Expression of stress response genes in wine strains with different fermentative behavior

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Abstract

The response to adverse growth conditions in yeast depends on the activation of signal transduction pathways which result in transcriptional changes and synthesis of protective molecules. During wine production, yeast cells are affected by a plethora of stress situations. In this work we have analyzed the fermentative behavior in synthetic must for six different wine yeast strains. In addition, we followed the expression of several stress response genes during the first half of the vinification. Our results indicate that common patterns of stress response are found among all the strains, but also that a subset of genes are differentially expressed according to the fermentative behavior of the various strains. Particularly, in the strains with the most severe fermentative problems, higher (and in some cases maintained) mRNA levels of many genes were found. The relevance of an equilibrium between stress response and growth efficiency during wine fermentation is discussed.

Keywords: Gene expression; Stress response; Vinification; Saccharomyces cerevisiae; Fermentative behavior; Wine yeasts

1. Introduction

Throughout the alcoholic fermentation step in wine production, yeasts belonging mainly to Saccharomyces cerevisiae produce ethanol, carbon dioxide and minor metabolites (relevant to the flavour of the final product) from the grape must. Nowadays, in most wineries, selected strains are inoculated into the must as active dry yeast. The inoculated strain is usually responsible for the fermentation and constitutes most of the total yeast population at the end of the process [1]. This strategy of inoculation from starters has several advantages, such as a decrease in the lag phase, significant reduction of the influence of naturally occurring yeast strains, rapid and complete grape must fermentation, and hence allows for a higher degree of wine reproducibility [2,3].

In wine production (not only in the fermentation step) yeast cells are exposed simultaneously and sequentially to a plethora of stress conditions [2,4]. Throughout the preparation of the dry active yeast to be inoculated into the must, cells are affected by oxidation, osmotic stress, ionic stress, raised temperatures, organic acids, alcohols, nutrient limitation and desiccation. When these yeast cells are inoculated into the must, they have to resist a considerable hyperosmotic stress, due to the high sugar concentration in this medium (approximately 200 g l⁻¹ of an equimolecular mixture of glucose and fructose). Moreover, as fermentation proceeds, they must cope with progressive nutrient depletion, and ethanol production. Depending on the winemaking process, other stress factors during this stage can be temperature increase, cold stress, high CO₂ levels, high SO₂ concentrations, and the presence of competing organisms [2]. In order to carry out wine production, yeast cells should be able to detect and respond to these stress conditions without substantial viability loss [2].

Stress response mechanisms involve sensor systems and signal transduction pathways which activate transcription factors, resulting in significant changes in the
mRNA levels of many genes [5–8]. Yeast cells develop a common response to different environmental conditions, that has been named environmental-stress response (ESR) [7]. In fact, microarray studies have indicated that the expression of approximately 900 genes is altered in S. cerevisiae in response to a variety of stressful environmental transitions. Many of the genes induced during this response are targets of Msn2p and Msn4p zinc finger proteins [9]. Under specific stress conditions, other transcription factors are also involved in the modulation of the gene expression. The heat shock response in yeast is primarily governed by the heat shock factor [10], which activates the transcription of genes containing heat shock elements in their promoter, such as those encoding heat shock proteins (Hsps). The levels of these proteins increase under several conditions (i.e. heat shock, osmotic, ethanol and acetaldehyde stress [11–13]), depending on the activity of Msn2/4p and/or Hsf1p transcription factors. The response to hyperosmotic stress is mainly controlled by the high-osmolarity glycerol (HOG) pathway [14], which determines the activation of several transcription factors (Hot1p, Sko1p, Msn2/4p and Smp1p [8,15]). This activation results in increased expression of osmo-induced genes, such GPD1 [7,15]. On the other hand, the Yap1p transcription factor co-regulates, in association with Skn7p, the expression of several stress genes (for instance TRX2) under conditions of oxidative stress [16,17].

Several gene expression studies have been performed during vinifications carried out by a selected wine yeast, considering the whole transcriptome or a particular subsets of genes [18–21]. In this work, we analyzed the expression of stress-related genes during vinifications carried out by six different S. cerevisiae strains previously used in our laboratory [22]. We found, for all the strains, some common patterns of gene expression, mainly related to the response to osmotic stress at the beginning of the fermentation and to the entry in stationary phase. However, we also detected among strains with different fermentative behavior some interesting differences in the steady-state levels of a subset of the mRNAs considered.

### Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>Origin</th>
<th>Fermentative behavior</th>
</tr>
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<tbody>
<tr>
<td>48</td>
<td>S. cerevisiae</td>
<td>Commercial (Pris Mouse Biostar)</td>
<td>Fermentation completed</td>
</tr>
<tr>
<td>16</td>
<td>S. cerevisiae</td>
<td>Commercial (Gist Brocades)</td>
<td>Fermentation completed</td>
</tr>
<tr>
<td>27</td>
<td>S. cerevisiae</td>
<td>Commercial (Lallemand Inc.)</td>
<td>Fermentation stopped (15–30 g l(^{-1}))(^a)</td>
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<tr>
<td>IFI 278</td>
<td>S. cerevisiae</td>
<td>Albariño (9.7(^{\circ}) in grape must)(^a)</td>
<td>Fermentation stopped (20–30 g l(^{-1}))(^b)</td>
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<tr>
<td>IFI 391</td>
<td>S. cerevisiae</td>
<td>Serrada (7(^{\circ}) in grape must)(^a)</td>
<td>Fermentation stopped (10–20 g l(^{-1}))(^b)</td>
</tr>
<tr>
<td>IFI 367</td>
<td>S. cerevisiae</td>
<td>Mentrida (8.7(^{\circ}) in grape must)(^a)</td>
<td>Fermentation stopped (40–60 g l(^{-1}))(^b)</td>
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</tbody>
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\(^a\) Information supplied by the Instituto de Fermentaciones Industriales.

\(^b\) Amount of residual sugar remaining in the must when glucose and fructose consumption was finished.
(1–3 days), and the number of colony-forming units per milliliter of cell culture was determined.

2.4. RNA analysis

RNA isolation was carried out as described previously [24]. RNA was spectrophotometrically quantified and the quality and integrity were checked by agarose gel electrophoresis. To obtain labeled cDNA, a solution containing 9 µg of total RNA, 360 pmol of primer for cDNA synthesis (p(dT)15, Roche), 40 U of RNaseOUT (Invitrogen) and 1.5 µl of a dNTPs mixture (containing a 16 mM concentration for dATP, dGTP and dTTP and 100 µM for dCTP), was initially incubated at 72 °C for 10 min and then quickly transferred to ice. Afterwards 6 µl of 5 × RT buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂) and 3 µl of 0.1 M DTT were added. The mix was incubated for 2 min at 42 °C and, after adding 200 U of reverse transcriptase (Invitrogen) and 1 µl of [33P]dCTP (10 µCi µl⁻¹, Amersham), for another 2 h at 42 °C. The enzyme was inactivated by adding to the solution 1 µl of 0.5 M EDTA. Labeled cDNA was purified by MicroSpin S-300 columns (Amersham).

2.5. Genomic DNA isolation and labeling

The genomic DNA isolation for each strain was carried out following the protocol “Yeast DNA miniprep (40 ml)” described in Kaiser et al. [25]. For this purpose, yeast strains were grown in YPD liquid medium overnight. DNA was spectrophotometrically quantified and the quality and integrity were checked by agarose gel electrophoresis. Labeling with radioactive [33P]dCTP (10 µCi µl⁻¹, Amersham) and random oligonucleotides (High Prime kit, Roche) was carried out following the manufacturer’s instructions. Labeled DNA was purified by MicroSpin S-300 columns (Amersham).

2.6. Analysis of the cDNA and genomic DNA levels

Analysis of the cDNA and genomic DNA levels obtained was performed by hybridization on nylon filters containing duplicated PCR products corresponding to the genes used for our study (filters fabricated by the DNA chip service of the Universitat de València, Spain, http://scsie.uv.es/scsie-docs/chipsdna/chipsdna.html). These genes (Table 2) were selected for their high induction under stress treatments in different laboratory conditions. Each filter also contained eight positive controls of yeast genomic DNA used for signal normalization and eight negative controls of E. coli DNA used for background substraction. To hybridize filters, 3 × 10⁶ dpm (determined in a liquid scintillation counter Wallac 1409) of purified labeled cDNA or 5 × 10⁶ dpm of purified labeled genomic DNA were used. Prehybridization of the filters was

<table>
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<th>Genes analyzed in this work and their induction by stress</th>
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<td><strong>Heat shock</strong></td>
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<tr>
<td><strong>Genes encoding Hsps</strong></td>
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<td>HSP26</td>
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<td>SSA3</td>
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<td><strong>Osmotic response genes</strong></td>
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<td>GPD1</td>
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<td>GLO1</td>
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<td>HOR7</td>
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<td><strong>Oxidative response genes</strong></td>
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<td>GRE2</td>
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<td>CUP1</td>
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<td>TXR2</td>
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<td><strong>Nutrient starvation response genes</strong></td>
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<td>SPI1</td>
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<td>COX6</td>
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<td>YGP1</td>
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<td>CAR1</td>
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<td><strong>Glycogen and trehalose metabolism genes</strong></td>
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<td><strong>Genes involved in protein degradation</strong></td>
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<td>UBI4</td>
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<td>UBC5</td>
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<tr>
<td><strong>Others</strong></td>
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<td>ACT1</td>
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* indicates induction reported by global gene expression analysis; × indicates induction detected by Northern analysis or reporter fusions expression.
carried out for 3-4 h at 42 °C in prehybridization solution (5 × SSPE, 5 × Denhart, 0.5% (w/v) SDS, 200 μg ml⁻¹ of sonicated salmon sperm DNA). SSPE (20 ×) contained 3.6 M NaCl, 0.2 M Na₂HPO₄, 0.02 M EDTA, pH 7.7. Denhart composition (100 ×) was as follows: 2% (w/v) BSA, 2% (w/v) Ficoll, 2% (w/v) PVP. The hybridization was carried out at 42 °C overnight in 5-ml tubes containing 1 ml of hybridization solution (prehybridization solution with 50% (v/v) deionized formamide and 10% (w/v) dextran sulfate). The filters were then washed once with 0.1% (w/v) SDS, 2 × SSPE at 65 °C for 20 min, and twice with 0.1% (w/v) SDS, 0.2 × SSPE at 65 °C for 30 min each.

Quantification of the signal was performed by exposing the filters on imaging plates (Fujifilm), processing in an Image Reader FLA 5000 (Fujifilm), and detection of the signal in each spot using the Image Gauge V4.0 software. Several exposures were carried out for each hybridization, avoiding saturation of the signals.

For hybridization with other samples, the labeled probe was stripped after quantitation. For this purpose filters were boiled in a solution containing Na₂HPO₄ 5 mM and 0.1% (w/v) SDS.

2.7. Data normalization

Microarray data were downloaded into a MicroSoft Excel file. An average of the quantification of the signal obtained from E. coli genomic DNAs was used as background and was subtracted from the value corresponding to each spot (values lower than 1.5 times the background were not considered for further analysis). The result obtained was then divided by an average of the values corresponding to the yeast genomic DNAs to normalize the signal intensity of each hybridization. The same filter was used to hybridize all the cDNA samples from the same culture and one sample of genomic DNA in order to eliminate possible variations due to different quantities of probes in each filter. Thereafter, the data obtained after division of each spot along the vinification by each spot of the filter hybridized with genomic DNA were used to obtain an average of the duplicated spots for each gene, and then for each strain an average for the different vinification cultures. All calculations involving expression ratios were carried out on the Log₂ scale by computing averages and standard deviations (SDs). A negative or positive Log₂ value indicated reduced or induced transcript levels (relative to genomic DNA), respectively.

3. Results

3.1. Fermentative behavior of yeast strains

Six strains (Table 1) were selected because of their differences in fermentative behavior in vinifications carried out with synthetic must of low nitrogen content [22]. According to those experiments and conditions, strains 16 and 48 were able to complete the vinification, while strains 27 and IFI 278 left some residual sugar, and strains IFI 367 and IFI 391 consumed very low amounts of glucose.

In the present work microvinification experiments in MS300 medium [20] were carried out with all these strains, and the data obtained are shown in Fig. 1. On the basis of this analysis, they were separated into two groups, although the second one was quite heterogeneous. The first group contained strains 16 and 48, and the second strains 27, IFI 278, IFI 367 and IFI 391. Strains in the first group were able to complete vinification, while strains in the second group left different amounts of residual sugar in the must (from 15 to 50 g l⁻¹, panel A). For all strains analyzed, over 10 mM assimilable nitrogen was left at the end of the vinification, indicating that nitrogen limitation was not a problem under the conditions tested.

For strains 16 and 48, the highest OD₆₀₀ was found around 240 and 358 h after inoculation, respectively (panel B), the highest number of viable cells appeared approximately at 149 h (panel C), and the nitrogen source was consumed at a higher rate during the first 149 h (panel D).

Regarding the second group, strain 27 showed a profile of growth, number of viable cells and nitrogen consumption similar to that found for group 1, but the vinifications were not completed.

The case of strain IFI 278 is quite particular because the highest OD₆₀₀ was found at 149 h (with a lower growth rate beginning at 118 h), and an important drop occurred between 240 and 358 h. This result is consistent with the rapid decrease in the number of viable cells after 118 h (in fact the number of viable cells after 358 h was very low). This strain also consumed nitrogen compounds more quickly than the others during the first 45 h, and no significant amounts were taken up later on.

The IFI 391 strain showed another particular behavior. On average, the growth pattern was similar to that of strains 16, 48 and 27, but it consumed lower nitrogen amounts than these strains. Besides, after 149 h, the number of viable cells was lower than for the other strains, and extremely low after 358 h (as for IFI 278).

In the case of strain IFI 367 important fermentative problems were found. As in the case of strains 27, IFI 278 and IFI 391, strain IFI 367 was not able to complete vinifications under these conditions, but it left the highest amount of residual sugar (approximately 50 g l⁻¹). The optical density was the lowest among the strains tested, and the viability was extremely low from the beginning of the process.

3.2. Selection of time points during vinification for gene expression analysis

Samples were taken at several time points to follow changes in gene expression and characterize the stress
response during vinification and the particular traits of these strains regarding fermentative behavior. According to the results mentioned above, the main differences among strains in maximal nitrogen consumption, OD$_{600}$, and number of viable cells were detected during the first half of the vinification. Thus, the following time points were selected: 1 h (when cells were affected by osmotic stress due to the high sugar concentration in the must), 6 h (the cells were beginning exponential growth), 45 h (exponential growth, with approximately 160–190 mg l$^{-1}$ of residual sugar), 149 h (entry in stationary phase for most of the strains and approximately 85–130 g l$^{-1}$ of remaining sugar) and 240 h. At the last time point, most of the strains showed the maximal OD$_{600}$ and viability loss, and a considerable sugar consumption (from 50 g l$^{-1}$ of sugar left in the medium for strains 16, IFI 278 and IFI 391 to 95 g l$^{-1}$ for IFI 367) and ethanol production (6–8% (v/v) depending on the strain, data not shown).

As the stress response should play a role in the ability to survive during the vinification and to carry out the process properly, we focused on the expression of several stress-induced genes at these time points. 19 Genes induced by several forms of stress and with peculiarities in their regulatory mechanisms were selected, and filters containing duplicated PCR products corresponding to these genes were fabricated (see Section 2). An ACT1 gene PCR product was also included in these filters because of its constitutive expression during vinification [20]. Table 2 contains the list of genes analyzed and references describing their regulation and/or induction by stress. We used the information in the literature to choose genes involved in the response to several forms of stress, although in some cases they are associated with the response to specific conditions such osmotic stress, oxidative stress, or nutrient starvation. Genes involved in glycogen and trehalose metabolism, processes also

Fig. 1. Sugar consumption (panel A), OD$_{600}$ (panel B), number of viable cells (panel C), and nitrogen consumption (panel D) throughout vinifications carried out with several commercial and non-commercial strains (see Table 1) in synthetic must with the composition and growth conditions described in Section 2.
related to stress responses, were also considered. Still other stress genes were used in preliminary experiments, but due to the very low detection levels they were not considered for the complete analysis.

3.3. Expression of genes encoding heat shock proteins

The expression during vinification of five genes encoding heat shock proteins was considered: *HSP26*, *HSP82*, *SSA3*, *STI1* (Fig. 2) and *SSA4* (data not shown). The *HSP26*, *SSA3*, *STI1* genes encode cytoplasmic heat shock proteins involved in protein folding and stress response [26]. *HSP82* encodes the chaperone Hsp90, an ATPase transporter located in the plasma membrane.

Comparison of the data obtained about the expression of these genes during vinification indicated some common traits. The maximal levels were detected for most genes and strains at 240 h, when the culture was in stationary phase, the only exception being *SSA3*. On the other hand the lowest mRNA levels were usually found 6 h after inoculation, although the differences with other times and among strains depended on the gene considered. For *HSP26* the levels were much lower than at 45 h, but in the case of *HSP82* and *STI1* they were quite similar at 6 and 45 h (except for the IFI 367 strain). Again *SSA3* showed a different pattern than the other genes in this sense.

Regarding differences between the strains, IFI 391 showed the highest mRNA steady-state levels, practically at any time considered and for any of these genes, with the exception of *SSA3*. For IFI 367 high levels similar to those found for IFI 391 were detected in some cases, for instance at 6 h for *HSP26* and at 1 h for *STI1*. It is worth mentioning that for all these genes, strain 27 showed lower levels at several time points. In the case of *HSP26* at 45, 149 and 240 h; for *HSP82* at 149 and 240 h and for *SSA3* and *STI1* at 149 h.

In the case of *SSA3* and *SSA4* we found clear differences in the expression pattern between the two groups of strains classified according to their fermentative behavior (as shown for *SSA3* in Fig. 2). Strains of group 1 showed lower levels at 1 h than the strains of group 2. In the former strains (16 and 48), mRNA levels were quite similar during the period considered, and a significant increase was detected at 240 h only for strain 16. In the strains of the second group, levels were higher at 1 h and lower at 149 h; constant levels were observed only in IFI 391.

3.4. Expression of osmotic and oxidative stress-induced genes

We considered three genes induced by osmotic stress (*GPD1*, *GLO1* and *HOR7*), two genes activated by oxidative stress (*CUP1* and *TRX2*), and one gene that responds to both stress conditions (*GRE2*). The *GPD1* and

![Fig. 2. Expression during the first half of vinification of some genes encoding heat shock proteins. Data show the mRNA levels for each strain, time and gene. Quantitation and normalization has been carried out as described in Section 2.](https://academic.oup.com/femsyr/article-abstract/4/7/699/512192)
GLO1 genes are involved in glycerol metabolism [27], CUP1 participates in copper ion binding, TRX2 encodes thioredoxin, and GRE2 a protein similar to plant dihydroflavonol-4-reductase. The molecular function of HOR7 is unknown. Results of these experiments are shown in Fig. 3.

The general consideration of the data indicates that the highest mRNA levels of osmotic response genes were found at 1 h (for GPD1, which correlates with previous results obtained in our laboratory [28], and for GRE2) or 149–240 h (for HOR7 and GLO1), and for most genes and strains the lowest values were detected at 6 h. The decrease in most of the strains between 1 and 6 h indicates that the glycerol production was able to counteract the osmotic stress. However, the difference in the mRNA levels at these two time points depends on the gene and strain considered, being lower in the case of GPD1 gene and for IFI 391 and IFI 367 strains. For IFI 367, no significant changes in GPD1 expression were detected during the first two days. In the case of IFI 391, the mRNA levels of this gene remained quite constant during the entire experiment (as happened for GRE2) and were even higher at 6 h than at 1 h.

In the case of the CUP1 gene, the results were clearly different. During vinification, its mRNA levels were higher at 6 h than at 1 h, especially for IFI 367 and IFI 391, that show the highest levels among the strains tested. Interestingly, the CUP1 and TRX2 (data not shown) steady-state mRNA levels were very low during most of the time points analyzed in the IFI 278 strain.

3.5. Expression of nutrient starvation response genes

Many yeast genes are regulated by sugar or nitrogen starvation and by entry into stationary phase [7]. In this work we considered the genes SPI1, COX6, CAR1 (Fig. 4) and YGP1 (data not shown) as indicative of the response to this stress condition. SPI1 and YGP1 encode cell wall proteins and are induced in mutants affecting cell wall construction [29]. COX6 encodes mitochondrial cytochrome oxidase, and the CAR1 gene arginase, whose enzymatic activity is induced under nitrogen limiting conditions [30].

The pattern of expression of these genes during vinification was quite similar to that found for osmotic response genes: the lowest mRNA levels were usually found at 6 h, and the highest around 149–240 h (or at 1 h, in the case of the CAR1 gene for IFI 367 and IFI 391 strains).

Regarding differences between genes and strains, IFI 391 and IFI 367, together with 16, presented the highest values among all strains. The strains IFI 278 and 27 showed the lowest expression levels of COX6 and CAR1, respectively.

Fig. 3. Expression during the first half of vinification of some genes induced by osmotic and/or oxidative stress. Data shown, quantitation and normalization as in Fig. 2.
3.6. Other stress-induced genes involved in different cellular processes

Many yeast genes involved in stress responses participate in processes related with protection mechanisms (acetate, acetaldehyde, glycogen and trehalose metabolism, protein degradation, signal transduction pathways). Particularly glycogen and trehalose have been implicated in the response to a wide variety of stressful environments, and genes affecting the synthesis and degradation of these carbohydrates are concomitantly induced as part of the ESR [7]. For this reason, we selected *GSY2* (encoding the predominant form of glycogen synthase) and *ATH1* (encoding the acidic trehalase) for further analysis.

According to the data obtained in our experiments (Fig. 5), the *GSY2* mRNA steady-state levels increased as fermentation proceeded. The highest values were found for strain 16 during the first hours and for strain...
IFI 391 beginning at 149 h. In this case, it is interesting to note the low levels found for strain IFI 278 at all the time points considered, and especially at 1h. For the ATP1 gene, we observed differences between the various strains during the vinification. However, the most significant result was that strains IFI 367 and IFI 391 showed higher levels than the other strains at 6 h and, especially for IFI 391, these levels were maintained during the whole time course. As for GSY2, the lowest expression levels were found in the IFI 278 strain.

In this work we have also studied the expression of two genes involved in protein degradation: UBC5 (encoding a ubiquitin-conjugating enzyme) and UBI4 (encoding a ubiquitin-tagging enzyme). For these genes the mRNA levels were significantly lower in the IFI 278 strain than in the others considered (data not shown).

We analyzed the expression of the ACT1 gene during the first half of the vinification. The mRNA levels of this gene were quite similar for all the strains, in agreement with the results obtained by Riou et al. [20] no significant changes in the expression of this gene were detected throughout the period considered (data not shown).

4. Discussion

In the last few years several studies have focused on gene expression analysis during vinification. In some cases, particular subsets of genes [19,20,20,21,28,31-33] have been considered. In other cases, global gene expression experiments under defined growth conditions have been carried out [18,21,34]. In each of these studies one particular yeast strain adapted to the process of winemaking has been used. In this work, we considered the changes in the expression of a number of stress response genes during the first half of vinifications conducted by several commercial and non-commercial yeast strains with different fermentative behavior. This approach provides a better understanding of the overall response to stress during this industrial process, and of differences in the patterns of gene expression between strains with different fermentative capabilities.

To better understand the most relevant findings of this work, we will discuss the data obtained with the strains that are able to complete vinification, and then the differences found in each one of the strains in the second group will be introduced.

The overall consideration of the data obtained in our analyses with the strains 16 and 48 indicates that the lowest levels for most of the stress response genes considered were found around 6 h after inoculation, and the highest levels approximately 149 h after inoculation and thereafter. The interpretation of these results is facilitated by considering the stress conditions taking place during vinification. With the approach followed for yeast inoculation in this work, cells were affected at the beginning of the vinification by dilution, changes in aeration, and hyperosmotic environment [22,28]. Under conditions of hyperosmotic stress, one of the adaptive responses of yeast cells is the production and accumulation of glycerol [8,35]. Indeed, in our experiments genes involved in glycerol metabolism (GLO1 and GPD1) showed very high levels 1 h after inoculation. This same pattern was observed with other stress response genes (HSP26, SSA3/4, STI1, HOR7, GRE2, SPIII, COX6, CAR1, YGP1). The transcriptional response to this form of stress is quick and transient [36]. Probably for this reason the expression of all these genes was significantly lower a few hours later during vinification (6 h in our experiments), when yeast cells had adapted to this adverse situation by increasing the intracellular levels of glycerol. Actually, a recent analysis of gene expression during the beginning of vinification indicated a maximal induction of GPD1 during vinification 1 h after yeast inoculation and a significant decrease in the mRNA levels of this gene 2 h later [28].

On the other hand, an increase in the mRNA levels occurred approximately 149 h after inoculation for most genes, and was especially clear in the case of SPIII, YGP1 and COX6. This result can be explained by the entry into stationary phase (Fig. 1), as most of these genes are induced by this growth stage under laboratory conditions [7] and during vinifications [19,20]. Particularly, the analysis of gene expression by Rossignol et al. [21] has revealed the induction of many stress genes (including some considered in our work, such HSP26, SSA3, SPIII, YGP1) at the entry into stationary phase or just after. These authors consider that stationary phase corresponds to a stress condition which is likely amplified due to the ethanol accumulation. Under the conditions used in our work, the reason for entry into stationary phase is not clear, given that it took place when neither sugar nor nitrogen was limiting. However, ethanol production could affect the assimilation of nitrogen compounds during vinification [37], or, due to its toxicity for several cellular processes, could provoke the transcriptional activation of several genes [11,13].

The information obtained with the other strains considered in this work reveals the general patterns described for strains 16 and 48, but interesting peculiarities appear. In the case of strain 27 (another commercial strain), growth rate, nitrogen consumption and viability were similar to those found for 16 and 48, although a small amount of residual sugar was left in the final product (Fig. 1, panel A). This strain has been selected for the elaboration of several wines because the fermentative problems are not substantial, and it provides interesting flavour properties to these wines (Anne Julien Ortiz and Antonio Palacios, personal communication). The differences in the expression profiles with the other two strains were small. However, for CUP1, CAR1 and all the HSP
genes considered, strain 27 showed lower levels than strains 16 and 48 at some time points.

The case of the IFI 278 strain is very interesting. This strain showed a considerable increase in viability and sugar and nitrogen consumption up to 118 h. However, after this time, the growth diminished and the viability drops. Our analysis indicates that for some stress genes, the expression levels were significantly lower in this strain than in the others considered. For instance, as shown in Fig. 5, the expression of GSY2 (encoding glycogen synthase) and ATIII (encoding acidic trehalase) was significantly lower in this strain. Production of storage carbohydrates is important for yeast cells to respond to stress conditions and is regulated at the transcriptional level in the ESR [7]. This result could hence indicate an unfavorable situation for this strain to cope with stress conditions which could result in viability loss. On the other hand, the mRNA levels of genes related with protein degradation (UBI4 and UBC5), or other stress conditions (SPI1, COX6, CUP1 and TRX2) were also lower at some time points than in the case of the other strains discussed so far.

In the case of the IFI 367 and IFI 391, the fermentative behavior shows some significant differences in viability during the first hours and in the final amount of residual sugar. However the pattern of nitrogen uptake and the low number of viable cells after 240 h is similar. Besides, both strains consumed very low amounts of glucose in vinifications carried out with synthetic must of low nitrogen content [21]. The data obtained in gene expression analyses could provide some explanations for these observations. In these strains, higher mRNA levels of almost all genes considered were found for IFI 367, and especially, IFI 391. Although this could indicate that these strains are capable of executing a stress response to cope with adverse conditions, the levels achieved, and especially their maintenance over long time periods, in some cases (for instance in the osmotic stress response genes GPD1, GLO1, HOR7 and GRE2) could have adverse effects. Under stress conditions, growth repression also occurs, probably by Msn2/4p-dependent Yak1p activation [38]. Interestingly, wine yeast cells in which Msn2p has been overexpressed, show higher resistance to stress under laboratory conditions, but also growth defects during vinification (P. Carrasco, J.E. Pérez-Ortín and M. del Olmo, unpublished data).

It is worth mentioning that hybridization experiments with genomic DNA isolated from these strains indicated differences in the number of copies of some of the genes considered (data not shown). This is the case for GPD1, GRE2, CUP1, STII, SPI1, GLO1, YGP1 and CAR1. Of particular interest is the lower copy number found for CUP1 and SPI1, in the IFI 367 and IFI 391 strains, and for GRE2 and STII in IFI 391. This result suggests that the higher mRNA levels found for these genes in these strains could be related to regulatory mechanisms involved in controlling the expression of these genes (HOG and PKA pathways, for instance).

Recently we have found a correlation between the fermentative behavior of several yeast strains and the resistance to two stress conditions relevant to wine production: oxidative and ethanol stress [22]. In fact, we have proposed the determination of the resistance to these stress situations under laboratory conditions as a method for the initial selection of wine yeasts, together with other physiological traits suggested by Degré [39]. The data obtained in the experiments shown in this work indicate that the expression of stress response genes during vinification can help to understand differences in fermentative behavior among strains. According to these results we suggest that the ability to properly conduct wine fermentation may depend on a complicated equilibrium between the plethora of stress conditions to which yeast cells should respond, and the requirement of growth efficiency. In the case of strains with slight defects in fermentation (i.e. 27 and IFI 278), the low levels of expression of some genes (mainly related to nutrient starvation, oxidative stress and protein degradation) could impair the necessary synthesis of protective molecules under adverse conditions. On the other hand, in the case of strains with problems during vinification (IFI 367 and IFI 391), the high mRNA levels of these genes and the incapacity to restore the physiological non-stressed condition (for instance after hyperosmotic shock) could lower the growth rate during vinification. It would be of great interest to continue experiments of gene expression in other wine yeast strains to further explore this possibility. Since in the present experiments we only have considered the expression of stress response genes, we cannot rule out the possibility that variations in the mRNA levels of other genes could be related with fermentative differences among these strains.

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