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Saccharomyces cerevisiae isolates with extreme hydrogen sulfide production showed different oxidative stress resistances responses during wine fermentation by RNA sequencing analysis

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ABSTRACT

In the wine industry, off-odors occurring during fermentation and bottling caused by hydrogen sulfide (H_2S) produced by *Saccharomyces cerevisiae* are still a major problem. Here, two native *S. cerevisiae* isolates possessing extreme H_2S production capacities were isolated from 166 strains in Chinese vineyards. Significant differences were observed in RNA sequencing analyses between the two strains. The results showed that sulfur metabolism was highly repressed and genes involved in the NADPH-dependent thioredoxin and glutaredoxin oxidoreduction systems were highly expressed in the non-H_2S producer; pathways associated with S-adenosyl methionine, and thiamine syntheses, which were related to oxidative stress resistance in yeast, were upregulated in the high H_2S producer. Oxidative stress tolerance experiment confirmed that the high H_2S producer possessed better oxidative stress tolerance, suggesting a potential connection between H_2S production and cellular stress resistance. Our findings provide insights into the differences in molecular and metabolic mechanisms between non- and high H_2S producers, highlighting the relationships between H_2S production and maintenance of the balance of oxidation potential in *S. cerevisiae* under wine fermentation conditions.

1. Introduction

Hydrogen sulfide (H_2S) produced by *Saccharomyces cerevisiae* has been extensively studied to achieve complete removing during wine fermentation because of undesirable reductive odors. H_2S can also form volatile sulfur compounds, and metal-complexed and oxidized forms of H_2S can be reduced to H_2S during bottling (Ferreira et al., 2018). Most commercial strains used for wine fermentation show excessive H_2S production (Kumar et al., 2010), especially under conditions of high elemental sulfur or low assimilable nitrogen levels leading to an increase of H_2S liberation (Jiranek et al., 1995).

There have been numerous researches over the last decade to identify the genomic determination of *S. cerevisiae* during wine fermentation. It has been found that strain variations in H_2S production are caused by different expression and mutations in genes involve in the sulfate assimilation pathway (SAP). Overexpression of *MET14*, *MET16*, and *SSU1* increase H_2S production (Donalies and Stahl, 2002), whereas mutations in *MET10* and *MET2* reduce H_2S production (Huang et al., 2014; Linderholm et al., 2010). Notably, genes in the SAP exhibit various functions under different genetic backgrounds. For example, *MET17* overexpression does not always reduce H_2S production (Spiropoulos and Bisson, 2000), and *MET10* mutations in high H_2S producers were found to be nonfunctional in a non-H_2S producing strain (Linderholm et al., 2010). Additionally, the amount of H_2S produced by the strain varies greatly in different fermentation conditions (Cordente et al., 2009; Donalies and Stahl, 2002; Spiropoulos and Bisson, 2000), whereas non-H_2S producers are not (Linderholm et al., 2010; Spiropoulos et al., 2000). In our previous experiments, we showed multi-gene participation traits among indigenous yeast in mechanism of H_2S production (Wang et al., 2018), which also indicated metabolic variation caused by genetic background (Kumar et al., 2010; Ugliano et al., 2011) among strains. Therefore, differences in genetic background are the main cause of variations in H_2S production (Mendes-Ferreira et al., 2002, 2009; Nowak et al., 2004; Spiropoulos et al., 2000). Although numerous efforts have been made to uncover the relationships between SAP and H_2S production, the mechanisms under wild strains exhibited different patterns of gene regulation during natural fermentation conditions are still unclear. It is hard to unequivocally uncover the mechanism that leads to H_2S overflow if only SAP variation is considered in high-H_2S producer.

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H_2S has been studied as a physiological mediator with a variety of functions in mammals, including synaptic transmission, vascular tone, angiogenesis, and protecting cells from oxidative stress (Kimura, 2014), and was shown to be not only a byproduct of sulfur metabolism but also have a function of stress resistance in bacteria (Shatalin et al., 2011; Mironov et al., 2017). Although mechanism of H_2S production in yeast has been studied for years, it may inspire us to think that pathways related to cellular roles of H_2S may also affect H_2S liberation.

This study was designed to investigate metabolic difference between H_2S-free strain and H_2S producer and to explain H_2S overflow mechanism in indigenous *S. cerevisiae* strain by enriching the genetic difference of RNA-Seq comparisons. Our findings provide new insights into our understanding of the mechanisms of sulfur regulation and the relationships between cell defense and H_2S production behavior. The results would aid understanding H_2S generation regulation during wine fermentation from the perspective of H_2S formation roles in oxidative stress resistance.

2. Materials and methods

2.1. S. cerevisiae strains and cultivation

The *S. cerevisiae* isolates used in this study were obtained from the College of Enology, Northwest A&F University (China). All 166 strains were isolated from different stages in spontaneous fermentation using various grapes among Chinese vineyards over numerous years. Sequencing of the 26S ribosomal DNA D1/D2 domain and Interdelta sequence analysis were used to identify isolates, as described by Sun et al. (2014). Strains 112y4 and 32y12 used in this study are diploid. YPD synthetic medium (1% yeast extract, 2% peptone, 2% glucose) was used for cultivation of all strains.

2.2. Screening of strains with extreme H_2S production

Overnight culture in 96-well plates was performed, followed by growth on BiGGY agar for 48 h to identify the color of each strain. The strains were classified as white, cream, brownish yellow, light brown, brown, or dark brown on BiGGY agar medium, corresponding to H_2S production from low to high yield. Fermentations were carried out in at least triplicate in 1-L conical flasks containing 600 mL Triple M medium: 100 g/L glucose, 100 g/L fructose, 1 g/L $(NH_4)_3PO_4$, 2 g/L casamino acids, 1 g/L L-proline, 0.8 g/L L-arginine, 0.1 g/L tryptophan, 6 g/L tartaric acid, 3 g/L malic acid, 0.5 g/L citric acid, and 1.7 g/L yeast nitrogen base without amino acids, pH 3.25 (Spiropoulos et al., 2000). Fermentation was performed using an inoculation of 5×10^5 cells/mL at 20 °C with a shaking speed of 150 rpm. H_2S detection were performed using H_2S detector tubes as described by Wang et al. (2018). Samples were collected at 16 h after inoculation and subsequently every 24 h until the end of fermentation. The collected samples were used directly to monitor biomass ($OD_{600\ nm}$) and residual sugar using the 3,5-dinitrosalicylic acid assay (Wang et al., 2018).

2.3. RNA extraction, cDNA library construction, and RNA sequencing

Two strains, i.e., the non-H_2S producer 112y4 and the high H_2S producer 32y12, were used to dissect the physiological mechanisms of H_2S production. Samples for RNA extraction were collected after 40, 88, and 160 h of fermentation with three replications. Sampling and RNA extraction were carried out according to a study by Wang et al. (2018). The RNA samples were then characterized on 1% agarose gels and examined with a spectrophotometer (NanoDrop ND1000, USA). The RNA integrity numbers (> 8.0) of these samples were assessed using a Bioanalyzer (Agilent 2100, USA). The construction of cDNA libraries and sequencing of RNA were performed by the Beijing Genomics Institute (Shenzhen, China) using standard protocols.

2.4. Data processing and analysis of differentially expressed genes (DEGs)

After trimming of adaptor sequences, more than 5.82 million clean reads were obtained from 18 samples, which were filtered from raw reads by removing reads in which the percentage of unknown bases was greater than 10% and low-quality reads with quality values of less than or equal to 5 were greater than 50% in a read. Clean reads were mapped onto the reference sequences for *S. cerevisiae* S288c (http://downloads.yeastgenome.org/sequence/S288C_reference/genome_releases/) using SOAP aligner (Li et al., 2009), with average mapping ratios of 94% for the genome of each sample, among which the unique alignment ratios were higher than 84% (Supplementary File 1-Table 1). Gene expression levels were calculated using the reads per kb per million reads (RPKM) method (Mortazavi et al., 2008). The NOISeq method was used to screen DEGs between the two groups (Tarazona et al., 2011), according to the following default criteria: fold change ≥ 2 and diverge probability ≥ 0.8. Two patterns were used to analyze DEGs between the two strains (Supplementary File 1-Table 2), and DEG data for the nine groups are shown in Supplementary Files 2–10. The results from BLAST sequences were annotated to the Nr database of NCBI according to gene ontology (GO) terms using BLAST2 GO (default parameters). The assembled sequences were annotated to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways also using BLAST. GO enrichment analysis was used to filter the DEGs that corresponded to specific biological functions by applying a strict algorithm. The calculated *p* values were subjected to Bonferroni corrections, and corrected *p* values of 0.05 or less were considered significant.

2.5. Survival tests against reactive oxygen species on solid medium

The two strains stored at −80 °C grown overnight in liquid YPD to an optical density at 600 nm (OD_{600}) of 1, and the cultures were incubated in normal saline for 2 h after diluted to OD_{600} 0.1. Yeast cells were centrifuged and resuspended with distilled water to final OD_{600} = 0.1, then serially diluted 10-fold three times and spotted (4 μL of each spot) onto YPD and Triple M plates containing 3–6 mM H_2O_2. Plates were incubated at 28 °C for 72 h and for plate images analysis.

3. Results

3.1. Phenotypic assays for H_2S production by native yeasts

To select two strains with extreme phenotypes among yeasts, we first used BiGGY agar to quantify the potential production of H_2S. 166 strains were separated into six levels (I–VI) according to the colony color (Fig. 1A), with 3, 6, 20, 20, 47, and 70 strains of type I, II, III, IV, V, and VI colonies, respectively (Supplementary File 1-Table 3). Strains with brownish yellow and dark color accounted for 90% of all strains, whereas strains with white or cream color accounted for only 10% of strains. To obtain strains with extreme H_2S production, strains with colors I (using three strains) and II–VI (using two strains each for colors II–V and three strains for color VI) were chosen for H_2S rescreening in Triple M condition. As expected, strains with color I showed lower H_2S production during fermentation; in particular, strain 112y4 showed no detectable H_2S during fermentation (Supplementary File 1-Table 4). H_2S production in strains with colors II–V were quantified between 15 and 400 ppm and strain 32y12 of color VI showed the highest H_2S production, with an H_2S yield of 2301.3 ppm. All strains, except for 112y4, were H_2S producers, with H_2S production becoming detectable at 40 h, peaking at 64–80 h, and then becoming undetectable at around 160 h (Fig. 1B). With regard to fermentation traits, the results showed strain-dependent variations. All fermentations were completed within 280 h, except for that of strains 112y4 and 112y14. Lower OD_{600} values were observed for high producers 32y15, 32y13, 32y9, and 32y12 (Supplementary File 1-Fig. 1), which were contradictory to the findings for UCD522, a widely studied high H_2S producer, which was shown to

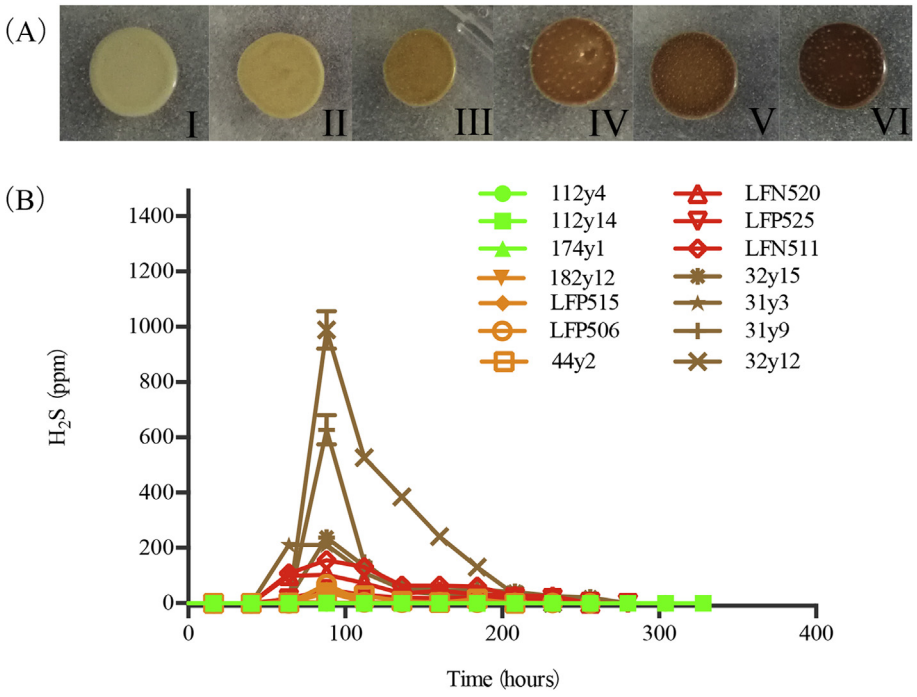

Fig. 1. Colony colors in BiGGY agar (A) and data points for H_2S production for each strain every 24 h with SD (B). 166 strains in this research were separated into six levels, I-VI represent white, cream, brownish yellow, light brown, brown and dark brown, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

have a higher OD_{600} than lower producer (Mendes-Ferreira et al., 2009). These results indicated that the relationship between H_2S yield and biomass was not relevant.

3.2. RNA-sequencing and data analysis

To elucidate the molecular mechanisms associated with H_2S production, we performed a RNA-seq analysis of strains 112y4 and 32y12, which exhibited extreme H_2S production capabilities. The DEGs of both strains were analyzed using two comparison patterns (comparison pattern A compared DEGs of the same stages between the two strains; comparison pattern B compared DEGs of the same strain between the different stages, explained in Supplementary File 1-Table 2) in three stages during wine fermentation. Strain comparison (pattern A) revealed 507, 1193, and 754 DEGs in three stages (Fig. 2A), suggested a distinct mechanism of gene expression regulation among three stages between the two strain. From time-points comparison (pattern B), we found that more DEGs were highly expressed in strain 32y12, particularly at 88 h of fermentation, and that most DEGs in strain 112y4 were downregulated during this stage but upregulated during stage III (Fig. 2B). To further analyze the distribution of DEGs in metabolic pathways between the two strains, GO analysis and KEGG pathway enrichment were used. GO functional enrichment analysis revealed significant enrichment of DEGs with oxidoreductase activity (Fig. 3 and Supplementary Table S1), including enriched carbohydrate metabolism (carboxylic acid, oxoacid, organic acid) and glucose and hexose metabolism (Supplementary Table S1). Additionally, sulfur compound, alpha-amino acid, and amino sugar metabolism were enriched during stage I; ribosome biogenesis, gene expression and translation, thiamine-containing compound metabolic metabolism, pyrimidine-containing compound metabolism, and cellular aromatic compound metabolism were enriched during stage II (Supplementary Table S1). KEGG metabolic pathway analysis showed fewer enriched pathways with corrected *p*-values less than 0.05; however, the enriched pathways were consistent with the GO analysis (Supplementary Table S1). Therefore, we further evaluated these pathways and their relationships with oxidoreductase and sulfur compound metabolism.

3.2.1. Divergence of oxidoreductase activity regulation in the two extreme H_2S production strains in wine fermentation stress

Oxidoreductase activity differed dramatically between the two strains according to GO functional analysis. The DEGs related to oxidoreductase activity (Fig. 3) were listed in Table 1.

In strain 112y4, these highly expressed genes were mainly enriched in oxidative stress response, including *CTA1*, *CTT1*, and *SOD2*, encoding catalase T and mitochondrial matrix superoxide dismutases, which are important enzymatic defense systems to maintain the cellular redox state (Jamieson, 1998). The remaining set of DEGs overexpressed in oxidative stress response was associated with thioredoxin and glutaredoxin oxidoreduction systems, including *TSA2*, *AHP1*, *SCO2*, and *PRX1*, encoding thioredoxin-dependent peroxiredoxins; *MXR1/2*, encoding peptide-methionine (R)−S-oxide reductase (Boschi-Muller et al., 2008); *RNR2*, encoding a small subunit of ribonucleotide reductase, a thioredoxin-specific reductase (An et al., 2006); and *GTT1*, *GTO1*, and *ECM4*, encoding enzymes involved in the glutaredoxin oxidoreduction system. This suggested that in strain 112y4, oxidative stress response systems were more susceptible to the stress caused by wine fermentation conditions. However, in strain 32y12, DEGs involved in oxidoreductase activity were enriched in cellular amino acid biosynthesis and were almost all associated with methionine and lysine (Table 1).

Notably, the thioredoxin and glutaredoxin oxidoreduction systems as well as the syntheses of methionine and lysine all require NADPH (Celton et al., 2012; Ljungdahl and Daignan-Fornier, 2012), which is also directly associated with H_2S production as a cofactor of sulfite reductase (Celton et al., 2012). Therefore, we analyzed DEGs associated with regeneration and consumption of NADPH (Table 2). Cytosolic NADPH is regenerated by glucose-6-phosphate dehydrogenase (Zwf1p), 6-phosphogluconate dehydrogenase (Gnd1/2p), isocitrate dehydrogenase isozyme 2 (Idp2p), and cytosolic acetaldehyde dehydrogenase encoded by *ALD6*. *GND2* was highly expressed in strain 32y12 during stage I; *ZWF1*, *GND2*, and *IDP2* were overexpressed in strain 112y4; *ALD6* was upregulated in strain 32y12 during stage II; and *GND2* and *IDP2* were highly expressed in strain 112y4 during the final stage. These results suggested that the NADPH regeneration pathway was activated during the early stages of fermentation in strain 32y12 and during later stages of fermentation in strain 112y4. DEGs involved in NADPH consumption (e.g., *ADH7*, *ERG5*, *ETR1*, *GDH1*, *GLN1*, *HMX1*,

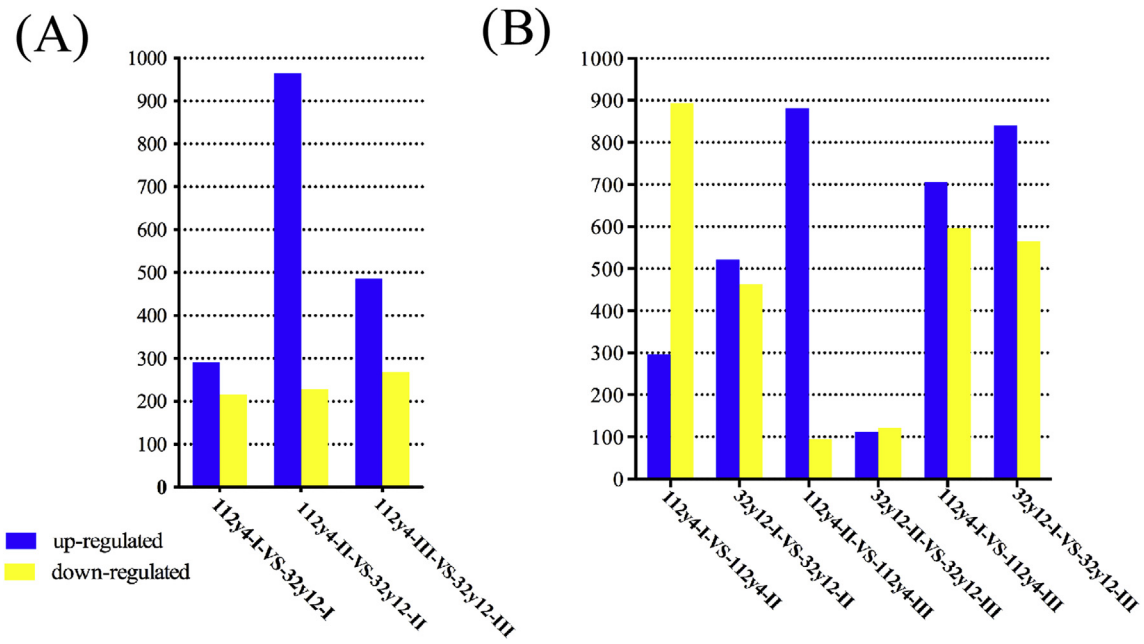

Fig. 2. Chart of groups of differentially expressed genes using two comparison patterns. (A): Numbers of DEGs in comparison of gene transcripts in the three stages between the two strains, respectively (comparison pattern A). (B): Numbers of DEGs in comparison of gene transcripts between different stages of one strain (comparison pattern B). The numbers I, II, and III next to the strain names indicate fermentation times of 40, 88, and 160 h, respectively. In the group pairwise comparison, X-vs-Y, X is considered as the control group, and Y is considered as the treatment group. "Up-regulated" indicated the gene expression of the treatment group was upregulated than the control group; "Down-regulated" indicated the gene expression of the treatment group was downregulated than the control group.

ILV5, *MET16*, and *OYE3*) were highly expressed in strain 112y4 during stage I; DEGs associated with lysine, S-containing amino acids, glutamic acid, fatty acids, and ergosterol synthesis were highly expressed in strain 32y12 during stage II. These data could indicate that NADPH accumulated at the beginning of H_2S release and was consumed mainly for the synthesis of amino acids in strain 32y12. However, strain 112y4 induced NADPH regeneration in stage II to mediate the oxidative stress response.

3.2.2. *Differences in transcriptional profiles of genes related to SAP*

As expected, DEGs of SAP had different expression patterns in most comparisons (Fig. 4 and Supplementary Files 2–10) because H_2S production was most vulnerable to gene expression and mutations in SAP. Most genes involved in sulfate assimilation and sulfide discharge in strain 112y4 were upregulated in stage I (e.g., upregulation of *SUL2* and *MET16* involved in sulfate assimilation and upregulation of *SSU1* involved in sulfide discharge by 2.14-, 2.8-, and 9.7-fold, respectively, compared with strain 32y12), whereas there was a more significant decrease in stages II and III compared with that in stage I (Fig. 4). These results suggested that sulfate was discharged as SO_2 in strain 112y4 in stage I and that SAP was downregulated during the remaining time of fermentation. In contrast, in strain 32y12, the set of genes related to SAM synthesis (e.g., *SUL1*, *MET14*, *MET16*, *MET10*, and *MET12*) was upregulated during stages II and III, indicating a demand for SAM in strain 32y12. Additionally, serine synthesis pathway genes (*SER1*, *SER2*, *SER3*, and *SER33*, 2–3.8-fold) and *O*-acetyl homoserine-related genes (*HOM3*, 2.1-fold) were significantly upregulated in strain 112y4 at 40 h. The relationship between serine anabolism and H_2S production is still unclear. However, not all genes in SAP showed significant discrepancies between the two strains, such as genes involved in the synthesis of cysteine, glutathione, and multifunctional factors, except for Cbf1p (Supplementary Files 2–9).

3.2.3. *Strain 32y12 exhibited an enhanced thiamine demand during wine fermentation*

The de novo thiamine synthesis pathway was one of the most

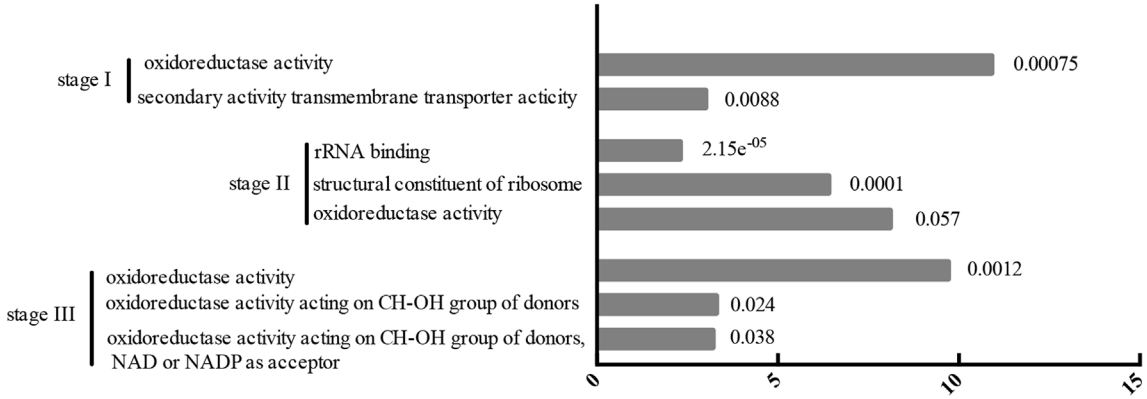

Fig. 3. GO functional enrichment analysis of DEGs between strains 112y4 and 32y12 in three stages. Cross bands represent DEG percentages, and corrected *p* values of less than 0.05 are indicated for each category.

Table 1
Gene functional category from the DEGs (comparison A) of oxidoreductase activity on enriched GO analysis.

	Stage I (40 h)	Stage II (88 h)	Stage III (160 h)
Gene functional category	DEGs higher in strain 112y4		
oxidative stress response	*ECM4 CTA1 MXR1 SOD2 TSA2 HMX1 OYE3 YHB1*	*AHP1 CTT1 ECM4 GRX2 GTO1 GTT1 GRE3 MXR2 PRX1 SCO2 SOD2 TSA2 YHB1*	*AHP1 CTT1 GRE3 GRX2 GRX4 GTT1 MXR2 PRX1 SOD2 SRX1 RNR2 YHB1*
cellular amino acid biosynthetic process	*GDH1 ILV5 LYS1 MET16 PUT1 SER3 SER33 MAE1 GCV1 GCV2*	*ALD3 GDH2 GDH3 PUT1*	*ARG5,6 GCV1 GCV2 GCV3 GDH1 GDH3 SER3*
aerobic respiration	*COX12 COX13 COX15 COX7 COX5B QCR10 QCR6*	*COX12 COX13 COX15 COX4 COX5B COX6 COX7 QCR6 QCR7*	*COX7*
aldehyde metabolic process	*AAD3 AAD4 AAD6*	*AAD3 AAD4 AAD6 YJR096W*	*AAD3 AAD6 YJR096W*
TCA (tricarboxylic acid cycle)		*KGD1 SDH2 SDH4 YJL045W*	*KGD1 SDH4 IDH2 YJL045W*
alcohol metabolic process	*ADH3 ADH7*	*ADH7*	*ADH1 ADH2 ADH7 BDH1*
NADH oxidation	*NDE2*	*NDE2 GUT2*	*GUT2 NDE2*
NAD biosynthetic process			*BNA4*
NADPH regeneration		*GND2 IDP2 ZWF1*	*GND2 IDP2*
formate catabolic process	*FRD1*		*FED1*
gluconeogenesis		*MDH2 MDH3 TDH1*	*MDH2*
fatty acid metabolism	*ETR1*	*POX2 SPS19*	*SPS19*
ergosterol biosynthesis	*ERG5*	*MCR1*	
other biological process	*CAT5 TDH1 AST1 MAN2 XYL2 TDA3 YKL071W*	*EMI5 MTC3 SHH3 YKL107W YPR127W YPR172W*	*ARI1 AIM33 SHH3 YKL107W YPR127W*
	DEGs higher in strain 32y12		
cellular amino acid biosynthesis	*MET13*	*LYS1 LYS12 LYS2 LYS9 MET10 MET12 PRO2 GCV2*	*LYS1 LYS2 LYS9 MET1 MET10 MET16 MET8*
oxidative stress response		*GRX3 GRX7 OYE2*	
NAD biosynthetic process	*BNA4*		
NADH oxidation		*ADH5 ADH6 NDE1 GPD2*	*ADH5*
NADPH regeneration	*GND2*		
acetate biosynthetic process	*ALD5 ALD6*		*ALD2 ALD3*
ergosterol biosynthesis		*ERG27*	*ERG27*
antibiotics and metal resistance	*ARR2*	*ARR2 TYW1*	*ARR2*
aerobic respiration	*CBR1*	*CBR1 OAR1*	*CBR1 OAR1*
protein folding	*EUG1 MPD1*		*ERO1*
fatty acid metabolism	*FAS1 POX1*	*FAS1 FAS2*	*FAS2 OLE1*
cellular aldehyde metabolic process	*YPL088W*	*YPL088W YGL039W*	*YGL039W*
gluconeogenesis		*TDH3*	
folic acid-containing compound biosynthetic process		*MTD1 MIS1*	
GTP biosynthetic process		*IMD2 IMD3 IMD4*	
mRNA binding		*MTR4 DUS3*	*MTR4*
other biological process	*SHH3 YCR102C SUR2 FDH1 HEM13*	*DSF1 MSC7 ARO1 LIA1 YGR017W YJR111C YKL071W*	*DSF1 RFS1 ARA2 AIM17 YGR017W*

Gene function involved in GO process was obtained from http://www.yeastgenome.org.

significantly altered pathways between the two strains. As shown in Fig. 5, almost all genes involved in thiamine synthesis, transport, and regulation were upregulated by at least 2-fold in strain 32y12. In addition, genes associated with the biosynthesis of pyridoxal 5′-phosphate (PLP) were also up-regulated in strain 32y12. *THI5* family genes showed prominent changes (2–238-fold), whereas *THI13* was significantly downregulated. Consistent with this, regulators of this pathway (*THI2*, *THI3*, and *PDC2*) were also highly expressed in this strain (2–2.4-fold change). The expression of genes encoding enzymes involved in thiamin biosynthesis was repressed by exogenous thiamin and induced in the absence of thiamin by an autoregulation complex composed of Thi2p, Thi3p, and Pdc2p (Nosaka et al., 2005; Nosaka, 2006). Accordingly, the dramatic increase in thiamine synthesis during the fermentation process suggested a great aggregated demand in strain 32y12. The active form of thiamine, thiaminediphosphate (TDP), is a cofactor for many enzymes, including pyruvate dehydrogenase *(PDA1)*, ketoglutarate dehydrogenase (*KGD1*), pyruvate decarboxylase (*PDC1/5/6*), and transketolase (*TKL1/2*), and two genes (*ILV2* and *ARP10*) related to isoleucine or valine synthesis and the Ehrlich pathway (Mojzita and Hohmann, 2006). As shown in Fig. 5, *PDA1*, *ILV2*, *PDC1*, and *PDC6* expression levels did not change, whereas *PDC5* and *TKL1* were highly expressed in strain 32y12. Overall, there were no significant differences among the conventional pathways needing TDP as cofactor. Therefore, excessive production of TDP may have functions in pathways other than the conventional pathway.

Moreover, most genes involved in PLP synthesis were highly expressed in strain 32y12 (Fig. 5). In particular, PLP is a coenzyme for cystathionine β-synthase, cystathionine γ-lyase, cystathionine γ-synthase, and cystathionine β-lyase (Cys4p, Cys3p, Str2p, and Str3p, respectively, in yeast) in cysteine synthesis (Messerschmidt et al., 2003; Aitken and Kirsch, 2005), however, the effects of intracellular PLP on H_2S production in yeast are still unclear.

3.3. The sensitivities of the two strains to oxidative stress

Metabolism of oxidative defense factors was differentially regulated in the two strains, which may be related to H_2S production capabilities. We therefore examined the sensitivities of the two strains to the

Table 2
DEGs (comparison A) related in NADPH regeneration and consumption.

		Stage I	Stage II	Stage III
Genes	Gene Annotation	Fold changes		
NADPH REGENERATION				
GND2	6-phosphogluconate dehydrogenase	2.4	6.0	10.1
ZWF1	Glucose-6-phosphate dehydrogenase		2.0	
ALD6	Cytosolic aldehyde dehydrogenase		2.1	
IDP2	Isocitrate dehydrogenase		2.8	6.1
NADPH CONSUMING				
Aminoacids synthesis				
ARG5,6	Acetyl glutamate kinase			2.1
GDH1	Glutamate dehydrogenase	2.3		2.3
GDH3	Glutamate dehydrogenase		5.7	6.1
ILV5	Acetohydroxyacid reductoisomerase	4		
LYS2	L-aminoadipate-semialdehyde dehydrogenase		4.1	2.2
LYS9	Saccharopine reductase		3.9	2.8
LYS12	Homoisocitrate dehydrogenase		4.3	
MET10	Sulfite reductase		2.1	2.2
MET12	Methylenetetrahydrofolate reductase		2.3	
MET16	Phosphoadenylyl-sulfate reductase	2.9		2.7
MET13	Methylenetetrahydrofolate reductase	2.1		
PRO2	Gamma-glutamyl phosphate reductase		2.6	
Alcohol metabolism				
ADH7	Medium chain alcohol dehydrogenase	4.7	14.1	9.2
ADH6	Medium chain alcohol dehydrogenase		2.6	
GRE3	Xylose reductase activity	2.2		2.2
YJR096W	Aldo-keto reductase	2.4		
ARI1	NADPH-dependent aldehyde reductase			4.1
Fatty acid, etc. synthetase				
FAS1	Beta subunit of fatty acid synthetase	2.7	2.1	
FAS2	Alpha subunit of fatty acid synthetase		2.0	2.0
ERG5	C-22 sterol desaturase	2.0		
ERG27	3-keto sterol reductase		2.7	2.1
LIA1	Deoxyhypusine hydroxylase		2.23	
Oxidative stress response				
HMX1	Heme oxygenase	2.4		
OYE2	NADPH dehydrogenase		2.9	
OYE3	NADPH dehydrogenase	2.6		
SPS19	2,4-dienoyl-CoA reductase	2.3		2.3
Unknown pathway				
ARA2	D-arabinose 1-dehydrogenase			2.2
YGL039W	Carbonyl reductase			3.0

oxidative stress in both rich (YPD plates) and fermentation conditions (Triple M plates). Fig. 6 showed the results of plates analysis of the sensitivity of the two strains to oxidative stress caused by H_2O_2. Strain 112y4 was more sensitive to the oxidative stress than strain 32y12 in both rich and fermentation conditions. Considering the harsher environment encountered in wine fermentation conditions, strains were more sensitive to the oxidative stress in fermentation condition than YPD culture. This observation confirmed our hypothesis that strains with extreme H_2S production capacities possessed the different characteristics of oxidative stress responses, which may be tightly related to the H_2S production mechanism.

4. Discussion

The importance of variations in sulfur metabolism to H_2S production has been demonstrated in numerous studies because H_2S liberation can be greatly affected when gene of sulfur metabolism was disturbed (Huang et al., 2014; Linderholm et al., 2010; Donalies and Stahl, 2002; Spiropoulos and Bisson, 2000). In reality, these results were found in high producer and few studies have been investigated the mechanism of none producer. Therefore, examined metabolic differences between non- and high H_2S production strains may provide new insights into H_2S production. In order to investigate the differences between these two types of strains, 166 strains in China vineyards were screened, and two strains were found with extreme H_2S yield. We found that less than 2% of the wild strains possessed little or no H_2S production ability, consistent with the results of previous studies (Kumar et al., 2010; Spiropoulos et al., 2000). Notably, rescreening fermentation results showed that strains with different H_2S yields had similarities in H_2S formation patterns but variations in biomass and fermentation abilities. These results also supported that there is a weak relationship among H_2S production, biomass, and fermentation ability (Wang et al., 2018). Thus, differences in metabolic pathways related to H_2S production capacity can be observed, even when two strains possess different fermentation traits.

RNA-Seq analysis in three fermentation stages allowed us to compare the differences in gene regulation between the two strains. Previous gene regulation profiles using DNA microarray analysis revealed that only thiamine biosynthetic genes were expressed differentially in high and low H_2S production strains (Bartra et al., 2010). However, our results showed that not only this pathway but also genes related to oxidoreductase activity and sulfur metabolism were dysregulated. We found that the oxidative stress response contained the most DEGs in strain 112y4, whereas two set of genes (*MET* and *LYS*) catalyzing S-containing amino acids and lysine were enriched in strain 32y12. Interestingly, these enriched pathways in the two strains were connected by NADPH/$NADP^+$ metabolism, which differed dramatically between the two strains. NADPH is the reductive potential in cell, required for a variety of biosynthetic pathways (fatty acids, amino acids), redox reactions and protecting against oxidative stress (Pollak et al., 2007). In strain 112y4, DEGs related to NADPH consumption was involved in oxidative stress resistance via the glutaredoxin/glutathione and thioredoxin systems, which could eliminate reactive oxygen species (ROS) by oxidation of self-sulfhydryl groups (−SH) using NADPH as an electrons donor (Murray et al., 2011). Consistent with this, DEGs involved in the electron transport chain in the mitochondria were highly expressed in strain 112y4, which may promote the generation of cellular ROS. Thus, the standard level of NADPH may be reduced due to upregulation of glutaredoxin/glutathione and thioredoxin systems. However, in strain 32y12, DEGs related to NADPH consumption was mainly related to S-containg compounds and lysine biosynthesis.

Indeed, NADPH offers the reducing power of sulfide reductase encoded by *MET5* and *MET10*, which encodes the α-subunit of sulfide reductase and has been shown to be the single genetic determinant of undetectable H_2S production traits in strain UCD522 (Linderholm et al., 2010). These results indicated that a specific NADPH metabolic

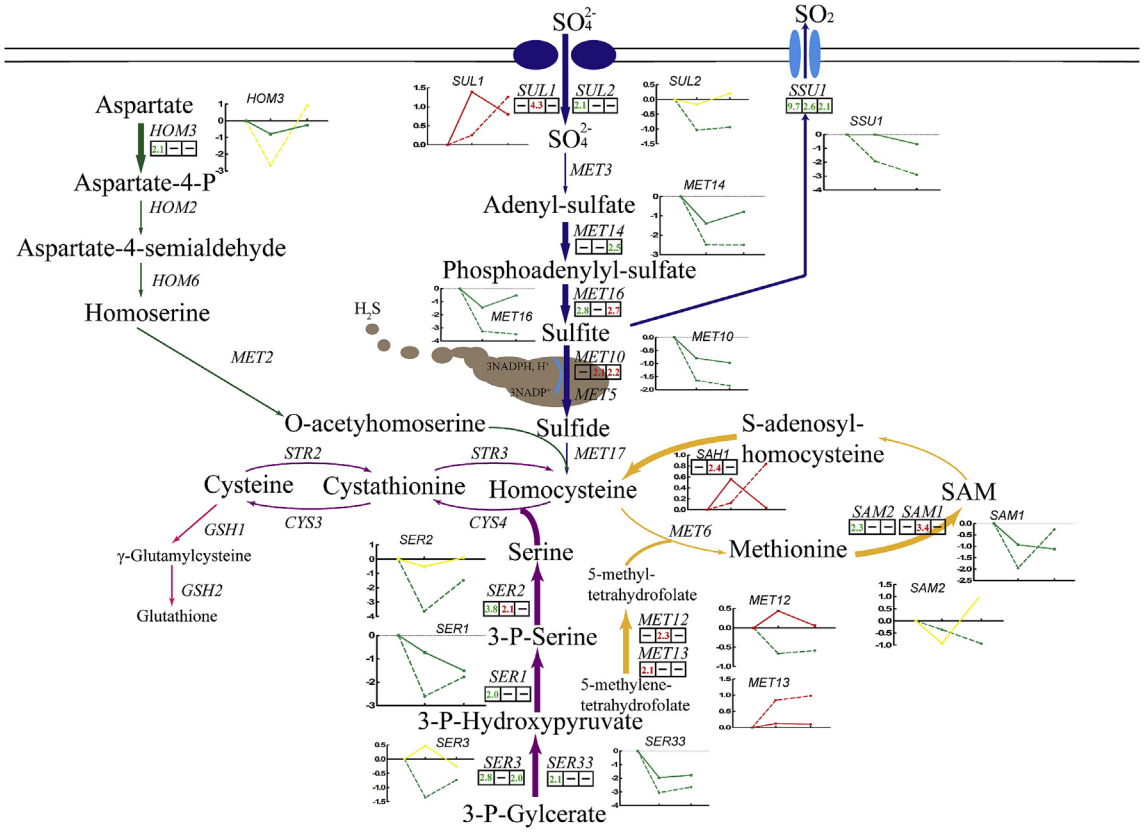

Fig. 4. Fold changes in DEGs related to sulfur metabolism in the two strains. The fold changes of the DEGs (Comparison pattern A, Supplementary Files 2–4) involved in sulfur metabolism were shown in the boxes. Highly expressed genes are shown in red for strain 32y12 and in green for strain 112y4. "-" indicates no significant difference (fold change < 2). Three boxes from left to right indicate stage I, II and III, respectively. The straight line charts in the figure represent the $\log_2$ ratios of DEGs (Comparison pattern B, Supplementary Files 5, 7, 8 and 9) at three stages for strain 112y4 (--●--) and strain 32y12 (—■—). Red indicates upregulation, and green indicates downregulation. Yellow indicates no significant change. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

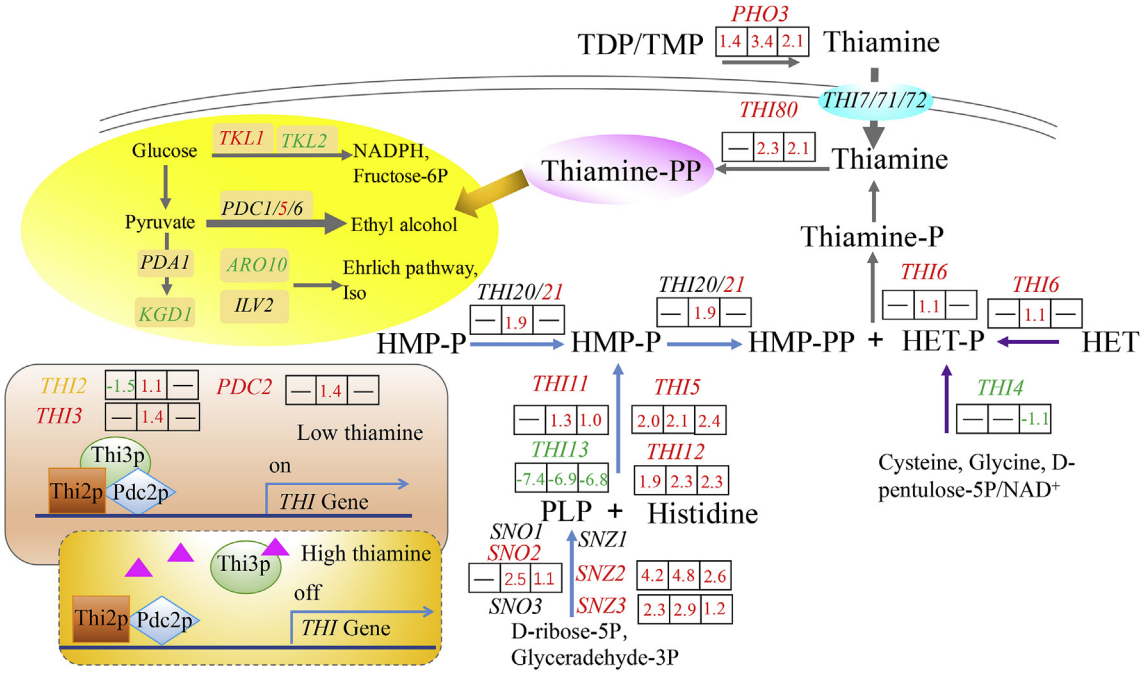

Fig. 5. Fold changes in DEGs related to thiamine metabolism. Expression ratios ($\log_2$ ratios in Supplementary Files 2–4) of the DEGs involved in thiamine metabolism, including biosynthesis, regulation, and utilization pathways, between the two strains were shown in the boxes. Highly expressed genes are shown in red for strain 32y12 and in green for strain 112y4. "-" indicates no significant difference ($\log_2$ ratio < 1). Three boxes from left to right indicate stage I, II and III, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

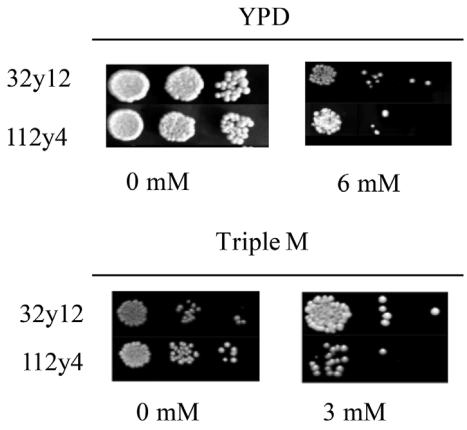

Fig. 6. Comparison of oxidative stress resistance analysis in strains 112y4 and 32y12. Normalized cultures were 10-fold serially diluted and spotted in YPD (contained 6 mM H_2O_2) and Triple M (contained 3 mM H_2O_2) plates, respectively. Plates containing free H_2O_2 were used as a control.

variations between the two types of strains may account for the different traits related to H_2S production. Meanwhile H_2S generation has been found to be related to NADPH in humans (Lin et al., 2013). It is likely that NADPH deficiency caused by highly expressed genes related to NADPH consumption in the beginning of H_2S release limit the reducing power of sulfite reduction in strain 112y4, leading to the low H_2S production. Redundant sulfite was exhausted extracellularly by overexpression of *SSU1*, which also be confirmed by high total SO_2 in wine after alcoholic fermentation finished in low or none-H_2S producers (data not shown). In contrast, in strain 32y12, the increased demand for SAM or SAM-related compounds induced the sulfur metabolic pathway leading to the high production of H_2S.

Apart from the upregulation of glutaredoxin/glutathione and thioredoxin systems in non-H_2S producer counteracted oxidative damage, our results also revealed that a greater oxidative tolerance may cause by a different oxidative resistance pattern in high producer. Based on the RNA-seq analysis, we found that genes involved in oxidative stress resistance, SAM synthesis, and thiamine synthesis pathways had dramatically different expressions between the two strains. Studies found that SAM is a precursor of polyamine synthesis, which is connected to stress resistance (Chattopadhyay et al., 2006), furthermore, thiamine has also been shown to have noncofactor-related functions in resistance to oxidative damage (Wolak et al., 2014). SAM is a methyl donor and precursor for the synthesis of polyamines, vitamins, phospholipids, and modified nucleotides, which are connected to stress resistance (Ljungdahl and Daignan-Fornier, 2012). In Particular, polyamines have been implicated in a variety of physiological processes and protect cells from ROS accumulation (Chattopadhyay et al., 2006). Thiamine is involved in the maintenance of the redox balance under oxidative stress conditions (Li et al., 2010; Wolak et al., 2014). These studies illustrate SAM or thiamine-mediated protection mechanism existed in *S. cerevisiae*. Because previous studies revealed that increasing thiamine and SAM biosynthesis expression levels alleviated redox stress (Zeng et al., 2017), we postulate that the upregulation of SAM and thiamine biosynthesis in high producer lead to higher oxidative stress tolerance. Indeed, strain 32y12 exhibited more tolerance than strain 112y4 (Fig. 6), confirming that upregulation of SAM and thiamine biosynthesis directly or indirectly confers to higher oxidative stress tolerance in *S. cerevisiae*. The mechanism of such SAM or thiamine-mediated protection remains unknown. Our results indicated an oxidative damage protection in high-H_2S producer, which may differ from normal oxidative defense system consisting of superoxide dismutases (Sod p), cytosolic catalase T (Ctt1p) and glutaredoxin/glutathione and thioredoxin systems (reviewed by Murray et al., 2011).

Besides, we speculated that such different oxidative damage protection may also related with high H_2S production because the upregulation of SAM and thiamine biosynthesis have a strong connection to high-H_2S production. Furthermore, it has been known that H_2S has a crucial function of cellular stress resistance in mammals and bacteria. Recently, a model of H_2S-mediated protection against oxidative stress was identified in *Escherichia coli* (Mironov et al., 2017). Although the detailed mechanism of H_2S-mediated protection in yeast remains unknown, these results suggested that SAM and thiamine biosynthesis upregulation in oxidative stress condition may be related to H_2S production to against the oxidative damage during wine fermentation in high-H_2S producer.

5. Conclusion

In this study, we highlighted the importance of gene expression patterns of NADPH metabolism in H_2S production and oxidative stress tolerance capacities of the two strains under wine fermentation conditions. A decreased number of expressed genes in involved in the SAP and a high level of NADPH consumption related to oxidative stress resistance were observed in strain 112y4, although these results have not yet been confirmed; these findings may account for the lack of H_2S production. Interestingly, we also found that the high expression of genes involved in the synthesis of S-containing amino acids and thiamine, which may be the mechanism that enabled the increase in oxidative stress tolerance in strain 32y12, but more work should be done to stand this mechanism. Considering the relationships among *MET*, *THI*, and stress resistance, variations in H_2S production may be related to different patterns of cell defense, a potential novel mechanism of H_2S production. Because the transcriptional data are not perfect, further studies are needed to determine the extent to which oxidative stress defense depends on sulfur metabolism or H_2S in both non- and high H_2S production strains and to elucidate the roles of NADPH in this mechanism.

Understanding the mechanism of H_2S-mediated protection in yeast has important implications for wine industry, which would not only diminish H_2S yield during wine fermentation but also, improve the stress resistance of yeast during wine fermentation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.10.021.

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