AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

GENETICS AND MOLECULAR BIOLOGY

Coordination of the Cell Wall Integrity and High-Osmolarity Glycerol Pathways in Response to Ethanol Stress in *Saccharomyces cerevisiae*

Nisarut Udom,[a,b] Pakkanan Chansongkrow,[a,c] Varodom Charoensawan,[c,d,e] Choowong Auesukaree[a,b,f,g]

[a]Department of Biology, Faculty of Science, Mahidol University, Bangkok, Thailand
[b]Center of Excellence on Environmental Health and Toxicology, CHE, Ministry of Education, Bangkok, Thailand
[c]Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand
[d]Integrative Computational BioScience Center, Mahidol University, Nakhon Pathom, Thailand
[e]Systems Biology of Diseases Research Unit, Faculty of Science, Mahidol University, Bangkok, Thailand
[f]Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok, Thailand
[g]Mahidol University-Osaka University Collaborative Research Center for Bioscience and Biotechnology, Faculty of Science, Mahidol University, Bangkok, Thailand

ABSTRACT During fermentation, a high ethanol concentration is a major stress that influences the vitality and viability of yeast cells, which in turn leads to a termination of the fermentation process. In this study, we show that the *BCK1* and *SLT2* genes encoding mitogen-activated protein kinase kinase kinase (MAPKKK) and mitogen-activated protein kinase (MAPK) of the cell wall integrity (CWI) pathway, respectively, are essential for ethanol tolerance, suggesting that the CWI pathway is involved in the response to ethanol-induced cell wall stress. Upon ethanol exposure, the CWI pathway induces the expression of specific cell wall-remodeling genes, including *FKS2*, *CRH1*, and *PIR3* (encoding β-1,3-glucan synthase, chitin transglycosylase, and O-glycosylated cell wall protein, respectively), which eventually leads to the remodeling of the cell wall structure. Our results revealed that in response to ethanol stress, the high-osmolarity glycerol (HOG) pathway plays a collaborative role with the CWI pathway in inducing cell wall remodeling via the upregulation of specific cell wall biosynthesis genes such as the *CRH1* gene. Furthermore, the substantial expression of CWI-responsive genes is also triggered by external hyperosmolarity, suggesting that the adaptive changes in the cell wall are crucial for protecting yeast cells against not only cell wall stress but also osmotic stress. On the other hand, the cell wall stress-inducing agent calcofluor white has no effect on promoting the expression of *GPD1*, a major target gene of the HOG pathway. Collectively, these findings suggest that during ethanol stress, the CWI and HOG pathways collaboratively regulate the transcription of specific cell wall biosynthesis genes, thereby leading to adaptive changes in the cell wall.

IMPORTANCE The budding yeast *Saccharomyces cerevisiae* has been widely used in industrial fermentations, including the production of alcoholic beverages and bioethanol. During fermentation, an increased ethanol concentration is the main stress that affects yeast metabolism and inhibits ethanol production. This work presents evidence that in response to ethanol stress, both CWI and HOG pathways cooperate to control the expression of cell wall-remodeling genes in order to build the adaptive strength of the cell wall. These findings will contribute to a better understanding of the molecular mechanisms underlying adaptive responses and tolerance of yeast to ethanol stress, which is essential for successful engineering of yeast strains for improved ethanol tolerance.

KEYWORDS ethanol, *Saccharomyces cerevisiae*, cell wall integrity pathway, cell wall remodeling, cell wall stress, high-osmolarity glycerol pathway

Citation Udom N, Chansongkrow P, Charoensawan V, Auesukaree C. 2019. Coordination of the cell wall integrity and high-osmolarity glycerol pathways in response to ethanol stress in *Saccharomyces cerevisiae*. Appl Environ Microbiol 85:e00551-19. https://doi.org/10.1128/AEM.00551-19.

Editor Irina S. Druzhinina, Nanjing Agricultural University

Address correspondence to Choowong Auesukaree, choowong.aue@mahidol.ac.th.

Received 6 March 2019
Accepted 9 May 2019
Accepted manuscript posted online 17 May 2019
Published 18 July 2019

The yeast *Saccharomyces cerevisiae* has been widely used in several fermentation industries, such as the production of alcoholic beverages and ethanol fuel. During fermentation, yeast cells are exposed to several environmental insults, such as high ethanol concentrations, high osmolarity, and oxidative stress (1). Among these, a high ethanol concentration is one of the major stress factors that influences the vitality and viability of yeast cells, resulting in the termination of the fermentation process (2). Ethanol stress has been shown to affect several cellular processes of yeast cells, including inhibition of glucose and amino acids transport systems, denaturation of crucial glycolytic enzymes such as pyruvate kinase and hexokinase, increased membrane permeability, and induction of reactive oxygen species (ROS) (2, 3). Many genes involved in cell wall biosynthesis and the cell wall integrity (CWI) mitogen-activated protein kinase (MAPK) signaling pathway (Fig. 1A) have been found to be essential for ethanol tolerance. These include the *ANP1*, *HOC1*, and *MNN10* genes encoding subunits of mannosyl polymerase complex II; the *KRE6* gene encoding a β-glucan synthase; and the *WSC1*, *MID2*, *BCK1*, *SLT2*, *SWI4*, and *SWI6* genes encoding the components of the CWI pathway (4–6). It is therefore likely that the cell wall plays a protective role against ethanol stress.

The cell wall is an essential cellular structure required for protecting yeast cells against environmental stresses in order to maintain cell shape and cell integrity. The yeast cell wall, which is a layered structure consisting of inner and outer layers, consists of four major components, i.e., β-1,3-glucan, β-1,6-glucan, chitin, and mannoproteins (7). The inner layer is mainly composed of a β-1,3-glucan network branched to β-1,6-glucan, which is covalently linked to chitin, whereas the outer layer is a lattice of glycosylated mannoproteins attached to a glucan network (7). In response to cell wall stress, the CWI signaling pathway is activated to upregulate the expression of genes involved in cell wall biosynthesis, leading to a remodeling of the cell wall architecture to be more robust. The cell wall-remodeling process includes an increase in the amount of cell wall components and a change in the cross-linking between the cell wall components (7).

When yeast cells are exposed to cell wall stress, the active cell surface sensors (Mid2p, Wsc1p, Wsc2p, and Wsc3p) stimulate the guanine nucleotide exchange factor (GEF) Rom2p to activate the small GTPase Rho1p, leading to the activation of the protein kinase C Pkc1p. Active Pkc1p phosphorylates MAPK kinase kinase (MAPKKK) Bck1p, which in turn phosphorylates the redundant MAPK kinases (MAPKKs) Mkk1p and Mkk2p, leading to the phosphorylation of MAPK Slt2p (8). The active MAPK Slt2p then activates transcription factors Rlm1p and the SBF (Swi4p-Swi6p cell cycle box-binding factor) complex to promote the expression of cell wall biosynthesis genes and G_1/S transition-regulating genes, respectively (8).

Although the CWI pathway is the main signaling pathway required for controlling cell wall stress responses, some other signaling pathways have been found to be involved in regulating the expression of several target genes of the CWI pathway. For instance, the high-osmolarity glycerol (HOG) pathway (Fig. 1B), another MAPK pathway that plays a major role in the osmotic stress response, has been shown to be involved in cross-signaling through its SHO1 branch with the CWI pathway in response to cell wall stress induced by the cell wall-degrading enzyme Zymolyase (9–11). However, since it has been reported that different types of cell wall-perturbing agents triggered distinct cellular responses (12, 13), it is still unknown whether the HOG pathway and/or some other signaling pathways are required for cooperative signaling with the CWI pathway in response to ethanol stress. The HOG pathway contains two signaling branches, i.e., the SLN1 and SHO1 branches, which appear to function independently (14). The SLN1 branch is controlled by the Sln1p osmosensor, which forms a phosphorelay signaling system with Ypd1p and Ssk1p. Under normal-osmolarity conditions, Sln1p is active and phosphorylates Ypd1p, which then transfers its phosphate to Ssk1p. The phosphorylated Ssk1p is inactive and unable to activate Ssk2p and Ssk22p, the redundant MAPKKKs of the HOG pathway. On the other hand, the SHO1 branch is comprised of two putative transmembrane osmosensors, Hkr1p and Msb2p, and a

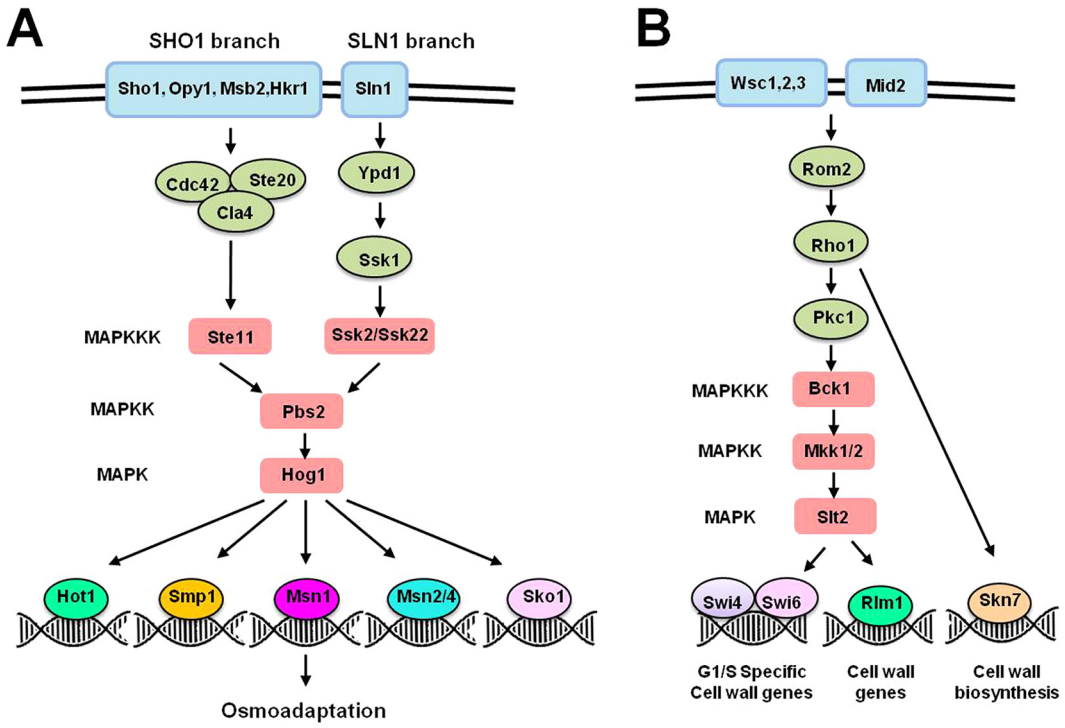

FIG 1 Schematic diagrams of the CWI pathway (A) and the HOG pathway (B) in *S. cerevisiae*.

plasma membrane-localized scaffold protein, Sho1p, which is involved in the recruitment of certain components of this pathway, including the membrane anchor protein Opy2p, the MAPKKK Ste11p, and the MAPKK Pbs2p. In response to hyperosmotic stress, both upstream branches are activated, and signals from both branches converge at the MAPKK Pbs2p, which then phosphorylates the MAPK Hog1p. Activated Hog1 rapidly translocates to the nucleus to stimulate the activities of several transcription factors, such as Hot1p, Smp1p, Msn2p, and Msn4p, leading to the upregulated expression of osmoresponsive genes (14).

In this study, the protective role of the CWI pathway against ethanol stress was investigated in *S. cerevisiae* by examining the growth of deletion mutants lacking genes encoding components of the CWI pathway in the presence of ethanol. Systemic transcriptome analysis was conducted to identify candidate cell wall biosynthesis genes that are the target genes of the response to ethanol-induced cell wall stress. We explore three main aspects: (i) the involvement of the CWI and HOG pathways in cooperative signaling in response to ethanol stress, (ii) the expression of candidate cell wall biosynthesis genes, and (iii) cell wall remodeling as determined in mutants impaired in either or both signaling pathways under ethanol stress conditions. In addition, the effect of the CWI pathway on the expression of the target gene of the HOG pathway was also determined.

RESULTS

The cell wall integrity pathway is important for ethanol tolerance. Previous studies of the yeast deletion mutant collection have revealed that a number of genes involved in the CWI signaling pathway and cell wall biogenesis were required for ethanol tolerance, suggesting the protective role of the cell wall against ethanol (4–6, 15). The CWI pathway is known to play an important role in controlling the transcriptional adaptive response to cell wall stress, leading to a remodeling of the cell wall architecture in order to improve resistance against cell wall stress (7). We therefore hypothesized that ethanol stress compromises the integrity of the cell wall. To test this hypothesis, we first examined the growth of mutants lacking genes encoding compo-

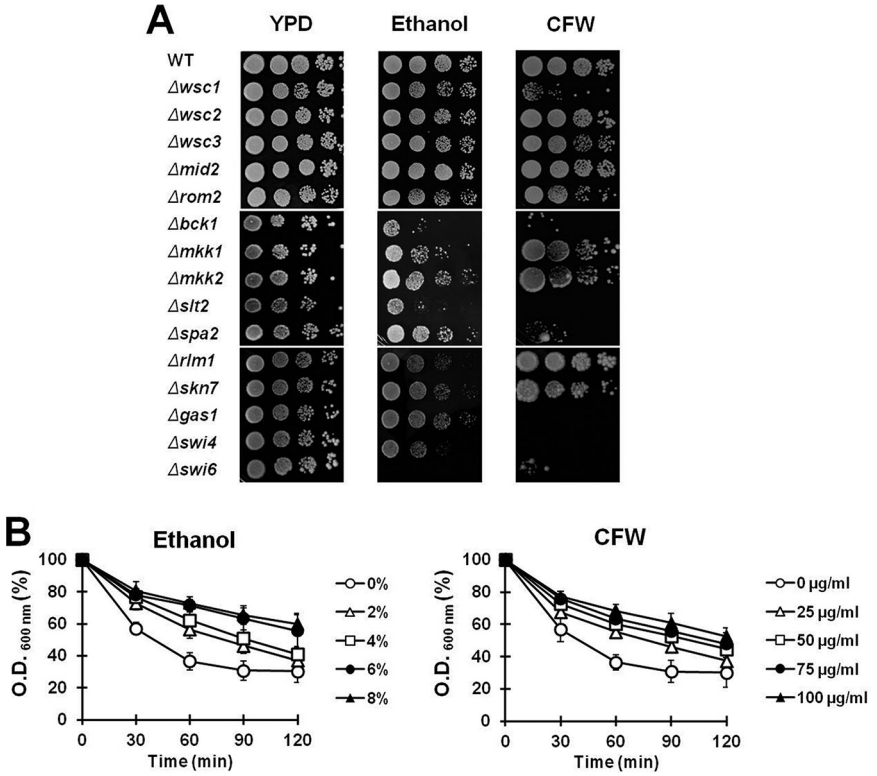

FIG 2 The CWI pathway is important for protecting yeast cells against ethanol-induced cell wall stress. (A) Growth of the wild-type strain (BY4742) and its isogenic deletion mutants lacking genes encoding components of the CWI pathway (i.e., Δ*wsc1*, Δ*wsc2*, Δ*wsc3*, Δ*mid2*, Δ*rom2*, Δ*bck1*, Δ*mkk1*, Δ*mkk2*, Δ*slt2*, Δ*spa2*, Δ*rlm1*, Δ*skn7*, Δ*gas1*, Δ*swi4*, and Δ*swi6* mutants) in the presence of ethanol or calcofluor white (CFW). Each strain was grown to log phase in YPD broth and serially diluted 10-fold from an initial OD_{600} of 1.0. Aliquots (3 μl) were spotted onto a YPD agar plate containing 12% (vol/vol) ethanol or 100 μg/ml CFW and incubated at 30°C for 3 days. (B) Susceptibility to Zymolyase of the wild-type strain after exposure to ethanol or CFW. Log-phase wild-type (BY4742) cells were incubated in YPD medium containing 0 to 10% ethanol or 0 to 100 μg/ml CFW at 30°C for 12 h. Cells were harvested and adjusted to an OD_{600} of 0.5 in TE buffer containing 100 μg/ml (1 U/ml) Zymolyase 20T. Susceptibility to Zymolyase was monitored by measuring the OD_{600} at the indicated times and is expressed as a percentage of the OD_{600} relative to that at the zero time point. Mean values ± SD are from three independent experiments.

nents of the CWI pathway on yeast extract-peptone-dextrose (YPD) plates containing 12% ethanol or 100 μg/ml of the cell wall-perturbing agent calcofluor white (CFW) by using a spot susceptibility test. We found that the Δ*bck1* and Δ*slt2* mutants lacking the core and nonredundant elements of the CWI pathway, i.e., MAPKKK and MAPK, respectively, were sensitive to both ethanol and CFW (Fig. 2A). Interestingly, for the mutants lacking components of the heterodimeric SBF transcription complex (Swi4p-Swi6p), which is the downstream target of the Slt2p MAPK, the Δ*swi6* mutant was severely sensitive to both ethanol and CFW, while the Δ*swi4* mutant was highly sensitive to only CFW (Fig. 2A). In addition, the Δ*wsc1* and Δ*spa2* mutants lacking the sensor and MAPK scaffold protein of the CWI pathway, respectively, also exhibited remarkable sensitivity to only CFW (Fig. 2A). These results suggest that although the MAPK module of the CWI pathway is involved in the response to ethanol stress, the ethanol-responsive signaling cascade through the CWI pathway is somewhat different from the typical CWI pathway responding to cell wall stress induced by CFW.

In response to ethanol challenge, previous studies reported that yeast cells showed increased resistance to cell wall-lytic enzymes, which suggests adaptive remodeling of the yeast cell wall (3, 6). We thus determined susceptibilities of wild-type cells to the cell wall-degrading enzyme Zymolyase, whose major activities are β-1,3-glucanase and β-1,3-glucan laminaripentaohydrolase, after treatment with ethanol or CFW. We found that the Zymolyase resistance of ethanol-treated cells was increased with increasing

concentrations of ethanol, similar to cells treated with CFW (Fig. 2B). Based on these results, it is likely that the CWI pathway is important for protecting yeast cells against ethanol stress, possibly through its role in the induction of cell wall remodeling in response to ethanol-induced cell wall stress.

Reinvestigation of cell wall and ethanol stress-responsive genes. To investigate the links between cell wall and ethanol stress-responsive pathways, we globally explored the repertoires of the two pathways in the yeast genome by reexamining existing transcriptomic data sets (see Materials and Methods for more details). Our reexamined transcriptomic data set reveals unique patterns of up- and downregulated transcripts under different cell wall and ethanol stress conditions (Fig. 3). Among the 14 groups of genes hierarchically clustered based on their transcriptomic patterns, we selected 5 groups that show interesting gene expression patterns under cell wall and ethanol stress conditions. Group 2 contains genes that exhibit increased transcript levels when treated with the cell wall-disturbing agent Congo red (for 2, 4, and 6 h), Zymolyase (for 2 h), and CFW (for 1.5 h) and under fermentation conditions but not under the other ethanol (3 to 7%) stress conditions (see Fig. S1 in the supplemental material). Key members of this group include the *KRE6* gene encoding β-1,6-glucan synthase, the *PIR1* gene encoding an O-glycosylated cell wall protein, the *SED1* gene encoding an glycosylphosphatidylinositol (GPI)-anchored cell wall glycoprotein, the *CHS3* gene encoding chitin synthase III, and the *SHO1* gene encoding a transmembrane osmosensor for the HOG pathway. When tested for functional enrichment using Gene Ontology (GO), the genes in this group are enriched for cell wall organization and biogenesis and carbohydrate metabolic processes. Group 14 is a small set of only 11 genes demonstrating strong upregulation (2- to 8-fold) after Congo red, Zymolyase, and ethanol treatments but not under CFW stress or fermentation conditions (Fig. S1). The group members are also enriched in cell wall organization and biogenesis, including the *FKS2* (or *GSC2*) gene encoding β-1,3-glucan synthase, the *CWP1* gene encoding a cell wall mannoprotein, and the *PIR3* gene encoding an O-glycosylated cell wall protein. In addition to the clusters of gene expression upregulated by both cell wall and ethanol stresses, we also observed group 3 genes that appear to be specifically upregulated by CFW treatment only, such as the *GPD1* and *HOG1* genes involved in the osmotic stress response (Fig. S1). Group 10 includes genes showing elevated transcription only after treatment with Congo red and ethanol (Fig. S1). A notable member of this group is the *RPI1* gene encoding a transcription factor mediating fermentation stress tolerance, which is also involved in cell wall organization and biogenesis. Group 11 genes are upregulated when treated with Congo red for 6 h, CFW for 1.5 h, and 15% ethanol for 2 h (Fig. S1). Examples of its members are the *BCK1* and *PTP2* genes encoding MAPKKK of the CWI pathway and a protein phosphatase involved in osmosensing, respectively. Based on this systematic transcriptome analysis, we selected seven notable cell wall biosynthesis genes whose expression is potentially upregulated in response to ethanol-induced cell wall stress under the control of the CWI pathway for further investigation. These included the *KRE6*, *PIR1*, *SED1*, and *CHS3* genes from group 2 and the *FKS2*, *CWP1*, and *PIR3* genes from group 14.

The CWI pathway is involved in regulating the expression of a set of cell wall biosynthesis genes in response to ethanol-induced cell wall stress. To investigate whether the candidate cell wall biosynthesis genes obtained from the systemic transcriptome analysis are the target genes of the response to ethanol-induced cell wall stress, we examined the expression levels of the *FKS2*, *CWP1*, and *PIR3* genes (group 14); the *KRE6*, *PIR1*, *SED1*, and *CHS3* genes (group 2); and also the *CRH1* gene encoding chitin transglycosylase, which is selected based on the literature (9), in the wild-type strain after exposure to 8% ethanol or 100 μg/ml CFW for 4 h. The expression of the *FKS2*, *CRH1*, *PIR3*, and *SED1* genes was markedly upregulated in response to both ethanol and CFW, whereas the upregulation of *KRE6* expression was observed only after exposure to CFW but not ethanol (Fig. 4A). These results suggest that the *FKS2*, *CRH1*, *PIR3*, and *SED1* genes are target genes of the response to ethanol stress. To determine whether the

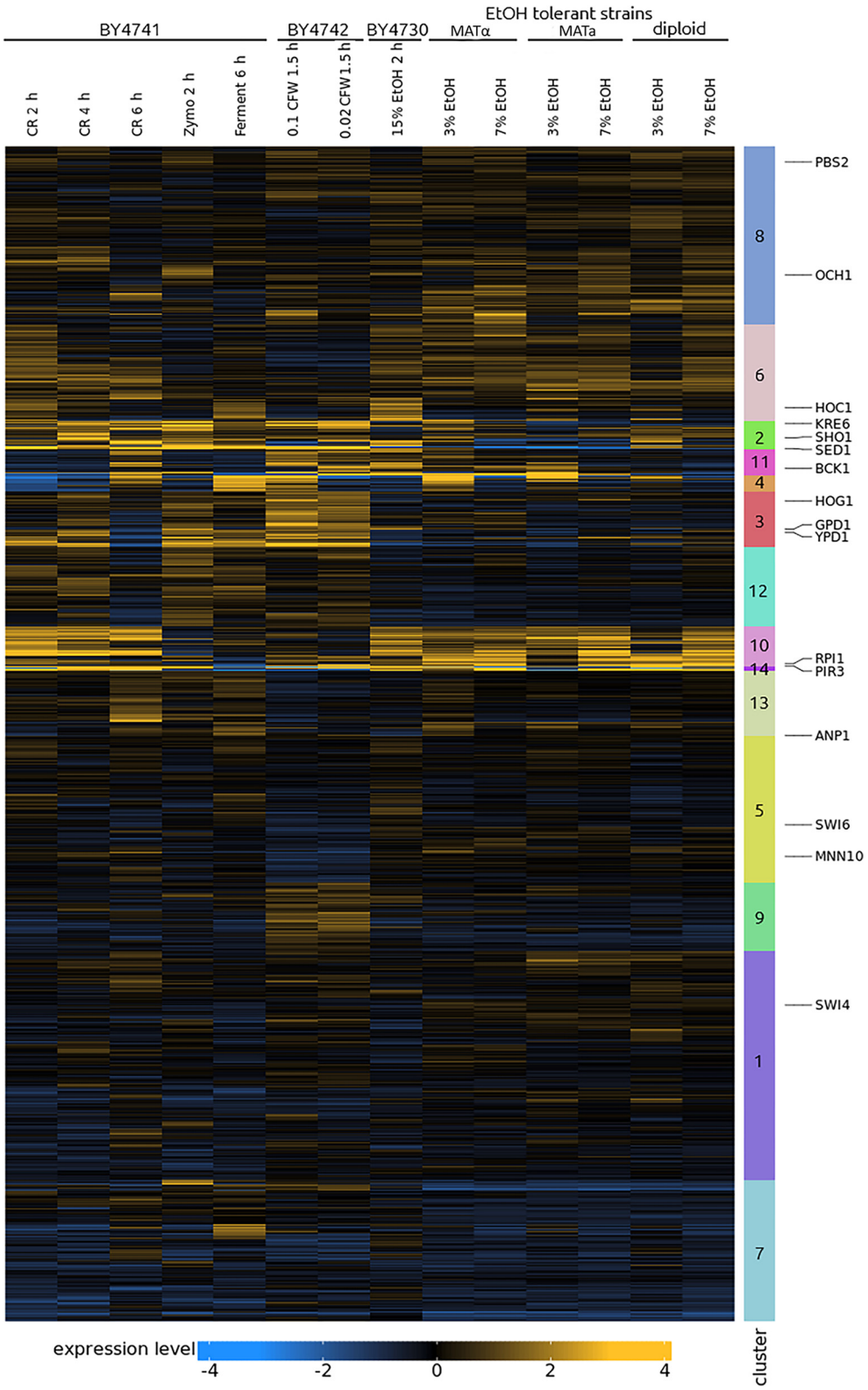

FIG 3 Heat map demonstrating relative transcriptional levels during cell wall and ethanol stresses. The conditions are, from left to right, BY4741 treated with Congo red (CR) for 2, 4, and 6 h; BY4741 treated with Zymolyase (Zymo) for 2 h; BY4741 under fermentation conditions (ferment) for 6 h; BY4742 treated with 0.1 and 0.02 mg/ml CFW for 1.5 h; BY4730 treated with 15% ethanol (EtOH) for 2 h; and ethanol-tolerant CGMCC2758 haploid *MAT*α, haploid *MAT***a**, and diploid *MAT***a**/α strains treated with 3% or 7% ethanol in fermentors. Data were normalized within and between arrays (see Materials and Methods for more details).

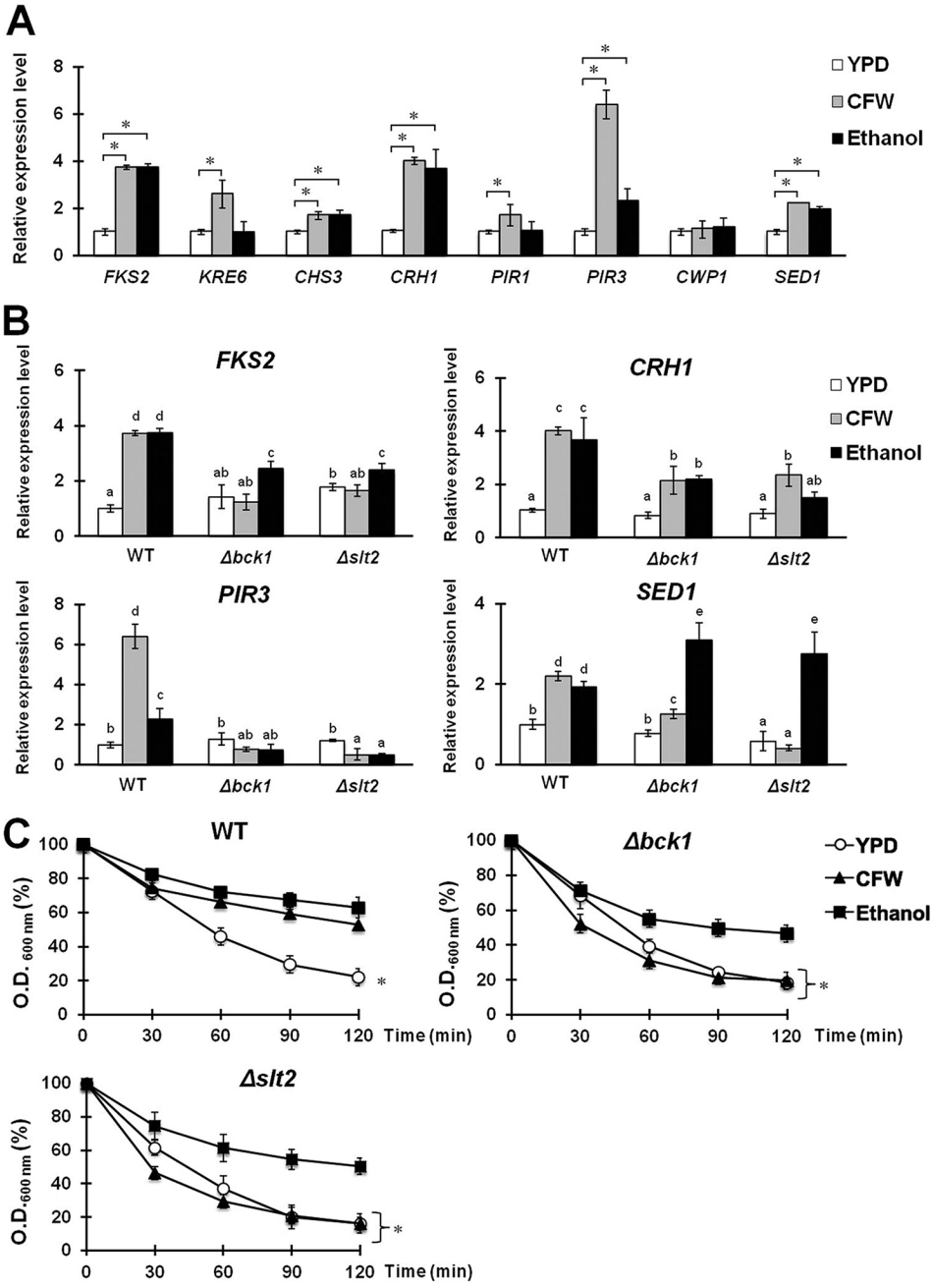

FIG 4 Bck1p MAPKKK and Slt2p MAPK regulate the expression of specific cell wall biosynthesis genes in response to ethanol stress, leading to the induction of cell wall remodeling. (A) Expression levels of cell wall biosynthesis genes (i.e., the *FKS2*, *KRE6*, *CHS3*, *CRH1*, *PIR1*, *PIR3*, *CWP1*, and *SED1* genes) of the wild-type (BY4742) strain after exposure to ethanol or CFW. Log-phase wild-type (BY4742) cells were treated with 8% ethanol or 100 μg/ml CFW for 4 h. Total RNA from each culture was used for quantitative RT-PCR. The mRNA levels of the *FKS2*, *KRE6*, *CHS3*, *CRH1*, *PIR1*, *PIR3*, *CWP1*, and *SED1* genes were normalized to that of the *ACT1* gene in the same sample. Mean values ± SD are from three independent experiments. *, $P < 0.05$. (B) Expression levels of the *FKS2*, *CRH1*, *PIR3*, and *SED1* genes of the Δ*mkk2* and Δ*slt2* mutants after treatment with 8% ethanol or 100 μg/ml CFW for 4 h. Mean values ± SD are from three independent experiments. Values with different superscript letters indicate statistically significant differences at a *P* value of <0.05. (C) Susceptibility to Zymolyase of the Δ*mkk2* and Δ*slt2* mutants after exposure to ethanol or CFW. Δ*mkk2* and Δ*slt2* cells were incubated in YPD medium containing 8% ethanol or 100 μg/ml CFW for 12 h. Susceptibility to Zymolyase was monitored by measuring the OD_{600} at the indicated times and is expressed as a percentage of the OD_{600} relative to that at the zero time point. Mean values ± SD are from three independent experiments. *, $P < 0.05$. WT, wild type.

expression of these four cell wall biosynthesis genes, as a response mechanism to ethanol challenge, is under the regulation of the CWI pathway, we monitored the expression levels of the *FKS2*, *CRH1*, *PIR3*, and *SED1* genes in the Δ*bck1* and Δ*slt2* mutants with impaired CWI MAPK signaling after exposure to 8% ethanol or 100 μg/ml CFW for 4 h. As expected, in contrast to the wild-type strain, the Δ*bck1* and Δ*slt2* mutants were unable to fully induce the expression of these four cell wall biosynthesis genes during exposure to the cell wall-perturbing agent CFW (Fig. 4B). On the other hand, after ethanol treatment, the expression levels of the *FKS2*, *CRH1*, and *PIR3* genes (but not the *SED1* gene) in the Δ*bck1* and Δ*slt2* mutants were significantly lower than those in the wild-type strain (Fig. 4B). Based on these observations, it appears that the CWI pathway is important for protecting yeast cells against ethanol-induced cell wall stress through upregulating the expression of some cell wall biosynthesis genes such as *FKS2*, *CRH1*, and *PIR3*.

Since the activation of the CWI pathway eventually leads to remodeling of the cell wall architecture to strengthen its structure (16, 17), an impaired CWI pathway is then supposed to hinder cell wall remodeling, thereby resulting in high susceptibility to cell wall-degrading enzymes, including Zymolyase. We then investigated whether ethanol-induced cell wall remodeling is regulated through the CWI pathway by monitoring the Zymolyase susceptibility of the Δ*bck1* and Δ*slt2* mutants after exposure to 8% ethanol or 100 μg/ml CFW for 12 h. Consistent with this idea, the Zymolyase resistance of the Δ*bck1* and Δ*slt2* mutants, unlike that of the wild-type strain, was not increased after CFW treatment (Fig. 4C). Interestingly, in contrast to CFW treatment, the Δ*bck1* and Δ*slt2* mutants exhibited increased Zymolyase resistance after ethanol treatment (Fig. 4C), suggesting their ability to induce cell wall remodeling in response to ethanol. In accordance with these findings, a slight upregulation of the expression of the *FKS2* and *CRH1* genes, which are target genes of the CWI pathway, was detected in the Δ*bck1* and Δ*slt2* mutants after ethanol exposure (Fig. 4B). Taken together, these data show that it is likely that, in addition to the CWI pathway, other signaling pathways are also involved in the cooperative induction of cell wall remodeling in response to ethanol stress.

The CWI and HOG pathways are involved in controlling cell wall remodeling in response to ethanol stress through their cooperative role in the upregulation of specific cell wall biosynthesis genes. It was previously shown that both the CWI and HOG pathways coordinately regulate gene expression in response to Zymolyase-mediated cell wall damage (11). It is therefore possible that both pathways may be involved in controlling the expression of some cell wall biosynthesis genes in response to ethanol-induced cell wall stress. The HOG pathway contains two signaling branches, i.e., SLN1 and SHO1, which are known to function independently (14). We thus examined the growth of the Δ*sho1*, Δ*ssk1*, and Δ*hog1* mutants lacking the osmosensor of the SHO1 branch, the signal transducer of the SLN1 branch, and the MAPK of the HOG pathway, respectively, on YPD plates containing ethanol or CFW. In contrast to the Δ*bck1* and Δ*slt2* mutants with the impaired CWI pathway, the Δ*sho1*, Δ*ssk1*, and Δ*hog1* mutants were not hypersensitive to either ethanol or CFW (Fig. 5A). In addition, after treatment with CFW or ethanol, Zymolyase resistance of the Δ*sho1*, Δ*ssk1*, and Δ*hog1* mutants was at the same level as that of the wild-type strain (Fig. 5B). Although dysfunction of the HOG pathway had no effect on growth and cell wall remodeling under cell wall stress conditions, the level of expression of the *PIR3* gene in the Δ*sho1*, Δ*ssk1*, and Δ*hog1* mutants treated with CFW or ethanol was strikingly lower than that in the wild-type strain, while significant inhibition of *FKS2* and *CRH1* gene expression was also observed in the ethanol-treated Δ*hog1* mutants (Fig. 6A to C). These results suggest the involvement of the HOG pathway in regulating the transcriptional response to ethanol, possibly ethanol-induced cell wall stress.

To test the role of the CWI and HOG pathways in cooperative signaling to regulate the adaptive response against cell wall stress, we first examined the growth of double mutants lacking the components of both pathways (i.e., the Δ*bck1* Δ*sho1*, Δ*bck1* Δ*ssk1*, Δ*bck1* Δ*hog1*, Δ*slt2* Δ*sho1*, Δ*slt2* Δ*ssk1*, and Δ*slt2* Δ*hog1* mutants) on YPD agar plates

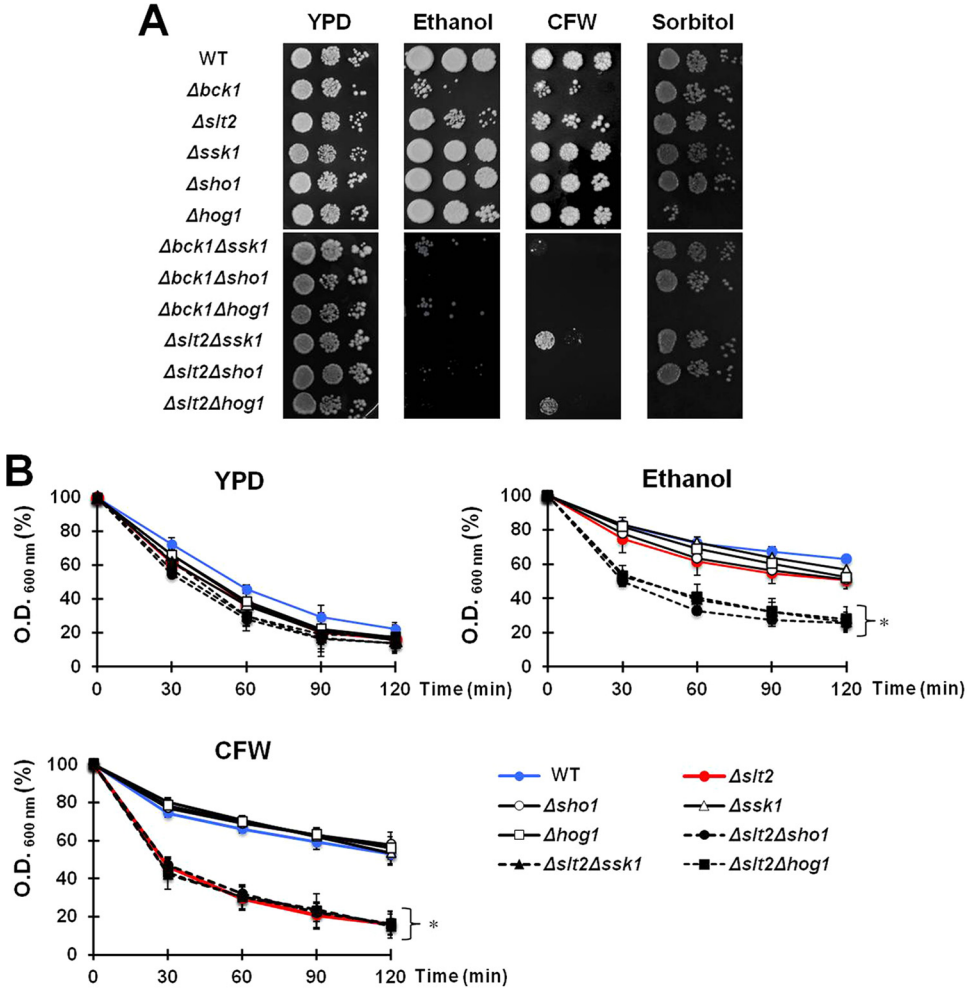

FIG 5 Both the CWI and HOG pathways are required for ethanol tolerance through their cooperative role in regulating cell wall remodeling in response to ethanol stress. (A) Growth of the mutants defective in either the CWI pathway, the HOG pathway, or both pathways in the presence of ethanol, CFW, or sorbitol. Wild-type (BY4742), Δ*bck1*, Δ*slt2*, Δ*ssk1*, Δ*sho1*, Δ*hog1*, Δ*bck1* Δ*ssk1*, Δ*bck1* Δ*sho1*, Δ*bck1* Δ*hog1*, Δ*slt2* Δ*ssk1*, Δ*slt2* Δ*sho1*, and Δ*slt2* Δ*hog1* cells were serially diluted and spotted onto a YPD agar plate containing 10% ethanol, 20 μg/ml CFW, or 1.5 M sorbitol. Plates were incubated at 30°C for 3 days. (B) Susceptibility to Zymolyase of the wild-type (BY4742), Δ*slt2*, Δ*sho1*, Δ*ssk1*, Δ*hog1*, Δ*slt2* Δ*sho1*, Δ*slt2* Δ*ssk1*, and Δ*slt2* Δ*hog1* strains after exposure to ethanol or CFW. Log-phase cells of each strain were incubated in YPD medium containing 8% ethanol or 100 μg/ml CFW at 30°C for 12 h. Cells were harvested and adjusted to an OD_{600} of 0.5 in TE buffer containing 100 μg/ml (1 U/ml) Zymolyase 20T. Susceptibility to Zymolyase was monitored by measuring the OD_{600} at the indicated times and is expressed as a percentage of the OD_{600} relative to that of the zero time point. Mean values ± SD are from three independent experiments. *, $P < 0.05$.

supplemented with CFW or ethanol. We found that these double-deletion mutants, compared with their parental single-deletion strains, displayed high sensitivities to both CFW and ethanol (Fig. 5A). Furthermore, when the *SHO1*, *SSK1*, or *HOG1* gene was further deleted in the Δ*bck1* and Δ*slt2* mutants, the increase of Zymolyase resistance, which was detected in the ethanol-treated Δ*bck1* and Δ*slt2* mutants, was significantly inhibited (Fig. 5B and Fig. S2). These results suggest that the CWI and HOG pathways are required for protecting yeast cells against ethanol-induced cell wall stress through their cooperative role in inducing cell wall remodeling.

To determine the role of the CWI and HOG signaling pathways in the coordinated transcriptional regulation of cell wall biosynthesis genes during cell wall stress, we monitored *FKS2*, *CRH1*, and *PIR3* mRNA levels in the double-deletion mutants defective in both the CWI and HOG pathways (i.e., the Δ*bck1* Δ*sho1*, Δ*bck1* Δ*ssk1*, Δ*bck1* Δ*hog1*, Δ*slt2* Δ*sho1*, Δ*slt2* Δ*ssk1*, and Δ*slt2* Δ*hog1* mutants) after treatment with CFW or ethanol.

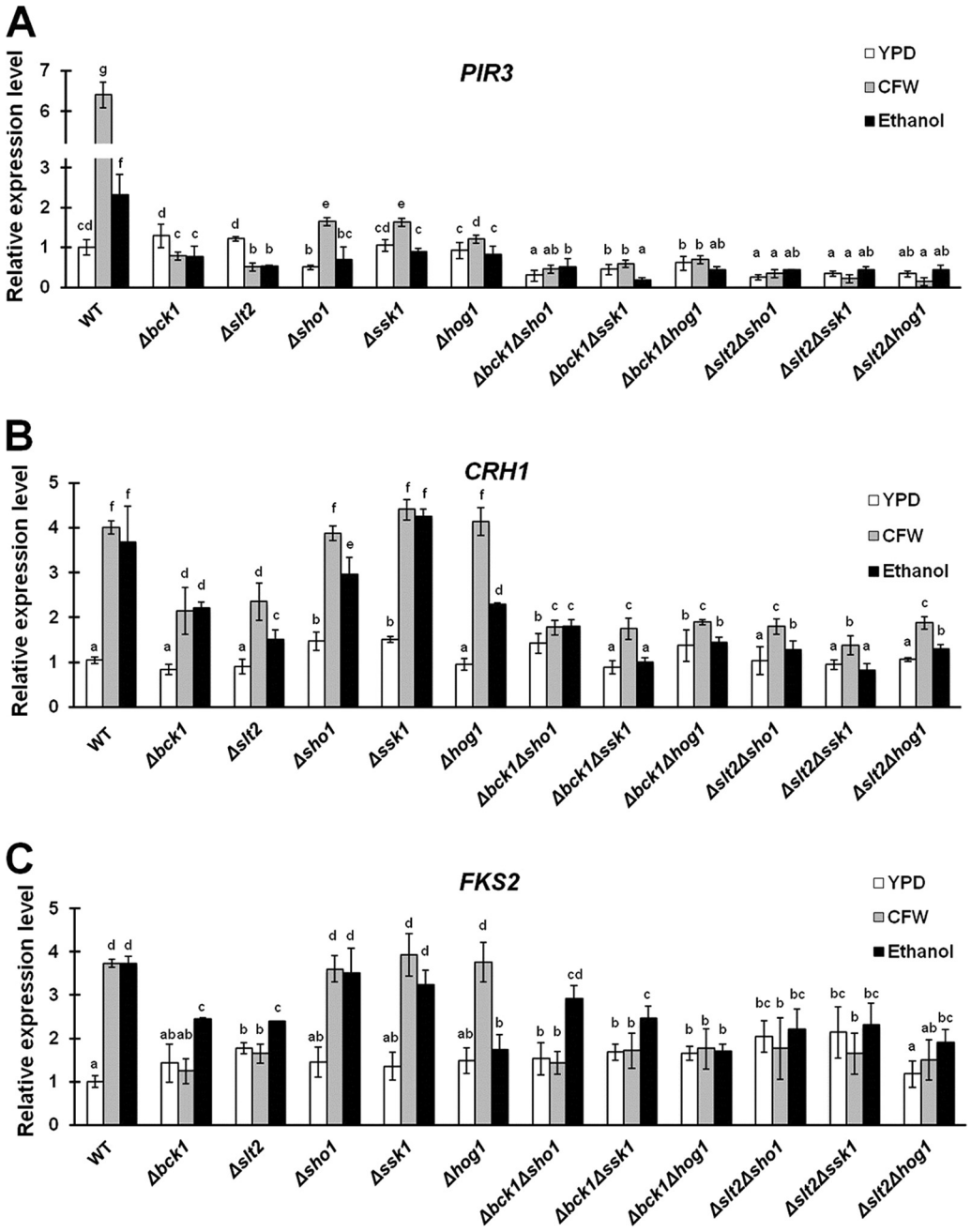

FIG 6 Both the CWI and HOG pathways are involved in the coordinated transcriptional regulation of specific cell wall biosynthesis genes in response to ethanol stress. Log-phase wild-type (BY4742), Δ*bck1*, Δ*slt2*, Δ*sho1*, Δ*ssk1*, Δ*hog1*, Δ*bck1* Δ*sho1*, Δ*bck1* Δ*ssk1*, Δ*bck1* Δ*hog1*, Δ*slt2* Δ*sho1*, Δ*slt2* Δ*ssk1*, and Δ*slt2* Δ*hog1* cells were treated with 8% ethanol or 100 μg/ml CFW for 4 h. Total RNA from each culture was used for quantitative RT-PCR. The mRNA levels of the *PIR3* (A), *CRH1* (B), and *FKS2* (C) genes were normalized to that of the *ACT1* gene in the same sample. Mean values ± SD are from three independent experiments. Values with different superscript letters indicate statistically significant differences at a *P* value of <0.05.

Compared to the expression levels of cell wall biosynthesis genes in the mutants impaired in the CWI pathway only (i.e., Δ*bck1* or Δ*slt2* mutant), *CRH1* mRNA levels in the mutants defective in both the CWI and HOG pathways were further decreased after treatment with CFW and ethanol (Fig. 6B). On the other hand, although the deletion of the *SHO1*, *SSK1*, or *HOG1* gene in the Δ*bck1* and Δ*slt2* mutants caused further significant reductions in *PIR3* expression under nonstress conditions, the *PIR3* expression level in these double-deletion mutants treated with CFW or ethanol was only slightly lower

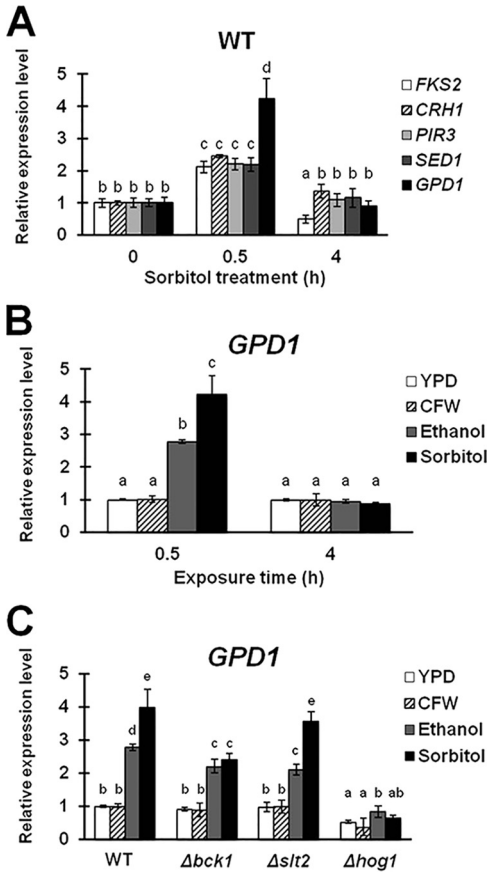

FIG 7 The expression of CWI-responsive genes is slightly induced by osmotic stress, but the expression of HOG-responsive gene is not induced by cell wall stress. (A) Expression levels of the *FKS2*, *CRH1*, *PIR3*, *SED1*, and *GPD1* genes of the wild-type (BY4742) strain after treatment with 1.5 M sorbitol for 0.5 and 4 h. (B) Expression levels of the *GPD1* gene of the wild-type (BY4742) strain after treatment with 100 μg/ml CFW, 8% ethanol, or 1.5 M sorbitol for 0.5 and 4 h. (C) Expression levels of the *GPD1* gene of the wild-type (BY4742), Δ*bck1*, Δ*slt2*, and Δ*hog1* strains after treatment with 100 μg/ml CFW, 8% ethanol, or 1.5 M sorbitol for 0.5 h. The mRNA levels of these genes were normalized to that of *ACT1* in the same sample. Mean values ± SD are from three independent experiments. Values with different superscript letters indicate statistically significant differences at a *P* value of <0.05.

than that in their parental single-deletion strains (i.e., the Δ*bck1* and Δ*slt2* mutants) (Fig. 6A). This may be due to the fact that *PIR3* expression in these double-deletion mutants was almost completely blocked, even under nonstress conditions. In contrast to *CRH1* and *PIR3* expression, the *FKS2* expression levels in the double-deletion mutants defective in both the CWI and HOG pathways were not further decreased from the levels in the single-deletion strains defective in only the CWI pathway under all conditions tested (Fig. 6C). These results suggest that both the CWI and HOG pathways cooperate in regulating the transcription of certain cell wall biosynthesis genes, especially the *CRH1* gene, in response to CFW- and ethanol-induced cell wall stress.

Since both the CWI and HOG signaling pathways were involved in upregulating certain cell wall biosynthesis genes during cell wall stress, it is possible that the expression of these cell wall biosynthesis genes may also be triggered by external hyperosmolarity. To test this possibility, we monitored the expression levels of the *FKS2*, *CRH1*, *PIR3*, and *SED1* genes in the wild-type strain after treatment with 1.5 M sorbitol for 4 h. However, the expression of these cell wall biosynthesis genes was not increased by a 4-h exposure to osmotic stress (Fig. 7A). Since it has been shown that the HOG pathway rapidly induces a transient upregulation of osmotic-stress-responsive genes, such as the *GPD1* gene encoding glycerol-3-phosphate dehydrogenase, within 30 min upon exposure to external hyperosmolarity, we then measured the expression levels of

these cell wall biosynthesis genes after treatment with sorbitol for 30 min. As expected, *GPD1* expression was strikingly upregulated upon short-term exposure to sorbitol (Fig. 7A). In addition to the osmotic-stress-responsive *GPD1* gene, the transcript levels of these four cell wall biosynthesis genes were slightly increased (approximately 2-fold) upon sorbitol treatment (Fig. 7A). These findings therefore suggest the effect of external hyperosmolarity on inducing a slight upregulation of the expression of some cell wall biosynthesis genes, including those that are targets of the CWI pathway.

Upregulation of *GPD1* gene expression upon ethanol challenge is induced by ethanol-mediated water stress. To investigate whether the activation of the CWI pathway by cell wall stress can induce the expression of target genes of the HOG pathway, we determined the expression levels of the *GPD1* gene in the wild-type strain treated with CFW or ethanol for 30 min and 4 h. Our results revealed that *GPD1* expression was remarkably induced only after a 30-min exposure to 8% ethanol, similar to its expression pattern in response to sorbitol-induced hyperosmotic stress (Fig. 7B). Since a considerable amount of ethanol, 8% (vol/vol), was supplemented into the medium, it is possible that increased *GPD1* expression levels after short-term ethanol treatment may result from reduced water activity caused by high concentrations of ethanol. To test this possibility, we measured *GPD1* transcriptional levels in the Δ*bck1*, Δ*slt2*, and Δ*hog1* mutants treated with ethanol for 30 min. Inhibition of *GPD1* transcription upon ethanol exposure was observed only in the Δ*hog1* mutant with an impaired HOG pathway but not in the Δ*bck1* and Δ*slt2* mutants lacking the functional CWI pathway (Fig. 7C). These results further suggest that the upregulation of *GPD1* gene expression upon ethanol exposure is a consequence of HOG pathway-mediated osmoadaptation in response to ethanol-induced water stress rather than ethanol-induced cell wall stress. In conclusion, our results suggest the cooperative role of both the CWI and HOG pathways in regulating the expression of certain cell wall biosynthesis genes in response to cell wall stress, in particular ethanol-induced cell wall stress.

DISCUSSION

In this study, we demonstrate that the CWI and HOG pathways play a cooperative role in response to ethanol-induced cell wall stress, resulting in the upregulation of the expression of specific cell wall biosynthesis genes such as the *CRH1* gene encoding chitin transglycosylase. This transcriptional activation induces cell wall remodeling, which strengthens the cell wall structure against ethanol-induced cell wall stress. So far, it is still unknown how ethanol disturbs the yeast cell wall structure. Although direct binding of ethanol to any cell wall components, such as glucan, chitin, and mannoprotein, has not been reported, ethanol has been shown to directly bind to protein molecules by forming hydrogen bonds and hydrophobic interactions with amino acid residues, which in turn leads to a disruption of the protein structure (18, 19). Based on this concept, it is possible that cell wall proteins, especially glycosylated mannoproteins, the major component of the outer cell wall layer, may be a target for ethanol. If this is the case, direct binding of ethanol to cell wall proteins may disturb the cell wall structure and cause cell wall stress. In support of this notion, we found that the expression levels of the *PIR3* and *SED1* genes encoding cell wall glycoproteins were upregulated after ethanol exposure, possibly to compensate for dysfunctional cell wall proteins caused by ethanol stress. Consistent with this idea, the expression of the *SPI1*, *TIP1*, *TIR1*, *TIR2*, and *TIR3* genes encoding cell wall mannoproteins has been reported to be upregulated during ethanol stress and/or ethanol fermentation (20–22). Furthermore, a number of genes involved in the biosynthesis of cell wall proteins, such as the *SPI1* gene encoding a GPI-anchored cell wall protein; the *ANP1* gene encoding subunit of the alpha-1,6-mannosyltransferase complex; and the *HOC1*, *MNN10*, *MNN11*, and *OCH1* genes encoding subunits of a Golgi mannosyltransferase complex, have been shown to be important for ethanol tolerance (5, 6, 15). Nevertheless, since increased expression levels of the *CRH1* gene encoding chitin transglycosylase and the *FKS2* gene encoding β-1,3-glucan synthase were also observed after ethanol treatment, the possibility that chitin and glucan of the cell wall are also the targets of ethanol cannot be ruled out.

Although we found that the core and nonredundant elements of the CWI pathway, such as Bck1p (MAPKKK) and Slt2p (MAPK), are important for tolerating both ethanol and CFW, some components of this pathway (i.e., the Wsc1p sensor, the Spa2p scaffold protein, and the Swi4p transcription cofactor) are required for tolerance to CFW only. Our findings therefore suggest that the CWI pathway plays an important role in transducing ethanol-induced cell wall stress signals. However, the signaling elements involved in adaptive responses to cell wall stresses induced by ethanol and CFW are somewhat different. Previously, deletome analysis for the identification of genes required for tolerance to three compounds that interfere with the yeast cell wall by different mechanisms, i.e., Congo red (chitin-binding dye), Zymolyase (an enzymatic cocktail with a predominant β-1,3-glucanase activity), and caspofungin (an inhibitor of β-1,3-glucan synthase), was performed (23). In agreement with our results, the deletion of genes encoding key elements of the CWI pathway, i.e., *BCK1* and *SLT2*, induced hypersensitivity to these three cell wall-interfering compounds, whereas single-deletion mutants of genes with redundant function, such as the Δ*mkk1* and Δ*mkk2* mutants lacking only one of the two redundant MAPKKs, exhibited no apparent sensitivities to Congo red and Zymolyase. In addition, it was also shown that the loss of only one sensor or transcription factor of the CWI pathway resulted in sensitivity to only some cell wall-interfering compounds. For instance, in the case of mutants lacking cell wall stress sensors, the Δ*wsc1* mutant was highly sensitive to only Congo red and slightly sensitive to Zymolyase, while the Δ*mid2* mutant was subtly sensitive to only caspofungin. Similarly, regarding the transcription factors of the CWI pathway, deletion of either the *SWI4* or *SWI6* gene caused hypersensitivity to Congo red and caspofungin but had no effect on Zymolyase sensitivity, whereas *RLM1* deletion slightly induced sensitivity to only caspofungin. In addition, the Δ*spa2* mutant was slightly sensitive to caspofungin only. Based on these observations, it is likely that in response to different types of cell wall stress, signal transduction via the CWI pathway is mediated through distinct signaling components, especially a specific sensor(s) and/or transcription factor(s). Further studies are necessary to elucidate the precise mechanisms of CWI pathway-mediated signal transduction in response to ethanol-induced cell wall stress.

Here, although deletion of the *SWI4* gene encoding the DNA-binding component of the SBF complex did not induce ethanol hypersensitivity, the Δ*swi6* mutant lacking the transcriptional activator of the SBF complex was severely sensitive to ethanol. This may be due to the fact that Swi6p can complex with two DNA-binding components, i.e., Swi4p and Mbp1p, to form the redundant SBF and MBF (MluI cell cycle box-binding factor) complexes, respectively, which coordinately regulate the transcription of late-G_1-specific genes, such as genes encoding cyclins and genes required for DNA synthesis and repair (24). Therefore, the loss of SBF activity in the Δ*swi4* mutant may be compensated for by MBF activity to control the expression of their targets. In agreement with this hypothesis, the Δ*mbp1* mutant was insensitive to ethanol (data not shown). If this is the case, some G_1-specific cyclins and/or proteins involved in DNA synthesis and repair, whose expression is under the control of the SBF and/or MBF complex, may play an important role in protection against ethanol stress. However, among the target genes of the SBF and/or MBF complex, only the *RNR4* gene, encoding one of the two DNA damage-inducible small subunits of the ribonucleotide reductase (RNR) complex, was found to be required for ethanol tolerance (15). Thus, the possible role of the SBF and/or MBF complex during ethanol stress warrants further exploration.

In response to ethanol-induced cell wall stress, we show here that the CWI and HOG pathways cooperate in regulating the transcription of specific cell wall biosynthesis genes such as the *CRH1* gene. Although the CWI and HOG pathways have been reported to also play a cooperative role in regulating cellular adaptation to Zymolyase-mediated cell wall damage (9, 11), the Zymolyase-responsive signaling mechanism coordinated by these two pathways is slightly distinct from that for the ethanol stress response. For instance, only the SHO1 branch of the HOG pathway is involved in the response to Zymolyase, whereas adaptation to ethanol-induced cell wall stress seems to require both the SHO1 and SLN1 branches. Furthermore, it is likely that concentra-

TABLE 1 Yeast strains used in this study

Strain	Relevant genotype and/or description	Source or reference
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 uraΔ0*	Open Biosystems
Δ*wsc1*	BY4742 isogenic; *wsc1*::*KanMX4*	Open Biosystems
Δ*wsc2*	BY4742 isogenic; *wsc2*::*KanMX4*	Open Biosystems
Δ*wsc3*	BY4742 isogenic; *wsc3*::*KanMX4*	Open Biosystems
Δ*mid2*	BY4742 isogenic; *mid2*::*KanMX4*	Open Biosystems
Δ*rom2*	BY4742 isogenic; *rom2*::*KanMX4*	Open Biosystems
Δ*bck1*	BY4742 isogenic; *bck1*::*KanMX4*	Open Biosystems
Δ*mkk1*	BY4742 isogenic; *mkk1*::*KanMX4*	Open Biosystems
Δ*mkk2*	BY4742 isogenic; *mkk2*::*KanMX4*	Open Biosystems
Δ*slt2*	BY4742 isogenic, *slt2*::*KanMX4*	Open Biosystems
Δ*spa2*	BY4742 isogenic; *spa2*::*KanMX4*	Open Biosystems
Δ*rlm1*	BY4742 isogenic; *rlm1*::*KanMX4*	Open Biosystems
Δ*skn7*	BY4742 isogenic; *skn7*::*KanMX4*	Open Biosystems
Δ*gas1*	BY4742 isogenic; *gas1*::*KanMX4*	Open Biosystems
Δ*swi4*	BY4742 isogenic; *swi4*::*KanMX4*	Open Biosystems
Δ*swi6*	BY4742 isogenic; *swi6*::*KanMX4*	Open Biosystems
Δ*sho1*	BY4742 isogenic; *sho1*::*KanMX4*	Open Biosystems
Δ*ssk1*	BY4742 isogenic; *ssk1*::*KanMX4*	Open Biosystems
Δ*hog1*	BY4742 isogenic; *hog1*::*KanMX4*	Open Biosystems
Δ*bck1* Δ*sho1*	BY4742 isogenic; *bck1*::*KanMX4 sho1*::*CgHIS3*	This study
Δ*bck1* Δ*ssk1*	BY4742 isogenic; *bck1*::*KanMX4 ssk1*::*CgHIS3*	This study
Δ*bck1* Δ*hog1*	BY4742 isogenic; *bck1*::*KanMX4 hog1*::*CgHIS3*	This study
Δ*slt2* Δ*sho1*	BY4742 isogenic; *slt2*::*KanMX4 sho1*::*CgHIS3*	This study
Δ*slt2* Δ*ssk1*	BY4742 isogenic; *slt2*::*KanMX4 ssk1*::*CgHIS3*	This study
Δ*slt2* Δ*hog1*	BY4742 isogenic; *slt2*::*KanMX4 hog1*::*CgHIS3*	This study

tions of ethanol used in this study were relatively high and sufficient to induce water stress, leading to the rapid activation of the HOG pathway to upregulate the expression of several osmoresponsive genes such as the *GPD1* gene. In general, an increase in extracellular fluid osmolarity will cause water efflux and, hence, cell shrinkage (14, 25). In addition to the possible effect of ethanol on inducing water stress, ethanol is well known to disturb cellular membranes, especially the plasma membrane, which in turn causes increases in membrane permeability to ions and leakage of metabolites (2). On the other hand, all sensors of the CWI pathway are plasma membrane-spanning proteins that share a similar architecture. Each sensor contains a highly mannosylated serine/threonine-rich (STR) region extending into the cell wall, a single transmembrane domain (TMD), and a cytoplasmic C-terminal tail (26). The STR region of these sensors is thought to have nanospring properties that stretch if either cell wall polysaccharides or membrane lipids are dislocated by external stress (27). Therefore, sensors of the CWI pathway have been suggested to act as mechanosensors to detect mechanical changes in the cell wall and/or the plasma membrane through their STR regions, which in turn transmit the stress signal to the cytoplasmic tail of the sensors to activate downstream signaling components of the CWI pathway. Based on these notions, it is possible that any conformational changes in either the cell wall or the plasma membrane may be detected by these CWI sensors. Consistent with this idea, we found that the expression of several target genes of the CWI pathway, including the *FKS2*, *CRH1*, *PIR3*, and *SED1* genes, was also upregulated in response to sorbitol-mediated osmotic stress. On the contrary, cell wall stress caused by CFW had no effect on the expression of targets of the HOG pathway such as the *GPD1* gene, suggesting that the plasma membrane-localized HOG sensors are not responsible for detecting cell wall stress. However, it remains unclear whether, in response to ethanol stress, the CWI pathway is activated by the cell wall-damaging effect and/or the plasma membrane-disturbing effect of ethanol. Further research is required to clarify the precise mechanism of the ethanol stress response mediated by the CWI and HOG pathways.

MATERIALS AND METHODS

Yeast strains and growth conditions. *S. cerevisiae* strains used in this study are listed in Table 1. The double-deletion strains were constructed by replacing the *SSK1*, *SHO1*, or *HOG1* gene with the *Candida*

glabrata HIS3 gene (*CgHIS3*) (28) in the Δ*bck1* and Δ*mpk1* mutants using a PCR-based method as described previously (29), resulting in the deletion of the entire open reading frame. Gene deletions were verified by colony PCR. Culture media used in this study were YPD (1% yeast extract, 2% peptone, and 2% glucose) and synthetic medium (SD) (0.67% yeast nitrogen base without amino acids plus 2% glucose) supplemented with the required amino acids, with the optional addition of 200 mg/liter G418 (Geneticin; Sigma-Aldrich). The culture was incubated at 30°C.

Spot susceptibility assay. Exponential-phase cells were diluted to an optical density at 600 nm (OD_{600}) of 0.1 and then serially diluted 10-fold. An aliquot (3 μl) of each dilution was spotted onto YPD agar plates and YPD agar plates supplemented with ethanol, calcofluor white (CFW), or sorbitol at the indicated concentrations. Growth was monitored after incubation at 30°C for 3 days.

Zymolyase susceptibility test. Susceptibility to Zymolyase was assayed as described previously (3), with some modifications. Briefly, exponential-phase cells were diluted to an OD_{600} of 0.5 in TE buffer (10 mM Tris-HCl and 1 mM EDTA [pH 7.5]) containing 1 U/ml Zymolyase 20T (a β-1,3-glucanase from *Arthrobacter luteus*) (Zymo Research, USA) and incubated at 30°C. Sensitivity to Zymolyase was monitored by measuring the OD_{600} at 30-min intervals for 2 h by using an automated microplate reader (Wallace Victor 1420; PerkinElmer, USA).

Comparative transcriptomic analysis of cell wall and ethanol stress-responsive genes. Comparative transcriptomic analysis of cell wall and ethanol stress-responsive genes was performed using publicly available data obtained from the Gene Expression Omnibus (GEO) under accession numbers GSE959 (BY4741 treated with Congo red for 2, 4, and 6 h and Zymolyase for 2 h) (30), GSE2224 (BY4730 treated with 15% ethanol for 2 h) (31), GSE4049 (BY4742 treated with 0.1 and 0.02 mg/ml CFW for 1.5 h) (32), GSE20108 (CGMCC2758 diploid *MAT***a**/α, haploid *MAT***a**, and haploid *MAT*α strains treated with 3% or 7% ethanol in fermentors and collected at the exponential phase) (33), and GSE42433 (BY4741 collected after fermentation for 6 h). The data sets in raw files were preprocessed by using R packages (34) according to their platforms and normalized across transcriptomes using the loess (locally estimated scatterplot smoothing) method, with the exception of data under GEO accession number GSE4049, which were processed manually. Those without available raw files (GEO accession numbers GSE959 and GSE2224) were processed by the authors. Only the genes whose transcript levels are present in all data sets were included for further analyses. After preprocessing, all data sets were separately scaled and then combined into one table, median centered in the statistical program R (34), using colMedians() from the miscTools package, and normalized with the cyclic loess method in order to minimize biases between different experiments by using normalizeBetweenArrays() from the Limma package (35), with the additional parameter cyclic.method="pair." Hierarchical clustering was performed using the hclust() function with the ward.D method. Data visualization was done using the ComplexHeatmap package and the built-in function boxplot(), all in the statistical program R (34).

RNA isolation and quantitative real-time RT-PCR assays. Total RNA was isolated by using the RNeasy minikit (Qiagen, USA), according to the manufacturer's instructions. One microgram of each RNA sample was converted to cDNA by using the iScript cDNA synthesis kit (Bio-Rad, USA), according to the manufacturer's instructions. Quantitative real-time reverse transcription-PCR (RT-PCR) was performed on an ABI 7500 instrument (Applied Biosystems, USA) using a Kapa SYBR fast qPCR kit (Kapa Biosystems, USA) and 200 nM specific primer pairs (see Table S1 in the supplemental material). The reaction conditions were as follows: 95°C for 180 s, followed by 35 cycles of 95°C for 3 s, 60°C for 20 s, and 72°C for 20 s. The relative gene expression level was calculated using the $2^{-\Delta\Delta C_T}$ method and normalized to *ACT1* mRNA levels.

Data analysis. All experiments were independently performed at least three times, and data are expressed as means with standard deviations (SD). All results were analyzed by one-way analysis of variance (ANOVA) using the least significant difference (LSD) method in the SPSS statistical package (version 18.0 for Windows; SPSS Inc., Chicago, IL, USA). The level of statistical significance was set at a P value of <0.05.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00551-19.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

ACKNOWLEDGMENTS

This research was supported by grants from the Faculty of Science, Mahidol University; the Thailand Research Fund (MRG5380030); and the Center of Excellence on Environmental Health and Toxicology, Science and Technology Postgraduate Education and Research Development Office (PERDO), Ministry of Education. N.U. was financially supported by a scholarship from the Science Achievement Scholarship of Thailand (SAST), Office of the Higher Education Commission. V.C. acknowledges the TRF grant for new scholar (MRG6080235); the Royal Society-OHEC Newton mobility grant (NI160206), Faculty of Science, Mahidol University; and the Crown Property Bureau Foundation (through the Integrative Computational BioScience [ICBS] Center, Mahidol University).

We thank Laran T. Jensen, Puey Ounjai, and Nitnipa Soontorngun for helpful suggestions and Noel Pabalan for editing the manuscript.

REFERENCES

1. Auesukaree C. 2017. Molecular mechanisms of the yeast adaptive response and tolerance to stresses encountered during ethanol fermentation. J Biosci Bioeng 124:133–142. https://doi.org/10.1016/j.jbiosc.2017.03.009.
2. Stanley D, Bandara A, Fraser S, Chambers PJ, Stanley GA. 2010. The ethanol stress response and ethanol tolerance of *Saccharomyces cerevisiae*. J Appl Microbiol 109:13–24. https://doi.org/10.1111/j.1365-2672.2009.04657.x.
3. Charoenbhakdi S, Dokpikul T, Burphan T, Techo T, Auesukaree C. 2016. Vacuolar H^+-ATPase protects *Saccharomyces cerevisiae* cells against ethanol-induced oxidative and cell wall stresses. Appl Environ Microbiol 82:3121–3130. https://doi.org/10.1128/AEM.00376-16.
4. Fujita K, Matsuyama A, Kobayashi Y, Iwahashi H. 2006. The genome-wide screening of yeast deletion mutants to identify the genes required for tolerance to ethanol and other alcohols. FEMS Yeast Res 6:744–750. https://doi.org/10.1111/j.1567-1364.2006.00040.x.
5. Auesukaree C, Damnernsawad A, Kruatrachue M, Pokethitiyook P, Boonchird C, Kaneko Y, Harashima S. 2009. Genome-wide identification of genes involved in tolerance to various environmental stresses in *Saccharomyces cerevisiae*. J Appl Genet 50:301–310. https://doi.org/10.1007/BF03195688.
6. Teixeira MC, Raposo LR, Mira NP, Lourenco AB, Sa-Correia I. 2009. Genome-wide identification of *Saccharomyces cerevisiae* genes required for maximal tolerance to ethanol. Appl Environ Microbiol 75:5761–5772. https://doi.org/10.1128/AEM.00845-09.
7. Orlean P. 2012. Architecture and biosynthesis of the *Saccharomyces cerevisiae* cell wall. Genetics 192:775–818. https://doi.org/10.1534/genetics.112.144485.
8. Levin DE. 2011. Regulation of cell wall biogenesis in *Saccharomyces cerevisiae*: the cell wall integrity signaling pathway. Genetics 189:1145–1175. https://doi.org/10.1534/genetics.111.128264.
9. Bermejo C, Rodriguez E, Garcia R, Rodriguez-Pena JM, Rodriguez de la Concepcion ML, Rivas C, Arias P, Nombela C, Posas F, Arroyo J. 2008. The sequential activation of the yeast HOG and SLT2 pathways is required for cell survival to cell wall stress. Mol Biol Cell 19:1113–1124. https://doi.org/10.1091/mbc.e07-08-0742.
10. Fuchs BB, Mylonakis E. 2009. Our paths might cross: the role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways. Eukaryot Cell 8:1616–1625. https://doi.org/10.1128/EC.00193-09.
11. Garcia R, Rodriguez-Pena JM, Bermejo C, Nombela C, Arroyo J. 2009. The high osmotic response and cell wall integrity pathways cooperate to regulate transcriptional responses to Zymolyase-induced cell wall stress in *Saccharomyces cerevisiae*. J Biol Chem 284:10901–10911. https://doi.org/10.1074/jbc.M808693200.
12. Boorsma A, Nobel H, Riet B, Bargmann B, Brul S, Hellingwerf KJ, Klis FM. 2004. Characterization of the transcriptional response to cell wall stress in *Saccharomyces cerevisiae*. Yeast 21:413–427. https://doi.org/10.1002/yea.1109.
13. Martin H, Shales M, Fernandez-Piñar P, Wei P, Molina M, Fiedler D, Shokat KM, Beltrao P, Lim W, Krogan NJ. 2015. Differential genetic interactions of yeast stress response MAPK pathways. Mol Syst Biol 11:800. https://doi.org/10.15252/msb.20145606.
14. Hohmann S. 2015. An integrated view on a eukaryotic osmoregulation system. Curr Genet 61:373–382. https://doi.org/10.1007/s00294-015-0475-0.
15. Kubota S, Takeo I, Kume K, Kanai M, Shitamukai A, Mizunuma M, Miyakawa T, Shimoi H, Iefuji H, Hirata D. 2004. Effect of ethanol on cell growth of budding yeast: genes that are important for cell growth in the presence of ethanol. Biosci Biotechnol Biochem 68:968–972. https://doi.org/10.1271/bbb.68.968.
16. Klis FM, Boorsma A, De Groot PW. 2006. Cell wall construction in *Saccharomyces cerevisiae*. Yeast 23:185–202. https://doi.org/10.1002/yea.1349.
17. Lesage G, Bussey H. 2006. Cell wall assembly in *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 70:317–343. https://doi.org/10.1128/MMBR.00038-05.
18. Dwyer D, Bradley R. 2000. Chemical properties of alcohols and their protein binding sites. Cell Mol Life Sci 57:265–275. https://doi.org/10.1007/PL00000689.
19. Thode AB, Kruse SW, Nix JC, Jones DN. 2008. The role of multiple hydrogen-bonding groups in specific alcohol binding sites in proteins: insights from structural studies of LUSH. J Mol Biol 376:1360–1376. https://doi.org/10.1016/j.jmb.2007.12.063.
20. Ogawa Y, Nitta A, Uchiyama H, Imamura T, Shimoi H, Ito K. 2000. Tolerance mechanism of the ethanol-tolerant mutant of sake yeast. J Biosci Bioeng 90:313–320. https://doi.org/10.1016/S1389-1723(00)80087-0.
21. Rossignol T, Dulau L, Julien A, Blondin B. 2003. Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. Yeast 20:1369–1385. https://doi.org/10.1002/yea.1046.
22. Wu H, Zheng X, Araki Y, Sahara H, Takagi H, Shimoi H. 2006. Global gene expression analysis of yeast cells during sake brewing. Appl Environ Microbiol 72:7353–7358. https://doi.org/10.1128/AEM.01097-06.
23. Garcia R, Botet J, Rodriguez-Pena JM, Bermejo C, Ribas JC, Revuelta JL, Nombela C, Arroyo J. 2015. Genomic profiling of fungal cell wall-interfering compounds: identification of a common gene signature. BMC Genomics 16:683. https://doi.org/10.1186/s12864-015-1879-4.
24. Hendler A, Medina EM, Buchler NE, de Bruin RAM, Aharoni A. 2018. The evolution of a G_1/S transcriptional network in yeasts. Curr Genet 64:81–86. https://doi.org/10.1007/s00294-017-0726-3.
25. Adya AK, Canetta E, Walker GM. 2006. Atomic force microscopic study of the influence of physical stresses on *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. FEMS Yeast Res 6:120–128. https://doi.org/10.1111/j.1567-1364.2005.00003.x.
26. Rodicio R, Heinisch JJ. 2010. Together we are strong—cell wall integrity sensors in yeasts. Yeast 27:531–540. https://doi.org/10.1002/yea.1785.
27. Kock C, Dufrene YF, Heinisch JJ. 2015. Up against the wall: is yeast cell wall integrity ensured by mechanosensing in plasma membrane microdomains? Appl Environ Microbiol 81:806–811. https://doi.org/10.1128/AEM.03273-14.
28. Kitada K, Yamaguchi E, Arisawa M. 1995. Cloning of the *Candida glabrata TRP1* and *HIS3* genes, and construction of their disruptant strains by sequential integrative transformation. Gene 165:203–206. https://doi.org/10.1016/0378-1119(95)00552-H.
29. Sakumoto N, Mukai Y, Uchida K, Kouchi T, Kuwajima J, Nakagawa Y, Sugioka S, Yamamoto E, Furuyama T, Mizubuchi H, Ohsugi N, Sakuno T, Kikuchi K, Matsuoka I, Ogawa N, Kaneko Y, Harashima S. 1999. A series of protein phosphatase gene disruptants in *Saccharomyces cerevisiae*. Yeast 15:1669–1679. https://doi.org/10.1002/(SICI)1097-0061(199911)15:15<1669::AID-YEA480>3.0.CO;2-6.
30. Garcia R, Bermejo C, Grau C, Perez R, Rodriguez-Pena JM, Francois J, Nombela C, Arroyo J. 2004. The global transcriptional response to transient cell wall damage in *Saccharomyces cerevisiae* and its regulation by the cell integrity signaling pathway. J Biol Chem 279:15183–15195. https://doi.org/10.1074/jbc.M312954200.
31. Caba E, Dickinson DA, Warnes GR, Aubrecht J. 2005. Differentiating mechanisms of toxicity using global gene expression analysis in *Saccharomyces cerevisiae*. Mutat Res 575:34–46. https://doi.org/10.1016/j.mrfmmm.2005.02.005.
32. Kuranda K, Leberre V, Sokol S, Palamarczyk G, Francois J. 2006. Investigating the caffeine effects in the yeast *Saccharomyces cerevisiae* brings new insights into the connection between TOR, PKC and Ras/cAMP signalling pathways. Mol Microbiol 61:1147–1166. https://doi.org/10.1111/j.1365-2958.2006.05300.x.
33. Li BZ, Cheng JS, Ding MZ, Yuan YJ. 2010. Transcriptome analysis of differential responses of diploid and haploid yeast to ethanol stress. J Biotechnol 148:194–203. https://doi.org/10.1016/j.jbiotec.2010.06.013.
34. R Core Team. 2017. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.
35. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. 2015. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 43:e47. https://doi.org/10.1093/nar/gkv007.