Global Phenotypic Analysis and Transcriptional Profiling Defines the Weak Acid Stress Response Regulon in *Saccharomyces cerevisiae*

Christoph Schüller,* Yasmine M. Mamnun,* Mehdi Mollapour,[†]
Gerd Krapf,* Michael Schuster,* Bettina E. Bauer,* Peter W. Piper,[†] and
Karl Kuchler*[‡]

*Department of Medical Biochemistry, Division of Molecular Genetics, Max F. Perutz Laboratories, University and Biocenter of Vienna, A-1030 Vienna, Austria; and [†]Department of Biochemistry and Molecular Biology, University College London, London WC1E 6BT, United Kingdom

Submitted May 22, 2003; Revised October 3, 2003; Accepted October 23, 2003

Monitoring Editor: Chris Kaiser

Weak organic acids such as sorbate are potent fungistatic agents used in food preservation, but their intracellular targets are poorly understood. We thus searched for potential target genes and signaling components in the yeast genome using contemporary genome-wide functional assays as well as DNA microarray profiling. Phenotypic screening of the EURO-SCARF collection revealed the existence of numerous sorbate-sensitive strains. Sorbate hypersensitivity was detected in mutants of the shikimate biosynthesis pathway, strains lacking the *PDR12* efflux pump or *WAR1*, a transcription factor mediating stress induction of *PDR12*. Using DNA microarrays, we also analyzed the genome-wide response to acute sorbate stress, allowing for the identification of more than 100 genes rapidly induced by weak acid stress. Moreover, a novel War1p- and Msn2p/4p-independent regulon that includes *HSP30* was identified. Although induction of the majority of sorbate-induced genes required Msn2p/4p, weak acid tolerance was unaffected by a lack of Msn2p/4p. Ectopic expression of *PDR12* from the *GAL1–10* promoter fully restored sorbate resistance in a strain lacking War1p, demonstrating that *PDR12* is the major target of War1p under sorbic acid stress. Interestingly, comparison of microarray data with results from the phenotypic screening revealed that *PDR12* remained as the only gene, which is both stress inducible and required for weak acid resistance. Our results suggest that combining functional assays with transcriptome profiling allows for the identification of key components in large datasets such as those generated by global microarray analysis.

INTRODUCTION

Short-chain weak organic acids such as sorbate, benzoate, and propionate are naturally occurring compounds that prevent microbial growth. They are therefore widely used in food and beverage preservation. They enter the cell by passive diffusion and dissociate at the higher intracellular pH generating protons and acid anions that accumulate. The mechanisms underlying the antimicrobial effects of these acids are still obscure but may be attributed, at least in part, to a cytoplasmic acidification that inhibits essential metabolic functions (Krebs *et al.*, 1983; Pearce *et al.*, 2001). Furthermore, lipophilic compounds such as sorbate and benzoate are thought to impact membrane permeability (Holyoak *et al.*, 1999). Several genes have been implicated in the weak acid stress response and adaptation so far. The ATP-binding cassette (ABC) efflux pump Pdr12p (Piper *et al.*, 1998) is a major factor counteracting intracellular anion accumulation. Cells lacking Pdr12p, which is highly induced upon weak acid stress, are hypersensitive to weak acids with a chain length from C3 to at least C8 (Wolfger, H., unpublished observations; Holyoak *et al.*, 1999; Hatzixanthis *et al.*, 2003). Moreover, the plasma membrane H⁺-ATPase Pma1p as well

as the heat shock proteins Hsp30p and Hsp26p appear modulated during weak acid stress (Holyoak *et al.*, 1996; Piper *et al.*, 1997; de Nobel *et al.*, 2001).

Saccharomyces cerevisiae cells can only cope with weak acid stress by inducing Pdr12p, leading to stress adaptation (Piper *et al.*, 1998). Therefore, genes upregulated by weak acid stress are prime candidates for important resistance factors. Indeed, cells adapted for growth on sorbate (de Nobel *et al.*, 2001) display distinct mRNA profiles, displaying enhanced expression levels of many genes. This set of genes overlaps partly with transcriptional patterns seen in the responses to several other adverse conditions (Rep *et al.*, 1999; Gasch *et al.*, 2000; Causton *et al.*, 2001). Interestingly, the spectrum of genes upregulated in sorbate-adapted cells includes many genes regulated by Msn2p and Msn4p (de Nobel *et al.*, 2001). These are the main regulators of the general stress response, and both also appear activated by sorbic and benzoic acid stress (Görner *et al.*, 1998).

Drastic changes of environmental contexts such as exposure to stressful conditions lead to a rapid and broad transcriptional response with up to hundreds of genes induced or repressed. DNA microarray mRNA profiling methods (DeRisi *et al.*, 1997; Gasch *et al.*, 2000; Young, 2000; Causton *et al.*, 2001) allow for efficient monitoring of these dynamic changes of gene expression regulation. Interestingly, global expression profiles as a consequence of a given condition or specific for a distinct cell type represent a remarkable stable diagnostic phenotype. For

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E03-05-0322. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E03-05-0322.

[‡] Corresponding author. E-mail address: Karl.Kuchler@univie.ac.at.

Table 1. Yeast strains used in this study

Strains	Genotype	Source
W303-1A	<i>MATa ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100</i>	Rothstein (1983)
YBB14	<i>MATa pdr12Δ::hisG-URA3-hisG</i> (otherwise isogenic to W303-1A)	Kren <i>et al.</i> (2003)
YBB24	<i>MATa hsp30Δ::HIS3</i> (otherwise isogenic to W303-1A)	This study
YBB25	<i>MATa pdr12Δ::hisG-URA3-hisG hsp30Δ::HIS3</i> (otherwise isogenic to W303-1A)	This study
YAK120	<i>MATa war1Δ::HIS3MX6</i> (otherwise isogenic to W303-1A)	Kren <i>et al.</i> (2003)
W303 <i>msn2 msn4</i>	<i>MATa msn2::TRP1 msn4::HIS3</i> (otherwise isogenic to W303-1A)	Görner <i>et al.</i> (1998)
YCS105	<i>MATa msn2::TRP1 msn4::HIS3 war1Δ::HIS3MX6</i> (otherwise isogenic to W303-1A)	This study
YAK2	<i>MATa ura3::PDR12-lacZ</i> (otherwise isogenic to W303-1A)	Kren <i>et al.</i> (2003)
YBB27	<i>MATa ura3::PDR12-lacZ war1Δ::HIS3MX6</i> (otherwise isogenic to W303-1A)	This study
FY1679-28c	<i>MATa ura3-52 leu2Δ1 his3Δ200 trp1Δ63</i>	EUROSCARF Collection
FY-GAL-PDR12	<i>MATa pdr12::GAL1-10-PDR12</i> (otherwise isogenic to FY1679-28c)	Hatzixanthis <i>et al.</i> (2003)
FY-GAL-PDR12war	<i>MATa pdr12::GAL1-10-PDR12 war1Δ::HIS3</i> (otherwise isogenic to FY1679-28c)	This study
BY-4741	<i>MATa ura3-Δ0 his3-Δ1 leu2-Δ0 met15-Δ0</i>	EUROSCARF Collection
BY-Deletions	<i>GEN::kanMX4</i> deletions of <i>HSP12</i> , <i>HSP26</i> , <i>HSP104</i> , <i>PDR12</i> , <i>TPO1</i> , <i>YBL059W</i> , <i>AUT7</i> , <i>FUN34</i> , <i>YKL051W</i> , <i>ECM39</i> , <i>TPO4</i> , <i>RPB4</i> , <i>GAL11</i> , <i>SWI3</i>	EUROSCARF Collection

example, transcriptome analysis can be used to classify tumor subtypes (Golub *et al.*, 1999) and sometimes even enable assignment of functional roles to uncharacterized genes (Hughes *et al.*, 2000). However, many genes whose functions are entirely unrelated to a given physiological or nonphysiological condition are usually also modulated in their expression. To distinguish functionally relevant genes from “innocent bystanders,” it is therefore necessary to combine global microarray profiling with functional assays or biochemical experiments (Shoemaker *et al.*, 1996; Hughes *et al.*, 2000). Functional assays at the genomic scale would be most informative, because such evidence complements microarray datasets. In this work, we used a two-pronged approach to identify and characterize components important for the molecular defense against weak organic acid stress. Thus, we combined a genome-wide phenotypic screening using the EUROSCARF deletion strain collection with global transcriptome microarray analysis.

The induction of *PDR12* by weak acid stress is fast and mainly regulated at the level of transcription (Kren *et al.*, 2003). Importantly, *Pdr12p* induction is specific for weak acid stress but not for other stress types and is independent of *Msn2p/4p* (Piper *et al.*, 1998). A novel transcription factor of the *Zn₂Cys₆* family, *War1p*, was recently identified as a stress regulator of *PDR12* (Kren *et al.*, 2003). Cells lacking *War1p* are weak acid hypersensitive, suggesting that genes induced by this factor are important under these conditions. To identify the genes dependent on *War1p*, we exploited genome-wide mRNA profiles to determine the global transcriptional response in weak acid-stressed cells. Our results suggest *War1p*, *Msn2p/4p*, and a third pathway as major players in the weak acid stress response. We can distinguish the regulons of *War1p* and *Msn2p/4p*, and we show that these are largely nonoverlapping. Strikingly, comparison of the results from the functional assays with microarray datasets indicated *PDR12* as the only gene that is both sorbate-induced and essential for stress adaptation. Although we identified many genes whose expression levels are modulated by weak acid stress, our results nicely illustrate that only a combination of functional genomics and whole-genome expression profiling can efficiently identify key players in biological response pathways.

MATERIALS AND METHODS

Yeast Strains, Culture Conditions, and Cytotoxicity Assays

All yeast strains used in this study are listed in Table 1. Rich medium (YPD) and synthetic medium (SC), supplemented with appropriate auxotrophic components, were prepared as described elsewhere (Kaiser *et al.*, 1994). Unless otherwise indicated, all yeast strains were grown routinely at 30°C. Tests for weak acid resistance phenotypes were performed with cells grown to the exponential growth phase and diluted. Identical volumes of cultures as well as 1:10 and 1:100 serial dilutions were spotted onto agar plates containing the indicated concentrations of weak acids. Colony growth was inspected after a 48-h incubation. Gene deletions were carried out by a PCR-based method using disruption cassettes derived from plasmids *pFA6a-HIS3MX6* and *pFA6a-KanMX6* (Wach *et al.*, 1997). YCS105 (*war1Δ msn2Δ msn4Δ*) was obtained by crossing the isogenic strains YAK120 (*war1Δ*) (Kren *et al.*, 2003) and W303-1A *msn2 msn4* (Görner *et al.*, 1998). The correct genotypes of progeny were verified by PCR analysis using appropriate primers. The plasmid *YCplac22* (Gietz and Sugino, 1988) was used to deliver the *TRP1* gene into the strains W303-1A, YBB14, and YAK120 for growth assays in the presence of sorbate. *WAR1* was deleted from strain FY-GAL-PDR12 carrying a *PDR12* gene under control of the *GAL1-10* promoter (Hatzixanthis *et al.*, 2003), using the *hphMX4* cassette system (Goldstein and McCusker, 1999), resulting in strain FY-GAL-PDR12 *war1Δ*.

Preparation of Cell Extracts and Immunoblotting

The preparation of cell-free extracts for immunoblotting was performed exactly as described elsewhere (Egner and Kuchler, 1996). Cell lysates equivalent to 0.5 A₆₀₀ units were resolved by SDS-PAGE in a 7.5% gel and transferred to nitrocellulose membranes. *Pdr12p* was visualized on immunoblots using the ECL chemiluminescence detection system (Amersham Bioscience, Piscataway, NJ) and a polyclonal anti-*Pdr12p* antiserum (Piper *et al.*, 1998).

β-galactosidase Measurements and RNA Methods

Cells carrying a *PDR12-LacZ* reporter gene were grown in YPD medium to the midexponential growth phase. Weak acids were dissolved in water as 0.5 M potassium salt stock and added to the culture medium up to desired concentrations. Cells were harvested by centrifugation after 30 min. β-galactosidase enzyme measurements were carried out exactly as described elsewhere (Rose, 1990). All experiments were done at least in triplicate. Preparation of RNA for Northern analysis and fractionation by agarose gel electrophoresis was done exactly as described previously (Wolfger *et al.*, 1997). DNA probes were radiolabeled with a Megaprime Labeling kit according to the manufacturer (Amersham). Signals were detected by exposure to phosphor imager screens (Molecular Dynamics, Sunnyvale, CA) and analyzed with ImageQuant software (Molecular Dynamics).

Phenotypic Screening

EUROSCARF (Frankfurt, Germany) deletion strains representing haploid (viable) single deletions of 4873 yeast ORFs were grown on solid YPD medium in 96-well microtiter plates. Strains were transferred into 96-well plates containing 100 μl YPD and grown overnight at 30°C. Next, cells were spotted onto YPD, pH 4.5, plates containing 1 mM sorbic acid and YPD pH4.5

reference plates, using a sterile spotting device. Growth was inspected after a 48-h incubation and compared with the reference plate to identify sensitive strains, all of which were subjected to a second screening round to avoid spotting artifacts.

DNA Microarray Profiling Experiments

Culture conditions: Cells from overnight cultures in YPD were diluted in fresh YPD to an OD₆₀₀ of 0.1 and grown at 30°C until an OD₆₀₀ of 1–1.1 was reached. Cultures were split and potassium sorbate (Sigma St. Louis, MO) was added at a final concentration of 8 mM to one half of the culture. After 20 min, both untreated and treated cultures were harvested by centrifugation at room temperature (2 min, 4000 × g), and cells were immediately washed in ice-cold water, reharvested at 4°C, and frozen at –80°C. Total RNA was prepared by the hot phenol method and quantified by spectrophotometry at 260 nm in TE buffer exactly as described elsewhere (Kren *et al.*, 2003). For cDNA synthesis, 20 µg of total RNA from treated and untreated cells was converted to cDNA using 200 U SuperscriptII reverse transcriptase (Invitrogen, Carlsbad, CA). Reactions included either Cy3dCTP or Cy5dCTP (Amersham Biosciences). Labeled cDNAs were pooled and RNA was hydrolyzed for 20 min in 50 mM NaOH at 65°C, neutralized with acetic acid, precipitated with an equal volume of isopropanol, washed briefly in 70% ethanol, and finally dissolved in 5 µl sterile distilled water. Glas microarrays (OCI Toronto; <http://www.microarrays.ca/>) containing PCR fragments of 6144 predicted *S. cerevisiae* ORFs spotted in duplicates were used for the expression profiling. Hybridization was done in a total volume of 60 µl in DigEasyHyb solution (Roche Diagnostics, Basel, Switzerland) with 0.1 mg/ml salmon sperm DNA (Sigma) as carrier solution at 37°C for 14–16 h. Microarrays were disassembled, washed three times in 1× SSC, and 0.1% SDS at 50°C for 15 min, followed by a 1-min wash in 1× SSC at room temperature. Slides were spun-dry for 5 min at 500 rpm in a table-top centrifuge at room temperature, scanned on an Axon4000B scanner (Axon Instruments, Foster City, CA) and analyzed using the GenePix Pro4.1 software (Axon Instruments). Microarrays and experimental protocols were obtained from the Ontario Cancer Institute (OCI Toronto; <http://www.microarrays.ca/>).

Computational Analysis

Microarray data were collected and analyzed with GenePix Pro4.1 (Axon Instruments). The entire raw dataset is available at <http://www.at.embnet.org/molg/kuchler/repository/>. Spots not found automatically by GenePix Pro4.1 because of low signal intensities were flagged absent and subsequently excluded from further analysis. Microarrays were normalized using GenePix Pro4.1 such that the sum of the Median of Ratios of unflagged values was 1. Median of Ratios of individual experiments were filtered using conventional spreadsheets. Median of Ratios of the wild-type, *war1*, *msn2/4*, and *war1 msn2/4* sets were log-transformed for averaging, omitting flagged values and ORFs with less than four values. ORFs with least one average Median of Ratios value of induction to repression above two in all four datasets were further analyzed. The assignment of ORFs into clusters that are regulated by *WAR1*, *MSN2/4*, or both was based on the induction relative to the wild-type control using a cutoff value of 0.6 (see Figure 2). Furthermore, clustering was performed using the cluster3 software (Eisen *et al.*, 1998). Uncentered correlation and complete linkage for genes and arrays, and subsequent visualization with Java TreeView revealed similar groups (see Figure 2). Detection of short common sequences in promoters was done with AlignACE version 2.2, (Roth *et al.*, 1998), using 800 base pairs of upstream sequence, sampling in a maximum window of 30, using 10 columns, 10 expected elements, including the following input ORFs: *YNR002C*, *YPL058C*, *YPL122C*, *YNR030W*, *YOL032W*, and *YGR046W*. A logo generator (<http://weblogo.berkeley.edu/logo.cgi>) was used for graphical presentation of the sequence logo (Schneider and Stephens, 1990).

RESULTS

To identify nonessential genes required for growth in the presence of sorbic acid, we used the haploid deletion strains of the EUROSCARF collection (EUROSCARF; <http://www.uni-frankfurt.de/fb15/mikro/euroscarf/>). Strains were grown overnight in microtiter plates in liquid YPD and spotted onto YPD plates, pH 4.5, containing 1 mM sorbic acid as well as YPD control plates lacking sorbate. After incubation for 48 h, sorbate sensitivity was scored. Eighty-six of 4827 strains tested, reproducibly showed impaired growth in the presence of sorbate (Table 2). Furthermore, this experiment has since been repeated independently with the corresponding homozygous diploid mutants from the EUROSCARF collection using higher sorbate concentrations (M. Mollapour, unpublished results), revealing a somewhat larger set of 194 genes, of which our set represents a subgroup. The results of the 1 mM sorbate screen

Figure 1. (A) *FUN34* is induced by sorbic acid in a War1p-dependent manner. Northern analysis of *FUN34* under sorbate stress. Sorbic acid, 10 mM, was added to exponentially growing cultures of W303-1A and YAK120 (*war1Δ*). mRNA was prepared from aliquots taken at the indicated time points. Blots were probed for *FUN34* and *HSP30* mRNAs, methylene blue-stained rRNA served as a loading control. (B) Sequence logo of a putative consensus War1p DNA-binding motif. An alignment of related sequences detected by AlignACE in the promoter regions of War1p-dependent genes is displayed as a sequence logo. (C) Pdr12p levels are strongly reduced in *rpb4Δ* mutants. Wild-type and the indicated mutant strains were grown in YPD to the exponential growth phase, stressed with 10 mM sorbic acid and Pdr12p levels were assayed on Western blots. A cross-reacting band served as a loading control.

at pH 4.5 are listed in Table 2, including the relevant gene ontology assignments (GO) listed in the *Saccharomyces* genome database (ftp://genome-ftp.stanford.edu/yeast/data_download/literature_curation/orf_geneontology.tab).

The mutant strains identified by our phenotypic analysis are involved in diverse cellular functions (Table 2). A major group belongs to energy metabolism, including genes such as phosphofructokinase alpha and beta subunits (*PFK1*, *PFK2*), as well as pyruvate decarboxylase 1 (*PDC1*) and alcohol dehydrogenase (*ADH1*). Another group appears involved in mitochondrial function, although no general effect of petite mutants was evident. Interestingly, several genes of the ergosterol biosynthesis pathway (*ERG2,3,6,28*) were identified, perhaps suggesting that perturbations of mem-

Table 2. List of ORFs implicated in weak acid resistance listed by canonical name

ORF name	Gene	Pathway	Biological function
YAL016w	TPD3	Protein biosynthesis	Protein phosphatase type 2A
YBL079w	NUP170	mRNA-nucleus export	Structural molecule
YBR036c	CSG2	Calcium ion homeostasis	Molecular function unknown
YBR096w		Biological process unknown	Molecular function unknown
YBR127c	VMA2	Vacuolar acidification	Hydrogen-transporting two-sector ATPase
YBR132c	AGP2	Response to osmotic stress	Hydrogen:amino acid symporter
YBR133c	HSL7	G2/M transition of mitotic cell cycle	Protein-arginine N-methyltransferase
YBR173c	UMP1	Protein catabolism	Chaperone
YBR267w		Biological process unknown	Molecular function unknown
YBR282w	MRPL27	Protein biosynthesis	Structural constituent of ribosome
YCR081w	SRB8	Negative regulator of Pol-II transcription	RNA polymerase II transcription mediator
YDL005c	MED2	Transcription from Pol-II promoter	RNA polymerase II transcription mediator
YDR007w	TRP1	Tryptophan biosynthesis	Phosphoribosylanthranilate isomerase
YDR017c	KCS1	Stress response	Protein kinase
YDR027c	LUV1	Not yet annotated	Molecular function unknown
YDR069c	DOA4	Deubiquitination	Ubiquitin-specific protease
YDR173c	ARG82	Arginine metabolism	Inositol/phosphatidylinositol kinase
YDR364c	CDC40	Not yet annotated	Molecular function unknown
YDR484w	SAC2	Actin filament-based process	Molecular function unknown
YER017c	AFG3	Protein complex assembly	Adenosinetriphosphatase
YER044c	ERG28	Ergosterol biosynthesis	Molecular function unknown
YER083c	RMD7	Cell wall organization and biogenesis	Molecular function unknown
YER090w	TRP2	Tryptophan biosynthesis	Anthranilate synthase
YER169w	RPH1	DNA repair	Not yet annotated
YFL017w-a	SMX2	mRNA splicing	Pre-mRNA splicing factor
YGL026c	TRP5	Tryptophan biosynthesis	Tryptophan synthase
YGL070c	RPB9	Transcription from Pol-II promoter	DNA-directed RNA polymerase II
YGL148w	ARO2	Not yet annotated	Chorismate synthase
YGL173c	KEM1	35S primary transcript processing	5'-3' Exoribonuclease
YGR063c	SPT4	DNA-dependent transcription regulation	Pol-II transcription elongation factor
YGR064w		Biological process unknown	Molecular function unknown
YGR076c	MRPL25	Protein biosynthesis	Structural constituent of ribosome
YGR104c	SRB5	Transcription from Pol-II promoter	RNA polymerase II transcription mediator
YGR112w	SHY1	Respiratory gaseous exchange	Molecular function unknown
YGR135w	PRE9	Ubiquitin-dependent protein catabolism	Proteasome endopeptidase
YGR159c	NSR1	rRNA processing	RNA binding
YGR162w	TIF4631	Translational initiation	Translation initiation factor
YGR222w	PET54	Protein biosynthesis	Molecular function unknown
YGR229c	SMI1	Cell wall organization and biogenesis	Not yet annotated
YGR240c	PFK1	Glycolysis	6-Phosphofructokinase
YGR272c		Biological process unknown	Molecular function unknown
YHR060w	VMA22	Protein complex assembly	Molecular function unknown
YIL018w	RPL2B	Protein biosynthesis	Structural constituent of ribosome
YJL063c	MRPL8	Protein biosynthesis	Structural constituent of ribosome
YJL075c		Biological process unknown	Molecular function unknown
YJL140w	RPB4	Transcription from Pol-II promoter	DNA-directed RNA polymerase II
YJL176c	SWI3	Chromatin modeling	RNA polymerase II transcription factor
YJL189w	RPL39	Protein biosynthesis	Structural constituent of ribosome
YKL169c		Biological process unknown	Molecular function unknown
YKL212w	SAC1	Dephosphorylation	Inositol/phosphatidylinositol phosphatase
YLR044c	PDC1	Ethanol fermentation	Pyruvate decarboxylase
YLR056w	ERG3	Ergosterol biosynthesis	C-5 sterol desaturase
YLR148w	PEP3	Non-selective vesicle docking	Protein binding
YLR399c	BDF1	Sporulation (sensu <i>Saccharomyces</i>)	Transcription factor
YLR402w		Biological process unknown	Molecular function unknown
YML008c	ERG6	Ergosterol biosynthesis	Delta(24)-sterol C-methyltransferase
YML061c	PIF1	DNA recombination	DNA helicase
YML076c	WAR1	Biological process unknown	Molecular function unknown
YMR060c	TOM37	Mitochondrial translocation	Protein transporter
YMR183c	SSO2	Non-selective vesicle fusion	t-SNARE
YMR202w	ERG2	Ergosterol biosynthesis	C-8 sterol isomerase
YMR205c	PFK2	Glycolysis	6-Phosphofructokinase
YMR231w	PEP5	Non-selective vesicle docking	Molecular function unknown
YNL084c	END3	Cytokinesis	Cytoskeletal adaptor
YNL225c	CNM67	Microtubule nucleation	Structural constituent of cytoskeleton
YNR032w	PPG1	Not yet annotated	Protein serine/threonine phosphatase
YNR037c	RSM19	Protein biosynthesis	Structural constituent of ribosome
YOL023w	IFM1	Translational initiation	Translation initiation factor
YOL051w	GAL11	Transcription from Pol-II promoter	RNA polymerase II transcription mediator

Table 2. Continued

ORF name	Gene	Pathway	Biological function
YOL086c	ADH1	Fermentation	Alcohol dehydrogenase
YOL143c	RIB4	Vitamin B2 biosynthesis	Riboflavin synthase
YOR235w		Biological process unknown	Molecular function unknown
YOR291w		Biological process unknown	Molecular function unknown
YPL042c	SSN3	Protein amino acid phosphorylation	RNA polymerase II transcription factor
YPL045w	VPS16	Protein-vacuolar targeting	Molecular function unknown
YPL058c	PDR12	Transport	Xenobiotic-transporting ATPase
YPL059w	GRX5	Response to osmotic stress	Thiol-disulfide exchange intermediate
YPL097w	MSY1	Not yet annotated	Tyrosine-tRNA ligase
YPL193w	RSB1	Ribosomal large subunit assembly	Molecular function unknown
YPL268w	PLC1	Pseudohyphal growth	1-PI-4,5-bisphosphate phosphodiesterase
YPR067w	ISA2	Iron transport	Molecular function unknown
YPR100w		Biological process unknown	Molecular function unknown

brane ergosterol can lead to dramatic sorbate hypersensitivity. Interestingly, strains lacking genes involved in the aromatic amino acid biosynthesis (e.g., *TRP1*, *TRP2*, *TRP5*, *ARO2*) also scored as sorbate sensitive. However, none of them was required for, or implicated in, transcriptional *PDR12* stress regulation (our unpublished results). Further, several mutants affected in RNA polymerase II (Pol-II)-dependent transcription were also identified, including *SWI3*, *SSN6*, *MED2*, *SRB5*, and *GAL11*, as well as *RPB9* and *RPB4*, two subunits of Pol-II. Notably, *RPB4* was previously shown to be required for heat and starvation stress tolerance (Choder, 1993). The sorbate hypersensitivity of these mutants emphasizes the importance of major transcriptional adjustments to elicit weak acid resistance and stress adaptation. Finally, weak organic acids might also cause oxidative stress (Piper, 1999; de Nobel *et al.*, 2001), consistent with the sorbate sensitivity of mutants lacking the *GRX5* mitochondrial glutaredoxin, an important determinant of oxidative stress resistance (Rodriguez-Manzanique *et al.*, 1999).

To identify genes directly involved in *PDR12* regulation, we also screened all 86 hypersensitive strains (Table 2) present in EUROSCARF collection for Pdr12p induction using immunodetection of Pdr12p levels after sorbate challenge (Piper *et al.*, 1998). Interestingly, *war1Δ* was the only mutant that completely lacked Pdr12p induction upon sorbate stress (Figure 1C). War1p is a Zn(II)₂Cys₆ transcription factor recently identified as a dedicated stress regulator of *PDR12* (Kren *et al.*, 2003). Notably, we found also a significantly reduced amount of Pdr12p protein levels in the *rpb4Δ* mutant strain, suggesting a transcriptional defect as a cause of its increased sorbate sensitivity (Figure 1C). In summary,

our functional genetic analysis revealed a plethora of different cellular activities reflecting the pleiotropic impact weak acid stress can exert on yeast cells. However, a sensitivity phenotype of mutants does not imply that the encoded wild-type gene is required for a weak acid-specific detoxification process, but merely indicates the inability to oppose weak acid stress.

War1p Activates a Small Msn2p/4p-independent Regulon

S. cerevisiae can adapt to weak organic acids in a process requiring rapid adjustment of its transcriptional program. To identify potentially protective genes induced by sorbate, we determined the whole-genome transcriptional profile during acute stress conditions. Wild-type cells were cultivated to the early exponential growth phase and treated with 8 mM sorbate for 20 min. The isolated mRNA was transcribed to fluorescent-labeled cDNA by incorporation of Cy3- or Cy5-conjugated dCTP and hybridized to *Saccharomyces cerevisiae* whole-genome microarrays. Each experiment was done at least in triplicate, revealing a set 100 representative genes that were significantly induced during short-term exposure to sorbate (Tables 3–7).

Additional experiments were performed with propionate-treated (30 mM) cells to exclude a possible sorbate-specific response as opposed to a general weak acid response. The sets of genes induced by sorbate and propionate were very similar, except for a less pronounced but detectable induction of Msn2p/4p-dependent genes by propionate (our unpublished results). A slightly acidic medium favors the protonated form of these weak acids (pK_a 4.6–4.7) and yeast is more sensitive to weak acids at pH 4.5. However, 1 mM

Table 3. Selection of genes induced by sorbic acid: *WARI*-dependent

Gene	ORF name	Biological process	Wild type	SDEV	<i>war1Δ</i>	SDEV	<i>msn2Δ msn4Δ</i>	SDEV	<i>war1Δ msn2/4Δ</i>	SDEV	a	STRE	b
<i>PDR12^c</i>	<i>YPL058C</i>	Transport	7.09	2.13	0.92	0.26	14.25	9.45	1.55	0.29	a		
<i>TFB2</i>	<i>YPL122C</i>	Transcription initiation Pol-II	3.18	1.33	1.03	0.18	4.16	0.86					
<i>FUN34^c</i>	<i>YNR002C</i>	Transport	2.60	0.58	1.02	0.05					a		b
<i>ECM39^c</i>	<i>YNR030W</i>	Protein amino acid glycosylation	2.24	0.31	1.27	0.29	4.39	2.46	1.34	0.20			

Values are *x*-fold induction. STRE: STRE-like sequences detected in the promoter region (Treger *et al.*, 1998; Moskvina *et al.*, 1998).

^a Genes induced in sorbic acid-adapted cells (de Nobel *et al.*, 2001).

^b Genes assigned *MSN2/4*-dependent by acidic stress (Causton *et al.*, 2001).

^c Putative War1p consensus element (5'-C G G c/t T g/c/t/g T A a/t-3'). The entire set of genes in each cluster are found in the supplementary material.

Figure 3. War1p, but not Msn2p/4p, is required for weak acid resistance. (A) Lack of *MSN2* and *MSN4* fails to influence growth properties on sorbic acid. Sensitivity of W303-1A and the mutant strains YBB14 (*pdr12Δ*), YAK120 (*war1Δ*), W303 *msn2 msn4* (*msn2Δ msn4Δ*), and YCS105 (*war1Δ msn2Δ msn4Δ*) was tested on YPD pH4.5 plates. Strains W303-1A, YBB14, and YAK120 carried the *TRP1* gene on the centromeric plasmid YCplac22. Growth was monitored after 48 h at 30°C. (B) *HSP12*, *HSP26*, and *HSP104* are not required for sorbic acid resistance. BY4741 derivatives were spotted onto sorbate-containing plates. Colony growth was recorded after 48 h.

sorbic acid in pH 4.5 medium resulted in the same pattern of induced genes, indicating that the intracellular weak acid accumulation is causing these changes rather than the pH of the growth medium (our unpublished results). Notably, the pattern of sorbate-inducible genes during short-term stress was quite distinct from the one reported earlier, derived from sorbate-adapted cells (de Nobel *et al.*, 2001). Interestingly, the pattern of rapidly induced genes showed a striking resemblance to the regulons modulated by the environmental stress response (Gasch *et al.*, 2000; Causton *et al.*, 2001).

To distinguish target genes of the general stress response pathway, which requires the transcription factors Msn2p/Msn4p (Martinez-Pastor *et al.*, 1996; Gasch and Eisen, 2002), from the War1p-dependent weak acid-specific regulon (Kren *et al.*, 2003), we repeated the microarray analysis in *msn2Δ msn4Δ*, *war1Δ*, and *war1Δ msn2Δ msn4Δ* mutants. The assignment into different clusters representing tentative regulons was done manually as well as by cluster analysis (Figure 2). We choose a cutoff value of 0.6 for the ratio of the

Figure 2. Clustering of transcript profiles. Hierarchical clustering of induction values representing average inductions over three replicate profiles of each the wild-type (W303-1A) and the isogenic derivatives (*war1Δ*, *msn2Δ msn4Δ*, *war1Δ msn2Δ msn4Δ*) after 20 min of 8 mM potassium sorbate treatment. Color codes refer to different regulatory groups after selection of differently regulated genes as revealed by ratio discrimination.

Figure 4. Pdr12p is necessary and sufficient for sorbic acid resistance. (A) Pdr12p levels as derived from a *GAL1-10-PDR12* gene are independent from War1p and sorbate challenge. Cells growing on YPD and YPGalactose were exposed to sorbic acid for 20 min. Immunoblots were decorated using polyclonal anti-Pdr12p antibodies. A cross-reacting band served as a loading control. (B) Cells from logarithmically growing cultures were spotted onto YPD, pH 4.5, and YPGal, pH 4.5, with and without 1 mM sorbic acid. Plates were monitored after a 48-h incubation at 30°C.

respective induction factors between *war1*, *msn2/4*, *war1 msn2/4*, and the wild-type. Following this scheme, War1p-dependent genes are required to have a fold induction of <0.6 of the wild-type but >0.6 of the *msn2/4* mutant (Figure 2). A permutation of this scheme yielded five gene clusters: War1p-dependent, Msn2/4p-dependent, War1p- and Msn2/4p-dependent, and War1p- and Msn2/4p-independent (*HSP30*) as well as "No-category." Except for a small group of four genes including *PDR12* (4%), the induction of most weak acid-inducible genes was unaffected by the absence of War1p. However, a large set with 35 genes (35%) was highly dependent upon Msn2p/4p. Using a *war1*Δ *msn2*Δ *msn4*Δ mutant strain (YCS105), we found 13 genes apparently coregulated by War1p and Msn2p/4p. Out of the remaining genes, 21 were assigned to a group regulated by neither War1p nor Msn2p/4p, thus forming an unexpected and as yet unknown regulon that included *HSP30*. Hence these results indicate the existence of a third weak acid sensing system besides War1p and Msn2p/4p.

Alternatively, we also used hierarchical clustering (Eisen *et al.*, 1998) and found a very similar distribution of the coregulated genes in different branches (Figure 2). Genes from the unassigned group mainly clustered with the *HSP30* branch, whereas genes from the War1p Msn2/4p-dependent group clustered to the Msn2/4p branch. However, War1p-dependent genes were clearly separated from the other groups (Figure 2). Northern analysis also demonstrated that induc-

tion of *HSP30* mRNA by sorbic acid is independent from War1p and partly independent from Msn2p/4p (see Figure 6A). Furthermore, the expression of 27 genes could not be placed in a regulatory context (Tables 3–7). The 25 genes previously identified in sorbate-adapted cells (de Nobel *et al.*, 2001) did not show a bias of distribution toward Msn2p/4p- or War1p-dependent genes. To confirm the microarray-based results, we tested several representative genes such as *FUN34* and *HSP30* by Northern analysis. Indeed, both genes were induced by sorbic acid stress, and as predicted, only *FUN34* induction required War1p (Figure 1A).

Finally, several genes were strongly and rapidly repressed by weak acid treatment (Table 8). However, the repression was independent of War1p and Msn2p/4p, indicating that these factors can only function as positive regulators. Interestingly, a number of genes encoding transporters and major facilitators were also strongly repressed (Table 8). For instance, repressed genes included those involved in cellular uptake of different nutrients and metals, such as phosphate (*PHO84*), zinc (*ZRT1*), amino acids (*GAP1*), purines (*FCY2*), sulfur amino acids (*MMP1*), and uridine (*FUI1*), but also amino acid biosynthesis genes (*HIS1*, *ILV5*) as well as genes of the ergosterol biosynthesis pathway (*ERG1*, *ERG11*).

War1p and Msn2p/4p Act through Distinct cis-acting Motifs in Target Genes

To identify a possible common *cis*-acting motif in various promoters of genes present in the respective groups, we used the AlignACE program (Roth *et al.*, 1998). This analysis indicated a shared motif (5'-CGG c/t T g/c t/g TA a/t -3') among the strongly War1p-dependent genes. This motif appears in the following genes: Target genes containing this motif are marked by an asterisk (Tables 3–7) and are represented graphically as a sequence logo reflecting the relative frequency of each base in the motif (Figure 1B). Related sequences appear five times in the *PDR12* promoter, overlapping with *cis*-acting sequences previously shown to be recognized by War1p *in vivo* (Kren *et al.*, 2003). Furthermore, the distribution of STRE motifs (5'-WAGGGG-3') was determined according to previously published analysis (Moskvina *et al.*, 1998; Treger *et al.*, 1998). This motif was clearly enriched in both Msn2p/4p- and War1p-Msn2p/4p-dependent groups (34 and 38%, respectively, compared with 14 and 19% in the *HSP30* and No-category groups), strongly supporting our assignment. Finally, the assignment of Msn2p/4p dependency under sorbate stress showed a remarkable overlap of >40% (Tables 3–7), with the one of Msn2p/4p-dependent genes under acid stress conditions (Causton *et al.*, 2001). These correlations confirmed our analysis to a large extent. However, we were unable to identify a common motif shared by most of the War1p-Msn2p/4p-independent genes.

To assess individual roles of War1p-regulated genes in weak acid resistance, we investigated growth of the respective viable EUROSCARF mutants on sorbate-containing plates and found only the *pdr12*Δ mutant strain to be highly sensitive, whereas all other mutants failed to show differences to the wild-type control in the plate assay (our unpublished results). Nevertheless, the functional screening and the microarray profiling essentially addressed two related issues. What are the genes required for coping with a given stress, and which genes are activated as a consequence of the subsequent response? Strikingly, the comparison of genes required for stress adaptation to genes induced by the same stress revealed only one gene that appeared exclusively in both datasets, namely *PDR12*. It was therefore tempting to

Table 4. Genes induced by sorbic acid: *MSN2/MSN4*-dependent

Gene	ORF name	Biological process	Wild type	SDEV	<i>war1Δ</i>	SDEV	<i>msn2Δ msn4Δ</i>	SDEV	<i>war1Δ msn2/4Δ</i>	SDEV	a	STRE	b
<i>SPI1</i>	<i>YER150W</i>	Biological process unknown	8.19	5.77	6.16	1.43	1.60	0.37	1.42	0.74	a		b
	<i>YHR087W</i>	Biological process unknown	6.48	5.00	8.58	4.89	1.04	0.10	0.80	0.25			b
<i>DDR2</i>	<i>YOL053C-A</i>	Response to stress	5.23	1.46	3.71	2.91			1.16	0.28		STRE	
<i>HSP26</i>	<i>YBR072W</i>	Response to stress	5.22	4.89	7.56	1.63	2.25	2.79	0.89	0.39	a	STRE	b
<i>HXK1^c</i>	<i>YFR053C</i>	Fructose metabolism	4.95	3.10	5.81	3.82	1.05	0.34	0.93	0.27		STRE	b
<i>GRE3</i>	<i>YHR104W</i>	Response to stress	4.50	3.04	5.18	3.03			1.47	0.76			b
<i>PHM7</i>	<i>YOL084W</i>	Biological process unknown	4.43	3.48	2.99	2.56	1.66	0.15	1.03	0.38			
<i>CTT1</i>	<i>YGR088W</i>	Response to stress	4.21	1.51	5.91	0.23			1.21	1.27	a	STRE	b
	<i>YER067W</i>	Biological process unknown	4.13	0.22	4.16	2.28			0.85	0.38			b
<i>TFS1^c</i>	<i>YLR178C</i>	Regulation of proteolysis	4.10	1.91	2.52	1.97	1.29	0.67	1.50	0.50		STRE	b
<i>ACH1^c</i>	<i>YBL015W</i>	Acetate metabolism	3.76	1.41	3.04	1.01	1.98	2.26	1.06	0.75		STRE	
<i>ALD3</i>	<i>YMR169C</i>	Response to stress	3.61	3.14	2.65	3.45			0.93	0.51	a		
<i>HXT6</i>	<i>YDR343C</i>	Hexose transport	3.34	0.72	5.13	2.61	0.43	0.17	0.46	0.09	a		
	<i>YOR173W</i>	Biological process unknown	3.27	2.84	4.75	2.12			0.80	0.43		STRE	b
<i>CYC7</i>	<i>YEL039C</i>	Electron transport	2.98	1.80	3.57	2.51	1.19	0.84	1.29	0.35		STRE	b
<i>UGP1</i>	<i>YKL035W</i>	Glycosylation ^c	2.94	1.28	2.11	2.46	1.07	0.21	0.95	0.22			
<i>HXT7</i>	<i>YDR342C</i>	Hexose transport	2.87	0.31	1.96	2.37	0.47	0.16	0.55	0.08	a		
<i>NCE103</i>	<i>YNL036W</i>	Biological process unknown	2.81	2.66	2.57	2.90	1.14	0.32	1.38	0.42			
<i>SSE2</i>	<i>YBR169C</i>	Protein folding	2.81	1.79	1.81	1.65			1.48	1.22			b
<i>GPX1</i>	<i>YKL026C</i>	Response to oxidative stress	2.45	2.16	1.67	2.17	1.00	0.27				STRE	
<i>OSH2</i>	<i>YDL019C</i>	Steroid biosynthesis	2.42	1.25	1.45	0.37	1.21	0.13	1.08	0.28			
	<i>YFL030W</i>	Glyoxylate cycle	2.40	1.14	2.02	0.80	0.97	0.16	1.29	0.73			b
<i>GAT1^c</i>	<i>YFL021W</i>	Transcription initiation Pol-II	2.30	1.27	1.89	1.31	0.94	0.35	0.73	0.11			
	<i>YPL004C</i>	Biological process unknown	2.27	0.66	2.54	1.42	1.35	0.37	1.24	0.26		STRE	b
<i>MCR1</i>	<i>YKL150W</i>	Response to oxidative stress	2.26	1.20	1.49	0.93			1.15	0.56	a	STRE	b
<i>MSS11</i>	<i>YMR164C</i>	Pseudohyphal growth	2.24	2.05	3.34	0.67	0.73	0.33	0.83	0.17			
<i>HXT3</i>	<i>YDR345C</i>	Hexose transport	2.22	0.24	1.81	0.77	0.63	0.18	0.49	0.12			
	<i>YLR251W</i>	Biological process unknown	2.17	0.63	1.58	0.35	1.25	0.38	1.28	0.66			b
<i>HXT4</i>	<i>YHR092C</i>	Hexose transport	2.16	0.31	2.56	0.52	1.12	1.13	1.04	0.63			
<i>PMC1</i>	<i>YGL006W</i>	Calcium ion transport	2.07	0.77	1.92	0.79	1.20	0.49	1.10	0.57			b
<i>YPS6</i>	<i>YIR039C</i>	Biological process unknown	2.07	0.75	2.49	0.61			1.11	0.25			
<i>PDR15</i>	<i>YDR406W</i>	Transport	2.06	0.48	1.80	0.36	0.64	0.32	0.56	0.16			b
	<i>YGR043C</i>	Biological process unknown	2.02	1.06	1.91	0.60	1.18	0.60	0.91	0.43		STRE	
	<i>YNR065C</i>	Biological process unknown	2.01	0.23	1.67	0.46			1.01	0.29			
<i>TRS120</i>	<i>YDR407C</i>	ER to Golgi transport	1.86	0.36	2.23	0.18			0.85	0.32			

Values are *x*-fold induction. STRE: STRE-like sequences detected in the promoter region (Treger *et al.*, 1998; Moskvina *et al.*, 1998).

^a Genes induced in sorbic acid-adapted cells (de Nobel *et al.*, 2001).

^b Genes assigned *MSN2/4*-dependent by acidic stress (Causton *et al.*, 2001).

^c Putative War1p consensus element (5'-C G G c/t T g/c t/g T A a/t-3'). The entire set of genes in each cluster are found in the supplementary material.

speculate that Pdr12p is the major cellular determinant of weak acid resistance.

Pdr12p Is Necessary and Sufficient for Weak Acid Resistance

To address this question, and to analyze the contribution of Msn2p/4p versus War1p-regulated genes to stress relief, we constructed a set of isogenic strains carrying *war1Δ* (YAK120), *pdr12Δ* (YBB14), *msn2Δ msn4Δ* (W303 *msn2 msn4*) as well as *war1Δ msn2Δ msn4Δ* (YCS105) deletions. At 1 mM sorbate, the *msn2Δ msn4Δ* strain grew almost like the W303-1A wild-type control, whereas the *pdr12Δ* strain failed to grow (Figure 3A). The *war1Δ* and *war1Δ msn2Δ msn4Δ* cells displayed the same degree of resistance. A low level of growth was perhaps due to a very low basal Pdr12p production. Therefore, absence of Msn2p/4p did not further exacerbate the hypersensitivity of *war1Δ* cells. Furthermore, we were unable to demonstrate weak acid hypersensitivity in mutants lacking the *HSP12*, *HSP26*, and *HSP104* genes, all of which are otherwise strongly regulated by Msn2p/4p (Martinez-Pastor *et al.*, 1996; Amoros and Estruch, 2001; Figure 3B).

These data as well as published results indicate that the weak acid sensitivity of cells lacking War1p is only due to its function in controlling Pdr12p induction. To test this assumption, we placed the *PDR12* gene under the control of the *GAL1-10* promoter, thus rendering Pdr12p inducible by galactose but not weak acids (Figure 4A). As expected, expression of Pdr12p driven by the *GAL1-10* promoter was undetectable in glucose-grown cells, but was strongly induced on galactose. The absence of War1p did not influence Pdr12p levels on galactose (Figure 4A). Hence, *PDR12* expression is completely independent of War1p function in this strain. The levels of Pdr12p due to galactose-triggered induction fully compensated for the absence of War1p on sorbate medium (Figure 4B) as well as on propionate (our unpublished results). Such induction restored almost wild-type levels of weak acid resistance to *war1Δ* cells (Figure 4B). These results demonstrate that ectopic expression levels of *PDR12* are sufficient to confer weak acid stress resistance in *war1Δ* cells, firmly establishing *PDR12* as the single most important and perhaps only relevant target gene of War1p.

Figure 5. Northern analysis of *HSP12* and *PDR12* expression during weak acid stress. (A) Cells were grown to the logarithmic growth phase in rich medium and exposed to the indicated weak acids for 20 min. Methylene blue staining of rRNA served as a loading control. (B) Weak acid stress-induced *PDR12* promoter activity is dependent on *WAR1*. Logarithmically growing cells were treated with the indicated concentrations of weak organic acids for 45 min. β -galactosidase activity was determined in crude extracts. Wild-type: YAK3 *PDR12-lacZ*, *war1Δ*: YBB27 *PDR12-lacZ war1Δ::HisMX6c*.

The War1p and Msn2p/4p Regulons Are Activated by Weak Organic Acids

One plausible explanation for the different roles of Msn2p/4p and War1p could be a distinct response to different, but possibly overlapping, spectra of inducing cues. To test this idea, we used the fact that weak acid induction of *HSP12* only demands Msn2p/4p, whereas *PDR12* was only regulated by War1p (Figure 5A). To test the weak acid substrate spectrum for War1p eliciting activation of the *PDR12* gene, we analyzed the activity of an integrated *PDR12-lacZ* fusion construct in response to various weak acids. We detected a rapid activation of this reporter construct by weak acids ranging from C3 to C8 chain length, which was completely absent in the *war1Δ* control reporter

Figure 6. Northern analysis of sorbic acid stress. (A) Exponentially growing cells of the indicated background (W303-1A, YAK120 (*war1Δ*), W303 *msn2 msn4* (*msn2Δ msn4Δ*), and YCS105 (*war1Δ msn2Δ msn4Δ*)) were stressed for 20 min with 10 mM sorbic acid. Strains W303-1A and YAK120 carried a *TRP1* gene on the centromeric plasmid YCplac22. mRNA levels of *HSP12*, *HSP30*, and *PDR12* were determined by simultaneous Northern hybridization. Methylene blue staining of rRNA served as a loading control. (B) *HSP30* does not modulate sorbate sensitivity of *pdr12Δ* cells. Strains lacking the *HSP30* and *PDR12* genes in all combinations were tested for growth phenotypes on sorbic acid medium pH 4.5. Cells were spotted onto YPD, pH 4.5, with and without 0.3 mM sorbic acid and plates inspected after a 48-h incubation at 30°C.

strain (Figure 5B). As also shown earlier (Hatzixanthis *et al.*, 2003), acetate (C2) was unable to induce Pdr12p (Figure 5B).

We then determined the mRNA levels of both *HSP12* and *PDR12* after addition of propionate, butyrate, and sorbate as well as hexanoic, heptanoic, octanoic acids, myristylate, and palmitate (Figure 5A). Both Msn2p/4p and War1p activate their respective target genes by organic acids with a chain length ranging from C3 to C10. Induction of *HSP12* by C14 and C18 acids pointed to marked differences in the War1p- and Msn2p/4p-dependent response mechanisms. Acetic acid and dodecanoic acid failed to induce the *PDR12* promoter, demonstrating that only weak acids of a certain chain length can activate War1p. Msn2p/4p activation as detected by *HSP12* induction seemed to include weak acids of higher chain length

Table 5. Genes induced by sorbic acid: *MSN2/MSN4*- and *WAR1*-dependent

Gene	ORF name	Biological process	Wild type	SDEV	<i>war1Δ</i>	SDEV	<i>msn2Δ msn4Δ</i>	SDEV	<i>war1Δ msn2/4Δ</i>	SDEV	a	STRE	b
<i>GLK1</i>	<i>YCL040W</i>	Carbohydrate metabolism	3.66	1.07	5.22	2.96	2.27	1.70	1.74	0.47		STRE	b
<i>NCE102</i>	<i>YPR149W</i>	Protein secretion	3.31	0.23	3.13	0.29	2.03	0.90	1.58	0.45		STRE	
	<i>YCL042W</i>	Biological process unknown	2.83	0.88	2.82	0.25	2.44	2.10	1.22	0.63			
<i>ALD4^c</i>	<i>YOR374W</i>	Ethanol metabolism	2.66	0.42	1.77	0.48	1.71	0.89	0.94	0.27	a	STRE	
	<i>YNL335W</i>	Dubious ORF	2.46	0.78	2.47	1.06	1.55	1.47					
<i>TPS1</i>	<i>YBR126C</i>	Response to stress	2.32	0.84	2.76	0.80	1.42	0.73	1.17	0.24		STRE	b
<i>RPA135</i>	<i>YPR010C</i>	Transcription Pol-I	2.30	1.69	3.16	1.52	2.30	1.13	1.08	0.52			
	<i>YNL224C</i>	Biological process unknown	2.16	1.15	1.98	1.22	1.90	0.50	1.25	0.66			
	<i>YBR134W</i>	Dubious ORF	2.09	0.47	1.90	0.41	1.39	0.92	0.93	0.25			
<i>LSM4</i>	<i>YER112W</i>	Nuclear mRNA splicing	2.08	0.43	1.78	0.31	2.06	1.40	0.74	1.01			
	<i>YMR090W</i>	Biological process unknown	2.06	0.55	1.73	0.57	1.51	0.80	0.88	0.39			b
<i>TPS3</i>	<i>YMR261C</i>	Response to stress	2.04	1.18	1.90	0.14	1.68	1.46	1.00	0.41		STRE	b
<i>GPD1</i>	<i>YDL022W</i>	Accumulation of glycerol	1.98	1.23	3.81	1.06	1.48	1.06	0.76	0.21			b

Values are *x*-fold induction. STRE: STRE-like sequences detected in the promoter region (Treger *et al.*, 1998; Moskvina *et al.*, 1998).

^a Genes induced in sorbic acid-adapted cells (de Nobel *et al.*, 2001).

^b Genes assigned *MSN2/4*-dependent by acidic stress (Causton *et al.*, 2001).

^c Putative War1p consensus element (5'-C G G c/t T g/c t/g T A a/t-3'). The entire set of genes in each cluster are found in the supplementary material.

as well (Figure 5A). Because Msn2p-GFP is known to accumulate rapidly in the nucleus of stressed cells, its activation was also monitored in living cells by visualization of the nuclear-cytoplasmic distribution of a Msn2-GFP fusion protein (Görner *et al.*, 1998). Except for acetic acid, all weak acids tested caused a rapid nuclear accumulation of Msn2p in <5 min (C. Shüller, unpublished results).

Finally, and in agreement with our microarray results, *HSP30* seemed activated through an as yet unknown mechanism, because we detected its mRNA induction by sorbate in a *war1Δ msn2Δ msn4Δ* strain (Figure 6A). Although *PDR12* and *HSP12* were strongly and exclusively affected by absence of War1p and Msn2p/4p, respectively, we found a partial effect of Msn2p/4p, but no effect of War1p on *HSP30* expression (Figure 6A). Because

Table 6. Genes induced by sorbic acid: *MSN2/MSN4*- and *WAR1*-independent

Gene	ORF name	Biological process	Wild type	SDEV	<i>war1Δ</i>	SDEV	<i>msn2Δ msn4Δ</i>	SDEV	<i>war1Δ msn2/4Δ</i>	SDEV	a	STRE	b
<i>HSP30</i>	<i>YCR021C</i>	Response to stress	11.88	6.42	10.35	1.86	4.82	1.75	8.89	6.75			
	<i>YER053C</i>	Biological process unknown	5.01	4.88	6.71	3.06	3.05	2.40	4.93	2.38			b
<i>YGP1</i>	<i>YNL160W</i>	Response to stress	4.48	0.92	5.00	1.97	4.92	2.92	6.75	2.95			
<i>YPC1</i>	<i>YBR183W</i>	Ceramide metabolism	4.15	1.80	3.27	1.53	3.31	1.95	2.57	1.12		STRE	b
<i>YRO2</i>	<i>YBR054W</i>	Biological process unknown	3.97	0.52	2.67	2.08	9.63	6.61	6.55	2.63			
<i>CIT1</i>	<i>YNR001C</i>	Tricarboxylic acid cycle	3.52	0.55	4.09	2.11	4.81	4.41	4.90	2.39		STRE	
	<i>YDR533C</i>	Biological process unknown	3.07	0.55	2.31	1.77	2.04	1.12	2.39	0.79	a		
	<i>YBR053C</i>	Biological process unknown	2.91	1.52	2.97	0.82	5.32	2.41	5.42	2.40			b
<i>ARO10</i>	<i>YDR380W</i>	Leucine catabolism	2.61	2.09	2.20	2.12	5.46	3.83	5.21	2.57			
<i>SDH2</i>	<i>YLL041C</i>	Tricarboxylic acid cycle	2.46	0.89	1.58	0.50	1.93	1.09	1.58	0.81			
	<i>YMR181C</i>	Biological process unknown	2.37	1.73	1.78	0.23	1.75	0.74	2.49	1.12		STRE	
<i>YPS1</i>	<i>YLR120C</i>	Protein processing	2.17	0.75	1.89	1.00	3.00	0.85	2.85	0.38			
	<i>YNL305C</i>	Biological process unknown	2.11	0.14	2.01	0.12	1.53	0.44	1.72	0.38			
<i>TPO2</i>	<i>YGR138C</i>	Polyamine transport	2.09	0.29	1.85	0.37	6.89	3.68	9.73	1.78			
<i>YDC1</i>	<i>YPL087W</i>	Response to heat	2.08	0.14	1.61	0.21	3.14	1.37	2.94	0.73			b
<i>PDC5</i>	<i>YLR134W</i>	Pyruvate metabolism	2.00	0.52	1.97	0.67	3.83	4.69	2.33	0.73			
<i>SFK1</i>	<i>YKL051W</i>	Actin cytoskeleton	2.00	0.30	1.37	0.27	3.26	1.04	5.25	1.68			
<i>BDH1</i>	<i>YAL060W</i>	Butanediol fermentation	1.95	0.98	3.42	1.63	2.66	2.53	1.79	0.64			
<i>TIP1</i>	<i>YBR067C</i>	Cell wall organization	1.91	0.25	2.18	0.21	1.53	0.63	2.37	0.83	a		
<i>TPO4</i>	<i>YOR273C</i>	Polyamine transport	1.91	1.28	1.62	0.53	1.73	0.71	2.43	1.04			b
<i>SDH4</i>	<i>YDR178W</i>	Tricarboxylic acid cycle	1.85	0.06	2.28	0.48	1.31	0.80	1.43	0.43			

Values are *x*-fold induction. STRE: STRE-like sequences detected in the promoter region (Treger *et al.*, 1998; Moskvina *et al.*, 1998).

^a Genes induced in sorbic acid-adapted cells (de Nobel *et al.*, 2001).

^b Genes assigned *MSN2/4*-dependent by acidic stress (Causton *et al.*, 2001).

^c Putative War1p consensus element (5'-C G G c/t T g/c t/g T A a/t-3'). The entire set of genes in each cluster are found in the supplementary material.

Table 7. Genes induced by sorbic acid: “No-category”

Gene	ORF name	Biological process	Wild type	SDEV	<i>war1Δ</i>	SDEV	<i>msn2Δ msn4Δ</i>	SDEV	<i>war1Δ msn2/4Δ</i>	SDEV	a	STRE	b
<i>RTN2</i>	<i>YDL204W</i>	Biological process unknown	5.55	2.77	3.16	2.23			1.08	0.69	a		b
<i>CAK1</i>	<i>YFL029C</i>	Protein phosphorylation	3.95	1.72	2.15	1.28	1.01	0.33	1.01	0.29			
	<i>YAL061W</i>	Biological process unknown	3.78	2.67	2.04	0.95	1.94	0.95	0.83	0.92		STRE	b
	<i>YDL222C</i>	Cell wall organization	3.37	0.89	1.87	1.24	1.42	1.59	0.75	0.47			
<i>TPO1^c</i>	<i>YLL028W</i>	Polyamine transport	3.25	1.81	1.81	0.79	4.92	1.64	3.48	0.96			
<i>ARO9</i>	<i>YHR137W</i>	Arom. amino acid metabolism	2.87	0.64			10.26	3.49	6.54	1.93			
<i>GAD1</i>	<i>YMR250W</i>	Response to oxidative stress	2.80	1.12			1.85	0.93	1.06	0.70			b
<i>RAD9</i>	<i>YDR217C</i>	DNA repair ^c	2.65	1.24	1.55	0.66	1.29	0.69	1.46	0.67			
	<i>YOR161C</i>	Biological process unknown	2.61	0.53	1.43	1.36	0.91	0.59	0.58	0.11			b
<i>CBP2</i>	<i>YHL038C</i>	Group I intron splicing	2.57	2.36	0.91	0.13							b
	<i>YGR248W</i>	Biological process unknown	2.50	1.58	1.81	0.97							
	<i>YKL036C</i>	Dubious ORF	2.40	1.29	1.64	1.24							
<i>HOT1</i>	<i>YMR172W</i>	Transcription factor	2.36	1.34	2.03	2.01							
	<i>YOR052C</i>	Biological process unknown	2.35	1.31			1.13	0.35	1.17	0.30			
<i>YJU2</i>	<i>YKL095W</i>	Biological process unknown	2.34	1.57	1.67	0.79							
	<i>YNL194C</i>	Biological process unknown	2.23	0.72	1.63	0.40						STRE	
	<i>YNL200C</i>	Biological process unknown	2.18	0.41			1.21	0.17	1.51	0.55			b
	<i>YOL048C</i>	Biological process unknown	2.18	0.47	1.36	0.43			1.72	0.55			b
<i>OM45</i>	<i>YIL136W</i>	Biological process unknown	2.16	0.98	2.48	1.51							b
	<i>YOL032W</i>	Biological process unknown	2.14	1.66	1.19	0.32			1.12	0.29			
<i>SSA4</i>	<i>YER103W</i>	Response to stress	2.12	1.97			2.24	2.21	1.42	0.38		STRE	b
	<i>YBR116C</i>	Dubious ORF	1.99	0.47			3.50	3.12	1.66	0.94	a		
<i>TPO3</i>	<i>YPR156C</i>	Polyamine transport	1.84	0.65			3.33	4.32	4.51	3.81			
	<i>YGR046W</i>	Biological process unknown	1.84	1.00	1.11	0.08	2.18	2.14					
	<i>YPL247C</i>	Biological process unknown	1.83	0.53	2.42	0.91						STRE	
<i>BAG7</i>	<i>YOR134W</i>	Signal transduction	1.80	0.74	2.03	0.14			1.18	0.47		STRE	
<i>YPR157W</i>	<i>YPR157W</i>	Biological process unknown	1.80	0.63	1.52	0.71			4.72	3.47			

Values are *x*-fold induction. STRE: STRE-like sequences detected in the promoter region (Treger *et al.*, 1998; Moskvina *et al.*, 1998).

^a Genes induced in sorbic acid-adapted cells (de Nobel *et al.*, 2001).

^b Genes assigned *MSN2/4*-dependent by acidic stress (Causton *et al.*, 2001).

^c Putative War1p consensus element (5'-C G G c/t T g/c t/g T A a/t-3'). The entire set of genes in each cluster are found in the supplementary material.

war1Δ cells were sorbate sensitive and because certain *HSP30* mutants display a longer lag-phase on sorbate (Piper *et al.*, 1997), we investigated a possible synthetic phenotype of cells lacking both *HSP30* and *PDR12*. However, strains devoid of *HSP30* failed to display significant viability defects in the plate assay on media over a range of sorbic acid concentrations (Figure 6B, our unpublished results). Therefore, the *war1Δ msn2Δ msn4Δ* cells were not compromised in their response to sorbate.

Taken together, our data show that it is not only the inducibility of War1p and Msn2p/4p by different weak acids that determines their physiological role. Instead, their specific physiological function in stress response and adaptation is determined at the level of the relevant downstream target genes. Moreover, the identification of all key genes of the weak acid stress response regulons was only possible by combining microarray profiling with functional screening approaches.

DISCUSSION

Adjustment of the transcriptional programs enables yeast cells to rapidly tune expression patterns in response to environmental challenges. With the advent of genome-wide analysis of gene expression, transcriptome changes can be easily identified because they relate to great many different conditions (see e.g., <http://genome-www4.stanford.edu/cgi-bin/SGR/publication/publicRef>). A major message of

these results is that only a fraction of a given genomic response is actually necessary to cope with any adverse condition. In other words, the transcriptional profile typical for a given stress situation usually includes many more genes than immediately required for stress relieve. Nonetheless, transcriptome patterns are remarkably stable phenotypes, which can be exploited in many ways. Another major achievement of the genomic age is the availability of large-scale systematic functional assays. Genome-wide knockout strain collections are available for *S. cerevisiae*. Similar screens are now also possible in higher eukaryotes using the siRNA methodology (Lum *et al.*, 2003). Although resources like that allow for rapid genetic screens, they cannot completely replace classical mutagenesis approaches. In this study, we pursued both strategies to identify genes involved in the response and adaptation to the fungistatic effects of short-chain weak organic acids such as sorbic acid or propionate. Our analysis leads us to the conclusion that a combination of phenotypic screening or functional assays (i.e., functional genomics) with datasets from mRNA profiles can effectively pinpoint crucial genes operating in complex biological pathways.

Weak acids have a potent but transient fungistatic effect on yeast cells. In our search for genes important for recovery during sorbate stress, we identified many genes with very different physiological roles or metabolic functions. Perhaps the most unexpected finding was the identification of a class of mutants in the aromatic amino acid biosynthesis path-

Table 8. Genes repressed by sorbic acid stress

Gene	ORF name	Biological process	Wild type	SDEV	<i>war1Δ</i>	SDEV	<i>msn2Δ msn4Δ</i>	SDEV	<i>war1Δ msn2/4Δ</i>	SDEV
<i>PHO84</i>	<i>YML123C</i>	Phosphate transport	0.12	0.06	0.26	0.64	0.08	0.01	0.04	0.02
<i>GLY1</i>	<i>YEL046C</i>	Threonine catabolism	0.18	0.05	0.23	0.13	0.57	0.08	0.60	0.14
<i>ILV5</i>	<i>YLR355C</i>	Mitoch. genome maintenance	0.20	0.10	0.35	0.80	0.38	0.02	0.47	0.11
<i>GAP1</i>	<i>YKR039W</i>	Amino acid transport	0.24		0.48	0.09	0.25	0.03	0.24	0.06
<i>NSR1</i>	<i>YGR159C</i>	rRNA processing	0.26	0.25	0.19	0.03	0.76	0.72	0.38	0.13
<i>FUI1</i>	<i>YBL042C</i>	Uridine transport	0.29	0.15	0.48	0.05	0.71	0.86	0.30	0.24
<i>ERG3</i>	<i>YLR056W</i>	Ergosterol biosynthesis	0.29	0.16	0.51	0.69	0.51	0.09	0.75	0.13
<i>SEO1</i>	<i>YAL067C</i>	Transport	0.31	0.26	0.87	0.53	2.39	1.83	0.68	0.71
<i>SAM1</i>	<i>YLR180W</i>	Methionine metabolism	0.31	0.09	0.61	0.66	0.50	0.16	0.47	0.07
<i>ENA2</i>	<i>YDR039C</i>	Sodium ion transport	0.33	0.19			1.45	1.92	0.80	0.16
<i>RHR2</i>	<i>YIL053W</i>	Response to osmotic stress	0.33	0.25	0.33	0.05	0.91	0.86	0.75	0.24
<i>YLR419W</i>	<i>YLR419W</i>	Biological process unknown	0.33	0.03	0.52	0.38	0.52	0.17		
<i>HIS1</i>	<i>YER055C</i>	Histidine biosynthesis	0.34	0.18	0.43	0.12	0.70	0.39	0.57	0.09
<i>CBF5</i>	<i>YLR175W</i>	rRNA modification	0.34	0.32	0.56	0.47	0.67	0.42	0.44	0.16
<i>EXG1</i>	<i>YLR300W</i>	Cell wall organization	0.35	0.06	0.58	0.32	0.90	0.52	0.57	0.12
<i>YNL300W</i>	<i>YNL300W</i>	Biological process unknown	0.35	0.08	0.35	0.19	0.24	0.12	0.27	0.06
<i>ZRT1</i>	<i>YGL255W</i>	High-affinity zinc ion transport	0.35	0.20	0.34	0.07	0.52	0.17	0.38	0.33
<i>HPT1</i>	<i>YDR399W</i>	Purine nucleotide biosynthesis	0.35	0.23	0.28	0.27				
<i>PTR2</i>	<i>YKR093W</i>	Peptide transport	0.36	0.11	0.60	0.27	0.40	0.15	0.51	0.08
<i>YBR043C</i>	<i>YBR043C</i>	Biological process unknown	0.36	0.24			1.46	0.91	0.79	0.38
<i>SUR4</i>	<i>YLR372W</i>	Sphingolipid biosynthesis	0.36	0.03	0.51	0.22	0.67	0.10	0.60	0.26
<i>ECM1</i>	<i>YAL059W</i>	Cell wall organization	0.36	0.08					0.64	0.92
<i>YDR492W</i>	<i>YDR492W</i>	Biological process unknown	0.37	0.10	0.57	0.14	1.04	0.80	0.47	0.23
<i>ERG1</i>	<i>YGR175C</i>	Ergosterol biosynthesis	0.37	0.04	0.39	0.08	0.77	0.50	0.60	0.14
<i>CRH1</i>	<i>YGR189C</i>	Biological process unknown	0.37	0.03	0.46	0.13	0.77	0.13	0.79	0.16
<i>VTC4</i>	<i>YJL012C</i>	Vacuole fusion (non-autophagic)	0.37	0.04	0.39	0.12	0.45	0.06	0.36	0.08
<i>URA7</i>	<i>YBL039C</i>	Phospholipid biosynthesis ^a	0.38	0.08	0.69	0.77	0.89	0.67	0.41	0.10
<i>PDR5</i>	<i>YOR153W</i>	Response to drug ^a	0.38	0.31	0.29	0.25	0.53	0.05	0.43	0.16
<i>YFR055W</i>	<i>YFR055W</i>	Copper ion homeostasis ^a	0.38	0.11	0.38	0.15	0.93	0.30	0.77	0.25
<i>CPA1</i>	<i>YOR303W</i>	Arginine biosynthesis	0.38	0.08	0.45	0.08	0.49	0.28	0.41	0.13
<i>ENA5</i>	<i>YDR038C</i>	Sodium ion transport	0.38	0.21	0.97	0.95	1.27	1.07	0.50	0.11
<i>MET6</i>	<i>YER091C</i>	Methionine biosynthesis	0.38	0.16	0.49	0.12	0.80	0.73	0.65	0.15
<i>YBR033W</i>	<i>YBR033W</i>	Biological process unknown	0.38	0.32	0.93	1.34	0.50	0.15	0.72	0.45
<i>ITR1</i>	<i>YDR497C</i>	Myo-inositol transport	0.39	0.16	0.54	0.09	0.67	0.53	0.71	0.25
<i>MEP2</i>	<i>YNL142W</i>	Pseudohyphal growth ^a	0.39	0.20	0.64	0.28	0.64	0.51	0.31	0.10
<i>HTA2</i>	<i>YBL003C</i>	Chromatin	0.39	0.06	0.47	0.19	0.58	0.21	0.50	0.15
<i>ERG11</i>	<i>YHR007C</i>	Ergosterol biosynthesis	0.40	0.08	0.39	0.06	0.85	0.59	0.69	0.21

A cutoff ratio of 0.4 was arbitrarily chosen to visualize repressed genes. The complete list of repressed genes is found in the supplementary materials.

way. Closer inspection of this phenotype revealed that an inability to synthesize tryptophan increases the sorbate sensitivity (Y. Mamnun, unpublished results). Because these mutants show normal sorbate-sensing and signaling as judged by induction of *Pdr12p*, the effect is most likely caused by reduced tryptophan uptake (Bauer *et al.*, 2003) through mechanisms that are unknown at present. Interestingly, the number of sorbate-sensitive genes appears proportional to the amount of sorbate used for phenotypic selection (M. Mollapour, unpublished results). The stringency of selection therefore has a strong effect on the resulting number of candidate genes implicated in susceptibility phenotypes. The main players identified by this approach include *PDR12*, an ABC transporter previously identified to confer weak acid resistance, probably through its ability to efflux acid anions (Piper *et al.*, 1998), as well as *WAR1*. The latter is a typical Zn(II)₂Cys₆ transcription activator recently shown to be a major regulator of *PDR12* (Kren *et al.*, 2003).

In a second approach, we analyzed genome-wide changes in mRNA levels following weak acid stress (see below). A comparison of the results from both approaches used in this study recovered only one gene, *PDR12*. We therefore reasoned that *Pdr12p* is the prime player in weak acid stress

response and that *War1p* represents its main stress regulator. We tested this idea by conditional expression of *Pdr12p*, thereby circumventing *War1p*-dependent induction of *Pdr12p*. As expected, placing *Pdr12p* under the control of the *GAL1-10* promoter eliminates the requirement for *War1p* in weak acid response and stress adaptation. This suggests that *Pdr12p* is necessary and sufficient for resistance, suggesting that *PDR12* is the main target gene of *War1p* at least in the context of weak acid resistance. Therefore, our hypothesis drawn from the genomic data are valid, and as shown here, the combination of two genome-wide assays readily identified the most important target gene.

The deletion of *War1p* results in weak acid hypersensitive cells (Kren *et al.*, 2003). Hence, identification of *War1p* targets might allow us to identify additional genes important for weak acid resistance. Genome-wide mRNA changes in sorbate-treated cells involve the rapid induction of ~170 genes, with a quite similar pattern of modulated genes in propionate-treated cells. The sorbate results might thus be of general applicability regarding other weak acids, at least in terms of target genes. Many sorbate-induced genes included those previously reported under the control of *Msn2p* and *Msn4p*, which govern the general stress response (Estruch,

2000; Gasch *et al.*, 2000). Expression profiling of strains lacking War1p and/or Msn2p/4p allowed us to identify the War1p-dependent genes. More than 50% of the sorbate-induced genes require Msn2p/4p. Weak acids also trigger a rapid nuclear accumulation of Msn2p/Msn4p, probably due to inhibition of their nuclear export signals (Görner *et al.*, 2002), followed by an increased occupancy of their cognate DNA binding sites (STREs) in the target promoters (Görner *et al.*, 1998). As for War1p, it is unclear how and why weak acids activate Msn2p/4p. A possible trigger might be the drop of the intracellular pH as caused by weak acid dissociation. Nevertheless, our data show that both War1p and Msn2p/4p respond to a broad range of weak acids, leading to the modulation of many downstream target genes. However, differences in the response patterns (Figure 5) make it unlikely that both are activated by the same mechanism.

The experiments reported in this study address the immediate early transcriptional response, similar to the environmental stress response (ESR) dataset (Gasch *et al.*, 2000) and the common environmental response (CER; Causton *et al.*, 2001). Short-term ethanol stress (Alexandre *et al.*, 2001) or salt stress (Rep *et al.*, 1999) has been reported to also induce many Msn2p/4p-dependent genes. The situation is different when following long-term exposure to weak acids (de Nobel *et al.*, 2001), because only 25 genes of the 167 genes described here are detected under both the acute response and the adapted response (de Nobel *et al.*, 2001). Interestingly, these 25 genes are overrepresented in War1p- and Msn2p/4p-dependent regulons, suggesting that long-term responses are also mediated by these factors.

A strong *HSP30* induction by sorbate and heat shock has been demonstrated in *msn2Δ msn4Δ* cells and in a *hsf1Δ* heat shock transcription factor mutant (Seymour and Piper, 1999). Here we provide evidence that *HSP30* may be part of a larger regulon, which is independent from War1p and Msn2p/4p, thus establishing a third regulatory mechanism activated by sorbate stress. But what then is the role of this regulon in weak acid resistance? Interestingly, several genes of this regulon are involved in processes on the level of the plasma membrane. Hsp30p is the only membrane-bound heat shock protein (Piper *et al.*, 1994); therefore, it could be involved in sensing membrane damage as exerted by weak organic acid exposure. Interestingly, we found that all four genes coding for polyamine transporters (*TPO1,2,3,4*) were also activated (Albertsen *et al.*, 2003). Tpo1p may also act as an herbicide and quinidine-resistance determinant (do Valle Matta *et al.*, 2001; Teixeira and Sa-Correia, 2002). Other genes in this group include the *HSP30* homologue *YRO2* (Wu *et al.*, 2000) as well as *CIT2* encoding a nonmitochondrial citrate synthase that participates in the glyoxylate cycle (Kim *et al.*, 1986). The demonstration of a functional significance for this regulon and the identification of upstream factors is clearly a next important step.

Although the pattern of upregulated genes is characteristic for a given environmental condition, this is also true for repressed genes. During the environmental stress response, twice as many genes are repressed when compared with the induced ones (Gasch *et al.*, 2000; Gasch and Werner-Washburne, 2002). In the case of weak acids, a number of genes encoding transporters localized to the plasma membrane are dramatically reduced. These genes are involved in diverse cellular functions such as phosphate (*PHO84*), sulfur amino acids (*MMP1*), zinc (*ZRT1*), amino acids (*GAP1*), and purine (*FCY2*) uptake. The physiological role of this downregulation is currently unclear, but it may indicate changes in the protein composition of the plasma membrane. Alternatively, this repression might reflect a growth-related effect, as cells

are in a stasis and thus might downregulate transport processes related to secondary metabolism. Moreover, downregulation of *GPA1* might contribute to the growth defect of strains lacking genes of aromatic amino acid biosynthesis identified in our functional screen.

The results give a fairly clear picture about the genes comprised in the War1p-dependent regulon. Although the precise mechanism of War1p activation is not known, it might directly bind and thus sense the activating agents such as weak acids. War1p, which constitutively decorates the *PDR12* promoter (Kren *et al.*, 2003), could become activated through a conformation change induced by posttranslational modification such as phosphorylation. Indeed, War1p is rapidly and phosphorylated after sorbate stress (Kren *et al.*, 2003), and mutations that prevent phosphorylation lead to nonfunctional War1p (Bauer *et al.*, unpublished results). In the uninduced state, self-masking could prevent constitutive activity of War1p. For example, self-masking has been described for Leu3p, a transcription factor reacting to α -isopropylmalate (Wang *et al.*, 1997, 1999), Hap1p that recognizes heme (Zhang and Guarente, 1995), and Put3p responding to proline levels (Des Etages *et al.*, 2001). Stress-induced War1p phosphorylation and its link to *PDR12* induction might be a cause or consequence of conformational changes. In any case, the kinase mediating War1p phosphorylation has escaped discovery as yet. Further, neither the genetic nor the functional screen uncovered candidate kinases, implying that they could be essential for viability or functionally redundant.

Our data illustrate that the general stress response, although induced by the same cues as the specific response through War1p, is not required for weak acid resistance. War1p and Msn2p/4p might function as upstream receivers for weak acid stress signals, but a distinct downstream physiological response is brought about by striking differences in target gene specificity. Why then do yeast cells require two partially redundant signaling mechanisms for weak organic acids? A *PDR12* gene under Msn2p/4p control would presumably result in Pdr12p induction under many adverse conditions such as starvation, heat shock, or osmotic stress. One reason for a second and yet specific signaling mechanism may be a need to avoid unwanted effects of higher Pdr12p levels under general environmental or metabolic stress. At any rate, our studies show that multiple mechanisms contribute to at least three distinct sorbate-inducible regulons. More importantly, our results indicate that only a combination of functional genomic analysis and global expression profiling represents an efficient way to identify key players operating in different complex biological stress response pathways.

ACKNOWLEDGMENTS

We thank Sabine Enzinger for excellent technical assistance and Gustav Ammerer for stimulating discussions. This work was supported by a grant from the Fonds zur Förderung der wissenschaftlichen Forschung (P-15934-B08) and in part by funds from the Verein zur Förderung der Genomforschung in Österreich to C.S. and K.K. P.P. was supported through project grants from the Biotechnology and Biological Sciences Research Council (BBSRC 31/D17868).

REFERENCES

- Albertsen, M., Bellahn, I., Kramer, R., and Waffenschmidt, S. (2003). Localization and function of the yeast multidrug transporter Tpo1p. *J. Biol. Chem.* 278, 12820–12825.
- Alexandre, H., Ansanay-Galeote, V., Dequin, S., and Blondin, B. (2001). Global gene expression during short-term ethanol stress in *Saccharomyces cerevisiae*. *FEBS Lett.* 498, 98–103.

- Amoros, M., and Estruch, F. (2001). Hsf1p and Msn2/4p cooperate in the expression of *Saccharomyces cerevisiae* genes *HSP26* and *HSP104* in a gene- and stress type-dependent manner. *Mol. Microbiol.* 39, 1523–1532.
- Bauer, B.E., Rossington, D., Mollapour, M., Mamnun, Y., Kuchler, K., and Piper, P.W. (2003). Weak organic acid stress inhibits aromatic amino acid uptake by yeast, causing a strong influence of amino acid auxotrophies on the phenotypes of membrane transporter mutants. *Eur. J. Biochem.* 270, 3189–3195.
- Causton, H.C. *et al.* (2001). Remodeling of yeast genome expression in response to environmental changes. *Mol. Biol. Cell* 12, 323–337.
- Choder, M. (1993). A growth rate-limiting process in the last growth phase of the yeast life cycle involves *RPB4*, a subunit of RNA polymerase II. *J. Bacteriol.* 175, 6358–6363.
- de Nobel, H., Lawrie, L., Brul, S., Klis, F., Davis, M., Alloush, H., and Coote, P. (2001). Parallel and comparative analysis of the proteome and transcriptome of sorbic acid-stressed *Saccharomyces cerevisiae*. *Yeast* 18, 1413–1428.
- DeRisi, J.L., Iyer, V.R., and Brown, P.O. (1997). Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* 278, 680–686.
- Des Etages, S.A., Saxena, D., Huang, H.L., Falvey, D.A., Barber, D., and Brandriss, M.C. (2001). Conformational changes play a role in regulating the activity of the proline utilization pathway-specific regulator in *Saccharomyces cerevisiae*. *Mol. Microbiol.* 40, 890–899.
- do Valle Matta, M.A., Jonniaux, J.L., Balzi, E., Goffeau, A., and van den Hazel, B. (2001). Novel target genes of the yeast regulator Pdr1p: a contribution of the *TPO1* gene in resistance to quinidine and other drugs. *Gene* 272, 111–119.
- Egner, R., and Kuchler, K. (1996). The yeast multidrug transporter Pdr5 of the plasma membrane is ubiquitinated prior to endocytosis and degradation in the vacuole. *FEBS Lett.* 378, 177–181.
- Eisen, M.B., Spellman, P.T., Brown, P.O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. USA* 95, 14863–14868.
- Estruch, F. (2000). Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. *FEMS Microbiol. Rev.* 24, 469–486.
- Gasch, A.P., and Eisen, M.B. (2002). Exploring the conditional coregulation of yeast gene expression through fuzzy k-means clustering. *Genome Biol.* 3, 1–22.
- Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D., and Brown, P.O. (2000). Genomic expression programs in the response of yeast cells to environmental changes. *Mol. Biol. Cell* 11, 4241–4257.
- Gasch, A.P., and Werner-Washburne, M. (2002). The genomics of yeast responses to environmental stress and starvation. *Funct. Integr. Genomics* 2, 181–192.
- Gietz, R.D., and Sugino, A. (1988). New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* 74, 527–534.
- Goldstein, A.L., and McCusker, J.H. (1999). Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* 15, 1541–1553.
- Golub, T.R. *et al.* (1999). Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science* 286, 531–537.
- Görner, W., Durchschlag, E., Martinez-Pastor, M.T., Estruch, F., Ammerer, G., Hamilton, B., Ruis, H., and Schüller, C. (1998). Nuclear localization of the C₂H₂ zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes Dev.* 12, 586–597.
- Görner, W., Durchschlag, E., Wolf, J., Brown, E.L., Ammerer, G., Ruis, H., and Schüller, C. (2002). Acute glucose starvation activates the nuclear localization signal of a stress-specific yeast transcription factor. *EMBO J.* 21, 135–144.
- Hatzixanthis, K., Mollapour, M., Seymour, I.J., Bauer, B., Krapf, G., Schüller, C., Kuchler, K., and Piper, P.W. (2003). Moderately lipophilic carboxylate compounds are the selective inducers of the *Saccharomyces cerevisiae* Pdr12p ATP-binding cassette transporter. *Yeast* 20, 575–585.
- Holyoak, C.D., Bracey, D., Piper, P.W., Kuchler, K., and Coote, P.J. (1999). The *Saccharomyces cerevisiae* weak-acid-inducible ABC transporter Pdr12 transports fluorescein and preservative anions from the cytosol by an energy-dependent mechanism. *J. Bacteriol.* 181, 4644–4652.
- Holyoak, C.D., Stratford, M., McMullin, Z., Cole, M.B., Crimmins, K., Brown, A.J., and Coote, P.J. (1996). Activity of the plasma membrane H(+)-ATPase and optimal glycolytic flux are required for rapid adaptation and growth of *Saccharomyces cerevisiae* in the presence of the weak-acid preservative sorbic acid. *Appl. Environ. Microbiol.* 62, 3158–3164.
- Hughes, T.R. *et al.* (2000). Functional discovery via a compendium of expression profiles. *Cell* 102, 109–126.
- Kaiser, C., Michaelis, S., and Mitchell, A. (1994). *Methods in Yeast Genetics. A Laboratory Course Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Kim, K.S., Rosenkrantz, M.S., and Guarente, L. (1986). *Saccharomyces cerevisiae* contains two functional citrate synthase genes. *Mol. Cell Biol.* 6, 1936–1942.
- Krebs, H.A., Wiggins, D., Stubbs, M., Sols, A., and Bedoya, F. (1983). Studies on the mechanism of the antifungal action of benzoate. *Biochem. J.* 214, 657–663.
- Kren, A. *et al.* (2003). War1p, a novel transcription factor controlling weak acid stress response in yeast. *Mol. Cell Biol.* 23, 1775–1785.
- Lum, L., Yao, S., Mozer, B., Rovecalli, A., Von Kessler, D., Nirenberg, M., and Beachy, P.A. (2003). Identification of Hedgehog pathway components by RNAi in *Drosophila* cultured cells. *Science* 299, 2039–2045.
- Martinez-Pastor, M.T., Marchler, G., Schüller, C., Marchler-Bauer, A., Ruis, H., and Estruch, F. (1996). The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). *EMBO J.* 15, 2227–2235.
- Moskvina, E., Schüller, C., Maurer, C.T., Mager, W.H., and Ruis, H. (1998). A search in the genome of *Saccharomyces cerevisiae* for genes regulated via stress response elements. *Yeast* 14, 1041–1050.
- Pearce, A.K., Booth, I.R., and Brown, A.J. (2001). Genetic manipulation of 6-phosphofructo-1-kinase and fructose 2, 6- biphosphate levels affects the extent to which benzoic acid inhibits the growth of *Saccharomyces cerevisiae*. *Microbiology* 147, 403–410.
- Piper, P., Mahé, Y., Thompson, S., Pandjaitan, R., Holyoak, C., Egner, R., Mühlbauer, M., Coote, P., and Kuchler, K. (1998). The Pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. *EMBO J.* 17, 4257–4265.
- Piper, P.W. (1999). Yeast superoxide dismutase mutants reveal a prooxidant action of weak organic acid food preservatives. *Free Radic. Biol. Med.* 27, 1210–1227.
- Piper, P.W., Ortiz-Calderon, C., Holyoak, C., Coote, P., and Cole, M. (1997). Hsp30, the integral plasma membrane heat shock protein of *Saccharomyces cerevisiae*, is a stress-inducible regulator of plasma membrane H(+)-ATPase. *Cell Stress Chaperones* 2, 12–24.
- Piper, P.W., Talreja, K., Panaretou, B., Moradas-Ferreira, P., Byrne, K., Praekelt, U.M., Meacock, P., Recnacq, M., and Boucherie, H. (1994). Induction of major heat-shock proteins of *Saccharomyces cerevisiae*, including plasma membrane Hsp30, by ethanol levels above a critical threshold. *Microbiology* 140, 3031–3038.
- Rep, M., Albertyn, J., Thevelein, J.M., Prior, B.A., and Hohmann, S. (1999). Different signalling pathways contribute to the control of *GPD1* gene expression by osmotic stress in *Saccharomyces cerevisiae*. *Microbiology* 145, 715–727.
- Rodriguez-Manzanique, M.T., Ros, J., Cabiscol, E., Sorribas, A., and Herrero, E. (1999). Grx5 glutaredoxin plays a central role in protection against protein oxidative damage in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 19, 8180–8190.
- Rose, M.D., Winston, F., and Hieter, P. (1990). *Methods in Yeast Genetics. A Laboratory Course Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Roth, F.P., Hughes, J.D., Estep, P.W., and Church, G.M. (1998). Finding DNA regulatory motifs within unaligned noncoding sequences clustered by whole-genome mRNA quantitation. *Nat. Biotechnol.* 16, 939–945.
- Schneider, T.D., and Stephens, R.M. (1990). Sequence logos: a new way to display consensus sequences. *Nucleic Acids Res.* 18, 6097–6100.
- Seymour, I.J., and Piper, P.W. (1999). Stress induction of *HSP30*, the plasma membrane heat shock protein gene of *Saccharomyces cerevisiae*, appears not to use known stress-regulated transcription factors. *Microbiology* 145, 231–239.
- Shoemaker, D.D., Lashkari, D.A., Morris, D., Mittmann, M., and Davis, R.W. (1996). Quantitative phenotypic analysis of yeast deletion mutants using a highly parallel molecular bar-coding strategy. *Nat. Genet.* 14, 450–456.
- Teixeira, M.C., and Sa-Correia, I. (2002). *Saccharomyces cerevisiae* resistance to chlorinated phenoxyacetic acid herbicides involves Pdr1p-mediated transcriptional activation of *TPO1* and *PDR5* genes. *Biochem. Biophys. Res. Commun.* 292, 530–537.
- Treger, J.M., Magee, T.R., and McEntee, K. (1998). Functional analysis of the stress response element and its role in the multistress response of *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* 243, 13–19.
- Wach, A., Brachat, A., Alberti-Segui, C., Rebischung, C., and Philippsen, P. (1997). Heterologous *HIS3* marker and GFP reporter modules for PCR-targeting in *Saccharomyces cerevisiae*. *Yeast* 13, 1065–1075.

- Wang, D., Hu, Y., Zheng, F., Zhou, K., and Kohlhaw, G.B. (1997). Evidence that intramolecular interactions are involved in masking the activation domain of transcriptional activator Leu3p. *J. Biol. Chem.* 272, 19383–19392.
- Wang, D., Zheng, F., Holmberg, S., and Kohlhaw, G.B. (1999). Yeast transcriptional regulator Leu3p. Self-masking, specificity of masking, and evidence for regulation by the intracellular level of Leu3p. *J. Biol. Chem.* 274, 19017–19024.
- Wolfger, H., Mahé, Y., Parle-McDermott, A., Delahodde, A., and Kuchler, K. (1997). The yeast ATP binding cassette (ABC) protein genes *PDR10* and *PDR15* are novel targets for the Pdr1 and Pdr3 transcriptional regulators. *FEBS Lett.* 418, 269–274.
- Wu, K., Dawe, J.H., and Aris, J.P. (2000). Expression and subcellular localization of a membrane protein related to Hsp30p in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* 1463, 477–482.
- Young, R.A. (2000). Biomedical discovery with DNA arrays. *Cell* 102, 9–15.
- Zhang, L., and Guarente, L. (1995). Heme binds to a short sequence that serves a regulatory function in diverse proteins. *EMBO J.* 14, 313–320.