Modification of the *TRX2* gene dose in *Saccharomyces cerevisiae* affects Hexokinase 2 gene regulation during wine yeast biomass production

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Running title: Wine yeast transcriptomics and *TRX2* dose

Research paper headed to Applied Microbial and cell Physiology category

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Abstract

In the industrial yeast biomass production process, cells undergo an oxidative and other stresses which worsens the quality of the produced biomass. The overexpression of the thioredoxin codifying gene *TRX2* in a wine *Saccharomyces cerevisiae* strain increases resistance to oxidative stress and industrial biomass production yield. We observed that variations in the *TRX2* gene dose in wine yeast strains are relevant to determine the fermentative capacity throughout the industrial biomass production process. So we studied the molecular changes using a transcriptomic approach under these conditions. The results provide an overview of the different metabolic pathways affected during industrial biomass production by *TRX2* gene manipulation. The oxidative stress-related genes, like those related with the glutathione metabolism, presented outstanding variations. The data also allowed us to propose new thioredoxin targets in *S. cerevisiae*, such as Hexokinase 2, with relevance for industrial fermentation performance.

Keywords: Wine yeast propagation, *TRX2* gene, Oxidative stress, Omics, Hexokinase 2.

Introduction

In the yeast biomass production process, growth conditions are optimized to obtain maximum yields. However, yeast cells are affected by oxidative and other stresses which worsen the quality of the produced biomass (Pérez-Torrado et al. 2005). The relevance of the *TRX2* gene during industrial yeast biomass propagation has been recently demonstrated by the construction of an overexpressing strain (T*TRX2*) which increases biomass yield and fermentative capacity, and improves the oenological properties of the final product by improving the oxidative stress response (Gómez-Pastor et al. 2010a; Pérez-Torrado et al. 2009). The *TRX2* gene codifies for one of the two cytoplasmic thioredoxins in *Saccharomyces cerevisiae*, which is a small protein (11 kDa) with thiol-disulphide oxidoreductase activity (Holmgren 1989). *TRX2* was one of the first gene targets of the main oxidative stress transcription factor Yap1p to be identified, and it is among the most highly induced genes to respond to oxidative stress (Kuge and Jones 1994).

Thioredoxins are involved in protein protection against oxidative and reductive stress (Trotter and Grant 2002). They are also responsible for the negative regulation of Yap1p (Izawa et al. 1999), which is one of the eight members of the Yap-bZIP family of transcription factors, the major regulon in the oxidative stress response (Fernandes et al. 1997; Rodrigues-Pousada et al. 2004). Furthermore, they participate in the catalytic cycle of Orp1p (*GPX3*), which is itself a positive regulator of Yap1p activity (Dealunay et al. 2002; Mason et al. 2006). Yap1p rapidly localizes in the nucleus after H_2O_2 treatment (Kuge et al. 1997; Mason et al. 2006). Once inside the nucleus, Yap1p can also cooperate with other transcriptional factors, like Skn7p, to regulate the transcriptional activation of several oxidative stress response genes (He et al. 2009). In

addition, thioredoxins are also involved in the regulation of other transcription factors like Msn2p and Msn4p (Boisnard et al. 2009), which control most of the genes involved in the stress response (Martínez-Pastor et al. 1996).

During yeast growth, the presence of at least one thioredoxin is important to maintain redox homeostasis. However, *TRX2* is more specialized than *TRX1* in protection against ROS as the single mutant *trx2*Δ is more sensitive to H_2O_2 (Kuge and Jones 1994), and Trx2p is thought to be the physiological reducing agent for Yap1p (Kuge and Jones 1994). However, mechanisms alternative to nuclear localization have been described for Yap1p-dependent activation of several genes (Demasi et al. 2006; Inoue et al. 1999).

Other functions of thioredoxins depend on their oxidoreductase activity. They act as hydrogen donors for thioredoxin peroxidases and 3´-phosphoadenosine 5´-phosphosulfate reductase (PAPS), which is a key enzyme in the sulphate metabolism (Muller 1991). Besides, yeasts lacking thioredoxins cannot withstand the high dNTP synthesis rate required for efficient DNA replication, as shown by the prolonged S phase and the shortened G1 interval. These results provide in vivo evidence for thioredoxin as a physiologically relevant electron donor for ribonucleotide reductase during DNA precursor synthesis (Koc et al. 2006). Approximately 80 proteins associated with thioredoxins have been identified in *Escherichia coli* and plants, thus supporting the involvement of thioredoxins in at least 26 cellular processes (Kumar et al. 2004; Montrichard et al. 2009). Other studies have demonstrated that, in plants, thioredoxins directly interact with glycolytic proteins like enolase, pyruvate descarboxylase, glyceraldehyde 3-phosphate dehydrogenase, and fructose bisphosphatase, by controlling their enzymatic activity by redox regulation (Montrichard et al. 2009; Gómez-Pastor et al. 2010c). In mammal cells, it has been

recently described that the expression of a thioredoxin-interacting protein (Txnip) correlates well with the glycolytic flux, which is regulated by oxidative phosphorylation status (Yu et al. 2010).

In this work, we studied the cellular changes and abilities produced by variations in the *TRX2* gene dose in wine yeast strains growing under industrial conditions. We used a transcriptomic profile analysis approach in order to understand the improved fermentative capacity previously observed for a *TRX2* gene overexpressing strain. The data also contribute to highlight the transcriptional regulation of wine yeasts oxidative stress related-genes under industrial conditions and allow us to propose new putative thioredoxin targets in *S. cerevisiae*, such as hexokinase 2, with direct implications for fermentative capacity performance.

Materials and methods

Yeast strains, plasmids and cultivation conditions

We used the *S. cerevisiae* strain T73 (CECT 1894) isolated from Alicante (Spain) musts (Querol et al. 1992) that has been commercialized by Lallemand Inc. (Montreal, Canada). This strain has been widely used in several studies and has proven to be a good wine yeast model. This strain was genetically modified previously to T73ura3 (Puig et al. 1998) to construct other strains because of the absence of auxotrophies in wine natural yeasts. These strains are also aneuploids, and have a chromosome number that is not a multiple of the haploid number, and they require several transformation rounds for deletion gene construction.

The YEp-TRX2 plasmid was obtained by subcloning a 0.7 kb *Eco*RI fragment containing the yeast *TRX2* gene and promoter into the episomal yeast plasmid Yep352

carrying the selectable marker *URA3*. The T*TRX2* strain (Pérez-Torrado et al. 2009) is a T_{73}*ura3* strain transformed with the YEp-TRX2 plasmid following the lithium acetate procedure (Gietz et al. 1995).

Strain *trx2*$^{-}$ was obtained by the sequential deletion of the two copies of the *TRX2* gene in strain T_{73}*ura3*. Disruption was carried out by homologous recombination at both ends of the *TRX2* open reading frame of an integration cassette carrying a *kanR* marker gene flanked by *loxP* sites. Excision of the marker is inducible by the expression of Cre recombinase introduced into the same strain (Güldener et al. 1996) to allow repeated disruptions. Integration of the cassette at the *TRX2* locus and further excision of the *kanR* marker were confirmed by PCR analysis. The absence of any *TRX2* gene product was confirmed by northern and western blot analysis. Uracil prototrophy was restored by introducing a 1.1-kb *Hin*dIII linear fragment containing the *URA3* gene.

Industrial production conditions

Biomass propagation experiments were carried out as previously described (Gómez-Pastor et al. 2010a, b; Pérez-Torrado et al. 2005; Pérez-Torrado et al. 2009). Molasses medium used for biomass propagation was diluted to a final sucrose concentration of 60 g L^{-1} and was supplemented with 7.5 g L^{-1} of $(NH_4)_2SO_4$, 3.5 g L^{-1} of KH_2PO_4, 0.75 g L^{-1} of $MgSO_4 7H_2O$, 10 ml L^{-1} of vitamin solution (50 mg L^{-1} of D-biotin, 1 g L^{-1} of calcium pantothenate, and 1 g L^{-1} of thiamine hydrochloride), and 1 mL L^{-1} of antifoam 204 (Sigma, St. Louis, Mo.). Biomass propagation experiments were designed with two growth stages (batch and fed-batch) in a bioreactor BIOFLO III (NBS, New Jersey, USA), and technical parameters (agitation, pH and fed rate) were established as previously described (Pérez-Torrado et al. 2005). The bioreactor containing 2 liters of sterilized molasses medium at pH 4.5 was inoculated to an initial optical density of 0.05

(OD_{600} = 0.05) from overnight YPD (1% yeast extract, 2% peptone, 2% glucose) precultures incubated at 30ºC with shaking (250 rpm) for 12 h. During the batch phase, cells fermented the sucrose present in molasses to ethanol. When sucrose was exhausted (12-18 h), the cell metabolism shifted to respiration of the previously produced ethanol, until approximately 40 h of the process. During this phase, pH was allowed to freely vary between 4 and 5. When the ethanol finished, the fed-batch phase started by continuously feeding the reactor with the molasses medium at the desired flow rate to avoid a fermentative metabolism. During the fed-batch phase, the reactor pH was maintained at 4.5 by the automatic addition of 42.5% H_3PO_3 or 1 M NaOH. Dissolved oxygen was maintained above 20% by a PID automatic control system. Three independent production experiments were carried out for each strain.

Total RNA preparation and cDNA synthesis

Total yeast RNA was obtained from yeast cells (50 mg) by the hot phenol method (Kohrer and Domdey 1991). cDNA synthesis and labeling reactions were carried out under the same conditions previously described for control strain T_{73} (Gómez-Pastor et al. 2010b).

Microarray hybridization and analysis

We used *S. cerevisiae* microarray slides from the Microarray Center of University Health Network (Toronto, Canada), comprising 6240 yeast ORFs. The microarrays hybridization conditions were identical to those previously described (Gómez-Pastor et al. 2010b). The obtained data were analyzed using the GenePix Pro 6.1 software package (Axon Instruments, California, USA).

Data were ratio-based normalized and processed using Acuity 4.0 (Axon Instruments). A 5% False Discovery Rate and Bonferroni correction were used to select the statistically significant data. Only the $\log_2$ ratios showing a variation greater than a 1.5-fold in comparison to the previous T_{73} data (Gómez-Pastor et al. 2010b) were considered. A functional group analysis was done using the GOstat and GO term finder (SGD database; http://www.yeastgenome.org) online applications. The presented data correspond to the averages of three biological replicates for strain T*TRX2* and of two biological replicates for strain *trx2*Δ. The microarray data [E-TABM-1143] are available in the EMBL database (http://www.ebi.ac.uk/arrayexpress/).

Northern blot analysis

Total RNA was obtained by resuspending cells in 0.5 mL LETS buffer (200 mM LiCl, 20 mM EDTA, 20 mM Tris-HCl (pH 8.0), 0.4% SDS), 0.5 mL of phenol and 0.5 mL of glass beads (acid-washed, 0.4-mm diameter) and cells were broken in FastPrep (Mp Bio) 3 x 30 sec at 4ºC. Equal amounts of RNA (20 µg) were separated in 1% (w/v) agarose gels containing formaldehyde (2.5% v/v), and were transferred to a nylon membrane. The DNA probes were obtained and labeled by using the specific oligonucleotides indicated in Table 1 and the non radioactive PCR digoxigenin probe synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany). Membrane pre-hybridizations and hybridizations were also performed with Digoxigenin Easy Hyb solution from Roche (Roche Diagnostics GmbH). After stringency washes, blots were subjected to immunological detection using an anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche Diagnostics), followed by CDP-Star detection (Roche Diagnostics). Images were captured with the Las-1000 Plus imaging system (Fuji, Kyoto, Japan).

181 Western blot analysis

182 Cell extracts were separated in 10% SDS-polyacrylamide gels and transferred to
183 polyvinylidene difluoride membranes. A rabbit polyclonal antibody against hexokinase
184 2 (Randez-Gil et al. 1998) and a mouse antibody against actin (MP Biomedicals, Clone
185 C4, Ohio, USA) were used at the 1/3500 and 1/500 dilutions, respectively. Peroxidase-
186 conjugated secondary antibodies (GE Healthcare, New Jersey, USA) were used for
187 detection purposes according to the manufacture's recommendations. Image acquisition
188 was performed with a ChemiDoc CCD camera (Bio-Rad, California, USA).

189 Hexokinase activity assay

190 Hexokinase activity was assayed in crude extracts as described in the Worthington
191 Enzyme Manual (http://www.worthington-biochem.com/HK/assay.html). The assay is
192 based on reduction of NAD^+ through a coupled reaction with glucose-6-phosphate
193 dehydrogenase and is determined spectrophotometrically by measuring the increase in
194 absorbance at 340 nm. Each reaction mix contained the following: 0.05 M Tris-HCl
195 buffer (pH 8.0) containing 13.3 mM $MgCl_2$, 0.67 M glucose, 16.5 mM adenosine
196 5'triphosphate, and 6.8 mM NAD^+. All the reagents were dissolved in
197 Tris·$MgCl_2$ buffer. Protein content was determined by the Lowry method (Lowry et al.
198 1951). One unit of enzyme activity (U) was defined as the amount of enzyme catalyzing
199 the production of 1 μmol of NADH per minute and enzymatic activity was expressed as
200 mU (mg of total protein) $^{-1}$.

201 Measurement of fermentative capacity

202 To determine fermentative capacity, the biomass collected at 0 h, 18 h and 80 h was
203 dehydrated under air flux in an oven at 30ºC until approximately 8% relative humidity.

10^7cells/mL were inoculated in YPGF medium (1% yeast extract, 2% peptone, 10% glucose, 10% fructose) and incubated with shaking at 30ºC and 65 rpm. The exact number of cells was determined by recounting in a Neubauer camera. CO_2 production was measured every 20 min over a 300-min period with a Chittick instrument (American Association of Cereal Chemists, 12-10, Minnesota, USA) and fermentative capacity was expressed in mL CO_2 $(10^7$ cells$)^{-1}$ min^{-1}.

Results

Fermentative capacity properties are enhanced in T*TRX2* mutant during the whole process

Fermentative capacity is considered one of the most important properties of the produced yeast biomass as it is mainly used to ferment high sugar content media. This parameter can also be used as an indicator of the yeast biomass physiology and glycolytic flux as it is evaluated as the amount of CO_2 produced by fermentation. Fig. 1 shows the fermentative capacity evaluated for all the strains at 0 h, 18 h and 80 h of growth. It is interesting to observe that the T73 control strain displayed an approximate 50% decrease in fermentative capacity at 80 h compared to initial industrial propagation steps, which can be directly correlated with the high oxidative stress response noted throughout the biomass production process (Gómez-Pastor et al. 2010a, b). The thioredoxin-deficient mutant also displayed a similar behaviour to the control strain, although the fermentative capacity detriment was higher (61.2%) than for the control strain. In contrast, the T*TRX2* strain maintained its fermentative properties throughout the process and displayed only a 17% decrease at the end of the process. These results may be associated with the differential expression profile observed for specific glycolytic genes, like *HXK2*, in the *TRX2* gene modified strains.

228 Transcriptional profile of strain TTRX2 during the biomass propagation process

229 To study the global gene expression changes due to *TRX2* gene dose variation, we
230 performed bench-top trials of the industrial biomass propagation process to compare
231 wild-type T73 to strains T*TRX2* and *trx2*Δ. The global gene expression experiments
232 were done with three representative samples taken at 14 h (early-batch phase), 18 h
233 (late-batch) and 80 h (late-fed batch) under the identical fermentation conditions and
234 sampling stages to those previously reported by Gómez-Pastor et al., (2010b). mRNA
235 was obtained from each sample and microarray hybridizations were performed using
236 three biological replicates for each time point. Significantly expressed genes were
237 obtained after microarray scanning and data normalization. Expression changes for a
238 selected set of genes were confirmed by northern blot analysis as shown in Fig. 2, where
239 a quantitative comparison of microarray and northern analysis data is also included. As
240 can be seen, expression profiles are identical and the highest quantitative differences are
241 20%. Gene functional category analyses were performed among the differentially
242 expressed genes for the T*TRX2* strain compared to control strain T_{73} by using the
243 GOstat online tools (http://gostat.wehi.edu.au/).

244 Table 2 shows the expression ratios for the up-regulated genes corresponding to the
245 averaged $\log_2$ signal intensity among the different microarrays. In comparison to control
246 strain T_{73}, a total of 36 ORFs were differentially expressed during the biomass
247 propagation process. Nine ORFs were up-regulated at 14 h of growth, 14 ORFs at 18 h
248 and 18 ORFs at 80 h. The fact that the highest number of up-regulated genes appeared
249 in the fed-batch phase suggests the importance of *TRX2* during respiratory metabolism.
250 Although several genes were up-regulated as a result of *TRX2* gene overexpression

251 throughout the propagation process, no significant functional categories were found
252 among the up-regulated genes.

253 As expected, the greatest induction was observed for *URA3* and *TRX2,* both the genes
254 present in the episomal plasmid carrying *TRX2*. Whereas *URA3* was up-regulated
255 throughout the process, the *TRX2* gene was up-regulated only during the 14-18 h growth
256 period and it showed no differences with the control strain at the end of the propagation
257 process. An explanation for this is that the *TRX2* gene was cloned into the Yep352
258 plasmid under the control of its own promoter. These results indicate that only the gene
259 dose, and not the expression pattern, of *TRX2* was modified in the T*TRX2* strain as a
260 result of gene overexpression as it displayed a similar expression profile to that
261 previously observed for control strain T_{73} (Pérez-Torrado et al. 2009).

262 Among the overall up-regulated genes, we noted *YAP3* which encodes a Yap1p-related
263 transcription factor. It presented a similar expression pattern to the *TRX2* gene, and was
264 up-regulated between 14 h and 18 h of growth. We also observed the induction of
265 *HXK2* and *PDC6* during the metabolic transition. *HXK2* codifies hexokinase isoenzyme
266 2, which catalyzes the phosphorylation of glucose in the cytosol and it is the
267 predominant hexokinase during growth on glucose, whereas *PDC6* codifies a minor
268 isoform of pyruvate decarboxylase involved in alcoholic fermentation, and its
269 transcription is glucose- and ethanol-dependent. The up-regulation of them both might
270 explain the high fermentative capacity observed for the T*TRX2* strain (Gómez-Pastor et
271 al. 2010a).

272 Table 3 offers the ratios for the down-regulated genes during the propagation process. A
273 total of 45 ORFs were differentially expressed in comparison to control strain T_{73}.
274 These genes were grouped into four statistically significant functional categories:

oxidoreductase activity (p<6.02E-11), stress response (p<5.01E-4), carbohydrates metabolism (p<2.10E-3) and the protein catabolic process (p<7.60E-3), where most were specifically down-regulated at 80 h of growth.

In the oxidoreductase activity group, some Yap1p-dependent genes were down-regulated (*PRX1*, *GTT1* and *GRX2*), and a more specialized functional subcategory corresponding to glutathione peroxidase activity was seen (p<1.09E-3), which includes *GTT1*, *GPX1*, *GLO2*, *GRX2* and *GPX3*, all of which are involved in not only ROS detoxification through glutathione, but also in membrane protection from oxidative damage (Avery and Avery 2001). Among the genes grouped in this category, the presence of the *GPX3* gene is highlighted, which codifies a glutathione peroxidase (GPx)-like protein that fulfils the sensor function for Yap1p activation by H_2O_2 exposure (Delaunay et al. 2002).

Another representative functional category was the stress response-related genes (p<5.01E-4), which were highly down-regulated at 80 h**.** Among these genes, there are some important stress-responsive heat shock genes (*HSP33*, *HSP70* and *HSP12*), which encode the proteins primarily involved in protein folding and membrane protection.

In the final fed-batch stage, we also observed the down-regulation of several genes related to carbohydrates metabolism (*TPI1*, *GPM2* and *HXT5)*, as well as transcription factors *HAP2* and *TYE7*, which activate the expression of the respiratory- and glycolytic-related genes, respectively. Furthermore, many of the genes encoding proteins of unknown functions were significantly repressed in the T*TRX2* strain (Supplementary material).

Transcriptional profile for the trx2Δ strain during the biomass propagation process

298 A total of 41 ORFs were up-regulated in the mutant lacking the two copies of the *TRX2*
299 gene. Seventy-five percent of total over-represented genes were expressed during the
300 metabolic transition at 18 h of growth (Table 4). All the up-regulated genes were
301 grouped into five functional categories: Ribosomal subunit (p<1.39E-5), DNA
302 metabolic process (p<1.45E-4), Intracellular transport (p<1.95E-4), Stress response
303 (p<5.23E-3) and Sulphur metabolism (p<8.10E-3) (Table 4).

304 A strong down-regulation of the cytoplasmic ribosomal proteins codified by the RPLs
305 and RPSs genes has been described during the metabolic transition and the diauxic shift,
306 indicating repression of protein synthesis to reprogram the gene expression profile for
307 adaptation to new environmental conditions (Ashe et al. 2000; DeRisi et al. 1997;
308 Gómez-Pastor et al. 2010b). However in the *trx2*Δ strain, we observed an up-regulation
309 of several genes encoding ribosomal proteins for both the 60S and 40S ribosomal
310 subunits. Those genes involved in DNA metabolic processes were also up-regulated; for
311 instance, *CLB6* encoding a B-type cyclin involved in DNA replication during the S-
312 phase (Schwob et al. 1993). In addition, some of the genes involved in the stress
313 response by DNA damage were up-regulated at different growth times, such as the
314 *HEX3* gene at 14 h and *RFX1* at 80 h.

315 Among those genes grouped in the stress response functional category, we found *YBP1*,
316 which codifies a positive regulator of Yap1p and acts in the same pathway as Gpx3p
317 (Veal et al. 2003). Although its specific role is not understood, it is clear that the
318 interaction of this protein with Yap1p is required for Yap1p oxidation by Gpx3p.

319 The genes related to the traffic between the endoplasmic reticulum and the Golgi
320 apparatus (*SSS1, TRS20, RET2, VRG4* and *SUR2*), and those encoding proteins for
321 sulphur metabolism (*SPE2, JLP1, SAM1, YNL092W* and *YDR140W*), were also up-

322 regulated, although sulphur was not seen to be limited in molasses (Shima et al. 2005).
323 Regulation of them all seems to be affected by the absence of the *TRX2* gene in the
324 batch phase.

325 Similarly to the up-regulated genes, the greatest difference observed in the expression
326 pattern of the down-regulated genes compared to the control strain occurred during the
327 metabolic transition at 18 h of growth (Table 5). We observed how 38 of the 53 total
328 down-regulated genes localized at 18 h. The genes were grouped into four functional
329 categories: Polyubiquitination process ($p<2.09E-9$), Hexose transport ($p<1.07E-5$),
330 Stress response ($p<1.94E-4$) and Carbohydrates metabolism ($p<3.45E-3$).

331 The fact that a large number of the genes encoding proteins with polyubiquitination
332 functions were down-regulated correlates with the up-regulation of the protein
333 biosynthesis-related genes observed at 18 h of growth (Table 5). In addition, the
334 expression of some polyubiquitination genes has been reported to be essential for
335 resistance to high temperatures, starvation and other stress types (Finley et al. 1987).
336 Hence, the down-regulation of polyubiquitination genes could be understood as an
337 attenuated stress response.

338 Unexpectedly, the genes activated by several stresses were down-regulated at 18 h of
339 the process. Some were involved in the glutathione metabolism (*GLO1, GLO2, GSH2*
340 and *GTT1*), although no differences in total GSH were observed in the *trx2*Δ mutant
341 compared to the control strain (data not shown). The most down-regulated genes in
342 *trx2*Δ were *FDH1* and *FDH2,* two $NAD(^{+})$-dependent formate dehydrogenases which
343 are related to glutathione-dependent formaldehyde oxidation.

Regarding the genes related to glycolysis, the *HXK2* gene was down-regulated in *trx2*Δ during the metabolic transition at 18 h, whose expression profile was the opposite to that in the T*TRX2* strain. In addition, the hexose transport-related genes also displayed a specific repression during the metabolic transition in the absence of thioredoxin, even for the high-affinity glucose transporters (*HXT2* and *HXT4*), unlike the observed behaviour displayed by control strain T_{73} (Gómez-Pastor et al. 2010b).

Hexokinase 2 gene expression, protein level and protein activity are regulated by thioredoxin 2

One of the most interesting gene expression changes observed in the transcriptomic analysis was *HXK2,* which displayed an opposite gene expression profile among the *TRX2* gene-modified strains. As already shown in Fig. 2A, we checked the *HXK2* gene expression by northern blot analysis in the three T73, *trx2*Δ and T*TRX2* strains at 0 h and 18 hours of growth in the biomass propagation medium. The obtained results corroborated the microarrays data as the expression level of gene *HXK2* increased in the T*TRX2* strain and *trx2*Δ showed a reduced expression level when compared to the control strain.

To analyze whether alterations in *HXK2* gene expression are associated with an increased amount of protein, we performed a western blot analysis against Hxk2p at 0 h and 18 of growth (Fig. 3A). As expected, Hxk2p is detected at the 0 h time point, corresponding to cells from the YPD stationary preculture where no transcriptional induction of *HKX2* gene occurs due to glucose exhaustion (Herrero et al. 1995), but the level of protein is still detectable. All the strains increased the Hxk2p level between 0 h and 18 h growth. However, the T*TRX2* strain presented higher levels of Hxk2p than the control strain at both time points. Similar results were obtained for the hexokinase

activity assay (Fig. 3B) where the T*TRX2* gene displayed a significantly increased enzyme activity at 18 h of growth. Conversely, the *trx2*Δ strain exhibited a slight reduction in the Hxk2p amount and reduced enzyme activity.

Discussion

We have studied the effect of the *TRX2* gene dose on wine yeasts under industrial conditions using a transcriptomic approach to understand the improved phenotype previously described for the *TRX2* gene-overexpressing strain (Pérez-Torrado et al. 2009; Gómez-Pastor et al. 2010a). The compared analysis using both *TRX2* gene-modified strains, one overexpressing and other lacking the *TRX2* gene, also provided new insights into the relationship between thioredoxins, the main transcriptional factors involved in the oxidative stress response (Yap1p and Msn2/4p), and glycolytic flux regulation.

It has been recently reported that oxidative stress plays a critical role during the yeast biomass propagation process, and that it is at its highest during the metabolic transition at 18 h of growth (Gómez-Pastor et al. 2010a, b; Pérez-Torrado et al. 2009). At this stage, several oxidative-stress related genes are up-regulated in the T_{73} wine yeast strain. An important up-regulated gene in T*TRX2* is *YAP3,* which encodes one of the eight members of the Yap-bZIP family (Fernandes et al. 1997). For laboratory yeast strains, it has been observed that *YAP3* is down-regulated by hydrogen peroxide and heat shock treatments (Cohen et al. 2002), although it is slightly up-regulated in the presence of menadione (Gasch et al. 2000). Despite the genomic microarray analysis data available for multiple forms of environmental insults and cellular stresses, very little is known about the Yap3p molecular function (Rodrigues-Pousada et al. 2004). Our results indicate that *YAP3* gene expression may be controlled by a high *TRX2* gene dose and

392 that it may play an important role in industrial wine yeasts in response to endogenous
393 oxidative stress.

394 The *TRX2* overexpressing strain shows several relevant down-regulated antioxidant
395 genes (*PRX1*, *GTT1*, *GLO2*, *GPX1*, *GPX3* and *GRX2*), some of which are directly
396 regulated by Yap1p (Izawa et al. 1999). The down-regulation of several general stress
397 response genes in the T*TRX2* strain at the end of the process, such as heat shock
398 proteins (*HSP33*, *SBA1* and *HSP12*) and those genes related to multistress response
399 (*DDR2*), nutrient starvation (*TOR1*) and osmotic stress (*SIP18*), also indicate that these
400 cells are less affected by not only oxidative stress (Gómez-Pastor et al. 2010a), but also
401 other stressing conditions. Some of the down-regulated genes, like *HSP12*, *GPM2*,
402 *PRX1* and *GRX2*, are also directly controlled by Msn2/4p (Hasan et al. 2002; Praekelt
403 and Meacock 1990), which evidences the interaction between Trx2p and these major
404 general stress transcription factors, just as Boisnard and colleagues (2009) previously
405 reported for laboratory yeast strains.

406 In contrast, deletion of the *TRX2* gene also lowers the expression of several stress
407 response genes (*TIR2*, *GRE2*, *GLO1*, *ALD3* and *GCN4*) during the metabolic transition
408 at 18 h (Table 5), when a high expression is expected. However, we also found that the
409 *YBP1* gene is up-regulated (Table 4), which positively regulates Yap1p activity (Veal et
410 al. 2003), and that many genes involved in the glutathione metabolism (*GRE1*, *GRE2*,
411 *GLO1*, *GLO2*, *GSH2* and *GTT1*) and formaldehyde oxidation (*FDH1* and *FDH2*) are
412 down-regulated. These data evidence the relationship between thioredoxins and
413 glutathione and the NADH metabolism, and suggest alternative pathways for oxidative
414 stress response regulation which are not directly related with Yap1p.

For laboratory yeast strains, it has been described that *TRX1* and *TRX2* are required for the reduction and activation of important enzymes like PAPS, a key enzyme in sulphate metabolism, and ribonucleotide reductase, which is essential for dNTP synthesis during DNA replication (Koc et al. 2006; Muller 1991). In relation with these results, lack of *TRX2* in wine yeasts leads to the increased expression at 80 h of several of the genes involved in the sulphur metabolism (*SPE2, JLP1, SAM1, YNL092W* and *YDR140W*), even when no sulphate limitation occurs during the process (Shima et al. 2005). These results correlate with the requirement of thioredoxins for PAPS reduction and activation (Muller 1991). In addition, lack of the *TRX2* gene in industrial yeast strains may also induce DNA damage in the S phase since some of the genes involved in DNA replication in the S phase (*CLB6*) and in the DNA damage stress response (*HEX3* and *RFX1*) are up-regulated (Table 4).

Alteration of the *TRX2* gene levels in wine yeasts also has an impact on the expression levels of the genes related to the carbohydrates metabolism. It has been previously described in plants that thioredoxins interact with glycolytic enzymes, such as enolase, pyruvate descarboxylase, glyceraldehyde 3-phosphate dehydrogenase, fructose bisphosphatase, by controlling their enzymatic activity by redox regulation (Montrichard et al. 2009; Gómez-Pastor et al. 2010c). One interesting result of this study is the different expression pattern observed for the *HXK2* gene when comparing T*TRX2* (3.1 fold increase) and *trx2*Δ (2-fold decrease). These results suggest a putative role of Trx2p in *HXK2* gene transcriptional regulation, which may have an important function in sugar consumption and biomass production in these strains under industrial conditions. Indeed, *TRX2* overexpression not only increases *HXK2* mRNA levels, but also Hxk2p protein amount and enzyme activity. On the other hand, the down-

439 regulation of *HXK2* in *trx2*Δ can explain the down-regulation observed for HXT genes
440 since the *HXK2* gene is required for the full induction of the *HXT* expression (Özcan
441 and Johnston 1999).

442 The glucose phosphorylating enzyme hexokinase 2 (Hxk2) plays a pivotal regulatory
443 role in glucose-sensing and repression pathways by controlling the glycolytic flux
444 (Randez-Gil et al. 1998; de la Cera et al. 2002; Ahuatzi et al. 2004). Hxk2p also
445 regulates the expression of *HXK1* and *GLK1*, and controls its own expression
446 (Rodriguez et al. 2001). However, it has been also described that when Hxk2p is not
447 present, hexokinase 1 (Hxk1) can also maintain glucose repression (Rose et al. 1991; De
448 Winde et al. 1996).

449 There is some evidence to connect glycolytic flux reconfiguration with oxidative stress
450 response (Ralser et al. 2007; Grant, 2008). Under oxidative stress conditions, organisms
451 can redirect their metabolic flux from glycolysis to the pentose phosphate pathway
452 (PPP) which provides the reducing power (NADPH) for the main cellular redox system
453 (Grant, 2008). This phenomenon might explain the fermentative capacity detriment
454 observed in control strain T73 as a result of rerouting the glycolytic flux under oxidative
455 stress conditions. Thus, the improved oxidative stress resistance observed in the T*TRX2*
456 strain (Gómez-Pastor et al. 2010a) might well prevent the drop in NADPH levels during
457 oxidative stress, thus avoiding not only a rerouting to the PPP, but also an increase in
458 glycolytic flux and fermentative capacity, as we propose in the model depicted in Fig. 4.
459 In agreement with these results, it has been recently described in mammal cells that a
460 thioredoxin-interacting protein (Txnip) expression correlates well with glycolytic flux,
461 which is regulated by oxidative phosphorylation status (Yu et al. 2010).

462 The analysis of the transcriptomic data from the *TRX2* gene-modified strains provides
463 an overview of the different metabolic pathways that are affected as a result of *TRX2*
464 gene level manipulation, where the oxidative stress- and carbohydrates-related genes
465 alter the most. This study contributes to a better understanding of oxidative stress
466 response regulation during a complex industrial process which may differ from the
467 information established for laboratory yeast strains (Fig. 4). The results obtained also
468 allow us to not only establish a relationship between fermentative capacity and
469 oxidative stress response, but to also propose *HXK2* as a new target of thioredoxins in
470 yeast.

471 **Acknowledgments and funding**

472 This work has been supported by grants AGL 2005-00508 and AGL 2008-00060 from
473 the Spanish Ministry of Education and Science (MEC). R.G-P. was a predoctoral fellow
474 of the I3P program from the CSIC (Spanish National Research Council). We thank the
475 Proteomic and Transcriptomic Service of the Institute of Agrochemistry and Food
476 Technology (IATA, CSIC) for their support in the transcriptomic analysis.

477

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604

605 **Figure legends**

606 **Fig. 1**. Fermentative capacity of the dried biomass from strains T73, *trx2*Δ and T*TRX2*
607 collected at 0 h (A), 18 h (B) and 80 h (C) of growth. Fermentative capacity was

608 expressed as mL of CO_2 produced per 10^7 cells and it was measured for 6h in YPGF
609 medium. Loss of fermentative capacity (D) was expressed as the percentage of values
610 between 0-18h and 80 h.

611 **Fig. 2**. Confirmation of expression changes for selected genes. **A)** The northern blot
612 analysis of several genes detected in the microarray studies in strains T73, *trx2*Δ and
613 T*TRX2* at 0 h and 18 h of growth under industrial conditions. B**)** Comparison of
614 quantitative transcriptional changes detected by microarrays and northern analysis.

615 **Fig. 3**. Hexokinase 2 expression and activity are dependent of the *TRX2* gene dose. **A)**
616 The Western blot analysis of Hxk2p and actin as the protein loading control, in strains
617 T73, *trx2*Δ and T*TRX2* at 0 h and 18 h of growth under industrial conditions. B**)**
618 Hexokinase activity was assayed under industrial conditions in a molasses medium and
619 normalized by using the total amount of protein. * p-value < 0.05.

620 **Fig. 4**. Model of the Trx2p interaction between the main oxidative stress transcription
621 factors, the glycolytic flux through Hxk2p regulation and fermentative capacity.

622

623

Table 1**. Genes and primers used for the amplification of DNA probes**

Probe	Primer	Sequence (5´- 3´)	Length (bp)
GSH2	***GSH2-1***	**CAAGATATGGCCCAACCTG**	**300**
	GSH2-2	**AGGATCGTACTTGTTTGCC**	
GTT1	***GTT1-1***	**GATGCTAACTTCCGTGCTC**	**530**
	GTT1-2	**GCTCAACTTCCCATCAACC**	
TRX2	***TRX2-1***	**AAATCCGCTTCTGAATAC**	**300**
	TRX2-2	**CTATACGTTGGAAGCAATAG**	
GLO1	***GLO1-1***	**GAACACTTCGGTATGAAG**	**400**
	GLO1-2	**GGTATTCCTGACCCTCTC**	
ALD3	***ALD3-1***	**CCACTCATCTTAAATCCGCC**	**1060**
	ALD3-1	**CTTACAAGATACTATGCGGG**	
ATG7	***ATG7-1***	**GAAGAACAAGCCACCACATG**	**330**
	ATG7-2	**CACAGTAAGTGGTTGGCATG**	
HXK2	***HXK2-1***	**GGTGGTAACATTCCAATGATTC**	**398**
	HKX2-2	**CAACATTGGAACAACATCGTG**	

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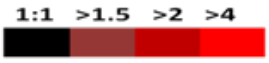
625

Table 2: **Expression profile of the up-regulated genes in T*TRX2* strain during biomass propagation process in comparison to T_{73} mRNA values previously published by Gómez-Pastor et al. (2010b).**

Gene name	Time(h) 14 18 80	Gene description	Molecular function
URA3		Orotidine-5'-phosphate decarboxylase	Biosynthesis of pyrimidines
TRX2		Thioredoxin II	Oxidoreductase activity
YGR251W		Uncharacterized protein	Maturation of 18S rRNA
STP22		Component of the ESCRT-I complex	Ubiquitin-dependent sorting of proteins
HUL5		Ubiquitin-conjugating enzyme (E4)	Transport of misfolded proteins
CTR3		Copper uptake transporter	Copper ion import
BUD19		BUD site selection	Unknown function
IKI3		Subunit of Elongator complex	Modification of nucleosides in tRNA
HXK2		Hexokinase isoenzyme 2	Glycolysis
YAP3		Basic (bZIP) transcription factor	Related to oxidative stress reponse
NAM2		Mitochondrial leucyl-tRNA synthase	Splicing of mitochondrial I introns
NCA3		Nuclear Control of ATPase	Regulates expression of ATP synthase
OLE1		Delta(9) fatty acid desaturase	Monounsaturated fatty acid synthesis
MET8		Ferrochelatase	Sulfate assimilation
POL2		Subunit of DNA polymerase (II) ε	Involved in DNA repair
ARP1		Actin-related protein	Spindle orientation
CDC37		Essential Hsp90p co-chaperone	Stabilizes protein kinase nascent chains
SHE4		Swi5p-dependent HO Expression	Polarization of the actin cytoskeleton
RHC18		Mms21-Smc5-Smc6 complex	DNA repair
YBR028C		Putative protein kinase	Unknown function
ILV5		Acetohydroxyacid reductoisomerase	Branched-chain amino acid biosynthesis
RRP14		Part of 66S pre-ribosomal particles	Ribosomal RNA Processing
PRY2		Pathogen Related in Yeast	Unknown function
UTH1		Mitochondrial outer membrane prot.	Involved in the oxidative stress response
BOP3		Potential Cdc28p substrate	Confers resistance to methylmercury
YGR250C		Putative RNA binding protein	Localizes to stress granules
YOL098C		Putative metalloprotease	Unknown function
ILV6		Subunit of acetolactate synthase	Branched-chain amino acid biosynthesis
PIM1		ATP-dependent Lon protease	Degradation of misfolded proteins
CBP1		Cytochrome B mRNA Processing	Stabilization of 5´-untranslated mRNAs
SWC1		Component of the SWR1 complex	Unknown function
PDC6		Pyruvate decarboxylase	Alcoholic fermentation
LAP4		Leucine AminoPeptidase	Cytosol to vacuole targeting pathway
SRB4		Suppressor of RNA polymerase B	Essential for transcriptional regulation
MSB2		Multicopy Suppressor of Budding	Sensor in the Sho1p mediatedpathway
UTP9		Nucleolar protein	processing of pre-18S rRNA

626



627

Table 3: **Expression profile of the down-regulated genes in T*TRX2* strain during biomass propagation process in comparison to T_{73} mRNA values previously published by Gómez-Pastor et al. (2010b).**

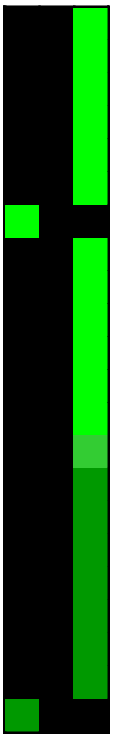
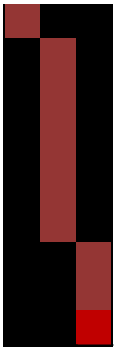
Gene name	Time (h)	Gene description	Molecular function
	14 18 80		**Oxidoreductase activity (p<6.02E-11)**
GPX1		Glutathione peroxidase I	Phospholipid hydroperoxides protection
GPX3		Glutathione peroxidase III	Phospholipid hydroperoxides protection
PRX1		Mitochondrial peroxiredoxin	Reduction of hydroperoxides
GLO2		Cytoplasmic glyoxalase II	Hydrolysis of S-D-lactoylglutathione
GTT1		Glutathione transferase I	Glutathione S-transferase
ERO1		Thiol oxidase	Oxidative protein folding
SFA1		Glutathione-dependent formaldehyde DH	Formaldehyde detoxification
ALD4		Mitochoondrial Aldehyde DH	Conversion of acetaldehyde to acetate
BDH1		NAD-dependent butanediol DH	Use of 2,3-butanediol as a carbon source
GPD1		NAD-dependent glycerol-3-P DH	Glycerol synthesis
AYR1		NADPH-dependent DHA reductase	Phosphatidic acid biosynthesis
FOX2		Fatty acid oxidation enzyme	Fatty acid beta-oxidation pathway
MDH3		Peroxisomal malate DH III	Glyoxylate cycle
FDH1		NAD(+)-dependent formate DH I	Protection from exogenous formate
FDH2		NAD(+)-dependent formate DH II	Protection from exogenous formate
FDH2-B		NAD(+)-dependent formate DH II	Protection from exogenous formate
MXR1		Methionine-S-sulfoxide reductase	Iron sulphur cluster protection
ETR1		2-enoyl thioester reductase	Fatty acid synthesis
GCY1		Putative NADP(+) coupled glycerol DH	Glycerol catabolism
GRX2		Cytoplasmic glutaredoxin II	Maintenance of redox state of proteins
ZTA1		NADPH-dependent quinone reductase	Detoxify alcohols and related compounds
MPD1		Protein disulfide isomerase	Inhibits the chaperone activity of Cne1p
			Stress response (p<5.01E-4)
HSP33		Possible chaperone and cysteine protease	Protein folding
RVS161		Amphiphysin-like lipid raft protein	Regulates actin polarization
SBA1		Co-chaperone Hsp70	Regulates Hsp90 family chaperones
PEP4		Vacuolar aspartyl protease	Protein turnover after oxidative damage
DDR2		DNA Damage Responsive protein	Multistress response protein
TOR1		PIK-related protein kinase	Controls growth in response to nutrients
SSY1		Sulfonylurea Sensitive on YPD protein	Expression of amino acid permease genes
RNY1		Vacuolar RNase of the T(2) family	Promotes apoptosis
SIP18		Phospholipid-binding protein	Induced by osmotic stress
LSP1		Primary component of eisosomes	Stimulates phosphorylation
HSP12		Heat shock protein	Protects membranes from stress
			Carbohydrates metabolism (p<2.1E-3)
GPM2		Glycerate PhosphoMutase	Glycolysis
TPI1		Triose phosphate isomerase	Glycolysis
AMS1		Vacuolar alpha mannosidase	Involved in oligosaccharide degradation
GIP2		Regulatory subunit of the protein Glc7p	Glycogen metabolism
GLC8		Regulatory subunit of Glc7p	Glycogen metabolism
HAP2		Heme Activator Protein	Transcription of respiratory genes
TYE7		Serine-rich protein	Binds E-boxes of glycolytic genes
HXT5		Hexose transporter V	Hexose transport
			Protein catabolic process (p<7.6E-3)
CDC34		Ubiquitin-conjugating enzyme (E2)	Regulates cell cycle progression
PAI3		Proteinase A (Pep4p) inhibitor	Osmotic genes induction
SCL1		Alpha 1 subunit of the 20S proteasome	Degradation of ubiquitinated substrates
RPN8		Regulatory subunit of the 26S proteasome	Protein degradation

Table 4: **Expression profile of the up-regulated genes in *trx2Δ* strain during biomass propagation process in comparison to T_{73} mRNA values previously published by Gómez-Pastor et al. (2010b).**

Gene name	Time (h) 14 18 80	Gene description	Molecular function
			Intracellular transport (p<1.95E-4)
CTR3		Copper uptake transporter	Copper ion import
DIC1		Dicarboxylic acid transporter	dicarboxylic acid transport
DTR1		Amine/polyamine transporter	Multidrug transporter activity
SFC1		Succinate-fumarate antiporter	Succinate-fumarate transport
SEO1		Putative permease of allantoate	Confers resistance to ethionine sulfoxide
SSS1		Subunit of the Sec61p complex	Protein secretion through the RE
TRS20		Transport protein particle (TRAPP)	ER to Golgi transport
RET2		COPI vesicle coat	ER to Golgi transport
VRG4		Golgi GDP-mannose transporter	Lipid glycosylation
SUR2		Fatty acid elongase	Post-Golgi transport
			Sulphur metabolism (p<8.10E-3)
YNL092W		Methyltransferase	Biological process unknown
YDR140W		Methyltransferase	Methylates translation release factor Sup45p
SPE2		S-adenosylmethionine decarboxylase	Biosynthesis of spermidine and spermine
JLP1		Sulfonate dioxygenase	Involved in sulfonate catabolism
SAM1		S-adenosylmethionine synthetase	Methionine metabolism
			Stress response (p<5.23E-3)
HEX3		SUMO-targeted ubiquitin ligase	Response to DNA damage
YBP1		Yap1p Binding Protein	Activation of the Transcription Factor Yap1
PFD1		Chaperone	Protein folding
PET100		Chaperone	Assembly of cytochrome c oxidase
RDS3		Regulator of drug senstivity	Involved in pre-mRNA splicing
TRR1		Thioredoxin reductase	Protects cells against oxidative stress
RFX1		Transcriptional repressor	Response to DNA damage
SVS1		Suppressor of Vanadate Sensitivity	Response to stress
			DNA metabolic process (p<1.45E-4)
DPB4		Subunit of DNA polymerase (II) ε	Involved in DNA replication
HTB1		Histone H2B	Required for chromatin assembly
RPC10		DNA-directed RNA polymerase	Transcription from Pol II promoter
TAF12		Part of TFIID and SAGA complexes	Chromatin modification
CCR4		CCR4-NOT transcriptional complex	Regulation of gene expression
SPT4		SuPpressor of Ty	Regulation of transcription
CLB6		B-type cyclin	Involved in DNA replication during S phase
			Ribosomal subunit (p<1.39E-5)
RPS16A		Cytosolic small ribosomal subunit	Protein biosynthesis
RPS18		Cytosolic small ribosomal subunit	Protein biosynthesis
RPS20		Cytosolic small ribosomal subunit	Protein biosynthesis
RPS25A		Cytosolic small ribosomal subunit	Protein biosynthesis
RPS28A		Cytosolic small ribosomal subunit	Protein biosynthesis
RPL2A		Cytosolic large ribosomal subunit	Protein biosynthesis
RPL27A		Cytosolic large ribosomal subunit	Protein biosynthesis
RPL34A		Cytosolic large ribosomal subunit	Protein biosynthesis
MRPS8		Mitochondrial small ribosomal subunit	Protein biosynthesis
MRPL1		Mitochondrial large ribosomal subunit	Aerobic respiration and Protein biosynthesis
MRPL36		Mitochondrial large ribosomal subunit	Protein biosynthesis

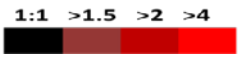
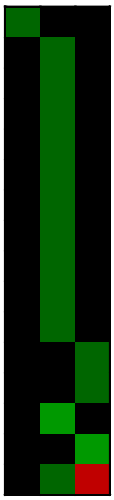
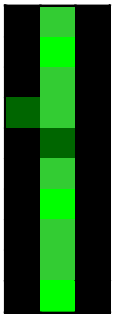
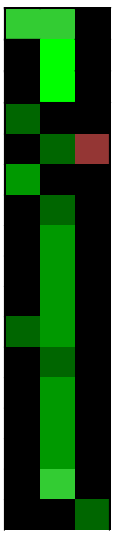
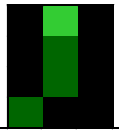


Table 5: **Expression profile of the down-regulated genes in *trx2Δ* strain during biomass propagation in comparison to T_{73} mRNA values published by Gómez-Pastor et al. (2010b)**

Gene name	Time (h) 14 18 80	Gene description	Molecular function
			Polyubiquitination process (p<2.09E-9)
UBS1		Ubiquitin-conjugating suppressor	Positive regulator of Cdc34p activity
UBP9		Ubiquitin hydrolase	Cleaves ubiquitin-protein fusions
UFO1		E3 ubiquitin ligase complex	Ho endonuclease degradation
YLR224W		F-box protein of SCF ubiquitin ligase	Ubiquitin-dependent protein catabolism
PIB1		RING-type ubiquitin ligase	Ubiquitin-dependent protein catabolism
SCL1		α1 subunit of the 20S proteasome	Degradation of ubiquitinated substrates
CDC23		Ubiquitin-protein ligase	Degradation of anaphase inhibitors
ATG7		Ubiquitin-activating enzyme	Autophagosome formation
RPT3		19S regulatory particle of the 26S	Degradation of ubiquitinated substrates
ASI3		Putative E3 ubiquitin ligase	Control of gene expression
RPT1		19S regulatory particle of the 26S	Degradation of ubiquitinated substrates
DEF1		RNAPII degradation factor	Ubiquitination and proteolysis of RNAPII
ATE1		Arginyl-tRNA-protein transferase	ubiquitin-dependent protein catabolism
MET30		F-box protein	Methionine biosynthesis
RPN5		Subunit of the 26S proteasome lid	Degradation of ubiquitinated substrates
UBP15		Ubiquitin-specific Protease	Ubiquitin precursor processing
			Hexose transport (p<1.07E-5)
HXT1		Hexose transporter	Low-affinity glucose transporter
HXT2		Hexose transporter	High-affinity glucose transporter
HXT3		Hexose transporter	Low affinity glucose transporter
HXT4		Hexose transporter	High-affinity glucose transporter
HXT9		Putative hexose transporter	Hexose transport
HXT10		Putative hexose transporter	Hexose transport
HXT11		Putative hexose transporter	Pleiotropic drug resistance
HXT12		Possible pseudogene in strain S288C	Hexose transport
HXT15		Hexose transporter	Hexose transport
GAL2		Galactose permease	Transport of glucose and galactose
			Stress response (p<1.94E-4)
TRX2		Thioredoxin II	Oxidoreductase activity
FDH1		Formate dehydrogenase I	NADH regeneration
FDH2		Formate dehydrogenase II	NADH regeneration
TIR1		Cell wall mannoprotein	Nucleocytoplasmic transport
TIR2		Putative cell wall mannoprotein	Response to stress
HOR7		HyperOsmolarity-Responsive	Response to osmotic stress
WHI2		Phosphatase activator	Regulation of growth
GRE1		Hydrophilin I	Response to stress
GRE2		NADPH-methylglyoxal reductase	Response to stress
GLO1		Monomeric glyoxalase I	Glutathione metabolism
GLO2		Cytoplasmic glyoxalase II	Glutathione metabolism
GSH2		Glutathione synthethase II	Biosynthesis of glutathione
GTT1		Glutathione transferase	Glutathione metabolism
ZRC1		Vacuolar membrane zinc transporter	Resistance to zinc shock
GCN4		Basic (bZIP) transcriptional activator	Response to amino acid starvation
ALD3		Cytoplasmic aldehyde dehydrogenase	Response to stress
BNI1		Cytoskeletal regulatory protein	Response to osmotic stress
			Carbohydrates Metabolism (p<3.45E-3)
HXK2		Hexokinase isoenzyme 2	Glycolysis
PGK1		3-phosphoglycerate kinase	Glycolysis
ERR1		phosphopyruvate hydratase	Glycolysis
TYE7		Transcription factor	Positive regulation of glycolysis