



Overlapping responses between salt and oxidative stress in *Debaryomyces hansenii*

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Abstract

Debaryomyces hansenii is a halotolerant yeast of importance in basic and applied research. Previous reports hinted about possible links between saline and oxidative stress responses in this yeast. The aim of this work was to study that hypothesis at different molecular levels, investigating after oxidative and saline stress: (i) transcription of seven genes related to oxidative and/or saline responses, (ii) activity of two main anti-oxidative enzymes, (iii) existence of common metabolic intermediates, and (iv) generation of damages to biomolecules as lipids and proteins. Our results showed how expression of genes related to oxidative stress was induced by exposure to NaCl and KCl, and, vice versa, transcription of some genes related to osmotic/salt stress responses was regulated by H₂O₂. Moreover, and contrary to *S. cerevisiae*, in *D. hansenii* *HOG1* and *MSN2* genes were modulated by stress at their transcriptional level. At the enzymatic level, saline stress also induced antioxidative enzymatic defenses as catalase and glutathione reductase. Furthermore, we demonstrated that both stresses are connected by the generation of intracellular ROS, and that hydrogen peroxide can affect the accumulation of in-cell sodium. On the other hand, no significant alterations in lipid oxidation or total glutathione content were observed upon exposure to both stresses tested. The results described in this work could help to understand the responses to both stressors, and to improve the biotechnological potential of *D. hansenii*.

Keywords *Debaryomyces hansenii* · Gene expression · Oxidative defenses · ROS content · Stress response

Introduction

Living cells are continuously exposed to various types of stress factors. This is especially relevant in the case of unicellular organisms, such as yeasts, since they are very metabolically versatile and possess an extraordinary ability to adapt to adverse environments. In order to cope with the different stresses and to protect themselves from cellular

damages, yeasts have developed highly complex response networks that vary depending on the stress types (Hohmann and Mager 2003). Up to date, most of the information available on these adaptive changes comes from the model yeast *Saccharomyces cerevisiae*. In this yeast, the Yap1-mediated regulatory pathway controls the oxidative stress response, and the High Osmolarity Glycerol (HOG) pathway regulates the response to Osmotic stress (Auesukaree 2017; Herrero et al. 2008). Additionally, *S. cerevisiae* also presents a global non-specific stress response induced by different environmental stresses (osmotic, ethanol, heat, oxidative, nitrogen starvation, etc.) that can happen separately or simultaneously, e.g. during ethanol fermentation (Auesukaree 2017; Gibson et al. 2007), although the molecular mechanisms that trigger this global response are not fully understood. The modulated genes are activated by the binding of two zinc fingers transcriptional activators, Msn2p and Msn4p, to a *cis*-acting sequence within their promoters, called Stress Responsive Element (STRE) (Gibson et al. 2007). Additionally, it is usually assumed, but not frequently demonstrated,

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that the same mechanisms work in a similar manner in the so-called nonconventional yeasts.

Debaryomyces hansenii is a hemiascomycetous yeast of importance in basic and applied research. On the one hand, it has a high biotechnological potential and, on the other hand, it shows physiological and molecular peculiarities not observed in other yeasts (Breuer and Harms 2006; Prista et al. 1997). *D. hansenii* has been described as a halotolerant/halophilic yeast found in saline environments such as sea water, salty foods or dry meat products (Cocolin et al. 2006; Encinas et al. 2000; Norkrans 1966, 1968; Ramos et al. 2017). In those environments, *D. hansenii* cells can be simultaneously exposed to other stresses that cause biochemical and physiological perturbations, and, for that reason, it is conceivable to think on the existence of common or coordinated responses to different external conditions as those recently proposed in *S. cerevisiae* (Taymaz-Nikerel et al. 2016) or in other pluricellular organisms as higher plants (see (Liang et al. 2018) for a recent review).

Saline stress main perturbations are related to ionic toxicity and low water activity. Halotolerance has been studied during decades in *D. hansenii*, and physiological adaptations to high salt have been reported at different levels as ion homeostasis (Norkrans and Kylin 1969; Prista et al. 1997), plasma membrane composition (Turk et al. 2007) or mitochondrial characteristics (Cabrera-Orefice et al. 2010, 2014). Furthermore, enzymes and cation transporters specifically involved in sodium tolerance have also been identified (Aggarwal et al. 2005; Carcia-Salcedo et al. 2007; Chawla et al. 2017; Martinez et al. 2011; Minhas et al. 2012).

Oxidative stress is a term associated to the generation of Reactive Oxygen Species (ROS) which are oxygen-derived molecules with unpaired electrons (Herrero et al. 2008; Toledano et al. 2004). ROS are highly toxic and mutagenic as they can damage all biomolecules, plus drastically alter the in-cell balance between reduced and oxidized glutathione (Sies 1986). In contrast to what mentioned for saline stress, little work has been devoted to the study of oxidative stress in *D. hansenii* (Michan et al. 2013; Navarrete et al. 2009). Recently, a transcriptomic analysis identified genes differentially expressed in the response of *D. hansenii* to heavy metals that are associated to oxidative stress production. Congruently, the authors concluded that the main response of *D. hansenii* to cobalt exposure was the activation of non-enzymatic oxidative stress response mechanisms and of metabolic processes that prevent biological production of reactive oxygen species (Guma-Cintrón et al. 2015).

Remarkably, in *D. hansenii* the presence of salt protects against different stress factors such as temperature, pH or oxidative stress inducers (Almagro et al. 2000). Nevertheless the final reasons for this phenomenon are not fully understood and this behavior does not seem to be observed in the model *S. cerevisiae* (Almagro et al. 2000). Hints of the

linkage between saline and oxidative stress responses in *D. hansenii* have also been reported (Garcia-Neto et al. 2017; Michan et al. 2013; Navarrete et al. 2009; Segal-Kischinevsky et al. 2011), although the stress conditions used in those works were either chronic or highly toxic.

The aim of this work was to study the molecular links between salt and oxidative responses in the halotolerant yeast *D. hansenii*. Our results show a relationship between both stress responses at: (i) the transcriptional level, as the expression of genes related to oxidative stress was affected by salt exposure and, vice versa, transcription of some genes related to osmotic/salt stress responses was regulated by oxidative stress inducers, and (ii) the enzymatic level, as ROS-related catalase and glutathione reductase activities were modulated by saline stress. Furthermore, we investigated the molecular mediators beneath these links and demonstrated that intracellular ROS levels increased upon exposure to oxidative stress and salts, and that hydrogen peroxide can enhance sodium accumulation inside the cells. All together our results suggest a molecular connection in *D. hansenii* between the responses to oxidative and saline stresses.

Materials and methods

Strains, media and growth conditions

Debaryomyces hansenii CBS767 (genotype wild-type, Netherland collection, <http://www.westerdijkinstituut.nl/collections/>) was used as wild-type strain throughout this work (Navarrete et al. 2009). Yeast cells were routinely grown in YPD with constant agitation (180 r.p.m.) at 26 °C. For exposure experiments, cultures were grown until exponential phase ($OD_{600nm} = 0.6–0.7$), and NaCl (200 mM), KCl (200 mM) or H₂O₂ (50 μM) were added.

RNA isolation and reverse transcription

Total RNA was extracted using the TRI-REAGENT reagent (Sigma-Aldrich). Briefly, cells in exponential phase were harvested, washed with cold water and resuspended in 1 ml of TRI-REAGENT plus approximately 200 μl 0.5-mm glass beads. For disruption, yeasts were vortexed 10 times for 1 min with intervals of at least 1 min on ice, incubated 5 min at 70 °C, and followed by other 10 times 1 min vortexing with cooling intervals. Afterward, the standard TRI-REAGENT protocol for RNA isolation was followed. Isolated RNA samples were treated using DNase I to remove contaminating DNA until no PCR amplification as observed without prior cDNA synthesis. RNA sample quality and quantification was performed spectrophotometrically. At least two RNA preparations were isolated for each experimental condition. 1 μg from each RNA sample was retrotranscribed

with Kit iScript™ cDNA Synthesis Kit (Bio-Rad) on three separate occasions that were pooled together before PCR amplification.

Real-time PCR

Sequences of genes for primer design were obtained from Gene Ontology Consortium (www.geneontology.org) and Genolevures Consortium (<http://igenolevures.org/>). Primers with high T_m (> 80.7 °C) and optimal $3'\Delta G$ (< -6.8 kcal mol⁻¹) values were made with OLIGO 7.60 program (Molecular Biology Insights) (Supplementary Table 1). All primer pairs specifically amplified the desired target sequence, no primer dimers were detected. All amplification efficiencies were close to 100%.

The PCR amplification was carried out in a mixture (25 μ l final volume) with IQ™ SYBR® Green Supermix (Bio-Rad), 1 μ l of cDNA, plus 0.1 μ M of the specific primers. PCR reactions were performed at least in triplicate. Real-time PCR conditions were an initial denaturation step, 95 °C 3 min, followed by forty PCR cycles consisting of 15 s of denaturation at 95 °C, and 30 s of annealing plus elongation at 70 °C. Finally, melting curves were determined.

Cell free extracts

Cells were harvested by centrifugation (5000 \times g, 4 °C, 10 min), and washed once with sterile water. Extracts were then prepared resuspending yeasts in 10 mM Tris–HCl pH 7.4, 1 mM PMSF, and grounded by vigorous shaking for 1 min with 0.5-mm glass beads. The process was repeated with at least a 1-min interval on ice until breakage, which was checked microscopically. Cell extracts were separated from cell debris and glass beads by centrifugation (20,000 \times g, 4 °C, 15 min).

Enzymatic antioxidant activities and total glutathione determination

These determinations were assayed spectrophotometrically using a DU®650 Spectrophotometer (Beckman Coulter), according to protocols habitually used in our group (e.g. Alhama et al. 2018; Navarrete et al. 2009).

Catalase (CAT) activity was determined by the breakdown of hydrogen peroxide measured at 240 nm. The reaction was performed in 17 mM potassium phosphate buffer, pH 7.0 and 20 mM H₂O₂. Specific activity is expressed as nmol consumed H₂O₂ min⁻¹ (mU) and further referred to protein content (mg⁻¹) (extinction coefficient = 0.04 mM⁻¹ cm⁻¹).

Glutathione reductase (GR) activity was detected by β -NADPH consumption at 340 nm in the presence of oxidized glutathione (GSSG). The reaction mixture contained 120 mM potassium phosphate buffer, pH 7.2, 0.275 mM

EDTA, 2.125 mM GSSG and 0.1625 mM β -NADPH. Specific activity is given in nmol β -NADPH consumed per min (mU) and mg protein using an extinction coefficient of 6.22 mM⁻¹ cm⁻¹.

Total glutathione was determined using 5-5-dithio-bis-2-nitrobenzoic acid (DTNB) for color development after protein precipitation of the samples with metaphosphoric acid. Briefly, glutathione reductase was added to transform GSSG into reduced glutathione (GSH). Then, glutathione reacts with DTNB in the presence of NADPH to produce a yellow colored 5-thio-2-nitrobenzoic acid that absorbs at 412 nm. The reaction mixture contained 90 mM potassium phosphate buffer, pH 7.0, 0.5 mM DTNB, 0.22 mM β -NADPH, and 1 μ l/ml Glutathione Reductase (Sigma-Aldrich). Total glutathione concentration was extrapolated using a standard curve generated by performing the assay using known concentrations of GSSG.

Protein contents of the extract were determined by the Bradford method (Bio-Rad) using bovine serum albumin (BSA) as a standard.

ROS levels

The intracellular ROS content was determined as described (Chattopadhyay et al. 2006; Nomura and Takagi 2004). Briefly, cells were grown until OD_{600nm} reached values of 0.6–0.7. Thirty minutes before collection, cells were supplemented with 10 μ g ml⁻¹ of 2',7'-dichlorofluorescein diacetate (DCFA) (Sigma-Aldrich). Ten minutes before collecting the cells, they were further supplemented with H₂O₂ (50 μ M or 80 mM) or NaCl (200 mM or 1 M). Only DCFA was added to controls. After centrifugation, cells were broken in water following the same procedure used for generating cell-free extracts (see above). Measurements were performed using a fluorescence spectrophotometer (LS 50B, Perkin Elmer) with λ_{ex} 504 nm and λ_{em} 529 nm. Samples were normalized by their protein concentration.

Sodium content analysis

To estimate internal sodium content, cells of *D. hanse-nii* were grown in YPD medium until exponential phase (OD_{600nm} = 0.6–0.7), and 50 μ M H₂O₂ or/and 200 mM NaCl were added to the medium. Cell samples (5 ml) were collected on Millipore nitrocellulose 0.8 μ m filters after 0, 10, 20 or 30 additional minutes of incubation at 26 °C. Cells were then rapidly washed with 20 mM MgCl₂, and treated with 165 mM chloridric acid during several hours (3–5 h). Finally, extracts were centrifuged, and the supernatants were analyzed by atomic emission spectrophotometry (Ramos et al. 1990).

Oxidative damage to lipids

Malondialdehyde (MDA) was detected fluorometrically using the thiobarbituric acid (TBA) reactivity assay. Briefly, 15 μl of each lysate was mixed with 125 μl 0.5% (w/v in methanol) butylated hydroxytoluene (BHT), 50 μl 0.66 N H_2SO_4 and 37.5 μl 0.4 M Na_2WO_4 . Final volume was adjusted to 1 ml with milli-Q water. After centrifugation ($5000\times g$, 5 min, room temperature), 250 μl 1% (w/v in NaOH) TBA were added to the supernatants, and the mixture was placed at 95 $^\circ\text{C}$ for 1 h. Once at room temperature, fluorescence was determined at Ex/Em 515/550 nm, 15 nm 230 slit width, using an LS 50B Fluorescence Spectrometer. MDA concentrations were determined according to a standard curve generated by 1,1,3,3-tetramethoxypropane (TMP) and expressed in pmol mg^{-1} protein.

Redox proteomics

100 μg of protein was used to label reduced and oxidized thiols. Reduced thiols were labeled with monobromobimane (mBbm) (200 mM) in the presence of SDS (1%). For oxidized protein thiol determinations, extracts were homogenized in iodoacetamide (200 mM) and SDS (1%) to blocked originally reduced thiols. After 10 min at room temperature, trichloroacetic acid (TCA) (20%) was added and the extract was washed twice with cold acetone (500 μl) to eliminate iodoacetamide excess. Oxidized thiols were reduced with DTT (1 mM) and SDS (1%), and labeled with mBbm (200 mM).

Samples were analyzed by SDS–polyacrylamide gel electrophoresis on 4% (w/v) stacking gel and 15% resolving gel using the Laemmli buffer system, and separated at 200 V constant in a Mini-PROTEAN 3 Cell (Bio-Rad). To diminish the background fluorescence, gels were washed in 50% methanol for 30 min. Fluorescent protein bands were detected in a Gel Doc EQ system (Bio-Rad), and further stained with Coomassie Blue R250 to visualize total proteins. Image Lab software (Bio-Rad) was used for acquisition of gel images and all subsequent analyses.

Statistical analysis

Statistical significance was evaluated using ANOVA, followed by post hoc multiple comparison according to Dunnett for parametric analysis or Kruskal–Wallis for non-parametric ones, using InStat software. Experiments were performed in at least 3 biological replicates. Statistically significant differences are expressed as *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Results

Transcriptional regulation upon saline or oxidative stress

Debaryomyces hansenii is a non-conventional yeast with increasing applications in food biotechnology due to its halotolerance and its heterogeneous metabolism (Prista et al. 2016). Several studies suggesting the existence of overlapping points in the regulatory pathways for oxidative and saline stress responses (Garcia-Neto et al. 2017; Michan et al. 2013; Navarrete et al. 2009; Segal-Kischinevzky et al. 2011) prompted us to investigate if the responses to salts and to hydrogen peroxide could transcriptionally modulate a common set of genes in *D. hansenii*. Initially, *CTA1* and *HOG1* were selected as marker genes for each stress pathway, as although their response to oxidative and salt stress, respectively, had not been previously studied in *D. hansenii*, both had been related to these stresses in several organisms. *CTA1* encodes peroxisomal catalase A, the enzyme that catalyzes decomposition of H_2O_2 ROS, thus, it is directly related to the oxidative stress response and constitutes one of the main barriers against intracellular oxidative damage. Furthermore, in *D. hansenii*, catalase activity has been described to be modulated by carbon sources (Segal-Kischinevzky et al. 2011). *HOG1* encodes a Mitogen-Activated Protein (MAP) kinase that plays a pivotal role as a transcriptional regulator in the response to saline stress by the High Osmolarity Glycerol (HOG) pathways in several yeasts including *D. hansenii*, although in *S. cerevisiae* its activation mechanism occurs at the post-transcriptional level via phosphorylation (Garcia-Neto et al. 2017; Ma and Li 2013; Melamed et al. 2008; Posas et al. 2000; Saito and Posas 2012). Also, in *C. albicans*, Ssk1p, an homologue of a member of this pathway in *S. cerevisiae*, has a minor role in osmotic adaptation but is essential for oxidative adaptation (Chauhan et al. 2003). Gene expression for *CTA1* and *HOG1* genes was determined after exposing *D. hansenii* exponential cells to non growth-inhibitory levels of H_2O_2 (oxidative stress), NaCl or KCl (saline stress), according to the data shown in Supplementary Fig. 1. Both transcripts showed statistically significant increments upon exposure to both types of stress, although top levels for *CTA1* mRNA were obtained after H_2O_2 exposure, and for *HOG1* after NaCl exposure (Supplementary Fig. 2).

Five more genes were then selected to deeper study transcription changes upon stress exposure in *D. hansenii*, among those previously related to oxidative or saline responses in other yeasts. *GSH1* codes for the first enzyme in the synthesis of glutathione, the key metabolite for intracellular redox state control (Navarrete et al. 2009). *MSN2*, together with *MSN4*, are homologues to *S. cerevisiae* genes

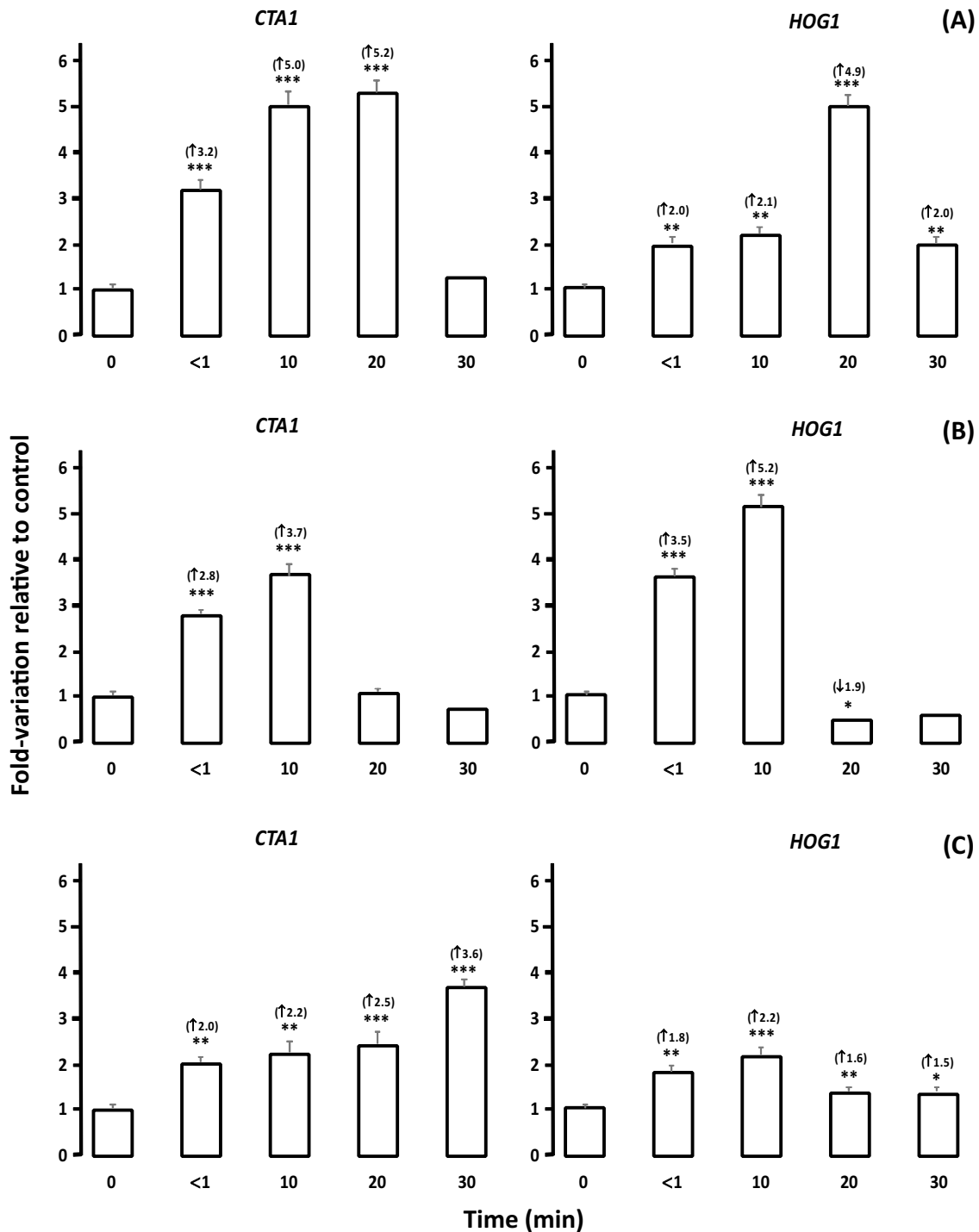


Fig. 1 Time-course effect of oxidative and saline stress on *CTA1* and *HOG1* mRNA levels. *D. hansenii* cells were grown in YPD medium to exponential phase and treated during <1, 10, 20 and 30 min with **a** 50 μ M H₂O₂, **b** 200 mM NaCl and **c** 200 mM KCl. 0 refers to untreated cells. Samples were then collected for RNA isolation, and

CTA1 and *HOG1* transcripts were determined by qRT-PCR. Data are the means of $n \geq 6$. Fold variation is given in *parentheses*. Statistically significant differences are expressed as * $p < 0.01$; ** $p \leq 0.001$; and *** $p \leq 0.0001$

coding for transcriptional regulators that play pivotal roles in the responses to oxidative, heat shock and starvation stresses (Kuang et al. 2018; Morano et al. 2012), and have also

been associated to the HOG pathway (Capaldi et al. 2008). Thioredoxins, represented by *TRX2* gene, are small redox proteins that play central roles in the response to oxidative

stress. *ENA1* codes for a plasma membrane Na^+, K^+ -ATPase transporter that is a key factor for survival to saline and alkaline stresses in *S. cerevisiae* (Arino et al. 2019). Finally, RCN proteins regulate the Ser/Thr phosphatase calcineurin, the protein that couples Ca^{2+} signals to cellular responses. In *S. cerevisiae* *RCN1* codes for an inhibitor linked to osmotic, heat, ethanol and cell wall stresses (Anderson et al. 2012; Kingsbury and Cunningham 2000). The regulation of all

these genes by oxidative or saline stress in *D. hansenii* was investigated by examining the changes of expression over time upon exposure to sub-lethal levels of H_2O_2 , NaCl or KCl (Figs. 1, 2, 3). In general, NaCl provoked the biggest changes in the analyzed gene transcription levels although H_2O_2 and KCl also affected the expression of most of the studied genes (Figs. 1, 2, 3). Those coding for proteins directly related to oxidative defenses in *D. hansenii* and/or

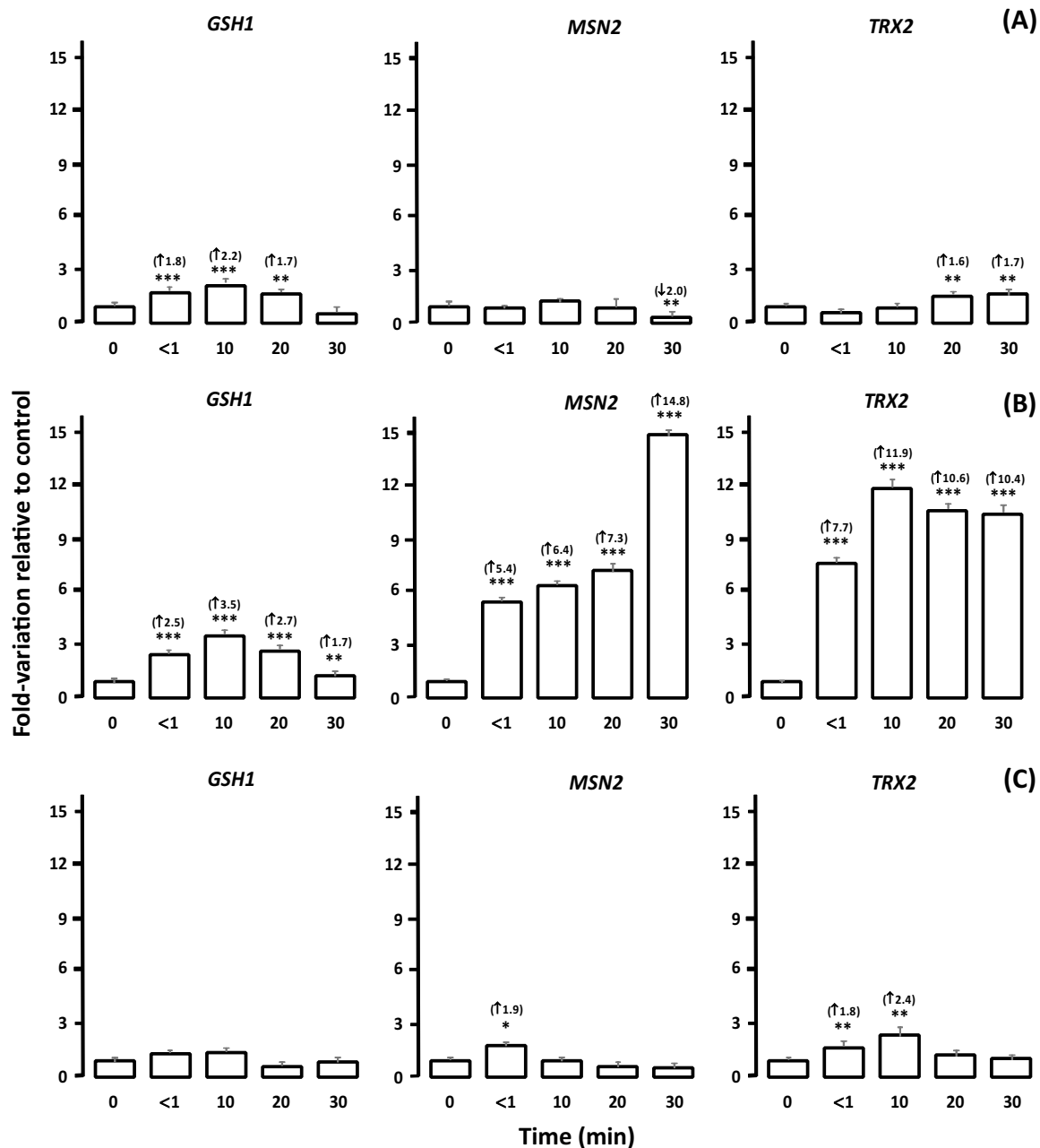


Fig. 2 Time-course effect of oxidative and saline stress on *GSH1*, *MSN2* and *TRX2* mRNA levels. *D. hansenii* cells were grown in YPD medium to exponential phase and treated during <1, 10, 20 and 30 min with **a** 50 μM H_2O_2 , **b** 200 mM NaCl and **c** 200 mM KCl. 0

refers to untreated cells. Samples were then collected for RNA isolation, and *GSH1*, *MSN2* and *TRX2* transcripts were determined by qRT-PCR. Further description as in Fig. 1

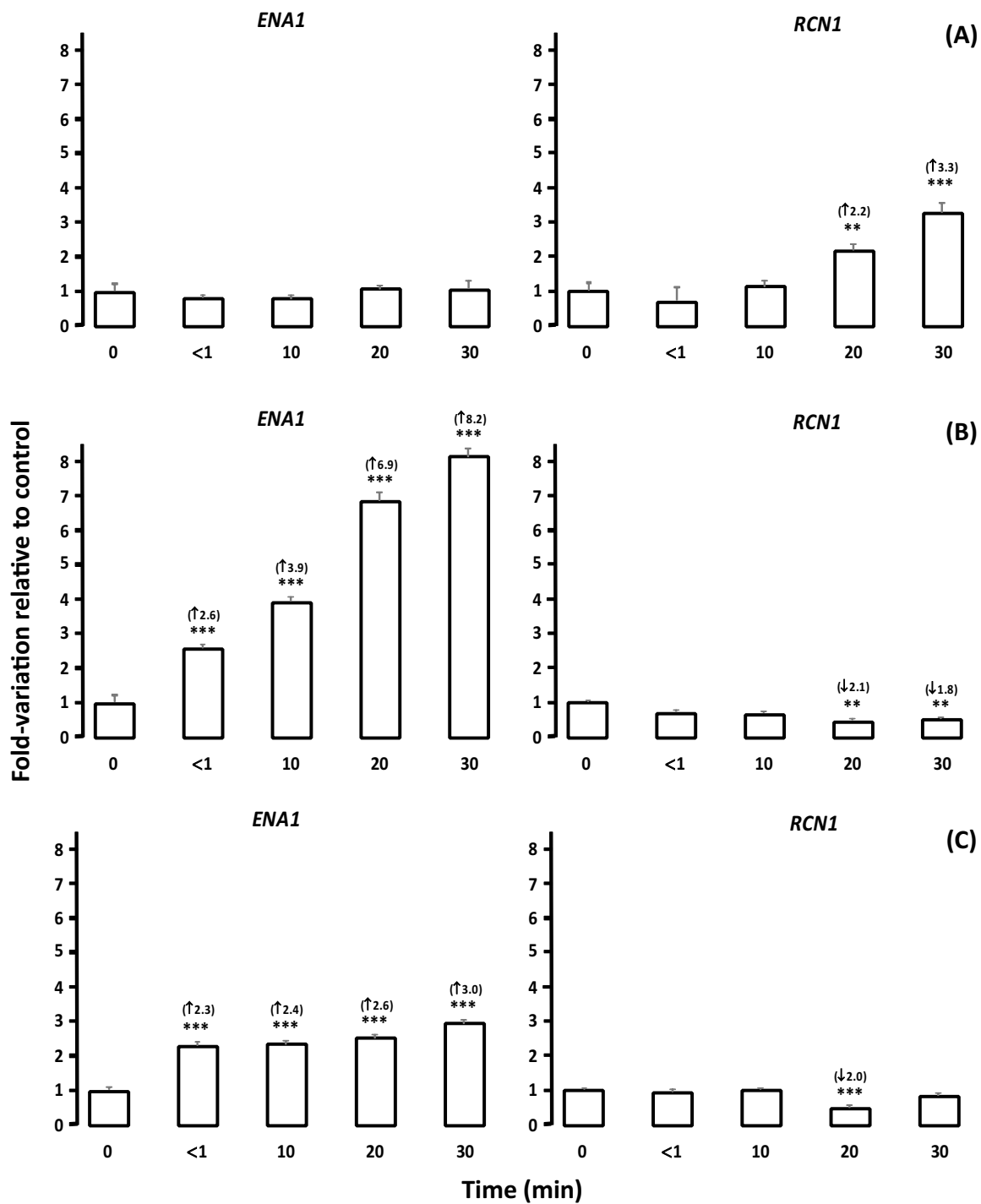


Fig. 3 Time-course effect of oxidative and saline stress on *ENA1* and *RCN1* mRNA levels. *D. hansenii* cells were grown in YPD medium to exponential phase and treated during <1, 10, 20 and 30 min with a 50 μM H₂O₂, b 200 mM NaCl and c 200 mM KCl. 0 refers to

untreated cells. Samples were then collected for RNA isolation, and *ENA1* and *RCN1* transcripts were determined by qRT-PCR. Further description as in Fig. 1

S. cerevisiae (*CTA1*, *TRX2* and *GSH1*) were induced upon H₂O₂ as expected, but also incremented their expression in the presence of salts (Figs. 1, 2). Even more, *GSH1* and *TRX2* transcripts, which products are typically related to the

control of intracellular redox state, reached top levels after NaCl exposure (Fig. 2). On the other hand, the expression of genes associated to osmotic/saline stress *HOG1*, *MSN2*, and *ENA1* was as well strongly induced upon expression to

NaCl, while repressor-coding *RCNI* transcript levels were reduced (Figs. 1, 2, 3).

Effect of saline and oxidative levels on enzymatic and non-enzymatic antioxidative defenses

To investigate if the detected transcriptional stress response in *D. hansenii* described in the previous section could affect

its antioxidative responses, the activity of two main antioxidant enzymes, catalase (CAT) and glutathione reductase (GR), was followed upon exposure to the stress inducers (Figs. 4, 5). As expected from their role in the antioxidative response, hydrogen peroxide increased the activity for both enzymes. Furthermore, CAT, in agreement with the transcriptional data, and GR activities also increased in the presence of NaCl and KCl to levels similar as those determined

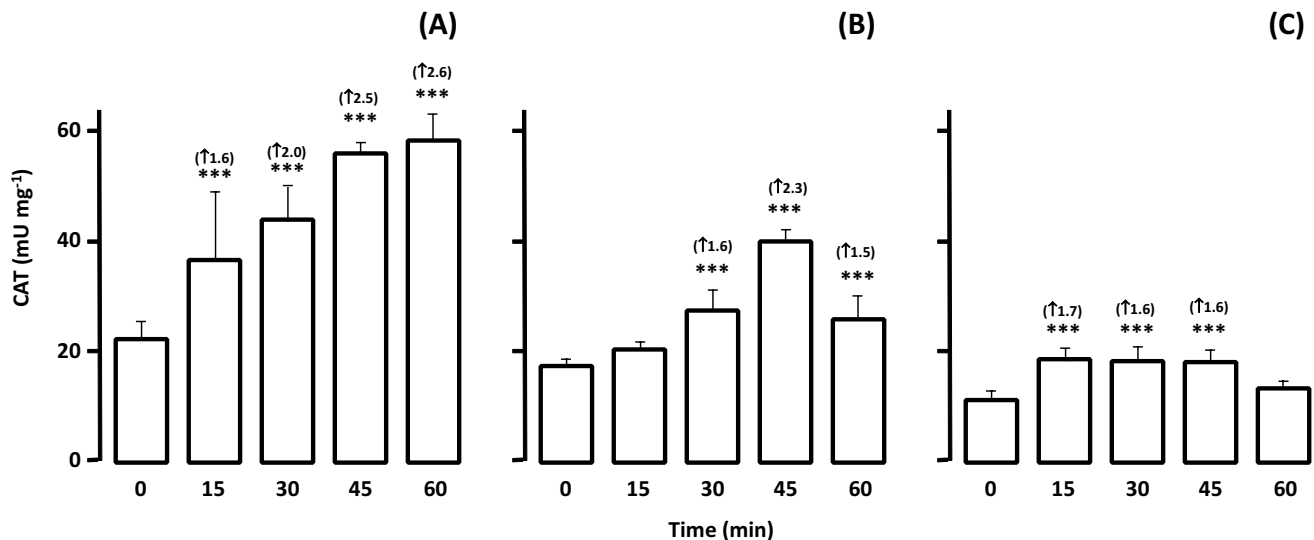


Fig. 4 Oxidative and saline stress effect on catalase activity. *D. hansenii* cells were grown in YPD to exponential phase and treated during 15, 30, 45 or 60 min with **a** 50 μM H₂O₂, **b** 200 mM NaCl, or **c** 200 mM KCl, collected and extracted for measuring catalase activity

(mU per microgram of total protein) ±SD from three independent experiments. 0 refers to untreated cells. Further description as in Fig. 1

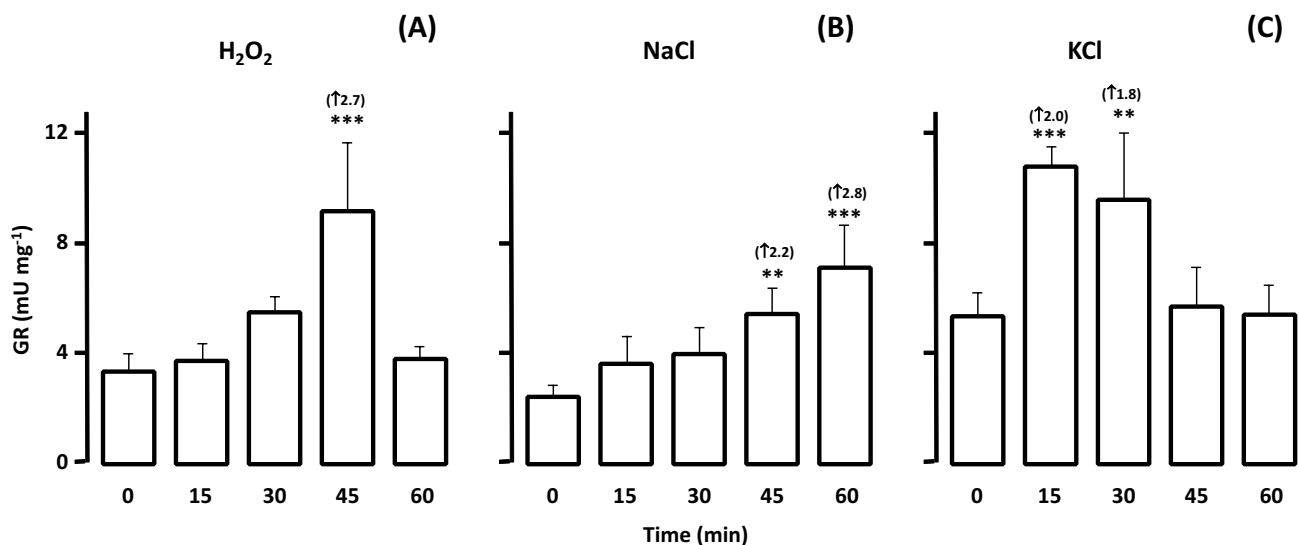


Fig. 5 Oxidative and saline stress effect on glutathione reductase activity. *D. hansenii* cells were grown in YPD to exponential phase and treated during 0, 15, 30, 45 or 60 min with **a** 50 μM H₂O₂, **b**

200 mM NaCl, or **c** 200 mM KCl, collected and extracted for measuring glutathione reductase activity (mU per mg of total protein) ±SD from three independent experiments. Further description as in Fig. 1

after oxidative stress (Figs. 4, 5). Nevertheless, the patterns observed were not similar neither for both enzymes nor for the different stressors. CAT activity response to hydrogen peroxide increased up to 60 min after induction while response to salts decreased after 45 min exposure. Also, CAT activity increments in response to the analyzed stressor were slightly inferior to those detected at the transcriptional level. Furthermore, induction of this activity in the presence of hydrogen peroxide or NaCl could be observed up to 60 min after adding the stressors while their corresponding mRNA increments were not observed after 20 and 10 min respectively.

Glutathione is a low molecular-weight thiol, highly abundant in the cytosol, that plays a pivotal role as a non-enzymatic antioxidant. Total glutathione content was determined in *D. hansenii* after exposure to oxidative or saline stress, but no statistically significant changes were detected (data not shown).

Intracellular ROS and Na⁺ levels upon exposure to saline or/and oxidative stress

To check if saline stress was affecting oxidation-related genes by inducing oxidative stress inside cells, intracellular ROS levels were estimated with 2',7'-dichlorofluorescein (DCFDA), a widely used technique for directly measuring the generalized oxidative stress in cell (Fig. 6). Low levels of hydrogen peroxide and NaCl used for the transcriptional and enzymatic analysis did not significantly increment fluorescence (data not shown). Nevertheless, a second analysis using higher concentrations of NaCl and H₂O₂ showed an

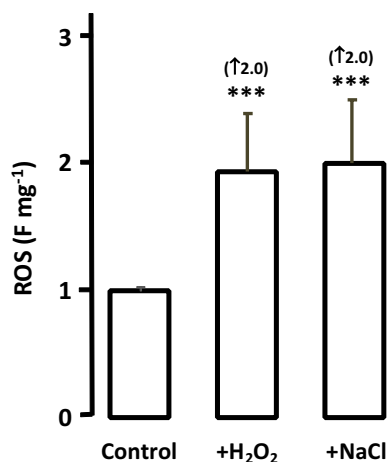


Fig. 6 ROS content. *D. hansenii* cells were grown in YPD to exponential phase and treated with 80 mM H₂O₂ or 1 M NaCl for 10 min. DCFDA fluorescence was determined as described in Materials and Methods. Data are means (arbitrary fluorescence units per mg wet weight) ± SD from three independent experiments. Further description as in Fig. 1

increase in the ROS levels to roughly the same extent for both stressors (Fig. 6).

Growing *D. hansenii* under saline stress can impact its intracellular Na⁺ levels (Herrera et al. 2017; Montiel and Ramos 2007). To investigate if oxidative stress could alter Na⁺ uptake in *D. hansenii*, cation intracellular accumulation was followed during 30 min after addition of H₂O₂, NaCl and both stressors (Fig. 7). No changes were detected after adding just hydrogen peroxide as under these conditions the sodium concentration present in the complex YPD medium is low (≈ 10 mM). As expected, intracellular Na⁺ concentration raised 50% after supplementing the culture media with NaCl. But, when both stressors were added simultaneously to the media, intracellular Na⁺ levels reached higher levels than those measured only in the presence of the salt (Fig. 7).

Oxidative and saline stress damage to lipids and proteins

ROS can deeply damage biomolecules, such as lipids or proteins. We investigated if oxidative and saline stresses were affecting those biomolecules by determining lipid peroxidation and the redox state of protein thiols. *D. hansenii* lipid peroxidation was determined analyzing MDA content after both oxidative and saline stress at the sub-lethal concentrations used for the transcriptional and enzymatic analysis. No lipid alterations were observed at the conditions assayed in agreement with the absence of ROS induction observed at those stressor levels (data not shown).

ROS content can also affect the reversible formation of disulphide bonds in proteins and, by that manner, alter the physiology of the cell (Paulsen and Carroll 2013). For that reason, one of the main ways to determine oxidative damage levels in proteins is to estimate the global “redox state” of their Cys residues as these reversible changes determine disulphide bonds’ formation (Alhama et al. 2018; Paulsen and Carroll 2013). Reduced and oxidized Cys residues were globally quantified after mBbm labeling and normalized by Coomassie Blue staining of total proteins (see Materials and Methods for details). Again, no significant variations in the Cys redox state were observed after saline exposure to *D. hansenii* cells at the concentrations used. Nevertheless, low-dose hydrogen peroxide exposure was able to produce a clear increase in protein oxidation (Fig. 8).

Discussion

In *S. cerevisiae* and other yeasts, responses to different stresses in general, and to oxidative and osmotic stress in particular, can be physiologically connected, e.g. osmotic stress can increment antioxidative defenses, and antioxidants can attenuate cell sensitivity to osmotic stress (Dolz-Edo

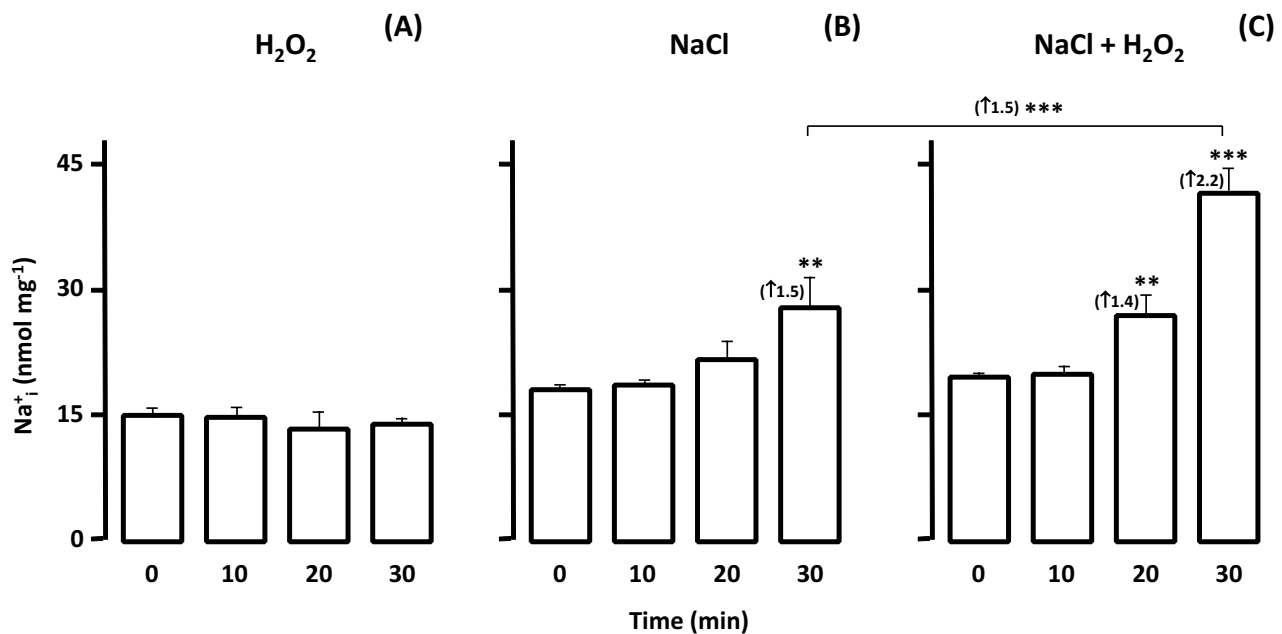


Fig. 7 Na⁺ content. *D. hansenii* cells were grown in YPD to exponential phase and treated during 0, 10, 20 or 30 min with **a** 50 μ M H₂O₂, **b** 200 mM NaCl, or **c** 50 μ M H₂O₂+200 mM NaCl. Cells were then collected on Milipore filters and treated as described in

Materials and Methods. Intracellular Na⁺ content was determined by atomic emission spectrophotometry. Data are means (nmol per mg of dry weight) \pm SD from three independent experiments. Further description as in Fig. 1

et al. 2013; Estruch 2000; Ma et al. 2015; Paumi et al. 2012). Regarding saline stress, in halophilic fungi, these interactions are not so clear as although in *D. hansenii* sodium

can modulate expression of catalase (Michan et al. 2013; Navarrete et al. 2009; Segal-Kischinevzky et al. 2011) or protect against oxidative stress (Navarrete et al. 2009), in *Hortaea werneckii*, oxidative survival was not affected by salt exposure (Petrovic 2006).

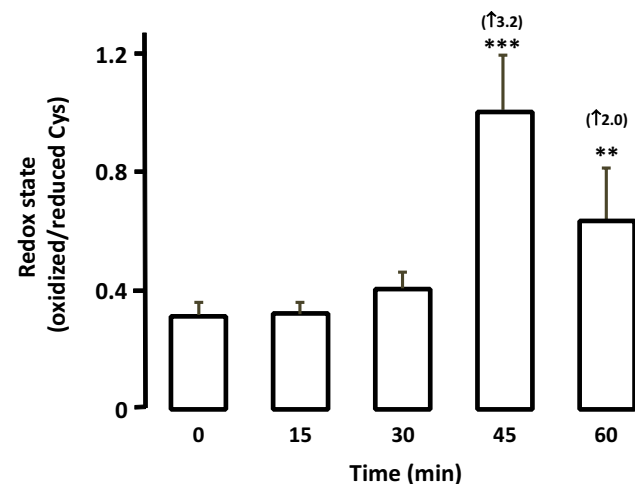


Fig. 8 Electrophoresis-based evaluation of Cys redox state. *D. hansenii* cells were grown in YPD to exponential phase and treated with 50 μ M H₂O₂ during 0, 15, 30, 45 and 60 min. Cells were then collected by centrifugation and processed as described in Materials and Methods. Labeled proteins were separated by SDS-PAGE and quantified in a Gel Doc EQ system (Bio-Rad). Total protein levels were inferred by Coomassie blue staining. Image Lab software (Bio-Rad) was used for analyses. Data are mean \pm SD from three independent experiments. Further description as in Fig. 1

Seven genes encoding proteins previously related to oxidative and saline stress responses were selected for this study. To our knowledge this is the first transcriptional study in *D. hansenii* on the selected genes. All genes but *ENAI* modulated their transcription upon sodium/potassium salts and hydrogen peroxide stressors, and, in general, NaCl provoked the biggest changes (Figs. 1, 2, 3). Those coding for proteins directly related to oxidative defenses in *D. hansenii* and/or *S. cerevisiae* (*CTAI*, *TRX2* and *GSH1*) were induced upon H₂O₂ as expected, but also incremented their expression in the presence of salts (Figs. 1, 2). Even more, *GSH1* and *TRX2* transcripts, which products are typically related to the control of intracellular redox state, reached top levels after NaCl exposure (Fig. 2). *CTAI* results somehow are in contradiction with those reported (Segal-Kischinevzky et al. 2011), showing that this gene transcription increased upon ethanol stress and stationary growth but was hindered when cells were grown on NaCl. Nevertheless, a chronic exposure to saline stress was evaluated in that study, while in our work an acute response is analyzed (Fig. 1) (Segal-Kischinevzky et al. 2011). Strikingly, the patterns of these changes were not always coincident suggesting different types/levels of transcriptional regulation. For example, *CTAI* or *HOG1*

transcript alterations were observed quickly after exposures and disappeared at longer times, but, on the contrary, *MSN2* and *ENAI* reached the highest transcript levels in the presence of NaCl at the last time-point analyzed (30 min). Furthermore, to our knowledge, this is the first report of *HOG1* transcriptional induction upon exposure to stress in yeasts, suggesting that the regulation of this pathway in *D. hansenii* is different to that described for *S. cerevisiae*. Finally, *RCN1* transcripts present a heterogeneous regulatory pattern as their level increased upon oxidative stress but diminished after exposure to salts.

In fact, homologs to several of these genes have been investigated in *S. cerevisiae* and other yeasts with mixed results. Transcriptional changes reported for the studied genes in previous reports were usually higher than the ones described here, but comparison is difficult as most of those studies used either chronic or elevated levels of stressors (Brombacher et al. 2006; Dormer et al. 2002; Kavitha and Chandra 2014; Posas et al. 2000; Saijo et al. 2010) while in this work, we have used mild concentrations of the stressors to avoid non-specific damage responses (see Supplementary Fig. 1). We would like to highlight that, although *Msn2* has been previously described as a general stress regulator and linked to oxidative stress in *S. cerevisiae* and other yeasts, in *D. hansenii* *MSN2* expression does not increase upon hydrogen peroxide exposure and even seems to be repressed at longer exposure times (Fig. 2). In *S. cerevisiae*, *Msn2/4* transcription factors reside in the cytoplasm in unstressed cells, and, due to active export, migrate to the nucleus upon stress exposure (Morano et al. 2012), involving a kinase cascade regulation as for *Hog1* activation (Saito and Posas 2012). Further experiments need to be done to confirm a similar mechanism in *D. hansenii* although our data show that, contrary to in *S. cerevisiae*, both *HOG1* and *MSN2* genes are clearly regulated at the transcriptional level (Figs. 1, 2). *ENAI* expression is strongly induced by NaCl, mildly by KCl and not by H₂O₂ (Fig. 3). Transcriptional induction of this gene in response to Na⁺ or Li⁺ cations has been described before for other yeasts and regulation of this gene in *S. cerevisiae* seems to follow a complex pattern linked to several different pathways: calcineurin, nutrient availability, Rim101, etc. (see (Arino et al. 2019) for a recent review). Finally, studies in *S. cerevisiae* and *C. albicans* show that *RCN1* expression can be induced by calcium in a calcineurin-dependent way, but, on the other hand, *Rcn1* can also inhibit this pathway (Kingsbury and Cunningham 2000; Reedy et al. 2010). Furthermore, the fact that, in *D. hansenii* the alterations in *RCN1* transcript levels always occurred at longer exposure times, suggest an indirect effect that may be related to variations in intracellular Ca²⁺ homeostasis.

CAT activity increments in response to the analyzed stressor were slightly inferior to those detected at the transcriptional level. Furthermore, induction of this activity

in the presence of hydrogen peroxide or NaCl could be observed up to 60 min after adding the stressors, while its corresponding mRNA returned to basal levels after 20 and 10 min, respectively. These differences could be explained by the fact that proteins are usually more stable than transcripts. As with *CTAI* transcriptional regulation, previous reports of CAT activity modulation by stress did not show the clear induction pattern after oxidative and saline exposure described in this work, probably due to the differences in the experimental conditions used (different amount of hydrogen peroxide, chronic exposure to NaCl, etc.) (Michan et al. 2013; Segal-Kischinevzky et al. 2011). To our knowledge, there are no previous reports studying GR activity in *D. hansenii*.

The combined presence of H₂O₂ and NaCl in the culture media favors sodium accumulation in *D. hansenii* (Fig. 7), although *ENAI* transporter mRNA levels were not induced by the ROS. The fact that hydrogen peroxide can influence in-cell sodium levels and that NaCl can influence in-cell ROS levels suggests the existence of common metabolite intermediates in both stress responses. Moreover, we would like to highlight that an increase in ROS has also been observed in *S. cerevisiae* after non-oxidative-related temperature stress (Zhang et al. 2003). On the other hand, at non-growth inhibitory H₂O₂ exposure, no ROS intracellular levels and no lipid damage could be detected, but the redox state of proteins was significantly altered. This discrepancy could be related to the fact that, although lipids and proteins are major targets of oxidative stress, the extent of oxidative damage to different biomolecules varies depending on the type of ROS, the location/concentration of target, or the levels of repair reactions (Davies 2005). Furthermore, DCFA-determined ROS levels may even be bias by cytochrome c, peroxidases or transition metal levels (Eruslanov and Kusmartsev 2010; Murphy et al. 2011). We are aware of the importance of the membrane lipid profile in stress responses (Turk et al. 2007), and further studies are needed to evaluate its role upon oxidative and saline stress in *D. hansenii*.

This report significantly improves our understanding of the stress response in *D. hansenii*. Our results clearly show that responses to salt and oxidants influence common elements at transcriptional, enzymatic and metabolic levels. Also, our results show that stress pivotal regulators as *Hog1* and *Msn2* are differently regulated in *D. hansenii* than in the model *S. cerevisiae*, opening new pathways for stress response modulation in yeasts. Our hypothesis is that physiological changes occurring by sodium accumulation inside cells may trigger a ROS-related metabolite able to activate antioxidative defenses before proper oxidative damage in *D. hansenii*. Additionally, the fact that no changes in *ENAI* transcript levels were detected upon H₂O₂ exposure suggests that the response to saline stress can be modulated through more than one pathway, including the one proposed above

and at least another one not connected to the oxidative stress response.

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