GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

A C-type lectin fold gene from Japanese scallop *Mizuhopecten yessoensis*, involved with immunity and metamorphosis

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Genet. Mol. Res. 14 (1): 2253-2267 (2015)
Received March 21, 2014
Accepted September 7, 2014
Published March 27, 2015
DOI http://dx.doi.org/10.4238/2015.March.27.11

ABSTRACT. C-type lectins are a superfamily of Ca^{2+}-dependent carbohydrate-recognition proteins that are well known for their participation in pathogen recognition and clearance. In this study, a putative C-type lectin fold (MyCLF) gene was identified from the Japanese scallop *Mizuhopecten yessoensis*. The full-length of MyCLF was 645 bp, encoding a polypeptide of 167 amino acids. MyCLF carried a signal peptide of 20 amino acid residues, and a single carbohydrate recognition domain, having relatively high amino acid sequence conservation with C-type lectins reported for other bivalves. The expression of MyCLF mRNA transcripts in adult tissues, after bacterial challenge and during different developmental stages was determined using real-time quantitative RT-PCR. MyCLF was mainly distributed in the mantle, gill, and kidney. The expression of MyCLF clearly increased 3 h after *Vibrio anguillarum* challenge, and dropped to a minimum level after 9 h compared to the control group. During

embryonic development, the expression level increased in the gastrulae, trochophore and early D-shaped larvae, decreased in D-shaped larvae, and then increased hundreds of times in metamorphosing larvae. The results suggested that MyCLF was involved in an immune response and it may play important roles during the metamorphosis phase of *M. yessoensis*.

Key words: C-type lectin fold; Cloning; Metamorphosis; *Mizuhopecten yessoensis*; mRNA expression

INTRODUCTION

Lectins, widely distributed throughout living organisms, are known to be important factors of the immune system in bivalves. Like most invertebrate animals, mollusks lack an adaptive immune system, and therefore rely mainly on their innate immune response to defend against various pathogens (Fujita, 2002; Loker et al., 2004). The innate immune system is composed of cellular and humoral components that have the ability to bind the conserved structures on the surfaces of pathogens, of which C-type lectins represent one family that recognize and bind to specific carbohydrate structures on the surface of microorganisms and take part in mediating the non-self antigen (Yu et al., 1999; Christophides et al., 2002). C-type lectins are Ca^{2+}-dependent glycan-binding proteins that have a C-type lectin fold, and a C-type lectin fold is a ligand-binding motif not necessarily restricted to binding sugars. Actually, proteins with C-type lectin fold could bind other proteins, lipids, inorganic molecules or ice by the motif (Varki et al., 2009).

In some cases, lectins have been suggested to participate in mechanisms other than defense. Early in the 1980s, lectins were suggested to be involved with metamorphosis of the larvae of the spriorbid polychaete *Janua (Dexiospira) brasiliensis* (Grube), and a biochemical model for settlement and metamorphosis of the larvae involving lectins was proposed (Kirchman and Mitchell, 1981; Kirchman et al., 1982). Recently, four lectins have been investigated in different larval stages of the marsh frog *Pelophylax ridibundus* (Kaptan et al., 2013), and the results showed that the changes in lectin binding in metamorphosis may be an indication of some cellular events occurring in larval metamorphosis, such as cell differentiation. In marine invertebrates, there are usually several metamorphosis events in early development, during which the internal structure, external morphology, physiological processes and life behavior of larvae change a lot. Studies have been carried out to probe into the processes of metamorphosis in some marine organisms, and lectins have been suggested to be involved with settlement, metamorphosis, and tissue remodeling (Davidson and Swalla, 2002; Woods et al., 2004; Roberts et al., 2007; Grasso et al., 2008). However, there are few preceding analogous reports in bivalves.

In bivalves, C-type lectins have been identified and implicated in defense mechanisms or pathogen clearance. For example, a C-type lectin (CfLec-1) cloned from the Zhingkong scallop *Chlamys farreri* is able to aggregate bacteria and inhibit bacterial growth (Wang et al., 2007). AiCTL1, a C-type lectin identified from the bay scallop *Argopecten irradians* is believed to participate in wound healing (Zhu et al., 2008). Furthermore, a C-type lectin (CvML) cloned from the eastern oyster *Crassostrea virginica* has revealed its potential role in particle capture or mucosal immunity (Jing et al., 2011). Additionally, C-type lectins have been identified in the flat oyster *Ostrea chilensis* (Minamikawa et al., 2004), manila clam *Ru-*

ditapes philippinarum (Kang et al., 2006), horse mussel *Modiolus modiolis* (Tunkijjanukij et al., 1997), Pacific oyster *Crassostrea gigas* (Yamaura et al., 2008) and other bivalves.

Up to now, the corresponding work on the Japanese scallop has not been reported and little is known about C-type lectin in this species. The Japanese scallop, *Mizuhopecten yessoensis*, is an economically important bivalve which is distributed widely in the cold seas along the coasts of the northern islands of Japan, the northern part of the Korean Peninsula, Russian Primorye, and the Sakhalin and Kuril Islands (Nagashima et al., 2005). Since its introduction into China in 1982, *M. yessoensis* has become one of the most favorite commercial species in northern China. In this study, a gene with C-type lectin fold (designated MyCLF) was cloned from the Japanese scallop. The level of MyCLF mRNA transcripts expression in adult tissues, following stimulation by *Vibrio anguillarum,* and the expression pattern of the scallop during embryonic development were measured by QRT-PCR, with the hope of enriching the study of lectins and contributing to the understanding of the possible potential function of C-type lectins.

MATERIAL AND METHODS

Scallops and bacterial challenge

Adults of *M. yessoensis* (shell length 7.2-7.6 cm) used in this study were collected from Zhangzidao Fishery Group Co., Dalian, China, and kept in tanks containing 2000 L aerated fresh seawater at 15°C for 1 week before processing. The seawater was refilled 100% daily and the scallops were fed with *Isochrysis galbana* at a concentration of 30,000 cells/mL a day. After one week, different tissues (hemocytes, gill, kidney, hepatopancreas, mantle, and adductor muscle) were collected and flash frozen at -80°C for investigating the expression of tissue-specific mRNA transcripts of MyCLF, and the rest of the scallops were used for bacterial challenge experiment. Each tissue from 5 scallops was pooled together as one sample, and three replicates were employed.

V. anguillarum used in the bacterial challenge experiments was grown in Luria-Bertani (LB) medium at 30°C. The cells were harvested and washed three times with PBS using centrifugation, then resuspended in sterilized seawater to a concentration of 10^8 cell/mL, and directly used to challenge the scallops. The scallops were divided into control and experimental groups. Individuals from the experimental group were each injected with 50 μL *V.anguillarum* at the adductor muscle, whereas individuals from the control group were each injected with 50 μL sterilized seawater. The injected scallops were returned to the tanks containing seawater and the hemolymphs were collected with a syringe from the adductor muscles at 0, 3, 6, 9, 12, 24, and 36 h (hemolymphs from 5 individuals pooled together as one sample, with three replicates for each time point) post injection. The samples were centrifuged at 1000 *g*/4°C for 10 min to harvest the hemocytes and frozen at -80°C.

Embryonic and larval stage specimens of *M. yessoensis* (taken from zygote to metamorphosing stage) were obtained from the scallop culture base of Liaoning Ocean and Fisheries Science Research Institute, China. The embryo had been hatched at 11°C and the temperature was raised to 15°C once the larvae were in the early D-shaped larval stage. The specimens of zygote (0 h), blastula (25 h), gastrula (40 h), trochophore (58 h), early D-shaped larva (80 h), D-shaped larva (5 days), and metamorphosing larva (veliger prior to settlement, 23 days) were collected and flash frozen at -80°C for RNA extraction. Specimens of zygote, blastula,

gastrula, trochophore, early D-shaped larva contained about 50,000 to 80,000 individuals, while the quantity of D-shaped larvae was ~5000 and metamorphosing larvae ~60. Three extractions were performed for each developmental stage.

RNA extraction and cDNA synthesis

Total RNA was isolated from *M. yessoensis* using RNAprep Pure Tissue kit (Tiangen Biotech, Beijing). The quality and quantity of total RNA were assessed by both agarose gel electrophoresis and UV-spectrophotometry. For rapid amplification of cDNA ends (RACE) technology, the first strand cDNA was synthesized with SMART™ RACE cDNA Amplification kit (Clontech, Mountain View). A 5-μL reaction mixture containing ~450 ng total RNA, 1 μL 5'-CDS primer A, 1 μL SMART II A oligo for 5'-RACE-Ready cDNA and RNase-free dH_2O was incubated at 70°C for 2 min, followed by cooling on ice for 2 min. First-Strand buffer, DTT, dNTP mix and MMLV reverse transcriptase were then added according to manufacturer instructions to a final volume of 10 μL. The mixture was kept at 42°C for 1.5 h to obtain the RACE-Ready first-strand cDNA. For real time PCR, the cDNA was synthesized using PrimeScript RT Reagent kit (Takara, Dalian). A 10-μL reaction mixture containing ~450 ng total RNA, 50 pM Random 6 mers, 25 pM Oligo dT Primer, 1 pM specific primer, appropriate amounts of PrimeScript buffer and RNase-free dH_2O was kept at 37°C for 15 min, then at 85°C for 5 s.

Cloning of the full-length cDNA

The expressed sequence tag (EST) sequence with the complete 3'-end of MyCLF was obtained from the *M. yessoensis* cDNA library (GenBank accession No. GR867931), and RACE technology was employed to confirm the known sequence and to obtain the full-length MyCLF gene. 5'-RACE was carried out with the SMART™ RACE cDNA Amplification kit, with the specific primers Lec5out, Lec5in listed in Table1. Following the manufacturer instructions, nested PCR using corresponding outer primer and inner primer provided by the kit was performed. The conditions for PCR with outer primers were as follows: 5 cycles at 94°C for 30 s and 72°C for 1.5 min; 5 cycles at 94°C for 30 s, 70°C for 30 s, and 72°C for 1.5 min; 25 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 1.5 min. The second round of PCR with inner primers consisted of 25 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 1.5 min.

The PCR products were separated by agarose gel electrophoresis and purified using the Agarose Gel DNA Fragment Recovery kit (Takara, Dalian). The purified DNA was cloned into pMD-19T Vectors (Takara, Dalian) and subjected to DNA sequencing (Takara, Dalian).

Sequence analysis

The predicted amino acid sequence obtained from ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi) was analyzed by the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to search for similar sequences. Multiple sequences of C-type lectins were aligned using the Clustalx1.83 program with default settings, and the results were displayed by GeneDoc. Phylogenetic tree of C-type lectins was constructed by the neighbor-joining (NJ) method and drawn with the MEGA4 software with 1000 bootstraps. The structure of MyCLF was acquired from SWISS-MODEL (http://swissmodel.expasy.org/), a fully automated pro-

tein structure homology-modeling sever. ProtParam (http://web.expasy.org/protparam/) was used to compute the physico-chemical parameters of the protein sequence, and SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the presence and location of signal peptide cleavage sites.

QRT-PCR analysis

QRT-PCR analysis was carried out with an Mx3000p real-time thermal cycler using the PrimeScript RT Reagent kit (Takara, Dalian). The expression of MyCLF mRNA was measured in different adult tissues (hemocytes, gill, kidney, hepatopancreas, mantle, and adductor muscle), following bacterial challenge (0, 3, 6, 9, 12, 24, and 36 h) and during different stages (zygote, blastula, gastrula, trochophore, early D-shaped larva, D-shaped larva, and metamorphosing larva). We had previously evaluated the stability of six reference genes in *M. yessoensis* QRT-PCR using geNorm (http://medgen.ugent.be/genorm/), which helped to determine the most stable reference genes from β-actin, glyceraldehydes-3-phosphate dehydrogenase, α-tubulin, cytochrome b, TATA box binding protein-associated factor and elongation factor 1α, and verified the variation in their stability under different experimental conditions (Bao et al., 2011). According to the results obtained, 2 reference genes were used simultaneously for a more accurate quantitative result; α-tubulin and TATA box binding protein-associated factor genes were used as the reference genes to analyze the expression in different tissues and developmental stages (Table 1), whereas glyceraldehydes-3-phosphate dehydrogenase and α-tubulin were chosen for the bacterial challenge experiment (Table 1).

Two MyCLF gene-specific primers CQLectF and CQLectR (Table 1) were designed to amplify a product of 147 bp from cDNA. Reverse transcription was carried out with the same reaction volume and same experimental conditions described earlier to minimize experimental variation. For real-time PCR, the sample contained 10 μL SYBR Premix Ex Taq, 0.8 μL gene-specific primers and 1 μL of diluted cDNA template, and made up to a final volume of 20 μL. The PCR amplification was performed under the following conditions: initial denaturation at 95°C for 30 s, 40 cycles at 95°C for 15 s, 58°C for 25 s and 72°C for 25 s. The specificity of QRT-PCR products was checked by dissociation curves.

Following the geNorm program manual, Ct values of reference genes and MyCLF were transformed to quantities by using the comparative Ct method. After the quantities were inputted, the normalization factor values of the two reference genes (the geometric mean of the two reference genes) were automatically calculated by geNorm, and the normalized MyCLF expression levels could be calculated by dividing the quantities for each sample by the appropriate normalization factor. There were three replicates and the standard deviation was calculated according to the mathematical formulas in the geNorm manual (Vandesompele et al., 2002). The data of bacterial challenge were analyzed by the Student *t*-test, and data of adult tissues and during different developmental stage were analyzed by one way-ANOVA with the SPSS 16.0 software. Differences were considered to be statistically significant if $P < 0.05$.

RESULTS

cDNA cloning and sequence analysis of MyCLF

The full-length cDNA of MyCLF obtained (GenBank accession No. KF805984) con-

sisted of a 33-bp 5' untranslated region (UTR), 504-bp open reading frame (ORF) and 108-bp 3' UTR that included the polyadenylation signal (AATAAA). The ORF encoded a polypeptide of 167 amino acids, which contained a putative signal peptide of 20 amino acids. The deduced MyCLF had a theoretical mass of 19.79 kDa and a theoretical isoelectric point of 9.21. The nucleotide and deduced amino acid sequences of MyCLF are shown in Figure 1.

```
1     CAT TCC TCT TGG TAA CCA CTG ACT ACA TCG AAG ATG AAT GTC CTG   45
                                          M   N   V   L          4
46    TTG ATG TCC CTG GTG TTT AGT GCC ATC ATG GTC ACA GAA GTG GCT   90
5      L   M   S   L   V   F   S   A   I   M   V   T   E   V   A  19
91    GGC TGG GGC TGT CCG AGG GGA TGG GTG CCT TTC AGG AAA CAT TGC   135
20    G↓  W   G   C   P   R   G   W   V   P   F   R   K   H   C   34
136   TAT TAC TTC AGC AAA TCA AAC GAA AAA CAG TCC TGG TTC CAA GCA   180
35     Y   Y   F   S   K   S   N   E   K   Q   S   W   F   Q   A  49
181   TCG TTT ACC TGT GCT GCT GCG GGA GGG GCT CTC GTC TCA TTT AAC   225
50     S   F   T   C   A   A   A   G   G   A   L   V   S   F   N  64
226   AGT CCA TCG GAG TTA TGG TGG GTC AAG AGA TAC GCC AGA AGA TTA   270
65     S   P   S   E   L   W   W   V   K   R   Y   A   R   R   L  79
271   CGC CGG GGA GAT TTC TGG CTC GGC GGA AAT GAC ATC AGA CAA GAG   315
80     R   R   G   D   F   W   L   G   G   N   D   I   R   Q   E  94
316   GGA AAG TGG GTG TGG AAT CCG ACT AAC GGA TTC CCT GTC AAC AGA   360
95     G   K   W   V   W   N   P   T   N   G   F   P   V   N   R  109
361   GAC TGT ACA GAC TGG GCC AGA GGG GAA CCA AAC AGC TAT AGA GGT   405
110    D   C   T   D   W   A   R   G   E   P   N   S   Y   R   G  124
406   AGA AAC CAG GAC TGT ATG CTG ATG TGG GCC AGG AGG GAC TTC CGC   450
125    R   N   Q   D   C   M   L   M   W   A   R   R   D   F   R  139
451   TGG GAC GAC GAC GAG TGC CAC CTT CAA AGG TCG TTC ATT TGT GAA   495
140    W   D   D   D   E   C   H   L   Q   R   S   F   I   C   E  154
496   AGA TTC GTG TTT TCA GAA AAG GTG TGC AGA TGT CGG GTG TAG GAG   540
155    R   F   V   F   S   E   K   V   C   R   C   R   V   *      
541   GTT GAC ACG CTC ACA GTA TAT ATC AAG TAC GTG TAT AGG CAG AGT   585
586   CAC GGG ATG TAA CAG TAG AAC AAT AAA GAG TCC TGA AGA CAT TAA   630
631   AAA AAA AAA AAA AAA   645
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Figure 1. Nucleotide and deduced amino acid sequences of MyCLF. The putative signal peptide and its cleavage site are indicated by double-underlines and vertical arrow, respectively. Conserved cysteine residues are shaded, whereas two extra cysteine residues are marked with a dot. The EPN motif involved in mannose-binding is underlined. Asterisk indicates the stop codon, and boxed nucleotides indicate the consensus polyadenylation signal.

Homology analysis and characterization of MyCLF

The sequence of MyCLF was blast against the NCBI database, and the results showed a good match with the C-type lectin superfamily proteins (Figure 2). MyCLF displayed several

characteristics of C-type lectins, including a CRD consisting of 132 residues (from Cys^{23} to Glu^{154}) bearing the "EPN" motif (Glu^{118}-Pro^{119}-Asn^{120}), determinant for mannose specificity, and the four conserved cysteine residues (Cys^{53}-Cys^{153},Cys^{129}-Cys^{145}) involved in the formation of the disulfide bonds (Drickamer and Taylor, 1993). In addition, MyCLF showed the conserved WDD residues (Trp^{140}-Asp^{141}-Asp^{142}) considered to be the principal site for calcium binding, and the QDC residues (Gln^{127}-Asp^{128}-Cys^{129}) thought to represent the secondary calcium binding site (Drickamer, 1988, 1993). To compare the sequence of MyCLF with those of C-type lectins from other bivalves, MyCLF was aligned with the C-type lectins of *C. farreri*, *Crassostrea gigas* and *C. virginica.* Among these, the highest identity was found with *C. virginica* (32% identity, 47% positive) and the lowest with *A. irradians* (29% identity, 45% positive).

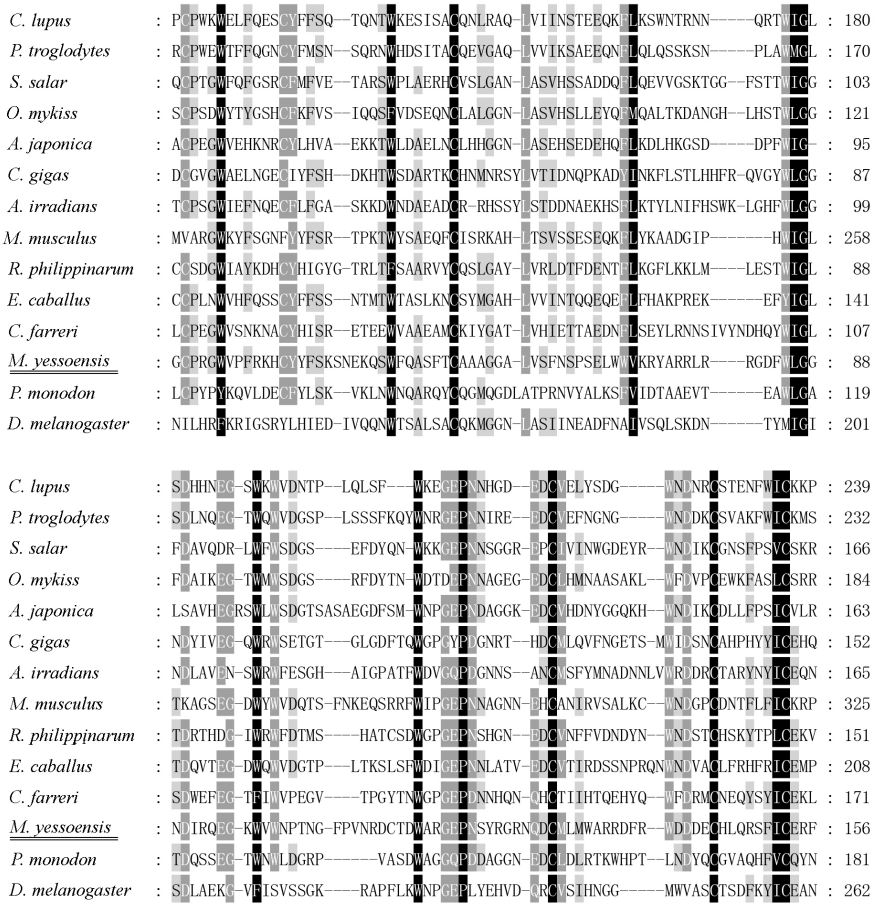

Figure 2. Multiple alignment of the carbohydrate recognition domains (CRDs) of MyCLF with the CRDs of other C-type lectin genes deposited in GenBank. All the conserved amino acid residues are shaded for homology comparison. The species and the GenBank accession numbers are as follows: NP_001124304.1 (*Canis lupus familiaris*), NP_001123945.1 (*Pan troglodytes*), AAO43604.1 (*Salmo salar*), AAM21196.1 (*Oncorhynchus mykiss*), BAB47156.2 (*Anguilla japonica*), BAF75353.1 (*Crassostrea gigas*), ABZ89710.1 (*Argopecten irradians*), AAK01952.1 (*Mus musculus*), ACU83213.1 (*Ruditapes philippinarum*), XP_001492844.2 (*Equus caballus*), ABB71672.1 (*Chlamys farreri*), AAZ29608.1 (*Penaeus monodon*), AAY34943.1 (*Drosophila melanogaster*).

As shown in Figure 3, the highly conserved cysteines C1 (Cys^{153}) and C4 (Cys^{53}) form the disulfide bridge linking β5 and α1, and the disulfide bridge formed by C2 (Cys^{129}) and C3 (Cys^{145}) links β3 and β5. Two extra cysteines, C0 (Cys^{23}) and C0' (Cys^{34}), also form a disulfide bridge, which is specific for the long-form C-type lectins (so-called because of a short N-terminal extension that forms a β-hairpin at the base of the domain) (Zelensky and Gready, 2005).

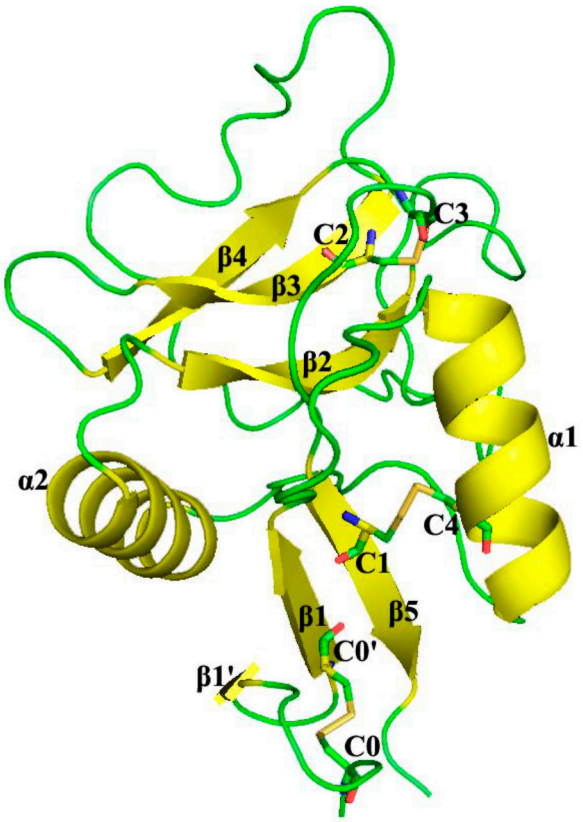

Figure 3. Predicted three-dimensional structure of MyCLF. α-helics and β-strands were in yellow. Cys^{153}, Cys^{53}, Cys^{129}, Cys^{145}, Cys^{23}, and Cys^{34} are shown in stick representation.

Phylogenetic analysis of MyCLF

To analyze the genetic relationship between MyCLF and other C-type lectins, a phylogenetic tree was constructed using the NJ method (Figure 4). The tree depicted the C-type lectins from 14 different species as two distinct groups. The first cluster consisted of some vertebrates and *R. philippinarum,* whereas the second cluster consisted of some invertebrates. The relationship between mammals and fishes depicted in the phylogenetic tree is consistent with the concept of classical taxonomy. However, little similarity was found between the C-type lectins of the mollusks, suggesting that there may be different evolutionary pathways or functions.

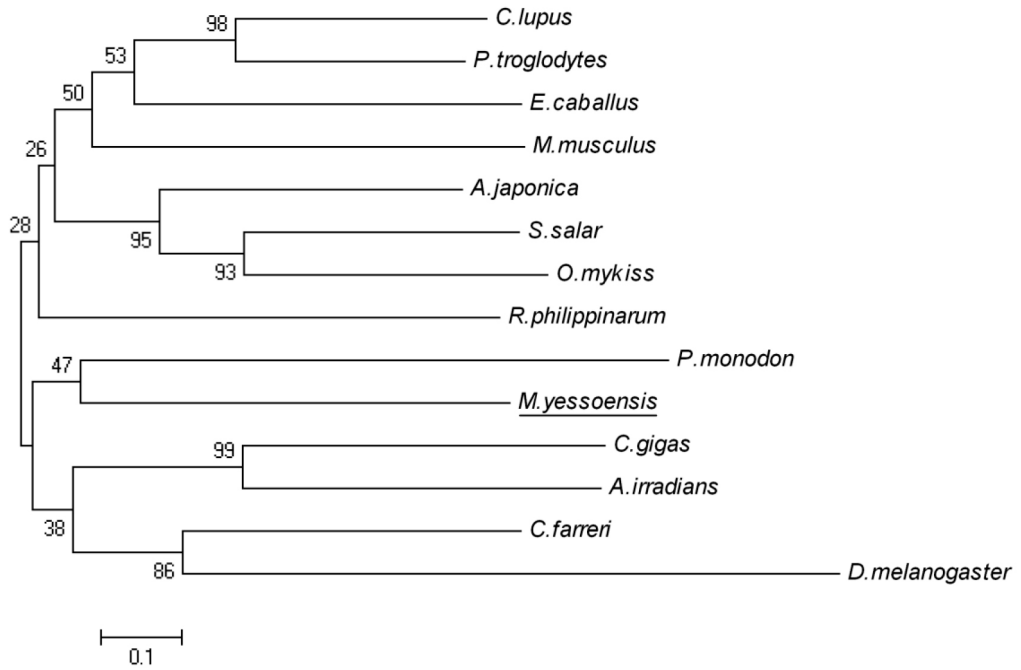

Figure 4. Consensus neighbor-joining tree based on the sequences of MyCLF and other C-type lectins. The numbers at the forks represented the bootstrap values for 1000 replications. The protein sequences used are the same as those in Figure 2.

QRT-PCR of MyCLF expression

The expression of MyCLF in *M. yessoensis* was investigated by measuring the levels of MyCLF mRNA transcripts in adult tissues, after bacterial challenge and during developmental stages of the scallops. The expression levels were measured using QRT-PCR. The melting curves of MyCLF and reference genes showed only one peak, and the amplification efficiencies ranged from 99.6 to 102.3% (Table 1), which indicated specific and highly efficient amplifications.

Table 1. Oligonucleotide primers used in the experiments.

Primer name	Sequence (5'-3')	Primer information	Amplification efficiency (%)
Lec5'OT	ACGATGCTTGGAACCAGGACTGT	5'RACE outer primer	-
Lec5'IN	AGCAATGTTTCCTGAAAGGCACCC	5'RACE inner primer	-
CQLectF	CATCGTTTACCTGTGCTGCTG	QRT-PCR for MyCLF	101.1
CQLectR	CCCACTTTCCCTCTTGTCTGA	QRT-PCR for MyCLF	
CQTubuF	AACACCTTCTTCAGTGAGACG	QRT-PCR for α-tubulin	99.8
CQTubuR	ATTGTGTAGTGACCTCTTGCG	QRT-PCR for α-tubulin	
CQTaF	TGATGTGGAAGAGGTACTCCTGC	QRT-PCR for TATA box binding protein-associated factor	99.6
CQTaR	ATTCCAGTTCCTCTCAAGGTGG	QRT-PCR for TATA box binding protein-associated factor	
CQGpdF	TGGTATGGCTTTCCGTGTGC	QRT-PCR for Glyceraldehydes-3-phosphate dehydrogenase	102.3
CQGpdR	TCCTCTGTGTAACCAAGGAACC	QRT-PCR for Glyceraldehydes-3-phosphate dehydrogenase	

MyCLF could be detected widely in all the tissues examined, including the hemocytes, gill, kidney, hepatopancreas, mantle, and adductor muscle (Figure 5). The highest expression level was found in mantle ($P < 0.05$). By contrast, the MyCLF mRNA transcripts in gill and kidney were at a medium level, which was about 0.4-fold of that in mantle. The transcripts in hepatopancreas, hemocytes and adductor muscle were at a low level, and the expression level in hepatopancreas was significantly lower than in hemocytes. No significant difference was observed between hepatopancreas and adductor muscle ($P < 0.05$).

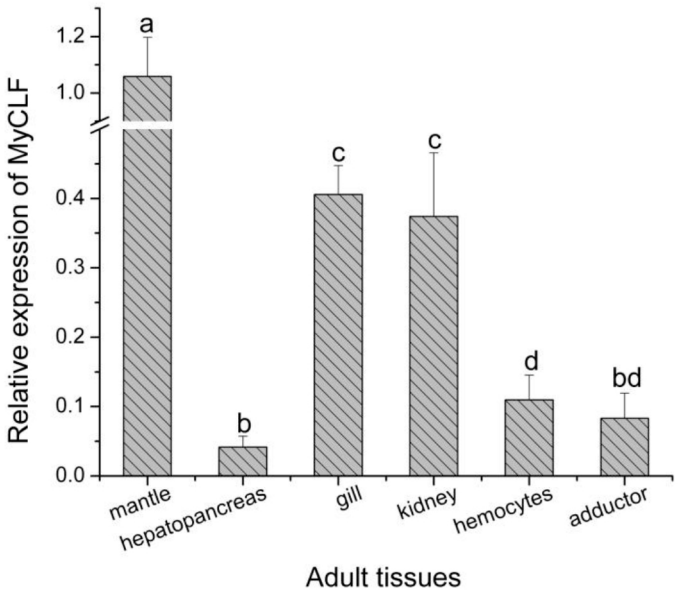

Figure 5. Tissue distribution of MyCLF mRNA level in *Mizuhopecten yessoensis* as measured by QRT-PCR. Data are reported as means ± SD (N = 3). Letters above the columns indicate significant differences ($P < 0.05$) among different tissues.

The expression of MyCLF in *M. yessoensis* in response to bacterial challenge was also investigated. The temporal expression of MyCLF after *V. anguillarum* challenge is shown in Figure 6. At 3 h after injection, the expression level of MyCLF increased in both the control group and *V. anguillarum*-challenged group, and was the highest level among the different time points. Afterwards, the expression level of MyCLF in both groups decreased, but started to rise again at 9 h in the case of the control group and 12 h in the case of *V. anguillarum*-challenged group. Interestingly, the levels attained by both groups at these time points were similar. Thereafter, both groups showed a rapid decrease to less than 30% of the initial expression level, with the *V. anguillarum*-challenged group maintaining a higher level compared to control group right up to 36 h. The data between control group and *V. anguillarum*-challenged group showed a significant difference only at 3 h among the experimental time points.

During the different developmental stages, no expression of MyCLF was found in the zygote or blastula. The expression level increased gradually in the gastrula, trochophore and early D-shaped larva, and then decreased in the D-shaped larva with the expression level lower

than that of the trochophore but higher than the expression level of the gastrula. The expression level of MyCLF mRNA transcripts in the trochophore and early D-shaped larva were significantly higher than that of the gastrula. When the larvae entered the metamorphosing stages, the expression level increased substantially, as much as a few hundred times (Figure 7).

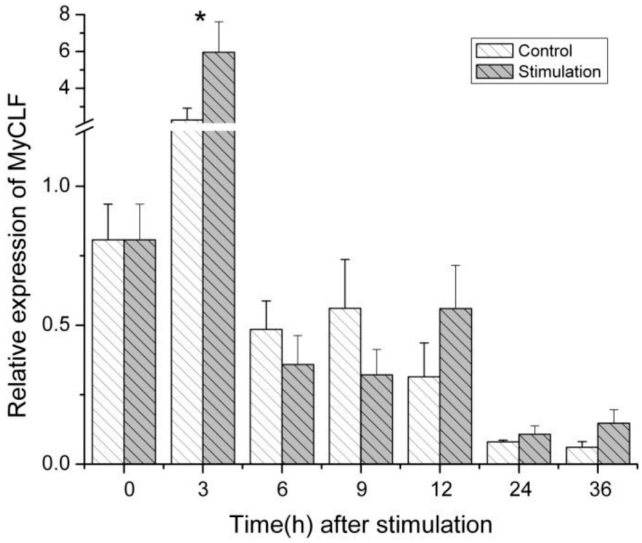

Figure 6. Temporal expression of MyCLF in the haemocytes of *Mizuhopecten yessoensis* after *Vibrio anguillarum* challenge. LSD *t*-test was used to perform statistical analysis of the results. *P < 0.05 indicated the significant difference between stimulation and control group.

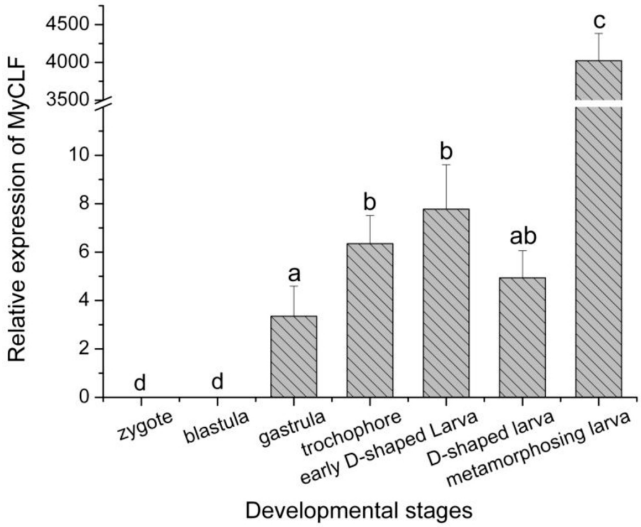

Figure 7. QRT-PCR analysis of MyCLF mRNA expression during the development of *Mizuhopecten yessoensis*. Specimens were collected in some classical developmental stages. Letters above the columns indicate significant differences (P < 0.05) among different developmental stages.

DISCUSSION

With the rapid development of transcriptomics, genomics and proteomics, studies about genes have attracted more attention. C-type lectins have been demonstrated to play important roles in the immunity of invertebrate. In this study, a C-type lectin fold of 645 bp was identified from the Japanese scallop *M. yessoensis* for the first time. The predicted signal peptide in the N-terminus of MyCLF indicated that it is likely to be a secretory protein that functions mainly as an extracellular protein. This is consistent with C-type lectin superfamily proteins, which is also a group of extracellular proteins (Zelensky and Gready, 2005).

Intracellular lectins mainly function in protein trafficking and sorting, while extracellular lectins are important for cell signaling and pathogen recognition (Wang and Wang, 2013) The 167-aa MyCLF also contained a CRD. Most C-type lectins recently characterized from invertebrates have only one CRD. Some members also have two or more CRDs, such as LPS-binding protein and multi-binding protein from *Bombyx mori* (Koizumi et al., 1999; Watanabe et al., 2006), immunolectins from *Manduca sexta* (Yu et al., 1999, 2005), Fclectin from *Fenneropenaeus chinensis* (Liu et al., 2007), multi-domain lectins from *C. farreri* (Zhang et al., 2009) and *A. irradians* (Wang et al., 2012). In the CRD region, MyCLF showed high conservation. Consistent with other known long-form C-type lectins, MyCLF contained four conserved cysteine residues involved in the formation of the disulfide bridges in CRD, two additional disulfide-bonded cysteine residues at the N-terminus specific for the long-form C-type lectins (Zelensky and Gready, 2005) and a ligand-binding motif "EPN" (Drickamer and Taylor,1993). In the CRDs of most known C-type lectins, the "EPN" or "QPD" motifs appear to determine the specificity of carbohydrate binding, and they are critical for the binding of carbohydrates (Fujita et al., 2004; Zelensky and Gready, 2005). Mannose (or similar sugars with the equatorial 3-OH and 4-OH) is preferred by "EPN", whereas galactose (or similar sugars with the axial 3-OH and equatorial 4-OH) is preferred by "QPD" (Weis et al., 1998; Sujatha and Balaji, 2004). In addition, the CRD of MyCLF also had two putative sites, "QDC" and "WDD", for calcium binding (Mullin et al., 1997; Gourdine et al., 2007) The structure of CRD and the dependence of calcium suggested that MyCLF is a typical C-type lectin.

According to the expression stability of reference genes reported in a previous study (Bao et al., 2011), we used different reference genes to measure the expression level of MyCLF mRNA transcripts, which led to more accurate and reliable quantitative results. In adults, MyCLF was expressed in all tissues examined, but mainly distributed in the mantle, gill, and kidney. The mantle and gill are involved in the process of continuous water exchange and food uptake from the outside environment, and the kidney is an important tissue for ion metabolism, making these tissues more susceptible to pathogen infection. Compared to other scallops in China, the tissue distribution of MyCLF in *M. yessoensis* differed from that of AiCTL1 in *A. irradians*, which shows the highest level in the hepatopancreas (Zhu et al., 2008). In the case of *C. farreri* CfLec-1, the expression was mainly detected in the gill and gonad, and marginally detectable in the mantle (Wang et al., 2007). Nevertheless, the tissue distribution of MyCLF suggested that it may provide an initial response to an invasion initiated by marine pathogenic microorganisms.

After *M. yessoensis* was challenged with *V. anguillarum*, a change in the level of MyCLF mRNA transcripts was observed. The direct injection of *V. anguillarum* caused an increase in MyCLF expression level in a short time, reaching a maximum at 3 h, and the expression level of the challenged group was significantly higher than that of the control group.

Afterwards, no significant difference in MyCLF expression level was detected between the bacteria-challenged and control groups. The expression level at 3 h significantly showed a response to *V. anguillarum* challenge, where the results still indicated the involvement of MyCLF in the mediation of pathogen recognition.

As lectins have been suggested to participate in the process of metamorphosis, the expression of MyCLF during embryonic development was also investigated here. During the embryonic stage, the expression of MyCLF was first observed in gastrulae, at which a series of cellular differentiation began, the rotating cilia appeared and the embryo started to rotate in the egg envelope. Based on the new cell-relationship formed in gastrulae, the primitive mouth appeared as an invagination on the ventral surface leading to the stomodaeum in the trochophore, while the larva began to elongate on its longitudinal axis and while the apical region became broader than the posterior region. At the stage of early D-shaped larva, the organism was enveloped by a transparent shell, through which the well-developed velum and newly formed digestive tract could be observed (Sastry, 1965). Of these stages, the expression level increased continuously. In the D-shaped larva, the morphology showed no great difference, except the enlargement of the individual. When entering the veliger prior to settlement stage (described as metamorphosing larvae), with the loss of velum and development of gill, the larvae crawled with the developed foot and began their filter-feeding. The expression level at this phase sharply increased. The expression level of MyCLF showed an interesting pattern during the developmental stages. In the gastrula, trochophore, early D-larva and veliger prior to settlement stage, there was a series of cell proliferation, migration, apoptosis and some visible development in internal structures and external morphology of the larvae. Meanwhile, the expression level of MyCLF showed a progressively increasing trend at these stages.

In recent years, there have been attempts to elucidate the possible potential function of lectins in settlement, metamorphosis and tissue remodeling in marine organisms. In *Herdmania curvata* (Woods et al., 2004) and *Boltenia villosa* (Davidson and Swalla, 2002; Roberts et al., 2007), lectins are differentially expressed at metamorphosis. In *B. villosa*, four lectins are upregulated in the larva or newly settled adult (Davidson and Swalla, 2002), and lectins play an important role in the recognition of bacteria, which induce metamorphosis and tissue remodeling (Roberts et al., 2007). In the coral *Acropora*, lectins are upregulated at metamorphosis, which suggests an analogous role in activating tissue remodeling (Grasso et al., 2008). A previous study investigating a C-type lectin gene in the American cockroach (*Periplaneta americana*) found that it was synthesized by epidermal cells, and then secreted into the regenerating leg saccule, where it is assembled around the myoblasts to form leg muscle fibers *in situ* (Arai et al., 1998). In our study, the significantly increased expression level in metamorphosing larvae could be involved with the 'recognition' or 'adhesion' function of lectins, and combined with the expression level of other phases, we hypothesize that MyCLF may play a more important role in metamorphosis or processes such as cell rearrangement and regeneration, rather than defense during embryonic development.

Since there is no adaptive immunity in scallops, the innate immune system plays a crucial role in defense against pathogens. The abundance of C-type lectins in invertebrates is thought to be the result of the need for an effective repertoire of defense in the natural environment. However, we are far from understanding the entire immune response mediated by different lectins. Taken together, these results indicated that MyCLF is probably involved in mediating the recognition of the invading bacteria. On the other hand, the different levels of MyCLF mRNA transcripts detected during the different stages of *M. yessoensis* development

also indicated that MyCLF may fulfill an important function during the metamorphosis or development of the organism. Further insight into the biological function of MyCLF could be obtained by acquiring the recombinant MyCLF protein (such as expressing it in *Escherichia coli*) and examining its agglutination or antibacterial activity. Finally, the mechanism by which MyCLF is regulated during embryonic development may also be of great importance.

ACKNOWLEDGMENTS

Research supported by the Science and Technology Project of Liaoning Province (#2011203003) and the Natural Science Foundation of Liaoning Province (#201102094). The authors would like to thank all the laboratory members for technical advice and helpful discussions.

REFERENCES

Arai T, Kawasaki K, Kubo T and Natori S (1998). Cloning of cDNA for regenectin, a humoral C-type lectin of *Periplaneta americana*, and expression of the regenectin gene during leg regeneration. *Insect Biochem. Mol. Biol.* 28: 987-994.

Bao XB, Liu WD, Jiang B, Su H, et al. (2011). Expression stability of reference genes for quantitative PCR in Japanese Scallop *Patinopecten yessoensis*. *Fish. Sci*. 30: 603-608.

Christophides GK, Zdobnov E, Barillas-Mury C, Birney E, et al. (2002). Immunity-related genes and gene families in *Anopheles gambiae*. *Science* 298: 159-165.

Davidson B and Swalla BJ (2002). A molecular analysis of ascidian metamorphosis reveals activation of an innate immune response. *Development* 129: 4739-4751.

Drickamer K (1988). Two distinct classes of carbohydrate-recognition domains in animal lectins. *J. Biol. Chem.* 263: 9557-9560.

Drickamer K (1993). Evolution of Ca^{2+}- dependent animal lectins. *Prog. Nucleic Acid Res. Mol. Biol.* 45: 207-232.

Drickamer K and Taylor ME (1993). Biology of animal lectins. *Annu. Rev. Cell. Biol.* 9: 237-264.

Fujita T (2002). Evolution of the lectin-complement pathway and its role in innate immunity. *Nat. Rev. Immunol.* 2: 346-353.

Fujita T, Matsushita M and Endo Y (2004). The lectin-complement pathway-its role in innate immunity and evolution. *Immunol. Rev.* 198: 185-202.

Gourdine JP, Markiv A and Smith-Ravin J (2007). The three-dimensional structure of codakine and related marine C-type lectins. *Fish Shellfish Immunol.* 23: 831-839.

Grasso LC, Maindonald J, Rudd S, Hayward DC, et al. (2008). Microarray analysis identifies candidate genes for key roles in coral development. *BMC Genomics* 9: 540.

Jing X, Espinosa EP, Perrigault M and Allam B (2011). Identification, molecular characterization and expression analysis of a mucosal C-type lectin in the eastern oyster, *Crassostrea virginica*. *Fish Shellfish Immunol.* 30: 851-858.

Kang YS, Kim YM, Park KI, Kim Cho S, et al. (2006). Analysis of EST and lectin expressions in hemocytes of Manila clams (*Ruditapes philippinarum*) (Bivalvia: Mollusca) infected with *Perkinsus olseni*. *Dev. Comp. Immunol.* 30: 1119-1131.

Kaptan E, Sengezer Inceli M and Sancar Bas S (2013). Lectin binding properties of liver, small intestine and tail of metamorphosing marsh frog (*Pelophylax ridibundus* Pallas 1771). *Acta Histochem.* 115: 595-602.

Kirchman D and Mitchell R (1981). A biochemical mechanism for marine biofouling. *Oceans 81 IEEE* 13: 537-541.

Kirchman D, Graham S, Reish D and Mitchell R (1982). Lectins may mediate in the settlement and metamorphosis of *Janua* (*Dexiospira*) *brasiliensis* Grube (Polychaeta: Spirorbidae). *Mar. Biol. Lett.* 3: 131-142.

Koizumi N, Imamura M, Kadotani T, Yaoi K, et al. (1999). The lipopolysaccharide-binding protein participating in hemocyte nodule formation in the silkworm *Bombyx mori* is a novel member of the C-type lectin superfamily with two different tandem carbohydrate-recognition domains. *FEBS Lett.* 443: 139-143.

Liu YC, Li FH, Dong B, Wang B, et al. (2007). Molecular cloning, characterization and expression analysis of a putative C-type lectin (Fclectin) gene in Chinese shrimp *Fenneropenaeus chinensis*. *Mol. Immunol.* 44: 598-607.

Loker ES, Adema CM, Zhang SM and Kepler TB (2004). Invertebrate immune systems - not homogeneous, not simple, not well understood. *Immunol. Rev.* 198: 10-24.

Minamikawa M, Hine M, Russell S, Huber P, et al. (2004). Isolation and partial characterization of a calcium-dependent

lectin (chiletin) from the haemolymph of the flat oyster, *Ostrea chilensis*. *Fish Shellfish Immunol.* 17: 463-476.
Mullin NP, Hitchen PG and Taylor ME (1997). Mechanism of Ca^{2+} and monosaccharide binding to a C-type carbohydrate-recognition domain of the macrophage mannose receptor. *J. Biol. Chem.* 272: 5668-5681.
Nagashima K, Sato M, Kawamata K, Nakamura A, et al. (2005). Genetic structure of Japanese scallop population in Hokkaido, analyzed by mitochondrial haplotype distribution. *Mar. Biotechnol.* 7: 1-10.
Roberts B, Davidson B, MacMaster G, Lockhart V, et al. (2007). A complement response may activate metamorphosis in the ascidian *Boltenia villosa*. *Dev. Genes. Evol.* 217: 449-458.
Sastry AN (1965). The development and external morphology of pelagic larval and post-larval stages of the bay scallop, *Aequipecten irradians concentricus* Say, reared in the laboratory. *Bull. Mar. Sci.* 15: 417-435.
Sujatha MS and Balaji PV (2004). Identification of common structural features of binding sites in galactose-specific proteins. *Proteins* 55: 44-65.
Tunkijjanukij S, Mikkelsen HV and Olafsen JA (1997). A heterogeneous sialic acid-binding lectin with affinity for bacterial LPS from horse mussel (*Modiolus modiolus*) hemolymph. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 117: 273-286.
Vandesompele J, De Preter K, Pattyn F, Poppe B, et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3: 34.1-34.11.
Varki A, Cummings RD, Esko JD, Freeze HH, et al. (2009). Essentials of Glycobiology. In: C-type Lectins. 2 nd edn. (Cummings RD and McEver RP, eds.). Cold Spring Harbor Laboratory Press, Long Island. Available at [http://www.ncbi.nlm.nih.gov/books/NBK1943/].
Wang H, Song LS, Li CH, Zhao JM, et al. (2007). Cloning and characterization of a novel C-type lectin from Zhikong scallop *Chlamys farreri*. *Mol. Immunol.* 44: 722-731.
Wang LL, Wang LL, Yang JL, Zhang H, et al. (2012). A multi-CRD C-type lectin with broad recognition spectrum and cellular adhesion from *Argopecten irradians*. *Dev. Comp. Immunol.* 36: 591-601.
Wang XW and Wang JX (2013). Diversity and multiple functions of lectins in shrimp immunity. *Dev. Comp. Immunol.* 39: 27-38.
Watanabe A, Miyazawa S, Kitami M, Tabunoki H, et al. (2006). Characterization of a novel C-type lectin, *Bombyx mori* multibinding protein, from the *B. mori* hemolymph: mechanism of wide-range microorganism recognition and role in immunity. *J. Immunol.* 177: 4594-4604.
Weis WI, Taylor ME and Drickamer K 1998. The C-type lectin superfamily in the immune system. *Immunol. Rev.* 163: 19-34.
Woods RG, Roper KE, Gauthier M, Bebell LM, et al. (2004). Gene expression during early ascidian metamorphosis requires signalling by Hemps, an EGF-like protein. *Development* 131: 2921-2933.
Yamaura K, Takahashi KG and Suzuki T (2008). Identification and tissue expression analysis of C-type lectin and galectin in the Pacific oyster, *Crassostrea gigas*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 149: 168-175.
Yu XQ, Gan H and Kanost MR (1999). Immulectin, an inducible C-type lectin from an insect, *Manduca sexta*, stimulates activation of plasma prophenol oxidase. *Insect. Biochem. Mol. Biol.* 29: 585-597.
Yu XQ, Tracy ME, Ling E, Scholz FR, et al. (2005). A novel C-type immulectin-3 from *Manduca sexta* is translocated from hemolymph into the cytoplasm of hemocytes. *Insect Biochem. Mol. Biol.* 35: 285-295.
Zelensky AN and Gready JE (2005). The C-type lectin-like domain superfamily. *FEBS J.* 272: 6179-6217.
Zhang H, Wang H, Wang LL, Song LS, et al. (2009). Cflec-4, a multidomain C-type lectin involved in immune defense of Zhikong scallop *Chlamys farreri*. *Dev. Comp. Immunol.* 33: 780-788.
Zhu L, Song LS, Xu W and Qian PY (2008). Molecular cloning and immune responsive expression of a novel C-type lectin gene from bay scallop *Argopecten irradians*. *Fish Shellfish Immunol.* 25: 231-238.