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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Transcriptome analysis of acetic-acid-treated yeast cells identifies a large set of genes whose overexpression or deletion enhances acetic acid tolerance

Yeji Lee[1] · Olviyani Nasution[1] · Eunyong Choi[1] · In-Geol Choi[3] · Wankee Kim[4] · Wonja Choi[1,2]

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Abstract Acetic acid inhibits the metabolic activities of *Saccharomyces cerevisiae*. Therefore, a better understanding of how *S. cerevisiae* cells acquire the tolerance to acetic acid is of importance to develop robust yeast strains to be used in industry. To do this, we examined the transcriptional changes that occur at 12 h post-exposure to acetic acid, revealing that 56 and 58 genes were upregulated and downregulated, respectively. Functional categorization of them revealed that 22 protein synthesis genes and 14 stress response genes constituted the largest portion of the upregulated and downregulated genes, respectively. To evaluate the association of the regulated genes with acetic acid tolerance, 3 upregulated genes (*DBP2*, *ASC1*, and *GND1*) were selected among 34 non-protein synthesis genes, and 54 viable mutants individually deleted for the downregulated genes were retrieved from the non-essential haploid deletion library. Strains overexpressing *ASC1* and *GND1* displayed enhanced tolerance to acetic acid, whereas a strain overexpressing *DBP2* was sensitive. Fifty of 54 deletion mutants displayed enhanced acetic acid tolerance. Three chosen deletion mutants (*hsps82Δ*, *ato2Δ*, and *ssa3Δ*) were also tolerant to benzoic acid but not propionic and sorbic acids. Moreover, all those five (two overexpressing and three deleted) strains were more efficient in proton efflux and lower in membrane permeability and internal hydrogen peroxide content than controls. Individually or in combination, those physiological changes are likely to contribute at least in part to enhanced acetic acid tolerance. Overall, information of our transcriptional profile was very useful to identify molecular factors associated with acetic acid tolerance.

Keywords Transcriptome profile · Acetic acid · Overexpression · Deletion mutant · Stress tolerance

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✉ Wankee Kim
kinesin03@hanmail.net

✉ Wonja Choi
wjchoi@ewha.ac.kr

[1] Division of Life and Pharmaceutical Sciences, Ewha Womans University, Seoul 120-750, Republic of Korea

[2] Microbial Resources Research Center, Ewha Womans University, Seoul 120-750, Republic of Korea

[3] School of Life Sciences and Biotechnology, Korea University, Seoul 136-713, Republic of Korea

[4] Department of Pharmacology, School of Medicine, Ajou University, Suwon 442-749, Republic of Korea

Introduction

Acetic acid is produced during the fermentation or is often included in the starting material, for bioethanol production. As it is deleterious to the growth of fermenting cells and thereby decreases the fermentation productivity, so a deeper understanding of adaptation to acetic acid is of particular interest in the alcoholic fermentation by *Saccharomyces cerevisiae* cells. Accordingly, elucidation of the molecular mechanisms underlying the tolerance to acetic acid stress of *S. cerevisiae* is crucial for constructing more robust industrial yeast strains in the field of ethanologenic fermentation. To do this, two different approaches at a genome-wide level have been exploited in general: DNA microarray analysis and functional screening of the non-essential gene deletion collections. The former approach identifies genes upregulated or downregulated by the presence of acetic acid, which can be further

analyzed to search for regulons as in the case of the transcription factor Haa1, which is required for a rapid adaptation to acetic and propionic acids (Fernandes et al. 2005). Meanwhile, the latter approach aims mainly to identify genes required for the resistance to acetic acid by showing individual deletion mutants that become sensitive (Kawahata et al. 2006; Mira et al. 2010a). During this procedure, deletion mutants with enhanced tolerance can often be isolated (Kawahata et al. 2006). Independent of those approaches, disruption of *FPS1* (Mollapour and Piper 2007; Mollapour et al. 2008) or overexpression of *HAA1* (Tanaka et al. 2012) was found to confer tolerance to acetic acid. Identification of such molecular factors can help construct acetic-acid-tolerant strains of any genetic background, possibly including industrial strains. In addition, genome shuffling was employed for construction of acetic-acid-tolerant strains (Zheng et al. 2011).

Prior to the present study, five studies have reported transcriptional changes when *S. cerevisiae* cells are exposed to acetic acid, one that studied both short and long (Kawahata et al. 2006), two for only short (Li and Yuan 2010; Mira et al. 2010b), and two for only long exposures (Abbott et al. 2007; Bajwa et al. 2013). The data from those genome-wide profiles hardly agree well with each other, apparently because of different experimental conditions, including strains used, pH of the medium, and concentrations of acetic acid (Supplementary Table S1). Nevertheless, such profiles are very informative not only to reinforce the previous findings but also to understand regulatory networks underlying the weak acid adaptation process (Mira et al. 2010c). Further elucidation of molecular mechanisms of acetic acid adaptation still may rely on genome-wide analysis of transcriptional changes that occur upon exposure to acetic acid under various conditions. Here we present a transcriptome profile of cells exposed to 0.6 % acetic acid (pH 4.5) for 12 h, the time presumably between shock (exposure for usually less than 30 min) and adaptation (up to 30 h).

We revealed that 114 genes (56 upregulated and 58 downregulated) were differentially expressed. Based on spot assay, overexpression of two arbitrarily chosen upregulated genes (*ASC1* and *GND1*) and individual deletion of 50 downregulated genes resulted in enhanced tolerance to acetic acid. It is possible that additional genes would be identified if the rest of upregulated genes are tested for stress tolerance.

Materials and Methods

Strains

BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and W303-1A (*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11, 15 ybp1-1*) were used for yeast transformation. Deletion mutants of BY4741 origin were retrieved from the non-essential haploid deletion library (Open Biosystems, Pittsburgh, PA, USA).

Culture

Yeast cells were grown in YPD (1 % yeast extract, 2 % peptone, and 2 % dextrose and 1.5 % agar for solid plates) or synthetic complete medium (SC) composed of 0.67 % yeast nitrogen base without amino acids, complete or depleted amino acid supplement mixture, 2 % dextrose, and 1.5 % noble agar for solid plates. Unless otherwise mentioned, synthetic media were used, and the pH was adjusted to 5.8.

RNA-seq analysis

Total RNA was extracted from cells untreated or treated with 0.6 % acetic acid (pH 4.5) for 12 h using a commercial RNA extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. To retrieve messenger RNAs (mRNAs) and construct a complementary DNA (cDNA) library for next-generation sequencing, a TruSeq RNA sample preparation kit (Illumina, San Diego, CA, USA) was employed and used as recommended by the manufacturer. Sequencing of a cDNA library for RNA sequence analysis was carried out using the MiSeq platform (Illumina) with paired-end sequencing reagent kit (500 cycles). The quality of raw read data was examined using the FastQC program and preprocessed using the Bioconductor/R package (Gentleman et al. 2004). Reads were trimmed in 5′ and 3′ ends by removing bases showing low Phred quality score (<20) using the ShortRead package (Morgan et al. 2009). The genome sequence of *S. cerevisiae* strain S288c was used as a reference for read alignment by the Bowtie2 program (Langmead and Salzberg 2012). The transcript database (TxDb.Scerevisiae.UCSC.sacCer2.sgdGene) in the Annotation package was used to count reads mapped on genes with the Rsamtools option in Bioconductor/R package (Gentleman et al. 2004). Differentially expressed genes (DEGs) were identified with the edgeR package (Robinson et al. 2010). The normalized raw data have been registered in the NCBI Sequence Read Archive under the identification number 227050, wherein AA00pc and AA06pc stand for cells untreated and treated with 0.6 % acetic acid, respectively.

Functional categorization of DEGs

Enrichment of functional categories among DEGs was analyzed using the Munich Information Center for Protein Sequences (MIPS) Functional Catalogue (http://mips.helmholtz-muenchen.de/funcatDB). Annotations of specific gene function were based on the Saccharomyces Genome Database (http://www.yeastgenome.org).

Molecular methods

Plasmid preparation, cloning, and sequencing were performed as previously described (Sambrook and Russell 2001).

Escherichia coli strain DH5a (Stratagene, La Jolla, CA, USA) was used as a host for plasmid preparation.

Yeast transformation

Plasmids for yeast transformation were manually prepared without RNA digestion. The DNA concentration was roughly measured by comparing the band intensity with that of control DNA of known concentration. The mixture of DNAs and RNAs was used for yeast transformation as previously described (Yang et al. 2011).

Spot assay

Cells were grown to an optical density of 1.0 at 600 nm (OD_{600}). Tenfold serial dilutions were then carried out four times, replica-spotted onto solid YPD, and incubated at 30 °C.

Polymerase chain reaction

Oligonucleotides used for polymerase chain reaction (PCR) are listed in Supplementary Table S2. The amplification conditions were 95 °C for 1 min, 55–60 °C for 1 min, and 72 °C for the appropriate period of time depending on the length of DNA to be amplified for 30 cycles. When necessary, PCR products were purified by gel elution, cloned into the pGEM-T easy vector (Promega, Madison, WI, USA), and sequenced.

Analysis of RNA expression levels

RNA expression levels were analyzed by performing reverse transcription-PCR (RT-PCR). Total RNAs were prepared from exponentially growing cells according to the directions of the manufacturer (Qiagen, Austin, TX, USA) and treated with RNase-free DNase (New England Biolabs, Ipswich, MA, USA). One microgram of total RNA was directly amplified for 30 cycles with the *ACT1* primers to confirm no DNA or reverse-transcribed and amplified with gene-specific primers for appropriate cycles. Relative transcriptional levels were determined by comparing densitometric band intensities of electrophoresed RT-PCR products.

Proton efflux assay

Proton efflux was measured for cells treated with 0.6 % acetic acid (pH 4.5) for 3 h, as described previously (Stratford et al. 2013) with minor modifications. Exponentially growing yeast cells were obtained from 40 ml shaken cultures at OD_{600} of 2.0. Cells were harvested at 4 °C by filtration (3 μm, 50-mm filters), washed four times with cold water and two times with cold 100 mM glucose, and then resuspended in cold 10 ml 100 mM glucose. The resuspended yeast concentration was adjusted to an OD_{600} of 7.0 and equilibrated with rapid stirring within a water jacket at 25 °C for 3 min. Extracellular pH was manually recorded at intervals for 20 min.

Membrane permeability assay

Cells of 1.0 OD_{600} were adjusted to 0.6 % acetic acid (pH 4.5) and further cultured for 48 h. Aliquots of 1 ml were taken every 6 h, harvested at 10,000 rpm for 2 min at 4 °C, washed two times with cold phosphate buffered saline (PBS), and then resuspended in 1 ml cold PBS to prepare a master cell resuspension. Fifty microliters of this resuspension was mixed with 50 μl propidium iodide (PI) solution (1 mg/ml in water), incubated at room temperature in the dark for 15 min, washed, resuspended in 50 μl of cold PBS, and visualized by fluorescence microscopy. After counting the number of stained and unstained cells within a fixed microscopic field, the percentage of stained cells was determined.

Measurement of internal hydrogen peroxide content

Internal hydrogen peroxide (H_2O_2) content was measured by using the H_2O_2 assay kit from Cell Biolabs (San Diego, CA, USA). In principle, externally added sorbitol first converts aqueous peroxide to a peroxyl radical, which oxidizes Fe^{2+} into Fe^{3+}. Then, Fe^{3+} reacts with an equal molar amount of xylenol orange in the presence of acid to create a purple product that absorbs maximally between 540 and 600 nm. In a 96-well microtiter plate, 50 μl of the master cell resuspension was mixed with 200 μl of the color developing reagent (500 μM xylenol orange, 1.25 mM Fe^{2+}, and 500 mM sorbitol in 125 mM H_2SO_4) provided by the kit manufacturer and incubated for 30 min at room temperature. The internal H_2O_2 content was determined by comparing with the predetermined H_2O_2 standard curve. The OD_{540} values of yeast cells were converted to nmol/μg dry cell weight (DCW).

Results

Global transcriptional change during adaptation to acetic acid

RNA-seq analysis was performed with three independent mRNA samples prepared from untreated or acetic-acid-treated BY4741 cells at OD_{600} of 0.5, but sequencing of one untreated sample failed for an unknown reason. We compiled transcriptional profiles from two samples of untreated and three samples of acetic-acid-treated cells. Comparing those identified 114 genes (56 upregulated and 58 downregulated) as DEGs (p value threshold ≤0.01). Neither downregulation of *FPS1* nor overexpression of *HAA1* was observed (see above). Functional annotations of DEGs are listed in Table 1.

Table 1 Regulated genes in BY4741 treated with acetic acid

Gene or ORF	Description	Functional group[a]	Fold change
DBP2	ATP-dependent RNA helicase	ACDGHT	39.4
OLE1	Fatty acid desaturase	AGT	21.1
LEU1	Isopropylmalate isomerase	A	19.7
RPL18A	Ribosomal 60S subunit protein L18A	EGT	17.1
YGR160W	Dubious open reading frame	Unknown	17.1
PMA1	H^+-ATPase, pumps protons out of the cell	BILT	17.1
BSC1	Similar to cell surface flocculin Flo11p	ALQ	17.1
YNL103W-A	Dubious open reading frame	Unknown	17.1
ALD5	Mitochondrial aldehyde dehydrogenase	ABKP	16
YHR182C-A	Dubious open reading frame	Unknown	13.9
SAM1	S-adenosyl methionine synthetase	AG	12.1
PSA1	GDP-mannose pyrophosphorylase	AFP	11.3
SHM2	Cytosolic serine hydroxymethyl transferase	AT	11.3
EGT2	GPI-anchored cell wall endoglucanase	AFN	11.3
BNA1	3-Hydroxyanthranilic acid dioxygenase	A	11.3
ADE17	Enzyme of "de novo" purine biosynthesis	ABQ	10.6
YEF3	Gamma subunit of translational elongation factor eEF1B	AEGPT	10.6
RPL3	Ribosomal 60S subunit protein L3	EGT	10.6
INA1	Putative protein of unknown function	Unknown	10.6
RPL8A	Ribosomal 60S subunit protein L8A	EGT	10.6
ASC1	Guanine nucleotide dissociation inhibitor for Gpa2p	A-EGIJLMO-QST	10.6
PIR1	O-glycosylated protein required for cell wall stability	IKPQ	10.6
YNL174W	Dubious open reading frame	Unknown	10.6
YMR290W-A	Dubious open reading frame	Unknown	10.6
RPL20B	Ribosomal 60S subunit protein L20B	EGT	9.8
YJR071W	Dubious open reading frame	Unknown	9.8
AAH1	Adenine deaminase	AGT	9.8
RPL9A	Ribosomal 60S subunit protein L9A	EGT	9.8
RPS9A	Ribosomal 40S subunit protein	EGT	9.8
NSR1	Nucleolar protein	DEGKPT	9.8
RPS9B	Ribosomal 40S subunit protein	EGT	9.2
RPS5	Ribosomal 40S subunit protein	EGT	9.2
RPL15A	Ribosomal 60S subunit protein L15A	EGT	9.2
RPL31B	Ribosomal 60S subunit protein L31B	EGT	8.6
ARO4	(DAHP) synthase	A	8.6
MAE1	Mitochondrial malic enzyme	ABGT	8.6
BAT1	Mitochondrial amino acid aminotransferase	ACGT	8.6
RRP12	Protein required for export of the ribosomal subunits	DEGT	8.6
RPS18B	Ribosomal 40S subunit protein	EGT	8.6
OAC1	Mitochondrial inner membrane transporter	BILT	8.0
GND1	6-Phosphogluconate dehydrogenase	ABGT	8.0
RPL24A	Ribosomal 60S subunit protein L24A	EGT	8.0
YLR149C-A	Dubious open reading frame	Unknown	8.0
HO	Site-specific endonuclease	CFILO	8.0
YGR265W	Dubious open reading frame	Unknown	8.0
ILV3	Dihydroxyacid dehydratase	AT	7.5
RPL2A	Ribosomal 60S subunit protein L2A	EGT	7.5
RPS16B	Ribosomal 40S subunit protein	EGT	7.5
SSB1	Cytoplasmic ATPase	AEFGIKT	7.5

Table 1 (continued)

Gene or ORF	Description	Functional group[a]	Fold change
MET6	Cobalamin-independent methionine synthase	AFT	7.5
YMR307C-A	Dubious open reading frame	Unknown	7.5
ILV2	Acetolactate synthase	AGT	7.5
RPS3	Ribosomal 40S subunit protein	EGT	7.5
CYS4	Cystathionine beta-synthase	AT	7.5
RPL21A	Ribosomal 60S subunit protein L21A	EGT	7.5
RPS1B	Ribosomal 40S subunit protein	EGT	7.5
YOL153C	Hypothetical protein	Unknown	−8.0
YIL059C	Dubious open reading frame	Unknown	−8.0
STF1	Regulator of the mitochondrial F1F0-ATP synthase	BHI	−8.0
SSA3	ATPase for protein folding and the response to stress	AFGIK	−8.0
OPI3	Phospholipid methyl transferase	CT	−8.0
OM45	Mitochondrial outer membrane protein	Unclassified	−8.0
JLP1	Sulfonate/alpha-ketoglutarate dioxygenase	AK	−8.0
CAT8	Zinc cluster transcriptional activator	ABDT	−8.0
CAT2	Carnitine acetyl-CoA transferase	AILMOQST	−8.0
YOR292C	Putative protein of unknown function	Unclassified	−8.6
YIL060W	Putative protein of unknown function	Unclassified	−8.6
YCR007C	Putative integral membrane protein	GIOP	−8.6
HSP12	Plasma membrane protein	ACKL	−8.6
GLK1	Glucokinase	ABIQT	−8.6
CSR2	Nuclear ubiquitin protein ligase binding protein	DP	−8.6
YCR100C	Putative protein of unknown function	Unclassified	−9.2
SET4	Protein of unknown function	CF	−9.2
PGM2	Phosphoglucomutase	AB	−9.2
HSP82	Hsp90 chaperone	ACKL	−9.2
GSP2	GTP binding protein	ACDFGIJPT	−9.2
ATO2	Putative transmembrane protein	ACI	−9.2
YJL133C-A	Putative protein of unknown function	Unclassified	−9.8
YBR241C	Putative transporter	FIT	−9.8
PRM5	Pheromone-regulated protein	O	−9.8
PNC1	Nicotinamidase	ACN	−9.8
MSC1	Protein of unknown function	CGKT	−9.8
MAL11	Inducible high-affinity maltose transporter	IT	−9.8
GPH1	Non-essential glycogen phosphorylase	ABGT	−9.8
FRT2	Tail-anchored ER membrane protein	JK	−9.8
GDB1	Glycogen debranching enzyme	AB	−10.6
FMP33	Putative protein of unknown function	Unclassified	−10.6
RGI1	Protein of unknown function	Unclassified	−11.3
GAD1	Glutamate decarboxylase	AKT	−11.3
FIT3	Mannoprotein that is incorporated into the cell wall	I	−11.3
YKL133C	Putative protein of unknown function	Unclassified	−12.1
UIP4	Protein that interacts with Ulp1p	Unclassified	−12.1
MAL33	MAL-activator protein	ADG	−12.1
ATH1	Acid trehalase	ABKT	−12.1
ALD3	Cytoplasmic aldehyde dehydrogenase	ABGIKMOQ	−12.1
YER067C-A	Dubious open reading frame	Unknown	−13.0
HXT6	High-affinity glucose transporter	AI	−13.9
CYC7	Cytochrome c isoform 2	BFGIKST	−13.9

Table 1 (continued)

Gene or ORF	Description	Functional group[a]	Fold change
CTT1	Cytosolic catalase T	GIKT	−13.9
HXT7	High-affinity glucose transporter	AI	−14.9
DCS2	m(7)GpppXpyrophosphatase regulator	ADGT	−14.9
PUT1	Proline oxidase	AT	−17.1
HXK1	Hexokinase isoenzyme	ABIT	−17.1
GAP1	General amino acid permease	ILT	−17.1
ALD4	Mitochondrial aldehyde dehydrogenase	ABGIKPT	−17.1
AIM17	Putative protein of unknown function	A	−17.1
YFL054C	Putative channel-like protein	AIKOT	−17.1
TMA10	Protein of unknown function	BFIKP	−18.4
TSL1	Trehalose 6-phosphate synthase/phosphatase complex	ABHKT	−18.4
GUT2	Mitochondrial glycerol-3-phosphate dehydrogenase	ABGIJPT	−19.7
GLC3	Glycogen branching enzyme	ABG	−19.7
SPL2	Protein with similarity to CDK inhibitors	HK	−19.7
IGD1	Inhibitor of Gdb1p glycogen debranching activity	Unclassified	−32.0
DDR2	Multi-stress response protein	K	−90.5

[a] Classified according to MIPS FunCat. Alphabetical codes are used that stand for the MIPS FunCat codes in parentheses: A, Metabolism (01); B, Energy (02); C, Cell cycle and DNA processing (10); D, Transcription (11); E, Protein synthesis (12); F, Protein fate (folding, modification, destination) (14); G, Protein with binding function or cofactor requirement (structural or catalytic) (16); H, Regulation of metabolism and protein function (18); I, Cellular transport, transport facilitation and transport routes (20); J, Cellular communication/signal transduction mechanism (30); K, Cell rescue, defense and virulence (32); L, Interaction with the cellular environment (34); M, Systemic interaction with the environment (36); N, Cell fate (40); O, Development (Systemic) (41); P, Biogenesis of cellular components (42); Q, Cell type differentiation (43); R, Tissue differentiation (45); S, Organ differentiation (47); T, Subcellular localization (70)

Next, we validated the results of RNA-seq analysis by examining the mRNA levels of three upregulated genes (*DBP2*, *ASC1*, and *GND1*, whose fold changes are approximately 40, 11, and 8 in order, as shown in Table 1) chosen among those excluding genes encoding structural constituents of the ribosome. These genes are not apparently related to each other in terms of function: *DBP2*, *ASC1*, and *GND1* encode respective ATP-dependent RNA helicase, G-protein β subunit and guanine nucleotide dissociation inhibitor for Gpa2, and 6-phosphogluconate dehydrogenase. Total RNAs were prepared in a similar way to what was done for RNA-seq analysis and used for RT-PCR to determination the expression levels. Although not as high as those obtained from RNA-seq analysis of acetic acid treated cells, ectopic overexpression of three genes were evident (Fig. 1a) and were enough to gain enhanced acetic acid tolerance (see Fig. 3).

Of DEGs, 46 of 56 upregulated and 55 of 58 downregulated genes were functionally annotated (Table 1). Those genes were further functionally categorized (p value threshold ≤ 0.05). For the upregulated genes, categories of "Metabolism" (mostly anabolic), "Protein synthesis," "Protein with binding function," and "Subcellular localization" were overrepresented (Table 2). As the last two involve most all of protein synthesis genes (Table 1), they seem not to be significant to characterize the gain of function. For the downregulated genes, categories of Metabolism (mostly catabolic), "Energy", "Transport", and "Cell rescue, defense, and virulence" were overrepresented (Table 2). Individual gene matches are shown in Supplementary Table S3. This functional categorization may reflect the physiological status of yeast cells exposed to acetic acid for 12 h. Although representing 9.6 % of the genome-wide ribosomal genes, the enriched protein synthesis genes (22 genes) suggest that the protein synthesis may resurge from the initial inhibition by acetic acid (Silva et al. 2013). On the other hand, attenuation of the expression of many stress genes (14 of 17 genes in the category Cell rescue, defense, and virulence) suggests that cells may no longer be under stress. Together, these possibly suggest that cells may be on the verge of adaptation.

To date, five studies have reported the genome-wide gene expression altered by acetic acid during either shock or adaptation (Supplementary Table S1). Of those, the study of Abbott et al. (2007) was excluded for comparison, since only a small number of DEGs are listed. Although the exposure time was not specified in the adaptation study of Kawahata et al. (2006), we presumed it to be longer than 24 h, considering the time taken for cells to propagate from 0.1 to 1.0 OD_{600} in the presence of 0.3 % acetic acid (pH 3.2). The exposure time of 12 h in the current study may represent a stage between shock and adaptation. Figure 1b shows the results from comparing our dataset 4 with previously published datasets (datasets 1–3, 5, and 6). Nine upregulated genes were common with any one

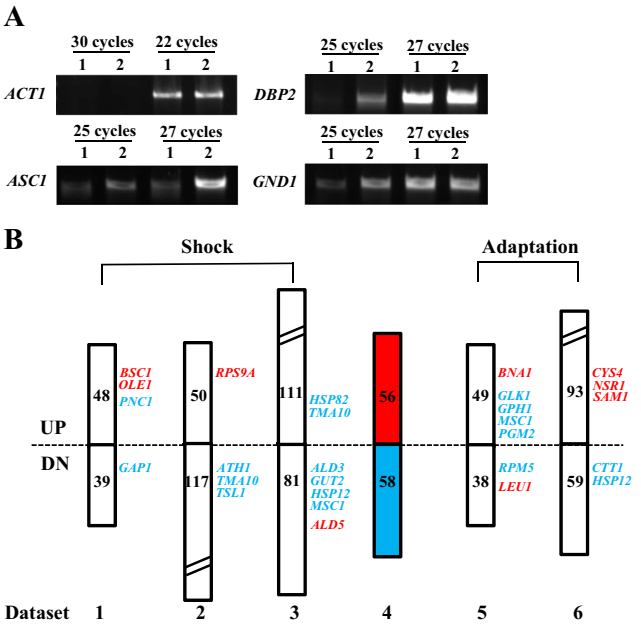

Fig. 1 Comparison of transciptome datasets compiled for acetic-acid-regulated genes. **a** RNA-seq data were validated by semi-quantitative RT-PCR of *DBP2*, *ASC1*, and *GND1*. Total RNAs were prepared from BY4741 cells untreated (lanes 1) and treated (lanes 2) with 0.6 % acetic acid (pH 4.5) for 12 h. One microgram of total RNA was directly amplified for 30 cycles with the *ACT1* primers to confirm no DNA contamination or reverse-transcribed and amplified for 22 (*ACT1*), or 25 and 27 cycles (*DBP2*, *ASC1*, and *GND1*) to measure relative transcriptional levels after acetic acid treatment. The *right most bars* show comparison of RNA-seq data with RT-PCR analysis of the transcriptional levels of the genes examined. *Shaded bars* correspond to RNA-seq and *white bars* to RT-PCR results. **b** Six datasets showing the transcriptionally regulated genes upon exposure to acetic acid are based on the studies of Kawahata et al. (2006) for datasets 1 and 5, Li and Yuan (2010) for dataset 2, Mira et al. (2010b) for dataset 3, this study for dataset 4, and Bajwa et al. (2013) for dataset 6. Conditions employed are briefly described in Supplementary Table S1. Genes in *red* and *blue* overlap with respective upregulated and downregulated genes of this study. *Numerals* inside the *box* indicate the number of upregulated or downregulated genes. *UP*, upregulated; *DN*, downregulated (Color figure online)

of them: seven as upregulated, but two as downregulated members. Meanwhile, 19 downregulated genes were common: 12 as downregulated members, but seven as upregulated members. Only three genes (*HSP12*, *MSC1*, and *TMA10*) appeared repeatedly in any two of the datasets. This data comparison shows that a large number of genes from our dataset are not common with those from other datasets, suggesting that the transcriptional programs altered by acetic acid may be significantly affected by experimental conditions employed.

Interaction network of the DEGs

Using the STRING web resource (http://string-db.org) (Szklarczyk et al. 2011), we conducted analysis on the predicted and known interactions among the DEGs identified. As shown in Fig. 2a, interactions between the upregulated genes were predominately two groups, one for 26 closely interrelated genes (22 protein synthesis genes and 4 others) and the other for 12 rather sparsely interrelated biosynthetic genes, consistent with the data of Table 2. Meanwhile, Fig. 2b shows that unlike the upregulated genes, the downregulated genes form a single dominant group composed of 27 closely interrelated genes. These data suggest the presence of a common regulatory factor(s) that represses various regulons whose activities may be required for shock recovery. If present, such a factor(s) must be induced at later times (post-exposure to acetic acid) for cells to adapt.

Effect of gene overexpression on acetic acid tolerance

Once adapted to a given stress, cells usually display enhanced tolerance to the same stress. To address the biological significance of the upregulated genes in adaptation, the effect of overexpression of some upregulated genes and individual deletion of the downregulated genes on enhanced tolerance were examined. For upregulated genes, we cloned the open reading frames (ORFs) of *DBP2*, *ASC1*, and *GND1* into pRS926, a pRS316 derivative in which the gene expression is controlled by the *TDH3* promoter, to generate plasmids pRS926-DBP2, -ASC1, and -GND1, respectively. As the ORFs of *DBP2* and *ASC1* are intervened by a single intron, their two coding sequences were separately amplified and joined by additional rounds of PCR. BY4741 cells were transformed with the control plasmid pRS926 and the other plasmids to yield strains BY/926, BY/DBP2, BY/ASC1, and BY/GND1. Similarly, strains W/926, W/DBP2, W/ASC1, and W/GND1 were constructed with W303-1A. It should be noted that the growth rate of BY/DBP2 and W/DBP2 was one third of the other BY4741- and W303-1A-derived strains (data not shown), indicating that overexpression of *DBP2* was detrimental for cell growth. When acetic acid tolerance of the resulting strains was examined based on the spot assay, enhanced acetic acid tolerance was observed in strains harboring the ORFs of *ASC1* or *GND1*, whereas strains harboring the ORF of *DBP2* were sensitive (Fig. 3a). Although tested in only two parental strains, it is very possible that the overexpression effect is not strain-specific. From this point, we focused on the overexpression effect in BY4741-derived strains only with excluding BY/DBP2 for further experiments because of its slow growth and sensitivity to acetic acid.

Once inside the near neutral cytoplasm of yeasts, weak acids, including acetic acid, dissociate into a proton and the corresponding anion, leading to intracellular acidification and anion accumulation, both of which limit the metabolic activities of yeasts (Mira et al. 2010c). Accordingly, it is very possible that strains with enhanced acetic acid tolerance also display a similar enhanced tolerance to other weak acids. The capacity of strains BY/926, BY/ASC1, and BY/GND1 to tolerate benzoic, propionic, and sorbic acids were examined

Table 2 Overrepresented functional categories in acetic acid regulated genes

Functional category[a]	Number of genes	% representation in the DEGs/genome[b]	*p* value
Up-regulated (46 out of 56 genes are found)			
01 Metabolism	23	50.0/33.6	1.56e-02
12 Protein synthesis	22	45.8/9.63	2.58e-11
16 Protein with binding function	30	65.2/41.7	1.10e-03
70 Subcellular localization	35	76/39.5	4.56e-07
Down-regulated (55 out of 58 genes are found)			
01 Metabolism	29	52.7/33.6	2.62e-03
02 Energy	16	29.0/8.18	4.67e-06
20 Transport	22	40/22.9	3.40e-03
32 Cell rescue, defense, virulence	17	30.9/15.6	3.23e-03

Functional categories with *p* value of ≤0.05 and gene match of ≥10 % are presented

[a] MIPS functional category number and description

[b] The percent value of genes represented in 48 upregulated or 53 downregulated genes and the whole yeast genome

using the spot assay. As shown in Fig. 3b, tolerance to the three weak acids of BY/ASC1 and BY/GND1 was not enhanced, demonstrating that overexpression of *ASC1* or *GND1* enhanced acetic acid tolerance only among weak acids examined.

Effect of gene deletion on acetic acid tolerance

We further investigated the effect of the downregulated genes on weak acid tolerance with mutants in which corresponding genes were deleted. Fifty-four viable

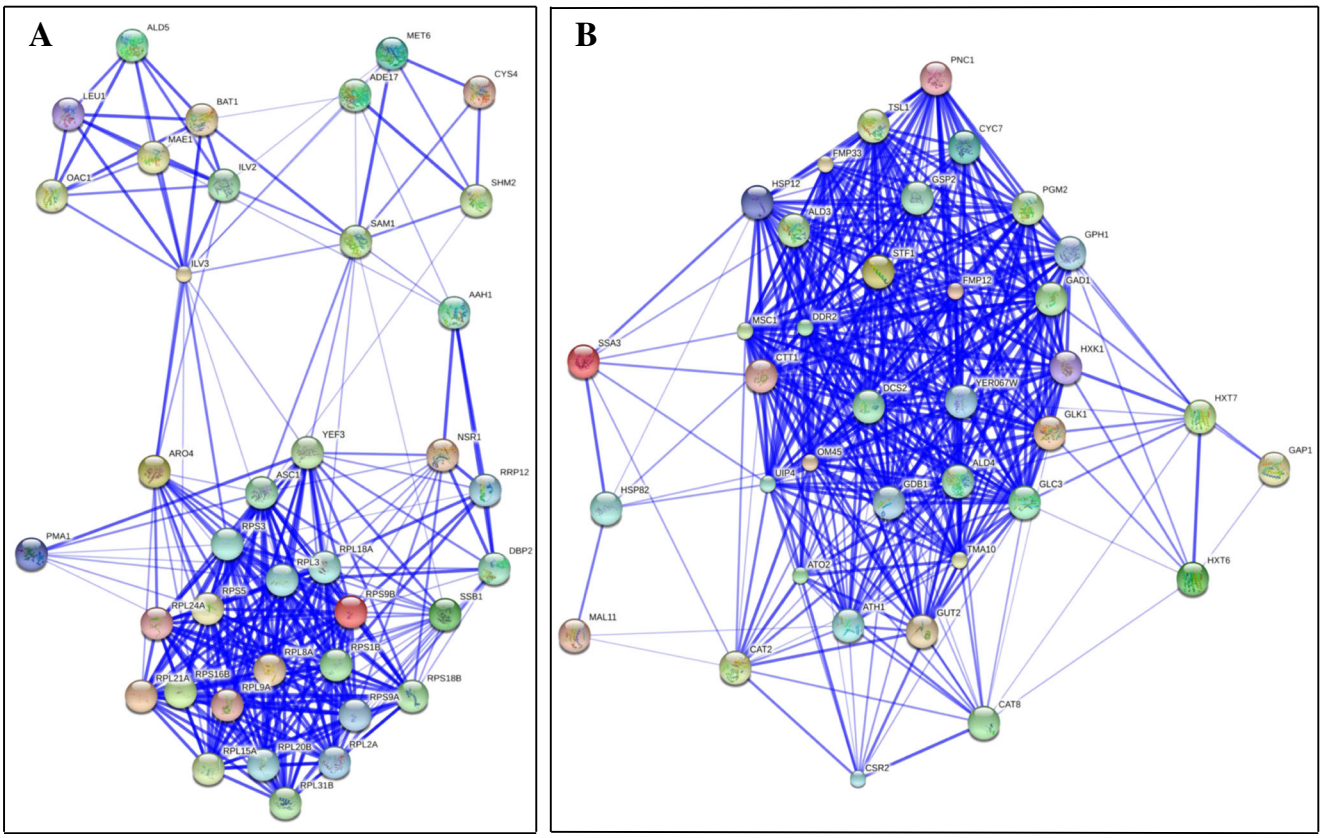

Fig. 2 Interaction network of the DEGs during adaptation to acetic acid. For functionally annotated genes (46 upregulated and 53 downregulated), their physical and functional interactions were identified computationally using the STRING web resource (version 9.1). Gene products are represented as nodes, whose colors have no meaning. Interactions are represented as node-connecting lines; *thicker lines* indicate stronger associations. Genes with less than three interactions are not shown, such that 39 upregulated and 37 downregulated genes remain

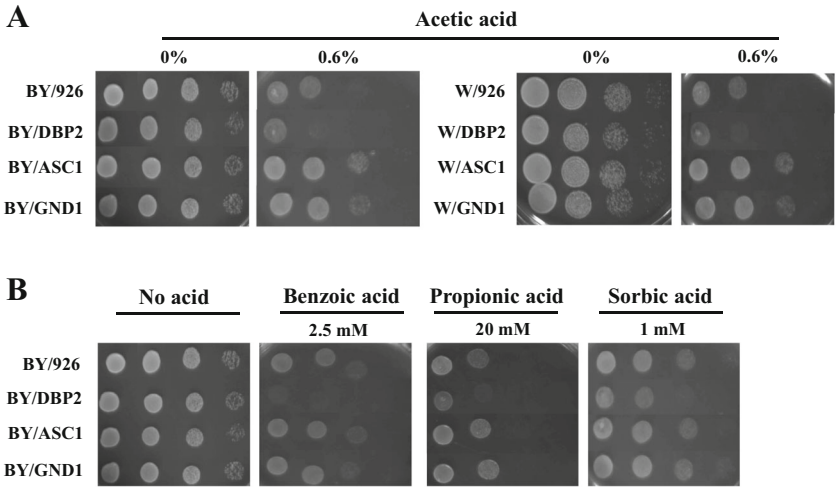

Fig. 3 Effect of gene overexpression on weak acid tolerance. BY4741 cells were transformed with the control plasmid pRS924, pRS924-DBP2, pRS924-ASC1, and pRS924-GND1 to yield respective strains BY/924, BY/DBP2, BY/ASC1, and BY/GND1. Similarly, strains W/924, W/DBP2, W/ASC1, and W/GND1 were generated using W 303-1A. Strains except for BY/DBP2 and W/DBP2 were grown to an OD_{600} of 1.0 in SC–Ura and 10-fold serial diluted. Since BY/DBP2 and W/DBP2 grew slowly, their cultures were concentrated to a final OD_{600} of 3.0 by centrifugation and resuspension. Aliquots (5 μl) were spotted onto the same solid SC unsupplemented or supplemented with 0.6 % acetic acid (**a**) and 2.5 mM benzoic, 20 mM propionic, and 1 mM sorbic acids (**b**). The pH of SC plates was 4.5

deletion mutants were retrieved from the deletion library. A preliminary spot assay showed that 50 deletion mutants, excluding *gph1Δ*, *msc1Δ*, *opi3Δ*, and *yer067-aΔ*, were tolerant to acetic acid (Supplementary Fig. S1). After grouping deletion mutants with the best acetic acid tolerance, one intentional (*hsp82Δ*) and two random (*ato2Δ* and *ssa3Δ*) were selected for further analysis. As Hsp82 (Hsp90 chaperone) is presumed to be a pro-death molecule involved in acetic-acid-induced apoptosis (Silva et al. 2013), the transcriptional decrease of *HSP82* may help cells to adapt to acetic acid instead of cell death. *ATO2* and *SSA3* encode a putative transmembrane protein and an ATPase for protein folding and the response to stress, respectively. Figure 4a shows that those three deletion mutants displayed enhanced acetic acid tolerance. We further examined the tolerance of *hsp82Δ*, *ato2Δ*, and *ssa3Δ* to other weak acids, including benzoic, propionic, and sorbic acids. As shown in Fig. 4b, all three mutants were tolerant to benzoic acid, but not propionic and sorbic acids, contrary to our expectation. There are several factors that determine the toxicity of weal acids, including lipophilicity. The lipophilic tendency of acetic, benzoic, propionic, and sorbic acids are respectively −0.24, 1.71, −0.32, and 1.63 (Mira et al. 2010b). Acetic and propionic acids can be classified into one group and benzoic and sorbic acids into another. Therefore, similar tolerance (or sensitivity) phenotypes, if present, would be found between identical group members rather than between different group members. The data of Fig. 4 suggest that the lipophilic tendency of weak acids had little influence on the weak acid tolerance of *hsp82Δ*, *ato2Δ*, and *ssa3Δ*.

Physiological changes in acetic-acid-tolerant strains

Acetic acid inhibits or prevents the growth of *S. cerevisiae*, at least in part, by causing intracellular acidification (Carmelo et al. 1996, 1997), oxidative damage (Piper 1999), and membrane integrity perturbation (Sikkema et al. 1995). Acetic-acid-tolerant yeast strains must overcome, to some extent, by reversing such growth inhibitory effects. As intracellular pH reduces rapidly below the pKa upon exposure to acetic acid (Carmelo et al. 1997), fast pH recovery (or proton efflux) is crucial for cells to maintain internal pH within physiological values. In *S. cerevisiae*, proton efflux depends predominantly on the activity of the plasma membrane H^+-ATPase proton pump (or Pma1) encoded by *PMA1* (Carmelo et al. 1996; Holyoak et al. 1996). We speculated that the enhanced weak acid tolerance of the examined strains might be attributed to an increased activity of Pma1. The Pma1 activity was determined directly by measuring the proton efflux rate in the presence of glucose in unbuffered suspensions of cells. The proton efflux rates over 20 min for BY/ASC1 and BY/GND1 cells treated with 0.6 % acetic acid (pH 4.5) for 12 h were approximately 85 and 95 nmol/mg DCW/h respectively, whereas the rate was 51 nmol/mg DCW/h for BY/926 (Fig. 5a). The proton efflux rates of BY/ASC1 and BY/GND1 were 40–46 % higher than that of BY/926. Similar patterns were observed for the deletion mutants *hsp82Δ*, *ato2Δ*, and *ssa3Δ* (95, 85, and 84 nmol/mg DCW/h in order compared to 59 nmol/mg DCW/h for BY4741; Fig. 5b). The proton efflux rates of the deletion mutants were 30–38 % higher than that of BY4741. It is very possible that the faster proton efflux of acetic-acid-tolerant strains helps to recover from low pH and therefore contributes to acetic acid tolerance to some extent.

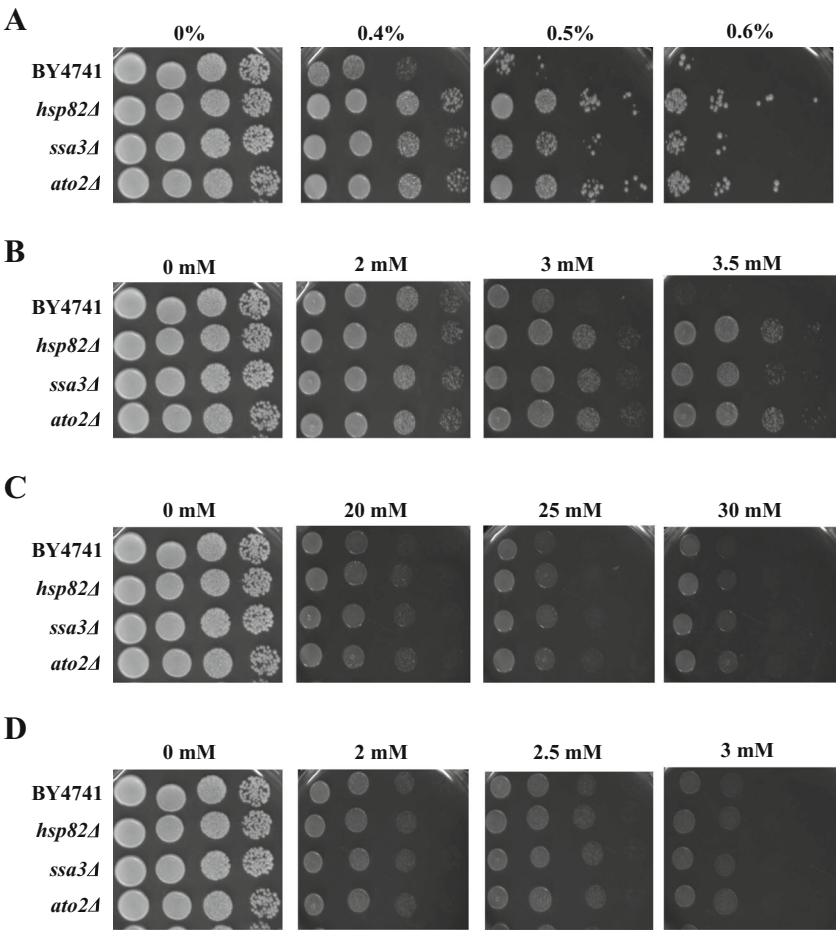

Fig. 4 Effect of gene deletion on weak acid tolerance. Weak acid tolerance of deletion mutants for *HSP82*, *ATO2*, and *SSA3* was assayed on SC unsupplemented or supplemented with the indicated concentrations of acetic (**a**), benzoic (**b**), propionic (**c**), and sorbic acids (**d**), as in Fig. 3

The plasma membrane is essential for maintaining the cell's integrity and plays a critical role in maintaining membrane potential (pH gradient) for the efficient uptake of nutrients and ions. Weak acids exert deleterious effects on the lipid organization and membrane function, including non-specific membrane permeabilization and perturbation of the function of membrane-embedded proteins (Stevens and Hofmeyr 1993; Teixeira et al. 2005). Membrane integrity can be monitored by measuring its permeability. PI enters the cell through damaged membranes, binds to nucleic acids, and fluoresces red when excited, whereas this does not occur in cells with intact membranes; staining of cells with PI is one of the indicators of membrane integrity. When the degree of PI staining was examined in cell populations harvested 12 h after treatment with 0.6 % acetic acid (pH 4.5), the percentage of stained cells in BY/ASC1 and BY/GND1 was lower than that in BY/926 (Fig. 5c) indicating that membranes in strains with enhanced weak acid tolerance were not damaged. Similar results were obtained for the deletion mutants *hsp82Δ*, *ato2Δ*, and *ssa3Δ* (Fig. 5d).

It was previously shown that reactive oxygen species (ROS) levels in acetic-acid-tolerant strains are considerably low compared with control strains (Zheng et al. 2011; Kim et al. 2013). Therefore, we were interested in the difference in internal levels of H_2O_2, the most abundant ROS in the cell, between BY/926, BY/ASC1, and BY/GND1, and between BY4741, *hsp82Δ*, *ato2Δ*, and *ssa3Δ* upon exposure to 0.6 % acetic acid (pH 4.5) over 48 h. In contrast to direct measurement of internal ROS in live cells by using fluorescent dye, the collective concentrations of H_2O_2 should be normalized by reflecting cell viability (live cells only). Based on the above PI staining data, correction factors (100 % divided by % cell viability) were calculated for each time point and used to measure internal H_2O_2 contents from live cells only (assay value multiplied by correction factor). Figure 5e shows the kinetic profile of internal H_2O_2 content in BY/926, BY/ASC1, and BY/GND1 cells over 48 h in the presence of acetic acid (0.6 %, pH 4.5). At 0 h, the internal H_2O_2 contents were 0.13 nmol/μg DCW for all. Upon exposure to acetic acid, the level of H_2O_2 slowly increased to approximately 0.6, 0.44, and 0.19 nmol/μg DCW at 48 h in BY/926, BY/ASC1, and BY/GND1 respectively. Similarly, Fig. 5f shows that those were 0.46, 0.32, 0.32, and 0.36 nmol/μg DCW for respective BY4741, *hsp82Δ*, *ato2Δ*, and *ssa3Δ*. These data indicate a difference in H_2O_2 levels between strains with enhanced acetic acid tolerance and the controls.

In conclusion, strains with gene overexpression- or deletion-mediated enhanced acetic acid tolerance were

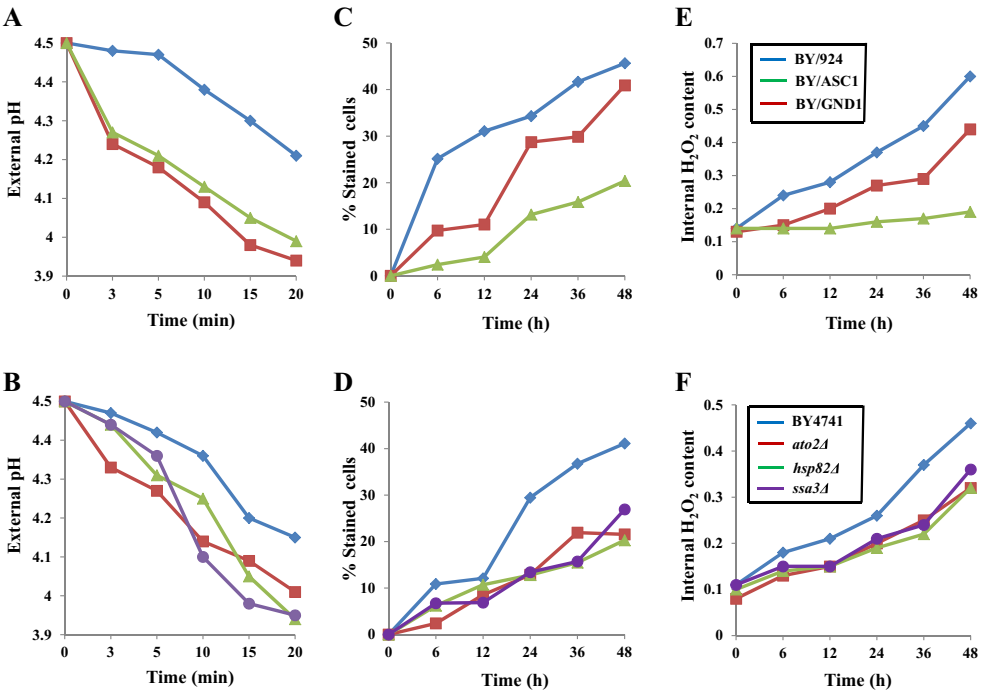

Fig. 5 Physiological changes in acetic-acid-tolerant strains. Strains BY/926, BY/ASC1, and BY/GND1 were for (**a**), (**c**), and (**e**), and strains BY4741, *hsp82Δ*, *ato2Δ*, and *ssa3Δ* for (**b**), (**d**), and (**f**). The data represent three independent experiments. Error bars are omitted for clarity. Cultures at 1.0 OD_{600} were treated with 0.6 % acetic acid (pH 4.5) for 3 h and harvested for proton efflux assay (**a** and **b**). Cultures at 1.0 OD_{600} were treated with 0.6 % acetic acid (pH 4.5) for 48 h with continuous shaking and harvested at the 6-h intervals for PI staining (**c** and **d**) and for determination of internal concentrations of H_2O_2 (**e** and **f**). The internal H_2O_2 content is expressed as nmol/μg DCW

superior in pumping protons out of the cell, maintaining healthy membranes, and lowering internal H_2O_2 contents compared to controls. These properties, individually or in combination, are likely to contribute at least in part to enhanced acetic acid tolerance.

Discussion

It is well established that yeast cells respond to stress by modifying the transcriptional program to upregulate and downregulate not only stress-specific genes but also hundreds of genes related to the commonly called environmental stress response (ESR) (Causton et al. 2001; Gasch et al. 2000). Through this reprogramed gene expression, cells may undergo a process called adaptation, recovering from various types of cell damage caused by stress. If cells fail to recover, their destiny is death—necrosis or apoptosis. It is likely that cells regulate different genes depending on the severity of stress, which can be determined by many environmental factors such as stress duration, width of physical change, and chemical concentration (Causton et al. 2001; Gasch et al. 2000; Marinho et al. 2014). With few exceptions, the global transcriptional changes are largely transient (Gasch et al. 2000). When cells adapt over time to a steady state, the levels of DEGs grossly decline close to those of unstressed cells. However, a number of genes seem to be newly regulated during adaptation. This holds true for acetic acid as demonstrated in the study by Kawahata and colleagues (2006), in which transcriptional profiles of acetic acid shock and adaptation were compiled simultaneously under identical conditions except for stress duration and, therefore, comparison of genes regulated during the two processes was possible (see Supplementary Table S1). Approximately 25 % overlapped among both upregulated and downregulated genes (11 among 48 shock and 49 adaptation for upregulated genes and 10 among 39 shock and 38 adaptation for downregulated genes). Thus, adaptation is likely to undergo a stepwise molecular process, i.e., transient (surge and recession) regulation of stress-specific and ESR genes, maintenance of most of regulated shock genes at a lower level, and additional regulation of a novel set of adaption genes. According to this notion, there are some differences in DEGs between shock and adaptation. Comparison of the shock datasets 1–3 of Fig. 1b with our dataset 4 is an example for acetic acid.

The data of Fig. 2 and Table 2 shows that under our experimental conditions (dataset 4), protein synthesis genes were upregulated and stress response genes were downregulated, which was unexpected. Examination of these two (sub)categories of Protein synthesis and "Stress response" in the other five datasets (Supplementary Table S4) raises interesting issues. Protein synthesis genes are upregulated in dataset 4, but downregulated in dataset 2. Meanwhile, stress response genes are upregulated in datasets 1, 3, 5, and 6, but downregulated in datasets 2 and 4. When cells are exposed to various stress conditions (not including acetic acid), global repression of protein synthesis is triggered at both the transcriptional (Causton et al. 2001; Gasch et al. 2000) and translational levels (Beilharz and Preiss 2004; Halbeisen et al. 2008; Simpson and Ashe 2012). Intriguingly, the shock dataset 2 has registered 28 genes (16.4 % of 177 downregulated genes) in the category Protein synthesis, whereas none in

two other shock datasets 1 and 3. The experimental conditions for the dataset 2 are unusual for shock (Li and Yuan 2010), in which although pH was not specified, the acetic acid concentration (1.8 %) was extremely high. Assuming the pH of the medium to be below the pKa of acetic acid, the conditions used were very toxic, such that cells might undergo acetic-acid-induced apoptosis, in which gene repression occurs at the genome-wide level except for pro-apoptotic genes. A significant repression of protein synthesis genes is an example (Almeida et al. 2009; Silva et al. 2013). Considering this, downregulation of protein synthesis and stress response genes shown in dataset 2 is quite sensible. In conclusion, regulation of protein synthesis and stress response genes of dataset 4 is the opposite of both shock datasets 1 and 3, and adaptation datasets 5 and 6. The reason for the lack of downregulation of protein synthesis genes in the shock datasets 1 and 3 may be that cells treated with 0.3 % acetic acid (pH 3.2 or 4.0) for 30 min were already in the "recession" stage (see above). As upregulation of protein synthesis genes and downregulation of stress response genes are rather unique to dataset 4 when compared with the other datasets (Supplementary Table S4), we speculate that dataset 4 may represent a status of gene expression between shock and adaptation. Thus, upregulation of protein synthesis genes and downregulation of stress response genes seem to be necessary to recover from shock and proceed to adaptation characterized by some physiological changes such as enhanced proton efflux, stabilized membrane integrity, and lessened internal H_2O_2 content.

In this study, we have identified acetic-acid-tolerant strains by overexpression of upregulated genes based on the transcriptional profile of acetic-acid-treated cell (Fig. 3). We presented examples of two genes (*ASC1* and *GND1*). There seems to be little correlation between upregulation fold and tolerance degree, suggesting that enhanced acetic acid tolerance is attributed to multiple factors. The results of *ASC1* and *GND1* overexpression agree with the concept that the upregulated genes under a given stress can confer resistance to the same stress, which is frequently proven not to be the case. We expect more acetic-acid-tolerant strains, if the rest of the upregulated genes are overexpressed. We also identified 50 acetic-acid-tolerant strains by screening 54 deletion mutants for the downregulated genes (Fig. 4). As the success rate is very high (2 out of 3 for overexpression and 50 out of 54 for deletion), information of the transcription profile we obtained under specific conditions (12-h exposure in particular) is quite straightforward in identifying molecular factors that confer enhanced acetic-acid-tolerant strains.

As mentioned earlier, acetic acid exerts various deleterious effects: damage to cell membranes, delays in cell growth, intracellular acidification, and ROS accumulation. For yeast cells to adapt to acetic acid, cell wall and membrane reorganization, pH recovery, efflux of anions, and ROS detoxification are required (Mira et al. 2010c). Several protein products have been specified for some of them: Haa1 downstream effector Spi1 for cell wall membrane reorganization (Simões et al. 2006), Pma1 for proton efflux (Carmelo et al. 1997), and Haa1 downstream effectors Tpo2/3 for anion efflux (Fernandes et al. 2005). Thus, *HAA1* plays an important role in acetic acid tolerance. Its ectopic overexpression confers enhanced tolerance to acetic acid (Tanaka et al. 2012), which opens a door for genetic manipulation of polyploidy wild-type yeast strains with enhanced acetic acid tolerance. Although both overexpression and deletion of genes can be used to construct acetic-acid-tolerant yeast strains of any genetic background in the laboratory, the former is more adequate to manipulate polyploidy industrial strains. One round of successful integration of a gene to be overexpressed into the genome has an advantage over at least two rounds of deletion. Accordingly, the more genes like *HAA1* are identified, the higher are the possibilities of constructing acetic-acid-tolerant industrial strains. In this sense, identification of *ASC1* and *GND1* as molecular factors that confer enhanced acetic acid tolerance is of industrial significance. Understanding how their overexpression enhances acetic acid tolerance is a future challenge.

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Conflict of interest The authors declare that they have no conflict of interest.

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