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Identification and expression analysis of the *MSP130-related-2* gene from *Hyriopsis cumingii*

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ABSTRACT. *MSP130-related-2* is thought to play a role in biomineralization as revealed in *Crassostrea gigas* and sea urchins. In this study, an *MSP130-related-2* gene was isolated from *Hyriopsis cumingii* (*HcMSP130-related-2*) and characterized for the first time. The *HcMSP130-related-2* cDNA was 2307 bp in length and consisted of a 572-bp 5'-untranslated region (5'-UTR), a 1239-bp open reading frame encoding 430-amino acid residues, and a 439-bp 3'-UTR. The molecular weight of the peptide was predicted to be 48551.3 Da, with a theoretical isoelectric point of 4.78 and instability index of 32.74, indicating that the protein is stable. The *HcMSP130-related-2* amino acid residues included a signal peptide and several potential N-glycosylation sites. NCBI BLAST analysis indicated that this full-length amino acid sequence showed the highest similarity with *HcMSP130-related-2* from *C. gigas* (45%) and about 38% identity with that from *SpMSP130-rel-2* and *Strongylocentrotus purpuratus*. A phylogenetic tree showed that *HcMSP130-rel-2* clustered with *MSP130* from *C. gigas*. *HcMSP130-related-2* was expressed in various tissues, including the mantle, blood, gill, foot, liver, kidney, intestine, and muscle, with the highest

transcripts found in the mantle. Quantitative real-time polymerase chain reaction was used to analyze the expression of the *HcMSP130-related-2* gene in grass carp after inducing shell damage. *HcMSP130-related-2* expression was upregulated significantly in the mantle within 7 days ($P < 0.05$) after damage; however, the expression remained unchanged in the adductor muscle tissues ($P > 0.05$). These data suggest that *HcMSP130-related-2* might be involved in shell formation in *H. cumingii.*

Key words: *MSP130-related-2* gene; *Hyriopsis cumingii*; Inductive expression; Shell formation

INTRODUCTION

The triangle sail mussel (*Hyriopsis cumingii*) is a freshwater pearl mussel that is distributed mainly in large lakes and rivers (Liu, 1979). This mussel produces pearls of good quality in terms of color, purity, and shape. Since the artificial propagation of *H. cumingii* was achieved in 1979, this species has become the most important mussel for commercial freshwater pearl production, particularly in China. Currently, the annual production of freshwater pearl is 1800 metric tons in China, and this accounts for almost 95% of the total world output. In China, more than 90% of Chinese freshwater pearls are produced from the triangle sail mussel (Xu et al., 2011). Pearl production continues to increase but, at the same time, the quality of freshwater pearls is reducing, and one important reason for this is that little is known about the molecular genetic mechanisms underlying pearl growth. Therefore, investigating the genes that are involved in pearl formation is important.

Pearl is a bio-mineral made up of calcareous rods. Scientists have been exploring the mechanical design of bio-minerals for many years (Stephen, 2001), and the mollusk shell, especially the nacre, is a masterpiece of natural design. The pearl consists of 2 layers: one non-calcified and one calcified. The non-calcified layer is composed of organic materials secreted from the mantle. The main component of the calcified layer is calcium carbonate, calcite, or aragonite (Lowenstaum and Weiner, 1989). Growth of the crystal is strictly regulated throughout mussel development, and various experimental systems have indicated that the proteins associated with shell formation differ on the basis of the genetic background of each particular species. A wide variety of bio-minerals play a key role in regulating the growth, shape and physical properties of materials, such as shells and pearls (Butler, 1998; Wilt, 1999; Fincham et al., 1999; Myllyharju and Kivirikko, 2001; Wang, et al., 2012).

MSP130-related-2 was identified in the sea urchin, *Strongylocentrotus purpuratus*. Two new bio-mineralization-related proteins related to the primary mesenchyme cell (PMC)-specific cell surface glycoprotein *MSP130* (*MSP130-related-1* and *MSP130-related-2*) were identified by analyzing a large database of gene products expressed by PMCs (Illies et al., 2002). Seven genes similar to *MSP130* were identified. These included the 3 previously indentified *MSP130* genes plus 4 others (Livingston et al., 2006). The cell surface protein *MSP130* plays a role in bio-mineralization, and it is expressed specifically in calcified tissues of both the embryo and adult (Leaf et al., 1987; Drager et al., 1989). *MSP130* was identified in *Crassostrea gigas* (Zhang et al., 2012). It contains a signal sequence, 2 glycine-rich do-

mains, and a C-terminal glycosylphosphatidyl inositol (GPI) anchor (Parr et al., 1990). There is evidence that the N-linked oligosaccharide chain on *MSP130* could bind divalent cations, including Ca^{2+} (Farach-Carson et al., 1989). A monoclonal antibody (1223) that recognizes this oligosaccharide chain blocks the uptake of radiolabeled calcium and the formation of spicules by cultured micromeres (Carson et al., 1985). On the basis of these findings, we suggest that *MSP130* sequesters Ca^{2+} ions from the blastocoel fluid and facilitates the uptake into PMCs. The SpMSP130-related-2 protein is similar to *MSP130.* Like *SpMSP130*, *SpMSP130-related-2* contains a signal sequence, a C-terminal GPI anchor domain, and several potential N-glycosylation sites. Thus, *MSP130-related-2* is thought to play a role in bio-mineralization as well. This study investigated the *MSP130-related-2* gene in *H. cumingii*.

MSP130-related-2 was cloned from *H. cumingii* by using a defined expressed sequence tag. To further investigate the role of *MSP130-related-2*, we characterized the gene and determined its potential physiological functions by assessing tissue-specific expression and expression in response to shell damage. Quantitative real-time polymerase chain reaction (qRT-PCR) data indicated that *HcMSP130-related-2* might function in shell formation in *H. cumingii*.

MATERIAL AND METHODS

Animal treatment and total RNA extraction

Adult *H. cumingii* individuals of the same age were cultured separately by the Wei-Wang Company of Jin-Hua (Zhejiang Province, China). Animals were raised at 28°C in 400-L aerated aquaria for 2 weeks before experimentation and fed green algae. The mantle, blood, liver, gill, kidney, intestine, foot, and adductor muscle tissues were collected from each of 3 healthy mussels, and total RNA was isolated from these tissues for qRT-PCR. In addition, 54 healthy mussels were used in a shell damage experiment, in which the shell of half of the mussels was damaged according to a published method with some modifications (Mount et al., 2004). A V-shaped notch was cut into the shell margin close to the adductor muscle of the mussels, and then they were divided into 9 groups at random with each group containing 3 individuals. At 2, 6, 12, 24, and 48 h and 4, 7, 15, and 30 days after injury, 3 mussels were selected from each group. About 1 cm^2 of the mantle tissue around the cut was collected. Meanwhile, the mantles of 3 individuals whose shell was not damaged were sampled as described above, and these mussels acted as controls for each group. The mantle and adductor muscle samples were collected from each mussel. All tissues were frozen in liquid nitrogen and then kept at -80°C. Total RNA was isolated using the RNAiso Plus (TaKaRa, Japan), incubated with RNase-free gDNA Eraser (TaKaRa), and stored at -80°C.

Cloning the full-length cDNA of *MSP130-related-2*

A 606-bp fragment of *MSP130-related-2* was obtained from a normalized full-length cDNA library of mantle tissues of *H. cumingii* (Bai et al., 2009), and this was used to clone the full-length cDNA of *MSP130-related-2* by rapid amplification of cDNA ends (RACE) by using the SMART RACE cDNA Amplification Kit (5'-RACE; Clontech, USA) and RNA PCR Kit (AMV) Ver. 3.0 (3'-RACE; TaKaRa) according to manufacturer instructions. RACE PCR was performed using primer sets designed to the adapter sequences of the kit mentioned above

and gene-specific primers that had been designed against the cloned PCR fragments (Table 1). The 5'-RACE reactions were carried out in 25-µL reaction volumes containing 17.25 µL PCR grade water, 2.5 µL 10X Advantage 2X PCR buffer, 0.5 µL 50X dNTP Mix (10 mM), 0.5 µL 50X Advantage 2 polymerase mix, 1.25 µL 5'-RACE-Ready cDNA, 2.5 µL 10X Universal Primer A mix (10 µM), and 0.5 µL gene-specific primer (10 µM). PCR was performed under the following conditions: 94°C for 30 min; 5 cycles of 94°C for 30 s and 72°C for 3 min; 5 cycles of 94°C for 30 s, 70°C for 30 s and 72°C for 3 min; and 37 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 3 min; and 72°C for 10 min. The 3'-RACE reactions were carried out in a 50-µL reaction volume containing 2.0 µL 3'-RACE-Ready cDNA, 8 µL 1X cDNA Dilution Buffer II, 10 µM 2 µL gene-specific primer, 10 µM 2 µL 3'-RACE Outer Primer, 4 µL 10X LA PCR Buffer II (Mg^{2+} free), 25 µM 3 µL $MgCl_2$, 5 U/µL 0.25 µL TaKaRa LA Taq, and 28.75 µL dH_2O under the following conditions: 94°C for 3 min; 5 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 2 min; 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min; and finally 72°C for 10 min. The PCR product was ligated into PMD19-T vector (TaKaRa), transformed into competent *Escherichia coli* DH5α cells (Tiangen, China), seeded on Luria-Bertani agar plates (containing ampicillin, isopropylthio-β-galactoside, and X-gal), and incubated overnight at 37°C. Positive clones containing the insert of the expected size were identified by colony PCR. Next, 4 of the positive clones were picked and sequenced on an ABI PRISM 3730 Automated Sequencer by using Big-Dye terminator v3.1 (Applied Bio-Systems, USA).

Sequence analysis

The open reading frame (ORF) of *MSP130-related-2* cDNA was determined using ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The physical parameters of the amino acid sequence were predicted using the Protparam program (http://www.expasy.org/tools/protparam.html). The watershed was predicted using the ProtScale program (http://www.expasy.ch/tools/protscale.html). The putative amino acid sequence was analyzed for the presence of signal peptides by using the SignalP3.0 Server (http://www.cbs.dtu.dk/services/SignalP/; Bendtsen et al., 2004). Nucleotide and amino acid sequence identities and the prediction of conserved domains in the peptide were performed using the BLAST program (GenBank, NCBI). A phylogenetic tree was constructed using the deduced full-length amino acid sequence by the neighbor joining (NJ)-embedded algorithm and maximum likelihood method in the Mega 5.0 program. The reliability of the estimated tree was evaluated using the bootstrap method with 1000 pseudo-replications. The accession numbers of the primer sequences used in this analysis are listed in Table 1.

Table 1. Primer sequences used in this study.

Primer name	Primer sequence (5'-3')	Application
MSP130-related-2-GSP for 5'-RACE	GCGACCACCCACGACTATTTTGTGAACT	5'-RACE PCR
5'-RACE-UPM	CTAATACGACTCACTATAGGGC	5'-RACE PCR
MSP130-related-2-GSP for 3'-RACE	CCCGCAATCTGACTAACACT	3'-RACE PCR
3'race-Primer M4	GTCGTGACTGGGAAAAC	3'-RACE PCR
MSP130-related-2-qRT-F	CGGCTTCAAGGATTGGTCA	Primers for qPCR
MSP130-related-2-qRT-R	GCGTCTCCTTCATTGGCAG	Primers for qPCR
β-actin-F	ACGGATAACACAAGGAAAGGAAAC	Housekeeping gene
β-actin-R	ATGGATGGAAACACGGCTCT	Housekeeping gene

F and R = forward and reverse primers, respectively.

qRT-PCR analysis

Mantle, blood, liver, gill, kidney, intestine, adductor muscle, and foot tissues were collected and used to assess tissue expression by qRT-PCR (mantle; blood; gill; foot; liver; kidney; intestines; muscle). Expression of *MSP130-related-2* after shell damage was investigated in the mantle and adductor muscle tissue of adult *H. cumingii* individuals, and this was performed using a CFX96 Real-Time PCR System (Bio-Rad). Total RNA was isolated after shell damage as described above. About 1 µg RNA from each sample was reverse transcribed using the Prime-Script RT reagent kit with the gDNA Eraser (TaKaRa). The first-strand cDNA was subsequently used as the template for PCR by using the primer pair listed in Table 1. *β-actin* was amplified to serve as an internal control for cDNA normalization (accession No. HM045420). qRT-PCR was performed in 20-µL reactions consisting of 1 µL 50 ng cDNA, 8.2 µL nuclease-free water, 10 µL 2X SYBR Premix Ex-Taq™ (TaKaRa), and 10 µM 0.4 µL of each gene-specific primer. The PCR cycling conditions were 95°C for 30 s; 40 cycles of 95°C for 5 s, and 60°C for 34 s; followed by dissociation curve analysis at 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s to verify the amplification of a single product. The threshold cycle (Ct) value was determined using the 7500 v2.0 software, and data were exported to Microsoft Excel for subsequent analysis, where the relative expression ratios of the target gene in treated and control groups were calculated by using the $2^{-\Delta\Delta Ct}$ method. Each experiment was repeated three times.

Statistical analysis

Data from the qRT-PCR experiments are reported as means ± SE. Differences among groups were analyzed in SPSS 19.0 by using one-way analysis of variance tests with the *post hoc* Dunnett T3 test. Significance was accepted at the level of $P < 0.05$.

RESULTS

Cloning and sequence analysis of *H. cumingii MSP130-related-2* cDNA

A full-length cDNA of *MSP130-related-2* from *H. cumingii* was obtained from a normalized full-length cDNA library and 5'-RACE and 3'-RACE experiments. The *HcMSP130-related-2* cDNA was 2307 bp in length, and this consisted of a 572-bp 5'-untranslated region (5'-UTR), a 1239-bp ORF encoding 430-amino acid residues, and a 439-bp 3'-UTR. The molecular weight of the peptide is predicted to be 48551.3 Da, with a theoretical isoelectric point of 4.78 and instability index of 32.74, which indicates that the protein is stable. The protein has no obvious watershed sites and does not exist across any membrane. NCBI BLAST analysis revealed that the deduced amino acid sequence of *HcMSP130-related-2* shared 45% identity with *MSP130* of *C. gigas* and about 38% identity with *SpMSP130-rel-2* of *S. purpuratus*. This sequence homology is relatively low, which suggests that *MSP130-related-2* has not been highly conserved during evolution. Thus, focusing on its potential functional domains, evolutionary relationships, and biological roles is important (Zhou et al., 2011). Similar to *SpMSP130-related-2*, *HcMSP130-related-2* is predicted to contain a signal sequence and several potential N-glycosylation sites (Figure 1), but it lacks the

extended glycine-rich domain found in MSP130 and the C-terminal GPI anchor found in the *S. purpuratus MSP130-related-2*, which is a valuable model system for analyzing the cellular and molecular regulation of bio-mineralization (Decker and Lennarz, 1988; Benson and Wilt, 1992; Ettensohn et al., 1997; Wilt, 1999). A phylogenetic tree was constructed using the neighbor joining and maximum likelihood methods (Figure 2). The data showed that the *MSP130s* from 3 species of sea urchins, *Hyriopsis erythrogramma*, *Hyriopsis tuberculata*, and *S. purpuratus* cluster together, and then with *SpMSP130-rel-1* and *SpMSP130-related-2* of *S. purpuratus*. The *HcMSP130-rel-2* in this study clustered with the *MSP130* of *C. gigas*, and then separated from sea urchins. This indicates that *HcMSP130-rel-2* belongs to the *MSP130* family.

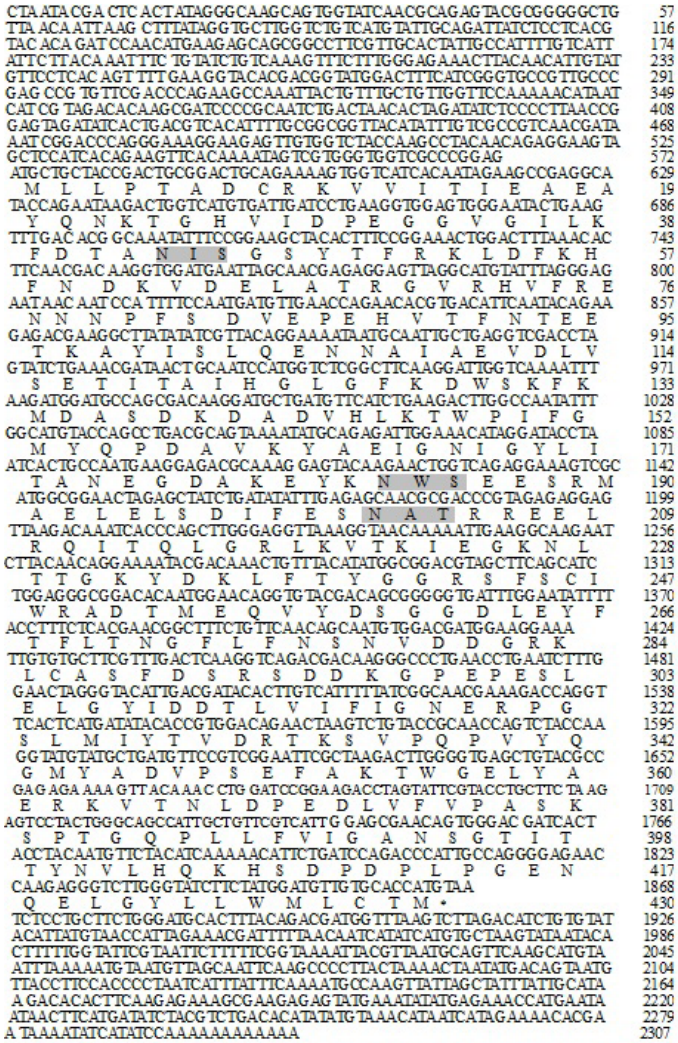

Figure 1. Nucleotide and deduced amino acid sequences of the *MSP130-related-2* cDNA in the triangle sail mussel (*Hyriopsis cumingii*). The N-glycosylation sites are shaded in gray.

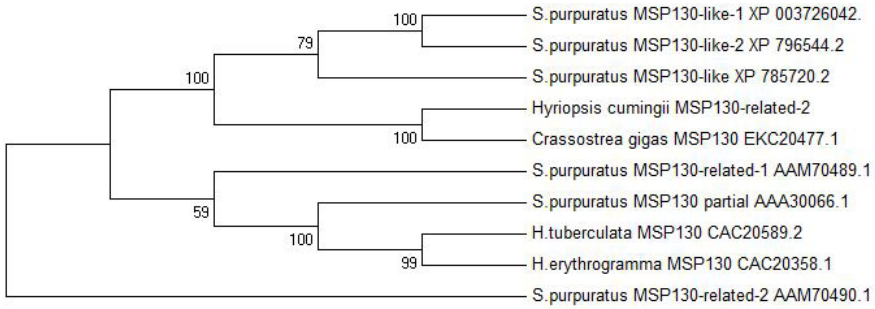

Figure 2. Neighbor-joining (NJ) phylogenetic tree. Amino acid sequences were analyzed using the NJ method in the Mega 5.0 program. The protein sequences used for phylogenetic analysis were as follows: *HcMSP130-rel-2* clustered with the *MSP130* from *Crassostrea gigas*, the MSP130 from three species of sea urchins, *Hyriopsis erythrogramma*, *H. tuberculata* and *Strongylocentrotus purpuratus*, clustered together, then with *SpMSP130-rel-1* and *SpMSP130-related-2* of *S. purpuratus* clustered.

Constitutive expression of the *HcMSP130-related-2* gene in different tissues

To gain insight into the possible physiological functions of *HcMSP130-related-2*, we analyzed the expression of *HcMSP130-related-2* in different tissues by using qRT-PCR. The *HcMSP130-related-2* mRNA was detected in the mantle, blood, gill, foot, liver, kidney, intestine, and muscle tissues (Figure 3). Although *HcMSP130-related*-2 was expressed in all tissues, its expression levels varied across these tissues. *HcMSP130-related*-2 showed significantly higher expression levels in the mantle than in other tissues ($P < 0.05$) and the lowest in blood. There were no statistically significant differences among its expression in gill, foot, liver, kidney, intestine, and muscle. This indicates that *HcMSP130-related-2* might play an important role in bio-mineralization, including pearl production and nacreous shell growth.

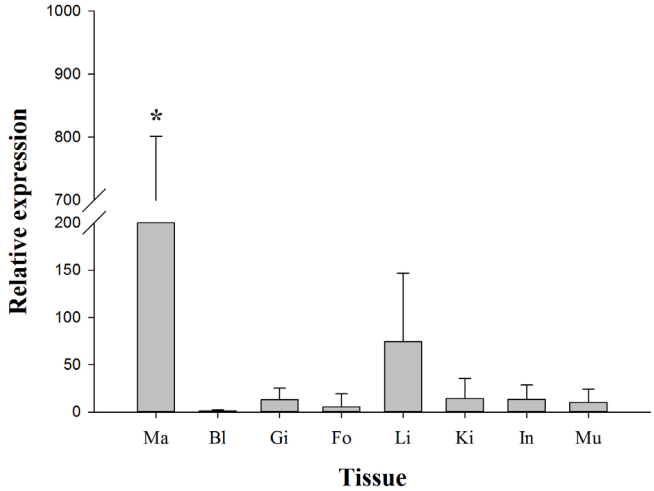

Figure 3. Relative expression levels of the *HcMSP130-rel-2* gene. Amounts of mRNA levels obtained by quantitative real-time PCR (qRT-PCR) are indicated. The relative expression of each particular gene transcript was calculated based on the standard curve and normalized to the *β-actin* mRNA level. *Indicates a significant difference ($P < 0.05$). Ma = mantle; Bl = blood; Gi = gill; Fo = foot; Li = liver; Ki = kidney; In = intestines; Mu = muscle.

Inductive expression of *HcMSP130-related-2* after shell damage

Inductive expression of *HcMSP130-related-2* was investigated after shell damage. After shell damage to *H. cumingii*, the expression of *MSP130-related-2* was investigated in the mantle and muscle. During the experiment, expression of *β-actin* was almost stable in each of the tissues under examination, which excluded the possibility that any differences in *HcMSP130-related-2* expression were caused by variations in the input RNA, efficiency of cDNA synthesis, or any other PCR artifact. In the mantle, the expression of *MSP130-related-2* was upregulated and then decreased (Figure 4). *MSP130-related-2* transcript levels in the mantle were upregulated significantly 7 days after shell damage ($P < 0.05$); thus, the expression had changed in response to this injury. However, no significant difference in expression was detected in the adductor muscles after shell damage ($P > 0.05$).

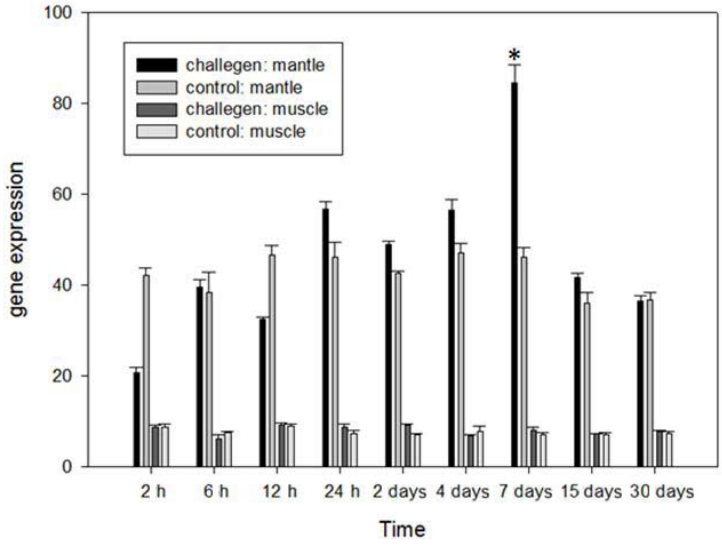

Figure 4. Quantification by qRT-PCR of the *MSP130-related-2* mRNA in the mantle and adductor muscle after shell damage. The X-axis is the time after injury. Transcript quantities were normalized to *β-actin* mRNA. The expression of *HcMSP130-related-2* was up-regulated significantly in the mantle 7 days after damage ($P < 0.05$). *Indicates a significant difference.

DISCUSSION

In this study, a *MSP130-related-2* was cloned and characterized for the first time in *H. cumingii.* Sequence alignment revealed that the deduced amino acid sequence of *MSP130-related-2* shared approximate identity with *MSP130* of *C. gigas* and 38% identity with *MSP130-related-2* of *S. purpuratus*. The *HcMSP130-rel-2* in this study clustered with the *MSP130* in *C. gigas*. The *HcMSP130-related-2* peptide was predicted to contain a signal sequence and several potential N-glycosylation sites. *MSP130-related-2* has been identified previously in the sea urchin and is known to be related to the PMC-specific cell surface glycoprotein *MSP130*. The primary sequences of *MSP130* proteins offer a few clues concerning their possible functions, but relatively little is known about MSP130-related-2 proteins. There is evidence that N-

linked oligosaccharides on *MSP130* bind Ca^{2+} and, while the precise role of *MSP130* remains unclear, the specific expression of this protein by PMCs in calcified tissues of the adult and its calcium-binding properties indicate strongly that it has a role in bio-mineralization. The carbohydrate epitope borne by *SpMSP130* is recognized by a monoclonal antibody and is shared by 2 other PMC-specific GPI-linked cell surface proteins. These proteins may be *SpMSP130-related-1* and *SpMSP130-related-2* (Kabakoff et al., 1992). *MSP130-related-2* probably contributes to this complex pattern and plays a role in bio-mineralization.

MSP130-related-2 can regulate the process of bio-mineralization in the endoskeleton of the sea urchin larva (Illies et al., 2002). In this study, *HcMSP130-related-2* was detected in the mantle, blood, gill, foot, liver, kidney, intestine, and muscle tissues, with the greatest expression detected in the mantle ($P < 0.05$) and at very low levels in the other tissues, especially in blood. The mantle is a key tissue involved in bio-mineralization (Beirao and Sorenson, 1985; Beirao and Nascimento, 1989). The epithelial cells of the inner and middle folds communicate directly with the ambient medium and are responsible for absorbing Ca^{2+} from the sea water (Simkiss and Wilbur, 1989). The mantle is also considered to be an important tissue for calcium storage (Pekkarinen and Valovirta, 1997). The shell and nacre of the mollusc are bio-mineralization products for which Ca^{2+} is one of the most important components. The high expression of *HcMSP130-related-2* in the mantle indicates that this gene might participate in Ca^{2+} absorption and storage and is involved in bio-mineralization.

qRT-PCR was used to quantify *HcMSP130-related-2* expression kinetics after shell damage. The *HcMSP130-related-2* transcript levels increased with time and, 7 days after induction of injury, the transcript levels were the greatest in the mantle, while they were lowest at 2 h (Figure 4). *HcMSP130-related-2* expression was significantly upregulated in the mantle 7 days after shell damage ($P < 0.05$). In contrast, *MSP130-related-2* transcript levels in the muscle decreased with time but not significantly ($P > 0.05$). The mantle is positively involved in the regulation and maintenance of the extrapallial fluid where calcium carbonate is crystallized, suggesting that the synthesis of matrix proteins present in the calcified layer of the shell depends on the mantle (Gong et al., 2008). *HcMSP130-related-2* expression before 24 h was probably high due to stress response, and then the expression levels increased significantly. The significant change of *HcMSP130-related-2* expression in the mantle after shell damage indicates that this gene might play a role in shell formation in *H. cumingii*. *HcMSP130-related-2* belongs to the *MSP130* family, and these cell surface proteins play key roles in bio-mineralization. *MSP130* is expressed specifically in calcified tissues in both the embryo and adult (Drager et al., 1989; Leaf et al., 1987). According to the results of the shell damage tests, we propose that *MSP130-related-2* has similar functions to *MSP130* and is involved in the bio-mineralization of the shell in *H. cumingii* and plays a role in calcium metabolism. The pearl and shell of *H. cumingii* are the result of calcium metabolism; thus, we speculate that *HcMSP130-related-2* plays a role in pearl formation. In this study, *HcMSP130-related-2* expression profiles in response to shell damage were determined only in the mantle and adductor muscle of adult *H. cumingii* individuals. Our data initially suggest that *HcMSP130-related-2* plays a role in the formation of the shell, but this requires further validation. Whether the contribution of *HcMSP130-related-2* to pearl production is the same as its role in shell growth also requires further investigation.

In conclusion, *HcMSP130-related-2* was cloned and characterized for the first time from *H. cumingii*. The present study is an initial investigation identifying the function of *HcMSP130-related-2* in *H. cumingii*. Further studies concerning the role of *MSP130-related-2* in the formation of pearl is warranted.

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