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Cloning and differential expression of a novel toll-like receptor gene in noble scallop *Chlamys nobilis* with different total carotenoid content

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ABSTRACT

To investigate whether toll like receptors (TLRs) genes do have an immune influence on noble scallop *Chlamys nobilis* under pathogen stress, acute challenges lasting 48 h to *Vibrio parahaemolyticus*, lipopolysaccharide (LPS), polyinosinic polycytidylic acid (Poly I:C), and PBS were conducted in two scallop stains of orange and brown with different carotenoids content. A novel toll-like receptor gene called *CnTLR-1* was cloned and its transcripts under different challenges were determined. Meantime, total carotenoids content (TCC) of different immune responses were determined to investigate whether there was a relationship between gene expression and carotenoids content. The full length cDNA of *CnTLR-1* is 2982 bp with an open reading frame (ORF) of 1920 bp encoding 639-deduced amino acids, which contains five leucine-rich repeats (LRR), two LRR-C-terminal (LRRCT) motifs and a LRR-N-terminal (LRRNT) motif in the extracellular domain, a transmembrane domain and a Toll/Interleukin-1 Receptor (TIR) of 138-amino acids in the cytoplasmic region. Phylogenetic tree analysis showed that *CnTLR-1* could be clustered with mollusk TLRs into one group and especially was related closely to *Crassostrea gigas* and *Mytilus galloprovincialis* TLRs. *CnTLR-1* transcripts were detected in decreasing levels in the mantle, hemocytes, gill, kidney, gonad, hepatopancreas, intestines and adductor. Compared with PBS control group, *CnTLR-1* transcripts were up-regulated in *V. parahaemolyticus*, LPS and Poly I:C groups. Further, *CnTLR-1* transcripts were significantly higher in orange scallops than that of brown ones with and without pathogenic challenges. TCC, which is higher in orange scallops, was initially increased and then decreased during a 48 h immune challenge in the hemocytes. The present results indicate that *CnTLR-1* is an important factor involved in the immune defense against pathogens in the noble scallop.

1. Introduction

Pattern recognition receptors (PRRs) are a set of proteins of the innate immune system involved in the recognition of pathogen-associated molecular patterns (PAMPs), which are associated with microbial pathogens [1–3]. One types of the PRR, toll like receptors (TLRs), are important transmembrane proteins that connect innate immunity and adaptive immunity [4]. They detect microorganisms based on conserved PAMPs like peptidoglycans, lipoproteins, double-strand viral RNA, lipopolysaccharide (LPS), unmethylated bacterial CpG DNA and so on [5]. TLRs mainly have three functional domains, an ectodomain of tandem leucine-rich repeats (LRRs) which participates in specific identification of pathogenic microorganisms, a transmembrane region, and an intracellular region that possesses a globular cytoplasmic Toll/Interleukin-1 Receptor (TIR) homology domain involving in meditating protein-protein interactions between TLRs and signal-transduction components, as well as directing localization of receptor [6,7]. The downstream signal components are pro-inflammatory cytokines including interleukins, interferon, and TNF, which are responsible for immediate innate response and for triggering adaptive immune cells [7].

The first toll-like receptor was identified in *Drosophila melanogaster* for its host defense against fungal infection [8] and then nine TLRs had been confirmed later [9]. As researchers paid more attention recent years, TLRs had been well-characterized [10]. In non-mammalian animals, from vertebrates to invertebrates, many species have also confirmed numerous TLRs [11]. The mollusk TLRs are mainly investigated in the bivalves, such as *Chlamys farreri* [12], *Crassostrea gigas* [13], *Mytilus galloprovincialis* [14], *Mya arenaria*

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[15], the cephalopod *Euprymna scolopes* [16], and *Sepia officinalis* [17]. The first TLR gene (CfToll-1) of *Crassostrea farreri* was identified in 2007 [12], then the TLR signaling pathway core components, MLR, CfMyd88, CfTRAF6, CfNF kappa B and Cfl kappa B have been characterized [18]. A TLR gene (CgToll-1) was acquired in *C. gigas* [13], the downstream genes Ref [19] and I kappa B [20], the upstream genes, MyD88 [21,22] and TRAF6 [23] were confirmed to be the signaling molecules. The application of Illumina technology in mussel, *M. galloprovincialis* help to identify 23 Toll-like receptors (TLRs) and 3 MyD88 adaptors [14]. *In vivo* infection with *Vibrio splendidus* in *M. arenaria* indicated that TLR2 and IRAK4 were regulated to participate in host immune response [15]. Other mollusks, for example, *E. scolopes* [16], *Euprymna tasmanica* [24], *S. officinalis* [17], bivalvias like the pearl oyster *Pinctada martensii* [25], Japanese scallop *Mizuhopecten yessoensis* [26] and manila clam *Ruditapes philippinarum* [27] only have been described several components of the TLR signaling pathway.

Carotenoids are yellow-red isoprenoid polyene pigments extensively existed in nature [28]. A variety of biological activities and much medicinal values have been found in carotenoids, such as scavenging free radicals, resisting oxidative damage, delaying senescence, quenching singlet oxygen, increasing the vitality of B cells in the immune system, enhancing humoral immune response in animals and humans, acting as the precursor of vitamin A, treating photosensitivity diseases and preventing against cataracts [29–33]. Carotenoids also have recognized functions in gene expression and regulation [3–5,34,35], for examples, carotenoids can up-regulate *connexin 43* to increase cell-to-cell communication and thus decrease cell proliferation [34], and can also up-regulate Vg gene to increase scallops' immunity [35].

The noble scallop *Chlamys nobilis*, an important edible marine bivalve belongs to the family Pectinidae, is widely distributed in Japan, Indonesia and the Southern Sea of China. The scallop not only displays its polymorphism in shell colors including orange, brown, orange-purple and purple, but also polymorphism in muscle colors including white and orange [36]. Since 1980′, noble scallop has been cultured and developed into a large-scale industry because of the obvious advantages of quick growth, short culturing period, high profit, good taste and nutrition [37,38]. However, the cultured scallop often suffers from large-scale mortality in the past two decades [35,39,40]. Several potential reasons including reproduction pressure, environmental factors, inbreeding, opportunistic invaders and pathogens have been proposed by researchers. Vibrio is one of the most harmful pathogens in cultured shellfish distributed in all coastal waters around the world, whose outbreaks usually are concentrated during the summer and early fall when higher water temperatures favor higher levels of bacteria [41]. The typical processes of vibrio infection in shellfish include pathogenic vibrio adhesion, invasion, large-scale tissue damage and then death of shellfish [42–44]. In Shantou, Guangdong Province, *Vibrio parahaemolyticus* is one of the most dominant bacterial pathogens that contribute to mass morality of farming areas [22].

A genetic and breeding project on the noble scallop has been carried out by artificial selection in our laboratory since 2008. We first found that the orange scallops with orange shell, orange mantle and orange adductor had significantly higher total carotenoids content (TCC) than the brown ones with brown shell, white mantle and adductor [38]. Then, by establishing different strains, we found that the scallops' shell and muscle colors were both consistently inherited in this species [36,38]. More importantly, TCC contained in orange scallops could be enhanced by selection breeding [36,38]. After four generations selection and two generations cultured demonstration, a new variety named "Nan'ao Golden Scallop" (ID: GS-01-009-2014) was bred and authorized in 2014 by the National Aquatic Protospecies and Improved Variety Approval Committee of China. Moreover, our previous study indicated that carotenoids content might be positively correlated with the expression levels of some genes of the immune system, which play an important role against pathogens in noble scallop *C. nobilis* [35].

Cellular immunity and humoral immunity are two main immune systems in scallops' host defense [45]. Like many other invertebrates, scallops relies more on the non-specific immune responses for defence against pathogens, such as hydrolytic enzyme activity, phagocytosis of cells, cell-mediated cytotoxic effect against pathogens [46].

To investigate whether toll like receptors (TLRs) genes have an immune influence on noble scallop *C. nobilis* under pathogen stress, acute challenges lasting 48 h to *V. parahaemolyticus*, LPS, polyinosinic-polycytidylic acid (Poly I:C), and PBS were conducted in two scallop stains of orange and brown with different carotenoids content. A novel TLR gene was cloned and its transcripts under different challenges were determined. Moreover, TCC of different immune responses mentioned above at each timing were also determined.

2. Materials and methods

2.1. Experimental animals and microbes

12-month old orange and brown scallops with different carotenoids content that originated from same population of noble scallop *C. nobilis* were used in the present study. The scallops were cultured at 500 L experimental tanks, fitted with circulatory pumps, maintained in the seawater at 18.0 °C–19.5 °C during the experiment, changing filtering seawater twice a day after feeding with diatom (*Nitzschia closterium f. minutissima*) and tetraselmis (*Platymonas subcordiformis*) which were cultured by our laboratory.

The bacterium *V. parahaemolyticus* was preserved in our laboratory. The method of bacterium culture had been reported by Zhang et al. [35].

2.2. Immune challenge and tissue collection

To investigate the distribution of *CnTLR-1* mRNA expression, seven tissues including the intestine, adductor, mantle, gonad, gill, kidney, hepatopancreas and hemolymph were collected from six orange and brown scallops, respectively. All these tissue samples were stored at −80 °C after addition of 1 ml Trizol reagent (Invitrogen) for subsequent RNA extraction. The rest adductor, gill, mantle tissues and hemolymph of the 12 scallops were collected and stored at −20 °C before using for determination of TCC.

To explore the different immune responses between orange and brown scallops, 300 individuals (equally between the two scallop strains) were chosen and randomly divided into eight groups (36 scallops each group). After 24 h acclimatization, 100 μl *Vibrio parahemolyticus* suspension (5×10^7 cfu ml^{-1} in PBS) were injected into two groups of scallops of one orange and one brown. Meanwhile, 100 μl LPS (Sigma Aldrich, 0.5 mg ml^{-1} in PBS) and Poly I:C (Sigma Aldrich, 1 mg ml^{-1} in PBS) were also applied to the scallops, respectively. For blank control, the scallops received an injection of 100 μl PBS. Then, the scallops were return to seawater tanks after treatment, six individuals were randomly sampled at 3, 6, 12, 24, 36 and 48 h post-challenge from each group. 6 orange or brown individuals without any treatment were randomly sampled at 0 h for control. The hemolymph was drawn from each scallop with a disposable syringe (1 ml) on ice and centrifuged at 800*g* and 4 °C for 10 min to harvest the hemocytes for RNA isolation. Additional hemolymph was withdrawn for the determination of TCC. All these