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Analysis of differentially expressed genes in the sea cucumber *Apostichopus japonicus* under heat stress

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Abstract

The sea cucumber *Apostichopus japonicus* plays important roles in marine benthic ecosystem as environmental cleaners, and it is the important aquaculture species in China. High water temperature poses critical threat for the survival of *A. japonicus*, which has resulted in extensive death in summer. To explore the genes expression profiles under different levels of heat stress, the high-throughput RNA-seq was applied in this study. Our results revealed a total of 1 371, 1 225 and 1 408 differentially expressed genes (DEGs) in 26℃ for 6 h, 26℃ for 48 h and 30℃ for 6 h respectively in comparison with Control group. The pathway analysis suggested "Protein processing in endoplasmic reticulum (ER)" was significantly enriched in all these heat stress (HS) treatment groups. The expression results of key DEGs in this pathway (Hsp70, Derlin, NEF, PDI, GPR94 and ERP57) by qRT-PCR was in accordance with the RNA-seq data. The subcluster analysis of DEGs revealed that a variety of heat shock proteins (HSPs) and calcium ion binding proteins had an obvious up-regulated expression in 26℃ for 6 h, comparatively low expression in 26℃ for 48 h, and the highest expression in 30℃ for 6 h. The other DEGs subcluster, consisting of critical components of extracellular matrix (ECM) and a subset of peptidases and proteases, showed significantly rising tendency in 30℃ for 6 h. Additionally, the expression of matrix metalloproteases (MMP1, MMP16 and MMP19) was prominently affected by HS, and peaked in 30℃ for 6 h. This study provides a series of candidate genes for further study about heat shock response in *A. japonicus*, especially genes associated with protein processing in ER and regulation of ECM, which also offers new insights into cellular homeostasis under stressful conditions in marine invertebrates.

Key words: *Apostichopus japonicus*, heat stress, RNA-seq, protein processing, extracellular matrix

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1 Introduction

Echinoderms, an ancient phylum of marine invertebrates, are constantly exposed to environmental stressors, such as global warming, ocean acidification, hypoxia, pathogens, metals, and pollutants (Pinsino and Matranga, 2015). These stressors usually had deleterious impacts on the fertilization, development, growth and distribution of echinoderms, as well as marine biota (Lamare et al., 2011; Pinsino and Matranga, 2015). Particularly in the context of climate change, adaptation to rising temperature was inevitable for echinoderms (Nguyen et al., 2011). Sea cucumbers, belonging to the phylum Echinodermata and the family Holothuroidea, play a central role in the maintenance of ecosystem integrity as environmental cleaners because they swallowed the sediments, organic matter extracts, protozoa, benthic microalgae, and detritus of macroalgae (Slater and Chen, 2015). The sea cucumber *Apostichopus japonicus* (Selenka), inhabiting the coastal sea from a latitude of 35°–44°N, is one of the highest commercially valuable species as seafood (Han et al., 2016; Oh et al., 2017). *Apostichopus japonicus* occupies huge Asia seafood market, particularly in China, which motivated the boom of large-scale aquaculture and artificial breeding (Han et al., 2021; Xu et al., 2015; Zhu et al., 2022). Water temperature is key environmental factor for *A. japonicus*, and heat in summer limits the survival rates and aquaculture production of this species (Huo et al., 2017). Breeding of heat-resistance varieties has been the hotspot of stimulating further progress of *A. japonicus* industry (Ru et al., 2019). Understanding the gene regulation mechanism of heat shock response will provide foundation for molecular breeding in this species.

The critical survival temperature for marine species usually varied from body size, development stages and acclimation ways (Byrne, 2010; Dong and Dong, 2008). Generally, *A. japonicus* survived in a temperature range of 0–30℃, and the optimum temperature for growth was 15–18℃ (An et al., 2007). Researches so far have studied a lot about the effect of high temperature in sea cucumbers. Remarkably, heat shock proteins (HSPs) were vital conserveds families to respond heat stress in *A. japonicus* (Dong and Dong, 2008; Lewis et al., 1999; Xu et al., 2018). Increasing

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evidences suggested that the regulation of heat shock response covered many cellular processes, including detoxification, metabolism, apoptosis, energy production and immune response, which played critical roles in adaptation and surviving under high temperature (Wang et al., 2008; Xu et al., 2016). It was worth noting that when water temperature increased to the lethal threshold, 30℃ for most adult sea cucumbers, autolysis was obviously observed. Autolysis was typical morphological response in sea cucumbers under severe environment and pathological status, which resulted in tissue degradation and proteolysis (Mukundan et al., 1986; Song et al., 2018; Sowmya et al., 2011). Studies to date indicated the damage and degradation of collagen fibres and fibrils, critical components of extracellular matrix (ECM), were associated with the autolysis of body wall in sea cucumbers (Liu et al., 2018, 2019). Several kinds of endogenous proteases from sea cucumbers have been reported to hydrolyse collagen and non-collagen proteins effectively, such as serine proteases, cysteine proteases and matrix metalloproteases (MMPs) (Liu et al., 2016, 2019; Yan et al., 2021). However, it is largely unknown about similarities and differences of the gene expression profiles under different levels of HS, and how the thermal stress triggered the autolysis remained unclear so far.

In the present study, the high-throughput RNA sequencing was applied under two levels of HS (26℃ and 30℃ respectively) in *A. japonicus*. The expression of key differential expressed genes in the pathway "protein processing in endoplasmic reticulum (ER)" was validated by the quantitative real-time PCR (qRT-PCR). Additionally, the expressions of four MMPs (MMP1, MMP2, MMP16 and MMP19) from *A. japonicus* were examined to illustrate the roles under these two levels of HS environments. The results will provide a resource for further mechanistic studies about the regulation of ER and ECM under HS, and offer new insights into cellular homeostasis under stressful conditions.

2 Materials and methods

2.1 *Samples*

A total of 40 sea cucumbers, weighing (100 ± 20) g, were collected from a farm in Yantai, Shandong Province, China in October of 2022. Before the beginning of experiment, these sea cucumbers had been accumulated in the tank of the volume of 2 m^3 30 (2 m × 1 m × 1 m) with the seawater of 18℃, salinity and pH 7.9–8.1 for 2 weeks. During the accumulation period, sea cucumbers were regularly fed with a mixture diet (*Sargassum thunbergii* and sea mud) twice a day, and residual feeds were disposed timely (Xia et al., 2012).

After accumulation, 30 healthy individuals were distributed randomly and equally to five tanks of the volume of 0.3 m^3 (100 cm × 60 cm × 50 cm). The water temperature of Control group was kept at 18℃ all through, and the intestine tissue of five sea cucumbers in this group was sampled. For the HS treatment groups, two levels of high temperature (26℃ and 30℃) were chosen because 26℃ was endurable while 30℃ was lethal for most adult sea cucumber. In the present study, 6 h and 48 h were set in sampling as many stress genes had a peak expression value after 6 h at 26℃ in *A. japonicus* and the 48 h group was widely set in studies on stress response (Xu et al., 2016; Ackerman et al., 2000; Randall and Tsui, 2002). The detailed thermal treatment was as followed. The water temperature went through an increasing process from 18℃ to 26℃ or to 30℃ respectively, with a rate of 2℃ per hour with heating rods. The initial time was set with the moment when the temperature reached 26℃ or 30℃, and then the water maintained at each treatment temperature. Five sea cucumbers at 26℃ treatment were respectively sampled with intestine tissue after 6 h and 48 h from initial time, which were regarded as the group 26℃ for 6 h and 26℃ for 48 h respectively. After 6 h from initial time, sea cucumbers at 30℃ treatment were sampled with intestine tissue, which was regarded as the group 30℃ for 6 h. By contrast, the individuals died after 48 h with exposure of 30℃ thereby no samples in this group. All the samples were quickly frozen in liquid nitrogen, and then stored at –80℃ for further usage.

2.2 *Construction of cDNA library and RNA-Seq transcriptome*

As described above, intestine tissue was respectively collected in Control, 26℃ for 6 h, 26℃ for 48 h and 30℃ for 6 h groups. For the high-throughput RNA sequencing, three biological replications were prepared in each group. The procedure was followed as described previously (Xu et al., 2018). Briefly, the total RNA was extracted from each sample using the Trizol Kit (Invitrogen, USA). After detection for RNA degradation, RNA integrity number and RNA purity, qualified mRNA samples were prepared to build cDNA libraries. First and second strand cDNA were successively synthesized. After adenylation of 3′ ends of DNA fragments, ligation with NEBNext adaptor, purification with AMPureXP beads and amplification, the generated high quality cDNA libraries were sequenced by an Illumina Hiseq platform at Novogene Bioinformatics Technology (Beijing, China).

2.3 *Data processing and identification of differentially expressed genes (DEGs)*

Clean reads were obtained from the raw reads by removing the adaptor sequences, low quality sequences and poly-N reads. All the downstream analyses were based on the clean data with high quality. Paired-end clean reads were aligned to the reference genome with the NCBI accession number GCA_002754855.1 using Hisat2 v2.0.5. Gene expression profiling was counted based on the number of reads, and fragments per kilobase million (FPKM) values were calculated for estimating the expressed values and transcript levels. The resulting *P*-values were corrected by the approach of Benjamini and Hochberg to limit the false discovery rate (FDR) (Benjamini and Hochberg, 1995). DEGs were filtrated with a *P*-value < 0.05 by DESeq2. The analysis of subclusters and hierarchical heat maps was respectively based on the centered and normalized $\log_2$(FPKM+1) and $\log_{10}$(FKPM+1) (Li et al., 2019).

2.4 *Gene Ontology (GO) annotations and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment*

To obtain functional classification and the involved pathways of the genes, GO and KEGG annotations were respectively determined by Blast2GO (http://www.BLAST2go.org/) and KEGG database (http://www.genome.jp/kegg/). The adjusted *P*-value (padj) < 0.05 was taken as a threshold after Bonferroni correction.

2.5 *Validation of DEGs in the pathway "Protein processing in ER" by qRT-PCR*

Five biological replications were examined for qRT-PCR validation in each group. Six genes (HSP70, Derlin, PDI, ERP57, GPR94 and NEF) involved in the pathway "protein processing in ER" were validated by qRT-PCR while the gene *β*-actin was selected for internal standardization. The mRNA of each sample was extracted, and first strand cDNA was synthesized using reverse transcriptase (Takara, Japan). SYBR Green® real-time PCR assay (SYBR PrimeScrip™ RT-PCR Kit II, Takara) and the $2^{-\triangle\triangle CT}$ method were respectively used to measure and analyze the com-

parative mRNA expression levels. One-way analysis of variance with SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical test with the significance level of $P < 0.05$. All the specific primers were listed in Table S1.

2.6 *The mRNA expression of MMPs under HS*

The mRNA expression profiles of four MMPs (MMP1, MMP2, MMP16 and MMP19) were examined by qRT-PCR, according to the known sequences so far (Lv et al., 2022; Miao et al., 2017; Zhang et al., 2016). The same control gene β-actin was used. The specific primers were also showed in Table S1. The procedure was the same as the description above. Circos graph was performed for MMPs expression levels by R (Windows release 4.1.0) (Liu et al., 2022).

3 Results

3.1 *Summary of transcriptomic sequencing data*

High-throughput RNA sequencing was performed through the sequencing platform Illumina HiSeq6000. The number of clean reads in each sample ranged from 39.84 million to 45.79 million (Table S2). The percentage of phred quality score larger than 20 (Q20) and percentage of phred quality score larger than 30 (Q30) in all samples were higher than 96.95% and 91.96%, respectively. Both the raw data and processed data were submitted to the NCBI database with the Gene Expression Omnibus Accession GSE205975.

3.2 *Quantification of gene expression and DEGs among groups*

The gene expression analysis revealed that a total of 1 371, 1 225 and 1 408 genes were differentially expressed in HS treatment conditions (26℃ for 6 h, 26℃ for 48 h and 30℃ for 6 h) in comparison with Control group (P-value < 0.05) (Fig. 1). Among these comparisons, the number of DEGs was the most abundant in 30℃ for 6 h vs. Control, with 715 up-regulated DEGs and 1 033 down-regulated DEGs respectively (Fig. 1a). While some overlapping of DEGs existed in every comparison group, the majority of these DEGs was unique to a single time point (Figs 1b and c). All the DEGs in five comparisons were listed in Table S3.

According to the gene expression characteristics among groups, we highlighted two gene subclusters with up-regulated response under HS. Genes in each subcluster showed similar trend of gene expression. Specifically, the genes in the first subcluster exhibited rapid response under both 26℃ and 30℃ HS: an obvious up-regulated expression in 26℃ for 6 h, comparatively low expression in 26℃ for 48 h, and the highest expression in 30℃ for 6 h (Fig. 2a). The heat map analysis also presented the consistent expression trend, and suggested that the highest expression of these genes occurred in the group 30℃ for 6 h (Fig. 2b). The gene description and the fold change of gene expression in each comparison were listed in Table 1. Most genes in

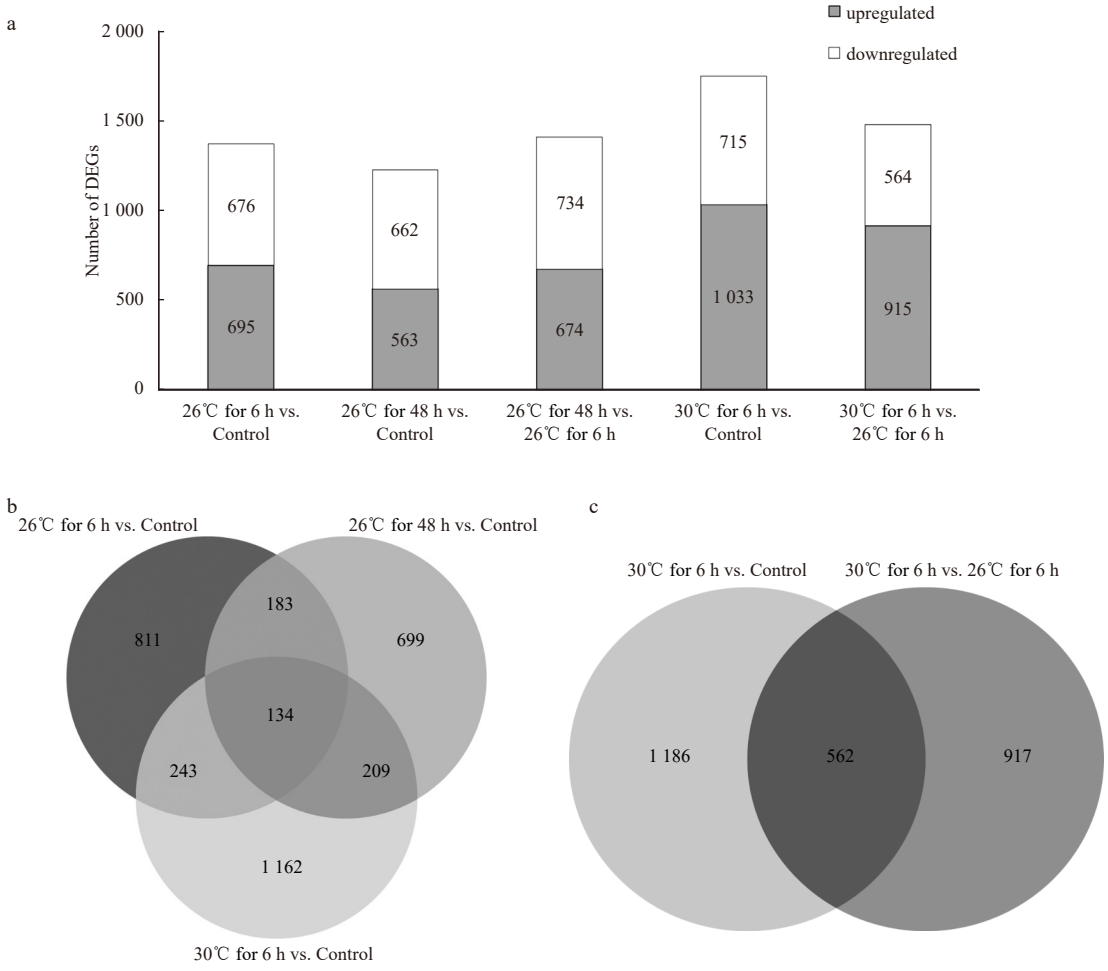

Fig. 1. Overview of the DEGs among the groups. Number of up-regulated and down-regulated DEGs ($P < 0.05$) (a). Venn plot of three HS groups compared with Control group (26℃ for 6 h vs. Control, 26℃ for 48 h vs. Control and 30℃ for 6 h vs. Control) (b). Venn plot of 30℃ for 6 h vs. Control, and 30℃ for 6 h vs. 26℃ for 6 h (c).

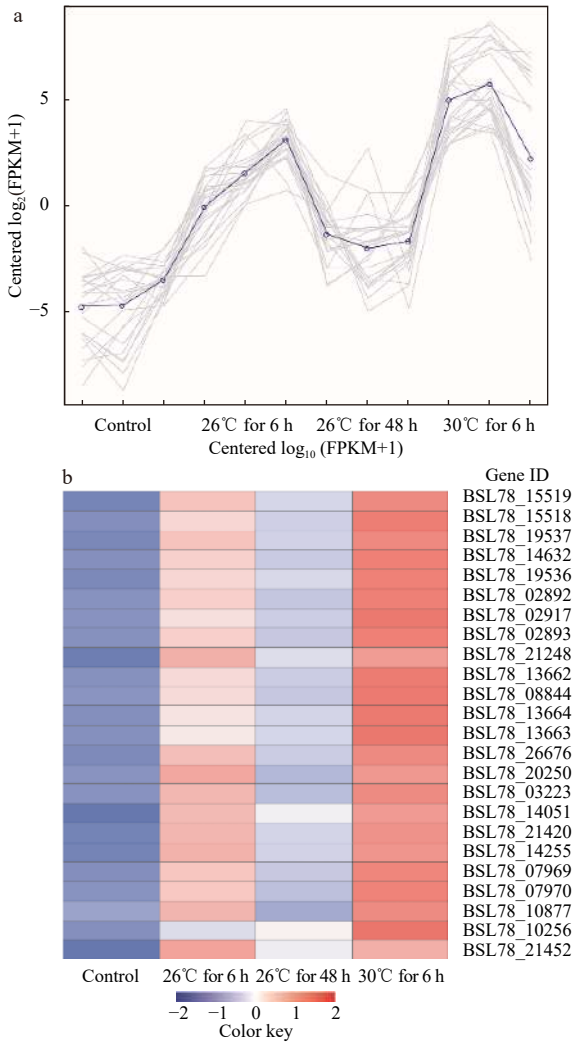

Fig. 2. Expression profiles of genes in subcluster 1. Expression patterns of four clustered profiles based on the centered and normalized $\log_2$(FPKM+1) (a). Hierarchical heat map of all expressed genes based on the centered and normalized $\log_{10}$(FPKM+1) (b); red color represents high expression value while blue color represents low one.

this subcluster were related to protein folding, in particular HSPs (Hsp70, Hsp26 and Hsp90). Besides, three calcium ion binding proteins (putative calumenin isoform X4 and 2 EF-hand calcium-binding domain-containing protein 7) were classified in this subcluster.

By contrast, the genes in the second subcluster appeared to just respond extreme high temperature because the gene expression in this subcluster showed significantly rising tendency only in 30℃ for 6 h (Fig. 3a). In this subcluster, many genes involved in ECM, including fibrinogen, ficolin, collagen and tenascin-R, presented significant up-regulated expression in 30℃_6 h vs. Control comparison (P-value < 0.05) (Fig. 3b, Table 2). Moreover, a subset of peptidases and proteases were found in this subcluster, such as several carboxypeptidases and serine proteases.

3.3 *GO and KEGG enrichment*

GO enrichment analysis revealed that "unfolded protein binding" was the only GO term in 26℃ for 6 h vs. Control comparison with the threshold of P-value (padj) < 0.05 (Fig. 4). A total of 19 GO terms were significantly enriched in 26℃ for 48 h vs. control comparison, among which the number of down-regulated DEGs overweighed the up-regulated ones, such as "metal ion transmembrane transporter activity" and "neurotransmitter: sodium symporter activity". Two GO terms "DNA integration" and "DNA metabolic process" were significantly enriched in 30℃ for 6 h vs. Control comparison.

The pathway analysis suggested "protein processing in ER" (pathway: spu04141) was significantly enriched in all the three HS groups compared with Control group (P-value (padj) < 0.05). Further analysis suggested that many genes involved in this pathway presented up-regulation expression in three HS groups compared with Control group (Figs S1–S3). Remarkably, sHSF/HSPs and molecular chaperones (Hsp70, Hsp40, Hsp26, and NEF) were significantly upregulated under HS, as well as several genes for protein processing such as Sec62/63, PDIs, GPR94, BiP and MKK7. The RNA-seq data showed that ERP57 was significantly up-regulated in both 26℃ for 6 h and 26℃ for 48 h while Derlin was significantly up-regulated in 30 ℃ for 6 h (P < 0.05).

3.4 *Validation of DEGs in the pathway "protein processing in ER" by qRT-PCR*

In order to validate the expression profiles from RNA-seq data, six genes involved in the pathway "protein processing in

Table 1. Fold change of DEGs in the first subcluster for each comparison

Gene ID	Gene description	$\log_2$fold change (26℃ for 6 h/ Control)	$\log_2$fold change (26℃ for 48 h/ Control)	$\log_2$fold change (26℃ for 48 h/ 26℃ for 6h)	$\log_2$fold change (30℃ for 6 h/ Control)	$\log_2$fold change (30℃ for 6 h/ 26℃ for 6 h)
Heat shock protein families and related						
BSL78_15519	heat shock protein 70	8.351 1 ↑	4.149 1 ↑	−4.156 9 ↓	11.074 2 ↑	2.728 7 ↑
BSL78_15518	heat shock protein 70	7.135 0 ↑	3.391 3	−3.689 3 ↓	11.419 ↑	4.284 6 ↑
BSL78_19537	heat shock protein 70	7.569 6 ↑	3.907 1 ↑	−3.632 6 ↓	9.856 2 ↑	2.287 2 ↑
BSL78_14632	heat shock protein 70	6.227 1 ↑	3.202 9 ↑	−2.989 6 ↓	8.865 8 ↑	2.634 8 ↑
BSL78_19536	heat shock protein 70	7.662 4 ↑	5.066 7 ↑	−2.533 3 ↓	10.7570 ↑	3.085 6 ↑
BSL78_02892	heat shock protein 70	7.217 2 ↑	2.654 6 ↑	−4.496 9 ↓	11.151 1 ↑	3.952 8 ↑
BSL78_02917	heat shock protein 70	6.846 7 ↑	3.230 4 ↑	−3.554 9 ↓	12.140 5 ↑	5.317 2 ↑
BSL78_02893	heat shock protein 70	7.452 2 ↑	2.856 8 ↑	−4.526 6 ↓	11.427 9 ↑	3.998 4 ↑
BSL78_21248	heat shock protein 26	6.389 6 ↑	3.179 9 ↑	−3.166 0 ↓	7.013 8 ↑	0.614 5
BSL78_13662	heat shock protein 26	7.361 0 ↑	3.254 9 ↑	−4.049 7 ↓	12.344 7 ↑	4.992 9 ↑
BSL78_08844	heat shock protein 26	7.498 6 ↑	2.929 7 ↑	−4.507 0 ↓	12.865 5 ↑	5.395 6
BSL78_13664	heat shock protein 26	7.464 5 ↑	4.130 6 ↑	−3.281 2 ↓	13.150 8 ↑	5.701 5 ↑
BSL78_13663	heat shock protein 26	7.193 0 ↑	4.062 3 ↑	−3.071 9 ↓	13.287 0 ↑	6.098 4 ↑
BSL78_26676	heat shock protein 90-alpha 3	5.219 4 ↑	2.123 8 ↑	−3.064 4 ↓	6.899 8 ↑	1.655 9

to be continued

continued from Table 1

Gene ID	Gene description	Log_2fold change (26℃ for 6 h/ Control)	Log_2fold change (26℃ for 48 h/ Control)	Log_2fold change (26℃ for 48 h/ 26℃ for 6h)	Log_2fold change (30℃ for 6 h/ Control)	Log_2fold change (30℃ for 6 h/ 26℃ for 6 h)
BSL78_20250	activator of 90 kDa heat shock protein ATPase-like 1-like	5.191 0 ↑	1.108 5	−4.041 4 ↓.	5.599 5 ↑	0.412 4
BSL78_03223	97 kDa heat shock protein	5.197 9 ↑	1.422 1 ↑	−3.738 0 ↓	6.575 0 ↑	1.383 4
BSL78_07969	putative stress-induced-phosphoprotein 1-like	4.875 9 ↑	1.911 0	−2.916 6 ↓	6.818 9 ↑	1.927 1
BSL78_07970	putative stress-induced-phosphoprotein 1-like	4.330 6 ↑	1.358 3 ↑	−2.933 3 ↓	6.305 3 ↑	1.961 9
Calcium ion binding proteins						
BSL78_14051	putative calumenin isoform X4	6.828 6 ↑	4.507 9 ↑	−2.297 0 ↓	7.833 4 ↑	0.963 7
BSL78_21420	putative EF-hand calcium-binding domain-containing protein 7	8.222 2 ↑	4.590 6 ↑	−3.600 8 ↓	9.496 5 ↑	1.270 6
BSL78_14255	putative EF-hand calcium-binding domain-containing protein 7	10.839 3 ↑	6.551 3 ↑	−4.319 8 ↓	12.039 6 ↑	1.129 6
Others						
BSL78_10877	starfish, testis, sperm, peptide transposon	8.094 3 ↑	–	−7.012 4 ↓	9.649 9 ↑	1.542 7
BSL78_10256	integrase catalytic domain-containing protein	4.196 2 ↑	5.750 0 ↑	1.560 3 ↓	9.837 0 ↑	5.603 1 ↑
BSL78_21452	Fes1 domain-containing protein	6.690 5 ↑	3.824 4 ↑	−2.823 2 ↓	6.358 5 ↑	−0.345 4

Note: The arrows " ↑ " and " ↓ " mean significantly up-regulated and down-regulated respectively ($P < 0.05$).

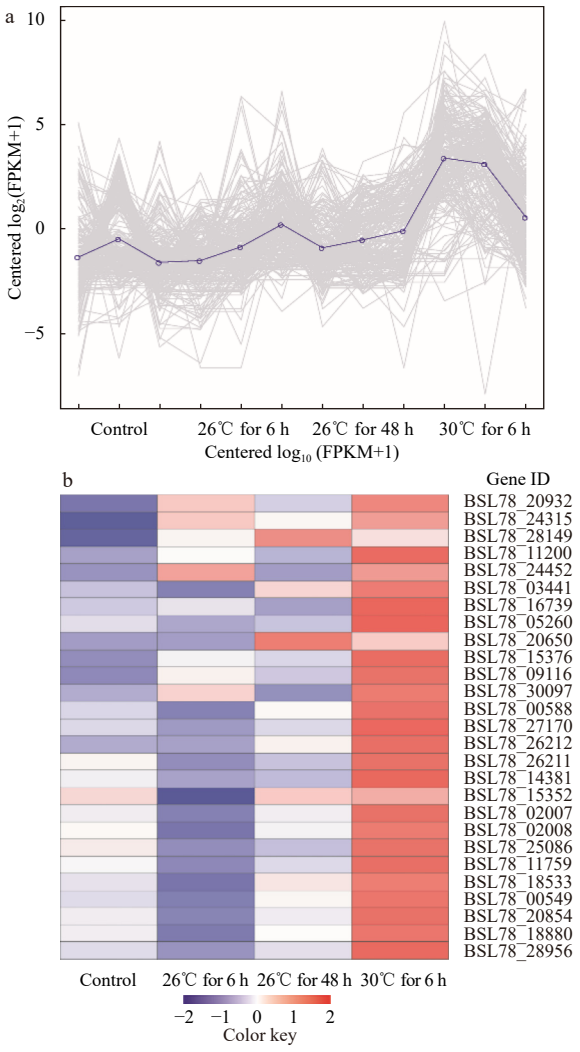

Fig. 3. Expression profiles of genes in subcluster 2. Expression patterns of four clustered profiles based on the centered and normalized log_2(FPKM+1) (a). Hierarchical heat map of all expressed genes based on the centered and normalized log_{10}(FPKM+1) (b). Red color represents high expression value while blue color represents low one.

ER" were applied with qRT-PCR, which included Hsp70, Derlin, NEF, PDI, GPR94 and ERP57. Generally, the results of qRT-PCR were in accordance with the RNA-seq data (Fig. 5). Both the qRT-PCR and RNA-seq data showed that the mRNA expression of these six genes was dysregulated along with the time and temperature of HS. The expression of Hsp70, Derlin and NEF were significantly elevated in 26℃ for 6 h and 30℃ for 6 h ($P < 0.05$). Strikingly, the highest expression of NEF in 30℃ for 6 h reached and 53.22- and 95.34- fold change by the qRT-PCR and RNA-seq analysis respectively. As for PDI, GPR94 and ERP57, the expression peak occurred in 26℃ for 6 h.

3.5 *The mRNA expression of four MMPs under HS*

The mRNA expression of MMP1, MMP2, MMP16 and MMP19 were examined by qRT-PCR. The results revealed that the expression of MMP1, MMP16 and MMP19 were prominently affected by HS (Fig. 6a). Strikingly, the expression of three MMPs peaked in 30℃ for 6 h ($P < 0.05$). As for the gene MMP2, there was no significant difference under HS (26℃ for 6 h, 26℃ for 48 h and 30℃ for 6 h) in comparison with Control group ($P > 0.05$). Additionally, the circos graph analysis suggested that the expression levels of MMP1 and MMP19 occupied large proportion, especially in the group 30℃ for 6 h (Fig. 6b).

4 Discussion

4.1 *HSPs acted as sensitive markers of HS*

HSPs play an important role to defend thermal stress in various species by helping protein folding, assembly, translocation, and degradation (Karouna-Renier and Zehr, 1999). Previous studies showed that a variety of HSPs genes (including Hsp70, Hsp26, Hsp90, Hsp10 and Hsp60) had highest expression before 6h exposure and decreased gradually (Xu et al., 2014, 2018). In the present study, the mRNA expression of most HSPs genes showed higher rise at 26℃ for 6 h in comparison with that at 26℃ for 48 h. It was noteworthy that fold-change of many HSPs genes at 30℃ for 6 h was generally larger than that at 26℃ for 6 h, which revealed that the response of HSPs genes under 30℃ was stronger than that under 26℃. HSPs have been used as biomarkers for stress in a range of algae, invertebrates, fish and other higher vertebrates (Lewis et al., 1999). Hsp70 was especially excellent tool for the analysis for heat stress, oxidative stress and di-

Table 2. Fold change of DEGs in the second subcluster for each comparison

Gene ID	Gene description	Log_2fold change (26℃ for 6 h/Control)	Log_2fold change (26℃ for 48 h/Control)	Log_2fold change (30℃ for 6 h/Control)	Log_2fold change (30℃ for 6 h/26℃for 6 h)
ECM component					
BSL78_20 932	fibrinogen-like protein A	5.461 5 ↑	3.066 8 ↑	7.164 4 ↑	1.748 2
BSL78_24315	putative ficolin-2-like	3.718 6 ↑	3.017 1 ↑	4.495 2 ↑	0.781 9
BSL78_28 149	fibrinogen-like protein A	3.769 9 ↑	5.479 3 ↑	4.062 2 ↑	0.272 2
BSL78_11200	fibrinogen-like protein A	3.155 4	1.196 7	6.263 8 ↑	2.985 4 ↑
BSL78_24452	fibrinogen-like protein A	2.516 7	0.126 4	2.642 7 ↑	0.138 0
BSL78_03441	putative ficolin-2	−4.599 5	2.835 4	5.579 3 ↑	10.177 6 ↑
BSL78_16739	putative ficolin-2-like	1.417 1	−3.257 3	5.158 5 ↑	4.285 4 ↑
BSL78_05260	putative short-chain collagen C4-like	−3.496 3	−1.321 3	5.267 1 ↑	8.782 5 ↑
BSL78_20650	putative collagen alpha-4(VI) chain-like	−0.036 8	1.964 9 ↑	1.230 6	1.248 5
BSL78_15376	putative tenascin-R-like	1.396 9	1.009 9	3.784 1 ↑	2.387 6
Peptidase and protease					
BSL78_09116	glutamyl aminopeptidase	2.300 9	1.174 6	4.500 1 ↑	2.197 9
BSL78_30097	putative lysosomal Pro-X carboxypeptidase	1.851 2	−0.512 1	3.253 5 ↑	1.406 0
BSL78_00588	putative carboxypeptidase A2-like	−2.416 6	0.997 7	4.223 4 ↑	6.602 9 ↑
BSL78_27170	carboxypeptidase A	−1.561 3	−0.038 7	4.046 3 ↑	5.537 6 ↑
BSL78_26212	putative carboxypeptidase B	−0.207 2	1.754 4	4.302 2 ↑	4.508 7 ↑
BSL78_26211	putative carboxypeptidase B	−3.904 1	−1.707 8	2.694 6 ↑	6.566 9 ↑
BSL78_14381	putative carboxypeptidase B	−1.132 7	−0.719 6	2.537 7	3.673 7 ↑
BSL78_15352	putative angiotensin-converting enzyme isoform X4	−3.649 8	0.206 4	0.638 6	4.251 5 ↑
BSL78_02007	proprotein convertase subtilisin/kexin type 9	−2.244 9	0.014 2	3.394 0	5.640 4 ↑
BSL78_02008	proprotein convertase subtilisin/kexin type 9	−2.859 2	−0.329 5	2.693 1	5.557 2 ↑
BSL78_25086	proprotein convertase subtilisin/kexin type 9	−2.685 9	−1.575 1	2.702 1	5.397 2 ↑
BSL78_11759	putative proteinase T	−2.353 3	−0.492 8	2.900 1	5.231 4 ↑
BSL78_18533	putative tolloid-like protein 1	−2.419 1	0.953 1	3.165 6	5.586 1 ↑
BSL78_00549	trypsin-like serine protease	−1.672 3	0.700 7	3.137 2	4.817 0 ↑
BSL78_20854	serine proteinase	−2.030 4	−0.025 7	3.152 7	5.185 6 ↑
BSL78_18880	serine proteinase	−2.070 9	0.260 3	2.860 9	4.939 2 ↑
BSL78_28956	peptidase_S8 domain-containing protein	−1.339 8	0.049 0	3.454 4	4.803 6 ↑

Note: The arrow " ↑ " and " ↓ " means significantly up-regulated and down-regulated respectively ($P < 0.05$).

verse environmental contamination (El Golli-Bennour and Bacha, 2011; Moreira-de-Sousa et al., 2018; Wang et al., 2018b). The characteristics of HSPs were similar among other species under HS. Our results revealed that the fold change of HSPs under acute HS (26℃ for 6 h and 30℃ for 6 h) were much stronger than that in summer in northern China (21℃ in June and 25℃ in July), according to the previous study in *A. japonicus* (Li et al., 2019). The differences resulted from the thermal treatment and final temperature. Our results reinforced the notion that HSPs members were sensitive markers of HS and had potential applications in monitoring and management of stress (Mohanty et al., 2018).

4.2 *Protein processing in ER under HS*

ER is a subcellular organelle where newly synthesized peptides are folded with the help of luminal chaperones. Our results presented that this pathway "protein procession in ER" was significantly enriched under HS in *A. japonicus* among those three comparisons. The evidence indicated that protein processing in ER was of vital importance for the protein folding and homeostasis under HS. This result was in accordance with that in other species, including plants (tomato, blueberry and algae) and fish (rainbow trout and pikeperch) (Callwood et al., 2021; Ding et al., 2020; Huang et al., 2018; Wang et al., 2018a, 2019). These studies reported DEGs involved in protein processing in ER such as protein folding machinery (HSPs and PDIs), protein homeostasis (sHSP), luminal chaperon (NEF and GRP94), chaperon binding protein (BiP), protein translocation (Secs), ubiquitination (Derlin) and other mechanisms involved in the HS response. In our study of *A. japonicus*, sHSF/HSPs, Sec62/63, BiP and PDIs were significantly up-regulated among the three HS comparisons with Control.

Secs proteins, including Sec61 and Sec62/63, located in the membrane of ER, were central proteins in the translocation process of nascent and newly synthesized precursor polypeptides into the ER (Linxweiler et al., 2017). The up-regulation of Secs implied that the nascent and newly synthesized precursor polypeptides increased in ER under HS, which could trigger unfolded protein response (UPR). On one hand, the chaperones and folding enzymes in ER were up-regulated in order to relieve the damage from the nascent polypeptides in ER. BiP, also referred to as the 78-kDa glucose-regulated protein (GRP78), was a major ER chaperone and master regulator of UPR (Li et al., 2008). BiP could translocate newly synthesized polypeptides across the ER membrane, facilitate the folding and assembly of newly synthesized proteins and maintain them in a state competent for subsequent folding and oligomerization (Hendershot, 2004). PDIs could cata-

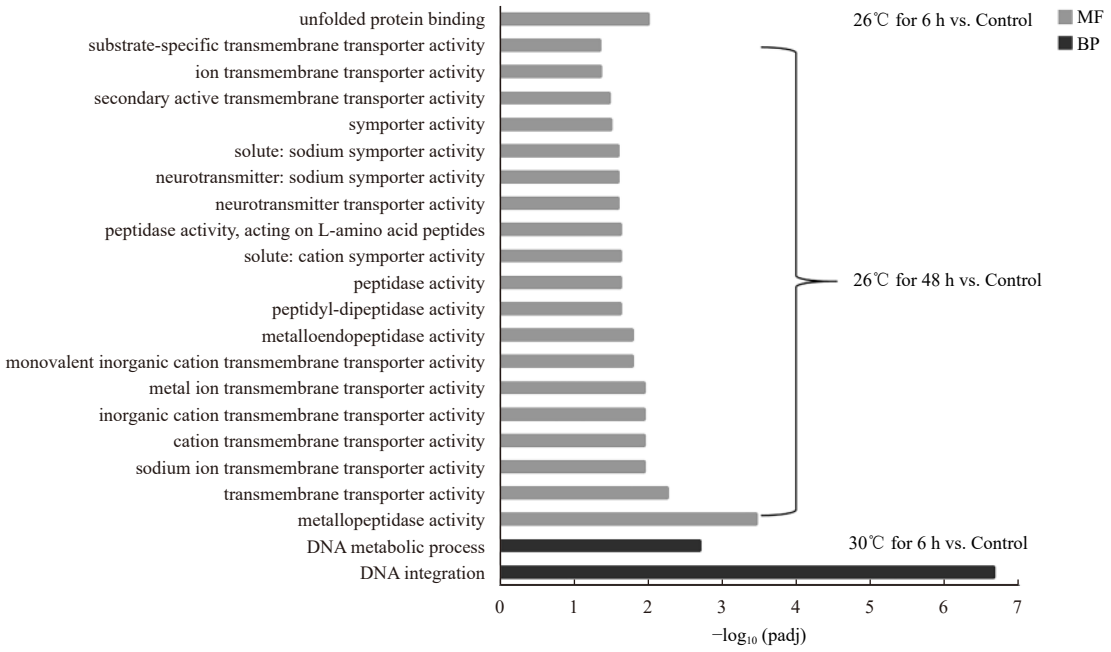

Fig. 4. Enriched Gene Ontology (GO) in three HS groups compared with Control group. "MF" and "BP" were short for "Molecular Function" and "Biological Process" respectively. Adjusted *P*-value (padj) < 0.05.

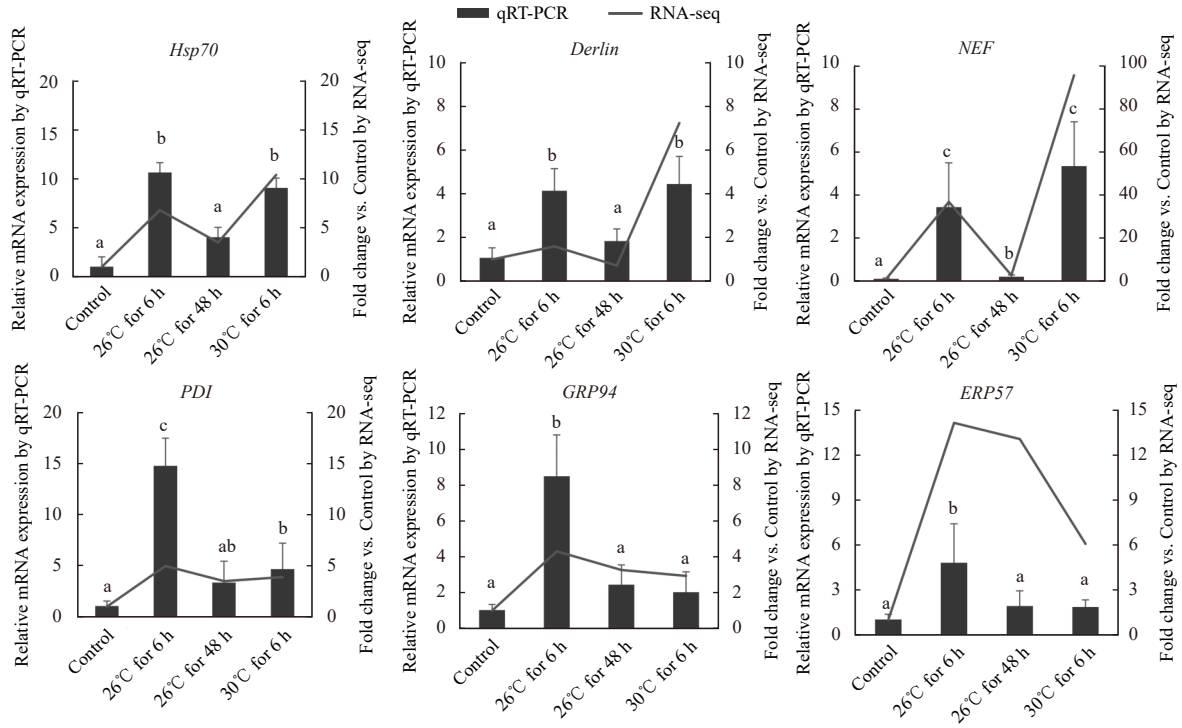

Fig. 5. Validation of the expression of six DEGs in the pathway "protein processing in endoplasmic reticulum" by qRT-PCR. The histogram represented the qRT-PCR analysis, in which the $2^{-\triangle\triangle CT}$ method was used. In the qRT-PCR analysis, the letters indicated significant differences ($P < 0.05$) and values were represented as mean ± standard deviation (SD) ($N = 5$). The line represented the fold change by RNA-seq data.

lyze disulfide bond formation and rearrangements in the substrate proteins and facilitate the folding of nascent polypeptides in ER (Feige and Hendershot, 2011). Both BiP and PDIs were key regulators of ER stress (Bertolotti et al., 2000). In our study, the up-regulation of BiP and PDI indicated that unfolded proteins in the ER accumulated under HS, and cells were adapted to ER stress by the way of UPR. In order to deal with the uncorrected-fold proteins, the system of ER-associated degradation (ERAD) was activated (Ruggiano et al., 2014). Our research revealed various HSPs and NEF played key roles in ERAD under both two HS conditions. Derlin1 was key component protein in the ubiquitin ligase complexes (Zattas et al., 2013). Derlin1 was significantly higher in the group 30℃ for 6 h, which implied more requirement for protein ubiquitination and degradation. Taken together,

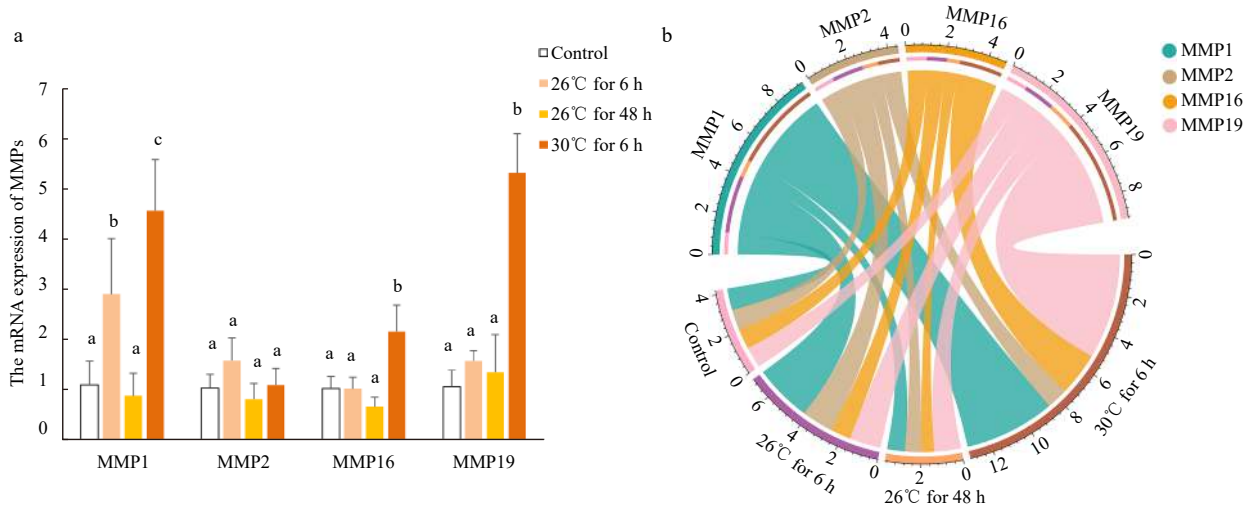

Fig. 6. The mRNA expression of four MMPs (MMP1, MMP2, MMP16 and MMP19) by qRT-PCR. a. The histogram represented the qRT-PCR analysis, in which the $2^{-\triangle\triangle CT}$ method was used. The letters indicated significant differences ($P < 0.05$) and values were represented as mean ± SD ($N = 5$). b. Circos graph shows the expression levels of four MMPs.

the differential expression patterns of DEGs in ER suggested possible protein homeostasis and repair mechanisms under HS.

4.3 *Extracellular matrix-associated genes under HS*

Our high-throughput RNA-seq data revealed that a subset of genes associated with fibrinogen, ficolin and collagen were significantly up-regulated at 30℃ for 6 h, which were the major proteins of ECM. In addition to providing physical support for cells, ECM actively participated in regulating the development, function and homeostasis of cells (Mouw et al., 2014). Our data suggested that the variation of ECM was significant feature in the group 30℃ for 6 h in *A. japonicus*. It was worth noting that the tissue autolysis was observed in 30℃ during our experiment. Previous study revealed that the degradation of collagen fibres was observed during the autolysis of sea cucumber body wall, as well as the structural damage of collagen (Liu et al., 2018). It was speculated that up-regulation of fibrinogen, ficolin and collagen components was related with the degradation and rebuilding of ECM under HS.

Our RNA-seq data suggested that several carboxypeptidases and serine proteases were significantly abundant in 30℃ for 6 h. These peptidase and proteases, which could cleave residues from a range of peptide and protein substrates, were identified to be critical regulators in the extracellular environment (Atiakshin et al., 2022; Lyons et al., 2008; Novikova et al., 2000; Wilkinson et al., 2017). MMPs, a large family of calcium-dependent zinc-containing endopeptidases, played significant roles in degrading ECM and reconstructing tissues (Das et al., 2003). Previous study revealed that the endogenous MMP degraded collagen fibres and fibrils in the body wall of sea cucumbers, and the treatment with MMP inhibitor prevented the autolysis (Liu et al., 2019). Our results showed that the mRNA expression of MMP1, MMP16 and MMP19 was significantly up-regulated by short-time HS, and the highest expression occurred in 30℃ for 6 h. Above all, our results suggested critical roles of these peptidases and proteases in ECM degradation resulted from HS, especially the temperature 30℃.

5 Conclusions

Our results revealed a total of 1 371, 1 225 and 1 408 DEGs in 26℃ for 6 h, 26℃ for 48 h and 30℃ for 6 h in comparison with Control group. The pathway "protein processing in ER" was significantly enriched, which included several DEGs, such as Hsp70, Derlin, NEF, PDI, GPR94 and ERP57. A variety of HSPs and calcium ion binding proteins had an obvious up-regulated expression in 26℃ for 6 h, comparatively low expression in 26℃ for 48 h, and the highest expression in 30℃ for 6 h. Some critical components of ECM and a subset of peptidases and proteases showed significantly rising tendency in 30℃ for 6 h. Additionally, the expression of MMP1, MMP16 and MMP19 peaked in 30℃ for 6 h. In the future, some works about the roles of MMPs and remodeling of ECM under HS, and the relationship between heat shock response and ER stress could be interesting and challenging.

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Supplementary information:

Fig. S1. Map of alignment of DEGs in the pathway "protein processing in endoplasmic reticulum" pathway in the comparison 26℃ for 6h vs Control. The colored box represents DEGs, and their degree of expression is depicted by color variations, with red the most upregulated and green the most downregulated ($P < 0.05$), white box represents no significant differences in gene expression.

Fig. S2. Map of alignment of DEGs in the pathway "protein processing in endoplasmic reticulum" pathway in the comparison 26℃ for 48h vs Control. The colored box represents DEGs, and their degree of expression is depicted by color variations, with red the most upregulated and green the most downregulated ($P < 0.05$), white box represents no significant differences in gene expression.

Fig. S3. Map of alignment of DEGs in the pathway "protein processing in endoplasmic reticulum" pathway in the comparison 30℃ for 6h vs Control. The colored box represents DEGs, and their degree of expression is depicted by color variations, with red the most upregulated and green the most downregulated ($P < 0.05$), white box represents no significant differences in gene expression.

Table S1. Primers used for qRT-PCR.

Table S2. Summary of transcriptomic sequencing data.

Table S3. All DEGs in each comparison.

The supplementary information is available online at https://doi.org/10.1007/s13131-023-2196-4 and http://www.aosocean.com/. The supplementary information is published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.