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Short communication

Molecular cloning of complement component C3 gene from pearl mussel, *Hyriopsis cumingii* and analysis of the gene expression in response to tissue transplantation

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ABSTRACT

Complement component C3 is well recognized as the central mediator of complement system, whose activation is responsible for the immune surveillance and elimination of non-self-antigens. In this study, C3 gene (HcC3) from a pearl making mussel, *Hyriopsis cumingii*, was successfully identified. The putative HcC3 possessed the canonical domains and highly conserved functional residues of C3 family members. In phylogenetic analysis, HcC3 was also clustered into C3 subfamily and separated from α2 macroglobulin clade. HcC3 gene was constitutively expressed in a wide range of tissues of pearl mussels, among which the immune-related tissues like hemocytes got highest expression. After allograft surgery of mantle tissues for aquaculture pearl production, the gene expression of HcC3 exhibited a rapid upregulation on day 1, dropped back on day 3, peaked the value on day 7, and restored to the level similar to control samples on day 14 after mantle allograft. The biphasic expression within the two weeks post the surgery suggests the important roles for HcC3 in alloimmune responses and an intricate complement activation mechanism in mollusks during tissue allograft.

1. Introduction

Complement refers to a tightly regulated network of proteins originally identified from mammalian serum, which alerts host cells of the presence of infectious agents and regulates the elimination of potential pathogens [1]. Among all the complement proteins, complement component 3 (C3) has been well recognized as the central mediator in keeping the complement system alert, activating all the known complement activation pathways, fueling the amplification of the complement responses, exerting direct opsonic and cytolytic effects on microbial pathogens, and coordinating downstream innate and adaptive immune effectors to provide animals a forceful defense against pathogens [2,3]. In recent years, C3 attracts more attention to its functional roles in maintaining vertebrate cell homeostatic process such as tissue regeneration and clearance of cellular debris, and its clinical potential as a therapeutic target to improve graft survival and patient health [4].

As the data of invertebrate C3 studies accumulates, it has been suggested that C3 preserves its immune sentinel function like the non-self discrimination and elimination of alien antigens throughout animal species ranging from primitive invertebrates to diverse lineage of the metazoans. C3 homologous genes have been successfully identified from various invertebrate species including mollusks [5–7]. In several bivalve mollusk species, gene expression analysis of C3s in response to lipopolysaccharides challenge or bacterial exposure provided molecular evidence that invertebrate C3 recognizes pathogenic microbials [8–10]. Moreover, a significant hemolytic activity of razor clam C3 has been demonstrated and that suggests the presence of a direct capability of mollusk C3 to eliminate exogenous blood cells [11]. However, to date it is still largely unknown how mollusk C3 responds to tissue transplantation.

Mantle tissue allograft in certain mollusk species has been worldwide employed for the cultivation of pearls and understanding of its regulatory mechanism is highly desirable for pearl aquaculture industry. Considered the known functions of C3, we performed cloning

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and gene expression analysis of C3 in triangle-shell pearl mussel, *Hyriopsis cumingii*, an important pearl aquaculture species in China, to get a better understanding of the mollusk alloimmune mechanism in response to mantle tissue transplantation.

2. Materials and methods

2.1. Triangle-shell pearl mussel culture and sampling

Thirty healthy triangle-shell pearl mussels with an average shell length of 9.5 cm (± 0.5 cm) were collected from Weiwang Pearl Farm of Jinhua City, Zhejiang Province, China. The mussels were maintained in one water tank containing 120 L of aerated freshwater at 24 °C and fed *Chlorella vulgaris*. The water was exchanged twice daily. After the culture of two weeks to acclimate the laboratory culture environment, mantle tissue allograft surgery, tissue anatomy, and hemocytes sampling were performed as described in Ref. [12,13]. For the study of C3 gene tissue distribution, the mussels without mantle transplantation were sacrificed first to collect adductor muscle, foot, gill, mantle, and hepatopancreas tissues. To investigate the time course expression of C3 gene in response to mantle allograft, the grafting mantle pieces and hemocytes from recipient mussels were sampled at 1, 3, 7, 14 days post the mantle grafting operation. At the same sampling time points, the mantle tissues and hemocytes from the mussels without tissue transplantation were collected as control samples. All the samples were harvested in triplicates and were stored in −70 °C.

2.2. C3 gene cloning and sequencing

Total RNA of mussel hemocytes was isolated using Isogen (Takara, Japan). The isolated RNA was further purified by Dynabeads® Oligo (dT) 25 (Invitrogen, USA) to synthesize cDNA. The cDNA was sequenced at a scale of 3 Gbase by an Illumina Hiseq™ 2500 system (Illumina, USA). Subsequently, *de novo* assembly of the raw sequence data were carried out with the assistance of CLC Genomics Workbench software 6.0.4 (GLC Bio, Denmark). The assembled sequences were further clustered by using CAP3 software to get non-redundant unigenes. BlastX alignment (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the obtained unigene sequences identified one gene with the best alignment score to carpet shell clam C3 (e-value: 3e–27). The resulting partial sequences were used to design specific primers for the amplification of the full cDNA. The primers used for this study were listed in Table 1. Rapid amplification of cDNA ends (RACE) was conducted using SMARTer ™ RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer's instructions. The full cDNA sequence of C3 of *H. cumingii* (HcC3) was sequenced and registered at GenBank under accession number MK648113.

2.3. Bioinformatic analysis of HcC3

Open reading frame search and deduced amino acid sequence analysis of HcC3 were conducted with DNAMAN 8.0 software (Lynnon Biosoft, USA). The protein domains on HcC3 amino acid sequences were identified by NCBI Conserved Domains Database (http://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi). Signal peptide was predicted using SignalP 3.0 server (http://www.cbs.dtu.dk/services/). Pairwise and multiple sequence alignments of HcC3 were performed with the Clustal Omega web server (http://www.ebi.ac.uk/Tools/msa/clustalo/). The phylogenetic relationship between HcC3 and its homologues was analyzed by using the maximum likelihood method based on the JTT matrix-based model with the MEGA 7.0 program and bootstrapping of 1000 replicates [14]. The sequence and characterization data of alpha-2-macroglobulin gene in *H. cumingii* were retrieved from Ref. [15].

Table 1
Primers used in the present study.

Name	Sequence (5′-3′)
HcC3 3′ RACE primer	CTGATGGACAAGGCATGTGATC
HcC3 real-time PCR forward primer	GCCAGTGCCCCATAATCAA
HcC3 real-time PCR reverse primer	TCAGTCGGACAGAATCATCC
EF1α real-time PCR forward primer	GGAACTTCCCAGGCAGACTGTGC
EF1α real-time PCR reverse primer	TCAAAACGGGCCGCAGAGAAT

EF1α, elongation factor 1 alpha, GenBank accession number: GW696134.

2.4. Real-time quantitative PCR assay of HcC3 transcripts

Total RNA from each test tissue or hemocyte sample was extracted by using Isogen (Takara, Japan) according to the manufactures' instruction manual. The isolated total RNA was first treated with Turbo DNA-free™ kit (Applied Biosystems, USA) at 37 °C for 1 h to remove any contaminating genomic DNA. Then 5 μg total RNA of each sample was used to synthesize cDNA template with superscript III reverse transcriptase (Invitrogen, USA) as described in Refs. [16,17]. After the cDNA synthesis, real-time quantitative PCR was carried out in 96-well optical plates with a CFX-96 touch™ real-time PCR detection system (Bio-Rad, USA). The house keeping gene of elongation factor 1 alpha was amplified as an internal reference. The transcript quantity of each target gene was calculated as the relative expression level of each target gene to the internal reference gene using the $2^{-\Delta\Delta Ct}$ method [18]. The differential transcription of the HcC3 gene among samples was statistically evaluated by using one-way analysis of variance in the Sigma Plot 10.0 package (Systat Software Inc., USA). A probability of 0.05 and less was taken as statistically significant.

3. Results and discussion

3.1. Sequence analysis of HcC3

The cloning and sequencing of HcC3 gene yielded the full cDNA sequence of 5592 base pairs (bp), which contained a 5′ untranslated region of 162 bp, an open reading frame of 4955bp encoded 1665 amino acid residues, and a 3' untranslated region of 432 bp (GenBank Accession No. MK648113). Signal peptide analysis predicted a secretion leader sequences from the deduced amino acids of 1–23. The result of conserved domain analysis of HcC3 revealed the presence of seven feature domains including A2M_N domain (131–230 amino acids of HcC3), α 2-macroglobulin family N-terminal region (A2M_N_2) (453–584 amino acids of HcC3), the anaphylatoxin-like domain (ANATO) (660–677 amino acids of HcC3), the α 2-macroglobulin family domain (A2M) (741–820 amino acids of HcC3), the α 2-macroglobulin complement component (A2M_comp) (1031–1259 amino acids of HcC3), the α 2-macroglobulin receptor (A2M_recep) (1403–1497 amino acids of HcC3), and the C345C domain (1546–1628 amino acids of HcC3). The homologous sequence alignment showed HcC3 had a β-α junction site, RRKR, and a α-γ junction site, RKRR. The C3 convertase cleavage site of HcC3 was predicted as "EER" (709–711). The thioester motif of HcC3 is "YCGEQ". The catalytical His-1108 and Gln-1110 in HcC3 are same as those of other C3 homologs (shown as Fig. 1).

The deduced amino acid sequence analysis revealed HcC3 was highly conserved in the canonical features of C3 and identified HcC3 as one member of C3 family. For instance, HcC3 possessed the C345C domain, which was not found in other members of thioester containing protein (TEP) superfamily like α 2-macroglobulins and CD109 [19]. HcC3 also had putative cleavage site characteristic of C3 molecule. The recognition of conserved β-α junction site and α-γ junction site indicated that matured HcC3 shared the constitution of three chains after posttranslational processing. One more noteworthy characteristic feature of HcC3 in the C3 sequence alignment was the highly conservation of thioester motif and two catalytical residues namely His-1108 and Gln-1110, which are usually described as the key regions guiding C3 fix

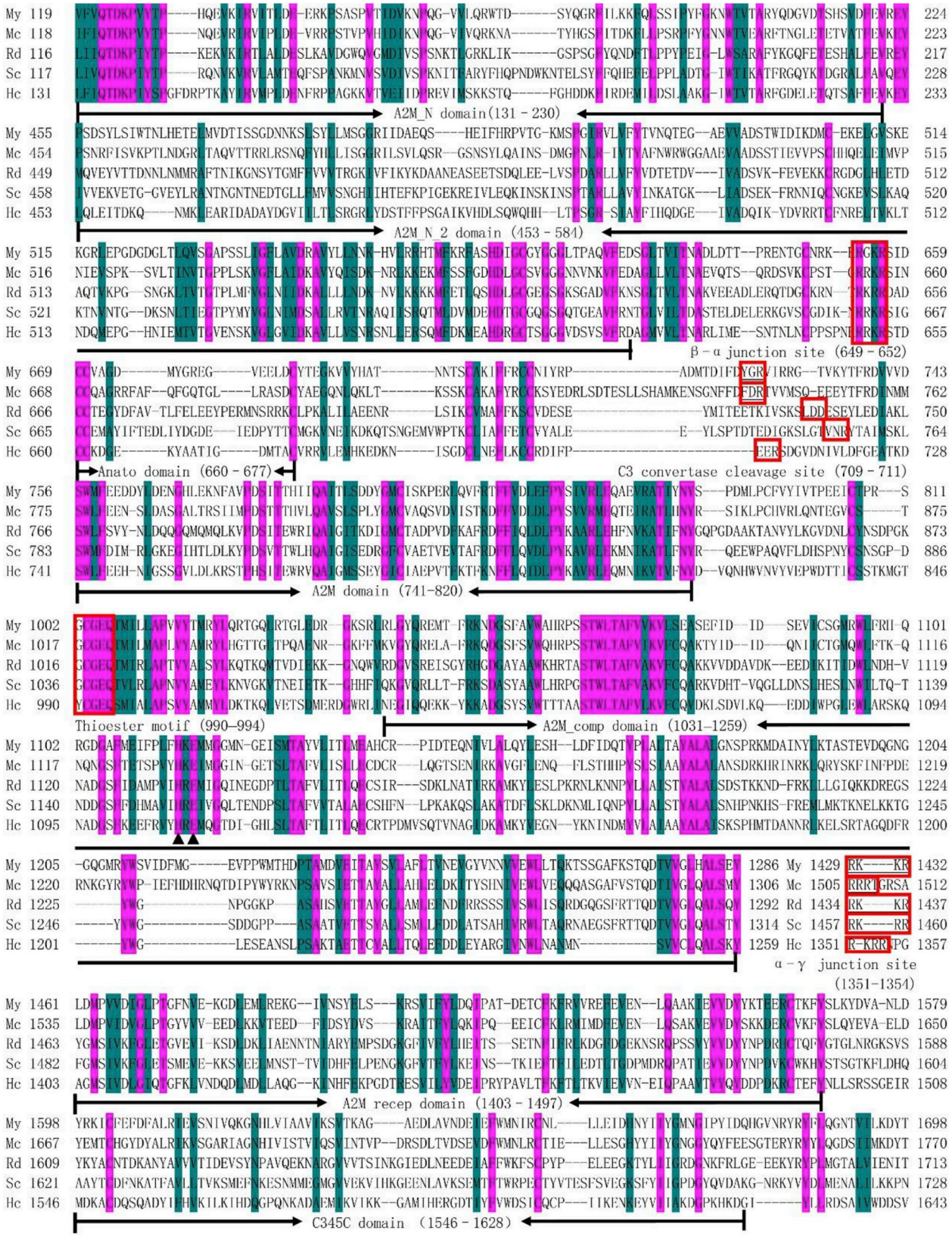

Fig. 1. Multiple alignments of partial amino acid sequences of HcC3 with other bivalve C3s. Identical and similar amino acid residues are highlighted in pink and cyan, respectively. Dashes indicate gaps. The seven conserved domains of C3 are labeled by bidirectional arrows. The posttranslational modification sites namely β-α junction site, C3 convertase cleavage site, thioester motif, and α-γ junction site are boxed. The highly conserved His and Glu are labeled with triangles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

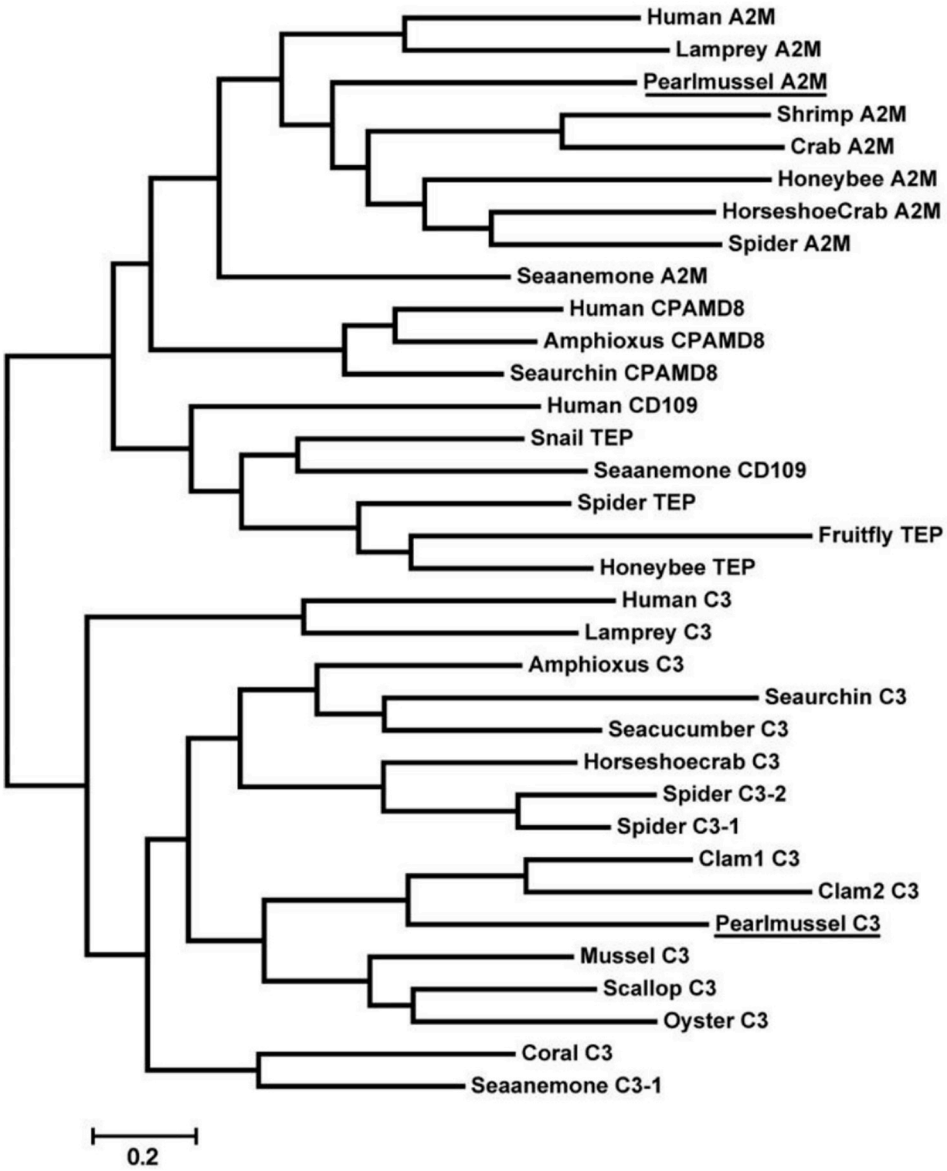

Fig. 2. Phylogenetic analysis of HcC3 and other TEP family proteins by maximum likelihood method based on JTT matrix-based model. The HcC3 and A2M protein from triangle-shell pearl mussel are underlined. The scientific name of animals, taxon names, and accession numbers of the analyzed sequences are: Human (*Homo sapiens*), A2M (P01023.3), C3(NP_000055.2), CD109 (NP_598000.2), CPAMD8 (NP_056507.3); Lamprey (*Lethenteron camtschaticum*), A2M (BAJ05271.1), C3 (Q00685.1); Pearl mussel (*Hyriopsis cumingii*), A2M (ABJ89824), C3 (MK648113); Shrimp (*Penaeus chinensis*), A2M (ABP97431.1); Crab (*Eriocheir sinensis*), A2M (ADD71943.1); Honeybee (*Apis mellifera*), A2M (XP_006565503.2), TEP (XP_026297267.1); Horseshoe crab (*Tachypleus tridentatus*), A2M (BAA19844.1), C3 (BAH02276.1); Spider (*Hasarius adansoni*), A2M (BAK64111.1), C3-1 (BAK64109.1), C3-2 (BAK64110.1), TEP (AB622471.1); Sea anemone (*Diadumene lineata*), A2M (BAJ05271.1), C3-1 (BAJ05269.1), CD109(BAJ05272.1); Amphioxus (*Branchiostoma floridae*), C3 (XP_002248496), CPAMD8 (XP_002239366); Sea urchin (*Strongylocentrotus purpuratus*), CPAMD8 (XP_785018.3), C3 (NP_999686.1); Snail (*Euphaedusa tau*), TEP (BAE44110.1); Fruit fly (*Drosophila melanogaster*), TEP (NP_523578.1); Sea cucumber (*Apostichopus japonicus*), C3 (ADN97000.1); Clam1 (*Ruditapes decussatus*), C3(ACN37845.1); Clam2 (*Sinonovacula constricta*), C3 (ANI85912.1); Mussel (*Mytilus coruscus*), C3 (AXS68445.1); Scallop (*Mizuhopecten yessoensis*), C3 (OWF37722.1); Oyster (*Crassostrea gigas*), C3 (NP_001292308.1); Coral (*Swiftia exserta*), C3 (AAN86548.1).

on the surface of a foreign body [20]. It is believed the thiol group of Cys-991 and the side-chain amide of Gln-993 (the numbering is based on HcC3 amino acid sequence) at the thioester motif form a thioester bond in native C3. Once native C3 is activated, the thioester bond will be broken and a transient chlormidazole intermediate between the conserved His-1108 and Gln-1110 can form covalent ester and amide bonds to restrict covalent deposition of C3b toward the site of activation [21].

3.2. Phylogenetic analysis of HcC3

A phylogenetic tree of TEPs was constructed to demonstrate the evolutionary status of HcC3 among the Eumetazoan TEPs. The resulting tree differentiated into two major clusters, one with all the C3s and the other with the proteins from alpha 2-macroglobulin (A2M) subfamily including the CPAMD8s, the CD109s, and the insect thioester binding proteins as shown in Fig. 2. HcC3 first clustered with other bivalve C3s and following grouped with the C3s from Arthropoda (such as spider), Echinodermata (such as sea urchin), Cephalochordata (amphioxus) and Vertebrata (such as human) in the C3 cluster in accordance with conventional taxonomy and phylogeny, while the A2M from triangle-shell pearl mussel was clustered with other protostome A2Ms together into one subbranch of the A2M cluster. The separation of their phylogenetic clusters conclusively identified HcC3 as a member of C3 subfamily, not as A2M. Furthermore, the presence of both C3 and A2M in triangle-shell pearl mussel was a straightforward evidence that strengthened the certainty of the possession of both C3s and A2M in mollusk, and deepened the notion of C3 occurrence in protostomes. Previously, it was suggested that protostomes experienced differentially the loss of C3 and A2M during TEPs evolution, since genomic sequencing data of many arthropods did not contain identifiable genes of C3 or A2M [6]. Now it appears that the loss of some TEPs may only happen in some specific Arthropoda lineages.

3.3. Tissue distribution of HcC3 transcripts

Gene expression level of HcC3 in the various tissues of *H.cumingii* were examined by quantitative PCR. A constitutive expression of HcC3 gene in all the examined tissues including adductor muscle, foot, gill, mantle, hemocytes, and hepatopancreas was detected (Fig. 3). The expression of HcC3 gene in hemocytes and hepatopancreas was significantly higher than that of other examined tissues.

The tissue distribution profile of HcC3 transcripts suggests that hemocytes and hepatopancreas are the major sites producing HcC3 and

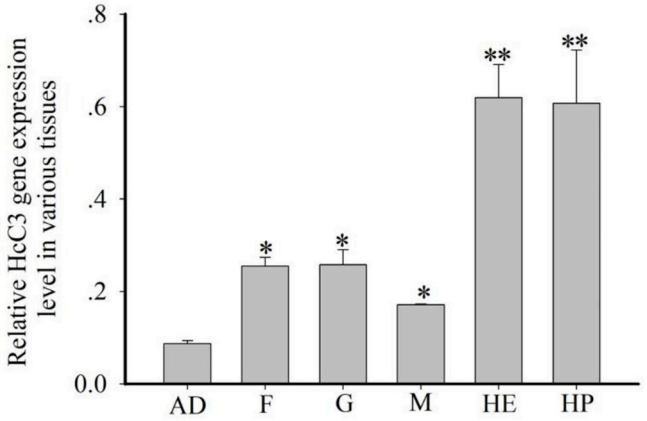

Fig. 3. Tissue distribution of HcC3 transcripts in pearl mussels. The expression of HcC3 gene in adductor muscles (AD), foots (F), gills (G), mantles (M), hemocytes (HE), and hepatopancreas (HP) is determined by real-time PCR. The expression level of HcC3 gene in each sample is normalized by the expression of a housekeeping gene, EF-1α. Each value is represented by the mean of triplicates and standard deviation is represented by the error bar. Significant differences from the AD samples are indicated with one asterisk at $P < 0.05$ and two asterisks at $P < 0.01$.

a wide range of tissues in triangle-shell pearl mussels are also able to secrete HcC3. That is consistent with the general understanding of C3 synthesis in mammalian studies. Liver is believed as the primary synthesis site of mammalian C3, while other tissues produce tissue-localized C3s on site [6,22]. In mollusks, hepatopancreas functions as the mammalian liver. Hemocytes are the fundamental immunocytes responsible for the recognition and elimination of infected pathogens in mollusks [23]. Therefore, the high expression of HcC3 in those two tissues hinted their corresponding physiological roles.

3.4. Expression profiles of HcC3 gene in response to mantle tissue allograft

After the mantle allograft surgeries, the gene expression of HcC3 in the hemocytes of recipient mussels underwent an immediate induction on day 1, declined on day 3, approached the highest level among all the test hemocyte samples on day 7, and decreased to the approximate control gene expression level within two weeks (Fig. 4). At the same

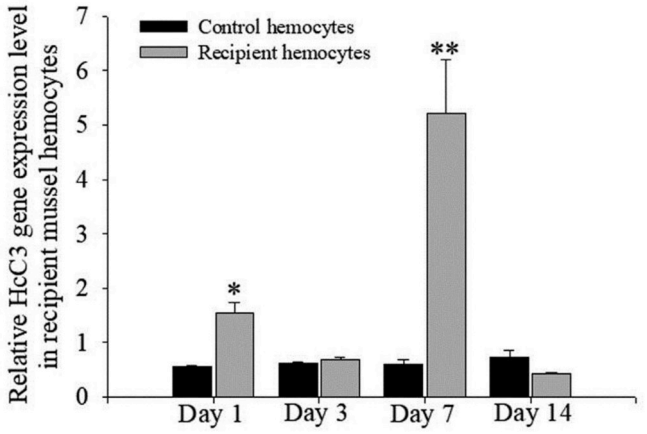

Fig. 4. Time-course expression analysis of HcC3 gene in hemocytes of recipient mussels after mantle allograft surgery. The expression level of HcC3 gene in each sample is normalized by the expression of a housekeeping gene, EF-1α. Each value is presented by the mean of triplicates and standard deviation is represented by the error bar. Significant differences from the control donor mantle tissues are indicated with one asterisk at $P < 0.05$ and two asterisks at $P < 0.01$.

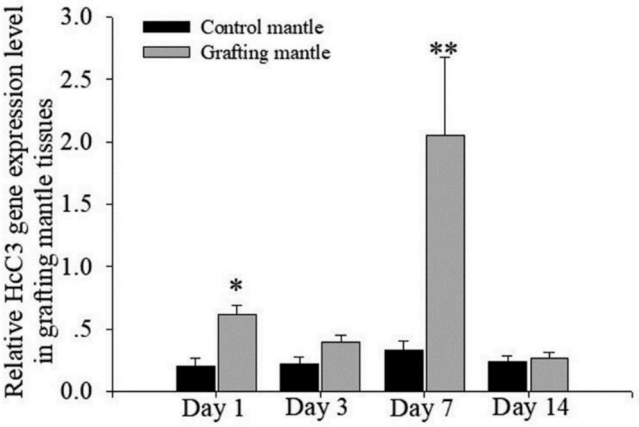

Fig. 5. Time-course expression analysis of HcC3 gene in donor mantle tissues after mantle allograft surgery. The expression level of HcC3 gene in each sample is normalized by the expression of a housekeeping gene, EF-1α. Each value is presented by the mean of triplicates and standard deviation is represented by the error bar. Significant differences from the control donor mantle tissues are indicated with one asterisk at $P < 0.05$ and two asterisks at $P < 0.01$.

time, thee grafting mantle tissues also displayed a similar HcC3 gene expression pattern. On the first day after grafting, the donor mantle tissues got significantly higher expression of HcC3 than that of control mantle. Then the HcC3 expression level dropped back on day 3, peaked the value on day 7, and restored to the level similar to control samples on day 14 (Fig. 5).

The upregulated expression of HcC3 genes in both the recipient hemocytes and donor tissues within 1 day after the mantle transplantation suggests a rapid activation of the complement system in triangle-shell pearl mussels. In mammals, complement activation can be achieved by the classical pathway, the alternative pathway and the lectin pathway [24,25]. In the classical pathway, non-self recognition by immunoglobulins activates C3 first and then C3 triggers the activation of other complement components. The lectin pathway activation is due to antigen surface carbohydrate recognition by lectins and lectin associated serine proteases, which in turn convert native C3 into its activation fragments leading to the further activation of the complement system [26]. In the alternative pathway, C3 directly binds to the hydroxyl group on antigen surface and attracts factor B generating C3 convertase, which catalyzes the production of more active C3 and thereby upregulates the complement responses [27]. Although the classical pathway mediated by immunoglobulins seems not applicable to mollusks, which lack adaptive immunocytes, complement activation by the alternative pathway or lectin pathway in mollusks have been supported by emerging experiment data. Numerous key homologous genes in those two pathways such as B factor, collectin, ficolin, and mannose binding lectin from various mollusk species have been identified and functionally characterized [28–30]. Therefore, as the central component of complement system, HcC3 is highly demanded to discriminate foreign cells for both the donor and recipient cells of mussels and triggers the attack to allograft tissues as other C3s do [4,11].

After the induced expression of HcC3 on day 1, a second elevated expression of HcC3 gene was detected on day 7 post the mantle transplantation. The repetitive activation suggests a great influence of HcC3 on mollusk tissue transplantation and the underlying activation mechanism is intricate. A similar biphasic expression of C3 was also observed in a mammalian tissue transplantation study. It is believed the first phase of C3 expression is due to allograft rejection and the second phase induction resulted from the regulation of proinflammatory cytokines like interleukin and interferon γ [31]. Moreover, tumor necrosis factor α is also demonstrated to stimulate rat C3 activation and production [32]. In triangle-shell pearl mussels, our previous study showed that interleukin-17 (HcIL-17) was strongly expressed in both the donor

mantle tissues and recipient hemocytes on day 7 after mantle allograft [6]. It appears that the concurrent upregulation of HcIL17 and HcC3 gene expression is more than a coincidence.

In conclusion, both the highly conserved gene structure and phylogenetic analysis results supported HcC3 as a typical C3 homolog. HcC3 got expressed in a wide range of triangle-shell pearl mussels. Biphasic expression of HcC3 in both the grafting tissue and recipient hemocytes suggests complicated alloimmune responses in pearl mussels during aquaculture pearl production.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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