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RESEARCH PAPER

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Comparative Transcriptome Analysis of the Gills from the Chinese Mitten Crab (*Eriocheir japonica sinensis*) Exposed to the Heavy Metal Cadmium

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Abstract

Heavy metal Cadmium (Cd) pollution has become a potential environmental problem, and it threatens aquatic organisms including economic crabs and shrimps. Chinese mitten crab is a native species in East Asia, and it is the most important economic crab species in China. However, there are only a few studies about gills responding to Cd stress in the Chinese mitten crab at a molecular level. To gain better understanding of the oxidative stress system (antioxidant defense) of the *E. j. sinensis*, in this study, RNA-Seq was applied to obtain gene expression profile from the mitten crab's gills in response to Cd for 24h, and yielding 39,612,948 high-quality clean reads from which 570,847 transcripts and 504,889 unigenes are assembled by removing redundancy and transcripts with low coverage. A total of 1544 differential expression genes (DGEs) are identified. GO functional analysis and KEGG pathway analysis indicated some vital pathways and genes in response to the challenge. Four genes related to immune response and detoxification are selected to validate the DGE results by qRT-PCR. This comparative transcriptome provides valuable molecular information about on the mechanisms of the Chinese mitten crab responding to Cd that lays the foundation for further understanding of heavy metals stress.

Introduction

The Chinese mitten crab (*Eriocheir japonica sinensis*) is a widely-distributed group in streams and rivers along the eastern pacific coast of China to the Korean Peninsula (Wang *et al*., 2018; Xiong *et al*., 2001). The Chinese mitten crab has abundant nutrition and delicate taste, making it becomes a common food today and has high market demand and price in China (Li *et al*., 2018; Wu *et al*., 2007). The annual production of *E. j. sinensis* has increased sharply during the past two decades which is approximately 812,103 tons in 2016 (China Fisheries Yearbook, 2017). Thus, *E. j. sinensis* has become one of the most prevalent and commercially important aquaculture crabs in China. In addition, *E. j. sinensis* always was considered as a model organism in aquaculture studies (Cui *et al.*, 2013).

Over the past few decades, with the accelerating industrialization and anthropogenic activities, more and more waters have been polluted by high levels of heavy metals (Wang *et al*., 2015). As a consequence, heavy metal pollution has become a serious environmental problem, producing deleterious impacts on the ecosystem. Particularly, it threatens aquatic organisms including economic crabs and shrimps (Fu and Qi 2011). As one of the most hazardous heavy metals, Cadmium (Cd) is a non-essential and potentially toxic metal because it cannot be degraded in bioaccumulation and can be accumulated in humans via food chain (Gaspic *et al*., 2002), which would may result in morphological deformities, physiological dysfunctions and even death (Sun *et al*., 2016). Cd can cause the production and accumulation of reactive oxygen species (ROS) which is known to induce oxidization of biological

macromolecules and variously physiological damage to animal tissues and organs (Thévenod 2009). Furthermore, Cd also generates DNA strand breaks and gene inactivation (Manna and Sil 2009), and alters gene expression to regulate activities of various enzymes (Overstreet 2000). Previous studies showed that effects of Cd exposure in many crustaceans which including *Sinopotamon henanense* (Sun *et al*., 2016), *Macrobrachium rosenbergii* (HAKaoud and ARezk 2011), and *Gammarus fossarum* (Silvestre *et al*., 2006). However, up to date, the research about *E. j. sinensis* is limited on the knowledge about the molecular basis of responding to Cd stress.

In crustaceans, the gills are multifunctional and generally regarded as a major organ of respiration (Burnett and Mcmahon 1985) and osmoregulation (Hu *et al*., 2015; Weihrauch *et al*., 2001). Additionally, the gills also play a critical role in xenobiotics entering organisms, material absorption and biological transformation because it associates with ambient environment directly (Wang *et al*., 2013). Previous studies have reported *E. j. sinensis* are threatened by Cd, but mostly focused on tissue-specific cadmium accumulation (Silvestre *et al*., 2005; Wang *et al*., 2001). Silvestre *et al*., (2005) found that the anterior gill in *E. j. sinensis* has highest level of cadmium accumulation, followed by the hepatopancreas and posterior gill. However, the studies on the molecular responses of differentially expressed genes are scarce in Chinese mitten crab gills exposed to Cd.

In recent years, with the development of next-generation sequencing, *de novo* transcriptomic approaches have been increasingly used to investigate physiological process and molecular characteristics of model and non-model organisms (Lou *et al*., 2017). Additionally, *de novo* transcriptome provides great breadth and depth of information that can be used to catalog all the genes expressed in the tissue, and facilitates detailed functional research of various proteins (Bain *et al*., 2016). To get better understanding about the molecular basis responses to Cd stress in *E. j. sinensis*, we used the comparative transcriptome of the gills to analyze the differentially expressed genes (DEGs) between exposed and unexposed to Cd by using Illumina sequencing platform and quantitative real-time PCR (RT-qPCR). The goal of this study is to improve a better understanding of molecular responses of *E. j. sinensis* when exposed to Cd and to provide a theoretical basis for the further researches on heavy metals stress.

Materials and Methods

Animals and Exposures

E. j. sinensis were purchased from Yangguang market in Yancheng, Jiangsu Province, China, on October 18th, 2017. Before experiments, the crabs were acclimated 2 days. Crabs were randomly divided to the treated group and the control group. The treated group of three crabs was exposed to Cd for 24 hours at a concentration of 1mg/L prepared by dissolving 2.036 mg of $CdCl_2 \cdot 2.5\ H_2O$ in 1L of clean water (without Cd). The control group of three crabs was maintained under clean water for 24 hours at 20 ± 0.5°C. The crabs of each group were settled at tanks (36 cm × 20 cm × 18 cm) which contain one third water. During the whole processes, crabs were not fed. Then the gills samples of the *E. j. sinensis* from each group were rapidly removed, snap-frozen in liquid nitrogen, placed into 1.5 ml RNase-free tubes and immediately stored at -80 °C prior to the RNA extraction, respectively.

RNA Extraction, cDNA Library Preparation and Illumina Sequencing

Total RNA was isolated from the composite sample using a standard TRIzol Reagent Kit according to the manufacturer's instructions. RNA purity was confirmed spectrophotometrically using a NanoDrop 2000 and integrity were assessed using an Agilent 2100 Bioanalyser (Agilent Technologies, USA). Briefly, mRNA was purified using oligo (dT) magnetic beads. Purified mRNA was fragmented into smaller fragments (about 200 bp) by using a fragmentation buffer. Then, random-hexamer primers and reverse transcriptase were used to synthesize the first-strand. Next, Second-strand cDNA synthesis was subsequently performed as a stable double-stranded structure, after which end repair and addition of a base at 3’ ends were conducted. The suitable fragments were purified by 2% agarose gel electrophoresis and enriched by PCR amplification for 15 cycles to create a cDNA library. Finally, the library was conducted by Illumina HiSeq™ 2000 platform (Illumina Inc.).

Assembly and Annotation

In this study, the SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickles) software were used to filter raw reads in FASTQ format by removing reads with sequencing adaptors, low quality (quality scores ≤ 20) and unknown nucleotides (N ratio > 10%). Following quality filtering, all downstream analyses were based on clean, high-quality data, and then clean reads were *de novo* assembled using Trinity software package (Haas *et al*., 2013). Longest transcripts of each gene were pooled as “unigenes”.

To search for the likely homologues of proteins with known function, all the unigenes matched with sequences in the BLAST against the Non-redundant (NR) database, String, Swiss-Prot protein database, Protein family database (Pfam), Kyoto Encyclopedia of Genes and Genome (KEGG) classification, Clusters of Orthologous Groups (COG) of proteins and Gene ontology (GO) databases, with a cut-off E-value of 1e-5 (Altschul *et al*., 1997). GO databases were mapped to

sequences based on their alignment using the Blast2GO software (Delanghe 2005).

Identification of Differentially Expressed Unigenes

To identify differential expression genes (DEGs) between two samples, the expression level of each unigene was measured by using the Fragments Per Kilobase of exon model per Million mapped reads (FPKM) method (Mortazavi *et al*., 2008). Differential expression analysis between samples was performed using edgeR (http://www.bioconductor.org/packages/2.12/bioc/html/edgeR). Genes were considered significantly differentially expressed (DEGs) when FDR (false discovery rate) < 0.05 and $\log_2|FC| >= 1$. GO term enrichment analysis and KEGG pathway functional enrichment analysis of the differentially expressed genes to determine the DEG functions and biological pathways by Goatools (https://github.com/tanghaibao/GOatools) and KOBAS (http://kobas.cbi.pku.edu.cn/home.do), respectively. We defined the corrected p value <= 0.05 as significantly enriched (Kanehisa *et al.*, 2008).

Expression Analysis by Real-Time Quantitative PCR (qRT-PCR)

A total of 4 up-regulated genes associated with Cd stress were selected for qRT-PCR analysis to validate the Illumina sequencing result and the expression pattern. The gene-specific primers were designed by Primer Premier 5.0 (Table 1). Total RNA was extracted from the gills of three crabs in each group using TRIzol (Invitrogen). qRT-PCR analysis was performed on an Applied Biosystem 7500 real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) with 2 × SYBR Green qPCR Mix (Aidlab Biotechnologies Co., Ltd., China). The qRT-PCR reaction system (25 µl) consisted of 12.5 µL of 2 × SYBR qPCR Mix, 1 µL of forward and reverse primers, 1 µL of cDNA, and 10.5 µL of RNase-free H_2O. β-actin was used as the reference gene to normalize target gene expression. The PCR program was set with an incubation step: 3 min at 95 °C, 40 cycles of 15 s at 94 °C, 15 s at 55 °C and 25 s at 72 °C, followed with the standard dissociation cycle. Each gene was repeated three technique replications and $2^{-\triangle\triangle CT}$ method was used for analyzing relative quantification (Thomas *et al*., 2008).

Results and Discussion

Illumina Sequencing and *De Novo* Assembly

To get an overview of the transcriptomic characteristics of the Cd stress, two cDNA libraries were constructed between the gills of exposed and unexposed *E. j. sinensis*. We obtained 39,612,948 total raw reads and 34,265,370 total raw reads from treated group and control group samples, respectively. The raw reads were further filtered into clean reads. After assembly, we obtained 570,847 transcripts, which were further clustered into 504,889 unigenes. The unigenes comprised 188,995,620 bases, and the N50 length of 364bp with an average GC content of approximately 44.11%. The average length, largest length and smallest length were 374.33bp, 50681bp and 201bp, respectively. Detailed information relevant to overall assembly quality was summarized in Table 2.

Functional Annotation and Classification

All the unigenes were aligned to NR, String, Swiss-Prot, Pfam, GO and KEGG database, with a cut-off E-value of 1e-5. A total of 71,968 unigenes (13.11% of all unigenes) had significant hits in at least one of these databases, including 60,587 (84.19%), 47,038 (65.36%), 42,963 (59.70%) and 42,357 (58.86%) were mapped to String, NR, Swiss-Prot and GO databases, respectively. However, comparing to four databases, only 25738 (35.76%) and 24773 (34.42%) of the total sequences were matched against Pfam and KEGG databases. These annotation and classification will facilitate the following interpretation of the gene function in *E. j. sinensis*.

From the annotated information of six databases, all unigenes were annotated through Blast against the NCBI non-redundant (nr) protein database built for the invertebrate. The Blast top-hit species distribution of the annotated unigenes showed the highest homology to the *Hyalella azteca* (9838, 20.92%), followed by *Zootermopsis nevadensis* (1547, 3.29%), *Trichuris trichiura* (1121, 2.38%), *Capitella teleta* (993, 2.11%) and *Plutella xylostella* (950, 2.02%). Except for the top 20 species with sequence hits, the largest number of the unigenes was hits in the other species (23619, 50.21%) due to limited genome information in curstaceans (Figure 1).

Table 1. Real-time quantitative PCR primers used in this study

Gene Name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
β-actin	GATGGTGGGAATGGGTCA	CCAACCGTGAGAAGATGACT
TRX	TGATTTAGGTGGATGAGTTGG	TTCCTCAATATGCCGTACCT
Prx3	GAGCCGAGAAGCAGACGA	ACTCGCACTTCTCCCACC
HSP70	CTGGTTGTCGGAGTAGGTG	ATTTCTCCACAGGCTCCA
ATP6L	CCCTCTTCAGTCTTTCCAGTCA	ATTGGTATCGTGGGTGACGC

Table 2. Summary of transcriptome characterization of *E. j. sinensis*

Sequencing		
Type	Treated Group	Control Group
Raw reads num	39612948	34265370
Clean read num	38301346	32916952
Assembly		
Type	Unigene	Transcripts
Total sequence num	504889	570847
Total sequence base	188995620	228410682
Percent GC	44.11	44.59
Largest	50681	50681
Smallest	201	201
Average	374.33	400.13
N50	364	396
N90	224	227
Annotation		
Type	Unigene	Transcripts
GO	42357	53002
KEGG	24773	34443
NR	47038	62284
PFAM	25738	34761
STRING	60587	73117
SWISSPROT	42963	53706

As an internationally standardized database of predicting the possible functions, COG analysis could help us understand the gene function distribution characteristics of the species. The results showed that of the 504,889 unigenes, 46,552 sequences had a COG classification. From the 25 COG categories, the cluster for "Amino acid transport and metabolism" (4116, 8.84%) was the largest group, followed by the group for "Replication, recombination and repair" (3993, 8.58%), "Energy production and conversion" (3482, 7.48%), "Inorganic ion transport and metabolism" (3095, 6.65%) and "Carbohydrate transport and metabolism" (3064, 6.58%) (Figure 2).

Besides COG analysis, 24773 unigenes were successfully mapped to 380 KEGG pathways within 46 categories. Among these pathways, the highly represented categories were "Metabolic pathways" (ko01100: 6637, 26.79 %), "Biosynthesis of secondary metabolites" (ko01110: 2869, 11.58%), "Biosynthesis of antibiotics" (ko01130: 2215, 8.94 %), "Microbial metabolism in diverse environments" (ko01120: 1914, 7.72 %) and "Carbon metabolism" (ko01200: 1450, 5.85 %).

Differentially Expressed Genes (DEGs) in the Gill of *E. j. sinensis*

To identify genes display significant changes in expression during Cd stress, we analyzed differentially expressed genes by comparing the Cd treated library with the control library. The expression level of each unigene was measured by FPKM method. We judged the statistical significance of gene corrected *p*-value < 0.05 and the $\log_2$ |FC| >= 1. A total of 1544 genes were significant differentially expressed in gills of crabs. Among these DEGs, 1084 genes were up-regulated and 460 genes were down-regulated compared with the control group (Figure 3). This analysis showed Cd stress had significant effect on the gene expression in *E. j. sinensis*, and the larger number of up-regulated genes suggested that most of the DEGs were mainly associated with stress response.

GO Analysis of DEGs

To obtain a better understanding of biological functions and gene interaction of DEGs, all DEGs were annotated in GO terms and significantly enriched in three GO categories, including Biological Process (BP, 29 sub-categories), Cellular Component (CC, 20 sub-categories), and Molecular Function (MF, 15 sub-categories) (Figure 4). Within the Biological Process, "cellular process" (GO: 0009987, 275 DEGs), "metabolic process" (GO: 0008152, 239 DEGs), "biological regulation" (GO: 0065007, 211 DEGs), "regulation of biological process" (GO: 0050789, 203 DEGs) and "cellular component organization or biogenesis" (GO: 0071840, 193 DEGs) were mostly prevalent term. Under Cellular Component category, "cell" (GO: 0005623, 285 DEGs) comprised the largest proportion, followed by "cell part" (GO: 0044464, 284DEGs), "organelle" (GO: 0043226, 271 DEGs), "organelle part" (GO: 0044422, 247 DEGs) and "membrane" (GO: 0016020, 222 DEGs). In Molecular Function category, "binding" (GO: 0005488, 229 DEGs) was the predominant GO term, followed by "catalytic activity" (GO: 0003824, 171 DEGs), "transporter activity" (GO: 0005215, 45 DEGs), "molecular function regulator" (GO: 0098772, 39 DEGs) and "structural molecule activity" (GO: 0005198, 28 DEGs).

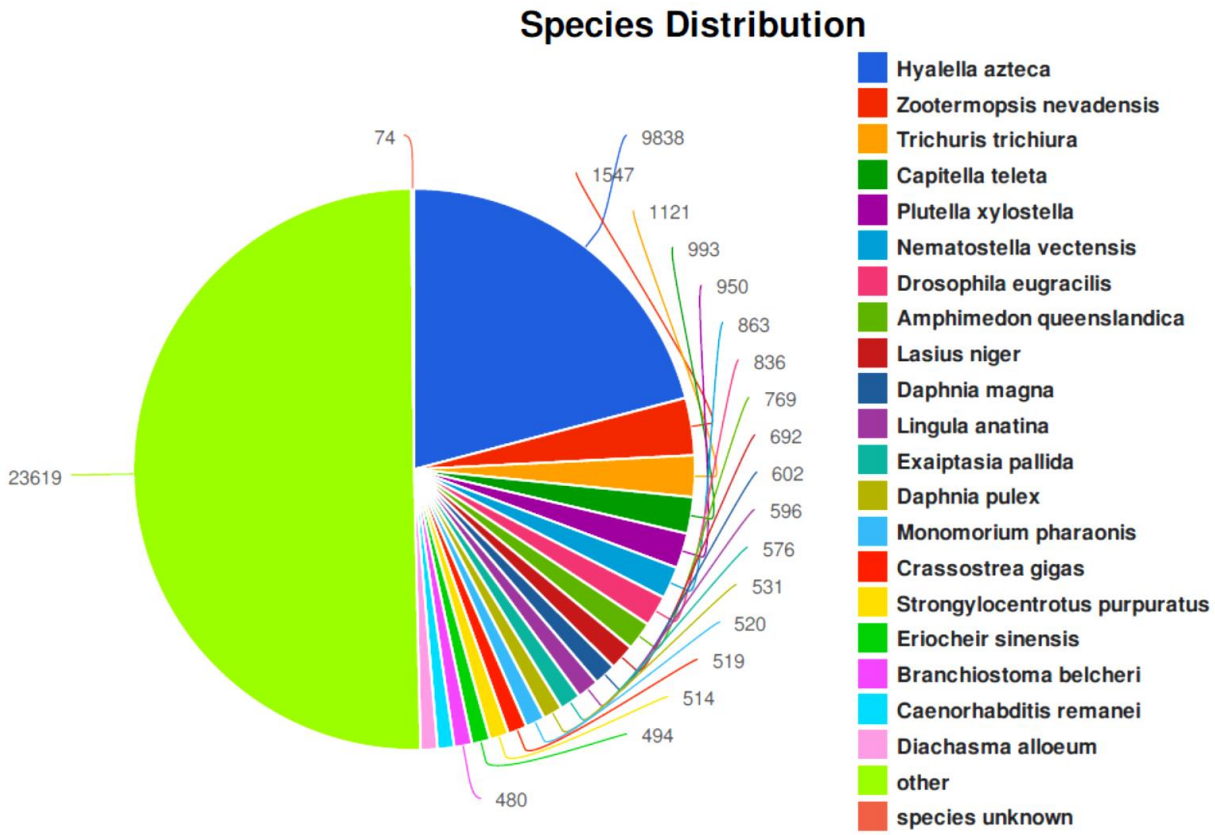

Figure 1. Species distribution of BLASTx matches the hepatopancreas transcriptome unigenes. Each piece of fan indicates the number of top BLAST matches against the Genbank non-redundant (Nr) protein database for various species.

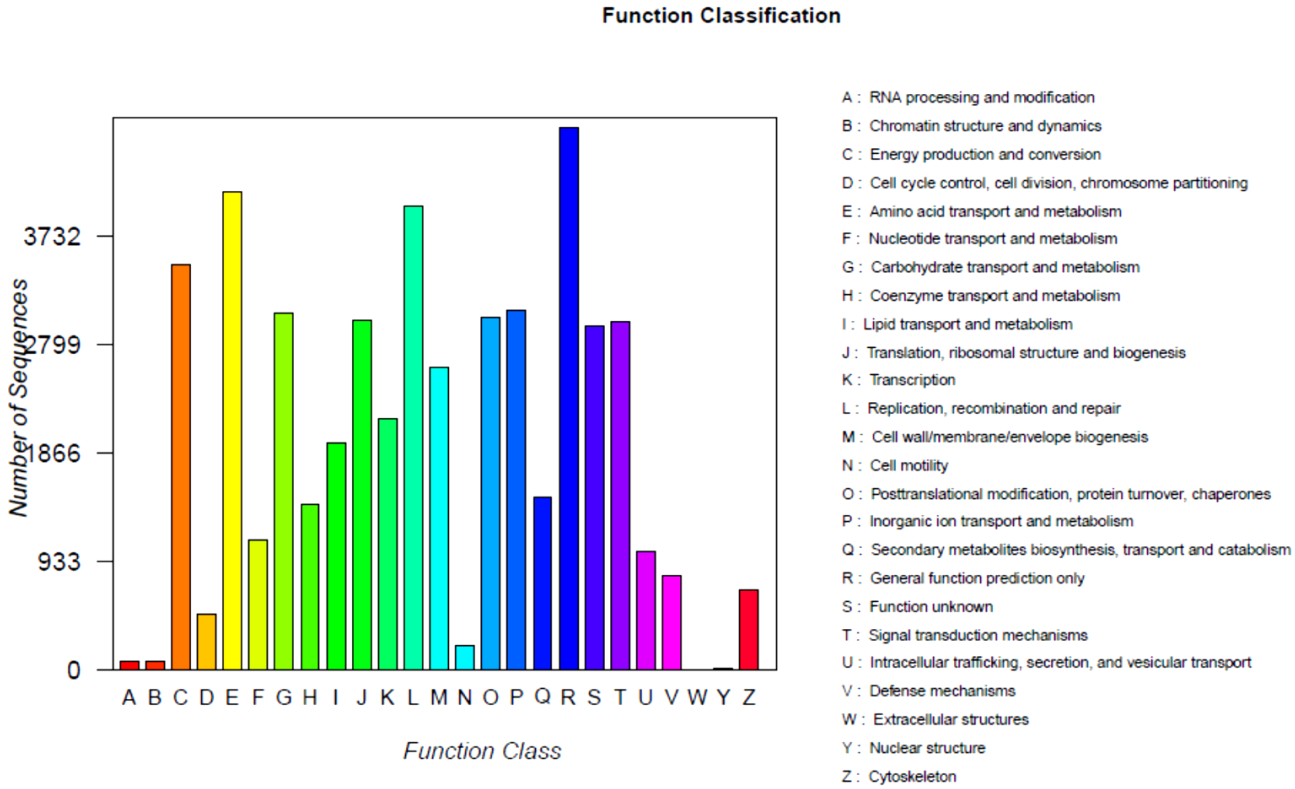

Figure 2. COG classification of all-unigenes. Unigenes were classified into 25 function classes. The columns represents the number of unigenes in each class.

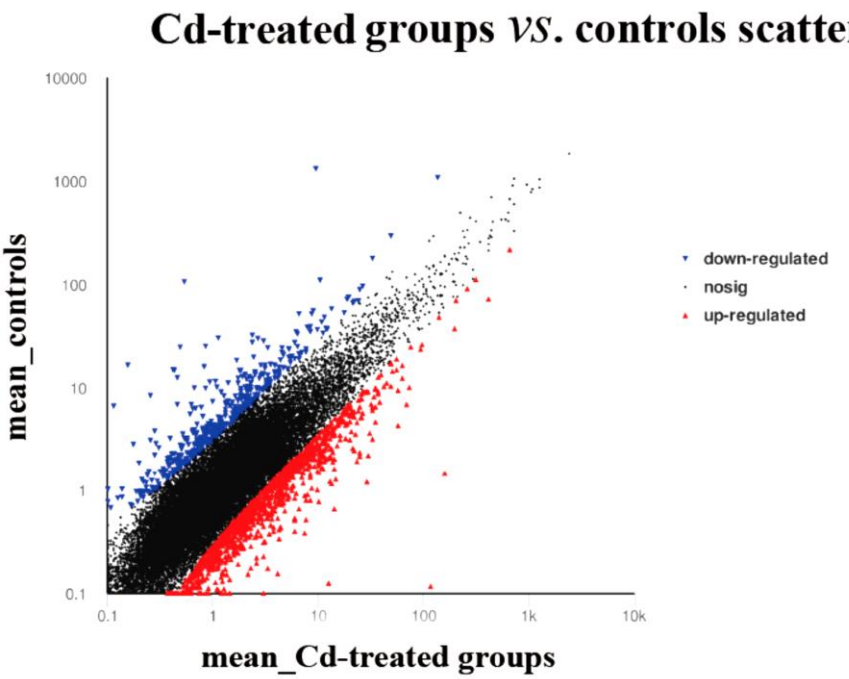

Figure 3. Analysis of DEGs following exposure of the gills to Cd. The change pattern is displayed as a Scatter plot. The X axis represents the FPKM value [log2(fold change)]. Red points represent upregulated genes and blue points represent downregulated genes based on the discriminative significance values (|fold change| ≥4.0 and FDR ≤ 0.001) adopted in this study.

In addition, among all the 64 GO terms, "synapse part", "electron carrier activity", "signal transducer activity", "transcription factor activity, protein binding", "nucleoid", "pigmentation", "presynaptic process involved in chemical synaptic transmission", "cell aggregation", "virion", "virion part", "metallochaperone activity", "detoxification", "translation regulator activity", "carbohydrate utilization", "nitrogen utilization", "protein tag" and "toxin activity" from three GO categories were all up-regulated DEGs with no down-regulated DEGs (Figure 5). It is worth noting that the "synapse part" (14), "electron carrier activity" (11) and "signal transducer activity" (10) categories had the most participating genes in these GO terms. Synapse is a structure that permits a neuron to pass an electrical or chemical signal to another neuron or to the target effector cell. Both "electron carrier activity" and "signal transducer activity" are involved in stress signal transduction (Liu *et al*., 2017). These findings indicate the importance of signal transduction in the stress response of the *E. j. sinensis* caused by Cd stress.

Pathway Analysis of DEGs

KEGG is a database for biological systems that integrates genomic, chemical, and systemic functional information (Kanehisa *et al*., 2007). In this study, all DEGs were assigned into KEGG metabolic and regulatory pathways to identify the biological pathways involved in Cd stress. These genes were mapped into 238 pathways in the KEGG database and 43 pathways were significantly enriched (corrected P-value<0.05) (Table 3). The DEGs were mainly concentrated in the pathways of "Metabolic pathways" (67 up-regulated, 3 down-regulated), "Biosynthesis of secondary metabolites" (27 up-regulated, 1 down-regulated), "Oxidative phosphorylation" (26 up-regulated, 0 down-regulated), "Biosynthesis of antibiotics" (23 up-regulated, 0 down-regulated) and "Microbial metabolism in diverse environments" (21 up-regulated, 0 down-regulated) (Table 3). These pathways were all classified with metabolites pathway and most DEGs were up-regulated. Remarkably, our study found that 29 DEGs were all significant up-regulated in the Oxidative phosphorylation pathway (Figure 4). As we all know, oxidative phosphorylation in mitochondria is a major pathway to supply energy in the basic activities of life through electron transport chain to generate 3'-adenosine triphosphate (ATP) (Saraste 1999). DEGs involved in the Oxidative phosphorylation pathway were all significant up-regulated, indicating that *E. j. sinensis* needs more ATP to provide more energy for biochemical activities caused by Cd stress.

Gill associates with ambient environment directly, and it is one of the most important organs involved in osmoregulation in crustaceans (Li *et al*., 2014). As we all know, Na^+/K^+-ATPase (NKA) is a widespread P-type ATPase that plays an important role in the regulation of hematopoietic osmotic pressure (Ahearn *et al*., 1999; Crambert *et al.*, 2000). In general, alterations to the ion environment could increase membrane fluidity, which may contribute to the changes of NKA enzyme. Here, several pathways directly related to the active regulation of the NKA enzyme were detected, such as "Collecting duct acid secretion" (ko04966), "Dopaminergic synapse" (ko04728) and "Bile secretion"

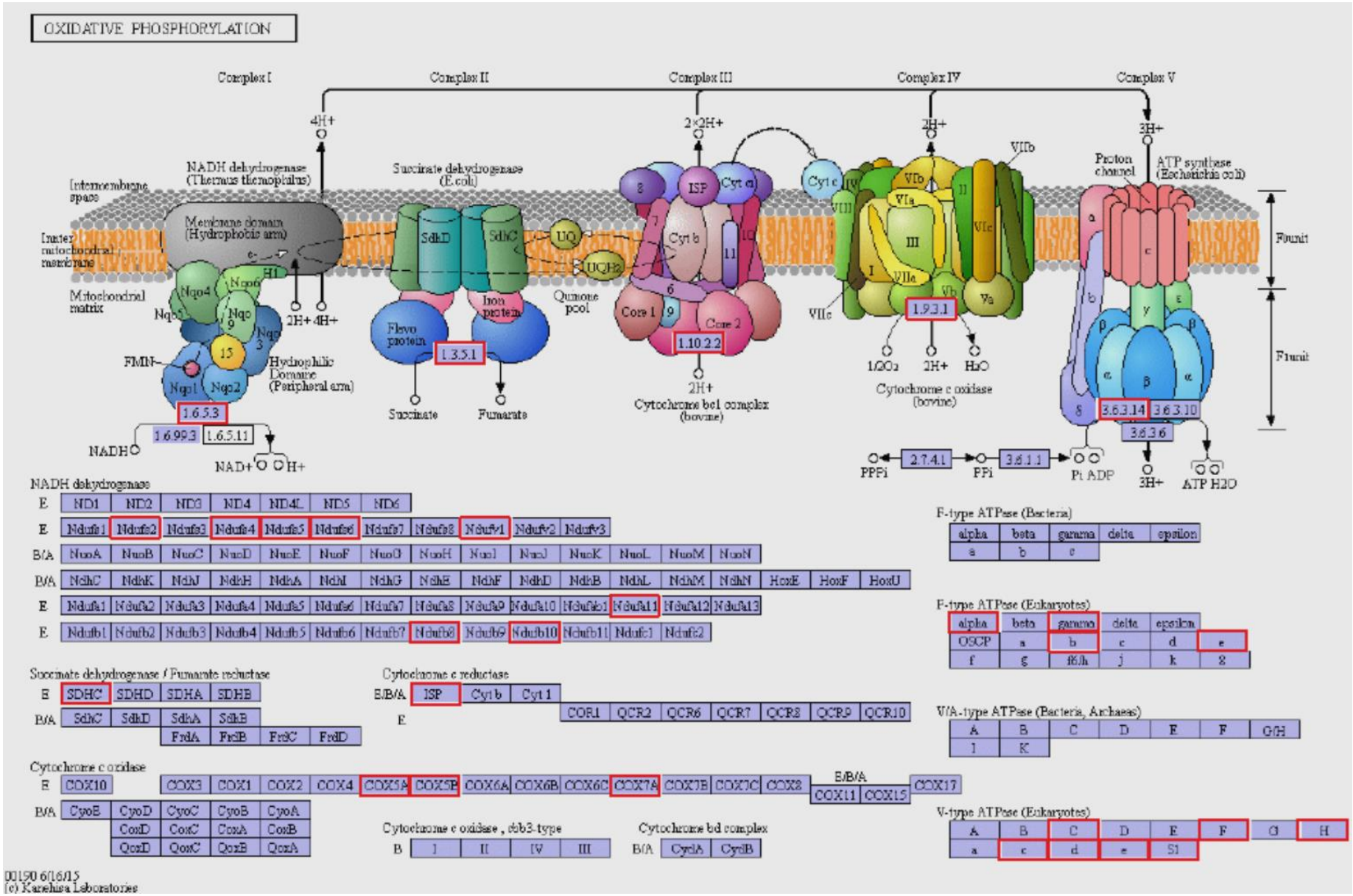

Figure 4. The KEGG pathway of metabolism of Oxidative phosphorylation. The enzymes in the red boxes are associated with the upregulated genes.

(ko04976) (Table 3). The regulatory genes of NKA in these pathways were all up-regulated, suggesting that the NKA strengthened the ion exchange function necessary to maintain the osmotic balance required for normal survival after Cd stress. Other regulators were also found in these pathways, including solute carrier organic anion transporter family member 5A1 (SLC5A1), calcineurin B-like protein 1 (CBL1) and serine proteinase inhibitor 2 (SPI2) which may related to metal ion transport (Fe^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}) and were expressed in the gill (Hui *et al*., 2017). In addition, three pathways were found that connected with the amino acid metabolism, including "Biosynthesis of amino acids" (ko01230), "Arginine and proline metabolism" (ko00330) and "Arginine biosynthesis" (ko00220) (Table 3). Free amino acids also played a vital role in osmoregulation, the results implied these pathways might all take part in resistant to Cd stress. In addition, we used cadmium in the form of Cadmium chloride. Chloridion is an essential element for organisms and participate in various kinds of biological function, such as cellular immunologic response, cellular proliferation, differentiation and apoptosis (Xu *et al.*, 2017). Therefore, the active uptake of chloridion also affects various physiological functions of crabs, particularity in osmoregulation. Chinese mitten crab must be required to maintain the proper ion balance, which is need more changes in the adaptive process. Certainly, the mechanism of adaptive process is very complex, and need more in-depth research in the future work.

Identification of Putative Genes for Responding Cd Stress

We examined the gills transcriptome of Cd-treated and untreated *E. j. sinensis* primarily to identify a variety of candidate genes that might be functionally associated with Cd toxicity response mechanism. Considering the significance of immune genes in the toxic environment,

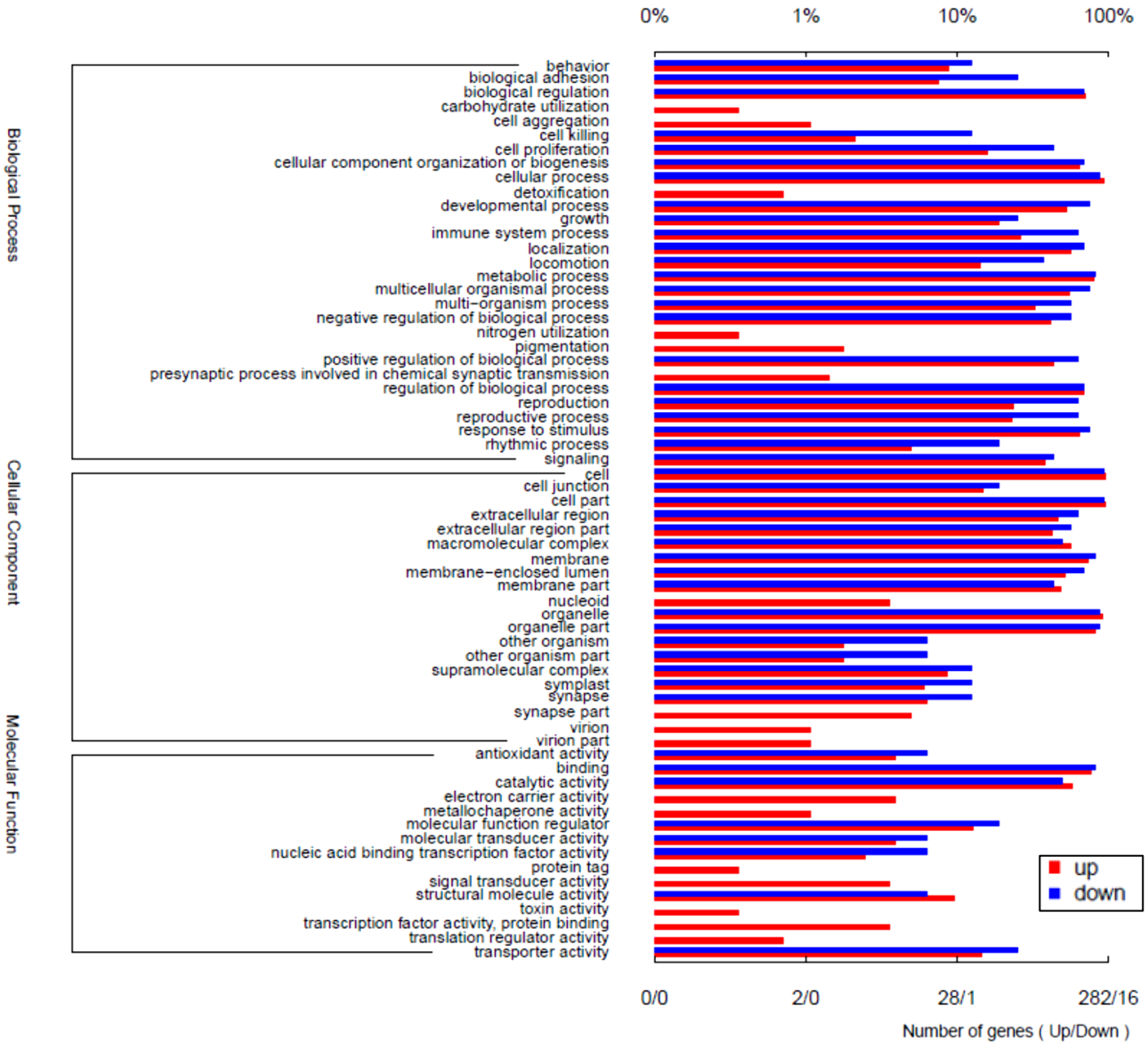

Figure 5. Up- and down-regulated unigenes of the top terms in comparisons between each Cd-treated group and the control.

Table 3. Significant enrichment of pathways for DEGs between Cd treated crabs and the control. The pathway ID was obtained from the KEGG database. Pathways with a corrected P-value of <0.05 were significantly enriched.

Pathway ID	Pathway	DEGs associated with the pathway	Up-regulated	Down-regulated	Corrected P-value
ko01100	Metabolic pathways	70	67	3	0.000072
ko00190	Oxidative phosphorylation	29	29	0	0.0000165
ko01110	Biosynthesis of secondary metabolites	28	27	1	0.000813172
ko01130	Biosynthesis of antibiotics	23	23	0	0.002590833
ko01120	Microbial metabolism in diverse environments	21	21	0	0.000336719
ko04141	Protein processing in endoplasmic reticulum	21	20	1	0.000997325
ko01200	Carbon metabolism	19	19	0	0.000142948
ko05016	Huntington's disease	19	19	0	0.006954576
ko05012	Parkinson's disease	17	17	0	0.008106575
ko01230	Biosynthesis of amino acids	16	15	1	0.0000127
ko05010	Alzheimer's disease	16	16	0	0.02056226
ko04932	Non-alcoholic fatty liver disease (NAFLD)	14	14	0	0.024560612
ko05418	Fluid shear stress and atherosclerosis	12	12	0	0.002404538
ko04145	Phagosome	12	12	0	0.002976426
ko05110	Vibrio cholerae infection	11	11	0	0.0000426
ko05152	Tuberculosis	11	11	0	0.000428232
ko05120	Epithelial cell signaling in Helicobacter pylori infection	10	10	0	0.000293024
ko04723	Retrograde endocannabinoid signaling	10	9	1	0.038736997
ko05323	Rheumatoid arthritis	9	9	0	0.000448045
ko00710	Carbon fixation in photosynthetic organisms	8	8	0	0.000370655
ko04721	Synaptic vesicle cycle	8	8	0	0.005633931
ko05164	Influenza A	8	7	1	0.01375675
ko01212	Fatty acid metabolism	7	7	0	0.005633931
ko05134	Legionellosis	7	5	2	0.010796747
ko04260	Cardiac muscle contraction	7	7	0	0.01375675
ko04966	Collecting duct acid secretion	6	6	0	0.004238181
ko00020	Citrate cycle (TCA cycle)	6	6	0	0.017624738
ko00071	Fatty acid degradation	6	6	0	0.017624738
ko00620	Pyruvate metabolism	6	6	0	0.022152894
ko00062	Fatty acid elongation	5	5	0	0.004238181
ko00330	Arginine and proline metabolism	5	4	1	0.022152894
ko05204	Chemical carcinogenesis	5	4	1	0.028603463
ko00030	Pentose phosphate pathway	4	4	0	0.010891196
ko01210	2-Oxocarboxylic acid metabolism	4	4	0	0.017720229
ko04976	Bile secretion	4	4	0	0.017720229
ko04146	Peroxisome	3	3	0	0.006983939
ko00515	Mannose type O-glycan biosynthesis	3	3	0	0.006926518
ko00220	Arginine biosynthesis	3	3	0	0.028603463
ko00650	Butanoate metabolism	3	3	0	0.028603463
ko02020	Two-component system	3	3	0	0.045535561
ko00930	Caprolactam degradation	2	2	0	0.042557678
ko00960	Tropane, piperidine and pyridine alkaloid biosynthesis	2	2	0	0.042557678
ko04614	Renin-angiotensin system	2	2	0	0.042557678

we identified 74 up-regulated unigenes and 10 down-regulated unigenes in “immune system process”, including HSP family members and Proteasome family members. To our knowledge, HSP plays a critical role in molecular chaperones of innate immune responses (Jiang *et al*., 2009; Wu *et al*., 2014). Notably, HSP60 play a crucial role in the process of pathogenic and protective immune responses and is implicated in autoimmune disease (Yang *et al*., 2013). HSP70 could suppress apoptosis by directly binding to Apaf-1, which could prevent recruitment of caspases from the apoptosome complex (Lu *et al*., 2016). In our research, we noted that many HSP members related to immune resistance had higher levels of expression in treated *E. j. sinensis*, including HSP60 and HSP70. The increased expression of these HSP genes is in line with previous reports (Sun *et al*., 2014), which tends to confirm the important role of these proteins in protecting this species from the stress induced by Cd challenge. Peroxisomes are related to immune responses and antioxidant defence (Blander and Medzhitov 2004; Zhou *et al*., 2015). Here, proteasome subunit alpha type-4 (*PSMA4*), proteasome alpha 3 (*PSMA3*) and proteasome subunit alpha type-5 (*PSMA5*) were all up-regulated, this increase suggesting the enhancement in immune responses and antioxidant defence of the crab caused by Cd stress

Exposure to heavy metals can promote the generation of ROS in crabs, and ROS production

promotes oxidation of cellular components and oxidative stress (Zenteno-Savín *et al*., 2006). Thus, crabs generate some antioxidant enzymes to clear the ROS and avoid oxidative damage. In order to understand the potential molecular mechanism for the damage of Cd stress, DEGs that associated with "detoxification" and "antioxidant activity" were considered to be potential candidate genes which are important for the response to acute Cd stress (Table 4). Among the "antioxidant activity" category, 11 DEGs were identified, which comprised 10 up-regulated genes, including glutathione peroxidase (*GPx*, c398007_g1), glutathione S-transferase (*GST*, c404726_g2), thioredoxin (*TRX*, c405622_g2), peroxiredoxin 5 (*Prx5*, c407190_g1), peroxinectin (*Pxt*, c407949_g1), peroxiredoxin 3 (*Prx3*, c412223_g2), prostaglandin D synthase (*PGDS*, c411308_g2), lipoxygenase (*LOX*, c411883_g2), glutathione S-transferase (*GST*, c396511_g1) and protein Smaug homolog 1 (*SAM1*, c405735_g4). Of which, glutathione S-transferase 1 (*GST1*, c396511_g1) and protein Smaug homolog 1 (*SAM1*, c405735_g4) also belonged to "detoxification" category. Among these up-regulated genes, GST plays a critical role in protecting organisms against the toxicity of ROS [superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-)] (Roch 1999). GST also is considered as major superfamily of antioxidant enzymes which facilitates the elimination of electrophiles. The increasing of GST activity has also been reported in the swimming crab (*Portunus trituberculatus*) treated with the elevated ambient ammonia-N (Ren and Pan 2014). Recently, GST has been cloned from the hemolymph of *E. j. sinensis* and this research also investigates the distribution of GST mRNA in different tissues in haemocytes of crabs challenged with *Aeromonas hydrophila*, providing a better understanding of the function of GST gene in this

Table 4. List of identified candidate genes potentially involved in Cd response in *E. j. sinensis* based on gene ontology (GO)

Terms from blast hits.	Seq_id	Gene description	Rgulate
detoxification	c396511_g1	glutathione S-transferase	up
	c405735_g4	protein Smaug homolog 1	up
toxin activity	c407817_g1	clip domain serine proteinase 2	up
antioxidant activity	c398007_g1	glutathione peroxidase	up
	c404726_g2	glutathione S-transferase	up
	c405622_g2	thioredoxin	up
	c407190_g1	peroxiredoxin 5	up
	c407949_g1	peroxinectin	up
	c412223_g2	peroxiredoxin 3	up
	c411308_g2	prostaglandin D synthase	up
	c411883_g2	lipoxygenase	up
	c411065_g1	cyclooxygenase	down

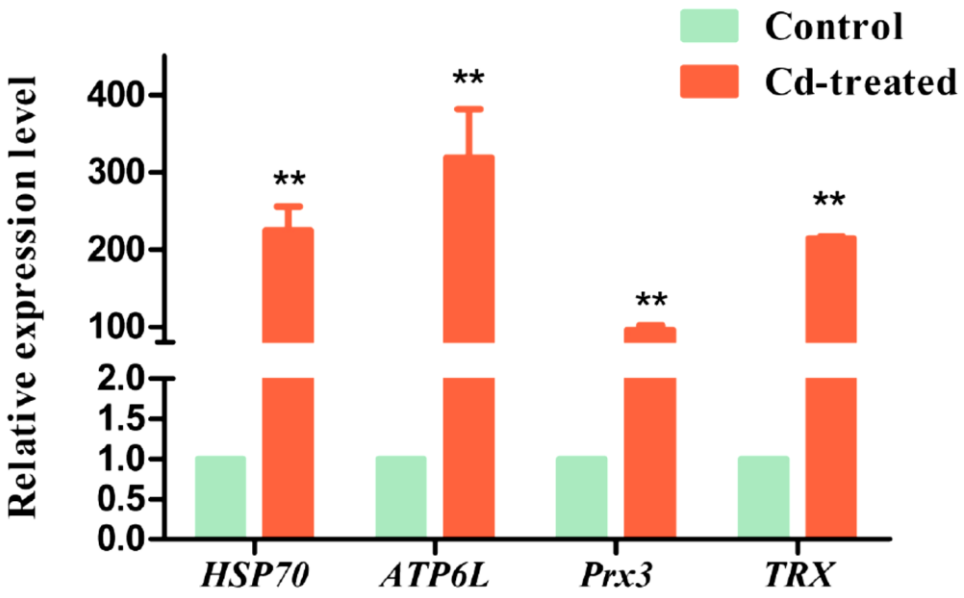

Figure 6. qRT-PCR analysis of the expression level of four Key genes associated with Cd stress.

species to resist oxidative stress (Zhao *et al.*, 2010). According previous research, GPx is anti-oxidative enzyme that reduces organic and inorganic hydroperoxides to the corresponding alcohols at the expense of reduced glutathione (Borchert *et al.*, 2018). *GPx* can keep the cellular redox balance and protect against oxidative damage by reducing hydroperoxides at the expense of glutathione (Bain *et al*., 2016). These results revealed that the upregulation of *GST* and *GPx* was involved in stress response and adaptation, and similar upregulation was observed in *Sinopotamon henanense* (Sun *et al*. 2016). These genes could be major targets for Cd tolerance, and further experiments are necessary to determine the exact function of these proteins.

Validation of RNA-Seq by qRT-PCR

To verify the gene expression profile identified by RNA-Seq, 4 genes associated with Cd response in each comparison were selected for further detection of the relative mRNA expression level by qRT-PCR. The results showed that the expressions of the four genes （*HSP70*, *ATP6L*, *Prx3*, *TRX*） were more than control group, which were in agreement with the RNA-Seq expression profile (Figure 6). This result also indicates that these genes were involved in the response to Cd.

Conclusion

In this study, we successfully constructed comparative gills transcriptomes dataset in treated group and control group of Chinese mitten crab and functionally annotated a total of 504,889 unigenes. Most of these genes had an annotation with matches in the NR, String, Swiss-Prot, Pfam, GO and KEGG database. Additionally, we identified 1544 DEGs in the gill after Cd stress. The results of the GO and KEGG pathway enrichment analyses indicated that the DEGs mainly related to signal transduction, oxidative phosphorylation and osmoregulation were enriched after Cd stress. A number of genes that are potentially relevant to immune responses, antioxidant, and detoxification were also identified. These results set the stage for expanding the genetic resources available for *E. j. sinensis*, and also provide important information for further research on the molecular basis of osmoregulation, detoxification and immune response resulting from Cd stress in crabs.

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