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RESEARCH ARTICLE

Transcriptomic analysis provides insight into the mechanism of salinity adjustment in swimming crab *Portunus trituberculatus*

Baoquan Gao[1,2] · Dongfang Sun[1] · Jianjian Lv[1,2] · Xianyun Ren[1,2] · Ping Liu[1,2] · Jian Li[1,2]

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Abstract

Background Low salinity is one of the main factors limiting the distribution and survival of marine species. As a euryhaline species, the swimming crab (*Portunus trituberculatus*) is adaptive to relatively low salinity. However, the mechanisms underlying salinity stress responses in *P. trituberculatus* is not very clear.

Objectives The primary objective of this study was to describe the salinity adaptation mechanism in *P. trituberculatus*.

Methods The crabs were exposed to low salinity stress, and gill tissue was sampled at 0, 12, 36, 48 and 72 h and subjected to high throughput sequencing. Subsequently, we tested the accuracy and quality of the sequencing results, and then carried out GO and KEGG bioinformatics on the differentially expressed genes (DEG).

Results Each sample yielded more than 1.1 Gb of clean data and 23 million clean reads. The process was divided into early (0–12 h), middle (12–48 h), and late phase (48–72 h). A total of 1971 (1373 up-regulated, 598 down-regulated), 1212 (364 up-regulated, 848 down-regulated), and 555 (187 up-regulated, 368 down-regulated) DEGs with annotations were identified during the three stages, respectively. DEGs were mainly associated with lipid metabolism energy metabolism, and signal transduction from the three stages, respectively.

Conclusion A substantial number of genes were modified by salinity stress, along with a few important salinity acclimation pathways. This work provides valuable information on the salinity adaptation mechanism in *P. trituberculatus*. In addition, the comprehensive transcript sequences reported in this study provide a rich resource for identification of novel genes in this and other crab species.

Keywords Transcriptomics · Salinity adjustment · Swimming crab · *Portunus trituberculatus*

Introduction

The swimming crab, *Portunus trituberculatus*, is an important aquaculture species in coastal China due to its rapid growth and widely distributed throughout the coastal waters of Asian-Pacific nations. In China, crab production reached 119,777 tons in 2017, accounting for more than 7.34% of total crustacean production (1,631,185 tons), and the breeding area covered 24,648 ha, accounting for 8.2% of crustacean acreage (299,053 ha) (Fishery Bureau, Ministry of Agriculture, China 2018). It is well known that salinity is one of the most important environmental factors affecting the growth and development of crustaceans and playing important roles in feeding, molting, growth, metabolism and immunity (Huni and Aravindan 1985; Morgan and Iwama 1991; Zhou et al. 2001). At present, cultivation of *P. trituberculatus* mainly utilises earthen ponds in China. In summer, frequent rainstorms are easy resulting in a sharp decline in salinity to 13–18 ppt (unpublished data). Decreased salinity can lead to imbalanced osmotic pressure in crabs, causing slow growth and disease outbreak (Sun et al. 2018), resulting in huge economic losses in the aquaculture industry. Hence, breeding varieties resistant to low salinity is of great

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✉ Ping Liu
gaobq@ysfri.ac.cn

[1] Key Laboratory of Sustainable Development of Marine Fisheries, Ministry of Agriculture, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, People's Republic of China

[2] Laboratory for Marine Fisheries and Aquaculture, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266071, People's Republic of China

significance to the sustainable development of *P. trituberculatus* aquaculture.

Recent studies on adaption to salinity have mainly focused on the effects of salinity on individual development (Zhang and Li 1992), growth and metabolism (Lu et al. 2012). They found that salinity had a significant effect on growth and energy utilization. At salinities of 20, 25, and 30, food intake (FId) was lower and food conversion efficiency (FCEd) was higher than in the control (Lu et al. 2012). Other studies indicated that Na^+, K^+, $2Cl^-$ cotransporter (NKCC) and Na^+/K^+-ATPase played an important role in the salinity acclimation of *P. trituberculatus* (Lv et al. 2016; Han et al. 2015). And, some salinity-related genes have been found by transcriptomic sequencing (Lv et al. 2013). However, the mechanisms of salinity stress remain underexplored and poorly understood.

In the present study, crabs were exposed to 11 ppt (the median lethal salinity LD_{50} of *P. trituberculatus* for 72 h), sampled at 0, 12, 48 and 72 h post low salinity stress, and subjected to transcriptomic sequencing. Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) enrichment analysis were performed on differentially expressed genes (DEG) identification at different stages of salinity stress. The findings presented here will expand our understanding of the mechanisms underlying salinity stress responses in *P. trituberculatus*.

Materials and methods

Experimental animals

Swimming crabs with initial weight of 20.24 ± 3.12 g were obtained from a local farm in Weifang, China. Crabs were fed with living *Potamocorbula laevis* for 7 days and acclimated to laboratory condition (33 ppt, 23 °C). The seawater salinity in two cement pools was adjusted to 11 ppt using a water quality meter and left to stand for 4 h. Thirty randomly selected crabs were placed in each pool when the seawater salinity had stabilised. During the experiment, the crabs were not fed, and water was not changed. Dead crabs were continually removed and mortality statistics were recorded at 12, 48 and 72 h.

Tissue fixation and biopsy

Gills were fixed with 4% paraformaldehyde for 2 h at 0, 12, 48 and 72 h after salinity stress, dehydrated with 30, 50 and 75% alcohol in series for 5 min each, and stored at 75% alcohol at − 20 °C. Tissue samples were stained with hematoxylin and eosin (HE). Tissue samples were first soaked in absolute ethanol for 10 min each time, then twice in xylene for 15 min each time, followed by paraffin embedding, sectioning, baking, and staining prior to microscopy analysis.

RNA isolation, library construction, and sequencing

Gills were sampled at 0, 12, 48 and 72 h after salinity challenge (C0, C12, C48 and C72 groups, respectively) from crabs in the other pool, and rapidly frozen in liquid nitrogen. Total RNA was extracted with TRIzol reagent (Invitrogen, USA). Library preparations were sequenced on a BGISEQ-500 platform (BGI, Shenzhen, China), and 50 bp single-end (SE) reads were generated. The BGISEQ-500 platform is powered by combinatorial probe-anchor synthesis (cPAS) and improved DNA Nanoballs (DNBs) technology. The cPAS chemistry works by incorporating a fluorescent probe into a DNA anchor on the DNBs, followed by high-resolution digital imaging. This combination of linear amplification and DNB technology reduces the error rate while enhancing the signal. In addition, the size of the DNBs is controlled so that only one DNB is bound per active site. This patterned array technology not only provides sequencing accuracy, but also increases chip utilisation and sample density.

De novo assembly and annotation

Prior to de novo transcriptome assembly, raw reads were subjected to quality control (QC) and filtered to generate clean reads by discarding reads with adaptors, unknown bases present at > 10%, and low-quality reads (low quality bases > 50% per read). Clean reads were aligned to the *P. trituberculatus* transcriptome database using HISAT (Kim et al. 2015), and relative expression levels for each sample were quantified using RSEM (Li and Dewey 2011) and calculated using the FPKM method.

BLAST was used to annotate transcripts over 200 bp in length, searching sequence according to NCBI non-redundant (NR) protein database, and HMMER was used to search sequence according to PFAM protein database (Finn et al. 2008). Using E-value threshold $1e^{-5}$ in BLAST and E-value threshold $1e^{-10}$ in HMMER, the transcripts were functionally annotated as identified proteins or nucleic acids with the highest sequence similarity. We use transcription codes to predict coding sequences (CDS) and convert them into corresponding amino acid sequences. The functional classification of gene ontology (GO) was performed by Blast2GO (Ana et al. 2005).

Differential expression analysis

Differential expression analysis using data collected at different time points was performed using the DESeq package (Audic and Claverie 1997). The resulting p values were

adjusted using the Benjamini and Hochberg approach to control the false discovery rate. Genes with an adjusted p value < 0.05 (q value < 0.05) and fold change > 2 in DESeq analysis were assigned as differentially expressed genes (DEGs) (Benjamini and Yekutieli 2001).

GO and KEGG analysis

GO functional significance enrichment analysis identifies GO functional terms that are significantly enriched in differentially expressed genes (DEGs) compared with the genomic background, thereby predicting related biological functions. After Bonferroni correction (Abdi 2007), p values are calculated to identify GO terms significantly enriched among DEGs, with a corrected p value < 0.05 as the threshold. Thus, the main biological functions of DEGs can be predicted by GO functional significance enrichment analysis.

The Kyoto Encyclopaedia of Genes and Genomes (KEGG) database was used to indicate the location of DEGs in different pathways. Pathways with q values < 0.05 were considered significant enriched in terms of DEGs. Identification of enriched pathways can assist prediction of the main biochemical/metabolic pathways and signal transduction pathways in which DEGs participate.

Quantitative real-time reverse-transcription PCR (qPCR) confirmation of transcriptome sequencing data

To confirm the reliability and accuracy of the transcriptome sequencing data, eight DEGs related to ion transfer and regulation were selected for qPCR verification. Specific primers designed by Primer Premier 5 for each of the DEGs and listed in Table S5. The β-actin gene was selected as an internal control for qPCR analysis. First-strand cDNA was generated following the protocol provided in the PrimeScript RT reagent Kit (TaKaRa, Japan). The cDNA was amplified with specific primers in 20 μL reactions. PCR was performed using an ABI 7500 RT-PCR instrument with an initial denaturation at 95 °C for 30 s, followed by 40 cycles at 95 °C for 15 s and 60 °C for 34 s, and a final dissociation curve analysis of one cycle at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Each cDNA sample was assayed three times and relative expression was determined using the $2^{-\Delta\Delta CT}$ method. Data are expressed as mean ± standard error (SE) following analysis by Student's t tests in Microsoft Excel, and data were standardised against the expression quantity at 0 h (given a value of 1). Results were plotted using Microsoft Excel.

Results

Effect of salinity stress on gill structures

The results of HE staining revealed that low salt stress altered the structure of gill filaments. The branchial cavity was increased after 12–48 h of salt stress. Meanwhile, gill filaments were thickened, and some of the epithelial cells disintegrated, leaving only cuticle. Interestingly, gill filament structure was essentially restored after 72 h (Fig. 1).

Post-salinity mortality statistics

As shown in Fig. 2, the mortality of crabs in normal sea water was zero. However, the mortality was 33.3%, 50% and 53.3% after low salinity stress for 12, 48 and 72 h, respectively. Thus, the results of HE staining and mortality analysis clearly showed that salinity affected crabs.

Preliminary analysis of transcriptome sequencing data

As shown in Table 1, four samples were tested by RNA-Seq sequencing (Mortazavi et al. 2008), yielding an average of 23,957,606 original reads. After removal of low-quality reads, the average number of remaining clean reads was 23,941,904. Filtered data were compared with the reference sequence of *P. trituberculatus* using HISAT(Kim et al. 2015) and Bowtie (Langmead et al. 2009). According to the statistics, the average ratio of clean reads to reference genes was 87.255%. We simultaneously subjected to sample data to QC from various aspects, and high quality was confirmed. All sequencing data have been deposited in the Short Read Archive of the National Center for Biotechnology Information (NCBI) under accession numbers SRR8187066 (C0), SRR8187067 (C12), SRR8187068 (C48) and SRR8187137 (C72).

Identification of differently expressed genes (DEGs)

To better understand salinity stress, the process was divided into three phases, early (0–12 h of salinity stress), middle (12–48 h of salinity stress), and late (48–72 h of salinity stress). As is shown in Table 2, 1971 (1373 up-regulated, 598 down-regulated), 1212 (364 up-regulated, 848 down-regulated) and 555 (187 up-regulated, 368 down-regulated) annotated DEGs were identified during the early, middle and late stages of salinity stress, respectively.

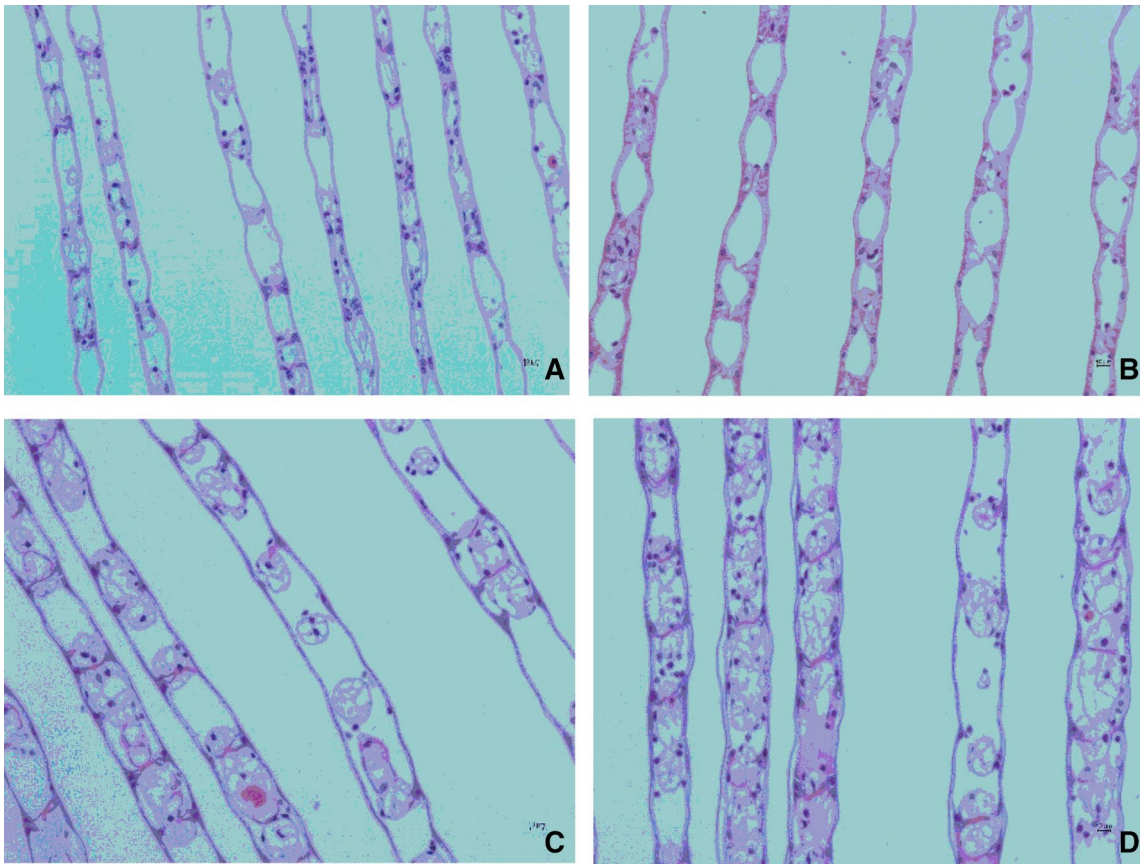

Fig. 1 The gills of crab after low salinity stress. **a**–**d** Represent gills at 0, 12, 48, 72 h after low salinity, respectively. During the 12 and 48 h of salinity stress, the branchial cavity increased, the gill filaments thickened, and some of the epithelial cell disintegrated, leaving only cuticle (**b**, **c**). After 72 h of stress, the gill filament structure was basically restored (**d**)

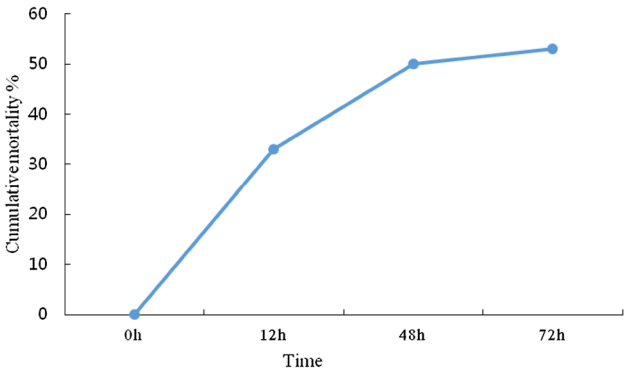

Fig. 2 Change of cumulative mortality after low salinity stress. The mortality is 33.3%, 50%, 53.3% after low salinity stress at 12, 48, 72 h, respectively

Detailed information related to these DEGs is included in Table S1. DEGs that could not be functionally annotated are listed as 'unknown'.

GO and KEGG enrichment analyses

GO enrichment analysis was performed to investigate the putative roles of DEGs. The early stage of salinity stress was mainly related to membrane-bound organelle, intracellular membrane-bound organelle, and aminoglycan metabolic process categories. The middle stage of salinity stress was linked to membrane-bound organelle and intracellular membrane-bound organelle categories. The late stage was mainly associated with membrane, extracellular region, and sulfotransferase activity categories. The top five GO terms in each of the three stages are listed in Table 3, and details of the GO terms are included in Table S2.

KEGG enrichment analysis was also performed for DEGs identified in the three stages. During the early stage, DEGs were mainly involved in pathways related to arachidonic acid metabolism, linoleic acid metabolism, and glycosaminoglycan biosynthesis-chondroitin sulfate/dermatan sulfate. In the middle stage, DEGs were linked to glycosaminoglycan biosynthesis-chondroitin sulfate/dermatan sulfate, endocytosis, and ribosome biogenesis in eukaryotes. In the late stage, cell adhesion molecules (CAMs), PI3K-Akt signalling, and

Table 1 Summary of sequencing data obtained in this study

Sample	Raw reads	Clean reads	Clean data (bp)	Q20%	Mapped (%)
C0	23,957,781	23,931,819	1,196,590,950	96.4	85.84
C12	23,957,547	23,951,554	1,197,577,700	96.3	86.63
C48	23,957,397	23,941,727	1,197,086,350	96.5	88.53
C72	23,957,697	23,942,517	1,197,125,850	96.6	88.02
Average	23,957,606	23,941,904	1,197,095,213	96.45	87.255

Table 2 Summary of identified DEGs

Case	Control	Total DEGs	Up-regulated genes	Down-regulated genes
C12	C0	1971	1373	598
C48	C12	1212	364	848
C72	C48	555	187	368

ECM-receptor interaction pathways were the most enriched. The main enriched KEGG terms in each of the three stages are listed in Table 4, and details of the KEGG terms are included in Table S3.

Expression of DEGs in key KEGG pathways

Expression patterns of DEGs in the key KEGG pathways (aldosterone-regulated sodium reabsorption, mineral absorption, and cGMP-PKG signalling) were further evaluated. Proteins associated with ion transport were mainly enriched mineral absorption and cGMP-PKG signalling pathways. As shown in Table 5, DEGs related to ion transporter genes, including those encoding Na^+/K^+-ATPase, Na^+/K^+-α, Na^+/K^+-8, Cl^--3, Na^+/K^+-β and Na^+/H^+-7, were up-regulated during the early stage of salinity stress, but down-regulated during the middle stage, except for Na^+/K^+-α. There were no significant differences for ion transporter genes in the late stage. Genes related to the cGMP-PKG signalling pathway were down-regulated in the early stage of salt stress, but there were no significant differences in the last two stages.

Expression patterns of DEGs in the three stages of salinity stress

The more significant DEGs may play important roles in responses to changes in the environment, hence they were identified and annotated. Expression pattern of twelve salinity regulation related genes including Na^+/K^+-ATPase, Cl^- channel (chloride intracellular channel), Na^+/H^+ exchanger 8 (sodium/hydrogen exchanger 8), $Na^+/K^+/2Cl^-$-C ($Na^+/K^+/2Cl^-$ contransporter), aquaporin, α-CAg (alpha-carbonic anhydrase), caspase, HSP70 (heat shock protein 70), CHH2 (crustacean hyperglycemic hormone 2), chitinase, cyc oxidase (cytochrome *c* oxidase subunit 3), arginine kinase were examined. As shown in Fig. 3, most of the DEGs up-regulated between 0 and 12 h of salt stress, such as Na^+/K^+-ATPase, potassium channel, chloride intracellular channel, Na^+/H^+-exchanger, calcium-transporting ATPase, chloride channel, and $Na^+/$

Table 3 Result of GO enrichment during the three stages of salinity stress

Time	Category	GO-term	Gene num.
0–12 h	Cellular component	Membrane-bounded organelle	139
	Cellular component	Intracellular membrane-bounded organelle	131
	Molecular function	Membrane-bounded organelle	139
	Biological process	Aminoglycan metabolic process	12
	Biological process	Actin filament organization	7
12–48	Cellular component	Membrane-bounded organelle	91
	Cellular component	Intracellular membrane-bounded organelle	88
	Molecular function	Helicase activity	8
	Biological process	Enzyme linked receptor protein signaling pathway	6
	Biological process	Nervous system development	13
48–72	Cellular component	Membrane	42
	Cellular component	Extracellular region	4
	Molecular function	Sulfotransferase activity	3
	Biological process	Sulfur compound metabolic process	6
	Biological process	Wing disc morphogenesis	5

Table 4 Result of KEGG enrichment during the three stages of salinity stress

Time	Pathway	DEGs	p value
0–12 h	Arachidonic acid metabolism	25	0.000413958
	Linoleic acid metabolism	15	0.000658866
	Ovarian steroidogenesis	28	0.000952417
	Glycosaminoglycan biosynthesis—chondroitin sulfate/dermatan sulfate	16	0.001586288
	Cytosolic DNA-sensing pathway	24	0.002553888
12–48 h	Glycosaminoglycan biosynthesis—chondroitin sulfate/dermatan sulfate	16	2.92E–05
	Endocytosis	90	0.000207212
	Ribosome biogenesis in eukaryotes	55	0.000220946
	Vitamin digestion and absorption	26	0.000369495
	Malaria	35	0.00081991
48–72 h	Cell adhesion molecules (CAMs)	64	5.18E–07
	PI3K-Akt signaling pathway	62	9.52E–06
	Prion diseases	30	1.17E–05
	ECM-receptor interaction	38	2.30E–05
	Malaria	24	5.45E–05

$K^+/2Cl^-$-cotransporter, were associated with ion transport, whereas down-regulated DEGs included aquaporin, carbonic anhydrase and heat shock protein 90 (HSP90). Genes related to ion regulation were primarily down-regulated between 12 and 48 h of salt stress, whereas energy metabolism-related genes including chitinase, chitinase 5, arginine kinase and cytochrome were mainly up-regulated between 12 and 48 h. Finally, expression levels of most genes remained steady between 48 and 72 h of salt stress.

Validation of selected DEGs by qPCR

To confirm the reliability of the RNA-seq data, nine DEGs related to ion channels and their regulation were selected for qPCR validation. These genes included Calprotectin, $Na^+/K^+/2Cl$-cotransporter ($Na^+/K^+/2Cl^-C$), sodium/hydrogen exchanger 8 (Na^+/H^+-E8), aquaporin, alpha-carbonic anhydrase (α-CA), V-ATPase subunit D (V-ATPase D), chloride channel protein 2 isoform X2 (Cl-channel 2), Na^+ K^+-ATPase, crustacean hyperglycemic hormone 2 (CHH2). Expression levels of these DEGs are shown in Fig. 4. Overall, the expression patterns of all eight DEGs obtained by qPCR were similar to those derived from RNA-Seq analysis, although the relative expression levels were not completely consistent. Therefore, the results of qPCR analysis confirmed the reliability and accuracy of the RNA-Seq data.

Discussion

Transcriptome profiles of *P. trituberculatus* in response to low salinity stress have been investigated previously (Liu et al. 2009; Xu 2011), most of which investigated salinity-related genes and/or salinity regulation mechanisms.

Table 5 DEGs associated with three major pathways

Pathway	Gene name	Log2 fold change		
		12 h	48 h	72 h
Aldosterone-regulated sodium reabsorption	Na^+/K^+-α (sodium/potassium-transporting ATPase subunit alpha-like)	3.94	1.75	−0.35
	Na^+/K^+ 8 (sodium/potassium-transporting ATPase subunit alpha isoform X8)	3.30	−0.35	−0.76
	Na^+/K^+-ATPase	2.77	−0.43	−0.14
	Na^+/K^+ β (sodium–potassium ATPase beta)	2.14	−0.21	0.24
Mineral absorption	Cl^-3 (chloride channel protein 2 isoform X3)	8.28	−3.21	−0.07
	Cl^-2 (chloride channel protein 2 isoform X2)	5.94	−2.26	0.12
	Na^+/H^+ 7 (Na(+)/H(+) exchanger protein 7-like)	4.27	−0.98	−1.41
	Na^+/K^+-α (sodium/potassium-transporting ATPase subunit alpha-like)	3.93	−1.75	−0.36
	Cl^-C (Chloride channel)	3.91	−0.33	−1.13
	Na^+/H^+-E (sodium/hydrogen exchanger)	3.72	−1.40	−0.17
	Na^+/K^+ 8 (sodium/potassium-transporting ATPase subunit alpha isoform X8)	3.30	−0.35	−0.76
	Na^+/K^+-ATPase	2.77	−0.44	−0.14
	Na^+/K^+ β (sodium–potassium ATPase beta)	2.14	−0.22	0.24
cGMP-PKG signaling pathway	Na^+/K^+-ATPase	2.77	−0.44	−0.14
	Na^+/K^+ β (sodium–potassium ATPase beta)	2.14	−0.22	0.24
	CA 8(adenylate cyclase type 8 isoform X2)	−2.89	0.37	0.07
	Na^+/K^+-α (sodium/potassium-transporting ATPase subunit alpha-like)	3.93	−1.75	−0.36
	Na^+/K^+ 8 (sodium/potassium-transporting ATPase subunit alpha isoform X8)	3.30	−0.35	−0.76

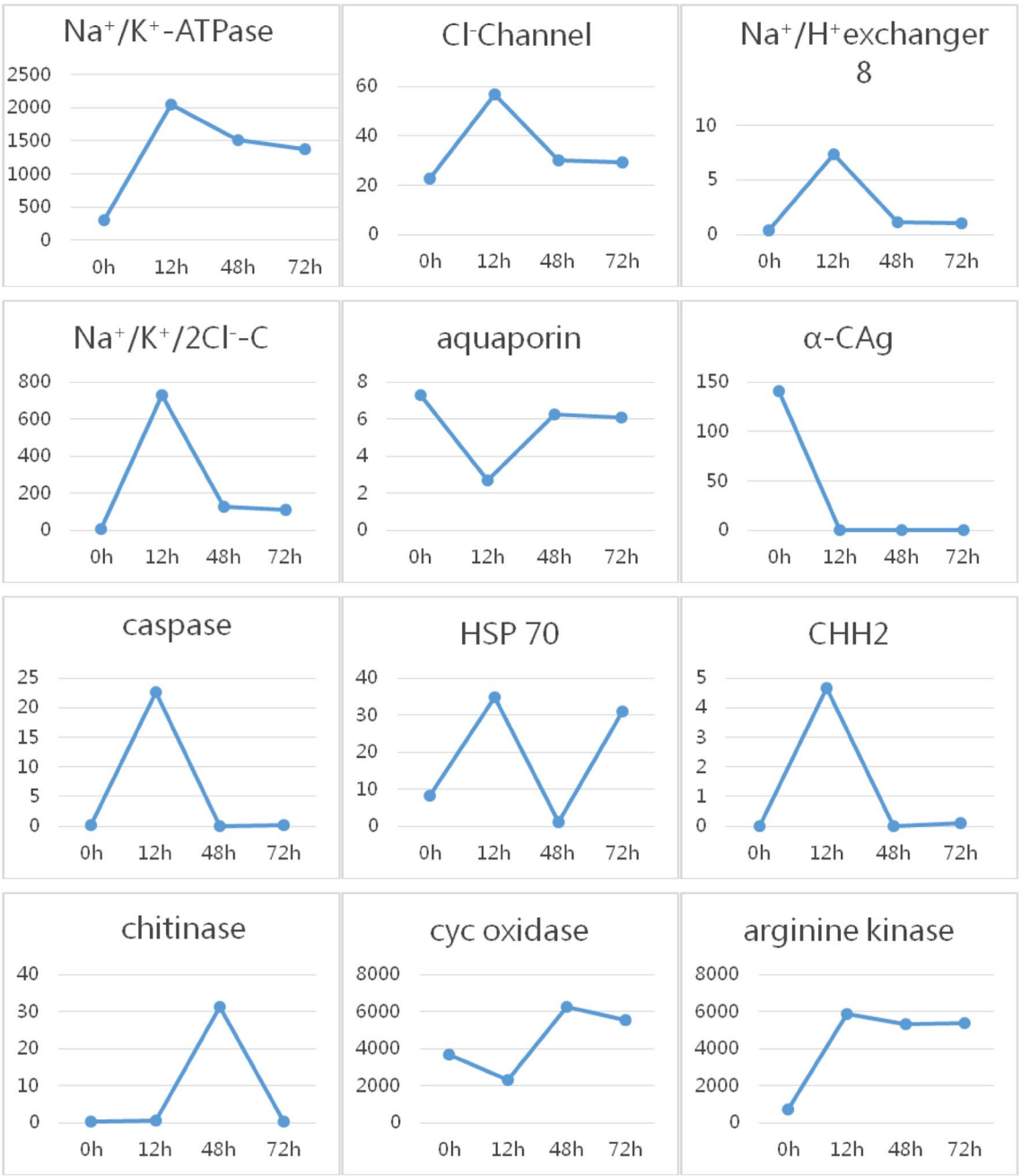

Fig. 3 Expression patterns of key genes involved in salinity stress. The control group was set at 0 h, the x-coordinate represents time of low salinity stress and the vertical axis represents the relative quantity of expression

However, the purpose of our study was to understand the overall process of salinity stress in this species.

The gills of crustaceans are important organs for controlling osmotic pressure and ions (Freire et al. 2008; Mcnamara and Faria 2012), and are regulated by two main strategies: limiting and compensatory processes. Limiting processes mainly alter the structure and permeability of the gill cuticle, reducing the passive diffusion of ions and water inflow to maintain osmotic pressure stability. These processes are important for allowing crustaceans to permanently inhabit constantly changing aquatic environments. Mechanisms involve adjusting gill permeability by closing stomata or changing the composition of fatty acids in the stratum corneum of the gill to facilitate adaptation to osmotic stress in the short and long term (Morris et al. 1982; Porter et al. 1996). Compensation processes allow organisms to maintain osmotic pressure and ion levels in haemolymph by activating ion transmembrane transport in response to external osmotic

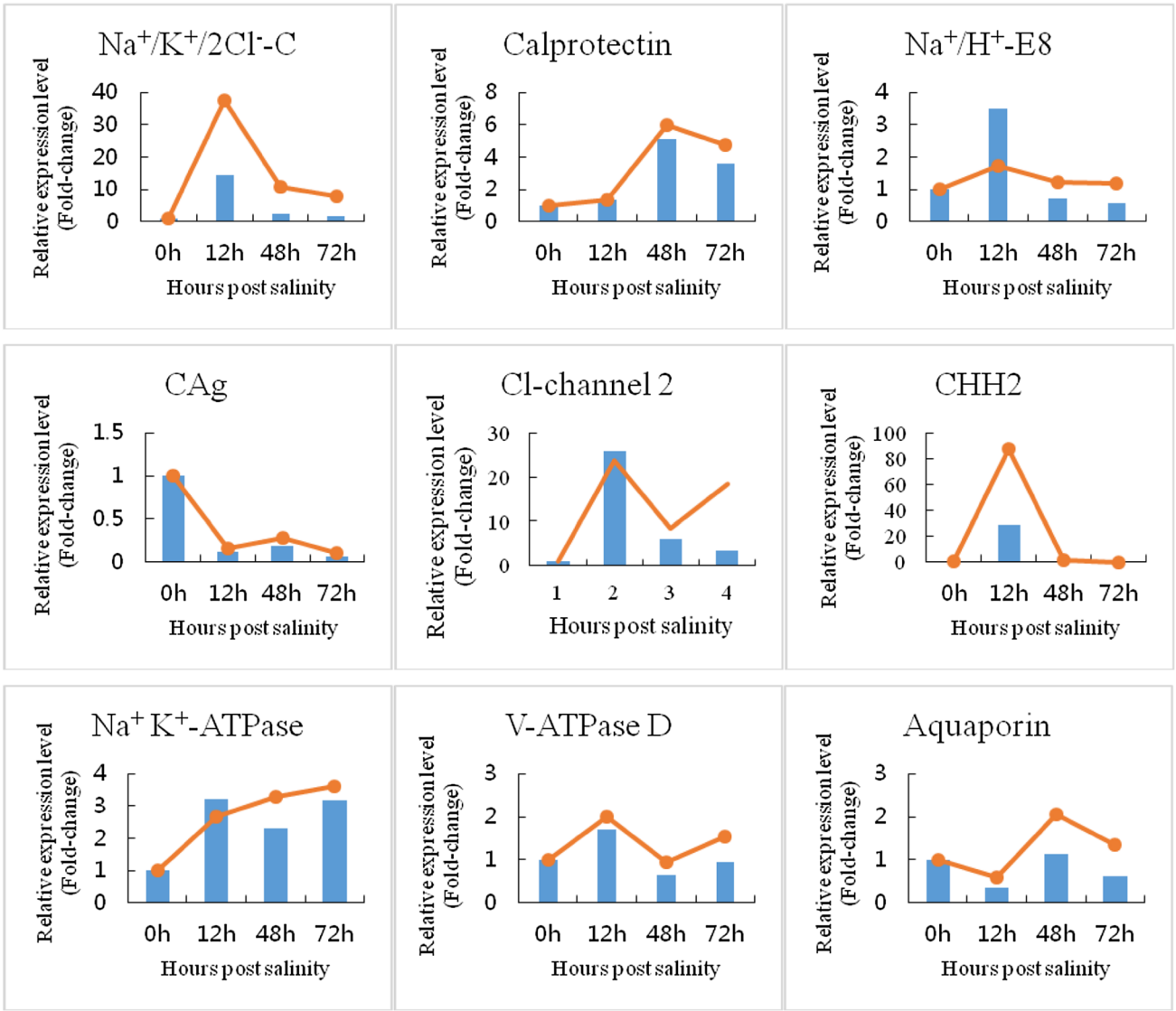

Fig. 4 Confirmation of transcriptomics sequencing data by RT-qPCR. Nine DEGs were randomly selected for qRT-PCR verification of samples. The relative expression levels of the genes at different time points were calculated as the ratio of gene expression level (qPCR) or normalized RPKM level (RNA-seq) relative to that 0 h (control) post-salinity. All data represent the mean ± SD of three replicates. The polygraph represents the result of sequencing, and the columnar graph represents the result of qRT-PCR

pressure stress, thereby balancing the passive diffusion caused by external osmotic pressure stress (Andre 1995).

In the present study, crabs were exposed to low salinity, and gill tissue was sampled at 0, 12, 48 and 72 h. In order to better understand the overall mechanism of crabs respond to low salinity, the process was divided into early (0–12 h), middle (12–48 h), and late (48–72 h) stages based on previous research. Data from the latter time points of each stage were compared with data from the earlier time points to identify DEGs. DEGs in the 0–12 h stage were mainly associated with lipid metabolism, DEGs in the 12–48 h stage were linked to energy metabolism, and DEGS in the 48–72 h stage were mainly related to signal transduction.

In the early stage, 1971 DEGs (1373 up-regulated and 598 down-regulated) were identified, more than in the other two stages. The content of sphingomyelin in gill epithelial cells of crabs is decreased significantly during this stage, while total phospholipids in the last three pairs of gill epithelial cells are increased significantly (Brichon et al. 1996), and the synthesis and renewal of sphingomyelin is accelerated (Whitney 1974; Chapelle et al. 1976). However, changes in phospholipids affect membrane permeability and water infiltration, resulting in an increase in the branchial cavity which represents a limiting process. Meanwhile, expression of some ion transporter genes, such as those encoding Na^+/K^+-ATPase, Cl^- channel, Na^+/H^+-E, $Na^+/K^+/2Cl^-$-C and

V-ATPase, were up-regulated significantly and peaked at 12 h. The sudden overexpression of the gene encoding crustacean hyperglycaemic hormone (CHH2) during this stage implies a regulatory role in salinity, consistent with previously reported studies (Webster et al. 2012; Tiu et al. 2007) and results from our laboratory (unpublished data). It was interesting to note that genes in the cGMP-PKG signalling pathway, such as Na^+/K^+-ATPase 8 and Na^+/K^+-ATPase, were enriched during this stage. For the ion regulation pathway in *Litopenaeus vannamei*, Na^+/K^+-ATPase is activated by the second messenger cGMP which assistant CHH to regulate ion transporters through cGMP (Li et al. 2017). Thus, CHH2 may be related to the activity of ion transporters in *P. trituberculatus*. Under low salt stress, gill filament cell volume is increased, and biogenic amine synthases such as ornithine decarboxylase are activated to produce biogenic amines (Watts et al. 1996). Biogenic amines stimulate adenylate cyclase, which increases the intracellular concentration of cGMP, a second messenger coupled to protein kinase A (PKA) (Benoit and Debauche 1994) This activates ion transporters such as Na^+/K^+-ATPase through PKA phosphorylation, promotes Na^+ to enter the hemolymph through the basal plasma membrane, and ultimately achieves osmotic balance (Mo et al. 2003). Aquaporin plays an important role in regulating water entry and exit (Kim et al.2010). Overexpression of apoptotic genes (caspases) stimulates apoptosis, hence the high mortality among crabs in the 0–12 h stage.

In the middle stage, 1212 (364 up-regulated and 848 down-regulated) DEGs were identified, 759 fewer than in the early stage. Expression levels of ion transporter genes were significantly lower than in the previous period, suggesting the synthesis of ion transporters was saturated following active regulation. Cellular accommodation is an energetic process that occurs in crab gills following osmotic stress (Engel et al. 1975). Moreover, many of these 364 up-regulated genes are associated with energy metabolism, including cytochrome *c* oxidase, ATP synthase, arginine kinase and chitinase. The activity of Na^+/K^+-ATPase peaked during this stage (Jiang and Xu 2011). Thus, we can speculate that active transport regulates osmotic pressure to facilitate adaptation to changes in external salinity via regulation of energy consumption during this period, which represents a compensatory process. According to previous reports, chitinase has many physiological functions (Söderhäll and Cerenius 1992; Li et al. 2015; Danulat 2010; Zhang et al. 2015). In our study, numerous chitinase genes related to amino sugar and nucleotide sugar metabolism were up-regulated during this stage, such as chitinase and chitinase 5 in particular appear to be related to energy metabolism in *P. trituberculatus*.

In the final stage (48–72 h), only 555 (187 up-regulated and 368 down-regulated) DEGs were identified, most of which regulate active transport through energy metabolism. After a long period of adjustment, the gill filament structure is restored (Shires et al. 1994) and crabs adapted successfully to the low salt environment, hence enzyme activity decreases (Jiang and Xu 2011). However, Na^+/K^+-ATPase remains the main ion transporter, suggesting that crabs require energy to maintain osmotic pressure, even after stabilisation (Engel et al. 1975).

Conclusions

In conclusion, the results of this study provide insight into the mechanisms by which crabs respond and adapt to salinity stress. During the initial passive stress stage, low salt stimulation altered the gill tissue structure, some ion transporters were involved, and the mortality rate was high. During the subsequent active transport stage, regulation by osmotic pressure proceeded mainly through active transport by ion transporters, and mortality declined during this stage. During the final adaptive phase, Na^+/K^+-ATPase remains the main ion transporter, indicating that crabs require energy to maintain osmotic pressure, even after stabilisation, the gill tissue structure was restored, and the mortality rate declined. These results revealed that a substantial number of genes were modified by salinity stress, along with a few important salinity acclimation pathways. The findings will serve as an invaluable resource for revealing the molecular basis of osmoregulation in *P. trituberculatus*. In addition, the comprehensive transcript sequences reported in this study provide a rich resource for identification of novel genes in this and other crab species.

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Compliance with ethical standards

Conflict of interest Baoquan Gao, Dongfang Sun, Jianjian Lv, Xianyun Ren, Ping Liu, Jian Li declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors. The study protocol was approved by the Experimental Animal Ethics Committee, Yellow Sea Research Institute, Chinese Academy of Fishery Sciences, China.

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