J Ind Microbiol Biotechnol (2010) 37:313–322
DOI 10.1007/s10295-009-0676-y

ORIGINAL PAPER

Microarray analysis of *p*-anisaldehyde-induced transcriptome of *Saccharomyces cerevisiae*

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Received: 5 June 2009 / Accepted: 29 November 2009 / Published online: 19 December 2009

Abstract *p*-Anisaldehyde (4-methoxybenzaldehyde), an extract from *Pimpinella anisum* L. seeds, is a potential novel preservative. To reveal the possible action mechanism of *p*-anisaldehyde against microorganisms, yeast-based commercial oligonucleotide microarrays were used to analyze the genome-wide transcriptional changes in response to *p*-anisaldehyde. Quantitative real-time RT-PCR was performed for selected genes to verify the microarray results. We interpreted our microarray data with the clustering tool, T-profiler. Analysis of microarray data revealed that *p*-anisaldehyde induced the expression of genes related to sulphur assimilation, aromatic aldehydes metabolism, and secondary metabolism, which demonstrated that the addition of *p*-anisaldehyde may influence the normal metabolism of aromatic aldehydes. This genome-wide transcriptomics approach revealed first insights into the response of *Saccharomyces cerevisiae* (*S. cerevisiae*) to *p*-anisaldehyde challenge.

Keywords *p*-Anisaldehyde · Antimicrobial · Gene expression profile · DNA microarray

Introduction

In the food industry, there is a growing interest in naturally occurring compounds that show antimicrobial activity and that, therefore, provide a potential source of novel preservatives [1]. In fact, plants probably represent the most diverse source of potential antimicrobials, such as thymol (thyme), carvacrol (oregano), *p*-anisaldehyde and so on. However, their application to food products as preservatives has not been fully exploited.

Understanding how antifungal compounds such as *p*-anisaldehyde function will be desirable if they are to be used as natural preservatives in commercial applications. *p*-Anisaldehyde (4-methoxybenzaldehyde) is extracted from anisal seed oil, which is derived from *Pimpinella anisum* L. seeds, and it is regarded as one of the world's principal flavoring compounds. *p*-Anisaldehyde exhibits strong antifungal activity against a number of yeast and mold strains in laboratory media, fruit-based agar systems, fruit purees, and fruit juices [2–4].

In previous studies, the antifungal properties of *p*-anisaldehyde were usually evaluated by the minimum inhibition concentration (MIC) value [5]. However, to our knowledge,

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Electronic supplementary material The online version of this article (doi:10.1007/s10295-009-0676-y) contains supplementary material, which is available to authorized users.

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no reports have yet elucidated the antifungal mechanism of *p*-anisaldehyde from a whole genome perspective. Measurement of changes in gene expression as an adaptive response upon exposure to a drug or chemical can help us to understand the mechanism of how drugs and drug candidates work in cells and organisms [6].

In the past few years, DNA microarray technology has been used to discover gene's functions, to understand biochemical pathways and to discover drug targets [7, 8]. The yeast *S. cerevisiae* is a eukaryotic cell model for the study of antifungal action because it adapts easily to changes in its environment and mimics many of the properties of higher organisms. Most notably, the genome sequence of *S. cerevisiae* has already been completed, and the functions of almost 70% of the genes are known [9]. In this study, yeast-based commercial oligonucleotide microarrays were used to analyze the genome-wide transcriptional changes in response to *p*-anisaldehyde.

Materials and methods

Fungal strains and materials

S. cerevisiae strain L1190 (*MAT*a/α) was kindly provided by Dr. Liang Zhang, CapitalBio. The components of YPD broth (1% yeast extract, 2% peptone, 2% glucose) were obtained from BD Biosciences, Inc., Sparks, MD. *p*-Anisaldehyde was purchased from the China Medical Culture Collection Center (CMCC), and stock solutions of varying concentrations were made in dimethyl sulfoxide (DMSO, Sigma-Aldrich).

Antifungal susceptibility testing

MIC of *p*-anisaldehyde against *S. cerevisiae* strain L1190 (*MAT*a/α) was determined by broth microdilution using twofold serial dilutions in RPMI 1640 medium as described by NCCLS (M27-A) [10]. Three rounds of experiments were performed, and the average MIC value was calculated.

Growth curves

S. cerevisiae strain L1190 (*MAT*a/α) was grown at 30°C with the shaking speed of 200 rpm in YPD broth to an optical density of 0.8 at 600 nm (OD_{600}). It was then divided into six Erlenmeyer flasks containing 100 ml of cell suspension. Five flasks were treated with *p*-anisaldehyde (dissolved in DMSO) to obtain the final concentrations of 1/4 × MIC, 1/2 × MIC, 1 × MIC, 2 × MIC, and 4 × MIC. The final DMSO concentration used was 1% (vol/vol). The sixth culture was used as a control with YPD culture alone. These cultures were then incubated further. A 2-ml sample of each culture was taken immediately from each flask after the addition of chemicals (*t* 0, 15, 30, 45, 60, 75, 90, 105, 120, 180, 240, 300, 360, 420, and 480 min) and was monitored at OD_{600}. Sequential OD measurements were used to generate growth curves in triplicate.

Cell culture and drug exposure for microarray experiment

S. cerevisiae strain L1190 (*MAT*a/α) was grown overnight in 10 ml of YPD broth in a rotary shaker at 30°C and 200 rpm. A 500-ml Erlenmeyer flask that contained 150 ml of YPD broth was inoculated with an overnight culture to an initial OD_{600} of 0.05. Then, the *S. cerevisiae* cells were grown at 30°C at 200 rpm to an OD_{600} of 0.8. Subsequently, 150 ml of YPD broth was divided into two flasks, each of which contained 75 ml of culture; *p*-anisaldehyde stock solution prepared in dimethyl sulfoxide (DMSO) was added to one of the cultures, giving a final concentration of 2 × MIC (128 μg/ml). Hence, the final concentration of *p*-anisaldehyde treatment was 1% (vol/vol) DMSO; this amount of DMSO did not alter the pH of the medium [11]. The other culture containing 1% (vol/vol) DMSO lacking *p*-anisaldehyde was used as the control. All fungal suspensions (both experimental and control suspensions) were further incubated for 90 min at 30°C. Yeast cells were harvested by concentration at 5,000 rpm for 5 min at room temperature, washed with fresh YPD broth, snap-frozen in liquid nitrogen, and then stored at −80°C until just before use. Three independent 150-ml cultures were prepared for biological repeats, and another 150-ml culture was prepared for the dye swap experiment.

Fabrication of DNA microarray

A yeast genome 70-mer oligonucleotide microarray was obtained from CapitalBio Corporation (Beijing, China). Briefly, a yeast genome oligo set consisting of 5′ amino acid-modified 70-mer probes was obtained from operon (http://www.Operon.com). Then, oligonucleotides were dissolved in EasyArray™ spotting solution (CapitalBio Corp.) at 40-μM concentrations. They were then printed on a PolymerSlide (75 × 25 mm) that had a surface covered by a thin layer of aldehyde group-modified three-dimensional polymer chain (CapitalBio Corp.). A set of microarrays containing a total of 23,184 spots [including over 6,400 clones in the form of PCR products and controls, including blank, negative (DMSO) and positive controls (*Hex, ACT1*, *RPL32*, *SSA4*, *TUB4* and *UB14*)] were spotted in triplicate on slides (CapitalBio Corp.). Each slide consisted of 48 blocks, and every block had 21 columns and 23 rows.

RNA preparation, labeling and hybridization

Total RNA was extracted by a hot acidic phenol method [12]. The RNA concentration and purity were determined spectrophotometrically by measuring absorbance at 230, 260, and 280 nm. The purity and integrity of the RNA were confirmed by agarose gel electrophoresis. cDNA labeled with a fluorescent dye (Cy5 and Cy3-dCTP) was produced by Eberwine's linear RNA amplification method and subsequent enzymatic reaction [13]. Double-stranded cDNAs (containing the T7 RNA polymerase promoter sequence) were synthesized from 5 μg total RNA, using the cDNA synthesis system according to the manufacturer's protocol (Promega, Madison, WI). A T7-oligo(dT) primer (5′-AA ACGACGGCCAGTGAATTGTAATACGACTCACTATA GGCGCTTTTTTTTTTTTTT-3′) was used instead of the poly-T primer provided in the kit. Labeled controls and test samples were quantitatively adjusted based on the efficiency of Cy5-dCTP or Cy3-dCTP incorporation and dissolved in 80 μl hybridization solution containing 25% formamide. Array hybridization was preformed in a CapitalBio BioMixer™ II Hybridization Station overnight at a temperature of 42°C and washed with two consecutive solutions (0.2% SDS, 2 × SSC at 42°C for 5 min, and 0.2 × SSC at room temperature for 5 min). The experimental samples of three biological repeats were labeled with Cy3, and the control samples were labeled with Cy5, whereas, in the another experiment for dye swap, the labeling of samples with these dyes was reversed.

Microarray imaging and data analysis

Arrays were scanned with a confocal LuxScan™ scanner (scanning resolution is 10 μm), and the images obtained were analyzed using LuxScan™ 3.0 software (both from CapitalBio). For channels data extraction, faint spots [with intensities below 800 units after background subtraction in both channels (Cy3 and Cy5)] were removed. A space- and intensity-dependent normalization based on a LOWESS (locally weighted scatter plot smoothing regression) program was employed [14]. Average relative fold changes were calculated from the average of the signal log ratio (SLR) from three separate experiments by using the following equations: for an SLR of ≥ 0, average relative fold change = 2^{SLR}; for an SLR of <0, average relative fold change = $-1 \times 2^{(-1 \times SLR)}$. Relative changes of $\geq$twofold in independent experiments were considered indicative of differences in transcript amounts [15].

Significant associations with either gene ontology (GO) terms or transcription factors were collected with the T-Profiler [16]. The T-profiler algorithm is a useful tool to analyze the relative expression of groups of genes in relation to the total genome and to define the probable activity of specific transcription factors by analysis of gene groups that share common motifs in the 600 bp of their upstream region [17]. This algorithm uses an unpaired *t*-test to classify a difference between the mean of a set of a specific class of genes and the mean of the remaining genes of the total gene expression profile [18]. Classes of genes containing a similar promoter element were studied. Gene ontology has been used to classify genes into specific categories. The experiments were separately analyzed, and no arbitrary cutoffs were applied before T-profiler analysis. T-profiler can be found at http://www.t-profiler.org.

Quantitative real-time RT-PCR assays

Aliquots of the RNA preparations from *p*-anisaldehyde-untreated and control samples used in the microarray experiments were saved for follow-up quantitative real-time RT-PCR. Quantitative real-time RT-PCRs were performed in triplicate using the 7,000-sequence detection system (Applied Biosystems, Foster City, CA) according to a previously described procedure [11]. Primer sequences are listed in Table 1.

Rhodamine 6G efflux by *S. cerevisiae* cells

The efflux of rhodamine 6G (R6G, Sigma) from *S. cerevisiae* cells was determined by adapting the method described by Nakamura et al. [19]. Yeast cells from YPD cultures in the exponential growth phase (OD_{600}, 0.5) were collected by centrifugation (3,000 × *g*, 5 min, 20°C) and washed three times with water. The cells were resuspended at a concentration of 0.5×10^6–1.0×10^7 cells per ml in HEPES–NaOH (50 mM; pH 7.0) containing 5 mM 2-deoxyglucose and 10 μM R6G. In some experiments *p*-anisaldehyde (32 μg/ml) was added. Cell suspensions were incubated at 30°C with shaking (200 rpm) for 90 min to allow rhodamine accumulation under glucose starvation conditions. The starved cells were washed twice in HEPES–NaOH, and portions (400 μl) were incubated at 30°C for 5 min before the addition of glucose (final concentration, 2 mM) to initiate rhodamine efflux. At specified intervals after the addition of glucose, the cells were removed from triplicate assays by centrifugation, and triplicate 100-μl volumes of the cell supernatants were transferred to the wells of 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark). The rhodamine fluorescence of the samples was measured with a Wallac Victor 3 1,420-multilabel counter (Perkin Elmer, Wallac Oy Inc., Turku, Finland). The excitation wavelength was 530 nm, and the emission wavelength was 590 nm.

Table 1 Primers used in real-time RT-PCR with SYBR green probes

Primer	ORF	Sequence (5′–3′)	Reference
18S rRNAfor		CGGCTACCACATCCAAGGAA	Lu et al. [60]
18S rRNArev		GCTGGAATTACCGCGGCT	Lu et al. [60]
*FEN1*for	YCR034W	GCATACTTATCACCATGGCGCT	This study
*FEN1*rev	YCR034W	CATAACCACGTGAACACCAAGG	This study
*LAG1*for	YHL003C	GGTGATACAGTTCCTGGCTTTG	This study
*LAG1*rev	YHL003C	CTTTTGACGATGCTCGGAAGTA	This study
*ADE6*for	YGR061C	TGGTCCTTGGCAAGTACCTGTT	This study
*ADE6*rev	YGR061C	GCGTTAACTGGTTTTTCACCCA	This study
*RAD59*for	YDL059C	AGCCCAGTTCGAGCATATCGTA	This study
*RAD59*rev	YDL059C	TGGACTGTAGAAGCCCAATCCT	This study
*RPN4*for	YDL020C	AAACCTTCGACTGCATCACCAT	This study
*RPN4*rev	YDL020C	GCACCACACGGTTCATTTGTAA	This study
*PDR3*for	YBL005W	ACCGCAGAAGGAGGATAGTTCC	This study
*PDR3*rev	YBL005W	CGCAGTGTCCAGATGCTGTACA	This study
*YGP1*for	YNL160W	GATCCAAGATAAGTCATCCGCC	This study
*YGP1*rev	YNL160W	GCATAAGCGGAATCTTCAGTGA	This study
*PRS1*for	YKL181W	TTAGCGCTGCTGAACATTTGGT	This study
*PRS1*rev	YKL181W	CAATGGCATCGGACTTTTCAAG	This study
*MET2*for	YKR069W	GCGACATCAGCAAGACATTCTG	This study
*MET2*rev	YKR069W	ACCGGATAGTACCCGTCCAAGT	This study
*RPA135*for	YPR010C	TTCCAAGTTCGTTCCACTGGTC	This study
*RPA135*rev	YPR010C	CGGATGCCTGTGTGTAGTCTGA	This study
*ADH6*for	YMR318C	CATTTTGTGGTGCCTATCCCAG	This study
*ADH6*rev	YMR318C	CGCAACCGTTACGAACCAAT	This study

ORF open reading frame

Results

MIC of *p*-anisaldehyde and influence of *p*-anisaldehyde on growth curves

In this experiment, the median MIC value of *p*-anisaldehyde against *S. cerevisiae* L1190 (*MAT*a/α) was 64 μg/ml. We determined growth curves of *S. cerevisiae* with *p*-anisaldehyde (16, 32, 64, 128, and 256 μg/ml) at the time point when $OD_{600} = 0.8$. The growth curves of *S. cerevisiae* are shown in Fig. 1.

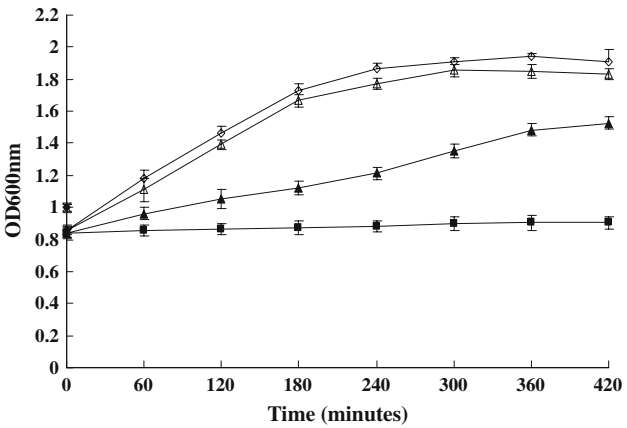

Fig. 1 Growth curve for *S. cerevisiae* strain L1190 (*MAT*a/α) in the presence or absence of *p*-anisaldehyde. *Open diamond* untreated *S. cerevisiae* L1190; *open triangle S. cerevisiae* L1190 plus 32 μg/ml *p*-anisaldehyde; *filled triangle S. cerevisiae* L1190 plus 128 μg/ml *p*-anisaldehyde; *filled square S. cerevisiae* L1190 plus 256 μg/ml *p*-anisaldehyde. Data are expressed as mean ± standard deviation. Statistical analysis was done using one-way analysis of variance (ANOVA) in SPSS 13.0 for Windows. *Error bars* represent standard deviation

Overview of expression changes in response to *p*-anisaldehyde treatment in *S. cerevisiae*

Microarray data analysis showed that a large number of genes (1,309) were differentially expressed after 90 min of exposure to *p*-anisaldehyde compared with untreated *S. cerevisiae* cells. A total of 593 genes increased in expression, and 716 genes were inhibited. The microarray-related data were submitted to Gene Expression Omnibus (GEO) under accession number GSE9409. A complete list of all of the genes differentially expressed due to *p*-anisaldehyde treatment can be found in the supplementary material (Table S1).

In previous studies, microarray analysis revealed that growth rate greatly modified the expression of many yeast genes [20], and exposure of yeast cells to 7 types of stress [21], 11 environmental changes [22], lithium [23],

Table 2 Real-time RT-PCR analysis of gene expression

ORF	Gene	Description	Fold change	
			RT-PCR	Microarray
YDL059C	*RAD59*	Protein involved in the repair of double-strand breaks in DNA during vegetative growth via recombination and single-strand annealing	+11.8	+5.8
YDL020C	*RPN4*	May act as common transcription factor on proteasomal and proteasome-related genes	+3.1	+2.7
YBL005W	*PDR3*	Zinc-finger transcription factor related to Pdr1p	+17	+6.6
YNL160W	*YGP1*	gp37, a glycoprotein synthesized in response to nutrient limitation that is homologous to the sporulation-specific SPS100 gene	+43.1	+23.7
YKR069W	*MET2*	Homoserine O-trans-acetylase	+29.6	+13.1
YMR318C	*ADH6*	Medium chain alcohol dehydrogenase	+7.8	+5.4
YCR034W	*FEN1*	Fatty acid elongase, involved in sphingolipid biosynthesis	−2.4	−2.1
YHL003C	*LAG1*	Ceramide synthase component, involved in synthesis of ceramide from C26(acyl)-coenzyme A and dihydrosphingosine or phytosphingosine	−1.9	−2.1
YGR061C	*ADE6*	5-phosphoribosylformyl glycinamidine synthetase	−23.7	−6.1
YKL181W	*PRS1*	Ribose-phosphate pyrophosphokinase	−5.5	−2.5
YPR010C	*RPA135*	RNA polymerase I subunit	−24.8	−7.9

rapamycin [24], and 3-aminotriazole led to the change of expression of STRE genes, covering a core of 1,000 environmental stress response (ESR) genes. We compared the genes that were differentially regulated in the present study with those identified by the studies mentioned above. We found 40 genes required for cell viability were significantly regulated by treatment with *p*-anisaldehyde. Of these, 5 showed an increase in expression and 35 showed a decrease in expression (see Table S1). Herein, our interest was focused mainly on specific genes that may allow the organism to survive in the presence of *p*-anisaldehyde.

Functional categories of the expression changes response to *p*-anisaldehyde treatment in *S. cerevisiae*

Transcriptome data were analyzed using the gene ontology (GO) categories in T-profile. The selected categories have a number of ORFs $\leq$1,000, and *t*-values of 4 or more or −4 or less are considered to be significant [25]. We observed 5 gene groups that were significantly up-regulated, whereas 11 categories were significantly repressed in response to *p*-anisaldehyde (Table 3).

Table 3 Selected results from T-Profiler analysis (http://www.t-profiler.org/) for GO terms associated with *p*-anisaldehyde data sets

Category	*t*-value	Mean	ORFs
Proteasome complex (sensu Eukarya) (46)	8.4	1.092	46
Sulfate assimilation (8)	7.04	2.376	8
Oxidoreductase activity (239)	6.03	0.405	239
Multidrug transporter activity (10)	5.94	1.729	10
Sulfur amino acid biosynthesis (9)	5.48	1.695	9
Amine biosynthesis (109)	−4.94	−0.279	109
Translation factor activity, nucleic acid binding (47)	−5.1	−0.503	47
Nucleosome (12)	−5.37	−1.231	12
Thiamin metabolism (19)	−5.94	−1.037	19
Aromatic compound metabolism (53)	−6.18	−0.575	53
Cell proliferation (576)	−7.08	−0.146	576
Biosynthesis (838)	−7.34	−0.094	838
tRNA modification (59)	−7.84	−0.757	59
Nucleolus (192)	−10.17	−0.526	192
Cytosolic ribosome (sensu Eukarya) (153)	−14.76	−0.933	153
Ribosome biogenesis and assembly (217)	−20.68	−1.161	217

The number of ORFs in each category is shown in parentheses. All functional categories were tested. The selected categories have a number of ORFs of $\leq$1,000 and at least a *t*-value of −4 or 4. *t*-value measuring the up-regulation ($t > 0$) or down-regulation ($t < 0$) in units of the standard error of the difference. *t*-values of 4 or more and −4 or less are considered to be significant

Validation of microarray data by real time RT-PCR

In order to validate results obtained from our microarray data, real-time quantitative RT-PCR was performed with the same RNA from the original microarray experiment. Eleven genes of interest were selected (*RAD59*, *RPN4*, *PDR3*, *YGP1*, *MET2*, *ADH6*, *FEN1*, *LAG1*, *ADE6*, *PRS1*, and *RPA135*). Overall, there was great accordance between microarray data and real time RT-PCR data for all 11 (Table 2) genes. Of the 11 genes, 6 were induced, and 5 were reduced, in response to *p*-anisaldehyde. However, for some genes (*RAD59*, *PDR3*, *YGP1*, *MET2*, *ADE6*, *PRS1*, and *RPA135*), the numerical values were much higher than

those obtained from our microarray. In situations where poor or no hybridization signals are generated for one of the samples tested, *n*-fold induction values can be under- or overestimated as a result of the higher efficiency of the RT step (no cyanine dye incorporation) and/or the higher sensitivity of the real-time PCR. For others (*RPN4*, *ADH6*, *FEN1*, and *LAG1*), the levels of genes in expression did not change markedly between microarray data and real-time RT-PCR data. Collectively, the real-time RT-PCR results provide independent verification of our DNA microarray results.

Discussion

The growth variation of *S. cerevisiae* cells treated by *p*-anisaldehyde

In Fig. 1, within 15 min after *p*-anisaldehyde treatment, the growth curves showed that there was an increase in the OD_{600} except at a concentration of 256 μg/ml. After 15 min, at the concentrations of 16, 32, 64, and 128 μg/ml *p*-anisaldehyde and in the control, we observed that the OD increased steadily; at 256 μg/ml *p*-anisaldehyde, we did not find a significant change in OD_{600} with time. After 420 min, at 16, 32, 64, 128, and 256 μg/ml *p*-anisaldehyde, the OD of the culture was approximately 97, 95, 93, 79, and 47% of the OD of untreated (control) cultures, respectively. A sample of 128 μg/ml *p*-anisaldehyde was prepared for microarrays because the concentration of 128 μg/ml was enough to inhibit yeast cell growth in large-scale cultures. Weak inhibition may not lead to any detectable change, whereas strong inhibition may lead to cell death. Moreover, in previous reports, one doubling time (90 min) was sufficient to detect specific gene expression changes in response to various antifungal drugs [26].

The variation of genes related to the sulfur assimilation after exposure to *p*-anisaldehyde

We found that the genes *MET1*, *MET3*, *MET8*, *MET10*, *MET14*, *MET16*, *MET17*, and *MET22*, which are related to the sulfur assimilation category, were up-regulated by *p*-anisaldehyde; the expression of genes *CBF1*, *CYS4*, *MET6*, *STR3*, *MET2*, *MET13*, and *MET28*, which are involved in the sulfur amino acid biosynthesis category, was also increased. Up-regulation of the methionine biosynthetic pathway and alterations in sulfur assimilation are cardinal transcriptional indicators of the metabolic phenotype [27]. Aranda et al. [28] also reported that the genes involved in sulfur metabolism were induced by acetaldehyde using microarray. Thomas et al. [29] indicated that the repression of *MET* gene expression occurs when high concentrations of methionine are added to the growth medium. Aranda et al. [28] stated that the transcription induction of sulfur metabolism genes by acetaldehyde seems to be a process that is independent of *MET* gene repression. This suggests that the presence of a high aldehyde concentration causes a certain depletion in sulfur sources that can be at least partially compensated by the addition of sulfur-containing organic compounds.

The gene involved in the oxidoreductase activity category was regulated by *p*-anisaldehyde

We found that several putative aryl-alcohol dehydrogenases, *AAD6*, *AAD10*, *AAD4*, *AAD14*, and *AAD16*, were induced by a factor of 4.1, 1.8, 1.7, 3.2, and 2.6, respectively. However, there is a very high nucleotide sequence similarity among all seven genes *AADs*, *AAD3*, *AAD4*, *AAD6*, *AAD10*, *AAD14*, *AAD15*, and *AAD16*. From the oligonucleotide microarray used in this study, *AAD6*, *AAD4*, and *AAD10* probe only matches to the *AAD6*, *AAD4*, and *AAD10* ORF, respectively; the *AAD14* probe has one mismatch to the *AAD4* ORF; the probe for *AAD16* (YFL057C_01) matches to the *AAD3* ORF and *AAD16* ORF and has one mismatch to the *AAD15* ORF, but fluorescent intensity of *AAD3* and *AAD15* was very low. Thus, the significant up-regulation of the *AAD6* and *AAD16* genes and slight induction of *AAD4* and *AAD10* gene by *p*-anisaldehyde, as observed by oligo microarray analysis, were correct, whereas the induction of the *AAD14* in oligo DNA microarray was due to cross hybridization. Yeast cultures grown to stationary phase display a significant aryl alcohol dehydrogenase (AAD) activity by degrading aromatic aldehydes to the corresponding alcohols [30]. Prior studies showed that diamide and diethyl maleic acid ester induced AAD genes, and those genes were induced by oxidative stress in a Yap1p-dependent manner [31]. Kitagawa et al. [32] demonstrated that dithiocarbamate fungicides, thiuram, zineb, and maneb, and other fungicides, TPN and PCP, also induced AAD genes. Thus, up-regulation of AADs may not be directly related to induction of aromatic aldehyde metabolism.

The categories related to cytosolic ribosome and ribosome biogenesis and assembly were effected by *p*-anisaldehyde

In this study, the significantly down-regulated categories were functionally related to cytosolic ribosome (sensu Eukarya) and ribosome biogenesis and assembly, including a series of *RPL* and *RPS* genes. As previous statements showed, the repression of the ribosomal genes is a general feature of the environmental stress response (ESR) [21]. It was demonstrated that, under environmental stresses, almost all ESR genes respond similarly to stress and

decreased growth rate. Conditions known to induce ESR genes often inhibit growth, indicating that the stress response shares a component with the response to changes in the specific growth rate [20]. Similar results have also been observed: the effects of 5-flucytosine on protein synthesis [33] and chitosan on plasma membrane [34] were reflected in the repression of ribosomal protein genes.

The category of sulfur amino acid biosynthesis was influenced by *p*-anisaldehyde

The up-regulation of sulfur amino acid biosynthesis often indicates activation of the glutathione biosynthesis pathway. Glutathione, γ-L-glutamyl-L-cysteinylglycine, is an important molecule that plays a major role in protecting cells against damage caused by oxidants and is therefore an important antioxidant molecule. In response to oxidative stress caused by compounds such as H_2O_2 and superoxide agents, the *S. cerevisiae* induces stress responses that result in protection against subsequent toxic levels of oxidants [35, 36]. The previous study indicated that exposure of yeast cells to oxidants results in an increase in the steady state level of *GSH1* mRNA, which encodes γ-glutamylcysteine synthetase, the first enzyme in the GSH biosynthetic pathway [37]. Consistent with previous study [28], in our microarray data, the glutathione synthesis was not one of the most induced pathways, because GSH1 was just induced by 4.1-fold and no strong induction for GSH2 after exposure on *p*-anisaldehyde, but we could not rule out the induction of this well-known stress protective molecule.

Genes involved in the repair of DNA double-stranded breaks were regulated by *p*-anisaldehyde

We found *that RAD59*, a member of the *RAD52* epistasis group, was involved in the repair of DNA double-stranded breaks and was strongly up-regulated by the addition of *p*-anisaldehyde, and two other RADs genes (RAD54 and RAD52) of this group were also up-regulated 2.4-fold and 2.2-fold, respectively. In *S. cerevisiae*, single-strand annealing (SSA) is dependent on the function of *RAD52* and *RAD59* [38], and *RAD52* and *RAD54* genes are essential for conservative double-strand break (DSB) repair [39]. In addition, some other ORFs related to DNA repair showed significant induction by *p*-anisaldehyde: MAG1 (4.4-fold), DDR48 (4.0-fold), MND1 (3.1-fold), IMP2' (2.7-fold), SOH1 (2.1-fold), DOA1 (2.0-fold), DDC1 (2.0-fold), and TDP1 (2.0-fold). Previous reports have shown that anisaldehyde and its structurally related compound vanillin have antimutagenic properties [40]. Further, vanillin has been shown to be anticarcinogenic in several model systems and species [41, 42]. It has been proposed that vanillin exerts its antimutagenic activity in damaged cells by promoting recombination and rejoining of DNA at homologous sites [43]. Gustafson et al. [44] demonstrated that the antimutagenic activity of vanillin is consistent with it modulating a process(es), such as DNA repair; these are associated with our results showing that genes involved in repair of DNA double-stranded breaks were induced by *p*-anisaldehyde.

The pleiotropic drug resistance (PDR) network was regulated by *p*-anisaldehyde

In *S. cerevisiae*, the pleiotropic drug resistance (PDR) network involved in multidrug resistance (MDR) is mainly regulated by the transcription factors Pdr1p and Pdr3p. *PDR1* and *PDR3* can control the expression of the ATP-binding cassette (ABC) transporters *PDR5*, *SNQ2*, and *YOR1* [45]. In this study, it was found that *SNQ2*, *PDR5*, and *YOR1* were strongly induced by a factor of 11.6, 10.1, and 14.6, respectively. We postulate that *p*-anisaldehyde possibly triggers the PDR network in yeast cells. Glucose-dependent efflux of R6G from *S. cerevisiae* was performed to assay a phenotypic correlation between ABC transporter overexpression induced by *p*-anisaldehyde and efflux of R6G in this study. It is known that fluorescent R6G is the substrate of ABC transporters Yor1p and Pdr5p [46, 47]; efflux from deenergized (by incubation with 2-deoxyglucose), R6G-preloaded cells required the presence of glucose. We took advantage of these properties of R6G to directly examine the action of *p*-anisaldehyde on R6G efflux using fluorescence techniques. The results (Fig. 2) show that the addition of *p*-anisaldehyde (32 μg/ml) resulted in a 44.0% increase in the concentration of released fluorescence 15 min following the addition of glucose; this is consistent with the elevated expression levels of the genes Pdr5 and Yor1 induced by *p*-anisaldehyde treatment.

Rpn4p is required for basal and stress-induced expression levels of proteosome sub-units; its own expression depends on PDR regulators, including Pdr1p, Pdr3p, and Yap1p. Significantly, RPN4 is also required for cell tolerance to various cytotoxic compounds [48]. The significant induction of RPN4 in the present study indicated it may play a role in PDR regulation under *p*-anisaldehyde stress. Notably, the FLR1 gene, which encodes a multidrug resistance (MDR) transporter of the major facilitator superfamily (MFS) that confers resistance to the drugs such as fungicide mancozeb and other inhibitory chemicals in *S. cerevisiae* [49], was up-regulated 3.6-fold upon exposure to *p*-anisaldehyde. A recent study suggested that Yap1, Rpn4, Pdr3, and Yrr1 coordinate control of the up-regulation of *FLR1* under the yeast adaptation to mancozeb [49]. In our study, the genes *FLR1*, *YAP1*, *RPN4*, *PDR3*, and *YRR1* were up-regulated by a factor of 3.6, 2.4, 2.7, 6.6, and 2.0, respectively.

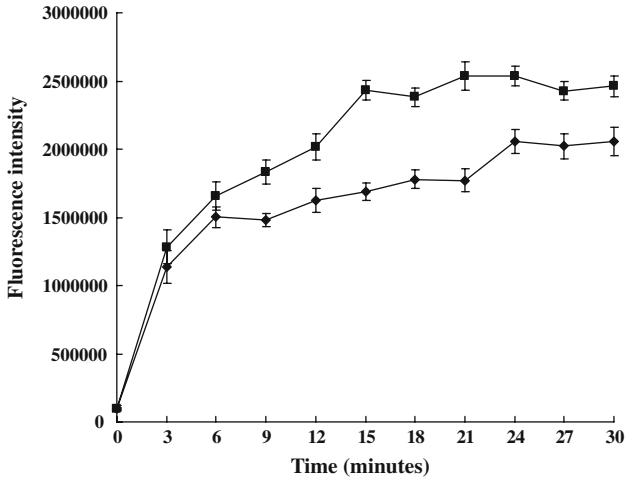

Fig. 2 Energy-dependent ABC transporter-mediated efflux of rhodamine 6G from yeast cells. Deenergized cells were preloaded with R6G as described in Materials and Methods. The efflux of rhodamine was followed by direct measurement of the fluorescence in cell supernatants following the addition of glucose (2 mM) to suspensions of *S. cerevisiae* cells. *Filled diamond* 10 μM R6G; *filled square* 10 μM R6G and 32 μg/ml *p*-anisaldehyde. Data were expressed as mean ± standard deviation. Statistical analysis was done using one-way analysis of variance (ANOVA) in SPSS 13.0 for Windows. *Error bars* represent standard deviation

Motifs regulated by *p*-anisaldehyde

In the present experiment, some genes associated with the TATA-binding protein (TBP) motif were induced by *p*-anisaldehyde. In *S. cerevisiae*, TATA-binding protein (TBP), an essential general transcription factor involved in directing the transcription of genes by the three nuclear RNA polymerases I, II, and III, is encoded by *SPT15* [50]. TBP is one of the few transcription factors highly conserved among all eukaryotes and is essential for viability.

In eukaryotes, the periodic expression of genes plays an important role in the regulation of the cell cycle. Two motifs, MBP1 and SWI4, associated with the cell cycle are members of transcription factors MBF and SBF, respectively [51]. We found *MBP1* and *SWI4* were repressed by *p*-anisaldehyde exposure in this study. In budding yeast, induction of early cell cycle genes is predominantly attributed to MBF and SBF [51]. The previous study indicated that loss of Mbp1 leads to a 20% increase in modal cell volume compared to that in controls; this volume increase is associated with a 5% increase in the proportion of budded cells, suggesting a possible delay in the replicative (budded) part of the cell cycle [52]. The absence of *SWI4* causes a notable phenotype, including slow growth, problems with morphogenesis, and large cell size [53]. These results suggested that *p*-anisaldehyde may have an effect on the cell cycle. In contrast to the above-mentioned transcription factor complexes, MBF and SBF, that are expressed early in the cell division cycle [54], the forkhead proteins Fkh1 and Fkh2 regulate cell cycle-dependent expression of a group of genes, named the *CLB2* gene cluster, that includes *CLB1* and *CLB2*, which encode mitotic cyclins in the G2 and M phases [55]. Our microarray data analyzed by T-profiler also identified the *FKH1* motif, GTAAACA. In addition, *FKH1* and *FKH2* also have redundant functions in preventing pseudohyphal growth [56] and silencing. *CLB2* cluster genes are transcribed in the late S and G2/M phases of the cell cycle [57], Clb2 plays the central role of transcriptional activator, and *CLB2* is its own target gene [58]. The identification of a large number of yeast Fkh-controlled genes of the CLB2 cluster in the fkh1 fkh2 mutant by DNA microarray analysis has been done by [59]; comparing their results with our dataset, we found that most of the 33 genes of the CLB2 cluster were repressed. The significantly down-regulated genes were *ALK1*, *BUD3*, *CLB2*, *KIP2*, *YIL158w*, *BUD8*, *YCL063w*, *YLR084c*, *CHS2*, and *YPR156c*. The genes *FKH1* and *FKH2* were down-regulated by a factor of 4.1 and 3.3, respectively. Taken together, our results demonstrate that the normal late S and G2/M phases of the cell cycle in yeast may be influenced by *p*-anisaldehyde treatment.

In conclusion, this study demonstrated that *p*-anisaldehyde possessed potent antifungal activity; transcriptional profiling by DNA microarray analysis revealed that some important genes and regulatory motifs were affected by *p*-anisaldehyde in *S. cerevisiae*. These findings may have important implications for understanding response mechanisms of *S. cerevisiae* to *p*-anisaldehyde, and this lays the groundwork for further development of *p*-anisaldehyde.

Acknowledgments We are grateful to Dr. LiangZhang (CapitalBio) for oligonucleotide microarray analysis. This work was supported by the national basic research program (program 973) (2006CB504402).

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