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Cardiac Transcriptome and Histology of the Heart of the Male Chinese Mitten Crab (*Eriocheir sinensis*) under High-Temperature Stress

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Abstract: High temperatures are important environmental stressors affecting the metabolism, growth, immunity, and mortality of Chinese mitten crabs (*Eriocheir sinensis*). In this study, Chinese mitten crabs were divided into two groups and exposed to temperatures of 35 °C (thermal stress group) or 25 °C (control group) for 24 h, and the transcriptome of the heart was analyzed. There were 4007 differentially expressed genes (DEGs) between the thermal stress and the control groups, including 2660 upregulated and 1347 downregulated genes. Heat shock proteins (HSPs) and transcription factors (TFs) were temperature-sensitive DEGs in Chinese mitten crabs. DEGs mainly focused on protein processing in the endoplasmic reticulum, ribosome biogenesis, glycine, serine, and threonine metabolism, protein export, and insect hormone biosynthesis pathways. A total of 28,916 SSRs and 59 TF families, including 851 TFs, were detected among all unigenes of *E. sinensis* transcripts. The qRT-PCR results for the HSPs and apoptotic DEGs from the heart exhibited the same trends as those in the *E. sinensis* transcriptome data. Results of light microscopy analyzing histological sections of the heart indicated that most myocardial fibers were lysed, and the number of nuclei and the connective tissue contents between the myocardial layers were both reduced following 35 °C exposure for 24 h.

Keywords: *Eriocheir sinensis*; transcriptome; heat shock protein; histology; immune

Key Contribution: Four thousand and seven DEGs and protein processing in the endoplasmic reticulum pathway are modulated at high temperatures in Chinese mitten crabs, thus indicating their importance in crab adaptation to high-temperature stress.

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1. Introduction

Temperature is an important environmental factor that affects the physiological processes of aquatic organisms [1]. With global warming, extremely high temperatures occur in China and other regions during the summer [2]. Aquatic organisms are ectothermic animals whose temperatures fluctuate depending on the water temperature [3]. Heat stress commonly occurs in aquaculture, ultimately resulting in high mortality rates. However, the mechanisms underlying thermal stress in aquatic organisms are not fully understood. When water temperature fluctuates, the synthesis and release of stress hormone-related genes in European sea bass (*Dicentrarchus labrax*) can be affected [4]. Furthermore, acute or chronic thermal stress can alter the stress axis functions and other stress responses [5]. Once the water temperature exceeds the normal range for crustaceans and fish, their growth, survival, and immunity are negatively affected [6–9].

The Chinese mitten crab *Eriocheir sinensis* is one of the most popular crustaceans in China due to its economic and nutritional properties and its pleasant aroma [10]. The

production of *E. sinensis* reached 815,318 tons in 2022 [11]. With the Earth getting warmer in recent years, water temperatures have also increased and this may significantly affect the immunity, metabolism, and survival of *E. sinensis*. In China, most *E. sinensis* are cultured in earthen ponds filled with submerged macrophytes (*Hydrilla verticillata*) and Nuttall's waterweed (*Elodea nuttallii*), and the depth of the water is approximately 1.5 m. Chinese mitten crabs rest at the bottom of the pond and are surrounded by aquatic weeds. Although the water temperature on the surface of the pond can reach 37 °C or even higher in the summer, the water temperature at the bottom of the pond surrounding aquatic weeds can be 35 °C or even lower. A previous study demonstrated that *E. sinensis* could molt normally at 35 °C, and the changes in diel water temperature from 28 °C to 35 °C did not affect molting [12]. Thus, 35 °C can be used as the thermal stress temperature. According to Li et al. [13], immune-related enzymes peaked at 12 h or 24 h and then decreased following thermal stress. When the temperature was elevated from 18 °C to 30 °C, *E. sinensis* grew faster, the molting period shortened, and the survival rate decreased from 100% to 97.2% [14]. Recent research revealed that all *E. sinensis* died after 10 min in 40 °C water, while in response to 35 °C water temperature stress, they began to die at 3 d [15]. Thus, water temperature should be controlled in *E. sinensis* aquaculture during the high-temperature season.

The heart is an important organ in fish and crustaceans and pumps blood or hemolymph containing cytokines, nutrition, and oxygen to the entire body [16]. To adapt to heat or cold stress, heart morphology may change to maintain its function. When male rainbow trout (*Oncorhynchus mykiss*) were cultured in warm water (20 °C) for eight weeks, the thickness of the compact myocardium increased, and connective tissue contents and spongy myocardium number decreased [17]. Temperature can also alter the heart rate and stroke volume. As the temperature increases, the heartbeat strength decreases and the frequency increases [18,19]. A previous study demonstrated that the stroke volume of Dungeness crabs (*Cancer magister*) decreased as the temperature changed from 4 °C to 12 °C but remained stable between 12 °C and 20 °C [20].

Transcriptome analysis is widely used to analyze differentially expressed genes (DEGs) in various biological processes [21,22]. A previous study revealed that *EsTreh* transcript levels are inhibited under hot or cold stress in Chinese mitten crabs [23]. To date, transcriptome analysis in response to thermal stress remains scarce, and DEGs responding to heat stress in the heart require further study. Therefore, it is essential to conduct transcriptome analyses to identify DEGs in response to heat stress.

In this study, the effects of heat stress on the transcriptome of the Chinese mitten crab heart were investigated. Eight DEGs were used to validate the transcriptome results by qRT-PCR, and histological sections of the heart were analyzed.

2. Materials and Methods

2.1. Crabs

The experiment was conducted at the Fisheries Institute, Anhui Academy of Agricultural Sciences, in June 2022. Healthy male *E. sinensis* individuals (60.1 ± 2.6 g) were obtained by cage traps from our experiment station ponds and acclimated in 20,000 L plastic tanks containing recycling aerated tap water. The temperature of the water was 24 °C, and one-quarter of the water volume was changed daily. The crabs were fed with sinking pellets twice daily. This study was conducted according to the Experimental Animal Welfare and Ethical Review Board of Anhui Academy of Agricultural Sciences guidelines of animals for research (AAAS2022-20).

2.2. Experimental Design and Sampling

After acclimation for seven days, the crabs were randomly divided into two groups, including the thermal stress group (TSG) and the control group (CG), each with three replicate tanks (185 L) with 20 individuals each. The water temperatures in the thermal stress group and the control group were maintained at 35 °C and 25 °C, respectively, using aquarium heaters and air conditioners. Water temperature was increased at a rate

of 1 °C/24 h. The temperature of the water in the control group was increased from 24 °C to 25 °C and maintained at 25 °C for 24 h. In the thermal stress group, water temperature was increased from 24 °C to 35 °C and maintained at each integer temperature for 24 h. One-quarter of the water in the six experimental tanks was replaced with same-temperature water each day and aerated continuously. Food was not provided during the study period. The complete hearts of 5 individuals from each tank were collected from the control group and the thermal stress group 24 h after the water temperature reached 25 °C and 35 °C, respectively. Five hearts from each group were separately placed in liquid nitrogen and then stored at −80 °C for transcriptome analysis. The other hearts were placed in 4% paraformaldehyde for histology.

2.3. Total RNA Extraction and cDNA Library Construction

Total RNA was extracted from the hearts of *E. sinensis* using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the quantity was assessed using a NanoPhotometer spectrophotometer and agarose gel electrophoresis. RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Oligo(dT) beads were used to extract total RNA. Total mRNA was fragmented using ultrasound, and the fragmented mRNA was reverse-transcribed into first-strand cDNA using M-MuLV reverse transcriptase (Promega, Madison, WI, USA) and random primers (Promega, Madison, WI, USA). Total RNA was then degraded using RNaseH. Second-strand cDNA was synthesized using DNA polymerase I (Promega, Madison, WI, USA), and the double-stranded cDNAs were purified. After adding poly(A), the cDNAs were connected to sequencing adapters and approximately 200 bp ligations were acquired and amplified.

2.4. RNA Sequencing and Transcriptome De Novo Assembly

The amplified cDNA was sequenced using an Illumina sequencing platform at GeneDenovo Biotechnology Co., Ltd. (Guangzhou, China). Fastp (version 0.18.0) [24] was used to filter the original data of adapters, unknown nucleotides exceeding 10%, or low-quality bases (q value ≤ 20) exceeding 50% before assembly and analyses. After filtering, high-quality reads were prepared to assemble a de novotranscriptome using Trinity (version v2.8.4) [25]. BUSCO (version 3) [26] was used to evaluate the completeness of the *E. sinensis* de novo transcriptome.

2.5. Transcription Factor (TF) and Structure Analysis

Protein-coding sequences in unigenes were aligned to the Animal TFdb (http://www.bioguo.org/AnimalTFDB/, accessed on 2 March 2023) to predict TF families using BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome, accessed on 2 March 2023). Simple sequence repeats (SSR) in the transcriptome were predicted using the MIcroSAtellite (v2.1) (http://pgrc.ipk-gatersleben.de/misa/, accessed on 2 March 2023).

2.6. Correlation and PCA Analysis

Pearson's correlation coefficient and principal component analysis (PCA) for sample replicates and sample stability were performed using the cor() function [27] in the R environment.

2.7. DEGs and Their Functional Analysis

Unigenes were identified using BLASTx (http://www.ncbi.nlm.nih.gov/BLAST/, accessed on 3 March 2023) with an e-value < 0.00001, and this aligned sequence was queried against databases of nonredundant proteins (Nr) in NCBI, Kyoto Encyclopedia of Genes and Genomes (KEGG), COG/KOG, and Swiss-Prot protein. Protein functional annotation was then conducted according to the best alignment results. Unigenes and their expression levels were calculated and normalized to RPKM (reads per kilobase per million reads) [28]. Differential RNA expression between the thermal stress and the

control groups was analyzed using DESeq [29]. The genes with a parameter of false discovery rate (FDR) below 0.05 and absolute fold-change (FC) ≥ 2 were considered to be differentially expressed genes (DEGs). To further analyze the biological functions of the DEGs, all DEGs were blasted to the Gene Ontology (GO) terms in the GO database (http://www.geneontology.org/, accessed on 3 March 2023). Significantly enriched GO terms for the DEGs were analyzed using the Omicsshare platform (https://www.omicshare.com/, accessed on 3 March 2023).

2.8. Quantitative Real-Time PCR (qRT-PCR)

Eight DEGs associated with immunity or apoptosis were selected to verify the RNA-seq results using qRT-PCR. RNA used for heart qRT-PCR was the same as that used for transcriptome analysis. qRT-PCR was performed using a SYBR Premix Ex Taq kit (Invitrogen) according to the manufacturer's instructions. All reactions were performed in triplicate. β-actin was selected as a housekeeping gene. The relative expression of each DEG was calculated using the $2^{-\Delta\Delta Ct}$ method [30]. The primer sequences for the eight DEGs and β-actin are listed in Table 1.

Table 1. Primer sequences used for qRT-PCR.

Full Name of Gene	Abbreviated Name	Primer Sequences
cytochrome *c′*	*Cyt-c-p*	F: CAAGGCGTCGGGTTATGTGT R: AGGTAGGCGATGAGGTCTGC
Eukaryotic translation initiation factor 5	*eIF5*	F: CAAGGTAGAGGGCAAAGGCA R: GGTCTCAGGGTTCTCGCACT
Apoptosis inhibitor 5	*api5-b*	F: GCCCCCAAAGGAACGCT R: ACGAGATGAGGCGATGAACC
Cytochrome c	*CYC*	F: CCAACAAGTCCAAGGGCATC R: CGAGGTAGGCGATAAGGTCTG
Member of heat shock protein 60 family	*Hsp60A*	F: ACCCCAGCCAATGAAGACC R: ATGATTCCCGCCTCCACAAG
Heat shock 70 kDa proteincognate 5	*Hsc70-5*	F: ATGCCCAAGGTGATTTCGCT R: TCGTGTTCCTGTTGATGAGC
Lysosome-associated membrane glycoprotein 1	*Lamp1*	F: CATCTGCCGCTCCTCCATTAC R: CCGACTGGTATGCTCCCGAC
Member of the transmembrane tetraspanin protein family	*Tspan11*	ACGACATCTGGGAGGAAGC ACATCCAGACGCCGACCA
beta-Actin	*β-Actin*	F: GGCTCGGGGATGGTCAA R: CCAGTTGGTAATGATGCCGT

2.9. Analyses of Histological Sections of the Heart

Chinese mitten crab hearts that were used for histological sections were fixed in 4% paraformaldehyde for 12 h, embedded in paraffin wax, and serially sectioned using a microtome (Leica Microsystems, Ankara, Turkey). Cut sections (5 µm thick) were immersed in sequence in Environmentally Friendly Dewaxing Transparent Liquid I (Servicebio, Wuhan, China) for 20 min, Environmentally Friendly Dewaxing Transparent Liquid II (Servicebio, Wuhan, China) for 20 min, Anhydrous ethanol I for 5 min, anhydrous ethanol II for 5 min, 75% ethyl alcohol for 5 min, and finally rinsed with tap water. The sections were then placed in a hematoxylin solution for 3–5 min and then rinsed with tap water. We then placed the sections, in sequence, in 85% ethanol for 5 min, 95% ethanol for 5 min, and eosin dye for 5 min. Sections were then placed in absolute ethanol I for 5 min, absolute ethanol II for 5 min, absolute ethanol III for 5 min, xylene I for 5 min, and xylene II for 5 min. All sections were mounted on glass slides with neutral gum. An Olympus light microscope (BX51) was used to microscopically examine all stained sections, and digital images were photographed using a cellSens Entry (Olympus Corporation, Tokyo, Japan).

3. Results and Discussion

3.1. Transcriptome and Unigene Annotation

When low-quality sequences were removed from the thermal stress group and the control group libraries, the raw reads number ranged from 37.73 M to 55.73 M, the clean reads numbers ranged from37.33 M to 55.48 M, and clean bases ranged from 5.57 G to 8.28 G. Values for Q20 and Q30 of the sequenced libraries ranged from 96.82% to 98.14% and from 91.67% to 94.31% individually, and the GC content ranged from 38.54% to 43.95% (Table S1), thus indicating that the sequence data were high quality and reliable for further study.

High-quality clean sequences were first assembled into contigs, and the assembled contigs were further assembled into unigenes. In this study, 43,606 unigenes were identified. The maximum, minimum, and average unigene lengths were 36,785 bp, 201 bp, and 1190 bp, respectively. The number and length of the N50 for unigenes were 6491 and 2190 bp, respectively. Of all unigenes, 14,800 (33.93%) surpassed 1 kb in length and 7353 (16.86%) surpassed 2 kb in length (Figure S1). In the *E. sinensis* transcriptomic assembly, BUSCO (Benchmarking Universal Single-Copy Orthologs) analysis revealed 904 (92.4%) complete, 34 (3.5%) fragmented, and 40 (4.1%) missing genes (Table 2).

Table 2. BUSCO analysis of the *Eriocheir sinensis* transcriptome.

Number of Genes	BUSCO Categorization
904	Complete (C)
810	Complete and single copy (S)
94	Complete and duplicated (D)
34	Fragmented (F)
40	Missing (M)
978	Total BUSCO groups searched (n)

The sequences of 43,606 unigenes were compared to those of genes in four protein databases: Swiss-Prot, KEGG, KOG, and Nr. There were 11,687 (26.80%), 17,141 (39.31%), 10,395 (23.84%), and 17,048 (39.10%) unigenes annotated using the Swiss-Prot, KEGG, KOG, and Nr databases, respectively (Figure 1). Raw reads from *E. sinensis* have been deposited in the NCBI database (accession number: PRJNA1064616).

3.2. SSR

SSR markers are codominant, multi-allelic, and useful tools for genetic, evolutionary, and polymorphism studies [31]. In this study, 28,916 SSRs were detected within *E. sinensis* unigenes. The most abundant repeat motifs were dinucleotides (13,356, 46.16%), followed by trinucleotides (11,925, 41.08%), tetranucleotides (2834, 9.8%), and hexanucleotides (136, 0.66%). Among the detected SSRs, the AC/GT motif was the most abundant (31.36%), followed by AG/CT (13.34%), AGG/CCT (11.37%), and ACC/GGT (7.32%) motifs (Figure 2).

3.3. TFs

In this study, 59 TF families, including 851 TFs, were detected among all unigenes of *E. sinensis* transcripts. The most abundant TF family was zf-C2H2 (473), followed by bHLH (35), HMG (30), ZBTB (29), MYB (28), TF_bZIP (24), THAP (23), homeobox (19), GCNF-like (18), and ETS proteins (12).

3.4. DEG Analysis

Genes that were differentially expressed between the thermal stress group (35 °C) and the control group (25 °C) were selected according to p-value < 0.05 and $|\log_2 \text{foldchange}| \geq 1$. A total of 4007 DEGs (Table S2) were identified between the two groups, and these included 2660 upregulated and 1347 downregulated genes. When the temperature was elevated to 35 °C for 24 h, DEGs of heat shock proteins and transcription factors were shown to be sensitive to temperature. DEG_S mainly focused on protein processing in the endoplasmic reticulum,

ribosome biogenesis in eukaryotes, glycine, serine, and threonine metabolism, protein export, and the insect hormone biosynthesis pathway. Molting and metamorphosis are essential processes in the growth and development of Chinese mitten crabs. In insects, molting and metamorphosis are primarily controlled by two hormones: ecdysone and juvenile hormones. Ecdysone and juvenile hormones are conserved between crustaceans and insects [32,33]. In this study, ecdysone and juvenile hormones in the insect hormone biosynthesis pathway were downregulated under thermal stress, possibly due to the observation that more energy was used for survival and not growth. When grass carp (*Ctenopharyngodon idellus*) were cultured at 34 °C for 48 h and infected with *Aeromonas hydrophila*, 3355 DEGs in the spleen were identified, including heat shock proteins and immune-related genes [34]. Transcriptome analysis demonstrated that when turbot (*Scophthalmus maximus*) was maintained at normal temperature (14 °C) or three different high temperatures (20 °C, 25 °C, 28 °C) for 24 h, DEGs were enriched in seven different pathways, and the numbers of DEGs increased with the increase in temperature [35]. After *E. sinensis* was exposed to hot (32 °C) or cold water (4 °C) for 0, 2, 6, 12, or 24 h, *EsTreh* expression was gradually downregulated [23].

Figure 1. Distribution of 43,606 *E. sinensis* unigenes in four protein databases: Swiss-Prot, KEGG, KOG, and Nr.

Heat shock proteins (HSPs) are conserved proteins that are important for protein folding, refolding, translocation, and degradation. HSP expression is regulated by thermal stress [36], pH, ammonia–N stress [37], salinity, and bacteria [38]. In the present study, the DEGs associated with HSPs included HSP60A, HSC70-3, HSP90AA1, HSP22, HSP110, AHSA1, HSPA8, HSPe1, HSPbp1, andenpl-1. HSP60A, HSP90AA1, HSP110, HSP22, Hsc70-3, AHSA1, HSPA8, HSPe1, HSPbp1, and enpl-1 (Table S2) were significantly upregulated (p <0.05) in the thermal stress group, and the $\log_2$fold-changes were 4.94, 4.76, 7.61, 4.95, 3.27, 5.32, 7.62, 4.33, 3.65, and 3.14, respectively. According to Oksala et al. [39], HSPs (HSP60, HSP70, HSP90, and HSC70) from doctor fish (*Garra rufa*) muscle were more highly elevated in high-temperature water (34.4 °C) than in normal-temperature water (25.4 °C). When pool barb (*Puntius sophore*) were fed curcumin and thermal-treated (36 °C) for 6 h, the expressions of HSP60, HSP70, HSP90, and HSP110 were elevated in the gills, while the expressions of HSP70 and HSP 110 were elevated in the liver [40]. When rainbow trout (*Oncorhynchus mykiss*) were stressed by high temperature (24 °C), the expressions of HSPa4L, HSPa8a, HSPa5, and HSP70a in the liver and HSPa4, HSPa4L, HSPa5, HSPa8b,

HSPa8a, HSPa9 and HSP70a in the head kidney were upregulated compared with levels in the normal temperature group (18 °C) [41]. HSP22 is a small heat shock protein that protects organisms against high temperature [42] and apoptosis [43]. AHSA1 is a cofactor of the HSP90 that can be upregulated by heat stress (24 °C) in rainbow trout [44]. HSPe1 is a member of the HSP10 family and ismore highly expressed in the liver, brain, and head kidney than in the gills, heart, and spleen under heat shock stress [45]. HSPbp1 is an HSP70-binding protein that affects the expression of chaperones by inhibiting HSP70 proteasome degradation and ubiquitylation [46]. HSPbp1 can be significantly upregulated in zebrafish (*Danio rerio*) during gonadal differentiation when they are exposed to high temperatures (35 °C) [47].

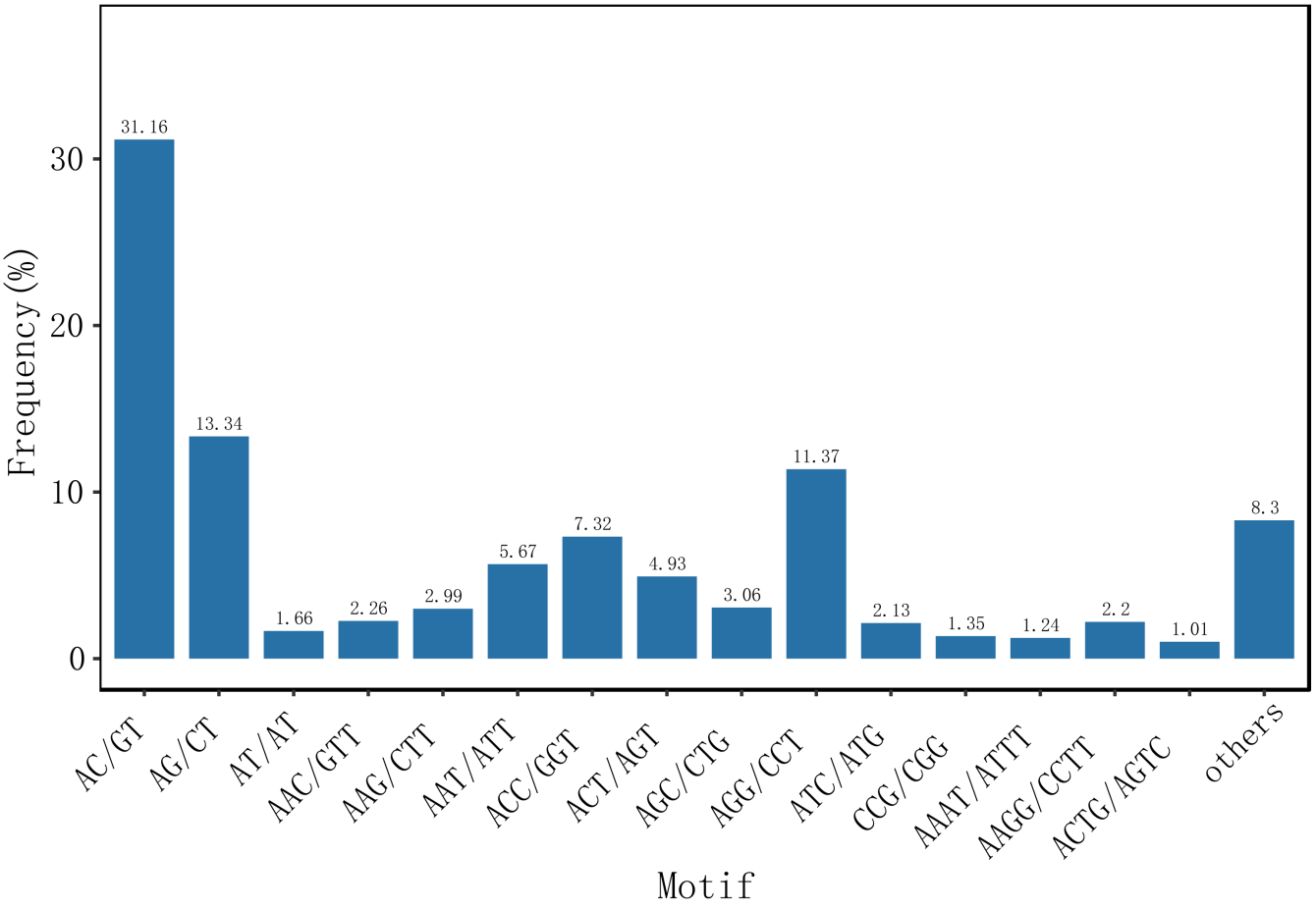

Figure 2. Frequenciesof detected SSRs in *Eriocheir sinensis* transcripts.

A previous study demonstrated that high temperature upregulates the expression of apoptotic genes [48]. In this study, *Cyt-c-p*, *CYC*, and *eIF5* (Table S2) were detected as apoptotic DEGs and were significantly upregulated in the thermalstress group, with $\log_2$fold-changes of 3.07, 2.09, and 1.62, respectively. *Cyt-c-p* belongs to the *cyt c* family of proteins. Deficiency of *cyt-c-p* in *Drosophila* can result in embryonic death due to its respiratory function in the mitochondria [49]. *Cytc* is released from mitochondria and binds to the apoptotic protease activating factor in the cytoplasm to activate the apoptosome [50]. Cytochrome c (CYC) is part of the respiratory chain and can be up-regulated in heart tissue. For example, this can be observed in rainbow trout maintained at 4 °C for four weeks compared with levels in the control group (18 °C) [51]. Eukaryotic translation initiation factor 5 (*eIF5*) is a GTPase responsible for the initiation of protein translation, and reduced *eIF5B* expression may disrupt proteostasis and trigger cellular processes associated with stress responses. *eIF5* was upregulated when *eIF5B* was knocked down in 293 T and HepG2 cells using the CRISPR/cas9 system [52].

3.5. PCA

After quality control, Pearson's correlation analysis was conducted on the samples to compare the thermal stress and control groups, and the results revealed that the samples were reliable (Table S3). PCA demonstrated that five samples in the same group clustered together and that the two groups were clearly separated (Figure 3).

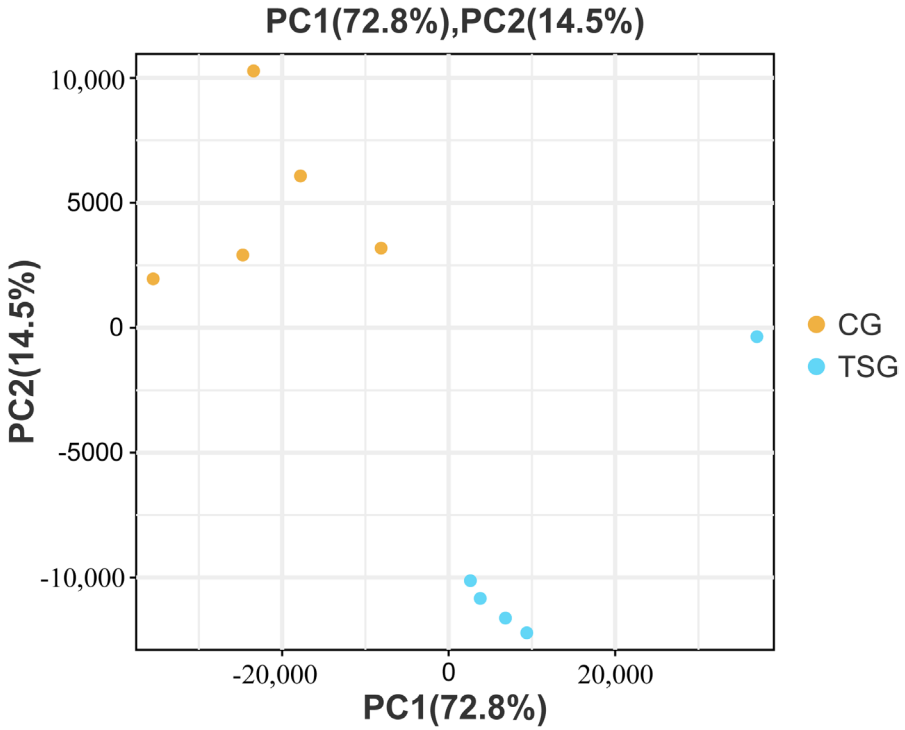

Figure 3. Principal component analysis of *Eriocheir sinensis* in the thermal stress group (35 °C) and the control group (25 °C). TSG and CG indicate the thermal stress group and the control group, respectively.

3.6. Functional Annotation

DEGs in the hearts of samples in the thermal stress group were significantly enriched ($p < 0.05$) for 1377 GO terms. The three most-enriched biological process (BP) items were "cellular process", "metabolic process", and "biological regulation". The three most-enriched cellular component (CC) items were "cellular anatomical entity", "protein-containing complex", and "virion component". A comprehensive sequence and structure analysis of major virion proteins indicate that they evolved on about 20 independent occasions, and in some of these cases, likely ancestors are identifiable among the proteins of cellular organisms. Although the replication modules of at least some classes of viruses might descend from primordial selfish genetic elements, bona fide viruses evolved on multiple independent occasions throughout the course of evolution by the recruitment of diverse host proteins that became major virion components [53]. The three most-enriched molecular function (MF) items were "binding", "catalytic activity", and "transporter activity" (Figure 4).

The top 20 KEGG pathways included protein processing in the endoplasmic reticulum, ribosome biogenesis in eukaryotes, peroxisome, glycine serine and threonine metabolism, aminoacyl-tRNA biosynthesis, arginine and proline metabolism, pyruvate metabolism, N-glycan biosynthesis, and tryptophan metabolism (Figure 5).

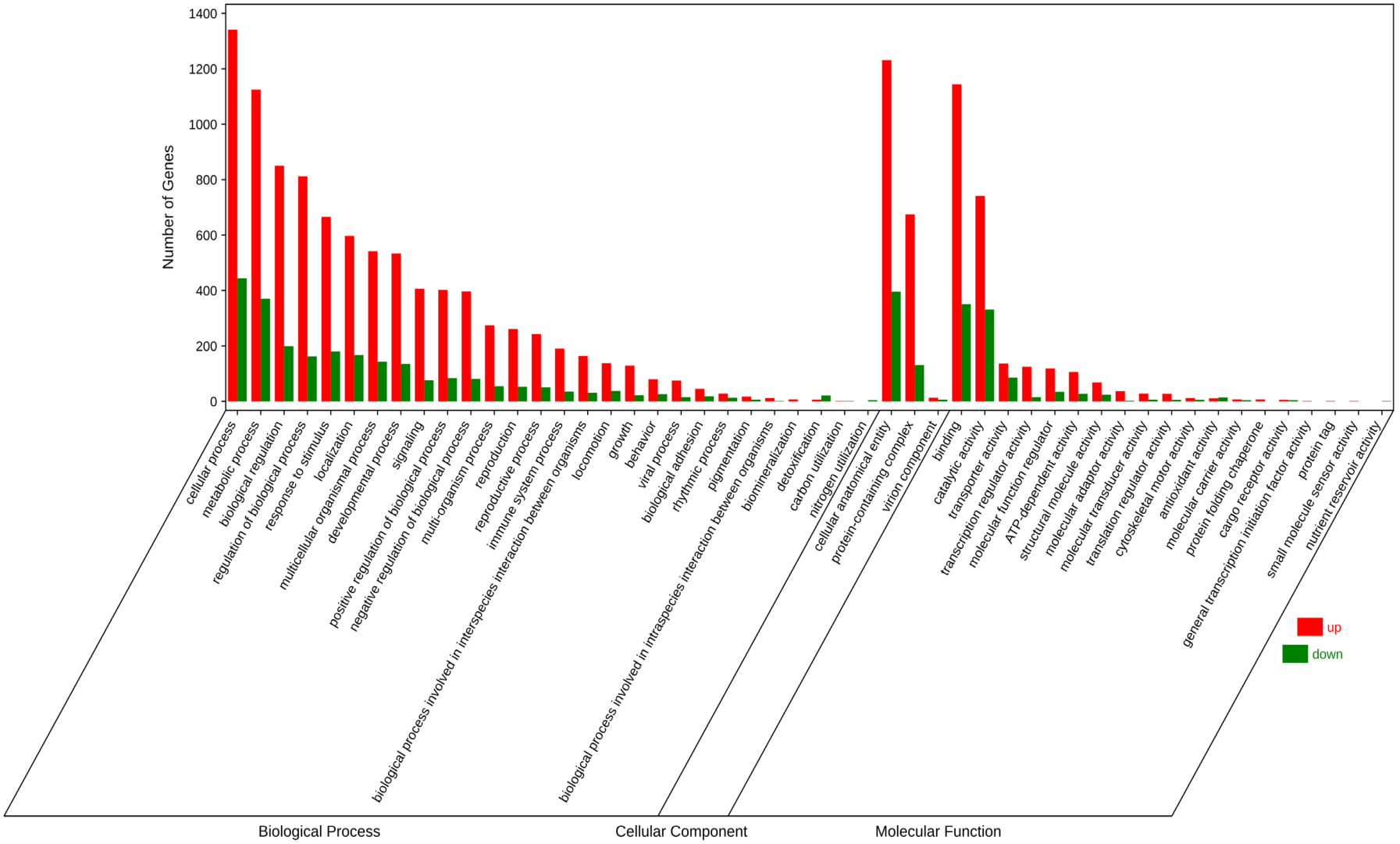

Figure 4. Gene ontology (GO) terms for DEGs between the thermal stress group (35 °C) and the control group (25 °C).

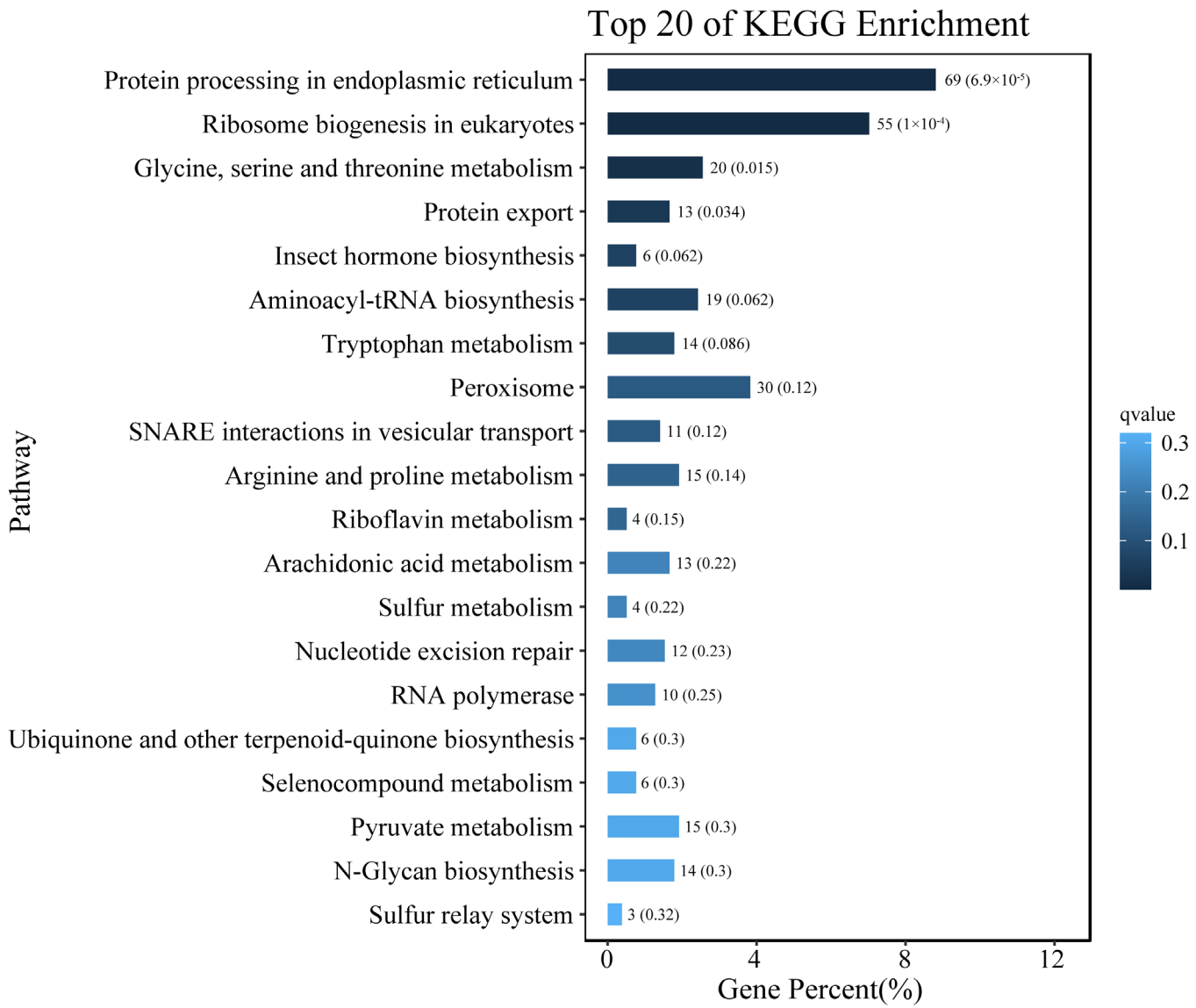

Figure 5. Top 20 KEGG pathways ofDEGs. The x and y axes indicate the percentage of transcripts and pathway names, respectively.

3.7. qRT-PCR

Eight DEGs (*Cyt-c-p*, *eIF5*, *api5-b*, *CYC*, *Hsp60A*, *Hsc70-5*, *Lamp1*, and *Tspan11*) involving apoptosis or heat stress proteins were selected for qRT-PCR analysis. The qRT-PCR results revealed that the expression levels of the eight DEGs in the heart (Figure 6B) were in accordance with the results of RNA-Seq analysis (Figure 6A). These results indicated that the *E. sinensis* transcriptome data were reliable.

3.8. Correlation between RN-seq and qRT-PCR Analyses

The correlation between the RNA-seq and qRT-PCR results for the eight DEGs in *E. sinensis* heart tissue was analyzed using the cor() function in R. The R^2 values were 0.50, 0.63, 0.47, 0.66, 0.44, 0.63, 0.70, and 0.63 for *Cyt-c-p*, *eIF5*, *api5-b*, *CYC*, *Hsp60A*, *Hsc70-5*, *Lamp1*, and *Tspan11*, respectively. All eight DEGs in the *E. sinensis* heart demonstrated a linear correlation between qRT-PCR and RNA-seq (Figure 7).

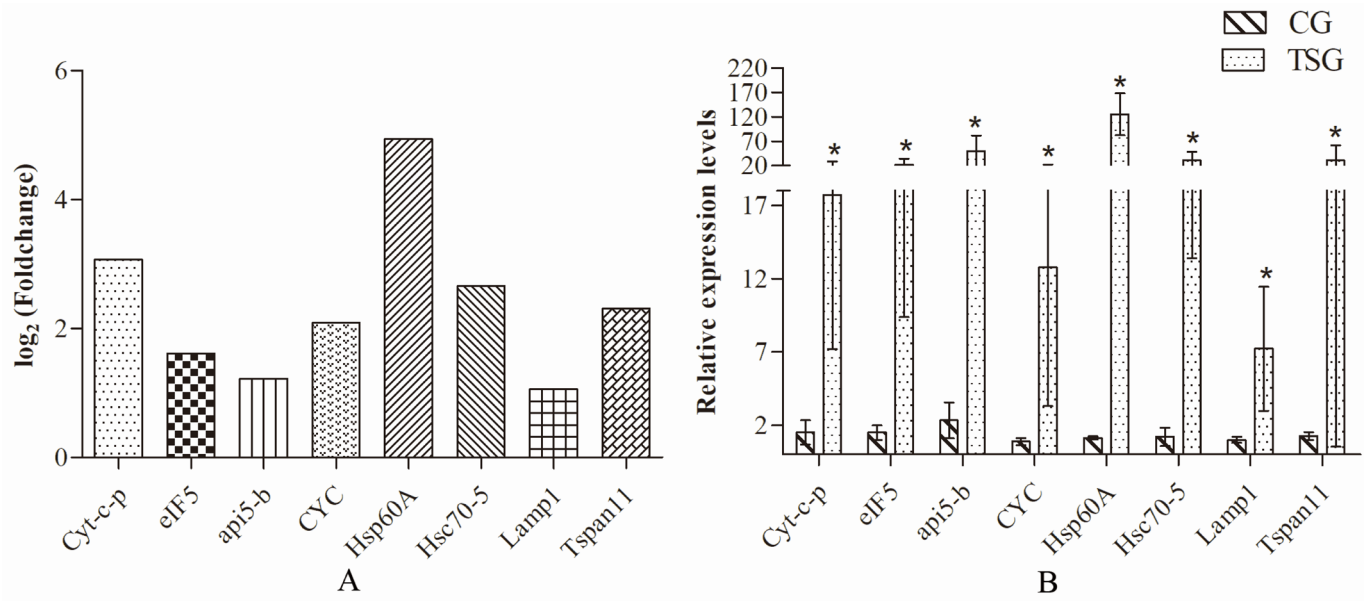

Figure 6. RNA-seq and qRT-PCR analyses of eight differentially expressed genes (DEGs). (**A**) fold-change of eight apoptosis or immune-related DEGs between thethermal stress group (35 °C) and the control group (25 °C)based onRNA-Seq analysis. (**B**) qRT-PCR expression of the *Eriocheir sinensis* DEGs in the heart; * indicates $p < 0.05$; TSG and CG represent the thermal stress group (35 °C) and the control group (25 °C), respectively.

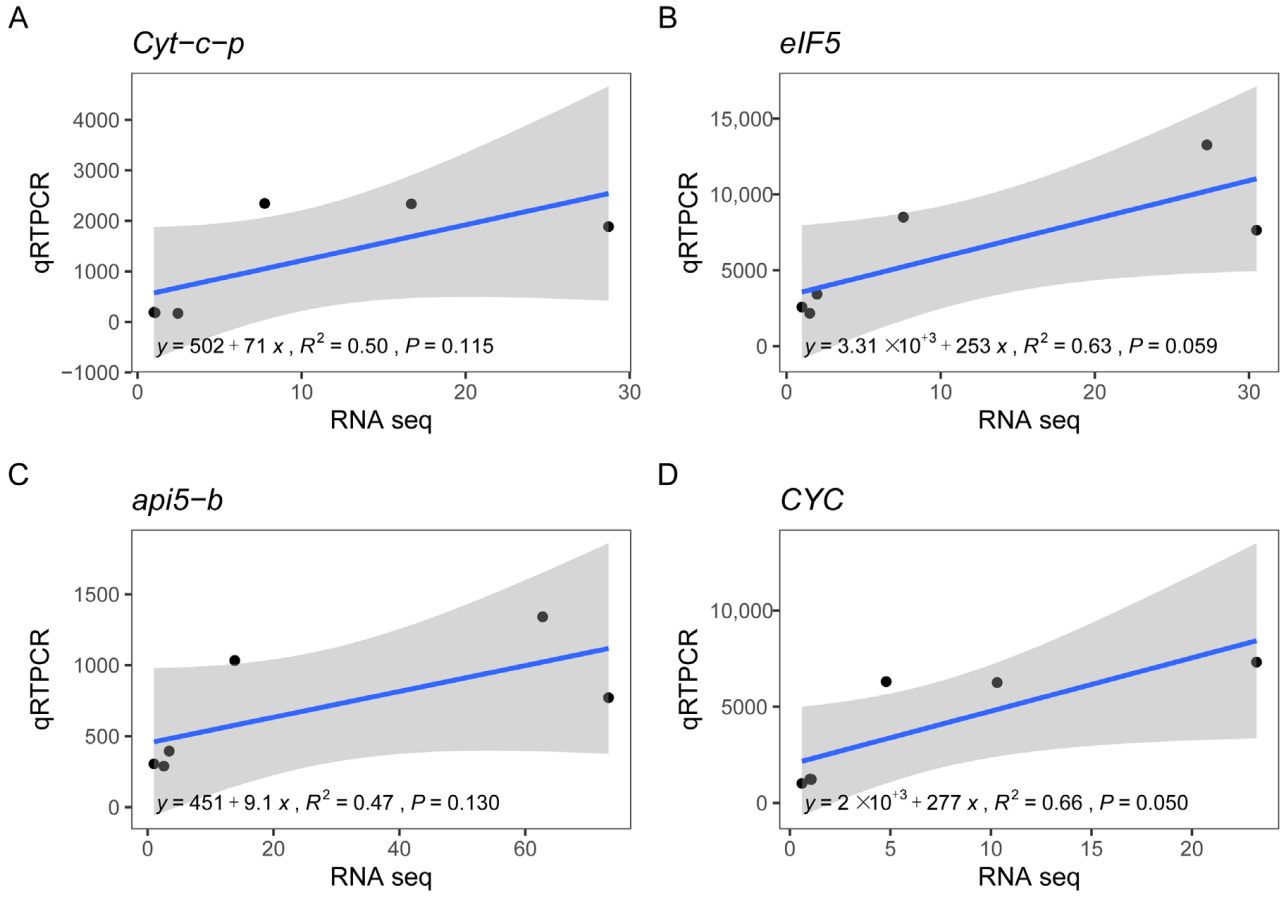

Figure 7. *Cont.*

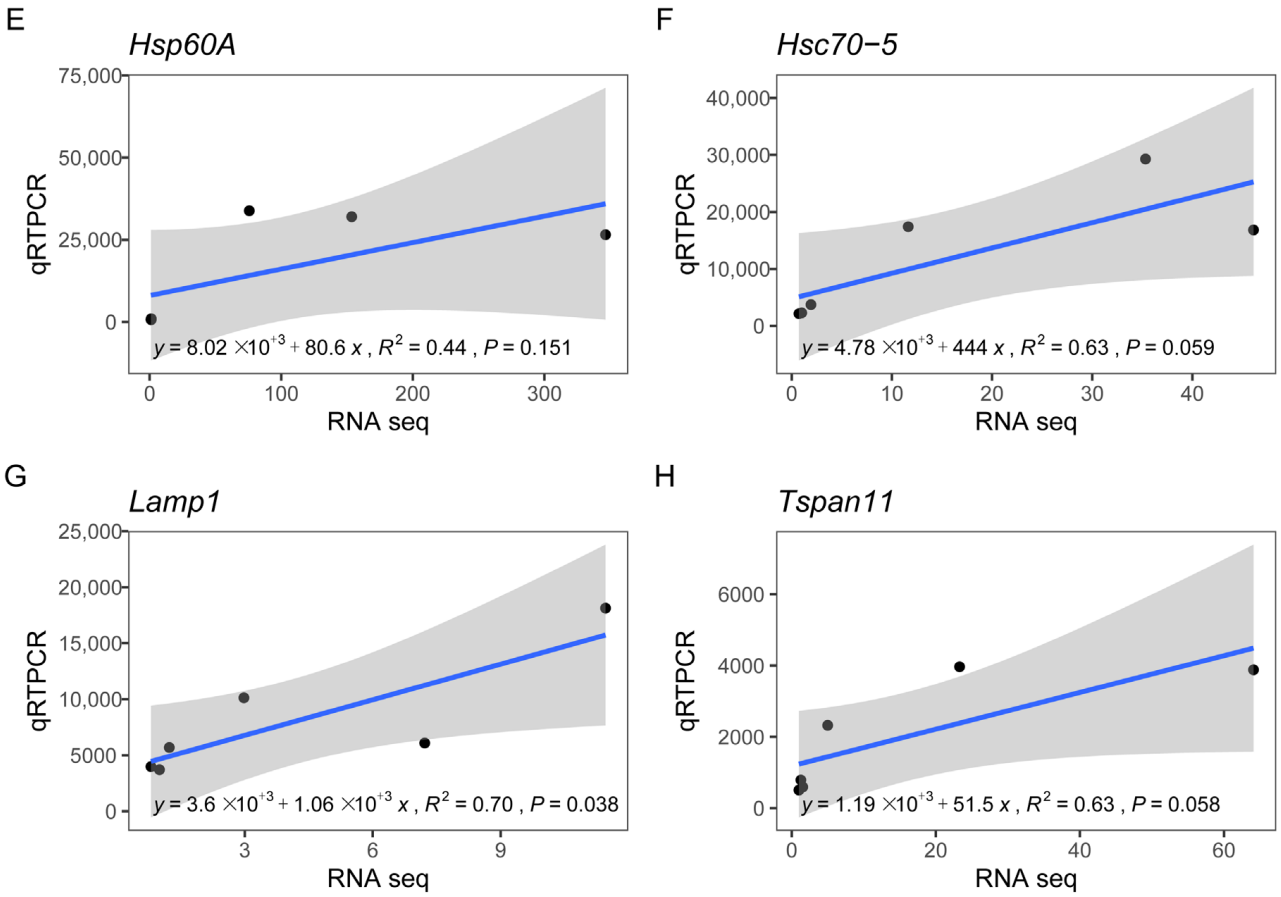

Figure 7. Correlation between qRT-PCR and RNA-seqresults for the eight DEGs in the *Eriocheir sinensis* heart. The x axis and y axis indicate RNA-seq and qRT-PCR results, respectively. (**A**), *Cyt-c-p*. (**B**), *eIF5*. (**C**), *api5-b*. (**D**), *CYC*. (**E**), *Hsp60A*. (**F**), *Hsc70-5*. (**G**), *Lamp1*. (**H**), *Tspan11*.

3.9. Heart Tissue Histology

For male *E. sinensis* cultured at 25 °C for 24 h, horizontal strips of myocardial fibers and nuclei were clear in histological sections of heart tissue (Figure 8A). Most myocardial fibers were lysed, and the number of nuclei and the connective tissue contents between the myocardial layers reduced after 24 h at 35 °C (Figure 8B). This result indicates that high temperatures damaged myocardial fibers and cells. When rainbow trout were acclimated in warm water (17 °C) for eight weeks, cardiac muscle cross-sectional areas decreased 0.8-fold and the thickness of the myocardium increased [17].

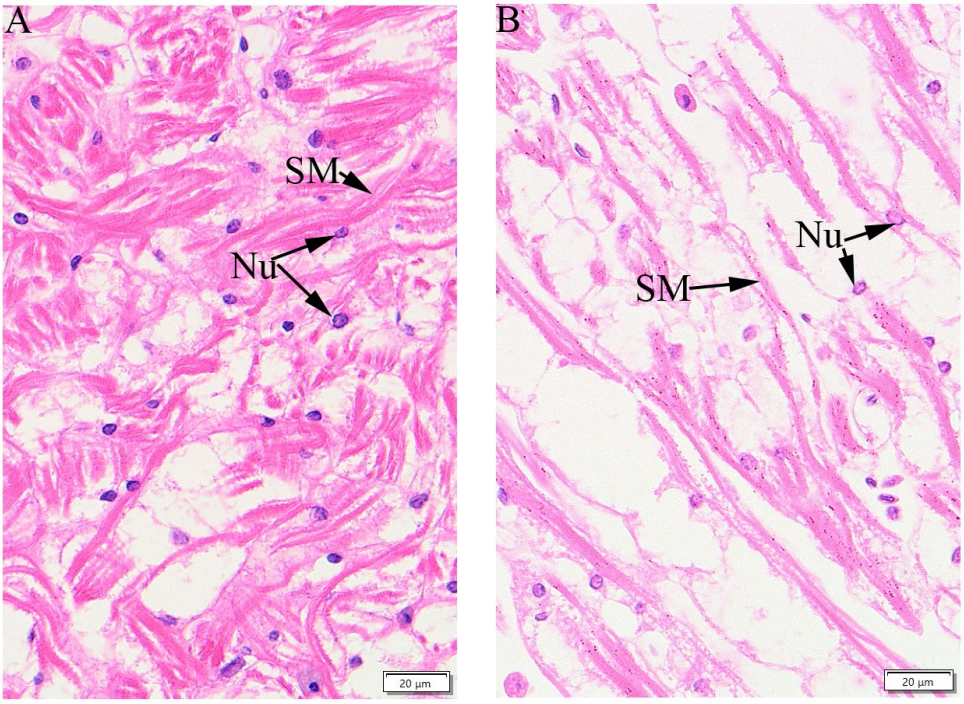

Figure 8. Light microscopic image of a stained *Eriocheir sinensis* histological cardiac section (HE-staining). (**A**), crab myocardium at 25 °C, horizontal strips of myocardial fiber and nuclei are clear; (**B**), crab myocardium at 35 °C, some myocardial fibers appear degraded, and horizontal strips are not clear. Bar markers = 20 μm in (**A**,**B**). SM: striated muscle; Nu: nuclei.

4. Conclusions and Prospects

In conclusion, transcriptome data from the hearts of male *E. sinensis* were analyzed at 35 °C and 25 °C. There were 4007 DEGs between the thermal stress group and the control group, and these included 2660 upregulated and 1347 downregulated genes. Heart DEGs in the thermal stress group were significantly enriched for 1377 GO terms, and the top 20 KEGG pathways included protein processing in the endoplasmic reticulum. Transcriptome quality, SSR, and TFs were also analyzed. RNA-seq and qRT-PCR revealed a significant ($p < 0.05$) correlation for the eight DEGs. This study demonstrated that DEGs of heat shock proteins, transcription factors, and pathways of protein processing in the endoplasmic reticulum were sensitive to high temperatures in male *E. sinensis*. These results will contribute to our understanding of the adaptation of Chinese mitten crabs to high temperatures.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes9030092/s1, Table S1: Quality of heart transcriptome in thermal stress group (35 °C) (TSG) and the control group (25 °C) (CG).; Table S2: DEGs identified between thermal stress group (35 °C) and the control group (25; Table S3: Sample correlation between the thermal stress group (35 °C) and the control group (25 °C). Figure S1: Length distribution of the assembled unigenes.

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