Fish & Shellfish Immunology 43 (2015) 219–229

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

A novel vertebrates Toll-like receptor counterpart regulating the anti-microbial peptides expression in the freshwater crayfish, *Procambarus clarkii*

Zheng Wang [a, 1], Yi-Hong Chen [b, 1], Yun-Jia Dai [c, 1], Jing-Min Tan [a], Ying Huang [a], Jiang-Feng Lan [c, **], Qian Ren [a, *]

[a] Jiangsu Key Laboratory for Biodiversity & Biotechnology and Jiangsu Key Laboratory for Aquatic Crustacean Diseases, College of Life Sciences, Nanjing Normal University, 1 Wenyuan Road, Nanjing 210046, PR China
[b] MOE Key Laboratory of Aquatic Product Safety/State Key Laboratory of Biocontrol, School of Marine Sciences, Sun Yat-sen University, Guangzhou, PR China
[c] College of Fisheries, Huazhong Agricultural University, Wuhan, Hubei 430070, PR China

ARTICLE INFO

Article history:
Received 2 September 2014
Received in revised form
24 December 2014
Accepted 27 December 2014
Available online 5 January 2015

Keywords:
Toll
RNAi
Over-expression
Innate immunity
Procambarus clarkii

ABSTRACT

Toll-like receptors (TLRs) play an important role in regulation of anti-microbial peptides (AMPs) expression. A novel vertebrates TLR counterpart named *PcToll*, was firstly identified from the freshwater crayfish, *Procambarus clarkii*. Phylogenetic analysis showed that PcToll together with *Drosophila melanogaster* and *Anopheles gambiae* Toll9 were clustered with human Tolls. *PcToll* was mainly expressed in hepatopancreas and gills and it also could be detected in hemocytes, heart, stomach and intestine. *PcToll* was upregulated in hemocytes and gills post 24 h *Vibrio anguillarum* challenge. In hepatopancreas and intestine, the highest expression level of *PcToll* could be observed at 12 h *V. anguillarum* challenge. In hemocytes, *PcToll* went up post 24 h *Staphylococcus aureus* challenge and in gills, the expression level of *PcToll* showed no obvious change from 2 to 24 h *S. aureus* challenge. In hepatopancreas post 12 h *S. aureus* challenge, *PcToll* was upregulated and it showed obvious upregulation post 12 h *S. aureus* challenge in intestine. RNAi results showed that *PcToll* was involved in regulation of *crustins* (*Cru*1, *Cru*2), *anti-lipopolysaccharide factor* 2 (*ALF*2) and *lysozyme* 1 (*Lys*1) expression. Overexpression of *PcToll* in *Drosophila* S2 cells could induce *Drosophila Attacin* (*Atta*), *Metchnikowin* (*Mtk*), *Drosomycin* (*Drs*) and shrimp *Penaeidin* (*PEN4*) expression. From the results, it could be speculated that *PcToll* might play important roles in crayfish innate immune defense.

1. Introduction

The freshwater crayfish, *Procambarus clarkii*, is one of the most important commercially cultivated crustaceans in China, especially in Jiangsu Province since the 90's. With the rapid development of crayfish aquaculture, diseases have become a major constraint and the most limiting factor for the crayfish culture industry. Various pathogens, such as spiroplasma and white spot syndrome virus (WSSV), have been reported to cause disease in crayfish *P. clarkii* in China [1,2]. Shrimp infected with WSSV become more susceptible to other pathogens, such as *Vibrio* species than that of WSSV free shrimp. So, there is an urgent need to develop strategies against the diseases and it was necessary to get further insight on crayfish innate immunity first.

Host defense in crustaceans including the crayfish *P. clarkii* is believed to rely largely on innate immunity [3]. Innate immunity is a sensitive non-self recognizing system triggered by components of pathogens, called pathogen-associated molecular patterns (PAMPs). PAMPs are recognized by pattern-recognition receptors (PRRs). During the recent years, Toll and its mammalian homologs Toll-like receptors (TLRs) have been recognized as major PRRs and they play an essential role in recognition of microbes during host defense [4–6]. Toll is initially identified in *Drosophila melanogaster*

* Corresponding author. Tel.: +86 25 85891955; fax: +86 25 85891526.
** Corresponding author.
E-mail address: renqian0402@126.com (Q. Ren).
[1] Zheng Wang, Yi-Hong Chen and Yun-Jia Dai are equally contributed to this paper.

http://dx.doi.org/10.1016/j.fsi.2014.12.038
1050-4648/

(named dToll) and dToll was thought to be involved in *D. melanogaster* dorsal–ventral development [7]. Later, it was shown that dToll plays a critical role in antifungal and antibacterial response of flies [8]. Recently, the Toll pathway was also identified as a vital part of the *D. melanogaster* antiviral response [9]. Among the 9 Tolls in *D. melanogaster*, only DmToll-1 regulates the expression of AMPs [10]. DmToll1 is not able to directly bind to the PAMPs and the Toll pathway activation need the participation of cytokine-like ligand Spatzle [10–12].

Since the first report of *D. melanogaster* Toll, more and more Tolls or TLRs from vertebrates to invertebrates were identified. Mammalian TLRs are type 1 transmembrane proteins with typical domain organization including leucine-rich repeats (LRRs) ecto-domains, transmembrane domains and intracellular Toll/interleukin-1 receptor (TIR) domains that are required for down-stream signal transduction [13]. Mammalian LRRs of TLRs could directly recognize the PAMPs, such as lipopolysaccharides, lipoproteins, single-stranded RNA, double-stranded RNA and unmethylated CpG-containing DNA, originated from diverse pathogens including bacteria, fungi, parasites and viruses [14]. Among the 10 human TLRs, TLR1, TLR2, TLR5, TLR6 and TLR10 could recognize microbial lipids, sugars and proteomes and related TLR3, TLR7, TLR8 and TLR9 recognize nucleotide derivatives originated from virus or bacteria [13,15].

Different number of Tolls or TLRs were found in different species. There are 222 and 72 TLR-related gene candidates existed in the sea urchin and amphioxus genome, respectively [16]. Ascidian *Ciona intestinalis* has only 2 *TLR* genes [16]. Only one Toll has been found in the *Caenorhabditis elegans* genome [11]. Up to 16 TLRs have been identified in the lamprey genome [17] and 17 different TLRs have also been found in fish [15]. In *Crassostrea gigas*, 4 sccTLRs (CgTLR1 to CgTLR4) have been reported [18]. CfTLR from *Chlamys farreri* belongs to mccTLR [19]. In Crustaceans, a number of TLRs were found in the previous research. Two novel Tolls (EsToll1 and EsToll2) belong to mccTLR and sccTLR respectively were identified from *Eriocheir sinensis* [20]. In shrimp, there are also some TLRs reported including a TLR from *Fenneropenaeus chinensis* [5], 3 TLRs from *Litopenaeus vannamei* [21], 1 Toll receptor from *Penaeus monodon*, *Marsupenaeus japonicus* and *Macrobrachium rosenbergii* [22–24].

Although some TLRs from crustaceans were identified in the previous research, no report of TLR could be obtained in crayfish such as *P. clarkii*. In this study, a novel vertebrate TLR counterpart from the freshwater crayfish, *P. clarkii* was firstly identified. Its transcript could be induced by *Vibrio anguillarum* challenge and the AMPs expression was regulated by *PcToll*. Our research may indicate its role in innate immune of the freshwater crayfish.

2. Materials and methods

2.1. Animals, immune challenge, hemocyte and tissue collection

Red swamp crayfish (*P. clarkii*) (about 10 g each), purchased from an aquatic product market in Nanjing, Jiangsu Province, China, and then were cultured in fresh water in tanks at a temperature of 25 °C in a laboratory. For bacteria challenge, over-night culture of *V. anguillarum* or *Staphylococcus aureus* was washed with PBS buffer twice and then was diluted 100 times with PBS. Diluted *V. anguillarum* or *S. aureus* (100 μl, About 3×10^7 cells) was injected into the abdominal segment of crayfish using a microliter syringe. At 0, 6, 12, and 24 h after *V. anguillarum* or *S. aureus* injection, hemolymph was collected from the crayfish ventral sinus by mixing with 1/10 volume of anticoagulant buffer (10% sodium citrate, pH 7.0) containing 200 mM phenylthiourea as melanization inhibitor and then immediately centrifuged at 800 *g* at 4 °C for 10 min to isolate the hemocytes. At the same specific time after the *V. anguillarum* or *S. aureus* injection, the tissues including hepatopancreas, gills and intestine were also collected from 3 crayfish for RNA isolation.

2.2. RNA isolation, cDNA synthesis for qRT-PCR analysis

RNAs from all above samples were extracted using an RNApure high-purity total RNA rapid extraction kit (Spin-column, BioTeke, Beijing, China) according to the manufacturer's protocol. RNA quality and the total RNA concentration were determined by Nanodrop (Thermo). The first-strand cDNA was synthesized from RT-PCR and qRT-PCR analysis using the PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) with the Oligo-d(T) Primer. The detailed methods were according to the manufacturer's protocol.

2.3. Gene cloning and sequence analysis of PcToll

When searching the transcriptome data of hepatopancreas from the crayfish, an EST sequence similar to TLR was found and was named *PcToll*. RACE methods were used to clone the full length of PcToll gene. First, the Clontech SMARTer™ RACE cDNA Amplification Kit from TAKARA (Dalian, China) was used to synthesize the 5′-RACE Ready cDNA with 5′-CDS Primer A and SMARTer IIA oligo and 3′-RACE-Ready cDNA with 3′-CDS primer A for amplification of 5′ and 3′ end of *PcToll*. Second, based on the EST sequences, gene specific forward and reverse primer was designed. For 5′ end

Table 1
Sequences of the primers used in this study.

Primers	Sequence (5′–3′)
PcToll-R	GTGGTTTGCGTAGTGACTTGTGGAGC
PcToll-F1	CGGTGGTGACGGTCTACTCTAAATG
PcToll-F2	GGCAGTGGGTGTTCGAACATCTCCTGTTG
PcToll-F3	TGTTGGGATGGCTTGGGCTCTCAGG
UPM Long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
UPM Short	CTAATACGACTCACTATAGGGC
5′-CDS Primer A	T25VN
3′-CDS primer A	AAGCAGTGGTATCAACGCAGAGTAC(T)30VN
SMARTer IIA oligo	AAGCAGTGGTATCAACGCAGAGTACXXXXX
PcToll-RT-F	GACTTGTCCAAAAACGATATACG
PcToll-RT-R	TGCGTTACAGTAGTGAGCGAAT
PcGAPDH-RT-F	CAATGTTCGTCTGTGGAGTGA
PcGAPDH-RT-R	GAAGATGGGATGATGTTCTGv
*PcCru1*F	TATTCCTCGCTGCACAAACA
*PcCru1*R	CACATAGCACCTCCCTCTTCA
*PcCru2*F	GGGAAGAAAAGCACAATGGT
*PcCru2*R	GGTATGGAGGTCGAGACAGG
*PcALF1*F	GAAGCGATGACGAGGAGCAAT
*PcALF1*R	GACGGGTTGGCACAAGAGC-
*PcALF2*F	CAAACTGGGCGGGTTATGG
*PcALF2*R	TGACGAAGTCCCTGGTGGC
*PcLysi1*F	GTCAACCCACCCTCAATAAC
*PcLysi1*R	CTTGTGAATCAGGGCGTA
PcToll-RI-F	GCGTAATACGACTCACTATAGGTATCCCAGAGGAGAAGC
PcToll-RI-R	GCGTAATACGACTCACTATAGGTATCCCAGAGGAGAAGC
GFP-Ri-F	GCGTAATACGACTCACTATAGGTGGTCCCAATTCTCGTGGAAC
GFP-Ri-R	GCGTAATACGACTCACTATAGGCTTGAAGTTGACCTTGATGCC
PcToll-ex-F	TATGCGGCCGCATGCTCTACGACACCTTCGTCTGC
PcToll-ex-R	TATGCGGCCGCATGCTCTACGACACCTTCGTCTGC

X = undisclosed base in the proprietary SMARTer oligo sequence.
N = A, C, G, or T; V = A, G, or C.