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Transcriptomics and metabolomics reveal the molecular and metabolic adaptation to heat stress in Manila clam *Ruditapes philippinarum*

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Temperature is an important environmental factor affecting metabolism, growth and life activities of marine bivalves. To reveal the molecular and metabolic responses to heat stress, comparative transcriptomics and metabolomics were performed in the commercially important bivalve, Manila clam *Ruditapes philippinarum*. Comparative transcriptomics revealed a total of 3980 differential genes (DEGs) mainly involved in energy metabolism, protein processing, glycerolphospholipid metabolism, inoxidizability and anti-apoptosis. Comparative metabolomic analysis identified 293 differential metabolites (DEMs) in the heat-stressed clams, including Acetyl-CoA, Beta-D-Glucose, phosphatidylcholine and free amino acids. For the heat-stress clams, they showed the significantly increased enzyme activities of AKP and ACP, as well as antioxidant-related enzyme activities (SOD and CAT) and oxidative damage substance (MDA). The changes in gene expression of glycolysis-related genes (GLUT1, GLK, HK1 and GPI6) and metabolites (Beta-D-Glucose and Acetyl-CoA) reflect the adjusted energy metabolism in the heat-stressed clams. The protein processing in endoplasmic reticulum may play the important roles in the stability of cell membrane structure during heat adaptation. The increased contents of glycerophospholipid (PC) may be essential to maintain the stability and permeability of cell membrane in the heat-stressed clams. Molecular chaperones (HSP70 and HSP90) may participate in the degradation of misfolded proteins to maintain cell homeostasis during the heat stress. To defense the heat stress, clams also initiate the protection and defense

mechanisms by activating antioxidant system, innate immune system and anti-apoptotic system. These findings will shed new lights on molecular and metabolic adaptation to heat stress in the intertidal mollusks in a warming climate at the global scale.

KEYWORDS

heat stress, *Ruditapes philippinarum*, transcriptomic, metabolomic, enzymatic activity

1 Introduction

Temperature is one of the major environmental stressors in marine organism. As the frequency of global extreme weather increases, the temperature in the sea area increases year by year (Bindoff et al., 2019). The molecular and physiological responses of aquatic animals to heat stress in their living environment has drawn more and more attention (Pörtner and Farrell, 2008; Somero, 2010). As previously reported, the metabolic activities, growth development and physiological conditions of aquatic animals have been remarkably affected by the heat stress, especially for the intertidal mollusks (Freytes-Ortiz and Stallings, 2018; Jiang et al., 2019; Dong et al., 2022a). Relative to the swimming ability of fish and crustaceans, most of intertidal mollusks have poor locomotion activity to escape from their local habitat (Trueman, 1983). For mollusks, the appropriate temperature can promote their growth and development, but when temperature exceeds a certain limit, it will negatively affect the normal metabolism and even cause their death (Velasco and Barros, 2008).

The potential effects of heat stress on survival, behavior, physiological and metabolic changes have been widely evidenced in a variety of mollusk species, contributing to the current status of species distribution patterns (Dong et al., 2022a). For mollusks, when temperature exceeded their optimal range, they have to decrease oxygen consumption rate and ammonia removal rate, resulting in the disordered physiological function (Ansell and Sivadas, 1973; Aldridge et al., 1995; Bougrier et al., 1995). As indicated, high temperature can significantly affect the oxygen consumption, ammonia-N excretion, enzyme activities, as well as valve closing behavior of scallop *Mizuhopecten yessoensis* (Hao et al., 2014). The aerobic metabolism of abalone *Haliotis iris* was remarkably limited under the heat stress, mainly due to the reduced binding ability of hemocyanin to oxygen (Wells et al., 1998). In order to cope with heat stress, the clam *Chamelea gallina* increased their metabolic intensity, leading to aggravated oxidative damage (Monari et al., 2007). The network analysis of oyster transcriptome revealed a cascade of gene responses during recovery after heat shock, including senescence-associated protein, inhibitor of apoptosis protein, cAMP response element-binding protein, and heat shock protein 70 (Zhang et al., 2012b). Despite this, the capacity of mollusks to adapt to heat stress from molecular to metabolic functions remains largely unknown in most species. Therefore, it is essential to conduct the integrative studies on different levels to illustrate the adaptive strategy in response to heat stress in mollusks.

Transcriptomics is a discipline that reveals complex biological pathways and molecular mechanisms of character regulatory networks through comprehensive research on gene expression and regulatory laws (Manalo et al., 2005). Transcriptomics has been widely used in molecular research of mollusks, and has been reported in oyster *Crassostrea gigas* (Meng et al., 2013), clam *Ruditapes philippinarum* (Nie et al., 2020), clam *Panopea globose* (Juárez et al., 2018), and scallop *Patinopecten yessoensis* (Jiang et al., 2018). Owing to recent technical advances, metabolomics has been utilized extensively for the identification of single metabolites, representing the apogee of the omics trilogy (Patti et al., 2012). However, metabolomics has been rarely applied to in the field of mollusks studies. Association analysis of different omics helps reveal more complex biological regulation mechanisms (Gracey and Connor, 2016; Xu et al., 2016). Therefore, the combination of multiple omics such as transcriptomics and metabolomics may help to further clarify the molecular and metabolic mechanisms of bivalve mollusks in response to heat stress.

The Manila clam *R. philippinarum* has the advantages of fast growth and high nutritional value, and is an important economic mollusk cultivated in coastal areas (Zhao and Zhang, 2016; Nie et al., 2017; Yan et al., 2019). Due to the rising of global temperatures, summer temperatures can frequently threaten the survival of this species (Zhang and Yan, 2006; Dong et al., 2022a). Although gene expression responses to thermal stress has been reported, the critical information about thermal effects at different levels is still lacking in clams (Nie et al., 2017; Ding et al., 2018). In this study, comparative transcriptome and metabolomics were combined to reveal the molecular and metabolic mechanisms of clams in response to heat stress, and further understand the thermal sensitivities of clams and their capacities to adapt to temperature changes. The findings of this study are of great significance to the study of molecular mechanism of aquatic organisms and the practice of aquaculture.

2 Materials and methods

2.1 Animal collection and acclimation

In this study, clams were collected from aquaculture ponds in Qingdao, and healthy individuals were selected for subsequent

experiments. The clams (shell length 31.83 ± 1.94mm) were raised in a seawater bath for seven days before the start of the experiment. After feeding, the clams were randomly divided into two groups, including a control group (20°C) and a heat stress group (30°C). The heat stress experiment was set up according the previous studies, which showed the decreased survival and depressed physiological condition of heat-stressed clams at 30°C, compared to the healthy condition at 20°C (Han et al., 2008; Munari et al., 2011; Zhang et al., 2023). After 24 h of stress, whole-tissue samples from nine clams in each group were used for metabolite extraction in the metabolome (nine biological repetitions), and three samples of the same amount in two group were mixed for RNA extraction in the transcriptome (three biological repetitions). The whole-tissue sampling was performed to investigate transcripts and metabolite changes of clams in response to heat stress at the whole-organism level. According to the results of transcriptomics, we selected six enzyme activities and nine genes for subsequent experiments. The gill tissues of clam under heat stress (three biological repetitions) for 24 h, 48 h, 72 h, 96 h and 120 h were taken for enzyme activity determination, reference gene screening and gene expression determination. Ethical review and approval was not required for the study on clams in accordance with the local legislation and institutional requirements.

2.2 RNA extraction and transcriptome sequencing

The total RNA from the entire tissue sample was extracted using Trizol reagent. The concentration and purity of total RNA were detected by NanoPhotometer spectrophotometer and agarose gel electrophoresis. The qualified total RNA was used in subsequent experiments. In short, RNA was randomly disrupted at elevated temperature and the fragmented RNA was used as a reverse transcription template to produce a second cDNA strand, which was sequenced using the Illumina HiSeqTM 4000 platform. After filtering the original data, the remaining clean reads were further mapped to the reference genome. Differential expressed genes (DEGs) between the two groups were screened using DESeq2 software. We selected DEGs according to the following criteria: FDR < 0.05, $|\log_2 FC| > 1$ (FDR: false discovery rate; FC: fold change). All the DEGs were then mapped to the GO term (FDR < 0.05) to characterize its primary biological function. Enrichment of the KEGG pathway was performed to identify the major biochemical and signal transduction pathways in DEGs.

2.3 Metabolome sample treatment and LC-MS/MS detection

Metabolites were extracted from 50 mg treated clam samples. Metabolites were extracted conventionally using a mixture of 1 ml of acetonitrile and methanol according to our previous study (Sun et al., 2021). After milling, low-temperature ultrasonic treatment and centrifugation, the sample was transferred to the sample bottle for subsequent analysis. LC-MS/MS analysis was performed using an UHPLC (1290, Agilent Technologies). For the positive mode, mobile phase A consists of 0.1% aqueous formic acid, and mobile phase B consists of acetonitrile. For the negative mode, mobile phase A was composed of 5mM ammonium acetate in water and mobile phase B was set as acetonitrile. Isolate the compound at 0.5 mL/min using the gradient method. The XCMS software was used for the correction of retention time correction, peak recognition, peak extraction, peak integration and peak alignment. The OPLS-DA (Orthogonal projection to latent structures-discriminant analysis) model was used in the following analysis. The differential metabolites (DEMs) were screened according to the combination of *P* value of t-test and VIP (variable importance in the projection) of the OPLS-DA model. The screening criteria were set as *P* value < 0.05 and VIP ≥ 1. The KEGG analysis was then used for the pathway enrichment of DEMs.

2.4 The integrated analysis of transcriptomic and metabolomic data

The transcriptomic and metabolomic datasets were combined for the integrated analysis. For transcriptomics and metabolomics association analyses, we used KEGG pathway analysis to determine the association between the transcript and the metabolite. Based on previous studies, we also analyzed the association between the transcript and the metabolite using different methods, including Two-way Orthogonal PLS O2PLS (Bylesjö et al., 2007), the joint loading plots (Bouhaddani et al., 2016) and pearson correlation coefficients (Copley et al., 2017).

2.5 Enzyme activity analysis

The enzyme activities including catalase (CAT), malondialdehyde (MDA), Na^+K^+-ATPase (NAK), alkaline phosphatase (AKP), acid phosphatase (ACP) and superoxide dismutase (SOD) were determined in gill of clams. All these enzyme activities were detected using the corresponding kits (Aidisheng, Jiangsu, China).

2.6 Selection of reference gene

In order to obtain accurate gene expression results, the optimal internal reference gene for clam under heat stress was screened out. The total RNA in the gills of clam at five sampling time points in the two groups was extracted by TRIzol. The purity and quality of RNA were confirmed by NanoPhotometer spectrophotometer (Implen, CA, USA). The cDNA was synthesized using the HiScript III RT SuperMix for qPCR (+gDNA wiper) (Vazyme, China) according to the instructions of the manufacture. In this study, the most appropriate internal reference gene was selected from seven common internal reference genes, including β-actin (ACT), elongation factor 1-α (EF1A), tubulin (TUB), ribosomal protein L31 (RPL31), ribosomal protein S23 (RPS23), histone (HIS) and

growth factor receptor-binding protein 2 (GFRP2). The primers were designed by Primer3 online (https://bioinfo.ut.ee/primer3-0.4.0/) and synthesized by Sangon Biotech (Shanghai) (Supplementary Table S1).

The qRT-PCR reactions were amplified in CFX Connect Real-Time PCR Detection System. The reaction solution included 10 μL 2×ChamQ STBR Color qPCR master mix (Vazyme, China), 0.4 μL each for forward and reverse primer, 2 μL cDNA template, 7.2 μL ddH_2O. The reaction procedure of qRT-PCR was 95°C for 30 s, 40 cycles at 95°C for 10 s and 60°C for 30 s. The reverse transcribed cDNA was diluted into five gradients to obtain a standard curve and calculate the amplification efficiency in qRT-PCR instrument. GeNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) were used to statistically analyze the stability of gene expression for each candidate reference gene. The best candidate reference gene ranking was obtained by RefFinder (http://www.leonxie.com/reference gene.php).

2.7 Gene expression level determination

In order to verify the expression patterns of important genes, nine genes were selected for qRT-PCR analysis. The selected genes include energy metabolism genes, including succinate dehydrogenase (SDH), phosphoenolpyruvate carboxykinase (PEPCK) and ADP-dependent glucokinase (ADPGLK). Four genes involved in protein processing, including nucleotide exchange factor SIL1 (SIL1), heat shock protein 70 (HSP70), GTP-binding protein SAR1B (SAR1B) and heat shock protein 90 (HSP90). Furthermore, there are also antioxidation gene cytochrome P450 (CYP450) and immune gene nuclear factor kappa-B (NF-κB). The screened optimal reference gene was used as an internal control, and relative gene expression levels were analyzed by the comparative $2^{-\Delta CT}$ method. The related information on the selected genes and relevant primers was listed in Supplementary Table S2.

2.8 Data analysis

Significant differences in enzyme content and gene expression between control group and heat stress group were assessed by t-tests. Results are presented as mean ± standard error of the mean.

3 Results

3.1 Transcriptome analysis of clam under heat stress

A total of 3980 DEGs including 1876 up-regulated genes and 2104 down-regulated genes were identified between the control and heat stress groups. In order to directly observe the expression pattern of DEGs between the two groups, a volcanic map with FDR and log_2FC values was drawn (Figure 1A). The genes with the greatest difference in expression between the heat stress group and control group were located at both ends of the horizontal axis, including lymphocyte antigen 6D, c-type lectin, serine/threonine-protein kinase ULK4, receptor-type tyrosine-protein phosphatase mu and serine protease inhibitor Cvsi. The heat map shows the DEGs expression levels between the control group (C1, C2 and C3) and the heat stress group (T1, T2 and T3) (Figure 1B). Gene expression levels in different samples were shown as light blue (low expression level) to dark red (high expression level). Three samples in the control group were divided into one category, and three samples in the heat stress group were divided into another category.

GO annotation and KEGG enrichment analysis were carried out to further explore the biological processes and metabolic pathways for these DEGs. For the biological process, most genes were enriched in three GO term processes: cellular process, metabolic process and single-organism process. For the cellular component, most DEGs were concentrated in cell, cell part and

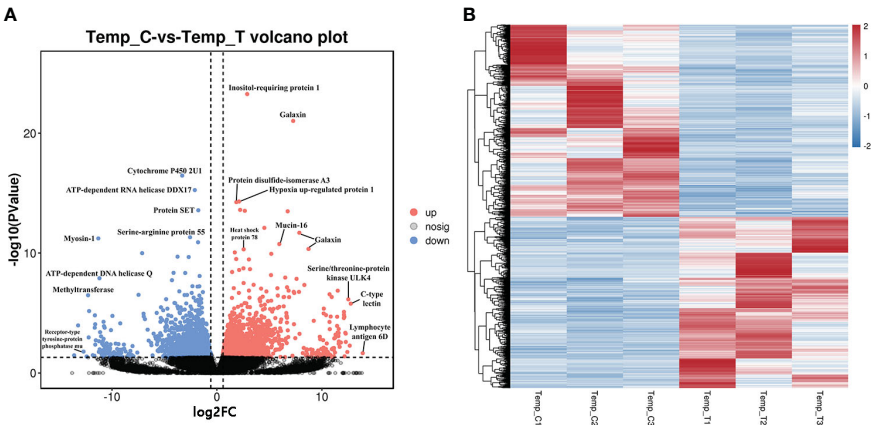

FIGURE 1
The volcanic plot and heat map of DEGs for the clam *R. philippinarum* in response to heat stress. **(A)** The volcanic plot shows the distribution of FDR and log_2FC values of the DEGs, Red dots, significantly up-regulated genes; Blue dots, significantly down-regulated genes; Black dots, no significant difference. **(B)** The heat map of DEGs of the clam, the expression level of genes in different samples is represented by different colors. The scale at the top right denoted normalized expression levels.

membrane. For the molecular functions, most of DEGs were significantly enriched in the subcategory of binding and catalytic activity (Figure 2). The KEGG analysis enriched several important metabolic pathways, such as “protein processing in endoplasmic reticulum”, “olfactory transduction”, “inflammatory mediator regulation of TRP channels”, “glucagon signaling pathway”, “cGMP - PKG signaling pathway” and “spliceosome” (Figure 3).

According the comparative transcriptomics, many DEGs were involved in energy metabolism, including phosphoenolpyruvate carboxykinase (PEPCK), succinate dehydrogenase cytochrome b (SDHB), glucose transporter type 1 (GLUT1), ADP-dependent glucokinase (ADP-GLK), glucose-6-phosphate isomerase (GPI6), and etc. (Table 1). A lot of DEGs related to protein processing were also detected in the heat-stressed clams, such as calreticulin (CRT), protein transport SEC24C (SEC24C), heat shock protein 70 (HSP70B2), X-box-binding protein 1 (XBP1), and DnaJ homolog subfamily C member 3 (DNAJ3; Table 2). As summarized in Table 3, multiple DEGs were found to be involved in substance metabolism (e.g. glycerophospholipid), including phosphoethanolamine N-methyltransferase 1 (PEMT1), Stearoyl-CoA Desaturase (SCD) and fatty acid synthase (FAS). Additionally, we detected some DEGs involved in inoxidizability, immunology and anti-apoptotic, such as cytochrome P450, glutathione S-transferase 1 (SGST), C-type lectin (CLEC), and E3 ubiquitin-protein ligase XIAP (Table 4).

3.2 Metabonomic analysis of clam under heat stress

In the metabolome analysis, positive ion (POS) and negative ion (NEG) modes were used to collect LC-MS information. First, basal peak chromatograms (BPC) of all samples were visually examined to provide an overall analysis of the metabolites in the control and heat stress groups. Based on OPLS-DA model analysis, Figures 4A, B show significant separation of each sample cluster between the two groups. For positive modes, values for categorical parameters were R2X = 0.235, R2Y = 0.868, Q2Y = 0.108, and for negative modes, R2X = 0.473, R2Y = 0.762, Q2Y = 0.003.

There are 293 kinds of DEMs detected in positive and negative ion modes, including 213 in the positive mode and 80 in the negative mode. For the positive mode, 176 up-regulated and 37 down-regulated metabolites were found in the heat-stressed group compared with that in the control group. For the negative mode, 37 up-regulated and 43 down-regulated metabolites were detected in the heat-stressed group compared with that in the control group. As a result, the top 20 enrichment pathways of KEGG enrichment analysis of DEMs mainly related to “fatty acid metabolism”, “glycerophospholipid metabolism”, “linoleic acid metabolism”, “valine, leucine and isoleucine biosynthesis”, “fatty acid biosynthesis” and “HIF-1 signaling pathway” (Figure 5). The energy metabolism-related DEMs related to heat stress was summarized in Table 1, including succinic semialdehyde, L-Isoleucine and Beta-D-Glucose and Acetyl-CoA. In addition, there were many DEMs involved in substance metabolism, including phosphatidylcholine (PC), phosphocholine and CDP-choline (Table 3).

3.3 Correlation analysis of transcriptome and metabolome of clam under heat stress

KEGG analysis showed that a total of 53 functional pathways might be involved in heat stress. The top 20 of enrichment pathway are mainly associated with “glucagon signaling pathway”, “alanine aspartate and glutamate metabolism”, “purine metabolism”, “arachidonic acid metabolism”, “glycolysis” and “pentose phosphate pathway”. The effect of association analysis was

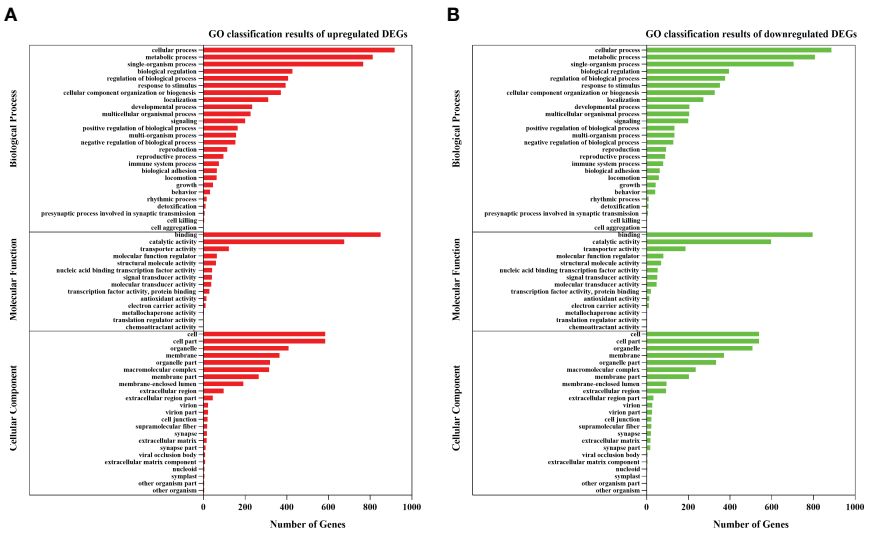

FIGURE 2
GO classification results for the DEGs of the clam *R. philippinarum* in response to heat stress. **(A)** GO classification results of upregulated DEGs. **(B)** GO classification results of downregulated DEGs.

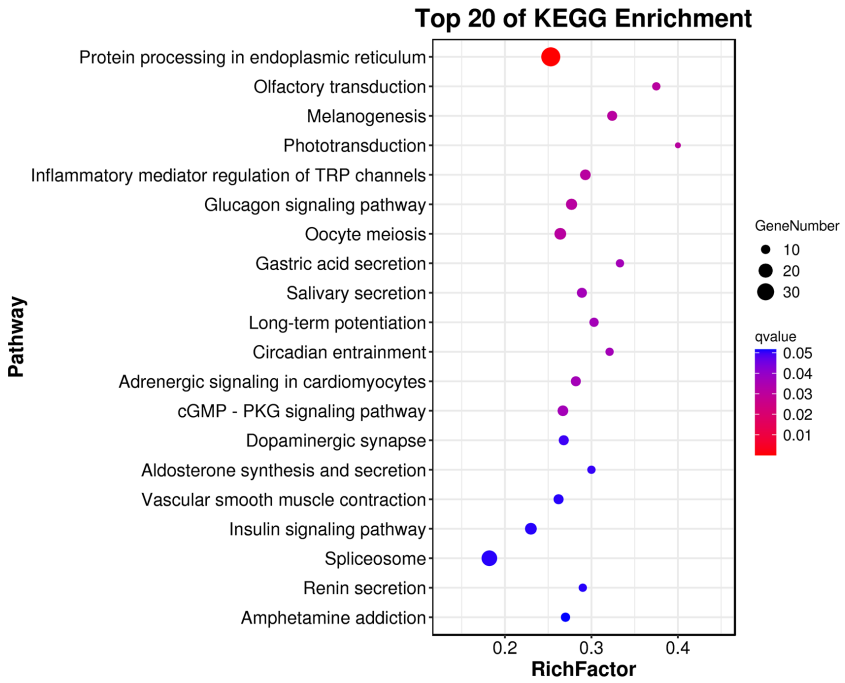

FIGURE 3
The top20 KEGG enrichment results for the DEGs of the clam *R. philippinarum* in response to heat stress.

assessed using an O2PLS model based on previous studies (Bouhaddani et al., 2016). The O2PLS model calculations (control vs. heat stress) were calculated as R2Xcorr = 0.953, R2Ycorr = 0.926, which reflected that the obtained O2PLS model was a reliable model for correlation analysis of transcriptome and metabolome. The joint loading plot is shown in Figures 6A, B. The metabolite joint loading revealed many metabolites closely related to genes, such as phosphatidylcholine, Homo-L-arginine, Acetyl-CoA, L-Histidine and L-Isoleucine (Figure 6A). The transcript joint loading uncovered thousands of genes closely related to metabolites, such as small heat shock protein, heat shock protein 70, inhibitor of apoptosis 1, Cyclic GMP-AMP synthase, glutamine–tRNA ligase and cytochrome family genes (Figure 6B). In this study, pearson correlation coefficient of DEGs and DEMs was calculated. In order to show the correlation characteristics, the top 250 DEGs and DEMs correlations of correlation coefficients are selected to show in heat map (Supplementary Figure S1). Metabolite and gene data from the heat map are presented in Supplementary File S1.

3.4 Selection for the optimal internal reference gene under heat stress

After analysis, the amplification efficiency of the candidate reference gene is between 90.4% and 93.8% (Supplementary Table S1). After qRT-PCR analysis, a single peak in the melting curve was observed for each reference gene. Because GFRP2 could not detect Ct values in some samples, it was excluded from the ranking. The Ct values of the remaining candidate reference genes ranged from 21.58 to 35.49, with the greatest change in ACT and the smallest change in RPS23 (Supplementary Figure S2). Stability of candidate reference genes was ranked by geNorm: RPL31/HIS > TUB > RPS23 > EF1A > ACT; Normfinder: RPL31 > TUB > HIS > EF1A > RPS23 > ACT; Bestkeeper: RPS23 > HIS > TUB > RPL31 > EF1A > ACT. The RefFinder was used to consolidate these results from different programs, resulting in the final ranking as following: RPL31 > HIS > TUB > RPS23 > EF1A > ACT (Supplementary Table S3).

3.5 Gene expression pattern by qRT-PCR analysis

According to qRT-PCR analysis, it was found that the expression levels of all target genes tended to increase after heat stress (Figure 7). For instance, SDH expression was significantly increased at 48 h, 72 h and 120 h. In contrast, PEPCK and ADPGLK were significantly increased at 24 h and 48 h. Moreover, the significantly up-regulated expression of SAR1B was found at 24 h, 48 h, and 120 h, and SIL1 at 24 h. For HSP70, HSP90 and CYP450, the significantly increased expression was detected at 24 h, 48 h and 72 h. For the immune gene (NF-κB), it was found to be significantly up-regulated at all of the time points.

3.6 Enzyme activity

According to enzyme activity analysis in clams, enzymes related to substance metabolism and energy metabolism were significantly affected by the heat stress. For instance, the enzyme activity of NAK was significantly decreased at 48 h and 72 h (Figure 8). AKP content

TABLE 1 Energy metabolism-related DEMs and DEGs list of clam *R. philippinarum* under heat stress.

	Metabolite/gene name	log_2FC
Metabolite	Acetyl-CoA	1.023758139
	S-Acetyldihydrolipoamide-E	1.57603009
	Succinic semialdehyde	-0.383241345
	L-Isoleucine	-0.471123684
	Beta-D-Glucose	-0.897291843
Gene	Phosphoenolpyruvate carboxykinase (PEPCK)	1.049623136
	Succinate dehydrogenase cytochrome b (SDHB)	1.358041788
	Isocitrate dehydrogenase 2 (IDH2)	0.923336596
	Glucose transporter type 1 (GLUT1)	1.199805736
	ADP-dependent glucokinase (ADP-GLK)	2.929790998
	Aldose 1-epimerase (AEP1)	1.644668747
	Alcohol dehydrogenase class-3 (ADH3)	1.098299321
	Hexokinase-1 (HK1)	-1.3
	Transposable element Tcb1 transposase (TCB1)	-3.098403704
	Glucose-6-phosphate isomerase (GPI6)	-0.633964696

increased significantly at 48 h and 72 h and decreased at 120 h, while ACP content increased significantly at 24 h and 48 h. Regarding to antioxidant defenses, we found that antioxidant enzymes of SOD and CAT were significantly increased at 48 h, 72 h and 96 h. The MDA content of oxidative damage substances increased significantly at 48 h and 72 h (Figure 8).

TABLE 2 Protein processing-related DEGs list of clam *R. philippinarum* after heat stress.

Gene name	log_2FC
Protein disulfide-isomerase A3 (PDIA3)	1.85544
Calreticulin (CRT)	1.701498
Calnexin (CNX)	1.007896
Protein transport SEC24C (SEC24C)	0.783708
GTP-binding protein SAR1B (SAR1B)	4.360481
Heat shock protein 70 (HSP70)	5.177193
Heat shock protein 78 (HSP78)	2.546135
Heat shock protein 90 (HSP90)	1.202487
Inositol-requiring protein 1 (IER1)	2.881703
X-box-binding protein 1 (XBP1)	1.446717
Nucleotide exchange factor SIL1 (SIL1)	2.528189
Glucose-regulated protein 94 (GRP94)	2.213494
Hypoxia up-regulated protein 1 (HYOU1)	2.107178
DnaJ homolog subfamily B member 11 (DNAJ11)	1.337801
DnaJ homolog subfamily C member 3 (DNAJ3)	0.99686

4 Discussion

4.1 Energy metabolism

Energy absorption and energy distribution of aquatic invertebrates will be affected by heat stress (Sokolova et al., 2012; Han et al., 2013). The energetic costs of mollusks under heat stress may be substantial and vary sharply with intensity of heat stress. For mollusks, these energy costs are likely to be instrumental in governing the suitability of different habitats and may play key roles in defining the distribution pattern over latitudinal and vertical temperature gradients (Dong et al., 2022a). Under heat and hypoxia stress, mollusks maintain the energy balance in a specific way, promoting carbohydrate metabolism by stimulating glycolysis (Sun et al., 2021; Hu et al., 2022a). As well-known, glycolysis is the first stage of carbohydrate metabolism, an anaerobic process producing two molecules of ATP from one molecule of glucose through enzymatic reactions (Lauer et al., 2012). For instance, air exposure could induced the significantly higher expression of glycolysis-related genes in oyster *C. gigas*, including hexokinase (HK), phosphofructokinase (PFK), glucose transporter 1 (GLUT1), phosphoenolpyruvate carboxykinase (PEPCK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Meng et al., 2018). After the heat and hypoxia stress of clam *Mercenaria mercenaria*, the glycolysis process was remarkably increased, showing the content of fructose and mannose significantly higher than that in the control group (Hu et al., 2022a). Consistently, our present study also revealed the significant enrichment of the glycolysis pathway in the heat-stressed clams. The metabolite of Beta-D-Glucose in the heat-stressed clams showed the significantly decreased pattern under heat stress, while Acetyl-CoA displayed increased (Table 1). Similarly, gene expression levels showed the significant increasing for the glycolysis-related genes, including GLUT1, PEPCK, ADP-dependent glucokinase (ADP-GLK), aldose 1-epimerase (AEP1) and alcohol dehydrogenase class-3 (ADH3). Moreover, we also detected the significantly increased expression levels of SDH, PEPCK and ADPGLK in the heat-stressed clams. In contrast, gene expression of hexokinase-1 (HK1), transposable element tcb1 transposase (TCB1) and glucose-6-phosphate isomerase (GPI6) was significantly decreased in the heat-stressed clams (Table 1). Therefore, the changes in glycolysis-related metabolites (Beta-D-Glucose and Acetyl-CoA) and gene expression suggests that glycolysis may serve as the main energy source in the heat-stressed clams. This may represent the survival strategy of the hard-stressed clams, which may help them to maintain the energy balance by promoting glycolysis during heat stress adaptation.

TCA cycle is the ultimate metabolic pathway, serving as the metabolic hub of carbohydrates, lipids and amino acids in aerobic organisms (Lu et al., 2020). In TCA cycle, pyruvic acid, a glycolysis product, will be gradually oxidized and decomposed into CO_2 and H_2O, generating NADH, $FADH_2$ and ATP. In this study, the metabolites of Acetyl-CoA and S-Acetyldihydrolipoamide-E involved in TCA cycle were found to be significantly increased after heat stress, while succinic semialdehyde was significantly decreased in the heat-stress clams. Acetyl-CoA is served as the

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TABLE 3 The substance metabolism-related DEMs and DEGs list of clam *R. philippinarum* under heat stress.

	Metabolite/gene name	log_2FC
Metabolite	PC(15:0/15:0)	0.489924261
	PC(18:1(9Z)/18:0)	0.361211051
	PC(18:3(6Z,9Z,12Z)/P-18:1(11Z))	0.648505776
	PC(20:2(11Z,14Z)/20:4(5Z,8Z,11Z,14Z))	0.510727891
	PC(22:4(7Z,10Z,13Z,16Z)/P-18:0)	0.84671402
	PC(22:5(4Z,7Z,10Z,13Z,16Z)/P-18:0)	0.456077126
	PC(22:5(7Z,10Z,13Z,16Z,19Z)/16:0)	1.068188073
	PC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/20:1(11Z))	0.727981142
	PC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/20:2(11Z,14Z))	0.768993293
	Phosphocholine	0.248528771
	CDP-choline	1.913617946
Gene	Choline/ethanolaminephosphotransferase 1 (CEPT1)	1.012022528
	Phosphoethanolamine N-methyltransferase 1 (PEMT1)	2.522226745
	Phosphoethanolamine N-methyltransferase 3 (PEMT3)	2.185069207
	Stearoyl-CoA Desaturase (SCD)	-2.460575253
	Fatty acid synthase (FAS)	-2.546994651

PC, phosphatidylcholine.

TABLE 4 Inoxidizability, immunology and anti-apoptotic-related DEGs list of clam *R. philippinarum* under heat stress.

	Gene name	log_2FC
Inoxidizability	Copper-zinc superoxide dismutase (CSOD)	4.35110099
	Glutathione S-transferase 1 (SGST)	1.986186154
	Cytochrome P450 3A9	5.017135912
	Cytochrome P450 2J6	2.227805918
	Cytochrome p450 family 4	1.394440595
	Cytochrome P450 CYP44	1.130232471
	Cytochrome P450 family 1	-1.31985078
	Cytochrome P450 3A28	-1.640907134
	Cytochrome P450 2E1	-1.740121326
	Cytochrome P450 2U1	-3.309820324
Immunology	C-type lectin (CLEC)	12.75377388
	C-type lectin 2 (CLEC2)	4.190627707
Anti-apoptotic	Baculoviral IAP repeat-containing protein 7 (BIRC7)	2.390893561
	Baculoviral IAP repeat-containing protein 3 (BIRC3)	1.900790396
	Baculoviral IAP repeat-containing protein 2 (BIRC2)	1.694874087
	Baculoviral IAP repeat-containing protein 1 (BIRC1)	-2.762258332
	E3 ubiquitin-protein ligase XIAP (E3)	-10.94153674

key metabolic node in TCA, allowing energy supply to match energy requirement in animal tissues (Shi and Tu, 2015). S-Acetyldihydrolipoamide-E is a key metabolite linking the TCA cycle, pyruvate metabolism, and gluconeogenesis (Ma et al., 2021). Succinic semialdehyde can be converted to succinic acid, which is a key intermediate product of the TCA cycle. In this study, our finding confirmed that genes involved in TCA cycle (succinate dehydrogenase cytochrome b, SDHB; isocitrate dehydrogenase 2, IDH2) were significantly up-regulated after heat stress (Table 1). SDH is a key enzyme linking oxidative phosphorylation and electron transfer, and its activity can generally be used as an indicator to evaluate the degree of TCA operation (Rutter et al., 2010). Furthermore, IDH is a key metabolic enzyme in TCA cycle, which catalyzes the conversion of isocitrate acid into α -ketoglutarate and plays an important role in cell energy metabolism and biosynthesis (Zheng et al., 2013). The present findings suggest that heat-stressed clams might strengthen their TCA flux to produce more energy to maintain energy balance for heat adaptation. This phenomenon is also supported by a previous study, which revealed the enhanced TCA cycle in scallop *Chlamys farreri* under heat stress (Dong et al., 2022b). Arginine and histidine are anaplerotic substrates, which are converted to α-ketoglutarate acid and then enter TCA cycle (Hara et al., 2021). In contrast, isoleucine enters TCA cycle by converting into succinyl-CoA, and then decomposes to produce ATP (Hara et al., 2021; Dong et al., 2022b). In this study, the increasing of arginine and histidine, accompanied by the reduction of isoleucine, suggests the important roles of free amino acids involved in TCA flux to produce more energy in the heat-stressed clams.

4.2 Protein processing

In this study, protein processing in endoplasmic reticulum (ER) pathway is the most significantly enriched pathway revealed by comparative transcriptomics analysis in clam *R. philippinarum* under the heat stress. In eukaryotic cells, ER plays an important role in the synthesis of a series of important substances such as protein, lipids and carbohydrate (Ellgaard and Frickel, 2003). The protein of ER is supervised by the quality control (QC) system, which ensures that the protein is correctly folded. The QC system includes several important genes, including calnexin (CNX), calreticulin (CRT) and glycoprotein specific thiol-disulfide oxidoreductase (ERP57; Ellgaard and Frickel, 2003; Gupta, 2012). In this research, comparative transcriptomics revealed the significantly up-regulated expression of CRT, protein disulfide-isomerase A3 (PDIA3) and CNX genes in the heat-stressed clams, probably involved in promoting the protein folding in ER (Table 2). Furthermore, secretory proteins of eukaryotic cells will be transported from the ER to Golgi apparatus through ER-Golgi intermediate compartment (Appenzeller-Herzog and Hauri, 2006). Proteins synthesized within the ER are transported to the Golgi *via*

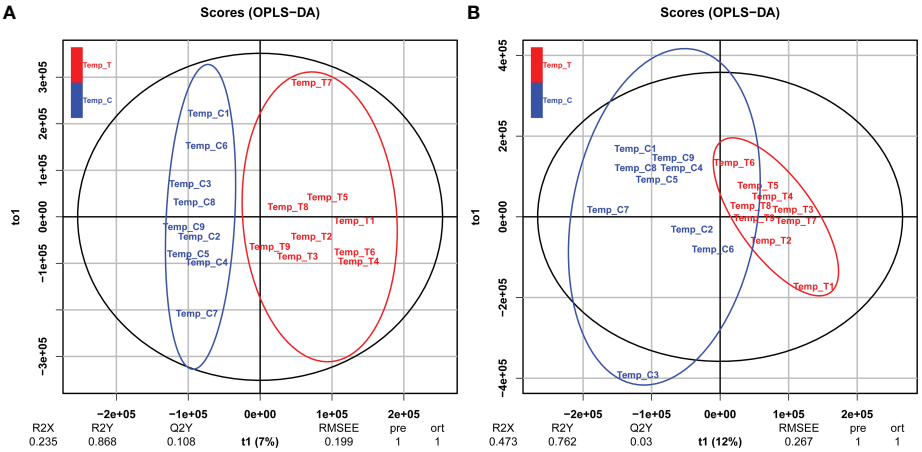

FIGURE 4
OPLS-DA score plots for the clam *R. philippinarum* in response to heat stress. **(A)** positive mode; **(B)** negative mode.

coat protein complex II (COPII)-coated vesicles, which comprise small GTPase Sar1, the inner-coat complex (SEC23/SEC24) and the outer-coat complex (SEC13/SEC31) (Saito et al., 2017). In this study, the significantly increased expression of protein transport SEC24C (SEC24C) and GTP-binding protein SAR1B (SAR1B) suggests that clams may adapt the heat stress by regulating protein transporting and processing in endoplasmic reticulum.

To cope with environmental stress, ER proteins will misfold and accumulate in the cells, affecting cell normal function, and even causing diseases (Ruggiano et al., 2014; Almanza et al., 2019).

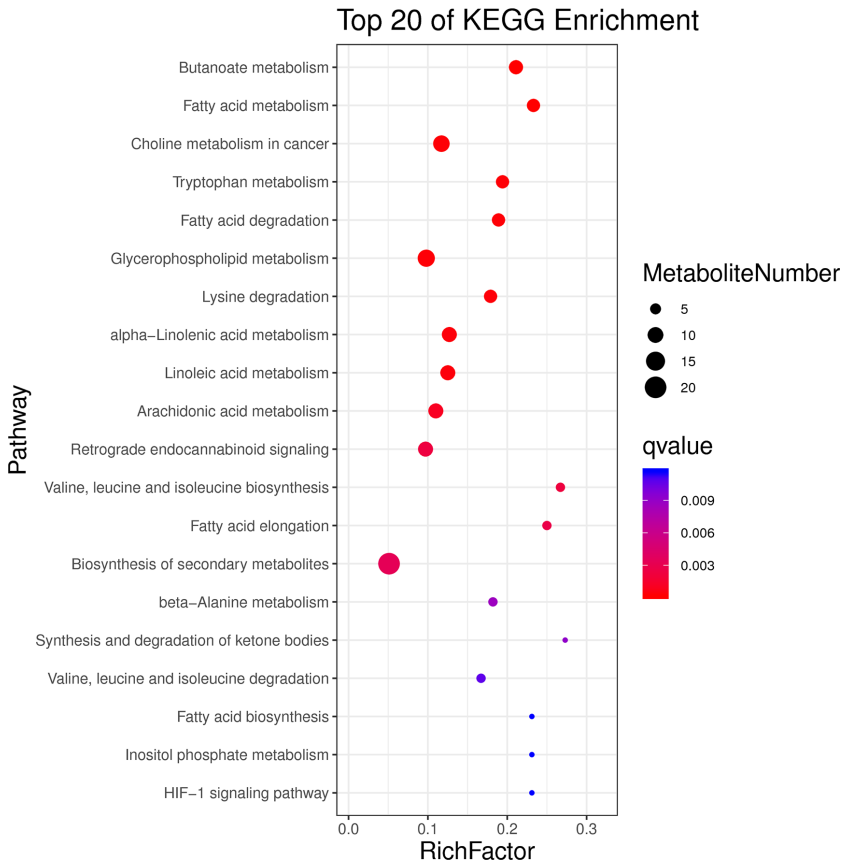

FIGURE 5
The top20 KEGG enrichment results for the DEMs of the clam *R. philippinarum* in response to heat stress.

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FIGURE 6
The loading plots for the differential genes and metabolites of the clams in response to heat stress. **(A)** metabolite joint loadings; **(B)** transcript joint loadings. Each dot represents a metabolite or a gene. The horizontal axis is the first-dimension coordinate of the joint part, and the ordinate axis of is the second-dimension coordinate of the joint part. The absolute values of an element in the coordinate represent the degree of association between this element and another omics.

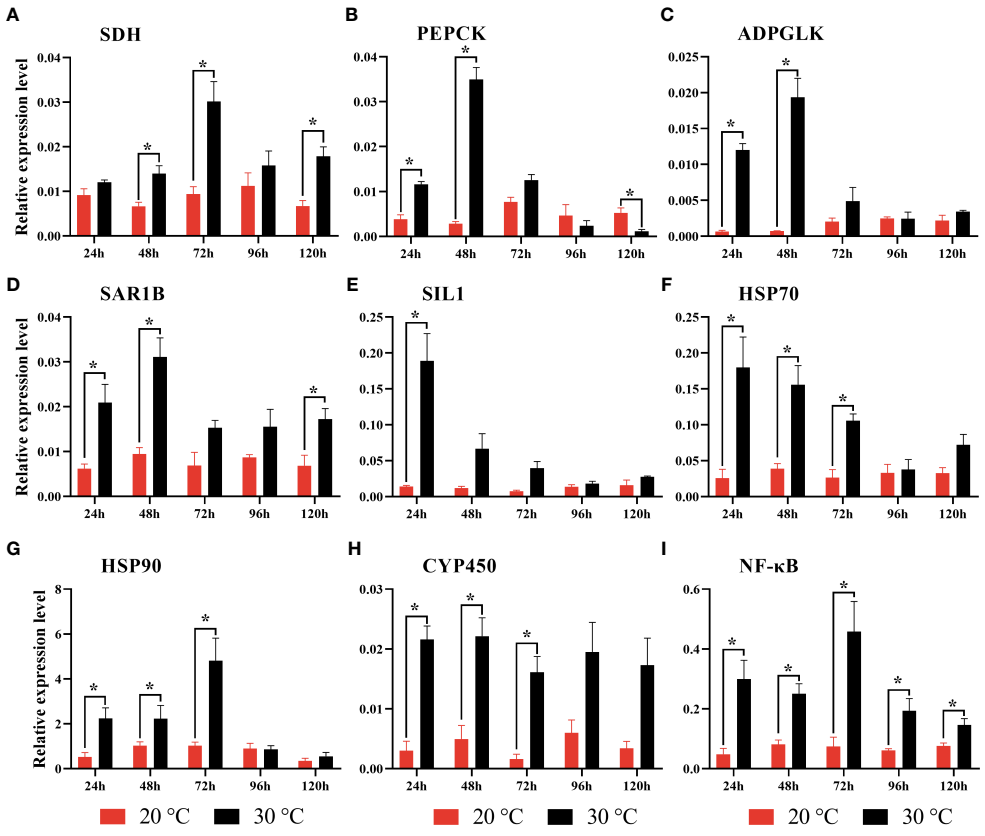

FIGURE 7
Gene expression revealed by qRT-PCR in gill tissue of clam *R. philippinarum* after heat stress. Values are mean ± standard error of the mean. Results are normalized against the RPL31 gene. Significance was considered at $*p < 0.05$. **(A)** Succinate dehydrogenase (SDH); **(B)** Phosphoenolpyruvate carboxykinase (PEPCK); **(C)** ADP-dependent glucokinase (ADPGLK); **(D)** GTP-binding protein SAR1B (SAR1B); **(E)** Nucleotide exchange factor SIL1 (SIL1); **(F)** Heat shock protein 70 (HSP70); **(G)** Heat shock protein 90 (HSP90); **(H)** Cytochrome P450 (CYP450); **(I)** Nuclear factor kappa-B (NF-κB).

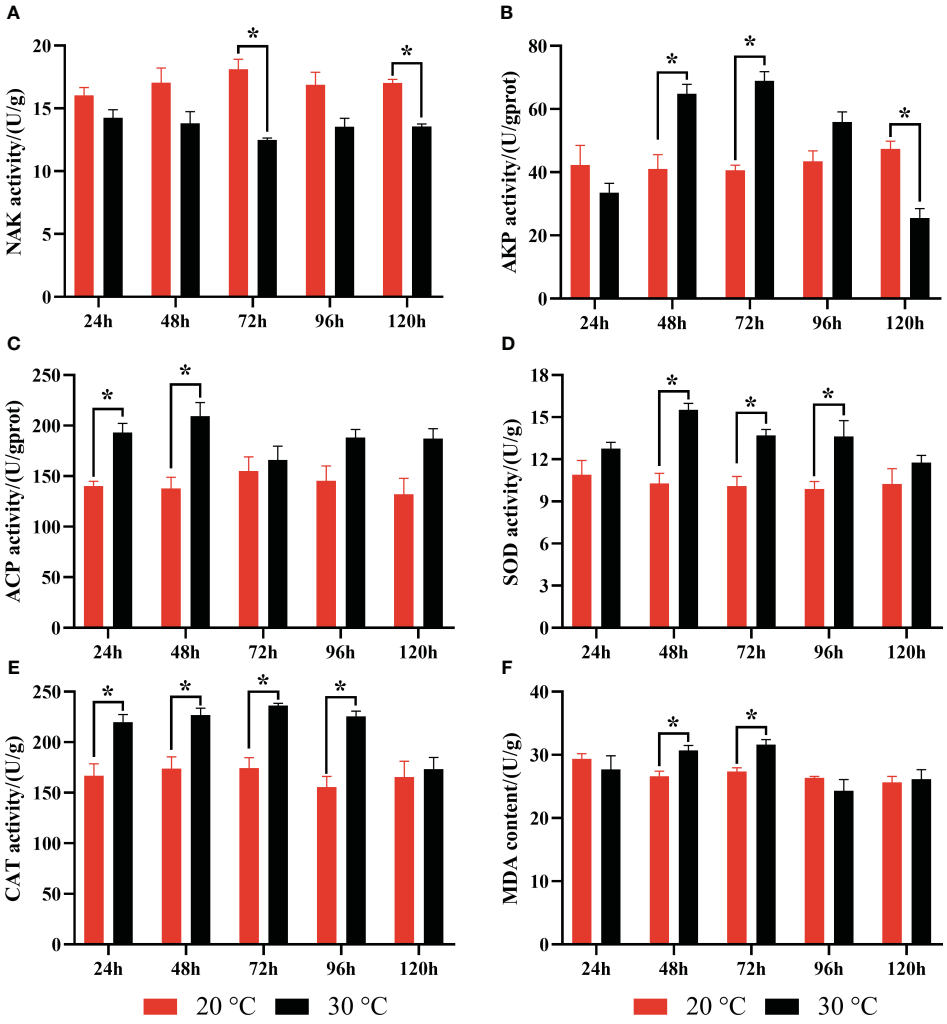

FIGURE 8
Effect of heat stress on enzyme activities in the gill of clam *R. philippinarum*. Values are mean ± standard error of the mean. Significance was considered at $*p < 0.05$. **(A)** Na+K+-ATPase (NAK); **(B)** Alkaline phosphatase (AKP); **(C)** Acid phosphatase (ACP); **(D)** Superoxide dismutase (SOD); **(E)** Catalase (CAT); **(F)** Malondialdehyde (MDA).

Endoplasmic reticulum-associated protein degradation (ERAD) is one of the most important pathways regulating ER pressure in eukaryotic cells. As indicated, multiprotein complexes in the ER are able to identify, remove, ubiquitinate and deliver misfolded proteins to the 26S proteasome for degradation in the cytosol (Needham et al., 2019). During the process, molecular chaperones of heat shock protein (HSP) family participate in ERAD process by combining with ubiquitin ligases (Goldberg, 2003). In this study, it is evidenced by the significantly increased expression levels of HSP70 and HSP90, probably aiming to promote the degradation of misfolded proteins and maintain cell homeostasis (Table 2). Moreover, molecular chaperones of GRP94 and HSP40 in ER are capable of recognizing misfolded protein and assisting in targeting malformed protein to ERAD (Haas, 1994; Plemper et al., 1997; Tyedmers et al., 2003; Eletto et al., 2010). In the present study, we also detect the up-regulation of molecular chaperones in clams after the heat stress, such as nucleotide exchange factor SIL1, glucose-regulated protein 94 (GRP94), dnaJ homolog subfamily C member 3 (DNAJ3), and DNAJ11 (Table 2). This suggests that molecular chaperones in the ER may participate in the degradation of misfolded proteins and maintain cell homeostasis during the heat stress in clam tissues.

Additionally, IRE1α can specifically cut the mRNA of X-box binding protein (XBP1), which regulates protein folding, transport and degradation at the transcriptional level, and thereby controls lipid synthesis and restores ER homeostasis by increasing ER volume (Tirasophon et al., 1998; Hetz et al., 2020). In this study, the up-regulated expression of inositol-requiring protein 1 (IER1) and XBP1 may be involved in maintaining the stability of ER by the degradation of misfolded proteins in the heat-stressed clams.

4.3 Substance metabolism

Glycerophospholipid (phosphatidylcholine, PC; phosphatidylethanolamine, PE), as the important component of cell membranes, is closely related to cell membrane permeability and ATPase activity (Overgaard et al., 2008; Nemova et al., 2017;

Nguyen et al., 2020). Glycerophospholipid has been recently recognized as an important metabolite of cell membrane damage caused by high temperature stress (Calzada et al., 2016). For instance, the increasing of PC is an adaptive response to heat stress to maintain biofilm stability and function in animal tissues (Qu and Ajuwon, 2018). The increased PE plays an important role in the heat tolerance of heat-resistant intertidal zone snail *Echinolittorina malaccana* (Chen et al., 2021). Moreover, the hard clam *M. mercenaria* increased their glycerophospholipid content after heat stress to enhance cell membrane stability (Hu et al., 2022a). Similarly, the razor clam *Sinonovacula constricta* also showed the increased contents of PC and PE under heat stress (Zhang and Dong, 2021). In this study, the increasing of PC contents in the heat-stressed clams was consistently detected by comparative metabonomics (Table 3).

As recently reported, PC biosynthesis occurs mainly *via* the CDP-choline pathway in eukaryotes (Islam et al., 2022). Choline can be converted to phosphocholine through phosphorylation by the activity of cytosolic enzyme choline kinase (CK). Phosphocholine is catalytically converted to CDP-choline by a rate-limiting enzyme such as phosphocholine cytidylyltransferase (CT), which is then catalyzed by c choline/ethanolaminephosphotransferase (CEPT1) to produce PC (Henneberry et al., 2002). In our study, we consistently find the significantly increased metabolites of CDP-choline and phosphocholine in the heat-stressed clams, supporting the conclusion of PC biosynthesis by the CDP-choline pathway. Moreover, another possible pathway of PC synthesis occurs when PE converts to PC through three successive methylation reactions catalyzed by phosphatidylethanolamine N-methyltransferase (PEMT) (van der Veen et al., 2017). In this study, PC synthesis derived from PE is also evidenced by the increased gene expression of CEPT1 and PEMT in the heat-stress clams (Table 3). Taken together, the increasing of PC synthesis is essential to maintain the stability and permeability of cell membrane during the heat stress in clams. These findings provide the molecular and metabolite evidences for heat stress adaptation in clams, as well as other bivalves.

Stearoyl-CoA Desaturase (SCD) is an important enzyme in fat metabolism and plays a central regulatory role in fatty acid biosynthesis (Ntambi, 1995; Kim and Ntambi, 1999; Ntambi et al., 2004). As reported, SCD plays an important role in membrane composition and fluidity regulation (Castro et al., 2011). The changes in SCD gene transcripts has been considered an important adaptative mechanism of aquatic organisms in response to temperature fluctuations (Hsieh and Kuo, 2005; Hsieh et al., 2007). In addition, fatty acid synthase (FAS) can catalyze the synthesis of long-chain fatty acids, which form as the important membrane substrates for cellular energy metabolism (Wakil, 1989; Smith et al., 2003). In the present study, the decreased gene expression of SCD and FAS suggests that clams may adapt to the heat stress by the regulation of membrane composition and fluidity.

In this study, the activities of several enzymes (e.g. NAK, AKP and ACP) were significantly affected by the heat stress in clams (Figure 8). As reported, NAK is an integral membrane protein that couples the hydrolysis of ATP to ion transport across the membranes in animal cells (Jorgensen and Pedersen, 2001). In this study, NAK activity in clams was significantly decreased at 72 h and 120 h after the heat stress, suggesting the reduction of ion transport across cell membranes during the heat stress. In addition to NAK, two important phosphatases (AKP and ACP) play an important role in stress signal transduction and physiological adaptation to the environment stress, probably by the degradation of exogenous protein, carbohydrates and lipids (Liu et al., 2004). More recently, ACP and AKP have also been reported to be involved in heat stress responses in scallop *P. yessoensis* and mussel *Mytilus coruscus* (Hu et al., 2015; Jiang et al., 2019). In this study, we consistently reveal that the activities of ACP and AKP were significantly increased in the heat-stressed clams. These findings suggests that the increased activities of ACP and AKP may be essential for stress signal transduction, and thereby play an important role in physiological adaptation to the heat stress in bivalves.

4.4 Inoxidizability

For mollusks, the increasing of seawater temperature can accelerate metabolic rates, resulting in the accumulation of reactive oxygen species (ROS) and activation of anti-oxidative enzymes (De Zoysa et al., 2009). However, excess ROS (including superoxide anion, peroxide and hydroxyl radical) may affect the normal cellular function and even cause the damages of DNA and protein function (Song et al., 2006; Kong et al., 2022). To control ROS production and accumulation, aquatic organisms usually activate antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferases (GST) and cytochrome P450 (CYP) (Rosic et al., 2010; Zhang et al., 2021). Different from those antioxidant enzymes, malonyldialdehyde (MDA) is usually served as a signature product of antioxidant damage (Wang et al., 2011). In mollusks, these antioxidant enzymes are usually activated by environmental stress, especially for the heat stress. For instance, gene expression of GST and CYP in clam *Sinonovacula constricta* was activated after the heat shock (Kong et al., 2022). Similarly, the increased GST expression was also observed in bivalve *Laternula elliptica* and scallop *P. yessoensis* under heat stress (Truebano et al., 2010; Jiang et al., 2018). Additionally, the mRNA levels of SOD and GST were found to be substantially increased in the mussels *Mytilus galloprovincialis* after the exposure of acute heat stress (Banni et al., 2014). In this study, we also confirm the activation of antioxidant system in the heat-stressed clams by the elevated gene expression of SOD, GST and CYP, and the increased enzyme activities of SOD, CAT and MDA (Table 4). These findings reflect the important roles of antioxidant enzymes in physiological adaptation to the heat stress in bivalves.

4.5 Immunology/anti-apoptotic

Under the infection of external pathogens, bivalve mollusks can improve the expression of immune-related genes, relying on innate immune system to defense against invaders (Manfredi et al., 2008; Huang et al., 2013; Thornton et al., 2017; Shen et al., 2018). As reported in the clam *S. constricta*, C-type lectins (CLEC) are served as pattern

recognition receptors, playing the crucial roles in innate immune response to thermal stress (Kong et al., 2022). Similarly, CLEC expression was found to be significantly up-regulated after the bacteria infection in bay scallop *Argopecten irradians* and clam *Sinonovacula constricta* (Huang et al., 2013; Shen et al., 2018). In addition to CLEC, NF-κB is an important transcription factor for regulating inflammation and innate immune function (Ghosh et al., 1998; Dixit and Mak, 2002; Wang et al., 2006). For instance, the increased expression level of NF-κB reflects the immune responses in the clam *R. philippinarum* under hypoxia stress (Nie et al., 2020). In this study, we consistently detect the significantly increased expression of CLEC and NF-κB in the heat-stressed clams, indicating that the clams might activate the innate immune system in response to the heat stress.

Besides the innate immune system, bivalves may also respond to the external environmental pressure through an effective anti-apoptotic system (Li et al., 2017; Song et al., 2021; Hu et al., 2022b). Inhibitors of apoptosis (IAP) and E3 ubiquitin-protein ligases (E3) are critical regulators of programmed cell death and apoptosis (Broemer and Meier, 2009; Song et al., 2021). For instance, IAPs and E3 played the important roles in survival of hard clam *M. mercenaria* and oyster *C. gigas* under air exposure and heat stress (Zhang et al., 2012a; Zhou et al., 2021; Hu et al., 2022b). In this study, the changes in expression levels of IAP and E3 genes suggest that clams may adapt to heat stress by regulating its anti-apoptotic system.

4.6 The predicted model for clams in response to heat stress

Based on the obtained data, we proposed a hypothetical pathway for clam adaptation to heat stress, as shown in Figure 9. During the heat stress, glycolysis may serve as the main energy source to maintain energy balance in the heat-stressed clams. Another important energy source may be provided by the TCA cycle linking with amino acid metabolism, which can facilitate energy production *via* the TCA cycle for heat adaptation. As illustrated, the changes in gene expression of glycolysis-related genes (GLUT1, GLK, HK1, GPI6) and metabolites (Beta-D-Glucose and Acetyl-CoA) reflect the adjusted energy metabolism in the heat-stressed clams. The protein processing in endoplasmic reticulum plays the important roles in the stability of cell membrane structure during heat adaptation. Molecular chaperones (HSP70 and HSP90) participate in the degradation of misfolded proteins to maintain cell homeostasis during the heat stress. The increased contents of glycerophospholipid are essential to maintain the stability and permeability of cell membrane in the heat-stressed clams. To defense the heat stress, clams also initiate the protection mechanisms by activating antioxidant system, innate immune system and anti-apoptotic system.

For aquatic invertebrates, moderate stress may cause metabolic and ATP turnover acceleration, allowing to compensate for additional energy cost for increased activity and cellular maintenance (Sokolova et al., 2012). However, extreme stress may give rise to the slow-down of activity and suppression of metabolic rate for time-limited survival. In this study, a large number of genes and metabolites, such as GLUT1, HSP, Beta-D-Glucose and Acetyl-CoA, were enriched in the energy metabolism pathway of clam under heat stress, indicating that clam may produce more energy to maintain the survival under heat stress.

To sum up, glycolysis, TCA cycle and amino acid metabolism may serve as the main energy sources to maintain energy balance in clams during heat stress. Protein processing in endoplasmic reticulum and glycerophospholipid may play an important role in

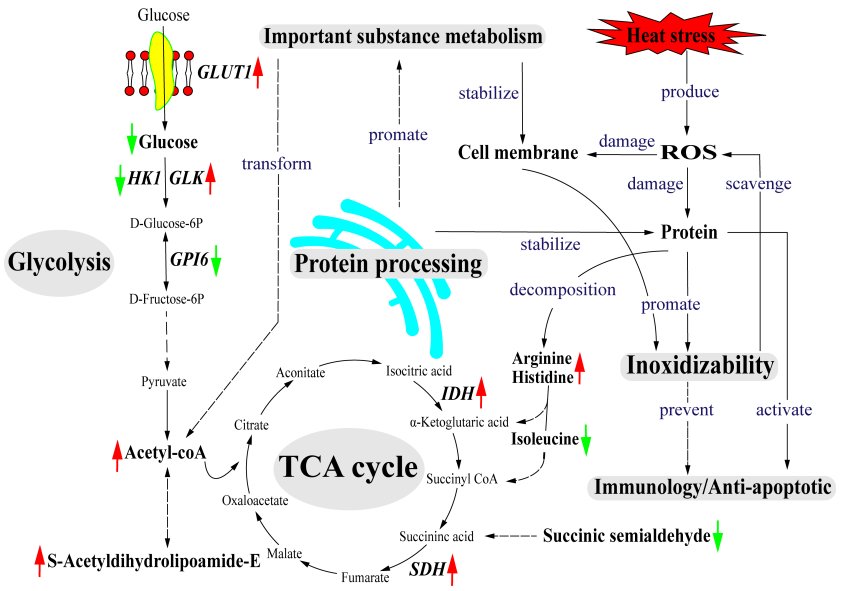

FIGURE 9
The predicted model for clams in response to heat stress. Solid line represents having a direct association and das lines represent having a potential association. The red arrows represent up-regulated and the green arrows represent down-regulated. GLUT1, glucose transporter type 1; HK1, hexokinase-1; ADP-GLK, ADP-dependent glucokinase; GPI6, glucose-6-phosphate isomerase; IDH, isocitrate dehydrogenase; SDH, succinate dehydrogenase cytochrome.

heat stress adaptation, especially for maintaining the stability of cell membrane. Furthermore, the protection mechanism during heat stress will be maintained by antioxidant system, accompanied by innate immune system and anti-apoptotic system. Therefore, the time-limited survival of clams during heat stress may result from the adjusted energy metabolism, cell membrane stability and activated protection mechanisms. These novel findings may also contribute to global distribution pattern of clams over temperature gradients from an ecological perspective.

5 Conclusion

The transcriptome results indicated that DEGs were mainly involved energy metabolism, protein processing, glycerolphospholipid metabolism, inoxidizability and anti-apoptosis. Metabolome data revealed that DEMs are mainly involved in energy supply and glycerophospholipid metabolism. According the present findings, we proposed the graphical model of molecular and metabolic mechanism for heat stress adaptation in clams. As illustrated, the protein processing, substance metabolism, inoxidizability and immunology/anti-apoptosis play the important roles in the physiological adaptation of clams in response to heat stress. The present finding will shed lights on the adaptive strategies to heat stress in clams, as well as other mollusks, in a warming climate at the global scale.

Data availability statement

The datasets presented in this study can be found in online repositories. The BioProject for the clam transcriptomes has been registered as PRJNA933718, and the transcriptome sequencing data has been deposited under the accession numbers from SRR23410264 to SRR23410269. This information has been provided in the Supplementary Material.

Ethics statement

Ethical review and approval was not required for the study on clams in accordance with the local legislation and institutional requirements.

Author contributions

XS, HJ, and LZ contributed to conception and design of the study. HJ and YG wrote the first draft of the manuscript. LZ, KT, ZL and BW wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1204598/full#supplementary-material

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