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Quantitative expression of shell matrix protein genes and their correlations with shell traits in the pearl oyster *Pinctada fucata*

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ABSTRACT

Shell matrix proteins play an important role in the regulation of shell growth and formation in the pearl oyster, *Pinctada fucata*. This study compared the expression levels of seven shell matrix protein genes (*aspein, prismalin-14*, *n16*, *n19*, *nacrein*, *msi7*, and *efcbp*) in pearl oysters of various sizes [large (L), medium (M), and small (S)] and analyzed the correlations between expression level and shell size. Except for *efcbp* and *msi7*, significant correlations were detected between the expression levels of matrix protein genes and shell size: *n19* expression displayed a negative correlation ($P<0.01$) with increasing shell size, whereas expression levels of *aspein*, *prismalin-14*, *n16*, and *nacrein* were greater in oysters with larger shell sizes. Furthermore, stronger correlations were found with shell height for genes related to prismatic layer of shell than shell weight; and with shell weight for genes related to nacreous layer of shell, which makes sense because prismatic proteins are considered to contribute to the extension of the shell length; and nacreous proteins are responsible for the shell thickness. These results contribute to a better understanding of the shell growth mechanisms of the pearl oyster.

1. Introduction

Shell growth in mollusks is a highly controlled process. Although they constitute less than 5% of shell weight, matrix proteins secreted from mantle tissues are believed to play a key role in crystal nucleation, regulation of crystal growth, crystal morphology and microstructure in molluscan shell formation (Marin et al., 2007; Wilt et al., 2003). They work as positive or inhibitory factors in shell formation, and affect the surface morphology of calcium carbonate crystals (Zhang and Zhang, 2006).

The shell of the pearl oyster *Pinctada fucata* consists mainly of two mineralized layers: the inner nacreous layer and the outer prismatic layer. A number of matrix proteins have been identified from these layers of the molluscan shell. For example, Aspein (Tsukamoto et al., 2004), Prismalin-14 (Suzuki et al., 2004), and MSI31 (Sudo et al., 1997) occur in the prismatic layer; N16 (Samata et al., 1999), N19 (Yano et al., 2007), and MSI60 (Sudo et al., 1997) are found in the nacreous layer; and Nacrein (Miyamoto et al., 1996), MSI7 (Zhang et al., 2003), and EFCBP (Huang et al., 2007) occur in both layers. Aspein is responsible for directed formation of calcite in the prismatic layer of the shell (Tsukamoto et al., 2004). Prismalin-14 and MSI31 may constitute the framework of the prismatic layer, and MSI60 may provide the framework of the nacreous layer (Sudo et al., 1997; Suzuki et al., 2004). When alone, N16 inhibits crystal growth, but it induces aragonite crystal formation when fixed onto a substrate (Samata et al., 1999). N19 functions as a negative factor, predominantly in the nacreous layer formation (Yano et al., 2007). Nacrein has carbonic anhydrase activity and supplies the shell with carbonate ions (Miyamoto et al., 1996). MSI7 induces the nucleation of aragonite and inhibits the crystallization of calcite (Feng et al., 2009). EFCBP may be an important regulator of shell formation (Huang et al., 2007). The gene expression patterns of several shell matrix proteins in mantle tissues and the pearl sac have been studied previously by Takeuchi and Endo (2006), Wang et al. (2009), and Inoue et al. (2011, 2010).

In the past few decades, a breeding program aimed at genetic improvement of the pearl oyster has been underway in China. Improvement to the growth of pearl oysters and pearl formation capacity of pearl oysters is an important part of the program. Mollusk growth involves growth of both soft tissues and the shell. Many researchers have studied the genetic improvement of shell growth and its heritability in *P. fucata* (e.g. He et al., 2006, 2008; Jiang et al., 2007). However, despite so many related works of the past decades, knowledge on the growth mechanisms of pearl oyster shell remains very limited. The prismatic layer is built along the edge of the shell and attains its final thickness when it is covered by the nacreous layer. The nacreous layer is continuously deposited onto the inner surface of the shell. Because some of the shell matrix proteins secreted from mantle tissues have been reported to play key roles in shell growth and formation, it is important to investigate how matrix protein genes

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perform *in vivo* in order to understand their relationship with shell growth. The purpose of this study was to compare the gene expression levels of these shell matrix proteins in mantle tissues of pearl oysters of various sizes to identify correlations between matrix proteins and shell growth traits. Herein, we selected two genes encoding Aspein and Prismalin-14 in the prismatic layer, which may induce or inhibit calcite precipitation, respectively; two genes encoding N16 and N19 in the nacreous layer, which may play positive or negative regulatory role in the nacre calcification. Another three genes *nacrein*, *msi7* and *efcbp* that exist in both layers were also selected. Nacrein supplies the shell with carbonate ions, MSI7 participates in the framework formation, and EFCBP is an extensive participator in the whole shell formation process with calcium-binding domain. They may play different roles in the shell formation.

2. Materials and methods

2.1. Experimental animals and tissue sampling

A *P. fucata* F_2 family was initiated on 29 May, 2009, by artificial insemination using a male and a female randomly chosen from one F_1 family. The parents of these F_1 oysters came from the Daya Bay cultured population, and the oysters of this F_2 family were cultivated under the same conditions at Daya Bay in Shenzhen, Guangdong, China. After almost 1 year of growth, 200 live specimens were collected randomly from this F_2 family on 20 May, 2010, and their shell heights and total weights were measured. Based on the shell heights, the largest (those in the top 5%; L) and smallest (those in the bottom 5%; S) individuals were selected as test groups in our research. Another 5% of oysters closing to the average shell height (medium; M) were chosen as the reference group. The left whole mantle tissue from each oyster in each of the three groups was removed and stored in Sample Protector (TaKaRa) at −80 °C. The adductor muscle, digestive gland and gill tissues from members of the L groups also were sampled and stored in the same manner. After the soft tissues were removed, the shell of each oyster was also weighed.

2.2. RNA preparation and cDNA synthesis

Total RNA was extracted using the RNAiso Reagent (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. The preliminary quantity and purity of the extracted RNA were measured at 260 and 280 nm using a UV1700 spectrophotometer (Shimadzu Corporation, Tokyo, Japan) and RNA integrity was checked using 1% agarose gel electrophoresis. The RNA preparation was treated with RNase-free DNase I (Fermentas, Vilnius, Lithuania) to remove any possible remnant genomic DNA, and the isolated RNA was stored at −80 °C for cDNA synthesis.

For the relative quantitative analysis of real-time PCR, 0.5 μg of RNA was applied to the reverse transcription reaction using the PrimeScript™ reagent kit (TaKaRa) following the manufacturer's instructions. The cDNA mix was diluted with double-distilled water to 1:6 and stored at −20 °C.

2.3. Real-time quantitative PCR analysis

Using Primer Premier version 5.0, real-time PCR primers were designed for the genes encoding following seven *P. fucata* shell matrix proteins: Aspein, Prismalin-14, N16, N19, Nacrein, MSI7, and EFCBP. Two housekeeping genes encoding glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and elongation factor 1 alpha (EF-1α) were selected as references for the calculation of relative expression levels of the genes of interest. For tissue expression analysis, only the *gapdh* gene was used.

The fluorescent real-time assay was carried out in a Rotor-Gene 3000 Real-time PCR system (Corbett Research, Sydney, Australia). The amplifications were performed in triplicate in a total volume of 20 μl containing 10 μl of 2× SYBR Premix Ex Taq™ (TaKaRa), 1 μl of diluted cDNA, 0.4 μl of each primer (Sangon, Shanghai, China) and 8.2 μl of double-distilled water. The cycle conditions were as follows: one initial denaturation cycle at 95 °C for 1 min, followed by 40 cycles at 95 °C for 5 s, 58 °C for 15 s, and 72 °C for 30 s. A dissociation analysis (50 to 99 °C) was performed to determine the absence of nonspecific products at the end of each PCR reaction. To estimate PCR efficiency, a standard curve was generated for 10-fold serial dilutions of cDNA for each primer pair. Table 1 lists the GenBank accession number of each studied gene, primers, amplicon size of each gene, and the value of PCR efficiency for each primer pair.

2.4. Statistical analysis

The comparative Ct method was used to analyze the expression levels of the genes of interest. All analyses were based on the Ct values of the PCR products. The Ct was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve. The Ct values for the genes of interest and the housekeeping genes were determined for each sample. The relative quantification of the expression of all genes was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

To compare the expression levels of each given gene among oysters of different sizes, the data obtained from real-time PCR analysis were subjected to one-way analysis of variance (ANOVA) with PASW Statistics version 18 software (SPSS, Chicago, USA), followed by the Tukey HSD test to determine differences in the mean value among the treatments. The data are presented as relative mRNA expressed as mean ± standard deviation (SD). Differences between groups were considered significant at $P<0.05$. The correlations between gene expression levels and shell parameters were subjected to bivariate correlation test with PASW Statistics 18 software. Correlations were considered significant at $P<0.05$.

3. Results

3.1. Morphological traits of P. fucata and PCR efficiencies

The mean shell height, shell weight, and total weight differed significantly among the three groups ($P<0.01$) (Table 2). The efficiencies of the quantitative RT-PCR were uniformly high and

Table 1
Real-time PCR primers used in this study.

Gene	Primer sequence	Access no.	Amplicon size (bp)	PCR efficiency
aspein	F CACCAAAATGAAGGGGATAGC R CATCACTGGGCTCCGATACTA	AB094512	149	105%
prismalin-14	F CAATGCGATCTCTGCTAGTCC R ATAGGAGAAACGCGGGAAATA	AB159512	143	106%
n16	F TACTGCTGGATACCCTACGACA R CATTCCACATCTAAGCCACTCA	AB023067	135	106%
n19	F TGGCAACAAAGCAGTCATAACCG R GGCGTCGTTGTAGCATTGAAGG	AB332326	124	107%
nacrein	F TGTTCATCTAACACCGGAGATG R TGAAGAACCCTTCTTGACACCT	D83523	150	102%
efcbp	F GACAATGACGGGAAACTCTCA R CTTACGTGACCATCACCGTCT	DQ494416	110	103%
msi7	F GATAAAAGGTCGGTGCCCAAC R AAGGTTGATGCCAGGTCCGTA	AF516712	145	102%
gapdh	F TGGCATTGAGGAAGGTTTG R GTGGAGGATGGTATGATGTTAGA	AB205404	129	104%
ef-1α	F GGCCACAGAGATTTCATCAAGAAC R CAACACCAGCAGCAATAATCAACAC	AB205403	70	106%