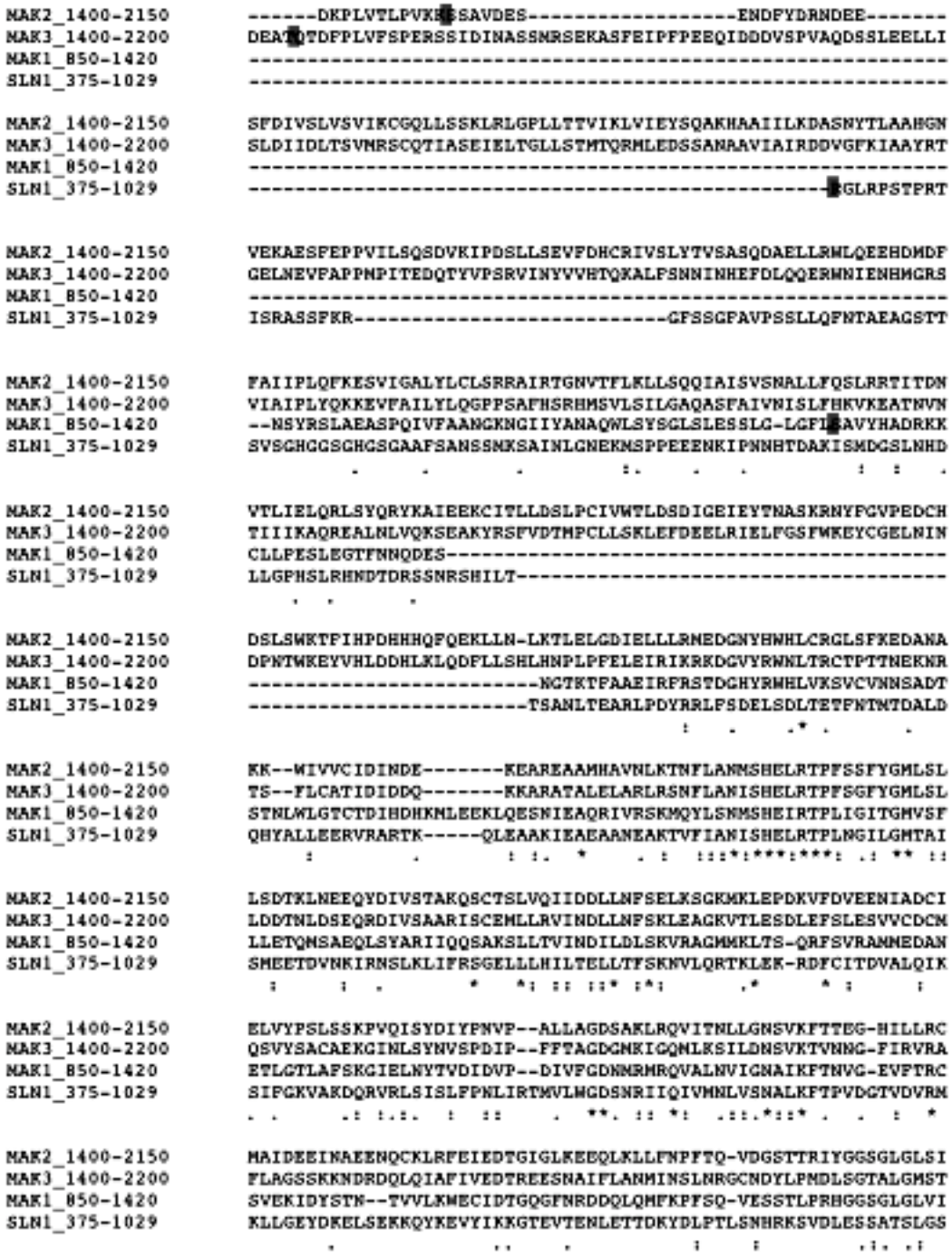

Figure 3-2 (A). Alignment of four HKs from *S. pombe* and *S. cerevisiae*. The HK domains of SLN1, MAK1-3 were aligned. The identical amino acid residues are indicated by *. The highly and less conserved residues are indicated by : and . respectively. The first and last amino acid residues of each HK are highlighted in gray (also see Figure 3-2 II). The sequence compared is indicated in the name of each of the HKs. The alignment was accomplished using ClustalW (European Bioinformatics Institute).

Figure 3-2 (B). Alignment of four HKs from *S. pombe* and *S. cerevisiae* (continued).

```
LGLGFLSAVYHADRKKCLLPESLEGTFNNQDESNGTKTFAAEIRFRSTDGHYRWH
LLL.......LL.....LL...HHHHHHHH....LLL....EEEEE..LL..EEE

LVKSVCVNNSADTSTNLWLGTCTDIHDHKMLEEKLQESNIEAQRIVRSKMQYLSN
EEEEEE....LLL...EEEEE......HHHHHHHHHHHHHHHHHHHH..HHHH..

MSHEIRTPLIGITGMVSFLLETQMSAEQLSYARIIQQSAKSLLTVINDILDLSKV
......L...HHHHHHHHH..LLLL..HHHHHHHHHH.HHHHHHHHHH......

RAGMMKLTSQRFSVRAMMEDANETLGTLAFSKGIELNYTVDIDVPDIVFGDNMRM
..L.........LHHHHHHHHHHHHHHH...LL.EEEEE.LLLLL.....L.HHH

RQVALNVIGNAIKFTNVGEVFTRCSVEKIDYSTNTVVLKWECIDTGQGFNRDDQL
HHHHHHHH.......LLLL.EEEE.......LLL..EEEEEEE.LLLL.L...HH

QMFKPFSQVESSTLPRHGGSGLGLVISKELVELHNGSMSCQSRRGVGTRFMWTAT
HHH......LL......L...HHHHHHHHHHHH.LL.EEEE..LL...EEEEE..

FTMDKTPLKFEPPDGCCPVCFCPYEKSKQSTEDYYCADDGNDKSATNFVKLAVNK
...LLLLLLLLL.............LLLLL.EEEE.LL...........EE..

ADPGRESNRRKLESDKNVQSNKYVNPFASESEFCRCGASADPYTVLFWRLYRNKP
.L..HHHHHHHH..LLL.EEE...LLLLLHHHHHHH...LLLL.EEEEE...LLL

SGIKLDKSALAVVVSHTKYSSEAIGNMLQSIIDISSFKDIVRYGNTYEAFEELLE
L.............LL.HHHHHHHHHHH..................HHHHH...

NPMQSKVTHIILNLP
LLLLL..EEEE..LL
```

Figure 3-3. Secondary structure prediction of HK-MAK1. H=helix, E=extended (sheet), L: is loop .: means that no prediction is made for this residue. The PROF program was employed to do the prediction (Rost *et al*., 1993).

```
DKPLVTLPVKKSSAVDESENDFYDRNDEESFDIVSLVSVIKCGQLLSSKLRLGPL
LLL.......LL..LL.L.....LLLL...LL.EEEE............LLLLL.

LTTVIKLVIEYSQAKHAAIILKDASNYTLAAHGNVEKAESFEPPVILSQSDVKIP
HHHHHHHH.....HHHHHHHHH......HH..LL......LLLL.........LL

DSLLSEVFDHCRIVSLYTVSASQDAELLRWLQEEHDMDFFAIIPLQFKESVIGAL
..HHH.......EEEEEE.L...HHHHHHHHHHH.LLL..EE........HHHHH

YLCLSRRAIRTGNVTFLKLLSQQIAISVSNALLFQSLRRTITDNVTLIELQRLSY
HH.....HH..LL..HHHHH.HHHHH....HHHHHHHH...LLL.LHHHHHH..H

QRYKAIEEKCITLLDSLPCIVWTLDSDIGEIEYTNASKRNYFGVPEDCHDSLSWK
HHHHHHHHHHH.....LL.EEEEE.LLLL.EEE........LLLLL.L..L.L..

TFIHPDHHHQFQEKLLNLKTLELGDIELLLRMEDGNYHWHLCRGLSFKEDANAKK
....LL...HHHHHH.LLL...LLL.EEEEE..LL.EEEE...LLLLL.......

WIVVCIDINDEKEAREAAMHAVNLKTNFLANMSHELRTPFSSFYGMLSLLSDTKL
.EEEEE....HHHHHHHHHHHHHHHHHHHH......LLL.HHHHHHHHH...LLL

NEEQYDIVSTAKQSCTSLVQIIDDLLNFSELKSGKMKLEPDKVFDVEENIADCIE
LHHHHHHHHHHHH.HHHHHHHHHH.........L....LLLL....HHHHHHHHH

LVYPSLSSKPVQISYDIYPNVPALLAGDSAKLRQVITNLLGNSVKFTTEGHILLR
H.......L..EEEE..LLLL.....LLHHHHHHHHHHH.........LL.EEEE

CMAIDEEINAEENQCKLRFEIEDTGIGLKEEQLKLLFNPFTQVDGSTTRIYGGSG
EEE....LLLL.L..EEEEEEE..L..LL...HHHH..............L...

LGLSICLQICKIMDGDIGVQSVYGEGSTFWFHVQLRNVTSKLSQKHFEESHERFA
HHHHHHHHHHH..LL.EEEE...LLL.EEEEEE.............HHHHHHHH

NIRQSLKNAKILVVKSFTTSRSIFRSLFSLAVVDTTTIYSDIEQQLIDSLDKRQP
HHHH....LL.EEE..LLL..HHHH.H.............HHHHH......LLL

YDFLCIEAASGQTEQIITQILSNQKLNKVLLIVLLPSIQRTKVRSDGDPFITSLN
L...E.....LL.HHHHHHH..LL..LL..EEEE.L.........LLL.....LL

KNQSRIFCFREPIRISKLLQNFPALLSKWSTPTKLV
LL..EEE...L.L.HHHH....H.....LLLLLLLL
```

Figure 3-4. Secondary structure prediction of HK-MAK2. H=helix, E=extended (sheet), L: is loop .: means that no prediction is made for this residue. The PROF program was employed to do the prediction (Rost *et al*., 1993).

```
DEATQTDFPLVFSPERSSIDINASSMRSEKASFEIPFPEEQIDDDVSPVAQDSSL
LLLLLLLLL...LLL.....................LLLL....LLLLL...LLLLH

EELLISLDIIDLTSVMRSCQTIASEIELTGLLSTMTQRMLEDSSANAAVIAIRDD
HHHH...L..........HHHHHHHHHH.....HHHHHH...LL.L........L

VGFKIAAYRTGELNEVFAPPMPITEDQTYVPSRVINYVVHTQKALFSNNINHEFD
LL.EEEEE..L......LLLLLLL.........EE.......HHHH.LLLL...L

LQQERWNIENHMGRSVIAIPLYQKKEVFAILYLQGPPSAFHSRHMSVLSILGAQA
..HHHHHHHH.LLL.EEEEE..LL..........LL..........HHHHH....

SFAIVNISLFHKVKEATNVNTIIIKAQREALNLVQKSEAKYRSFVDTMPCLLSKL
.................LLL...HHHHHHHH......HHH........H...L.L

EFDEELRIELFGSFWKEYCGELNINDPNTWKEYVHLDDHLKLQDFLLSHLHNPLP
LLLHHHHHHHH.HHHHHH....LLLL.L.............HHHHHHH...LLLL

FELEIRIKRKDGVYRWNLTRCTPTTNEKNRTSFLCATIDIDDQKKARATALELAR
L..EEEE...LL..EE....LLLLL........EEEEE...HHHHHHHHHHHHHH

LRSNFLANISHELRTPFSGFYGMLSLLDDTNLDSEQRDIVSAARISCEMLLRVIN
HHHHHHH.......LL..HHHHHHHH...LLLLHHHHHHHHHHHH.HHHHHHHH

DLLNFSKLEAGKVTLESDLEFSLESVVCDCMQSVYSACAEKGINLSYNVSPDIPF
HH........L..E..LL.....HHHHHHHHHHHHH...LL..EEEE.LLLL..

FTAGDGMKIGQMLKSILDNSVKTVNNGFIRVRAFLAGSSKKNDRDQLQIAFIVED
...LL..HHHHHHHHH..L......L..........LLLLLL.......EEE..

TREESNAIFLANMINSLNRGCNDYLPMDLSGTALGMSTCLQLCKIMGGSVSVEVS
L.....HHHHHHHHH............LLL.....HHHHHHHHH.LL..EEEE.

QNNPTFKICYDLKIHELGKERYDIIATPLFQNLTEFNDLIKSKVAIRVSKTSTEY
.LL..EEEEE.......LL.....EE..................EEE...LL...

DNITTYLQAARKVLHVFKGLQDLASIFDLSPDSALLRCSVVVVDVYSMDDVKAVE
..HHHHHHHHHHHHH......HH.H..LLL.........EEEEE..L.HHHHH

KILKSYPDVHVIYLCCDPSRLNIEQELQKPSGRSFACKKRWGFLQMPCTRENFLK
HHHH.LLL..EEEE...LL.LL.HHH..LLLLL..............LL......

VTLQVFKSNEDTCNFYSYVNEYGESPKPDDD
.......LL.............LLLLLLLLL
```

Figure 3-5. Secondary structure prediction of HK-MAK3. H=helix, E=extended (sheet), L: is loop .: means that no prediction is made for this residue. The PROF program was employed to do the prediction (Rost *et al*., 1993).

Since MPR1 was known to interact with and transfer phosphoryl groups to MCS4 *in vitro* (Aoyama *et al*. 2000), MPR1 and MCS4-R4 were chosen to do a pilot

assay to determine which vector MPR1 would be cloned into. When MPR1 was cloned into the pPC86 vector and MCS4-R4 into the pDBleu vector, no interaction was observed. When MPR1 was cloned into pDBleu and MCS4-R4 into pPC86, weak interaction was detected, which is very similar to that observed for YPD1/SSK1-R2 interaction (Porter 2003). Thus MPR1 was cloned into the pDBleu, while all of the other components were cloned into pPC86. Negative controls were assayed in parallel, including vectors alone (pDBleu, or pPC86), one vector with the other plasmid (for example pDBleu/pPC86-MCS4 for pDBleu-MPR1/pPC86-MCS4 pair), and vise versa (for example pDBleu-MPR1/pPC86 for pDBleu-MPR1/pPC86-MCS4 pair). These negative controls showed no interactions. Positive controls were provided by the ProQuest two-hybrid system (BRL/Invitrogen), including controls of various interaction strengths for determinations of the relative strength of interaction between the proteins (domains) tested. The cDNA for the weak interaction control was the human *RB* and *E2F1* genes, while the cDNA for the moderately strong interaction was the *Drosophila DP* and *E2F* genes.

PRR1-RR did not exhibit any obvious interaction with MPR1, as shown in Chapter 2. To find out which component(s) interact with PRR1-RR, the HK domains of MAK1-3 were put in yeast two-hybrid system to test their interaction with PRR1-RR. When the *PRR1-RR* gene was cloned into pPC86 vector and *MAK1-3-HK* into pDBleu vector, no interaction could be detected. When *PRR1-RR* gene was clone into pDBleu, it showed weak interaction with pPC86 vector control (the negative control). The *MAK1-3-HK* genes were cloned into pPC86. The β-galactosidase value was calculated after subtracting the β-galactosidase value of the negative control. Table 3-1 showed that all

of the three HKs have a weak interaction with PRR1-RR. The β-galactosidase value of the MAK1-HK was twice that of MAK2-HK, and almost three times that of MAK3-HK. To prove that PRR1-RR can be phosphorylated by one or more of the three HK domains, attempts were made to express the three HK domains *in vitro*, but failed.

To ensure that the all of the yeast two-hybrid results obtained above were not due to differences in protein expression, but an accurate representation of the strength of protein-protein interaction, Western blot assays were performed using anti-MPR1 antibody (Cocolica) and anti-AD from Sigma (Figure 3-6, 7, 8).

Prey protein/domain	Bait protein
	PRR1-RR
MAK1-HK	1.66 ± 0.49
MAK2-HK	0.77 ± 0.13
MAK3-HK	0.47 ± 0.05

Table 3-1. Yeast two-hybrid results in β-galactosidase unit. PRR1-RR was cloned into pDBLeu vector, while the HK domains of MAK1-3 were cloned into pPC86 vector. All of the three HKs showed a weak interaction with PRR1-RR, with MAK1-HK giving the strongest signal, twice of that of MAK2-HK and three times that of MAK3-HK. The cDNA for the weak interaction control was the human *RB* and *E2F1* genes, while the cDNA for the moderately strong interaction was the *Drosophila DP* and *E2F* genes. The weak interaction control (provided by ProQuest™ Two-Hybrid System) value (Control C) was 0.39 ± 0.08.

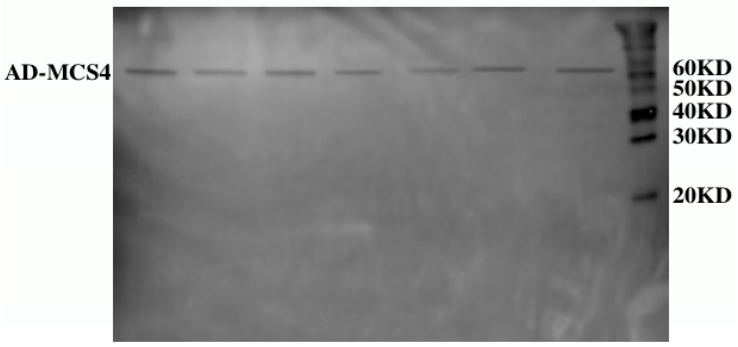

Figure 3-6. Western blot result I. AD-MCS4 was detected using anti-AD antibody. From left to right, the cells contain the following plasmid(s) respectively: pPC86-MCS4, pPC86-MCS4/pDBleu (sample 1), pPC86-MCS4/pDBleu-MPR1 (sample 1), pPC86-MCS4/pDBleu-MPR1ΔN160, pPC86-MCS4/pDBleu-MPR1ΔC135, pPC86-MCS4/pDBleu (sample 2), pPC86-MCS4/pDBleu-MPR1 (sample 2). The last lane is the molecular weight marker provided by Invitrogen.

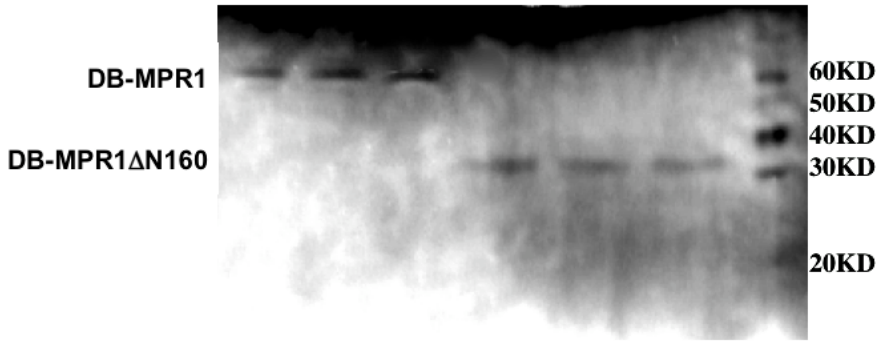

Figure 3-7. Western blot result II. AD-MPR1 was detected using anti-AD antibody. Comparable level of proteins for AD-MPR1 and AD-MPR1N160 were detectable. From left to right, the cells contain the following plasmid(s) respectively: pDBleu-MPR1, pDBleu-MPR1/pPC86, pDBleu-MPR1/pPB86-PRR1-RR, pDBleu-MPR1ΔN160, pDBleu-MPR1ΔN160/pPC86, pDBleu-MPR1ΔN160/pPC86-PRR1-RR. The last lane is the molecular weight marker provided by Invitrogen.

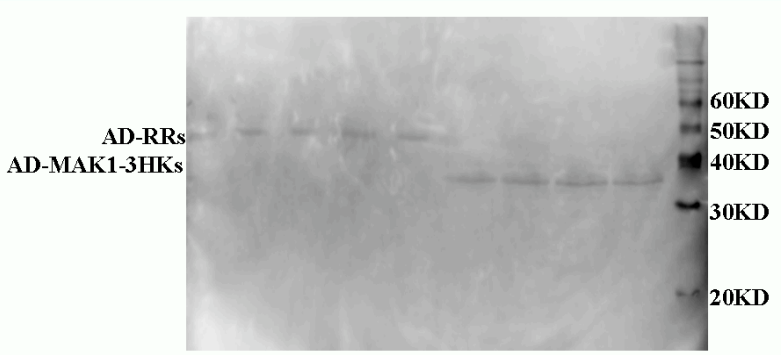

Figure 3-8. Western blot result III. The expression of AD-MAK1-3-HKs and the RR domains of MAK1~3 and PRR1 were detected using anti-AD antibody. From left to right, the cells contain the following plasmids respectively: pDBleu-MPR1/pPC86-MAK1-RR, pDBleu-MPR1/pPC86-MAK2-RR, pDBleu-MPR1/pPC86-MAK3-RR, pDBleu/pPC86-MAK3-RR, pPC86-MAK1-RR, pDBleu-MPR1/pPC86-MAK1-HK, pDBleu-MPR1/pPC86-MAK2-HK, pDBleu-MPR1/pPC86-MAK3-HK, pDBleu/pPC86-MAK1-HK. The last lane is the molecular weight marker provided by Invitrogen.

3.4. Discussion

All cellular processes ranging from signal transduction, DNA replication, cell cycle control to enzymatic reactions, employ protein-protein interactions. A large number of these interactions that control and regulate cellular processes are transient, including substrate interactions with kinases, phosphatases, glycosyl transferases and proteases. Studying protein-protein interactions is important to our understanding of biological processes. An important and popular tool to study protein-protein interactions is the yeast two-hybrid method, reconstructing the two domains of transcription factor GAL4 via the proteins of interest to detect interactions between the proteins. More refined functional studies can then be carried out to characterize and manipulate the identified proteins. These follow-up methods include chemical cross-linking, co-immunoprecipitation, pull-down assays, nuclear magnetic resonance (NMR), mass spectroscopy, and X-ray crystallography.

Why do we use the yeast two-hybrid system? There are multiple reasons. First, it is an *in vivo* method that employs yeast as the host, giving an environment closer to higher eukaryotic organisms. Second, only full-length protein or domains of the protein of interest are needed, making the system a relatively easy screening method. Lastly, it is a possible to detect weak and transient interactions, since the reporter gene strategy enables a significant magnification.

Two distinct phosphorelay pathways may exist in *S. pombe*, regulating oxidative stress responses, one from MAK1 to PRR1, the other one from MAK2/MAK3 to MPR1 then to MCS4. The advantage of having more than one histidine kinase or branches is that different signals can be sensed and transferred to the same regulatory pathway if the phosphorylation state of a response regulator is controlled by more than one histidine kinases. Both MAK2 and MAK3 are upstream of MCS4, indicating that they may feed different signals into this pathway. But as shown in Figure 3-9, MAK2 and MAK3 have similar domain structures, both having a GAF domain, a PAS/PAC motif and an atypical serine/threonine kinase domain beside the conserved HK domain and the RR domain. The sequence similarity between the two proteins is quite high, with an overall 24% identity and 43% homology. The domain resemblance and the sequence similarity between MAK2 and MAK3 suggests that the reason for the presence of two similar HKs in the same pathway is to input different signals. It is possible that MAK2 and MAK3 function as a dimer to activate MCS4.

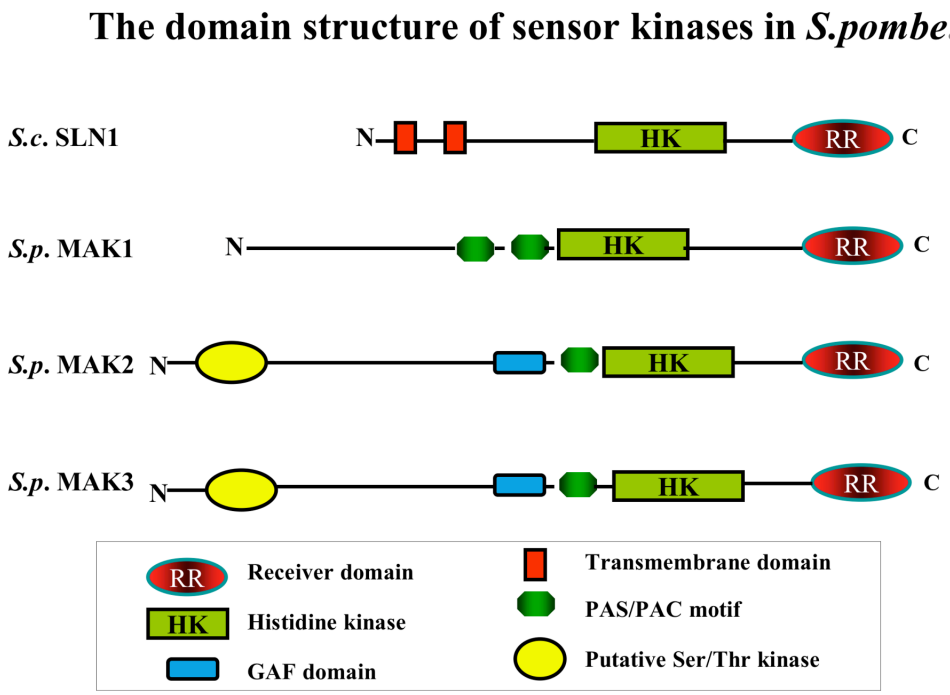

Figure 3-9. Domain structures of sensor kinases from *S. pombe* and *S. cerevisiae*. The domain structures of SLN1 from *S. cerevisiae* (*S.c.*) and MAK1-3 from *S. pombe* (*S.p.*) were depicted using colored shapes. Beside an RR domain and an HK domain at the C-terminal end of the four proteins, SLN1 has two transmembrane domains, MAK2 and MAK3 have a putative Ser/Thr kinase domain that does not exist on others.

If MPR1 does not function between the histidine kinase MAK1 and response regulator PRR1, the MAK1→PRR1 pathway fits into a category with more than one RR (reviewed by Stock *et al*., 2002), one is the RR domain of MAK1, the other one is the RR domain of PRR1. A possible mechanism of having two RR under one cognate kinase is that one of the two RRs would provide a sink for phosphoryl groups that are passed from the HK to the other RR, as do CheY1 and CheY2 in the *Rhizobium meliloti* chemotaxis system. Another possibility is that the two RRs simply compete for the

phosphoryl groups from the HK protein, like CheB and CheY in bacterial chemotaxis (Stock *et al*., 2002).

3.5. Summary

The yeast two-hybrid assay result showed that MAK1 may directly control the activity of PRR1. This evidence further supports the idea that there are two branches in the phosphorelay in *S. pombe*, one from MAK1 to PRR1, the other one from MAK2/MAK3 to MPR1 then to MCS4.

4. An *In vitro* Comparative Study of YPD1- and MPR1-Response Regulator Binding Specificity

4.1. Introduction

To avoid cross talk during signal transduction, the HPt proteins need to interact with the appropriate RR in reaction to a specific stress. YPD1, the HPt protein in *S. cerevisiae*, has two RRs downstream, SSK1 and SKN7, each being phosphorylated under different circumstances. *In vitro* phosphorylation assays have shown that YPD1 transfers phosphoryl groups more efficiently to SSK1-R2 than to SKN7-R3 (Janiak-Spens *et al*., 2005; Tan *et al*., in press), while in contrast MPR1 the HPt protein in *S. pombe* exhibits a preference for SKN7-R3 over SSK1-R2 (Tan *et al*., in press). Studying the differences between YPD1 and MPR1 may help us to understand how the HPt proteins distinguish downstream RRs when transducing different signals.

Based on results using the yeast two-hybrid system and the YPD1/SLN1-R1 co-crystal structure, it was found that the hydrophobic patch around the site of phosphorylation is the primary binding surface for RRs on YPD1, while the peripheral residues surrounding the hydrophobic patch may be responsible for the specificity of binding to RRs (Porter *et al*., 2003; Xu *et al*., 2003; Porter *et al*., 2005). This hydrophobic patch is located between H64 (the phosphorylation site) and the αA helix of YPD1, and the patch is conserved among other HPt proteins from fungi, bacteria and plants (Xu *et al*., 1999; Porter *et al*., 2005). The YPD1 αA helix plays an important role in binding to response regulators (Porter *et al*., 2003; Xu *et al*., 2003; Porter *et al*., 2005).

Among the 4 helices on YPD1 that form the RR binding surface area with SLN1-R1, αA has the least number of identical residues between YPD1 and MPR1 (the percentage identity for αA is 9%, αB is 37%, αC is 73.7%, αD is 61.5% identical). The αA helix also has a significant number of intermolecular contacts with RRs (Porter *et al*., 2005). The X-ray structure $P2_12_12_1$ complex of YPD1/SLN1-R1 shows that 27% of amino acid residues on αA helix on YPD1 forms hydrophobic interactions with SLN1-R1, 18% forms hydrogen bond interaction with SLN1-R1, compared with 7%/15% residues on αB helix, 16%/21% on αC helix, and 6%/12% on αD helix (Porter *et al*., 2003) (Table4-1).

	% of identical residues between YPD1 and MPR1	% of hydrophobic contacts in $P2_12_12_1$ YPD1/SLN1-R1 complex	% of H-bond interactions in $P2_12_12_1$ YPD1/SLN1-R1 complex
αA helix	9	27	18
αB helix	37	7	15
αC helix	73.7	16	21
αD helix	61.5	6	12

Table 4-1. Comparison of the four loops that are in contact with YPD1 on SLN1-R1 in the YPD1/SLN1-R1 co-crystal structure. The calculations of percentage were done by dividing the numbers of identical residues (or hydrophobic interacted residues, H-bonded residues) by the total number of residues on that certain helix.

In the YPD1/SLN1-R1 co-crystal structure (Xu *et al*., 2003), four loops in SLN1-R1 (β1-α1, β3-α3, β4-α4, β5-α5) were observed to contact YPD1 in the crystal form $P2_12_12_1$ and 2 loops (β1-α1, β5-α5) in the crystal form $P3_2$. The β2-α2 loop in SLN1-R1

is located at the edge of the interface and has no direct contact with YPD1. The β4-α4 loop is not observed to make contact in the $P3_2$ crystal form of the complex. Among the three loops that do make contact with YPD1 in both crystal forms, the β1-α1 loop is completely conserved between SSK1-R2 and SKN7-R3, while the β5-α5 loop is highly conserved between the two response regulator domains (Porter *et al*., 2003). The β3-α3 is the largest loop and least conserved one (Table 4-2). I hypothesize that contact between YPD1 and this 3rd loop on different RRs may lead to different strength of binding between HPt proteins and RRs. The E58 residue on YPD1 may be part of the contact between YPD1 and the 3rd loop on RRs since the E58 residue is in a very close proximity to the 3rd loop of the RR (Figure 4-1). Instead of E58 on YPD1, the corresponding position is K237 on MPR1. Thus, I further hypothesize that the E58 residue on YPD1 may be responsible for RR binding specificity.

Loop	RR domain	Residues on the loop
β1-α1	SSK1-R2	E D
	SKN7-R3	E D
β3-α3	SSK1-R2	L Q L P V L S
	SKN7-R3	I V M P N L D
β5-α5	SSK1-R2	K P V
	SKN7-R3	K P F

Table 4-2. The alignment of three loops of the two response regulator domains in *S. cerevisiae*. The residues highlighted in grey are in close proximity to E58 on YPD1. Their contact with E58 may be responsible for binding specificity with YPD1.

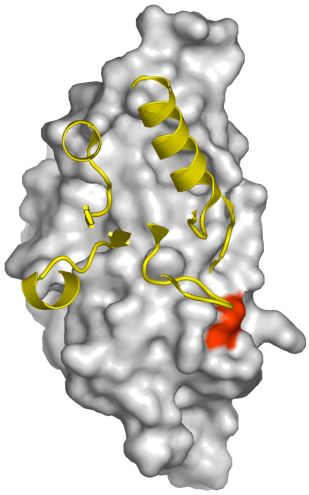

Figure 4-1. Molecular surface representation of YPD1 with a partial ribbon diagram of SLN1-R1. The YPD1 E58 residue is highlighted in red, and a partial model for SLN1-R1 is shown in yellow ribbon diagram. YPD1 E58 is close to 3rd loop of SLN1-R1. The figure was generated using the program PYMOL (DeLano, 2002).

Figure 4-2. Alignment of MPR1 and YPD1 proteins. The alignment shows that the residues involving in RR core binding and binding specificity are highly conserved between YPD1 and MPR1. The residues highlighted in pink are responsible for the core binding (the only one that is not conserved between the two proteins is in green). The residues in dark blue are putatively responsible for RR specificity, with the nonconserved residues indicated by yellow arrows. The αA helix of YPD1 (also the predicted MPR1 corresponding helix) is underlined with a green box.

On YPD1, the histidine-containing phosphotransfer protein in *S. cerevisiae*, the residues responsible for the core binding to response regulators are: M20, F27, L31, F65,

G68, and L73 (hydrophobic), E16, D21, D60 and S69 (hydrophilic). The residues that are predicted to be involved in interactions with specific response regulators are: S19 (with SLN1-R1), D23, E58, K67, S70, Q86 (with SKN7-R3), W80, E83 (with SLN1-R1 and SKN7-R3), I13, D24, Q38, Q76 (with SSK1-R2 and SKN7-R3). The sequence alignment shows that with the exception of D60 (S on MPR1), all other core residues are identical or highly conserved between MPR1 and YPD1. Even the residues responsible for putative RR-binding specificity are conserved as well, except three residues, E58, Q76 and W80, which are K215, T233 and K237 respectively on MPR1 (Porter 2004), as showed in Figure 4-2.

In order to find out what is responsible for the difference in HPt-RR phosphotransfer specificity, YPD1 and MPR1 were mutated to study the changes in phosphotransfer *in vitro*. The mutations include switching the αA helix and site-directed mutatgenesis of the amino acid residues that are putatively involved in binding specificity (E58 and Q76 on YPD1).

4.2. Materials and methods

4.2.1 Construction of YPD1 and MPR1 mutants

YPD1 and *MPR1* were mutated using the Quikchange site-directed mutagenesis method (Stratagene). *MPR1* in pDEST17 or *YPD1* in pUC12 served as templates in the PCR reactions. The primer pairs used in PCR were listed in Table 4-3. All of the procedures are similar to that described in "Construction of deletion mutants of MPR1" in Chapter 2. The mutagenesis design is shown in Figure 4-3. The resultant plasmids are pUC12-YPD1-swap-E58K (OU plasmid$^{\#}$284, abbreviated as OU pl$^{\#}$284), pUC12-

YPD1-swap (OU pl#300), pUC12-YPD1-Q76T (OU pl#301), pUC12-YPD1-swap-Q76T (OU pl#302) and pDEST17-MPR1-swap (OU pl#250).

The PCR protocol was as follows (final volume was 25 μl):

Pfu cloned buffer (10X)	2.5 μl
Template	~40 ng
5' primer	~400 ng
3' primer	~400 ng
dNTP	10 nmol
Pfu Turbo DNA polymerase	1 unit

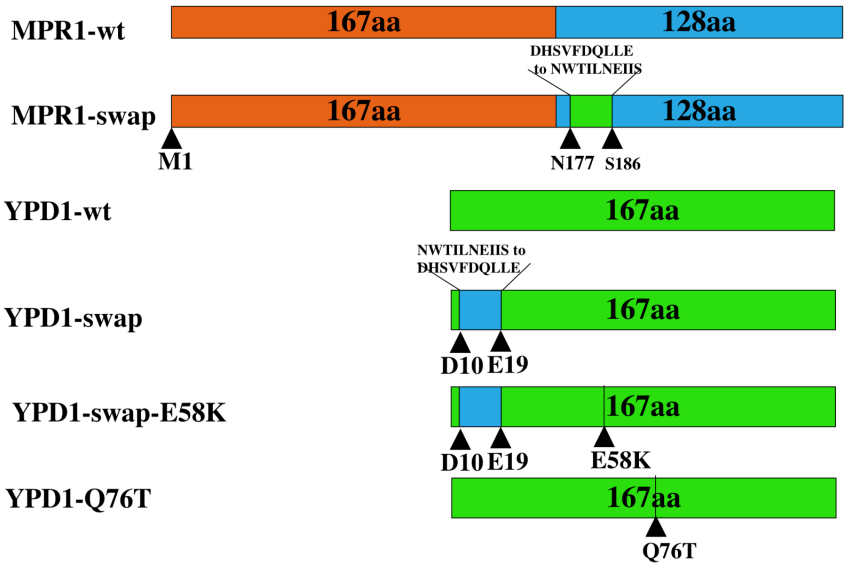

Figure 4-3. MPR1 and YPD1 mutagenesis scheme. The mutations on YPD1 and MPR1, together with wild type (wt) YPD1 and MPR1, are shown in this figure. The αA helix of YPD1 was swapped with the corresponding helix on MPR1, and vice versa. Single amino acid residue mutations were also done on YPD1 and YPD1 swap using Quikchange site-directed mutagenesis method.

AW404 MPR1-swap-1f	5′GACAAACAGCTCATTAATTGGACCATTTTAGACCAGTTGCTTGAG 3′
AW405 MPR1-swap-1r	5′CTCAAGCAACTGGTCTAAAATGGTCCAATTAATGAGCTGTTTGTC 3′
AW412 MPR1-swap-2f	5′AATTGGACCATTTTAAACGAGATTATTTCGATGGATGATGATGAT 3′
AW413 MPR1-swap-2r	5′ATCATCATCATCCATCGAAATAATCTCGTTTAAAATGGTCAAATT 3′
AW454 YPD1 E58K f	5′GAAAAAAATCTTACCAAATTAGACAATC 3′
AW455 YPD1 E58K r	5′GATTGTCTAATTTGGTAAGATTTTTTTC 3′
AW456 YPD1 Q76T f	5′CTGCATTAGGCTTAACAAGAATTGCCTGGG 3′
AW457 YPD1 Q76T r	5′CCCAGGCAATTCTTGTTAAGCCTAATGCAG 3′
AW416 YPD1-swap-1f	5′CCGTCAGAAATCATCGATCATTCCGTTTTTAATGAAATTATATCA 3′
AW417 YPD1-swap-1r	5′AGATATAATTTCATTAAAAACGGAATGATCGATGATTTCTGACGG 3′
AW418 YPD1-swap-2f	5′GATCATTCCGTTTTTGACCAGTTGCTTGAGATGGATGACGATGATTC 3′
AW419 YPD1-swap-2r	5′GAATCATCGTCATCCATCTCAAGCAACTGGTCAAAAACGGAATGATC 3′

Table 4-3. Primers used for mutagenesis in Chapter 4. f is forward primer, r is reverse primer. MPR1 swap and YPD1 swap were mutated in two steps, each step using one pair of the primers.

4.2.2 Purification of YPD1 and MPR1 mutants

The purification of the YPD1 mutants (OU strain 344, 348, 349 for YPD1 swap, swap E58K, Q76T respectively) was the same as YPD1 wild type purification as described previously (Xu *et al*., 1999). The MPR1-swap protein was purified from inclusion bodies as described for purification of rTEV (Lucast *et al*., 2000). OU strain 505 (OU pl$^{\#}$250 in BL21(DE3)StarRIL host cell) was induced at 37 ^{o}C for 6 hours using 0.5 mM IPTG (Isopropyl-β-D-thiogalactoside). After sonication, the pellet was resuspended in suspension buffer (6 M guanidine-HCl, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH8.0) by heating in a 65^{o}C water bath for 4-6 hours. The sample was then pelleted at 10000 rpm for 20 minutes, using JA-20 rotor at 4^{o}C. The supernatant was loaded on a 5 ml Ni^{2+} resin column, which has been equilibrated with equilibration buffer (6 M Urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8.0). The resin was washed with 20 ml equilibration buffer, followed by 30 ml wash buffer (6 M Urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH6.3). The protein was eluted with elution buffer (6 M Urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH4.5). 10 fractions were collected at 1 ml each. The fractions that contain His6-MPR1 were combined and the sample was neutralized to

pH8.0 by adding NaOH. Then it was dialyzed in a 4-liter dialysis buffer (100 mM Tris-HCl, 500 mM NaCl, 5 mM βME, 10% glycerol, pH8.0), using dialysis membrane MWCO 6000-8000 (Spectra/Por®). After ~8 hours dialysis, the sample was centrifuged for 30 minutes to remove any precipitated protein. The supernatant was stored in aliquots at -80^{o}C or -20^{o}C.

4.2.3 β-galactosidase assay

The plasmid pJF1416 (a kind gift from Dr. Jan Fassler, the University of Iowa) was transformed into OU yeast strain 140 cell (Matα, can^{R}, cyh^{R}, his3Δ200, leu2Δ1, ura3-52, trp1Δ63, lys2Δ202, *ypd1*Δkan, 2μ-*PTP2*(*URA3*$^{+}$), also a gift from Dr. Fassler, using the PEG/LiAc method described in Materials and Methods in Chapter 2. SC-Leu-Trp plates were used to plate the newly transformed cells. After 48 hours in a 30^{o}C incubator, colonies were picked into 2.5 ml SC-Leu-Trp liquid media and were shaken at 30^{o}C to prepare an overnight culture. One mL of the culture was used to inoculate 4 mL of YPAD (Yeast extract, peptone, adenine sulfate, dextrose) media at 30^{o}C until an OD_{600} of 1.0 was reached. Cells were collected, lysed and treated according to the procedure described in the "Yeast two-hybrid assay" in Chapter 2, except that ONPG (o-nitrophenyl-β-D-galactopyranoside) was used instead of CPRG (Chlorophenol-red-β-D-galactopyranoside), and that OD_{420} was measured instead of OD_{574}. β-galactosidase activity was also calculated as β-gal unit = 1000 x OD_{420}/ (t x V x OD_{600}), where t is time in minutes and V is the volume of the initial aliquot.

4.2.4 *In vitro* phosphorylation assay

See Materials and Methods in Chapter 2.

4.3. Results

Changing the hydrophobicity and/or side chain volume of the amino acid residues peripheral to the RR docking site may change the binding strength of the HPt proteins to the RRs (Porter, 2004). By using the Quik-change Mutagenesis method, the αA helix of YPD1 was switched with the corresponding helix of MPR1. The amino acid residues were mutated from "NWTILNEIIS" to "DHSVFDQLLE" in YPD1, and vice versa on MPR1 (Table 4-4). The resultant mutants were named *ypd1 swap* and *mpr1 swap*. The proteins were expressed as YPD1 swap and His6-MPR1 swap. In addition, YPD1 swapE58K and YPD1 Q76T were constructed to test the binding specificity to SSK1-R2/SKN7-R3.

α of Wt-MPR1	D	H	S	V	F	D	Q	L	L	E	M
αA of Wt-YPD1	N	W	T	I	L	N	E	I	I	S	M
				13	14		16	17			

Table 4-4. The amino acid residues on the αA helix of YPD1 and the corresponding α helix on MPR1.

The mutants were expressed in *E. coli* and an *in vitro* phosphorylation assay was performed for each mutant, and compared to wild type YPD1 and MPR1. GST-HK was incubated with SLN1-R1 and [γ-^{32}P]-ATP for 40 minutes to generate SLN1-R1~P, then SLN1-R1~P was separated from the mixture and incubated with wild type YPD1 and

SSK1-R2 (or SKN7-R3). Aliquots were taken at designated time intervals. The samples were analysed on 15% SDS-polyacrylamide gels and by autoradiography. The mutated proteins were treated in the same way.

4.3.1 Phosphotransfer from phospho-SLN1-R1 to YPD1 mutants

HPt proteins (domains) have been shown to interact with and transfer phosphoryl groups from and to non-cognate response regulators *in vivo* and *in vitro* (Yaku, *et al.,* 1997; Chang, *et al.,* 1998; Janiak-Spens *et al.,* 1999). Both YPD1 and MPR1 were able to accept phosphoryl groups from phospho-SLN1-R1 (Figure 4-4, Figure 4-8), but the phosphorylation level was different between the two HPt proteins. MPR1 was less efficiently phosphorylated than YPD1 using phospho-SLN1-R1 as a donor. After mutating YPD1 to make it more like MPR1, the mutants (YPD1 Q76T, YPD1 swap, YPD1 swapE58K) could all accept phosphoryl group from phospho-SLN1-R1, albeit to a lesser amount. Figures 4-4 through 4-8 show the phosphortransfer between SLN1-R1 and HPt proteins (YPD, YPD1 mutants, and MPR1) as a function of time. All of them reached steady state phosphorylation levels at the earliest time point. YPD1 received almost half of the phosphoryl groups from phospho-SLN1-R1 at 1 minute and 3 minutes time point (Figure 4-4). Compared to wild type YPD1, the YPD1 mutants and MPR1 received less than half of the phosphoryl group from phospho-SLN1-R1 (Figure 4-5, 6, 7, 8). Phospho-SLN1-R1 has a half-life of 13 minutes (Janiak-Spens *et al.,* 1999), which can explain the decrease in intensity of phospho-SLN1-R1 in the figures. Compared with YPD1 swap (Figure 4-7) which received around one third of the total phosphoryl group

from phospho-SLN1-R1, YPD1 swapE58K (Figure 4-6) received about one fourth of the total amount, which is obviously less than that of YPD1 swap.

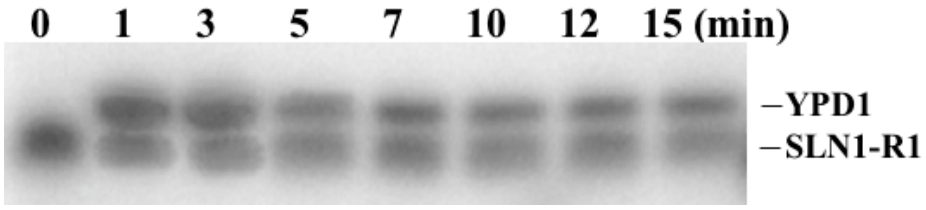

Figure 4-4. Phosphoryl transfer from SLN1-R1 to YPD1. YPD1 was incubated at room temperature with phospho-SLN1-R1. YPD1 received about 50% phosphoryl group from SLN1-R1~P before 1 minute reaction.

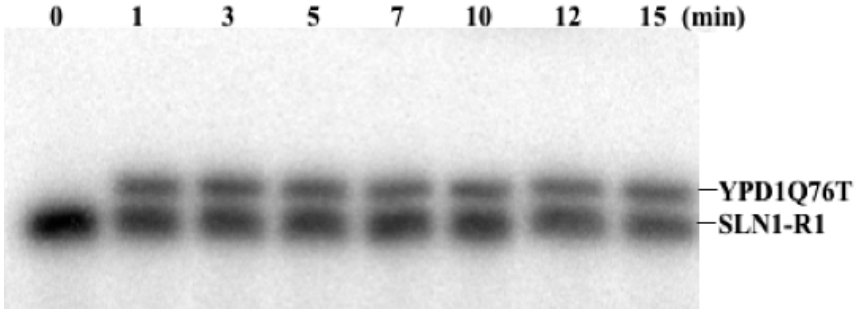

Figure 4-5. Phosphoryl transfer from SLN1-R1 to YPD1 Q76T. YPD1 Q76T still could receive phosphoryl group from phospho-SLN1-R1, The steady state was reached before 1 minute reaction. About 30-40% phosphoryl group was transferred to YPD1 Q76T from SLN1-R1.

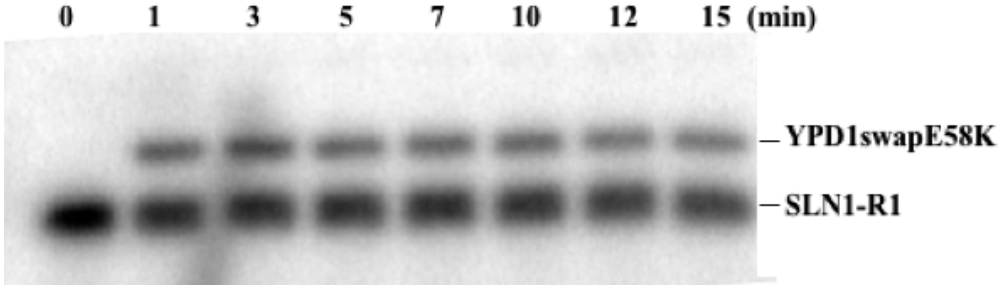

Figure 4-6. Phosphoryl transfer from SLN1-R1 to YPD1 swapE58K. SLN1-R1 could pass phosphoryl group to YPD1 swapE58K, like to YPD1. Only about 30% phosphoryl group was transferred.

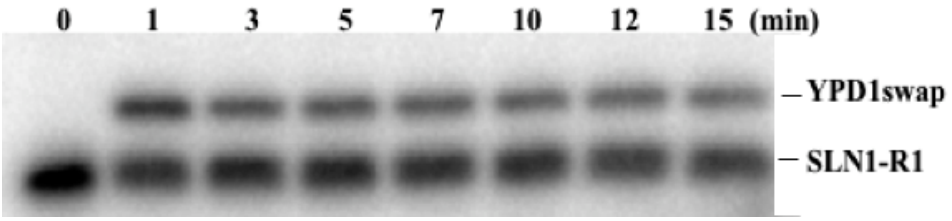

Figure 4-7. Phosphoryl transfer from SLN1-R1 to YPD1 swap. YPD1 swap could receive 50% phosphoryl group from phospho-SLN1-R1 around 1 minute point.

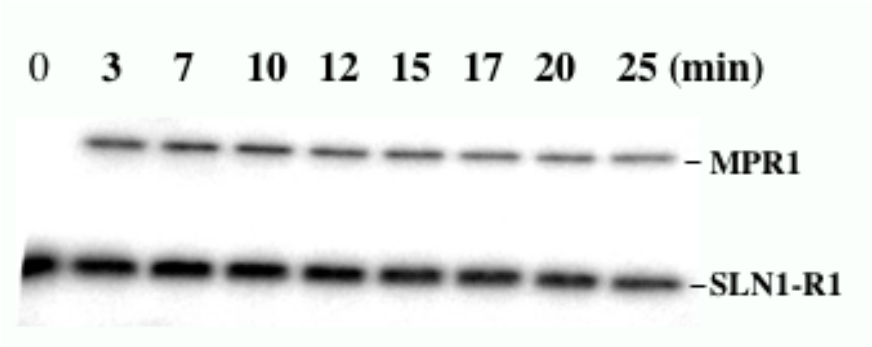

Figure 4-8. Phosphoryl transfer from SLN1-R1 to MPR1. MPR1 could receive phosphoryl group from non-cognate RR phospho-SLN1-R1 *in vitro*..

4.2.2 *In vitro* phosphotransfer from YPD1 mutants to SSK1-R2 and SKN7-R3

Phosphorylated YPD1 is capable of donating phosphoryl groups to both SSK1-R2 and SKN7-R3 *in vitro*, as reported by Janiak-Spens *et al.*, 1999. The amount of phosphoryl groups transferred by YPD1 is significantly different between the two response regulator domains. From the earliest time point (1 minute), SSK1-R2 received the majority of the phosphoryl group from YPD1 (Figure 4-9). Phosphoryl group transfer between phospho-SLN1-R1 and SSK1-R2 in the presence of wild-type YPD1 was complete within seconds and the majority of the phosphoryl groups were transferred to SSK1-R2 (F. Janiak-Spens, 2000). In contrast, the transfer between YPD1 and SKN7-R3 is much slower (Figure 4-9).

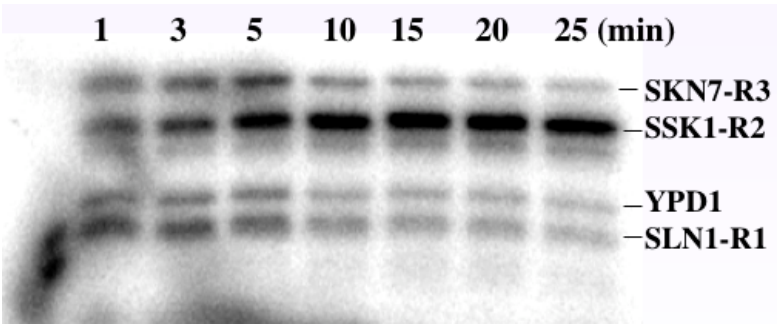

Figure 4-9. Competition between SSK1-R2 and SKN7-R3 in presence of YPD1 and SLN1-R1~P. YPD1 transfers phosphoryl groups to SSK1-R2 and SKN7-R3 *in vitro*. The amount of phosphoryl group transferred by YPD1 is significantly different between the two response regulator domains.

The phosphodonor activity of the YPD1 mutants was also tested. YPD1 swap behaved differently from wild-type YPD1 in several ways. First of all, the reaction time was slower (minutes in the case of HPt~P toward SSK1-R2) (Figure 4-10). The percentage of phosphoryl group transferred was much lower at early time points (less than half toward SSK1-R2). The change in phosphotransfer was before 3 minutes when YPD1 swap transferred more phosphoryl group to SKN7-R3 than to SSK1-R2. Before 3 minutes, the percentage of phosphoryl group transferred from YPD1 swap to SKN7-R3 was more than 50% of the total amount transferred from YPD1 swap (i.e. SKN7-R3~P/{SKN7-R3~P+SSK1-R2~P}), while wild type YPD1 transferred more phosphoryl groups to SSK1-R2 from the earliest time point. Although the phosphotransfer rate from YPD1 swap to SKN7-R3 was faster than from wild type YPD1 to SKN7-R3, YPD1 swap could not stabilize phospho-SKN7-R3.

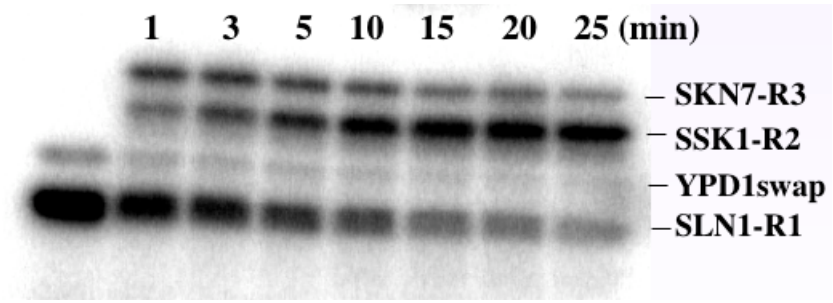

Figure 4-10. Competition between SSK1-R2 and SKN7-R3 in presence of YPD1 swap and SLN1-R1~P. SKN7-R3 received more phosphoryl groups from YPD1 swap than SSK1-R2 at the 1 minute time point, but over time, accumulation of phospho-SSK1-R2 occurs.

The YPD1 swapE58K mutation further changed the phosphotransfer from HPt protein to SSK1-R2 and to SKN7-R3 (Figure 4-11). The transfer toward SKN7-R3 was even more intense around 3 minutes' time point comparing that from YPD1 wild type. More than 60% phosphoryl group was transferred to SKN7-R3 from YPD1 swapE58K, while 40% was transferred to SSK1-R2. Although longer than when wild type YPD1 and YPD1 swap was present, the half life of phospho-SKN7-R3 was still shorter than that of SSK1-R2 in presence of YPD1 swapE58K (Figure 4-11).

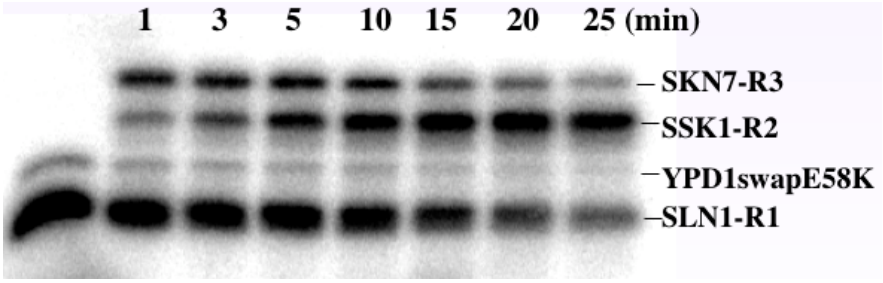

Figure 4-11. Competition between SSK1-R2 and SKN7-R3 in presence of YPD1 swapE58K and SLN1-R1~P. YPD1 swapE58K showed phosphotransfer specificity to SKN7-R3 at least before 3 minutes' reaction, then accumulation of phospho-SSK1-R2 occured.

The YPD1 Q76T mutant displayed similar changes in phosphorelay as that caused by YPD1 swap, accumulating phospho-SKN7-R3 only at early time points (Figure 4-12). The phosphotransfer rate from YPD1 Q76T to SSK1-R2 seemed to be slightly slower than from wild type YPD1 to SSK1-R2 (Figure 4-12).

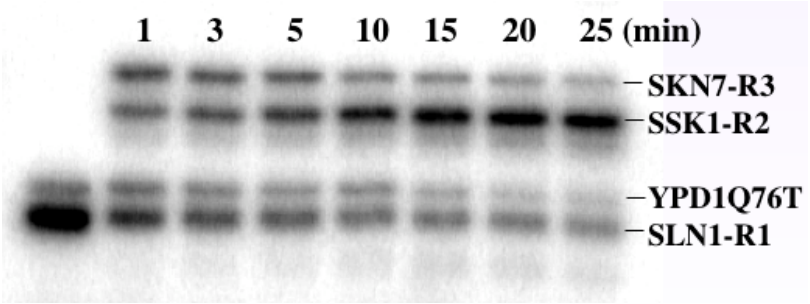

Figure 4-12. Competition between SSK1-R2 and SKN7-R3 in presence of YPD1 Q76T and SLN1-R1~P. YPD1 Q76T also transfers phosphoryl group toward SSK1-R1 and SKN7-R3 *in vitro*. At 1 minute time point, SKN7-R3 received more phosphoryl group from YPD1~P. All of the components were of equal molar concentration.

4.2.3 *In vitro* phosphotransfer activity of MPR1 mutant

Attempts were also made to swap the α helix on MPR1 with αA helix on YPD1, named MPR1 swap. When the protein was expressed in BL21(DE3)StarRIL cell as an expression host, the protein was insoluble and had to be purified from inclusion bodies. To compare the phosphorelay capacity of MPR1 and MPR1 swap, the two proteins were incubated with phospho-SLN1-R1 at room temperature for 10 minutes. Both MPR1 and MPR1 swap received phosphoryl groups from phospho-SLN1-R1, only that MPR1 swap received less phosphoryl groups (Figure 4-13, lanes 2 and 3). MPR1 and MPR1 swap were also incubated with SSK1-R2 and SKN7-R3 in presence of phospho-R1 for 10 minutes. Compared with MPR1, MPR1swap transferred more phosphoryl groups to

SSK1-R2 (Figure 4-13, lanes 4 and 5) and SKN7-R3 (Figure 4-13, lanes 6 and 7) in terms of percentage. MPR1 swap transferred 50% of its phosphoryl group to SSK1-R2 (Figure 4-13 lane 5), almost 80% phosphoryl group to SKN7-R3 (Figure 4-13, lane 6). Both percentages are obviously higher than those of wild type MPR1. In the time course of MPR1 swap's phosphotransfer to SKN7-R3, the capability of MPR1 swap to transfer phosphoryl groups to SKN7-R3 was enhanced significantly (Figure 4-14). Almost 100% of the phosphoryl group was passed on to SKN7-R3 within 10 minutes from MPR1 swap.

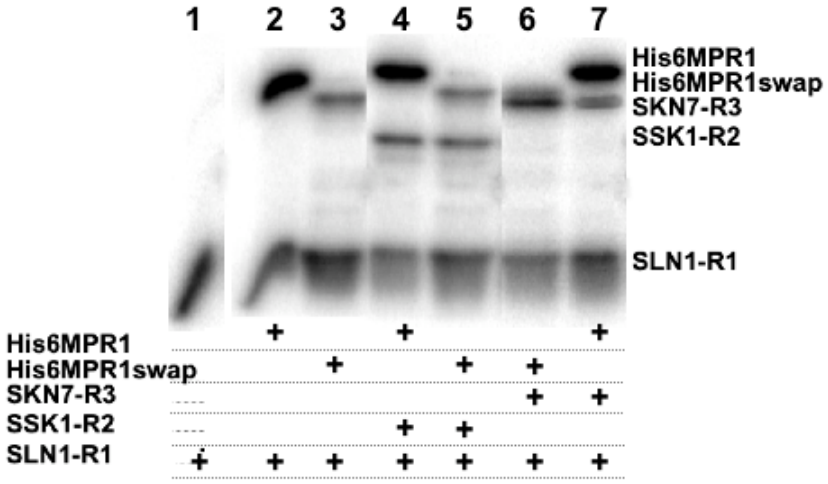

Figure 4-13. Phosphoryl transfer from MPR1/MPR1 swap to SSK1-R2/SKN7-R3 in presence of SLN1-R1. Phospho-SLN1-R1 was incubated with MPR1 , with (lane 4 and 7) or without (lane 2) SSK1-R2 and SKN7-R3, at room temperature for 10 minutes. MPR1 swap was treated in the same way (lane 3 was with phospho-SLN1-R1 only, lane 5 was with phospho-SLN1-R1 and SSK1-R2, lane 6 was with phospho-SLN1-R1 and SKN7-R3). The concentration of reactants was equal except SLN1-R1, which is one third of the others'.

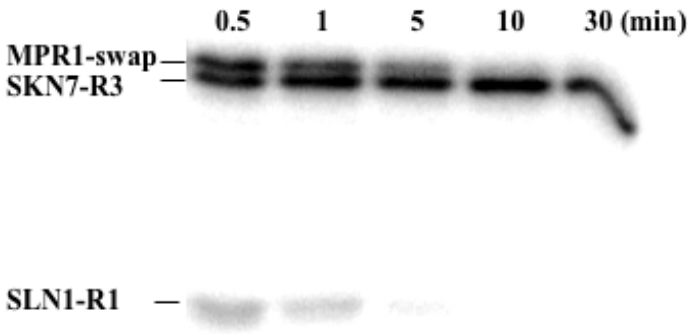

Figure 4-14. Time course of phosphotransfer from MPR1 swap to SKN7-R3 in presence of SLN1-R1. MPR1 swap was incubated with phospho-SLN1-R1 at room temperature for 2 minutes. Then SKN7-R3 was added into the reaction.

The decrease on MPR1 swap's ability to receive phosphoryl groups from phospho-SLN1-R1 may be due to disruption of the overall binding capacity to SLN1-R1. But swapping did enhance MPR1's phosphorelay capacity toward SSK1-R2 and SKN7-R3, meaning that the αA helix of YPD1 might be more important in HPt/SSK1-R2 and HPt/SKN7-R3 interactions than in HPt/SLN1-R1 interaction.

4.2.4 *In vivo* assay for testing phosphotransfer activities of the YPD1 mutants to SKN7

A quantitative β-galactosidase assay, an *in vivo* assay to test phosphotransfer from HPt proteins to full length SKN7, was used to compare phosphorelay capacity of YPD1, MPR1 and YPD1 mutants. The yeast plasmid pJF1416 has an OCH1 promoter, a promoter that can be activated by phosphorylated transcription factor SKN7 (SKN7~P), and the activation is SKN7~P concentration dependent (Figure 4-15). The LacZ gene is the reporter gene under the control of the OCH1 promoter. When both plasmids pJF1416 and pRS315-(YPD1, MPR1, YPD1 mutants) were transformed into the yeast strain

OU[#]140, the phosphotransfer ability of the HPt proteins toward SKN7 can be estimated by measuring β-galactosidase activity of the cell lysate (Figure 4-16). The more SKN7 be phosphorylated by the HPt protein, the higher the β-galactosidase activity.

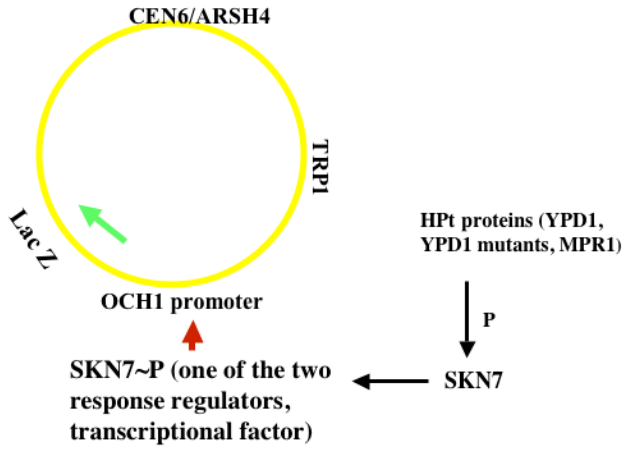

Figure 4-15. Plasmid pJF1416 used in β-galactosidase assay. The yeast plasmid pJF1416 has an OCH1 promoter that can be activated by phosphorylated transcription factor SKN7 (SKN7~P). The activation of the promoter is SKN7~P concentration dependent. The LacZ gene is the reporter gene under the control of the OCH1 promoter.

β-galactosidase assay result showed that MPR1 generated more β-galactosidase activity than YPD1 (Figure 4-16), meaning that MPR1 transferred more phosphoryl groups to full length SKN7 *in vivo*. This result agrees with the observation from *in vitro* phosphorylation assay that SKN7-R3 could receive more phosphoryl group from MPR1 than from YPD1. YPD1 swap, YPD1 Q76T and YPD1 swapE58K gave similar β-galactosidase value as that from YPD1, indicating that the mutations on YPD1 did not alter YPD1 phosphotransfer ability toward SKN7. This again agrees with the observation in Figures 4-10, 11, and 12.

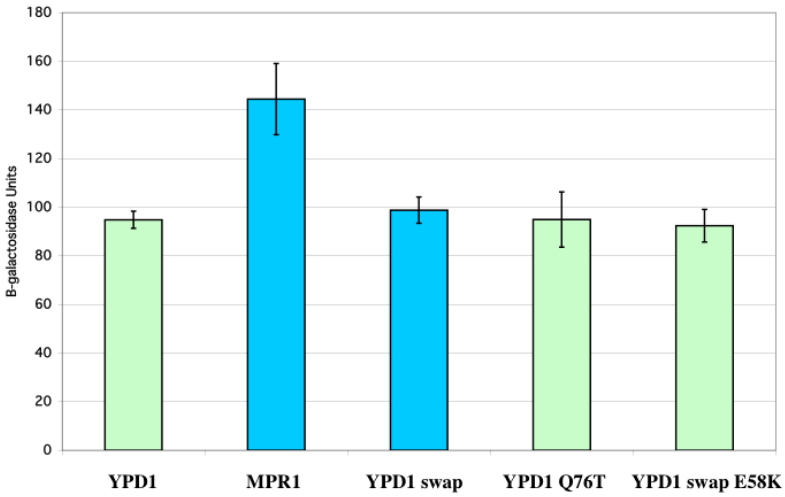

Figure 4-16. β-galactosidase assay results. β-galactosidase activity was calculated as: β-gal unit = 1000 x OD_{420}/ (t x V x OD_{600}), where t is time in minutes and V is the volume of the initial aliquot, OD_{600} is the cell density at the start of the assay, OD_{420} is absorbance by o-nitrophenol.

4.3. Discussion

YPD1 and MPR1 displayed different phosphotransfer specificity toward the two response regulator domains from *S. cerevisiae*. MPR1 transferred more phosphoryl groups to SKN7-R3 than to SSK1-R2, while YPD1 favored SSK1-R2 over SKN7-R3. In order to investigate the reason that leads to this difference, YPD1 and MPR1 were mutated based on sequence alignment and Porter *et al*.'s study on response regulator binding site on YPD1 (Porter *et al*., 2003; Porter *et al*., 2005).

The αA helix on YPD1 has the least number of identical amino acid residues with MPR1, yet it makes the most hydrophobic interaction and hydrogen bonding with SLN1-R1. Based on the fact that the response regulator domains are highly similar to each other, we presumed that the YPD1/SLN1-R1 interaction may represent YPD1/SSK1-R2 and YPD1/SKN7-R3 interactions to some degree. That was why the

αA helix on YPD1 was chosen to be mutated to the corresponding helix on MPR1, and vice versa. In the YPD1/SLN1-R1 crystal structure (Xu *et al*., 2003), E58 on YPD1 is located adjacent to the β3-α3 loop, which is the least conserved loop between SSK1-R2 and SKN7-R3. The E58 residue may play a role in phosphotransfer specificity between HPt proteins and RRs. It was mutated to K, which is the corresponding amino acid residue on MPR1. Q76 and W80 are the only two residues on YPD that are putatively involved in YPD1/RRs specific interactions but they are not conserved on MPR1. Q76 was mutated to T. W80 was planned to be mutated originally, but was not since other mutations (swapping, Q76T and E58K mutations) did not change HPt-RR phosphotransfer specificity.

YPD1 swap, YPD1 swapE58K and YPD1 Q76T did slightly change phosphotransfer ability compared with wild type YPD1. The phosphotransfer rate from the mutated HPt protein to SKN7-R3 was increased in all three mutations, while the phosphotransfer rate toward SSK1-R2 was slightly decreased in the YPD1 Q76T mutant. These changes in phosphotransfer rate upon mutation may be due to the change of binding of RRs to YPD1. But YPD1-RR phosphotransfer specificity was not altered. It is highly possible that it is not a single amino acid residue's responsibility, but the combined differences on MPR1 and YPD1 that determines HPt proteins' phosphotransfer specificity toward RRs.

One of the possible reasons that swapping the αA helix of YPD1 to the counter part helix on MPR1 caused decreased phosphotransfer from phospho-SLN1-R1 to YPD1 could be increased steric hindrance at the protein-protein interface, since L14 on YPD1 was mutated to phenylalanine. Another reason that contributed to the decrease could be

that one of the hydrogen-bond interactions between YPD1 and SLN1-R1 was disrupted after the YPD1 E16 residue was mutated to glutamine. The YPD1/SLN1-R1 co-crystal structure (both $P2_12_12_1$ complex and $P3_2$ complex) showed that E16 of YPD1 formed hydrogen-bond interaction with R1199 on SLN1-R1 (Xu *et al.,* 2003). The third possible explanation for decrease could be that the surface area of hydrophobic patch that is critical for YPD1/SLN1-R1 interaction was changed after I13 of YPD1 was mutated to valine and I17 was mutated to leucine. I13 of YPD1 with P1196 of SLN1-R1, and I17 of YPD1 with V1102 of SLN1-R1 are two of the hydrophobic interactions observed on YPD1/SLN1-R1 co-crystal structure (Xu *et al.,* 2003).

YPD1 swapE58K received even less phosphoryl group than YPD1 swap from phosphoryl SLN1-R1. A possible reason that the E58K mutation further disrupted phosphotransfer from SLN1-R1 to YPD1 could be that E58K mutation decreased intermolecular contacts between YPD1 and SLN1-R1.

Although swapping decreased MPR1's ability to receive phosphoryl group from phospho-SLN1-R1, the mutation increased MPR1's ability to transfer phosphoryl group to both SSK1-R2 and SKN7-R3. It may indicate that the αA helix on YPD1 might be more important in interaction with SSK1-R2 and SKN7-R3.

4.4. Summary

The C-terminal domain of MPR1 shares high identity with full length YPD1. The two HPt proteins displayed different phosphotransfer specificity *in vitro* toward the two response regulator domains from *S. cerevisiae*, SSK1-R2 and SKN7-R3. MPR1 transferred more phosphoryl groups to SKN7-R3 than to SSK1-R2 when the two RRs

competed for them, while YPD1 showed opposite specificity, transferring more phosphoryl groups to SSK1-R2 than to SKN7-R3. YPD1 mutants and a MPR1 mutant were tested for specificity changes to find out the structural basis behind the specificity of HPt proteins toward RRs. All of the YPD1 mutants failed to convert YPD1's overall preference for SSK1-R2 in phosphodonor activity. Neither the the αA helix, nor the E58 residue, nor the Q76 residue on YPD1 alone are responsible for HPt-RR phosphotransfer specificity, but may be responsible for binding affinity of RRs to YPD1 since these muations changed phosphotransfer rate from YPD1 to RRs. The mutant MPR1-swap had decreased efficiency in receiving phosphoryl group from phospho-SLN1-R1, but the protein had increased ability to transfer phosphoryl group to both SSK1-R2 and SKN7-R3. It may indicate that the αA helix on YPD1 might be more important in interacting with SSK1-R2 and SKN7-R3 than with SLN1-R1. It is highly possible that it is not a single amino acid residue's responsibility, but the combined differences on MPR1 and YPD1 that determines the HPt proteins phosphotransfer specificity toward RRs.

References

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W. & Hoskins, R.A. (2000). The genome sequence of *Drosophila melanogster*. *Science.* **287**: 2185-2195.

Alberts, A.S., Bouquin, N., Johnston, L.H. & Treisman, R. (1998). Analysis of RhoA-binding proteins reveals an interaction domain conserved in heterotrimeric G protein b subunits and the yeast response regulator protein Skn7. In: *J. Biol. Chem.* **273**: 8616-8622.

Alexander, M. R., Tyers, M., Perret, M., Craig, B. M., Fang, K. S. & Gustin, M. C. (2001). Regulation of cell cycle progression by Swe1p and Hog1p following hypertonic stress. *Mol. Biol. Cell* **12**: 53-62.

Alexandrov, N. N., Nussinov, R. & Zimmer, R. M. (1996). Fast protein fold recognition via sequence to structure alignment and contact capacity potentials. In: Pacific Symposium on Biocomputing (Hunter L&Klein TE, Eds.), Vol. pp. 53-72. World Scientific Publishing Co., Singapore.

Aono, T., Yanai, H., Miki, F., Davey, J. & Shimoda, C. (1994). Mating pheromone-induced expression of the mat1-Pm gene of *Shizosaccharomyces pombe*: Identification of signaling components and characterization of upstream controlling elements. *Yeast*, **10**: 757-770.

Aoyama, K., Aiba, H. & Mizuno T. (2001). Genetic analysis of the His-to-Asp phosphorelay implicated in mitotic cell cycle control: involvement of histidine-kinase genes of *Schizpsaccharomyces pombe. Biosci. Biotechnol. Biochem*., **65**(100): 2347-2352.

Aoyama, K., Mitsubayashi, Y., Aiba, H. & Mizuno T. (2000). Spy1, a histidine-containing phosphotransfer signaling protein, regulates the fission yeast cell cycle through the Mcs4 response regulator. *J. Bacteriol*. **182**: 4868-4874.

Aravind, L. & Ponting, C.P. (1997). The GAF domain: an evolutionary link between diverse phototransducing proteins. *Trends Biochem. Sci*. **22**: 458-459.

Arricau, N., Hermant, D., Waxin, H., Ecobichon, C.,Duffey, P. S. & Popoff, M. Y. (1998). The RcsB-RcsC regulatory system of *Salmonella typhi* differentially modulates the expression of invasion proteins, flagellin and Vi antigen in response to osmolarity. *Mol. Microbiol* **29**: 835-850.

Asakura, Y., Hagino, T., Ohta, Y., Aokil, K., Yonekura-Sakakibara, K., Deji, A., Yamaya, T., Sugiyama, T. & Skakibara, H. (2003). Molecular characterization of His-Asp phosphorelay signaling factors in maize leaves: Implications of the signal divergence by cytokinin-inducible response regulators in the cytosol and the nuclei. *Plant Mol. Biol*. **52**(2): 331-341.

Av-Gay, Y., Jamil, S. & Drews, S. J. (1999). Expression an characterization of the Mycobacterium tuberculosis serine/threonine protein kinase PknB. *Infect. Immun*. **67**(11): 5676-5682.

Bartel, P. L. & Fields, S., Eds. (1997). The yeast two-hybrid system. New York: Oxford University Presss.

Baunuett, F. (1998). Signalling in the Yeasts: an informational cascade with links to the filamentous fungi. *Microbiol. Mol. Boil. Rev*. **62**: 249-274.

Besant, P. G. & Attwood, P. V. (2005). Mammalian histidine kinases. *Biochem. Biophys. Acta* **1754**: 281-290.

Bilwes, A. M., Alex, L. A., Crane, B. R. & Simon, M. I. (1999). Structure of CheA, a signal-transducing histidine kinase. *Cell.* **96**: 131-141.

Booher, R. & Beach, D. (1987). Interaction between *cdc13*$^+$ and *cdc2*$^+$ in the control of mitosis in fission yeast: dissociation of the G1 and G2 roles of the *cdc2*$^+$ protein kinase. *EMBO J*. **6**: 3441-3447.

Bouquin, N., Johnson, A. L., Morgan, B. A. & Johnston, L. H. (1999). Association of the cell cycle transcription factor Mbp1 with the Skn7 response regulator in budding yeast. *Mol. Biol*. *Cell* **10**: 3389-3400.

Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E. & Gustin, M. C. (1993) An osmosensing signal transduction pathway in yeast. *Science* **259**: 1760-1763.

Brown, J., North, L. S. & Bussey. H. (1993). SKN7, a yeast multicopy suppressor of a mutation affecting cell wall beta-glucan assembly, encodes a product with domains homologous to prokaryotic two-component regulators and to heat shock transcription factors. *J. Bacteriol*. **175**: 6908-6915.

Brown, J. L., Bussey, H. & Stewart, R. C. (1994). Yeast Skn7p functions in a eukaryotic two-component regulatory pathway. *EMBO J.* 13, 5186-5194.

Buck, V., Quinn, J., Pino, T. S., Martin, H., Saldanha, J., Makino, K., Morgan, B. A. & Millar, J. B. A. (2000). Peroxide sensors for the fission yeast stress-activated mitogen-activated protein kinase pathway. *Mol. Bio. Cell.* **12**: 407-419.

Burbulys, D., Trach, K. A. & Hoch, J. A. (1991). Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. *Cell.* **64**: 545-552.

Calera, J. A. & Calderone, R. A. (1999). Identification of a putative histidine kinase two-component phosphorelay gene (CaSSK1) in *Candida albicans. Yeast*. **15**: 1243-1254.

Calera, J. A., Choi, G. H. & Calderone, R. A. (1998). Identification of a putative histidine kinase two-component phosphorelay gene (CaHK1) in *Candida albicans. Yeast*. **14**: 665-674.

Calera, J. A., Herman D. & Calderone, R. A. (2000). Identification of YPD1, a gene of *Candida albicans* which encodes a two-component phosphohistidine intermediate protein. *Yeast*. **16**: 1053-1059.

Chang, C., Kwok S.F., Bleecker A.B. & Meyerowitz, E.M. (1993). Arabidopsis ethylene-response gene ETR1: similarity of product to two-component regulators. *Science.* **262**: 539-544.

Chang, W. T., Thomason, P. A., Gross, J. D. & Newell, P. C. (1998). Evidence that the RdeA protein is a component of a multistep phosphorelay modulating rate of development in *Dictyostelium EMBO J.* **17**: 2809-2816.

Charbonneau, H., Prusti, R.K., LeTrong, H., Sonnenburg, W.K., Mullaney, P.J., Walsh, K.A. & Beavo, J.A. (1990). Identification of a noncatalytic cGMP-binding domain conserved in both the cGMP-stimulated and photoreceptor cyclic nucleotide phosphodiesterases. *Proc. Natl. Acad. Sci.* USA **87**: 288-292.

Charizanis, C., Juhnke, H., Krems, B. & Entian. K. D. (1999). Expression of bacterial mtlD in *Saccharomyces cerevisiae* results in mannitol synthesis and protects a glycerol-defective mutant from high salt and oxidative stress. *J. Bacteriol.* **179**: 157-162.

Charizanis, C., Juhnke, H., Krems, B. & Entian. K. D. (1999). The oxidative stress response mediated via Pos9/Skn7 is negatively regulated by the Ras/PKA pathway in *Saccharomyces cerevisiae*. *Mol. Gen. Genet*. **261**: 740-752.

Chien, C. T., Bartel, P. L., Sternglanz, R. & Fields, S. (1998). The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. *Proc. Natl., Acad. Sci.* (USA), **88**: 9578-9582.

Cottare, G. (1997). Mcs4, a two-component system response regulator homologue, regulates the *Schizosaccharomyces pombe* cell cycle control. *Genetics* **147**: 1043-1051.

Crews, S.T. & Fan, C.M. (1999). Remembrance of things PAS: regulation of developmemt by bHLH-Pas proteins. *Curr. Opin. Gnet*. **9**: 580-587.

Estojak, J., Brent, R. & Golemis, E. A. (1995). Correlation of two-hybrid affinity data with in vitro measurements. *Mol. Cell. Biol*. **15**: 5820-5829.

Falke, J. J., Bass, R. B., Butler, S. L., Chervitz, S. A. & Danielson, M. A. (1997). The two-component signaling pathway of bacterial chemotaxis: a molecular view of signal transduction by receptors, kinases, and adaptation enzymes. *Annu. Rev. Cell Dev. Biol* **13**: 457-512.

Fields, S. & Song, O. K. (1989). A novel genetic system to detect protein-protein interactions. *Nature.* **340**: 245-246.

Freeman, J. A. & Bassler B. L. (1999). A genetic analysis of the function of LuxO, a two-component response regulator involved in quorum sensing in *Vibrio harveyi. Mol. Microbiol.* **31**: 665-667.

Freeman, J. A. & Bassler, B. (1999). Sequence and function of LuxU: a two-component phosphorelay protein ahtat regulates quorum sensing in *Vibrio harveyi. J. Bacteriol.* **181**: 899-906.

Friedman, D. I. & Gottesman, M. (1982). Lytic mode of *lambda* development. In *Lambda II*. Cold Spring Harbor Laboratory. New York. 21-51.

Gacto, M., Soto, T., Vicente-Soler, J., Villa, T. G. & Cansado, J. (2003). Learning from yeasts: intracellular sensing of stress conditions. *Int. Microbiol.* **6**: 211-219.

Greenall, A., Hadcroft, A. P., Malakasi P., Jones, N., Morgan, A. B., Hoffman, C. S. & Whitehall, S. K. (2002). Role of fission yeast TUP1-like repressors and PRR1 transcription factor in response to salt stress. *Mol. Biol. Cell*. **13**: 2977-2989.

Gustin, M. C., Albertyn, J., Alexander, M. & Davenport, K. (1998). MAP kinase pathways in the yeast S. cerevisiae. *Micro. Mol. Biol. Rev*. **62**: 1264-1300.

Hoch, J. A. (1993). Regulation of the phosphorelay and the initiation of sporulation in *Bacillus subtilis. Annu. Rev. Microbiol*. **47**: 441-465.

Hohmann, S. (2002). Osmotic stress signaling and osmoadaptation in yeasts. *Micro. Mol. Biol. Rev*. **66**: 300-372.

http://megasun.bch.umontreal.ca/People/lang/species/spo/spogeneral.html From GOBASE, a taxonomically broad organelle genome database that organizes and integrates diverse data related to organelles.

Ishige, K., Nagasawa, S., Tokishita, S. H. & Mizuno, T. (1994). A novel device of bacterial signal transducers. *EMBO J*., **13**: 5195-5202.

Jang, M. J., Jwa, M., Kim, J. & Song, K. (2001). Selective inhibition of MAPKK Wis1 in the stress-activated MAPK cascade of *Schizosaccharomyces pombe* by novel berberine derivatives. *J. Biol. Chem*., **277**(14): 12388-12395.

Janiak-Spens, F., Sparling, J. M., Gurfinkel, M. & West, A. H. (1999). Differential stabilities of phosphorylated response regulator domains reflect functional roles the yeast osmoregulatory SLN1 & SSK1 proteins. *J Bacteriol* **181**: 411-417.

Janiak-Spens, F. & West, A. H. (2000). Functional roles of conserved amino acid residues surrounding the phosphorylatable histidine of the yeast phosphorelay protein YPD1. *Mol. Micro*. **37**(1): 136-144.

Janiak-Spens, F., Cook, P. F. & West, A. H. (2005). Kinetic analysis of YPD1-dependent phosphotransfer reactions in the yeast osmoregulatory phosphorelay system. *Biochemistry* 44: 377-386.4

Jeanmougin, F, Thompson, J. D., Gouy, M., Higgins, D. G. & Gibson, T. J. (1998) Multiple sequence alignment with Clustal X. *Trends Biochem. Sci.* **23**: 403-405.

Kato, M., Mizuno, T., Shimizu, T. & Hakoshima, T. (1997). Insights into multistep phosphorelay from the crystal structure of the C-terminal HPt domain of ArcB. *Cell* **88**: 717-723.

Kehoe, D. M. & Grossman, A. R. (1997). New classes of mutants in complementary chromatic adaptation provide evidence for a novel four-step phosphorelay system. *J. Bacteriol*. **179**: 3914-3921.

Ketela, T., Brown, J. L., Stewart, R. C. & Bussey, H. (1998). Yeast Skn7p activity is modulated by the Sln1p-Ypd1p osmosensor and contributes to regulation of the HOG1 pathway. *Mol. Gen. Genet*. **259**: 372-378.

Ketela, T., R. Green, & H. Bussey. (1999). *Saccharomyces cerevisiae* Mid2p is a potential cell wall stress sensor and upstream activator of the PKC1-MPK1 cell integrity pathway. *J. Bacteriol*. **181**: 3330-3340.

Krantz, M., Becit, E. & Hohmann, S. (2006). Comparative analysis of HOG pathway proteins to generate hypotheses for functional analysis. *Curr. Genet*. **49**: 152-165.

Li, S., Ault, A., Malone, C. L., Raitt, D., Dean, S., Johnston, L. H., Deschenes, R. J. & Fassler, J. S. (1998). The yeast histidine protein kinase, Sln1p, mediates phosphotransfer to two response regulators, Ssk1p and Skn7p. *EMBO J*. **17**: 6952-6962.

Li, S., Dean, S., Li, Z., J. Horecka, J., Deschenes, R. J. & Fassler. J. S. (2002). The eukaryotic two-component histidine kinase Sln1p regulates OCH1 via the transcription factor, Skn7p. *Mol. Biol*. *Cell* **13**: 412-424.

Lorenz, M. C. & Heitman, J. (1998). Regulators of pseudohyphal differentiation in *Saccharomyces cerevisiae* identified through multicopy suppressor analysis in ammonium permease mutant strains. *Genetics* **150**: 1443-1457.

Lucast, L. J., Batey, R.T. & Doudna, J. A. (2000). Large-scale purification of a stable form of recombinant tobacco etch virus protease. *Biotechniques*. **30**: 544-554.

Madhusudan, Zapf J., Hoch, J. A., Whiteley, J. M., Xuong, N. H. & Varughese, K. I. (1997) A response regulatory protein with the site of phosphorylation blocked by an arginine interaction: crystal structure of Spo0F from Bacillus subtilis. *Biochemistry* **36**: 12739-45.

Madhusudan, Zapf J., Whiteley, J. M., Hoch, J. A., Xuong, N. H. & Varughese, K. I. (1996). Crystallization and preliminary X-ray analysis of a Y13S mutant of Spo0F from *Bacillus subtilis*. *Acta Cryst* **52**: 589-590.

Maeda T., Wurgler-Murphy, S. M. & Saito, H. (1994). A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature* **239**:242-245.

Maher, B. A. (2002). A tale of two-hybrid. *The Scientist*: 38-39.

Makino, S., Kiba, T., Imamura, A., Hanaki, N., Nakamura, A., Suzuki, T., Taniguchi, M., Ueguchi, C., Sugiyama, T. & Mizuno, T. (2000). Genes encoding pseudo-response regulators: insight into His-to-Asp phosphorelay and circadian rhythm in Arabidopsis thaliana. *Plant Cell Physiol*. **41**: 791–803.

Melnikova, M., Nazzaruolo, B. & Xie, H. B. (1997). The evolution of fungi. *Evolution* V23.0058 Dr. Fitch.

Miyata, S., Urao, T., Yamaguchi-Shinozaki, K. & Shinozaki, K. (1998). Characterization of genes for two-component phosphorelay mediators with a single HPt domain in Arabidopsis thaliana. *FEBS Lett*. **437:**11-14.

Mizuno, T. (1998). His-Asp phosphot6ransfer signal transduction. *J Biochem* (Toyko) **123**(4): 555-563.

Mloz, L., R. Booher, P. Young, & D. Beach. (1989). cdc2 and the regulation of mitosis: six interaction mcs genes. *Genetics* **122**:773-782.

Morgan, B. A., Bouquin, N., Merrill, G. F. & Johnston. H. (1995). A yeast transcription factor bypassing the requirement for SBF and DSC1/MBF in budding yeast has homology to bacterial signal transduction proteins. *EMBO J*. **14**: 5679-5689.

Morgan, B. A., Banks, G. R., Toone, W. M., Raitt, D., Kuge, S. & Johnston, L. H. (1997). The Skn7 response regulator controls gene expression in the oxidative stress response of the budding yeast Saccharomyces cerevisiae. *EMBO J.* **16**: 1035-1044.

Mourey, L., Da Re, S., Pelelacq, J. D., Tolstykh, T., Faurie, C., Guillet, V., Stock, J.B., & Samama, J. P. (2001). Crysal structure of the CheA histidine phosphotransfer domain that mediates response regulator phosphorylation in bacterial chemotaxis. *J. Biol. Chem*. **276**: 31074-31082.

Nakamichi, N., Yanada, H., Aiba, H., Aoyama, K., Ohmiya, R. & Mizuno, T. (2003). Characterization of the Prr1 response regulator with special reference to sexual development in *Schizosaccharomyces pombe. Biosci. Biotechnol. Biochem*. **67**(3): 547-555.

Nguyen, N.A., Lee, A., Place, W. & Shiozaki, K. (2000). Multistep phosphorelay proteins transmit oxidatiove stress signals to the fission yeast stress-activated protein kinase. *Mol. Biol. Cell* **11**: 1169-1181.

Ohmiya, R., Kato, C., Yamada, H., Aiba, H. & Mizuno, T. (1999). A fission yeast gene (prr1+) that encodes a response regulator implicated in oxidative stress response. *J. Biochem*. **125**:1061-1066.

Ohmiya, R., Yamada, H., Kato, C., Aiba, H. & Mizuno, T. (2000). The Prr1 response regulator is essential for transcription of *stl1*$^{+}$ and for sexual development in fission yeast. *Mol. Gen. Genet.* **264**: 441-451.

Ota, I.M. & Varshavsky, A. (1993). A yeast protein similar to bacterial two-component regulators. *Science*. **262**(22): 566-569.

Pao, G.M. & Saier, M.H.Jr. (1995). Response regulators of the bacterial signal transdution system systems: selective domain shuffling during evolution. *J. Mol. Evol*. **40**: 136-154.

Parkinson, J. S. & E. C. Kofiod, (1992). Communication modules in bacterial signaling proteins. *Annu. Rev. Genet*. **26**: 71-112.

Parkinson, J. S. & Kofold, E. C. (1992). Communication modules in the bacteria

Perraud, A.L., Weiss, V. & Gross, R. (1999). Signaling pathways in two-component phosphorelay systems. *Trends Microbiol*. **7**: 115-120.

Popov, K. M., Kedishvili, N. Y., Zhao, Y., Shimomura, Y., Crabb, D. W. & Harris, R. A. (1993). Primary structure of pyruvate dehydrogenase kinase establishes a new family of eukaryotic protein kinases. J. Biol. Chem. **268**: 26602–26606.

Porter, S. W., Xu, Q., & West, A. H. (2003). Ssk1p resonse regulator binding surface on histidine-containing phosphotransfer protein Ypd1p. *Euk. Cell*, **2**: 27-33.

Porter, S. W. (2004). Identification and analysis of the response regulator binding site on the surface of the histidine-containing phosphotransfer protein YPD1 from *Saccharomyces cerevisiae*. Ph.D Dissertation of University of Oklahoma.

Porter, S. W. & West, A. H. (2005). A common docking site for response regulators on the yeast phosphorelay protein YPD1. *Biochim. Biophys. Acta* **1748**:138-145.

Posas, F., Wurgler-Murphy, S. M., T. Maeda, E. A. Witten, T. C. Thai, & H. Saito. (1996). Yeast HOG11 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 "two-component osmosensor. *Cell* **86**: 885-875.

Potts, M., Sun, H., Mockaitis, K., Kennelly, P. J., Reed, D. & Tonks, N. K. (1993). A protein-tyrosine/serine phosphatase encoded by the genome of the cyanobacterium Nostoc. *J. Boil. Chem*. **268**: 7632-7635.

Raitt, D. C., Johnson, A. L., Erkine, A. M., Makino, K., Morgan, B., Gross, D. S. & Johnston, L. H. (2000). The Skn7 response regulator *of Saccharomyces cerevisiae* interacts with Hsf1 in vivo and is required for the induction of heat shock genes by oxidative stress. *Mol. Biol. Cell.* **11**: 2335-2347.

Rhodes G. (1998). Crystallography made crytal clear. *Adcdemic Press, INC.*

Robinson, L. L. & Brasch, M. A. (19980. Identification of interacting proteins with the ProQuest two-hybrid system. *Focus*, **20**: 19-23.

Robinson, V. L., Buckler, R. D. & Stock, A. M. (2000). A tale of two components: a novel kinase and a regulatory switch. *Nat. Struct. Biol*. 7(8): 626-633.

Rost, B. & Sander, C. (1993). Prediction of protein secondary structure at better than 70% accuracy. *J Mol Biol*, **232**:584-599.

Saito, H (2001) Histidine phosphorylation and two-component signaling in eukaryotic cells. *Chem. Rev.* **101**: 2497-2509.

Santos, J. L. & Shiozaki, K. (2001). Fungal histidine kinases. Science's Stke. www.stke.org/cgi/content/full/OC_sigtrans:2001/98/re1.

Schaller, G. E., Mathews, D.E., Gribskov, M. & Walker, J. C. (2002). Two-component signaling elements and histidyl-aspartyl phosphorelays. *The Arabidopsis Book*. 1-9.

Schaller, G. E. (2000). Histidine kinases and the role of two-component systems in plants: *Adv. Bot. Res.* **32**: 109–148.

Serebriiskii, I. G. & Golemis, E. A. (2000). Uses of *lac*Z to study gene function: Evaluation of –galactosidase assays employed in the yeast two-hybrid system *Anal. Biochem*. **285**: 1-15.

Shieh, J. C., Wilkinson, M. G., Buck, V., Morgan, B. A., Makino, K. & Millar, J. B. (1997). The Mcs4 response regulator coordinately controls the stress-activated Wak1-Wis1-Sty1 MAP kinase pathway and fission yeast cell cycle. *Genes Dev*. **11**: 1008-1022.

Shiozaki, K., Shiozaki, M. & Russell, P. (1997). Mcs4 mitotic catastrophe suppressor regulates the fission yeast cell cycle through the Wak1-Wis1-Spc1 kinase cascade. *Mol. Bio. Cell* **8**: 409-419.

Song, H. K., Lee, J. Y., Lee, M. G., Min, J., Yang, J. K., & Suh, S. W. (1999). Insights into eukaryotic mutistep phosphorelay signal transduction revealed by the crystal structure of YPD1 from *Saccharomyces cerevisiae*. *J. Mol. Biol*. **293**: 753-761.

Stewart R. C. (1996). Kinetic characterization of phosphotransfer between CheA and CheY in the bacterial chemotaxis signal transduction pathway. *Biochemistry*. **36**: 2030-2040.

Stewart R. C., Jahreis, K. & Parkinson, J. S. (2000). Rapid phosphotransfer to CheY from a CheA protein lacking the CheY-binding domain. *Biochemistry* **39**: 13157-13165.

Stock, A. M., Robinson, V. L. & Goudreau, P. N. (2000). Two-component signal transduction. *Annu. Rev. Biochem***. 69**: 183-215.

Stock, A. M. & West, A. H. (2002). Response regulator proteins and their interactions with histidine protein kinases. Histidine kinases in Signal transduction. *Elsevier Science* (USA). 237-253.

Stock, J. (1999). Signal transduction: gyration protein kinases. *Curr. Biol*. **9**: R364-R367.

Stock, J. B., Surette, M. G., Levit, M. & Park, P. (1995). "Two-component signal transduction systems: Structure function relationships and mechanisms of catalysis", in Two-component signal transduction, Hoch, J.A. & Silhavy, T.J. Editors American Society for Microbiology: Washington, D.C. pp25-51.

Sugawara, H., Kawano, Y., Hatakeyama, T., Yamaya, T., Kamiya, N., & Kakibara, H. (2005). Crystal structure of the histidine-containing phosphotransfer protein ZmHP2 from maize. *Protein Science*. **14**: 202-208.

Sugimoto, A., Iino, Y., Maeda, T., Watanabe, Y. & Yamamoto, M., (1991). *Schizosaccharomyces pombe stell*+ encodes a transcription factor with an HMG motif that is a critical regulator of sexual development. *Genes Dev*., **5**: 1990-1999.

Suzuki, T., Imamura, A., Ueguchi, C. & Mizuno, T. (1998). Histidine-containing phosphotransfer(HPt) signal transducers implicated in His-to-Asp phosphorelay in *Arabidopsis. Plant Cell Physiol*. **39**: 1258-1268.

Suzuki, T., Sakurai, K., Imamura, A., Nakamura, A., Ueguchi, C. & Mizuno, T. (2000). Compilation and characterization of histidine-containing phosphotransmitters implicated in His-to-Asp phosphorelay in plants: AHP signal ransducers of *Arabidopsis thaliana. Biosci. Biotechnol. Biochem.* **64**: 2486-2489.

Takeda, S., Fujisawa, Y., Matsubara, M., Aiba, M. & Mizuno, T. (2001). A novel feature of the multistep phosphorelay in Escherichia coli: a revised model of the RcsC-YojN-RcsB signaling pathway implicated in capsular synthesis and swarming behavior. *Mol. Microbiol*. **40**(2): 440-450.

Tan, E., Besant, P.G. & Attwood, P.V. (2002). Mammalian histidine kinases: Do they really exist? *Biochem*. **41**: 3843-3851.

Tan, H., Janiak-Spens, F. & West, A. H. (2007). Functional Characterization of the Phosphorelay Protein Mpr1p from *Schizosaccharomyces pombe*. *FEMS Yeast Research*. In press.

Tanaka, T., Saha, S., Tomomori, C., Ishima, R., Lir, D., Tong, K., Park, H., Dutta, R., Qin, L., Swindells, M., Yamazaki, T., Ono, A., Kainosho, M., Inouye, M. & Ikura, M. (1998). NMR structure of the histidine kinased domain of the E. Coli osmosensor Envz. *Nature* **396**: 88-92.

Thomason, P. & Kay, R. (2000). Eukaryotic signal transduction via histidine-aspartate phosphorelay. *J. Cell Sci*. **113**: 3141-3150.

Thomason, P. A., Traynor, D., Cavet, G., Chang, W. T., Harwood, A. J. & Kay, R. R. (1998). An intersection of the camp/PKA and two-component signal transduction systems in *Dictyostelium. EMBO. J.* **17**: 2838-2845.

Urao, T., Yakubov, B., Satoh, R., Yamaguchi-Shinozaki, K., Seki, M., Hirayama, T. & Shinozaki, K. (1999). A transmembrane hybrid-type histidine kinase in *Arabidopsis* functions as an osmosensor. *Plant Cell* **11**: 1743-1754.

Varughese, K. I., Madhusudan, Zhou, X. Z., Whiteley, J. M. & Hoch, J. A. (1998). Formation of a novel four-helix bundle and molecular recognition sites by dimerization of a response regulator phosphotransferase. *Mol Cell* **2**: 485-493.

Vilella, F., Herrero, E., Torres, J. & de la Torre-Ruiz, M. A. (2005). Pkc1 and the upstream elements of the cell integrity pathway in Saccharomyces cerevisiae, Rom2 and Mtl1, are required for cellular responses to oxidative stress. *J Biol Chem* **280**(10): 9149-59.

Villarino, A., Duran, R., Wehenkel, A., Fernandez, P., England, P., Brodin, P., Cole, S. T., Zimny-Arndt, U., Jungblut, P. R., Cervenansky, C & Alzari, P. M. (2005). Proteomic identification of M. tuberculosis protein kinase substrates: PknB recruits GarA, a FHA domain-containing protein, through activation loop-mediated interactions. *J. Mol. Biol.* **350**(5): 953-963.

Voltz, K. (1993). Structural conservation in the CheY superfamily. *Biochemistry* **32**: 11741-11753.

Warbrick, E. & Fantes. P. A. (1991). The wis1 protein kinase is a dosage-dependent regulator of mitosis in *Schizosaccharomyces pombe*. *EMBO J*. **10**: 4291-4299.

Watanabe, Y., Iino, Y., Furuhata, K., Shimoda, C. & Yamamoto, M. (1998). The *S. pombe mei2* gene encoding a crucial molecule for commitment to meiosis in under the regulation of camp. *EMBO J.* **7**: 761-767.

West, A. H. & Stock, A. M. (2001). Histidine kinases and response regulator proteins in two-component signaling systems. *Trends Biochem. Sci*. **26**: 369-376.

White, M. A. (1996). The yeast two-hybrid system: forward and reverse *Proc. Natl. Acad. Sci*. (USA). **93**: 10001-10003.

Wood, V., Gwilliam, R., Rajandream, M. A., Lyne, M., Lyne, R., Stewart, A., Sgouros, J., Peat, N., Hayles, J., Baker, S., *et al*., (2003). The genome sequence of *Schizosaccharomyces pombe*. *Nature***. 415**: 871-880.

Wurgler-Murphy, S. M. & Saito, H. (1997). Two-component signal transducers and MAPK cascades. *Trends Biochem Sci*. **22**(5): 172-176.

Xu, Q. & West, A. H. (1999). Conservation of structure and function among histidine-containing phosphotransfer (HPt) domains as revealed by the crystal structure of YPD1. *J. Mlo Biol*. **292**: 1039-1050.

Xu, Q., Porter, S. W. & West, A. H. (2003). The yeast YPD1/SLN1 complex: Insights into molecular recognition in two-component systems. *Structure* **11**:1569-1581.

Yahu, H. & Mizuno, T. (1997). The membrane-located osmosensory kinase, EnvZ, that contains a leucine zipper-like motif functions as a dimer in *Escherchiac coli. FEBS Lett*. **417**: 409-413.

Yhomason, P. & Kay, R. (2000). Ecukaryotic signal transduction via histidine-aspartate phosphorelay. *J. Cell Science*. **113**: 3141-3150.

Zaitsevskaya-Carter, T. & Cooper, J. A. (1997). Spm1, a stress-activated MAP kinase that regulates morphogenesis in S. pombe. *EMBO J.* **16**: 1318-1331.

Zhang, C. C. (1996). Bacterial signaling involving eukaryotic-type protein kinases. *Mol. Microbiol*. **20**: 9-15.