# Analysis of low temperature-induced genes (LTIG) in wine yeast during alcoholic fermentation

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**Running head**: Cold adaptation in a wine yeast

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**ABSTRACT**

Fermentations carried out at low temperatures, i.e. 10–15 °C, enhance production and retention of flavour volatiles, but also increase the chances of slowing or arresting the process. In this study, we determined the transcriptional activity of ten genes, that were previously reported as induced by low temperatures and involved in cold adaptation, during fermentation with the commercial wine yeast strain QA23. Mutant and overexpressing strains of these genes were constructed in a haploid derivative of this strain to determine the importance of these genes in growth and fermentation at low temperature. In general, the deletion and overexpression of these genes did affect fermentation performance at low temperature. Most of the mutants were unable to complete fermentation, while overexpression of *CSF1, HSP104* and *TIR2* decreased the lag phase, increased the fermentation rate and reached higher populations than the control strain. Another set of overexpressing strains were constructed by integrating copies of these genes in the delta regions of the commercial wine strain QA23. These new stable overexpressing strains again showed improved fermentation performance at low temperature, especially during the lag and exponential phases. Our results demonstrate the convenience of carrying out functional analysis in commercial strains and in an experimental set up close to industrial conditions.

**Keywords:** *Saccharomyces cerevisiae*, cold response, transcriptional activity, fermentation rate, mutants, overexpressing strains

## Introduction

Winemakers effectively control the temperature of fermentation to avoid shifts (above 30 °C), that may result in stuck fermentations, and ensures not only a more efficient fermentation but also products with better sensory quality. Low-temperature fermentations (10-15 ºC) prevent the loss of primary (varietal) aromas by evaporation, but increase the synthesis of secondary aromas (mainly ethyl and acetate esters) (Torija *et al*., 2003; Beltran *et al*., 2007; Beltran *et al*., 2008). However, temperature affects both the rate of yeast growth and fermentation; lower temperatures often result in stuck or sluggish fermentations. Therefore, we have been studying how *S. cerevisiae* adapts to low temperatures (cryotolerance) (Beltran *et al*., 2007; Beltran *et al*., 2008; Salvado *et al*., 2008). This knowledge will improve future performance of wine yeast through the development of genetically modified yeast or by the adaptation of yeast during industrial production.

Low temperature affects a variety of cellular processes and characteristics in *S. cerevisiae*. Previous studies found that protein translation, cell membrane fluidity, RNA secondary structure stability, enzymatic activity, protein folding and heat shock protein regulation are significantly affected by growth at low temperatures (Schade *et al*., 2004; Aguilera *et al*., 2007; Tai *et al*., 2007; Pizarro *et al*., 2008). The physiological consequences are a decrease in transport, an accumulation of misfolded proteins and reduced enzymatic activity. The cells respond to these physiological and biochemical changes by modifying cellular processes such as protein phosphorylation and degradation and through other longer term effects that involve transcriptional changes (Schade *et al*., 2004). However, the type of response depends on the length of exposure to stressful conditions. Sudden exposure to environmental changes (e.g., cold shock) is likely to trigger a rapid, highly dynamic stress-response (adaptation). Prolonged

exposure to non-lethal stimuli leads to acclimation, i.e., establishment of a physiological state in which regulatory mechanisms, like gene expression, fully adapt to suboptimal environmental conditions (Tai *et al.*, 2007).

Some studies have analyzed the genome-wide transcriptional response of *S. cerevisiae* both during adaptation (to an abrupt decrease of temperature from 30 to 10ºC) (Sahara *et al.*, 2002; Schade *et al.*, 2004) and during the acclimation to sub-optimal temperatures that are not restrictive for growth (13 ºC) (Tai *et al.*, 2007). Schade *et al.* (2004) identified two distinct groups of transcriptionally-modulated genes that are grouped into two phases during the cold-shock response: 1) an early cold-shock response (ECR) that occurs within the first 2 h after exposure to low temperature and 2) a late cold response (LCR) that occurs 13 h or later after exposure to low temperature. The ECR induced genes are implicated in RNA and lipid metabolism, whereas genes induced during LCR mainly encode proteins involved in protecting the cell against a variety of stresses. In fact, the LCR response is very similar to the general stress response mediated by the transcription factors MSN2/MSN4 (Estruch, 2000). This data on transcription during cold adaptation was compared to the transcriptome analysis obtained during cold acclimation (chemostat-cultures at 13 ºC) by Tai *et al.* (2007). Only a group of genes involved in lipid metabolism showed a similar regulation both during adaptation (cold-shock) and acclimation (continuous growth at low temperature).

During wine fermentation, yeast needs to adapt to a new environment when they are inoculated into the grape-must (osmotic-, pH- and temperature-shock). But yeast cells also need to acclimatize to low temperature, being able to grow and ferment at the same time. Therefore, we are interested in both the genes regulated after a cold-shock (adaptation) and after a continuous exposure to low temperature (acclimation). With this aim of studying genes involved in cold adaptation and acclimation during wine

fermentation, we selected genes whose transcriptional activity has been previously reported as strongly regulated by cold (Sahara *et al*., 2002; Homma *et al*., 2003; Schade *et al*., 2004; Beltran *et al*., 2006; Murata *et al*., 2006; Tai *et al*., 2007; Pizarro *et al*., 2008).

A total of ten cold-shock or low temperature-induced genes (LTIG) were analyzed. These included *HSP104*, *HSP26* and *TCP1*, which encode molecular chaperones that are involved in protein-folding or protein aggregation prevention (Ursic & Culbertson, 1991; Haslbeck *et al*., 1999; Bösl *et al*., 2006); *HSP12*, *TIP1* and *TIR2*, which encode proteins involved in the maintenance of the plasma membrane and cell-wall (Kondo & Inouye, 1991; Sales *et al*., 2000; Abramova *et al*., 2001); *NSR1* and *LOT2* (*RPL2B*), which are involved in pre-rRNA processing and ribosome biogenesis (Kondo & Inouye 1992; Zhang *et al*., 2001); *LTE1*, which encodes the Cdc25 family guanine-nucleotide exchanging factors (Zhao *et al*., 2007); and *CSF1*, which was described by Tokai *et al*. (2000) to associate with the nutrient transport system and is only required for fermentation at low temperature.

The transcriptional pattern of these genes in a commercial wine yeast strain was monitored during wine fermentation. Additionally, we evaluated growth and fermentative capacity of constructed strains with either deletion or over-expression of these genes in a derivative haploid of an industrial strain of *S. cerevisiae*. Finally, the genes showing an improved phenotype at low temperature were overexpressed by integrating several copies in the delta regions of the genome of this commercial wine yeast strain.

## Material and Methods

### Strains, fermentations and sampling

The commercial *S. cerevisiae* wine strain QA23 (Lallemand S.A., France) was used to study the transcriptional profile of the selected genes during wine fermentation. The fermentation was performed on the synthetic grape-must (pH 3.3) described by Riou *et al.* (1997) but with 100 g $L^{-1}$ of glucose and 100 g $L^{-1}$ of fructose (henceforth SM). The Yeast Assimilable Nitrogen (YAN) content was 300 mg N $L^{-1}$: ammoniacal nitrogen ($NH_4Cl$) 120 mg N $L^{-1}$ and amino acids 180 mg N $L^{-1}$.

Fermentations were performed at 25 ºC and 13 ºC in laboratory-scale fermenters using 2 L bottles filled with 1.8 L of media and fitted with closures that enabled the carbon dioxide to escape and samples to be removed. The population inoculated in every flask was 2 x $10^6$ cells $mL^{-1}$ from an overnight culture in YPD. Fermentation was monitored by measuring the relative density of the media (g $L^{-1}$) using a Densito 30PX densitometer (Mettler Toledo, Switzerland). Residual sugars were determined using enzymatic kits (Roche Applied Science, Germany). Fermentation was considered to be completed when residual sugars were below 2 g $L^{-1}$. Yeast cell biomass was determined by absorbance at 600 nm and by plating on YPD. Five-millilitre samples were harvested at different points during the fermentation process so that mRNA could be analyzed. Cell pellets were transferred to 1.5 mL Eppendorf tubes and frozen immediately in liquid nitrogen. They were then kept at -80 ºC until they were analyzed.

In order to simplify the generation of mutant strains, we constructed the derivative haploid *ho*QA23 of the wine strain by disrupting the *HO* gene and substituting it with the KanMX4 cassette (explained with more detailed below) (Walker *et al*., 2003). The transformants were sporulated, and the spores were selected by resistance to geneticin. We carried out several tests to confirm the haploid state of the spores. Firstly we verified that the segregants were unable to sporulate due to successful disruption of *HO*. We also determined the MAT locus of the segregants by PCR (Huxley *et al*., 1990).

These haploid segregants were *MAT*a or *MAT*α. Finally we determined the ploidy of the cells by flow cytometry (Bradbury *et al*., 2006). After screening the growth and fermentation capacity of twenty *HO* disruptants, the haploid strain most like the parental wine strain was selected for the construction of the mutants. The KanMX4 marker of the selected haploid strain, *ho*QA23, was excised using the Cre-*lox* system. This strain was then transformed with the plasmid YEp351-Cre-Cyh (Güldener *et al*., 1996), which carries both the positive marker for CYH$^R$, which confers resistance to cycloheximide, and for *CRE* under the control of the inducible *GAL1* promoter. CRE recombinase expression was induced by shifting the cells from YPD to YPG (galactose) medium. Thus, the *ho*QA23 strain was free of the geneticin resistance. However, as this strain was prototrophic, we introduced auxotrophy by deleting the *LEU2* gene with the same strategy explained above to construct the strain *ho*QA23Δ*leu*. The introduction of auxotrophy was necessary to allow for the selection of transformants that contained the plasmid YEp181, which carries the *LEU2* gene.

*Escherichia coli* strain DH5α was used for the construction and amplification of plasmids employed in this study.


**RNA extraction and cDNA synthesis**

Total RNA was isolated from yeast samples as described by Sierkstra *et al*. (1992) and re-suspended in 50 µL of DEPC-treated water. Total RNA was purified using the High Pure Isolation kit (Roche Applied Science, Germany) according to the manufacturer's instructions. This RNA isolation kit also includes a DNase treatment for genomic DNA removal. RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA), and the quality of the RNA was

verified electrophoretically on a 0.8% agarose gel. Solutions and equipment were treated so that they were RNase free as outlined in Sambrook *et al.* (1989).

Total RNA was reverse-transcribed with Superscript$^{TM}$ II RNase H$^-$ Reverse Transcriptase (Invitrogen, USA) in a GenAmp PCR System 2700 (Applied Biosystems, USA). The reaction contained 0.5 µg of Oligo (dT)$_{12-18}$ Primer (Invitrogen, USA) and 0.8 µg of total RNA as template in a total reaction volume of 20 µL. As directed by the manufacturer, after denaturation at 70 ºC for 10 min, cDNA was synthesized at 42 ºC for 50 min, and then the reaction was inactivated at 70 ºC for 15 min.

**Real-time quantitative PCR**

The PCR primers used in this study are listed in Table S1. The primers were designed with GenBank sequence data and Primer Express software (Applied Biosystems, USA) in accordance with the Applied Biosystems guidelines for designing PCR primers for quantitative PCR (with the exception of the housekeeping gene *ACT1*, which was previously described by Beltran *et al.* (2004). All amplicons were shorter than 100 bp, which ensured maximal PCR efficiency and the most precise quantification.

For each gene, a standard curve was generated using yeast genomic DNA. DNA extraction was performed as described by Querol *et al.* (1992), digested by RNase and isolated by two-fold phenol-chloroform extraction and ethanol precipitation. The concentration was determined using a GeneQuant spectrophotometer (Pharmacia, Canada). Ten-fold serial dilutions of DNA were created to yield DNA concentrations ranging from 400 to 4 x $10^{-2}$ ng µL$^{-1}$. This dilution series was amplified (in triplicate) for each gene with SYBR GREEN PCR to obtain standard curves (see above). The standard curve is displayed as the Ct value *vs.* log $_{10}$ of the starting quantity of each standard. The starting quantity of the unknown samples was calculated against the

standard curve by interpolation. Gene expression levels are shown as the concentration of the studied gene normalized to the concentration of the housekeeping gene, *ACT1*.

Real-Time Quantitative PCR was performed using SYBR$^{®}$ GREEN I PCR (Applied Biosystems, USA). The SYBR PCR reactions contained 300 nmol L$^{-1}$ of each PCR primer, 1 µL cDNA (or 5 µL of each DNA serial dilution for standard curves) and 12.5 µL of SYBR GREEN master mix (1X) (Applied Biosystems, USA) in a 25 µL reaction. All PCR reactions were mixed in 96-well optical plates (Applied Biosystems, USA) and cycled in a PE Applied Biosystems 5700 thermal cycler using the following conditions: 50 ºC for 2 min, 95 ºC for 10 min, and 40 cycles at 95 ºC for 15 s and at 60 ºC for 60 s. Each sample had two controls which were run in the same quantitative PCR: NAC (No Amplification Control; sample without reverse transcriptase reaction) to avoid the interference by contaminant genomic DNA and NTC (No Template Control; sample without RNA template) to avoid interference by primer-dimer formation. All samples were analyzed in triplicate and the expression values were averaged by the analysis software (Applied Biosystems, USA).

**Molecular biology techniques**

DNA manipulations were performed as described by Sambrook *et al*., (1989). *E. coli* was transformed by electroporation (Dower *et al*., 1988) and plasmids were purified from *E. coli* cells by using the Illustra plasmidPrep Mini Spin kit (GE Healthcare, UK). DNA fragments resolved in agarose gels were purified with the high pure PCR product purification kit (Roche Applied Science, Germany). Transformation of *S. cerevisiae* strain was carried out using the lithium acetate method (Gietz & Woods, 2002).

**Construction of mutant and overexpressing strains**

All genes were deleted using the short flanking homology (SFH) method based on the KanMX4 deletion cassette (Güldener *et al*., 1996). The primers used for amplification of the *loxP-KanMX4-loxP* cassette from the plasmid pUG6 are shown in Table S1. The primers have 60-nucleotide extensions corresponding to regions upstream of the target gene start codon (forward primer) and downstream of the stop codon (reverse primer). The PCR fragments were used to transform the haploid *ho*QA23 strain. Total DNA from transformants that were resistant to G418 geneticin were analyzed by PCR using primers upstream and downstream of the deleted region combined with primers of the *KanMX4* gene (Table S1). The absolute lack of transcription for each of the deleted genes was further verified by real-time quantitative PCR (QPCR).

The same cold-induced genes were overexpressed by constructing and cloning multicopy vectors carrying these genes in the haploid *ho*QA23 strain. All genes were amplified from approximately 600 nucleotides upstream of the start codon to 400 nucleotides downstream of the stop codon to ensure that the promoter and terminator regions were included. The PCR protocol involved an initial denaturation at 94 ºC (5 min), followed by 30 cycles of 30 s at 94 ºC, 30 s at 55-60 ºC (depending on the different primers) and 1 min at 72 ºC. The last cycle was followed by a final extension step of 10 min at 72 ºC. The primers used for amplification of the genes are shown in Table S1. Two multicopy vectors (YEp181 and pGREG505) were used to construct the over-expressing strains. One of these plasmids was chosen for the different strains due to methodological reasons as, after numerous attempts, we were unable to insert genes *CSF1*, *HSP104*, *LOT2* and *LTE1* into YEp181. Thus, the centromeric pGREG505 plasmid was the alternative used for overexpressing these genes. The *in vivo* homologous recombination between the gene and the plasmid enabled a quicker and

more straightforward construction than the *in vitro* insertion used for the episomal plasmid.

The PCR products of the *HSP12*, *HSP26*, *NSR1*, *TCP1*, *TIP1* and *TIR2* genes were cloned into the pGEM T-easy cloning vector (Promega Corporation, USA). The plasmids obtained were transformed into *E. coli* for their amplification. The PCR products were isolated from plasmids by digestion with *Sal*I and *Sph*I (these restriction sites were included in the primers used for the gene amplification; Table S1) and inserted by ligation into YEp181 previously digested with the same restriction enzymes. The resulting plasmids were designated YEp*HSP12*, YEp*HSP26*, YEp*NSR1*, YEp*TCP1*, YEp*TIP1* and YEp*TIR2*. These plasmids were finally transformed into the *ho*QA23*leu*Δ strain (auxotrophic for leucine). Transformants were selected on SD plates (0.67% yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI), 2% dextrose, 2% agar) incubated for 2 days at 30 °C. The correct insertion of the gene was confirmed by digestion of the plasmid with *Sal*I and *Sph*I and by PCR using primers upstream and downstream of the cloning site.

Alternatively, the PCR products of the genes *CSF1*, *HSP104*, *LTE1* and *LOT2* were cloned into the plasmid pGREG505 using the "Drag and Drop" method of cloning (Jansen *et al*., 2005). This method consists of co-transformation of the gene PCR product and the linearized plasmid and a subsequent *in vivo* homologous recombination between both fragments. The plasmid was linearized by *Sal*I digestion to make the recombination process easier. The PCR fragments were generated with oligonucleotides that contained the short sequences rec5 (forward) and rec2 (reverse), which are homologous to the sequences in the plasmid. The transformants were selected by geneticin resistance, which is encoded by the *KanMX4* gene on the plasmid. The correct

insertion of the gene was confirmed by digestion of the plasmid with *Sal*I and *Eco*RV and by PCR using primers upstream and downstream of the cloning site.

Transcriptional activity in the overexpressing strains was determined 24 hours after the yeast was inoculated into synthetic grape-must at 25 ºC by real-time QPCR and these activities were compared to the respective control strains (*ho*QA23YEp181 and *ho*QA23pGREG505). The strains overexpressing the target genes displayed increased activity ranging from 5 to 15 times higher for the plasmid YEp181 and from 2-5 times higher for the plasmid pGREG505 (data not shown). We also tested plasmid stability in the overexpressing strains after wine fermentation and found that it never dropped below 75%.

The fermentations with the mutant and overexpressing strains (and their respective control strains) were performed as described above with the exception that the synthetic grape must contained ammonium as sole nitrogen source (henceforth $SM_{NH4}$). We got the highest rate of YEp181 plasmid stability by removing the amino acids from the SM. Geneticin was also added (200 mg $L^{-1}$) to the $SM_{NH4}$ for the overexpressing strains constructed with the plasmid pGREG505 for the same purposes. Fresh geneticin was periodically added (every 72 hours) to the fermentation medium to keep the selective pressure and plasmid stability.


**Construction of stable overexpressing strains by chromosomal integration in the commercial wine yeast QA23**

The cold-induced genes *CSF1*, *HSP104* and *TIR2* were stably overexpressed by integrating several copies of these genes into the genome of the commercial wine yeast QA23. To this end, the method proposed by Guerra *et al* (2006) was followed with some modifications. This novel system of genetic transformation allows multiple

integrations of a desired gene in the delta repetitive elements of the *S. cerevisiae* genome.

In a first step, the KanMX4 cassette was integrated approximately 400 bp downstream of the stop codon of the gene of interest. After checking for correct integration, a new PCR product incorporating the gene of interest, with its own promoter, and resistance to geneticin (KanMX4) was obtained. This PCR product was flanked by the delta sequences. The primers used were D1-Forward and KanD2-R (Table S1), which contain tails homologous to the δ element of Ty1 (Guerra *et al.*, 2006). The expression cassettes for the *CSF1*, *HSP104* and *TIR2* genes (12244, 5741 and 3491 bp respectively) were used to transform the wine strain QA23. Transformants were selected on plates of YPD with geneticin and PCR was used to test the correct insertion of the cassettes in the delta elements. The overexpression of these genes in the transformants was confirmed by real-time quantitative PCR (QPCR).

These new stable overexpressing strains, denominated δ*CSF1*δ, δ*HSP104*δ and δ*TIR2*δ, were used to perform wine fermentations in complete SM as described above. No selective pressure was needed in the fermentation medium to promote gene overexpression.

**Calculation of generation time**

The generation time is a measure of the number of generations (or doublings) that occur per unit of time in an exponentially growing culture. In our experiments, $2 \times 10^6$ cells $mL^{-1}$ were inoculated into 35 mL of synthetic must. The culture was incubated at 13 °C or 25 °C, and the $OD_{600nm}$ value was monitored. The calculations used to determine the generation time were based on exponential fits to the growth curves using the equation $t_d=\ln2/\mu$, where $\mu$ is the slope from the exponential phase of the growth curves.

**Statistical data processing**

All experiments were repeated at least three times, and the data is reported as the mean value ± SD. Significant differences between the control strain, the mutant and the overexpressing strains were determined by ANOVA and $t$-tests (SPSS 13 software package). The statistical level of significance was set at $P \leq 0.01$.

# Results

**Gene expression during wine fermentation at low temperature**

The commercial wine strain QA23 was used to ferment synthetic grape must at 25 ºC (control temperature) and 13 ºC (low temperature). Fermentation time course, measured as the reduction in media density and yeast growth, is shown in Figure 1. Fermentation finished after 120 h and 288 h at 25 ºC and 13 ºC, respectively. The maximum population was similar in both fermentations (approximately $2 \times 10^8$ CFU mL$^{-1}$). However, this maximum was reached after 24 h and 120 h of fermentation at 25 ºC and 13 ºC, respectively. The exponential phase was followed by a lengthy non-proliferating phase (stationary phase) in which the culture, at 13 ºC, retained high viability, while a gradual decrease in viability was measured at 25 ºC.

Samples were taken at different times during fermentation to analyze the transcriptional evolution of the 10 genes studied (Figure 2). All of the transcriptional changes are expressed relative to time zero, which is the level of gene expression in the wine yeast before inoculation (after overnight growth in YPD). The expression levels and transcription profiles of these genes varied greatly. However, most of the genes can be grouped into two transcriptional patterns: genes whose transcriptional activity increased during the lag and exponential phases and those that were repressed during the growth

phase and were activated during the non-proliferative or stationary phase (Figure 2). Thus, the *LOT2*, *HSP104*, *NSR1* and *TCP1* genes showed higher activity during the lag and exponential phase, and the *CSF1*, *HSP12*, *HSP26*, *TIP1* and *TIR2* genes were repressed during this growth phase. This pattern of induction/repression was independent of the temperature, although at low temperature, a delay (or shift) in time was observed as a consequence of slow growth. The *LTE1* gene had higher activity during the lag and log phase at the control temperature and higher transcriptional activity during the stationary phase at low temperature.

The genes *LOT2* and *NSR1* had the strongest induction of the studied genes in the first hours after yeast inoculation and strong repression when the cells entered the stationary phase. The activity of *NSR1* increased 200-fold a half-hour and two hours after inoculation at 25 ºC and 13 ºC, respectively. Both genes are involved in ribosome biogenesis and are connected with the cell's preparation for growth in a new medium. The genes *LTE1* and *TCP1* also had a transcriptional pattern very similar to the former genes but with two differences: the induction level was lower and these genes had higher transcriptional activity during the stationary phase at low temperature.

The induction of *TIP1* and *TIR2* coincided with the cellular growth arrest. The inoculation repressed the activity of the *HSP12* and *HSP26* until the stationary phase when transcriptional levels similar to that in cells previous to inoculation (time 0) were reached.

The genes *CSF1* and *HSP104* deserve special attention because these genes were the only ones that had higher activity at low temperature in most of the samples and in which the transcriptional activity was growth-independent. *HSP104* was strongly induced during the first two hours after inoculation into fermentation at low temperature. At the beginning of subsequent growth, the activity of this gene was again

repressed. Conversely, *CSF1* reached maximum transcriptional activity when the cells entered the stationary phase during fermentation at low temperature. Induction was not as strong in the cells growing at optimum temperature.

**Phenotypic screening of mutant and overexpressing strains of LTIG**

In order to determine the importance of low temperature-induced genes (LTIG) on growth and fermentation at low temperatures, mutant and overexpressing strains of LTIG were constructed in the derivative haploid wine strain, with the exception of Δ*tcp1,* which was unviable. Both mutant and overexpressing strains were phenotypically evaluated according to their growth and fermentation capacity. The relative generation times (GT) of the mutant and overexpressing strains are shown in Figure 3. The mutants for the genes *HSP12*, *NSR1*, *CSF1*, *LOT2* and *LTE1* showed significantly increased GT but, unexpectedly, this reduction in growth rate primarily affected the cells growing at optimum temperature. In fact, only the Δ*hsp12* and Δ*nsr1* mutants had significant differences in GT when grown at low temperature. The overexpression of the LTIG genes did not always have the opposite effect to their deletion. In fact, only the strains overexpressing *HSP12* and *TIR2* displayed improved growth at low temperature (GT shorter than 20% of that of the control strain). At optimum temperature, the strains overexpressing *TIP1* and *TIR2* also decreased their generation time. Conversely, the overexpression of *CSF1*, *HSP104*, *LOT2* and *LTE1* impaired growth of their respective strains at 25 ºC, but not at low temperature.

The other parameter used in this screening consisted of determining the time needed to consume 50% of total sugars (T50) in a synthetic grape must during fermentation at 25 ºC and 13 ºC (Figure 4). This fermentation point matched the period of maximum fermentation activity and the end of the exponential phase, where the differences among

strains were more evident. The deletion of some genes impaired the fermentation performance of the wine strain. This was especially remarkable for Δ*csf1* and Δ*hsp104*, which showed stuck fermentations at both temperatures. The Δ*lte1* strain also showed a significant delay in fermentation activity at low temperature, but not at 25 ºC. Conversely, the Δ*hsp12* and Δ*nsr1* strains improved fermentation performance at 25 ºC, reducing the T50 value. Overexpression of the genes *CSF1*, *HSP104*, *NSR1*, *TIP1* and *TIR2* increased fermentation activity at this point of fermentation. The growth (CFU mL$^{-1}$) of the mutant and overexpressing strains was also determined during fermentation. The mutant strains did not show remarkable differences in the maximum population as compared to the control strain at this fermentation point (data not shown). Conversely, the strains overexpressing *CSF1*, *HSP104* and *TIR2* reached significantly higher populations than the control strains (data not shown).


**Stable overexpression of the LTIG selected genes in a commercial wine yeast**

On the basis of these results, we selected the genes *CSF1*, *HSP104* and *TIR2* to construct stable overexpressing strains in the genetic background of the commercial wine yeast QA23. Gene overexpression stability was obtained by inserting several copies of the desired gene into the yeast chromosome. These copies were integrated by homologous recombination into the repetitive delta elements of Ty1 and Ty2. This overexpression was confirmed by QPCR. Gene expression was analysed at the fermentation point of major activity according to the transcriptional data shown above. The maximum overexpression detected was 2, 3.3 and 6 times higher than the commercial wine strain for δ*CSF1*δ, δ*HSP104*δ and δTIR2δ strains, respectively (data not shown).

Fermentation kinetics for overexpressing strains compared with the industrial wine strain are shown in Figure 5. The overexpression of these genes resulted in a rapid start of the fermentation process (lower values at T5) and most of the overexpressing strains maintained this significant higher activity at T50 and T100. Likewise, these strains also showed significant higher growth rate and higher maximum populations in comparison with the wine strain QA23.

## Discussion

The availability of the complete genomes of many organisms has created the need to assign a function to many genes of unknown phenotype. The determination of transcriptional activity and the modification of the gene dosage by altering copy number are two of the most powerful methods for studying and verifying the function of gene products. However, because industrial strains have higher ploidy than other strains, most of these functional studies have been performed in haploid laboratory-derived strains that are not suitable for industrial production. In this study, we studied the transcriptional activity of ten genes that were previously reported to be induced by low temperature and to be involved in cold adaptation, throughout the fermentation process in a commercial wine yeast. Moreover, strains that were either mutant for or overexpressed these genes were constructed in a derivative haploid of a commercial strain to determine the importance of these genes in yeast growth and fermentation at low temperature. Finally, three out of the ten genes were selected for stable overexpression by integrating several copies in the genome of the same commercial wine strain.

Despite previous descriptions of these genes, which found that they were induced by low temperature, most of them did not show clear regulation by low temperature but

were affected by the growth phase. These growth-dependent genes were down- or up-regulated with the beginning of growth (exponential phase) or with the entrance into the stationary phase (growth arrest) regardless of the fermentation temperature. However, as a consequence of slower growth at low temperature, there is a shift in the growth phases and in the transcriptional profile. Thus, the higher activity of these genes could not be related to low temperature regulation whereas the transcriptional differences are consequence of different growth rate. In our experimental conditions, the increase in GT of the control strain *ho*QA23 at 13 ºC and 25 ºC is greater than 3-fold. Tai *et al*. (2007) found that it was difficult to separate the effects of temperature from the effects of growth rate in batch cultures at low temperature on transcription. They proposed the use of chemostat continuous cultures that permit accurate control of growth rate independent of other culture conditions. Two recent chemostat studies (Regenberg *et al*., 2006; Castrillo *et al*., 2007) also found that the growth rate itself has a strong effect on transcriptional activity. However, *NSR1* was up-regulated in both batch and chemostat cultures at low temperatures (Sahara *et al*., 2002; Schade *et al*., 2004; Tai *et al*., 2007). This gene is required for pre-rRNA processing and ribosomal biogenesis and has previously been identified as a marker of cold-shock (Kondo *et al*., 1992). Tai *et al*. (2007) also indicated that this gene was regulated by temperature in a growth-independent manner. We detected a strong up-regulation of this gene at the beginning of fermentation (regardless of temperature) and a down-regulation when the cells approached the stationary phase (decrease in growth rate). The specific experimental set-up of our study compared to previous studies should be taken into account. We used synthetic grape-must, which has a low pH and high sugar and low nitrogen concentrations. In this experimental context, Δ*nsr1* had the most severe growth defect of all the mutant strains analyzed. However, this growth defect was larger at optimum

temperature than at 13 ºC. This delay in growth did not affect fermentation activity because the Δ*nsr1* strain consumed sugar quicker than the control strain at low temperature. Surprisingly, the overexpressing strain also performed better at low temperature.

*LTE1* is the acronym for "Low Temperature Essential" and this gene, along with *TEM1* and *CDC15*, is considered essential for the exit from M phase at low temperatures (Shirayama *et al*., 1994). In this case, although its transcriptional profile showed a clear growth-dependence at 25 ºC (induction and repression during exponential and stationary phases respectively), at 13 ºC this gene had sustainable activity throughout the process. Many genes are controlled by multiple transcriptional regulation systems that act in a hierarchical manner; therefore, *LTE1* activity may be regulated by both growth rate and temperature. During a global transcriptomic analysis of wine fermentation in this same commercial strain, Beltran *et al*. (2006) reported higher *LTE1* activity in the middle and late phases of fermentation at 13 ºC than at 25 ºC. They concluded that the higher abundance of transcripts was related to cell-cycle progression at low temperature due to an increased ability of yeast cells to survive at this fermentation temperature. Consistent with this idea, the viability of the yeast cells during fermentation was higher at 13 ºC than at 25 ºC. Moreover, the Δ*lte1* strain had a significant decrease in fermentation activity, which resulted in incomplete fermentation at 13 ºC, but was unaffected at 25 ºC.

As mentioned above, Lte1p is a part of the mitotic exit network that governs the transition from late M to G1, which is a strongly regulated network. The increase of one unit of this protein complex would produce an imbalance in this network, which may explain why the strain that overexpressed *LTE1* did not have improved fermentation activity and had impaired growth (higher GT than the control strain).

A similar co-regulation between growth phase and low temperature can also be suggested for the genes *CSF1* and *HSP104*. However, low temperature seemed to be a more important activator than growth phase. *HSP104* encodes a general anti-stress chaperone of the *HSP100* gene family (Bösl *et al.*, 2006), which helps to disassemble protein aggregates that have accumulated due to stress (Glover & Lindquist, 1998). Hsp104p is required to restore crucial protein functions that were lost upon stress-induced aggregation (Bösl *et al.*, 2006). This role in dissolving protein aggregates must be important to start growth in a new medium because, in this study, the highest gene activity was observed after yeast inoculation. Moreover, low temperature provokes misfolded proteins and aggregates (Al-Fageeh & Smales, 2006); therefore, this protein may be necessary for stimulating growth at low temperature. Regarding *CSF1*, little information is available regarding the function of the gene. Tokai *et al.* (2000) reported that this gene was required for growth and fermentation, but only at low temperatures. They postulated that this gene was associated with nutrient transport at low temperatures. The importance of both genes in cold adaptation and acclimation is supported by the observed fermentation activity of the mutant and overexpressing strains. The mutant strains were unable to ferment grape-must, producing a stuck fermentation when the medium contained half of the sugars, whereas the overexpressing strain had a better fermentation rate and produced higher populations than the control strain.

*TIR2* was the other gene selected for stabilizing its overexpression in the wine yeast. The overexpressing *TIR2* strain grew faster (GT values) and reached a higher maximum population during wine fermentation than the control strain. It should be highlighted that inoculation of yeast into refrigerated grape-must (13 ºC) can result in up to 90% mortality. This decrease in inoculum viability produces a longer lag phase during

fermentations at 13 ºC (Beltran *et al.*, 2006). The overexpression of *TIR2* significantly decreased this mortality and decreased the lag or adaptation phase. Tir2p is a cell wall mannoprotein induced by cold shock and anaerobiosis (Kowalski *et al.*, 1995; Cohen *et al.*, 2001). In this overexpressing strain, higher growth rate and maximum population were correlated with higher fermentation rate.

Temperature fluctuations are an inevitable aspect of microbial life in exposed natural environments, but sub-optimal temperatures are also common in industrial processes like winemaking, in which the fermentation is cooled to retain volatile compounds and increase the wine aroma. Although the response of *S. cerevisiae* to heat-shock stress has been investigated widely, little is known about the molecular mechanisms that govern adaptation to cold (Al-Fageeh & Smales, 2006). In the present study, we have examined 10 genes described as cold induced and crucial for low temperature adaptation. Our results did not confirm the low temperature regulation for most of these genes. However, we did find that they were regulated in a growth-dependent manner. Only *CSF1*, *HSP104* and *TIR2* were up regulated at low temperature. This higher transcriptional activity at low temperature was corroborated by the growth and fermentation activity of the disrupted or overexpressing genes in the derivative haploid of the wine strain. Thus, several copies of these genes were integrated in the genome of the commercial wine strain QA23 to confirm their overexpression improves fermentation performance at low temperature. Overexpression of these genes decreased lag phase, increased fermentation rate and reached higher populations than the control strain. For industrial application, chromosomal integration is preferred over expression of non-integrative plasmids, because the latter usually requires cultivation of the recombinant strain in the presence of antibiotics or in a chemically defined medium in order to maintain selection pressure. In addition, episomal and centromeric plasmids,

both used in this study, exhibit a certain degree of structural instability (Flagfeldt et al., 2009). These results are a consequence of one round of integrative transformation but we can increase this overexpression by integrating more copies of the desired genes in successive rounds of transformations of the same wine strain. The *S. cerevisiae* genome contains about 280 delta elements, representing remnants of LTR-retrotransposons (Ty1 and Ty2) previously integrated at these sites (Kim et al., 1998). Our results demonstrate the advantages of carrying out functional analysis in industrial strains and in an experimental context that approximates industrial conditions. In our opinion, such studies are valuable not only in terms of basic research but are also beneficial for application to the productive sector. The identification of key genes that are useful for particular aspects of the biotechnological industry might improve the process either through the selection of proper strains or through the genetic modification of current industrial yeasts. Currently, overexpressing strains of the abovementioned genes are being developed using clean and safe integrating methods, which could be used in the wine industry for future fermentations at low temperature.

## Acknowledgements

## References

Abramova NE, Cohen BD, Sertil O, Kapoor R, Davies KJA & Lowry CV (2001) Regulatory mechanisms controlling expression of the *DAN/TIR* mannoprotein genes

during anaerobic remodeling of the cell wall in *Saccharomyces cerevisiae*. *Genetics* **157**:1169–1177.

Aguilera J, Randez-Gil F & Prieto JA (2007) Cold response in *Saccharomyces cerevisiae*: new functions for old mechanisms. *FEMS Microbiol Rev* **31**:327–341.

Al-Fageeh MB & Smales CM (2006) Control and regulation of the cellular responses to cold shock: The responses in yeast and mammalian systems. *Biochem J* **397**:247–259.

Beltran G, Novo M, Guillamón JM, Mas A & Rozès N (2008) Effect of fermentation temperature and culture media on the yeast lipid composition and wine volatile compounds. *Int J Food Microbiol* **121**:169–177.

Beltran G, Novo M, Rozès N, Mas A & Guillamón JM (2004) Nitrogen catabolite repression in *Saccharomyces cerevisiae* during wine fermentations. *FEMS Yeast Res* **4**:625–632.

Beltran G, Novo M, Leberre V, Sokol S, Labourdette D, Guillamón JM, Mas A, François J & Rozès N (2006) Integration of transcriptomic and metabolic analyses for understanding the global responses of low-temperature winemaking fermentations. *FEMS Yeast Res* **6**:1167–1183.

Beltran G, Rozès N, Mas A & Guillamón JM (2007) Effect of low-temperature fermentation on yeast nitrogen metabolism. *World J Microbiol Biotechnol* **23**:809–815.

Bradbury JE, Richards KD, Niederer HA, Lee SA, Dunbar PR & Gardner RC (2006) A homozygous diploid subset of commercial wine yeast strains. *Anton Leeuw Int J* **89**:27–38.

Bösl B, Grimminger V & Walter S (2006) The molecular chaperone Hsp104-A molecular machine for protein disaggregation. *J Struct Biol* **156**:139–148.

Castrillo JI, Zeef LA, Hoyle DC, Zhang N, Hayes A, Gardner DCJ *et al* (2007) Growth control of the eukaryote cell: A systems biology study in yeast. *J Biol* **6**(2).

Cohen BD, Sertil O, Abramova NE, Davies KJA & Lowry CV (2001) Induction and repression of *DAN1* and the family of anaerobic mannoprotein genes in *Saccharomyces cerevisiae* occurs through a complex array of regulatory sites. *Nucleic Acids Res* **29**:799–808.

Dower WJ, Miller JF & Ragsdale CW (1988) High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res* **16**:6127–6145.

Estruch F (2000) Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. *FEMS Microbiol Rev* **24**:469–486.

Flagfeldt DB, Siewers V, Huang L & Nielsen J (2009) Characterizatin of chromosomal integration sites for heterologous gene expression in *Saccharomyces cerevisiae*. *Yeast* **26**: 545–551.

Guerra OG, Rubio IG., da Silva Filho CG, Bertoni RA, dos Santos Govea RC & Vicente EJ (2006). A novel system of genetic transformation allows multiple integrations of a desired gene in *Saccharomyces cerevisiae* chromosomes. *J Microbiol Meth* **67**: 437–445.

Gietz RD & Woods RA (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods enzymol* **350**:87–96.

Glover JR & Lindquist S (1998) Hsp104, Hsp70, and Hsp40: A novel chaperone system that rescues previously aggregated proteins. *Cell* **94**:73–82.

Güldener U, Heck S, Fiedler T, Beinhauer J & Hegemann JH (1996) A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res* **24**:2519–2524.

Haslbeck M, Walker S, Stromer T, Ehrnsperger M, White HE, Chen S, *et al.* (1999) Hsp26: A temperature-regulated chaperone. *EMBO J* **18**:6744–6751.

Homma T, Iwahashi H & Komatsu Y (2003) Yeast gene expression during growth at low temperature. *Cryobiology* **46**:230–237.

Huxley C, Green ED & Dunham I (1990) Rapid assessment of *S. cerevisiae* mating type by PCR. *Trends Genet* **6**:236.

Jansen G, Wu C, Schade B, Thomas DY & Whiteway M (2005) Drag&Drop cloning in yeast. *Gene* **344**:43–51.

Kim MD, Vanguri S, Boeke JD & Gabriel A (1998) Transposable elements and genome organization: a comprehensive survey of retrotransposons revealed by the complete *Saccharomyces cerevisiae* genome sequence. *Genome Res* **8**: 464–478.

Kondo K & Inouye M (1991) *TIP1*, a cold shock-inducible gene of *Saccharomyces cerevisiae*. *J Biol Chem* **266**:17537–17544.

Kondo K & Inouye M (1992) Yeast *NSR1* protein that has structural similarity to mammalian nucleolin is involved in pre-rRNA processing. *J Biol Chem* **267**:16252–16258.

Kowalski LRZ, Kondo K & Inouye M (1995) Cold-shock induction of a family of TIP1-related proteins associated with the membrane in *Saccharomyces cerevisiae*. *Mol Microbiol* **15**:341–353.

Murata Y, Homma T, Kitagawa E, Momose Y, Sato MS, Odani M, *et al*. (2006) Genome-wide expression analysis of yeast response during exposure to 4 °C. *Extremophiles* **10**:117–128.

Pizarro FJ, Jewett MC, Nielsen J & Agosin E (2008) Growth temperature exerts differential physiological and transcriptional responses in laboratory and wine strains of *Saccharomyces cerevisiae*. *Appl Environ Microb* **74**:6358–6368.

Querol A, Barrio E, Huerta T & Ramon D (1992) Molecular monitoring of wine fermentations conducted by active dry yeast strains. *Appl Environ Microb* **58**:2948–2953.

Regenberg B, Grotkjær T, Winther O, Fausbøll A, Åkesson M, Bro C, *et al*. (2006) Growth-rate regulated genes have profound impact on interpretation of transcriptome profiling in *Saccharomyces cerevisiae*. *Genome Biology* **7**(11).

Riou C, Nicaud J, Barre P & Gaillardin (1997) Stationary-phase gene expression in *Saccharomyces cerevisiae* during wine fermentation. *Yeast* **13**:903–915.

Sahara T, Goda T & Ohgiya S (2002) Comprehensive expression analysis of time-dependent genetic responses in yeast cells to low temperature. *J Biol Chem* **277**:50010–50021.

Sales K, Brandt W, Rumbak E & Lindsey G (2000) The LEA-like protein *HSP12* in *Saccharomyces cerevisiae* has a plasma membrane location and protects membranes against desiccation and ethanol-induced stress. *BBA-Biomembranes* **1463**:267–278.

Salvadó Z, Chiva R, Rodríguez-Vargas S, Rández-Gil F, Mas A & Guillamón JM (2008) Proteomic evolution of a wine yeast during the first hours of fermentation. *FEMS Yeast Res* **8**:1137–1146.

Sambrook J, Fritsch EF & Maniatis T (1989) Methods in yeast genetics: a laboratory manual. *Cold Spring Harbor Laboratory Press*, Cold Spring Harbor, NY.

Schade B, Jansen G, Whiteway M, Entian KD & Thomas DY (2004) Cold adaptation in budding yeast. *Mol Biol Cell* **15**:5492–5502.

Shirayama M, Matsui Y, Tanaka K & Toh-e (1994) Isolation of a *CDC25* family gene, *MSI2/LTE1*, as a multicopy suppressor of *IRA1*. *Yeast* **10**:451–461.

Sierkstra LN, Verbakel JMA & Verrips CT (1992) Analysis of transcription and translation of glycolytic enzymes in glucose-limited continuous cultures of *Saccharomyces cerevisiae*. *J Gen Microbiol* **138**(12).

Tai SL, Daran-Lapujade P, Walsh MC, Pronk JT & Daran J (2007) Acclimation of *Saccharomyces cerevisiae* to low temperature: A chemostat-based transcriptome analysis. *Mol Biol Cell* **18**:5100–5112.

Tokai M, Kawasaki H, Kikuchi Y & Ouchi (2000) Cloning and characterization of the *CSF1* gene of *Saccharomyces cerevisiae*, which is required for nutrient uptake at low temperature. *J Bacteriol* **182**:2865–2868.

Torija MJ, Rozès N, Poblet M, Guillamón JM & Mas A (2003) Effects of fermentation temperature on the strain population of *Saccharomyces cerevisiae*. *Int J Food Microbiol* **80**:47–53.

Ursic D & Culbertson MR (1991) The yeast homolog to mouse *Tcp-1* affects microtubule-mediated processes. *Mol Cell Biol* **11**:2629–2640.

Walker ME, Gardner JM, Vystavelova A, McBryde C, Lopes MDB & Jiranek V (2003) Application of the reuseable, KanMX selectable marker to industrial yeast: Construction and evaluation of heterothallic wine strains of *Saccharomyces cerevisiae*, possessing minimal foreign DNA sequences. *FEMS Yeast Res* **4**:339–347.

Zhang L, Ohta A, Horiuchi H, Takagi M & Imai R (2001) Multiple mechanisms regulate expression of low temperature responsive (*LOT*) genes in *Saccharomyces cerevisiae*. *Biochem Biophys Res Commun* **283**:531–535.

Zhao X, Chang AY, Toh-e A & Arvan P (2007) A role for Lte1p (a low temperature essential protein involved in mitosis) in proprotein processing in the yeast secretory pathway. *J Biol Chem* **282**:1670–1678.

**FIGURE LEGENDS**

**Figure 1.** Fermentation kinetics and growth was measured as density reduction and CFU mL $^{-1}$ (filled symbols indicate fermentation at 13 ºC and open symbols indicate the control fermentation).

**Figure 2.** Relative expression of the selected genes at different stages of alcoholic fermentation at 25 ºC (open bars) and 13 ºC (filled bars). Changes in gene activity are shown relative to the expression at time zero of fermentation (set as value 1). Growth curves were measured by OD increases (right axis) throughout fermentation at 25 ºC (open circles) and at 13 ºC (shaded circles).

**Figure 3.** Generation time (GT) of mutant (A) and overexpressing (B) strains at 13 ºC (filled bars) and 25 ºC (open bars). Time zero represents the time required by the control strains (*ho*QA23 for the mutants and *ho*QA23pGREG505 or *ho*QA23YEp181 for the overexpressing strains) to double their population. Positive and negative values represent the increases and decreases in the generation time (in hours) of the mutant and overexpressing strains regarding their control strains. The duplication time for the control strains was the following: 17 h and 4 h for *ho*QA23, 21 h and 5 h for *ho*QA23YEp181 and 13 h and 4 h for *ho*QA23pGREG505 at 13 ºC and 25 ºC, respectively. * Indicates results with statically significant differences (P-value $\leq$ 0.01).

**Figure 4.** Fermentation activity of mutant (A) and overexpressing (B) strains at 13 ºC (filled bars) and 25 ºC (open bars). Time zero represents the time required by the control strains (*ho*QA23 for the mutants and *ho*QA23pGREG505 or *ho*QA23YEp181 for the overexpressing strains) to ferment 50% (T50) of the sugars in a synthetic must. Positive

and negative values represent the increases and decreases in T50 (in hours) of the mutant and overexpressing strains regarding their control strains. The T50 for the control strains were the following: 170 h and 60 h for *ho*QA23, 194 h and 72 h for *ho*QA23YEp181 and 295 h and 70 h for *ho*QA23pGREG505 at 13 ºC and 25 ºC, respectively. * Indicates results with statically significant differences (P-value ≤ 0.01).

**Figure 5**. Fermentation kinetics at 13 ºC of the overexpressing strains δ*CSF1*δ, δ*HSP104*δ and δ*TIR2*δ and the control strain QA23 measured as (A) density reduction, (B) Optical Density (OD) and (C) time required to ferment 5% (T5; white bars), 50% (T50; grey bars) and 100% (T100; black bars). Time zero represents the T5, T50 and T100 of the control strain QA23 (59, 173 and 341 h respectively). Positive and negative values represent the increases and decreases (in hours) regarding the control strain. *Indicates results with statistically significant differences (P-value ≤ 0.01).

**Table S1**. List of the DNA sequences of the oligonucleotides that were used in this study.

| Primer | SEQUENCE (5´→ 3´) |
|---|---|
| HOm-F [a] | AGACATCGCAAACGTCACGGCTAACTCTTACGTTATGTGCGCAGATGGCT CGTACGCTGCAGGTCGACA |
| HOm-R [a] | ACTTTATGAGGCCCGCGGACAGCATGAAACTGTAAGATTCCGCCACATT ACTAGTGGATCTGATATC |
| CSF1mF [a] | TAGATTGGATTCTCACGGTAGTAGTATGTTTGACAATGATATTCTACATG CGTACGCTGCAGGTCGACA |
| CSF1mR [a] | CATTATGAAGATGGAAGTGTATGTTACTTATAGTGCCGATTAAGAACATA ACTAGTGGATCTGATATC |
| HSP12mF [a] | ATGTCTGACGCAGGTAGAAAAGGATTCGGTGAAAAAGCTTCTGAAGCTTT CGTACGCTGCAGGTCGACA |
| HSP12mR [a] | TTACTTCTTGGTTGGGTCTTCTTCACCGTGGACACGACCGGAAACATATT ACTAGTGGATCTGATATC |
| HSP26mF [a] | ACAAATTAACATGTCATTTAACAGTCCATTTTTTGATTTCTTTGACAACA CGTACGCTGCAGGTCGACA |
| HSP26mR [a] | TTCTTGAGAAGAAACCTCAATCTTCTTGACGTGGTTCTTACCATCCTTCA ACTAGTGGATCTGATATC |
| HSP104mF [a] | GCGTTATTCAGCATCATTGGAAGTGAAATGGACTGGATCGACGACAAGTT CGTACGCTGCAGGTCGACA |
| HSP104mR [a] | ATTACTTCCTTTAAACTTTAGTTATCAACGCCATATGTCCCAAATACAAT ACTAGTGGATCTGATATC |
| LOT2mF [a] | TTGTCATAAACTCACCAAGAACCACAAAGTTATTGAACAATGGGTATGT CGTACGCTGCAGGTCGACA |
| LOT2mR [a] | AAAGATTAATCTTGGGTCTTTTGAGAACCACGTAGTAAACCGGTTCTTCT ACTAGTGGATCTGATATC |
| LTE1mF [a] | ATGGAAATATTTAGCCAGAA  GATTACTACCCGACTCCATCCTCAAATG |

|  |  |
|---|---|
|  | T<u>CGTACGCTGCAGGTCGACA</u> |
| LTE1mR [a] | ATAGTTCATTAATCTCTTCTTGTGTTAATGTGCTAATATAAACACACATTA <u>ACTAGTGGATCTGATATC</u> |
| NSR1mF [a] | CAGACAGGATTTCACAGGCAGCTAAGTAATAACGAATATGCTTGCTTCAC <u>CGTACGCTGCAGGTCGACA</u> |
| NSR1mR [a] | CAAATAGAGATGGTGAATGAAAGGTTTAAGAATGTATCCTATTTACTTAC <u>ACTAGTGGATCTGATATC</u> |
| TIP1mF [a] | ATGTCCGTTTCCAAGATTGCTTTCGTTTTAAGTGCCATTGCCTCTTTGGC <u>CGTACGCTGCAGGTCGACA</u> |
| TIP1mR [a] | TAACAATAAAGCAGCTGCACCTGCAACAACAGCACCGAAAGAGGCAGCG C<u>ACTAGTGGATCTGATATC</u> |
| TIR2mF [a] | AGAAACATCTTTATAAATCTTGGCCGGCCAAGATTCCCAAACGAGACTC <u>CGTACGCTGCAGGTCGACA</u> |
| TIR2mR [a] | ACGGTAAATCATTTCCGTTTTCAAAACGTACACTACAGCCTCCAGTTGAT <u>ACTAGTGGATCTGATATC</u> |
| HOc-F [b] | GAGGTTTGCAGAAGCTTGTTGA |
| HOc-R [b] | TTGGCGTATTTCTACTCCAGCAT |
| K2 [b] | GGGACAATTCAACGCGTCTG |
| MAT [b] | AGTCACATCAAGATCGGTTATGG |
| MATF [b] | GCACGGAATATGGGACTACTTCG |
| MATa [b] | ACTCCACTTCAAGTAAGAGTTTG |
| CSF1c-F [b] | TAAAGTGAGAGGTTCACGATGCGT |
| CSF1c-R [b] | AATGATTACTATGGGTGGAAATGCT |
| HSP12c-F [b] | TGGCATCTGTTAAGGGGATA |
| HSP12c-R [b] | AAACAGGTTGAAAAGACGCGA |
| HSP26c-F [b] | CTCGTAACAGTAAGGTATTCGCACTTA |
| HSP26c-R [b] | CTTTCCCAGAAGTAGAAGTAGGCAAG |
| HSP104c-F [b] | AATCCAAACTCCAAGGGCACAT |

| | |
|---|---|
| HSP104c-R [b] | GTAGTCATATATATGAGTCATATGAGTCGC |
| LOT2c-F [b] | ACCGCCTACGAGTAATGTTGGATA |
| LOT2c-R [b] | GAATGCACAGAACTCGTAGTATTTCC |
| LTE1c-F [b] | AATATCATTTGGTATGGAGGGACC |
| LTE1c-R [b] | GCAATATCTGCCTCTATACCAATCACT |
| NSR1c-F [b] | TAGCTGTTGCTGTCTGCACTT |
| NSR1c-R [b] | CACAACGCCCAAGCTTTTAT |
| TIP1c-F [b] | TGATTGTTCCGGGAAATGTG |
| TIP1c-R [b] | AAGGAGAAAGACTCTTCGCTG |
| TIR2c-F [b] | TTGCAGAAGGAGTGCAGAGTT |
| TIR2c-R [b] | TGTCGAAAATGTTCGTTCCG |
| CSF1rec5-F [c] | AACAAAAGCTGGAGCTCGTTTAAACGGCGCGCCGAGCGTGCATTACACCGTACAAT |
| CSF1rec2-R [c] | GCGTGACATAACTAATTACATGACTCGAGGTCGACAATTGATACTATGGGTGGAAATGCT |
| HSP104rec5-F [c] | AACAAAAGCTGGAGCTCGTTTAAACGGCGCGCCGTGGATGTTCAGGACTAACGT |
| HSP104rec2-R [c] | GCGTGACATAACTAATTACATGACTCGAGGTCGACCGCTGTAACATTATATTGGCA |
| LOT2rec5-F [c] | AACAAAAGCTGGAGCTCGTTTAAACGGCGCGCCTCGTATCAGTGGAGGCGAGAG |
| LOT2rec2-R [c] | GCGTGACATAACTAATTACATGACTCGAGGTCGACTACAAGAACACGTGCCACGG |
| LTE1rec5-F [c] | AACAAAAGCTGGAGCTCGTTTAAACGGCGCGCCTTCGTCCTATACGCCGGATTC |
| LTE1rec2-R [c] | GCGTGACATAACTAATTACATGACTCGAGGTCGACTCGTCGGACATATCCTCGTTG |
| Rec5-F [c] | AACAAAAGCTGGAGCTCGTTTAAACGGCGCGCC |

| | |
|---|---|
| HSP12t-F [d] | CATGTAgtcgacTGGCTCTTGGGACAAGACTA |
| HSP12t-R [d] | ATTATCgcatgcAAGAAGCAACCGCCATGTTTA |
| HSP26t-F [d] | CATGTAgtcgacGCACCGTTGAACCTGTAGCTTA |
| HSP26t-R [d] | ATTATCgcatgcTCTTCGTCGAGGTTTAACGCTT |
| NSR1t-F [d] | CATGTAgtcgacCCTCATACGCAGAGAAGTGT |
| NSR1t-R [d] | ATTATCgcatgcTTTGAGTATTTTCCAAGGCCT |
| TCP1t-F [d] | CATGTAgtcgacTGCTGACGAAGAATCTTCCTCA |
| TCP1t-R [d] | ATTATCgcatgcTTCATGGAATACACTCCGCATT |
| TIP1t-F [d] | CATGTAgtcgacGGCTTGCGATCCACATTGATA |
| TIP1t-R [d] | ATTATCgcatgcGTTGGCGATGCCCATATTTAG |
| TIR2t-F [d] | CATGTAgtcgacCAATCATATTTCATGGCCTCC |
| TIR2t-R [d] | ATTATCgcatgcTGTTCGTTCCGCTTATGATTA |
| Actq-F [e] | TGGATTCCGGTGATGGTGTT |
| Actq-R [e] | CGGCCAAATCGATTCTCAA |
| CSF1q-F [e] | TTTAGCGACTACTGAGGCATGGT |
| CSF1q-R [e] | AATAGCGGTTACTAATTGCTCAGTTG |
| HSP12q-F [e] | GGCAGACCAAGCTAGAGATTACATG |
| HSP12q-R [e] | AACATATTCGACGGCATCGTT |
| HSP26q-F [e] | CTGGTGAAATTCCATCTACCTTGA |
| HSP26q-R [e] | TTACCAGAGCTGCTCTCCTTGAC |
| HSP104q-F [e] | TTCTGGATAGCGCCTTAGTCACT |
| HSP104q-R [e] | TGGCAATCTTCTATATGGCAAGTAA |
| LOT2q-F [e] | GGTGCTGCCAAGTTGAGAACT |
| LOT2q-R [e] | TGGACAATTTGCTTAACGATACCA |
| LTE1q-F [e] | ACCACTGCAGGACATTTCAGAA |
| LTE1q-R [e] | AAGTCGCCTGGTTCAATCAGA |
| NSR1q-F [e] | TTCAATGCTGACAGAGACGCTATT |

| | |
|---|---|
| NSR1q-R [e] | GATACGGACGGAAACAACTTCAC |
| TCP1q-F [e] | GGTGCCAGGTTATGCTTTGAA |
| TCP1q-R [e] | CGGCGATACGTTTAGGCATT |
| TIP1q-F [e] | ATTCCATCTGATGTCTTGAGTGTGTAT |
| TIP1q-R [e] | AGTGATAGCATCAAAGTCCAATTCAC |
| TIR2q-F [e] | GTCAAGTTCAAGCCACTAGCACTGT |
| TIR2q-R [e] | CCATGACACCAGCACCCATA |
| H104kan-F [f] | TTCCATGCGCTTGGAAAATTGAATGATAGAAAATTCGAACAGCGATGATT <u>CGTACGCTGCAGGTCGACA</u> |
| H104kan-R [f] | TTATAACTATCACTATATTATCATATACGGTGTTAGATGATGACATAGGT <u>ACTAGTGGATCTGATATC</u> |
| CSF1kan-F [f] | TCTTTTAGGGTCATTGATATGATATTAAAGCCAGCCATAAATTCATCAAA <u>CGTACGCTGCAGGTCGACA</u> |
| CSF1kan-R [f] | AAATAAATGAATTGTCATATTATGTCGAAGAGACTAACGTGGATATTGGA <u>ACTAGTGGATCTGATATC</u> |
| TIR2kan-F [f] | AATCATAGTAGCATGGCGGCAACAGCAGCTAAAGCACCGGCACCCATAC C<u>CGTACGCTGCAGGTCGACA</u> |
| TIR2kan-R [f] | CTCTTCAACATCTGCCGTTCCAGCTTCCACCACCGGTTACAACAATTCTA <u>ACTAGTGGATCTGATATC</u> |
| kanD2-R [g] | AAATGGGTGAATGTTGAGATAATTGTTGGGATTCCATTGTTGATAAGGC <u>ACTAGTGGATCTGATATC</u> |
| CSF1D1F [g] | TGTTGGAATAGAAATCAACTATCATCTACTAACTAGTATTTACATTACTA<u>G AGCGTGCATTACACCGTACAAT</u> |
| H104D1F [g] | TGTTGGAATAGAAATCAACTATCATCTACTAACTAGTATTTACATTACTA<u>G TGGATGTTCAGGACTAACGT</u> |
| TIR2D1F [g] | TGTTGGAATAGAAATCAACTATCATCTACTAACTAGTATTTACATTACTA <u>CAATCATATTT CATGGCCTCC</u> |

[a] Primers used for amplification of the knockout cassette. The sequence with homology to the *loxP-kanMX4-loxP* cassette from the plasmid pUG6 is underlined. The remainder of the primer sequences is homologous to the flanking region of the deleted ORF.

[b] Primers used to check the correct insertion of the deletion cassette.

[c] Primers used for the construction of the overexpressing strains in the plasmid pGREG505. The recombination sequences homologous to the plasmid ends are underlined.

[d] Primers used for the construction of overexpressing strains in the plasmid YEp181. Lower case sequences show restriction sites designed for cloning of the PCR products.

[e] Primers used for transcriptomic analysis by RT-PCR.

[f] Primers used for the construction of the overexpressing cassette (gene of interest-kanMX4) in QA23 strain. The sequence with homology to the *loxP-kanMX4-loxP* cassette from the plasmid pUG6 is underlined.

[g] Primers used for the construction of the overexpressing strains by integration into the delta regions. The recombination sequences homologous to delta regions are not underlined.

**Figure 1**
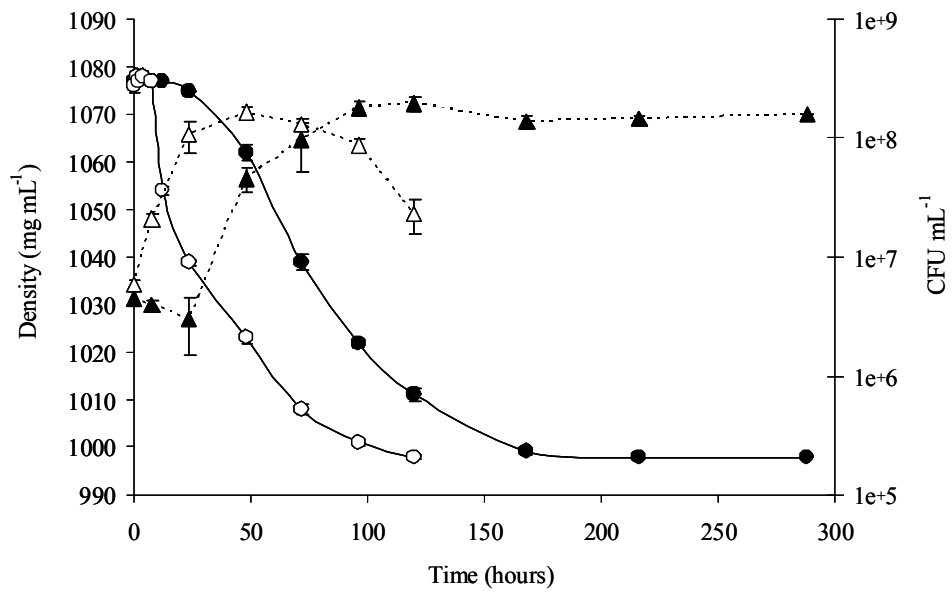
**Figure 2**
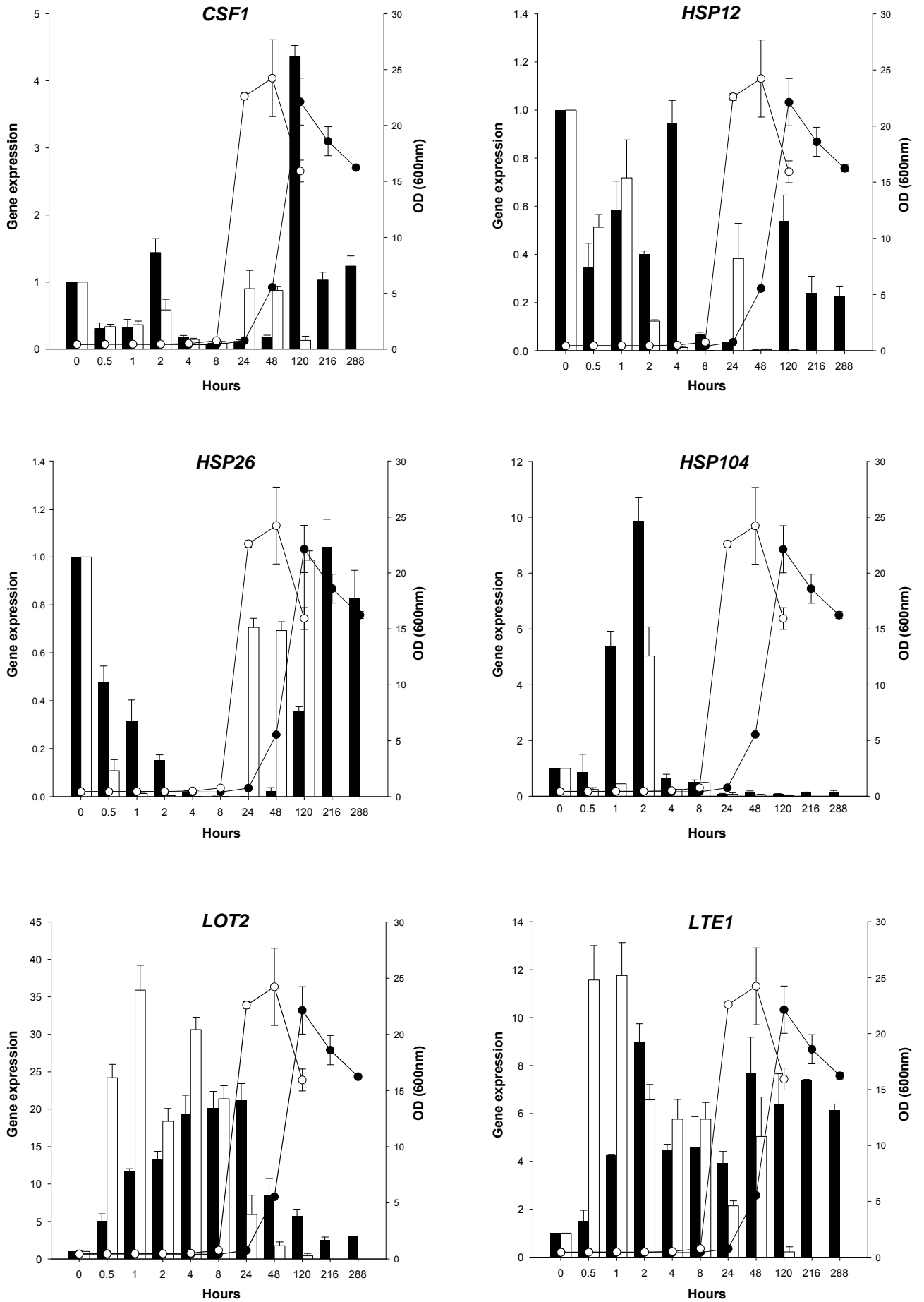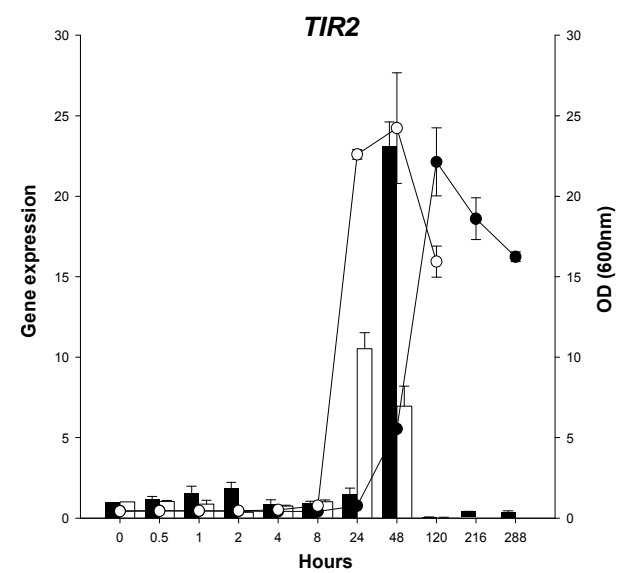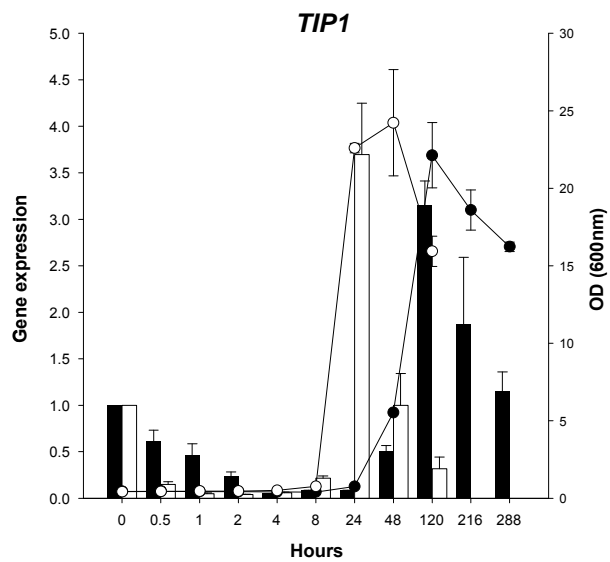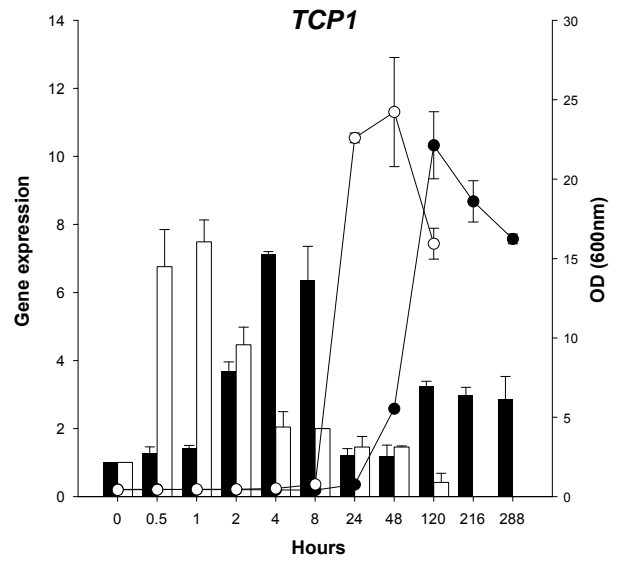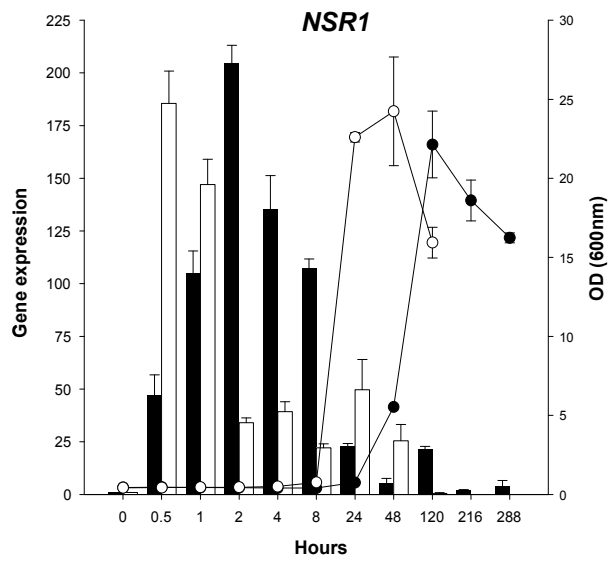
**Figure 3**

**Figure 4**

**Figure 5**

**A**

Density (mg mL$^{-1}$)

1080
1070
1060
1050
1040
1030
1020
1010
1000
990

0    50    100    150    200    250    300    350    400

**Hours**

**B**

OD (600 nm)

14
12
10
8
6
4
2
0

0    50    100    150    200    250

**Hours**

δ*CSF1*δ      δ*HSP104*δ      δ*TIR2*δ      QA23

**C**

*

*

*

δ*CSF1*δ

*

*

δ*HSP104*δ

*

*

δ*TIR2*δ

-30    -25    -20    -15    -10    -5    0

**Hours**

☐ T5      ▨ T50      ■ T100

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