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Original Article

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Transcription factor Pf-Rel regulates expression of matrix protein genes *Prismalin-14* and *MSI60* in the pearl oyster *Pinctada fucata*

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Abstract

Molluscan shell is a biomineral that consists of a highly organized calcium carbonate composite. Organisms mainly use matrix proteins to elaborately control the biomineralization process, but knowledge of their regulatory mechanisms is limited. The transcription factor Pf-Rel, which belongs to the Rel/nuclear factor-κB family, was shown to regulate transcription at the *Nacrein* promoter in the pearl oyster *Pinctada fucata*. Here, we further explored the transcriptional regulation mechanisms of Pf-Rel on the matrix proteins Prismalin-14 and MSI60. The relative expression levels of *Prismalin-14* and *MSI60* were high in the mantle edge and mantle pallial tissues of *P. fucata*. These three genes were significantly up-regulated after shell notching, suggesting that they might play important roles during shell formation. Importantly, *Pf-Rel* gene knockdown by RNA interference led to down-regulation of *Prismalin-14* and *MSI60* expression. In transient co-transfection assays, Pf-Rel significantly up-regulated the promoter activities of the *Prismalin-14* and *MSI60* genes in a dose-dependent manner. Furthermore, the promoter regions of *Prismalin-14* (−1794 to −1599 bp) and *MSI60* (−2244 to −1141 bp) were required for the activation by Pf-Rel. Altogether, these results suggest that the transcription factor Pf-Rel can up-regulate the expression of the matrix protein genes *Prismalin-14* and *MSI60* during shell formation in *P. fucata*, which improves our understanding of transcription regulation at the molecular level during molluscan shell development.

Key words: Pf-Rel, *Prismalin-14*, *MSI60*, shell formation, biomineralization

Introduction

Biomineralization, a ubiquitous phenomenon in nature, is the process by which organisms secrete bio-organic macromolecules such as proteins, glycoproteins, and polysaccharides, to regulate the nucleation, growth, and molecular arrangement of inorganic crystals in specific parts of the organism to form an ordered deposition [1–4]. As the exoskeleton of mollusks, shells are the main products of biomineralization of soft organisms such as shellfish.

The pearl oyster *Pinctada fucata*, the shell of which is composed of an inner nacre and an outer prismatic layer, is widely used as the model to investigate the mechanism of biomineralization. Molluscan shells consist of more than 95% calcium carbonate crystal by weight, while the remaining 5% consists of organic macromolecules,

including matrix proteins, polysaccharides, and lipids [5]. Although the proportion of the organic matrix in the shell is low, the oysters are able to direct this 95% of inorganic material into the formation of regular crystals during biomineralization [6]. Matrix proteins, such as Nacrein [6], Pif [7], the KRMP family [8], and the Shematrin family [9], have been proven to be the major components responsible for nucleation, orientation, morphology, and organization during the shell formation process in *P. fucata* [10]. In this study, we focused on the matrix proteins Prismalin-14 and MSI60.

The matrix protein Prismalin-14, which is mainly found in the acid-insoluble components of the prismatic layer, can inhibit calcium carbonate precipitation and bind ionic calcium [11]. *In vitro* crystallization experiments using truncated protein bodies revealed that the Gly/Tyr-rich region of Prismalin-14 is a chitin-binding region, and the N- and C-terminal Asp-rich regions are key regions for the inhibition of calcium carbonate precipitation. These results indicated that Prismalin-14 may be involved in the formation of shells as a framework protein [12,13].

The matrix protein MSI60 is present in the water-insoluble component of the pearl layer. The sequence of MSI60 is rich in poly-Gly and poly-Ala blocks, which have the ability to bind with calcium ions. It is considered a framework protein that participates in the formation of the reverse β-sheet structure of the pearl layer [14]. Although the mechanisms of calcification that matrix proteins participate in have been sought by many investigators, our understanding of how the upstream transcription factors regulate the downstream matrix protein genes is limited. Research has shown that the transcription factors Pf-POU3F4 and SOX9 may up-regulate the expression of *Prismalin-14* [15,16]. At present, there have been few studies on the regulation of MSI60 matrix protein.

The nuclear factor-kappa B (NF-κB) transcription factor is found in both vertebrates and invertebrates [17,18] and is known as a classic and evolutionarily conserved mediator of immune responses in vertebrates [19]. The core elements of the NF-κB signaling pathway include the activating inhibitor of nuclear factor kappa B kinase (IKK) complex, the inhibitory IκB protein, and the transcription factor Rel/NF-κB [20,21]. Since its initial discovery as a B-cell-specific transcription factor [22], previous research has shown that, in mammals, the NF-κB family of transcription factors regulates the expression of a wide array of genes involved in various physiological processes [23–26]. In recent years, scientists have found that the NF-κB signaling pathway not only regulates genes involved in the inflammatory and immune responses but also plays important role in bone homeostasis, osteoclast differentiation, and vertebrate bone formation [27–30]. Importantly, our previous studies revealed that the NF-κB signaling pathway is involved in shell formation and that the transcription factors Pf-IKK and Pf-Rel are involved in transcription regulation at the *Nacrein* promoter in *P. fucata* [21,31].

To better understand the mechanisms that regulate shell formation, and to investigate whether the transcription factor Pf-Rel regulates expression of the matrix protein genes *Prismalin-14* and *MSI60* in pearl oysters, the *Pf-Rel* gene was silenced by RNA interference (RNAi) in *P. fucata* and changes in the expression levels of *Prismalin-14* and *MSI60* were observed. For the *in vitro* assays, the promoters of *Prismalin-14* and *MSI60* were cloned into luciferase reporter vectors and co-transfected with the *Pf-Rel* gene into the human embryonic kidney cell line HEK293T. Measurement of luciferase levels clarified that Pf-Rel was involved in the regulation of the promoter activities of *Prismalin-14* and *MSI60*. Furthermore, to explore the activation sites for Pf-Rel on the *Prismalin-14* and *MSI60* promoters, different truncations of each promoter were cloned into the luciferase reporter systems and co-transfected with a Pf-Rel expression plasmid into HEK293T cells. Our results provide a foundation for understanding the transcriptional regulation of matrix proteins and shed light on the mechanisms of shell formation at the molecular level.

Materials and Methods

Experimental animals

Pinctada fucata oysters were purchased from the Zhanjiang Pearl Farm (Zhanjiang, China) and maintained in an artificial seawater tank for 5 days prior to experimentation. The use of animals and experimental procedures followed the Guidelines of Animal Use and Care of the National Institute of Health and were approved by the Animal Experimental Ethics Committee of Tsinghua University, Beijing, China.

Tissue distribution detected by real-time polymerase chain reaction

Samples of seven tissues (gonad, gill, foot, heart, mantle pallial, mantle edge, and shell muscle) were collected from three healthy pearl oysters of similar size. The distributions of *Pf-Rel*, *Prismalin-14*, and *MSI60* mRNA in each of the tissues were detected using real-time polymerase chain reaction (PCR). Total cDNA was synthetized using the PrimeScript™ RT reagent from a gDNA Eraser Kit (Takara, Kusatsu, Japan). Real-time PCR primers were designed via SnapGene and the nucleotide sequences are shown in **Table 1**. Real-time PCR analysis was carried out using SYBR Premix Ex Taq™ (Tli RNaseH Plus; Takara). PCR amplification was carried out as 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 30 s in duplicate.

Shell notching

We collected 40 *P. fucata* oysters of similar size and randomly divided them into eight groups of five individuals each. We used scissors to cut out a 'V'-shaped gap on the edge of the shell, taking care to cut into the shell pearls and prismatic layers only, without damaging the mantle tissues. At eight specific time points (0, 6, 12, 24, 36, 48, 72, and 96 h) after shell notching, we collected the mantle tissues from the five individuals of one group for cryopreservation in liquid nitrogen.

Silencing of the Pf-Rel transcription factor

RNAi experiments were performed to determine the variation tendencies of *Prismalin-14* and *MSI60* expression under the depression of *Pf-Rel in vivo*. The synthesis and purification of *Pf-Rel* dsRNA and green fluorescent protein (GFP) dsRNA were conducted using a T7RiboMAX™ Express RNAi System Kit (Promega, Madison, USA) according to the manufacturer's instructions. The vector pEGFP-C1 (Clontech, Shiga, Japan) was used as the template to amplify GFP. Primer sequences were designed (Ruibiotech, Beijing, China) with a T7 promoter and are listed in **Table 1**. Purified dsRNA was diluted to 20 μg/200 μl and 60 μg/200 μl using MilliQ water (Tiangen Biotech, Beijing, China). We collected 36 *P. fucata* oysters of similar size and randomly divided them into four groups of nine individuals each. The four groups were sequentially injected by syringe with MilliQ water, GFP dsRNA, and a low or high concentration of *Pf-Rel* dsRNA. After injection, the mantle tissues from three individuals were collected separately from each group and cryopreserved at

Table 1. Nucleotide sequences of primers used in the RNAi, real-time PCR, and truncation experiments

Group	Gene	Primer name	Nucleotide sequence
RNAi	*Pf-Rel*	dsRel-F	5′-GGATCCTAATACGACTCACTATAGGTACACTGGTACTGCTGTAATCGTTGTG-3′
		dsRel-R	5′-GGATCCTAATACGACTCACTATAGGCTGTTCTCCTGGTAAAACCTCACTCC-3′
	GFP	dsGFP-F	5′-GGATCCTAATACGACTCACTATAGGATGGTGAGCAAGGGCGA-3′
		dsGFP-R	5′-GGATCCTAATACGACTCACTATAGGACTTGTACAGCTCGTCCATG-3′
Real-time PCR	*Pf-Rel*	Rel-F	5′-GGCAGCGCAAAGAGATTAATG-3′
		Rel-R	5′-TGCGAGTGATTTTACCCTGG-3′
	Prismalin-14	P14-F	5′-GTGTTAGTAAGTCTGTGAGGGTG-3′
		P14-R	5′-TGCTGTTCAAAAGGATCCGG-3′
	MSI60	MSI60-F	5′-CCAAATGTCTTAACCTTGAACCC-3′
		MSI60-R	5′-TGTTATGCGTTACGTTCCAGG-3′
	β-*Actin*	Actin-F	5′-CTCCTCACTGAAGCCCCCCTCA-3′
		Actin-R	5′-ATGGCTGGAATAGGGATTCTGG-3′
Truncation	*Prismalin-14*	P14-2-PGL-F	5′-CCGCTCGAGAGCTTTAAAGGTCATTTCCTTAC-3′
		P14-3-PGL-F	5′-CCGCTCGAGCTACACCGACATGGAATTTCGC-3′
		P14-PGL-R	5′-CATGCCATGGCGGACAGTCTGTAAACTTACC-3′
	MSI60	M60-1-PGL-F	5′-CCGCTCGAGGATTGTGAAAAGAGGGATTCCAT-3′
		M60-2-PGL-F	5′-CCGCTCGAGAGAAAGAAAGAAAGAAAAACCCA-3′
		M60-PGL-R	5′-CCCAAGCTTTCTTACCTATGTTATGTGGGGAG-3′

2 days, 4 days, and 6 days. Preparation of the cDNA templates and determination of relative gene expression levels using real-time PCR were performed as previously described.

Cell culture, transfection, and dual luciferase assay

The PCR-amplified promoters of *Prismalin-14* (GeneBank number KM519601) and *MSI60* (GeneBank number KM519604) were inserted into the multiple cloning sites of the pGL4.10-Basic vector (Promega) to construct *Prismalin-14* and *MSI60* promoter-luciferase reporters, designated pGL4.10-*Prismalin-14* and pGL4.10-*MSI60*. The pcDNA3.1(+)-myc vector plasmids for expression of *Pf-Rel* in eukaryotic cells were constructed using traditional molecular cloning methods [15].

HEK293T cells (China Infrastructure of Cell Line Resources, Beijing, China) were maintained in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, USA). The cells were transfected using VigoFect (Vigorous Biotechnology, Beijing, China) in accordance with the manufacturer's instructions. For co-transfection assays, cells were transfected with the following, added to each well of a 48-well plate: 500 ng, 250 ng, 125 ng, 62.5 ng, or 0 ng of pcDNA3.1(+)-myc-*Pf-Rel* or empty pcDNA3.1(+)-myc expression vector; 100 ng of pGL4.10-*Prismalin-14* or pGL4.10-*MSI60* or pGL4.10 empty luciferase vector; and 2 ng of pRL-TK (Promega), which was co-transfected as an internal reference to normalize the transfection efficiency. The empty pcDNA3.1(+)-myc expression vector was used to adjust the total transfected DNA volume in dose-effect experiments. Luciferase reporter analysis and data process [15] was performed using a Dual-Luciferase Reporter Assay system (Promega) and a Varioskan^{TM} Flash multimode reader (Thermo Scientific, Waltham, USA) 36 h after transfection.

Truncation plasmid construction and cell transfection

The coding region of *Pf-Rel* was cloned into the expression vector pcDNA3.1(+)-myc (Invitrogen, Carlsbad, USA) for the expression of myc epitope fusion proteins and GFP fusion proteins. Using a transcription factor and promoter activation site prediction website (http://jaspar.genereg.net/), we found possible activation sites of *Pf-Rel* on the promoters of the *Prismalin-14* and *MSI60* genes, which were used to design two truncations of each promoter. The *Prismalin-14* gene promoter-defined luciferase reporter plasmids designated as pGL4.10-*Prismalin-14*-2 (−1599 to +47 bp), and pGL4.10-*Prismalin-14*-3 (−1577 to +47 bp), and two truncations of the *MSI60* gene promoter-defined luciferase reporter plasmids, pGL4.10-*MSI60*-2 (−1141 to +28 bp), pGL4.10-*MSI60*-3 (−1188 to +28 bp), were cloned. All constructs were verified by sequencing. The primers used are listed in **Table 1**. Co-transfections contained 500 ng of pcDNA3.1(+)-myc-*Pf-Rel*, 100 ng of pGL4.10-*Prismalin-14*-2/3, or pGL4.10-*MSI60*-2/3 or pGL4.10 empty luciferase vector, and 5 ng of pRL-TK was added to each well of 48-well plate.

Statistical analysis

Statistical Package for the Social Sciences version 18.0 software (SPSS Inc., Chicago, USA) was used for the statistical analysis. Values are shown as the mean±SD of three independent experiments and were analyzed by Student's *t*-test to identify the differences between groups. $P<0.05$ was considered statistically significant. Spearman's rank correlation was used to analyze the correlations between Pf-rel and Prismalism-14 or MSI60 during shell notching. $P<0.05$ were considered statistically significant.

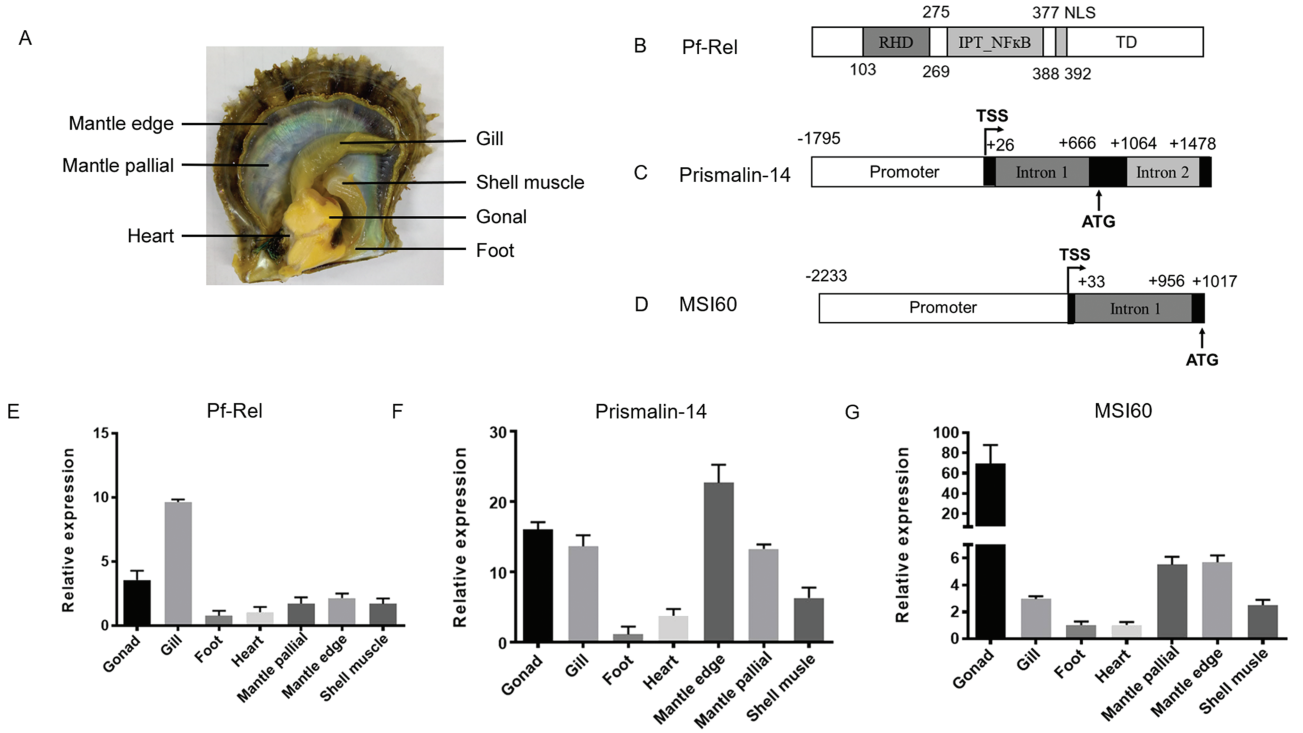

Figure 1. Tissue distribution of *Pf-Rel*, *Prismalin-14*, and *MSI60* mRNAs in pearl oysters (A) Anatomy map of *Pinctada fucata*. (B) Schematic diagram of the functional domains of the Pf-Rel protein. Schematic diagrams of the transcriptional regulatory regions of *Prismalin-14* (C) and *MSI60* (D). TSS: transcription start site. (E–G) Relative expression levels of *Pf-Rel*, *Prismalin-14*, and *MSI60* mRNA in seven pearl oyster tissues (gonad, gill, foot, heart, mantle pallial, mantle edge, and shell muscle), calculated using the $2^{-\Delta\Delta Ct}$ method. Data are shown as the mean±SD of five samples.

Results

Tissue distribution of *Pf-Rel*, *Prismalin-14*, and *MSI60* mRNA in pearl oysters

The expression levels of *Pf-Rel*, *Prismalin-14*, and *MSI60* in seven different tissues of *P. fucata* were determined using real-time PCR (**Fig. 1**). Although mRNAs from *Pf-Rel*, *Prismalin-14*, and *MSI60* were distributed widely across the tissues analyzed, the expression patterns were different. The expression of *Pf-Rel* was highest in gill tissue, followed by gonad, mantle edge, mantle pallial, shell muscle, heart, and foot. *Prismalin-14* mRNA was mainly expressed in mantle edge, gonad, mantle pallial, and gill, while *MSI60* was mainly expressed in gonad, mantle edge, and mantle pallial. The relative higher expressions of *Prismalin-14* and *MSI60* in mantle tissues implied that they may play important roles in the biomineralization process, as mantle tissues play a key role in shell formation.

Shell notching and the variation tendencies of *Pf-Rel*, *Prismalin-14*, and *MSI60* mRNA expression in pearl oysters

Following an injury caused by external stimuli, it is generally believed that the shell itself will respond to the damaged stimulus and repair the damaged parts. At this time, the expression of genes related to mineralization would be expected to change in accordance with the restoration of the shell.

The shell notching experiments (**Fig. 2A**) suggested that injury led to a sudden increase in the expression level of the Pf-Rel transcription factor at 6 h, followed by a sharp decrease (**Fig. 2B**). The expression level gradually increased from the 12 h time point until it reached a peak at 36 h; a gradual decrease followed, but the high expression level was maintained at the 96 h time point. The expression level of the *Prismalin-14* gene (**Fig. 2C**) suddenly increased at 6 h after notching, and then decreased, gradually rising between 12 h and 36 h and peaking at 36 h. There was a gradual decrease, with a return to essentially normal levels at 96 h. The expression level of the *MSI60* gene (**Fig. 2D**) also suddenly increased at 6 h after notching, and then decreased, followed by a gradual increase from 24 h to 48 h and a peak at 48 h. The expression gradually decreased but was still maintained at a high level. In general, the expressions of these three genes changed in a synchronized manner, and statistical analysis results showed that the expression of the matrix protein Prismalin-14 was significantly correlated with Pf-Rel (**Table 2**). The expression correlation between MSI60 and Pf-Rel showed no statistical significance (**Table 2**). These data indicated that matrix protein Prismalin-14 and transcription factor Pf-Rel showed more consistent response to shell regeneration, compared to that between matrix protein MSI60 and transcription factor Pf-Rel.

Pf-Rel knockdown leads to a decrease in *Prismalin-14* and *MSI60* gene expression

After observing that the expression patterns of *Pf-Rel*, *Prismalin-14*, and *MSI60* were similar in response to shell notching, we speculated that Pf-Rel might regulate the expression of *Prismalin-14* and *MSI60*. To confirm this speculation, we knocked down *Pf-Rel in vivo* using dsRNA. As shown in **Fig. 3**, the injection of 20 μg of *Pf-Rel* dsRNA led to a down-regulation in the expression of *Pf-Rel* mRNA to 50% after 2 days, 30% after 4 days, and 15% after 6 days, compared with the group injected with MilliQ water. The expression level of

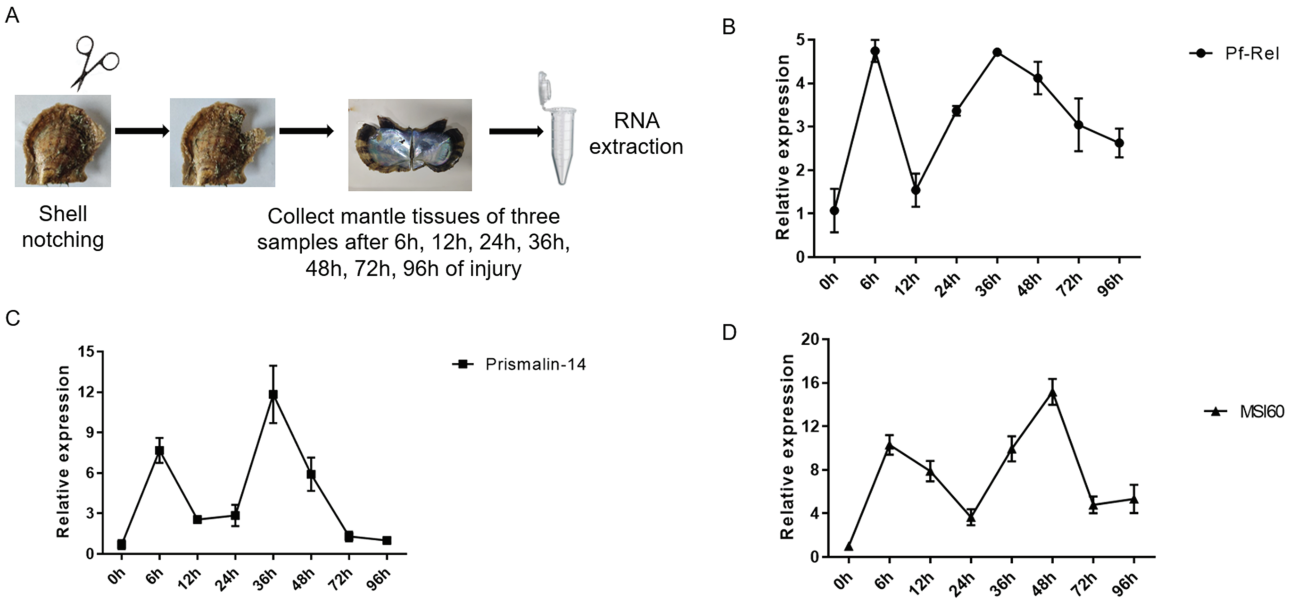

Figure 2. Shell notching and the variation tendency of *Pf-Rel*, *Prismalin-14*, and *MSI60* mRNA expression in pearl oysters (A) Schematic diagram of shell notching and sample collection. (B–D) Relative expression of *Pf-Rel*, *Prismalin-14*, and *MSI60* mRNA in shell notching experiments calculated using the $2^{-\Delta\Delta Ct}$ method. Data are shown as the mean±SD for five samples.

Table 2. Correlation assay between Pf-Rel and Prismalin-14 or MSI60

Item	Pf-Rel vs Prismalin-14	Pf-Rel vs MSI60
R	0.905	0.690
P	0.002	0.058

R represents the coefficient of correlation. Correlation was considered statistically significant at $P<0.05$.

Prismalin-14 mRNA was down-regulated to 70%, 35%, and 30%, respectively, and the expression level of *MSI60* mRNA was down-regulated to 85%, 45%, and 40%, respectively. The expression levels of all three genes decreased over time. These results suggested that the RNAi method not only inhibited *Pf-Rel* at the mRNA level but also affected the mRNA expression of matrix protein *Prismalin-14* and *MSI60*. After 60 μg of *Pf-Rel* dsRNA was injected, *Pf-Rel* expression was suppressed to 30%, 20%, and 20% after 2, 4, and 6 days, respectively, while the expression of *Prismalin-14* was down-regulated to 60%, 25%, and 20%, respectively, and that of *MSI60* to 65%, 20%, and 20%, respectively. Compared with the group injected with MilliQ water, the expression levels of *Pf-Rel*, *Prismalin-14*, and *MSI60* mRNA in the group injected with an equal quantity of GFP dsRNA showed no significant changes, which meant that GFP dsRNA group was a successful negative control.

Pf-Rel activates the promoters of *Prismalin-14* and *MSI60*

To further confirm that *Pf-Rel* could be a regulator of both *Prismalin-14* and *MSI60* gene expression, a dual *in vitro* luciferase assay was conducted. After co-transfection of HEK293T cells with dual luciferase reporters and 0 ng, 62.5 ng, 125 ng, 250 ng, or 500 ng of *Pf-Rel* expression vector, we found that Pf-Rel significantly increased

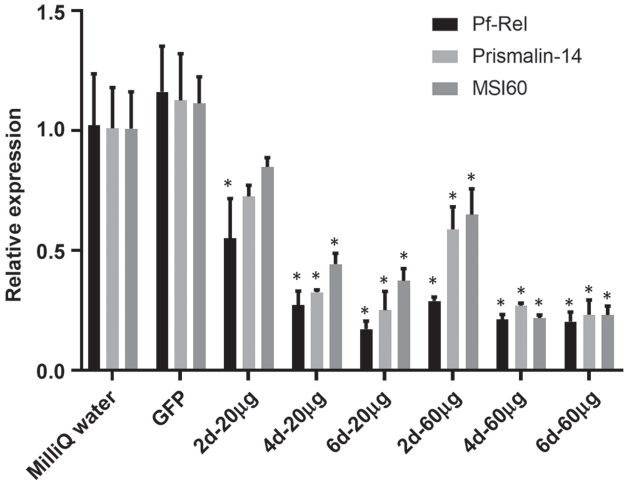

Figure 3. *Pf-Rel* knockdown decreased the expression levels of *Prismalin-14* and *MSI60* The relative expression levels of *Pf-Rel*, *Prismalin-14*, and *MSI60* mRNA in *P. fuctata* mantle at 2 days, 4 days, and 6 days after low-dose or high-dose injection of *Pf-Rel* dsRNA. Three samples were used in each experiment. The *Pf-Rel*, *Prismalin-14*, and *MSI60* mRNA expression levels in controls are attributed a relative value of 1.0. Data are shown as the mean±SD of three samples. Asterisks indicate statistically significant reduction. *$P<0.05$, Student's *t*-test.

the promoter activities of *Prismalin-14* and *MSI60* in a dose-dependent manner (**Fig. 4A,B**). This demonstrated that Pf-Rel could regulate the expression of *Prismalin-14* and *MSI60* by activation of their promoters.

Prismalin-14 and *MSI60* promoter sites are directly activated by Pf-Rel

To further explore which regions of the *Prismalin-14* and *MSI60* promoters are specifically activated by Pf-Rel, we constructed two

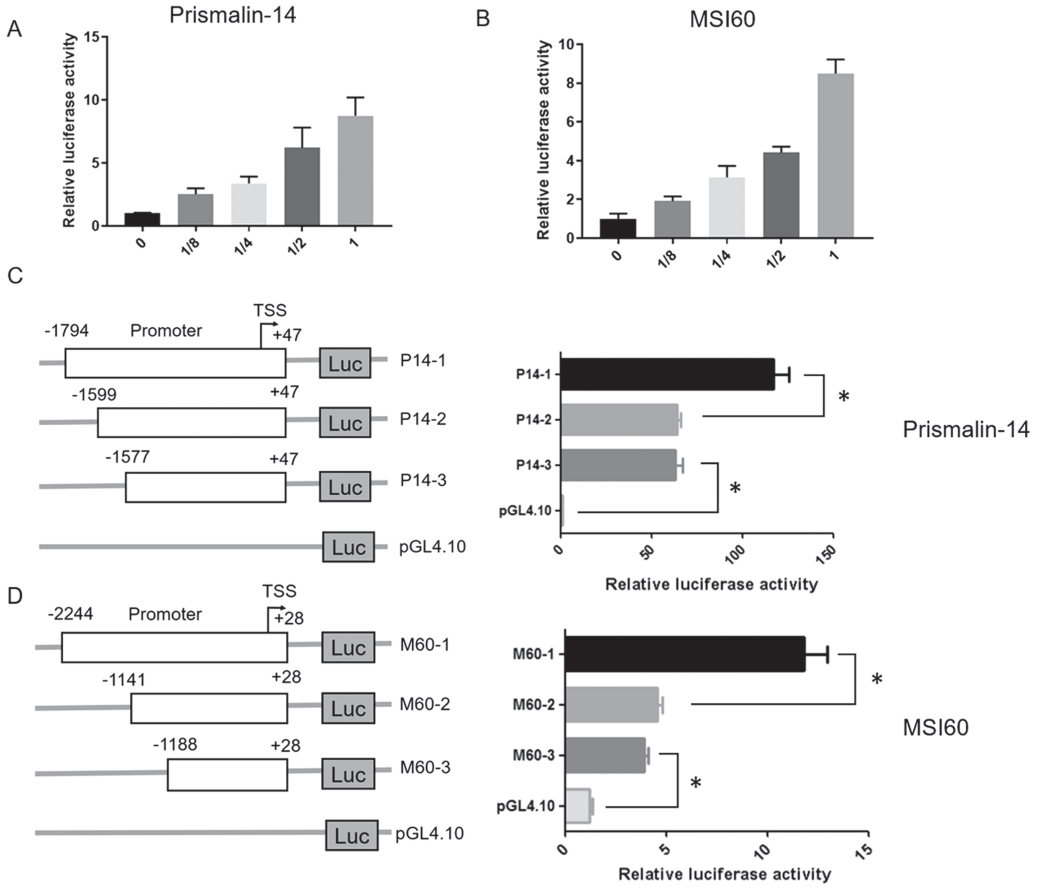

Figure 4. *Pf-Rel* directly activated the sites of the *Prismalin-14* and *MSI60* gene promoter (A,B) HEK293T cells were transfected with different doses of pcDNA3.1(+)-myc-*Pf-Rel*. The '1' indicates 500 ng of recombinant expression vector per well of a 48-well plate. Data are shown as the mean±SD of three independent experiments. Promoter truncations revealed that (C) the −1794 to −1599 bp region of the *Prismalin-14* gene promoter and (D) the −2244 to −1141 bp region of the *MSI60* gene promoter are essential for activation by *Pf-Rel*. Asterisks indicate statistically significant differences between the adjacent two promoter truncations. * $P<0.05$, Student's *t*-test.

promoter truncations with progressive upstream deletions for each gene as follows: pGL4.10-*Prismalin-14*-2 (−1577 to +47 bp) and pGL4.10-*Prismalin-14*-3 (−1599 to +47 bp), and pGL4.10-*MSI60*-2 (−1141 to +28 bp) and pGL4.10-*MSI60*-3 (−1188 to +28 bp). As shown in **Fig. 4**, the relative luciferase activity of pGL4.10-*Prismalin-14*-2 was significantly decreased compared with the full length *Prismalin-14* gene promoter (pGL4.10-*Prismalin-14*-1), whereas there is almost no difference between pGL4.10-*Prismalin-14*-2 and pGL4.10-*Prismalin-14*-3. Moreover, there still existed significant difference between the relative luciferase activity of pGL4.10-*Prismalin-14*-2 and that of vector pGL4.10. This showed that the sites from −1794 to −1599 bp and from −1577 to +47 bp in the *Prismalin-14* gene promoter might be possible regions that transcriptional factor Pf-Rel could activate. Regarding the *MSI60* promoter truncations, the relative luciferase activity of pGL4.10-*MSI60*-2 was obviously decreased compared with the full length gene promoter (pGL4.10-*MSI60*-1), and there was no difference between the expression of pGL4.10-*MSI60*-2 and pGL4.10-*MSI60*-3. In addition, the relative luciferase activity of pGL4.10-*MSI60*-2 was also significantly higher than that of pGL4.10. This showed that Pf-Rel could activate transcription at the possible sites between −2244 and −1141 bp as well as −1188 to +28 bp in the *MSI60* gene promoter.

Discussion

The *P. fucata* species is the main source of pearl production in aquaculture. Shells and pearls are the typical products of biomineralization. Many experiments have shown that various matrix proteins play important roles in shell formation, making the matrix proteins a long-time research hotspot. Still, the upstream regulatory mechanisms of transcription factors of the matrix protein genes are worthy of further investigation. This study focused on the regulatory mechanism of the transcription factor Pf-Rel on matrix protein genes *Prismalin-14* and *MSI60* in *P. fucata*.

Matrix proteins are secreted by the mantle tissues, which cover the inner surface of the shell and are responsible for the formation of the prismatic layer and the nacreous layer [32]. The high expression of *Prismalin-14* and *MSI60* in mantle edge and mantle pallial tissues suggested their important roles in shell formation. Though the expression of transcriptional factor *Pf-Rel* in mantle tissues was relatively lower, we could not deny the participation of Pf-Rel in biomineralization process considering the RNAi as well as shell notching experiments. Besides, Pf-Rel, an upstream transcriptional factor, might cooperate with other factors or activate the downstream effect factors to play roles in the biomineralization process indirectly.

In the shell notching assay, the expression of Pf-Rel, Prismalin-14, and MSI60 all up-regulated after shell injury, indicating their vital

roles in shell formation. The expression changes of these three genes seemed highly consistent; the expression level increased immediately after shell notching, and then decreased sharply, which might be a stress reaction, and followed by another increased expression, and then decreased slowly. At 96 h after shell notching, the relative expression of *Pf-Rel*, *Prismalin-14*, and *MSI60* genes returned to nearly normal level but was still higher than 0 h. The correlation assay showed that the expression of the matrix protein Prismalin-14 was significantly correlated with Pf-Rel, while the correlation between the expression of matrix protein MSI60 and transcriptional factor Pf-Rel showed no statistical significance. We inferred that MSI60 might be regulated by several other transcriptional factors as the regulation network was rather complex during shell formation. Previous studies had shown that matrix proteins Prismalin-14 was mainly found in prismatic layer while MSI60 was mainly found in nacreous layer, and the prismatic layer formed firstly and then the nacreous layer [11,14]. As the expression level of Prismalin-14 gene reached a peak at 36 h and the expression level of MSI60 gene reached a peak at 48 h, it is possible that Prismalin-14 might response earlier than MSI60 during shell formation, which is an extra proof that there is temporal and spatial difference among the expression of different matrix proteins.

The comprehensive analysis showed that the expression levels of *Prismalin-14* and *MSI60* were significantly decreased in both low-dose and high-dose RNAi injection experiments, with the high-dose injection of *Pf-Rel* dsRNA exhibiting a more effective suppression of *Pf-Rel*, *Prismalin-14*, and *MSI60* mRNA expression than the low-dose injection. Therefore, these results further indicated that the *Pf-Rel* gene has a regulatory effect on the *Prismalin-14* matrix protein and the *MSI60* matrix protein. In general, the inhibition effect of *Prismalin-14* mRNA expression was relatively stronger than that of *MSI60* mRNA expression when detected at the same time and injected with the equal amount of *Pf-Rel* dsRNA. This observation was in accordance with the above correlation analysis during shell notching, which once more suggested that matrix protein *MSI60* could be regulated by more than one transcriptional factor. Finally, using a dual luciferase reporter system in co-transfection experiments in HEK293T cells, we proved that Pf-Rel could activate the promoters of *Prismalin-14* and *MSI60* genes. Although we had achieved our initial objective, we sought to explore where Pf-Rel binds to the *Prismalin-14* and *MSI60* gene promoters to activate transcription. Our truncations of the *Prismalin-14* and *MSI60* gene promoters allowed us to narrow the possible activation regions from −1794 to −1599 bp and from −1577 to +47 bp in the *Prismalin-14* promoter, and from −2244 to −1441 bp as well as from −1188 to +28 bp in the *MSI60* promoter. The exact activation sites need to be proved by further explorations.

Based on our previous studies that Pf-Rel could also regulate the expression of the matrix protein Nacrein [21], we propose that Pf-Rel has a general regulatory effect on matrix proteins and thus plays a very important role in shell formation. In addition, the *Pf-POU3F4* transcription factor was also shown to activate the promoter of *Prismalin-14* and to have a certain regulatory effect on the matrix protein *Prismalin-14* [15]. Taken together, these results demonstrate that the transcriptional regulation network in the *P. fucata* was rather complex, which need further studies and would help us understand biomineralization mechanism better.

In summary, transcription factor Pf-Rel can regulate expression of matrix protein genes Prismalin-14 and MSI60 by promoter activation in the pearl oyster *P. fucata*. We plan to use genetic engineering methods to combine the precise promoter activation sites into a stronger promoter, and therefore regulate the expression of the *Prismalin-14* and *MSI60* genes more effectively. Ultimately, this will facilitate the creation of transgenes to accelerate the formation of pearls and increase the economic value of *P. fucata* oysters.

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