Transcriptome analysis reveals the effect of residual chlorine on immunity in the *Cyclina sinensis*

Si-ting Wang[1,2], Guo-liang Ren[1,2], Su-su Yan[1,2], Mei-mei Liu[1,2,3], Zhi-guo Dong[1,2,3,*], Hong-xing Ge[1,2,3]

1 Co-Innovation Center of Jiangsu Marine Bio-industry Technology, Jiangsu Institute of Marine Resources Development, Lianyungang, China

2 Jiangsu Key Laboratory of Marine Bioresources and Environment, Jiangsu Ocean University, Lianyungang, China

3 Jiangsu Institute of Marine Resources Development, Lianyungang, China

*Corresponding author: dzg7712@163.com (Z. Dong)

Introduction

The clam (*Cyclina sinensis*) is a common buried shellfish, also known as the ring clam and black clam, and is one of the main cultured shellfish in the aquaculture industry in China. The clam is very widely distributed in the ocean, from Korea to the north and south coasts of China, mainly in coastal mudflat areas, mostly in river mouths and other places, and is a common shellfish species in coastal areas of China. Due to the advantages of strong resistance to adversity, tasty meat and high farming output rate, the clam has become an ideal cultured seafood enrichment shellfish with huge production and sales in China.

Chlorine residual is a common by-product of warm water discharge from coastal nuclear power plants. In order to prevent damage to the cooling system from organisms in seawater, seawater needs to be electrolyzed so that the cooling water contains a certain concentration of chlorine to kill all kinds of organisms in the seawater. The seawater enters the cooling system and takes away the waste heat from the nuclear reaction to become warm water, but the residual chlorine in it also enters the ocean with the warm water. This issue is also one of the research hotspots of scholars in various countries nowadays. However, there are few studies on the effect of residual chlorine on shellfish in warm water drainage of coastal nuclear power plants in China, which needs further investigation and research.

In the current studies, there are relatively few studies on the mechanism of toxicity of residual chlorine to aquatic organisms, and most of the studies on shellfish are on the effects of residual chlorine on gills, hepatopancreas, blood and other indicators. In the available studies, the explanation of fish mortality due to residual chlorine stress is mostly biased towards asphyxiation mortality. This explanation is also consistent with the mechanism of toxicity of chlorine damage to the respiratory system of humans and animals. Residual chlorine causes lesions in the gill tissue of aquatic organisms, such as hyperplasia, hypertrophy, and accumulation of mucus. Lesions in gill tissues cause swelling of gill filaments, which separates the gill filaments from the capillaries and prevents the normal entry of oxygen into the capillaries, thus causing a decrease in oxygen supply and a decrease in the blood oxygen content in the organism, which elevates the respiratory rate in order to provide the oxygen content required for the activity of the organism.

Methods

1. Sample preparation

One-year-old clam with an average shell length 1.20 ± 0.10 cm and body weight of 0.70 g ± 0.2 g was purchased from Lianyungang Zhongchuang Aquaculture Co, Lianyungang, China, and then acclimated for one week in an indoor circulating water system.

2. Sample and data analysis

2.1. The clams under 0 hour of stress were used as the control group, and were recorded as H-0; the clams under 96 hours of stress at three different concentrations (20mg/L, 50 mg/L, 100 mg/L) were used as the treatment groups, which were recorded as H-96a, H-96b, and H-96c, respectively. The hepatopancreas of six clams were taken from each group, three were used for testing and three were used as backups, which were frozen in liquid nitrogen and stored in -80°C refrigerator.

2.2. RNA extraction, cDNA library preparation and next-generation sequencing: Total RNA was extracted from the hepatopancreas of C. sinensis using TRIzol Reagent (Takara) according to the manufacturer's recommendations, and genomic DNA was removed with DNase I (Takara).

2.3. De novo assembly and analysis of the transcriptome: The gene expression profiles were compared among the four groups, control (H-0, 0 mg/L), a (H-96a, 20mg/L), b, (H-96b, 50 mg/L) and c, (H-96c, 100 mg/L) treatments, and then all DEGs in each comparison were submitted to GO functional and KEGG pathway enrichment analysis using the GO database and KEGG database, respectively.

2.4. Validation of the RNA-seq profiles by quantitative real-time PCR (qPCR).

Results

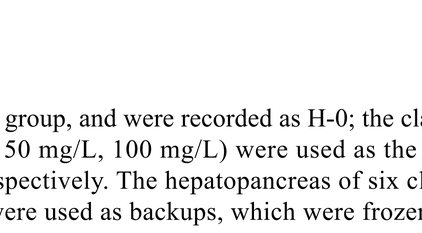
1. Transcriptome sequence assessment and annotation

After transcriptome sequencing, the 12 libraries prepared from the three concentration groups at 0 h and 96 h of stress yielded 5.6-7 billion Raw Reads. 5.6-6.9 billion Clean Reads were obtained after removing the low quality reads, with GC content of 43.66-45.80%, Q20 96.91-97.50%, and Q30 91.59-92.82% of the sequences. 92.82% (Table 1).

Table 1 Data quality of sample sequencing

Sample	RawData(bp)	CleanData(bp)	Q20(%)	Q30(%)	N(%)	GC(%)
H-0-1	5857228500	5807035294	97.18	92.14	0.00	44.5
H-0-2	5877342900	5820804760	97.24	92.29	0.00	44.09
H-0-3	7048446300	6977114096	97.25	92.27	0.00	43.79
H-96-a1	6683467800	6626230908	97.24	92.24	0.00	43.66
H-96-a2	6790605300	6732578082	97.5	92.82	0.00	44.6
H-96-a3	6442787100	6382515585	97.2	92.19	0.00	44.74
H-96-b1	6248469900	6193389106	97.33	92.47	0.00	44.7
H-96-b2	6652345500	6600088333	97.11	91.99	0.00	44.9
H-96-b3	5932570500	5885252093	96.91	91.59	0.00	45.8
H-96-c1	6426845100	6382143885	97.36	92.5	0.00	45.52
H-96-c2	6066531300	6018951829	97.14	92.09	0.00	45.29
H-96-c3	5642172000	5602841327	97.37	92.55	0.00	45.09

Fig. 1 Statistical chart of DEGs



2. Analysis of differentially expressed genes (DEGs)

The number of DEGs in the three groups of a (20 mg/L), b (50 mg/L) and c (100 mg/L) were counted (Fig. 1). The results showed that compared with the control group, 13, 17 and 16 unigenes were up-regulated and 23, 34 and 13 unigenes were down-regulated in group a, b and c, respectively. Compared with group a at 96 hours of residual chlorine stress, 19 and 23 unigenes were up-regulated and 17 and 16 unigenes were down-regulated in group b and group c, respectively. Finally, 23 and 27 unigenes were up-regulated and down-regulated in group c, respectively, compared with group b at 96 hours. Most of the genes in groups a, b, and c were down-regulated compared with the control group, and most of the genes in groups a, b, and c were up-regulated compared with each other.

To analyse the functions of these DGEs, GO assignments were made. These DGEs were assigned to major GO categories (level 3), i.e., biological process, cellular component, and molecular function (Fig. 2).

The annotation of the number of differential genes is shown in Figure 3: In KEGG annotation, the most single gene pathways among groups were: cofactors in metabolic pathways and signal transduction in vitamin metabolism and environmental information processing; Transport and catabolism in cellular processes and Signal transduction in environmental information processing.

Fig. 2 GO annotation map

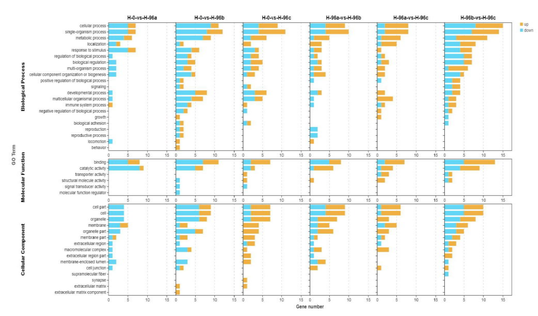
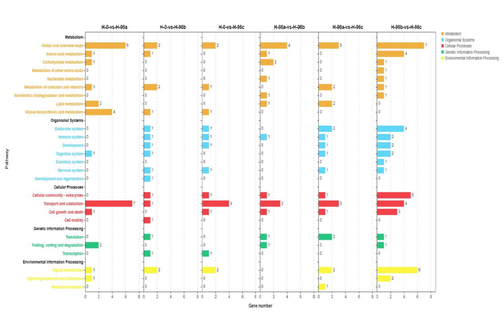


Figure 3 KEGG annotation map



5. Key genes involved in immunity response to residual chlorine stress in *Cyclina sinensis*

In this study, DEGS associated with immune defense were divided into 5 groups. There were two genes related to protein coding, two genes related to signal transduction, two pattern recognition proteins/receptors, one gene related to apoptosis, and five other immune molecules (Table 2).

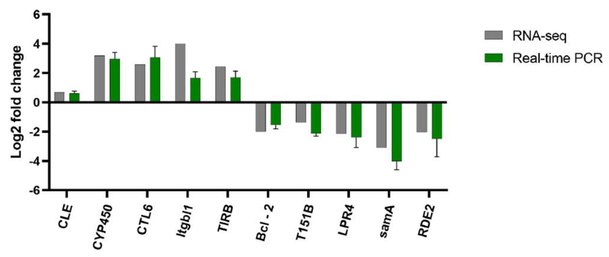
Table 2 Summary of differential expressed genes related to immune in transcriptome of *C. sinensis*.

Gene Name	Gene ID	Treatment	log2(fc)	PValue	Description	Up/Down
CASP9	MSTRG.1925	H-0vsH-96a	1.631505087	0.035496024	caspase 9, partial	Up
		H-0vsH-96b	1.620009448	0.005040889	APY24036.1 caspase 9, partial [Corbicula fluminea]	Up
TUBB4B	MSTRG.26046	H-0vsH-96a	-2.79316902	0.025012132	Tubulin beta chain	Down
		H-0vsH-96c	-4.66106548	0.009999854	EZA61695.1 Tubulin beta chain [Ooceraea biroi]	Down
		H-96avsH-96c	-2.72024221	0.008293093	AFV81455.1 beta-tubulin [Scrobicularia plana]	Down
		H-96bvsH-96c	-2.30030782	0.041063401	AFV81455.1 beta-tubulin [Scrobicularia plana]	Down
CLEC4E	MSTRG.16577	H-0vsH-96a	-1.93481927	0.006990145	C-type lectin 5	Down
		H-96avsH-96b	1.963583334	0.001601805	AZS54112.1 C-type lectin 5 [Ruditapes philippinarum]	Up
		H-96avsH-96c	2.153116714	7.72554E-05	AZS54112.1 C-type lectin 5 [Ruditapes philippinarum]	Up
FURIN	MSTRG.24813	H-0vsH-96a	-9.54432052	0.001425931	neuroendocrine convertase 2-like	Down
		H-96avsH-96b	9.013089999	0.015262546	XP_022786340.1 neuroendocrine convertase 2-like [Stylophora pistillata]	Up
		H-96avsH-96c	8.17990909	0.022493285	XP_022786340.1 neuroendocrine convertase 2-like [Stylophora pistillata]	Up
PCSK1	MSTRG.24808	H-0vsH-96a	-4.33327307	0.00073803	neuroendocrine convertase 1 isoform X3	Down
		H-96avsH-96b	3.622437206	0.00827055	XP_031785643.1 neuroendocrine convertase 1 isoform X3 [Nasonia vitripennis]	Up
LRP2	MSTRG.24803	H-0vsH-96b	-2.86507042	0.031284607	XP_030836080.1 low-density lipoprotein receptor-related protein 6 [Strongylocentrotus purpuratus]	Down
		H-96avsH-96b	-3.89862298	0.026152278	XP_030836080.1 low-density lipoprotein receptor-related protein 6 [Strongylocentrotus purpuratus]	Down
REG1	MSTRG.29191	H-0vsH-96b	-4.49825087	0.005354497	XP_022109148.1 macrophage mannose receptor 1-like [Acanthaster planci]	Down
		H-0vsH-96c	-7.14210706	0.001428206	XP_022109148.1 macrophage mannose receptor 1-like [Acanthaster planci]	Down
FCER2	MSTRG.14827	H-0vsH-96c	-5.45669665	0.044461356	ANG56319.1 C-type lectin [Meretrix meretrix]	Down
		H-96bvsH-96c	-6.21988557	0.004401987	ANG56319.1 C-type lectin [Meretrix meretrix]	Down
SLC16A12	MSTRG.25223	H-0vsH-96c	2	0.041965987	XP_021355715.1 monocarboxylate transporter 12-like [Mizuhopecten yessoensis]	Up
		H-96bvsH-96c	2.447458977	0.035346079	XP_021355715.1 monocarboxylate transporter 12-like [Mizuhopecten yessoensis]	Up
CLEC10A	MSTRG.27186	H-96avsH-96b	2.470532612	0.014231177	AZS54109.1 C-type lectin 2 [Ruditapes philippinarum]	Up
		H-96avsH-96c	2.417245819	0.003157778	AZS54109.1 C-type lectin 2 [Ruditapes philippinarum]	Up
TUBB4	MSTRG.11519	H-96avsH-96b	9.321928095	0.01272045	NP_001279339.1 tubulin beta-4A chain [Callorhinchus milii]	Up
		H-96bvsH-96c	-9.32192809	0.033238793	NP_001279339.1 tubulin beta-4A chain [Callorhinchus milii]	Down
AGAP007347	MSTRG.9304	H-96avsH-96b	-3.33479286	0.001252323	AJQ21538.1 C-type lysozyme 3 [Mytilus galloprovincialis]	Down
		H-96bvsH-96c	2.185380151	6.57215E-05	AJQ21538.1 C-type lysozyme 3 [Mytilus galloprovincialis]	Up

6. Validation of transcriptomic sequencing by quantitative PCR analysis

As shown in Figure 4, the transcriptome results were verified by qRT-PCR, and 10 immune-and antioxidation-related genes (5 up-regulated and 5 down-regulated) were randomly selected. The verification results were consistent with the sequencing results, indicating that the transcriptome sequencing results were authentic and reliable.

Fig. 4 Transcriptome result validation



Conclusion

Through this study, it was found that acute stress with residual chlorine significantly altered the expression levels of immune-related molecules associated with signal transduction, microbial agglutination, apoptosis, pattern recognition proteins/receptors, and protein coding. Taken together, the present study provides valuable information for understanding the effects of acute residual chlorine stress on the molecular mechanisms of immune function in *Cyclina sinensis*.

Reference

Li M, Gong S, Li Q, et al. Ammonia toxicity induces glutamine accumulation, oxidative stress and immunosuppression in juvenile yellow catfish *Pelteobagrus fulvidraco*[J]. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 2016, 183: 1-6.

Sıkdokur E, Belivermiş M, Sezer N, et al. Effects of microplastics and mercury on manila *Cyclina sinensis Ruditapes philippinarum*: Feeding rate, immunomodulation, histopathology and oxidative stress[J]. Environmental Pollution, 2020, 262: 114247.

Huang W, Li H, Cheng C, et al. Analysis of the transcriptome data in *Litopenaeus vannamei* reveals the immune basis and predicts the hub regulation-genes in response to high-pH stress[J]. Plos one, 2018, 13(12): e0207771.

Cheng C H, Ma H L, Su Y L, et al. Ammonia toxicity in the mud crab (*Scylla paramamosain*): the mechanistic insight from physiology to transcriptome analysis[J]. Ecotoxicology and Environmental Safety, 2019, 179: 9-16.

Bertucci A, Pierron F, Gourves P Y, et al. Whole-transcriptome response to wastewater treatment plant and stormwater effluents in the Asian *Cyclina sinensis*, Corbicula fluminea[J]. Ecotoxicology and Environmental Safety, 2018, 165: 96-106.