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Peroxiredoxin 6 modulates Toll signaling pathway and protects DNA damage against oxidative stress in red swamp crayfish (*Procambarus clarkii*)

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ABSTRACT

Peroxiredoxin 6 (Prx6) is an important member of the peroxiredoxin family that plays critical roles in protecting host against the toxicity of oxidative stress and participates in cell signaling. Herein, we report Prx6 gene from red swamp crayfish, *Procambarus clarkii*. The cDNA fragment of *PcPrx6* was 660 bp, encoding a 219 amino acid residues protein. The quantitative real time PCR analysis showed ubiquitous expression of *PcPrx6* mRNA in the tested tissues. The challenge with peptidoglycan and Poly I:C remarkably suppressed the mRNA level of *PcPrx6* in hepatopancreas at 3, 12, 48 h compared with the PBS control. However, the expression level significantly increased after 36 h of their treatment. The knockdown of *PcPrx6* by small interference RNA significantly enhanced the transcript levels of Toll pathway-responsive genes at 24 h. Recombinant PcPrx6 protein was purified using affinity chromatography and analyzed for its biological role. The results revealed that the recombinant PcPrx6 protein manifested the ability to protect supercoiled DNA damage from oxidative stress elicited by mixed function oxidative assay. Altogether, *PcPrx6* may have multiple functional roles in the physiology of *P. clarkii*, since it negatively regulates the Toll signaling transduction and protects supercoiled DNA damage from oxidative stress.

1. Introduction

Peroxiredoxins (Prxs) are a large family of thiol-specific antioxidant proteins that are broadly spread in living organisms. The members of this family play a vital function in cellular oxidative stress by decreasing the peroxynitrite, hydrogen peroxide (H_2O_2) and wide range of organic hydroperoxide (ROOH) into non-toxic forms [1–3]. So far, several members of the Prx family have been identified and molecularly characterized in various organisms [4]. In mammals, there are six isoforms of the Prx (Prx1-Prx6), which are classified into three subgroups (1-Cys, 2-Cys and atypical 2-Cys) based on the position and number of cysteine residues [4–6]. The Prx1-Prx4 is classified into the 2-Cys subgroup, and Prx5 comprising two additional cysteine residues contains the second subgroup, atypical 2-Cys. The Prx6 belongs to 1-Cys Prx family for its one conserved cysteine residue. Several 1-Cys Prx have been identified from both invertebrates and vertebrates, including *Anguila japonica* [7], *Oplegnathus fasciatus* [8], mice [9], *Scophthalmus maximus* [10], *Haliotis discus* and so on [11]. However, the Prx6 is rarely investigated in crustaceans.

Reactive oxygen species (ROS) including superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO•) are generated as a natural byproduct of the aerobic respiration. Exogenous sources of ROS include heavy metals, heat and ultraviolet light [12–14]. Lower concentration of ROS is essential for the routine cellular functions such as host defense against microbial infection, signal transduction, regulation of gene expression and so on [15]. However, excessive generation of ROS in intracellular environment cause variety of harmful effects, including lipid peroxidation, protein oxidation, lipoprotein modification and DNA damage [16,17]. Therefore, maintenance of optimum level of intracellular ROS is crucial for the regulation of normal cellular functions. Antioxidants have been shown to play a vital biological role in regulating the level of ROS by detoxifying harmful

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oxygen intermediates without effecting the routine cellular functions [13,18].

In addition, many studies with human and mammalian cells suggest that Prxs can also regulate immune related signaling, besides cellular oxidative stress responses. Thus, it was discovered that both Prx1 and Prx2 can inhibit NF-κB signaling, whereas Prx1 can also inhibit c-Jun *N*-terminal kinase (JNK) activation, thereby modulates cellular responses to pro-inflammatory particles [19–21]. In contrast, Prx4 can stimulate both the NF-κB and JNK signaling and can also induce inflammation [22]. Prx1 also interacts with the inhibitory factor of macrophage migration, an important cytokine in the regulation of host inflammatory and immune responses [23]. The human Prx2 regulates TNF signaling transduction through mitogen-activated protein kinases subsequent to TNF-alpha stimulation [24]. A recent study suggests that Prx6 negatively regulate NF-κB pathway by disrupting TRAF6-ECSIT complex, which is formed by the stimulation of TLR4 receptor [15]. Similarly, Lee et al. [25] observed that Prx6 modulate the activities of p38 MAPK and JNK pathway in C57BL/6 J-Tg mice and protect them from the microbial pathogen induced damage. Thus, Prx6 controls different biological functions in living organisms, further there is growing evidence that Prxs regulate innate immune and pro-inflammatory responses in invertebrates and vertebrates.

Red swamp crayfish (*Procambarus clarkii*) is an economically important crustacean, with broad global distribution [26,27]. They are edible and contain high quality proteins, with all the amino acids needed for human nutrition. However, they are highly susceptible to microbial pathogens such as virus, fungus and bacteria, which greatly affect their culture. It follows that continued studies into the immunobiology of *P. clarkii* is essential. In the present study, we identified and molecularly characterized Prx6 (hereafter PcPrx6) from *P. clarkii*. The main objectives of this study were (1) to identify the full-length cDNA of *Prx6* from the *P. clarkii*., (2) to determine spatial and temporal expression patterns of *PcPrx6*, (3) to analyze physiological role of *PcPrx6* in innate immune responses and (4) to determine the DNA protecting activity of PcPrx6 protein.

2. Materials and methods

2.1. Experimental animals

P. clarkii (about 10 ± 0.5 g and 7–8 cm each) individuals were purchased from a local market and maintained in tanks at 25 °C for at least two weeks. The water was continuously circulating through tanks, and *P. clarkii* individuals were fed with commercial diet.

2.2. RNA isolation and amplification of the PcPrx6 gene

Total RNA was isolated from hepatopancreas of *P. clarkii* using Trizol Reagent (Invitrogen) following the manufacturer's instructions. First strand cDNA was prepared by reverse transcription using the TransScript Synthesis SuperMix (TransGen, Beijing, China). To amplify the cDNA fragment of *PcPrx6,* gene specific primers were designed by using the Primer Premier 5.0 software package (Table 1). Polymerase chain reaction (PCR) was performed by using an amplification program as follows: 3 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 55 °C for 35 s and 72 °C for 50 s. The ensuring PCR products were resolved on 1% agarose gel electrophoresis, cloned into the pMD 19-T vector (Promega, USA), and then sequenced by Invitrogen.

2.3. Homologous alignment and phylogenetic analysis

The Prx4, Prx5 and Prx6 amino acid sequences from invertebrate and vertebrate species were obtained from the GenBank database and used to determine phylogenetic relationship. Multiple sequence alignments were carried out using Clustal X software [28]. A phylogenetic tree was constructed using W-IQ-TREE with the best-fitting substitution model using maximum likelihood, and subsequently viewed and graphically edited with FigTree v1.4.2 [29]. This method was used to infer phylogenetic trees with 1000 bootstrap repetitions. The JTT + I + G4 model was found to be appropriate for the amino acid sequence dataset in the W-IQ-TREE web interface.

Table 1
Primers used in this study.

Primer No.	Primer sequences (5′–3′)	Purpose
F1	ATGGTTAACTTAGGCGATGTG	RT-PCR
R1	TTACTCTGGTTGTGGCGTTGTG	RT-PCR
F2	CCGGAATTCATGGTTAACTTAGGCGATGTG	Protein Expression
R2	CCGCTCGAGTTACTCTGGTTGTGGCGTTGT	Protein Expression
F3	CGGATCACTGGAGGGTCAAACACTT	qPCR
R3	GCAATTTTCATCCTCGGCATCAT	qPCR
Toll-F	GCTGTTGCTGCTTAGGCTCA	qPCR
Toll-R	TCCTCCACAGCTCTTCATTCC	qPCR
Sptäzle-F	GTCGGCAGCAACGACATACA	qPCR
Sptäzle-R	GGTGTCATGGTTGGCTGTGA	qPCR
Cactus-F	CTTGTGAGAGAGCCGTGTGG	qPCR
Cactus-R	CAGTACAAGCAGCAGCAGCA	qPCR
ALF-F	TCCTGAATTGCTCCTCGTCA	qPCR
ALF-R	GCGGTGGCAACTGTACTTCA	qPCR
F18S	CTGTGATGCCCTTAGATGTT	qPCR
R18S	GCGAGGGGTAGAACATCCAA	qPCR
si-Per6-F	GCCAUCAAGCUGCACACCUTT	RNAi
si-Per6-R	AGGUGUGCAGCUUGAUGGCTT	RNAi
si-NC-F	UUCUCCGAACGUGUCACGUTT	RNAi
si-NC-R	ACGUGACACGUUCGGAGAATT	RNAi

Note: “ ” present Restriction Enzyme cutting site: *EcoR* I.
“___” present Restriction Enzyme cutting site: *Xho* I.

2.4. Protein expression and purification

To express the recombinant PcPrx6 protein in the prokaryotic expression system. The ORF sequence, which was previously cloned into pMD 19-T vector and then digested with *EcoR* I and *Xho* I, was then ligated into the pGEX-4T-1 vector (Novagen, USA). The insert (i.e., pGEX-4T-1-Pc-Prx-6), was then confirmed by DNA sequencing and transformed into Escherichia *coli* (BL21 DE3) (Novagen, USA) for protein expression. For recombinant protein expression, initially we used different concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG) to determine induction level (concentration) of IPTG in bacterial culture. Then, to produce recombinant protein for purification, after short-term culturing and an IPTG induction at a final concentration of 0.8 mM, the cells were harvested by centrifugation at 8000 × *g* for 5 min. The cell pellets were suspended in a binding buffer composed of 20 mM Tris–HCl, 500 mM NaCl, 5 mM imidazole, pH 7.9 and disrupted by sonication on ice. After centrifugation at 12,000 × *g* for 20 min at 4 °C, the recombinant protein was purified by using the GSTBind™ Resin Kit (Novagen, Hilden, Germany) according to the manufacturer's instructions. To analyze the recombinant proteins, we used both 15%-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. The purified recombinant proteins were used to determine the biological functions of PcPrx6.

2.5. Spatial distribution of PcPrx6 gene

Total RNA was isolated from muscle, stomach, gut, heart, hepatopancreas, gill and hemocyte, and by using TRIzol Reagent (Invitrogen) following the manufacturer's instructions. The isolated RNA samples were reverse transcribed into cDNA. Oligonucleotide primers specific for the *PcPrx6* sequence were designed by Primer 5.0 software based on the known sequence. Quantitative Real-time PCR was performed in 25 μL reactions that comprises 1 μL of each of the forward and reverse primer, 2 μL of cDNA, 12.5 μL of 2 × SYBR Premix Ex TaqII (Takara) and 8.5 μL of RNase-free H_2O. The amplification program went as

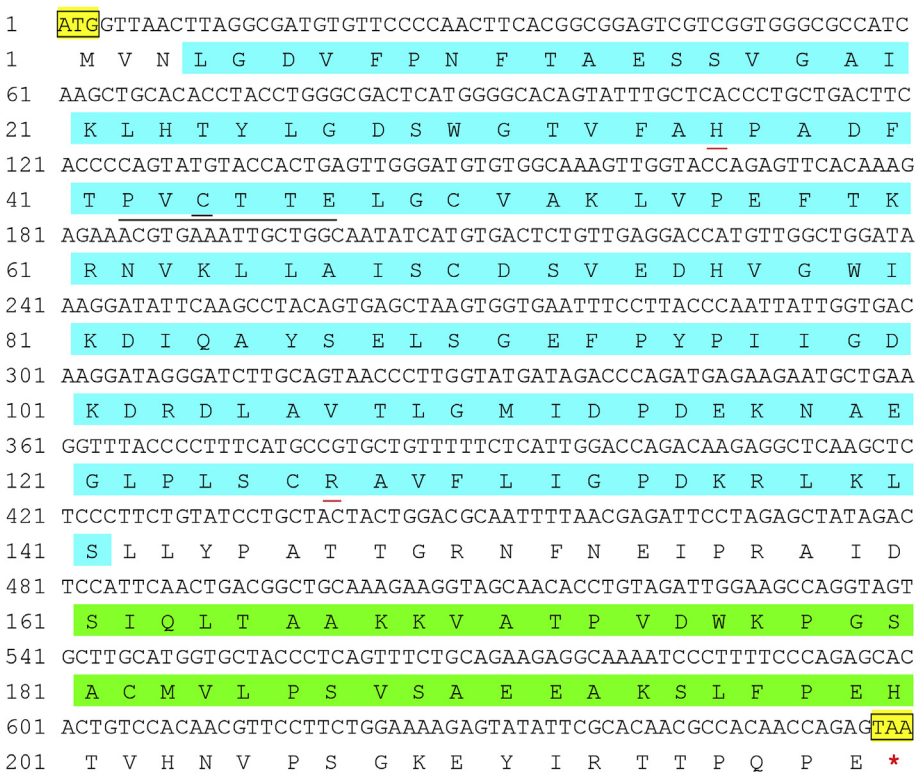

Fig. 1. Nucleotide and deduced amino acid sequences of PcPrx6 cDNA of *Procambarus clarkii*. The start codon (ATG), and the stop codon (TAA) were enclosed in solid lines. The Pfam Redoxin domain was cyan couloured and Pfam 1-cysPrx_C domain was green coloured. The active cysteine residue was black-double underlined, and the catalytic center was black-underlined. The conserved residues (His36 and Arg127) were red-underlined. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

follows: initial denaturation at 95 °C for 3 min; followed by 40 cycles of amplification (95 °C for 10 s, 55 °C for 30 s, 72 °C for 30 s). The continuous fluorescence acquisition 65–95 °C with an increase of 0.5 °C per 10s was used for melting curve analysis. The relative expression level of *PcPrx6* gene was calculated according to the $2^{-\triangle\triangle CT}$ method [30] and 18S rRNA gene was used internal control.

2.6. Temporal distribution of PcPrx6 gene

The *P. clarkii* individuals (adult) were randomly divided into three groups. They were administrated with Poly I:C or PGN. Administration with only phosphate-buffered saline (PBS) served as control. These injections were delivered with a microliter syringe (Gaoge, Beijing, China). Hepatopancreas samples were collected at 3, 6, 12, 24, and 48 h after injection. Three organisms were taken as one sample and biological sampling protocol was repeated three times. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. The transcript expression profiles of *PcPrx6* was determined by qRT-PCR as described in our previous studies [e.g. Refs. [31,32]].

2.7. RNA interference assay

Based on the *PcPrx6* sequence, BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST) was executed to avoid off-target influences on other gene sequences. The siRNAs (Table 1) were chemically synthesized by the Shanghai GenePharma Co., Ltd. (Shanghai, China). The siRNAs were dissolved in diethylpyrocarbonate treated water (Milli-Q-grade), and then (10 μL, 1 μg/μL) administrated into the last abdominal segment of each the *P. clarkii* using microliter syringes. To avoid leakage of siRNA from the body, needles were kept still at the injection point for 30 s. One group of the *P. clarkii* was administrated with a set of siRNAs while random sequences (*NC*-F, *NC*-R) were used as negative controls. The hepatopancreas of the *P. clarkii* were sampled at two different time points (24 and 48 h) for qRT-PCR analysis.

2.8. DNA protecting activity of PcPrx6

To determine whether recombinant PcPrx6 can protect DNA against oxidative stress. The mixed-Function Oxidase (MFO) assay, which is used to determine the degree of DNA breakage caused by the ROS produced from the thiol/Fe3 + /O2 MFO system and to what extent the PcPrx6 could inhibit the DNA breakage and was performed according to Ref. [8] with modifications. In short, 1 μg of substrate supercoiled plasmid (pMD 19-T) DNA was treated with freshly prepared MFO mix (0.825 mM of DTT + 8.25 μM of FeCl3) and varying concentrations of recombinant PcPrx6 fusion protein in a total reaction volume of 100 μL. Thereafter the reaction mixtures were incubated at 37 °C for 3 h. Then the reaction mixtures were purified using a PCR purification kit (Bioneer, Korea) and the samples were subjected to 1% agarose gel at 100 mV for 25 min at room temperature. The agarose gel was stained with ethidium bromide and visualized under UV light. Triplicate assays were conducted to confirm the reliability.

2.9. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. All data were represented as the mean ± standard error (S.E.). The differences were considered significant at $P < 0.05$.

3. Results

3.1. Bioinformatics analysis

The *PcPrx6* contained a complete open reading frame (ORF) of 660 bp, which encoded a 219 amino acid residues protein. The calculated molecular weight of the deduced mature *PcPrx6* was 23.85 kDa, and the protein had theoretical isoelectric point of 5.24. The deduced amino acid sequence of *PcPrx6* is presented in Fig. 1. Consistent with other peroxiredoxins, the newly identified *PcPrx6* contained typical Peroxiredoxin (PRX) domains, including Pfam Redoxin domain (from Leu 4 to

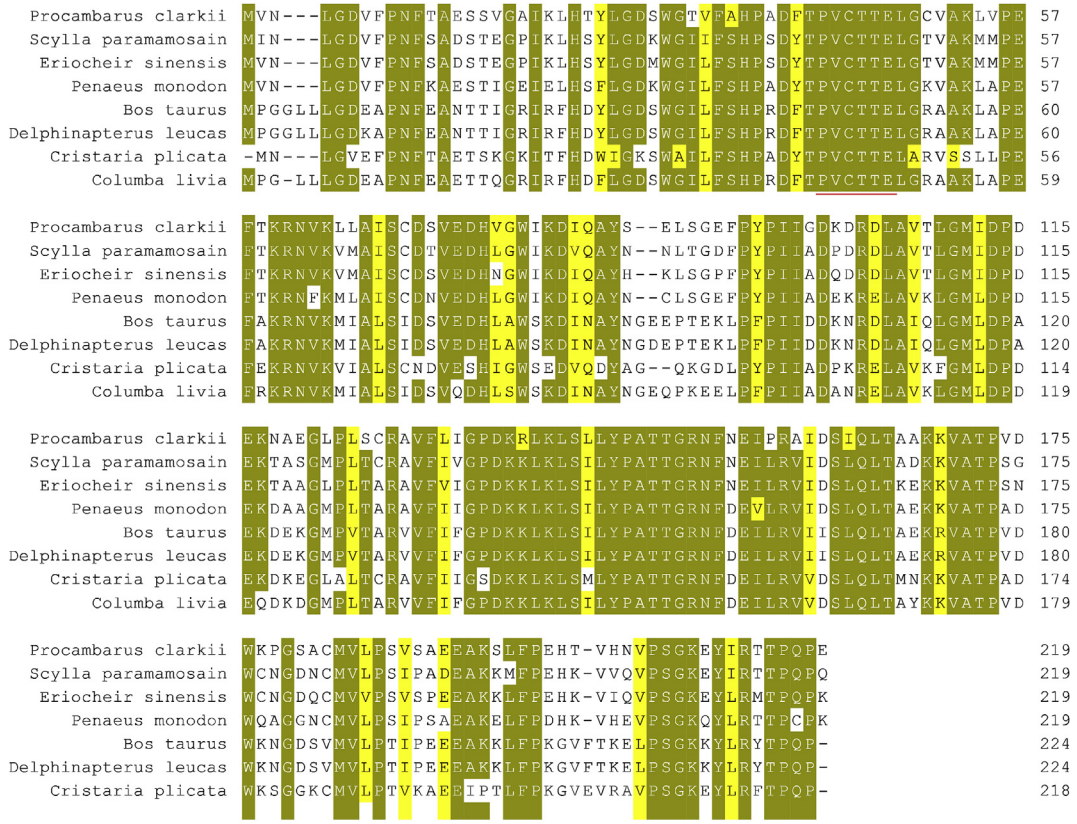

Fig. 2. Alignment of the PcPrx6 protein with other homologous proteins. The deduced amino acid sequence of PcPrx6 was aligned with Prx from *Scylla paramamosain* (ACJ53746), *Ericheir sinensis* (ACF35639), *Penaeus monodon* (AQW41375), *Bos Taurus* (NP_777068), *Delphinapterus leucas* (XP_022420938), *Cristaria plicata* (ADN06076), *Columba liva* (PKK27212). The catalytic center was red-underlined. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Ser 141) and Pfam 1-cysPrx_C domain (from Ser 161 to His 200). The *PcPrx6* sequence was found to lack signal peptides.

The deduced amino acid sequence of *PcPrx6* was highly identical (77%) to that of prx6 of *Ericheir sinensis* (GenBank ID: ACF35639) and (75%) *Scylla paramamosain* (GenBank ID: ACJ53746), while to a lesser extent (63%) to that of *Delphinapterus leucas* (GenBank ID: XP_022420938). Multiple sequence alignments showed that cysteine residue (Cys 44) was highly conserved in all the examined Prx6, and one histidine (His 36) and one arginine (Arg 127) residue were also highly conserved in them. In addition, all of the analyzed Prx6 had peroxidase catalytic center, PVCTTE, in their deduced amino acids sequence (Fig. 2). The phylogenetic analysis was performed to examine evolutionary relationship of PcPrx6 with known members of Prx from invertebrates and vertebrates (Fig. 3). As expected, three subgroups corresponding to Prx4, Prx5, Prx6 were observed. This clustering pattern showed that Prx6 is distantly related to the Prx4 and Prx 5 subgroups. Additionally, Prx6 counterpart from vertebrates was separately clustered in their corresponding sub-groups. The PcPrx6 was in the same subgroup as the Prx6 from *Scylla paramamosain*, *Ericheir sinensis, Penaeus monodon* and *Cristaria plicata* (Fig. 3).

3.2. Prokaryotic protein expression

The recombinant PcPrx6 protein was expressed in pGEX-4T-1 (+) vector in *E. coli* (BL21 DE3) cells. SDS-PAGE analysis revealed that recombinant PcPrx6 protein was successfully expressed, and expression level was approximately equal with different concentrations of IPTG (Fig. 4A). A purified PcPrx6 × GST fusion protein was extracted using affinity chromatography and a protein band corresponding to the predicted molecular weight of approximately 48 kDa was observed (Fig. 4B). Western blotting analysis of the recombinant proteins using an *anti*-GST-tag antibody confirmed the presence of recombinant PcPrx6 (Fig. 4C). The purified recombinant protein was then used to determine the physiological functions of PcPrx6.

3.3. Spatial expression patterns of PcPrx6

The expression pattern of *PcPrx6* gene in various tissues was evaluated at the transcriptional level by qRT-PCR. The results revealed that the *PcPrx6* gene was ubiquitously expressed in all tested tissues, with higher level of expression detected in hepatopancreas and muscles. Furthermore, lower expression levels were observed in the gills and stomach (Fig. 5).

3.4. Temporal expression patterns of PcPrx6

The temporal expression patterns of *PcPrx6* were determined in hepatopancreas after Poly I:C and PGN administration by qRT-PCR. The *PcPrx6* transcript expression profiles varied remarkably in hepatopancreas following Poly I:C and PGN treatment, and change in the transcript level further depended on the type of pathogen. As shown in Fig. 6, Poly I:C and PGN greatly affected *PcPrx6* expression in hepatopancreas; however, both the mRNA level and the time of maximum mRNA level of this gene varied in the examined tissue. Compared with the PBS control, the transcript level of *PcPrx6* in the hepatopancreas remarkably downregulated following Poly I:C and PGN infection. At 6 and 24 h after their challenge, there was no remarkable difference between the treated group and the control group. At 3, 12 and 48 h post injection, the *PcPrx6* transcript level was remarkably low than that of the control group. However, the mRNA expression of *PcPrx6* strongly increased after 36 h of their administration compared with the PBS control.

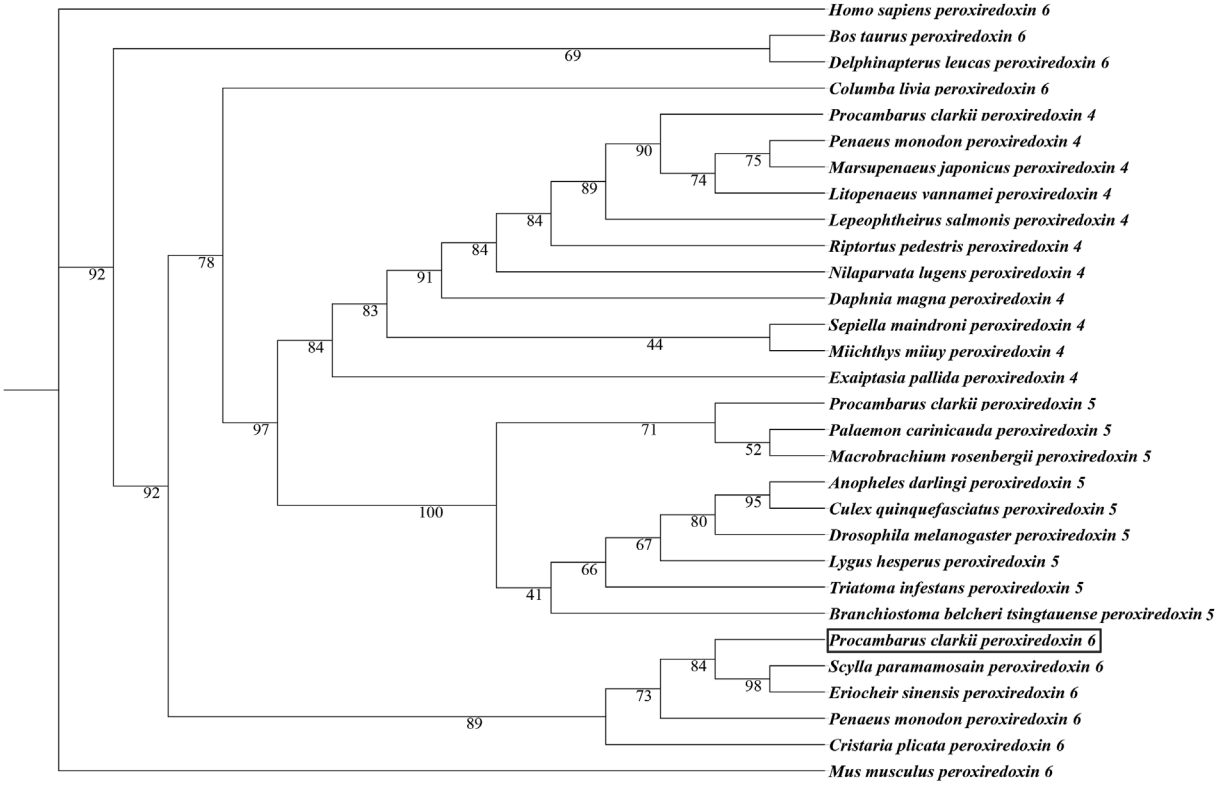

Fig. 3. Consensus neighbor joining tree based on the amino acid sequences of Prx from various animal species. The amino acid sequences used to construct phylogenetic tree are as follows: *Penaeus monodon* (ABZ80828), *Litopenaeus vannamei* (AET36895), *Marsupenaeus japonicus* (AGV39321), *Daphnia magna* (JAN72511), *Riptortus pedestris* (BAN20936), *Sepiella maindroni* (AKN23454), *Miichthys miiuy* (AGT56737), *Lepeophtheirus salmonis* (ACO12581), *Nilaparvata lugens* (AMM72617), *Exaiptasia pallida* (KXJ22794), *Homo sapiens* (EAW98996), *Mus musculus* (AAH19578), *Palaemon carinicauda* (AGJ03548), *Macrobrachium rosenbergii* (AET34923), *Triatoma infestans* (JAC17556), *Lygus hesperus* (JAG54910), *Drosophila melanogaster* (XP_017040176), *Anopheles darlingi* (ETN58721), *Culex quinquefasciatus* (XP_001849745), *Branchiostoma belcheri tsingtauense* (AAM18076), *Homo sapiens* (AAF17200) and *Mus musculus* (AAF27532), *Scylla paramamosain* (ACJ53746), *Eriocheir sinensis* (ACF35639), *Penaeus monodon* (AQW41375), *Bos Taurus* (NP_777068), *Delphinapterus leucas* (XP_022420938), *Cristaria plicata* (ADN06076), *Columba liva* (PKK27212). The deduced amino acid sequences were aligned and a phylogenetic tree was constructed by W-IQ-TREE using the maximum likelihood analysis method. The studied species (*Procambarus clarkii*) is boxed.

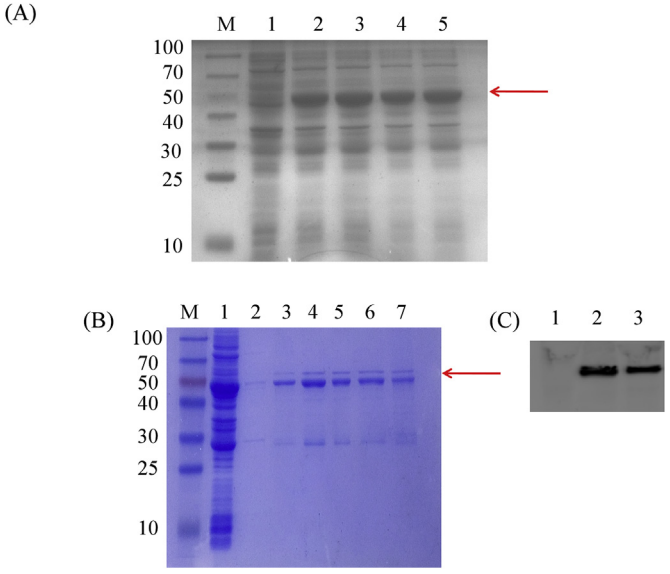

Fig. 4. Protein expression and western blot analysis. (a) The SDS-PAGE of expressed PcPrx6 protein in *E. coli* induced by different IPTG concentration. Lane M: Protein molecular weight marker. Lane 1: non-induced expression; lane 2, 3, 4 and 5, after 4 h induction with 0.5, 1.0, 1.5 and 2.0 mM IPTG, respectively. (b) The SDS-PAGE of expressed and purified PcPrx6 protein. (c) Western blotting of recombinant PcPrx6 protein with *anti*-GST tag antibodies. Lane 1, No IPTG induction. Lane 2 and 3, after IPTG induction.

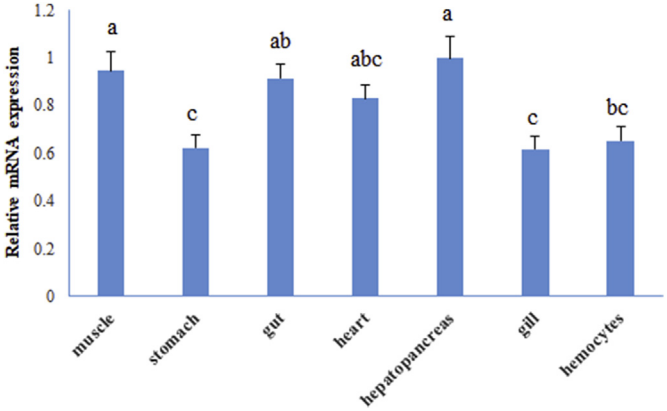

Fig. 5. Spatial distribution of *PcPrx6* mRNA quantified by SYBR Green qRT-PCR. The tissues including muscle, stomach, gut, heart, hepatopancreas, gill and hemocyte sampled from *Procambarus clarkii.* Vertical bars were represented as the means ± standard error (SE). Bars labeled with different letters are significantly different (one-way ANOVA followed by Tukey's test, $P < 0.05$).

3.5. *PcPrx6 regulates Toll signaling pathway*

To determine the physiological role of *PcPrx6,* we quantified the expression profiles of Toll pathway responsive genes (Toll, Sptazle, Cactus and Anti-lipopolysaccharide factor) after the suppression of

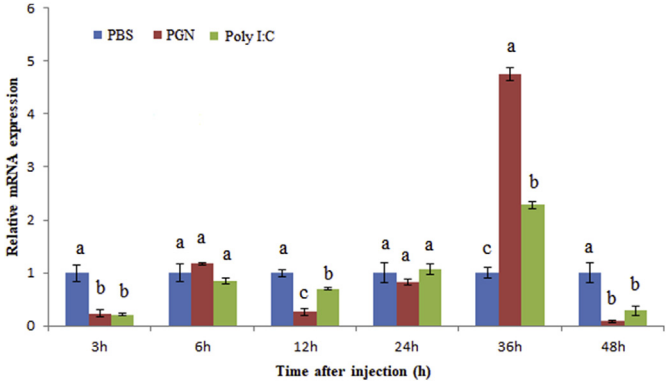

Fig. 6. Expression profiles of PcPrx6 in hepatopancreas at different time points (3 h to 48 h) after peptidoglycan and Poly I:C administration. Data were represented as the means ± standard error (SE). Bars labeled with different letters are significantly different (one-way ANOVA followed by Tukey's test, $P < 0.05$).

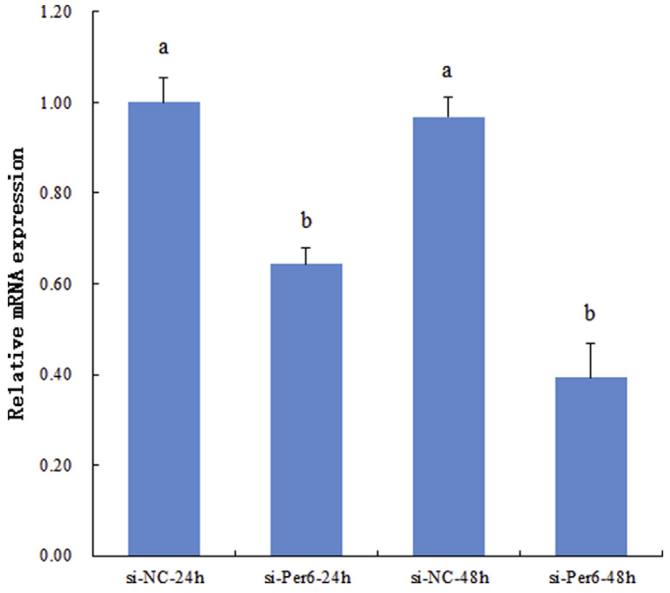

Fig. 7. RNAi efficiency estimation of *PcPrx6*. Relative mRNA expression levels of *PcPrx6* in the negative control-treated *Procambarus clarkii* hepatopancreas and *PcPrx6* si-RNA-treated *Procambarus clarkii* hepatopancreas after 24 h and 48 h by qRT PCR. The data were analyzed using one-way analysis of variance. Data were represented as the means ± standard error (SE). Differences were considered significant at *P* value of < 0.05.

PcPrx6. The efficiency of siRNAs injection at 24 and 48 h was analysed by executing qRT-PCR, and the result revealed that the *PcPrx6* gene was successfully down regulated compared with the control group (Fig. 7). The expression levels of all the Toll pathway responsive genes were remarkably upregulated at 24 h and 48 h after *PcPrx6* depletion (Fig. 8), although the sptazle and anti-lipopolysaccharide factor transcript levels were approximately equal to the control group at 48 h. Interestingly, all of the examined Toll pathway responsive genes expression remarkably decreased at 48 h compared with the expression at 24 h in the *PcPrx6* depleted groups, even the *PcPrx6* greatly interfered over the time.

3.6. *In vitro* DNA protecting activity of rPcPrx6 protein against oxidative damage

To determine the supercoiled DNA protection activity of rPcPrx6 protein, we executed DNA protection assay using pMD19-T plasmid. As shown in Fig. 9, supercoiled DNA nicking (by MFO system) and supercoiled pMD19-T plasmid DNA protection (by Pc-Prx-6) were determined simultaneously by shifting gel mobility following the treatment. The results revealed that separately incubating MFO components were not influenced to supercoiled DNA compared to negative control (lane 2 and 3). Maximum supercoiled DNA nicking (OC DNA) was found with complete MFO system and also with the lack of the recombinant PcPrx6 protein (Lane 4). Nicked form of supercoiled DNA (OC DNA) was approximately same in the presence of recombinant PcPrx6 at the concentration of 1.5 μg (Lane 5). However, with the increase of recombinant PcPrx6 protein dose from 2.5 μg to 10 μg formation of nicked form DNA suppressed in dose dependent manner (Lane 6–8), which shows the supercoiled DNA protection. Supercoiled DNA was not protected in the control experiment.

4. Discussion

Peroxiredoxins, a ubiquitous peroxide family, have been shown to be involved in various biological processes such as redox signaling, antioxidant defense, kinase modulation and apoptosis control in animals including *P. clarkii* [14,18,33]. In the present study, we demonstrated the molecular characterization of a Prx6 from *P. clarkii*. The deduce amino acid sequence of *PcPrx6* shared 77% homology with the previously described prx6 from *Ericheir sinensis* [34]. The complete sequence and physiological functions of *PcPrx6* had never been studied before.

The full-length cDNA encoding *PcPrx6* was identified from the *P. clarkii*. The ORF (660 bp) of the cDNA encoded a 219 amino acid residues protein. Like the other previously reported Prx6, *PcPrx6* shared *N*-terminus cystein residue (Cys 44), which is the representative characteristics of Prx6 [35–37], one histidine (His 36) and one arginine (Arg 127). Additionally, it shared peroxidase catalytic center, PVCTTE with other characterized Prx6. Phylogenetic analysis revealed *PcPrx6* was in the same subgroup as the Prx6 from *Scylla paramamosain, Ericheir sinensis*, *Penaeus monodon, Cristaria plicata,* and this subgroup was different from the Prx4 and Prx5 subgroups. Altogether, these results suggest that *PcPrx6* is a member of Prx family, and may have similar functions like other known Prx6 [34].

The spatial distribution assay revealed that *PcPrx6* ubiquitously expressed in different tissues, although the expression profile of this gene slightly varied in them, implying that *PcPrx6* might play a key physiological role in the tissues of the *P. clarkii*. The expression profile is consistent with the previous studies in terms of ubiquitous distribution [34,38]. Additionally, the *PcPrx6* transcript level varied in all the tested tissues, the highest expression level was observed in hepatopancreas and muscles (Fig. 5). The hepatopancreas is the major metabolic center for ROS production [39]. Moreover, hemocyte also plays a key physiological role in the innate immune responses against microbial pathogens, and also contribute in phagocytosis and cellular encapsulation [40–44]. These results suggest that *PcPrx6* might be involved in tissue specific biological functions e.g. immune responses.

In crustaceans, the hepatopancreas plays a vital role in the metabolism process and innate immune responses, which is considered to be homologous to pancreas and liver of mammals and is responsible for key events on immunity and metabolism [45–48]. Additionally, it was always used as candidate tissue for evaluating the variation of immune related genes [49,50]. In this study, hepatopancreas of the *P. clarkii* was selected as our target tissue to study the transcript levels of *PcPrx6* after Poly I:C and PGN challenge. Both Poly I:C and PGN were administrated to the *P. clarkii*, and transcript profiles of *PcPrx6* was investigated by qRT-PCR. The expression of *PcPrx6* remarkably decreased after 3, 12 and 48 h of Poly I:C and PGN challenge. However, the expression level significantly increased after 36 h of their treatment. Altogether, our data shows that both bacterial and viral pathogens could suppress the expression of *PcPrx6;* but late phase infection could be enhancing transcript level of this gene. However, our previous studies on Prx4 and Prx5 showed different expression profiles in hepatopancreas,

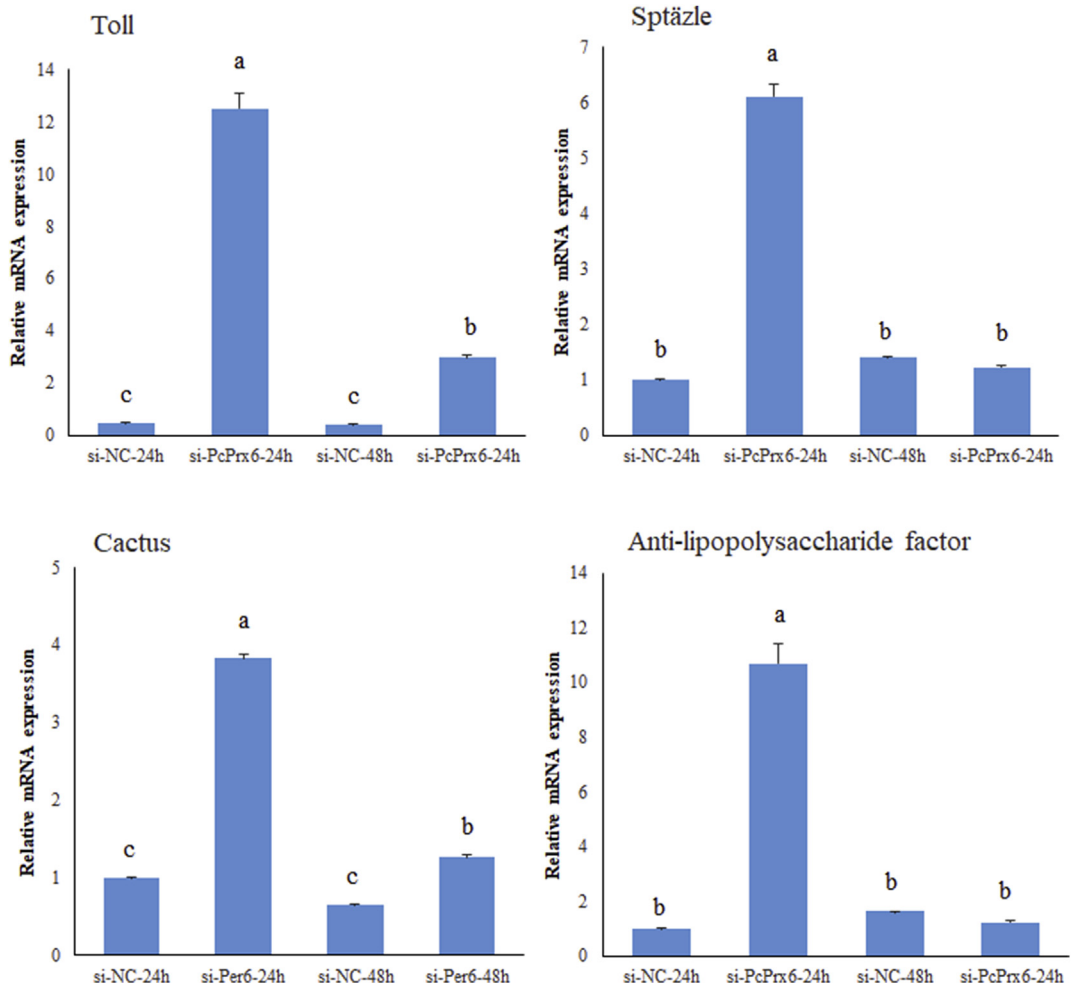

Fig. 8. Expression levels of the Toll pathway-associated genes in response to the *PcPrx6* knockdown. Relative mRNA expression levels of Toll pathway-associated genes, namely (A) Toll, (B) spatzle, (C) cactus, and (D) anti-lipopolysaccharide. Si-NC: negative control; si-Prx6: treated with PcPrx6 si-RNA and peptidoglycan. The data were analyzed using one-way analysis of variance. Data were represented as the means ± standard error (SE). Bars labeled with different letters are significantly different (one-way ANOVA followed by Tukey's test, $P < 0.05$).

hemocytes and gills in the same organism when challenged with LPS and Poly I:C. Contrary to the present study, both of these genes showed quick response against bacterial and viral pathogens [14,18]. These difference in their expression patterns may indicate their particular response against the infectious agents. Furthermore, Prx6 response against pathogens have also been reported in other organisms. For instance, in Chinese mitten crab Prx6 down-regulated in hemocytes after bacterial challenge [34]. Whereas, Prx6 expression in *Anguilla japonica* gradually enhanced in liver and spleen, however varied with the type of pathogen [51]. These observations suggest that Prx6 responses against infectious agents may vary with type of tissue or with type of pathogen.

Toll signaling plays a vital role the innate immune response, which is responsible for eliminating invading microbial pathogens through the induction of inflammatory molecules in the host [52,53]. The Toll signaling is suppressed and abolished by various negative regulators to avoid an injurious response towards the immune system of host [54–57]. In the present study, we observed that depletion of *PcPrx6* in *P. clarkii* enhanced the production of Toll pathway responsive genes, suggesting PcPrx6 has suppressive role in the activation of Toll signaling. Recently, Min et al. [15] demonstrated that overexpression of Prx6 leads to suppress the activity of NF-κB pathway stimulated by TLR4. The Prx6 inhibits the formation of TRAF6-ECSIT complex in LPS infected Prdx6^{KD} THP-1 cells, thereby negatively regulates the NF-κB activation. Most probably, PcPrx6 is working in an analogous manner. Additionally, in the present study, the Toll pathway responsive genes expression decreased in the *PcPrx6* depleted groups at 48 h compared with the 24 h, even the *PcPrx6* greatly interfered over the time. There are some other factors, which likely to control the extensive activation of Toll pathway and should be determined in future research.

Many studies have shown that Prx6 is an important cytoprotective protein that has ability to reduce ROS thereby protect cell components from oxidative damage in eukaryotes [7,8]. Sharapova et al. [58] compared the antioxidant properties of Prx6 from more than six species of vertebrate and invertebrate (human, Xenopus, rat and *Drosophila*) using the supercoiled plasmid DNA. They determined the Prx6 concentration at which approximately 50% of supercoiled plasmid DNA remained intact were 0.005 mg/mL for *Drosophila,* 0.3 mg/mL for Xenopus, 0.15 mg/mL for rat and 0.16 mg/mL for human. In the present study we determine the antioxidant activity of recombinant PcPrx6. The results revealed that recombinant PcPrx6 effectively scavenge ROS produced by metal catalyzed reaction, thereby protect supercoiled plasmid DNA from oxidative damage. Additionally, the recombinant PcPrx6 suppressed plasmid DNA damage in dose dependent manner. Moreover, Nikapitiya et al. [11] and De Zoysa et al. [8] have also estimated the antioxidant potential of recombinant Prx6 in mollusk and fish under oxidative stress conditions. Taken together, we suggest that PcPrx6 as a cytoprotective enzyme may play a vital role to protect DNA damage from oxidative stress; however, further studies are required to assess the level of concentration which provides maximum protection to DNA.

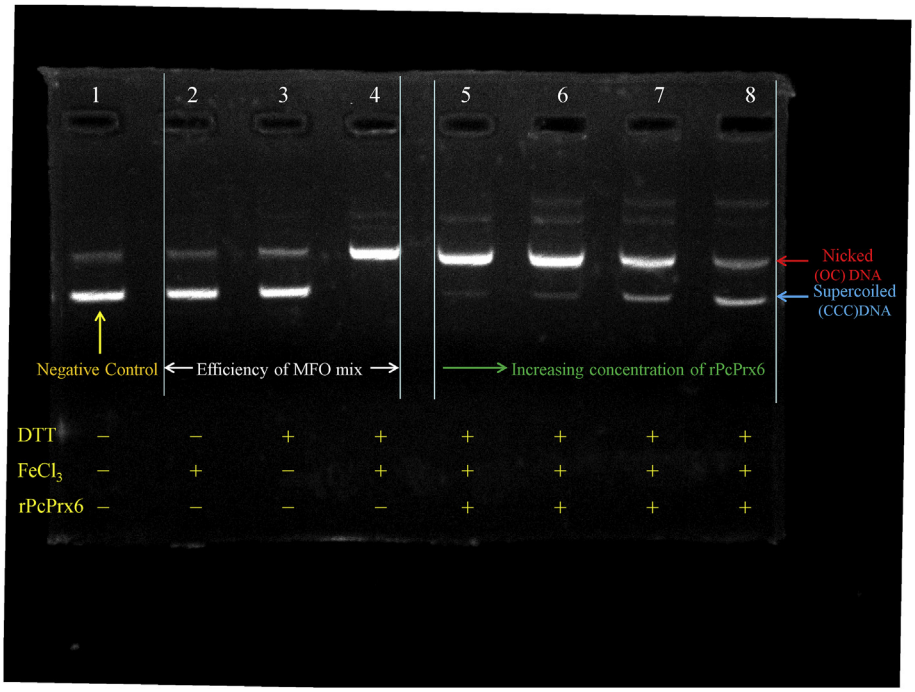

Fig. 9. Protection of supercoiled DNA cleavage by recombinant PcPrx6 in mixed-function oxidase (MFO) assay. (1) pMD 19-T (1 μg) supercoiled plasmid DNA without incubation; (2) pMD 19-T + $FeCl_3$ (50 mM); (3) pMD 19-T + DDT (10 mM); (4) pMD 19-T + MFO mix (50 mM, $FeCl_3$ +10 mM DDT); (5) pMD 19-T + MFO mix + 1.5 μg of rPcPrx6; (6) pMD 19-T + MFO mix + 2.5 μg of rPcPrx6; (7) pMD 19-T + MFO mix + 5 μg of rPcPrx6; (8) pMD 19-T + MFO mix + 10 μg of rPcPrx6. OC: open circular plasmid DNA; CCC: covalently closed circular DNA.

Altogether, peroxiredoxin 6 from *P. clarkii* was identified and characterized. The findings of the present study demonstrate that *PcPrx6* is certainly a member of 1-Cys peroxiredoxin subfamily. Like, our previous studies, where we characterized Prx4 and Prx5 using approximately same methods in the same organism [14,18], *PcPrx6* ubiquitously expresses in all of the tested tissues under normal physiological conditions. These genes show different behavior with the type of pathogens and with the type of tissue. However, they protect supercoiled plasmid DNA against oxidative stress. Furthermore, the present study showed that *PcPrx6* can also regulate the activity of Toll signaling pathway. Our data suggest that *PcPrx6* play a vital role in defense of cellular oxidative stress and host immune responses against microbial pathogen infection like other peroxiredoxins.

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References

[1] C.A. Neumann, D.S. Krause, C.V. Carman, S. Das, D.P. Dubey, J.L. Abraham, R.T. Bronson, Y. Fujiwara, S.H. Orkin, R.A. Van Etten, Essential role for the peroxiredoxin Prdx1 in erythrocyte antioxidant defence and tumour suppression, Nature 424 (2003) 561–565.
[2] S. Kawazu, K. Komaki-Yasuda, H. Oku, S. Kano, Peroxiredoxins in malaria parasites: parasitologic aspects, Parasitol. Int. 57 (2008) 1–7.
[3] M.E. Perez-Perez, A. Mata-Cabana, A.M. Sanchez-Riego, M. Lindahl, F.J. Florencio, A comprehensive analysis of the peroxiredoxin reduction system in the Cyanobacterium *Synechocystis sp.* Strain PCC6803 reveals that all five peroxiredoxins are thioredoxin dependent, J. Bacteriol. 191 (2009) 7477–7489.
[4] Z.A. Wood, E. Schroder, J.R. Harris, L.B. Poole, Structure, mechanism and regulation of peroxiredoxins, Trends Biochem. Sci. 28 (2003) 32–40.
[5] H.R. Ellis, L.B. Poole, Roles for the two cysteine residues of AhpC in catalysis of peroxide reduction by alkyl hydroperoxide reductase from *Salmonella typhimurium*, Biochemist 36 (1997) 13349–13356.
[6] H.Z. Chae, S.W. Kang, S.G. Rhee, Isoform of mammalian peroxiredoxin that reduce peroxides in presence of thioredoxin, Methods Enzymol. 300 (1999) 219–226.
[7] T.T. Priyathilaka, Y. Kim, H.M.V. Udayantha, S. Lee, H.M.L.P.B. Hearth, H.H.C. Lakmal, D.A.S. Elivitigala, N. Umasuthan, G.I. Godahewa, S. Kang, H.B. Jeong, S.K. Kim, D.J. Kim, B.S. Lim, Identification and molecular characterization of peroxiredoxin 6 from Japanese eel (*Anguilla japonica*) revealing its potent antioxidant properties and putative immune relevancy, Fish Shellfish Immunol. 51 (2016) 291–302.
[8] M. De Zoysa, J.H. Ryu, H.C. Chung, C.H. Kim, C. Nikapitiya, C. Oh, H. Kim, K.S. Revathy, I. Whang, J. Lee, Molecular characterization, immune responses and DNA protection activity of rock bream (*Oplegnathus fasciatus*) peroxiredoxin 6 (Prx6), Fish Shellfish Immunol. 33 (2012) 28–35.
[9] J.H. Pak, W.C. Son, S.B. Seo, S.J. Hong, W.M. Sohon, B.K. Na, T.S. Kim, Peroxiredoxin 6 expression is inversely correlated with nuclear factor-kB activation during *Clonorchis sinesis* infestation, Free Radic. Biol. Med. 99 (2016) 273–285.
[10] W.J. Zheng, Y.H. Hu, M. Zhang, L. Sun, Analysis of the expression and antioxidative property of a peroxiredoxin 6 from *Scophthalmus maximus*, Fish Shellfish Immunol. 29 (2010) 305–311.
[11] C. Nikapitiya, M. De Zoysa, I. Whang, C.G. Kim, Y.H. Lee, S.J. Kim, J. Lee, Molecular cloning, characterization and expression analysis of peroxiredoxin 6 from disk abalone *Haliotis discus discus* and the antioxidant activity of its recombinant protein, Fish Shellfish Immunol. 27 (2009) 239–249.
[12] H. Kamata, H. Hirata, Redox regulation of cellular signaling, Cell. Signal. 11 (1999) 1–14.
[13] J. Nordberg, E.S. Arner, Reactive oxygen species, antioxidants, and the mammalian thioredoxin system, Free Radic. Biol. Med. 31 (2001) 1287–1312.
[14] L.S. Dai, X.M. Yu, M.N. Abbas, C.S. Li, S.H. Chu, S. Kausar, T.T. Wang, Essential role of the peroxiredoxin 4 in *Procambarus clarkii* antioxidant defense and immune responses, Fish Shellfish Immunol. 75 (2018) 216–222.
[15] Y. Min, S.M. Wi, D. Shin, E. Chun, K.Y. Lee, Peroxiredoxin-6 negatively regulates bactericidal activity and NF-κB activity by interrupting TRAF6-ECSIT complex, Front. Cell. Infect. Microbiol. 7 (2017) 94.
[16] T.C. Karlenius, K.F. Tonissen, Thioredoxin and cancer: a role for thioredoxin in all states of tumour oxygenation, Cancers 2 (2010) 209–232.
[17] E.M. Hanschmann, J.R. Godoy, C. Berndt, C. Hudemann, C.H. Lillig, Thioredoxins, glutaredoxins, and peroxiredoxins-Molecular mechanisms and health significance: from cofactors to antioxidants to redox signaling, Antioxidants Redox Signal. 19 (2013) 1539–1605.
[18] L. Wu, Y. Zhou, M.N. Abbas, S. Kausar, Q. Chen, C.X. Jiang, L.S. Dai, Molecular structure and functional characterization of the peroxiredoxin 5 in *Procambarus clarkii* following LPS and Poly I:C challenge, Fish Shellfish Immunol. 71 (2017) 28–34.
[19] D.Y. Jin, H.Z. Chae, S.G. Rhee, K.T. Jeang, Regulatory role for a novel human thioredoxin peroxidase in NF-kappa B activation, J. Biol. Chem. 272 (1997) 30952–30961.
[20] Y.J. Kim, W.S. Lee, C. Ip, H.Z. Chae, E.M. Park, Y.M. Park, Prx1 suppresses radiation-induced c-Jun NH2 terminal kinase signaling in lung cancer cells through interaction with the glutathione S-transferase Pi/c-Jun NH2-terminal kinase complex, Cancer Res. 66 (2006) 7136–7142.
[21] J.M. Hansen, S. Moriarty-Craige, D.P. Jones, Nuclear and cytoplasmic peroxiredoxin-1 differentially regulate NF-kappa B activities, Free Radic. Biol. Med. 43 (2007) 282–288.
[22] V. Haridas, J. Ni, A. Meager, J. Su, G.L. Yu, Y. Zhai, H. Kyaw, K.T. Akama, J. Hu,

L.J. Van Eldik, B.B. Aggarwal, TRANK, a novel cytokine that activates NF-kappa B and c-Jun N-terminal kinase, J. Immunol. 161 (1998) 1–6.
[23] H. Jung, T. Kim, H.Z. Chae, K.T. Kim, H. Ha, Regulation of macrophage migration inhibitory factor and thiol-specific antioxidant protein PAG by direct interaction, J. Biol. Chem. 276 (2001) 15504–15510.
[24] S.W. Kang, T.S. Chang, T.H. Lee, E.S. Kim, D.Y. Yu, S.G. Rhee, Cytosolic peroxiredoxin attenuates the activation of JNK and p38 but potentiates that of ERK in Hela cells stimulated with tumor necrosis factor-alpha, J. Biol. Chem. 279 (2004) 2535–2543.
[25] D.H. Lee, J.H. Park, S.B. Han, D.Y. Yoon, Y.Y. Jung, J.T. Hong, Peroxiredoxin 6 overexpression attenuates lipopolysaccharide-induced acute kidney injury, Oncotarget 8 (2017) 51096–51107.
[26] A.F. Helfrich, J. Distelano, Crayfish Biodiversity and Conservation, Sustaining American Aquatic Biodiversity, 2003, pp. 420–524.
[27] M.R. Fishar, Red swamp crayfish (*Procambarus clarkii*) in river nile Egypt, Biodiversity Monoitoring and Assessment Project (Bio Map), Nature Conversion Sector, 2006.
[28] J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin, D.G. Higgins, The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, Nucleic Acids Res. 25 (1997) 4876–4882.
[29] Trifinopoulos, L.T. Nguyen, A. von Haeseler, B.Q. Minh, W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis, Nucleic Acids Res. 44 (2016) 232–235.
[30] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(T) (-Delta Delta C) method, Methods 25 (2001) 402–408.
[31] M.N. Abbas, S. Kausar, Y.X. Sun, J.W. Tian, B.J. Zhu, C.L. Liu, Suppressor of cytokine signaling 6 can enhance epidermal growth factor receptor signaling pathway in *Bombyx mori* (Dazao), Dev. Comp. Immunol. 81 (2018) 187–192.
[32] S. Kausar, M.N. Abbas, C. Qian, B.J. Zhu, Y. Sun, Y.X. Sun, L. Wang, G.Q. Wei, I. Maqsood, C.L. Liu, Serpin-14 negatively regulates prophenoloxidase activation and expression of antimicrobial peptides in Chinese oak silkworm *Antheraea pernyi*, Dev. Comp. Immunol. 76 (2017) 45–55.
[33] Y. Mu, F.M. Lian, Y.B. Teng, J. Ao, Y.L. Jiang, Y.X. He, Y. Chen, C.Z. Zhou, X. Chen, The N-terminal b-sheet of peroxiredoxin 4 in the large yellow croaker *Pseudosciaena crocea* is involved in its biological functions, PLoS One 8 (2013) e57061.
[34] C. Mu, J. Zhao, L. Wang, L. Song, H. Zhang, C. Li, L. Qiu, Y. Gai, Molecular cloning and characterization of peroxiredoxin 6 from Chinese mitten crab *Eriocheir sinensis*, Fish Shellfish Immunol. 26 (2009) 821–827.
[35] H.A. Woo, H.Z. Chae, S.C. Hwang, K.S. Yang, S.W. Kang, K. Kim, S.G. Rhee, The inactivation of peroxiredoxins caused by cysteine sulfinic acid formation, Science 300 (2003) 653–656.
[36] X. Wang, S.A. Phelan, C. Petros, E.F. Taylor, G. Ledinski, G. Jurgens, K. Forsman-Semb, B. Paigen, Peroxiredoxin 6 deficiency and atherosclerosis susceptibility in mice: significance of genetic background for assessing atherosclerosis, Atherosclerosis 177 (2004) 61–70.
[37] Q. Wang, K.P. Chen, Q. Yao, Y. Zhao, Y.J. Li, H.X. Shen, R.H. Mu, Identification and characterization of a novel 1-Cys peroxiredoxin from silkworm, *Bombyx mori*, Comp. Biochem. Physiol. B 149 (2008) 176–182.
[38] D.D. Tu, Y.L. Zhou, W.B. Gu, Q.H. Zhu, B.P. Xu, Z.K. Zhou, Z.P. Liu, C. Wang, Y.Y. Chen, M.A. Shu, Identification and characterization of six peroxiredoxin transcripts from mud crab *Scylla paramamosain*: the first evidence of peroxiredoxin gene family in crustacean and their expression profiles under biotic and abiotic stresses, Mol. Immunol. 93 (2018) 223–235.
[39] S. Arun, P. Subramanian, Antioxidant enzymes in freshwater prawn *Macrobrachium malcolmsonii* during embryonic and larval development, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 121 (1998) 273–277.
[40] L. Cerenius, K. Söderhäll, The prophenoloxidase-activating system in invertebrates, Immunol. Rev. 198 (2004) 116–126.
[41] M.N. Abbas, B.J. Zhu, S. Kausar, L.S. Dai, Y.X. Sun, J.W. Tian, C.L. Liu, Suppressor of cytokine signalling 2-12 regulates antimicrobial peptides and ecdysteroid signaling pathways in *Bombyx mori* (Dazao), J. Insect Physiol. 103 (2017) 47–56.
[42] S. Kausar, M.N. Abbas, C. Qian, B.J. Zhu, J. Gao, Y. Sun, L. Wang, G.Q. Wei, C.L. Liu, Role of *Antheraea pernyi* serpin 12 in prophenoloxidase activation and immune responses, Arch. Insect Biochem. Physiol. (2017) e21435.
[43] X. Song, X. Xin, M. Dong, W. Wang, L. Wang, L. Song, The ancient role for GATA2/3 transcription factor homolog in the hemocyte production of oyster, Dev. Comp. Immunol. 82 (2018) 55–65.
[44] Z. Jia, L. Wang, S. Jiang, M. Sun, M. Wang, Q. Yi, L. Song, Functional characterization of hemocytes from Chinese mitten crab *Eriocheir sinensis* by flow cytometry, Fish Shellfish Immunol. 69 (2017) 15–25.
[45] J. Du, H. Zhu, P. Liu, J. Chen, Y. Xiu, W. Yao, T. Wu, Q. Ren, Q. Meng, W. Gu, W. Wang, Immune responses and gene expression in hepatopancreas from *Macrobrachium rosenbergii* challenged by a novel pathogen spiroplasma MR-1008, Fish Shellfish Immunol. 34 (2013) 315–323.
[46] S.M. Sun, X.P. Ge, H.T. Fu, J. Zhu, S.Y. Zhang, Molecular cloning and gene expression of peroxiredoxin (Prx) in oriental river pawn (*Macrobrachium nipponense*) in response to environmental hypoxia and reoxygenation, J. Fish. Sci. China 21 (2014) 474–483.
[47] Z. Liang, R. Liu, D. Zhao, L. Wang, M. Sun, M. Wang, L. Song, Ammonia exposure induces oxidative stress, endoplasmic reticulum stress and apoptosis in hepatopancreas of pacific white shrimp (*Litopenaeus vannamei*), Fish Shellfish Immunol. 54 (2016) 523–528.
[48] R. Sun, L. Qiu, Q. Yi, M. Wang, F. Yue, L. Wang, L. Song, CgNrdp1, a conserved negative regulating factor of MyD88-dependent Toll like receptor signaling in oyster *Crassostrea gigas*, Fish Shellfish Immunol. 74 (2018) 386–392.
[49] P. Zhang, Y. Liu, M. Wang, M. Dong, Z. Liu, Z. Jia, W. Wang, A. Zhang, L. Wang, L. Song, Chinese mitten crab (*Eriocheir sinensis*) iron-sulphur cluster assembly protein 2 (EsIscA2) is differentially regulated after immune and oxidative stress challenges, Dev. Comp. Immunol. 84 (2018) 343–352.
[50] C. Yang, L. Wang, H. Zhang, Q. Yi, L. Wang, H. Wang, L. Song, The first CUB-domain containing serine protease from *Chlamys farreri* which might be involved in larval development and immune response, Dev. Comp. Immunol. 76 (2017) 163–168.
[51] T.T. Priyathilaka, Y. Kim, H.M.V. Udayantha, S. Lee, H.M.L.P.B. Herath, H.H.C. Lakmal, D.A.S. Elvitigala, N. Umasuthan, G.I. Godahewa, S.I. Kang, H.B. Jeong, S.K. Kim, D.J. Kim, B.S. Lim, Identification and molecular characterization of peroxiredoxin 6 from Japanese eel (*Anguilla japonica*) revealing its potent antioxidant properties and putative immune relevancy, Fish Shellfish Immunol. 51 (2016) 291–302.
[52] R. Liu, Q. Cheng, X. Song, H. Wang, X. Wang, L. Wang, B. Zhu, L. Song, A vital ubiquitin-conjugating enzyme CgUbe2g1 participated in regulation of immune response of Pacific oyster *Crassostrea gigas*, Dev. Comp. Immunol. 91 (2019) 132–142.
[53] M. Wang, L. Wang, Y. Guo, R. Sun, F. Yue, Q. Yi, L. Song, The broad pattern recognition spectrum of the Toll-like receptor in mollusk Zhikong scallop *Chlamys farreri*, Dev. Comp. Immunol. 52 (2015) 192–201.
[54] J.A. Hoffmann, The immune response of *Drosophila*, Nature 426 (2003) 33–38.
[55] M.A. Anwar, S. Basith, S. Choi, Negative regulatory approaches to the attenuation of Toll-like receptor signaling, Exp. Mol. Med. 45 (2013) 11.
[56] M. Wang, L. Wang, Z. Jia, Q. Yi, L. Song, The various components implied the diversified Toll-like receptor (TLR) signaling pathway in mollusk *Chlamys farreri*, Fish Shellfish Immunol. 74 (2018) 205–212.
[57] W. Wang, T. Zhang, L. Wang, J. Xu, M. Li, A. Zhang, L. Qiu, L. Song, A new non-phagocytic TLR6 with broad recognition ligands from Pacific oyster *Crassostrea gigas*, Dev. Comp. Immunol. 65 (2016) 182–190.
[58] M.G. Sharapov, V.I. Novoselov, V.K. Ravin, The cloning, expression and comparative analysis of peroxiredoxin 6 from various sources, Mol. Biol. 43 (2009) 465–471.