Aquaculture 442 (2015) 125–131

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

SNP E4-205 C/T in C-type lectin of *Portunus trituberculatus* is association with susceptibility/resistance to *Vibrio alginolyticus* challenge

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ARTICLE INFO

Article history:
Received 17 December 2014
Received in revised form 4 February 2015
Accepted 6 February 2015
Available online 14 February 2015

Keywords:
Portunus trituberculatus
C-type lectin
Polymorphism
Susceptibility/resistance
High resolution melting (HRM) analysis

ABSTRACT

C-type lectins play an important role in innate immunity of invertebrates, especially in defending them against bacterial pathogens. In this study, the full-length genomic DNA of C-type lectin (designated as *CTL* gene) was isolated from the swimming crab *Portunus trituberculatus*. The genomic DNA of *CTL* consists of 1473 bp, containing four exons and three introns. Polymorphisms of the *CTL* gene were identified to explore their association with susceptibility/resistance to *Vibrio alginolyticus* infection. Four sites of single nucleotide polymorphisms (SNPs) and one site of insertion/deletion (ins/del) polymorphism were identified in *CTL*. Three of these SNPs were synonymous and one was non-synonymous. The distribution of these polymorphisms in the susceptible and resistant stocks was identified, according to the survival time after *V. alginolyticus* challenge. The non-synonymous SNP E4-205 C/T, a dimorphism caused from C to T transition that resulted in a Threonine to Isoleucine substitution at position 152 in the peptide of CTL protein, showed significant difference between the two stocks according to Chi-squared test ($P < 0.05$). The SNP site has three genotypes that are C/T, C/C and T/T. The T/T genotype frequency was 6.1% in susceptible stock, and 21.2% in resistant stock, with a significant difference between them ($P = 0.022$). Therefore, the T/T genotype was suggested to be associated with increased resistance to *V. alginolyticus* ($P < 0.05$). The presumption was confirmed by another challenge experiment, in which high resolution melting (HRM) analysis was used to analyze the three genotypes distribution. The cumulative mortality of genotype T/T (57.1%) was significantly lower than C/C (82%) and T/C (81.4%) ($P = 0.001$). These results suggested that the E4-205 genotype T/T is a potential marker for selection of swimming crabs resistant to *V. Alginolyticus* challenge.

1. Introduction

Portunus trituberculatus is widely distributed along the coast of China, Japan, Korea, and other Southeast Asian countries. This species is one of the most common edible marine crabs found in China, and has also been artificially cultured since the 1990s. However, the uncontrolled expansion in the scale of farming, increase in stocking density, and the polluted breeding environment, all result in the frequent outbreaks of diseases. Some of the most recurrent of these are the infections caused by the *Vibrio* family, such as *Vibrio metschnikovii* (Wan et al., 2011), and *Vibrio alginolyticus* (Liu et al., 2007). The "emulsification disease" caused by *V. alginolyticus* has brought considerable economic losses, and has seriously restricted the sustained and healthy development of crab aquaculture (Wang et al., 2006). Select breeding of new strains resistant to *V. alginolyticus* is a very important step towards controlling this disease.

Traditional breeding techniques are expensive, time-consuming, and easily influenced by the environment (Collard et al., 2005). One of the methods that could be used to improve breeding strategies is marker assisted selection (MAS), a molecular method successfully used in the improvement of agricultural population (Dekkers and Hospital, 2002). It is a method where a marker is used for the indirect selection of the target trait. The marker used is closely linked to the target trait. Some studies have reported on the use of DNA markers in MAS of livestock in order to cultivate novel disease-resistant varieties (Maillard et al., 2003; Masoudi et al., 2007). However, the use of MAS on *P. trituberculatus* is strategically difficult, as there are very few markers associated with specific traits (Liu et al., 2012; Lee et al., 2013). Therefore, it is important to identify the marker associated with resistance to *V. alginolyticus* in the swimming crab to develop strategies for MAS.

Immune-related genes are considered as the optimal candidates for the selection of markers associated with resistance to pathogens. Gene

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http://dx.doi.org/10.1016/j.aquaculture.2015.02.007
0044-8486/

polymorphism could improve the genetic quality or quantity, enhancing the ability of the immune system in protecting itself against infection. In recent years, polymorphisms in some immune-related genes have been reported to affect pathogen or disease resistance in aquatic animals. Examples of such genes include the lysozyme gene in Zhikong scallop (*Chlamys farreri*) (Li et al., 2009), the superoxide dismutase gene family in the bay scallop (*Argopecten irradians*) (Bao et al., 2010), the serine protease inhibitor gene in the eastern oyster (*Crassostrea virginica Gmelin*) (He et al., 2012; Yu et al., 2011), the MDA5 gene in grass carp (Wang et al., 2012), and the mannose binding lectin (MBL) in Zebra fish (*Danio rerio*) (Jackson et al., 2007). Polymorphism in these immune genes yielded different genotypes, which exhibited immunity phenotypes. Many genes involved in immune response of swimming crab, such as the C-type lectin (Kong et al., 2008), C-type lysozyme (Pan et al., 2010), crustins (Yue et al., 2010), prophenoloxidase (Chen et al., 2010) and anti-lipopolysaccharide (Y. Liu et al., 2011, 2012), were reported. However, polymorphisms in these genes, and the correlation between these gene polymorphisms and disease susceptibility and resistance are not yet well understood (Li et al., 2013).

The C-type lectins play an important role in pattern recognition and innate immune response in invertebrates (McGreal et al., 2004). They generally contain one or more carbohydrate recognition domains. In the presence of Ca^{2+}, they specifically recognize the oligosaccharides expressed on the cell surface of exogenous microbes, and initiate an immune reaction against the invasion of pathogenic microorganisms (Wang and wang, 2013). A number of C-type lectins have been identified in crustaceans, most of which display anti-bacterial and anti-viral properties (Huang et al., 2014; Wang et al., 2013; Wei et al., 2012). In *P. trituberculatus*, the cDNA of a C-type lectin-like domain (CTLD)-containing protein with one carbohydrate recognition domain (CRD) was cloned and designated as PtLP (CTLD-containing protein) (Kong et al., 2008). In our previous study, a high level of PtLP transcripts was found in the hemocytes and hepatopancreas of *P. trituberculatus* following injection of *V. alginolyticus*. To elucidate the role of PtLP in susceptibility or resistance of *P. trituberculatus* to *V. alginolyticus*, the complete DNA sequence of PtLP was cloned, designated as *CTL*. Our objective was to identify polymorphisms in *CTL* and to investigate the possible association of these polymorphisms with susceptibility/resistance to infection by the *V. alginolyticus*.

2. Materials and methods

2.1. Crab breeding and V. alginolyticus challenge

One hundred and sixty swimming crabs with an average weight of 25 ± 3 g were collected from a commercial farm (Xiangshan, China) and acclimatized in aerated seawater at 25 °C for a week before processing. During the entire period of the experiment, the crabs were fed with clam meat once every night, and seawater changed every day.

For the bacterial challenge experiment, the crabs were divided into four groups (40 crabs in each group). These were cultured in four cement pools (2 m × 5 m × 0.9 m, width × length × depth). Crabs from the bacteria-challenged groups were injected of 200 μL live *V. alginolyticus* suspended in 0.01 mol/L Phosphate Buffer Saline (PBS) (pH 7.2, 7.8×10^7 cfu/mL) and the injection site is the arthrodial membrane of the last walking leg. The control group crabs received an injection of 200 μL PBS. All the crabs were observed every hour for the first 24 h, followed by observation every 3 h after 120 h post-challenge. The crabs that died in the first 24 h were classified as the susceptible stock, and the crabs that survived 120 h post-challenge were regarded as being relatively resistant to *V. alginolyticus* (resistant stock). Muscles in the last walking leg of all crabs from these two stocks were removed and stored at −80 °C for DNA extraction.

2.2. Acquiring of CTL DNA sequence

Genomic DNA was extracted from the muscle of *P. trituberculatus* using Genomic DNA Purification Kit (Axygen Biosciences, Corning, NY, USA). The primers *CTL-F1* and *CTL-R1* (Table 1) were designed, according the full length cDNA of *PtLP* in *P. trituberculatus* (GenBank accession no. EU477491.1), to acquire the DNA sequence [21]. The reaction was carried out in a 50 μL reaction mixture, containing 5 μL of 10× LA PCR buffer (TaKaRa), 8 μL of dNTP (2.5 mM), 1 μL each of forward primer and reverse primer (10 μM), 0.5 μL (2.5 U) of LA Taq (TaKaRa), 3 μL of target DNA and 31.5 μL of double distilled water. The reaction was performed in a Tprofessional Thermocycler (Biometra, Goettingen, Germany) under the following conditions; 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 50 s, 72 °C for 2.5 min, and a final extension at 72 °C for 10 min. The PCR products were purified by the manufacturer's protocol of PCR gel purification kit (Axygen) and cloned into pMD19-T simple vector (TaKaRa Bio Inc., Otsu, Japan). The vector was transformed into the Trans1-T1 phage resistant chemically competent cell (TransGen, Beijing, China), and the positive recombinants identified through anti-Amp selection and PCR screening using *M13-47* and *RV-M* primers (Table 1). Three of the positive clones were sequenced using an ABI3730 Automated Sequencer (Applied Biosystems, Carlsbad, CA, USA).

2.3. Identification of polymorphic loci in the CTL

One pair of gene specific primer *CTL-F2* and *CTL-R2* (Table 1) was designed based on the obtained *CTL* DNA sequence to amplify the whole *CTL* gene fragment. The reaction was carried out in a 50 μL reaction mixture, containing 5 μL of 10× PCR buffer (TaKaRa), 6 μL of dNTP (2.5 mM), 1 μL each of forward primer and reverse primer (10 μM), 0.5 μL (1.25 U) of PrimeSTAR HS DNA Polymerase (TaKaRa), 3 μL of target DNA and 33.5 μL of double distilled water. The reaction was performed following conditions; one cycle of 94 °C for 4 min, followed by 35 cycles of 94 °C for 50 s, 55 °C for 50 s, and 72 °C for 1.5 min, and a final 10 min extension at 72 °C.

The PCR products from three susceptible and resistant crabs each were analyzed on 1% agarose gels and purified. The objective fragments were cloned into pMD19-T vector (TaKaRa) and at least three clones were sequenced from every sample. The objective nucleotide sequence of *CTL* was aligned using the Vector NT1 Suite 9 (Life Technologies, Carlsbad, CA, USA). The polymorphisms in *CTL* gene were detected from the sequence alignments of different crabs.

2.4. Analysis of SNPs in the CTL and association with V. alginolyticus resistance

Three pairs of gene-specific primers (*CTL-F3*/*R3*, *CTL-F4*/*R4* and *CTL-F5*/*R5*) (Table 1) were designed based on the sites of polymorphism

Table 1
Primers used in this study.

Name	Sequence (5′-3′)	PCR objective
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	Sequencing
RV-M	GAGCGGATAACAATTTCACACAGG	Sequencing
CTL-F1	TCCTGTTCTGACAACCACCAA	Obtaining DNA sequence
CTL-R1	CTGTGCCCGAGTCAAGAAGTA	Obtaining DNA sequence
CTL-F2	AGCAGCACCGATAAAAGAGGCACC	Finding SNPs
CTL-R2	AAGCTGGTTAGCACATTACAC	Finding SNPs
CTL –F3	TCAAATCATTTCCTTGTGAGGG	Sequencing SNPs in two stocks
CTL –R3	ACAAAAGGATGTCACCGTAGAA	Sequencing SNPs in two stocks
CTL –F4	GCAATTCTACGGTGACATCCTT	Sequencing SNPs in two stocks
CTL –R4	TTATCGCCATAGTTAGCCCAAA	Sequencing SNPs in two stocks
CTL –F5	ACTCCTTTTTGGGCTAACTATGGC	Sequencing SNPs in two stocks
CTL –R5	TCCAAAGTAAGCTGGTTAGCACAT	Sequencing SNPs in two stocks
CTL-HRM-F	AAATGCCTACTGGAGGAGAGAAACA	Genotyping amplification
CTL-HRM-R	TTTCCTCGTTACTTCTCACAAATCG	Genotyping amplification