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RESEARCH

Transcriptomic Analysis Reveals Dynamics of Gene Expression in Liver Tissue of Spotted Sea Bass Under Acute Thermal Stress

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Abstract

The spotted sea bass (*Lateolabrax maculatus*), a eurythermal species, exhibits strong adaptability to temperature variations and presents an ideal model for studying heat stress-responsive mechanisms in fish. This study examined the liver transcriptome of spotted sea bass over a 24-h period following exposure to elevated temperatures, rising from 25 to 32 °C. The results revealed significant alterations in gene expression in response to this thermal stress. Specifically, we identified 1702, 1199, 3128, and 2636 differentially expressed genes at 3, 6, 12, and 24 h post-stress, respectively. Weighted Gene Co-expression Network Analysis (WGCNA) was used to identify specific gene modules responsive to heat stress, containing hub genes such as *aco2*, *eci2*, *h6pd*, *suclg1*, *fgg*, *fga*, *fgb*, *f2*, and *apoba*, which play central roles in the heat stress response. Enrichment analyses via KEGG and GSEA indicated that upregulated differentially expressed genes (DEGs) are predominantly involved in protein processing in the endoplasmic reticulum, while downregulated genes are primarily associated with the AGE-RAGE signaling pathways. Additionally, 272 genes exhibited differential alternative splicing, primarily through exon skipping, underscoring the complexity of transcriptomic adaptations. These findings provide deeper insights into the molecular responses to thermal stress and are crucial for advancing the breeding of heat-resistant strains of spotted sea bass.

Keywords Thermal stress · *Lateolabrax maculatus* · Liver · RNA-Seq · Alternative splicing

Introduction

Globally, temperatures have changed significantly in recent decades, influencing the frequency, intensity, and duration of extreme weather events, such as high temperatures (Islam et al. 2020). These extreme temperature changes, resulting from climate change, pose one of the most significant challenges to aquaculture productivity, especially for fish in aquaculture facilities (Cascarano et al. 2021). When water temperatures exceed the physiological thresholds of fish, they exhibit various physiological, metabolic, and behavioral adaptations to cope with thermal stress (Roychowdhury et al. 2021). Therefore, analyzing the metabolic processes of fish under thermal stress would provide insights into the molecular mechanisms of responses to abiotic stress.

In recent decades, the rapid advancement of high-throughput sequencing technology has made transcriptome analysis a crucial tool for studying fish responses to environmental changes, providing essential data for aquaculture (Logan and Somero 2010). Numerous transcriptomic studies have been conducted on thermal stress in fish and revealed various different expressed genes. These genes are mainly involved in apoptosis, oxidative stress, protein synthesis, energy metabolism, protein processing in the endoplasmic reticulum, and immune functions (Lewis et al. 2010; Sara et al. 2018). Additionally, heat shock proteins (Hsps) have been found to be significantly upregulated in response to thermal stress (Basu et al. 2002). The spotted sea bass (*Lateolabrax maculatus*), as a eurythermic and euryhaline species, is widely distributed along the Chinese coasts (Wang et al. 2016; Cai et al. 2020) and is known for its remarkable adaptability to temperature variations, with an optimal temperature range of 14 to 25 °C and a thermal tolerance extending from 3 to 34 °C. Its remarkable adaptability to

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temperature variations, high nutritional value, and delightful taste make the spotted sea bass one of the most important and promising species for mariculture in China (Liu et al. 2013). Global warming and increasingly frequent extreme temperature events have exacerbated the challenge of spotted sea bass survival (Sun et al. 2021).

This study investigates the response of spotted sea bass liver tissues to heat stress using dynamic transcriptome analysis to (1) identify the dynamic changes of DEGs in the liver of sea bass after heat stress, (2) determine modules responsive to heat stress and explore the functions of associated genes, (3) explore the existence of two distinct expression patterns of DEGs in the upregulated protein processing in the endoplasmic reticulum pathway during heat stress, and (4) characterize the differential alternative splicing (DAS) events and DAS genes induced by thermal stress. The results of this study will contribute to understanding the response and tolerance mechanisms of spotted sea bass to heat stress, providing new insights into fish adaptation to thermal stress.

Materials and Methods

Experimental System Design and Animals

The thermal stress experiment was conducted at Shuangying Aquatic Seedling Co., Ltd., Lijin, Shandong, China. All the spotted sea bass (*Lateolabrax maculatus*) used in this study were artificially cultured and came from the same batch, ensuring genetic uniformity. Specifically, we randomly selected 60 individuals with an average body length of 13.33 ± 0.24 cm and an average body weight of 38.96 ± 2.01 g. Sixty fish were evenly distributed among three 120-L tanks. Each tank was equipped with a recirculating water supply, a biofiltration system, and a temperature control system. Before the experiment, the water temperature in the tanks was maintained at 25 °C. During the thermal stress experiment, the water temperature was gradually increased from 25 °C to the target temperature of 32 °C at a rate of 1 °C/h and maintained at this temperature until the end of the experiment. To ensure consistency and representativeness, we selected fish of similar age, size, and physiological condition from the same environment. Nine fish from each tank were quickly anesthetized with MS-222 (200 mg/L), and liver samples were collected at 0 h, 3 h, 6 h, 12 h, and 24 h after exposure to high temperature stress. Liver samples were immediately placed in liquid nitrogen and subsequently stored at − 80 °C for RNA extraction.

Quality Filtering and Mapping

Sequencing data quality was assessed using FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). RNA data were quality filtered using Trimmomatic with default parameters (Bolger et al. 2014). The obtained clean reads were mapped to *L. maculatus* reference genome (PRJNA408177) using Hisat2 v2.2.1 (Kim et al. 2015). These reads were summarized at the gene level using featureCounts (Liao et al. 2014).

RNA Extraction, Library Construction, and Sequencing

Total RNA from liver tissues was extracted using TRIzol reagent (Invitrogen, USA). The concentration of total RNA was determined using a NanoDrop 2000 (Thermo Scientific, Waltham, MA), and RNA integrity was checked by the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). To minimize data variation caused by individual differences, equal amounts of RNA from three fish in the same tank were pooled into one sample, with three replicate samples generated for each time point. A total of 15 sequencing libraries (3 replicate samples × 5 sampling time points) were constructed. The Illumina Novaseq™ 6000 was used as a double-end sequencing platform, generating 150-bp paired-end reads.

Identification of DEGs

Counts data were entered into DESeq2 for normalization, and differentially expressed genes were identified using DESeq2. Genes with FDR < 0.05 and |log2(Fold Change (FC)|> 1 at the transcript level between stages were considered differentially expressed (Love et al. 2014).

WGCNA Analysis

To understand the correlations between genes, the expression data were standardized, and FPKM values were calculated. Weighted correlation network analysis (WGCNA) was performed using the WGCNA package in R (Langfelder and Horvath 2008). A topological overlap matrix (TOM) with a soft Power of 14, a minimal module size of 30, and a branch merge cut height of 0.25 was used to construct the co-expression network. The significant modules correlated with heat stress time were analyzed. Module membership (MM) was employed to estimate the association between gene expression profiles and their respective modules. KEGG enrichment analyses were performed using KOBAS (v 3.0) (http://kobas.cbi.pku.edu.cn/) (Bu et al. 2021) to show the functions of the modules. The co-expression network patterns and interactions of key hub genes in the "MMblue" module were visualized with Cytoscape (v 3.8.2) (Shannon et al. 2003).

Time-Series Analysis

The gene expression time-series data were clustered using the fuzzy c-means algorithm in the Mfuzz package (Kumar and Futschik 2007), and consistent expression gene changes were grouped into a cluster.

Functional Enrichment Analysis

The clustered differentially expressed genes (DEGs) were subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis using KOBAS (v 3.0) (Bu et al. 2021).

Protein–Protein Interaction (PPI) Network Analysis

The protein–protein interaction (PPI) network was constructed using String (v 11.5) (https://www.stringdb.org/), with an interaction confidence score threshold greater than 0.4 considered significant for interconnections. Interactions among proteins were exported based on this threshold. Subsequently, the cytoHubba (v 0.1) algorithm was employed to identify hub genes within each module using four methods (MNC, MCC, Degree, EPC) (Doncheva et al. 2019). The protein–protein interaction network was visualized in Cytoscape (v 3.8.2). The hub genes were determined based on the connectivity degree by cytoHubba (v 0.1).

Gene Set Enrichment Analysis (GSEA)

Gene set enrichment analysis (GSEA) was performed using GSEA software (v 4.2.3) (Subramanian et al. 2005) with KEGG gene sets (C2) (https://www.gsea-msigdb.org/gsea/msigdb/collections.jsp) from MSigDB. The enrichment of functional gene sets within ranked gene lists was assessed, and NES was calculated using normalized data. Significance thresholds were set at |NES|> 1, NOM P-value < 0.05, and FDR q-value < 0.25.

Identification of Differential Alternative Splicing (DAS) Events

DAS events generated from transcriptome sequencing data were analyzed using rMATS (v 4.0.1) (http://rnaseq-mats.sourceforge.net/) (Shen et al. 2014). Five types of AS events—skipped exon (SE), alternative 5′ splice site (A5SS), alternative 3′ splice site (A3SS), mutually exclusive exons (MXE), and retained intron (RI)—were tested. The software employs a likelihood-ratio test to calculate the P-value and false discovery rate (FDR) of inclusion levels between two sets of RNA-Seq datasets (Katz et al. 2010). An FDR threshold of < 0.05 was applied to identify significant DAS events. KEGG enrichment analyses using KOBAS (v 3.0) were conducted to elucidate the functions of DAS genes. Co-expression network patterns and interactions among DAS genes were visualized using Cytoscape (v 3.8.2).

Quantitative Real-Time PCR (qPCR) Validation

To validate the reliability of the transcriptome sequencing results, we randomly selected six significantly upregulated genes (*vlacsl*, *hsp4a*, *cysteine dioxygenase type 1*, *hsp60*, *armc2*, and *kcnj10l*) and six significantly downregulated genes (*cephalotoxin-like protein*, *arrdc3*, *hbbb2*, *gch1*, *apodl*, and *m1ip1*) for qPCR validation (Supplementary Table 1). Specifically, the reaction mixture consisted of 2 μL of cDNA template, 0.4 μL of forward and reverse primers (each), 10 μL of 2 × ChamQ SYBR Color qPCR Master Mix, and 7.2 μL of ddH_2O, resulting in a total volume of 20 μL. The qPCR was performed in triplicate with the following conditions: 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s. The 18S ribosomal RNA served as the internal reference gene, and the relative expression levels were calculated using the $2-\Delta\Delta Ct$ method. Primer amplification efficiencies exceeded 90%.

Results

Overview of High-Throughput Sequencing Data

In this study, RNA-Seq analysis was performed on the liver tissues of spotted sea bass at 0, 3, 6, 12, and 24 h following heat stress**.** All clean reads were aligned to the spotted sea bass reference genome, with mapping ratios ranging from 90.98 to 93.51%, and unique mapping ratios varied between 77.62 and 84.00% (Supplementary Table 2). Calculating the Pearson coefficient between samples can reveal the correlation among transcriptome samples (Supplementary Fig. 1). A principal component analysis (PCA) of the transcriptome data illustrated the variance distribution in gene expression across the samples (Fig. 1a).

Identification of DEGs

The results revealed an increase in the total number of DEGs with prolonged heat stress duration. Comparisons between T3 and T0, T6 and T0, and T12 and T0, as well as T24 and T0, identified 1702, 1199, 3128, and 2636 DEGs, respectively (Fig. 1b). Volcano plots displayed expression profiling of DEGs between 3 h vs 0 h, 6 h vs 0 h, 12 h vs 0 h, and 24 h vs 0 h groups (Fig. 1c). As the heat stress duration extended from 0 to 12 h, the number of DEGs significantly increased, with a slight decrease observed from 12 to 24 h. Notably, 409 DEGs coexisted were common across all four comparison groups.

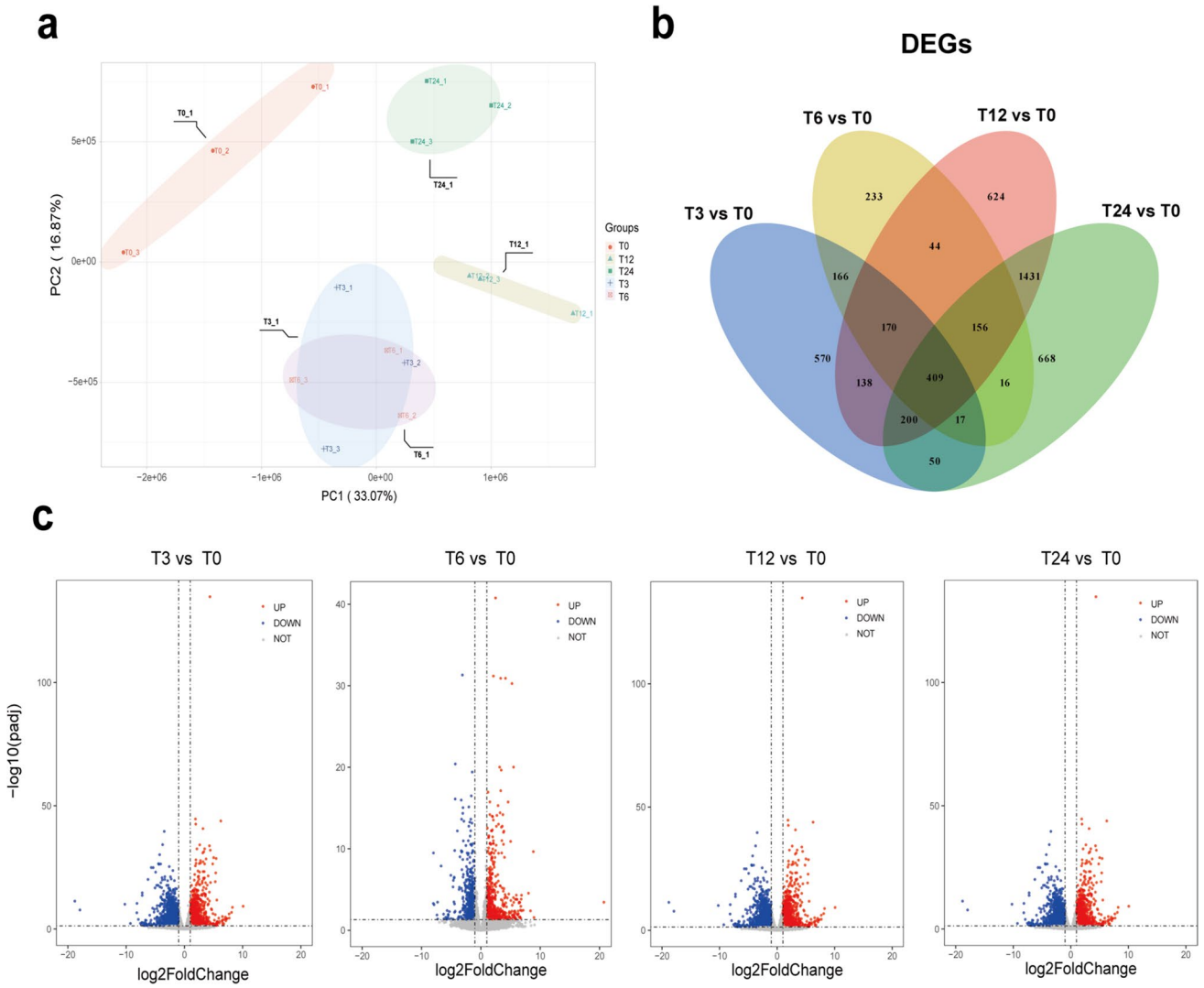

Fig. 1 Identification of differentially expressed genes in transcriptome data of liver tissue of spotted sea bass under acute thermal stress. **a** PCA analysis of sea bass liver transcriptomic data. T0_1, T0_2, and T0_3 represent the three sample groups at the T0 time point, with a similar representation for subsequent time points. **b** Total differentially expressed genes of sea bass liver. **c** Volcano plots displayed expression profiling of DEGs between 3 h vs 0 h, 6 h vs 0 h, 12 h vs 0 h, and 24 h vs 0 h groups. Red dots represented upregulated genes, blue dots represented downregulated genes, and gray dots represented genes with no significant expression differences. The x-axis indicated log2 fold change, and the y-axis indicated − log10 *P*-value

WGCNA Analysis

To identify genes associated with the heat stress response, Weighted Gene Co-expression Network Analysis (WGCNA) was conducted. After filtering out low-expressed genes (fragments per kilobase of transcript per million; FPKM < 0.01) across 15 transcriptome sequencing libraries, WGCNA analysis was performed on the top 5000 genes based on FPKM values. Using the WGCNA package, gene weights were calculated to achieve a scale-free network distribution, and the soft-thresholding power β was determined using the function "sft$powerEstimate," with $\beta = 7$ selected for constructing the co-expression network (Supplementary Fig. 2). Visualization of the gene network is achieved using a heatmap plot, which displays the topological overlap matrix of genes included in the analysis (Fig. 2a). The constructed gene co-expression network successfully divided into six modules, with each module represented by a different color indicating genes clustered based on similar expression patterns, among which the MMblue module was the most significant (Fig. 2b).

To explore the potential biological functions of genes within the MMblue module, KEGG enrichment analysis was performed, and a protein–protein interaction (PPI) network was constructed to identify hub genes within the module. The KEGG enrichment analysis revealed that genes in the

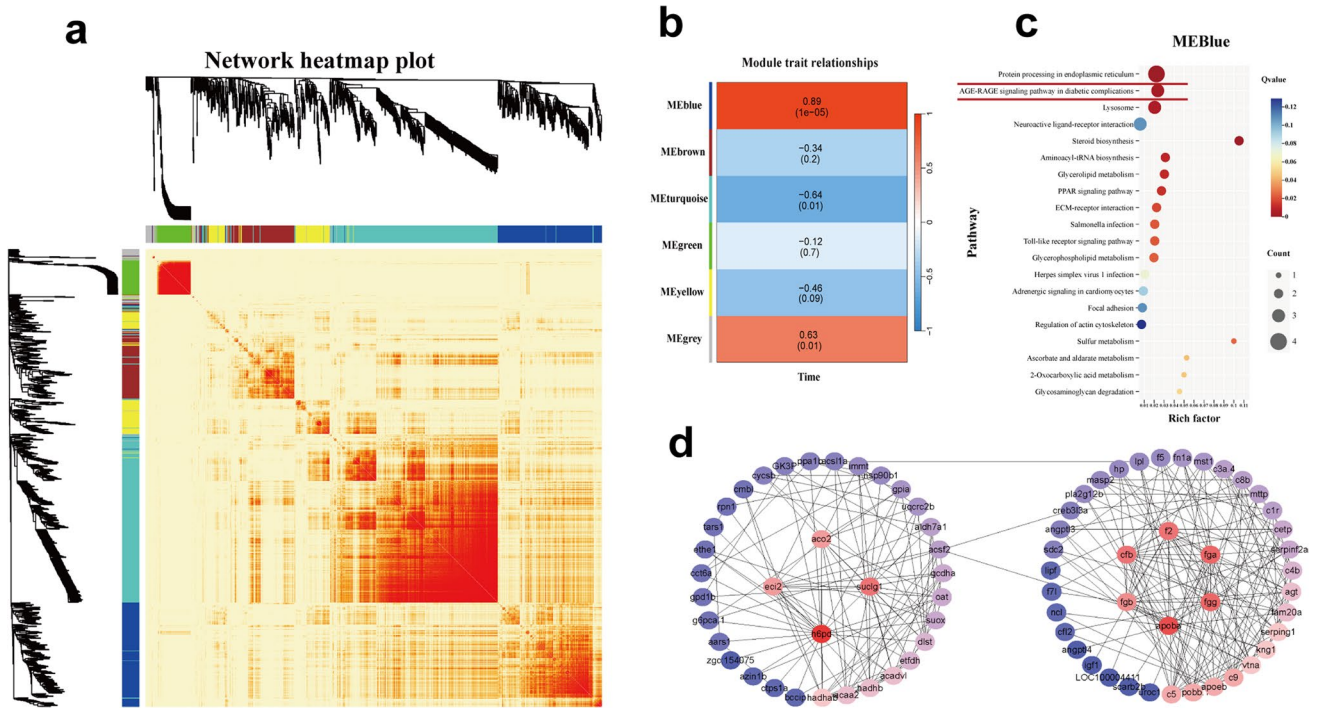

Fig. 2 WGCNA analysis and KEGG enrichment with PPI network analysis for the MMblue module in the transcriptome of liver tissue from spotted sea bass under acute thermal stress. **a** Visualization of the gene network is achieved using a heatmap plot, which displays the topological overlap matrix of genes included in the analysis. Lighter colors indicate a lower overlap, while progressively darker shades of red signify a higher overlap. Blocks of darker colors along the diagonal represent distinct modules. The gene dendrogram and module assignments are depicted along the left side and top of the heatmap. **b** Module trait relationships of WGCNA analysis. **c** KEGG pathway enrichment analysis for the MMblue module; **d** protein–protein interactions (PPI) network of DEGs and the identified hub genes for MMblue module. The color of the nodes represents the degree and the hub gene is located in the middle of the PPI networks

MMblue module were predominantly enriched in pathways protein processing in the endoplasmic reticulum and AGE-RAGE signaling pathway in diabetic complications (Fig. 2c). The PPI network identified nine key hub genes, including *aco2*, *eci2*, *suclg1*, *h6pd*, *f2*, *cfb*, *fga*, *fgb*, and *fgg*, which were ranked based on degree (Fig. 2d).

Dynamic Gene Expression Patterns

The six clusters represent six distinct patterns of expression trajectories over the time course, with the detected clusters of co-expressed genes indicating co-regulation (Fig. 3). As our primary focus is on the most relevant regulatory networks (genes) interacting with the heat stress environment, we further analyzed four clusters—designated as cluster 1, cluster 2, cluster 4, and cluster 5—which are enriched with heat stress-responsive genes and pathways.

PPI Network Construction and Hub Genes Identification

The genes within the six clusters were screened based on criteria of FDR < 0.05 and |log2(Fold Change (FC)) |> 1, to exclude those with minimal fold changes. This process yielded the DEGs distributed across these clusters. Combined with the results of dynamic gene expression patterns, we generated PPI networks using the String database to explore the correlations of these DEGs within cluster 1, cluster 2, cluster 4, and cluster 5. Subsequently, hub genes from the constructed PPI network were identified using four algorithms (MNC, MCC, Degree, EPC) of the cytoHubba plugin within Cytoscape (Fig. 4a).

As a result, ten hub genes including *aldh18a1*, *vars*, *yars*, *pwp2h*, *polr3b*, *iars*, *tars*, *ddx18*, *larsb*, and *aars* were identified for cluster 1. Nine genes including *lum*, *lox*, *tg*, *col14a1a*, *pdgfrb*, *fn1a*, *mapk12a*, and *col1a1a* were identified as hubs for cluster 2. Nine genes including *hsp90aa1.1*, *hspa5*, *hsp90b1*, *hsp70*, *hspd1*, *hspa4b*, *stip1*, *sgut1*, and *dnajb1a* were identified as hubs for cluster 4. Eight genes including *hsp90b1*, *abce1*, *pdcd11*, *diexf*, *gnl2*, *nol6*, *ctps1a*, and *nom1* were recognized as hub genes for cluster 5.

Subsequently, KEGG enrichment analysis was performed on the DEGs within these clusters to explore their potential functions (Fig. 4b). In the downregulated cluster 2, the significantly enriched pathways included the AGE-RAGE signaling pathway in diabetic complications.

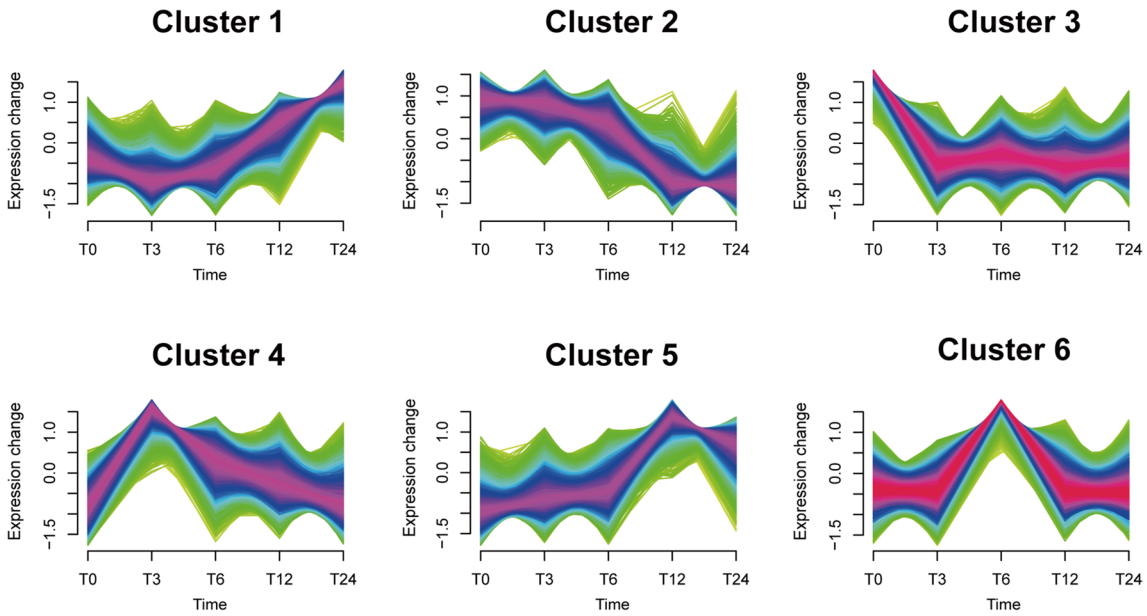

Fig. 3 Time-series analysis of gene expression in the transcriptome of liver tissue from spotted sea bass under acute thermal stress. Six clusters (clusters 1–6) were identified based on the similarity in gene expression patterns. Different colors indicate the match degrees between changes in genes and the major changes in the clusters. Fuchsia, blue, and green represent high, moderate, and low match degrees, respectively

Additionally, although clusters 1, 3, and 5 exhibited different expression trends, the KEGG enrichment results consistently showed that the most significantly enriched pathway in all three clusters was protein processing in the endoplasmic reticulum. This indicates that protein processing in the endoplasmic reticulum may be the primary pathway through which the liver responds to heat stress. Furthermore, there were distinct expression patterns among the DEGs involved in protein processing in the endoplasmic reticulum.

Functional Gene Set Enrichment Analysis

In addition to performing KEGG enrichment analysis on these DEGs, we also conducted GSEA (Fig. 5). By utilizing gene sets from the Molecular Signatures Database (MsigDB), we interpreted the gene expression data of liver tissues under heat stress conditions.

We identified the most significantly enriched gene sets positively correlated with the heat stress groups (gene sets upregulated in 3-h, 6-h, 12-h, and 24-h groups), including "protein export," "RNA degradation," and "spliceosome pathways." The glycine, serine, and threonine metabolism pathways were downregulated in the 3-, 6-, and 12-h groups, but upregulated in the 24-h group. This may be due to the fact that the adjustment of amino acid metabolism in response to heat stress is a transient process, and the gradual adaptation of the spotted sea bass to the heat stress environment leads to different expression trends in the glycine, serine, and threonine metabolism pathway. This is also similar to the findings in protein processing in the endoplasmic reticulum pathway observed in this study.

Identification of DAS Events and DAS Genes

The rMATS software was used to identify genes undergoing alternative splicing (AS) under heat stress (Fig. 6a), and the DAS genes in different comparison groups were statistically analyzed (Supplementary Table 3). The predominant alternative splicing events were skipped exon (SE). To further investigate the potential functions of these genes, KEGG enrichment analysis (Fig. 6b) and PPI analysis were conducted (Fig. 6c). The results revealed that these genes were primarily enriched in the adherens junction, focal adhesion, and protein processing in the endoplasmic pathway, which is consistent with the previous findings from the analysis of DEGs. A PPI network analysis was performed on these DAS genes, further identifying hub genes undergoing alternative splicing. These hub genes include *hnmpc*, *mrps16*, *mrpl58*, *tfb1m*, *aldh18a1*, *stat3*, *rptor*, *rac1a*, *ctnnb1*, and *ykt6*.

An Overview of Genes Involved in Protein Processing in the Endoplasmic Reticulum

Finally, we depicted a schematic diagram illustrating the relevant regulatory genes and their involvement in biological processes in the liver tissue of spotted sea bass under

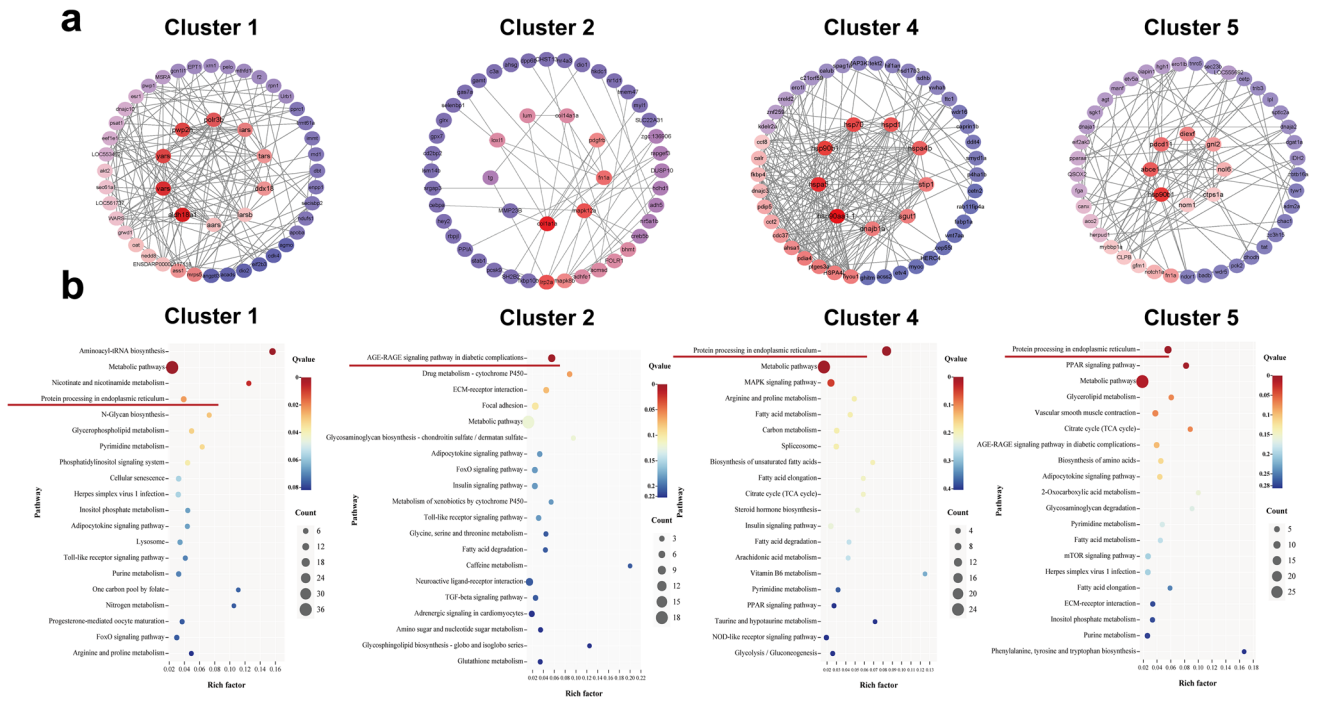

Fig. 4 PPI analysis and KEGG enrichment analysis for cluster 1, cluster 2, cluster 4, and cluster 5. **a** Protein–protein interactions (PPI) network of DEGs and the identified hub genes for clusters 1, 2, 4, and 5, respectively. The top 50 nodes by degree are visualized. The color of the nodes represents the degree and the hub gene is located in the middle of the PPI networks. **b** KEGG pathway enrichment analysis for cluster 1, cluster 2, cluster 4, and cluster 5

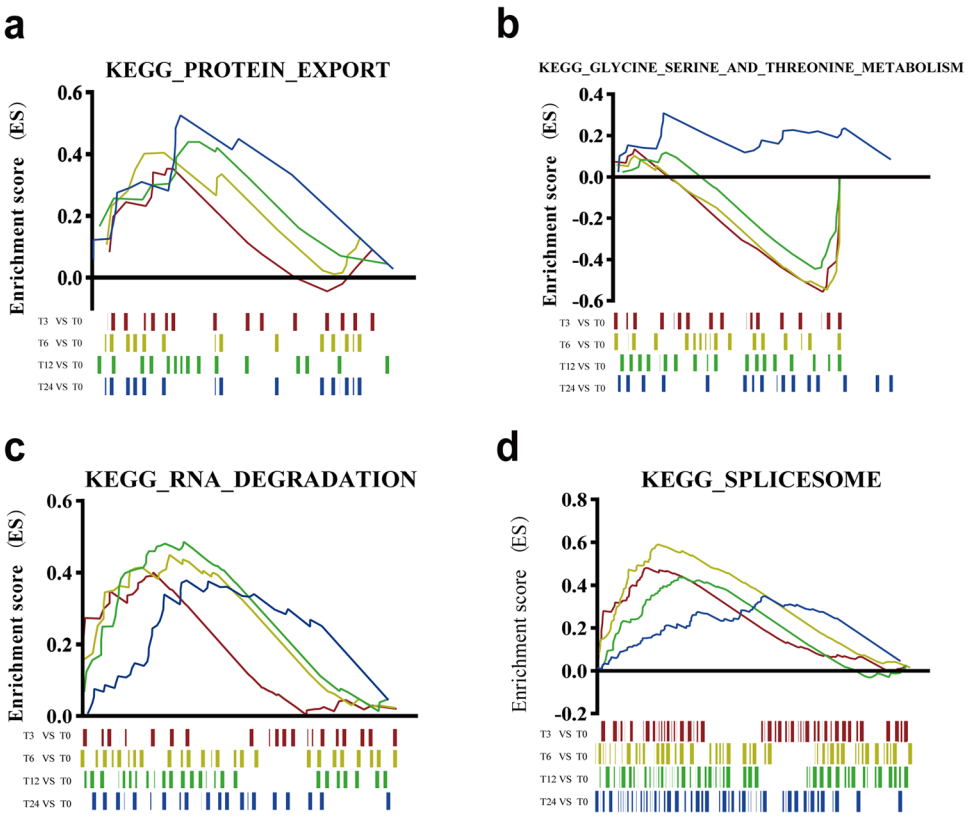

Fig. 5 Gene set enrichment analysis in the transcriptome of liver tissue from spotted sea bass under acute thermal stress. Gene set enrichment analysis (GSEA) for the 3 h vs 0 h, 6 h vs 0 h, 12 h vs 0 h, and 24 h vs 0 h groups, respectively, marked with red, yellow, green, and blue

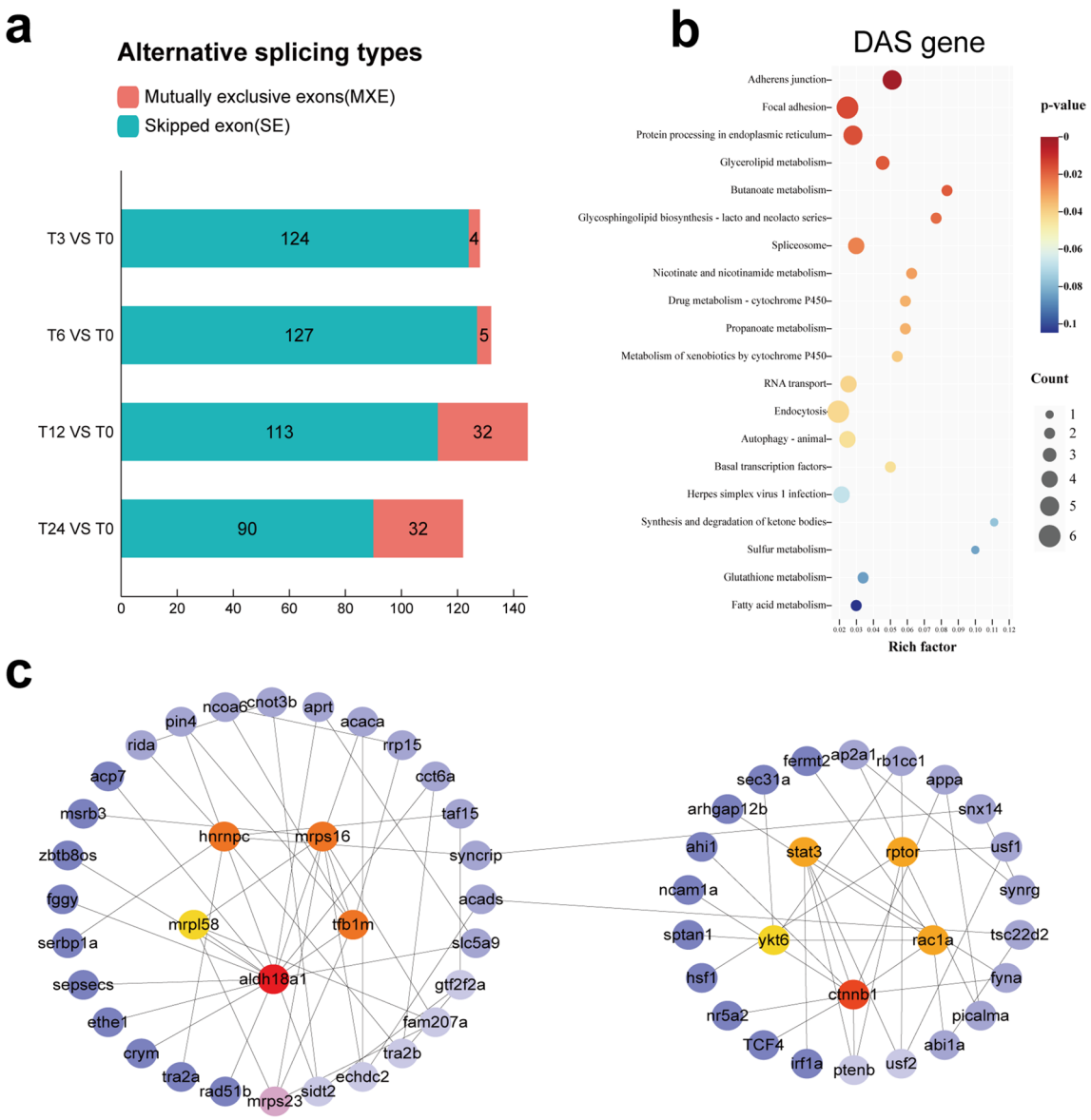

Fig. 6 Analysis of differential alternative splicing events in the transcriptome of liver tissue from spotted sea bass after heat stress. **a** Statistics of alternative splicing event types. **b** KEGG pathway enrichment analysis of DAS genes. **c** Protein–protein interactions (PPI) network of DAS genes

heat stress conditions (Fig. 7). Based on the above bioinformatic analysis results, in combination with manual literature searches about the functions of DEGs, we conducted an in-depth analysis of the expression patterns of genes within protein processing in the endoplasmic reticulum pathways and AGE-RAGE signaling pathway (Fig. 8); the mRNA expression values of genes involved were illustrated (Supplementary Table 4).

The study found that although these genes in protein processing in the endoplasmic reticulum pathways are upregulated in the liver of spotted sea bass under heat stress (except for *traf2*), there are two distinct expression patterns. The genes exhibiting the first expression pattern are highly expressed in the T3 vs T0 comparison and continuously decrease over the 3- to 24-h period. These genes include *pdia4*, *dnajc3*, *ero1l*, *calr*, *hyou1*, *hspa5*, *pdia6*, *hsp90b1*, *dnajb1*, *hspa4l*, *hsp90aa1*, and *hspa1a*. The genes belonging to the second type continuously increase throughout the entire 24-h heat stress process. These genes include *traf2*, *sec61a1*, *eif2ak3*, *canx*, *ssr1*, *sec23b*, *dnaja2*, *rad23a*, *bcap31*, *dnajc10*, *rpn1*, *bcl2*, *ero1lb*, *dnaja1*, and *herpud1*. Additionally, genes including *tgfb*, *fn1a*, *mapk8b*, *mapk12b*, *col1a1a*, *col1a2*, and *col1a1b* showed a downregulated trend in the AGE-RAGE signaling pathway. Their potential biological functions are discussed in detail in the "Discussion" section.

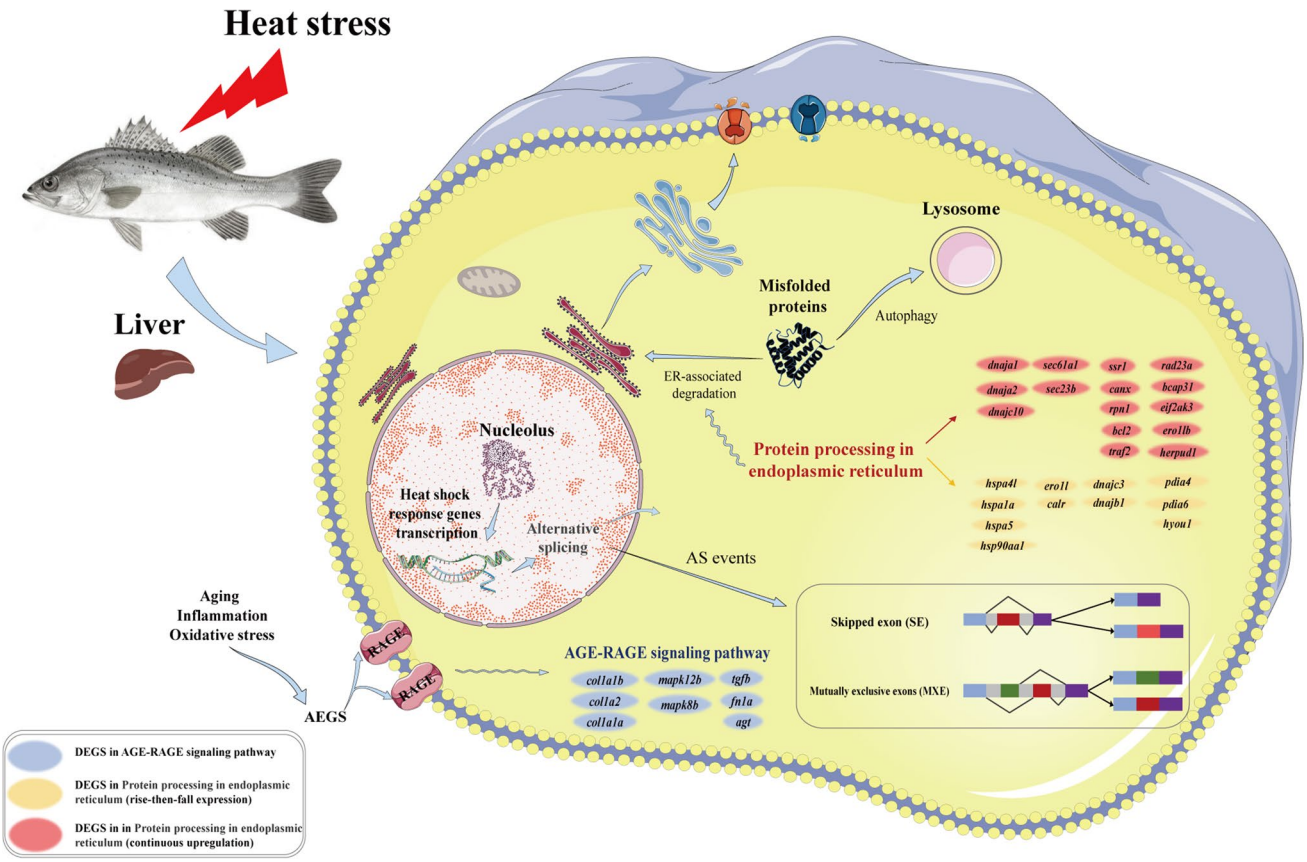

Fig. 7 Schematic diagram of the potential molecular mechanisms under heat stress in the spotted sea bass. The diagram illustrates the changes in gene expression levels and alternative splicing events related to the protein processing in the endoplasmic reticulum and AGE-RAGE signaling pathway pathways in the liver of spotted sea bass under environmental heat stress

Quantitative Real-Time PCR (qPCR) Validation

To verify the reliability of the transcriptome sequencing results, 12 genes were randomly selected from the screening results of DEG assay (Supplementary Fig. 3). The experimental results showed that the expression trends of the DEGs obtained by qPCR at 3 h, 6 h, 12 h, and 24 h after high-temperature stress were generally consistent with those at 0 h, indicating the accuracy and reliability of the transcriptome sequencing results.

Discussion

Fish are excellent vertebrate models and have been widely used in studying the physiological and molecular responses after thermal stresses (Basu et al. 2002; Dawood et al. 2020; Alfonso et al. 2021). During heat stress, genes associated with the heat shock response are often tissue-specific (Råbergh et al. 2000). The liver has important physiological functions, such as metabolism and detoxification (Qian and Xue 2016; Roychowdhury et al. 2021), and is also an important response organ of fish in response to heat stress (Rendell et al. 2006; Roychowdhury et al. 2021; Qian and Xue 2016). In this study, we identified the DEGs and pathways involved in heat stress through transcriptome analysis of the liver tissues of sea bass. The study found that high temperatures affected various metabolism-related pathways, such as protein processing in the endoplasmic reticulum pathway and AGE-RAGE signaling pathway. This indicates that heat stress-related genes are involved in multiple biological processes. The following section will discuss in detail the relevant genes and pathways, as well as their potential functions in response to heat stress.

Protein Processing in the Endoplasmic Reticulum Is the Main Biological Response Process of Spotted Sea Bass to Heat Stress

High temperatures can stimulate cells, leading to oxidative stress, energy shortages, and various other changes (Riezman 2004). The combination of Weighted Gene Co-Expression Network Analysis (WGCNA) and time-series

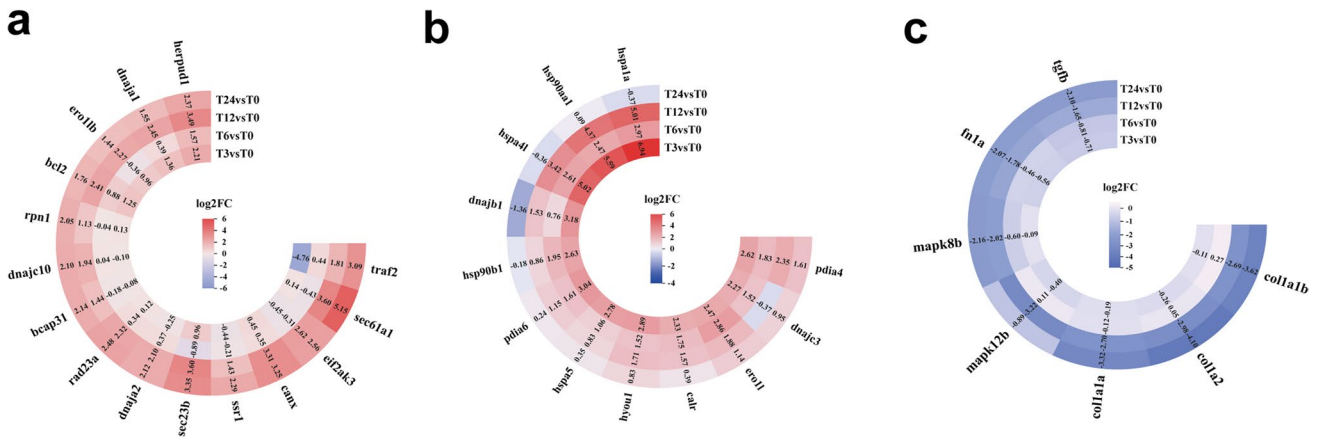

Fig. 8 The diagram illustrates the three different gene expression patterns in the protein processing in the endoplasmic reticulum and AGE-RAGE signaling pathways in the liver of spotted sea bass under heat stress. **a** Genes exhibiting a persistent increase in expression levels. **b** Genes with expression levels that initially increase before subsequently decreasing. **c** Genes demonstrating a consistent decrease in expression levels

analysis provides a comprehensive and precise approach to understanding the dynamic changes in gene expression in the liver of spotted sea bass under heat stress. WGCNA can analyze the correlation patterns among genes across samples (Langfelder and Horvath 2008), identifying modules associated with biological responses to heat stress (Huang et al. 2020), while time-series analysis can reveal the dynamic gene expression patterns in the transcriptome (Kumar and Futschik 2007).

In this study, by combining WGCNA analysis and MFUZZ cluster analysis, the screened modules and expression trends of different clusters were identified. It was found that protein processing in the endoplasmic reticulum was the most important biological process in the liver of spotted sea bass in response to heat stress. Many researches indicate that when fish are subjected to environmental stressors, such as high temperatures, significant changes occur in the structure and function of structural proteins and enzymes (Madeira et al. 2014; Shi et al. 2019). Researchers have studied the liver transcriptome of Atlantic salmon and largemouth bass following heat stress (Shi et al. 2019; Zhao et al. 2022b), which is similar to the findings of this study. This also suggests the presence of endoplasmic reticulum stress and the unfolded protein response (UPR) (Choi et al. 2010). Under such conditions, fish synthesize a large quantity of molecular chaperones, such as heat shock proteins, to assist in the folding of proteins and molecular assembly within cells (Basu et al. 2002; Shi et al. 2019; Sun et al. 2021; Zhao et al. 2021). In this study, through analyzing the two distinct patterns of gene expression changes in the protein processing in the endoplasmic reticulum pathway, differences in the expression patterns of members of the HSP gene family and the DnaJ gene family were identified. Among them, *hspa5*, *hsp90b1*, *hspa4l*, *hsp90aa1*, and *hspa1a* exhibited a rapid increase in expression levels at 3 h of heat stress, followed by a gradual decline. In contrast, *dnaja1*, *dnaja2*, and *dnajc10* showed a continuous increase in expression levels throughout the 24-h heat stress period.

HSPs are molecular chaperones that exhibit a high degree of conservation across different species in terms of their sequences (Padmini 2010), involved in numerous cellular processes (Wu 2011; Hoter et al. 2018). They are classified into five families based on their molecular weights (MWs): HSP90, HSP70, HSP60/HSP10, HSP40, and small heat shock proteins (sHSP) (Park and Seo 2015; Sun et al. 2021). When organisms are exposed to environmental stress, HSPs facilitate the folding of cellular proteins and the degradation of misfolded proteins, thereby preventing the accumulation of unfolded proteins and maintaining cellular homeostasis, thus protecting the organism from damage (Parcellier et al. 2003; Higgins et al. 2018). The *dnaj* gene encodes the DnaJ protein (HSP40) (Genest et al. 2019; Pobre et al. 2019), which acts as a co-chaperone for HSP70. When cells are exposed to environmental stressors like heat shock, they recognize and bind to damaged or unfolded proteins, facilitating their refolding by stimulating the ATPase activity of HSP70 (Liu et al. 2022; Li et al. 2024). Subsequently, some of these refolded proteins are transferred to HSP90 for final folding and maturation, while other misfolded proteins are degraded by the lysosome (Zhou et al. 2009; Rashid et al. 2015). This coordinated chaperone network is crucial for maintaining protein homeostasis and function under heat stress, ensuring that proteins can correctly fold and perform their functions properly in high-temperature environments (Pobre et al. 2019).

Heat Stress Activates the AGE-RAGE Signaling Pathway and Related Genes

Through transcriptome data analysis, it was found that genes in the "MMblue" module from WGCNA analysis and in cluster 2 from MFUZZ clustering analysis both showed a significant downregulation trend. These genes were significantly enriched in the AGE-RAGE signaling pathway. The AGE-RAGE signaling pathway involves the interaction between advanced glycation end-products (AGEs) and their receptor (RAGE) (Asadipooya and Uy 2019). This interaction plays a crucial role in the pathogenesis of various diseases, including diabetes, cardiovascular diseases, and inflammation. AGEs are formed through non-enzymatic reactions between reducing sugars and the amino groups of proteins, lipids, or nucleic acids (Tobón-Velasco et al. 2014; Asadipooya and Uy 2019). RAGE is a multi-ligand receptor widely expressed in various cell types, including endothelial cells, macrophages, neurons, and muscle cells (Hudson and Lippman 2018). Under heat stress, the metabolic rate of cells accelerates, leading to increased formation and accumulation of advanced glycation end-products (AGEs). MAPK (mitogen-activated protein kinase) genes encode a series of protein kinases involved in cell signaling transduction and play a critical role in responding to various cellular stresses, including heat stress. In this study, after 6 h of heat stress, the expression of *mapk12b* and *mapk8b* genes in the AGE-RAGE signaling pathway was significantly downregulated, which may be due to the fact that in the early stages of heat stress, the MAPK pathway is rapidly activated, allowing cells to quickly respond to heat stress by inducing the expression of stress response genes, thereby protecting the cells from heat-induced damage (Winkler et al. 2002; Liu et al. 2016). However, to prevent overactivation of the stress response pathway, cells may reduce the expression of MAPK genes through a negative feedback mechanism. This regulation helps the cells restore homeostasis and avoid an excessive stress response.

Other Response Pathways of Spotted Sea Bass to Heat Stress

By analyzing the dynamic changes in transcriptome data of the liver in spotted sea bass during 0–24 hof heat stress, we found that the heat stress response in the organism is a transient process. By analyzing the types and numbers of DEGs, we observed that the gene expression patterns in spotted sea bass during the 0- to 12-h period of heat stress are markedly distinct from those during the 12- to 24-h period. The GSEA of the glycine, serine, and threonine metabolism pathway in the T24 vs T0 group also shows significant differences compared to other groups. This dynamic change in gene expression patterns has also been noted in a transcriptome study of heat stress in Atlantic salmon (Shi et al. 2019). At the same time, the identified cluster is also enriched with pathways such as the PPAR signaling pathway and fatty acid metabolism. These pathways have also been proven to be related to heat stress in other fish species such as *Ctenopharyngodon Idella* and *Scophthalmus maximus* (Bao et al. 2018; Zhang et al. 2022; Zhao et al. 2022a).

DAS Genes Identified Under Heat Stress Are also Involved in the Protein Processing in the Endoplasmic Reticulum Process

Alternative splicing (AS) is a crucial regulatory mechanism of gene expression that generates multiple mRNA transcripts from a single pre-mRNA by selectively including or excluding different exons and introns (Blencowe 2006). This mechanism plays a vital role at the post-transcriptional level by producing functionally diverse protein isoforms (Lee and Rio 2015; Wright et al. 2022). In recent years, increasing evidence has demonstrated that alternative splicing mechanisms play a significant role in gene regulation in fish in response to environmental stress. These studies include the liver of catfish after heat stress (Tan et al. 2019), the liver of rainbow trout following heat stress (Sun et al. 2022), the brain of carp during cold acclimation (Long et al. 2020), and the gill tissue of spotted sea bass under alkaline stress (Zhang et al. 2023).

In this study, GSEA revealed that significantly enriched pathways include the spliceosome. Consequently, rMATS software was used to analyze potential alternative splicing events. The results indicated a substantial number of alternative splicing events under heat stress, with the majority of DAS events being exon skipping, accounting for over 80% of all alternative splicing events (over 85% of DAS events). Studies have shown that the incidence of exon skipping events is higher in animals compared to other eukaryotes (Patthy 2019), and ES (exon skipping) is also the most abundant type of AS (alternative splicing) event in spotted sea bass (Tian et al. 2020). Notably, KEGG enrichment analysis of DAS genes revealed significant enrichment in the protein processing in the endoplasmic reticulum pathway. This suggests that these DAS events may also impact the processing of misfolded proteins at the post-transcriptional level. However, the potential regulatory mechanisms underlying these observations warrant further investigation in future studies.

Conclusions

This study investigated the gene expression patterns of spotted sea bass under heat stress through an in-depth analysis of the transcriptomic dataset. While analyzing DEGs at various time points, WGCNA analysis identified gene

modules responsive to heat stress in spotted sea bass, with upregulated genes mainly involved in the protein processing in the endoplasmic reticulum and downregulated genes in the AGE-RAGE signaling pathway. Time-series analysis grouped these changes into six clusters, and further, KEGG and GSEA revealed two distinct expression patterns in the protein processing pathway. Additionally, we discovered 272 genes showing differential alternative splicing, primarily through exon skipping, further implicated in protein processing. This study provides theoretical insights into the biological response processes of eurythermal fish to heat stress and holds potential value for the selective breeding of heat-tolerant spotted sea bass.

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Data Availability The data presented in the study are deposited in the NCBI Sequence Read Archive (SRA) repository, accession number SRR30002608-SRR30002622.

Declarations

Ethical Approval and Consent to Participate This study obtained ethics approval from the Institutional Review Board at Ocean University of China (Permit Number: 20141201). All participants provided written informed consent. The study did not involve endangered or protected species, and experiments were conducted in accordance with relevant guidelines.

Competing Interests The authors declare no competing interests.

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