Transcriptomics unravels the adaptive molecular mechanisms of *Brettanomyces bruxellensis* under SO_2 stress in wine condition

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Abstract

Sulfur dioxide is generally used as an antimicrobial in wine to counteract the activity of spoilage yeasts, including *Brettanomyces bruxellensis*. However, this chemical does not exert the same effectiveness on different *B. bruxellensis* yeasts since some strains can proliferate in the final product leading to a negative sensory profile due to 4-ethylguaiacol and 4-ethylphenol. Thus, the capability of deciphering the general molecular mechanisms characterizing this yeast species' response in presence of SO_2 stress could be considered strategic for a better management of SO_2 in winemaking. A RNA-Seq approach was used to investigate the gene expression of two strains of *B. bruxellensis*, AWRI 1499 and CBS 2499 having different genetic backgrounds, when exposed to a SO_2 pulse. Results revealed that sulphites affected yeast culturability and metabolism, but not volatile phenol production suggesting that a phenotypical heterogeneity could be involved for the SO_2 cell adaptation. The transcriptomics variation in response to SO_2 stress confirmed the strain-related response in *B. bruxellensis* and the GO analysis of common differentially expressed genes showed that the detoxification process carried out by *SSU1* gene can be considered as the principal specific adaptive response to counteract the SO_2 presence. However, nonspecific mechanisms can be exploited by cells to assist the SO_2 tolerance; namely, the metabolisms related to sugar alcohol (polyols) and oxidative stress, and structural compounds.

Keywords: *B. bruxellensis*, RNA-Seq, SO_2 stress, *SSU1* gene

1. Introduction

Brettanomyces bruxellensis is one of the yeast species causing wine spoilage, in particular red wines aged in contact with wood (Fabrizio et al., 2015). It can cause several types of defects, including biofilm formation (Fugelsang et al., 1993), loss of colour (Mansfield et al., 2002), production of acetic acid (Vigentini et al., 2008) thus leading to high volatile acidity (Fugelsang et al., 1993), mousy off-flavours, biogenic amines (Grbin and Henschke, 2000), and volatile phenols (VPs) (Chatonnet et al., 1995; Loureiro and Malfeito-Ferreira, 2003; Oelofse et al., 2009). The latter, in particular, can have a detrimental effect on wine aroma conferring undesirable notes associated to descriptors such as “leather”, “horse sweat”, “medicinal”, “barnyard” and “bacon” (Chatonnet et al., 1995). The VPs derive from a two-step enzymatic reaction involving free hydroxycinnamic acids present in wine (Gerbaux et al., 2002; Oelofse et al., 2008). Red wines are more susceptible to the growth of *B. bruxellensis* because of their lower acidity and the frequent aging in wood containers (Campolongo et al., 2014), where a semi-anaerobic environment can be established. However, *B. bruxellensis* has also been isolated in bottled wine indicating its ability to survive even in anaerobic condition (Oelofse et al. 2008).

In order to prevent the production of these off-flavours, the growth of *B. bruxellensis* needs to be controlled. Although it displays adverse effects on human health above a certain concentration (Pozo-Bayón et al. 2012), sulfur dioxide (SO_2) is the most common preservative used in winemaking also known for its antioxidant and antioxidasic properties (Divol et al., 2012). Its antiseptic activity against *B. bruxellensis* has been well documented (Agnolucci et al., 2014). Nevertheless, *B. bruxellensis* displays a certain level of resistance to SO_2, which is variable among yeast species, strains and physiological state (in connection to growth phase), besides also being a heritable feature (Beech and Thomas 1985; Warth 1985; Pilkington and Rose 1988; Divol et al. 2006; Ventre 1934). A better understanding of the molecular mechanisms conferring SO_2 resistance to *Brettanomyces* would be useful to fine-tune the winemaking practices in order to reduce the spoilage risks. Resistance mechanisms previously observed in the reference wine yeast, *Saccharomyces cerevisiae*, as a response to SO_2 stress include the production of acetaldehyde and upregulation of *SSU1,* a gene encoding a plasma-membrane SO_2 efflux pump (Stratford et al., 1987; Pilkington and Rose 1988; Casalone et al., 1992; Park and Bakalinsky 2000). Nardi et al. (2010) highlighted sulphite as the main regulator of *SSU1* expression in *S. cerevisiae.* Recently, Nadai et al. (2016) through a transcriptomic

approach pointed out that in *S. cerevisiae* the effects of sulphite stress involve adaptation mechanisms based on a higher basal gene expression level, rather than a specific gene induction. As for *B. bruxellensis*, previous investigations (Curtin et al., 2012b; Vigentini et al., 2013) suggested that the response to SO_2 in *B. bruxellensis* is also a strain-dependent trait. In this yeast species, variations in *SSU1* expression were observed. Indeed, Varela et al. (2019) showed that four different *SSU1* haplotypes contribute to the strain-dependent character observed upon SO_2 exposure and this could explain why differences in both culturability and viability can arise depending on the amount of SO_2 and on the haplotype of the strain investigated (Agnolucci et al., 2014). *B. bruxellensis* has indeed been observed to enter into a Viable but not Culturable (VBNC) state upon SO_2 exposure (Millet et al. 2000). The molecular bases of this state were recently studied by Capozzi et al. (2016). The latter authors observed the induction of genes related to carbohydrate metabolism, heat shock proteins, amino acid transport and transporter activity during recovery.

Although several studies investigated the *B. bruxellensis* tolerance toward the SO_2 stress, information on the species-associated specific and/or general adaptive molecular mechanisms shared by different strains to counteract the presence of SO_2 is still fragmented.

In this study, two strains of *B. bruxellensis*, AWRI 1499 and CBS 2499, were investigated under oenological conditions using a transcriptomic approach. In order to standardize the environmental conditions and to generate consistent data, the growth of both strains was performed in a bioreactor and the RNA-Seq analysis was carried out at pertinent sampling times to determine possible short- and long-term stress responses. Considering the diversity of genetic backgrounds within the species *B. bruxellensis*, the yeast strains were carefully chosen for (1) the availability of their complete genome sequence, (2) their different ploidy, and (3) their distinct sensitivity to SO_2 (i.e. AWRI 1499 is more resistant than CBS 2499) (Avramova et al., 2018). The aim was to describe the molecular mechanisms allowing strains across this yeast species to survive and grow under SO_2 stress. The information generated can be considered strategic for an optimized management of SO_2 during wine fermentation and ageing.

2. Materials and Methods

2.1. Yeasts, media and culturing conditions

Two strains of *B. bruxellensis*, AWRI 1499 (Curtin et al., 2012a; https://www.ncbi.nlm.nih.gov/genome/11901?genome_assembly_id=40324) and CBS 2499 (Piskur et al., 2012; https://genome.jgi.doe.gov/Dekbr2/Dekbr2.home.html), were used. Yeasts cultures were maintained in YPD medium [10 g/L Yeast extract, 20 g/L Peptone, 20 g/L Glucose, pH 5.6] supplemented with 20% (v/v) glycerol, at -80°C. Yeast pre-cultures were prepared by inoculation into YPD medium and incubation at 25°C for 3 days in shaking flasks. Cells were collected by centrifugation (2900 *g* x 15 min - Hettich, Rotina 380R, Tuttlingen, Germany) and washed with 0.9% saline solution (sodium chloride in distilled water). Fifty mL of a synthetic wine medium (SWM) [2.5 g/L Glucose, 2.5 g/L Fructose, 5 g/L Glycerol, 5 g/L Tartaric acid, 0.5 g/L Malic acid, 0.2 g/L Citric acid, 4 g/L L-lactic acid, 1.7 g/L Yeast Nitrogen Base w/o AA and Ammonium sulphate (Difco, Sparks, USA), 1.5 g/L Ammonium sulphate, 0.5 mL/L Tween 80, 20 mg/L Uracil, 10 mg/L *p*-Coumaric acid, 10 mg/L Ferulic acid, 15 mg/L Ergosterol, 5 mg/L Oleic acid, pH 3.5] plus 5% ethanol (v/v) were distributed into flasks (100 mL). The flasks were inoculated with the yeast pre-culture at an 0.1 OD_{600nm} at 25°C in aerobic conditions. At about 5 OD_{600nm} units, cells were collected by centrifugation at 2900 *g* for 15 min (Hettich, Rotina 380R) and inoculated at 0.1 OD_{600nm} unit in batches (800 mL) filled with SWM plus 10% ethanol (v/v).

2.2. Batch cultivations

Triplicate batch cultures for both strains were carried out in a Biostat-Q system (B-Braun, Melsungen, Germany). Anaerobic conditions were obtained with N_2 sparging before the inoculum. During the experiment the concentration of the dissolved oxygen was maintained at about 5 ± 2 mg/L simulating a semi-anaerobic condition (Smith and Divol, 2018) by introducing nitrogen gas into the batch. Temperature was maintained at 22°C with a continuous stirring speed of 200 rpm. Cellular growth was monitored daily by measuring the OD_{600nm} until biomass reached 1 ± 0.1, then SO_2 was added in the form of sodium metabisulphite (prepared according to Valdetara et al., 2017). The concentration corresponded to a calculated molecular SO_2 (mSO_2) concentration of 0.35 mg/L (Ribéreau-Gayon et al., 2006; Usseglio-Tomasset and Bosia, 1984). Thereafter, sugar consumption and yeast growth were monitored daily. The experiments were terminated when the concentration of residual sugars reached 0.5 g/L. For transcriptomic analysis, a cell

amount corresponding to 20 OD_{600nm} units per culture was collected immediately before the SO_2 pulse (T0), 5 hours after addition (T5) and once sugar consumption resumed (Tr), depending on the strain.

2.3. Microbial and chemical analysis

Cell enumeration and chemical analysis were performed on each sample, namely each time point (T0/T5/Tr) of each triplicate for both strains. Moreover, the quantification of volatile phenols and organic acids was carried out at the inoculation time (Ti). Samples were centrifuged at 18,000 *g* for 3 min (Hettich, MIKRO 200) and the supernatants were stored at -20°C until further analysis. The concentrations of ethanol, glycerol, glucose and fructose were determined using Megazyme's enzymatic assay kits (Wicklow, Ireland) according to the manufacturer's instructions. Culturability was determined by plating 100 µL of an appropriate tenfold serial dilution on WL nutrient agar medium (Sharlau, Sentmenat, Spain) and then incubating the plates for 5-7 days at 30°C.

The concentrations of hydroxycinnamic acids, namely *p*-coumaric and ferulic acids, vinyl-phenol, vinyl-guaiacol, ethyl-phenol and ethyl-guaiacol were determined using a UPLC as described by Valdetara et al. (2017).

The organic acids, namely tartaric, malic, lactic, citric and acetic acids, were quantified as described by Fracassetti et al. (2019).

The concentrations of free and total SO_2 were determined by direct titration with iodine in accordance to the OIV-MA-AS323-04B method (Compendium OIV, 2009).

2.4. Transcriptomic analysis

A volume of cell culture corresponding to 20 OD_{600nm} units per sample was frozen with liquid nitrogen immediately after a centrifugation step (adaptors for 50-mL tubes were previously cooled down in order to maintain RNA integrity) at 28,000 *g* for 1 min at 4° C (Hettich, ROTINA 380R). All pellets were stored at -80°C until further use. Samples were collected from triplicate. RNA extractions were carried out using the Presto Mini RNA Yeast Kit protocol (Geneaid, New Taipei City, Taiwan) following the manufacturer's instructions with few modifications, as previously reported in Valdetara et al. (2017). After extraction, RNAs were quantified by measuring the absorbance at 260 nm in a PowerWave XS2 spectrophotometer (BioTek, Winooski, Vermont, United States). The integrity of RNA samples was assessed by electrophoresis on 1.2% agarose-FA gel. The electrophoretic run was carried out at 100 V for 1 hour and then bands were visualized

under UV irradiation (Bio-Rad, Berkeley, California). RNAs were maintained at -80°C until samples were sequenced. Transcriptome analysis were conducted by CNR, Istituto di Biomembrane e Bioenergetica, Bari, Italy. RNAs were purified and then submitted to NGS-sequencing [NextSeq® 500/550 Mid Output Kit, v2 (150 cycles), FC-404-2001Illumina].

2.5. RNA-seq data analysis

First, raw reads obtained from sequencer were submitted to FastQC for quality evaluation, then reads were mapped to a reference genome of *B. bruxellensis* (obtained from the strain AWRI1499, Curtin et al., 2012a) with hisat2 (v2-2.1.0) (Kim et al., 2015) and subsequently quantified using the Cufflinks package (v2.2.1) (Trapnell et al., 2013). Results obtained from quantification (Cuffquant) were normalized (Cuffnorm) and tested for differential expression (Cuffdiff), thus obtaining FPKM (Fragments per Kilobase of Million mapped reads) gene expression and $\log_2$fold-change values, respectively. TPM (Transcript Per Million) values were calculated from FPKM values: the formula for TPM calculation was derived from Pachter (2011). Genes statistically (FDR-adjusted p-value < 0.05) differentially expressed more than twofold were used to identify Gene Ontology (GO) categories significantly (Bonferroni corrected p-value < 0.01) enriched. Gene Ontology enrichment analysis was performed and visualised using http://go.princeton.edu/cgi-bin/GOTermFinder (Boyle et al., 2004) and REVIGO (Supek et al., 2011) e-tools.

3. Results

In order to evaluate the genetic mechanisms activated by *B. bruxellensis* to counteract the stress caused by the addition of SO_2 during winemaking, an RNA-seq approach was used. The study was conducted on two *B. bruxellensis* strains, namely AWRI 1499 and CBS 2499, exposed to a sub-lethal dose of SO_2 supplied in oenological conditions. As reported by Avramova et al. (2018) these two strains present a triploid and a diploid genome, respectively, and they show different sensitivities to SO_2. Indeed, AWRI 1499 is more tolerant than CBS 2499. Briefly, cells in late exponential phase of growth were treated with sodium metabisulphite and RNA-sequencing was performed on samples collected 5 h after the SO_2 exposure (T5) and when sugar consumption resumed (Tr). Samples collected immediately before SO_2 addition (T0) were used as the reference condition. Unless otherwise specified, the term “response” is used here for the comparison between transcriptomes obtained from the cells at T5 and T0 (T5 response) or at Tr and T0 (Tr

response). Five hours was deemed an appropriate time period to analyse the stress response, considering the slow duplication time of *B. bruxellensis* before the SO_2 addition (approx. 18h and 40h in the exponential phase of growth for AWRI 1499 and CBS 2499 strains, respectively) (Murata et al., 2006; Nardi et al., 2010). Preliminary data showed that CBS 2499 strain was unable to proliferate at 0.50 mg/L of mSO_2 in our conditions and confirmed that 0.55 mg/L mSO_2 represented the growth/no growth threshold for AWRI 1499 strain (Curtin et al., 2012b). Thus, with the aim to study the adaptive response against SO_2 stress in both strains, a concentration of 0.35 mg/L mSO_2 was applied in our experiments and common Differentially Expressed Genes (DEGs) between the 2 strains were processed in the GO analysis.

3.1. SO_2 affected yeast culturability and metabolism, but not VPs production

Cell growth was monitored daily until an OD_{600nm} of 1 ± 0.1 was reached (corresponding to 1.5 ± 0.3 x 10^7 and 1.2 ± 0.3 x 10^7 CFU/mL for AWRI 1499 and CBS 2499 strains, respectively), and SO_2 added (Fig. 1). The strains were inoculated around 10^6 CFU/mL and the populations increased with one log unit within 160 h. CBS 2499 displayed a biomass increase of about half a log unit within the first 80 h which correlated with a faster consumption of sugars. The addition of SO_2 had an immediate impact on the cell culturability of both strains with a decrease of 2-3 log units measured after 5 hours (T5) with a further decrease of 1-1.5 log units in the following three days, depending on the strain. Plate counts increased at a constant rate thereafter ending again at around 10^7 CFU/mL. Both strains consumed completely the glucose and almost all the fructose. However, statistically significant differences ($p < 0.05$) were detected at strain level in sugar consumption rate, as illustrated in Fig. 1. Before the SO_2 pulse, about 0.5 g/L of glucose and 2 g/L of fructose were still available for AWRI 1499 strain. On the contrary, for the CBS2499 strain, glucose was almost depleted and fructose concentration resulted in 1 g/L. For both strains, no sugar consumption was observed during the 5 h after the SO_2 stress exposure up to approximatively 7 days (from 165 to 330 h) until cell numbers again reached 10^6 CFU/mL. With regard to SO_2, the total amount remained stable until the end of the fermentation at a value of 16.4 ± 4.8 mg/L, while the free fraction decreased to about 25% of the initial concentration, namely 2.1 ± 0.9 mg/L.

The concentrations of ethanol, glycerol, lactic, tartaric, acetic, malic, citric, *p*-coumaric, ferulic, acids, vinyl- and ethyl-phenol and guaiacol, in the medium were determined at the same time points where RNA extraction was performed.

The concentrations of ethanol, glycerol, and lactic and tartaric acids did not show any significant differences between strains or time points (data not shown). The concentration of acetic acid differed significantly for both strains only between Ti and T0 (Table 1), i.e. before the SO_2 pulse. Overall, CBS 2499 produced almost double the amount of acetic acid compared to AWRI 1499 (Table 1). Malic acid and citric acid concentrations increased significantly during the fermentation process only for CBS 2499 (Table 1). To the best of our knowledge, *B. bruxellensis* has never been reported to release malic or citric acids, even in small amounts. Further investigations are required to clarify this finding.

Despite an expected initial concentration at Ti of 10 mg/L hydroxycinnamic acids (each), 8 mg/L *p*-coumaric acid was measured for both strains, while the quantification of ferulic acid revealed a lesser amount of this acid in the medium, 4.35 ± 0.43 and 5.11 ± 0.19 mg/L for AWRI 1499 and CBS 2499, respectively (Table 1). Both *p*-coumaric and ferulic acids decreased during the fermentation; nonetheless, the difference detected before and after the SO_2 addition was negligible and not significant, thereby indicating that this decrease was not correlated to the addition of SO_2 (Table 1). The two strains were characterized by a different uptake of hydroxycinnamic acids (Table 1): in the CBS 2499 fermentations, the final amount of these acids was significantly ($p < 0.05$) lower than in AWRI 1499 fermentations. As expected, VPs were not detected at inoculation time (Ti). While vinyl-guaiacol was not measured at any time, the other volatile phenols were all produced during the first part of the experiment (Ti-T0). The higher amount of VPs was produced by strain CBS 2499, where both vinyl-phenols and ethyl-phenols were approximately six and two folds more abundant than in AWRI 1499, respectively (Table 1).

3.2. The transcriptomic variation in response to SO_2 stress is strain-related

Three sample types, untreated cells (T0), cells collected 5 h after the SO_2 pulse (T5), and cells able to restore their growth (Tr), were analysed. A PCA analysis was carried out on TPM values and dispersion of the samples is reported in Fig. 2. The analysis covered almost 73% of the variability in the samples with more than 47% explained by component 1 and about 26% by component 2. The PCA indicated that a response to

SO_2 addition arose in a strain- and time-dependent manner. Strains were clearly differentiated on the basis of component 1 and samples corresponding to replicate measurements at the same growth condition grouped together, with a complete separation among groups only at the T0 and Tr conditions for both strains. Indeed, an overlap between groups of replicates at times T0 and T5 was detected suggesting that the 5 h exposure time did not induce a strong modulation of yeast transcriptome unlike that observed at the time of recovery of the cell growth (Tr).

3.3. *B. bruxellensis* strategy to counteract SO_2: 66 genes drive the global transcriptional response

T5 and Tr responses were evaluated by comparing transcriptomes from cells collected at the respective time points *versus* untreated cells (T0). In general, the two strains expressed a similar number of genes: 4855, 4854 and 4851 in AWRI 1499 strain (A) and 4835, 4834 and 4836 in the CBS 2499 strain (C) at T0, T5 and Tr, respectively. The number of genes showing a significant change (corrected p-value < 0.05) in their expression (DEGs), is reported in Table 2. Considering all the genes identified in the AWRI1499 genome (4861), 3589 are homologous to *S. cerevisiae* genes (73.8%). The outcome showed that few genes significantly changed their expression at T5, and they were mainly down-regulated. In particular, in the AWRI 1499 strain a significantly different transcriptome was observed comparing the number of DEGs after 2 h (Varela et al. 2019) and 5 h from the SO_2 pulse. The DEGs at 2h in Varela and co-authors were 536 in AWRI 1499 (287 up and 249 down regulated genes) whereas in the present study, genes were mainly down-regulated (19 up and 149 down expressed genes). On the other hands, at Tr the expression of a higher number of genes significantly changed. The list of genes that showed significant differences in their expression (increase or decrease) with the correspondent log_2FC values and the annotation to *S. cerevisiae* genome, is given as Supplementary material (Table S1), for both strains and times of sampling.

Considering DEGs of the two strains at the two time points, only one gene (AWRI1499_4045) of the Significantly Up-Regulated Genes (SURGs) was common between the two strains at T5, and it also resulted as the one with the highest up-regulation (log_2FC = 0.89, in AWRI 1499). This gene, homologous to *S. cerevisiae*'s *CPR3*, has been described as a mitochondrial peptidyl-prolyl cis-trans isomerase that catalyses the cis-trans isomerization of peptide bonds N-terminal to proline residues and has been observed to be involved in protein refolding after import into mitochondria (Matouschek, et al., 1995). Conversely, the gene

which was the most strongly down-regulated resulted in CBS 2499 strain for AWRI1499_3932 gene (log_2FC = -1.27) that has no homolog in *S. cerevisiae*. Results revealed no SURGs with $|log_2FC| > 1$ neither for AWRI1499 nor for CBS2499, and none of the few Significantly Down Regulated Genes (SDRGs) having $|log_2FC| > 1$ were shared between the strains. Nevertheless, in the AWRI 1499 strain, we confirmed the down-regulation of *S. cerevisiae* homologous *PCL1*, encoding a protein involved in cell cycle progression, and the absence in the regulation of *BbSSU1*, recently reported in *B. bruxellensis* upon 2 h sulphite exposure (Varela et al., 2019).

In the Tr response, the highest up-regulation (log_2FC = 7.05) and the lowest down-regulation (log_2FC = -4.23) were measured in the AWRI 1499 strain, for *RCF2* and *GAP1* genes, respectively. The two strains had in common 66 *S. cerevisiae* homologous SURGs/SDRGs, 57 of which had the same orientation in the change of expression in both strains, 38 increased and 19 decreased (Fig. 3). Among SURGs with the highest difference in the level of expression, *SSU1* can be linked to detoxification processes (and more specifically to active SO_2 efflux), *HXT13, HXK1, GAL1 GAL10, GAL7, ADH6, ADH7, YLR345W, FMP37, LSC1, LSC2, SUC2* and *MPH2* are related to carbon metabolism, while some of the down-regulated genes (*MAK5, RRP5, TRM2, UTP20)* are linked to RNA processes. The remaining 9 genes (*ALD4, ARO10, CYB2, DLD1, FLO1, HER2, JEN1, OXP1, YLR278C*) had decreased expression in the AWRI 1499 strain and increased expression in the CBS 2499 strain.

Considering the response at the strain level, in the AWRI 1499 strain, 5 of the SURGs at T5 were still up-regulated at Tr, with 1 (AWRI1499_3589) having a log_2FC slightly above the set threshold of 1. This gene is homologous with the *YBR096W* open reading frame of *S. cerevisiae*, which has been described as a protein of unknown function which localizes at the endoplasmic reticulum level (Huh et al., 2003). Regarding the SDRGs, out of the 149 genes (Table 2, Table S1), 38 were still down-regulated at Tr, with 9 of these having a log_2FC below -1. Three genes were down-regulated more than 2-fold at T5, the last two remained significantly down-regulated at Tr, but not as strongly, while one gene did not have significantly decreased expression. In the CBS 2499 strain, only 1 of the two SURGs at T5 was differentially expressed still in the Tr response, while 3 out of 7 SDRGs maintained the significant down-regulation (Table 2, Table S1).

Extreme changes in gene expression were in both strains measured at the Tr response and up- and down-regulated genes did not correspond among the two strains. In the AWRI 1499 strain, the gene with the

highest increase was the homolog of the *S. cerevisiae RCF2* gene, which codes for a cytochrome c oxidase subunit. This gene changed its expression more than 132-fold. On the other hand, the gene with the largest decrease in expression was the homolog of the *S. cerevisiae GAP1* gene, encoding a general amino acid permease. In the CBS 2499 strain, the gene with the highest increase in expression was the homolog of the *S. cerevisiae ACH1* gene, which codes for an acetyl CoA hydrolase. The gene with the largest decrease in expression was the homolog of the *S. cerevisiae OPT2* gene, encoding an oligopeptide transporter.

3.4. The general response of *B. bruxellensis* species against SO_2 from the GO analysis perspective

In order to obtain an overview of the general response associated to SO_2 stress adaptation in *B. bruxellensis* at the level of biological processes, cellular components and molecular functions involved, SURGs and SDRGs shared by the two strains at the Tr response (Fig. 3) were analysed according to their Gene Ontology (GO) annotation (Table 3, Fig. S2). Thirty-one significantly (p-value < 0.01) enriched biological processes in the SURGs set were found. Carbon metabolism was one of the most represented processes, together with representative GO term superclusters corresponding to monocarboxylic acid, acyl-CoA, cofactor, and sulfur compound metabolism. For the SDRGs, the only significantly enriched biological process was transmembrane transport. From the cellular component ontology, 4 terms were significantly (p-value < 0.01) enriched in the set of SURGs. Among them, the significance of the mitochondrial pyruvate dehydrogenase complex was noteworthily higher (p-value = 1.46e-06) than in the other (0.0045 and 0.00338). No terms from the cellular component ontology were significantly enriched. The analysis of the molecular function ontology revealed 9 significantly enriched terms in the SURGs set, corresponding to the representative superclusters of pyruvate dehydrogenase and carbohydrate kinase activities, and the catalytic and succinate-CoA ligase (ADP forming) activities. Six terms were significantly enriched in the SDRGs set, all corresponding to transporter activities.

3.5. GO analysis at strain level

A strain specific analysis of the enriched biological processes, cellular components and molecular functions was carried out on SURGs and SDRGs involved in the SO_2 adaptive response at Tr. The results are presented in an aggregate as tree maps in Fig. S2. Fifty significantly (p-value < 0.01) enriched biological processes in

the SURGs set of AWRI 1499 strain were found, with cellular carbohydrate metabolism, generation of precursor metabolites and energy, glucose import and pyridine-containing compound metabolism being the main representatives. On the other hand, organic hydroxy-compound metabolism was the principal biological process resulting from the analysis of the SURGs of CBS 2499 strain, which in total returned 28 significantly enriched processes. Considering the SDRGs, there were 31 and 7 significantly enriched processes in the AWRI 1499 and CBS 2499 strains, respectively. In the AWRI 1499 strain, the anion transmembrane transport emerged, together with the ribosomal small subunit biogenesis, the RNA 5'end-processing, and the oxidation-reduction process. On the other hand, results in the CBS2499 strain mainly indicated the involvement of genes from the supercluster of monocarboxylic acid metabolism; this latter term was also found among the processes enriched by SURGs, but different genes were involved. The cellular localization GO analysis of SURGs of AWRI 1499 strain detected 8 significantly enriched cellular components, while 4 were significant based on the analysis of SURGs in the CBS 2499 strain. In addition, SDRGs defined 14 and 2 significantly enriched cellular localization terms in AWRI 1499 and CBS 2499 strains, respectively. Among the enriched terms resulting from the AWRI 1499 strain analysis based on SURGs we identified superclusters of peroxisome and cytoplasm. Based on AWRI 1499 SDRGs, these are annotated to significantly enriched cellular localization terms pre-ribosome, nucleolus, the integral component of plasma membrane, the cell part and periphery, and the membrane-enclosed lumen. The enrichment analysis of SURGs and SDRGs derived from the CBS 2499 strain resulted in a single strain-specific cellular localization term, namely the nucleotide-excision repair complex supercluster, which was found based on the SDRGs. In the AWRI 1499 strain, the significantly enriched molecular functions based on SURGs and SDRGs displayed 6 and 21 terms, respectively. Molecular functions of the genes with increased expression level include carbohydrate binding, while among the enriched functions of the genes with decreased expression level the snoRNA and cofactor binding are notable, together with the oxidoreductase activity. In the CBS2499 strain, 9 terms were significantly enriched based on SURGs, while 8 such terms resulted from the analysis based on the SDRGs. The former group includes oxidoreductase activity and the cofactor binding, while the latter includes oxidoreductase and catalytic activity, and ion binding.

4. Discussion

The exposure of cells to suboptimal growth conditions or any environmental condition that negatively affects parameters such as cell viability or fitness can be considered a stress. Nonetheless, different kinds of stresses, defined as mild, chronic or acute stresses, occur. Cell responses depend on the organism, its physiological state and the environment in which the stress arises. Responses are usually defined by two components: a generic or environmental response, common to various types of stresses, and a specific adaptive response, characteristic of particular stress factors. Both general and stress-specific responses are generated as the consequence of mechanisms acting over a series of time scales; post-translational effects provide immediate responses, while regulation of gene expression is essential for the slower, long-term adaptation and recovery phases (de Nadal et al., 2011).

Our data showed an arrest in the growth of both analysed strains, characterised by a different genetic background (triploid and diploid), and a decrease in their cell culturability resulting from the exposure to the stress-inducing factor investigated in this study. However, strains recovered their growth at 80 h following the SO_2 pulse thereby demonstrating the capability to adapt to the stress applied. This result differs from that observed in the study of Varela et al. (2019) in which the AWRI 1499 strain (triploid) showed a culturable population at 48 h following the SO_2 pulse whereas the AWRI 1613 strain showed a culturability below the limit of detection (<10 CFU/mL) after 24 h following the SO_2 pulse and did not recover further. The discrepancy between the two studies could be the result of similar, but not identical, experimental conditions.

No statistical variations were recorded regarding lactic and tartaric acids, as well as glycerol and ethanol concentrations during the experiment. Conversely, the observed statistical difference on acetic acid concentration at T5 vs T0 confirms that this compound is produced during yeast growth. Moreover, its release in the medium is not affected by the SO_2 stress, since no differences in the amount were detected after the SO_2 pulse. Considering sugar utilization, the differences highlighted in the results between the two strains at the different time points indicate that the usage of sugars undergoes a strain-specific consumption dynamic.

Regarding the release of VPs they were not produced after the SO_2 pulse, in disagreement to what was observed by Serpaggi et al. (2012) who reported the cells can produce 4-ethyl-phenol, although in a lower amount than control cells, entering in a SO_2-induced VBNC state. The last observation suggests that a

VBNC state is not triggered by the SO_2 treatment under the investigated experimental conditions. Moreover, Serpaggi et al. (2012) defined the VBNC state as being characterised by a reduced glycolytic flux coupled with changes in redox homeostatis/protein turnover-related processes. Considering that at T5 cells did not undergo any significant change in the expression of genes, we could speculate that the SO_2 addition led to death of "sensitive cells" and that, the remaining "resistant cells" were able to adapt themselves to new environmental conditions. Besides being genetically identical, cells can exhibit different phenotypes: diversity in the phenotypical behaviour, defined "phenotypical heterogeneity", could be the determinant for the cell adaptation to changing environments, this conferring a significant competitive advantage to more heterogeneous isolates exposed to stressful conditions (Hewitt et al., 2016; Holland et al., 2014). However, more investigations are required to confirm this hypothesis.

The analysis of results arising from the study of a shorter (T5) and a longer-time (Tr) exposure response to SO_2 in *B. bruxellensis* evidenced that in both strains the outcome in terms of number of statistically differentially expressed genes is considerably smaller at T5 in comparison to Tr. Results obtained showed that only a low number of genes are differentially expressed at T5, with only a few genes changing their expression more than two-folds. Moreover, in the case of the AWRI 1499 strain, the difference observed in the modulation of the transcriptome in Varela and co-authors (2019) could derive by the different growth conditions applied in the two studies, mainly fermentation strategy and sampling time (i.e. 2 h - Varela et al. and 5 h - this study). On the other hand, at Tr, genes that were found significantly differentially expressed were around a thousand, with approximately 10% having a $\log_2$ fold-change greater than |1|, thus displaying a stronger effect.

The transcriptomic analysis of *B. bruxellensis* in the presence of SO_2 reveals that the cells reacted against the stress factor by activating a specific adaptive and a general response simultaneously (Fig. 4). The former could be identified as the sulphite detoxification mechanism, where the main gene involved in sulphite removal from cells in *S. cerevisiae* is *SSU1*. This gene encodes a plasma membrane sulphite pump that can determine the sensitivity/resistance to the toxic action of sulfur compounds at strain level (Avram and Bakalinsky, 1997; Divol et al., 2006, 2012; Nardi et al., 2010; Nadai et al., 2016). Ssu1p is part of the major facilitator superfamily involved in efflux of toxic compounds, specifically mediating efflux of the free form of sulphite (Park and Bakalinsky, 2000). Nardi and collaborators, in 2010, provided evidence that sulphite is

the main regulator of *SSU1* expression. The present study confirms that this protein exerts a strong detoxification role in *B. bruxellensis* cells as observed by other researchers (Valera et al., 2019; Capozzi et al., 2016; Godoy et al., 2016; Nadai et al., 2016). AWRI1499_0080, homologous of *SSU1*, is highly expressed at Tr, resulting in increases of more than 4 and 47 times in CBS 2499 and AWRI 1499, respectively. At the strain level, this reflects the higher SO_2 resistance of AWRI 1499 strain. In this strain, recently Varela et al. (2019) demonstrated that the presence of two copies of the most efficient *SSU1* haplotype, which are also preferentially expressed, conferring in this way its greater sulphite tolerance.

A more general response related to the SO_2 stress applied in this study includes genes related to sugar alcohol (polyols) metabolism, oxidative stress and, structural compounds (Fig. 4). MDR members (medium-chain dehydrogenase/reductase (MDR) family) are basic metabolic enzymes acting on alcohols or aldehydes (Riveros-Rosas et al., 2003), and thus these enzymes may have roles in detoxifying alcohols and related compounds, protecting against environmental stresses such as osmotic shock, reduced or elevated temperatures, or oxidative stress (Nordling et al., 2002). *ADH6* and *ADH7,* involved in the conversion of longer chain aldehydes and alcohols together with *BDH1*, the gene encoding for NAD-dependent (R,R)-butanediol dehydrogenase (González et al., 2000), were found up-regulated in response to vanillin stress (Ishida et al., 2016; Nguyen et al., 2015) in *S. cerevisiae*, and their homologues were found overexpressed in both *B. bruxellensis* strains.

The overexpression of genes related to oxidative stress, such as *PST2* and *CLD1*, was also detected; indeed, the fact that SO_2 exposure triggers an oxidative stress has been already reported (Vigentini et al., 2013; Capozzi et al., 2016). *PST2* is an oxidative stress-induced gene (Morano et al., 2012) encoding a flavodoxin-like protein and *CLD1*, the gene with the highest expression in CBS2499, encodes a mitochondrial cardiolipin-specific phospholipase that was observed to undergo up-regulation as a result of exposure to hydrogen peroxide and thus important for the decrease of the oxidative stress effects (Lou et al., 2018).

Genes related to fatty acids metabolism, like *ACH1, FOX2* and *SPS19,* and then possibly involved in the regulation of membrane permeability, were also found up-regulated as already observed in other studies (Beltran et al., 2006; Nadai et al., 2016; Zhu et al., 2013). In particular, Ach1p acts as a CoA-transferase by catalyzing the transfer of CoA from succinyl-CoA to acetate. A role in detoxifying mitochondria from

acetate has been reported in *S. cerevisiae* (Fleck and Bock, 2009). This role can be more important in CBS 2499 than in AWRI 1499, due to the higher acetate production of the former strain.

Regarding amino acid metabolism, some genes could be identified, albeit differently regulated. *DIP5* (dicarboxylic amino acid permease) and *LEU5*, encoding a mitochondrial inner membrane protein involved in CoA transport to the mitochondrial matrix (Prohl et al., 2001) were found to be upregulated. Alteration of amino acid metabolism has previously been reported as one of the principal effects of the response to sulphite exposure in *B. bruxellensis* (Vigentini et al, 2013). On the contrary, *GCV2*, codifying a glycine decarboxylase and *GAP1*, a general amino acids permease, were both down-regulated.

Regarding down regulated expression, the only biological process significantly affected in both strains was transmembrane transport (Fig. 4). Other genes referred to RNA processes also underwent a down-regulation, according to other studies where in response to different stresses the same trend of expression was observed for ribosomal biogenesis and assembly genes (Soontorngun, 2017; Yu et al., 2010).

A discussion is required for genes related to carbon metabolism. Upregulation of genes belonging to this category has been found after SO_2 treatment (Capozzi et al. 2016; Varela et al. 2019). In our study several genes resulted in significant up-regulation at the Tr response in both strains. Among them, *HXK1* was identified among genes related to different stress responses in *S. cerevisiae* (Beltran et al., 2006; Bereketoglu et al., 2017; Causton et al., 2001; Murata et al., 2006; Zhu et al., 2013), and particularly up-regulated in stationary phase of growth (Gasch et al., 2000). Furthermore, *SUC2*, *GAL10* and *YDR109C* were found up-regulated by Capozzi et al. (2016). *NTH1* was previously detected as over-expressed in response to different stresses (Zähringer et al., 1997). Nevertheless, we should consider that the low concentration of available sugars approaching Tr could have also contributed to trigger glucose/(carbon) de-repression, other than the adaptation to SO_2-related stress. In this situation, genes related to sugar transport and assimilation (*HXT13*, *GAL1/7/10*, *HXK1*, *YLR345W*, *YDR109C*) could increase their expression. The *HXT13* gene is, in both strains, up-regulated more than 15-fold. Tiukova et al. (2013) also related its expression in *B. bruxellensis* under conditions of oxygen limitation, similar to those of our cultivations. Moreover, in *S. cerevisiae*, it has been described as a putative transmembrane polyol transporter that can uptake mannitol and sorbitol and supports growth (Jordan et al., 2016), being induced by non-fermentable carbon sources and at low glucose

concentrations (Greatrix and van Vuuren, 2006). *SOR1*, highly up-regulated in this study, is reported to be induced in sorbitol or xylose containing media (Sarthy et al., 1994; Toivari et al., 2004).

Other genes that control the utilization of alternative carbon sources as well as genes related to the pyruvate dehydrogenase complex/carrier (*PDA1*, *PDB1*, *PDX1*, *FMP37* and *LAT1*) were also up-regulated, suggesting that the yeast was prepared to assimilate all the available carbon sources. Interestingly, the gene encoding the transcriptional regulator *CAT8*, that has been observed important in *S. cerevisiae* for the growth on non-fermentable carbon sources such as glycerol and ethanol (Mojardín et al., 2018) was found overexpressed in both strains.

The negative impact of VPs on wine sensory is well-known due to their detrimental effect caused by the appearance of leather, horse sweat, medicinal, barnyard and bacon, defined as *Brett*-character (Chatonnet et al. 1992). Stress conditions, i.e. high concentrations of ethanol and SO_2, and low pH and poor availability of nutrients, can limit the release of VPs but not completely prevent it due to the ability of *B. bruxellensis* to grow and survive in extreme environments (Steensels et al., 2015). Before the SO_2 pulse, increased concentrations of both ethyl-phenol and ethyl-guaiacol were found. Both strains released higher level of ethyl-guaiacol in comparison to ethyl-phenol. This results is in accordance to Valdetara et al. (2017) who investigated the volatile phenols produced by CBS 2499 strain. In particular, the amount of VPs was more than 2-fold higher for CBS 2499, further indicating the strain-dependent release of VPs. After the SO_2 pulse, at Tr, ethyl-phenol was significantly higher only for the AWRI 1499 strain. Genetically this could be due to its triploid state (Curtin et al., 2012), and physiologically the residual content of both sugars still present at the cell recovery may have favoured the release of ethyl-phenol as recently reported by Smith and Divol (2018).

In conclusion, according to the sulfur resistance of the two strains the transcriptomic response observed showed that the activated detoxification processes can be considered as the principal specific adaptive response to counteract the SO_2 presence. However, nonspecific mechanisms can be exploited by cells to assist the SO_2 tolerance behaviour.

Considering the climate change that is leading to the production of less acidic wine (Mozell and Thachn, 2014), the effectiveness of SO_2 can result further be limited as lower mSO_2 can be dissolved in wine for an equal level of total SO_2 due to a higher pH. In this case, as our study demonstrated, a sub-population of

adapted cells can resist the stressful environment resulting, in presence of some residual sugars, in the appearance of the *Brett* character. Thus, the general trend to produce low sulphite wines could determine in future an increase in the occurrence of volatile phenols in the final products due to the selection of more and more resistant *B. bruxellensis* strains.

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Figure captions:

Figure 1

Sugar (glucose and fructose) consumption over time for AWRI 1499 (light green) and CBS 2499 (dark blue). Continuous and dashed lines represent glucose and fructose fermentative trend, respectively. Dotted lines were used to represent culturablity results [Log_{10} (CFU/mL)]. Different letters (bold characters refer to glucose curves and standard characters refer to fructose consumption) correspond to significant differences ($p < 0.05$) across the sampling times. Average curves of the triplicate data. RNA-Seq sampling times: T0, red circle; T5, orange circle; Tf, light violet circles.

Figure 2

Principal Component Analysis (PCA) of the samples (TPM values) in the first two principal component space. Sample coding includes both the strain (A=AWRI 1499, C=CBS 2499), collection time point (T0 = untreated cells, collected immediately before the SO_2 addition, T5 = samples collected 5h after the SO_2 pulse, Tr = cells collected at the recovery phase of growth) and replicates (_1, _2, _3). Lines grouping the different time point are coloured differently, based on the strain (AWRI 1499 is light green, CBS 2499 is blue); within the same strain, different times of collection are represented by different hatching (lines grouping all time points are dotted lines, T0 are continuous lines, T5 are dotted-dashed lines and Tr are dashed lines). All the lines have been drawn to make the visualisation easier.

Figure 3

Bar-diagram representing the log_2FC value of common homologous SURGs (38)/SDRGs (19) in the response for cells collected at the recovery phase of growth (Tr) respect to T0. SURGs and SDRGs are listed in alphabetical order. Green bars: *B. bruxellensis* AWRI 1499. Blue bars: *B. bruxellensis* CBS 2499.

Figure 4

Map of the adaptive molecular mechanisms exploited by *B. bruxellensis* to assist the SO_2 tolerance. Colours of the squares indicates the main UP (yellow/orange) or DOWN (blue/light blue) regulated metabolisms or processes and genes.

Table 1

Determination of organic acids and volatile phenols (mg/L) reported as the average±standard deviation of triplicate fermentations. "Ti" indicates the sampling time concurrent to the inoculation. Different lowercase letters indicate the statistically significant variation per strain among sampling times. Different capital letters indicate the statistically significant variation per sampling time between strains ($p < 0.05$); LOD: limit of detection (Valdetara et al., 2017).

Strain	Time	Malic acid	Citric acid	Acetic acid	*p*-coumaric acid	Ferulic acid	Vinyl-phenol	Vinyl-guaiacol	Ethyl-phenol	Ethyl-guaiacol
AWRI 1499	Ti	0.49±0.08$^{a, A}$	0.34±0.09^{a}	< LOD	8.08±0.64$^{a, A}$	4.35±0.43$^{a, A}$	< LOD	< LOD	< LOD	< LOD
	T0	0.48±0.00$^{a, A}$	0.36±0.05^{a}	0.10±0.02$^{a, A}$	6.59±0.32$^{b, A}$	3.82±0.13$^{a,b, A}$	0.35±0.00$^{a, A}$	< LOD	0.72±0.08$^{a, A}$	1.12±0.31$^{a, A}$
	T5	0.48±0.07$^{a, A}$	0.41±0.07^{a}	0.13±0.01$^{a, A}$	6.45±0.81$^{b, A}$	3.57±0.48$^{b, A}$	0.33±0.07$^{a, A}$	< LOD	0.77±0.00$^{a, A}$	1.42±0.70$^{a, A}$
	Tr	0.58±0.06$^{a, A}$	0.46±0.08^{a}	0.14±0.05$^{a, A}$	6.69±0.46$^{b, A}$	3.88±0.15$^{a,b, A}$	0.09±0.01$^{b, A}$	< LOD	0.97±0.20$^{b, A}$	1.11±0.48$^{a, A}$
CBS 2499	Ti	0.47±0.03$^{a, A}$	0.34±0.02^{a}	< LOD	8.15±0.09$^{a, A}$	5.11±0.19$^{a, A}$	< LOD	< LOD	< LOD	< LOD
	T0	0.55±0.07$^{b, A}$	0.46±0.06^{b}	0.21±0.09$^{a, A}$	3.40±0.35$^{b, B}$	2.06±0.18$^{b, B}$	2.59±0.32$^{a, B}$	< LOD	1.81±0.22$^{a, B}$	2.43±0.13$^{a, B}$
	T5	0.55±0.03$^{b, A}$	0.47±0.05^{b}	0.18±0.02$^{a, B}$	3.24±0.27$^{b, B}$	2.09±0.18$^{b, B}$	2.32±0.30$^{a, B}$	< LOD	1.88±0.20$^{a, B}$	2.54±0.13$^{a, A}$
	Tr	0.64±0.02$^{c, A}$	0.49±0.02^{b}	0.21± 0.05$^{a, A}$	3.37±0.66$^{b, B}$	1.99±0.09$^{b, B}$	2.11±0.33$^{a, B}$	< LOD	2.06±0.30$^{a, B}$	2.57±0.15$^{a, B}$

Table 2

Number of genes with a significant log_2FC value (corrected *p*-value <0.05). The number of DEGs with a $|log_2FC| > 1$ is reported in brackets. For the Tr-T0 comparison, the number of genes having a correspondent homolog in the reference *S. cerevisiae* genome, is shown. (n.d. = not detected)

	T5-*vs*-T0		Tr-*vs*-T0			
			AWRI1499-identifiers		***S. cerevisiae* homolog**	
	UP	**DOWN**	**UP**	**DOWN**	**UP**	**DOWN**
AWRI1499	19 (n.d.)	149 (3)	571 (170)	573 (138)	448(126)	425(96)
CBS2499	2 (n.d.)	7 (1)	536 (107)	549 (85)	387(81)	388(52)

Table 3

Statistically significant (p-value <0.01) enriched categories, alphabetically listed, of Biological Process, Molecular Function and Cellular Component, with relative cluster and genome frequency, and gene annotated. Colours of the dots (yellow  , /blue ) indicates UP or DOWN regulated categories, respectively. Dimension of the dots refer to $\log_{10}$(p-value), in detail:  /  : 2 < $\log_{10}$(p-value) <3; /  :3 < $\log_{10}$(p-value) <4; / : $\log_{10}$(p-value) >4.

Enriched Biological Process in common between AWRI1499 and CBS2499 - Long response						
Gene Ontology term	**Cluster frequency**	**Genome frequency**	**Statistical significance**	**FDR%**	**False Positives**	**Genes annotated to the term**
acetyl-CoA biosynthetic process	4 of 38 genes, 10.5%	6 of 7166 genes, 0.1%		0.00	0.00	*LAT1, PDX1, PDA1, PDB1*
acetyl-CoA biosynthetic process from pyruvate	4 of 38 genes, 10.5%	4 of 7166 genes, 0.1%		0.00	0.00	*LAT1, PDX1, PDA1, PDB1*
acetyl-CoA metabolic process	5 of 38 genes, 13.2%	13 of 7166 genes, 0.2%		0.00	0.00	*LAT1, ACH1, PDX1, PDA1, PDB1*
acyl-CoA biosynthetic process	4 of 38 genes, 10.5%	8 of 7166 genes, 0.1%		0.00	0.00	*LAT1, PDX1, PDA1, PDB1*
acyl-CoA metabolic process	7 of 38 genes, 18.4%	20 of 7166 genes, 0.3%		0.00	0.00	*LAT1, LSC1, ACH1, PDX1, PDA1, LSC2, PDB1*

ATP generation from ADP	4 of 38 genes, 10.5%	33 of 7166 genes, 0.5%		0.00	0.00	*YLR345W, PDA1, PDB1, HXK1*
carbohydrate catabolic process	9 of 38 genes, 23.7%	120 of 7166 genes, 1.7%		0.00	0.00	*YLR345W, PDA1, GAL7, HXK1, GAL1, SUC2, GAL10, NTH1, PDB1*
carbohydrate metabolic process	13 of 38 genes, 34.2%	314 of 7166 genes, 4.4%		0.00	0.00	*YLR345W, CAT8, PDA1, GAL7, MPH2, HXK1, SUC2, GAL1, GAL10, SOR1, NTH1, YDR109C, PDB1*
carbohydrate phosphorylation	4 of 38 genes, 10.5%	14 of 7166 genes, 0.2%		0.00	0.00	*GAL1, YLR345W, YDR109C, HXK1*
carboxylic acid metabolic process	13 of 38 genes, 34.2%	433 of 7166 genes, 6.0%		0.00	0.00	*YLR345W, CAT8, ACH1, PDA1, HXK1, FOX2, SPS19, LAT1, LSC1, PDX1, LSC2, ETR1, PDB1*
cellular carbohydrate metabolic process	8 of 38 genes, 21.1%	215 of 7166 genes, 3.0%		0.00	0.00	*YLR345W, CAT8, MPH2, HXK1, GAL1, SUC2, NTH1, YDR109C*
coenzyme metabolic process	9 of 38 genes, 23.7%	185 of 7166 genes, 2.6%		0.00	0.00	*YLR345W, ACH1, PDA1, HXK1, LAT1, LSC1, LSC2, PDX1, PDB1*
cofactor metabolic process	9 of 38 genes, 23.7%	232 of 7166 genes, 3.2%		0.00	0.00	*YLR345W, ACH1, PDA1, HXK1, LAT1, LSC1, LSC2, PDX1, PDB1*
galactose catabolic process	3 of 38 genes, 7.9%	8 of 7166 genes, 0.1%		0.00	0.00	*GAL1, GAL10, GAL7*
galactose catabolic process via UDP-galactose	3 of 38 genes, 7.9%	5 of 7166 genes, 0.1%		0.00	0.00	*GAL1, GAL10, GAL7*
galactose metabolic process	3 of 38 genes, 7.9%	13 of 7166 genes, 0.2%		0.00	0.00	*GAL1, GAL10, GAL7*
glycolytic process	4 of 38 genes, 10.5%	33 of 7166 genes, 0.5%		0.00	0.00	*YLR345W, PDA1, PDB1, HXK1*
hexose catabolic process	3 of 38 genes, 7.9%	9 of 7166 genes, 0.1%		0.00	0.00	*GAL1, GAL10, GAL7*
hexose metabolic process	7 of 38 genes, 18.4%	88 of 7166 genes, 1.2%		0.00	0.00	*GAL1, YLR345W, GAL10, CAT8, SOR1, GAL7, HXK1*

monocarboxylic acid metabolic process	11 of 38 genes, 28.9%	185 of 7166 genes, 2.6%		0.00	0.00	*YLR345W, CAT8, ACH1, PDA1, HXK1, FOX2, SPS19, LAT1, PDX1, ETR1, PDB1*
monosaccharide metabolic process	8 of 38 genes, 21.1%	99 of 7166 genes, 1.4%		0.00	0.00	*YLR345W, CAT8, GAL7, HXK1, GAL1, GAL10, SOR1, YDR109C*
nucleoside diphosphate phosphorylation	4 of 38 genes, 10.5%	35 of 7166 genes, 0.5%		0.00	0.00	*YLR345W, PDA1, PDB1, HXK1*
organic acid metabolic process	13 of 38 genes, 34.2%	451 of 7166 genes, 6.3%		0.00	0.00	*YLR345W, CAT8, ACH1, PDA1, HXK1, FOX2, SPS19, LAT1, LSC1, PDX1, LSC2, ETR1, PDB1*
oxidation-reduction process	12 of 38 genes, 31.6%	454 of 7166 genes, 6.3%		0.00	0.00	*PDA1, ADH7, ADH6, FOX2, SPS19, SOR1, LSC1, BDH1, LSC2, PST2, ETR 1, PDB1*
oxoacid metabolic process	13 of 38 genes, 34.2%	450 of 7166 genes, 6.3%		0.00	0.00	*YLR345W, CAT8, ACH1, PDA1, HXK1, FOX2, SPS19, LAT1, LSC1, PDX1, LSC2, ETR1, PDB1*
pyruvate metabolic process	6 of 38 genes, 15.8%	52 of 7166 genes, 0.7%		0.00	0.00	*YLR345W, LAT1, PDX1, PDA1, PDB1, HXK1*
small molecule metabolic process	21 of 38 genes, 55.3%	812 of 7166 genes, 11.3%		0.00	0.00	*CAT8, ADH7, GAL1, ADH6, SPS19, LAT1, LSC1, BDH1, ETR1, YDR109C, YLR345W, ACH1, PDA1, GAL7, HXK1, GAL10, FOX2, SOR1, LSC2, PDX1, PDB1*
succinyl-CoA metabolic process	2 of 38 genes, 5.3%	2 of 7166 genes, 0.0%		0.00	0.00	*LSC1, LSC2*
sulfur compound metabolic process	7 of 38 genes, 18.4%	146 of 7166 genes, 2.0%		0.00	0.00	*LAT1, LSC1, ACH1, PDX1, PDA1, LSC2, PDB1*
thioester biosynthetic process	4 of 38 genes, 10.5%	8 of 7166 genes, 0.1%		0.00	0.00	*LAT1, PDX1, PDA1, PDB1*
thioester metabolic process	7 of 38 genes, 18.4%	20 of 7166 genes, 0.3%		0.00	0.00	*LAT1, LSC1, ACH1, PDX1, PDA1, LSC2, PDB1*
transmembrane transport	8 of 19 genes, 42.1%	471 of 7166 genes, 6.6%		0.00	0.00	*GAP1, STL1, TPN1, THI73, UGA4, YOR1, MCH2, PHO84*

Enriched Cellular Component in common between AWRI1499 and CBS2499 - Long response						
Gene Ontology term	**Cluster frequency**	**Genome frequency**	**Statistical significance**	**FDR%**	**False Positives**	**Genes annotated to the term**
mitochondrial pyruvate dehydrogenase complex	4 of 38 genes, 10.5%	7 of 7166 genes, 0.1%		0.00	0.00	*LAT1, PDX1, PDA1, PDB1*
Mitochondrion	17 of 38 genes, 44.7%	1210 of 7166 genes, 16.9%		0.67	0.02	*RCF2, TMA10, LAT1, LSC1, IMO32, ETR1, ACH1, PDA1, FMP37, HXK1, SUC2, CLD1, LEU5, LSC2, PDX1, PST2, PDB1*
oxidoreductase complex	4 of 38 genes, 10.5%	43 of 7166 genes, 0.6%		0.50	0.02	*LAT1, PDX1, PDA1, PDB1*
pyruvate dehydrogenase complex	4 of 38 genes, 10.5%	7 of 7166 genes, 0.1%		0.00	0.00	*LAT1, PDX1, PDA1, PDB1*
Enriched Molecular Function in common between AWRI1499 and CBS2499 - Long response						
Gene Ontology term	**Cluster frequency**	**Genome frequency**	**Statistical significance**	**FDR%**	**False Positives**	**Genes annotated to the term**
active transmembrane transporter activity	5 of 19 genes, 26.3%	170 of 7166 genes, 2.4%		2.00	0.08	*YOR1, UGA4, STL1, MCH2, PHO84*
carbohydrate kinase activity	4 of 38 genes, 10.5%	16 of 7166 genes, 0.2%		0.00	0.00	*GAL1, YLR345W, YDR109C, HXK1*
catalytic activity	26 of 38 genes, 68.4%	2439 of 7166 genes, 34.0%		0.00	0.00	*ADH7, GAL1, ADH6, SPS19, LAT1, NTH1, LSC1, IMO32, BDH1, ETR1, YDR109C, YLR345W, ACH1, PDA1, GAL7, HXK1, SUC2, YLL056C, GAL10, FOX2, CLD1, SOR1, PDX1, LSC2, PST2, PDB1*
oxidoreductase activity	10 of 38 genes, 26.3%	347 of 7166 genes, 4.8%		0.00	0.00	*PDA1, ADH7, ADH6, FOX2, SPS19, SOR1, BDH1, ETR1, PST2, PDB1*
oxidoreductase activity, acting on CH-OH group of donors	5 of 38 genes, 13.2%	84 of 7166 genes, 1.2%		0.00	0.00	*ADH6, FOX2, SOR1, BDH1, ADH7*
oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	5 of 38 genes, 13.2%	78 of 7166 genes, 1.1%		0.00	0.00	*ADH6, FOX2, SOR1, BDH1, ADH7*

pyruvate dehydrogenase (acetyl-transferring) activity	2 of 38 genes, 5.3%	2 of 7166 genes, 0.0%		0.00	0.00	*PDA1, PDB1*
pyruvate dehydrogenase activity	2 of 38 genes, 5.3%	3 of 7166 genes, 0.0%		0.22	0.02	*PDA1, PDB1*
secondary active transmembrane transporter activity	4 of 19 genes, 21.1%	93 of 7166 genes, 1.3%		1.60	0.08	*UGA4, STL1, MCH2, PHO84*
solute: proton symporter activity	3 of 19 genes, 15.8%	37 of 7166 genes, 0.5%		1.33	0.08	*UGA4, STL1, PHO84*
succinate-CoA ligase (ADP-forming) activity	2 of 38 genes, 5.3%	2 of 7166 genes, 0.0%		0.00	0.00	*LSC1, LSC2*
succinate-CoA ligase activity	2 of 38 genes, 5.3%	2 of 7166 genes, 0.0%		0.00	0.00	*LSC1, LSC2*
symporter activity	4 of 19 genes, 21.1%	50 of 7166 genes, 0.7%		0.00	0.00	*UGA4, STL1, MCH2, PHO84*
transmembrane transporter activity	7 of 19 genes, 36.8%	379 of 7166 genes, 5.3%		2.00	0.06	*GAP1, YOR1, UGA4, STL1, TPN1, MCH2, PHO84*
transporter activity	8 of 19 genes, 42.1%	476 of 7166 genes, 6.6%		0.00	0.00	*GAP1, STL1, TPN1, THI73, UGA4, YOR1, MCH2, PHO84*

Figure 1

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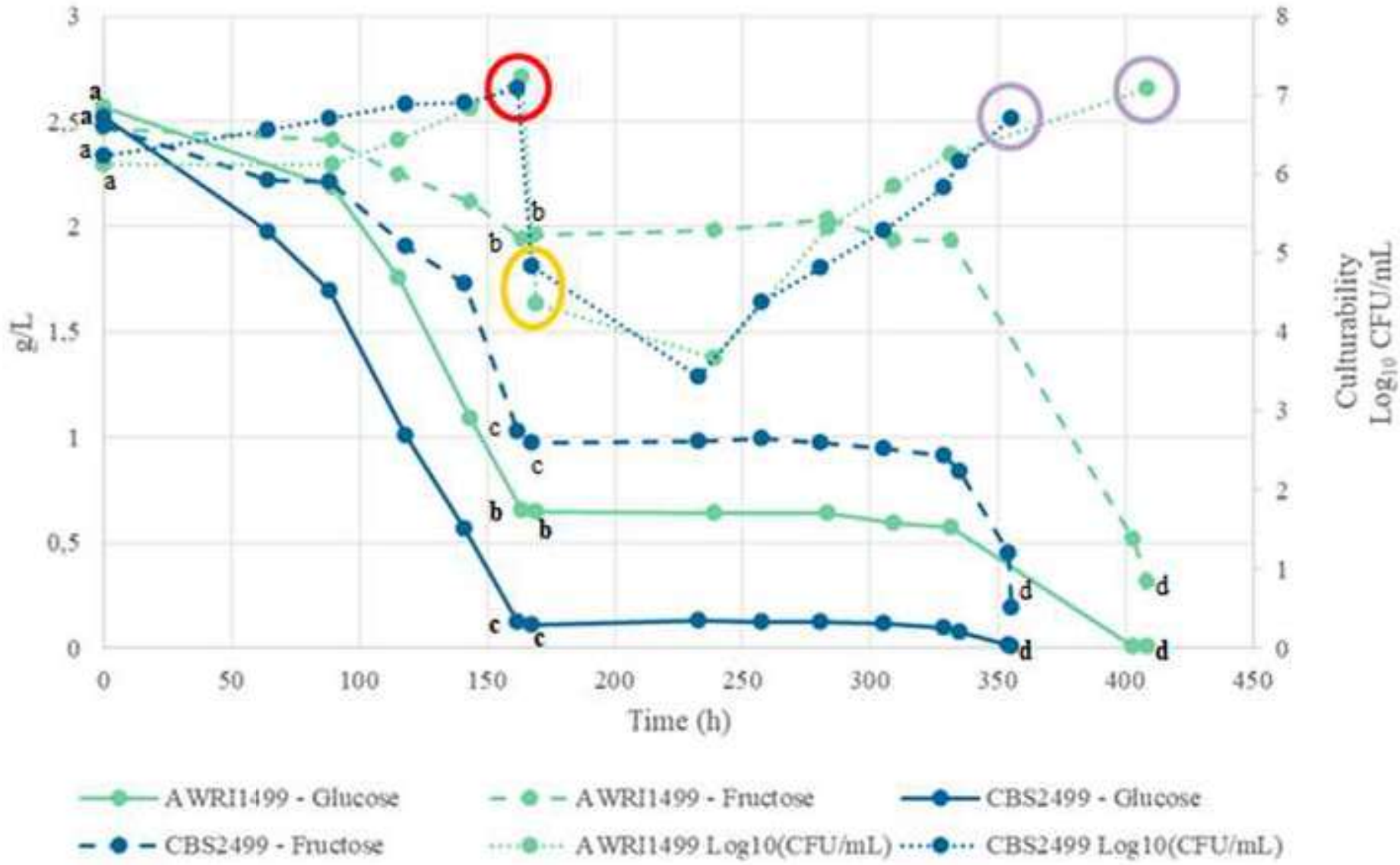

Figure 2

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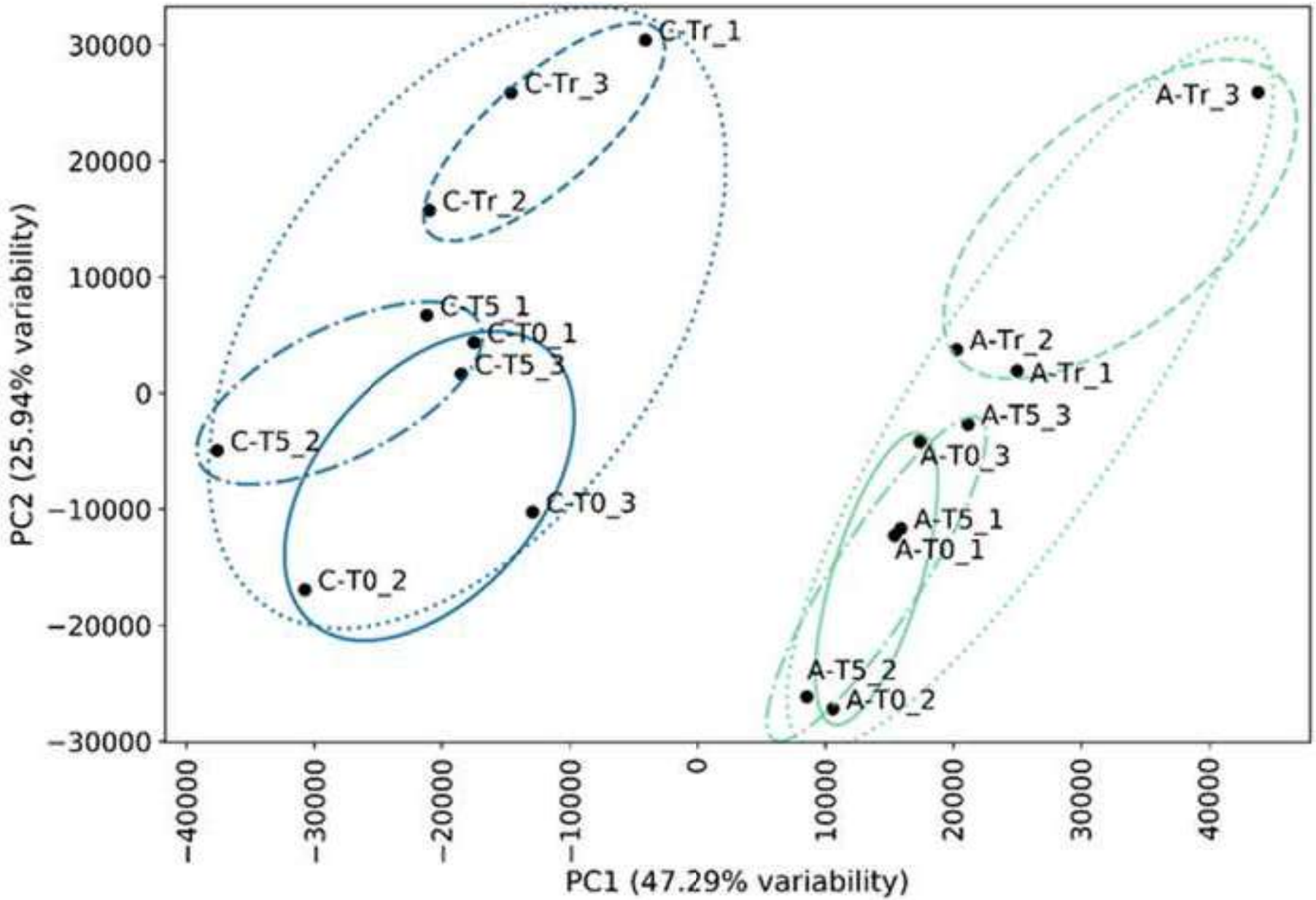

Figure 3

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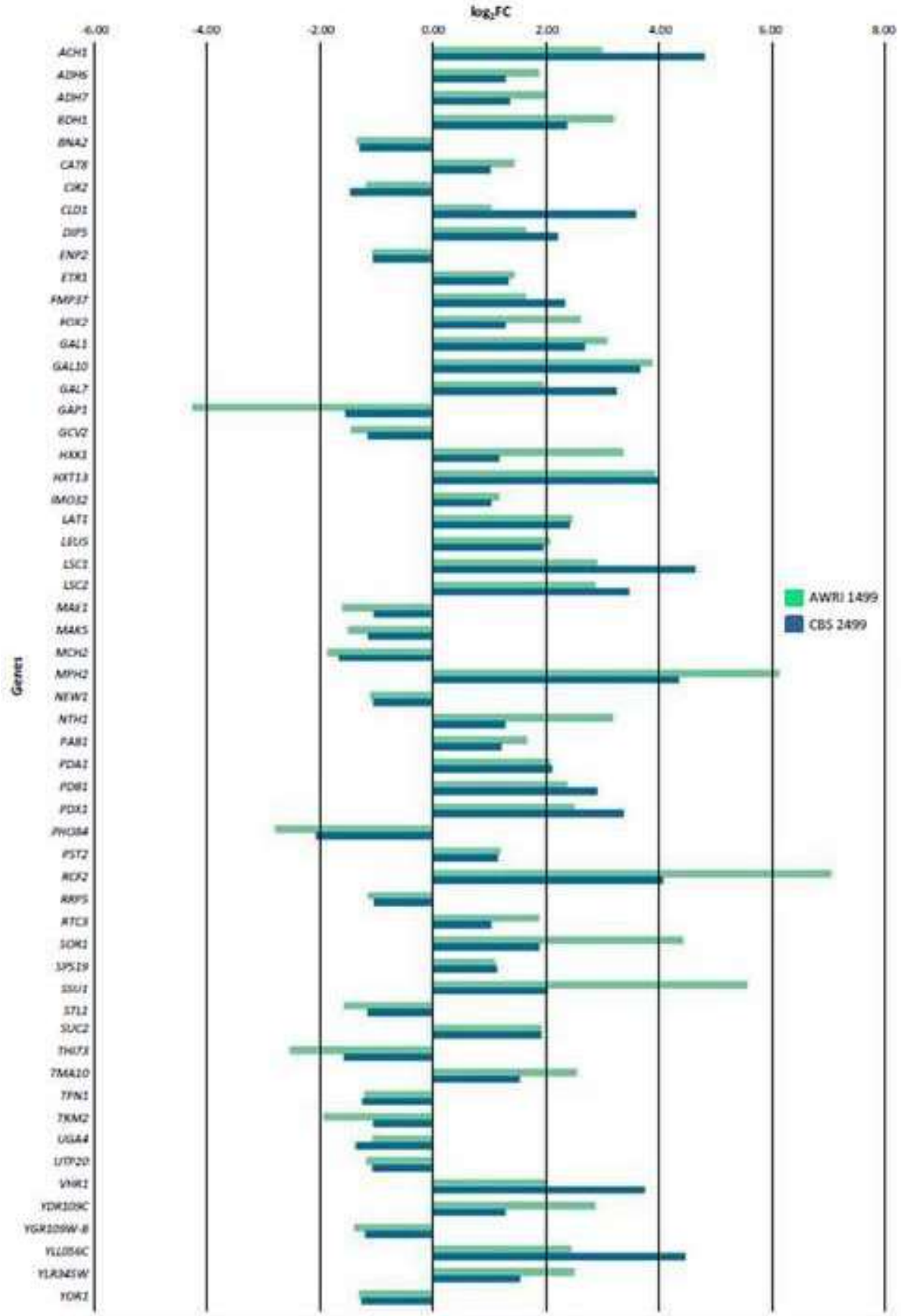


Figure 4

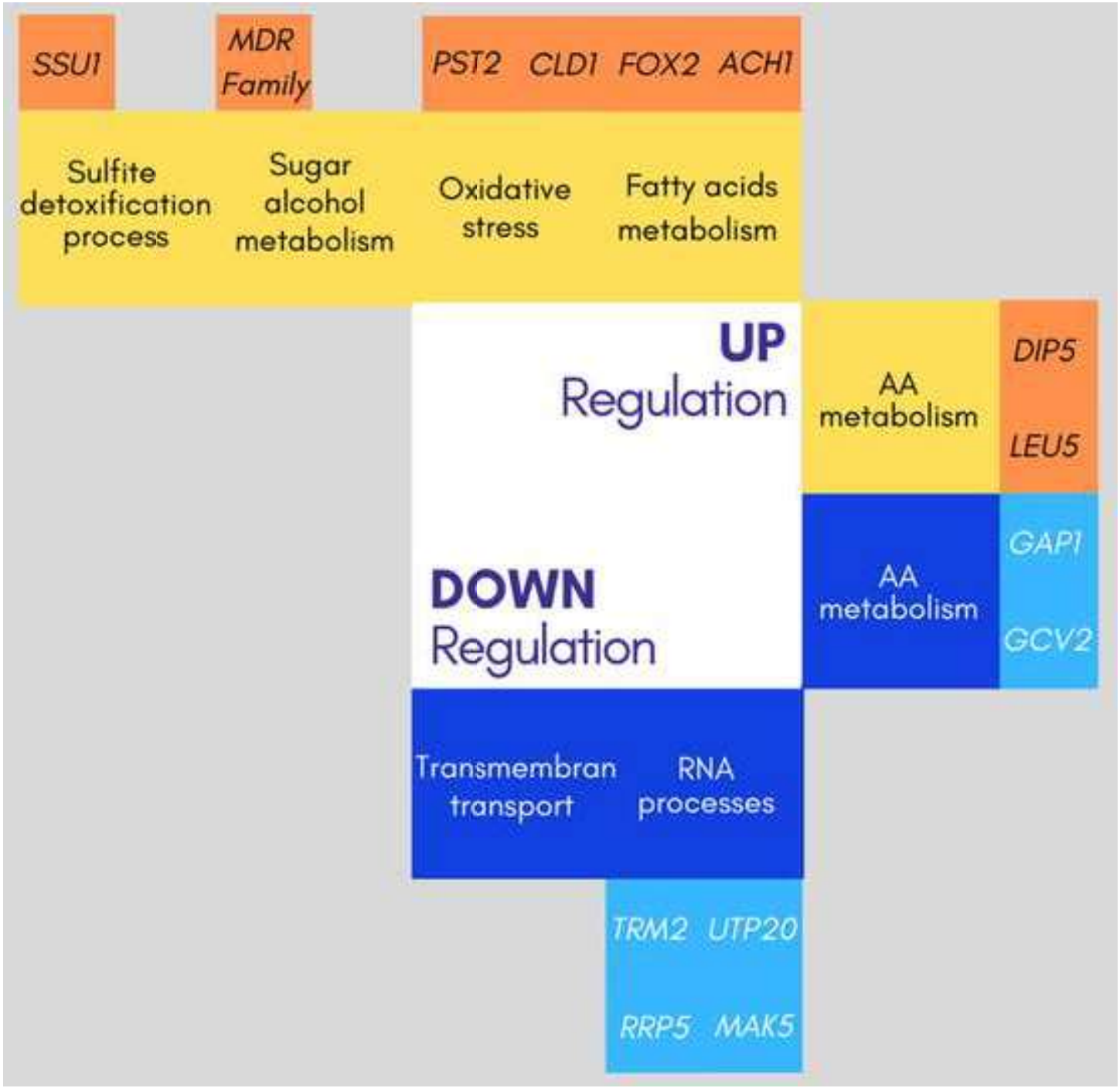
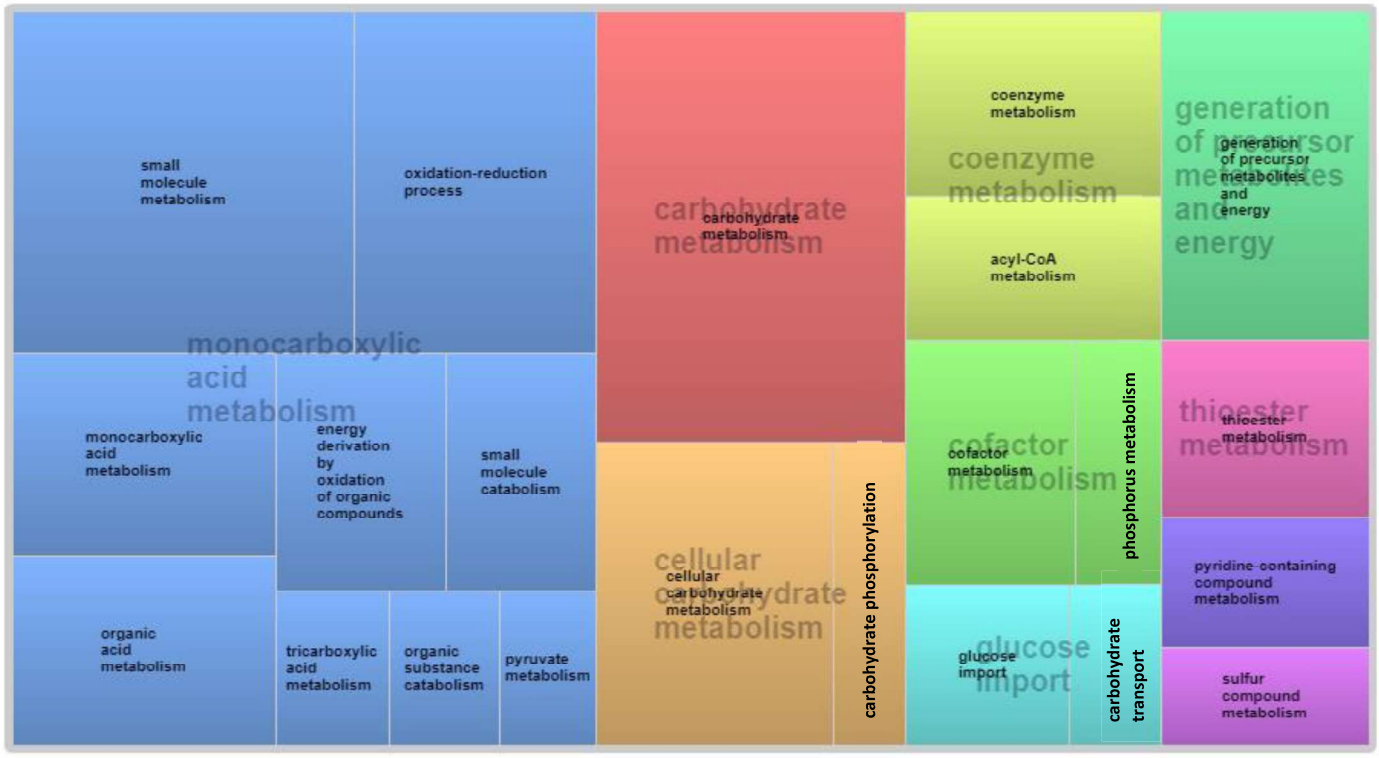
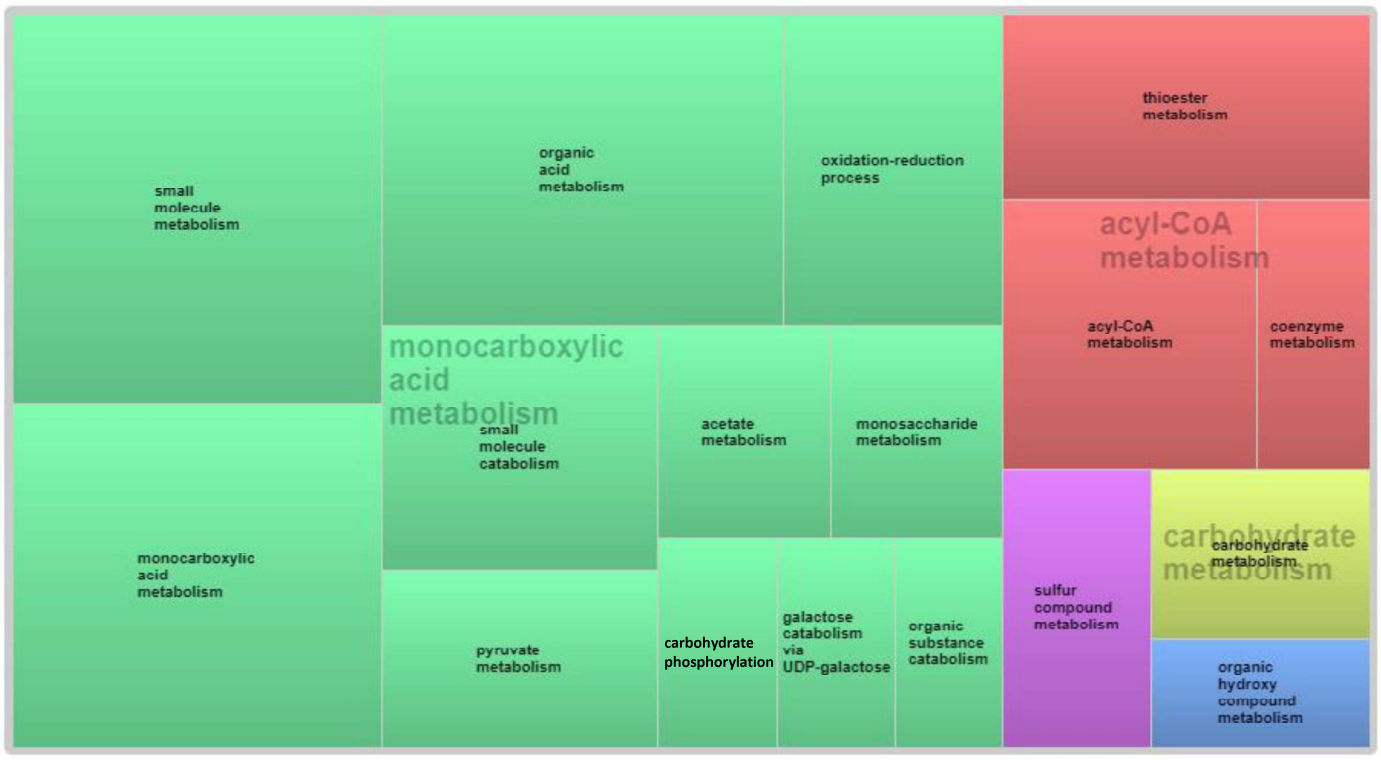


Figure S2

PROCESSES UP - AWRI 1499

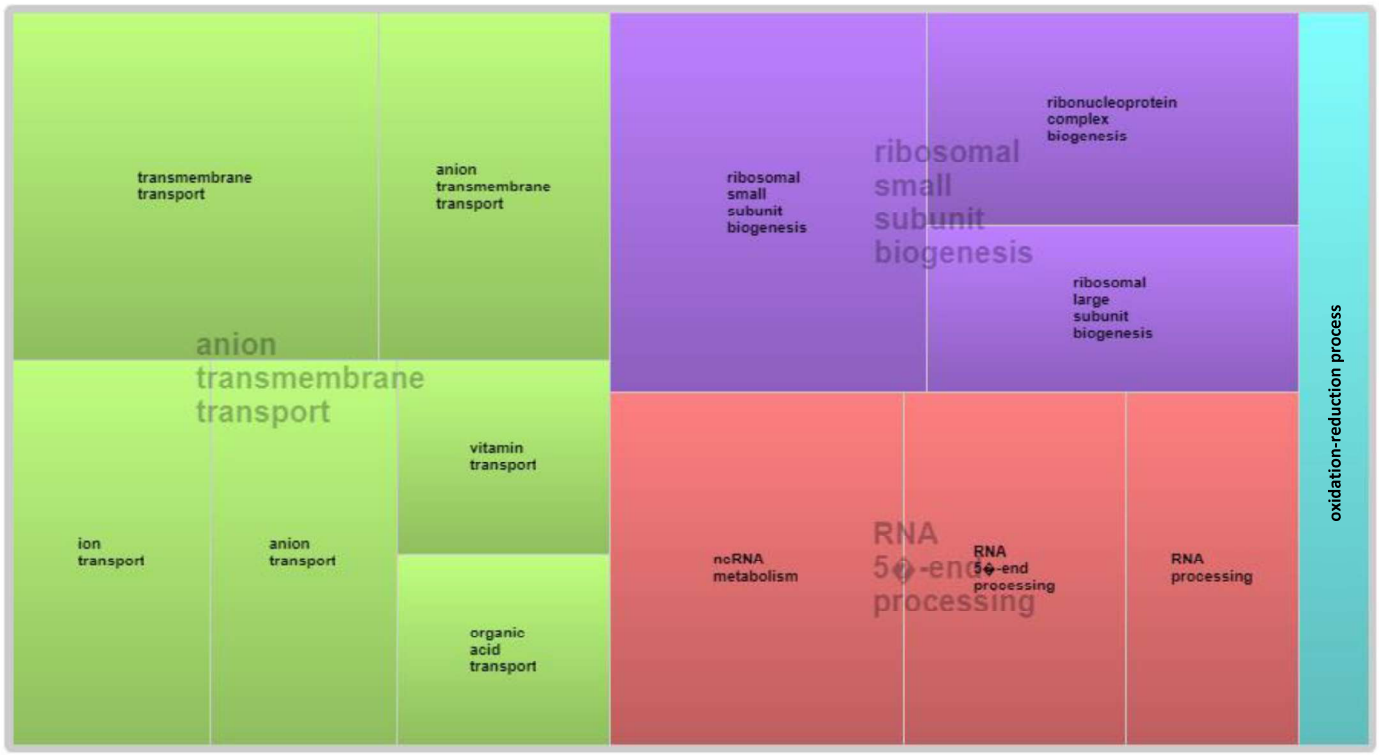


PROCESSES UP – CBS 2499

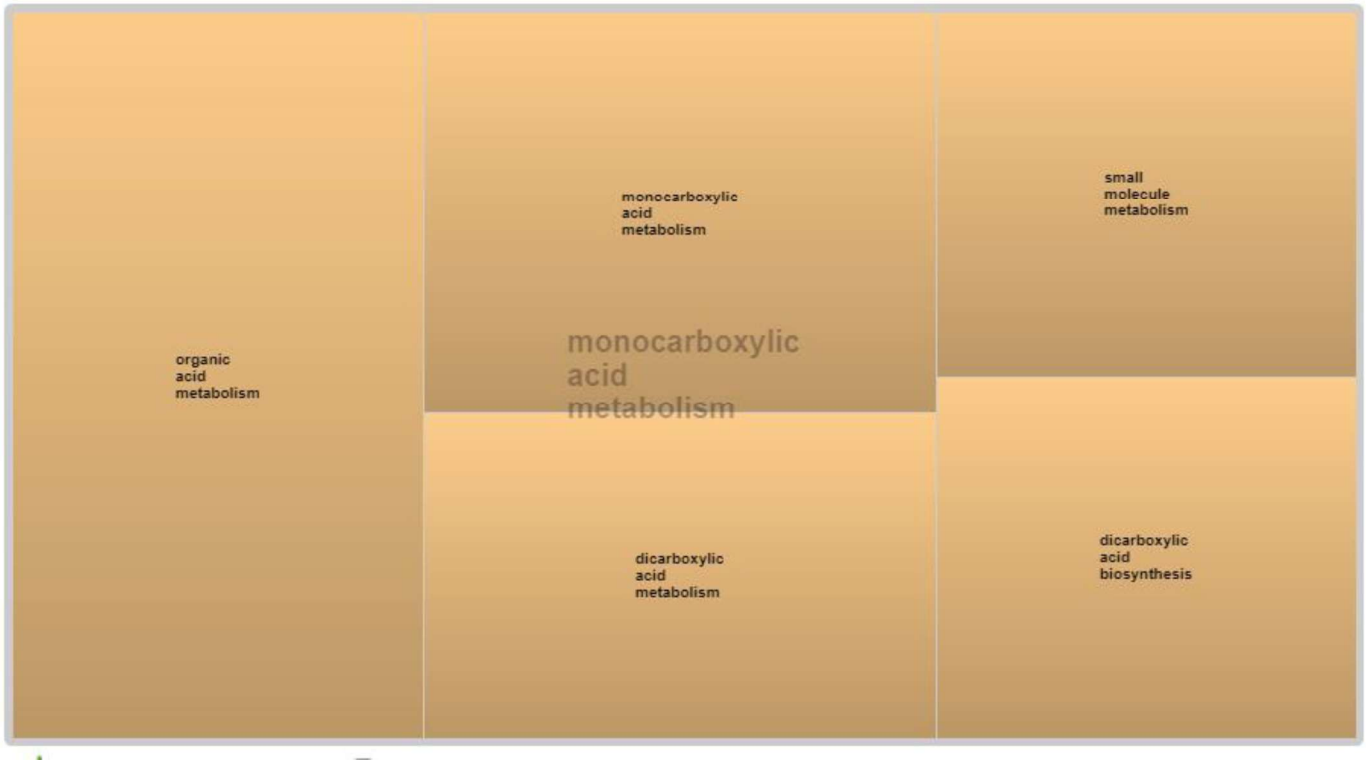


PROCESSES UP – GENERAL RESPONSE – AWRI1499 and CBS2499

PROCESSES DOWN – AWRI 1499

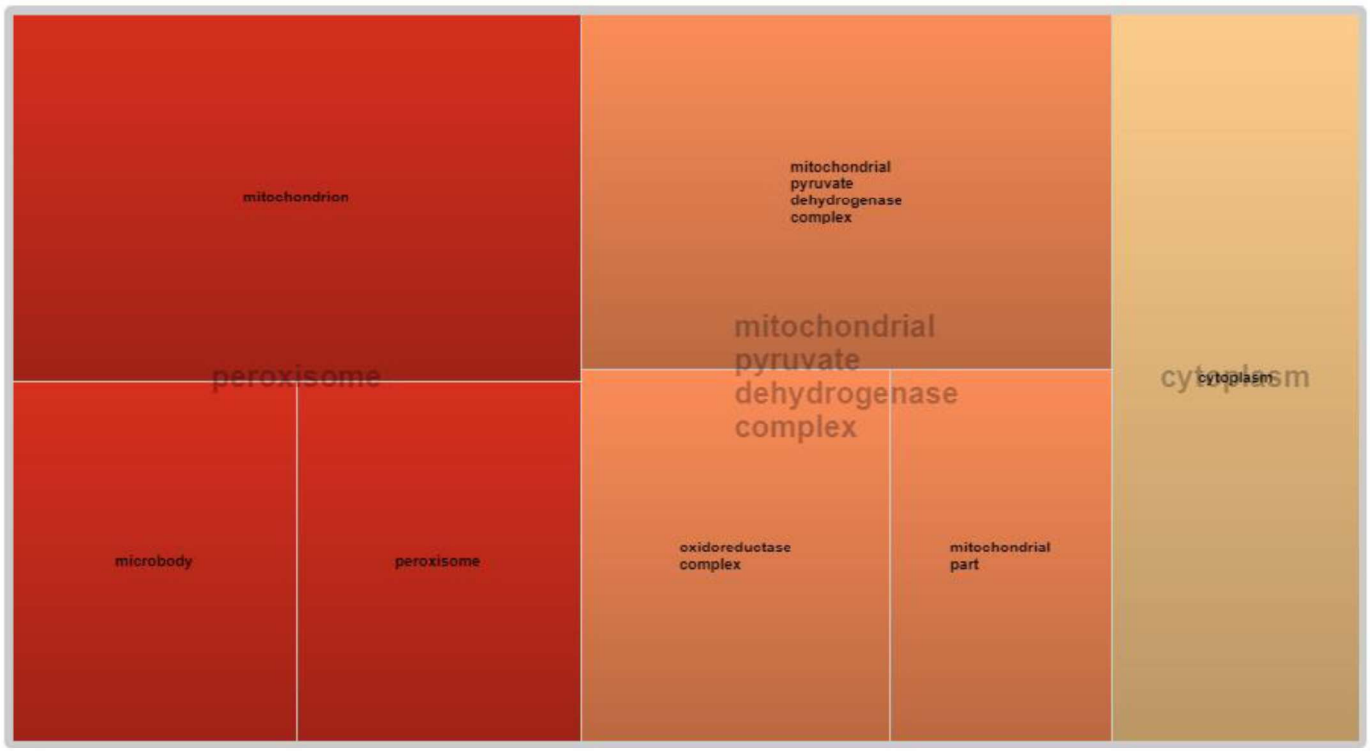


PROCESSES DOWN – CBS 2499



PROCESSES DOWN – GENERAL RESPONSE – AWRI 1499 and CBS 2499

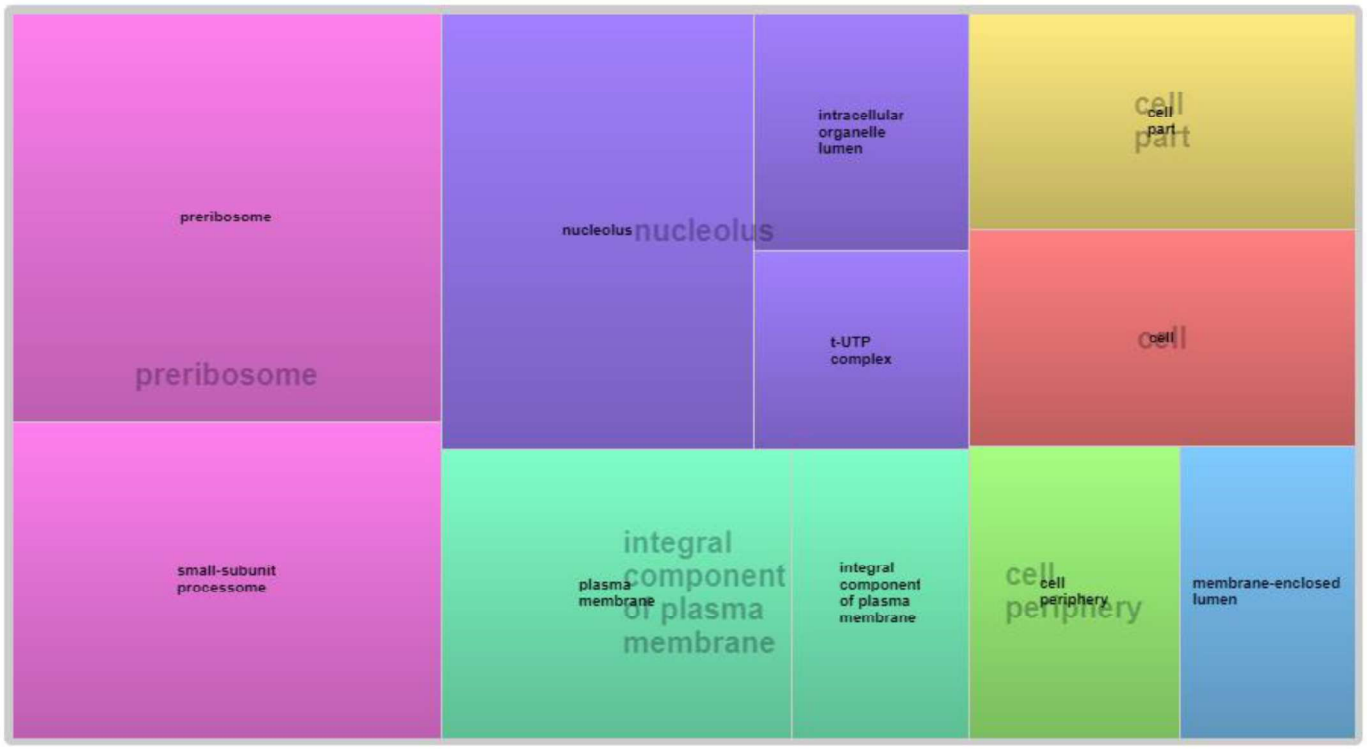
COMPONENT UP – AWRI 1499



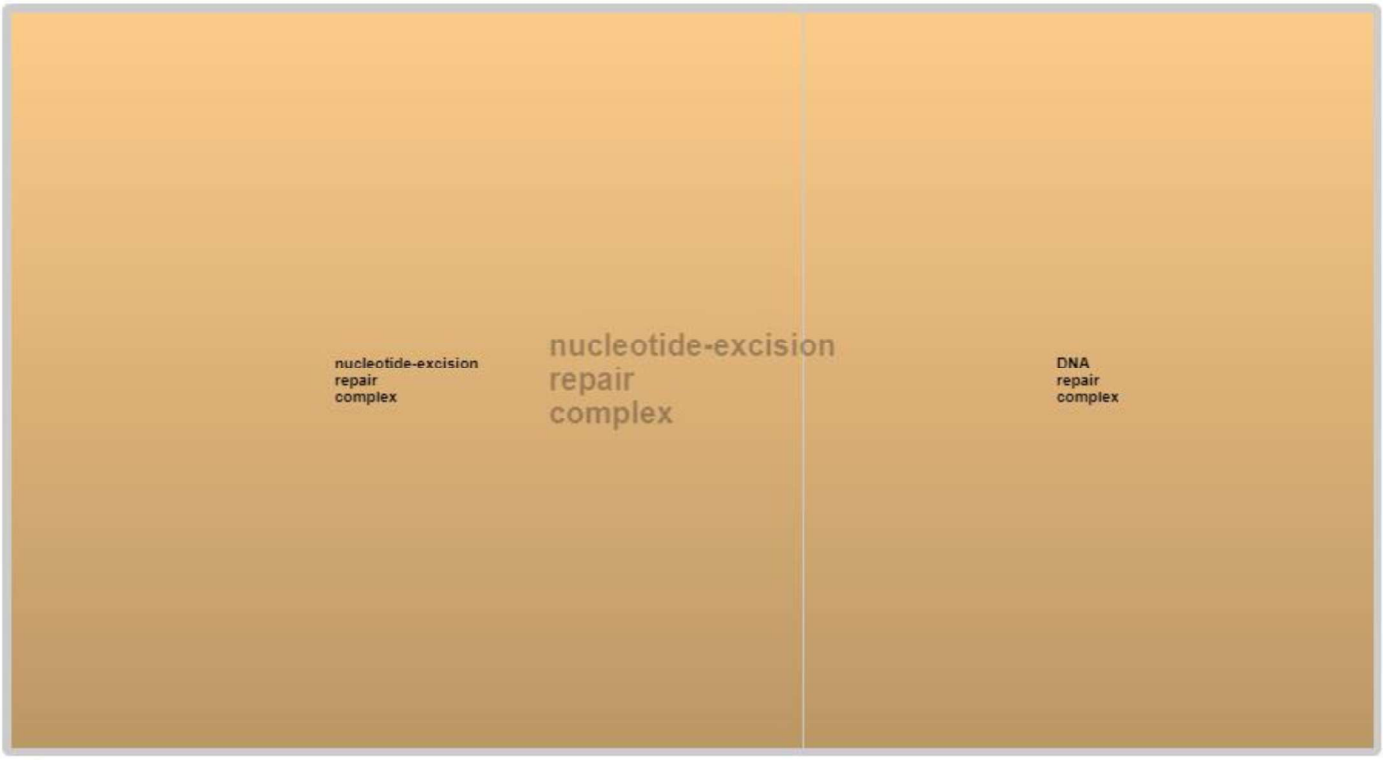
COMPONENT UP – CBS 2499

COMPONENT UP – GENERAL RESPONSE - AWRI 1499 and CBS 2499

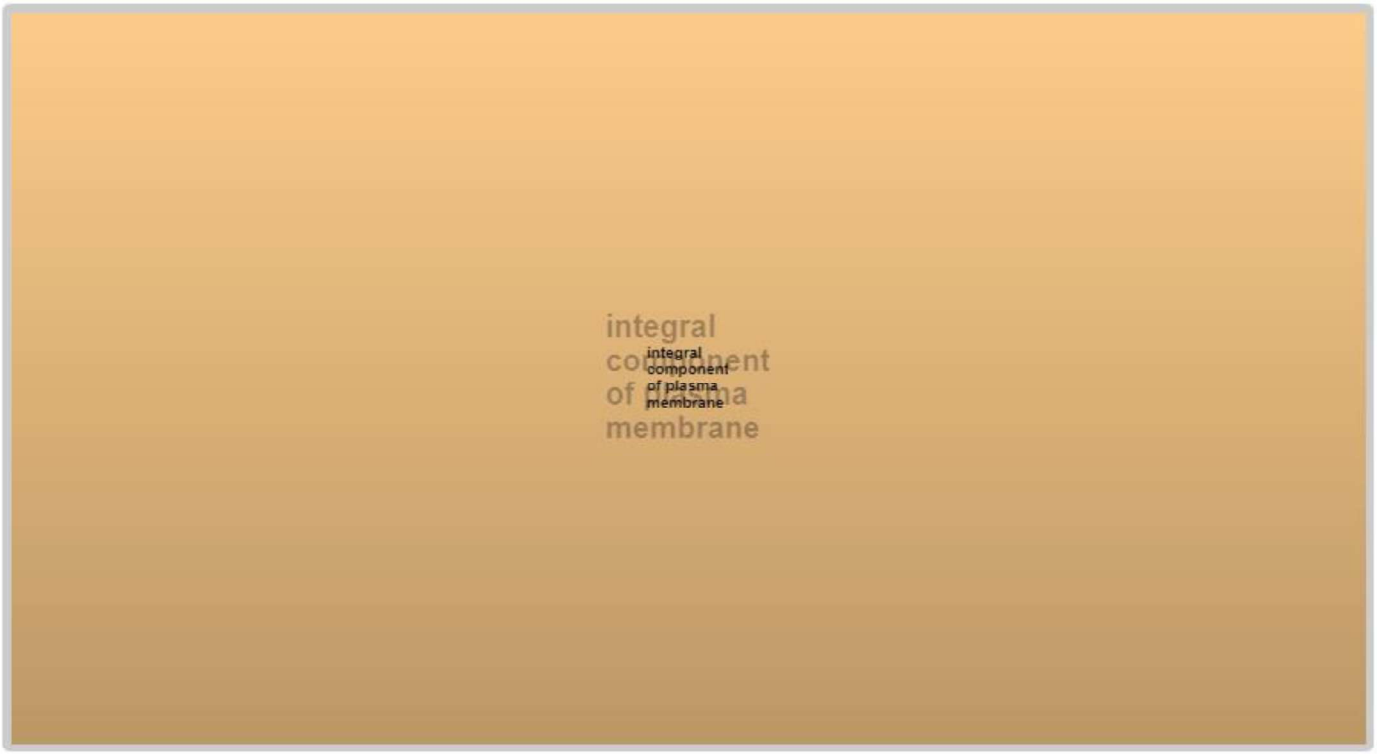
COMPONENT DOWN – AWRI 1499



COMPONENT DOWN – CBS 2499



COMPONENT DOWN – GENERAL RESPONSE – AWRI1499 and CBS 2499

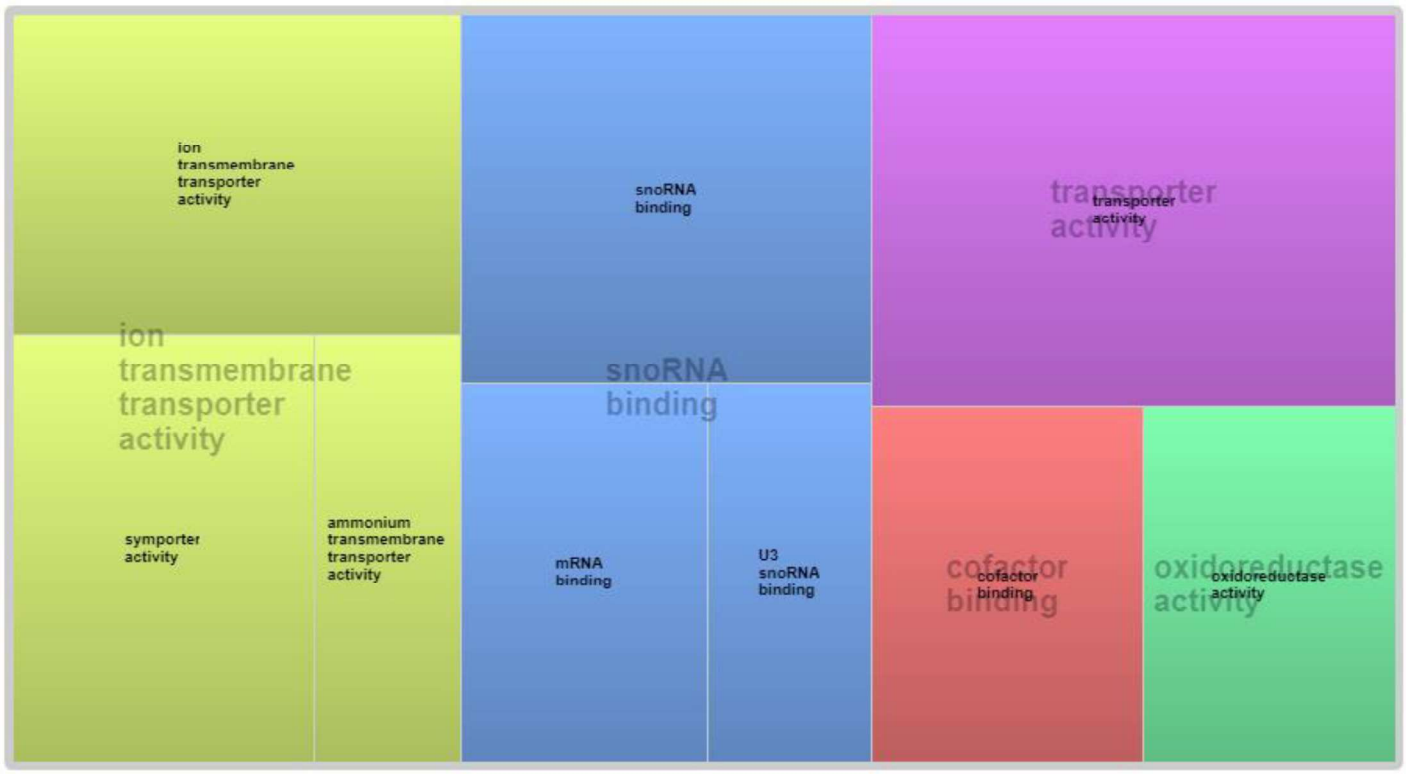


FUNCTION UP - AWRI1499

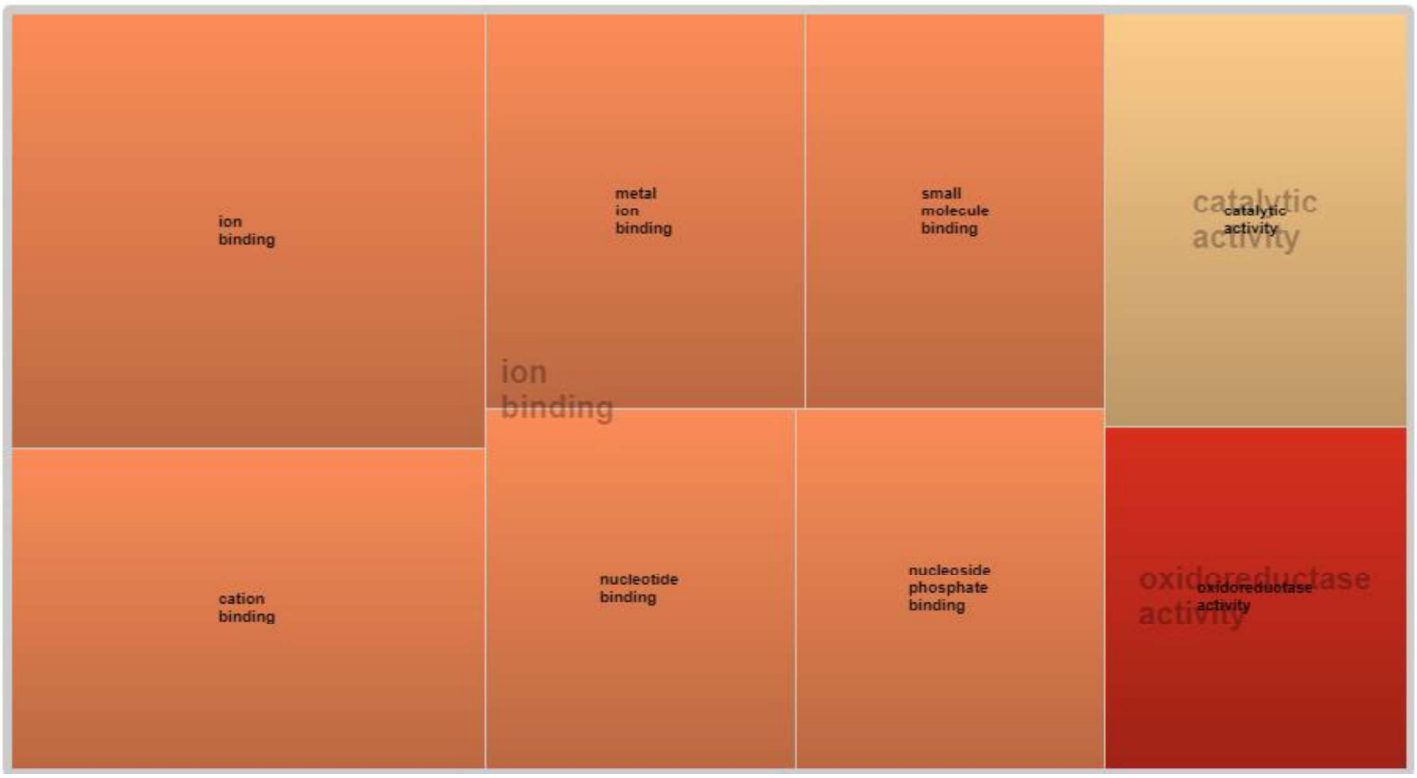
FUNCTION UP – CBS 2499

FUNCTION UP - GENERAL RESPONSE – AWRI 1499 and CBS 2499

FUNCTION – DOWN - AWRI1499



FUNCTION – DOWN – CBS 2499



FUNCTION – DOWN - GENERAL RESPONSE – AWRI 1499 and CBS 2499