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Two novel Toll genes (*EsToll1* and *EsToll2*) from *Eriocheir sinensis* are differentially induced by lipopolysaccharide, peptidoglycan and zymosan

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ABSTRACT

Tolls/Toll-like receptors (TLRs) play an essential role in initiating innate immune responses against pathogens and are found throughout the insect kingdom but have not yet been reported in the crustacean, *Eriocheir sinensis*. For this purpose, we cloned two novel Toll genes from *E. sinensis*, *EsToll1* and *EsToll2*. The full-length cDNA of *EsToll1* was 3963 bp with a 3042-bp open reading frame (ORF) encoding a 1013-amino acid protein. The extracellular domain of this protein contains 17 leucine-rich repeats (LRRs) and a 139-residue cytoplasmic Toll/interleukin-1 receptor (TIR) domain. The cDNA full-length of *EsToll2* was 4419 bp with a 2667-bp ORF encoding an 888-amino acid protein with an extracellular domain containing 10 LRRs and a 139-residue cytoplasmic TIR domain. By phylogenetic analysis, *EsToll*1 and *EsToll*2 clustered into one group together with Tolls from other crustaceans. Quantitative RT-PCR analysis demonstrated that a) both *EsToll1* and *EsToll2* were constitutively expressed in all tested crab tissues; b) *EsToll1* and *EsToll2* were differentially induced after injection of lipopolysaccharides (LPS), peptidoglycan (PG) or zymosan (GLU). Importantly, *EsToll2* expression was significantly upregulated at almost all time intervals post-challenge with LPS, PG and GLU. Our study indicated that *EsToll1* and *EsToll2* are differentially inducibility in response to various PAMPs, suggesting their involvement in a specific innate immune recognition mechanism in *E. sinensis*.

1. Introduction

Invertebrates do not have counterparts to mammalian B or T cells and, therefore, cannot induce acquired immune responses by producing antibodies and complement to pathogenic organisms [1]. Nonetheless, invertebrates can induce rapid and effective immune responses to clear the intruding pathogens relying largely on innate immunity. As the first-line host defense mechanism, innate immunity is mainly mediated by germ-line-encoded pattern-recognition receptors (PRRs) such as Toll/Toll-like receptors (TLRs), Nod-like receptors (NLRs) and RIG-like receptors (RLRs) [2]. These so-called PRRs can recognize pathogen-associated molecular patterns (PAMPs) [3] that are conserved molecular motifs on the surface of pathogenic microorganisms, such as lipopolysaccharides (LPS) from Gram-negative bacteria, peptidoglycans (PG) from Gram-positive bacteria, double-stranded RNA (dsRNA) and β-glucans (GLU) from fungi [4], in order to induce rapid and effective immune responses against intruding pathogens.

Tolls/TLRs possess important roles in the activation of innate immunity and are thought to function as cytokine receptors (Toll in *Drosophila*) or PRRs (TLRs in mammals), that can activate similar, albeit non-identical, signal-transduction pathways in flies and mammals [5]. Tolls or TLRs are type I transmembrane (TM) proteins with ectodomains containing leucine-rich repeats (LRRs) that mediate the recognition of PAMPs; TM domains and intracellular Toll/interleukin-1 (IL-1) receptor (TIR) domains required for downstream signal transduction [3]. In mammals, different PAMPs can act as ligands for diverse TLRs include LPS from the outer membrane of Gram-negative bacteria (TLR4), lipoproteins and PG from Gram-positive bacteria (TLR1, 2, and 6), flagellin from bacterial flagella (TLR5), dsRNAs generated during viral infection (TLR3), single-stranded viral RNA (TLR7, TLR8) and unmethylated CpG dinucleotide motifs commonly found in bacterial and viral genomes (TLR9) [6,7]. In *Drosophila*, the *Dm*Toll signaling pathway plays an important role in anti-positive bacterial and anti-fungal

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responses [8], and it is triggered by a proteolytically processed form of the secreted factor Spaetzle, thought to be the Toll ligand [9,10]. The cleavage of this molecule is the end point of a complex extracellular proteolytic cascade, which involves four distinct trypsin-like serine proteases [5]. Activated Toll signals via the Tube and Pelle proteins to the heterodimeric protein complex Cactus-Dorsal ultimate lead to the phosphorylation and degradation of Cactus [5]. The transcriptional factor Dorsal then translocates into the nucleus to regulate the expression of Toll-dependent genes, such as antimicrobial peptides and a substantial number of other innate immune responsive genes [11–13].

The Chinese mitten crab *Eriocheir sinensis* is one of the most important crustacean species widely cultivated in Southeast Asia [14], but the frequent outbreaks of diseases have caused decreased production and catastrophic losses in the past decade [15]. Therefore, studying the structure and transcriptional responses of potential immune-related genes such as TLRs to pathogenic antigens may facilitate a better understanding of the crab immune defense and recognition mechanisms and support the sustainable development of better disease management strategies in the Chinese mitten crab farming industry. Recently, several research labs, including our own, have made efforts to screen immune-related genes from *E. sinensis* by constructing cDNA libraries [16–24], with the aim of designing efficient strategies for disease control. The main objectives of this current study were (1) to clone the full-length cDNAs of *EsToll1* and *EsToll2* from *E. sinensis*; (2) to investigate the mRNA expression patterns of *EsToll1* and *EsToll2* in different tissues; and (3) to detect the temporal responses of *EsToll1* and *EsToll2* in hemocytes induced by LPS, PG and GLU challenge. Together, our results determined the potentially important roles of these novel genes in the innate immune recognition and response to exogenous pathogenic stimulation in the Chinese mitten crab.

2. Materials and methods

2.1. Animal immune challenge and sample collection

Healthy adult Chinese mitten crabs ($n = 200$; 80 ± 20 g wet weight) were collected from the Tong Chuan Aquatic Product Market in Shanghai, China. After acclimation for one week at 20–25 °C in filtered, aerated freshwater, crabs were placed in an ice bath for 1–2 min until lightly anesthetized. Hemolymph was drawn from the hemocoel in the arthrodial membrane of the last pair of walking legs using a syringe (~2.0 ml per crab), added to an equal volume of anticoagulant solution (2.05 g glucose, 0.8 g citrate and 0.42 g NaCl in 100 ml double distilled water) and centrifuged at 500× *g* at 4 °C to isolate hemocytes. The other tissues (hepatopancreas, gills, muscle, stomach, intestine, thoracic ganglia, brain and heart) were harvested, snap frozen in liquid nitrogen and stored at −80 °C prior to nucleic acid analysis. For cloning and subsequent in-depth analysis, tissues from five crabs were pooled and ground with a mortar and pestle prior to extraction.

For stimulation by PAMPs, 120 crabs were divided equally into four groups. The three experimental crab groups were injected into the arthrodial membrane of the last pair of walking legs with approximately 100 μl of LPS from *Escherichia coli* (Sigma–Aldrich, St. Louis, MO, USA), 100 μl of PG from *Staphylococcus aureus* (Sigma–Aldrich) and 100 μl of zymosan (GLU from *Saccharomyces cerevisiae*, Sigma–Aldrich) resuspended (500 μg/mL) in *E. sinensis* saline (ESS, 0.2 M NaCl, 5.4 mM KCl, 10.0 mM $CaCl_2$, 2.6 mM $MgCl_2$, 2.0 mM $NaHCO_3$; pH 7.4). Meanwhile, the control group crabs were each administered 100 μl ESS (pH 7.4) in the same manner. Five crabs were randomly selected at each time interval of 0 (as blank control), 2, 6, 12 and 24 h after injection of each type of PAMP. Hemocytes were harvested as described above and stored at −80 °C after the addition of 1 ml Trizol reagent (Invitrogen, Carlsbad, CA, USA) for subsequent RNA extraction.

2.2. Total RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from *E. sinensis* tissues sampled as described in Section 2.1 using Trizol® reagent (RNA Extraction Kit, Invitrogen) according to the manufacturer's protocol. The total RNA concentration and quality were estimated using spectrophotometry at an absorbance of 260 nm and agarose-gel electrophoresis, respectively.

Total RNA (5 μg) isolated from hemocytes was reverse transcribed using the SMARTer™ RACE cDNA Amplification kit (Clontech, Mountain View, CA, USA) for cDNA cloning. For RT-PCR and quantitative real-time RT-PCR (qRT-PCR) expression analysis, total RNA (4 μg) was reverse transcribed using the PrimeScript™ Real-time PCR Kit (Takara, Shiga, Japan).

2.3. EST analysis and cloning of full-length EsToll1 and EsToll2 cDNA

Partial cDNA sequences of *EsToll1* and *EsToll2* were obtained from the transcriptome data of the hepatopancreas [25,26], testis [27] from *E. sinensis*. The *E. sinensis EsToll1* and *EsToll2* partial cDNA sequences were extended using 5′ and 3′ RACE (SMARTer™ RACE cDNA Amplification kit, Clontech), and gene-specific primers (Table 1) were designed based on the original cDNA sequence. The 3′ RACE PCR reaction was carried out in a total volume of 50 μl containing 2.5 μl (800 ng/μl) of the first-strand cDNA reaction as the template, 5 μl of 10× Advantage 2 PCR buffer, 1 μl of 10 mM dNTPs, 5 μl (10 μM) of gene-specific primers (*EsToll1*-3′RACE and *EsToll2*-3′RACE, Table 1), 1 μl of Universal Primer A Mix (UPM; Clontech), 34.5 μl of sterile deionized water and 1 U 50× Advantage 2 polymerase mix (Clontech). For the 5′ RACE, UPM was used as the forward primer in PCR reactions in conjunction with the reverse gene-specific primers (*EsToll1*-5′RACE and *EsToll2*-5′RACE, Table 1).

Table 1
PCR primer sequences used for *EsTolls* analysis.

Primer name	Sequences (5′ → 3′)
5′-RACE	
EsToll1-5′-1	CACAGCGTTACTATCTTCAACTTCC
EsToll1-5′-2	CTGCGGTTGTTTGGCGGTGA
EsToll1-5′-3	ATTGTAGGTTCACAGTCTCCAAGTCAG
EsToll1-5′-4	TCACAGTCTCCAAGTCAGGACAGTT
EsToll1-5′-5	GCAAATAAGGTTTCGGGTA
EsToll2-5′-1	ACTCTGGTGGAAGTTCTACGATGT
EsToll2-5′-2	TGAGTAATGAACCTTCCTGTCCAC
EsToll2-5′-3	CCGCAACTCCTCGTCAGACA
3′-RACE	
EsToll1-3′-1	CTGGAAGTTGAAGATAGTAACGCTGTG
EsToll1-3′-2	GGAAGTTGAAGATAGTAACGCTGTG
EsToll1-3′-3	TCACCCTATCACAGTTCTC
EsToll1-3′-4	CCACTGTCTTGCTCGTCGTCTT
EsToll2-3′-1	TGCGTTTATTCTTTTCCTCGGTGTA
EsToll2-3′-2	GGCACTGTGACTGTGACCTCCT
EsToll2-3′-3	GCTCTCCTCGCTCAATGTCTCT
UPM-Long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
UPM-Short	CTAATACGACTCACTATAGGGC
qRT-PCR	
EsToll1-F	CCACTGTCTTGCTCGTCGTCTT
EsToll1-R	CAATGCTCTGGTCAATCTGGTTCTG
EsToll2-F	GCATACCAGGACGACGAACAAG
EsToll2-R	TCAAGGAGGTCACAGTCACAGT
β-actin-F	CTCCTGCTTGCTGATCCACATC
β-actin-R	GCATCCACGAGACCACTTACA
Sequencing	
T7	TAATACGACTCACTATAGG
SP6	ATTTAGGTGACACTATAGAA