Structural and functional analyses of calcium ion response factors in the mantle of *Pinctada fucata*.

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The authors declare that they have no conflicts of interest with the contents of this article.

Running Title: Calcium response factors in *Pinctada fucata*

Abstract

The pearl oyster, *Pinctada fucata*, is cultured for pearl production in Japan. The shell of the pearl oyster consists of calcium carbonate and a small amount of organic matrix. Despite many studies of the shell matrix proteins, the mechanism by which calcium elements are transported from the mantle to the shell remains unclear. Investigating the molecular mechanism of calcium transportation, we prepared artificial seawater with a high concentration of calcium ions (10ASW) to induce calcification in the pearl oyster. When pearl oysters were cultured in 10ASW, unusual nanoparticles were precipitated on the surface of the nacreous layer. SDS-PAGE and 2D-PAGE analyses revealed that some calcium-sensing proteins (Sarcoplasmic Ca-binding Protein (Pf-SCP) and Pf-filamin A) might be related to the synthesis of these nanoparticles. The recombinant proteins of Pf-SCP can bind to calcium ions and accumulate nanoparticles of calcium carbonate crystals. However, transcriptomic analysis of the pearl oysters grown in 10ASW showed that the matrix protein genes in the shell did not differ before and after treatment with 10ASW. These results suggest that, despite increasing calcium transportation to the shell, treatment with a high concentration of calcium ions does not induce formation of the organic framework in the shell microstructure. These findings offer meaningful insights into the transportation of calcium elements from the mantle to the shell.

1. Introduction

The shell of *Pinctada fucata* is more than 90% calcium carbonate, along with a small amount of organic matrix (Akira, 1995). The shell of *P. fucata* contains two layers, the nacreous and the prismatic, with completely different microstructures. They are made up of organic matrices that interact with inorganic substances to precisely regulate crystal morphology and orientation (Belcher et al., 1996; Weiner and Addadi, 1997). In the nacreous layer, thin aragonite tablets are stacked on thin organic membranes, whereas each calcite prism in the prismatic layer is surrounded by an

intercrystalline organic framework in a honeycomb structure (Bevelander and Nakahara, 1969; Tong et al., 2002). The components of these layers, including inorganic and organic substances, are secreted from mantle epithelial cells (Marie et al., 2012). Between the shell and the mantle epithelial cells is a space filled with extrapallial fluid, which is separate from the external seawater but has a salinity almost identical to that of seawater (Koji, 1978). The inorganic and organic shell materials are secreted into the extrapallial fluid, and the organic matrix induces precipitation of the inorganic substances to form the shell. Many researchers have identified various organic compounds related to shell calcification and described their functions; for example, prismalin-14 (Suzuki et al., 2004), aspein (Isowa et al., 2012; Tsukamoto et al., 2004), and shematrin (Yano et al., 2006) are from the prismatic layer; nacrein (Miyamoto et al., 2005, 1996), MSI60 (Asakura et al., 2006; Sudo et al., 1997), N16 (Metzler et al., 2010; Samata et al., 1999), N19 (Yano et al., 2007; Zhang and He, 2011), and Pif (Bahn et al., 2015; Suzuki et al., 2009) are from the nacreous layer.

Furthermore, proteomic analysis of shell proteins, transcriptomic analysis of the mantle, and genomic sequencing of *P. fucata* have been performed. Proteomic analysis allowed identification of novel proteins, resulting in 9 non-specific proteins isolated from both the prismatic and the nacreous layers, 10 nacreous-specific proteins, and 16 prismatic-specific proteins (Liu et al., 2015). A transcriptomic and genomic sequence database indicated that *P. fucata* has more than 23,000 genes (Takeuchi et al., 2012). In addition, 700 proteins were manually annotated so far (Kawashima et al., 2013). Although many proteins and genes related to shell calcification have been analyzed, the mechanism and regulation of calcium response factors, such as calcium transportation from the mantle to the shell, remain unclear.

Previous reports have shown that calcium ions secreted from the mantle are derived from the external medium, whereas most of the carbon sources for bicarbonate ions and the organic matrices are derived from metabolic carbon dioxide (Robertoson, 1941). Research has also demonstrated that

low-salinity seawater improves the quality of pearls, which have the same microstructure as nacre (Hayashi, 2008). Additionally, it has been reported that gene expression changes depending on the pH of the external seawater (Liu et al., 2012). Therefore, seawater conditions, especially the concentration of calcium ions, have major effects on shell calcification. We suggest that calcium absorption from the shell occurs when the external medium contains a low calcium level, whereas calcium emission onto the shell occurs when the calcium level is high, maintaining a constant blood level of calcium through regulation of calcium response factors related to shell calcification.

In this study, we reared *P. fucata* in artificial seawater containing a high calcium concentration to identify the calcium response factors. Rearing oysters in artificial seawater with a high calcium concentration has not been attempted previously because the calcium concentration in natural seawater (NSW) is already supersaturated, with calcium carbonate and calcium sulfate precipitating at higher calcium concentrations. Therefore, we decreased the concentrations of both bicarbonate and sulfate ions to prevent precipitation. Using a combination of SDS-PAGE and LC-MS/MS, we identified two proteins that respond to changes in the calcium concentration, whereas variations in the gene expression levels of *P. fucata* were comprehensively analyzed via next-generation sequence analysis.

2.   Materials and methods

2.1. Culture of living P. fucata in the artificial seawater

The living pearl oysters, *P. fucata*, (the size is 5-6 cm.) were transported from the Ago Bay (Mie Prefecture, Japan) by the Fisheries Research Division, Mie Prefectural Science and Technology Promotion Center, Japan. The living pearl oysters were cultured in the aquarium containing the normal artificial sea-water for one day at 20°C. Then, 4-6 living pearl oysters were cultured in the 10 L aquarium containing the experimental artificial seawater with various concentrations of calcium

ion for one week at 20°C. The components of the experimental artificial seawater were shown in Table 1. After culture for one week, the shells were collected and washed by distilled water. On the other hand, live tissues were separated into each part and stored at -80°C. The tissues were crushed and powdered in liquid nitrogen to extract the RNA or protein samples.

Only the shells from the pearl oyster were also incubated in the experimental artificial seawater for one week at 20°C. After culture for one week, the shells were collected and washed by distilled water.

2.2. Observations of the shells by electron microscope

The washed shells from each condition were dried and attached on aluminum stages using a carbon tape. The microstructures of shell were observed with secondary electron images (SEI) at the acceleration voltage of 2-15 kV using the scanning electron microscope (SEM) (Hitachi S-4800). The samples were coated with Pt–Pd for high-resolution SEI recording. The cross sections of shell microstructure were prepared using Hitachi FB-2100 FIB system. Transmission Electron Microscope (TEM) observations were performed using a JEOL JEM-2010 TEM operated at 200 kV. All images and selected-area electron diffraction patterns were also recorded by the CCD camera (Gatan ESW-500W, Pleasanton, CA, U.S.A.).

2.3. Extraction of proteins from the mantle

The frozen mantle tissues from each condition were homogenized in the buffer (50 mM HEPES-KOH (pH 7.8)/420 mM KCl/0.1 mM EDTA/5 mM $MgCl_2$/20% glycerol) containing cOmplete mini, EDTA free (1 tablet/10 mL), and the buffer extract was collected. After concentration and desalting by ultrafiltration tube (M. W. 10000 cut off), the protein concentration of each extract was measured and applied to SDS-PAGE and two dimensional -PAGE.

2.4. Identification of proteins

The protein extract solution was distilled using swelling buffer (8 M urea/4% CHAPS/18 mM dithiothreitol (DTT)/0.002% Coomassie Brilliant Blue (CBB)). Prior to isoelectric focusing, IPG strips (pH 4–7, 11 cm, GE Healthcare) were passively rehydrated with 200 µl of protein solution (1 mg/ml of protein concentration) in wells for 12 h. Isoelectric focusing was conducted using the following protocol: 500 V for 2 h, gradient voltage increase to 1000 V for 1 h, and gradient voltage increase to 6000 V for 2.5 h, 6000 V for 40 min, and IEF Parameters at 50 µA/strip (Ettan IPGphor, GE Healthcare). To prepare for the second dimension SDS-PAGE, strips were incubated in equilibration buffer (50 mM Tris–HCl (pH 8.8)/6 M urea/30% glycerol/1% SDS) for 15 min at two times, first with 0.25% DTT and second with 4.5% iodoacetamide. IPG strips were then placed on top of 12.5% polyacrylamide gels at 30 mA. Gels were subsequently stained with CBB or silver staining (Invitrogen, silver quest).

After visualization of protein bands, the intensely stained portion was excised and analyzed by LC-MS/MS. The gel bands or spots were cut into pieces and transferred into 1.5 ml tube. The pieces were vortexed with 50 µL of 50% acetonitrile in 100 mM ammonium bicarbonate to decoloration and washed with 100 mM ammonium bicarbonate. Then 100 µL of acetonitrile was added and kept stand for 15 min, tapping every 5 min. After discarding supernatant and drying pieces, the pieces were incubated with 50 µL of 10 mM DTT/100 mM ammonium bicarbonate at 56°C for 60 min to be reduced. The supernatant was discarded and 50 µL of 55 mM iodoacetamide/100 mM ammonium bicarbonate for 45 min at room temperature to be alkylated. The pieces were washed by being incubated with 100 mM ammonium bicarbonate for 15 min and then 100% of acetonitrile for 15 min respectively twice. After discarding the supernatant and drying pieces, the pieces were incubated with 250 ng Trypsin Gold (Promega) and 10 µL of 50 mM ammonium bicarbonate for 6 h to digest

the proteins. The supernatant was transferred to a new tube. 20 µL of 5% formic acid/50% acetonitrile was added to the pieces, followed by vortex for 20 min, and then the supernatant was transferred to the previous same tube. This supernatant was completely dried and finally, 40 µL of 0.1% trifluoro acetic acid (TFA) /2% acetonitrile was added, followed by application to LC-MS/MS (Thermo Fisher, Orbitrap Velos). The data from LC-MS/MS was analyzed using the soft of Proteome Discover 1.4 and genome database of Pfu_aug1.0 (OIST).

2.5. Gene expression analyses

The frozen powder of the mantle was put into 1 ml Sepasol®-RNA Super G (Nacalai) for 5 min, and then chloroform (0.2 mL) was added. After centrifugation, the supernatant was collected, and RNA was purified by ethanol precipitation. The extracted RNA solution was stored at -80°C. First-strand cDNA was synthesized with 0.5 µg of total RNA using a PrimeScript™ RT reagent Kit (Takara, Perfect Real Time) according to the manufacturer's instructions.

Real-time quantitative PCR (qPCR) was used to quantity the expression levels of the genes. qPCR primers were designed using the Primer3Plus software (Table 2). Actin was used as an internal reference for qPCR. qPCR was carried out on the Light Cycler (Roche) using a SYBR Premix Ex Taq II (Takara). To compare the expression levels of these genes, ΔΔCT method was used in the experiment. The qPCR reaction mixture (10 µl) was adjusted according to the manufacturer's instructions. Cycling parameters were: 1 cycle of 0.5 min at 95°C, 40 cycles of 5 s at 95°C and 20 s at 60°C. Dissociation curves were analyzed at the end of each run to determine the purity of the product and specificity of amplification. qPCR measurements was repeated three times using three different individuals.

2.6. Knockdown experiments

The primers used for generating the Pf-filamin and Pf-SCP dsRNA were designed (Table 3). The sequence underlined is a T7 promoter sequence. Using these primers, the specific sequence was amplified using Ex taq® (Takara) from the first-strand cDNA as a template and was purified by ethanol precipitation. Both strands of RNA were synthesized from its PCR product using T7 RNA polymerase (Takara) according to the manufacturer's instructions. The synthesized RNAs were heated at 75°C for 5 min to anneal both strands of the RNA to form double strand RNA (dsRNA). Template DNA was digested with RNase free DNase I (Takara) at 37°C for 30 min, and the integrity of the dsRNA was verified by agarose gel electrophoresis. The GFP cDNA sequence from pEGFP-C1 (Clontech) was inserted into the pbluescript plasmid, and GFP dsRNA was generated using T7/T3 RNA polymerase as a negative control.

Regardless of the amount of dsRNA applied, the volume for all injections was 200 μl throughout this study. For sham injections, the same volume of PBS and 100 μg of GFP dsRNA in PBS were separately injected. Japanese pearl oysters with a shell length of 5-6 cm were used for this experiment. Samples were injected into the adductor muscle, using a NN-2333R syringe, with a G26 needle (Terumo). Four individuals were used for each treatment. Each group was injected with 100 μg of Pf-filamin and Pf-SCP dsRNAs. The shells were collected after one week of injection. Total RNA was extracted from the mantle after 24 hours of injection and first-strand cDNA was synthesized as described above. Real-time quantitative PCR (qPCR) was used to quantity the expression levels of the genes. qPCR measurements was repeated three times using three different individuals.

2.7. Preparation of recombinant Pf-SCP (rPf-SCP)

rPf-SCP was prepared as follows. Two oligonucleotide primers were designed based on the nucleotide sequence of the cDNA encoding Pf-SCP. The N-terminal primer (recom5:

GGGCATATGGACTACTTGACCGGAAA) contained the NdeI site (italics). The C-terminal primers (recom3: CCCGGATCCTTACAGCACTAATTTGAGCG) contained the BamHI site (italics) and a stop codon (bold characters). PCR was performed with recom5 and recom3 using the synthesized cDNA from the mantle as template. The amplified PCR products were cloned into a pGEM®-T Easy Vector plasmid (Promega), and the nucleotide sequence of a desired PCR product was checked. Subsequently, the Pf-SCP insert was released from the pGEM®-T Easy Vector plasmid by NdeI/EcoRI digestion, and then ligated into the NdeI/EcoRI site of an expression plasmid pET 28b (Invitrogen). E. coli, BL21(DE3) cells (Takara) were transformed with each expression plasmid with the insert and selected on LB plates containing kanamycin (12.5 μg/ml). Bacterial cells from a single colony were grown at 37°C overnight in an LB medium containing the same antibiotics, and the culture then diluted 50-fold with the same medium. The diluted culture was incubated at 37°C for 2 h, and then isopropyl thio-β-D-galactoside (Nacalai) was added to the culture to a final concentration of 1 mM. After incubation for another 2 h, bacterial cells were harvested by centrifugation and suspended in 1/20 culture volume of phosphate-buffered saline (10 mM Na_2HPO_4/150 mM NaCl (pH 7.5)). The cells were disrupted by sonication and centrifuged. After the supernatant was collected, the pellet was suspended in phosphate-buffered saline. Both the supernatant and the suspended insoluble material were subjected to SDS-PAGE under reducing conditions. The rPf-SCP was purified by Ni Sepharose 6 Fast Flow (GE Healthcare). 5 ml supernatant was applied to a 2.7 mL Ni Sepharose 6 Fast Flow equilibrated with binding buffer (20 mM Na_2HPO_4/500 mM NaCl/20 mM imidazole), the column was washed with 10 mL of binding buffer, and then the sample was eluted with a elution buffer (20 mM Na_2HPO_4/500 mM NaCl/100 mM imidazole). Analysis of the eluate by SDS-PAGE revealed a single band around 23 kDa. The purified rPf-SCP was washed by ultrafiltration (AmiconUltra-15(Merck)).

2.8. Isothermal Titration Calorimetry (ITC)

rPf-SCP was incubated in Chlex[®] 100 Resin (100-200 Mesh Sodium Form, BIO-RAD) for 12 hours at 4°C to remove the calcium ion from the protein residues. 200 μL of 25 μM rPf-SCP dissolved in 50 mM Tris-HCl (pH 7.4) was filled in sample cell. The reference cell was filled with distilled water. 1 mM $CaCl_2$ in 50 mM Tris-HCl (pH 7.4) was titrated into rPf-SCP solution at 25°C. Each titration was carried out with initial injection (0.4 μL) followed by 19 main injections (2 μL each) at intervals of 120 s. The first titration (0.4 μL) was excluded for the analysis. The data were analyzed according to a model for one set of sites provided in the Origin 7.0 software for MicroCal iTC200.

2.9. Measurements of activity on calcium carbonate crystallization

The activity on calcium carbonate crystallization of rPf-SCP was examined by the method described previously (Inoue et al., 2001). Formation of calcium carbonate precipitates was monitored by recording the turbidity of a solution containing 200 μl of 22 mM $NaHCO_3$ (pH 8.7) and 20 μl of sample solution after the addition of 200 μl of 22 mM $CaCl_2$ to the solution. Changes in the turbidity of the solutions were measured every 1 min for 6 min by the absorbance at 570 nm using a spectrophotometer (V-550, Jasco). The synthesized calcium carbonate crystals after 12 hours were washed with distilled water and ethanol. The dried calcium carbonate crystals were observed by SEM.

2.10. in vitro calcium carbonate crystallization on the nacre surface using rPf-SCP

We prepared the nacre (5 mm x 5 mm) washed by distilled water. The nacre was put on the bottom of 96-well plate. Solutions of $CaCl_2$ and $MgCl_2$ were added to wells of 96-well plate to a final concentration of 20 mM and 5 mM, respectively. Next, rPf-SCP was added to each well to a

final concentration of 10 μM, while only10 mM Tris-HCl buffer (pH8.0) was added to the other well as a control. Then, 5 g $(NH_4)_2CO_3$ was added to wells in the four corners. This 96-well plate was put in the desiccator to remove the air. Each total volume of the well was 200 μl. After precipitation, the resulting crystals in the solution were washed with distilled water and ethanol. The morphologies of crystals were observed by SEM.

2.11. Comprehensive expression analyses using next generation sequencer

RNA-seq was conducted using four biological replicate samples for experimental (10ASM) and control (1ASM) conditions. Total RNA was extracted following the instructions of the manufacturer using Sepasol®-RNA I Super G (Nacalai). Transcriptome libraries for paired-end (300bp for each read) sequencing were prepared using a TruSeq RNA Library Preparation Kit v2 (Illumina). RNAs were sequenced following the manufacturer's instructions for the Illumina Miseq (Illumina). Adapter sequences and low quality bases (PHRED ≤ 20) were trimmed using Trimmomatic 0.36 (Bolger et al., 2014). Each sequence was mapped in the gene database of *P. fucata* using Bowtie2 ver. 2.2.6 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) (Langmead and Salzberg, 2012). The read preprocessing, assembly, and mapping were performed in the read annotation pipeline of DDBJ using default parameters (Nagasaki et al., 2013). In order to analyze gene expression levels, transcripts per kilobase million (TPM) was calculated by eXpress ver. 1.5.1 (http://bio.math.berkeley.edu/eXpress/index.html) and visualized with R ver. 3.2.2 (https://www.r-project.org/) (Roberts and Pachter, 2013).

3. Results

Artificial seawater with a high concentration of calcium ions was prepared to examine the response of *P. fucata* to calcium ions. The components of each artificial seawater formula are

described in Table 1. Living pearl oysters were cultured in each type of artificial seawater for 1 week at 20°C. After 1 week, living tissues and the shell were collected to analyze differences among the 1ASW, 0ASW, and 10ASW treatments. To determine the non-physiological effects of abnormal artificial seawater, the shells of normal pearl oysters were also soaked in 0ASW and 10ASW for 1 week at 20°C.

The internal surface of the shell from each condition was observed using SEM. Normal tablets (2–3 μm in diameter) were grown and bound together into a sheet structure in the treatment with 1ASW (Fig. 1A). The tablets observed in 1ASW are almost same as the normal nacre surface in previous report (Saruwatari et al., 2009; Suzuki et al., 2009). Although tablets were observed in the nacre surface of living pearl oysters from the 0ASW treatment, a groove separated each sheet (Fig. 1C). The 0ASW treatment with only shells showed that the surface microstructure was dissolved in seawater with a low concentration of calcium ions (Fig. 1B). These results suggest that the 0ASW treatment caused both physical and physiological effects on the nacreous layer of the shell. On the other hand, the sheet structure was also disordered in 10ASW, and abnormal nanoparticles (10–100 nm in diameter) were precipitated on the surface of the tablets (Figs. 1E and F). When shells were soaked in 10ASW, the surface of the nacreous layer showed normal tablet growth (Fig. 1D) indicating that the abnormal nanoparticles observed in living pearl oysters cultured in 10ASW were not formed through physical precipitation but through a physiological phenomenon related to the mantle tissue.

The surface of the prismatic layer in each condition was also observed using SEM. The treatment with 1ASW had a honeycomb-like microstructure made up of a thick organic framework surrounding calcite prisms (10–50 μm in diameter) (Fig. S1A). The treatment with 0ASW etched the calcite surface, forming small holes (Figs. S1B–E). Similar holes were observed in the shells soaked in 0ASW and 10ASW. On the other hand, a thinned organic framework was observed in living pearl

oysters in the 10ASW condition (Fig. S1E). These results suggest that the 0ASW and 10ASW treatments had little effect on the prismatic layer of the shell. Thus, in this study, we focused on the nacreous layer under various calcium ion concentrations in abnormal artificial seawater.

To determine the detailed microstructure of the nanoparticles on the nacreous layer of the 10ASW condition, we observed the nanoparticles using TEM (Fig. 2). The organic framework separated aragonite tablets into a compartmental structure, and a granular layer covered the surface of this structure (Fig. 2A). The selected area electron diffraction (SAED) pattern of the aragonite tablet indicated that its c-axis was almost perpendicular to the surface (Fig. 2B). The SAED pattern in the granular layer showed the same SAED pattern as that of the aragonite tablet (Fig. 2C). These results suggest that the nanoparticles on the nacreous surface of 10ASW were not randomly precipitated from the solution but were formed in the process of epitaxial growth through a physiological mechanism.

Protein solution was extracted from the mantle tissues of pearl oysters living in 1ASW, 0ASW, and 10ASW using an extraction buffer. The protein solution was applied to SDS-PAGE to compare the band patterns under each condition (Fig. 3). A band around 40 kDa increased only under the 0ASW condition (lane 2, arrow). This band was cut out and digested with trypsin to identify its amino acid sequence using LC-MS/MS (Table S1). A BLAST search of the identified amino acid sequence (pfu_aug1.0_5192.1_45137.t1) revealed that the 40-kDa protein was arginine kinase (Fig. S2). The expression level of arginine kinase was often induced under stressful conditions (Horney et al., 2001). It was difficult to detect shell calcification factors under the 0ASW condition, because 0ASW causes great stress to the pearl oyster. A band around 130 kDa increased only under the 10ASW condition (lane 3, arrow). This band was also cut out, digested by trypsin, and sequenced using LC-MS/MS (Table S2). A BLAST search of its amino acid sequence (pfu_aug1.0_1756.1_51401.t1) revealed that the 130-kDa protein was filamin A (Pf-filamin A) (Fig.

4). As filamin A is known to be a calcification-related signal molecule in vertebrates (Awata et al., 2001; Leung et al., 2010), further analyses were performed on the protein solution from the 10ASW treatment.

The protein solution extracted under the 10ASW condition was applied to 2D-PAGE (Fig. 5). The spot patterns on the gels were detected using silver staining. The spot around 20 kDa and pI 5.0 (spot 1) was strong under the 10ASW condition. On the other hand, the intensity of a neighboring spot (spot 2) did not vary among treatments. Spots 1 and 2 were analyzed by LC-MS/MS (Tables S3 and S4). The results for both spots 1 and 2 indicated the same protein ID (pfu_aug1.0_320649.1_36017.t1). A BLAST search showed that the protein was Sarcoplasmic Ca-binding Protein (Pf-SCP) (Fig. 6). As the registered nucleotide sequence of Pf-SCP was a partial sequence, its whole-gene sequence was determined using transcriptomic data from the mantle (Kinoshita et al., 2011). Pf-SCP has two EF-hand motifs and a phosphorylated tyrosine residue at amino acid position 83. The difference in pI between spots 1 and 2 might be derived from the presence or absence of phosphorylation on 83Y.

To determine the gene expression levels of Pf-filamin A and Pf-SCP, quantitative real-time PCR was performed (Fig. S3). The actin gene was used as an internal standard for the $\Delta\Delta C_T$ method. The expression of both Pf-filamin A and Pf-SCP increased in the 10ASW treatment, suggesting that increased gene expression led to greater production of the protein.

To analyze the functions of Pf-filamin A and Pf-SCP *in vivo*, RNAi experiments were performed using dsRNA of the target genes. The dsRNAs of Pf-filamin A and Pf-SCP were designed from their nucleotide sequences and synthesized using transcriptase. The synthesized dsRNAs were injected into living pearl oysters. Twenty-four hours after injection, mantle tissue was collected to analyze the expression level of each gene. The Pf-filamin A expression level of Pf-filamin A dsRNA injection was significantly smaller than that of EGFP dsRNA injection (Fig. S4A). Four days after

injection, the shells were collected to observe the microstructure of the nacreous layer. The nacre surface of the EGFP dsRNA injection treatment showed tablets covered by granular particles (Figs. 7A and B). These particles were also observed in the nacreous layer of an oyster cultured in 10ASW, suggesting that EGFP dsRNA injection did not affect the artifacts observed in nacre formation. On the other hand, the nacre surface after Pf-filamin A dsRNA injection was smooth, suggesting that nacre calcification was stopped by RNAi (Figs. 7C and D). Next, the effects of Pf-SCP dsRNA injection were analyzed. The expression level of Pf-SCP after injection of Pf-SCP dsRNA was reduced to that of EGFP dsRNA injection (Fig. S4B). Four days after injection, shells were collected to observe the microstructure of the nacreous layer. Holes were observed in the center of tablets in the nacreous layer after Pf-SCP dsRNA injection (Figs. 7E and F). Thus, suppressed expression of Pf-SCP induced deficient tablet formation on the surface. Because these results suggest that Pf-SCP plays a role in the transportation of calcium ions or calcium carbonate to the tablets, the function of Pf-SCP was further investigated.

Recombinant Pf-SCP protein was prepared using *E. coli* (Fig. S5). The nucleotide sequence of Pf-SCP was cloned into the expression vector (pET-28a). After induction of expression using IPTG, precipitated *E. coli* cells were treated with sonication to extract the expressed protein (Fig. S6A). Recombinant Pf-SCP was purified using a Ni-agarose column (Fig. S6B). To determine the calcium ion binding activity of recombinant Pf-SCP, the binding potential was examined by ITC, a method for analyzing the interaction between two compounds by measuring the change in the caloric output when they are mixed (Fig. S7). When recombinant Pf-SCP was titrated with calcium ion solution, exothermic release of binding energy was detected. On the other hand, when the interactions between recombinant Pf-SCP and a buffer without calcium ions were examined as a control, an exothermic reaction was not detected under the same conditions. These results suggest that recombinant Pf-SCP has a function in calcium ion binding activity.

The calcium carbonate precipitation activity of recombinant Pf-SCP was measured (Fig. S8). A supersaturated solution of calcium carbonate was mixed with the sample solution. The turbidity of the resulting solution was measured as absorbance at 570 nm for 5 min using a spectrophotometer. The supersaturated calcium carbonate solution without recombinant Pf-SCP showed a gradient of increasing turbidity over 5 min. On the other hand, the supersaturated calcium carbonate solution with 2 μM recombinant Pf-SCP showed a sharp increase in turbidity, indicating that recombinant Pf-SCP induced precipitation of calcium carbonate. After the solution was stored for 24 h, precipitated calcium carbonate crystals were observed using SEM (Fig. S9). Calcium carbonate precipitated in the solution without recombinant Pf-SCP in the form of rhombohedral calcite (Figs. 9A and B), whereas the solution with recombinant Pf-SCP contained small granules and exhibited an increase in the amount of precipitation (Figs. S9C and D). These results suggested that Pf-SCP regulates the size of calcium carbonate crystals and induces precipitation of small calcium carbonate granules.

These experimental results suggested that recombinant Pf-SCP controlled precipitation of calcium carbonate *in vitro* and that calcium carbonate on the surface of the nacre was precipitated by recombinant Pf-SCP. The nacre from *P. fucata* was placed into a solution containing calcium chloride and magnesium chloride with or without recombinant Pf-SCP. The solution was incubated in a desiccator with powdered ammonium bicarbonate for 24 h. SEM images showed that long, needle-like aragonite crystals were synthesized on the surface of the nacre in the solution without recombinant Pf-SCP (Figs. S10A–C). On the other hand, the solution containing recombinant Pf-SCP induced formation of small granules on the nacre surface (Figs. S10D–F). The shape of the granules was very similar to that precipitated on the nacre in 10ASW.

Comprehensive expression analyses using next generation sequencer showed that we acquired 17,062,913 reads from four individuals in 1ASW and 15,151,426 reads from four individuals in

10ASW. After quality trimming, each fragment was mapped to a model gene in the genomic database (pfu_aug1.0_Nall.fasta). Total sequences, total read counts, and average read counts are shown in Table 4. The expression level of Pf-filamin A showed the significant difference between 1ASW and 10ASW, while the expression level of Pf-SCP did not change in both conditions (Fig. S11). The result of Pf-SCP is not corresponding to the result of qPCR. The length of nucleotide sequence of Pf-SCP is shorter than that of Pf-filamin A. The reading count by the next generation sequences probably did not work well in the short sequence. To determine the expression levels of shell matrix protein genes, the read counts of all genes were analyzed using eXpress. The expression levels of N16, nacrein, Pif177, N19, and MSI60 identified from the nacreous layer did not show significant differences under the 1ASW and 10ASW conditions (Fig. S12); this was also the case for the expression levels of aspein, prismalin-14, shematrin-1, shematrin-5, Pfty1, and KRMP-1 identified from the prismatic layer (Fig. S13). These results suggested that the gene expression levels of shell matrix proteins were not affected by the concentration of calcium ion in the environmental sea water.

4. Discussion

Previous reports have indicated that changes in the salinity of seawater affect the quality of pearls, suggesting that the concentration of minerals in the seawater has some effect on the calcification of the shell (Hayashi, 2008). However, the relationship between mineral concentration and shell mineralization has not been clarified. Few studies of the pearl oyster *P. fucata* involve cultivation in artificial seawater with high or low calcium ion concentrations. In this study, anion concentrations (carbonate and sulfate) were decreased to solubilize high concentrations of calcium ions. In place of carbonate and sulfate, the concentration of chloride was increased.

When living pearl oysters were cultured with a low concentration of calcium ions (0ASW) for 1

week, the nacre microstructure of the shell was disrupted, and many holes appeared in the prismatic layer. Because a solution with a low calcium concentration can dissolve solid calcium carbonate, we investigated whether the dissolution observed on the surface of the shells was derived from a physiological function or a physical effect. When shells collected from the pearl oysters were incubated in 0ASW, dissolution effects were observed in both the nacreous and the prismatic layers. Although the effects in the prismatic layer were almost identical in living pearl oysters and in empty shells, the patterns of dissolution in the nacreous layer differed. Random dissolution was observed in the shells, whereas the boundaries of the tablets in the nacreous layer of the living pearl oyster were preferentially dissolved, suggesting that the mantle probably absorbs calcium ions from the shell through a physiological mechanism.

When living pearl oysters were cultured with a high concentration of calcium ions (10ASW) for 1 week, numerous nanoparticles were precipitated on the surface of the nacreous layer, whereas calcite prisms in the prismatic layer showed overgrowth beyond the organic framework. A cross-section of the nacreous layer with nanoparticles showed that the crystal orientation of the nanoparticles was almost the same as that of the aragonite crystals in the nacre tablets, suggesting that the nanoparticles were produced by epitaxial growth on the surface. Such nanoparticles were not observed in the nacreous layer of a shell incubated in 10ASW, indicating that the nanoparticles were synthesized by a physiological process. These observations imply that oversupply of calcium ions can induce calcification of the shell to eliminate the extra calcium ions.

 SDS-PAGE analysis showed that the band around 40 kDa in the mantle tissue extract from pearl oysters cultured in 0ASW was thicker than that from 1ASW. LC-MS/MS analysis showed that this band was arginine kinase, which catalyzes the phosphate reaction of arginine residues in the protein. Previous studies have reported that expression of arginine kinase increased in the crayfish under stressful conditions caused by parasites and by stimulation of lipopolysaccharide levels in the scallop

(Horney et al., 2001; Shi et al., 2012). Arginine kinase may be a stress response to the conditions of 0ASW. As 0ASW was too harsh for the cultivation of living pearl oysters, it was difficult to identify the molecules related to calcification in this treatment.

SDS-PAGE analysis indicated that the band around 130 kDa in the mantle tissue extract from pearl oysters cultured in 10ASW was thicker than that from 1ASW. LC-MS/MS analysis showed that this band represented filamin A. Previous research revealed that filamin A interacts with the calcium sensing receptor (CaSR) in mammalian cells to promote the Rho signaling pathway and induce calcification of bone (Min et al., 2002). Human filamin A has two CH domains that bind to actin and 24 filamin repeat regions that bind to CaSR (Awata et al., 2001). These complexes prepare the scaffold for the interaction of signaling molecules. Filamin A in *P. fucata* (Pf-filamin A) also has two CH domains and 15 filamin repeat regions, suggesting that Pf-filamin A may have a role binding CaSR in *P. fucata*. 2D-PAGE analysis showed that the spot 1 around 20 kDa and pI 5.0 increased in 10ASW, and LC-MS/MS analysis identified that the spot 1 as SCP. The neighboring spot 2 at low pI was also SCP, with the difference in pI caused by the presence or absence of phosphorylation. The spot 2 with low pI was SCP phosphorylated at the 83rd tyrosine residue, and only this phosphorylated SCP increased in 10ASW. The results of RT-PCR showed that SCP gene expression also increased in 10ASW, suggesting that the rate of phosphorylation could not keep up with the rate of expression; thus, only SCP without phosphorylation increased. Previous research identified SCP from the muscle of crayfish, and found it to be widespread in invertebrates. As SCP typically has two EF-hand motifs that are calcium-binding domains, SCP is thought to have a role in the storage and transport of calcium ions in the sarcoplasmic reticulum (Hermann and Cox, 1995; Rossi and Dirksen, 2006). A homology search showed that Pf-SCP from *P. fucata* included two EF-hand motifs at the N-terminal and C-terminal domains, suggesting that Pf-SCP binds calcium ions in the sarcoplasmic reticulum and transports it to the shell.

To elucidate the functions of these genes *in vivo*, knockdown experiments were performed using RNAi under the 10ASW condition. The injection of EGFP dsRNA that is not present in the genome of *P. fucata* led to precipitation of nanoparticles on the nacre surface, indicating that the dsRNA

itself did not cause non-specific effects on calcification of the shell. The injection of Pf-filamin A dsRNA caused no change in the nacre. As filamin A probably plays important roles in the upstream signal transduction for calcification, calcium supply might have been stopped by the knockdown experiment. On the other hand, the dsRNA injection of Pf-SCP induced strange incomplete tablets, with a hole in the center and a granular structure, in the nacreous layer. These results suggest that Pf-SCP has a role in transport of calcium to the nacreous layer. To develop a detailed understanding of the function of Pf-SCP, we prepared recombinant Pf-SCP protein.

ITC analysis showed that an exothermic reaction occurred in the recombinant Pf-SCP solution during titration with calcium ions, indicating that Pf-SCP has calcium ion-binding activity. However, it is difficult to know the exact binding constant of recombinant Pf-SCP. As the solubility of recombinant Pf-SCP without calcium ion was very low, the intensity of ITC was too small to calculate the exact quantitative analysis.

Some previous researches suggested that amorphous calcium carbonate (ACC) cluster was precipitated inside the vesicle (Mahamid et al., 2010). The organic molecules stabilize the ACC cluster inside the vesicle. We guess that Pf-SCP probably exists in the vesicle to bind calcium ion and ACC clusters. To examine the interaction between Pf-SCP and calcium carbonate, the inhibitory activity on calcium carbonate precipitation was measured. Previous research showed that calcium carbonate-binding molecules inhibited the increase of turbidity from calcium carbonate (Inoue et al., 2008; Wheeler et al., 1981). Recombinant Pf-SCP induced turbidity from calcium carbonate in a dose-dependent manner. Increased turbidity might mean that SCP plays a role in the synthesis of nucleation or promotes the rate of crystal growth. After 24 hours of calcium carbonate precipitation, we observed the morphology of calcium carbonate using SEM. The crystal size in the solution containing Pf-SCP was smaller than that in the control solution. These small crystals had a granular shape, which increased the surface area and thus increased the speed of crystal growth. These

observations implied that Pf-SCP binds to both calcium ions and calcium carbonate to synthesize small granular crystals.

The nacre of the shell was incubated in supersaturated calcium carbonate solution with or without recombinant Pf-SCP. Elongated, needle-like calcium carbonate crystals were observed in the control, showing the typical morphology of aragonite crystals formed in supersaturated calcium carbonate solution containing magnesium ions. Recombinant Pf-SCP promoted the formation of granular calcium carbonate, suggesting that Pf-SCP inhibited crystal growth and induced the nucleation of small granular calcium carbonate.

High calcium concentration stimulates the signal transduction of calcium ions in the mantle cells. The calcium-sensing receptor may be altered by increased Pf-filamin A to promote signal transduction. This signal probably induces secretion of Pf-SCP from the sarcoplasmic reticulum. The secreted Pf-SCP binds to calcium ions and regulates the formation of the calcium carbonate that makes up the shell (Fig. S14). The results of the present study support this hypothesis (Fig. 8). The normal condition, 1ASW, showed a good balance of calcium signal transduction, expression of SCP, and expression of shell matrix proteins. This orchestrated system induced an ordered compartmental microstructure in the nacreous layer. On the other hand, at the high calcium ion concentration of 10ASW, calcium signal transduction and expression of SCP increased, whereas transcriptomic analysis indicated that the expression levels of shell matrix proteins were not changed. Thus, although the organic framework of the nacreous layer was synthesized at normal speed, the rate of calcium precipitation was significantly increased. Abnormal precipitation of calcium carbonate caused an overflowing of the organic framework, leading to nanoparticle formation on the nacre surface. These findings suggest that transport of calcium from the mantle to the shell and formation of the organic matrix are regulated separately by different mechanisms. Further analysis of the calcification process in the nacreous layer will lead to more efficient production of high-quality

pearls in the future.

Acknowledgements

TABLES

TABLE 1 Components of each seawater formula

	NSW	1ASW	0ASW	10ASW
Cl^-	0.54	0.54	0.54	0.54
Na^+	0.46	0.46	0.46	0.25
SO_4^{2-}	0.03	0.03	0.03	0.01
Mg^{2+}	0.05	0.05	0.05	0.05
Ca^{2+}	0.01	0.01	**0.00**	**0.10**
K^+	0.01	0.01	0.01	0.01

(M)

TABLE 2 Sequences of primers used for qPCR

Primer	Sequence
Actin-qF	CAGAAGGAAATCACCGCACT
Actin-qR	AGAGGGAAGCAAGGATGGAG
Filamin-qF	GTAGAACACAGCCAGCCAAG
Filamin-qR	CCACTTTACACGGACCTTCC
SCP-qF	TGACAACTCCAACGACACG

SCP-qR   CATTTCACTTGCTATCCTTCC

TABLE 3 Sequences of primers used for RNAi experiments

Primer	Sequence
Filamin-T7F	GCGTAATACGACTCACTATAGGGGTGGGCAAACGAACACCTG
Filamin-T7R	GCGTAATACGACTCACTATAGGGTCCCAGTCAGGGCACAAAC
SCP-T7F	GCGTAATACGACTCACTATAGGGGCTGGACGTAAACCATGACG
SCP-cT7R	GCGTAATACGACTCACTATAGGGGGTTGACGTAATCCGTGTCG

TABLE 4 Results of transcriptomic analysis using next-generation sequencing

		Total Sequences	Total Read Counts	Average of Mapped Read Counts Per Gene
1ASW	S1	4318320	869143	11.97
	S2	4450836	861440	11.87
	S3	3961805	718984	9.90
	S4	4331952	890550	12.27
	Total	17062913		
10ASW	S5	4086822	772792	10.64
	S6	4162447	707454	9.74
	S7	3574048	716607	9.87
	S8	3328109	682099	9.40
	Total	15151426		

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Figure legends

Fig. 1. **SEM observations of nacre surface.** (A) Nacre surface of living *P. fucata* treated with 1ASW. (B) Nacre surface of the shell from *P. fucata* treated with 0ASW. (C) Nacre surface of living *P. fucata* treated with 0ASW. (D) Nacre surface of the shell from *P. fucata* treated with 10ASW. (E) Nacre surface of living *P. fucata* treated with 10ASW. (F) The magnified image from the white rectangle region in (E).

Fig. 2. **TEM observation of nacre cross section from living *P. fucata* treated with 10ASW treated with 10ASW.** (A) Bright filed image of the cross section. The arrow head indicated the granular layer. The arrows indicated the organic framework in the nacre. White circles of a and b showed the selected areas for electron diffractions of (B) and (C), respectively. (B) SAED pattern from the white circle of a in (A). The white arrow indicated the direction of *c*-axis. (C), SAED pattern from the white circle of b in (A). The white arrow indicated the direction of *c*-axis.

Fig. 3. **SDS-PAGE of the mantle extracts from the living *P. fucata*.** Lane 1 showed the control sample treated with 1ASW. Lane 2 showed the sample treated with 0ASW. Lane 3 showed the sample treated with 10ASW. Arrow in lane 2 indicates 40-kDa protein and that in lane 3 130-kDa protein.

Fig. 4. **Amino acid sequence of Pf-filamin A.** The rectangle showed the detected peptide fragment in LC-MS/MS. The underlines showed the domain of SAC6 super family for the function of calcium ion binding. The bold characters show the filamin-type immunoglobulin domains that bind to actin.

Fig. 5. **2D-PAGE of the mantle extracts in the conditions of 1ASW and 10ASW.** (A) the

condition of 1ASW. (B) the condition of 10ASW.

Fig. 6. **Amino acid sequence of Pf-SCP.** The rectangle showed the detected peptide fragment in LC-MS/MS. The underlines showed the domains of EF-hand motif. Circled Y means the phosphorylated tyrosine.

Fig. 7. **SEM observations after RNAi experiments.** (A) The SEM image of nacre surface from *P. fucata* injected with EGFP dsRNA. (B) the magnified image from (A). (C) the SEM image of nacre surface from *P. fucata* injected with Pf-filamin dsRNA. (D) the magnified image from (C). (E) The SEM image of nacre surface from *P. fucata* injected with Pf-SCP dsRNA. (F) the magnified image from (E).

Fig. 8. **Schematic representation of nacre formation in the condition of 1ASW and 10ASW.** (A) The expression of genes in 1ASW kept the good balance to make the normal tablet growth in the nacreous layer. (B) 10ASW induced the expression of Pf-filamin A and Pf-SCP that regulate the calcium transport to the shell, while 10ASW did not change the expression level of the genes of shell matrix proteins. This unbalance promote the abnormal precipitation on the nacre surface and inhibited the normal tablet growth in the nacreous layer.