Appl Biochem Biotechnol (2010) 161:106–115
DOI 10.1007/s12010-009-8811-9

Identification of *Saccharomyces cerevisiae* Genes Involved in the Resistance to Phenolic Fermentation Inhibitors

Linda Sundström · Simona Larsson · Leif J. Jönsson

Received: 21 May 2009 / Accepted: 2 October 2009 /
Published online: 22 October 2009

Abstract *Saccharomyces cerevisiae* was exposed to inhibitory concentrations of the three phenolic phenylpropanoids: coniferyl aldehyde, ferulic acid, and isoeugenol. Deoxyribonucleic acid microarray analysis was employed as one approach to generate a set of candidate genes for deletion mutant analysis to determine the potential contribution of the corresponding gene products to the resistance against toxic concentrations of phenolic fermentation inhibitors. Three *S. cerevisiae* deletion mutants with increased sensitivity to coniferyl aldehyde were identified: *yap1Δ*, *atr1Δ*, and *flr1Δ*. The rate of reduction of coniferyl aldehyde to coniferyl alcohol decreased sixfold when the gene encoding the transcriptional activator Yap1p was deleted, and threefold when the Yap1p-controlled genes encoding Atr1p and Flr1p were deleted. Growth, glucose consumption, and ethanol formation progressed after a lag phase during which coniferyl aldehyde reduction and coniferyl alcohol formation occurred. The results link *ATR1*, *FLR1*, and *YAP1* by their ability to confer resistance to coniferyl aldehyde and show that deletion of any of these three genes impairs the ability of *S. cerevisiae* to withstand coniferyl aldehyde and detoxify it by reduction. Furthermore, the results suggest that overexpression of *ATR1*, *FLR1*, and *YAP1* is of interest for the construction of novel yeast strains with improved resistance against inhibitors in lignocellulose hydrolysates.

Keywords Liquid biofuel · Cellulosic ethanol · Fermentation inhibitors · *Saccharomyces cerevisiae* · Hyperresistance

Linda Sundström, née Björklund.

L. Sundström
Applied Microbiology, Lund University, SE-221 00 Lund, Sweden

S. Larsson
Faculty of Civil Engineering, Riga Technical University, Riga LV 10483, Latvia

L. J. Jönsson (✉)
Department of Chemistry, Umeå University, SE-901 87 Umeå, Sweden
e-mail: Leif.Jonsson@chem.umu.se

Present Address:
L. Sundström
AstraZeneca R&D Mölndal, SE-431 83 Mölndal, Sweden

Introduction

The yeast *Saccharomyces cerevisiae* is used extensively as a model system for eukaryotic cells and for industrial applications such as baking, brewing, wine making, and the production of industrial ethanol [1]. In both natural and industrial environments, *S. cerevisiae* is exposed to various lignocellulose-derived aromatic compounds, which may be inhibitory in high concentrations [2]. Many lignocellulose-related aromatic compounds are phenylpropanoids with a structure related to the basic building blocks of lignin. Lignin is formed mainly from the three phenylpropanoid precursors: coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol [3].

Lignocellulose is an abundant raw material that has a potential for utilization as a feedstock in industrial production of ethanol and other biofuels [4, 5]. However, the pretreatment required for efficient conversion of lignocellulose polysaccharides into fermentable sugars also results in by-product formation [6, 7]. When *S. cerevisiae* ferments lignocellulose hydrolysates, it encounters a wide variety of inhibitory phenolic compounds [2, 8]. The inhibitory effects of phenolic fermentation inhibitors have been demonstrated in experiments with hydrolysates from willow, softwood, and sugarcane bagasse [9–11].

With respect to phenylpropane derivatives, coniferyl alcohol is less toxic to *S. cerevisiae* than corresponding oxidized and reduced forms, such as ferulic acid, coniferyl aldehyde, and isoeugenol [2]. Heterologous expression of the phenol oxidase laccase and the overexpression of phenylacrylic acid decarboxylase result in transformants with increased resistance to phenolic compounds [12, 13].

The objective of this work was to identify proteins involved in the resistance to phenylpropane derivatives, particularly coniferyl aldehyde, which has been identified as a potent inhibitor of ethanolic fermentation of lignocellulose hydrolysates [2, 12]. Addition of toxic compounds to yeast cultures would trigger an enhanced messenger ribonucleic acid (mRNA) expression of genes encoding proteins involved in resistance. This was explored by using genome-wide expression analysis. Selected genes from the DNA microarray experiments and other genes of interest were investigated by deletion mutant analysis. By using this approach, three deletion mutants with increased susceptibility to coniferyl aldehyde were identified. Identification of genes involved in the resistance to inhibitors makes it possible to use genetic engineering to develop novel yeast strains with improved ability to ferment lignocellulose hydrolysates and contributes to knowledge of the function of the corresponding proteins.

Materials and Methods

Microbial Strains and Fermentation Procedures

All cultivations were performed at pH 5.5 and 30°C. Commercial baker's yeast (Jästbolaget AB, Rotebro, Sweden) was used. Inocula were prepared in two stages. The cells were inoculated from Yeast Peptone Dextrose agar plates [14] and grown with shaking overnight in 10 mL synthetic medium [15]. The cells were harvested by centrifugation at 1,200×*g* and 4°C for 10 min, washed with 0.9% (w/v) sodium chloride, and transferred to baffled shake flasks containing 250 mL of the same synthetic medium. When the cultures reached the late exponential phase (OD_{600} of 5.0–5.5), the cells were harvested and used as inoculum for the fermentors.

Fermentor vessels (1 L; Belach AB, Stockholm, Sweden) containing 800 mL of the synthetic medium were used for batch fermentations. Either coniferyl aldehyde or ferulic acid or isoeugenol was added to a final concentration of 1 mM. The conditions were selected based on previous studies [2]. All chemicals were of analytical grade and obtained from Fluka (Buchs, Switzerland), unless otherwise stated. Control fermentation without any of the phenylpropane derivatives added to the medium was also performed. Nitrogen gas (0.20 L/min) was flushed through the medium. The inoculum was added to a final OD of 5.0 corresponding to a dry weight of 1.5 g/L. To minimize the evaporation of the ethanol produced, the exhaust gas was passed through a reflux cooler. The pH was maintained at 5.5 by using 3 M sodium hydroxide and 3 M sulfuric acid. The stirring speed was 250 rpm. Samples for expression analysis (75 mL) were withdrawn after 1 and 2 h of cultivation. These samples were chilled on ice and the cells were then harvested by centrifugation at 1,200×*g* and 4°C for 15 min. The cells were washed once with 50 mL ice-cold water. Thereafter, the cells were washed twice, first with 25 mL and then with 10 mL ice-cold 50 mM sodium acetate buffer, pH 5.4, containing 10 mM ethylenediaminetetraacetic acid (EDTA). The final supernatant was decanted and the cells were flash-frozen in liquid nitrogen and stored at −80°C until the RNA purification was performed. Substrate consumption, product formation, and biomass concentration were monitored during the fermentation.

Twenty-six deletion mutants (Table 1) were studied in small-scale fermentations. The deletion mutants were obtained from European *Saccharomyces Cerevisiae* Archive for Functional Analysis (Institute for Microbiology, Johann Wolfgang Goethe University, Frankfurt, Germany) and were derivatives of the strain BY4741 (*MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*). Cells for inoculation were grown to the late exponential phase (OD_{620} of 4–10) and were harvested by centrifugation at 6,000×*g* and 4°C for 5 min. The cells were washed once with 0.9% (w/v) sodium chloride. The inoculum was added to an OD of 0.5. The volume of the cultures was 20 mL and synthetic medium [15] supplemented with either 1.0 mM coniferyl aldehyde, or 1.5 mM ferulic acid, or 1.5 mM isoeugenol was used. Control fermentations in synthetic medium were included for all deletion mutants and BY4741. The cultivations were performed for 24 h at 30°C in 25-mL glass vessels, sealed with rubber stoppers, and equipped with cannulas for carbon dioxide removal, and with constant stirring. Samples (1.0 mL) for analysis of OD, glucose, ethanol, coniferyl aldehyde, and coniferyl alcohol were taken at the beginning of the fermentations and after 4, 8, 12, and 24 h.

Analyses

The OD was measured spectrophotometrically at 620 nm (U-2000, Hitachi, Tokyo, Japan). Glucose and ethanol were determined by using a High-performance liquid chromatography (HPLC) system (Gilson Inc., Middleton, WI, USA) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) using a refractive index detector (RID-6A, Shimadzu, Kyoto, Japan). The separation was performed at 45°C using 5 mM sulfuric acid with a flow rate of 0.6 mL/min as the mobile phase. The consumption of coniferyl aldehyde and the formation of coniferyl alcohol were determined using an HPLC system (Waters, Milford, MA, USA) equipped with a BioSil-C18 column (Bio-Rad) and a UV detector (2487, Waters) set on a wavelength of 254 nm and operating at ambient temperature (approximately 25°C). The mobile phase consisted of 40% aqueous methanol, adjusted to pH 3 with concentrated hydrochloric acid, at a flow rate of 0.40 mL/min.

Table 1 Set of genes tested in deletion mutant experiments and results from the DNA microarray analysis[a].

ORF	Gene	Description[b]	Coniferyl aldehyde	
			1 h	2 h
YAL005C[d]	*SSA1*	Heat shock protein of HSP70 family, cytosolic	−2.0	n.c.
YBR008C[c]	*FLR1*	Putative H^+ antiporter regulated by Yap1p and involved in multidrug resistance	9.1	18
YBR244W	*GPX2*	Glutathione peroxidase	3.5	3.2
YCL026C		*YCL026Ca* FRM2 protein involved in fatty acid regulation, *YCL026Cb* protein of unknown function localized to cytoplasma and nucleus	8.2	9.6
YCR102C		Similarity to zinc-type alcohol dehydrogenase *Cochliobolus carbonum* toxD gene	34	22
YDL243C	*AAD4*	Aryl-alcohol dehydrogenase involved in the oxidative stress response	18	6.0
YDR011W	*SNQ2*	"Full size" ABC transporter involved in multidrug resistance	4.0	4.2
YDR135C	*YCF1*	Vacuolar "full size" ABC transporter responsible for vacuolar sequestration of glutathione S-conjugate transporter	3.3	2.3
YFL056C[c]	*AAD6*	Putative aryl-alcohol dehydrogenase, involved in the oxidative stress response	~6.9	~3.6
YGR192C	*TDH3*	Glyceraldehyde-3-phosphate dehydrogenase 3	~−26	n.c.
YGR213C	*RTA1*	Integral membrane protein involved in 7-aminocholesterol resistance	6.3	~3.4
YHR048W		Putative mediator of drug efflux	6.4	3.8
YHR179W[c]	*OYE2*	NADPH dehydrogenase (old yellow enzyme), isoform 1	n.c.	n.c.
YKL071W[c]		Protein of unknown function localized to cytoplasm	76	34
YLL025W[d]		Strong similarity to members of the Srp1p/Tip1p family	n.c.	n.c.
YLL056C		Weak similarity to *Y. pseudotuberculosis* CDP-3,6-dideoxy-D-glycero-L-glycero-4-hexulose-5-epimerase	30	26
YLL057C	*JLP1*	DnaJ-like protein 1	5.3	~2.0
YLL060C[c]	*GTT2*	Glutathione S-transferase	23	11
YLR346C		Protein of unknown function localized to mitochondria	20	9.7
YML007W	*YAP1*	Transcriptional activator involved in oxidative stress response	2.6	n.c.
YML116W[c]	*ATR1*	Putative substrate-H^+ antiporter conferring resistance to aminotriazole	3.2	3.9
YML131W[c]		Putative hydroxydehydrogenase	5.5	5.5
YPL091W	*GLR1*	Glutathione reductase (NADPH)	2.2	n.c.
YPL171C[c]	*OYE3*	NADPH dehydrogenase (old yellow enzyme), isoform 3	21	15
YPR125W	*YLH47*	Mitochondrial inner membrane protein	~30	n.c.
YPR200C	*ARR2*	Arsenate reductase	6.5	3.4

NADPH Nicotinamide adenine dinucleotide phosphate

[a] The increase or decrease in expression after 1 and 2 h of fermentation in the presence of coniferyl aldehyde is indicated. The values preceded by "~" are considered less confident due to low signal for the control sample (taken from a culture to which no inhibitory aromatic compound was added). No change or a change that was not considered sufficiently confident (<2 times) is indicated by "n.c."

[b] Descriptions from the MIPS Comprehensive Yeast Genome Database [33]

[c] Genes under control of *YAP1* [20]

[d] Increased expression only in the presence of ferulic acid and isoeugenol

Preparation of Nucleic Acids

Total RNA was prepared from the harvested cells (75 mL culture with an OD≈5) using a hot phenol protocol [16]. PolyA$^+$ mRNA was isolated from 0.5 mg of total RNA using the Oligotex oligo (dT) method (Qiagen, Hilden, Germany). The polyA$^+$ mRNA was concentrated by precipitation at −20°C after addition of 0.1 volumes of 3.0 M sodium acetate, pH 5.2, and 2.6 volumes of 95% ethanol. Double-stranded complementary DNA was synthesized from 2.5 to 4.5 μg polyA$^+$ mRNA with the SuperScript Choice System (Gibco BRL Life Technologies, Gaithersburg, MD, USA) and using an HPLC-purified T7-$(dT)_{24}$ primer (5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$-3') (Genset, Paris, France) for the first strand synthesis. In vitro transcription and biotin labeling was performed using the Enzo BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY, USA). The biotin-labeled cRNA was purified using RNeasy spin columns (Qiagen) and then fragmented at 94°C for 35 min.

Hybridization with DNA Microarrays

The hybridization was performed according to protocols supplied by the manufacturer of the DNA microarrays (Affymetrix). The samples for hybridization consisted of 50 ng/μL fragmented cRNA, 50 pM control oligonucleotide B2 (Eukaryotic Hybridization Control Kit, Affymetrix, Santa Clara, CA, USA), control cRNA cocktail containing staggered concentrations of *BioB*, *BioC*, *BioD*, and *cre* (1.5, 5, 25 and 100 pM, respectively; Eukaryotic Hybridization Control Kit, Affymetrix, Santa Clara, CA, USA), 0.1 mg/mL herring sperm DNA, 0.5 mg/mL acetylated BSA, 100 mM MES-NaOH, pH 6.5, 20 mM EDTA, 0.89 M sodium chloride, and 0.01% (v/v) Tween 20. The hybridization was performed using Yeast Genome S98 Arrays and a GeneChip Hybridization Oven 320, as described by the manufacturer (Affymetrix). A GeneChip Fluidics Station 400 was used for washing and staining and an HP GeneArray Scanner was used for scanning (both from Affymetrix). The microarray data were processed using the Microarray Suite software, version 4.0 (Affymetrix). Comparisons between the control fermentation and the three fermentations with coniferyl aldehyde, ferulic acid, or isoeugenol added to the medium were performed for samples taken after both 1 and 2 h.

Results

Coniferyl aldehyde was completely consumed during the cultivation of yeast cells for microarray analysis (Fig. 1). The yield of coniferyl alcohol was 0.44 g per gram consumed coniferyl aldehyde. Only a slight decrease in the concentrations of ferulic acid and isoeugenol occurred.

After a 1-h exposure to 1 mM coniferyl aldehyde, 11 open reading frames (ORFs) were induced tenfold or more (*YCR102C*, *YDL243C*, *YFL057C*, *YKL070W*, *YKL071W*, *YKL086W*, *YLL056C*, *YLL060C*, *YLR346C*, *YPL171C*, and *YPR125W*). After 2 h, seven ORFs displayed tenfold or higher expression level than the control (*YBR008C*, *YCR102C*, *YFL057C*, *YKL071W*, *YLL056C*, *YLL060C*, and *YPL171C*).

In the presence of 1 mM ferulic acid, three ORFs (*YAL005C*, *YER153C*, and *YPR125W*) displayed a tenfold or higher increase in expression level after 1 h. No ORFs showed that high an increase after 2 h. In the presence of 1 mM isoeugenol, the expression level of two ORFs (*YLL025W* and *YPR125W*) increased more than tenfold after 1 h. After 2 h with

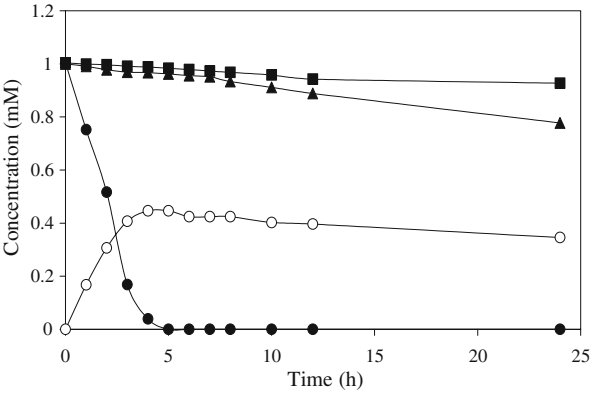

Fig. 1 The concentration of coniferyl aldehyde (*filled circle*), ferulic acid (*filled square*), and isoeugenol (*filled triangle*) in separate *S. cerevisiae* cultures used for taking samples for DNA microarray analyses. The concentration of coniferyl alcohol (*blank circle*) in the culture with coniferyl aldehyde is also shown

isoeugenol in the medium, no ORFs showing tenfold or higher increase in expression level were detected.

A set of 26 deletion mutants were selected (Table 1), in part on basis of the results from the DNA microarray study. Since several ORFs that were highly induced by coniferyl aldehyde are regulated by Yap1p, the ORF corresponding to Yap1p, *YML007W*, and additional ORFs under the control of Yap1p were included among the mutants. Further deletion mutants were selected on basis of involvement in multidrug resistance and detoxification. *YGR192C* was selected due to that it showed a very large decrease in the presence of coniferyl aldehyde. Other ORFs were selected since there was an increase after addition of more than one of the aromatic compounds or a high increase at both time points for the same aromatic compound.

The mutants displayed similar growth rate, glucose consumption rate, and ethanol formation rate as the control strain (BY4741) in medium without inhibitor added (data not shown). Three of the 26 deletion mutants, namely *yap1Δ*, *atr1Δ*, and *flr1Δ*, showed little or no growth in the presence of coniferyl aldehyde (Fig. 2). In addition, deletion of any of these three genes resulted in a very clear decrease in the consumption of glucose and formation of ethanol when coniferyl aldehyde was present (Fig. 2) and also led to a decrease in the rate of conversion of coniferyl aldehyde and the formation of coniferyl alcohol (Fig. 3, Table 2). Even though the conversion rate was threefold lower for *atr1Δ* and *flr1Δ* than for the control, nearly all coniferyl aldehyde was consumed after 24 h. For *yap1Δ*, the coniferyl aldehyde consumption rate was sixfold lower and more than half of the initial concentration remained even after 24 h (Fig. 3).

Six mutants with *YAL005C*, *YLL025W*, *YPR125W*, *YML007W*, *YML116W*, or *YBR008C* deleted were cultivated in the presence of 1.5 mM ferulic acid or 1.5 mM isoeugenol. However, none of the mutants showed any difference compared with the control under these conditions (not shown).

Discussion

The changes in the genome-wide expression pattern of an industrial strain of *S. cerevisiae* induced by three inhibitory phenylpropanoids, coniferyl aldehyde, ferulic acid, and isoeugenol, were used as one approach to generate a set of candidate genes for deletion mutant analysis. The same yeast strain has also been used to investigate the inhibitory effects of aliphatic acids [17], furan aldehydes [17], and aromatic compounds [2].

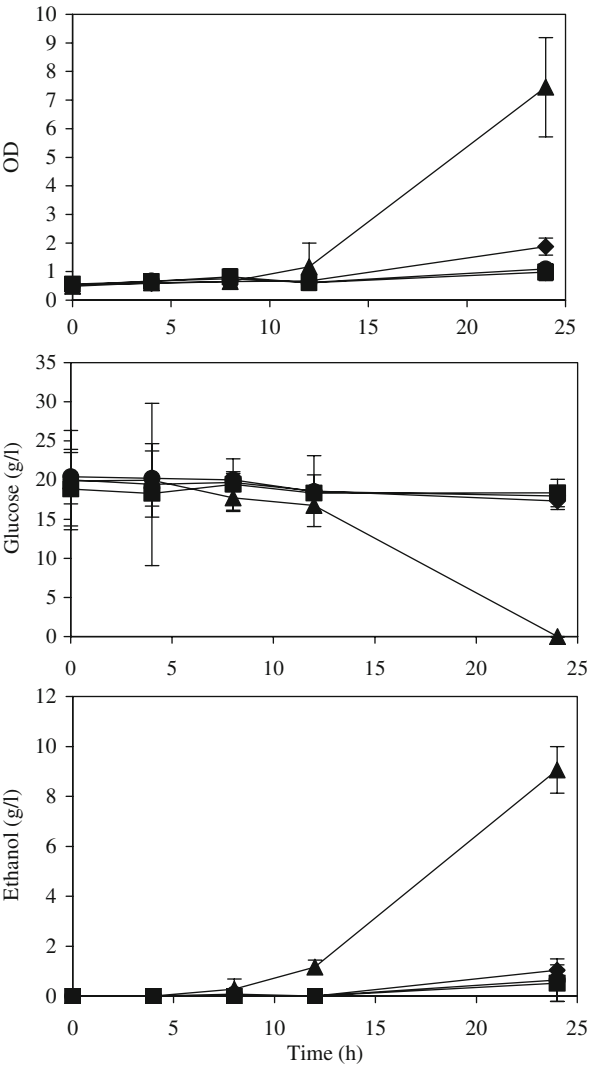

Fig. 2 Performance of the control strain (BY4741; *filled triangle*) and the *atr1Δ* (*filled diamond*), *flr1Δ* (*filled circle*), and *yap1Δ* (*filled square*) mutants in medium containing 1 mM coniferyl aldehyde (**a** cell growth; **b** glucose consumption; **c** ethanol formation). Mean values are based on two independent experiments

Coniferyl aldehyde was rapidly metabolized. *S. cerevisiae* reduces coniferyl aldehyde to coniferyl alcohol and dihydroconiferyl alcohol [2]. The identification of coniferyl alcohol as a major product from coniferyl aldehyde (Fig. 3) is consistent with previous findings. Coniferyl aldehyde increased the mRNA expression level of 11 ORFs more than tenfold in the 1-h sample. The concentration of coniferyl aldehyde added was sufficient to slightly delay growth and lower the initial ethanol productivity. The results from the DNA microarray experiments contributed to the selection of a set of candidate genes for analysis by using deletion mutants.

Among the 26 deletion mutants that were cultivated in the presence of 1 mM coniferyl aldehyde, *yap1Δ* showed decreased growth, glucose consumption, and ethanol formation as well as a major decrease in the rates of coniferyl aldehyde conversion and coniferyl alcohol formation. Yap1p is an extensively studied transcriptional activator protein involved in oxidative stress response and multidrug resistance and controls the expression of at least

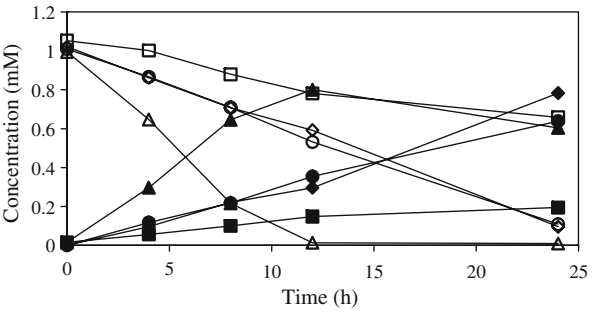

Fig. 3 Consumption of coniferyl aldehyde in fermentations with the control strain (BY4741; blank *triangle*), as well as the *atr1Δ* (*blank diamond*), *flr1Δ* (*blank circle*), and *yap1Δ* (*blank square*) mutants. The formation of coniferyl alcohol is shown with *filled symbols*

32 proteins [18, 19]. An increase (2.6 times) in the expression level of the gene encoding Yap1p was observed after 1 h when coniferyl aldehyde was present. The *yap1Δ* mutant was selected for analysis because of this increase but also because some genes known to be regulated by Yap1p were expressed at high levels when coniferyl aldehyde was present.

Both *ATR1* and *FLR1* are induced by overexpression of Yap1p [20]. Inclusion of 1 mM coniferyl aldehyde resulted in a 3.2-fold and 3.9-fold increase in the expression level of the gene encoding Atr1p after 1 and 2 h, respectively. The expression level of the gene encoding Flr1p increased 9.1-fold after 1 h and 18-fold after 2 h. One Yap1p-binding site (TTACTAA or TGACTAA) has been found upstream of the gene coding for Atr1p while the gene coding for Flr1p is preceded by three such sites. Atr1p and Flr1p encode plasma membrane proteins belonging to the drug:H^+ antiporters of the major facilitator superfamily and are involved in stress response and multidrug resistance [21–24]. Atr1p confers resistance to 3-amino-1,2,4-triazole, a herbicide, and both Atr1p and Yap1p contribute to Sn^{2+}-resistance [25]. Flr1p confers resistance to a range of drugs and chemicals, e.g., benomyl, fluconazole, and mancozeb [23, 26–28]. The *atr1Δ* and *flr1Δ* mutants showed decreased growth, glucose consumption, ethanol formation, and conversion rate of coniferyl aldehyde, but to a lesser extent than the *yap1Δ* mutant. Tenreiro et al. [26] found that deletion of the gene coding for Yap1p had a stronger effect on the growth under benomyl stress than the deletion of the gene coding for Flr1p. A likely explanation for these observations, both with respect to coniferyl aldehyde and benomyl, is that Yap1p stimulates expression of several proteins engaged in the resistance of the cell to these toxic compounds and that Flr1p is only one of these proteins. *FLR1* has been shown to be regulated not only by Yap1p, but also by Yrr1p and Pdr3p [27, 29]. Flr1p has been localized to the cytoplasmic membrane [30], and Flr1p as well as Atr1p have been suggested to act as efflux pumps eliminating toxic substances from the cell by facilitating their transfer across the membrane [30–32]. Flr1p has been given the dual role of being a major facilitator and a determinant of resistance to oxidants [23] and there are hypotheses that major facilitator

Table 2 Maximum conversion rate of coniferyl aldehyde and formation rate of coniferyl alcohol.

Deletion mutant	Coniferyl aldehyde, mM/h[a]	Coniferyl alcohol, mM/h[a]
Control[b]	0.097±0.009	0.075±0.005
atr1Δ	0.038±0.007	0.033±0.004
flr1Δ	0.038±0.0003	0.027±0.004
yap1Δ	0.017±0.005	0.008±0.001

[a] The standard deviations are indicated

[b] *S. cerevisiae* BY4741

transporters involved in multidrug resistance could have natural substrates [reviewed in [24]]. For both the *atr1Δ* and the *flr1Δ* mutants, the rate of the reduction was less than half of the original (Table 2). This might reflect a general decrease in metabolic activity due to poorer ability to withstand the coniferyl aldehyde, but the detailed mechanism behind the reduction of coniferyl aldehyde remains to be elucidated.

The selection of deletion mutants was mainly based on higher expression levels in the presence of coniferyl aldehyde. Most of the deletion mutants tested (23/26) did not show any difference compared to the control when cultivated in the presence of 1 mM coniferyl aldehyde. Some of these could have been expressed at higher levels due to the induction of transcriptional activators, such as Yap1p [18, 20]. For instance, *YKL071W* showed a 76-fold increased expression after 1 h of fermentation in the presence of coniferyl aldehyde, but no negative effects were observed in the deletion mutant experiment. Upstream of this ORF are five Yap1p-binding sites, which might explain the high expression level. Also the ORFs *YLL060C*, *YPL171C*, *YFL056C*, and *YHR179W* have Yap1p-binding sites upstream of the start codon. The results show that although the presence of coniferyl aldehyde leads to higher expression levels of many genes controlled by Yap1p, only few of the corresponding gene products are useful for the resistance of the cell to this toxic compound. Thus, although a large number of ORFs with increased expression in the presence of coniferyl aldehyde were identified using DNA microarray analysis, deletion of most of them resulted in no detectable difference with regard to resistance. This means that high expression levels observed with the DNA microarray analysis was not sufficient for identification of involvement in resistance. This result is not surprising considering that a toxic compound might trigger a general stress response and that few of the gene products induced may be dedicated to resistance to that specific compound. Despite that changes in the expression levels induced by a specific toxic compound would not serve as evidence for the involvement of the corresponding gene products in the resistance to that particular compound, DNA microarray analysis can still be employed to provide candidate genes to assay using other techniques.

The deletion mutant analysis revealed that Yap1p, Atr1p, and Flr1p contribute to the cellular defense against the naturally occurring compound coniferyl aldehyde. This is the first study in which specific *S. cerevisiae* proteins have been shown to be involved in the resistance to coniferyl aldehyde. The deletion of the gene coding for the transcriptional activator Yap1p affected the resistance of the cell more severely than deletion of the genes coding for Atr1p or Flr1p, the expression of which are controlled by Yap1p. The results should be useful for construction of hyperresistant yeast strains for production of liquid biofuels and other products from lignocellulose hydrolysates. Further studies of the role of Yap1p, Atr1p, and Flr1p in the resistance to inhibitory compounds and lignocellulose hydrolysates are underway.

Acknowledgements We thank Prof. Carl A. K. Borrebaeck for providing facilities for DNA microarray analysis. This work was supported by grants from The Swedish National Energy Administration.

References

1. Borneman, A. R., Chambers, P. J., & Pretorius, I. S. (2007). *Trends in Biotechnology, 25*, 349–355.
2. Larsson, S., Quintana-Sáinz, A., Reimann, A., Nilvebrant, N.-O., & Jönsson, L. J. (2000). *Applied Biochemistry and Biotechnology, 84–86*, 617–632.
3. Ralph, J., Lundquist, K., Brunow, G., Lu, F., Kim, H., Schatz, P. F., et al. (2004). *Phytochemistry Reviews, 3*, 29–60.

4. Ragauskas, A. J., Williams, C. K., Davison, B. H., et al. (2006). *Science, 311*, 484–489.
5. Lynd, L. R., Laser, M. S., Bransby, D., Dale, B. E., Davison, B., Hamilton, R., et al. (2008). *Nature Biotechnology, 26*, 169–172.
6. Wyman, C. E. (2007). *Trends in Biotechnology, 25*, 153–157.
7. Galbe, M., & Zacchi, G. (2007). *Advances in Biochemical Engineering, Biotechnology, 108*, 41–65.
8. Ando, S., Arai, I., Kiyoto, K., & Hanai, S. (1986). *Journal of Fermentation Technology, 64*, 567–570.
9. Jönsson, L. J., Palmqvist, E., Nilvebrant, N.-O., & Hahn-Hägerdal, B. (1998). *Applied Microbiology and Biotechnology, 49*, 691–697.
10. Larsson, S., Reimann, A., Nilvebrant, N.-O., & Jönsson, L. J. (1999). *Applied Biochemistry and Biotechnology, 77–79*, 91–103.
11. Martín, C., Galbe, M., Wahlbom, C. F., Hahn-Hägerdal, B., & Jönsson, L. J. (2002). *Enzyme and Microbial Technology, 31*, 274–282.
12. Larsson, S., Cassland, P., & Jönsson, L. J. (2001). *Applied and Environmental Microbiology, 67*, 1163–1170.
13. Larsson, S., Nilvebrant, N.-O., & Jönsson, L. J. (2001). *Applied Microbiology and Biotechnology, 57*, 167–174.
14. Sherman, F. (1991). *Methods in Enzymology, 194*, 3–21.
15. Verduyn, C., Postma, E., Scheffers, W. A., & van Dijken, J. P. (1992). *Yeast, 8*, 501–517.
16. Schmitt, M. E., Brown, T. A., & Trumpower, B. L. (1990). *Nucleic Acids Research, 18*, 3091–3092.
17. Larsson, S., Palmqvist, E., Hahn-Hägerdal, B., Tengborg, C., Stenberg, K., Zacchi, G., et al. (1999). *Enzyme and Microbial Technology, 24*, 151–159.
18. Lee, J., Godon, C., Lagniel, G., Spector, D., Garin, J., Labarre, J., et al. (1999). *Journal of Biological Chemistry, 274*, 16040–16046.
19. Rodrigues-Pousada, C. A., Nevitt, T., Menezes, R., Azevedo, D., Pereira, J., & Amaral, C. (2004). *FEBS Letters, 567*, 80–85.
20. DeRisi, J. L., Iyer, V. R., & Brown, P. O. (1997). *Science, 278*, 680–686.
21. André, B. (1995). *Yeast, 11*, 1575–1611.
22. Coleman, S. T., Tseng, E., & Moye-Rowley, W. S. (1997). *Journal of Biological Chemistry, 272*, 23224–23230.
23. Nguyen, D.-T., Alarco, A.-M., & Raymond, M. (2001). *Journal of Biological Chemistry, 276*, 1138–1145.
24. Sá-Correira, I., dos Santos, S. C., Teixeira, M. C., Cabrito, T. R., & Mira, N. P. (2009). *Trends in Microbiology, 17*, 22–31.
25. Viau, C., Pungartnik, C., Schmitt, M. C., Basso, T. S., Henriques, J. A., & Brendel, M. (2006). *BioMetals, 19*, 705–714.
26. Tenreiro, S., Fernandes, A. R., & Sá-Correia, I. (2001). *Biochemical and Biophysical Research Communications, 280*, 216–222.
27. Brôco, N., Tenreiro, S., Viegas, C. A., & Sá-Correia, I. (1999). *Yeast, 15*, 1595–1608.
28. Teixeira, M. C., Dias, P. J., Simões, T., & Sá-Correira, I. (2008). *Biochemical and Biophysical Research Communications, 367*, 249–255.
29. Le Crom, S., Devaux, F., Marc, P., Zhang, X., Moye-Rowley, W. S., & Jacq, C. (2002). *Molecular and Cellular Biology, 22*, 2642–2649.
30. Jungwirth, H., Wendler, F., Platzer, B., Bergler, H., & Högenauer, G. (2000). *European Journal of Biochemistry, 267*, 4809–4816.
31. Kanazawa, S., Driscoll, M., & Struhl, K. (1988). *Molecular and Cellular Biology, 8*, 664–673.
32. Oskouian, B., & Saba, J. D. (1999). *Molecular and General Genetics, 261*, 346–353.
33. Mewes, H. W., Frishman, D., Gruber, C., Geier, B., Haase, D., Kaps, A., et al. (2000). *Nucleic Acids Research, 28*, 37–40.