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Carnitine Uptake by AGP2 in Yeast *Saccharomyces cerevisiae* Is Dependent on Hog1 MAP Kinase Pathway

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The *AGP2* gene encodes a plasma membrane carnitine transporter in *S. cerevisiae*. Here, we report the identification of *AGP2* as an osmotic stress response gene. *AGP2* was isolated from mTn3 tagged mutants that contained in-frame fusions with *lacZ*. The expression of *AGP2* was down-regulated by osmotic stresses, including NaCl, sorbitol, and KCl. We also found that carnitine uptake was inhibited by NaCl. In the *ssk1Δ ste11Δ* double-mutant strain, the expression of *AGP2* and the uptake of carnitine were greatly reduced compared to the wild-type strain. Furthermore, carnitine uptake was inhibited by the constitutive expression of *PBS2*, which encodes a MAPKK that activates Hog1. We concluded, therefore, that the HOG pathway plays an important role in the regulation of carnitine uptake in *S. cerevisiae*.

Keywords: Carnitine Uptake; Expression; HOG Pathway; Osmotic Stress; *S. cerevisiae.*

Introduction

The growth of fungi and plants is greatly affected by external osmotic stress. Adjustment to these is essential for their continued existence. It was recently shown in the yeast, *S. cerevisiae*, that an increase in extracellular osmolarity causes a transient induction of the expression of stress protective genes, and the Hog1 MAP kinase cascade plays a pivotal role in this osmotic adjustment (Gustin *et al.*, 1998; Posas *et al.*, 1998) (Fig. 1). Under osmotic-stress conditions, the activation of Hog1 MAP kinase is regulated by two independent osmosensors - a Sln1-mediated two-component system and Sho1, a transmembrane protein. These sensing mechanisms activate a kinase cascade that involves the Ssk2, Ssk22, and Ste11 MAPK kinase kinases, the Pbs2 MAPK kinase, and the Hog1 MAPK. Tyrosine- and PP2C serine/threonine-phosphatases may also regulate this pathway by dephosphorylating these MAP kinases (Maeda *et al.*, 1994; Fig. 1). Once Hog1 is phosphorylated by Pbs2, it is translocated into the nucleus, where it induces diverse stress responses (Gustin *et al.*, 1998; Posas *et al.*, 1998). Finally, osmosenser activation of a MAP kinase cascade upregulates glycerol production and accumulation for osmotic adjustment in *S. cerevisiae*.

In *S. cerevisiae*, degradation of fatty acids takes place exclusively in the peroxisome (Kunau *et al.*, 1995). The acetyl-CoA that is produced by β-oxidation must be transported from the peroxisome to the mitochondrial matrix for complete oxidation of CO_2 and H_2O. However, long-chain acyl-CoA molecules do not readily traverse the inner mitochondrial membrane. Therefore, a special transport mechanism is needed, in which activated long-chain fatty acids are carried across the inner mitochondrial membrane by conjugating them to carnitine (3-hydroxy-4-*N*-trimethyl-aminobutyrate), a small and highly polar zwitterionic compound that is formed from lysine (Bremer, 1983). By functional complementation of mutants that are specifically defective in the carnitine-dependent acetyl-unit transport from peroxisomes to mitochondria, Van Roermund *et al.* (1999) cloned a yeast carnitine transporter, Agp2, which is essential for carnitine uptake from the medium into the cell.

Earlier, we isolated and characterized several mutants

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Abbreviations: HOG, high osmolarity glycerol; MAP, mitogen-activated protein.

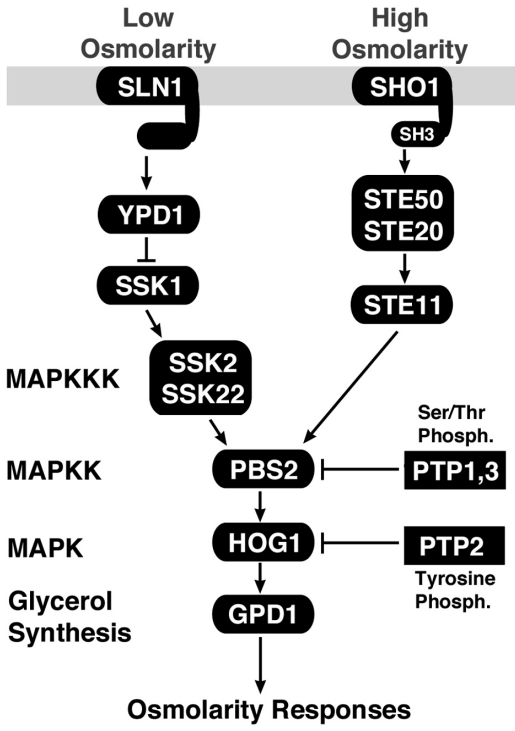

Fig. 1. Osmotic stress mediated signaling pathways in yeast *Saccharomyces cerevisiae* (see details in the **Introduction**).

that are defective in the osmotic stress response of *S. cerevisiae* (Lee *et al*., 2001). In this work, we report that one of the mutants has a defect in the *AGP2* gene. We show that osmotic stress greatly inhibits the uptake of carnitine across the plasma membrane. Furthermore, we provide evidence that the effect is mediated by the Hog1 MAP kinase pathway.

Materials and Methods

Media, strains, and plasmids Standard procedures for yeast culture and growth media preparation were followed (Sherman, 1991). Yeast transformation was performed by the lithium acetate method of Elble (1992). The wild-type *S. cerevisiae* strains that were used in this study were BWG7a (Yun *et al*., 1998) and W303-1a (Mendoza *et al*., 1996). Strain *agp2Δ* (isogenic to W303-1a with *agp2::URA3*) was obtained by the one-step gene disruption method (Rothstein, 1985) after the coding region of *AGP2* was replaced with the *URA3* gene (Van Roermund *et al*., 1999). Disruption of the *AGP2* gene was confirmed by Southern blot hybridization. The SO622 strain (isogenic to W303-1a with *ssk1::HIS3 ste11::LEU2*) was obtained from Dr. Orouke (Herskowitz's Lab, University of California, USA). The *ssn6Δ* was obtained from Ramon Serrano (Institude de Biologia Molecular Celuar de Plantas, Universidad Politecnica de Valencia-CSIC). Strain TM260 (*MAT*a *ura3 leu2 trp1 pbs2::LEU2*) was obtained from Dr. T. Maeda (University of Tokyo, Japan). The plasmid, 903CU-*PBS2*, was constructed by inserting the PCR product of *PBS2* into the vector 903CU. Dr. Maeda (University of Tokyo, Japan) provided the plasmid, pGDG21, carrying PBS2DD, an active form of PBS2.

Construction of a yeast::mTn3 fusion library and cloning of *AGP2* The mTn3 insertion library DNA (Rothstein, 1985) (kindly provided by Dr. Snyder, Yale University, USA) was digested with *Not*I to enable homologous recombination with host DNA. Linearized DNA was transformed into the BWG7a strain, and mTn3 tagged yeast mutant library pools were obtained by selecting transformants on SC-Leu media. Leu+ transformants were selected on YPD plates that were supplemented with 1.5 M NaCl to identify salt-resistant colonies. To determine the genomic DNA sequence adjacent to the mTn3 insertion, a plasmid rescue procedure was performed as described elsewhere (Ross-Macdonald *et al*., 1995).

Southern and Northern blot analyses Yeast genomic DNA, plasmid DNA, restriction endonuclease analysis, gel electrophoresis, and DNA hybridization analysis were prepared or performed as described (Jeong *et al*., 2001). DNA probes were prepared by incorporation of [α-^{32}P] dCTP (Amersham, UK) by random priming of denatured DNA fragments that were isolated by gel electrophoresis. For Northern blot analysis, total RNA was extracted, and probed with ^{32}P-labeled DNA fragments that corresponded to the coding region of *AGP2* and *ADL2* genes under standard high-stringency conditions (Sambrook and Russell, 2001).

β-Galactosidase assay For the β-galactosidase assay, 1.0 M NaCl was added to exponentially growing cells (absorbance at 600 nm of 0.8 to 1.2) in YPD, and the cultures were incubated at 30°C for 1 h. The values are normalized to the OD_{600} nm of control cultures that were grown without NaCl. Each value is means of three experiments. The enzyme assay was performed as described elsewhere (Gaxiola *et al*., 1992), and normalized to protein concentration.

Carnitine uptake Carnitine uptake assays were performed as described by Van Roermund *et al*. (1999). Briefly, the cells were grown on minimal media that contained a 0.67% yeast nitrogen base without amino acids (Difco, USA). They were supplemented with 0.3% glucose and adenine (20 μg/ml), histidine (20 μg/ml), uracil (20 μg/ml), and tryptophan (20 μg/ml) as needed, to a density of OD_{600} = 1.0. The samples were pre-warmed to 30°C for 10 min and 5 μM L-[methyl-14C] carnitine hydrochloride (Amersham, UK) was added. Freshly-growing cells were rapidly filtered through 0.45 μm-HAWP membranes after a salt treatment (Milipore, USA), and washed with a 10 ml 1× PBS buffer. The radioactivity that was retained on the filters was measured by liquid scintillation counting.

Results and Discussion

Isolation of the osmotic stress response gene, *AGP2* In order to identify the novel genes that are involved in NaCl sensitivity in yeast cells, we constructed a yeast::mTn3 tagging library (Burns *et al*., 1994) and isolated 10 mutants that displayed a resistant growth phenotype on YPD media that contained 1.5 M NaCl (Lee *et al.*, 2001). Among the mutants, we could monitor the expression of an osmotic stress response gene (*OSR1*) by a β-galactosidase activity assay since the mTn3 insertion in the yeast *OSR1* gene generated an in-frame fusion product with *lacZ* (Fig. 2A). The expression of *OSR1* was down-regulated by NaCl stress (Fig. 2B). Genomic DNA was prepared from the *osr1* mutant. The *OSR1* sequence was identified by the plasmid rescue method (Graham *et al.*, 1994). Analysis of the tagged sequence revealed that the mutation was placed within *AGP2*, which encodes a plasma membrane carnitine transporter (Van Roermund *et al*., 1999). The isolated gene had an in-frame *lacZ* gene fusion at codon position 290 (Fig. 2A). Since it has been reported that some of the mutants that were obtained from the mutagenized library contained more than one mTn3 insertion (Burns *et al*., 1994), genomic DNA was extracted from an *osr1* mutant and Southern blot analysis was performed using the *lacZ* gene as a probe. As shown in Fig. 2C, we could detect only a single band from the *osr1* mutant, which indicates that the phenotype that is shown in Fig. 2B was mediated by a single insertion of *lacZ* in the *AGP2* locus (Fig. 2C).

Next, we examined whether or not other osmotic stresses also influenced the expression of the *AGP2* gene. Wild-type yeast cells were exposed to NaCl, KCl, and sorbitol, and Northern blot analysis was performed. In this experiment, *ALD2*, a Hog1-dependent gene, was used as a positive control. The transcription level of *ALD2* was increased by the osmotic stresses. It reached a maximum with 0.4 M NaCl, 0.4 M KCl, and 0.6 M sorbitol (Fig. 2D). However, the transcript level of *AGP2* was greatly reduced when the concentration of NaCl, KCl, and sorbitol increased from 0 to 1.0 M (Fig. 2D). These results indicate that the expression of *AGP2* is negatively regulated by osmotic stresses, and this stress response is not specific to NaCl.

Osmotic stress inhibits carnitine uptake by AGP2 It was recently reported that *AGP2* encodes a carnitine transporter in yeast cell (Van Roermund *et al*., 1999). To elucidate the relationship between carnitine uptake and osmotic stress, we first compared the ability of wild-type and *agp2Δ* cells to take up carnitine. The uptake of carnitine in wild-type cells increased with time and reached a maximum level at 120 min; however, in *agp2Δ* cells, there was very little uptake of carnitine (Fig. 3A). When the wild-type yeast cells were transformed with pVT-U::

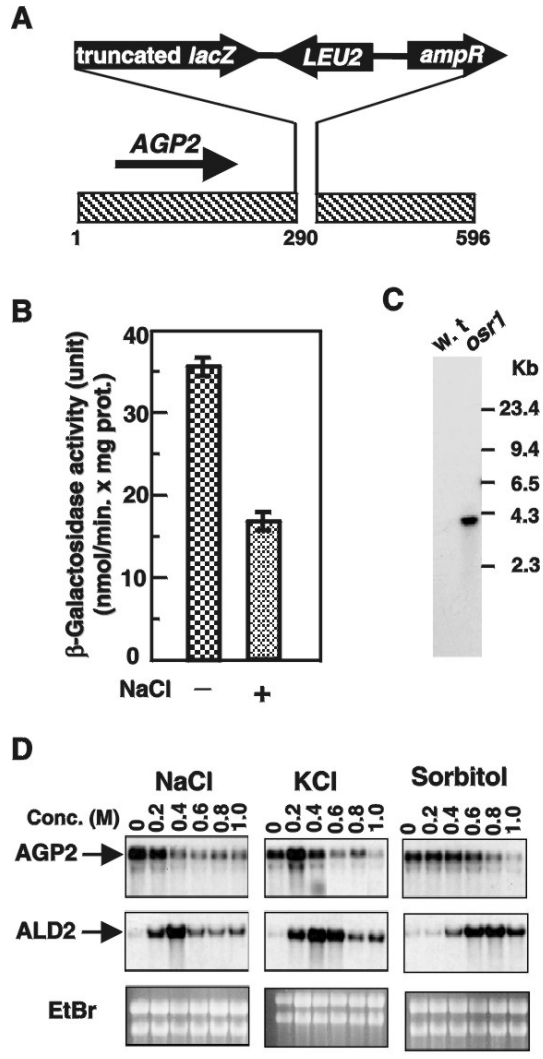

Fig. 2. Isolation of an osmotic stress response gene, *AGP2*. **A.** Scheme of the *AGP2* locus in the *osr1* mutant and site of the *lacZ* gene insertion. The arrows indicate the direction of the gene transcription. The numbers indicate the codon position of *AGP2*. **B.** Effect of NaCl stress on the expression of *OSR1*. The *osr1* mutant cells, growing exponentially in the YPD medium, were supplemented without (–) or with (+) 1.0 M NaCl for 1 h and β-Galactosidase activity was measured. The results are the mean values that were obtained from three independent experiments and bars in the figures that correspond to standard deviations. **C.** Analysis of the integrated mTn3 copy number in the *osr1* mutant. Genomic DNA was prepared from the wild-type and *osr1* mutant strain. After digestion with *Eco*RI, a Southern blot analysis was performed. The probe that was used in this experiment was a *Bam*HI/*Hpa*I fragment of *lacZ*, which was present in the mTn3 construct. **D.** Effect of different osmotic stresses on the expression of *AGP2*. W303-1a cells that grow exponentially in YPD medium were treated with the indicated amounts of NaCl, KCl, and sorbitol for 1 h. After total RNA was extracted from the samples, the transcript levels were determined by Northern analysis with probes for the genes that were listed on the left of each panel.

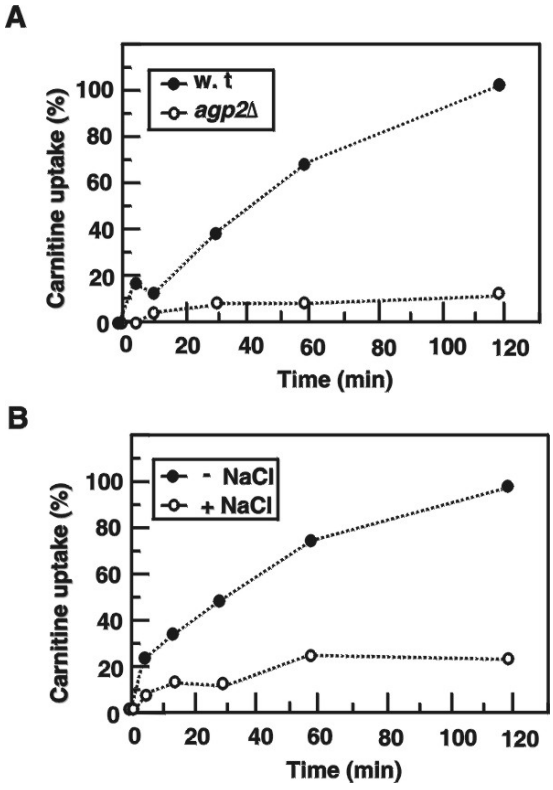

Fig. 3. Role of *AGP2* as a carnitine transporter and the effect of osmotic stress on carnitine uptake. **A.** The uptake of carnitine by *AGP2*. The W303-1a (wild-type) and *agp2Δ* mutant cells were incubated with 5 μM L-[methyl-14C]-carnitine, and the carnitine uptake activity was measured. The uptake rates at 120 min in wild-type cells were taken as a reference (100%). **B.** Suppression of carnitine uptake by NaCl stress. W303-1a (wild-type) cells growing exponentially in the YPD medium were treated without (open circle) or with (closed circle) 0.8 M NaCl for the indicated times, and carnitine uptake was determined, described in Fig. 3A.

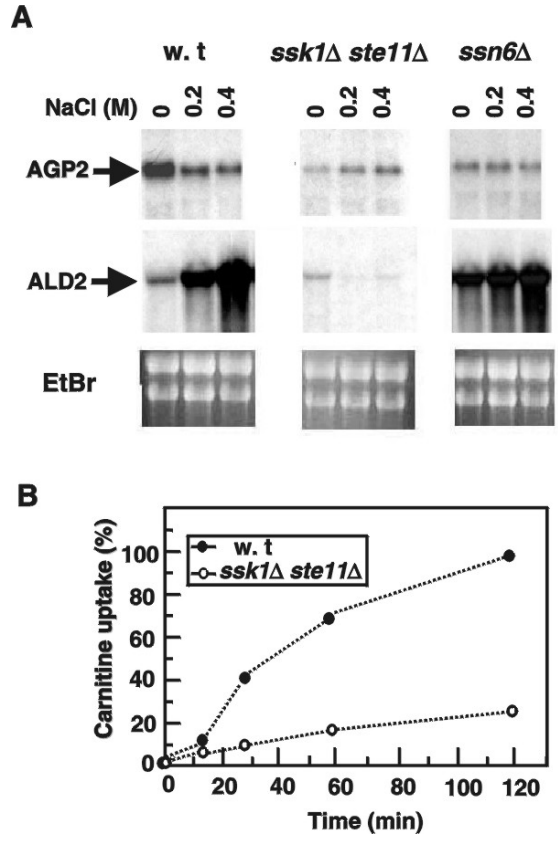

Fig. 4. Expression of *AGP2* and carnitine uptake in the osmotic stress signaling mutant. **A.** Expression of *AGP2* in the Hog pathway related mutants. The W303-1a (wild-type) cells and mutant cells (*ssk1Δste11Δ* and *ssn6Δ*) that grew exponentially in the YPD medium were treated with the indicated amounts of NaCl for 1 h. Northern analysis was performed as described in Fig. 2D. **B.** Carnitine uptake in Hog pathway mutant cells. W303-1a (open circle), and *ssk1Δste11Δ* (closed circle) cells were incubated with 5 μM L-[1-^{14}C]-carnitine. Carnitine uptake activity was measured as described in Fig. 3.

*AGP2 (*a multi-copy plasmid that contained full-length *AGP2)*, the uptake of carnitine was enhanced significantly compared to the cells that were transformed with the vector alone (data not shown). Based on these results, we confirmed an earlier report (Van Roermund *et al*., 1999) that Agp2 could function as a carnitine transporter. Next, we examined whether or not osmotic stress influenced the uptake of carnitine. When wild-type yeast cells were treated with NaCl over a period of time, we found that the uptake of carnitine was significantly decreased (Fig. 3B). From these results, we conclude that both the expression of the *AGP2* gene and the ability of the Agp2 protein to take up carnitine are highly dependent on extracellular osmolarity.

HOG pathway dependent inhibition of carnitine uptake by AGP2 The HOG pathway is activated by changes in the extracellular osmolarity, and is involved in the regulation of many genes (Gustin *et al*., 1998; Posas *et al*., 1998). Since the expression of *AGP2* and its ability to take up carnitine are dependent on osmotic stress, we examined the role of the HOG pathway in the regulation of *AGP2* transcription by using mutant strains (*ssk1Δste11Δ* and *ssn6)*. Ssn6, a negative regulator of osmotic stress responses, acts at the transcriptional level of both Hog-dependent and Hog-independent genes (Proft and Serrano, 1999). In this experiment, the *ALD2* gene was used as a positive control since its expression is HOG pathway-dependent and maximal at 0.4 M NaCl (Fig. 3A), the concentration corresponding to maximum phosphorylation of the Hog1 MAP kinase (Brewster *et al*., 1993). As shown in Fig. 4A, the expression of *AGP2* was significantly lower in the mutant strains (*ssk1Δste11Δ* and

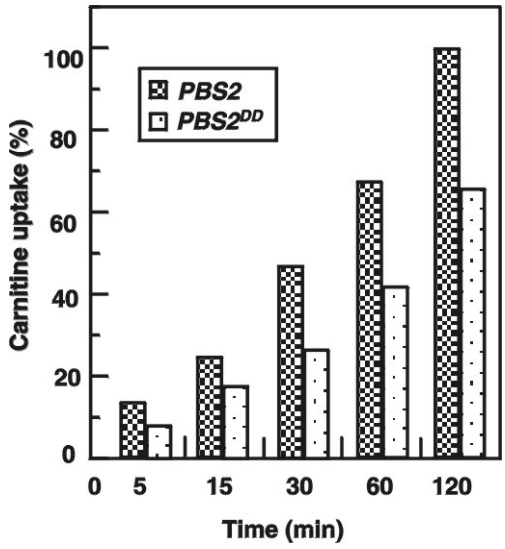

Fig. 5. Constitutive activation of Pbs2 suppresses carnitine uptake. TM260 (*pbs2Δ*) cells that contain 903CU-PBS2 (a plasmid that carries a functional *PBS2* gene and *URA3*) or pGDG21 (a plasmid that carries $PBS2^{DD}$, an active form of *PBS2* gene, and *URA3*) were grown in a SC-Ura medium to the mid-long phase, then transferred to a fresh YPG medium that contained 5 μM L-[1-^{14}C]-carnitine respectively, at time zero. After the samples were harvested at the indicated times, the carnitine uptake activity was measured as described in Fig. 3.

ssn6) compared to the wild-type strain. The level of uptake of carnitine correlated with the level of the *AGP2* gene expression (Fig. 4). The uptake of carnitine in wild-type cells increased with time and reached its maximum level at 120 min; however, the uptake was greatly reduced in *ssk1Δste11Δ* cells (Fig. 4B). Next, we examined the effect of $PBS2^{DD}$ (constitutively active form of *PBS2*) on the expression of *AGP2* and carnitine uptake. TM260 (*pbs2Δ*) cells that carried *PBS2* or $PBS2^{DD}$ that was regulated by the GAL promoter were grown in a glucose medium, then transferred to the galactose medium. The expression of $PBS2^{DD}$ reduced the level of both the *AGP2* mRNA expression (data not shown) and carnitine uptake compared to the cells that carried the wild-type *PBS2* gene (Fig. 5). We, therefore, conclude that the HOG pathway negatively regulates the expression of AGP2 and carnitine uptake.

The results that are described in this paper can be explained by the following model: since the activation of Hog1 is repressed by the Sln-1 mediated two-component system (Bremer, 1983; Brewster, 1993), then under conditions of low osmolarity, Hog1 no longer represses the expression of *AGP2* (Fig. 4A). When challenged by osmotic stress, the Sho1-mediated activation of Hog1 suppresses the expression of *AGP2* and leads to a decreased uptake of carnitine (Figs. 2, 3, and 5).

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References

Bremer, J. (1983) Carnitine metabolism and function. *Physiol. Rev*. **63**, 1420–1480.

Brewster, J. L., de Valoir, T., Dwyner, N. D., Winter, E., and Gustin, M. C. (1993) Anosmosensing signal transduction pathway in yeast. *Science* **259**, 1760–1763.

Burns, N., Grimwade, B., Ross-Macdonald, P. B., Choi, E.-Y., Finberg, K., Roeder, G. S., and Synder, M. (1994) Large-scale analysis of gene expression, protein localization, and gene disruption in *Saccharomyces cerevisiae*. *Genes Dev*. **8**, 1087–1105.

Elble, R. (1992) A simple and efficient procedure for transformation of yeast. *Biotechniques* **13**, 18–20.

Gaxiola, R., de Larrinoa, I. F., Villalba, J. M., and Serrano, R. (1992) A novel and conserved salt-induced protein is an important determination of salt tolerance in yeast. *EMBO J*. **11**, 3157–3164.

Graham, G. C., Mayers, P., and Henry, R. J. (1994) A simplified method for the preparation of fungal genomic DNA for PCR an RAPD analysis. *Biotechniques* **16**, 48–50.

Gustin, M. C., Alvertyn, J., Alexander, M., and Davenport, K. (1998) MAP kinase pathways in the yeast, *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev*. **62**, 1264–1300.

Jeong, M. J., Park, S. C., and Byun, M. O. (2001) Improvement of salt tolerance in transgenic potato plants by glyceraldehydes-3 phosphate dehydrogenase gene transfer. *Mol. Cells* **12**, 185–189.

Kunau, W. H., Dommes, V., and Schulz, H. (1995) Beta-oxidation of fatty acids in mitochondria, peroxisomes and bacteria; a century of continued progress. *Prog. Lipid Res.* **34**, 267–342.

Lee, J. Y., Shin, D. J., Lee, B. Y., and Yun, D. J. (2001) Isolation and characterization of salt stress signaling components from yeast *Saccharomyces cerevisiae*. *Korean J. Life Sci*. **11**, 30–31.

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.

Mendoza, I., Quintero, F. J., Bressan, R. A., Hasegawa, P. M., and Pardo, J. M. (1996) Activated calcineurin confers high tolerance to ion stress and alters the budding pattern and cell morphology of yeast cells. *J. Biol. Chem*. **271**, 23061–23067.

Posas, F., Takegawa, M., and Saito, H. (1998) Signal transduction by MAP kinase cascades in budding yeast. *Curr. Opin. Microbiol*. **1**, 175–18182.

Proft, M. and Serrano, R. (1999) Repression and upstream repressing sequence of the stress-regulated ENA1 gene in *Saccharomyces cerevisiae*: bZIP protein Sko1p confer HOG-dependent osmotic regulation. *Mol. Cell. Biol.* **19**, 537–546.

Ross-Macdonald, P., Burns, N., Malczynski, M., Sheehan, A., Roeder, S., and Snyder, M. (1995) Methods for large-scale analysis of gene expression, protein localization, and disrup-

tion phenotypes in *Saccharomyces cerevisiae*. *Methods Mol. Cell. Biol*. **5**, 298–308.

Rothstein, R. (1985) DNA Cloning, Glover, D. M. (ed.), pp. 45–66, IRL Press, Oxford, Washington D.C.

Sambrook, J. and Russell, D. W. (2001) *Molecular Cloning*: *A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sherman, F. (1991) Getting started with yeast. *Methods Enzymol*. **194**, 12–17.

Van Roermund, C. W. T., Hettema, E. H., Van Roermund den Berg, M., Tabak, H. F., and Wanders, R. J. A. (1999) Molecular characterization of carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria in *Saccharomyces cerevisiae* and identification of a plasma membrane carnitine transporter, Agp2p. *EMBO J*. **18**, 5843– 5852.

Yun, D. J., Ibeas, J. I., Lee, H., Coca, M. A., Narasimhan, M. L., Uesono, Y., Hasegawa, P. M., Pardo, J. M., and Bressan, R. A. (1998) Osmotin, a plant antifungal protein, subverts signal transduction to enhance fungal cell susceptibility. *Mol. Cell* **1**, 807–817.