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Molecular cloning and expression of a C-type lectin gene from *Venerupis philippinarum*

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Abstract C-type lectins have been demonstrated to play important roles in invertebrate innate immunity by mediating the recognition of pathogens and clearing the micro-invaders. In the present study, a C-type lectin gene (denoted as VpCTL) was identified from *Venerupis philippinarum* by expressed sequence tag and rapid amplification of cDNA ends approaches. The full-length cDNA of VpCTL consists of 904 nucleotides with an open-reading frame of 456 bp encoding a peptide of 151 amino acids. The deduced amino acid sequence of VpCTL shared high similarity with C-type lectins from other species. The C-type lectin domain and the characteristic EPN and WND motifs were found in VpCTL. The VpCTL mRNA was dominantly expressed in the haemocytes of the *V. philippinarum*. After *Listonella anguillarum* challenge, the temporal expression of VpCTL mRNA in haemocytes was increased by 97- and 84-fold at 48 and 96 h, respectively. With high expression level in haemocytes and hepatopancreas, and the up-regulated expression in haemocytes indicted that VpCTL was perhaps involved in the immune responses to *L. anguillarum* challenge.

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Introduction

As the first-line of defense against pathogens, the innate immune system of the invertebrates relied on the pattern-recognition proteins (PRPs) to recognize specific patterns of foreign pathogens [1]. Lectins are a family of single or multi-domain glycoproteins capable of binding sugar moieties through specific interaction with an intramolecular carbohydrate recognition domain, CRD [2–4], and are considered to be one of the important PRPs [5]. Researches have illustrated that lectins act as recognition molecules, mediating cell adhesion and signal transduction events through binding to carbohydrates [3, 6]. Lectins exist in almost all living organisms and can be divided into seven subgroups according to their structures and functions [7]. The C-type lectin family is one of the major groups that bind carbohydrates in a calcium dependent manner [2, 8, 9]. All the C-type lectins share the same structural features such as CRD sequences, disulfide-bond positions, and calcium binding sites [10].

In recent years, many C-type lectins have been characterized in various invertebrates, such as insects, tunicates, crustaceans and bivalves [11–14]. In bivalves, lectins have been identified in haemolymph/haemocytes and were suggested to be implicated in defense responses or pathogen uptake. For example, five C-type lectins from *Chlamys farreri* (CfLec-1–CfLec-5) and seven C-type lectins from bay scallop *Argopecten irradians* (AiCTL-1–AiCTL-7) have been characterized, and most of them were proved to be involved in the immune responses against certain Gram-positive or Gram-negative bacteria [13, 15–19]. In addition, a novel putative C-type lectin identified from

Crassostrea virginica was detected in the mucocytes lining the epithelium of the digestive gland and the pallial organs, and was involved in particle capture and in oyster mucosal immunity [20].

The Manila clam, *Venerupis philippinarum*, is an important marine bivalve for commercial fisheries, accounting for about 80 % of mudflat fishery production in China (China Bureau of Fisheries, 2004). However, clam culture in China has been severely plagued by pathogenic microorganisms, which has caused serious economic losses [21]. Therefore, understanding the immune responses of clams against pathogen challenge may be helpful to the sustainable development of clam culture. In the present study, the full-length cDNA encoding a C-type lectin was cloned from *V. philippinarum*, and the spatial and temporal expression profile of VpCTL transcript after bacterial challenge were also investigated, hopefully shedding light on the roles of C-type lectins in the immune responses of clam.

Materials and methods

Clams culture and challenge

Adult clams *V. philippinarum* (shell length: 3.0–4.0 cm, Zebra pedigrees) were collected from a local culturing farm and acclimatized in aerated seawater (32 psu) at 25 °C for 10 days before commencement of the experiment. Clams were fed with *Chlorella vulgaris* Beij ($1–2 \times 10^4$ CFU mL^{-1}) daily, and the seawater was renewed everyday. After the acclimatization, the clams were randomly divided into six groups and cultured in flat-bottomed rectangular tanks (80 L), each tanks containing 50 individuals.

For bacterial challenge experiment, five groups of clams were challenged with *Listonella anguillarum* (suspended in fresh sea water) with final concentration of 1.0×10^7 CFU mL^{-1}. The untreated group was employed as the control group. Five individuals were randomly sampled at 6, 12, 24, 48 and 96 h after challenge. The haemolymph from the control and the challenged groups was collected from adductor muscle using a syringe and centrifuged at $2{,}000 \times g$, 4 °C for 10 min to harvest the haemocytes. Meanwhile, hepatopancreas, gill, muscle, haemocytes, mantle and foot from four untreated clams were also collected to determine the tissue distribution of VpCTL. Total mRNA was immediately extracted using Trizol reagent according to the manufacture's protocol (Invitrogen, USA).

Cloning the full-length cDNA of VpCTL

BLAST analysis of all expressed sequence tag (EST) sequences from a cDNA library of *V. philippinarum* haemocytes revealed that one EST shared 36 % homology to the previously identified C-type lectin in *Oplegnathus fasciatus* (ACY66646.1). The 5′ and 3′ ends of VpCTL were obtained by rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA Amplification Kit (Clontech, USA) according to manufacturer's recommendations. The PCR products were gel-purified and subcloned into pMD18-T simple vector (Takara, Japan). After transformation into the competent cells of *Escherichia coli* Top10F', three positive clones were sequenced on an ABI3730 Automated Sequencer (Applied Biosystem, USA).

Sequence analysis of VpCTL

The cDNA and amino acid sequences of VpCTL was analyzed by using the BLAST algorithm at NCBI website (http://www.ncbi.nlm.nih.gov/blast), and the deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/). The protein domain features were determined by using Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/). The percentage of similarity and identity of VpCTL with C-type lectin proteins from other organisms was calculated by the Identity and Similarity Analysis program (http://www.biosoft.net/sms/index.html). Multiple alignments of VpCTL were performed with the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/) and Multiple Alignment Show program (http://www.biosoft.net/sms/index.html). A phylogenetic tree was constructed with Mega4.1 software using the neighbor-joining (NJ) method based on the alignment [22]. Bootstrap analysis was used with 1,000 replicates to test the relative support for the branches produced by the NJ analysis [23].

VpCTL mRNA distribution in different tissues and temporal expression profile in haemocytes after bacterial challenge

For the tissue distribution experiment, hepatopancreas, gill, muscle, haemocytes, mantle and foot were taken aseptically from four unchallenged clams and subjected to total RNA extraction. The haemocytes were selected to analyze temporal expression profile of VpCTL challenged by *L. anguillarum*. One microgram of total RNA was used in a reverse transcription reaction with M-MLV Reverse Transcriptase (Promega, USA). The resultant cDNA templates were diluted to 1:50 for subsequent SYBR Green assay. Real Time PCR was carried out in an ABI 7500 Real-Time Detection System by using the SYBR ExScript qRT-PCR Kit (Takara, Japan) and performed in a total volume of 50 μL, containing 25 μL of 2× SYBR Green Master Mix (Applied Biosystems), 15 μL of the diluted cDNA mix, 0.625 μL of each primer (10 μmol L^{-1}),

Table 1 Primers used in the present study

Primer sequence	(5′–3′)	Sequence information
P1 (forward)	CTCCCTTGAGAAGAGCTACGA	Real time actin primer
P2 (reverse)	GATACCAGCAGATTCCATACCC	Real time actin primer
P3 (forward)	CTGTAGCGATGGTTGGATAG	Real time VpCypA1 primer
P4 (reverse)	TGTCAGTCAGTCCAATCCAC	Real time VpCypA1 primer

8.75 μL of DEPC-treated water. Two clam β-actin primers, P1 and P2 (Table 1) were used to amplify a 121 bp fragment as an internal control to verify the successful reverse transcription and to calibrate the cDNA template. Two VpCTL gene-specific primers, P3 and P4 (Table 1), were designed to amplify a PCR product of 203 bp. In a 96-well plate, each sample was run in triplicate along with the internal control. The thermal profile for real-time PCR was 50 °C for 2 min and 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min.

At the end of each PCR reaction, dissociation curve analysis of amplification products was performed to confirm that only one PCR product was amplified and detected. The baseline was set automatically by the software to maintain consistency. The expression level of VpCTL was analyzed using $2^{-\Delta\Delta CT}$ method [24]. All data were given in terms of relative mRNA expression as mean ± SE.

Statistical analysis

One-way analysis of variance (one-way ANOVA) was performed on all data using SPSS 13.0 and *P* values less than 0.05 was considered statistically significant.

Results

cDNA cloning and sequence analysis of the VpCTL

A 904 bp nucleotide sequence representing the complete cDNA of VpCLT was deposited in GenBank under accession number GQ384389. The full nucleotide sequence and deduced amino acid sequence of VpCTL is shown in Fig. 1. The complete cDNA contained a 5′ untranslated region (UTR) of 151 bp, a 3′ UTR of 297 bp and an open reading frame of 456 bp encoding a polypeptide of 151 amino acids with the predicted molecular weight of 17.14 kDa and the theoretical isoelectric point of 5.79.

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   1 TCTTTGTAGATATAATTAGTTAAGATTAATATCCAAATACCAGTCTTACATCTTTTTCAT
  61 TTTATGAAATATATTTTTTTTTAATATTTCAGATACGGATTTTATTAGTTTGTGGAATAC
   1                                   M  K  S  A  I  V  L  L  L  V
 121 AAATTCAAATACGACGTGAAACTTAAGCGAAATGAAGTCTGCAATAGTTTTGCTGCTTGT
  11  V  A  C  L  A  P  G  I  N  S  R  A  C  C  S  D  G  W  I  A
 181 CGTTGCATGCCTGGCGCCAGGAATTAATTCAAGAGCTTGCTGTAGCGATGGTTGGATAGC
  31  Y  K  D  H  C  Y  H  I  G  Y  G  T  R  L  T  F  S  A  A  R
 241 CTATAAAGACCATTGTTACCACATTGGTTACGGAACACGTTTAACGTTTTCTGCTGCACG
  51  V  Y  C  Q  S  L  G  A  Y  L  V  R  L  D  T  F  D  E  N  T
 301 GGTGTATTGTCAAAGTCTTGGTGCTTACCTTGTCCGGCTTGACACGTTTGATGAAAACAC
  71  F  L  K  G  F  L  K  K  L  M  L  E  S  T  W  I  G  L  T  D
 361 TTTCCTTAAAGGATTTCTTAAGAAACTAATGCTGGAAAGTACGTGGATTGGACTGACTGA
  91  R  T  H  D  G  I  W  R  W  F  D  T  M  S  H  A  T  C  S  D
 421 CAGGACTCATGACGGAATCTGGAGATGGTTTGACACAATGAGTCATGCAACATGCTCTGA
 111  W  G  P  G  [E  P  N]  S  H  G  N  E  D  C  V  N  F  F  V  D
 481 CTGGGGTCCCGGAGAACCTAACAGTCATGGTAATGAAGATTGTGTGAACTTTTTTGTGGA
 131  N  D  Y  N  [W  N  D]  S  T  C  H  S  K  Y  T  P  L  C  E  K
 541 CAACGACTATAACTGGAATGACAGTACTTGTCATAGCAAGTACACCCCTTTGTGCGAAAA
 151  V  *
 601 AGTCTGAACTTTAACTCCTCAACGATATAAAAATTTACACAAAATATAATAATAATTTTA
 661 AAGGCAAGTTAAGAACTTATTTTCAACTATTATAGTAAGTAGTACAATGACGAATAACAA
 721 TTATTTGGAATTAGCTGTCAATAAAATTACGGATATTGCTAAAAAAAAAAAAAAAAAAAT
 781 AACAAAATTGTTTTGACCAATTATAATAGTAAGTTATTCTATGACGAATTTCAATTATTC
 841 GAAATTGGCTGTCAATAAAATTACTGACATTGCGAAAAAAAAAAAAAAAAAAAAAAAAA
 901 AAAA
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Fig. 1 Nucleotide and deduced amino acid sequences of VpCTL (GQ384389). The nucleotide and amino acid sequence is *numbered* on the *left*. The putative signal peptide is *underlined*. The start and stop codons are shown in *bold*. The classical polyadenylation signal is *wave underlined* and the EPN and WND motifs are *boxed*

A putative signal peptide was identified from 1 to 20 amino acid residues. SMART program analysis revealed that VpCTL contained a typical C-lectin domain ranging from Cys^{24} to Glu^{149}. Multiple alignments revealed that four cysteine residues (Cys^{24}, Cys^{35}, Cys^{53}, Cys^{148}) involved in the formation of the CRD internal disulphides, two motifs for Ca^{2+}-dependent carbohydrate binding, EPN (Glu-Pro-Asn) and WND (Trp-Asn-Asp), were well found in VpCTL (Fig. 2).

Homology and Phylogenetic analysis

BLAST analysis showed that VpCTL exhibited moderate similarities with C-type lectins from *O. fasciatus* (ACY66646.1, 36 % identity), *C. farreri* (ADF87943.1, 35 % identity), *Verasper variegatus* (BAE44114.2, 35 % identity), *Salmo salar* (NP_001140055.1, 34 % identity), and *Mus musculus* (NP_081232.2, 35 % identity).

To evaluate the molecular evolutionary relationships of VpCTL with other C-type lectins, a phylogenetic tree was constructed based on the amino acid sequences of 12 C-type lectins by the NJ method (Fig. 3). In the phylogenetic tree, the VpCTL was firstly clustered with C-type lectin from *C. farreri*, and then formed a sister group with *Haliotis discus discus*. Other species relationships

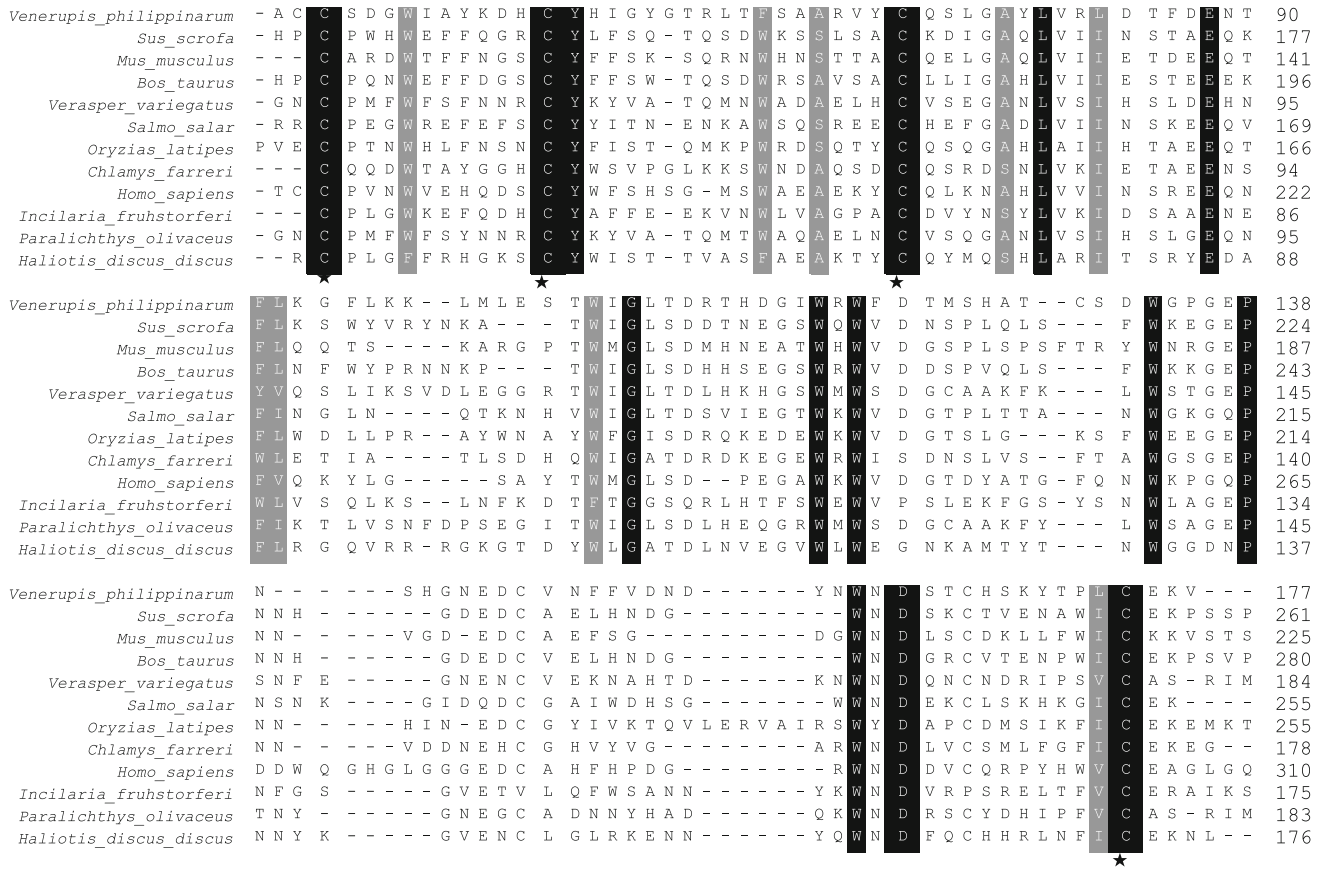

Fig. 2 Multiple alignment of different species of C-type lectin. The amino acid residues with *black* background are totally conserved, the *grey* background are conserved more than 80 %, and the *stars* are the conserved cysteine. The sequence were as follows: *Haliotis discus discus* (ABO26594), *Paralichthys olivaceus* (ACY70392), *Incilaria fruhstorferi* (BAA19862), *Homo sapiens* (NP 006335), *Chlamys farreri* (ADF87943), *Oryzias latipes* (BAD93253), *Salmo salar* (NP_001140055), *Verasper variegatus* (BAE44114), *Bos taurus* (NP 001139228), *Mus musculus* (AAI07190), *Sus scrofa* (NP 001123444)

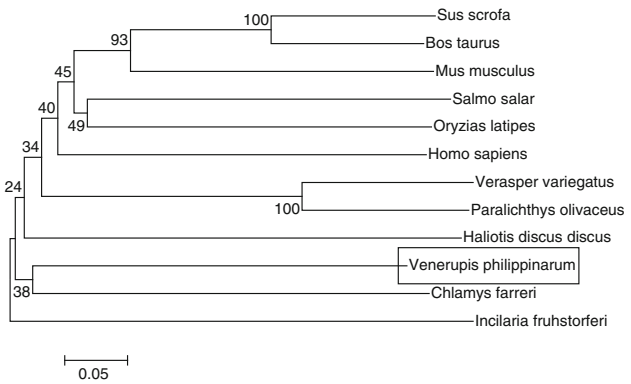

Fig. 3 Phylogenetic tree constructed by the neighbor-joining method. *Numbers* next to the branches indicated bootstrap value of each internal branch in the phylogenetic tree nodes from 1,000 replicates. The sequence were as follows: *Haliotis discus discus* (ABO26594), *Paralichthys olivaceus* (ACY70392), *Incilaria fruhstorferi* (BAA 19862), *Homo sapiens* (NP 006335), *Chlamys farreri* (ADF87943), *Oryzias latipes* (BAD93253), *Salmo salar* (NP_001140055), *Verasper variegatus* (BAE44114), *Bos taurus* (NP 001139228), *Mus musculus* (AAI07190), *Sus scrofa* (NP 001123444)

displayed in the phylogenic tree were in generally agreement with traditional taxonomy.

Tissue-specific expression of VpCTL

To examine the tissue distribution profile of VpCTL, total RNA was isolated from tissues of hepatopancreas, gill, muscle, haemocytes, mantle and foot. For VpCTL and β-actin genes, only one peak was detected at the corresponding melting temperature during the dissociation curve analysis, indicating the specificity of the PCR (data not shown). The mRNA transcript of VpCTL was found to be constitutively expressed in a wide range of tissues with the highest expression level in haemocytes and the lowest level in muscle (Fig. 4).

The expression level of VpCTL after bacterial challenge

The temporal expression of VpCTL gene in haemocytes after *L. anguillarum* challenge was detected by Real-time

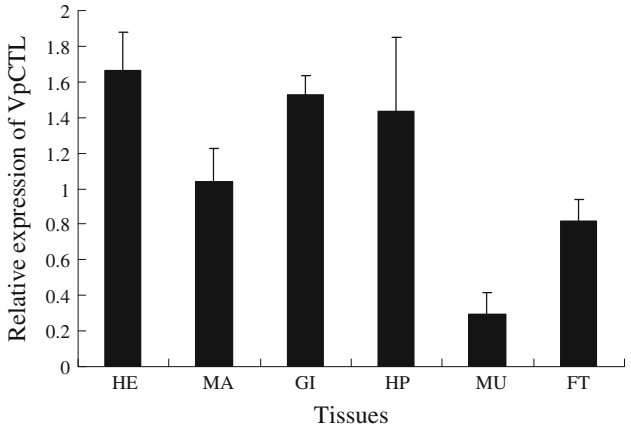

Fig. 4 VpCTL expression level in different tissues measured by quantitative real-time PCR. Each *symbol* and *vertical bar* represents the mean ± SE. HP, GI, MU, HE, MA and FT represented hepatopancreas, gill, muscle, haemocyte, mantle and foot, respectively

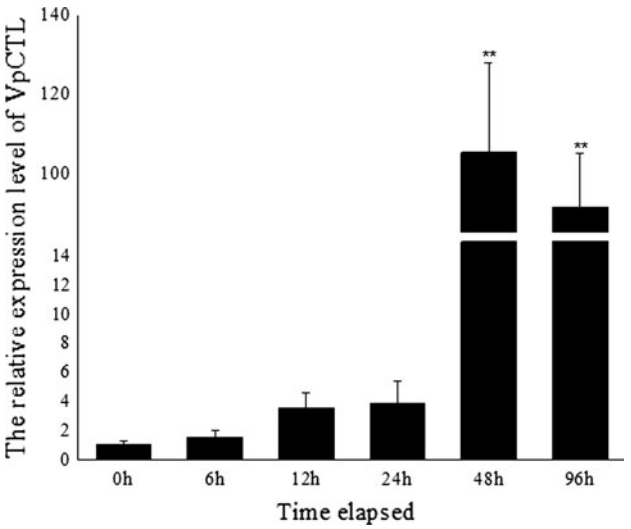

Fig. 5 Temporal expression of VpCTL transcript in haemocytes of clams after *Listonella anguillarum* infection measured by quantitative real-time PCR at 0, 6, 12, 24, 48 and 96 h. Each bar represented mean ± SE. Significant difference was indicated with two asterisks at $P < 0.01$

PCR assay (Fig. 5). In the *L. anguillarum* challenged group, the expression of VpCTL transcript increased gradually from 0 to 24 h, then the expression level was significantly up-regulated to 97-fold ($P < 0.01$) and 84-fold ($P < 0.01$) of the control group at 48 and 96 h.

Discussion

C-type lectins are a superfamily of carbohydrate-recognition proteins which play crucial roles in the innate immunity. Previous studies have demonstrated that C-type lectins play a key role in immune recognition in invertebrates as PRRs [14, 25, 26]. However, the research of C-type lectins in marine mollusks is still in the early stage. The most important feature of C-type lectins was to bind specific carbohydrate ligands on cell surface and agglutinate cells in a Ca^{2+}-dependent manner [27]. In each CRD, there is a typical double-loop stabilized by three or two pairs of disulfide bridges and four Ca^{2+}-binding sites, among which the Ca^{2+}-binding site 2, consisting of EPN (or EPT, EPH) and WND (or WCNT) motifs, is believed to be essential to the recognition of carbohydrate ligand [4]. Two binding sites containing an EPN motif and a WND motif were found in VpCTL. All these characterization suggested that VpCTL should be a new member of C-type lectin and may be involved in immune response to against bacterial challenge.

In the present study, the VpCTL transcript was constitutively expressed in the tissues of hepatopancreas, gill, muscle, haemocyte, mantle and foot, with the high expression level in haemocytes and hepatopancreas. The high expression level of VpCTL transcript in hepatopancreas conform to the demonstration that hepatopancreas played key roles in innate immunity for defense against the pathogen [28]. In addition, haemocytes were believed to play a central role in mollusk defense responses by many pathophysiological events, such as respiratory burst, phagocytosis and encapsulation of foreign bodies [29]. In order to better understand it‘s potential roles in clams, the spatial and temporal expression pattern of VpCTL in haemocytes was investigated by RT-PCR. After *L. anguillarum* challenge, the temporal expression of VpCTL transcript in haemocytes was up-regulated 97-fold ($P < 0.01$) and 84-fold ($P < 0.01$) compared to the control group at 48 and 96 h, respectively. Similar expression pattern of C-type lectins after microbe challenge has been reported in scallop. For example, the mRNA level of CfLec-3 in haemocytes of *C. farreri* was significantly up-regulated at 8 and 12 h post-challenge [16]. In the present study, the expression of VpCTL was perhaps up-regulated to minimize the negative effects with the accumulation of hazard effect caused by bacterial stimulation. However, the explicit roles of VpCTL in immune responses need to be explored in the future.

In conclusion, full-length cDNA of a C-type lectin (designated as VpCTL) was identified from *V. philippinarum*. The VpCTL mRNA was dominantly expressed in the haemocytes and hepatopancreas of the *V. philippinarum*. After *Listonella anguillarum* challenge, the temporal expression of VpCTL mRNA was significantly up-regulated, which suggested the involvement of VpCTL in the immune responses to *L. anguillarum* challenge.

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