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A novel approach for the improvement of stress resistance in wine yeasts

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Abstract

During wine production yeast cells are affected by several stress conditions that could affect their viability and fermentation efficiency. In this work we describe a novel genetic manipulation strategy designed to improve stress resistance in wine yeasts. This strategy involves modifying the expression of the transcription factor *MSN2*, which plays an important role in yeast stress responses. The promoter in one of the genomic copies of this gene has been replaced by the promoter of the *SPI1* gene, encoding for a cell wall protein of unknown function. *SPI1* is expressed at late phases of growth and is regulated by Msn2p. This modification allows self-induction of *MSN2* expression. *MSN2* gene transcription, Msn2p protein levels and cell viability increase under several stress conditions in the genetically modified strain. The expression of stress response genes regulated by Msn2p also increases under these situations. Cells containing this promoter change are able to carry out vinifications at 15 and 30 °C with higher fermentation rates during the first days of the process compared to the control strain.
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Keywords: Stress response; Wine yeast; Vinification; *MSN2* gene; *SPI1* gene; Genetic manipulation

1. Introduction

Wine production is a complex process both from the microbiological and from the biochemical point of view in which the yeast *Saccharomyces cerevisiae* plays a central role. As in other industrial processes, yeast cells never find a physiologically optimal environment during their use in wine production. In fact, they are exposed to a continuous mix of several stress conditions (Attfield, 1997; Bauer and Pretorius, 2000).

At the beginning of vinification, yeast cells are affected by osmotic stress due to the high sugar concentration in musts, as well as to low pHs (below 4). As fermentation progresses other stress conditions become relevant as ethanol accumulation and nutrient limitation (especially nitrogen, carbon and vitamins depletion). Depending on specific winemaking procedures, other stresses may occur, as those related to excessively high or low temperatures, high SO_2 concentrations, and the presence of competing microorganisms.

Taking all these data into account, the ability of yeast cells to detect and respond to the above described stress conditions is essential to avoid important losses of viability (Bauer and Pretorius, 2000). The mechanisms of stress response involve sensor molecules and signal transduction pathways which determine changes in the mRNA levels for many genes (Estruch, 2000; Gasch et al., 2000; Hohmann and Mager, 2003). Yeast cells develop a common response to environmental adverse conditions, the so-called environmental-stress response, ESR (Gasch et al., 2000). Global yeast gene expression analysis by DNA arrays indicates changes in the mRNA levels for about 900 genes in response to environmental stresses. Many of these genes are targets of the Msn2p and Msn4p zinc finger transcription factors (Martínez-Pastor et al., 1996), that recognize STRE elements in the promoter of these genes. The activity of these transcription factors is negatively regulated (at the level of subcellular localization) by the cAMP-dependent protein kinase A (PKA, Marchler et al., 1993; Martínez-Pastor

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et al., 1996; Boy Marcotte et al., 1999) and by the TOR ("Targets Of Rapamycin", Cardenas et al., 1999; Schmelzle and Hall, 2000) pathways which activate cellular growth. On the other hand, under stress conditions, the expression of growth related genes is repressed (Gasch et al., 2000), at least partly by the regulation of the Yak1p quinase by Msn2p (Smith et al., 1988; Garrett and Broach, 1989; Hartley et al., 1994). Several studies have allowed a better understanding of the expression of stress response genes throughout vinification (Riou et al., 1997; Ivorra et al., 1999; Puig and Pérez-Ortín, 2000; Perez-Torrado et al., 2002a; Erasmus et al., 2003; Rossignol et al., 2003; Zuzuarregui and del Olmo, 2004).

Several authors have proposed features of wine production in which genetic manipulation could allow an improvement (Dequin, 2001; Pretorius and Bauer, 2002; Pretorius, 2003; Schuller and Casal, 2005). A better understanding of the mechanisms involved in stress response during vinification may be also useful for the selection and genetic manipulation of yeast strains to improve their fermentative behaviour. Regarding stress response, several modifications have been already carried out and some of them are of special interest. One is the *GPD1* overexpression, which determines an increase in glycerol production and fermentation rate in stationary phase (Remize et al., 1999). Another one is the overproduction of glycogen (by overexpression of *GSY2*, which encodes glycogen synthase) which results in enhanced viability under glucose deprivation conditions (Pérez-Torrado et al., 2002b).

In order to improve stress response we have selected as a target the *MSN2* gene, due to its relevance in this process as a transcription factor. Overexpression of *MSN2* under the control of a constitutive promoter such as that of glyceraldehyde-3-phosphate dehydrogenase (*TDH1*) results in an increase in stress resistance in a laboratory strain but growth is affected (Martínez-Pastor et al., 1996). A similar result is found when *MSN2* is overexpressed under the control of its own promoter in an episomal plasmid (Carrasco, Pérez-Ortín and del Olmo, unpublished results). In this manuscript we propose another strategy of manipulation for *MSN2* expression regulation, based on the use of the *SPI1* promoter. This gene encodes a protein important in the structure and biogenesis of the cell wall (Shimoi et al., 1998; Keptein et al., 1999; Horie and Isono, 2001). The interest of this gene, that justifies its use in our strategy, is that it is induced in stationary phase (Puig and Pérez-Ortín, 2000), and under several stress conditions (Gasch et al., 2000). Besides, the promoter of this gene contains three STRE sequences for Msn2p transcription factor binding. Finally, during vinification its highest expression levels are found in later stages (Puig and Pérez-Ortín, 2000; Rossignol et al., 2003), when the growth problems derived from *MSN2* overexpression would be minimized. When the promoter of *MSN2* was changed by that of *SPI1* gene in a multicopy plasmid an improved stress resistance was detected but yeast growth was severely affected. However, substitution in the T73 wine yeast genome of the *MSN2* promoter in one of its copies by the *SPI1* promoter determined an improvement in resistance to several stress conditions, and a reduction in the lag phase in vinification.

2. Materials and methods

2.1. Yeast strains and growth conditions

All strains used in this work are derived from the commercial wine strain T73 (Lallemand Inc., Querol et al., 1992). T73-4 is a T73 derivative with both copies of *URA3* gene deleted (Puig et al., 1998).

In the experiments under laboratory conditions YPD medium (1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose), SD medium (0.17% (w/v) yeast nitrogen base without amino acids and ammonium sulphate, 0.5% (w/v) ammonium sulphate, 2% (w/v) glucose), YPGly medium (as YPD but containing 2% (w/v) glycerol instead of glucose), and MSM (Minimal Sporulation Medium; 1% potassium acetate) were used. Cultures were incubated at 30 °C with shaking.

To analyze the stress resistance, cells from exponential cultures in the appropriate medium for each strain (YPD or SD) were exposed to the following stress conditions: ethanol addition up to a 10% (v/v) final concentration and incubation for 1.5 h, heat shock at 42–45 °C for 1–2 h, change to a pH 2 in the same medium, shift (by centrifugation followed by resuspension) to the same medium containing 25% (w/v) glucose or 3 M KCl and incubation for 1 h (osmotic stress), change to the same medium containing 0.25 mM sulfite (added as potassium metabisulfite) and incubation for 24 h, and change to MSM and incubation for two days to test nutrient starvation resistance. In all these cases cell viability was determined by counting the number of colonies growing in YPD plates from appropriate dilutions of the cultures. To analyze the resistance to oxidative stress the diameter of the growth inhibition region produced by hydrogen peroxide was determined according to Stephen et al. (1995).

In the studies of gene expression and protein levels under stress conditions, cells from exponential cultures were treated as follows: for heat shock cells were incubated at 39 °C for 30 min; for ethanol stress, this alcohol was added to a 10% (v/v) final concentration and the incubation continued for 1 h; in the case of glucose starvation, cells from YPD cultures were shifted to YP medium and cultures were incubated for 1 h; finally to analyze expression in stationary phase, samples were obtained from cultures growing in YPD medium for 2 days. In all cases, after these treatments, cells were collected by centrifugation at 4 °C and washed with cold water previous to be chilled under liquid nitrogen and kept at −80 °C.

For microvinification experiments cells from two-day cultures in YPD were inoculated at a final concentration of 2 10^5 cells/ml in Sauvignon blanc natural must provided by Carrascal wineries (Requena, Spain, 2004). Incubation was carried out at several temperatures (15 °C, 22 °C or 30 °C) without shaking. The evolution of the vinifications was followed by the determination of OD_{600}, viability and sugar consumption, as previously described (Zuzuarregui and del Olmo, 2004).

All the experiments were carried out at least in triplicate.

2.2. Strain constructions

For the construction of the plasmid pMSNP (which contains *MSN2* under the control of *SPI1* promoter) a 464 bp PCR

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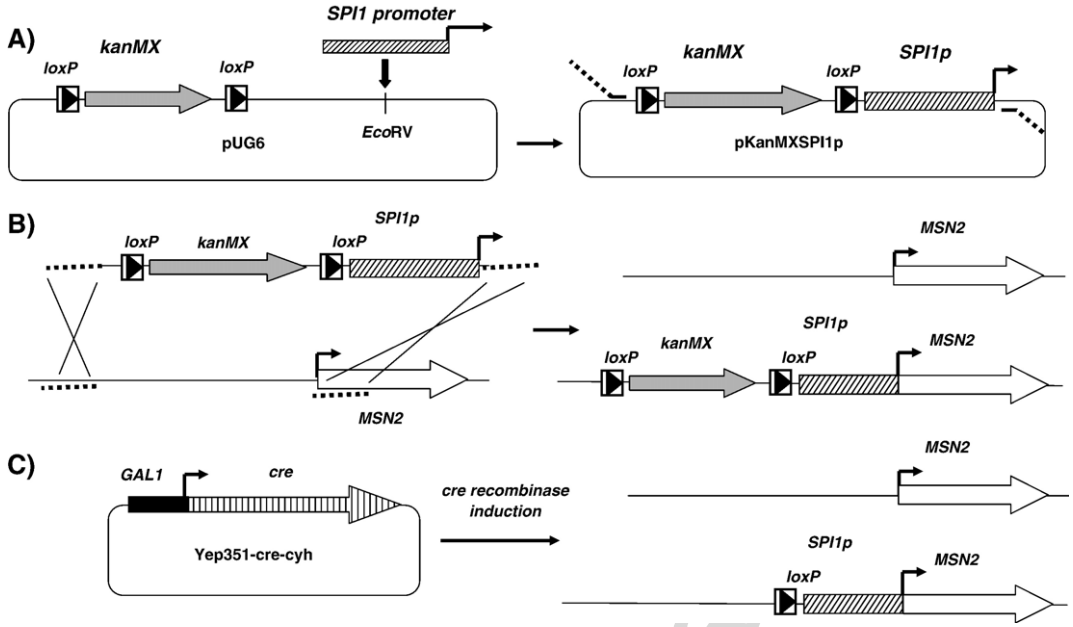

Fig. 1. Scheme of the construction of T73S strain. Details are explained in the Materials and methods section.

fragment with ends for recognition by restriction enzymes *Eco*RI and *Xba*I was obtained from genomic DNA by amplification with SPI1a and SPI1b primers. This fragment contains 3 STREs and the initiation codon for *SPI1* translation. It was introduced into YEp352 plasmid. Afterwards the *MSN2* coding region and terminator was amplified with the MSNORFa and MSNORFb primers, which contain recognition sites for *Sal*I and *Sph*I. This fragment was directionally cloned together with the *SPI1* promoter in the YEp352 derived plasmid. Strain resulting from the transformation of T73-4 with pMSNP plasmid (according to the protocol of Gietz et al., 1995) was called T73P.

For promoter substitution in one copy of *MSN2* the strategy shown in Fig. 1 was followed. In this case a 1.1 kb region of the *SPI1* promoter was amplified using SPI1b and SPI1c oligonucleotides. This PCR fragment was cloned in the *Eco*RV site of pUG6 plasmid to render pKanMX-SPI1p plasmid (A). The substitution cassette with flanking regions homologous to the *MSN2* promoter was obtained by amplification with MSN2a and MSNb oligonucleotides using pKanMX-SPI1p as template. T73 strain was transformed with this cassette and geneticin resistant colonies were isolated and tested by PCR with oligonucleotides K3 and SPI1b to select the ones with the right promoter replacement (B). To eliminate the antibiotic resistance gene, and therefore most prokaryotic sequences, the recombinant strain was transformed with the Cre recombinase containing plasmid Yep351-cre-cyh (Delneri et al., 2000) and

Table 1
Oligonucleotides used in this work

Name	Sequence	Purpose
SPI1a	CGGAATTCATTGAAATTAGTGCTTGTCTT	Amplification of *SPI1* promoter for MSNP construction and *SPI1* probe
SPI1b	GCTCTAGACATTATTAGTAATAGTAC	Amplification of *SPI1* promoter for MSNP and T73S constructions
MSNORFa	CGGACGACGGTCGACCATGATTTC	Amplification of *MSN2* ORF and terminator for MSNP construction
MSNORFb	CCACATGCATGCTGATATTACGGTGCCATATG	”
SPI1c	CGGAATTCGTCAATGGAAGTGTATGGTC	Amplification of *SPI1* promoter for T73S construction
MSN2a	TTTCGCTCTTGGTATCCGTACCTCACTTATCTC CAGCACCTTCGTACGCTGCAGGTCGAC	Amplification of the promoter substitution cassette for T73S construction
MSN2b	GGAATAAAATATCTTCGCTATTGAAATCATGGT CGACCGTCATTATTAGTAATAGTACTG	”
MSN2c	TTCCGTTACGGGCAGTGG	Confirmation of T73S construction
MSN2d	ACGCATTCCGTAGCAGCC	”
SPI1d	CTCGAAGTTCCCAGATGCCC	”
K3	CCTCGACATCATCTGCCC	”
SPI1-2	ACTCCCTATTGCACAACGGC	*SPI1* probe
MSN2-1	TGTCTTAGATAGTGCGG	*MSN2* probe
MSN2-2	AGATTCGAATTAGCACC	”
ALD3-1	CCACTCATCTTAAATCCGCC	*ALD3* probe
ALD3-2	CTTACAAGATACTATGCGGG	”

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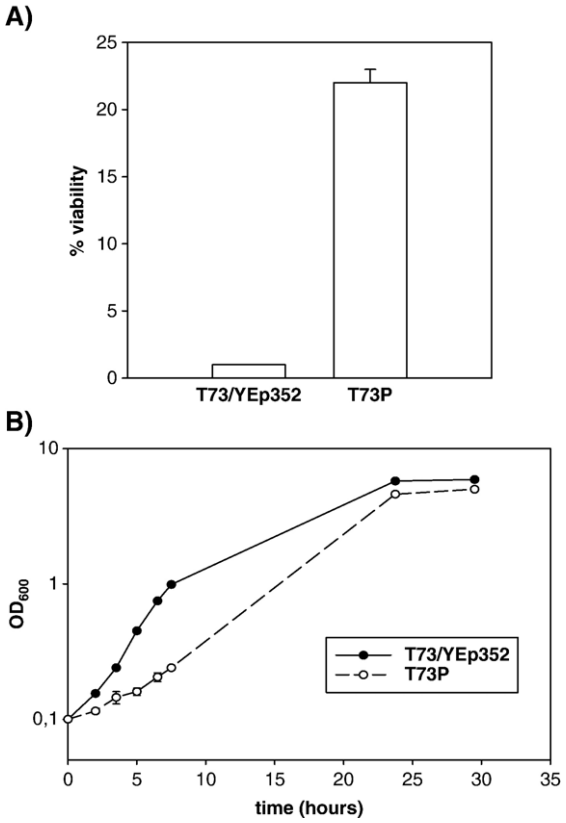

Fig. 2. Stress resistance and growth of the strain T73P compared with its parental T73-4 transformed with YEp352 plasmid. (A) Response to a heat shock stress of 2 h at 45 °C. (B) Growth kinetics in SD medium. At least three independent experiments were carried out. Figure shows the average and standard deviation from the whole data.

transformants were selected in cycloheximide plates. Cre recombinase was induced with galactose according to Delneri et al. (2000) and geneticin sensitive colonies were obtained and tested by PCR with the oligonucleotides MSN2c, MSN2d, and SPI1d to select a strain with only one copy of the *MSN2* gene modified (C). Yep351-cre-cyh was lost by growing cells in YPD for a large number of generations. The resulting strain was called T73S.

Oligonucleotides used for these constructions are shown in Table 1.

2.3. Analysis of gene expression

RNA was isolated by the hot-phenol procedure described by Kohrer and Domdey (1991). It was quantified and its quality and concentration were comproved in a 1% agarose gel in TAE buffer.

RNA analysis by Northern blot, hybridization and quantitation was carried out as previously described (Aranda et al., 2002). Detection of *SPI1*, *MSN2*, *CTT1* and *ALD3* mRNAs was carried out with probes generated by PCR using oligonucleotides SPI1a/SPI1-2, ALD3-1/ALD3-2 and MSN2-1/MSN2-2 respectively (Table 1). The *CTT1* probe was a 1.1 kb *Eco*RI fragment derived from plasmid pRB322-5109 (Spevak et al., 1983).

2.4. Analysis of Msn2p levels under stress conditions

Protein extracts were prepared from cultures of T73 and T73S strains under exponential growth in YPD and after a 30-min heat shock at 39 °C. For this purpose cells were collected by centrifugation and resuspended in 200 µL of isolation buffer (20 mM Tris-phosphate 20 mM pH 6.7, 5 mM EDTA, 1 mM PMSF, 2 µM pepstatin A and 0.6 µM leupeptin) and broken with glass beads. Total protein concentration was determined by the Bradford method (Bradford, 1976) and 60 µg of protein was used for each analysis. After SDS-PAGE electrophoresis in 7.5% polyacrylamide, proteins were transferred to a nitrocellulose membrane of 0.45 µm (Trans-Blot, 1620113, Bio-Rad) by electrotransference (Burnette, 1981). Immunodetection was carried out with an Msn2p antibody generated from the C-terminal region of Msn2p (provided by Dr. Francisco Estruch). This primary antibody was diluted 1/300 and the secondary antibody (peroxidase anti-rabbit antibody) 1/2000. Blocking and incubation with the antibodies were carried out in TBST buffer (20 mM Tris–HCl pH 8, 0.5 M NaCl, 0.05% (v/v) Tween 20) with 5% (w/v) nonfat dry milk. Washes were carried out in TBST. For detection, "ECF™ Western Blotting detection reagents" (RPN2106, Amersham) were used, following the instructions of the manufacturer.

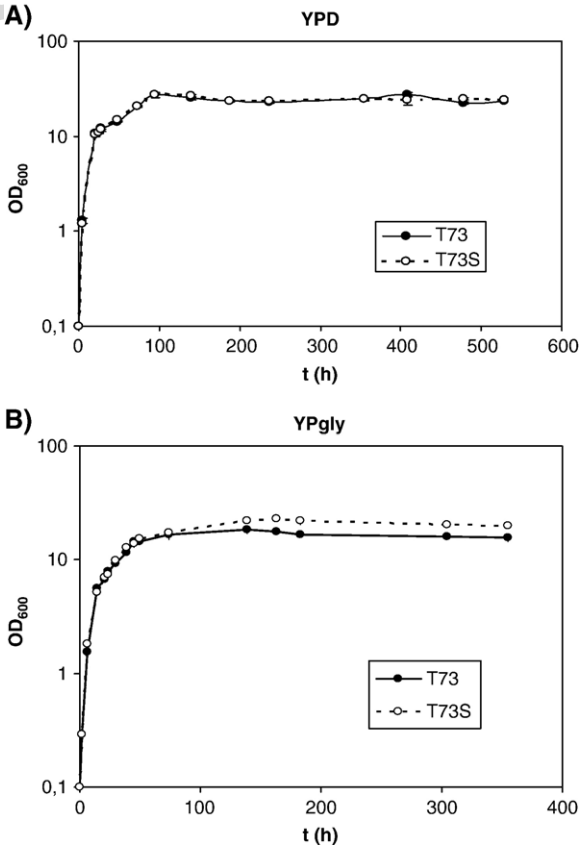

Fig. 3. Growth curve of the T73S and T73 strains. (A) Results obtained in YPD at 30 °C. (B) Growth in YPGly at the same temperature. At least three independent experiments were carried out. Figure shows the average and standard deviation from the whole data.

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3. Results

3.1. Effect of the expression of MSN2 under the control of the SPI1 promoter in a multicopy plasmid

Strain T73P was obtained as described in the Materials and methods section. As shown in Fig. 2A, the response of this strain to a heat shock stress (2 h, 45 °C) is clearly improved when compared with the wild type strain (T73-4 strain transformed with the YEp352 plasmid), but growth is severely affected during the first hours of growth in minimal medium (Fig. 2B). Similar results were found when the *MSN2* promoter was changed with a modified *CYC1* promoter containing STRE elements (Carrasco, Pérez-Ortín and del Olmo, unpublished results). Due to the undesired growth defects a new strategy was followed, consisting of the integration of the promoter substitution in the genome.

3.2. Substitution of the MSN2 promoter by SPI1 promoter in one copy of the T73 yeast genome. Effects on growth, gene expression and stress resistance

The strategy followed to obtain T73S strain has been described in the Materials and methods section and in Fig. 1. This kind of modification leads to the introduction of only 71 exogenous base pairs; basically a *loxP* site remains as a mark of the reporter gene integration.

Due to the growth defects detected previously in strains in which *MSN2* gene was overexpressed, studies with T73S strain were started considering its capability for growth under several conditions. Some of these results are shown in Fig. 3. In an optimal medium (YPD) and temperature (30 °C) for yeast growth, there was no significant difference when compared with the T73 strain (Fig. 3A), which demonstrates that the modification of *MSN2* in one of its copies does not result in negative effects in

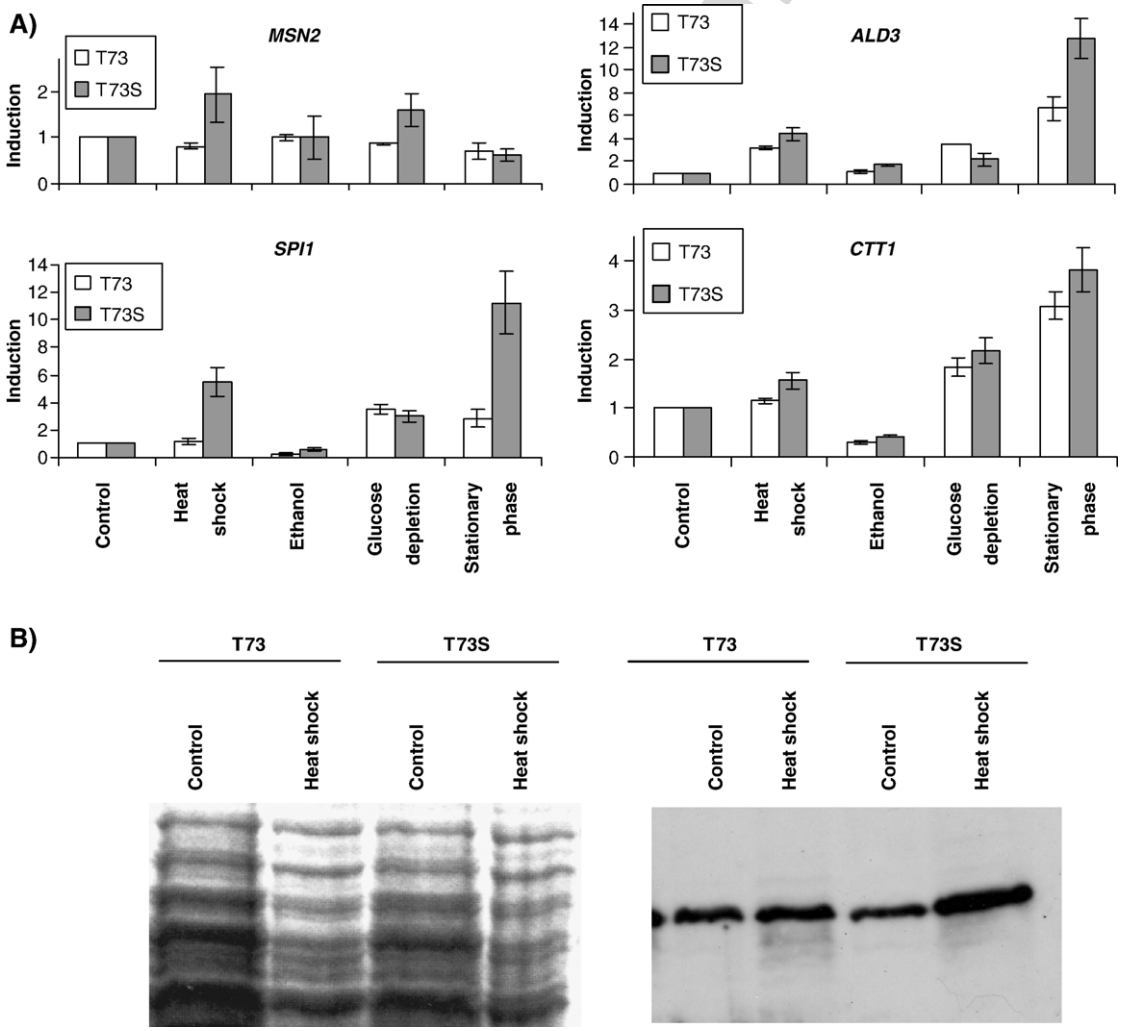

Fig. 4. Effects of the genetic manipulation on *MSN2*, *SPI1*, *ALD3* and *CTT1* gene expression and Msn2p levels under stress conditions. Yeasts cells of T73 and T73S strains under exponential growth were affected by stress treatments as described in the Material and methods section. (A) Quantification of the data obtained from three independent Northern hybridization experiments with selected probes (see Table 1), by determination of the values for each strain and condition relative to those under control conditions without stress. For normalization purposes, hybridization with a rDNA probe was used. (B) Western analysis of the Msn2p expression. Figure shows an image of a representative electrophoretic analysis (on the left) followed by immunodetection with Msn2p antibodies (on the right).

Table 2
Viability of T73S strain under stress conditions

	T73	T73S
Heat shock[1]	16.88±1.03	66.02±1.66
Acidic pH[2]	10.62±1.01	30.76±0.57
Sulfite[3]	14.14±0.86	3.43±1.04
Ethanol[4]	12.77±0.22	23.84±0.78
Starvation[5]	12.77±2.42	19.47±1.83

Table shows the percentage of viable cells after the stress treatments described in the Material and methods section: [1]42 °C for 1 h, [2]at pH 2 for 1 h, [3]0.25 mM sulfite for 24 h, [4]10% (v/v) ethanol for 90 min, [5]2 days after inoculation of the cells into sporulation medium. In the case of sulfite resistance, it was measured as the percentage of growth inhibition 24 h after inoculation at 0.1 OD_{600} from 2-day cultures. Experiments were carried out at least in triplicate. Data shown includes average and standard deviation.

growth. A similar result was detected when experiments where carried out at 37 °C or in minimal medium (SD) at 30 or 37 °C (data not shown). It is worth mentioning that under growth on glycerol as carbon source (YPGly medium) the modified strain is able to reach higher cell densities than the original strain (Fig. 3B).

To understand the effect of this genetic manipulation on the expression of stress related genes in strain T73S, experiments were carried out under several stress conditions (heat shock, ethanol, glucose depletion and after 2 days in stationary phase) and the expression of *MSN2*, *SPI1* and two other genes regulated by Msn2p (*CTT1* and *ADL3*) was analyzed. As shown in Fig. 4A, *MSN2* induction after heat shock and glucose starvation compared to the basal level (exponential growth, nonstress condition, control) is particularly increased in the modified strain, although the absolute levels of *MSN2* mRNA during exponential growth are approximately 1.5-fold lower in the modified strain (data not shown). Our results also indicate that the expression of the *SPI1* gene (a gene regulated by Msn2p) is induced by heat shock, glucose depletion and stationary phase, as already described in the literature (Gasch et al., 2000; Puig and Pérez-Ortín, 2000), and it shows a bigger induction in the engineered strain during heat shock and stationary phase. There is a clear correlation between *MSN2* and *SPI1* levels during heat shock but not under the other stress conditions tested. In the case of *ALD3*, another Msn2p-regulated gene (Gasch et al., 2000), the expression increases in the modified strain under conditions of heat shock and stationary phase, similar to the result found for *SPI1*. Finally, *CTT1* gene expression is higher in T73S strain under these conditions and also after glucose depletion, although the differences are less important than in the case of the other genes. These results point out that, in addition to *MSN2* mRNA levels, other factors are modulating the expression of those genes under particular stress conditions.

In the case of the control and heat shock samples, the Msn2p levels were determined by immunodetection. As shown in Fig. 4B, the higher expression of *MSN2* under this stress condition is followed by a similar increase in the protein levels. Also in correlation with the gene expression data, lower Msn2p levels are detected in the engineered strain under control conditions.

Finally the stress resistance of the T73 and T73S strains was analyzed. Table 2 shows the results of experiments carried out under several adverse conditions. Under 10% ethanol the viability of the T73S strain was about two-fold higher than that of the T73 strain. After 1 h at 42 °C, the viability in T73S was approximately 4-fold higher than in T73. After a 1-h incubation at a pH 2 or 24 h in the presence of 0.25 mM sulfite, this difference was around 3 times. Under nutrient starvation in minimal sporulation medium for two days, the engineered strain also shows a slightly better survival rate. Under the conditions of osmotic and oxidative stress tested, there were no significant differences among the two strains (data not shown).

3.3. Fermentative behaviour of T73S in natural must

Vinification experiments were carried out in Sauvignon must at three different temperatures (15, 22 and 30 °C) in order to cover variations that can be found in wineries depending on the kind of wine produced. In all these vinifications the behaviour was followed by cell viability and sugar determinations (Fig. 5).

Under all these conditions T73S strain showed a shorter lag phase and a higher fermentation rate during approximately the first two days, and was capable to complete the vinification

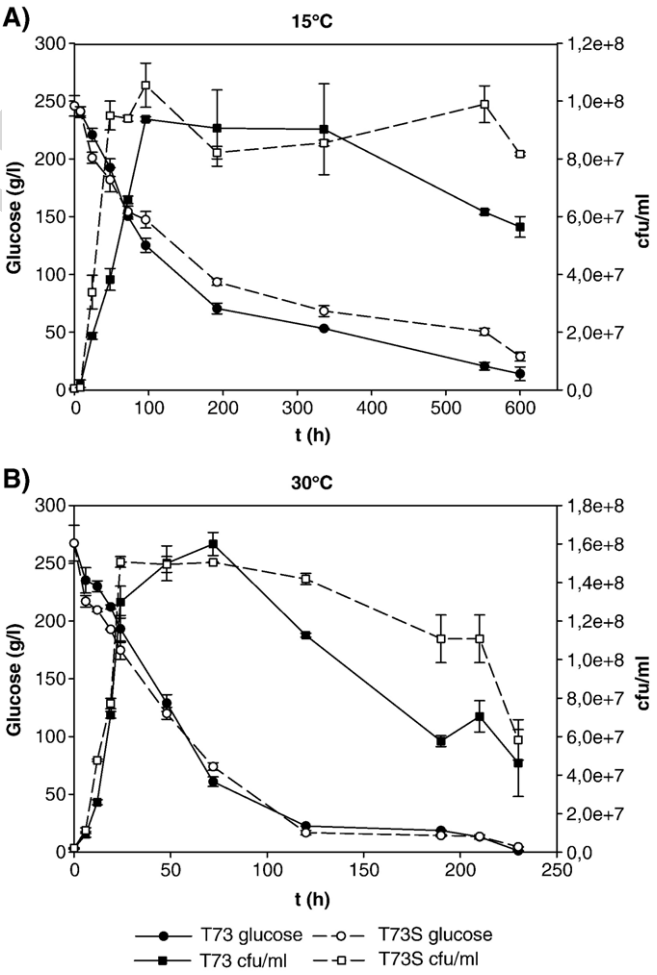

Fig. 5. Fermentative behaviour of T73 and T73S strains in Sauvignon must at 30 °C (A) and 15 °C (B). Vinification was followed by determining the number of viable cells and measuring sugars. Experimental details are described in the Material and methods section. Figure shows the average and standard deviation corresponding to at least three independent experiments.

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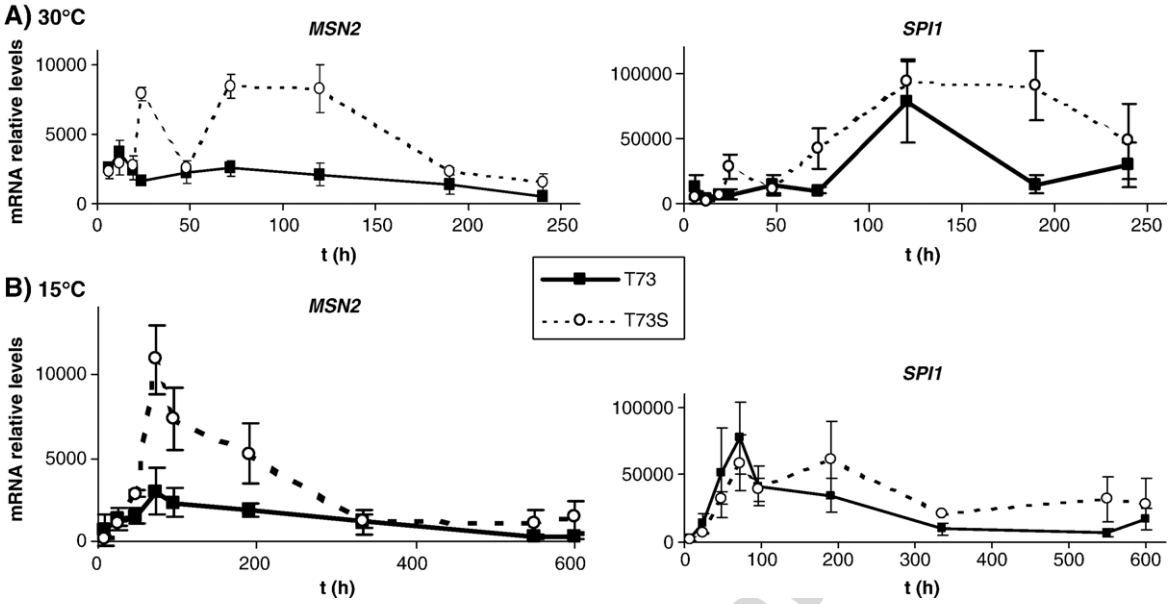

Fig. 6. *MSN2* and *SPI1* genes expression during vinifications carried out at 30 °C (A) and 15 °C (B) in Sauvignon must. Figure shows the average and standard deviation corresponding to at least three independent experiments.

approximately at the same time than the T73 strain (240 h at 30 °C, 360 at 22 °C and more than 600 h at 15 °C; only in the latter case the rate of glucose consumption at the end is delayed in T73S strain). Fig. 5 shows the results obtained at the two temperatures for which the effects are more significant: 30 °C (A) and 15 °C (B). According to our data, the number of viable cells is also higher for T73S strain during the first days of vinification at both temperatures. At 30 °C it also occurs after entering stationary phase and till the end of the vinification.

In order to understand the differences in the behaviour of the modified strain, expression of *SPI1* and *MSN2* genes was analyzed at several time points during the vinifications carried out at 15 and 30 °C. These results are shown in Fig. 6. The results obtained indicate that *MSN2* expression is higher in the modified strain in certain stages during the vinification. In the case of the experiments carried out at 30 °C, the expression of this gene is higher during the second day and in the central part of the vinification, when higher viability is observed for this strain (Fig. 5A). In the case of 15 °C, the highest *MSN2* mRNA levels are detected up to 300 h, with a partial coincidence with the higher viability (Fig. 5B). In the case of *SPI1*, as already described for this and other strains (Riou et al., 1997; Puig and Pérez-Ortín, 2000), the expression in T73 strain increases during the stationary phase, but this increase is higher in the engineered strain at both temperatures, as expected according to the results obtained for *MSN2* gene expression and the regulation of *SPI1* by Msn2p. A similar increase in the T73S strain was observed for other Msn2p-controlled stress response genes such *ALD3* (data not shown). There is a significant correlation in the gene expression data obtained for these genes, especially in the case of the T73S strain.

4. Discussion

During vinification yeast cells are affected by a plethora of stress conditions. Improvement of resistance to these conditions can be a useful way to get a better fermentative behaviour, although according to the information in the literature, growth efficiency can be affected. Recent data obtained in our laboratory indicates that accurate expression of stress response genes may be important for the fermentative behaviour of yeast strains: strains that are not capable to complete vinification usually show lower expression levels during vinification than strains capable of completing the process; besides strains that consume very low amounts of glucose show high and maintained levels of some stress response genes (Zuzuarregui and del Olmo, 2004).

One candidate for genetic manipulation in order to improve stress resistance is the transcription factor *MSN2*, which controls the expression under several stress conditions of an important number of genes. In our laboratory we have tried to increase the expression of this gene by overexpression in a multicopy plasmid under the control of its own promoter or a *CYC1* promoter containing STREs (data not shown) or with *SPI1* promoter. In the former case important viability loss occurred during vinification and in the others growth problems arose. It is worth mentioning that *YAK1* deletion in all its genomic copies resulted in a partial reduction of these effects (results not shown). In all these modifications stress resistance increased but the resulting strain appeared not to be convenient for vinification purposes.

In this work we show a novel strategy based on the replacement in one genomic copy of the *MSN2* promoter by that of the *SPI1* gene, which is induced under several stress conditions including stationary phase. With this strategy, we attempted to have only one of the *MSN2* promoter copies regulated by the *SPI1* promoter, which contains STRE elements and is a target of Msn2p. According to this, a higher but self-regulated expression of *MSN2* under stress conditions was expected and hence a better response under winemaking stages in which the stress is more important (stationary phase for instance) and the undesirable growth effects

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are less deleterious. Actually, with this approach *MSN2* mRNA levels increase under several stress conditions (mainly heat shock and glucose depletion, Fig. 4A), higher Msn2p levels are found under heat shock (Fig. 4B), the expression of Msn2p-regulated genes increases under certain adverse situations (Fig. 4A), and stress resistance under some conditions is also increased (Table 2). Besides, under regular laboratory or vinification growth conditions this genetic modification occurs without a significant effect on growth (Fig. 3).

The *SPI1* and *MSN2* mRNA levels are also higher in T73S strain during the alcoholic fermentation (Fig. 6), in partial or complete correlation with higher cell viability. Besides, the most relevant result achieved about fermentative behaviour was the higher glucose consumption during the first days of vinification, when the osmotic stress is more severe. This effect is more important in experiments carried out at 15 °C and 30 °C, more stressful conditions, for which the fermentation rate increased during the first days in the case of the engineered strain. The better fermentative behaviour displayed by the modified strain could be related with the higher resistance of this strain (see Table 2) to two stress conditions by which yeast cells are affected during this stage: acidic pH and presence of sulfite, used as a microbiological stabilizing agent. At the beginning of these vinifications, an increase in the *MSN2* mRNA levels is not found; probably yeast cells are better adapted to the stress conditions found at this time in the modified strain because of the previous growth in stationary phase in which higher *MSN2* mRNA levels are found (Fig. 4). These results point out a better adaptation of this strain to some vinification conditions and leads to the possibility to effectively use this strain for certain winemaking procedures. This strain could be potentially interesting to impose during the first stages of vinification over indigenous or inoculated strains with lower capacity of adaptation to the osmotic stress, the low pH or the sulfite concentration at the beginning of vinification, allowing a more rapid beginning of the process. Although compared to the parental strain the time of fermentation is the same, the ability to start the vinification more quickly is an important point regarding competition with other strains that could be present in musts or wineries but whose imposition is not adequate, for instance, for the organoleptic properties of the resulting wine.

These results indicate that in order to improve stress resistance in wine yeasts used in biotechnological processes it is essential to carry out minor genetic modifications to avoid growth problems and to favour the fermentation process. The approach introduced in this work leads to an increase in *MSN2* expression but only one of the copies of this gene is modified, the expression is self-regulated and the effects on growth are clearly different to those found in strains in which higher expression of this gene is achieved by overexpression in multicopy plasmids under the direct control of *SPI1* promoter or STREs. The strategy for this manipulation can be carried out easily in the laboratory and can be applied to other industrial strains, as the transformation steps are carried out on the basis of antibiotic resistances, not in auxotrophies. On the other hand other combinations of promoters and genes can be selected, depending on the interests in each case. It is also possible to inoculate strains modified in this way in certain stages of the vinification to improve fermentative behaviour.

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