Immunology Letters 206 (2019) 19–27

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

Identification and immune-related analysis of SNPs in *Litopenaeus vannamei* Toll3 receptor

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ARTICLE INFO

Keywords:
Litopenaeus vannamei
Toll3 receptors
SNPs
Immune response

ABSTRACT

Tolls and Toll-like receptors (TLRs), as innate immune-recognition receptors that recognize molecular patterns associated with microbial pathogens, play a critical role in antimicrobial immune responses. Here, we report on single nucleotide polymorphisms (SNPs) of *Litopenaeus vannamei* Toll3 (LvToll3). Multiple sequence alignment of the *L. vannamei* Toll3 Leucine rich repeat C-terminal domain (LvToll3-LRR-CT) with other *L. vannamei* Tolls LRR-CT domains showed 39.23% - 43.96% homology at the nucleic acid level and 20.31% - 30.00% identity at the amino acid level. Analysis of different shrimp tissues by polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) revealed that LvToll3-LRR-CT had genetic polymorphisms at both the genomic deoxyribonucleic acid (gDNA) and complementary deoxyribonucleic acid (cDNA) levels. Further, high-throughput sequencing analysis confirmed the presence of 8 non-synonymous SNP (nsSNP) and 1 nsSNPs with frequency greater than 1% at the gDNA level, while 13 nsSNPs and 2 nsSNPs with frequency greater than 1% at the cDNA level. *In silico* analysis revealed that the α-helix secondary structure and tertiary structure of LvToll3 changed when 3 SNPs (C2039T, T2041C, T2228C) were mutated. Interestingly, 2 novel bands on PCR-DGGE, which were identified as 2 nsSNPs (C2140A, T2186A) were observed following challenge with *Streptococcus iniae* but not with *Vibrio parahaemolyticus* or White spot syndrome virus (WSSV). Moreover, the secondary and tertiary structures of LvToll3 changed when the nsSNP T2186A was mutated. The present findings therefore provide novel insight into the molecular basis of shrimp innate immune response to pathogens through the generation of specific SNPs.

1. Introduction

Litopenaeus vannamei is the most important aquaculture shrimp species in the penaeid shrimp farming industry. However, in the past two decades the industry have had to deal with both infectious and non-infectious diseases thereby limiting productivity and consequently economic loses [1,2]. As invertebrates, shrimp do not have an adaptive immune system but relies mainly on its innate immunity to protect and defend itself against pathogens [3,4]. It is therefore of paramount importance to properly understand the innate immune system of shrimp in order to be able to develop and institute measures that would ensure the long term viability of the shrimp aquaculture industry.

Single nucleotide polymorphisms (SNPs), the most frequent type of genetic variation, are associated with diversity in the population, individuality, susceptibility to diseases, individual response to medicine, and can be used to identify common diseases and pharmacogenetic traits [5,6]. SNPs have therefore been used as a genetic marker for genome-wide association studies in various organisms, and have been applied in marker assisted selection of economic traits as well as resistance selective breeding in some aquatic species such as *Cyprinus carpio*, *Meretrix meretrix and Litopenaeus vannamei* [7–9]. Meanwhile, SNPs have been identified in several immune-related genes, and shown to affect the structure of the translated protein thereby altering the susceptibility and/or resistance of the host to pathogens infection. For instance, five SNPs were found in a fragment of L. *vannamei* Hsp70 gene after Taura syndrome virus (TSV) challenge, with the frequency of the 892C/T SNP genotypes significantly different between TSV-resistant and susceptible shrimp [10]. Similarly, polymorphism of the anti-lipopolysaccharide factor (ALF) has been reported to be related to WSSV-resistance in L. *vannamei*, with 18 SNPs found in a genomic fragment of

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https://doi.org/10.1016/j.imlet.2018.12.002
Received 12 September 2018; Received in revised form 15 November 2018; Accepted 10 December 2018
Available online 11 December 2018

397 bp located at the LPS-binding domain encoding sequence [11]. Our previous research also found that the C-terminus of L. *vannamei* hemocyanin possessed several SNPs related to shrimp resistance to different pathogens [12,13]. All these studies therefore suggest that SNPs play a key role in host resistance to pathogens.

Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) that have a unique and essential function in animal immunity, and comprise a family of type I transmembrane receptors [14,15]. So far, four types of Toll (LvToll 1, 2, 3 and 4) have been identified in L. *vannamei*, with LvToll1, LvToll2, and LvToll3 shown to play important roles in bacteria and virus infection [16,17]. A significant increase in shrimp mortality coupled with reduced bacterial clearance was observed in RNA interference (RNAi) of *L. vannamei* Toll (LvToll) followed by challenge with *Vibrio harveyi*, therefore suggesting that LvToll is an important factor in shrimp innate immune response to acute *V. harveyi* infection [18]. Notably, we previously identified SNPs in LvToll1 at the gDNA and cDNA level, and found that the genetic polymorphisms of LvToll1 was linked with the immune response to pathogen infections [19]. However, prior to this study, the genetic polymorphism of LvToll3 and how this relates to immune response had not been explored.In the present study, the genetic polymorphisms of LvToll3 and SNPs related to immune response were analyzed. The results revealed that the Leucine rich repeat C-terminal domain (LRR-CT) of LvToll3 had 8 and 13 non-synonymous single nucleotide polymorphisms (nsSNPs), at the gDNA and cDNA level, respectively. Most importantly, the amplified LvToll3-LRR-CT fragment generated 7, 9, 7 novel bands, when shrimps were challenged with *Streptococcus iniae, Vibrio parahaemolyticus* and WSSV, respectively. Two unique bands with 2 nsSNPs (C2140 A, T2186 A) were observed with *S. iniae* infection. Our findings here thus demonstrate that *L. vannamei* LvToll3 has genetic polymorphism that is linked with different pathogens infection.

2. Materials and methods

2.1. Experimental shrimps

Healthy shrimp (*L. vannamei*), weighting 8–10 g were obtained from Shantou Huaxun Aquatic Product Corporation (Shantou, China) and then cultured in laboratory tanks filled with aerated seawater of salinity 1 ppm at 25 °C. Shrimps were acclimatized for 3 days before they were used for the experiments, during which time they were fed commercial feed once daily. All animal experiments were carried out in accordance with the guidelines and approval of the Animal Research and Ethics Committees of Shantou University, China.

2.2. Bioinformatics analysis

The sequence of LvToll3 (Genbank accession number AEK86517.1) was obtained from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). The conserved domains of shrimp Toll receptors were analyzed with the Simple Modular Architecture Research Tool (SMART) program (http://smart.embl-heidelberg.de/). Multiple sequence alignment was performed using the DNAMAN program (version 6.0.3.99). The effect of non-synonymous SNP (nsSNP) on protein function was predicted using the Scale-Invariant Feature Transform (SIFT) algorithm (http://sift.jcvi.org/). The NPS@ server (https://npsa-prabi.ibcp.fr/) and SWISS-MODEL (https://www.swissmodel.expasy.org/) were used to predict the secondary and tertiary structure change after LvToll3 SNPs mutation, respectively.

2.3. Total RNA extraction, cDNA synthesis and gDNA extraction

Hemolymph from three individual shrimp was collected as previously described [20] via the pericardial sinus with a sterile needle and syringe into an equal volume of pre-chilled anti-coagulant buffer (27 mM sodium citrate, 9 mM EDTA, 115 mM glucose, 336 mM NaCl, pH 7.0), one tube per each shrimp. Hemocytes were harvested by centrifugation at 800 *g* for 10 min at 4 °C for immediate use while the hepatopancreas, stomach and gills tissues from each of the individual shrimp were collected, snap frozen in liquid nitrogen and then ground into fine powder before nucleic acid extraction. Total RNA was extracted using the RNAFAST 200 kit (FeiJie, China) according to the manufacturer's instruction. Extracted RNA was quantified using NanoDrop 2000 spectrophotometer (Nano-drop Technologies, Wilmington, DE) while the quality of the RNA was checked on 1% agarose gel electrophoresis. cDNA samples were synthesized using the PrimeScript TM RT reagent kit (TaKaRa, Japan), following the manufacturer's protocol. Genomic DNA (gDNA) was extracted using the marine animals DNA extraction kit (TiansGen, Beijing, China) as described by the manufacturer's protocol. The total RNA, cDNA and gDNA were used immediately or stored at −80 °C for later use.

2.4. Cloning of LvToll3-LRR-CT DNA and cDNA

To clone the LvToll3-LRR-CT fragment which spanning nucleic acid 1993–2280 bp (amino acids 665–760), PCR was carried out with template DNA or cDNA obtained in subsection 2.3 above. Gene specific primers LvToll3-LRR-CT-GC-F and LvToll3-LRR-CT-R (Table 1) designed by Primer Premier5 software according to the obtained sequence and a 40-bp GC-clamp added to the 5′-end of the LvToll3-LRR-CT-GC-F primer to avoid complete denaturing of the amplified products was used. The PCR reaction system contained 20 u L 2 × PCR Mix (GenStar, Beijing, China), 2 u L DNA or cDNA template, 1 u L primers (forward and reverse, respectively) and ddH_2O to a total volume of 40 u L. The PCR program was as follows: one cycle at 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s and one cycle at 72 °C for 10 min. The quality of the PCR products was checked on 2% agarose gel electrophoresis, bands excised and purified using the EasyPure Quick Gel Extraction Kit (TransGen, Beijing, China) according to the manufacturer's instruction.

2.5. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis

The PCR-DGGE analysis was performed as described previously [19]. Briefly, the PCR products from subsection 2.4 above were resolved on 8% polyacrylamide gel with urea formamide denaturing gradient of 30% – 50%. The gels were run in 1 × TAE buffer (2 M Tris-ethanoic acid and 100 mM EDTA, pH 8.5) using the DGGE-2001 system

Table 1
The primers used in this study.

Primers name	Sequence (5'-3')
For PCR-DGGE	
LvToll3-LRR-CT-GC-F	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGGTGCCCGTCGGAAAGTC
LvToll3-LRR-CT-R	GCACGTCATCTCGCAATCA
For the PCR and high-throughput sequencing	
LvToll3-LRR-CT-F	GTGCCCGTCGGAAAGTC
LvToll3-LRR-CT-R	GCACGTCATCTCGCAATCA

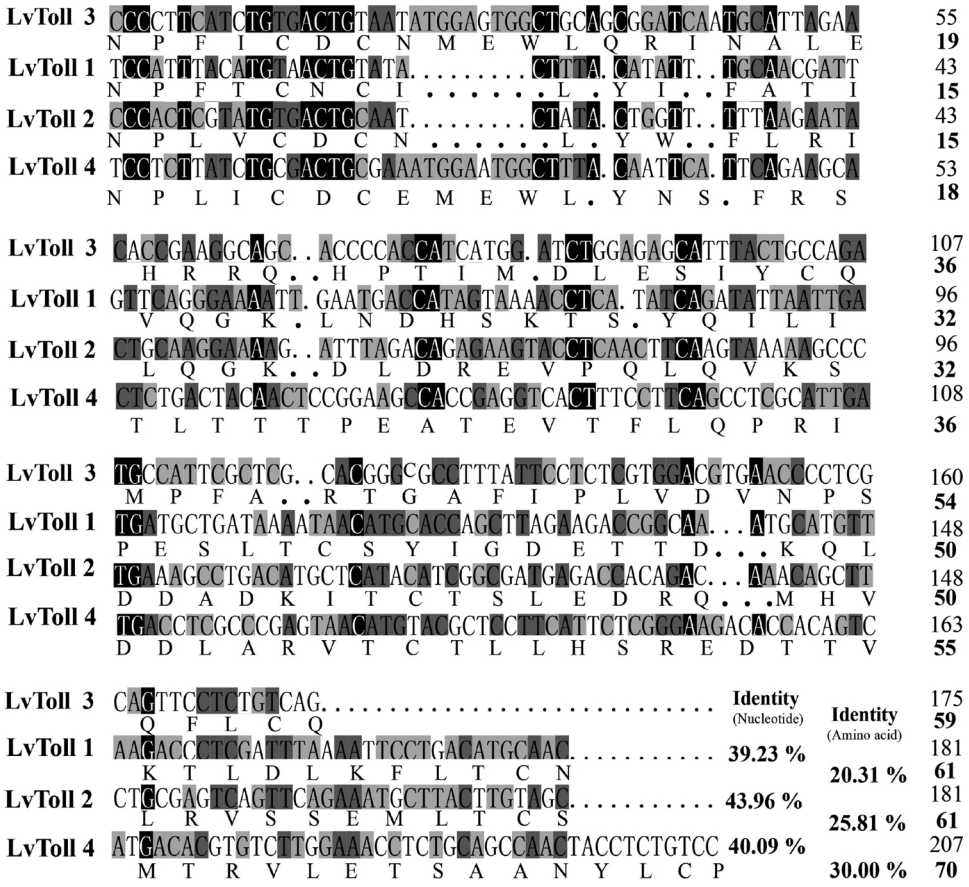


Fig. 1. Sequence analysis of the LvToll3-LRR-CT domain. LRR-CT domain sequences (amino acids 679–737) of *Litopenaeus vannamei* Toll3 (GenBank: AEK86517.1), LRR-CT domain sequences (amino acids 469–529) of *Litopenaeus vannamei* Toll1 (GenBank: ABK58729.1), LRR-CT domain sequences (amino acids 551–611) of *Litopenaeus vannamei* Toll2 (GenBank: AEK86516.1) and LRR-CT domain sequences (amino acids 749–818) of *Litopenaeus vannamei* LvToll4 (GenBank: AKF17613.1) were obtained from NCBI. The LRR-CT domains were predicted using the SMART program and the multiple sequence alignment was performed using the DNAMAN program (version 6.0.3.99). The LvToll3-LRR-CT multiple sequence alignment at nucleic acid level with percentage identity of 39.23%, 43.96%, and 40.09% compared with LvToll1, LvToll2 and LvToll4, respectively, and amino acid level with percentage identity of 20.31%, 25.81% and 30.00%, compared with LvToll1, LvToll2 and LvToll4, respectively. The one letter amino acid sequence of LvToll1, LvToll2, LvToll3 and LvToll4 are shown below, respectively.

(CBS Scientific, USA) at 120 V and 60 °C for 13 h. After the electrophoresis, gels were stained with GelRed® nucleic acid gel stain (Biotium, USA) for 30 min and visualized using a Gel Doc XR system (Bio-Rad, USA). The results of the DGGE were quantified using the Quantity One 4.6.2 program.

2.6. High-throughput sequencing

The LvToll3-LRR-CT fragments from the cDNA and gDNA of three individual shrimp gills were obtained as described above in subsection 2.3. The concentration and quality of PCR products were estimated using a Qubit Fluoromerer (Invitrogen, USA) and 1% agarose gel electrophoresis, respectively. To construct the cDNA and gDNA libraries for high-throughput sequencing, the PCR products were fragmented by ultra-sonication followed by the addition of an adaptor at the 5′ and 3′-terminus. Next, the libraries were submitted to Beijing Genomics Institute (BGI, Shenzhen, China) for paired-end sequencing. Adaptors and low-quality reads were removed from the raw reads, after which the clean reads were assembled, and the resulting sequences subjected to SNPs analysis by multiple sequence alignment using Clustal X according to previous reports [21].

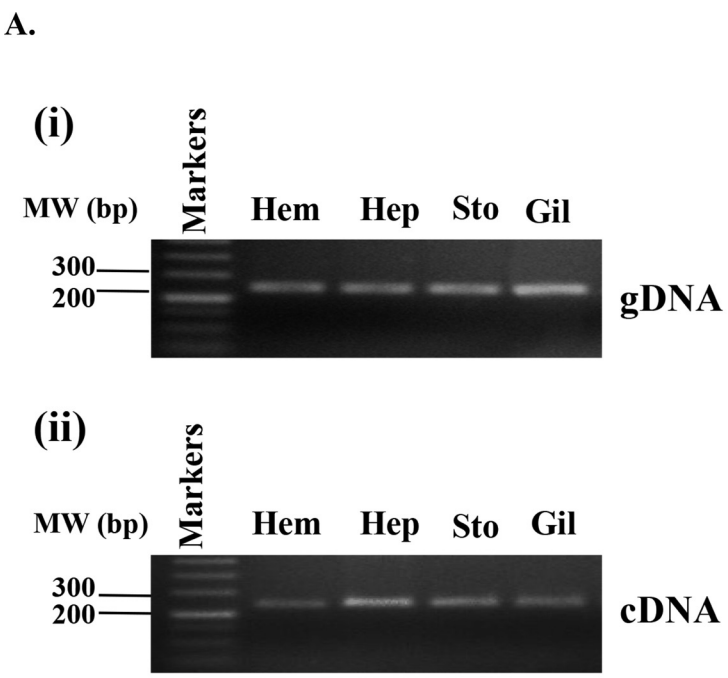
2.7. SNPs profiles of LvToll3 following pathogen challenge

For the challenge experiments, white spot syndrome virus (WSSV), the beta-hemolytic Gram-positive bacteria *S. iniae* [22] and the Gram-negative bacteria *V. parahaemolyticus* were used. Both *S. iniae* and *V. parahaemolyticus* were prepared as described previously [23] and then suspended in 0.85% saline at concentrations of 1×10^7 CFU/mL and 2×10^5 CFU/mL, respectively. The WSSV stock used in this study was prepared from WSSV-infected crayfish as described previously [24], and then diluted to 3×10^4 copy/mL with 0.85% saline before challenge experiments. Eighty randomly selected pre-acclimatized shrimp divided into 4 groups (20 shrimp per group), were each intramuscularly injected at the second abdominal segment with 50 μL of the corresponding pathogen prepared above or with an equivalent volume of saline as control. At 0, 3, 6, 9, 12 and 24 h post injection, gills from three individual shrimp were collected into separate tubes for RNA extraction and cDNA synthesis. The synthesized cDNA was then used for PCR-DGGE analysis as described in subsection 2.5 above. Following this, the differential bands on the PCR-DGGE gels were excised and eluted with 50 μL deionized water overnight at 4 °C as reported previously [25]. Finally, the eluted samples were used for re-amplification with the primers Lv-Toll3-LRR-CT-F/R (Table 1). The PCR products were cloned and sequenced as described previously [13], after which the obtained sequences were used for SNPs detection by multiple sequence alignment as described in subsection 2.2 above. The results of DGGE were quantified using the Quantity One 4.6.2 program. Experiments were repeated at least three times with different batches of shrimp from the same aquaculture farm and cultured under the same laboratory conditions.

3. Results

3.1. Identification of SNPs in the variable region of LvToll3

To identify the variable regions of LvToll3, we analyzed the LvToll3-LRR-CT domain, as it has previously been reported that the LvToll1-LRR-CT domain was the site of potential SNPs [19]. The LRR-CT domain of LvToll3 spans amino acids 679–737 and the scope of our cloning in this study was from amino acids 665-760. Multiple sequence alignment based on the amino acids sequence of the LRR-CT domains of LvToll3 (GenBank: AEK86517.1), LvToll1 (GenBank: ABK58729.1), LvToll2 (GenBank: AEK86516.1) and LvToll4 (GenBank: AKF17613.1)



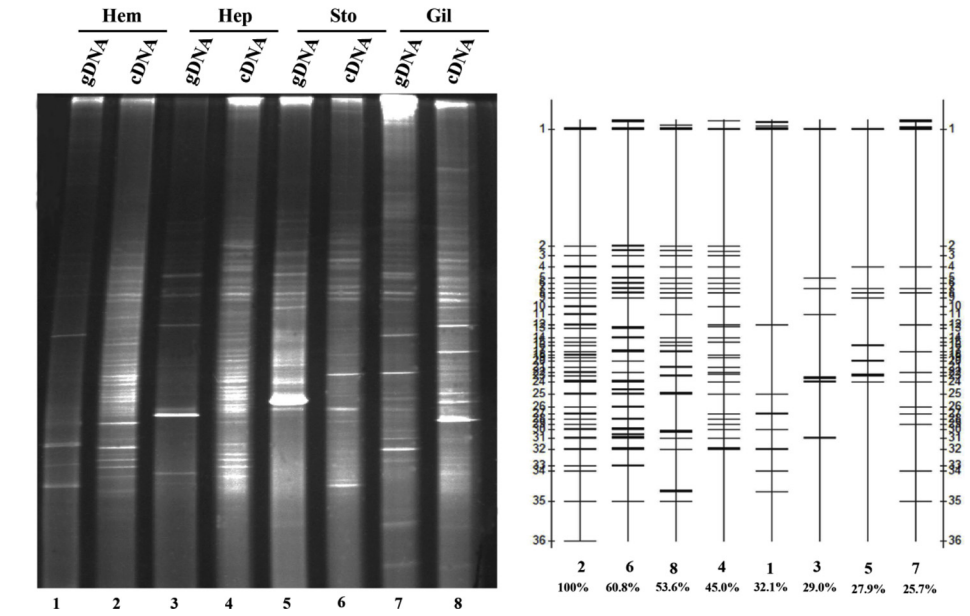


Fig. 2. PCR-DGGE analysis of the SNPs in LvToll3 LRR-CT. (A) PCR amplification of the LvToll3 spanning nucleic acid 1993–2280 bp (amino acids 665–760). The gDNA and cDNA from three individual shrimp hemocytes, hepatopancreas, stomach and gill tissues were prepared and then amplified by PCR. (B) DGGE analysis of the LvToll3 LRR-CT fragments in shrimp hemocytes, hepatopancreas, stomach and gill. The PCR products of LvToll3 LRR-CT were purified and then resolved on 8% polyacrylamide gel with denaturing gradient. The lanes are labeled at the bottom 1 to 8 from left to right. The DGGE results (bands) were quantified using the Quantity One 4.6.2 program. Hem: hemocytes, Hep: hepatopancreas, Sto: stomach and Gil: gill. The data shown represent one of three similar biological replicates.

was performed using the DNAMAN program (version 6.0.3.99). The results revealed that at the nucleic acid level, LvToll3-LRR-CT shared 39.23% identity with LvToll1-LRR-CT, 43.96% with LvToll2-LRR-CT and 40.09% with LvToll4-LRR-CT, while at the amino acid level it shared an identity of 20.31% with LvToll1-LRR-CT, 25.81% with LvToll2-LRR-CT and 30.00% with LvToll4-LRR-CT (Fig. 1). A comparison of the main structural domains of LvToll3, LvToll1, LvToll2 and LvToll4 is shown in Supplementary Fig.S1. These results therefore show that the LvToll3-LRR-CT domain has low homology among the Toll like receptors of *L. vannamei.*

3.2. PCR-DGGE analysis of the SNPs in the LvToll3-LRR-CT domain

The LvToll3-LRR-CT fragment was amplified by PCR using specific primers (Table 1) with gDNA and cDNA obtained from four shrimp tissues including hemocytes, hepatopancreas, stomach and gills used as templates. As shown in Fig. 2A, a specific band with size about 288 bp was obtained from both the gDNA and cDNA samples, which was consistent with the theoretical predicted size of LvToll3-LRR-CT. The results suggest that LvToll3-LRR-CT could be amplified from both gDNA and cDNA samples therefore implying that LvToll3-LRR-CT was located in an exon region. Next, when the PCR products were analyzed by DGGE, the results showed that LvToll3-LRR-CT generated 36, 29, 27 and 24 bands in hemocytes, hepatopancreas, stomach and gill,

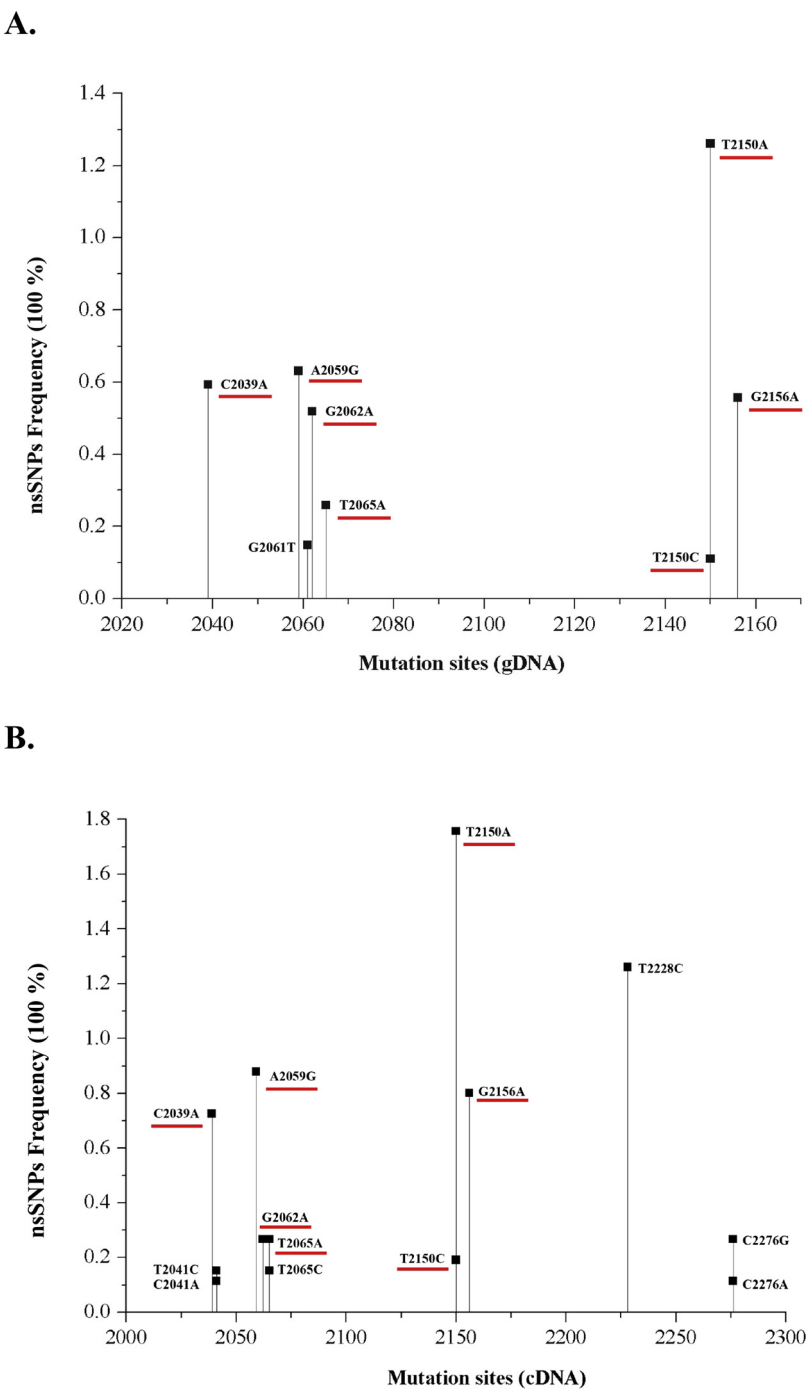


Fig. 3. High-throughput sequencing analysis of the nsSNPs in LvToll3 LRR-CT. Mutation sites and frequency at the gDNA level (A), cDNA level (B). The mutation site at gDNA level is T2150A, while the mutation sites at the cDNA level are T2150A and T2228C with are high frequency more than 1%. There were 7 common SNPs (C2039A, A2059G, T2050A, G2062A, T2065A, T2150C, G2156A) at both the gDNA and cDNA levels, which are underlined in red. The data shown represent one of three similar biological replicates (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

respectively (Fig. 2B). This data thus suggest that LvToll3-LRR-CT had molecular polymorphisms at both the gDNA and cDNA levels.

3.3. Identification of SNPs in LvToll3 by high-throughput sequencing

To further examine and identify the SNPs in LvToll3-LRR-CT, high-throughput sequencing was carried out using samples from the gills of a single shrimp. Gill samples were used because gill is considered an important immune organ in shrimp and the response of LvToll3 in gill was significantly increased upon WSSV infection [17], couple with the fact that it is much easier to collect enough gill tissue samples from a single shrimp for simultaneous extraction of RNA and DNA. After the PCR amplification of the LvToll3-LRR-CT fragment using gDNA and cDNA templates from gills, the samples were then subjected to high-throughput sequencing on the Illumina platform. The results of the high-throughput sequencing revealed that LvToll3-LRR-CT had 8 nsSNPs at the gDNA level, of which, 7 nsSNPs had a frequency ranging from 0.11% to 1.26%, while 1 nsSNP (T2150 A) possessed a high frequency of over 1% (Fig. 3A). In addition, a total of 13 nsSNPs were detected in LvToll3-LRR-CT at the cDNA level, of which, 11 nsSNPs had a frequency between 0.11% and 1.76%, while 2 nsSNPs (T2150 A and T2228C) showed a high frequency of over 1% (Fig. 3B). Further

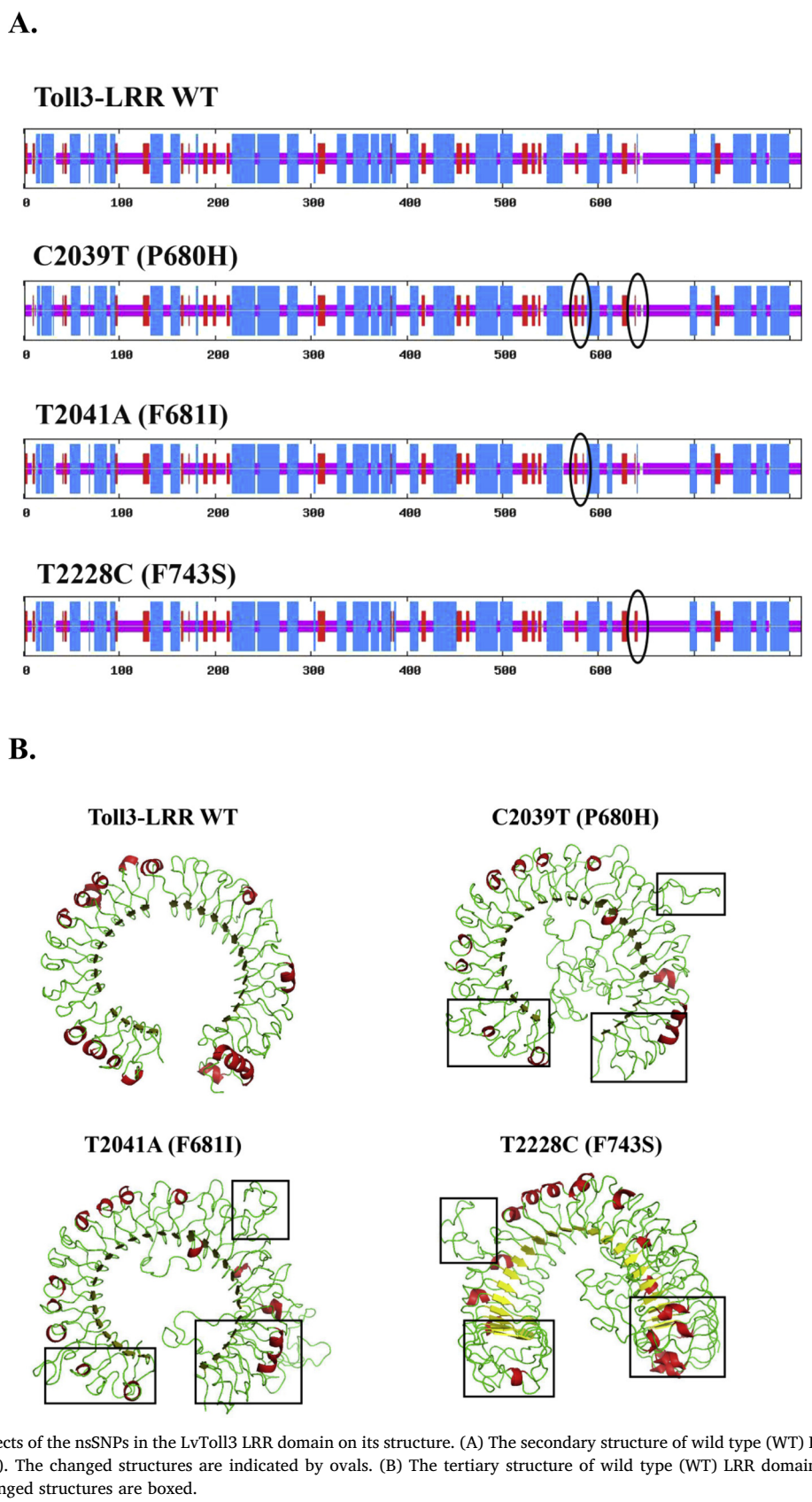


Fig. 4. Prediction of the effects of the nsSNPs in the LvToll3 LRR domain on its structure. (A) The secondary structure of wild type (WT) LRR domain and its mutants (C2039T, T2041A, T2228C). The changed structures are indicated by ovals. (B) The tertiary structure of wild type (WT) LRR domain and its mutants (C2039T, T2041A, T2228C). The changed structures are boxed.

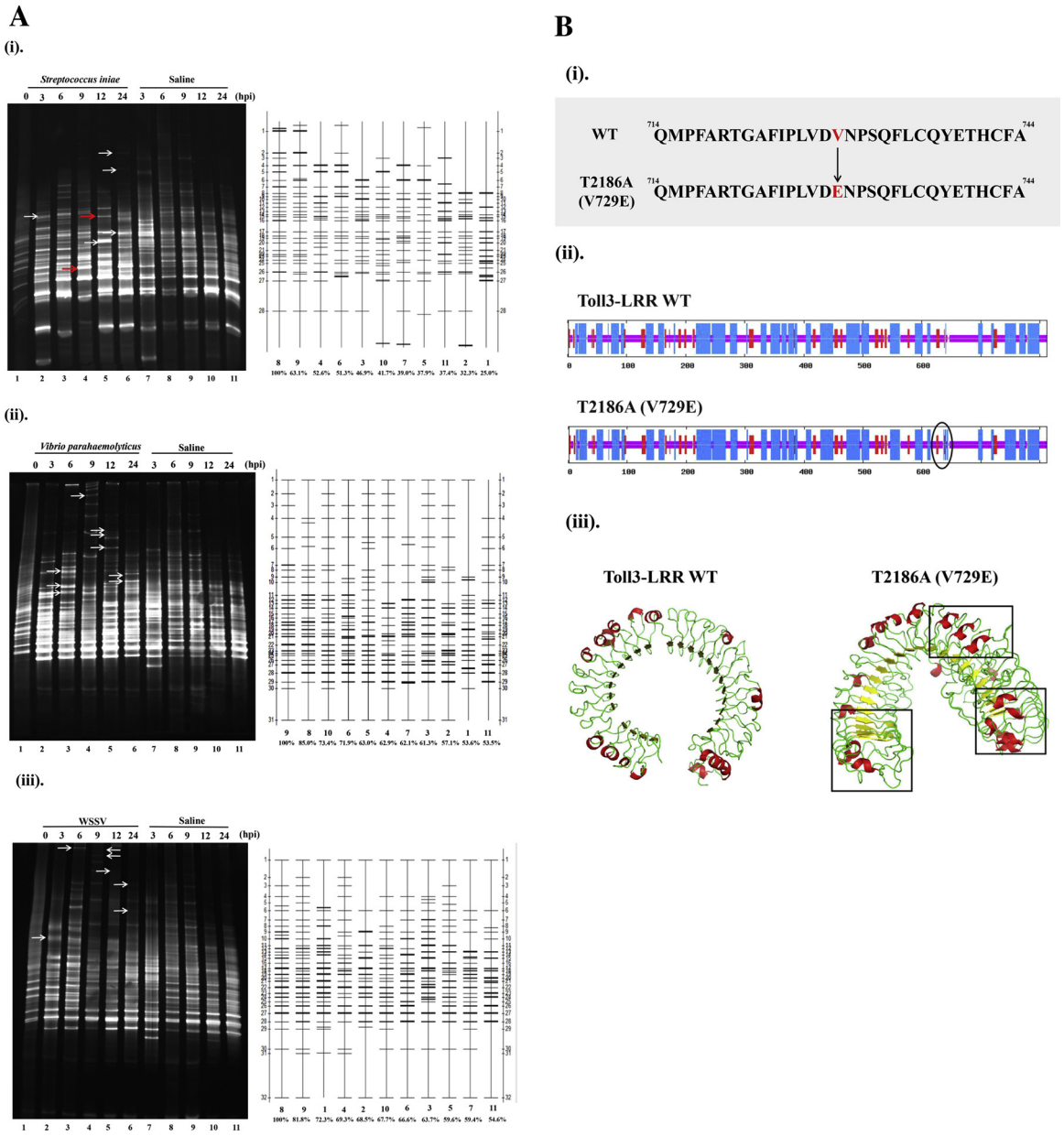


Fig. 5. Analysis of the LvToll3 SNPs related with immune response to pathogen infection. Shrimps were injected with *Streptococcus iniae* (A (i)), *Vibrio parahaemolyticus* (A (ii)), WSSV (A (iii)), and normal saline used as control. Shrimp gill samples collected at 0, 3, 6, 9, 12 and 24 h post infection were used for analysis. The novel bands are marked with arrows, with two prominent bands marked with red arrows while the other bands are marked with white arrows. Quantification of the bands in the figures revealed that there were 3, 1, 3, 2 novel bands at the 6, 9, 12, 24 h post injection. The gel lanes are labeled from 1 to 8 from left to right. The DGGE results were quantified using the Quantity One 4.6.2 program. B (i) Amino acid substitutions in LvToll3 when the sequence was mutated (T2186 A). B (ii) Secondary structure of WT LvToll3 LRR and mutant LvToll3, B (iii) Tertiary structure of WT and mutant LvToll3 LRR. The changed secondary structures are indicated by an oval while the changed tertiary structures are boxed. The data shown represent one of three similar biological replicates (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

analysis showed that 7 nsSNPs (C2039 A, A2059 G, T2050 A, G2062 A, T2065 A, T2150C and G2156 A) were common at both the gDNA and cDNA levels.

3.4. SNPs affect the structure of LvToll3 LRR

In order to analyze the effects of the identified nsSNPs on the function of LvToll3, bioinformatics tools (the NPS@ server and the SWISS-MODEL) were used to analyze the secondary and tertiary structures of LvToll3. The *in silico* results showed that the secondary structure of wild type (WT) LvToll3, which is α-helix (Fig. 4A) and the tertiary structure (Fig. 4B) changed when 3 nsSNPs (C2039T, T2041A and T2228C) were mutated.

3.5. SNPs in LvToll3 are a response to pathogens infection

In order to determine how the SNPs of LvToll3 were related with immune response to pathogen infection, shrimps were injected with various pathogens (*S. iniae*, *V. parahaemolyticus*, and WSSV). The SNPs profiles at the cDNA level from gills extracted at different time-points post infection were analyzed using PCR-DGGE. As shown in Fig. 5A (i), the amplified LvToll3-LRR-CT fragment generated 1, 1, 2, 3 novel bands (marked with arrows) at 3, 9, 12, 24 hpi, respectively, from samples challenged with *S. iniae* compared with control (Saline group). In

particular, two prominent bands at 9 h and 12 h (marked with red arrows) were observed. Quantification of the bands in the Fig. 5A (ii) revealed that there were 3, 1, 3, 2 novel bands (indicated with white arrows) at the 6, 9, 12, 24 hpi, respectively in the *V. parahaemolyticus* samples compared with the Saline group. On the other hand, the WSSV challenged samples generated 1, 1, 2, 1, 2 new bands at 3, 6, 9, 12, 24 hpi, respectively compared with control (indicated with white arrows in Fig. 5A(iii)). Next, the 2 bands, which are marked with red arrows in the *S. iniae* gels were excised and used for gene cloning and sequencing. The results from this indicated that these bands had 2 nsSNPs (C2140 A, T2186 A). Next, bioinformatics analysis of the effects of these 2 nsSNPs on the secondary and tertiary structure of LvToll3 was carried out. As shown in Fig. 5B (i), the results revealed an amino acid substitutions in the TLRs when the sequence was mutated (T2186 A). Moreover, the secondary (Fig. 5B (ii)) and tertiary structures (Fig. 5B (iii)) also changed when the nsSNPs (T2186 A) was mutated. However, the SNPs (C2140 A) did not affect the structure of LvToll3 (Fig. S2). These results thus suggest that the SNPs in LvToll3 could be related to immune response to various pathogens.

4. Discussion

Tolls and Toll-like receptors (TLRs) are evolutionarily conserved from insects to mammals, with an increased number of genes encoding TLR or TLR-like molecules identified in different species [26]. The discovery of TLRs as components of the immune system that recognize conserved structures in pathogens has greatly advanced our understanding of how invading pathogens are sensed, thereby triggering innate immune responses and primes antigen-specific adaptive immunity [27]. Recently, many studies have shown that Tolls/TLRs from various species have SNPs, which are associated with immune response [28,29], and therefore play an important role in host resistance to pathogens [19,30]. Particularly, SNPs have mostly been found in the Tolls/TLRs LRR domains of different species [31,32]; domains which provide an important structural framework for molecular interactions, and also for PAMP recognition by TLRs and NLRs [33]. Although SNPs in other Tolls/TLRs have been studied, the genetic polymorphisms of LvToll3, which has an extracellular domain containing 23 LRRs, and how these SNPs changed in response to infections have not been explored.

Our previous study revealed that the LRR-CT domain of LvToll1 has the lowest sequence homology compared to the LRR domain of other species and that this domain also has SNPs [19]. In the present study, analysis of LvToll3-LRR-CT revealed that it also had low homology compared to other Tolls of L. *vannamei* in terms of nucleic acids and amino acids (Fig.1), therefore suggesting that the LvToll3-LRR-CT domain might also have SNPs. The results from PCR-DGGE analysis using different shrimp tissue samples revealed that indeed LvToll3-LRR-CT had genetic polymorphisms at both the gDNA and cDNA levels (Fig. 2B). Moreover, high-throughput sequencing analysis further confirmed the presence of 8, 13 nsSNPs at the gDNA and cDNA level, respectively (Fig. 3). Since our previous studies had shown that LvToll1 had a total of 145 nsSNPs at the gDNA level and 38 nsSNPs at the cDNA level [19], it seems to suggest that the number and types of SNPs in L. *vannamei* Tolls differed. Given that SNPs can affect the structure of genes and therefore their function [34,35], we went about to explore if the SNPs in LvToll3 affected its structure. Using bioinformatics structural prediction, it was revealed that the secondary α-helix structure of LvToll3 and its tertiary structure (Fig. 4) changed when the 3 nsSNPs (C2039 T), (T2041 A), (T2228C) were mutated. Taken together, the data thus far suggest that the SNPs of LvToll3 might play a key role during shrimp immune response against pathogens.

Since polymorphism of the TLR gene has a considerable role in disease susceptibility [36,37], with SNPs in Tolls/TLRs implicated in immune response [38,39], we sought to further explore the SNPs of LvToll3 that might be related with immune response to pathogen infection. In shrimps challenged with *S. iniae*, *V. parahaemolyticus* and WSSV, the amplified LvToll3-LRR-CT fragment generated 7, 9, 7 novel bands, respectively. Among these novel bands, 2 prominent novel bands observed in the samples from the *S. iniae* challenged shrimps, had 2 nsSNPs, C2140 A and T2186 A. Importantly, the secondary and tertiary structures of LvToll3 changed when the SNP T2186 A was mutated by *in silico* analysis (Fig. 5). These observations seem to suggest that LvToll3 might play a pivotal role in the host immune response to *S. iniae* infection. This is synonymous with our previous study, where the SNPs in LvToll1 following challenge with *S. iniae*, *V. parahaemolyticus* and WSSV significantly changed the SNPs profiles, giving rise to 6, 4 and 4 novel bands, respectively [20]. Meanwhile, it has been reported that two sets of SNPs appeared in the Toll gene of *Scylla paramamosain* (SpToll) when crabs were challenged with *V. parahemolyticus* and that the c.1372 A > G mutation contributed to a low mortality after *V. parahemolyticus* infection [40]. It thus seems to suggest that different SNPs are generated in LvToll3 and LvToll1 in immune response to the same pathogen in L. *vannamei*. This phenomenon might be considered a type of 'simple specific innate immunity' [41,42], which is synonymous with adaptive immunity, as the adaptive immune response recognizes specific molecular structures on pathogens to generate large numbers of antigen receptors, i.e., T-cell receptors (TCRs) and immunoglobulins, by somatic rearrangement [43].

In conclusion, the current study demonstrated that LvToll3 had multiple SNPs, and that these genetic polymorphisms might be closely related to the shrimp innate immunity. It further suggests that different Tolls, such as LvToll1 and LvToll3, have different genetic polymorphisms, and therefore play different immunological functions in shrimp challenged with the same or similar pathogens.

Acknowledgments

This work was funded by the National Natural Science Foundation of China (No. 31672689 & 31872596), Natural Science Foundation of Guangdong Province (No. 2017A030311032) and Department of Education of Guangdong Province (No. 2017KZDXM033).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2018.12.002.

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