**Molecular characterization and functional analysis of
lysosomal cathepsin D-like gene in red swamp crayfish,
*Procambarus clarkii***

Journal:	<i>Genome</i>
Manuscript ID	gen-2020-0154.R2
Manuscript Type:	Article
Date Submitted by the Author:	26-Apr-2021
Complete List of Authors:	Huang, Long; Anhui Academy of Agricultural Sciences Wu, Ben-li; Anhui Academy of Agricultural Sciences He, Ji-Xiang; Anhui Academy of Agricultural Sciences Zhang, Ye; Anhui Academy of Agricultural Sciences Chen, Jing; Anhui Academy of Agricultural Sciences Chen, Xia-jun; Anhui Academy of Agricultural Sciences
Keyword:	cathepsins, crayfish, immune genes, immune system, microbial infection
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

SCHOLARONE™
Manuscripts

Molecular characterization and functional analysis of lysosomal *cathepsin D-like* gene in red swamp crayfish, *Procambarus clarkii*

Long Huang[#], Ben-li Wu [#], Ji-xiang He^{*}, Ye Zhang, Jing Chen, Xia-jun Chen

Anhui Province Key Laboratory of Aquaculture & Stock Enhancement, Fisher Institute of Anhui Academy of Agricultural Sciences, Hefei, China, 230031

^{*} Corresponding author: Ji-Xiang He

Tel/fax: +86 551 65160709

E-mail: friaaas@163.com (JX He)

Address: Anhui Province Key Laboratory of Aquaculture & Stock Enhancement, Fisher Institute of Anhui Academy of Agricultural Sciences, Hefei 230031, P.R. China.

[#] These authors contributed equally to this work.

Abstract

The aspartic proteinases are one of the four families of proteinase enzymes, which are widely present among living organisms. They are involved in different physiological events such as protein degradation, development, and host defense. However, the characterization and functional roles of aspartic proteinases remain to be elucidated in crustaceans. Here, we characterized a fragment of *cathepsin D-like* cDNA from red swamp crayfish, *Procambarus clarkii* (*Pc-cathepsin D-like*). The *Pc-cathepsin D-like* gene's open reading frame contained 1152-bp, encoding a protein of 383 amino acid residues. We also evaluated the immunological role of the *Pc-cathepsin D-like* gene in vivo. Spatial distribution analysis revealed that the *Pc-cathepsin D-like* mRNA was high in the hepatopancreas, followed by gut, gill, and hemocytes of *P. clarkii*. The expression levels of *Pc-cathepsin D-like* gene increased following challenge with viral (Polyinosinic: polycytidylic acid) and bacterial (lipopolysaccharides, peptidoglycan) PAMPs compared with PBS injection. The suppression of the *Pc-cathepsin D-like* gene by RNA interference significantly increased the expression of immune-associated genes. These results showed that the *Pc-cathepsin D-like* gene has an essential biological role in the innate immune responses since it regulates the expression of immune-associated genes.

Keywords: cathepsins; crayfish; immune genes; immune system; microbial infection

1. Introduction

A lysosome is generally a house of various cathepsin molecules, divided into three categories: cysteine proteases, serine proteases, and aspartate proteases (Turk et al., 2002; Sirikharina et al., 2010). Aspartic peptidases (cathepsin D and E) catalyze molecular reactions at acidic pH and are sensitive towards inhibition by Pepstatin A. Their active site is characterized by the well-conserved triad Asp-Thr-Gly around the two aspartic residues located between two lobes (Fusek and Vetvicka, 2005; Söderhäll et al., 2010). Aspartic peptidases have been described in a wide range of taxa and have extensively been studied in mammals, parasites, and insects (Söderhäll et al., 2005; Valle et al., 2017; Rebello et al., 2018).

Cathepsin D, aspartic proteinase, is an essential component of the lysosome, present in almost all mammalian cells and tissues. It is produced in the rough endoplasmic reticulum as a zymogen and transported to lysosomes, where it is transformed into a mature enzyme after proteolytic cleavage (Richo and conner, 1994; Cerenius and Söderhäll, 2018; Korkut et al., 2018). Due to the capability of degrading structural and functional proteins, cathepsin D modulates a wide range of physiological functions. In parasites, cathepsin D contributes to degradation of hemoglobin (Sojka et al., 2012; Mancilla-Ole et al., 2018), muscle (Ladtrat et al., 2003; Godiksen et al., 2009), and serum proteins in the host. Also, it plays a crucial role in different developmental stages in some parasites (Leyria et al., 2015). In humans, cathepsin D participates in various processes such as the pathogenesis of human neurodegenerative disorders (Zhang et al., 2010; Vidoni et al., 2016), the activation of growth factors, hormones (Jiravanichpaisal et al., 2009; Dubey et al., 2017), signaling pathways leading to cell death (Salvesen et al., 2001). Although cathepsin D has been studied intensively in the development of

cancer drugs in recent years, its role in the generation of antigenic peptides and MHC class-II-mediated antigen presentation has produced inconsistent results too small in different species (Tsukuba et al., 2000).

In contrast to mammals, the literature on aspartic peptidases is scanty in crustaceans (Meng et al., 2019; Liu et al., 2020). So far, only a few studies reported the physiological functions of aspartic peptidases in crustaceans. For example, a cathepsin D-like protein extracted from the American lobster's digestive tract (*Homarus americanus*) has been characterized for its physiological functions (Rojo et al., 2013; Rodriguez-Siordia et al., 2018). Therefore, it is much required to identify and characterize aspartic peptidases to improve our understanding of crustaceans' cathepsins. In this study, to the best of our knowledge, we first time identified the cathepsin D-like gene from red swamp crayfish, *P. clarkii* (*Pc-cathepsin D-like*), and determined its spatial and temporal distribution. We also analyzed the immunoregulatory role of the *Pc-cathepsin D-like* gene after knocked it down.

2. Materials and methods

2.1 Animals and sample collection

Red swamp crayfish, *P. clarkii* (average body weight: 10 ± 0.5 g) were obtained from an aquaculture farm, Zhejiang, China. They were transported to Wenzhou Medical University and maintained in tanks with running aerated water at ambient temperature (28 ± 2 °C) with salinity at 15 ppt. To acclimatize, they were kept in these tanks for at least two weeks, and the animals were fed with natural (insect larvae and water snail) and commercial diet (Chu et al., 2019; Abbas et al., 2019; Li et al., 2020). For the spatial analysis of *Pc-cathepsin D-like* gene, muscle,

heart, stomach, hepatopancreas, gut, hemocyte, and gill were sampled and stored at -80 °C until further use. Three experimental groups (injected with 1 mg/ mL PGN (bacterial peptidoglycan) or 1 mg/ mL LPS (bacterial lipopolysaccharide) or 1 mg/ mL poly I:C) and the control group (injected with 1 mg/ mL PBS) were established to investigate the response of *Pc-cathepsin D-like* after stimulation under biotic stress, with 20 individuals in each group. Hepatopancreas tissue from individuals at time points of 3, 6, 12, 24, 36, and 48 h after treatment were collected for *Pc-cathepsin D-like* mRNA level detection, separately. Three additional groups were used for the RNA interference test, with 20 individuals in each group.

2.2 Full-length cDNA cloning of the *Pc-cathepsin D-like* gene

For the full-length *Pc-cathepsin D-like* cDNA cloning, the total RNA was extracted from the hepatopancreas of *P. clarkii* using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and then purified using a QuickPrep Micro mRNA Purification Kit (GE Healthcare, USA). The first-strand cDNA was prepared using the TransScript Synthesis SuperMix (TransGen, Beijing, China) according to the manufacturer's instructions. The nucleotide sequence of the *Pc-cathepsin D-like* gene was obtained from the *P. clarkii* genome library, which was previously constructed in our laboratory (Dai et al., 2017; Zhou et al., 2017). For cloning the *Pc-cathepsin D-like* gene, gene-specific primers were designed using partial sequences in the *P. clarkii* database mentioned above using Oligo 7.0 software (Table 1). Thermal cycling for polymerase chain reaction (PCR) was as followed: 94 °C for 3 min, 35 cycles at 94 °C for 30 s, 55 °C for 35 s and 72 °C for 45 s. The PCR products were subjected to agarose gel (1%) electrophoresis, and the gene of interest was purified for further analysis. The purified fragment was then inserted into the pMD19-T Vector and sequenced by Invitrogen.

2.3 Bioinformatics analysis

For homology analysis, the *cathepsin D-like* cDNA was analyzed by the BLAST program ([http:// www.ncbi.nlm.nih.gov/blast](http://www.ncbi.nlm.nih.gov/blast)). The deduced amino acid sequence of *cathepsin D-like* was obtained from the Expert Protein Analysis System (<http://www.expasy.org/>). Multiple sequence alignments were performed in Clustal X software (Thompson et al., 1997). A phylogenetic tree was constructed with cathepsin D from different animal species using MEGA 6 software (1000 bootstrap replicates) by the Neighbor-Joining (NJ) method (Tamura et al., 2011).

2.4 Determination of the tissue localization of *cathepsin D-like* mRNA

A quantitative real-time PCR (qPCR) assay was used for mRNA expression level detection. The total RNAs of sampled tissues were reverse transcribed into cDNA using a TransScript Synthesis SuperMix (TransGen, Beijing, China). Oligonucleotide primers specific for the *cathepsin D-like* sequence were designed by using Primer 5.0 software (Table 1). The PCR was performed at a total volume of 25 μ L containing 12.5 μ L 2 $\times$ SYBR Premix Ex TaqII (Takara), 2 μ L of diluted cDNA template, 2 μ L primer mix, and 8.5 μ L RNase-free H₂O. The PCR programs were set as follows: initial denaturation at 95 $^{\circ}$ C for 3 min, followed by 40 cycles at 95 $^{\circ}$ C for 10 s, 55 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 30 s. The continuous fluorescence acquisition 65-95 $^{\circ}$ C with an increase of 0.5 $^{\circ}$ C per 10 s was used for melting curve analysis. The relative expression of the *cathepsin D-like* gene was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak, and Schmittg, 2001), and the 18S rRNA gene was used as an endogenous control.

2.5 Expression profiles of *cathepsin D-like* gene under biotic stress

Three of the four groups of red swamp crayfish were injected with PGN, LPS, or poly I:C, 1 mg/mL, respectively. The rest of the group treated with only PBS was used as the control. These injections were delivered with a Hamilton Microliter™ syringe (Sigma-Aldrich, China). Four organisms were taken as one sample, and the biological sampling protocol was repeated three times. At 3, 6, 12, 24, 36, and 48 h post-injection, hepatopancreas from twenty individual crayfish were collected into liquid nitrogen and stored at -80 °C until RNA isolation. The mRNA distribution of *cathepsin D-like* gene was analyzed by qRT-PCR using the same protocol as described in section 2.4.

2.6 Effect of *cathepsin D-like* silencing on the expression of immune-pathways associated genes

To investigate the effect of *cathepsin D-like* gene depletion on the expression of genes related to the immune pathway, double-stranded (ds) RNAs of *cathepsin D-like* gene and fluorescent protein (EGFP) were prepared by following the protocol of T7 RiboMAX Express RNAi System (Promega, USA). A template for *in vitro* transcription reactions was prepared by PCR amplification of a clone containing the *cathepsin D-like* gene ORF using gene-specific primers (Table 1) with a T7 polymerase binding sequence at 5' end, as described before in section 2.4, except for a change in the cDNA templates. After phenol-chloroform extraction and heat treatment, the dsRNA was diluted with nuclease-free water to a final concentration of 10 µL (1 µg/µL) for injection into red swamp crayfish. EGFP dsRNA and nuclease-free DEPC water were served as negative controls injected with the same dose. Twenty red swamp crayfish were treated in each independent experiment, respectively. The needles were kept still at the injection point for the 30 s. Hemocyte samples were collected from each treatment group at 24 and 48 h

after injection. Total RNA was isolated and used for the expression analysis of immune-pathway associated genes.

2.7 Data analysis

Statistical analysis was one-way analysis variance (ANOVA) followed by Tukey's test and performed using SPSS version 17.0. The difference with a p -value less than 0.05 were considered statistically for all treatments.

3. Results

3.1. Bioinformatics analysis of *Pc-cathepsin D-like*

The full-length cDNA sequence of *Pc-cathepsin D-like* comprised a 1152 bp open reading frame (ORF). The ORF sequence encoded a protein of 383 amino acid residues with a calculated molecular weight of 41.47 KDa and an isoelectric point of almost 4.35. The protein had a signal peptide of 16 amino acid residues and two active sites (D) motif at amino acid residues 81 and 268, respectively (Fig. 1). The deduced amino acid sequence of cathepsin D-like shared the highest identity (73.59 aa) to that of *P. vannamei* followed by *H. americanus* (74.09 aa), while lowest identity (47.37 aa) to that of *H. sapiens*. Five conserved cysteine residues dispersed throughout the *Pc-cathepsin D-like* sequence (shown as dots), and two active sites motif at amino acid residues 81 and 268 (shown as arrows) (Fig. 2).

To understand the evolutionary relationship of *Pc-cathepsin D-like* protein with cathepsin D or D-like proteins from other animal species, a phylogenetic tree was constructed using amino acid sequences of selected invertebrate and vertebrate species (Fig. 3). The results revealed that

crustaceans cathepsin D were clustered into one clade, which was separated obviously from the clade of mollusk, insects, mammals, and fishes. In the crustacean's clade, Pc-cathepsin D-like exhibited a close relationship with *Penaeus vannamei*, *Homarus americanus*, *Homarus Gammarus*, which is in line with the results of sequence (amino acid) identity analysis.

3.2. Expression profiles of *cathepsin D-like* gene in different tissues

To determine the expression profile of the *Pc-cathepsin D-like* gene in various tissues (hepatopancreas, muscle, gut, stomach, heart, hemocytes, and gill), we performed a qRT-PCR assay (Fig. 4). The *Pc-cathepsin D-like* gene was expressed constitutively in all the examined tissues; however, *Pc-cathepsin D-like* expression levels varied among them. It was most significant in the hepatopancreas, followed by the gut.

3.3. Expression profiles of Pc-cathepsin D-like genes under biotic stress

The temporal expression of the *Pc-cathepsin D-like* was determined in the hepatopancreas using the qRT-PCR assay. A significant change in *Pc-cathepsin D-like* expression was found in hepatopancreas after the poly I:C, LPS, and PGN treatment. However, both the mRNA level and the time of the highest mRNA expression were not synchronized among them. In the hepatopancreas, red crayfish challenged by poly I:C exhibited approximately equal transcript level to the control group after infection for 3 h (Fig. 5A). However, the level of expression reached its peak after 24h of treatment. The maximum expression of the *Pc-cathepsin D-like* gene was observed after 6 h of LPS treatment (Fig. 5B), and this occurred after 6 and 36 h of PGN treatment (Fig. 5C).

3.4. *Pc-cathepsin D-like* regulates the expression of genes associated with immune-pathways

To determine the possible role of *Pc-cathepsin D-like* gene in innate immune responses, the transcript patterns of different genes related to the immune pathways were quantified following the depletion of *Pc-cathepsin D-like* by dsRNA, and the dsEGFP treated individuals were used as control. The efficiency of dsRNA administration at 24 h and 48 h was analyzed by executing qRT-PCR (Fig. 6A). The expression of all the tested immune-pathway associated genes (Serine/threonine protein kinase, Anti-lipopolysaccharide factor, Sptäzle, Cactus, Lectin-like, and Toll3) was significantly increased in the *Pc-cathepsin D-like* silenced group compared with the dsEGFP control (Fig. 6B). These results suggest that *Pc-cathepsin D-like* has a crucial biological role in the immune responses of *P. clarkii* since it can modulate genes associated with immune pathways. To further ensure our results, we analyzed the expression levels of non-immunity genes, including oxysterol binding protein 1 and Ecdysteroid receptor. The results revealed that the depletion of *Pc-cathepsin D-like* did not affect the expression of non-immunity genes.

4. Discussion

In this study, we identified and characterized a cathepsin D-like cDNA from red swamp crayfish, *P. clarkii*. *Pc-cathepsin D-like* cDNA encoded a predicted protein of 383 amino acids, including the peptidase domain containing the active sites that are the characteristic feature of cathepsin D-like proteins. Amino acid sequence analyses revealed *Pc-cathepsin D-like* showed 47.37%-74.09% identity to cathepsin proteins from other species, including crustaceans and vertebrates. Phylogenetic analysis exhibited that *Pc-cathepsin D-like* was most closely related to

211 *P. vannamei* cathepsin D. In living organisms, cathepsin D participates in different molecular
212 reactions to maintain homeostasis. In parasites, cathepsin D controls the degradation of serum
213 proteins, haemoglobin, and muscle in the host (Godiksen et al., 2009; Sojka et al., 2012;
214 Mancilla-Olea et al., 2018). Moreover, it regulates various physiological processes, including
215 immune responses, cell death-related signaling pathways, etc., in invertebrates and vertebrates
216 (Dubey et al., 2017; Vidoni et al., 2016; Sun et al., 2017). Thus, it is much needed to explore the
217 biological role of cathepsin D to understand physiological functions and molecular mechanisms
218 in economically important animals, including crustaceans.

219 We investigated the spatial expression patterns of *Pc-cathepsin D-like* gene of *P. clarkii*
220 and found it to be very similar to those of cathepsins of other crustaceans (Apitanyasai et al.,
221 2016; Sun et al., 2018; Liu et al., 2020; Huang et al., 2020). In healthy and non-induced *P.*
222 *clarkii*, *Pc-cathepsin D-like* mRNA was expressed in all the tested tissues, including muscle,
223 heart, stomach, hepatopancreas, gut, hemocyte, and gill. However, the expression was
224 remarkably higher in hepatopancreas and gut, and the expression was lowest in muscle. The
225 hepatopancreas is implicated in the immune response by producing immune factors such as
226 lectins, nitric oxide, hemocyanin and antimicrobial peptides to prevent microbial diseases.
227 Additionally, it modulates secretion of digestive enzymes, digestion of carbohydrates and fat,
228 absorption of nutrients, and storage of energy in the form of lipid etc., suggesting the
229 multifunctional organ in crustaceans (Yasothornsrikul et al., 2003; Benes et al., 2008; Chen et
230 al., 2011; Liu et al., 2019). Thus, the high level of *Pc-cathepsin D-like* mRNA in the
231 hepatopancreas suggests that *Pc-cathepsin D-like* may play a similar role in *P. clarkii* like other
232 cathepsins.

Many authors reported the upregulation of cathepsins in tissues/organ of aquatic invertebrates infected with microbial pathogens. For example, cathepsin A and C gene expression were upregulated in the hepatopancreas of crayfish infected with bacterial and viral pathogens (Oidtmann et al., 2004; Liu et al., 2020; Huang et al., 2020). In both cases, the crayfish exposed to pathogens for different time periods to evaluate the change in the expression of cathepsins. Infection of *Spiroplasma eriocheiris* in *Eriocheir sinensis* has also been reported to increase the expression of cathepsin D gene (Ning et al., 2018). In this case, the crustacean was exposed to the pathogen for a long period (for nine days). In the present study, *P. clarkii* was infected with immune elicitors (virus and bacteria) for a short period (time points: 3, 6, 12, 24, 36, and 48 h). The expression of the *Pc-cathepsin D-like* gene in the hepatopancreas was strongly increased at critical time points of infection. *Pc-cathepsin D-like* expression profiles are consistent with the previous studies, although the time exposed to the microbial pathogen is different. A recent study suggested that recognition and regulator genes are usually enhanced after microbial infection (Xiong et al., 2015; Abbas et al., 2020a; Abbas et al., 2020b). Thus, we proposed that *Pc-cathepsin D-like* might implicate in the regulation of immune genes in the *P. clarkii*.

Immune-pathway associated genes are important molecules that indicate the immune-pathways activity, and their mRNA expression can be either ubiquitous in a basal level or stimulated following pathogen challenge (Min et al., 2017; Sun et al., 2017; Abbas et al., 2017). The IMD and Toll signaling pathways and their related molecules are the primary components of the innate immune system, which regulate immune effectors, e.g., the production of antimicrobial proteins (Min et al., 2017; Kausar et al., 2017, 2018). After the microbial invasion, Toll receptors detect microbial pathogens' components, thereby transfer signals to the

downstream molecules (Uematsu and Akira, 2006; Medzhitov et al., 2007; Kausar et al., 2019). Several studies reported the enhancement of expression of Toll receptors after challenge with LPS and *Vibrio anguillarum* in *F. chinensis*, *M. japonicus*, and *L. vannamei* (Xu et al., 2000; Yang et al., 2008; Dai et al., 2018). The stimulation of Toll receptors is associated with activation of immune pathways, subsequently, induce the expression of immune effectors such as ALF to inhibit infection (Ma et al., 2019; Zhu et al., 2019). However, the modulation of the expression of Toll receptors and other immune genes is less explored in crustaceans. In the present study, we found that *Pc-cathepsin D-like* silencing resulted in an upregulation of all the tested genes, including Serine/threonine protein kinase, Lectin-like, Sptäzle, Antilipopolysacchride, Toll3, and Cactus in the hepatopancreas of *P. clarkii*. The upregulated genes mainly contribute to immune functions in the crustacean. Toll receptors recognize of microbial pathogens and activate immune responses. Sptzale is an extracellular ligand that induces innate immunity via binding with Toll receptor after detecting pathogens, and cactus has antifungal functions, and so on. In addition, the expression levels of Toll3 and lectin-like were higher after 48 h of *Pc-cathepsin D-like* silencing; even the down-regulation of the gene was almost similar at both the time points, suggesting both the genes may follow time-dependent expression patterns or different animals may have a different response in similar conditions. Additionally, non-immunity genes expression remains unchanged in *Pc-cathepsin D-like* and control. Altogether, these results allowed us to infer that *Pc-cathepsin D-like* is involved in the regulation of immune-pathway associated genes. The involvement of cathepsins in the modulation of immune genes is not entirely a new phenomenon, and it has also been reported in many other studies (Liu et al., 2020; Huang et al., 2020). However, further research is required to elucidate molecular mechanisms by which cathepsins regulate immune genes.

In summary, the full-length cDNA sequence of *Pc-cathepsin D-like* from red swamp crayfish, *P. clarkii* was cloned and molecularly characterized. The *Pc-cathepsin D-like* is produced in all tested tissue, and challenge with immune elicitors induced its expression. We also observed that *Pc-cathepsin D-like* contributes to the regulation of immune-pathway associated genes.

Acknowledgements

This work was supported by the National Key Research and Development Project of China (2018YFD0300904), the central government Guiding Local Science and Technology Development for Anhui Province (201907d06020015), the Scientific and Technological Innovation Team of Anhui Academy of Agricultural Sciences (2020YL041).

Competing interests

The authors have declared that no competing interests exist.

References

- Abbas, M.N. Kausar, S., Zhao, E., Cui, H. 2020a. Suppressors of cytokine signaling proteins as modulators of development and innate immunity of insects. *Dev. Comp. Immunol.* 104, 103561.
- Abbas, M.N., Kausar, S. Sun, Y.X., Sun, Y., Wang, L., Qian, C., Wei, G.Q., Zhu, B.J., Liu, C.L. 2017. Molecular cloning, expression, and characterization of E2F transcription factor 4 from *Antheraea pernyi*. *Bull. Entomol. Res.* 1, 8.
- Abbas, M.N., Kausar, S., Cui, H., 2019. The biological functions of peroxiredoxins in innate immune responses of aquatic invertebrates. *Fish Shellfish Immunol.* 89, 91-97.
- Abbas, M.N., Liang, H., Kausar, S., Dong, Z., Cui, H. 2020b. Zinc finger protein RP-8, the *Bombyx mori* ortholog of programmed cell death 2, regulates cell proliferation. *Dev. Comp. Immunol.* 104, 103542.

- 303 Apitanyasai, K., Noonin, C., Tassanakajon, A., Söderhäll, I., Söderhäll, K., 2016.
304 Characterization of a hemocyte homeostasis-associated-like protein (HHAP) in the
305 freshwater crayfish *Pacifastacus leniusculus*. Fish Shellfish Immunol. 58, 429-435.
- 306 Benes, P., Vetvicka, V., Fusek, M., 2008. Cathepsin D: many functions of one aspartic protease.
307 Crit. Rev. Oncol. Hematol. 68, 12-28.
- 308 Cerenius, L., Söderhäll, K., 2018. Crayfish immunity: Recent findings. Dev. Comp. Immunol.
309 80, 94-98.
- 310 Chen, L., Zhang, M. Sun, L., 2011. Identification and expressional analysis of two cathepsins
311 from half-smooth tongue sole (*Cynoglossus semilaevis*). Fish Shellfish Immunol. 31, 1270-
312 1277.
- 313 Chu, S.H., Liu, L., Abbas, M.N., Li, Y.Y., Kausar, S. Qian, X.Y., Ye, Z.Z., Yu, X.M., Li, X.K.,
314 Liu, M., Dai, L.S., 2019. Peroxiredoxin 6 modulates Toll signaling pathway and protects
315 DNA damage against oxidative stress in red swamp crayfish (*Procambarus clarkii*). Fish
316 Shellfish Immunol. 89, 170-178.
- 317 Dai, L.S., Abbas, M.N., Kausar, S., Zhou, Y., 2017. Transcriptome analysis of hepatopancreas of
318 *Procambarus clarkii* challenged with polyribonucleosinic polyribocytidylic acid (poly I:C).
319 Fish Shellfish Immunol. 71, 144-150.
- 320 Dai, L.S., Yu, X.M., Abbas, M.N., C.S. Li, Chu, S.H., Kausar, S., Wang, T.T. 2018. Essential
321 role of the peroxiredoxin 4 in *Procambarus clarkii* antioxidant defense and immune
322 responses. Fish Shellfish Immunol. 75, 216-222.
- 323 Dubey, V., Luqman, S., 2017. Cathepsin D as a promising target for the discovery of novel
324 anticancer agents. Curr. Cancer Drug Targets. 17, 404-422.
- 325 Fusek, M., Větvicka V., 2005. Dual role of cathepsin D: ligand and protease. Biomed. Papers.
326 149, 43-50.
- 327 Godiksen, H., Morzel, M., Hyldig, G., Jessen, F., 2009. Contribution of cathepsins B, L and D to
328 muscle protein profiles correlated with texture in rainbow trout (*Oncorhynchus mykiss*).
329 Food Chem. 113, 889-896.
- 330 Huang, L., Dai, L.S., Wu, B.L., Zhang, Y., Chen, J., He, J.X., 2020. Identification and
331 immunoregulatory role of cathepsin A in the red swamp crayfish, *Procambarus clarkii*. Int.
332 J. Biol. Macromol. 153, 865-872.

- Jiravanichpaisal, P., Roos, S., Edsman, L., Liu, H., Söderhäll, K., 2009. A highly virulent pathogen, *Aeromonas hydrophila*, from the freshwater crayfish *Pacifastacus leniusculus*. J. Invert. Pathol. 101, 56-66.
- Kausar, S. Abbas, M.N., Zhao, Y., Cui, H. 2019. Immune strategies of silkworm, *Bombyx mori* against microbial infections. Invert. Surviv. J. 16, 130-140.
- Kausar, S., Abbas, M.N., Qian, C. Zhu, B.J., Gao, J., Sun, Y., Wang, L., Wei, G.Q., Liu, C.L. 2018. Role of *Antheraea pernyi* serpin 12 in prophenoloxidase activation and immune responses. Arch. Insect Biochem. Physiol. e21435.
- Kausar, S., Qian, C., Abbas, M.N., Zhu, B.J., Liu, Y., Wang, L., Wei, G.Q., Sun, Y., Liu, C.L. 2017. Characterization and functional analysis of serpin-10 gene from oak silkworm *Antheraea pernyi*. Eur. J. Entomol. 114, 430-438.
- Korkut, G.G., Söderhäll, I., Söderhäll, K., Noonin, C., 2018. The effect of temperature on bacteria-host interactions in the freshwater crayfish, *Pacifastacus leniusculus*. J. Invert. Pathol. 157, 67-73.
- Ladrat, V., Verrez-Bagnis, Noel, J., Fleurence, J., 2003. In vitro proteolysis of myofibrillar and sarcoplasmic proteins of white muscle of sea bass (*Dicentrarchus labrax* L.): effects of cathepsins B, D and L. Food Chem. 81, 517-525.
- Leyria, J., Fruttero, L.L., Nazar, M., Canavoso, L.E., 2015. The Role of DmCatD, a cathepsin D-like peptidase, and acid phosphatase in the process of follicular atresia in *Dipetalogaster maxima* (Hemiptera: Reduviidae), a vector of chagas' disease. PLoS One. 10, e0130144.
- Li, C.S., Kausar, S., Gul, I., Yao, X.X., Li, M.Y., Chen, C.C., Abbas, M.N., Dai, L.S., 2020. Heat shock protein 20 from *Procambarus clarkii* is involved in the innate immune responses against microbial infection. Dev. Comp. Immunol. 106, 103638.
- Liu, M., Liu L., Abbas, M.N., Kausar, S., Zhang, J.W., Ye, Z.Z., Qian, X.Y., Zhao, X.M., Chu, S.H., Dai, L.S., 2019. Involvement of gamma interferon inducible lysosomal thiol reductase in the innate immune responses of red swamp crayfish, *Procambarus clarkii*. Dev. Comp. Immunol. 99, 103405.
- Liu, Q.N., Kausar, S., Gul, I., Zhou, H.L., Abbas, M.N., Dai, L.S., 2020. The red swamp crayfish, *Procambarus clarkii* cathepsin C, participates in the innate immune response to the viral and bacterial pathogens. Fish shellfish Immunol. 100, 436-444

- 364 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time
365 quantitative PCR and the $2(-\Delta\Delta C(T))$ method. *Methods*. 25, 402-408.
- 366 Ma, H., Abbas, M.N., Zhang, K., Hu, X., Xua, M., Liang, H., Kausar, S., Yang, L., Cui, H. 2019.
367 20-Hydroxyecdysone regulates the transcription of the lysozyme via Broad Complex Z2
368 gene in silkworm, *Bombyx mori*. *Dev. Comp. Immunol.* 94, 66-72.
- 369 Mancilla-Olea, M.I., Ortega-López, J., Figueroa-Angulo, E.E., Avila-González, L., Cárdenas-
370 Guerra, R.E., Miranda-Ozuna, J.F.T., González-Robles, A., Hernández-García, M.S.,
371 Sánchez-Ayala, L. Arroyo, R., 2018. Trichomonas vaginalis cathepsin D like aspartic
372 proteinase (Tv-CatD) is positively regulated by glucose and degrades human hemoglobin.
373 *Int. J. Biochem. Cell Biol.* 97, 1-15.
- 374 Medzhitov, R. 2007. TLR-mediated innate immune recognition. *Semin. Immunol.* 19, 1-2.
- 375 Meng, X., Hong, L., Yang, T.T., Liu, Y., Jiao, T., Chu, X.H., Zhang, D.Z., Wang, J.L., Tang,
376 B.P., Liu, Q.N., Zhang, W.W., He, W.F., 2019. Transcriptome-wide identification of
377 differentially expressed genes in *Procambarus clarkii* in response to chromium challenge.
378 *Fish Shellfish Immunol.* 87, 43-50.
- 379 Min, Y.H., Zhu, B.J., Sun, Y., Wei, G.Q., Wang, L., Qian, C., Abbas, M.N., Liu, C.L. 2017.
380 Characterization and functional analysis of serpin-1 like gene from oak silkworm *Antheraea*
381 *pernyi*. *Bull. Entomol. Res.* 1, 7.
- 382 Ning, M., Yuan, M., Liu, M., Gao, Q., Wei, P., Gu, W., Wang, W., Meng, Q., 2018.
383 Characterization of cathepsin D from *Eriocheir sinensis* involved in *Spiroplasma*
384 *eriocheiris* infection. *Dev. Comp. Immunol.* 86, 1-8.
- 385 Oidtmann, B., Schaefer, N., Cerenius, L., Söderhäll, K., Hoffmann, R.W., 2004. Detection
386 of genomic DNA of the crayfish plague fungus *Aphanomyces astaci* (Oomycete) in clinical
387 samples by PCR. *Vet. Microbiol.* 100, 269-282.
- 388 Rebello, K.M., McKerrow, J.H., Mota, E.M., Donoghue, A.J. O., Neves-Ferreira, A.G.C., 2018.
389 Activity profiling of peptidases in *Angiostrongylus costaricensis* first-stage larvae and adult
390 worms. *PLoS Negl. Trop. Dis.* 31, e0006923.
- 391 Richo, G.R., Conner, G.E., 1994. Structural requirements of procathepsin D activation and
392 maturation. *J. Biol. Chem.* 269, 14806-14812.

- Rodriguez-Siordia, I., Rojo-Arreola, L., Navarrete Del Toro, M.L.A., García-Carreño, F., 2018. American lobster cathepsin D, an aspartic peptidase resistant to proteolysis and active in organic solvents, non-ionic detergents and salts. *Int. J. Biol. Macromol.* 107, 1501-1509.
- Rojo, L., García-Carreno, F., Navarrete Del Toro, M.L.A., 2003. Cold-adapted digestive aspartic protease of the clawed lobsters *homarus americanus* and *homarus gammarus*: biochemical characterization. *Mar. Biotechnol.* 15, 87-96.
- Salvesen, G.S., 2001. A lysosomal protease enters the death scene. *J. Clin. Invest.* 107, 21-22.
- Shi, H., Ruan, L., Söderhäll, I., Soderhall, K., Xu, X., 2018. Transfection of crayfish hematopoietic tissue cells. *Dev. Comp. Immunol.* 88, 70-76.
- Sirikharina, R., Söderhäll, I., Söderhäll, K., 2018. Characterization of a cold-active transglutaminase from a crayfish, *Pacifastacus leniusculus*. *Fish Shellfish Immunol.* 80, 546-549.
- Söderhäll, I., Kim, Y.A., Jiravanichpaisal, P., Lee, S.Y., Söderhäll, K., 2005. An ancient role for a prokineticin domain in invertebrate hematopoiesis. *J. Immunol.* 174, 6153-6160.
- Söderhäll, K., 2010. Invertebrate immunity. Preface. *Adv. Exp. Med. Biol.* 708, vii-ix.
- Sun, Y.X., Zhu, B.J., Tang, L., Sun, Y., Chen, C., Abbas, M.N., Wang, L., Qian, C., Wei, G.Q., Liu, C.L., 2017. Cathepsin O is Involved in the Innate Immune Response and Metamorphosis of *Antheraea pernyi*. *J. Invertebr. Pathol.* 150, 6-14.
- Sun, Y.X., Zhu, B.J., Tang, L., Sun, Y., Chen, C., Abbas, M.N., Wang, L., Qian, C., Wei, G.Q., Liu, C.L. 2017. Cathepsin O is involved in the innate immune response and metamorphosis of *Antheraea pernyi*. *J. Invertebr. Pathol.* 150, 6-14.
- Sun, Y.X., Tang, L., Wang, P., Abbas, M.N., Tian, J.W., Zhu, B.J., Liu, C.L., 2018. Cathepsin L-like protease can regulate the process of metamorphosis and fat body dissociation in *Antheraea pernyi*. *Dev. Comp. Immunol.* 78, 114-123.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731-2739.
- Turk, V., Turk, B., Guncar, G., Turk, D., 2002. Lysosomal cathepsins: structure, role in antigen processing and presentation, and cancer. *Adv. enzyme Regul.* 42, 285-304.
- Uematsu, S., Akira, S. 2006. Toll-like receptors and innate immunity. *J. Mol. Med.* 84, 712-725.

- Valle, R.S., Ramos, L.S., Reis, V.J., Ziccardi, M., Dornelas-Ribeiro, M., Sodré, C.L., Branquinho, M.H., Santos, A.L.S., 2017. *Trichosporon asahii* secretes a 30-kDa aspartic peptidase. *Microbiol. Res.* 205, 66-72.
- Vidoni, C., Follo, C., Savino, M., Melone, M.A., Isidoro, C., 2016. The role of cathepsin D in the pathogenesis of human neurodegenerative disorders. *Med. Res. Rev.* 36, 845-870.
- Xiong, G.H., Xing, L.S., Lin, Z., Saha, T.T., Wang, C., Jiang, H., Zou, Z., 2015. High throughput profiling of the cotton bollworm *Helicoverpa armigera* immuno-transcriptome during the fungal and bacterial infections. *BMC Genomics.* 16, 321.
- Xu, Y., Tao, X., Shen, B., Horng, T., Medzhitov, R., Manley, J.L., Tong, L. 2000. Structural basis for signal transduction by the Toll/interleukin-1 receptor domains. *Nature.* 408, 111-115.
- Yang, C., Zhang, J., Li, F., Ma, H., Zhang, Q., Jose Priya, T.A., Zhang, X., Xiang, J. 2008. A Toll receptor from Chinese shrimp *Fenneropenaeus chinensis* is responsive to *Vibrio anguillarum* infection. *Fish Shellfish Immunol.* 24, 564-574.
- Yasothornsrikul, S., eenbaum, D., Medzihradszky, K.F., Toneff, T., Bunday, R., Miller, R., 2003. Cathepsin L in secretory vesicles functions as a prohormone-processing enzyme for production of the enkephalin peptide neurotransmitter. *Proc. Natl. Acad. Sci. USA.* 100, 9590-9595.
- Zhang, Y., Söderhäll, I., Söderhäll, K., Jiravanichpaisal, P., 2010. Expression of immune-related genes in one phase of embryonic development of freshwater crayfish, *Pacifastacus leniusculus*. *Fish Shellfish Immunol.* 28, 649-653.
- Zhou, M., Abbas, M.N., Kausar, S., Jiang, C.X., Dai, L.S., 2017. Transcriptome profiling of red swamp crayfish (*Procambarus clarkii*) hepatopancreas in response to lipopolysaccharide (LPS) infection. *Fish Shellfish Immunol.* 71, 423-433.
- Zhu, J.J., Ye, Z.Z., Li, C.S., Kausar, S., Abbas, M.N., Xiang, G.H., Qian, X.Y., Dai, L.S. 2019. Identification and molecular characterization of a novel anti-lipopolysaccharide factor (ALF) from red swamp crayfish, *Procambarus clarkii*. *Int. J. Biol. Macromol.* 132, 43-50.

Figure legends

Fig. 1 Nucleotide and deduced amino acid sequences of *Pc-cathepsin D-like* gene. Signal peptides (1-16 aa) are underlined, the Peptidase domain is boxed, and active sites are shaded with yellow colour. The start and stop codons were showed by box and asterisk, respectively.

Fig. 2 Multiple sequence alignment of the *Pc-cathepsin D-like* protein from *Procambarus clarkii* with other cathepsin D proteins. The deduced amino acid sequence of *Pc-cathepsin D-like* was aligned with the cathepsin D proteins from *P. vannamei* (ROT61198.1), *H. americanus* (ACG70181.1), *P. carinicauda* (AGJ03549.1), *P. monodon* (ABQ10738.1), and *H. sapiens* (AAA51922.1). Cysteine residues and active sites are shown by dot and arrow symbol, respectively.

Fig. 3. Neighbour-joining phylogenetic for the *Procambarus clarkii* cathepsin D-like amino acid sequence, constructed by MEGA 6 software. The phylogenetic tree was investigated with 10,000 bootstrap replications, and the bootstrapping values were shown via numbers at the nodes. The *Procambarus clarkii* cathepsin D-like is marked with rhombus-shaped, and GenBank number is given in parenthesis.

Fig. 4. The expression patterns of *Pc-cathepsin D-like* in different tissues of *Procambarus clarkii* by qRT-PCR. Bars represent the mean $\pm$ S.E. (n = 3).

Fig. 5. The *Pc-cathepsin D-like* expression profiles in the hepatopancreas of *Procambarus clarkii* following challenge with immune elicitors. *Pc-cathepsin D-like* expression patterns in the hepatopancreas of *Procambarus clarkii* treated with LPS (A), PGN (B), and Poly I:C (C). PBS was used as control. Bars represent mean $\pm$ S.E. (n = 3). All data were analyzed by one-way

474 analysis of variance. It was considered statistically significant when p -values less than 0.05 and
475 indicated by an asterisk (*).

476 **Fig. 6. (A) RNA interference efficiency of *Pc-cathepsin D-like* in the hepatopancreas of**
477 ***Procambarus clarkii* by qRT-PCR.** The relative expression of *Pc-cathepsin D-like* was
478 quantified at 24 and 48 h after the dsRNA administration. The dsEGFP treated group was used as
479 a control. All data were analyzed by t-test analysis. It was considered statistically significant
480 when p -values were less than 0.05 and indicated by an asterisk (*). **(B) Effects of *Pc-cathepsin***
481 ***D-like* silencing on the expression levels of immune-pathways associated genes.** Expression
482 of immune-pathways related genes in the hepatopancreas of *Procambarus clarkii* following *Pc-*
483 *cathepsin D-like* RNA interference. All data were analyzed by t -test analysis. It was considered
484 statistically significant when p -values were less than 0.05 and indicated by an asterisk (*).

485 **Table 1** Primers used in this study

Fig. 1 Nucleotide and deduced amino acid sequences of *Pc-cathepsin D-like* gene.

```

1  ACGGGGGATCAACCCGTC AACATCACCCGCCACTCTCACACCAACATGAAGTCCTTCGCC
1  M K S F A
61  TGCCTCCTGCTCCTGGTGGCCCTGGTTCGCTGCCGAAATCCACAGAGTGCCGCTGAAGAAG
6  C L L L L V A L V A A E I H R V P L K K
121  GTGGAGCGGAAGAGGACCCCTGCAGGACCTCCGTCGCTCCCACGCCTTCCTGGCTCACCGT
26  V E R K R T L Q D L R R S H A F L A H R
181  TACGGCGCCAAGGACGACATCATCGTCATCGACGACTACCAAGACGCTCAGTACTATGGA
46  Y G A K D D I I V I D D Y Q D A Q Y Y G
241  CCCATTAGCATCGGGACGCCCCGGGCAAGGGTTTGATATCATCTTCGACACTGGATCGTCC
66  P I S I G T P G Q G F D I I F D T G S S
301  AACCTTTGGGTGCCGTCTGAACAGTGCAGCATCATCAACCTGGCCTGTCAGACGCACAAC
86  N L W V P S E Q C S I I N L A C Q T H N
361  CGCTACGACTCCACGCTCTCCTCCTCTACAAAGCCAACGGCACCGACTTCGCCATAGAG
106  R Y D S T L S S S Y K A N G T D F A I E
421  TACGGCTCTGGCTCCCTCACCGGCTTCCTTTCCAGCGACACGGTGGCAGTCGGGGACTTG
126  Y G S G S L T G F L S S D T V A V G D L
481  GCTGTCGTTGACCAGACCTTCGCTGAGGCCACCCACGAGCCTGGCATAGCCTTCCTGGCG
146  A V V D Q T F A E A T H E P G I A F L A
541  GCGTGTTCGACGGCATCTTGGGCATGGGCTTCGTCGAGATCTCCGTCATGGGAATCCCC
166  G V F D G I L G M G F V E I S V M G I P
601  ACAGTGTTCGACAATATGGTGGCTCAGGGCGTCGTCGATGAACCCATTTTCTCCTTCTTC
186  T V F D N M V A Q G V V D E P I F S F F
661  CTTAACCATGACATGACCGAGACGCTCGGAGGGGAAGTGGTGTGGGCGGGTCAGACCCC
206  L N H D M T E T L G G E L V L G G S D P
721  AACCACTACGAGGGAGAGTTCCACTACGTCCCAGTCTCCAGGGTGGGCTACTGGCAAATC
226  N H Y E G E F H Y V P V S R V G Y W Q I
781  ACAGCCGAGGCGATTAAGGTGCGGAGGCGTAGACACGACCTTCTGCAGCCCGTGTGAGGCC
246  T A E A I K V G G V D T T F C S P C E A
841  ATCGTCGACACCGGCACCTCCCTCATCGCTGGACCCAAAGATGAGGTCAAGGAGATCGTA
266  I V D T G T S L I A G P K D E V K E I V
901  ACCCAGTTAGGAGGGTTCGCCTTCCTTGCTGGAGAGTACCTCATCAACTGCGACAAGGTG
286  T Q L G G F A F L A G E Y L I N C D K V
961  GAGGAGATGCCGCTCTTCACCTTCACCCTCAACGGCCAGGACTTCGACCTGACAGGACCC
306  E E M P L F T F T L N G Q D F D L T G P
1021  GAGCTGGTCATCGAGAACGTGGACGAGGCCACCGGAGCCAAGACCTGCATTGTGGGCATC
326  E L V I E N V D E A T G A K T C I V G I
1081  CTGGGGCTCGACCTGGGCACCATCGAAGCCTGGATCCTTGAGACCCATTTCATCGCCGAC
346  L G L D L G T I E A W I L G D P F I A D
1141  TACTACACAGAGTTCGACGTTGGCCAGAAGCGCATTGGTTTTGCCAAATCGATTTAGACG
366  Y Y T E F D V G Q K R I G F A K S I *
1201  ATATTGTCTTCCAAGTTGCCACAACCTCTCCTCCCCAACCCCGACCTCCCTTATCTGCCCC
1261  CTACGTCCAAGTGGTTGAGCACCATTCCCTTTCCCCCGTCAAATCCCAAATCCTTATCCTG
1321  ACCCCTTCCCAGTGCTTAATGGTCATAATGGCTAAGTGTCTCCTCATAGTTCTCTCT
1381  CTCTCTCTCTCTCTCTCTCCCCACAGCCCTCTATAGCACCCATCCAGCCCCCTCCACTTT
1441  CAAAGGGTGAGGCCGGGATAGGTGGAATAAAAAAATTGGACTCTGTCTGTGATTAGATTT
1501  TCTTTATAGCGAAAAAAAAAAAAAAAAAAAAA

```

Fig. 2 Multiple sequence alignment of the *Pc-cathepsin D-like* protein from

Procambarus clarkii with other cathepsin D proteins.

Fig. 3. Neighbour-joining phylogenetic for the *Procambarus clarkii* cathepsin

***D-like* amino acid sequence, constructed by MEGA 6 software.**

Fig. 4. The expression patterns of *Pc-cathepsin D-like* in different tissues of

Procambarus clarkii by qRT-PCR.

Fig. 5. The *Pc-cathepsin D-like* expression profiles in the hepatopancreas of *Procambarus clarkii* following challenge with immune elicitors.

Fig. 6. (A) RNA interference efficiency of *Pc-cathepsin D-like* in the hepatopancreas of *Procambarus clarkii* by qRT-PCR. (B) Effects of *Pc-cathepsin*

***D-like* silencing on the expression levels of immune-pathways associated genes.**

Table 1 Primers used in this study.

Primer No.	Primer sequences (5'-3')	Purpose
F1	ATGAAGTCCTTCGCCTGCCTC	RT-PCR
R1	ACCAAAACGGTTTAGCTAAATC	RT-PCR
Serine/threonine protein kinase -F	TGCTATGTGAAGCTCGGCTCT	qPCR
Serine/threonine protein kinase -R	GCGATCTGATGCTCCTCCTCT	qPCR
ALF-F	TCCTGAATTGCTCCTCGTCA	qPCR
ALF-R	GCGGTGGCAACTGTACTTCA	qPCR
Sptäzle-F	GTCGGCAGCAACGACATACA	qPCR
Sptäzle-R	GGTGTGATGGTTGGCTGTGA	qPCR
Cactus-F	CTTGTGAGAGAGCCGTGTGG	qPCR
Cactus-R	CAGTACAAGCAGCAGCAGCA	qPCR
Lectin-like-F	TGCTCCTGGTGGTAGTGGT	qPCR
Lectin-like-R	GTAGAGGTGTTTGGGTGTGG	qPCR
Toll3-F	GCTGTTGCTGCTTAGGCTCA	qPCR
Toll3-R	TCCTCCACAGCTCTTCATTCC	qPCR
Ecdysone receptor-F	TTCCCGAGTCTCAATGCC	qPCR
Ecdysone receptor-R	GAAGAGTGCCGAAACCAG	qPCR
Oxysterol-binding protein 1-F	TTGCAACGATTAACGGAAGATTTT	qPCR
Oxysterol-binding protein 1-R	ATGGGCGGATGATGAGACACCTG	qPCR
F18S	CTGTGATGCCCTTAGATGTT	qPCR
R18S	GCGAGGGGTAGAACATCCAA	qPCR
dsRNA-EGFP-F	<u>GGATCCTAATACGACTCACTATAGGC</u> AGTGCTTCAGCCGCTACCC	RNAi
dsRNA-EGFP-R	<u>GGATCCTAATACGACTCACTATAGGA</u> CTCCAGCAGGACCATGTGAT	RNAi
dsRNA-Cathepsin D-Like-F	<u>GGATCCTAATACGACTCACTATAGGG</u> ACACTGGATCGTCCAACCT	RNAi
dsRNA-Cathepsin D-Like-R	<u>GGATCCTAATACGACTCACTATAGGT</u> GCAGAAGGTCGTGTCTACG	RNAi

Note: T7 promoters are in bold and underlined.