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Cloning and expression of the thioredoxin gene in *Cherax quadricarinatus* (Von Martens, 1868) (Decapoda: Astacidea: Parastacidae) under bacterial stress

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ABSTRACT

As a member of the thioredoxin (Trx) system, the Trx1 gene plays essential roles in the pathogenesis of various diseases. The function of Trx in bacterial infections in the freshwater crayfish *Cherax quadricarinatus* (Von Martens, 1868), however, has not been clarified. We cloned thioredoxin 1 (CqTrx1) from *C. quadrangularis*, and its expression was investigated. The results showed that the coding sequence of the CqTrx1 gene was 318 bp, encoding a polypeptide of 105 amino acids, contains the conserved motif CGPC (Cys-Gly-Pro-Cys), and was 75.24% and 66.67% identical to sequences of the shrimps *Macrobrachium rosenbergii* (De Man, 1879) and *Fenneropenaeus chinensis* (Osbeck, 1765), respectively. The CqTrx1 transcript existed in all organs tested, with the highest expression level in the intestinal tract and the lowest expression level in the gonads. Under the stress of *Vibrio algolyticus*, *V. parahaemolyticus*, or *Aeromonas hydrophila*, the expression level of CqTrx in the gills was 3.1, 4, and 7 times that in the control group at 3, 9, and 24 h, respectively, indicating that CqTrx1 plays an essential role in shrimp antibacterial immunity.

KEY WORDS: *Aeromonas hydrophila*, cloning, Crustacea, genetics, *Vibrio*

INTRODUCTION

As a disulphide reductase, the thioredoxin (Trx) system plays a significant effect in the regulation of the redox state (Zuo *et al.*, 2019). Genes of this system, including thioredoxin, thioredoxin reductase (Trxr), Nadph oxidase, and cytoplasm proteins, are present in most organisms (Zuo *et al.*, 2019). Three forms of thioredoxin have been characterized (Cunningham *et al.*, 2015; Kohárýová *et al.*, 2015) and all have a Cys-Gly-Pro-Cys-active motif, which is essential for its function as a general protein disulphide oxido-reductase (Léveillard *et al.*, 2017). The best-investigated isomer is cytosolic Trx1, which is 12 kDa and mainly exists in the cytosol. Under oxidation stress, thioredoxin1 can be secreted to the extracellular matrix or migrate to the nucleus (Li *et al.*, 2017). Another isoform, mitochondrial thioredoxin2, has a unique peptide at its N-terminal that makes a difference between it with thioredoxin1, and facilitates transfer trx2 into the mitochondria (Wei *et al.*, 2021). The third isotopic form Trx3 (SpTrx) plays a role in spermatozoa (Kim *et al.*, 2015).

Additional recent experimental evidence suggests that the Trx gene plays important function in multiple diseases and cellular homeostasis. They have been found to be involved in redox signaling and presented in a range of organisms from bacteria to higher eukaryotes. Not only do they provide antioxidant capacity, but they are also involved in various biological events in bacteria, such as DNA synthesis and protein folding (Liyanage *et al.*, 2022). They have been found to be effective in inhibiting the growth of GSH-deficient bacteria (Ren *et al.*, 2020). It was found that viral hemorrhagic septicemia virus and pathogen-associated molecular pattern molecules were observed in blood cells and gill tissues of the wrinkled disc abalone under bacterial stress (Liyanage *et al.*, 2022). To date, Trxs from *Eriocheir sinensis* H. Milne Edwards, 1853 and *Portunus trituberculatus* (Miers, 1876) have been cloned and their expression under pathogenic attack were analyzed (Song *et al.*, 2012).

Vibrio is a group of common foodborne pathogen (Li *et al.*, 2019) that is ubiquitously present in freshwater, estuarine, and marine environments, mainly in fish, shrimp, shellfish, and other

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products. These gram-negative, rod-shaped bacteria are natural constituents of the environment (Baker-Austin *et al.*, 2017). *Vibrio alginolyticus* is involved in attacking the host with a toxic substance (Huang *et al.*, 2019) and is a conditional pathogen of shrimps (Huang *et al.*, 2015). Previous results showed that the phagocytosis rate was decreased, causing acute hepatopancreatic necrosis and the survival rate, activity of phenol oxidase (PO), superoxide dismutase (SOD), and total blood count (THC) were significantly negatively affected by *V. phagocytosis* in *Marsupenaeus japonicas* (Spence Bate, 1888) (Hsieh *et al.*, 2008; Ma *et al.*, 2018; Pooljun *et al.*, 2020).

Aeromonas hydrophila is a facultative anaerobic, gram-negative, opportunistic aquatic pathogen (Stratev *et al.*, 2016). It is considered an important foodborne bacterial zoonotic pathogen in aquaculture. A virulent isolate phenotypically and molecularly identified as a hydrophilic strain was identified as the causative agent of diseased freshwater farmed white shrimp, leading to major economic losses (Zhou *et al.*, 2019a). Moreover, this pathogen can cause seafood-borne diarrheal disease in humans worldwide (Li *et al.*, 2019). Experimental evidence showed that injected *A. hydrophila*, which is highly virulent, causes 100% mortality of an infected freshwater crayfish within 1–6 h (Jiravanichpaisal *et al.*, 2009).

Cherax quadricarinatus (Von Martens, 1868) is a fast-growing and large-sized crayfish and has been studied intensely (Yang *et al.*, 2020). It is an important commercial species and a potential biological model. Trx1 is an important antioxidant gene that can maintain redox hemostasis in individuals during the immune response (Lee *et al.*, 2013). To investigate the role of CqTrx1 in the antiviral immunity of *C. quadricarinatus*, we first cloned the thioredoxin gene CqTrx1in *C. quadricarinatus*, and studied its expression under *V. alginolyticus, A. hydrophila* or *V. parahemolyticus* stress.

MATERIALS AND METHODS

Experimental materials

Tissues from 30 individuals of *C. quadricarinatus* (36 ± 4 g, 12 ± 1.2 cm), including hepatopancreas, muscle, gills, intestine, stomach, testis, abdominal nerve, ovary, heart, eye, and hemocytes were quickly frozen in liquid nitrogen after the crayfish were fully anaesthetized by placing them in an ice bath, and then conserved at −80 °C.

For stimulation by bacteria, 160 crayfish (7.5 ± 0.5 cm, 10.5 ± 1.5 g) were acclimated at 23.0 °C for three days, and then divided into four groups. Each individual was injected with 0.1 ml of *V. alginolyticus*, 0.1 ml of *V. parahemolyticus*, 0.1 ml of *A. hydrophila* in PBS, or 0.1 ml of PBS alone. Crayfish were sampled as soon as they died, and gills of three living crayfish in each group were randomly harvested at 3, 6, 9,12, 24, and 48 h after administration for RNA extraction.

Molecular cloning of the thioredoxin genes

One pair of CqTrx1-specific amplification primers was designed based on the transcripts in *C. quadricarinatus* using single-molecule long-read sequencing (Xu *et al.*, 2021) by Oligo 6.0 software (Table 1). PCR amplification was performed according to the experimental steps established in our laboratory as described by Lu *et al.* (2021), and the sequences were analyzed by using DNAStar 7.1 after sequencing by Sangon Biotech (Shanghai, China).

Bioinformatics analysis

Bioinformatics descriptions were performed as described by Wang *et al.* (2020). NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used in the analysis similar to that of thioredoxin with other CDSs in the nucleotide collection database by the megablast program. The amino acid sequence, protein functional sites, and open-reading frame were analyzed using Expasy (http://www.expasy.org) and NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). The signal peptides were predicted online using SingalP-5.0 (https://services.healthtech.dtu.dk/service.php?SignalP-5.0). Sequence alignment was analyzed by DNAMAN software (https://www.lynnon.com/). Phylogenetic analysis using bootstrap resampling with the maximum likelihood method was analyzed by MEGA10 (http://www.megasoftware.net/) (with 1,000 pseudoreplicates) and TBtools v1.098661software (Chen *et al.*, 2020).

Tissue distribution of CqTrx1

qRT-PCR was performed in a CFX96TM Real-Time System (Bio-Rad, Hercules, CA, USA) as described by Lu *et al.* (2021). All qRT-PCR reactions were completed in triplicate and the comparative CT method was used as previously described (Lu *et al.*, 2021). The relative expression level of CqTrx1 was analyzed using the formula $2^{-\Delta\Delta CT}$ by normalization to the control Ef1a gene. Duncan's method was used to statistically analyze and perform comparisons of the treatment's groups with SPSS software (https://www.ibm.com/products/spss-statistics).

Table 1. Primer sequences.

Primers	Sequence (5'–3') length(bp)	Amplification	Tm	
TrxF1	CTCGTCTCTGCTACCTTCATA	571	59	CDS cloning
TrxR1	TAACAGTGCCATCAAGTACA			
TrxdlF1	AGATTGAGTCCTTCTCTGGCG	93	61	qRT-PCR
TrxdlR1	GAGCATTTCTTGGAGGTAGCA			
EfdlF1	TCAAACTTCCAGAGGGCAATA	109	60	qRT-PCR
EfdlR1	AGTCAACAGAGATGGGCAAAG			

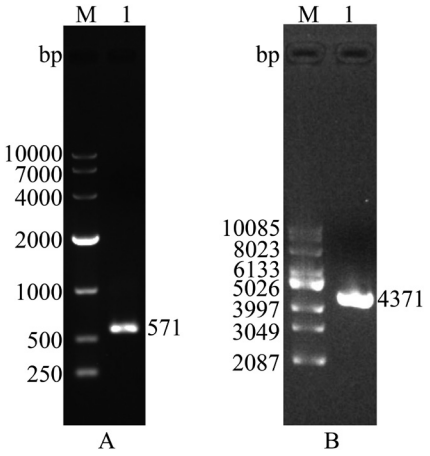

Figure 1. Cloning of the CqTrx1 genes of *C. quadricarinatus*: PCR product of CqTrx1 (**A**). The plasmid of PCR product cloned into the TA cloning vector (**B**).

RESULTS

Cloning of the CqTrx1 genes

The coding sequence (CDS) obtained by RT-PCR of the CqTrx1 gene of *C. quadricarinatus* was 571 bp (Fig. 1A), which was consistent with the expected results. The sequence of CqTrx1 had been submitted to NCBI (GenBank OM243912).

Sequence features of the CqTrx1 genes

The ORF Finder software in the NCBI website analysis indicated that the ORF length of the CqTrx1 genes was 318 bp, encoding 105 amino acids (Fig. 2A). The CqTrx1 protein domain prediction revealed that it contains a thioredoxin family active site (residues 24–42), a cAMP- and cGMP-dependent protein kinase phosphorylation site (13GRI16), a casein kinase II phosphorylation site (6GRI19), and other regions (Fig. 2B).

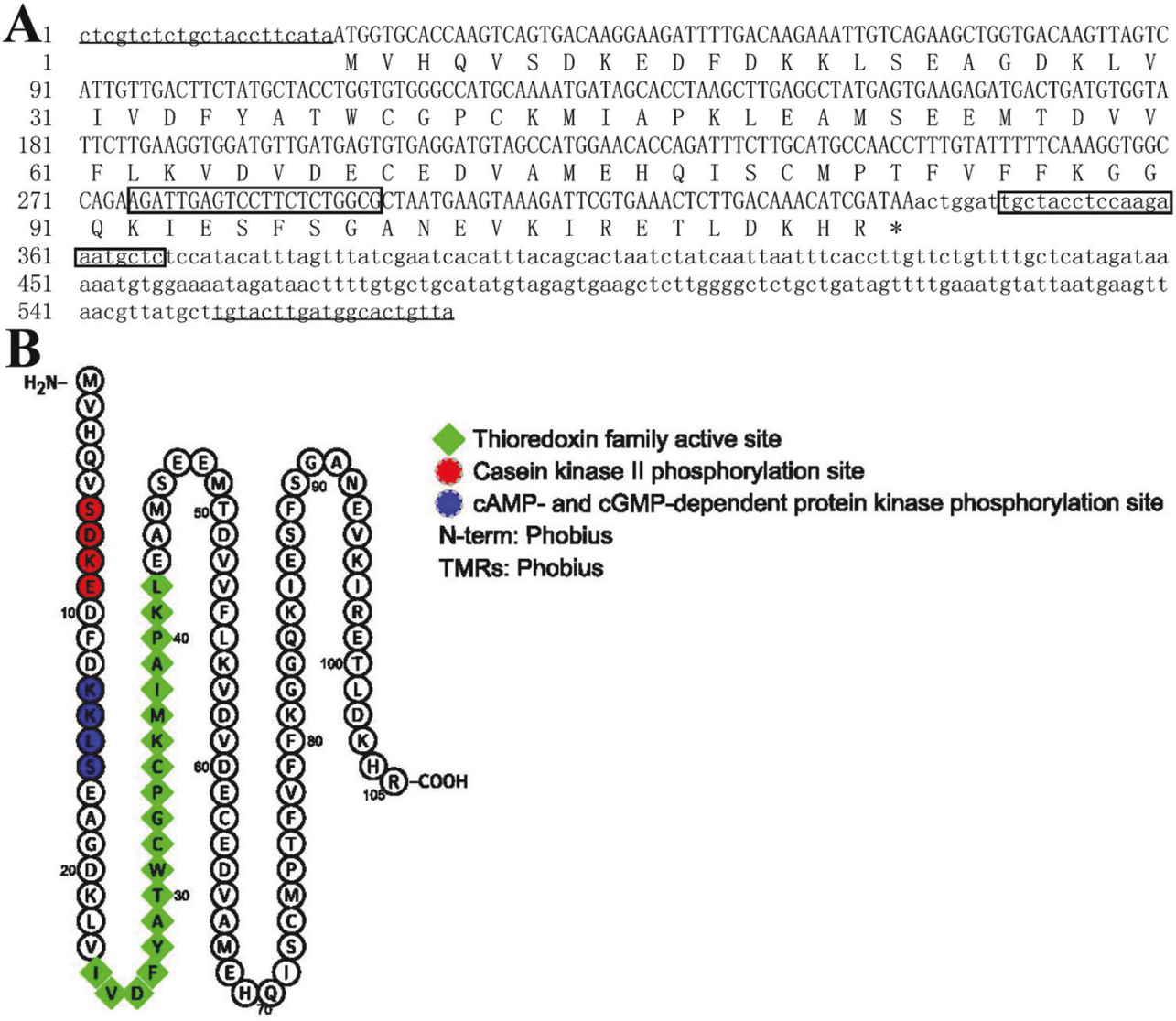

Figure 2. Sequences analysis of the CqTrx1 gene: nucleotide and amino acid sequences (**A**). TrxF1 and TrxR1 primers are underlined, TrxdlF1 and TrxdlR1 are boxed. Motif sites of the CqTrx1 gene (**B**).

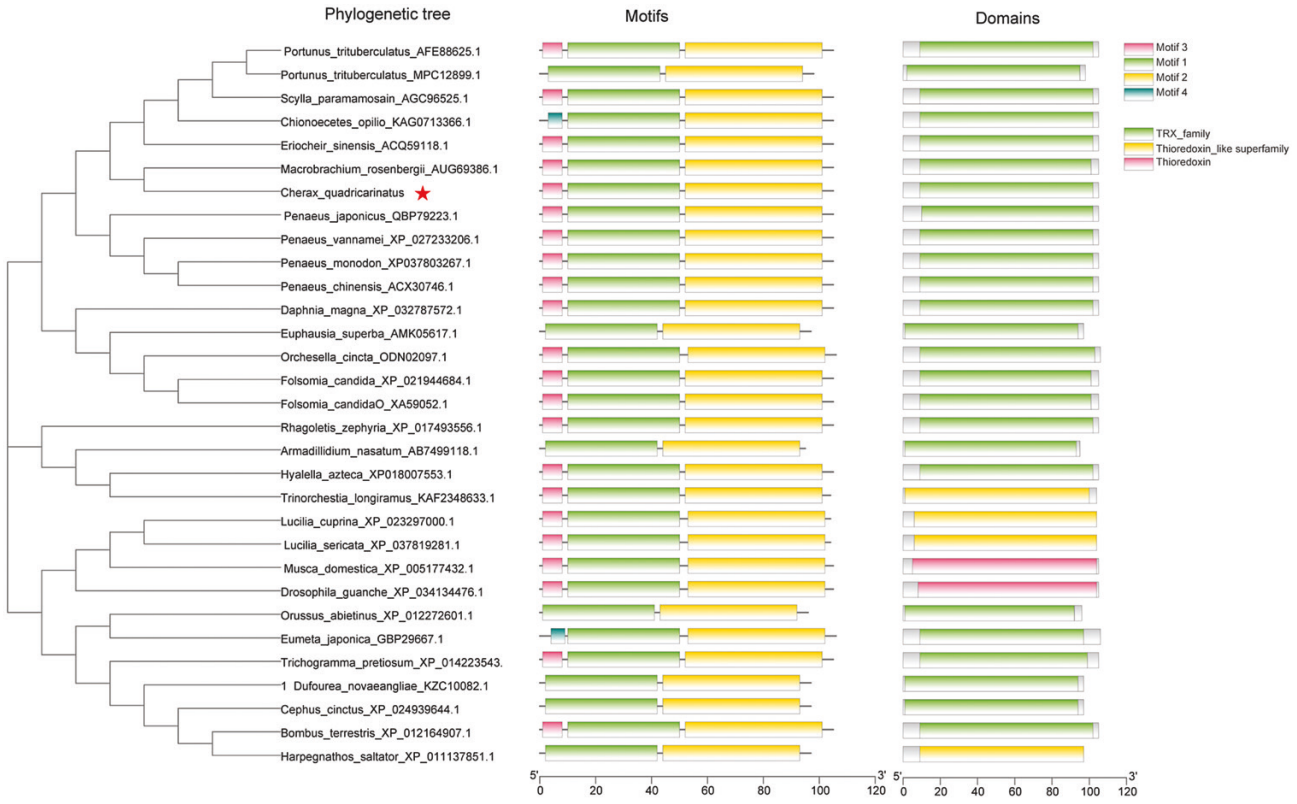

Figure 3. Phylogenetic analysis of the CqTrx1 gene.

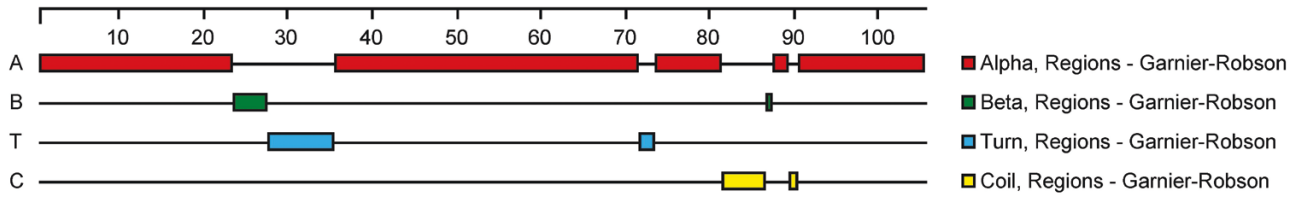

Figure 4. Secondary structures of the CqTrx1 gene of *Cherax quadricarinatus*.

The overall similarity of CqTrx1 in *C. quadricarinatus* was 75.24%, 67.62% and 66.67% identity with that from *Macrobrachium rosenbergii*, *Penaeus monodon* Fabricius, 1798, and *Fenneropenaeus chinensis*, respectively. The phylogenetic tree of Trx based on the amino acid sequences showed that CqTrx1 was located in independent branches (Fig. 3).

The molecular formula of this protein is $C_{527}H_{825}N_{133}O_{163}S_{10}$ and it contains 105 amino acids. Among the 105 amino acids, Glu, Lys, and Val had the highest content (10.5% each), followed by Asp (9.5%), and Tyr (1.0%); the isoelectric point of Cqtrx1 was 4.77. There was no signal peptide in the CqTrx1 N-terminus and it was located in the cytoplasm, containing five α-helices, five β-folds, five T-turns, and two irregular coils (Fig.4). Tissue distribution of the CqTrx1 gene and its expression

Melting curve analysis of qRT-PCR indicated that the primers of CqTrx1 were monomers (Fig. 5A), and the results showed that CqTrx1 transcription was widely found in the heart, gill, hepatopancreas, gonad, intestine, eyestalk, muscle, and nerve tissues (Fig. 5B), in the decreasing order of intestine > gill > heart> nerve > muscle > eyestalk > gonad > hepatopancreas (Fig. 5C), and the maximum difference between their expression levels in tissue can be up to seven times.

The transcription pattern of CqTrx1 mRNA in the gills after bacterial exposure is shown in Figure 6. After *V. alginolyticus* exposure, the expression of the CqTrx1 gene in the gill at 3, 6, and 9 h was significantly higher than that in the control group, and presented a downward trend. This expression value of CqTrx1 was 3.1 times higher at 3 h and 0.37 times lower at 48 h than that in the control group (Fig. 6A)

After exposure to *V. parahaemolyticus*, the expression first increased and then decreased compared with that of the control group, reaching the maximum value at 9 h, which was approximately four times that of the control group and 0.03 times lower at 24 h than that in the control group (Fig. 6B).

The expression level of the CqTrx1 gene under *A. hydrophila* stress was greater than that in the control group except at 6 h, and reached the highest value at 24 h, which was seven times the control expression value (Fig. 6C).

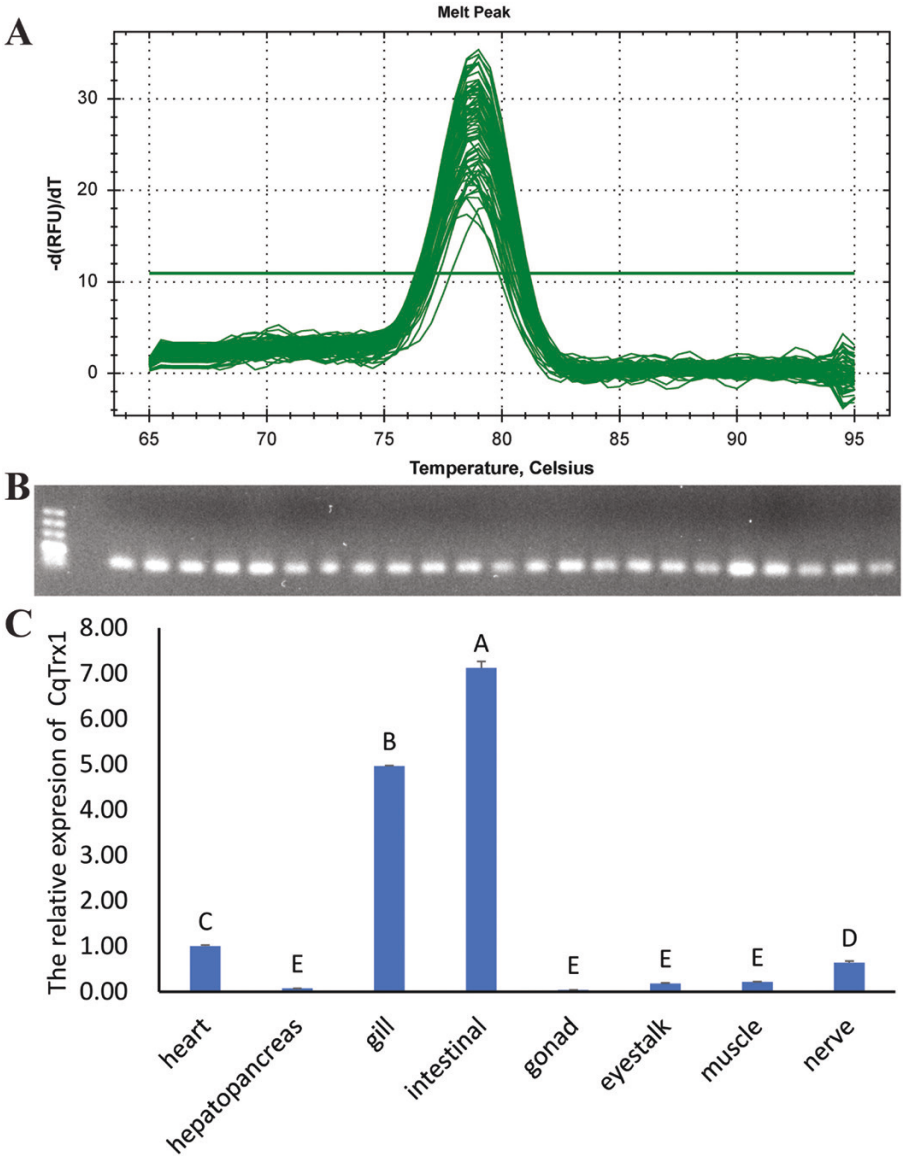

Figure 5. The expression level of the CqTrx1 gene in different tissues of *C. quadricarinatus*. Melt curve analysis of the CqTrx1 gene (**A**). Detection of PCR products using agarose gel electrophoresis (**B**). Relative expression level of CqTrx1 in different tissues (**C**). The same letters above the bars indicate no statistically significant differences, and different letters above the bars indicate statistically significant differences between the samples (one-way ANOVA followed by a post hoc Tukey test, $P < 0.05$).

DISCUSSION

The Trx system plays essential roles in maintaining cellular redox homeostasis and antioxidant defenses (Lu & Holmgren, 2014). It had been shown that the reactive oxygen species/reactive nitrogen species (ROS/RNS) play an important function in modifying thiols in proteins. The system can reversibly regulate thiol modifications, which regulate redox signaling involved in the central nervous system (Ren *et al.*, 2017). Thioredoxin is a redox protein with a broad role in intracellular signaling and regulation of redox homeostasis. This reducing protein can reverse the oxidative modification of intracellular protein cysteines (Zhou *et al.*, 2019b).

We undertook cloning and expression of the thioredoxin1 gene in *C. quadricarinatus,* and the results showed that it was 75.24%, 74.29%, and 66.67% similar to the Trx genes of *M. rosenbergii*, *Eriocheir sinensis* (Mu *et al.*, 2009), and *P. chinensis* (Ren *et al.*, 2010), respectively. $Multiple sequence comparison revealed the important CGPC-active site motif (Booze *et al.*, 2016). CqTrx1 contains five α-helices, two β-folds, two T-turns, and two random curls; however, compared with the predicted secondary structure of rhodopsin *Larimichthys crocea* Trx, our CqTrx1 lacked 2 β-folds at the N-terminus (Chen *et al.*, 2018). Since CqTrx1 also contains two β-folds, two T-turns and other active sites, the lack of a β-fold at the N-terminus may not directly affect the antioxidant activity. Meanwhile, no signal peptide was found within CqTrx1, suggesting that the CqTrx1 protein might be a cytosolic form.

CqTrx1 transcripts were expressed in all examined tissues, similar to other reports (Chen *et al.*, 2018; Cheng *et al.*, 2016). It was demonstrated that the expression of Trx1 was not all the same among species; for example in the fish golden pompano *Trachinotus ovatusin* (Linneaus, 1758), Trx1 expression was the

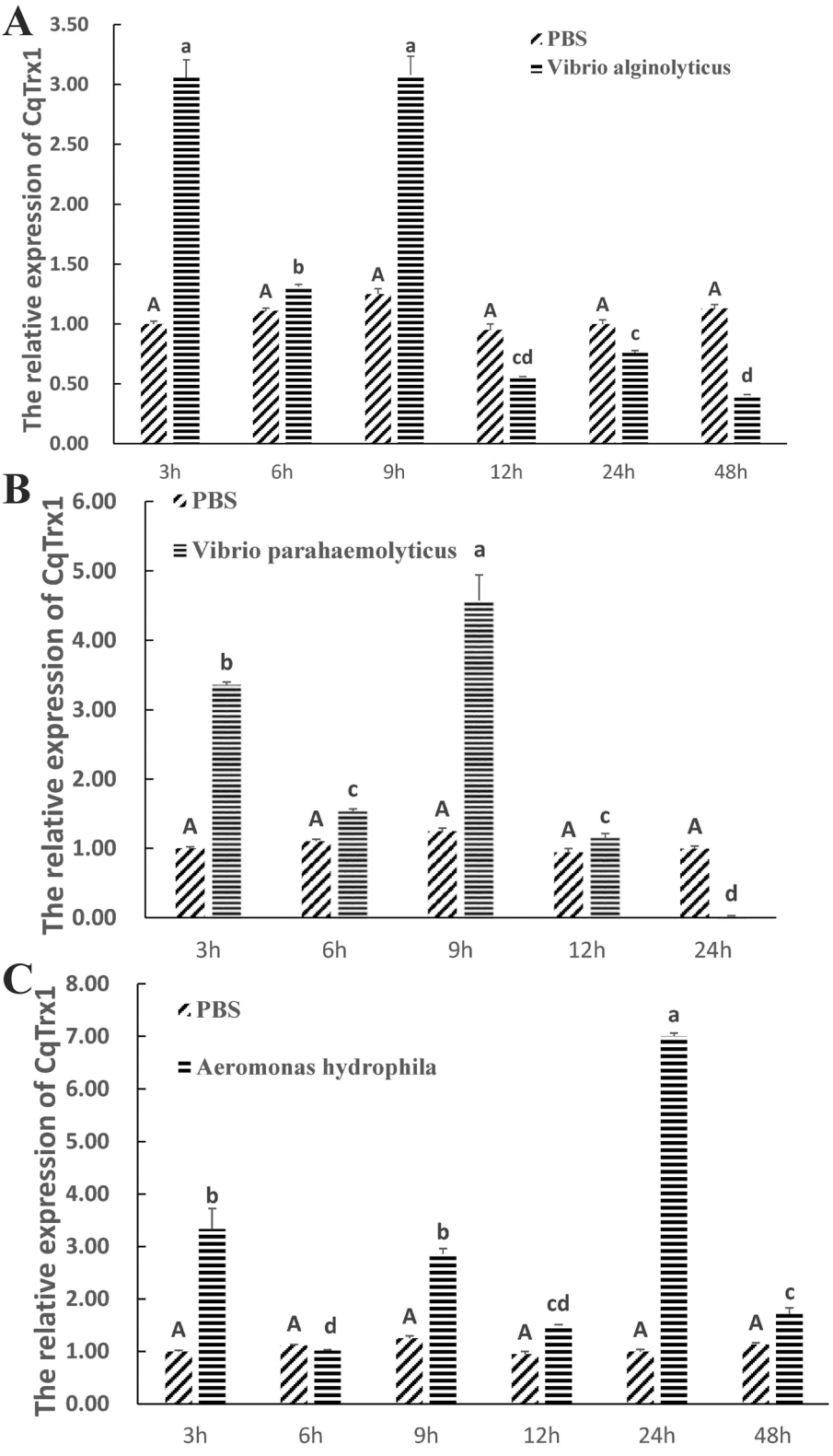

Figure 6. The expression pattern of the CqTrx1 gene in the gills of *C. quadricarinatus* after bacterial challenge. Relative expression of CqTrxq after *V. alginolyticus* challenge (**A**), relative expression of CqTrxq after *V. parahaemolyticus* challenge (**B**), and relative expression of CqTrxq after *A. hydrophila* challenge (**C**). The same letters above the bars indicate no statistically significant differences, and different letters above the bars indicate statistically significant differences between the samples (one-way ANOVA followed by a post hoc Tukey test, $P < 0.05$).

highest in the stomach, followed by gills and fins (Pourbasheer *et al.*, 2015;Wang *et al.*, 2015), but in the big-belly seahorse *Hippocampus abdominalis* Lesson, 1827, the highest expression was observed in muscle tissue. Herein, CqTrx1 gene expression in *C. quadricarinatus* was highest in the intestine followed by gills, heart, and hepatopancreas.

Shrimps farmed in pond water have always been affected by infectious diseases, mainly of bacterial and viral etiology (Flegel, 2012). After *V. parahaemolyticus* infection, Trx1 expression was downregulated significantly at 12 and 48 h post-infection in the liver and kidney of the fish *Larimichthys crocea* (Richardson, 1846) (Chen *et al.*, 2018), whereas *Chlamys nobilis* Trx transcript levels were found to be significantly upregulated after V. parahaemolyticus infection, significantly higher in the golden scallops than that in the brown scallop *Chlamys nobilis* (Reeve, 1852) (Zhang *et al.*, 2018). The knockdown of Trx1 in the *Marsupenaeus japonicus* (Spence Bate, 1888), however, significantly reduced mortality and virus-copy number (Guo *et al.*, 2019). We found that the expression of the CqTrx gene in the gills of *C. quadricarinatus* was significant within 3 h after bacterial exposure and reached the highest level within 9 h in the *V. algolyticus* and *V. parahaemolyticus* groups, which were three and five times higher than that of the control, respectively. In the gills of individuals were exposed to *A. hydrophila*, the expression of the CqTrx gene was significantly higher at 24 h ($P < 0.05$) and seven-fold higher than that of the control. Therefore, *A. hydrophila* remained in *Cherax quadricarinatus* longer and was more virulent than in the two *Vibrio* species. These results suggest that CqTrx has a major antioxidant function and plays an important role in regulating cellular redox homeostasis *in vivo* in this crayfish.

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