Cloning and characterization of the SpLRR cDNA from green mud crab, *Scylla paramamosain*

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ABSTRACT

Infectious diseases have seriously inhibited the aquaculture of mud crab *Scylla paramamosain* in southeastern China. Identification of the immune molecules and characterization of the defense mechanisms will be pivotal to the reduction of these diseases. Available data show that leucine-rich repeat (LRR) proteins play a crucial role in protein–protein interactions, recognition processes or immune reactions in both invertebrates and vertebrates. In the present study, we cloned and characterized a LRR cDNA from the mud crab *Scylla paramamosain* (SpLRR) by using the RACE strategy. Bioinformatics analysis predicted that SpLRR contains one open reading frame of 1893 bp and encodes a LRR protein of 630 amino acids with 17 LRR domains and 5 potential N-glycosylation sites. Further, SpLRR and other arthropod LRR proteins could be clustered into one branch in a phylogenetic tree. SpLRR transcripts were detected using RT-PCR from examined tissues including heart, gill, stomach, intestine, muscle and hemocytes, whereas not from hepatopancreas. More importantly, the SpLRR mRNA expression was up-regulated after infection with *Vibrio alginolyticus*, *Beta streptococcus* or Poly I:C for 12–48 h, suggesting a novel LRR homolog in crab might be associated with the resistance to pathogens. The result could be important for future investigation of crab immune defense mechanisms.

1. Introduction

Aquaculture of mud crab has been conducted for at least the past 100 years in China and for the past 30 years throughout Asia [1]. However, within the past decade crab farms have suffered from dramatic decreases in production due to infectious diseases [2,3], mainly of viral, bacterial and parasitical etiologies. For example, crab production in Shantou of China dropped nearly 60% in 2010 as a result of several infections [4]. Researches suggested that understanding of crab immunology will be very helpful to establish strategies for crab disease control [5].

Crabs lack an adaptive immune system, but they have developed various defense mechanisms that recognize antigens on the surface of potential pathogens [5–12]. Recently, a large variety of immune molecules have been characterized in crabs including antimicrobial peptide [6,7], ferritins protein [8], hemocyanin [9], peroxinectin [10], antioxidant enzyme [11] and anti-lipopolysaccharide factor (ALF) [12]. The evidences suggest that leucine-rich repeat (LRR) proteins are involved in protein–protein interactions, recognition processes or immune reactions in both invertebrates and vertebrates. The LRR EST in *Scylla paramamosain* has been noted by Chen et al. [13], whereas its complete cDNA sequence and role in immune reactions have not yet been reported.

In this study, SpLRR gene was cloned and characterized from the green mud crab *S. paramamosain*. Results also showed that SpLRR mRNA expression in hemocytes was up-regulated after infection with *Vibrio alginolyticus*, *Beta streptococcus* or Poly I:C for 12–48 h, suggesting a novel LRR homolog in crab might be associated with the resistance to pathogens. The result could be important for future investigation of crab immune defense mechanisms.

2. Materials and methods

2.1. Experimental animals

Live healthy mature green mud crabs (*S. paramamosain*) (average weight: 150 g) were obtained from Shantou Huaxun Aquatic Product Corporation (Shantou, China). They were acclimatized at room temperature for one week before the experiments were carried out.
2.2. RNA isolation and cDNA synthesis

Total RNA was extracted from hemocytes of *S. paramamosain* using RNAiso Plus (Takara, Japan) according to manufacturer instructions. The extracted RNA was treated with RNase-Free DNase (Takara, Japan) to remove contaminating DNA. cDNA was synthesized using the PrimeScriptRT reagent Kit with gDNA Eraser (for Real Time RT-PCR, Takara, Japan) or the SMART RACE cDNA Amplification Kit (for RACE, Clontech, Mountain View, CA) following the manufacturer’s instructions.

2.3. Rapid amplification of cDNA ends (RACE)

The EST of SpLRR was obtained from NCBI database (GenBank Accession: FJ774669), which was reported by Chen et al. [13]. cDNA ends of SpLRR were obtained using the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA). The universal primer mix (UPM) provided in the kit plus LRR-GSP5 and LRR-GSP3 (Table 1) were used for amplification of the 5’-end and 3’-end, respectively, of the SpLRR cDNA. The PCR was performed in a volume of 50 μl containing 2 μl 5’ RACE cDNA (or 3’ RACE cDNA), 5 μl LRR-GSP5’ (or LRR-GSP3’, 10 μM), 5 μl UPM (long UPM 0.4 μM, short UPM 2 μM) (Table 1), 0.5 μl LA Taq DNA Polymerase (5 u/μl), 28.5 μl sterilized ultrapure water, 5 μl 10× PCR buffer (MgCl2 plus) and 4 μl dNTPs (10 mM). The PCR program included an initial denaturation at 94 °C for 3 min, then 5 cycles of 94 °C for 30 s and 72 °C for 2 min, 5 cycles of 94 °C for 30 s and 70 °C for 2 min, then 27 cycles of 94 °C for 30 s and 68 °C for 30 s and 72 °C for 2 min, and a final 72 °C for 10 min. The PCR products were extracted and inserted into pMD-19T (Takara, Japan) and then transformed into *Escherichia coli* DH5α (Promega, Madison, WI). Recombinant clones grown on MacConkey agar (Sigma, St. Louis, MO) were identified using blue–white screening. Finally, positive clones were picked and confirmed by sequencing.

2.4. Bioinformatic analysis

The nucleotide and deduced amino acid sequence of SpLRR were analyzed using the BLAST algorithm (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/). Tertiary structure of SpLRR was predicted by Swiss MODEL (http://swissmodel.expasy.org/). Potential N-linked glycosylation sites were predicted by NetNGlyc 1.0 Serve (http://www.cbs.dtu.dk/services/NetNGlyc/).

2.5. mRNA expression of SpLRR

Total RNA was extracted from the *S. paramamosain* heart, gill, hepatopancreas, stomach, intestine, muscle and hemocytes. 1 μg of the total RNA was then used for the synthesis of cDNA. The SpLRR cDNA fragment was amplified using primers LRR-F and LRR-R (Table 1). The PCR was performed in a volume of 20 μl containing 10 μl PCR Mix, 1 μl SpLRR cDNA, 1 μl of 10 μM (each) LRR-F and LRR-R (Table 1) and 7 μl ddH2O. The PCR program included pre-denaturation at 94 °C for 5 min, 32 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and 30 s, followed by a final elongation at 72 °C for 5 min. As an internal loading control, the crab beta-actin cDNA fragment was amplified with 10 μM of primers Beta-actin-F and Beta-actin-R (Table 1) using the same PCR amplification conditions. The PCR mixtures bulk volume was 20 μl containing.

2.6. Immune challenge tests

For the immune challenge tests, three experimental groups and a control group were set up with each group having three crabs. Each individual crab was injected with 0.1 ml of *Vibrio alginolyticus* (10^9 CFU/100 g body weight), *Beta streptococcus* (10^9 CFU/100 g), poly I:C (polynosinic–polycytidylic acid, Sigma, 1 mg/kg) or 0.85% NaCl solution. Hemocytes were collected from each crab at 0, 6, 12, 24, 48 and 72 h for RNA extraction. After cDNA synthesis, a relative quantitative real-time RT-PCR assay was performed using an ABI PRISM-7300 (Applied Biosystems, Foster City, CA). A segment of 127 and 88 bp was amplified using primer sets LRR-F/LRR-R and Beta-actin-F/Beta-actin-R, respectively. The PCR was performed in a volume of 20 μl containing 10 μl 2× SYBR® Premix Ex Taq™ II (Tli RNaseH Plus), 0.4 μl ROX Reference Dye II (50×), 0.8 μl of 10 μM (each) LRR-F/LRR-R (or Beta-actin-F and Beta-actin-R) (Table 1), 2 μl SpLRR cDNA and 6.0 μl dH2O. The PCR program included 1 cycle of 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 31 s. Data from the quantitative real-time RT-PCR analysis were determined by the formula of 2^- Δ Δ Ct which the mean of Δ Δ Ct was [(treated crab SpLRR – treated crab Beta-actin)–(untreated crab SpLRR – untreated crab Beta-actin)], and subjected to the one-way analysis (one-way ANOVA) followed by an unpaired, two-tailed t-test.

3. Results

3.1. Cloning of the SpLRR gene from *S. paramamosain*

A full length cDNA of LRR in *S. paramamosain* (SpLRR) was amplified with RT-PCR after obtaining the sequences of the 5’- and 3’-end of SpLRR using RACE, which has been submitted to GenBank (GenBank Accession: JQ681527). Sequence analysis predicted that the full length cDNA of 2313 bp contains a 185 bp 5’ untranscribed region (UTR), a 1893 bp open reading frame (ORF) and a 235 bp 3’UTR, which includes a partial poly A-tail of 29 bp, and 5 potential N-linked glycosylation sites were also found (Fig. 1A). Alignment of the deduced 630 amino acids sequence with the LRRs consensus sequence identified 17 LRR domains (residues 171–612) (Fig. 1B), which were designed as a schematic diagram in Fig. 1C. Neither signal peptide nor transmembrane domain was found. In addition, the 3D structure of SpLRR predicted a horseshoe shape containing two of the five N-glycosylation sites. *Asn*^{70} and *Asn*^{332} in the convex face (Fig. 1D), which was further confirmed by the Ramachandran plot (Fig. 1E).

3.2. Phylogenetic analysis of SpLRR

A LRR phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis software (MEGA 5.0). The automatically generated phenogram showed that SpLRR and PmLRR (*Peneaus monodon*) were clustered into a clade (Fig. 2A). In addition, an amino acid sequence alignment between SpLRR and its cluster.

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**Table 1**

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<th>Primer</th>
<th>Sequence (5’–3’-3’)</th>
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<td>GCTACCGGCGCCCGAAGCTGCG</td>
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<tr>
<td>LRR-GSP3</td>
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<td>UPM (long)</td>
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<td>LRR-R</td>
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<tr>
<td>Beta-actin-R</td>
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* a The specific primers for 3’- and 5’- RACE were designed according to the *Scylla paramamosain* expressed sequence tags (EST), which were reported by Chen et al. [13] (GenBank accession: FJ774669).
members including CLRR (Camponotus floridanus), NvLRR (Nasonia vitripennis), HsLRR (Harpegnathos saltator), AeLRR (Acromyrmex echinatior) and PmLRR was shown in Fig. 2B. The identity rates ranged from 74.0% to 87.0%.

3.3. Expression of SpLRR mRNA

Expression of SpLRR mRNA was determined using RT-PCR. As shown in Fig. 3, a predominant band with an expected size of 127 bp was observed in examined tissues including heart, gill, stomach, intestine, muscle, and hemocytes, while not in hepatopancreas.

3.4. Responses of SpLRR to pathogens challenge

In order to test whether SpLRR respond to pathogen stimulation, a quantitative real-time RT-PCR assay was carried out to determine the transcript levels of SpLRR in hemocytes of S. paramamosain treated with V. alginolyticus, B. streptococcus or poly I:C. As shown in Fig. 4, after V. alginolyticus stimulation, the SpLRR mRNA level was increased by more than 100-fold compared to the control group.
increased significantly 3.3-fold at 48 h. Similarly, SpLRR transcripts in *B. streptococcus* and Poly I: C-treated group were increased to certain extent at different time points during the 72 h testing period.

4. Discussion

The LRR motif was first described in a human serum protein in 1985 [14]. Up to now, at least 60,000 LRR proteins have been identified from viruses, bacteria, archaea and eukaryotes [15]. In this study, a LRR homolog, SpLRR in crab *S. paramamosain*, was cloned and characterized (Fig. 1-A). SpLRR displayed a structural characteristic of the LRR family (Fig. 1-B), and could be clustered into one branch along with *PmLRR* in a phylogenetic tree (Fig. 2-A). In addition, consistent with the expression profiles of the *PmLRR* in *P. monodon* [16], the transcripts of SpLRR were found in multiple tissues except hepatopancreas of *S. paramamosain* (Fig. 3). Therefore, these results strongly suggest that SpLRR is a novel LRR homolog in crab.

LRR proteins contain several LRR motifs with a consensus “LxxLxLxxNxLx” sequence of about 20–30 amino acid residues in length [15]. Here, we found 17 LRR motifs “xxxLxxLxLxxNxLxxLx” located in SpLRR (Fig. 1-B, C). Similarly, 16, 13 and 11 tandem LRR motifs have been identified in *P. monodon*, *Manduca sexta* and *Toxoplasma gondii* LRR protein [16–18], respectively.

Furthermore, recent evidence indicates that the crystal structure of the LRR motif resembles a horseshoe-shaped structure with internal paired β-sheets and external helices [19]. In the tertiary structure, several ligand-binding sites locate on the concave surface of solenoid [20,21], suggesting that the region is involved in providing a versatile structural framework for protein–protein interactions [22]. In addition, glycosylation in the region may also influence receptor surface presentation, trafficking, and recognition [23,24]. Our analysis indicated that the 3D structure of SpLRR displayed a horseshoe shape with two of five N-glycosylation sites in its convex face (Fig. 1-D, E). Collectively, these findings suggest that SpLRR might participate in protein–protein interactions in crab. However, direct evidence for the binding of SpLRR to ligands is still missing.

Previous findings showed that LRR proteins play a crucial role in signal transduction and activation in defense responses [22,25,26]. Ng et al. reported that the human LRR protein LRSAM1 might act as a component of the antibacterial autophagic response [27]. Baxter et al. noted that two *Anopheles gambiae* LRR proteins, LRIM1 and APL1C, could form a heterodimer and regulate complement-like immunity [28]. Sriphaijit et al. suggested that the *P. monodon* LRR (*PmLRR*) was down-regulated after viral injection [16]. Thus, it is possible that SpLRR might be involved in crab immune response to pathogens.

To investigate the association of SpLRR with crab resistance to pathogenic infection, a quantitative real-time RT-PCR assay was carried out. As a result, SpLRR transcript levels were increased significantly after *V. alginolyticus*, *B. streptococcus* or Poly I: C treatment (Fig. 4). This is similar to the findings that *NtLRR1* (*Nicotiana tabacum* LRR1) and *NtLRR2* mRNA transcriptions are activated rapidly after infection of *Pseudomonas syringae pv. tabaci* or tobacco mosaic virus [29]. However, the data showed significant differences in comparison to that of *PmLRR* expression in YHV (yellow head virus, YHV)- or WSSV (white spot syndrome virus, WSSV)- treated shrimps *P. monodon* [16]. These differences may be
<table>
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<th>AeLRR</th>
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<th>NhLRR</th>
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Fig. 2. (continued)
SpLRR might also act as a signal-transducing receptor binding LPS hepatopancreas, gill, muscle, heart, hemocytes, stomach and intestine, respectively.

In conclusion, a novel LRR homolog in *Scylla paramamosain* (SpLRR) was cloned and characterized. To our knowledge, this is the first report showing that a LRR protein from crab, SpLRR, might be involved in crab resistance to pathogenic infection. Further investigation will be required to explore its exact role in the signaling pathway in crab immune responses. This research will be helpful in establishing strategies for disease control and the development of a sustainable crab aquaculture.

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