FUNCTIONAL CHARACTERIZATION IN VIVO OF ESSENTIAL SACCHAROMYCES CEREVISIAE'S HYDROPHILIN FOR DESICCATION TOLERANCE

Gema Isabel López Martínez

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Functional characterization *in vivo* of essential *Saccharomyces cerevisiae's* hydrophilin for desiccation tolerance

Doctoral Thesis

Department of Biochemistry and Biotechnology

Universitat Rovira i Virgili

Tarragona

2015

Gema López Martínez

Functional characterization *in vivo* of essential *Saccharomyces cerevisiae's* hydrophilin for desiccation tolerance

Doctoral Thesis

Departament of Biochemistry and Biotechnology

Tarragona, 2015

This doctoral research was carried out between 2011-2015 within the Food Biotechnology Microbiology research group (Department of Biochemistry and Biotechnology, Faculty of Oenology) at the Universitat Rovira i Virgili. The doctoral thesis was supervised by Professor Ricardo Cordero Otero.

The research was financially supported by Spanish Government projects (AGL2009-07933 and AGL2012-40018-C02-1).

The doctoral research was support by the Fundació Universitat Rovira i Virgili for one year (BRDI), and then a doctoral fellowship (FI-DGR 2012) from the AGAUR Agency of the Catalan Government. The researcher's three months stay at the Biomolecular Mass Spectromety and Proteomics (Netherlands Proteomics Centre, University of Utrecht) was funded by an AEE grant from Universitat Rovira i Virgili.

Editing of the thesis book was supported by Postgraduate and Doctoral School of the University Rovira i Virgili.

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I STATE that the present study, entitled "Functional characterization *in vivo* of essential *Saccharomyces cerevisiae's* hydrophilin for desiccation tolerance", presented by Gema López Martínez for the award of the degree of Doctor, has been carried out under my supervision at the Department of Biochemistry and Biotechnology of this university.

Tarragona, 22 of July, 2015

Doctoral Thesis Supervisor

Ricardo Cordero Otero

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"Caminante, son tus huellas
el camino y nada más;
caminante no hay camino,
se hace camino al andar".

Antonio Machado, 1917

A mis padres,
mi tío y Miquel

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OBJECTIVES AND OUTLINE OF THE THESIS

Objectives

Outline of the thesis

Objectives

Since I joined the group, the main target of study has been the functional characterization of *Saccharomyces cerevisiae* proteins involved in desiccation tolerance. Most yeast-based food industries are going to introduce Active Dry Yeast (ADY) due to its genetic stability at room temperature, reducing transport and storage costs. Unfortunately, most isolated strains and laboratory-developed industrial yeast strains have the biotechnological handicap of losing viability during the drying process. In order to understand dehydration cell tolerance, a number of stress-response pathways and molecules have been hypothesized as important, including osmoregulation, ion homeostasis, DNA damage repair, and protein folding. Indeed, several potential stress-response molecules appear to be induced in dried *S. cerevisiae* cells. Among them, proteins termed hydrophilin participate in the cellular tolerance to this stress condition (A. Garay-Arroyo 2000), reducing the oxidative damage during the stress imposition. A genetic screen of the *S. cerevisiae* deletion library for mutants sensitive to dehydration stress was carried out by our group. Among the genes characterized for overcoming dehydration stress, overexpression of five out of twelve genes encoding hydrophilic proteins were found essential for overcoming dehydration stress (*SIP18, STF2, GRE1, NOP6* and *YJL144w*) (Rodríguez-Porrata *et al.,* 2012b), demonstrating that the biochemical properties of hydrophilic proteins are not related to desiccation tolerance but instead to specific dehydration inducing genes. *SIP18*, which has been largely studied in our group, (Rodríguez-Porrata *et al.*, 2012a) is the only one that shows an early transcriptional response during dehydration stress. Furthermore, the protein expression increases in

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hyperosmotic stress, localized in the nucleus, and the *SIP18* overexpressing strain enhances the viability in both laboratory and industrial commercial wine strains (López-Martínez *et al.*, 2013), apparently by acting as an antioxidant molecule and minimising the late apoptotic cells (Rodríguez-Porrata *et al.*, 2012a). However, apart from Sip18p, only Stf2p of the other dehydration tolerant hydrophilin is involved in reducing the ROS cells while increase the viability after desiccation process (López-Martínez *et al.*, 2012). Moreover, the viability due to *STF2* overexpression is not related to the respiratory chain malfunction that correlates with other dehydration studies related to mitochondrial inheritance (Picazo *et al.*, 2015). This result suggests that other mechanisms and molecules are involved in the dehydration role of Sip18p and Stf2p. Within this framework, the working hypothesis of this thesis was:

Overexpression of hydrophilin proteins promote a molecular scenario in *Saccharomyces cerevisiae* that enhances desiccation tolerance.

In order to validate this hypothesis the following objectives were attained:

1. Characterization of STF2p hydrophilin role in overcoming the dehydration tolerance. We defined the relationship between overexpression of essential tolerant hydrophilin genes to desiccation and ROS damage after the stress imposition considering the hydrophilin artificial group to be protective molecules against oxidative damage.

2. Validation of *SIP18* role in desiccation tolerance in four wild and commercial wine industry strains overexpressing *SIP18* hydrophilin.

3. Identification of the specific yeast traits involved in dehydration stress tolerance using QTL (Quantitative Trait Locus) analysis across 96 segregates from three stable hybrid strains. It was analysed considering that other genes apart from *SIP18* could be involved in desiccation tolerance.

4. Analysis of the membrane proteomic profile of the overexpressed Sip18p strain in comparison to the wild type before cell dehydration and after rehydration in order to find other proteins involved in dehydration tolerance response.

The results of this thesis could enhance the development of a robust yeast strain that satisfies the requirements of the food industries, and provides a better understanding of

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desiccation-tolerance genetics for potential applications in plant biotechnology, bio-ethanol technology and biomedicine with successful long-term storage of living cells for tissue engineering, cell transplantation and genetic technology.

Outline of the thesis

The STF2p hydrophilin from *Saccharomyces cerevisiae* is required for dehydration stress tolerance

The yeast *Saccharomyces cerevisiae* is able to overcome cell dehydration; cell metabolic activity is arrested during this period but restarts after rehydration. The yeast genes encoding hydrophilin proteins were characterised to determine their roles in the dehydration-resistant phenotype, and STF2p was found to be a hydrophilin that is essential for survival after the desiccation-rehydration process. Deletion of *STF2* promotes the production of reactive oxygen species and apoptotic cell death during stress conditions, whereas the overexpression of *STF2*, whose gene product is localized in the cytoplasm, results in a reduction in ROS production upon oxidative stress as the result of the antioxidant capacity of the Stf2p protein.

Results are reported and discussed in **Chapter II**

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

In the last few decades spontaneous grape must fermentations have been replaced by inoculated fermentation with *Saccharomyces cerevisiae* strains as active dry yeast (ADY). Among the essential genes previously characterized to overcome the cell-drying/ rehydration process, six belong to the group of very hydrophilic proteins known as hydrophilins. Among them, only Sip18p has shown early transcriptional response during dehydration stress. In fact, the overexpression in *S. cerevisiae* of gene *SIP18* increases cell viability after the dehydration process. The purpose of this study was to characterize dehydration stress tolerance of three wild and one commercial *S. cerevisiae* strains of wine origin. The four strains were submitted to transformation by insertion of the gene *SIP18*. Selected transformants were submitted to the cell-drying–rehydration process and yeast viability was evaluated by both viable cell count and flow cytometry. The

antioxidant capacity of Sip18p was illustrated by ROS accumulation reduction after H_2O_2 attack. Growth data as cellular duplication times and lag times were calculated to estimate cell vitality after the cell rehydration process. The overexpressing *SIP18* strains showed significantly longer time of lag phase despite less time needed to stop the leakage of intracellular compounds during the rehydration process. Subsequently, the transformants were tested in inoculated grape must fermentation at laboratory scale in comparison to untransformed strains. Chemical analyses of the resultant wines indicated that no significant change for the content of secondary compounds was detected. The obtained data showed that the transformation enhances the viability of ADY without affecting fermentation efficiency and metabolic behaviour.

Results are reported and discussed in **Chapter III**

ATG18* and *FAB1* are involved in dehydration stress tolerance in *Saccharomyces cerevisiae

Recently, different dehydration-based technologies have been evaluated for the purpose of cell and tissue preservation. Although some early results have been promising, they have not satisfied the requirements for large-scale applications. The long experience of using quantitative trait loci (QTL) with the yeast *Saccharomyces cerevisiae* has proven to be a good model organism for studying the link between complex phenotypes and DNA variations. Here, we use QTL analysis as a tool for identifying the specific yeast traits involved in dehydration stress tolerance. Three hybrids obtained from stable haploids and sequenced in the Saccharomyces Genome Resequencing Project showed intermediate dehydration tolerance in most cases. The dehydration resistance trait of 96 segregants from each hybrid was quantified. A smooth, continuous distribution of the anhydrobiosis tolerance trait was found, suggesting that this trait is determined by multiple QTLs. Therefore, we carried out a QTL analysis to identify the determinants of this dehydration tolerance trait at the genomic level. Among the genes identified after reciprocal hemizygosity assays, *RSM22*, *ATG18* and *DBR1* had not been referenced in previous studies. We report new phenotypes for these genes using a previously validated test. Finally, our data illustrates the power of this approach in the investigation of the complex cell dehydration phenotype.

Results are reported and discussed in **Chapter IV**

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SIP18 overexpression increases dehydration stress tolerance by modulating the membrane protein load

We previously reported that overexpression of the gene encoding the yeast hydrophilin *SIP18* in *Saccharomyces cerevisiae* increases dehydration stress tolerance by 100%. The lipoprotein membranes of the cell suffer a greater amount of damage compared with other cellular compartments during the dehydration and rehydration process. In the present study, we characterised the putative pleiotropic effects caused by the intracellular accumulation of Sip18p during stress, which enhances dehydration tolerance. Therefore, we evaluated the changes in the membrane protein profiles during the time points before dehydration and after rehydration in the BY4742, *sip18* null mutant and *SIP18*-overexpressing strains. Of the proteins identified after comparing the proteomic changes among the strains, the Gvp36p, Gdp1p, Ald4p, Asc1p, Pma1p, Hsp30p and Lsp1p proteins had not been referenced in previous studies of dehydration stress. We discuss the putative roles of these proteins during stress. Finally, our data illustrates the power of this approach for investigating the complex cell dehydration phenotype.

Results are reported and discussed in **Chapter V**

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CHAPTER I

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1. Anhydrobiotes

2. General stress response mechanisms in *Saccharomyces cerevisiae*

2.1. Heat shock stress

2.2. Osmotic stress

2.3. Oxidative stress

References

ANHYDROBIOTES

Introduction

Defence mechanisms

Introduction

Desiccation tolerance, also referred to as anhydrobiosis, is the ability of an organism to withstand removal of its intracellular water content and then resume normal metabolism after rehydration (Crowe *et al.*, 1992). Desiccation-tolerant organisms, called anhydrobiots, are found across all three branches of life, including nematodes, rotifers, and tardigrades among animals, bryophytes among plants, and a number of bacteria, terrestrial microalgae, lichens, and yeast among microorganisms. Anhydrobiots are also classified according to the amount of water loss. While desiccation-sensitive anhydrobiots, which are almost all tested species, die if dried to 20% water content, desiccation-tolerant anhidrobiotes can survive drying to 10% water content. Desiccation-tolerant organisms can survive in the dry state for a very long time, ranging from 1,100 years in a seed of the sacred lotus *Nelumbo nucifera* (Shen-Miller *et al.*, 1995), to 20-40 years in mosses, liverworts and nematodes; and between 5 and 9 years in ptridophytes, rotifers and tardigrates (Alpert and Oliver, 2002; Jönsson and Guidetti, 2002). The recovery time also varies from a few minutes in some mosses, to about an hour in the larva of *Polypedilum* and about 1-2 days in most flowering plants (Alpert, 2000; Kikawada, 2005). Although anhydrobiots have similar physical characteristics such as length in animals which are no longer than 5 cm or flowering plants that grow up to about 3 m tall, they do not share all the dehydration defence mechanisms and some of them belong to a specific kingdom (Alpert, 2006).

Defence mechanisms

The structure and function of essential cell components are modified during the desiccation-rehydration process. Two major effects of desiccation are mechanical effects and macromolecule damage due to the stresses involved in desiccation, such as heat and osmotic stress, and oxidative damage. At least four mechanisms of desiccation tolerance have been found in common across desiccation-tolerant organisms, at least belonging to the same branch of life, which can reduce the two major effects of desiccation mention above. These four common mechanisms are: cell rigidity, accumulation of sugars, regulation of proteins and antioxidants, and the need to repair the damage accumulated while dry (Alpert, 2006). These strategies, which lead to desiccation tolerance, belong to mechanical stress or functional molecular mechanisms.

Mechanical stress mechanisms are partly related to cell shrinkage. During the first step of drying, the cell volume decreases by 40% compared to physiological conditions (Dupont *et al*, 2011). Even though the cell volume decreases, the plasmatic membrane area is maintained due to the poor lateral compressibility of the membrane, thereby leading to an increased area-to-volume ratio in cells and finally to deformation (Beney *et al*., 1998). As a consequence of this process, molecules that do not interact with each other in their hydrated state interact (Eggers and Valentine, 2001), promoting structural modifications such as the denaturation and aggregation of proteins (Carpenter and Crowe, 1989) or leading to the coexistence of phases in a mixture of membrane phospholipids (Shechter, 2004). Rigid skeletons can prevent the macromolecule dysfunction caused by dehydration in animals, but any of desiccation-tolerant animals have skeletons. Plants show a greater ability than animals to combine tolerance and rigidity. Some plants replace large vacuoles with numerous small ones and fill them with non-aqueous compounds as the cells dry, or pull up rather than push up through files of dead cells in the xylem (Thomson, 1997; Farrant, 2000).

Functional molecular mechanisms are the other defence activated by cells during dehydration-rehydration stress in combination with the mechanical mechanisms described above, in which some of them are activated in order to avoid structural damage. Progressive cell water loss increases the concentration of solutes in the medium due to water evaporation, while at the end of desiccation process the medium is transformed into a solid matrix increasing contact between the dried cells and the air, as well as molecular interactions. During this process, cytoplasmic hydric potential and the external potential

equilibrate very quickly, mainly by osmosis, but modify proteins and membranes (Gervais and Beney, 2001). Regardless of lipid composition, the immiscibility between the different structural phospholipid phases leads to the formation of domains with different fluidities in model membranes (Tokumasu *et al*,. 2003; Veatch and Keller, 2003). This can lead to membrane reorganization, aggregation of membrane proteins (Billi *et al*., 2000), and changes in membrane permeability (Fernández Murga *et al*., 1999; Hays *et al*., 2001). A common mechanism for preserving the macromolecular structure and decreasing the contact between membrane lipids in animal, plant and microorganism desiccation-tolerant organisms is trehalose (Billi *et al*., 2000). It reduces the mobility and reactivity of molecules during desiccation (Carpenter and Crowe, 1989; Levine and Slade, 1991; Allison *et al*., 1999; Buitink and Leprince, 2004). Trehalose also stabilizes both membranes and proteins (Golovina *et al*., 2010) and avoids crystallization effects due to the formation of glasses in the last step of desiccation. The formation of stable glasses during drying is due to its high glass transition temperature (Levine and Slade, 1991). Because of the high viscosity of glasses, this glassy state may maintain biomolecules in a form that allows them to return to their native structure and therefore be totally functional following rehydration (Buitink and Leprince, 2004). Furthermore, glasses considerably reduce the rates of chemical reactions that lead to the loss of viability of dry cells and deterioration in storage of dehydrated commercial products (Hoekstra *et al*., 2001; Sun and Leopold, 1997). Indeed, trehalose acts as an antioxidant since it is capable of reducing oxidant-induced modifications of proteins during exposure of yeast cells to H_2O_2 (Benaroudj *et al*, 2001).

S. cerevisiae can survive desiccation when drying in a stationary phase, in which cells synthesize large amounts of trehalose compared to exponential phase cells (Calahan *et al*., 2011; Welch *et al*., 2013; Dupont *et al*., 2014; Beker and Rapoport, 1987). Many industries make use of special treatments, such as heat shock or a rise in osmotic pressure (Eleutherio *et al*., 1997) in order to increase the trehalose content of yeast, prior to subjecting them to dehydration. Dehydration tolerance in mammalian cells, including human cells, also increases when they are treated with trehalose (Wolkers *et al*., 2001; Wolkers *et al*., 2002; Crowe *et al*., 2003; Gordon *et al*., 2001; Matsuo, 2001; Satpathy *et al*., 2004). Genetic engineering has been used to synthesize trehalose in a human primary fibroblast using a recombinant vector with desiccation-tolerant bacteria genes. However, these methods have not worked on mouse cells, whole plants or metazoans. In fact, some

rotifers tolerate dehydration without accumulating sugar trehalose, although this sugar enhances desiccation in a large number of anhydrobiots. As a result, even if trehalose plays a major role in the dehydration resistance of many desiccation-tolerant organisms, it does not appear to be the only protective mechanism in anhydrobiosis, and other molecular mechanisms are involved in the prevention of cell death during desiccation. Among these, some proteins appear to be involved in desiccation tolerance. Hydrophilic proteins (late embryogenesis abundant proteins, LEA) and heat shock proteins (HSP) have often been reported in anhydrobiots, increasing viability and/or reducing cell damage (Collins and Clegg, 2004; López-Martínez *et al*., 2012; Garay-Arroyo, 2000). Some LEA proteins, which are found in animals, plants and microorganisms during water limitation, act as antioxidants, reducing oxidative damage after dehydration (Pereira *et al*., 2003; Gechev *et al*., 2012). In animals, LEA proteins may act as molecular chaperones for DNA or counter physical stress during desiccation (Wise, 2003). In plants they may increase the transition temperature and hydrogen bonding strength of sucrose glasses (Wolkers *et al*., 2001), helping to inhibit membrane fusion, protein denaturation, and the effects of free radicals (Oliver *et al*., 2001). However, desiccation tolerance is not induced in *S. cerevisiae* by the overexpression of all the hydrophilin proteins described. As a result, not all hydrophilin-like proteins belonging to this artificial (non-functional) group enhance desiccation tolerance, even in presence of trehalose (López-Martínez *et al*., 2012). This leads us to hypothesize that several discovered and as yet undescribed mechanisms could be involved, and probably induce cross-protection against stresses in the complex desiccation process.

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GENERAL STRESS RESPONSE MECHANISMS IN *SACCHAROMYCES CEREVISIAE*

Introduction

2.1 Heat shock stress

2.2 Osmotic stress

2.3 Oxidative stress

Introduction

In nature, as well as under laboratory conditions and in industrial processes, yeast is subjected to changing environmental conditions, such as desiccation, to which it must adapt in order to survive. Environmental stresses include osmotic stress, ionic stress, temperature, pH, oxidative stress, starvation and chemical solvents. In general, cells are susceptible to a stress imposition in the exponential phase, whereas in the stationary phase cells are able to overcome stress impositions. To date, comprehensive studies on yeast environmental stress responses at stationary phase have been conducted to understand the molecules and mechanisms involved in dehydration, using genomic transcriptional analysis and more recently proteomic techniques such as two-dimensional electrophoresis, mass spectrometry, and chromatography-based proteomics. According to previous studies, two major pathways regulate the general stress response in *S. cerevisiae*: stress proteins, known as HSP and stress response elements (STRE). HSP characterization leads to the description of several pathways of signal transduction and transcription factors involved in heat, nutrient limitations, sporulation, nitrogen limitation, hyperosmolarity and oxidative stress among others (Burnie *et al*., 2006).

In addition to HSP genes, another stress response element is STRE, a cis-regulatory element with a sequence that is common in the promoter region of a large amount of

genes induced by stress that enable a cell tolerant state to be acquired. Among these genes, we found those that codified for transporters, proteases or proteins that protect cells against different types of stress such as nitrogen starvation, carbon starvation, osmotic, oxidative, acidic pH, ethanol and heat stress conditions. However, some genes contain the STRE element in the promoter region and it is not functional.

Apart from the general stress response against several conditions, some stresses are also cross-protected by pathways that are activated by particular stresses. Two examples are oxidative damage and hyperosmolarity, which can be reduced or prevented during stress imposition by the combination of both specific and general stress response mechanism.

Dehydration and rehydration stress includes several stresses. Of these, temperature, osmotic and oxidative stresses are found the most representative in desiccation tolerance. Cells under each particular condition can induce cross-protection between each other and against other types of stresses, suggesting that a general stress response against osmotic, temperature or oxidative damage may be involved in desiccation tolerance. In order to elucidate which molecules and molecular mechanisms could participate in dehydration tolerance, we will deal specifically with each stress belonging to desiccation (heat, osmotic and oxidative stress) in the following section.

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2.1. HEAT SHOCK STRESS

Introduction

Heat shock response pathways

Introduction

Cells grow optimally within a relatively narrow temperature range but tolerate moderate deviations - some of which impinge upon cell structure and function – by means of rapid physiological adaptations. Heat shock could be induced directly by temperature upshift or indirectely as a consequence of other stresses such as dehydration. A heat treatment differs from thermal drying mainly in that the material to be dried experiences a decrease in water content, leading to osmotic and oxidative stresses among other factors, which are detrimental to dehydration tolerance. Desiccation could take place by freezing-drying (Santivarangkna *et al*., 2007; Desobry *et al*., 1997), convective drying (spray drying, fluidized bed, air drying in an oven, tunnel drying, spouted-bed drying or vacuum) (Elizondo and Labuza, 1974; Corcoran *et al*., 2004; Strasser *et al*., 2009; Di Salvo *et al*., 2007; Dimitrellou *et al*., 2008; Alpas *et al.*, 1996; Oliveira *et al*., 2007a) or conductive drying (vacuum, Berk, 2009). In a convective drying process, an air stream or gas such as nitrogen is passed around or through the material being dried and the moisture content inside the material is removed by evaporation and diffusion. In our group, desiccation is carried out by streaming air at 28ºC. The temperature increase during air drying is influenced by factors including the initial water content of the material containing yeast, the exposure time, the water removal rate, the rate of temperature increase and the temperature of the drying air, among other important factors.

Even though starving cells are significantly more tolerant of dehydration and less sensitive to heat shock than cells in exponential phase, dehydration significantly damages the membranes. The coexistence of different phases in a mixture of phospholipids (Shechter, 2004) leads to an increase in the membrane gel to fluid phase transition

temperature (TM), increasing not only the damage to the cell wall and membrane, but also the other cellular compartments and molecules such as sphingolipids. These lipids are involved in the formation of P bodies during autophagy in the oxidative stress response (Cowart *et al*., 2010), and are required for signalling, activating the transcription not only of the *TPS2* gene which synthetizes trehalose (Figure 1); but also STRE that reports on the activity of the Msn2/4p general stress pathway (Jenkins *et al*., 1997; Dickson and Lester, 2002). Preserving membranes during dehydration is thus an important feature of dehydration tolerance.

Heat shock response pathways

Three response pathways are induced by heat shock in order to overcome the stress: the CWI (cell wall integrity), the ESR (environmental stress response), and the HSR (heat shock response).

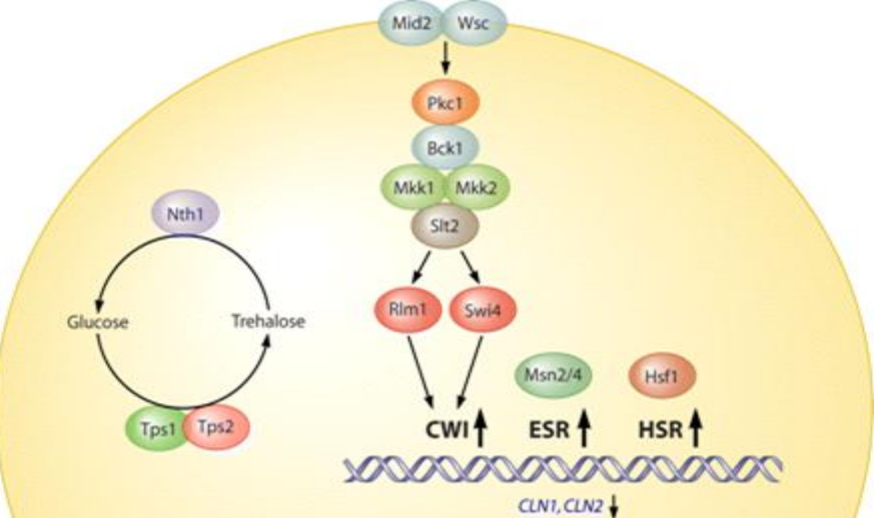

Figure 1. Physiological effects of heat shock. Three response pathways are shown to be induced by heat shock: the CWI (cell wall integrity) pathway, the ESR (environmental stress response), and the HSR (heat shock response) (Modified from Verghese *et al*., 2012).

The CWI pathway is induced by transient heat shock or growth at 37ºC in response to perturbations in the cell ultrastructure. The mechanism is unknown but requires at least one member of the putative sensors Sho1p (required for the activation of the HOG pathway in hyperosmolarity stress, Winkler *et al*., 2002), Mid2p and Wsc1p to Wsc4p which lead the activation of Slt2 (also present in osmotic stress response pathway, and a “client” of Hsp90p) (Figure 1). CWI pathway appears to be cross-talked to ESR, which

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is mainly focused on Msn2p/Msn4p. However, one of the most powerful adaptations to increased temperature is the HSR which is primarily governed by the action of Hsf1p (Figure 1).

As a consequence of heat shock pathway activation, gene expression programs become altered. They include HSPs, metabolic genes, and genes of unknown function (Gasch and Werner-Washburne, 2002). Some regulated genes appear not only with heat shock, but also in the osmotic and oxidative stress response pathways, suggesting a crosstalk between these three responses in dehydration cell survival. Among transcription factors, Hsf1p and Msn2/4p appear to be the most important regulators in heat shock activating heat shock elements (HSE) and STREE (Figure 2).

Array studies examining the contributions of each factor (Msn2/4p and Hsf1p) revealed a significant overlap in target gene expression. The Hsf1p regulon was found to comprise approximately 165 genes (Hahn *et al.*, 2004). Hsf1p is essential for cell viability at all temperatures, and it is constitutively bound on promoter of *HSP* genes as a trimer in the absence of stress. However, after a shift between 15ºC, 20ºC and 30ºC, Hsf1p is rapidly phosphorylated coincident with the transient induction of *HSP* genes in order to avoid destabilisation of cellular proteins that could cause misfolding (Ellis, 1987), a consequence of the desiccation process. A large number of misfolding proteins is known to be able to inhibit the ubiquitin-dependent proteasome system (UPS) or lead to the formation of toxic protein aggregates that could cause cell death. However, little to no protein misfolding occurs at temperatures between 36ºC and 37ºC, suggesting that major protein misfolding aggregation in our dehydration system might be due to oxidative stress and not mainly due to heat shock. Under oxidative stress, Yap1p transcription factor selectively induces Hsf1p-dependent expression leading to induction of HSP to protect nascent or misfolded proteins (Ahn, 2003; Lee *et al.*, 2000; Liu and Thiele, 1996). Thus, Hsf1p might be activated during dehydration probably by oxidative stress, rather than heat stress, leading to induction of HSPs.

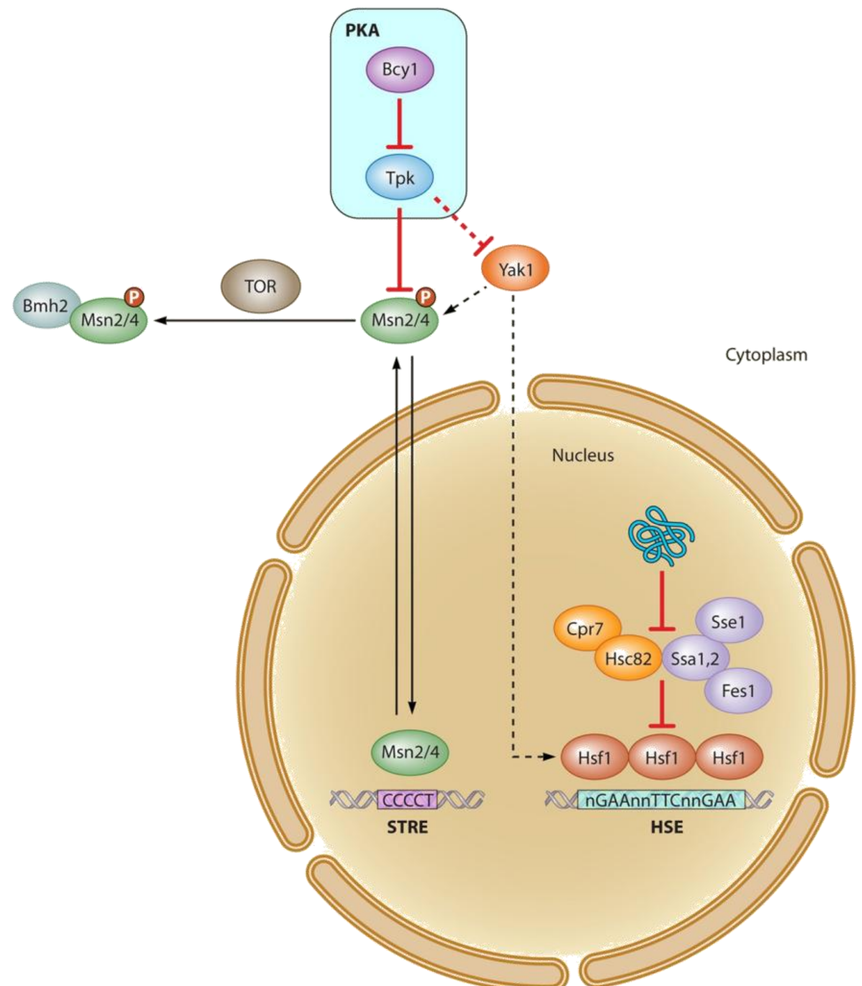

Figure 2. Hsf1p and Msn2/4p, primary modulators of the heat shock response. Dashed lines represent postulated interactions of the Yak1p kinase in the regulation of both Msn2/4p and Hsf1p. Red lines indicate regulatory interactions of protein kinase A. P, phosphorylation; STRE, stress response element; HSE, heat shock element (Verghese *et al*., 2012).

The yeast genome contains more than 60 chaperones or HSP. Among them, Hsp90, Hsp70, Hsp104 and the small Hsp (sHsp) are the main chaperones activated by Hsf1p in conjunction with co-chaperone proteins. These HSPs are named according to their apparent molecular masses. Hsp70 are found in ER, mitochondria and cytoplasm and interacts with almost any partially unfolded protein it encounters. They protect nascent polypeptides as they emerge from the ribosome, assisting in targeting and translocation, and play important roles in either refolding damaged proteins or shepherding their ubiquitylation and degradation.

Unlike the Hsp70 chaperon system, the Hsp90 chaperone is much more selective and interacts with a list of protein “clients” which rely on Hsp90 for the final steps of

maturation after initial interactions with Hsp70. Both chaperone machines effectively link in an "assembly line" of protein maturation. These two HSP are ATP binding protein with a chaperone cycle governed by nucleotide cycling, and a number of co-chaperones regulating the steps in this cycle.

Unlike most chaperones, Hsp104 are capable of extracting misfolded proteins from aggregates, followed by translocation through the central channel. However, Hsp104 cannot refold proteins alone and relies on Hsp70 to fully rescue substrates and return them to the appropriate native conformation. Moreover, Hsp104 is one of the few yeast protein chaperones absolutely required for thermotolerance (Sanchez and Lindquist, 1990).

The last main group of chaperones induced by heat shock consists of sHSP which represents a diverse family of proteins with passive activity that requires the action of the ATP-dependent Hsp70 and Hsp104 chaperones. Instead of preventing the aggregation of damaged proteins, sHSPs appear to co-aggregate with their substrates in a mixed oligomeric agglomeration that can be resolubilized with the help of the additional chaperones (Haslbeck *et al*., 2005). Two major sHSPs have been characterized in yeast: Hsp26 and Hsp42. Another highly abundant sHSP is Hsp12 which partly associates with cellular membranes, increasing membrane stability. Cells lacking Hsp12p are hypersensitive to severe heat shock and osmotic stress (Welker *et al*., 2010). Moreover, Hsp12p belongs to the artificial group of hydrophilin proteins involved in desiccation tolerance in anhydrobiote organisms (Garay-Arroyo, 2000).

After the termination of the activation of the heat shock response that leads to the transcription of HSPs, Hsf1p remains hyperphosphorylated until the replacement of the serine domain with alanine (Sorger, 1990), which causes a completely derepression of Hsf1p activity and returns it to the inactive state in the attenuation phase. Two main classes of HSP are also involved in repression of the transcriptional factor: Hsp70 and Hsp90. Moreover, cAMP-protein kinase A (PKA) indirectly inhibits Hsf1 activity (Ferguson, 2004).

In addition to the heat shock gene transcription mediated by Hsf1p, a parallel pathway in *S. cerevisiae* senses and responds to a remarkable variety of stresses besides heat shock such as oxidative stress. These are the transcription factors Msn2p and Msn4p involved in ESR pathway, which mediate STRE-mediated gene expression (Schmitt and McEntee, 1996). ESR contains between 300 and 600 genes up- or downregulated depending on the

type of stress. Genes related to DNA damage, heat shock, oxidative stress and osmostress belong to ESR. Most of the genes upregulated by ESR are involved in carbohydrate metabolism, protein metabolism, intracellular signalling, and defence against ROS, while the downregulated genes include protein synthesis and growth-related processes (Gasch *et al*., 2000; Causton *et al*., 2001; Capaldi *et al*., 2008; Berry and Gasch, 2008; López-Martínez *et al*., 2012; Martínez-Montañés *et al*., 2010). The multistress response mediated by Msn2/4p is generally transient, and the intensity and duration of the response are dependent on the strength of the stresses (Gasch and Werner-Washburne, 2002). Of the two genes, *MSN2* seems to play a more pronounced role in heat shock, as the overexpression of *MSN4* can only partially suppress phenotypes of an *Δmsn2* mutant under stress imposition (Schmitt and McEntee, 1996).

Two nutrient-sensing pathways have been described as playing important regulatory roles in controlling Msn2/4p: the cAMp-protein kinase A pathway and the TOR pathway (Figure 2). In the absence of PKA, Msn2p is located in the nucleus while Msn4p oscillates between cytoplasm and nucleus (Jacquet *et al*., 2003). Under stress circumstances such as temperature upshift, dehydration by ethanol, sorbate or osmotic stress (Görner *et al*., 1998), PKA activity is downregulated by Pp1p or Bcy1p. At this point PKA activates Yak1p (involved in oxidative and heat stress) which in turns phosphorylates Msn2/4p, which remain in nucleus. Accumulation of the two factors consequently leads to an induction of STRE-mediated gene expression (Malcher *et al*., 2011). In contrast to this scenario, under no stress, PKA levels increase and *MSN2* is exported to the cytoplasm compartment by *MSN5* (Jacquet *et al*., 2003).

The TOR pathway also impacts the activities of Msn2/4p controlling different cellular responses (Thomas and Hall, 1997). Unlike the cAMP-PKA pathway, which appears to primarily regulate nuclear exports, TOR prevents the nuclear import of Msn2p and Msn4p (Beck and Hall, 1999) by stimulating the association of Msn2/4p with the cytoplasmic protein Bmh2p (Figure 2).

After synthesis of transcripts in the nucleus by Msn2/4p, mRNA is accumulated in the nucleus (Saavedra *et al*., 1996) and therefore efficiently exported. The mRNA sequestered in response to stress appears to be concentrated in P bodies or stress granules (SGs).

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2.1. OSMOTIC STRESS

Introduction

HOG pathway

Introduction

Osmotic stress is caused by changes in the concentration of dissolved molecules in the medium surrounding a cell. Hyperosmolarity and hypo-osmolarity are different forms of osmotic stress that have different control mechanisms, but both involve an alteration in the intracellular water content. While an increase in external osmolarity (hyperosmotic stress) will lead to an outflow of water fromthea cell, a decrease in external osmolarity (hypo-osmotic stress) will cause a water inflow (Wood, 1999). Hyperosmotic stress is one of the stresses caused by dehydration which induces the intracellular protective mechanisms involved in osmoregulation. We therefore focus on the molecular response of *S. cerevisiae* in a hyperosmotic condition for further discussion.

The osmoregulation caused by hyperosmotic stress includes transcriptional and translational responses, as well as accumulation of osmolites in order to overcome the stress imposed. These adaptive responses are mostly governed by the high osmolarity glycerol (HOG) signalling pathway, of which the core is the Hog1p MAP kinase (MAPK) cascade. However, osmoregulation is not only performed by the HOG pathway and it induces many genes that are considered to be part of general stress responses such as ESR. Among general stress responses oxidation and detoxification and the stabilization of cellular proteins and structures are included (Mager and Varela, 1993; Yancey, 2005), thus creating a cross-protection during desiccation imposition. As a result, when cells are subjected to a mild stress, STRE mediated responses are induced even in the absence of Hog1p (Berry and Gasch, 2008). However, this protection is not sufficient for cells lacking Hog1p to survive higher levels of osmolarity. In fact, approximately 80% of the genes that are induced upon osmostress depend on Hog1p MAPK for full induction

(Posas *et al*., 2000; Rep *et al*., 2000; O'Rourke, 2003; Capaldi *et al*., 2008). Taking into account all these considerations, we will therefore proceed with the explanation of the dynamics of the HOG pathway and downstream Hog1p adaptive responses.

HOG pathway

Adaptation to hyperosmolarity through the HOG pathway includes two phases: sensing osmotic changes and activating appropriate cellular responses (Figure 3).

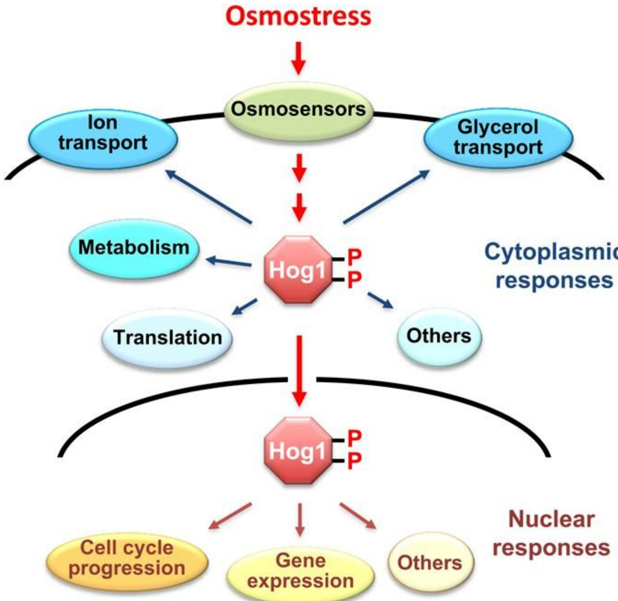

Figure 3. Osmo-adaptive responses in yeast. In response to an increase in extracellular osmolarity, the Hog1p MAPK is activated, which leads to the induction of cytoplasmic and nuclear adaptive responses. Cytoplasmic responses include the control of ionic fluxes and glycerol transport, metabolic enzymes, and protein translation. Nuclear responses include the modulation of cell-cycle progression and the control of gene expression (Saito and Posas, 2012).

During the first period of hyperosmotic shock, it causes disassembly of the actin cytoskeleton, water loss and consequent cell shrinkage. These first dehydration consequences are followed by a stimulation of the HOG pathway, which induces transcriptional and translational responses. As a consequence, osmolite glycerol starts to accumulate among the other osmoprotectants, such as trehalose, induced by some other minor pathways. At this point a feedback on HOG pathway takes place, as well as transcriptional responses. Reassembly of the actin cytoskeleton occurs in this stage and on condition of osmotic balance is re-established (Brewster and Gustin, 1994). By the end

of the osmoregulation process, glycerol accumulation reaches its maximum levels and the final cell volume is restored, which leads to cell proliferation (Figure 4).

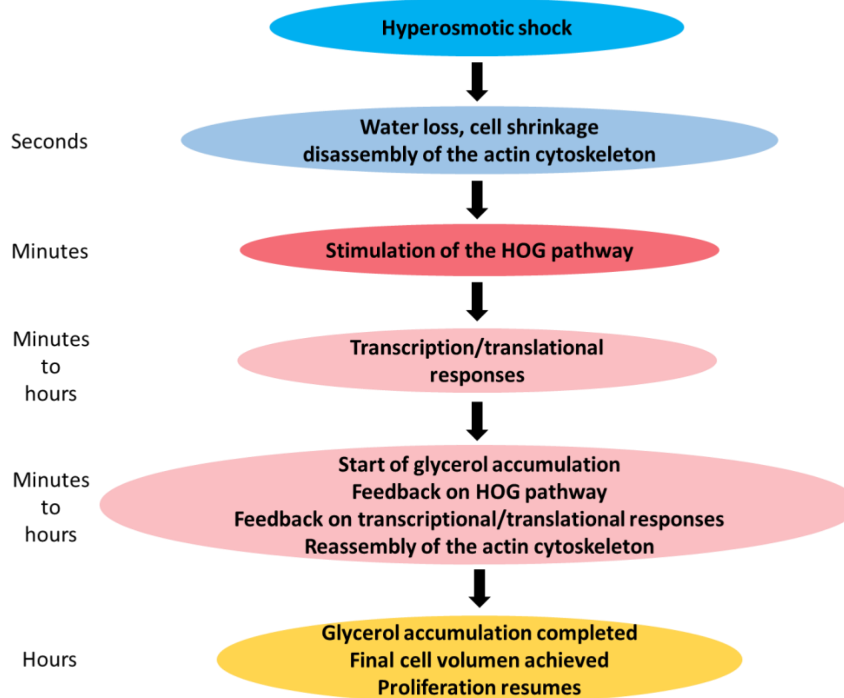

Figure 4. Time course of different responses to hyperosmotic shock (adapted from Tamás and Hohmann, 2003).

The HOG pathway is activated by extracellular stimuli that subsequently initiates a multistep phosphorelay and interactive system bound to MAP kinase cascades. The HOG pathway core, Hog1p MAPK, is activated by Pbs2p, a MAPK kinase (MAPKK), by dual phosphorylation of the conserved Thr and Tyr residues. The MAPKK is similarly activated, but this could be carried out by a different MAPKK kinase (MAPKKK) which comprises two functionally redundant signal branches: Sln1p and Sho1p (Figure 5). Sln1p branch is well understood, while the activation of the Sho1p branch is still only vaguely defined.

Sln1p governs two distinct signalling pathways: the Sln1p-Ypd1p-Ssk1p multistep phophorelay, which regulates hyper-osmolarity responses, and the Sln1p-Ypd1p-Skn7p multistep phophorelay, which makes a contribution to hypo-osmolarity responses. Moreover, Skn7p is involved in oxidative and heat shock response through Hf1p. Regarding the Sln1-Ypd1-Ssk1 signalling pathway, the first sensor is thought to respond to changes in the plasma membrane due to osmotic stress (Reiser *et al.*, 2003; Hayashi and Maeda, 2006; Panadero *et al.*, 2006). Under normal osmotic conditions Sln1p remains

active and it interacts with Ypd1p which constitutively phosphorylates Ssk1p. (Maeda *et al*., 1994; Fassler and West, 2010). However, under hyperosmotic conditions, unphosphorylated Ssk1-OH is accumulated, and it binds and activates Ssk2/Ssk22p which exclusively phosphorylates Pbs2p MAPKK, and thereby activates Hog1p MAPK (Maeda *et al*., 1994; Reiser *et al*., 2000).

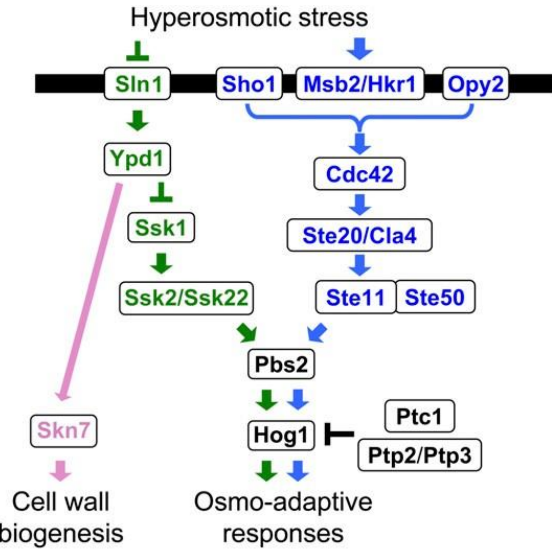

Figure 5. A schematic diagram of the yeast HOG pathway. The protein names separated by a thrash (/) are functionally redundant. Proteins that are specific to the Sln1p branch are colored green, those that are specific to the Sho1p branch are colored blue, and those that are common are colored black. The black horizontal bar represents the plasma membrane. Arrows indicate activation, whereas the T-shaped bars represent inhibition (Saito and Posas, 2012).

Unlike Sln1p branch, signalling response in the Sho1p branch is initiated by the putative osmosensors Msb2p and Hkr1p, which are highly glycosylated single-pass transmembrane proteins. Through an as yet undefined mechanism, this response seems to lead the activation not only of the Ste20p kinase but also the Cla4p kinase, which partially compensates for the function of the Ste20p (Tatebayashi *et al*., 2006), by inducing their association with the membrane-bound small protein Cdc42p (Lamson *et al*., 2002). Activated Ste20/Cla4p then phosphorylates and activates the Ste11p MAPKKK (Raitt *et al*., 2000; Kikawada, 2005; Drogen *et al*., 2000) which is dependent on Ste50p (Ramezani-Rad, 2003) recruited to the membrane by the anchor protein Opy2 (Wu *et al*., 2006; Yamamoto *et al*., 2010). Ste11p, as well as other signalling elements, can be activated by several MAPK cascades involved in the mating response, filamentous and invasive growth (FIG) besides hyperosmolarity. In order to activate Ste11p by HOG pathway, which in turns phosphorilates Pbs2p, crosstalk from one MAK pathway to

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another can be prevented by a number of mechanisms, including negative regulation and cross-inhibition between the same signalling element that activates strongly in the HOG pathway under osmotic stress conditions but weakly in the FIG or mating pathway. However, the mechanism of cross-inhibition remains obscure.

After the activation of Hog1p by either the Sln1p branch or the Sho1p branch under hyperosmosis or other non-osmotic stresses, including cold (Hayashi and Maeda, 2006; Panadero *et al*., 2006), heat *(Winkler et al*., 2002), and low pH (Kapteyn *et al*., 2001), Hog1p is rapidly accumulated in the nucleus in order to activate a program for cell adaptation that includes long- and short-term responses. Long-term adaptation involves transcriptional and translational regulation. Hog1p can induce activation of a subset of osmoresponsive genes by phosphorylation of promoter-specific transcription factors and binding to RNA Pol II *(*Alepuz *et al*., 2003) concomitantly with the fact that Hog1p is present in the coding regions of these genes and it travels with elongating RNA Pol II (Pascual-Ahuir *et al*., 2006; Pokholok *et al*., 2006; Proft *et al*., 2006). In addition to phosphorylating components of the transcriptional and cell-cycle machineries, the Hog1p MAPK also phosphorylates other cytoplasmic and nuclear proteins that have been found in a recent phospho-proteomic study (Soufi *et al*., 2009). Short-term adaptation is accomplished by the accumulation of compatible solutes, also known as osmolites. Glycerol seems to be the most important osmolyte for the growth of *S. cerevisiae* in the presence of high osmolarity, although trehalose, amino acids, and ions contribute differently to adaptation to osmostress (Hohmann *et al*., 2007).

The production and accumulation of glycerol maintains the water balance and restablishes the volume and the turgor of the cells (Blomberg and Adler, 1989; Hohmann *et al*., 2007; Westfall *et al*., 2008; de Nadal *et al*., 2011). Glycerol accumulates by several mechanisms: GPD1 expression via the Hog1p pathway, Fps1p mediated channel and by metabolic adjustments. The first takes at least 15 minutes to achieve the maximum glycerol levels through the induction of Gpd1p, Gpp2p and other genes involved in downregulation of the Hog1p pathway (Hirayarna *et al*., 1995; Hohmann, 2002). Meanwhile, specific aquaporin transmembrane channel Fps1p mediates the rapid import and export of glycerol. Fps1p closes apparently independently of Hog1p in response to osmostress (Tamás *et al*., 1999). Nevertheless, Fps1p is regulated by Rgc2p which is partially controlled by the Hog1p MAPK. Finally, glycerol accumulation causes metabolic adjustments and cells must redirect carbon resources toward the enhanced

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production of glycerol, leading to significant modulation of central carbon metabolism during osmo-adaptation (Klipp *et al*., 2005; Mollapour and Piper, 2007; Westfall *et al*., 2008; Beese *et al*., 2009; Bouwman *et al*., 2011; Dihazi *et al*., 2004).

As adaptation proceeds, and osmotic balance is re-established, Hog1p activity falls to near basal levels, and it is exported back to the cytoplasm under conditions of adaptation to high osmolarity or after returning to an iso-osmotic environment. Finally, it is still necessary to inactivate the kinases by dephosphorylation to bring the system to a prestimulation state. In order to achieve this state, the two activating phosphorylation sites in Hog1p are dephosphorylated by different enzymes, of which Ptc1p phosphatase is the most important (Warmka *et al*., 2001; Saito and Tatebayashi, 2004; Martín *et al*., 2005). Another negative feedback mechanism of Hog1p is focused on the Ste50-Opy2p complex. Phosphorylation of Ste50p by Hog1p reduces the affinity for the membrane anchor Opy2p, and leads to deactivation of the HOG pathway.

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2.3. OXIDATIVE STRESS

Introduction

Sources of oxidative stress

Intracellular degradation of oxidized biomolecules

Transcriptional regulation of gene expression

Antioxidant defences

Translational regulation of gene expression and metabolic reconfiguration

Late Embryogenesis Proteins

Yeast hydrophilin proteins

Introduction

All organisms exposed to radical-generating compounds or normal aerobic metabolism accumulate reactive oxygen species (ROS) (Halliwell, 2006). ROS are continuously produced and eliminated and their concentration is therefore a dynamic parameter. When the balance between ROS production and elimination is disturbed, cells accumulate ROS, leading to the state of oxidative stress which can either activate stress responses for cell survival or program cell death (PCD) (Figure 6). Briefly, stress signals relayed by ROS themselves (such as H_2O_2) can activate transcription factors which upregulate the expression of genes encoding enzymatic (such as catalases) and non-enzymatic antioxidants (such as GSH). These systems will be explained below. These response mechanisms, together with the targeted removal of small, oxidized proteins by the ubiquitin-dependent proteasome system (UPS), help to ensure the survival of cells.

Additionally, cells can activate cytoprotective autophagic pathways that remove irreparably oxidized macromolecules or dysfunctional organelles, such as mitochondria. However, an abnormally high degree of autophagy might also mediate PCD. Finally, exposure of cells to severe oxidative insults can elicit lethal response pathways such as apoptosis, necrosis, and possibly other forms of PCD which have yet to be discovered (Farrugia and Balzan, 2012).

Then, PCD is activated under severe oxidative insults leading cell death, which can proceed via apoptosis, necrosis or autophagy depending on the physiological state of the cells and the nature of the oxidative insult. We will focus on each PCD response in the following section.

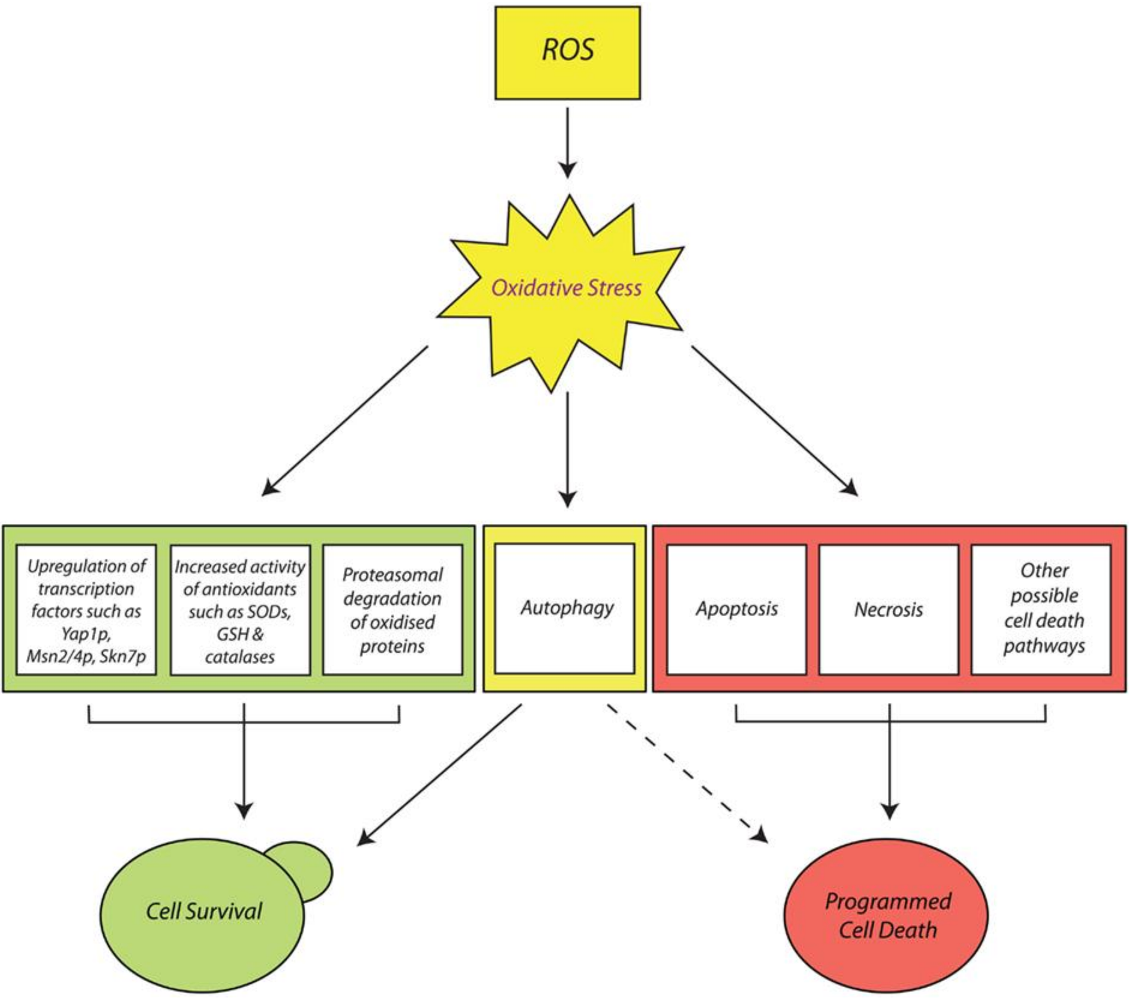

Figure 6 Cellular responses to oxidative stress in *S. cerevisiae***.** Oxidative stress, induced by the accumulation of ROS, can elicit a range of stress responses in budding yeast cells, that either result in cell survival (shown in green) or cell death (shown in red). Additionally, cells can activate cytoprotective autophagic pathways (bordered in yellow) (Farrugia and Balzan, 2012).

Apoptosis, which is typically induced by exposure to low doses of oxidants such as H_2O_2 (Madeo *et al.*, 1999), can be detected by morphological hallmarks such as phosphatidylserine externalization, chromatin condensation and DNA fragmentation, among others (Rodríguez-Porrata *et al.*, 2012b) (Figure 6 and 7).

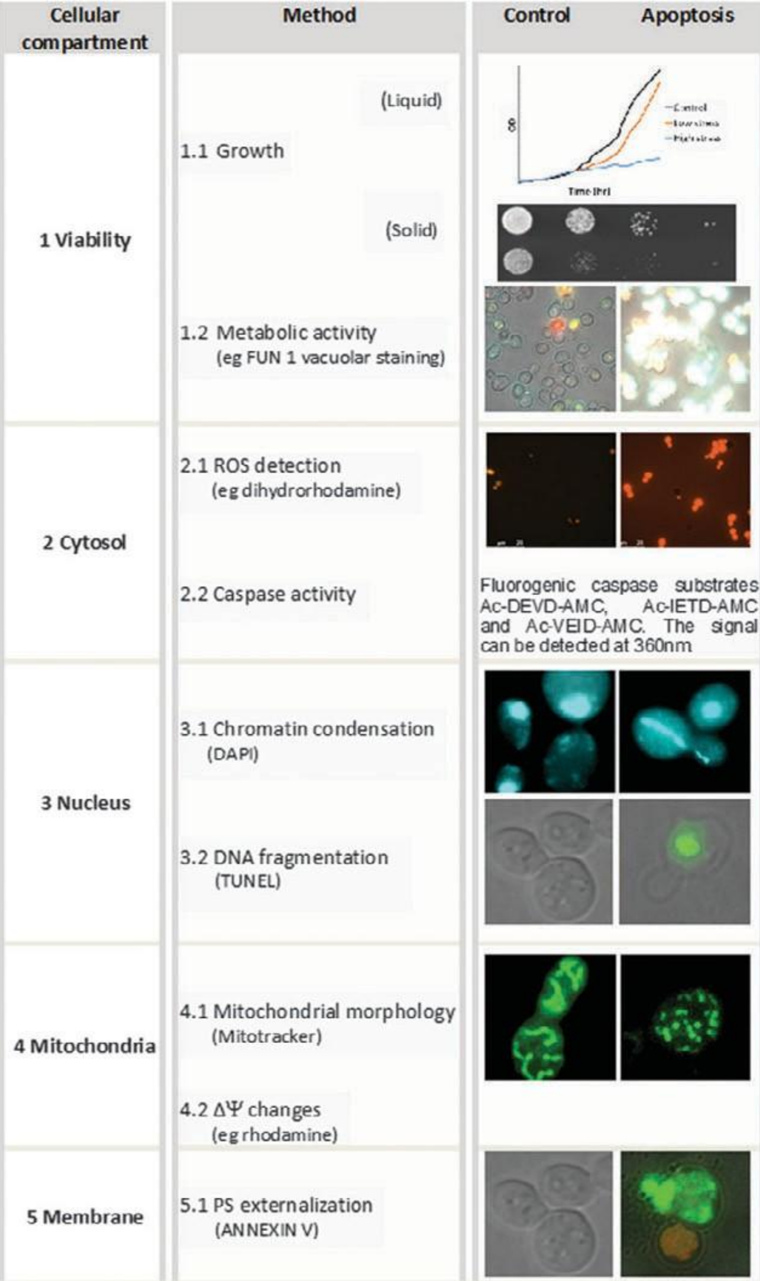

Figure 7 Overview of methods to study apoptotic hallmarks in yeast *S. cerevisiae* (Muñóz *et al.*, 2012).

Despite morphological hallmarks, the most compelling evidence of apoptosis in yeast is derived from the discovery of several apoptotic regulators. Yeast cell death induced by ROS-associated stress stimuli can proceed via either caspase-dependent apoptosis or caspase-independent apoptosis (Madeo *et al.*, 2009). Accumulation of ROS by salt, heat

or hyperosmotic stress leads to a caspase-mediated apoptotic phenotype attributed to the yeast caspase protein 1 (Yca1p) with the participation of other caspase-like proteases (Wilkinson and Ramsdale, 2011). One such protein is Esp1p, which induces disruption of the mitochondrial membrane potential and cytochrome c release, followed by apoptosis (Reyes *et al*., 2008). Another yeast protein associated with caspase-like activity is Kex1p, which mediates apoptotic cell death by defective N-glycosylation, acetic acid and chronological aging (Hauptmann and Lehle, 2008). Apoptotic cell death in yeast caused by ROS-associated stress stimuli can also take place with no need for Yca1p activity. Caspase-independent apoptosis requires functional mitochondria because it is the source of important yeast apoptotic regulators capable of operating independently of Yca1p. Among the regulators, Aif1p and Nuc1p (Wissing, 2004; Büttner *et al*., 2007) are translocated from the mitochondrion to the nucleus, in response to oxidative pro-apoptotic stimuli such as H_2O_2, resulting in a caspase-independent apoptotic phenotype. Additionally, the yeast nucleus itself contains the Yca1p-independent death regulator, Nma111p, which in response to H_2O_2, starts to aggregate in the nucleus and induces yeast cell apoptosis (Fahrenkrog *et al*., 2004).

Necrosis can also occur in response to oxidative stress (Madeo *et al*., 1999) such as exposure to H_2O_2, acetic acid and heavy metals (Madeo *et al*., 1999; Ludovico *et al*., 2001; Liang and Zhou, 2007) (Figure 6). Necrosis is characterized by bioenergetics failure and morphological features such as random DNA fragmentation, an increase in cell volume and loss of cell plasma membrane integrity and subsequent leakage of intracellular contents (Zong and Thompson, 2006). As a result, necrosis can be detected by specific hallmarks such as rupture of the plasma membrane, disintegration of subcellular structures, or activation of cathepsins (Wloch-Salamon and Bem, 2013). Necrosis was long dismissed as an accidental form of cell death (Galluzzi *et al*., 2011) which generally occurs in response to extreme environmental stresses (Madeo *et al*., 1999). However, this paradigm has changed, since necrotic cell death is regulated by factors such as signalling and catabolic proteins (Baines, 2010) as well as the heat shock protein Hsp90 (Dudgeon *et al*., 2008). Peroxisomes, which generate ROS, can also act as key regulators of necrosis in yeast through important proteins such as Pex6p (Jungwirth *et al*., 2008) and Pmt20p (Bener Aksam *et al*., 2008). Another prominent regulator of necrosis in yeast, which also participates in caspase-independent apoptosis

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system, is Nuc1p. Moreover, Pep4p is also a common regulator in both types of PCD in chronologically aging yeast cells (Carmona-Gutiérrez *et al*., 2011).

Autophagy might also act as a mediator of cell death when it occurs at high levels due to ROS accumulation (Pattingre *et al*., 2005; Kissová *et al*., 2004) (Figure 6). There is a complex interplay between autophagy and cell death - two distinct stress responses which depending on the circumstances of the cells, can either compete against each other or cooperate in a manner which is probably at least partially regulated by the ROS-dependent mitophagic turnover of mitochondria (Carmona-Gutierrez *et al*., 2010).

Various disease processes, including cancer, neurological diseases and aging have been shown to involve oxidative damage (Hansen *et al*., 2006) causing cell death or cell survival due to positive stress response activation. In fermented food industries, such as baking and brewing, *S. cerevisiae* is exposed to oxidative stresses during processing, transportation and storage (especially in the dry state). Oxidation damage can ruin the industrial end product due to lipid peroxidation, protein oxidation or genetic damage (Richards *et al*., 2002). Appropriate activation of molecular mechanisms against death could partially prevent these dehydration consequences. For this reason, we will now focus on the ROS sources and the stress response for cell survival during induced oxidative stresses, which could elucidate the molecular mechanisms and cell components involved in dehydration tolerance.

Sources of oxidative stress

Yeast cells can become exposed to ROS production after exposure to numerous exogenous agents including xenobiotics, carcinogens and ionizing radiations. However, the main source of ROS is provided by leakage of the electrons in the respiratory chain used to produce ATP and water from oxygen consumption. All these sources of oxidative stress generate different forms of ROS: free radicals such as superoxide anion (O_2^-) and hydroxyl radical ($^{\bullet}HO$), non-radical reactive species such as hydrogen peroxide (H_2O_2) and singlet oxygen (Figure 8). Other less studied reactive species can enhance oxidative stress, such as reactive nitrogen species (RNS) and their derivatives (Li and Moore, 2007).

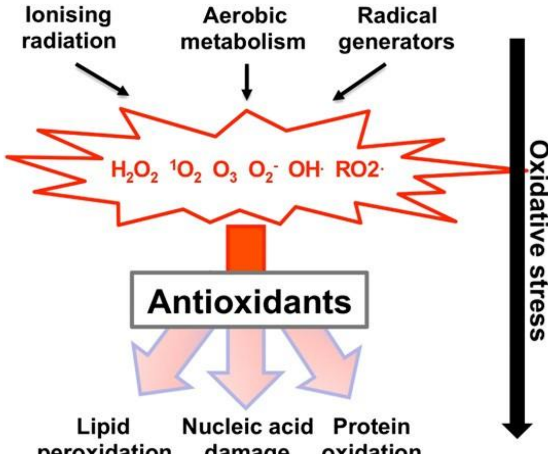

Figure 8 Oxidative stress. Different sources of oxidative stress stress can damage a wide variety of cellular components resulting in a lipid peroxidation, protein oxidation, and genetic damage through the modification of DNA (Morano *et al.*, 2011).

Of all the ROS sources, the most widely used as a model of oxidative stress is H_2O_2. This non-radical reactive species plays a role as a signalling molecule in the regulation of many biological processes (Veal *et al.*, 2007). However, its cellular production from the superoxide anion precursor (O_2^-), the major ROS product from electron leakage, increases oxidative damage, and it must therefore be removed. Otherwise, H_2O_2 leads to the formation of highly reactive hydroxyl radicals (•HO) from the Fenton and Haber-Weis reactions (Halliwell, 2006) (Figure 9).

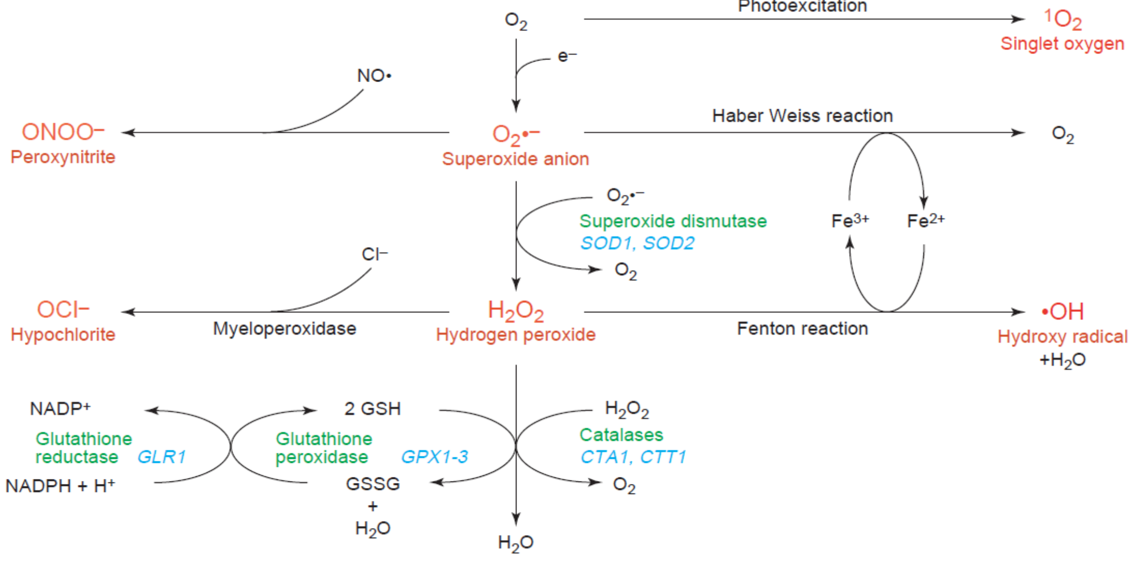

Figure 9 ROS and other radicals generated in cells. Intermediates are in red and antioxidant enzymes in green (Temple *et al.*, 2005)

•HO can also be generated by free redox active metals such as iron or copper via the Fenton reaction by oxidation of Fe^{2+}, or via Haber-Weiss by reduction of Fe^{3+} (Figure 9) (Liochev and Fridovich, 1999; Freitas *et al*., 2003). Apart from H_2O_2 and free redox active metals, other complexes are frequently used to induce oxidative stress, such as thiol-reactive compounds. These include indirect oxidative stress inducers by binding to and depleting thiol groups, as well as compounds which directly oxidize thiol groups (Sheehan *et al*., 2001).

Under normal metabolic conditions, all the free radicals mentioned above would be scavenged by antioxidant defence systems, but under dehydration these mechanisms might not function, and oxidative damage becomes one of the most deleterious effects of water depletion. In fact, yeast cells showed a more than tenfold increase in oxidation after dehydration, confirming the oxidative damage produced during the dehydration-rehydration process (Rodríguez-Porrata *et al*., 2011; López-Martínez *et al*., 2012; Pereira *et al*., 2003).

Free radical accumulation during desiccation causes damage to lipids, proteins and DNA (Figure 8). In lipids, the cell membranes become fluidized and perturbed during dehydration, and may be susceptible to beattack by ROS (Carpenter and Crowe, 1989). These radicals often cause an extensive peroxidation and de-esterification of membrane lipids at intermediate ranges of water loss (Senaratna *et al*., 1987). Moreover, water deficiency produces a large rise in the levels of malondialdehyde (MDA), a product of lipid peroxidation that in combination with free chlorine produces hypochlorite, increasing the oxidative cell damage (Espindola *et al*., 2003; Pereira *et al*., 2003; França *et al*., 2005a). As a result of lipid oxidation and the increase in the phase transition temperature in phospholipids due to heat shock, membranes become a crucial cell component for repair after rehydration in order to overcome the stress.

During desiccation, proteins can denature and misfold principally as a consequence of oxidative stress, resulting in a loss of biological activity upon rehydration. One of the difficulties in maintaining the viability of fragile proteins is to ensure their chemical integrity, which could be disturbed due to the oxidation of the functional groups. After that, proteins become sensitive to proteolysis and may be inactivated or may show reduced activity (Dalle-Donne *et al*., 2003). Another oxidative injury caused by dehydration is the protein carbonylation produced during storage of dried *S. cerevisiae* cells (França *et al*., 2005b). In genetic damage, DNA chemical bonds can be impaired by

oxidative degradation in the native DNA structure, causing a possible mutation or aging (Finkel and Holbrook, 2000; Nohmi *et al*., 2005).

In order to avoid cell damage due to exposure to oxidants, *S. cerevisiae* has an oxidative stress response that ensures the survival of the cell through the reestablishment of an appropriate redox balance during dehydration-rehydration and other stresses. The defence systems that detoxify ROS include reduction of their rate of production, and repair of the damage caused by them (Figure 6). We will therefore discuss each oxidative stress response below.

Intracellular degradation of oxidized biomolecules

Cell biomolecules exposed to stress can sustain severe oxidative damage that cannot be repaired, such as carbonylated proteins and oxidatively damaged mitochondria. Yeast has two stress response mechanisms that facilitate the removal of irreparably oxidized biomolecules when overcoming oxidative stress: the ubiquitin-dependent proteasome system (UPS) and autophagy.

The ubiquitin-dependent proteasome system facilitates the removal of small oxidized proteins from cells via proteasome with the participation of ubiquitin receptor proteins such as Rad23p, Dsk2p, Ddilp and Rad4p (Schauber *et al*., 1998; Chen and Madura, 2002; Wilkinson *et al*., 2001; Bertolaet *et al*., 2001; Elsasser *et al*., 2004; Li *et al*., 2010). Because the UPS mainly operates in the cytosol, oxidized proteins located within cell compartments segregated by membranes cannot be degraded by this mechanism unless they are exported into the cytosol. In the case of ER, the ER-associated degradation (ERAD) pathway facilitates the translocation, while in the case of mitochondria, it is the mitochondria-associated degradation (MAD) pathway that carries out the translocation for UPS degradation.

Autophagy involves the intracellular degradation and recycling of long-lived biomolecules such as macromolecular proteins and organelles via vacuoles (Klionsky *et al*., 2011). Autophagy plays a crucial pro-survival role not only in response to ROS accumulation but also in nutrient starvation. This is important during dehydration stress, since cells are in a stationary state before the dehydration process. The autophagy pathway is activated by the expression of autophagy-related genes (ATG), of which there are at least 33 in yeast (Reggiori and Klionsky, 2002; Goldman *et al*., 2010). Two types

of autophagy exist: non-selective sequestration of intracellular macromolecules and portions of cytosol, and selective autophagy for the degradation of large oxidized biomolecules. The first is performed by a double-membraned vesicle called the autophagosome, which then delivers its enclosed material to a vacuole (Klionsky *et al.*, 2011). Non-selective autophagy often takes place in conjunction with selective autophagy. The second one, selective degradation of biomolecules, takes place in organelles. As an example, during oxidative stress, mitochondrial damage stimulates the specific and selective degradation of irreparably damaged mitochondria by mitophagy (Goldman *et al.*, 2010; Lemasters, 2005; Kissová *et al.*, 2004) which is dependent on an outer micochondrial membrane protein, Uth1p. Two mitogen-activated protein kinases (MAPKs) have been discovered in this process, Slt2p and Hog1p (Mao *et al.*, 2011), and are both involved in osmoregulation. Selective autophagic degradation of specific biomolecular targets can also take place in Golgi apparatus (crinophagy), ER (reticulophagy), ribosomes (ribophagy) and peroxisomes (pexophagy), as well as mitochondria (Glaumann, 1989; Hamasaki *et al.*, 2005; Kraft *et al.*, 2008; Sakai *et al.*, 1998).

Transcriptional regulation of gene expression

As in osmotic stress regulation, some ROS responses belong to general stress, but there are also many ROS specific responses which also cross-talk. A key feature in this response is the transcriptional reprogramming of gene expression to provide the necessary changes in proteins to return the redox status of the cell to an acceptable range. The most important transcriptional regulators (some of them involved in heat shock responses) are Yap1p, Skn7p and Msn2/4p.

Yap1p is critical for tolerance to oxidants such as H_2O_2 and diamide, as well as heavy metals like cadmium (Schnell and Entian, 1991; Kuge and Jones, 1994; Wu and Moye-Rowley, 1994), although it is involved in heat shock response through Hsf1p. Yap1 belongs to a family of AP-1 type transcription factors that bind to AP-1 binding sequences of the DNA molecule (Wiatrowski and Carlson, 2003; Kuge *et al.*, 2001; Netto *et al.*, 2007; Lushchak, 2010)

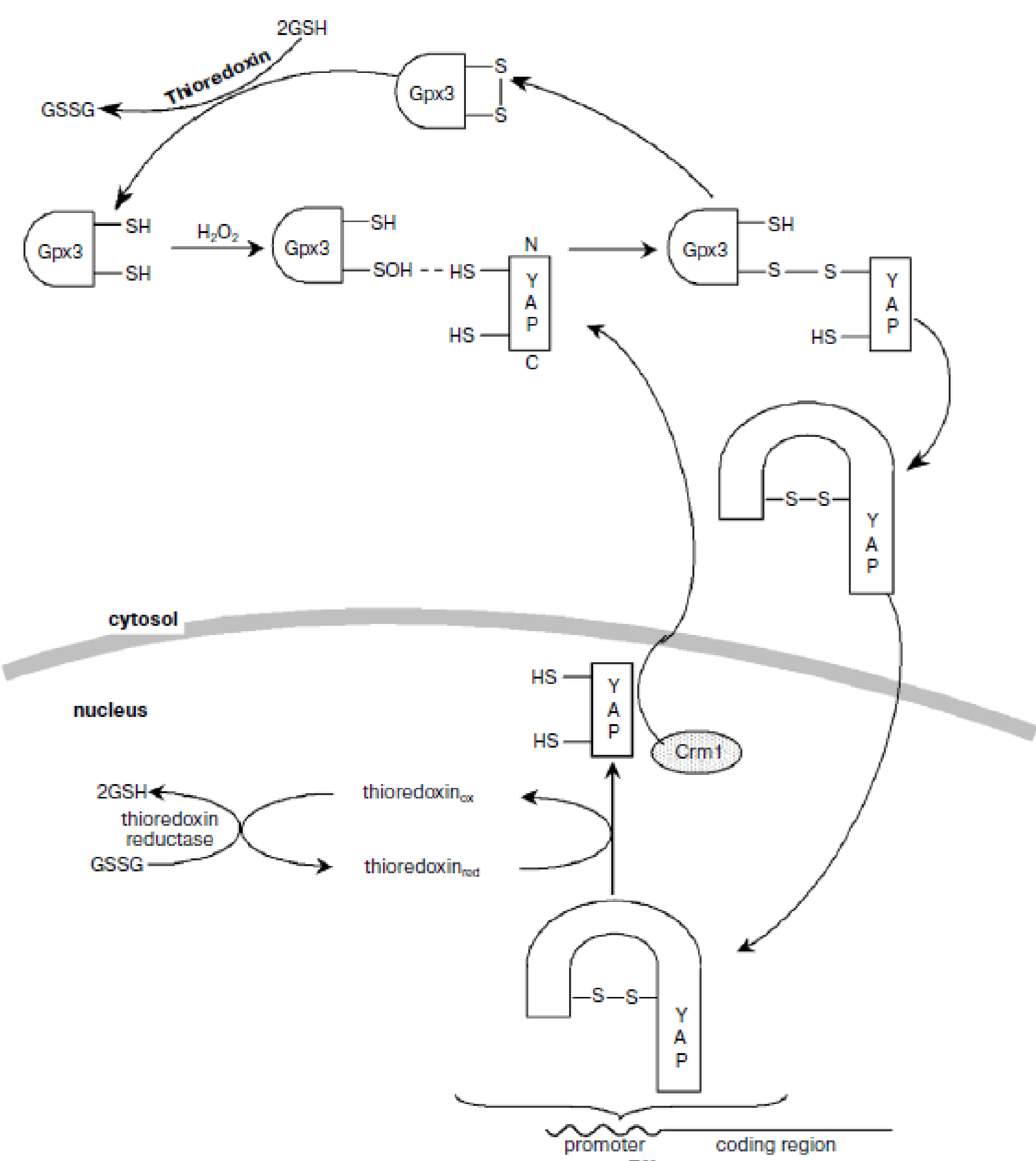

Figure 10 Operation of Yap1p regulatory protein during treatment of *S. cerevisiae* with hydrogen peroxide (Lushchak, 2010).

In yeast cells, at least eight Yap proteins have been found, named Yap1-Yap8p respectively. They have slightly different although similar specificities to bind with DNA. However, their function may differ (Fernandes *et al.*, 1997; Moye-Rowley *et al.*, 1989; Stephen *et al.*, 1995). The activity of Yap1p is mainly regulated at the level of its export from the nucleus (Figure 10).

Under normal conditions Yap1p is mostly localized in cytosol, due to its active export from the nucleus by the participation of protein Crm1p (Kuge *et al.*, 1998; Yan *et al.*, 1998) which interacts with Yap1p at the C-terminal domain (CRD-cysteine rich domain)

UNIVERSITAT ROVIRA I VIRGILI
FUNCTIONAL CHARACTERIZATION IN VIVO OF ESSENTIAL SACCHAROMYCES CEREVISIAE'S HYDROPHILIN FOR DESICCATION TOLERANCE
Gema Isabel López Martínez
Dipòsit Legal: T 1354-2015

(Kudo *et al*., 1999). Under hydrogen peroxide and other oxidant agents, the cysteine 36 of the sensor Gpx3p is oxidized to sulfoxide, which further interacts with the cysteine residue 598 of Yap1p, forming an intermolecular disulfide bond between cysteine 303 and cysteine 598 (Figure 10). In addition, a disulfide bond linking C310-C629 seems to be required for dissociation between Yap1p and Crm1p (Coleman *et al*., 1999; Delaunay *et al*., 2000; Wood *et al*., 2004). Finally, both Ybp1p and Ybp2p are required for the correct folding of Yap1p in conjuction with Gpx3. While overproduction of Ybp2p can bypass a *Δgpx3*, overproduction of Ybp1p cannot (Veal *et al*., 2003; Gulshan *et al*., 2004; Okazaki *et al*., 2007).

The oxidation of cysteine residues of Yap1p and consequent prevention of interaction with Crm1p results in an accumulation of Yap1p in the nucleus, with concomitant induction of target gene expression via binding with the specific DNA sequences localized in promoter regions of regulated genes (Toone and Jones, 1999). Many of these genes are known to respond to oxidative stress, including *TRX2* encoding thioredoxin, *GSH1*-glutamylcysteine synthase, *GSH2*-glutathione synthase, *TRR1*-thioredoxin reductase 1, *GPX2*-glutahione peroxidase 2, *TSA1*-thioredoxin peroxidase 1, *GLR1*-glutaredoxin 1 and *AHP1*-alkylhdroperoxide reductase 1, as well as genes encoding transporters *YCF1*, *ATR1* and *FLR1* (Kuge and Jones, 1994; Wu and Moye-Rowley, 1994; Sugiyama *et al*., 2000; Morgan *et al*., 1997; Grant *et al*., 1996; Inoue *et al*., 1999; Lee *et al*., 1999a; Lee *et al*., 1999b; Wemmie *et al*., 1997; Coleman *et al*., 1999; *Alarco et al*., 1997). Finally, after recovering the redox balance, the nucleus oxidized Yap1p may be reduced by thioredoxin and become sensitive once again to hydrogen peroxide (Carmel-Harel *et al*., 2001). Furthermore, thioredoxin is reduced by thioredoxin reductasee (EC 1.8.1.9) at the expense of NADPH.

Skn7p is another transcriptional factor in oxidative stress. Skn7p is also phosphorylated in the Sln1p branch of the HOG pathway under hypo-osmotic stress (Saito and Posas, 2012), and thus cannot interfere with the oxidative stress response in dehydration-rehydration stress (Figure 5). Skn7p regulates the transcription of *TRX2* as well as Yap1p under hydrogen peroxide resistance, but Yap1p is bound to the *TRX2* promoter at a different site to Skn7p (Figure 11). Nevertheless, *TRX2* is not the only case where Yap1p and Skn7p act at a common promoter to induce oxidative stress tolerance. Of the 32 different polypeptide chains where Yap1p control their expression, 15 require the presence of both Yap1p and Skn7p (Lee *et al*., 1999b). In this group, we found, two

SOD1 and *SOD2* dismutases, a *TSA1* peroxiredoxin and the alkyl hydroperoxide reductase, among others. Other factors are required to support the transcriptional activation of Skn7p, such as Ccp1p, a mitochondrial cytochrome c peroxidase (Charizanis *et al*., 1999) or Fap7p that is also implicated in ribosome subunit processing (Juhnke *et al*., 2000; Granneman *et al*., 2005). Skn7p could also interact independently of Yap1p with a wide range of genes such as *SSA1*, transcriptional regulators of cell cycle progression (Mbp1p) (Machado *et al*., 1997) or general transcription regulators Cdk8p and Ccr4p (Cooper *et al*., 1999; Vincent *et al*., 2001; Lenssen *et al*., 2007; Lensen *et al*., 2002). The constitutive nuclear protein Skn7p presents homology to heat shock factor protein Hsf1p. In fact, both Skn7p and Hsf1p upregulate heat shock proteins by binding to heat shock elements (HSE) in H_2O_2-treated *S. cerevisiae* cells exposed to heat shock (Raitt *et al*., 2000).

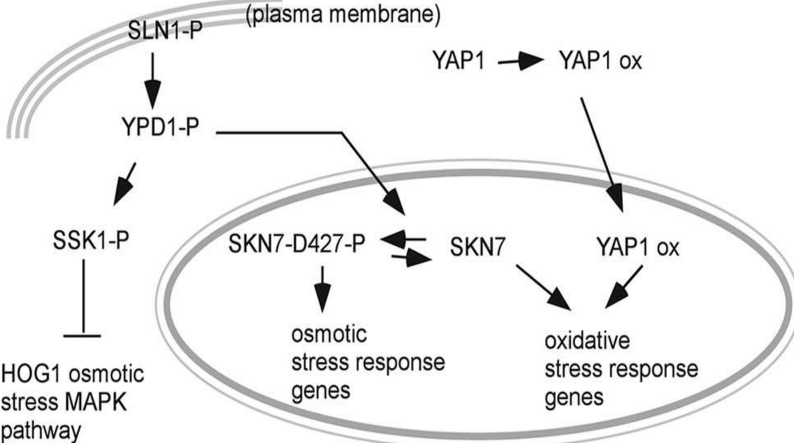

Figure 11 The role of Skn7. Skn7 transcription factor is involved in two different signal transduction pathways (He *et al*., 2009).

Msn2/Msn4p are considered important participants in heat shock tolerance (Figure 2), yet also play roles in resistance to oxidative stress in conjunction with Yap1p and Snk7p and upon osmotic stress (Görner *et al*., 1998; Boisnard *et al*., 2009). Msn2p and Msn4p are reversibly translocated into the nucleus and recruited by Trx1/2p thioredoxins (Boisnard *et al*., 2009) in response to desiccation-related stresses (oxidation, heat and hyperosmolarity). Msn2/4p can then activate about 30 proteins such as the catalase Ctt1p (Toledano *et al*., 2007; Izawa *et al*., 1996) by binding Msn2p and Msn4p to DNA at STRESS regions due to their Zn-type DNA-binding domains (Cys2-His2).

Additional levels of regulation, different sorts of effectors or intermediate shuffling between sensors and targets provide a very accurate response by yeast to oxidative and other stresses.

Antioxidant defences

In response to ROS, cells alter global transcription patterns of stress protective molecules such as metabolic enzymes (Gasch *et al*., 2000; Causton *et al*., 2001) and genes encoding antioxidants (Shenton *et al*., 2006), some of which are transcriptionally activated by Skn7p, Yap1p and other transcriptional regulators.

Antioxidant defences include a number of protective enzymes and non-enzymatic molecules that are present in different subcellular compartments and can be upregulated in response to ROS exposure (Kuge and Jones, 1994; Izawa *et al*., 1999). Enzymatic ROS detoxification includes two groups (Table 1, Figure 13). One group of enzymes act directly as ROS detoxifiers, while members of the second group consists of enzymes which act as redox regulators of protein thiols and contribute to maintaining the redox balance of the cells. However, both types of strategies overlap. Non-enzymatic defences typically consist of small molecules that can act as free radical scavengers. To date, only ascorbic acid and GSH have been extensively characterized in yeast.

Table 1 Protective enzymes that can be upregulated in response to ROS exposure (Morano *et al*., 2011)

Antioxidant	Gene	Location	Activity
The thioredoxin system			
Thioredoxin	*TRX1, TRX2*	Cyt	Disulfide oxidoreductase activity
	TRX3	Mit	Mitochondrial disulphide oxidoreductase activity
Thioredoxin reductase	*TRR1*	Cyt	Reduces oxidized thioredoxins (Trx1p, Trx2p)
	TRR2	Mit	Reduces oxidized thioredoxin (Trx3p)
Peroxiredoxin	*TSA1, TSA2*	Cyt	2-Cys Prx, thioredoxin peroxidase and chaperone activity
	AHP1	Cyt	2-Cys Prx, thioredoxin peroxidase particularly with alkyl hydroperoxides
	DOT5	Nuc	Nuclear 2-Cys Prx, functions in telomeric silencing
	PRX1	Mit	Mitochondrial 1-Cys Prx, thioredoxin peroxidase activity
The glutathione system			
GSH synthesis	*GSH1, GSH2*	Cyt	Catalyzes two ATP-dependent steps in GSH biosynthesis
Glutathione reductase	*GLR1*	Cyt/Mit	Recycles oxidized GSSG to reduced GSH, co-localizes to Cyt and Mit
Glutathione transferase	*GTT1*	ER	Catalyzes the conjugation of GSH to various electrophiles
	GTT2	Mit	
	GTO1	Per	Omega class glutathione transferase
	GTO2, GTO3	Cyt	Omega class glutathione transferase
Glutathione peroxidase	*GPX1, GPX2*	Cyt	Phospholipid hydroperoxide glutathione peroxidase
	GPX3	Cyt	Phospholipid hydroperoxide glutathione peroxidase, Yap1p signal transducer

continue

Antioxidant	Gene	Location	Activity
Glutaredoxin	*GRX1*	Cyt	Glutathione disulfide oxidoreductase activity
	GRX2	Cyt/Mit	Glutathione disulfide oxidoreductase activity
	GRX3, GRX4	Nuc	Monothiol glutaredoxin
	GRX5	Mit	Monothiol glutaredoxin, synthesis/assembly of iron-sulfur centers
	GRX6, GRX7	Gol	Cis-Golgi localized monothiol glutaredoxins
	GRX8	Cyt	Glutathione disulfide oxidoreductase activity
Superoxide dismutase	*SOD1*	Cyt/Nuc	Catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide
	SOD2	Mit	
Catalase	*CTT1*	Cyt	Catalyzes the reduction of hydrogen peroxide to water and oxygen
	CTA1	Per	
Methionine sulphoxide reductase	*MXR1*	Cyt	Catalyzes thiol-dependent reduction of methionine (S)-sulfoxide
	MXR2	Mit	Catalyzes thiol-dependent reduction of methionine (R)-sulfoxide
	FRMsr	Cyt	Catalyzes thiol-dependent reduction of free Met-R-SO
Erythroascorbate	*ALO1*	Mit	D-arabinono-1,4-lactone oxidase, final step of erythroascorbate synthesis

Although many studies have shown that tolerance to drying is correlated with an increase in the antioxidant potential in anhidrobiotes, regulation of the antioxidant defence system is complex and its role in desiccation tolerance has not yet been firmly established (Figure 13).

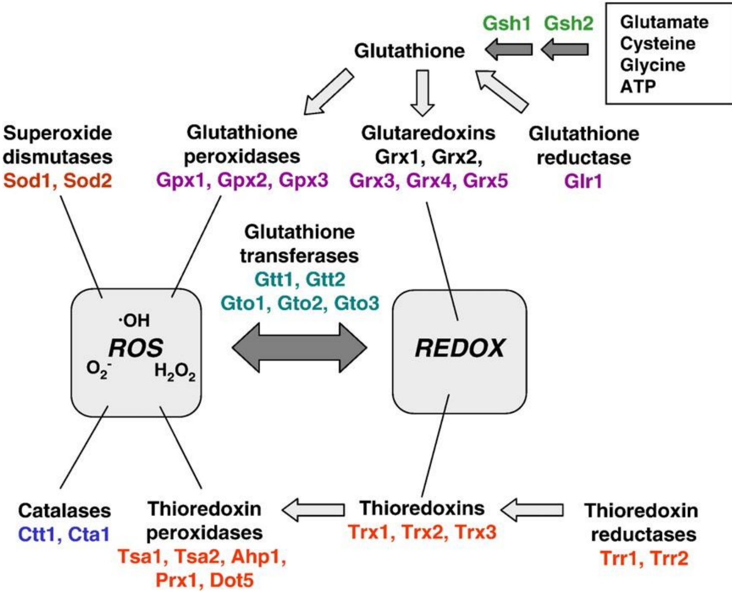

Figure 13 Enzymatic systems involved in ROS detoxification and in control of the redox state of protein sulphydryl groups in *S. cerevisiae*, with their interrelationship (Herrero *et al*., 2008).

The thioredoxin system includes thioredoxin, thioredoxin reductase and peroxiredosin (Prx) and can be found in cytoplasm, nucleus or mitochondria (Table 1). Two thioredoxins (*TRX1* and *TRX2*), a thioredoxin reductase (*TRR1*) and the peroxiredoxin *TSA1*, *TSA2* and *AHP1* are localized in cytoplasm (Gan, 1991). *TRX2* expression is strongly upregulated in response to oxidative stress conditions, whereas *TRX1* may serve an ancillary or back-up role when *TRX2* is insufficient to provide an antioxidant defence (Garrido and Grant, 2002). Oxidized thioredoxins are reduced by NADPH in conjunction with the cytoplasmic thioredoxin reductase *TRR1* forming the oxidized disulphide form (Figure 14).

A major part of the antioxidant function of thioredoxins is mediated by peroxiredoxins that have multiple roles in stress protection, acting as antioxidants, molecular chaperones, and in the regulation of signal transduction (Wood, *et al*., 2003). Prx have been divided into two classes, the 1-Cys and 2Cys Prx's, on the basis of the number of Cys residues directly involved in catalysis to reduce peroxides. The 1-Cys peroxiredoxin class includes the mitochondrial Prx1p, which is active as a peroxidase (Pedrajas *et al*., 2000), while 2-Cys group has three cytoplasmic and a nuclear peroxiredoxin. The three cytoplasmic 2-Cys Prx's are: Tsa1p, Tsa2p and Ahp1p (Figure 14). All of them play distinct physiological roles apart from the detoxification of hydroperoxides (Garrido and Grant, 2002; Wong *et al*., 2004; Jang *et al*., 2004; Lee *et al*., 1999a; Park *et al*., 2000). Tsa2p is highly homologous to Tsa1p and possesses similar peroxidase and chaperone activities, but it is expressed at significant lower levels than Tsa1p (Jang *et al*., 2004). Ahp1p is active as an antioxidant, but in contrast to Tsa1p, its catalytic efficiency is greater with alkyl hydroperoxides than with H_2O_2 (Lee *et al*., 1999a; Park *et al*., 2000). Finally, the nuclear 2-Cys Prx, Dot5p, is mostly active against alkyl hydroperoxides (Cha *et al*., 2003).

The thioredoxin reductase *TRR2* and the thioredoxin *TRX3* are localized in mitochondria besides *PRX1*. *TRR2* has an antioxidant role independently of mitochondria thioredoxin *TRX3* (Pedrajas *et al*., 2000; Trotter and Grant, 2005) which is reduced by *TRR2* and NADPH to regenerate (Figure 14). The redox states of the cytoplasmic and mitochondrial thioredoxin systems are independently maintained, and cells can survive in the absence of both systems (Trotter and Grant, 2005).

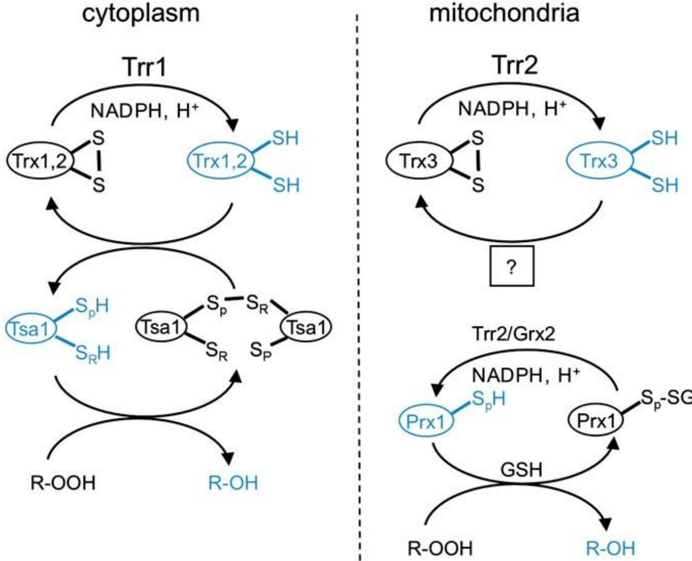

Figure 14 Comparison of cytoplasmic and mitochondrial thioredoxin systems. Reduced components are shown in blue. Among all three peroxiredoxin, only Tsa1p is shown for simplicity (Morano *et al*., 2011).

The glutathione system seems to be the most important in desiccation resistance among all antioxidants, with special emphasis on catalase, superoxide dismutase and GSH synthesis (Figure 13). The glutathione system comprises the following enzymes:

Catalase catalyzes the dismutation of H_2O_2 into H_2O and O_2 (Figure 9). Yeast has two such enzymes, Cta1p and Ctt1p. The first may function in the detoxification of H_2O_2 generated from fatty acid β-oxidation (Hiltunen *et al*., 2003), while Ctt1p is thought to play a more general role, since *CTT1* expression is induced by various stress conditions including heat, osmotic, starvation, and hydrogen peroxide stress (Martínez-Pastor *et al*., 1996). Focussing on dehydration, tolerance is dependent on catalase since strain deficient in cytosolic catalase showed a more oxidized intracellular environment and thus higher sensitivity to water loss (França *et al*., 2005a) suggesting that this enzyme plays a role in the maintenance of the intracellular redox balance during dehydration.

Superoxide dismutases (SODs) convert the superoxide anion to hydrogen peroxide, which can then be reduced to water by catalases or peroxidases (Figure 9). Yeast has two SODs which differ in location and the metal cofactor requirement for their activation, Cu, Zn-DOS (Sod1) and Mn-SOD (Sod2). The cytoplasmic Sod1 is also localized to the mitochondrial inner membrane space (Sturtz *et al*., 2001) and the mitochondrial matrix Mn-SOD (Sod2) is particularly required during stationary phase growth (Longo *et al*., 1996). However, the absence of only one of them does not impair desiccation tolerance (Pereira *et al*., 2003). Moreover, overexpression of *SOD1* in yeast

cells leads to a fivefold increase desiccation tolerance, whereas overexpression of both enzymes, *SOD1* and *SOD2*, increase eightfold (Pereira *et al*., 2001).

Methionine sulfoxide reductase (MSR) protects against methionine oxidation by ROS, catalysing thiol-dependent reduction of oxidized amino acid residues especially in methionine, avoiding the formation of Met-S-sulfoxide and Met-R-sulfoxide in cells (Dean *et al*., 1997; Stadtman *et al*., 2003). Yeast contains three MSR: *FRMsr* is thought to be the main enzyme responsible for the reduction of free Met-R-SO, whereas *MXR1* and *MXR2* are active with Met-S-SO and Met-R-SO (Le *et al*., 2009).

GSH synthesis takes place via two ATP-dependent steps (Figure 15). Gsh1p catalyzes the first step (Lisowsky *et al*., 1993) whereas the second is catalysed by glutathione synthetase Gsh2p (Grant *et al*., 1997). GSH is involved in a variety of cellular processes apart from ROS, including amino acid transport, synthesis of nucleic acids and proteins, modulation of enzyme activity, and the metabolism of carcinogens and xenobiotics (Schafer and Buettner, 2001). During dehydration, glutathione plays a key role in protecting cell membranes and in maintaining redox homeostasis under water deficiency, favouring tolerance to the dry state (Espindola *et al*., 2003). Glutathione is predominantly present in its reduced GSH form in yeast due to the constitutive action of glutathione reductase (Glr1p) which transfers electrons from NADPH to glutaredoxins (López-Barea *et al*., 1999). However, oxidative stress converts glutathione to its oxidized disulphide form (GSSG) (Figure 15). In addition, GSH can be oxidized in reactions catalysed by Grx1-8p and Gto1-3p.

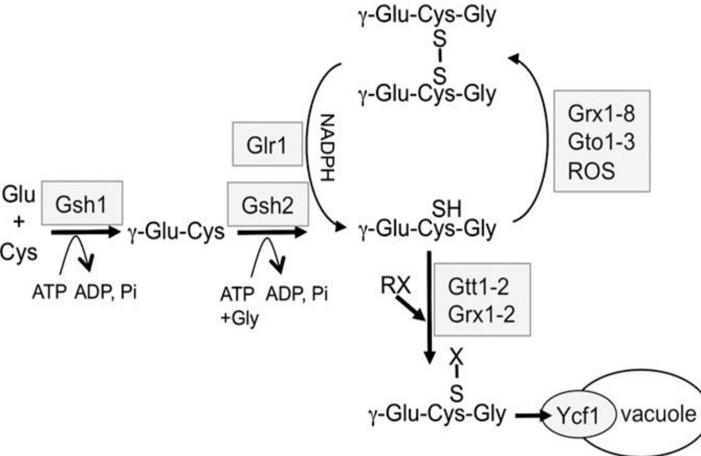

Figure 15 The glutathione system (Morano *et al*., 2011)

Glutaredoxins (Grx) are small heat-stable oxidoreductases. They have been proposed as having roles in protein folding and the regulation and reduction of dehydroascorbate (Holmgren, 1989) besides protection against ROS. Yeast contains eight

Grx (Grx1-8). Grx1p is induced by superoxide anion, whereas Grx2p is induced by hydrogen peroxide (Luikenhuis *et al*., 1998). Both are regulated in response to oxidative stress conditions via stress-responsive STRE elements, although the induction of *GRX2* is much more rapid and stronger than *GRX1* (Grant *et al*., 2000). Grx3-8p are found in different subcellular compartments and are important during the oxidative stress response (Rodríguez-Manzaneque *et al*., 1999; Molina *et al*., 2004; Izquierdo *et al*., 2008; Mesecke *et al*., 2008; Eckers *et al*., 2009; Mühlenhoff *et al*., 2010), except for Grx8p, which is not thought to function in the oxidative stress response (Eckers *et al*., 2009).

Glutathione transferases (GST) catalyze the conjugation of electrophilic substrates to GSH prior to their removal from cells via glutathione conjugate pumps (Figure 15). Yeast contains two classes of GSTs. On the one hand, *GTT1* and *GTT2* have an overlapping function with *GRX1* and *GRX2* under cell exposure to xenobiotics, heat and oxidants (Collinson *et al*., 2002; Collinson and Grant, 2003). On the other, *GTO1*, *GTO2* and *GTO3* are induced in response to oxidants such as hydroperoxides and thiol oxidants, under the control of Yap1p and STRE-responsive elements (Barreto *et al*., 2006; Garcerá *et al*., 2006).

Glutathione peroxidase (Gpx's) provides the major enzymatic defence against oxidative stress caused by hydroperoxides. Yeast expresses three Gpx's encoded by *GPX1-3* (Avery and Avery, 2001; Inoue *et al*., 1999) that are able to protect membrane lipids against peroxidation. Gpx3p has an additional function as a peroxide sensor and activator of Yap1p (Delaunay *et al*., 2002) (Figure 10).

Ascorbic acid is a non-enzymatic defence (Winkler *et al*., 1994). *ALO1* encodes D-arabinono-1,4-lactone oxidase, which catalyzes the final step in erythroascorbate biosynthesis and is induced by hydrogen peroxide and the superoxide anion (Huh *et al*., 1998). However, the extremely low levels of erythroascorbate detected in yeast make its functional role as an antioxidant questionable (Spickett *et al*., 2000). Moreover, supplementation of ascorbic acid during rehydration period of desiccation process does not enhance the yeast vitality, indicating that this antioxidant agent has no beneficial effects on some desiccated strains (Rodríguez-Porrata *et al*., 2008).

Translational regulation of gene expression and metabolic reconfiguration

Although some genes are translationally active, most mRNAs are inhibited in response to oxidative stress conditions, when the number of P bodies is significantly increased, suggesting their storage (Teixeira *et al*., 2005; Mazzoni *et al*., 2007). Indeed, under low adaptive concentrations of hydrogen peroxide, more than 60% of the proteins inhibit their synthesis in a reversible way within 15 min (Godon *et al*., 1998; Shenton and Grant, 2003; Shenton *et al*., 2006).

The inhibition is largely mediated by the Gcn2p protein kinase which can be activated by exposure to an organic hydroperoxide, a thiol oxidant and a heavy metal (cadmium) (Mascarenhas *et al*., 2008). Gcn2p phosphorylates the α-subunit of the translation initiation factor 2 (elF2p) (Shenton *et al*., 2006) resulting in a decrease in elF2Bp activity which leads to reduced ternary complex levels and concomitantly inhibits translation initiation (Pavitt *et al*., 1998) (Figure 12). The depletion of amino acids leads to an accumulation of uncharged tRNA which activates Gcn2p. Nevertheless, a significant number of genes that are translationally downregulated in response to high H_2O_2 concentrations are increased at the transcript level and viceversa (Shenton *et al*., 2006).

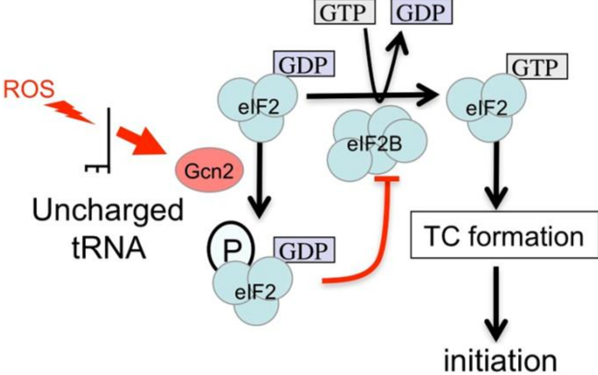

Figure 12 Control of translation initiation by Gcn2p (Morano *et al*., 2011).

Apart from mRNA and proteins, a number of metabolic genes are also up- or downregulated in response to low concentrations of H_2O_2, consistent with significant metabolic reconfiguration during oxidative stress conditions. In fact, metabolic changes are detected within seconds of an oxidative stress, before slower changes in gene expression are measured (Chechik *et al*., 2008; Ralser *et al*., 2009).

The key to these metabolic changes appears to be the reprogramming of the carbohydrate metabolism, and specifically the metabolic flux from glycolysis to the pentose phosphate pathway, with the concomitant generation of NADPH in response to oxidative stress

(Ralser *et al*., 2007). NADPH is particularly important since it provides the reducing potential for most antioxidant and redox regulatory enzymes that have been explained in the previous section. Additionally, glucose 6-phosphate dehydrogenase and 6-phophogluconate, which catalyse the first two steps of the pentose phosphate pathway, maintain their activities during oxidant exposure, confirming their role in the oxidative stress response (Izawa *et al*., 1998; Shenton and Grant, 2003). Furthermore, reduction in the activity of glycolytic enzymes such as triosephosphate isomerase and glyceraldehyde 3-phosphate dehydrogenase confers resistance against oxidative stress conditions (Ralser *et al*., 2007; Ralser *et al*., 2006). Modification of glycolytic enzymes is thought to be at post-transcriptional level and then causes rapid and reversible changes in enzyme activity, not only under oxidative stress but also during glycerol production in HOG pathway due to hyperosmolarity (Biswas *et al*., 2006).

Late Embryogenesis Abundant proteins

Stress response against desiccation includes gene regulation in heat shock, osmotic stress and oxidative stress as it was explained above. However, regulation of other important protective macromolecules also correlates with desiccation resistance. Among these, late embryogenesis abundant (LEA) proteins can act as an antioxidant molecules and their abundance can also increase tolerance against heat shock and osmotic individual stresses. Most LEA proteins are part of a more widespread group of proteins called hydrophilins. However, a distinguishing feature of the hydrophilins is a high glycine content and therefore not all LEA proteins are included in the hydrophilins. However, some non-LEA hydrophilins are essential for yeast desiccation tolerance (Dang and Hincha, 2011; López-Martínez *et al*., 2012) suggesting that a large number of molecules and molecular systems may be involved in such a complex stress besides LEA proteins. However, knowledge of hydrophilin proteins can lead to biotechnology engineering not only in microorganisms, but also in eukaryotic cells such as tissue preservation in drying state.

LEA proteins were first described about 30 years ago as accumulating in cotton seed development, when the embryo becomes desiccation-tolerant (Dure *et al*., 1981). They have been classified in different groups according to sequence motifs in plant LEA, but the groupings and nomenclature of the groups have not been consistent in the literature (Table 2). Although LEA have been discovered and classified in plants, closely related

proteins have also been found in bacteria (Dure, 2001; Stacy and Aalen, 1998; Battista *et al*., 2001), cyanobacteria (Close and Lammers, 1993), slime molds (Eichinger *et al*., 2005) and fungi (Abba *et al*., 2006; Katinka *et al*., 2001; Sales *et al*., 2000; Garay-Arroyo, 2000). In addition, more than 30 protein sequences for LEA and LEA-like proteins appear in animals such as nematodes (Browne *et al*., 2004; Browne *et al*., 2002; Solomon *et al*., 2000; Tyson *et al*., 2007; Gal *et al*., 2003; Haegeman *et al*., 2009; Gal *et al*., 2004), rotifers (Denekamp *et al*., 2010; Denekamp *et al*., 2009; Tunnacliffe *et al*., 2005) and tardigrades (Förster *et al*., 2009).

Table 2 Main classifications of LEA proteins with time-introduction of class nomenclature (Amara *et al*., 2014)

Pfam	**Dure *et al.* 1989**	**Bray 1993**	**Tunnacliffe and Wise 2007**	**Battaglia *et al.* 2008**	**Bies-Esthève *et al.* 2008**	**Hundermark and Hincha 2008**	**LEAPdb 2010**
PF00257	D11	Group 2	Group 2	Group 2	Group 2	Dehydrin	Classes 1 to 4
	D19	Group 1	Group 1	Group 1	Group 1	LEA_5	Classes 5
PF00477	D132						
	D7	Group 3	Group 3	Group 3A	Group 6	LEA_4	Classes 6
PF02987	D29	Group 5		Group 3B			
PF03168	D95			Group 5C	Group 7	LEA_2	Classes 7 and 8
PF03242	D73		LEA_5	Group 5B	Group 6	LEA_3	Classes 9
PF03760		Group 4	Group 4	Group 4A	Group 4	LEA_1	Classes 10
	D113			Group 4B			
PF04927	D34	Group 6	Group 6	Group 5A	Group 5	SMPO	Classes 11
				Group 6	Group 8	PvLEA18	Classes 12
PF03168				Group 5A			

While the nomenclature is unsatisfactory in plants, the nature and categorisation of LEA proteins became even more problematic when they were discovered in organisms outside the plant kingdom and were classified in the established plant groups. Nevertheless, here we will adopt Battaglia's classification to explain each group (Battaglia *et al*., 2008).

LEA Group 1 contains an internal 20-mer sequence present in several copies arranged in tandem, from one to four in plant species, and up to eight in other organisms (Hundertmark and Hincha, 2008; Galau *et al*., 1986; Dure, 1986; Goday *et al*., 1988;

Campos *et al*., 2013). Group 1 LEA proteins are mostly found in seeds, while they are not induced by stress conditions in vegetative tissues (Manfre *et al*., 2006; Manfre *et al*., 2009). Moreover, they have also been found in *Bacillus subtilis* (Borovskii *et al*., 2002). The second group of LEA proteins, also known as “dehydrins”, are a Lys-rich 15-residue motif. This motif is named the K-segment (Campbell and Close, 1997) which can be found in one to 11 copies within a single polypeptide. Additional motifs are also found, such as the Y-segment, which is usually found in one to 35 copies or Ser residues segment, the S-segment, acting as a site for protein phosphorylation (Campbell *et al*., 1998). This group of proteins are accumulated in response to low temperature and salinity, as well as water deficit and seed desiccation (Ismail, 1999; Nylander *et al*., 2001; Brini *et al*., 2007). Many accumulate in the cytoplasm, and some are also located in the nucleus in plants.

In contrast to group 1 and 2, LEA Group 3 are characterized by a motif of 11 amino acids repeated several times across the polypeptide (Dure, 1993). The variability in the 11-mer motif leads to a sub-classification of the group 3 LEA: proteins. The first subclass, 3A, have almost two of the motifs corresponding to the same 11-mer, whereas 3B is characterized by four variations of the 11-mer. Group 3 LEA proteins are localized in the cytoplasm and vacuoles, as is the case for HVA1 from barley, *Hordeum vulgare* (Marttila *et al*., 1996). The expression of group 3 LEA proteins in plants appears to be regulated by abscisic acid (ABA) during specific developmental stages (Curry *et al*., 1991; Ried and Walker-Simmons, 1993) and is one of the expression hallmarks for LEA proteins. Proteins similar to group 3 LEA are accumulated in several non-plant organisms in response to dehydration, in the fungi, microbial and animal kingdoms. They have been found in prokaryotes and in nematodes, where the expression is correlated with the survival of the organism under conditions of desiccation, osmosis, and heat stress (Battista *et al*., 2001; Dure, 2001; Gal *et al*., 2004). LEA Group 4 has the motif named 1 located in the N-terminal region (Battaglia *et al*., 2008). However, four additional motifs can be distinguished in many group 4 LEA proteins. The presence or absence of the motif defines two subgroups within the family: the first, 4A, consist of small proteins (80-124 residues long) and 4B has longer representatives (108-180 residues). Plants deficient in one, two or three members of group 4 LEA are more susceptible than wild-type plants to water deficit.

Unlike all LEA defined groups, LEA Group 5 contains a higher proportion of hydrophobic residues leading to an atypical LEA group. The smaller LEA proteins belong to LEA Group 6 (7-14KD) (Battaglia *et al*., 2008) and 36 genes of this family have been described from vascular plants to date. LEA Group 7 includes Abscisic acid-stress-and ripening induced proteins (ASR) which are small, heat-stable and unstructured (69, 70 de Amara 2014). However, no ASR-like genes are found in *Arabidopsis*, the LEA grouping plant model. This group shares physiochemical properties with other LEA proteins and they consequently accumulate in seeds during late embryogenesis and in response to water-limiting conditions (Silhavy *et al*., 1995).

Even though LEA proteins have been classified in different groups, they share common biochemical and structure characteristics, and similar functions, although very little is known about how they act. In the next section we will focus on these features, which in some cases will be related to LEA plant proteins because even though LEA proteins are spread widely across different kingdoms, LEA plants are the most extensively studied and knowledge of them could be useful in the investigation of LEA proteins' properties and functions in yeast.

The **physicochemical properties** of LEA proteins are: a hydrophilicity index greater than 1, a glycine content that differs from hydrophilin which is 6%, and a lack of or low proportion of Cys and Trp residues (Raghavendra *et al*., 2010; Baker *et al*., 1988; Garay-Arroyo, 2000; Dure, 1993; Oliveira *et al*., 2007b; Anchordoguy and Carpenter, 1996). The hydrophilicity is likely to be responsible for their lack of a conventional secondary structure in the fully hydrated state. As a result, LEA proteins are considered members of the broader classification of intrinsically disordered proteins (Tompa and Kovacs, 2010; Uversky and Dunker, 2010). This phenomenon was also observed in animals as well as plants (Goyal *et al*., 2003; Wolkers *et al*., 2001; Boudet *et al*., 2006; Mouillon *et al*., 2006; Tolleter *et al*., 2007). In the fully hydrated state, most LEA proteins are predominately unstructured with a preponderance of random coils, and become more ordered as dehydration proceeds, developing a secondary structure. Li and He (2009) utilized a 66-amino-acid fragment of AavLEA1, where they showed that the protein assumes a more folded conformation at different water contents from 83.5 to 2.4 wt% H_2O. This group concludes *in vitro* that at below 50.4 wt% H_2O the protein starts to fold, and at less than 20 wt% the protein begins to adopt a significant amount of the secondary structure (Figure 16). The random coil structure is predominantly found at a water content

of more than 20%, whereas α-Helical is mainly present in the dehydrated state. Consistent with the gain of secondary structure as LEA proteins are dehydrated, the numbers of intraprotein hydrogen bonds are projected to increase and the number of hydrogen bonds present between the protein and water are projected to markedly decrease as the water is removed.

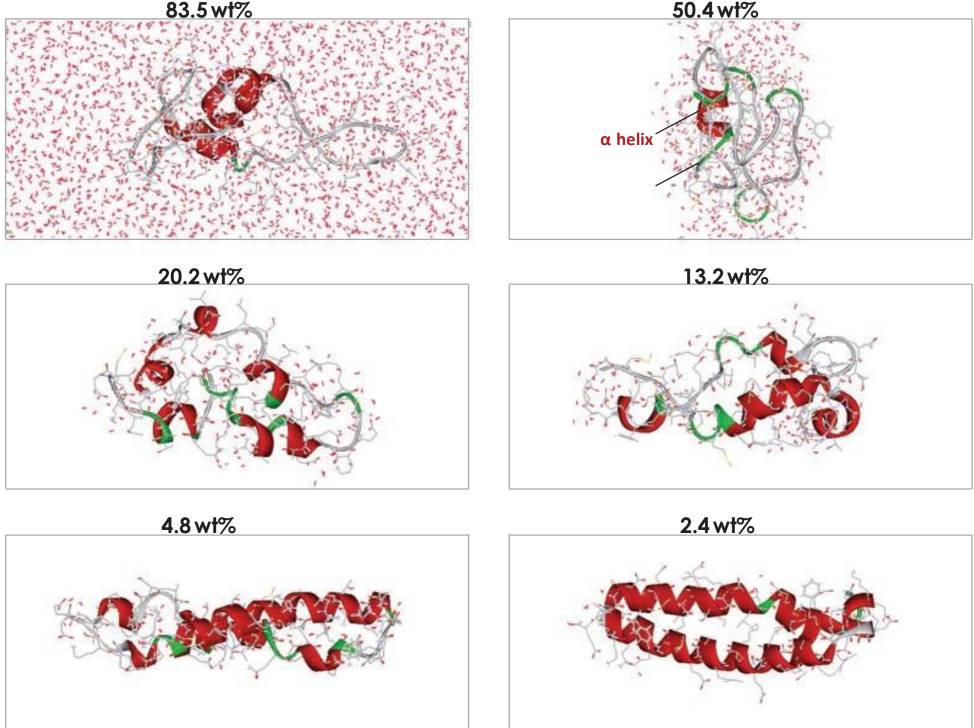

Figure 16 Representative conformations of the 66-amino-acid fragment of a LEA protein (AavLEA1) from the nematode *Aphelenchus avenae* are shown at different water contents. The smaller water molecules (*gray* and *red*) are depicted in the line style, and the larger LEA protein molecules are denoted using the solid ribbon style (αhelix, *red*; βsheet, *green*; random coil, *gray*). NN wt% means water content expressed in % (Hand *et al*., 2011).

Although LEA proteins have similar biochemical properties and structure, they vary in size, ranging from 5 to 77kDa for the same LEA protein in different organisms, and the net charge, which may be acid, neutral or basic. Moreover, subcellular localization also depends on each LEA protein. Indeed, they are expressed in a number of subcellular compartments depending on the cell organism to protect critical cellular components from desiccation-induced damage such as maintaining the integrity of mitochondria (Hand and Menze, 2008).

Functional role. Several possible activities of LEA proteins in the dry state have been described. These include RNA, DNA, water or ion binding, antioxidant activity,

stabilization of enzymes, proteins and membranes, molecular shield, chaperone role, hydration buffer and sugar glass stabilization. Although LEA proteins appear to carry out similar functions, the existence of distinctive groups is indicative of functional diversity. However, these functional roles are widespread among animals and microorganism anhydrobiots, with the most experimental evidence of their functional role in plants (Tunnacliffe and Wise, 2007; Wise, 2003; Wolkers *et al*., 2001; Oliver *et al*., 2001; Reyes *et al*., 2005; Goyal *et al*., 2005; Sales *et al*., 2000; Tunnacliffe *et al*., 2010).

Enzyme protection is one of the functions observed in various LEA proteins in plant groups 1, 2 and 3. They can preserve the activity of isolated enzymes such as citrate synthase and LDH after desiccation or freezing by preventing conformational enzyme changes that otherwise may lead to loss of their activity and under more severe water restriction conditions, to denaturation and consequently to protein aggregation (Figure 17) (Boucher *et al*., 2010; Chakrabortee *et al*., 2007; Goyal *et al*., 2005; Kovacs *et al*., 2008).

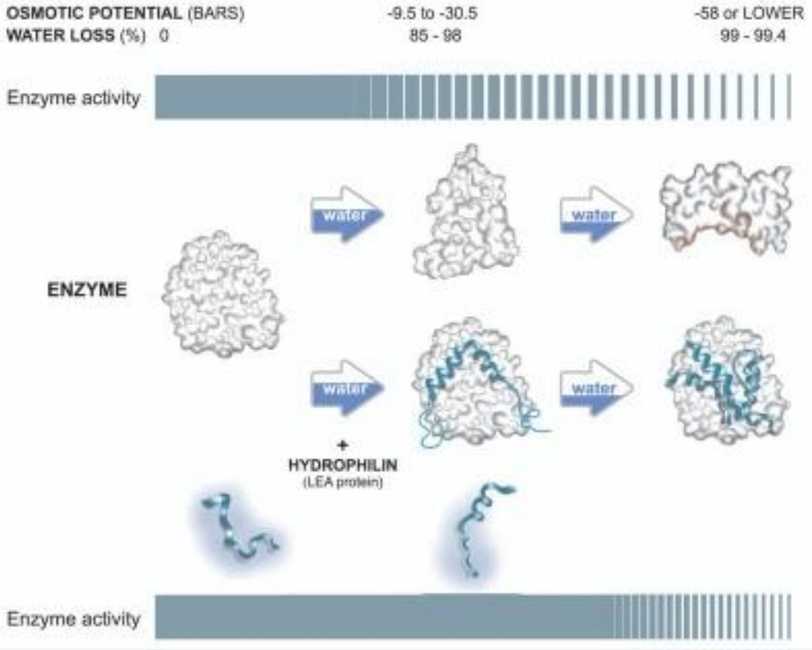

Figure 17 This scheme illustrates a hypothetic model for the function of LEA proteins and other hydrophilins. Hydrophobic residues are shown in red and LEA proteins in blue (Olvera-Carrillo *et al*., 2011).

The enzyme protection role can be achieved at a 1:1 hydrophilin:enzyme ratio under moderate water stress, suggesting that protein-protein interactions are necessary for their function to be accomplished; however, during severe dehydration the action of more than one hydrophilin per enzyme is needed (Reyes *et al*., 2005).

The enzyme protection activity of LEA proteins is also apparent in non-plant groups such as bacterial, yeast and nematode hydrophilin during desiccation (Goyal *et al*., 2003; Reyes *et al*., 2005). Moreover, this role of LEA protein could be linked to a molecular chaperone role without the ATP requirement. Furthermore, Tunnacliffe's group proposed that LEA proteins may exert a "molecular shield" activity (Tunnacliffe *et al*., 2005; Goyal *et al*., 2005) with a space-filling role, helping to prevent the collapse of the cell as its water is lost, thus decreasing the interaction between partially denatured polypeptides and preventing their aggregation.

Membrane protection is another role attributed to LEA proteins during drying. Since LEA proteins contain no transmembrane segments, they are unlikely to be able to integrate into membranes. However, LEA proteins that form amphipathic α-helices during drying may enable peripheral interaction with membranes, contributing to membrane protection. This membrane-stabilizing function has been shown *in vitro* for both rotifer and plant proteins (Popova *et al*., 2011; Thalhammer *et al*., 2010; Pouchkina-Stantcheva *et al*., 2007; Rahman *et al*., 2010; Tolleter *et al*., 2010), but it has been shown in few LEA proteins *in vivo* (Artus *et al*., 1996).

Ion binding is another LEA function, since they can sequestrate ions that concentrate in intracellular components as a result of dehydration damage (Danyluk *et al*., 1998; Dure, 1993). Group 2 and 3 LEA proteins are known to bind a number of metal ions that may otherwise increase ROS damage and liposome peroxidation. Moreover, LEA proteins are not only linked to antioxidant properties due to metal ion sequestration, but also due to scavenging for hydroxyl and peroxyl radicals that could also increase ROS species (Hara *et al*., 2004; Danyluk *et al*., 1998; Dure, 1993; Tunnacliffe and Wise, 2007; Iturbe-Ormaetxe *et al*., 1998).

Stabilization of sugar glasses was reported by Wolkers (Wolkers *et al*., 2001) in an *in vitro* assay, concluding that LEA plant proteins can stabilize vitrified sugar glasses (trehalose and sucrose), an important property for substantial dehydration tolerance (Crowe *et al*., 1998; Crowe *et al*., 1997). This effect is enhanced by the presence of non-disacharide molecules. The mixture increases the density of the sugar glasses by strengthening the hydrogen-bonding of the sugar/LEA mixture (Buitink and Leprince, 2004). As an example, the combination of trehalose and LEA-like peptides exhibits a 7ºC increase in T_g compared with mixtures using the control peptide (Timasheff, 2002).

Hydrogen buffer is the last suggested function for LEA proteins, whereby unstructured hydrophilic proteins bind greater numbers of water molecules in their hydration shells than a typical globular protein (Mouillon *et al*., 2006; Roberts *et al*., 1993; Garay-Arroyo, 2000; McCubbin *et al*., 1985). This suggests that LEA proteins might delay water loss during dehydration (Garay-Arroyo, 2000; McCubbin *et al*., 1985; Cuming, 1999) and could retain a protein function.

LEA across different domains is now an established fact, even though LEA proteins were first discovered in plants. The homology of some hydrophilin genes are found in animals and also microbes in the eubacterial and eukaryotic domains.

Plant LEA proteins are overexpressed during the last stage of seed formation, during periods of water shortage in vegetative organs and osmotic and/or low temperature stress (Bray, 1997; Bray, 1993; Bies-Ethève *et al*., 2008; Hundertmark and Hincha, 2008; Dure *et al*., 1989). Plant LEA mRNAs are maintained at high levels in the dehydrated mature embryos, while transcripts of storage protein genes are completely degraded during the last embryogenesis stage (Goldberg *et al*., 1989). Expression of LEA in plants is thus important in overcoming not only seed formation but also dehydration after the plant is adult. In the wine industry, LEA proteins in the berry are also important because water deficit stress causes a tissue-specific accumulation of enzymes that catalyse the formation of key flavour and aroma compounds in wine, including organic acids, specific sugars, phenylpropanoids, proanthocyanins and volatile compounds. LEA plants are mainly found in cytoplasm and nuclear regions (Zahn and Zhao, 2003); however, they are also located in the mitochondrion, chloroplast, endoplasmic reticulum, vacuole, peroxisome, and plasma membrane (Tunnacliffe and Wise, 2007).

Many transgenic plants expressing some LEA proteins enhance tolerance against environmental stress such as desiccation, hyperosmolarity or freezing. As an example, in group 3, *HVA1* confers water stress and salt stress in transgenic rice (Xu *et al*., 1996; Rohila *et al*., 2002; Chandra Babu *et al*., 2004). Another group 3 LEA protein from rapeseed was used to make transgenic lines of Chinese cabbage, resulting in improved salt and drought tolerance (Park *et al*., 2005). Although most of the evidence concerning the participation of LEA proteins in plants' tolerance to water deficit has been obtained by overexpression experiments, these data should be interpreted with caution, since ectopic expression may be misleading evidence for the function of the endogenous protein. Rather than enhancing tolerance stresses, some reports showed only a slight

effect or no effect on stress tolerance in transgenic plants expressing group 2 proteins. For example, the introduction of two dehydrins and a group 3 LEA protein from *Craterostigma plantagineum* did not improve the drought tolerance of transgenic tobacco (Iturriaga *et al*., 1992). Likewise, overexpression of *RAB18* in *Arabidopsis thaliana* did not improve freeze or drought tolerance (Lång, 1993).

Animal LEA protein cannot be related to seed maturation. However, on the basis of relatedness to plant sequences (Table 2), all non-plant LEA proteins have been categorised as group 3, with the single exception of LEA protein from *Bacillus subtilis*, which belongs to group 1.

In animals, a link to tolerance of water stress is maintained in invertebrates (McGee, 2006). As an example, a gene encoding group 3 LEA protein in the nematode *Aphelenchus avenae* is upregulated by desiccation and osmotic upshift, but not by cold, heat or oxidative independent stresses (Browne *et al*., 2004; Browne *et al*., 2002). Other examples are found in the model nematode *Caenorhabditis elegans* which increases desiccation resistance associated with the expression of an LEA-like protein. However, many other nematode species possess LEA proteins. Group 3 LEA proteins are also found in desiccation tolerant rotifers such as *Brachionus plicatilis* (Tunnacliffe *et al*., 2005; Denekamp *et al*., 2009). In the arthropod *Polypedilum vanderplanke* there are also several LEA proteins that are expressed during desiccation or osmotic stress (Kikawada *et al*., 2006). Finally, group 3 LEA proteins have also been described in the crustacean *Artemia franciscana* (Hand *et al*., 2007).

Experiments in human cells have also recently been carried out (Li *et al*., 2012). In these experiments, an LEA protein naturally expressed in embryos of the brine shrimp *A. franciscana*, AfrLEA3m, were transfected into human HepG2 cells and dried. Immediately after rehydration, control cells without the LEA protein exhibited 0% membrane integrity, compared with 94% in cells expressing AfrLEA3m (Li *et al*., 2012). Moreover, the *in vivo* coexpression of aggregation-prone proteins containing long polyglutamine or polyalamine sequences with a group 3 AavLEA1 LEA protein in mammalian cells showed a reduction in the expression of protein aggregates associated to neurodegenerative diseases (Chakrabortee *et al*., 2010). The mechanisms by which animals protect cellular structure and function in the state of anhydrobiosis, or "life without water" (Keilin, 1959; Crowe and Clegg, 1978; Crowe *et al*., 1997), are not only

of fundamental interest, but also of potential biomedical importance for cell stabilization (Crowe *et al*., 2005; Huang and Tunnacliffe, 2007; Hand and Hagedorn, 2008).

Microorganism LEA proteins appear to be homolog to those in plants (Garay-Arroyo, 2000; (Sales *et al*., 2000; Stacy and Aalen, 1998) and in animals, but with the characteristic that these organisms can easily be manipulated by genetic engineering due to their genetic properties. This advantage led us to discover the functional role and the mechanism in which both microscopic organisms and higher eukaryote cells may be involved.

Bacteria, and specifically the genome of *Escherichia coli*, contains five encoding proteins with the characteristics of hydrophilins (*YCIG*, *PRTL*, *YJBJ*, *YHDL*, *RMF*) (Garay-Arroyo, 2000), while *B. subtillis* contains two (*GSB* and *COTT*). All the *E. coli* hydrophilin transcripts mentioned above, except for *YHDL*, accumulated in response to osmotic stress, with the highest accumulation for the *YCIG* occurring after 20 minutes' treatment (Garay-Arroyo, 2000). Transgenic strains transfected with original plant LEA also demonstrate tolerance against environmental stresses in bacteria, such as recombinant *PM2* group 3 LEA proteins from soybeans (Liu and Zheng, 2005) and other LEA proteins from group 2, 3 and 4 from *A. thaliana* (Campos *et al*., 2006).

Yeast is a good eukaryotic model organism for testing the functional role of the artificial LEA group proteins in order to ascertain their role in higher eukaryotes. Hincha's group (Dang *et al*., 2014) tested 15 candidate genes from six *A. thaliana* LEA protein families (group 2, 3, 4 and 5) expressing them in *S. cerevisiae*. Of these selected LEA proteins, three of four dehydrin and all the groups 3 and 5 enhance tolerance to desiccation, but not to hyperosmotic or oxidative stress. The recombinant proteins showed enzyme but not membrane protection during drying. This experiment showed that even though the biochemical properties of these proteins are similar, not all of them play a role in desiccation tolerance in yeast. Twelve important hydrophilin have been discovered in *S. cerevisiae* genome. They have been also tested for possible functions. However, only 6 have been functionally characterized. Only some of the twelve selected genes containing the characteristics of hydrophilins are involved in desiccation tolerance, as explained below. Knowledge of these can be useful for the wine industries in order to enhance the desiccation tolerance of Active Dry Wine Yeast (ADWY) and save transport and storage costs, as well as medical engineering future investigations.

Yeast hydrophilin proteins

The twelve yeast hydrophlin proteins are small (from 79 to 225 amino acids) and by definition highly hydrophilic (hydrophatic index from 1.02 to 1.87) (Garay-Arroyo, 2000) (Table 3). However, they share no common sequence features. The charge distribution also varies widely, from uncharged to basic and acidic. Regarding subcellular localization, six out of twelve are localized in cytoplasm, even though some of them are also shown in other compartments (Table 3). Only Stf2p, Sip18p, Gre1p and Yjl144wp show exclusive cytoplasmic localization under no stress conditions. The nucleus is also well represented by Hsp12p, Wwm1p and Gon7p. Whereas the first two proteins are also localized in the cytoplasm and plasma membrane or mitochondrion, Gon7p is shown only in the nucleus. Ribosomal subunits and membrane localizations are the other compartments where yeast hydrophilin are present. While the first includes Tif11p, Nop6p and Rpl42ap; Hsp12p, Ybr016wp, Wwm1p and Ynl190wp belong to membrane localizations.

Table 3 *S. cerevisiae* hydrophilin proteins. Function and subcellular localization from yeastgenome.org

Hydrophilin protein	Function	Subcellular localization
Hsp12p	Lipid binding	Cytoplasm, nucleus, plasma membrane
Tif11p	Translational initiation factor activity	43S preinitiation complex
Nop6p	RNA binding	90S preribosome, nucleus
Gon7p	Chromatin DNA binding	Nucleus
Rpl42ap	Structural constituent of ribosome	60S ribosomal subunit
Stf2p	Unknown	Cytoplasm
Sip18p	Unknown	Cytoplasm
Ybr016wp	Unknown	Plama membrane
Wwm1p	Unknown	Nucleus, cytoplasm, mitochondrion
Gre1p	Unknown	Cytoplasm
Ynl190wp	Unknown	Cell wall
Yjl144wp	Unknown	Cytoplasm

Among the twelve hydrophilin yeast proteins, only Hsp12p, Tif11p, Nop6p, Gon7p and Rpl42ap have been functionally characterized, yet the other yeast hydrophilin proteins have specific and different roles from each other. The expression pattern of 8 of 12 *S. cerevisiae* hydrophilin transcripts showed an accumulation in response to osmotic stress: *GRE1, STF2, SIP18, YBR016W, HSP12, YJL144W, GON7* and *YNL190W* (Garay-

Arroyo, 2000). Moreover, *HSP12* and *GRE1* are induced by oxidative stress such as H_2O_2 as well as heat shock. Transcriptional gene screening in response to desiccation and rehydration also showed an upregulation of some hydrophilin genes, *GRE1* (7.6 fold), *SIP18* (9.3 fold) and *YJL144W* (3 fold) (Singh *et al*., 2005).

Under no circumstances can *S. cerevisiae* strains survive to desiccation stress if cells are not in a stationary state. At this point transcripts of *HSP12, STF2, SIP18, GRE1, YNL190W* and *YJL144W* are upregulated, showing the same pattern in osmotic stress. By contrast, *TIF11* and *RPL42A* are downregulated and *NOP6* and *YBR016* showed no significant up- or downregulation (Gasch *et al*., 2000).

Nevertheless, even though a transcription level of some hydrophilins in a stationary state and/or desiccation related stresses suggests the participation of some yeast hydrophilin in desiccation tolerance, it does not mean an appropriate accumulation of hydrophilin protein to overcome the stress, which could in fact be more closely linked to cell survival. Regarding this possibility, Soufi *et al*. (2009) characterized more than one hundred proteins that increase in abundance after osmotic stress. Of the 3,383 identified proteins, three hydrophilins were included in the first 25 changed proteins (Stf2p, Sip18p and Hsp12p), yet Sip18p showed the highest upregulation, with a more than sevenfold change.

Regardless of the transcriptional and translational level of some hydrophilins, only some of them are definitely involved in desiccation stress phenotype, although other proteins not belonging to the LEA proteins are also involved in desiccation stress to a certain extent . Focusing on this hypothesis, our group analyzed the viability of the complete collection of non-essential genes in *S. cerevisiae*, deleting each one upon dehydration and rehydration stress. A group of 102 deletion mutants with viability of less than 10% were detected, which is a very stringent cutoff. Most belong to the protein synthesis and biogenesis of cellular component functional classes (Rodríguez-Porrata *et al*., 2011). In this 102 protein group, only *SIP18* appear among all *S. cerevisiae* hydrophilins, while three more hydrophilin genes showed less than 20% viability (López-Martínez *et al*., 2012). In contrast to this pattern, 12 deletion mutants with viability values higher than those of the reference strain were also found, some of which are directly connected to the PCD (Rodríguez-Porrata *et al*., 2012a). These results may suggest that other proteins apart from hydrophilin may be involved in cell survival under desiccation stress.

Characterization of the rest of *S. cerevisiae* hydrophilin proteins, only null strains in *NOP6, STF2, YJL144W* and *YNL190W* hydrophilins decrease their mortality besides *SIP18* (López-Martínez *et al*., 2012), yet *YNL190W* showed no variation in desiccation tolerance in yeast hydrophilin screening by the Hincha group (Dang and Hincha, 2011). Furthermore, overexpression of all of them except *YNL190W* significantly increases desiccation tolerance (López-Martínez *et al*., 2012; Dang and Hincha, 2011). Moreover, overexpression of *GRE1* also increases the viability, even though its knock-out strain showed no difference in desiccation tolerance compared to the reference strain (López-Martínez *et al*., 2012; Dang and Hincha, 2011). Since the proteins with a positive effect on desiccation are predicted to be unstructured and since they share no sequence similarity, it is unfortunately not possible to identify any sequence or structural features that may be related to their ability to stabilize cells during desiccation. Thus, enhancement of the desiccation tolerance is not due to the biochemical properties of hydrophilin proteins. On the other hand, the unchanged desiccation tolerance of the other hydrophilin proteins overexpressing and/or knockout strains (Hsp12p, Tif11p, Gon7p, Rpl12ap, Ybr016wp, Wwm1p and Ynl190wp) (López-Martínez *et al*., 2012; Dang and Hincha, 2011b) may either indicate that the respective hydrophilins play no functional role in cellular desiccation tolerance or that their absence can be compensated by other proteins or even that they indirectly regulate other molecules involved in desiccation tolerance.

We can divide *S. cerevisiae* hydrophilin proteins into two groups since not all *S. cerevisiae* hydrophilin-like proteins could thus enhance desiccation tolerance besides the conclusion that other proteins non-hydrophilin proteins could enhance cell survival against desiccation. The first group includes *NOP6*, *STF2*, *SIP18*, *GRE1* and *YJL144W* whose overexpression increases the viability after desiccation stress by up to 30 to 60% compared to the reference strain (López-Martínez *et al*., 2012). Second, we classified the rest other hydrophilins with no effect on desiccation tolerance: *HSP12*, *TIF11*, *GON7*, *RPL42A*, *YBR016W*, *WWM1* and *YNL190W*. We will therefore deal specifically with the first group below.

Of the proteins belong to the firt group, *GRE1* is the only that has a *SIP18* paralog but maintaining the same role in desiccation. The cytoplasmic gene *GRE1* is regulated by the HOG pathway and it is regulated negatively by the cAMP-PKA transduction pathway and positively by the transcriptional factors Msn2p and Msn4p (Garay-Arroyo and Covarrubias, 1999).

NOP6 is localized on chromosome IV and is the longest *S. cerevisiae* hydrophilin protein (225 amino acids). In addition, it is highly basic (p*I* >10.0) since it is rich in Lys and Arg. *NOP6* is a transactivating factor involved in 40S ribosomal subunit biogenesis, and has been predicted to function in rRNA processing due to its RNA recognition motif (Samanta and Liang, 2003; Sigrist *et al*., 2010). Indeed *NOP6* interacts with 90S pre-ribosomal particle, and specifically 35S and 32S.

YJL144W is another hydrophilin gene with overexpression increasing desiccation tolerance and also freezing tolerance. Localized on chromosome X, its transcriptionally upregulation during desiccation (3 fold) (Singh *et al*., 2005), also correlates with positive dehydration tolerance phenotype (López-Martínez *et al*., 2012; Dang and Hincha, 2011). Moreover *YJL144W* is also upregulated more than tenfold in five different starvation treatments (glucose, ethanol, ammonium, phosphate and sulphate limiting nutrient) (Wu *et al*., 2004).

Of the *STF2, SIP18, GRE1, NOP6* and *YJL144W* overexpressing strains, accumulation of Sip18p showed the highest increase in viability after rehydration in comparison to its knock out strain (80%) (López-Martínez *et al*., 2012). Even if the abundance of these five proteins increase desiccation tolerance, only *STF2* and *SIP18* contribute to reducing the cell damage done by ROS in oxidative stress imposition (H_2O_2) and during desiccation, exhibiting antioxidant properties (López-Martínez *et al*., 2012). On the other hand, *GRE1, NOP6* and *YJL144W* overexpression do not decrease ROS damage during desiccation, even though *GRE1* is induced transcriptionally by oxidative stress and desiccation (Singh *et al*., 2005).

Overexpression of *SIP18* or *STF2* could lead to a reduction of apoptotic or necrotic cells since its abundance decrease ROS counting cells. We showed that while *SIP18* reduces the secondary necrotic cells (Rodríguez-Porrata *et al*., 2012a), *STF2* reduces the number of apoptotic cells during stress induction (López-Martínez *et al*., 2012) as a putative consequence of the reduction of ROS accumulation. The cytoplasmic gene *STF2*, localized on chromosome VII, has no functional role conferred as yet. Stf2p is the major modulator of Inh1p, which acts as the inhibitor of the hydrolytic activity of the F1F0-ATP synthase, preventing the hydrolysis of ATP to ADP and P_i. During oxygen deprivation or cytosol acidification, the Inh1 binds directly to the F1 sector promoting the inhibition of ATP hydrolysis and thereby preserving cellular ATP. This mechanism is carried out thanks to the modulation of Inh1p, but also Stf1p by Stf2p which binds to the

F0 sector of mitochondrial FIF0 ATPase (Dienhart *et al*., 2002). Nevertheless, we found that viability values in the overexpressing strain were not a consequence of ATP levels (López-Martínez *et al*., 2012), which does not correlate with the literature.

SIP18 not only enhances desiccation tolerance but also freezing stress. It may be part of an osmotic stress response locus on chromosome XIII, with regulation occurring through the HOG signaling cascade (Miralles and Serrano, 1995) as well as through the cyclin-dependent kinase Ssn3p (Holstege *et al*., 1998). Indeed osmotic study based on proteomics found Sip18p to have the highest upregulation value among whole proteome, showing more than a sevenfold change (Soufi *et al*., 2009). Analysis of Sip18p demonstrated that the carboxy-terminal lysisne residues of the protein are essential for binding to phospholipids *in vitro* (Scheglmann *et al*., 2002), which are involved in transcriptional regulation. The participation of Sip18p in desiccation tolerance is also corroborated by the upregulation of the transcript during the stress imposition (9.3 fold) (Singh *et al*., 2005). As well as *YJL144W*, *SIP18* is also upregulated more than tenfold in five different starvation treatments (glucose, ethanol, ammonium, phosphate and sulphate limiting nutrient) (Wu *et al*., 2004). Although Sip18p is localized in cytoplasm, it appears in the nucleus under osmotic and dehydration stress. Taking into account the specific cytolocalization of this protein and the reduction of necrotic cells during stress imposition acting as an antioxidant molecule, we can hypothesize that Sip18p may be involved in preventing cell death-regulating factors (Rodríguez-Porrata *et al*., 2012a). The enhancement of desiccation tolerance due to the overexpression of *SIP18* is apparent not only in haploid and diploid laboratory strains (López-Martínez *et al*., 2012; Dang and Hincha, 2011b), but also in commercial and wild wine strains used in alcoholic fermentation products with the same viability pattern under desiccation stress, without affecting the fermentative parameters (López-Martínez *et al*., 2012).

Hydrophilin research in different organisms has enabled us to make significant advances towards understanding some of their biological properties, including their roles as antioxidants and as membrane and protein stabilizers during water stress, either by direct interaction or by acting as a molecular shield among other roles. However, further studies will be necessary to completely understand their function. In addition, the knowledge of the cellular mechanisms involved in water deficit periods is of particular interest for the development of long-term preservation strategies for functional biomolecules, cells, organisms, tissues, and organs. We can assume that not all LEA proteins are necessarily

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Dipòsit Legal: T 1354-2015

involved in desiccation tolerance due to their biochemical properties because it is an artificial group and not a functional group. Furthermore, other non-LEA related proteins could also participate independently of hydrophilin proteins, and some of them might be activated under one of the related desiccation stresses or PCD. Otherwise, hydrophilin may act as a target gene of these, or have physical roles instead of functional ones, or a mix of both.

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UNIVERSITAT ROVIRA I VIRGILI
FUNCTIONAL CHARACTERIZATION IN VIVO OF ESSENTIAL SACCHAROMYCES CEREVISIAE'S HYDROPHILIN FOR DESICCATION TOLERANCE
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CHAPTER II

The STF2p Hydrophilin from *Saccharomyces cerevisiae* Is Required for Dehydration Stress Tolerance

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PLoS one 7 (2012): e33324

Abstract

The yeast *Saccharomyces cerevisiae* is able to overcome cell dehydration; cell metabolic activity is arrested during this period but restarts after rehydration. The yeast genes encoding hydrophilin proteins were characterised to determine their roles in the dehydration-resistant phenotype, and Stf2p was found to be a hydrophilin that is essential for survival after the desiccation-rehydration process. Deletion of *STF2* promotes the production of reactive oxygen species and apoptotic cell death during stress conditions, whereas the overexpression of *STF2*, whose gene product localises to the cytoplasm, results in a reduction in ROS production upon oxidative stress as the result of the antioxidant capacity of the Stf2p protein.

Introduction

The kingdoms of bacteria, fungi and plants contain anhydrobiotic organisms that can survive during water-deficient periods (Reyes *et al.*, 2005). During dehydration, the metabolic processes of these organisms are in a suspended state. However, during desiccation stress, genes are expressed that promote cellular tolerance to dehydration through protective functions in the cytoplasm, an alteration in the cellular water potential to promote water uptake, the control of ion accumulation, and the further regulation of gene expression (Bray, 1993). Some of the proteins termed hydrophilins participate in the cellular tolerance to this stress condition and are biochemically characterised by a Gly content greater than 6%, a hydrophilicity index of .1.0, and a secondary structure of 50–80% coils. The genome of *S. cerevisiae* contains genes (*GON7, GRE1, HSP12, NOP6, RPL42A, STF2, SIP18, TIF11, WWM1, YBR016w, YJL144w*, and *YNL190w*) that code for proteins with the characteristics of hydrophilins. The fact that the transcripts of all of these genes accumulate in response to osmotic stress suggests that the expression of hydrophilins represents a widespread adaptation to water deficit (Garay-Arroyo *et al.*, 2000). The properties of hydrophilins include their roles as antioxidants and as membrane and protein stabilisers during water stress, either by direct interaction or by acting as molecular shields (Tunnacliffe and Wise, 2007). Although the functional role of most *S. cerevisiae* hydrophilins remains speculative, there is evidence supporting their participation in the acclimation or adaptive response to stress (Battaglia *et al.*, 2008). The ectopic expression of some hydrophilins in yeast confers tolerance to water-deficit

conditions (Imai *et al.*, 1996; Swire-Clark and Marcotte, 1999; Zhang *et al.*, 2000), and the presence of these proteins has been associated with chilling tolerance (Ismail *et al.*, 1999). In this study, we evaluated the role of the aforementioned *S. cerevisiae* hydrophilins in dehydration stress. Five strains overexpressing *SIP18, STF2, GRE1, YJL144w* or *NOP6* were identified as dehydration tolerant. For *STF2*, we found that the cell viability after desiccation and rehydration process was due to the antioxidant capacity of this protein, which reduced the number of apoptotic cells during stress conditions by minimising the accumulation of ROS in the cells.

Materials and Methods

Strains and plasmids

Table 1 summarises the yeast strains and plasmids used in this study. The single null mutant strains and the reference strain, all in the BY4742 genetic background, were purchased from EUROSCARF (Frankfurt, Germany). The yeast strain expressing the *GFP-STF2* chromosomal fusion was purchased from Invitrogen. Recombinant DNA techniques were performed according to standard protocols (Sambrook and Russell, 2001). The synthetic genes (*GON7*, *GRE1*, *HSP12*, *INH1*, *NOP6*, *RPL42A*, *SIP18*, *STF2*, *WWM1*, *YBR016w*, and *YNL190w*) were obtained by PCR and cloned into the pGREG505 yeast expression vector (under the control of the *GAL1* promoter) digested with *Sal*I. The plasmids were then used to transform a yeast strain in which the corresponding gene had been deleted. The pGREG575 vector was used to express GFP-tagged proteins. Transformants were selected by plating on synthetic glucose media lacking leucine. Leu^+ transformants were selected and re-streaked to obtain single colonies, which were confirmed by PCR using the primer pair GALFw and CYCRv (Table 2) and by testing for the loss of the *LEU* marker. The PCR fragments were obtained using BY4742 genomic DNA as a template together with the primer pairs shown in Table 2. The amplification reactions contained single-strength PCR buffer (Roche, Mannheim, Germany), 1.25 mM dNTPs, 1.0 mM $MgCl_2$, 0.3 μM of each primer, 2 ng μl^{-1} template DNA and 3.5 U DNA polymerase (Roche) in a total volume of 100 μl. All of the reactions were carried out using a PCR Express thermal cycler for 15 cycles, as follows: denaturation, 2 min at 94°C; primer annealing, 30 s at 55°C; and primer extension, 1 min at 68°C.

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Table1 Plasmids and yeast strains used in this study

Strains	Relevant characteristics	References
BY4742	MATα, *his3*Δ1, *leu2*Δ0, *lys2*Δ0, *ura3*Δ0	Brachmann *et al.*,1998
STF2-GFP	MATα, *his3*Δ1, leu2Δ0, *lys2*Δ0, *ura3*Δ0, *STF2-GFP-KanMX*	Invitrogene
Δ*stf2*	BY4742, *stf2::kanMX4*	EUROSCARF
Δ*sip18*	BY4742, *sip18::kanMX4*	EUROSCARF
Δ*hsp12*	BY4742, *hsp12::kanMX4*	EUROSCARF
Δ*YBR016w*	BY4742, *YBR016w::kanMX4*	EUROSCARF
Δ*wwm1*	BY4742, *wwm1::kanMX4*	EUROSCARF
Δ*gre1*	BY4742, *gre1::kanMX4*	EUROSCARF
Δ*YJL144w*	BY4742, *YJL144w::kanMX4*	EUROSCARF
Δ*nop6*	BY4742, *nop6::kanMX4*	EUROSCARF
Δ*gon7*	BY4742, *gon7::kanMX4*	EUROSCARF
Δ*YNL190w*	BY4742, *YNL190w::kanMX4*	EUROSCARF
Δ*inh1*	BY4742, *inh1::kanMX4*	EUROSCARF
BY4742, GAL_p	BY4742+pGREG505Δh	This work
BY4742, GAL_pG	BY4742+pGREG575Δh	This work
Δ*stf2*, GAL_p-*STF2*	Δ*stf2*+pGREG505st	This work
Δ*stf2*, GAL_pG-*STF2*	Δ*stf2*+pGREG575gst	This work
Δ*sip18*, GAL_p-*SIP18*	Δ*sip18*+pGREG505si	This work
Δ*hsp12*, GAL_p-*HSP12*	Δ*hsp12*+pGREG505hs	This work
Δ*YBR016w*, GAL_p-*YBR016w*	Δ*rif2*+pGREG505yb	This work
Δ*wwm1*, GAL_p-*WWM1*	Δ*wwm1*+pGREG505ww	This work
BY4742, GAL_p-*TIF11*	BY4742+pGREG505tf	This work
Δ*gre1*, GAL_p-*GRE1*	Δ*gre1*+pGREG505gr	This work
Δ*YJL144w*, GAL_p-*YJL144w*	Δ*gre1*+pGREG505yj	This work
Δ*nop6*, GAL_p-*NOP6*	Δ*nop6*+pGREG505np	This work
Δ*gon7*, GAL_p-*GON7*	Δ*gon7*+pGREG505gn	This work
Δ*gon7*, GAL_p-*YNL190*	Δ*gon7*+pGREG505yl	This work
BY4742, GAL_p-*RPL42A*	BY4742+pGREG505rp	This work
Δ*inh1*, GAL_p-*INH1*	Δ*inh1*+pGREG505ih	This work
Plasmids		
pGREG505Δh	$GAL1_p$-*SalI*-$CYC1_t$-*KanMX4-LEU2-bla*	Jansen *et al.*, 2005
pGREG575Δh	$GAL1_p$-*GFP-SalI*-$CYC1_t$-*KanMX4-LEU2-bla*	Jansen *et al.*, 2005
pGREG505st	$GAL1_p$-*STF2*-$CYC1_t$-*KanMX4-LEU2-bla*	This work
pGREG575gst	$GAL1_p$-*GFP-STF2*-$CYC1_t$-*KanMX4-LEU2-bla*	This work
pGREG505si	$GAL1_p$-*SIP18*-$CYC1_t$-*KanMX4-LEU2-bla*	Jansen *et al.*, 2005
pGREG505hs	$GAL1_p$-*HSP12*-$CYC1_t$-*KanMX4-LEU2-bla*	This work
pGREG505yb	$GAL1_p$-*YBR016*-$CYC1_t$-*KanMX4-LEU2-bla*	This work
pGREG505ww	$GAL1_p$-*WWM1*-$CYC1_t$-*KanMX4-LEU2-bla*	This work
pGREG505tf	$GAL1_p$-*TIF11*-$CYC1_t$-*KanMX4-LEU2-bla*	This work
pGREG505gr	$GAL1_p$-*GRE1*-$CYC1_t$-*KanMX4-LEU2-bla*	This work
pGREG505yj	$GAL1_p$-*YJL144w*-$CYC1_t$-*KanMX4-LEU2-bla*	This work
pGREG505np	$GAL1_p$-*NOP6*-$CYC1_t$-*KanMX4-LEU2-bla*	This work
pGREG505gn	$GAL1_p$-*GON7*-$CYC1_t$-*KanMX4-LEU2-bla*	This work
pGREG505yl	$GAL1_p$-*YNL190*-$CYC1_t$-*KanMX4-LEU2-bla*	This work
pGREG505rp	$GAL1_p$-*RPL42A*-$CYC1_t$-*KanMX4-LEU2-bla*	This work
pGREG505ih	$GAL1_p$-*INH1*-$CYC1_t$-*KanMX4-LEU2-bla*	This work

Table 2 Primers designed for PCR

Primer	Oligonucleotide sequence[a]
STF2Fw	5′-**GAATTCGATATCAAGCTTATCGATACCGTCGACA**TGACGAGAACAAACAAG-3′
STF2Rv	5′-**GCGTGACATAACTAATTACATGACTCGAGGTCGAC**TCATTCCTTTTGGACGT-3′
HSP12Fw	5′-**GAATTCGATATCAAGCTTATCGATACCGTCGACA**TGTCTGACGCAGGTAG-3′
HSP12Rv	5′-**GCGTGACATAACTAATTACATGACTCGAGGTCGAC**TTACTTCTTGGTTGGGTC-3′
YBRFw	5′-**GAATTCGATATCAAGCTTATCGATACCGTCGACA**TGTCTGCTAACGATTAC-3′
YBRRv	5′-**GCGTGACATAACTAATTACATGACTCGAGGTCGAC**TTAGAATAGCATATCCATG-3′
WWM1Fw	5′-**GAATTCGATATCAAGCTTATCGATACCGTCGACA**TGGCTCAAAGTAAAAGTAAT-3′
WWM1Rv	5′-**GCGTGACATAACTAATTACATGACTCGAGGTCGAC**CCATGGATATGCTATTCTAA-3′
TIF11Fw	5′-**GAATTCGATATCAAGCTTATCGATACCGTCGACA**TGGGTAAGAAAAACAC-3′
TIF11Rv	5′-**GCGTGACATAACTAATTACATGACTCGAGGTCGAC**TTAAATGTCATCAATATC-3′
GRE1Fw	5′-**GAATTCGATATCAAGCTTATCGATACCGTCGACA**TGTCCAATCTATTAAACAAG-3′
GRE1Rv	5′-**GCGTGACATAACTAATTACATGACTCGAGGTCGAC**CTACCAGACGCCTTG-3′
YJL144wFw	5′-**GAATTCGATATCAAGCTTATCGATACCGTCGACA**TGTTAAGGAGGGAAACTT-3′
YJL144wRv	5′-**GCGTGACATAACTAATTACATGACTCGAGGTCGAC**TTATCATGAACAACGGCGAG-3′
NOP6Fw	5′-**GAATTCGATATCAAGCTTATCGATACCGTCGACA**TGGGGTCCGAGGAAG-3′
NOP6Rv	5′-**GCGTGACATAACTAATTACATGACTCGAGGTCGAC**TCATTTAAGTAGTTTGGCT-3′
GON7Fw	5′-**GAATTCGATATCAAGCTTATCGATACCGTCGACA**TGAAACTACCGGTAGC-3′
GON7Rv	5′-**GCGTGACATAACTAATTACATGACTCGAGGTCGAC**TAAACAGCATCTTCGTC-3′C
YNL190wFw	5′-**GAATTCGATATCAAGCTTATCGATACCGTCGACA**TGAAGTTCTCTTCTGTTA-3′C
YNL190wRv	5′-**GCGTGACATAACTAATTACATGACTCGAGGTCGAC**TTATAATAGTAATAAGGCACC-3′
RPL42Fw	5′-**GAATTCGATATCAAGCTTATCGATACCGTCGACA**TGGGTATGTTATAACC-3′
RPL42wRv	5′-**GCGTGACATAACTAATTACATGACTCGAGGTCGAC**TCAGAATTGCAAAGCTTGAC-3′
INH1Fw	5′-**GAATTCGATATCAAGCTTATCGATACCGTCGACA**TGTTACCACGTTCAGC-3′
INH1Rv	5′-**GCGTGACATAACTAATTACATGACTCGAGGTCGAC**TTATTTGGTCATCGAGTC-3′
GALFw	5′-GAAAAAACCCCGGATTCTAG-3′
CYCRv	5′-ATAACTAATTACATGACTCGAG-3′

Growth conditions and desiccation-rehydration process

Yeast strains were grown in shake flasks at 150 rpm in SC medium containing 0.17% yeast nitrogen base (Difco), 2% glucose, 0.5% $(NH_4)_2SO_4$, 25 $mg \cdot l^{-1}$ uracil, and 42 $mg \cdot l^{-1}$ lysine and histidine. The desiccation-rehydration process and yeast viability determinations were performed as described previously (Rodríguez-Porrata *et al.*, 2011).

Tests for apoptotic markers

The DHE staining, Annexin V/PI co-staining and TUNEL staining were performed as described in Buttner *et al.* (2007). The same samples were analysed by fluorescence microscopy. To determine the frequencies of the morphological phenotypes revealed by the TUNEL, DHE and Annexin V/PI staining, at least 10^6 cells from three independent experiments were evaluated using flow cytometry and FloMax software (Partec GmbH, Germany).

Microscopy

Cultures of strains harbouring the *GFP*-tagged genes were grown to the stationary phase in SC medium. The cells were washed with 1 x PBS buffer (pH 7.4) and fixed with 70% ethanol for 10 min at R.T. Fluorescence was viewed using a Leica fluorescence

UNIVERSITAT ROVIRA I VIRGILI
FUNCTIONAL CHARACTERIZATION IN VIVO OF ESSENTIAL SACCHAROMYCES CEREVISIAE'S HYDROPHILIN FOR DESICCATION TOLERANCE
Gema Isabel López Martínez
Dipòsit Legal: T 1354-2015

microscope (DM4000B, Germany). A digital camera (Leica DFC300FX) and the Leica IM50 software were used for the image acquisition. Confocal images were obtained using a laser microscope (Nikon TE2000-E) equipped with a digital camera (Nikon DXM1200C), and overlaid images using NIS-Elements software (Nikon).

Determination of ATP and pyruvate concentrations

The cellular pyruvate concentration was determined using the Pyruvate Assay Kit (BioVision Research Products, USA), and the ATP content was assessed with the ATP Bioluminescence Assay Kit HS II (Roche Applied Science, Germany). The quantification was carried out using a POLARstar Omega microplate reader equipped with two reagent injectors (BMG LABTECH, USA).

Assessment of mitochondrial changes

The changes in mitochondrial mass and $\Delta\Psi_m$ were assessed using JC-1 (Molecular Probes Inc.) as previously described (Pina-Vaz *et al.*, 2001). JC-1 allows the simultaneous quantification by flow cytometry of both the mitochondrial mass (green fluorescence) and $\Delta\Psi_m$ (red fluorescence). We defined the relative mitochondrial function (RMF) as the ratio of JC-1 red:green, which reflects the changes in $\Delta\Psi_m$ per unit mitochondrial mass.

Statistical analysis

The results were statistically analysed by one-way ANOVA and the Scheffé test using the SPSS 15.1 statistical software package. The statistical significance was set at $p \leq 0.05$ and $p \leq 0.01$.

Results

Hydrophilins from S. cerevisiae enhance dry stress tolerance

Among the 12 proteins of the hydrophilin group found in *S. cerevisiae*, Tif11p and Rpl42Ap are encoded by essential genes. Therefore, the desiccation tolerance capacity of a set of 10 viable mutant haploid strains (BY4742) for the genes encoding these hydrophilins was assessed using a colony-counting assay. The mean CFU (colony-forming units) ml^{-1} value for survival after rehydration was calculated after taking into account the viability before drying. Only the Δ*stf2* and Δ*sip18* strains (BY4742 background) exhibited lower values of viability after stress induction, ~10% and <10%,

respectively (Figure 1). The viability of the Δ*hsp12*, Δ*YBR016w*, Δ*wwm1*, Δ*gre1*, Δ*nop6*, Δ*gon7*, and Δ*YNL190w* strains did not exhibit statistically significant differences from the reference strain (at ~35%).

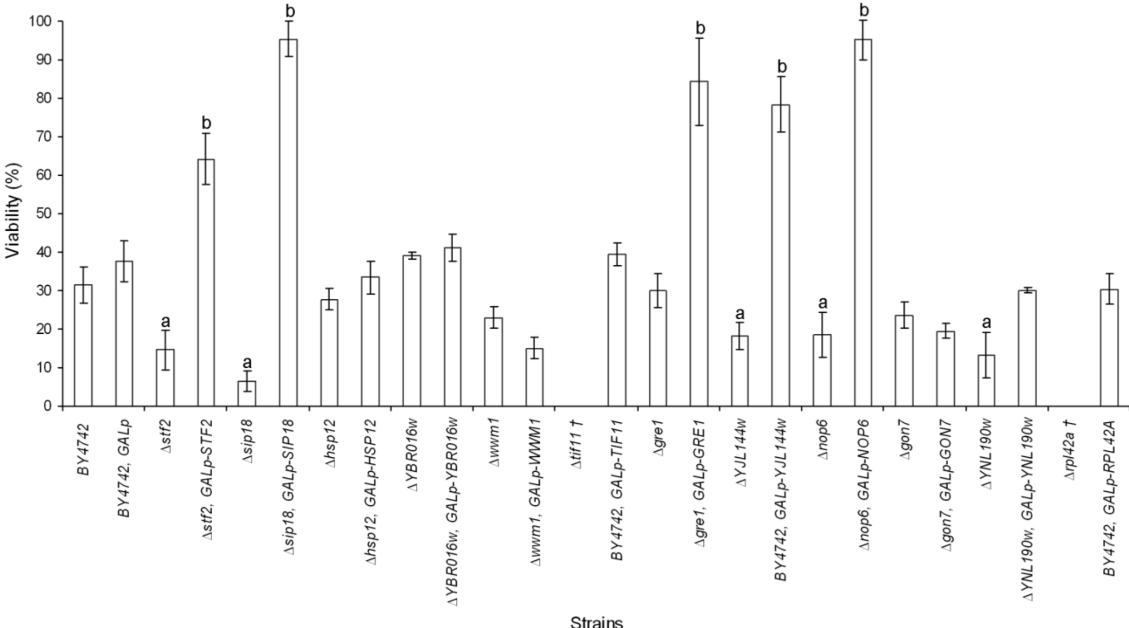

Figure 1. Effect of over-expressing hydrophilin genes on the yeast viability after the drying and rehydration process. The scale of viability (%) indicates the percentage of experimental values for the different strains. Values shown are the means of at least $n=3$ independent samples ± the standard deviation. [a,b]Significant differences ($p \leq 0.05$) with respect to the BY4742 and to the BY4742, *GALp* strain, respectively.

We next characterised the effects of increasing the Stf2p, Sip18p, Hsp12p, YBR016wp, Wwm1p, Tif11p, Gre1p, YJL144wp, Nop6p, Gon7p, YNL190wp and Rpl42ap expression levels in stationary-state cells using a plasmid that allows expression of these genes under the control of the *GAL1* promoter (*pGAL1*) in the corresponding yeast gene-deletion strain (except for the two essential genes, *TIF11* and *RPL42*, which were over-expressed in the BY4742 strain). After rehydration, the following strains exhibited approximately 50% higher viability than the BY4742, *GALp* strain: Δ*stf2, GALp-STF2*; Δ*sip18, GALp-SIP18*; Δ*gre1, GALp-GRE1*; and Δ*nop6, GALp-NOP6* (Figure 1). Furthermore, the other transformant strains showed cell viability values similar to that of the reference strain harbouring the empty vector (i.e., BY4742, *GALp*). These results allowed us to conclude that the *STF2* and *SIP18* genes, this last was the subject of a previous study (Dang and Hincha, 2011), are essential to overcome the simple stress of the desiccation-rehydration process. Moreover, the increased levels of *STF2*, *SIP18*,

GRE1, and *NOP6* gene products before stress induction might enhance the dehydration stress tolerance.

Overexpression of STF2 prevents cellular ROS accumulation

Based on the reported antioxidant role of hydrophilins in different organisms, as reviewed by Tunnacliffe and Wise (2007), we wanted to ascertain, in stationary-state cells, whether the higher viability rate of the Stf2p, Gre1p, YJL144wp and Nop6p over-expressing strains relative to the wild type after the de- and rehydration process could be due to differences in ROS accumulation (Szeto *et al.*, 2007). Yeast cells in the stationary phase and after rehydration were incubated in the presence of dihydroethidium (DHE) to quantify the ROS accumulating cells (Figure 2).

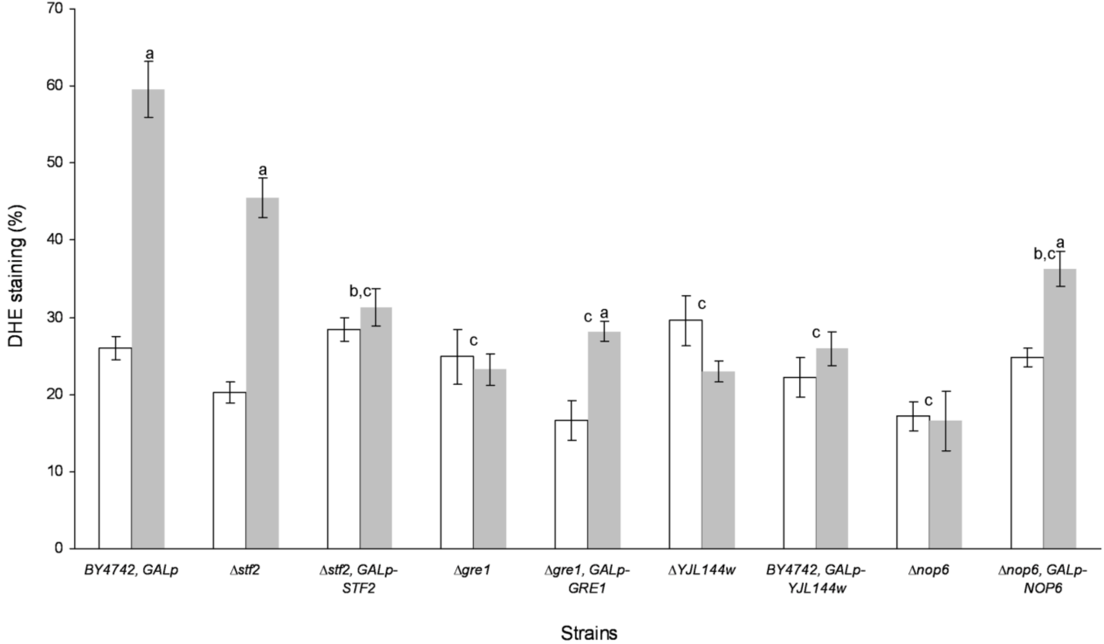

Figure 2. Yeast cells accumulate ROS during stress induction. Quantification of the ROS accumulation using DHE staining before cell dehydration (BD, white bars) and after the rehydration process (AR, grey bars). Values are the means of at least n= 3 determinations ± the SD. [a]Significant differences ($p\leq0.05$) during stress induction with respect to the BD step. [b]Significant differences ($p\leq0.05$) during stress induction of overexpressing strain with respect to the knock-out strain. [c]Significant differences ($p\leq0.05$) during stress induction with respect to the BY4742, *GALp* strain. In each experiment 500 cells were evaluated.

Before dehydration, approximately 29% of the cells of each evaluated strain showed fluorescence after DHE incubation. After rehydration, the cultures of BY4742, *GALp* and *Δstf2* strains contained up to 35% more cells that exhibited intense intracellular DHE staining. During stress induction, the *Δstf2, GALp-STF2* cells (30%) showed a statistically significant reduction in ROS accumulation in comparison to the *Δstf2* cells (45%).

However, the *Δgre1, GALp-GRE1*; *ΔYJL144w, GALp-YJL144w*; and *Δnop6, GALp-NOP6* cells did not show statistically significant reduction in fluorescence in comparison to *Δgre1*, *ΔYJL144w* and *Δnop6* cells. Notably, around 32% of *Δgre1; Δgre1, GALp-GRE1; ΔYJL144w, GALp-YJL144w; Δnop6;* and *Δnop6, GALp-NOP6* cells showed 50% lower ROS levels during stress induction than BY4742, *GALp* cells. Considering the cell viability results for the over-expressing strains (Figure 1) and their ROS reduction values in comparison to the corresponding knockout strain (Figure 2), we suggest that only Stf2p overexpression correlates with the increase in the desiccation survival rate and the reduction in ROS levels after stress induction.

Therefore, we explored whether the changes in the cell viability observed in the *Δstf2, GALp-STF2* strain with elevated dehydration tolerance correlated with other apoptotic processes, such as phosphatidylserine externalisation (Annexin V/PI staining) and DNA strand breaks (TUNEL assay) (Figure 3). Using flow cytometry, we were able to quantify apoptotic (Annexin V+ /PI^2), secondary necrotic (Annexin V+ /PI+), and true necrotic (Annexin V^2/PI+) cells. After stress induction, the *Δstf2* strain showed amounts of apoptotic (12%, as BY4742, *GALp* strain) and secondary necrotic (29%) fluorescent cells significantly higher than *Δstf2, GALp-STF2* cells, 5% and 15% respectively, whereas the reference strain and the dehydration-tolerant clone had similar percentages of Annexin V/PI and PI cells, 15% and 29%, respectively. Additionally, before dehydration, the percentages of Annexin V, Annexin V/PI and PI cells for the BY4742, *GALp*; and *Δstf2* strains did not exhibit significant differences, with staining levels of 15% for Annexin, 5% for Annexin V/PI and, 11% for PI, respectively for both strains, whereas the *Δstf2, GALp-STF2* strain showed similar percentages Annexin V/PI and PI cells but only 5% for Annexin V cells. These results suggest that the overexpression of Stf2p minimised the number of apoptotic cells during stress induction as a putative consequence of the reduction of ROS accumulation in the cells. By the contrary, cell death of *Δgre1, ΔYJL144w* and *Δnop6* strains might be linked to some molecular pathway in a ROS accumulation-independent way.

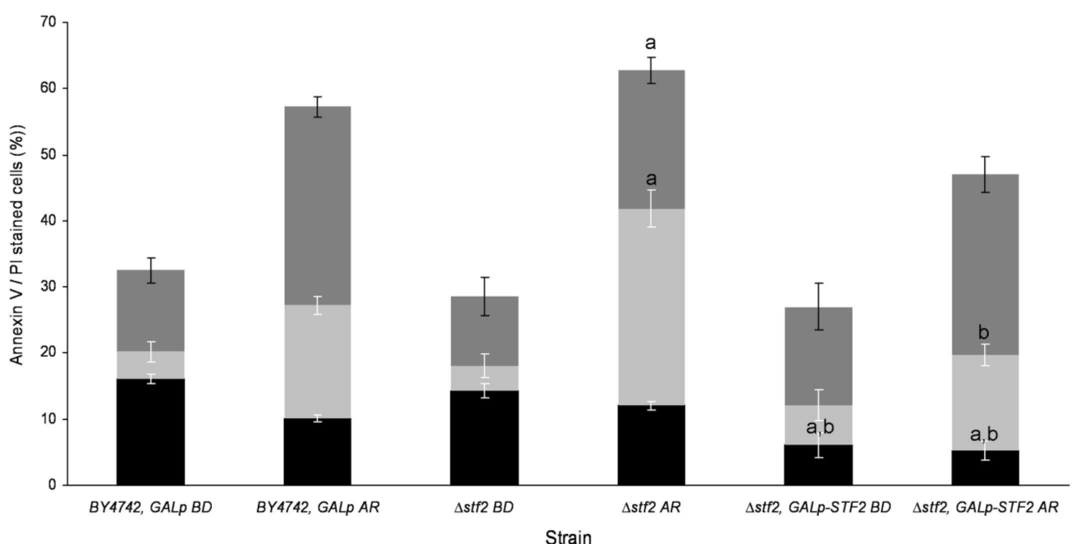

Figure 3. Apoptotic hallmarks in the STF2p-over-expressing strain. Stained cells: light grey, necrotic; dark grey, secondary necrotic; and black, apoptotic cells. The scale of Annexin V/PI-stained cells (%) indicates the percentage of experimental values for the different strains BD and AR. The represented values are the means of n= 3 determinations ± the SD. [a,b]Significant differences ($p \leq 0.05$) with respect to BY4742, *GALp* and *Δstf2* strains, respectively. In each experiment, 16105 cells were evaluated.

GFP-STF2 fusion protein accumulates in the cytoplasm

With the aim of investigating the localisation of Stf2p, a strain carrying a fusion of Stf2p and green fluorescent protein (GFP) integrated in the *STF2* locus (*GFP-STF2*) was analysed by microscopy after 2 days of growth [*STF2* is mainly expressed during the stationary phase (Gasch *et al.*,2000)]. After 48 h of growth, a culture of the Δ*stf2* strain with the plasmid expressing *GFP-STF2* under *GALp* (Δ*stf2*, *GALpG-STF2*) was divided, and the stationary-state cells were observed after a 4 h supplementation with 2% galactose or 2% glucose. The fusion protein was expressed at a very low level in the presence of glucose, resulting in the diffuse labelling of the cells, mainly due to the low activity of *GALp* even after glucose starvation. However, the Δ*stf2*, *GALpG-STF2* cells with galactose exhibited a high fluorescent signal, with most cells exhibiting green fluorescence in the cytoplasm (Figure 4A).

In order to better note the localization of the full protein in the cytoplasm, cells of the over-expressing GFP-Stf2p fusion strain were observed after 4 h of galactose induction, using a confocal microscope. Labelling of the cell surface, nucleus or vacuolar system was not observed in any case (Figure 4B). Additionally, cells of both the Δ*stf2*, *GALpG-STF2* and Δ*stf2*, *GALp-STF2* strains (Figure 4C) showed the same increase in viability

after rehydration in comparison with the reference strains harbouring the empty vectors (Figures. 1 and 4C). Therefore, the GFP tag did not result in any phenotypic defect in the viability of the BY4742 strain after the dehydration and rehydration process.

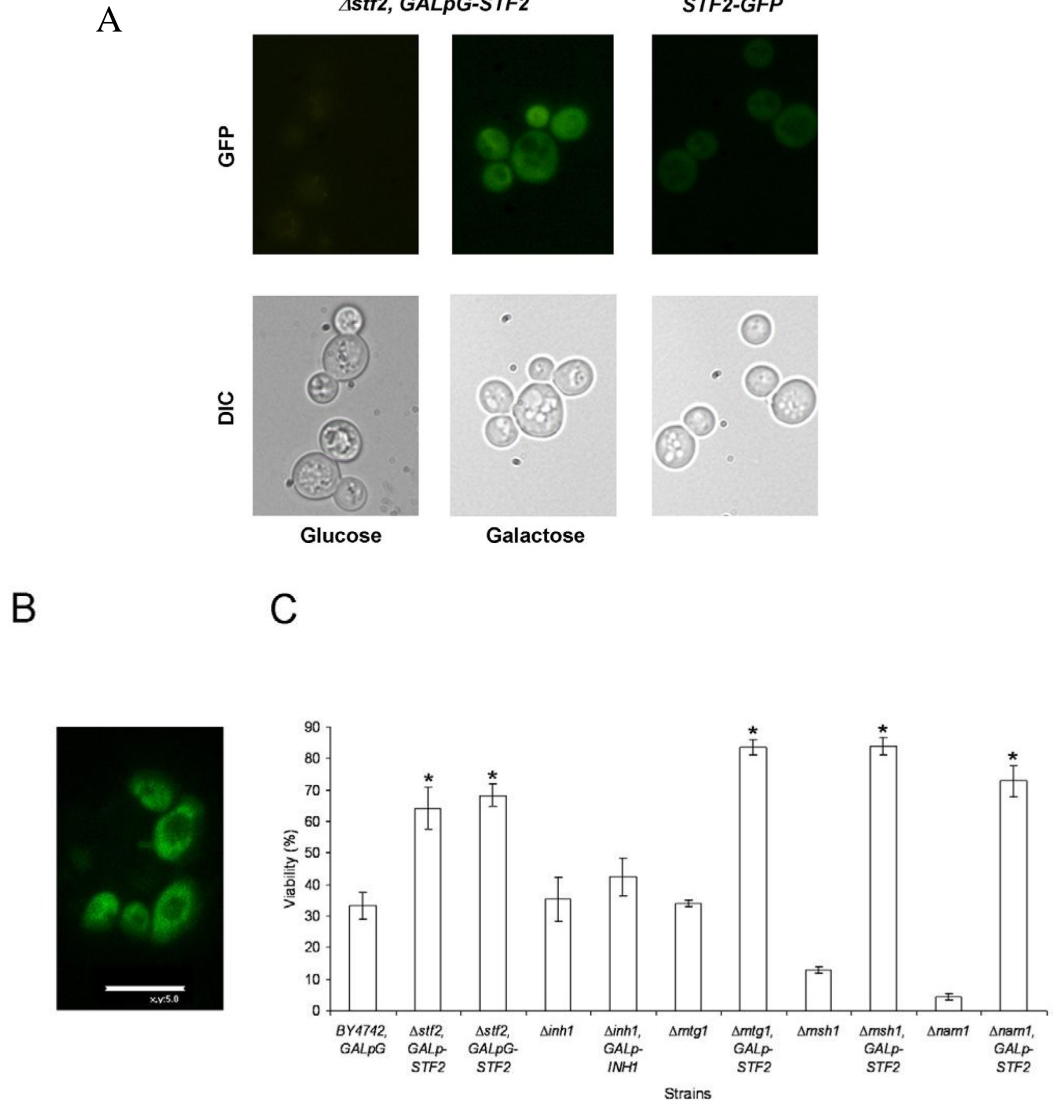

Figure 4. *GFP-STF2* fusion localises to the cytoplasm. A) Each column shows images of the same field, with the fluorescence of the green fluorescent protein (GFP) in the top row and the differential interference contrast (DIC) images of the cultured yeast cells in the bottom row. The Δ*stf2* cells transformed with the vector expressing *GFP-STF2* under the control of the *GAL1* promoter were photographed after 4 h of galactose or glucose supplementation. Cells expressing the *GFP-STF2* fusion protein under the *STF2* promoter were photographed after 24 h in the stationary phase. B) Analysis of cells expressing *GFP-STF2* using the confocal microscope image generated by the average of a pile of five optical sections. C) The scale of viability (%) indicates the percentage of the experimental values for the different strains after the dehydration and rehydration process relative to the highest value for the fresh cultures before the induction of the stress. Values are the means of $n = 3$ determinations ± the SD. *Significant differences ($p \leq 0.01$) of overexpressing strains with respect to the BY4742, *GALp* strain.

Respiration deficiency in the GALp-STF2 strain does not promote dehydration tolerance

Mitochondria are both the source of and the site for the detoxification of ROS in yeast. A physiological stimulus for ATP synthesis can become a pathological stimulus for ROS generation (Brookes *et al.*, 2004). The Stf2p protein may act as stabilising factor that enhances the inhibitory action of the Inh1p protein in the F1F0-ATP synthase; homodimers of the Inh1p protein bind directly to the F1-sector, allowing the maintenance of intracellular ATP levels (Bienhart *et al.*, 2002). Therefore, we evaluated whether changes in the regulation of ATP hydrolysis correlated with the enhancement of the cell dehydration tolerance. Both the Δ*inh1* and Δ*inh1*, *Galp-INH1* strains did not show improved survival relative to the reference strain (at ~35%) (Figure 4C). The increase in the cellular ATP level reduces the flux through the glycolytic pathway, thus inducing a reduction in pyruvate accumulation (Larsson *et al.*, 2000). Figure 5 shows the evaluation of the cellular pyruvate and ATP concentrations for the Δ*stf2* and Δ*stf2*, *GALp-STF2* strains before cell dehydration (BD) and after the rehydration process (AR). The cells of the *BY4742*, *GALp* and Δ*stf2* strains showed similar patterns of ATP content BD and AR, increasing 4 fold and 2 fold, respectively. In the Δ*stf2*, *GALp-STF2* cells AR, less than 90% of the ATP level of the reference strain was observed (Figure 5A). The mutated strains did not exhibit statistically significant changes in their pyruvate concentrations relative to the reference strain BD and AR (Figure 5B). With regard to BD and AR, a minor discrepancy was observed between the mitochondrial mass and ΔΨm in the cells of the *BY4742*, *GALp*; Δ*stf2* and Δ*stf2*, *GALp-STF2* strains, but no significant change in their RMFs was observed (Figure 5C), suggesting that variations in the mitochondrial function did not play a significant role in the cellular ATP content during the dehydration process. Therefore, we evaluated three petite mutant strains (Δ*mtg1*, Δ*msh1*, and Δ*nam1*) overexpressing the *STF2* gene to exclude the possibility that the increase in cell dehydration tolerance was a consequence of lack of synchronicity between ATP and pyruvate metabolism (Figure 4C). Stationary cells from the Δ*mtg1*, *GALp-STF2*; Δ*msh1*, *GALp-STF2*; and Δ*nam1*, *GALp-STF2* strains after galactose induction showed survival rates (75%) similar to that of the Δ*stf2*, *GALp-STF2* strain (Figure 1).

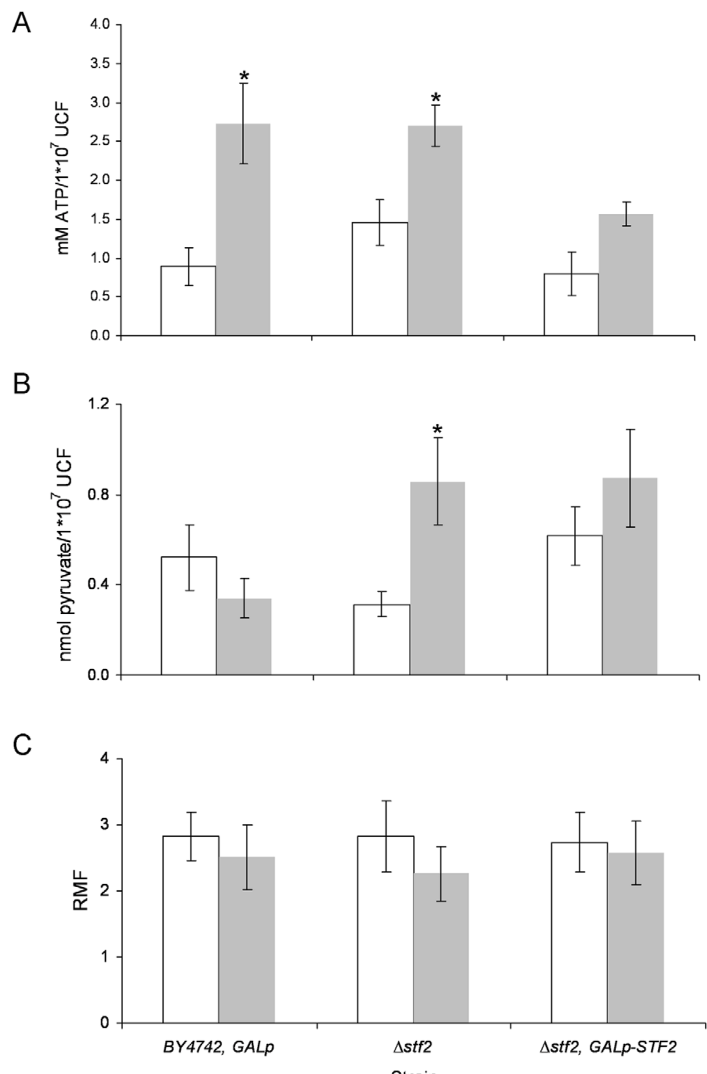

Figure 5. Quantification of ATP (A), pyruvate (B), and the relative mitochondrial function (C) BD (white bars), AR (grey bars) and relative mitochondrial function (RMF). Value shown are the means of at least $n = 3$ independent samples ± the SD. *Significant differences ($p \leq 0.05$) with respect to the BD step.

Cells over-expressing Stf2p show a reduction in DHE fluorescence after H_2O_2 stress

Stationary-state cells of the BY4742, *GALp* and Δ*stf2*, *GALp-STF2* strains were induced for 4 h with 2% galactose and exposed to 4 mM H_2O_2 (Figure 6). The Stf2p-overexpressing strain showed a reduction in the number of DHE-positive cells. As shown in figure 6, after 10 min of H_2O_2 treatment, the percentage of cells accumulating ROS was 10% less of the value for the reference strain, supporting the hypothesis that Stf2p acts as an antioxidant. However, after 20 min and 40 min of H_2O_2 stress, the number of DHE-positive cells did not exhibit significant differences, suggesting that Stf2p does not have a strong positive effect on H_2O_2 clearance.

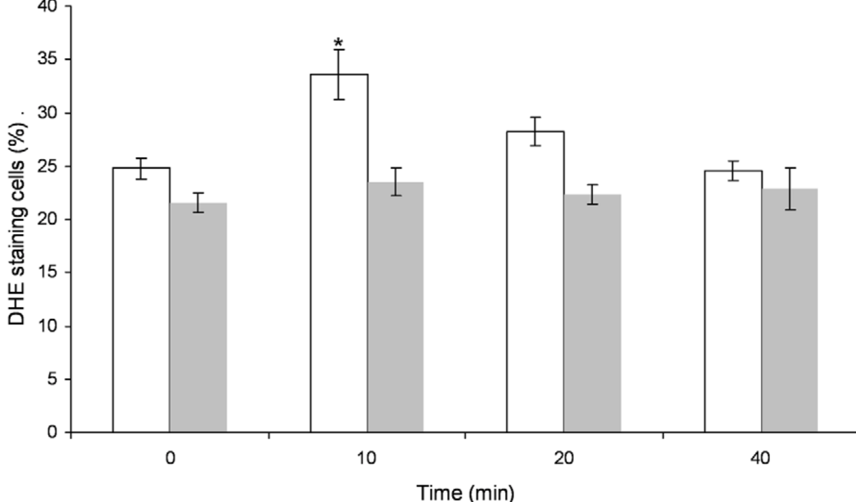

Figure 6. Levels of DHE accumulation after oxidative stress induced by H_2O_2. BY4742 (white bars) and Δ*stf2*, *pGAL-STF2* (grey bars) cells were exposed to 4 mM H_2O_2, and at the indicated times, aliquots were collected to determine the number of DHE-positive cells. The represented data are the means of $n = 6$ determinations ± the SD. *Significant differences ($p \leq 0.05$) for each time with respect to the Δ*stf2*, *pGAL-STF2* strain.

Discussion

It has been reported previously that some highly hydrophilic proteins are commonly induced during water-deficit conditions (Garay-Arroyo *et al.*, 2000). Among the 12 *S. cerevisiae* hydrophilin proteins, we found that only Stf2p, Sip18p, Gre1p, YJL144wp and Nop6p are necessary for the cells to overcome dehydration stress; only Stf2p (involved in the regulation of the mitochondrial F1F0-ATP synthase) and Nop6p (an rRNA-binding protein required for 40S ribosomal subunit biogenesis) have known functions in yeast (Lebowitz and Pedersen, 1996; Buchhaupt *et al.*, 2007). The overexpression of the above proteins significantly enhanced cell viability under stress conditions. Therefore, considering the cell viability results and the apparently uncoupled cellular roles of the yeast Stf2p, Sip18p, Gre1p, YJL144w, and Nop6p hydrophilins, we suggest that the roles as putative intracellular cell protectors might not be their only activity, as was shown for the group 3 late embryogenesis abundant (LEA) proteins, which prevent both protein aggregation and membrane fusion (Török *et al.*, 2001). In this present study, we characterised the role of Stf2p in dehydration stress and examined the possible physiological relationship between the overexpression of Stf2p and the enhancement of viability after the induction of stress. The prevention

of ROS accumulation in cells of the Δ*stf2*, *GALp- STF2* strain, during both the desiccation-rehydration process and H_2O_2 oxidative stress, indicates that STF2p is a protein with antioxidant capabilities, as has been reported for some plant LEA proteins (Battaglia *et al.*, 2008). Thus, the overexpression of *STF2* prevents ROS accumulation and, consequently, cell apoptosis (Mazzio and Soliman, 2004; Li *et al.*, 2003). The strains with highly significant viability rates, the Gre1p-, YJL144wp- and Nop6p-overexpressing strains, yielded viability and ROS results that were contrary to those for the *Δstf2*, *GALp- STF2* strain. Perhaps, as was suggested for the LEA proteins, Gre1p, YJL144wp and Nop6p behave as molecular shields that prevent protein aggregation by steric or electrostatic repulsion, analogous to the polymer stabilisation of colloidal suspensions (Chakrabortee *et al.*, 2007); however, no direct evidence of functional mechanisms were described. It is well documented that mitochondrial function is necessary to maintain low intracellular ROS levels under both saline and osmotic stress conditions (Koziol et al., 2005). In addition, a physiological stimulus for ROS generation can become a stimulus for ATP synthesis in growing cells (Brookes *et al.*, 2004). Most of the mechanisms of cellular tolerance to harsh conditions are driven via plasma membrane ATPase and vacuolar ATPase functions, processes that require large amounts of ATP to overcome acidic or osmotic stress; thus, a low ATP concentration could compromise cell viability during stress conditions (Martínez-Muñoz and Kane, 2008; Hamilton *et al.*, 2002). However, the accumulation of ATP reduces *S. cerevisiae* glycolytic activity, preventing pyruvate formation (Larsson *et al.*, 2000). During the drying and rehydration process, the Δ*stf2*, *GALp-STF2* strain showed lower levels of accumulated ATP than the *Δstf2* strain, and both strains had similar pyruvate concentrations after the stress induction, supporting the idea that the different viability values were not a consequence of achieving critical values for ATP and pyruvate. Stf2p is a modulator of the Inh1p regulatory peptide, which acts on the F1F0-ATP synthase complex (Hong and Pedersen, 2002), and the deletion or altered expression of the *INH1* gene could decrease the ATP supply or enhance cell growth and pyruvate production, respectively (Zhou *et al.*, 2009). Considering the lack of correlation between the ATP and pyruvate concentrations in the *STF2* strains during the dehydration-rehydration process and the similar viabilities of the *Δinh1*, *GALp-INH1* and *Δinh1* strains, we conclude that the lack of synchronicity between the glycolytic pathway and ATP synthesis did not have a major role in the improved survival rate of the *Δstf2*,

GALp-STF2 strain. Moreover, the overexpression of the *STF2* gene in the petite and non-petite strains resulted in similar viability rates. This result provides evidence that Stf2p may allow the cell to survive by stabilising other cellular proteins rather than by interacting with apoptotic proteins, such as Nuc1p, shuttling from the mitochondria to the nucleus or by a reconfiguration of metabolism via the mitochondrial retrograde signal that is involved in nutrient sensing and cell aging (Büttner *et al.*, 2006; Liu and Butow, 2006). The present work provides evidence that Stf2p allows yeast cells to survive during dehydration stress by contributing to the cellular antioxidant capacity that prevents ROS accumulation rather than by the inhibition of apoptotic proteins.

Further studies will be necessary to establish the functional mechanisms of yeast hydrophilins which provide dehydration stress tolerance to the cells. With recent advances in tissue engineering, cell transplantation and genetic technology, the successful long-term storage of living cells is of critical importance. Studies in yeast may provide a better understanding of desiccation-tolerance genetics for potential applications in biomedicine, plant biotechnology, and beverage and bio-ethanol technology.

Acknowledgements

The authors would like to thank the Microscope Unit at the *Universitat Rovira i Virgili* for their assistance with confocal microscopy.

Author contributions

Conceived and designed the experiments: RCO. Performed the experiments: GLM BRP MMC RCO. Analyzed the data: GLM and RCO. Contributed reagents/materials/analysis and tools: RCO. Wrote the paper: GLM and RCO.

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CHAPTER III

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

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Yeast 30 (2013): 319-330

Abstract

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

Introduction

The inoculum of grape must with selected *S. cerevisiae* strains is nowadays a general winemaking practice because the use of starters reduces the risk of sluggish fermentations and contributes to reproducible sensorial properties and quality in wine. Actually, the most widely used starter formulation in this sector is represented by ADWY. The performance of dry yeast products, including their fermentation capacity and flavour release, depends by factors related to the production, such as industrial practice during biomass propagation and desiccation (Attfield *et al.*, 2000; Pretorius, 2000). The ADWYs, used in most yeast-based food industries, undergo several stress conditions

during technological processes production. In *S. cerevisiae*, strain genetic constitution plays a fundamental role in desiccation tolerance. Among the genes required by the yeast to overcome dehydration stress, some of the genes encoding for the proteins termed hydrophilins are essentials (Rodríguez-Porrata *et al*., 2012). On the other hand, the overexpression of genes encoding hydrophilins in some yeasts confers tolerance to water-deficit conditions (Dang and Hincha, 2011; López-Martínez *et al*., 2012). Hydrophilin research in different organisms has allowed significant advances to be made towards the understanding of some of their biological properties, including their roles as antioxidants and as membrane and protein stabilizers during water stress, either by direct interaction or by acting as a molecular shield (Tunnacliffe and Wise, 2007). Among yeast hydrophilin proteins, SIP18p was characterized as an inhibitor for cell apoptosis during the dehydration-rehydration process, by its antioxidative capacity through the reduction of ROS accumulation after an H_2O_2 attack (Rodríguez-Porrata *et al*., 2012).

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

The objectives of this study were to increase the dehydration tolerance in *S. cerevisiae* strains of wine origin. For this purpose, four *S. cerevisiae* strains were transformed with *SIP18* gene from the strain BY4742 (Brachmann *et al*., 1998), transcriptionally bonded to the promoter of the *GAL1* gene, in order to enhance its expression during biomass

production before ADWY preparation. The consequences of overexpression of gene *SIP18* for yeast viability and fermentative performance were investigated. The results obtained showed that the transformation improved the viability of ADWY without affecting fermentation efficiency and metabolic behaviour.

Materials and methods

Microbial strains, plasmids and media

Table 1 summarizes the *S. cerevisiae* strains and plasmids used in this study. Recombinant DNA techniques were performed according to standard protocols (Sambrook and Russell, 2001). The synthetic *SIP18* gene was obtained by PCR and cloned into the pGREG505Δh yeast expression vector (under the control of the *GAL1* promoter) digested with *Sal*I. The plasmids, containing the *KanMX* (geneticin resistance; Gt^R) marker gene, were then used to transform the wine yeast strains. Transformants were selected by plating on synthetic glucose medium with 200 mg/ml geneticin. Gt^R transformants were selected and restreaked to obtain single colonies, which were confirmed by PCR using the primer pair: GALFw, 5′-GAAAAAACCCCGGATTCTAG-3′; and CYCRv, 5′-ATAACTAATTACATGACTCGAG-3′ and by testing for the loss of the *KanMX* marker. The PCR fragments were obtained using BY4742 genomic DNA as a template together with the primer pairs:

SIP18F,5′GAATTCGATATCAAGCTTATCGATACCGTCGACAATGTCTAACATG ATGAATAA-3′

SIP18R,5′GCGTGACATAACTAATTACATGACTCGAGGTCGACTTATTTTTCA TGTTTTCGT-3′.

The amplification reactions contained single-strength PCR buffer (Roche, Mannheim, Germany), 1.25 mm dNTPs, 1.0 mm $MgCl_2$, 0.3 μm each primer, 2 ng/μl template DNA and 3.5 U DNA polymerase (Roche) in a total volume of 100 μl. All the reactions were carried out using a PCR Express thermal cycler for 15 cycles, as follows: denaturation, 2 min at 94°C; primer annealing, 30 s at 55°C; and primer extension, 1 min at 68°C.

Table1. *Saccharomyces cerevisiae* strains and plasmids used in this study

Strain	Genotype/description	Source/reference
BY474	MATa, *his3Δ1*, *leu2Δ0*, *lys2Δ0*, *ura3Δ0*	EUROSCARF/Brachmann *et al.* (1998)
4LB2	Wild wine strain	UBYC/Capece *et al.* (2011)
F15	Commercial wine strain	Laffort
RB3-7Sc2	Wild wine strain	UBYC/Capece *et al.* (2010)
Sc9-11	Wild wine strain	UBYC/Siesto *et al.* (2013)
LB, GAL_p	4LB + pGREG505Δh	This work
F, GAL_p	F15 + pGREG505Δh	This work
RB, GAL_p	RB3-7Sc2 + pGREG505Δh	This work
Sc, GAL_p	Sc9-11 + pGREG505Δh	This work
LB, GAL_p-*SIP18a*	4LB + pGREG505si	This work
LB, GAL_p-*SIP18b*	4LB + pGREG505si	This work
F, GAL_p-*SIP18a*	F15 + pGREG505si	This work
F, GAL_p-*SIP18b*	F15 + pGREG505si	This work
RB, GAL_p-*SIP18a*	RB3-7Sc2 + pGREG505si	This work
RB, GAL_p-*SIP18b*	RB3-7Sc2 + pGREG505si	This work
Sc, GAL_p-*SIP18a*	Sc9-11 + pGREG505si	This work
Sc, GAL_p-*SIP18b*	Sc9-11 + pGREG505si	This work
Plasmids		
pGREG505Δh	$GAL1_p$-*Sal*I-*Sal*I-$CYC1_t$-*KanMX4-LEU2-bla*	Rodríguez-Porrata *et al.* (2012)
pGREG505si	$GAL1_p$-*SIP18*-$CYC1_t$-*KanMX4-LEU2-bla*	Rodríguez-Porrata *et al.* (2012)

Dehydration and rehydration treatment

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

Flow cytometry analysis

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

Tests for intracellular ROS accumulation

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

Measurement of intracellular nucleotide leakage

The rehydrated yeast cells were harvested by centrifugation at 5000 rpm for 3 min at 4ºC. The supernatant absorbance values at 260 and 280 nm were used to calculate the

nucleotide equivalents in mg/ml = (0.063 A_{260}) - (0.036 A_{280}) (Herbert *et al.*, 1971). The total intracellular nucleotide calculated was around 3 mg/g rehydrated cells. These analyses were done at least in triplicate and standard deviations (SDs) were < 10%.

Determination of biological parameters

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

Fermentation at the laboratory scale

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

Measurement of volatile compounds

Higher alcohols, ethyl acetate, acetaldehyde and acetic acid were determined by direct injection gas chromatography, using an Agilent 7890A gas-liquid chromatograph fitted

with a flame ionization detector (FID) and a split-splitless injector, and provided with an automatic sampler and a Supelco glass column packed with 80/120 Carbopack BAW/5% Carbowax 20 M (180 cm x 2 mm i.d.). Chromatographic conditions entailed the following: helium carrier gas, head pressure of 140 kPa; total flow of 20 ml/min; purge flow of 7.0 ml/min; injector and detector temperature of 250ºC; initial column temperature of 80ºC, held for 2 min, then raised to 200ºC at 4ºC/min; make-up gas He at 30 ml/min; detector FID, H_2 at 30 ml/min; air 300 ml/ min; injected volume, 1 ml. The identification and quantification of volatile compounds were determined by comparing each chromatographic peak with the retention times and relative areas of standard solutions. Volatile compounds were determined by solid-phase microextraction (SPME). Ten ml wine samples were transferred to 20 ml glass vials with 2 g NaCl, and 100 ml isoctane (concentration 10000 mg/l) was added as internal standard. The equilibration was performed by stirring for 20 min at 46ºC, whereas the adsorption phase was carried out at 50ºC for 15 min under agitation. A carboxenpolydimethylsiloxane-coated fibre (100 mm) was used. After extraction, the fibre was placed in the injector of the GC for 10 min. A DB-WAXTER (Agilent) column was used (length 30 m, i.d. 0.250 mm). The analysis was performed in splitless mode and the following conditions were used: 220ºC as injection temperature; 250ºC as detector temperature; helium as carrier gas with a flow rate of 20 ml/min. The initial temperature was 40ºC and then it was raised to 240ºC at 7ºC/min.

Statistical analysis

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

Results

SIP18p hydrophilin enhances wine yeast dry stress tolerance

In the first step, the effects of increasing the SIP18p expression levels were evaluated in stationary phase cells of four different *S. cerevisiae* wine strains (Table 1). For this purpose, a plasmid was used that allows expression of this gene under the control of the

GAL1 promoter (*GAL1p*), which is less active than the endogenous *SIP18* promoter in the stationary phase. Both kinds of transformant strains, harbouring the empty vector or the plasmid expressing *SIP18* under *GALp*, after 48 h cultivation in selective dropout (SD) medium with 400 mg/ml geneticin were dried after 4 h supplementation with 2% galactose. The desiccation tolerance capacity of the yeast LB, *GALp*; Sc, *GALp*; RB, *GALp*; and F, *GALp* strains after cell rehydration with pure water at 37ºC exhibited viability values of 20%, 30%, 55% and 60%, respectively (Figure 1).

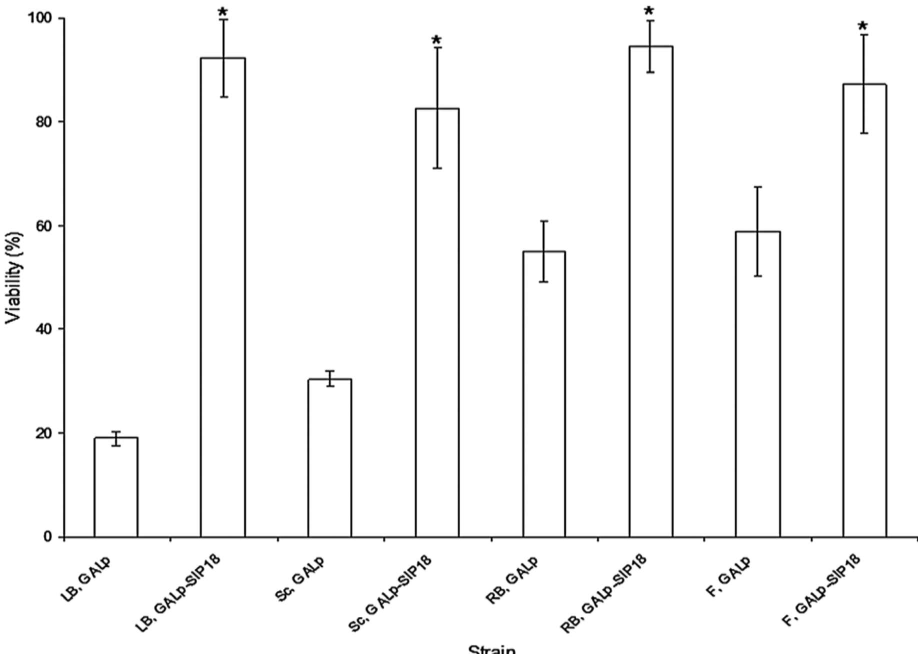


Figure 1. Effect of overexpressing *SIP18* hydrophilin gene on yeast viability after stress induction. The scale of viability indicates the experimental values (%) for the different strains. Values shown are mean ± SD of at least three independent samples. *Significant differences ($p \leq 0.05$) with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

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

Overexpressing SIP18 gene strains show reduced ROS accumulation

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

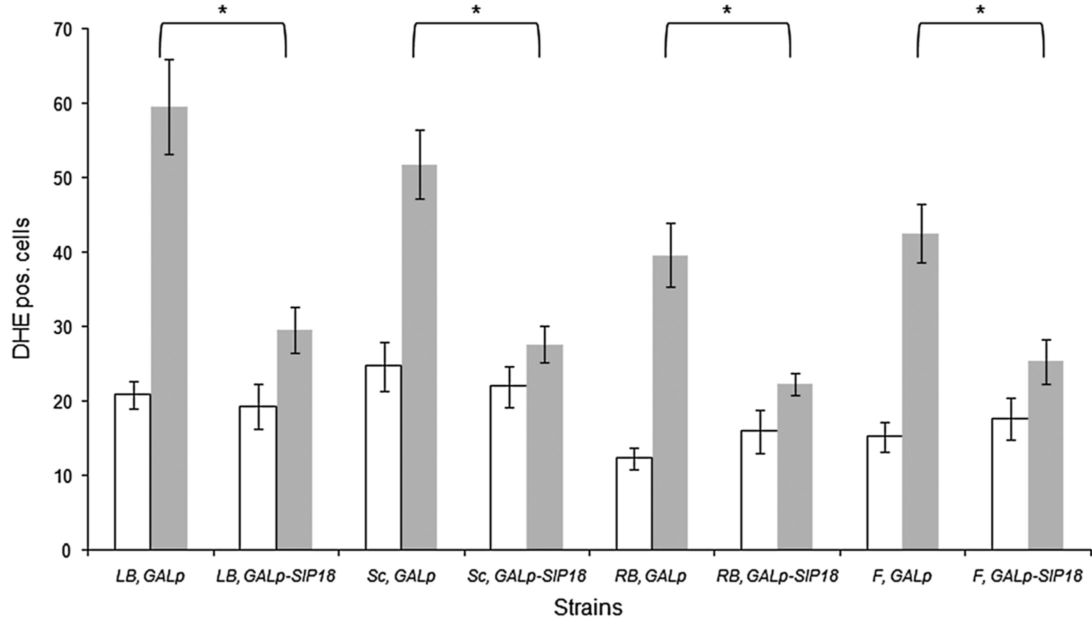


Figure 2. ROS accumulation by yeast cells during stress induction. (A) Quantification of ROS accumulation using DHE staining before drying (white bars) and after rehydration (grey bars). Values are mean ± SD of three determinations. DHE pos., DHE- positive cells. *Significant differences ($p \leq 0.05$)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

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

Cells from LB, *GALp*; LB, *GALp-SIP18*; Sc, *GALp*; Sc, *GALp-SIP18*; RB, *GALp*; RB, *GALp-SIP18*; F, *GALp* and F, *GALp-SIP18* strains, after 4 h galactose induction, were subjected to 4 mM H_2O_2. After this treatment, the *SIP18*-overexpressing strains showed

40% reduction in the number of DHE cells after 10 or 20 min (Figure 3), whereas for all the strains at 30 and 40 min, the number of DHE-positive cells was similar. These results confirmed antioxidant properties by Sip18p, validating the previous results obtained in strain BY47472 by Rodríguez-Porrata *et al*. (2012), also in these four different wine yeast strains.

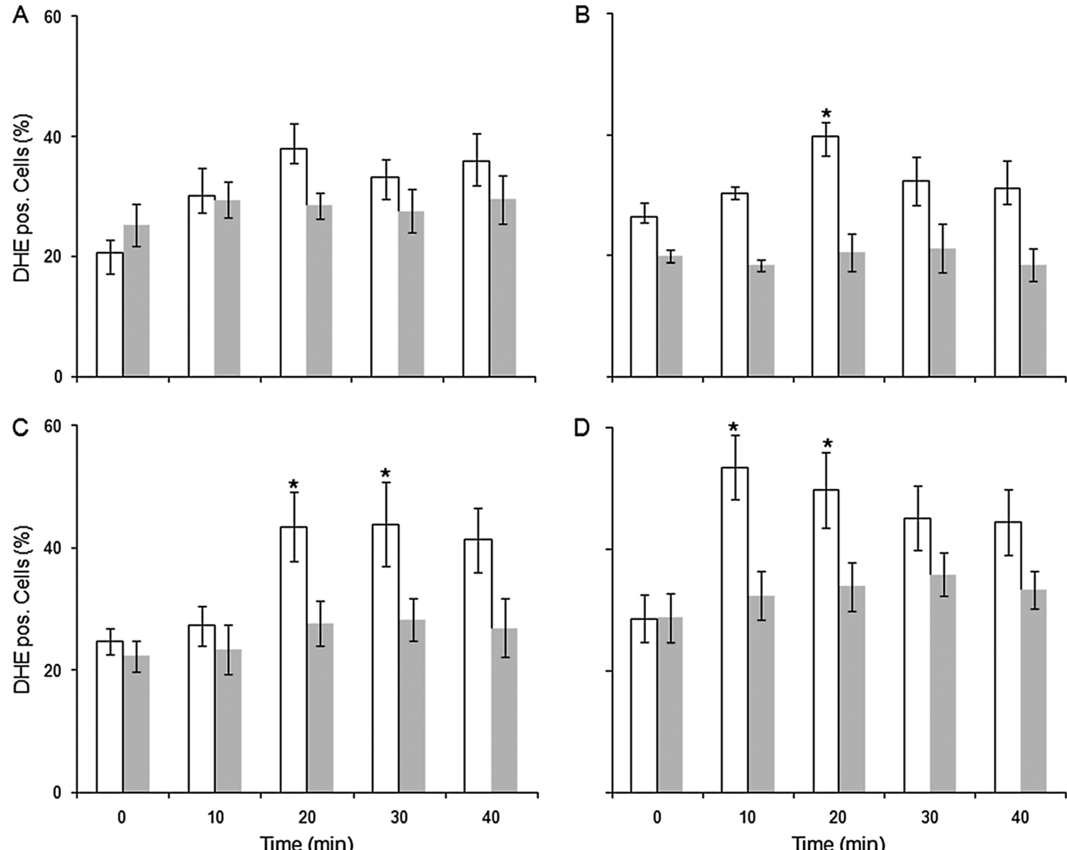
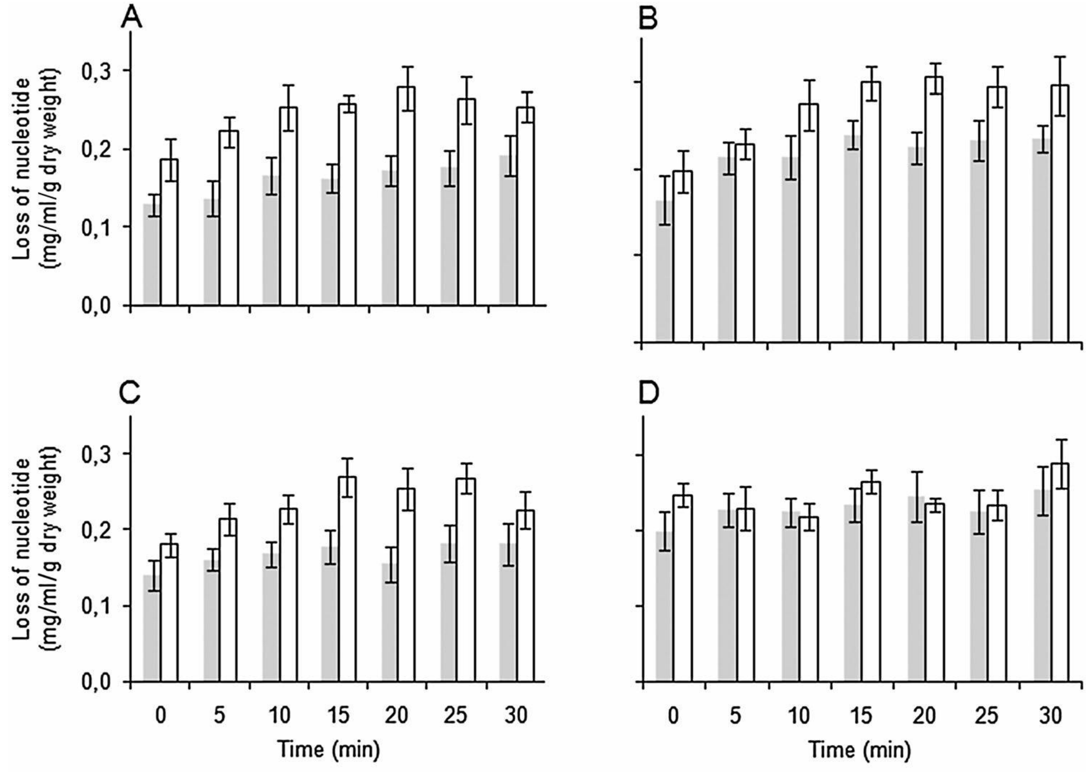


Figure 3. Levels of DHE accumulation after oxidative stress by H_2O_2. Cells in stationary state from the transformant reference (white bars) and the overexpressing *SIP18* strains (grey bars) were exposed to 4 mM H_2O_2 at the indicated times; aliquots were taken to evaluate DHE-positive (DHE pos.)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

Cell leakage during cell rehydration and ADY vitality

Dehydrated yeast can lose up to 30% of soluble cell compounds when rehydrated, which proves the non-functionality of the cell membrane. A faster reduction in leakage may therefore be beneficial for the vitality of rehydrated yeast cells. The degree of intracellular compound leakage was assessed by evaluating 260 nm light absorbtion, at each point in

time, of nucleotide concentration in the rehydrating supernatants of the transformant wine yeast strains (Figure 4A-D). On the other hand, after the rehydration process, cells were inoculated into YPD at 28°C and evaluated biomass time course production (Figure 4a-d). For the experimental rehydration of LB, GAL_p; Sc, GAL_p; and RB, GAL_p strains, the nucleotide concentration time course in the supernatant appeared to exhibit two periods that were delimited at the inflection points 20, 15 and 15 min, respectively, where cell leakage rate was inhibited (Figure 4A-C). The leakage trend exhibited by the LB, *GALp-SIP18*; Sc, *GALp-SIP18*; and RB, *GALp-SIP18* strains showed a first period of ~5 min and a relative total nucleotide leakage of ~16%. Neither two leakage trend periods nor relative total nucleotide leakage differences between F, GAL_p and F, GAL_p-*SIP18* strains were observed (Figure 4D). These experiments reveal that most of the overexpressing *SIP18* strains show at least 25% lower relative leakage than the transformant reference strains (Fig 4A-C).



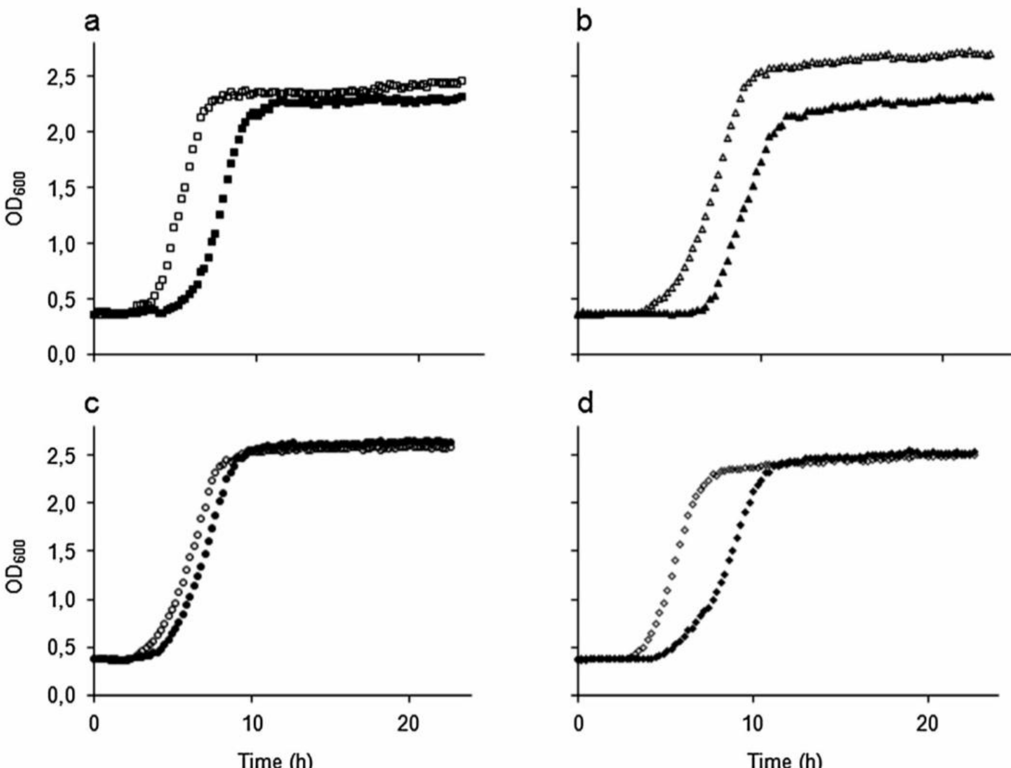


Figure 4. Time course of extracellular nucleotide concentration at the rehydration process (A, B, C and D, page121) and growth curve of rehydrated cells (a, b, c and d). Overexpressing *SIP18* strains (white bars) and their respective transformant reference strains (grey bars) were incubated at 37°C in pure water. The data represented are mean ± SD of triplicate rehydration experiments. The graphs are a representative example of growth experiments performed, with two independent transformants for each strain. (A,a) LB, *GALp-SIP18* and LB, *GALp* strains; (B,b) Sc, *GALp-SIP18* and Sc, *GALp* strains; (C,c) RB, *GALp-SIP18* and RB, *GALp* strains; and (D,d) F, *GALp-SIP18* and F, *GALp* strain.

Strains overexpressing SIP18 gene show longer lag phase after rehydration process

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

(Figure 4a-d). The overexpressing *SIP18* strains exhibited an increase of λ phase even though, in general, they stopped the intracellular compounds leakage earlier after stress induction.

SIP18p hydrophilin did not affect fermentative performance

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

SIP18p hydrophilin did not affect metabolic behaviour of the strains

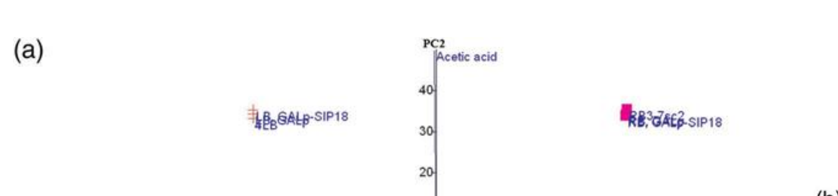
The successive step was to verify the effect of *SIP18* hydrophilin on the metabolic behaviour of the transformants in comparison to the wild strains. The amounts of the principal secondary compounds determined in the experimental wines by gas chromatography are reported in Table 2. The four original strains exhibited a very similar metabolic behaviour in these fermentations; in fact, the contents of all the determined compounds were similar among the wines obtained from the four original strains, even though some differences occurred. In particular, the *n*-propanol level detected in the wine produced by 4LB was statistically significant different from the content of the wine produced by F15, whereas the acetaldehyde content in the wine produced by Sc9-11 differed significantly from the level found in the wine produced by RB3-7Sc2. However, it must be underlined that all the compounds tested were present at acceptable levels, including acetic acid, which was below the critical threshold of about 0.7 g/l (range 201−330 mg/l). As regards the wines produced by transformant strains, obtained by each of the four *S. cerevisiae* wild strains, generally, the secondary compounds were present at levels comparable to the wines obtained by the non-

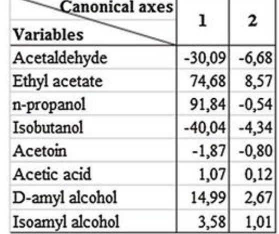
transformant strains. Only two transformants (LB, *GALp* derived from 4LB and RB, *GALp* obtained from RB3-7Sc2) showed concentrations of acetic acid significantly higher than those of the original strains (p<0.05) although also in this case the values were below the threshold value (Table 2).

Table 2. Main volatile compounds produced during laboratory-scale fermentations by four *S. cerevisiae* strains and two transformants for each strain. Data are expressed in mg/l and are mean ±SD of three independent experiments. *Values significantly different from the control (p<0.05), represented by non-transformant strains. Different letters (a, b) in the same column correspond to statistically significant differences for each non-transformant strain (p<0.05).

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

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





(b)

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

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

Furthermore, the effect of strain transformation on yeast metabolic behaviour was evaluated by analysing the experimental wines for the content of volatile organic compounds (VOCs), present at low level but known to influence the final organoleptic quality of wine. These compounds, determined by SPME analysis, are represented mainly by terpenes, esters and higher alcohols. Figure 6 reports the comparison in VOC number between wines produced by inoculating both kinds of transformant strains and wines produced by the wild strains. Each compound is indicated with a different number. In this context, it is interesting to notice that the number and the VOCs determined in wines obtained by original and corresponding transformant strains were very similar. The wines containing the highest number of VOCs were those obtained by inoculating strains 4LB and F15 (the original strains and both the transformants). The main percentage of compounds is common to all the wines, whereas some compounds were present only in some cases. For example, eugenol was present in all the wines except those derived by fermentation with Sc9-11 (original and transformants), whereas dimethyl sulphone was found only in wines obtained by inoculating 4LB (original and both the transformant strains). This means that these compounds are related to the specific metabolic activity of 4LB strain and, in addition, the presence of this compound also in wines produced with its transformants, demonstrates that the treatment had not affected this metabolism in 4LB strain. Therefore, the results obtained by both gas

chromatographic analyses suggest that the transformation did not significantly affect the production of the secondary compounds involved in wine aroma.

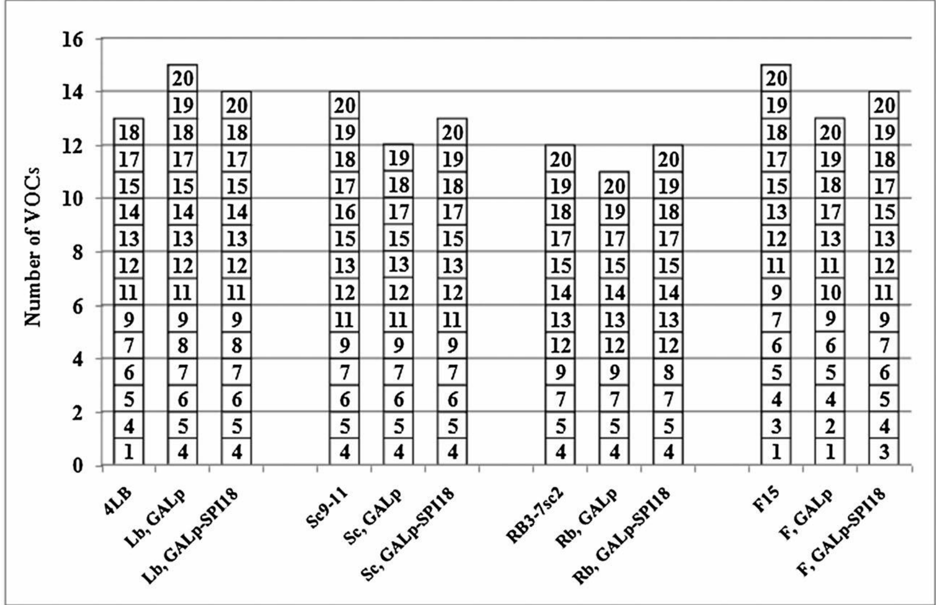


Figure 6. Volatile organic compounds (VOCs) determined by SPME in wines obtained by non-transformant and transformant strains: 1, acetone; 2, dimethyl sulphone; 3, b-pinen; 4, *n*-butanol; 5, limonen; 6, isobutyl formate; 7, isobutyl acetate; 8, ethyl-butyrate; 9, ethylhexanoate; 10, 2,3 butanediol;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

Discussion

Desiccation tolerance by the wine yeast *S. cerevisiae* has enabled the food industry to work with a more technologically secure product, but still excluding those yeast strains of higher interest and the newly isolated or created hybrid strains for beverage industry (*Saccharomyces* sp. and non-*Saccharomyces*) that cannot cope with the treatment of drying and rehydration (Rodríguez-Porrata *et al.*, 2011). Natural yeasts possessing high survival to desiccation are not very diffused and the genetic manipulation of strains possessing interesting oenological properties, but low tolerance to desiccation, could represent an interesting tool. In the present study, we analysed four different wine strains. Three of them (4LB, RB3-7Sc2 and Sc9-11) were wild *S. cerevisiae* strains, isolated during spontaneous fermentation of grapes collected in different Italian regions and selected on the basis of interesting oenological characteristics, whereas the last one (F15) was a commercial strain, widely used as ADY in Italian cellars. On the basis of previous results reporting that the overexpression in *S. cerevisiae* of gene *SIP18* increases cell

viability after the dehydration process (Rodríguez-Porrata *et al*., 2012), the four strains were submitted to transformation by insertion of the gene *SIP18*. In the case of transformed strains, it is very important to verify whether the introduced modifications should not change the characteristics essential in the fermentation process (Schuller and Casal, 2005). For most genetic modifications it was shown that, apart from the introduced metabolic change, no significant differences were found between wines produced with non-modified strains and the corresponding transformed strain, whereas in other cases genetic modification affected the characteristics of the final wines (Michnick *et al*., 1997; Remize *et al*., 2000). In this study, different techniques were used to evaluate the influence of transformation on the characteristics of analysed strains. The 'fitness' of active dried wine yeast cultures is related to the maintenance of cell 'viability' and 'vitality' during the process of yeast manufacture, including desiccation and storage (Pretorius, 2000). In our research, yeast 'viability' was assessed both directly, by determining loss of cell viability (plate counts), and indirectly, by assessing the preventing ROS accumulation effect of *SIP18* even after an H_2O_2 attack, as was already shown in a laboratory haploid strain by Rodríguez-Porrata *et al*. (2012). On the other hand, we also evaluated the 'fitness' of the modified strains by simulating real vinification conditions. After grape must inoculation, during biomass formation, the absence of both the selection pressure by geneticin and galactose activation (in glucose-less medium) reduces at a very low level the cellular Sip18p content during vinification. In this way, the putative Sip18p impact is negligible in the organoleptic profile of wines, elaborated with strains for which *SIP18* was overexpressed during ADY production. The transformants obtained in this study did not negatively impact wine profile, although at the beginning of the fermentation they carry on a high level of the *SIP18* stress peptide. Our results demonstrated that, apart from the introduced change related to improved dehydration tolerance, no significant differences were found between original and modified strains as regards the fermentative performance and production of secondary compounds influencing wine aroma. These findings indicate that strain oenological characteristics are not affected by genetic modifications used in this study.

In conclusion, the transformation of wine strains by overexpression of the *SIP18* gene could represent an useful tool to improve strains tolerance to dehydration. Further studies are in progress in order to test the behaviour of these modified strains, in particular by

evaluating the strain imposition capacity during real vinification trials, where the inoculated starter has to compete with the indigenous microflora.

Acknowledgements

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

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UNIVERSITAT ROVIRA I VIRGILI
FUNCTIONAL CHARACTERIZATION IN VIVO OF ESSENTIAL SACCHAROMYCES CEREVISIAE'S HYDROPHILIN FOR DESICCATION TOLERANCE
Gema Isabel López Martínez
Dipòsit Legal: T 1354-2015

CHAPTER IV

ATG18 and *FAB1* Are Involved in Dehydration Stress Tolerance in *Saccharomyces cerevisiae*

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PLoS One 10 (2015): e0119606

Abstract

Recently, different dehydration-based technologies have been evaluated for the purpose of cell and tissue preservation. Although some early results have been promising, they have not satisfied the requirements for large-scale applications. The long experience of using quantitative trait loci (QTLs) with the yeast *Saccharomyces cerevisiae* has proven to be a good model organism for studying the link between complex phenotypes and DNA variations. Here, we use QTL analysis as a tool for identifying the specific yeast traits involved in dehydration stress tolerance. Three hybrids obtained from stable haploids and sequenced in the *Saccharomyces* Genome Resequencing Project showed intermediate dehydration tolerance in most cases. The dehydration resistance trait of 96 segregants from each hybrid was quantified. A smooth, continuous distribution of the anhydrobiosis tolerance trait was found, suggesting that this trait is determined by multiple QTLs. Therefore, we carried out a QTL analysis to identify the determinants of this dehydration tolerance trait at the genomic level. Among the genes identified after reciprocal hemizygosity assays, *RSM22*, *ATG18* and *DBR1* had not been referenced in previous studies. We report new phenotypes for these genes using a previously validated test. Finally, our data illustrates the power of this approach in the investigation of the complex cell dehydration phenotype.

Keywords: anhydrobiosis, dehydration stress, *FAB1*, *ATG18*, yeast

Introduction

Almost all yeast-based food industries are steadily expanding their use of active dry yeast (ADY) because of its greater genetic stability at room temperature and lower transport and storage costs. Unfortunately, most laboratory-developed industrial yeast strains, as well as strains isolated from industrial environments, have the biotechnological handicap of losing viability during the drying process (Dupont *et al.*, 2014). Therefore, such strains are excluded from the commercial catalogues of yeast manufacturers, awaiting a breakthrough that would allow their desiccation to be optimized. In a previous study, we performed a genetic screen of the *Saccharomyces cerevisiae* deletion library for mutants sensitive to dehydration stress (Rodríguez-Porrata *et al.*, 2012b). Among the genes characterized as essential for overcoming

dehydration stress, only five (*SIP18*, *STF2*, *GRE1*, *YJL144w*, and *NOP6*) were found to have protective effects against dehydration stress when overexpressed (Rodríguez-Porrata *et al.*, 2012b; López-Martínez *et al.*, 2012). Recent studies investigating whether the response to desiccation involves regulation at the transcriptional and/or translational level detected changes in genes involved in lipid binding and synthesis, protein synthesis and mobility, and metabolism (Novo *et al.*, 2007; Singh *et al.*, 2005; Miermont *et al.*, 2007; Rossignol *et al.*, 2006; Nakamura *et al.*, 2008). However, correlations were rare between these transcriptomic studies and genetic screens using the *S. cerevisiae* deletion library of mutants sensitive to dehydration stress (Rodríguez-Porrata *et al.*, 2012b; Ratnakumar *et al.*, 2011; Cubillos *et al.*, 2011). In contrast, haploid strains overexpressing yeast genes encoding hydrophilic proteins (Stf2p, Sip18p, Gre1p, Yjl144wp, and Nop6p), which are essential for overcoming dehydration stress, are tolerant of dry conditions (Rodríguez-Porrata *et al.*, 2012b; López-Martínez *et al.*, 2012).

On the other hand, Rodríguez-Porrata *et al.* (2012a) showed that the knockout mutants for four nuclear apoptotic-related genes with mitochondrial functions (Δ*aif1*, Δ*nuc1*, Δ*cpr3*, and Δ*qcr7*) were hyper-tolerant of dehydration stress. Most *S. cerevisiae* genes involved in qualitative traits related to their basic biology have been identified using recombinant DNA techniques. However, many phenotypes important to industrially appear to be quantitative traits that are determined by quantitative trait loci (QTLs), such as growth temperature, ethanol tolerance, acetic acid production, sporulation rate, sake aromatic compounds production, and nitrogen utilization (Cubillos *et al.*, 2011; Yang *et al.*, 2013; Hu *et al.*, 2007; Marullo *et al.*, 2007; Deutschbauer *et al.*, 2005; Katou *et al.*, 2008; Ambroset *et al.*, 2011). Considering the large amount of genetic variability in industrial yeast, a characteristic as crucial as dehydration tolerance is likely controlled by multiple QTLs that cannot be identified by conventional molecular genetic approaches.

In this paper, we performed QTL analysis on 96 segregants derived from a cross between two haploid strains derivatives of two strains of wine yeast using statistical linkage analysis between dehydration tolerance characteristics and DNA marker genotype data. We functionally characterized two QTLs encompassing six genes involved in dehydration stress tolerance that contribute to the natural phenotypic variation in the paternal strains (Cubillos *et al.*, 2011).

Materials and Methods

Strains and plasmids

Table 1 summarizes the yeast strains and plasmids used in this study. The *RIM15*, *BST1*, *BUD27*, *BLM10*, *YFH7*, *FAB1*, *ATG18*, *CBT1*, *MRP49*, *RSM22*, and *DBR1* genes were deleted using a short-flanking homology PCR technique in which *URA3* was the selectable marker (Figure S1B) in the *Mat α* and *Mat a* versions of the WA (Hyg^R), WA (Nat^R), WE (Hyg^R), and WE (Nat^R) strains (Schiest and Gietz, 1989). Degenerative primers (shown in Table S1) were used to amplify the *URA3* deletion module from the pNSU114 plasmid (Louis and Borts, 1995). Transformants were obtained using the lithium acetate transformation protocol and selected by plating on synthetic glucose media lacking uracil (Schiest and Gietz, 1989). URA^+ transformants were selected and restreaked to obtain single colonies, for which integrations were confirmed by PCR using the primer pair URA3Fw and GENERv, a reverse primer that anneals at the downstream region of the deleted gene (Table S1). The URA3 module was deleted from the WE, *Δatg18* strain by transforming single mutant strains with the PCR DNA fragment obtained using the ATGufw-ATGurv primer pair from the *atg18*::*URA3* locus. The transformants, which were able to grow in the presence of 5FOA and unable to grow on SC^{-ura} medium, were further evaluated by PCR. The validated WE, *Δatg18u* strain was further transformed, as mentioned previously, to obtain the WE, *Δatg18u*, *Δfab1* strain. Haploid strains with opposite mating types were crossed on yeast peptone dextrose agar (YPDA) medium supplemented with 100 $\mu g \cdot ml^{-1}$ hygromycin B and 200 $\mu g \cdot ml^{-1}$ nourseothricin sulfate. Diagnostics for isolates from individual colonies were made with the *MAT* locus by PCR using WA (Nat^R) and WE (Hyg^R) as tester strains (Huxley *et al.*, 1990). Recombinant DNA techniques were carried out according to standard protocols (Sambrook *et al.*, 2001). The amplification reactions contained a 1x PCR buffer, 1.25 mM dNTPs, 1.0 mM $MgCl_2$, 0.3 μM of each primer, 2 $ng \cdot \mu l^{-1}$ template DNA, and 3.5 U DNA Polymerase in a total volume of 100 μl. All reactions were performed using a PCR thermal cycler for 25 cycles, as follows: denaturation, 2 min at 94°C; primer annealing, 30 s at 55°C; and primer extension, 1.5 min at 68°C.

Table 1. Strains and plsmid used in the study

Strain	Relevant characteristics	References
BY4742	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*	Bryant and Stevens, 1998
DBVPG6044 (WA *Hyg*^{R})	*MATα, ho::HygMX, ura3::KanMX*	Kim *et al.*, 2000
DBVPG6044 (WA *Nat*^{R})	*MATα, ho::NatMX, ura3::KanMX*	Kim *et al.*, 2000
DBVPG6765 (WE *Hyg*^{R})	*MATα, ho::HygMX, ura3::KanMX*	Kim *et al.*, 2000
DBVPG6765 (WE *Nat*^{R})	*MATα, ho::NatMX, ura3::KanMX*	Kim *et al.*, 2000
Y12 (SA *Hyg*^{R})	*MATα, ho::HygMX, ura3::KanMX*	Kim *et al.*, 2000
YPS128 (NA *Hyg*^{R})	*MATα, ho::HygMX, ura3::KanMX*	Kim *et al.*, 2000
WE/NA	WE *Nat*^{R}/NA *Hyg*^{R}	Cubillos *et al.*, 2011
WE/WA	WE *Nat*^{R}/ WA *Hyg*^{R}	Cubillos *et al.*, 2011
WA/WE	WA *Nat*^{R}/ WE *Hyg*^{R}	This work
WE/SA	WE *Nat*^{R}/ SA *Hyg*^{R}	Cubillos *et al.*, 2011
96 spores WE/NA	F1 from WE *Nat*^{R}/NA *Hyg*^{R}	Cubillos *et al.*, 2011
96 spores WE/WA	F1 from WE *Nat*^{R}/ WA *Hyg*^{R}	Cubillos *et al.*, 2011
96 spores WE/SA	F1 from WE *Nat*^{R}/ SA *Hyg*^{R}	Cubillos *et al.*, 2011
WA, *Δrim15*	*MATα, ho::NatMX, rim15::URA3*	This work
WA, *Δbst1*	*MATα, ho::NatMX, bst1::URA3*	This work
WA, *Δbud27*	*MATα, ho::NatMX, bud27::URA3*	This work
WA, *Δblm10*	*MATα, ho::NatMX, blm10::URA3*	This work
WA, *Δyfh7*	*MATα, ho::NatMX, yfh7::URA3*	This work
WA, *Δfab1*	*MATα, ho::NatMX, fab1::URA3*	This work
WA, *Δatg18*	*MATα, ho::NatMX, atg18::URA3*	This work
WA, *Δcbt1*	*MATα, ho::NatMX, cbt1::URA3*	This work
WA, *Δmrp49*	*MATα, ho::NatMX, mrp49::URA3*	This work
WA, *Δrsm22*	*MATα, ho::NatMX, rsm22::URA3*	This work
WA, *Δdbr1*	*MATα, ho::NatMX, dbr1::URA3*	This work
WE, *Δrim15*	*MATα, ho::NatMX, rim15::URA3*	This work
WE, *Δbst1*	*MATα, ho::NatMX, bst1::URA3*	This work
WE, *Δbud27*	*MATα, ho::NatMX, bud27::URA3*	This work
WE, *Δblm10*	*MATα, ho::NatMX, blm10::URA3*	This work
WE, *Δyfh7*	*MATα, ho::NatMX, yfh7::URA3*	This work
WE, *Δfab1*	*MATα, ho::NatMX, fab1::URA3*	This work
WE, *Δatg18*	*MATα, ho::NatMX, atg18::URA3*	This work
WE, *Δrpl2a*	*MATα, ho::NatMX, rpl2a::URA3*	This work
WE, *Δcbt1*	*MATα, ho::NatMX, cbt11::URA3*	This work
WE, *Δmrp49*	*MATα, ho::NatMX, mrp49::URA3*	This work
WE, *Δrsm22*	*MATα, ho::NatMX, rsm22::URA3*	This work
WE, *Δdbr1*	*MATα, ho::NatMX, dbr1::URA3*	This work
WE, *Δatg18u*	*MATa, ho::HygMX, atg18::ura3*	This work
WE, *Δatg18u*, Δ*fab1*	*MATa, ho::HygMX, atg18::ura3, fab1::URA3*	This work
WA/*Δrim15*^{WE}	WA *Hyg*^{R}/WE, *Δrim15*	This work
WA/*Δbst1*^{WE}	WA *Hyg*^{R}/WE, *Δbst1*	This work
WA/*Δbud27*^{WE}	WA *Hyg*^{R}/WE, *Δbud27*	This work
WA/*Δblm10*^{WE}	WA *Hyg*^{R}/WE, *Δblm10*	This work
WA/*Δyfh7*^{WE}	WA *Hyg*^{R}/WE, *Δyfh7*	This work
WA/*Δfab1*^{WE}	WA *Hyg*^{R}/WE, *Δfab1*	This work
WA/*Δatg18*^{WE}	WA *Hyg*^{R}/WE, *Δatg18*	This work
WA/*Δrpl2a*^{WE}	WA *Hyg*^{R}/WE, *Δrpl2a*	This work
WA/*Δcbt1*^{WE}	WA *Hyg*^{R}/WE, *Δcbt1*	This work
WA/*Δmrp49*^{WE}	WA *Hyg*^{R}/WE, *Δmrp49*	This work
WA/*Δrsm22*^{WE}	WA *Hyg*^{R}/WE, *Δrsm22*	This work
WA/*Δdbr1*^{WE}	WA *Hyg*^{R}/WE, *Δdbr1*	This work
WE/*Δrim15*^{WA}	WE *Hyg*^{R}/WA, *Δrim15*	This work
WE/*Δbst1*^{WA}	WE *Hyg*^{R}/WA, *Δbst1*	This work
WE/*Δblm10*^{WA}	WE *Hyg*^{R}/WA, *Δblm10*	This work
WE/*Δyfh7*^{WA}	WE *Hyg*^{R}/WA, *Δyfh7*	This work
WE/*Δfab1*^{WA}	WE *Hyg*^{R}/WA, *Δfab1*	This work
WE/*Δatg18*^{WA}	WE *Hyg*^{R}/WA, *Δatg18*	This work
WE/*Δcbt1*^{WA}	WE *Hyg*^{R}/WA, *Δcbt1*	This work
WE/*Δmrp49*^{WA}	WE *Hyg*^{R}/WA, *Δmrp49*	This work
WE/*Δrsm22*^{WA}	WE *Hyg*^{R}/WA, *Δrsm22*	This work
WE/*Δdbr1*^{WA}	WE *Hyg*^{R}/WA, *Δdbr1*	This work
WA/*Δatg18u*^{we}, Δ*fab1*^{we}	WA *Nat*^{R}/WE, *Δatg18u*, *Δfab1*	This work
Plasmid		
pNSU114		Weedon *et al.*, 2008

Growth conditions and desiccation-rehydration process

Yeast strains were grown in shake flasks at 150 rpm in SC medium containing 0.17% yeast nitrogen base, 2% glucose, 0.5% $(NH_4)_2SO_4$, and 25 $mg \cdot l^{-1}$ uracil. The desiccation-rehydration process and yeast viability assays were performed as previously described (Rodríguez-Porrata *et al.*, 2011).

Linkage analysis

Linkage analysis was performed using the rQTL software, and the LOD score was calculated using a normal model (Cubillos *et al.*, 2011; Broman *et al.*, 2003; Salinas *et al.*, 2012). Briefly, the significance of a QTL was determined from permutations. For each trait and cross, we permuted the phenotype values within tetrads 1,000 times and recorded the maximum LOD score each time. A QTL was considered significant if its LOD score was greater than the 0.05 tail of the 1,000 permuted LOD scores.

RNA isolation and cDNA synthesis

The total RNA was obtained from: WE, WA, WE,*Δatg18*, WE,*Δfab1*, WA,*Δatg18*, WA,*Δfab1*, and WA/*Δatg18u*^{WE}, *Δfab1*^{WE} yeast cells using a GeneJET RNA Kit (Thermo Scientific, Lithuania) according to the manufacturer's protocol. The RNA was resuspended in 100 µL RNase-free water. The DNase I RNAase free kit (Fermentas, Thermo Scientific) was used to remove the 16 genomic DNA from the RNA preparations. The RNA was quantified with a spectrophotometer (Nanodrop 1,000 Spectrophotometer, Thermo Scientific) at an absorbance of 260 nm and tested for purity (by the A260/280 ratio) and integrity by denaturing gel electrophoresis. The first strand of cDNA was reverse transcribed from 1 µg total RNA from each sample using a First Strand cDNA Synthesis Kit (Fermentas, Thermo Scientific) according to the manufacturer's protocol. An identical reaction without the reverse transcription was performed to verify the absence of genomic DNA. The cDNA was subsequently amplified by PCR using yeast strain specific couple of primers forward-reverse for: *ATG18*, *FAB1*, *ALG9* and *TAF10* genes (Table S1).

Real-time RT-PCR

Quantitative PCR for *ATG18* and *FAB1*, was carried out using a Real Time qPCR kit according to the manufacturer's protocol and was analysed on a Real-Time PCR Detection System. The thermal cycling was composed of an initial step at 50°C for 2 min followed by a polymerase activation step at 95°C for 10 min and a cycling step

with the following conditions: 40 cycles of denaturation at 95°C for 15s, annealing at 63°C for 1 min, and extension at 72°C for 1 min. Oligonucleotides of varying lengths produce dissociation peaks at different melting temperatures. Therefore, at the end of the PCR cycles, the PCR products were analysed using a heat dissociation protocol to confirm that a single PCR product was detected by the SYBR Green dye. The fluorescence data was acquired at the 72°C step. The threshold cycle (C_t) was calculated using a software to indicate significant fluorescence signals above the noise during the early cycles of amplification. The software calculated copy numbers for the target samples from the Ct using interpolation from the standard curve. The relative levels of expression of the target genes were measured using *ALG9* and *TAF10* mRNA as an internal control and calculated according to the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

Microscopy

Cultures of strains were grown to the stationary phase in selective medium. The cells were washed with 1× PBS buffer (pH 7.4) and fixed in 70% ethanol for 10 min at room temperature. Fluorescence was viewed using a fluorescence microscope. A digital camera and a software were used for image acquisition.

Statistical analysis

To determine the statistical significance of data the results were analysed by one-way ANOVA, the Shapiro-Wilk test and the Scheffé test were carried out using a statistical software package. Statistical significance was set at $p<0.001$.

Results

Variation in dehydration stress tolerance in recombinant yeast populations

Using a colony-counting assay, desiccation tolerance was assessed for a set of three recombinant populations of 96 segregants generated from a cross of divergent *S. cerevisiae* isolates (WE [Wine European] x WA [West African], WE x NA [North American], and WE x SA [Sake]) previously described (Figure S1A) (Cubillos *et al.*, 2011). The mean CFU (colony-forming units) per ml value for survival after rehydration was calculated, taking into account the viability before drying (Figure 1A-C). The *W* value obtained from the Shapiro-Wilk test carried out with the three sets of

segregants were lower than 0.5, therefore, for an α level of 0.05, the phenotypic distributions of segregants did not show a normal distribution, suggesting a polygenic contribution to cellular desiccation tolerance (Figure 1A-C). The highest number of transgressive segregants (24%) was observed in the cross between the low dehydration stress-resistant strains WE (20.3%) and WA (49.4%) (Figure 1A). However, when the highly sensitive WE strain was crossed with the resistant SA and NA strains (75.9% and 70.5%, respectively), approximately 5.5% of segregants exceeded the phenotypic range of their parents by at least 2 SD, criteria previously used to name these segregants as transgressive, (Figure 1B-C) (Marullo *et al.*, 2006).

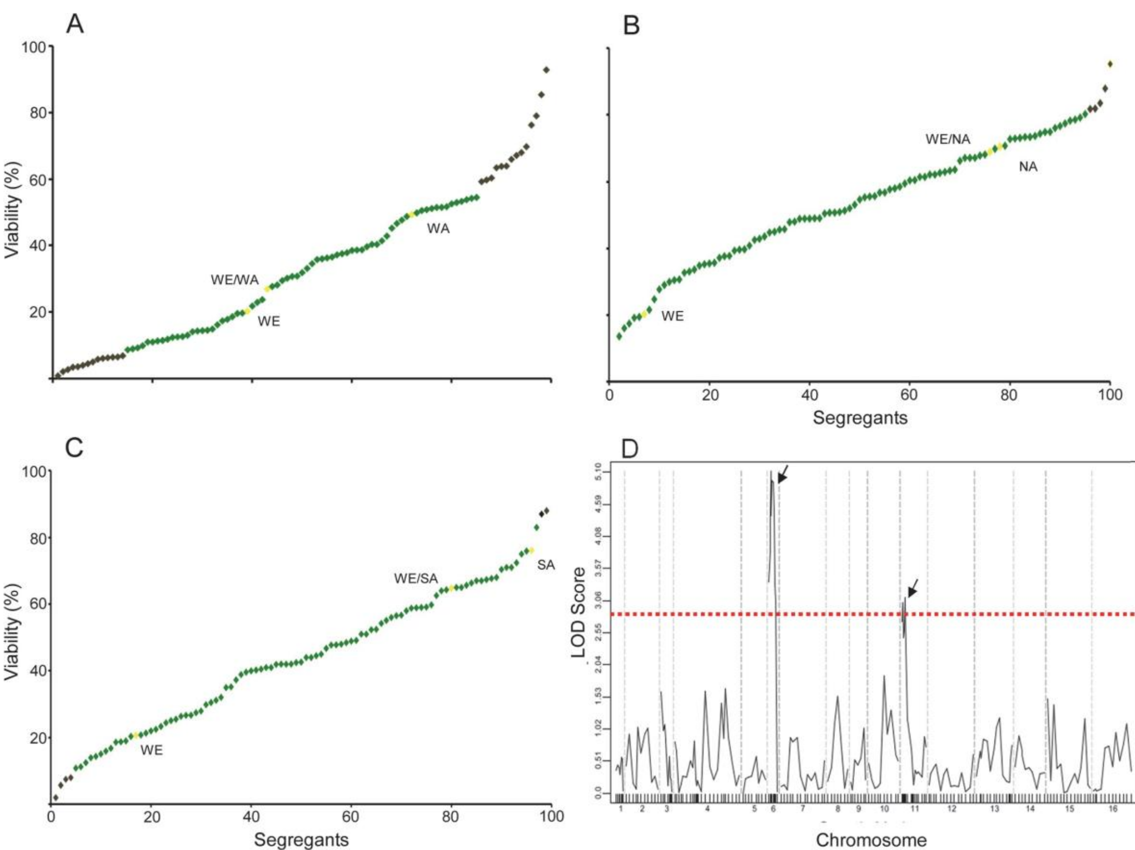

Figure 1. Viability rate variation after dehydration stress. Viability rate values are shown on the *y*-axis for the 96 ranked segregants of the WE x WA cross (A), WE x NA cross (B), and WE x SA cross (C). Dots indicate segregants with transgressive phenotypes (exceeding two parental standard deviations, black), parental and hybrid strains (yellow), and segregants within the phenotypic range of the parental strains (green). D) Linkage analysis for dehydration stress tolerance from WE/WA segregants. The chromosomes are displayed on the *x*-axis, and LOD viability values, according to each molecular marker across the 16 yeast chromosomes, are displayed on the *y*-axis. The significant LOD score threshold is indicated by a red line and was determined by a permutation test. The significant QTLs are indicated by arrows.

By running a linkage analysis using ~200 previously reported genotype markers, we evaluated whether the different genotypes correlated with the viability trend observed in the WE/WA strain segregants (Cubillos *et al.*, 2011). Only the genetic markers *Y034W*, *BST1*, *FRS2*, *RPN11*, *ROG3*, *TRP3*, and *FAS1* showed significant differences ($p<0.005$). The same analysis performed for the segregants from the WE/NA and WE/SA strains did not show any correlation between genomic region and cell viability.

Identification of QTLs involved in dehydration tolerance

To identify the QTL intervals responsible for natural phenotypic variations in dehydration stress, linkage analysis was performed based on the cellular viability after stress induction and the genotypes of the 96 F1 segregants (Cubillos *et al.*, 2011, Louis and Borts, 2009). In total, two significant regions were mapped using the marker regression model and permutation method in the WE x WA cross, allowing the identification of 15 candidate genes (Figure 1D; Table 2). A region in chromosome XI (from 37 to 137 kb) with a peak LOD score of 3.10 was identified and after further inspections, we identified seven candidate genes (*CBT1*, *YKT6*, *FAS1*, *MRP49*, *RSM22*, *DBR1* and *AVT3*) within this QTL. In the second QTL (Chr VI, LOD 5.1), eight candidate genes (*RIM15*, *BST1*, *BUD27*, *BLM10*, *YFH7*, *FAB1*, *ATG18* and *ROG3*) were identified between 65 KB and 196 KB. After a sequence alignment, only 11 of the genes encompassed by either QTL interval (*RIM15, BST1 BUD27, BLM10, YFH7, FAB1, ATG18, CBT1, MRP49, RSM22* and *DBR1*) contained single nucleotide polymorphisms (SNPs) (Table 2). Furthermore, the SNPs did not create premature stop codons in the coding sequence of the WE and WA strains. Among these genes, only *BUD27*, *FAB1*, and *CBT1* were found to be necessary for the yeast to overcome desiccation stress (Rodríguez-Porrata *et al.*, 2012b; Ratnakumar *et al.*, 2011; Salinas *et al.*, 2012).

Table2. The position in the genome, significance value, genes in the respective regions and the differences in the amino acid sequence for each gene in WE strain versus WA are described. – allele without mismatch

Chromosome	QTL's	Position (cM)	LOD	Gene / Position	Position of amino acid change *WA* allele→*WE* allele
VI	Y034w	65	3.85	*RIM15* / 69.11	161 E → K; 240 S→G; 249 E→D; 251 T→S; 366 T→S; 399 V→A; 771 R→P; 1020 T→I; 1022 C→Y
	BST1	84	5.11	*BST1* / 84.14	202 A→T; 221 N→D; 253 A→P; 432 N→D; 438 K→M; 506 Q→L; 610K→R; 636S→W; 849 D→V
				BUD27 /90.9	32 Δ→E; 33D→Y; 75 S→F; 177 E→G; 182 D→E
	HTX10	111	4.95	*BLM10* / 123.47	99 Q→R; 220 T→A; 258 G→A; 729 S→N; 759 I→V; 791 N→D; 902 C→Y; 1102 R→K; 1315 G→S; 1444 D→N; 1586 P→A; 1592 R→C; 1698 T→A; 1782 G→D; 1861 D→Y; 1900 I→V; 1971 M→I
	ARS605	136	4.93	-	
	RPN11	153	4.50	*YFH7* / 159.29	109 V→I; 138 A→T; 149 V→A
	YFR016c	180	3.32	*FAB1* / 184.50	120 S→N; 126 N→S; 333 A→S; 583 Δ→N; 1273 N→D; 1300 Y→H; 1524 G→E; 1604 R→M; 1780 P→S; 1878 I→M; 1882 S→A; 1884 Q→Δ
				ATG18 / 194.81	195 N→S
	ROG3	196	2.40	-	
XI	TRP3	37	2.72	*CBT1* / 47.15	29 S→G; 109 T→A
	ARS1103	58	3.03	-	
	YKT6	75	2.46	-	
	FAS1	103	2.58	-	
	TP05	121	3.10	*MRP49* / 133.72	131 G→R
	PIR1	142	2.34	*RSM22* / 159.45	228 E→K; 474 D→S; 619 S→N
				DBR1 / 167.61	223 Q→R; 286 K→E; 325 N→D
	AVT3	173	1.16	-	

Dissection of the QTLs associated with stress tolerance

To identify causative genes within the mapped QTL intervals on chromosomes VI and XI, we generated a set of haploid strains with deletions in the candidate genes (Table 1). Then, their desiccation tolerance capacity was assessed (Figure 2). After rehydration, four strains (WA, Δ*bud27*; WA, Δ*fab1*; WA, Δ*atg18*; and WA, Δ*cbt1*) exhibited a similar reduction in cell viability values, which were ~20% lower than in the WA strain (49%). Surprisingly, the same set of gene deletions in the WE genetic background showed the opposite effect, with viability values ~30% higher than the WT. In addition, both versions of the Δ*dbr1* strain showed significantly higher viability values after dehydration stress compared with the WT WA and WE strains (20% and 80%, respectively). Furthermore, the WE, Δ*rsm22* strain displayed 30% higher viability than its reference strain, whereas the WA, Δ*rsm22* strain had similar viability to the WA strain. The viabilities of the Δ*rim15*, Δ*bst1*, Δ*blm10*, Δ*yfh7*, and Δ*mrp49* strains were not significantly different from the WT strains, WA and WE,

suggesting that these genes are not involved in desiccation-rehydration stress resistance. Therefore, two-thirds of the WE mutants enhanced dehydration stress tolerance, suggesting that the *BUD27*^{WE}, *FAB1*^{WE}, *ATG18*^{WE}, *CBT1*^{WE}, and *RSM22*^{WE} alleles have a detrimental effect on the ability of the WE strain to overcome this type of stress.

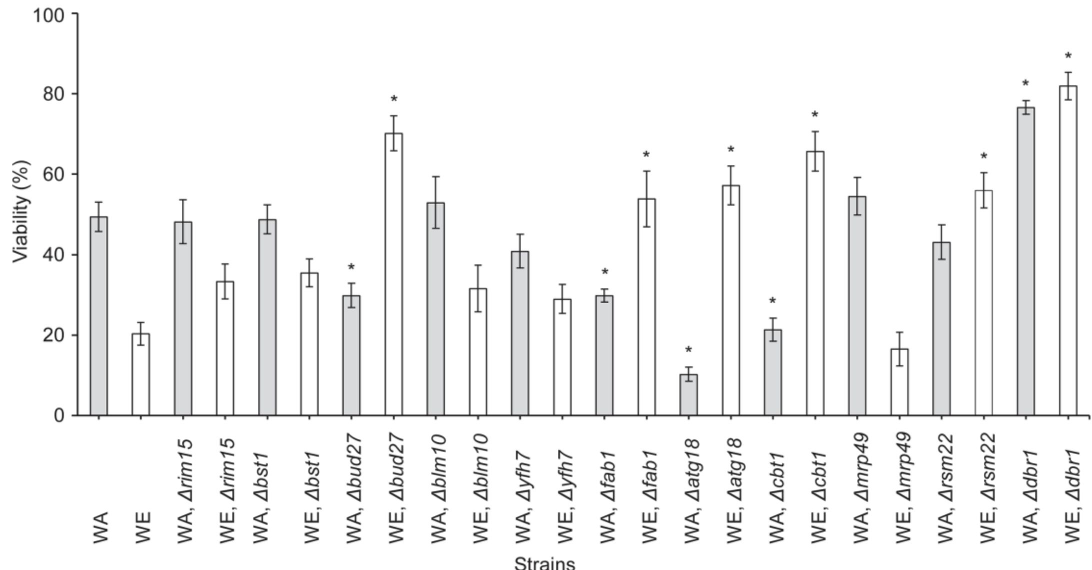

Figure 2. Effect of knockout haploid strains on yeast viability after dehydration-rehydration stress (DRS). The scale of viability (%) indicates the percentage of experimental values for the different strains. The values shown are means of n = 3 independent samples ± SD. *Significant differences (p<0.01) between knockout and its own parental strains.

To confirm the impact of these alleles on dehydration stress, we used a reciprocal hemizygosity analysis (Figure S1B) (Marullo *et al.*, 2006). A set of isogenic hybrid strains was developed by crossing the haploid knockout strains with the complementary WA (*Nat*^{R}) or WE (*Hyg*^{R}) strain [e.g., WA (*Nat*^{R}) x WE Δ*rim15* (*Hyg*^{R}) or WA Δ*rim15* (*Hyg*^{R}) x WE (*Nat*^{R}), Table 1]. The desiccation tolerance of the hemizygous strains was measured (Figure 3). The WA/Δ*bud27*^{WE} strain showed ~40% higher viability than the WA/WE strain, which correlated with the increased viability of the WE, Δ*bud27* strain after stress induction, suggesting an adverse effect of the *BUD27*^{WE} allele on stress resistance. Additionally, the WE/Δ*bud27*^{WA} strain could not be obtained, suggesting a certain level of incompatibility between the *BUD27*^{WE} allele and the WA genetic background. After dehydration stress induction, the hybrid strains carrying *FAB1*^{WA}, *ATG18*^{WA}, *CBT1*^{WE}, and *RSM22*^{WA} showed viability values nearly 30% higher than the hybrids carrying *FAB1*^{WE}, *ATG18*^{WE}, *CBT1*^{WA}, and *RSM22*^{WE} and the reference strains. The detrimental effects of the *FAB1*^{WE}, *ATG18*^{WE}, *CBT1*^{WA}, and

$RSM22^{WE}$ alleles on overcoming dehydration stress were concomitant with the enhanced viability values obtained for the WE, Δ*fab1*, WE, Δ*atg18*, WA, Δ*cbt1*, and WE, Δ*rsm22* strains (Figure 2). Furthermore, hybrids carrying either the $DBR1^{WE}$ or $DBR1^{WA}$ allele exhibited 30% higher viability than the heterozygous strains (Figure 3). From the cell viability results for the WA, Δ*dbr1*, WE, Δ*dbr1* and heterozygous strains, a correlation can be assumed between the increasing number of *DBR1* allele copies per cell and the decreasing desiccation survival rate. The desiccation tolerances of a collection of 4,850 viable mutant haploid strains (BY4742) were previously assessed (Rodríguez-Porrata *et al.*, 2012b; Shima *et al.*, 2008). For the genes above, only the Δ*rsm22* and Δ*dbr1* strains (BY4742 background) exhibited significantly higher viability values after stress induction (73% and 77%, respectively) compared with the BY4742 strain. The viability of the Δ*rim15*, Δ*bst1*, Δ*bud27*, Δ*yfh7*, Δ*fab1*, Δ*atg18*, and Δ*cbt1* strains did not significantly differ from the reference strain (34%) (Rodríguez-Porrata *et al.*, 2012a). However, the BY4742, Δ*mrp49* strain showed 20% viability, which contrasts with the unchanging viability values for the WA, Δ*mrp49* and WE, Δ*mrp49* strains. These results confirm that $RSM22^{WE}$, which has 98% sequence identity to the $RSM22^{BY4742}$, $DBR1^{WA}$, $DBR1^{WE}$, and $DBR1^{BY4742}$ gene products, has a detrimental effect on dehydration stress tolerance.

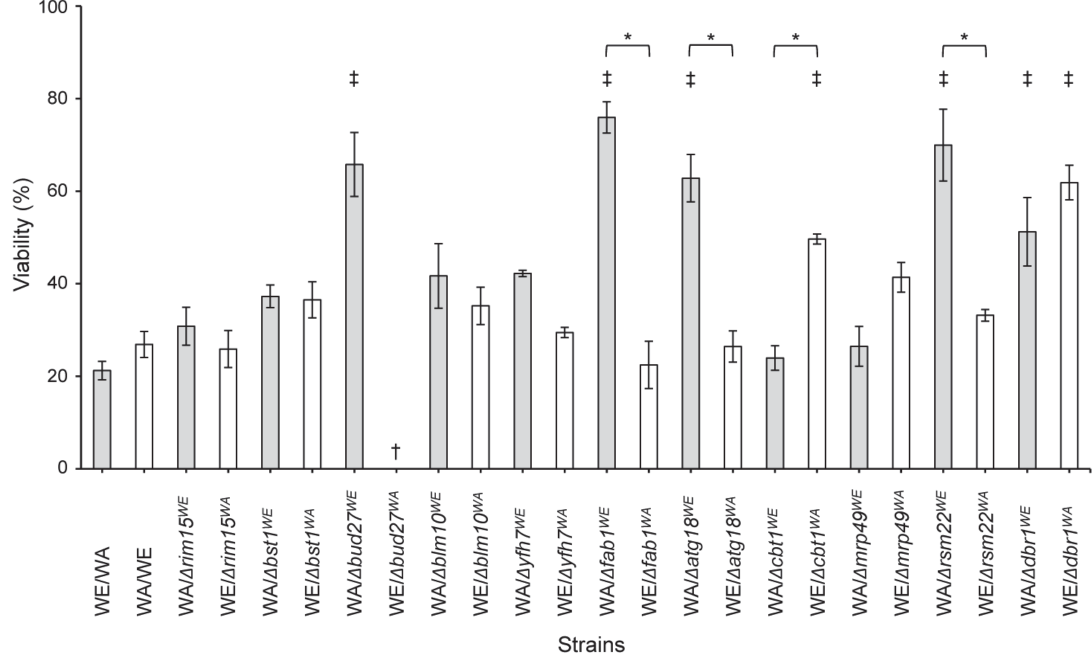

Figure 3. Hybrid viability after DRS. The scale of viability (%) indicates the percentage of experimental values for the different strains. The values shown are the means of at least $n = 3$ independent samples ± SD. † Non-viable strain. *Significant differences at $p<0.01$ between hemizygous strains. ‡ Significant differences at $p<0.01$ between the hemizygous and reference strains.

The ATG18^{WE} allele compromises vacuole function

Atg18p is a key component in retrograde membrane trafficking from the vacuole to the Golgi apparatus via the endosome and is also an apparent effector and modulator of phosphatidylinositol (3,5)-bisphosphate [PtdIns(3,5)*P*2] (Efe *et al.*, 2007). It should be noted that the vacuole is responsible for amino acid storage and therefore represents the cellular reserve of nitrogen and phosphate. When yeast cells are exposed to starvation conditions, such as upon entrance into the stationary phase or during sporulation, vacuolar hydrolases are upregulated to obtain recycled nutrients through the turnover of macromolecules (Klionsky *et al.*, 1990). It follows then that malfunctions in the nutrient storage or recycling machinery are likely to compromise cell viability. Homozygous diploid Δ*atg18* is defective in autophagy prior to vacuole fusion of autophagosomes, causing the development of cell sensitivity to nitrogen starvation and non-sporulating cells (Barth *et al.*, 2001). The hybrid carrying *ATG18^{WA}* showed 35% higher asci formation than the WE (*Nat^R*)/WA (*Hyg^R*) and WA (*Nat^R*)/WE (*Hyg^R*) strains, at 7% and 3%, respectively. However, the hybrid carrying *ATG18^{WE}* showed the lowest asci formation, at 0.5% of the total cells (Figure 4A). The wild-type and hemizygous strains were first grown to the mid-log phase and then shifted to nitrogen starvation conditions, and their viability was determined over time (Figure 4B). The hybrid strains survived nine days of nitrogen starvation with no significant decrease in viability. In contrast, the number of viable cells for the hybrid carrying *ATG18^{WE}* and the hybrid carrying *ATG18^{WA}* decreased by up to 60% and 20%, respectively, over the same time period. Additionally, Δ*atg18* cells exhibited phenotypic defects, including non-acidic and conspicuous vacuoles and the loss of osmotic stress tolerance (Yamamoto *et al.*, 1995). To determine putative changes in vacuole morphologies, samples of aerated wild-type, WA/Δ*atg18^{WE}*, and WE/Δ*atg18^{WA}* cells in the stationary phase were analysed by fluorescence microscopy using FM4-64 and the blue fluorescent dye Arg-CMAC, which accumulates in acidic vacuoles (Figure 4D).

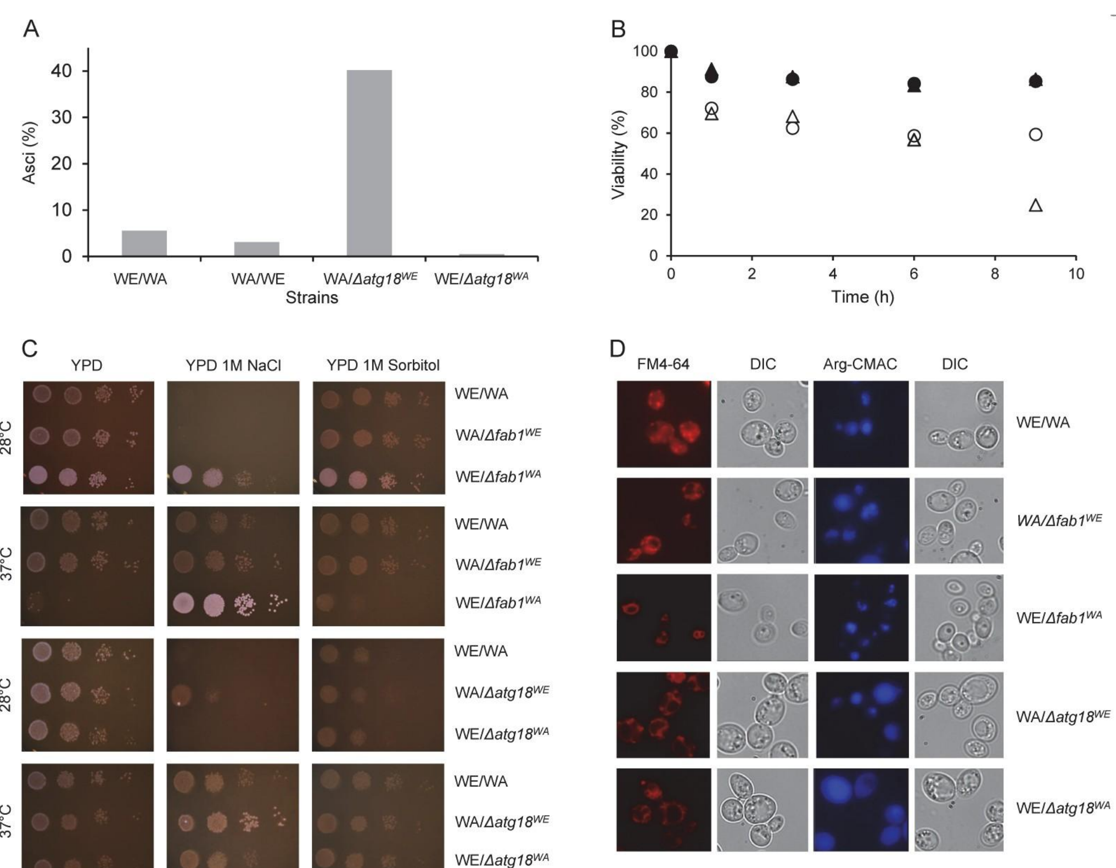

Figure 4. Phenotypic characterization of *ATG18* and *FAB1* alleles. A) After 48 hours on 1% K-acetate, the count of asci were expressed as a percentage of total cells. B) Effect of nitrogen starvation on cell viability of the Δ*atg18* strains. The hybrid WE (*Nat^R*)/WA (*Hyg^R*) (●), WA (*Nat^R*)/WE (*Hyg^R*) (▲), WE/*ATG18* ^{WA} (o), and WA/*ATG18* ^{WE} (Δ). The scale of viability (%) indicates the percentage of viable cells for the different strains against the time in starvation medium. Values are the mean of triplicate measurements, and the standard deviation was less than 15%. C) *FAB1*^{WA} and *ATG18*^{WE} rescue cells from ionic-hyperosmotic stress at 37°C plated on YPD medium, YPD medium containing 1 M NaCl, and 1 M sorbitol. D) Hemizygous cells show vacuole fragmentation and vacuole acidification deficiency. Each pair of image columns show phase microscopy of the same field, which shows cells stained with FM4-64 to visualize vacuole membrane, pH vacuolar dye cell blue Arg-CMAC, and the differential interference contrast (DIC) images.

Both Δ*atg18* hemizygous strains had larger vacuoles than the WE/WA cells, but the hybrid carrying *ATG18*^{WE} showed abnormal vacuolar acidification compared with the hybrid carrying *ATG18*^{WA} and the WE/ WA strains. To assess the consequences of the *ATG18*^{WE} allele, the osmotic sensitivity was tested when the cells were grown on media containing 1 M NaCl or 1 M sorbitol at 28°C and 37°C (Figure 4C). On the 1 M NaCl plates, the hybrid carrying *ATG18*^{WA} showed better growth performance at 37°C and 28°C relative to the hybrid carrying *ATG18*^{WE}. No significant growth differences were exhibited between hybrids for the other serial dilutions grown on YPD and 1 M

sorbitol at 37°C and 28°C. The data indicates that *ATG18*^{WE} may not provide adequate nutrient storage to tolerate starvation conditions, thereby inducing both low cell viability under nitrogen starvation conditions and impaired asci formation. The *ATG18*^{WE} allele was more sensitive to osmotic stress at high temperatures than the *ATG18*^{WA} allele, which correlated with the differences in dehydration tolerance observed for these alleles. Furthermore, the ionic osmotic sensitivity showed by the hybrids carrying either the *ATG18*^{WA} or the *ATG18*^{WE} allele reverted to a resistant phenotype when the cells were grown at a high temperature.

The FAB1^{WE} *allele enhances osmotic ionic stress tolerance*

Retrograde membrane traffic from the vacuole to the Golgi apparatus via the endosome depends on PtdIns(3,5)*P*2 (Gary *et al.*, 2002; Dove *et al.*, 2002). The kinase FAB1p generates PtdIns(3,5)*P*2 via phosphatidylinositol (3)-phosphate phosphorylation (Gary *et al.*, 1998; Dove *et al.*, 2004). Abnormal levels of PtdIns(3,5)*P*2 were observed in Δ*atg18* yeast cells, suggesting that Atg18p is an inhibitor of the Fab1p kinase (Merz and Westermann, 2009). Yamamoto *et al.* (1995) suggested that *fab1* mutations in yeast cells cause aberrant chromosome segregation, defects in cell surface integrity, and deficiencies in vacuole morphology and function. To determine the incidence of *FAB1* alleles in vacuole activity, WA/Δ*fab1*^{WE} and WE/Δ*fab1*^{WA} cells were grown on medium containing 1 M NaCl or 1 M sorbitol at 28°C and 37°C (Figure 4C). The hybrid carrying *FAB1*^{WE} grew on 1 M NaCl at 28°C, whereas the hybrid carrying *FAB1*^{WA} and the WE/WA strain did not. However, all of the strains grew similarly on 1 M sorbitol. At 37°C, the hybrid carrying *FAB1*^{WE} was osmoremediated on 1 M NaCl but was not recovered on 1 M sorbitol. The data indicates that ionic osmotic stress rescues the growth of *FAB1*^{WE} hemizygous cells at this non-permissive temperature. The vacuolar morphology and activity of hybrid-carrying *FAB1*^{WA} or *FAB*^{WE} in the stationary phase were analysed using FM4-64 and Arg-CMAC dyes, respectively (Figure 4D). The vacuolar acidity Arg-CMAC dye profile of the hemizygote cells was similar to that of the reference cells. However, Arg-CMAC and FM4-64 staining revealed vacuolar fragmentation in the hybrid carrying *FAB1*^{WE}, which contrasts with the single large vacuole per cell observed in both the hybrid carrying *FAB1*^{WA} and the WE/WA strain. The *FAB1*^{WE} allele is more sensitive than the *FAB1*^{WA} allele to osmotic stress at high temperatures, which correlates with the differences in dehydration tolerance observed for these alleles. Alternatively, an

isogenic strain was developed by crossing the haploid double knockout strain WE, Δ*atg18u*, Δ*fab1* with the complementary WA (*Nat*^{R}) strain (Table 1). The WA/Δ*atg18u*^{WE}, Δ*fab1*^{WE} strain showed ~60% higher viability than the WA/WE strain, which was correlated with the increase in viability of the WE, Δ*atg18u*, Δ*fab1* strain after dehydration stress, which showed 65% viability (data not shown). Surprisingly, the double knockout WA, Δ*atg18u*, Δ*fab1* strain could not be obtained. To exclude putative artificial regulatory effect of the deletions over the genes *ATG18* or *FAB1*, which are in the same chromosome at a distance of 3.5 kb, we quantified their expression in samples from WA; WE; WA, Δ*fab*1; WA, Δ*atg18*; WE, Δ*fab*1; WE, Δ*atg18* and WA/Δ*atg18u*^{WE}, Δ*fab*1 strains (Figure S2). Our data showed no statistically significant differences between the controls and the strain samples in the expression of any of the tested genes.

The CBT1 and RSM22 alleles do not show respiratory deficiencies

From a gene pool identified after a large-scale functional analysis of respiratory-deficient yeast, the mutant Δ*cbt1 and* Δ*rsm22* strains showed impaired respiratory performance (Merz and Westermann, 2009). The mitochondrial small ribosomal subunit protein Rsm22p participates in mitochondrial mRNA translation, and Cbt1p is involved in mt mRNA stabilization. Both of these proteins are essential for respiratory growth. To assess the putative effects of these alleles on respiration activity, serial dilutions of the wild-type, WA/Δ*cbt1*^{WE}, WE/Δ*cbt1*^{WA}, WA/Δ*rsm22*^{WE}, and WE/Δ*rsm22*^{WA} strains were plated on YPD and YPG media and incubated at 28°C for 24 h and48 h. No significant differences in growth were observed between the different hybrids on YPG medium with glycerol as the respiratory carbon source (Figure 5A), suggesting that the *CBT1* and *RSM22* alleles do not significantly affect the respiratory activity of hybrid cells. Therefore, both the hybrid carrying *CBT1*^{WE} and the hybrid carrying *RSM22*^{WA} enhance dehydration tolerance with no apparent variation in cellular respiration.

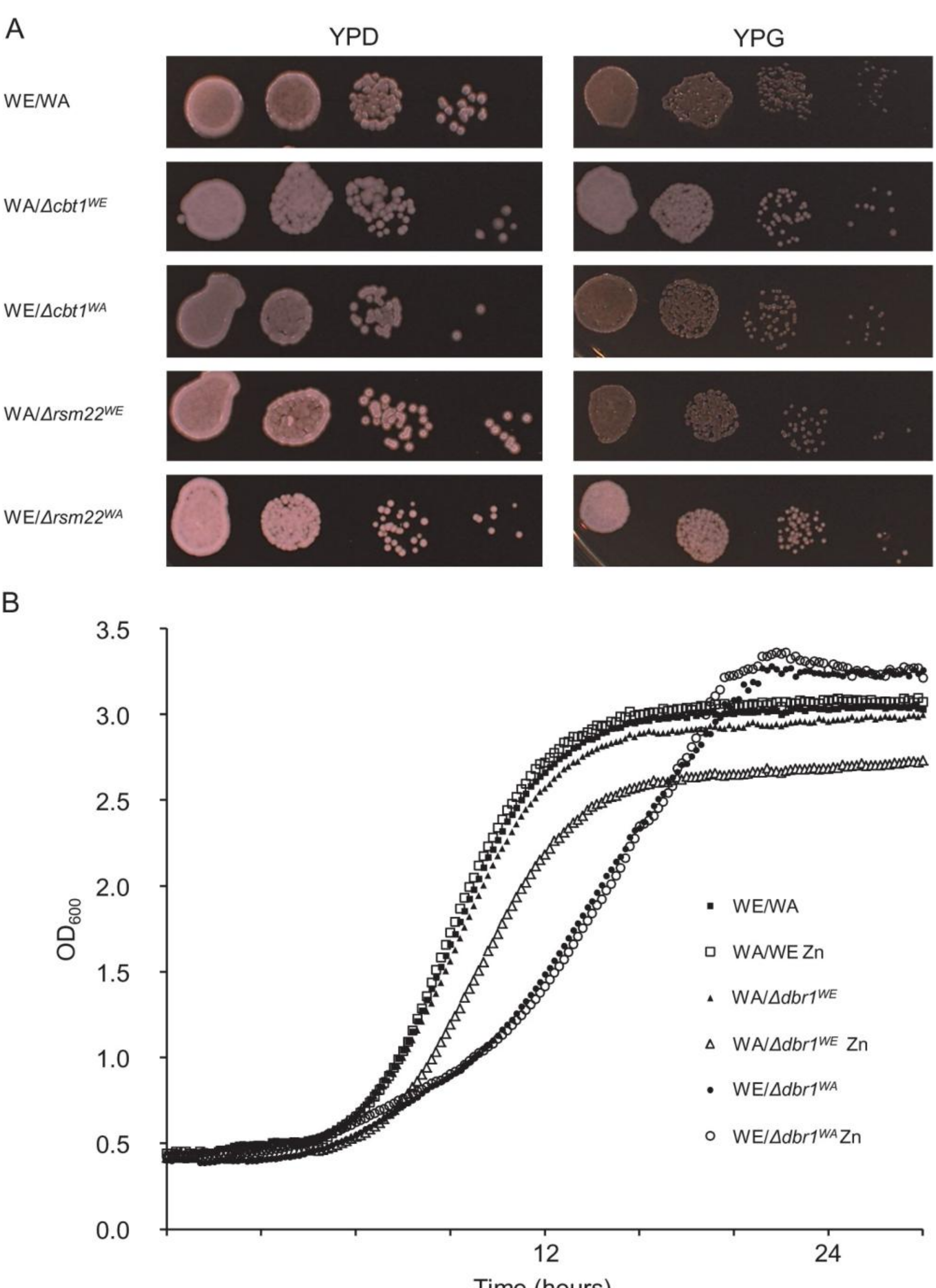

Fig 5. Phenotypic characterization of *CBT1*, *RSM22*, and *DBR1* alleles. A) *CBT1* and *RSM22* alleles did not show respiratory deficiency. Serial dilutions of heterozygous and hemizygous strain cells were plated on YPD medium and YPG medium containing 2% glycerol, which were grown at 28°C for one and two days, respectively. B) The hybrid carrying *DBR1^{WA}* shows defective competitive fitness. Optical density at 600 nm (OD_{600}) was monitored every 10 min as a growth measure at 28°C of the strains in SD medium and SD medium containing 3.5 mM $ZnCl_2$.

The DBR1^{WA} allele provides competitive disadvantages to yeast cells

The RNA lariat debranching enzyme Dbr1p is involved in intron turnover, which is required for efficient Ty1p transposition (Chapman and Boeke, 1991). The

phenotypes already described for the Δ*dbr1* strain include decreasing competitive fitness and lower resistance to zinc deficiency (Breslow *et al.,* 2008; North *et al.*,2012). We aimed to ascertain the growth performance of the Δ*dbr1* hemizygous strains in minimal medium and minimal medium supplemented with 1 μM, 3.5 mM, or 7 mM zinc dichloride (Figure 5B shows the growth with 3.5 mM $ZnCl_2$). Hybrids carrying $DBR1^{WA}$ and $DBR1^{WE}$ exhibited doubling times (DT) that were 5.8 min and 67.7 min higher, respectively, than the WE/WA strain. Both the hybrid carrying $DBR1^{WE}$ and the reference strain showed similar DT in media with or without Zn, but the hybrid carrying $DBR1^{WA}$ exhibited a 24.8 min higher DT in the presence of Zn than when grown in minimal medium alone.

Discussion

Most of the genetic determinants of dehydration tolerance in yeast are still unknown. In this paper, two dehydration-tolerant QTLs were identified using a segregating population. By analysing strains with deleted genes in each QTL and by reciprocal hemizygosity assays, six genes have been confirmed to affect the capacity of yeast cells to survive dehydration and rehydration, namely the *BUD27*, *FAB1*, and *ATG18* genes mapped to QTLs on chromosome VI and the *CBT1*, *RSM22*, and *DBR1* genes in QTLs on chromosome XI. Furthermore, their phenotypic effects have been estimated. The genes *ATG18*, *RSM22*, and *DBR1* were not found to be necessary for desiccation tolerance in yeast cells (Rodríguez-Porrata *et al.*, 2012b; Ratmakumar *et al.*, 2011). The fact that the genes mapped in our results do not fully coincide with previous genetic studies carried out with the *S. cerevisiae* deletion libraries of mutants sensitive to dehydration stress may indicate that different cellular mechanisms for overcoming stress imposition were caused by dissimilar selective forces exerted during the evolution of the yeast strains, or because the mutations present in the laboratory strains used for these studies are the effectors of these particular phenotypes (Lettre *et al.*, 2008; Maher, 2008; Weedon *et al.*, 2008; Romano *et al.*, 2010). Therefore, small discrepancies among the genes associated with cell dehydration tolerance from different studies support the idea that different allelic combinations exert different effects. The nitrogen-deficient sporulation medium contains acetate as a carbon source to promote high levels of respiration, which induce sporulation in diploid yeast strains. In *S. cerevisiae*, the Δ*atg18* mutant is defective in sporulation but does not

exhibit impaired vacuolar acidification (Barth *et al.*, 2001). The sequences of the *ATG18*^{WA} and *ATG18*^{WE} alleles revealed seven non-identical nucleotides. However, only one point mutation at nucleotide 584, from G to A, causes a single amino acid change of a serine to an asparagine residue (S195N; Table 2). Multiple sequence alignment of the WE and WA ATG18 alleles with 25 sequences of the *ATG18* gene from different *S. cerevisiae* strains annotated in the *Saccharomyces* Genome Database (SGD), as well as the Atg18p sequence characterized in this study, showed that the S residue is present in 16 genes, the N in eight genes, and the R in only one. This residue is located in the N-terminal region before the two WD40 domains and within a patch of highly conserved residues in Atg18p from *Pichia pastoris*, *Schizosaccharomyces pombe*, and *Homo sapiens* (Guan *et al.*, 2001). The immediate response of yeast cells to osmotic challenge involves the release of calcium from the vacuole and the formation of fragmented vacuoles (Li and Kane, 2008). Our results suggest that the *FAB1*^{WE} allele principally affects vacuolar morphology, which might allow the hybrid carrying *FAB1*^{WE} to adapt quickly to ionic stress. However, 1 M sorbitol osmotic stress at 37°C is lethal to these cells when the WE/ WA strain and the hybrid carrying *FAB1*^{WA} are adapted. The *FAB*^{WA} and *FAB1*^{WE} allele sequences revealed 15 non-identical nucleotides, producing differences in 12 residues (Table 2); however, only the N1273D and Y1300H mutations are located in a region of conserved residues within the Zn-finger domain (Shaw *et al.*, 2003). Furthermore, none of these residues have a high identity ratio among the Fab1p sequences from the 28 *S. cerevisiae* strains (SGD). Fab1p governs vacuole homeostasis by generating PtdIns(3,5)*P2* on the vacuolar membrane. Atg18p colocalizes with Fab1p, and its deletion causes an abnormal elevation in the levels of PtdIns(3,5)*P2*, which suggests that Atg18p is also a negative regulator of the Fab1p kinase pathway (Efe *et al.*, 2007). The hybrid carrying *FAB*^{WA} and the hybrid carrying *ATG18*^{WE} exhibit an osmotic pressure-dependent growth phenotype (Figure 4C), indicating that the genes are essential for growth only at high temperatures in the presence of osmotic ionic stress. At the permissive temperature, the hybrids carrying *FAB1*^{WA} and the hybrid carrying *ATG18*^{WE} exhibited extremely defective growth. These phenotypes are comparable to the ones exhibited by some of the temperature-sensitive isolated vacuolar protein sorting (*vps*) mutants, which require one or more vacuolar functions at the permissive temperature that cannot be provided at 37°C by other vacuolar components in these mutant cells (Bryant and Stevens, 1998).

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FUNCTIONAL CHARACTERIZATION IN VIVO OF ESSENTIAL SACCHAROMYCES CEREVISIAE'S HYDROPHILIN FOR DESICCATION TOLERANCE
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Dipòsit Legal: T 1354-2015

The *DBR1* gene is conserved in humans (*hDBR1*) and maintains the same function in both human and yeast cells (Kim *et al.*, 2000). Among other phenotypes of the Δ*dbr1* strain, decreases in competitive fitness and Zn deficiency stress resistance have been previously described (Breslow *et al.*, 2008; North *et al.*, 2012). The growth fitness of a strain with the *DBR1*^{WE} allele is affected and this strain is less sensitive to Zn stress than the *DBR1*^{WA} allele, for which the opposite effect on growth is observed. The *DBR1*^{WA} allele had K^{286} and N^{325} residues in the putative HMM domain, replacing E^{286} and D^{325}, respectively (Table 2), which are 100% conserved in other Dbr1p deduced from the genomic sequences of 26 different *S. cerevisiae* strains (SGD). The deduced sequence of Cbt1^{WA} showed two residue differences with Cbt1^{WE}, S29G, and T109A. In addition, three mutations were observed between the deduced peptide sequences of the *RSM22*^{WA} and *RSM22*^{WE} genes: E228K, D474S, and S619N (Table 2). These mutations do not affect the respiratory capacity of the different strains, thus enabling the separation of dehydration stress tolerance from respiration capacity. However, the above-mentioned variations in the sporulation efficiency of the *ATG18* hemizygous strains are not due to a pleotropic effect of the *RSM22* or *CBT1* alleles that affects cellular respiration.

The genetic approach used in this study, with a population of 96 segregants, allowed the detection of yeast dehydration resistance QTLs. The *RSM22* and *ATG18* genes enclosed within these QTLs that provide dehydration tolerance to the cell were not referenced in previous studies. Additionally, a detrimental effect on dehydration stress tolerance was shown to be provided by *DBR1* gene products. Our results further the understanding that dehydration stress tolerance is not a phenotype that results from the individual addition of independent genes. Furthermore, the monogenic approach is not suitable for summarizing all of the epistatic effects driven by a group of alleles. Currently, the successful long-term storage of living cells is of critical importance, but the contradictory results with complex eukaryotic cells make the application of a simpler model desirable. There are a number of advantages, including ease of growth and modification and well-characterized cell physiology, genetics and biochemistry, which make yeast cells the model of choice for anhydrobiotic engineering.

Supporting information

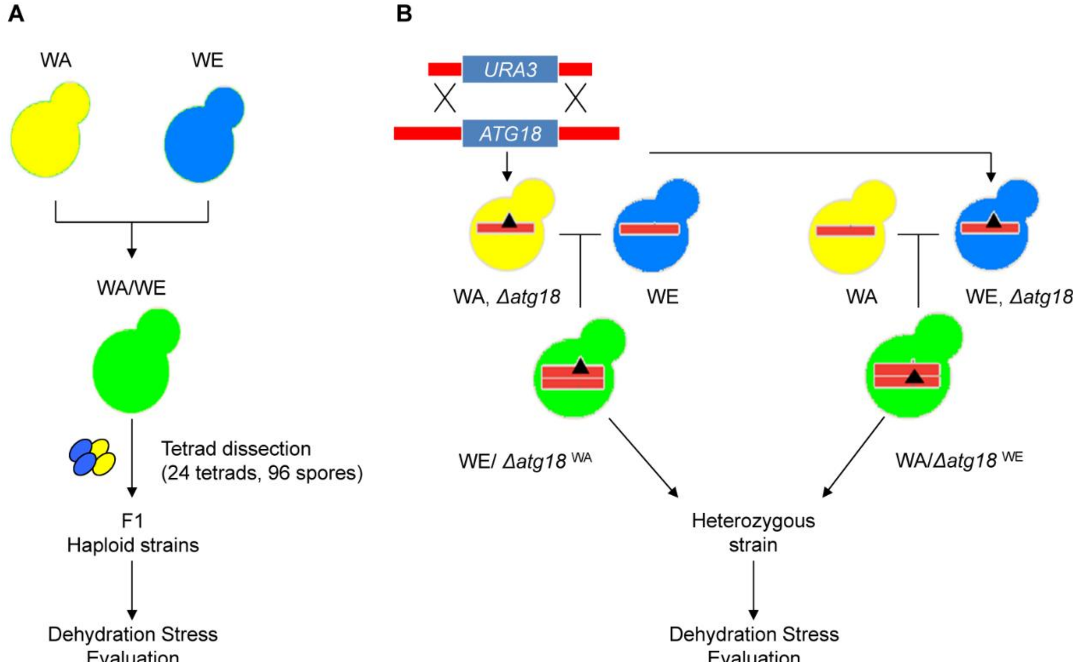

Figure S1. Diagrams of strain generation. A) Production of F1 population (Liti *et al.*, 2009). B) Haploid strains were disrupted for the identified genes (e.g., *ATG18*) using *URA3* and used to develop heterozygous diploid strains by reciprocal hemizygous crossover.

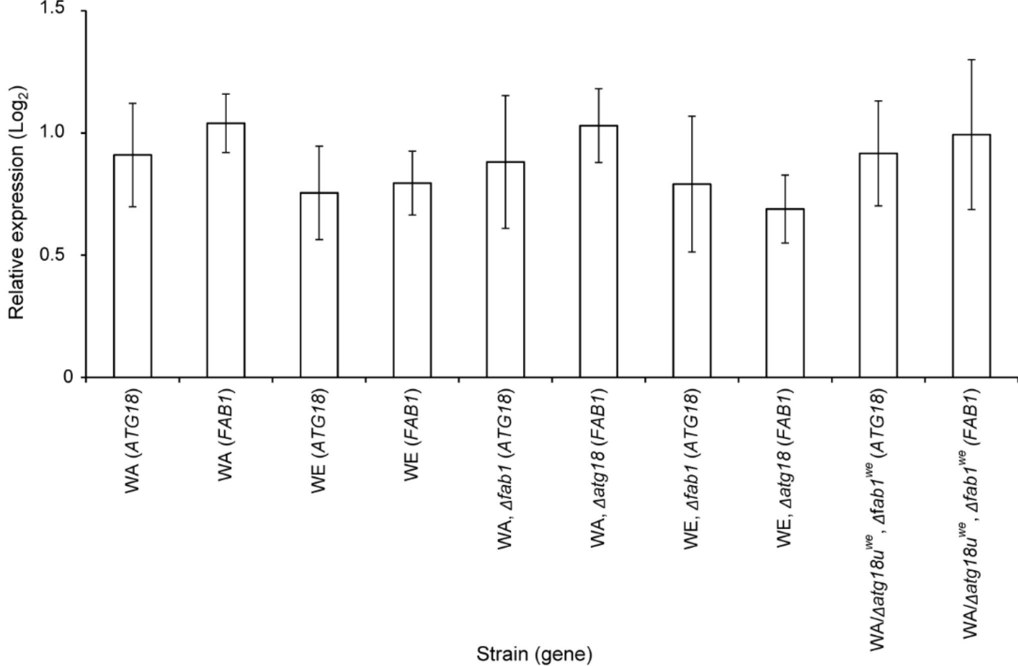

Figure S2. Quantitative real-time PCR analysis of gene expression before stress. Data represent mean relative expression ± SD (y axis, Log_2 values) of each individual gene (show at the bottom) before dehydration of different strains. Genes *ALG9* and *TAF10* were simultaneously used as constitutive reference genes as determined by the geNorm algorithm (Vandesompele *et al.*, 2002). Relative expression was calculated using REST-MCS v2 software (Pfaffl *et al.*, 2002)].

Table S1. Primers used in this study.

Primer	Nucleotide sequence (5'-3')
HXT10fw	ACGAAATCCCATACGCAAAT[1]
HXT10rv	AGCCGATGAGATGAGGATTG[1]
ARS605fw1	CCATGTAGCTTATCGCAGCA[1]
ARS605rv1	CCAAGCAGTTCTCTAGCTCCA[1]
ARS605fw2	TGGCACTTCGTATATGCAACA[1]
ARS605rv2	AGGAACCAAAAATCGCCAAC[1]
YFR016Cfw2	TGACCTTTCAATTTAGAAGATTTCC[1]
YFR016Crv2	CTGGTGTGCTTGATTACTCTGG[1]
ARS1103fw	TGGGCTATTTCATCCCATTG[1]
ARS1103rv	GAGAAGGAGGCAGCAGGAG[1]
YKT6fw	CGCCACCCAATAAGAAAAA[1]
YKT6rv	CTTGTTTGGTGTCGGCATAA[1]
TP05fw	TCGTCAGCTAAAGCAGGTAACA[1]
TP05rv	ACAAAGTCTGTGTTTATTGGATCA[1]
PIR1fw	TGAGAATTGTAGCATTACGATCTTCT[1]
PIR1rv	GCCTTTTTATGTCCTGCCAAT[1]
PIR3fw1	AGGTATGTGCGCAGCTCTTT[1]
PIR3rv1	CCGGTGACCTTACCGATCTA[1]
PIR3fw2	GAGCAACCATTTCCGAATGT[1]
PIR3rv2	ACGGCGAAATATGCCAAA[1]
RIM15fw	ATTATTCTCAGATTTGCTTTCAAGCAAAGTTTTTATTCAGTTATTTTTTTAATTATCTTTATCTTAAAATTTATAGCTTTTCAATTCAATTCATCAT[1]
RIM15rv	AGCATTTTCCCTTTTTTTTTTTCCCTTTCCTTTCTCTTGCCTCATTTGATAGAATAGATAAGCCCAGTAGAGGAAGACAGAAGCTTTTTCTTTCCAATT[1]
RIM15A1fw	ATTCTGCTTTTAATATTTCCAGATTT[1]
BST1fw	CAAGCTTTTTTCTTGCCATGATCTAGAACTCTCAGGCAATATATACAGTTAATCTTTTTTTACTGGGTTGTAGTTCAGCTTTTCAATTCAATTCATCAT[1]
BST1rv	CACACTCGAAATACTCCCTCTACTTTAAAGCATTGGCCTATATCTTAGGCTTACCATCATACAAAAATCTTCATTTCGTTAAGCTTTTTCTTTCCAATT[1]
BST1A1fw	TGATCAAAATTTACGGCTTTGA[1]
BUD27fw	GAATTTTATAGTAAACAGGTATCCTCAGACTGTAATAGCCAAGCTTTTCAATTCAATTCATCAT[1]
BUD27rv	GTTAATATAGATTCTGATTTACTTTCTGTCTCCATATGGGTAGCTTTTTCTTTCCAATT[1]
BUD27A1fw	AATTTTGTGGTCGGATCGTG[1]
BLM10fw	TGTATTTGCATACATAAACTTTATCATTGTTCGTTAGCTAGCTTTGCACATTAATTTTTCGATTTGTTACCGCCAAAGCTTTTCAATTCAATTCATCAT[1]
BLM10rv	AATCAGCAGATAGCTCCAGCTATTTGTTTAGATGTACATATATGTCTAGATATGTGCTTAATATCCTATACTAATATGAATAGCTTTTTCTTTCCAATT[1]
BLM10A1fw	GGCCGAGGTATCCCTTAGAA[1]
YFH7fw	AACCTTGTTAGGTTAATTTCACTAGTACTATACATATTTTATCCTGTATCATACCAGAGGATCATTCTAGCCACAAAGCTTTTCAATTCAATTCATCAT[1]
YFH7rv	TGCCGTTTTTGCTTGCGCGCCTTAATTATCTGTATTCAGTTCGATTTTACAAAAATATATACAAGGTTCCGCCTAACCTTCAGCTTTTTCTTTCCAATT[1]
YFH7A1fw	TGGGCTTATCAGACTTGTCCA[1]
FAB1fw	ATAAAGGGCCAAACAAAAAACTATTTCGAATAGCAAGGTAGCTTCCATCCTGTACATGCAAGACCGTCACACAGCAAGCTTTTCAATTCAATTCATCAT[1]
FAB1rv	TACTGAAAGTTAAAGAACACTAATGTGCGTGATAGTGTATAAAAAAAAGTTACAGAATATAACTTGTACACGTTTATGTATAGCTTTTTCTTTCCAATT[1]
FAB1A1fw	TAACTCTCCCTCTCCCCCTCT[1]
ATG18fw	CACGACCCTCCCTTATTAATCAGTTAGTAATAGTGTTCCAGTTAACTCTGTATCCTTTTCTTCTTCGGCCTGACAAAGCTTTTCAATTCAATTCATCAT[1]
ATG18rv	AGATTATACGCAGGAGTTTATATAAACTATATTGTGTATGCGTTGTGACGTACGGAAGGCAGCGCGAGACACTTCCGTGATAGCTTTTTCTTTCCAATT[1]
ATG18A1fw	CATTCGGAAGTGCGACAATA[1]
RPL2Afw	ACAATCACATGGTTGTTAAATCACGGTGCTGACATACCCATAGCTTTTCAATTCAATTCATCAT[1]
RPL2Arv	GAACTGGTTTGTTACGTGGTTCTCAAAAGACCCAAGATTAGAGCTTTTTCTTTCCAATT[1]
RPL2AA1fw	AACTTGGCAGCACCTTGTCT[1]
CBT1fw	GATTGATCAGAAGTTTACTGCGCTTTTGGGTAAAGAAGCATTAAACAAAGGAGAGAGAAATATTGCAAGGAAAAAAAGCTTTTCAATTCAATTCATCAT[1]
CBT1rv	AGCCAGTGCTATAGTCACCAAATAATACGCATTATATATGGATATGTACAGTTCGCAGATCTTTATGGCATATTTATCGTTAGCTTTTTCTTTCCAATT[1]
CBT1A1fw	GCCATTTGCCTATAGCTTGG[1]
MRP49fw	AGTTTTGAATTTACATATTTCCATGAAGGGCAATGTTTTTTGATATATACATGAACAAACTTATCGAGAGAAAGCTAGCTTTTCAATTCAATTCATCAT[1]
MRP49rv	CAGGATATCTGTAAGAATCGGCCATAAAACTCATTAATAGAAGAACAGTATAACATAAGTGAGCCTGCTACAATAAGAAGAAGCTTTTTCTTTCCAATT[1]
MRP49A1fw	TCTCCTCCTGCATTACCATTG[1]
RSM22fw	ATATTCACGTATGTAGAATATTAAAGTATTGAATATATTAATATTATTACTTTATTTCCAGTTACTTACAATTTCCAGCTTTTCAATTCAATTCATCAT[1]
RSM22A1fw	GTTACCTGCGAATCCTGCTC[1]
DBR1fw	GTATGACTAAAAATTCTCTCAAGAAGGCTTGGCTTTAAGCTCTAATTCCGTCTGCATTCGTAATAGAAATATCTCTAGCTTTTCAATTCAATTCATCAT[1]
DBR1rv	AAATGAGCAGGAGAAAGTCATATGGCGAACGTAAATATGTAACTAAAAATTAAGATGGGCAGACATTTATCATTTTGCTTAAGCTTTTTCTTTCCAATT[1]
DBR1A1fw	GTCCCCCACCATTTATGAAC[1]
ATGufw	CACGACCCTCCCTTATTAATCAGTTAGTAATAGTGTTCCAGTTAACTCTGTATCCTTTTCTTCTTCGGCCTGACAAATCACGGAAGTGTCTCGCGCTGC
ATGurv	AGATTATACGCAGGAGTTTATATAAACTATATTGTGTATGCGTTGTGACGTACGGAAGGCAGCGCGAGACACTTCCGTGATTTGTCAGGCCGAAGAAG
S8rv	CCTCTAGGTTCCTTTGTTACTTCT[2]
MATaspe	ACTTCCACTTCAAGTAAGAGTTTG[3]
MATαspe	GCACGGAATATGGGACTACTTCG[3]
MATfla	AGTCACATCAAGATCGTTTATGG[3]
ATG18fw	GAAACTTCCCGTTGAAACCA[1]
ATG18rv	CCGGATACTCGGATGTGTCT[1]
FAB1fw	TTGATCGCATTTTGCTTGAG[1]
FAB1rv	TTGGGCATTCAAGTTCATCA[1]
ALG9fw	GCCGTCTACGAGCAATTTTC[1]
ALG9rv	TCTGGCAGCAGGAAAGAACT[1]
TAF10fw	CCAGGATCAGGTCTTCCGTA[1]
TAF10rv	AGCTCTCGCCTGACTGTTGT[1]

Author contributions

Conceived and designed the experiments: GL and RCO. Performed the experiments: GLM, MMC andFS. Analyzed the data: GLM, GL and RCO. Contributed reagents/materials/analysis tools: GLM,FS, GL, RCO. Wrote the paper: GLM, GL and RCO.

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CHAPTER V

SIP18 overexpression increases dehydration stress tolerance by modulating the membrane protein load

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In progress

Absract

We previously reported that overexpression of the gene encoding the yeast hydrophilin *SIP18* in *Saccharomyces cerevisiae* increases dehydration stress tolerance two-fold. The lipoprotein membranes of the cell suffer a greater amount of damage compared with other cellular compartments during the dehydration and rehydration process. In the present study, we characterised the putative pleiotropic effects caused by the intracellular accumulation of Sip18p during stress, which enhance dehydration tolerance. Therefore, we evaluated the changes in the membrane protein profiles during the time points before dehydration and after rehydration in the BY4742, *sip18* null mutant and *SIP18*-overexpressing strains. Of the proteins identified after comparing the proteomic changes among the strains, the Gvp36p, Gdp1p, Ald4p, Asc1p, Pma1p, Hsp30p and Lsp1p proteins had not been referenced in previous studies of dehydration stress. We discuss the putative roles of these proteins during stress. Finally, our data illustrate the power of this approach for investigating the complex cell dehydration phenotype.

Keywords: *Saccharomyces cerevisiae*, dehydration, membrane proteome, SIP18p

Introduction

The yeast *Saccharomyces cerevisiae*, along with many plants and bacteria, is part of a group of anhydrobiotic organisms that are able to overcome extreme dehydration stress through various molecular mechanisms and metabolites (Reyes *et al*., 2005). These metabolites include sugars (trehalose) and proteins (hydrophilins) that stabilise macromolecules and membranes, as well as antioxidants that protect against reactive oxygen species (ROS) damage (Leslie *et al*., 1994, Rodríguez-Porrata *et al*., 2012b). Dehydration causes a rapid water efflux through the membrane, resulting in cytoskeletal collapse (Walker and van Dijck, 2006) that arrests cellular metabolism. The dehydration of cells alters the structure and function of the vacuole and the integrity and functionality of nuclear and cell membranes (Walker and Van Dijck, 2006). Therefore, lipoprotein membranes are one of the targets damaged during the dehydration-rehydration process. Our group previously reported that phosphatidylcholine (PC) is the only lipid among fatty acids, phospholipids and sterols that plays a primary role in yeast dehydration tolerance. By increasing the amount of intracellular PC in commercial yeast strains before

dehydration (BD), cells viability was increased by approximately 55% (Rodríguez-Porrata *et al.*, 2011). Hydrophilins accumulate in response to water deficits in plants, bacteria and yeast (Garay Arroyo *et al*., 2000, Yale and Bohnert 2001). Among the 12 hydrophilin proteins described for *S. cerevisiae*, we demonstrated previously that the overexpression of *GRE1*, *NOP6*, *YJL144W*, *STF2* and *SIP18* in laboratory and commercial yeast strains increases cell viability by a range of 30% to 60% after stress treatment (López-Martínez *et al*., 2012, López-Martínez *et al.* 2013). In the present study, we hypothesised that hydrophilins protect the proteomic profile of cell membranes to overcome dehydration stress. To validate this hypothesis, we evaluated the yeast membrane proteome of the haploid strain BY4742, *sip18* null mutant (*Δsip18, pGAL*) and a *SIP18*-overexpressing strain (*Δsip18, pGAL-SIP18*) at time point zero, (cells in stationary phase after 4 h of galactose induction, BD) and after 30 min of rehydration (AR). By comparing their proteomic profiles, we identified 19 membrane proteins that were significantly up- or downregulated among the three strains during stress. The proteomic approach used in this study provides the first demonstration of the putative roles of the Gvp36p, Gdp1p, Ald4p, Asc1p, Pma1p, Hsp30p and Lsp1p proteins in yeast dehydration stress tolerance.

Material and methods

Strains and plasmids

The *S. cerevisiae* BY4742 (*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*) and *sip18* null mutant (BY4742, *sip18::kanMX4*) strains were purchased from EUROSCARF (Frankfurt, Germany) (Brachmann et al. 1998). The *SIP18*-overexpressing strain (Δ*sip18, pGAL-SIP18*) and its reference strain transformed with an empty vector (Δ*sip18, pGAL)* were developed previously by our group (Rodríguez-Porrata *et al.*, 2012a).

Growth conditions and the dehydration-rehydration process

The yeast BY4742 strain was grown in shake flasks (150 rpm, 28°C) in complete drop-out synthetic glucose media, and the Δ*sip18*, *pGAL* and Δ*sip18, pGAL-SIP18* strains were grown in complete synthetic glucose media devoid of leucine. All strains were cultivated for 48 h, and cells were harvested after 4 h supplementation with 2% galactose. The dehydration-rehydration process was performed as described by Rodríguez-Porrata *et al.* (2011).

Lysis and digestion

Cells from three independent cultures of BY4742; Δ*sip18*, *pGAL*; and Δ*sip18, pGAL-SIP18* strains were harvested at time point BD and AR. The cells from each triplicate sample were harvested, immediately washed with ice-cold water and pelleted. The membrane protein fractions were isolated in the presence of protease inhibitor tablets (Roche, Basel, Switzerland) using a FOCUS Membrane Proteins Kit according to the manufacturer's instructions (BD Biosciences, USA). The protein amount was determined using a 2D Quant Kit (GE Healthcare, Zeist, The Netherlands). Next, the proteins were reduced, carbamidomethylated and digested using an adaptation of the FASP method (Wisniewski *et al.*, 2009, Wisniewski *et al.*, 2012). Briefly, 250 µg of sample was mixed with 0.1 mL of UA solution (8 M urea in 0.1 M Tris-HCl [pH 8.5]) and 10 mM DTT. After the mixtures were incubated at 56°C for 20 min at 600 rpm, they were loaded into Microcon-30 kDa filtration devices (Millipore, Darmstadt, Germany) and centrifuged at 14,000×*g* for 15 min. The concentrates were diluted in the devices with 0.2 mL of UA solution and centrifuged again. Then, the concentrates were mixed with 0.1 mL of 50 mM iodoacetamide in UA solution and incubated in the dark at room temperature for 20 min. After the concentrates were centrifuged for 15 min, they were diluted with 0.1 mL of UB solution (8 M urea in 0.1 M Tris-HCl [pH 8]) and concentrated again. This step was repeated twice. Subsequently, 5 µg of Lys-C in 40 µL of UB was added to the filter, and the samples were incubated at 37°C for 4 h. Next, 5 µg of trypsin in 180 µL of NH_4HCO_3 was added to the sample and incubated at 37°C overnight. The peptides were collected by centrifuging the filter units, followed by the addition of 50 µL of 0.5 M NaCl and centrifugation at 14,000×*g* for 15 min. The collected samples were acidified with 1% formic acid, and peptides were desalted using reverse-phase Sep-Pak C18 cartridges (Waters Corporation, Milford, MA, USA) and a Supelco Visiprep DL system (Sigma, The Netherlands).

LC-MS/MS analysis

LC-MS/MS was used to analyse the digested and purified samples. The samples were analysed using a Proxeon Easy-nLC 1000 (Thermo Scientific) connected to a Q Exactive mass spectrometer (Thermo Scientific). The injected samples were first trapped (Dr. Maisch Reprosil C18, 3 µm, 2 cm, 100 µm) at a maximum pressure of 600 bar with solvent A (0.1% formic acid in water) before being separated on an analytical column (Agilent Zorbax SB-C18, 1.8 µm, 40 cm, 50 µm) at a stable temperature of 40°C. Peptides

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were separated chromatographically by a 90 min gradient from 7% to 30% solvent B (0.1% formic acid in acetonitrile) at a flow rate of 100 nL·min^{-1}. The column eluent was introduced directly into the electrospray source of the mass spectrometer. The electrospray voltage was set to 1.7 kV using a fused silica capillary (360 μm o.d., 20 μm i.d., 10 μm tip i.d., constructed in-house). The mass spectrometer was used in data-dependent mode, which automatically switched between MS and MS/MS using a Top10 method (higher-energy collision dissociation fragmentation).

MS data analysis

The mass spectrometry raw data were quantified in a label-free manner using the MaxQuant software (version 1.4.1.2) with the integrated Andromeda search engine and with the match between runs and label-free quantification (LFQ) options selected (Cox and Mann, 2008; Cox *et al*., 2011). Analysis was performed against a decoy database of the predicted proteome from *S. cerevisiae* downloaded from the SGD homepage (www.yeastgenome.org). Peptide tolerance was set initially to 20 ppm for the first search and to 4.5 ppm after recalibration. Enzyme specificity was set as C-terminal to Arg and Lys for full tryptic digestion with a maximum of two missed cleavages. Carbamidomethylation of cysteine was set as mixed modification, and N-terminal protein acetylation and methionine oxidation were set as variable modifications. All peptide spectrum matches (PSMs) and proteins were validated with a 1% false discovery rate. Only PSMs with a minimum length of 6 amino acids were kept.

Post-acquisition data analysis

Before analysis using the Perseus software, the "proteingroups.txt" table generated by MaxQuant was filtered for contaminants, reverse hits and hits only identified by site. Subsequently, Perseus software analysis was performed to identify specific proteins involved in dehydration tolerance that showed a significant change at both the BD and AR time points in the Δ*sip18, pGAL-SIP18* strain versus the Δ*sip18, pGAL* and BY4742 strains. First, the LFQ values were transformed to a logarithmic scale ($\log_2$), and the resulting Gaussian distribution of the data was used to replace missing values based on the normal distribution. Then, statistical analysis was performed using an ANOVA or *t*-test with a highly stringent cut-off value (FDR 0.001 for BD and FDR 0.01 for AR) in which low-intensity peptides and proteins were efficiently removed from the "significant"

data set. The ANOVA test was applied to LFQ for BD samples, and *t*-test-based statistics were applied to LFQ for AR samples.

Gene Ontology enrichment, protein interaction and co-expression analyses

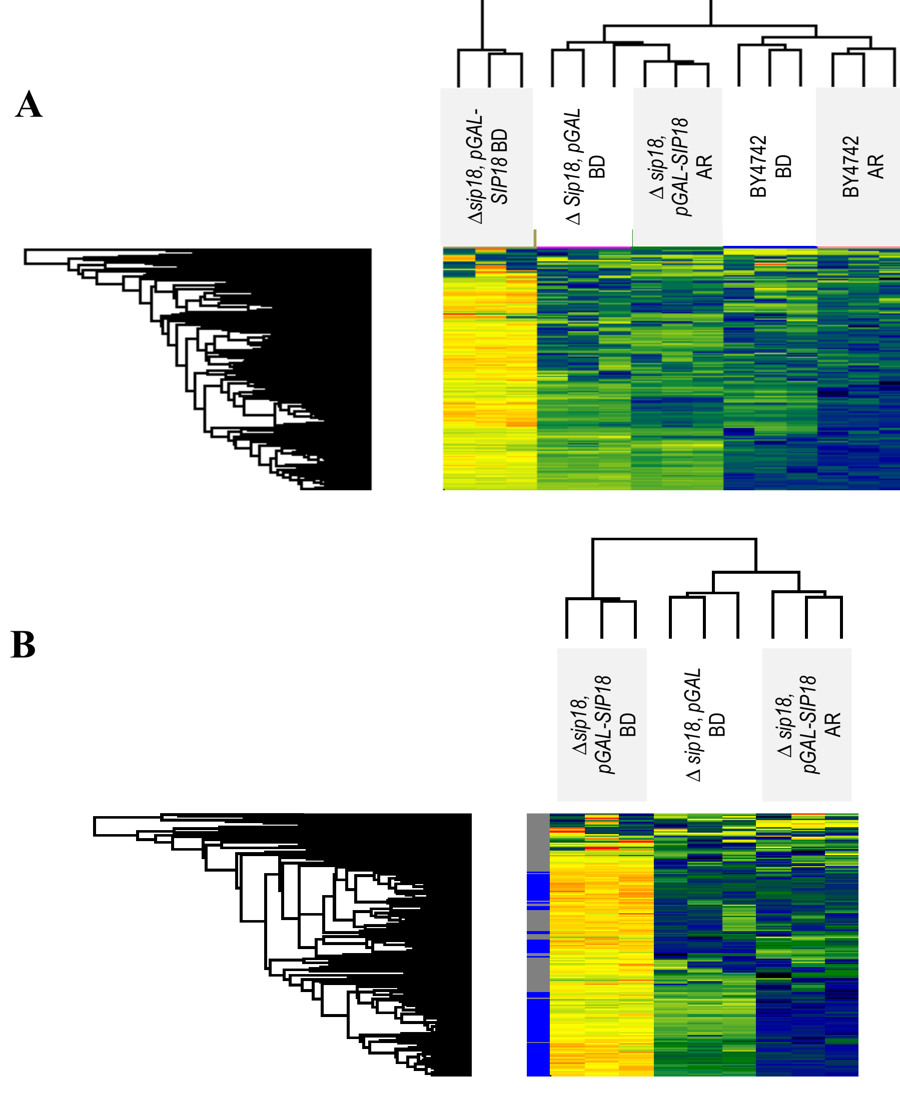
Gene Ontology categories for molecular function and cellular component were based on the *Saccharomyces* Genome Database (SGD) for the presence-absence pattern of each category (Cherry *et al.*, 2012). We examined the enrichment of pathways using the DAVID database (Huang *et al.*, 2009a and 2009b). All *P* values below 0.015 were reported. To show the relation among proteins in terms of co-expression and protein-protein interactions, we used the STRING network database at confidence 0.400 (Jensen *et al.*, 2009).

Results and discusion

LC-MS/MS

We compared the yeast membrane proteome among the strains BY4742; Δ*sip18*, *pGAL*; and Δ*sip18, pGAL-SIP18* to identify proteins that are significantly involved in the observed higher dehydration tolerance in response to *SIP18* overexpression, as was shown previously in different yeast strains (Rodríguez-Porrata *et al.*, 2012a; López-Martínez *et al.*, 2013). The protein profiles were examined BD and AR. For each condition, three biological replicates were analysed. Protein samples were subjected to LS-MS/MS-based proteomic analysis and then Perseus data analysis, in which the obtained values were filtered for contaminants, reverse hits and hits only identified by site; after data transformation, we obtained 534 proteins. Unexpectedly, a higher abundance of the hydrophilin Gre1p was observed in Δ*sip18, pGAL-SIP18* cells compared with the Δ*sip18, pGAL* and BY4742 cells BD (1.060±0.040, -0.456±0.034, and -0.238±0.016, respectively). Among the other identified proteins, the overexpression of *GRE1* enhanced dehydration tolerance similar to *SIP18* (López-Martínez *et al.*, 2012). Nonetheless, Gre1p showed a significant decrease in abundance AR in Δ*sip18, pGAL-SIP18* cells compared with BY4742 cells (-0.565±0.284 and 0.181±1.343, respectively). In additional analyses, we accounted for the 313 proteins that met the condition of having a quantitative value for the three replicates of at least one of the three evaluated strains. BD samples were statistically analysed using ANOVA at FDR 0.001, whereas AR samples were analysed using a *t*-test at FDR 0.01. In both cases, Pearson correlation

coefficient and R-squared values were determined for each sample and plotted on a scatter plot (data not shown). Significant data in all the combinations of biological replicates were defined as those between 0.97 and 0.99 for Pearson correlation coefficients and between 0.96 and 0.98 for R-squared values, except for Δ*sip18, pGAL* AR that was excluded of this study because did not show good correlation values. After statistical analysis, 158 proteins BD and 112 proteins AR were found to be significantly up- or downregulated among the three strains for each condition (Figure 1A, B and C).



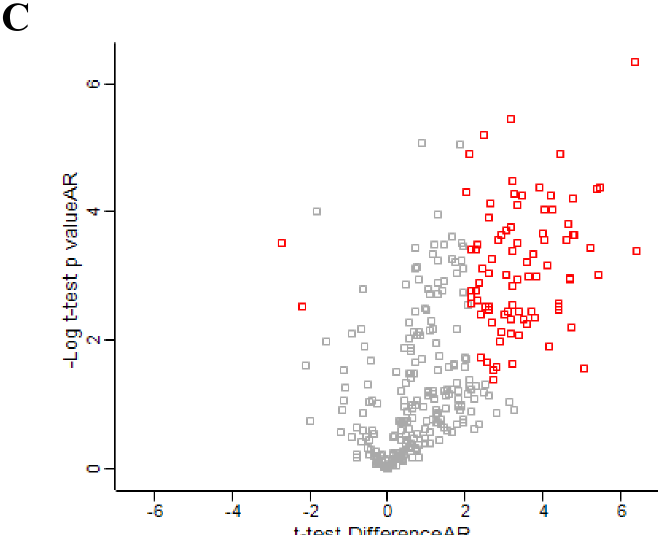


Figure 1. Clustering of all identified proteins. (A, page 163) Hierarchical clustering of all identified proteins at the BD and AR time points. (B, page 163) and (C) Proteins that were significantly up- or downregulated upon dehydration/rehydration stress. (B) Hierarchical clustering of all 158 proteins that changed at the BD time point. Significance was determined by ANOVA analysis at FDR 0.001. (C) Volcano plot showing t-test *P* values ($-\log_{10}$) versus protein change ($\log_2$) of all 112 altered proteins at the AR time point (empty red squares). Significance was determined by a two-sample test at FDR 0.01.

The statistical analysis of the AR samples was also performed with a more stringent cut-off (FDR 0.001) in which only 38 proteins were significant. Thus, we continued our study of the 112 AR proteins that were defined (data not shown). Next, we evaluated which of these 158 significant proteins in the BD condition were common to those that were significant in the AR condition. When comparing BD samples with AR samples at the FDR 0.01 cut-off, 78 significant proteins were common in both conditions (Figure 2A), whereas only 29 were identified with the highly stringent cut-off (data not shown). Of the 78 common significant proteins in the BD and AR conditions, we investigated which of these proteins have a putative role in overcoming dehydration stress in the cell. We clustered these proteins into one of the following profiles: i) those showing at least two-fold differences in the BD and AR conditions of *Δsip18, pGAL-SIP18* cells compared with BD-*Δsip18, pGAL* cells and for BD and AR time points in the BY4742 strain (Table 1) and ii) those showing the highest values in the BD condition for *Δsip18, pGAL-SIP18* cells but with AR values lower than those in AR BY4742 cells (Table 2). According to these profiles, 45 proteins were highlighted. In the first profile, 27 proteins were found to be up-regulated in AR-*Δsip18, pGAL-SIP18* cells (Figure 2B, Table 1). These results

suggest that although these proteins decrease in abundance during dehydration, the accumulation of some of these proteins in the *Δsip18, pGAL-SIP18* strain may contribute to the 90% viability observed for this strain after rehydration. In contrast, the BY4742 and *Δsip18, pGAL* strains showed 40% and 10% viability, respectively, after rehydration (López-Martinez *et al.*, 2012). The 18 proteins that were categorised into the second profile may be cellular background noise in the *Δsip18, pGAL-SIP18* strain during stress (Figure 2B, Table 2). Additionally, the faster elimination of some of these proteins, which may have a toxic effect during the dehydration/rehydration process, may have a positive effect on cellular reorganisation after the rehydration process.

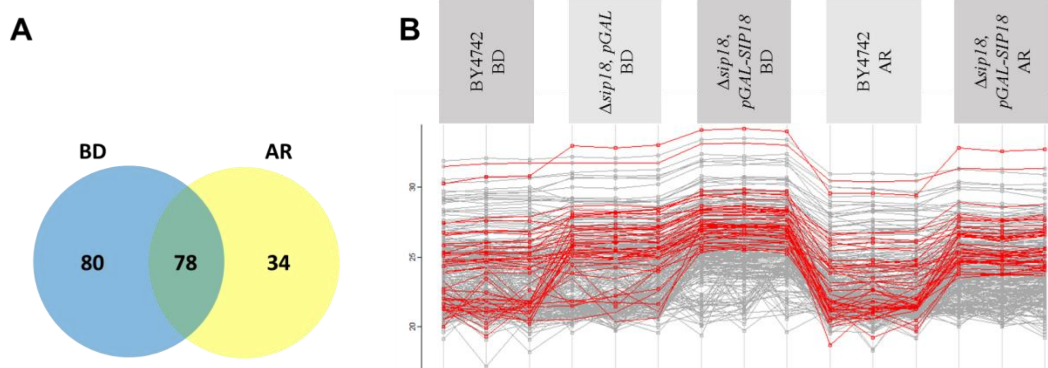


Figure 2. (A) Overlap between significant proteins expressed BD and AR. (B) Clustering of 45 common proteins that were altered between the BD and AR time points that belong to both profiles.

Table 1: LFQ of overlapped significant of the first protein profile proteins between BD and AR

Accession number	Name	Name description	BD			AR	
			BY4742	*Δsip18, pGAL*	*Δsip18, pGAL-SIP18*	BY4742	*Δsip18, pGAL-SIP18*
YBR039W	Atp3p	ATP synthase	-0,840±0.098	-0.739±0,114	1,288±0,061	-0,540±0,501	0,000±1,000
YBR230C	Om14p	Outer Membrane Protein of 14 kDa	-1,226±0.024	0,209±0,055	1,038±0,022	-0,525±0,590	0,000±1,000
YCL040W	Glk1p	GLuco Kinase	-0,954±0.064	-0,329±0,031	1,283±0,002	-0,550±0,430	0,000±1,000
YCR004C	Ycp4p	Unknown function	-1,392±0.102	0,321±0,055	0,953±0,044	-0,576±0,101	0,560±0,343
YDL022W	Gpd1p	Glycerol-3-phosphate dehydrogenase	-0,814±0.003	-0,436±0,083	1,265±0,020	-0,535±0,534	0,565±0,295
YDL067C	Cox9p	Cytochrome c OXidase	-1,310±0,094	0,236±0,052	1,080±0,039	-0,554±0,400	0,000±1,000
YDR032C	Pst2p	Protoplasts-Secre Ted	-0,855±0,020	0,000±0,109	1,121±0,031	-0,577±0,074	0,577±0,056
YDR155C	Cpr1p	Cyclosporin A-sensitive Proline Rotamase	-1,144±0,059	0,202±0,048	1,158±0,101	-0,577±0,038	0,571±0,206
YEL054C	Rpl12ap	Ribosomal Protein of the Large subunit	0,108±0,024	-1,416±0,152	1,086±0,053	-0,574±0,151	0,573±0,175
YFL014W	Hsp12p	Heat Shock Protein	-0,910±0,027	0,023±0,201	1,389±0,031	-0,570±0,222	0,000±1,000
YGL008C	Pma1p	Plasma Membrane ATPase	-0,965±0,024	0,091±0,091	0,883±0,010	0,000±1,000	0,543±0,480
YGL191W	Cox13	Cytochrome c OXidase	-0,859±0,077	-0,793±0,165	1,269±0,005	-0,569±0,245	0,565±0,290
YGL255W	Zrt1p	Zinc-Regulated Transporter	-0,970±0,001	-0,527±0,093	1,345±0,017	-0,576±0,090	0,577±0,035
YGR043C	Nqm1p	Non-Quiescent Mutant	-1,216±0,024	0,281±0,042	1,010±0,007	-0,532±0,552	0,548±0,445
YGR180C	Rnr4p	RiboNucleotide Reductase	-1,152±0,037	-0,077±0,012	1,273±0,025	-0,577±0,060	0,512±0,653
YGR234W	Yhb1p	Yeast HemogloBin-like protein	-1,116±0,034	0,423±0,098	0,867±0,041	-0,567±0,262	0,000±1,000
YIL041W	Gvp36p	Golgi Vesicle Protein	-0,511±0,039	-0,840±0,058	1,123±0,187	0,000±1,000	0,556±0,385
YIR038C	Gtt1p	GlutaThione Transferase	-0,858±0,086	0,334±0,068	0,940±0,013	-0,577±0,022	0,571±0,202
YJL034W	Kar2p	KARyogamy	-0,115±0,293	-1,030±0,459	0,997±0,341	-0,559±0,355	0,000±1,000

continue

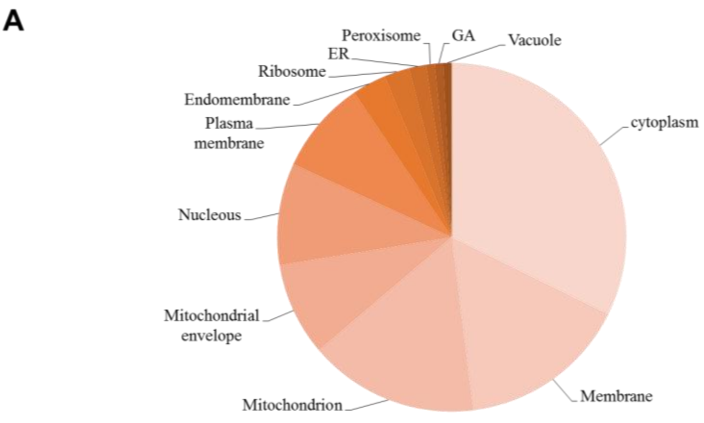
Accession number	Name	Name description	BD			AR	
			BY4742	Δ*sip18, pGAL*	Δ*sip18, pGAL-SIP18*	BY4742	Δ*sip18, pGAL-SIP18*
YKL085W	Mdh1p	Malate DeHydrogenase	-1,237±0,004	0,471±0,013	0,842±0,019	-0,577±0,058	0,000±1,000
YLR044C	Pdc1p	Pyruvate DeCarboxylase	-0,641±0,018	-0,586±0,034	1,384±0,031	0,552±0,418	0,558±0,359
YLR058C	Shm2p	Serine HydroxyMethyltransferase	-1,129±0,027	0,304±0,101	0,854±0,027	0,000±1,000	0,539±0,505
YNR034W	Sol1p	Suppressor Of Los1-1	-1,558±0,187	0,275±0,044	1,034±0,016	-0,562±0,326	0,567±0,271
YOR374W	Ald4p	ALdehyde Dehydrogenase	-0,751±0,059	0,049±0,042	1,033±0,024	-0,535±0,529	0,573±0,175
YPL106C	Sse1p	Heat Shock Protein component	-0,870±0,111	-0,494±0,007	1,351±0,042	-0,574±0,160	0,530±0,563
YPL281C	Err2p	Enolase-Related Repeat	-0,603±0,239	-0,996±0,062	1,266±0,033	-0,511±0,657	0,532±0,547
YPR191W	Qcr2p	QH2:cytochrome-C oxidoReductase	-1,223±0,018	0,462±0,007	0,849±0,038	-0,577±0,073	0,000±1,000

Table 2: LFQ of overlapped significant of the second protein profile proteins between BD and AR

Accession number	Name	Name description	BD			AR	
			BY4742	Δ*sip18, pGAL*	Δ*sip18, pGAL-SIP18*	BY4742	Δ*sip18, pGAL-SIP18*
YAL003W	Efb1p	Elongation Factor Beta	-0,851±0,001	-0,417±0,061	1,144±0,112	0,564±0,307	-0,527±0,578
YBL015W	Ach1p	Acetyl CoA Hydrolase	-1,066±0,043	0,382±0,062	0,949±0,044	0,541±0,494	0,000±1,000
YBL075C	Ssa3p	Stress-Seventy subfamily A	-0,967±0,099	0,134±0,077	1,191±0,011	0,577±0,038	-0,567±0,269
YBR072W	Hsp26p	Heat Shock Protein	-1,144±0,015	0,286±0,036	1,002±0,060	0,571±0,209	0,000±1,000
YCL035C	Grx1	GlutaRedoXin	-1,195±0,327	-0,126±0,042	1,012±0,135	0,575±0,124	-0,532±0,549
YCR021C	Hsp30p	Heat Shock Protein	-1,091±0,046	-0,167±0,047	1,243±0,015	0,000±1,000	-0,541±0,492
YDR513W	Grx2p	GlutaRedoXin	-0,952±0,052	-0,031±0,052	1,029±0,035	0,566±0,279	-0,543±0,480
YDR533C	Hsp31p	Heat-Shock Protein	-0,972±0,156	-0,339±0,173	1,283±0,078	0,556±0,382	-0,524±0,596
YEL060C	Prb1p	PRoteinase B	-0,882±0,001	-0,002±0,148	1,127±0,014	0,000±1,000	-0,577±0,037
YER177W	Bmh1p	Brain Modulosignalin Homologue	-0,152±0,010	-1,065±0,012	1,161±0,009	0,563±0,316	-0,562±0,327
YJR121W	Atp2p	ATP synthase	-1,238±0,032	0,209±0,008	1,047±0,039	0,000±1,000	-0,566±0,280
YKR097W	Pck1p	Phosphoenolpyruvate CarboxyKinase	-1,166±0,052	0,488±0,031	0,821±0,013	0,529±0,564	-0,571±0,207
YLR340W	Rpp0p	Ribosomal Protein P0	-1,190±0,205	0,036±0,071	0,990±0,061	0,577±0,045	-0,576±0,089
YLR355C	Ilv5p	IsoLeucine-plus-Valine requiring	-1,061±0,003	0,383±0,054	0,945±0,065	0,576±0,082	-0,524±0,594
YMR116C	Asc1p	Absence of growth Suppressor of Cyp1	-1,069±0,032	-0,228±0,231	1,167±0,003	0,566±0,282	-0,576±0,078
YOR065W	Cyt1p	Cytochrome c OXidase	-1,060±0,483	-0,515±0,050	1,203±0,059	0,000±1,000	-0,576±0,092
YPL004C	Lsp1p	Long chain bases Stimulate Phosphorylation	-1.589±0,125	0,316±0,025	0,880±0,128	0,550±0,432	-0,572±0,183
YPL078C	Atp4p	ATP synthase	-1.038±0,107	0,448±0,009	0,877±0,008	0,570±0,219	-0,509±0,669

Gene Ontology enrichment, protein interactions and co-expression analysis

To determine whether these 45 proteins were involved in maintaining and/or restoring the cell from the damage produced by dehydration stress, we searched for their molecular function GO term and subcellular distribution using the *Saccharomyces* Genome Database (Figure 3). Of the 45 proteins, only 19 are membrane proteins (Figure 3a), which belong to the first established profile, except for Atp2p, Atp4p, Cyt1p and Lsp1p (Figures 4a and 5a, Table 2). Some of these 19 membrane proteins localised specifically to the plasma membrane (Glk1p, Ycp4p, Hsp30p, Pst2p, Bmh1p, Pma1p, Zrt1p, and Lsp1p), whereas others localised to endomembranes (Hsp12p, Gtt1p, Kar2p and Gvp36p) or to mitochondrial envelopes (Atp2p, Atp3p, Atp4p, Cox9p, Cox13p, Cyt1p, Om14p and Qcr2p) (Figure 6a).



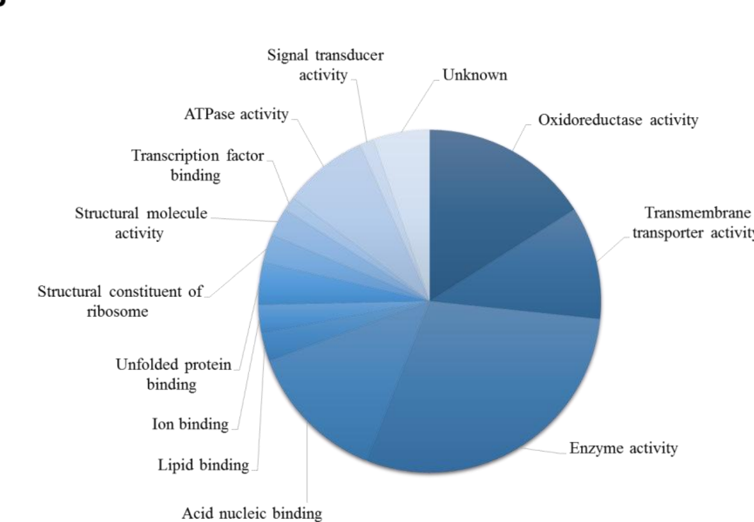
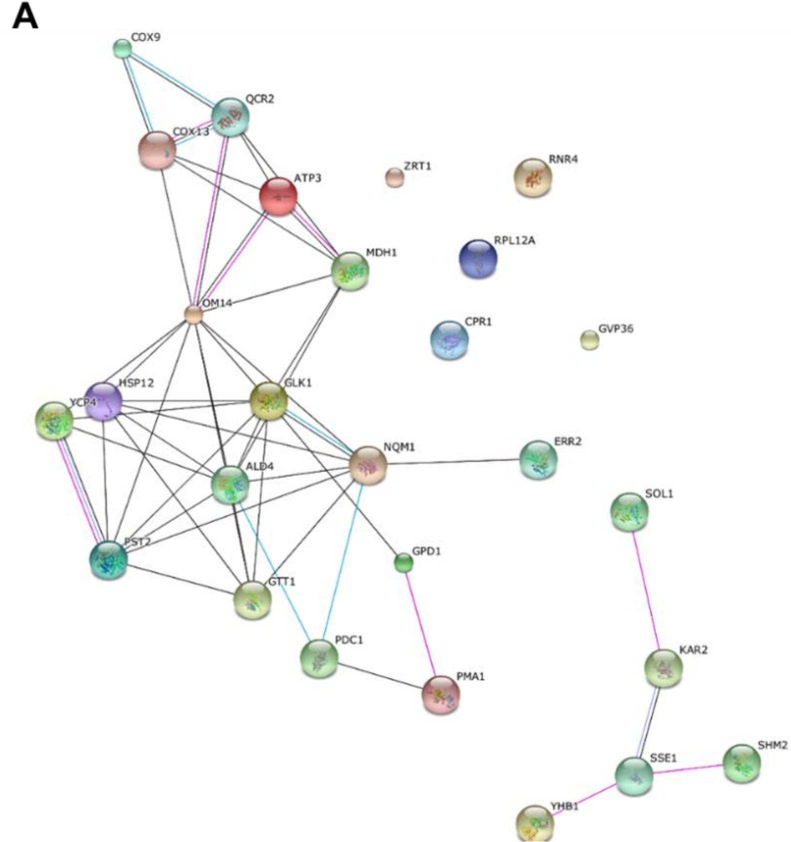


Figure 3. Functional category analysis of the 45 common proteins that were altered BD and AR that belong to both profiles. (A) subcellular distribution and (B) functional annotation.

Next, we investigated the putative relations of the abundance of the proteins of each profile by co-expression and protein-protein interaction data using the STRING database. Figure 4 shows the network analysis for the first profile of 27 selected proteins, of which 22 show co-expression and/or interaction links; the exceptions include Zrt1p, Rnr4p, Rpl12ap, Cpr1p and Gvp36p. According to the co-expression data, Om14p had the highest association score and a major protein interaction (Figure 4). Om14p showed co-expression with Glk1p, Hsp12p, Cox13p, Mdh1p, Ald4p and the transaldolase of unknown function Nqm1p (Figure 4B). Additionally, Om14p is co-expressed with Sip18p with a medium-high association score (data not shown). Om14p is a mitochondrial outer membrane receptor for cytosolic ribosomes that participates in co-translational mitochondrial import by interacting with Por1p and Om45p (Lesnik *et al.*, 2014; Lauffer

et al., 2012). Nevertheless, the Δ*om14* strain shows high resistance to osmotic stress; therefore, the accumulation of Om14p is unexpected in cells subjected to the osmotic stress that occurs during the dehydration/rehydration process (Troppens *et al.*, 2013). However, the cytoplasmic protein Gpd1p and the membrane protein Gvp36p contribute to high tolerance to hyperosmotic stress, but only *GPD1* shows co-expression with *GLK1* (Ansell *et al.*, 1997). The BAR domain protein Gvp36p localises to Golgi vesicles, and the glycerol-3-phosphate dehydrogenase Gpd1p synthesises glycerol (Yoshikawa *et al.*, 2009; Albertyn *et al.*, 1994). The apparent contradiction of Omp14p suggests that its major role may be related to the resumption of mitochondrial activity and that Gvp36p and Gpd1p provide osmotic robustness to *Δsip18, pGAL-SIP18* cells during stress. The second highest score was exhibited by Glk1p (Figure 4A), which is co-expressed with Gpd1p, Pst2p, Hsp12p, Mdh1p and Ald4p (Figure 4B). Additionally, in an anti-tag co-immunoprecipitation assay, interactions between Glk1p and Cpr1p, Pma1p, Pdc1p, Shm2p and Sol1p were detected, but the only member of the first protein profile that showed a high score value with Pdc1p was Pma1p (Figure 4B) (Ossareh-Nazari *et al.*, 2010). The glucokinase Glk1p catalyses the phosphorylation of glucose in the first irreversible step of glycolysis as sucrose or trehalose degradation. This specific function may be related to the idea that intracellular trehalose glycolysis supplies the *Δsip18, pGAL-SIP18* cells with ATP earlier by Glk1p accumulation after stress compared with the BY4742 strain (Herrero *et al.*, 1995). The endomembrane protein Hsp12p accumulated during all processes in *Δsip18, pGAL-SIP18* cells and is an *S. cerevisiae* hydrophilin protein similar to Sip18p; with Lsp1p (present in the second profile). Hsp12p is a constituent of the GO functional annotation term lipid binding (López-Martínez *et al.*, 2012) (Figure 3B). Hsp12p is involved in pathways that respond to water deficit and acts in an analogous manner to trehalose by protecting membrane integrity and stability against dehydration without altering the overall plasma membrane lipid composition (Mtwisha *et al.*, 1998; Sales *et al.*, 2000). Despite this membrane protection, an *HSP12*-overexpressing strain exhibited detrimental effects after the dehydration process (López-Martínez *et al.*, 2012). However, a *Δhsp12* strain shows low tolerance to oxidative stress driven by the dehydration/rehydration process (Welker *et al.*, 2010, Rodríguez-Porrata et al., 2012b). The apparent contradiction between Hsp12p accumulation in *Δsip18, pGAL-SIP18* cells during stress and the detrimental effect of *HSP12* overexpression suggests that a dose-dependent effect of Hsp12p allows the cells to overcome dehydration stress.

The null mutant of the mitochondrial aldehyde dehydrogenase, the Δ*ald4* strain, shows low tolerance to oxidative stress; therefore, Ald4p accumulation in Δ*sip18, pGAL-SIP18* cells contributes to the mitigation of the oxidative stress caused by stress imposition. On the other hand, the null mutant of the ATPase component of heat shock protein Hsp90 chaperone complex, the Δ*sse1* strain, shows low tolerance to dehydration; consequently, it suggests that Δ*sip18, pGAL-SIP18* cells accumulation of Sse1p allow to reduce cell damages during dehydration (Shima *et al*., 2008). The mitochondrial malate dehydrogenase Mdh1p is involved in the tricarboxylic acid (TCA) cycle, which Ratnakumar *et al*. (2011) showed decreases in the dehydration-resistant Δ*mdh1* strain. Therefore, Mdh1p accumulation induced by *SIP18* overexpression may play a role in overcoming cell dehydration stress.



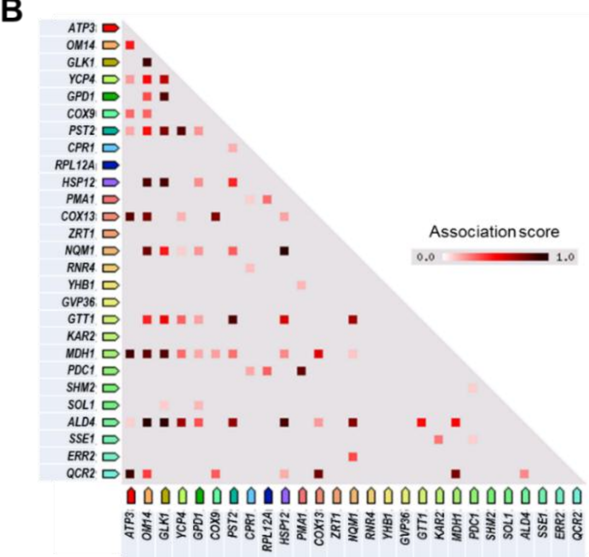
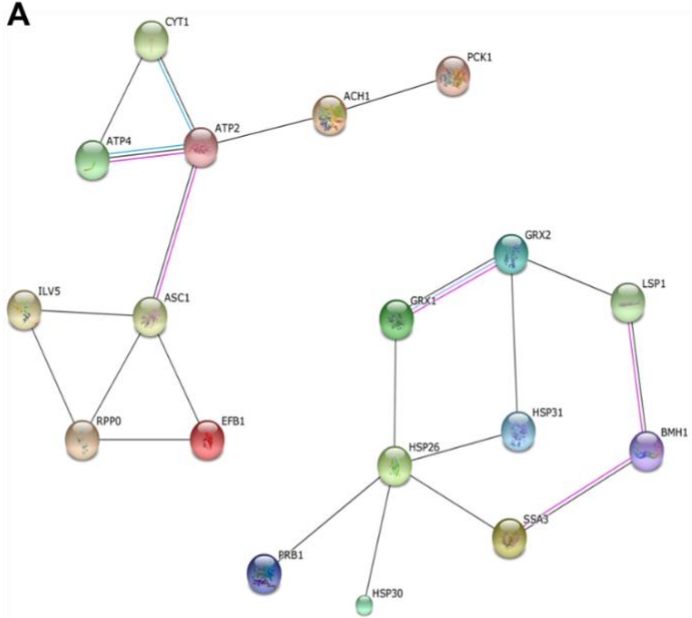


Figure 4. Enriched protein network analysis of the first profile. STRING network analysis (A, page 169). STRING interaction confidence was set at 0.4. The dark line corresponds to co-expression prediction, the pink line corresponds to a protein-protein interaction, and turquoise corresponds to a protein interaction group. Protein co-expression association score (B).

Figure 5 shows the network analysis for the 18 selected proteins in the second profile. The co-expression data showed two primary non-interconnected sub-groups that include the following proteins: i) Cyt1p, Atp4p, Atp2p, Ach1p, Pck1p, Asc1p, Ilv5p, Rpp0p and Efb1p; and ii) rx1p, Grx2p, Lsp1p, Bmh1p, Hsp26p, Hsp30p, Hsp31p, Ssa3p and Prb1p (Figure 5A). The proteins from each sub-group exhibited varying levels of co-expression, but only the members that belong to the first sub-group showed a high association score.



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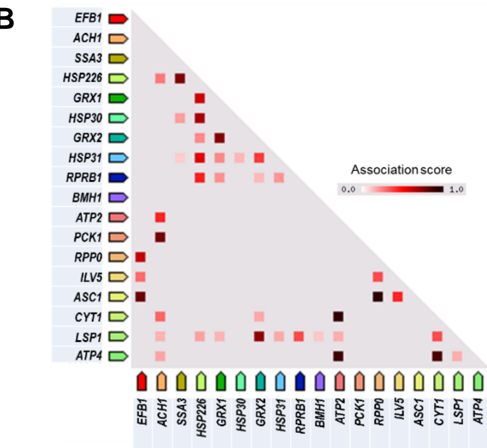

Figure 5. Enriched protein network analysis of the second profile. STRING network analysis (A, page 170). STRING interaction confidence was set at 0.4. The dark line corresponds to co-expression prediction, the pink line corresponds to a protein-protein interaction, and turquoise corresponds to a protein interaction group. Protein co-expression association score (B).

Atp2p showed high co-expression with Atp4p and Cyt1p, and Rpp0p showed high co-expression with Asc1p. Co-immunoprecipitation studies have shown protein-protein interactions among Efb1p, Bmh1p, Rpp0p, Ilv5p and Asc1p (Ossareh-Nazari *et al.*, 2010; Lee *et al.*, 2011) (Figure 5B). The ribosomal protein P0 (Rpp0p), which is involved in the interaction between translational elongation factors and the ribosome, did not show any protein-protein interactions but was highly co-expressed with the gene that encodes the core component of the small (40S) ribosomal subunit Asc1p (Krokowski *et al.*, 2006). The Δ*asc1* strain shows an enhanced oxidative tolerance phenotype, which correlates with the second established profile for Δ*sip18, pGAL-SIP18* cells and which contributes to the moderation of the oxidative stress induced by the dehydration/rehydration process (Brown *et al.*, 2006)**.** However, single null strain characterisation studies for stress dehydration showed that the presence of Bmh1p or Rpp0p has a deleterious effect on dehydration-stressed cells (Welch *et al.*, 2013, Ratnakumar *et al.*, 2011).

The 14-3-3 proteins form a family of conserved eukaryotic proteins that bind to over 200 different proteins involved in nearly all cellular processes. The yeast *S. cerevisiae* has two genes encoding 14-3-3 proteins, Bmh1p and Bmh2p (Bruckmann *et al.*, 2007). Co-expression between Bmh1p and the rest of the membrane proteins has not been established (Figure 6B), but Bmh1p has a documented protein-protein interaction with the membrane protein Lsp1p, which is the second constituent of the GO functional annotation term lipid binding (Figure 3B).

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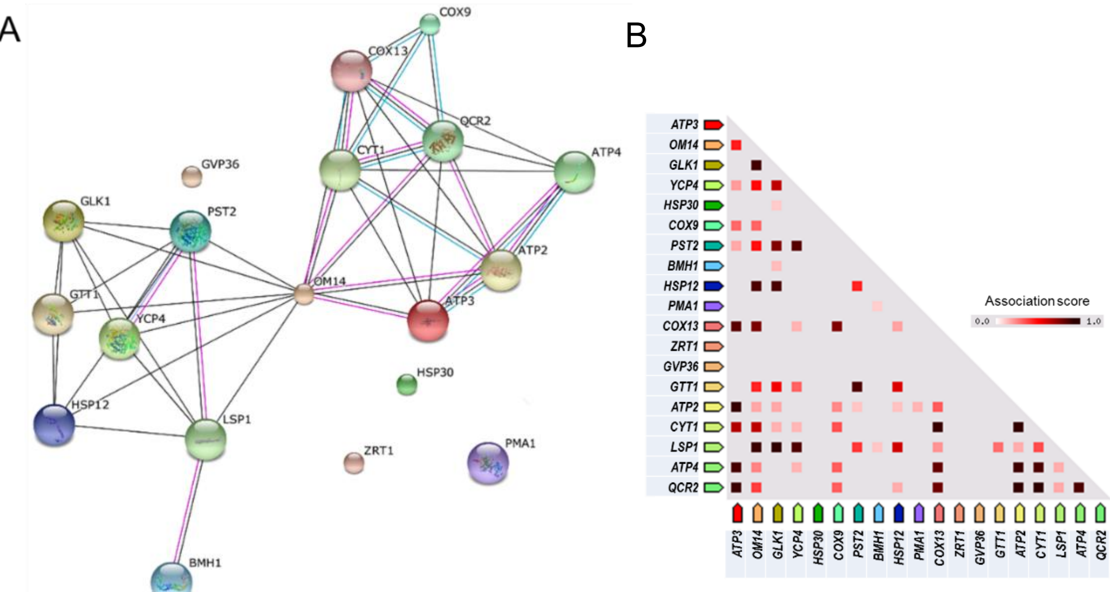

Figure 6. Enriched protein network analysis of membrane proteins. STRING network analysis (A). STRING interaction confidence was set at 0.4. The dark line corresponds to co-expression prediction, the pink line corresponds to a protein-protein interaction, and turquoise corresponds to a protein interaction group. Protein co-expression association score (B).

Lsp1p downregulates resistance to heat stress and specifically participates in down-regulating the activity of the Pkc1p-MAP kinase cascade and the partially parallel Ypk1/2p pathway that control growth and cell integrity (Zhang *et al*., 2004). Therefore, the over-degradation of Lsp1p contributes to the enhanced stress tolerance of *Δsip18, pGAL-SIP18* cells (Table 2). Nevertheless, Lsp1p was highly co-expressed and showed protein-protein interactions with membrane proteins with unknown functions, Ycp4p and Pst2p, which are induced by oxidative stress and which are found in the first established profile (Lee *et al*., 1999) (Figure 6). In contrast, *PST2* was co-expressed with the endoplasmic reticulum-associated glutathione S-transferase *GTT1*, for which little information is available on which to base a hypothesis regarding its effect during stress (Figure 6B). Considering our data, we suggest that the faster degradation of proteins such as Bmh1p, Lsp1p and Rpp0p in *Δsip18, pGAL-SIP18* cells compared with BY4742 cells after stress may help to resume growth after stress, although these proteins are involved in important cell metabolic processes. Seven of the eight members of the GO functional annotation term transmembrane transporter activity (Atp2p, Atp3p, Atp4p Cox9p, Cox13p, Pma1p and Qcr2p) are involved in the oxidative phosphorylation KEEG pathway (Figure 3B). Among them, Qcr2p, Cox9p, Cox13p and Cyt1p showed protein-protein interactions as detected by PSI-MI assay (Claypool *et al*., 2008) (Figure 6A).

Cytochrome c1 (Cyt1p) is contained in the second profile and is a component of the mitochondrial respiratory chain. Furthermore, the Δ*cyt1* strain shows severe mitochondrial deficiencies. However, the cytochrome c oxidase subunits Cox9p and Cox13p and the cytochrome c reductase subunit Qcr2p, which are essential to for energy generation showed accumulation in Δ*sip18, pGAL-SIP18* cells during stress (Hunte *et al*., 2003). The same profile was exhibited by the H^+-ATPase plasma membrane efflux pump Pma1p, which is the major regulator of cytoplasmic pH and plasma membrane potential and which is essential for rehydrated cells to recover membrane selectivity and, indirectly, vacuolar activity by restoring cytoplasmic pH (Ambesi *et al*., 2000). However, Hsp30p, which is a negative regulator of Pma1p, showed an opposite profile in Δ*sip18, pGAL-SIP18* cells that correlated with the dehydration sensitivity phenotype described for the Δ*hsp30* strain (Rodriguez-Porrata *et al*., 2012a). Atp2p, Atp3p and Atp4p are subunits of the mitochondrial F1F0 ATP synthase complex that is required for ATP synthesis (Devenish *et al*., 2000). Both Atp2p and Atp4 are contained in the second established profile (Table 2), which contrasts with the published phenotype of the single mutants Δ*atp2* and Δ*atp4*, which show decreased dehydration stress tolerance (Ratnakumar *et al*., 2011). Thus, we suggest that the significant reduction of Atp2p, Atp4 and Cyt1p in Δ*sip18, pGAL-SIP18* cells may create a bottleneck in ATP stock recovery to resume growth after the rehydration process. The disruption of the cellular energy supply after stress may be lethal for the cell or may delay growth by causing a 120 min longer lag phase compared with the reference strain as was observed by López-Martínez *et al*. (2013). The final member of the transmembrane transporters group is the high-affinity plasma membrane zinc transporter Zrt1p, which was part of the first established profile but which did not show protein-protein interactions or co-expression with any of the other 45 proteins (Figure 4 and 6). Curiously, the Δ*asc1* strain showed high resistance to growth under Zn-limited conditions, and in our study of Asc1p, the Δ*sip18, pGAL-SIP18* cells displayed the second established profile (North *et al*., 2012).

Conclusion

The present work provides evidence that the *SIP18*-overexpressing strain creates a synergetic abundance of certain proteins that allows yeast cells to overcome dehydration stress. From the first protein profile, we obtained 534 proteins expressed in cells BD and AR. We found 158 statistically significant proteins BD and 112 proteins AR, but only 78

proteins were common for both time points. Finally, we pooled 45 of these proteins into two profiles: the 1st profile contained 27 proteins that showed at least a two-fold change in BD and AR Δ*sip18, pGAL-SIP18* cells compared with BD Δ*sip18, pGAL* cells and BD and AR BY4742 cells. The 18 proteins showing the highest values in BD Δ*sip18, pGAL-SIP18* cells but lower AR values than AR BY4742 cells were classified into the 2nd profile. Of these 45 proteins, only 19 were membrane proteins. In particular, by combining the published data of co-immunoprecipitation and co-expression assays and the phenotype of null mutants, we were able to explain the putative contribution of variations in protein abundance among Δ*sip18, pGAL-SIP18*; BY4742; and Δ*sip18, pGAL* cells and their viabilities, which were 90%, 40% and 10%, respectively, after dehydration stress (López-Martinez *et al*., 2012). When cells are subjected to dehydration stress, they are actually being subjected to a variety of stresses, such as oxidative, heat, osmotic and pH, which alter the structure and function of the vacuole and the integrity and functionality of nuclear and cell membranes (Walker and van Dijck, 2006; Porrata-Rodríguez *et al*., 2012b). In previous studies of null and overexpressing strains, genes that were essential for the cell to overcome dehydration stress could be characterised (Rodríguez-Porrata *et al*., 2012a; Ratnakumar *et al*., 2011; Shima *et al*., 2008; López-Martínez *et al*., 2012). Consistent with the null mutant strain studies, we found that the over-degradation of Bmh1p, Lsp1p and Rpp0p in Δ*sip18, pGAL-SIP18* cells correlated with enhanced dehydration stress tolerance. Consistent with the increase in osmotic stress tolerance, the *SIP18*-overexpressing cells accumulated Gvp36p and Gdp1p. The accumulation of Ald4p and the over-degradation of Asc1p in Δ*sip18, pGAL-SIP18* cells correlated with increased oxidative stress tolerance. Additionally, the accumulation of Pma1p and the degradation of its negative regulator Hsp30p are consistent with the idea of rapid restoration of cytoplasmic pH and membrane potential. However, the significant reduction of Lsp1p during stress supports the improvement of the Pkc1p/Ypk1p stress resistance pathways. This study is the first to demonstrate the putative roles of Gvp36p, Gdp1p, Ald4p, Asc1p, Pma1p, Hsp30p and Lsp1p proteins in yeast dehydration stress tolerance.

Acknowledgements

This work was financially supported by grants from the Spanish government (Project AGL2012-40018-C02-1, awarded to RC). GL would also like to thank the Rovira i Virgili

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University and AGAUR for the doctoral fellowship FI-DGR 2012 and Rovira i Virgili University for the short-term fellowship AEE2013. This work has been partly funded within the framework of PRIME-XS, grant number 262067, funded by the European Union 7th Framework Program and the MICINN BIO2011-29233-C02-01 project. GL and RC acknowledge additional support from the Netherlands Proteomics Centre.

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CHAPTER VI

Conclusions and future outlook

Conclusions and future outlook

Anhydrobiote organisms can remain in dry state for years and then restart their cellular activity after rehydration. They have been described among animals, plants and microorganisms belonging to both the eukaryote and prokaryote domains (Crowe *et al.*, 1993). The yeast *S. cerevisiae* is an excellent eukaryotic anhydrobiote for research (Garay-Arroyo, *et al.*, 2000) because it is easy to manipulate and the genetics are simpler in comparison with other eukaryotic organisms. These features have led us to study the structural and functional cell damage and molecular responses caused by desiccation stress. Genetic engineering studies of *S. cerevisiae* not only deepen our understanding of this process, but also serve as a model for future research in tissue storage without the need for a hydric solution. Moreover, *S. cerevisiae* in ADWY formulations is widely used in the food industry in the production of fermented products such as beverages because in this dried format the yeast has greater genetic stability at room temperature, resulting in savings in transport and storage costs. However, the loss of cell viability during the industrial drying and rehydration processes and the resulting lower activity have to some extent hindered the development of a high-quality inoculum (Rodríguez-Porrata *et al.*, 2011). For this reason, a better understanding of the molecular processes involved in yeast desiccation tolerance is required.

ADWY production results in several stress situations for the cell. Heat, osmotic and oxidative stresses cause particular damage to cells. Although desiccation is carried out in the optimum conditions of the stationary phase, adaptive responses are needed to ensure cell survival. Of all the cross-talking molecules and pathways involved in tolerance to desiccation-related stresses, yeast hydrophilin proteins have been described as the most important because they enhance viability after rehydration. Hydrophilin proteins form an artificial rather than functional group and share common physicochemical properties such

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as Gly content and hydrophilicity (Battaglia *et al.*, 2008). This biochemical group is well represented not only among yeast species but also bacterial, animal and plant anhydrobiots (Alpert, 2006). LEA genes may act as binding molecules, antioxidants, molecular shields, chaperons, sugar glass stabilizers, membrane protector and in other roles that have yet to be discovered (Olvera-Carrillo, *et al*., 2011; Hand *et al*., 2010). Twelve yeast hydrophilin proteins were identified in desiccation-related stresses at different omic levels (Garay-Arroyo *et al*., 2000; Gash *et al*., 2000; Singh *et al*., 2005). Indeed, previous research by our group showed that the over-expression of the *SIP18* hydrophilin gene enhances dehydration tolerance by acting as an antioxidant (Rodríguez-Porrata *et al*., 2012a). In the present doctoral thesis we have found that some *S. cerevisiae* hydrophilin proteins are involved in dehydration and rehydration tolerance (see chapters II and III) through the regulatory expression of other metabolites (see chapter V). However, desiccation tolerance is a complex mechanism involving not only hydrophilin proteins but also other molecules identified in our results for the first time (see chapter IV).

We characterized the twelve *S. cerevisiae* hydrophilins in a laboratory strain (see chapter II). Despite possessing biochemical features, not all hydrophilins play a role in yeast desiccation tolerance. Over expression of *STF2* and *SIP18* has a positive effect on desiccation tolerance because they both reduce ROS damage without causing apoptotic cell fraction (Rodriguez-Porrata *et al*., 2012a, see chapter II). Consequently, it is possible that the two hydrophilins have not shared functional role against desiccation, although, it is also possible that they have a common structural role such as protecting nucleic acid or acting as a molecular shield arresting intracellular water content. These hypothesis should be evaluated in the future.

Over expression of *SIP18* in wild and commercial wine yeast strains produced the same desiccation phenotype as in the laboratory strain without altering fermentative performance (see chapters II and III). These common results lead us to propose the over expression of Sip18 as a possible way of reducing inoculum expenses since it would enhance the viability of dried commercial wine strains (see chapter III). These strains were treated with H_2O_2 and it was found that not all of them reduced ROS accumulation as they did after rehydration. These results led us to hypothesize that Sip18p reduces not only non-radical reactive species, but also other minor reactive species such as RNS (Li

and Moore, 2007) and free radicals such as O_2^- (Sheehan *et al*., 2001) that might remain in the cell before Haber-Weis reaction (Halliwell, 2006).

Although the mechanism by which over-expression of *SIP18* decreases ROS and thus increases viability in *S. cerevisiae* remains unclear, we have shown that desiccation tolerance involves other molecules and molecular mechanisms belonging to desiccation related stresses (see chapters IV and V). Among them, membrane proteins could be one of the most important molecules since membrane is damaged primarily during stress imposition even though the starvation-induced stationary phase allows to the cell to withstand different stresses (Werner-Washburne *et al*., 1993). Nevertheless, prior over-expression of the *SIP18* hydrophilin 'preadapts' cells by increasing their capacity to overcome and enhance dehydration and rehydration stress. This cell preadaptation could be due to both *SIP18* accumulation and changes in the expression of the membrane's proteomic profile as a consequence of *SIP18* accumulation (see chapter V). Among the 78 fold-changed proteins, 45 increase in numbers after stress imposition and 27 were found to be downregulated, some of which were identified for the first time in this study. It is worth to note that Gre1p hydrophilin protein appears significantly changed due to overexpression of Sip18p. These results lead us to hypothesize that these 78 proteins could be activated or inhibited as a result of transcriptional regulation by different cross-talked pathways related to desiccation stresses in which *SIP18* might participate or by specific degradation of some proteins by *SIP18* action. This hypothesis is strengthened by the co-expression of *SIP18* leads to the up and downregulation of certain proteins. However, the role of *SIP18* in signaling and inducing degradation needs to be studied in the future.

The results shown in chapters II, III and V confirm that other metabolites in addition to hydrophilins are involved in desiccation stress tolerance. We used QTL analysis to identify which other molecules play a leading role in enhancing dehydration tolerance (see chapter IV). This technique has also been used in several studies to link genotype and phenotype (Jara *et al*., 2014). We evaluated the link between dehydration phenotype and DNA variations in 96 segregants derived from a cross between two haploid strains (WAxWE) created from two strains of wine yeast. Characterization of genes with sequence changes between alleles showed that only five out of eleven genes had different viability for both genetic backgrounds. These results led us to suggest that dehydration tolerance is not gene induced but rather depends on the specific amino acid sequence of

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each allele, possibly in conjunction with other genes activating or inhibiting their function (see chapter V). However, it would be interesting to carry out further studies for the genes which did not show any sequence evaluation in the QTL analyses. Evaluating these genes in other backgrounds is needed to confirm the sequence similarity and their role in dehydration tolerance. This would enable possible molecular markers to be identified that could be used in for selecting high-quality yeast strains for the food industry.

Finally, it would be worth to evaluate the molecular scenario involving overexpression of Gre1p, Stf2p, YJL144wp and Nop6p, which also increase yeast desiccation tolerance, in order to find common molecules and molecular mechanisms with a Sip18p molecular scenario or find some of the proteins described in chapter IV. Taking all into account could be useful for producing robust ADWY or for further investigation in tissue engineering.

The results presented in this doctoral thesis deepen knowledge of the cross-talked desiccation tolerance mechanism and the metabolites involved in it.

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ANNEX

Currivulum Vitae

Publications

Currivulum Vitae

Gema was born on 17 October 1987 in Barcelona, Spain. In 2005 she took up studies in Biotechnology and Biochemistry at the Universitat Rovira and Virgili (URV) in Tarragona. She obtained her degrees in 2009 and 2010 consecutively. During her thesis research at the Abteilung für Tierzucht und Genetics (University of Veterinarian Medicine, Austria) she worked in the field of embryo membrane permeabilization of medaka fish under the supervision of Prof. Thomas Czerny. In 2010 she did a Master's Degree of Oenology. In the course of writing her master's thesis at the URV's Department of Biochemisty and Biotechnology she worked under the supervision of Dr Ricardo Cordero Otero on characterising the hydrophilin proteins of *Saccharomyces cerevisiae* in response to dehydration tolerance. During her master's degree she was introduced to yeast molecular biology mechanisms and she decided to take her career in this direction. After graduation in 2011 Gema started work as doctoral researcher in the Food Microbial Biotechnology Group under supervision of Dr Ricardo Cordero Otero. Her research mainly focused on describing the metabolites and molecular mechanisms involved in yeast dehydration tolerance. At the same time she was awarded a fellowship so she could broaden her knowledge of biomolecular mass spectrometry and proteomics at the Netherlands Proteomics Centre, University of Utrecht. During her short stay she worked in the field of yeast membrane proteomics during dehydration stress under supervision of Dr Maarten Altelaar and Albert J. R. Heck. The results of her work are presented in this thesis.

Publications

López-Martínez, G., Margalef-Català, M., Salinas, F., Liti, G., Cordero-Otero, R. (2015) *ATG18* and *FAB1* Are Involved in Dehydration Stress Tolerance in *Saccharomyces cerevisiae*. *PLoS ONE* **10**: e011.

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Acknowledgements

agradecimientos / agraïments

Antonio Machado escribió "Caminante no hay camino, se hace camino al andar". Es en ese camino que lleva a la cumbre de mi tesis doctoral donde siempre he tenido el apoyo científico y personal de los que me rodean. Aquellos a los que ya quería antes de iniciar la ruta pero también a las personas que después de horas de laboratorio se han hecho querer.

A mi director de tesis, el Dr. Ricardo Cordero Otero, quiero agradecer el eterno aprendizaje que he recibido de su mano durante estos cuatro años, ya que a través de nuestras charlas he aprendido a aprender a hacer ciencia. En definitiva, gracias por ser mi guía sobre el terreno de la investigación.

Asimismo, agradezco al grupo *Biomolecular Mass Spectrometry and Proteomics* liderado por Albert Heck, y a mi tutor durante mi estancia doctoral, Maarten Altelaar, por permitir ser parte de un equipo excepcional en investigación durante algunos meses y poder impregnarme de sus conocimientos en el área. Esta estancia no solo se ha saldado con una próxima publicación sino con una amistad. Gracias a Alba y Aitor por toda la ayuda prestada personal y científica.

A todo el personal de dentro y fuera de los laboratorios del Departamento de Biotecnología y Bioquímica y especialmente a los miembros del grupo Biotecnología Microbiana de los Alimentos. Especial mención es también para los miembros del tribunal de mi tesis así como los revisores externos.

Durant el doctorat hi ha tantes vivències que es podria fer un annex al llibre de la tesi amb un rànquing amb tot el que he viscut, però en cada una d'elles sempre han estat les meves amigues. Gràcies Anna, Mar i Maria José. Tornaria a fer aquest camí si vosaltres estiguéssiu al meu costat, compartint el dia a dia. Gràcies SS. I com no a Gemma i Eugènia que ara ja també formeu part d'aquest camí.

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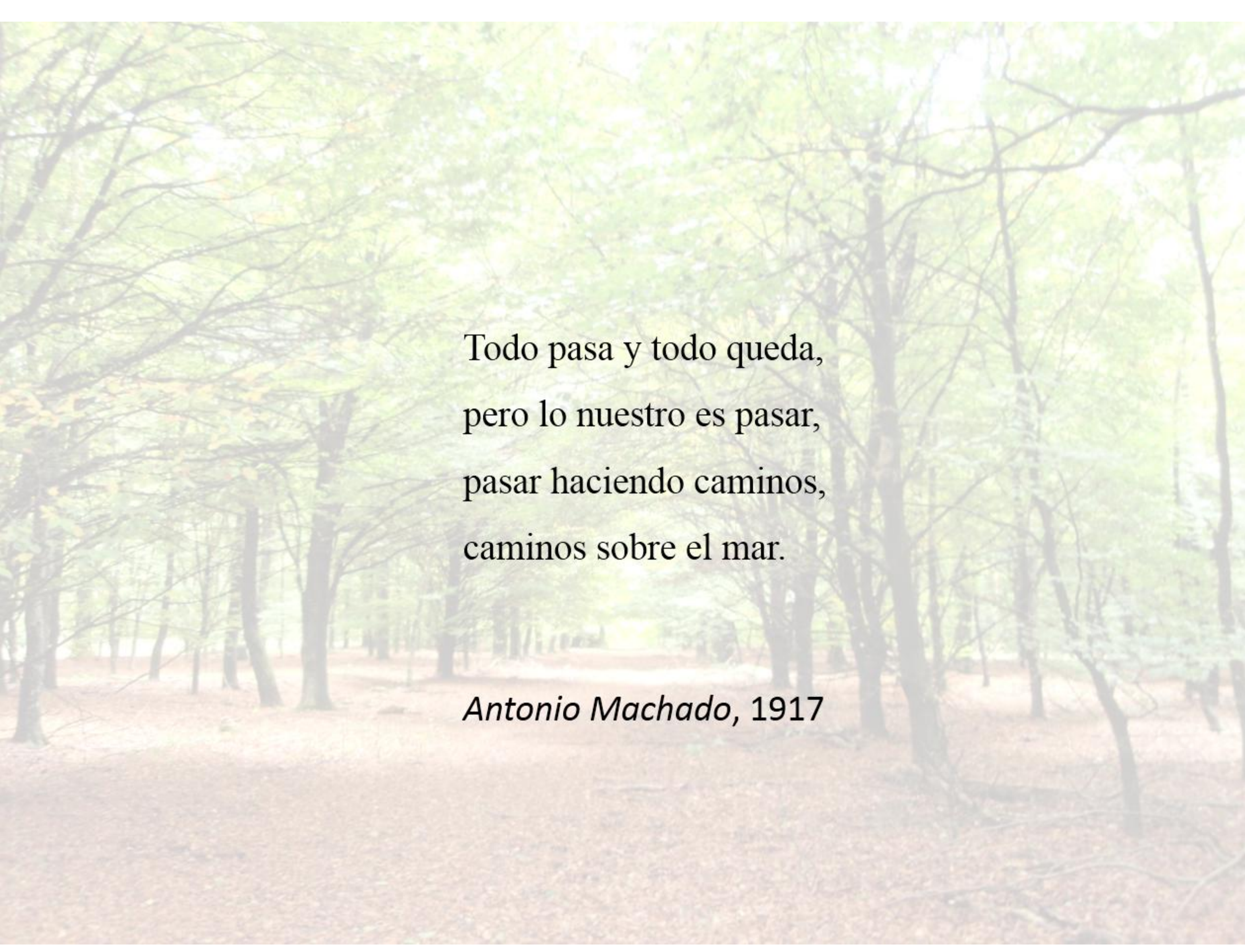
A Carmen y Vanessa por todos los ánimos que siempre me han dado en esta ruta, etapa a etapa y siempre con una gran sonrisa. A las chicas de mi cuadrialla de la Uni por vuestro apoyo cada vez que nos juntanos y comprobamos que el tiempo no pasa entre nosotras.

Finalmente, muchas gracias a los que ya estabais en la meta mucho antes de iniciar el camino y que a pesar de no pertenecer a éste mundo, habéis sufrido como los que más. Mamá, Papá, Nene y Miquel, gracias por el quinteto familiar que formamos. En especial a Miquel por la paciencia diaria y la comprensión y a mi padre por enseñarme que las metas realmente no existen, siempre hay que seguir caminando a la siguiente etapa de la ruta.


Todo pasa y todo queda,
pero lo nuestro es pasar,
pasar haciendo caminos,
caminos sobre el mar.
Antonio Machado, 1917

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