



Transcriptomic Signatures of Pearl Oyster *Pinctada Maxima* in Response to Acute Salinity Stress

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Acute salinity stress can impact many physiological processes of marine shellfish. The responses of *Pinctada maxima* to salinity stress, especially the osmotic pressure regulation and immune response, are of great significance to health. To investigate the molecular changes in response to acute salinity stress, the pearl oysters were transferred from 30 ppt (C) to 40 ppt (HS) and 20 ppt (LS) for 12 h, and the transcriptome analysis was conducted on the gills. Compared to the control, there were 6613 (3253 up-regulated and 3360 down-regulated) differentially expressed genes (DEGs), 4395 (2180 up-regulated and 2215 down-regulated) DEGs observed in HS and LS, respectively. The related molecular biological processes and potential functions were explored from enrichment analysis. A total of 332 KEGG pathways (including 1514 genes) and 308 KEGG pathways (including 731 genes) were enriched in C vs. HS and C vs. LS, respectively. In addition, there are 1559 DEGs shared by C vs. HS group and C vs. LS group, and the results of the KEGG function annotation showed that 7 DEGs were involved in membrane transport, and 34 DEGs were involved in the immune system. The correlation network for expression of genes shows that the expression of 3 genes was significantly correlated with each other in membrane transport, and there were significant correlations between the expression of 27 genes in immune response. The results of this study will be of great value in understanding the molecular basis of salinity stress adaptation in the pearl oyster *P. maxima*.

Keywords: bivalves, *Pinctada maxima*, molecular response, biochemical response, salinity

INTRODUCTION

During their lifetime, marine bivalve mollusks are not only exposed to biotic stressors, but also affected by varied environment such as high fluctuation of light intensity, pH, water temperature, dissolved oxygen, and salinity. Salinity is an important ecological factor that has a profound impact on the survival, distribution, growth, and development of marine organisms (Pourmozaffar et al., 2019). It is predicated that the human activities and global warming will eventually lead to changes in the salinity of ocean water by the melt of glaciers and ice sheets (Velez et al., 2016a;

Velez et al., 2016b). In addition, intertidal zone and coastal waters are vital marine areas for aquaculture, where organisms may be subjected to salinity stress due to the ebb and flow of the tide, and the severe weather (Coughlan et al., 2009; Chen et al., 2021). The osmotic pressure of most marine mollusks is similar to the seawater because they are osmotic conformers and lack the ability to regulate the osmotic pressure of the internal medium (Hochachka and Somero, 2002; Larsen et al., 2014). Marine mollusks can live in water with salinity ranging from 75–80 ppt to 4–5 ppt, and they can be divided into stenohalinity and euryhalinity (Hedgpeth et al., 1997). For the past few years, more and more attention has been paid to the effects of salinity changes on marine organisms. Many studies have shown that salinity changes have a great influence on the life activities, physiological processes, growth and development, molecular response, and metabolic physiology of shellfish (Ertl et al., 2019; Tanner et al., 2019; Zhang H. et al., 2020; Balogh and Byrne, 2021; Garçon et al., 2021; Li et al., 2021; Ni et al., 2021; Sun et al., 2021).

Osmoregulatory organs in marine organisms play an important role in maintaining osmotic pressure stability, such as gills can secrete ions in high salinity water and absorb ions in low salinity water (Cui et al., 2019). In addition, the tight closure of the shell to separate the external environment from the internal fluid could protect the shellfish from changes in salinity for a short time, and the free amino acid pool could help marine bivalves maintain the osmotic equilibrium (Solan and Whiteley, 2016). However, extreme fluctuations in salinity can cause damage and even death to marine mollusks (Vanessa et al., 2014; Gajbhiye and Khandeparker, 2017). Previous studies have shown that regulating ionic balance and osmotic pressure requires a large amount of energy in response to salinity stress (Li et al., 2014). In addition, there are many studies showing the effects of salinity stress on behavioral response, hemolymph chemistry and histopathological changes, and immune response of marine mollusks such as *Ruditapes decussatus*, *Crassostrea gigas*, *C. virginica*, *Pteria penguin*, and *Venerupis corrugate* (Knowles et al., 2014; Casas et al., 2018; Vasquez et al., 2020; Woodin et al., 2020; Zhang M. et al., 2020). However, the genes and pathways involved in salinity adaptation remain largely unknown, and more information is needed about the molecular effects of acute salinity stress on marine mollusks. RNA-seq based transcriptome is a transcriptomics technique used to analyze the structure and expression level of transcripts, which is profiling developed rapidly and has been widely used in aquatic animals for understanding the transcriptomic signatures to changes in the environment (Wang et al., 2018; Ertl et al., 2019; Jeffries et al., 2019). In addition, transcriptome analysis techniques have also been applied to determine the molecular response of marine mollusks in response to salinity stress, such as *C. gigas* (Zhao et al., 2012), *C. hongkongensis* (Xiao et al., 2018), *C. virginica* (Eierman and Hare, 2014), *C. nippona* (Gong et al., 2021), *Ostrea lurida* (Maynard et al., 2018), *Saccostrea glomerata* (Ertl et al., 2019), *Sinonovacula constricta* (Li et al., 2021), *Cyclina sinensis* (Ni et al., 2021), and *R. philippinarum* (Nie et al., 2020). However, there is still a blank about the

transcriptomic signatures in response to acute salinity stress of pearl oyster *P. maxima*.

Pearl oyster *Pinctada maxima* is mainly distributed in the Indo-Pacific region, and it is known for producing large-sized pearls with high economic value (Liang et al., 2016). Previous studies have shown that *P. maxima* can tolerate a wide range of salinities, and the optimal growth salinity is 30 ppt (Taylor et al., 2004; Deng et al., 2013). In comparison with salinity 30 ppt, Taylor et al. (2004) pointed out that the growth of *P. maxima* was significantly depressed at a salinity of 25 ppt, 40 ppt, and 45 ppt, and the results of Deng et al. (2013) showed that the growth and survival rate of *P. maxima* larvae declined at the salinity of 27 ppt, 24 ppt, and 21 ppt. Furthermore, in recent years, the research about genetic diversity, pearl cultivation, larval reproduction, growth performance, shell materials, and disease have been documented in *P. maxima* (Lind et al., 2009; Nayfa and Zenger, 2016; Ompi et al., 2018; Anggraini et al., 2019; Hao et al., 2019; Wang et al., 2019a; Wullur et al., 2020; McDougall et al., 2021). Therefore, more information is needed on the effects of salinity stress on the *P. maxima*, as *P. maxima* is often cultured in offshore areas that are subject to fluctuating salinity.

Previous studies have shown that acute salinity stress can significantly affect the osmotic pressure and antioxidant system of marine shellfish (Casas et al., 2018; Zhang M. et al., 2020; Sun et al., 2021). In this study, biochemical indexes [$\text{Na}^+\text{-K}^+\text{-ATPase}$, reduced glutathione (GSH), and glutathione peroxidase (GSH-Px)] at the control group (30 ppt), different time points (1 h, 3 h, 6 h, 12 h, 24 h, and 48 h) after being exposed to different environmental salinity (40 ppt and 20 ppt) were measured first to evaluate the effect of the acute salinity stress on *P. maxima* and determine the most suitable time point for transcriptomics analysis. Then, transcriptome analysis techniques were applied to determine the responsive genes and pathways in gills of pearl oyster *P. maxima* after acute salinity stress, aiming to better understand the transcriptomic signatures in response to acute salinity stress of pearl oyster *P. maxima*.

MATERIALS AND METHODS

Experimental Samples for Salinity Stress and Tissues Collection

Pearl oysters *P. maxima* that were 2.5 years old with a comparable size (body mass of 114.11 ± 13.65 g and shell width of 22.08 ± 1.10 mm, $n = 40$) were collected from the cultured stock suspended in Weizhou Island of Beihai, China ($109^{\circ}06'N$, $21^{\circ}01'E$). *P. maxima* was allowed to acclimatize to holding conditions in a cement tank (5000 L) with filtered seawater (salinity of 30 ppt, $21\text{--}22^{\circ}C$) for 7 days before the salinity exposure experiments. During the acclimation, the *P. maxima* was fed with *Platymonas subcordiformis* twice a day, and half seawater was replaced every day. The optimal salinity for growth is 30 ppt, and the growth of *P. maxima* was significantly depressed at low salinity and high salinity (Taylor et al., 2004). In this study, salinity 30 ppt (C) was served as the control group, and two salinity treatments were set at salinity 20 ppt (LS) and 40 ppt (HS), respectively. Salinity of

experiment water was adjusted to the desired by mixing filtered seawater with sea salts or freshwater.

A total of 140 pearl oysters *P. maxima* were divided into three groups, 60 individuals were exposed to the acute low salinity of 20 ppt for 48 h, 60 individuals were exposed to the acute high salinity of 40 ppt for 48 h, and the remaining 20 individuals were kept at the acclimation tank as the control group (salinity of 30 ppt). Gills of 9 individuals were sampled into triplicate at the control group, different time points (1 h, 3 h, 6 h, 12 h, 24 h, and 48 h) after acute salinity stress, and stored at -80°C after freezing in liquid nitrogen for further analysis.

Biochemical Indexes Analysis

Biochemical indexes ($\text{Na}^{+}\text{-K}^{+}$ -ATPase, glutathione peroxidase, and reduced glutathione) of gills at control group and different time points (1 h, 3 h, 6 h, 12 h, 24 h, and 48 h) after acute salinity stress were measured with commercial kits from Abbkine Reagent Company and Nanjing Jiancheng Institute of Biological Engineering to evaluate the effect of the acute salinity stress on *P. maxima* and determine the most suitable time point for transcriptomics analysis (there should be significant differences among the control group, low salinity group, and high salinity group). The activities of $\text{Na}^{+}\text{-K}^{+}$ -ATPase (Item no. KTB1800) and glutathione peroxidase (Item no. KTB1640) were determined in accord with the instructions of kits from Abbkine Reagent Company. The total protein (Item no. A045-4-4) and reduced glutathione (Item no. A006-2-1) were determined according to the instructions of kits from Nanjing Jiancheng Institute of Biological Engineering.

RNA Extraction and Library Construction

In this study, the control group and the samples of 12 h after acute salinity stress were selected for transcriptomics analysis. Total RNA was extracted with the TRIzol Reagent (Invitrogen, US) according to the manufacturer's instructions. RNA degradation and contamination were monitored on 1% agarose gels. RNA purity and concentration were checked using a NanoDrop2000 (Thermo, MA). RNA integrity number (RIN) was measured by Agilent2100 with RNA 6000 Nano kit (Agilent, CA). Only RNA samples with RIN, A260/A280, and A260/A230 > 8.0 , 1.8, and 1.0, respectively, were used to create cDNA libraries for sequencing. Triplicate RNA samples ($>5 \mu\text{g}$ each) of each group were used to construct RNA-seq libraries, and a total of 3 biological libraries were established.

RNA-seq Data and Differential Expression Analysis

The sequenced image signal from Illumina high throughput sequencing platform converts into text signal via CASAVA base-calling, and stores in FASTQ format as the raw data. Before analysis, low-quality reads and reads containing poly-N and adaptors from raw data have been removed by FASTP (Version 0.19.5, <https://github.com/OpenGene/fastp>) to obtain clean data. In addition, the Q20, Q30, and GC content of the clean data was calculated. For transcriptome research without reference genome, after obtaining high-quality RNA SEQ sequencing data, all sequencing reads were assembled from

scratch to generate contigs and singletons by Trinity (Version v2.8.5). Based on the quantitative results of gene expression, the differentially expressed genes between the two groups were analyzed by *deseq2*, and the screening threshold was set as $|\log_2 \text{fold-change}| \geq 1$ and $p\text{-adjust} < 0.05$. In addition, the RSEM software (version 1.3.1) was used for the principal components analysis (PCA).

GO and KEGG Analysis

The assembled transcripts were mapped using the databases Nr, Swiss-prot, Pfam, COG, GO, and KEGG to obtain differentially expressed genes' function information. BLAST2GO software (version 2.5.0) was used to implement the GO enrichment analysis of the differentially expressed genes, and the statistical enrichment of DEGs in KEGG pathways were carried out by KOBAS software (version 2.1.1). GO terms and KEGG pathways with corrected p value < 0.05 were considered significantly enriched by DEGs (Wei et al., 2019).

Confirmation Using Quantitative Real-Time PCR

Nine typical DEGs and β -actin (reference gene) were randomly selected for quantitative real-time PCR (qRT-PCR) to verify the accuracy of the transcriptome and DGEs data, and the primers were designed using the Primer 5 (Table 1). RT-PCR was conducted using an RT-PCR system (Analytik Jena GmbH, Germany) with SYBR Green (Tiangen Biotech Co., Ltd., China), and the reaction conditions were referred to in Han et al. (2021). The $2^{-\Delta\Delta\text{CT}}$ method was used to calculate the relative expression levels of the DEGs.

Statistical Analysis

The data were processed by Excel 2019 and expressed as mean \pm standard deviation (SD), and the statistical significance was set at $P < 0.05$. Significant differences were analyzed by SPSS 18.0 (IBM SPSS Inc. Chicago, IL) software with one-way analysis of variance (ANOVA), followed by Tukey's s -b multiple comparison tests.

RESULTS

Biochemical Response After Acute Salinity Stress

The changes of $\text{Na}^{+}\text{-K}^{+}$ -ATPase, glutathione peroxidase, and reduced glutathione in *P. maxima* gill are shown in Figure 1. The maximum value of $\text{Na}^{+}\text{-K}^{+}$ -ATPase, glutathione peroxidase, and reduced glutathione were all found at 12 h after salinity stress of 40 ppt, while the minimum value of $\text{Na}^{+}\text{-K}^{+}$ -ATPase, glutathione peroxidase, and reduced glutathione were found at 1 h after salinity stress of 40 ppt, 24 h after salinity stress of 40 ppt, and 6 h after salinity stress of 20 ppt, respectively. The value of $\text{Na}^{+}\text{-K}^{+}$ -ATPase, glutathione peroxidase, and reduced glutathione in *P. maxima* gill significantly varies with different salinity stress and time point. Under normal circumstances, after 48 h salinity stress of 40 ppt, the value of the $\text{Na}^{+}\text{-K}^{+}$ -ATPase, glutathione peroxidase, and reduced glutathione stood at the same level as the control group, while the value of glutathione

TABLE 1 | Primer sequences of selected genes for quantitative real-time PCR.

Gene ID	Gene name	Primers (5'–3')
TRINITY_DN130_c0_g1_i2	UNP1	F: TCATCACTCATCACTGTTCCCATCATC R: TTTCCGGAGTCTGTGGTATTTGTGCTAG
TRINITY_DN137_c0_g1_i2	UNP2	F: TCACAACCATCAATCACAACAACAACC R: CTACGACTACACGGCGACCATTC
TRINITY_DN345_c0_g1_i2	DNAJB4	F: CACAAACTTTGGAGGTCTCTGGAGAG R: TCGAACACACAGAGAATCTTGACG
TRINITY_DN656_c0_g1_i6	Hsp90	F: AAGCCGGAGCAGACATCTCC R: AACCCACAGCGCTAGACTCC
TRINITY_DN6533_c0_g1_i19	PYCR2	F: CTCGGTATTGGCCCCAGGTT R: ACTGCATCCAGCTGGTCTC
TRINITY_DN5650_c0_g1_i4	HP	F: AAGAACGAAGAATTACGCTCAGAGG R: CTCACCTTACGCCGACTGTTAC
TRINITY_DN4923_c0_g2_i1	EF2	F: CAATAACAACCTTACGAGCACGCACAC R: CATCAACCGCCACCCTGACAAC
TRINITY_DN4457_c0_g1_i2	Hsp68	F: GCTCCTCTGTCCTCGGTATCG R: TGGATGTTGACACCTGGCTGATTG
TRINITY_DN30234_c0_g2_i2	ERF3A	F: CCACCACCTCCACAGAAAATACAG R: TCAGCCTCCTTTCATCTTCATCTCC
β -actin	β -actin	F: CGGTACCACCATGTTCTCAG R: GACCGATTTCATCGTATCC

peroxidase and reduced glutathione after 48 h 20 ppt salinity stress were extremely higher than that of the control group.

As there were significant differences in all three biochemical factors between any two groups of control group, the high salinity group (40 ppt) and the low salinity group (20 ppt) only at 12 h, samples of the control group, 12 h after salinity stress of 40 ppt and 20 ppt in triplicate were picked for transcriptomics analysis.

Transcriptome Sequence Assembly and Functional Annotation

The description of the sequencing data is presented in **Table 2**. After preprocessing and removing the low-quality sequences, a total of 64.25 Gb of clean data were obtained, and the clean data of each sample were above 6.2 Gb. The Q20 and Q30 in all treatments were > 98% and 93%, respectively, and the GC was 40.81%–41.39%. After filtering, 74.16%–72.39% clean reads were successfully matched to the genomes of *C. gigas*, *C. virginica*, *Pecten maximus*, *Mizuhopecten yessoensis*, *P. fucata*, and other species within the NR database (<ftp://ftp.ncbi.nlm.nih.gov/blast/db/>).

The results of transcriptome expression analysis and functional annotation of the *P. maxima* gill are shown in **Figure 2**. The number of unigenes expressed in the control group (C), high salinity group (HS), and low salinity group (LS) were 33,419, 34,751, and 36,859, respectively, and there were 24,320 unigenes shared by each group (**Figure 2A**). The correlation coefficients between parallel samples were over 0.91, and the principal components analysis (PCA) revealed significant differences in metabolism of pearl oysters between the three groups (**Figure 2B**). Gene Ontology (GO) categorization of the unigenes in the gill transcriptome of *P. maxima* for all three major GO functional domains (biological processes, cellular components, and molecular function) are presented in **Figure 2C**. For biological processes, transcripts

involved in cellular process (35.24%, GO:0009987) and metabolic process (26.11%, GO:0008152) were the most represented categories; for cellular component, transcripts involved in the membrane part (38.59%, GO:0044425) and the cell part (27.71%, GO:0044464) were highly represented, whereas, regarding molecular function, binding (48.62%, GO:0005488) was the most represented GO term, followed by catalytic activity (41.04%, GO:0003824).

DEGs in Response to Acute Salinity Stress

The results of the statistics and GO categorization of the DEGs between the control group, high salinity group, and low salinity group are shown in **Figure 3**. A total of 11,793 DEGs were obtained, and there were 6613 (3253 up-regulated genes and 3360 down-regulated genes) DEGs (**Supplementary Table S1**), 4395 (2180 up-regulated genes and 2215 down-regulated genes) DEGs (**Supplementary Table S2**), and 6836 (3280 up-regulated genes and 3556 down-regulated genes) DEGs for C vs. HS, C vs. LS, and LS vs. HS, respectively (**Figures 3A, B**). In C vs. LS and C vs. HS group, it is worth noting that the genes whose expression was most significantly decreased were neuroglian isoform X2 and inositol oxygenase, respectively; and the most significantly elevated genes were heat shock protein 68 and cysteine sulfinic acid decarboxylase, respectively (**Supplementary Table S1 and Supplementary Table S2**). C vs. HS and C vs. LS shared 1559 DEGs (contains 1480 genes with consistent regulatory trends, **Supplementary Table S3**), while C vs. HS, C vs. LS, and LS vs. HS shared 240 DEGs (**Figure 3A**). **Figure 3C** shows the GO categorization of the DEGs in C vs. HS, C vs. LS, and LS vs. HS. In C vs. HS, binding (12.72%, GO:0005488) and catalytic activity (41.04%, GO:0003824), membrane part (9.51%, GO:0044425) and cell part (27.71%, GO:0044464), and cellular process (8.54%, GO:0009987) and metabolic process (26.11%, GO:0008152) were the most represented GO terms for molecular function, biological processes, and cellular components, respectively. Coincidentally,

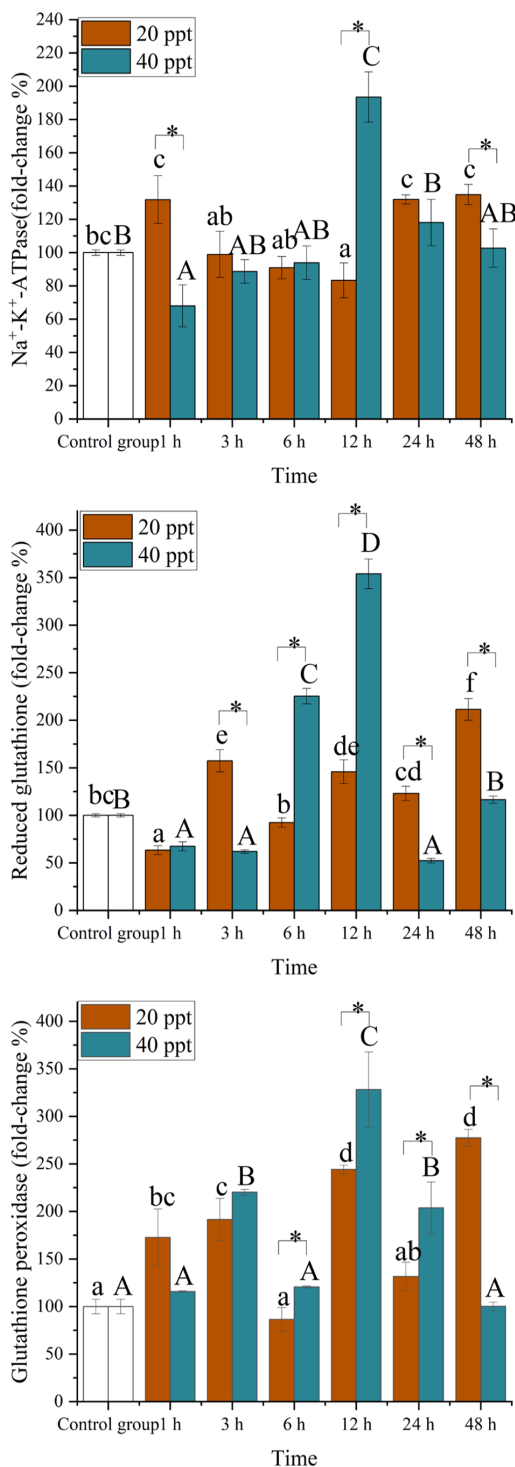


FIGURE 1 | Effect of acute salinity stress on Na⁺-K⁺-ATPase, reduced glutathione, and glutathione peroxidase of *Pinctada maxima*. Data were expressed as mean ± SD (n = 3). Different lowercase letters (a–e) in the figure show significant differences (P < 0.05) among the salinity 20 ppt group, different capital letters (A–D) in the figure show significant differences (P < 0.05) among the salinity 40 ppt group, and * shows significant differences (P < 0.05) among treatment groups.

the most represented GO terms in C vs. LS and LS v.s. HS are exactly the same as in C vs. HS.

KEGG Enrichment Analysis of DEGs

In C vs. HS, a total of 332 KEGG pathways were enriched, including 1514 genes. The first category with the largest number of pathways was ‘Organismal Systems’ (86) and ‘Human Diseases’ (86), followed by ‘Metabolism’ (83), ‘Environmental Information Processing’ (35), ‘Cellular Processes’ (22), and ‘Genetic Information Processing’ (20). In addition, 27 KEGG pathways were significantly (P < 0.05) enriched (Figure 4A), including ‘Metabolism’ (11, ‘Retinol metabolism’ as a representative), ‘Human Diseases’ (6, ‘Small cell lung cancer’ as a representative), ‘Organismal Systems’ (4, ‘Toll and Imd signaling pathway’ as a representative), ‘Cellular Processes’ (4, ‘Apoptosis - multiple species’ as a representative), and ‘Environmental Information Processing’ (2, ‘NF-kappa B signaling pathway’ as a representative). The expression levels of most DEGs in the top 10 significantly enriched pathways (‘Small cell lung cancer’, ‘NF-kappa B signaling pathway’, ‘Platinum drug resistance’, ‘Retinol metabolism’, ‘Toxoplasmosis’, ‘Apoptosis - multiple species’, ‘TNF signaling pathway’, ‘Chemical carcinogenesis’, ‘Toll and Imd signaling pathway’, and ‘Peroxisome’) in C vs. HS were up-regulated (Figure 4B).

In C vs. LS, a total of 308 KEGG pathways were enriched, including 731 genes. The first category with the largest number of pathways was ‘Organismal Systems’ (85), followed by ‘Human Diseases’ (84), ‘Metabolism’ (64), ‘Environmental Information Processing’ (34), ‘Cellular Processes’ (21), and ‘Genetic Information Processing’ (20). In addition, 27 KEGG pathways were significantly (P < 0.05) enriched (Figure 5A), including ‘Human Diseases’ (14, ‘MicroRNAs in cancer’ as a representative), ‘Organismal Systems’ (5, ‘Hematopoietic cell lineage’ as a representative), ‘Environmental Information Processing’ (4, ‘NF-kappa B signaling pathway’ as a representative), ‘Cellular Processes’ (3, ‘Cell cycle’ as a representative), and ‘Metabolism’ (1, ‘Retinol metabolism’ as a representative). The expression levels of most DEGs in the top 10 significantly enriched pathways (‘NF-kappa B signaling pathway’, ‘MicroRNAs in cancer’, ‘Cell cycle’, ‘Hematopoietic cell lineage’, ‘Pancreatic cancer’, ‘Inflammatory bowel disease (IBD)’, ‘Rheumatoid arthritis’, ‘Axon regeneration’, ‘Cell adhesion molecules (CAMs)’, and ‘Toxoplasmosis’) in C vs. LS were up-regulated (Figure 5B).

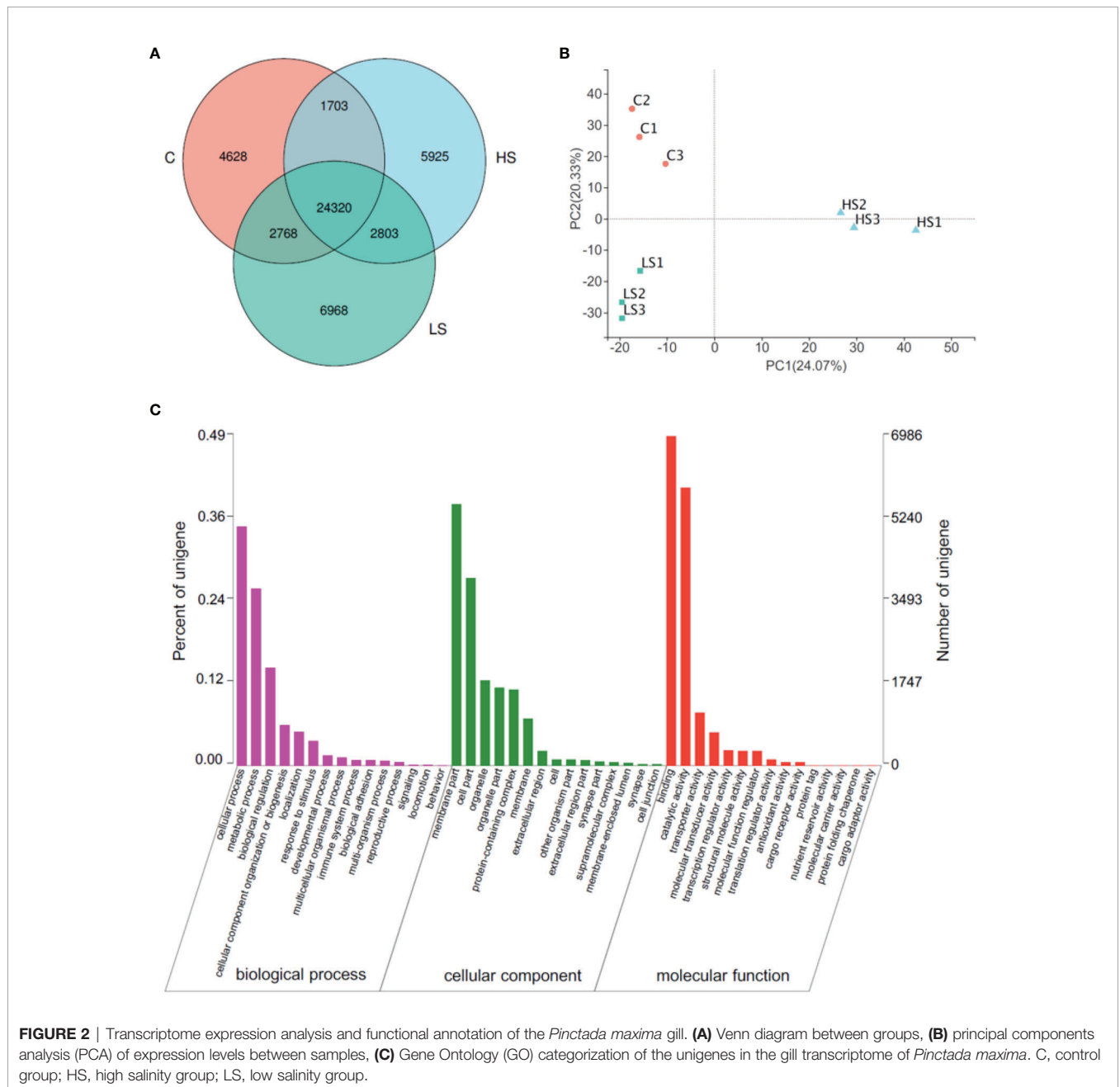
DEGs Shared by C vs. HS Group and C vs. LS Group

There were 1559 DEGs shared by the C vs. HS group and the C vs. LS group. The results of KEGG function annotation showed that 7 DEGs were involved in membrane transport, which includes 2 uncharacterized proteins and 5 function proteins (2 PLL, TTX1, ABCB1-3, and ABCB10) (Figure 6A). In addition, 34 DEGs were observed in the immune system, including 6 uncharacterized proteins and 28 function proteins (Figure 6A). The results of expression pattern clustering heat map analysis showed that salinity stress would down-regulate most membrane transport related genes, and only one up-regulated gene (ABCB10) was

TABLE 2 | Statistics for transcriptome sequencing data from the *Pinctada maxima* gill.

Sample	Raw reads	Raw bases	Clean reads	Clean bases	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)	Mapped reads (Mapped ratio%)
C1	45220194	6828249294	44929360	6635515730	0.024	98.47	95.10	40.81	33809722 (75.25)
C2	54795396	8274104796	54516450	8034990552	0.0239	98.53	95.23	41.13	41351534 (75.85)
C3	52016166	7854441066	51718844	7586813806	0.0239	98.52	95.19	41.01	39031934 (75.47)
HS1	44376036	6700781436	44139488	6528187653	0.0253	98.03	93.75	40.89	33277932 (75.39)
HS2	43368242	6548604542	43055216	6330131948	0.0241	98.43	94.98	41.11	33130654 (76.95)
HS3	42818842	6465645142	42565506	6263938962	0.024	98.49	95.12	40.81	32281480 (75.84)
LS1	53501674	8078752774	53184406	7851925620	0.0242	98.43	94.91	40.97	39443504 (74.16)
LS2	52085874	7864966974	51768660	7609577192	0.0241	98.45	94.99	41.39	39460452 (76.22)
LS3	50757344	7664358944	50432914	7409536061	0.0239	98.54	95.24	41.36	38505996 (76.35)

C, control group; HS, high salinity group; LS, low salinity group; Q20% means percentage of bases with Phred value > 20, Q30% means percentage of bases with Phred value > 30, and GC% means percentage of G and C bases among total bases.



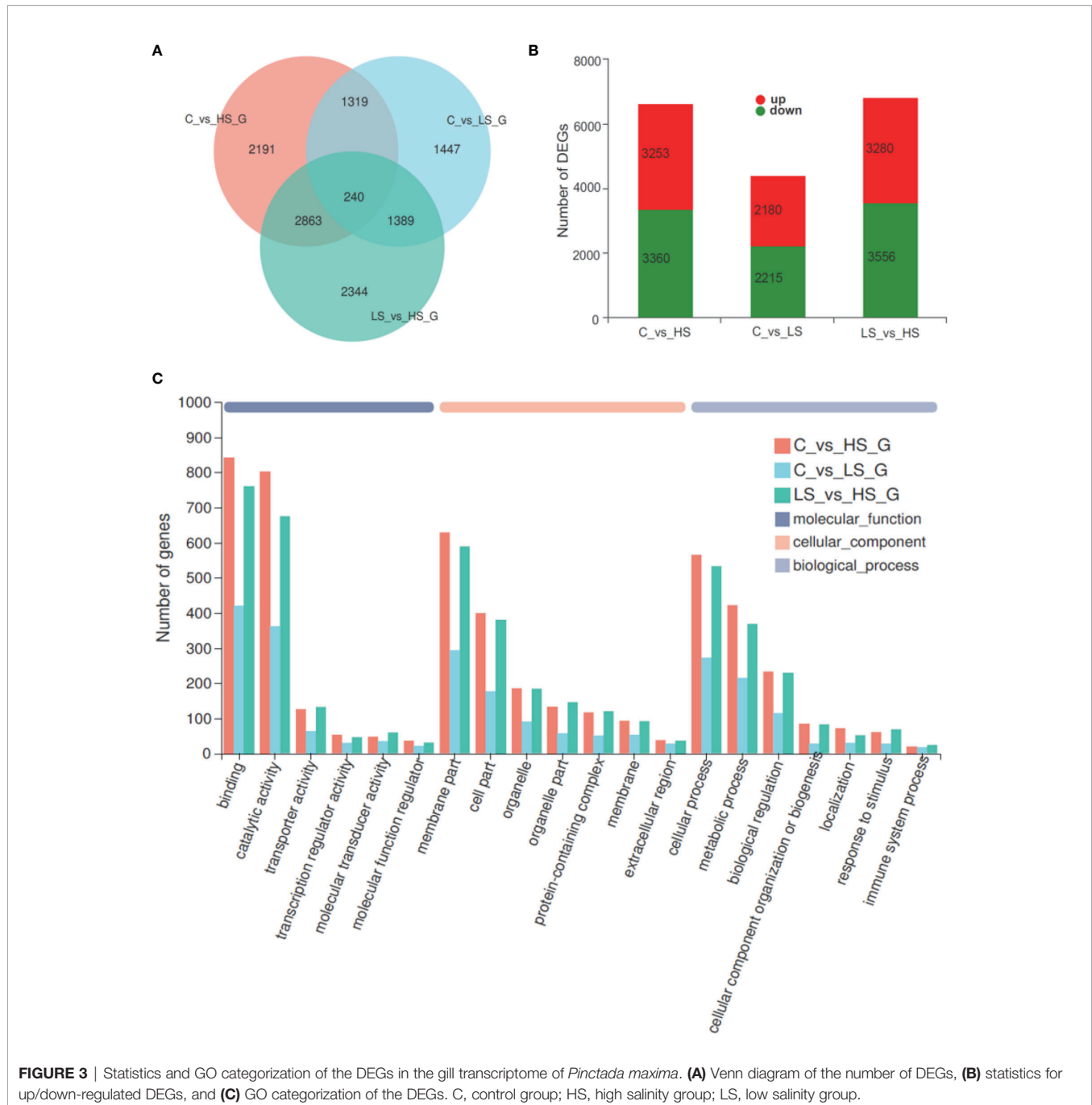


FIGURE 3 | Statistics and GO categorization of the DEGs in the gill transcriptome of *Pinctada maxima*. **(A)** Venn diagram of the number of DEGs, **(B)** statistics for up/down-regulated DEGs, and **(C)** GO categorization of the DEGs. C, control group; HS, high salinity group; LS, low salinity group.

found under high salinity stress (**Figure 6B**). For the immune system related genes, there were 9 genes down-regulated both in the C vs. HS group and the C vs. LS group, 7 genes were up-regulated in the C vs. HS group, and 10 genes were up-regulated in the C vs. LS group (**Figure 6B**). The correlation network for expression of genes shows that the expression of 2 PLL was significantly correlated with the expression of ABCB1-3 in function genes of membrane transport (**Figure 6C**), and there were more than 10 genes related to QRFPR, pepN-L1, TLR2-1,

HSP68, RHOU, MYD88-1, NOTCH2, CDC42, and Sli-1 in function genes of the immune system (**Figure 6D**).

Verification of the Expression Changes of Some DEGs

Figure 7 shows the results about the fold changes detected by qRT-PCR and RNA-Seq expression profiles of 9 randomly selected DEGs. The results showed that the variation trends of the 9 randomly selected DEGs from qRT-PCR were consistent

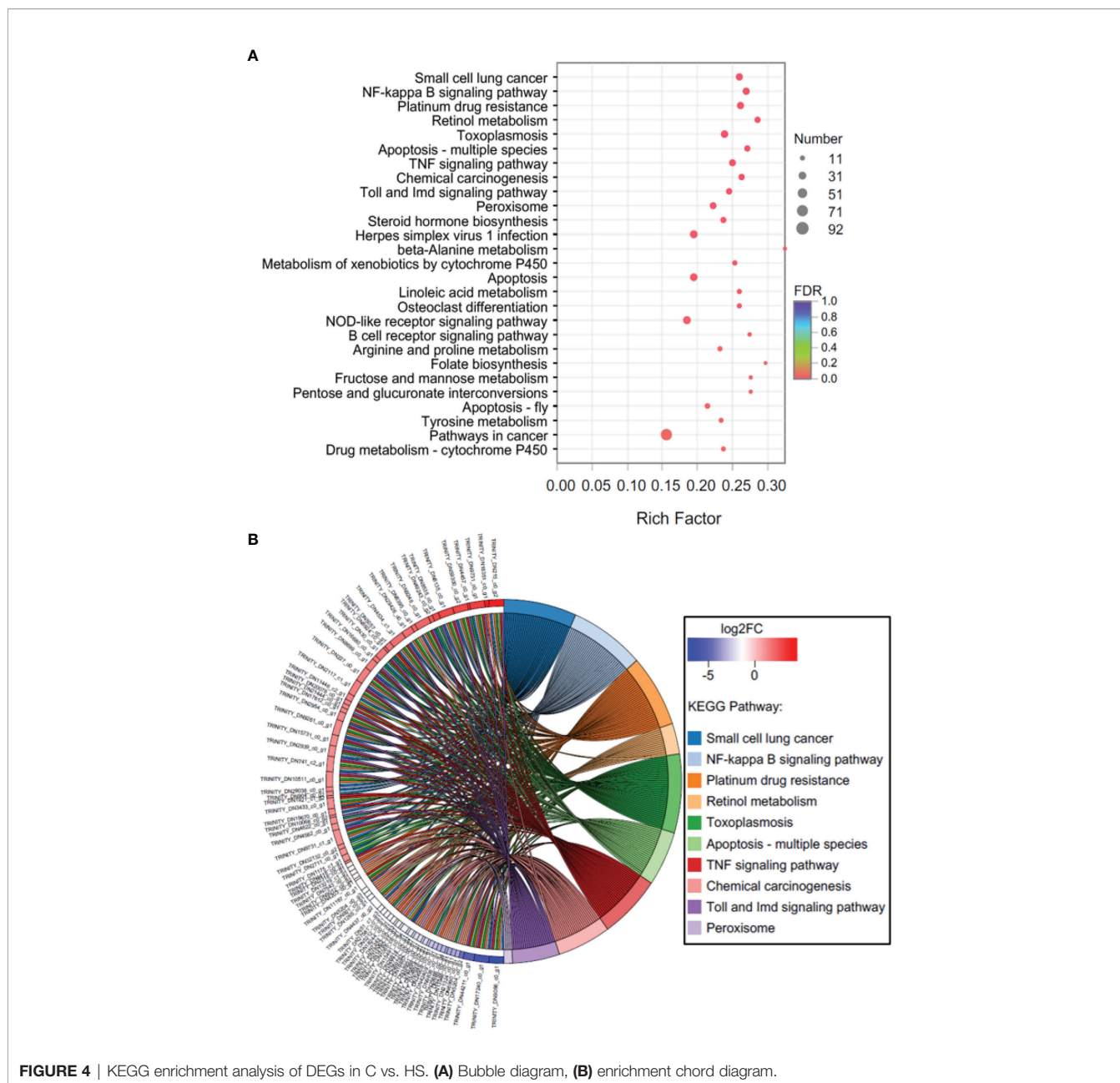


FIGURE 4 | KEGG enrichment analysis of DEGs in C vs. HS. **(A)** Bubble diagram, **(B)** enrichment chord diagram.

with those determined by RNA-Seq, indicating that transcriptome data were accurate and reliable.

DISCUSSION

In this study, Na⁺-K⁺ -ATPase, reduced glutathione (GSH), and glutathione peroxidase (GSH-Px) were measured to evaluate the effect of the acute salinity stress on *P. maxima* and determine the most suitable time point for transcriptomics analysis. Na⁺-K⁺ -ATPase is critical for osmotic regulation, it is an ion channel that could use the energy generated by ATP hydrolysis to drive three Na⁺ outlets and two K⁺ intents to maintain membrane potential

(Evans and Lambert, 2015; Abdel-Mohsen, 2016). GSH is a tripeptide α-amino acid that participates in the detoxification of hydrogen peroxide by various glutathione peroxidases to protect cells from oxidation, which is the most abundant antioxidant in aerobic cells. (Browne and Armstrong, 1998; Owen and Butterfield, 2010). GSH-Px is an important peroxidase that plays a key role in scavenging of free radicals and cell metabolism, and its main function is to catalyze the reduction of lipid peroxides, free hydrogen peroxide derivatives, and the generation of oxygen (Peña-Llopis et al., 2003; Brigelius-Floh'e and Maiorino, 2013). In this study, the highest value of Na⁺-K⁺ -ATPase, GSH, and GSH-Px appeared at 12 h after high salinity stress and then the value returned to the level of the control group at 48 h, while the highest

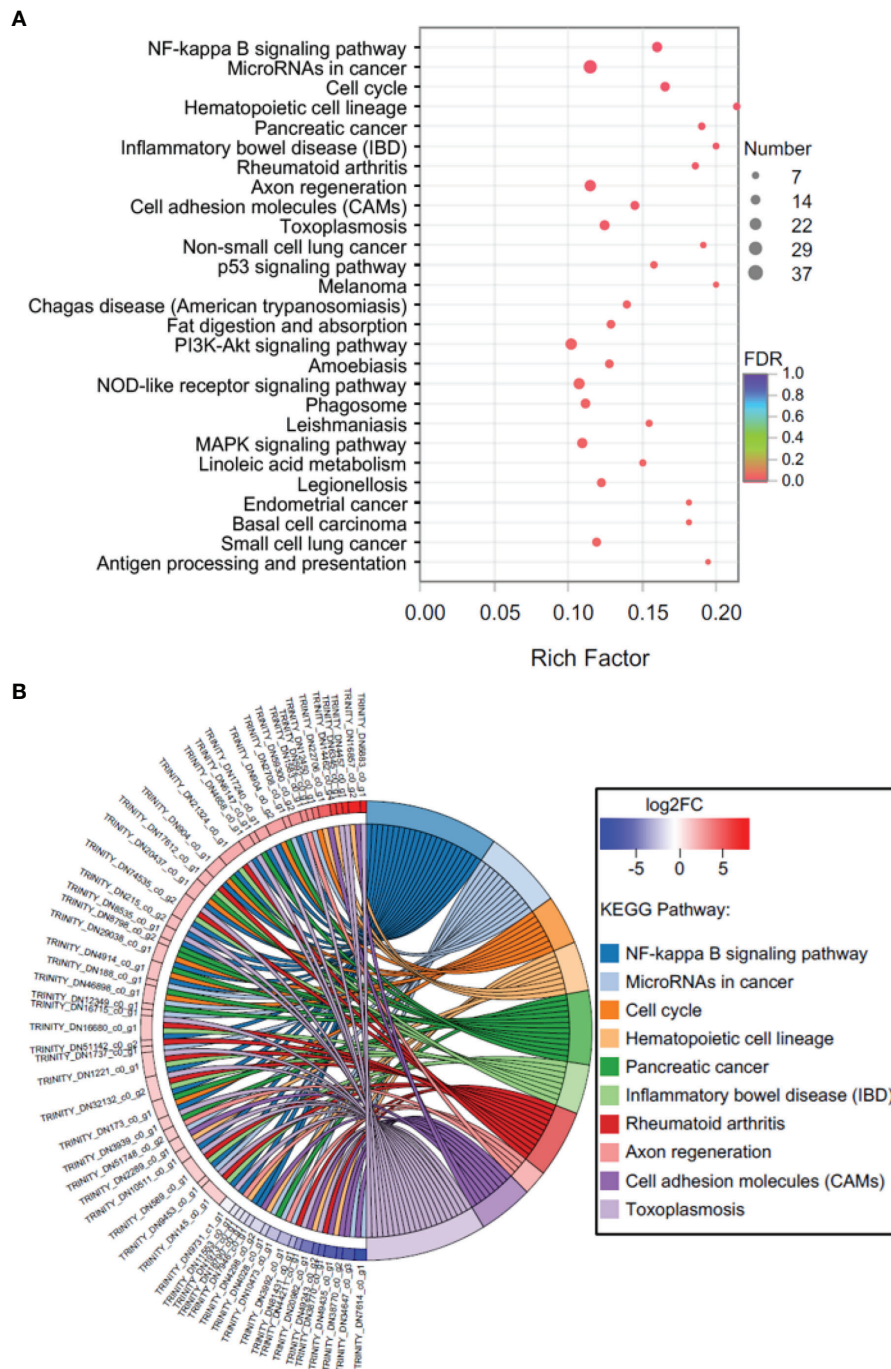


FIGURE 5 | KEGG enrichment analysis of DEGs in C vs. LS. **(A)** Bubble diagram, **(B)** Enrichment chord diagram.

value of Na⁺-K⁺-ATPase, GSH, and GSH-Px appeared at 48 h after low salinity stress. This indicated that *P. maxima* is more adaptable to high salinity than low salinity and suggested that more attention should be paid to prevent *P. maxima* from the low salinity stress during the culture of *P. maxima*. In addition, the time point of 12 h after acute salinity stress was selected for transcriptomics analysis to clarify the transcriptome

characteristics of pearl oyster *P. maxima* in response to acute salinity stress.

Response Characteristics of *P. Maxima* to Acute Salinity Stress

Many studies have shown that marine shellfish undergo a series of physiological reactions including signal transduction,

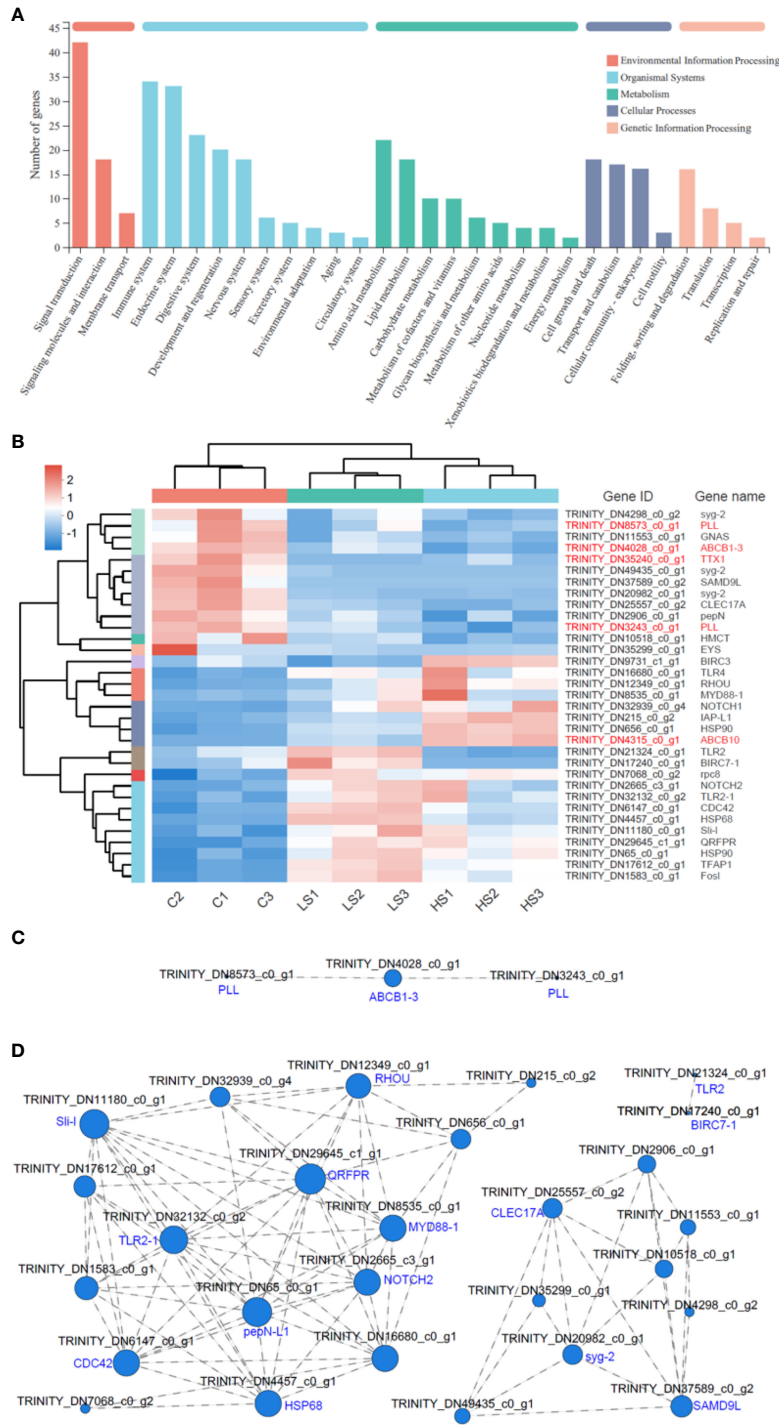


FIGURE 6 | Clustering heat map analysis and the correlation network for expression pattern of genes in membrane transport and the immune system. **(A)** KEGG functional annotation of DEGs shared by C vs. LS and C vs. HS groups. **(B)** Expression pattern clustering heat map analysis of genes in membrane transport and the immune system (the red words show the genes involved in membrane transport). **(C)** Correlation network for expression of genes in membrane transport. **(D)** Correlation network for expression of genes in the immune system. In **(C)** and **(D)**, the larger the gene node, the more genes that have expression correlation with the gene.

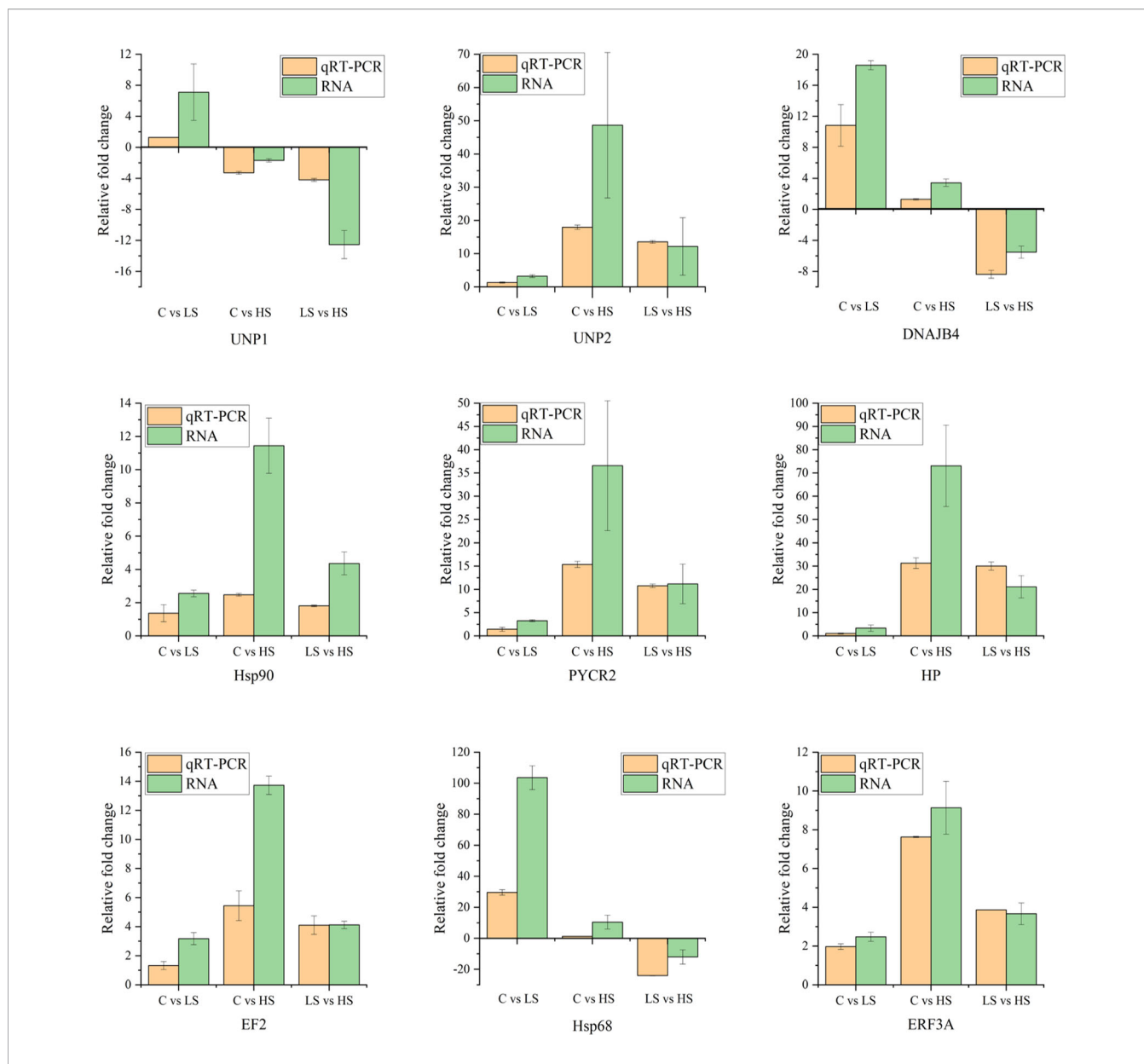


FIGURE 7 | Verification of the selected 9 DEGs by quantitative real-time PCR as compared with RNA-seq data.

osmoregulation, metabolism, immune response, etc. to response salinity changes, and maintain bodily homeostasis (Eierman and Hare, 2014; Gong et al., 2021; Ni et al., 2021). The changes in the salinity will influence the physiological process of marine shellfish, and the changes of osmoregulatory organ deserve special attention because osmotic regulation is related to the adaptation and survival of marine shellfish under salinity stress. In the present study, we analyzed the transcriptional changes of pearl oyster *P. maxima* under acute high-salinity and low-salinity stress in 12 h from the gill, a critical organ in the osmotic regulation system. The results of this study showed that there were 6613 DEGs and 4395 DEGs in C vs. HS and C vs. LS groups, respectively, and the results of the GO function annotation and KEGG pathway enrichment

showed that the annotated DEGs and enriched pathways in C vs. HS group were more than those in the C vs. LS group. In addition, the expression levels of most DEGs in pathways were up-regulated in both the C vs. HS group and C vs. LS group. The above results indicated that there was significant difference in the transcriptional response of *P. maxima* between the high-salinity group and low-salinity group and advised that the transcriptional response of *P. maxima* in the high-salinity group was stronger than that in the low-salinity group. In this study, the transcriptional response of the gill of *P. maxima* under acute salinity stress was described in detail, and the changes of common DEGs in membrane transport and immune function were analyzed with emphasis. The results of this study can provide more ideas and inspiration for future

research on the adaptability of pearl oysters to salinity stress. However, how the salinity change is adapted by pearl oyster through the coordination of various systems or organs throughout the whole body still needs further research.

Although most GO terms in the C vs. LS group are the same as the GO terms in the C vs. HS group, the response strategies of *P. maxima* with different salinity stress were significantly different. Many signaling pathways were observed in the C vs. HS group and the C vs. LS group. Strikingly, NF-kappa B signaling pathway and NOD-like receptor signaling pathway were the common significant enrichment up-regulated signaling pathways in the C vs. HS group and C vs. LS group. Those signaling pathways play a key role in cell response to external stimuli, mainly participation in pathogen recognition and immune response (Kleniewska et al., 2013; Wen et al., 2013). This indicated that both low-salinity stress and high-salinity stress would activate the immune response of *P. maxima*. In addition, the p53 signaling pathway which participates in differentiation and apoptosis was significant enrichment in the C vs. LS group (up-regulated). Instead, in the C vs. LS group, 'TNF signaling pathway' and 'Toll and Imd signaling pathway' which participate in inflammatory response and immune response were up-regulated and significant enrichment. The above results indicated that the low-salinity stress would cause greater damage to *P. maxima*, and *P. maxima* under high-salinity stress would respond by activating more immune responses.

It is reported that aquatic animals redistribute energy metabolism to regulate physiological homeostasis and cope with environmental stress (Petitjean et al., 2019; Morrell and Gobler, 2020). In aquatic organisms, adenosine-5'-triphosphate (ATP) is the main energy source of cells, which is provided by a series of metabolic pathways, including glycolysis, citrate cycle (TCA cycle), and the electron transport chain (Nelson and Cox, 2005). In this study, several up-regulated DEGs involved in the citrate cycle (TCA cycle) which is one of the most important energy metabolism pathways observed. For example, ATP-citrate synthase isoform X2 (ACLY) and isocitrate dehydrogenase [NADP] cytoplasmic-like (IDH1) were up-regulated in both the C vs. HS group and C vs. LS group. However, we also observed many down-regulated metabolic pathways in this study. For example, most DEGs involved in significantly enriched metabolic pathways like linoleic acid metabolism were down-regulated in both the C vs. HS group and C vs. LS group. Furthermore, DEGs involved in retinol metabolism, steroid hormone biosynthesis, beta-alanine metabolism, metabolism of xenobiotics by cytochrome P450, arginine and proline metabolism, folate biosynthesis, fructose and mannose metabolism, pentose and glucuronate interconversions, tyrosine metabolism, and drug metabolism - cytochrome P450 were down-regulated in the C vs. HS group. These down-regulated genes may substantially impact overall energy metabolism (Anni et al., 2016). This is because salinity stress may lead to the down-regulation of most genes involved in energy material metabolism pathways (Yang et al., 2019). Solan and Whiteley (2016) reported that shellfish would isolate the external environment and internal fluid by tightly closing the shell to provide short-time protection from stressful salinity. The behavior caused by salinity stress

would lead to a decrease in the clearance rate of shellfish (Jahromi et al., 2020), resulting in an insufficient oxygen supply, which may be the main reason for the down regulation of the metabolic pathway. In addition, we also found that most genes in the digestive system were down-regulated in this study, which may be because shellfish close their shells, resulting in reduced food intake. Of course, these viewpoints need further experiments to demonstrate. In addition, in marine mollusks, proline and alanine are usually the important FAAs to promote the process of osmotic pressure regulation in cells (Hosoi et al., 2003), and the down-regulated DEGs involved in beta-alanine metabolism and arginine and proline metabolism may be to adapt to the high salinity of the aquatic environment.

The Common Membrane Transport-Related Genes Response to High-Salinity Stress and Low-Salinity Stress

Many aquatic animals had the adaptability to the salinity change of the aquatic environment (Kültz, 2005). The adaptive process, such as osmotic pressure regulation in gills is achieved by several ion transporters and ion transport channels, in which lots of genes are involved (Tseng and Hwang, 2008). In the present study, 7 (including 2 uncharacterized protein) common membrane transport-related DEGs were both observed in the C vs. HS group and C vs. LS group. Several DEGs are up-regulated in both the C vs. HS group and C vs. LS group. For example, ABAB10 were up-regulated, which encodes transporters and channel proteins that act as efflux pumps responsible for intracellular homeostasis (Fletcher et al., 2010). However, we also observed many down-regulated DEGs involved in membrane transport. For instance, ABCB1-3 and PLL were down-regulated in both the C vs. HS group and C vs. LS group, and the correlation network for expression of genes in membrane transport shows that the expression of 2 PLL was significantly correlated with the expression of ABCB1-3. These down-regulated DEGs shows that the membrane transport is decreased, and this further confirms that shellfish can isolate the external environment by closing their double shells. In addition, under salinity stress, shellfish may resist the change of salinity through other reactions, such as activating the immune system to regulate the stability of the internal environment (Ertl et al., 2019; Li et al., 2021; Ni et al., 2021).

The Common Immune System-Related Genes Response to High-Salinity Stress and Low-Salinity Stress

In addition to their typical roles in osmotic pressure regulation and seawater filtration, gills are also the key organ of oyster innate immunity (Dautremepuits et al., 2009; Zhang et al., 2015). Pearl oyster *P. maxima* is an invertebrate metazoan, and its innate immunity is the only immunological defense mechanism (Hoffmann and Reichhart, 2002). In addition, phagocytosis by immune cells is the predominant mechanism of *P. maxima* defense (Ellis et al., 2011). In this study, we observed that immune response was elicited when pearl oyster *P. maxima* encountered hyper-salinity and hypo-salinity, and there were 34

(including 6 uncharacterized proteins) DEGs involved in the immune system shared by the C vs. HS group and the C vs. LS group. In addition, 27 DEGs of 28 function DEGs were found to have significant correlations with the other ($P < 0.05$), and there were more than 10 genes related to QRFPR, pepN-L1, TLR2-1, HSP68, RHO, MYD88-1, NOTCH2, CDC42, and Sli-1. Significantly, QRFPR, TLR2-1, and NOTCH2 are upstream genes of Chemokine signaling pathway, P13K-Akt signaling pathway, and Notch signaling pathway, respectively, which have certain regulatory effects on many downstream genes of the pathway. This may be one of the reasons why QRFPR, TLR2-1, and NOTCH2 become key nodes in the expression network in this study. CDC42 and RHO are both small GTPase of the Rho-subfamily. CDC42 is the canonical member of the family, and usually regulates many cell functions including cell migration, cell polarity, and cell proliferation (Jaffe and Hall, 2005). RHO belongs to atypical Rho GTPase, previous studies have shown that RHO could activate PAK1 and JNK1, and can induce filopodium formation and stress fiber dissolution (Saras et al., 2004; Ory et al., 2007; Brady et al., 2009). In this study, we observed that RHO and CDC42 are simultaneously enriched in Ras signaling pathway and Chemokine signaling pathway for activating JNK and PAK1, respectively. In addition, QRFPR were also observed in Chemokine signaling pathway, and we also found that RHO and CDC42 are simultaneously enriched in the T cell receptor signaling pathway and leukocyte transendothelial migration. Those results suggest that RHO and CDC42 play a vital role in regulation of cell motility and morphology, regulation of actin cytoskeleton, phagocytosis, and inflammatory response of *P. maxima* under salinity change. MAPK signaling pathway is a signal transduction system for eukaryotic cells to mediate extracellular signals to intracellular responses, and MAPK families play a vital role in regulating complex cellular programs like apoptosis, transformation, development, differentiation, and proliferation (Yue and José, 2020). A plethora of studies have demonstrated that MAPK signaling pathway is important in response to osmoregulation, freezing, and anoxia (Cowan and Storey, 2003; Huang et al., 2009). In this study, RHO, CDC42, HSP68, and MYD88-1 were up-regulated and enriched in the MAPK signaling pathway, indicating that the pathway was induced. Similar results were also found in the hemocyte of *Litopenaeus vannamei* under salinity change (Zhao et al., 2015). Heat shock proteins (HSPs) participate in a variety of immune responses and regulate apoptosis in response to environmental stress (Park et al., 2009; Wang et al., 2019b). Many studies have shown that the HSP family genes play a significant role in shellfish response to acute salinity stress (Meng et al., 2013; Hui et al., 2014). In the present study, other than MAPK signaling pathway, we also observed 9 HSP genes (including up-regulated HSP68) enriched in the endocytosis pathway. The main function of HSP68 is uncoating in clathrin-dependent endocytosis. It is noteworthy that RHO and CDC42 were also enriched in the endocytosis pathway, and they play a critical role in regulating clathrin-independent endocytosis.

CONCLUSION

In the present study, *P. maxima* was exposed to acute salinity stress (high-salinity, or low-salinity) and their changes of physiological indexes and molecular response to the salinity stress examined with test kits and RNA-Seq. *P. maxima* is more adaptable to high salinity than low salinity, and this suggests that more attention should be paid to prevent *P. maxima* from the low salinity stress. Overall, our results provided large transcriptome data and revealed 11,793 DEGs related to adaption of salinity changes in *P. maxima*. These findings indicated that the adaptation of *P. maxima* to changes of salinity is a complex process involving multiple organs and genes, and acute salinity stress has a greater impact on genes involved in signaling pathways, metabolism, membrane transport, and immune system. The results of this study will be of great value in understanding the molecular basis of salinity changes adaptation in the pearl oyster.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI SRA (accession: SRP365859).

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences.

AUTHOR CONTRIBUTIONS

HW and MC conceived and designed the project and wrote the manuscript. ZD, YW, GY, and ZM cultured the pearl oyster and collected the samples. WZ, JS, JY, and YL carried out analysis and visualized data. All listed authors have read, edited, and approved the final manuscript.

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South China Sea, and China Agriculture Research System of MOF and MARA.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.902595/full#supplementary-material>

Supplementary Table 1 | A total of 6613 DEGs of *P. maxima* exposed to high salinity (40 ppt).

Supplementary Table 2 | A total of 4395 DEGs of *P. maxima* exposed to low salinity (20 ppt).

Supplementary Table 3 | A total of 1559 DEGs of *P. maxima* shared by C vs. HS and C vs. LS group.

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