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Genome-Wide Screening of *Saccharomyces cerevisiae* Genes Regulated by Vanillin

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During pretreatment of lignocellulosic biomass, a variety of fermentation inhibitors, including acetic acid and vanillin, are released. Using DNA microarray analysis, this study explored genes of the budding yeast *Saccharomyces cerevisiae* that respond to vanillin-induced stress. The expression of 273 genes was upregulated and that of 205 genes was downregulated under vanillin stress. Significantly induced genes included *MCH2*, *SNG1*, *GPH1*, and *TMA10*, whereas *NOP2*, *UTP18*, *FUR1*, and *SPR1* were down regulated. Sequence analysis of the 5′-flanking region of upregulated genes suggested that vanillin might regulate gene expression in a stress response element (STRE)-dependent manner, in addition to a pathway that involved the transcription factor Yap1p. Retardation in the cell growth of mutant strains indicated that *MCH2*, *SNG1*, and *GPH1* are intimately involved in vanillin stress response. Deletion of the genes whose expression levels were decreased under vanillin stress did not result in a notable change in *S. cerevisiae* growth under vanillin stress. This study will provide the basis for a better understanding of the stress response of the yeast *S. cerevisiae* to fermentation inhibitors.

Keywords: *Saccharomyces cerevisiae*, DNA microarray, vanillin, stress response

Introduction

Lignocellulosic biomass such as crop residues and wood chips is an abundant energy source that has the potential for utilization as a feedstock for the production of bioethanol [27]. The production of fuel ethanol from biomass has attracted attention as part of efforts to prevent global warming, protect the environment, and improve energy reserves [23]. The major components of lignocellulosic biomass are cellulose, hemicellulose, and lignin. Therefore, to obtain fermentable sugars, physical or chemical pretreatment processes such as steam explosion, dilute-acid hydrolysis, ammonia freeze explosion, and enzymatic hydrolysis are necessary [20]. The degradation products formed by pretreatment of lignocellulose depend on both the biomass and pretreatment conditions such as temperature, time, pressure, pH, and addition of catalysts [6]. For economic reasons, dilute-acid hydrolysis is commonly used to prepare lignocellulose for enzymatic saccharification and fermentation [11]. As a result of the pretreatment, various inhibitory compounds are released from lignocellulose biomass, which reduce the ethanol yield and productivity during fermentation by microorganisms [1, 20]. These inhibitory compounds belong to three groups: furans, weak acids, and phenolic compounds. The furan compounds 5-hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde, which are formed by dehydration of hexoses and pentoses, respectively [7, 33], have been shown to reduce the enzymatic activity of alcohol dehydrogenase, aldehyde dehydrogenase, and pyruvate dehydrogenase [28]. Formic and levulinic acids are products of HMF breakdown, whereas acetic acid is formed by de-acetylation of hemicelluloses [1, 7]. A wide range of phenolic compounds are generated due to lignin breakdown; vanillin is a major phenolic compound [1, 8].

Recent reports have shown that vanillin can effectively inhibit the growth of yeast and mold when tested in fruit purees and fruit-based agar systems [4, 5]. Vanillin inhibited the growth of *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Debaryomyces hansenii*, and *Z. rouxii* [24, 32].

Vanillin has been suggested to be a stronger inhibitor of growth and bioethanol fermentation than other inhibitors

because it acts at low concentrations [9, 20]. Improvement of *S. cerevisiae* tolerance to vanillin is important in the development of industrial strains for fuel ethanol production and vanillin synthesis [30]. Since vanillin is toxic to yeast, the development of vanillin-tolerant *S. cerevisiae* strains is a critical prerequisite for efficient bioethanol production. A mitochondrial superoxide dismutase mutant exhibits an enhanced resistance to vanillin [14]. Vanillin causes nuclear accumulation of Yap1, an oxidative stress–responsive transcription factor, and subsequent transcriptional activation of Yap1-regulated genes in *S. cerevisiae* [30]. These data indicate that a variety of factors affect *S. cerevisiae* resistance to vanillin, although the mechanism of vanillin tolerance remains obscure. Genome-wide, fitness-based screening methods have identified mutations that confer vanillin sensitivity and non-essential deletion mutants exhibiting vanillin sensitivity [9, 14, 19]. Despite the increasing interest in the inhibitory effects of vanillin, little is known about the genes regulated by vanillin and the mode of action of vanillin in *S. cerevisia*e.

In this study, *S. cerevisiae* genes whose expression levels are notably changed by vanillin were explored by DNA microarray analysis. In addition, the phenotypes of *S. cerevisiae* strains harboring deletions of the genes deregulated by vanillin stress were examined.

Materials and Methods

Chemicals, Strains, and Cultivation Conditions

S. cerevisiae strains (Table 1) were purchased from European *Saccharomyces cerevisiae* Archive for Functional Analysis and grown in YEPD (1% bacto-yeast extract, 2% bacto-proteose peptone, and 2% glucose) at 30°C unless stated otherwise. Vanillin was purchased from Sigma-Aldrich (USA). To determine *S. cerevisiae* sensitivity to vanillin, various vanillin concentrations were added to the medium.

Table 1. *S. cerevisiae* strains used in this study.

Name	Genotype
BY4742	*MAT α his3Δ 1 leu2Δ0 lys2Δ 0 ura3Δ0*
Y15070	*Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 Δmch2::kanMX4*
Y15047	*Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 Δsng1::kanMX4*
Y15236	*Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 Δtma10::kanMX4*
Y13529	*Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 Δhsp42::kanMX4*
Y15575	*Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 Δgph1::kanMX4*
Y16498	*Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 Δtsl1::kanMX4*
Y13717	*Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 Δgpm2::kanMX4*
Y13354	*Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 Δsds24::kanMX4*
Y15867	*Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 Δhxk1::kanMX4*

Cell growth was quantified by measuring the optical density at 600 nm (OD_{600}). For microarray experiments, exponentially growing cells were harvested by filtration [31] and resuspended in YEPD containing 4 mM vanillin for 2 h. Cells were then cultured in 500 ml baffled flasks (Nalgene, USA) containing 100 ml of YEPD supplemented with various concentrations of vanillin at 30°C for 30 h with shaking at 200 rpm. For plate growth assay, *S. cerevisiae* was grown in YEPD overnight and diluted to an OD_{600} of 0.2. Then, 5-fold serial dilutions were prepared, and 10 µl of each dilution was spotted onto a YEPD plate containing 6 mM vanillin. The plates were incubated at 30°C for 2 or 3 days [25].

Analytical Methods

Ethanol concentrations were determined by a QuantiChrom Ethanol Assay Kit (BioAssay Systems, USA). Glucose was measured by high-performance liquid chromatography (Shimadzu, Japan) on an HPX-87H column (Bio-Rad, USA); the mobile phase was 0.005 N H_2SO_4.

RNA Extraction and DNA Microarray Analysis

Total RNA was extracted from cells exponentially growing in YEPD containing 4 mM vanillin. mRNA was purified using a PureLink RNA Kit (Ambion, USA) according to the manufacturer's recommendations. Gene expression analysis was performed using a GeneChip Yeast Genome 2.0 Array (Affymetrix, USA) and GenPlex software (Istech, Korea). Statistical analysis for the normalization of microarray data was performed using methods described elsewhere [17, 18].

Results and Discussion

Effects of Vanillin on Growth of *S. cerevisiae*

To determine the appropriate concentration of vanillin for DNA microarray analysis, the sensitivity of *S. cerevisiae* BY4742 strain to vanillin was examined. *S. cerevisiae* growth was inhibited by vanillin at concentrations above 4 mM (Fig. 1). At a vanillin concentration of 4 mM, the specific growth rate of the BY4742 strain was reduced by approximately 38%. Inhibition of cell growth by vanillin was also observed in shake-flask cultures (Fig. 2). Inhibition of growth of the haploid BY4742 strain treated with vanillin was highly correlated with ethanol production, which is in line with the data reported for the diploid BY4743 strain [3]. The rates of both cell growth and ethanol production from glucose were significantly affected at 4 mM vanillin. Based on these results, RNA was prepared from wild-type *S. cerevisiae* BY4742 cells grown in the presence of 4 mM vanillin.

DNA Microarray Analysis

Vanillin-regulated genes were identified using DNA

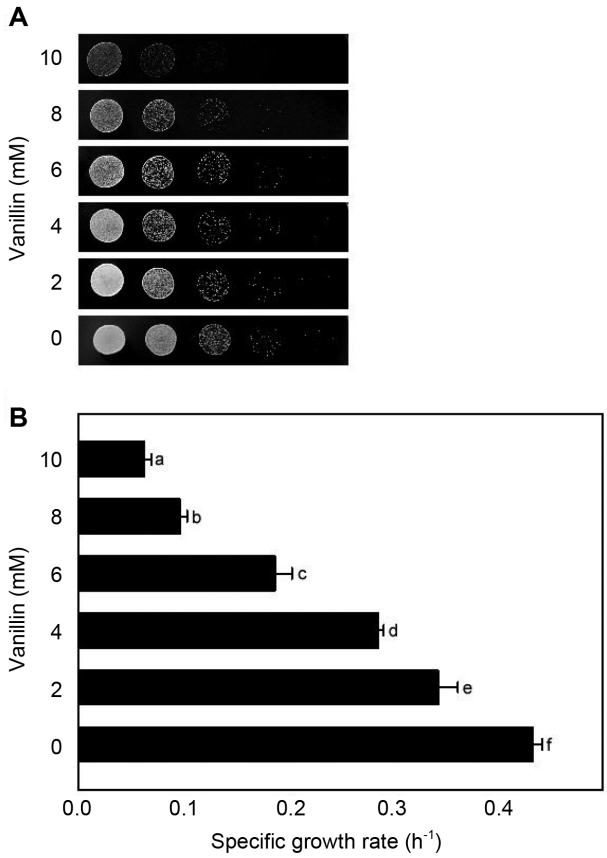

Fig. 1. Plate growth assay (**A**) and specific growth rate (**B**) of *S. cerevisiae* strain BY4742 in vanillin-containing media.
For plate growth assay, cells were grown in YEPD overnight and diluted to an OD_{600} of 0.2. Then, 5-fold serial dilutions were prepared, and 10 μl of each dilution was spotted onto a YEPD plate. The plates were incubated at 30°C for 2 days. Specific growth rates were determined at an exponential growth phase. Different letters in (**B**) indicate a significant difference between means.

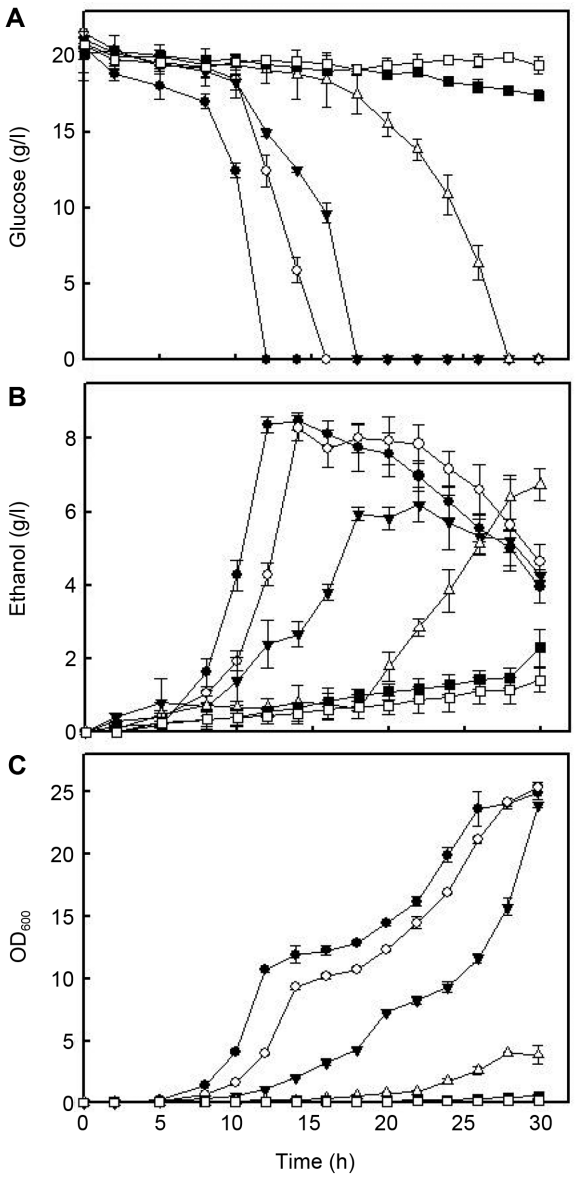

Fig. 2. Cell growth profiles (OD_{600}), glucose consumption, and ethanol production by *S. cerevisiae* BY4742.
Shake-flask cultures were grown in YEPD supplemented with 0 mM (●), 2 mM (○), 4 mM (▼), 6 mM (△), 8 mM (■), or 10 mM (□) vanillin. Averages and standard errors from three independent experiments are shown.

microarrays. Based on the comparison of statistically normalized test and control data from 5,744 probe sets for 5,845 genes present in *S. cerevisiae*, it was found that the expression of 273 genes was upregulated over 2-fold, whereas the expression of 205 genes was downregulated over 2-fold under vanillin stress (Table 2). Expression of the genes involved in the citrate cycle was significantly affected by vanillin (Fig. 3); 46% of the genes of the citrate cycle (such as *IRC15*, *CIT3*, *PYC1*, *SDH1*, *SDH2*, *LSC2*, and *CIT1*) were upregulated. However, the number of genes involved in glyoxylate and dicarboxylate metabolism whose expression was upregulated under vanillin stress was relatively small: out of 61 genes, only *ICL2*, *CIT3*, *MLS1*, and *CIT1* were upregulated. Microarray analysis also revealed that the expression of the glycogen phosphorylase 1 (*GPH1*) gene, involved in glycogen metabolism, was increased 5.4-fold. The expression of *GPH1* is known to be regulated by the stress-response element (STRE) [22] and Hog1p-mitogen-activated protein kinase-dependent pathway [34].

The expression of 24 genes involved in ribosome

Table 2. *S. cerevisiae* genes deregulated by vanillin.

Gene symbol	Gene title	Average log2 (fold change)
MCH2	Monocarboxylate permease	3.57 ± 0.83
SNG1	Protein involved in resistance to nitrosoguanidine and 6-azauracil	3.22 ± 0.77
TMA10	Unknown function	2.82 ± 0.51
HSP42	Small heat shock protein (sHSP) with chaperone activity	2.46 ± 0.54
GPH1	Glycogen phosphorylase required for the mobilization of glycogen	2.43 ± 0.47
TSL1	Large subunit of trehalose 6-phosphate synthase (Tps1p)/phosphatase (Tps2p) complex	2.30 ± 0.52
GPM2	Tetrameric phosphoglycerate mutase	2.26 ± 0.35
SDS24	APC/cyclosome regulation protein	2.19 ± 0.22
HXK1	Hexokinase isoenzyme 1	2.19 ± 0.35
NOP2	Probable RNA m5C methyltransferase	-1.00 ± 0.05
UTP18	Small-subunit processing some protein involved in pre-18S rRNA maturation	-1.00 ± 0.04
FUR1	Uracil phosphoribosyltransferase	-1.00 ± 0.04
SPR1	Sporulation-specific exo-1, 3-β-glucanase	-1.00 ± 0.05
HMT1	Nuclear SAM-dependent mono- and asymmetric methyltransferase	-1.00 ± 0.05
IPI1	Essential component of the Rix1 complex	-1.00 ± 0.03
IZH4	Membrane protein involved in zinc ion homeostasis, member of the four-protein IZH family	-1.00 ± 0.04
LOH1	Protein of unknown function with proposed roles in maintenance of genome integrity	-1.03 ± 0.04
PWP1	Protein with WD-40 repeats involved in rRNA processing	-1.03 ± 0.05

biogenesis, including *NOP2*, *UTP18*, *FUR1*, *SPR1*, *NOB1*, *NMD3*, *RNT1*, and *IMP3*, was notably downregulated under vanillin stress. Nop2p is an RNA methyltransferase, which plays a role in rRNA processing and large ribosomal subunit biogenesis [16]. Vanillin was reported to affect ribosome assembly [19], a process that also involves *NOP2*, *UTP18*, and *PWP1* [8]. Four genes involved in autophagy [21], *ATG3*, *ATG7*, *ATG8*, and *ATG13*, were slightly upregulated.

It is interesting to note that *MCH2*, *SNG1*, and *TMA10* were strongly upregulated under vanillin stress. Mch2p, a monocarboxylate transporter-homologous (MCH) family protein, is involved in the uptake or secretion of monocarboxylates such as lactate, pyruvate, and acetate across the plasma membrane [26]. However, the function of Mch2p in stress response has not been studied intensively. It might be involved in the transport of vanillin, a phenolic aldehyde that has aldehyde, hydroxyl, and ether functional groups [26]. *SNG1* has been previously described as a gene involved in nitrosoguanidine resistance [13]; its overexpression causes resistance to 6-azauracil in *S. cerevisiae* [12]. The expression of *TMA10*, which is increased under DNA-replication stress [10], was also upregulated by vanillin. Transcription of the *HSP42* gene, which encodes a cytosolic small heat shock protein with chaperone activity [15], was also increased. The *HSP42* transcript is detectable at the basal level under all culture conditions, but its expression is induced by stresses such as heat shock, salt shock, and starvation [15, 36]. This upregulation of *HSP42* transcription is mediated by the transcription factors Hsf1p and Msn2p/Msn4p that respectively bind to heat shock elements [2] and stress response elements in the *HSP42* promoter [35]. Semiquantitative polymerase chain reaction confirmed that the expression of the genes listed in Table 2 was significantly changed by vanillin (data not shown).

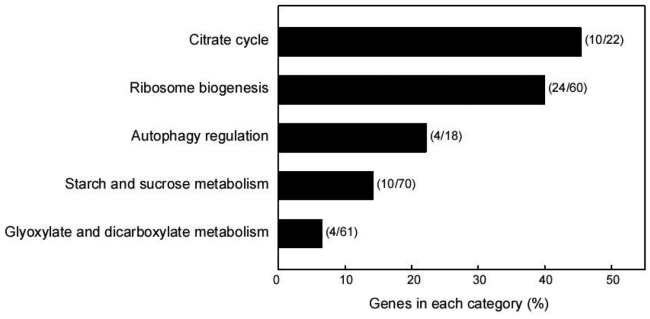

Fig. 3. Functional categories of the genes whose expression levels are deregulated over 2-fold by vanillin (4 mM).
The denominators and numerators in parentheses indicate the numbers of total genes and affected genes in each category, respectively.

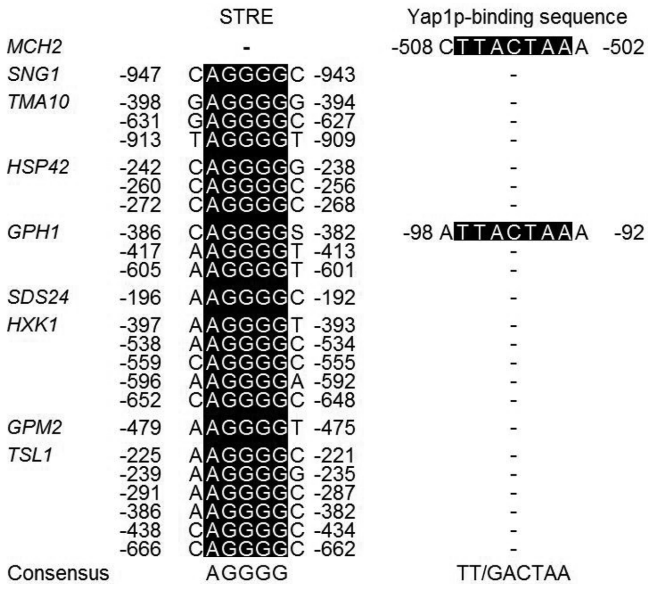

Fig. 4. Alignment of stress response elements (STRE) and Yap1p-binding sequence in the 5′-flanking regions of the genes regulated by vanillin in *S. cerevisiae*.

Conserved nucleotides are shaded in black. Numbers indicate nucleotide positions upstream of the translation initiation codon (ATG) of each gene.

Vanillin regulates gene expression in a Yap1p-dependent manner [30]. Sequence analysis indicated that the 5′-flanking region of *MCH2* contains one putative Yap1p-binding sequence (5′-TT/GACTAA-3′) [29], 508 nucleotides upstream of the initiation codon (Fig. 4). A putative Yap1p-binding sequence was also found 98 nucleotides upstream of the initiation codon of *GPH1* (Fig. 4). These observations suggest that the expression of *MCH2* and *GPH1* may be regulated in a Yap1p-dependent manner. Three putative STRE sequences were found in the 5′-flanking region of *GPH1*, but not in that of *MCH2*, suggesting that regulation of *GPH1* expression is more complex. An interesting finding of this study is that the expression of genes that have no Yap1p-binding sequences in their 5′-flanking regions was strongly induced by vanillin. It cannot be excluded that other transcription factors such as Msn2p/Msn4p related to the STRE-mediated pathway play a crucial role in vanillin stress. Identification of the transcription factors and their roles would also be important for understanding the response of *S. cerevisiae* to other stresses.

Phenotypes of Mutant *S. cerevisiae* Strains

The role of the genes whose expression was affected by vanillin stress was examined in a plate growth assay using mutant strains of *S. cerevisiae* BY4742 (Fig. 5). Deletion of *MCH2*, *SNG1*, or *GPH1*, but not of any other genes listed in Table 1, resulted in notable growth defects at an elevated vanillin concentration (8 mM). These data suggest that the elevated expression of *MCH2*, *SNG1*, and *GPH1* under vanillin stress may be required for the response to vanillin. Deletion of the genes whose expression was downregulated under vanillin stress did not result in a notable change in growth of *S. cerevisiae* strains either in a haploid or a diploid genetic background (data not shown). These results warrant further studies to unveil the genetic regulation underlying the vanillin stress response in *S. cerevisiae*.

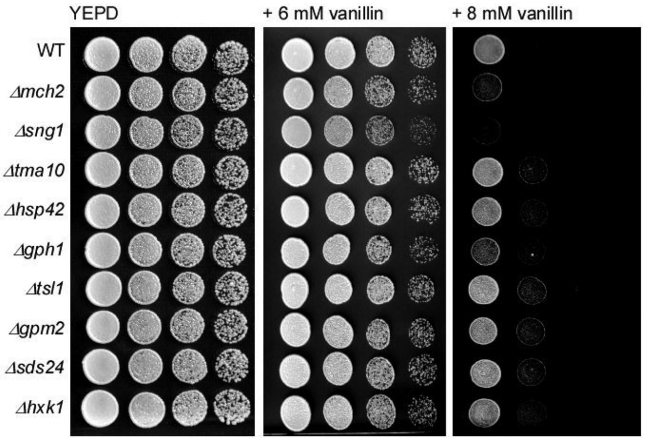

Fig. 5. Growth phenotypes of *S. cerevisiae* strains on vanillin-containing medium.

S. cerevisiae strains with the indicated mutation in a haploid genetic background were used for the growth assay. Cells were grown to mid-log phase in YEPD and diluted to an OD_{600} of 0.2 with the same medium. Then, 5-fold serial dilutions were spotted onto a YEPD plate and cells were incubated at 30°C for 3 days.

In conclusion, the expression of genes involved in a wide range of cellular processes was affected by vanillin. The results of this study will be useful for developing more stress-tolerant *S. cerevisiae* strains for bioethanol fermentation.

Acknowledgments

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