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

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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 (*TTRX2*) 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 H₂O₂ 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 *trx2Δ* is more sensitive to H₂O₂ (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 decarboxylase, glyceraldehyde 3-phosphate dehydrogenase, and fructose biphosphatase, 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 Lallemend 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 *EcoRI* fragment containing the yeast *TRX2* gene and promoter into the episomal yeast plasmid Yep352

carrying the selectable marker *URA3*. The *TTRX2* strain (Pérez-Torrado et al. 2009) is a *T_{73ura3}* 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_{73ura3}*. 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 *HindIII* 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 (NH₄)₂SO₄, 3.5 g L⁻¹ of KH₂PO₄, 0.75 g L⁻¹ of MgSO₄·7H₂O, 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

(OD₆₀₀ = 0.05) from overnight YPD (1% yeast extract, 2% peptone, 2% glucose) precultures incubated at 30°C 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% H₃PO₃ 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 T₇₃ (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 *TTRX2* and of two biological replicates for strain *trx2Δ*. 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 4°C. Equal amounts of RNA (20 μ g) 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).

Western blot analysis

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

Hexokinase activity assay

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

Measurement of fermentative capacity

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

10⁷ cells/mL were inoculated in YPGF medium (1% yeast extract, 2% peptone, 10% glucose, 10% fructose) and incubated with shaking at 30°C and 65 rpm. The exact number of cells was determined by recounting in a Neubauer camera. CO₂ 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 CO₂ (10⁷ cells)⁻¹ min⁻¹.

Results

Fermentative capacity properties are enhanced in *TTRX2* 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 CO₂ 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 *TTRX2* 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.

Transcriptional profile of strain TTRX2 during the biomass propagation process

To study the global gene expression changes due to *TRX2* gene dose variation, we performed bench-top trials of the industrial biomass propagation process to compare wild-type T73 to strains TTRX2 and *trx2*Δ. The global gene expression experiments were done with three representative samples taken at 14 h (early-batch phase), 18 h (late-batch) and 80 h (late-fed batch) under the identical fermentation conditions and sampling stages to those previously reported by Gómez-Pastor et al., (2010b). mRNA was obtained from each sample and microarray hybridizations were performed using three biological replicates for each time point. Significantly expressed genes were obtained after microarray scanning and data normalization. Expression changes for a selected set of genes were confirmed by northern blot analysis as shown in Fig. 2, where a quantitative comparison of microarray and northern analysis data is also included. As can be seen, expression profiles are identical and the highest quantitative differences are 20%. Gene functional category analyses were performed among the differentially expressed genes for the TTRX2 strain compared to control strain T₇₃ by using the GOstat online tools (<http://gostat.wehi.edu.au/>).

Table 2 shows the expression ratios for the up-regulated genes corresponding to the averaged log₂ signal intensity among the different microarrays. In comparison to control strain T₇₃, a total of 36 ORFs were differentially expressed during the biomass propagation process. Nine ORFs were up-regulated at 14 h of growth, 14 ORFs at 18 h and 18 ORFs at 80 h. The fact that the highest number of up-regulated genes appeared in the fed-batch phase suggests the importance of *TRX2* during respiratory metabolism. Although several genes were up-regulated as a result of *TRX2* gene overexpression

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

As expected, the greatest induction was observed for *URA3* and *TRX2*, both the genes present in the episomal plasmid carrying *TRX2*. Whereas *URA3* was up-regulated throughout the process, the *TRX2* gene was up-regulated only during the 14-18 h growth period and it showed no differences with the control strain at the end of the propagation process. An explanation for this is that the *TRX2* gene was cloned into the Yep352 plasmid under the control of its own promoter. These results indicate that only the gene dose, and not the expression pattern, of *TRX2* was modified in the *TTRX2* strain as a result of gene overexpression as it displayed a similar expression profile to that previously observed for control strain T₇₃ (Pérez-Torrado et al. 2009).

Among the overall up-regulated genes, we noted *YAP3* which encodes a Yap1p-related transcription factor. It presented a similar expression pattern to the *TRX2* gene, and was up-regulated between 14 h and 18 h of growth. We also observed the induction of *HXK2* and *PDC6* during the metabolic transition. *HXK2* codifies hexokinase isoenzyme 2, which catalyzes the phosphorylation of glucose in the cytosol and it is the predominant hexokinase during growth on glucose, whereas *PDC6* codifies a minor isoform of pyruvate decarboxylase involved in alcoholic fermentation, and its transcription is glucose- and ethanol-dependent. The up-regulation of them both might explain the high fermentative capacity observed for the *TTRX2* strain (Gómez-Pastor et al. 2010a).

Table 3 offers the ratios for the down-regulated genes during the propagation process. A total of 45 ORFs were differentially expressed in comparison to control strain T₇₃. 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 *TTRX2* strain (Supplementary material).

Transcriptional profile for the *trx2Δ* strain during the biomass propagation process

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

A strong down-regulation of the cytoplasmic ribosomal proteins codified by the RPLs and RPSs genes has been described during the metabolic transition and the diauxic shift, indicating repression of protein synthesis to reprogram the gene expression profile for adaptation to new environmental conditions (Ashe et al. 2000; DeRisi et al. 1997; Gómez-Pastor et al. 2010b). However in the *trx2Δ* strain, we observed an up-regulation of several genes encoding ribosomal proteins for both the 60S and 40S ribosomal subunits. Those genes involved in DNA metabolic processes were also up-regulated; for instance, *CLB6* encoding a B-type cyclin involved in DNA replication during the S-phase (Schwob et al. 1993). In addition, some of the genes involved in the stress response by DNA damage were up-regulated at different growth times, such as the *HEX3* gene at 14 h and *RFX1* at 80 h.

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

The genes related to the traffic between the endoplasmic reticulum and the Golgi apparatus (*SSSI1*, *TRS20*, *RET2*, *VRG4* and *SUR2*), and those encoding proteins for sulphur metabolism (*SPE2*, *JLP1*, *SAM1*, *YNL092W* and *YDR140W*), were also up-

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

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

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

Unexpectedly, the genes activated by several stresses were down-regulated at 18 h of the process. Some were involved in the glutathione metabolism (*GLO1*, *GLO2*, *GSH2* and *GTT1*), although no differences in total GSH were observed in the *trx2Δ* mutant compared to the control strain (data not shown). The most down-regulated genes in *trx2Δ* were *FDH1* and *FDH2*, two NAD⁽⁺⁾-dependent formate dehydrogenases which are related to glutathione-dependent formaldehyde oxidation.

Regarding the genes related to glycolysis, the *HXK2* gene was down-regulated in *trx2Δ* during the metabolic transition at 18 h, whose expression profile was the opposite to that in the *TTRX2* 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 T₇₃ (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, *trx2Δ* and *TTRX2* 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 *TTRX2* strain and *trx2Δ* 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 h 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 *HXK2* 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 *TTRX2* 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 *TTRX2* gene displayed a significantly increased enzyme activity at 18 h of growth. Conversely, the *trx2Δ* 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 T₇₃ wine yeast strain. An important up-regulated gene in *TTRX2* 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

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

The *TRX2* overexpressing strain shows several relevant down-regulated antioxidant genes (*PRX1*, *GTT1*, *GLO2*, *GPX1*, *GPX3* and *GRX2*), some of which are directly regulated by Yap1p (Izawa et al. 1999). The down-regulation of several general stress response genes in the *TTRX2* strain at the end of the process, such as heat shock proteins (*HSP33*, *SBA1* and *HSP12*) and those genes related to multistress response (*DDR2*), nutrient starvation (*TOR1*) and osmotic stress (*SIP18*), also indicate that these cells are less affected by not only oxidative stress (Gómez-Pastor et al. 2010a), but also other stressing conditions. Some of the down-regulated genes, like *HSP12*, *GPM2*, *PRX1* and *GRX2*, are also directly controlled by Msn2/4p (Hasan et al. 2002; Praekelt and Meacock 1990), which evidences the interaction between Trx2p and these major general stress transcription factors, just as Boissard and colleagues (2009) previously reported for laboratory yeast strains.

In contrast, deletion of the *TRX2* gene also lowers the expression of several stress response genes (*TIR2*, *GRE2*, *GLO1*, *ALD3* and *GCN4*) during the metabolic transition at 18 h (Table 5), when a high expression is expected. However, we also found that the *YBP1* gene is up-regulated (Table 4), which positively regulates Yap1p activity (Veal et al. 2003), and that many genes involved in the glutathione metabolism (*GRE1*, *GRE2*, *GLO1*, *GLO2*, *GSH2* and *GTT1*) and formaldehyde oxidation (*FDH1* and *FDH2*) are down-regulated. These data evidence the relationship between thioredoxins and glutathione and the NADH metabolism, and suggest alternative pathways for oxidative 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 decarboxylase, glyceraldehyde 3-phosphate dehydrogenase, fructose biphosphatase, 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 *TTRX2* (3.1 fold increase) and *trx2Δ* (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-

regulation of *HXK2* in *trx2Δ* can explain the down-regulation observed for HXT genes since the *HXK2* gene is required for the full induction of the *HXT* expression (Özcan and Johnston 1999).

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

There is some evidence to connect glycolytic flux reconfiguration with oxidative stress response (Ralser et al. 2007; Grant, 2008). Under oxidative stress conditions, organisms can redirect their metabolic flux from glycolysis to the pentose phosphate pathway (PPP) which provides the reducing power (NADPH) for the main cellular redox system (Grant, 2008). This phenomenon might explain the fermentative capacity detriment observed in control strain T73 as a result of rerouting the glycolytic flux under oxidative stress conditions. Thus, the improved oxidative stress resistance observed in the *TTRX2* strain (Gómez-Pastor et al. 2010a) might well prevent the drop in NADPH levels during oxidative stress, thus avoiding not only a rerouting to the PPP, but also an increase in glycolytic flux and fermentative capacity, as we propose in the model depicted in Fig. 4. In agreement with these results, it has been recently described in mammal cells that a thioredoxin-interacting protein (Txnip) expression correlates well with glycolytic flux, which is regulated by oxidative phosphorylation status (Yu et al. 2010).

The analysis of the transcriptomic data from the *TRX2* gene-modified strains provides an overview of the different metabolic pathways that are affected as a result of *TRX2* gene level manipulation, where the oxidative stress- and carbohydrates-related genes alter the most. This study contributes to a better understanding of oxidative stress response regulation during a complex industrial process which may differ from the information established for laboratory yeast strains (Fig. 4). The results obtained also allow us to not only establish a relationship between fermentative capacity and oxidative stress response, but to also propose *HXX2* as a new target of thioredoxins in yeast.

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Figure legends

Fig. 1. Fermentative capacity of the dried biomass from strains T73, *trx2Δ* and *TTRX2* collected at 0 h (A), 18 h (B) and 80 h (C) of growth. Fermentative capacity was

expressed as mL of CO₂ produced per 10⁷ cells and it was measured for 6h in YPGF medium. Loss of fermentative capacity (D) was expressed as the percentage of values between 0-18h and 80 h.

Fig. 2. Confirmation of expression changes for selected genes. **A)** The northern blot analysis of several genes detected in the microarray studies in strains T73, *trx2Δ* and *TTRX2* at 0 h and 18 h of growth under industrial conditions. **B)** Comparison of quantitative transcriptional changes detected by microarrays and northern analysis.

Fig. 3. Hexokinase 2 expression and activity are dependent of the *TRX2* gene dose. **A)** The Western blot analysis of Hxk2p and actin as the protein loading control, in strains T73, *trx2Δ* and *TTRX2* at 0 h and 18 h of growth under industrial conditions. **B)** Hexokinase activity was assayed under industrial conditions in a molasses medium and normalized by using the total amount of protein. * p-value < 0.05.

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

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

Probe	Primer	Sequence (5' - 3')	Length (bp)
<i>GSH2</i>	<i>GSH2-1</i>	CAAGATATGGCCCAACCTG	300
	<i>GSH2-2</i>	AGGATCGTACTTGTGTTGCC	
<i>GTT1</i>	<i>GTT1-1</i>	GATGCTAACTTCCGTGCTC	530
	<i>GTT1-2</i>	GCTCAACTTCCCATCAACC	
<i>TRX2</i>	<i>TRX2-1</i>	AAATCCGCTTCTGAATAC	300
	<i>TRX2-2</i>	CTATACGTTGGAAGCAATAG	
<i>GLO1</i>	<i>GLO1-1</i>	GAACACTTCGGTATGAAG	400
	<i>GLO1-2</i>	GGTATTCCTGACCCTCTC	
<i>ALD3</i>	<i>ALD3-1</i>	CCACTCATCTTAAATCCGCC	1060
	<i>ALD3-1</i>	CTTACAAGATACTATGCGGG	
<i>ATG7</i>	<i>ATG7-1</i>	GAAGAACAAGCCACCACATG	330
	<i>ATG7-2</i>	CACAGTAAGTGGTTGGCATG	
<i>HXK2</i>	<i>HXK2-1</i>	GGTGGTAACATTCCAATGATTC	398
	<i>HXK2-2</i>	CAACATTGGAACAACATCGTG	

Table 2: Expression profile of the up-regulated genes in TTRX2 strain during biomass propagation process in comparison to T₇₃ mRNA values previously published by Gómez-Pastor et al. (2010b).

Gene name	Time(h)	Gene description	Molecular function
	14 18 80		
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) &	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 mediated pathway
UTP9		Nucleolar protein	processing of pre-18S rRNA

Table 3: Expression profile of the down-regulated genes in TTRX2 strain during biomass propagation process in comparison to T₇₃ mRNA values previously published by Gómez-Pastor et al. (2010b).

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

1:1 >1.5 >2 >3 >4

Table 4: Expression profile of the up-regulated genes in *trx2Δ* strain during biomass propagation process in comparison to T₇₃ mRNA values previously published by Gómez-Pastor et al. (2010b).

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

1:1 >1.5 >2 >4

Table 5: Expression profile of the down-regulated genes in *trx2Δ* strain during biomass propagation in comparison to T₇₃ mRNA values published by Gómez-Pastor et al. (2010b)

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