Fish and Shellfish Immunology 84 (2019) 885–893

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Identification of immune-related genes in gills of Chinese mitten crabs (*Eriocheir sinensis*) during adaptation to air exposure stress

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ARTICLE INFO

Keywords:
Transcriptome
Eriocheir sinensis
Air exposure
Gill
Immunity

ABSTRACT

The Chinese mitten crab, *Eriocheir sinensis*, is the most important crab in China. Air exposure is regarded as one of the crucial restriction factors in the crab cultivation and transportation process. Numerous studies have shown that air exposure stress can cause many negative effects on aquatic farming animals. However, the molecular mechanisms of drying on Chinese mitten crabs are still poorly studied. In this study, gill reference transcriptome was assembled and differentially expressed gene (DGE) analysis was conducted between air exposure 16 h and normal dissolved oxygen of Chinese mitten crab. A total of 76075 transcripts were generated and 50800 unigenes with a mean length of 1090 bp and N50 length of 1584 bp were observed. Transcriptomic comparison revealed 352 DEGs between air exposure 16 h group and control group, including 122 up-regulated genes and 230 down-regulated genes. Gene ontology (GO) analysis revealed that these DEGs involved in 16 biological process subcategories, 8 cellular component subcategories and 6 molecular function subcategories. Further Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis annotated 352 DEGs to 85 pathways, and some pathways were regarded as related with immune system and diseases, such as phagosome, systemic lupus erythematosus, and alcoholism. Eight genes involved in multiple KEGG signaling pathways were validated by qRT-PCR. This study demonstrates the first gill transcriptomic analysis challenged with air exposure stress in Chinese mitten crab and provides valuable gene resources for understanding the crab gill immunity, which can provides insight into the immune response of crab against air exposure stress.

1. Introduction

The Chinese mitten crab, *Eriocheir sinensis*, is one of the most important aquaculture crustacean species in China. Annual production was more than 800,000 m t., with a value of over 5 billion US dollars in 2014 [1]. Both yield and value were the highest in crab aquaculture in China. However, during the cultivation process, crabs will frequently encounter various physical and chemical challenges, such as air exposure, acidification, hypoxia, and high temperature. Among these, air exposure is regarded as one of the most important restriction factors in the aquatic crustacean cultivation and transportation process [2]. When the water environment has deteriorated or there is a food shortage, crabs will escape from the water and put themselves under the air exposure stress. To deal with the challenge of air exposure, *Holthuisana transversa* forms a lung-like structure. This lung structure is located inside the diverticulum. A large number of blood vessels pass through the bottom to form a damp film that can effectively maintain moisture and exchange gas [3]. However, once Chinese mitten crabs are forced to leave the water, they can only rely on the residual moisture in the lid to obtain dissolved oxygen. If out of water for a long time, most of the water in the gill will evaporate, and as the gill gradually dries the crabs experience expiratory dyspnea. At the same time, crabs face high temperatures, hunger, oxygen deficiency, and irradiation [4], which likely hinder the normal metabolism of crabs and may eventually cause death.

Numerous studies have shown that air exposure stress can cause many negative effects on aquatic farming animals, including reductions in growth, resistance to diseases, and the survival rate of the culture [5,6]. To adapt to air exposure, a series of responses were regulated by immune factors in the body [7]. Compared with vertebrates,

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https://doi.org/10.1016/j.fsi.2018.10.085
Received 14 August 2018; Received in revised form 22 October 2018; Accepted 30 October 2018
Available online 01 November 2018

crustaceans lack an acquired immune system in the true sense. They do not have B or T lymphocytes and cannot be induced to acquire an immune response by complement and antibody systems; thus, they must rely on a very effective non-specific immune mechanism to deal with environmental stresses and invasion of pathogens.

Previous research on immunity of Chinese mitten crabs has mainly focused on several genes, such as SOCS2 [8], CTLs [9], RACK1 [10], EsALF-2 [11], and CatL [12]. Recently, transcriptome sequencing has become an efficient way to evaluate immune responses of aquatic organisms. Different gene expression patterns across tissue types and environmental stressors can be detected using transcriptome analysis. The progress of high-throughput RNA-Seq technology now provides opportunities to explore transcriptome information and the whole gene expression pattern involved in crab immunity [13,14]. Using this sequencing technique, many immune-related genes were identified in several immune-related organs in crabs, including *Portunus trituberculatus* [15], *Scylla paramamosain* [16], and *Carcinus maenas* [17]. However, elucidation of the molecular mechanisms against air exposure in Chinese mitten crabs is still lacking. The gill is an important organ involved in crustacean innate immunity because it is directly exposed to the external aqueous environment that contains a large microbial biomass. To the best of our knowledge, there is only a study that uses the RNA-seq method to illustrate the differential gene expression profiles in *P. trituberculatus* gills during aerial exposure [18]. In this study, we represented the transcriptional profiles of gills in Chinese mitten crabs following an air exposure challenge. RNA-Seq was performed using Illumina Hiseq platform. Differential expressed genes (DEGs) were further analyzed by Gene Ontology (GO) functional enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Furthermore, eight genes that play important roles in response to air exposure challenge were validated by qRT-PCR. These results will provide a meaningful gene resource for further understanding of the molecular mechanism following air exposure of Chinese mitten crabs and other invertebrates.

2. Materials and methods

2.1. Air exposure

Healthy farmed Chinese mitten crabs were obtained from Guanghe Crab Industry Co. Ltd in Panjin, Liaoning Province, China, and were transported to the laboratory at the Shenyang Agricultural University. In the laboratory, Chinese mitten crabs were distributed into 300 L aquariums. Constant aeration was provided by air pump to ensure the oxygen content was not lower than 6 mg L^{-1}. The pH was 7.0–7.2, temperature was 17 °C, and the natural photoperiod (12 h:12 h light/darkness) was used. Chinese mitten crabs were fed the artificial feed, which was offered twice per day. Leftovers were removed 2 h after feeding. Water was renewed at 100% per day. Over a 2-week acclimation period, the crabs suffered from air exposure after starvation for 24 h. Twenty crabs were placed in a glass tank (50 cm × 35 cm × 20 cm) and the experiment had 4 replications. Mean crab weight was 3.7 ± 0.3 g. The water temperature was 17 °C. They were inspected for deaths every 2 h, and the observation interval was shortened to half hour after a dead individual was found. The criterium for crab death was no response after being touched by tweezers. The time and the number of crab deaths were accurately recorded.

2.2. Experiment settings

According to the results obtained from the air exposure experiment, the first dead individual appeared at 20 h. Thus, 16 h was selected as the longest air exposure time. We also verified that the crabs were in good condition after 16 h of air exposure and then returned them to the water. Ten crabs were placed in an empty glass tank (50 cm × 35 cm × 20 cm) for air exposure, ten crabs remained in water as the control, and the experiment was replicated 3 times. After 16 h air exposure, five crabs per tank were randomly sampled. The gill tissue was sampled and frozen immediately in liquid nitrogen, and then transferred into a freezer at −80 °C. The samples were used for RNA extraction and transcriptome sequencing.

2.3. RNA quantification and qualification

RNA concentration was measured using the NanoDrop 2000 (Thermo). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

2.3.1. Library preparation for transcriptome sequencing

A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's recommendations and index codes were added to attribute sequences in each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis ReactionBuffer (5×). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase. Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3′ ends of DNA fragments, NEBNext Adaptor with hairpin loop structures were ligated to prepare for hybridization. To select cDNA fragments of preferentially 240 bp in length, the library fragments were purified with the AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Then, PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers, and Index (X) Primer. Finally, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

2.3.2. Clustering and sequencing

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using a TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq 2000 platform and paired-end reads were generated.

2.3.3. Quality control

Raw data (raw reads) of fastq format were firstly processed through in-house perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N, and low-quality reads from the raw data. At the same time, Q20, Q30, GC-content, and sequence duplication levels of the clean data were calculated. All the downstream analyses were based on clean data with high quality.

2.3.4. Transcriptome assembly

Trinity was used for transcriptome assembly with a min_kmer_cov set to 2 by default and all other parameters set by default [19].

2.3.5. Gene functional annotation

Gene function was annotated based on the following databases: NR (NCBI non-redundant protein sequences); Pfam (Protein family); KOG/COG/eggNOG (Clusters of Orthologous Groups of proteins); Swiss-Prot (a manually annotated and reviewed protein sequence database); KEGG (Kyoto Encyclopedia of Genes and Genomes); and GO (Gene Ontology).

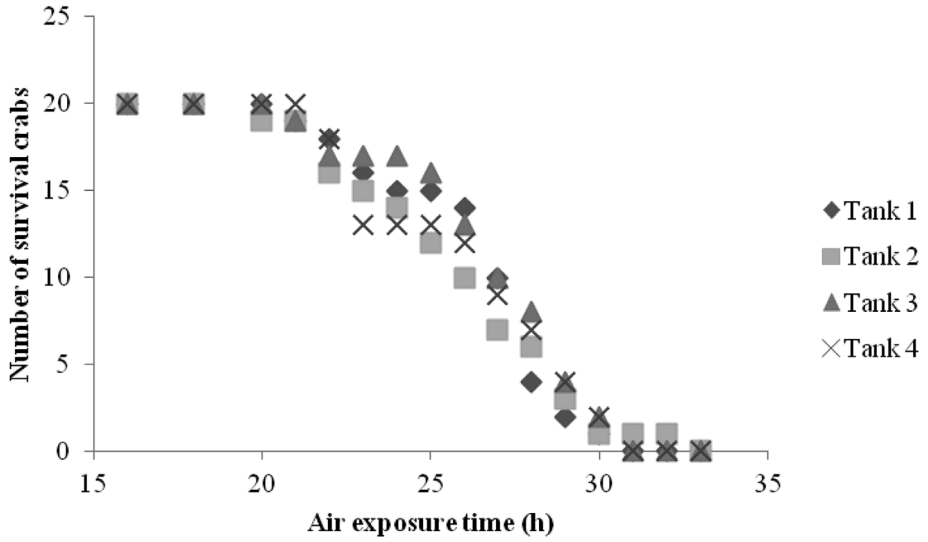

Fig. 1. The number of survival Chinese mitten crabs under different air exposure times.

2.3.6. Quantification of gene expression levels

Gene expression levels were estimated by RSEM for each sample [20]. Clean data were mapped back onto the assembled transcriptome. Readcount for each gene was obtained from the mapping results.

2.3.7. Differential expression analysis

Differential expression analysis of two conditions/groups was performed using the DESeq R package (1.10.1). DESeq provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate [21]. Genes with an adjusted P-value < 0.05 found by DESeq were assigned as differentially expressed.

2.3.8. GO enrichment analysis

Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) was implemented by the topGO R package based on the Kolmogorov–Smirnov test.

2.3.9. KEGG pathway enrichment analysis

KEGG is a database resource for understanding high-level functions and utilities of the biological system [22], such as the cell, the organism, and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/). We used KOBAS software to test the statistical enrichment of DEGs in KEGG pathways [23].

2.4. Identification and analysis of DEGs

FRKM (fragments per kilobase of transcripts per million fragments mapped) was used as the unit of measurement to estimate the expression level of each transcript. False discovery rate (FDR) was conducted to correct for the E-value. Genes with FDR ≤0.05 and an FPKM ratio larger than 2 or smaller than 0.5 were considered DEGs between samples. With the DEGs, GO, and KEGG pathway classifications, functional enrichments were also performed, as mentioned in section 2.3.8 and 2.3.9.

2.5. Confirmation using qRT-PCR

qRT-PCR was used to validate the expression levels of DEGs by Illumina sequencing. Specific primers for qRT-PCR were designed using Premier primer 5 and the sequences are listed in Supplementary Table 1. Stable expression of gene screened from transcriptome project was used as a reference gene. The cDNA used as a template for qRT-PCR was synthesized using the *TransScript* II All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (One-Step gDNA Removal) (TransGen Biotech, China) following the manufacturer's instructions. qRT-PCR was performed using a *TransStart* Top Green qPCR SuperMix kit (TransGen Biotech, China). Each reaction was performed in triplicate, and the following conditions were used: 94 °C for 2 min followed by 45 cycles of 94 °C for 5 s, 60 °C for 15 s, and 72 °C for 10 s. A 20 μl reaction mixture contained 10 μl 2 × 2 × *TransStart* Top Green qPCR SuperMix, 0.8 μl forward and reverse primers, 2 μl cDNA, and 7.2 μl RNase-free H_2O. The melting curve was analyzed to detect single amplification and the relative gene expression level was analyzed using the $2^{-\triangle\triangle CT}$ method [24]. Means ± standard errors (standard error of the mean, SEM) were used to report the data.

3. Results

3.1. Air exposure

The results showed that the vitality of the Chinese mitten crab decreased gradually with prolonged air exposure time, and death and an individual appeared at 20 h air exposure. The time of a 50% mortality rate was 26.5 h, and all of the crabs were dead at 32.5 h (Fig. 1).

In this experiment, the first death occurred at 20 h; thus, 16 h air exposure time was selected. Furthermore, the crab was in good condition after air exposure for 16 h then returned to the water.

3.2. Assembly and splicing

A total of 20.68 million clean reads in the control group and 20.82 clean reads from the air exposure group were obtained. The Q30 was higher than 90.96%, and the G/C content was approximately 45–49% (Table 1). A total of 76075 transcripts were generated by Trinity software with average lengths of 1075 bp and N50 lengths of 1630 bp. Furthermore, 50800 unigenes with a mean length of 1090 bp and N50 length of 1584 bp were observed. Among these unigenes, 19,244 (37.88%) were within the range of 300–500 bp, 14,502 (28.55%) were within 500–1 kbp, 9747 (19.19%) were within 1–2 kbp, and 6587 (12.97%) were longer than 2 kbp. The length distribution of all the unigenes were showed in Fig. 2. These results demonstrated that the data obtained from transcriptome sequencing were high-quality, and the unigenes could be used for subsequent annotation analysis.

Table 1
Sequencing data output statistics.

Samples	Read Number	Base Number	GC Content	%≥Q30	Clean Reads	Mapped Reads	Mapped Ratio
C1	22,487,746	6,713,331,432	45.58%	91.48%	22,487,746	14,774,171	65.70%
C2	22,226,863	6,628,365,148	49.02%	90.96%	22,226,863	14,394,200	64.76%
C3	24,594,218	7,339,124,496	47.44%	91.09%	24,594,218	16,515,988	67.15%
E1	21,622,461	6,451,868,834	46.83%	91.34%	21,622,461	14,134,252	65.37%
E2	24,327,438	7,254,867,410	48.22%	91.27%	24,327,438	15,299,310	62.89%
E3	23,874,781	7,123,476,102	47.46%	91.36%	23,874,781	15,481,248	64.84%

A Venn diagram of gene expression was mapped to display the number of shared and exclusively expressed genes between the two groups. There had 16,327 genes shared, 1569 genes were exclusive to the control group, and 1858 genes were present only in the air exposure group (Fig. 3).

3.3. Identification and analysis of differentially expressed genes

According to the results of statistical analysis, a total of 352 genes were found to be differentially expressed after air exposure (Supplementary Table 2), including 122 up-regulated and 230 down-regulated genes in the air exposure group. The distribution of DEGs and non-DEGs are shown in Fig. 4. In the air exposure group, down-regulated genes, such as Catalase (CAT), Cathepsin L (CatL), and HSP90, and up-regulated genes, such as Peroxinectin, HSP70, and HSP20, were associated with immune response. Furthermore, some genes in 352 DEGs were relative to energy production and conversion, material transport and metabolism. In these genes, Fumarate hydratase (FH), Succinyl-CoA ligase (SUCL), Pyruvate dehydrogenase E1 component subunit beta (PDHE1B) and Isocitrate dehydrogenase (ICDH) were down-regulated genes; FAD-dependent oxidoreductase (FOXRE), Glycogen synthase kinase 3 binding (GSK-3B), Inositol polyphosphate kinase (IPK) and Amino acid permease (AAP) were up-regulated genes in the air exposure group.

3.4. Unigene functional annotation and classification

After assembly, 8 functional databases (COG, GO, Swissprot, KEGG, KOG, NR, eggNOG, and Pfam) for unigenes were used to perform functional annotation; the annotation summary is shown in Supplementary Table 3. Out of 50800 unigenes, 22136 unigenes (43.57%) were annotated in at least one database. The number of unigenes with a significant similarity to sequences in COG, GO, Swissprot, KEGG, KOG, NR, eggNOG, and Pfam databases were 8264 (16.26%), 6900 (13.58%), 12190 (24.00%), 11328 (22.30%), 14406 (28.36%), 20097 (39.56%), 19904 (39.18%), and 17725 (34.89%), respectively (Table 2).

The GO functional enrichment analyses of 352 DEGs were classified into three categories: biological process (BP), cellular component (CC), and molecular function (MF). BP was the most prevalent, followed by CC and MF. In this study, BP contains 16 subcategories, and CC and MF each contain 8 and 6 subcategories, respectively (Fig. 5). Among the various categories of BP, the top three go terms were regulation of transcription, cellular response to extracellular stimulus, nucleosome assembly. Within the CC category, integral component of membrane, nucleus and ribosome were the top three go terms. In the MF category,

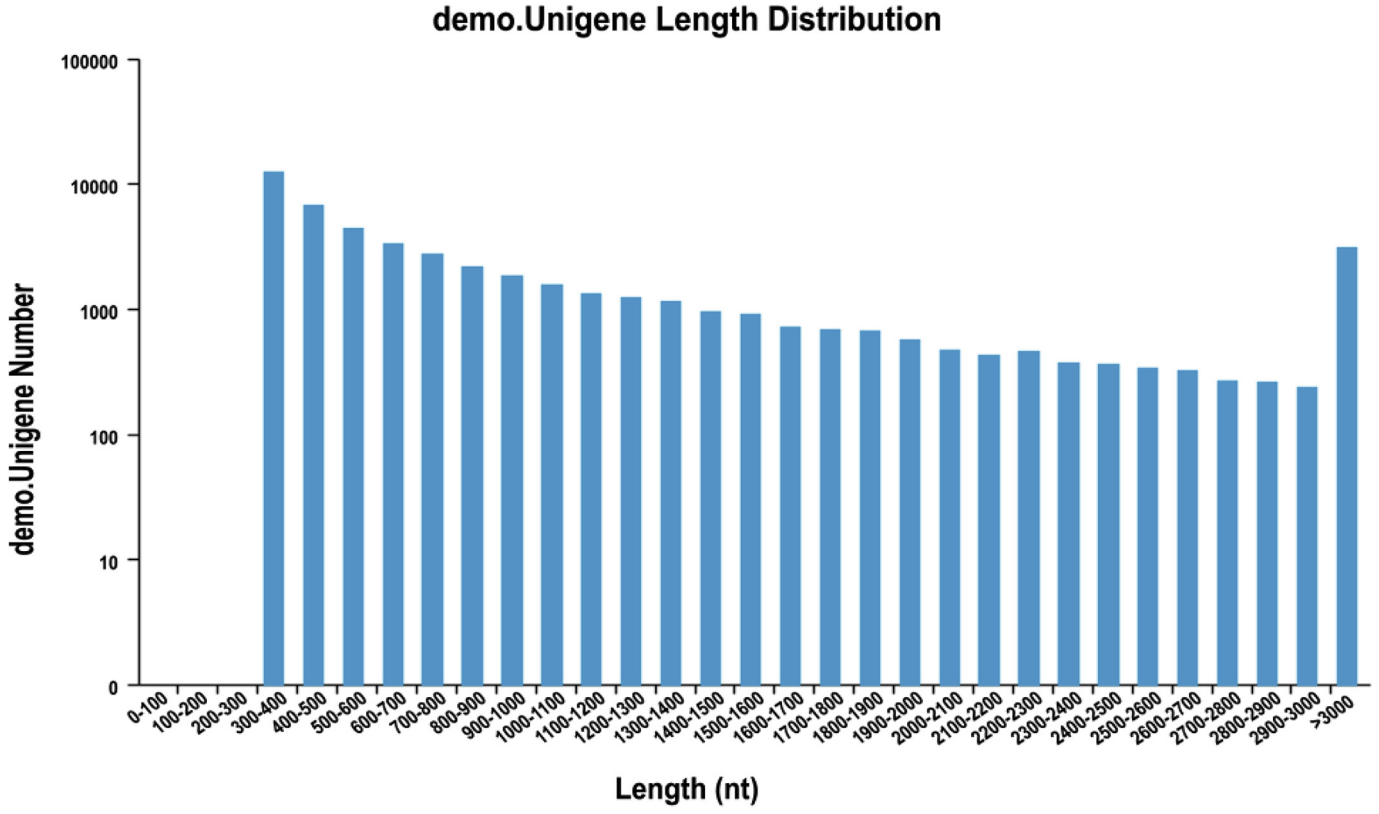

Fig. 2. Length distribution of all the unigenes.

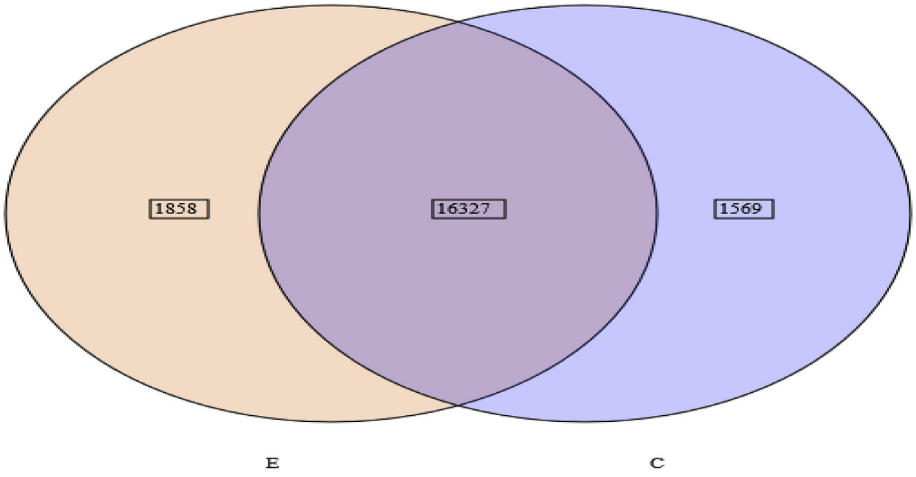

Fig. 3. The number of differentially expressed genes (DEGs) between control group and air exposure group showed in Venn diagram. Abbreviations: C, control group; E, air exposure group.

zinc ion binding, sequence-specific DNA binding transcription and protein heterodimerization activity constituted the top three go terms.

KEGG functional enrichment analysis was identified based on the 352 DEGs. The top 20 KEGG pathways are shown in Fig. 6 and all of the pathways are shown in Supplementary Table 4. Among them, phagosome was related to immune responses. Ribosome was associated with genetic information processing. Systemic lupus erythematosus and alcoholism were associated with diseases.

3.5. Validation of DEGs using qRT-PCR

To validate the DEGs by Illumina sequencing, eight DEGs were randomly selected based on up-regulated, down-regulated and biological functions. Four genes, including CatL, HSP90, CAT, Pro-resilin, were down-regulated in air exposure group, and four genes, including Tyrosine-protein phosphatase vhp-1 (TPP), Facilitated trehalose transporter (Tret1), Oxidoreductase, and $Na^+/K^+/2Cl^-$ cotransporter (Cotransporter) were up-regulated in air exposure group. There were four genes correlated with immune, including CatL, HSP90, CAT and TPP. Three genes were correlated with material absorption and metabolism, among them, Tret 1 was related to carbohydrate transport and metabolism, Oxidoreductase and Cotransporter were related to amino acid transport and metabolism. Pro-resilin was known to be responsible for the generation of deformability and flexibility in arthropoda. The DEGs and primers for qRT-PCR analysis are listed in Supplementary Table 1. The $\log_2$ (fold change) of each gene value are seen in Fig. 7 (qRT-PCR vs RNA-seq). In general, the expression trends in the qRT-PCR were consistent with the sequencing results, indicating that results were reliable. The DEGs and primers for qRT-PCR analysis are listed in Supplementary Table 1. The $\log_2$ (fold change) of each gene value are seen in Fig. 7 (qRT-PCR vs RNA-seq). In general, the expression trends in the qRT-PCR were consistent with the sequencing results, indicating that results were reliable.

4. Discussion

In this study, when crabs were stressed by air exposure for 16 h, a total of 50080 unigenes and 352 DEGs were obtained. These DEGs were enriched to 85 metabolic pathways and demonstrated involvement in biological functions, such as phagosomes, ribosome, systemic lupus erythematosus, alcoholism, suggesting that complicated molecular mechanisms were involved in air exposure. Meanwhile, many immune-related genes were also identified in this study.

The HSP family plays an important role in protein–protein

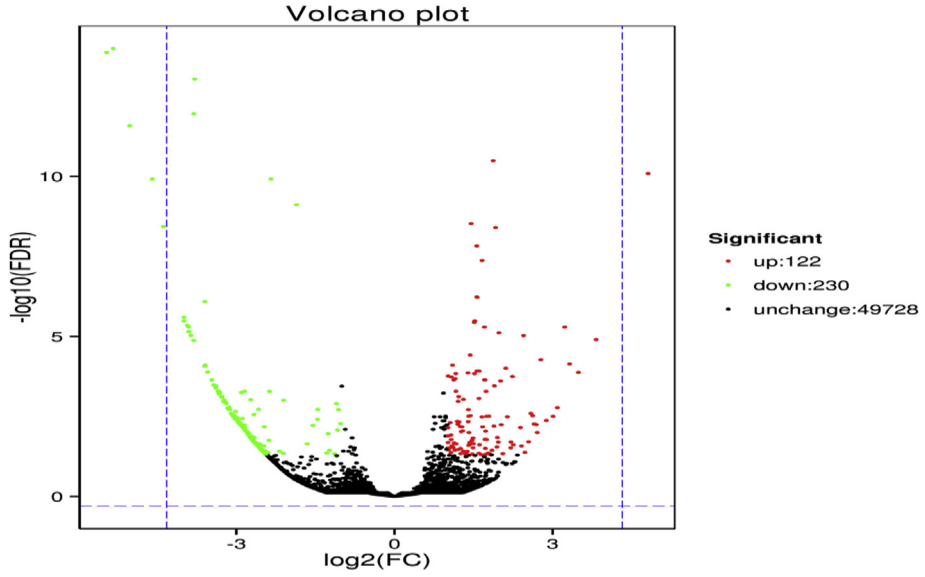

Fig. 4. Volcano plot of differently expressed gene distribution trends between the control group and air exposure group.

Table 2
Summary statistics of Chinese mitten crab transcriptome annotation.

	Annotated_Number	Percentage (%)	300 ≤ length < 1000	length ≥ 1000
COG_Annotation	8264	16.26	3998	4266
GO_Annotation	6900	13.58	3516	3384
KEGG_Annotation	11328	22.30	5057	6271
KOG_Annotation	14406	28.36	6426	7980
Pfam_Annotation	17725	34.89	7527	10198
Swissprot_Annotation	12190	24.00	5278	6912
eggNOG_Annotation	19094	39.18	9202	9892
NR_Annotation	20097	39.56	9602	10495
All_Annotated	22136	43.57	11324	10812
Total unigenes	50800	100		

interactions and is named according to their molecular weight in kilodaltons (kDa). In crabs, there is evidence that the HSPs are highly changed in response to pathogen infection [25,26]. HSP90, as one of crucial heat-related proteins in bacteria and all branches of eukaryote, has important function in assisting folding, intracellular transport, maintenance, and degradation of proteins as well as facilitating cell signaling [27,28]. HSP90 was reported to be involved in immune response and stress in crabs [29,30]. For example, the HSP90 mRNA level was significantly up-regulated in the hepatopancreas and gills of *P. trituberculatus* after cold treatment [29]. In one recent report, researchers found that the expression patterns of HSP90 in *S. paramamosain* were enhanced after challenged by the *Staphylococcus aureus*, *Vibrio harveyi,* and white spot syndrome virus (WSSV) [30]. In this study, HSP90 was also significantly changed after air exposure, further illustrating that HSP90 plays a crucial role in crustacean innate immunity. However, HSP90 was significantly down-regulated after air exposure. Therefore, its detailed function should be elucidated by further research. HSP70 may exert protective effects in the immune system by contributing to the processing and presentation of bacterial and tumoral antigens [31]. HSP20 in *Babesia bovis* was identified as an immunostimulatory antigen present in low-molecular-weight protein fractions of merozoites that induced proliferation and the likelihood of inducing a cross-reactive immune response [32]. In this study, HSP70 and HSP20 were significantly up-regulated after air exposure, indicating that both of them were involved in the crab immune response.

Phagosomes, which are vesicles formed around a particle engulfed during the phagocytic process, were also significantly changed after environmental stressed, as was reported by Ramachandra et al. [33]. In our transcriptome data, unigenes annotated as phagosomes exhibited significant change after air exposure, including cathepsin L (CatL), actin, tubulin beta chain, and tubulin alpha chain. CatL is believed to degrade proteins in the lysosome of vertebrates and is also crucial for immune response [34,35]. To date, little information has been obtained regarding its function in crustacean innate immunity. In a previous study, researchers found that CatL was up-regulated in WSSV-resistant *F. chinensis* [36]. CatL mRNA expression in Chinese mitten crabs was tissue-specific and responsive to hemocytes in a *V. anguillarum* challenge, after 8 h post *V. anguillarum* challenge, the CatL expression level was the highest [37]. In this study, CatL was also significantly changed after air exposure, further illustrating that CatL plays a crucial role in crustacean innate immunity. Different with pathogenic infection, CatL was significantly down-regulated after air exposure. Therefore, its detailed function should be elucidated after environmental stress. As cytoskeletals, actin and tubulin expression are unstable during

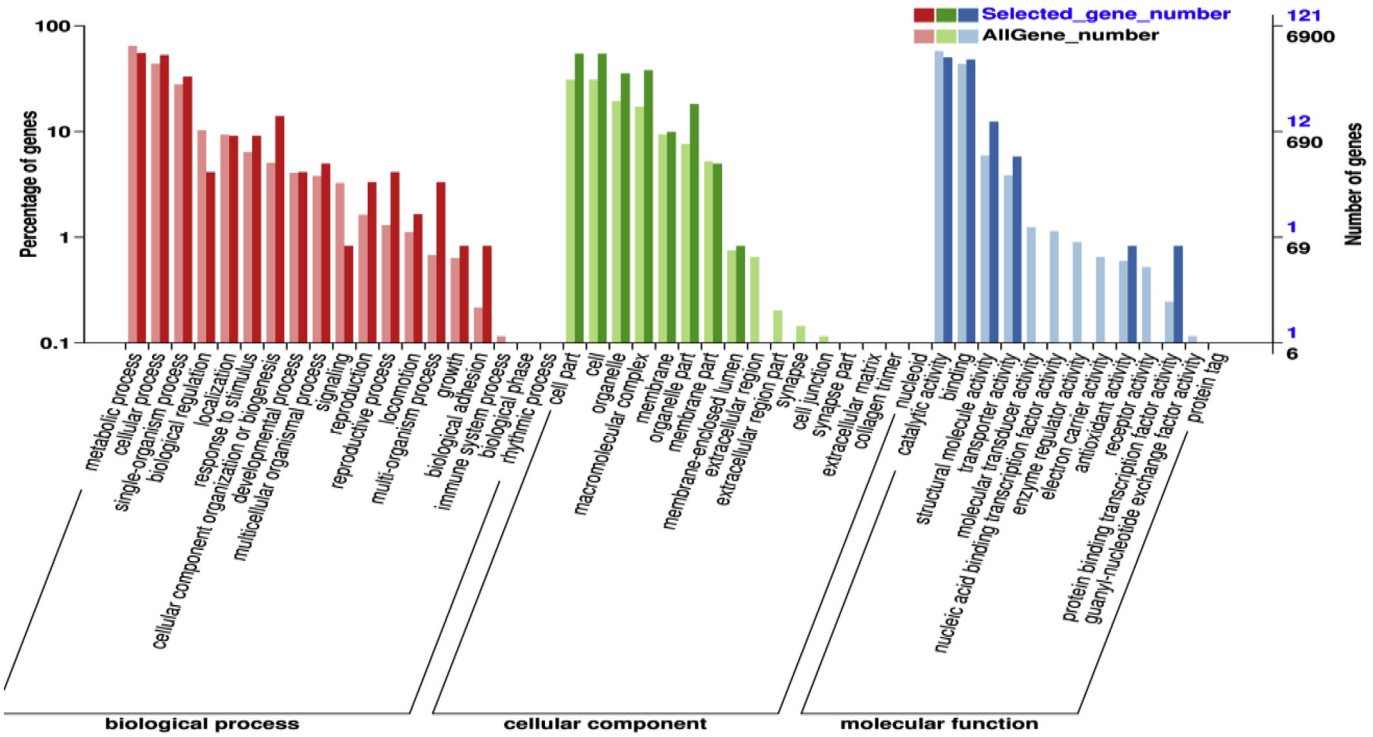

Fig. 5. Histogram description of Gene Ontology enrichment of DEGs. All the DEGs are classified into three categories: biological processes (BP), cellular components (CC), or molecular functions (MF). The X-axis represents various gene functions and the Y-axis corresponds to the number of DEGs.

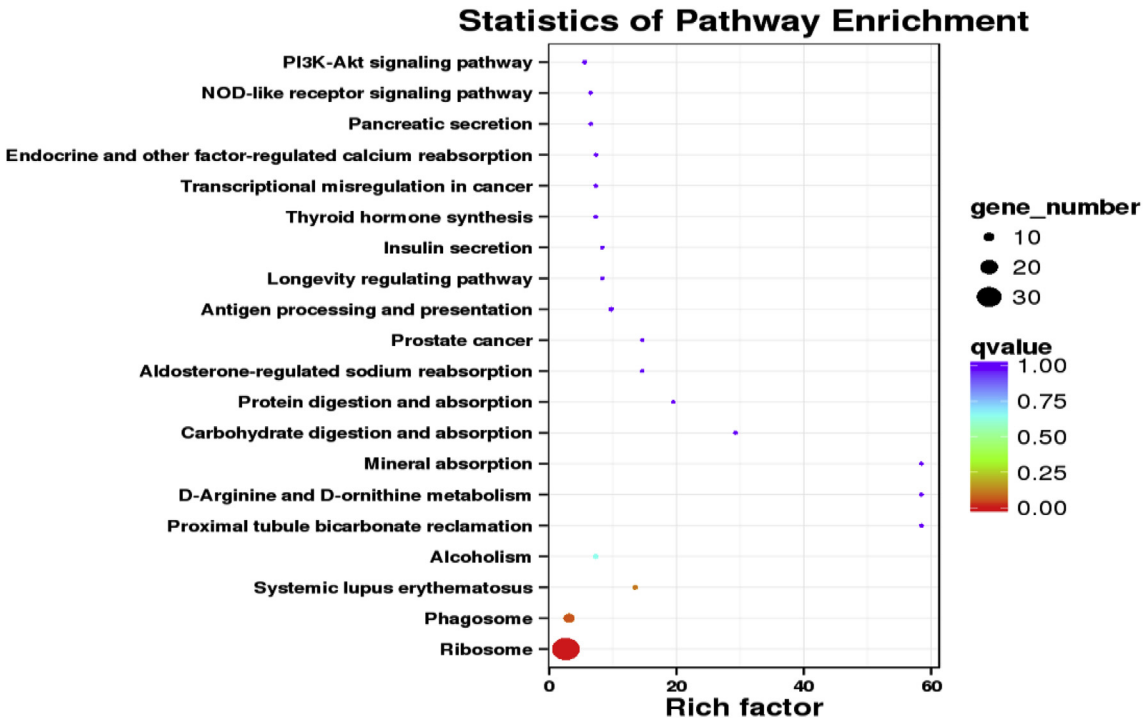

Fig. 6. Scatter diagram of pathway enrichment for DEGs following air exposure. In this scatter diagram, the top 20 pathways are listed. The rich factor is the ratio of DEGs in this pathway relative to all the genes in this pathway. The X-axis represents the rich factor of pathway, and the Y-axis corresponds to different pathways. The magnitude of the dots displays gene number, which ranged from 10 to 30, and the q-value is described by the color classification. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

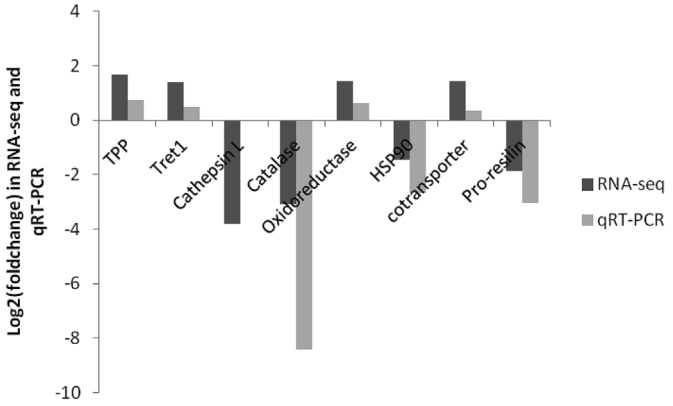

Fig. 7. Comparison of the expression profiles of 8 selected genes as determined by Hiseq2000 sequencing and qRT-PCR. Gene abbreviations are as follows: TPP, tyrosine-protein phosphatase vhp-1; Tret1, Facilitated trehalose transporter; CatL, Cathepsin L; CAT, Catalase; HSP90, Heat shock protein 90; Cotransporter, $Na^+/K^+/2Cl^-$ cotransporter.

environmental change and they may be involved in the immune defense process in crustacean. In *Litopenaeus vannamei*, expressions of three actin genes were significantly down-regulated after WSSV infection [38]. Similarly, actin genes were significantly down-regulated in the hemolymph of *S. serrate* [39] and the lymphoid organ of *F. chinensis* after *V. anguillarum* infection [40]. In this study, actin genes were also significantly down-regulated in the gills of Chinese mitten crabs after air exposure, which indicated that actin is involved in the immune defense of Chinese mitten crabs. Tubulins were significantly down-regulated in the gills of Chinese mitten crabs after air exposure. This was consistent with the research of Li et al. [41]. He found that the level of the alpha-tubulin-like gene (EsTUBA) mRNA expression in the heart, intestine, and gill was down-regulated after *V. parahaemolyticus* challenge. These results suggest that tubulin may be involved in immune defense of crabs. On the contrary, a previous study has shown the enhancement in the level of α-tubulin in influenza a virus-infected cells [42]. The reason may be that transcriptional expression of tubulin showed a clear time-dependent response [41]. Tubulin expression significantly decreased after air exposure for 16 h, but whether it had an increasing trend after short-term air exposure and specific roles in immune defense are still unclear.

Research has confirmed that peroxinectin possesses the features of both peroxidase activity and adhesive property and plays important roles in the innate immune system of crustaceans. Lv et al. [43] found that the expression of peroxinectin in Chinese mitten crabs was susceptible to exterior stimulation, and that the highly expressional peroxinectin would be released into the extracellular matrix by the stimulation of LPS and beads. A similar result was observed in *L. vannamei* when the expression level of the peroxinectin gene was significantly up-regulated after injection of *V. alginolyticus* [44]. Kobayashi et al. [45] thought that the up-regulation of peroxinectin was associated with enhanced encapsulation and phagocytic activity to boost pathogen resistance. Likewise, the expression of peroxinectin in the air exposure group was 3.27 times higher than that of the control group. This study further confirmed that peroxinectin was an important immune gene that responds to environmental stress and microbial invasion in crustaceans. Catalase (CAT) can effectively eliminate H_2O_2 and maintain the redox balance of the immune system, which is essential for innate immunity. The relative expression level of CAT mRNA in hemocytes was continuously up-regulated and reached the peak level at 48 h post *V. anguillarum* challenge in Chinese mitten crabs [46]. However, we found that the relative expression level of CAT mRNA in gills was down-regulated in Chinese mitten crabs after being challenged with air exposure. All these results demonstrate that CAT is an efficient antioxidant enzyme and potentially involved in the regulation of redox and innate immune responses of crabs. Meanwhile, this study indicated that the long air exposure had a negative influence on the immune system of Chinese mitten crabs. Recent research showed that serine protease

inhibitor (Serpin) was relevant to the crustacean immune responses [47]. The expression pattern of serpin was significantly up-regulated after challenged with WSSV in *F. chinensis* and challenged by *V. anguillarum* and *Pichia pastoris* in Chinese mitten crabs [48,49]. Likewise, the expression of serpin following air exposure group was 2.17 times higher than that of the control group. This study further confirmed that serpin was an important immune gene that answers to environmental stress and further functional studies are needed for this to be clarified. The clip-domain serine proteinase was also significantly up-regulated after being challenged with air exposure. This was consistent with the research of Jia et al. [50]. He found that the clip domain serine proteinase was up-regulated after *V. anguillarum* and *P. pastoris* stimulations in Chinese mitten crab. These results collectively suggest that clip domain serine proteinase plays important roles in immune defense against bacterial infection and environmental stress in crabs.

As crabs lack an acquired immune system, their defense depends entirely on an innate, non-adaptive mechanism to defend against environmental stress and invasion of pathogens [51]. Aberrant mechanistic target of rapamycin inhibition (mTOR) signaling is involved in many diseases, including tumorigenesis, prostate cancer, and Alzheimer's disease [52]. Inhibiting the mTOR signaling pathway with rapamycin has been shown to lead to suppression of induced autophagy and correlative regulation in *L. vannamei* [53]. In the present study, many genes involved in the mTOR signaling pathway were found to undergo expression changes in the gills following air exposure. For instance, PTKs (protein tyrosine kinases), RpS6 (40 S ribosomal protein S6), Vha-11(V-type proton ATPase subunit C), and VAPB (V-type proton ATPase subunit B) were down regulated significantly, hence indicating that they might play an important role in response to air exposure. However, the underlying molecular mechanism also remains unclear. In mammals, PTKs has been verified as immune targets of viruses [54]. However, the immune function of PTKs in crustaceans is still unknown. This study is the first report showing the changes in the PTK levels after air exposure stress in crabs. Meanwhile, the functions of other genes involved in the mTOR pathway in innate immunity of crustaceans need further study.

In this study, some genes involved in material transport and metabolism, such as FOXRE, GSK-3B, IPK and AAP were up-regulated in the air exposure group. These genes were conducive to resisting oxidative stress [55–58]. Meanwhile, some genes were significantly down-regulated in the air exposure group, especially involved in citrate cycle (TCA) pathway. TCA is the most crucial central pathway in aerobic organisms and is the ultimate metabolic pathway of carbohydrate, lipid and protein. About 2/3 of the total energy is produced by TCA [59]. Under stress conditions, the organism will increase the energy demand to meet metabolic consumption. In the present study, many genes involved in this pathway, including FH, SUCL, PDHE1B and ICDH, were down-regulated after air exposure, suggesting that long time air exposure will cause severely damage in Chinese mitten crab because of energy deficiency due to suppressed TCA pathway [60–63].

5. Conclusions

In this study, transcriptome sequencing libraries were constructed with the gills of Chinese mitten crabs following the immune response to air exposure. Most genes were induced compared to controls as measured by qRT-PCR. Some genes observed here are reported for the first time in the Chinese mitten crab. The results revealed that various processes were involved in the crab immune response and provided a better understanding of the immune defense to air exposure stress, proved that long duration air exposure had negative effects on immunity of crustaceans, and provided insights into the potential immune mechanisms of Chinese mitten crabs.

Conflicts of interest

The authors declare no competing interests.

Acknowledgements

This work was supported by the earmarked fund for Modern Agro-industry Technology Research System (CARS-48), Natural Science Foundation of Liaoning Province of China (201602654), Startup Foundation for Doctors of Liaoning Province (201601103).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.10.085.

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