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Transcriptional Response of Multi-Stress-Tolerant *Saccharomyces cerevisiae* to Sequential Stresses

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Abstract: During the fermentation process, yeast cells face different stresses, and their survival and fermentation efficiency depend on their adaptation to these challenging conditions. Yeast cells must tolerate not only a single stress but also multiple simultaneous and sequential stresses. However, the adaptation and cellular response when cells are sequentially stressed are not completely understood. To explore this, we exposed a multi-stress-tolerant strain (BT0510) to different consecutive stresses to globally explore a common response, focusing on the genes induced in both stresses. Gene Ontology, pathway analyses, and common transcription factor motifs identified many processes linked to this common response. A metabolic shift to the pentose phosphate pathway, peroxisome activity, and the oxidative stress response were some of the processes found. The *SYM1*, *STF2*, and *HSP* genes and the transcription factors Adr1 and Usv1 may play a role in this response. This study presents a global view of the transcriptome of a multi-resistance yeast and provides new insights into the response to sequential stresses. The identified response genes can indicate future directions for the genetic engineering of yeast strains, which could improve many fermentation processes, such as those used for bioethanol production and beverages.

Keywords: fermentation process; RNA-Seq; stress response; acquired stress resistance; yeast

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1. Introduction

Fermentation is a process in which a complex organic substance is converted into a simpler one usually due to the metabolic activity of microorganisms, such as yeast and bacteria [1]. This process is widely used in industrial settings to produce chemicals, bioethanol, and beverages. During fermentation, yeast cells encounter different stresses that have a significant influence on their microbial physiology. Initially, yeast cells experience osmotic stress caused by high solute concentrations, temperature shock due to exothermic reactions, and oxidative stress. As fermentation progresses, they encounter other stresses, such as starvation and increased ethanol concentration [2]. These environmental changes presented during fermentation processes might cause a reduced growth rate, an extended lag phase, and metabolic cessation, with some yeast surviving and others dying [3].

To survive these stressful environments, yeast cells can maintain their biological homeostasis through transcription regulation, activating genes associated with cell survival and function restoration. The efficiency of this process in a given yeast strain can determine its robustness [4–6]. While the stress response has been extensively studied, most of those studies focused on a single stress response [7–9]. Although such studies have intrinsic and fundamental value, they disregard issues widely observed in industrial environments, such as the stress fluctuations and variations during the fermentation process.

A few studies have explored the effects of multiple and sequential stresses and have shown that yeast subjected to different mild stresses followed by lethal stress exhibit little

overlap in the genes involved in the response [10]. Further analyses of yeast cellular memory in response to short, pulsed hyperosmotic stress also demonstrated diverse gene behavior [11]. However, a comprehensive evaluation of the common response to sequential stresses that considers the conditions of industrial environments, focusing on genes induced in all stresses, remains to be thoroughly explored, particularly in yeast strains that already possess a multi-tolerant profile.

A transcriptome analysis can provide insights into the stress response in yeast, leading to a better understanding of cellular metabolism and the related pathways that play a role in the response [12]. In this study, we employed RNA sequencing to identify a cluster of genes that comprise a common response to sequential stresses and the essential pathways, genes, and transcription factors (TFs) associated with that response. We investigated the *Saccharomyces cerevisiae* strain BT0510 due to its high flocculation capacity and high tolerance to ethanol, osmotic, and heat shock stresses, as well as high fermentation rates [13]. A whole-genome analysis of BT0510 revealed substantial genetic differences between it and the *Saccharomyces cerevisiae* reference genome, S288c, as well as distinct genomic endowments when compared with two other cachaça fermentation strains. That analysis highlighted a collection of intragenic SNPs that could influence cellular processes, such as transport and the stress response, and it identified a set of gene loss and gene fragmentations that could directly influence fermentation processes [14]. This current transcriptomic study can reveal how these genomic changes affect the gene expression in this strain, as well as providing baseline knowledge on the response of this multi-tolerant yeast strain to different sequential stresses. This analysis also provides targeted genetic information to enhance stress tolerance in yeast that could improve a variety of biotechnological processes, such as beverage production, baking, and biofuel production.

2. Materials and Methods

2.1. Yeast Strain, Culture Conditions, and Experimental Design

The industrial yeast strain used in this study was previously isolated from a cachaça distillery in the State of Espírito Santo, Brazil, and it is described in Bravim et al. [13]. This strain (URM 6670) was selected for its flocculation ability and tolerance to ethanol, osmotic, and heat shock stresses and has been fully sequenced [14]. URM 6670 is stored at the Federal University of Pernambuco Culture Collection (URM604). In this and previous studies, the strain is designated BT0510.

Yeast cells were grown at 30 °C with aeration in a liquid synthetic complete medium (SC) (yeast nitrogen base without amino acids, drop-out mix, 2% glucose) to the exponential growth phase (OD600 = 1.0) before being exposed to sequential stresses. Stress treatments were selected based on the chronological progression of stress events during fermentation, with ethanol stress increasing towards the end of the fermentation process. Accordingly, the yeast cells were subjected to three different treatments: (1) osmotic stress followed by ethanol stress; (2) oxidative stress followed by ethanol stress; and (3) glucose withdrawal followed by ethanol stress. For each treatment, samples were collected before the first stress (control), after the first stress, and after the second stress (ethanol stress) for RNA extraction. The control was used to identify differentially expressed genes after the first and sequential stresses.

For osmotic stress, cells were incubated in liquid SC containing 1 M sorbitol for 30 min at 28 °C. The cells were subjected to oxidative stress by incubating them in SC containing 0.6 mM hydrogen peroxide for 30 min. Glucose withdrawal was performed by transferring the cells in SC + 2% glucose to SC + 0.05% glucose for 30 min. After each stress, the cells were collected via filtration, washed with deionized water, and reinoculated in SC supplemented with 8% (*v/v*) ethanol for 30 min at 28 °C. Several studies have demonstrated that, upon exposure to stress for 30 min, cells are capable of initiating a complex signaling network and displaying robust gene expression patterns [15,16]. The experiments were performed in triplicate.

2.2. RNA Preparation, Library Construction, and Sequencing

The total RNA from three biological replicates for each treatment was extracted using a Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quality and quantity of the total RNA samples were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The integrity of the RNA was evaluated on an Agilent Bioanalyzer 2100 system using an RNA Nano 6000 Assay Kit (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's protocol. Poly-A-tailed RNA was captured and reverse-transcribed to complementary DNA (cDNA), which was used for library preparation with the Stranded Total RNA Prep (Illumina, San Diego, CA, USA) following the manufacturer's protocol.

2.3. Reads Mapping, Annotation, and Gene Expression Analysis

After trimming and filtering, paired-end reads were aligned to the reference genome of *S. cerevisiae* S288c via Hisat2 software (v2.0.1). Fragments per kilobases per million reads (FPKM) values were calculated as the metric of gene expression using Cufflinks [17] and were used for a principal component analysis (PCA) and a K-means clustering analysis via iDEP.91 [18]. The 2000 most variable genes were included and mean-normalized in the K-means analysis. The data for K = 4 are shown since higher values of K yielded no further distinct expression patterns. The assembled transcripts generated by Cufflinks were used to obtain the differential gene expression via Cuffdiff [17]. After adjustment using Benjamini and Hochberg's approach for controlling the false discovery rate, p-value < 0.05 and the value of log2 fold change >1 were aggregated to detect differentially expressed genes in both sequential stresses.

2.4. Enrichment Analysis

DAVID 6.8 [19] was used to annotate functions and identify enriched metabolic pathways. The pathways enriched were highlighted in the DAVID Functional Annotation Tool and retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The GO direct category was used to select GO mapping directly annotated by the source database (no parent terms included). GO terms with FDR < 0.05 were considered significantly enriched within the gene set. The enriched KEGG pathways (FDR < 0.05) identified are reported.

2.5. Enriched Promoter Motifs

ShinyGO v0.65 [20] was used to enrich the motifs in the 300 bp region upstream of the genes in the gene list.

2.6. Quantitative Real-Time PCR Validation

Seven representative genes were analyzed in all different sequential stresses using the real-time PCR method to validate the RNA-Seq analysis. The total RNA was extracted from yeast cells using phenol/chloroform and precipitated with 3 M sodium acetate/absolute ethanol. The extracted RNA was treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA) and reverse-transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). PCR reactions were carried out using a 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). The relative expression levels of the target genes were measured using the $2-\Delta\Delta CT$ method, and ALG9 was used as the reference gene [21]. All tests were performed at least three times. The primers are listed in Table 1.

Table 1. Oligonucleotides used as primers for qRT-PCR analysis.

Target mRNA	Primer Sequence 5′–3′	Amplicon Size (bp)	PCR Efficiency [a] (%)
HSP12	Forward, 5′CGCAGGTAGAAAAGGATTCG3′ Reverse, 5′TCAGCGTTATCCTTGCCTTT3′	194	105
YGP1	Forward, 5′TGACGGTGGTTACTCTTCCA3′ Reverse, 5′GAACGGCAGAACTCAAGGAG3′	49	104
SYM1	Forward, 5′ACGGGTAGCTGTCGATCAAT3′ Reverse, 5′AGGCCACCATTGCTCTTTTA3′	126	97
STF2	Forward, 5′CGGTGAATCTCCAAATCACA3′ Reverse, 5′CACTGGGGGTATTTCACCAT3′	108	99
HSP26	Forward, 5′ATGCTGGCGCTCTTTATGAT3′ Reverse, 5′TTCTAGGGAAACCGAAACCA3′	95	95
SSE2	Forward, 5′CACTGGGGTCAAGGTTCCTA3′ Reverse, 5′GGTAAAGGCACTGGCTCTTG3′	137	94
HSP42	Forward, 5′TGAACGCATTATCCAACCAA3′ Reverse, 5′TTGTCCATAATGGGGATGGT3′	94	100
ALG9	Forward, 5′ACATCGTCGCCCCAATAAAT3′ Reverse, 5′GATTGGCTCCGGTACGTAAA3′	142	93

[a] The PCR efficiency of each primer was evaluated using the dilution series method using a mix of sample cDNAs as the template and based on the slope of the standard curve.

3. Results

3.1. Global Analysis of the Transcriptome

The principal component analysis (PCA) performed on the normalized FPKM data showed that the samples clustered together in each treatment, except for one replicate after the treatment with glucose withdrawal (Figure S1). We identified this outlier and removed it from subsequent analyses. Overall, the clustering indicates a consistent response to different sequential stresses in the yeast cell population.

3.2. Identification of Common Genes in Response to Sequential Stresses

The K-means clustering of the FPKM data identified groups of genes that displayed a distinct expression pattern. Our aim was to identify these stress-induced genes and their corresponding cellular activities that lead to sequential stress adaptation in yeast, namely, genes whose transcript levels increased in abundance in response to both sequential stresses. A cluster of genes with this behavior was identified in all treatments (Figure 1). To select the responsive genes that were differentially expressed, we selected the ones that were upregulated (log2 > 1; adjusted $p < 0.05$) in both stresses. This yielded 746 differentially expressed genes (DEGs) with the pattern of interest when osmotic stress was applied as the first stress, 342 DEGs with oxidative stress treatments, and 588 DEGs in glucose withdrawal experiments. A total of 219 genes were upregulated in all treatments (Figure 2). This list of 219 genes was analyzed using DAVID's Functional Annotation Tool using *Saccharomyces cerevisiae* S288c as a background to functionally annotate and identify enriched pathways in KEGG.

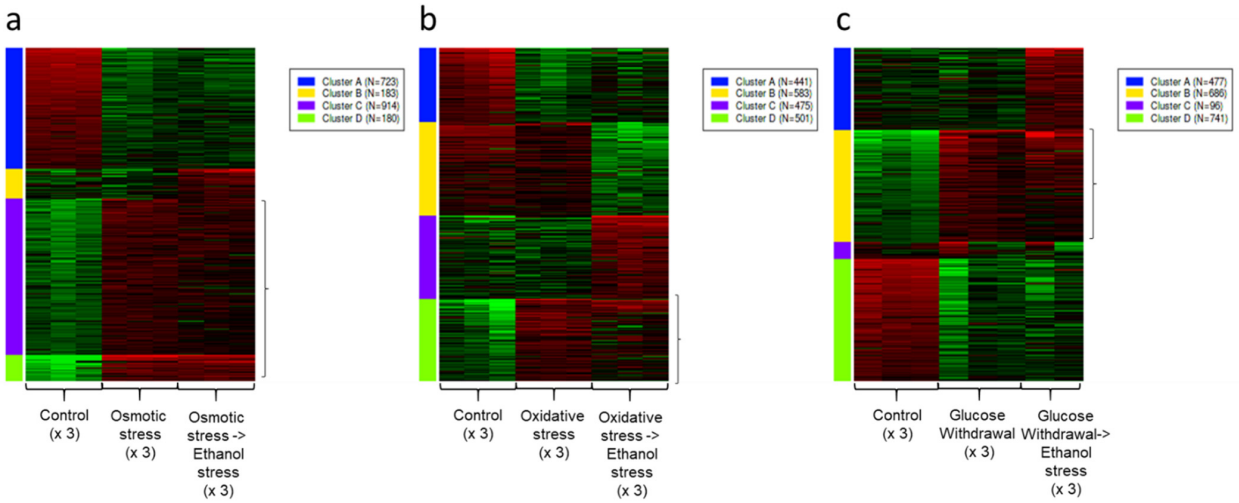

Figure 1. K-means clustering analysis of FPKM data after different sequential stresses. (**a**) Gene clusters after osmotic stress followed by ethanol stress. (**b**) Gene clusters after oxidative stress followed by ethanol stress. (**c**) Gene clusters after glucose withdrawal followed by ethanol stress. For each treatment, four clusters were identified and are color-coded in blue, yellow, purple, and green. The cluster with the gene pattern of interest was highlighted in each treatment. The analysis included the 2000 most variable genes and was mean-normalized. Values below the mean are shown in green, and those above are shown in red. The number of replicates analyzed from each treatment is identified in parentheses.

Figure 2. Venn diagram showing overlap between upregulated genes in different sequential stresses.

3.3. Gene Ontology Functional Annotation and Enrichment of Gene Cluster

The 10 most significant terms annotated in all three Gene Ontology (GO) categories are listed in Figure 3. The biological processes included oxidation–reduction (41 genes), metabolism (27 genes), cellular response to oxidative stress (17 genes), carbohydrate metabolism (15 genes), glutathione metabolism (7 genes), negative regulation of gluconeogenesis (5 genes), carbohydrate phosphorylation (5 genes), D-xylose catabolism (4 genes), arabinose catabolism (4 genes), and NADPH regeneration (4 genes). The enriched processes indicate diverse cellular activities, especially those involved in catabolism and defense against oxidative stress. The cellular components included the cytoplasm (113 genes), mitochondria (58 genes), vacuole (14 genes), peroxisome (9 genes), glucose-induced degradation (GID) complex (4 genes), peroxisomal importomer complex (4 genes), and membrane raft (4 genes). Regarding molecular functions, genes were involved in oxidoreductase activity (38 genes); transferase activity (33 genes); catalytic activity (29 genes); hydrolase activity, acting on glycosyl bonds (7 genes); aldehyde dehydrogenase (NAD) activity (6 genes); alditol: NADP+ 1-oxidoreductase activity (5 genes); oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD, or NADP as acceptor (5 genes); aldo–keto reductase (NADP) activity (4 genes); glutathione transferase activity (4 genes); and oxidoreductase activity, acting on the CH-OH group of donors, NAD, or NADP as acceptor (4 genes). The major molecular function category was oxidoreductase activity, indicating redox-balance-related gene expression alterations under sequential stresses. Molecular functions involving nicotinamide adenine dinucleotide (NAD+), an essential cofactor involved in various cellular processes, were also highlighted.

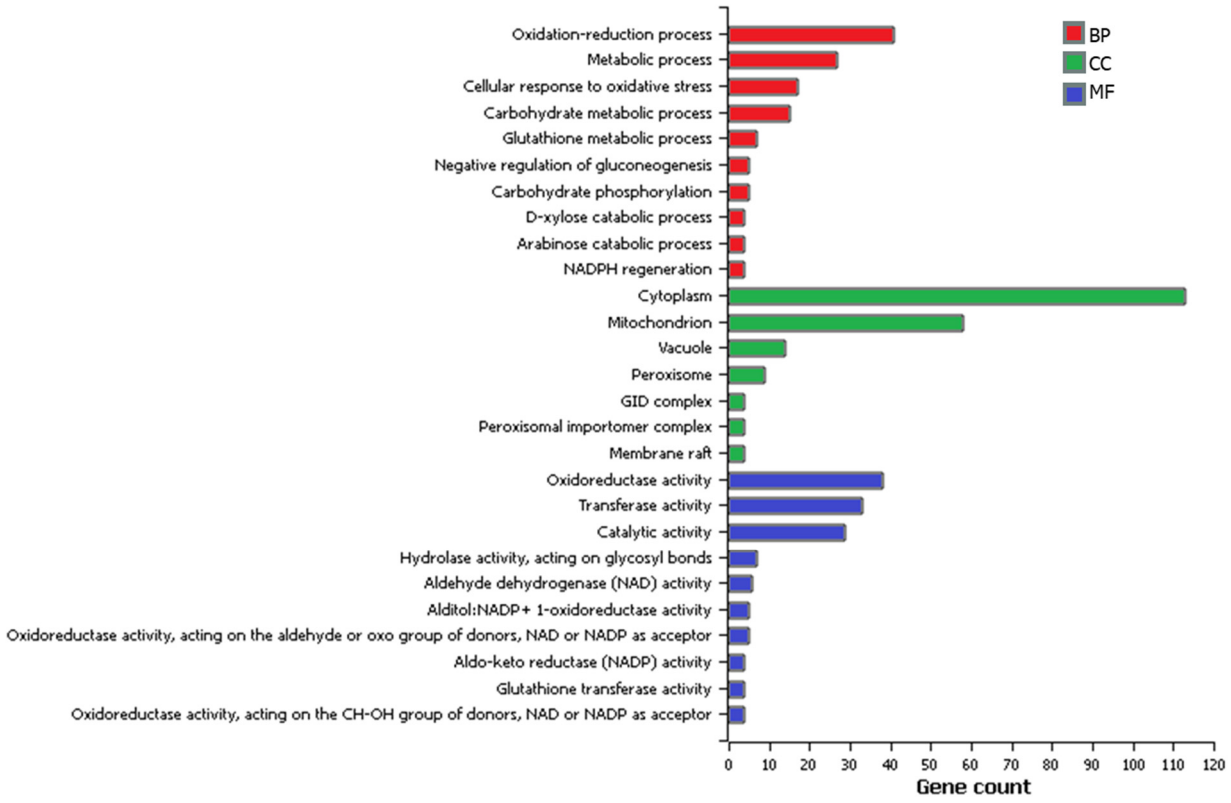

Figure 3. Gene Ontology (GO) enrichment of genes common to the sequential stress response. All the genes were annotated with regard to three categories, namely, biological process (BP), cellular component (CC), and molecular function (MF); these categories are represented on the Y-axis, and the number of genes enriched in each category is indicated on the X-axis. The figure presents the 10 GO terms from each category that contained the largest number of enriched genes.

3.4. KEGG Enrichment Analysis of DEGs

The KEGG pathway database provides molecular-level information about the interactions, reactions, and relation networks of the yeast biological system. A pathway enrichment analysis can reveal metabolic or signal transcription pathways in our gene set that may improve the understanding of yeast's response to sequential stresses. Among the 219 common genes, 79 (36%) were assigned to 18 pathways (Table 2), all of them significantly enriched. This result suggests the activation of several main pathways, including carbon metabolism, glycolysis/gluconeogenesis, the pentose phosphate pathway, amino sugar and nucleotide sugar metabolism, peroxisome, and glutathione metabolism.

Table 2. Significantly enriched KEGG pathways obtained via DAVID 6.8.

Pathways	Count	%	*p*-Value	FDR
Metabolic pathways	48	21.9	6.8×10^{-8}	1.8×10^{-6}
Biosynthesis of secondary metabolites	23	10.5	3.8×10^{-4}	3.6×10^{-3}
Biosynthesis of antibiotics	19	8.7	3.6×10^{-4}	3.6×10^{-3}
Carbon metabolism	16	7.3	6.3×10^{-6}	1.1×10^{-4}
Starch and sucrose metabolism	12	5.5	5.9×10^{-8}	1.8×10^{-6}
Glycolysis/gluconeogenesis	8	3.7	3.9×10^{-3}	1.9×10^{-2}
Peroxisome	7	3.2	1.9×10^{-3}	1.1×10^{-2}
Alanine, aspartate, and glutamate metabolism	6	2.7	3.6×10^{-3}	1.9×10^{-2}
Amino sugar and nucleotide sugar metabolism	6	2.7	4.8×10^{-3}	2.1×10^{-2}
Phenylalanine metabolism	5	2.3	4.1×10^{-4}	3.6×10^{-3}
beta-Alanine metabolism	5	2.3	8.4×10^{-4}	6.4×10^{-3}
Tyrosine metabolism	5	2.3	1.1×10^{-3}	7.6×10^{-3}
Fructose and mannose metabolism	5	2.3	5.7×10^{-3}	2.3×10^{-2}
Glutathione metabolism	5	2.3	9.3×10^{-3}	3.2×10^{-2}
Galactose metabolism	5	2.3	9.3×10^{-3}	3.2×10^{-2}
Pentose phosphate pathway	5	2.3	1.6×10^{-2}	4.7×10^{-2}
Methane metabolism	5	2.3	1.6×10^{-2}	4.7×10^{-2}
Butanoate metabolism	4	1.8	9.7×10^{-3}	3.2×10^{-2}

Sixteen genes were enriched in the carbon metabolism pathway: *SOL4*, *YJL068C*, *AGX1*, *SFA1*, *CTT1*, *CIT3*, *DAK1*, *ZWF1*, *HXK1*, *IDP3*, *MET13*, *GND2*, *GPM2*, *EMI2*, *NQM1*, and YJL045W. The induction of *HXK1*, a glucokinase that catalyzes the phosphorylation of glucose at C6 in the first irreversible step of glucose metabolism [22], may indicate the maintenance of internal levels of glucose. Two genes encoding a hexose transporter, HXT5 and YGL104C, were also upregulated upon the sequential stresses. The genes involved in steps 1, 2, and 3 of the oxidative phase of the pentose phosphate pathway (PPP) (*ZWF1*, *SOL4*, and *GND2*) were also overexpressed after the stresses, as well as *NQM1*, a *TAL1* paralog that arose from whole-genome duplication [23]. These results suggest a metabolic shift from glycolysis to the PPP to increase NADPH production. NADPH regeneration by the pentose phosphate shuttle is one fate of glucose in response to stressful environments, along with trehalose synthesis and glycogen storage [24], and genes from the last two processes were also recovered in our analysis.

The genes enriched in the peroxisome pathway were *PXA1*, *POX1*, *AGX1*, *CAT2*, *CTT1*, *SYM1*, and *IDP3*. This gene set attends numerous functions, such as β-oxidation (*POX1*, *PXA1*, and *IDP3*) [25], ethanol metabolism (*SYM1*) [26], and amino acid metabolism (*AGX1*) [27]. The results show stress-induced genes linked to other organelles: the Golgi apparatus (*CCC2*, *EMP46*, *ATG9*, *TRX2*, *GGA1*) and vacuole (*BXI1*, *HOR7*, *PHM7*, *ATH1*, *ECM38*, *SGA1*, *YJL132W*, *YNL115C*, *APE1*, *PEP4*, *PRB1*, *SPO1*, *RNY1*, *TXR2*, and others). The peroxisome and vacuole are required after oxidative damage and possibly play a role in the response to sequential stresses. The upregulation of five genes (*CUZ1*, *UBC8*, *FYV10*, *GID7*, and *VID28*) that were related to proteasome-mediated ubiquitin-dependent protein and glutathione metabolism (*GTT1*, *GRX2*, *ECM38*, *GLO1*, *ECM4*, *GTO3*, and *TRX2*) may

also indicate mechanisms of protein homeostasis and redox balance that may occur in the cell upon sequential stresses.

Five genes encoding heat shock proteins (Hsps) were upregulated upon sequential stresses: *HSP12*, *HSP26*, *HSP31*, *HSP42*, and *SSE2*. These proteins are involved in maintaining organization and resistance during stress conditions, as cytoplasmatic, membrane, and mitochondrial chaperones [28], and they might be required by the cell machinery in the response to sequential stresses. Moreover, several genes involved in transcription regulation were upregulated: *CSR2*, *SIP4*, *USV1*, *XBP1*, *RPN4*, and *ADR1*. *ROM1*, one of the upstream regulatory factors that modulate Pkc1 activity [29], was also found to be induced in all sequential stresses.

3.5. Transcription Factors Enriched in Clustered Genes

We identified the promoter motifs in the gene list that could be involved in the TFs that coordinate the response to sequential stress. The motifs and TFs are listed in Table 3. Most of the enriched TFs, such as Msn2, Msn4, Usv1, and Gis1, were related to different stress responses [30,31]. Two of them, Usv1 and Adr1, were enriched in the motif analysis and were genes upregulated during both stresses. Accordingly, they could be crucial to a common response to sequential stresses.

Table 3. The enriched motif in the promoter's area (upstream 300 bp) from the gene list. Transcription factors (TFs) marked with (*) were both enriched in the analysis and present in the list of DEGs.

Enriched Motif in Promoter	TF	TF Family
GGGG	Adr1 (*)	C2H2 ZF
TAGGGG	Gis1	C2H2 ZF
AGGGG	Gis1	C2H2 ZF
AGGGG	Gis1	C2H2 ZF
GCGGGG	Mig3	C2H2 ZF
TAT	Mot2	RRM
AAGGGG	Msn2	C2H2 ZF
AGGGG	Msn2	C2H2 ZF
AGGG	Msn2	C2H2 ZF
AGGGG	Msn4	C2H2 ZF
AGGGG	Msn4	C2H2 ZF
AGGGG	Msn4	C2H2 ZF
TCAGGGG	Rei1	C2H2 ZF
TCAGGGG	Rei1	C2H2 ZF
TCAGGGG	Rei1	C2H2 ZF
AGGGG	Rgm1	C2H2 ZF
AGGGG	Rgm1	C2H2 ZF
TCAGGGG	Rgm1	C2H2 ZF
TTAGGGGT	Rph1	C2H2 ZF
ATTTAGGGGG	Rph1	C2H2 ZF
TTAGGGGT	Rph1	C2H2 ZF
TTAGGGGT	Rph1	C2H2 ZF
TCAGGGG	Usv1 (*)	C2H2 ZF
AGGGG	Usv1 (*)	C2H2 ZF
TCAGGGG	Usv1 (*)	C2H2 ZF
ATAGGGG	YER130C	C2H2 ZF
ATAGGGG	YER130C	C2H2 ZF
ATAGGGG	YER130C	C2H2 ZF
ATAGGGG	YER130C	C2H2 ZF
GTGGGGGG	YGR067C	C2H2 ZF

3.6. RNA-Seq Expression Validation via Quantitative Real-Time PCR

The expressions of seven genes (*HSP12*, *YGP1*, *SYM1*, *STF2*, *HPS26*, *SSE2*, and *HSP42*), which were shown to exhibit the behavior of interest in all sequential stresses, were measured using RT-qPCR to confirm the transcriptome results. The RT-qPCR results indicated

that these genes were upregulated after glucose withdrawal followed by ethanol stress and oxidative stress followed by ethanol stress, as illustrated in Figure 4.

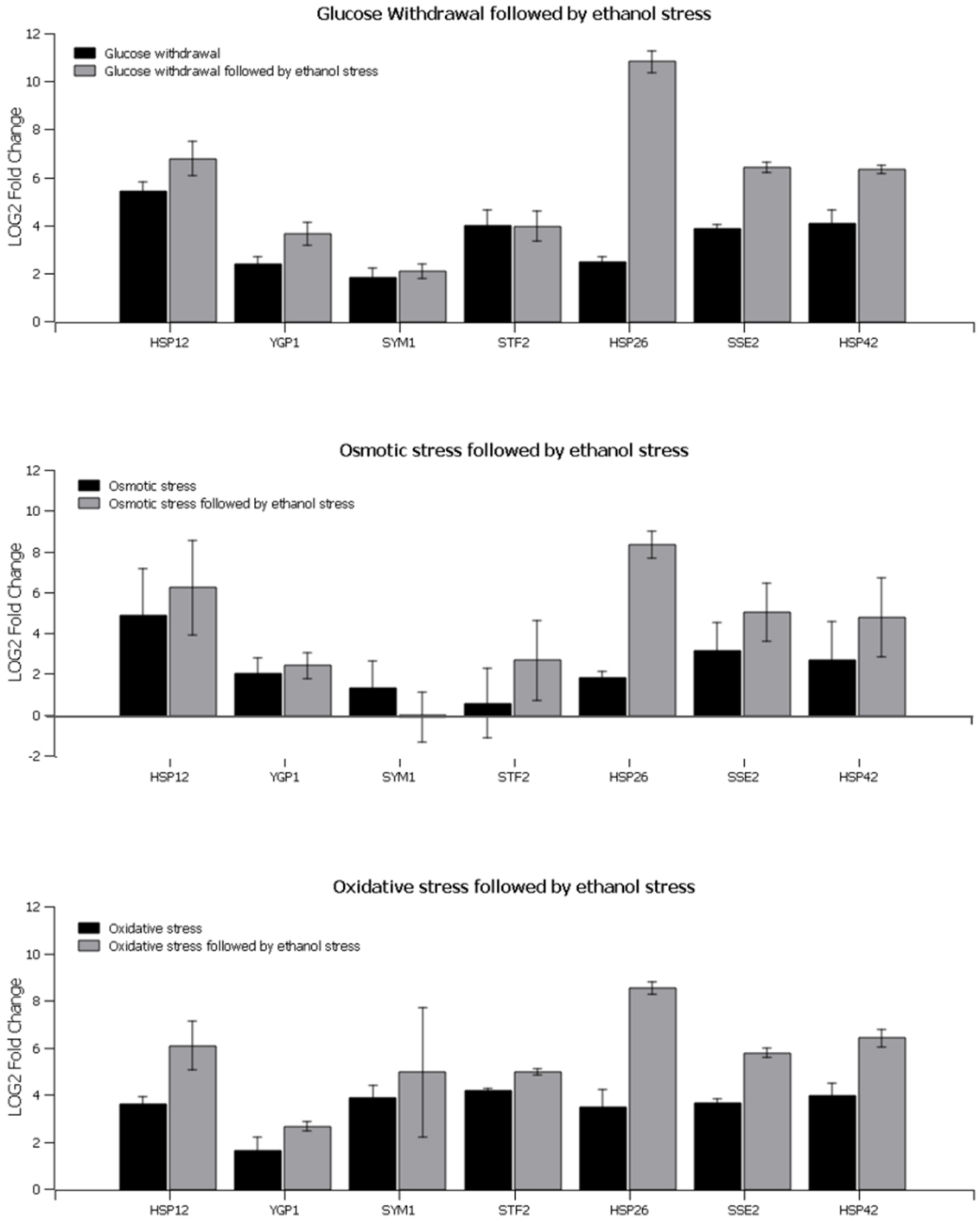

Figure 4. Validation of differentially expressed genes using quantitative real-time PCR (qPCR). Relative expressions of 7 genes were analyzed in all different sequential stresses: glucose withdrawal followed by ethanol stress, osmotic stress followed by ethanol stress, and oxidative stress followed by ethanol stress. The data are expressed as mean (SD) (n = 3).

Among the HPSs analyzed, *HSP26* displayed the highest overexpression, particularly after glucose withdrawal followed by ethanol stress, which may be associated with cellular protection and efficient refolding after sequential stresses. Similar behavior was observed for all HPSs analyzed, indicating a robust activity of this class of genes during the treatments.

YGP1, a gene that plays a role in the response to nutrient limitation and weak acids [32], also demonstrated consistent upregulation across all treatments. Conversely, five genes analyzed showed similar behavior during osmotic stress followed by ethanol stress, while *SYM1* and *STF2* exhibited more heterogeneous expression patterns. Previous studies have linked both *SYM1* and *STF2* to cell recovery and stress tolerance in the BT0510 strain, with the overexpression of *SYM1* also resulting in enhanced ethanol production [33].

4. Discussion

Our RNA-Seq analysis revealed that genes display different behaviors in response to sequential stresses with several distinct patterns. The pattern of interest in this study was that of the genes induced in both stresses regardless of the initial stress applied. We were able to identify genes with this specific pattern in all treatments, and hundreds of genes were common in all sequential stresses applied. Through this data, we were able to identify shared processes and metabolic pathways in all treatments (Figure 5).

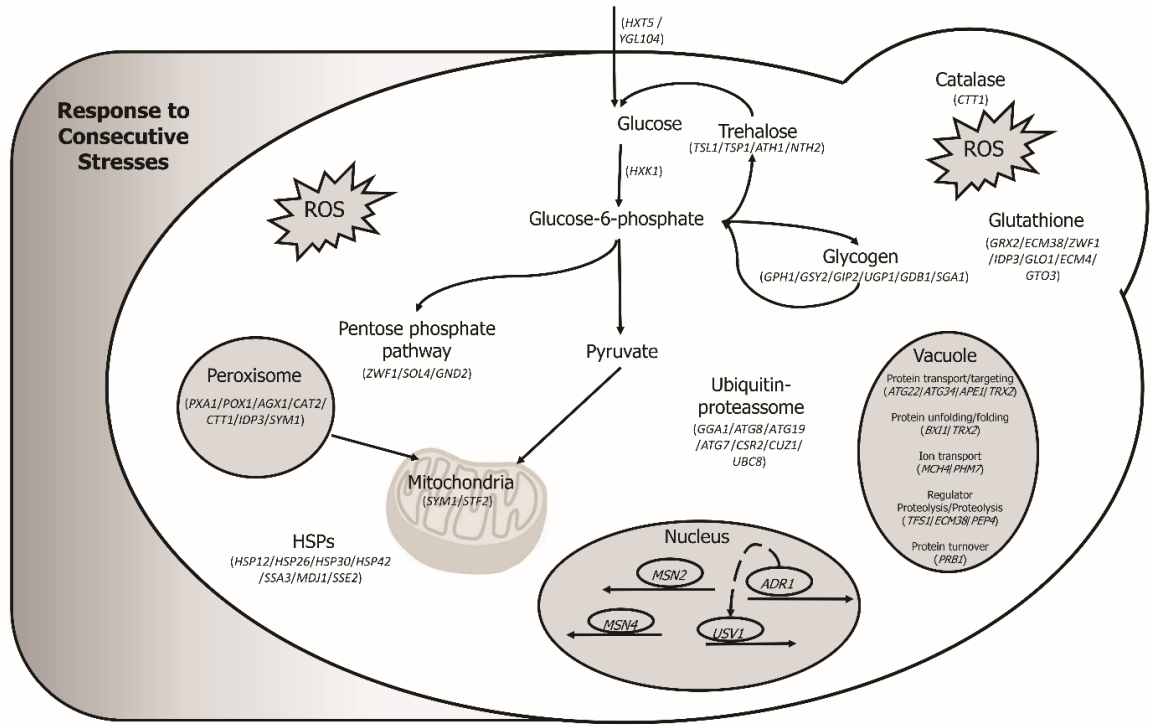

Figure 5. Schematic representation of the yeast response to sequential stresses. Upon sequential stresses, several genes (some of them represented within parenthesis) from different pathways and mechanisms were induced. The transcriptome analysis showed the induction of genes related to carbohydrate metabolism, trehalose syntheses, glycogen storage, and NADPH regeneration by the pentose phosphate pathway. Some organelles also played a role in the response, such as the peroxisome, mitochondria, and vacuole. Several genes that are associated with oxidative stress response and with chaperone functions were also upregulated. Gene motif analysis indicated several TFs coordinating the response, including Msn2 and Msn4, as well as ADRdr1 and Usv1.

A general stress response is important during the bioethanol production process since multiple stresses act together in different combinations depending on the time of the fermentation, the process conditions, and the type of biomass used [34]. Any abrupt change in the yeast cells' environment leads to a universal genomic expression program known as the environmental stress response (ESR) [5,24]. Stressful environments often occur in combinations or sequentially, especially in the fermentation process inside fermentative vats. The ESR likely operates the successive abrupt changes during sequential stresses and the transcriptional reprogramming of metabolic processes. The genes induced in the

ESR are involved in carbohydrate metabolism, metabolite transport, fatty acid metabolism, the maintenance of the cellular redox potential, the detoxification of reactive oxygen species, protein folding and degradation, DNA-damage repair, vacuolar and mitochondrial functions, and others [24]. Many genes that belong to these processes were highlighted in our analysis, as well as two TF motifs from this general response mediated by Msn2 and Msn4. We also identified two other TF motifs in the promoters of genes induced in the response, as well as the genes themselves in the list of activated genes by sequential stresses: Usv1(YPL230W) and Adr1. Both TFs affect the transcriptional regulation of genes involved in growth on non-fermentable carbon sources [30,35].

Usv1 mediates transcriptional changes in response to various stress conditions and alcoholic fermentation processes. It relocates to the nucleus in response to either gluconeogenic conditions or the presence of salt stress and affects the transcriptional activation of several carbon- or salt-regulated genes. Usv1-mediated transcriptional activation is partially dependent on the Snf1 signaling pathway that controls the activity of several downstream transcription regulators, including Adr1. Two copies of the consensus Adr1 binding site were identified in the promoter of *USV1,* and its transcription is decreased in *adr1*Δ mutants growing in glycerol, indicating that Adr1 plays a role in *USV1* activation [30]. Adr1 encodes a transcriptional activator involved in the expression of genes that are regulated by glucose repression and is one of many genes controlling the fermentation/respiration balance [35,36]. *ADR1* deleterious mutants are unable to switch between fermentative and respiratory metabolism [37]. This gene is also related to the activation of genes involved in ethanol metabolism (e.g., *ALD4*), glycerol metabolism (e.g., *GUT2*), fatty acid utilization (e.g., *POX1* and *FOX2*), and peroxisome biogenesis [38,39]. Koerkamp et al. [40] suggested that Adr1 could act be a globally acting factor in the response to transient oxidative stress, and our transcriptome analysis also indicates a role of Adr1 along with Usv1 in the response to sequential stresses.

The signal transduction that is mediated by the single yeast isozyme of protein kinase C (Pkc1p) is also critical for maintaining the integrity of yeast cells and plays a role in the response to oxidative stress [29,41]. The upstream regulators of Pkc1p include Rom1p and Rom2p, which are GDP/GTP exchange proteins for the small GTP-binding protein Rho1p [42]. The activation of Rho1p triggers the activation of Pkc1 and ensures cellular integrity through multiple mechanisms. Firstly, it activates the MAP kinase cascade via Pkc1p, and secondly, it acts as a regulatory subunit of the 1,3-β-glucan synthase complex involved in cell wall biosynthesis [29]. While previous research has suggested that Rom2p plays a major role in signaling [29], our results indicate that *ROM1* rather than *ROM2* may be involved in this response, as all sequential stresses led to the upregulation of *ROM1*. A cellular process recurrent in our results was the modulation of glucose metabolism, and this modulation is largely studied upon yeast stress. The regulated genes in this response include genes with different functions ranging from intracellular glucose transporters to subsequent catabolism [24]. We noticed the modulation of many genes responsible for the phosphorylation of glucose and the conversion from glucose-1-phosphate to glucose-6-phosphate, as well as genes related to trehalose synthesis, glycogen storage, and NADPH regeneration by the pentose phosphate shuttle (Figure 5).

We observed that sequential stresses promoted the redirection of metabolic flux from glycolysis to the pentose phosphate pathway through the induction of three genes (*ZWF1*, *SOL4*, and *GND2*) that catalyze the first steps of the pentose phosphate pathway. This adjustment was previously described as a response to oxidative stress through the increased production of reducing power for cellular redox systems [43]. The ability to maintain intracellular redox homeostasis is important and is directly associated with stress tolerance. Kitichantaropas et al. [44] showed that, in response to simultaneous multi-stresses mimicking the fermentation process, redox homeostasis appeared to be the primary mechanism required for cell protection, and multiple stress-tolerant strains may have the ability to minimize intracellular ROS levels. Previous studies by Bravim et al. [45] showed that the gene expression pattern in response to high hydrostatic pressure (HHP) displays an

oxidative stress response profile in the BT0510 strain and that the overexpression of *STF2*, a gene whose upregulation prevents cellular ROS accumulation [46], led to increased tolerance to HHP stress. *STF2*, as well as *PRX1* and *GND2*, which play a role in the oxidative response [24], was recovered in our gene set. The oxidative damage response and the increase in cellular redox systems could be important to the yeast response to sequential stresses.

The NADPH regenerated by the PPP is an essential cofactor for glutathione- and thioredoxin-dependent enzymes that protect cells against oxidative damage [47]. These proteins are located in the yeast peroxisomes. Peroxisomes are found in most eukaryotic cells and are important in cell metabolism, especially in different catabolic processes, including fatty acid β-oxidation, the glyoxylic shunt, and methanol metabolism, as well as in osmotic stress tolerance [48,49]. Our analysis identified upregulated genes throughout the β-oxidation pathway, including *PXA1* encoding the ATP-binding cassette transporter that transports long-chain fatty acids to the peroxisome; *POX1* responsible for the first step of B-oxidation; and *CAT2* required for transporting acetyl-CoA generated from β-oxidation into the mitochondria, where it enters the TCA cycle [50]. Genes from this pathway are induced through Adr1 triggered by Hog1 MAP kinase upon salt stress, demonstrating that yeast adaptation to environmental stresses involves the dynamic modulation of peroxisomal activity to reshape cellular energy metabolism [51]. Peroxisomal proteins might also play a role in cell survival through the spatial regulation of the redox potential [52]. Our RNA-Seq analysis revealed that the genes related to these organelles were highly responsive to sequential stresses, and the modulation of peroxisomal genes and activities could be promising targets in the biotechnological enhancement of yeast that could lead to multi-stress tolerance.

SYM1, also highlighted in our analysis, could provide another strategy for cellular defense and a target to enhance ethanol fermentation. Even though our analysis showed its heterogeneous expression in response to osmotic stress, the same behavior was not presented in the other sequential stresses. Bravim et al. [33] showed that *SYM1* over-expression enhanced the fermentative capacity of BT0510. Sym1 is homologous to the mammalian peroxisomal membrane protein Mpv17 and was found to be required for both the metabolism and tolerance of ethanol during heat shock. In contrast to the peroxisomal localization of Mpv17p, Sym1 is an integral membrane protein of the inner mitochondrial membrane and was suggested to play an important metabolic role in mitochondrial function during heat shock [26]. We propose that *SYM1* may be part of a global response affecting cellular adaptation, not only in response to heat shock but also to other fermentative stresses.

The vacuole is another organelle enhanced after cell exposure to environmental stresses. A fundamental role of the yeast vacuole is the recycling of biological macromolecules. Proteins, small molecules, and even organelles are degraded and then recycled in the vacuoles [24]. A previous electron microscopic analysis showed that this organelle changes morphology after chronic heat stress, with an accelerated invagination of vacuoles as a stress response, perhaps due to the loss of cell surface proteins as the temperature increases [53]. Our analysis implicates a role of the vacuole in the sequential stress response, with genes associated with protein degradation, protein turnover after oxidative damage, and cytosol-to-vacuolar targeting (CVT) upregulated in all treatments. Gene set enrichment indicates the induction of cellular machinery for protein degradation in both the vacuole and glucose-induced degradation complex. A genome analysis of BT0510 has shown a loss of genes linked to vesicle formation and COPII binding that could alter intracellular transport [14], but we demonstrated that, in the face of sequential stresses, this yeast strain regulates other genes that could mitigate this loss, such as *EMP46* and *ATG9*, both present in our gene set.

Genes encoding protein folding chaperones are also induced by a variety of stressful conditions [24]. *HPS12* encodes a heat shock protein highly expressed after stress and a well-established reporter for the ESR pathway [24,54]. *HSP12* overexpression provided resistance to both freezing storage and heat shock, as well as an increase in intracellular levels of

glutathione peroxidase and reductase activity [55,56]. Other genes from the *HSP* family that were also in the gene set analyzed included *HSP31*, *HSP42*, *HSP78*, and *SSE2*, which influence yeast tolerance to different stresses, such as ethanol and high hydrostatic pressure [57]. These genes encode proteins that have varied functions, although most of them operate as chaperones, inhibiting protein aggregation [58,59]. Kitichantaropas et al. [44] suggested that the continuous expression of some *HSP* genes during multi-stress treatments may be involved in the acquisition of the thermotolerant phenotype. They showed that thermotolerant *S. cerevisiae* strains maintained *HSP* gene expression even after long-term exposure to heat stress, suggesting that the continuous expression of these genes may contribute to thermotolerance. Yeasts may face a series of physiological changes upon sequential stresses, and a way to adapt to these stresses could be through protein stabilization by the *HSP* gene family.

5. Conclusions

In this study, an RNA-Seq analysis provided a global overview of the response to sequential stresses in a tolerant strain, presenting new insights into how cells coordinate their metabolic pathways during fermentative stresses. Processes involving NADPH regeneration, peroxisome activity, and genes (such as *SYM1*, *STF2*, and *HSP*s) could be essential for tolerance acquisition, and the genes highlighted in our results could be used to construct more tolerant strains. These responsive genes were shared in all treatments, indicating a common defense strategy to different sequential stresses, an interesting attribute in biotechnological processes involving yeast cells and fermentation. This analysis can improve all fermentation-based processes and enhance the understanding of the response to stress in higher eukaryotes, as *S. cerevisiae* is a recognized study model.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9020195/s1, Figure S1. Principal component analysis (PCA) of RNA-Seq data.

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