Fish & Shellfish Immunology 32 (2012) 1117–1123

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

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

Association of *Cf*LGBP gene polymorphism with disease susceptibility/resistance of Zhikong scallop (*Chlamys farreri*) to *Listonella anguillarum*

Vinu S. Siva [a,b], Chuanyan Yang [a,b], Jialong Yang [a,b], Lingling Wang [a,*], Leilei Wang [a,b], Zhi Zhou [a,b], Limei Qiu [a], Linsheng Song [a,*]

[a] Key laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, 7 Nanhai Rd., Qingdao 266071, China
[b] Graduate University of Chinese Academy of Sciences, Beijing 100049, China

ARTICLE INFO

Article history:
Received 16 February 2012
Received in revised form
15 March 2012
Accepted 15 March 2012
Available online 24 March 2012

Keywords:
Chlamys farreri
Lipopolysaccharide and β-1
3-glucan binding protein
Single nucleotide polymorphism
Disease resistance
Disease susceptibility

ABSTRACT

Lipopolysaccharide and β-1, 3-glucan binding protein (LGBP) is a pattern recognition receptor (PRR) recognizing and binding both LPS and β-1, 3-glucan, playing important roles in innate immunity. In the present study, the single nucleotide polymorphisms (SNPs) were assessed in LGBP gene from scallop *Chlamys farreri* (designated *Cf*LGBP), and eight SNPs were found in its potential LPS and glucanase binding motif. The locus +7679 with the transition of G-A, which produced an amino acid substitution at codon 360 from a non polar Glycine to polar Serine, was selected to inspect their association with disease resistance/susceptibility to *Listonella anguillarum*. Three genotypes G/G, G/A and A/A, were revealed at locus +7679, and their frequencies were 89.7%, 7.7% and 2.6% in the resistant stock, while 63.2%, 34.2% and 2.6% in the susceptible stock, respectively. The frequency of genotypes G/G and G/A were significantly different ($P < 0.05$) between the two stocks. The pathogen-associated molecular patterns (PAMP) binding activity of two recombinant proteins, r*Cf*LGBP (G) with G variant at locus +7679 and r*Cf*LGBP (S) with A variant at locus +7679, were elucidated by ELISA assay. The binding affinities of both LPS and β-glucan binding affinity were varied in a dose-dependent manner, where the binding affinity of r*Cf*LGBP (G) was significantly higher than that of r*Cf*LGBP (S) ($P < 0.05$). The results collectively suggested that the polymorphism of +7679 G/G in *Cf*LGBP possibly enhances the binding activity of LPS and β-glucan, and was associated to disease resistance of scallop against *L. anguillarum*, which could be a potential marker applied in future selection of scallop with enhanced resistance to *L. anguillarum*.

1. Introduction

Innate immune system is the first line of defense against various pathogens for both invertebrates and vertebrates, and its recognition mainly relies on germ-line-encoded pattern recognition receptors (PRRs). These PRRs evolved to recognize pathogen and conserved metabolic products of pathogen, and this specific recognition permits the host immune system to distinguish self and non-self molecular discrimination [1]. In general, PRRs also called pattern recognition proteins (PRPs) recognize common epitopes of pathogen consisting carbohydrate moieties [2,3], and the major PRPs include Lipopolysacharide- and beta, 1-3-glucan-binding proteins (LGBPs) [4], Peptidoglycan binding proteins (PGRPs) [5], β-1, 3-glucan binding proteins (BGBP) [6], gram-negative binding proteins (GNBPs) [7] and so on. Among them, LGBP can recognize and bind LPS and beta-1, 3-glucan, and plays a crucial role as PRRs in innate immunity.

LGBP, also known as Gram-negative Binding Protein (GNBP), was firstly identified from silkworm *Bombyx mori* [8] as one of the constitutive, inducible acute-phase proteins for pathogen interaction. It has been considered as a significant molecule responsible for recognition of invaders and induction of downstream innate immune response including activation of proPO cascade [9,10], opsonization [11–13], agglutination [14] and induction of antimicrobial peptides [7]. By now, several LGBP genes have been characterized in invertebrates especially in crayfish, earthworms, sea urchins, insects and crustaceans [7,10,15–20]. In our previous study, one LGBP was identified from scallop *Chlamys farreri* (designated *Cf*LGBP), and it could recognize and bind not only LPS and β-glucan, but also PGN. *Cf*LGBP also exhibited obvious agglutination activity toward Gram-negative, Gram-positive bacteria and fungi, acted as an important molecule in innate immunity of scallop [4,14].

* Corresponding authors. Tel.: +86 532 82898552; fax: +86 532 82880645.
E-mail addresses: wanglingling@ms.qdio.ac.cn (L. Wang), lshsong@ms.qdio.ac.cn (L. Song).

1050-4648/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2012.03.017

The polymorphisms in PRRs and their association with disease resistance have been reported in human [21–23], and also in some other animals including pig, cattle, chicken [24–26]. However, the information about gene polymorphism and its association with immune phenotype are still quite inadequate in mollusk. Because of its significance in immunity, LGBP is one of the handy candidate genes to disclose immunological information as well as for polymorphism analysis and association studies. For example, the mutation variants in *Drosophila* GNBP 1, 3 genes affected Toll pathway considerably, and associated with susceptibility toward bacteria and fungi [27]. But to our knowledge, there is no report about the association of polymorphism and disease resistance on mollusk LGBP gene.

Understanding the phenotypic consequences of SNPs for immunologically important PRRs like *Cf*LGBP is an urgent concern because of the increasing severity of disease problems in Zhikong scallop aquaculture. Furthermore, the identification of gene polymorphism responsible for disease resistance should be helpful to develop disease resistant stocks. In the present study, the polymorphisms in the potential LPS and glucanase binding motif of *Cf*LGBP were screened and the association of SNP +7679 G-A with disease resistance/susceptibility of Zhikong scallop to *Listonella anguillarum* infection was investigated to elucidate information concerning functional variability which might be helpful for disease management, and probably used as a potential marker for future selective breeding.

2. Materials and methods

2.1. Bacterial challenge and stock identification of scallop

One hundred and seventy five scallops with approximately 60 mm in shell length were randomly collected from several farms (Qingdao, China), and kept in aerated seawater at 18 ± 1 °C for a week before processing. The scallops were divided into five groups (35 scallops in each group) for the bacterial challenge experiment, where four of the groups were cultured in 24 L tanks containing aerated seawater with high density of *L. anguillarum* (5.0×10^7 CFU ml^{-1}) and the rest group was still kept in aerated seawater and used as the control group. All groups were observed at an interval of 2-h until 120-h post challenge in order to monitor the mortality of scallops after bacterial challenge. The scallops that died during the early period of the challenge were considered as the susceptible stock and the individuals that survived throughout the challenge experiment were considered as the resistant stock. The samples were collected regularly and the adductor muscle of each individual scallop from two stocks was removed, and stored immediately at −80 °C until the DNA was extracted. About 100 mg adductor muscle from each scallop was homogenized in 500 μl buffer containing 100 mmol/L EDTA, 10 mmol/L Tris–HCl, pH 8.0, 1% SDS and 0.1 mg/mL Proteinase K (Merck). The genomic DNA was extracted by proteinase K and phenol method as previously described [28].

2.2. Gene structure and analysis of polymorphisms in the LPS and glucanase binding motif of CfLGBP

Three pair primers (*Cf*LGBP F1, F2, F3; R1, R2, R3) (Table 1) were designed corresponding to various positions of the *Cf*LGBP cDNA (GenBank accession NO. AY259542) and used to amplify the full length genomic DNA sequence of *Cf*LGBP. The total genomic sequence of the *Cf*LGBP was approximately 9720 bp (data not shown) and the structure was consisted of 13 exons interrupted by 12 introns (Fig. 1). The potential LPS and glucanase binding motif region of *Cf*LGBP was selected for further polymorphism analysis.

Based on the genomic sequence data (data not published), a pair of gene specific primers, *Cf*LGBP GSP-F and *Cf*LGBP GSP-R (Table 1) were designed and used to amplify a 569-bp fragment of the potential motif region located in exon 10 and exon 11 of *Cf*LGBP. PCR reaction was performed using LA taq polymerase (TaKaRa) in a 25 μl reaction volume as previously described [28] on a PTC-100 Programmable Thermal Controller Cycler (TaKaRa) with the following temperature profile: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min; a further 10 min elongation at 72 °C. The PCR products were detected by agarose gel electrophoresis, and were excised and purified. The target fragments of six representative samples from susceptible and resistance stock were cloned into pMD18-T vector (TaKaRa) and transformed into *Escherichia coli* Top10. The positive clones were sequenced by using an ABI3730 Automated Sequencer (Applied Biosystem). Sequences of the objective *Cf*LGBP region were aligned by using Vector NTI suite 9 and the polymorphism sites in the coding region were analyzed by using Primer Premier 5.0. The possible structural changes of polymorphic variants were predicted by using Project HOPE program (http://www.cmbi.ru.nl/hope/home).

2.3. Screening of SNP at locus +7679 in susceptible/resistant scallops

The SNP +7679 G/A was screened by using tetra ARMs PCR analysis to find the possible association for susceptibility of Zhikong scallop to *L. anguillarum*. The tetra ARMs PCR primers (Table 1) were designed by using a computer program by Ye et al. (2001) [29], and the PCR reaction was performed in a 25 μl reaction volume as previously described [28]. The reaction was carried out with the

Table 1
Names and sequences of primers.

Primer name	Sequence (5′-3′)
*Cf*LGBP F1	ACTAAACGTCGCATTGTGTATTCA
*Cf*LGBP R1	TGCTTATCCCCGATGATGTATG
*Cf*LGBP F2	CATACATCATCGGGGATAAGCAAG
*Cf*LGBP R2	CCCCATAAGTCTAGTGTGCCTTTT
*Cf*LGBP F3	AAGGCACACTAGACTTATGGGGC
*Cf*LGBP R3	GCCAGGGTTTAGCGTAAGGTGA
*Cf*LGBP GSP-F	TAAGAAGGAGAAAACGGAAGGCA
*Cf*LGBP GSP-R	TCCTACTTTATATGGTCTTCCGTCC
GA − Mutagenesis primer	CCGTCACTACACAGCTCCTGA**TAG**CACTCACAAAGGTGTCG
	CGACACCTTTGTGAGTG**CTAT**CAGGAGCTGTGTAGTGACGG
A-Forward inner primer (A allele)	GGTAACCGTCACTACACAGCTCCTGCTA
B - Reverse inner primer (G allele)	GAAGGAGTCGACACCTTTGTGAGTTCC
P - Forward outer primer	TGACATTTTGTTGCTGGAATTGGATACC
Q - Reverse outer primer	GAACTGACTTTTCAACGTGTGCTGTCTG