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TMT-based proteomic and transcriptomic analysis reveal new insights into heat stress responsive mechanism in edible mushroom *Grifola frondosa*

Hongyan Xie [a], Luzhang Wan [a], Jiandong Han [a], Chunyan Huang [a], Jin Li [a], Qiang Yao [a], Peng Yang [a], Yan Zhang [b], Zhiyuan Gong [a,*], Hao Yu [c,*]

[a] State Key Laboratory of Nutrient Use and Management, Key Laboratory of Wastes Matrix Utilization, Ministry of Agriculture and Rural Affairs, Institute of Agricultural Resources and Environment, Shandong Academy of Agricultural Sciences, Jinan, Shandong 250100, China
[b] Shandong Agricultural Technology Extending Station, Jinan, Shandong 250100, China
[c] Shandong Provincial Key Laboratory of Applied Mycology, School of Life Sciences, Qingdao Agricultural University, 700 Changcheng Road,Chengyang District, Qingdao, Shandong 266109, China

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ABSTRACT

High temperature can lead to severe retardation in the growth and development of mushrooms, which influence the quality of fruiting bodies. *Grifola frondosa* is a popular and wide-cultivated mushroom in Asia; however, little information about the heat response mechanism in this commercially cultivated mushroom has been reported. The growth test was performed to determine the heat tolerance of different strains. Then, the TMT-based proteome and transcriptome analysis of major *G. frondosa* cultivar Qinghui-151 under different temperatures were performed. High temperature seriously affected the growth and recovery growth of mycelia. Thirty-four heat shock proteins (HSPs) were identified in the multiomics data and the expressions of most HSPs were induced by high temperature. The accumulation of HSPs under the control of HSFs is assumed to play a central role in the heat stress response (HSR) in *G. frondosa*. mTOR and Ca^{2+} signaling pathways were found to be activated under high temperature and might involved in heat stress signaling transduction. Polyamines synthesis enzymes were also up-regulated, suggesting the accumulation of stress protector polyamines under heat stress. In addition, expression of an L-phenylalanine ammonia-lyase *(GfPAL)* gene was significantly up-regulated under high temperature, which might be related to secondary metabolites synthesis. Taken together, these findings improve our understanding of the molecular mechanisms underlying the response to heat stress in *G. frondosa*, which could promote the breeding of new heat-tolerant *G. frondosa* cultivars.

1. Introduction

Environmental factors, including temperature, light, gas, and nutrients, play key roles in the growth and fruiting body development of edible mushrooms (Sakamoto 2018). Heat stress is an important adverse environmental stress that influences all levels of biological organization including the growth and development of mushrooms (Kotak et al., 2007; Richter et al., 2010; Wesener et al., 2023). Heat stress has a harmful influence on the growth and development of edible mushroom mycelia and fruiting body (Sakamoto 2018; Xu et al., 2021). Huang et al. (2011) reported that high temperature (40 °C) could induce the autolysis of the *Pleurotus tuber-regium* mycelia in submerged cultivation. Song et al. reported that heat stress-induced apoptotic-like cell death in *Pleurotus ostreatus* and *Pleurotus pulmonarius* (Song et al., 2014). Autolysis and apoptosis under heat stress were also observed in *Hypsizygus marmoreus* mycelia cultivated in PDA plates (Xu et al., 2021). Besides, high-temperature could increase the contamination rate in the vegetative and reproductive stages. One of the reports for this is that extracellular metabolites of *P. ostreatus* induced by high-temperature has positive effects on the growth of *Trichoderma asperellum*, one of the major edible mushroom pathogen (Qiu et al., 2018). High temperature can also influence the metabolic and secondary metabolite synthesis in edible mushrooms. Zhang et al. (2016) reported that heat stress could induce the accumulation of ganoderic acid, one of the major bioactive compounds in *Ganoderma lucidum*. The increase of NO, Ca^{2+}, and reactive oxygen species (ROS) concentration might participate in the regulation of heat stress-mediated regulation of ganoderic acid biosynthesis (Liu et al., 2018a, 2018b; Zhang et al., 2016).

* Corresponding authors.
E-mail addresses: sdgzy2656@126.com (Z. Gong), yuhaosunshine@163.com (H. Yu).

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During the development, edible mushrooms produced conservative and effective defending systems to respond to high-temperature stress (Hu et al., 2023; Huang et al., 2011; Kong et al., 2012; Lei et al., 2019; Liu et al., 2016; Xu et al., 2021). Trehalose accumulation ubiquity response mechanism for edible mushroom. Kong et al. (2012) detected the accumulation of trehalose in *Pleurotus eryngii* var. *tuoliensis* under heat stress. Liu et al. (2016) reported that trehalose played an important role in the protection of *Flammulina velutipes* against high-temperature stress. High temperature significantly stimulated the activities of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase and promote the accumulation of trehalose for the first 2–6 hours after heat shock at 37 °C (Liu et al., 2016). Wang et al. reported that the silence of a heat shock protein (HSP) 40 (LeDnaJ) gene in *Lentinula edodes* caused defects in mycelial growth and resistance to heat stress (Wang et al., 2018a, 2018b). Up-regulation of HSPs under heat stress was also reported in other mushrooms, such as *H. marmoreus* (Xu et al., 2021, 2020), *G. lucidum* (Tan et al., 2018), *Wolfi-poria cocos* (Hu et al., 2023), and *P. ostreatus* (Zou et al., 2018). ROS is an universal signal in stress response in organisms including heat stress response (Liu et al., 2018b; Xu et al., 2021). Accordingly, mushrooms activate the ROS scavenging systems, including superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and glutathione reductases, to eliminate the damages caused by increased ROS (Tan et al., 2018; Xu et al., 2021). Several molecules and pathways were involved in the heat stress signal transduction and gene expression regulation, including MAPK signal pathway, Ca^{2+}, IAA, and NO (Liu et al., 2018a, Tan et al., 2018; Wang et al., 2018a). To date, several studies have been performed to investigate the heat stress response mechanism of edible mushrooms; however, little information has been reported for the important edible and medicinal mushroom *Grifola frondosa*.

G. frondosa, generally known as "Huishuhua" in Chinese and "maitake" in Japanese, is a Basidiomycetes edible mushroom that belongs to the family of *Grifolaceae* and the order of Polyporales (Mayuzumi et al., 1997; Ren et al., 2022). *G. frondosa* has both nutritional and medicinal properties, long history of edibility, and is widely accepted in China, Japan, and other Asian countries (He et al., 2017; Ren et al., 2022; Wu et al., 2021). *G. fronodosa* attracted our attention due to its high-value bioactive, mainly polysaccharides, that has various physiological activities, including antioxidant, immunomodulatory, antidiabetic activities, anti-tumor activities, as well as their influence on gut microbiota (Chen et al., 2018; Ding et al., 2016; Li et al., 2017, 2019; Ren et al., 2022; Tripodi et al., 2022). Most of the researches focus on the production and characterization of bioactive compounds in *G. frondosa* (Guo et al., 2022; Liu et al., 2021) to date, while little information has been reported on the genetic research and environmental factor response in this mushroom. Kawaguchi et al. identified a tyrosinase gene that played a key role in the phenotype of albinism in *G. frondosa* (Kawaguchi et al., 2019a, 2019b). The role of tyrosinase in mushroom color has also been reported in other mushrooms (Zhang et al., 2022). Loshchinina et al. (2016) reported that the NO synthase system might be a response to the abiotic stress factors in *G. frondosa*. However, the mechanism underlying the response and adaptation to heat stress in *G. frondosa* is still obscure.

With the genome sequence of the *G. frondosa* genome, (Kawaguchi et al., 2019a; Li et al., 2018; Song et al., 2022) comprehensive analysis of the heat stress response in *G. frondosa* based on multi-omics data becomes feasible. In the present study, we aimed to investigate the effects of heat stress on *G. frondosa*. Integrated transcriptome and proteome analysis was used to assess the gene and protein expression profile of *G. frondosa* at normal temperature and elevated temperature to clarify the adaptive mechanism. The study will provide useful information for the rational design to improve the thermotolerance of this important commercially cultivated mushroom.

2. Materials and methods

2.1. G. frondosa cultivation and strain selection

The *G. frondosa* strains were maintained on potato dextrose agar (PDA, BD Difco) plates at 4 °C. For heat treatment experiments, the 5-mm diameter mycelia block was cut from the edge of the seed plate and transferred to new PDA plates. The mycelia were cultured at 25 °C in the dark until the mycelial diameter reached 4 cm. Then the cultivation temperature was changed to 37 °C for 48 h and then the temperature was adjusted back to 25 °C. The plates were cultured at 25 °C until the mycelia recovered and grew for another 7 days. The growth rate of each stage and the time needed for regrowth after heat shock were recorded.

2.2. Heat treatment of G. frondosa mycelia for multi-omics analysis

Three *G. frondosa* strains were cultivated and analyzed for their heat stress responses in this study. After inoculation, the PDA plates were cultivated at 25 °C in the dark for 14 days (Fig. 1a) and mycelia on the plates were sampled as day 0 (Gf0 group). Thereafter, the left plates were transferred to an incubator at 37 °C for heat shock treatment. Mycelia that were treated with high temperature for 8 hours (Gf8 group) and 24 hours (Gf24 group) were collected as the heat treatment groups (Fig. 1a). Each biological replicate at each time point (0, 8, and 24 h) were treated independently. Three plates were randomly selected for one sample for a total of 9 plates for one group. Half of the mycelia scratched from the plates were transferred to a 2 mL centrifugation tube and the samples were snap-frozen in liquid nitrogen for RNA extraction. The other half mycelia were treated the same way for protein extraction.

2.3. Transcriptomic analysis

Samples for RNA-Seq analysis were sent to Novogene Ltd. (Beijing, China), where the libraries were produced and sequenced. Briefly, total RNA was extracted from *G. frondosa* samples using Trizol reagent (Takara, Japan) according to the manufacturer's protocols. The RNA purification and completeness were determined using a NanoPhotometer spectrophotometer (ThermoFisher, USA) and Agilent Technologies 2100 Bioanalyzer (Agilent, CA, USA), respectively. mRNA was enriched from total RNA using Oligo(dT) magnetic beads and disrupted to 250–300 bp fragments by ion interruption in NEB Fragmentation Buffer. The first strand of cDNA fragments was synthesized using RNA fragments as the template with 6-base random primers and reverse transcriptase. The second strand was synthesized using the first strand as the template. Paired-end sequencing libraries' quality was evaluated using the Agilent Technologies 2100 Bioanalyzer. Sequencing was performed on an Illumina NovaSeq 6000 platform (2 × 150 bp, paired-end) (Illumina, CA, USA).

2.4. Reads mapping, quantification, and differential expression identification

Adaptors were removed from raw data, and then low-quality reads were filtered using fastp (version 0.18.0) software to generate clean reads. The clean reads were mapped to the *G. frondosa* strain 9006–11 genome downloaded from GenBank using Hisat2 software (Kim et al., 2015). The sam files obtained from Hisat2 was sorted and changed to bam file using samtools software. Map count was calculated with the R package Rsubread software. Abundance estimation (TMM) was performed using tools in the Trinity software suite (http://trinityrnaseq.github.io). DESeq2 software was employed to estimate the fold change (FC) and differentially expressed genes (DEGs) based on the read counts of each gene. The DEGs were determined with the parameters using FDR below 0.05 and absolute fold-change > 2.

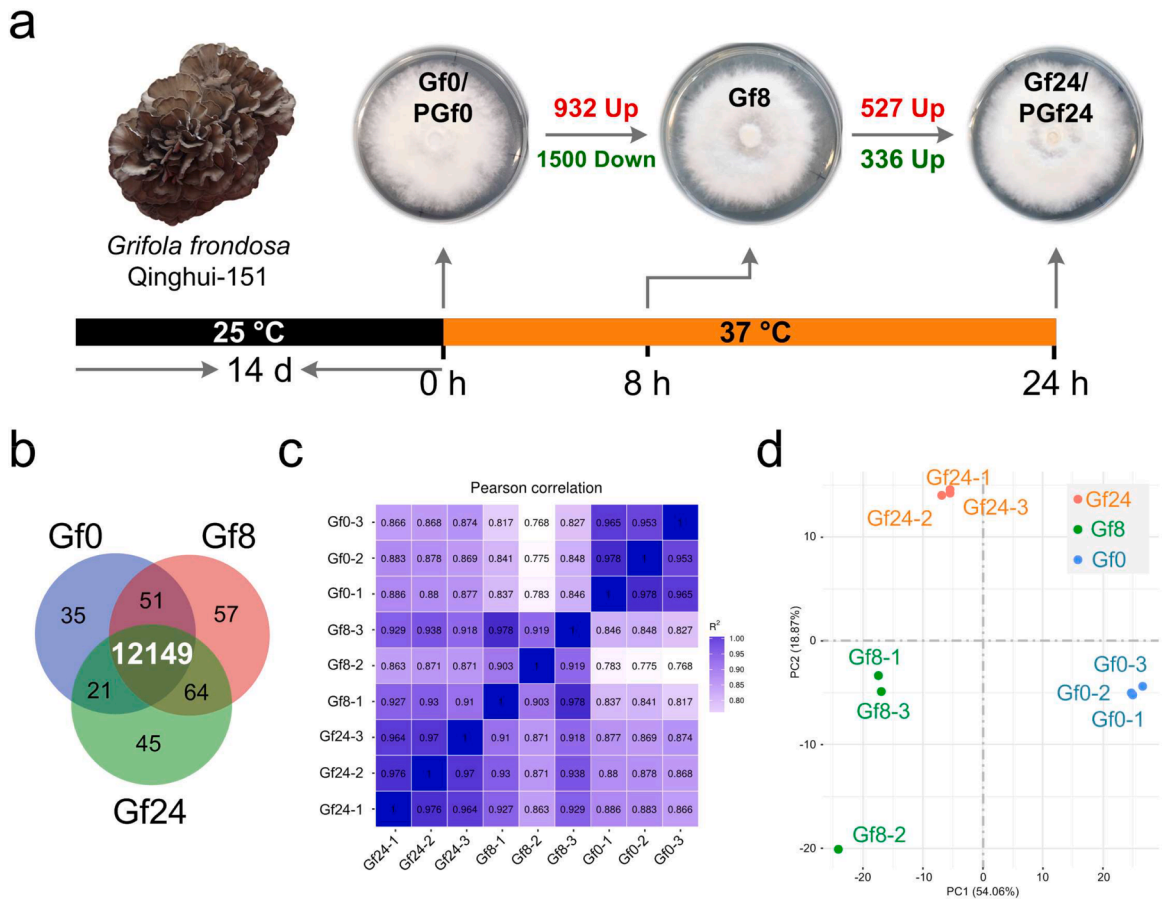

Fig. 1. Heat treatment and trancriptomic analysis of *G. frodosa* mycelia. a, Sample treatment procedures and the mycelia after treatment. b, Venn plot showing the genes identified in the transcriptomic analysis of three groups. c, Pearson correlation analysis of the 9 samples based on the expression of genes that were identified in all three groups. d, The expression profiles of the samples were shown based on PCA analysis.

2.5. Protein extraction, trypsin digestion, and LC-MS/MS analysis

Total mycelia protein was extracted as our previous description (Lin et al., 2022; Zhu et al., 2022) with slight modification. The mycelia were grounded to the powder in a grinder (Shanghai Jingxin) at a low temperature, 60 Hz for 240 s. Then the pre-heated (95 °C) ST buffer (4 % SDS, 10 mM DTT, pH 7.6) was added and the sample was vortexed for 5 min. Protein extraction was enhanced by bath-ultrasonication for 30 min and then the tubes were centrifuged at 15,000 g for 10 min. The supernatant was transferred to a new tube and stored at −80 °C for further analysis. For trypsin digestion, the protein was reduced by DTT and alkylated using iodoacetamide. Digestion was performed using the STrap method as our previous description (Xu et al., 2023b). Digested peptides were desalted using the C18 column. The peptide content was measured and 50 μg peptide mixture of each sample was labeled using TMT reagent according to the manufacturer's instructions (Thermo Fisher Scientific, USA). Peptides were fractionated on a Waters BEH C18 column (4.6 × 250 mm, 5 μm) using the U3000 HPLC system and divided into ten fractions, which were lyophilized respectively.

The fractions were dissolved by adding 0.1 % formic acid and injected for Nano LC-MS/MS analysis. Peptides were loaded onto a reversed-phase trap column connected to a C18-reversed-phase analytical column (15 cm long, 75 μm inner diameter, 3 μm resin). The peptides were separated with a linear gradient at a flow rate of 3 μL/min for 60 min. LC-MS/MS analysis was performed on a Q Exactive HF mass spectrometer that was coupled to Easy NanoLC. The mass spectrometer was operated in positive ion mode using a data-dependent top10 method. The survey scan is from 350 to 1500 *m/z*, the AGC target was set to 3e6, and the maximum inject time was 20 ms. Survey scans were acquired at a resolution of 60,000.

Protein identification and quantification were performed using Proteome Discoverer 2.2 against *G. frondosa* proteome. Abundances of each sample were normalized by adjusting to the same sum. Differentially expressed proteins (DEPs) were screened based on the following criteria: p-value < 0.05 and fold-change > 1.5 or $< 1/1.5$.

2.6. Function annotation and enrichment

Correlation analysis, Venn plot, volcano plot, heat map, and PCA analysis were performed and visualized using R packages corrplot, venn, ggplot2, factoextra, and FactoMineR, respectively. Gene ontology (GO) annotation was performed using the information from the UniProt database (proteome: UP000092993). Protein pathways were annotated by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al., 2016).

2.7. RT-qPCR analysis

RNA extraction and cDNA synthesis were performed as described above. The qPCR reaction system was prepared using FastStart Universal SYBR Green Master (Rox) Roche according to the manufacturer's instruction in a total volume of 20 μL for each tube. The qPCR reactions were performed using a 7500 Real-Time PCR System (ABI, USA). Gene encoding glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were used as internal control gene. Relative expression levels were calculated using the relative $2^{-(\Delta\Delta Ct)}$ method, and the expression level was normalized to the expression level of the control gene.

3. Results

3.1. Response to heat stress in G. frondosa strain Qinghui-151

G. frondosa Qinghui-151 was the major cultivar in Zhejiang Qingyuan, one of the major *G. frondosa* producing areas in China. To identify the differences in high-temperature tolerance, the mycelial growth rates of several *G. frondosa* strains cultivated in PDA plates with high-temperature treatment were measured. The growth rates of three strains, the main major cultivar Qinghui-151, the heat stress-sensitive strain Luqian-3, and the heat stress tolerant strain CGMCC5.404, were listed in Table 1. None strain could grow under high-temperature (37 °C) treatment or even several days after the high-temperature treatment (Table 1). High-temperature tolerance strain CGMCC5.404 has a higher mycelial growth rate under normal temperature (25 °C) and less recovery growth time (4 days). In contrast, the high-temperature sensitive strain Luqian-3 has the lowest growth rate under normal temperature and the longest recovery time (12 days) for re-growth after high-temperature treatment (Table 1). The results suggested that high temperature has an obvious inhibitory effect on the growth of *G. frondosa* mycelia.

3.2. Transcriptome analysis revealing heat stress response in G. frondosa

The mycelia in PDA plates were cultivated in the dark for 14 days, which was set as a control group (Gf0). Then the mycelia were treated with high temperature at 37 °C for 8 hours (Gf8 group) and 24 hours (Gf24 group), respectively (Fig. 1a). The gene expression profiles of mycelia in the three groups were analyzed using RNA-Seq technology (Supplementary Table S1). After data filtering and trimming, a total of 506 M clean reads corresponding to 75.95 Gbp were obtained from the 9 sequencings. A total of 12,422 genes were identified in three groups and 12,149 genes were shared by all three groups (Fig. 1b). Pearson correlation analysis indicated that samples from the same group have a high correlation (>0.9) with each other (Fig. 1c). PCA showed the same result that samples from same group clustered with each other (Fig. 1d). Interestingly, samples between Gf0 group and Gf24 group showed higher correlations than those between Gf0 and Gf8 groups. The results indicated that cell response to heat stress in gene expression level was done in the first few hours. Compared with the Gf0 group, 932 genes were significantly up-regulated in the Gf8 group and 1500 genes were significantly down-regulated. Compared with the Gf8 group, 527 genes were significantly up-regulated and 336 genes were down-regulated in the Gf24 group (Fig. 1a).

To investigate the molecular basis of heat stress response, gene ontology (GO) enrichment of the DEGs was performed (Supplementary Tables S2 and S3). As we can see from Fig. 2a, up-regulated DEGs in the Gf8 group (compared with the Gf0 group) were enriched in GO terms such as nucleolus, intracellular organelle, unfolded protein binding, ribosome biogenesis, carbamoyl-phosphate synthase (glutamine-hydrolyzing) activity, methylenetetrahydrofolate dehydrogenase ($NADP^+$) activity, alternative oxidase activity, amino binding, and fatty acid biosynthetic process. Most of the GO terms enriched for up-regulated DEGs in the Gf24 group (compared with the Gh0 group) were different from those of the Gf8 group (Fig. 2a,c). Only one GO term, fatty acid biosynthetic process, was enriched in the up-regulated DEGs of both groups. In addition, GO terms of spermine biosynthetic process and spermidine biosynthetic process were enriched in Gf24 up-regulated DEGs, which indicated that *G. frondosa* might synthesize polyamines (a stress response compound) to resist high temperature. The results indicated that the heat shock response or defense system in *G. frondosa* is done by different steps. The Gh8 group represented the early response to heat shock stress while the gene expression in the Gf24 group represented the latter response to heat shock.

Unlike the up-regulated DEGs, most of the enriched GO terms were shared by the down-regulated DEGs of Gf8 and Gf24 groups (Fig. 2b,d). The common GO terms were metal ion binding, oxidoreductase activity, proteolysis, carbohydrate metabolic process, extracellular region, structural constituent of ribosome, hydrolase activity, hydrolyzing O-glycosyl compounds, translation, ribosome, carbohydrate binding, cellulose binding, oxidoreductase activity, acting on CH—OH group of donors, serine-type endopeptidase activity, and polysaccharide catabolic process. Clearly, the expression of CAZymes was inhibited by high temperature. CAZymes enzymes were involved in carbon metabolism and substrate utilization in fungi, therefore, the inhibition of CAZymes suggested that the growth of *G. frondosa* was inhibited at high temperature. Similar to the GO enrichment results, most of the KEGG pathways enriched in down-regulated DEGs in Gf8 and Gf24 groups are the same (Fig. 3).

The KEGG pathways of protein processing in the endoplasmic reticulum, carbon metabolism, peroxisome, fatty acid metabolism, and autophagy were shared by the up-regulated DEGs in Gf8 and Gf24 groups (Fig. 3a,c). Most of the proteins in pathways of protein processing in the endoplasmic reticulum pathway were HSPs, which indicated that HSPs play important roles in the response to heat stress in this mushroom. The enrichment of autophagy indicated that heat stress-induced the autophagy signaling in *G. frondosa* as some other mushrooms (Song et al., 2014; Xu et al., 2021). Most of the genes in fatty acid metabolism and peroxisome are related to fatty acid oxidation, which indicated that the energy production system might be influenced by the high temperature.

3.3. Proteomic analysis and the relationship between gene and protein expression levels

Proteins are executors of most biological processes and the expression level of proteins was not always consistent with those of the gene expression. Therefore, TMT-based quantitative proteomic analysis were carried out to identify the protein expression profile in *G. frondos*a under high-temperature treatment. Considering that the protein translation process is later compared with the transcription process. Mycelia that were treated with high temperature at 37 °C for 24 h (PGf24) and without high-temperature treatment (PGf0) were used for proteomic analysis. A total of 4397 proteins were identified from the six samples (Supplementary Table S4). Compared with the PGf0 group, 150 proteins were significantly (fold-change > 1.5 and p-value < 0.05) upregulated and 278 proteins were significantly down-regulated in the PGf24 group.

Results of correlation coefficiency analysis with the fold change value of each gene (or protein) of Gf8, Gf24, and PGf24 groups compared with the control group (Gf0 or PGf0) were shown in Fig. 4a (Supplementary Table S5). Correlation between RNA-Seq groups (0.66) was higher than those between transcriptome and proteome groups (0.12 and 0.23). Lack of correlation between transcriptomics and proteomics data was also reported in many other studies, which might due to multiple factors such as different levels of regulation, stability of mRNA, rate of mRNA transcription and protein translation as well as protein stability and the biochemical diversity of proteins (Fournier et al., 2010; Vogel et al., 2012). However, the correlation between PGf24 and Gf8 groups was higher than that between PGf24 and Gf24, in line with the above speculation (delayed correlation of mRNA and protein expression).

Table 1
Growth rate of three *G. frondosa* strains under heat stress.

Strain name	Average growth rate at 25 °C (mm/d)	Growth under 37 °C treatment in 48 hours (mm)	Recovery time needed for regrowth at 25 °C (d)
Luqian-3	2.14	0.00	12
Qinghui-151	2.81	0.00	5
CGMCC5.404	3.60	0.00	4

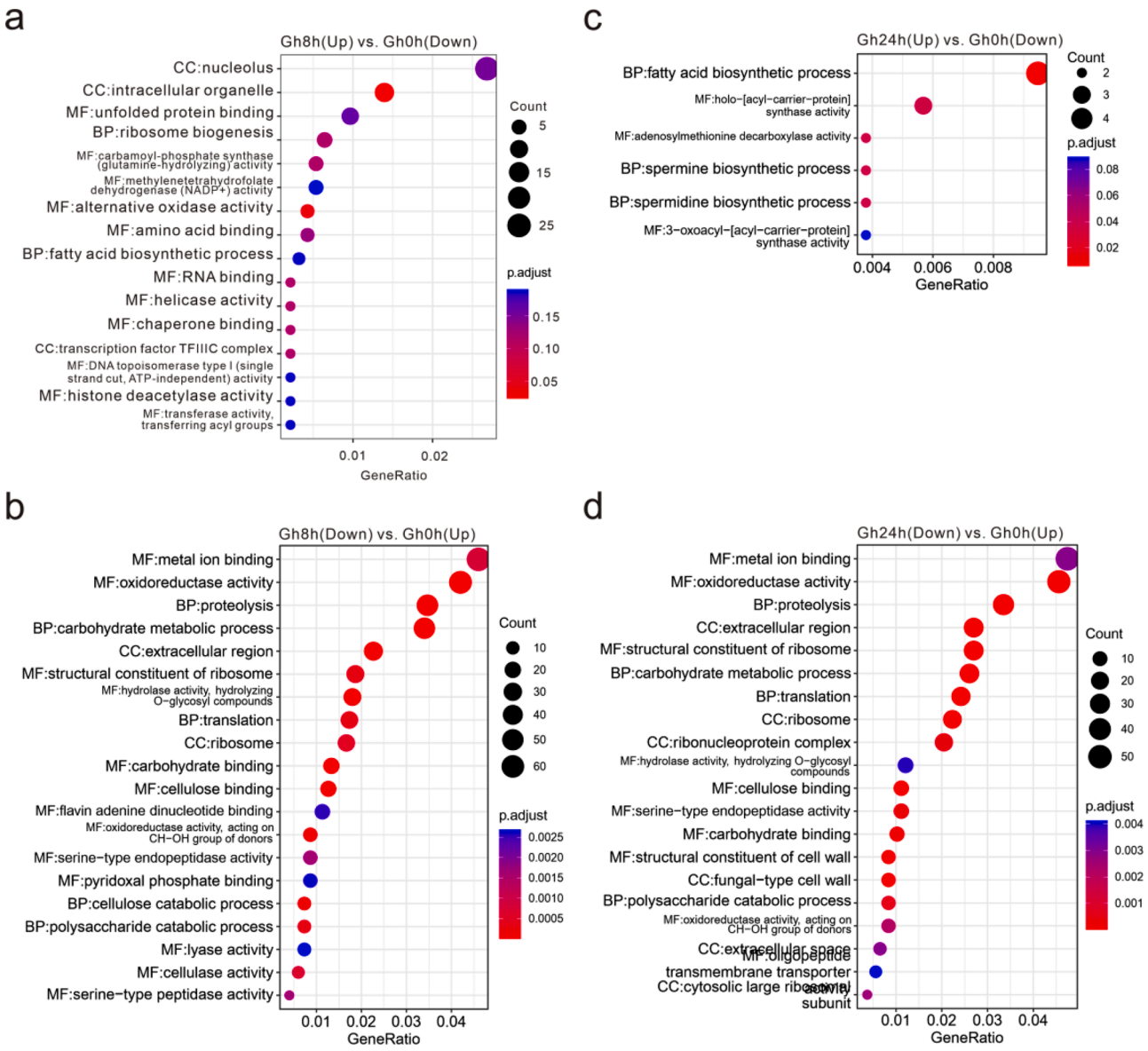

Fig. 2. Enriched GO terms of up-regulated and down-regulated DEGs between different groups. a and b, Enriched GO terms of up-regulated and down-regulated DEPs between the Gf8 group and the Gf0 group. c and d, Enriched GO terms of up-regulated and down-regulated DEPs between the Gf24 group and the Gf0 group.

The Venn diagram analysis further pinpointed 48 differential expressed genes (proteins) that up-regulated upon heat treatment in both transcriptional and translational levels (Fig. 4b). Among the 48 genes, thirteen genes were chaperons including sHSP, HSP40, HSP70, and HSP90 (Table 2, Supplementary Table S6). The results are consistent with those of the transcriptome results, which confirm that heat shock proteins play a pivotal role in heat stress response in *G. frondosa*. Two genes involved in ion homeostasis (A0H81_07426, Store-operated calcium entry-associated regulatory factor and A0H81_03367, Vacuolar iron transporter) and one kinase (A0H81_06148, Serine/threonine-protein kinase TOR) were also significantly up-regulated. These genes might contribute to the heat stress signal transduction in *G. frondosa*. Four genes (A0H81_00358, A0H81_06020, A0H81_04680, and A0H81_12,196) related to energy metabolism were overrepresented upon heat treatment. This indicated the requirements of energy for mycelia under high-temperature.

3.4. Gene expression of L-phenylalanine ammonia-lyase (GfPAL) after heat treatment

The *pal* gene has been widely studied in plants and participates in plant growth, development, and defense systems. The study of Hou et al. showed that expressions of the two *pal* genes were higher in the reproductive growth stage than in the vegetative growth stage and both *pal* genes were significantly up-regulated upon high-temperature treatment (Hou et al., 2019). In this study, we identified a *pal* gene (*GfPAL*, A0H81_10,452) from the genome of *G. frondosa*. The amino acid sequence identity between GfPAL and two Pal from *P. ostreatus* were 64 % and 68 %, respectively. Transcriptome data showed that the *GfPAL* gene was significantly up-regulated (with a fold-change of 3.06) at 8 h and also up-regulated (with a fold-change of 1.66) at 24 h (Supplementary Tables S2 and S3). The temporal expression of *GfPAL* in three *G. frondosa* strains (Qinghui-151, Luqian-3, and CGMCC5.404) was further determined using RT-qPCR. The expression of *GfPAL* was significantly up-regulated in all three strains at 8 h after high-temperature treatments (Fig. 5). The heat-tolerant strain CGMCC5.404 showed the highest induction level with a fold-change of

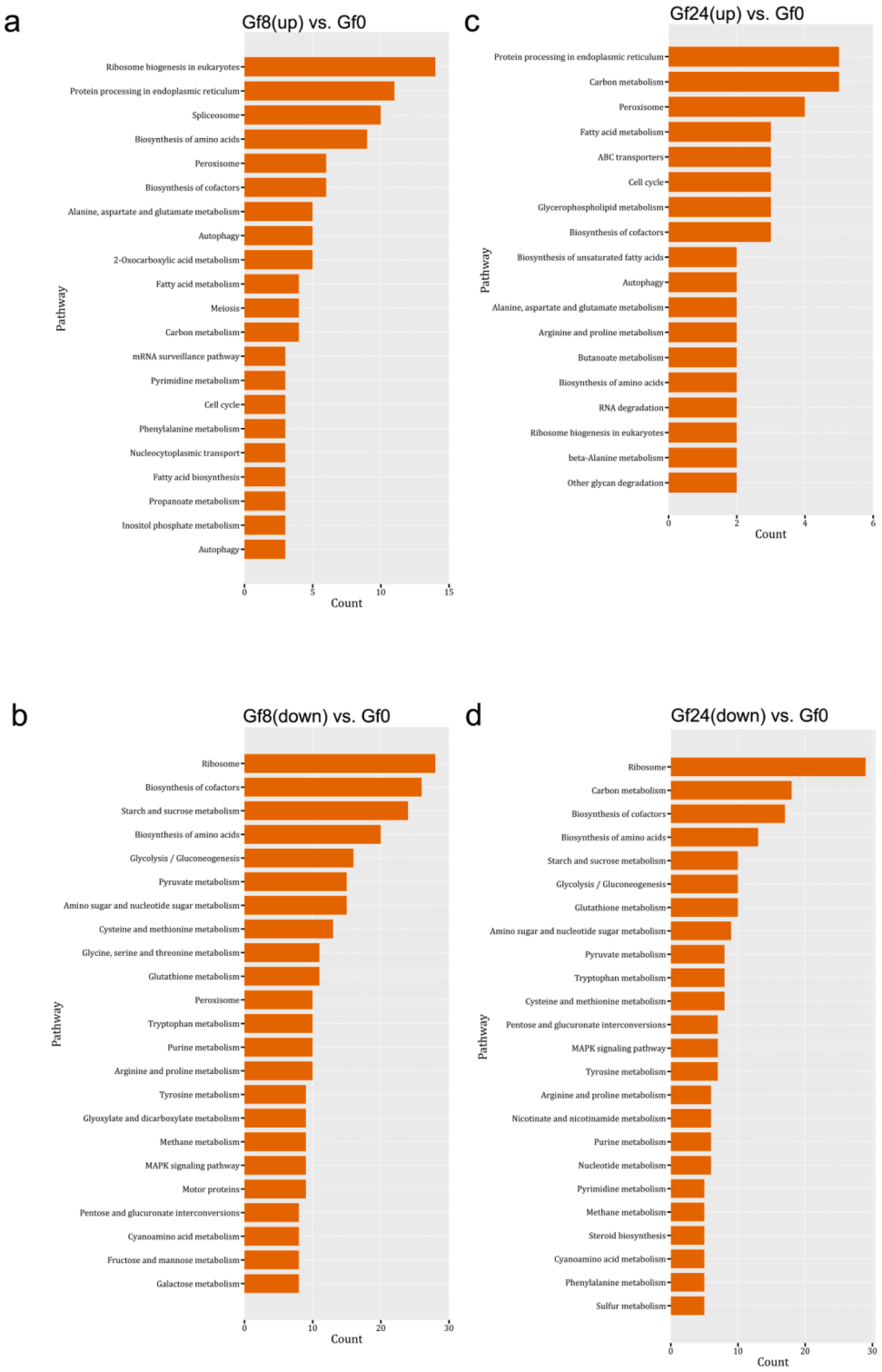

Fig. 3. Enriched KEGG pathways of up-/down-regulated DEGs in Gf8 and Gf24 groups. a and b, KEGG pathways enriched in up-regulated (a) and down-regulated (b) DEGs in the Gf8 group compared with the Gf0 group. c and d, KEGG pathways enriched in up-regulated (c) and down-regulated (d) DEGs in the Gf24 group compared with the Gf0 group.

22.6. The fold-change between *GfPAL* expression at 8 and 0 h in Qinghui-151 was 13.9, while that for heat-sensitive strain Luqian-3 was only 1.8. The expressions of *GfPAL* gene were decreased at 24 h compared with those at 8 h, which is in line with the transcriptome data. Overall, the inducible expression of *GfPAL* upon heat stress was related to the heat tolerance ability of this strain.

3.5. mRNA expression of selected HSP genes

To confirm the expression of *HSP* genes that were involved in heat stress response, the mRNA expression of 6 differentially expressed *HSP* genes, which was significantly up-regulated in Gf8, Gf24, and PG24 groups, were analyzed. Primers for RT-qPCR were listed in supplementary Table S7. The results showed that the mRNA expression levels of the

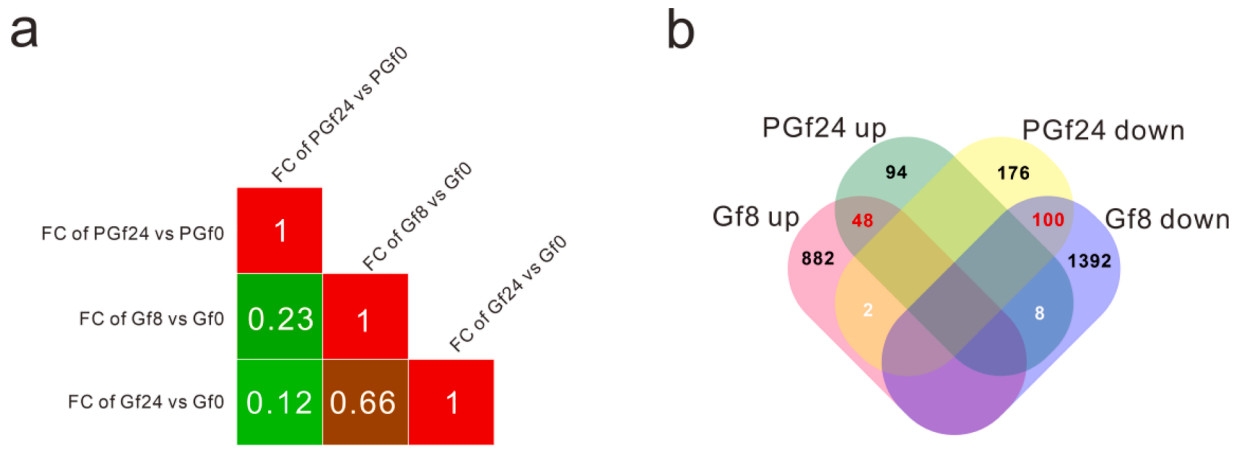

Fig. 4. Correlation and Venn diagram analysis of multi-omics analysis of transcriptome and proteome. a, Correlation analysis between Gf8, Gf24, and PGf24 groups based on the fold change of these groups compared with the corresponding group at 0 h (Gf0 or PGf0 groups); b, Venn diagram analysis of the differentially expressed genes or proteins in Gf8 and PGf24 groups (compared with Gf0 and PGf0, respectively).

Table 2
Differential expressed genes (proteins) in both transcriptomic and proteomic data upon heat treatment.

Gene	Functional description	Fold Change		
		PGf24 vs. PGf0	Gf8 vs. Gf0	Gf24 vs. Gf0
A0H81_04236	molecular chaperone HSP82	2.17	4.76	2.34
A0H81_10,394	Heat shock protein	1.68	5.07	2.79
A0H81_05124	Heat shock protein 16	3.78	26.40	4.11
A0H81_11,685	Heat shock protein 16	5.08	20.93	3.05
A0H81_07337	Heat shock protein 60, mitochondrial	1.90	4.65	3.38
A0H81_06494	Heat shock protein 78, mitochondrial	2.27	6.13	2.57
A0H81_10,815	Heat shock protein HSS1	1.59	2.55	2.31
A0H81_08205	Heat shock protein sti1	1.95	3.21	2.09
A0H81_07352	Heat shock protein, mitochondrial	3.18	5.53	3.20
A0H81_05512	Hsp70 nucleotide exchange factor FES1	1.98	2.80	2.15
A0H81_00495	DnaJ 1, mitochondrial	1.81	2.94	1.94
A0H81_14,588	DnaJ subfamily C member 3	1.69	2.22	1.87
A0H81_08328	Small heat shock protein C4	3.34	14.24	6.24
A0H81_06148	Serine/threonine-protein kinase TOR	1.72	2.41	1.87
A0H81_07426	Store-operated calcium entry-associated regulatory factor (Transmembrane protein 66)	1.55	2.70	1.60
A0H81_03367	Vacuolar iron transporter 1.1	1.53	3.08	1.80
A0H81_00358	Protein ATP11, mitochondrial	1.51	2.04	1.43
A0H81_06020	Mitochondrial fission 1 protein	1.65	2.04	1.68
A0H81_04680	Mitochondrial protein import protein mas5	1.58	2.94	1.62
A0H81_12,196	Putative NADPH dehydrogenase C23G7.10c	2.58	8.28	2.25

6 *HSP* genes were significantly up-regulated at 8 and 24 h compared with samples at 0 h (Fig. 6), which exhibited the same trends at the transcriptome data. As results of transcriptome data, the expressions of the 6 genes were higher at 8 h than those at 24 h The results confirmed that *HSP* genes play important roles in heat stress response in *G. frondosa*.

4. Discussions

Heat stress is an important adverse environmental stress that influences the growth and development of mushrooms (Kotak et al., 2007; Wesener et al., 2023; Xu et al., 2021; Zhao et al., 2020). In this study, the

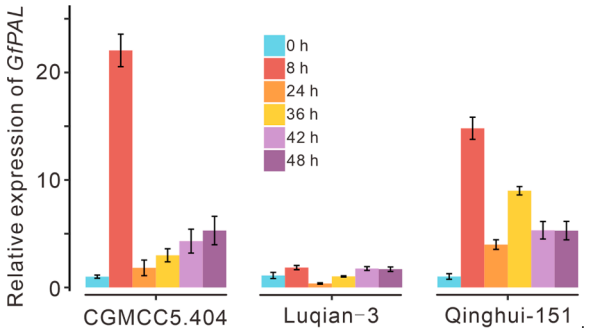

Fig. 5. The RT-qPCR analysis of *GfPAL* gene expression in Qinghui-151, Luqian-3, and CGMCC5.404 after heat treatment at 37 °C.

growth of *G. frondosa* Qinghui-151 was completely inhibited at 37 °C. After the temperature was changed back to 25 °C, it took 5 days to recover the growth. However, the growth rate of Qinghui-151 at 25 °C after heat treatment was still lower than that of the original growth rate without heat treatment. Growth-rate homeostasis at different parts of the fruiting body is crucial for the normal development of the mushroom fruiting body. Therefore, understanding heat stress response mechanism and thereby breeding the heat-tolerance cultivars is necessary. Identification of genes/proteins responsive to heat stress is the first step toward understanding the molecular mechanisms. In this study, transcriptomic and proteomic analysis were performed to study the heat stress response in *G. frondosa*.

The accumulation of Heat shock proteins (HSPs) is assumed to play a central role in the heat stress response and acquired thermo-tolerance in all organisms (Kotak et al., 2007). HSPs are representative molecular chaperons that can prevent the formation of misfolded protein structures and rescue the previously aggregated or denatured proteins (Richter et al., 2010). HSPs play critical roles in regulating organism growth and development as well as defense mechanisms under environmental stress, including high-temperature stress (Richter et al., 2010). Here, 34 heat shock proteins were identified in transcriptome and proteome data (Fig. 7). Among which, 28 HSPs were upregulated in at least one group upon high-temperature treatment, and 11 HSPs were significantly upregulated in all Gf8, Gf24, and PGf24 groups (Fig. 7). The up-regulation expression of six of the 11 HSPs were further confirmed by RT-qPCR (Fig. 6). HSPs play the central role in the heat stress response in many other mushrooms. Hu et al. Identified 16 HSPs in *Wolfi-poria cocos* and most of the HSPs were induced by high temperature. Heterologous expression of some HSPs in *Escherichia coli* enhanced the survival rate of

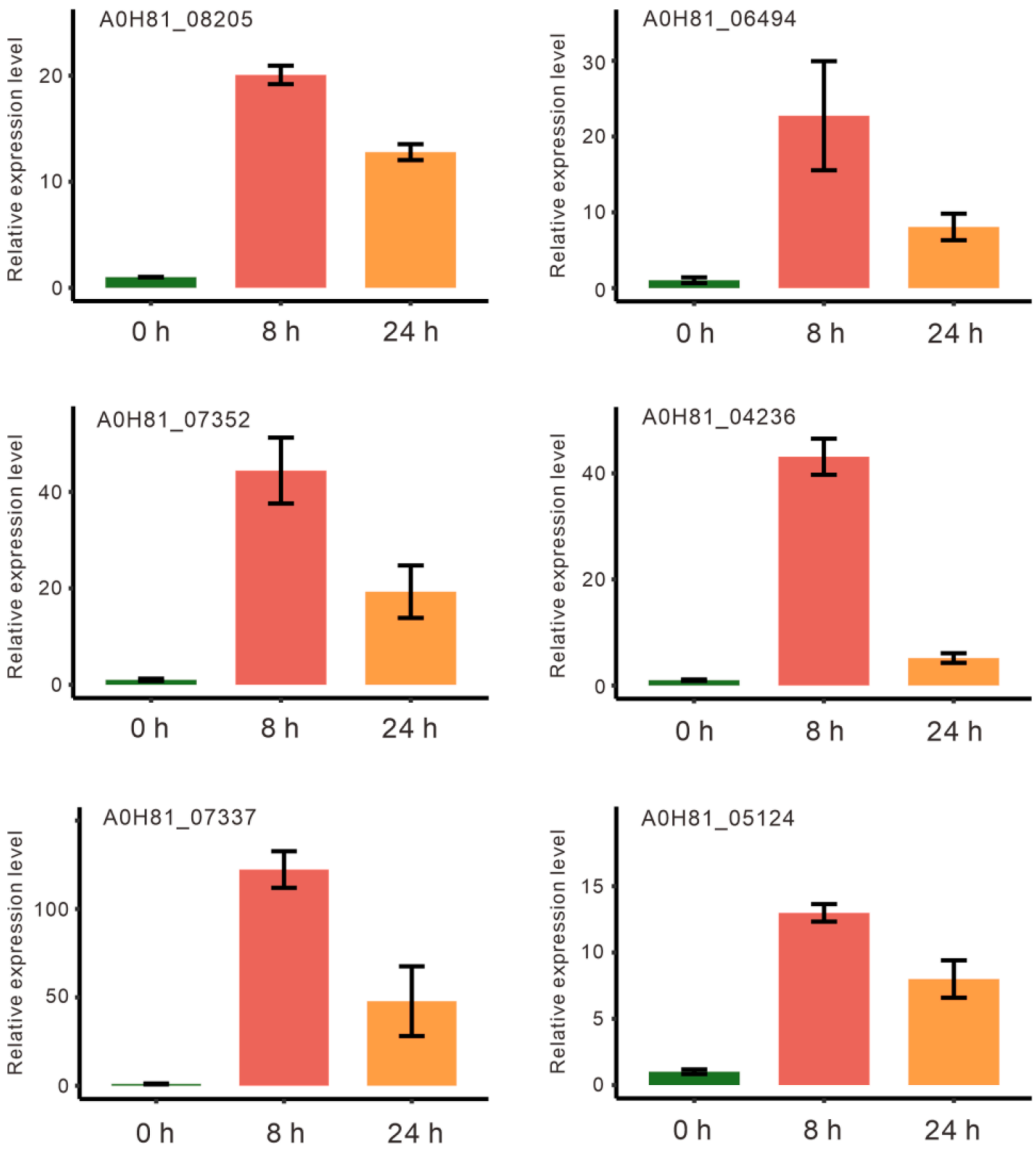

Fig. 6. RT-qPCR analysis of transcriptional expression analysis of representative HSP coding genes. The expression level of genes in Gf0 group was set as 1.

E. coli during heat stress. These findings suggest that HSPs play key roles in the high-temperature stress tolerance in *W. cocos* (Hu et al., 2023). Tan et al. (2018) reported that 15 *HSPs* were upregulated or only expressed in heat treatment *G. lucidum* mycelia and only one of them was down-regulated. Xu et al. (2021) identified 20 HSPs in the proteome data of *H. marmoreus* mycelia in heat treatment experiments and 18 of them were upregulated in the heat stress group, which indicated that HSPs involved in the heat response in *H. marmoreus*. We think that HSPs could prevent the formation of misfolded protein structures and rescue the aggregated or denatured proteins when *G. frondosa* cells are exposed to high-temperature. HSPs play central roles in heat stress response in this mushroom (Fig. 8).

HSPs were expressed under the control of heat stress transcription factors (HSFs). HSF is an ancient eukaryotic transcription factor that adjusts the cellular proteostasis system to changing stress loads by inducing the expression of a large set of genes including HSPs (Kotak et al., 2007). In *Saccharomyces cerevisiae*, surplus Hsp70 can inhibit HSF DNA-binding activity by binding to HSF to form the HSF-Hsp70 complex. During heat shock, Hsp70 is out-titrated by misfolded proteins, and the released HSF could bind to the heat-shock elements (HSEs) in the promoter of downstream genes, including HSPs, and induce the expression of these genes (Masser et al., 2019). We identified 4 HSFs (A0H81_14,313, A0H81_06454, A0H81_10,872, and A0H81_03562) in the genome of *G. frondosa* by sequence alignment. None of them was upregulated in the gene or protein expression level under heat stress. The results indicated that *G. frondosa* might have a similar regulation strategy to *S. cerevisiae*. The up-regulating expression of HSPs genes was induced by the release of HSFs instead of the upregulating of HSFs. However, we cannot exclude the new HSFs, which need further investigation.

Response the heat stress rapidly is one of the key factors for *G. frondosa* cells to survive in high-temperature. Signal transduction systems have been reported involved in the response to heat stress in other mushrooms. The study of Liu et al. revealed that the NO and Ca^{2+} signals promoted each other in response to heat stress, which could further regulate the synthesis of ganoderic acid in *G. lucidium* (Liu et al., 2018a). Here, a store-operated calcium entry-associated regulatory factor and vacuolar iron transporter were significantly upregulated upon high-temperature treatment in both transcriptome and proteome analysis (Table 1). Cation transporters are involved in the movement of various ions in microorganisms and are known to play pivotal roles in many physiological processes including stress tolerance (Shabala et al., 2016). The upregulation of the two proteins indicated that the Ca^{2+} signal might be involved in the heat stress signal transduction in

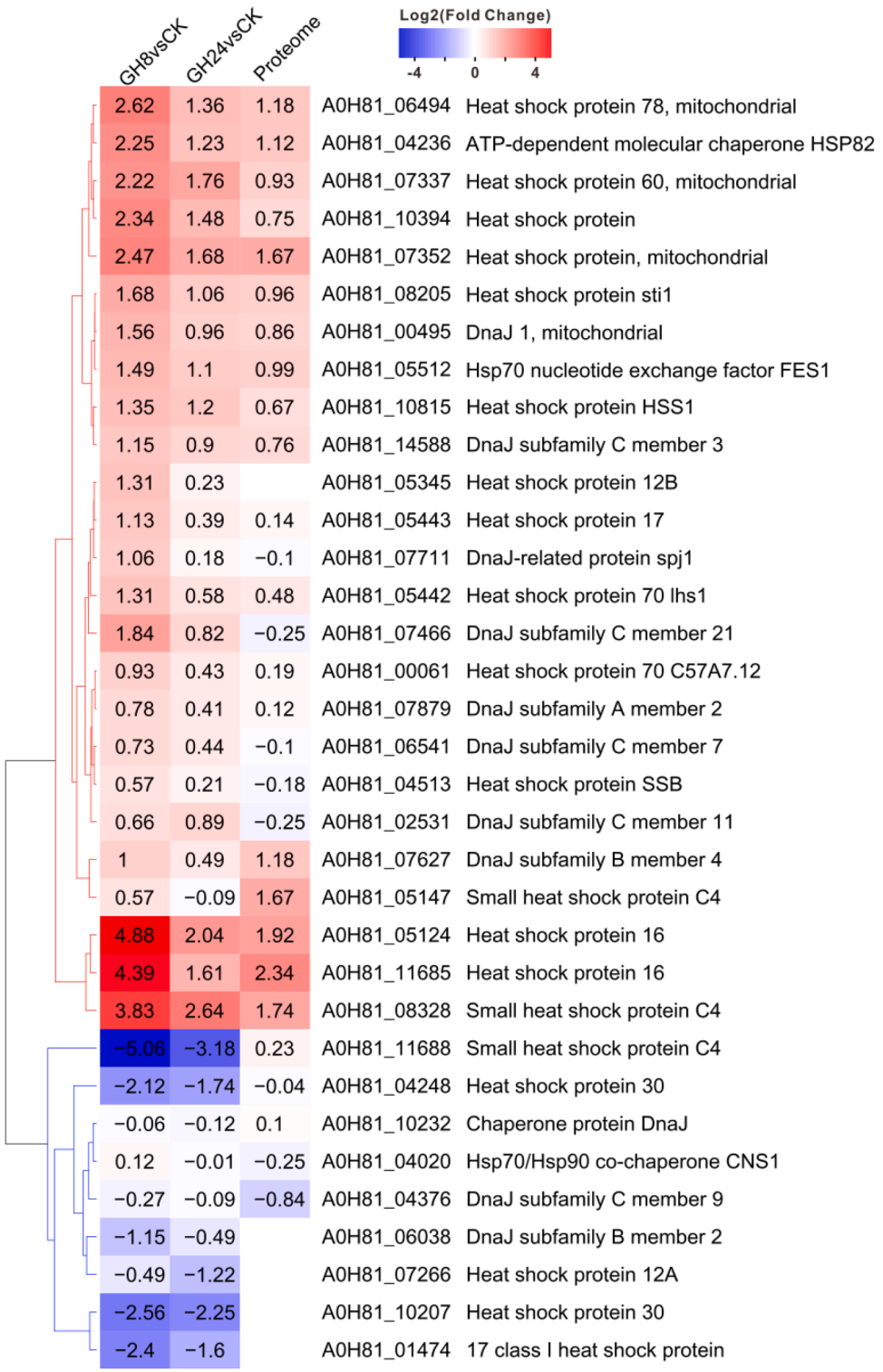

Fig. 7. Heat map representing the Log2(fold-change) of heat shock proteins (coding genes) expression in Gf8, Gf24, and PGf24 groups compared with those at the group at 0 h (Gf0 and PGf0 groups), respectively.

G. frondosa (Fig. 8).

Changes in protein phosphorylation are crucial for signal transduction in fungal (Albataineh and Kadosh, 2016). MAPK pathways (Ortiz-Urquiza and Keyhani, 2015) have been reported to be involved in the development (Xu et al., 2023a), environmental factor signal transduction (Zhu et al., 2022) as well as heat stress response (Wang et al., 2018a; Xu et al., 2021; Zou et al., 2018). Here, we identified six *MAPK* genes in transcriptome and proteome data. Two of them were significantly down-regulated at the transcriptional level while the expression of other genes has no significant difference. However, a Serine/threonine-protein kinase TOR (A0H81_06148) was significantly up-regulated in both transcriptional and translational levels upon heat treatment (Table 1). This protein was predicted as the component of TORC1 complex 1, which is the central regulator of the mTOR signaling pathway. mTOR signaling pathway might be involved in the heat stress signaling transduction in *G. frondosa* such as the signaling to induce

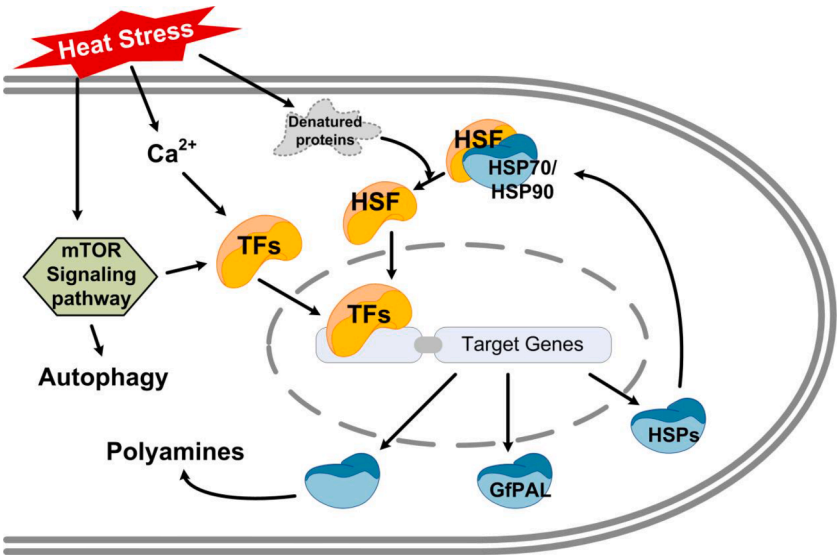

Fig. 8. Schematic model for the role of HSPs, signaling pathway, and protectants in heat stress response in *G. frondosa*.

cellular autophagy (Fig. 3). mTOR signaling pathway plays a central role in regulating many fundamental cell processes, from protein synthesis and autophagy to the aging process (Liu et al., 2020). It was also reported to respond to the nutrients in fungi (Rutherford et al., 2019; Zhang et al., 2015). Here, our results indicated that the mTOR signaling pathway, but MAPK, played an important role in transmitting heat stress signal transduction in *G. frondosa* (Fig. 8).

Endogenous or exogenous protectants could increase the thermotolerance of organisms. *Streptomyces coelicolor* could synthesize the compatible solutes ectoine and 5-hydroxyectoine in response to heat stress (39 °C) (Bursy et al., 2008). Polyamines with cytoprotective activities were induced by diverse stresses in plants raising the hypothesis that they might play a role in inducing stress response, including heat stress response (Rhee et al., 2007). In mushrooms, trehalose and ROS scavenging were reported as protectants to resist heat stress. The accumulation of trehalose and upregulating of trehalose synthesis genes upon high-temperature treatment were reported in different mushrooms such as *P. eryngii* (Kong et al., 2012), *F. velutipes* (Liu et al., 2016), *P. ostreatus* (Lei et al., 2017), *P. pulmonarius* (Liu et al., 2019), and *H. marmoreus* (Xu et al., 2021). Here, five trehalose synthesis-related genes were identified in transcriptome and proteome data. Only one trehalase gene (A0H81_08066) was upregulated in transcriptome data, while all the other genes were down-regulated upon heat treatment in both transcriptional and translational levels. Similar results were observed in ROS scavenging system. Several catalases, superoxide dismutases, peroxidases, glutathione synthases, and glutathione S-transferases were identified in transcriptome and proteome data. However, no clear expression trends were observed upon heat treatments. The results were in consistent with the experimental results. These results suggested that trehalose synthesis and ROS scavenging system were not the major heat stress response system in *G. frondosa*.

GO analysis showed that terms of spermine biosynthetic process and spermidine biosynthetic process were enriched in Gf24 up-regulated DEGs. The two genes (A0H81_11,908, adenosylmethionine decarboxylase, and A0H81_11,909, S-adenosylmethionine decarboxylase proenzyme) were involved in polyamines synthesis. The protection effect of polyamines to heat stress has been reported in plants. Jing et al. reported the alleviating effect of exogenous polyamines on wheat upon heat stress (Jing et al., 2020). The study of Sagor et al. Showed that the polyamine spermine could protect *Arabidopsis* from heat stress-induced damage by increasing the expression of heat shock-related genes (Sagor et al., 2013). The biosynthesis of polyamines in post-harvest *Agaricus bisporus* was reported by different studies (Yang et al., 2020), which indicated that polyamines played roles in mechanical damage or cold stress response in *A. bisporus*. These results suggested that *G. frondosa* use polyamines instead of trehalose as the protectants to alleviate the damage caused by high-temperature (Fig. 8).

High-temprature could induce the synthesis of secondary metabolites. Liu et al. reported that heat stress could induce the biosynthesis of ganoderic acid in *G. lucidum* (Liu et al., 2018b). In plants, heat stress could also induce the accumulation of soluble phenolics such as flavonoids and phenylpropanoids (Cingoz et al., 2016; Pan et al., 2006; Rivero et al., 2001; Wen et al., 2008). PAL is considered to be the principal enzyme of the phenylpropanoid pathway and the up-regulated expression upon heat stress was observed in several plants and fungi (Hou et al., 2019). In this study, the expression of *GfPAL* was significantly up-regulated upon heat treatment and the expression level was dependent on the thermal tolerance ability. The results suggested that the GfPAL gene was involved in heat stress response in *G. frondosa*. Wen et al. reported that the activation of PAL and accumulation of phenolics might lead to the development of thermotolerance (Wen et al., 2008), which was also reported in other plants (Cingoz et al., 2016; Pan et al., 2006; Rivero et al., 2001). Interestingly, *pal* genes (*pal1* and *pal2*) were also up-regulated in *P. ostreatus*. However, the inhibition of *pal1* expression by RNAi promoted the growth of *P. ostreatus* mycelia under high-temperature, which suggested that *pal1* had a growth inhibition effect in this mushroom (Hou et al., 2019). In sum, the high temperature could induce the expression of *GfPAL* in *G. frondosa*. The *GfPAL* gene might be involved in the secondary metabolites synthesis in this mushroom; however, further studies are required to investigate its role in heat tolerance (Fig. 8).

5. Conclusion

High temperature severely inhibit the growth of *G. frondosa* mycelia. Heat stress response in the major cultivar *G. frondosa* strain Qinghui-151 was characterized using transcriptome and TMT-based proteome analysis. HSPs played a central role in response to heat stress in *G. frondosa*. mTOR and Ca^{2+} signaling pathways to sense and respond to heat stress signals and autophagy. Polyamines, but trehalose, might act as protection agents upon high-temperature treatment. The up-regulation of *GfPAL* genes was related to the thermal tolerance ability of *G. frondosa* strains and might be involved in secondary metabolites synthesis under high-temperature (Fig. 8). The unique features of *G. frondosa* in response to heat stress will be the focus of future studies.

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The authors declare that they did not use generative AI and AI-assisted technologies in the writing process.

CRediT authorship contribution statement

Hongyan Xie: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Funding acquisition. **Luzhang Wan:** Methodology, Supervision, Project administration. **Jiandong Han:** Formal analysis, Investigation, Data curation. **Chunyan Huang:** Formal analysis, Resources. **Jin Li:** Investigation, Software. **Qiang Yao:** Formal analysis, Data curation. **Peng Yang:** Investigation. **Yan Zhang:** Investigation. **Zhiyuan Gong:** Conceptualization, Supervision, Project administration, Funding acquisition. **Hao Yu:** Conceptualization, Methodology, Formal analysis, Software, Data curation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Data availability

RNA-Seq raw data have been deposited in the Genome Sequence Archive in National Genomics Data Center under accession number CRA011647 (https://bigd.big.ac.cn/gsa/browse/CRA011647).

The raw data for the proteome analysis reported in this paper have been deposited in the OMIX, China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Science under accession number OMIX004402.

Other data will be made available at https://file.mushroomlab.cn or on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2023.112542.

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