

# Genetic Basis for Osmosensitivity and Genetic Instability of the Wine Yeast *Saccharomyces cerevisiae* VIN7

Daniel J. Erasmus<sup>1,2</sup> and Hennie J.J. van Vuuren<sup>3\*</sup>

**Abstract:** *Saccharomyces cerevisiae* is exposed to sugar-induced osmotic stress in fermenting grape must. As part of the osmotic stress response, yeast strains display different phenotypes for acetic acid and glycerol formation in wine. We investigated why an osmosensitive industrial wine yeast, VIN7, produces high amounts of acetic acid and glycerol compared with the industrial wine yeast ST. Comparison of global gene expression patterns revealed that Hog1p and Msn2/Msn4p-regulated genes are expressed at higher levels in VIN7. Furthermore, data suggests that acetic acid production by wine yeast is the result of the reaction catalyzed by Ald6p and influenced by the pentose phosphate pathway and lipid biosynthesis. PCR analyses on the genome of VIN7 revealed that ~30 Kb on the left arm of chromosome XV, close to the telomere, is absent. Among the genes absent is YOL159C, a gene whose deletion causes osmosensitivity and an increase in Ty1 retrotranspositions, which may cause genetic instability in *S. cerevisiae*.

**Key words:** *Saccharomyces cerevisiae*, osmotic stress, wine, volatile acidity

During the alcoholic fermentation of grape must, *Saccharomyces cerevisiae* is exposed to multiple stresses, including osmotic shock, ethanol toxicity, nutrient availability, and temperature (Marks et al. 2008). Osmotic stress during wine fermentations is caused by the high sugar concentrations (16 to 26% w/v) of grape must (Margalit 1997). In some extreme cases such as icewine fermentations, the sugar concentration in the must is at least 35% (w/v) and can be as high as 50% (w/v). For the production of icewine, grape berries are left on the vine to freeze; the berries can only be harvested at temperatures equal to or below -8°C. This freezing process concentrates sugar and other grape constituents, resulting in must with a thick syrupy appearance. The high sugar concentration in icewine must results in the production of relatively high levels of glycerol and acetic acid by yeast (Erasmus et al. 2004). Acetic acid levels in icewines often exceed the legal limit in Canada (0.13% w/v; Canadian Food and Drug Act section B.02.101) resulting in financial losses to wineries.

The adaptation of *S. cerevisiae* to osmotic stress has been studied in great detail (for a review see Hohmann 2002). Yeast senses high osmolarity mainly through the

HOG MAP kinase cascade, resulting in a transcriptional response to allow for the production of glycerol as compatible solute. Glycerol formation prevents the efflux of water from the cell into the environment, thereby preventing dehydration of the yeast. The key enzyme in glycerol formation is a NADH-dependent glycerol-3-phosphate dehydrogenase, encoded by *GPD1* that converts dihydroxyacetone phosphate to glycerol-3-phosphate with the concomitant oxidation of NADH to NAD<sup>+</sup>. High levels of glycerol formation during osmotic stress lead to a redox imbalance in the yeast cell (NADH:NAD<sup>+</sup>). To compensate for this redox shift, it has been suggested that yeast may produce acetic acid as a redox sink to convert NAD<sup>+</sup> back to NADH (Blomberg and Adler 1989).

Wine yeasts produce acetic acid by the oxidation of acetaldehyde to acetate by NAD(P)<sup>+</sup>-dependent (acet)aldehyde dehydrogenases (Remize et al. 2000). *Saccharomyces cerevisiae* has five *ALD* isogenes that encode acetaldehyde dehydrogenases that possibly catalyze the oxidation of acetaldehyde to acetate: *ALD2*, *ALD3*, *ALD4*, *ALD5*, and *ALD6* (Navarro-Avino et al. 1999). Ald2p and Ald3p are both cytosolic and use NAD<sup>+</sup> as cofactor. During NaCl-induced osmotic stress, the NAD-dependent aldehyde dehydrogenase activity increases in wild-type cells but not in an *ald2ald3* mutant (Navarro-Avino et al. 1999). Furthermore, both *ALD2* and *ALD3* are regulated by the two general stress transcription factors Msn2/4p (Martinez-Pastor et al. 1996). Ald4p and Ald5p are located in mitochondria and require NAD(P)<sup>+</sup> and NADP<sup>+</sup>, respectively. Ald6p is located in the cytoplasm and is NADP<sup>+</sup>-dependent (Wang et al. 1998). For acetic acid to function as redox sink, *ALD2* and *ALD3* have to respond to the shift in redox balance caused by excess glycerol formation. Indeed, acetic acid formation has been linked to increased *ALD2* and *ALD3* mRNA and protein levels in response to the redox imbalance caused by glycerol formation (Bro et al. 2003). Furthermore, acetaldehyde,

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Acknowledgments: This research was supported by NSERC/AAFC/BCWI Grant 240132 to H.J.J.V.V.

The authors are grateful to Russ Morris of the University of British Columbia Media Group for preparing the artwork.

Manuscript submitted Jun 2008, revised Nov 2008, accepted Dec 2008

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the precursor of acetic acid, causes induction of *ALD2* and *ALD3* (Aranda and Del Olmo 2003). Since *S. cerevisiae* lacks transdehydrogenases (Bruinenberg et al. 1983), it has been assumed that the cytosolic NAD<sup>+</sup>-dependent genes *ALD2* and *ALD3* are mainly responsible for the formation of acetic acid during the fermentation of high-sugar grape musts. However, it has recently been shown that *ALD4* and *ALD6* are the major contributors of acetate formation during wine fermentations (Remize et al. 2000). *ALD2*, *ALD3*, *ALD4*, and *ALD6* are induced in response to sugar-induced osmotic stress (Erasmus et al. 2003). The current literature, therefore, is confusing and even contradictory as to which *ALD* genes are responsible for acetic acid formation.

Depending on wine style, acetic acid levels >0.7 g/L detract from wine quality (Lambrechts and Pretorius 2000). Therefore, it is important to use wine yeast strains that produce relatively low amounts of acetic acid, especially in high-sugar musts. We have previously identified two industrial wine yeast strains of *S. cerevisiae*, ST and VIN7, that have opposite phenotypes in a sugar-induced osmotic stress environment; ST grows faster than VIN7, but produces significantly less acetic acid and glycerol than VIN7 (Erasmus et al. 2004). ST and VIN7 seem to ferment 40% (w/v) sugar in a synthetic grape must at similar rates, but at lower sugar concentrations (20% w/v) VIN7 ferments at a faster rate than ST. The purpose of this study was to investigate the genetic basis for osmosensitivity and genetic instability of *S. cerevisiae* VIN7.

## Materials and Methods

**Media and yeast strains.** Riesling grape juice (Okanagan Valley, BC, Canada, 2000) containing ~22% (w/v) sugar (equimolar amounts of glucose and fructose), 112 mg/L free amino nitrogen, and 79 mg/L ammonia was used. Grape must was treated with 0.02 mL pectinase (Pec5L; Scott Laboratories, Petaluma, CA) for 3 hr at 40°C. Gelatine was added to a final concentration of 0.2 g/L and incubated at 7°C for 12 hr to precipitate particulate matter. The grape must was then filter-sterilized using a 0.22-µm filter (Millipore, Billerica, MA). Equimolar amounts of glucose and fructose were added to a portion of the grape juice (22% w/v sugar) to obtain a grape juice with 60% (w/v) sugar.

Synthetic grape must contained equimolar amounts of glucose and fructose at final concentrations of 20% or 60% (w/v sugar), 4.5 g/L L-malic acid, 0.3 g/L citric acid, 4.5 g/L tartaric acid, 2 g/L ammonium sulfate (515 mg/L N), 1.7 g/L yeast nitrogen base (without ammonium sulfate or amino acids), 1 mL/L Tween 80, and 5 mg/L oleic acid. The synthetic must was adjusted to pH 3.2 with 0.5 N KOH and filter-sterilized using a 0.22-µm filter (Millipore).

*Saccharomyces cerevisiae* strains ST (J. Laffort & Cie, Bordeaux, France) and VIN7 (Anchor Yeast, Industria, South Africa) were used. To rehydrate active dry yeast (ADY), 10 mL 22% (w/v) grape juice was diluted with sterile deionized water (1:2), and ADY was rehydrated at 40°C for 30 min. Yeast deletion strains in the S288C genetic background were obtained from Invitrogen (Carlsbad, CA) (Table 1); these

yeast strains were grown in YEPD (yeast extract, peptone, dextrose) with either 2% or 40% (w/v) dextrose.

**Growth conditions for microarray analysis.** Active dry yeast of ST and VIN7 were used to inoculate 500 mL Riesling grape juice containing 22% (w/v) sugar to a final concentration of  $6 \times 10^6$  cell/mL. Yeast cultures were grown at 20°C in 1-L Kimax bottles fitted with fermentation locks to midlog phase ( $A_{600nm} = 2.0$ ), after which 500 mL grape juice containing 60% (w/v) sugar was added to both bottles to yield a final concentration of ~40% (w/v) sugar. Both flasks were further incubated stationary at 20°C for 2 hr. Yeast cells were harvested by centrifugation for 5 min at 5000 rpm at 4°C. Cells were then washed once at 4°C with diethylpyrocarbonate (DEPC)-treated ddH<sub>2</sub>O and stored at -80°C until RNA extraction. Microarray analysis was done in triplicate, each time with independently grown cells (n = 3).

**RNA extraction and sample preparation.** Total RNA was extracted using the hot phenol method (Ausubel et al. 1995). Methods for poly(A)<sup>+</sup> RNA purification, amplification, and labeling and cRNA fragmentation were previously described (Erasmus et al. 2003).

**Hybridization, fluidics, and scanning procedures.** Oligonucleotide yeast genome arrays (YGS98; Affymetrix, Santa Clara, CA) were used as targets for hybridization. Preparation of hybridization solution, hybridization, and washing, staining, and scanning of yeast arrays were according to manufacturer protocols (Eukaryotic Arrays GeneChip Expression Analysis and Technical Manual; Affymetrix). The EukGE-WS2v4 fluidics protocol of the Affymetrix MASv5.0 software was used to perform staining and washing procedures. Experiments were conducted in triplicate, with independently grown cells.

**Data analyses.** Data were analyzed using MASv5.0 and DMT (Affymetrix). All tenable parameters were set to default values (Affymetrix Statistical Algorithm Reference Guide). Genes with change values of  $p < 0.003$  (genes with an increased call) or  $p > 0.997$  (genes with a decreased call) were considered reproducible and statistically significant. The average of the signal log (base 2) ratio (SLR) values were used to calculate the fold change. Genes were linked to their gene ontology (GO) annotations using the “orf\_geneontology.tab” table ([www.yeastgenome.org/gene\\_list.shtml](http://www.yeastgenome.org/gene_list.shtml)). Microarray data obtained were compared to previ-

**Table 1** *Saccharomyces cerevisiae* yeast strains used in the study.

Yeast strain	Source	Genotype
<b>Enological</b>		
<i>S. cerevisiae</i> VIN7	Anchor Yeast (South Africa)	unknown
<i>S. cerevisiae</i> ST	J. Laffort & Cie (France)	unknown
<b>Laboratory</b>		
BY4743	Invitrogen (USA)	<i>mata</i> $\alpha$ (BY4741/BY4742)
BY4741	Invitrogen (USA)	<i>mata his3 leu2 met1 ura3</i>
BY4741 $\Delta$ <i>ald6</i> ::G418	Invitrogen (USA)	<i>mata his3 leu2 met1 ura3</i>
BY4741 $\Delta$ <i>zwf1</i> ::G418	Invitrogen (USA)	<i>mata his3 leu2 met1 ura3</i>
BY4741 $\Delta$ <i>zms1</i> ::G418	Invitrogen (USA)	<i>mata his3 leu2 met1 ura3</i>

ously published data to determine the number of Msn2/4p-regulated genes (Gasch et al. 2000), sugar-induced osmotic stress genes (Erasmus et al. 2003), and Hog1p-regulated genes (Rep et al. 2000) that were expressed differently in VIN7 and ST. Genes whose expression levels decreased by more than 75% in a *hog1* deletion strain during osmotic stress (Rep et al. 2000) were considered *HOG1*-dependent.

**Semiquantitative reverse transcriptase RT-PCR.** Yeasts were cultured in triplicate as described for Riesling grape must except that synthetic grape juice was used. Yeast cells were rapidly harvested, washed, and stored at  $-80^{\circ}\text{C}$  until RNA extraction. Total RNA was extracted using the hot phenol method followed by on-column treatment with DNase I according to manufacturer protocol using a Qiagen RNeasy Mini Kit (cat. 74104; Qiagen, Valencia, CA). A total of 2  $\mu\text{g}$  RNA was used to synthesize cDNA using Omniscript RT and a random hexamer primer mix (2.5  $\mu\text{M}$ ) according to manufacturer protocol (cat. 205110; Qiagen). At the end of the reaction, 480  $\mu\text{L}$  deionized water was added and then stored at  $-30^{\circ}\text{C}$  until analyzed. Semiquantitative reverse transcriptase real-time (RT) PCR was conducted using QuantiTect SYBR Green PCR kit (cat. 204243; Qiagen) and an ABI PRISM 7000 instrument (Applied Biosystems, Carlsbad, CA). PCR reactions were conducted in triplicate using 2  $\mu\text{L}$  cDNA as template for both genes of interest. *IPPI* was used as a control gene, as this gene is not affected by osmotic stress (Rep et al. 1999). *IPPI* was also expressed at the same levels in ST and VIN7. Samples were cycled 40 times between  $95^{\circ}\text{C}$  for 15 sec and  $57^{\circ}\text{C}$  for 1 min. To calculate the fold change, the CT value of *IPPI* was subtracted from the gene of interest in the same cDNA sample (e.g.,  $\text{GeneX}_{\text{ST}} - \text{IPPI}_{\text{ST}}$ ) to yield  $\Delta\text{CT}$  value. The CT value was set manually by selecting the cycle number where a clear increase (logarithmic increase) in fluorescence was observed. The  $\Delta\text{CT}$  value was then subtracted from the  $\Delta\text{CT}$  value of the gene of interest from the other cDNA sample (e.g.,  $\text{GeneX}_{\text{VIN7}} - \text{GeneX}_{\text{ST}}$ ) to yield the  $\Delta\Delta\text{CT}$  value. Primers used for RT-PCR are listed in Supplementary Table 1.

**Resistance of ST, VIN7, and BY4741 to rapamycin.** *Saccharomyces cerevisiae* strains ST, VIN7, and BY4741 were inoculated from freshly streaked YEPD-agar plates into 5 mL YEPD media. Cultures were grown overnight at  $30^{\circ}\text{C}$ . Cells were then diluted to  $\text{OD}_{600\text{nm}} = 0.1$  from which 10-fold serial dilutions were made. A total of 10  $\mu\text{L}$  was taken from each serial dilution and spotted onto YEPD plates with or without 100 ng/mL rapamycin, followed by incubation at  $30^{\circ}\text{C}$ . Plates without rapamycin were photographed after one day and plates with rapamycin after two days.

**Sequencing of TOR2 in VIN7.** Sequencing of *TOR2* in Vin7 was done using PCR fragments generated from genomic DNA isolated from VIN7. The following primers pairs were used to generate PCR fragments: set 1, TOR25FULL and TOR2Rseq to generate a 2050 bp PCR fragment; set 2, TOR2Fseq and TOR2B to generate a 1289 bp PCR fragment; set 3, TOR2A and TOR2D to generate a 1561 bp PCR fragment; set 4, TOR2C and TOR2E to generate a 1449 bp PCR fragment; and set 5, TOR2FRBF and

TOR23FULL to generate a 1654 bp PCR fragment. The primers used to generate the PCR fragments were also used for sequencing, which was done on both strands at the Nucleic Acid Protein Service (NAPS), University of British Columbia. Primers used for sequencing are listed in Supplementary Table 1.

**PCR analysis of the telomeric region on chromosome XV from ST and VIN7.** Genomic DNA from BY4743 (S288C derivative), ST, and VIN7 was used as template for PCR. Genomic DNA from BY4743 was used as control for the PCR reactions. Taq DNA polymerase (MBI Fermentas) was used to generate DNA fragments (using primers listed in Supplementary Table 1). Primers YOL164WF and YOL164WR were used to generate a 1997 bp PCR product for YOL164W; Sth-YOL163w-5 and Sth-YOL163W-3 to yield a 420 bp PCR fragment for YOL163W; 160-W-5' and 160-W-3' to yield a 400 bp PCR product for YOL160W; 159-A-5' and 159-A-3' to yield a 342 bp PCR product for YOL159C-A; YOL159C F1000 and YOL159CR1000 to produce a 2515 bp fragment for YOL159C; ENB1F and ENB1R to yield a 1871 bp PCR product for *ENB1*; YOL157CF and YOL157CR to yield a 1818 bp PCR product for YOL157C; HXT11-5' and HXT11-3' to yield a 1758 bp PCR product for *HXT11*; SthZPS1-5 and SthZPS1-3 to produce a 467 bp PCR product for *ZPS1*; and DCP1-5' and DCP1-3' to yield a 757 bp PCR product for *DCP1*. DNA fragments were fractionated and visualized on 0.7% agarose gels.

**Effect of *ald6*, *zms1*, and *zwf1* deletions on acetic acid, glycerol, and ethanol formation.** The laboratory yeast strain BY4741 and its isogenic null mutants *ald6*, *zms1*, and *zwf1* (Table 1), were grown overnight in 5 mL YEPD. Cells from these cultures were used to inoculate 10 mL YEPD containing 40% (w/v) glucose in test tubes to a final concentration of  $1 \times 10^6$  cell/mL. Cultures were then grown aerobically at  $30^{\circ}\text{C}$  for 3 days. Media were harvested by centrifugation at 5000 rpm for 5 min., followed by filter sterilization using 0.22- $\mu\text{m}$  syringe filters. Filtered media were stored at  $4^{\circ}\text{C}$  until samples were analyzed by HPLC. Cultures were grown twice, each in duplicate.

## Results

Global gene expression analyses were conducted on two commercial *S. cerevisiae* wine yeast strains with different phenotypes; ST cells grow faster and produce less glycerol and acetic acid than VIN7 in synthetic must containing 40% (w/v) sugar (Erasmus et al. 2004). However, these two yeast strains ferment at similar rates in synthetic must containing 40% (w/v) sugar (data not shown).

Comparison of high-density DNA microarray data revealed that 337 genes were expressed two-fold or more differently in ST and VIN7 when grown in Riesling grape must containing 40% (w/v) sugar; 169 genes were expressed at higher levels in VIN7 and 168 genes were expressed at lower levels than in ST. Microarray data were highly reproducible; regression analysis between ST replicates were ( $n = 3$ )  $0.956 \pm 0.021$ , between VIN7 replicates ( $n = 3$ )  $0.977 \pm 0.002$ , and VIN7 compared to ST ( $n = 3$ )  $0.882 \pm 0.010$ .

Robustness of the data was confirmed by semiquantitative reverse transcriptase RT-PCR of *ACS1*, *ALD6*, *GPD1*, *GLK1*, *HSP26*, *HSP30*, *YOR315W*, and *ZMS1* when VIN7 and ST were grown in synthetic grape must (Table 2).

**Osmosensitivity of VIN7 and ST.** Global gene expression profiles of VIN7 and ST fermenting Riesling grape must containing 40% (w/v) sugar were compared to previously published data sets to identify their transcriptional response to sugar-induced osmotic stress, environmental stress, and salt stress (Erasmus et al. 2003, Gasch et al. 2000, Rep et al. 2000) (Figure 1). Sixty of the 169 genes that were expressed at higher levels in VIN7 are known to be up-regulated in response to sugar-induced osmotic stress (Erasmus et al. 2003) (Figure 1; Supplemental Table 2). In contrast, only 10 genes responsive to sugar-induced osmotic stress (Erasmus et al. 2003) were expressed at higher levels in ST than in VIN7. Of the 168 genes expressed at a lower level in VIN7 than in ST, 42 genes are known to be

down-regulated by sugar-induced osmotic stress (Figure 1; Supplementary Table 3).

Researchers identified 181 genes that are regulated by the two transcriptional activators Msn2/4p in response to environmental stress (Gasch et al. 2000). Thirty-three of the 169 genes that were expressed more than two-fold higher in VIN7 are regulated by Msn2/4p; only one Msn2/4p-regulated gene was expressed at a lower level in VIN7 than in ST (Figure 1; Supplementary Table 4).

The *HOG1* gene encodes the MAP kinase in the signal transduction pathway for osmotic stress (for a review see Hohmann 2002). Of the 49 genes known to be Hog1p-regulated during osmotic stress (Rep et al. 2000), seven (*GRE1*, *SPS100*, *TFS1*, *YAL061W*, *YGR043C*, *YKL151C*, and *YMR090W*) were expressed at higher levels in VIN7 than in ST; only one Hog1p-regulated gene (*ARO9*) was expressed at a lower level in VIN7 than in ST (Supplementary Table 5).

**Expression of genes involved in biosynthesis.** Genes involved in several major biological processes were expressed at lower levels in VIN7 than in ST; 78 genes involved in translation and protein biosynthesis had lower transcript levels in VIN7. Eleven of these genes were expressed at least two-fold lower: *MRPL31* (-2.0-fold), *NAM9* (-2.0-fold), *RPL13A* (-2.1-fold), *RPL17B* (-2.0-fold), *RPL18B* (-2.7-fold), *RPL40B* (-2.0-fold), *RPL7B* (-2.0-fold), *RPS10B* (-3.5-fold), *RPS16A* (-2.0-fold), *RPS26B* (-2.8-fold), and *SRO9* (-2.5-fold).

The *TOR1* and *TOR2* (target of rapamycin) gene products play a central role in the transcriptional regulation of genes encoding ribosomal proteins (Heitman et al. 1991, Powers and Walter 1999). Twenty-two of the 78 genes involved in translation and protein biosynthesis that are expressed at lower levels in VIN7 than in ST encode for ribosomal proteins. VIN7 was more resistant to rapamycin than both ST and the laboratory strain BY4741 (Figure 2). Sequencing of *TOR2* in VIN7 revealed a single nucleotide polymorphism at position 3187; this C to T substitution caused an amino acid change from proline to serine. In addition *TOR1* (-1.62-fold) and *TOR2* (-2.1-fold) transcript levels were lower in VIN7 than in ST.

In addition, 33 genes involved amino acid biosynthesis and 17 genes involved in nucleotide biosynthesis were also down-regulated in VIN7. However, six genes involved in amino acid metabolism were expressed at higher levels in VIN7: *LYS5* (1.7-fold), *HIS2* (1.6-fold), *CPAI* (4.2-fold), *ARG1* (2.9-fold), *ARG3* (3.2-fold), and *CARI* (2.0-fold). Four of these six genes (*CPAI*, *ARG1*, *ARG3*, and *CARI*) are involved in arginine metabolism. Furthermore, eight genes involved in thiamine metabolism were expressed at least two-fold lower in VIN7. Although metabolic pathway genes involved in arginine or thiamine metabolism were expressed lower in VIN7, the addition of these two compounds to VIN7 or ST fermenting 40% (w/v) synthetic grape must had no influence on acetic acid, glycerol, or fermentation rate (data not shown).

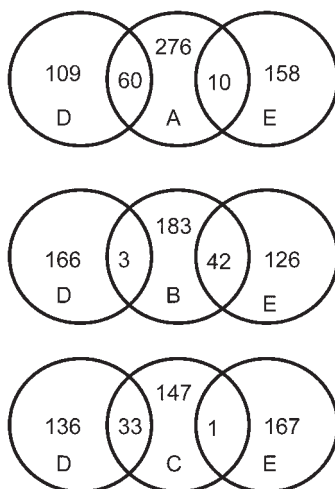
Phosphatidylcholine, a diacylglycerol (DAG), is a major phospholipid in cellular membranes. Ten genes that encode enzymes required for the biosynthesis of phosphatidylcho-

**Table 2** Comparative expression of *ACS1*, *ALD6*, *GPD1*, *GLK1*, *HSP26*, *HSP30*, *YOR315W*, and *ZMS1* in VIN7 and ST grown in Riesling and synthetic grape musts containing 40% (w/v) sugar. Values indicate fold differences (VIN7 vs ST).

Gene	Grape must	
	Riesling <sup>a</sup>	Synthetic <sup>b</sup>
<i>ACS1</i>	-2.4	-2.1
<i>ALD6</i>	1.9	3.7
<i>GPD1</i>	1.4	2.2
<i>GLK1</i>	2.0	2.0
<i>HSP26</i>	3.1	6.0
<i>HSP30</i>	6.5	-1.6
<i>YOR315W</i>	-22.2	-1.3
<i>ZMS1</i>	-1.8	7.8

<sup>a</sup>High-density DNA microarray data.

<sup>b</sup>Semiquantitative reverse transcriptase RT-PCR data



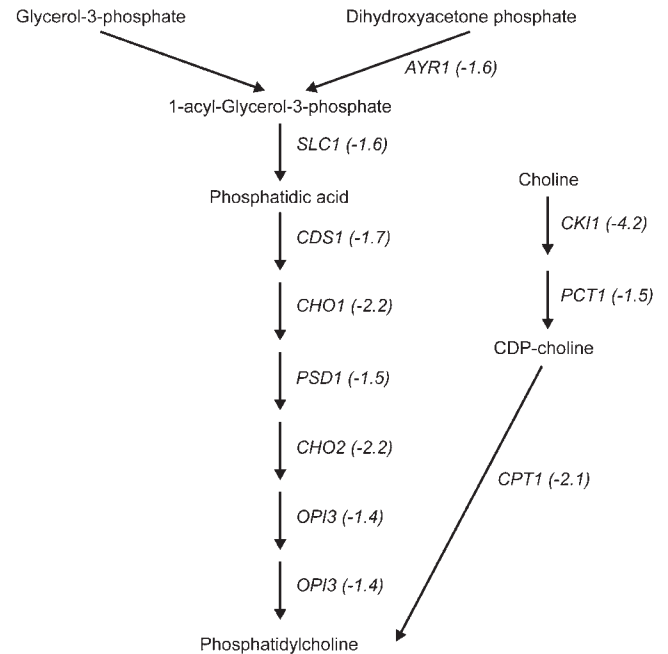
**Figure 1** Stress-related genes that were expressed two-fold or more differently between VIN7 and ST. (A) Genes up-regulated by sugar-induced osmotic stress. (B) Genes down-regulated by sugar-induced osmotic stress. (C) Genes regulated by Msn2/4p. (D) Genes with higher transcript levels in VIN7 compared to ST. (E) Genes with lower transcript levels in VIN7 compared to ST.

line from glycerol-3-phosphate, dihydroxyacetone phosphate (DHAP), and choline were expressed at lower levels in VIN7 than in ST: *AYR1*, *SLC1*, *CDS1*, *CHO1*, *PSD1*, *CHO2*, *OPI3*, *CK11*, *PCT1*, and *CPT1* (Figure 3).

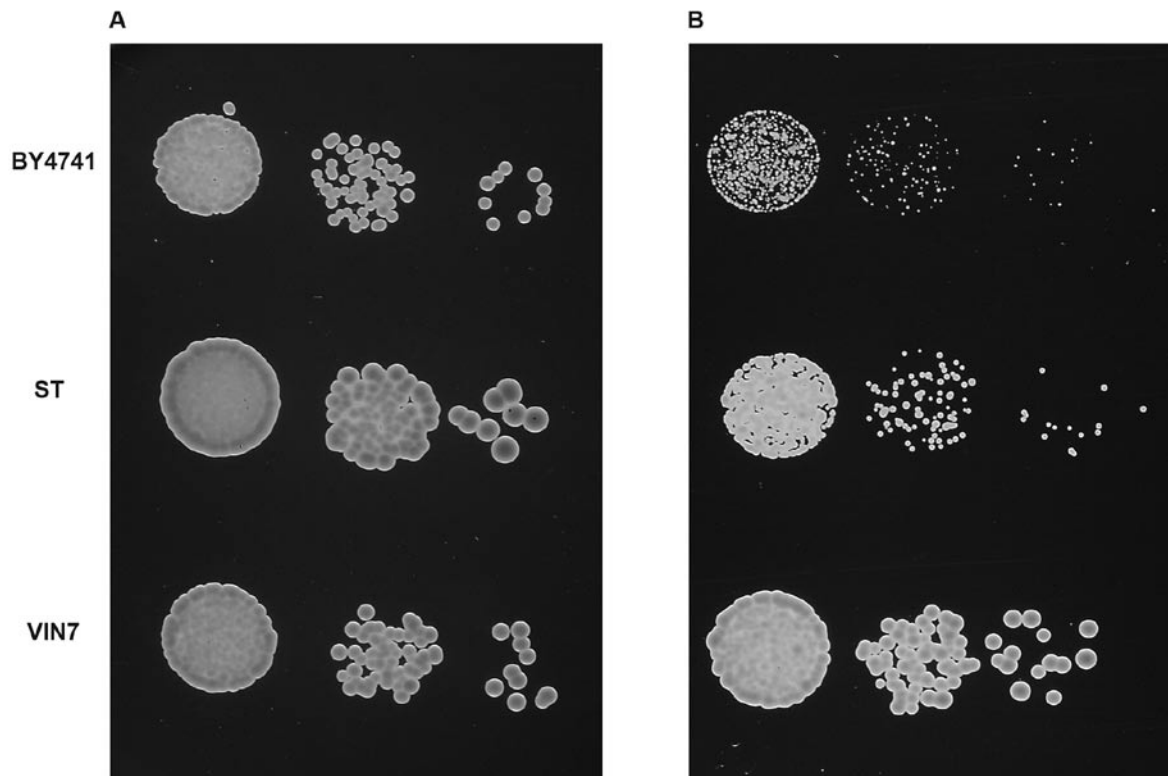
**PCR of the telomeric region of chromosome XV of ST and VIN7.** An arbitrary cut-off of five-fold difference in expression levels (VIN7 vs ST) was used to identify orphan genes that may contribute to the VIN7 phenotype. Four orphan genes that responded to osmotic stress were found: YAL061W, YKL071W, YOL159C, and YPL222W (Table 3). Three of the four genes were expressed in both ST and VIN7. However, no transcripts were found for YOL159C in VIN7. The transcriptional response of these orphan genes obtained with microarray technology in Riesling grape must were confirmed in synthetic grape must using semiquantitative reverse transcriptase RT-PCR (Table 2). The expression of YAL061W, YKL071W, and YPL222W was confirmed, but no transcript was detected for YOL159C, implying that it was not expressed or was absent in the genome of VIN7. PCR analyses on the genomes of VIN7, ST, and BY4743 using primers specific for YOL164W, YOL163W, YOL160W, YOL159C-A, YOL159C, *ENB1*, YOL157C, *HXT11*, *ZPS1*, and *DCP1* suggest that the left arms on chromosome XV of ST and VIN7 were depleted (Figure 4). YOL164W, YOL163W, and *HXT11* were absent in both ST and VIN7. YOL164W, YOL163W, YOL160W, YOL159C-A, YOL159C, *ENB1*, and YOL157C were absent in VIN7.

**Transcription of structural genes in glycerol and acetic acid production.** VIN7 produces significantly more

glycerol (13.8 vs 9.62 g/L,  $p < 0.0001$ ) and acetic acid (1.72 vs 0.932 g/L,  $p < 0.0001$ ) than ST in Riesling icewine must containing ~40% sugar (40 Brix) (Erasmus et al. 2004).



**Figure 3** Microarray data indicate that genes involved in biosynthesis of phosphatidylcholine are expressed at lower levels in VIN7 than in ST during the fermentation of Riesling grape must containing 40% (w/v) sugar. Fold difference is given in parenthesis (VIN7 vs ST).



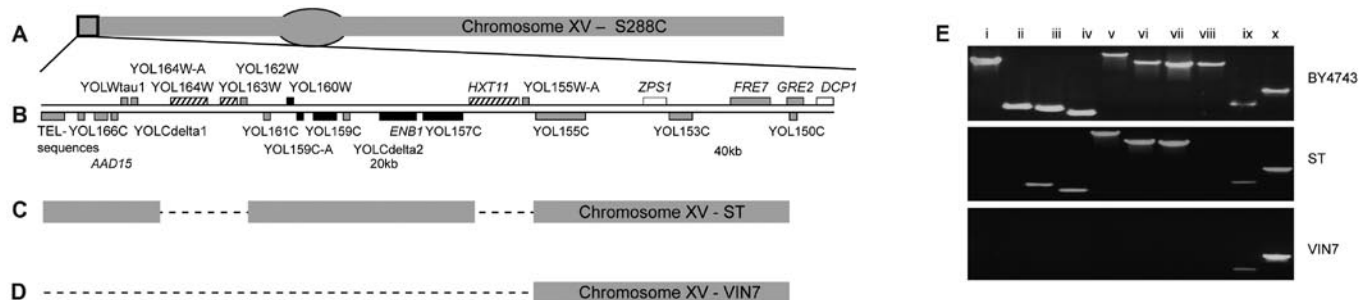
**Figure 2** VIN7 is more rapamycin-resistant than ST and BY4741. (A) Growth on YEPD without rapamycin after one day. (B) Growth after two days on YEPD containing 100 ng/mL rapamycin.

**Table 3** Comparison of four orphan gene transcripts in VIN7 and ST. Values indicate fold differences (VIN7 vs ST).

Gene	Grape must		Phenotype or transcriptional response
	Riesling <sup>a</sup>	Synthetic <sup>b</sup>	
YAL061W	6.3	3.4	Induced by salt (Yale and Bohnert 2001) and sugar-induced osmotic stress (Erasmus et al. 2003)
YKL071W	11.2	4.5	Induced by sugar-induced osmotic stress (Erasmus et al. 2003)
YOL159C	Absent in VIN7	Absent in VIN7	Null mutant is osmosensitive (Giaever et al. 2002)
YPL222W	7.0	16.4	Null mutant is osmosensitive (Giaever et al. 2002); transcription by sugar-induced osmotic stress (Erasmus et al. 2003)

<sup>a</sup>Fold difference derived from high-density DNA microarray data.

<sup>b</sup>Fold difference derived from semiquantitative reverse transcriptase RT-PCR data.

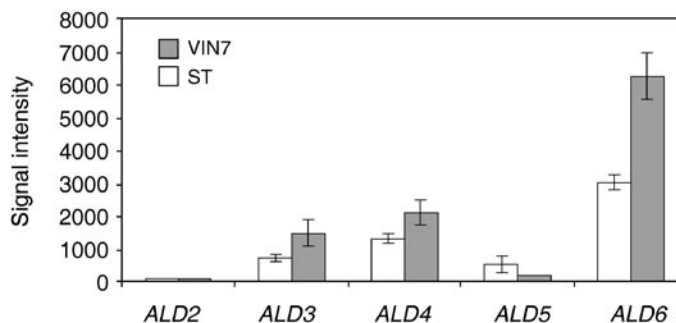


**Figure 4** (A) Telomeric region on the left arm of chromosome XV in *S. cerevisiae*. (B) Genes present in both ST and VIN7 in white. Genes absent in both ST and VIN7 in stripes. Genes absent in VIN7 but present in ST in black. PCR analyses not done on genes in gray because of high AT content in the ORFs (>60%). Structure of chromosome XV in ST (C) and VIN7 (D). Dotted lines indicate a 3.9 Kb and a 1.7 Kb deletion in chromosome XV of ST (C) and a 30 Kb deletion in VIN7 (D). (E) PCR amplification of (i) YOL164W, (ii) YOL163W, (iii) YOL160W, (iv) YOL159C-A, (v) YOL159C, (vi) *ENB1*, (vii) YOL157C, (viii) *HXT11*, (ix) *ZPS1*, and (x) *DCP1* located on chromosome XV of *S. cerevisiae* strains BY4743, ST, and VIN7.

Consistent with these observations, we found that *GPD1* (glycerol production) and *ALD3*, *ALD4*, and *ALD6* (acetic acid production) were expressed at higher levels in VIN7 than in ST (Table 2, Figure 5). However, transcript levels of *GPP2* and *ALD2* were similar in these two yeast strains, but *ALD5* was expressed at a lower level in VIN7 than in ST. *ACSI*, which encodes for acetyl-CoA synthetase, was expressed at a lower level in VIN7. *ZMS1* is a putative transcriptional activator of *ALD6*. This gene was expressed at a lower level in VIN7 according to the microarray data; however, semiquantitative reverse transcriptase RT-PCR indicated that *ZMS1* was expressed at a higher level in VIN7 than in ST (Table 2).

## Discussion

The transcriptional response of *S. cerevisiae* to osmotic stress, including the formation of glycerol as compatible solute, is mainly regulated through the HOG MAP kinase cascade pathway. The MAP kinase, Hog1p, has been shown to function under enological conditions where high sugar concentrations present an osmotic stress environment for *S. cerevisiae* (Remize et al. 2003). High levels of glycerol formation during osmotic stress lead to a redox imbalance in the yeast cell (NADH:NAD<sup>+</sup>) (Blomberg and Adler 1989). Furthermore, several studies have linked acetic acid formation to glycerol formation (Remize et al. 1999). It has been suggested that yeast may use the oxidation of acetaldehyde to acetic acid as a redox sink to convert NAD<sup>+</sup> back to



**Figure 5** Expression levels of *ALD* genes in VIN7 and ST during fermentation of 40% (w/v) sugar Riesling grape must. Expression levels determined by DNA microarrays. Results are the mean values  $\pm$  standard deviation ( $n = 3$ ). *ALD6* was significantly different ( $p < 0.003$ ) between VIN7 and ST.

NADH (Blomberg and Adler 1989, Remize et al. 1999). Furthermore, there is a direct correlation between the sugar concentration in grape juice and the amounts of glycerol and acetic acid produced (Erasmus et al. 2004).

**Transcriptional adaptation of VIN7 and ST to sugar-induced osmotic stress.** To adapt to osmotic stress and changing conditions during wine fermentations, *S. cerevisiae* has to rapidly reprogram its transcriptional response in order to adjust its metabolism accordingly. Genes involved in glycerol formation (*GPD1* and *GPP2*) and genes involved in acetic acid formation (*ALD2*, *ALD3*, *ALD4*, and *ALD6*) are up-regulated by osmotic stress when sugar,

salt, or sorbitol is present as an osmolyte (Erasmus et al. 2003, Rep et al. 2000). VIN7 produced the highest levels of glycerol (13.8 g/L) and acetic acid (1.72 g/L), whereas ST produced the lowest levels (9.62 and 0.932 g/L, respectively) in icewine (Erasmus et al. 2004). The differential expression of genes between VIN7 and ST can be attributed mainly to genetic polymorphisms in the two yeast strains that result in differential expression of genes. A total of 589 genes were identified to respond either positively or negatively to sugar-induced osmotic stress (Erasmus et al. 2003). VIN7 was more sensitive to sugar-induced osmotic stress (transcriptional response to 40 vs 22% [w/v] sugar) than ST (Figure 1); transcript levels of most Hog1p and Msn2/4p regulated genes were higher in VIN7 than in ST (Figure 1).

In addition to the HOG MAP kinase cascade pathway that regulates Msn2/4p-dependent gene expression in response to osmotic stress (Rep et al. 2000, Schuller et al. 1994), the cAMP-PKA pathway and Tor1/2p (TOR, target of rapamycin) also regulate Msn2/4p-dependent transcription (Gorner et al. 1998). TOR regulates several essential growth-related biological processes in yeast, including organization of the actin cytoskeleton, membrane trafficking, protein degradation, PKC signalling, regulation of nitrogen metabolism, initiation of translation, and transcriptional regulation of ribosomal protein genes (for a review see Schmelzle and Hall 2000). When TOR activity increases, the expression of ribosomal protein genes increases; a lack of TOR activity results in a decrease in the expression of ribosomal protein genes (Powers and Walter 1999). The *TOR1* and *TOR2* genes were originally identified in mutants that were resistant to the immunosuppressant rapamycin (Heitman et al. 1991), which inhibits TOR activity causing, among other changes, the repression of the transcription of ribosomal protein genes (Powers and Walter 1999). Several domains have been identified in the N-terminal region of Tor1p and Tor2p, including two HEAT repeat motifs that mediate protein-protein interactions and cellular localization of TOR and a FAT domain for which no function has been assigned. On the C-terminal side of the FAT domain, the FRB-domain, the putative kinase domain, and a FATC domain were identified (for a review see Schmelzle and Hall 2000). The resistance of VIN7 to rapamycin suggests that TOR activity might be lower in VIN7 than in ST (Figure 2). A lower TOR activity is supported by numerous ribosomal protein genes expressed at lower levels in VIN7 than in ST. A single nucleotide polymorphism was found in Tor2p at position 1063, resulting in an amino acid substitution on the N-terminal side of the FRB-domain; the proline at position 1063 was substituted by a serine in VIN7. Proline is a hydrophobic amino acid, often found in beta turns, whereas serine is polar and often the target site of phosphorylation. This mutation occurred in the second HEAT repeat motif of Tor2p spanning residues 560 to residues 1220. Protein modeling did not reveal any secondary structural changes in Tor2p because of the amino acid substitution, but the impact of

the amino acid change in Tor2p on TOR activity in Vin7 needs to be further investigated. The lower activity of TOR in VIN7 might also be ascribed to the fact that both *TOR1* and *TOR2* were expressed at lower levels in VIN7. Lower TOR activity in VIN7 might be responsible for the higher number of Msn2/4p-regulated genes expressed at higher levels in VIN7 than in ST.

Chromosome length polymorphisms are common in wine yeast strains; this phenomenon is routinely used in the karyotyping of strains. PCR analyses on the left arm of chromosome XV confirmed microarray data and indicated that YOL159C is absent in the genome of VIN7. This finding prompted us to investigate if any other genes flanking YOL159C were absent. PCR data revealed that ~30 Kb was absent in VIN7, including the following genes: YOL164W, YOL163W, YOL160W, YOL159C-A, YOL157c, *ENBI*, and *HXT11* (Figure 4). The absence of the orphan genes YOL160W and YOL159C causes slower growth in laboratory strains when exposed to osmotic stress (Giaever et al. 2002). It is, therefore, likely that deletion of YOL160W and YOL159C in the genome of VIN7 could contribute to this strain being more sensitive to osmotic stress; the slower growth rate of VIN7 in media containing 40% (w/v) sugar is consistent with these data. The function of YOL160W and YOL159C in *S. cerevisiae* has not yet been elucidated, but it is documented that deletion of YOL159C in laboratory strains increases Tyl retrotranspositions (Griffith et al. 2003). The increase in retrotranspositions may increase genetic drift and affect the genetic stability of a strain; VIN7 is considered to be genetically unstable. The orphan genes YOL164W, YOL163W, YOL159C-A, and YOL157C, as well as *ENBI* that encodes for an endosomal ferric enterobactin transporter and *HXT11* that encodes for a hexose transporter, have not been linked to osmosensitivity in laboratory strains.

*HXT11*, YOL164W, and YOL163W were absent in ST (Figure 4). None of these three genes have been implicated to play a role during osmotic stress. The function of YOL164W and YOL163W has not been elucidated. A *hxt11*, *yol164w*, *yol163w* triple mutant may be more sensitive to osmotic stress. However, the absence of these three genes did not affect the ability of ST to ferment icewine must.

Transcription of *GPD1*, *ALD3*, *ALD4*, and *ALD6* has been reported to be up-regulated by sugar, salt, or sorbitol-induced osmotic stress (Erasmus et al. 2003, Rep et al. 2000). The elevated expression of *GPD1*, *ALD3*, *ALD4*, and *ALD6* in VIN7 is therefore consistent with the greater osmosensitivity of VIN7 and its tendency to produce increased amounts of glycerol and acetic acid (Erasmus et al. 2004). The formation of glycerol from the glycolytic pathway intermediate DHAP, however, is catalyzed by two enzymes encoded by *GPD1* and *GPP2* (Albertyn et al. 1994, Norbeck et al. 1996). Both *GPD1* and *GPP2* have been shown to respond to sugar-induced osmotic stress (Erasmus et al. 2003). However, only *GPD1* was expressed at higher levels in VIN7 than in ST (Table 2); *GPP2* was expressed at a similar level in VIN7 and ST (data not shown).

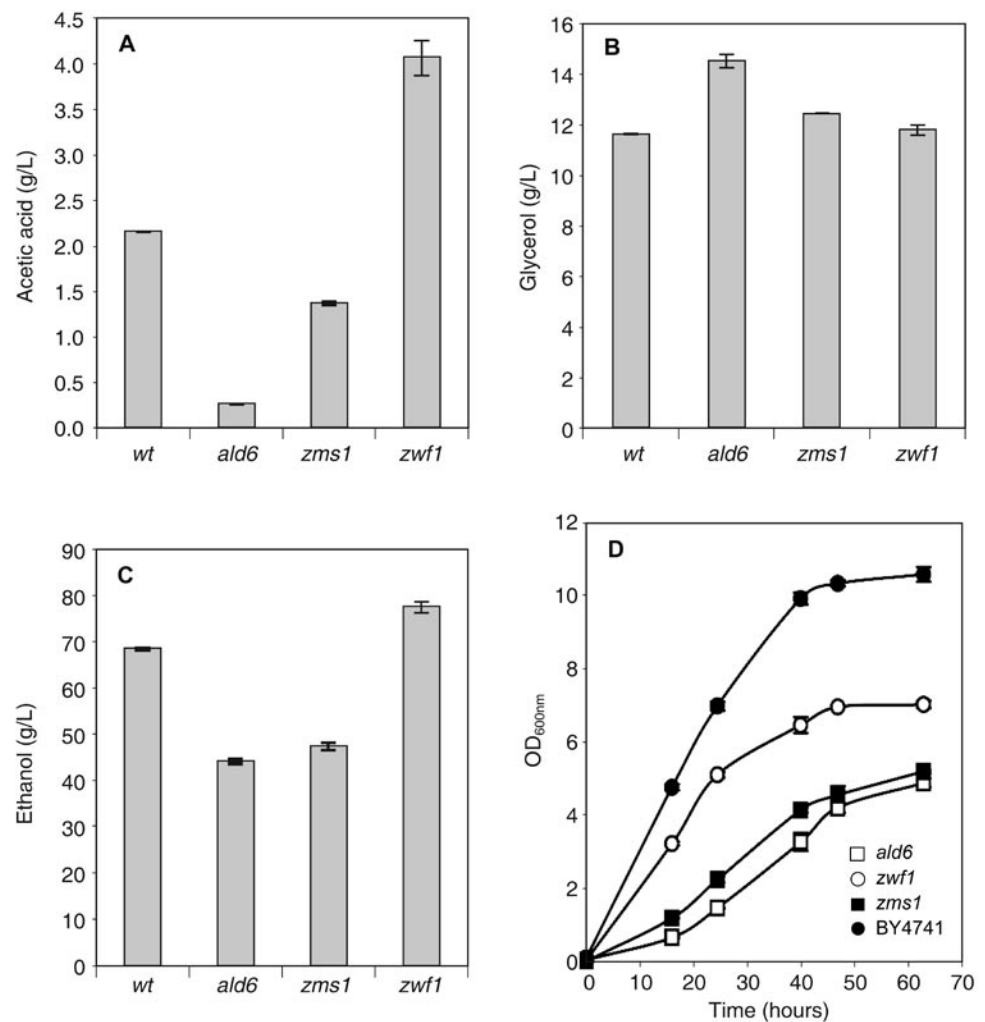
*GPD1* encodes for the key enzyme in this two-step process, not only in laboratory conditions but also in enological conditions (Albertyn et al. 1994).

The most widely accepted hypothesis of acetic acid formation by yeast during osmotic stress is that the formation of acetic acid from acetaldehyde acts as a redox sink to reduce  $\text{NAD}^+$  back to  $\text{NADH}$  (Blomberg and Adler 1989). *Saccharomyces cerevisiae* has five *ALD* isogenes that encode acetaldehyde dehydrogenases that can possibly catalyze the oxidation of acetaldehyde to acetate: *ALD2*, *ALD3*, *ALD4*, *ALD5*, and *ALD6* (Navarro-Avino et al. 1999). Ald2p and Ald3p are both cytosolic and use  $\text{NAD}^+$  as cofactor. Ald4p and Ald5p are mitochondrial and use  $\text{NAD(P)}^+$  and  $\text{NADP}^+$ , respectively. Ald6p is cytosolic and uses  $\text{NADP}^+$  as cofactor. Ald6p has been reported as responsible for a major portion of acetic acid produced during wine fermentations (Remize et al. 2000). *ALD3*, *ALD4*, and *ALD6* were expressed at higher levels in VIN7 than in ST, with *ALD6* expressed at the highest level in both strains (Figure 5). If transcript levels correlate with enzyme levels of the particular *ALD* genes, then *ALD6* seems to be a major contributor to acetic acid production in icewines. However, Ald6p uses  $\text{NADP}^+$  and not  $\text{NAD}^+$  (Wang et al. 1998). If the purpose of acetic acid formation under conditions of osmotic stress is to reset the redox balance and reduce  $\text{NAD}^+$  produced during glycerol formation back to  $\text{NADH}$ , then the  $\text{NAD}^+$ -dependent Ald2p and/or Ald3p should be the major enzymes involved in production of acetic acid. Based on transcript levels, our data indicate that *ALD2* and *ALD3* in VIN7 may not be responsible for the high levels of acetic acid produced by VIN7. It raises an intriguing question of how  $\text{NAD}^+$  produced during glycerol formation is reduced back to  $\text{NADH}$ .

**Deletion of *ZMS1*, *ALD6*, and *ZWF1*.** *ZMS1* encodes for a putative zinc-finger transcription factor that increases the expression of *ALD6* (Grabowska and Chelstowska 2003). Although *ZMS1* does not respond to sugar-induced osmotic stress in Vin13 (data not shown), its deletion has been reported to cause osmosensitivity in *S. cerevisiae* (Giaever et al. 2002). Furthermore, *zms1* deletion mutants produce ~50% less acetic acid than the corresponding wild-type strain (Figure 6). *ZMS1*, therefore, seems to be indirectly involved in

the formation of acetic acid. Contradictory data for *ZMS1* transcript levels were obtained by microarray and RT-PCR techniques in this study: -1.8-fold and 7.8-fold, respectively. Higher levels of *ZMS1* mRNA in VIN7 would be consistent with higher *ALD6* mRNA levels and higher acetic acid levels observed under conditions of osmotic stress in this study.

During osmotic stress, the  $\text{NAD}$ -dependent aldehyde dehydrogenase activity increases in wild-type cells, but not in an *ald2ald3* mutant when grown in 2% (w/v) glucose (Navarro-Avino et al. 1999). The redox link between glycerol and acetic acid formation is supported by the fact that when *S. cerevisiae* is grown in a galactose-containing medium and then exposed to 15 mM LiCl, glycerol and acetic acid formation increase in conjunction with *GPD1*, *ALD2*, and *ALD3* mRNA and protein levels, whereas *ALD6* mRNA and protein levels decrease (Bro et al. 2003). However, the roles of *ALD2* and *ALD3* when *S. cerevisiae* is exposed to osmotic stress under enological conditions were not investigated. In contrast, our data obtained with deletion mutants of individual *ALD* genes in the S288C genetic background revealed that *ALD6*, the  $\text{NADP}^+$ -dependent isoform, was



**Figure 6** Formation of (A) acetic acid, (B) glycerol, and (C) ethanol and (D) growth of laboratory wild-type strain BY4741 and its isogenic deletion mutant strains *ald6*, *zwf1*, and *zms1* in YEPD containing 40% (w/v) glucose. Cultures were grown twice, each in duplicate.



responsible for the major portion of acetic acid when yeast cells are exposed to sugar (40% w/v)-induced osmotic stress (Figure 6). Furthermore, deletion of the cytosolic NAD<sup>+</sup>-dependent genes *ALD2* and *ALD3* does not affect acetic acid formation during sugar-induced osmotic stress, suggesting that biological processes other than increased glycerol formation influence acetic acid formation as well. *ALD6* uses NADP<sup>+</sup> and glycerol formation yields NAD<sup>+</sup>. With no transdehydrogenase identified in yeast (Meaden et al. 1997, Wang et al. 1998), it raises the question of how increased glycerol formation results in an increase in acetic acid formation if Ald6p requires NADP<sup>+</sup>.

Deletion of *ALD6* and *ZWF1* is synthetically lethal in *S. cerevisiae* (Grabowska Chelstowska 2003). *ZWF1* encodes for glucose-6-phosphate dehydrogenase, which catalyzes the first irreversible step in the oxidative part of the pentose phosphate pathway (PPP). The oxidative part of the PPP is the major source of NADPH in the yeast cell (Alberts et al. 1994). NADPH is primarily used in biosynthetic pathways such as nucleotide, amino acid, and phospholipid biosynthesis. Deletion of *ZWF1* will result in no formation of NADPH by the PPP. Both Ald6p and Zwf1p are involved in NADPH formation (Grabowska and Chelstowska 2003). Under conditions of osmotic stress, *S. cerevisiae* produce more acetic acid when *ZWF1* is deleted (3.3 vs 2.5 g/L) (Figure 6).

Transcription of several genes encoding enzymes in the oxidative and nonoxidative part of the PPP was affected when *S. cerevisiae* was exposed to osmotic stress (Erasmus et al. 2003). If these changes in mRNA transcript levels result in increased or decreased enzyme levels that affect the flow of carbon in the PPP and reduce NADPH production, yeast cells could experience a shortage of NADPH. The conversion of acetaldehyde to acetic acid by Ald6p under conditions of osmotic stress might, therefore, compensate for this shortage of NADPH. Indeed, differences have been reported in the PPP metabolic flux and concomitant NADPH production among strains of *S. cerevisiae* (Heux et al. 2008). Further research on proteins and metabolites and the flux of carbon in the PPP will yield valuable data on the possible role of the PPP in the generation of NADPH that might force yeast cells to produce more acetic acid mediated by Ald6p under conditions of osmotic stress. Differences in the metabolic flux in the PPP pathway could therefore account for differences in acetic acid production among strains.

Genes involved in phospholipid biosynthesis are expressed at lower levels in VIN7 than in ST and may contribute to increased concentrations of glycerol and acetic acid produced under conditions of osmotic stress.

The link between fatty acid synthesis and acetic acid production has previously been established; a lack of free fatty acids in strongly clarified grape musts increases acetic acid formation by wine yeast (Garcia Moruno et al. 1993). In contrast, addition of unsaturated free fatty acids causes a reduction in acetic acid formation. The formation of acetyl-CoA from acetic acid and Co-enzyme A, catalyzed by acetyl-CoA synthetase encoded by *ACSI*, is the first step in the formation of the long-chain aliphatic fatty acids.

Data suggest that yeast strains that produce more acetic acid have lower acetyl-CoA synthetase activity than yeast strains that produce low levels of acetic acid (Verduyn et al. 1990). Furthermore, *ACSI* transcripts were lower in VIN7 than in ST (Table 2). However, *ACSI* does not respond to osmotic stress in an industrial strain of *S. cerevisiae* Vin13 subjected to high sugar concentrations (Erasmus et al. 2003). Lower transcript levels of *ACSI* in VIN7 under conditions of osmotic stress may be due to the osmosensitivity of VIN7 caused by the deletion of YOL159C and YOL160W (Figure 4). If transcript levels correlate with protein activity and the concentration of metabolic intermediates in VIN7 and ST, then a lower rate of phospholipid biosynthesis in VIN7 may result in the consumption of less acetic acid for the synthesis of phospholipids. Furthermore, high *ALD6* transcript levels might also stimulate acetic acid production. The high acetic acid levels produced by VIN7 might thus be due to a combination of less acetic acid consumed by Acs1p for fatty acid synthesis and enhanced formation of acetic acid by Ald6p, which would yield more NADPH.

## Conclusions

Yeast strains have a significant impact on the final chemical composition and quality of wine. Global gene expression patterns revealed differential expression of 337 genes between the wine yeast strains ST (low acetate producer) and VIN7 (high acetate producer). VIN7 was more sensitive to sugar-induced osmotic stress and transcript levels of most Hog1p- and Msn2/4p-regulated genes were higher in VIN7 than in ST.

Chromosomal length polymorphisms are common in most yeast strains. PCR data revealed that ~30 Kb was absent on the left arm of chromosome XV in VIN7. Genetic polymorphisms in chromosomal structure, absence of YOL159C, and single nucleotide polymorphism in *TOR2* whose protein play a central role in regulating metabolism may increase osmosensitivity in *S. cerevisiae* VIN7, leading to the production of high amounts of acetic acid. Our data suggest that acetic acid production by wine yeast is the result of the reaction catalyzed by Ald6p and influenced by the pentose phosphate pathway and lipid biosynthesis.

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