Gene 610 (2017) 140–147

Contents lists available at ScienceDirect

Gene

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

Research paper

Newly identified *PcToll4* regulates antimicrobial peptide expression in intestine of red swamp crayfish *Procambarus clarkii*

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ARTICLE INFO

Article history:
Received 29 December 2016
Received in revised form 22 January 2017
Accepted 13 February 2017
Available online 16 February 2017

Keywords:
Procambarus clarkii
WSSV
Toll-like receptor
Anti-lipopolysaccharide factor
RNAi
Overexpression

ABSTRACT

Tolls or Toll-like receptors (TLRs) have an essential role in initiating innate immune responses against pathogens. In this study, a novel Toll gene, *PcToll4*, was first identified from the intestinal transcriptome of the freshwater crayfish, *Procambarus clarkii*. The *PcToll4* cDNA is 4849 bp long with a 3036 bp open reading frame that encodes a 1011-amino acid protein. PcToll4 contains a signal peptide, 13 LRR domains, 3 LRR TYP domains, 2 LRR CT domains, an LRR NT domain, a transmembrane region, and a TIR domain. Quantitative RT-PCR analysis revealed that *PcToll4* mRNA was detected in all tested tissues, and the expression of *PcToll4* in the intestine was significantly upregulated after white spot syndrome virus (WSSV) challenge. Overexpression of PcToll4 in *Drosophila* Schneider 2 (S2) cells activates the antimicrobial peptides (AMPs) of *Drosophila*, including *metchnikowin*, *drosomycin*, *attacin* A, and shrimp *Penaeidin-4*. Results of RNA interference by siRNA also showed that *PcToll4* regulates the expressions of 5 anti-lipopolysaccharide factors (ALFs) in the intestine of crayfish. Our findings suggest that *PcToll4* is important for the innate immune responses of *P. clarkii* because this gene regulates the expressions of AMPs against WSSV.

1. Introduction

The freshwater crayfish, *Procambarus clarkii*, is one of the most important commercial species extensively distributed in China (Liang et al., 2010). Similar to other invertebrates, crayfish lack an adaptive immune system and mainly rely on their innate immune responses to resist pathogen invasion (Soderhall and Thornqvist, 1997). The innate immune system is the first line of the host defense against invading pathogens, especially in invertebrates, and is conserved throughout evolution (Medzhitov and Janeway, 2000). Upon infection, the specific molecular motifs known as pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS), peptidoglycan (PGN), double-stranded RNA (dsRNA), and β-glucans (GLU) of microbes, are directly recognized by germ-line receptors called pattern recognition receptors (PRRs) (Janeway and Medzhitov, 2002). The recognition process activates multiple signaling pathways, thereby resulting in rapid and effective cellular and humoral defenses (Smale, 2012).

Toll or Toll-like receptors (TLRs) are the most extensively studied PRRs, which play a fundamental role in pathogen recognition and activation of the innate immunity (Akira et al., 2006; Yang et al., 2008). Tolls/TLRs are type I integral membrane glycoproteins that are characterized by their domain organization. Tolls and TLRs contain an ectodomain, which encompasses leucine-rich repeats (LRRs), a transmembrane domain, and an intracellular Toll/interleukin-1 receptor (TIR) domain required for downstream signal transduction (Bowie and O'Neill, 2000). Toll, the founding member of TLRs, was originally identified in *Drosophila melanogaster* (called dToll) and was proven involved in the development of embryonic dorsoventral polarity in *D. melanogaster* (Anderson and Nüsslein-Volhard, 1983). The role of dToll in the antifungal and antibacterial responses of flies was later identified (Rutschmann et al., 2002). At present, a large number of Tolls/TLRs from vertebrates to invertebrates are widely investigated (Coscia et al., 2011). Multiple Tolls are also found in shrimp or crayfish, including 1 Toll receptor from *Penaeus monodon* (Arts et al., 2007), *Marsupenaeus japonicus* (Mekata et al., 2008), and *Macrobrachium rosenbergii* (Srisuk et al., 2014), 3 Tolls from *Litopenaeus vannamei*

Abbreviations: TLR, Toll-like receptor; LRR, leucine-rich repeat; LRR TYP, Leucine-rich repeats, typical (most populated) subfamily; LRR CT, Leucine rich repeat C-terminal; LRR NT, Leucine rich repeat *N*-terminal; WSSV, white spot syndrome virus; S2, *Drosophila* Schneider 2; AMP, antimicrobial peptide; LPS, lipopolysaccharides; PGN, peptidoglycan; dsRNA, double-stranded RNA; GLU, β-glucans; PRR, pattern recognition receptor; TM, transmembrane; TIR, intracellular Toll/interleukin-1 receptor; NF-κB, nuclear factor–kappa B; ALF, anti-lipopolysaccharide factor; EST, expressed sequence tag; PBS, phosphate buffer saline; RNAi, RNA interference; qRT-PCR, quantitative real-time PCR; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; S. E, standard error; SDM, serum-free medium; PEN4, *Penaeidin*-4; Mtk, *metchnikowin*; Drs, *drosomycin*; Atta, *attacin* A; UTR, untranslated region; aa, amino acids.

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http://dx.doi.org/10.1016/j.gene.2017.02.018
0378-1119/

(Wang et al., 2012; Yang et al., 2007), and 1 Toll from *Fenneropenaeus chinensis* (Yang et al., 2008) and *P. clarkii* (Wang et al., 2015). Toll receptors of invertebrates induce their innate immune responses (Kawai and Akira, 2011). Signal pathway activation leads to the translocation of the nuclear factor–kappa B (NF-κB) into the nucleus, which finally induces the expression of immune effector molecules such as antimicrobial peptides (AMPs) (Lemaitre and Hoffmann, 2007).

AMPs, one of the major components of the invertebrate immune system, function as the front line of host defenses against microbial infection and have a broad spectrum of antimicrobial activities against bacteria, fungi, parasites, and viruses (Hancock et al., 2006; Silva et al., 2013). The major crustacean AMPs are represented by three cationic peptide families: penaeidins, crustins, and anti-lipopolysaccharide factors (ALFs) (Tassanakajon et al., 2010). ALFs represent a family of basic proteins of around 100 amino acids, which bind and neutralize the activity of LPS (Morita et al., 1985). They mediate the degranulation of hemocytes and activate the intracellular coagulation cascade (Morita et al., 1985). The first ALF, LALF, was isolated from the hemocytes of the horseshoe crab *Limulus polyphemus* and has a strong antibacterial effect on gram-negative R-type bacteria (Tanaka et al., 1982; Warren et al., 1992). >100 ALF sequences were reported in shrimps, lobsters, crayfish, and crabs (Supungul et al., 2004; Beale et al., 2008; Zhang et al., 2010; Sun et al., 2011). Although distinct ALF isoforms usually coexist in one species, their similarities are low (Li et al., 2013). These isoforms are regulated by different signal pathways and exhibit different expression patterns in response to bacterial or viral infection. However, the transcriptional levels of most identified ALFs in crustaceans are usually upregulated, thereby suggesting that ALFs have important roles in the defense against bacterial and viral pathogens (Li et al., 2014).

In this study, a novel invertebrate Toll *PcToll4* was identified from *P. clarkii*. Its transcription can be induced by white spot syndrome virus (WSSV) challenge, and the five ALF expressions were regulated by *PcToll4*. Our study reveals the role of *PcToll4* in the innate immunity of the red swamp crayfish.

2. Materials and methods

2.1. Identification of PcToll4 and ALF genes and sequence analysis

An expressed sequence tag (EST) in *P. clarkii* that is similar to the Toll gene and 11 ESTs that are similar to ALF genes were obtained from the intestinal transcriptome data (unpublished). The 5′ and 3′ cDNA were synthesized following the instructions of Clontech SMARTer RACE cDNA Amplification Kit from Takara (Dalian, China) using 5′-CDS primer A, SMARTer IIA oligo, and 3′-CDS primer A. The following gene-specific forward and reverse primers were designed based on the EST: PcToll4-F and PcToll4-R (Table 1). A Clontech Advantage 2 PCR Kit from Takara (Dalian, China) was used for gene cloning. PCR reaction was conducted using the following conditions: 5 cycles at 94 °C for 30 s and 72 °C for 2 min; 5 cycles at 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 2 min; and 20 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 2 min. The corresponding amplified fragments were subcloned into T3 vectors prior to the sequencing by TransGen Biotech (Beijing, China). Genomic DNA from the intestine of *P. clarkii* was isolated using NucleoSpin Tissue (Clontech). The full length of *PcToll4* was obtained by overlapping the EST sequences and the 5′ and 3′ fragments. Three pairs of gene-specific primers (i.e., gPcALF1-*F*1, gPcALF1-R1; gPcALF1-F2, gPcALF1-R2; gPcALF3-F, gPcALF3-R; gPcALF4-*F*1, gPcALF4-R1; and gPcALF4-F2, gPcALF4-R2) were designed based on the obtained cDNA sequences of *PcALF1*, *PcALF3*, and *PcALF4* to clone the genomic sequences of *PcALF1*, *PcALF3*, and *PcALF4* (see primer sequence in Table 1).

The BLASTP algorithm at NCBI (http://www.ncbi.nlm.gov/blast) was used to search the homology of the protein sequences. Translations of cDNAs and predictions of the deduced amino acid sequences were conducted using ExPASy (http://www.au.expasy.org/). MEGA 5.05 was used to construct phylogenetic trees, and the neighbor-joining method was selected for phylogenetic analysis (Kumar et al., 2008).

Table 1
Primer sequences used in this study.

Primers name	Sequences (5′-3′)
PcToll4-F	GCCGAACCATTGTGATACTGTCGTCCA
PcToll4-R	TGAAGGGTTGAAGTTGGATGTGGGAATG
UPM	
Long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
Short	CTAATACGACTCACTATAGGGC
5′-CDS Primer A	T25VN
SMARTer II A oligo	AAGCAGTGGTATCAACGCAGAGTACXXXXX
3′-CDS primer A	AAGCAGTGGTATCAACGCAGAGTAC(T)30VN
gPcALF1-*F1*	GGTCTCCCCGGAGCTCATCAATCTCA
gPcALF1-R1	CTATTGCTTGAGCCAAGCTGCAGC
gPcALF1-F2	AGAGCCTCAGGCGTGGCCAGGTG
gPcALF1-R2	CCCACCCAGTTTGTTGATGATGAGATTG
gPcALF3-F	ATGAAGTGGTCGGTGTTGGTGG
gPcALF3-R	CTAATTCTTTATCCAAGCTGAAG
gPcALF4-*F1*	ATGTCAGCAGCGCCGTCAGG
gPcALF4-R1	TCATCAAAGCCACGCTGATGCCTC
gPcALF4-F2	ACACTGACTTGAGAATGGTCCAG
gPcALF4-R2	CGTTGTCAGCGACGGTGGAGATG
PcToll4-RT-F	AGGAACAAGACGCTGACAAG
PcToll4-RT-R	TCACGGTAATGGAGACACAC
PcALF1-RT-F	CGAGAGGCTGTAGAGGATGC
PcALF1-RT-R	CCCAGTTTGTTGATGATGAG
PcALF2-RT-F	CGTGGGAGTGTTTGTGGTGGT
PcALF2-RT-R	TTGGACTGTAACTGTAGCGGC
PcALF3-RT-F	AGGTGTTGAAGATGAAGTGGT
PcALF3-RT-R	GCTTGTTGATAATGAGGGTGA
PcALF4-RT-F	CCAGATCATCTCCACCGTCG
PcALF4-RT-R	GTAGCCTTGAGCTTTTCCCA
PcALF5-RT-F	ATGGGGAGGTGAGGCTACT
PcALF5-RT-R	CCTTCCTGCTCGGTGATGA
PcALF6-RT-F	ACAAATGAACACAAGCCACCC
PcALF6-RT-R	TGATAAACCTGTCCTCCCAAC
PcALF7-RT-F	CCAGCCATTGCGGAAAAAC
PcALF7-RT-R	GGGGCACCACATGCGACCC
PcALF8-RT-F	GCGGAACGGTGAGGTGGAG
PcALF8-RT-R	TGATGAGGCCGGCTTGGAA
PcALF9-RT-F	AGTGGCGTCATACAGGAAGGGG
PcALF9-RT-R	CCAAAGGATGGCGAGAAATAGT
PcALF10-RT-F	AGAGAAGATCGCTCAACGCC
PcALF10-RT-R	CACACTCGCCCAAACAGACG
PcALF11-RT-F	CACTCTCTCGGCTTCCATCC
PcALF11-RT-R	GCTCCACCTCACCGTTCTTC
PcGAPDH-RT-F	CAATGTTCGTCTGTGGAGTGA
PcGAPDH-RT-R	GGAAGATGGGATGATGTTCTG
PcToll4-pAc-F	CCCGGATCGGGGTACCATGGCAAGTACACCAAACCTCACAG
PcToll4-pAc-R	TTCGAACCGCGGGCCCTCCTTCTCCCAGAACTTGGGATCA

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

2.2. Animal, virus, immune challenge, and tissue collection

Adult *P. clarkii* (about 15 g each) were purchased from an aquatic product market in Nanjing, Jiangsu Province, China and were then cultured in fresh water in tanks at a temperature of 25 °C in a laboratory. PCR using WSSV-specific primers (5′-TATTGTCTCTCCTGACGTAC-3′ and 5′-CACATTCTTCACGAGTCTAC-3′) were conducted to ensure that the crayfish were WSSV-free prior to experimental infection. The WSSV inoculums used in this study were obtained from Zhejiang University. For the experimental group, WSSV (10^5 copies/mL, 100 μL/crayfish) was injected into the abdominal segment of the crayfish using a microliter syringe. For the control group, the crayfish were inoculated with 100 μl of PBS (0.14 M NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). At 0, 12, 24, 36, 48, 60, and 72 h post-injection, the intestine was randomly collected from the experimental and control groups. Hemolymph was collected from the crayfish ventral sinus by