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Leucine-rich repeats containing protein functions in the antibacterial immune reaction in stomach of kuruma shrimp *Marsupenaeus japonicus*

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ABSTRACT

Leucine rich repeat (LRR) motif exists in many immune receptors of animals and plants. Most LRR containing (LRRC) proteins are involved in protein-ligand and protein-protein interaction, but the exact functions of most LRRC proteins were not well-studied. In this study, an LRRC protein was identified from kuruma shrimp *Marsupenaeus japonicus*, and named as *Mj*LRRC1. *MjLRRC*1 was consistently expressed in different tissues of normal shrimp with higher expression in gills and stomach. At the transcriptional level, there were no significant changes of *MjLRRC*1 after injection of *Vibrio anguillarum* or *Staphylococcus aureus* in gills and hepatopancreas. While in *V. anguillarum* oral infection, *MjLRRC*1 was upregulated in stomach but not in intestine. The recombinant *Mj*LRRC1 protein could bind to Gram-positive and Gram-negative bacteria, bacterial cell wall components including peptidoglycan, lipoteichoic acid, and lipopolysaccharide. *Mj*LRRC1 regulated the expression of some antimicrobial peptide (AMP) genes and participated in bacteria clearance of stomach. All these results suggested that *Mj*LRRC1 might play important roles in antibacterial immune response of kuruma shrimp.

1. Introduction

Innate immunity is the first line defense for animals to eliminate the nonself pathogenic invaders [1]. Invertebrates totally depend on innate immunity as they lack adaptive immune system. Similar to vertebrates, the innate immunity of invertebrates includes humoral responses, such as melanization, coagulation, and production of AMPs, and cellular responses, including encapsulation, phagocytosis, and autophagy [2]. The nonself recognition is accomplished by germline-encoded pattern recognition receptors (PRRs), which recognized some conserved pathogen-associated molecular patterns (PAMPs) [1,3]. The recognition could initiate immune reactions. More and more PRRs have been identified and studied in *Drosophila*, such as Toll receptors, peptidoglycan recognition proteins, Gram-negative binding proteins, thioester-containing proteins, scavenger receptors and C-type lectins [4]. At least 11 PRRs have been characterized in shrimp till now, and they show binding specificities and function in different immune reactions [5].

LRR containing (LRRC) proteins have two or more LRR motifs. LRRs are characterized for the first time from a leucine rich alpha 2-glycoprotein in human serum [6]. LRRs are conserved in evolution [7]. Most LRR proteins contain 2-45 LRR motifs and each repeat has about 20–30 amino acids with the consensus sequence of LxxLxLxxNxL [7–10]. By protein-protein interaction, LRR proteins function in the signal transduction and host defense of plants and animals [7,11]. In plants, LRRC proteins such as LRR receptor protein (LRR-RP) and LRR receptor kinase (LRR-RK) play important roles by recognition of PAMPs in the plant-microbe interaction of innate immunity [12,13]. In mammals, Toll-like receptors (TLR) and NOD-like receptors (NLR) are two major PRRs of innate immune reaction, and the ligand recognition domains of TLR and NLR both contain several LRR motifs [14,15]. In human, 375 LRRC proteins are categorized by genomic wide bioinformatics analysis and characterized in innate immunity, and almost half of them contain only LRR motifs [16]. Some LRR proteins are implicated in human diseases: LRR and immunoglobulin-like domain proteins are associated with human cancers as double-edged swords [17]; LRR containing protein LRRK2 and LINGO1 function in Parkinson's disease [18,19]; LRR repeat protein 7 is involved in Alzheimer's disease [20].

In *Drosophila*, ligand Spaetzle binding to ectodomain LRR motifs

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of Toll receptor could activate Toll signal pathway which is important in innate immunity and embryonic development [21]. Two LRR containing proteins in mosquito *Anopheles gambiae* could antagonize the *Plasmodium* parasite infection by forming complex and interacting with TEP1 [22]. An LRR containing protein leureptin shows the binding activity to bacterial lipopolysaccharide and participates in antibacterial response of hemocytes [23]. In *Caenorhabditis elegans*, a transmembrane LRR protein is characterized for the Microsporidia infection [24], and gene pan-1 encoding the transmembrane and cytoplasmic LRR protein could regulate larval development [25]. Three LRR-only proteins are identified in scallop *Chlamys farreri*, and act as PRRs or immune effectors of innate immune reaction [26,27]. An LRR homolog identified in green mud crab *Scylla paramamosain*, plays a role in resistance of pathogens [28]. In black tiger shrimp *Penaeus monodon*, an LRR protein is highly expressed in hemocytes and lymphoid organ, and functions in the immune reaction against pathogens [10]. However, the exact functions of LRRC proteins in kuruma shrimp are still not well-studied. Here in this study, an LRRC protein was identified from kuruma shrimp *M. japonicus*, and designated as *Mj*LRRC1. The bioinformatic analysis of *Mj*LRRC1 showed that it belonged to LRR protein soc-2 homolog family. *MjLRRC*1 was highly expressed and upregulated after bacteria oral infection in stomach. In vitro analysis demonstrated the binding activities of recombinant *Mj*LRRC1 to Gram-negative and Gram-positive bacteria and bacterial cell wall components. After knocking down *MjLRRC*1 expression, the upregulation of some AMP genes in response to *V. anguillarum* oral infection was impaired in shrimp. Bacterial clearance assay showed that *Mj*LRRC1 participated in extraneous bacteria clearance of shrimp stomach. All the results indicated that *Mj*LRRC1 might function in the antibacterial immune reaction of kuruma shrimp.

2. Materials and methods

2.1. Shrimp immune challenge and sample collection

The kuruma shrimp *M. japonicus* (body weight 8–12 g) were obtained from an aquatic product market in Jinan, Shandong Province, China, and then cultured in lab tanks with air-pumped circulating seawater at 22 °C for one week before processing. For bacterial injection immune challenge, each shrimp was injected at the abdominal segment with *V. anguillarum* or *S. aureus* (3×10^6 CFU). For the bacteria oral infection, each shrimp was fed with 10^9 CFU *V. anguillarum* following the previous feeding method [29]. The control groups were the shrimp injected or oral fed with the same volume of phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4).

Hemolymph was extracted from the ventral sinus of at least three shrimp using a sterile syringe preloaded with 1/3 volume of anticoagulant buffer (450 mM NaCl, 10 mM KCl, 10 mM EDTA, and 10 mM Hepes pH 7.45). Then hemocytes were collected by centrifugation at 800×*g* for 10 min at 4 °C. Tissues including gills, heart, hepatopancreas, intestine, and stomach were collected from at least three shrimp for total RNA extraction.

2.2. Sequence cloning and bioinformatics analysis

The full length sequence of *MjLRRC*1 cDNA was obtained from the transcriptome sequencing of hemocytes. Hemocytes were collected from pathogen challenged shrimp and total RNA was extracted for transcriptome sequencing by BGI (Beijing Genomics Institute, Shenzhen, China). Several cDNAs of LRRC proteins were obtained by the sequencing. We chose *Mj*LRRC1 for further study. The nucleotide sequence translation was performed by Expasy Translate tool (http://web.expasy.org/translate/), and the isoelectric point and molecular weight were computed by Expasy Compute pI/Mw tool (http://web.expasy.org/compute_pi/). The signal peptide and LRR domain were predicted through the simple modular architecture research tool (http://smart.embl-heidelberg.de). The sequence alignment of LRR domains was performed by the ClustalW and weblogo program (http://weblogo.berkeley.edu/logo.cgi) [30]. The phylogenetic tree was constructed by MEGA 5.2 software [31]. The amino acid sequences of LRRCs from different species were searched by the BLASTp program in National Center for Biotechnology Information server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/) for phylogenetic analysis.

2.3. Expression pattern analysis by real time quantitative RT-PCR

Tissue distribution at the transcriptional level of *MjLRRC*1 in hemocytes, heart, hepatopancreas, gills, stomach, and intestine was analyzed by real time quantitative PCR (qRT-PCR). The first strand cDNAs were reversely transcribed using RNase-free DNase I digested total RNAs from tissues of normal shrimp. Then cDNAs were diluted 20-fold in nuclease-free sterile water as templates for qRT-PCR. *MjLRRC*1RTF (5′-TCT TCC CTC ACT CTG TCA CGC-3′) and *MjLRRC*1RTR (5′-CCC AAG TTC AGT TCC ACC ATA-3′) were used as primers. *β-actin* was used as control with primers *Mjactin*F (5′-GCA TCA TTC TCC ATG TCG TCC CAG T-3′) and *Mjactin*R (5′-TAC GGC TGC GAG AAG ACG ACA GAA-3′). C1000 thermal cycler (Bio-Rad, USA) was used to perform qRT-PCR. The total volume of qRT-PCR was 20 μL including 2 μL template, 4 μL forward primer (1 μM) and 4 μL reverse primer (1 μM), and 10 μL Ultra SYBR mixture (2×, containing ROX, CWBio, Beijing, China). The qRT-PCR program was as follows: 10 min at 95 °C; followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min; and melting curve analysis from 65 °C to 95 °C. The qRT-PCR was repeated at least three times. $2^{-\Delta\Delta CT}$ method and Graphpad Prism software (San Diego, CA, USA) were used to analyze data and construct figures.

Total RNAs from gills and hepatopancreas after PBS, *V. anguillarum*, or *S. aureus* injection or from stomach and intestine post oral feeding of PBS or *V. anguillarum* at different time point were reversely transcribed to first strand cDNAs. The PBS-injected or PBS-fed shrimp were used as controls to eliminate injury or solution effect for bacterial infection at the same time point. The qRT-PCR program was the same as tissue distribution analysis. Results were calculated for the relative expression profile of *MjLRRC*1 after bacterial infection compared with the PBS group.

2.4. Recombinant expression and protein purification of MjLRRC1

*Mj*LRRC1 was recombinant expressed in *Escherichia coli*. The nucleotide sequence encoding full length *MjLRRC*1 was amplified using primers *MjLRRC*1ExF (5′-ATA CCATGG CTA TGA TGA GAA AGA CAG CTA AA-3′) and *MjLRRC*1ExR (5′-ATA CTCGAG TCA CAT TGT GCG GTA TGG-3′) and ligated into pET32a (+) vector. The recombinant plasmid was transformed into *E. coli* Rosetta competent cells and induced with 1 mM isopropyl-1-thio-β-D-galactopyranosid (IPTG). The recombinant protein was expressed in the form of insoluble inclusion body. After denatured and refolded, it was purified as the method described previously [32]. The purified recombinant protein was named as r*Mj*LRRC1.

2.5. Microorganism and bacterial cell wall component binding assay

Five Gram-positive bacteria (*Bacillus megaterium* AS1.223, *B. subtilis* ATCC 9372, *B. thuringiensis, Micrococcus luteus*, and *S. aureus* ATCC 6538) and four Gram-negative bacteria (*E. coli* ATCC