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Cloning and functional characterization of thioredoxin gene from kuruma shrimp *Marsupenaeus japonicus*

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ABSTRACT

As an important disulfide reductase of the intracellular antioxidant system, Thioredoxin (Trx) plays an important role in maintaining oxidative stress balance and protecting cells from oxidative damage. In recent years, there is increasing evidence that Trx is a key molecule in the pathogenesis of various diseases and a potential therapeutic target for major diseases including lung, colon, cervical, gastric and pancreatic cancer. However, few knowledge is known about the function of Trx in virus infection. In this study, we reported the cloning and functional investigation of a Trx homologue gene, named *MjTrx*, in shrimp *Marsupenaeus japonicus* suffered white spot syndrome virus (WSSV) infection. *Mj*Trx is a 105-amino acid polypeptide with a conservative Cys-Gly-Pro-Cys motif in the catalytic center. Phylogenetic trees analysis showed that *Mj*Trx has a higher relationship with Trx from other invertebrate and clustered with Trx1 from arthropod. *MjTrx* transcripts is abundant in the gill and intestine tissues and can be detected in the hemocytes, heart, stomach, and hepatopancreas tissues. The transcription levels of *MjTrx* in hemocytes, gills and intestine tissues of shrimp were significantly up-regulated after white spot syndrome virus infection. *Mj*Trx was recombinant expressed in vitro and exhibited obvious disulfide reductase activity. In addition, overexpression *Mj*Trx in shrimp resulted in the increase of hydrogen peroxide (H_2O_2) concentration in vivo. All these results strongly suggested that *Mj*Trx functioned in redox homeostasis regulating and played an important role in shrimp antiviral immunity.

1. Introduction

The normal metabolism of aerobic organisms produces superoxide anion, singlet oxygen, hydroxyl radicals, peroxyl radicals and hydrogen peroxide components which were termed reactive oxygen species (ROS) during their physiological metabolism [1]. Low concentration of ROS may be beneficial or even essential for intracellular signaling or intrusive pathogen clearance [2,3]. However, excessive accumulation of intracellular ROS can cause oxidative damage to macromolecules resulting in physiological dysfunction [4]. Therefore, several efficient redox systems including glutathione, thioredoxin and pyridine nucleotide were developed in aerobic organisms to maintain the redox homeostasis [5–8].

Thioredoxin is a 12-kDa multi-functional protein that is found ubiquitously in prokaryotes and eukaryotes [9]. Trx has a pair of redox-active Cys (Cys-XX-Cys) at its catalytic center (Trx motif) which reduces disulfide bonds in target proteins directly, thus involved in several cellular responses including gene expression/regulation, cell proliferation, cellular signaling, and apoptosis [10,11]. Then the oxidized active-site cysteines of Trx are regenerated by Trx reductase and nicotinamide adenine dinucleotide phosphate (NADPH) [12–14].

The shrimp aquaculture industry of the world has developed rapidly in recent years but huge economic losses occurs every year due to the disease [15] caused by bacteria and viruses [16]. In shrimp culture, pathogenic viruses include white spot syndrome virus (WSSV), yellow head virus (YHV) and Taura syndrome (TSV). WSSV virus is the most dangerous pathogen and can cause 90–100% mortality in shrimp. WSSV is a double-stranded DNA virus and belongs to *Nimaviridae* virus family [17], whose virion contains nucleocapsid, tegument and envelope [18–21]. WSSV is highly invasive [22–24], with a broad host range [25,26] from amphipods, ostracods, crabs, lobsters, copepods, waterflies to shrimp. WSSV targets a variety of tissues including gills,

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hepatopancreas, heart, stomach, ovaries, spermary and intestine, which causing rapid onset and significant mortality.

Kuruma shrimp *Marsupenaeus japonicus* is a kind of economical shrimp species [27]. Infectious diseases caused by WSSV have became a huge threat to this industry and have caused huge economic losses. Trx is one of the important antioxidant families which functioning in keeping redox hemostasis during the immune response of animals [28]. To understand the function of Trx in antivirus immunity of *M. japonicus*, we identified a Trx gene and studied of its function in vitro and in vivo.

2. Materials and methods

2.1. Virus inoculum, shrimp, and virus challenge

Virus stock was prepared as described previously [29] and was stored at – 20 °C until use. The WSSV inoculum was prepared from the supernatant of the virus stock by centrifugation at 3000*g* (10 min) and further diluted to 10^7 copies/mL with PBS as described previously [30].

Adult shrimp *M. japonicus* were purchased from Jinan seafood market in Jinan, Shandong Province, China and cultured in laboratory tanks filled with air-pumped seawater temporarily. Before the experiment, the shrimp were acclimatized for 24 h at room temperature (25 °C) under laboratory conditions. Shrimp in the experimental groups were challenged by intramuscular injection with virus inoculum of WSSV (2.5×10^5 copies per shrimp), while shrimp in the control group were injected with the same volume of phosphate buffer saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). At various time points (0, 6, 12, 24 and 48 h) after injection, Hemolymph was extracted from the ventral sinus of the shrimp using 1/10 volume of anticoagulant buffer (10% sodium citrate, pH 7.0), then centrifuged at 800 × *g* for 15 min at 4 °C to isolate the hemocytes immediately. Afterward, the gills, hepatopancreas, heart, stomach and intestines of the experimental shrimp (n = 3) and control shrimp (n = 3) were collected and frozen immediately in liquid nitrogen and stored at – 80 °C.

2.2. Total RNA isolation and cDNA synthesis

Total RNA was extracted from shrimp tissues by Unizol reagent (TransGen Biotech, Beijing, China). The first-strand cDNA was synthesized by TransScript All-in-One First-Strand cDNA Synthesis kit (TransGen Biotech, Beijing, China).

2.3. Gene cloning and sequence analysis

The full-length cDNA sequence was obtained by sequencing of the ovary cDNA library of shrimp *M. japonicus*. The cDNA sequence was confirmed by sequencing the open reading frame (ORF) fragment amplified with the gene specific primers *Mj*SF (5′-ATGGTTTACCAAGTGAAAG -3′) and *Mj*SR (5′- TTACTTGTTCTTCTGAATG -3′) from another cDNA template. The PCR conditions were as follows: 1 cycle at 94 °C for 3 min; 35 cycles at 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 50 s; and 1 cycle at 72 °C for 10 min. Nucleotide sequence homology and amino acid sequence was analyzed using on-line program BLAST (http://blast.ncbi.nlm.nih.gov/). Protein translation and deduced protein prediction was obtained using ExPASy (http://www.au.expasy.org/) while SMART (http://www.mart.embl-heidelberg) was used for signal sequencing and domain prediction. Phylogenetic analysis was performed using the MEGA 5.0 via contiguous method and multiple alignments were performed by the GenDoc software [31].

2.4. Detection the expression patterns of MjTrx

The tissue distribution of *MjTrx* in the hemocytes, gills, intestines, heart, hepatopancreas and stomach was analyzed using semi-quantitative RT-PCR with the primers *MjTrx*RTF(5′-CTGATGTCGTGTTCCTGAAGGTAGA-3′), *MjTrx*RTR (5′- CTTGGCTTCGTTGGCACCAGTCAGG-3′), *β-actin* was amplified as the internal control with primer pairs *MjActin*RTF (5′-CAGCCTTCCTTCCTGGGTATGG-3′) and *MjActin*RTR (5′-GAGGGAGCGAGGGCAGTGATT -3′). The temporal expression profile of *MjTrx* in hemocytes, gills and intestine were detected by quantitative RT-PCR (qRT-PCR). The reaction was conducted in a 10 μL mixture contained 1 μL of 1:10 diluted original cDNA, 5 μL of SYBR Green Master mix(TransStart Tip Green qPCR superMix, TRANS, China), 1 μL (1 mM) of each primer and 2 μL ddH_2O. The PCR parameters were as follow: a initial step at 95 °C for 10min; 40 cycles of 95 °C for 10s, 60 °C for 50s and 75 °C for 2s. The specific amplification of PCR products was confirmed by melting curve. The relative mRNA levels of *MjTrx* were analyzed using the $2^{\triangle\triangle Ct}$ methods. The data were presented as the relative expression levels (means ± S.D), and significant differences ($P < 0.05$) were analyzed by one-way Analysis of Variance (ANOVA).

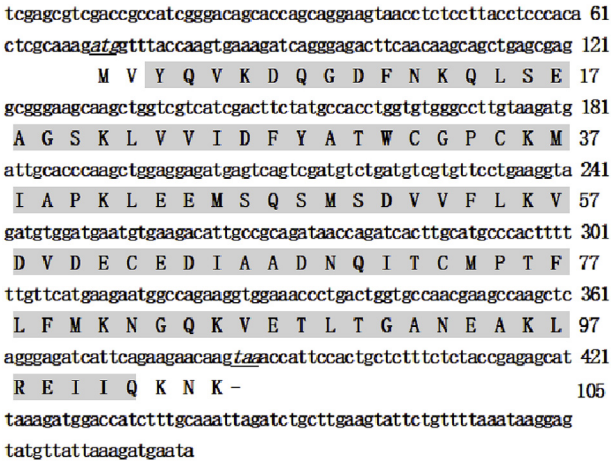


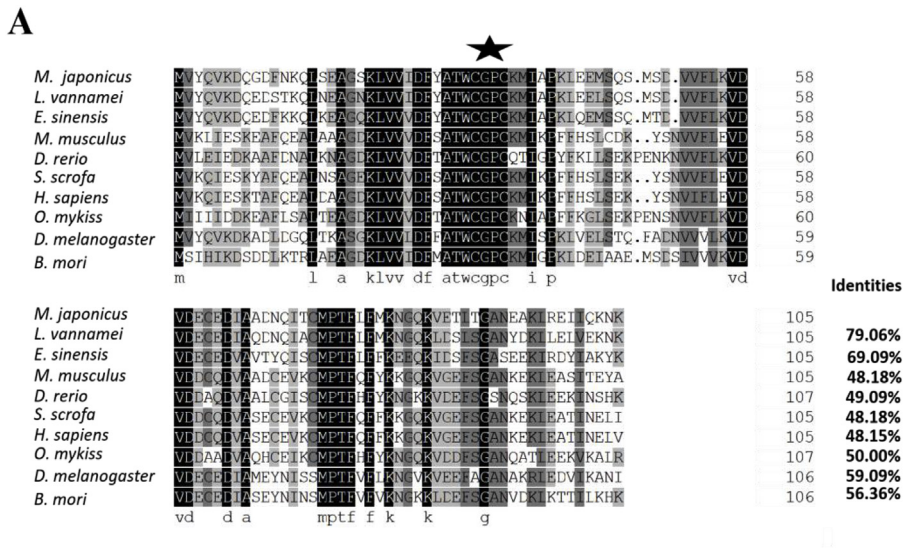
Fig. 1. Complete cDNA sequence and deduced amino acid sequence of *Trx* gene from *M. japonicus*. The start (ATG) and stop (TAA) codons are italicized and underlined. The Ahpc-TSA domain from 2 to 102 amino acids is shaded.

2.5. Recombinant expression and purification of MjTrx

The primer pair *MjTrx*ExF (5′-TACTCAGGATCCATGGTTTACCAAGTGAAA-3′) and *MjTrx*ExR (5′-TACTCACTCGAGTTACTTGTTCTTCTGAAT-3′) was used to amplify a cDNA fragment encoding the mature *Mj*Trx peptide. The underlined are the *Bam*H I and *Xho*I cleavage sites. The amplified PCR product fragment and the pET-30a (+) vector were digested with the same restriction enzyme (FastDigest, Thermo Scientific), ligated with T4 ligase, and transformed into competent *Escherichia. Coli* Rosetta cells for recombinant expression. The recombinant proteins were induced expression by adding 0.5 mM Isopropyl β-D-1-thiogalactopyranoside(IPTG) at 28 °C and purified by His-Bind Affinity column (Roche Inc., USA) following the instructions.

2.6. Detection the rMjTrx activity in vitro

Insulin disulfide reduction assay was performed to detect the r*Mj*Trx activity. In brief, dithiothreitol (DTT) was added to a final concentration of 2 mM in 1 ml of reaction mixture which containing 50 mM Tris-HCl (pH 7.5), 10 mM ethylene diamine tetraacetic acid (EDTA), 0.2 mM insulin (Biotopped) and r*Mj*Trx with three concentration (5 μM, 25 μM, 50 μM) to started the reaction. The equal volume PBS was added as the blank control group. Then the optical density (OD) value was measured at 690 nm at room temperature every 1 min to draw the reaction curve. The all treatments were performed in triplicate, and data were presented as means ± SE.



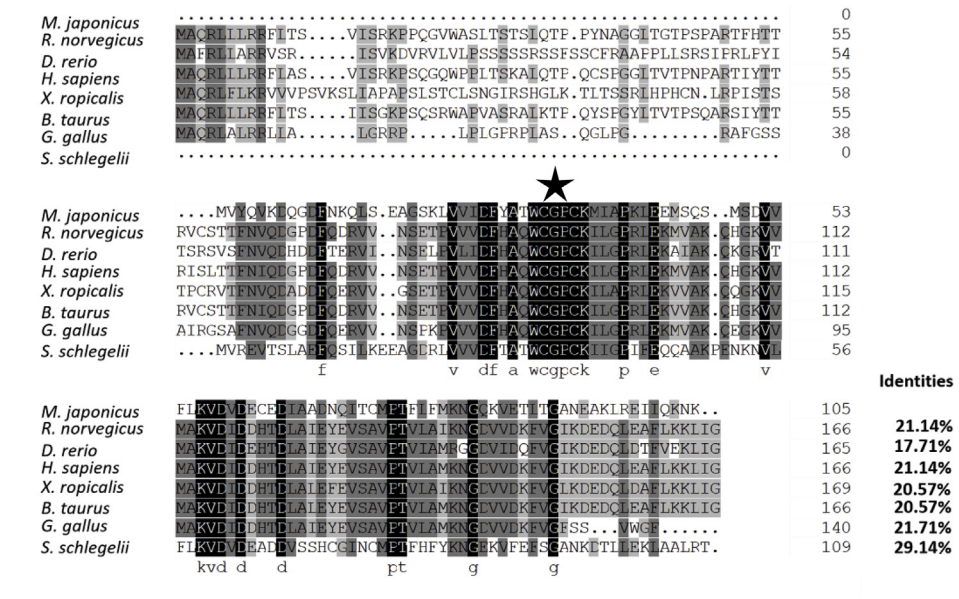


Fig. 2. Amino acids sequence alignment of *Mj*Trx with Trxs from other organism. (A)Amino acids sequence alignment of *Mj*Trx with Trx1 from other organisms (*Penaeus vannamei* ACA60746.1, *Eriocheir sinensis* ACQ59118.1, *Mus musculus* NP_035790.1, *Danio rerio* NP_001002461.1, *Sus scrofa* NP_999478.1, *Homo sapiens* AAF87085.1, *Oncorhynchus mykiss* ACO08786.1, *Drosophila melanogaster* AAF37263.1, *Bombyx mori* ABM92269.1). (B)Amino acid sequence alignment of *Mj*Trx with Trx2 from other organisms (*Rattus norvegicus* NP_445783.1, *Danio rerio* NP_991204.1, *Homo sapiens* AAF86467.1, *Xenopus tropicalis* NP_001008161.1, *Bos taurus* NP_776633.1, *Gallus gallus* NP_001026581.1, *Sebastes schlegelii* BAK82164.1). Numbers on the right indicate the amino acid position of different sequences. The black star indicates the completely conserved sequence: CGPC. The data under "Identities" represent the sequence similarity between *Mj*Trx and Trx from other species.

2.7. Detection the rMjTrx activity in vivo

To confirm the effect of injecting r*Mj*Trx in vivo, the amount of H_2O_2 in shrimp gills were evaluated. Shrimp (*M. japonicas*, 10–15 g) were randomly divided into four groups. The first group was normal without any stimulation, the second group was challenged with WSSV (2.5×10^5 per shrimp), the third group was injected with r*Mj*Trx mixed with WSSV (20 μg of protein per shrimp), while the fourth group was injected with rGFP mixed with WSSV (20 μg of protein per shrimp) as the control. For tissues collection, at 12 h after challenge, 3 individuals were randomly selected from each of the groups. The gills of shrimp were dissected and the H_2O_2 concentration of the gills was measured according to the kit (S0038, Beytime). The experiment was repeated three times with 3 batches of shrimp.

3. Results

3.1. Gene cloning of Trx from M. japonicus

The full-length *MjTrx* (GenBank accession No. MH161183) was 518 bp including a 71 bp 5′ untranslated region (UTR), a 315 bp ORF and a 134 bp 3′ UTR that includes a stop codon (TAA) (Fig. 1). The *MjTrx* ORF encodes a 105 amino acid sequence with a theoretical molecular weight of 11.8 kDa. A typical redoxin/Ahpc-TSA domain was existed in *Mj*Trx by SMART program analysis.

3.2. Multiple sequence alignment and phylogenetic analysis and of Trx

A score of Trx including Trx1 and Trx2 from different species were selected for multiple sequence alignment and phylogenetic analysis. Multiple sequence alignment revealed that the Trx1 from various species have highly sequence similarities, especially in the thioredoxin family active site which contains the CGPC motif (Fig. 2A). The *Mj*Trx

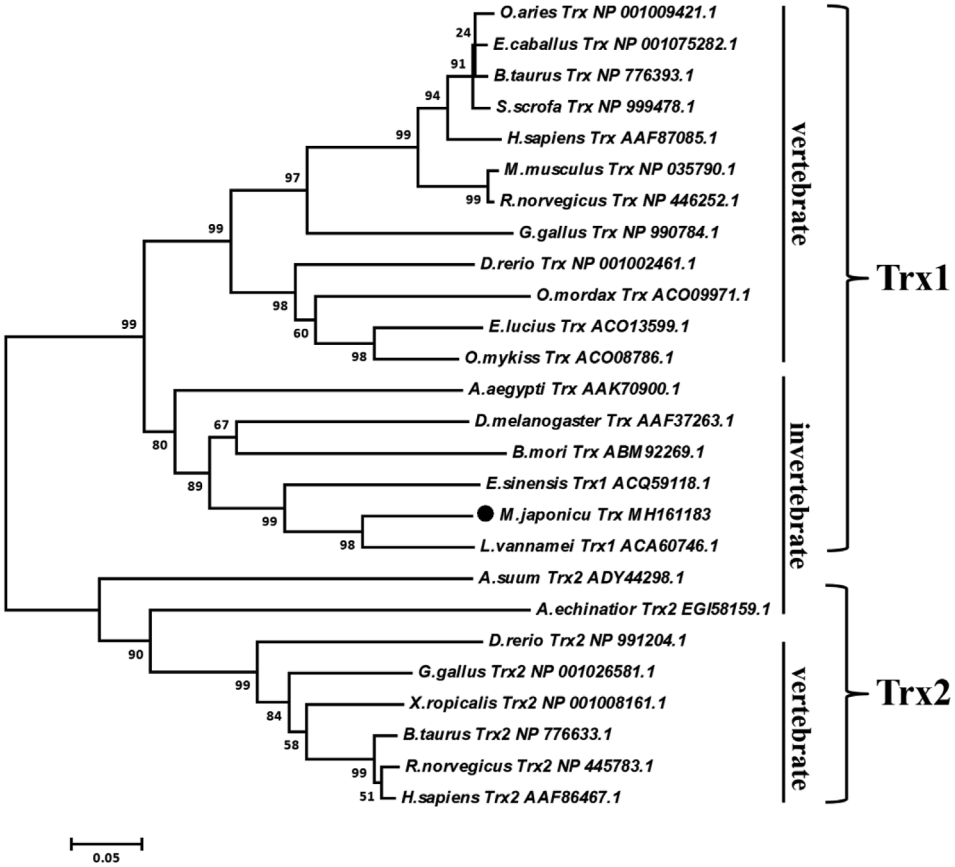


Fig. 3. Phylogenetic analysis of *Mj*Trx from various animals. The GenBank accession numbers are shown after the scientific names. Neighbor-joining trees were constructed using MEGA 5.0. One thousand bootstraps were performed to determine the reproducibility of the results.

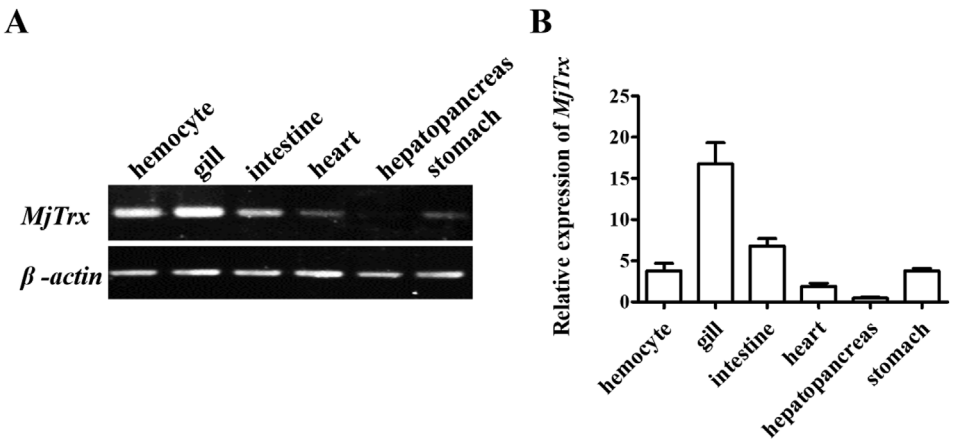


Fig. 4. Tissue distribution of *MjTrx*. (A) Semiquantitative RT-PCR detection of relative expression of *MjTrx* in different tissues. Each tissues sample of hemocyte, gill, intestine, heart, hepatopancreas and stomach were collected from three individual *M. japonicus*. (B)Statistic analysis of the relative expression of *MjTrx*. The *β-actin* gene was used as an internal control. Vertical bars represented the means ± SD (N = 3).

shared higher sequence identity with Trx1 (from 49% to 79%) than Trx 2 (no more than 20%) from other species (Fig. 2B). Phylogenetic analysis showed that Trx from vertebrate and invertebrate were clustered into two different groups. Interestingly, Trx proteins were also clustered into Trx1 and Trx2 subgroups in invetebrate, and the *Mj*Trx clustered with Trx1 from arthropod (Fig. 3).

3.3. Tissue distribution MjTrx

To determine the expression level of *MjTrx* mRNA in shrimp tissues, semiquantitative RT-PCR was used to detect the transcription level of *MjTrx* in hemocytes, gills, intestine, heart, hepatopancreas and stomach of shrimp. The results showed that *MjTrx* mRNA could be detected in all examined tissues, and the highest expression level of *MjTrx* mRNA was detected in gills tissues, followed by the intestine, hemocytes and stomach. The lowest expression level was detected in hepatopancreas tissue (Fig. 4).

3.4. Gene expression pattern of MjTrx in shrimp tissues during virus infection

Three tissues (gills, hemocytes and intestine) were selected according to the tissue distribution of *MjTrx* for investigating the change of *MjTrx* expression during WSSV infection. The transcription of *MjTrx* was enhanced in all the tissues while the time were different. The *MjTrx* mRNA reached a peak at 12 h post infection (hpi) in hemocytes and gills tissues (Fig. 5A and B), nevertheless, a high transcription was observed after 24 hpi and reached to a peak at 48 hpi in intestine tissue (Fig. 5C).

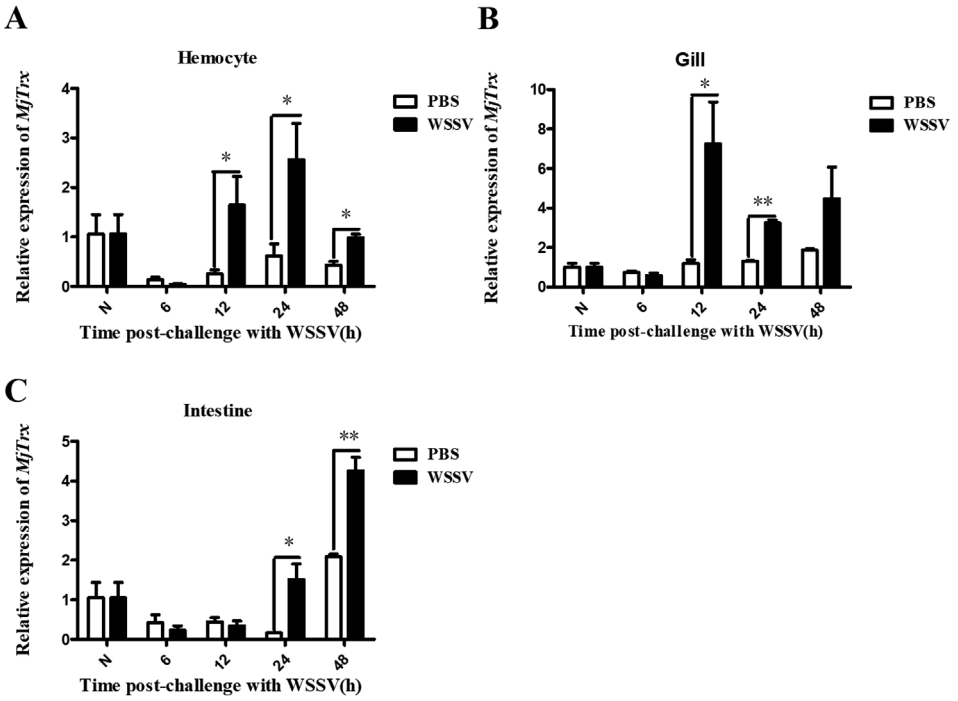


Fig. 5. Temporal expression profile of the *MjTrx* in shrimp tissues: hemocyte (A), gills (B), and intestine (C) after the WSSV infection. *β-Actin* gene was used as an internal control. Values are given as mean ± SD, n = 5. Asterisks indicate significant differences: *P < 0.05, **P < 0.01.

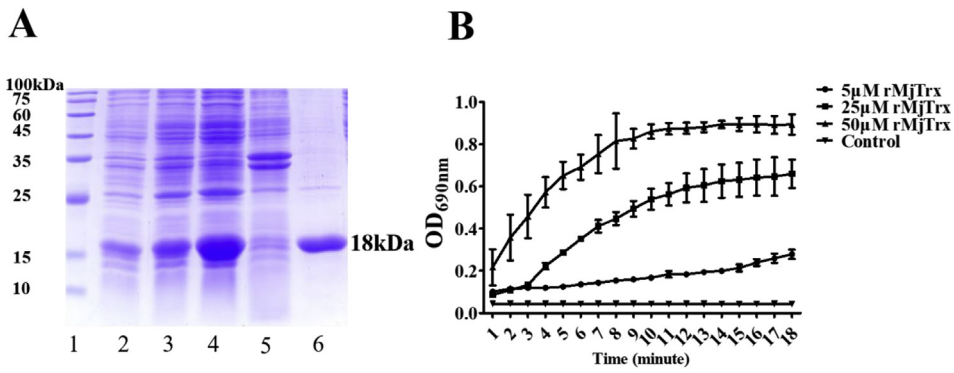


Fig. 6. Expression, purification and function detection of recombinant *Mj*Trx. (A) Expression and purification of r*Mj*Trx. Lane 1, molecular mass marker; Lane 2, lysate of *E. coli* with *Mj*Trx -pET30 without induction; lane 3, lysate of *E. coli* with MjTrx-pET30 induced with IPTG; Lane 4, the supernatant of lysate; Lane 5, the pellet of lysate; lane 6, purified recombinant *Mj*Trx by His Bind resin chromatography; (B) r*Mj*Trx catalyzed reduction of insulin by dithiothreitol. The incubation mixture contained in a final volume of 1.0 mL with 0.2 mM insulin, 10 mM EDTA, 50 mM HEPES, 2 mM DTT and different concentration refolded r*Mj*Trx. Insulin reduction was determined by measuring absorbance at OD690, which was plotted against the reaction time.

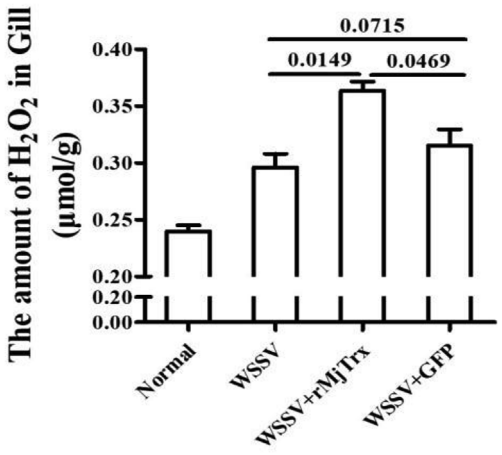


Fig. 7. r*Mj*Trx injection increased the H_2O_2 concentration in vivo. Each bar represents the H_2O_2 concentrations in three pooled samples of shrimp gill lysates. The data on the top of the column means the *p* value between different groups. Bars represent the mean ± SD.

3.5. Recombinant expression, purification and reductive activity detection of MjTrx

Mature *Mj*Trx was recombinant expressed in *E. coli* system as soluble protein, the recombinant *Mj*Trx (r*Mj*Trx) was purified with His-bind resin (Fig. 6A). The activity of r*Mj*Trx was investigated by insulin-disulfide reduce assay. As shown in Fig. 6B, there was no change in the control group until reaction finished. In contrast, the absorbance of r*Mj*Trx at 690 nm increased rapidly in a concentration dependent way after the start of the experiment, which was stabilized after 10 min (Fig. 6B).

3.6. rMjTrx injection increase the H_2O_2 concentration in vivo

To investigate the Trx function in the redox balance of shrimp during WSSV infection, the purified r*Mj*Trx and the control protein (GFP) was injected into the shrimp along with the WSSV, and concentration of ROS in shrimp tissues was detected. As shown in Fig. 7, WSSV infection increased the ROS concentration in detected tissues, the r*Mj*Trx injection further enhanced the production of H_2O_2 while the

control protein had no effect on the ROS concentration.

4. Discussion

Trx is a ubiquitous 12 kDa small thioldisulfide oxidoreductases protein that exist in all kingdoms of life from archaebacteria to mammals, which was first identified in *Escherichia coli* in 1964 [32]. As so far as now, three isoforms have been identified in mammalian cell, including the cytosolic Trx1, the mitochondrial (Trx2) types [11,33] and the truncated form of Trx (Trx 80) [34,35]. In vertebrate, the main Trx isoform is Trx1, which can be secreted out of the cell or translocated into the nucleus under certain circumstances. To date, although Trx was identified in several invertebrate species, most of them were classified as Trx1 isoform [36–39]. In our research, the *Mj*Trx has the archetypical active site sequence -Cys-Gly-Pro-Cys- motif and high sequence similarities with Trx1 from other species. In addition, the *Mj*Trx clustered with other invertebrates in the Trx1 branches and separated with the Trx2 banches which mostly belonged to vetebrates (Fig. 3). On the basis of these typical characteristics, *Mj*Trx was proposed to be a new member of the thioredoxin-1 family.

The transcription of *MjTrx* was detected in all the tissues examined, which was similar to the other reports [37,38]. However, the high mRNA level of *Trx* in tissues was different in various reports. For example, the high expression of *Trx* mRNA was in stomach, followed by gill and fin in golden pompano *Trachinotus ovatus*in [40,41] but in bigbelly seahorse *Hippocampus abdominalis,* the highest expression level was observed in the muscle tissue. In our research, the high expression of *MjTrx* mRNA was detected in gill tissues and was up-regulated by WSSV infection [36]. The discrepancy expression pattern of Trx in different researches may due to the species character and the reference gene they used. Anyhow, Trx was highly expressed in the organs that need high antioxidant efficiency and played important roles in keeping redox homeostasis of them [42,43].

Moreover, the r*Mj*Trx was recombinant expressed in vitro and purified (Fig. 6A). The activity of r*Mj*Trx was investigated by the insulin-disulfide reduction assay according to the classical method described by Holmgren et al. [44,45]. The results showed that the insulin reductive activity of rMjTRX increased quickly in a dose-dependent manner, it reached the peak in a short time at high concentration. These results are in agreement with the observations in other species. For example, the recombinant thioredoxin protein of sea cucumber *Apostichopus japonicas* and large yellow croaker *Larimichthys crocea* displayed higher reducing activity in high concentration although the data were incomparable because of the wavelength of the absorbance and the concentration gradient of rTrx are different [37,38,42]. Those results demonstrated that r*Mj*Trx was expressed as a biologically active protein which can be used for further function investigation.

Bacteria and virus are two kinds of pathogens for multicellular animals. To date, most researches focus on investigating the Trx function in anti-bacterial immunity of animas while few literate reports the function of Trx in anti-virus immunity of them [37]. In our research, the oxidative stress in shrimp that indicated by H_2O_2 concentration was enhanced by WSSV infection and WSSV + r*Mj*Trx injection at 12 hpi, which was in accordance with the research in *Fenneropenaeus indicus* but different from that in shrimp *Penaeus monodon* [46,47]. In *P. monodon,* the oxidative stress was indicated by the ratio of reduced glutathione to oxidized glutathione (GSH/GSSG), which was only significantly increased at 48 h postinfection. The difference maybe caused by the different indicator. Knockdown *Penaeus monodon Trx* (*PmTrx*), a homologue gene of *Trx*1 in shrimp *P. monodon,* led to significant decreases in mortality and viral copy numbers. The author further proved that oxidative stress promotes *Pm*Trx binding to WSSV IE1 in vitro in a catalytic site independent way and supposed that up-regulated expression of *Pm*Trx at 48 hpi was utilized to restore the DNA binding activity of IE1 and benefit to virus amplification [46]. Interestingly, overexpression of *Mj*Trx in vivo caused a steady increase of oxidative stress which maybe benefit to the binding of Trx and IE1, thus facilitating the amplification of virus. Whether this mechanism existed in *M. japonicus* is still need further research.

Although some components of redox system in *M. japonicus* have been reported previously, such as Prx4 [48], thioredoxin has not been studied in *M. japonicus*. In this study, we reported the cloning and the temporal expression study of Trx1 homologue gene in shrimp. We also expressed and purified the r*Mj*Trx protein and analyzed its biological activity in vivo and in vitro. Our results indicated that *Mj*Trx was a functional disulfide reductase enzyme in shrimp and played an important role in shrimp antiviral immunity.

Acknowledgments

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