CHAPTER VI
DISCUSSION

6.1 Roles of the MAPK pathways in response to ethanol stress in S. cerevisiae

Although the ethanol toxicity has been widely studied, the ethanol stress signaling is still unknown. Several reports have suggested that the genes involved in the MAPK signaling pathways are required for ethanol tolerance in S. cerevisiae (Auesukaree et al., 2009; Fujita et al., 2006; Kubota et al., 2004). Consistent with the previous reports, our results revealed that the genes involved in the cell wall integrity pathway, i.e. BCK1 encoding MAPKKK, MPK1 encoding MAPK, SWI4 and SWI6 encoding the transcription cofactors, are required for ethanol tolerance (Fig. 5.1). The cell wall integrity pathway is activated by cell wall stresses caused by cell wall-disturbing agent such as Calcofluor white and Congo red, and cell wall-degrading enzyme such as Zymolyase (Cid et al., 1995; Firon et al., 2004; Levin, 2005). It has been reported that defects in signal transduction through the cell wall integrity pathway resulted in a lack of ability to induce cell wall remodeling process required for recovery of yeast cell wall damage (de Nobel et al., 2000). However, we found that the mutants lacking the other components of the cell wall integrity pathway such as the Δwsc1, Δwsc2, Δwsc3, Δmid2, Δrom2, Δmkk1, Δmkk2, and Δspa2 mutants did not confer ethanol-sensitive phenotype (Fig. 5.1), possibly due to a redundant role of these genes. For instance, deletion of either MKK1 or MKK2 gene did not inhibit growth during heat stress, whereas the double deletion mutant of these genes exhibited sensitivity to high temperature (Irie et al., 1993). Interestingly, our results showed that the Δwsc1 and Δspa2 mutants were sensitive to Calcofluor white but grew normally in the presence of 12% ethanol (Fig.5.1). These results suggested that Wsc1p sensor and Spa2p scaffold protein are important for signal transduction in response to cell wall damage but the response to ethanol may be mediated by alternative components. Moreover, deletion of SWI4 gene resulted in hypersensitivity to Calcofluor white but

less sensitive to ethanol, whereas deletion of SWI6 caused a high sensitive to both ethanol and Calcofluor white (Fig.5.1). These findings suggested a role of the transcription complex of Swi4p/Swi6p in the regulation of cell wall remodeling in response to Calcofluor white stress but, in response to ethanol, SWI6 may form the alternative complex with another transcription factors to provide a specific response.

Furthermore, our results showed that only STE3encoding a transmembrane receptor for pheromone and AKR1 encoding a negative regulator of the pheromone signaling pathway, but not the other components of this pathway, are required for ethanol tolerance (Fig. 5.2). In addition, the Δste3 and Δakr1 mutants were also sensitive to Calcofluor white. These findings suggested that STE3 and AKR1may play an important role not only in pheromone signaling but also in maintaining cell wall structure during ethanol stress. However, we also found that the Δste20 and Δbem1 mutants lacking components involved I the pheromone signaling pathway were sensitive to Calcofluor white but not ethanol (Fig.5.2). This may be the fact that Ste20p and Bem1p are important for promoting polarized morphogenesis in mating response which may activate the cell wall integrity pathway (Buehrer & Errede, 1997). It has been reported that in the Δste20 mutant, the phosphorylation of Mpk1p was not observed in response to mating pheromone (Zarzov et al., 1996), although the association between Ste20p and actin that regulates polarized rearrangement of the actin required Bem1p during yeast mating (Leeuw et al., 1995).

6.2 The cell wall remodeling process is activated in response to ethanol stress in S. cerevisiae

Does ethanol exposure trigger the activation of the cell wall integrity pathway, leading to the induction of cell wall remodeling process? Recently, it has been reported that 6% ethanol induced cell wall remodeling in yeast wild-type strain (Teixeira et al., 2009). Based on our results of Zymolyase sensitivity test, we found that ethanol and Calcofluor white induced cell wall remodeling in dose- and time-dependent manner in the wild-type strain (Fig. 5.3 and 5.4). These findings suggested that ethanol causes cell wall damage and the cell wall remodeling process is one of cellular adaptations to ethanol stress. It is known that in response to cell wall stresses

caused by cell wall-perturbing agent such as Calcofluor white and Congo red or cell wall-degrading enzyme such as Zymolyase, the cell wall integrity pathway is activated, leading to a change in cell wall structure. Thus, a lack of signaling proteins of this pathway such as Bck1p and Mpk1p is supposed to result in the inhibition of cell wall remodeling process, thereby leading to increased sensitivity to Zymolyase. However, when the *Δ*bck1 and *Δ*mpk1 mutants lacking MAPKKK and MAPK, respectively, were pretreated with ethanol and Calcofluor white, they exhibited increased resistance to Zymolyase (Fig 5.5 B and C), similar to that observed in the wild-type strain, suggesting the important role of the alternative pathways in transducing signal of cell wall stresses caused by ethanol and calcoflour white. Since a cross-talk between the cell wall integrity pathway and the HOG pathway in response to zymolyase-induced cell wall stress has been reported (Bermejo et al., 2008), it is possible that ethanol-induced cell wall stress needs an activation of the both pathways and the HOG pathway to induce cell wall remodeling. Moreover, the *Δ*swi4 and *Δ*swi6 mutants showed increased resistance to Zymolyase after exposure to ethanol Calcofluor white, suggesting that SWI4 and SWI6 is not required for cell wall remodeling in response to ethanol stress.

In addition, the Δste3 exhibited increased resistance to Zymolyase after exposure to sublethal concentrations of ethanol or Calcofluor white (Fig 5.6 B and C), suggesting that STE3 is not required for cell wall remodeling during ethanol stress. By contrast, after exposure to ethanol or Calcofluor white for 12 hours, the *Δ*akr1 mutant was more sensitive to Zymolyase than that the observed in wild-type strain (Fig 5.6 B and C), suggesting the role of Akr1p, but not Ste3p, in signal transduction involved in cell wall remodeling during ethanol stress.

6.3 The important role of the expression of cell wall-related genes in response to ethanol stress.

Does the activation of the cell wall integrity pathway results in induced the expression of cell wall-related genes which important for ethanol tolerance? Our results revealed that the expression of cell wall-related genes, i.e. FKS2, CHS3, CRH1,

and SED1, which are target genes of the cell wall integrity pathway, was upregulated in response to not only Calcofluor white but also ethanol and the expression of FKS2 and CHS3 seemed to be rapidly induced by ethanol (Fig 5.7 B). However, in the presence of Calcofluor white the expression reached the maximum levels after exposure to ethanol for 4 hours (Fig. 5.7 B). These results suggested that ethanol may cause a severe effect on cell wall organization, thereby inducing a rapid response through the cell wall integrity pathway to increase the expression of specific cell wall-related genes such as FKS2 and CHS3.

The expression levels of FKS2 and CRH1 in the *Δ*bck1 and *Δ*mpk1 mutants, were increased after exposure to ethanol and Calcofluor white (Fig 5.8 A and C). These findings suggested the role of alternative signaling pathways in response to ethanol when 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, 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. Recently, two transcription factors have been identified as the major target of Mpk1p in response to cell wall stress; the transcription factor complex of Swi4p/Swi6p and Rlm1p (Bermejo et al., 2008; Firon et al., 2004; Jung & Levin, 1999; Jung et al., 2002), therefore ethanol induce cell wall stress lead to the activation of the Rlm1p transcription factor to increase expression of some specific cell wall-related genes.

6.3 The hyperosmolarity is not involved in the induction cell wall remodeling in response to ethanol stress.

In this study, we found that the addition of sorbitol restored the growth defects of the *Δ*bck1, *Δ*mpk1, and *Δ*swi4 mutant lacking genes involved in the cell wall integrity pathway under ethanol and Calcofluor white stress conditions (Fig. 5.9). Consistent with our findings, it has been reported that the deleterious effect on cell growth caused by several stresses such as cell wall stress, high temperature, and high

pH was mitigated by the addition of sorbitol into the growth media (de Nobel et al., 2000; Klis et al., 2006; Levin, 2005; Serrano et al., 2006). We therefore investigated the role of increased osmolarity in improving growth during cell wall stresses caused by ethanol and Calcofluor white on the expression of GPD1 encoding glycerol-3-phosphate dehydrogenase and cell wall-related genes, we found that the expression level of GPD1, a target of the HOG pathway, was rapidly increased after exposure to 1.5 M sorbitol and 8% ethanol for 30 minutes (Fig. 5.7 B and D). Consistent with our result, the GPD1 expression has been shown to be induced after short-term exposure to ethanol (Alexandre et al., 2001). This may be due to the role of sorbitol in inducing adaptation program in response to cell wall stress through the rapid and transient activation of the HOG pathway to increase intracellular glycerol concentration (Hohmann et al., 2007; Schuller et al., 1994), which is important for suppression of the deleterious effect on cell growth. We also found that sorbitol slightly induced the expression of cell wall-related genes including FKS2, CRH1, and SED1 within 30 minutes (Fig. 5.7 D). Moreover, in the presence of ethanol with sorbitol or Calcofluor white with sorbitol, the expression of FKS2, CRH1 and SED1 after 12-hour incubation were induced to the levels higher than that incubated without sorbitol supplementation (Fig 5.7 B, C, E and 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. It is possible that the signaling of cell wall stresses caused by ethanol and Calcofluor white is mediated through the cell wall integrity pathway and the HOG pathway. Thus, increased osmolarity may also induce the activation of the cell wall integrity pathway to remodel cell wall structure in response to cell wall stress. However, our result clearly revealed that sorbitol has no effect on the activation of cell wall remodeling in response to cell wall stress caused by ethanol and Calcofluor white (Fig. 5.10 A-D). However, the increased intracellular glycerol levels may protect the damaged cell wall from rupture.

However, in response to adverse conditions, more than one appropriate mechanism might be required for better and faster adaptation. Recently, it has been reported that unfolded protein response (UPR) was activated in response to cell wall stress mediated through the cell wall integrity pathway but the presence of sorbitol

with cell wall-disturbing agent led to decreased UPR activity (Scrimale et al., 2009). The UPR pathway might be activated by ethanol-induced cell wall stress through the cell wall integrity pathway and the addition of osmotic stabilizer such as sorbitol may somehow reduce protein unfolding rates, resulting in a less UPR activity and increased ethanol tolerance.