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Improved Acetic Acid Resistance in *Saccharomyces cerevisiae* by Overexpression of the *WHI2* Gene Identified through Inverse Metabolic Engineering

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Development of acetic acid-resistant *Saccharomyces cerevisiae* is important for economically viable production of biofuels from lignocellulosic biomass, but the goal remains a critical challenge due to limited information on effective genetic perturbation targets for improving acetic acid resistance in the yeast. This study employed a genomic-library-based inverse metabolic engineering approach to successfully identify a novel gene target, *WHI2* (encoding a cytoplasmatic globular scaffold protein), which elicited improved acetic acid resistance in *S. cerevisiae*. Overexpression of *WHI2* significantly improved glucose and/or xylose fermentation under acetic acid stress in engineered yeast. The *WHI2*-overexpressing strain had 5-times-higher specific ethanol productivity than the control in glucose fermentation with acetic acid. Analysis of the expression of *WHI2* gene products (including protein and transcript) determined that acetic acid induced endogenous expression of Whi2 in *S. cerevisiae*. Meanwhile, the *whi2*Δ mutant strain had substantially higher susceptibility to acetic acid than the wild type, suggesting the important role of Whi2 in the acetic acid response in *S. cerevisiae*. Additionally, overexpression of *WHI2* and of a cognate phosphatase gene, *PSR1*, had a synergistic effect in improving acetic acid resistance, suggesting that Whi2 might function in combination with Psr1 to elicit the acetic acid resistance mechanism. These results improve our understanding of the yeast response to acetic acid stress and provide a new strategy to breed acetic acid-resistant yeast strains for renewable biofuel production.

Lignocellulosic biomass from nonfood stocks such as agricultural and forestry residues has been identified as the prime source for production of renewable biofuels to substitute for conventional fossil fuels in the face of growing demand for energy and rising concerns about greenhouse gas emissions (1–4). Bioconversion of plant cell wall materials by microbial fermentation is typically preceded by harsh (physico)chemical hydrolysis designed to release sugars; this hydrolysis treatment also generates by-products that are toxic to fermenting microorganisms (5, 6). Since hemicellulose and lignin in the plant cell wall are ubiquitously acetylated (7, 8), the typical acidic pretreatment of lignocellulosic biomass generates substantial amounts of acetic acid (with concentrations ranging from 1 g/liter to 15 g/liter) in the resulting hydrolysates (9, 10). Acetic acid severely inhibits cell growth and fermentation activity in *Saccharomyces cerevisiae* (5, 6, 11–13), the predominant microorganism used in industrial fermentation (14, 15). Therefore, improvement in *S. cerevisiae* resistance to acetic acid is highly desired and critical for achieving efficient and economically viable bioconversion of cellulosic sugars to biofuels.

The toxic effects of acetic acid in *S. cerevisiae* have been intensively characterized, and toxicity mechanisms have been proposed (10, 11, 16–20). When the external pH is lower than the pK_a of acetic acid (4.7), the undissociated form of acetic acid prevails and can enter the cells simply by diffusion through plasma membranes. The dissociation of acetic acid at neutral cytosolic pH can lead to intracellular acidification (6). As a result, the intracellular pH needs to be recovered by pumping out protons at the expense of ATP hydrolysis, which may induce cell growth arrest and reduced fermentation performance (21). In addition, intracellular anion accumulation can reach high levels and decrease the activity of some key enzymes for glycolysis (16).

However, the genetic basis of the yeast stress response to acetic acid remains unclear, making it difficult to improve the acetic acid resistance in *S. cerevisiae*. It is known that the yeast response to acetic acid stress involves genome-wide transcriptional changes (22–25). For example, upregulation of various genes involved in glycolysis, the Krebs cycle, and ATP synthesis was identified in yeast cells cultivated in the presence of acetic acid, indicating substantial alterations in carbohydrate and energy metabolism in acetic acid-stressed cells (26). Genome-scale transcriptional analyses suggested that the transcriptional activators Haa1 and Msn2 might be involved in regulating yeast adaptation to acetic acid (21, 26, 27). A large-scale chemical genomics study identified 648 genes whose deletion increased the susceptibility of yeast to acetic acid, and these gene determinants were found to be involved in carbohydrate metabolism, cell wall assembly, amino acid metabolism, internal pH homeostasis, biogenesis of mitochondria, and signaling and uptake of various nutrients (26). Recent studies reported cell-to-cell heterogeneity in acetic acid tolerance (28, 29). It was found that only a fraction of the cells within an isogenic *S. cerevisiae* population resumed growth under acetic acid stress (28)

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TABLE 1 Plasmids and strains

Plasmid or strain	Description	Reference or source
Plasmids		
pRS424	*TRP1*, a multicopy episomal plasmid	65
pRS425	*LEU2*, a multicopy episomal plasmid	65
pRS424GPD	pRS424 with the GPD promoter	65
pRS425GPD	pRS425 with the GPD promoter	65
pRS424GPD-*WHI2*	*WHI2* expressed in pRS424GPD	This study
pRS425GPD-*WHI2*	*WHI2* expressed in pRS425GPD	This study
pRS424GPD-*HAA1*	*HAA1* expressed in pRS424GPD	This study
pRS424GPD-*MSN2*	*MSN2* expressed in pRS424GPD	This study
pRS424GPD-*PSR1*	*PSR1* expressed in pRS424GPD	This study
pRS425GPD-*PSR1*	*PSR1* expressed in pRS425GPD	This study
Strains		
D452-2	*MATα leu2 his3 ura3 can1*	66
SR8	D452-2 expressing *XYL1*, *XYL2*, and *XKS1* through integration, evolutionary engineering in xylose-containing media, and *ALD6* deletion	31
SR8-*trp*	SR8 with *trp1* as the auxotrophic marker	Developed in Yong-Su Jin's lab
S-*msn2*Δ	*msn2* knockout mutant of SR8-*trp*	This study
SR8-*4*	SR8 with *trp1*, *leu2*, *his3*, and *ura3* as auxotrophic markers	Developed in Yong-Su Jin's lab
S-C1	SR8-*trp* harboring pRS424GPD	This study
S-*WHI2*	SR8-*trp* harboring pRS424GPD-*WHI2*	This study
S-*HAA1*	SR8-*trp* harboring pRS424GPD-*HAA1*	This study
S-*MSN2*	SR8-*trp* harboring pRS424GPD-*MSN2*	This study
S-*msn2*Δ-C1	S-*msn2*Δ harboring pRS424GPD	This study
S-*msn2*Δ-*WHI2*	S-*msn2*Δ harboring pRS424GPD-*WHI2*	This study
S-C2	SR8-*4* harboring pRS424GPD and pRS425GPD, as a control	This study
S-*WHI2*-c	SR8-*4* harboring pRS424GPD-*WHI2* and pRS425GPD	This study
S-*PSR1*-c	SR8-*4* harboring pRS424GPD-*PSR1* and pRS425GPD	This study
S-*WHI2-PSR1*	SR8-*4* harboring pRS424GPD-*WHI2* and pRS425GPD-*PSR1*	This study
BY4742	*MATα his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*	
BY2-*WHI2*	BY4742 harboring pRS425GPD-*WHI2*	This study
BY2-C1	BY4742 harboring pRS425GPD	This study

and that variations in the cytosolic pH of individual cells might contribute to the differences between cells (29). The findings suggested that genes related to cell-to-cell heterogeneity might be a potential pool for the search of genetic targets for improving acetic acid resistance. These prior results illustrate that the acetic acid stress response mechanism in *S. cerevisiae* is rather complex and involves coordinated regulations of multiple genes. Due to the currently incomplete understanding of relevant genetic and biomolecular networks for the acetic acid stress response, it is challenging to develop acetic acid-resistant yeast strains through knowledge-based rational metabolic engineering (30). In particular, information regarding the genetic targets and perturbation strategy for effectively engineering acetic acid-resistant yeast strains is needed.

This study employed a genomic-library-based inverse metabolic engineering approach to develop *S. cerevisiae* strains for improved fermentation of cellulosic sugars under acetic acid stress and identify genetic perturbation targets for enhancing the acetic acid resistance in the yeast. Specifically, we first introduced a genome-wide library into a parent *S. cerevisiae* strain containing an optimized xylose fermentation pathway, so that we could evaluate the transformants in the fermentation of glucose and/or xylose (the two most abundant sugars from lignocellulosic biomass) under acetic acid stress. We then characterized the screened transformants to identify the target of gene perturbation. Here we report on a novel gene target, *WHI2*. Overexpression of *WHI2* substantially enhanced the acetic acid resistance of *S. cerevisiae*. We show that acetic acid-induced endogenous expression of Whi2 and that deletion of the *WHI2* gene resulted in hypersensitivity to acetic acid. We further determined the function of Whi2 and its binding partner Psr1 in eliciting the acetic acid resistance mechanisms in *S. cerevisiae*. Last, we characterized improved performance of an engineered strain for glucose and/or xylose fermentation in the presence of toxic levels of acetic acid under industrially relevant conditions. The results improve our understanding of the stress response to acetic acid in *S. cerevisiae* and provide a new strategy for breeding acetic acid-resistant yeast strains for renewable biofuel production from lignocellulosic biomass.

MATERIALS AND METHODS

Strains and plasmids. All of the strains and plasmids used in this study are summarized in Table 1. The recombinant xylose-fermenting *S. cerevisiae* strain SR8 (31) was used in this study for glucose and xylose fermentation. The strain was constructed previously (31) through heterologous expression of *XYL1* (coding for xylose reductase [XR]), *XYL2* (coding for xylitol dehydrogenase [XDH]), and *XYL3* (coding for xylulokinase [XK]) from *Scheffersomyces stipitis* in *S. cerevisiae* D452-2 (*MATα leu2 ura3 his3 can1*) and optimization of the expression levels of XR, XDH, and XK, laboratory evolution on xylose, and deletion of *ALD6*, coding for acetaldehyde dehydrogenase. The auxotrophic marker genes in the strains SR8-*trp* and SR8-*4* (Table 1) were recovered by the CRISPR-cas9 method (32, 33). These strains were kindly provided by Yong-Su Jin's lab. The *Escherichia*

coli TOP10 strain was used for gene cloning and manipulation. The *whi2* knockout mutant derived from the laboratory strain BY4741 (GE Healthcare Dharmacon) was used for evaluating the susceptibility of the null mutant versus that of the wild type. The *msn2* knockout mutant derived from the strain SR8-*trp* was used for testing the hypothesis regarding the interaction of Whi2 and Msn2 in eliciting acetic acid resistance.

Enzymes, primers, and chemicals. Restriction enzymes, DNA-modifying enzymes, and other molecular reagents were obtained from New England BioLabs (Beverly, MA). The reaction conditions were set up following the manufacturer's instructions. All general chemicals and medium components were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Primers for both PCR and sequencing were synthesized by Integrated DNA Technologies (Coralville, IA) and are listed in Table S1 in the supplemental material.

Media and culture conditions. Yeast strains were routinely cultivated at 30°C in YP medium (10 g/liter of yeast extract and 20 g/liter of peptone) or synthetic complete (SC) medium (6.7 g/liter of yeast nitrogen base and appropriate amino acids or nucleotides) containing 20 g/liter of D-glucose (SCD). SC medium containing 20 g/liter agar and glucose without tryptophan and/or leucine amendment was used to select transformants using *TRP1* and/or *LEU2* as auxotrophic markers. The *E. coli* strains were grown in Luria-Bertani medium at 37°C, and 100 μg/ml of ampicillin was added to the medium when required.

Construction of *S. cerevisiae* genomic library and yeast transformation. The genomic DNA of the *S. cerevisiae* S288C strain was used to construct the genomic library as previously described (34). Briefly, genomic DNA fragments (2 to 5 kb) were generated by sonication and ligated into a multicopy plasmid, pRS424 (a yeast episomal plasmid), with *TRP1* as an auxotrophic selection marker (35, 36). Plasmid extraction was performed using the QIAprep spin miniprep kit (Germantown, MD). The genomic library was transformed to the *S. cerevisiae* strain SR8-*trp* using lithium acetate-polyethylene glycol (LiAc-PEG) methods (37).

Selection of transformants with acetic acid resistance. Yeast genomic library transformants were inoculated on SC medium agar plates (15-mm diameter), which contained glucose (20 g/liter) or xylose (20 g/liter) and different concentrations of acetic acid (2.0 g/liter, 2.5 g/liter, 3.0 g/liter, and 3.5 g/liter). The pH of the agar plates was adjusted to be 4.0 so that acetic acid was predominantly undissociated. The strain SR8-*trp* harboring the pRS424GPD plasmid (i.e., the strain S-C1 in Table 1) was used as the control under all conditions. Transformants were isolated from the plates with acetic acid concentrations at which the corresponding plates inoculated with the control strain had no colony grown. Then, the cell growth and sugar consumption rates of the isolated transformants were evaluated in liquid SC medium containing a toxic level of acetic acid and 20 g/liter glucose or 20 g/liter xylose. The initial cell biomass was adjusted to an optical density at 600 nm (OD_{600}) of 0.05. Plasmids from the selected transformants were isolated by a Zyppy plasmid miniprep kit (Zymo Research) and amplified by *E. coli* transformation. The isolated plasmids were retransformed into the strain SR8-*trp* to verify the effects of the plasmids on acetic acid resistance.

Insert identification and plasmid and strain construction. The verified plasmids showing effects in improving acetic acid resistance were sequenced using T3 and T7 primers (see Table S1 in the supplemental material) to determine the nucleotide sequences of the inserted genomic DNA fragments. Sequences were compared to the *S. cerevisiae* genome sequence to identify the insert sizes and open reading frames (ORFs). To construct overexpression plasmids, the complete open reading frames of the target genes (*WHI2*, *PSR1*, *HAA1*, or *MSN2*) were amplified by PCR with the primers listed in Table S1. The PCR products were subsequently digested and ligated to appropriate multiple cloning sites of the plasmid pRS424GPD or pSR425GPD. To construct the *msn2* mutant strain from SR8-*trp*, the *kanMX* marker gene flanked by about 200 bp homologous to upstream and downstream regions of the *MSN2* gene was PCR amplified from the genomic DNA of an *msn2* knockout strain derived from the laboratory strain BY4741 (GE Healthcare Dharmacon) with primers msn2-F and msn2-R, listed in Table S1. Transformation of the PCR product into SR8-*trp* was performed using an EZ yeast transformation kit (BIO 101). Positive transformants were selected on YP medium containing 20 g/liter glucose and 300 μg/ml Geneticin (G418). A diagnostic PCR was performed to confirm successful deletion, yielding the strain S-*msn2*Δ. The overexpression plasmids were transformed into the strain SR8-*trp*, SR8-*4*, or S-*msn2*Δ using an EZ yeast transformation kit.

Protein expression experiments and data analysis. A yeast green fluorescent protein (GFP) clone collection of *S. cerevisiae* BY4742 strains (no. 95702; Life Technologies), developed by oligonucleotide-directed homologous recombination to tag each ORF with *Aequorea victoria* GFP (S65T) in its chromosomal location at the 3′ end (38), was used in protein expression analysis in response to acetic acid stress. Precultured yeast cells with the GFP fusion protein Whi2 were grown in SCD medium to early exponential phase and then were inoculated into 200 μl SCD medium (OD_{600} = 0.2) in clear-bottom black 96-well plates (Costar, Bethesda, MD) containing different concentrations of acetic acid (0 g/liter, 2 g/liter, and 3 g/liter [pH 4.0]). The plates were then incubated in a microplate reader (Synergy HT multimode; Biotech, Winooski, VT) at 30°C with fast shaking, and the OD_{600} and GFP signal (fluorescence readings were excitation at 485 nm and emission at 528 nm) were simultaneously measured every 1 h for 6 h. All experiments were performed in triplicate.

The protein expression levels based on GFP signals were calculated using the method previously described (39, 40). The raw data from the OD_{600} and GFP measurements were corrected by considering the background signals in control with medium only, and the protein expression per cell unit for each measurement was calculated by normalizing the GFP data to the cell number (OD_{600}), with P equal to the GFP measurement over the OD_{600}. The expression of the *PGK1* gene product was used as the reference, and the P values for each protein of interest were normalized based on the mean P level of *PGK1* corresponding to the same condition on the same plate. The relative expression (RE) for the protein of interest at each time point under each acetic acid exposure condition versus the control condition without acetic acid was calculated as $RE = P_{aa}/P_c$, where P_{aa} refers to the normalized P in the experimental condition with acetic acid and P_c represents the corrected and normalized P in the control condition without acetic acid.

Reverse transcription-qPCR. The wild-type strain in early exponential phase was grown in SC medium containing glucose (20 g/liter) and acetic acid (2 g/liter) or in medium without acetic acid (control condition). After 6 h of incubation, cells from triplicate experiments were harvested by centrifugation for 30 s at 1,000 rpm under 4°C, and RNA was immediately extracted by using a PureLink RNA minikit (Life Technologies, NY) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories, CA). The cDNA was then used for quantitative PCR (qPCR) analysis using the primers in Table S1 in the supplemental material on a CFX Connect real-time system (Bio-Rad Laboratories) and by using iQ SYBR green supermix (Bio-Rad Laboratories). To confirm the amplification specificity, the PCR products were subjected to melting curve analysis and gel electrophoresis. All of the measurements were performed in triplicate for each biological triplicate ($n = 3 \times 3$). Gene expression was calculated by the quantification method (41) as $(1 + E)^{Cq}/(1 + E_{ref})^{Cq,ref}$, where E refers to application efficiency and Cq refers to the quantification cycle value.

Fermentation experiments. Batch fermentation experiments under oxygen-limited conditions were performed in 125-ml Erlenmeyer flasks containing 20 ml medium, and anaerobic batch fermentation experiments were performed in 160-ml serum bottles that contained 20 ml medium and were sealed with butyl rubber stoppers, at 30°C and 100 rpm. The anaerobic fermentation media were prepared by flushing with nitrogen which had passed through a heated, reduced-copper column to remove trace oxygen. Precultured yeast cells in SCD medium were centrifuged and washed with sterilized water and then inoculated into fermentation media containing glucose/xylose and acetic acid. The initial cell

densities were adjusted to an OD_{600} of 1. The initial pH of the medium was adjusted to 4.0. For the anaerobic fermentation experiments, ergosterol and Tween 80 were added to final concentrations of 0.01 g/liter and 0.42 g/liter, respectively (42). Culture samples were taken from fermentation experiments to measure the OD_{600} values and concentrations of metabolites. For anaerobic fermentation experiments, samples were collected by sterile syringes and 26-gauge BD needles. Yeast cell dry weight was determined using a microwave method as described previously (43). All of the fermentation experiments were performed in duplicate.

As for fermentation with cellulosic hydrolysates, the corn stover hydrolysate was prepared by the National Renewable Energy Laboratory (NREL) (http://www.nrel.gov/biomass/pdfs/47764.pdf) through dilute acid pretreatment. The hydrolysate liquid fraction contained 10.9 g/liter acetic acid, 115 g/liter xylose, and 17 g/liter glucose. The hydrolysate was mixed with YP medium at a 50% (vol/vol) ratio to result in a hydrolysate mixture containing around 3.5 g/liter acetic acid, 40 g/liter xylose, and an adjusted glucose concentration of 20 g/liter. The pH was adjusted to 4.0. The fermentation experiments were set up in flasks under oxygen-limited conditions as described above.

Analytical methods. Cell growth was monitored by measuring the OD_{600} using a UV-visible spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). Glucose, xylose, xylitol, glycerol, acetic acid, and ethanol were quantified by a high-performance liquid chromatograph (Agilent Technologies 1200 series) equipped with a refractive index detector and a Rezex ROA-organic acid H^+ (8%) column (Phenomenex Inc., CA). The column was eluted with 0.005 N H_2SO_4 as the mobile phase under the flow rate of 0.6 ml/min at 50°C.

RESULTS

Screening of transformants exhibiting improved acetic acid resistance. With the goal of obtaining transformants that grew faster with acetic acid stress under both glucose- and xylose-consuming conditions, a method that combined selection on agar plates and screening in liquid medium containing toxic levels of acetic acid was developed. The plates inoculated with the genomic-library transformants had colonies grown on glucose- or xylose-containing agar plates with up to 3 g/liter acetic acid (pH 4.0) within 3 to 5 days. In contrast, the plates inoculated with the control strain S-C1 (Table 1) did not have any colony grown at acetic acid concentrations of >2 g/liter through 2 weeks of incubation. Then 130 fast-growing transformants were isolated from the plates containing 2.5 g/liter or 3 g/liter acetic acid (65 from the glucose-containing plates and 65 from the xylose-containing plates).

We further screened the isolated transformants in liquid SC medium to identify strains with superior resistance to acetic acid under both glucose-consuming and xylose-consuming conditions. Among the 65 transformants isolated from xylose-containing plates, 10 transformants (numbers 7, 32, 34, 35, 36, 38, 41, 49, 52, and 59) grew much faster than the control strain S-C1 in liquid medium containing glucose plus acetic acid (see Fig. S1A in the supplemental material), and 5 out of these 10 (numbers 34, 35, 38, 52, and 59) also exhibited significant improvements in liquid medium containing xylose plus acetic acid (Fig. S1B). As for the 65 transformants isolated from glucose-containing plates, while some transformants showed improved cell growth in medium containing glucose plus acetic acid, no significant improvements were observed when these transformants were grown in medium containing xylose plus acetic acid (data not shown). A possible reason is that the yeast was more sensitive to acetic acid when grown on xylose than on glucose; a similar observation was also reported in a previous study (44).

Plasmids from each of the five selected transformants were isolated and retransformed into the parental strains, respectively, to confirm the effects. Four plasmids (numbers 34, 38, 52, and 59) showed positive effects in the retransformed strains comparable to those in the original transformants in terms of cell growth and sugar consumption under acetic acid stress. The number 35 plasmid did not show improved acetic acid resistance in the retransformed strain, indicating that the increased resistance in the original transformant might be due to genome mutations. Sequence analysis of the four confirmed plasmids revealed that three (numbers 34, 38, and 59) had genome coordinates similar to those of the identified targets (chromosome XV [chrXV], nucleotide [nt] positions 409259 to 412369 for numbers 34 and 38; chrXV, nt positions 410675 to 412722 for number 59), while plasmid number 52 did not harbor any intact open reading frame (chrIX, nt positions 4597 to 5728).

All of the three inserts harbored a complete sequence of the gene *WHI2* (chrXV, nt positions 410870 to 412330). The *WHI2* gene product in *S. cerevisiae* is a 55-kDa cytoplasmatic globular scaffold protein (38). Whi2 is involved in coordinating cell growth and proliferation and plays an important role in nutrient-dependent cell cycle arrest (45–48). The protein has also been reported to be required for activation of the general stress response in the yeast (49–51).

Enhanced acetic acid resistance by overexpression of the *WHI2* gene. The effect of overexpression of *WHI2* on acetic acid resistance in *S. cerevisiae* has not been reported before, and the role of Whi2 in the acetic acid stress response is unclear. To determine the effect of *WHI2* as a gene perturbation target for improving acetic acid resistance, we introduced a multicopy plasmid overexpressing *WHI2* under the control of the constitutive *GPD* (*TDH3*) promoter and *CYC1* terminator (Table 1) into *S. cerevisiae* strain SR8-*trp* or BY4742 (i.e., *WHI2* overexpression in different strain backgrounds), yielding strains S-*WHI2* and BY2-*WHI2* (Table 1), and the corresponding control strains S-C1 and BY2-C1 harbored the plasmid without the *WHI2* insert. The strain S-*WHI2* had noticeably higher cell growth under acetic acid stress than the control strain S-C1, according to the results of yeast spotting assays on SC agar plates containing glucose (20 g/liter) and different concentrations of acetic acid (Fig. 1A). When grown on plates with xylose as the substrate, the strain S-*WHI2* also had substantially higher acetic acid resistance than the control strain (Fig. 1B). Overexpression of *WHI2* in another strain background (BY-*WHI2*) also showed improvement (see Fig. S2 in the supplemental material).

Batch experiments were conducted to characterize the glucose/xylose fermentation performances of the strain S-*WHI2* versus those of the control strain S-C1 under acetic acid stress. Figure 2 shows the ethanol production and sugar consumption profiles of the two strains. In glucose fermentation under oxygen-limited conditions, glucose (20 g/liter) was completely consumed within 39 h by S-*WHI2* with the presence of 2.5 g/liter acetic acid, while the control strain S-C1 consumed only 3.2 g/liter glucose (Fig. 2A). The specific sugar consumption rate and specific ethanol productivity of the strain S-*WHI2* were both >5 times higher than those of the control strain (see Table S2 in the supplemental material). The pH of the fermentation medium was 4.0 initially and decreased to 3.3 at the end of S-*WHI2* fermentation; a toxic level of the protonated form of acetic acid prevailed during the fermentation process. It was noted that the fermentation profiles of S-*WHI2* and S-C1 had no significant differences under the condi-

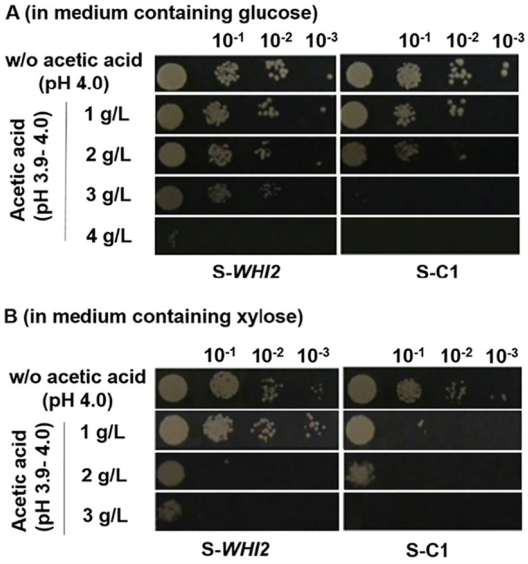

FIG 1 Increased cell growth conferred by overexpression of *WHI2*. Cells were grown on minimal medium agar plates containing glucose (20 g/liter) (A) or xylose (20 g/liter) (B), amended with various concentrations of acetic acid or without (w/o) acetic acid. Cells of the strain S-*WHI2* or the control strain S-C1 were spotted in serial dilutions (diluted by a factor of 10).

tion without acetic acid (with a pH of <4.0 during fermentation) (Fig. 2B), suggesting that the improvement brought by *WHI2* overexpression is associated with the cellular response to acetic acid stress. It was noted that the ethanol yields in glucose fermentation with acetic acid were higher than the yields without acetic acid for both S-*WHI2* and S-C1 (Table S2), probably due to the stimulating effects on glucose fermentation by low concentrations of weak acids as reported in previous studies (52, 53). A similar level of improvement in the fermentation by the strain S-*WHI2* versus that of S-C1 was also observed under strict anoxic conditions (Table S2).

Additionally, xylose fermentation under acetic acid stress also showed significant improvement by overexpression of *WHI2*. The strain S-*WHI2* consumed xylose with a specific rate of 0.245 ± 0.004 g/g cells (dry weight)/h under the oxygen-limited condition, while the control strain did not consume xylose in the experimental time frame (Fig. 2C). In the absence of acetic acid, the two strains had similar xylose fermentation profiles (Fig. 2D), suggesting that *WHI2* overexpression did not have a significant impact on the xylose fermentation pathway itself. Xylose fermentation under anaerobic conditions proceeded slowly due to the intrinsic limitation of the xylose-assimilating pathway of the fungi. No cell growth was observed with 2.5 g/liter acetic acid, and thus the lower level of acetic acid (1.5 g/liter) was used in the experiment. There were significant improvements (t test, $P < 0.05$) in the sugar consumption rate (0.23 ± 0.03 g sugar/g cells]dry weight]/h) and ethanol productivity (0.074 ± 0.007 g ethanol/g cells [dry weight]/h) in S-*WHI2* compared to that in S-C1 (0.195 ± 0.01 g sugar/g cells [dry weight]/h and 0.066 ± 0.001 g ethanol/g cells [dry weight]/h) (see Table S2 in the supplemental material). Overall, the data showed that overexpression of *WHI2* might improve the yeast growth and ethanol fermentation from glucose or xylose under acetic acid stress.

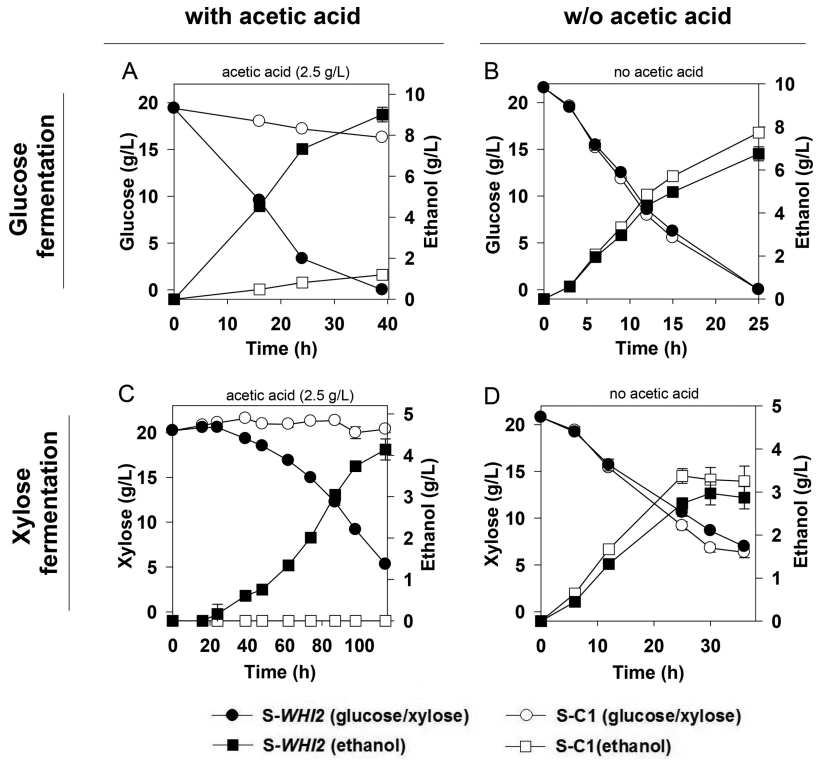

FIG 2 Improved fermentation by the strain S-*WHI2* compared to that of the control strain S-C1 in SC medium containing glucose (20 g/liter) (A and B) or xylose (20 g/liter) (C and D) under oxygen-limited conditions with or without acetic acid. Sugar consumption and ethanol production are shown. The results are the means from duplicate experiments; error bars indicating standard deviations are not visible when smaller than the symbol size.

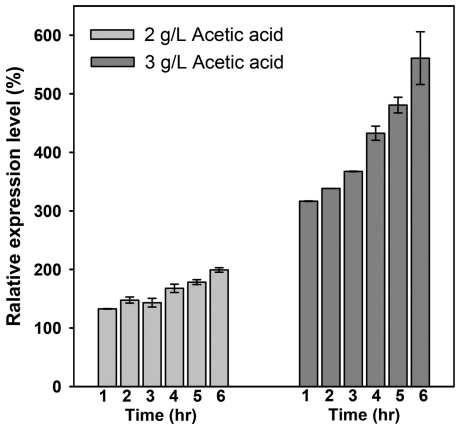

FIG 3 Effects of acetic acid on the expression of Whi2. Yeast strains with GFP fusion proteins in exponential phase of growth were incubated in SCD medium amended with acetic acid (2 g/liter or 3 g/liter [pH 4.0]) for 6 h. The protein expression level of Pgk1 was used as the reference for normalization. The normalized protein level in cells without acetic acid stress was taken as 100%. The results are the means from triplicate experiments; error bars represent standard deviations.

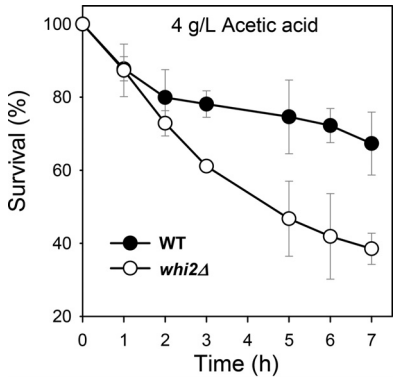

FIG 4 Stress susceptibility of the *whi2*Δ mutant versus that of the wild-type strain *S. cerevisiae* BY4741 to acetic acid. Cells in exponential growth phase in minimal medium were treated with acetic acid (4 g/liter [pH 4.0]), and the CFU were counted as a function of time. The results are the means from triplicate experiments; error bars represent standard deviations.

The acetic acid concentration in glucose fermentation did not change significantly, most likely due to carbon catabolite repression by glucose, while the acetic acid concentration in xylose fermentation started to decrease after 40 h and was reduced by ~1 g/liter at the end of the fermentation experiment. Under anoxic conditions, no acetic acid consumption occurred in glucose or xylose fermentation, as *S. cerevisiae* does not metabolize acetic acid without oxygen (43). Noticeably, when no acetic acid consumption occurred, there was still significant improvement in fermentation by strain *S-WHI2* versus that by the control strain S-C1 (Fig. 2; see also Table S2 in the supplemental material), suggesting that the positive impact of *WHI2* overexpression was not due to acetic acid consumption.

Acetic acid-induced expression of Whi2. The positive effect brought by overexpression of *WHI2* motivated us to examine how the endogenous expression of Whi2 would change in response to acetic acid. The *S. cerevisiae* strains with GFP fusion proteins (no. 95702; Life Technologies) related to each target were employed to examine protein expression levels. A detailed description of the methods and data analysis was provided in Materials and Methods. The relative expression levels of Whi2 in the presence of acetic acid (2 g/liter or 3 g/liter) versus those under the control condition without acetic acid over time are shown in Fig. 3. Under the condition with 2 g/liter acetic acid (pH 4.0), expression of Whi2 was induced, and the expression level increased during the exposure time period (Fig. 3). At the higher concentration of acetic acid (3 g/liter), the Whi2 protein had substantially increased expression levels. The longer the exposure time was, the higher the expression level was (Fig. 3), and the expression level of Whi2 with 6 h of incubation with acetic acid (3 g/liter) was 550% of that without acetic acid. The results showed that acetic acid activated the endogenous expression of Whi2 in *S. cerevisiae*.

To further confirm the expression level of the *WHI2* gene product, we determined the transcriptional changes of *WHI2* in response to acetic acid stress through reverse transcription-quantitative PCR. The transcriptional level of *WHI2* in the wild-type strain under the condition of 2 g/liter acetic acid was (2.84 ± 0.07)-fold of that under the control condition without acetic acid. The transcriptional analysis together with the protein expression results confirmed that endogenous expression of the *WHI2* gene was induced by acetic acid stress.

Hypersensitivity to acetic acid in a deficient mutant of Whi2. To evaluate the importance of Whi2 in yeast resistance to acetic acid, we characterized the susceptibility of the *whi2*Δ mutant to acetic acid stress compared to that of the wild type. The cell viability under high acetic acid stress was quantified as a function of time in the presence of 4 g/liter acetic acid (pH 4.0) (Fig. 4). From the viability data from 1 h to 7 h, the cell death rates were calculated based on first-order kinetics. The death rate for the *whi2*Δ mutant strain was determined to be 0.143 ± 0.042 h^{-1}, which was nearly 3 times higher than that of the wild-type strain (death rate, 0.047 ± 0.012 h^{-1}). These results suggested that Whi2 might play a critical role in the yeast response to acetic acid stress.

Whi2 and its binding partner Psr1 were both involved in eliciting acetic acid resistance. It has been suggested in the existing literature that Whi2 can interact with the plasma membrane phosphatase Psr1 as the binding partner for full activation of the stress response in *S. cerevisiae* (49, 54). Thus, we hypothesized that Whi2 might function in combination with Psr1 to induce acetic acid resistance. To test the hypothesis, we investigated the effect of perturbation of the *PSR1* gene on acetic acid tolerance. We constructed a new group of recombinant yeast strains that overexpressed *WHI2*, *PSR1*, or *WHI2* plus *PSR1* or contained a control plasmid (i.e., the strains S-*WHI2*-c, S-*PSR1*-c, S-*WHI2-PSR1*, and S-C2, respectively, listed in Table 1). The batch fermentation results showed that the strain S-*WHI2-PSR1* had the highest cell growth rate (Fig. 5A), glucose consumption rate (Fig. 5B), and ethanol productivity (Fig. 5C) among the four strains. The strain S-*PSR1-c* showed fermentation performance comparable to that of the strain S-*WHI2*-c. Noticeably, overexpression of *WHI2* and *PSR1* simultaneously resulted in substantially higher acetic acid resistance than overexpression of each gene individually (Fig. 5A to C). Such a synergistic effect indicated that the pathway involving both Whi2 and Psr1 might contribute to eliciting endogenous acetic acid resistance in *S. cerevisiae*. A possible mechanism for Whi2/Psr1 involving the stress response to acetic acid is proposed in Fig. 5D and is addressed in more depth in Discussion.

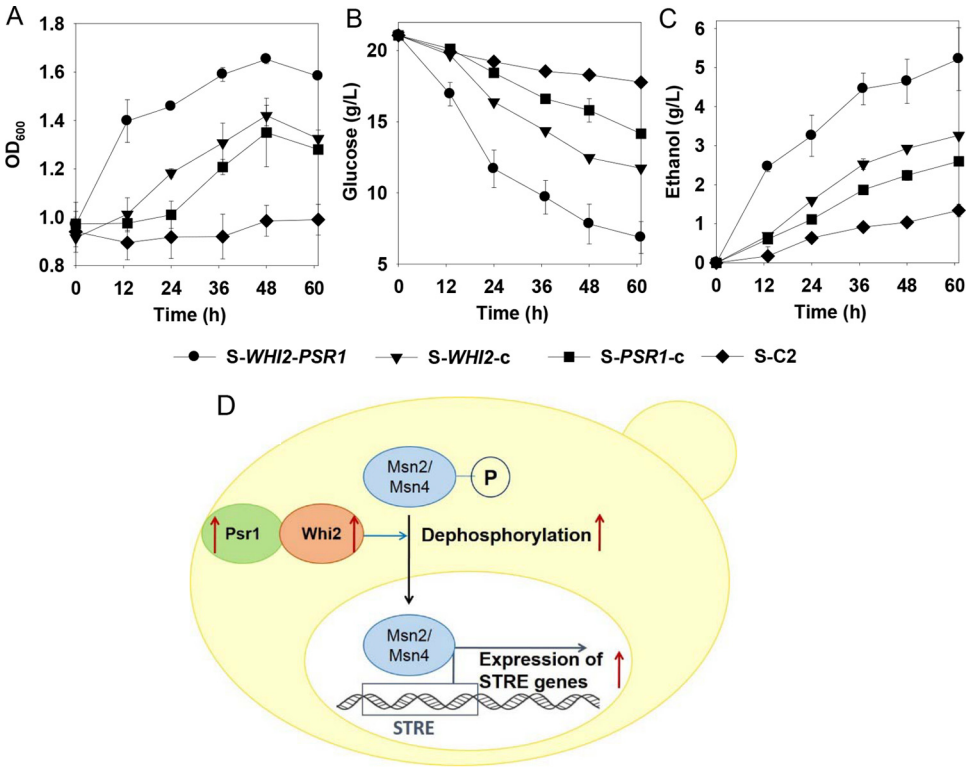

FIG 5 Improved acetic acid resistance by overexpression of *WHI2* and/or *PSR1* gene targets. Cell growth (A), glucose consumption (B), and ethanol production (C) were determined during fermentation in SC medium containing glucose (20 g/liter) and acetic acid (2.5 g/liter [pH 4.0]). The results are the means from duplicate experiments; error bars indicating standard deviations are not visible when smaller than the symbol size. (D) Proposed mechanism involving Whi2 and Psr1 in eliciting the acetic acid stress response in *S. cerevisiae*.

Improved fermentation by the engineered acetic acid-resistant strain. The *WHI2*-overxpressing strain S-*WHI2* was further characterized under industrially relevant conditions, including fermentation of a high glucose concentration (80 g/liter) or of mixed sugars (40 g/liter glucose plus 40 g/liter xylose) with acetic acid (2.5 g/liter [pH 4.0]), and fermentation with real cellulosic hydrolysates. As expected from the results of earlier experiments, the strain S-*WHI2* grew better and had much faster sugar fermentation under conditions with a high glucose concentration (Fig. 6A) or a glucose-plus-xylose mixture (Fig. 6B) with acetic acid stress than the control strain. The control strain did not have observable cell growth and consumed only a small portion of glucose under both conditions (10 g/liter and 18 g/liter, respectively). In the fermentation by S-*WHI2*, the acetic acid concentration did not decrease substantially until the glucose concentration became low after 24 h (Fig. 6A) or almost depleted in a mixed-sugar fermentation (Fig. 6C). A possible explanation is that acetic acid was consumed as an alternative substrate due to alleviated carbon catabolite repression as glucose was fermented in the yeast. In addition, the results indicated that the positive impact by *WHI2* overexpression might not be attributed to acetic acid consumption, although the reduced acetic acid concentration during the later phase of sugar fermentation might contribute to detoxification of the medium. The improved sugar fermentation was also demonstrated in corn stover hydrolysates, where fermentation by the control strain was severely inhibited (see Fig. S3 in the supplemental material).

DISCUSSION

Improving the resistance of *S. cerevisiae* to acetic acid, a major fermentation inhibitor, is highly desirable and important for achieving efficient and cost-effective biofuel production from lignocellulosic biomass (5, 30). Prior research efforts have significantly advanced the understanding of the toxic effects of acetic acid and the stress response in *S. cerevisiae* (5, 6, 22, 26). However, engineering of yeast strains with superior acetic acid resistance remains a challenge due to the limited information regarding which genes can be effective perturbation targets. The present study applied an inverse metabolic engineering approach and identified a novel gene overexpression target, *WHI2*, that substantially improved the acetic acid resistance in *S. cerevisiae*. Results from the characterization experiments, including induced expression of Whi2 under acetic acid stress, increased the susceptibility of the *whi2*Δ mutant versus that of the wild type, and the synergistic effects of coexpressing Whi2 and its binding partner Psr1 clearly suggested that Whi2 could play an important role in eliciting endogenous acetic acid resistance in *S. cerevisiae*. The engineered strain developed herein demonstrated improved glucose and/or xylose fermentation in the presence of toxic levels of acetic acid. Results from this study contribute to the breeding of yeast strains with superior acetic acid resistance for achievement of efficient and cost-effective biofuel production from lignocellulosic biomass.

The remarkable improvement in acetic acid resistance by overexpression of the *WHI2* gene was observed in different strain

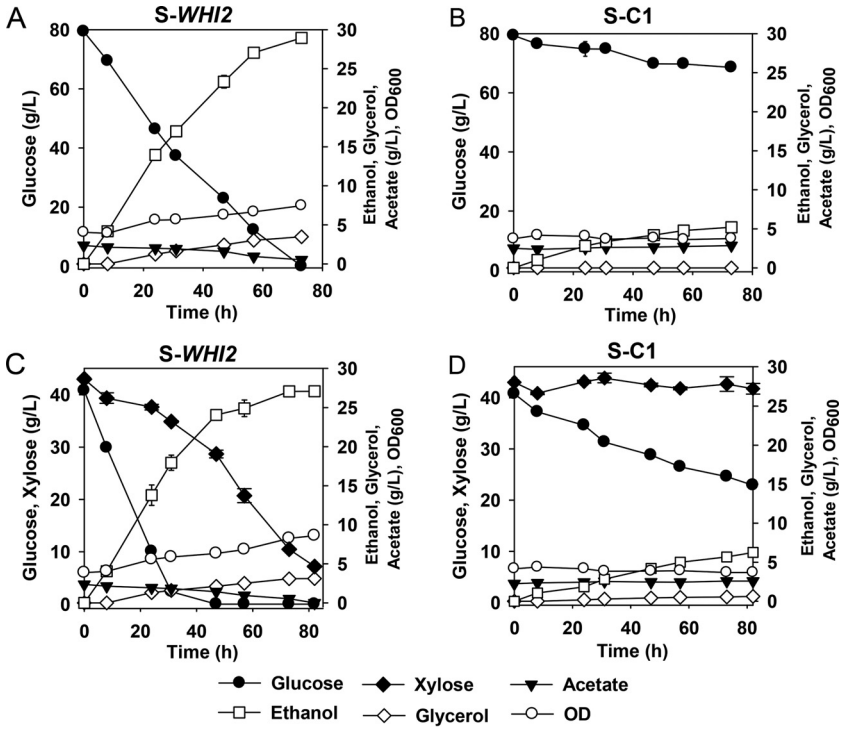

FIG 6 Improved fermentation by the strain S-*WHI2* compared to that of the control strain S-C1 in minimal medium containing 80 g/liter glucose (A and B) and 40 g/liter glucose plus 40 g/liter xylose (C and D), both with acetic acid (2.5 g/liter [pH 4.0]). The results are the means from duplicate experiments; error bars indicating standard deviations are not visible when smaller than the symbol size.

backgrounds in our study, suggesting that Whi2 could provide a protective effect against acetic acid stress in *S. cerevisiae*. Whi2 is a 55-kDa cytoplasmatic globular scaffold protein in *S. cerevisiae* (38); it is involved in coordinating cell proliferation and nutrient-dependent cell cycle arrest (45–47). For example, *whi2*Δ mutant cells failed to cease cell division with nutrient depletion (45, 55) and had much smaller cell sizes than the wild-type cells (56). Deletion of *WHI2* resulted in cells that failed to accumulate storage carbohydrate compounds and had reduced resistance to environmental stress, such as heat shock (46, 57). This study found that the *whi2*Δ mutant had significantly lower cell viability than the wild type in the presence of acetic acid stress, which is consistent with a previous observation that deletion of *WHI2* in *S. cerevisiae* led to increased cell susceptibility to propionic acid and acetic acid (22).

While overexpression of *WHI2* substantially enhanced acetic acid resistance in the yeast, it was noted that the induction level of endogenous *WHI2* expression by 2 g/liter acetic acid was relatively low. Similarly, the *HAA1* gene was reported in a previous study to be an overexpression target for improving acetic acid resistance in *S. cerevisiae* (58), but the induction of *HAA1* expression in the wild-type strain under acetic acid stress (6 g/liter acetic acid) was only 2.1-fold of that in the absence of acetic acid (25). These findings suggested that an effective gene overexpression target for improving yeast acetic acid resistance does not necessarily have high endogenous expression in response to acetic acid. The relatively low induction level of *WHI2* expression with acetic acid stress could also be a reason why it was not reported in previous transcriptomic analysis, which generally focused on analyzing genes with substantially different expression levels. Furthermore, the exposure time to acetic acid stress had a considerable impact on the induction level of Whi2 expression. The short timing of the response in previous transcriptomic analysis (e.g., sampling after only 30 min of exposure) (26) might be another reason why the gene *WHI2* was not identified. Further work is needed to elucidate the mechanism through which Whi2 is involved in the stress response to acetic acid.

The synergistic effect by overexpression of *WHI2* and *PSR1* together on enhancement of acetic acid resistance provided new insight to possible endogenous stress response mechanisms to acetic acid in *S. cerevisiae*. Psr1 is a plasma membrane phosphatase which was first identified to be required for the sodium osmostress response (54). It was found that Whi2 can bind Psr1 and form a functional complex to interact with Msn2/Msn4 for full activation of gene expressions controlled by stress response elements (STREs) (49, 59). The transcription factors Msn2/Msn4 were regarded to be localized in the cytoplasm in the phosphorylated form under nonstress conditions and to be dephosphorylated and translocated into the nucleus under stress conditions, where they can activate STRE-mediated gene expression (60). Genetic studies on the function of Whi2/Psr1 showed that *whi2* or *psr1* null mutant strains had hyperphosphorylation of Msn2/Msn4 and reduced transcription of STRE-controlled genes (49). As such, a possible mechanism to explain the observed synergistic effect of overexpression of *WHI2* and *PSR1* in improving yeast acetic acid resistance is illustrated in Fig. 5D. Since Whi2 can bind the phosphatase Psr1 to mediate dephosphorylation of Msn2/Msn4, increases in the expression levels of both *WHI2* and *PSR1* might facilitate the formation of the Whi2-Psr1 functional complex to a larger extent than overexpression of either target alone and thus greatly enhance the dephosphorylation of the phosphorylated

forms of Msn2/Msn4. The increased level of dephosphorylated Msn2/Msn4 might induce the expression of stress response genes related to acetic acid tolerance and consequently improve the resistance phenotype. The hypothesized mechanism was supported by the observation that the improvement in acetic acid tolerance brought by overexpression of *WHI2* in the *msn2*Δ mutant strain (i.e., S-*msn2*Δ-*WHI2* versus S-*msn2*Δ-C1) was smaller than that in the wild-type strain (i.e., S-*WHI2* versus S-C1) (see Fig. S4 in the supplemental material). Deletion of *MSN2* did not completely eliminate the gain in acetic acid tolerance by overexpression of *WHI2*, probably because the activity of Msn4 (the Msn2 homolog) still remained. Our ongoing work is focused on the transcriptomic and metabolomic analysis of the engineered resistant strain versus the control strain to further determine the molecular mechanisms for improved acetic acid resistance in yeast.

It is worth mentioning that overexpression of *WHI2* had an effect on improving acetic acid resistance superior to that of overexpression of *HAA1* or *MSN2* (see Fig. S5 in the supplemental material). Haa1 and Msn2 were identified as two important transcription factors that regulate genes associated with acetic acid responses in previous genome-wide analysis studies (21, 26). In particular, Haa1 regulates approximately 80% of the genes induced in acetic acid-stressed cells (26), and the effect of overexpression of *HAA1* on acetic acid tolerance was reported previously (58). However, overexpression of *HAA1* had a considerably smaller positive effect than overexpression of *WHI2* on improving acetic acid resistance in the yeast (see Fig. S5 in the supplemental material). On the other hand, although the transcriptional activator Msn2 (and its homolog Msn4) was found to regulate many genes induced by weak acids (22, 23, 26), overexpression of *MSN2* did not result in observable improvement in acetic acid resistance. It was also noted before that activation of the Msn2p/Msn4p regulon did not necessarily result in enhanced resistance to weak acids (22, 23). Based on our proposed mechanism, a possible explanation for no observable improvement in the strain overexpressing *MSN2* is that increasing *MSN2* transcription alone does not necessarily mean a higher level of dephosphorylated Msn2 and its appropriate localization to the nucleus for activating the STRE-mediated gene expression. All of these observations suggested the need for identifying effective gene targets through methods that directly select resistant yeast strains with traceable genetic perturbations. Successful application of inverse metabolic engineering in the present study and some previous works (34, 61–64) demonstrated the effectiveness of this approach in discovering novel gene perturbation targets for improving the desirable target phenotypes.

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