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Short communication

cDNA cloning and expression analysis of glutaredoxin (Grx) 2 in the Pacific white shrimp *Litopenaeus vannamei*

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ABSTRACT

Glutaredoxin (Grx) is a class molecule oxidoreductase, which can regulate the redox state of proteins and plays a key role in antioxidant defense. However, the informations of Grx cDNA sequences and their functions are lack in decapod crustacea. In the present study, the cDNA of LvGrx 2 was cloned from the Pacific white shrimp, *Litopenaeus vannamei*. The open reading frame (ORF) of LvGrx 2 was 360 bp, which encoded a polypeptide of 119 amino acids. The molecular mass of the predicted protein is 12.87 kDa with an estimated pI of 8.22. Sequence alignment showed that the amino acid sequence of LvGrx 2 shares 59%, 59% and 58% identity with that of the coelacanth *Latimeria chalumnae,* the plateau frog *Nanorana parkeri* and the half-smooth tongue sole *Cynoglossus semilaevis*, respectively. Quantitative real-time PCR analysis revealed that LvGrx 2 were detected in a wide range of tissues, with highest expression in gill, hepatopancrea and intestine, and weakest expression in muscle. The expression responses of LvGrx 2 were analyzed in hepatopancrea and gill after ammonia-N stress or lipopolysaccharide (LPS) injection. During ammonia-N exposure, the LvGrx 2 transcriptions in hepatopancrea and gill significantly up-regulated, and the peak value appeared after 12 h and 24 h exposure respectively. After LPS injection, expression levels of LvGrx 2 in hepatopancrea obviously increased in the early and late stages, while LvGrx 2 transcription in gill sharply up-regulated in the middle period. These results suggest that LvGrx 2 may play a vital role in shrimp defense system against environmental stress and pathogen infection. RNA interference experiment was designed to further probe roles of LvGrx 2 during ammonia-N exposure. Ammonia-N induced obvious improvement in expression levels of LvGrx 2, LvGrx 3, GPx, GST and Trx, accompanied by increases of protein carbonyl and malondialdehyde (MDA) contents. However, transcription of GPx and GST were much weaker in LvGrx 2 interfered-shrimp, and oxidative damage in both lipid and protein were more serious. These results further suggest that LvGrx 2 in shrimp participates in oxidative defence and regulation of antioxidant system.

1. Introduction

The Pacific white shrimp (*Litopenaeus vannamei*), as the most lucrative penaeid shrimp, were farmed across the world [1]. Its aquaculture has greatly improved the economies and livelihoods of world to some extent. Nevertheless, because of pathogen infection and environmental deterioration, this industry was subjected to high mortality and huge economic losses [2]. Study in immune defense mechanisms of shrimp is crucial for disease control and prevention strategies.

Glutaredoxin (Grx) is a specific molecule oxidoreductase of the thioredoxin (Trx) superfamily, which plays an important role in redox system [3]. It catalyzes the reduction of disulfides in both prokaryotes and eukaryotes, and glutathione (GSH) serves as a cosubstrate [4,5]. Similar to the structure of Trx, Grx have highly conserved active sites which are spatially exposed to the surface of the protein, and generally has GSH binding sites in the vicinity [6]. According to the structure and conserved domain, Grxs can be roughly divided into three categories. The first category is a classical Grx of about 9–14 kDa with two

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Table 1
Summary of primers used in the present research.

Primers	Sequences
Grx2-F1	ATGAAGGGCCCAGTTGCT
Grx2-R1	CTGGACCAAATCTGCTAATTTTC
Grx2-3′F1	GGCATTTAGTGATATCGGAG
Grx2-5′R1	CAAACACTCTGGGTACAGTTCTGGCA
3′RACE Outer Primer	TACCGTCGTTCCACTAGTGATTT
UPM Long primer	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
Short primer	CTAATACGACTCACTATAGGGC
Grx2-RT-F1	TGATAAGCAGCCTGATGGTG
Grx2-RT-R1	GCCTTGTTTCTGTTCCTCCA
Grx 2i-F (with T7)	GGATCCTAATACGACTCACTATAGGGGCTCACCGAGTTGTCA
Grx 2i-F	GGCTCACCGAGTTGTCA
Grx 2i-R (with T7)	GGATCCTAATACGACTCACTATAGGTGCTGGACCAAATCTGC
Grx 2i-R	TGCTGGACCAAATCTGC
GFPi-F (with T7)	TAATACGACTCACTATAGGGAGAGTGCCCATCCTGGTCGAGCT
GFPi-F	GTGCCCATCCTGGTCGAGCT
GFPi-R (with T7)	TAATACGACTCACTATAGGGAGATGCACGCTGCCGTCCTCGAT
GFPi-R	TGCACGCTGCCGTCCTCGAT
β-Actin F	GCCCATCTACGAGGGATA
β-Actin R	GGTGGTCGTGAAGGTGTAG
GPx-F	AGGGACTTCCACCAGATG
GPx-R	CAACAACTCCCCTTCGGTA
GST-F	AAGATAACGCAGAGCAAGG
GST-R	TCGTAGGTGACGGTAAAGA
Trx-F	TTAACGAGGCTGGAAACA
Trx-R	AACGACATCGCTCATAGA
LvGrx 3-F	TTCAGCCGCACAACCATA
LvGrx 3-R	AGTCCTTGTCGCACTTCCTC

cysteines in their active motif, such as *Escherichia coli* Grx 1 and the human Grxs. The second category, represented by *E. coli* Grx 2, corresponds to a very special type of Grx including more homologies with glutathione S-transferase (GST) than other types. It has a dimercapto active site C—P—Y—C and the oxidoreductase activity of Grx. The third category mainly consists of the monothiol glutaredoxin, which typically has an active motif C-G-F—S, like yeast Grx 3, Grx 4, and Grx 5 [6–9]. In recent years, more and more functions of Grx are demonstrated by many studies. It was reported that Grx play a crucial role in oxidative stress, protein modification, signal transduction, apoptosis and cell differentiation [10,11]. However, these researches mainly focus on plants, mammals, *Escherichia coli*, yeast and clam [12–14], and it has not been studied in shrimp yet. In our research, the full-length ORF of Grx 2 was cloned from *L. vannamei*. To further investigate the functions of LvGrx2, the expression responses of LvGrx2 were determined in *L. vannamei* under ammonia-N stress or after LPS injection.

2. Materials and methods

2.1. Experimental shrimp

Pacific white shrimp *L. vannamei* (body weight 12.2 ± 2.1 g) were purchased from a local shrimp farm in Haikou and reared in cycling-filtered plastic tanks in salinity (25‰), temperature (24 ± 2 °C) and pH (7.9–8.0) conditions. Prior to experiment, shrimp were fed twice daily with commercial shrimp diet (40.0% protein, 5.0% fat, 5.0% fiber and 16.0% ash) until 24 h before the experiment began.

Hemolymph (400 μl) was collected from the pericardial sinus of each shrimp with 25 gauge needle and 1.5 ml syringe absorbing an equal volume of ice-cold anticoagulant solution (AS, glucose 20.5 g L^{-1}, sodium citrate 8 g L^{-1}, sodium chloride 4.2 g L^{-1}, pH 7.5) [15]. Hemocyte were separated by centrifugation at 800*g* and 4 °C for 5 min, and the hemocyte pellets were immediately used for RNA extraction. Eyestalk, gill, hepatopancrea, muscle and intestine were dissected out and preserved in liquid nitrogen for RNA extraction [16].

2.2. Ammonia-N stress and LPS challenge

Expression responses of LvGrx 2 in shrimp under ammonia-N stress or after LPS injection were determined. Two doses of ammonia-N (zero control and 20 mg L^{-1}) were set in the ammonia-N exposure experiment. The ammonia-N solution was prepared by adding NH_4Cl to 25‰ seawater. The actual mean doses for test and control groups were 0.01 and 20.53 mg L^{-1} respectively. Test and control groups were conducted in triplicate with 25 shrimp per replicate in plastic tanks with 80 L water (24 ± 2 °C, pH 7.9–8.0, salinity 25‰) aerated continuously using an air stone. The seawater was renewed daily.

LPS (2 mg ml^{-1}, from *Escherichia coli* 055:B5, Sigma) was dissolved in physiological saline solution (0.85% NaCl) to give a dose of 2 μg $μl^{-1}$. Shrimp were randomly divided into two groups with 25 shrimp per replicate. According to the previous study [17], the LPS injection dose was 8 μg g^{-1} wet weight, and the shrimp injected with the same amount of sterile physiological saline solution were maintained as control group.

Nine shrimp were collected from each group after 0, 3, 6, 12, 24 and 48 h ammonia-N or LPS injection. The hepatopancrea and gill tissues of shrimp were dissected quickly and separately preserved in liquid nitrogen immediately, and then store at −80 °C before RNA extraction.

2.3. RNA extraction and cDNA synthesis

Briefly, tissues were grinded in liquid nitrogen, then total RNA was extracted with TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The extracted RNAs were treated with RNase-free DNase I (TakaRa, Japan). The quantity and quality of each RNA were measured by NanoDrop 2000 spectrophotometer (NanoDrop Technologies, USA), and RNA integrity was verified on agarose electrophoresis. First-strand cDNA was synthesized from total RNA using PrimeScript RT reagent Kit With gDNA Eraser (Takara, Dalian, China).

2.4. Cloning of Grx cDNA fragment and sequence analysis

A middle fragment of Grx 2 cDNA was initially obtained from our previous transcriptome data of *L. vannamei*. BLAST analysis of all

expressed sequence tags (ESTs) from a cDNA library of many species revealed that EST was highly similar to the previously identified Grx 2. The first-round polymerase chain reaction (PCR) was performed using primers Grx2-F1 and Grx2-R1 (Table 1) with 1 cycle of denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, followed by a 10 min extension at 72 °C.

All PCR products were cloned into pMD18-T Vector (Takara, Dalian) and sequenced by the Beijing Genomics Institute (BGI) (Guangzhou, China).

2.5. Rapid amplification of cDNA ends (RACE)

Based on the obtained middle sequence of *L. vannamei* Grx 2, specific primers were designed to obtain the 3′ and 5′ ends of Grx 2 using the 3′-Full RACE Core Set with PrimeScript RTase (Takara, Dalian) and SMART RACE cDNA Amplification Kit (Clontech, USA). For the 3'end, the PCR was performed by Gene-specific primer Grx2-3′F1 and 3′ RACE Outer Primer. The PCR protocol was as follows: 1 cycle of denaturation at 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, followed by a 10 min extension at 72 °C.

The 5′-untranslated regions (UTR) were amplified initially with primers Grx2-5′R1 and universal primer mix (UPM). The PCR was carried out using 30 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min. 5′-and 3′-RACE PCR products were gel purified, cloned, and sequenced as described. All primer sequences are provided in Table 1.

2.6. Alignments of sequences and construction of a phylogenetic tree

The nucleotide and amino acid sequence of Grx 2 cDNA were analyzed via the BLAST algorithm at NCBI web site (http://www.ncbi.nlm.nih.gov/blast) and Expasy search program (http://au.expasy.org/tools/). Multiple sequence alignment was performed with Clustal X software. A neighbor-joining phylogenic tree was made by the deduced amino acid sequences of Grx 2 through using MEGA 6.0 software. Bootstrap sampling was used with 1000 replicates.

2.7. Tissue expression of Grx 2

Relative expression levels of LvGrx 2 in different tissues were determined by quantitative real-time PCR. The house-keeping gene β-actin was used as internal control.

2.8. Real-time qPCR analysis of LvGrx 2 mRNA expression

A real-time qPCR detection method using Stratagene Mx3005P (Agilent, USA) was applied to study the expression of LvGrx 2 in hepatopancrea and gill after ammonia-N stress and LPS injection. RNA extraction and cDNA synthesis were followed by the method described previously. The amplification was performed with the primers of target gene Grx2-RT-F1/Grx2-RT-R1, and internal control gene β-actin F/β-actin R (Table 1). The thermal cycling parameters were 94 °C for 3 min to activate the polymerase, followed by 40 cycles of 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 20 s. Results were calculated by the $2^{-\Delta\Delta Ct}$ method and all of data were given in terms of relative mRNA expressed as means ± SD.

2.9. LvGrx 2 gene silence experiment

2.9.1. Double strands RNA (dsRNA) synthesis

DNA templates for dsRNA preparation were performed using the gene-specific primers Grx 2i-F and Grx 2i-R with a T7 promoter sequence at the 5′ end (Table 1). As a control, green fluorescent protein (GFP) dsRNA was amplified using a pGFP vector as template and primers with T7 promoter sequences shown in Table 1. The PCR products were purified by QIAquick PCR Purification Kit (QIAGEN, Germany) and used as template for synthesis of LvGrx 2 or GFP dsRNA with the T7 RiboMAXTM Express RNAi System (Promega, USA). The synthesized dsRNAs were verified by agarose electrophoresis, their concentrations were estimated by using spectrophotometry at an absorbance of 260 nm.

2.9.2. Gene silence and semi-quantitative RT-PCR analysis

To silence the expression of LvGrx 2, dsRNA was dissolved in protective buffer (10 mM Tris-HCl, pH 7.5, 400 mM NaCl) to a final concentration of 2.5 μg/μl 12.5 μg dsRNA was injected rapidly into the lateral area of the fourth abdominal segment of each shrimp [18]. At various times after injection (0, 12, 24 and 48 h), hepatopancreas was collected individually from each shrimp. First-strand cDNA was synthesized as the method mentioned above. The level of Grx 2 gene transcript in each RNA sample was examined by semi-quantitative RT-PCR using specific primers pair Grx2-RT-F1 and Grx2-RT-R1 (Table 1). *β-actin* gene was served as the internal control for cDNA template normalization. First-strand cDNA (1 μl) synthesized from total RNA was subjected to PCR in a 25 ml reaction volume. The PCR protocol was: 94 °C for 3 min, 25 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, followed by a 10 min extension at 72 °C. The product was analyzed by TAE-1.0% agarose gel electrophoresis.

2.9.3. Effects of ammonia-N stress on LvGrx 2-interfered shrimp

To assess the effects of ammonia-N on shrimp following suppression of the LvGrx 2 gene expression, one set of shrimp was injected with GFP dsRNA and another set was injected with Grx 2 dsRNA. After 24 h injection, both sets of shrimp were exposed to ammonia-N (20 mg L^{-1}), then samples were collected after 0, 1.5, 3, 6, 12 and 24 h exposure. At each sampling time, hepatopancreas of nine shrimp from each group were collected, and the hepatopancreas was frozen in liquid nitrogen separately, and then store at −80 °C before RNA extraction.

2.9.4. Expression levels of LvGrx 2 and antioxidant enzymes

Relative expression levels of LvGrx 2 and some other antioxidant enzymes (GPx, GST, TRx and LvGrx 3, Table 1) were assayed in LvGrx 2-silenced and control (dsGFP) shrimp using real-time qPCR.

2.9.5. Protein carbonyl content

Protein carbonylation content in hepatopancreas of shrimp was quantified using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions. Carbonyl content was calculated based on its molar absorption coefficient as 22,000 M^{-1} cm^{-1}. Results were expressed as nmol protein carbonyl per mg protein.

2.9.6. MDA (malondialdehyde) content

Content of MDA were determined by monitoring the formation of thiobarbituric acid reactive substances to evaluate lipid peroxidation in hepatopancreas of shrimp. Measurement was using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions. Briefly, hepatopancreas were homogenized and then centrifuged at 11,000 × *g* for 10 min. Supernatant was mixed with thiobarbituric acid solution following incubation at 95 °C for 40 min. After cooling, the mixture was centrifuged (4000 r/min, 10 min) and absorbance of supernatant was determined at 532 nm. The protein of supernatant was quantified based on the Bradford method using total protein quantification kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), with bovine serum albumin as the standard. Results were expressed as nmol MDA per mg protein.

2.10. Statistical analyses

All data are presented as means ± standard deviation (SD). Normality test of the data was checked by the Shapiro-Wilk test. One way analysis of variance (ANOVA) was used to analyze the data and a multiple comparison (Tukey) test was conducted to compare the

Fig. 1. Nucleotide and deduced amino acid sequences of Grx 2 gene of *Litopenaeus vannamei*. The letters in box indicate the start codon (ATG), the stop codon (TAG) and the polyadenylation signal sequence (AATAAA). The 3′-UTR instability motifs (ATTTA) are underlined. The catalytic amino acids (C—P—Y—C) are shaded by gray and the active cysteine residues (C) are underlined. The other cysteine residues Cys^{7}, Cys^{30}, Cys^{92} and Cys^{116} in Grx 2 are also underlined.

significant differences among treatments using SPSS 18.0 program (SPSS Inc., Chicago, IL, USA). A P value < .05 was considered significant.

3. Results

3.1. Full-length cDNA cloning of Grx 2 in L. vannamei

The full-length cDNA of LvGrx 2 was 735 bp, including 11 bp 5′-UTR and 364 bp 3′-UTR with an AATATA repeat and a poly (A) tail. The open reading frame (ORF) of LvGrx 2 was 360 bp, which encoded a putative protein of 119 amino acids with calculated molecular weight of 12.87 kDa with an estimated pI 8.22. The GSH binding sites of *Lv*Grx 2 are formed by Lys^{36}, Cys ^{39}, Tyr^{41}, Arg ^{81}, Thr $^{82, 97}$, Val^{83}, Pro^{84}, $Gly^{95, 96}$ and Glu^{98} (Fig. 1).

3.2. Homology and phylogenetic analysis

Blastx analysis revealed that *Lv*Grx 2 shared the highest similarity with Grx 2 from the coelacanth *Latimeria chalumnae* (XP_006012994.1, 59% identity) and the plateau frog *Nanorana parkeri* (XP_018423419.1, 59% identity). The second similarity in homology is *Cynoglossus semilaevis* (XP_008336650.1, 58% identity), *Sinocyclocheilus anshuiensis* (XP_016311131.1, 58% identity) and *Cyprinus carpio* (XP_018920444.1, 58% identity). The other species with a similarity of 57% were *Rana catesbeiana* (ACO51594.1), *Astyanax mexicanus* (XP_007233296.1), *Cyprinodon variegatus* (XP_015227435.1), *Oryzias latipes* (XP_004068148.1), *Sinocyclocheilus rhinocerous* (XP_016377366.1) and *Crocodylus porosus* (XP_019386257.1). The last three species used for homologous alignments were *Kryptolebias marmoratus* (XP_017284588.1), *Saccoglossus kowalevskii* (XP_002730955.2) and *Acanthisitta chloris* (XP_009080205.1), which shares 55%, 54% and 53% identity with that of *Lv*Grx 2 respectively.

Multiple sequence alignment demonstrated that *Lv*Grx 2 contains conserved domains which are critical to the Grx protein in fundamental structure and function (Fig. 2). The dimercapto active site C—P—Y—C was highly conserved in all the alimented sequence of Grx 2. In addition, Grx 2 possessed 11 GSH binding sites. These feature suggested that *Lv*Grx 2 was a new member of Grx 2 family.

The phylogenetic tree showed that the Grx 2 members were mainly divided into two branches by their vertebrate or invertebrate origin (Fig. 3). *Lv*Grx 2 was firstly formed a closest group with Grx 2 from the acorn worm *Saccoglossus kowalevskii*, and then grouped with those from original vertebrate like *Crocodylus porosus* and *Acanthisitta chloris*, and frog *Rana catesbeiana* and *Nanorana parkeri*, and further grouped with those from fish species.

3.3. Tissue expression of Grx 2 in L. vannamei

Real time-qPCR result showed that LvGrx 2 was detected in all examined tissues of *L. vannamei* and was expressed most strongly in gill, intestine and hepatopancrea, followed by hemocyte, but poorly in eyestalk and muscle (Fig. 4).

3.4. Expression profile of LvGrx 2 in hepatopancrea and gill during ammonia-N stress

Expression profiles of LvGrx 2 in hepatopancrea of *L. vannamei* during ammonia-N exposure are shown in Fig. 5A. The transcription of LvGrx 2 in hepatopancrea significantly increased from 6 to 48 h (P <

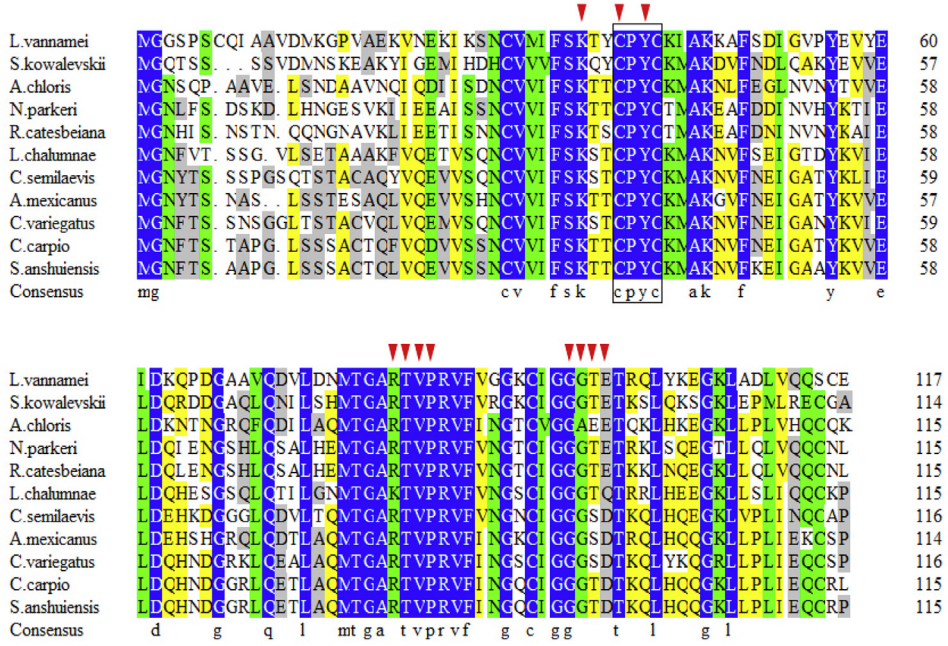

Fig. 2. Multiple sequence alignment of the Grx 2 in *Litopenaeus vannamei*. The blue region indicates all sequences share the same amino acid residue. Gaps are indicated by spots to improve the alignment. Square shows the special catalytic residues and arrows red triangle represents the eleven GSH binding sites. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

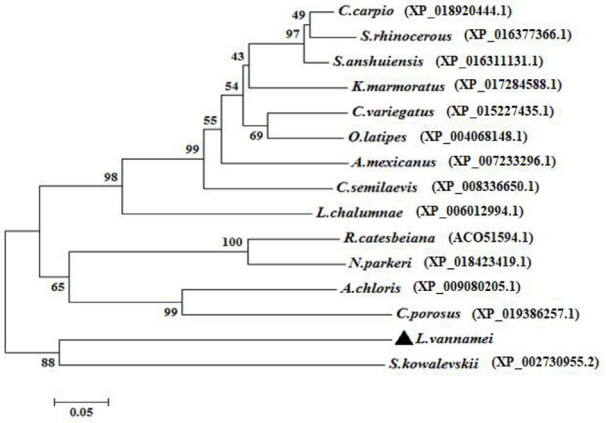

Fig. 3. Phylogenetic tree consists of 15 sequences of Grx 2 from different animals. Amino acids sequences were aligned by using CLUSTALW, and the tree was constructed with NJ method in MEGA 4. The numbers at the forks indicate the bootstrap values (in %) out of 1000 replicates. The scale bar was 0.05.

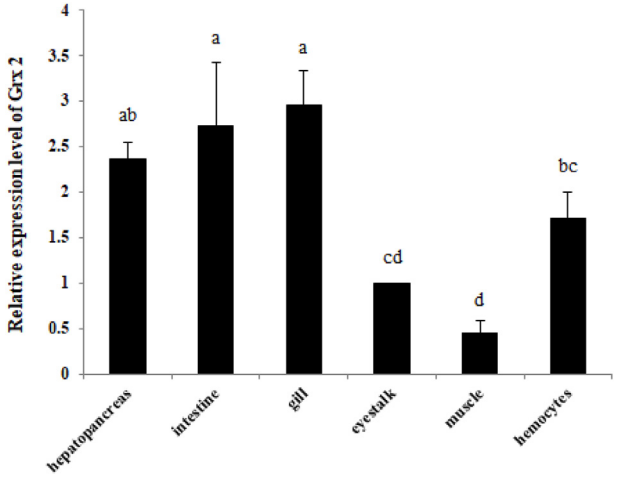

Fig. 4. Relative expression of Grx 2 in different tissues of *Litopenaeus vannamei*, including hepatopancrea, intestine, gill, eyestalk, muscle and hemocyte, respectively. (β-actin was as internal control, $P < .05$).

.05), and reached the highest level after 12 h exposure, which was about 3.20 times higher than the control group. The expression response of LvGrx 2 in gill is similar to that in hepatopancrea. Expression level of LvGrx 2 in gill was up-regulated during 6–48 h exposure ($P < .05$). The peak value of LvGrx 2 expression in gill was found after 24 h exposure, which was 17.58 times higher than the control (Fig. 5B).

3.5. Expression profiles of LvGrx 2 in hepatopancrea and gill after LPS injection

The expression profile of LvGrx 2 in hepatopancrea of LPS-injected shrimp was presented in Fig. 6A. The transcriptional level of LvGrx 2 in hepatopancrea increased after 3–6 h injection ($P < .05$), but fell back to the control level after 12–24 h ($P > .05$), and then increased again after 48 h ($P < .05$). The highest level of LvGrx 2 in hepatopancrea was presented after 48 h LPS injection. LvGrx 2 expression level in gill significantly upregulated after 12–24 h injection ($P < .05$), and the peak expression level was observed after 24 h (Fig. 6B).

3.6. Efficiency of LvGrx 2 dsRNA interference

The dsGrx 2 and dsGFP, which were noted at approximately 400 and 700 bp respectively, were successfully synthesized (Fig. 7A). As revealed by electrophorogram, a decrease in mRNA expression level of LvGrx 2 was observed after 12 h injection with LvGrx 2 dsRNA (Fig. 7B). At 24 h, the transcript of LvGrx 2 was the least. The express levels of LvGrx 2 did not change significantly in shrimp injected with the same amount of GFP dsRNA until the end of the experiment (Fig. 7B).

3.7. Expression levels in LvGrx 2-interfered shrimp under ammonia-N stress

In dsGFP-injected shrimp, LvGrx 2 expression level did not change after 24 h injection (the 0 h of ammonia-N exposure) ($P > .05$). After 1.5–24 h ammonia-N stress, significant increases were observed in LvGrx 2 expression ($P < .05$). In dsGrx 2-injected shrimp, after 24 h injection, LvGrx 2 transcription was significantly inhibited ($P < .05$). And then, after 1.5–24 h ammonia-N stress, transcriptional level of

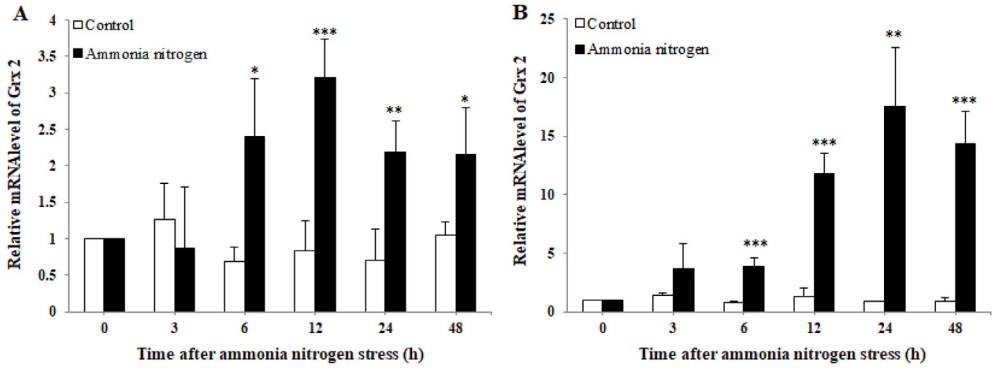

Fig. 5. Analysis of Grx 2 expression in hepatopancrea (A) and gill (B) of ammonia nitrogen stress group and the control group by real-time PCR at 0, 3, 6, 12, 24 and 48 h post-stress (n = 9). Statistical significance was calculated using SPSS 18.0 (* stands for $P < .05$, ** stands for $P < .01$, *** stands for $P < .001$).

LvGrx 2 recovered to the level of pre-injection ($P > .05$). From 0 to 24 h ammonia-N exposure, mRNA expressions of LvGrx 2 in LvGrx 2-suppressed shrimp kept observably lower than those in dsGFP-injected shrimp ($P < .05$) (Fig. 8A).

LvGrx 3 mRNA levels in dsGFP-injected shrimp significantly enhanced from 1.5 to 24 h ammonia-N exposure ($P < .05$). In dsGrx 2-injected shrimp, significant increase in expression of LvGrx 3 was found after 1.5 and 3 h ammonia-N stress ($P < .05$), and then LvGrx 3 expression level reduced to the pre-injection level ($P > .05$). Compared to dsGFP shrimp, transcriptional level of LvGrx 3 in LvGrx 2-interfered shrimp was higher after 1.5 h exposure ($P < .05$), but lower from 6 to 24 h ($P < .05$) (Fig. 8B).

In dsGrx 2 shrimp, GPx mRNA level rapidly upregulated after 1.5 h exposure ($P < .05$), peaked at 3 h, then returned to PI levels at 6–24 h ($P > .05$), while increased at 1.5 h, 3 h and 24 h in dsGFP shrimp ($P < .05$). GPx expression levels in LvGrx 2-interfered shrimp were lower than those in dsGFP shrimp after 6–24 h exposure ($P < .05$) (Fig. 8C).

GST mRNA levels upregulated from 1.5 to 12 h ammonia-N stress in dsGFP shrimp ($P < .05$), whereas no significant change could be observed in dsGrx 2 shrimp ($P > .05$). Compared to dsGFP shrimp, expression levels of GST in dsGrx 2 shrimp were continuously lower from 0 to 24 h exposure ($P < .05$) (Fig. 8D).

Trx transcriptional levels in dsGFP shrimp upregulated after 1.5–6 and 24 h stress ($P < .05$), while those in LvGrx 2-interfered shrimp increased from 1.5 to 12 h ($P < .05$), and peaked at 12 h. Compared to dsGFP shrimp, expression levels of Trx in dsGrx 2 shrimp were higher after 0 and 12 h exposure ($P < .05$) (Fig. 8E).

3.8. Protein carbonyl content in LvGrx 2-interfered shrimp under ammonia-N stress

As showed in Fig. 9, in dsGFP shrimp, protein carbonyl content increased significantly after exposure to ammonia-N from 1.5 to 12 h ($P < .05$), and then dropped to the initial level after 24 h ($P > .05$). In LvGrx 2-suppressed shrimp, protein carbonyl content rised continuously from 1.5 to 24 h ($P < .05$), and the peak occurred after 12 h stress. Compared to dsGFP shrimp, levels of protein carbonyl in dsGrx 2 shrimp were higher after 12 and 24 h exposure ($P < .05$) (Fig. 9).

3.9. MDA content in LvGrx 2-interfered shrimp under ammonia-N stress

Obvious increase of MDA levels was noted after 3–24 h ammonia-N stress in both dsGFP shrimp and dsGrx 2 shrimp ($P < .05$). Compared to dsGFP shrimp, MDA content in dsGrx 2 shrimp were higher from 1.5 to 24 h exposure ($P < .05$) (Fig. 10).

4. Discussion

Grx 2 is a special, heat-stable type oxidoreductase of Grx, which possess a dimercapto active site Cys–Ser–Tyr–Cys and more homologies with GST than other types [19,20]. The sequence of Grx 2 has been obtained in human, mouse, amphibian, some species of fish, waterflea *Daphnia pulex* and clam *Venerupis philippinarum*, but there is still no information about Grx in decapod crustacean. In this study, the complete full-length ORF of Grx 2 was obtained from the Pacific white shrimp, *L. vannamei*. The full-length cDNA of LvGrx 2 was 735 bp, including the open reading frame (ORF) 360 bp which encoded a putative protein of 119 amino acids. Moreover, a dimercapto active site C−⊜P−Y−C and 11 GSH binding sites were found in LvGrx 2, which were

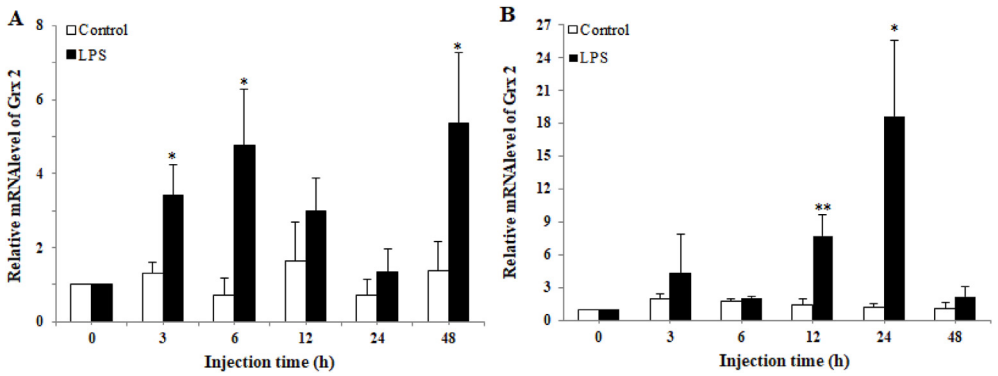

Fig. 6. Analysis of Grx 2 expression in hepatopancrea (A) and gill (B) of LPS injection group and the control group by real-time PCR at 0, 3, 6, 12, 24 and 48 h post-injection (n = 9). Statistical significance was calculated using SPSS 18.0 (* stands for $P < .05$, ** stands for $P < .01$).

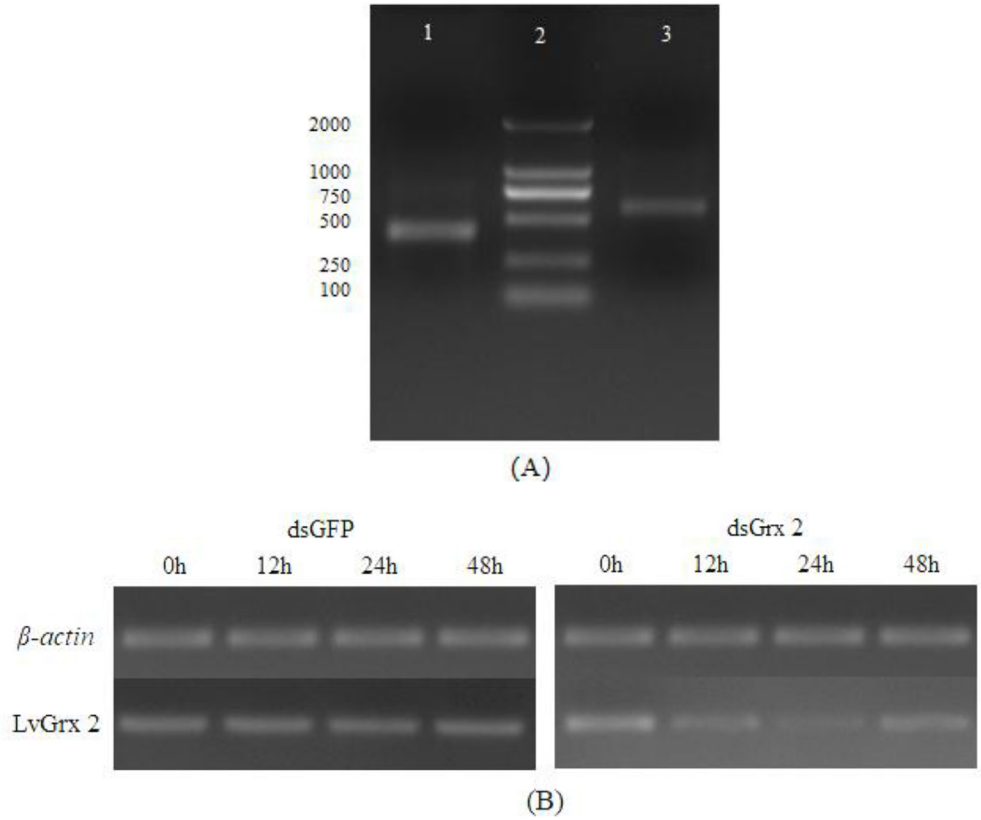

Fig. 7. The quality of dsRNA synthesized in vitro and the silencing efficiency of LvGrx 2 dsRNA injection. (A) Detection of purified dsRNA of GFP and LvGrx 2 genes by gel electrophoresis. Lane 1, LvGrx 2 dsRNA. Lane 2, DNA marker (DS, 2000). Lane 3, GFP dsRNA. (B) LvGrx 2 expression in shrimp of dsGFP-injection and dsGrx 2-injection by semi-quantitative RT-PCR. The *β-actin* gene was used as a control.

highly conserved in members of Grx 2 from other species. Phylogenetic analysis showed that LvGrx 2 was closest with that from *S. kowalevskii* which belonged to a hemichordate class of invertebrates and lived in the sediment on the seabed. Noteworthily, the second closer group was the ancient vertebrate (*C. porosus* and *A. chloris*), suggesting that LvGrx 2 was ancient in origin and slow development in evolution.

The expressions of Grx 2 in different tissues have been studied in some aquatic species. In zebrafish, Grx 2 was improved to express in heart even play an indispensable role in the development of the heart [21]. In clam *Venerupis philippinarum*, the mRNA transcripts of Grx 2 could be detected in all the examined tissues, including hepatopancrea, hemocyte, adductor muscle, mantle and gill, while the highest expression level was presented in gill and hepatopancrea [13]. In our study, LvGrx 2 expression was the first report in tissues of decapoda crustacean, finding that the LvGrx 2 mRNA was detected in all examined tissues of untreated *L. vannamei*, suggesting that there is a basal expression of Grx 2 in all the detected tissues. The greatest LvGrx 2 expression was observed in gill, intestine and hepatopancrea, which is similar to that of *V. philippinarum* [11], but differs from zebrafish [13,21,22]. Grx 2 highly expressed in digestive organs and gill of both *L. vannamei* and *V. philippinarum*, suggesting that Grx 2 might play similar roles among aquatic invertebrates.

More and more researches reported that Grx 2 play a crucial role in oxidative stress, signal transduction, protein modification, cell differentiation and apoptosis [10,11]. For example, Grx 2 of *Saccharomyces cerevisiae* participates in the apoptotic death guided by cadmium, which was considered as a mode of cellular suicide causing the removal of damaged cells [23]. Furthermore, mammalian Grx 2 enables to catalyze the reduction of GSH-mixed disulfides effectively, which was proved to contribute to redox regulation and antioxidant defences of mitochondria [24–26]. However, the specific functions and characterization of Grx 2 in crustacean are poorly understood. The present study determined the expression responses of LvGrx 2 to ammonia-N stress and LPS injection, in order to investigate its roles in defence system against environmental stress and pathogen infection.

Ammonia-N, which derived from nitrogenous wastes excretion of aquatic animals and food decomposition, is a common water pollutant in aquaculture. It has been reported to be toxic to aquatic animals, including decapod crustacean [27–29]. Previous studies of crustacean demonstrated that ammonia-N would cause toxic ROS/RNS overproduction and subsequent oxidative damage of tissues, and antioxidant activity would be induced to defense against oxidative stress [30–33]. In the present ammonia-N experiment, up-regulation of LvGrx 2 expression could be found in both hepatopancrea and gill of the challenged shrimp from 6 h to 12 h post-stress, suggesting that LvGrx 2 play a vital role in defence mechanism against ammonia-N stress, due to its common function in redox regulation and antioxidant defence. On the other hand, though the response patterns of LvGrx 2 between hepatopancrea and gill was similar, we found that the induced LvGrx2 transcription in gill was much higher than that in hepatopancrea. Under ammonia-N exposure, the highest expression level of LvGrx 2 in gill was about 17.58 fold, while that was about 3.20 fold in hepatopancrea. These facts indicate that LvGrx 2 is more important in antioxidant defence of gill during ammonia-N exposure. Gill is the organ that contacts with the external environment directly. Ambient toxic factors, such as ammonia, enter into the aquatic animals mainly through the gill [33], so gill might be the most vulnerable site under environmental stress. Drastic up-regulation of LvGrx 2 in gill would protect this important organ involved in respiratory.

LvGrx 2 RNA interference experiment was carried out to further analyzed its function under ammonia-N stress. Results showed that compared to the control group, expression levels of LvGrx 2 and some other antioxidant enzymes, including GPx and GST, downregulated in LvGrx 2-interfered shrimp, whereas significant upregulation in mRNA expression of LvGrx 3 and Trx could be found at several exposure time. On the other hand, contents of both protein carbonyl and MDA in LvGrx 2-interfered shrimp were higher than those in shrimp of control group. These findings demonstrate that LvGrx 2 expression have a close

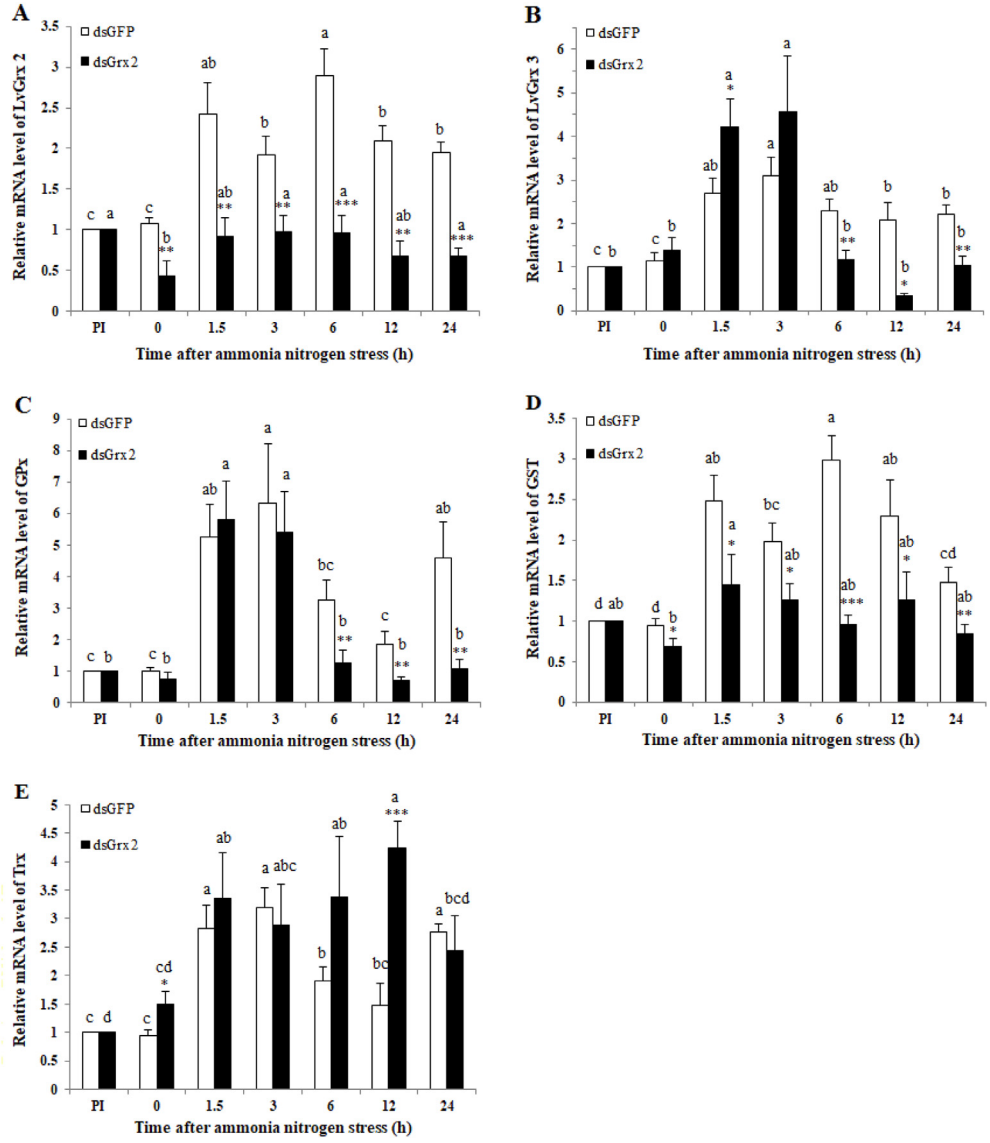

Fig. 8. Relative expression levels of LvGrx 2 (A), LvGrx 3 (B), GPx (C), GST (D) and Trx (E) in hepatopancreas of LvGrx 2-interfered shrimp after exposure to ammonia-N (dsGFP group was used as control) (n = 9). Significant differences between dsGrx 2 group and dsGFP group at the same exposure time are indicated by asterisks (* stands for $P < .05$, ** stands for $P < .01$). Dates at the same group with different letters are signifcantly different ($P < .05$).

connection to other antioxidant enzymes, and LvGrx 3 and Trx seemed to compensate the function of LvGrx 2 to some extent. These results also indicate that LvGrx 2 play a nonsubstitutable capability in oxidative defence.

LPS is an integral component in the outer membrane of Gram-negative bacteria. It is the highly antigenic and cytotoxic substance, and is also known as endotoxin [34]. The cellular toxicity of LPS on shrimp hemocyte has been described clearly [30,34,35]. Our previous research demonstrated its toxic mechanism that LPS caused ROS/RNS-induced Ca^{2+}-mediated hemocyte apoptosis and subsequent THC decline [15]. In the molecular level, LPS was shown to affect the expression levels of immune related genes in shrimp [36,37]. In this study, variation of LvGrx 2 expression could also be observed in hepatopancrea and gill of shrimp after LPS injection. Transcription of LvGrx 2 in hepatopancrea seemed more sensitive to LPS stimulation than that in gill. The expression of LvGrx 2 in hepatopancrea was significantly up-regulated in the early and late stages after LPS injection, while its expression level in gill sharply increased in the middle period. These facts suggest that LvGrx 2 play a critical role in immune defence against pathogenic bacteria infection. The expression intensity of LvGrx 2 was also different between hepatopancrea and gill after LPS stimulation. The highest relative expression level of LvGrx 2 was about 18.65 fold in gill, while that was about 5.36 fold in hepatopancrea. This observation is similar to that of ammonia-N stress experiment, indicating that LvGrx 2 play a more vital role in defence mechanism of gill.

In summary, the full-length ORF of LvGrx 2 was cloned from *Litopenaeus vannamei* and proved as a member of thioredoxin superfamily. LvGrx 2 was expressed in all tested tissues in white shrimp with the highest expression in the gill, intestine and hepatopancrea. The mRNA expression levels of LvGrx 2 in hepatopancrea and gill were obviously induced in shrimp when challenged by ammonia-N stress or LPS injection, suggesting that LvGrx 2 plays an important role in shrimp defense system against environmental stress and pathogen infection. LvGrx 2 expression silence experiment demonstrated that under ammonia-N stress, suppression of LvGrx 2 transcription caused obvious increases of oxidative damage level in both lipid and protein, and inhibited the expression levels of some antioxidant enzymes, indicating that LvGrx 2 in shrimp participates in oxidative defence and regulation of antioxidant system.

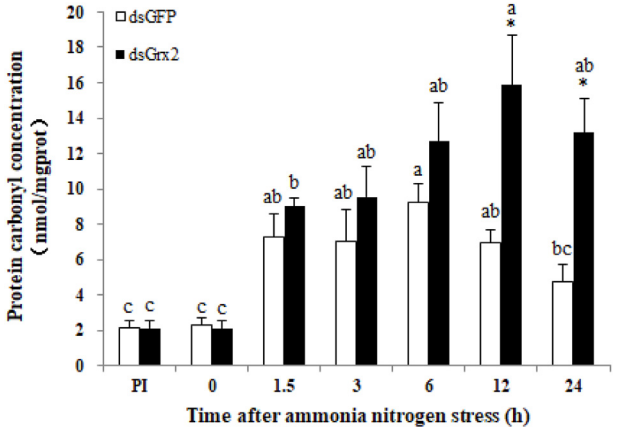

Fig. 9. Protein carbonyl content in hepatopancreas LvGrx 2-interfered shrimp after exposure to ammonia-N (dsGFP group was used as control) (n = 9). Significant differences between dsGrx 2 group and dsGFP group at the same exposure time are indicated by asterisks (* stands for $P < .05$, ** stands for $P < .01$). Dates at the same group with different letters are signifcantly different ($P < .05$).

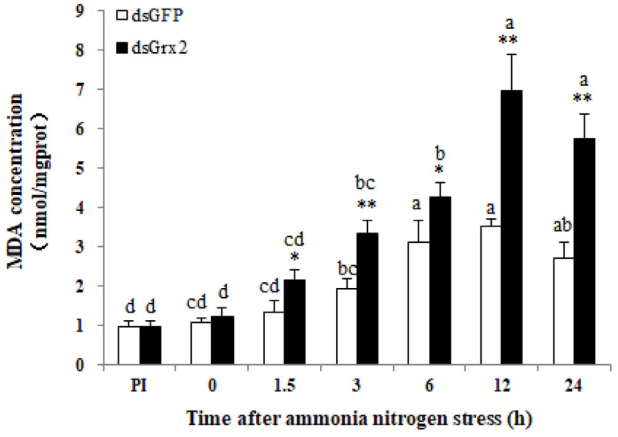

Fig. 10. MDA content in hepatopancreas LvGrx 2-interfered shrimp after exposure to ammonia-N (dsGFP group was used as control) (n = 9). Significant differences between dsGrx 2 group and dsGFP group at the same exposure time are indicated by asterisks (* stands for $P < .05$, ** stands for $P < .01$). Dates at the same group with different letters are signifcantly different ($P < .05$).

Acknowledgements

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