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Nuclear Localization of Haa1, Which Is Linked to Its Phosphorylation Status, Mediates Lactic Acid Tolerance in *Saccharomyces cerevisiae*

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Improvement of the lactic acid resistance of the yeast *Saccharomyces cerevisiae* is important for the application of the yeast in industrial production of lactic acid from renewable resources. However, we still do not know the precise mechanisms of the lactic acid adaptation response in yeast and, consequently, lack effective approaches for improving its lactic acid tolerance. To enhance our understanding of the adaptation response, we screened for *S. cerevisiae* genes that confer enhanced lactic acid resistance when present in multiple copies and identified the transcriptional factor Haa1 as conferring resistance to toxic levels of lactic acid when overexpressed. The enhanced tolerance probably results from increased expression of its target genes. When cells that expressed Haa1 only from the endogenous promoter were exposed to lactic acid stress, the main subcellular localization of Haa1 changed from the cytoplasm to the nucleus within 5 min. This nuclear accumulation induced upregulation of the Haa1 target genes *YGP1*, *GPG1*, and *SPI1*, while the degree of Haa1 phosphorylation observed under lactic acid-free conditions decreased. Disruption of the exportin gene *MSN5* led to accumulation of Haa1 in the nucleus even when no lactic acid was present. Since Msn5 was reported to interact with Haa1 and preferentially exports phosphorylated cargo proteins, our results suggest that regulation of the subcellular localization of Haa1, together with alteration of its phosphorylation status, mediates the adaptation to lactic acid stress in yeast.

Plastics made from chemicals derived from renewable carbon sources like polylactic acid have been receiving increasing attention in relation to controlling atmospheric CO_2 emissions (1). Lactic acid bacteria are generally used for the production of lactic acid, the building block chemical for polylactic acid. These bacteria, however, are sensitive to low pH (2) and have complex nutritional requirements (3), and the lactic acid that they produce is often not of high optical purity (3), which makes them suboptimal for the bulk production of lactic acid needed to make renewable plastics. A strain of *Saccharomyces cerevisiae* that is tolerant of lower pH than lactic acid bacteria and has fewer nutritional requirements (2, 4–6) has been genetically modified, partly by integration of a heterologous L-lactate dehydrogenase gene, and found to produce high levels of L-lactic acid (7) of extremely high optical purity (8), even without pH control (7). Also costly procedures to neutralize the lactic acid produced and desalination of the resulting lactate (6, 9), which are necessary when bacteria are used to produce lactic acid, can possibly be bypassed in the case of lactic acid formation by yeast because of its higher tolerance for low pH. Still, below pH values of 2.8, lactic acid production with the engineered yeast strain dropped (7). Therefore, conferring higher lactic acid resistance on *S. cerevisiae* would improve lactic acid productivity under nonneutralized conditions (10).

Lactic acid stress affects the plasma membrane and leads to the activation of enzymes involved in iron metabolism. In the presence of lactic acid, the levels of two unsaturated fatty acids, palmitoleic and oleic acids, decrease, changing the fatty acid composition of the cell membrane, while the plasma membrane H^+-ATPase activity decreases significantly (11). In addition, a vacuolar membrane H^+-ATPase is associated with the lactic acid stress response in *S. cerevisiae* (12). Lactate chelates metal cations, especially the iron that is present in culture media, which are required for growth (13, 14). As a response, Aft1, a transcriptional activator involved in iron utilization, is translocated to the nucleus upon exposure to lactic acid (12) and induces its target genes (12, 13). Correspondingly, a histone acetyltransferase complex involved in transcription has been found to take part in this mechanism to counteract lactic acid stress (12).

Another transcriptional factor, Haa1, has been implicated in the mechanisms of *S. cerevisiae* resistance to acid stress (13, 15, 16). Haa1, a homolog of the copper-regulated transcriptional factor Ace1 (17), was initially reported to be required for rapid adaptation of yeast cells to acetic, propionic, and butyric acids (18). Among genes upregulated by Haa1 (16, 17), those coding for the plasma membrane multidrug transporters Tpo2 and Tpo3 and for the cell wall glycoprotein Ygp1, which is also synthesized in response to nutrient limitation or cell wall perturbation (19, 20), were found to be essential for this adaptation (18). Furthermore, in response to acetic acid stress, Haa1 is required for the activation of *SPI1*, a gene involved in the decrease of cell wall porosity (21); *SAP30*, encoding a subunit of a histone deacetylase complex; and *HRK1*, coding for a protein kinase probably involved in the regulation of plasma membrane transport (15). By determination of the Haa1-binding sites in promoters of putative target genes, a transcriptional-regulatory network could be deduced (16). The Haa1-mediated acetic acid resistance could be regulated by Yak1, as this protein kinase, which activates the stress-responsive tran-

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TABLE 1 Primers used in this study

Name	Sequence (5′–3′)[a]
HAA1-1	CTCGGATCCACGTTAACGCTTCCGGTAAT
HAA1-2t	CTCGGATCCTCATGATCTACTTCTGGAAGTTA
HAA1-3	CTCGGATCCTTCGGACTTGCCTTCCTAGT
TEF2–p1	CTCCTCGAGTTACCCATAAGGTTGTTTGT
TEF2–p2	CTCGTCGACGTTTAGTTAATTATAGTTCG
HAA1–p1	GAGAAAAGAAACAAGAGAGGGCTACAGATTTCTAAATATCCTTTTGCCACTAGTGGATCT
HAA1–p2 (TEF2)	TATGCACCTCTCACAGGCATACTTTATGCCATTTATCAAGACCATGTTTAGTTAATTATAGTTCG
HAA1-5d	AAGAAAAAGGAAACAAAAGTATAGAAAAAAAAAACCTAAAAAATACTTCGTACGCTGCAG
HAA1-6d	GGAAAACTACAGTTACAGAGAAGCAAGAGACGAAAAGCAAATTTAGCCACTAGTGGATCT
MSN5–d1	ATGGATTCCACAGGCGCTTCTCAGATTGTTAGCGCACTAGATGTTCTTCGTACGCTGCAG
MSN5–d2	TCAGTTGTCATCAAAGAGATTACCCACAGCACCGTCTTCAATGTTGCCACTAGTGGATCT
HAA1-GFP-1	ACCGATCAAGGATTTGCGGATTTGGATAATTTCATGTCTTCGTTAAGTAAAGGAGAAGAA
HAA1-GFP-2	GGAAAACTACAGTTACAGAGAAGCAAGAGACGAAAAGCAAATTTAAAAACGACGGCCAGT
HAA1-13Myc-1	TCAAGGATTTGCGGATTTGGATAATTTCATGTCTTCGTTACGGATCCCCGGGTTAATTAA
HAA1-13Myc-2	ACTACAGTTACAGAGAAGCAAGAGACGAAAAGCAAATTTAGAATTCGAGCTCGTTTAAAC
ACT1-RT1	TGGTATGTGTAAAGCCGGTTTTG
ACT1-RT2	CATGATACCTTGGTGTCTTGGTCTA
HAA1-RT1	TCCACCTATGGCACCAACAA
HAA1-RT2	GATTGCTTGCTACGGCATTG
YGP1-RT1	AGCCCAATCGAAAACCTATTCA
YGP1-RT2	CGGCAAAGTGGAGTTGATATTCT
GPG1-RT1	CCGTTGTCGGAGAGTTGCA
GPG1-RT2	CCTTGATTCAGGCTCAATTTTGT
SPI1-RT1	CGCTAAGCTCCTTCTATCATTGG
SPI1-RT2	CCACGATTACAGAGGAACTAGAATTAGAT

[a] The underlined sequences indicate restriction enzyme sites.

scriptional factors Hsf1 and Msn2/Msn4, was suggested to be involved (22). Analysis of transcriptional responses to high concentrations of lactic acid of *S. cerevisiae* at pH 3, when more undissociated acid is present, and at pH 5, when lactate is the prominent form, indicated that Haa1 regulates the response to undissociated lactic acid, while at pH 5, the main response was related to iron homeostasis (13).

Although a growing number of factors involved in tolerance for lactic acid have been identified, the precise mechanisms that underlie the lactic acid adaptation response remain elusive, which hampers the development of effective approaches to improve the lactic acid resistance of yeast. In order to enhance our understanding of the adaptation response to lactic acid, we screened for *S. cerevisiae* genes that, in increased copy numbers, confer improved lactic acid resistance. Our screening results showed that overexpression of the transcriptional activator Haa1 has a prominent effect on conferring resistance against lactic acid. In the absence of lactic acid, we found that Haa1 was spread throughout the cell and appeared to be phosphorylated. Under conditions of lactic acid stress, however, Haa1 accumulated in the nucleus while the degree of phosphorylation seemed to decrease. In the absence of Msn5, a nuclear export factor, Haa1 is retained in the nucleus irrespective of exposure to lactic acid. As Msn5 interacts with Haa1 (23) and preferentially exports the phosphorylated forms of a variety of transcriptional factors from the nucleus into the cytoplasm (24–27), our observations suggest that nuclear accumulation of Haa1 might be linked to a reduction in the degree of phosphorylation of Haa1. The increase in nuclear localization of Haa1 could be one of the adaptation mechanisms in yeast to protect against lactic acid stress.

MATERIALS AND METHODS

Strains, plasmids, and media. The *S. cerevisiae* wild-type strains used in this study were BY4741 (*MAT***a** *leu2*Δ0 *his3*Δ1 *ura3*Δ0 *met15*Δ0) and BY4742 (*MAT*α *leu2*Δ0 *his3*Δ1 *ura3*Δ0 *lys2*Δ0) (28). Plasmid pLAT5, isolated from the YEp13-based genomic library (see below) as conferring lactic acid resistance, harbored a fragment of chromosome XVI (sequence coordinates 568899 to 574330) (see Fig. S1A in the supplemental material). Plasmids pLAT5-1 and pLAT5-2 were constructed by self-ligation of pLAT5 after digestion with ApaI and PshAI (within YEp13), followed by Klenow treatment to remove the ApaI overhangs, or after digestion with HindIII (one HindIII site was on YEp13), respectively. Plasmid p565-HAA1 was made by cloning the complete *HAA1* gene (nucleotides [nt] −650 to +2585), amplified with primers HAA1-1 and HAA1-3 (Table 1), from BY4741 genomic DNA, into p565 (a YEp-type vector with a *LEU2* marker). Similarly, p565-HAA1ΔC was p565 containing the *HAA1* gene missing 768 nucleotides from its 3′ end (nt −650 to +1314), which was amplified with primers HAA1-1 and HAA1-2t (Table 1) from BY4741 genomic DNA. Plasmid pUG6-CgLEU2-TEF2p containing a loxP-CgLEU2-loxP-$TEF2_{promoter}$ cassette was constructed by inserting a SalI-XhoI fragment containing the *TEF2* gene promoter (nt −403 to −1), prepared by PCR using primers TEP2–p1 and TEF2–p2 (Table 1) and BY4741 genomic DNA as a template, into the SalI site of pUG6-CgLEU2 (29). By homologous recombination, an *HAA1* overexpression strain, TEF2-HAA1, was constructed in BY4741 by replacing the native promoter (nt −400 to −1) on the chromosome with the loxP-CgLEU2-loxP-$TEF2_{promoter}$ cassette amplified by PCR using pUG6-CgLEU2-TEF2p as a template and primers HAA1–p1 and HAA1–p2 (TEF2) (Table 1). A Δ*haa1* disruptant was made in BY4741 by transformation of a disruption cassette prepared by PCR using pUG6-CgHIS3 (29) as a template and primers HAA1-5d and HAA1-6d (Table 1). Analogously, a Δ*msn5* strain was obtained by introducing a disruption cassette prepared by PCR using pUG6-CgLEU2 (29) as a template and primers MSN5–d1 and MSN5–d2 (Table 1) into strain BY4741. A strain expressing Haa1 with green fluo-

rescent protein (GFP) fused to its C terminus was constructed as follows. A GFP-ADH1$_{terminator}$-CgHIS3 cassette was amplified with plasmid p2446 (30) as a template and primers HAA1-GFP-1 and HAA1-GFP-2 (Table 1) and used to replace the original stop codon of *HAA1* in BY4741 or in Δ*msn5*, yielding strains HAA1-GFP and HAA1-GFP Δ*msn5*. Analogously, two strains, HAA1-MYC and TEF2-HAA1-MYC, in which Haa1 with 13 Myc epitopes at its C terminus is expressed from either the native or the TEF2 promoter, were obtained by homologous recombination in BY4741 or TEF2-HAA1, respectively. For this, a 13Myc-ADH1$_{terminator}$-kanMX6 cassette was used, which was amplified with plasmid pFA6a-13Myc-kanMX6 (31) as a template and primers HAA1-13Myc-1 and HAA1-13Myc-2 (Table 1). The response to lactic acid exposure of strain HAA1-GFP, HAA1-MYC, or TEF2-HAA1-MYC was confirmed to be indistinguishable from that of the parental strain BY4741 or TEF2-HAA1. All strains were propagated on YPD medium (1% yeast extract, 2% peptone, 2% glucose [Sigma-Aldrich], and 0.04% adenine, pH 6.2), synthetic complete (SC) medium (2% glucose, 0.67% yeast nitrogen base without amino acids [Becton, Dickinson and Co.] and the required auxotrophic supplements), or SC medium lacking leucine (SC-leu) at 30°C. Lactic acid medium (pH 2.6) was prepared by adding 6.2% (wt/vol) L-lactic acid (Nacalai Tesque, Inc.) to YPD medium. The pHs of other lactic acid media used in this study were 2.8 (4% lactic acid medium), 2.7 (5% lactic acid medium), and 2.6 (6% lactic acid medium). All solid media contained 2% agar. Quantitative comparison of growth differences between yeast strains were done by spotting serial 10-fold dilutions, starting from 10^6 cells, alongside each other on the same agar plates, which were incubated at 30°C.

Screening for genes that confer tolerance for lactic acid. A yeast genomic DNA library in the multicopy vector YEp13 (ATCC 37323) was prepared and transformed into BY4742. Transformants were selected on SC-leu plates at 30°C, replica plated onto YPD plates containing 6% L-lactic acid, and then kept at 30°C to yield lactic acid-resistant colonies.

Fluorescence microscopy. Yeast cells were grown in 10 ml of YPD medium at 30°C to mid-log phase (optical density at 600 nm [OD_{600}] = 1.0) and subjected to 4% lactic acid stress by adding L-lactic acid to a final concentration of 4%. After 0, 5, and 30 min of cultivation, cells were mounted with Vectashield containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) and observed under a BX61 fluorescence microscope (Olympus) equipped with a UPlan Apo 100×/1.35 oil iris objective with a U-MWU2 mirror/filter unit (Olympus) for DAPI and a U-MWIB2 mirror/filter unit (Olympus) for GFP. Images were acquired with a cooled mono-12-bit charge-coupled device (CCD) EXi camera with MetaMorph software (Universal imaging Corp.). Image overlays were done with Photoshop software (Adobe Systems).

RNA isolation, cDNA preparation, and quantitative real-time PCR. Yeast cells grown in 10 ml of YPD medium at 30°C to mid-log phase (OD_{600} = 1.0) were harvested, resuspended in 10 ml of YPD medium containing 4% L-lactic acid, and incubated for 30 min at 30°C. Total RNA was isolated using an RNeasy Midi kit (Qiagen), and cDNA was prepared by using a QuantiTech reverse transcription kit (Qiagen). Quantitative real-time PCR was performed using SYBR premix *Ex Taq* II (TaKaRa Bio) in triplicate in a Dice real-time thermal cycler system (TaKaRa Bio) according to the manufacturer's instructions. Relative mRNA levels were normalized to *ACT1* mRNA levels. The primers used for quantitative real-time PCR were as follows: ACT1-RT1 and ACT1-RT2 for *ACT1* mRNA, HAA1-RT1 and HAA1-RT2 for *HAA1* mRNA, YGP1-RT1 and YGP1-RT2 for *YGP1* mRNA, GPG1-RT1 and GPG1-RT2 for *GPG1* mRNA, and SPI1-RT1 and SPI1-RT2 for *SPI1* mRNA (Table 1).

Protein extraction, Western blot analysis, and phosphatase treatment. Yeast cells grown in YPD medium at 30°C to mid-log phase (OD_{600} = 1.0) were harvested, resuspended in YPD medium containing 4% L-lactic acid, and incubated for ~60 min at 30°C. Cells from 5 ml of culture were harvested, washed with 0.5 ml of 20% ice-cold trichloroacetic acid (TCA), and resuspended in 100 μl of 20% TCA. Cells were disrupted with a Multibeads Shocker (Yasui Kikai), and 100 μl of 20% TCA was added to the recovered cell extract. Following centrifugation at 3,000 rpm for 10 min at 4°C, the pellets were resuspended in 25 μl of 4× lithium dodecyl sulfate (LDS) sample buffer plus 10 μl of 0.5 M dithiothreitol, 60 μl of H_2O, and 5 μl of 1 M Tris base to adjust the pH. Samples were heated for 10 min at 70°C, followed by centrifugation at 12,000 rpm for 10 min. Protein concentrations were determined by an RC/DC (Bio-Rad) or a Qubit (Invitrogen) protein assay, and samples with 30 μg protein were separated on a 3 to 8% NuPAGE SDS-PAGE gel (Invitrogen) or a 10% SDS-PAGE gel (for actin) (TaKaRa) and electroblotted to polyvinylidene difluoride (PVDF) membranes. Haa1-Myc protein and actin were visualized with mouse monoclonal anti-c-Myc antibody (sc-40 horseradish peroxidase [HRP]; dilution, 1:2,000; Santa Cruz Biotechnology) and mouse monoclonal anti-actin antibody (MAB1501; 1:5,000 dilution; Millipore), respectively, followed by addition of horseradish peroxidase-conjugated anti-mouse IgG whole antibody (NA931; 1:10,000 dilution; GE Healthcare) and developed with the Western Lightning Plus-ECL reagent (PerkinElmer). For phosphatase treatment, crude cell extracts were prepared by beat beating using a Multibeads Shocker (Yasui Kikai) in lysis buffer (25 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 0.1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail [P8215; Sigma], and phosphatase inhibitor cocktail [07574-61; Nacalai Tesque]) from cells exposed to 4% lactic acid as described above. Haa1-Myc was immunoprecipitated from 1 mg crude cell extract with mouse monoclonal anti-c-Myc antibody bound to protein G beads. After washing with lysis buffer without phosphatase inhibitor cocktail, the Haa1-Myc immunocomplex was incubated with buffer alone or in the presence of 400 units λ protein phosphatase (New England BioLabs) with or without phosphatase inhibitor (Nacalai Tesque) for 20 min at 30°C and analyzed by Western blotting as described above.

RESULTS

Haa1 enhances lactic acid resistance when overexpressed. Despite its tolerance for acid, high levels of undissociated lactic acid (>6%) are severely toxic to yeast (13) (Fig. 1). In order to enhance our understanding of the adaptation response against lactic acid in the yeast *S. cerevisiae*, we screened for genes that at an increased dosage would enable growth on media containing 6% lactic acid (pH 2.6), on which the wild-type strain was not able to grow. Strain BY4742 was transformed with a YEp13-based multicopy genomic library, and over 65,000 transformants were screened for tolerance for 6% lactic acid. Three transformants were found to have plasmid-dependent lactic acid tolerance. Each of these transformants was found to harbor a plasmid carrying an identical genomic region, as revealed by restriction digestion and partial sequence analysis. This plasmid, named pLAT5, rendered clear resistance to 6.2% lactic acid (pH 2.6) and harbored a continuous region of chromosome XVI (with sequence coordinates 568899 to 574330) that contained a 3′-truncated form of *ICL2* encoding a 2-methylisocitrate lyase, the meiotic cohesin gene *REC8*, a glycine tRNA (*tG*[*GCC*]*P1*), and a 3′-truncated form of the transcriptional activator *HAA1* (http://www.yeastgenome.org/), which is involved in the adaptation to weak acid stress (see Fig. S1A in the supplemental material). As could be expected, subcloning analysis demonstrated that the observed lactic acid tolerance was provided only by the region containing *HAA1* (see Fig. S1B in the supplemental material), although 768 nucleotides were missing from its 3′ end, which would result in a C-terminally truncated protein lacking 256 amino acids out of the 694 of the wild-type version. The missing C-terminal region does contribute to Haa1 function, as a multicopy plasmid harboring a full-length copy of *HAA1* conferred higher lactic acid resistance than was obtained with a plasmid containing the 3′-truncated version of *HAA1* originally isolated in the screen (see Fig.

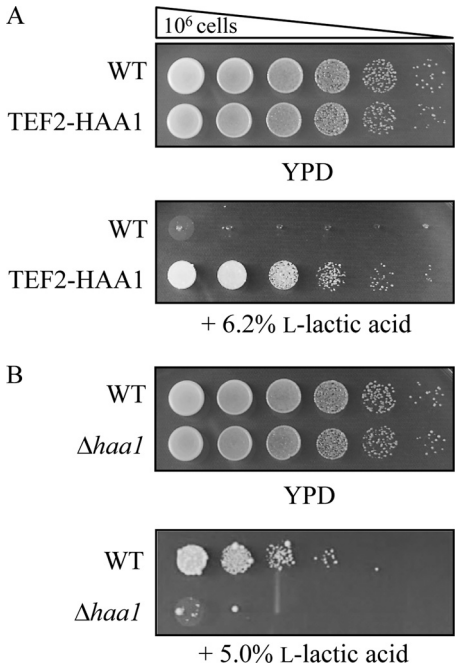

FIG 1 Lactic acid tolerance of strains in which *HAA1* is overexpressed or disrupted. Ten-fold serial dilutions of wild-type (BY4741) (WT), *HAA1* overexpression (TEF2-HAA1), and *HAA1* disruption (Δ*haa1*) strains were spotted onto YPD medium containing 0% (pH 6.2), 5% (pH 2.7) (B), or 6.2% (pH 2.6) (A) lactic acid and incubated at 30°C.

S1C in the supplemental material). In order to ensure that overexpression of *HAA1* enabled the cells to adjust to high levels of lactic acid, we constructed a strain in which the *HAA1* allele was placed under the control of the promoter region of *TEF2*, a gene that is constitutively highly expressed. This TEF2-HAA1 strain was resistant to 6.2% lactic acid in the medium (pH 2.6) (Fig. 1A), and the growth characteristics suggested that its lactic acid tolerance was comparable to that of the wild-type strain harboring a multicopy vector with *HAA1* (cf. Fig. 1A to Fig. S1C in the supplemental material, lane p565-HAA1). We also confirmed that disruption of *HAA1* caused severe lactic acid sensitivity (Fig. 1B), as reported previously (13). Furthermore, overexpression of *HAA1* also improved lactic acid resistance on a synthetic minimal medium (see Fig. S1D in the supplemental material). We conclude that overexpression of *HAA1* can confer resistance to high levels of undissociated lactic acid at low pH. To examine whether overexpression of *HAA1* leads to increased tolerance for other stresses, the TEF2-HAA1 strain was exposed to heat shock (55°C for 1 h), oxidative stress (15 mM H_2O_2), acetic acid (0.5%; pH 4.1), sulfuric acid (0.5%; pH 2.6), hydrochloric acid (0.3%; pH 2.3), ethanol (12%), and high salt (1 M NaCl). We found that, of these stresses, only the exposure to acetic acid was tolerated due to *HAA1* overexpression, as described recently (reference 32 and data not shown).

Nuclear localization of Haa1 occurs in response to lactic acid stress. Transcriptional activation of Haa1 target genes occurs under conditions of lactic or acetic acid stress, and some of these genes are required to provide resistance to acetic acid (13, 15, 33). To examine whether overexpression of *HAA1* enhanced the expression of its target genes, transcriptional induction of *YGP1*, *GPG1*, and *SPI1*, encoding a secretory glycoprotein, a gamma subunit of the heterotrimeric G protein, and a glycosylphosphatidylinositol (GPI)-anchored cell wall protein, respectively (16), was analyzed in the presence of lactic acid (Fig. 2). In the wild-type strain, expression of *YGP1* mRNA was induced about 4-fold in response to 4% lactic acid stress (pH 2.8) (Fig. 2A), indicating that 4% lactic acid activates the lactic acid adaptation response. After exposure of the TEF2-HAA1 strain to 4% lactic acid stress, *YGP1* mRNA synthesis was induced 7-fold more than in the case of the wild-type strain grown under the same conditions. Expression of *GPG1* mRNA was also induced approximately 6-fold in the wild-type strain in response to 4% lactic acid stress (Fig. 2B). In the TEF2-HAA1 strain, 2.5-fold-higher induction of *GPG1* mRNA was observed than in the wild-type strain under lactic acid conditions. Similarly, 4% lactic acid activated expression of *SPI1* approximately 3-fold in the wild-type strain (Fig. 2C), and this transcriptional activation was further enhanced (approximately 2-fold) in the TEF2-HAA1 strain under lactic acid conditions. These results indicate that overexpression of *HAA1* leads to enhanced induction of its target genes under lactic acid conditions.

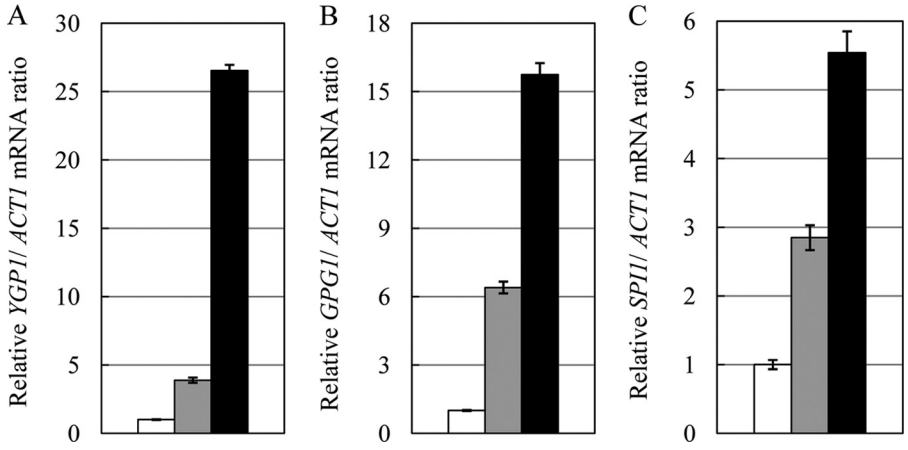

FIG 2 Expression analysis of *YGP1*, *GPG1*, and *SPI1* mRNAs. Expression levels of *YGP1* (A), *GPG1* (B), and *SPI1* (C) mRNAs in wild-type (BY4741; gray bars) and *HAA1* overexpression (TEF2-HAA1; black bars) strains incubated in YPD medium containing 4% lactic acid (pH 2.8) for 30 min were analyzed by quantitative real-time PCR and expressed as relative mRNA values (normalized to *ACT1* expression), with the value for the wild-type (BY4741; white bars) strain grown in YPD medium (pH 6.2) set to 1.0. The error bars show the standard deviations.

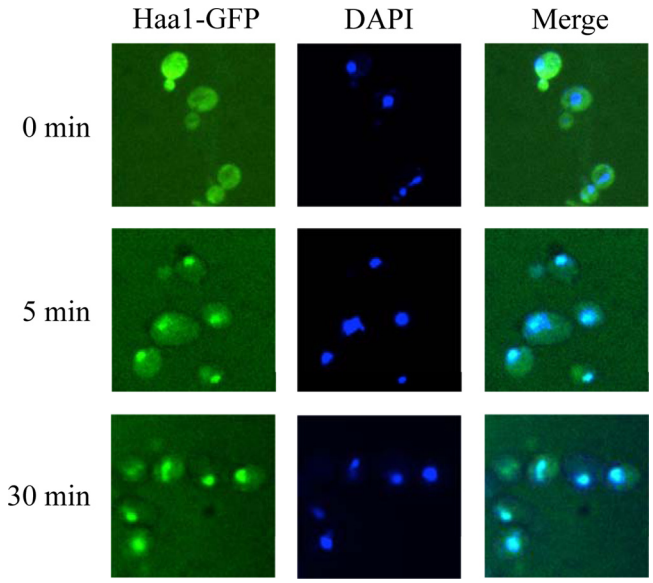

FIG 3 Localization of Haa1 in response to lactic acid. Localization of Haa1-GFP was analyzed by fluorescence microscopy after exposure of the cells to YPD medium containing 4% lactic acid (pH 2.8) for 0, 5, and 30 min (green). Nuclear DNA was stained with DAPI (blue).

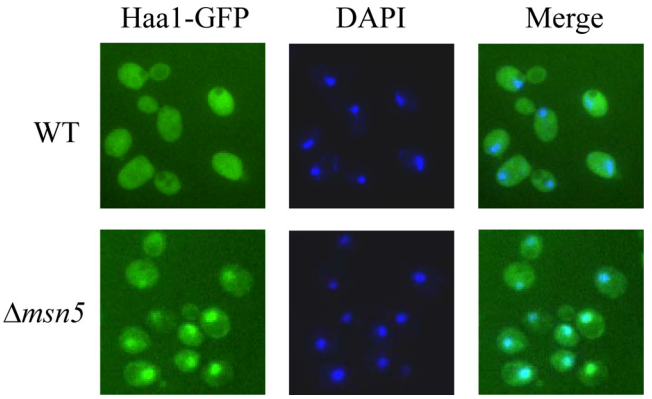

FIG 4 Nuclear localization of Haa1 in the absence of Msn5. Shown is fluorescence microscopy of HAA1-GFP Δ*msn5* cells grown in YPD medium (pH 6.2) (green). Nuclei were stained with DAPI (blue).

In order to search for Haa1 target genes important for lactic acid resistance, we constructed in an *HAA1* overexpression strain strains with single deletions of 10 of the Haa1 target genes (*GRE1*, *SPI1*, *TPO2*, *TPO3*, *YGP1*, *YRO2*, *PHM8*, *COM2*, *TDA6*, and *YLR297W*), some of which are reported to be essential for resistance to weak organic acids, including acetic, propionic, butyric, benzoic, and octanoic acids (17, 18, 21). However, all 10 single-deletion strains did not show decreased lactic acid resistance compared to the *HAA1* overexpression strain (data not shown). Similarly, deletion of the 10 target genes in some double and triple combinations (Δ*tpo2* Δ*tpo3*, Δ*spi1* Δ*ygp1*, Δ*yro2* Δ*ygp1*, Δ*com2* Δ*ygp1*, Δ*com2* Δ*yro2*, Δ*tpo2* Δ*tpo3* Δ*spi1*, Δ*tpo2* Δ*tpo3* Δ*ygp1*, Δ*tpo2* Δ*tpo3* Δ*yro2*, and Δ*com2* Δ*ygp1* Δ*yro2*) in the *HAA1* overexpression strain did not cause decreased lactic acid resistance (data not shown), indicating that these genes are dispensable for the lactic acid resistance conferred by *HAA1* overexpression, at least singly or in some combinations.

To understand more about the Haa1-mediated lactic acid adaptation response, we analyzed the regulation mechanisms of target gene expression in more detail in the wild-type strain. Since increased levels of transcriptional induction of the target genes was found to occur when *HAA1* was highly expressed, the response to lactic acid stress could occur by enhanced transcription of *HAA1* in the wild-type strain. When we tested the possibility of transcriptional upregulation of *HAA1* in response to lactic acid stress by quantitative real-time PCR, we found, however, that the expression level of *HAA1* in the wild-type cells under lactic acid-free conditions was not much different from that in the wild-type cells after exposure to 4% lactic acid, suggesting other possibilities (see Fig. S2 in the supplemental material).

It is well known that the activities of a variety of transcriptional activators or repressors are regulated by their subcellular localization (34–36). We therefore examined the possibility of a shift in the localization of Haa1 in response to lactic acid stress. For this, we constructed a strain in which Haa1 carried a C-terminal GFP tag and examined by fluorescence microscopy the subcellular localization of Haa1 over time upon exposure to 4% lactic acid. In the absence of lactic acid, Haa1 was distributed throughout the cell (Fig. 3, top row). After 5-min exposure to lactic acid, however, the bulk of Haa1 had translocated from the cytoplasm to the nucleus (Fig. 3, middle row), and this nuclear localization was maintained throughout the 30-min cultivation in lactic acid medium (Fig. 3, bottom row). These results indicate that the main subcellular localization of Haa1 shifted from the cytoplasm to the nucleus in response to lactic acid stress and suggest that this is one of the molecular mechanisms to induce its target genes in response to lactic acid stress. When *HAA1* was overexpressed, nuclear localization of Haa1 and approximately 1.5- to 8-fold-higher expression of *SPI1*, *GPG1*, and *YGP1* mRNAs than in the wild-type strain were also observed, even in the absence of lactic acid stress (see Fig. S3 in the supplemental material), suggesting that overexpression of *HAA1* caused mislocalization of Haa1, which may induce expression of the target genes even in the absence of lactic acid, although the expression levels of the target genes differ depending on the genes.

Msn5 is required for nuclear export of Haa1. A nuclear import factor for Haa1 has not yet been identified, but Haa1 has been reported to interact with the nuclear exportin Msn5 (23), which is essential for the export of various transcriptional factors from the nucleus (24–27). Therefore, we tested whether Msn5 was involved in nuclear export of Haa1 by analyzing the subcellular localization of Haa1 in an *msn5* disruptant by fluorescence microscopy. In the absence of Msn5, Haa1 was retained inside the nucleus even when the cells were not exposed to lactic acid (Fig. 4). Thus, our results indicate that Haa1 is shuttling between the nucleus and cytoplasm and, while it is retained in the nucleus upon exposure to lactic acid, Msn5 is essential for its export from the nucleus to the cytoplasm.

Haa1 is phosphorylated, and a hypophosphorylated form of Haa1 appears immediately upon exposure to lactic acid stress. Msn5 is known to preferentially export the phosphorylated forms of transcriptional factors from the nucleus (24–27). Several (phospho)proteomic approaches using mass spectrometry showed that Haa1 was a phosphoprotein and that several serine residues, such as those at positions 271 and 274, were phosphorylated (37–40).

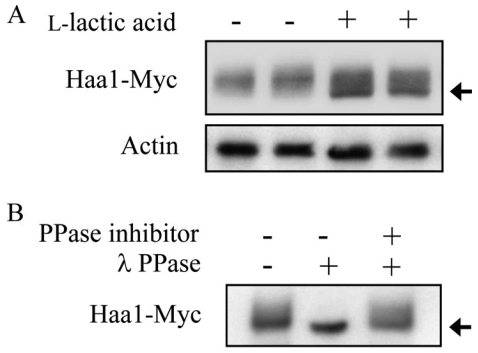

FIG 5 Lactic acid-dependent changes in the phosphorylation status of Haa1. (A) Western blot analysis of Haa1-Myc using anti-Myc antibody. Extracts were isolated from HAA1-MYC cells after exposure to YPD medium containing 4% lactic acid (pH 2.8) for 0 (−) and 5 (+) min. Actin was used as a loading control. Samples from both conditions were analyzed in duplicate. (B) Phosphatase treatment of Haa1-Myc immunoprecipitated from HAA1-MYC cells that had been subjected to lactic acid stress for 5 min. Immunocomplexes were treated with λ protein phosphatase (PPase) in the presence or absence of PPase inhibitors and analyzed on immunoblots using anti-Myc antibody. The arrows indicate the faster-migrating form of Haa1-Myc that accumulates upon exposure to lactic acid.

Therefore, we analyzed the phosphorylation status of Haa1 after exposure to 4% lactic acid for 0 and 5 min, since the main localization of Haa1 differed under these conditions, i.e., cytoplasm and nucleus (Fig. 3). For this, we prepared cell extracts from a yeast strain in which a functional, C-terminally Myc-tagged version of Haa1 was expressed under the control of the endogenous promoter. Western blots with anti-myc antibody revealed that in the absence of lactic acid stress, Haa1-Myc protein migrated as a broad band (Fig. 5A). However, a major fraction of Haa1-Myc with higher electrophoretic mobility was present upon exposure to lactic acid stress, although it still appeared as a diffuse band. Since the diffused electrophoretic mobility of Haa1-Myc might be due to proteins that differ in their degrees of modification, it is possible that Haa1 is phosphorylated under lactic acid-free conditions and that hypophosphorylated or dephosphorylated forms of Haa1 accumulate in response to lactic acid stress. To test this idea, Haa1-Myc was immunoprecipitated from extracts of cells exposed to 4% lactic acid for 5 min and incubated with λ phosphatase (Fig. 5B). Upon phosphatase treatment, the diffuse bands detected by anti-Myc antibody in the untreated sample were converted to a single band showing the same electrophoretic mobility as the fastest-migrating band that is prominent in samples from cells subjected to lactic acid exposure. In the presence of phosphatase inhibitors, no change in the banding pattern of Haa1-Myc was observed. Time course Western blot analysis of Haa1-Myc also showed that Haa1-Myc with higher electrophoretic mobility appeared and predominated for 60 min under 4% lactic acid conditions (see Fig. S4 in the supplemental material). These results demonstrated that Haa1 is only modified by phosphorylation. Under normal conditions, when cells are not exposed to lactic acid, Haa1 is phosphorylated and localized throughout the cell, but upon exposure to lactic acid, Haa1 accumulates in the nucleus, which is linked to a reduction in the degree of phosphorylation of Haa1. Taken together, our data suggest that subcellular localization of Haa1 or its activity required for the induction of its target genes is regulated by its degree of phosphorylation.

DISCUSSION

Our analysis demonstrated that one of the adaptation responses to lactic acid stress is the nuclear localization of Haa1 to induce expression of its target genes, such as *YGP1*, *GPG1*, and *SPI1*, and also suggested not only that Haa1 activity is regulated by its subcellular localization, but also that this is linked to the extent of phosphorylation, which appeared to be important for Msn5-mediated nuclear export of Haa1. A similar result where overexpression of *HAA1* improved yeast resistance to acetic and lactic acids was also very recently reported in an ethanol production study (41). Since nuclear accumulation of transcription factors is one of the efficient strategies for cells to rapidly enhance expression of their target genes, our data suggest that nuclear localization of Haa1 that is linked to its phosphorylation status mediates lactic acid adaptation. It was reported very recently that Haa1 was also accumulated in the nucleus by exposure to hydroxyurea or methyl methanesulfonate (42). Such DNA replication stress also caused transcription factors Aft1 and Msn2 (but not Hsf1 or Msn4) to translocate from the cytoplasm to the nucleus. Whether the nuclear localization of Aft1 and Msn2 is induced by the same mechanism as during acid exposure or whether these DNA-binding factors can protect DNA as part of their capacity to resist acid stress remains to be seen.

Identification of genes responsible for rendering lactic acid resistance in the *HAA1* overexpression strain is important, not only for understanding the lactic acid adaptation mechanism mediated by Haa1, but also for further improvement of lactic acid resistance for biotechnological applications. Our analysis of the disruption of 10 target genes (17, 18, 21) singly or in some combinations suggests that other Haa1 target genes having functions redundant with those of the 10 genes tested in this study were also overexpressed in the *HAA1* overexpression strain. Thus, disruptions of a single gene or some combinations of the 10 genes might not have caused decreased lactic acid resistance in the *HAA1* overexpression strain. Genes specifically involved in the Haa1-mediated lactic acid adaptation response might also be involved in the enhanced lactic acid resistance, although we cannot rule out the possibility of involvement of some of the Haa1 target genes that were not tested in this study but were reported to be involved in acetic acid resistance (15). However, since high transcript levels of Haa1 target genes, such as *YGP1*, *GPG1*, and *SPI1*, was observed in the *HAA1* overexpression strain, overexpression of its target genes might confer stronger protective effects on yeast cells so that they can survive exposure to toxic levels of lactic acid. In support of this, we originally identified a C-terminally truncated version of Haa1 that conveyed this resistance. Over one-third of the protein was missing, but the regions that bind DNA (16) or are phosphorylated (37–40) were retained, so that its function as a transcriptional activator would not be affected and it conferred enhanced lactic acid resistance.

Interestingly, Haa1, which was extended with a TAP epitope, was found by chromatin immunoprecipitation to bind upstream of one its target genes, *TPO3*, irrespective of exposure to acetic acid stress, but still under acidic conditions (pH 4.0) (16). Our results showed that nuclear localization of Haa1 is induced after exposure to lactic acid stress, which was accompanied by a pH change from 6.2 to 2.8. In addition, Haa1 target genes were found to be strongly expressed at pH 3 but only moderately at pH 5, despite the presence of high levels of lactic acid (mostly in the form

of lactate) (13). Therefore, it is possible that pH sensing is part of the response mechanism in which Haa1 is involved. It is also possible that lactic acid activates its response mechanism in a different way from acetic acid. For example, lactic acid stress might induce nuclear localization of Haa1 to increase the amount of Haa1 that can bind to the promoter region and enhance the expression of the target genes. On the other hand, acetic acid stress might induce a response to enhance a transcriptional activator activity of Haa1 that had been bound to the promoter region of the target genes (16). Alternatively, there might also be a possibility that Haa1 regulates the expression of a variety of its target genes in different ways.

Several serine residues of Haa1 were reported to be phosphorylated (37–40) and were found to be part of the phosphorylation motifs recognized by protein kinase A and casein kinase I (43; http://www.phosphogrid.org). Our data suggest that upregulation of a phosphatase activity or downregulation of a kinase activity with Haa1 as the substrate is linked to nuclear localization of Haa1 in response to lactic acid stress. Therefore, identification of factors controlling the phosphorylation status of Haa1 will further promote our understanding of the adaptation response to lactic acid stress in yeast.

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