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PHYSIOLOGY

Involvement of the Cell Wall Integrity Pathway of *Saccharomyces cerevisiae* in Protection against Cadmium and Arsenate Stresses

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ABSTRACT Contamination of soil and water with heavy metals and metalloids is a serious environmental problem. Cadmium and arsenic are major environmental contaminants that pose a serious threat to human health. Although toxicities of cadmium and arsenic to living organisms have been extensively studied, the molecular mechanisms of cellular responses to cadmium and arsenic remain poorly understood. In this study, we demonstrate that the cell wall integrity (CWI) pathway is involved in coping with cell wall stresses induced by cadmium and arsenate through its role in the regulation of cell wall modification. Interestingly, the Rlm1p and SBF (Swi4p-Swi6p) complex transcription factors of the CWI pathway were shown to be specifically required for tolerance to cadmium and arsenate, respectively. Furthermore, we found the *PIR2* gene, encoding cell wall O-mannosylated heat shock protein, whose expression is under the control of the CWI pathway, is important for maintaining cell wall integrity during cadmium and arsenate stresses. In addition, our results revealed that the CWI pathway is involved in modulating the expression of genes involved in cell wall biosynthesis and cell cycle control in response to cadmium and arsenate via distinct sets of transcriptional regulators.

IMPORTANCE Environmental pollution by metal/metalloids such as cadmium and arsenic has become a serious problem in many countries, especially in developing countries. This study shows that in the yeast *S. cerevisiae*, the CWI pathway plays a protective role against cadmium and arsenate through the upregulation of genes involved in cell wall biosynthesis and cell cycle control, possibly in order to modulate cell wall reconstruction and cell cycle phase transition, respectively. These data provide insights into molecular mechanisms underlying adaptive responses to cadmium and arsenate.

KEYWORDS cadmium, arsenate, cell wall integrity pathway, cell wall remodeling, cell cycle control, *Saccharomyces cerevisiae*

Due to rapid industrialization and urbanization, several anthropogenic activities, such as mining, industrial manufacturing, and fossil fuel combustion, have accelerated worldwide contamination of soil and water with heavy metals and/or metalloids (1–3). Among metal/metalloid contaminants, cadmium and arsenic are of global concern due to their extensive contamination levels and high toxicities (4, 5). Long-term exposure to cadmium leads to cancer, renal dysfunction, liver damage, osteoporosis, and pulmonary disease (4, 6). Chronic arsenic exposure causes various adverse health effects such as cancer, dermatitis, and severe systemic toxicity (such as nervous, cardiovascular, renal, reproductive, and respiratory system toxicities) (5).

In the model eukaryote *Saccharomyces cerevisiae*, cellular effects of metal/metalloid are generally caused by direct interaction of a metal/metalloid with the thiol group of

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proteins, a replacement of essential metal cofactors with toxic metal/metalloid, or a metal/metalloid-induced oxidation of biomolecules (7). Major molecular mechanisms involved in metal/metalloid detoxification in yeast include extrusion by the plasma membrane efflux system, vacuolar sequestration, and chelation by specific peptides and proteins such as glutathione and metallothionein (7). However, the precise molecular mechanisms of metal/metalloid toxicity and cellular response to metal/metalloid toxicity remain unclear. Several genes encoding the components of the cell wall integrity (CWI) mitogen-activated protein kinase (MAPK) signaling pathway (e.g., *BCK1* and *SLT2* genes, encoding MAPK kinase kinase [MAPKKK] and MAPK of the MAPK cascade, respectively) have been reported to be required for tolerances to cadmium and arsenate (8–10). In addition, the Slt2p MAPK of the CWI pathway has been shown to be activated in response to cadmium and arsenate (10, 11). Based on these observations, it seems that the CWI pathway is required for protecting yeast cells against metal/metalloid, especially cadmium and arsenate, possibly through its role in the transduction of cell wall stress signals induced by metal/metalloids.

The yeast CWI pathway is crucial for the regulation of adaptive response to cell wall stress through its role in inducing the expression of several cell wall-related genes, leading to the reconstruction of the cell wall in order to strengthen the wall structure (12). The cell wall is a rigid cellular structure required for the maintenance of cell shape and protection against environmental stresses. The major components of yeast cell wall are β-1,3-glucan, β-1,6-glucan, β-1,4-linked polymer of *N*-acetylglucosamine (chitin), and mannoproteins, which are covalently linked to form macromolecular complexes arranged into inner and outer layers. The inner layer is composed of a large network of β-1,3-glucan cross-linked to β-1,6-glucan and chitin, whereas the outer layer comprises densely packed glycosylated mannoproteins (12). The cell wall remodeling provoked by the activated CWI pathway in response to cell wall stress includes the increased synthesis of cell wall components and the cross-linking of newly synthesized components into the existing cell wall (12). In addition to the role in cell wall stress response, the CWI pathway is also involved in the regulation of cell growth and cell division processes such as cell cycle progression, bud formation, mating pheromone-induced morphogenesis, sporulation, and pseudohyphal growth (13). The cell-surface sensors of the CWI pathway (i.e., Wsc1p, Wsc2p, Wsc3p, Mid2p, and Mtl1p) detect cell wall damage and transmit cell wall stress signals to activate the guanine nucleotide exchange factor (GEF) Rom2p, leading to the activation of the small GTPase Rho1p. The active Rho1p stimulates the protein kinase C Pkc1p to activate the MAPK cascade (MAPKKK Bck1p, the redundant MAPK kinase [MAPKK] Mkk1p and Mkk2p, and the MAPK Slt2p), which in turn leads to the activation of transcription factors (Rlm1p, SBF [Swi4p-Swi6p] complex, and MBF [Swi6p-Mbp1p] complex) in order to induce the expression of specific cell wall genes (13). The major target genes of the CWI pathway include *FKS2* and *CRH1*, encoding catalytic subunits of β-1,3-glucan synthase and chitin transglycosylase, respectively (13). Changes in expression of cell wall-related genes, including those involved in cell wall biosynthesis, cell wall cross-linking, and translocation of cell wall components, may lead to a rearrangement of cell wall composition and architecture during cell wall stress and cell division (12, 13).

In this study, we demonstrate that the CWI pathway is important for protecting yeast cells against cell wall stress induced by cadmium and arsenate through its role in the upregulation of genes involved in cell wall biosynthesis, leading to the remodeling of cell wall architecture. Among the CWI target genes tested, we found that *PIR2* (encoding cell wall O-mannosylated heat shock protein) was overexpressed under cadmium and arsenate stress conditions, suggesting it may play an important role in stabilizing cell wall structure. Furthermore, we also show that distinct sets of transcriptional regulators of the CWI pathway are required for the upregulation of specific cell wall genes in response to cadmium and arsenate stresses.

RESULTS

CWI pathway is required for cadmium and arsenate tolerances. To investigate the role of the CWI pathway in protecting yeast cells against heavy metals and/or

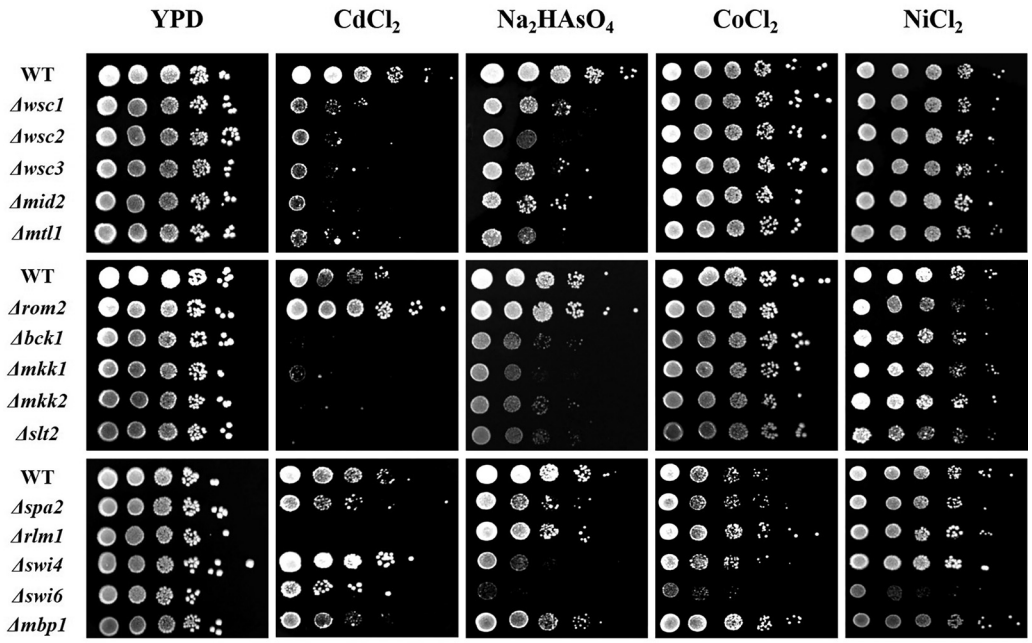

FIG 1 Growth of the mutants lacking components of the CWI pathway under cadmium, arsenate, cobalt, and nickel stress conditions. The wild-type (BY4742), Δ*wsc1*, Δ*wsc2*, Δ*wsc3*, Δ*mid2*, Δ*mtl1*, Δ*rom2*, Δ*bck1*, Δ*mkk1*, Δ*mkk2*, Δ*slt2*, Δ*spa2*, Δ*rlm1*, Δ*swi4*, Δ*swi6*, and Δ*mbp1* cells grown to log phase in YPD broth were adjusted to an OD_{600} of 1.0 and then serially diluted 10-fold to a 10^{-5} dilution. Aliquots (3 μl) were spotted onto YPD agar plates containing 30 μM $CdCl_2$, 2 mM Na_2HAsO_4, 2 mM $CoCl_2$, or 2 mM $NiCl_2$, and incubated at 30°C for 3 days.

metalloids, we examined the growth of mutants lacking components of the CWI signaling pathway on yeast extract-peptone-dextrose (YPD) agar plates containing $CdCl_2$, Na_2HAsO_4, $CoCl_2$, or $NiCl_2$ by spot susceptibility assay. Our results revealed that the mutants lacking sensors of the CWI pathway (i.e., the Δ*wsc1*, Δ*wsc2*, Δ*wsc3*, Δ*mid2*, and Δ*mtl1* mutants) and the mutants lacking components of the CWI MAPK cascade (i.e., the Δ*bck1*, Δ*mkk1*, Δ*mkk2*, and Δ*slt2* mutants) were sensitive to $CdCl_2$ and Na_2HAsO_4 but not to $CoCl_2$ and $NiCl_2$ (Fig. 1). Interestingly, for the mutants lacking transcription factors in the CWI pathway, the Δ*rlm1* mutant was sensitive only to $CdCl_2$ (to which it was remarkably sensitive), whereas the Δ*swi4* and Δ*swi6* mutants were sensitive to Na_2HAsO_4. Nevertheless, it should be noted that only the Δ*swi6* mutant was sensitive to $CoCl_2$ and $NiCl_2$. These results therefore suggest that the CWI pathway is required for protecting yeast cells against cadmium and arsenate stresses. Nevertheless, it appears that the CWI transcription factors Rlm1p and SBF (Swi4p-Swi6p) complex are involved in the regulation of adaptive response to cadmium and arsenate, respectively.

CWI pathway is involved in the regulation of cell wall modification in response to cadmium and arsenate stresses. It was reported that the CWI pathway is the main mechanism involved in the regulation of adaptive response to cell wall stress. This signaling pathway plays a key role in inducing the expression of genes involved in cell wall biosynthesis and remodeling, thereby leading to a rearrangement of cell wall architecture in order to strengthen cell wall structure (12, 13). Since we found that the components of the CWI pathway are important for tolerances to cadmium and arsenate, it is possible that the CWI pathway may be involved in regulating the modifications of cell wall structure in response to metals and/or metalloids, especially cadmium and arsenate. To test this hypothesis, we first examined the cell wall robustness of the wild-type strain after challenge with $CdCl_2$, Na_2HAsO_4, $CoCl_2$, $NiCl_2$, or a cell wall-perturbing agent calcofluor white (CFW) (as a positive control) for 12 h by monitoring susceptibilities to the cell wall-degrading enzyme Zymolyase, whose major activities are β-1,3-glucanase and β-1,3-glucan laminaripentaohydrolase. Our results revealed that, similar to the case of CFW treatment, the Zymolyase resistance of wild-type cells was

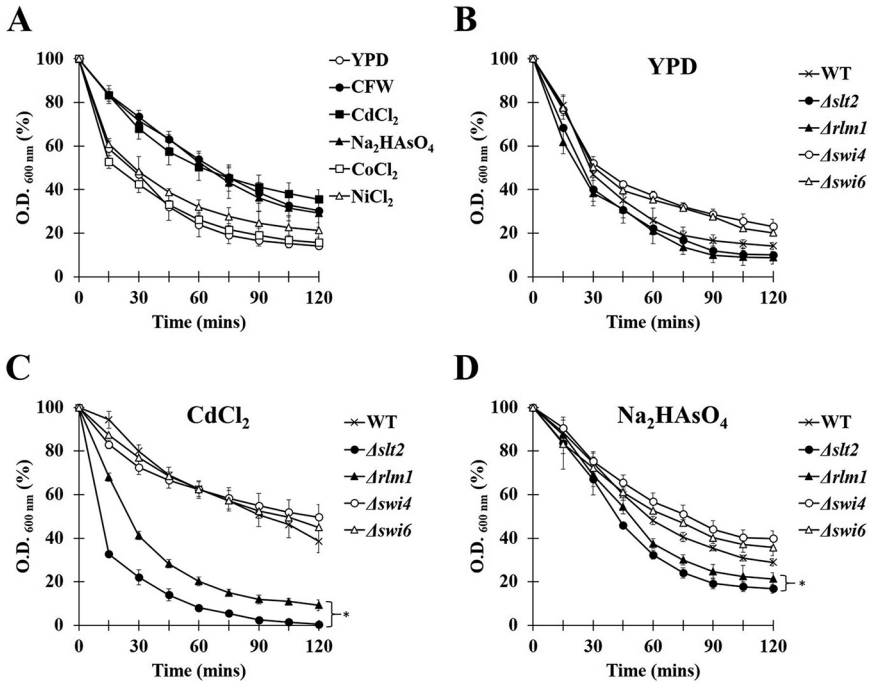

FIG 2 Susceptibility to Zymolyase of the wild-type strain and mutants lacking components of the CWI pathway after exposures to metals and metalloid. (A) The log-phase wild-type (BY4742) cells were incubated in YPD medium containing 100 μg ml^{-1} calcofluor white (CFW), 30 μM $CdCl_2$, 2 mM Na_2HAsO_4, 2 mM $CoCl_2$, or 2 mM $NiCl_2$ at 30°C for 12 h. Cells were harvested and resuspended at an OD_{600} of 0.5 in TE buffer containing 100 μg ml^{-1} (1 U) Zymolyase 20T. Susceptibility to Zymolyase was monitored by measuring the OD_{600} at 15-min intervals for 2 h and expressed as a percentage of the OD_{600} relative to that of the zero time point. (B to D) The log-phase wild-type (BY4742), Δ*slt2*, Δ*rlm1*, Δ*swi4*, and Δ*swi6* cells were incubated in YPD medium (B) or YPD medium containing either 30 μM $CdCl_2$ (C) or 2 mM Na_2HAsO_4 (D) at 30°C for 12 h. Zymolyase susceptibility was monitored as described above. Mean ± SD values are from three independent experiments; *, $P < 0.05$.

increased after the treatments with $CdCl_2$ and Na_2HAsO_4 but not $CoCl_2$ or $NiCl_2$ (Fig. 2A). These results suggest that the modification of cell wall structure was induced in response to cadmium and arsenate stresses, thereby leading to a more robust cell wall. To determine the alterations in the contents of cell wall components during cadmium and arsenate stresses, we next determined the levels of β-1,3-glucan and chitin, which are the major components of yeast cell wall, in the wild-type strain after treatment with $CdCl_2$ or Na_2HAsO_4. We found that the levels of β-1,3-glucan and chitin were increased after $CdCl_2$ exposure, whereas the Na_2HAsO_4 treatment had no significant effect on the β-1,3-glucan and chitin contents of the wild-type strain (Fig. 3).

To investigate the role of the CWI pathway in regulating cell wall remodeling in response to cadmium and arsenate, we determined Zymolyase susceptibilities and cell wall components (i.e., β-1,3-glucan and chitin) of the wild type, Δ*slt2*, Δ*rlm1*, Δ*swi4*, and Δ*swi6* strains after treatments with CFW, $CdCl_2$, or Na_2HAsO_4 for 12 h. After $CdCl_2$ exposure, despite the fact that the levels of β-1,3-glucan and chitin of the cadmium-sensitive Δ*slt2* and Δ*rlm1* mutants were significantly increased, the Zymolyase resistances of these mutants were not improved (Fig. 2C and 3). These findings suggest that, during cadmium stress, the signal transduction through the CWI pathway via the Rlm1p transcription factor is important for increasing cell wall robustness, which was found to be independent of cell wall β-1,3-glucan and chitin levels. On the other hand, although the Swi4p-Swi6p complex is important for arsenate tolerance, only the Δ*slt2* and Δ*rlm1* mutants, and not the Δ*swi4* and Δ*swi6* mutants, were more sensitive to Zymolyase than the wild-type strain after treatment with Na_2HAsO_4 (Fig. 2D). In addition, similar to the case of the wild-type strain, the Na_2HAsO_4 treatment did not alter the cell wall β-1,3-glucan and chitin levels of all mutants tested (except the chitin content of the

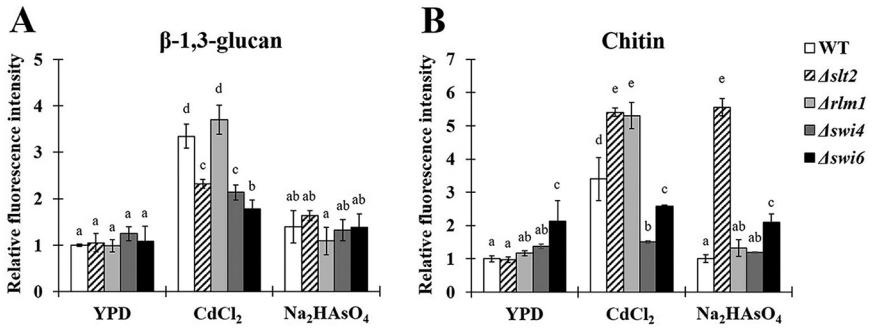

FIG 3 Levels of β-1,3-glucan and chitin of the wild-type strain and mutants lacking components of the CWI pathway after exposures to cadmium and arsenate. The log-phase wild-type (BY4742), Δ*slt2*, Δ*rlm1*, Δ*swi4*, and Δ*swi6* cells were incubated in YPD medium alone or YPD medium containing either 30 μM $CdCl_2$ or 2 mM Na_2HAsO_4 at 30°C for 12 h. The contents of β-1,3-glucan (A) and chitin (B) were measured as described in the Materials and Methods. The fluorescence intensity values were normalized to the OD_{600} of each sample. Mean ± SD values are from three independent experiments. Values with different superscript letters indicate statistically significant differences at $P < 0.05$.

Δ*slt2* mutant) (Fig. 3). It is therefore likely that the enhancement of cell wall robustness by the rearrangement of cell wall structure in response to arsenate may be partially mediated through the CWI pathway via the Rlm1p transcription factor, whereas the role of the SBF (Swi4p-Swi6p) complex in arsenate tolerance seems to be unrelated to the induction of cell wall remodeling.

Since the cell wall is the first line of defense against foreign substances and environmental stresses, the increased cell wall robustness caused by metal(loid)s may be attributed to increased resistances against antifungal drugs. To test this idea, the wild-type cells were pretreated with $CdCl_2$ or Na_2HAsO_4 for 12 h prior to the assessments of resistances against three major antifungal drugs, i.e., amphotericin B (polyene antifungal agent), ketoconazole (azole antifungal agent), and caspofungin (echinocandin antifungal agent). Our results revealed that the pretreatment with cadmium or arsenate did not improve the resistances of yeast cells against these antifungal agents (Fig. 4).

Pir2p is required for maintaining yeast cell wall integrity during cadmium and arsenate stresses. To explore the role of cell wall components and cell wall-related proteins in tolerances to cadmium and arsenate, we examined the growth of mutants lacking genes involved in the biosynthesis of cell wall components in the presence of $CdCl_2$ or Na_2HAsO_4 (Fig. 5A). The cell wall mutants tested include the following: Δ*fks1* and Δ*fks2* mutants, lacking catalytic subunits of β-1,3-glucan synthase; the Δ*fks3* mutant, lacking a protein with similarity to Fks1p and Fks2p; the Δ*kre6* mutant, lacking a protein involved in β-1,6-glucan biosynthesis; the Δ*chs3* mutant, lacking chitin synthase; the Δ*skt5 and* Δ*shc1* mutants, lacking activators of Chs3p; the Δ*crh1* and Δ*crh2* mutants, lacking chitin transglycosylases; the Δ*crr1* mutant, lacking a protein with similarity to Crh1p; the Δ*bgl2* mutant, lacking endo-β-1,3-glucanase; the Δ*gas1* mutant, lacking β-1,3-glucanosyltransferase; the Δ*exg1* and Δ*exg2* mutants, lacking exo-β-1,3-glucanases; the Δ*pir1*, Δ*pir2*, and Δ*pir3* mutants, lacking O-glycosylated cell wall proteins; and the Δ*sed1*, Δ*cwp1*, Δ*ygp1*, Δ*pst1*, Δ*ssr1*, and Δ*cis3* mutants, lacking cell wall glycoproteins (12, 14). Among the cell wall-defective mutants tested, we found that only the Δ*pir2* mutant, lacking a cell wall O-mannosylated heat shock protein required for cell wall stability, was sensitive to cadmium and arsenate. To determine the role of Pir2p in maintaining cell wall integrity during cadmium and arsenate stresses, we examined the Zymolyase susceptibility of the Δ*pir2* mutant after challenge with $CdCl_2$ or Na_2HAsO_4 for 12 h. We found that, unlike that of the wild-type strain, the Zymolyase resistance of the Δ*pir2* mutant was not significantly increased after treatment with either $CdCl_2$ or Na_2HAsO_4 (Fig. 5B). Furthermore, irrespective of the presence or absence of cadmium and arsenate, the Δ*pir2* mutant was more sensitive to Zymolyase than the wild-type strain (Fig. 5B). These findings suggest the important role of Pir2p

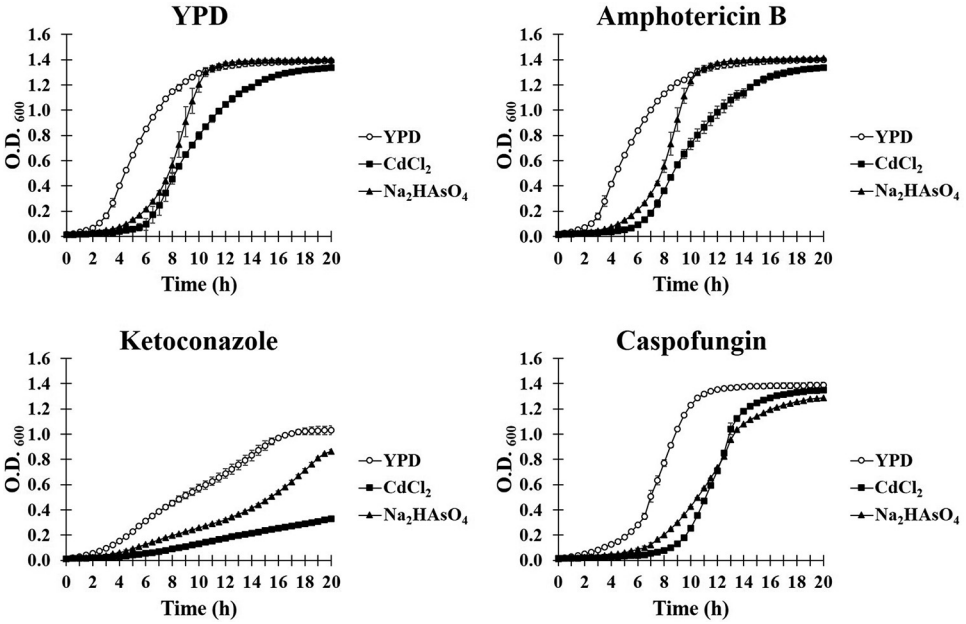

FIG 4 Susceptibilities to antifungal drugs of the wild-type strain after exposures to cadmium and arsenate. The log-phase wild-type (BY4742) cells were either not given pretreatment or pretreated with 30 μM $CdCl_2$ or 2 mM Na_2HAsO_4 at 30°C for 12 h. Cells were harvested and transferred to YPD medium or YPD medium containing 0.2 μg ml^{-1} amphotericin B, 4 μg ml^{-1} ketoconazole, or 0.04 μg ml^{-1} caspofungin, and incubated with shaking (200 rpm) at 30°C. Cell growth was monitored at 0.5-h intervals for 20 h by measuring the OD_{600}.

in maintaining cell wall integrity not only under cadmium and arsenate stress conditions but also under physiological conditions.

CWI pathway is involved in the upregulation of *PIR2* gene expression in response to cadmium and arsenate stresses. The expression of the *PIR2* gene has been shown to be induced by heat shock, oxidative stress, and aluminum (15–18). Since we found that Pir2p is important for tolerances to cadmium and arsenate through its role in maintaining cell wall integrity, we next examined the expression of the *PIR2* gene in the wild-type strain after challenge with $CdCl_2$ or Na_2HAsO_4 for 12 h. We found that *PIR2* gene expression was significantly increased after exposures to cadmium and arsenate (Fig. 5C), suggesting that the upregulation of *PIR2* gene expression in response to cadmium and arsenate stresses may contribute to cell wall remodeling in order to protect yeast cells against cadmium and arsenate. Since cell wall remodeling is mainly controlled by the CWI pathway and our aforementioned results revealed that Rlm1p and the SBF (Swi4p-Swi6p) complex, the transcriptional activators of the CWI pathway, were specifically required for tolerances to cadmium and arsenate, respectively, we next investigated the role of the CWI pathway and its transcription factors in regulating the expression of the *PIR2* gene in response to cadmium and arsenate stresses. In contrast to the wild-type strain, the *PIR2* expression levels of the Δ*slt2*, Δ*rlm1*, Δ*swi4*, and Δ*swi6* mutants were not induced after cadmium treatment (Fig. 5C), suggesting that the upregulation of *PIR2* gene expression during cadmium stress is under the control of the CWI pathway via both transcriptional activators, Rlm1p and the SBF complex. On the other hand, after arsenate treatment, the *PIR2* expression levels of the Δ*slt2*, Δ*rlm1*, and Δ*swi4* mutants were slightly increased but significantly lower than that of the wild-type strain (Fig. 5C). In contrast, *PIR2* gene expression in the Δ*swi6* mutant was completely inhibited during arsenate stress (Fig. 5C). These findings suggest that, under arsenate stress conditions, *PIR2* gene expression is mainly controlled by Swi6p and partially activated by the CWI signaling pathway through Rlm1p and Swi4p transcription factors.

Expression of cadmium and/or arsenate-responsive genes is regulated by the CWI pathway through distinct sets of transcription factors. According to the

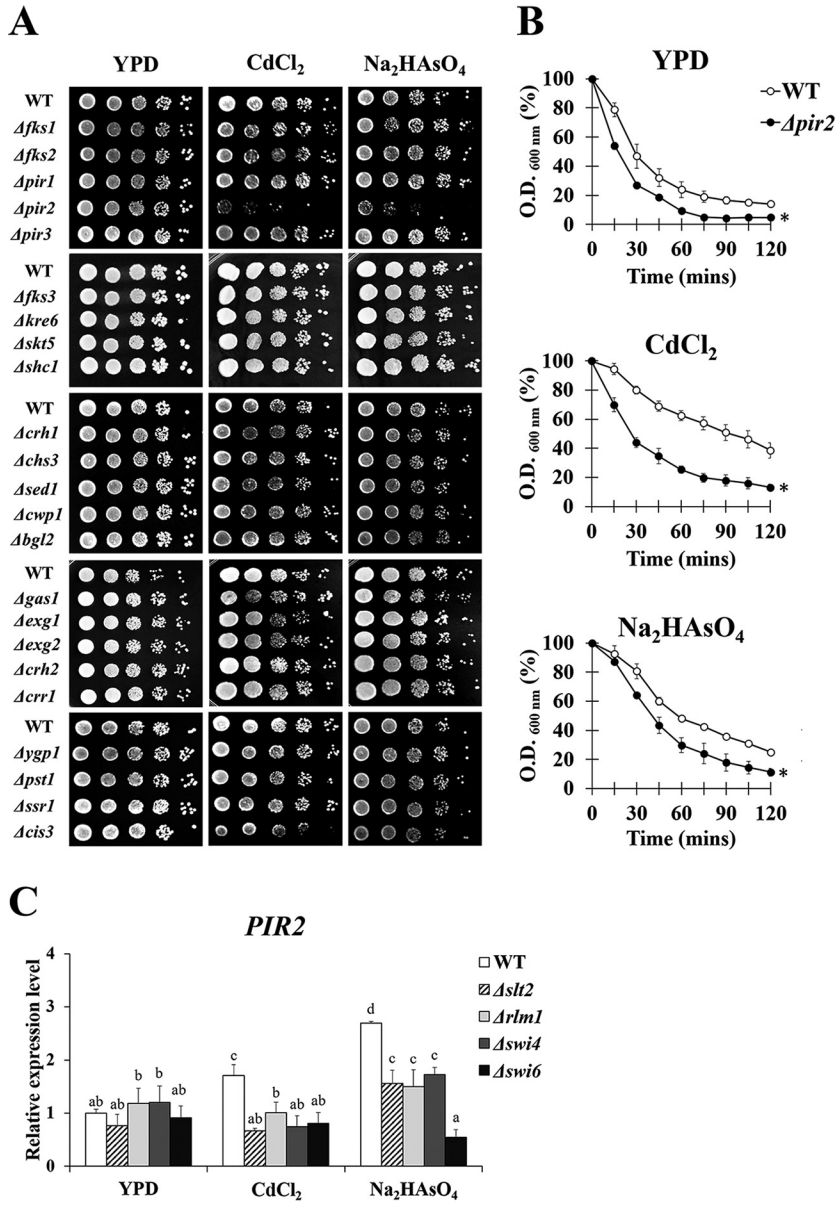

FIG 5 Protective role of *PIR2* expression against cadmium and arsenate stresses. (A) Growth of the mutants lacking genes involved in the biosynthesis of cell wall components. The wild-type (BY4742), Δ*fks1*, Δ*fks2*, Δ*pir1*, Δ*pir2*, Δ*pir3*, Δ*fks3*, Δ*kre6*, Δ*skt5*, Δ*shc1*, Δ*crh1*, Δ*chs3*, Δ*sed1*, Δ*cwp1*, Δ*bgl2*, Δ*gas1*, Δ*exg1*, Δ*exg2*, Δ*crh2*, Δ*crr1*, Δ*ygp1*, Δ*pst1*, Δ*ssr1*, and Δ*cis3* cells grown to log phase in YPD broth were adjusted to an OD_{600} of 1.0 and then serially diluted 10-fold to a 10^{-5} dilution. Aliquots (3 μl) were spotted onto YPD agar plates containing 30 μM $CdCl_2$ or 2 mM Na_2HAsO_4 and incubated at 30°C for 3 days. (B) Susceptibility to Zymolyase of the Δ*pir2* mutants after exposure to cadmium or arsenate. The log-phase wild-type (BY4742) and Δ*pir2* cells were incubated in YPD medium containing 30 μM $CdCl_2$ or 2 mM Na_2HAsO_4 at 30°C for 12 h. Cells were harvested and resuspended at an OD_{600} of 0.5 in TE buffer containing 100 μg ml^{-1} (1 U) Zymolyase 20T. Susceptibility to Zymolyase was monitored by measuring the OD_{600} at 15-min intervals for 2 h and expressed as a percentage of the OD_{600} relative to that of the zero time point. Mean ± SD values are from three independent experiments; *, $P < 0.05$. (C) *PIR2* expression levels of the wild-type (BY4742), Δ*slt2*, Δ*rlm1*, Δ*swi4*, and Δ*swi6* strains after treatment with 30 μM $CdCl_2$ or 2 mM Na_2HAsO_4 for 12 h. The mRNA levels of *PIR2* gene were normalized to that of the *ACT1* gene in the same sample. Mean ± SD values are from three independent experiments. Values with different superscript letters indicate statistically significant differences at $P < 0.05$.

above-described results, Rlm1p and SBF (Swi4p-Swi6p) transcription factors of the CWI pathway were specifically required for protecting yeast cells against cadmium and arsenate, respectively. On the other hand, we found that Pir2p was important for tolerances to both cadmium and arsenate and that *PIR2* gene expression was controlled

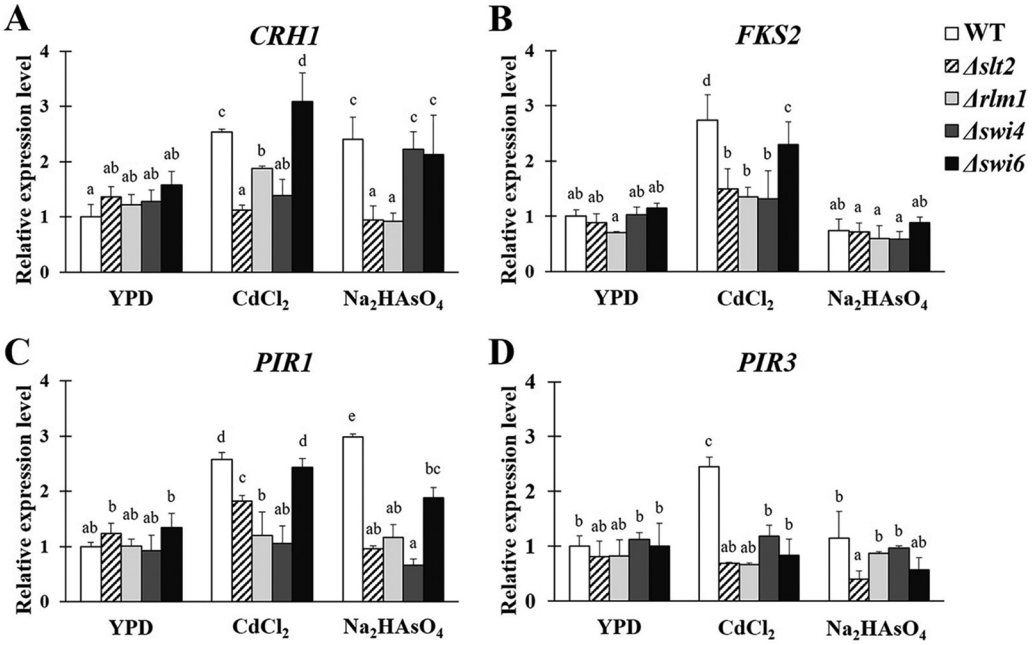

FIG 6 Expression profiles of cell wall-related genes in response to cadmium and arsenate stresses. Relative expression levels are shown for *CRH1* (A), *FKS2* (B), *PIR1* (C), and *PIR3* (D) genes of the wild-type (BY4742), Δ*slt2*, Δ*rlm1*, Δ*swi4*, and Δ*swi6* strains after treatment with 30 μM $CdCl_2$ or 2 mM Na_2HAsO_4 for 12 h. The mRNA levels of these genes were normalized to that of the *ACT1* gene in the same sample. Mean ± SD values are from three independent experiments. Values with different superscript letters indicate statistically significant differences at $P < 0.05$.

by both Rlm1p and SBF (Swi4p-Swi6p) transcription factors. To identify the CWI target genes whose expression is specifically regulated by Rlm1p and Swi4p-Swi6p complex in response to cadmium and arsenate, respectively, we first monitored the expression of four cell wall-related genes, i.e., *CRH1* (encoding chitin transglycosylase), *FKS2* (encoding the catalytic subunit of β-1,3-glucan synthase), and *PIR1* and *PIR3* (encoding O-glycosylated cell wall proteins), which are the major target genes of the CWI pathway (13, 19), after challenge with $CdCl_2$ or Na_2HAsO_4 for 12 h. In the wild-type strain, the expression levels of these four cell wall genes were significantly increased after cadmium treatment, whereas the arsenate treatment induced the upregulation of only *CRH1* and *PIR1* expression (Fig. 6A to D). These results therefore suggest the differential requirements of cell wall proteins in response to cadmium and arsenate stresses.

To examine the role of the CWI pathway and its transcription factors, i.e., Rlm1p and the SBF (Swi4p-Swi6p) complex, in regulating the expression of these cell wall genes in response to cadmium and arsenate stresses, we next measured the transcript levels of *CRH1*, *FKS2*, *PIR1*, and *PIR3* genes in the Δ*slt2*, Δ*rlm1*, Δ*swi4*, and Δ*swi6* mutants after treatment with $CdCl_2$ or Na_2HAsO_4 for 12 h. After exposures to $CdCl_2$, the upregulation of *PIR3* gene expression was completely inhibited in all mutants tested, whereas significant inhibition of *CRH1*, *FKS2*, and *PIR1* gene expression was observed in the Δ*slt2*, Δ*rlm1*, and Δ*swi4* mutants, but not in the Δ*swi6* mutant (Fig. 6A to D). In the case of arsenate stress, the complete inhibition of *CRH1* upregulation was observed only in the Δ*slt2* and Δ*rlm1* mutants (Fig. 6A). On the other hand, the upregulation of *PIR1* gene expression upon arsenate exposure was not detected in the Δ*slt2*, Δ*rlm1*, and Δ*swi4* mutants but was slightly induced in the Δ*swi6* mutant (Fig. 6C). These findings suggest that, in response to cadmium and arsenate stresses, the CWI pathway plays an important role in controlling the expression of specific cell wall-related genes through distinct sets of CWI transcriptional regulators.

CWI pathway is involved in regulating expression of cell cycle genes in response to cadmium and arsenate. The MAPK Slt2p has been reported to play a critical role in the activation of Rlm1p and SBF (Swi4p-Swi6p) transcriptional regulators for

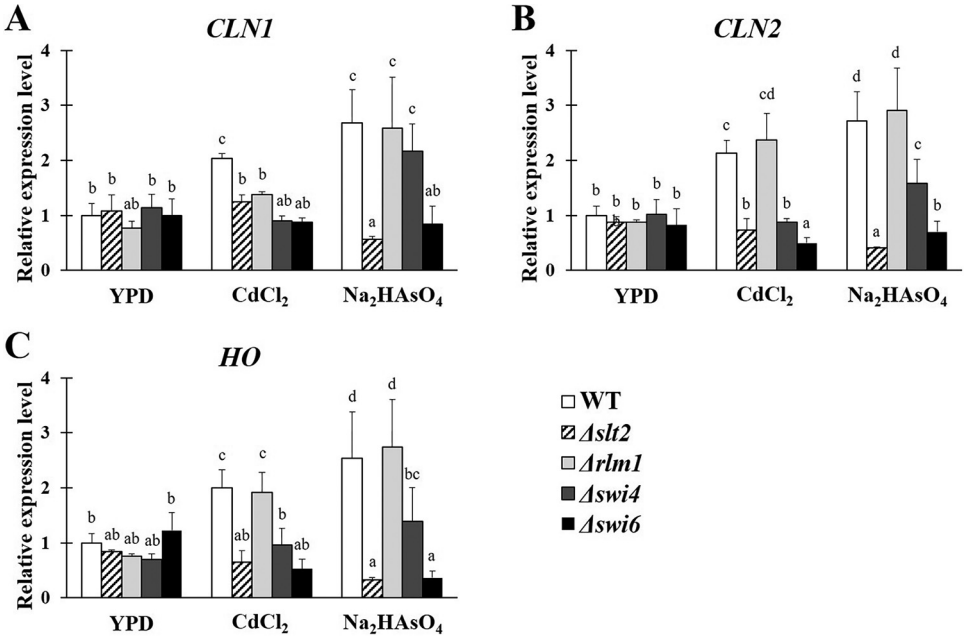

FIG 7 Expression profiles of cell cycle genes in response to cadmium and arsenate stresses. Relative expression levels of the *CLN1* (A) *CLN2* (B), and *HO* (C) genes of the wild-type (BY4742), Δ*slt2*, Δ*rlm1*, Δ*swi4*, and Δ*swi6* strains after treatment with 30 μM $CdCl_2$ or 2 mM Na_2HAsO_4 for 12 h. The mRNA levels of these genes were normalized to that of the *ACT1* gene in the same sample. Mean ± SD values are from three independent experiments. Values with different superscript letters indicate statistically significant differences at $P < 0.05$.

initiating cell wall reconstruction and G1/S mitotic transition, respectively (13, 20). The target genes of the SBF complex include the *CLN1* and *CLN2* genes, encoding G1 cyclins involved in the G1/S transition, and the *HO* gene, encoding DNA endonuclease involved in mating type switching (13, 21). Since it has been shown that some cell cycle-related genes were required for tolerance against arsenite, which induces a transient delay in G1 and G2 phases (22, 23), it is possible that the SBF complex may be important for cell cycle regulation during arsenate stress. We thus examined the expression levels of *CLN1*, *CLN2*, and *HO* genes in the wild-type strain after challenge with cadmium or arsenate and found that the expression of all three genes was upregulated upon exposures to cadmium and arsenate (Fig. 7A to C). These findings suggest the effects of cadmium and arsenate on cell cycle control.

To explore the potential role of the CWI pathway and its transcription factors in controlling the expression of *CLN1*, *CLN2*, and *HO* genes during exposures to cadmium and arsenate stresses, we monitored the transcript levels of these genes in the Δ*slt2*, Δ*rlm1*, Δ*swi4*, and Δ*swi6* mutants treated with $CdCl_2$ or Na_2HAsO_4 for 12 h. Our results revealed that the upregulation of *CLN1*, *CLN2*, and *HO* gene expression upon exposures to cadmium and arsenate was completely inhibited in the Δ*slt2* and Δ*swi6* mutants, lacking MAPK and the transcription cofactor of the SBF complex, respectively (Fig. 7A to C). Although the Δ*swi4* mutant, lacking the DNA binding component of the SBF complex, was unable to induce the expression of *CLN1*, *CLN2*, and *HO* genes during cadmium stress, this mutant exhibited increased expression levels of these three genes upon arsenate exposure (Fig. 7A to C). In contrast, the expression levels of *CLN1*, *CLN2*, and *HO* genes in the Δ*rlm1* mutant, lacking another major transcription factor of the CWI pathway, were similar to those in the wild-type strain under all conditions tested (except the *CLN1* gene expression level under cadmium-stress conditions) (Fig. 7A to C). Based on our observations, it is likely that, in response to cadmium and arsenate stresses, distinct sets of the CWI transcriptional activators (i.e., the SBF [Swi4p-Swi6p] complex and only Swi6p, respectively) are required for inducing the expression of genes involved in cell cycle control and mating process.

DISCUSSION

In this study, we demonstrate that the CWI signaling pathway plays an important role in inducing cell wall remodeling during cadmium and arsenate stresses via the upregulation of genes involved in cell wall biosynthesis, possibly in response to cell wall stress caused by cadmium and arsenate. However, the mechanisms by which metal/metalloid, especially cadmium and arsenate, induce cell wall damage are still unclear. The direct binding of chemicals to the yeast cell wall may disturb cell wall structure and result in cell wall damage. For instance, some cell wall-disturbing agents, including Congo red and calcofluor white, are known to directly bind to chitin and cause cell wall damage. The exposures to these cell wall-disturbing agents will lead to the activation of the CWI pathway and the induction of cell wall remodeling by increasing chitin content (13, 24, 25). Since it has been shown that cadmium can directly bind to chitin in the cell wall of *Neurospora crassa* (26), cadmium may be capable of binding to chitin of the *S. cerevisiae* cell wall, which in turn induces cell wall damage. So far, direct binding of arsenate to the yeast cell wall has not been reported. It is well known that arsenate is a structural analog of phosphate and the outer cell wall mannoprotein layers of yeast cells contain phosphate groups (27, 28). It is therefore possible that arsenate may replace phosphate in cell wall mannoproteins and cause cell wall damage.

Among the cell wall-defective mutants tested, we found that only the Δ*pir2* mutant, lacking a cell wall O-mannosylated heat shock protein, displayed growth defects under cadmium and arsenate stress conditions. In addition, *PIR2* expression was upregulated upon exposure to cadmium and arsenate. Previously, it has been reported that the *PIR2* gene is required for tolerance to several stresses, such as cell wall-perturbing agents, heat stress, and nitrogen limitation (15, 16, 29, 30). Furthermore, Pir2p has been shown to bind to β-1,3-glucan of the cell wall by alkali-sensitive ester linkages via its repetitive sequences (17, 18, 31). Based on these findings, it is likely that the cross-linking of Pir2p to β-1,3-glucan may be important for maintaining cell wall stability during exposures to cell wall-damaging stresses, including cadmium and arsenate stresses. Since cadmium and arsenate are known to induce oxidative stress (32, 33) and Pir2p is essential for tolerances to oxidative stress-inducing agents such as H_2O_2 and diamide (16), it is also possible that Pir2p is important for protecting yeast cells against cadmium- and arsenate-induced oxidative stresses through its role in maintaining cell wall integrity. Since the mutants lacking essential genes are inviable, the deletion mutants lacking essential cell wall genes were not included in this analysis. Examples of such essential genes are *KRE5*, encoding a protein required for β-1,6-glucan biosynthesis, (34), *KRE9*, encoding a glycoprotein involved in cell wall β-glucan assembly (35), and *CHS2*, encoding chitin synthase II required for chitin synthesis in the primary septum during cytokinesis (36). Therefore, the roles of these essential genes in cadmium and arsenate tolerances could not be excluded. Moreover, only 38 mutants lacking genes involved in the CWI pathway and cell wall biosynthesis were tested, the requirement of other untested genes for yeast adaptive response to cadmium and arsenate stresses cannot be ruled out.

In addition to *PIR2*, the upregulated expression of four CWI-responsive cell wall genes, i.e., *CRH1*, *FKS2*, *PIR1*, and *PIR3*, was observed in the wild-type strain after cadmium exposure, whereas arsenate treatment induced the upregulation of only *CRH1* and *PIR1* in the wild-type strain. *CRH1*, which is functionally redundant with *CRH2*, encodes chitin transglycosylase involved in the transfer of chitin to β-1,3- and β-1,6-glucans in the cell wall (37). The *FKS2* gene encodes a stress-inducible catalytic subunit of β-1,3-glucan synthase (38). The *PIR1* and *PIR3* genes encode O-glycosylated cell wall proteins covalently bound to the cell wall via β-1,3-glucan, which are required for maintaining cell wall stability (39). Consistent with the expression levels of *FKS2*, the cell wall β-1,3-glucan level of the wild-type strain was significantly increased after exposure to cadmium but not arsenate. Nevertheless, we found that the resistance of wild-type cells to the β-glucan-lytic enzyme Zymolyase was elevated after treatments with cadmium and arsenate, suggesting that the modification of cell wall structure, espe-

cially the β-glucan network structure, was induced in response to both cadmium and arsenate stresses. Based on our results, the strengthening of cell wall structure by increased cross-linking of chitin and cell wall proteins to the glucan network seems to be an important mechanism for protecting yeast cells against cadmium and arsenate stresses.

On the other hand, the cadmium- and arsenate-sensitive Δ*slt2* mutant, lacking MAPK of the CWI pathway, was unable to upregulate the expression of five cell wall-related genes tested (i.e., *CRH1*, *FKS2*, *PIR1*, *PIR2*, and *PIR3*) upon exposures to cadmium and/or arsenate due to defective CWI signal transduction. In agreement with these findings, the Zymolyase resistance of this mutant was improved by neither cadmium nor arsenate, probably due to the lack of ability to induce cell wall remodeling via the CWI pathway. After cadmium exposure, although the upregulated expression of the *FKS2* gene, encoding the catalytic subunit of β-1,3-glucan synthase, was not detected in the Δ*slt2* mutant, increased levels of cell wall β-1,3-glucan were observed. Since the catalytic subunits of β-1,3-glucan synthase are encoded by two functionally redundant genes, i.e., the constitutive *FKS1* and the stress-inducible *FKS2* (38), the increased glucan biosynthesis in the CWI-defective Δ*slt2* mutant may be mainly mediated through the Fks1p-dependent mechanism.

Rlm1p and the SBF (Swi4p-Swi6p) complex, two major transcriptional activators of the CWI pathway, have been reported to be predominantly involved in the regulation of gene expression during cell wall stress and G1/S mitotic transition, respectively (12, 13). However, in the case of the *FKS2* gene, encoding the catalytic subunit of β-1,3-glucan synthase, it has been shown that *FKS2* gene expression is activated by both Rlm1p and the SBF complex in response to cell wall stress agents Congo red and calcofluor white (40), suggesting the cooperative role of Rlm1p and SBF complex in regulating the expression of some specific cell wall genes, including *FKS2*, during the adaptive response to cell wall stress. Our results revealed that, under cadmium stress conditions, the expression of *PIR2* and its paralog *PIR3*, encoding O-glycosylated cell wall proteins, was under the control of both Rlm1p and the SBF (Swi4p-Swi6p) transcriptional activators, while only Rlm1p and Swi4p were required for the expression of the other cell wall genes tested (i.e., *CRH1*, *FKS2*, and *PIR1*). These findings suggest that Rlm1p, Swi4p, and Swi6p transcription factors may coordinately modulate the expression of cell wall genes in response to cadmium-induced cell wall stress. Previously, it has been shown that Swi4p complexed with phosphorylated Slt2p can bind to the SBF-binding sites within the *FKS2* promoter independently of Swi6p (40). It is therefore possible that only Swi4p, possibly in the form of Swi4p-Slt2p complex, is sufficient to initiate the expression of some cell wall genes, including *CRH1*, *FKS2*, and *PIR1*, during cadmium stress. In the case of cell cycle regulation-associated genes, we found the expression of *CLN1*, *CLN2*, and *HO* genes under cadmium stress conditions was mainly controlled by the SBF (Swi4p-Swi6p) complex. It has been previously reported that the SBF complex is required for the transcriptional regulation of genes involved in cell cycle control and the DNA repair system during the G1/S transition, and also in response to DNA damage (41). In addition, cadmium is known to induce DNA damage, which in turn leads to cell cycle arrest to prevent cells with damaged DNA from further dividing and to allow time for DNA repair (42–44). Taken together, it is possible that the SBF (Swi4p-Swi6p) complex is involved in the transcriptional regulation of cell cycle regulation-associated genes, including *CLN1*, *CLN2*, and *HO*, in response to cadmium-induced DNA damage.

On the other hand, under arsenate stress conditions, we found that only Swi6p was required for the upregulation of *PIR2*, *CLN1*, *CLN2*, and *HO* genes. Although Swi6p can form complexes with three transcriptional coactivators (i.e., Swi4p, Mbp1p, and Stb1p), it has been reported that the cell wall stress-induced unfolded protein response (UPR) activity is regulated by the CWI pathway via Swi6p in a manner that is independent of its known coregulators (45). The UPR is essential for alleviation of endoplasmic reticulum (ER) stress caused by the accumulation of unfolded proteins in the ER through its role in enhancing protein folding and clearance of misfolded proteins (46). In budding

TABLE 1 Yeast strains used in this study

Strain	Relevant genotype	Deleted gene	Function of relevant gene product	Source	References
BY4742	*MATα; his3Δ1; leu2Δ0; lys2Δ0; uraΔ0*			Open Biosystems	
Cell wall integrity pathway					
Δ*wsc1*	BY4742 isogenic, *wsc1::KanMX4*	*WSC1*	Cell surface sensor	Open Biosystems	(13, 54)
Δ*wsc2*	BY4742 isogenic, *wsc2::KanMX4*	*WSC2*	Cell surface sensor	Open Biosystems	(13, 54)
Δ*wsc3*	BY4742 isogenic, *wsc3::KanMX4*	*WSC3*	Cell surface sensor	Open Biosystems	(13, 54)
Δ*mid2*	BY4742 isogenic, *mid2::KanMX4*	*MID2*	Cell surface sensor	Open Biosystems	(13, 54)
Δ*mtl1*	BY4742 isogenic, *mtl1::KanMX4*	*MTL1*	Cell surface sensor	Open Biosystems	(13, 54)
Δ*rom2*	BY4742 isogenic, *rom2::KanMX4*	*ROM2*	Guanine nucleotide exchange factor	Open Biosystems	(13, 55)
Δ*bck1*	BY4742 isogenic, *bck1::KanMX4*	*BCK1*	MAPKKK	Open Biosystems	(13, 56)
Δ*mkk1*	BY4742 isogenic, *mkk1::KanMX4*	*MKK1*	MAPKK	Open Biosystems	(13, 56)
Δ*mkk2*	BY4742 isogenic, *mkk2::KanMX4*	*MKK2*	MAPKK	Open Biosystems	(13, 56)
Δ*slt2*	BY4742 isogenic, *slt2::KanMX4*	*SLT2*	MAPK	Open Biosystems	(13, 56)
Δ*spa2*	BY4742 isogenic, *spa2::KanMX4*	*SPA2*	Scaffolding protein	Open Biosystems	(13, 57)
Δ*rlm1*	BY4742 isogenic, *rlm1::KanMX4*	*RLM1*	Transcription factor	Open Biosystems	(13, 58)
Δ*swi4*	BY4742 isogenic, *swi4::KanMX4*	*SWI4*	Component of SBF complex	Open Biosystems	(13, 59)
Δ*swi6*	BY4742 isogenic, *swi6::KanMX4*	*SWI6*	Component of SBF and MBF complexes	Open Biosystems	(13, 59)
Δ*mbp1*	BY4742 isogenic, *mbp1::KanMX4*	*MBP1*	Component of MBF complex	Open Biosystems	(13, 59)
Glucan synthesis and processing					
Δ*fks1*	BY4742 isogenic, *fks1::KanMX4*	*FKS1*	Subunit of β-1,3-glucan synthase	Open Biosystems	(12, 60)
Δ*fks2*	BY4742 isogenic, *fks2::KanMX4*	*FKS2*	Subunit of β-1,3-glucan synthase	Open Biosystems	(12, 60)
Δ*fks3*	BY4742 isogenic, *fks3::KanMX4*	*FKS3*	Protein with similarity to Fks1p and Fks2p	Open Biosystems	(12, 60)
Δ*kre6*	BY4742 isogenic, *kre6::KanMX4*	*KRE6*	Protein required for β-1,6-glucan synthesis	Open Biosystems	(12, 61)
Δ*bgl2*	BY4742 isogenic, *bgl2::KanMX4*	*BGL2*	Endo-β-1,3-glucanase	Open Biosystems	(12, 62)
Δ*gas1*	BY4742 isogenic, *gas1::KanMX4*	*GAS1*	β-1,3-Glucanosyltransferase	Open Biosystems	(12, 63)
Δ*exg1*	BY4742 isogenic, *exg1::KanMX4*	*EXG1*	Exo-β-1,3-glucanase	Open Biosystems	(12, 62)
Δ*exg2*	BY4742 isogenic, *exg2::KanMX4*	*EXG2*	Exo-β-1,3-glucanase	Open Biosystems	(12, 62)
Chitin synthesis and cross-linking					
Δ*chs3*	BY4742 isogenic, *chs3::KanMX4*	*CHS3*	Chitin synthase III	Open Biosystems	(12, 64)
Δ*skt5*	BY4742 isogenic, *skt5::KanMX4*	*SKT5*	Activator of Chs3p	Open Biosystems	(12, 64)
Δ*shc1*	BY4742 isogenic, *shc1::KanMX4*	*SHC1*	Activator of Chs3p	Open Biosystems	(12, 64)
Δ*crh1*	BY4742 isogenic, *crh1::KanMX4*	*CRH1*	Chitin transglycosylase	Open Biosystems	(12, 62)
Δ*crh2*	BY4742 isogenic, *crh2::KanMX4*	*CRH2*	Chitin transglycosylase	Open Biosystems	(12, 62)
Δ*crr1*	BY4742 isogenic, *crr1::KanMX4*	*CRR1*	Protein with similarity to Crh1p	Open Biosystems	(12, 62)
Cell wall proteins					
Δ*pir1*	BY4742 isogenic, *pir1::KanMX4*	*PIR1*	O-glycosylated cell wall proteins	Open Biosystems	(12, 65)
Δ*pir2*	BY4742 isogenic, *pir2::KanMX4*	*PIR2*	O-glycosylated cell wall proteins	Open Biosystems	(12, 65)
Δ*pir3*	BY4742 isogenic, *pir3::KanMX4*	*PIR3*	O-glycosylated cell wall proteins	Open Biosystems	(12, 65)
Δ*sed1*	BY4742 isogenic, *sed1::KanMX4*	*SED1*	GPI-cell wall glycoprotein	Open Biosystems	(12, 65)
Δ*pst1*	BY4742 isogenic, *pst1::KanMX4*	*PST1*	GPI protein	Open Biosystems	(12, 65)
Δ*cwp1*	BY4742 isogenic, *cwp1::KanMX4*	*CWP1*	Cell wall mannoprotein	Open Biosystems	(12, 65)
Δ*ygp1*	BY4742 isogenic, *ygp1::KanMX4*	*YGP1*	Cell wall glycoprotein	Open Biosystems	(12, 65)
Δ*ssr1*	BY4742 isogenic, *ssr1::KanMX4*	*SSR1*	Cell wall glycoprotein	Open Biosystems	(12, 65)
Δ*cis3*	BY4742 isogenic, *cis3::KanMX4*	*CIS3*	Cell wall glycoprotein	Open Biosystems	(12, 65)

yeast, the UPR signaling pathway is composed of an ER transmembrane sensor, Ire1p, which detects the extent of misfolded proteins in the ER, and a UPR-specific transcriptional activator, Hac1p, which controls the transcription of genes encoding ER-resident proteins, especially ER chaperones. During ER stress, the activated Ire1p removes the translation-inhibitory intron from *HAC1* mRNA and induces *HAC1* mRNA splicing, thereby leading to increased production of Hac1p. The transcription factor Hac1p is then translocated to the nucleus, where it activates the transcription of UPR target genes to enhance the capacity of the ER protein folding machinery (46). It has been reported that either the loss of the UPR sensor Ire1p or the hyperaccumulation of a misfolded protein leads to cell wall defects (45). Altogether, these findings suggest the interaction between the CWI and UPR signaling pathways in adaptive responses to extracellular cell wall stress and intracellular ER stress. Since arsenic has been reported to trigger ER stress (47–49), Swi6p may play an important role in controlling the expression of cell cycle-regulating genes in response to arsenate-induced ER stress.

TABLE 2 Primers used in this study

Target gene	Primer name	Sequence
CRH1	CRH1-qRT-F	5′-TGGTGACCAATCTGGTTCTTGGGA-3′
	CRH1-qRT-R	5′-ACTGCGAAATCTTCTTGGGCTTGG-3′
FKS2	FKS2-qRT-F	5′-CCGTCAAATCCGTCCTCCTAT-3′
	FKS2-qRT-R	5′-GTACAAGCTGCAATACCTCCTAACC-3′
PIR1	PIR1-qRT-F	5′-CAGCTCCAGCTCCAATTACT-3′
	PIR1-qRT-R	5′-CCTTTACCGTCAGTCAGGATAC-3′
PIR2	PIR2-qRT-F	5′-CTTTGGTTGCCTCTGCTTTG-3′
	PIR2-qRT-R	5′-GTAACACCACCGCTGTAAGT-3′
PIR3	PIR3-qRT-F	5′-CCATTAGTCGTCTCCGCTTTAG-3′
	PIR3-qRT-R	5′-CCACCCTTGTAAGTAGCTGATG-3′
CLN1	CLN1-qRT-F	5′-CTCGTATTCCACGCCTTTCT-3′
	CLN1-qRT-R	5′-AAACGTCCCAGTTCAGAGTATC-3′
CLN2	CLN2-qRT-F	5′-GTCTCTGGTTGGCTGCTAAA-3′
	CLN2-qRT-R	5′-CCTTGGGTTGGGACCATAAA-3′
HO	HO-qRT-F	5′-GTCACACAGGGCTATCAGAAA-3′
	HO-qRT-R	5′-TGTTCTACGCCTGGGATCTA-3′
ACT1	ACT1-458F	5′-TGGATTCCGGTGATGGTGTT-3′
	ACT1-529R	5′-TCAAAATGGCGTGAGGTAGAGA-3′

Nevertheless, to clarify the precise mechanism of the CWI-mediated regulation of gene expression in response to cadmium and arsenate, further studies on the expression profiles of cadmium- and arsenate-responsive genes under the control of the CWI pathway are indispensable.

MATERIALS AND METHODS

Yeast strains and growth conditions. *S. cerevisiae* strains used in this study are listed in Table 1. Cells were cultured at 30°C in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) with the optional addition of 200 mg liter^{-1} G418 (Geneticin; Sigma-Aldrich).

Spot susceptibility assay. Log-phase cells were adjusted to an optical density at 600 nm (OD_{600}) of 1.0 and then serially diluted 10-fold to a 10^{-5} dilution. An aliquot (3 μl) of each dilution was spotted onto YPD agar plates, and YPD agar plates containing 30 μM $CdCl_2$, 2 mM Na_2HAsO_4, 2 mM $CoCl_2$, or 2 mM $NiCl_2$ and incubated at 30°C for 3 days.

Zymolyase susceptibility test. Susceptibility to Zymolyase was carried out as described previously (50). Briefly, log-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 100 μg ml^{-1} (1 U) Zymolyase 20T (Zymo Research, USA). The susceptibility to Zymolyase was monitored by measuring OD_{600} at 15-min intervals for 2 h by the Wallace Victor 1420 microplate reader (PerkinElmer, USA).

Measurement of β-1,3-glucan content. The amount of β-1,3-glucan was measured as described previously (51) with some modifications. Briefly, log-phase cells were harvested, washed twice with TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.5), and resuspended in 250 μl of TE Buffer. After the addition of 6 N NaOH to a final concentration of 1 N, the cells were incubated at 80°C for 30 min followed by the addition of 1.05 ml of AB solution (0.03% aniline blue, 0.18 N HCl, and 0.49 M glycine/NaOH [pH 9.5]). The samples were incubated at 50°C for 30 min and then placed at room temperature for 30 min. The fluorescence intensity of each sample was measured at an excitation wavelength of 400 nm and an emission wavelength of 460 nm by the Infinite M200 multifunctional microplate reader (TECAN, Switzerland).

Measurement of chitin content. The amount of chitin was measured as described previously (52). Log-phase cells were harvested, washed twice with phosphate-buffered saline (PBS), and resuspended in PBS. Cells were then incubated with 20 μg ml^{-1} calcofluor white (CFW) at room temperature for 5 min, washed twice with PBS, and resuspended in PBS. The fluorescence intensity of each sample was measured at an excitation wavelength of 325 nm and an emission wavelength of 435 nm by the Infinite M200 multifunctional microplate reader (TECAN, Switzerland).

Antifungal susceptibility test. Log-phase cells were harvested, resuspended in YPD broth with and without the supplementation of 30 μM $CdCl_2$ or 2 mM Na_2HAsO_4, and incubated at 30°C for 12 h. Cells were then harvested, adjusted to an OD_{600} of 0.1 in YPD medium alone or YPD medium containing 0.2 μg ml^{-1} amphotericin B, 4 μg ml^{-1} ketoconazole, or 0.04 μg ml^{-1} caspofungin, and incubated with shaking (200 rpm) at 30°C. Cell growth was monitored at 0.5-h intervals for 20 h by measuring the OD_{600}.

RNA isolation and quantitative real-time RT-PCR assays. Total RNA isolation and quantitative real-time RT-PCR were performed as described previously (53). Briefly, total RNA from log-phase cells was isolated by using a FavorPrep Tissue Total RNA minikit (Favorgen, Taiwan) according to the manufacturer's instructions. The concentration and quality of RNA were determined by measuring the absorbance ratios at 260 nm/280 nm ($A_{260/280}$) and 260 nm/230 nm ($A_{260/230}$) and by gel electrophoresis. One microgram of each RNA sample was converted to cDNA by using the iScript cDNA synthesis kit (Bio-Rad, USA) following the manufacturer's instructions. Quantitative real-time PCR experiments were performed in the ABI 7500 instrument (Applied Biosystems, USA) using KAPA SYBR FAST qPCR kit (Kapa Biosystems, USA)

and 200 nM specific primers (Table 2). The reaction conditions were as follows: 95°C for 180 s, followed by 40 cycles of 95°C for 3 s, 60°C for 20 s, and 72°C for 20 s. A melting curve was generated at the end of each PCR to verify specific amplification of the target cDNA. The negative control contained no DNA template. Relative gene expression was calculated using the threshold cycle ($2^{-\Delta\Delta CT}$) method and normalized to *ACT1* mRNA levels.

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

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