

**Regulation of *DOG2* Gene Expression and Signal Transduction in
Environmental Stress Response of *Saccharomyces cerevisiae***

Yoshiyuki Tsujimoto

1998

CONTENTS

INTRODUCTION	1
Chapter 1 SCREENING AND IDENTIFICATION OF THE OXIDATIVE STRESS- RESPONSIBLE GENES BY SNYDER LIBRARY	5
Chapter 2 GLUCOSE REPRESSION OF <i>DOG2</i> GENE	18
Chapter 3 REGULATION OF <i>DOG2</i> GENE EXPRESSION UNDER HIGHLY OSMOTIC CONDITIONS	32
Chapter 4 REGULATION OF <i>DOG2</i> GENE EXPRESSION UNDER OXIDATIVE STRESS CONDITIONS	40
CONCLUSION	50
ACKNOWLEDGMENTS	52

INTRODUCTION

Several environmental stresses are known to trigger intracellular alterations in organisms. For example, the synthesis of some stress proteins is enhanced by changes of intracellular or extracellular environment to respond to a number of types of stress including oxidative stress, osmotic stress, heat shock, nutrients starvation, and so on. In recent years, the environmental stress responses of the budding yeast *Saccharomyces cerevisiae* have been the focus of attention, yet they remain obscure (for reviews, see Mager and de Kruijff, 1995; Kullik and Storz, 1994; Moradas-Ferreira *et al.*, 1996; Ruis and Schuller, 1995).

Heat shock response is one of stress responses which have been understood most advanced. When yeast cells are exposed to elevated temperature, they synthesize a set of heat shock proteins (HSPs). A heat shock factor (HSF), which is constitutively synthesized and binds to a *cis*-element (HSE, heat shock element), is modified by heat shock to induce the expression of several *HSP* genes (for reviews, see Mager and de Kruijff, 1995). Some *HSP* genes such as *HSP12*, *HSP26* and *HSP104* contain stress response element (STRE, 5'-AGGGG-3' or 5'-CCCCT-3') in their 5'-upstream regions, and their expressions are independent of HSF (Ruis and Schuller, 1994). The *CTT1* gene encoding cytosolic catalase also contains STREs in its 5'-upstream region, and its expression is induced by various types of environmental stresses such as osmotic stress, oxidative stress, and so on as well as heat shock (Schuller *et al.*, 1994). The expression of *CTT1* gene is controlled by HOG (high osmolarity glycerol) response pathway which is one of the mitogen-activated protein (MAP) kinase cascades in yeast (Martines-Pastor *et al.*, 1996; Schuller *et al.*, 1994).

Osmotic stress response in yeast has also been well characterized (for review, see Varela and Mager, 1996). The yeast cell has two osmosensing transmembrane proteins, Sln1p and Sho1p, both of which regulate HOG-MAP kinase cascade to adapt to osmotic stress. The Sln1p, which contains both a histidine kinase domain and a receiver domain in the same protein, constitutes a two-component system with Ssk1p (Maeda *et al.*, 1994). The Sho1p has an SH3 domain that can bind to a Pbs2p to activate the HOG-MAP kinase cascade (Maeda *et al.*, 1995). Recently, it has been found that Msn2p and Msn4p are required for transcriptional induction through STRE (Martinez-Pastor *et al.*, 1996). The *MSN2* and *MSN4* genes encode the zinc-finger transcriptional factor, and were initially identified as multicopy suppressors for glucose repression in the Snf1p (Ser/Thr protein kinase) deficient mutant (Estruch and Carlson, 1993). Expression of the *GLO1* gene encoding glyoxalase I is induced by osmotic stress, and its induction is abolished in a double disruptant of the *MSN2* and

MSN4 gene as well as a disruptant of the *HOG1* gene (Inoue *et al.*, 1998).

Glucose represses the expression of *SUC*, *GAL*, and other genes, whose products are not required for glucose metabolism but required for assimilation of sucrose, galactose, and other carbon source, respectively. In this glucose repression, Snf1p protein kinase plays a central role (Celenza and Carlson, 1986). One function of the Snf1p protein kinase is to relieve glucose repression by Mig1p/Ssn6p/Tup1p complex at the *SUC*, *GAL*, and other genes (Johnston *et al.*, 1994; Treitel and Carlson, 1995). This regulatory mechanism strictly controls the expression of many genes involved in carbon metabolism.

On the other hand, oxygen respiration as well as carbon metabolism plays an important role to acquire the energy efficiently. Because the formation of reactive oxygen species (ROS), which can damage many cell components such as DNA, lipid membranes and proteins, is commonplace in aerobic cells, they possess diverse antioxidant defense systems (for reviews, see Cadenas, 1989; Kullik and Storz, 1994; Moradas-Ferreira *et al.*, 1996). It has been known that Yap1p is a key regulator in oxidative stress response of yeast and regulates the expression of *TRX2* gene (Kuge and Jones, 1994), *GSH1* gene (Wu and Moye-Rowley, 1994), and *GLR1* gene (Grant *et al.*, 1997) under oxidative stress conditions.

As mentioned above, the environmental stress responses are diversified and complicated. To understand the stress response of *S. cerevisiae* more detailedly, the author has sought oxidative stress-responsible genes by using Snyder library (Burns *et al.*, 1994), which is a genomic library containing random *lacZ* insertions throughout the yeast genome. As a result, expression of the *DOG2* gene encoding 2-deoxyglucose-6-phosphate phosphatase was found to be induced by oxidative stress. Expression of the *DOG2* gene was also induced by osmotic stress and glucose starvation, but not by heat shock. The *DOG2* gene had *cis*-element termed STRE as well as the consensus sequence to which the Mig1p transcriptional repressor binds adjacent to the STRE. In this study, the author disrupted several genes involved in oxidative stress response, osmotic stress response and glucose repression, and demonstrated that expression of the *DOG2* gene was regulated by both HOG-MAP kinase cascade and Snf1p-Mig1p pathway under environmental stress conditions.

References

- Burns, N., Grimwade, B., Ross-Macdonald, P. B., Choi, E.-Y., Finberg, K., Roeder, G. S., and Snyder, M. (1994) Large-scale analysis of gene expression, protein localization, and gene disruption in *Saccharomyces cerevisiae*. *Genes Dev.* **8**:1087-1105
- Cadenas, E. (1989) Biochemistry of oxygen toxicity. *Annu. Rev. Biochem.* **58**:79-110
- Celenza, J. L., and Carlson, M. (1986) A yeast gene that is essential for release from glucose repression encodes a protein kinase. *Science* **233**:1175-1180
- Estruch, F., and Carlson, M. (1993) Two homologous zinc finger genes identified by multicopy suppression in a SNF1 protein kinase mutant of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **13**:3872-3881
- Grant, C. M., Collinson, L. P., Roe, J. -H., and Dawes, I. W. (1996) Yeast glutathione reductase is required for protection against oxidative stress and is a target gene for YAP-1 transcriptional regulation. *Mol. Microbiol.* **21**:171-179
- Inoue, Y., Tsujimoto, Y., and Kimura, A. (1998) Expression of the glyoxalase I gene of *Saccharomyces cerevisiae* is regulated by high osmolarity glycerol mitogen-activated protein kinase pathway in osmotic stress response. *J. Biol. Chem.* **273**:2977-2983
- Johnston, M., Flick, J. S., and Pexton, T. (1994) Multiple mechanisms provide rapid and stringent glucose repression of *GAL* gene expression in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **14**:3834-3841
- Kuge, S., and Jones, N. (1994) YAP1 dependent activation of *TRX2* is essential for the response of *Saccharomyces cerevisiae* to oxidative stress by hydroperoxides. *EMBO J.* **13**:655-664
- Kullik, I., and Storz, G. (1994) Transcriptional regulators of the oxidative stress response in prokaryotes and eukaryotes. *Redox Report* **1**:23-29
- Maeda, T., Takekawa, M., and Saito, H. (1995) Activation of yeast PBS2 MAPKK by MAPKKs or by binding of an SH3-containing osmosensor. *Science* **269**:554-558
- Maeda, T., Wurgler-Murphy, S. M., and Saito, H. (1994) A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature* **369**:242-245
- Mager, W. H., and de Kruijff, A. J. J. (1995) Stress-induced transcriptional activation. *Microbiol. Rev.* **59**:506-531
- Martinez-Pastor, M. T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H., and Estruch, F. (1996) The *Saccharomyces cerevisiae* zinc finger protein Msn2p and Msn4p are required for transcriptional induction through the stress-response element

(STRE). *EMBO J.* **15**:2227-2235

Moradas-Ferreira, P., Costa, V., Piper, P., and Mager, W. H. (1996) The molecular defenses against reactive oxygen species in yeast. *Mol. Microbiol.* **19**:651-658

Ruis, H., and Schuller, C. (1995) Stress signaling in yeast. *BioEssays* **17**:959-965

Schuller, C., Brewster J. L., Alexander, M. R., Gustin, M. C., and Ruis, H. (1994) The HOG pathway controls osmotic regulation of transcriptional *via* the stress response element (STRE) of *Saccharomyces cerevisiae* *CTT1* gene. *EMBO J.* **13**:4382-4389

Treitel, M. A., and Carlson, M. (1995) Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. *Proc. Natl. Acad. Sci. USA* **92**:3132-3136

Verela, J. C. S., and Mager, W. H. (1996) Response of *Saccharomyces cerevisiae* to changes in external osmolarity. *Microbiol.* **142**:721-731

Wu, A.-L., and Moye-Rowley, W. S. (1994) *GSH1*, which encodes γ -glutamylcysteine synthetase, is a target gene for yAP-1 transcriptional regulation. *Mol. Cell. Biol.* **14**:5832-5839

Chapter 1

SCREENING AND IDENTIFICATION OF THE OXIDATIVE STRESS-RESPONSIBLE GENES BY SNYDER LIBRARY

Cells respond to oxidative stress to acquire adaptive advantage. In all aerobic cells, oxygen respiration plays an important role to acquire the energy efficiently, and leads to formation of harmful reactive oxygen species (ROS) such as superoxide radicals, hydrogen peroxide and hydroxyl radicals, which can damage many cell components such as DNA, lipid membranes and proteins (for review, see Cadenas, 1989). As ROS is commonplace in aerobic cells, they possess diverse antioxidant defense systems (for reviews, see Mager and de Kruijff, 1995; Bunn and Poyton, 1996). Cells use both enzymatic defense, which includes catalase catalyzing the decomposition of hydrogen peroxide, superoxide dismutase (SOD) catalyzing the disproportionation of superoxide radicals, and nonenzymatic ones such as glutathione (GSH) and thioredoxin (Trx). GSH plays a very important role in oxidative stress response of yeast as known in mammals (Grant *et al.*, 1996b; Izawa *et al.*, 1995). Utilization of GSH in antioxidant defense mechanism results in its conversion to the oxidized form (GSSG), and it must be recycled to reduced one (GSH) to maintain intracellular redox state. Glutathione reductase catalyzes the reduction of GSSG to GSH using NADPH as a reducing power.

Many antioxidant defense systems are known to be drastically regulated at transcriptional step (for reviews, see Kullik and Storz, 1994; Jamieson and Storz, 1997; Moradas-Ferreira *et al.*, 1996). Yap1p belongs to Jun family of transcriptional activators including mammalian AP-1 (Moye-Rowley *et al.*, 1989). Yap1p regulates the expression of *TRX2* gene encoding thioredoxin (Kuge and Jones, 1994), *GSH1* gene encoding γ -glutamylcysteine synthetase (Wu and Moye-Rowley, 1994), and *GLR1* gene encoding glutathione reductase (Grant *et al.*, 1996a). Yap1p also functions as a sensor in the response to oxidative stress (Kuge *et al.*, 1997). The induction of Yap1p-dependent transcription is not due to an induction in synthesis of Yap1p, but to an induction of its localization to nucleus. Localization of Yap1p molecule is mediated by a cysteine-rich domain at the C-terminus of Yap1p.

It is known that regulators of oxidative stress response are Msn2p, Msn4p, Pos9p/Skn7p, Mac1p, Ace1p, Hap1p, and so on besides Yap1p (for reviews, see Kullik and

Storz, 1994; Jamieson and Storz, 1997; Moradas-Ferreira *et al.*, 1996). It is thought that the expression of *SOD1* gene encoding Cu,Zn-SOD and *CTT1* gene encoding cytosolic catalase is regulated by Ace1p and Mac1p, respectively (Gralla *et al.*, 1991; Jungmann *et al.*, 1994). Expression of the *CTT1* gene, which has STREs in its 5'-upstream region, is induced by various types of environmental stresses, such as osmotic stress, heat shock and so on, as well as oxidative stress (Schuller *et al.*, 1994), and is controlled by HOG (high osmolarity glycerol) response pathway which is one of the mitogen-activated protein (MAP) kinase cascades in yeast (Martines-Pastor *et al.*, 1996; Schuller *et al.*, 1994).

To analyze the response mechanism against oxidative stress in yeast cells, the author has sought oxidative stress-responsible genes by using Snyder library (Burns *et al.*, 1994), which is a genomic library containing random *lacZ* insertions throughout the yeast genome. As a result, expression of the *DOG2* gene encoding 2-deoxyglucose-6-phosphate phosphatase was found to be induced by oxidative stress. The expression of *DOG2* gene was also induced by osmotic stress and glucose starvation, but not by heat shock.

Materials and Methods

Yeast strains

The diploid yeast strain (YPH274) was used to generate Snyder library. Plasmids of Snyder library were kindly provided by A. Dancis at NICHD/NIH, Bethesda. The construction of a yeast genomic library were carried out as described by Burns *et al.* (1994). Tetrad analysis was carried out by a standard method.

Screening

Plasmids of Snyder library (pool #22 and #23) were introduced to *E. coli* strain DH10B, and kanamycin (Km)-resistant clones (6.9×10^5) were obtained. All transformants were collected from the plates, and cultured in LB medium (1% peptone, 0.5% yeast extract, 1% NaCl; pH 7.2) containing 25 µg/ml Km at 37°C for 16 h. Plasmids were prepared by Qiagen kit, and digested with *NotI*. DNA fragments carrying yeast chromosome with the *lacZ-LEU2-ampC* cassette was purified by low-melting agarose gel electrophoresis, and introduced to *S. cerevisiae* YPH274 by electroporation method. Approximately 3,000 Leu⁺ clones were obtained. Each clone was replica plated to SD (2% glucose, 0.67% yeast nitrogen

base without amino acids)-agar plate without Leu.

To screen the oxidative stress-responsible clones, each clone was replica plated on nylon membrane (Hybond-N, Amersham) on the SD-agar plate containing 0.8 mM *tert*-butyl hydroperoxide, and cultured at 28°C for 1 day. Nylon membrane with yeast colonies was peeled off from the plate, dipped in the liquid nitrogen for 10 sec, and then put on to the filter paper previously soaked in the Z-buffer (16.1g/liter Na₂HPO₄•7H₂O, 5.5g/liter NaH₂PO₄•H₂O, 0.75g/liter KCl, 0.246g/liter MgSO₄•7H₂O) containing 330 µg/ml X-gal. Nylon membrane was incubated at room temperature for 12-24 h, then colonies turned to be blue were selected as first candidates.

For the second screening, first candidates were replica plated triplicate on the nylon membrane from the master plate, and each membrane was put on the SD-agar plate with 0 mM, 0.08 mM and 0.8 mM *tert*-butyl hydroperoxide. Cells were incubated at 28°C for 1 day. After colonies were appeared, nylon membranes were peeled off from each plate, and dipped in the liquid nitrogen, and then put on to the filter paper containing X-gal as described above. Clones that mostly turned to be blue in the presence of higher concentration of *tert*-butyl hydroperoxide were selected as "up-regulated" clones, and clones that mostly turned to be blue without *tert*-butyl hydroperoxide were selected as "down-regulated" clones. The author used "up-regulated" clones as second candidates.

For the third screening, the second candidates were cultured in test tubes containing 5 ml SD medium without *tert*-butyl hydroperoxide at 28°C for 16 h, and a small portion of the culture was transferred to 2-liter Sakaguchi flask containing 1-liter SD medium with or without 0.6 mM *tert*-butyl hydroperoxide. Cells were cultured at 28°C with reciprocal shaking until OD₆₁₀ of the culture reached approximately 1.0. Cells were harvested by centrifugation, washed three times with 0.85% NaCl solution. Cell extracts were prepared as described below. Eventually, the author isolated a clone named SET8 as a final candidate.

Plasmid rescue and DNA sequencing

To rescue the *lacZ-LEU2-ampC* cassette from the genome DNA of a SET8 clone, a plasmid YIp5 was introduced to the SET8 clone. The Ura⁺ transformants were isolated, and chromosomal DNA was prepared as described by Cryer *et al.* (1975). The chromosomal DNA was digested with *NsiI*, the digestion mixture was self-ligated, and introduced to *E. coli* JM 109. Ampicillin (Ap)-resistant transformants were isolated by LB-agar plate containing 50 µg/ml Ap. To sequence the 5'-flanking region of the *lacZ-LEU2-ampC* cassette, a primer (5'-CGTTGTAAAACGACGGGATCCCCCT-3') was synthesized as described by Burns *et al.* (1994), and nucleotide sequence was determined by using an automated DNA sequencer

(Applied Biosystems model 373A) by dideoxy chain termination method of Sanger *et al.* (1977). Homology search analysis was done using BLAST and FAST programs (Altschul *et al.*, 1990; Pearson and Lipman, 1988) against *Saccharomyces* Genome Database at Stanford University.

Stress experiments

Yeast cells were cultured in a test tube containing 5 ml YPD medium (2% glucose, 2% peptone, 1% yeast extract; pH 5.5) at 28°C with shaking for overnight. A small portion of the culture was transferred to 200ml Erlenmeyer flask containing 50 ml YPD medium to obtain the OD₆₁₀ = 0.05. Cells were cultured at 28°C with reciprocal shaking until OD₆₁₀ reached 0.8-1.0, and then concentrated solutions of various agents were added. In the case of osmotic stress experiment, solid NaCl was added to the culture. After the addition of agents, culture was continued for another 1 h, and then cells were collected by centrifugation. Preparation of cell extracts were described below.

Preparation of cell extracts

Cells cultured in an appropriate medium were collected by centrifugation, washed three times with 0.85 % NaCl solution, and resuspended in 10 mM potassium phosphate buffer (pH 7.0). Cells were disrupted with glass beads by using a vortex mixer or Braun Homogenizer at 0°C for 2.5 min, and cell homogenates were centrifuged at 14,000 rpm for 15 min at 4°C. The resultant supernatants were used as cell extracts.

β-Galactosidase assay

β-Galactosidase activity was measured as described by Miller (1972). One unit of the activity was defined as the amount of enzyme increasing A₄₂₀×1000 per min at 30°C. Protein was determined by the method of Lowry *et al.* (1951).

Table 1 Yeast Strains used in this study : YGSG, Yeast Genetic Stock Center, University of California at Berkeley

Strain	Genotype	Reference or source
YPH274	<i>MAT a α trp1-Δ1/trp1-Δ1 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 lys2-Δ801/lys2-Δ801 ade2-101/ade2-101 ura3-52/ura3-52</i>	YGSG
YPH250	<i>MAT a trp1-Δ1 his3-Δ200 leu2-Δ1 lys2-Δ801 ade2-101 ura3-52</i>	YGSG
SET8	Isogenic of YPH274, except for <i>DOG2/dog2Δ::lacZ</i>	this study
SET8-1-A	<i>MAT a trp1-Δ1 his3-Δ200 leu2-Δ1 lys2-Δ801 ade2-101 ura3-52 dog2Δ::lacZ-LEU2</i>	this study
SET8-1-B	<i>MAT a trp1-Δ1 his3-Δ200 leu2-Δ1 lys2-Δ801 ade2-101 ura3-52 DOG2</i>	this study
SET8-1-C	<i>MAT α rp1-Δ1 his3-Δ200 leu2-Δ1 lys2-Δ801 ade2-101 ura3-52 dog2Δ::lacZ-LEU2</i>	this study
SET8-1-D	<i>MAT α trp1-Δ1 his3-Δ200 leu2-Δ1 lys2-Δ801 ade2-101 ura3-52 DOG2</i>	this study
SCP2	Isogenic of SET8-1-C, except for <i>pbs2-Δ1::URA3</i>	this study
SCH1	Isogenic of SET8-1-C, except for <i>hog1Δ::URA3</i>	this study
SCM2	Isogenic of SET8-1-C, except for <i>msn2-Δ3::HIS3</i>	this study
SCM24	Isogenic of SET8-1-C, except for <i>msn2-Δ3::HIS3 msn4Δ::URA3</i>	this study
SCM4	Isogenic of SET8-1-C, except for <i>msn4Δ::URA3</i>	this study
SCS1	Isogenic of SET8-1-C, except for <i>snf1Δ::HIS3</i>	this study
SCMG1	Isogenic of SET8-1-C, except for <i>mig1Δ::HIS3</i>	this study
SCM241	Isogenic of SET8-1-C, except for <i>msn2-Δ3:: HIS3 msn4Δ::ADE2 mig1Δ::URA3</i>	this study
SCSP12	Isogenic of SET8-1-C, except for <i>snf1Δ::HIS3 pbs2-Δ1::URA3</i>	this study
SCSH11	Isogenic of SET8-1-C, except for <i>snf1Δ::HIS3 hog1Δ::URA3</i>	this study
SCYP1	Isogenic of SET8-1-C, except for <i>yap1-Δ1::HIS3</i>	this study

Results

Molecular cloning of oxidative stress-responsive genes from *S. cerevisiae* by Snyder library

In Snyder library, the *lacZ* gene without its original promoter was randomly inserted into the genome of *S. cerevisiae*. This library has several advantages for screening the genes that are expressed under restricted conditions, such as stressful conditions, growth phase, and differences of nutrients, because it is possible to evaluate the strength of promoter or timing of expression of the corresponding gene by measuring β -galactosidase activity. Since X-gal is one of the substrates of β -galactosidase and reaction product of this substrate turned to be blue, expression pattern of the *lacZ*-inserted gene can be visualized. The author is interested in the systematic study on the yeast genes whose expression is responsible for the oxidative stress, thus he used the Snyder library to achieve this objective.

In the first screening, the author replica plated approximately 3,000 *Leu*⁺ transformants on to the nylon membrane on SD-agar plate containing 0.8 mM *tert*-butyl hydroperoxide (*t*-BHP) to roughly screen the genes which can be expressed even in the presence of *t*-BHP. The author could obtain 320 candidates by this screening method. Since the objective of this study is to isolate the genes whose expression is responsible to oxidative stress, the author then subjected these clones to the second screening. In the second screening, the author replica plated the candidates triplicate on nylon membrane on the SD-agar plate containing 0 mM, 0.08 mM, and 0.8 mM *t*-BHP. By this screening, the author could exclude the clones whose *lacZ* gene is expressed constitutively, and could obtain 62 candidates. Finally, to quantify the β -galactosidase activity, candidates were cultured in liquid medium with or without 0.6 mM *t*-BHP. The author could obtain several clones whose β -galactosidase activity increased in the presence of *t*-BHP. Figure 1 showed a result of SET8 clone as a representative. β -Galactosidase activity of the SET8 was detected when the cells were cultured without *t*-BHP, although it increased approximately 3-fold if the cells were cultured in the presence of 0.6 mM *t*-BHP. In this study, the author chose the SET8 to study the regulation mechanism of the SET8 gene expression further.

To identify the SET8 locus, the *lacZ*-*LEU2*-*ampC* cassette was rescued by using YIp5, and 5'-flanking region of the cassette corresponding to the SET8 locus was sequenced. The SET8 locus was found to be the *DOG2* gene, namely the *lacZ*-*LEU2*-*ampC* cassette was inserted near the C-terminus of the *DOG2* gene. The *DOG2* gene was initially cloned as multicopy suppressor for the toxicity of 2-deoxyglucose in *S. cerevisiae* (Randez-Gill *et al.*,

1995). The *DOG2* gene product has been reported to have 2-deoxyglucose-6-phosphate phosphatase activity. However, 2-deoxyglucose is not a natural substance, therefore, it is still vague that what is an intrinsic substrate for and actual function of the Dog2p.

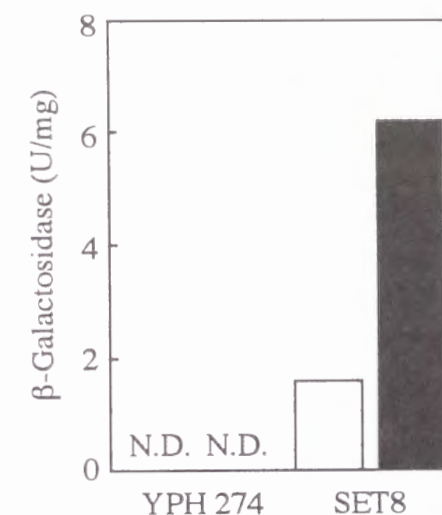


Fig. 1 Induction of SET8-lacZ fusion by *tert*-butyl hydroperoxide. Experimental procedures were described in the text. White bar indicates β -galactosidase activity that were assayed in cells grown in SD medium. Black bar indicates β -galactosidase activity that were assayed in cells grown in SD medium containing 0.6 mM *tert*-butyl hydroperoxide. N. D., not detected.

Effect of disruption of *DOG2* gene on cell growth

Since the host cell for construction of Snyder library is diploid strain, the *DOG2*-*lacZ* insertion of SET8 clone is heterozygous. To determine the phenotype of haploid cells carrying the insertion mutation, the SET8 was sporulated and tetrads were dissected. All spores from 17 tetrads could germinated, thus the *DOG2* gene was not essential. The *LEU2* marker and β -galactosidase activity was completely linked and separated in 2:2 (data not shown). Using a set of resultant four haploids, effect of the *DOG2* gene disruption on growth was monitored. As shown in Fig. 2, no difference was observed in the growth of cells with *t*-BHP, while the cell growth of *dog2* Δ disruptant decreased compared with that of wild type cell in the presence of 2-deoxyglucose. No difference in cell growth was observed between wild type and *dog2* Δ disruptant under highly osmotic conditions (data not shown). Because overexpression of the *DOG2* gene confer the resistance against 2-deoxyglucose (Randez-Gill *et al.*, 1995), the results of growth experiments herewith reconfirmed that the *DOG2* gene is disrupted.

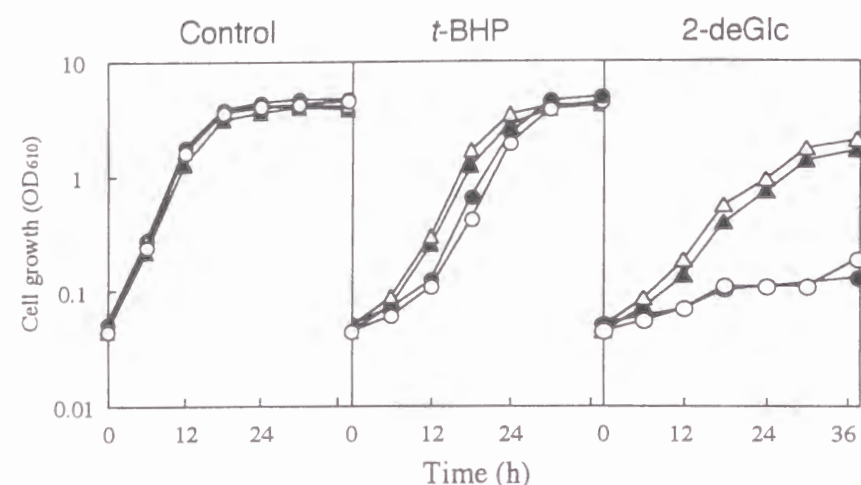


Fig. 2 Effect of disruption of *DOG2* gene on cell growth in *t*-BHP or 2-deoxyglucose-containing medium. Yeast strains used were: SET8-1-A(*dog2Δ*),○; SET8-1-B (*DOG2*),△; SET8-1-C (*dog2Δ*),●; SET8-1-D (*DOG2*),▲. Yeast cells were grown in test tubes containing 5 ml fructose medium (2% fructose, 0.67% yeast nitrogen base without amino acids, and appropriate amino acids) at 28°C with shaking for overnight. A small portion of the preculture was transferred to fresh fructose medium containing 0.4 mM *tert*-buthyl hydroperoxide (*t*-BHP) or 0.1% 2-deoxyglucose (2-deGlc). Cells were cultured at 28°C with shaking, and OD₆₁₀ was monitored.

Expression of *DOG2* gene under environmental stress conditions

In Fig. 1, the SET8 clone was cultured in the presence of *t*-BHP. The author then tested whether expression of the *DOG2-lacZ* is induced transiently if the cells were exposed to environmental stress. A haploid strain obtained by tetrad analysis (*dog2Δ::lacZ*) was used for analysis. As shown in Fig. 3, expression of the *DOG2* gene was transiently increased by the addition of *t*-BHP in the culture.

Besides oxidative stress caused by lipid hydroperoxide, the *DOG2* gene expression was found to be induced by glucose starvation. Cells were cultured in YPD medium to mid-log phase (OD₆₁₀ = 0.8-1.0), harvested by centrifugation, and then resuspended in YPD medium without glucose (named YP medium). After 1 h, cell extracts were prepared and β-galactosidase activity was measured. As shown in Fig. 3, expression of the *DOG2* gene was induced by glucose starvation. 2-Deoxyglucose is a non-metabolizable glucose analogue and usually used as a blocker for glycolysis, therefore, addition of 2-deoxyglucose into the medium mimics glucose starvation. 2-Deoxyglucose could also induce expression of the *DOG2* gene (Fig. 3). Expression of the *DOG2* gene was also induced by osmotic stress. β-Galactosidase activity was increased if 0.3 M NaCl, 0.3 M KCl, and 0.6 M sorbitol was

added to the culture in which the cells were growing logarithmically. Since sorbitol could also induce the *DOG2* gene expression, change of osmolarity is a stimulus to enhance the gene expression rather than the change of specific solute. Among environmental stresses tested, heat shock did not induce the *DOG2* gene expression (Fig. 3)

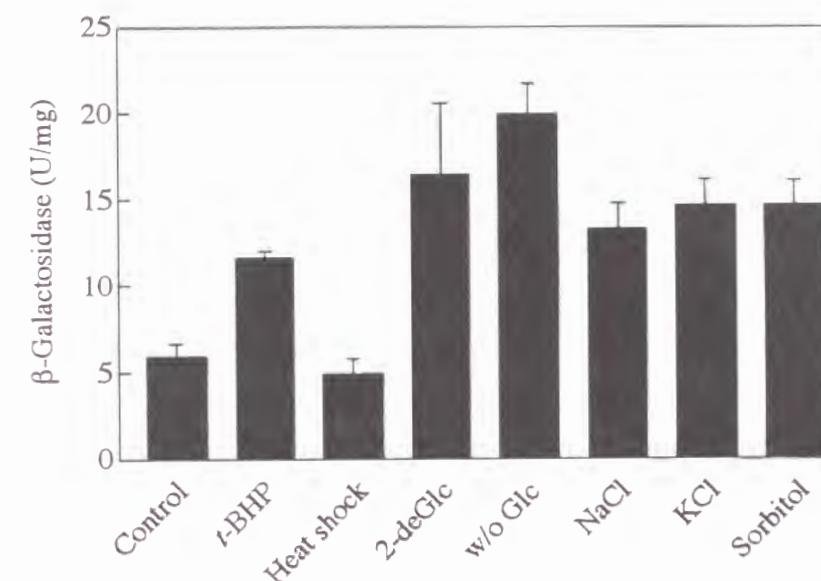


Fig. 3 Induction of *DOG2-lacZ* fusion by several chemicals. Yeast stain used in this experiment was SET8-1-C. Cells were cultured at 28°C in YPD medium until OD₆₁₀ reached approximately 0.8-1.0, then each chemical was added. Cells were cultured for another 1 h, and cell extracts prepared as described in the text. Concentrations of chemicals added were: *tert*-butyl hydroperoxide (*t*-BHP), 0.6 mM; 2-deoxyglucose (2-deGlc), 0.1%; NaCl, 0.3 M; KCl, 0.3 M; and sorbitol, 0.6 M. For heat shock experiment, flask containing yeast culture at 28°C was transferred to an incubator preheated at 37°C. Glucose starvation experiment (w/o Glc) was done by changing the culture medium to YPD medium without glucose (YP medium) after collecting the cells by centrifugation.

Discussion

The author cloned some oxidative stress-responsible genes other than the *DOG2* gene by using Snyder library. Expression of the *DOG2* gene was found to be induced by oxidative stress. The *DOG2* gene, which is able to confer resistance to 2-deoxyglucose when overexpressed, encodes 2-deoxyglucose-6-phosphate phosphatase (Randez-Gil *et al.*, 1995). Expression of the *DOG2-lacZ* fusion gene was induced by osmotic stress and glucose starvation as well as oxidative stress, but not by heat shock (Fig. 3). By an analysis of the 5'-upstream region of *DOG2* gene, the author found an STRE between 186 and 182 nucleotide (nt) upstream of the initiation codon (ATG) and the consensus sequence of Mig1p-binding site (AT-rich+GGGG) between 206 and 188 nt upstream of ATG (Lundin *et al.*, 1994). The expression of *CTT1* gene, which has two STREs in its 5'-upstream region, is induced by various types of environmental stresses, such as osmotic stress, heat shock, oxidative stress, and so on (Schuller *et al.*, 1994). In the case of osmotic stress response, the *CTT1* gene expression is controlled by HOG-MAP kinase cascade (Martinez-Pastor *et al.*, 1996; Schuller *et al.*, 1994). On the other hand, expression of the *GLO1* gene, which also has two STREs in its 5'-upstream region, is induced by only osmotic stress, but not by oxidative stress (Inoue *et al.*, 1998). Induction by heat shock was not observed in the expressions of *DOG2* and *GLO1* gene, but observed in the expression of *CTT1* gene. It was reported that Msn2p and Msn4p were required for transcriptional induction through STRE, and that they were regulated by the HOG-MAP kinase cascade (Martinez-Pastor *et al.*, 1996; Schuller *et al.*, 1994). The *MSN2* and *MSN4* genes encode homologous zinc-finger transcriptional factor, and were initially identified as multicopy suppressors for derepression of the *SUC2* gene in the Snf1p (Ser/Thr protein kinase) deficient mutant (Estruch and Carlson, 1993). The Snf1p is required to relieve glucose repression by Mig1p/Ssn6p/Tup1p complex (Johnston *et al.*, 1994; Treitel and Carlson, 1995). Both of the transcriptional activator (Msn2p and/or Msn4p) and the transcriptional repressor (Mig1p) may bind to the promoter region of the *DOG2* gene. Thus, the regulation of *DOG2* gene expression in environmental stress response is of considerable interest.

Using a set of tetrads, effects of the *DOG2* gene disruption on the cell growth to environmental stresses were tested (Fig. 2). The differences of cell growth under oxidative stress were not observed, while the cell growth of *dog2Δ* disruptants decreased compared to that of wild type in 2-deoxyglucose-containing medium (Fig. 2). It is known that twin genes, the *DOG1* and *DOG2* gene are able to confer resistance to 2-deoxyglucose when they are

overexpressed (Randez-Gil *et al.*, 1995). The homology of these genes is very high, and these genes share 92% identity at amino acids level. In the *dog2Δ* disruptant, Dog1p may substitute for Dog2p function under environmental stress conditions. Yeast cells exposed to environmental stresses must enhance the generation of ATP as energy to survive. The Dog2p might be functioning in energy generating process.

References

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**:403-410
- Burns, N., Grimwade, B., Ross-Macdonald, P. B., Choi, E., Finberg, K., Roeder, G. S., and Snyder, M. (1994) Large-scale analysis of gene expression, protein localization, and gene disruption in *Saccharomyces cerevisiae*. *Genes Dev.* **8**:1087-1105
- Bunn, H. F., and Poyton, R. O. (1996) Oxygen sensing and molecular adaptation to hypoxia. *Physiol. Rev.* **76**: 839-885
- Cadenas, E. (1989) Biochemistry of oxygen toxicity. *Annu. Rev. Biochem.* **58**:79-110
- Cryer, D. R., Eccleshall, R., and Marmur, J. (1975) Isolation of yeast DNA. *Methods Cell Biol.* **12**, 39-44
- Estruch, F., and Carlson, M. (1993) Two homologous zinc finger genes identified by multicopy suppression in a SNF1 protein kinase mutant of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **13**:3872-3881
- Grant, C. M., Collinson, L. P., Roe, J. -H., and Dawes, I. W. (1996a) Yeast glutathione reductase is required for protection against oxidative stress and is a target gene for yAP-1 transcriptional regulation. *Mol. Microbiol.* **21**:171-179
- Grant, C. M., MacIver, F. H., and Dawes, I. W. (1996b) Glutathione is an essential metabolite required for resistance to oxidative stress in the yeast *Saccharomyces cerevisiae*. *Curr. Genet.* **29**:511-515
- Gralla, E. B., Thiele, D. J., Silar, P., and Valentine, J. S. (1991) ACE1, a copper-dependent transcription factor, activates expression of the yeast copper, zinc superoxide dismutase. *Proc. Natl. Acad. Sci. USA* **88**:8558-8562
- Inoue, Y., Tsujimoto, Y., and Kimura, A. (1998) Expression of the glyoxalase I gene of *Saccharomyces cerevisiae* is regulated by high osmolarity glycerol mitogen-activated

- protein kinase pathway in osmotic stress response. *J. Biol. Chem.* **273**:2977-2983
- Izawa, S., Inoue, Y., and Kimura, A. (1995) Oxidative stress response in yeast: effect of glutathione on adaptation to hydrogen peroxide stress in *Saccharomyces cerevisiae*. *FEBS Lett.* **368**:73-76
- Jamieson, D. J., and Storz, G. (1997) Transcriptional regulators of oxidative stress responses. In *Oxidative stress and the molecular biology of antioxidant defenses*: 91-115, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Johnston, M., Flick, J. S., and Pexton, T. (1994) Multiple mechanisms provide rapid and stringent glucose repression of *GAL* gene expression in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **14**:3834-3841
- Jungmann, J., Reins, H. A., Lee, J., Romeo, A., Hassett, R., Kosman, D., and Jentsch, S. (1994) MAC1, a nuclear regulatory protein related to Cu-dependent transcription factors is involved in Cu/Fe utilization and stress resistance in yeast. *EMBO J.* **13**:5051-5056
- Kuge, S., and Jones, N. (1994) YAP1 dependent activation of *TRX2* is essential for the response of *Saccharomyces cerevisiae* to oxidative stress by hydroperoxides. *EMBO J.* **13**:655-664
- Kuge, S., Jones, N., and Nomoto, A. (1997) Regulation of yAP-1 nuclear localization in response to oxidative stress. *EMBO J.* **16**:1710-1720
- Kullik, I., and Storz, G. (1994) Transcriptional regulators of the oxidative stress response in prokaryotes and eukaryotes. *Redox Report* **1**:23-29
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275
- Lundin, M., Nehlin, J. O., and Ronne, H. (1994) Importance of a flanking AT-rich region in target site recognition by the GC box-binding zinc finger protein MIG1. *Mol. Cell. Biol.* **14**:1979-1985
- Mager, W. H., and de Kruijff, A. J. J. (1995) Stress-induced transcriptional activation. *Microbiol. Rev.* **59**:506-531
- Martinez-Pastor, M. T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H., and Estruch, F. (1996) The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress-response element (STRE). *EMBO J.* **15**:2227-2235
- Miller, J. H. (1972) *Experiments in Molecular Genetics.*, 352-356, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Moradas-Ferreira, P., Costa, V., Piper, P., and Mager, W. H. (1996) The molecular defences against reactive oxygen species in yeast. *Mol. Microbiol.* **19**:651-658
- Moye-Rowley, W. S., Harshman, K. D., and Parker, C. S. (1989) Yeast *YAP1* encodes a novel form of the jun family of transcriptional activator proteins. *Genes Dev.* **3**:283-292
- Pearson, W. R., and Lipman, D. J. (1988) Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA* **85**:2444-2448
- Randez-Gil, F., Blasco, A., Prieto, J. A., and Sanz, P. (1995) *DOG^R1* and *DOG^R2* : Two genes from *Saccharomyces cerevisiae* that confer 2-deoxyglucose resistance when overexpressed. *Yeast* **11**:1233-1240
- Sanger, F., Nicklen, S., and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463-5467
- Schuller, C., Brewster J. L., Alexander, M. R., Gustin, M. C., and Ruis, H. (1994) The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of *Saccharomyces cerevisiae* *CTT1* gene. *EMBO J.* **13**:4382-4389
- Treitel, M. A., and Carlson, M. (1995) Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. *Proc. Natl. Acad. Sci. USA* **92**:3132-3136
- Wu, A. -L., and Moye-Rowley, S. (1994) *GSH1*, which encodes γ -glutamylcysteine synthetase, is a target gene for yAP-1 transcriptional regulation. *Mol. Cell. Biol.* **14**:5822-5839

Chapter 2

GLUCOSE REPRESSION OF *DOG2* GENE

As mentioned in chapter 1, expression of the *DOG2* gene was induced by glucose starvation and osmotic stress as well as oxidative stress. The *DOG2* gene was found to have a *cis*-element termed stress response element (STRE; 5'-AGGGG-3') and the consensus sequence to which the Mig1p transcriptional repressor binds. The aim of this chapter is to clarify the regulation of *DOG2* gene expression under the glucose starved conditions.

Yeast cells grown in glucose as a carbon source repress the expression of a large number of genes which are dispensable for glucose utilization. For example, *GAL*, *SUC*, and *MAL* genes, which are indispensable for utilization of galactose, sucrose, and maltose, respectively, are subject to glucose repression. Many genes involved in glucose repression have been identified by a variety of genetic methods (for review, see Johnston and Carlson, 1992). The *MIG1* gene was isolated as a multicopy inhibitor of the *GAL1* promoter, and Mig1p is involved in glucose repression (Nehlin and Ronne, 1990), which requires both Ssn6p and Tup1p (Trumbly, 1992; Treitel and Carlson, 1995). The Snf1p is required to relieve glucose repression by the Mig1p/Ssn6p/Tup1p complex (Johnston *et al.*, 1994; Treitel and Carlson, 1995). The *SNF1* gene encodes a Ser/Thr protein kinase (Celenza and Carlson, 1986), and its expression is not repressed by glucose (Celenza and Carlson, 1984; Schuller and Entian, 1987). The Snf1p protein kinase forms the complex with other proteins, including the activating subunit Snf4p (Celenza *et al.*, 1989; Schuller and Entian, 1988), and the Sip1p, Sip2p, and Gal83p (Yang *et al.*, 1992; 1994). The Sip1p/Sip2p/Gal83p family anchors Snf1p and Snf4p into a complex, and its conformational change is regulated by glucose (Jiang and Carlson, 1997). The Snf1p has 64% identity to mammalian AMP-activated protein kinase within the catalytic core (Gao *et al.*, 1996; Michelhill *et al.*, 1994). A high AMP:ATP ratio

may trigger the derepression of glucose-repressed genes through the Snf1p (Wilson *et al.*, 1996).

On the other hand, it is suggested that expression of the *CTT1* gene, which encodes cytosolic catalase and has STREs in its 5'-upstream region, is negatively regulated by cAMP-dependent protein kinase (PKA) through STRE (Martinez-Pastor *et al.*, 1996; Ruis and Schuller, 1995). Additionally, it was reported that Msn2p and Msn4p were required for transcriptional induction through STRE, and that they were regulated by HOG-MAP kinase cascade in osmotic stress response (Martinez-Pastor *et al.*, 1996; Schuller *et al.*, 1994). The HOG-MAP kinase cascade is defined by the *PBS2* and *HOG1* gene products, a member of MAP kinase kinase and MAP kinase, respectively (Boguslawski, 1992; Brewster *et al.*, 1993). To confirm whether Snf1p-Mig1p pathway and HOG-MAP kinase cascade are involved in the expression of *DOG2* gene under glucose starved conditions, the author used a gene targeting method. As a result, induction of the *DOG2* gene expression by glucose starvation was abolished in the *snf1Δ* disruptant, but not in several gene disruptants involved in HOG-MAP kinase cascade. In *mig1Δ* disruptant, basal level of the *DOG2* gene expression increased even though glucose was present in the medium. Thus, expression of the *DOG2* gene was found to be regulated by the Snf1p-Mig1p pathway. Unexpectedly, induction of the *DOG2* gene expression was still observed in *snf1Δ/hog1Δ* double disruptant under the glucose starved conditions.

Materials and Methods

Yeast strains, plasmids and gene disruptions

Yeast strains used in this work are listed in Table 1. Disruption of each gene was done by one-step gene replacement method.

The *PBS2* gene was disrupted using a plasmid pJB4D as described by Brewster *et al.* (1993).

The *HOG1* gene with its 5'- and 3'-regions was cloned by PCR using primers: HOG1S, 5'-GTTGTTAGGAAAGCATGCTTTATCTCCAAG-3' and HOG1R, 5'-CCTTTT-ATGGGATCCTAATTTCTTAAGGAG-3'. Both primers were designed to contain recognition site for *SphI* and *BamHI*, which were shown by underline, respectively. The PCR fragment (2340 bp) was cloned between *SphI* and *BamHI* site of pUC19 to form pUCHOG1. To construct the *hog1Δ::URA3* disruptant, pUCHOG1 was digested with *BalI* and *HincII*, and 400 bp fragment in the open reading frame (ORF) of the *HOG1* gene was replaced with the *URA3* gene to yield pUHOGΔUra3. The resultant plasmid was then digested with *SphI* and *BamHI* and then the *hog1Δ::URA3* fragment was introduced to *S. cerevisiae* to disrupt the *HOG1* gene (Inoue *et al.*, 1998).

The *MSN2* gene was disrupted using a plasmid pt32-DXB::HIS as described by Estruch and Carlson (1993).

The *MSN4* gene was cloned by PCR using primers: MSN4S, 5'-CGCCACA-CCAACATGCAACTTCTCCCAAGA-3' and MSN4R, 5'-GCTCTTCCAACCAAGCCTCA-TTGCTCCTTG-3'. Primer MSN4S corresponded to the region between 533 and 562 nt downstream from ATG codon, and the MSN4R corresponded to the region between 2653 and 2682 nt from ATG codon of *MSN4* gene, respectively. The PCR fragment (2150 bp) was digested with *SphI* and *EcoRI*, then cloned between the *SphI* and *EcoRI* site of pUC19. The resultant plasmid (pUCmsn4) was digested with *EcoRV* and *AflIII* to delete 593-bp fragment,

which contained zinc-finger motif of Msn4p (Estruch and Carlson, 1993), and then replaced with the *URA3* or *ADE2* gene to construct pUmsn4ΔUra3 (Inoue *et al.*, 1998) and pUmsn4ΔAde2, respectively. The pUmsn4ΔUra3 was digested with *SphI* and *EcoRI*, and DNA fragment containing the *msn4Δ::URA3* cassette was introduced to *S. cerevisiae*. To disrupt the *MSN4* gene with *ADE2* marker, the pUmsn4ΔAde2 was digested by *SphI* and *EcoRI*, and the resultant fragment carrying the *msn4Δ::ADE2* cassette was used.

The *SNF1* gene was cloned by PCR using primers: 5'-GCGCAAGAAACGGCA-GAAC-AGAAGCTGCTC-3' and 5'-TCCCGATAACGCTCTGGAATTC-AGTGTTGG-3'. Both primers were designed to contain the *EcoRI* site, thus the PCR fragment (3376bp) was digested with *EcoRI*, and cloned into the *EcoRI* site of pUC19. The resultant plasmid (pUCSNF1) was digested with *AflIII* and *MluI*, and then 816bp fragment corresponding to the ORF of the *SNF1* gene was replaced with the *HIS3* gene to yield pUSNF1ΔHis3. The plasmid was digested with *EcoRI*, and DNA fragment containing the *snf1Δ::HIS3* cassette was introduced to *S. cerevisiae*.

The *MIG1* gene was obtained by PCR using primers: 5'-GCATATCAACGC-ATGCGTTACACAAGATAT-3' and 5'-GGGATTATGTCGACCTGAAGATTAACCCAC-3', which were designed to contain recognition site for *SphI* and *SalI* site, respectively (underlined). The PCR product carrying the *MIG1* gene (4436bp) was cloned between *SphI* and *SalI* site of pUC19 to give pUCMIG1. The region between *XhoI* site and *StyI* site within the *MIG1* ORF was replaced with *HIS3* gene to construct pUMIG1ΔHis3. To disrupt the *MIG1* gene, pUMIG1ΔHis3 digested with *Clal* and *PvuII*, and *mig1Δ::HIS3* cassette was introduced to *S. cerevisiae*. The *MIG1* gene was also disrupted with *URA3* marker. The *MIG1* gene has three *StyI* sites in its ORF, therefore, the pUCMIG1 was digested with *StyI*, then the *URA3* gene was cloned to yield pUCmig1ΔUra3. To disrupt the *MIG1* gene,

pUMIG1ΔUra3 digested with *SphI* and *Sall*, and *mig1Δ::URA3* cassette was introduced to *S. cerevisiae*.

The *YAP1* gene disruption was carried out by using a plasmid pSM27 as described by Wu *et al.* (1993).

Disruption of each gene was verified by PCR and corresponding phenotype.

Stress experiments and preparation of cell extracts

Experimental procedures were described in chapter 1. Yeast cells grown to mid-log phase were collected at 28°C, resuspended in YPD (with glucose) or YP (without glucose) medium, and then cultured at 28°C with shaking for another 1 h. Cell extracts were prepared to assay β-galactosidase activity as described in chapter 1.

Results and Discussion

Expression of *DOG2* gene is repressed by Snf1p-Mig1p pathway

Depletion of nutrients is one of the stresses to the cells. For example, in *E. coli* cell expression of several stress-inducible gene are enhanced when the cells entered the stationary phase. In this case, a sigma factor of RNA polymerase is changed from vegetative sigma factor (σ^{70}) to σ^S encoded by the *katF* (*rpoS*) gene, and resultant RNA polymerase changes to recognize specific promoter sequence of the stress-inducible genes (for review, see Loewen and Hengge-Aronis, 1994). Depletion of nutrients in culture can also induce the change of sigma factor. On the other hand, glucose repression was also observed in *E. coli* cell (for reviews, see Gottesman, 1984; Ribaud and Schwartz, 1984). The *lac* operon has been well documented. If the *E. coli* cells were cultured in the medium containing glucose, expression of the *lac* genes was repressed by an *lacI*-repressor. Once the cells are shifted to lactose medium, intracellular cAMP level increases, and cAMP-receptor protein (CRP)-cAMP complex binds to the promoter region of *lac* promoter to activate transcription of *lac* genes as well as release of lactose-bound *lacI* repressor from the *lac* operator region. Glucose repression (catabolite repression) can be seen in *S. cerevisiae*. Mechanisms for glucose repression in yeast have been extensively studied by Johnston, Carlson, and their colleagues (for review, see Johnston and Carlson, 1992).

In the case of *DOG2* gene, basal expression level was increased if the cells are exposed to glucose-starved conditions (Fig. 3). Analysis of 5'-upstream region of the *DOG2* gene revealed that a consensus sequence to which a Mig1p/Ssn6p/Tup1p complex can recognize and bind (AT-rich + GGGG; MBS=Mig1p binding site) was located between 206 nt and 188 nt upstream of translational initiation codon (ATG). The Mig1p/Ssn6p/Tup1p is one of the global repressors in *S. cerevisiae*, and expression of the *SUC2* gene has been known to be negatively regulated by this complex in the presence of glucose. To assess whether the

glucose repression of the *DOG2* gene was regulated by Mig1p/Ssn6p/Tup1p complex, the *MIG1* gene encoding a zinc-finger protein was disrupted. As shown in Fig. 4, basal level of the *DOG2* gene expression was increased in the *mig1Δ* disruptant compared with that of wild type strain. It has been reported that repression of the *SUC2* gene by the Mig1p/Ssn6p/Tup1p complex was derepressed by Snf1p Ser/Thr protein kinase if the cells are cultured in glucose-starved medium. To confirm whether the same mechanism is working on to the *DOG2* gene expression, the *SNF1* gene was disrupted and then the cells were cultured in YP medium. As shown in Fig. 4, induction of *DOG2* gene expression in YP medium was not observed in the *snf1Δ* disruptant. These results suggest that glucose repression of the *DOG2* gene is regulated by Snf1p-Mig1p pathway.

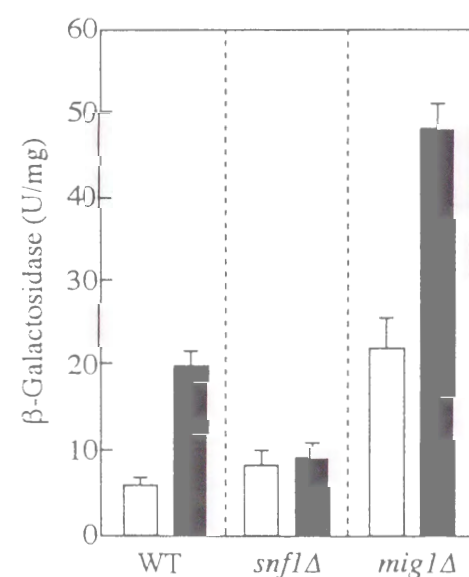


Fig. 4 Repression of *DOG2-lacZ* fusion by Snf1p-Mig1p pathway. White bars indicate β -galactosidase activity that were assayed in cells transferred to YPD (with 2% glucose) medium. Black bars indicate β -galactosidase activity that were assayed in cells transferred to YP (without glucose) medium. Detailed conditions for experiments are described in the text. WT represents SET8-1-C.

Snf1p-Mig1p pathway interacts with HOG-MAP kinase cascade as regulator system of *DOG2* gene

The *MIG1* gene encodes a zinc-finger protein, and the gene product can recognize a consensus sequence (MBS) consisted of AT-rich sequence followed by the G-cluster, and bind to the GGG motif. The *DOG2* gene has the similar sequence in its promoter region, and actually basal expression level of the *DOG2* gene was increased in the *mig1Δ* disruptant (Fig. 4). Interestingly, the *DOG2* gene expression was further induced by glucose starvation in the *mig1Δ* disruptant. This observation suggests that there may be other factor(s) or mechanism(s) that can regulate glucose repression of the *DOG2* gene.

As shown in Fig. 3, expression of the *DOG2* gene was also induced by osmotic stress. In *S. cerevisiae*, hyperosmotic signal is transduced through HOG-MAP kinase cascade (Boguslawski, 1992; Brewster *et al.*, 1993). Two transcriptional factors has been identified so far that are under the control of the HOG-MAP kinase pathway in the osmotic stress response; *i. e.*, Msn2p and Msn4p. Initially the *MSN2* and *MSN4* genes were identified as a multicopy suppressor for derepression of the *SUC2* gene under the glucose-starved conditions in the *snf1*-deficient mutant (Estruch and Carlson, 1993; Martinez-Pastor *et al.*, 1996; Schuller *et al.*, 1994). Both of the *MSN2* and *MSN4* gene encode Mig1p-like C2H2 type zinc-finger protein, and Msn2p and Msn4p have been proved to bind to the STRE (5'-AGGGG-3') sequence. These three transcriptional factors, Mig1p, Msn2p and Msn4p have similar structure (zinc-finger protein) and recognize similar DNA sequence. The Mig1p complex is phosphorylated by Ser/Thr protein kinase encoded by the *SNF1* gene. On the other hand, it has been thought that Msn2p and Msn4p is functionally regulated by Hog1p, which is also a Ser/Thr protein kinase. Expression of the *GLO1* gene is specifically induced by osmotic stress, and its induction is strictly regulated by HOG-MAP kinase cascade, because no induction was found in the *hog1Δ* disruptant. Furthermore, its induction by osmotic stress was completely abolished in the *msn2Δ/msn4Δ* double disruptant (Inoue *et al.*,

1998). These observations strongly suggest that both Msn2p and Msn4p are regulated by Hog1p. From these circumstances, the author speculated that induction of the *DOG2* gene expression in the *mig1Δ* disruptant might be performed by Msn2p and/or Msn4p, both of which have potential to bind G-cluster of MBS. To confirm this hypothesis, the author constructed *msn2Δ/msn4Δ/mig1Δ* triple disruptant, and analyze its phenotype. In disagreement with this hypothesis, induction of the *DOG2* gene expression by glucose starvation was still observed in this triple disruptant as well as in the *msn2Δ*, *msn4Δ* and *msn2Δ/msn4Δ* disruptants (Fig. 5A).

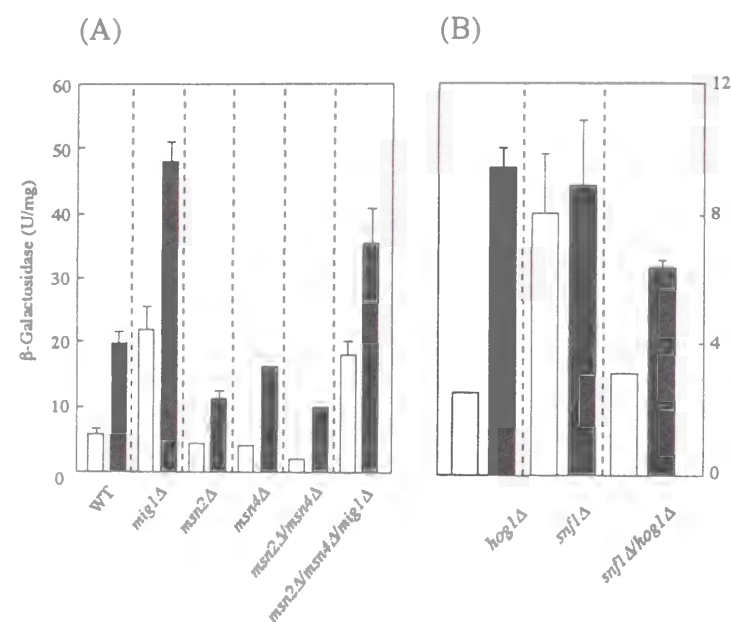


Fig. 5 Expression of *DOG2-lacZ* by glucose starvation in various disruptants. Cells were cultured at 28°C with shaking until OD₆₁₀ reached 0.8-1.0, then collected by centrifugation. Cells were resuspended in YPD medium (with 2% glucose, white bars), or YPD medium (without glucose, black bars), and incubated at 28°C with shaking for another 1 h. Transcriptional regulators (A) or Ser/Thr protein kinase (B) were disrupted. Detailed genotype of each disruptant was given in Table 1.

Disruption of the *SNF1* gene abolished derepression of the *DOG2* gene expression under glucose-starved conditions. This suggests that a glucose-starved signal is focused on

the Snf1p in the *DOG2* gene expression. On the other hand, the *DOG2* gene has the STRE in its promoter region. It has been reported that expression of the *CTT1* gene, which has two STREs in its promoter, was negatively regulated by protein kinase A (PKA), and it was thought to be performed through STRE. In the case of *S. cerevisiae*, cAMP level is high when glucose is present in the medium. Thus, PKA is thought to be in the "active form" in log phase. Actually, catalase T (*CTT1* gene product) activity is extremely low in log phase and it increases when the cells enter the stationary phase. Increase of catalase T activity in stationary phase is thought to be derepression of the *CTT1* gene expression, because cAMP level decreases in stationary phase being due to depletion of glucose and PKA turns to be the "inactive form". In the case of *DOG2* gene, disruption of the *SNF1* gene prevented the inducible expression of the *DOG2* gene under the glucose-starved conditions. If PKA also participates in the repression of *DOG2* gene, induction of *DOG2* gene expression should have been observed in the *snf1Δ* disruptant under glucose-starved conditions. However, the author could not find the increase of β-galactosidase activity derived from the *DOG2-lacZ* gene. Therefore, the author ruled out the possibility that the PKA repressed the expression of *DOG2* gene in the presence of glucose.

Interestingly, expression of the *DOG2* gene was induced in the *snf1Δ/hog1Δ* double disruptant when the cells were exposed to glucose-starved medium. Disruption of the *HOG1* gene alone did not inhibit induction of the *DOG2* gene expression in YP medium, whereas disruption of the *SNF1* gene alone did. Since *msn2Δ/msn4Δ/mig1Δ* triple disruptant could still respond to glucose starvation (Fig. 5A), the author speculated the 4th factor that could regulate the expression of *DOG2* gene. The 4th factor is likely to be constitutively and negatively regulated by Hog1p. Furthermore, the 4th factor seems to be sensing the glucose-starvation if both Snf1p and Hog1p are absence. The author thought that the Adr1p could be a candidate for such a factor. Adr1p is known to regulate the expression of *CTA1* gene encoding peroxisomal catalase A (Simon *et al.*, 1991). Expression of the *CTA1* gene is also

repressed when glucose is present in the medium. This repression is performed by PKA, although the *CTA1* gene does not have the STRE in its promoter region. In the case of *CTA1* gene, Adr1p is a positive regulator and it is phosphorylated by PKA if glucose is present in the medium. When glucose is eliminated from the medium or exhausted by entering the stationary phase, the Adr1p is dephosphorylated and it turns to be an "active form". PKA as well as Snf1p and Hog1p is a Ser/Thr protein kinase. From these backgrounds, the author speculated a putative model for explanation for the induction of *DOG2* gene expression in the *snf1Δ/hog1Δ* double disruptant under the glucose-starved conditions. If glucose is present in the medium, Snf1p is in the "inactive form", thus Mig1p/Ssn6p/Tup1p complex binds to the MBS of the *DOG2* gene promoter. PKA is in the active form, and it phosphorylates the Adr1p to be in the inactive form. Therefore, the phosphorylated Adr1p does nothing on the *DOG2* gene expression. If glucose is starved, the Snf1p turns to be an active form, and it phosphorylates the Mig1p/Ssn6p/Tup1p complex, and derepression is performed. On the other hand, PKA changes to be an inactive form, and Adr1p is no longer phosphorylated, thus the active-form-Adr1p enhances expression of the *DOG2* gene. This mechanism is independent from Mig1p/Ssn6p/Tup1p complex, because inducible expression of the *DOG2* gene was still observed even in the *mig1Δ* disruptant and *msn2Δ/msn4Δ/mig1Δ* triple disruptant. If the Adr1p could be a substrate for Snf1p and Hog1p, the author can explain why the *DOG2* gene expression was induced by glucose starvation in the *snf1Δ/hog1Δ* double disruptant. If the *snf1Δ/hog1Δ* disruptant is exposed to glucose-starved medium (YP medium), PKA turns to be an "inactive form", thus Adr1p is not phosphorylated by PKA any longer. Furthermore, Snf1p and Hog1p are not existed in the cells, so the Adr1p can activate transcription of the *DOG2* gene. Computer analysis predicted several putative Adr1p-binding-site in the promoter region of *DOG2* gene. Analysis the phenotype of *adr1Δ* mutant will answer the rationality of this model.

References

- Boguslawski, G. (1992) *PBS2*, a yeast gene encoding a putative protein kinase, interacts with the RAS2 pathway and affects osmotic sensitivity of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **138**:2425-2432
- Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E., and Gustin, M. C. (1993) An osmosensing signal transduction pathway in yeast. *Science* **259**:1760-1763
- Celenza, J. L., and Carlson, M. (1984) Structure and expression of the *SNF1* gene of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **4**:54-60
- Celenza, J. L., and Carlson, M. (1986) A yeast gene that is essential for the release from glucose repression encodes a protein kinase. *Science* **233**:1175-1180
- Celenza, J. L., Eng, F. J., and Carlson, M. (1989) Molecular analysis of the *SNF4* gene of *Saccharomyces cerevisiae*: evidence for physical association of the SNF4 protein with SNF1 protein kinase. *Mol. Cell. Biol.* **9**:5045-5054
- Estruch, F., and Carlson, M. (1993) Two homologous zinc finger genes identified by multicopy suppression in a SNF1 protein kinase mutant of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **13**:3872-3881
- Gao, G., Fernandez, C. S., Stapleton, D., Auster, A. S., Widmer, J., Dyck, J. R. B., Kemp, B. E., and Witters, L. A. (1996) Non-catalytic β - and γ -subunit isoforms of 5'-AMP-activated protein kinase. *J. Biol. Chem.* **271**:8675-8671
- Gottesman, S. (1984) Bacterial regulation: regulatory networks. *Annu. Rev. Genet.* **18**:415-441
- Inoue, Y., Tsujimoto, Y., and Kimura, A. (1998) Expression of the glyoxalase I gene of *Saccharomyces cerevisiae* is regulated by high osmolarity glycerol mitogen-activated protein kinase pathway in osmotic stress response. *J. Biol. Chem.* **273**:2977-2983
- Jiang, R., and Carlson, M. (1997) The Snf1 protein kinase and its activating subunit, Snf4,

interact with distinct domains of the Sip1/Sip2/Gal83 component in the kinase complex. *Mol Cell. Biol.* **17**:2099-2106

Johnston, M., and Carlson, M. (1992) Regulation of carbon and phosphate utilization. In *The molecular and cellular biology of the yeast Saccharomyces cerevisiae: Gene Expression*, 193-281. Cold Spring Harbor Laboratory Press, Cold spring Harbor, NY.

Johnston, M., Flick, J. S., and Pexton, T. (1994) Multiple mechanisms provide rapid and stringent glucose repression of *GAL* gene expression in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **14**:3834-3841

Loewen, P. C., and Hengge-Aronis, R. (1994) The role of the sigma factor σ^S (Kat F) in bacterial global regulation. *Annu. Rev. Microbiol.* **48**:53-80

Martinez-Pastor, M. T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H., and Estruch, F. (1996) The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress-response element (STRE). *EMBO J.* **15**:2227-2235

Mitchelhill, K. I., Stapleton, D., Gao, G., House, C., Michell, B., Katsis, F., Witters, L. A., and Kemp, B. E. (1994) Mammalian AMP-activated protein kinase shares structural and functional homology with the catalytic domain of yeast Snf1 protein kinase. *J. Biol. Chem.* **269**:2361-2364

Nehlin, J. O. and Ronne, H. (1990) Yeast MIG1 repressor is related to the mammalian early growth response and Wilms' tumor finger proteins. *EMBO J.* **9**:2891-2895

Ribaud, O., and Schwartz, M. (1984) Positive control of transcription initiation in bacteria. *Annu. Rev. Genet.* **18**:173-206

Ruis, H. and Schuller, C. (1995) Stress signaling in yeast. *BioEssays* **17**:959-965

Schuller, H., Brewster, J. L., Alexander, M. R., Gustin, M. C., and Ruis, H. (1994) The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the *Saccharomyces cerevisiae* *CTT1* gene. *EMBO J.* **13**:4382-4389

Schuller, H. -J., and Entian, K.-D. (1987) Isolation and expression analysis of two yeast

regulatory genes involved in the depression of glucose-repressible enzymes. *Mol. Gen. Genet.* **209**:366-373

Schuller, H. -J., and Entian, K.-D. (1988) Molecular characterization of yeast regulatory gene *CAT3* necessary for glucose derepression and nuclear localization of its product. *Gene* **67**:247-257

Simon, M., Adam, G., Raevak, W., Spevak, W., and Ruis, H. (1991) The *Saccharomyces cerevisiae* *ADR1* gene is a positive regulator of transcription of gene encoding peroxisomal proteins. *Mol. Cell. Biol.* **11**:699-704

Trumbly, R. J. (1992) Glucose repression in the yeast *Saccharomyces cerevisiae*. *Mol. Microbiol.* **6**:15-21

Treitel, M., and Carlson, M. (1995) Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. *Proc. Natl. Acad. Sci. USA* **92**:3132-3136

Wilson, W. A., Hawley, S. A. and Hardie, G. (1996) Glucose repression/derepression in budding yeast: SNF1 protein kinase is activated by phosphorylation under derepressing conditions, and this correlates with a high AMP:ATP ratio. *Curr. Biol.* **6**:1426-1434

Yang, X., Hubbard, J. A., and Carlson, M. (1992) A protein kinase substrate identified by the two-hybrid system. *Science* **257**:680-682

Yang, X., Jing, R., and Carlson, M. (1994) A family of proteins containing a conserved domain that mediates interaction with the yeast SNF1 protein kinase complex. *EMBO J.* **13**:5878-5886

Chapter 3

REGULATION OF *DOG2* GENE EXPRESSION UNDER HIGHLY OSMOTIC CONDITIONS

As mentioned in chapter 1, the expression of *DOG2* gene was induced by osmotic stress and glucose starvation as well as oxidative stress. The *DOG2* gene had a *cis*-element termed stress response element (STRE; 5'-AGGGG-3') and the consensus sequence to which the Mig1p transcriptional repressor binds. In this chapter, the author describes regulation of the *DOG2* gene expression and signal transduction under highly osmotic conditions.

Osmotic stress response in yeast has been well documented (for reviews, see Ruis and Sculler, 1995; Varela and Mager, 1996). When yeast cells are exposed to highly osmotic pressure, they synthesize and accumulate glycerol in cytosol to adapt to osmotic stress. The yeast cell has two osmosensing transmembrane proteins, Sln1p and Sho1p, which regulate HOG (high osmolarity glycerol) response pathway. The HOG pathway is defined by the *PBS2* and *HOG1* gene products, a member of MAP kinase kinase and MAP kinase, respectively (Boguslawski, 1992; Brewster *et al.*, 1993). The Sln1p, which contains both a histidine kinase domain and a receiver domain in the same protein, constitutes a two-component system with Ssk1p (Maeda *et al.*, 1994). The Sho1p contains an SH3 domain that can bind to the Pbs2p and activate the HOG pathway (Maeda *et al.*, 1995). Recently, it has been found that Msn2p and Msn4p are required for transcriptional induction through STRE (Martinez-Pastor *et al.*, 1996). Expression of the *GLO1* gene, which encodes glyoxalase I and contains two STREs in its 5'-upstream region, is induced by osmotic stress, and its induction is abolished in a double disruptant of *MSN2* and *MSN4* gene as well as in a disruptant of *HOG1* gene (Inoue *et al.*, 1998). The *MSN2* and *MSN4* genes encode homologous zinc-finger transcriptional factor, and were initially identified as multicopy suppressors for glucose repression in the Snf1p (Ser/Thr protein kinase) deficient mutant (Estruch and Carlson, 1993). It has been proposed that Msn2p and Msn4p might recognize and bind to the same sequence to which the Mig1p/Ssn6p/Tup1p complex binds. On the other hand, the *ENA1* gene encodes an ATPase involved in sodium exclusion. Induction of the *ENA1* gene expression by low NaCl concentration (0.3 M) is mediated by the HOG-MAP kinase cascade, while induction by high NaCl concentration (0.8 M) is controlled by the protein phosphatase calcineurin (Marquez and Serrano, 1996). Additionally, it has been shown that the expression of *ENA1* gene is also regulated by Snf1p-Mig1p pathway (Alepuz

et al., 1997). In this chapter, the author analyzes whether HOG-MAP kinase cascade and Snf1p-Mig1p pathway are involved in induction of the *DOG2* gene expression under highly osmotic conditions. As a result, induction of the *DOG2* gene expression by 0.7 M NaCl was abolished in the *pbs2Δ*, *hog1Δ*, and *snf1Δ* disruptant, whereas induction of the *DOG2* gene expression by 0.3 M NaCl was not abolished in *pbs2Δ*, *hog1Δ*, and *snf1Δ* disruptant. This was not the case for the *snf1Δ/hog1Δ* and *snf1Δ/pbs2Δ* double disruptants.

Materials and Methods

Stress experiments and preparation of cell extracts

Yeast strains used in this chapter are summarized in Table 1. Experimental procedures are described in chapter 1. Yeast cells were cultured at 28°C in YPD medium until OD₆₁₀ reached approximately 0.8-1.0, and then various concentrations of NaCl were added. Cells were cultured for another 1h, and cell extracts were prepared to assay β-galactosidase activity.

Results and Discussion

Effect of NaCl concentration on the expression of *DOG2* gene

It has been known that *S. cerevisiae* cells responds to the change of osmolarity of environment. *S. cerevisiae* has two osmosensors that mediate hyperosmotic stress, and the signal activates the HOG-MAP kinase cascade. The Hog1p is one of the MAP kinases in *S. cerevisiae*, and the Msn2p and Msn4p, both of which are zinc-finger protein, and bind to the STRE sequence, are thought to be under the control of HOG-MAP kinase cascade. The *DOG2* gene has an STRE sequence in the promoter region. As shown in Fig. 3, expression of the *DOG2* gene was enhanced by osmotic stress caused by NaCl, KCl and sorbitol. These results indicated that a change of osmolarity of environment is a stimulus that activates transcription of the *DOG2* gene, rather than the change of concentration of a specific solute.

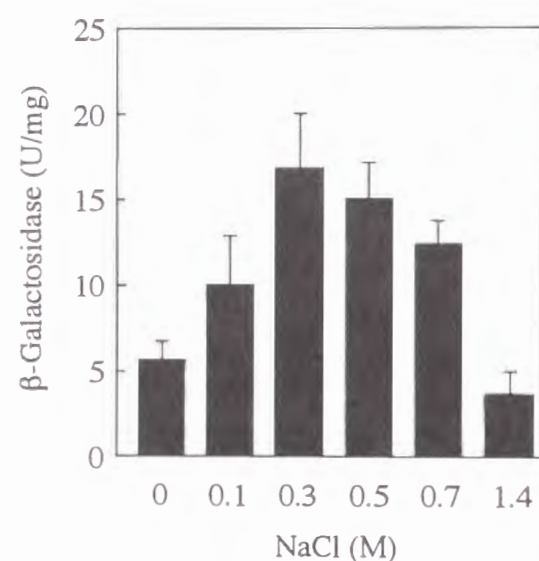


Fig. 6 Effect of NaCl concentration of *DOG2* gene expression. SET8-1-C was cultured at 28°C in YPD medium until OD₆₁₀ reached approximately 0.8-1.0, and then various concentrations of NaCl were added. Cells were cultured for another 1h, and β-galactosidase activity in cell extracts were assayed.

Figure 6 shows the effect of NaCl concentrations on expression of the *DOG2* gene. Induction of the *DOG2* gene expression was observed between 0.1 M and 0.7 M NaCl, maximum at 0.3 M, but its induction was not seen at 1.4 M. It has been reported that maximum concentration of NaCl that activates Hog1p was 0.3 M (Brewster *et al.*, 1993).

Norbeck and Blomberg (1997) identified several genes whose expression was enhanced by 1.4 M NaCl in *S. cerevisiae*. They presented a putative *cis*-element that responded to 1.4 M NaCl stress, *i. e.*, they proposed 5'-TATGCCTCT-3' as the consensus sequence. However, the *DOG2* gene does not contain such a sequence in its 5'-upstream region. Therefore, the expression of *DOG2* gene was thought to be regulated by the HOG-MAP kinase cascade through STRE under highly osmotic conditions.

Regulation of *DOG2* gene expression by HOG-MAP kinase cascade

To assess whether osmotic stress response of the *DOG2* gene expression is dependent on the HOG-MAP kinase pathway, the author disrupted the genes involved in this pathway, such as *PBS2* and *HOG1* genes. The Hog1p is one of the mitogen-activated protein kinases (MAPKs) in *S. cerevisiae*. Both residues of Thr174 and Tyr176 of the Hog1p are phosphorylated by a MAPK kinase (MAPKK), Pbs2p. Pbs2p is phosphorylated by redundant MAPKK kinases (MAPKKKs), Ssk2p and Ssk22p. The *hog1Δ* knockout mutant showed lethality under highly osmotic conditions (Schuller *et al.*, 1994; Brewster *et al.*, 1993). Induction of the *DOG2* gene expression was completely repressed in the *pbs2Δ* and *hog1Δ* disruptants (Fig. 7A). These results indicate that the expression of *DOG2* gene was regulated by HOG-MAP kinase cascade.

As described in previous chapters, both of the *MSN2* and *MSN4* gene products are thought to be under the control of Hog1p. In the case of *GLO1* gene, inducible transcription by osmotic stress was reduced in *msn2Δ* and *msn4Δ* disruptants, and it was completely repressed in the *msn2Δ/msn4Δ* double disruptant (Inoue *et al.*, 1998). To clarify these zinc-finger proteins are involved in the osmotic stress response of the *DOG2* gene, these genes were disrupted. As shown in Fig. 7A, basal level of the *DOG2* gene expression was decreased by disruption of *MSN2* and/or *MSN4* gene, although the *msn4Δ* disruptant, still responded to the osmotic stress and fold increase of β-galactosidase activity derived from *DOG2-lacZ* was comparable with that of wild type strain. The *msn2Δ/msn4Δ* double disruptant could respond to osmotic stress with regard to the *DOG2* gene expression. This was the case for *CTT1* gene. Expression of the *CTT1* gene was regulated by the HOG-MAP kinase cascade, however, induction of gene expression still occurred in *msn2Δ/msn4Δ* double mutant (Martinez-Pastor *et al.*, 1996; Inoue *et al.*, 1998). These results suggest that the HOG-MAP kinase cascade may control other unknown-factor(s) in addition to the Msn2p

and Mns4p.

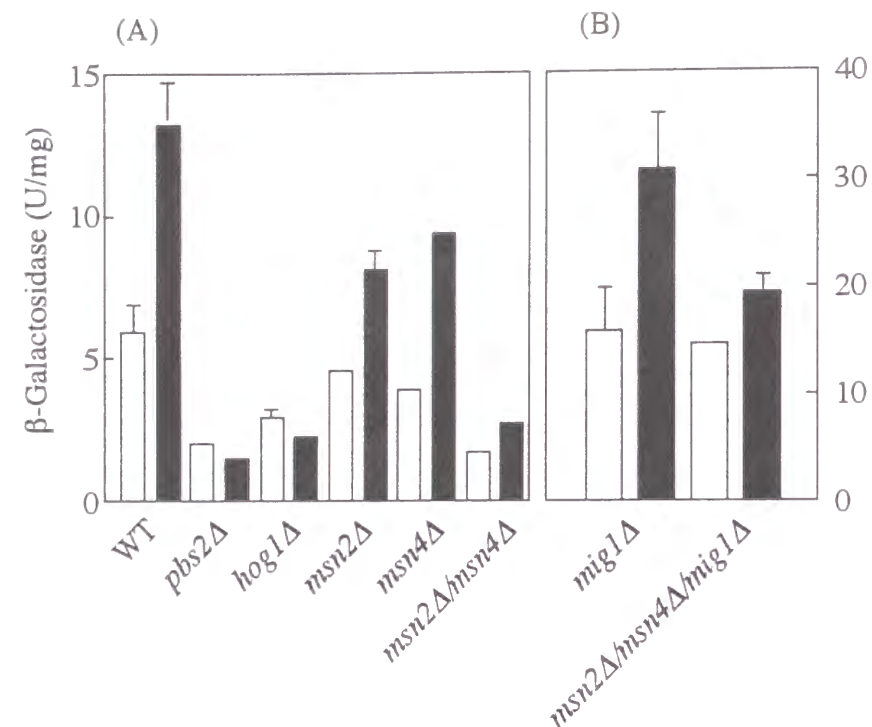


Fig. 7 Expression of *DOG2-lacZ* under highly osmotic conditions. Genotype of each strain was given in Table 1. Cells were cultured at 28°C with shaking until OD₆₁₀ reached 0.8-1.0, and solid NaCl was added to the culture to bring final concentration to be 0.7 M. Cells were then cultured for another 1 h, and β-galactosidase activity in cell extracts were assayed. White bars, no addition of NaCl; back bars, 0.7 M NaCl.

As described in Chapter 2, the *DOG2* gene has MBS (Mig1p/Ssn6p/Tup1p complex binding site) in its promoter region just one-base pair adjacent to the STRE. The *MSN2* and *MSN4* were originally cloned as a multicopy suppressor that enabled to derepress the *SUC2* gene repression in the *snf1*-deficient mutant (Estruch and Carlson, 1993). The Mig1p/Ssn6p/Tup1p complex recognizes and binds to the AT-rich followed by G-cluster; while the Msn2p and Msn4p bind to the 5'-AGGGG-3' sequence. The results that the *DOG2* gene expression was still observed in the *msn2Δ/msn4Δ* double disruptant, the author suspected that the Hog1p might phosphorylate the Mig1p/Ssn6p/Tup1p complex to release the repression of *DOG2* gene under highly osmotic conditions. However, as shown in Fig. 7B, the *msn2Δmsn4Δ/mig1Δ* triple disruption could not repress induction of the *DOG2* gene expression by osmotic stress. Thus, as the author mentioned above, other factor(s), which should have a positive effect on the *DOG2* gene expression, might be present in the osmotic stress response of the *DOG2* gene.

Combinational regulation of *DOG2* gene by HOG-MAP kinase pathway and Snf1p-Mig1p pathway under highly osmotic conditions

Osmotic stress-inducible increase of the *DOG2* gene expression was completely repressed in the *pbs2Δ* and *hog1Δ* disruptant by treatment with 0.7 M NaCl (Fig. 7). Therefore, the author concluded that expression of the *DOG2* gene was regulated by HOG-MAP kinase pathway. On the other hand, as shown in Fig. 6, optimal concentration of NaCl for induction of *DOG2* gene expression was 0.3 M. As shown in Fig. 8, the *DOG2* gene expression was still observed in the *pbs2Δ* and *hog1Δ* disruptants by treatment of the cells with 0.3 M NaCl, although basal level of the *DOG2* gene expression was decreased by disruption of the *PBS2* and *HOG1* gene. These results suggest that there may be another regulation system that alters the expression of *DOG2* gene in osmotic stress response; *i. e.*, at 0.7 M HOG-MAP kinase cascade mediates the osmotic stress to *DOG2* gene, while at 0.3 M unknown regulation system is working in addition to HOG-MAP kinase cascade.

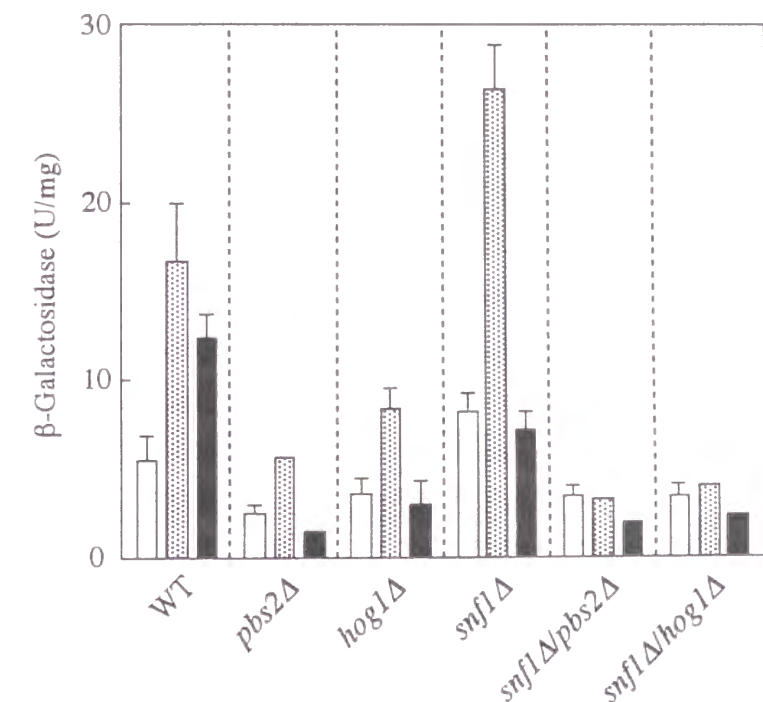


Fig. 8 Expression of *DOG2-lacZ* fusion by osmotic stress in various disruptants. Genotype of each disruptant was given in Table 1. Cells were cultured at 28°C, until OD₆₁₀ reached 0.8-1.0, and solid NaCl, was added to final concentrations to be 0 M (white bars), 0.3 M (shaded bars), and 0.7 M (black bars), respectively. Cells were cultured at 28°C for another 1 h, and β-galactosidase activity in cell extracts were assayed.

As shown in Fig. 8, disruption of the *SNF1* gene also abolished induction of the *DOG2* gene expression at 0.7 M NaCl, but not at 0.3 M. If the *SNF1* gene was disrupted in the *pbs2Δ* or *hog1Δ* background, resultant mutants could not respond to the osmotic stress at all, even though the cells were treated by 0.3 M NaCl. These results implied a hypothesis that the Snf1p might be another mechanism that regulates transcription of *DOG2* gene under highly osmotic conditions. One possibility is that the osmosensor proteins, Sln1p and Sho1p, activate Snf1p to derepress the *DOG2* gene repression through the Mig1p/Ssn6p/Tup1p complex. However, as shown in Fig. 7, the *msn2Δ/msn4Δ/mig1Δ* triple disruptant could still respond to 0.7 M NaCl-treatment; suggesting a possibility that unknown transcriptional factor, which might be a target for Snf1p, is involved in osmotic stress response of *DOG2* gene.

References

- Alepuz, P. M., Cunningham, K. W., and Estruch, F. (1997) Glucose repression affects ion homeostasis in yeast through the regulation of the stress-activated *ENA1* gene. *Mol. Microbiol.* **26**:91-98
- Boguslawski, G. (1992) *PBS2*, a yeast gene encoding a putative protein kinase, interacts with the RAS2 pathway and affects osmotic sensitivity of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **138**:2425-2432
- Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E., and Gustin, M. C. (1993) An osmosensing signal transduction pathway in yeast. *Science* **259**:1760-1763
- Estruch, F., and Carlson, M. (1993) Two homologous zinc finger genes identified by multicopy suppression in a SNF1 protein kinase mutant of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **13**:3872-3881
- Inoue, Y., Tsujimoto, Y., and Kimura, A. (1998) Expression of the glyoxalase I gene of *Saccharomyces cerevisiae* is regulated by high osmolarity glycerol motogen-activated protein kinase pathway in osmotic response. *J. Biol. Chem.* **273**: 2977-2983
- Maeda, T., Takekawa, M., and Saito, H. (1995) Activation of yeast PBS2 MAPKK by MAPKKs or by binding of SH3-containing osmosensor. *Science* **269**:554-558
- Maeda, T., Wurgler-Murphy, S. M., and Saito, H. (1994) A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature* **369**:242-245
- Martinez-Pastor, M. T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H., and Estruch, F. (1996) The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress-response element (STRE). *EMBO J.* **15**:2227-2235
- Marquez, J., and Serrano, R. (1997) Multiple transduction pathways regulate the sodium-extrusion gene *PMR2/ENA1* during salt stress in yeast. *FEBS Lett.* **382**:89-92
- Norbeck, J., and Blomberg, A. (1997) Metabolic and regulatory changes associated with growth of *Saccharomyces cerevisiae* in 1.4 M NaCl. *J. Biol. Chem.* **272**, 5544-5554
- Ruis, H., and Schuller, C. (1995) Stress signaling in yeast. *BioEssays* **17**:959-965
- Schuller, C., Brewster J. L., Alexander, M. R., Gustin, M. C., and Ruis, H. (1994) The HOG pathway controls osmotic regulation of transcriptional via the stress response element (STRE) of *Saccharomyces cerevisiae* *CTT1* gene. *EMBO J.* **13**:4382-4389
- Verela, J. C. S., and Mager, W. H. (1996) Response of *Saccharomyces cerevisiae* to changes in external osmolarity. *Microbiol.* **142**:721-731

Chapter 4

REGULATION OF *DOG2* GENE EXPRESSION UNDER OXIDATIVE STRESS CONDITIONS

Adenosine triphosphate (ATP) is an energy for all types of organisms. *S. cerevisiae* is a facultative anaerobe, thus this organism can live both aerobically and anaerobically. Under anaerobic conditions, *S. cerevisiae* produces ATP by EMP (Embden-Myerhof-Parnas) pathway. The EMP pathway, namely glycolysis, can be defined as an anaerobic oxidation of glucose. In glycolysis, glucose is oxidized to pyruvate and NAD^+ is an acceptor of electrons derived from glucose. During glycolysis, 4 mol of ATP is generated from 1 mol of glucose. Pyruvate is decarboxylated by pyruvate decarboxylase, and acetaldehyde thus formed is reduced to ethanol eventually. On the other hand, under aerobic conditions, pyruvate enters the TCA cycle and then completely oxidized. Upon one cycle of TCA cycle, 5 pairs of protons are extracted and these reducing powers are transferred to the electron chain (respiration chain) in mitochondria. Flux of one electron in this respiration chain produces 3 mol of ATP. Electrons are finally accepted by molecular oxygen by means of cytochrome c oxidase. All aerobic organisms can produce ATP efficiently by respiration. The molecular oxygen is reduced to water through the acceptance of four electrons. During the reduction of molecular oxygen, several reactive oxygen species are formed; *i. e.*, acceptance of one, two and three electrons to form, respectively, the superoxide radical, hydrogen peroxide and hydroxyl radical. Such reactive oxygen species have been reported to be causative agents in several degenerative diseases. They attack almost all cell components, DNA, protein and lipid membrane, and sometimes cause lethal damage to the cells (for review, see Cadenas, 1989).

Lipid hydroperoxides are also one of the reactive oxygen species. Radiation, halocarbons, some drugs and herbicides have been known to be causative of oxidative stress being able to peroxidize the biological membrane *in vivo*. Among the reactive oxygen species, hydroxyl radical as well as perhydroxyl radical ($\text{HOO}^\bullet$) can extract bis-allylic hydrogen atom of unsaturated fatty acid (LH) to form lipid alkyl radical ($\text{L}^\bullet$). The $\text{L}^\bullet$ is oxidized by molecular oxygen to generate a lipid peroxy radical ($\text{LOO}^\bullet$), and the $\text{LOO}^\bullet$ thus formed react with LH to give lipid hydroperoxide (LOOH) and $\text{L}^\bullet$. Radical chain reaction is then propagated. Occurrence of the lipid hydroperoxides in the biological membrane may be one of the major oxidative damages to the cells.

Both prokaryotic and eukaryotic microbial cells have defensive mechanisms against such oxidative damage (for reviews, see Kullik and Storz, 1994; Jamieson and Storz, 1997; Moradas-Ferreira *et al.*, 1996). *E. coli* and *Salmonella typhimurium* cells have the OxyR-controlled regulon of hydrogen peroxide-inducible genes. *E. coli* cells also have a *soxRS*-regulon which is induced by the superoxide radicals. Voluminous studies of the defensive mechanisms against reactive oxygen such as superoxide radicals and hydrogen peroxide in bacterial cells have been done by many investigators. Oxidative stress response of yeast has also been receiving an increasing attention. It has been reported that the Yap1p is critical for oxidative stress response in *S. cerevisiae*. The *YAP1* gene has been cloned by many investigators as multicopy suppressors to the toxicity of various structurally unrelated chemicals, such as 1,10-phenanthroline (*PAR1*) (Schnell and Entian, 1991), cycloheximide (*PDR4*) (Hussain and Lenard, 1991), nitrosoguanidine (*SNQ3*) (Hertle *et al.*, 1991), cadmium (*YAP1*) (Wu *et al.*, 1993), and H_2O_2 (*YAP1*) (Hirata *et al.*, 1994). The Yap1p is transcriptional regulator for the *GSH1* gene encoding g-glutamylcysteine synthetase (Wu and Moye-Rowley, 1993), *GLR1* gene encoding glutathione reductase (Grant *et al.*, 1996a), *TRX2* gene encoding thioredoxin (Kuge and Jones, 1994), and *YCF1* encoding an ABC transporter localized on the vacuole membrane that transports glutathione S-conjugates into vacuole (Li *et al.*, 1996; Wemmie *et al.*, 1994). Therefore, the Yap1p is important for *de novo* synthesis as well as recycling of glutathione, and xenobiotics detoxification with glutathione. Glutathione is one of the antioxidants in organisms, therefore, it is reasonable that a transcriptional factor which can regulate glutathione metabolism is crucial for oxidative stress response.

As the first objective of this study, the author tried to add new knowledge to the mechanism of oxidative stress response in *S. cerevisiae*. The author used lipid hydroperoxide as oxidant, and has screened for the oxidative-stress-responsible genes by using Snyder library. As a result, the author cloned the *DOG2* gene and described its expression pattern in several environmental stress responses, such as glucose starvation and osmotic stress. In this chapter, the author describes a possibility that induction of the *DOG2* gene expression by oxidative stress may be synergistically regulated by Snf1p and Hog1p.

Materials and Methods

Stress experiment

S. cerevisiae strains used in this chapter are summarized in Table 1. Cells were cultured in YPD medium at 28°C with shaking until OD₆₁₀ reached 0.8-1.0, *tert*-butyl hydroperoxide (*t*-BHP, Yoshitomi co.) was added to the medium to bring final concentration of 0.6 mM. Cells were incubated for another 1 h, then cell extracts were prepared to measure β -galactosidase activity as described in Chapter 1.

Results and Discussion

Yap1p is not involved in oxidative stress response in *DOG2* gene expression

The Yap1p has been known to be a critical transcriptional factor for oxidative stress response in *S. cerevisiae*. Deficiency in *YAP1* gene increased sensitivity to several oxidants such as hydrogen peroxide, *t*-BHP in addition to glutathione-depleting agent such as diamide. The Yap1p controls transcription of the genes whose gene products are involved in the biosynthesis and recycling of glutathione, such as *GSH1*, *GLR1* and *TRX2*. The author constructed the *yap1Δ* disruptant by gene disruption method and analyzed the *DOG2* gene expression under oxidative stress conditions. As shown in Fig. 9, the *DOG2* gene expression was still induced by *t*-BHP in the *yap1Δ* disruptant. In the case of *yap1Δ* disruptant, treatment with 0.6 mM *t*-BHP killed the cells, thus 0.1 mM *t*-BHP was used. At this concentrations, the *DOG2* gene expression was also induced in the wild type (data not shown).

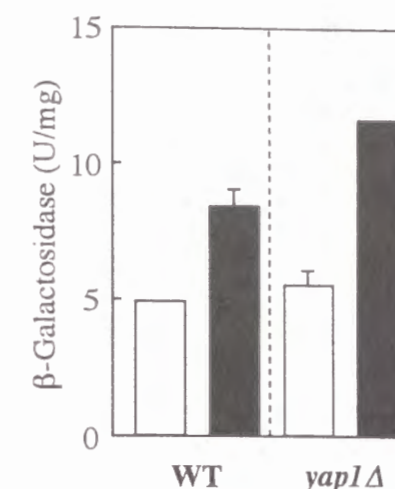


Fig. 9 Expression of *DOG2-lacZ* in *yap1Δ* background. SET8-1-C (WT) was cultured at 28°C in YPD medium until OD₆₁₀ reached approximately 0.8-1.0, and then 0.6 mM *t*-BHP were added (black bar). In the case of *yap1Δ* disruptant (SCYP1), 0.1 mM *t*-BHP (black bar) was added when OD₆₁₀ of the culture reached approximately 0.8-1.0. Cells were cultured for another 1 h. Cell extracts were prepared as described in the text to measure β -galactosidase activity.

The name of Yap was originally come from yeast AP-1 factor. AP-1 is the bZip (basic leucine zipper) protein consisting of hetero-complex of c-Fos and c-Jun or the homodimer of c-Jun family in mammalian cells, and enhances transcription of target genes under several environmental stresses. Two different activation mechanisms for c-Jun have been identified so far; *i. e.*, redox signal activates AP-1 in the presence of thioredoxin and Ref1, or Jun N terminal kinase (JNK)/stress activated protein kinase (SAPK), a member of MAP kinase, phosphorylates c-Jun. The target sequences of AP-1 are TRE (TPA responsible element; TGACTCA) and CRE (cAMP responsible element; TGACGTCA). The Yap1p has been reported to bind the sequence of TGACTAA that is appeared in the SV40 promoter sequence, and the AP-1 can also bind to this sequence. The *GSH1* gene has this motif in its promoter region and the Yap1p could bind to this sequence. However, the Yap1p has more affinity to the sequence of TTA⁺CTAA which is found in the promoter regions of *TRX2* and *GLR1* gene. *In vitro* binding assay clearly demonstrated that the Yap1p had more affinity to this sequence. On the other hand, *S. cerevisiae* has several bZip proteins, and Struhl and his colleagues

recently reported that 14 bZip proteins including Yap1p, are a complete set of bZip proteins in *S. cerevisiae* (Fernandes *et al.*,1997). Gcn4p is also a bZip protein and it binds most efficiently to the sequence of TGACTCA. The *GCN4* gene has been originally cloned as transcriptional factor that regulates the "general control" of amino acid synthesis in *S. cerevisiae*, and later it was also reported that the Gcn4p is involved in the signal transduction of UV response in RAS pathway. *S. cerevisiae* has 8 Yap family proteins, and all of them are bZip protein. Amino acid residues in the basic region critical for specificity to target sequence of DNA of these proteins are conserved among these 8 proteins, however, those are not conserved in other bZip proteins including mammalian AP-1. The amino acid residues appeared in the basic region of Gcn4p are a typical type of AP-1. Struhul and his colleagues, therefore, insisted that the Gcn4p rather than Yap family is a conventional AP-1 factor in *S. cerevisiae*. However, it has not yet been reported that disruption of *GCN4* gene causes hypersensitivity to oxidative stress. At any rate, the *DOG2* gene does have neither TGACTAA nor TTACTTA sequences in its promoter region. Therefore, the author concluded that the Yap1p is not involved in the regulatory machinery in the oxidative stress response of *DOG2* gene.

Expression of *DOG2* gene under oxidative stress conditions is regulated by both Hog1p and Snf1p

In the previous chapters, the author showed that expression of the *DOG2* gene was enhanced by osmotic stress, glucose starvation, and oxidative stress caused by lipid hydroperoxide. Osmotic stress response of the *DOG2* gene expression was regulated by HOG-MAP kinase pathway, while the glucose repression was performed by Snf1p-Mig1p pathway. The *DOG2* gene has the STRE (stress response element) as well as MBS (Mig1p/Ssn6p/Tup1p complex binding site) in its promoter region. The STRE has been documented that it mediates various kinds of stresses such as oxidative stress, heat shock stress, osmotic stress, ethanol stress, and so on. Expression of the *CTT1* gene is actually increased by these stresses. In the case of osmotic stress response, Msn2p and Msn4p, a zinc-finger transcriptional activator, are controlled by Hog1p-MAP kinase, and both factors bind to the STRE. Therefore, the author analyzed expression of the *DOG2* gene in the *hog1Δ* mutant under oxidative conditions. As shown in Fig. 10, induction of the *DOG2* gene was still observed in the *pbs2Δ* and *hog1Δ* disruptants. Therefore, the HOG-MAP kinase pathway alone seemed not to regulate the oxidative-stress-inducible transcription of *DOG2* gene. The author also investigated the induction of *DOG2* gene expression in the *snf1Δ* mutant. As shown in Fig. 10, transient increase of the expression of *DOG2* gene by *tert*-butyl

hydroperoxide (*t*-BHP) was seen in the *snf1Δ* mutant. This result suggests that the Snf1p kinase alone is not likely to be involved in the oxidative-stress-inducible transcription of *DOG2* gene.

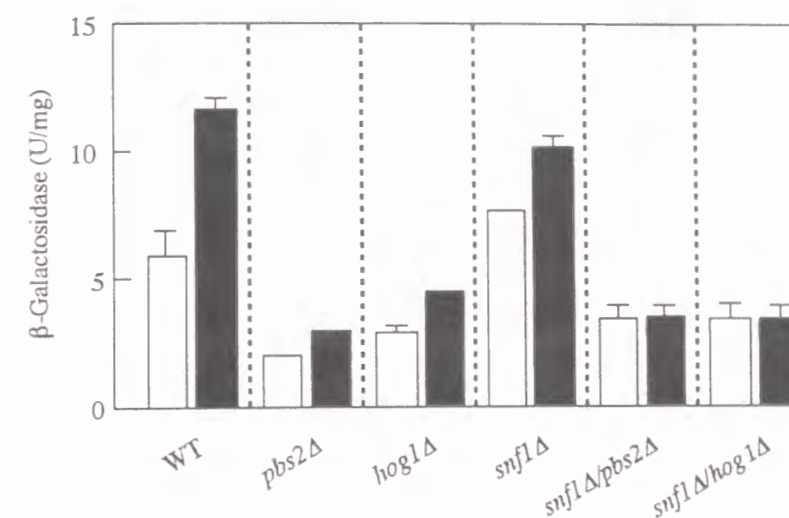


Fig. 10 Expression of *DOG2-lacZ* by oxidative stress in various disruptants. Yeast strains used are listed in Table 1. Cells were cultured at 28°C in YPD medium until OD₆₁₀ reached approximately 0.8-1.0, and 0.6 mM *t*-BHP were added. Cells were cultured for another 1 h, and cell extracts were prepared as described in the text. White bars and black bars indicate β-galactosidase activity of the cells treated by 0 mM and 0.6 mM *t*-BHP, respectively.

In mammalian system, two transcriptional factors have been known to mediate the oxidative stress; *i. e.*, AP-1 and NF-κB. The former is heterodimer of c-Fos and c-Jun, or a homodimer of c-Jun, and Jun N-terminal kinase (JNK)/stress activated protein kinase (SAPK), a member of MAP kinase, phosphorylates Jun protein to activate its function. The latter is heterodimer of p50 and p65 proteins, and NF-κB makes a complex with I-κB. The NF-κB/I-κB complex is retained in the cytosol, whereas once the cells are exposed to oxidative stress the I-κB kinase phosphorylates I-κB, and phosphorylated I-κB dissociates NF-κB. The NF-κB is then localized to nucleus, and activates transcription of target genes, such

as *IL-2R*. Therefore, protein kinases have been reported to play an important role in mediating oxidative stress response in mammalian cell. As shown in Fig. 10, disruption of *HOG1* gene or *SNF1* gene alone did not affect the induction of the *DOG2* gene expression in oxidative stress response. The author then examined the inducibility of *DOG2* gene expression by *t*-BHP in the *snf1Δ/pbs2Δ* and the *snf1Δ/hog1Δ* double disruptants. Interestingly, these mutants did not increase the transcription of *DOG2* gene under oxidative conditions.

The Snf1p (Ser/Thr protein kinase) has been suggested to be activated by elevation of the intracellular AMP:ATP ratio. The author suspects that *S. cerevisiae* requires energy to adapt to the oxidative stress. Because, for example, *S. cerevisiae* enhances glutathione biosynthesis and recycling when the cells are exposed to oxidative stress. Glutathione-synthesizing enzymes (Gsh1p and Gsh2p) require ATP for their catalytic functions. Oxidized glutathione (GSSG, glutathione disulfide) is reduced by the actions of glutathione reductase and glucose-6-phosphate dehydrogenase, and reducing potential of NADPH is transferred to GSSG. In the recycling reactions of GSSG, NADPH is oxidized to NADP⁺, although it is reduced by glucose-6-phosphate dehydrogenase. Glucose-6-phosphate is synthesized from glucose and ATP by glucokinase. Therefore, consumption of intracellular ATP may, presumably, increase under the oxidative conditions, and activation of Snf1p kinase could occur by the elevation of intracellular AMP:ATP ratio.

However, disruption of *SNF1* gene alone did not abolish the induction of *DOG2* gene expression under oxidative conditions. Therefore, additional mechanism was thought to be existed in yeast cell. Simultaneous disruption of the *PBS2* gene or *HOG1* gene with *SNF1* gene suppressed the induction of *DOG2* gene expression. These results strongly suggest that expression of the *DOG2* gene is cooperatively controlled by Hog1p and Snf1p. The Hog1p is proved to be activated by osmotic stress, but not by hydrogen peroxide treatment. In this study, the author used *t*-BHP as a representative for lipid hydroperoxide. Expression of the *DOG2* gene was increased by 0.4 mM hydrogen peroxide (1.3 fold), although fold increase in β-galactosidase activity, derived from *DOG2-lacZ*, by hydrogen peroxide treatment was much smaller than that caused by 0.6 mM *t*-BHP treatment (2.0 fold). One possible explanation for this phenomenon is that *t*-BHP might activate the HOG-MAP kinase pathway by unknown mechanism. Because the *snf1Δ/pbs2Δ* disruptant could not induce the *DOG2* gene expression, the oxidative stress signal may accepted at least by Pbs2p or upstream of the Pbs2p. Osmotic stress sensors on the cell membrane, Sln1p and Sho1p, might mislead the signal by *t*-BHP treatment, or there might be other redundant MAP kinase kinases other than Ssk2p, Ssk22p, and Ste11p, that can interact with Pbs2p.

References

- Boguslawski, G. (1992) *PBS2*, a yeast gene encoding a putative protein kinase, interacts with the RAS2 pathway and affects osmotic sensitivity of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **138**:2425-2432
- Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E., and Gustin, M. C. (1993) An osmosensing signal transduction pathway in yeast. *Science* **259**:1760-1763
- Cadenas, E. (1989) Biochemistry of oxygen toxicity. *Annu. Rev. Biochem.* **58**:79-110
- Celenza, J. L., and Carlson, M. (1986) A yeast gene that is essential for release from glucose repression encodes a protein kinase. *Science* **233**:1175-1180
- Estruch, F., and Carlson, M. (1993) Two homologous zinc finger genes identified by multicopy suppression in a SNF1 protein kinase mutant of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **13**:3872-3881
- Fernandes, L., Rodrigues-Pousada, C., and Struhl, K. (1997) Yap, a novel family of eight bZIP proteins in *Saccharomyces cerevisiae* with distinct biological functions *Mol. Cell. Biol.* **17**: 6982-6993
- Gounalaki, N., and Thireos, G. (1994) Yap1p, a yeast transcriptional activator that mediates multidrug resistance, regulates the metabolic stress response. *EMBO J.* **13**:4036-4041
- Grant, C. M., Collinson, L. P., Roe, J. -H., and Dawes, I. W. (1996a) Yeast glutathione reductase is required for protection against oxidative stress and is a target gene for yAP-1 transcriptional regulation. *Mol. Microbiol.* **21**:171-179
- Grant, C. M., MacIver, F. H., and Dawes, I. W. (1996b) Glutathione is an essential metabolite required for resistance to oxidative stress in the yeast *Saccharomyces cerevisiae*. *Curr. Genet.* **29**:511-515
- Hertle, K. Haase, E., and Brendel, M. (1991) The *SNQ2* gene *Saccharomyces cerevisiae* confer hyper-resistance to several functionally unrelated chemicals. *Curr. Genet.* **19**:429-433
- Hussain, M., and Lenard, J. (1991) Characterization of *PDR4*, a *Saccharomyces cerevisiae* gene that confers pleiotropic drug resistance in high-copy number. *Gene* **101**:149-152
- Hirata, D., Yano, K., and Miyakawa, T. (1994) Stress-induced transcriptional activation mediated by *YAP1* and *YAP2* genes that encode the Jun family of transcriptional activators in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **242**:250-256

Inoue, Y., Tsujimoto, Y., and Kimura, A. (1998) Expression of the glyoxalase I gene of *Saccharomyces cerevisiae* is regulated by high osmolarity glycerol mitogen-activated protein kinase pathway in osmotic response. *J. Biol. Chem.* **273**: 2977-2983

Izawa, S., Inoue, Y., and Kimura, A. (1995) Oxidative stress response in yeast: effect of glutathione on adaptation to hydrogen peroxide stress in *Saccharomyces cerevisiae*. *FEBS Lett.* **368**:73-76

Jamieson, D. J., and Storz, G. (1997) Transcriptional regulators of oxidative stress responses. In *Oxidative stress and the molecular biology of antioxidant defenses*: 91-115, Cold Spring Harbor Laboratory Press

Johnston, M., Flick, J. S., and Pexton, T. (1994) Multiple mechanisms provide rapid and stringent glucose repression of *GAL* gene expression in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **14**:3834-3841

Kuge, S., and Jones, N. (1994) YAP1 dependent activation of *TRX2* is essential for the response of *Saccharomyces cerevisiae* to oxidative stress by hydroperoxides. *EMBO J.* **13**:655-664

Kullik, I., and Storz, G. (1994) Transcriptional regulators of the oxidative stress response in prokaryotes and eukaryotes. *Redox Report* **1**:23-29

Li, Z.-S., Szczypka, M., Lu, Y.-P., Thiele, D. J., and Rea, P. A. (1996) The yeast cadmium factor protein (YCF1) is a vacuolar glutathione S- conjugate pump. *J. Biol. Chem.* **271**:6509-6517

Maeda, T., Takekawa, M., and Saito, H. (1995) Activation of yeast PBS2 MAPKK by MAPKKs or by binding of an SH3-containing osmosensor. *Science* **269**:554-558

Maeda, T., Wurgler-Murphy, S. M., and Saito, H. (1994) A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature* **369**:242-245

Mager, W. H., and de Kruijff, A. J. J. (1995) Stress-induced transcriptional activation. *Microbiol. Rev.* **59**:506-531

Martinez-Pastor, M. T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H., and Estruch, F. (1996) The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress-response element (STRE). *EMBO J.* **15**:2227-2235

Moradas-Ferreira, P., Costa, V., Piper, P., and Mager, W. H. (1996) The molecular defenses against reactive oxygen species in yeast. *Mol. Microbiol.* **19**:651-658

Moye-Rowley, W. S., Harshman, K. D., and Parker, C. S. (1989) Yeast *YAP1* encoded a novel form of the jun family of transcriptional activator proteins. *Genes Dev.* **3**:283-292

Nehlin, J. O., Carlberg, M., and Ronne, H. (1990) Yeast MIG1 repressor is related to the mammalian early growth response and Wilms' tumor finger proteins. *EMBO J.* **9**:2891-2895

Randez-Gil, F., Blasco, A., Prieto, J. A., and Sanz, P. (1995) *DOG^{R1}* and *DOG^{R2}* : Two genes from *Saccharomyces cerevisiae* that confer 2-deoxyglucose resistance when overexpressed. *Yeast* **11**:1233-1240

Ruis, H. and Schuller, C. (1995) Stress signaling in yeast. *BioEssays* **17**:959-965

Schuller, C., Brewster J. L., Alexander, M. R., Gustin, M. C., and Ruis, H. (1994) The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of *Saccharomyces cerevisiae* *CTT1* gene. *EMBO J.* **13**:4382-4389

Schnell, N., and Entian, K.-D. (1991) Identification and characterization of *Saccharomyces cerevisiae* gene (*PAR1*) conferring resistance to iron chelators. *Eur. J. Biochem.* **200**: 487-493

Wilson, W. A., Hawley, S. A., and Hardie, G. (1996) Glucose repression/derepression in budding yeast: SNF1 protein kinase is activated by phosphorylation under derepressing conditions, and this correlates with a high AMP:ATP ratio. *Curr. Biol.* **6**:1426-1434

Wemmie, J. A., Szczypka, M. S., Thiele, D. J., and Moye-Rowley, W. S. (1994) Cadmium tolerance mediated by the yeast AP-1 protein requires the presence of an ATP-binding cassette transporter-encoding gene, *YCF1*. *J. Biol. Chem.* **269**:32592-32597

Wu, A. -L., and Moye-Rowley, S. (1994) *GSH1*, which encodes γ -glutamylcysteine synthetase, is a target gene for yAP-1 transcriptional regulation. *Mol. Cell. Biol.* **14**:5822-5839

Wu, A. -L., Wemmie, J. A., Edgington, N. P., Goebel, M., Guevara, J. L., and Moye-Rowley, W.S. (1993) Yeast bZip proteins mediate pleiotropic drug and metal resistance. *J. Biol. Chem.* **268**:18850-18858

CONCLUSION

In this thesis, the author studied the regulation of *DOG2* gene expression and signal transduction in environmental stress response of *Saccharomyces cerevisiae*. Expression mechanisms can be illustrated like Fig.11 from the results obtained from this study.

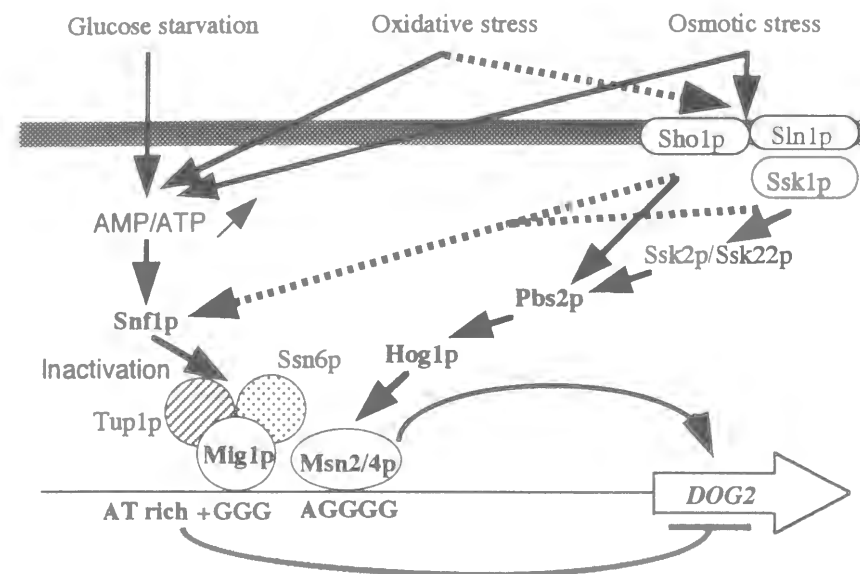


Fig. 11 Speculative model for *DOG2* gene expression and regulation of signal transduction in environmental stress response.

Conclusions in each chapter are:

Chapter 1

To analyze stress response of *S. cerevisiae*, the author screened the oxidative stress-responsive genes by using Snyder library, which is a genomic library containing random *lacZ* insertions throughout the yeast genome. As a result, expression of the *DOG2* gene encoding 2-deoxyglucose-6-phosphate phosphatase was found to be induced by oxidative stress. Expression of the *DOG2* gene was also induced by osmotic stress and glucose starvation, but not by heat shock. By an analysis of 5'-upstream region of the *DOG2* gene, the author found that it had a *cis*-element termed STRE (stress response element, 5'-AGGGG-3') as well as the consensus sequence to which the Mig1p/Ssn6p/Tup1p transcriptional repressor binds adjacent to the STRE.

Chapter 2

Induction of the *DOG2* gene expression by glucose starvation was abolished in *snf1Δ* disruptant, but not in several gene disruptants involved in HOG-MAP kinase cascade. In the *mig1Δ* disruptant, basal expression level of *DOG2* gene increased even though glucose was in the medium. These results indicated that the expression of *DOG2* gene was subjected to glucose repression by the Snf1p-Mig1p pathway. Unexpectedly, the double disruption of *SNF1* and *HOG1* gene did not abolish induction of the *DOG2* gene expression by glucose starvation. These observation suggested that Snf1p-Mig1p pathway interacts with HOG-MAP kinase cascade as a regulator system of *DOG2* gene. Unknown factor, which might be repressed by HOG-MAP kinase cascade, could receive a signal of glucose starvation and regulate the expression of *DOG2* gene.

Chapter 3

Induction of the *DOG2* gene expression by 0.7 M NaCl stress was abolished in the *pbs2Δ*, *hog1Δ*, and *snf1Δ* disruptants. Induction by 0.3 M NaCl stress was not abolished in these disruptants, but abolished in the *snf1Δ/hog1Δ* and *snf1Δ/pbs2Δ* double disruptants. These results indicated that expression of the *DOG2* gene was regulated by both HOG-MAP kinase cascade and Snf1p-Mig1p pathway under highly osmotic conditions.

Chapter 4

The author demonstrated that Yap1p did not regulate the expression of *DOG2* gene under oxidative stress conditions. In the *snf1Δ/hog1Δ* and *snf1Δ/pbs2Δ* double disruptants, the induction of *DOG2* gene by oxidative stress was abolished. However, its induction was not abolished in the *snf1Δ*, *hog1Δ* and *snf1Δ* disruptants. These results suggested that oxidative stress caused by lipid hydroperoxide might activate Snf1p and Hog1p, both of which are a Ser/Thr protein kinase.

ACKNOWLEDGMENTS

The author wishes to express his sincere gratitude to Dr. Akira Kimura, Professor of the Research Institute for Food Science, Kyoto University, for his kind guidance and encouragement throughout the course of this study.

The author is deeply grateful to Dr. Yoshiharu Inoue, Associate Professor of the Research Institute for Food Science, Kyoto University, for his valuable instruction and advice throughout the course of this study.

The author wishes to express his sincere thanks to Mr. Shingo Izawa, Instructor of the Research Institute for Food Science, Kyoto University, for his kind guidance and valuable advice throughout the course of this study.

The author wishes to express his sincere thanks to Dr. Junichi Mano, Instructor of the Research Institute for Food Science, Kyoto University, for his valuable discussion and advice throughout the course of this study.

The author is grateful to all the members of Department of Molecular Breeding of Microorganisms, the Research Institute for Food Science, Kyoto University, for their kind encouragements and cooperative supports.