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Short communication

RNAi knock-down of shrimp *Litopenaeus vannamei* Toll gene and immune deficiency gene reveals their difference in regulating *antimicrobial peptides* transcription

Fujun Hou [a], Shulin He [a], Yongjie Liu [a], Xiaowen Zhu [b], Chengbo Sun [b], Xiaolin Liu [a,*]

[a] College of Animal Science and Technology, Northwest A&F University, Shaanxi Key Laboratory of Molecular Biology for Agriculture, Yangling 712100, China
[b] Fisheries College, Guangdong Ocean University, Guangdong 524088, China

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ABSTRACT

NF-κB dependent antimicrobial peptides (AMPs) are of critical importance in protecting insects or mammals from microorganisms infection. However, we still do not make clear signaling pathways in regulating AMPs expression in shrimps. In this study, RNAi approach was used to study differences between Toll signaling pathway and immune deficiency signaling pathway in regulating the transcription of NF-κB dependent *AMPs* post bacteria challenge. Results showed that the transcription level of *anti-lipopolysaccharide factor* was highly suppressed in *Litopenaeus vannamei immune deficiency* (*LvIMD*) silenced shrimps by gene specific dsRNA compared to *Litopenaeus vannamei Toll* (*LvToll*) silenced shrimps with or without *Vibrio anguillarum* and *Micrococcus lysodeikticus* challenge. Conversely the transcription level of *penaeidin3a* was significantly suppressed in *LvToll* silenced shrimps compared to *LvIMD* silenced shrimps. However, no obvious difference was found in regulating the transcription of *CrustinP*. Meanwhile, we found that silencing *LvToll* both down regulated the transcription of *Dorsal* and *Relish* while silencing *LvIMD* only down regulated the transcription of *Relish*. At last, shrimp survival experiment showed that post *V. anguillarum* challenge high mortality was found both in *LvToll* and *LvIMD* silenced groups while post *M. lysodeikticus* challenge we saw high mortality only in *LvToll* silenced group. Hence, we conclude that shrimp *L. vannamei* Toll pathway and IMD pathway might be different in regulating the transcription of NF-κB dependent *AMPs* and responding to bacteria challenge but not independent of each other.

1. Introduction

Penaeid shrimp cultivation is an important economic activity in the world since the early 1980s. However, the global shrimp farming industry has been severely affected by infectious diseases caused by virulent bacteria or viruses such as *Vibrio anguillarum, Vibrio harveyi* and WSSV et al. (Bachère et al., 1995; Gross et al., 2001). Thus, understanding shrimp immune responses to pathogens is essential for the robust shrimp aquaculture industry.

As other crustaceans, shrimps only rely on innate immunity to protect themselves from pathogen infection which includes cellular defenses (phagocytosis, encapsulation, nodule) and humoral defenses (such as the prophenoloxidase system, hemolymph clotting mechanism and the release of antimicrobial peptides) (Jiravanichpaisal et al., 2006; Young Lee and Söderhäll, 2002). Antimicrobial peptides (AMPs) are one of the major components of shrimp immune system and the major AMPs are represented by the three cationic peptide families: penaeidins, crustins, and anti-lipopolysaccharide factors. They have a broad range of antimicrobial activity against Gram-positive and Gram-negative bacteria, filamentous fungi and, in some cases, viruses and protozoa (Bachère, 2003). In *Drosophila*, seven distinct AMPs (plus isoform) have been identified and their expressions are controlled by different NF-κB pathways. Diptericin, Cecropin, Attacin and Drosocin against Gram-negative bacteria are regulated by immune deficiency (IMD) pathway, while Drosomycin against fungi are controlled by Toll pathway and Defensin as well as Metchnikowin are controlled by both IMD and Toll pathways (Hoffmann and Reichhart, 2002; Lemaitre et al., 1995, 1996). Recent studies mainly focus on identification and cloning of genes participating in the two pathways and their responses to bacteria or virus challenge (see review (Li and Xiang, 2013)). RNAi knock-down *LvToll* increased shrimp mortality post *V. harveyi* injection but not WSSV injection (Han-Ching Wang et al., 2010). Besides, the importance of shrimp AMPs for defending bacteria infection was also found (see review (Tassanakajon et al., 2011)). However, to date, there are no any reports on whether shrimp Toll pathway and IMD pathway regulate the transcription of shrimp AMPs. Here, we observed the transcription level of three shrimp *AMPs* in *Litopenaeus vannamei*

* Corresponding author. Tel.: +86 029 87054333; fax: +86 029 87092164.
E-mail address: liuxiaolin@nwsuaf.edu.cn (X. Liu).

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Toll (*LvToll*) silenced shrimps and *Litopenaeus vannamei immune deficiency* (*LvIMD*) silenced shrimps using sequence specific dsRNA. To explore the potential functional relationship between Toll pathway and IMD pathway, we also detected the transcription of *Dorsal* and *Relish* in *LvIMD* silenced shrimps and *LvToll* silenced shrimps. At last, shrimp survival experiment showed the difference between Toll pathway and IMD pathway in response to Gram-negative bacteria and Gram-positive bacteria challenge.

2. Materials and methods

2.1. Preparation of dsRNA and bacteria cultivation

Preparation of dsRNA for *LvToll, LvIMD* and EGFP was done following our previous study (Hou et al., 2013). Briefly, we designed specific primers with T7 RNA polymerase binding site at the 5′ terminus to obtain sense and anti-sense strand separately by PCR (Table 1). After PCR products were purified and quantified, Single stranded RNAs were transcribed in vitro with T7 RNase polymerase (New England Biolabs) according to manufacture's protocol. The single stranded RNAs were then mixed and annealed to get double stranded RNA following manufacture's protocol. After purification, dsRNAs were verified, quantified and stored at −80 °C.

Gram-negative bacteria *Vibrio anguillarum* (*V. anguillarum*) and Gram-positive bacteria *Micrococcus lysodeikticus* (*M. lysodeikticus*) were gifted by professor Fuhua Li. The method for bacteria preparation and quantification was same as previous study (Hou et al., 2013).

2.2. RNAi knock-down of LvToll and LvIMD in vivo followed by bacteria challenge

Shrimps (mean body weight 8–10 g) used for RNAi experiment were presented by Guolian shrimp base (Guangdong, China) and were reared in water tanks for 5 days before experiment. Three shrimps without any treatment were collected as control. 160 shrimps were divided into four groups, 40 shrimps intramuscularly injected with *LvToll* dsRNA (dsToll) (1.8 μg/g shrimp, 20 μl dissolved in PBS), 40 shrimps intramuscularly injected with *LvIMD* dsRNA (dsIMD) (1.8 μg/g shrimp, 20 μl dissolved in PBS), 40 shrimps intramuscularly injected with *EGFP* dsRNA (dsEGFP) (1.8 μg/g shrimp, 20 μl dissolved in PBS) and 40 shrimps for PBS (20 μl) injection. At 48 and 72 h post injection (hpi), three shrimps were respectively randomly sampled from dsToll, dsIMD group, dsEGFP and PBS group and used for gene silencing efficiency examination. Then, the dsToll group were equally divided into two groups for bacteria infection, one group for *V. anguillarum* (1.6×10^8 CFU/shrimp, 20 μl) challenge, and the other group for *M. lysodeikticus* (1.0×10^{10} CFU/shrimp, 20 μl) challenge. The same method for bacteria challenge was also applied in shrimps of dsIMD group, dsEGFP and PBS group. Post bacteria injection, three shrimps were randomly sampled at time points 4, 12 h. 1 ml injector was used for shrimp hemolymph collection, then the hemolymph was mixed with pre-cooled equal volume of modified Alsevier solution (MAS) anticoagulant. After centrifuged for 10 min. at 800*g*, 4 °C, the supernatant of hemolymph was removed and the hemocytes were used for total RNA extraction using Trizol reagent (Invitrogen) according to the manufacturer's protocol. PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa) was used for cDNA synthesis.

2.3. The examination of silencing efficiency of LvToll and LvIMD

Real time PCR was used to detect the transcription level of *LvToll* or *LvIMD* at 48, 72 h post dsRNA injection and 4, 12 h post bacteria infection. Shrimps with PBS and dsEGFP were used as negative control. Specific primers were designed for *LvToll* (GenBank No. DQ923424.1), *LvIMD* (GenBank No. FJ592176.1) and *L. vannamei 18s rRNA* (*18s*, GenBank No. AF186250) which was used as the internal control (Table 1). Methods used in real time PCR and data analysis were described before (Hou et al.,

Table 1
PCR primers used in this study.

Target gene name	Primer name	Sequence (5′–3′)
dsRNA preparation[a]		
LvToll	dsLvToll-F	aatctgcgtcggtggaa
	dsLvToll-R	gtcggtcacattgaggtagtc
	dsLvToll-T7F	ggatcctaatacgactcactatagggagaaatctgcgtcggtggaa
	dsLvToll-T7R	ggatcctaatacgactcactatagggagagtcggtcacattgaggtagtc
LvIMD	dsLvIMD-F	aagacagattcggctccac
	dsLvIMD-R	cagcgactcatcatctcgtac
	dsLvIMD-T7F	ggatcctaatacgactcactatagggagaaagacagattcggctccac
	dsLvIMD-T7R	ggatcctaatacgactcactatagggagacagcgactcatcatctcgtac
EGFP	dsEGFP-F	gtgcccatcctggtcgagct
	dsEGFP-R	tgcacgctgccgtcctcgat
	dsEGFP-T7F	ggatcctaatacgactcactatagggtgcccatcctggtcgagct
	dsEGFP-T7R	ggatcctaatacgactcactataggtgcacgctgccgtcctcgat
qPCR analysis		
LvToll	qToll-F	gaccatccctttacaccagact
	qToll-R	cctcgcacatccaggacttta
LvIMD	qIMD-F	cggctctgcggttcacat
	qIMD-R	cctcgaccttgtctcgttcct
18s rRNA	18s-F	aacgctcgtagtttgacttctgc
	18s-R	cacgaccattcgggctgta
Anti-lipopolysaccharide factor	ALF-F	cgcttcaccgtcaaaccttac
	ALF-R	gccaccgcttagcatcttgtt
Crustin	Crustin-F	ggtgttggtggtggtttccc
	Crustin-R	cagtcgcttgtgccagttcc
Penaeidin3a	Pen3-F	atacccaggccaccaccctt
	Pen3-R	tgacagcaacgccctaacc
Dorsal	Dorsal-F	gatggaatgatagaatgggaagc
	Dorsal-R	cactggtactcttgtctggtggtc
Relish	Relish-F	ctacattctgcccttgactctgg
	Relish-R	ggctggcaagtcgttctcg

[a] T7 RNA polymerase binding site is underlined.