Yeast
Yeast 2013; **30**: 319–330.
Published online 22 May 2013 in Wiley Online Library
(wileyonlinelibrary.com) **DOI:** 10.1002/yea.2952

Special Issue Article

Genetic improvement of *Saccharomyces cerevisiae* wine strains for enhancing cell viability after desiccation stress

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Abstract

In the last few decades spontaneous grape must fermentations have been replaced by inoculated fermentation with *Saccharomyces cerevisiae* strains as active dry yeast (ADY). Among the essential genes previously characterized to overcome the cell-drying/rehydration process, six belong to the group of very hydrophilic proteins known as hydrophilins. Among them, only *SIP18* has shown early transcriptional response during dehydration stress. In fact, the overexpression in *S. cerevisiae* of gene *SIP18* increases cell viability after the dehydration process. The purpose of this study was to characterize dehydration stress tolerance of three wild and one commercial *S. cerevisiae* strains of wine origin. The four strains were submitted to transformation by insertion of the gene *SIP18*. Selected transformants were submitted to the cell-drying–rehydration process and yeast viability was evaluated by both viable cell count and flow cytometry. The antioxidant capacity of SIP18p was illustrated by ROS accumulation reduction after H_2O_2 attack. Growth data as cellular duplication times and lag times were calculated to estimate cell vitality after the cell rehydration process. The overexpressing *SIP18* strains showed significantly longer time of lag phase despite less time needed to stop the leakage of intracellular compounds during the rehydration process. Subsequently, the transformants were tested in inoculated grape must fermentation at laboratory scale in comparison to untransformed strains. Chemical analyses of the resultant wines indicated that no significant change for the content of secondary compounds was detected. The obtained data showed that the transformation enhances the viability of ADY without affecting fermentation efficiency and metabolic behaviour. Copyright © 2013 John Wiley & Sons, Ltd.

Received: 20 December 2012
Accepted: 16 March 2013

Keywords: **hydrophilin; dehydration stress; Saccharomyces *cerevisiae*; ADY; volatile organic compounds (VOCs)**

Introduction

The inoculum of grape must with selected *S. cerevisiae* strains is nowadays a general winemaking practice because the use of starters reduces the risk of sluggish fermentations and contributes to reproducible sensorial properties and quality in wine. Actually, the most widely used starter formulation in this sector is represented by active dry yeast (ADY). The performance of dry yeast products, including their fermentation capacity and flavour release, depends by factors related to the production, such as industrial practice during biomass propagation and desiccation (Attfield *et al.*, 2000; Pretorius, 2000). The ADYs, used in most yeast-based food industries, undergo several stress conditions during technological processes production. In *S. cerevisiae*, strain genetic constitution plays a fundamental role in desiccation tolerance. Among the genes required by the yeast to overcome dehydration stress, some of the genes encoding for the proteins termed hydrophilins are essentials (Rodríguez-Porrata *et al.*, 2012). On the other hand, the overexpression of genes

encoding hydrophilins in some yeasts confers tolerance to water-deficit conditions (Dang and Hincha, 2011; López-Martínez *et al*., 2012). Hydrophilin research in different organisms has allowed significant advances to be made towards the understanding of some of their biological properties, including their roles as antioxidants and as membrane and protein stabilizers during water stress, either by direct interaction or by acting as a molecular shield (Tunnacliffe and Wise, 2007). Among yeast hydrophilin proteins, SIP18p was characterized as an inhibitor for cell apoptosis during the dehydration–rehydration process, by its antioxidative capacity through the reduction of ROS accumulation after an H_2O_2 attack (Rodríguez-Porrata *et al*., 2012).

Due to the demanding nature of modern winemaking practice, there is a continuously growing quest for specialized *S. cerevisiae* strains (Capece *et al*., 2012), possessing a wide range of optimized or novel oenological properties. The great advances in yeast genetics has led wine microbiologists to look for alternative ways to exploit yeast natural genetic diversity or even to genetically manipulate yeast strains in order to improve specific properties. The publication of the complete *S. cerevisiae* genome (Goffeau *et al*., 1996), together with a growing arsenal of recombinant DNA technologies, led to major advances in the fields of molecular genetics, physiology and biotechnology and the construction of specialised strains, mainly by heterologous gene expression or by altered gene dosage (overexpression or deletion). Over the last 15 years, different genetically improved yeast strains useful for winemaking have been developed (reviewed by Blondin and Dequin, 1998; Dequin, 2001; Dequin *et al*., 2003; Pretorius, 2000; Pretorius and Bauer, 2002; Pretorius *et al*., 2003; Schuller and Casal, 2005). The most important target for strain improvement was related to enhancement of fermentation performance, higher ethanol tolerance, better sugar utilization and nitrogen assimilation and enhanced organoleptical properties.

The objectives of this study were to increase the dehydration tolerance in *S. cerevisiae* strains of wine origin. For this purpose, four *S. cerevisiae* strains were transformed with *SIP18* gene from the strain BY4742 (Brachmann *et al*., 1998), transcriptionally bonded to the promoter of the *GAL1* gene, in order to enhance its expression during biomass production before ADY preparation. The consequences of overexpression of gene *SIP18* for yeast viability and fermentative performance were investigated. The results obtained showed that the transformation improved the viability of ADY without affecting fermentation efficiency and metabolic behaviour.

Materials and methods

Microbial strains, plasmids and media

Table 1 summarizes the *S. cerevisiae* strains and plasmids used in this study. Recombinant DNA techniques were performed according to standard protocols (Sambrook and Russell, 2001). The synthetic *SIP18* gene was obtained by PCR and cloned into the pGREG505Δh yeast expression vector (under the control of the *GAL1* promoter) digested with *Sal*I. The plasmids, containing the *KanMX* (geneticin resistance; Gt^R) marker gene, were then used to transform the wine yeast strains. Transformants were selected by plating on synthetic glucose medium with 200 mg/ml geneticin. Gt^R transformants were selected and restreaked to obtain single colonies, which were confirmed by PCR using the primer pair: GALFw, 5′-GAAAAAACCCCGGATTCTAG-3′; and CYCRv, 5′-ATAACTAATTACATGACTCGAG-3′) and by testing for the loss of the *KanMX* marker. The PCR fragments were obtained using BY4742 genomic DNA as a template together with the primer pairs: SIP18F, 5′-GAATTCGATATCAAGCTTATCGATACCGTCGACAATGTCTAACATGATGAATAA-3′; and SIP18R, 5′-GCGTGACATAACTAATTACATGACTCGAGGTCGACTTATTTTTTCATGTTTTCGT-3′. The amplification reactions contained single-strength PCR buffer (Roche, Mannheim, Germany), 1.25 mM dNTPs, 1.0 mM $MgCl_2$, 0.3 μM each primer, 2 ng/μl template DNA and 3.5 U DNA polymerase (Roche) in a total volume of 100 μl. All the reactions were carried out using a PCR Express thermal cycler for 15 cycles, as follows: denaturation, 2 min at 94°C; primer annealing, 30 s at 55°C; and primer extension, 1 min at 68°C.

Dehydration and rehydration treatment

The desiccation–rehydration process was performed as described by Rodríguez-Porrata *et al*. (2011).

DOI: 10.1002/yea

Table 1. *Saccharomyces cerevisiae* strains and plasmids used in this study

Strain	Genotype/description	Source/reference
BY4742	MATα, *his3Δ1*, *leu2Δ0*, *lys2Δ0*, *ura3Δ0*	EUROSCARF/Brachmann *et al.* (1998)
4LB	Wild wine strain	UBYC/Capece *et al.* (2011)
F15	Commercial wine strain	Laffort
RB3-7Sc2	Wild wine strain	UBYC/Capece *et al.* (2010)
Sc9-11	Wild wine strain	UBYC/Siesto *et al.* (2013)
LB, GAL_p	4LB + pGREG505Δh	This work
F, GAL_p	F15 + pGREG505Δh	This work
RB, GAL_p	RB3-7Sc2 + pGREG505Δh	This work
Sc, GAL_p	Sc9-11 + pGREG505Δh	This work
LB, GAL_p-*SIP18a*	4LB + pGREG505si	This work
LB, GAL_p-*SIP18b*	4LB + pGREG505si	This work
F, GAL_p-*SIP18a*	F15 + pGREG505si	This work
F, GAL_p-*SIP18b*	F15 + pGREG505si	This work
RB, GAL_p-*SIP18a*	RB3-7Sc2 + pGREG505si	This work
RB, GAL_p-*SIP18b*	RB3-7Sc2 + pGREG505si	This work
Sc, GAL_p-*SIP18a*	Sc9-11 + pGREG505si	This work
Sc, GAL_p-*SIP18b*	Sc9-11 + pGREG505si	This work
Plasmids		
pGREG505Δh	$GAL1_p$-*SalI*-*SalI*-$CYC1_t$-*KanMX4-LEU2-bla*	Rodríguez-Porrata *et al.* (2012)
pGREG505si	$GAL1_p$-*SIP18*-$CYC1_t$-*KanMX4-LEU2-bla*	Rodríguez-Porrata *et al.* (2012)

Flow cytometry analysis

Flow cytometry was carried out using a CYFlow® space instrument (PARTEC GmbH, Germany) fitted with a 22 mW ion laser for excitation (488 nm), while monitoring with a single emission channel (575 nm band-pass filter). FloMax software (Quantum Analysis GmbH, Germany) was used for instrument control, data acquisition and data analysis. As control of full viability (99% by propidium iodide stain), an overnight YPD culture of each reference strain (4LB, F15, RB3-7Sc2 and Sc9-11) was used.

Tests for intracellular ROS accumulation

The dihydroethidium (DHE) staining was performed as described by López-Martínez *et al*. (2012). The samples were analysed by fluorescence microscopy. To determine the frequencies of the morphological phenotypes revealed by the DHE staining, at least 10^3 cells from three independent experiments were evaluated, using a Leica fluorescence microscope (DM4000B, Germany). A digital camera (Leica DFC300FX) and Leica IM50 software were used for the image acquisition.

Measurement of intracellular nucleotide leakage

The rehydrated yeast cells were harvested by centrifugation at 5000 rpm for 3 min at 4°C. The supernatant absorbance values at 260 and 280 nm were used to calculate the nucleotide equivalents in mg/ml = $(0.063 \cdot A_{260}) - (0.036 \cdot A_{280})$ (Herbert *et al*., 1971). The total intracellular nucleotide calculated was around 3 mg/g rehydrated cells. These analyses were done at least in triplicate and standard deviations (SDs) were $< 10\%$.

Determination of biological parameters

The growth data from microplate wells were monitored at 600 nm every 20 min, after 20 s shaking, for 24 h at 28°C in a POLARstar OMEGA instrument (BMG Labtech, Germany). Microplate wells, filled with 190 µl YPD medium, were inoculated with 10 µl rehydrated cells inoculum, measured by flow cytometry cell counting, to reach 0.4 OD (4.3×10^6 cells/ml), which is above the minimal limit detection previously established by calibration. Blanks were determined from quintuplicate non-inoculated wells for each experimental 96-well plate. Two independent transformants of each construction were evaluated, and each was evaluated in triplicate. The growth data from plate counts were enumerated as $\log_{10}$ values. The biological parameters, duplication time (DT) and lag phase time (λ), were estimated by fitting the growth curves into the model of Baranyi and Roberts (1994), using MicroFit software (Institute of Food Research, Norwich, UK).

DOI: 10.1002/yea

Fermentation at the laboratory scale

Small-scale fermentations were carried out in triplicate using natural grape must. After pasteurization for 20 min at 100°C, standard analyses (titratable acids, pH, assimilable nitrogen concentration,YAN, and sugar content) were done on the unfermented must. The yeast strains were grown for 24 h at 28°C in 150 ml YPD-containing culture flasks at 180 rpm, whereas the strains carrying the plasmid pGREG505 were grown in YPD with 400 mg/ml geneticin. After settling, 0.75 g/l diammonium phosphate (DAP) was added to the must to adjust the nitrogen concentration. The strains were inoculated into the grape must to a final concentration of 1×10^7 cells/ml and the fermentations were performed in 100 ml flasks at 25°C. The fermentation process was followed daily by measuring the decrease in weight, and the fermentation process was considered complete when the weight of the flasks was stabilized. Upon completion of fermentation, the wines were racked and then stored at 4°C until analytical evaluation.

Measurement of volatile compounds

Higher alcohols, ethyl acetate, acetaldehyde and acetic acid were determined by direct injection gas chromatography, using an Agilent 7890A gas–liquid chromatograph fitted with a flame ionization detector (FID) and a split–splitless injector, and provided with an automatic sampler and a Supelco glass column packed with 80/120 Carbopack BAW/5% Carbowax 20 M (180 cm × 2 mm i.d.).

Chromatographic conditions entailed the following: helium carrier gas, head pressure of 140 kPa; total flow of 20 ml/min[1]; purge flow of 7.0 ml/min; injector and detector temperature of 250°C; initial column temperature of 80°C, held for 2 min, then raised to 200°C at 4°C/min; make-up gas He at 30 ml/min; detector FID, H_2 at 30 ml/min; air 300 ml/min; injected volume, 1 µl. The identification and quantification of volatile compounds were determined by comparing each chromatographic peak with the retention times and relative areas of standard solutions.

Volatile compounds were determined by solid-phase microextraction (SPME). Ten ml wine samples were transferred to 20 ml glass vials with 2 g NaCl, and 100 µl iso-octane (concentration 10 000 mg/l) was added as internal standard. The equilibration was performed by stirring for 20 min at 46°C, whereas the adsorption phase was carried out at 50°C for 15 min under agitation. A carboxenpolydimethylsiloxane-coated fibre (100 µm) was used. After extraction, the fibre was placed in the injector of the GC for 10 min.

A DB-WAXTER (Agilent) column was used (length 30 m, i.d. 0.250 mm). The analysis was performed in splitless mode and the following conditions were used: 220°C as injection temperature; 250°C as detector temperature; helium as carrier gas with a flow rate of 20 ml/min. The initial temperature was 40°C and then it was raised to 240°C at 7°C/min.

Statistical analysis

The results were statistically analysed by one-way ANOVA and the Scheffé test, using SPSS 15.1 statistical software package (SPSS Inc., 2001). Furthermore, multivariate analysis of variance–canonical variants analysis (MANOVA/CVA) was carried out using the statistical package PAST, v. 1.90 (Hammer *et al*., 2001). The statistical significance was set at $p < 0.05$.

Results

SIP18p hydrophilin enhances wine yeast dry stress tolerance

In the first step, the effects of increasing the SIP18p expression levels were evaluated in stationary-state cells of four different *S. cerevisiae* wine strains (Table 1). For this purpose, a plasmid was used that allows expression of this gene under the control of the *GAL1* promoter ($GAL1_p$), which is less active than the endogenous *SIP18* promoter in the stationary phase. Both kinds of transformant strains, harbouring the empty vector or the plasmid expressing *SIP18* under GAL_p, after 48 h cultivation in selective dropout (SD) medium with 400 mg/ml geneticin were dried after 4 h supplementation with 2% galactose. The desiccation tolerance capacity of the yeast LB, GAL_p; Sc, GAL_p; RB, GAL_p; and F, GAL_p strains after cell rehydration with pure water at 37°C exhibited viability values of 20%, 30%, 55% and 60%, respectively (Figure 1). After rehydration, the strains LB, GAL_p-*SIP18*; Sc,

DOI: 10.1002/yea

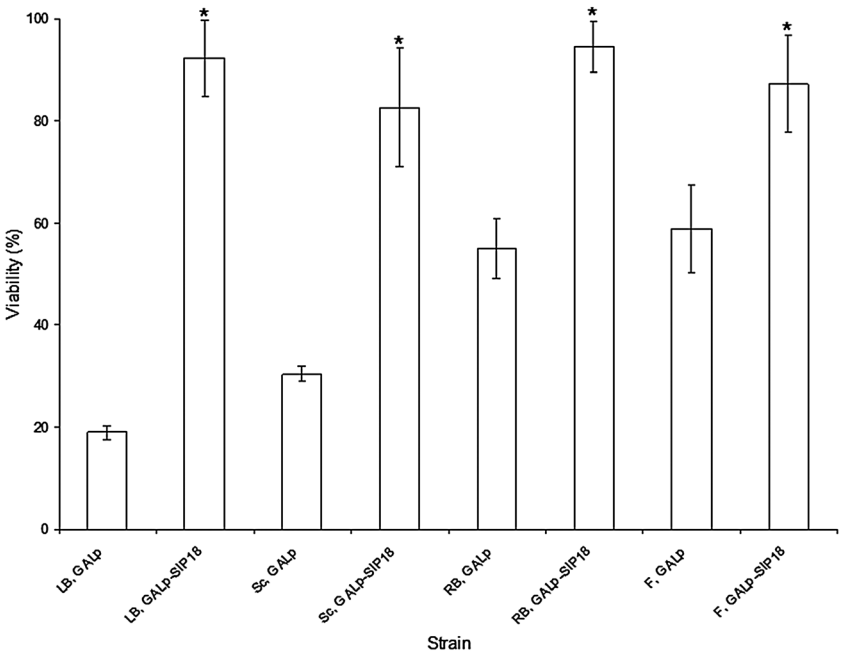

Figure 1. Effect of overexpressing *SIP18* hydrophilin gene on yeast viability after stress induction. The scale of viability indicates the experimental values (%) for the different strains. Values shown are mean ± SD of at least three independent samples. *Significant differences ($p \leq 0.05$) with respect to the respective transformant reference strain

GAL_p-*SIP18*; RB, GAL_p-*SIP18*, and F, GAL_p-*SIP18* exhibited 70%, 50%, 40% and 20% higher viability than the reference strains harbouring the empty vector, respectively (i.e. LB, GAL_p). Furthermore, the non-transformant yeast 4LB, F15, RB3-7Sc2 and Sc9-11 strains showed cell viability values similar to those of the transformant reference strains (data not shown). On the basis of these results, it is possible to conclude that the increased levels of *SIP18* gene product before stress induction in four different genetic backgrounds enhance the dehydration stress tolerance, as was previously shown in the laboratory haploid strain BY4742 (Rodríguez-Porrata *et al*., 2012).

Overexpressing *SIP18* gene strains show reduced ROS accumulation

The relationship between the increased viability rate of *SIP18p*-overexpressing strains after stress induction and differences in accumulating ROS cells was evaluated. Yeast strains were grown in SD medium with 400 mg/ml geneticin, and cells from the stationary phase before desiccation and after rehydration were analysed for the accumulation of reactive oxygen species (ROS). Before dehydration, around 17% of cells from all evaluated strains showed fluorescence after DHE incubation, whereas after rehydration the strains overexpressing *SIP18* showed DHE accumulation only reaching ~20% less than the strains harbouring pGREG505Δh. Taking into consideration the cell viability results of overexpressing SIP18p wine strains (Figure 1) and the ROS accumulation values (Figure 2), we can confirm, as previously observed in the haploid strain BY4742, that there is a correlation between the increase in desiccation survival rate and the reduction of intracellular ROS levels after stress imposition.

Tolerant strain dehydration shows reduction in DHE cells after oxidative stress by H_2O_2

Cells from LB, GAL_p; LB, GAL_p-*SIP18*; Sc, GAL_p; Sc, GAL_p-*SIP18*; RB, GAL_p; RB, GAL_p-*SIP18*; F, GAL_p and F, GAL_p-*SIP18* strains, after 4 h galactose induction, were subjected to 4 mM H_2O_2. After this treatment, the *SIP18*-overexpressing strains showed 40% reduction in the number of DHE cells after 10 or 20 min (Figure 3), whereas for all the strains at 30 and 40 min, the number of DHE-positive cells was similar. These results confirmed antioxidant properties by SIP18p,

DOI: 10.1002/yea

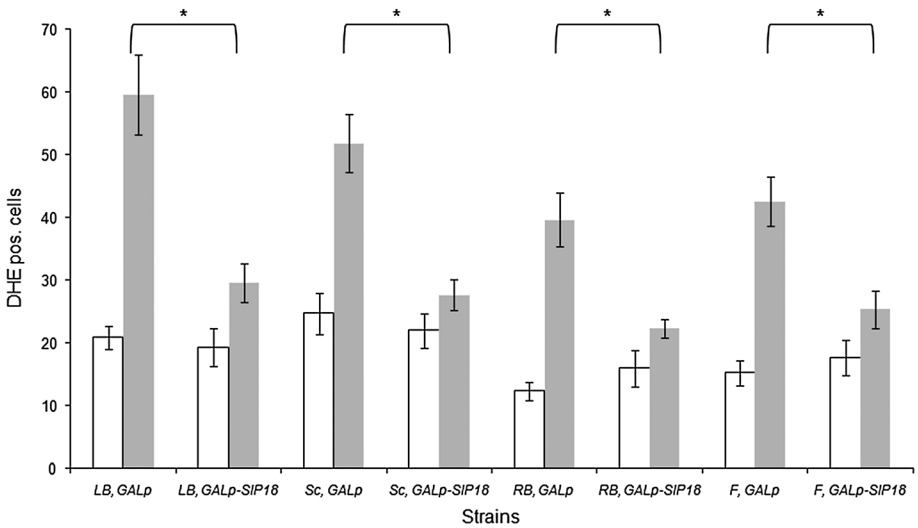

Figure 2. ROS accumulation by yeast cells during stress induction. (A) Quantification of ROS accumulation using DHE staining before drying (white bars) and after rehydration (grey bars). Values are mean ± SD of three determinations. DHE pos., DHE-positive cells. *Significant differences ($p \leq 0.05$) compared to the respective transformant reference strain after stress induction

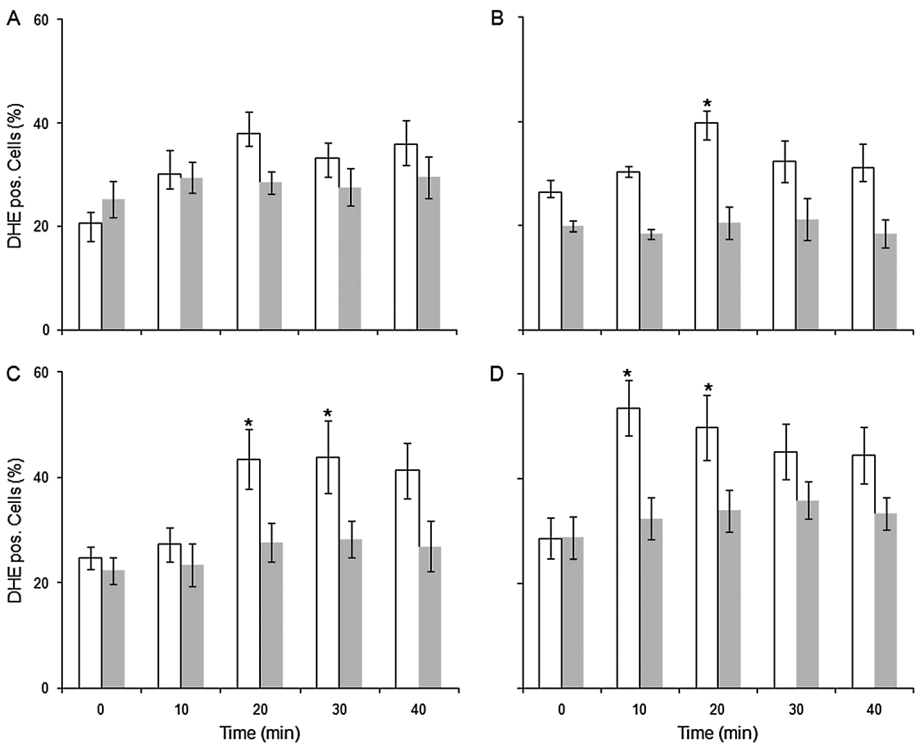

Figure 3. Levels of DHE accumulation after oxidative stress by H_2O_2. Cells in stationary state from the transformant reference (white bars) and the overexpressing *SIP18* strains (grey bars) were exposed to 4 mM H_2O_2 at the indicated times; aliquots were taken to evaluate DHE-positive (DHE pos.) cells. (A) LB, GAL_p-*SIP18* and LB, GAL_p strains; (B) Sc, GAL_p-*SIP18* and Sc, GAL_p strains; (C) RB, GAL_p-*SIP18* and RB, GAL_p strains; (D) F, GAL_p-*SIP18* and F, GAL_p strains. The represented data are values ± SD from at least three independent experiments. *$p < 0.05$ compared to the respective transformant reference strain at each time

DOI: 10.1002/yea

validating the previous results obtained in strain BY47472 by Rodríguez-Porrata *et al*. (2012), also in these four different wine yeast strains.

Cell leakage during cell rehydration and ADY vitality

Dehydrated yeast can lose up to 30% of soluble cell compounds when rehydrated, which proves the non-functionality of the cell membrane. A faster reduction in leakage may therefore be beneficial for the vitality of rehydrated yeast cells. The degree of intracellular compound leakage was assessed by evaluating 260 nm light absorbtion, at each point in time, of nucleotide concentration in the rehydrating supernatants of the transformant wine yeast strains (Figure 4A–D). On the other hand, after the rehydration process, cells were inoculated into YPD at 28°C and evaluated biomass time course production (Figure 4a–d). For the experimental rehydration of LB, GAL_p; Sc, GAL_p; and RB, GAL_p strains, the nucleotide concentration time course in the supernatant appeared to exhibit two periods that were delimited at the inflection points 20, 15 and 15 min, respectively, where cell leakage rate was inhibited (Figure 4A–C). The leakage trend exhibited by the LB, GALp-SIP18; Sc, GALp-SIP18; and RB, GALp-SIP18 strains showed a first period of ~5 min and a relative total nucleotide leakage of ~16%. Neither two leakage trend periods nor relative total nucleotide leakage differences between F, GAL_p and F, GAL_p-*SIP18* strains were observed (Figure 4D). These experiments reveal that most of the overexpressing *SIP18* strains show at least 25% lower relative leakage than the transformant reference strains (Fig 4A–C).

Strains overexpressing *SIP18* gene show longer lag phase after rehydration process

In this phase, it was evaluated whether the relative lower leakage of the overexpressing *SIP18* strains during the rehydration process was correlated with a shorter lag phase, compared to the transformant reference strains, once inoculated in complete medium. The LB, GAL_p-*SIP18*; Sc, GAL_p-*SIP18*; and RB, GAL_p-*SIP18* strains exhibited a λ which was 135, 160, 141 and 176 min longer than the transformant reference strains, respectively (Figure 4a–d). On the other hand, the LB, GAL_p-*SIP18* and F, GAL_p-*SIP18* strains showed 0.15 and 0.22 higher DTs than their transformant reference strains, respectively, whereas Sc, GAL_p-*SIP18* strain 0.21 lower DT than the transformant reference strains and the RB transformant strains did not show significant differences between them. The combination of these results with cell leakage data might confirm that there is not a correlation between faster-recovering membrane permeability and the strains showing shorter lambda phase (Figure 4a–d). The overexpressing *SIP18* strains exhibited an increase of λ phase even though, in general, they stopped the intracellular compounds leakage earlier after stress induction.

SIP18p hydrophilin did not affect fermentative performance

The wild strains and the corresponding transformants were tested during inoculated fermentation at the laboratory scale in order to evaluate the influence of transformation on strain fermentative performance. For all the strains, no statistically significant differences in fermentative vigour, expressed as amount of CO_2 produced when the strains fermented 15% of the total sugar present in the grape must, were found between the original and both kinds of transformant strains (data not shown). Furthermore, for all strains except Sc9-11, the transformants produced amounts of CO_2 slightly higher than those shown by non-transformant strains. The highest increase of fermentative vigour was exhibited by the transformants harbouring the empty vector (LB, GAL_p, RB,GAL_p and F,GAL_p), which produced about 0.2 g CO_2 more than non-transformant strains. These results revealed that the transformation did not negatively affect the fermentative performance of modified strains.

SIP18p hydrophilin did not affect metabolic behaviour of the strains

The successive step was to verify the effect of *SIP18P* hydrophilin on the metabolic behaviour of the transformants in comparison to the wild strains. The amounts of the principal secondary compounds determined in the experimental wines by gas chromatography are reported in Table 2. The four original strains exhibited a very similar metabolic behaviour in these fermentations; in fact, the contents of all the determined compounds were similar among the wines obtained from the four original strains, even though some differences

DOI: 10.1002/yea

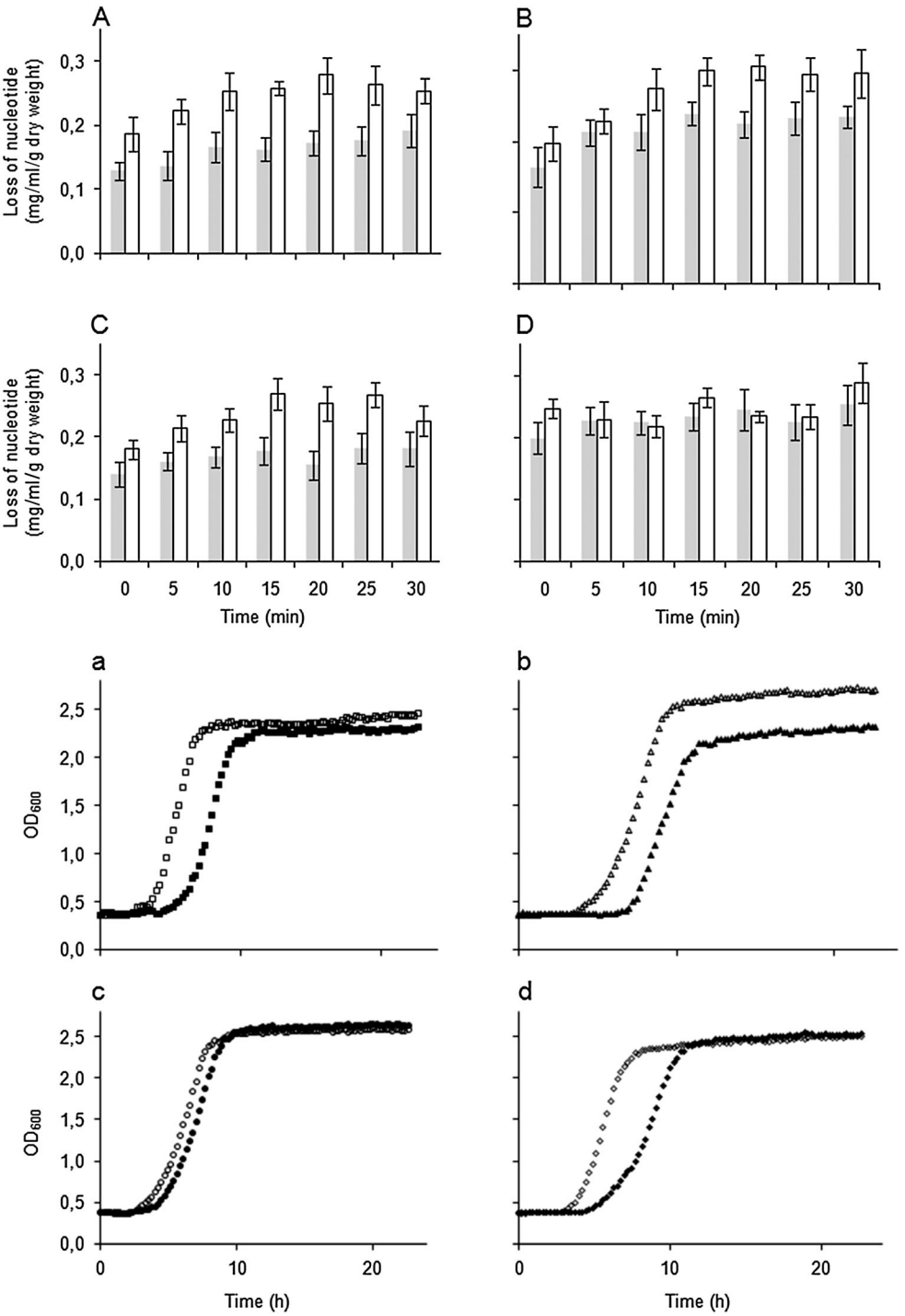

Figure 4. Time course of extracellular nucleotide concentration at the rehydration process and growth curve of rehydrated cells. Overexpressing *SIP18* strains (white bars) and their respective transformant reference strains (grey bars) were incubated at 37°C in pure water. (A) LB, GAL_p-*SIP18* and LB, GAL_p strains; (B) Sc, GAL_p-*SIP18* and Sc, GAL_p strains; (C) RB, GAL_p-*SIP18* and RB, GAL_p strains; and (D) F, GAL_p-*SIP18* and F, GAL_p strain. The data represented are mean ± SD of triplicate rehydration experiments. (a) LB, GAL_p-*SIP18* and LB, GAL_p strains; (b) Sc, GAL_p-*SIP18* and Sc, GAL_p strains; (c) RB, GAL_p-*SIP18* and RB, GAL_p strains; (d) F, GAL_p-*SIP18* and F, GAL_p strains growth curves. The graphs are a representative example of growth experiments performed, with two independent transformants for each strain

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Table 2. Main volatile compounds produced during laboratory-scale fermentations by four *Saccharomyces cerevisiae* strains and two transformants for each strain

Strains	Acetaldehyde	Ethyl acetate	*n*-Propanol	Isobutanol	Acetic acid	D Amyl alcohol	Isoamyl alcohol
4 LB	18.34 ± 3.35^{a}	57.70 ± 1.01	48.79 ± 0.39^{a}	17.79 ± 0.09	209.05 ± 38.39	46.76 ± 2.57	116.33 ± 1.48
LB, GAL_p	22.25 ± 2.56	51.74 ± 1.89	48.15 ± 0.25	17.31 ± 0.03	458.22 ± 55.10*	52.35 ± 1.54	123.39 ± 3.40
LB, GAL_p-*SIP18*	25.70 ± 2.98	54.45 ± 1.55	48.37 ± 0.61	19.77 ± 0.33	306.79 ± 80.51	58.96 ± 0.82	134.49 ± 0.94
Sc9-11	22.44 ± 0.29^{a}	53.24 ± 2.15	52.65 ± 0.04^{a}	26.33 ± 0.14	317.05 ± 54.26	44.18 ± 0.86	120.63 ± 0.48
Sc, GAL_p	19.13 ± 0.45	51.87 ± 0.06	53.28 ± 0.69	29.21 ± 0.08	360.05 ± 37.00	48.25 ± 0.40	111.05 ± 4.69
Sc, GAL_p-*SIP18*	16.37 ± 1.41	51.95 ± 0.37	52.54 ± 0.96	26.80 ± 2.92	393.39 ± 31.73	40.87 ± 2.52	98.88 ± 11.26
RB3-7Sc2	14.34 ± 0.06^{b}	54.15 ± 0.52	51.53 ± 2.04^{a}	14.78 ± 1.86	330.54 ± 54.00	39.41 ± 4.07	88.33 ± 10.95
RB, GAL_p	19.30 ± 4.71	54.29 ± 2.28	50.53 ± 0.76	14.84 ± 1.25	501.89 ± 17.24*	41.83 ± 0.16	93.63 ± 4.67
RB, GAL_p-*SIP18*	17.57 ± 1.36	53.71 ± 0.44	51.05 ± 0.06	14.82 ± 0.52	454.47 ± 33.20	41.74 ± 0.41	90.50 ± 2.81
F15	18.83 ± 0.08^{a}	53.57 ± 0.39	58.97 ± 4.04^{b}	24.47 ± 5.79	201.95 ± 76.10	43.71 ± 7.27	106.14 ± 20.76
F, GAL_p	18.76 ± 2.17	54.06 ± 1.42	57.58 ± 0.31	20.48 ± 0.51	250.91 ± 2.76	38.84 ± 0.99	91.59 ± 2.52
F, GAL_p-*SIP18*	19.20 ± 2.38	54.14 ± 1.29	57.26 ± 0.28	19.20 ± 2.24	273.64 ± 43.85	37.38 ± 2.18	87.56 ± 6.05

Data are expressed in mg/l and are mean ± SD of three independent experiments.

*Values significantly different from the control ($p < 0.05$), represented by non-transformant strains.

Different letters (a, b) in the same column correspond to statistically significant differences for each non-transformant strain ($p < 0.05$).

occurred. In particular, the *n*-propanol level detected in the wine produced by 4LB was statistically significant different from the content of the wine produced by F15, whereas the acetaldehyde content in the wine produced by Sc9-11 differed significantly from the level found in the wine produced by RB3-7Sc2. However, it must be underlined that all the compounds tested were present at acceptable levels, including acetic acid, which was below the critical threshold of about 0.7 g/l (range 201–330 mg/l).

As regards the wines produced by transformant strains, obtained by each of the four *S. cerevisiae* wild strains, generally, the secondary compounds were present at levels comparable to the wines obtained by the non-transformant strains. Only two transformants (LB, GAL_p derived from 4LB and RB, and GAL_p obtained from RB3-7Sc2) showed concentrations of acetic acid significantly higher than those of the original strains ($p < 0.05$), although also in this case the values were below the threshold value (Table 2).

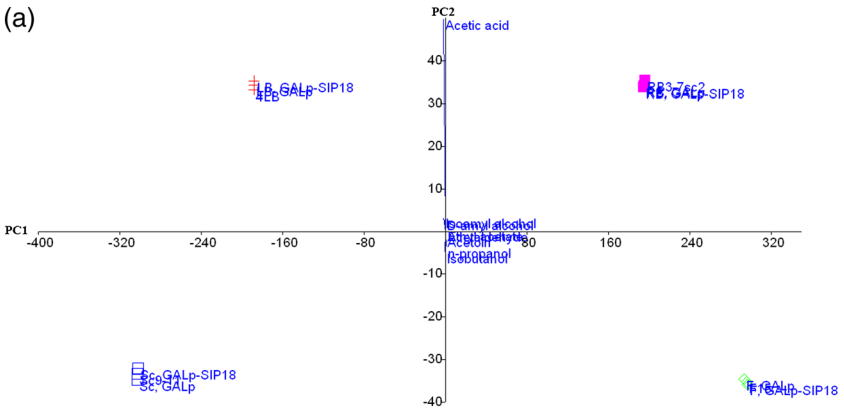
The data of secondary compounds determined in the experimental wines were submitted to MANOVA/CVA analysis in order to maximize the differences among the four predefined groups, represented by wines obtained by each strain and the corresponding transformants. Two tests were used in this analysis, the Wilks' λ and the Pillai trace, which yielded p values < 0.05 ($6.57E^{-5}$ and $2.31E^{-6}$, respectively), indicating that the variation among the four groups was highly significant. The scatter plot obtained by CVA analysis revealed that the four groups (each composed of wines obtained by original and corresponding transformant strains) are located in the four different quadrants (Figure 5a), indicating that the wines produced by the transformant strains were very similar to those produced by the corresponding wild strains. The analysis of loading values revealed that the first component explains 98% of the variance and the compounds mainly influencing the variance in this component were *n*-propanol and isobutanol (Figure 5b).

Furthermore, the effect of strain transformation on yeast metabolic behaviour was evaluated by analysing the experimental wines for the content of volatile organic compounds (VOCs), present at low level but known to influence the final organoleptic quality of wine. These compounds, determined by SPME analysis, are represented mainly by terpenes, esters and higher alcohols. Figure 6 reports the comparison in VOC number between wines produced by inoculating both kinds of transformant strains and wines produced by the wild strains. Each compound is indicated with a different number. In this context, it is interesting to notice that the number and the VOCs determined in wines obtained by original and corresponding transformant strains were very similar.

The wines containing the highest number of VOCs were those obtained by inoculating strains 4LB and F15 (the original strains and both the transformants). The main percentage of compounds is common to all the wines, whereas some

DOI: 10.1002/yea

(a)



(b)

Canonical axes / Variables	1	2
Acetaldehyde	-30,09	-6,68
Ethyl acetate	74,68	8,57
n-propanol	91,84	-0,54
Isobutanol	-40,04	-4,34
Acetoin	-1,87	-0,80
Acetic acid	1,07	0,12
D-amyl alcohol	14,99	2,67
Isoamyl alcohol	3,58	1,01

Figure 5. (a) Scatter plot and (b) loadings of MANOVA/CVA analysis of main secondary compounds determined in wines obtained by non-transformant and transformant strains. Values are reported as mean of three independent experiments. Each group, composed by wines obtained by non-transformant strain and corresponding transformants, is represented with a different symbol: +, wines by 4LB, LB, *GAL*$_p$*-SIP18* and LB, *GAL*$_p$ strains; ■, wines by RB3-7Sc2, RB, *GAL*$_p$*-SIP18* and RB, *GAL*$_p$ strains; □, wines by Sc9-11, Sc, *GAL*$_p$*-SIP18* and Sc, *GAL*$_p$ strains; ◊, wines by F15, F, *GAL*$_p$*-SIP18* and F, *GAL*$_p$ strains

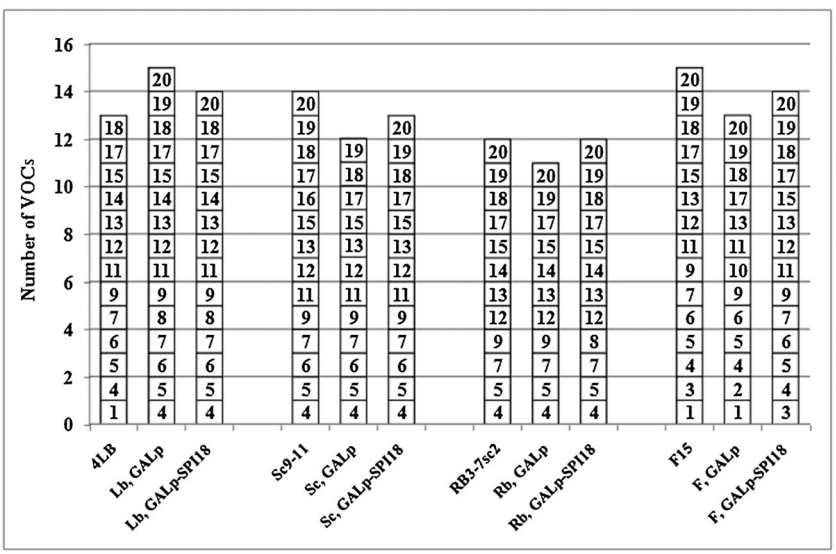


Figure 6. Volatile organic compounds (VOCs) determined by SPME in wines obtained by non-transformant and transformant strains: 1, acetone; 2, dimethyl sulphone; 3, β-pinen; 4, *n*-butanol; 5, limonen; 6, isobutyl formate; 7, isobutyl acetate; 8, ethyl-butyrate; 9, ethylhexanoate; 10, 2,3 butanediol; 11, terpinene; 12, exanol; 13, geranial; 14, β-citronellol; 15, decanol; 16, 2-phenylethanol; 17, β-jonon; 18, eugenol

DOI: 10.1002/yea

compounds were present only in some cases. For example, eugenol was present in all the wines except those derived by fermentation with Sc9-11 (original and transformants), whereas dimethyl sulphone was found only in wines obtained by inoculating 4LB (original and both the transformant strains). This means that these compounds are related to the specific metabolic activity of 4LB strain and, in addition, the presence of this compound also in wines produced with its transformants, demonstrates that the treatment had not affected this metabolism in 4LB strain.

Therefore, the results obtained by both gas chromatographic analyses suggest that the transformation did not significantly affect the production of the secondary compounds involved in wine aroma.

Discussion

Desiccation tolerance by the wine yeast *S. cerevisiae* has enabled the food industry to work with a more technologically secure product, but still excluding those yeast strains of higher interest and the newly isolated or created hybrid strains for beverage industry (*Saccharomyces* sp. and non-*Saccharomyces*) that cannot cope with the treatment of drying and rehydration (Rodríguez-Porrata *et al*., 2011). Natural yeasts possessing high survival to desiccation are not very diffused and the genetic manipulation of strains possessing interesting oenological properties, but low tolerance to desiccation, could represent an interesting tool. In the present study, we analysed four different wine strains. Three of them (4LB, RB3-7Sc2 and Sc9-11) were wild *S. cerevisiae* strains, isolated during spontaneous fermentation of grapes collected in different Italian regions and selected on the basis of interesting oenological characteristics, whereas the last one (F15) was a commercial strain, widely used as ADY in Italian cellars. On the basis of previous results reporting that the overexpression in *S. cerevisiae* of gene *SIP18* increases cell viability after the dehydration process (Rodríguez-Porrata *et al*., 2012), the four strains were submitted to transformation by insertion of the gene *SIP18*. In the case of transformed strains, it is very important to verify whether the introduced modifications should not change the characteristics essential in the fermentation process (Schuller and Casal, 2005). For most genetic modifications it was shown that, apart from the introduced metabolic change, no significant differences were found between wines produced with non-modified strains and the corresponding transformed strain, whereas in other cases genetic modification affected the characteristics of the final wines (Michnick *et al*., 1997; Remize *et al*., 2000). In this study, different techniques were used to evaluate the influence of transformation on the characteristics of analysed strains. The 'fitness' of active dried wine yeast cultures is related to the maintenance of cell 'viability' and 'vitality' during the process of yeast manufacture, including desiccation and storage (Pretorius, 2000). In our research, yeast 'viability' was assessed both directly, by determining loss of cell viability (plate counts), and indirectly, by assessing the preventing ROS accumulation effect of SIP18p even after an H_2O_2 attack, as was already shown in a laboratory haploid strain by Rodríguez-Porrata *et al*. (2012). On the other hand, we also evaluated the 'fitness' of the modified strains by simulating real vinification conditions. After grape must inoculation, during biomass formation, the absence of both the selection pressure by geneticin and galactose activation (in glucose-less medium) reduces at a very low level the cellular SIP18p content during vinification. In this way, the putative SIP18p impact is negligible in the organoleptic profile of wines, elaborated with strains for which *SIP18* was overexpressed during ADY production. The transformants obtained in this study did not negatively impact wine profile, although at the beginning of the fermentation they carry on a high level of the *SIP18* stress peptide. Our results demonstrated that, apart from the introduced change related to improved dehydration tolerance, no significant differences were found between original and modified strains as regards the fermentative performance and production of secondary compounds influencing wine aroma. These findings indicate that strain oenological characteristics are not affected by genetic modifications used in this study.

In conclusion, the transformation of wine strains by overexpression of the *SIP18* gene could represent an useful tool to improve strains tolerance to dehydration. Further studies are in progress in order to test the behaviour of these modified strains, in particular by evaluating the strain imposition capacity during real vinification trials, where the inoculated starter has to compete with the indigenous microflora.

DOI: 10.1002/yea

Acknowledgements

This work was supported by the Spanish Ministerio de Ciencia e Innovación (Grant No. AGL2009-07933). G.L.-M.'s doctoral fellowship was supported by the Agaur Agency of the Catalonian Government (Grant No. FI-DBR 2012), while R.-P. was the recipient of fellowship support by the Metapontum Agrobios s.c.r.l. (Italy). The authors are grateful to COST Action FA0907 'Bioflavour' for supporting this work.

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