CHAPTER V
RESULTS

5.1 Role of the MAPK pathways in ethanol tolerance in S. cerevisiae

To investigate the role of the MAPK pathways in response to ethanol stress, the growth of S. cerevisiae wild-type strain (BY4742) and its isogenic deletion mutants lacking genes encoding components of the MAPK pathways was examined on YPD plates containing 12% ethanol by using spot susceptibility test. Our results revealed that the mutants lacking components of the cell wall integrity pathway, i.e. the *Δ*bck1 mutant lacking MAPKKK, the *Δ*mpk1 mutant lacking MAPK, and the *Δ*swi4 and *Δ*swi6 mutants lacking the transcription cofactors, were sensitive to 12% ethanol (Fig. 5.1). However, the other mutants of this pathway, including the *Δ*wsc1, *Δ*wsc2, *Δ*wsc3, and *Δ*mid2 mutants lacking cell wall stress sensors, the *Δ*rom2 mutant lacking GDP/GTP exchange protein, and the *Δ*mkk1 and *Δ*mkk2 mutants lacking MAPKK, did not exhibit the ethanol-sensitive phenotype (Fig. 5.1), possibly due to redundant functions of these genes. These results suggested that the cell wall integrity pathway may play an important role in response to ethanol stress. In addition, the mutants lacking STE3 gene encoding a transmembrane receptor for yeast pheromone and AKR1 gene encoding a negative regulator of the pheromone signaling pathway, were also sensitive to 12% ethanol (Fig. 5.2). Since the other deletion mutants lacking downstream components of the pheromone signaling pathway were not sensitive to ethanol, it is possible that only Ste3p and Akr1p, but not the other components of this pathway, may also play a role in maintaining cell wall structure during ethanol stress. To test this possibility, the growth of the *Δ*ste3 and *Δ*akr1 mutants was examined on YPD plates containing 100µg/ml Calcofluor white, a cell wall-perturbing agent with a high affinity for chitin (Roncero & Duran, 1985), by using spot susceptibility test. Our results showed that the *Δ*ste3 and *Δ*akr1 mutants were also sensitive to Calcofluor white, suggesting that Ste3p and Akr1p are involved in maintaining integrity of cell wall, possibly through the transduction of cell wall stress signals (Fig.5.2).

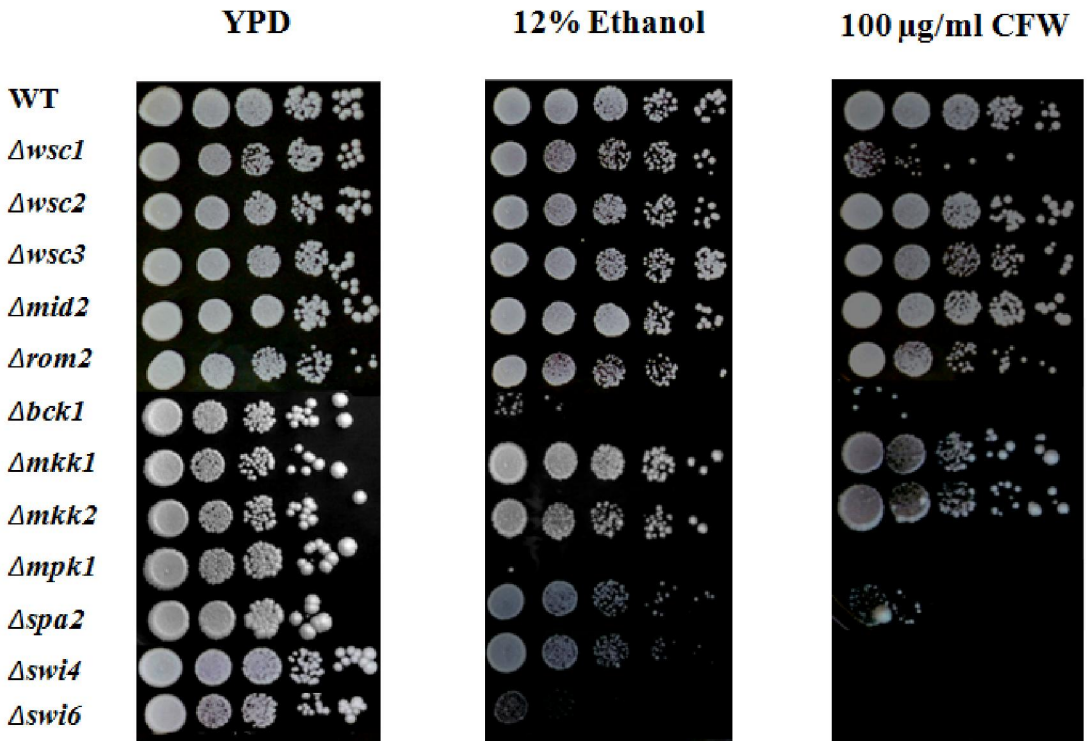


Figure 5.1 Growth of the mutants lacking genes encoding components of the cell wall integrity pathway under ethanol and cell wall stress conditions. The wild-type strain (BY4742) and its isogenic deletion mutants pregrown to log phase in YPD broth were serially diluted ten-fold from an initial OD_{600} of 1. Aliquots (3 µl) were spotted on the YPD agar plates and YPD agar plates containing 12% ethanol and 100µg/ml Calcofluor white (CFW), and incubated at 30°C for 2 days.

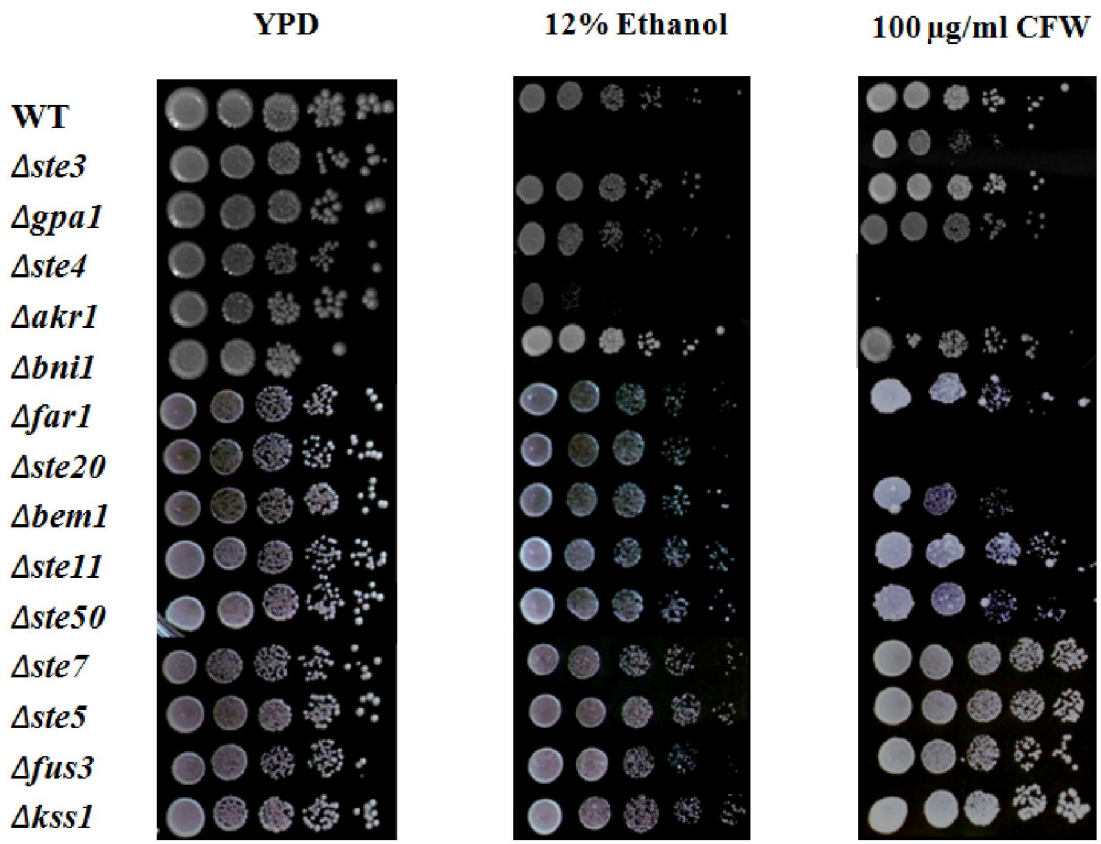


Figure 5.2 Growth of the mutants lacking genes encoding components of the pheromone signaling pathway under ethanol and cell wall stress conditions. The wild-type strain (BY4742) and its isogenic deletion mutants pregrown to log phase in YPD broth were serially diluted ten-fold from an initial OD_{600} of 1. Aliquots (3 µl) were spotted on the YPD agar plates and YPD agar plates containing 12% ethanol and 100µg/ml Calcofluor white (CFW), and incubated at 30°C for 2 days.

5.2 Effect of ethanol on cell wall remodeling in the yeast wild-type strain

Since BCK1, MPK1, SWI4, and SWI6, which encode components of the cell wall integrity pathway, are required for ethanol tolerance, it is possible that this signaling pathway is important for recovery from ethanol-induced cell wall damage through the activation of cell wall remodeling. The cell wall remodeling is a cellular mechanism important for maintaining cell wall integrity during stress (Aguilar-Uscanga & Francois, 2003; de Nobel et al., 2000). Changes in cell wall composition lead to a resistance to cell wall-disturbing agent and cell wall-degrading enzyme (Aguilar-Uscanga & Francois, 2003; de Nobel et al., 2000; Klis et al., 2006). Zymolyase is an enzyme, which has a strong lytic activity against living yeast cell wall catalyzing β-1,3 glucan hydrolyzing (Aguilar-Uscanga & Francois, 2003; Bermejo et al., 2008; Levin, 2005). To investigate whether cell wall remodeling occurs in response to ethanol stress, Zymolyase sensitivity test was used to monitor the cell wall alterations after ethanol challenge. The effect of concentrations of ethanol and Calcofluor white on cell wall remodeling was first examined in the wild-type strain grown in YPD, YPD containing 2%, 4%, 6%, 8%, and 10% (v/v) ethanol, and YPD containing 25, 50, 75, and 100 µg/ml Calcofluor white for 12 hours. We found that the sensitivity to Zymolyase was decreased with increasing concentrations of ethanol and Calcofluor white (Fig. 5.3 A and B). Among these, the wild-type cells exposed to 8% ethanol and 100 µg/ml Calcofluor white exhibited the highest resistance to Zymolyase (Fig. 5.3 A and B). However, the yeast cells exposed to 10% ethanol were more sensitive to Zymolyase than those challenges to 6% and 8% ethanol (Fig. 5.3). This may be due to the high toxicity of 10% ethanol that will inhibit proper cell wall remodeling process (Fig. 5.3). Based on our observations, it is likely that ethanol and Calcofluor white induce cell wall remodeling in dose-dependent manner.

To test whether the degree of cell wall alteration is correlated with exposure time, we examined the Zymolyase sensitivity test in the wild-type cells incubated in YPD, YPD containing 8% ethanol and 100 µg/ml calcofluor white for 1, 3, 6, and 12 hours. Our results revealed that the sensitivity to Zymolyase did not change after prolonged incubation in YPD media (Fig. 5.4 A). On the other and, in the presence of ethanol and Calcofluor white, the sensitivity to Zymolyase was decreased

with increasing exposure times (Fig. 5.4 B and C). The cells exposed to 8% ethanol and 100 µg/ml Calcofluor white for 12 hours, were more resistant to Zymolyase than those exposed for shorter periods (Fig. 5.4 B and C), indicating that ethanol and Calcofluor white induced cell wall remodeling in time-dependent manner.

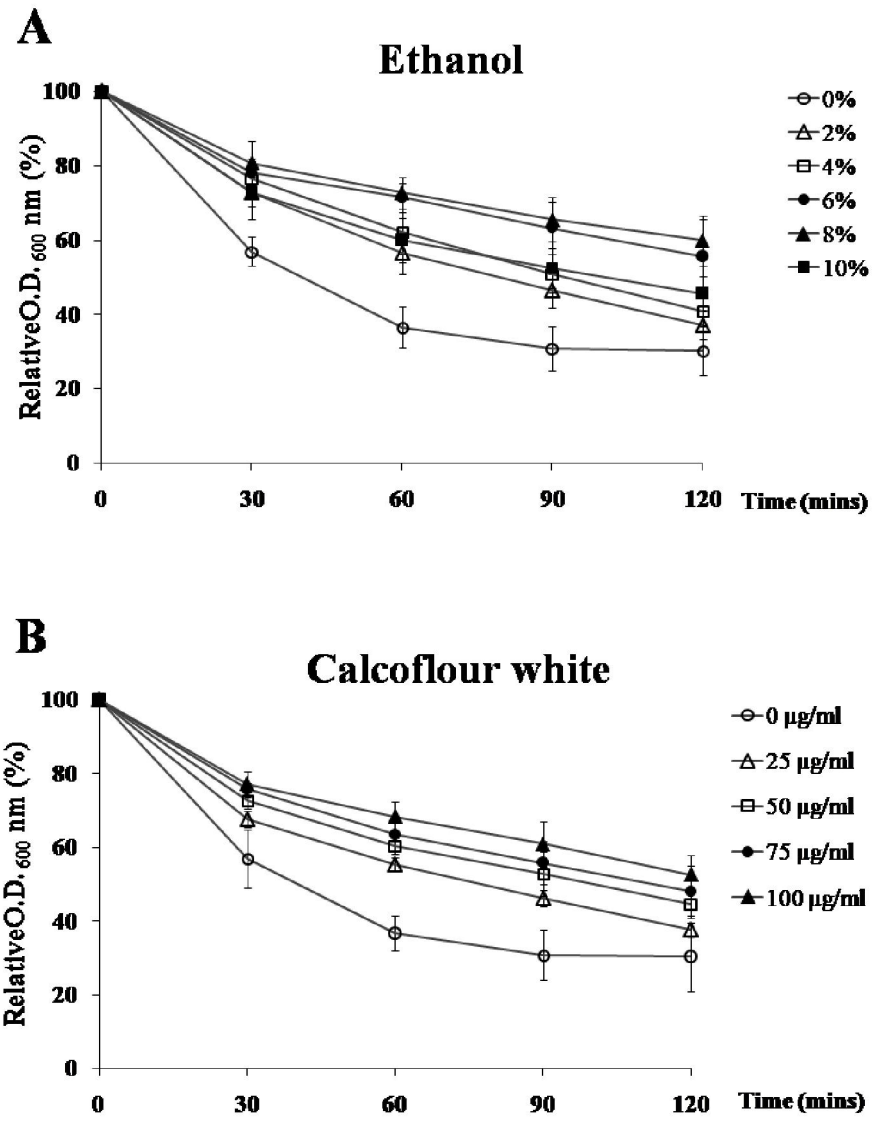
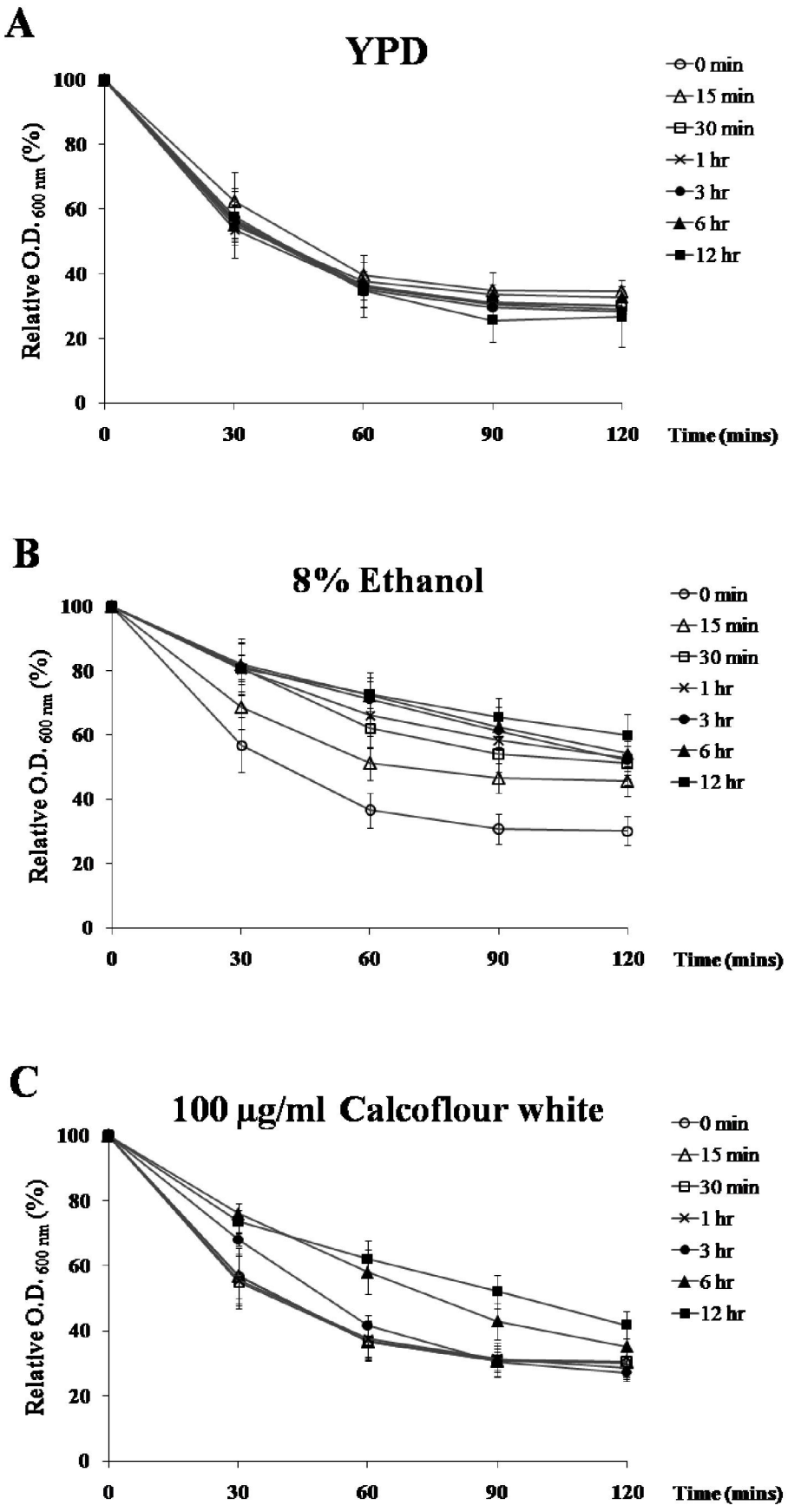


Figure 5.3 Zymolyase sensitivity of the wild-type strain after exposure to various concentrations of ethanol and Calcofluor white. Cells grown to log phase in YPD broth were inoculated into YPD (○) and YPD media containing 2% (Δ), 4% (□), 6% (●), 8% (▲) and 10% (¾) (v/v) ethanol (A) and 25 (Δ), 50 (□), 75 (●), or 100 µg/ml (▲) Calcofluor white (B). Cells were harvested, washed in TE buffer, and resuspended at an $O.D._{600}$ of 0.5 in the same buffer containing 50 µg/ml (0.5 U) zymolyase. The sensitivity to Zymolyase was measured using optical density at 600 nm at the indicated times. The sensitivity to Zymolyase is shown as a percentage of the optical density at 600 nm relative to that at t=0.

Figure 5.4 Zymolyase sensitivity of the wild-type strain after exposure to ethanol and Calcofluor white for various times. Cells grown to log phase in YPD broth were inoculated into YPD (A) and YPD media containing 8% (v/v) ethanol (B) and 100 µg/ml Calcofluor white (C) for 0 minute (○), 15 minutes (Δ), 30 minutes (□) , 1 hour (x), 3 hours (●), 6 hours (▲) , and 12 hours (■). Cells were harvested, washed in TE buffer, and resuspended at an $O.D._{600}$ of 0.5 in the same buffer containing 50 µg/ml (0.5 U) zymolyase. The sensitivity to zymolyase was measured using optical density at 600 nm at the indicated times. The sensitivity to Zymolyase is shown as a percentage of the optical density at 600 nm relative to that at t=0.


A
YPD
Relative O.D. 600 nm (%)
100
80
60
40
20
0
0
30
60
90
120
Time (mins)
0 min
15 min
30 min
1 hr
3 hr
6 hr
12 hr
B
8% Ethanol
Relative O.D. 600 nm (%)
100
80
60
40
20
0
0
30
60
90
120
Time (mins)
0 min
15 min
30 min
1 hr
3 hr
6 hr
12 hr
C
100 µg/ml Calcoflour white
Relative O.D. 600 nm (%)
100
80
60
40
20
0
0
30
60
90
120
Time (mins)
0 min
15 min
30 min
1 hr
3 hr
6 hr
12 hr

5.3 Effect of ethanol on cell wall remodeling in the mutants lacking genes encoding components of the MAPK pathways

Since the *Δ*bck1, *Δ*mpk1, *Δ*swi4, and *Δ*swi6 mutants lacking genes involved in the cell wall integrity pathway, and the Δste3 and Δakr1 mutants lacking genes involved in the pheromone signaling pathway, were sensitive to ethanol and Calcofluor white, it is possible that these components of the MAPK pathways are involved in response to cell wall damage caused by ethanol. To determine whether these components of the MAPK pathways are required for the induction of cell wall remodeling during ethanol stress, the Zymolyase sensitivity test was examined in the *Δ*bck1, *Δ*mpk1, *Δ*swi4, *Δ*swi6, Δste3, and Δakr1 mutants after exposure to 8% ethanol and 100µg/ml Calcofluor white. Lack of signaling proteins of the cell wall integrity pathway such as Bck1p and Mpk1p was supposed to result in the inhibition of cell wall remodeling process, thereby leading to increased sensitivity to Zymolyase. Consistent with this idea, after growth in YPD media for 12 hours, the *Δ*bck1 and *Δ*mpk1 mutants were more sensitive to Zymolyase than the wild-type strain (Fig 5.5 A), emphasizing that the cell wall remodeling is regulated by the cell wall integrity pathway. Furthermore, although, after exposure to Calcofluor white, the *Δ*bck1, *Δ*mpk1, and *Δ*swi4 mutants were more sensitive to Zymolyase than the wild-type strain, they were more tolerance to Zymolyase than those grown in YPD (Fig. 5.5B), suggesting the important role of these signaling molecules of the cell wall integrity pathway in response to Calcofluoe white-induce cell wall damage. Surprisingly, after exposure to 8% ethanol, the *Δ*bck1, *Δ*mpk1, *Δ*swi4, and *Δ*swi6 mutants exhibited increased tolerance to Zymolyase to the same level as in wild-type strain (Fig 5.5 C). These results suggested that ethanol may induce the cell wall remodeling through the alternative signaling pathways when the signaling moleucles of the cell wall integrity pathway are inactivated.

On the other hand, in the Δakr1 mutant, the pretreatment with 8% ethanol or 100µg/ml Calcofluor white did not significantly affected the enzyme activity (Fig 5.6 B and C), suggesting the role of Akr1p in signal transduction involved in cell wall remodeling during ethanol stress. Whereas, after exposure to ethanol and Calcofluor white, the Δste3 mutant lacking transmembrane receptor for mating pheromone did not

show a defect in cell wall remodeling (Fig 5.6 B and C), suggesting that Ste3p is not involved in cell wall remodeling in response to cell wall stress.

Figure 5.5 Cell wall remodeling of the yeast mutant strains after exposure to ethanol and Calcofluor white. The wild- type (BY4742) (○), Δbck1 (Δ), Δmpk1 (□), Δswi4 (●), and Δswi6 mutants (▲) grown to log phase in YPD broth were inoculated to YPD (A) and YPD media containing 100µg/ml Calcofluor white (B) or 8% (v/v) ethanol (C). Cells were harvested, washed in TE buffer, and resuspended at an $O.D._{600}$ of 0.5 in the same buffer containing 50 µg/ml (0.5 U) Zymolyase. The decrease in the optical density at 600 nm was measured at the indicated times. The sensitivity to Zymolyase is shown as a percentage of the optical density at 600 nm relative to that at t=0.

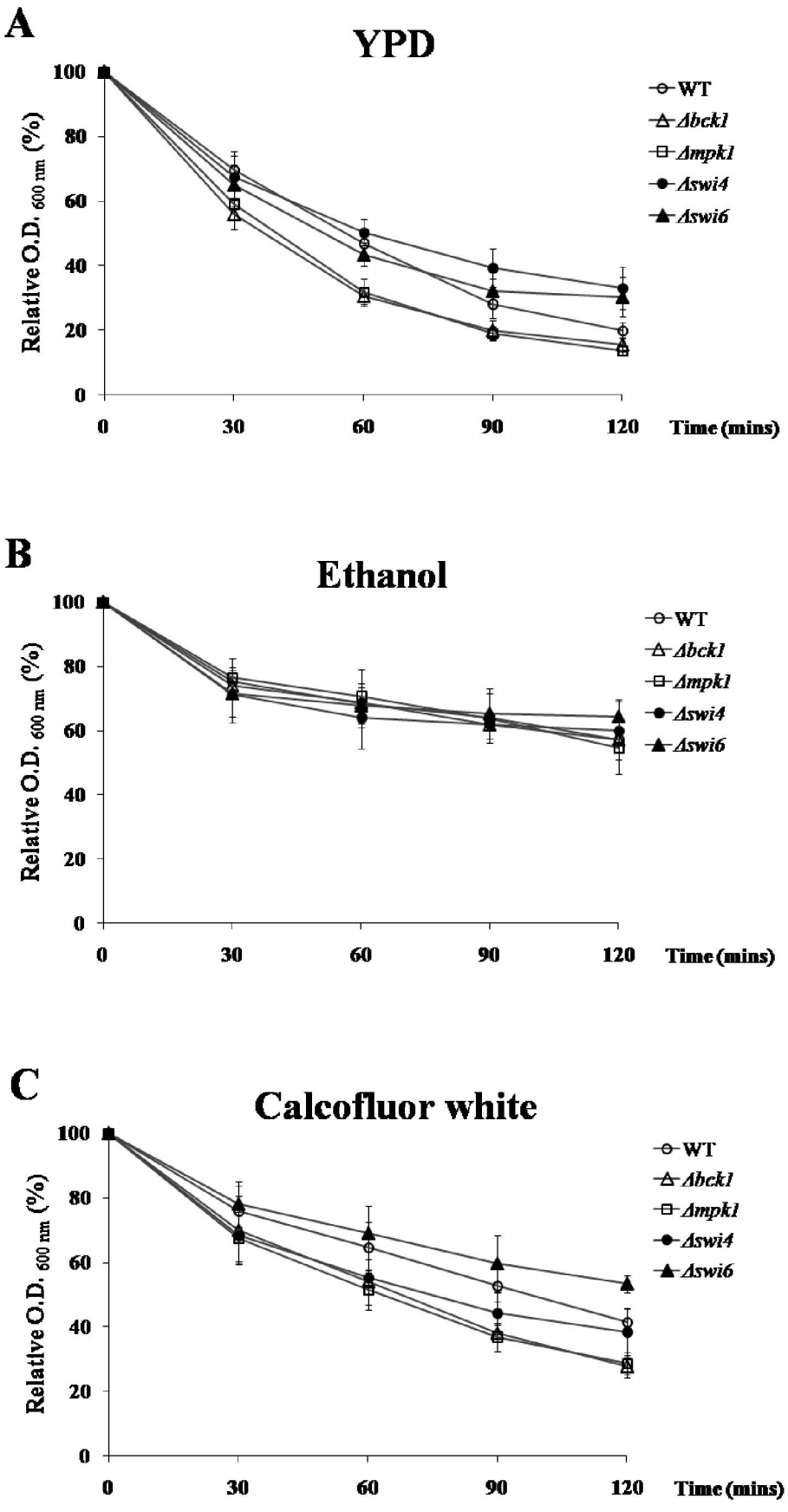
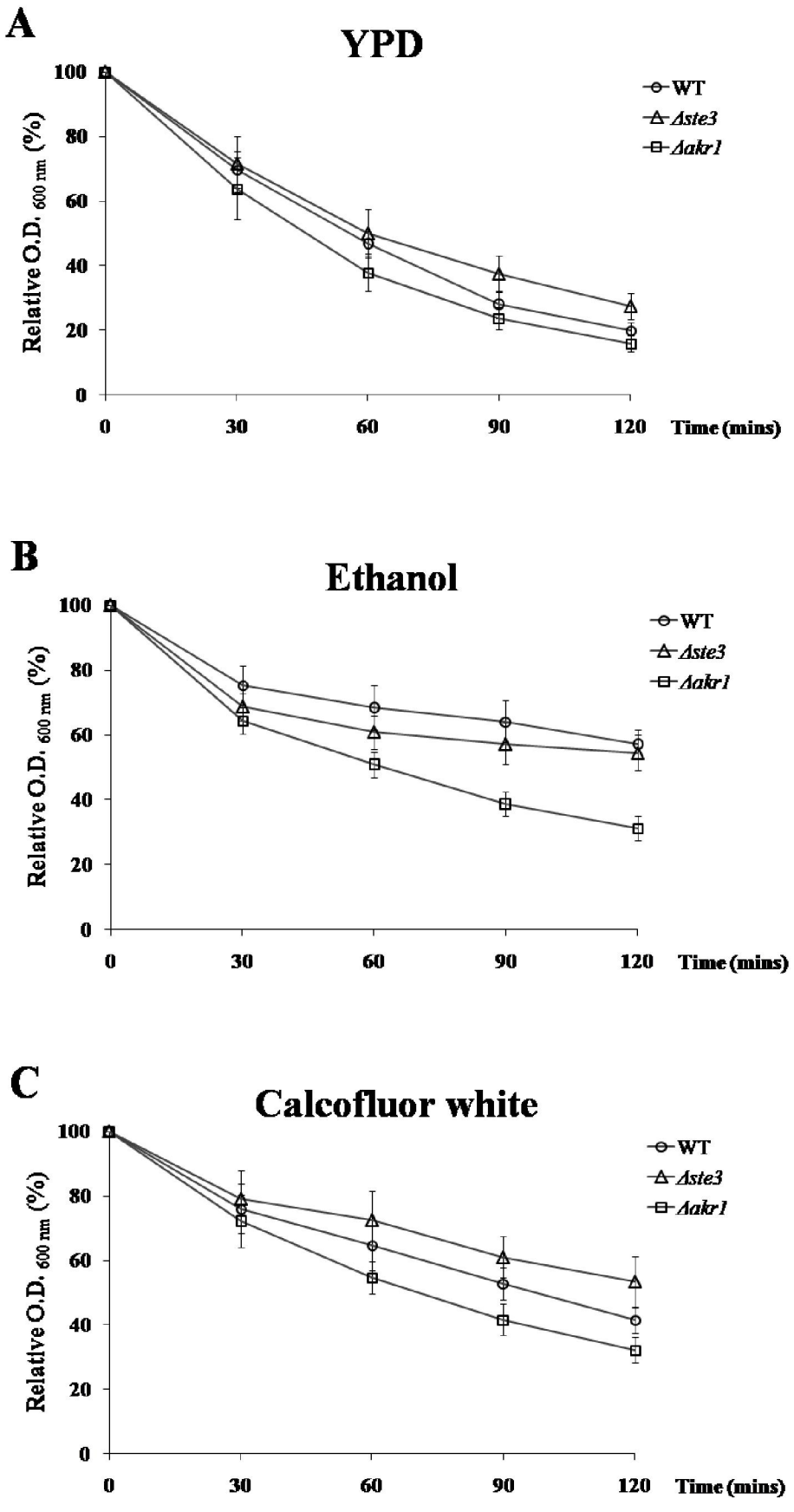
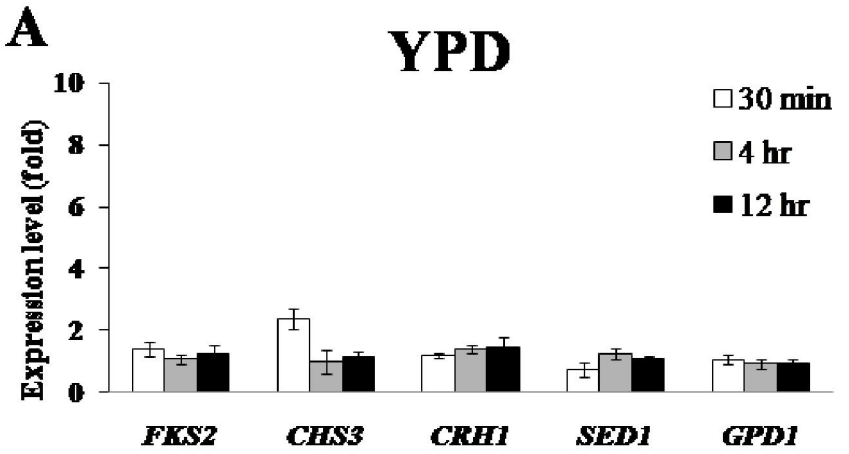

A
YPD
Relative O.D. 600 nm (%)
100
80
60
40
20
0
0
30
60
90
120
Time (mins)
WT
Δbck1
Δmpk1
Δswi4
Δswi6
B
Ethanol
Relative O.D. 600 nm (%)
Time (mins)
C
Calcofluor white
Relative O.D. 600 nm (%)
Time (mins)

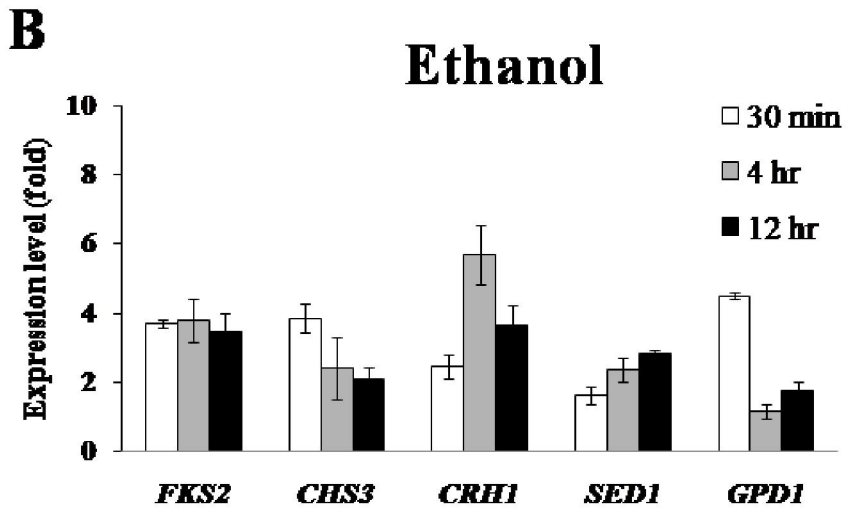
Figure 5.6 Cell wall remodeling of the yeast mutant strains after exposure to ethanol and Calcofluor white. The wild- type (BY4742) (○), Δste3 (Δ), and Δakr1 mutants (□) grown to log phase in YPD broth were inoculated to YPD (A) and YPD media containing 100µg/ml Calcofluor white (B) or 8% (v/v) ethanol (C). Cells were harvested, washed in TE buffer, and resuspended at an $O.D._{600}$ of 0.5 in the same buffer containing 50 µg/ml (0.5 U) Zymolyase. The decrease in the optical density at 600 nm was measured at the indicated times. The sensitivity to Zymolyase is shown as a percentage of the optical density at 600 nm relative to that at t=0.

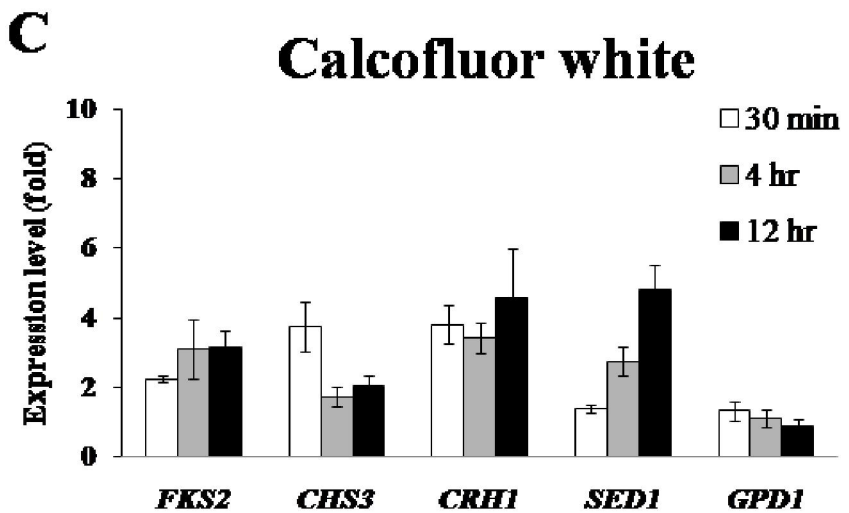

A
YPD
Relative O.D. 600 nm (%)
100
80
60
40
20
0
0
30
60
90
120
Time (mins)
WT
Δste3
Δakr1
B
Ethanol
Relative O.D. 600 nm (%)
100
80
60
40
20
0
0
30
60
90
120
Time (mins)
WT
Δste3
Δakr1
C
Calcofluor white
Relative O.D. 600 nm (%)
100
80
60
40
20
0
0
30
60
90
120
Time (mins)
WT
Δste3
Δakr1

5.4 The expression of cell wall related-genes in the yeast wild-type strain exposed to ethanol

Since Bck1p, Mpk1p, Swi4p, and Swi6p involved in the cell wall integrity pathway are required for ethanol tolerance, it is possible that this pathway is activated in response to ethanol stress, leading to induction of expression of cell wall-related genes that are important for modification of cell wall components. To investigate the expression of cell wall-related genes, which are target genes of the cell wall integrity pathway, after ethanol challenge, the expression of FKS2 encoding a subunit of β-1,3 glucan synthase, CHS3 encoding a chitin synthase III, CRH1 encoding chitin transglycosylase, and SED1 encoding GPI-cell wall glycoprotein in stationary-phase cells, was monitored in the wild-type strain after exposure to 8% ethanol and 100μg/ml Calcofluor white for 30 minutes, 4 and 12 hours by using real-time quantitative PCR analysis. We found that the expression levels of these cell wall-related genes, i.e. FKS2, CHS3, CRH1, and SED1, were significantly upregulated after exposure to ethanol and Calcofluor white (Fig 5.7 B and C), suggesting the induction of cell wall-related gene expression in response to ethanol-induced cell wall stress to modify cell wall structure. Interestingly, after exposure to ethanol and Calcofluor white for 30 minutes, the expression of FKS2 was increased approximately 3.7-fold and 2.2-fold, respectively (Fig. 5.7 B and C), whereas after prolonged incubation with ethanol and Calcofluor white for 4 and 12 hours, the expression levels of FKS2 reached a constant level (3.5-fold) (Fig. 5.7 B and C), suggesting a more rapid induction of FKS2 gene expression by ethanol. For CHS3, the expression levels is rapidly increased approximately 3.8-fold after exposure to ethanol and Calcofluor white for 30 minutes, and dropped to approximately 2-fold after prolonged incubation for 4 and 12 hours (Fig. 5.7 B and C). For CRH1, the expression reached the maximum levels after exposure to ethanol for 4 hours (Fig. 5.7 B), whereas, in the presence of Calcofluor white, the expression level of this gene was constant at about 4-fold (Fig. 5.7 C). For SED1, the expression levels were increased with increasing exposure time to both ethanol and Calcofluor white (Fig. 5.7 B and C).


A
YPD
Expression level (fold)
10
8
6
4
2
0
30 min
4 hr
12 hr
FKS2
CHS3
CRH1
SED1
GPD1


B
Ethanol
Expression level (fold)
10
8
6
4
2
0
30 min
4 hr
12 hr
FKS2
CHS3
CRH1
SED1
GPD1


C
Calcofluor white
Expression level (fold)
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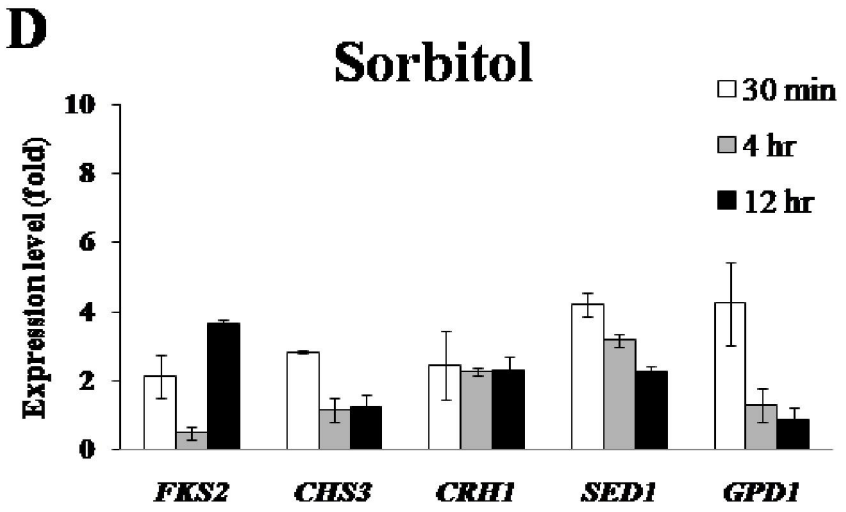
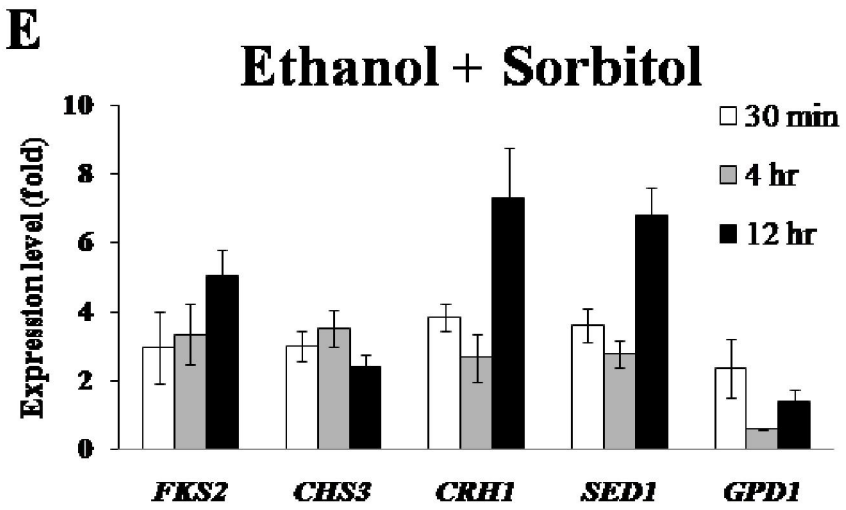
8
6
4
2
0
30 min
4 hr
12 hr
FKS2
CHS3
CRH1
SED1
GPD1


D
Sorbitol
30 min
4 hr
12 hr
Expression level (fold)
0
2
4
6
8
10
FKS2
CHS3
CRH1
SED1
GPD1


E
Ethanol + Sorbitol
30 min
4 hr
12 hr
Expression level (fold)
0
2
4
6
8
10
FKS2
CHS3
CRH1
SED1
GPD1

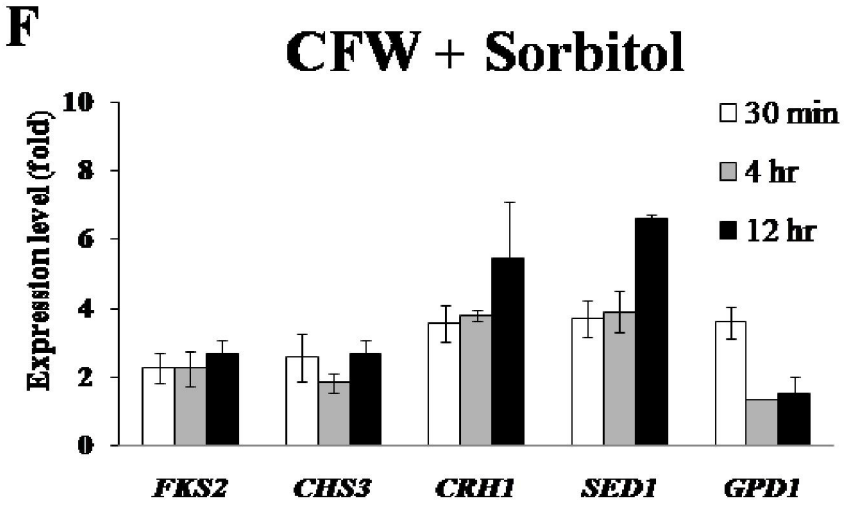
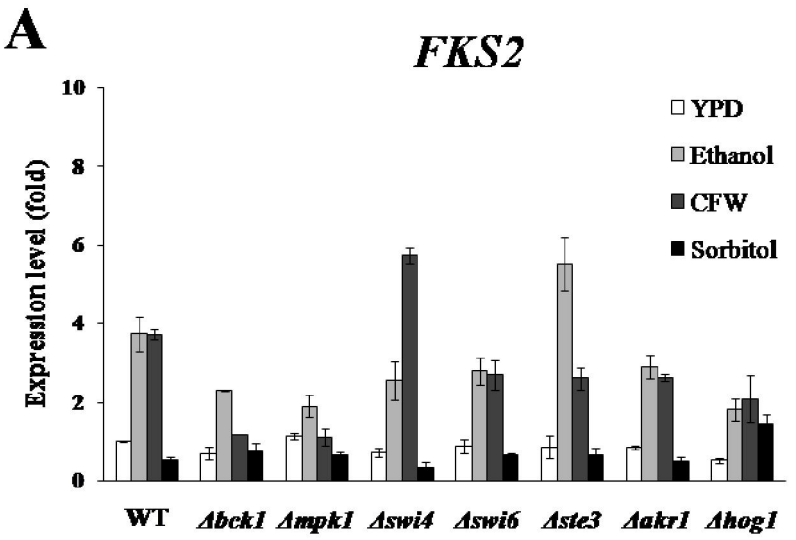

F
CFW + Sorbitol
30 min
4 hr
12 hr
Expression level (fold)
0
2
4
6
8
10
FKS2
CHS3
CRH1
SED1
GPD1

Figure 5.7 Expression of cell wall-related genes in the wild-type strain under ethanol and Calcofluor white stress conditions. The wild-type strain (BY4742) grown to log phase in YPD broth were inoculated into YPD (A), YPD media containing 8% (v/v) ethanol (B), 100µg/ml Calcofluor white (CFW) (C), 1.5 M sorbitol (D), 8% ethanol with 1.5 M sorbitol (EtOH + Sorbitol) (E), or 100 µg/ml Calcofluor white with 1.5 M sorbitol (CFW + Sorbitol) (F), and incubated for 30 minutes, 4 and 12 hours. Total RNA from each culture was used for real-time quantitative PCR. The mRNA levels of FKS2, CHS3, CRH1, SED1, and GPD1 were normalized to that of ACT1 in the same sample. Values are expressed as fold change relative to the 0 hour time point.

5.5 The expression of cell wall related-genes in the yeast strains exposed to ethanol

Since, the Δbck1 and Δmpk1 mutants lacking MAPK cascade of the cell wall integrity pathway were likely to be able to induce partial cell wall remodeling in response to cell wall stress caused by ethanol and Calcofluor white (Fig. 5.5 B and C) and the expression of cell wall-related genes, which are target genes of the cell wall integrity pathway, were upregulated after ethanol challenge, it is possible that the expression of some cell wll-related genes may be partially induced even in the absence of MAPK cascade. We monitored the expression of FKS2, CHS3, CRH1, SED1, and GPD1 in the *Δ*bck1, *Δ*mpk1, *Δ*swi4, *Δ*swi6, *Δ*ste3 and *Δ*akr1 mutants after exposure to 8% ethanol and 100µg/ml Calcofluor white for 4 hours by using real-time quantitative PCR analysis because the expression of cell wall related-genes in the wild-type strain in response to ethanol and Calcofluor white reached the similar levels after 4 hours exposure (Fig. 5.7 B and C). Our results revealed that, in the *Δ*bck1 and *Δ*mpk1 mutants, although the induction of expression of cell wall-related genes i.e. FKS2, CHS3, CRH1, and SED1, was significantly inhibited even after exposure to Calcofluor white, the induction of FKS2, CHS3, and SED1expression was observed after ethanol exposure (Fig 5.8 A-D). These findings suggested the activation of alternative signaling pathways in response to ethanol-induced cell wall stress when the MAPK cascade of the cell wall integrity pathway is inactivated. On the other hand, in the case of the Δswi4 and Δswi6 mutants lacking transcription factor complex of the cell wall integrity pathway (Fig 5.8 A-D), the expression of these cell wall-related genes was normally induced in response to ethanol and Calcofluor white, suggesting that the induction of expression of these cell wall-related genes does not depend on the Swi4p/Swi6p transcription factor complex. In addition, we also found that the Δste3 mutant lacking transmembrane receptor of pheromone signaling pathway did not exhibit defect in expression of cell wall-related genes in response to ethanol and Calcofluor white (Fig 5.8 A-E). For the Δakr1mutant, the expression of FKS2 and CHS3, but not SED1, in response to ethanol and Calcofluor white was partially inhibited (Fig 5.8 A, B and D). These results suggested that STE3 and AKR1 may not

be involved direcly in the regulation of the cell wall-related gene expression in response to cell wall stress caused by ethanol and Calcofluor white.


A
FKS2
Expression level (fold)
10
8
6
4
2
0
YPD
Ethanol
CFW
Sorbitol
WT
Δbck1
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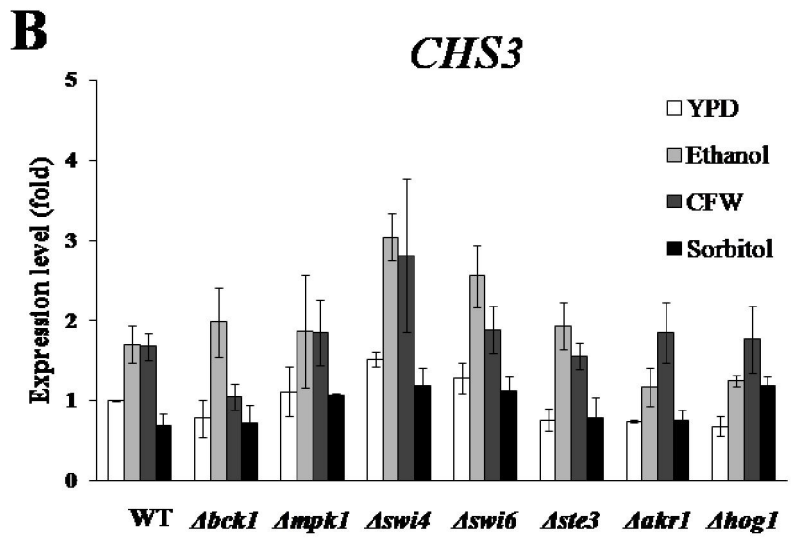
Δswi4
Δswi6
Δste3
Δakr1
Δhog1


B
CHS3
Expression level (fold)
5
4
3
2
1
0
YPD
Ethanol
CFW
Sorbitol
WT
Δbck1
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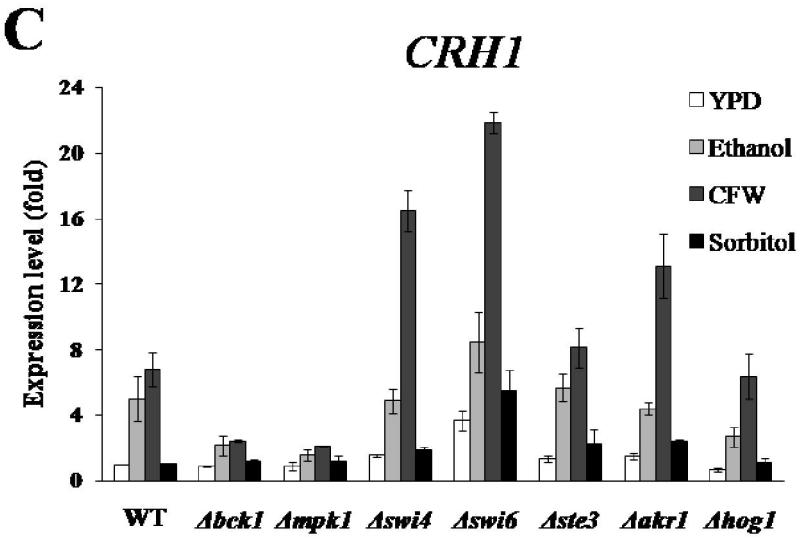
Δswi4
Δswi6
Δste3
Δakr1
Δhog1


C
CRH1
Expression level (fold)
24
20
16
12
8
4
0
YPD
Ethanol
CFW
Sorbitol
WT
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Δste3
Δakr1
Δhog1

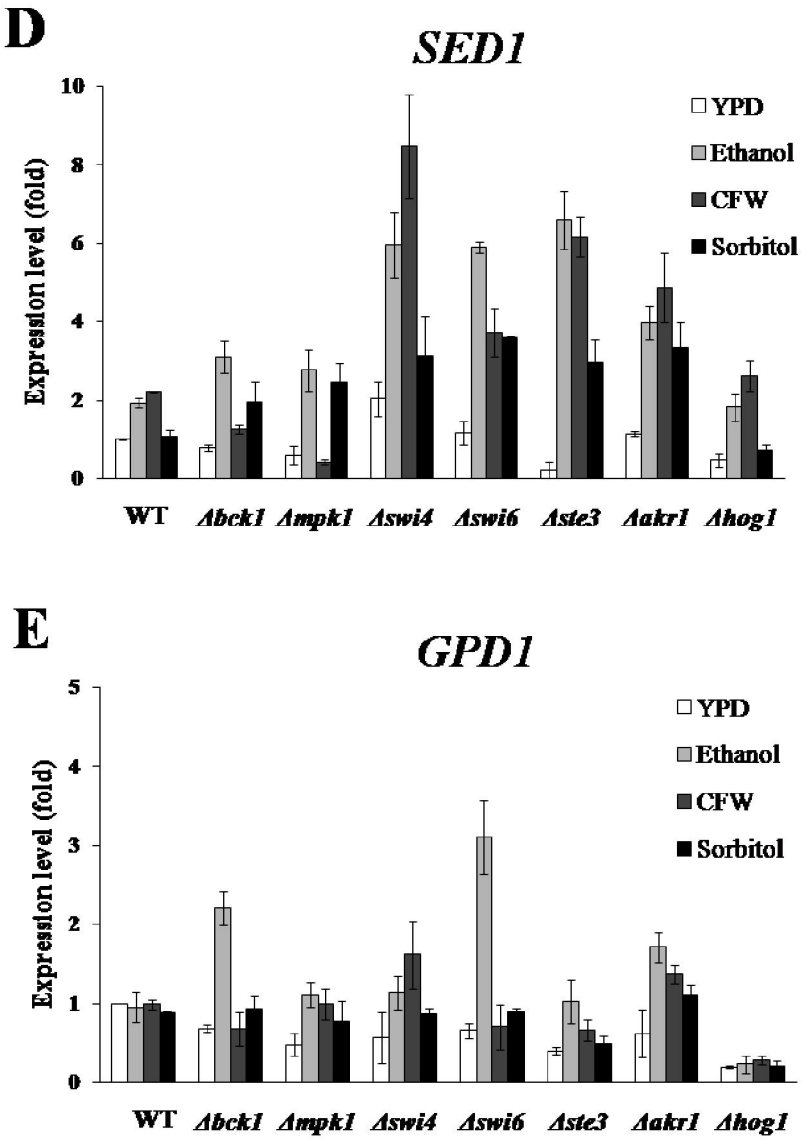


Figure 5.8 Expression of cell wall-related genes in the yeast mutant strains under ethanol stress condition. The wild-type (BY4742), *Δ*bck1, *Δ*mpk1, *Δ*swi4, *Δ*swi6, *Δ*ste3, *Δ*akr1, and Δhog1 strain grown to log phase in YPD broth were inoculated into YPD, YPD media containing 8% (v/v) ethanol, 100µg/ml Calcofluor white (CFW), or 1.5 M sorbitol, and incubated for 4 hours. Total RNA from each culture was used for real-time quantitative PCR. The mRNA levels of FKS2 (A), CHS3 (B), CRH1 (C), SED1 (D), and GPD1 (E), were normalized to that of ACT1 in the same sample. Values are expressed as fold change relative to control cells (untreated wild-type cells at 0 hour time point).

5.6 Effect of hyperosmolarity on cell growth during cell wall stress

Our results suggested the role of alternative pathway in partial activation of cell wall-related gene expression and cell wall remodeling in response to ethanol stress. Recently, it has been reported that the deleterious effect of cell wall-perturbing agents such as Calcofluor white and Zymolyase on cell growth is mitigated by sorbitol (de Nobel et al., 2000) and the response to Zymolyase-induced cell wall stress is mediated through not only the cell wall integrity pathway but also the cross-talk with the HOG pathway (Bermejo et al., 2008). Based on these findings, it is possible that the signaling of cell wall stress induced by ethanol is mediated through the cross-talk between the cell wall integrity pathway and the HOG pathway. If this is the case, increased osmolarity by sorbitol supplementation may somehow induce the activation of the cell wall integrity pathway in response to cell wall stresses caused by ethanol and Calcofluor white. To test this possibility, we examined the effect of sorbitol on growth of the yeast wild-type strain (BY4742) and its isogenic deletion mutants during ethanol and Calcofluor white stresses by using spot susceptibility test. We found that the addition of 1.5 M sorbitol resulted in suppression the ethanol-sensitive phenotype of the Δbck1, Δmpk1, Δswi4, Δswi6, Δste3, and Δakr1 mutants (Fig 5.9). On the other hand, the addition of sorbitol mitigated the Calcofluor white-induced growth inhibition of the Δbck1, Δmpk1, and, Δswi4 mutants, but not the Δste3, Δakr1, and Δswi6 mutants (Fig 5.9).

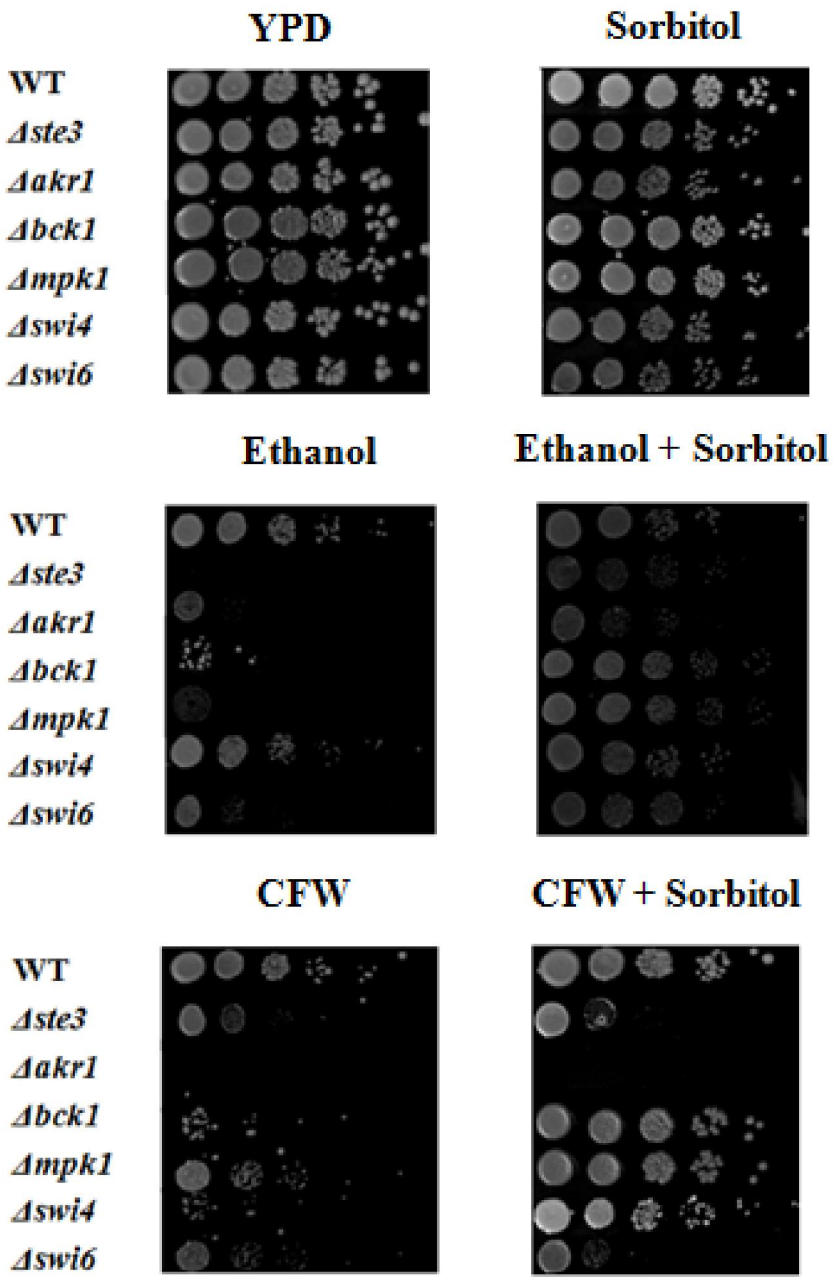
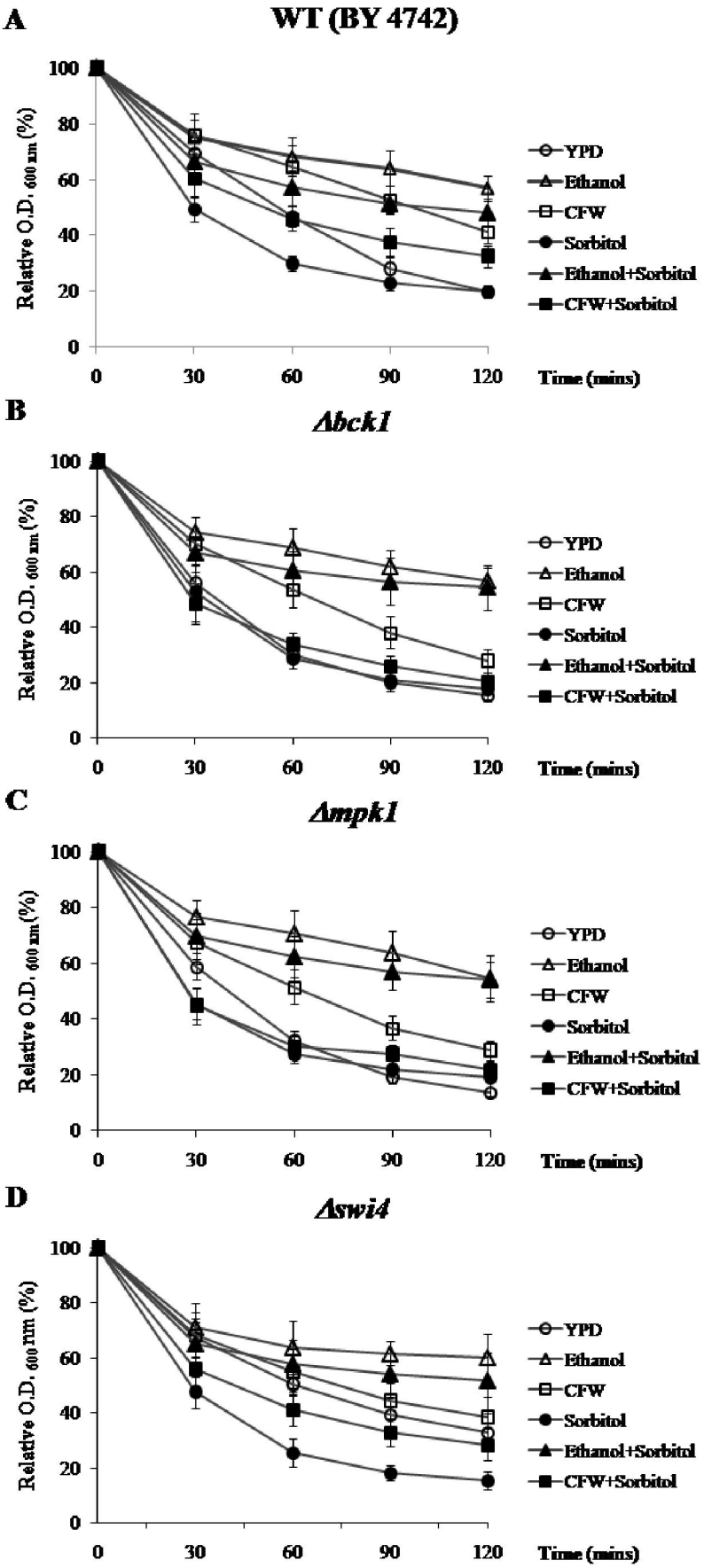


Figure 5.9 Growth of the yeast strains under ethanol and cell wall stress conditions with the supplementation of sorbitol. The wild-type strain (BY4742) and its isogenic deletion mutants pregrown to log phase in YPD liquid media were serially diluted ten-fold from an initial OD_{600} of 1. Aliquots (3 µl) were spotted on the YPD agar plates, YPD agar plates containing 12% ethanol, 100 µg/ml Calcofluor white (CFW), 12% ethanol with 1.5 M sorbitol, and 100µg/ml Calcofluor white with 1.5 M sorbitol, and incubated at 30°C for 2 days.

5.7 Effect of hyperosmolarity on cell wall remodeling and cell wall-related gene expression during cell wall stresses caused by ethanol and Calcofluor white.

To determine whether sorbitol induces the activation of the cell wall remodeling in response to cell wall stresses caused by ethanol and Calcofluor white, we first monitored the cell wall alteration in the wild-type, Δbck1, Δmpk1, Δswi4, Δswi6, Δste3, and Δakr1 strains exposed to either ethanol or Calcofluor white with the supplementation of sorbitol. Our result revealed that the addition of sorbitol did not lead to increased tolerance to Zymolyase, but rather resulted in increased sensitivity to Zymolyase (Fig. 5.10 A-G). These findings suggested that sorbitol has no effect on the activation of cell wall remodeling in response to cell wall stresses caused by ethanol and Calcofluor white. To further investigate the role of increased osmolarity in improving growth during cell wall stress, we examined on the expression of GPD1 encoding glycerol-3-phosphate dehydrogenase, a target gene of the HOG pathway, and cell wall-related genes in the yeast wild-type cells by using quantitative real-time PCR analysis. We found that the expression level of GPD1 was rapidly increased approximately 4.3-fold and 4.5-fold after 30-minute exposure to 1.5 M sorbitol and 8% ethanol, respectively, and dropped to the level before treatment after prolonged exposure more than 4 hours (Fig. 5.7 B and D). On the other hand, Calcofluor white seemed to have no effect on the expression of GPD1 (Fig. 5.7 C). Interestingly, we found that 1.5 M sorbitol slightly induced the expression of cell wall-related genes including FKS2, CRH1, and SED1 within 30 minutes (Fig. 5.7 D). However, the expression level of FKS2 was dropped after 4-hours exposure to sorbitol and increased again after 12-hour exposure (Fig. 5.7 D), whereas the expression levels of CHS3 and SED1 was dropped after long-term incubation (4-12 hours). When the wild-type cells were grown in the presence of 8% ethanol with 1.5 M sorbitol for 12 hours, the expression of FKS2, CRH1, and SED1 were greatly induced to the levels higher than that grown without sorbitol supplementation (Fig 5.7 E). Similarly, the wild-type cells grown in the presence of 100 µg/ml Calcofluor white with 1.5 M sorbitol for 12 hours exhibited higher expression levels of CRH1 and SED1 than that grown without sorbitol supplementation (Fig 5.7 F). These results suggested that increased osmolarity

by sorbitol is involved in inducing expression of some cell wall-related genes such as CRH1 and SED1 in response to prolonged exposure to ethanol and Calcofluor white, leading to increased tolerance to ethanol and Calcofluor white.


A
WT (BY 4742)
B
Δbck1
C
Δmpk1
D
Δswi4
Relative O.D. 600 nm (%)
Time (mins)
YPD
Ethanol
CFW
Sorbitol
Ethanol+Sorbitol
CFW+Sorbitol
0
20
40
60
80
100
0
30
60
90
120

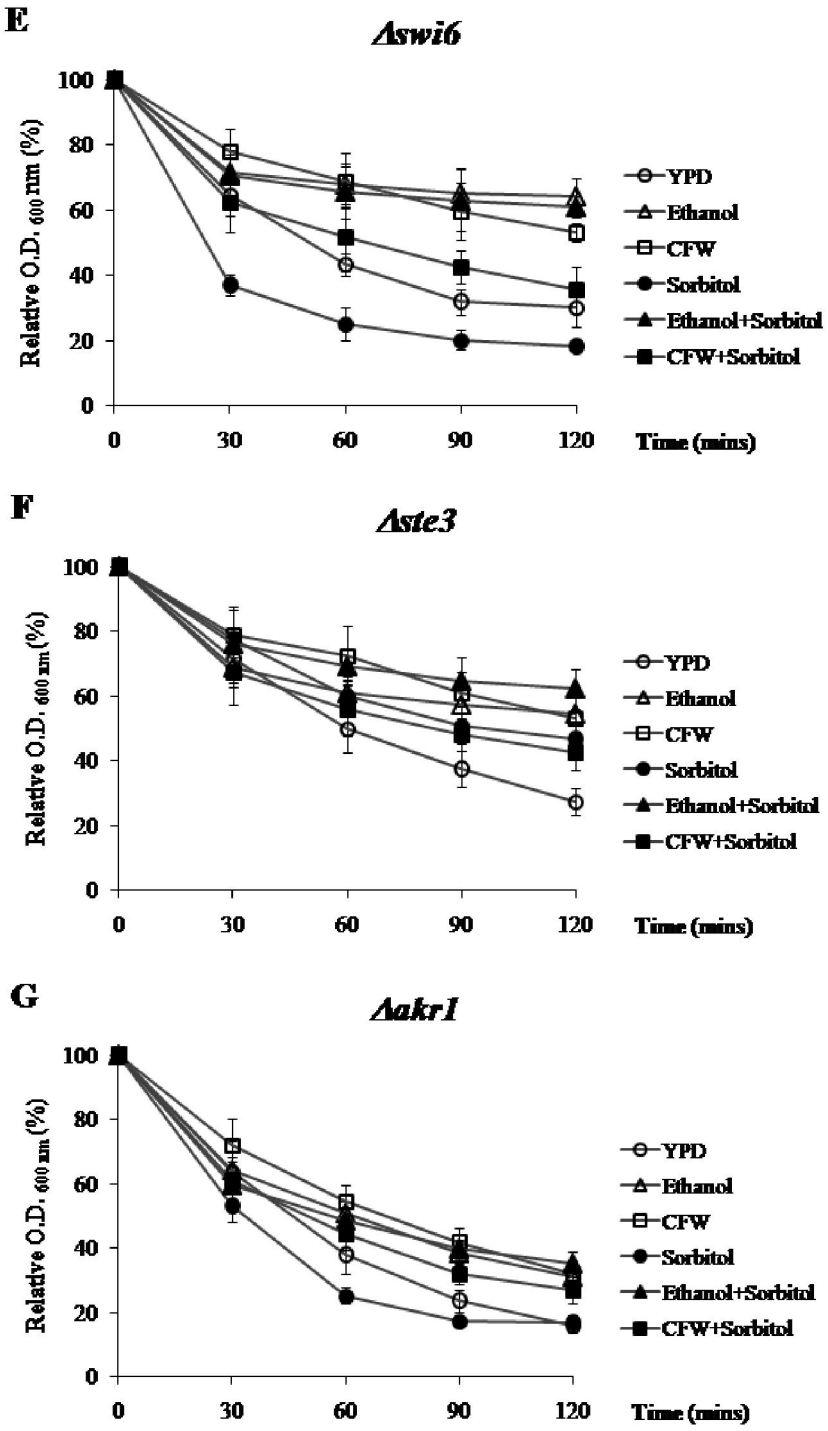

E
Δswi6
Relative O.D. 600 nm (%)
100
80
60
40
20
0
0
30
60
90
120
Time (mins)
YPD
Ethanol
CFW
Sorbitol
Ethanol+Sorbitol
CFW+Sorbitol
F
Δste3
Relative O.D. 600 nm (%)
Time (mins)
YPD
Ethanol
CFW
Sorbitol
Ethanol+Sorbitol
CFW+Sorbitol
G
Δakr1
Relative O.D. 600 nm (%)
Time (mins)
YPD
Ethanol
CFW
Sorbitol
Ethanol+Sorbitol
CFW+Sorbitol

Figure 5.10 Cell wall remodeling of the yeast mutant strains after exposure to ethanol and Calcofluor white with sorbitol supplementation. The wild-type (BY4742) (A), Δbck1(B), Δmpk1(C), Δswi4 (D), Δswi6 (E), Δste3 (F), and Δakr1 (G) grown to log phase in YPD broth were inoculated to YPD (○), YPD media containing 8% (v/v) ethanol (Δ), 100 µg/ml Calcofluor white (CFW) (□), 1.5M sorbitol (●), 8% (v/v) ethanol with 1.5M sorbitol (▲), or 100 µg/ml Calcofluor white with 1.5M sorbitol (■). Cells were harvested, washed in TE buffer, and resuspended at an $O.D._{600}$ of 0.5 in the same buffer containing 50 µg/ml (0.5 U) Zymolyase. The decrease in the optical density at 600 nm was measured at the indicated times. The sensitivity to Zymolyase is shown as a percentage of the optical density at 600 nm relative to that at t=0.