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*Sp*Toll1 and *Sp*Toll2 modulate the expression of antimicrobial peptides in *Scylla paramamosain*

Yan Chen[a,b,1], Jude Juventus Aweya[a,b,1], Wanwei Sun[a,b], Xiaoyuan Wei[a,b], Yi Gong[a,b], Hongyu Ma[a,b], Yueling Zhang[a,b], Xiaobo Wen[a,b], Shengkang Li[a,b,*]

[a] Guangdong Provincial Key Laboratory of Marine Biology, Shantou University, Shantou 515063, China
[b] Marine Biology Institute, Shantou University, Shantou 515063, China

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ABSTRACT

Tolls and Toll-like receptors (TLRs) were the first pattern recognition receptors (PRRs) identified to play key roles in host innate immunity. However, relatively little is known about other types of Toll-like receptors in *Scylla paramamosain*, although a Toll-like receptor (*Sp*Toll1) has recently been cloned. In this study, we cloned and characterized another novel Toll-like receptor 2 (*Sp*Toll2) from *S. paramamosain*. The full-length cDNA of *Sp*Toll2 is 3391 bp with a 2646 bp open reading frame (ORF) encoding a putative protein of 881 amino acids, and predicted to contain six extracellular leucine-rich repeat (LRR) domains, a transmembrane domain and an intracellular Toll/IL-1 receptor (TIR) domain. Phylogenetic analysis revealed that *Sp*Toll2 clustered with *Drosophila* Toll1, and shared high homology with *Pt*Toll4. Real-time qPCR analysis showed that *Sp*Toll2 was widely expressed in all tissues tested, with the highest level found in hemocytes and hepatopancreas while the lowest in heart and muscle. The transcript levels of both *Sp*Toll1 and *Sp*Toll2 in mud crabs hemocytes was induced following challenge with *Vibrio parahaemolyticus*, *Staphylococcus aureus*, Polyinosinic: polycytidylic acid (Poly I:C) and white spot syndrome virus (WSSV). In addition, recombinant *Sp*Toll1-LRR and *Sp*Toll2-LRR proteins could bind to *V. parahaemolyticus*, *S. aureus*, *Escherichia coli*, and Beta *Streptococcus*. In order to study the signaling pathway of AMPs' expression in mud crab, RNA interference were used to test the expression of *Sp*AMPs after the challenges with *V. parahaemolyticus* or *S. aureus*. The data suggested that *Sp*Toll1and *Sp*Toll2 could regulate the transcripts of several AMPs and four immune related mediators (*Sp*MyD88, *Sp*Tube, *Sp*Pelle and *Sp*TRAF6) at different scale. While silencing of *Sp*Toll1 post pathogens challenge attenuated the expression of *Sp*Histin, *Sp*ALF1 and *Sp*ALF5 in mud crab's hemocytes, depletion of *Sp*Toll2 post pathogens challenge inhibited the expression of *Sp*ALF1-6, *Sp*GRP, *Sp*Arasin and *Sp*Hyastastin. Furthermore, the results of overexpression assay also showed *Sp*Toll1 and *Sp*Toll2 could enhance the promoter activities of *Sp*ALFs in mud crab. Taken together, these results indicated that *Sp*Toll1 and *Sp*Toll2 might play important roles in host defense against pathogen invasions in *S. paramamosain*.

1. Introduction

Invertebrates including mud crab *S. paramamosain* lack an adaptive immune system and mainly rely on their innate immunity for protection against pathogens (Loker et al., 2004; Rowley and Powell, 2007). In the animal kingdom, innate immunity constitutes the first line of defense against microbial pathogens based on pattern recognition (Holmskov et al., 2003). Innate immunity in invertebrates is similar to that in vertebrates, which involve humoral responses including melanization, coagulation and production of AMPs and cellular responses like encapsulation, phagocytosis, and autophagy (Lemaitre and Hoffmann, 2007). All these responses are based on a small number of receptors called pattern recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs) derived from viruses, bacteria, mycobacteria, fungi, and parasites (Janeway and Medzhitov, 2002). There are currently four main classes of invertebrates PRRs, including Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-like receptors (RLRs) and C-type lectin receptors (CTLs) (Lester and Li, 2014; Kannaki et al., 2011).

In mammals, TLRs were the first PRRs identified and were also the best characterized, recognizing a wide range of PAMPs (Beutler, 2008; Hoffmann, 2003; Medzhitov, 2007). TLRs are type I transmembrane

* Corresponding author. Guangdong provincial key laboratory of marine biology, Shantou University, Shantou 515063, China.
E-mail address: lisk@stu.edu.cn (S. Li).
[1] These authors contributed equally to this work.

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proteins and comprise an ectodomain, which contains leucine-rich repeats (LRR) that mediate the recognition of PAMPs, a transmembrane region (TM) and cytosolic Toll-IL-1 receptor (TIR) domains, that activate downstream signaling pathways. Each TLR detects distinct PAMPs, such that TLR4 recognizes LPS, whereas TLR-3, -7, -8, and -9 recognize viral nucleic acids (Akira et al., 2006; Kawai and Akira, 2011). Upon direct activation by PAMPs, TLRs activate signaling pathways that provide specific immunological responses tailored towards the microbes expressing the PAMPs. In the MyD88-dependent pathway, MyD88 is recruited by TLRs, which thus further recruits IRAKs, TRAF6 and the TAK1 complex, leading to early-phase activation of NF-κB and MAP kinases, and finally induces the production of inflammatory cytokines (Kawai and Akira, 2010; Takeda and Akira, 2004). Different from mammalian TLRs pathway, Toll signaling pathway in *Drosophila* cannot recognize PAMPs directly, but can be recruited by a proteolytically activated form of Spätzle (Spz) protein. Subsequently, cleaved Spätzle binds to Toll, triggering a conformational change that generates an active Toll dimer (Schneider et al., 1994; Belvin and Anderson, 1996; Drier and Steward, 1997; Wasserman, 2000). The dimerized cytoplasmic domain of Toll interacts with an adaptor, *Dm*MyD88 (homologue of mammalian MyD88), which recruits a second adaptor Tube (regarded as IRAK4 homologue) and the protein kinase Pelle (homologue of human IRAK1) and *Dm*TRAF6, resulting in the translocation of Dif or Dorsal (homologue of human NF-kappa B) and the expression of antimicrobial peptides, cytokines as well as other immune mediators (Hoffmann, 2003; Imler and Bulet, 2005; Lemaitre and Hoffmann, 2007; Weber et al., 2003). However, in the last couple of years, some studies on invertebrate Tolls have revealed that Tolls can directly recognize foreign PAMPs, such as vesicular stomatitis virus, peptidoglycan (PGN) and lipopolysaccharide (LPS), just as mammalian TLRs (Nakamoto et al., 2012; Sun et al., 2017).

As of now, about 10 functional TLRs in humans and 12 in mice have been identified (Kawai and Akira, 2010), while about 17 TLRs in various teleost (Palti, 2011; Rebl et al., 2010) and 16 TLRs from lamprey genome (Armant and Fenton, 2002) have been identified. In the case of invertebrates, there exists 9 Tolls in *Drosophila* (Kasamatsu et al., 2010), with the sea urchin (*Strongylocentrotus purpuratus*), having 253 Toll loci, representing the zenith of documented duplication and divergence (Buckley and Rast, 2012; Buckley and Smith, 2007). For crustaceans, Arts et al. (2007) reported the first partial cDNA sequence of a Toll-like receptor (*Pm*Toll) in tiger shrimp *Penaeus monodon* (Arts et al., 2007), after which different types of full-length cDNAs of Toll-like receptors, including *Sp*Toll from *S. paramamosain* (Lin et al., 2012), *Pt*Toll1-3 from *Portunus trituberculatus* (Zhou et al., 2015), *Es*Toll1 and *Es*Toll2 from *Eriocheir sinensis* (Yu et al., 2013), *Lv*Toll1-3 from *Litopenaeus vannamei* (Wang et al., 2011b), *Mr*Toll from *Macrobrachium rosenbergii* (Srisuk et al., 2014; Feng et al., 2016), *Pc*Toll1 and *Pc*Toll3 from *Procambarus clarkii* (Wang et al., 2015; Lan et al., 2016), *Fc*Toll from *Fenneropenaeus chinensis* (Yang et al., 2008), and *Mj*Toll1 from *Marsupenaeus japonicus* (Mekata et al., 2008) were identified. Similarly, *Hc*Toll2 and *Hc*Toll3 from the Mollusca, *Hyriopsis cumingii* have been identified (Ren et al., 2014; Zhang et al., 2017).

Antimicrobial peptides (AMPs) are present in all living organisms, and are generally small (usually < 10 kDa or < 100 amino acids) cationic amphipathic molecules that kill a broad spectrum of microorganisms in a stoichiometric manner. The first AMP was isolated from *Carcinus maenas* in 1996, and was a proline-rich protein that shares homology to bovine bactenecin 7 (Schnapp et al., 1996). Over the past two decades, many types of AMPs have been identified and characterized from different crab species, such as single-domain AMPs scyllin (Chattopadhyay and Chatterjee, 1997), Callinectin (Khoo et al., 1999), anti-lipopolysaccharide factors (ALFs) (Liu et al., 2012; Sun et al., 2015), multi-domain AMPs *Sp*Hyastatin (Shan et al., 2016a, 2016b), *Sp*Crustin, *Sp*Arasin and *Sp*GRP (Imjongjirak et al., 2009, 2011), and the unconventional Histone derived AMP *Sp*Histin (Chen et al., 2015). Although numerous AMPs exhibit structural diversity, they could be classified into three major groups (Marshall and Arenas, 2003; Bulet et al., 2004): (i) linear peptides that form amphipathic α-helices, such as *Sp*Histin; (ii) cysteine-rich peptides containing single or several disulfide bridges, such as *Sp*Crustin, *Sp*GRP, and *Sp*ALFs; (iii) peptides with an overrepresentation of proline, arginine, glycine, tryptophan or histidine, such as *Sp*Arasin and *Sp*Hyastatin.

In the present study, another novel Toll, designated *Sp*Toll2, was isolated from *S. paramamosain*. The bioinformatic analysis of *Sp*Toll2 showed that it belonged to the Toll-like receptor family and shared great similarities with Toll4 from *P. trituberculatus*. *In vitro* analysis revealed that recombinant *Sp*Toll1 and *Sp*Toll2 could bind to Gram-negative and Gram-positive bacteria as well as cell wall components. Upon RNAi-mediated knockdown of *Sp*Toll1 and *Sp*Toll2 in *S. paramamosan*, the regulation of some AMPs and downstream mediators of immune response to *V. parahaemolyticus* and *S. aureus* infection decreased significantly. Bacterial clearance assay showed that *Sp*Toll1 and *Sp*Toll2 participated in extraneous bacteria clearance from hemolymph. Collectively, these results indicated that *Sp*Toll1 and *Sp*Toll2 might play a role in the antibacterial immune response of *S. paramamosan*.

2. Materials and methods

2.1. Immune challenge and tissue collection

One hundred healthy mud crabs (approximately 100 g each) were purchased from a crab farm in Niutianyang (Shantou, Guangdong, China), and acclimatized in laboratory tanks containing water with salinity 10‰ at 25 °C for one week before further processing. For challenge experiments, 200 μl V. *parahaemolyticus* (1×10^7 cfu ml^{-1}), 200 μl *S. aureus* (1×10^5 cfu ml^{-1}), Poly I:C (1 mg ml^{-1}), or 200 μl WSSV (containing 10^3 pfu ml^{-1}) were injected into the base of the fourth leg of each crab. For the control group, 200 μl 0.8% NaCl (normal saline, NS) (Sangon Biotech, Shanghai, China) was injected. Hemolymph was withdrawn from each anesthetized crab using a disposable needle and syringe (1 ml) at 0, 3, 6, 12, 24 and 48 hpi, and collected into tubes containing ice-cold acid citrate dextrose (ACD) anticoagulant buffer (1.32% sodium citrate, 0.48% citric acid, 1.47% glucose). Samples were immediately centrifuged at 800 × *g* for 20 min at 4 °C to isolate the hemocytes for RNA extraction. Other tissues including brain, muscle, subcuticular epidermis, gills, hepatopancreas, intestines and heart of healthy *S. paramamosain* were also quickly collected, rinsed with 0.1% diethylpyrocarbonate (DEPC)-treated water, immediately dipped into liquid nitrogen for subsequent total RNA extraction.

2.2. Total RNA extraction and first-strand cDNA synthesis

The total RNA from hemocytes was extracted using TRIzol reagent (Ambion, USA) following the manufacturer's protocol. For full-length *Sp*Toll2 cloning, 5 μg RNA was reverse transcribed with the M-MLV First-Strand cDNA Synthesis Kit (Invitrogen, USA), while for quantitative real-time PCR (qPCR) analysis, first-strand cDNAs synthesis was carried out using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China) after incubation for 30 min at 37 °C with 10 units of DNase I (Takara, Dalian, China) to remove residual genomic DNA. For the other *S. paramamosain* tissues (brain, muscle, subcuticular epidermis, gills, hepatopancreas, intestines and heart), total RNA was extracted using the RNAfast200 Total RNA rapid Extraction Kit (Feijie, Shanghai, China) following the manufacturer's protocol. The obtained RNA samples were used for first-strand cDNA synthesis as described above. The purity and quality of the RNA samples was ascertained using 1.0% (w/v) agarose-gel electrophoresis while the concentration was measured with a Nanodrop® ND-1000 spectrophotometer (LabTech, Holliston, MA) at absorbance 260 nm/280 nm (A260/280).