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Genome-wide gene expression of a natural hybrid between *Saccharomyces cerevisiae* and *S. kudriavzevii* under enological conditions

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ABSTRACT

The species *Saccharomyces cerevisiae* plays a predominant role in the wine making process. However, other species have been associated with must fermentation, such as *Saccharomyces uvarum* (*Saccharomyces bayanus* var. *uvarum*) or *Saccharomyces paradoxus*. Recently, yeast hybrids of different *Saccharomyces* species have also been reported as responsible for wine production. Yeast hybrids between the species *S. cerevisiae* × *S. kudriavzevii* isolated in wine fermentations show enhanced performance in low temperature enological conditions and increased production of interesting aroma compounds. In this work, we have studied the transcriptomic response in enological conditions of a *S. cerevisiae* × *S. kudriavzevii* hybrid strain and compared it with the reference species of *S. cerevisiae* and *S. kudriavzevii*. The results show that the hybrid strain presents an up-regulation of genes belonging to functional group translation and amino-acid metabolism. Moreover, key genes related to cold stress and production of glycerol and aroma compounds were also up-regulated. While some genes inherited regulation patterns from one of the parents, most of the up-regulated genes presented a new gene expression pattern, probably generated during the hybridization and adaptation process.

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1. Introduction

Wine fermentation is a complex ecological and biochemical process involving the sequential development of different yeast species. *S. cerevisiae* is the predominant yeast in most fermentation processes (Pretorius, 2000); however there are two other species belonging to this genus (*Saccharomyces bayanus* and *Saccharomyces paradoxus*) that have also been described as responsible or, at least, as playing an important role during wine fermentation (González et al., 2006). Recently, natural interspecific hybrid strains between *Saccharomyces* species have been related to wine fermentations. González et al. (2006) described wine yeast hybrids between the species *Saccharomyces cerevisiae* × *Saccharomyces kudriavzevii* and Lopandic et al. (2007) also found these hybrids in Austria wineries. It is interesting to note, that several commercial wine yeasts are also hybrids between *S. cerevisiae* × *S. kudriavzevii* (Bradbury et al., 2006; González et al., 2006). The presence of *S. cerevisiae* × *S. kudriavzevii* hybrids in brewing processes has also been described (González et al., 2008).

The fermentative power of *S. cerevisiae* has been used for the production of food and alcoholic beverages throughout man's history, and the industrial strains of this species are well adapted to stress conditions such as temperature, osmotic pressure and ethanol toxicity, present in the different fermentative processes. *S. kudriavzevii* has been isolated in Japan from decaying leaves and soil or in Europe from oak tree bark in Portugal (Sampaio and Gonçalves, 2008) and in different areas in Spain (Lopes et al., 2010). However, to date it has never been found in fermentative environments. The physiological characterization of the Japanese strains of *S. kudriavzevii* (Arroyo-López et al., 2009; Belloch et al., 2008; Naumov et al., 2000) would indicate that they grow very well at low temperatures, even at 4 °C. They also display high cellulolytic activity, inulin (fructose polymer) hydrolysis, galactitol utilization and synthesis of starch-like polymers. In spite of these distinctive characteristics, they can ferment grape must with 200 g l^{-1} sugar (González et al., 2007).

The *S. cerevisiae* × *S. kudriavzevii* hybrid strains are well adapted to stress conditions common to wine fermentations (low pH, high sugar and ethanol content) (Belloch et al., 2008), and their enological characterization reveals interesting properties according to new trends in winemaking (Gamero et al., 2011; Gang et al., 2009; González et al., 2007). They acquired physiological properties from both parents, for example the alcohol and glucose tolerance of *S. cerevisiae* and the low-temperature tolerance of *S. kudriavzevii*, which confer upon them selective advantages in intermediate or fluctuating conditions with respect to their reference or parent species (Greig et al., 2002; Masneuf et al., 1998; Serra et al., 2005; Zambonelli et al., 1997).

The enological characterization (González et al., 2007) showed that the *S. cerevisiae* × *S. kudriavzevii* hybrid strain W27 seemed to be well adapted to low and intermediate temperature conditions, producing more aromatic compounds than their reference strains,

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especially higher alcohols at 14, 18 and 22 °C (González et al., 2007). These results have been confirmed by Gang et al. (2009) who evaluated different *Saccharomyces* hybrids in wine fermentation conditions. The authors postulated that the physiology of the hybrids uses metabolic pathways of both parent species, but to a different extent depending on temperature.

The most interesting findings indicate that *S. cerevisiae* × *S. kudriavzevii* hybrids exhibit several better winemaking properties than strains of *S. cerevisiae* and *S. kudriavzevii* used as a reference: i) a significantly lower production of acetic acid, a compound that reduces wine quality; ii) higher glycerol synthesis, a favorable compound; and iii) the higher release and production of flavors and aromas. But the most important conclusion is that these hybrid strains maintain ethanol tolerance and the ability to grow in media containing high levels of sugar, as do the majority of the strains of *S. cerevisiae*, and clearly improved growth at lower temperatures, as does the type strain of *S. kudriavzevii*. This characteristic is of great value due to the current tendency in the wineries to perform wine fermentation at low temperatures to obtain fruity and aromatic wines.

Due to the interesting properties of these hybrids, the present work sets out to compare the global gene expression of *S. cerevisiae* × *S. kudriavzevii* hybrid strain W27 with the global gene expression of a representative *S. cerevisiae* (a commercial strain T73) and the type strain of *S. kudriavzevii* (IFO 1802) in enological conditions (Macabeo juice fermentation at 18 °C). To this end, we attempt to elucidate gene expression changes that enable hybrid strain W27 to adapt to cold temperature fermentation conditions producing different fermentation and volatile production profiles.

2. Materials and methods

2.1. Yeast strains and media

The hybrid strain W27 (*S. cerevisiae* × *S. kudriavzevii*) is an active dry yeast from Lallemand (Montreal, Canada). *S. cerevisiae* strain T73 (commercial wine strain Lalvin T73 from Lallemand Montreal, Canada) and *S. kudriavzevii* type strain IFO 1802T (obtained from Dr. G. I. Naumov) were used as reference species representing the parental strains. Yeast precultures were carried out in YPD (glucose 2%, yeast extract 1% and peptone, 2%).

2.2. Fermentations, enological determinations and volatile compound analysis

The yeast cells were harvested from previous fermentation and the enological and microbiological data are described in this previous work (González et al., 2007). All fermentations were carried out in triplicate by using 450 ml of Macabeo juice in sterile 500 ml vessels. The sterility conditions were always maintained working near a flame when flasks were opened. The musts employed contained 275 g of fermentable sugars/l, they were supplemented with 0.2 g l^{-1} of diammonium phosphate (Panreac, Barcelona, Spain) and 0.1 mg l^{-1} of thiamine (Sigma, Steinheim, Germany) and the pH was 3.5 ± 0.1. They were treated by adding 1 mg/l of dimethyl di-carbonate (Fluka, Buchs, Switzerland) to be sterilized and SO_2 free up to 20 ppm, and allowed to settle overnight. The musts were inoculated with a final concentration of 10^6 cell ml^{-1} of pure yeast culture. The vinification process was conducted at 18 °C until the alcoholic fermentation was completed. Samples were collected daily for assessing fermentation by measuring reducing sugars and for enumerating yeast populations. Reducing sugar concentration in musts and wines was automatically measured by enzymatic methods using a commercial kit adapted to an automated ECHO instrument (Logotech) following the manufacturer's instructions. Prior to sampling, the flasks were stirred for homogeneity.

2.3. Cell extract preparation and RNA extraction

Ten milliliters of each must fermentation culture was taken when 50% of sugars were consumed. Cells were harvested by centrifugation and resuspended in 0.5 ml of LETS buffer (200 mM LiCl, 20 mM EDTA, 20 mM Tris–HCl pH 8.0, 0.4% sodium dodecyl sulphate) and transferred to a screw-cap microcentrifuge tube containing 0.5 ml of phenol and 0.5 ml of glass beads (acid-washed beads, 0.4 mm diameter). The suspension was mixed vigorously three times for 1 min each time in a Mini Bead-Beater homogenizer (BioSpec). After centrifugation at 17,900 × *g* for 10 min (at 4 °C), the upper phase was extracted successively with phenol–chloroform–isoamyl alcohol (25:24:1) and chloroform–isoamyl alcohol (24:1). These steps were repeated until the interface between the aqueous and organic layers was clear after centrifugation. Total nucleic acids were precipitated with two volumes of ice-cold 100% ethanol and 0.1 volume of 3.0 M potassium acetate, left at −20 °C for 3 h, and pelleted at 15,000 rpm for 15 min at 4 °C. The pellet was washed with 70% ethanol, dried, and resuspended in 50 μl of sterile diethyl pyrocarbonate-treated water. Total RNA was purified with RNeasy mini column (QIAGEN) according to the manufacturer's instructions. RNA total concentration was quantified by A_{260}, the ratio of A_{260} to A_{280} was used to estimate RNA purity. Nucleic acid contamination was also checked on a 1% agarose gel.

2.4. Synthesis of [^{33}P] dCTP-labeled cDNA

Ten micrograms of purified RNA, 1 μl of oligo$(dT)_{15}$ (Roche Molecular Biochemical, Mannheim, Germany) and 1 μl of RNA inhibitor (RNA Guard, Amersham Biosciences, Roosendaal, The Netherlands) were mixed with water to obtain a final volume to 10 μl, heated for 10 min at 70 °C and then chilled on ice. The following components were added: first strand buffer (Invitrogen, Carlsbad, Canada), 0.1 M dithiothreitol (Invitrogen, Carlsbad, Canada), 0.8 mM dATP, dGTP and dTTP, 200 U SuperScript II reverse transcriptase, RNase H (Invitrogen, Carlsbad, Canada), 50 μCi [^{33}P]dCTP (Hartmann Analytic, Braunschweig, Germany) and water to give a final volume of 30 μl. The mixture was incubated at 43 °C for 1 h. The cDNA was then purified with the MicroSpin S300 HR columns (Amersham Biosciences, Roosendaal, The Netherlands) according to the manufacturer's instructions and quantified by liquid scintillation counter.

2.5. Macroarray hybridization

S. cerevisiae macrochip membranes made by the DNA chips service of the Universitat de Valencia, Spain (http://scsie.uv.es/chipsdna, Alberola et al., 2004) were washed 30 min in 0.5% SDS at 80 °C. Membranes were prehybridized for 3 h with 5 ml of saline sodium citrate (SSC) based hybridization solution (5× SSC, 5× Denhart's, 0.5% SDS, 50% deionized formamide and 100 μg herring sperm DNA/ml) at 42 °C in a roller oven. This temperature permits heterologous hybridization of closely related species or hybrids using *S. cerevisiae* chips, as calculated taking into account *S. kudriavzevii* homology (Belloch et al., 2009). The purified cDNA probe was denatured for 5 min at 100 °C, cooled on ice and 3×10^6 dpm/ml was added to the pre-hybridization mixture. After overnight hybridization at 35 °C, filters were rinsed with 2× SSC–0.1% SDS at 65 °C for 20 min. Filters were then transferred to a plastic container and washed with 0.2× SSC–0.1% SDS at room temperature for 15 min. Filters were exposed to a high-resolution BAS-MP 2040S imaging plate (Fuji, Kyoto, Japan) for 48 h and scanned in a phosphor-imager (FLA-3000; Fuji).

To diminish quantification and reproducibility problems, all filters used came from the same batch. Filters were stripped by pouring boiling stripping buffer (5.0 mM sodium phosphate, pH 7.5, 0.1% SDS) two or three times over the membrane. The first time, the stripping buffer was left at 65 °C for 20 min, while after the second and third washes the filters were left at room temperature. To ensure that

radioactivity had been eliminated, filters were checked with a Geiger counter. Membranes were not dried at any time to avoid permanent fixation of the radioactivity.

Hybridization experiments were performed in triplicate. The replicates were done with RNA samples from three different bottles of parallel experiments performed at the same time (see Section 2.2) to avoid differences in cell growth and handling.

2.6. Data analysis and spot validation

Spot intensities were quantified as artefact-removed-density (ARM), background and background-corrected ARM density (sARM) with the Arrays Vision Software (Imaging Research, Canada). Triplicate macroarray data were downloaded to Microsoft Excel files. To normalize the signal intensity of each replicate hybridization set, spot intensities were normalized against total spot intensity. To determine fold changes between pairs of yeast strains in the same hybridization conditions, average spots were normalized against highly conserved genes in the genus *Saccharomyces* as histones H2A and H2B (HTA1-2 and HTB1-2) and translation elongation factor EF-1α (TEF1-2) (Kurtzman and Robnett, 2003). These genes were chosen because previous studies carried out in our laboratory have shown that all strains used in this study (*Saccharomyces* species strains and hybrids) have the same number of copies from these genes and have more than 98% nucleotide homology. Log (base 2) average values were used to calculate the fold change. A False Discovery Rate of 5% was used to select for significant data using Bonferroni correction for false positives. Microarray data was validated with qPCR analyzing four randomly selected genes for each of the strains. The pair of primers used was AAGCACTTGGAAGGTATCTC-GTCCAAGTAGTAAGATGGCT for *GPM1*, GACACTTTGGCAGAACGTG-AGTAAGTACCTTCCTTACCG for *ILV3*, AGGGTGTCTTCCAAGGTG-TCGTTCAACTTGGACTTGG for *HSP12* and CAACTGGTGGTTTCTACC-CACCTAATCTCTTCTTGGC for *ADH4*. In all 24 comparisons except one (*ADH4* in T73), the values obtained with qPCR and microarray were not significantly different ($p<0.01$). The signal Log (base 2) ratio of ≥ 1.5 was used to select the increased data set and ≤ -1.5 for the decreased data set. "GO (Gene Ontology) terms" were investigated by using Term Finder online software from *Saccharomyces* Genome Database (http://www.yeastgenome.org). Functional groups with Bonferroni corrected p-values below 0.05 were considered significant.

3. Results

3.1. Global gene expression analysis of W27 hybrid and Saccharomyces reference species

We monitored global gene expression in the hybrid wine strain W27 in Macabeo juice alcoholic fermentation at 18 °C, compared with their reference species *S. cerevisiae* (Lalvin T73) and *S. kudriavzevii* (IFO 1802). Cells were harvested at the end of logarithmic phase just before entry into stationary phase, when 50% of sugar was consumed. Transcriptomic values were normalized and significant data was selected for further analysis. The expression comparison between cells of W27 hybrid and both reference species (T73 and IFO 1802) in enological condition identifies a relatively small number of genes with different expression levels (Supplementary Table 1). The ratios between W27 and the reference strains showed that the hybrid strain increased expression of 156 genes compared to *S. cerevisiae* and 55 genes compared to *S. kudriavzevii*, 48 of which are common in both reference strains. These 48 genes were similarly expressed between the reference strains, except for *DOT1*, *PGK1* and 4 ribosomal genes (*RPL23B*, *RPP1B*, *RPS9B*, *RPP2B*), which were up-regulated in *S. cerevisiae*. On the contrary, few genes were down-regulated in the hybrid strain W27 compared with the reference species, 36 for *S. kudriavzevii* and three for *S. cerevisiae*, two of which were common.

GO terms constitute an interesting tool to find significant overrepresented functional groups in a gene set. This functional analysis was done with up- and down-regulated genes in W27, compared with each reference species (Table 1). Up-regulated genes in the W27 hybrid compared to *S. kudriavzevii* IFO 1802 strain have functions related to cellular amino acid biosynthetic processes and cellular aromatic compound metabolic processes. Both functional groups shared many genes and are mainly involved in glycolysis, fermentation and amino acid biosynthesis. W27 also showed increased transcription of genes related to translational elongation. Interestingly, comparison of W27 with *S. cerevisiae* T73 strain, also revealed overrepresentation of functional groups related to translation (regulation of translation, maturation of SSU-rRNA from tricistronic rRNA transcript, peptidyl-amino acid modification and translational elongation) and amino acid biosynthesis (organic acid metabolic process).

Genes down-regulated in the W27 hybrid compared with both reference species did not show any significantly overrepresented functional group.

3.2. Expression analysis of genes related to cold adaptation, glycerol and aroma production

To better understand the phenotypic differences observed between the hybrid and reference strains of the species *S. cerevisiae* and *S. kudriavzevii* we focus on groups of genes. Table 2 shows the ratio of significantly up-regulated genes related with cold stress adaptation and membrane fluidity. The hybrid W27 induced eight genes related to these functional groups compared with *S. cerevisiae* reference species. When expression of these W27 hybrid genes was compared with *S. kudriavzevii*, significant differences were observed in three genes. The W27 up-regulated genes mainly belonged to the *PAU*, *DAN/TIR* families, linked to cold shock adaptation, while one gene (*ARE1*) is involved in sterol metabolic processes.

Two genes involved in glycerol biosynthesis showed a significantly higher expression in the hybrid W27 related to *S. cerevisiae* reference species (Table 3). *PGI1* and *TPI1* genes showed a four-fold increase in expression compared to *S. cerevisiae* reference strain (T73). The first gene (*PGI1*) encodes the glycolytic enzyme hexose monophosphate isomerase, which

Table 1
Enrichment of functional categories in genes up-regulated in W27 with respect to parental strains in Macabeo must fermentation at 18 °C.

Category	p-Value	Genes
W27 versus IFO 1802		
Cellular amino acid biosynthetic process (GO:0008652)	1.30E−08	*CYS3 ARO4 ILV6 LEU2 HIS4 LYS20 ARO1 UTR4 PRO3 SER3*
Translational elongation (GO:0006414)	9.72E−06	*CYS3 GCV3 TIP1 ADH5 ILV6 KAR4 RPP1B LYS20 RPP2B EFT2 YEL047C TIR1*
Cellular aromatic compound metabolic process (GO:0006725)	1.86E−05	*ADH5 ARO4 ILV6 HIS4 LYS20 ARO1*
W27 versus T73		
Regulation of translation (GO:0006417)	8.38E−12	*TPD3 CDC19 RPS8A OLA1 RPL4A RPS11B TEF2 GRS1 RPS9B PGI1 ARO4 HIS4 NAT1 RPL13A RPS16B RPL4B RPS11A ARO1 EFT2 RPL12B RPL12A RPS24A PAB1*
Maturation of SSU-rRNA from tricistronic rRNA transcript (GO:0000462)	1.20E−06	*RPS8A RPS11B RPS6B RPS9B RPS14A PWP2 RPS16B FAL1 RPS11A RPS13 RPS24A*
Peptidyl-amino acid modification (GO:0018193)	3.78E−06	*PRS4 HMT1 PHO88 SWD3 ARO4 SSH1 NAT1 RPP1A PHO13 GUK1 WBP1*
Organic acid metabolic process (GO:0006082)	1.81E−05	*CDC19 ADH5 PDB1 ARO4 ILV6 HIS4 PGK1 ARO1 HOM2 RIB3*
Translational elongation (GO:0006414)	1.94E−05	*EFB1 CYS3 GCV3 BNA4 TIP1 TEF2 ADH5 ILV6 KAR4 RPP1A RPP1B HEM13 TPI1 RPP2B EFT2 RIB3 SEC20 GEA2 YEL047C SIT1 TIR1*

Table 2
Upregulated genes involved in cold stress adaptation and sterol biosynthesis in W27 hybrid compared to *S. cerevisiae* and *S. kudriavzevii* reference species.[a]

Cold stress adaptation and sterol biosynthesis		Hybrid expression ratio with respect to reference species		Reference species expression ratio
ORF	Gene name	*S. cerevisiae* (W27/T73)	*S. kudriavzevii* (W27/IFO1802)	IFO1802/T73
Cold stress				
YBR301W	*DAN3*	**2.02**	−0.50	**2.52**
YEL049W	*PAU2*	**2.96**	−0.12	**3.08**
YCR104W	*PAU3*	**3.64**	0.40	**3.24**
YFL020C	*PAU5*	**3.34**	0.30	**3.04**
YAR020C	*PAU7*	**2.57**	0.01	**2.56**
YBR067C	*TIP1*	**2.64**	**1.87**	0.77
YER011W	*TIR1*	**4.29**	**3.45**	0.84
Sterol metabolism and membrane fluidity				
YCR048W	*ARE1*	**2.32**	**2.36**	−0.39

[a] Bold type: ratio <−1.5 or >1.5. Values represent Log_2(ratio).

catalyses the conversion of glucose-6-phosphate to fructose-6-phosphate. The second gene (*TPI1*), with triose-phosphate isomerase activity, is also involved in glycerol biosynthesis and could be responsible for the higher glycerol level in wine fermented by hybrid W27 compared with wine fermented by *S. cerevisiae* T73 strain.

Table 4 shows the genes related with the aroma compound biosynthetic pathways that were expressed more or less three-fold (Log ratio > 1.5) in the W27 hybrid, compared with each *Saccharomyces* reference species. A significant number of genes (19) involved in higher alcohol and ester biosyntheses were up-regulated in the hybrid strain compared with both reference species, showing increased levels when compared with the *S. cerevisiae* reference species. Three genes (*ADH5*, *ARO4* and *LEU2*) were expressed more than six-fold (Log ratio > 3) in W27 as compared to the *S. cerevisiae* reference species (Table 4). These genes were specifically involved in ester biosynthesis and in branched chain, sulfur, aroma and other amino acid metabolism.

4. Discussion

The *S. cerevisiae* × *S. kudriavzevii* hybrid strain W27 seemed better adapted to low and intermediate temperature fermentative conditions, with aromatic compound profile that differed from their reference strains. Genome composition and structure of W27 seem to be constituted by two copies of most of the chromosomes, and three copies of chromosomes V and XIV, with a partial loss of the *S. kudriavzevii* genome and recombinations in three chromosomes (Belloch et al., 2009; González et al., 2008; Querol and Bond, 2009). This finding suggests that *S. cerevisiae* × *S. kudriavzevii* hybrid strains tend to be genetically similar to *S. cerevisiae* with some additional genes (or chromosomes) from *S. kudriavzevii*, including mitochondrial DNA, which confers an adaptive advantage. In this work we described how all these genomic rearrangements have affected the transcriptional regulation allowing hybrid adaptation to a specific environment. Moreover, it will shed light on the changes in fermentation performance and wine chemical composition produced by the wine hybrid W27, as compared with reference strains of the species *S. cerevisiae* and *S. kudriavzevii*.

Global gene expression analysis showed that a significant number of genes were overexpressed in the hybrid compared with both reference species. Functional analysis showed significant overrepresentation of functional groups mainly involved in translation or carboxylic acid and amino acid metabolism. This analysis indicates that, under these enological conditions, W27 hybrid has a new transcriptional profile that is significantly different to the expression patterns of the reference species. Moreover, we observed up-regulation of special key genes involved in cold adaptation, glycerol production and aromatic compound biosynthesis. We propose that the genomic rearrangements that occurred after hybrid formation led to the appearance of this new transcriptional pattern in fermentative conditions. Although we cannot rule out the possibility that this new transcriptional pattern was present in the W27 ancient parent strains some data point against this idea. The wine strain population is very homogenous compared to the whole *S. cerevisiae* variants, suggesting an adaptation to specific conditions (Liti et al., 2009). Since W27 genome contains mainly *S. cerevisiae* genes and they are wine alleles (Belloch et al., 2009), we suggest that ancient parent wine strain has a very similar gene expression to wine reference strain T73.

Table 3
Upregulated genes involved in glycerol metabolism in W27 hybrid compared to *S. cerevisiae* and *S. kudriavzevii* reference species.[a]

Glycerol metabolism		Hybrid expression level ratio with respect to reference species		Reference species expression ratio
ORF	Gene name	*S. cerevisiae* (W27/T73)	*S. kudriavzevii* (W27/IFO1802)	IFO1802/T73
YBR196C	*PGI1*	**2.12**	1.33	0.79
YDR050C	*TPI1*	**2.21**	0.78	1.42

[a] Bold type: ratio <−1.5 or >1.5. Values represent Log_2(ratio).

Table 4
Upregulated genes involved in higher alcohol and ester biosyntheses in W27 hybrid compared to *S. cerevisiae* and *S. kudriavzevii* reference species.[a]

Higher alcohol and ester biosyntheses		Hybrid expression level ratio with respect to reference species		Reference species expression ratio
ORF	Gene name	*S. cerevisiae* (W27/T73)	*S. kudriavzevii* (W27/IFO1802)	IFO1802/T73
Acetaldehyde/acetate/ethanol metabolism				
YBR145W	*ADH5*	**3.03**	**2.40**	0.64
Branched chain amino acid metabolism				
YCL009C	*ILV6*	**2.17**	**2.02**	0.15
YCL018W	*LEU2*	**3.48**	**3.27**	0.20
Sulfur amino acid metabolism				
YAL012W	*CYS3*	**2.73**	**2.54**	0.18
YDR158W	*HOM2*	**1.61**	0.39	1.22
YER043C	*SAH1*	**2.00**	**1.65**	0.35
YDR502C	*SAM2*	**1.75**	0.40	1.35
Aromatic amino acid metabolism				
YDR127W	*ARO1*	**1.76**	**1.87**	−0.11
YBR249C	*ARO4*	**3.327**	**2.902**	0.43
Higher alcohol metabolism/carbonyl compounds				
YAL060W	*BDH1*	0.93	**1.62**	−0.69
YAL044C	*GCV3*	**1.90**	**2.11**	−0.21
YBR121C	*GRS1*	**1.90**	1.09	0.81
YBR115C	*LYS2*	**1.71**	−0.31	1.40
YER023W	*PRO3*	**2.49**	**1.74**	0.74
YER081W	*SER3*	**1.92**	**2.44**	0.52
YBR263W	*SHM1*	**2.53**	**2.11**	0.42
YDL244W	*THI13*	**1.63**	−0.66	**2.29**
YBR006W	*UGA2*	**2.57**	**1.90**	0.66
YEL038W	*UTR4*	**1.80**	**1.66**	0.14

[a] Bold type: ratio <−1.5 or >1.5. Values represent Log_2(ratio).

4.1. Cold adaptation and glycerol synthesis

Cold stress adaptation of the W27 hybrid has been confirmed (Belloch et al., 2008). In agreement with our results, these data suggest that wine yeast *S. cerevisiae* and *S. kudriavzevii* hybrids might have inherited the ability to grow at lower temperatures from the non-*S. cerevisiae* reference species. In our work, the hybrid W27 overexpressed eight genes widely described in *S. cerevisiae* as cold-shock genes. However, six

of them were also expressed in the reference species *S. kudriavzevii*, again suggesting that the hybrid has maintained cold stress transcriptional regulation and its consequent cold adaptation from this species. Interestingly, *ARE1* gene was up-regulated in W27 hybrid compared with both reference species. The gene *ARE1* encodes an acyl-CoA:sterol acyltransferase, an enzyme that contributes to sterol esterification activity in the absence of oxygen. This fact could represent a new mechanism to adapt the cellular membranes to cold conditions.

Glycerol is a beneficial by-product of ethanol fermentation, which also seems to play an important role for low-temperature tolerance in yeasts (Izawa et al., 2004). In fact, cryotolerant wine strains produce more glycerol than non-cryotolerant yeasts (Bertolini et al., 1996; Castellari et al., 1994). In a recent study (Arroyo-Lopez et al., 2010) we showed that commercial wine yeasts T73 and W27 exhibited an optimum for glycerol production at 24 and 21 °C, respectively. However, the response of the non-wine yeast *S. kudriavzevii* IFO1802 was totally different and its production decreased linearly as temperature increased. The different behavioral patterns exhibited by the wild yeast *S. kudriavzevii* IFO 1802 in response to temperature variations would suggest that this species could have a different regulation system for glycerol synthesis compared to wine yeasts T73 and W27, as a result of adaptation to growing at lower temperatures (Arroyo-Lopez et al., 2010). Indeed, glycerol concentration produced by W27 hybrid strain in Macabeo juice at 18 °C was an intermediate value compared with both reference species strains (González et al., 2007). In this work, we observed that two genes (*PGI1* and *TPI1*) involved in glycerol biosynthesis were overexpressed in the W27 hybrid related to *S. cerevisiae* (T73) reference species. These data support the idea that the W27 hybrid could have inherited different glycerol regulations than *S. cerevisiae* strains allowing an increase in the glycerol level in wine fermentations at 18 °C.

4.2. Aroma compounds

Several interesting aroma compounds such as higher alcohols and esters (Abbas, 2006; Bisson and Karpel, 2010), were found in higher or intermediate concentrations in wine fermented by the hybrid W27, as compared with wines fermented with each *Saccharomyces* reference species (González et al., 2007). Higher alcohols are produced from amino acid catabolism via the well characterized Ehrlich pathway. Esters are formed from the condensation reaction between an alcohol and an acyl group carried by coenzyme A (Abbas, 2006; Bisson and Karpel, 2010).

In our study, 19 genes involved in amino acid metabolism and ester formation showed significant changes in expression level in the hybrid W27 compared with their *Saccharomyces* reference species. One of them was *ADH5*, involved in ethanol, acetaldehyde and acetate production (Delneri et al., 1999; Hazelwood et al., 2008). Moreover, *ADH5* expression has been correlated with increased levels of isobutanol, isobutyric acid, propionic acid, acetic acid and 2-phenyl ethanol (Rossouw et al., 2008). One of the most up-regulated genes was *LEU2*, which encodes a beta-isopropylmalate dehydrogenase that catalyzes the third step in the leucine biosynthesis pathway (Andreadis et al., 1984; Hsu and Schimmel, 1984) and has been strongly correlated with compounds such as isobutanol (Rossouw et al., 2008). The W27 hybrid strain also overexpressed the gene *UGA2* encoding a succinate semialdehyde dehydrogenase, involved in the utilization of gamma-aminobutyrate (GABA) as a nitrogen source and part of the 4-aminobutyrate and glutamate degradation. This gene could be correlated with the high concentration of diethyl succinate observed in wines fermented with W27 hybrid strain (González et al., 2007). *EHT1* is another interesting gene overexpressed in the W27 hybrid. This gene encodes an acyl-coenzymeA:ethanol O-acyltransferase that plays a role in medium-chain fatty acid ethyl ester biosynthesis, but also exerts known esterase activity (Saerens et al., 2006). *EHT1* expression has been positively correlated with ethyl acetate, ethyl caprylate and isoamyl acetate. Interestingly, *EHT1* expression has been fairly strongly inversely correlated with 2-phenylethyl acetate and octanoic acid, as well as a weaker yet significant inverse correlation with decanoic acid (Rossouw et al., 2008). The up-regulation of these three genes (*ADH5*, *LEU2* and *EHT1*), and others, could explain the increased production of aroma compounds observed in W27 hybrid strain. It is interesting to highlight that, similarly to cold stress and glycerol genes, most of the 19 up-regulated genes showed an increase compared to both reference species. These data also support emergence of a new transcriptional pattern after the formation and adaptation of the hybrid to fermentative conditions.

Our results demonstrate that wine yeast natural hybrid W27 is well adapted to fermentation conditions at low temperatures and produces higher amount of aroma compounds than the reference species by up-regulation of some key genes involved in those physiological processes. Our results also support the idea that the construction of laboratory hybrids using selected reference *Saccharomyces* strains of interesting species represents a promising method for genetic improvement of wine yeasts.

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