CHAPTER IV
MATERIALS AND METHODS

4.1 Strains and media

Saccharomyces cerevisiae strains used in this study were the wild-type strain BY4742 (MATα his3Δ1 leu2Δ0 lys2Δ0 uraΔ0) and the isogenic deletion mutant of gene involved in the mitogen-activated protein kinase pathways as described in Table 4.1. All yeast strains were obtained from Research Genetics/Invitrogen (Winzeler et al., 1999). In each deletion mutant, the corresponding gene was completely deleted and replaced by the KanMX module.

Culture media used in this study was YPD media composed of 1% yeast extract (Bio Basic Inc), 2% peptone (Bio Basic Inc), and 2% glucose (Bio Basic Inc).

4.2 Chemicals and reagents

Chemicals and reagents used in this study are alphabetically ordered as follows:

(Hydroxmethyl) aminomethane	Research organics, Inc.
Bovine Serum Albumin (BSA)	Fermentas
Calcofluor white	Sigma- Aldrich
dNTP	iNtRON
DyNAmo™ cDNA Synthesis Kit	Finzymes
Dynamo™ SYBR® Green 2-Step qRT-PCR Kit	Finzymes
Ethanol	Merck
Ethylenediaminetetraacetic acid (EDTA)	Merck
PCR buffer	iNtRON
Primer	Bio basic, Inc.
RNeasy Mini kit	QIAGEN

Sorbitol	Bio basic, Inc.
Taq polymerase	iNtRON

4.3 Apparatuses

The apparatuses used in this study are alphabetically ordered as follows:

ABI 7500 instrument	Applied Biosystems
Analytical balance	Denver TR 403
Automated microplate reader	Wallace Victor 1420 PerkinElmer
Centrifuge	IEC B-22M
Gel Documentation	LightBIS Pro
Incubator	AMBI-HI-LO chamber
Lamina flow	ISSCO Model BVI-124S S
Orbital mixing incubator	Ratex
PCR Thermocycler	PTC-1148 MJ Mini Thermal Cycler
pH meter	Denver Model 215
Spectrofluorometer	Jasco FP-6200
Spectrophotometer	Shimadzu UV-1700
Sterilizer	Sanyo
Glassware	Duran

4.4 Methods

4.4.1 Growth of the mutants lacking genes encoding components of the MAPK pathways under ethanol and Calcofluor white stress conditions

4.4.1.1 Growth determination by spot susceptibility test

The effect of ethanol and Calcofluor white on cell growth was monitored by spot susceptibility test. To investigate the growth of the mutants lacking genes encoding components of the MAPK pathways under ethanol and cell wall stresses, the S. cerevisiae wild-type strain (BY4742) and its isogenic deletion mutants were precultivated in YPD liquid media with shaking at 200 rpm at 30°C for 12 hours.

The overnight culture was diluted to an initial O.D.$_{600}$ of 1.0 with sterile water and were then serially diluted 10-fold. Aliquots (3 µl) were spotted onto YPD agar plate and YPD agar plates containing 12% ethanol and 100 µg/ml of Calcofluor white. Growth was monitored after incubation at 30°C for 2 days.

4.4.2 The effect of ethanol and Calcofluor white on cell wall remodeling in the yeast wild-type strain

4.4.2.1 Zymolyase sensitivity test

To examine the effect of concentrations of ethanol and Calcofluor white on cell wall remodeling, the yeast wild-type strain (BY4742) pregrown to log phase in YPD medium was inoculated into YPD medium and YPD media containing 2, 4, 6, 8, and 10% ethanol or 25, 50, 75, and 100 µg/ml Calcofluor white and was incubated at 30 C for 12 hours. Cells were harvested, washed in TE buffer (10 mM Tris/HCl and 1 mM EDTA, pH 7.5), and resuspended at an O.D.$_{600}$ of 0.5 in the same buffer containing 50 µg/ml (0.5 U) Zymolyase 20T (a β-1,3 glucanase from Arthrobacter luteus) (Zymo research). The sensitivity to Zymolyase was monitored by measuring O.D.$_{600}$ at 30-minute intervals for two hours by an automated microplate reader (Wallace Victor 1420 PerkinElmer, USA).

To examine the effect of exposure times to ethanol and Calcofluor white on cell wall remodeling, the yeast wild-type strain (BY4742) pregrown to log phase in YPD medium was inoculated into YPD medium and YPD media containing 8% ethanol or 100 µg/ml of Calcofluor white and incubated at 30°C for 15 minutes, 1, 3, 6, and 12 hours. Cells were examined for Zymolyase sensitivity as mentioned above.

4.4.3 Cell wall remodeling in the yeast mutants lacking genes encoding components of the MAPK pathway after exposure to ethanol and Calcofluor white

4.4.3.1 Zymolyase sensitivity test

To examine the effect of ethanol and Calcofluor white on cell wall remodeling, the yeast mutants lacking genes encoding components of the MAPK pathway pregrown to log phase in YPD medium was inoculated into YPD medium and YPD media containing 8% ethanol or 100 µg/ml Calcofluor white and incubated at 30 C for 12 hours. Cells were examined for Zymolyase sensitivity as mentioned above.

4.4.4 The effect of hyperosmolarity on cell growth and cell wall remodeling of the yeast strains exposed to ethanol and Calcofluor white

4.4.4.1 Spot susceptibility test

The effect of hyperosmolarity on cell growth under ethanol and Calcofluor white stress conditions was monitored by spot susceptibility test. To investigate the growth of the mutants lacking genes encoding components of the MAPK pathways under ethanol and cell wall stresses, the S. cerevisiae wild-type strain (BY4742) and its isogenic deletion mutants were precultivated in YPD liquid media containing 1.5 M sorbitol with shaking at 200 rpm at 30 C for 12 hours. The overnight cultures were diluted to an initial O.D.$_{600}$ of 1.0 with sterile water and cells were then serially diluted 10-fold. Aliquots (3 µl) were spotted onto YPD agar plate and YPD agar plates containing 12% ethanol and 100µg/ml Calcofluor white. Growth was monitored after incubation at 30°C for 2 days.

4.4.4.2 Zymolyase sensitivity test

To examine the effect of hyperosmolarity on cell wall remodeling under ethanol and Calcofluor white stress conditions, the yeast wild-type strain (BY4742) and its isogenic deletion mutants pregrown to log phase in YPD media containing 1.5 M sorbitol was inoculated into YPD media and YPD media containing 8% ethanol or 100 µg/ml Calcofluor white and incubated at 30 C for 12 hours. Cells were examined for Zymolyase sensitivity as mentioned above.

4.4.5 The effect of ethanol and Calcofluor white on gene expression of the yeast wild-type strain

4.4.5.1 RNA isolation

Total RNA was isolated from the yeast wild-type cells cultivated in YPD liquid medium and YPD liquid media containing 8% ethanol or 100 µg/ml of Calcofluor white by using the RNeasy Mini kit (QIAGEN, USA) following the manufacturer's instructions. Briefly, the yeast wild-type strain (BY4742) precultivated to log phase in YPD medium was inoculated into YPD media and YPD media containing 8% ethanol or 100 µg/ml Calcofluor white and incubated at 30 C for 30 minutes, 4 and, 12 hours. Cells were harvested and resuspended in 100 µl of freshly prepared buffer Y1 (1M sorbitol, 0.1 M EDTA pH 7.4, 0.1% β-ME and zymolase 50 U/1 x 10^7 cells). The cell suspension was incubated for 30 minutes at 30°C with gentle shaking and then centrifuged for 5 minutes at 3,000 rpm before discarding supernatant. 350 µl of RLT buffer and 250 µl of 95% ethanol were added and mixed by pipetting. The sample was then transferred to RNeasy spin column placed in a 2 ml microcentrifuge tube and centrifuged for 15 seconds at 10,000 rpm. 700 µl of RW1 buffer was added to the RNeasy spin column before centrifugation for 15 seconds at 10,000 rpm. 500 µl of RPE buffer was added to the RNeasy spin column and centrifuged for 15 seconds at 10,000 rpm. This step was repeated for two times. A new 1.5 ml microcentrifuge tube was placed to the RNeasy spin column and 40 µl of RNase-free water was added before centrifugation for 1 minute at 10,000 rpm to elute the total RNA. Total RNA concentrations were determined by measuring O.D. $_{260\ nm}$.

4.4.5.2 Real-time quantitative PCR

First-strand cDNAs were synthesized from 1µg of total RNA using the cDNA synthesis kit for reverse transcription-PCR (DyNAmo™ cDNA Synthesis Kit), following the recommendations of the manufacturer. In brief, cDNA synthesis premix was prepared and dispensed into reaction tube. The reaction began with incubation at 25°C for 10 minutes to extend the random hexamer oligonucleotides, followed by incubation at 37°C for 60 minutes to synthesize cDNA. The reaction was terminated by incubation at 85°C for 5 minutes through the inactivation of the M-MuLV reverse transcriptase.

Real-time quantitative PCR was performed on an ABI 7500 instrument (Applied Biosystems) using Dynamo™ SYBR® Green 2-Step qRT-PCR Kit (Finzymes) and specific oligonucleotide primers as described in Table 4.2. The reaction mix (total volumn of 20 µl) contains 10 µl of 2x Master Mix, 0.5 µM each of forward and reverse primers, and 1 µg of cDNA template. The reaction condition was as followed: an initial incubation at 50°C for 2 minutes, 95°C for 15 minutes; 40 cycles of amplification including denaturation at 94 °C for 10 seconds, annealing at 50°C for 10 seconds, and extension at 72°C for 30 seconds. For each PCR product, melting curves were determined according to the supplier’s guidelines to ensure specific amplification of the target gene.

Table 4.1 Yeast strains used in this study

Strain	Genotype
BY4742	MATα; his3Δ1; leu2Δ0; lys2Δ0; uraΔ0
Δste3	BY4742 isogenic, ste3:: KanMX4
Δste4	BY4742 isogenic, ste4:: KanMX4
Δgpa1	BY4742 isogenic, gpa1:: KanMX4
Δste5	BY4742 isogenic, ste5:: KanMX4
Δste50	BY4742 isogenic, ste50:: KanMX4
Δbem1	BY4742 isogenic, bem1:: KanMX4
Δste20	BY4742 isogenic, ste20:: KanMX4
Δste11	BY4742 isogenic, ste11:: KanMX4
Δste7	BY4742 isogenic, ste7:: KanMX4
Δfus3	BY4742 isogenic, fus3:: KanMX4
Δfar1	BY4742 isogenic, far1:: KanMX4
Δgpa2	BY4742 isogenic, gpa2:: KanMX4
Δras2	BY4742 isogenic, ras2:: KanMX4
Δkss1	BY4742 isogenic, kss1:: KanMX4
Δptp2	BY4742 isogenic, ptp2:: KanMX4
Δptp3	BY4742 isogenic, ptp3:: KanMX4
Δwsc1	BY4742 isogenic, wsc1:: KanMX4
Δwsc2	BY4742 isogenic, wsc2:: KanMX4
Δwsc3	BY4742 isogenic, wsc3:: KanMX4
Δmid2	BY4742 isogenic, mid2:: KanMX4
Δrom2	BY4742 isogenic, rom2:: KanMX4
Δbck1	BY4742 isogenic,bck1:: KanMX4
Δmkk1	BY4742 isogenic, mkk1:: KanMX4
Δmkk2	BY4742 isogenic, mkk2:: KanMX4
Δmpk1	BY4742 isogenic, mpk1:: KanMX4
Δrlm1	BY4742 isogenic, rlm1:: KanMX4
Δswi4	BY4742 isogenic, swi4:: KanMX4
Δswi6	BY4742 isogenic, swi6:: KanMX4

Δmbp1	BY4742 isogenic, mbp1::KanMX4
Δfks1	BY4742 isogenic, fks1:: KanMX4
Δfks2	BY4742 isogenic, fks2:: KanMX4
Δsho1	BY4742 isogenic, sho1:: KanMX4
Δssk2	BY4742 isogenic, ssk2:: KanMX4
Δssk22	BY4742 isogenic, ssk22::KanMX4
Δpbs2	BY4742 isogenic, pbs2:: KanMX4
Δhog1	BY4742 isogenic, hog1:: KanMX4
Δmsn2	BY4742 isogenic, msn2:: KanMX4
Δmsn4	BY4742 isogenic, msn4:: KanMX4
Δgpd1	BY4742 isogenic, gpd1:: KanMX4
Δgpd2	BY4742 isogenic, gpd2:: KanMX4
Δgpp1	BY4742 isogenic, gpp1:: KanMX4
Δgpp2	BY4742 isogenic, gpp27::KanMX4

Table 4.2 Primers used in this study

Primer Name	Sequence
ACT1-RT-F	5´ TGGATTCCGGTGATGGTGTT 3´
ACT1-RT-R	5´ TCAAAATGGCGTGAGGTAGAGA 3´
CHS3-RT-F	5´ AGCATGGGTTCATTCACTCC 3´
CHS3-RT-R	5´ GCAATACTCGGTGACGTCCT 3´
CRH1-RT-F	5´ TGGTGACCAATCTGGTTCTTGGGA 3´
CRH1-RT-R	5´ ACTGCGAAATCTTCTTGGGCTTGG 3´
FKS2-RT-F	5´ CCGTCAAATCCGTCCTCCTAT 3´
FKS2-RT-R	5´ GTACAAGCTGCAATACCTCCTAACC 3´
GPD1-RT-F	5´ CCAGAAGTTTTCGCTCCAATAGTA 3´
GPD1-RT-R	5´ AGCAACCAAATTGTCGGGTAGA 3´
SED1-RT-F	5´ AACAAACCACAGCCAACCCAAGTC3´
SED1-RT-R	5´ AGCAACACCAGCCAAACCTAAAGC3´