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A novel Toll like receptor with two TIR domains (HcToll-2) is involved in regulation of antimicrobial peptide gene expression of *Hyriopsis cumingii*

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ABSTRACT

Animal Toll-like receptors (TLRs) are involved in innate immunity. Toll proteins are generally transmembrane proteins. In this study, an atypical Toll-like receptor (*HcToll-2*) was identified from the triangle-shell pearl mussel *Hyriopsis cumingii*, which belongs to phylum Mollusca. Unlike the typical Toll like receptors with extracellular leucine-rich repeats (LRRs), transmembrane, and intracellular Toll/interleukin-1 receptor (TIR) domains, HcToll-2 has two homologous TIR domains located at the C-terminal (designated as *Hc*TIR1 and *Hc*TIR2) and lacks a transmembrane domain. Phylogenetic analysis showed that HcTIR1 was clustered with TIR of sea anemone Toll, and HcTIR2 was clustered with TIR of *Drosophila* Toll. *HcToll-2* mRNA could be detected in the hepatopancreas and was upregulated after challenge with *Escherichia coli* and *Staphylococcus aureus*. Recombinant HcLRR protein with GST tag could bind to bacteria and also to LPS and PGN. Over-expression of both *Hc*TIR1 and *Hc*TIR2 induced drosomycin genes in *Drosophila* S2 cells. RNAi analysis showed that *HcToll-2* was required for the expression of theromacin, which is a cysteine-rich antimicrobial peptide (AMP) gene. This research is the first report of an atypical Toll-like receptor *HcToll-2* involved in antibacterial immunity through induction of AMP expression.

1. Introduction

Innate immunity constitutes the first line of defense against invading pathogens in vertebrates and invertebrates (Hoffmann et al., 1999; Medzhitov and Janeway, 2000). Recognition of pathogen-associated molecular patterns (PAMPs), the conserved structures of microbes, is the first step of innate immunity (Medzhitov and Janeway, 2002). Pathogen recognition is mediated by germ-line receptors, called pattern recognition receptors (PRRs) (Janeway and Medzhitov, 2002).

In *Drosophila melanogaster*, the immune deficiency (Imd) pathway is induced by Gram-negative bacteria and the Toll signaling pathway is activated by Gram-positive bacteria and fungi (Lemaitre et al., 1997). The Toll receptor plays an important role in *Drosophila* dorsal–ventral development and immune response (Anderson et al., 1985; Lemaitre et al., 1996). Tolls and Toll like receptors (TLRs) are type I membrane receptors characterized by their domain organization. Tolls and TLRs have an extracellular leucine-rich repeats (LRRs) domain necessary for the recognition of PAMPs, a transmembrane (TM) domain, and a cytoplasmic Toll/interleukin-1 receptor homology (TIR) domain mediating downstream signal transduction (Kawai and Akira, 2010). TLR signaling pathways are MyD88-dependent (Akira et al., 2001). MyD88, a TIR domain-containing molecule in the TLR pathways, interacts with IL-1 receptor-associated kinase (IRAK) through the death domain. Activated IRAK associates with TRAF6 and activates the NF-κB pathway (Takeda and Akira, 2004). In *Drosophila*, the Toll pathway is also MyD88-dependent. *Drosophila* Toll is activated by the cytokine-like ligand Spaetzle (SPZ). Gram-positive bacteria or fungi induce a proteolytic cascade and finally activate the pro-SPZ. Intracellular signal transduction in the *Drosophila* Toll pathway needs two death domain–containing adapter molecules called tube and pelle. Finally, the NF-κB transcription factor DIF or Dorsal is activated (Ferrandon et al., 2004). Recently, Nakamoto et al.

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(2012) reported that Toll-7 interacts with vesicular stomatitis virus, acting directly as a pattern recognition receptor which is similar to mammalian TLRs and induces the antiviral autophagy in *Drosophila* (Nakamoto et al., 2012).

TLRs are widely investigated from vertebrates to invertebrates. Up to 10 functional TLRs have been identified in humans and 12 in mice, and among these TLRs, TLR1–TLR9 are conserved (Kawai and Akira, 2010). Seventeen different TLRs have also been found in fish (Palti, 2011), and up to 16 TLRs have been identified in the lamprey genome (Armant and Fenton, 2002). In mammalian immune systems, TLRs discriminate between diverse PAMPs and then induce pathogen-specific immune responses (Kasamatsu et al., 2010).

Multiple TLRs are also found in invertebrates. About 222 TLRs have been implicated in the sea urchin genome (Leulier and Lemaitre, 2008; Valanne et al., 2011). In *Drosophila* genomes, nine Toll genes have been found (Kasamatsu et al., 2010). However, only one Toll has been found in the *Caenorhabditis elegans* genome (Leulier and Lemaitre, 2008). Shrimp TLRs have also been reported (Mekata et al., 2008; Yang et al., 2008; Arts et al., 2007). Three TLRs from *Litopenaeus vannamei* (LvToll, LvToll2, and LvToll3) have been identified (Wang et al., 2011). In mollusks, Toll receptors from *Chlamys farreri* and *Crassostrea gigas* have been reported (Qiu et al., 2007; Zhang et al., 2011a). A primitive MyD88-dependent TLR signaling pathway has been characterized in the mollusk *C. farreri* and this TLR signaling pathway is important in immune defense against *Listonella anguillara* (Wang et al., 2010).

Among Tolls and TLRs identified in invertebrates and vertebrates, most TLRs have a typical domain architecture, including an extracellular LRR domain, a TM domain, and a cytoplasmic TIR domain. In this study, a Toll-like receptor from *Hyriopsis cumingii* (HcToll-2) with two TIR domains but no TM domain at the C-terminus was identified. Our study showed that *HcToll-2* may participate in innate immunity through regulation of antimicrobial peptide (AMP) gene expression.

2. Materials and methods

2.1. Mussels and bacterial challenge

Approximately 1-year-old *H. cumingii* were obtained from Wuhu City, Anhui Province, China, and were cultured for a week in a recirculation system containing filtered freshwater at about 25 °C before the experiment. In the experimental groups, approximately 30 μl of *E. coli* (OD_{600} = 0.5) or *Staphylococcus aureus* were injected into the adductor muscles of *H. cumingii* (each group contained 12 mussels) using a 100 μl syringe. The hepatopancreas of three mussels were collected after 2, 6, 12, and 24 h for RNA isolation. Hemolymph was diluted (1:10) with anticoagulant buffer (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, and 10 mM ethylenediamine tetraacetic acid, pH 4.6) and collected into an RNAse free tube. Hemocytes were obtained through centrifugation at 800*g* for 10 min (4 °C) for RNA extraction. The hepatopancreas, gills and mantle of the healthy mussels were also dissected for RNA extraction. Total RNA was extracted from the aforementioned samples using an RNApure high-purity total RNA rapid extraction kit (spin-column; Bioteke, Beijing, China).

2.2. 3′ and 5′ Rapid amplification of cDNA ends (RACE) of HcToll-2 from the hepatopancreas

Analysis of the hepatopancreas transcriptome data of the healthy mussels revealed a sequence of the Toll-like receptor gene (*HcToll-2*). Based on the obtained 3348 bp sequences, a gene-specific forward primer (HcToll-2-F, Table 1) was designed to obtain the 3′-end sequence with the UPM primer from the kit using 3′ RACE method and a gene-specific reverse primer (HcToll-2-R, Table 1) was designed to obtain the 5′-fragment with the UPM primer using 5′ RACE method using Clontech SMARTer™ RACE cDNA amplification kit from Takara (Dalian, China). The cDNA transcribed from the hepatopancreas RNA was used as a template for gene cloning. The detailed transcription methods were based on a previous study (Ren et al., 2012). The full-length HcToll-2 cDNA was obtained by overlapping the 5′, 3′, and middle fragments (NHcToll-2-1-F1, Hc-Toll-32549-R1, Table 1).

2.3. Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignment was performed with the orthologous sequences of the Tolls and TLRs signaling pathway using MUSCLE with default parameters, which were manually curated when necessary. The gene structure and position of motifs were manually checked using data from Entrez, and the conservation of motifs was predicted using the SMART software (http://smart.embl-heidelberg.de/). Phylogenetic analysis was conducted using the MrBayes 3.1 software for Bayes analysis with the mixed amino acid substitution model. Phylogeny support was verified with the bootstrap consensus tree inferred from 1000 replicates.

2.4. Selection pressure analysis

The nonsynonymous/synonymous substitution rate ratio ($\omega = dn/ds$) can provide a measurement for the changes in selective pressures. $\omega = 1$, <1, and >1 indicate neutral evolution, purifying selection, and positive selection on the target gene, respectively. We used a codon-substitution model implemented in the CodeML program of the PAML 4.4 software package to analyze the changes in selective pressure using the M0 model. To explore the divergence of different branches of TLR genes in evolutionary history, we also used the branch model of the CodeML software to compute the nonsynonymous/synonymous substitution rate ratio of the foreground (branch of *HcToll-2*) and background branches.

2.5. Tissue distribution and expression pattern of HcToll-2 in hepatopancreas after bacterial challenge

First-strand cDNA was synthesized from the samples using the M-MLV First Strand Kit (Invitrogen, Shanghai, China) with oligo(dT)20 primer. Reverse transcription-polymerase chain reaction (RT-PCR) method was used to study tissue distribution of *HcToll-2* in hemocytes, hepatopancreas, gills, and mantle using gene-specific primers (HcToll-2-RT-F, HcToll-2-RT-R, Table 1). The expression pattern of *HcToll-2* in the hepatopancreas challenged with *E. coli* and *S. aureus* was determined using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) with primers HcToll-2-RT-F and HcToll-2-RT-R. A 2× SYBR real-time PCR premixture (Bioteke, Beijing, China) was used in the qRT-PCR experiment with Stratagene MX300P. Details of the methods were based on a previously published paper (Shi et al., 2008). β-Actin was used as the internal standard with primers Hc-Actin F and Hc-Actin R. All samples were in triplicate during the qRT-PCR analysis. The data were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and subjected to statistical analysis. An unpaired sample *t*-test was conducted, and $P < 0.05$ indicated statistical significance.

2.6. Recombinant HcLRR expression

The HcLRR fragments were amplified using primers (HcLRR-ex-F, HcLRR-ex-R, Table 1). A pGEX-4T1-HcLRR recombinant plasmid was generated by subcloning HcLRR cDNA into the pGEX-4T1 vector. The constructed plasmid was transformed into competent