Fermentative Conditions Modulating Sweetness in Dry Wines: Genetics and Environmental Factors Influencing the Expression Level of the *Saccharomyces cerevisiae* *HSP12* Gene

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Abstract:

Yeast lees influence the organoleptic properties of wines by increasing their sweet taste. This effect is in part due to the protein Hsp12p, which is regulated by different stress response pathways in *Saccharomyces cerevisiae*. This work investigated the genetics and environmental factors influencing the expression level of the *HSP12* gene in an enological context. RT-qPCR confirmed that the *HSP12* expression level is regulated by temperature change and ethanol content during the alcoholic fermentation but not by the sugar content. Moreover, this gene shows an important variation according to the yeast strain used. For the first time yeast strain is demonstrated to play an important role in the perception of sweetness in red wine due to post-fermentation lees autolysis. Interestingly, a correlation between the expression level of *HSP12* and the sweetness perception was found using yeast strains of different origins. All of the findings provide new insights on the contribution of yeast to wine taste.

Keywords: *HSP12*, wine sweetness, expression level, protein, strain, *Saccharomyces cerevisiae*, taste

INTRODUCTION

Wine quality depends on both its complexity of odors and its harmony of flavors. This taste balance is mainly due to interactions between sweet, sour, and bitter tastes. In particular, sweetness plays a decisive role in wine perception and is likely to contribute to the pleasure of the tasters.[1] Dry wines containing <2 g/L (below the perception threshold) of residual sugar exhibit paradoxically a subtle sweet taste.[2] Previous works have established that ethanol, glycerol,[3–5] polysaccharides,[4] and nucleotides[6] have no direct influence on sweetness perception in the concentration ranges at which they are found in dry wines. However, sensory studies have shown that wine sweetness increases during yeast lees autolysis[5] and oak wood aging;[7] therefore, the molecular determinants explaining these phenomena have been, at least in part, elucidated.[5,8]

In particular, we demonstrated in a previous work that *HSP12* has a direct or an indirect role in the increase of wine sweetness observed during yeast lees autolysis.[5] Biochemical and sensory experiments suggested that a peptide fraction of Hsp12p causes the sweet perception. We also demonstrated that sweetness intensity was linked to the amount of yeast lees in contact with wine during autolysis. All of these findings suggest that Hsp12p is involved in the gain of sweetness conferred by yeast lees.

The *HSP12* gene encodes a small heat-shock protein described as a LEA-like protein in *Saccharomyces cerevisiae*.[9] This protein is crucial for the survival of a variety of stress conditions including heat shock,[10] osmotic[11] and oxidative stresses,[10] freezing,[12] or desiccation.[13] Hsp12p protein is also implied in the extension of lifespan in dietary restriction[14] as well as in biofilm formation.[15] Despite numerous studies, the molecular function of this protein was not completely elucidated. Unlike other small heat shock proteins, *HSP12* displays a negligible molecular chaperone activity regarding protein aggregation.[14] In contrast, structural and biochemical studies suggested that *HSP12* interacts with lipid molecules and has a membrane-stabilizing role acting as a “lipid chaperone”.[10,14,16] The expression level of this gene has been widely investigated due to its important response to a wide set of environmental stresses.[17] Many studies reported an induction of *HSP12* expression related to stress in both laboratory[18] and biotechnological contexts.[12,19] In the wine alcoholic fermentation context, *HSP12* appears to be highly expressed[20] despite the high sugar concentration found in wine, which is supposed to down-regulate its expression.[21,22] The high expression level of *HSP12* in the enological context is likely due to high ethanol concentration[23] and the stationary growth

phase of fermenting yeast. Moreover, important physicochemical parameters in wine fermentation can modulate the expression level of this gene, including low and high temperatures,[24,25] nitrogen content,[26] and lipid concentration.[27] Besides the impact of environmental conditions, an important variability in global gene expression can be found within yeast strains[28,29] and species.[30] For *HSP12*, this general trend is verified both for the mRNA expression[31,32] and for the protein production level.[30]

These previous works emphasize that the great range of *HSP12* expression variation and the subsequent amount of protein may be linked to the resulting sweetness perception in wine.[5] However, these experiments were carried out in many different conditions (media, strain), impairing the elucidation of the main factors modulating the *Hsp12p* expression in wine context. This study proposes to investigate the effect of some genetic and environmental factors affecting *HSP12* expression.

MATERIALS AND METHODS

Yeast Strains and Growth Media Used. The *S. cerevisiae* strains used are listed in Table 1. The construction of the strain Δ°*hsp12* was described in Marchal et al.[5] This strain is isogenic to the commercial starter Zymaflore Fx10 (Laffort, France) referenced as H4-1D in a previous work.[33] The other strains are commercial starters form Laffort or homothallic monosporic clones previously referenced by Blein-Nicolas et al.[30]. Yeast strains were propagated at 28 °C on YPD medium (1% yeast extract, 2% peptone, dextrose 2%) containing 2% agar to prepare a solid medium.

Fermentation on KP Medium. Fermentations were carried out using the model synthetic medium (KP medium) buffered at pH 3.30 and containing 190 mg (N)/L of assimilable nitrogen.[34] Fermenting sugars are constituted by an equimolar mix of glucose and fructose and were set to 240 g/L. Other concentrations of sugars were used (120, 160, or 300 g/L) for one experiment as specified in the text. Precultures were run for 24 h at 24 °C under orbital agitation (150 rpm) in the fermentation media filtered through a 0.45 μm nitrate cellulose membrane (Millipore, Molsheim, France) and diluted 1:1 with Milli-Q water. The inoculum concentration was 10^6 viable cells/mL. Fermentations were run in closed 350 mL glass reactors, locked to maintain anaerobiosis, with permanent stirring (300 rpm). Fermentations were carried out at 26 °C, but other temperatures (12, 18, 22, or 31 °C) were used for an experiment as specified in the text.

The amount of CO_2 released (g/L) was monitored daily by the weight loss of the bioreactors. The raw fermentation kinetics data were smoothed by a Loess function and plotted using the R program as previously described.[35] All fermentations were done in triplicate.

Yeast Cell Collection and Quantification. Cells were collected from fermenting KP medium at nearly 46 g/L of CO_2 produced for analyzed cell expression. In the expression monitoring experiment, samples were collected at different fermentation points (30, 46, 55, 66, or 76 g/L of CO_2 produced). The cell concentration was quantified using a flow cytometer (QuantaSC MPL, Beckman Coulter, Fullerton, CA, USA), equipped with a 488 nm laser (22 mW) and a 670 nm long-pass filter. Samples were diluted in McIlvaine buffer, pH 4.0 (0.1 M citric acid, 0.2 M sodium phosphate dibasic), and propidium iodide was added (0.3% v/v) to stain dead cells (FL3 channel).

Quantification of *HSP12* Expression Level. Extractions of mRNA and cDNA synthesis were carried out as previously described (Thibon et al. 2008).[36] Briefly, 1×10^7 cells were harvested, washed, and lysed using a Fastprep FP120 apparatus (MP Biomedicals, Solon, OH, USA). RNA was extracted using Tri reagent (Sigma, L'Isle d'Abeau Chesnes, France), and DNA contamination was treated using a DNA-free Kit (Ambion Inc., Austin, TX, USA); RNAs were retrotranscribed into cDNAs using the iScriptTM cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The extracted RNA was quantified using the ND-1000 UV–visible light spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The absence of contaminant genomic DNA in RNA preparations was verified using RNA as a template in real-time PCR assays. Each cDNA sample was analyzed two times independently by quantitative real-time PCR using iCycler iQ (Bio-Rad). Gene transcripts were amplified by using the following primers: *HSP12* p321, GGGTGTCTTCCAAGGTGTC; p322, TTGGCGGCTCCCATGTAATC (151 bp); *ALG9* p605, CACGGATAGTGGCTTTGGTGAACAATTAC; p606, TATGATTATCTGGCAGCAGGAAAGAACTTGGG (163 bp); *ACT1* p323, TACCGGCCAAATCGATTCTC; p904, TACTGGTATTGTTTTGGATTCC (123 bp). The *ALG9* gene was used as a second reference gene as proposed previously.[37] Real-time quantitative PCRs (qPCRs) were carried out using the iQ SYBR Green Super Mix (Bio-Rad). Primers were added at a concentration of 0.3 mM each. The PCR program used was as follows: 3 min at 95 °C for initial denaturation, then 40 cycles of 10 s at 95 °C, 30 s at TM, and 30 s at 72 °C. A final melt curve was carried out for control specific amplification by 36 cycles of 10 s starting at 65 °C, with increasing steps of 0.5 °C at each cycle. The PCR efficiencies were 80.2, 89.7, and 88.7% for *HSP12*, *ACT1*, and *ALG9*, respectively. A standard curve was

determined for each gene using genomic DNA as template, where x is the threshold cycle and y is the log value of the starting quantity (ng): *HSP12* ($y = -3.90x + 21.44$, adjusted $R^2 = 0.995$); *ACT1* ($y = -3.62x + 22.44$, adjusted $R_2 = 0.985$); *ALG9* ($y = -3.65x + 23.81$, adjusted $R_2 = 0.994$). Standard curves were obtained from eight points in triplicate, and linearity was observed from 0.0366 to 183 ng of DNA. A threshold value for the fluorescence of all samples was set manually to adjust the Ct value of standard samples in each experiment. The relative amounts of *HSP12* with respect to each reference gene were calculated from the standard curve. Expression levels obtained from both reference genes were highly correlated (Pearson correlation test $r = 0.99$, $p_{val} = 1 \times 10^{-8}$); consequently, only the *HSP12*/*ACT1* ratios are graphically presented.

Production of Wine without *Hsp12p*. A red grape juice of Merlot (Bordeaux) harvested in 2011 was used for this study. This grape must obtained by thermoextraction was supplemented with ammonium sulfate and glucose plus fructose to obtain a final concentration of 210 mg (N)/L of assimilable nitrogen and 210 g/L of sugar. A batch of 6 L of this grape must was fermented with the strain Δ°hsp12 at 24 °C until completion of the fermentation. The final amount of residual sugar at the end of alcoholic fermentation was <2 g/L. This wine was then dispatched in 1 L glass vessel and supplemented with the biomass of different strains freshly harvested by centrifugation (5 min, 8000*g*) and washed two times with sterile water (4 °C). The concentration of the added biomass in wine was adjusted to 2×10^8 cells/mL to remain close to the yeast concentration usually found in enology.[35] After 24 h, the viability of the cell was controlled by platting 1 mL of wine on YPD. Fewer than 10^3 viable cells/mL were recovered.

Yeast Autolysis. Yeast autolysis of the different strains (Fx10, Δ°hsp12, D2, W1, B1, VL3, F33, and XMC30) took place at 32 °C during 10 days without light or stirring. At the end of this period, the wines were centrifuged (4000 rpm, 15 min) to eliminate yeast lees and kept at 4 °C for 24 h in sterilized glass bottles inerted with CO_2 until tasting.

Wine Tasting. All of the tasting sessions took place in a specific room equipped with individual booths and air-conditioned at 20 °C. The samples (20 mL) were presented at 18 °C in normalized dark glasses coded with random numbers. All of the panelists (17 tasters aged from 21 to 64 years) were wine-tasting specialists or winemakers and had been previously informed of the nature and risks associated with the investigation. The presentation order of the glasses was randomly changed between each taster.

Prior to the formal evaluation of wines, the panelists performed training sessions. In a first session, a hydroethanolic solution (12% alcohol by vol, 3 g/L of tartaric acid, pH adjusted to 3.5) with sucrose adjusted at different concentrations (0, 3, 6, and 12 g/L) was presented to

the tasters. In a second session, a red wine (Bordeaux 2011, 12.9% alcohol by vol, 0.3 g/L glucose + fructose) was used for a similar test. For both sessions, tasters were asked to classify the wines following the intensity of sweetness. A Page test was used to interpret these results.[38]

For the formal evaluation of the yeast strain impact on sweetness, the wines were tasted the day after centrifugation. The tasters were asked to rate the wines on a scale from 0 to 7. The statistical interpretation was based on an analysis of variance (ANOVA) according to the recommendations of the International Organization for Standardization.[39]

Statistical and Graphical Analyses. All of the statistical and graphical analyses were carried out using the R program version 2.15.1.[40] The variation of each trait analyzed was estimated by an analysis of variance (ANOVA) according to the model in figure captions. For each variable, the homogeneity of the variance was assessed using a Levene test by means of R's car package, as well as the normality of residues distribution using a Shapiro test. Duncan's multiple comparison was used to determine which means differ significantly (Duncan's multiple comparison, $\alpha = 0.05$) using the *agricolae* package. When required, a Wilcoxon test was used with at least three independent repetitions.

RESULTS AND DISCUSSION

***HSP12* Expression Correlates with the Progression of Alcoholic Fermentation.** Our study investigated the genetic and environmental factors affecting the expression level of *HSP12* during the alcoholic fermentation. First, we monitored by quantitative PCR the *HSP12* mRNA level of two commercial starters (Zymaflore VL3 and Fx10) during the alcoholic fermentation carried out at 26 °C in a synthetic grape juice containing 240 g/L of sugar. The expression level of both strains was compared nearly at the same fermentation advancement (30, 46, 55, 66, and 76 g/L of CO_2 produced) to compare the strains at the same physiological stage (Figure 1A). As shown in panel B, the expression level of *HSP12* steadily increased during the fermentation process. For both strains, significant changes were observed between the earliest and latest stages (Table 2). At 55 and 76 g/L of CO_2 produced, the *HSP12* expression level differs between VL3 and Fx10 (pairwise Wilcoxon test, $\alpha = 0.05$), indicating that the strains do not respond at the same time to their changing environment (pairwise Wilcoxon test $\alpha = 0.05$).

This first experiment confirms previous studies showing the steady increase of *HSP12* expression during the alcoholic fermentation.[20] Indeed, a linear correlation between CO_2

produced and *HSP12* expression was found (Spearman $\rho = 0.71$, $p_{val} = 2.89 \times 10^{-6}$), suggesting that ethanol concentration and fermentation progression might trigger in part *HSP12* expression. The strain VL3 seems to be generally more stressed than Fx10 with an earlier induction of *HSP12* expression. Accordingly, the strain VL3 showed a longer CO_2 production kinetics, finishing 3 days after Fx10 (data not shown). This first experiment suggested that the expression level of *HSP12* is concomitant with the increasing level of ethanol in wine, corroborating the results obtained in previous studies.[23]

In aqueous solution, ethanol exhibits a sweet taste but also an antagonist bitterness.[41,42] Previous works have showed that this compound has no significant effect on wine sweetness.[3,4] In accordance with these results, we have recently demonstrated that the addition of 1.5% v/v ethanol to a wine does not increase its sweet taste.[5] These data suggest that ethanol has no direct impact on wine sweet taste. Nevertheless, some tasters frequently point out that wines with high levels of alcohol often exhibit an intense sweet taste.[2] Consequently, this empirical relationship between ethanol level and sweetness intensity could be indirect and interpreted through the effect of Hsp12p. Indeed, a higher level of ethanol could lead to an increased expression of this protein at the end of alcoholic fermentation, thus increasing the sweet taste of wine.

Temperature and Sugar Concentration Differentially Influenced *HSP12* Expression. In this section, the transcriptional response of the *HSP12* gene for osmotic pressure and temperature was investigated using the same two yeast starters (VL3 and Fx10) in a synthetic grape must. For the osmotic stress response, fermentations were carried out at 26 °C with initial sugar amount varying from 120 to 300 g/L. Fermenting yeast was sampled when the CO_2 produced reached 46 g/L. In fact, at this fermentation point, both strains showed a similar expression level (Figure 1), allowing comparison of other factors. Therefore, this point was chosen to measure the specific effect of sugar concentration by dissociating it from ethanol impact. As expected, sugar content has a strong impact on the successful completion of fermentation. Strains Fx10 and VL3 left, respectively, 9 and 43 g/L of residual sugars in the highest sugar conditions (data not shown). In contrast, we did not detect any significant changes between *HSP12* expression fold for both strains. This result suggested that sugar content in must did not affect per se the expression of this gene (Figure 2). Although the *HSP12* gene is activated by the HOG pathway,[43] this lack of expression induction due to high sugar concentration has been previously described in industrial conditions.[21]

To analyze the *HSP12* response to temperature, isothermal fermentations (12, 18, 22, 26, or 31 °C) were carried out in a synthetic must containing 240 g/L sugars. Samples of both Fx10 and VL3 were taken when the CO_2 produced reached 46 g/L. Contrary to sugar content,

the temperature drastically modified the expression level of *HSP12* (Figure 3). For both strains, high *HSP12* expression values were found at low temperature (12 °C), confirming the strong induction of this gene in cold conditions.[24] With warmer temperatures, strains responded in a different manner. At 18 °C, VL3 and Fx10 showed similar expression profiles. However, when fermentation temperature was higher, the *HSP12* expression level strongly increased for VL3 (>10-fold). These findings confirmed that the white wine starter VL3 is quite sensitive to temperature as previously shown for heat shock resistance.[31] In contrast, Fx10 showed a steady expression level of *HSP12* between 18 and 31 °C, which corroborates the good adaptation of this strain to high temperatures and its use for red grape fermentation.[33] The strong strain × temperature effect found (24% of total variance explained) by analysis of variance highlighted that yeast strains and fermentation temperature might affect drastically both the expression level of *HSP12* mRNA and its related protein amount.

Even if fermentation temperature significantly influences the *HSP12* expression, this parameter does not appear to be really relevant as a possible tool to modulate the sweetness intensity in enological conditions. In fact, fermentation temperature deeply affects the overall organoleptic profile,[44,45] and high temperatures (>30 °C) are detrimental to completion of fermentation.[33] In white winemaking, the temperature must be controlled when fermentation is run in high-capacity fermenting tanks to avoid these risks. Furthermore, low temperatures (<18 °C) drastically affect the aromatic expression of wine due to the increase of ester synthesis by yeast.[44] Thus, low temperatures are not recommended for the elaboration of complex white wines.[46] In the case of white winemaking in oak barrels, the fermentation temperatures remain close to the cellar temperature and are most of the time not controlled. Therefore, they are often higher than in stainless steel tank fermentation (from 22 to 25 °C).[46] Such a winemaking process could therefore result in higher *HSP12* expression and, combined with the release of sweet compounds from oak wood,[8] may produce a more intense sweetness in the final product.

In red winemaking, whereas the wine temperature should be maintained at 26–28 °C to avoid stuck fermentation, post-fermentation maceration is sometimes achieved at higher temperatures (30–32 °C).[46] During this period, part of the yeast biomass is still metabolically active and may have a *HSP12* expression shift that could increase the concentration of the related protein.

Yeast Strain Background Affects *HSP12* Expression during Alcoholic Fermentation. The impact of yeast strains on the expression of *HSP12* was then assayed during alcoholic fermentation. For these studies we used four commercial starters (VL3, Fx10, XMC30, and F33), the Δ°hsp12 strains (isogenic to Fx10), and three yeast strains from other

biotopes (brewery = B1, distillery = D2, and oak exudate = W1). These three last strains showed sluggish CO_2 kinetics and were unable to ferment the totality of the synthetic must (data not shown). As *HSP12* expression is linked to the relative amount of produced ethanol, all of the fermenting yeasts were sampled when the accumulative produced CO_2 was close to 46 g/L. At this sampling point, all of the strains showed a high viability (>85%) and were fermenting actively. As shown in Figure 4, the *HSP12* expression ranged about 34-fold between extreme individuals. Four distinct groups were statistically determined (Duncan test, $\alpha = 0.05$). The nonenological strains D2 and W1 expressed the highest quantity of *HSP12* mRNA, whereas some wine starters F10, Fx10, and XMC30 showed the lowest values. Nevertheless, no significant correlations were found between fermentation phenotypes and *HSP12* expression level (data not shown), which was likely due to the small set of strains studied in this work. To our knowledge, few studies investigated the expression level of several strains during the wine alcoholic fermentation. In previous studies, similar variations were found between unrelated strains.[31] However, the *HSP12* expression level was estimated after the application of a heat shock situation not frequently found in controlled enological conditions. Our results confirmed that the mRNA expression level of *HSP12* is strongly affected by the strain used.

Impact of the Yeast Strain on the Sweetness Perception after Lees Aging. Our data confirmed a strong variability for *HSP12* gene expression within yeast strains. The role of this gene product on sweetness perception suggested that yeast strain may affect the sweetness of wine during lees aging. Because yeast strains affected wine taste for many factors (i.e., acetic acid, residual sugars, esters...[47]), the direct comparison of wines fermented with different strains does not allow verifying rigorously this hypothesis. To overcome this problem, we fermented a Merlot grape juice with the strain Δ°hsp12 to obtain a red wine exempted of Hsp12p protein and related peptides. Yeast biomass of eight different strains was added to this wine, and a post-fermentative maceration was carried out for 10 days as described under Materials and Methods. The biomass was harvested at the mRNA quantification point (46 g/L CO_2) from the eight strains previously described. Because of the small quantities of residual sugars (<2 g/L), yeasts were not able to develop and were degraded following autolysis mechanisms.

The application of Page methodology (Table 3) showed that the sensory training tests were significant at 1% in both matrices. The implementation of this methodology resulted in the successful creation of a panel trained to perceive differences of sweetness intensity in both a model solution and a red wine. The results demonstrate their ability to classify wines

following sweet intensity even in a matrix such as red wine containing numerous sapid compounds that are likely to interact with sweetness perception.

The eight resulting wines obtained after the post-fermentation maceration were submitted to sensory analysis to assay their sweetness intensity. Panelists were asked to rate sweetness on a scale from 0 to 7. A two-way analysis of variance was used (Figure 5) and demonstrated that there was no "panelist effect". In contrast, the results showed a strong "yeast strain effect", allowing the discrimination of five distinct groups of sweetness intensity among the eight modalities (Duncan's test, $\alpha = 0.05$). The base wine was the same for all of these modalities, and the only difference was the yeast strain used for lees aging. Consequently, this sensory study clearly establishes that yeast strain influences the sweet taste of wine. Previous works showed that yeast strain has a strong influence on white wine aroma,[48,49] and a recent study suggests a similar effect for fruity red wine aroma revelation.[50] Here, we demonstrated for the first time that the taste of wine was also affected by the yeast strain during autolysis. This parameter appears as an effective factor to modulate wine sweetness and such sweetening effect could therefore be taken into consideration as an additional relevant criterion to select industrial yeast strains.

Moreover, the wine aged with the strain Δ°hsp12 received the lowest rate (Figure 5), whereas the one aged with W1 was perceived as the sweetest. Among the commercial starters used for red winemaking, Fx10 and XMC30 provided more sweetness to wine than F33. More generally, we observed a weak correlation between *HSP12* expression and sweetness intensity (Kendall correlation test, $T = 22$, p value = 0.0601), suggesting that Hsp12p might contribute to the sweetness modulation consecutive to yeast autolysis. The imperfect correlation observed can be due to different causes. First, the correlation was established with yeast samples collected at mid fermentation, which does not reflect exactly the final expression level of this gene. In fact, as shown in Figure 1, the *HSP12* expression level can vary drastically during the alcoholic fermentation. However, we chose this sampling point to compare strains with an active fermentation and showing the same viability level (>85%). As some strains (W1, D2) used were unable to finish the alcoholic fermentation, a sampling point at mid fermentation was the most relevant choice. This correlation should be also much clearer using more strains or by measuring directly the expression level of Hsp12p protein instead of quantifying the mRNA level. However, independent experimental data suggest that in the case of the *HSP12* gene, the mRNA and protein expression are well correlated.[51] Moreover, in a previous work we quantified by shotgun proteomics the relative abundance of Hsp12p for some strains used in this study.[30] For example, strains W1 and D2 exhibited a high expression level of Hsp12p,

whereas strain B1 showed a much lower expression; these results sustain the results found in the present study.

This work provides new insights concerning the fermentative parameters modulating wine sweetness by means of *HSP12* expression. The exact implication of the Hsp12p protein and the factors influencing its release in wine need further investigations to better control and rationalize the post-fermentative processes.

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Figures

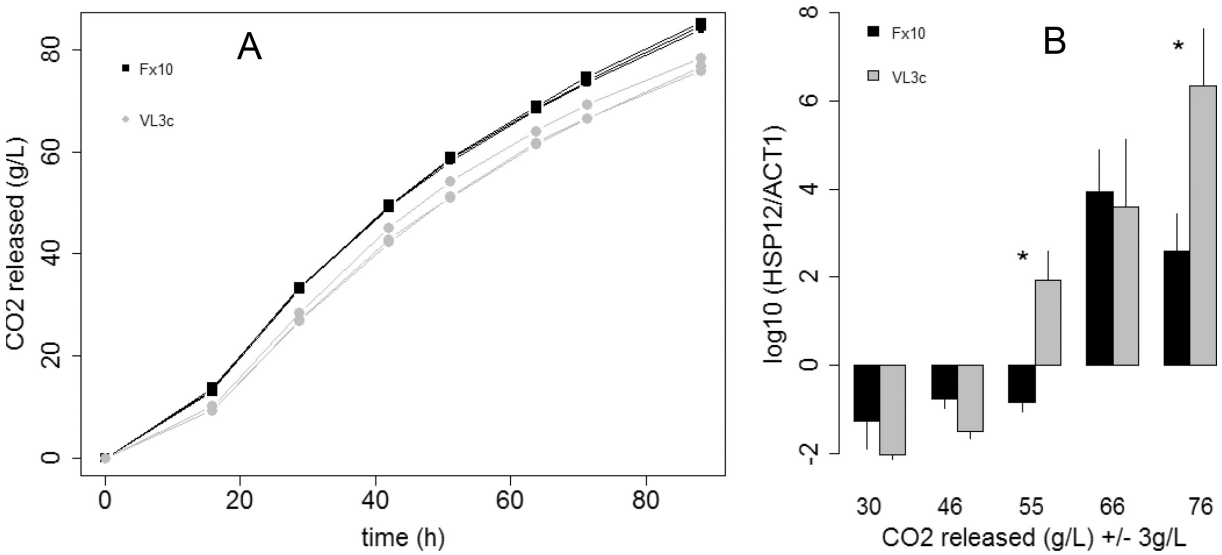

Figure 1. *HSP12* expression monitoring during alcoholic fermentation at 26 °C. (A) Yeast strains were cultivated in a synthetic medium containing 240 g/L sugar and fermented at 26 °C. The time course of CO_2 production is shown for each strain replicate. The black squares and gray circles represent the strains Fx10 and VL3, respectively. (B) Cell samples were collected at different fermentation points when the CO_2 produced reached 30, 46, 55, 66, and 76 g/L. The average of the *HSP12* expression at each time point is shown on the bar plot (expressed as the logarithm of *HSP12*/*ACT1* ratio). Bar errors indicate the standard error of the three replicates. Statistically relevant differences between strains were indicated for each collecting point by a (∗) Wilcoxon test ($\alpha = 0.05$).

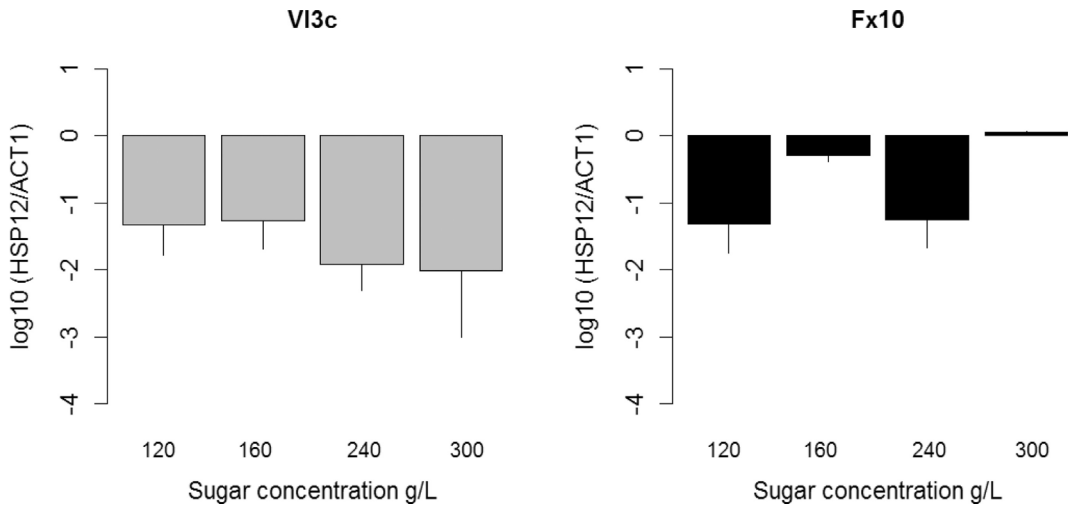

Figure 2. Sugar concentration does not affect the *HSP12* expression level at 26 °C. Fermenting biomass samples were collected at mid fermentation (46 g/L of CO_2 produced) from different synthetic musts containing 120, 160, 240 and 300 g/L initial sugar and fermented at 26 °C. The average of the *HSP12* expression is shown on the bar plot (expressed as the logarithm of *HSP12*/*ACT1* ratio). Bar errors indicate the standard error of the three replicates.

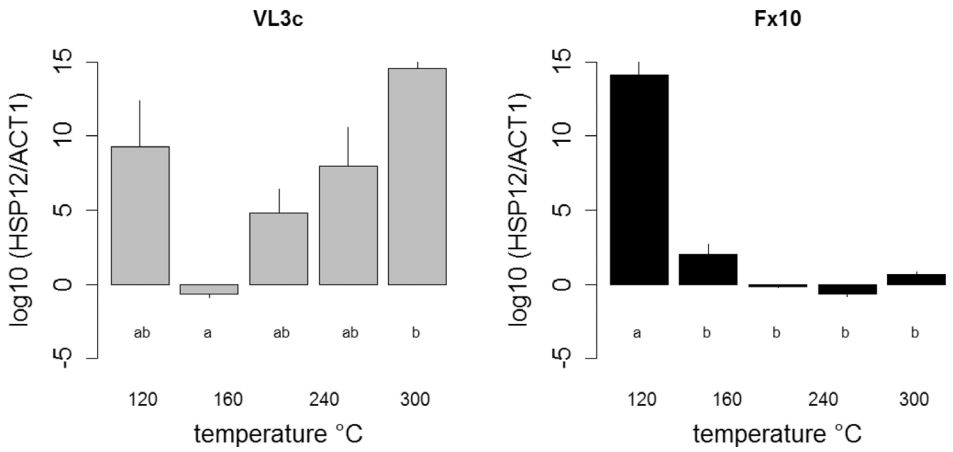

Figure 3. Temperature has a strong effect on *HSP12* expression level. Fermenting biomass samples was collected at mid fermentation (46 g/L of CO_2 produced) from a synthetic must containing 240 g/L initial sugar and fermented at different temperatures of 12, 18, 22, 26, and 31 °C. The average of the *HSP12* expression is shown on the bar plot (expressed as the logarithm of *HSP12*/*ACT1* ratio). Bar errors indicate the standard error of the three replicates. A two-way analysis of variance was carried out to estimate the strain and temperature and their interaction ($Y = \mu + \text{strain}^{i} + \text{temperature}^{j} + \varepsilon^{ij}$). A significant effect was found for yeast strains and temperature × strain interaction. The letters below the bar plot indicate groups with a statistical difference according to the linear model used (Duncan test, $\alpha = 0.05$).

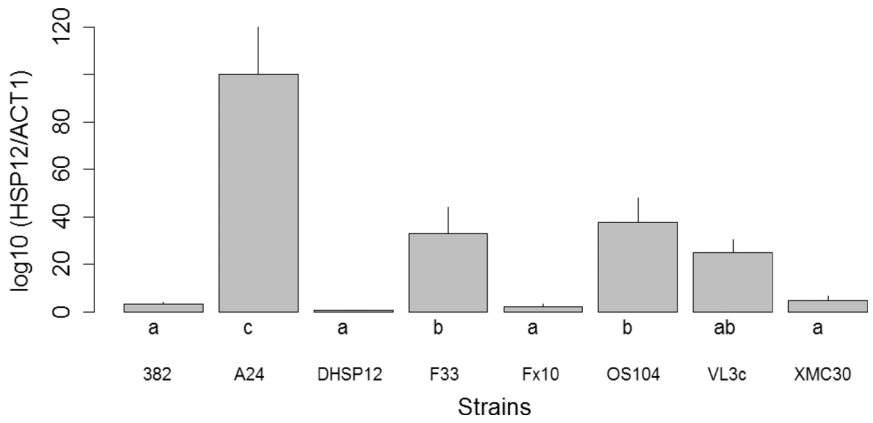

Figure 4. The *HSP12* expression level differs according to the strains at mid fermentation. Fermenting biomass of eight yeast strains was collected at mid fermentation (46 g/L of CO_2 produced) from a synthetic must containing 240 g/L initial sugar and fermented at 26 °C. The average of the *HSP12* expression is shown on the bar plot (expressed as the logarithm of *HSP12*/*ACT1* ratio). Bar errors indicate the standard error of three replicates. A one-way analysis of variance was carried out to estimate the strain effect ($Y = \mu + gene^i + \varepsilon^i$). The letters below the bar plot indicate groups with a statistical difference according to the linear model used (Duncan test, $\alpha = 0.05$).

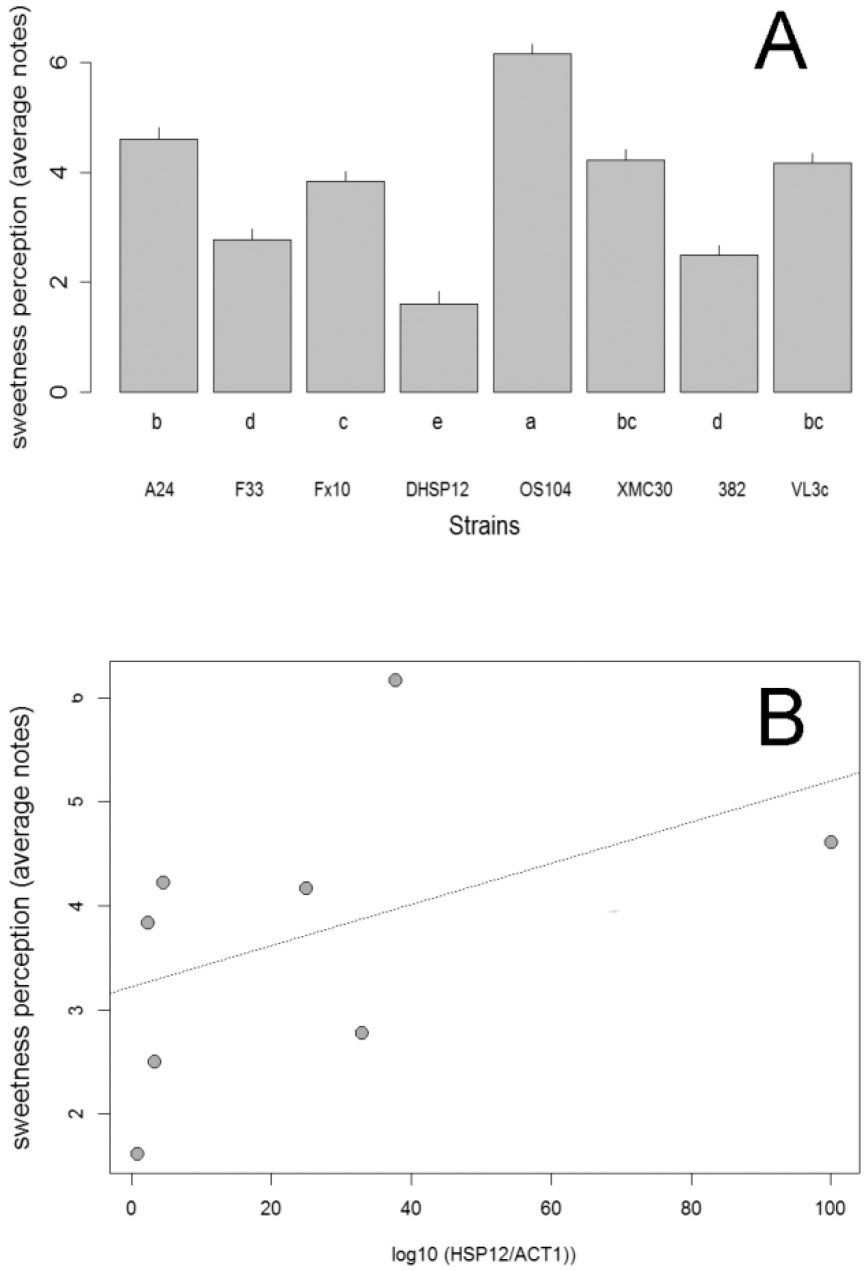

Figure 5. Wine in contact with lees obtained from different yeast strains showed marked difference in sweetness perception. (A) A wine without Hsp12p was obtained by fermenting a Merlot juice with Δ°hsp12. Yeast biomass of eight different strains was added to this wine, and aging on lees was carried out at 32 °C for 10 days. This biomass was collected from fermenting cells at 46 g/L of CO_2 produced. After wine centrifugation, a panel tasted the modalities, and the sweetness intensity was rated on a scale from 0 to 7. Error bars indicates the standard error of 17 notes. A two-way analysis of variance was used to estimate the strain effect ($Y = \mu + \text{strain}^i + \text{panel}^j + \varepsilon^{ij}$). The letters below the box plot indicate groups with a statistical difference according to the linear model used (Duncan test $\alpha = 0.05$). (B) Relationship between sweetness perception and expression level of *HSP12* (Kendall correlation test, $T = 22$, p value = 0.0601).

Tables

Table 1. Yeast Strains Used

name	genotype	origin	comment	reference
Zymaflore Fx10	diploid, HO/HO	Laffort	red wine specialized starter	Marullo et al., 2009
Δ°hsp12	diploid, *HO*/*HO*, *hsp12*Δ°/*hsp12*Δ°	lab collection		Marchal et al., 2011
D2	diploid, *HO*/*HO*	lab collection	derived from the distillery yeast alcotech 24	Blein et al., 2013
W1	diploid, *HO*/*HO*	lab collection	derived from the natural isolate YSP128	Blein et al., 2013
B1	diploid, *HO*/*HO*	lab collection	derived from the brewery yeast	Blein et al., 2013
Zymaflore VL3	diploid, *HO*/*HO*	Laffort	white wine specialized starter	
Actiflore F33	diploid, *HO*/*HO*	Laffort	generic starter	
XMC30	diploid, *HO*/*HO*	Laffort	red wine specialized starter	

Table 2. Multiple Test Comparison of the *HSP12* Expression during Alcoholic Fermentation[a]

	CO_2 produced in				
strain	**30 g/L**	**46 g/L**	**55 g/L**	**66 g/L**	**76 g/L**
Fx10	–1.25 cd	–0.75 bcd	–0.84 bcd	3.93 ab	2.58 bcd
VL3	–2.02 c	–1.50 bc	1.92 abc	3.59 ab	6.34 a

[a]Data presented are the mean of *HSP12* expression levels expressed in log 10(*HSP12*/*ACT1*) at different moments of alcoholic fermentation (CO_2 produced). For each strain, a one-way analysis of variance was carried out to estimate the sampling point effect ($Y = \mu + CO_2 i + \varepsilon(i)$. The letters beside each strain value indicate groups with a statistical difference according to the linear model used (Duncan test, $\alpha = 0.05$).

Table 3. Results of the Panel Training to Sweetness Perception[a]

matrix	R_1	R_2	R_3	R_4	L	L'[b]
model solution	30	36	47	57	471	3.86**
red wine	26	34	46	64	488	5.29**

[a]R_1, R_2, R_3, and R_4 are the sums of ranks for addition of, respectively, 0, 3, 6, and 12 g/L of sucrose. L and L' were calculated as described in ISO 8587:200638 for the Page test: $L = \sum i = 1^{p} i R_i$ and $L' = (12L - 3np(p+1)2)/(p(p+1)(n(p-1))1/2)$ (n is the number of panelists and p the number of modalities/treatments).

[b]Significance: ns, nonsignificant ($L' < 1.645$); *, significant at 5% ($L' \geq 1.645$); **, significant at 1% ($L' \geq 2.326$)