Original Paper

Proteome and Transcriptome Reveal Involvement of Heat Shock Proteins and Indoleacetic Acid Metabolism Process in *Lentinula Edodes* Thermotolerance

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Key Words

L. edodes • Heat stress • Thermotolerance • LeDnaJ • IAA

Abstract

Background/Aims: Heat stress could cause huge losses for *Lentinula edodes* in China and other Asian cultivation areas. Yet our understanding of mechanism how to defend to heat stress is incomplete. **Methods:** Using heat-tolerant and heat-sensitive strains of *L. edodes*, we reported a combined proteome and transcriptome analysis of *L. edodes* response to 40 °C heat stress for 24 h. Meanwhile, the effect of *LeDnaJ* on the thermotolerance and IAA (indoleacetic acid) biosynthesis in *L. edodes* was analyzed via the over-expression method. **Results:** The proteome results revealed that HSPs (heat shock proteins) such as Hsp40 (DnaJ), Hsp70, Hsp90 and key enzymes involved in tryptophan and IAA metabolism process LeTrpE, LeTrpD, LeTam-1, LeYUCCA were more highly expressed in S606 than in YS3357, demonstrating that HSPs and tryptophan as well as IAA metabolism pathway should play an important role in thermotolerance. Over-expression of *LeDnaJ* gene in S606 strains showed better tolerance to heat stress. It was also documented that intracellular IAA accumulation of S606 (8-fold up) was more than YS3357 (2-fold up), and exogenous IAA enhanced *L. edodes* tolerance to heat stress. **Conclusion:** Our data support the interest of LeTrpE, LeDnaJ, tryptophan and IAA could play a pivotal role in enhancing organism thermotolerance.

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Introduction

Lentinula edodes, which is one of most consumed mushrooms in the world and is famous for its nutritional properties and pharmacological effects and its invaluable effective bio-conversion for agricultural wastes, is the most widely cultivated edible mushrooms in the world and ranks second behind only *Agaricus bisporus* in terms of worldwide production [1-3]. For hundreds of years, medicinal mushrooms such as *L. edodes* have been used as decoctions and essences and are applied as alternative medicine in China, Korea, Japan and eastern Russia [4]. As a result, the cultivation areas of *L. edodes* have increased gradually in different regions of the world. China, where millions of tons of *L. edodes* account for two-thirds of the total world's production [5], spans different monsoon climates from south to north. According to different cultivation seasons, the cultivation mode of *L. edodes* involves spring, summer and fall planting. In the presence of high temperatures during the summer period, the mycelial viability of *L. edodes* inoculated in the spring becomes weak, causing areas of rotted logs or bags that are infected by other pathogens and a loss in yield and quality [6, 7]. Additionally, the thermotolerance capacities of *L. edodes* strains in PDA medium were tested and were shown to be obviously different [8]. Therefore, it is of both theoretical and practical significance to determine how to acquire thermotolerance under heat stress for *L. edodes*, which will eventually contribute to the mushroom cultivation industry.

Based on transcriptomic, proteomic and metabolomic analyses, the ways in which organisms respond to heat stress are diverse [9-11]. Based on the molecular level and metabolite network, the heat shock protein (HSP) family, plant hormones, redox proteins and signal transduction such as Ca^{2+} were positively correlated with the organism thermotolerance [12-15]. Among these factors, HSPs that play a crucial role in the thermotolerance of organisms are well known. Hsp100, Hsp90 and Hsp70 refold and reactivate misfolded proteins and prevent incorrect protein aggregation, improving the defense against the heat shock response in plants and fungi [16-18]. Small Hsps such as Hsp20 and Hsp40 (DnaJ) were combined with other chaperones to protect organisms from heat stress [19, 20]. Nevertheless, for higher basidiomycetes, the expression levels of Hsp9 and Hsp20, as well as that of Hsp70, were studied in *Grifola frondosa*, *A. bisporus* and *Pleurotus tuber* [21-23].

In addition, other factors, including the plant hormones ABA (abscisic acid) and SA (salicylic acid) as well as reactive oxygen, could improve organism thermotolerance [24, 25]. In *Saccharomyces cerevisiae*, the downstream gene activation of hypertonic glycerol signaling pathways could protect cells from heat stress damage, and gene mutation or silencing of the tryptophan metabolite pathway could improve mutant strain thermotolerance [26, 27]. Furthermore, exogenous trehalose and nitric oxide alleviated the heat stress-induced oxidative damage of *Pleurotus* sp. [28-30], and the overexpression of the methionine sulfoxide reductase A (*MsrA*) gene enhanced the heat tolerance of *P. ostreatus* [31]. Thus only few studies on the thermotolerance signal in higher basidiomycetes have been published.

Omics technology advances increasingly facilitate the comprehensive analysis of protein expression and mRNA transcript abundance of organisms subjected to different stressors at different subcellular levels. The transcriptome and proteome changes could provide an aid in determining the molecular pathways underlying the response to heat stress. The current study was undertaken: (a) to test the thermotolerance to sixty *L. edodes* strains to 24 h of high-temperature stress; (b) to characterize the molecular changes via exploiting the technologies of RNA-seq and LC/MS-MS to dissect the transcriptome and proteome, respectively; (c) the transgenic over-expression of *L. edodes* *LeDnaJ* enhanced resistance to heat stress, and (d) to detect the effects of o-aminobenzoic acid (OABA), tryptophan and indole acetic acid (IAA) on the thermotolerance of the *L. edodes* strains. Such an integrated approach elucidated the effects on the network of biological pathways and adaptive pathways, which regulate the *L. edodes* response to heat stress.

Materials and Methods

Mushroom strains, growth conditions and thermotolerance assay

Sixty *L. edodes* strains from wild and cultivation strains [32, 33] were used to screen thermotolerance in this study by primarily observing the mycelial recovery. The well-grown mycelial plugs were inoculated into sawdust plates (78 g of hardwood sawdust, 20 g of wheat bran, 2 g of lime and 20 g of agar in 1 L of

distilled water). According to our previous study [6], the experimental groups were subjected to 39 °C and 40 °C heat stress (HS) for 24h after 6 days of normal growth at 25 °C. Meanwhile, five strains screened in the thermotolerance assay were inoculated into a test tube containing the mixed sawdust medium containing 79% hardwood sawdust, 20% wheat bran, 1% gypsum, and 60% humidity [34]. After 6 days of normal growth at 25 °C, the mycelial elongation position in each test-tube was marked as the starting point, and the test-tubes were subjected to 39 °C, 40 °C and 41 °C heat stress for 24 hours. Subsequently, the test-tubes were placed in the incubators at 25°C. The groups grown at 25°C for the full time were treated as the control. In this assay, we used the mycelial recovery condition and growth rate to quantify the heat sensitivity of the *L. edodes* strains. Based on mycelial recovery and harm conditions, a heat-tolerant strain and a heat-sensitive strain were obtained. Furthermore, we observed the mycelium difference using a microscope and a scanning electron microscope as supplements to thermotolerance determination [7].

Heat treatment and sample collection

After growth in 20 mL of sawdust medium in a 12-cm Petri dish for 10 days at 25 °C, the mycelial cultures of the heat-tolerant strain and heat-sensitive strain were divided into the treatment and control groups. Both strains of the heat treatment groups were exposed to 40 °C stress temperature for 24 h. The strains in the control groups were cultured for the same period of time at 25 °C (NH, not heat stress). After heat stress, the mycelia in each group were collected and frozen in liquid nitrogen immediately and were then stored in -80 °C. The analyses of the proteomes and transcriptomes were carried out for the two comparative groups.

RNA sequencing and transcriptome data processing

Total RNA was isolated using the RNAiso Plus (TaKaRa, DaLian, China) and Fruit-mate™ for RNA Purification (TaKaRa) Plant Kit according to the manufacturer's instructions. The total RNA concentration and purity were detected using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) (A260:A280>2.0). The integrity of RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and only those samples with an RNA integrity number higher than 7.4 were used for RNA sequencing. Twenty micrograms of RNA was equally isolated from mycelia for mRNA isolation, cDNA library construction and sequencing according to the manufacturer's instructions (Illumina, San Diego, CA, USA). The four cDNA libraries were sequenced at Beijing Genomics Institute (BGI)-Shenzhen, Wuhan, China. Paired-end 100-bp deep sequencing was carried out on an Illumina HiSeq 4000 sequencer (BGI). Three independent biological replicates of control (Not Heat) and treatment (40°C, 24 h) were taken.

Twelve raw datasets of all samples were pre-treated by FastQC, filtered and trimmed via Trimmomatic (parameter, PE and phred 33). The indices were constructed via bowtie2 according to *L. edodes* genome files [35], followed by mapping RNA-seq reads to the *L. edodes* genome by tophat 2 using the default settings [36]. Subsequently, Samtools creates sam files for bam files [37]. An integrated and comprehensive transcript set was obtained via Cufflinks and Cuffmerge. We ran the Cuffdiff function to find differentially expressed genes and transcripts in the samples using the default settings [36]. One-way ANOVA was applied, and a step-up FDR p-value was calculated to sort out the statistically significant up/down regulated genes by filtering the data at $p < 0.05$ (FDR) and $|\log_2 \text{ratio}| \geq 1$ in the expression.

Functional analysis of the differentially expressed genes (DEGs) was carried out using Web Gene Ontology Annotation Plotting (WEGO: <http://wego.genomics.org.cn/cgi-bin/wego/index.pl>) [38] and Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways [39] as well as eukaryotic orthologous groups (KOGs) [40]. Transcription factors (TFs) were identified from significant DEG lists using the InterPro number for conserved domains via the Fungal Transcription Factor Database (FTFD) (<http://ftfd.snu.ac.kr/>) and Plant Transcription Factor Database (Plant TFDB) (<http://planttfdb.cbi.pku.edu.cn/>) with an E-value cut-off $< 10^{-5}$ [41, 42].

Protein isolation and proteomics data processing

Protein isolation was operated according to Cai's procedure [43] (Cai Y. et al. 2017). 0.1 g of mycelial powder from each group (four replicates for each group) was mixed with 0.5 mL of extraction buffer (0.5 mM Tris-HCl, 50 mM EDTA, 2% β -mercaptoethanol, 2 mM PMSF). The supernatants were collected by extraction for 10 min and centrifugation at 10,000×g for 15 min at 4 °C to remove the mycelia. The same volume of saturated Tris-phenol was added to the supernatants. The 5 volumes of pre-cooled 0.1 M ammonium acetate in methanol was added to precipitate the protein. The pelleted proteins were washed several times with pre-cooled 80% acetone and were re-solubilized and denatured in 40 μ L of 8 M Urea/100 mM NH_4HCO_3 for 10 minutes. Next, the pelleted proteins were diluted to 200 μ L for tryptic digestion as follows: 10 μ L of 25 mM DTT, 10 μ L of ACN, 110 μ L of 25 mM NH_4HCO_3 , pH 8.2, and 30 μ L of trypsin solution (100 ng/ μ L Trypsin Gold from PROMEGA Corp. in 25 mM NH_4HCO_3).

Mass spectrometric analysis of soluble intracellular proteins was undertaken as described by Gaskell J [44]. In brief, peptide extract in 100% CAN and 0.5% trifluoroacetic acid was analyzed by LC-MS/MS using an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific) coupled to a nano Easy high-performance liquid chromatography equipment. Raw MS/MS data were searched against the genome of *L. edodes* v1.0 [35], using the search engine MaxQuant (version 3. 2. 2. 0). The MS/MS search was undertaken according to the following protocols: (i) trypsin was used to digest the proteins allowing two missed cleavages; (ii) oxidation of methionine was chosen as the dynamic modification, and the carbamidomethyl of the cysteine residues was selected as the fixed modification; and (iii) FTMS was chosen as the mass analyzer, with a mass tolerance of 20 ppm and a fragment mass tolerance of 10 top peaks per 100 Da. Protein identifications were accepted if they could be established at greater than 95.0% probability ($p \leq 0.05$) and contained at least 2 identified peptides. The false discovery rate was set as 1% [45]. Proteomic data was subjected to univariate (one-way ANOVA; 5% FDR with an $\alpha = 0.05$) analyses via SPASS 24 (SPSS IBM., Chicago, IL, USA).

Proteins with a fold change (percent of the relative abundance for treatment group / percent of the relative abundance for control group) <0.5 or >2 in all of the groups were considered significantly differentially expressed proteins, and proteins with percent of the relative abundance $>0.1\%$ were treated as up-regulated proteins. Gene ontology (GO) analysis of the identified proteins was performed using the following databases: Swissport database, InterPro Database, Non-Redundant (NR) protein database of NCBI, and conserved domain database (CDD) of NCBI. Based on the GO categories, proteins were classified according to biological processes, molecular functions, and cellular components.

Quantitative reverse transcription-PCR analysis

Total RNA was reverse transcribed into complementary DNA using HiScript II One Step RT-PCR Kit (Vazyme, Nanjing, China) according to the manufacturer's instructions. Gene-specific primers were designed for the target genes and actin transcript (Table S1 - for all supplemental material see www.karger.com/10.1159/000494784/). Quantitative RT-PCR was set up in a 10- μ L total volume containing 5 μ L of AceQ qPCR SYBR Master SYBR (Vazyme, Nanjing, China), 1 μ L of five times diluted cDNA, 0.5 μ L of the forward and reverse primer (10 mM) and 3 μ L of nuclease-free water. The thermal cycling parameters of the reactions were as follows: 3 min at 95 °C for initial polymerase activation, followed by 40 cycles of 20 s at 95 °C for denaturation, 30 s at 60 °C for annealing and 30 s at 72 °C for extension in a 96-well reaction plate. Melting curve analysis of the PCR products was performed to verify their specificity and identity. The results were then analyzed using a CFX Connect real-time PCR system (BIO-RAD). The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method as described by Fan X [46]. The actin gene (*L. edodes* genome Gene ID: LE01Gene01050) expression was used as an internal reference.

Effect of some exogenous substances on *L. edodes* mycelium thermal capacity

Next, 0.01 mM of exogenous substances such as OABA, tryptophan, trehalose and IAA was added to 60 °C sawdust medium, and the group containing the reagent was treated as control. The mycelial plugs (6 mm in diameter) activated for 6 days were inoculated into the sawdust medium. After 5 days of normal growth at 25 °C, the experimental groups were subjected to 40 °C heat stress for 24 hours and were then returned to the incubators at 25 °C. Subsequently, we observed and documented the mycelial recovery condition every four days.

Transgenic manipulation, expression level analysis and thermotolerance test of transformants

For mushroom transgenic manipulation, the ORF of the *L. edodes* heat shock protein 40 (*LeDna*) gene was PCR amplified from the cDNA of strain S606, and the glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*, from reference strain W1-26) promoter was PCR amplified from the DNA of strain W1-26 with primers containing the EcoRI and KpnI restriction sites, respectively (Table S1). Subsequently, they were linked with the pCambia1300 vector via homologous recombination using the ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China) according to the vector construction method of *A. bisporus* [47]. Next, *L. edodes* S606 mycelial plugs (6 mm in diameter) growing for 7 d in malt yeast glucose (MYG) were infiltrated with the suspension of *Agrobacterium tumefaciens* strain AGL-1, including the *Legpd* promoter and *LeDna* gene [48, 49]. At least 10 independent lines were selectively grown on a MYG plate containing 6 μ g/mL hygromycin for further analysis. After five-round sub-cultured mycelia of the transformants in 6- μ g/mL hygromycin MYG plates, 10 stable transformants were used to test the expression change in the transcript level and response to 41 °C HS for 24 h.

Detection and measurement of IAA

To quantify IAA contents, mycelial samples were ground to fine powder in liquid nitrogen. For IAA extraction, it was undertaken according to IAA extraction in plant [50]: (a), 100mg of about 10 d growth

mycelia were extracted once with 750 μ L extraction buffer (methanol: ddH₂O: acetic acid = 80:18:2 (v/v)) for 16h ice bath; (b), transferred the supernatant into a new centrifugal tube and extracted again with 450 μ L extraction buffer (methanol: ddH₂O: acetic acid = 80:19:1 (v/v), 10ng/ml d5-IAA as internal standard) for 4h ice bath; (c), mixed two-time supernatants, dried with N-EVAP, and re-dissolved with 30% methanol (methanol:ddH₂O=3:7 (v/v)). Five concentration gradients (1, 2, 5, 10, 20 PPb) were used to plot the standard curve. Quantification was performed in an ABI 4000Q-TRAR LC-MS system with stable-isotope-labeled auxin as standards according to a method described previously with minor modification.

Results

Heat stress damaged *Lentinula edodes* mycelial growth

High temperatures had a bad effect on *L. edodes* mycelial activity, causing areas of rotted logs or bags that are infected by other pathogens and a loss in yield and quality. After heat stress for 24 h, sixty *L. edodes* strains showed different thermal capacities, and 40 °C was a lethal temperature for most of the *L. edodes* strains (Table 1). Based on the tube and plate experiments (Table S2 and Table 1), we documented that the recovery capacity of S606 had been proven to be stronger and that YS3357 had lost most of the growth capacity after heat stress, respectively. Therefore, the two strains S606 and YS3357 were screened as the thermo-tolerance strain and a heat-sensitive strain, respectively. As shown in Table 1 and Table S2, after 24 h of heat stress at 40 °C, S606 mycelia could regrow on the sawdust medium in the plate and tube, whereas YS3357 mycelia stopped growing (Fig. 1). According to the microscope and SEM (scanning electron microscope) results, the damage of the cell surface and loss of cytoplasmic inclusions could be observed after heat stress, and the damage level of S606 was weaker than that of YS3357 (Fig. 1 b, f and g, h). Thus, S606 and YS3357 provide a group of experimental models to study the mechanisms of intrinsic thermotolerance in *L. edodes*.

Transcriptome analysis of *L. edodes* strains S606 and YS3357 after heat stress

To investigate the role of genes involved in defending heat stress for *L. edodes*, the transcriptome profile of the *L. edodes* strains S606 and YS3357 at 25 °C and 40 °C were analyzed via high-throughput sequencing. Consequently, 26106469 and 19623606 100-bp paired-end reads were gained in YS3357 at NH (not heat stress) and HS (heat stress), whereas 24349141 and 27245155 reads were gained in S606 at NH and HS, respectively (Table S3). Using the splice-aware aligner Tophat2, approximately 50% of reads in YS3357 and 80% of reads in S606 were mapped to the *L. edodes* (WX1-206) genome sequence. The ratio mapping to the genome was related to the genetic characteristics of different *L. edodes* strains: WX1 (WX1-206 parent) and S606 were *L. edodes* cultivated strains, while YS3357 belongs to the wild-type strain. The expression levels of transcripts were measured as reads per kilobase of transcript per million mapped fragments (RPKM). According to the RPKM values, we calculated the Pearson correlation coefficients of different replicates per group. Their values were more than 96.2% (YS3357) and 82.9% (S606) in three replicates per group, respectively (Table 2 and Table 3), which indicated that our RNA-seq data

Table 1. Recovery conditions of sixty *L. edodes* strains subjected to high temperatures.. Note: ++, Strong resistance; +, Resistance; +-, Instability resistance in different experiment groups; -, Sensitive; --, High sensitive

Strain No.	High temperatures			Strain No.	High temperatures			Strain No.	High temperatures		
	39°C±1	40°C±1	40°C±0.3		39°C±1	40°C±1	40°C±0.3		39°C±1	40°C±1	40°C±0.3
Z9	++	+-	-	Z10	++	-	-	YS3357	--	--	--
Z88	++	-	-	YS94	++	-	-	YS3353	-	-	-
Z87	+-	-	-	L9319	+	-	-	YS3334	+-	+-	-
Z85	+	-	+	YS91	++	-	-	YS30	++	-	-
Z82	+	+	-	YS89	+	-	-	YS29	++	++	-
Z67	+-	-	-	YS88	-	-	-	YS234	-	-	-
Z64	+-	-	-	YS84	+	-	-	YS1518	++	-	-
Z6	++	++	-	YS8	+	+	-	YS1515	-	-	-
Z51	+	+	-	YS79	-	-	-	YS14	++	+	-
Z50	-	-	-	YS78	-	-	-	YS121	-	-	-
Z49	++	-	-	YS76	+	-	-	YS118	-	-	-
Z48	+	++	-	YS70	++	-	-	YS113	-	-	-
Z47	+-	-	-	YS7	-	-	-	YS111	+	+	-
Z42	-	-	-	YS55	--	--	--	YS110	-	-	-
Z31	+-	-	-	YS51	++	-	-	YS11	-	-	-
Z28	+	-	-	YS5	+	-	-	YS104	-	-	-
Z27	-	-	-	YS39	-	-	-	YS100	-	-	-
Z23	+	-	-	YS39	-	-	-	YS1	++	-	-
Z20	-	-	-	YS366	-	-	-	W1	+	+-	-
Z2	-	-	-	YS358	-	-	-	S606	++	++	+

were dependable and reflected the transcriptome profile of *L. edodes* at NH and HS.

The analysis identified the differential expression of 512 and 1375 (more than 2-fold) genes responsive to heat stress in S606 and YS3357, respectively (Fig. S1). Gene Ontology (GO) annotation of differentially expressed genes (DEGs) was carried out. The functional analysis of the GO term for cellular component in S606 and YS3357 demonstrated that many genes belonged to cell part, organelle and macromolecular complex (Fig. 2a). The enriched GO obtained via biological process functional categorization revealed that the largest numbers of genes for S606 and YS3357 were related to metabolic process, cellular process, localization,

biological regulation, pigmentation and response to stimulus. The contribution of other binding proteins, catalytic activity, transporter activity and structural molecule activity was high in functional categorization among all GO terms. The numbers with differentially expressed (less than 0.5-fold) genes were 524 and 1024 in S606 and YS3357, respectively (Fig. 2b). From the perspective of the GO term for molecular function, many genes were involved in binding containing protein binding, ion binding, nucleotide binding and cofactor binding, and catalytic activity such as hydrolase activity, transferase activity, oxidoreductase activity and ligase activity. Among the DEGs, many genes encoding *L. edodes* HSPs showed a dramatic up/down-regulation: in two strains after heat stress, the expression levels of many *sLeHsp* genes including *LeHsp10*, *LeHsp12*, *LeHsp16*, *LeHsp18.1*, *LeHsp18.2* and *LeHsp30* showed obvious upregulation; the expression levels of *LeHsp60* gene displayed no difference; most of *LeHsp70* upregulation levels were more obvious; the expression levels of *LeHsp90*, *LeHsp98* and *LeHsp104* were significant upregulation (Fig. 3a); a small amount of the *LeDnaJ* expression levels up-regulated (Fig. 3b),

Fig. 1. The morphology of two tested *L. edodes* strain mycelia response to heat stress. The upper of the Fig. shows schematic representation of heat stress experiment. Six-day-old mycelial colonies were subjected to a heat regime of 24 h, 40°C, followed by recovery at 25°C. Untreated groups were kept at 25°C during the whole time. Subsequently, mycelia were observed at 6 d before and after heat stress by microscope and scanning electron microscope (SEM), and mycelia colonies were photographed at 0 h and 15 days after heat stress, respectively. (a) and (c), S606 and YS3357 mycelia at 25 °C via microscope (100×). (e) and (g), S606 and YS3357 mycelia at 40 °C via microscope (100×). (b) (d) (f) and (h), S606 and YS3357 mycelia at 40 °C via SEM (10K ×).

Table 2. Pearson correlation coefficients distribution at NH and HS in S606. Note: NH and HS indicate not heat stress and heat stress, respectively. T1, T2 and T3 are three replicates for S606 at the same condition

Strains	62 NH T1	62 NH T2	62 NH T3	64 HS T1	64 HS T2	64 HS T3
S606NH T1	1	0.8372	0.973892	0.231608	0.209162	0.18048
S606NH T2	0.8372	1	0.829936	0.236572	0.201933	0.226888
S606NH T3	0.973892	0.829936	1	0.21951	0.200089	0.169767
S606HS T1	0.231608	0.236572	0.21951	1	0.996862	0.855491
S606HS T2	0.209162	0.201933	0.200089	0.996862	1	0.829758
S606HS T3	0.18048	0.226888	0.169767	0.855491	0.829758	1

Table 3. Pearson correlation coefficients distribution at NH and HS temperatures in YS3357. Note: NH and HS indicate not heat stress and heat stress, respectively. T1, T2 and T3 are three replicates for YS3357 at the same condition

Strains	YS3357NH T1	YS3357NH T2	YS3357NH T3	YS3357HS T1	YS3357HS T2	YS3357HS T3
YS3357NH T1	1	0.962039	0.980254	0.258038	0.183911	0.222963
YS3357NH T2	0.962039	1	0.963431	0.266816	0.194929	0.226559
YS3357NH T3	0.980254	0.963431	1	0.255209	0.178706	0.228453
YS3357HS T1	0.258038	0.266816	0.255209	1	0.986687	0.983421
YS3357HS T2	0.183911	0.194929	0.178706	0.986687	1	0.963181
YS3357HS T3	0.222963	0.226559	0.228453	0.983421	0.963181	1

demonstrating that the expression of HSP genes were response to heat stress in *L. edodes*.

To further assess the functions of DEGs against heat stress, the pathways were analyzed via the KEGG database. Our KEGG analysis (Table S4) found that most of the DEGs were involved in metabolic pathways, biosynthesis of secondary metabolites, biosynthesis of antibiotics, microbial metabolism in diverse environments, MAPK (mitogen-activated protein kinase) signaling pathway - yeast, and oxidative phosphorylation. As the secondary messenger, DEG expression levels involved in the calcium signaling pathway and cAMP-dependent pathway were higher after heat stress. However, we found that the expression levels of DEGs involved in the mTOR signaling pathway displayed complicated changes. After heat stress, the mTOR signaling pathway may regulate cell growth via the MAPK signaling pathway. Among those DEGs, we identified 19 and 21 TFs (transcript factors) (Table S5) for S606 and YS3357 from the FTFD and Plant TFDB pipeline, respectively. For two *L. edodes* strains, TFs were involved in the major TF families in most fungi and plants such as bHLH, zinc finger, bZIP, CBF1, NFYB_HMF, Homeobox, Myb_DNA-binding, and the special families in most fungi such as HMG_box TFs. These TFs may play an important role in the *L. edodes* response to heat stress. However, the TF encoded by the DEG HSF_DNA-bind was found in the thermotolerant strain S606 but not in thermosensitive strain YS3357, demonstrating that it should have a significant effect on the *L. edodes* defense against heat stress. KOG analysis showed that most of DEGs involved in signal transduction mechanisms, transcription, posttranslational modification and protein turnover and chaperones (Fig. S2).

Differential proteome analysis of L. edodes strains S606 and YS3357 after heat stress

To better understand *L. edodes* thermotolerance, the whole cell proteins of S606 and YS3357 after 24 h of heat stress were analysed by nano-arbitrap LC/MS-MS. From the LC/MS-MS results, 9394 peptides were identified by MaxQuant searching against *L. edodes* genome annotation files (Table S6); 1181 and 1298 proteins were obtained for the heat-tolerant strain S606 in the control and treatment groups, respectively; nevertheless, approximately

Fig. 2. Functional classification of gene orthology (GO) of transcriptome data. (a), GO term of differential up-regulation genes in two strains. (b), GO term of differential down-regulation genes in two strains. Y3357, YS3357.

1184 and 903 proteins were identified for the heat-sensitive strain YS3357 in the two groups (Fig. 4a). There were fewer identified proteins (1393) in our results, which could have been related to genetic differences between the reference strain W1-206 and the two tested strains of the study. For S606 after heat stress, up- and down-regulated proteins accounted for approximately 7.794% and 3.162% (Fig. 4b), respectively, whereas 3.47% of the proteins were up-regulated and 11.514% were down-regulated in YS3357 (Fig. 4b). The proportion of down-regulation proteins in S606 was lower than that in YS3357, indicating that S606 had a better thermotolerant capacity than YS3357. We found that 106 proteins in S606 (HS vs NH) were significantly up-regulated. In order to find critical protein accurately, Based on crossing part between upregulation proteins in S606 after heat stress and proteins of

which the relative abundances in S606 were higher than it in YS3357, 31 proteins were significantly different before and after heat stress for the S606 and YS3357 strains, and their relative protein abundance is shown in Fig. 3d and e. Surprisingly, it was documented that the anthranilate synthase (TrpE, LE01Gene02473) level in S606 had approximately 500-fold increases after heat stress, respectively, but their levels in YS3357 were down-regulation (Fig. 5 and Table S7), demonstrating that they possess the potential in *L. edodes* thermotolerance.

After heat stress, many heat shock proteins (HSPs) containing LesHsp, LeDnaJ (Le01Gene01273) and LeHsp98 were expressed inducibly in the two strains (Table S8). Among these LeHSPs, we found that in S606 and YS3357, the LesHsp such as LeHsp16, LeHsp18.1, LeHsp18.2 and LeHsp30 were induced significantly but showed no obvious difference (Fig. 3d and Table S8). The content of LeDnaJ (LE01Gene01273) in S606 (0.06256‰) was approximately ten-fold compared with that of YS3357 (0.008651‰) that had no significant change, indicating that this LeDnaJ could played an important role in the process of *L. edodes* thermotolerance. Among seven LeHsp70 proteins, only one protein was upregulated in S606 but down-regulated in YS3357; the expression of LeHsp98 showed an approximately 100-fold increase in S606 but an approximately 16-fold increase in YS3357. However, the LeHsp90 and LeHsp104 showed no obvious difference in both two strains after heat stress (Fig. 4d and Table S8).

Transcriptome and proteome data correlations

The increased coverage for proteins, encoded by more abundant transcripts, was observed. Only six proteins that were obtained by LC-MS/MS (liquid chromatography-tandem mass chromatography) showed no read signals. A large overlap of identifications was obtained (1394) in the two strains when LC-MS/MS and RNA-seq data were compared. Subsequently, whether the changes in protein levels correlated with changes in the corresponding transcripts was investigated. Based on differentially expressed proteins ($|\text{Fold}| \geq 2$), we found

Fig. 3. Heat map of HSPs' gene expression data after heat stress. (a), Heat map showing HSP gene expression change ($\log_2$ fold change) except for DnaJ in two tested *L. edodes* strains after heat stress compared with control groups (NH). (d), Heat map showing DnaJ gene expression change ($\log_2$ fold change) in two tested *L. edodes* strains after heat stress compared with control groups (NH). YS3357, YS3357; Red, upregulated; Green, downregulated; scale bar given at the top of the heat map.

Fig. 4. Venn diagram and heat-map representation of the proteomic dataset. (a), Venn diagram showing number of identified proteins before and after heat stress for two *L. edodes* strains. (b), Portion of up or down regulation proteins after heat stress. (c), Venn diagram showing 106 up-regulation proteins in S606 (HS vs NH) and 44 up-regulation proteins in YS3357 (HS vs NH) and 1005 proteins of which the relative abundances in S606 were higher than it in YS3357. (d), Heat-map is shown to indicate relative abundance for heat shock proteins among the 4 samples. The abundance of each protein was shown according to its portion (%) among all identified proteins for every strain at single treatment. The different colors correspond to the values of protein abundance as indicated by the bar at the top of the heat map. YS3357, YS3357; NH, not heat stress; HS, heat stress.

abundances in S606 were higher than it in YS3357. (d), Heat-map is shown to indicate relative abundance for heat shock proteins among the 4 samples. The abundance of each protein was shown according to its portion (%) among all identified proteins for every strain at single treatment. The different colors correspond to the values of protein abundance as indicated by the bar at the top of the heat map. YS3357, YS3357; NH, not heat stress; HS, heat stress.

that approximately 10% and 20% of the genes encoding the differentially expressed proteins showed a direct conflict between the transcript- and protein-level changes for the same gene, respectively (Fig. 6). After heat stress, most of heat shock protein genes included in *Leshsp* and *LeDnaJ* as well as *Lehsp98* were obviously upregulated based on our transcriptome data (Fig. 3a), which was consistent with heat shock protein content changes that most of heat shock proteins were induced dramatically or upregulated obviously (Fig. 4d). Nevertheless, most of genes related to tryptophan and IAA biosynthesis had an obvious upregulation in term of protein content, yet their transcription levels had no change or downregulation (Table S9). For *LeTrpE* after heat stress, it had a dramatical upregulation in protein changes but downregulation in transcription level. These results demonstrated that for the two tested *L. edodes* strains, the protein profile changes after heat stress might be controlled at the post-transcriptional level or might involve interacting proteins and that the mRNA expression changes provided only limited insight into changes in the protein expression.

Validation of proteome and RNA-seq data by qRT-PCR

According to the proteome and transcriptome data, we found that the expression levels of many genes were significantly up-regulated. To validate the results of the proteome and RNA-seq analysis, twenty-one genes included in heat shock protein and other genes related to tryptophan and IAA biosynthesis were selected to confirm differential expression by quantitative real-time PCR. For tryptophan and IAA biosynthesis, the expression levels of the anthranilate synthase gene *LetrpE* with an obvious increase in protein content were significantly down-regulated in the two tested strains after heat stress, and expression levels of tryptophan synthase *LetrpB-39* and indole-3-pyruvate monooxygenase *LeYUCCA* were upregulated obviously in S606 after heat stress, whereas the expression levels of other genes showed no changes (Fig. 7a and b). Those fifteen genes, including four *Leshsp* (*LeHsp16*, *LeHsp18.1*, *LeHsp18.2* and *LeHsp30*), genes, three *LeDnaJ* gene, one *LeHsp60* gene,

three *LeHsp70* genes, one *LeBAG* gene encoding Bcl-2-associated athanogene protein (a regulator of Hsp70), one *LeHsp90* gene, one *LeHsp98* gene and one *LeHsp104* gene, are known to be associated with heat stress. Our results indicated that most of these fifteen gene expression levels were significantly up-regulated in the two *L. edodes* strains after heat stress and showed that the expression patterns of those genes were similar to the differential analysis results from the RNA-seq (Fig. 7). Heat shock proteins, an important factor in the response to many stresses, showed diverse expression changes in the presence of heat stress. The different expression levels of *LeHsp* (except for *LeDnaJ*), *LeHsp70*, *LeHsp90* and *LeHsp104* genes in YS3357 were obviously higher than those in S606, demonstrating that they may strongly respond to heat stress but have no enough effect on the thermotolerance (Fig. 7c). For other Hsps such as *LeDnaJ* (*LeDnaJ-07*, LE01Gene01273), *LeBAG* and *LeHsp98*, their up-regulated levels were higher in S606 than in YS3357 after heat stress, demonstrating that they may have protectable effect on *L. edodes* mycelial against heat stress damage (Fig. 5d, e and f). Nevertheless, the gene expression level of *LeHsp60* changed little (Fig. 7f).

Key enzymes of Tryptophan and IAA biosynthesis related to the heat stress

In our proteomic results, we found that the protein expression level of OABA synthase, one rate-limiting enzyme in tryptophan biosynthesis, increased dramatically after 24 h of heat stress in S606 (Table S9) but decreased negligibly in YS3357 after 24 h heat stress. Except that, *LeYUCCA* (indole-3-pyruvate monooxygenase), an important enzyme for IAA biosynthesis, in S606 was upregulated but downregulated in YS3357 after 24 h heat stress (Table S9). Therefore, it was speculated that the tryptophan and IAA pathway played a significant role in the *L. edodes* mycelia defense to heat stress. According to transcriptome results, it was documented that *LetrpE* transcription levels in two strains were downregulated after 24 h heat stress, and *LeYUCCA* transcription levels in two strains were upregulated after 24

Fig. 5. Heat-map showing the relative abundance for 31DEPs among the two strains. The abundance of each protein was shown according to its portion (%) among all identified proteins for every strain at single treatment. The different colors correspond to the values of protein abundance as indicated by the bar at the top of the heat map. Y3357, YS3357; NH, not heat stress; HS, heat stress.

Fig. 6. The proportional of the protein changes and transcript level changes in *L. edodes* at 25°C and 40°C. Up or down, different expression genes or protein.

Fig. 7 qRT-PCR analysis. (a and b), qPCR validation of six key genes in tryptophan and IAA biosynthesis at 25°C compared with 40°C. (c, d, e and f), qPCR validation of the up-regulation of genes encoding LeHsp at 25°C compared with 40°C. c, Four genes encoding LeHsp (except for LeDnaJ). d, Three genes encoding LeDnaJ. e, Three genes encoding LeHsp70 and one gene encoding LeBAG. f, Four genes encoding LeHsp60, LeHsp90, LeHsp98, LeHsp104, respectively. *LearoC*, Chorismate synthase; *LetrpE*, Anthranilate synthase; *LetrpF*, N- (5'-phosphoribosyl) anthranilate (PRA) isomerase; *LetrpB*, Tryptophan synthase; *LeTam-1*, Tryptophan aminotransferase; *LeYUCCA*, Indole-3-pyruvate monooxygenase; *LeHsp*, Heat shock protein; 1, S606 NH; 2, S606 HS; 3, YS3357 NH; 4, YS3357 HS.

h heat stress (Table S9). Meanwhile, it was documented that IAA could be synthesized via indole-3-acetamide (IAM) and indole-3-pyruvic acid (IPA) pathways (Fig 8a). To further support the observed changes in the tryptophan and IAA biosynthesis-related protein levels, we chose six structural genes (*LearoC*, *LetrpE*, *LetrpF*, *LetrpB-39* (LE01Gene07739), *LeTam-1*, *LeYUCCA*) for additional analyses by quantitative real-time polymerase chain reaction (q-RT-PCR). The results showed that the *LetrpB-39* and *LeYUCCA* transcripts were up-regulated in S606 after 24 h of heat stress but that the *LetrpE* transcripts in the two strains were obviously down-regulated (Fig. 8a), a finding that was not associated with the proteomic data (Tables S9). Based on the above findings, we predicted that these enzymes underwent interaction with other proteins such as *LeHsp* or proteases to prevent *L. edodes* mycelia from heat stress damage. In the presence of abundant protective molecules, the enzyme activity and structure were stabilized, enhancing *L. edodes* mycelial thermotolerant activity against heat stress. And, expression levels of indole-3-pyruvate monooxygenase *LeYUCCA* were upregulated obviously in S606 after heat stress, and relative expression of *LeYUCCA* in S606 was obvious higher than it in YS3357 (Fig. 7b). Therefore, we speculated that IAA could play a key role in *L. edodes* thermotolerance, and detected intracellular IAA concentration after heat stress. Intracellular IAA concentration of S606 had a dramatical change (about 8-fold) and was about 2-fold higher than it of YS3357 after heat stress, while it of YS3357 had an about 2-fold increase (Fig. 8b), indicating that IAA should play a positive role in *L. edodes* thermotolerance.

Effect of OABA, tryptophan and IAA on L. edodes mycelial growth and thermotolerance

When OABA, tryptophan and IAA were added to the sawdust medium, different effects on mycelial growth were documented: for S606, 0.01mM OABA and IAA had a positive role in mycelial growth, while tryptophan led to a negative effect on mycelial growth; nevertheless, for YS3357, 0.01mM tryptophan was beneficial to mycelial growth (Table S10). Meanwhile, it was found that exogenous 0.01mM OABA, tryptophan and IAA had no obvious effect on the transcript expression levels of the genes related to IAA biosynthesis in S606; however, the transcript expression of *LetrpE* and *LeYUCCA* in YS3357 showed an obvious increase after the addition of OABA, tryptophan and IAA (Fig. S3). Mycelial recovery tests (Fig. 8c) demonstrated that for S606, all of tryptophan and OABA could enhance the *L. edodes* mycelial recovery activity after 24 h of heat stress; nevertheless, 0.01mM IAA could retard mycelial recovery growth but 0.1 nM had little effect on mycelial recovery growth, and the recovery activity of mycelia in the medium containing 0.01 mM trehalase was decreased slower than that of the control group. Regarding YS3357, 0.01 mM IAA and 0.1nM IAA played a positive

Fig. 8. Effect and Protein changes of OABA, tryptophan and IAA on *L. edodes* mycelia after heat stress. (a), IAA biosynthesis pathway in *L. edodes*. (b), IAA content changes in S606 and YS3357 after 40 °C heat stress. (c), OABA and tryptophan and IAA enhance *L. edodes* thermotolerance. Mycelial colonies growing for 6 d were subjected to 24 h heat stress of 40 °C, then photographed after 15 recovery growth at 25 °C. For IAA group, mycelial colonies growing for 4 d were subjected to 24 h heat stress of 40 °C, then photographed after 15 recovery growth at 25 °C. 1, S606 NH (not heat stress); 2, S606 HS (not heat stress); 3, YS3357 NH; 4, YS3357 HS; OABA, O-aminobenzoic acid; N-5PA, N-(5'-phosphoribosyl) anthranilate; CDRP, Enol-1-(O-carboxyphenylamino)-1-deoxy ribulose phosphate; IGP, Indole-3-glycerol phosphate; IPA, Indole-3-pyruvic acid; IAAid, Indole-3-glyoxylic acid; IAM, indole-3-acetamid; IAA, Indole-3-acetic acid; * indicates the difference; ** indicates the significant difference.

role in mycelial recovery activity, and colony diameters of mycelia were about 8.10 ± 0.20 cm (0.01mM IAA) and 8.30 ± 0.23 cm (0.1 nM IAA), respectively, demonstrating that 0.01mM IAA had a better effect than 0.1 nM IAA for mycelial recovery growth. However, other substrates produced little effect on mycelia recovery.

Over-expressing *LeDnaJ* enhances thermotolerance of *L. edodes* strain S606

In recent years, it has been found that plant DnaJ proteins (DnaJ) played important roles in response to both biotic and abiotic stresses, such as drought, bacterium disease, and heat [19, 51]. Based on *L. edodes* genome and omics results, one gene (Le01Gene01273) encoding *LeDnaJ* was dramatically upregulation (Fig. 3a and Fig. 4d). Full-length cDNA of *LeDnaJ* consists of 1119 nucleotides and encodes a protein of 372 amino acids, and contains a predicted J-domain conserved

among plant and yeast as well as *Escherichia coli* DnaJ proteins. To investigate the function of *LeDnaJ* in *L. edodes*, the over-expression vector was constructed and transformed into *L. edodes* mycelia. qRT-PCR analysis was undertaken to detect whether the *LeDnaJ* expression levels of ten transformants were upregulated. Among the ten transformants, the *LeDnaJ* expression levels in seven transgenic strains had a more two-fold upregulation, and two transformants displayed approximately 7.0- and 13.9-fold upregulation, respectively (Fig. 9a), verifying that the *LeDnaJ*-pCAMBIA1300 expression vector was transformed into *L. edodes* mycelia and that the *LeDnaJ* gene was overexpressed. At 25 °C, mycelial growth rates of two transformants had no significance to that of wild S606 (Fig. 9b). After 41 °C heat stress for 24 h, that two obvious overexpression transformants mycelia started to germinate approximately 15 d after 25 °C recovery, and mycelial colony diameter (cm) of two transformants were 7.05 ± 0.24 and 6.66 ± 0.26 respectively, but the wild-type S606 strain with 2.96 ± 0.17 cm colony diameter had no recovery trend 2.96 ± 0.17 (Fig. 9 c and d), which indicated that *LeDnaJ* could enhance *L. edodes* thermotolerance.

Fig. 9. qRT-PCR and phenotype analysis of over-expressed-*LeDnaJ* in S606. (a), qPCR validation of *LeDnaJ* over-expression transformants. 1-10, Ten *LeDnaJ* overexpression transformants. (b), Mycelial growth rate of wild and transgenic strains at 25°C. 1, *LeDnaJ*-o-3; 1, *LeDnaJ*-o-5. (c), Colony diameter of wild and transgenic strains for 15-day recovery growth after 41°C heat stress. 1, *LeDnaJ*-o-3; 1, *LeDnaJ*-o-5. (d), Colony characteristics of wild and transgenic strains for 15-day recovery growth after 41°C heat stress. For *LeDnaJ* overexpression transformants of S606 and wild S606, mycelial colonies growing for 6 d were subjected to 24 h heat stress of 42 °C, then photographed after 15 recovery growth at 25 °C. ** indicates the significant difference.

Fig. 10. IAA quantification and qRT-PCR analysis of the genes related to Tryptophan and IAA biosynthesis in *LeDnaJ* overexpression transformants. a, qRT-PCR analysis of the genes related to Tryptophan and IAA biosynthesis in *LeDnaJ* overexpression transformants. b, IAA quantification of the WT strain and *LeDnaJ* overexpression transformants. The lowercase (a-d) indicates the significant difference. * indicates the significant difference.

IAA quantification and qRT-PCR analysis of the genes related to Tryptophan and IAA biosynthesis in *LeDnaJ* over-expression transformants

The relationship of *LeDnaJ* with the IAA biosynthesis was investigated by analyzing the transcript level of the genes related to IAA biosynthesis. The qRT-PCR results indicated that the expression of *LetrpE* gene in two *LeDnaJ* overexpression transformants had an about 1.6-fold increase compared to the WT; the expression levels of *LetrpD* gene in two *LeDnaJ* overexpression transformants showed 1.7 and 1.3-fold increase, respectively; the expression levels of *Letam-1* in *LeDnaJ*-o-3 and *LeYUCCA* in *LeDnaJ*-o-5 displayed about 2-fold increases (Fig. 10a), indicating that *LeDnaJ* maybe participate in the IAA biosynthesis. In the absence of heat stress, S606 and *LeDnaJ* over-expression transformants showed a fine difference in the intracellular IAA concentration, but displayed an obvious difference in the presence of heat stress. After heat stress, the intracellular IAA concentration in the *LeDnaJ* over-expression transformants showed an obvious increase than WT S606 (Fig. 10b).

Discussion

Heat stress for several days inhibits mycelium growth, impairs fruiting and affects mushroom quality [52]. In terms of our previous study, many cultivated strains of *Lentinula edodes* showed a block in growth and decline in stress resistance in the presence of high temperature, causing rotted logs and yield loss during *L. edodes* production [6]. Based on the thermotolerance assay results, it was documented that most of the *Lentinula edodes* strains lost growth capacity after 24 h heat stress (Table 1). Via microscope and SEM, we observed that the two tested strains showed mycelial breakage, such as damage to the cell surface and a loss of cytoplasmic inclusions and cell death, similar to the symptoms of *Agaricus bisporus* mycelia and *Saccharomyces cerevisiae* after heat stress [21, 53, 54]. In particular, clamp connections, which are an important genetic characteristic for the differentiation between *L. edodes* monokaryons and dikaryons [55], showed more obvious differences in the two tested strains after 24 h of heat stress; the harm level of the thermotolerant strain S606 was lower than that of the heat-sensitive strain YS3357 (Fig. 1 and Table S2).

HSPs in response to heat stress

In the heat-shock protein family, the typical characteristics of the HSR (heat stress response), which is an important regulator of heat stress, was found in plants and fungi subjected to heat shock and stress [17, 18, 56]. Based on our proteomic and transcriptomic results, the expression levels of many *LeHsp* genes and proteins were strongly up-regulated: the transcript and protein levels of *LeHsp* and *LeHsp70* were induced or significantly up-regulated after heat stress; *LeHsp98* (LE01Gene03170), an important structural protein, showed remarkable changes in the thermotolerance strain (Fig. 2 and 4). Similarly, in terms of *A. bisporus* mycelia subjected to high temperature, Hsp20 and Hsp70 proteins, as well as Hsp90 protein, which is known to be potentially involved in *A. bisporus* thermo-tolerance, showed faster and more robust accumulation in the thermotolerance strain. Additionally, a

heat shock protein (*HSP70*) gene was found in the vicinity of the QTL (quantitative trait loci) on LG (linkage group) II [21, 57]. Furthermore, the hypocotyl of *Arabidopsis* transgenic plants in which an *A. bisporus Hsp20* gene, possessing more than 50% similarity to *L. edodes* in the amino acid sequence, was transformed could recover after 1 h of heat stress at 45°C [20]. However, most of *LesHsp* gene expression upregulation levels in the thermos-sensitive strain YS3357 after 24 h of heat stress at 40°C were more obvious than those of the thermotolerance strain S606, indicating that *LesHsp* proteins could only play a positive role in response to heat stress, a finding that was consistent with the heat-induced genes playing a negligible role in thermotolerance with little overlap with the heat-shock survival genes [58]. Additionally, it was documented that in the thermotolerant *L. edodes* strain S606, the protein and transcript levels of *LeDnaJ* (LE01Gene01273) were significantly induced and up-regulated after heat stress (Fig 3b and 4d); this relationship between *DnaJ* and thermotolerance has rarely been reported in basidiomycetes. *DnaJ* proteins are important for protein translation, folding, unfolding, translocation, and degradation, primarily by stimulating the ATPase activity of chaperone proteins *Hsp70s* [59, 60]. *Hsp42* required to maintain the solubility of various yeast proteins during heat shock was significantly up-regulated in the previous proteomic and transcriptomic analysis of HSR (heat shock response) [61]. In plants, Kong F. et al [19]. reported that the overexpression of *DnaJ* protein could facilitate plant heat tolerance by alleviating the PSII photoinhibition and protecting the antioxidant enzymatic activity in chloroplasts; the *DnaJ* proteins TMS1 and AtDJB1 play important roles in thermotolerance in *A. thaliana* [62, 63], which is consistent with our results that transgenic overexpression of *LeDnaJ* could enhance *L. edodes* thermotolerance (Fig. 8).

Signal transduction in response to heat stress

Heat signal transduction pathways are composed of some transcription factors and cascades of kinases. Transcription factors of different families were significantly regulated in response to heat stress. Heat shock factors, which is known as the major TF known to mediate HSR, plays a vital role in regulating HSP expression by recognizing a heat shock element located in the promoters of its target genes and providing thermotolerance increase for plants and fungi [64, 65]. And, heat shock factors could affect organism tolerance to high temperatures by controlling the expression levels of HSPs, and *Arabidopsis hsfA2* knockout mutants are defective in thermomemory [66]. According to our results, it was documented that the expression of the *HSF_DNA-bind* gene was obviously downregulated in the thermotolerant strain S606 after heat stress, which indicated that *L. edodes* tolerance to heat stress could be negatively controlled by *HSF_DNA-bind*. Besides, many genes encoding other transcription factors including in bHLH, Myb and C₂H₂ (Zinc finger family protein) regulated significantly in plant after heat stress [67, 68], were among the up- or down-regulated (Table S5). Additionally, the gene expression and protein levels of the BAG gene were dramatically induced and upregulated. The BAG3 family plays an important role in regulating disorders, selective autophagy and selective macroautophagy as an adaptive mechanism to maintain cellular homeostasis [69] and provides the concept that Bcl-2-associated athanogene protein (BAG, LE01Gene00075) may be relevant to *L. edodes* mycelial thermotolerance by regulating the activity of *Hsp70* to maintain cellular homeostasis. Proteases play a regulatory role in maintaining proteome homeostasis in most subcellular compartments. In *Arabidopsis*, FtsH6, a relatively well-known protease, could negatively control the *HSP21* expression level, causing a loss in thermomemory [70]. DEGs encoding proteases like FtsH protease were found in our transcriptome analysis (Table S11).

According to KOG and KEGG pathway analysis, it was documented that DEGs related to calcium signaling and MPKA pathway were significantly regulated (Fig. S2 and Table S4). In *Ganoderma lucidum*, heat stress induced a significant increase in the cytosolic Ca²⁺ concentration, regulating the accumulation of HSPs and downstream events in filamentous fungi [71]. In plants, it was reported that calcium-activated calmodulin (Ca²⁺-CaM) and calcium-dependent protein kinase (CDPK) were directly involved in the heat stress signal transduction pathway and up-regulated heatshock proteins (HSPs) under heat stress [72-74]. Gao J. et al [75]. documented that *Phytophthora sojae* transformants in which PsMPK7 expression was silenced were significantly more sensitive to osmotic and oxidative stress. Based on our results (Table S4), it was found that the expression levels of multiple genes involved in many pathways such as the MPKA signaling and calcium signaling pathways and transcript factors such as heat shock factors were obviously upregulated.

Tryptophan and IAA biosynthesis pathway in response to heat stress

In the presence of heat stress, the transcriptional level of PABA (Para-aminobenzoic acid) synthase in the *A. bisporus* thermotolerance strain was upregulated, and exogenous PABA could alleviate heat stress damage by removing H_2O_2 and elevating defense-related proteins [21]. Based on our proteomic and transcriptomic results, the transcriptional and protein levels of PABA showed little change. Nevertheless, the protein level of OABA synthase, an important enzyme in tryptophan biosynthesis, in the thermotolerant *L. edodes* strain S606 after heat stress had a dramatic upregulation, while that of the heat-sensitive strain YS3357 was not detected, indicating that *LetrpE* could play a pivotal role in *L. edodes* strain thermotolerance. In our recent paper [76], it was reported that the *LetrpE* RNAi decreased the thermotolerance of *L. edodes* strain S606. In *S. cerevisiae*, the overexpression of genes related to tryptophan biosynthesis or exogenous tryptophan could improve the tolerance of ethanol [77]. Jarolim et al [26] documented that in the presence of the proper concentration of tryptophan added to medium, the thermotolerance of *S. cerevisiae* tryptophan mutants was improved significantly: when the tryptophan concentration was higher than 10 $\mu\text{g/mL}$, the thermotolerance of the wild-type and mutant strains became weak and the silencing of enzyme genes related to tryptophan biosynthesis improved the thermotolerance of the mutants, but the key gene involved remains unclear. However, the thermotolerance of the *L. edodes* strain S606 was improved by the supplementation of exogenous tryptophan and OABA in the medium, indicating that the upstream and downstream events of tryptophan biosynthesis played a positive role in improving *L. edodes* thermotolerance. Meanwhile, in terms of the thermophile *Thermus thermophilus*, dimer formation of OABA synthases *trpE* and *trpG* stabilized the activity to keep normal growth at high temperature [78]. For the heat-sensitive strain YS3357, thermotolerance before and after supplementation with exogenous tryptophan showed no change (Fig. 9c), which indicated that proteins related to tryptophan biosynthesis may improve thermotolerance by maintaining enzyme activity interacting with other proteins.

Exogenous IAA maintained normal growth with the heat-sensitive strain YS3357 about seven days after 24 h of heat stress at 40 °C (Fig. 9c), suggesting that a tryptophan downstream event such as IAA biosynthesis may be another way by which *L. edodes* thermotolerance was improved. Nevertheless, it was documented that higher concentration IAA weakened the thermotolerance strain S606 mycelial recovery (Fig. 9c), indicating that IAA level was very vital to improve *L. edodes* mycelial thermotolerance. In plant, Kentucky bluegrass cultivars with relatively higher IAA content may have greater heat tolerance than the one with less IAA content under heat stress [79]; for the heat-treated barley, the anther development could be restored in the presence of two applications of auxin (1-naphthaleneacetic acid or 2, 4-dichlorophenoxyacetic acid) [80]; the heat stress effect on *Brassica juncea* seed and soybean pedicel treated by IAA was mitigated effectively [81]; exogenous IAA leads to a stronger male

Fig. 11. Schematic model for the role of tryptophan, IAA and HSPs in enhancing thermotolerance of *L. edodes*. The dotted arrows and question marks indicate raw data supported by our experiment and being explored to understand their interaction after heat stress. The solid arrows indicate data experimentally supported in other fungal and botanic systems.

sterility phenotype in *Gossypium hirsutum* under HT stress [82]. In fungi, IAA was detected by chromatography and synthesized via IPA (indole-3-pyruvic acid), IAM (tryptamine), and TAM (indole-3-acetonitrile) pathways, in *Phanerochaete chrysosporium*, *Lentinus sajor-caju*, yeast, *Pleurotus ostreatus*, *Tricholoma vaccinum* and *Fusarium graminearum* [83-88]. Based on our proteomic results, those genes encoding IAA via tryptophan and IPA pathway in S606 upregulated obviously after heat stress (Table S8), demonstrating that IAA in *L. edodes* could be synthesized via tryptophan and IPA pathway and accumulated during heat stress. Nevertheless, very little literature that moderate IAA could play a positive role in affecting fungus thermotolerance was found. Therefore, it was the first time that IAA via tryptophan and IPA pathway were effectively to enhance *L. edodes* mycelial thermotolerance, and an excess of IAA could retard *L. edodes* mycelium recovery growth (Fig. 8c). And, in our recent study, it was verified that LeDnaJ participate in the thermotolerance of *L. edodes* via regulating IAA biosynthesis [89].

Conclusion

We speculate a model for the role of tryptophan and IAA biosynthesis and other signals in enhancing *L. edodes* thermotolerance (Fig. 11). Multiple strategies have been employed to adapt to heat stress for *L. edodes*, including the metabolite upregulation of tryptophan and IAA biosynthesis. Experimentally increasing the levels of OABA and tryptophan only enhanced the heat-tolerant strain S606 tolerance against heat stress, whereas exogenous IAA enhanced the tolerance of the two tested strains against heat stress. Our findings provide a new view of enhancing mushroom thermotolerance via tryptophan and IAA biosynthesis. However, how to regulate thermotolerance via tryptophan and IAA remains unclear. To explore their effect on *L. edodes* thermotolerance, the overexpression and silencing of those genes related to tryptophan and IAA biosynthesis are important in identifying crucial genes, and mutants are being selected. Additionally, the mechanism how to affect *L. edodes* thermotolerance via IAA and the between trpE and other proteins need to be studied further. In summary, our data provide comprehensive and reliable transcriptome and proteome data of *L. edodes* at nonconductive temperatures.

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Disclosure Statement

The authors have declared no conflict of interests.

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