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Dermatopontin, a shell matrix protein gene from pearl oyster *Pinctada martensii*, participates in nacre formation

Yu Jiao, Huan Wang, Xiaodong Du *, Xiaoxia Zhao, Qingheng Wang, Ronglian Huang, Yuewen Deng

Fishery College, Guangdong Ocean University, Zhanjiang 524025, China

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ABSTRACT

Dermatopontin (DPT) is identified as a major component of the shell matrix protein. However, its exact function in the shell formation remains obscure. In this study, we described the characteristic and function of DPT gene from *Pinctada martensii*. DPT cDNA was 797 bp long, containing an open reading fragment (ORF) of 537 bp encoding a polypeptide of 178 amino acids with an estimated molecular mass of 21.4 kDa and theoretical isoelectric point of 5.97. The 5′ untranslated region (UTR) was 11 bp and the 3′UTR was 249 with 18 bp poly (A) tail. In the peptide, there was a signal sequence, six potential phosphorylation sites, one glycosylation site and eight cysteine residues. Moreover, a sequence motif (D-R-X-W/F/Y-X-F/Y/I/L/M-X_{1-2}-C) was contained and repeated itself three times in the entire sequence. DPT mRNA was constitutively expressed in all studied tissues with the most abundant mRNA in the mantle, which was nacre formation-related tissue. After decreasing DPT expression using RNA interference (RNAi) technology in the mantle, the nacreous layer showed a disordered growth; whereas the prismatic layer of the shells has no significant changes. These results suggested that DPT obtained in this study was a constitutive matrix protein and participated in nacre formation in *P. martensii*.

1. Introduction

Molluscan shells are extraordinarily stable organo-mineral composites. It is a characteristically layered structure composed of calcium carbonate crystals and an organic matrix consisting of polysaccharides, proteins, glycoproteins and proteoglycans [1,2]. Although the organic matrix typically constitutes less than 5 wt.% of the biomineralized shell, they play pivotal roles in crystal nucleation, crystal orientation, and mineral polymorph selection [3]. The shell of the bivalve oyster consists of two mineralized layers including an inner nacreous and an outer prismatic layer. These two layers own different crystal polymorphisms and microstructures which were controlled by the matrix proteins secreted from the epithelial cells of the mantle. Despite the obvious importance of matrix proteins in biomineralization processes, only a few matrix components have been found and functionally identified, such as prisilkin-39 and pif [3,4].

Dermatopontin (DPT), also named tyrosine-rich acidic matrix protein (TRAMP), is a widely distributed extracellular matrix macromolecule initially purified from bovine dermis with possible functions in cell-matrix interactions and matrix assembly [1]. In mammalian, DPT was reported to induce fibroblast cell adhesion, accelerate collagen fibrillogenesis and modify the newly formed collagen fibrils [5]. DPT-knockout mice showed an abnormal ECM architecture and tissue flexibility in response to mechanical forces [6].

In invertebrate, DPT was initially extracted from the crossed lamellar layer of the freshwater snail *Biomphalaria glabrata* [7]. Using immunological assays, DPT was found in the 1500 year old fossil shells of the extinct land snail *Mandarina luhuana* [8]. In-depth proteomic analysis of the shell matrix of the limpet *Lottia gigantea*, DPT was also identified [9]. Therefore, DPT was a shell matrix protein and participated in shell formation. However, it is obscure whether DPT is involved in nacreous layer formation or prismatic layer formation.

Pinctada martensii, the main species cultured for marine pearl production in China and Japan, is a remarkable research model for nacre formation. However, relatively few nacre-formation related matrix proteins in *P. martensii* have been identified and functionally characterized. In our previous research of the transcriptom of pearl oyster *P. martensii*, we have got a partial sequence of DPT gene [10]. The aims of this study were to get the full length of DPT and deliberate its exact functions *in vivo* using RNA interference (RNAi) technology.

2. Materials and methods

2.1. Experimental samples and total RNA extraction

Pearl oysters *P. martensii* (about 2 years of age) were obtained from Liushagang, Zhanjiang, Guangdong Province, and China.

* Corresponding author. Address: Fishery College, Guangdong Ocean University, 40 East Jiefang Road, Xiashan District, Zhanjiang City, Guangdong 524025, China. Fax: +86 0759 2382044.
E-mail address: duxd@gdou.edu.cn (X. Du).

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Table 1
Primers for gene amplification and characterization.

Primer	Sequence (5′–3′)	Application
Actin-F	5′-GACAATGCCGTGCTCAAT-3′	qRT-PCR
Actin-R	5′-TGGTATGGGACAGAAGGAC-3′	qRT-PCR
3′RACE-DPT1	5′-TGAGCCTTTTGATTTCACTTGT-3′	Inner PCR
3′RACE-DPT2	5’- TTACAAAGAAGACAGGAGGATG -3’	Outer PCR
5′RACE-DPT3	5’-CCTTTTGATTTCACTTGTCC-3′	Inner PCR
5′RACE-DPT4	5′-TTTTACAACCTGCTACTACA-3′	Outer PCR
DPT5	5′-GCTCATCCCAGTCATTCAC-3′	qRT-PCR
DPT6	5′-ATGTCACCATACCAAAACCA-3′	qRT-PCR
DPT-T7-R	5′-GCGTAATACGACTCACTATAGGGATGAGTTGTAACATATTTGG-3′	dsRNA
DPT-T7-F	5′-GCGTAATACGACTCACTATAGGGTCAGCACCGCATGTAGTAGC-3′	dsRNA
EGFP-T7-R	5′-GCGTAATACGACTCACTATAGGGATGGTGAGCAAGGGCGAGGA-3′	dsRNA
EGFP-T7-F	5′-GCGTAATACGACTCACTATAGGGTTACTTGTACAGCTCGTCCA-3′	dsRNA

Adductor muscle, gill, pearl sac, mantle, hepatopancreas, gonad, foot and haemocytes were collected from pearl oyster and immediately stored in liquid nitrogen until used. Total RNA was extracted from different tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The integrity of RNA was determined by fractionation on 1.2% formaldehyde-denatured agarose gel and staining with ethidium bromide. The quantity of RNA was determined by measuring OD260/OD280 with NanoDrop ND1000 Spectrophotometer.

2.2. Rapid amplification of cDNA Ends (RACE)

Single stranded cDNA for all RACE reactions were prepared from the mantle total RNA. 5′RACE and 3′RACE were conducted using SMART RACE cDNA Amplification Kit (Clontech) and Advantage 2 cDNA Polymerase Mix (Clontech) according to the manufacturer's instructions. The gene specific primers were prepared based on the partial sequence obtained from the transcriptome of *P. martensii* [10] (Table 1). To increase specificity and sensitivity, nested-PCR was performed. The inner and outer PCR primers were indicated in the Table 1.

2.3. DNA sequencing and sequence analysis

All amplified products, containing the 5′ and 3′ ends, were subcloned into pGEM-T Easy Vector (Promega) for sequencing. The sequence was spliced and the open reading fragment (ORF) was characterized using DNAMAN Version 5.2.2 (Lynnon Biosoft). The signal peptide was predicted using SignalP2.0 (http://www.cbs.dtu.dk/services/SignalP-2.0/). The physical and chemical properties were analyzed using Protparam tool (http://www.expasy.org/tools/protparam). Phosphorylation site was predicted using PhosphoSitePlus (http://www.phosphosite.org). Glycosylation site was predicted using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc).

2.4. Quantitative real-time pCR (qRT-PCR) validation

cDNA was synthesized using M-MLV First Strand cDNA Synthesis Kit (Invitrogen). Sequences of the specific primer sets were shown in Table 1. The qRT-PCR was performed using the SYBR Green I Hot Start Fluorescent PCR Core Reagent Kit (Sangon Biotech Co., Ltd., China) according to the manufacturer's protocol and were done on the C1000TM Thermal cycler CFX96TM Real-time system (Bio-Rad, USA). The results were normalized to beta-actin.

2.5. RNAi experiments

RNAi was performed as previously described with some modification [4]. Sequence specific primers (Table 1) were used to amplify the specific sequences from the synthesized cDNA. For GFP, pEGFP-C1 (Clontech) was used as the template. The PCR products were purified using Wizard PCR Prep DNA purification system (Progema). T7 RNA polymerase (TAKARA) was used to synthesize the dsRNA. RNase free DNase I (TAKARA) was used to digest the template DNA. The integrity and quantity of the dsRNA were verified as previously described [4]. The dsRNA-DPT were diluted to 5, 15 and 30 μg 100 μl^{-1} with PBS. Hundred micro liters solutions were injected into the adductor muscle of pearl oyster. PBS and dsRNA-GFP were used as controls. Six individuals were used in each treatment. These pearl oysters were injected the same solutions again after three days. Total RNA was extracted from the mantle from each oyster seven days after the first injection. QRT-PCR was used to evaluate the expression levels of DPT gene to test the effectiveness of RNAi experiment. The shells of the injection groups were collected after being washed with Mili-Q water and air-dried. The shells were cut into pieces, coated with carbon and observed by an FEI Quanta 200 scanning electron microscope (SEM) [4].

3. Results

3.1. cDNA cloning and characterization of DPT gene

Based on the sequence from the transcriptom of pearl oyster *P. martensii* [10], using gene-specific primers, two fragments of 358 and 540 bp were amplified by 3′-RACE and 5′-RACE. A 797bp nucleotide sequence representing the complete DPT cDNA sequence was obtained by overlapping the two fragments amplified above. The complete sequence of the DPT cDNA consisted of a 5′ terminal untranslated region (UTR) of 11bp, an open reading fragment (ORF) of 537bp and a 3′UTR of 249bp with 18bp poly (A) tail (Fig. 1). The sequence of *P. martensii* DPT showed 40.1% sequence identity to *the Haliotis diversicolor supertexta sequence*, 36.3% identity to *Haliotis discus discus*, 29.9% identity to *Suberites domunculaand* and 28.8% identity to *B. glabrata* (data not shown). The DPT sequence was deposited in GenBank under accession No. JQ734542.

3.2. Characterization of the deduced amino acid sequence of DPT gene

The deduced protein DPT encompassed 178 amino acids with an estimated molecular mass of 21.4 kDa and a theoretical isoelectric point of 5.94. DPT contained a high proportion of charged amino acid residues, Arg (13), Asp (15), Glu (7), and Lys (5). Totally, there were eight cysteine residues, which might form disulfide bridges to confer a rigid three dimensional conformation. Six potential phosphorylation sites, including four serines and two tyrosines, were found in its sequence. One glycosylation site, which was inferred to be responsible for biomineralization, was found at position 72 of the peptide. More importantly, a sequence motif of D-R-X-W/F/Y-X-F/Y/I/L/M-X_{1-2}-C, where X is any amino acid, repeated itself three times in the entire sequence and in the second repeat there was one mutation (W/F/Y → M) (Fig. 1).

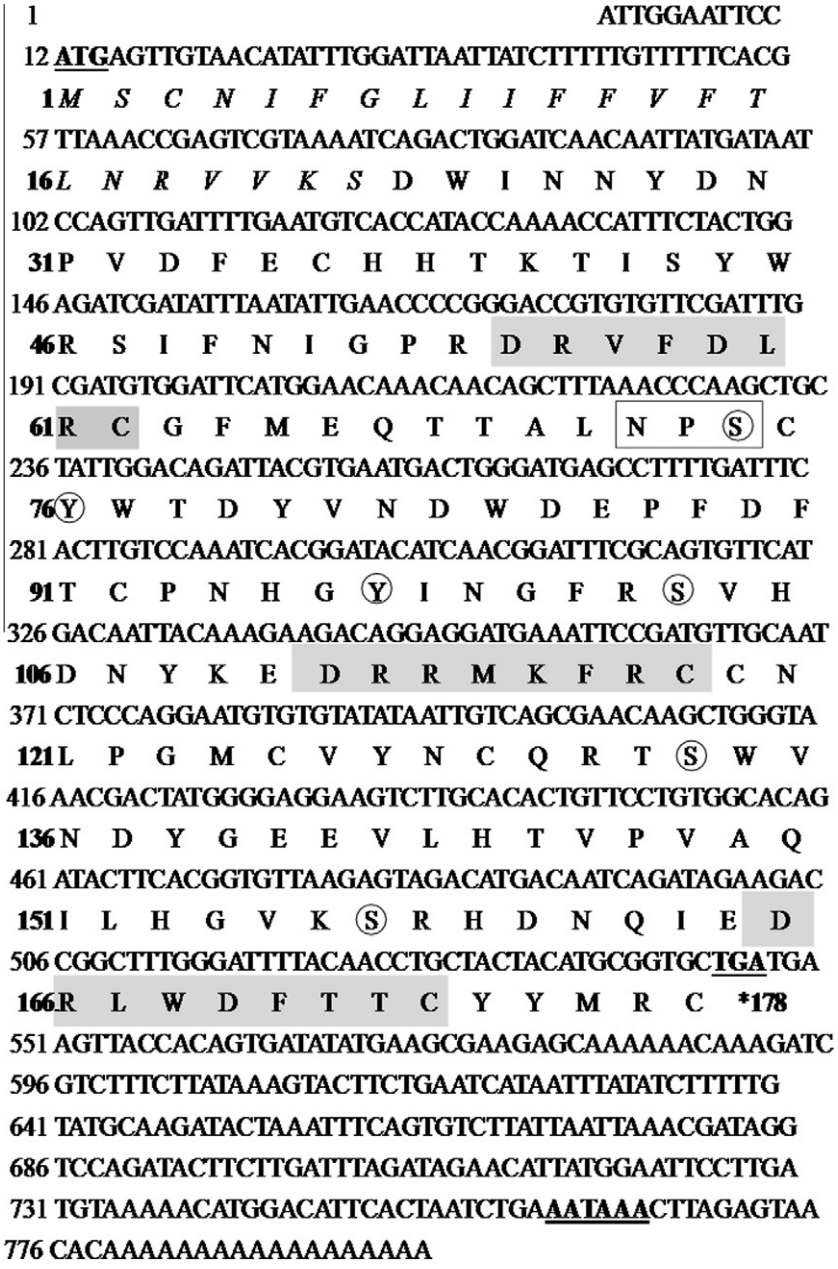

Fig. 1. Nucleotide and amino acid sequence of DPT. Numbers and boldface numbers on the left indicate the positions of the nucleotides in the DPT cDNA sequence and the amino acid residues in the deduced protein, respectively. The putative signal peptide (residue 1–22) is indicated in italic type. The initiation codon (ATG), the stop codon (TGA), and the putative polyadenylation signal (AATAA) are underlined. The putative phosphorylation sites are circled. The putative glycosylation site (NPS) is boxed. The sequence motifs of D-R-X-W/F/Y-X-F/Y/I/L/M-X_{1-2}-C are indicated in gray boxed.

3.3. DPT mRNA expression profile in different tissues

QRT-PCR analysis was employed to determine the tissue specific expression of DPT mRNA with beta-actin as an internal control. The results showed that the DPT mRNA was constitutively expressed in all detected tissues (adductor muscle, gill, pearl sac, mantle, hepatopancreas, gonad, foot and haemocytes). As shown in Fig. 2, DPT mRNA was highly expressed in the mantle and pearl sac, which were nacre formation-related tissues. These results suggested that DPT may be involved in nacre formation.

3.4. RNA interference (RNAi) knockdown of DPT

We then tested the functions of DPT using RNAi technology. Controls were PBS and dsRNA-GFP; GFP was not expressed in *P. martensii*. DsRNA was injected into the muscle of *P. martensii* (2 years old), and qRT-PCR was used to measure expression levels of DPT gene seven days after dsRNA injection. DPT expression levels of the 5 μg-dsRNA-DPT injected groups were suppressed approximately by 25% compared with the PBS or dsRNA-GFP injected groups, and decreased by approximately 95% in the 15 and 30 μg-injected groups (Fig. 3).

3.5. Changes of the inner surface structure of the shells after dsRNA-DPT injection

To further investigate the function of DPT gene in shell biomineralization *in vivo*, we observed the inner surface structure of the shells after dsRNA-DPT injection using SEM. The surfaces of shells in the control groups (PBS and dsRNA-GPF) had the same normal

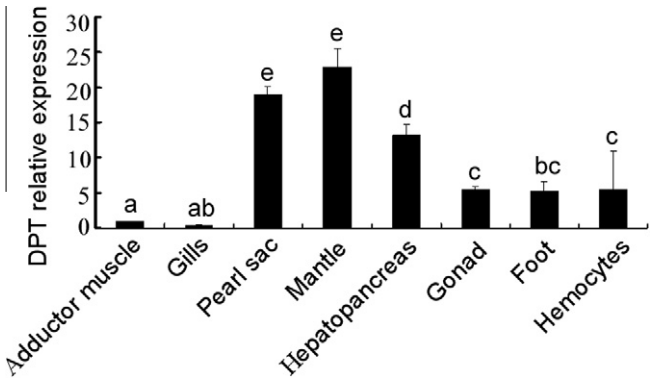

Fig. 2. Expression level of DPT mRNA in different tissues. qRT-PCR was done with RNA samples from adductor muscle, gill, pearl sac, mantle, hepatopancreas, gonad, foot and haemocytes. The pearl oyster beta-actin gene was used as an internal control.

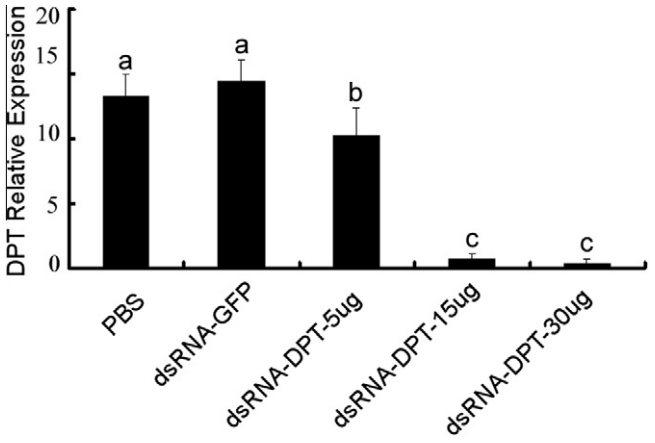

Fig. 3. Expression level of DPT mRNA knocked-down by RNAi. qRT-PCR was done with RNA samples from control (PBS and dsRNA-GFP) and dsRNA-DPT injection (5, 15 and 30 μg) seven days after dsRNA injection and six individuals were tested in each group. The pearl oyster beta-actin gene was used as an internal control.

well-defined type of microstructure. In nacreous layer, the small hexagonal flat tablets of aragonite were packed together to produce a stair-like growth pattern, and in the prismatic layer the prisms built up into a well-compacted smooth structure. In the dsRNA-DPT injected groups (30 μg), the prismatic layer of the shells did not have significant changes; whereas the nacreous layer showed a disordered growth like the phenomenon when DT_Cluster524.seq.Contig1 which participated in nacre formation was inhibited after the related-dsRNA injection (Fig. 4) [11].

4. Discussion

DPT is a widely distributed extracellular matrix protein. In invertebrate, it has been observed in 8 snail species belonging to the order Basommatophora and Stylommatophora, *H. discus* and Limpet *L. gigantea*. DPT was inferred to play a crucial rule in shell formation [12]. However, it is obscure whether DPT participates in nacreous layer formation or prismatic layer formation. In the DGE (different gene expression) profile of the pearl sac development stages, DPT mRNA was expressed to the highest level in the later stages when the nacreous layer has been formed (unpublished). So, we inferred DPT in *P. martensii* involved in nacre formation, but further studies are needed to deliberate its exact function.

DPT gene in *P. martensii* obtained in this study encoded 178 amino acids. The theoretical isoelectric point was 5.94. One of the most conspicuous features of the published skeletal matrix proteins was their acidic nature. Isao Sarashina had analyzed the characteristic of 77 invertebrate skeletal matrix proteins and found that 43 were acidic (pI < 6.0), 20 were neutral and 14 were basic (pI > 8.0). Therefore, Isao Sarashina concluded acidic skeletal matrix proteins were more common than basic ones [13]. The acidic nature of the matrix protein was mainly due to the high component of acidic amino acid residues. The high ratio of acidic amino acid residues may play a role in the regulation of crystal polymorph [14].

Sequence analysis indicated DPT amino acid sequence of *P. martensii* own eight cysteine residues, which were conserved in the position among other invertebrate DPT genes [10]. Cysteine residues were also observed in other shell matrix proteins, such as shematrin, prisilkin-39, n16 [3]; these cysteine residues could form disulfide bonds which mediate protein cross-linking and give the protein a good flexibility [15].

Six potential phosphorylation sites were observed in the entire DPT amino acid sequence of *P. martensii*. Several potential phosphorylation sites were also found in other molluscan DPT, for example, nine potential phosphorylation sites were contained in bassommatophoran DPT1 [12]. It was reported that the phosphorylation degree of the soluble matrix in the shell could regulate the carbonate crystal growth *in vivo* [16]. So, these potential phosphorylation sites may be crucial for the function of DPT in shell formation.

The sequence motif of D-R-X-W/F/Y-X-F/Y/I/L/M-X_{1-2}-C repeated itself three times in DPT entire sequence. Repeated sequences are usually found among skeletal matrix proteins, such

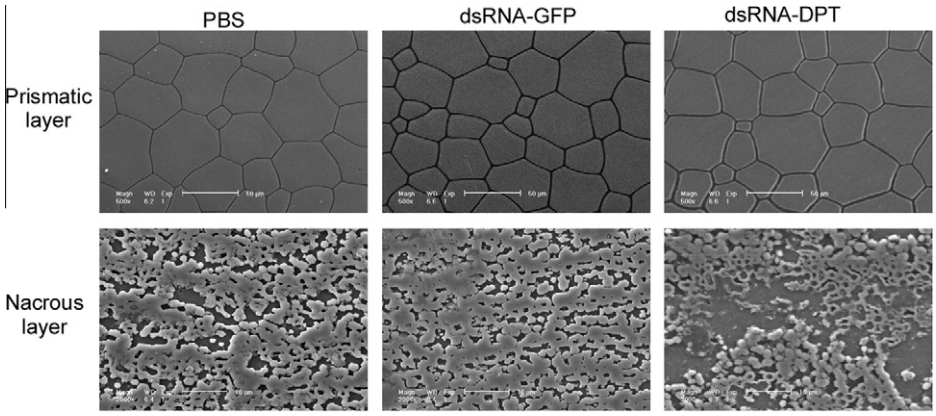

Fig. 4. Effect of DPT gene on shell growth. (A/B) SEM images of the internal prismatic/nacreous layer surface of the control groups (PBS and dsRNA-GFP injection) and dsRNA-DPT injection (30 μg) seven days after dsRNA injection.

as SM50, Galaxin and Mucoperlin [12]. Functions of these repeats have been proposed to bind some macromolecules [13]. This motif was conserved in all DPT examined so far; so this repeated sequence may be crucial for the general functions of DPT, but not specifically for shell formation.

One glycosylation site was observed at position 72 of the peptide. It was reported that glycosylation was a common modification of the skeletal matrix proteins and the shell's organic matrix always contained glycoproteins [1,2]. The general functions of protein glycosylation included protein folding and stability, cell adhesion and signal transduction [13]. The functions of glycoproteins in the shell matrix were proposed to stabilize different calcium carbonate polymorphs [17]. In bassommatophoran, there were three DPT types (DPT1, DPT2, DPT3); only DPT1 has a glycosylation site and was highly expressed in the mantle; so, DPT1 was suggested to be a shell matrix protein; DPT2 and DPT3, having no glycosylation sites, were proteins mainly for general functions. The DPT gene obtained from *P. martensii* in this study was also highly expressed in the mantle and contained one glycosylation site. These results suggested the obtained DPT gene was a shell matrix protein.

In order to elucidate the role of DPT in shell formation in *P. martensii*, dsRNA-DPT was injected into the oyster to knock down its expression level. The expression of DPT gene decreased by approximately 95% in 15 and 30 μg-injected groups. RNAi is a conserved biological response to dsRNA that mediates resistance to both endogenous parasitic and exogenous pathogenic nucleic acids. Once inside the cell, dsRNA is cleaved by a ribonuclease III homolog or Dicer into 21–23 nucleotide-long small interfering RNA (siRNA). siRNA is then targeted to a specific region of its complementary mRNA resulting in mRNA degradation. As it could regulate the expression of protein-coding genes by sequence-specific gene silencing, RNAi is a useful tool for investigating the function of specific gene [18]. It has been used successfully in a range of invertebrate species to date such as tsetse flies, *Tribolium castaneum*, aphids, sealice [19]. In *P. martensii*, RNAi has been used successfully to elucidate the function of specific gene [4,11]. The scilencing level achieved is dependent on various factors, such as the length of dsRNA, the target site of dsRNA [20]. DsRNA-DPT used in this study could achieve a higher scilencing level. We proposed the major reason was that the length of DPT was only 797bp and the dsRNA-DPT has contained the full length of its ORF.

Together with the SEM images of the inner surface of the shells, we found the prismatic layer of the shells did not have significant changes, whereas the nacreous layer showed a disordered growth. Therefore, these results suggested that the cloned DPT in our research played an important role in the nacre formation. Then, how does DPT function in nacre formation is still in question. Previous research has identified DPT had no Ca^{2+}-binding ability [21]. In mammals, DPT could bind to decorin and the DPT-decorin complex accelerates collagen fibillogenesis and modifies the behavior of TGF-β [22]. In *B. glabrata*, no collagen has been identified in the shell matrix, but the shell matrix contains many proteoglycans, thus, Marxen inferred DPT may interact with shell proteoglycans to organize an organic scaffold for mineralization [13]. While, in the shell matrix of *Haliotis asinina*, HasCL10contig2 protein containing 7 collagen-like-GGSGGxGFG-repeats were identified; thus, DPT may involved in nacre formation by interacting with the proteins containing collagen-like repeat [23]. In *P. martensii*, we found many collagen unigenes in the transcriptom of pearl sac [10], whereas the existence of collagen in the shell matrix was not clear. From these results, we could infer DPT participates in nacre formation by constructing the nacre organic matrix scaffold through connection with collagen or other proteins. The repeated domains (E-D-R-X-W/F/Y-X-F/Y/I/L/M-X_{1-2}-C) and glycosylation site may be crucial for this connection.

In conclusion, based on the partial sequence obtained from the transcriptom of *P. martensii* [10], we have got the full length of DPT cDNA from *P. martensii*, and analyzed the characteristic of its ORF and peptide sequence. Comparing with other tissues, the mantle has the highest expression level of DPT mRNA. Using RNAi technology, we have identified DPT participated in nacre formation in *P. martensii*. Further studies on the structure–function relationship are required to understand the physiological function of DPT in the nacre formation.

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