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RESEARCH REPORT

Cloning and expression analysis of a stomatin gene from the sea cucumber *Apostichopus japonicas*

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Abstract

Stomatin was the first member of the stomatin-prohibitin-flotillin-HflC/K (SPFH) superfamily proteins to be studied. It is also known as band 7 integral membrane protein. In this study, a stomatin gene in the sea cucumber *Apostichopus japonicus* (designated as *AjSto*) was identified and characterized. Its cDNA was 2085 bp in length including 131 bp of 5'-UTR, 1117 bp of 3'-UTR, and 837 bp of open reading frame (ORF) encoding a putative protein of 279 residues with a SPFH domain of band 7 family, a predicted molecular mass of 30.5 kDa and a theoretical pI of 5.25. Protein structure prediction and phylogenetic analysis showed that *AjSto* is highly conserved as compared to those from other vertebrate and invertebrate species. Analysis of *AjSto* expression in the tissues of *A. japonicas* showed that the respiratory tree and body wall had the highest expression, followed by the intestine, celomocytes, tube feet, and longitudinal muscle. Time-course analysis of *AjSto* expression in the celomocytes revealed obvious and significant inhibition of expression following *Vibrio splendidus* challenge, with a 0.18-fold reduction after 6 h of exposure to the bacteria compared to the control, but the expression was up-regulated by 2.12-fold after 72 h of exposure. These results suggested that *AjSto* might play critical roles not only by acting as the major integral protein of erythrocyte lipid rafts, but may also involved in the innate immune defense against bacterial infections.

Key Word: stomatin; *Apostichopus japonicus*; tissue distribution; temporal expression

Introduction

Stomatin was first identified in human erythrocyte in 1991 and was also termed band 7 membrane protein (Hiebl-Dirschmied *et al*., 1991a). Stomatin plays an important role in the modulation of K^+ and Na^+ permeability in red blood cells. Absence or partial deficiency of the stomain gene can cause overhydrated hereditary stomatocytosis, which is a form of autosomal dominant hemolytic anemia (Lande *et al*., 1983; Hiebl-Dirschmied *et al*., 1991b; Salzer *et al*., 1993). Stomatin belongs to the superfamily of stomatin-prohibitin-flotillin-HflC/K (SPFH) proteins, which also includes Prohibitin, Flotillin and Hflk/Hflc (Gehl and Blatt, 2009; Lapatsina *et al*., 2012; Chi and Hu, 2016). Members of this superfamily possess a representative SPFH domain that is involved in regulating targeted protein turnover between stomatins and other membrane-associated proteins (Tavernarakis *et al*., 1999; Browman *et al*., 2007). As a member of the SPFH superfamily, stomatin is the major membrane-bound protein that participates in the regulation of membrane-associated proteins. Stomatin in particular, has a single hydrophobic domain that plays a critical role in the regulation of ion transport (Stewart, 1997; Chen *et al*., 2005). Furthermore, proteins of the stomatin family contain a conserved core stomatin-domain spanning a region of 150 amino acids, and this domain defines a family of proteins that is found in an ancient duplication event which occurred early on in the evolution of prokaryotes (Green and Young, 2008). Five main members of the mammalian stomatin family have been found, and they include stomatin, stomatin-like protein 1(SLP-1), SLP-2, SLP-3 and podocin (Lapatsina *et al*., 2012; Chi and Hu, 2016). Moreover, the SPFH superfamily includes another branch named mechanosensory protein 2 (Mec-2). The mouse Mec-2 protein is similar to stomatin from *Caenorhabditis elegans*, as these two proteins share over 65 % sequence identity in the stomatin domain. At least nine stomatin-like proteins have been

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identified in *C. elegans* (Lapatsina *et al*., 2012). All the available data show that members of the SPFH superfamily are all homologous and the stomatin domains in these proteins are remarkably conserved.

Stomatin is widely expressed in various types of cells, such as erythrocyte, diseased cells including cancer cells and tumor cells, and nerve cells. Stomatin deficiency will lead to overhydrated hereditary stomatocytosis. (Hiebl-Dirschmied *et al*., 1991a; Fricke *et al*., 2000; Salzer and Prohaska, 2001; Arkhipova *et al*., 2014; Chen *et al*., 2016). Existing data show that stomatin is closely linked to diseases, but the precise function of stomatin remains unclear. Chi and Hu found that stomatin-like protein 2 of turbot play a vital role in host immune defense against bacterial and viral pathogens (Chi and Hu, 2016). Structurally, the protein contains a closed N-terminus and a single hydrophobic domain (Gehl and Blatt, 2009). In addition, it also as an independently organized higher order oligomer, which acts as a separate scaffolding component at the cytoplasmic face of erythrocyte lipid rafts (Salzer and Prohaska, 2001; Green and Young, 2008; Lapatsina *et al*., 2012). Functionally, stomatin binds to glucose transporter-1 (GLUT1) and modulates the rate of glucose uptake, a function that may involve the interaction with membrane-bound scaffolding protein modulating transport proteins (Zhang *et al*., 1999; Rungaldier *et al*., 2013). Stomatin exerts the most prominent effect on acid-sensing ion channels (ASICs), and it achieves this by inhibiting the acid-evoked current. ASICs as H^+ gated channels are involved in the sensing of acidosis associated with painful conditions such as skin and muscle inflammation (Brand *et al*., 2012; Moshourab *et al*., 2013). Although the wide distribution of stomatin indicates that it has an important role, its physiological function in invertebrates as well as the mechanisms associated with the diseases that it causes in these animals have never been studied before. It was necessary to explore the properties of stomatin in sea cucumber, an invertebrate of high economic value.

Since 2004, diseases like skin ulceration syndrome (SUS) have already caused mass mortalities and resulted in serious economic losses for the sea cucumber farming industry (Yan *et al*., 2014). The sea cucumber *Apostichopus japonicas* is an economically important aquaculture species in China, Japan, south Korea and Russia. However, the outbreak of SUS has severely limited the sustainable development of the industry (Chang *et al*., 2009). Analysis of the bacterial strain isolated from the lesions of sea cucumbers suffering from SUS revealed similarity to *Vibrio splendidus*. Although the main pathogenic bacterium responsible for SUS has been confirmed, there has been no effective measure to prevent the occurrence of SUS (Deng *et al*., 2009). Many innate immune genes of sea cucumber have been characterized, like mitogen-activated protein kinase kinases, TNF receptor associated factors and thioredoxin (Cheng *et al*., 2016; Wang *et al*., 2016; Yang *et al*., 2016), but so far there has been no study on the stomatin gene regarding its role in the immune responses. In this study, we described the identification and characterization of a stomatin gene from *A. japonicus* and analyzed its expression in six different tissues of healthy adult *A. japonicus* individuals and in the celomocytes of *A. japonicus* individuals that had been challenged with *V. Splendidus*.

Materials and Methods

Samples preparation and bacterial challenge experiment

Healthy *Apostichopus japonicus* individuals (body weight 68 ± 4.59 g) were collected from Dalian and kept at 16 - 17 ℃ in our laboratory for one week. Three animals were sacrificed and their tissues, including the body wall, intestine, respiratory tree, body wall, tube feet, celomocytes, and longitudinal muscle were extracted and subjected to spatial expression analysis. To harvest the celomocytes, the celomic fluid was collected and centrifuged immediately at 1,000*g* for 5 min at 4 ℃. The celomocytes were immediately snap-frozen in liquid nitrogen and stored at -80 ℃.

Table 1 Primer sequences used for *AjSto* cloning and expression analysis

Primers	Sequences (5′-3′)	Application	Melting temperatures
AjSto-5'-out	CAATGACCTTCGCACGGGCTT	5'-RACE	56℃
AjSto-5'-in	CCTGGTCCGTTGTCTTTCGCC	5'-RACE	56℃
AjSto-3'-out	TATTTCCCCTTTGCTTTTGCC	3'-RACE	56℃
AjSto-3'-in	GGGGTTTGCTTATTACGCTGG	3'-RACE	56℃
UMP-1	TAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	RACE	56℃
UMP-2	CTAATACGACTCACTATAGGGC	RACE	56℃
AjSto-F	CACGCTTCCCTTCTCTCTGTTCT	qPCR	60℃
AjSto-R	GAAGGAGTCAATGCAGGGTAGTATG	qPCR	60℃
Cytb-F	TGAGCCGCAACAGTAATC	Reference gene	60℃
Cytb-R	AAGGGAAAAGGAAGTGAAAG	Reference gene	60℃

Total RNA extraction and cDNA synthesis

Total RNA was extracted from *A. japonicus* using an RNAprep pure Tissue Kit (Tiangen, China) according to the instructions of the manufacturer. The quality and quantity of the RNA were assessed by 1 % agarose gel electrophoresis and UV spectrophotometry, respectively. UV spectrophotometry was performed on a NanoPhotometer (Munich, Germany). The first strand cDNA was synthesized in a 10-μL reaction mixture containing 1 g of total RNA, 2 μL of 5× PrimerScript buffer, 0.5 μL of Oligo dT Primer (50 μM), 0.5 μL of Random 6 mers (100 μM), and 0.5 μL of PrimerScript RT Enzyme Mix (PrimerScriptTM RT reagent Kit, TaKaRa, Japan) and RNase free dH_2O. The sample was incubated at 37 ℃ for 15 min, followed by heating at 85 ℃ for 5 s to denature the reverse transcriptase. All cDNA samples were stored at -20 ℃ until used.

Gene cloning and sequencing analysis

The partial cDNA sequence of stomatin was acquired from our transcriptome assembly data (unpublished data). Gene specific primers for stomatin were designed by Primer Premier 5.0. All primers are listed in Table 1. 5'- and 3'-RACE by the SMARTer®RACE 5'/3' Kit (TaKaRa,Japan) were performed according to the manufacturer's instructions. The polymerase chain reaction (PCR) was performed in a 25-μL reaction mixture containing 2.5 μL of 3'-RACE-Ready cDNA, 1 μL of gene specific primer (GSP, 10 μM),1 μL of 10×UPM, 12.5 μL of 2×TransStart® FastPfu PCR Supermix (Transgen Biotech, China) and 8 μL of ddH_2O. The PCR conditions were as follows: an initial denaturation step at 94 ℃ for 3 min followed by 35 cycles of denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 30 s and extension at 72 ℃ for 1 min, and a final extension step at 72 ℃ for 10 min. The PCR product was detected by 1.0 % agarose gel electrophotesis and purified using the EasyPure Quick Gel Extraction Kit (Transgen Biotech, China). The purified PCR product was ligated to PEASY®-1 Cloning Vector (Transgen Biotech, China) and Trans1-T1 Phage Resistant Chemically Competent Cells (Transgen Biotech, China) were transformed with the ligation products. Positive transformants were verified by colony PCR using M13 Primers (Transgen Biotech, China). Three independent clones were subjected to DNA sequencing to confirm the presence of the correct insert.

Bioinformatics analysis of AjSto

The full-length cDNA sequence of the *A. japonicas* stomatin gene was analyzed using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast) program. Open reading frame (ORF) was determined by ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/orfig.cgi). The amino acid sequence encoded by the open reading frame was analyzed by the online Expert Protein Analysis System (http://www.expasy.org/). The molecular weight of the encoded polypeptide chain was calculated with the Expasy compute pI/MW tool (http://www.expasy.org/), and the signal peptide was predicted by SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). Multiple sequence alignment of amino acid sequences was performed with DNAman program, while a phylogenetic tree constructed was by the neighbor-joining (NJ) method using the MEGA 7 program. Domain structure of the protein was analyzed using the Simple Modular Architecture Research Tool (SMART) program (http://smart.embl-heidelberg.de/) and InterPro: protein sequence analysis & classification (http://www.ebi.ac.uk/interpro/). Transmembrane region was predicted by TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0) and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). The secondary structure and three dimensional (3D) structure of *AjSto* protein were predicted using PSIPRED v3.3 software (http://bioinf.cs.ucl.ac.uk/psipred/) and the SwissModel Workspace (https://swissmodel.expasy.org/) which was evaluated by Swiss-PdbViewer (version 4.1).

Bacterial challenge experiment

Vibrio splendidus D4501 was obtained from our laboratory and cultured at 28 ℃ with shaking at 200 rpm for overnight. The bacterial cells were harvested at the following day by centrifugation at 4,000*g* for 1 min. For the *V. spendidus* challenge experiment, twenty sea cucumbers were immersed in a tank of sea water containing *V. splendidus* at a concentration of 1×10^7 CFU/mL. The same number of sea cucumbers were immersed in sea water without *V. splendidus* as controls. Three individuals from each group were removed at 0, 6, 12, 24, 48 and 72 h post-immersion, and their celomocytes were extracted for further experiment.

Expression analysis of A. japponicus *stomatin gene*

The expression profile of the stomatin gene in the celomocytes was analyzed by quantitative real time PCR (*q*RT-PCR), which was carried out using the Applied Biosystem 7500 Real-time System (Applied Biosystems, USA). The cytochrome b *(Cytb)* gene was used as a reference gene (Yang *et al*., 2010). Primers of the *q*RT-PCR assay are listed in Table 1. Quantitative RT-PCR was performed in a 20-μL reaction sample containing 1 μL cDNA, 10 μL of 2× SYBR Green Master mix (TaKaRa, Japan), 0.4 μL of ROX Reference DyeII, 7 μL PCR grade water and 0.8 μL (10 mM) of each primer (Table 1). Amplification was carried out under the following conditions: 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 60 °C for 32 s. At the end of the amplification, PCR melting curve analysis was conducted to confirm the presence of a single PCR product. The relative expression level of the stomatin gene was determined by the comparative $2^{-\Delta\Delta Ct}$ method. The concrete formula was: ΔΔCt = [Ct (sample) - Ct (internal reference)] - [Ct (control) − Ct (internal reference)]. All bacterial challenge experimental data were expressed as mean values ± standard deviations. Differences in stomatin expression among the various tissues were analyzed by one-way ANOVA and bacterial infection was tested by T-test contained in the SPSS software (version 16.0) package.

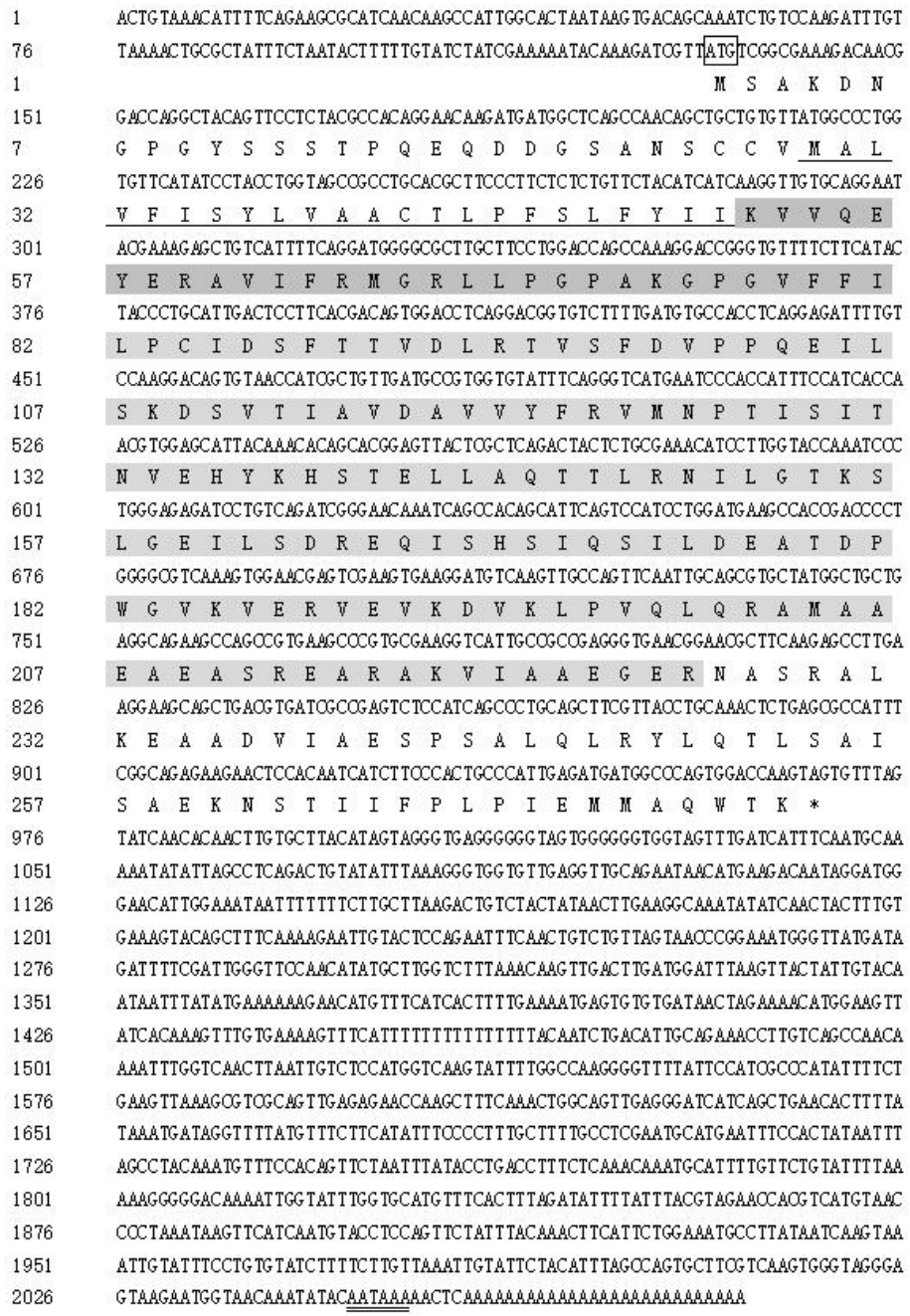


Fig. 1 Nucleotide and deduced amino acid sequences of *A. japonicus* stomatin cDNA. Start codon (ATG) is boxed, and asterisk represents the stop codon. The transmembrane domain is underlined. The SPFH domain of band 7 family is shaded. The polyadenylation signal (ATTAAA) is double-underlined.

Result and Discussion

Sequence analysis of AjSto *cDNA*

The complete cDNA sequence of the *A. japonicus* stomatin gene (designated as *AjSto*, and GenBank accession No. MG209701) was obtained through the assembling of EST from the transcriptome database and two amplified fragments, one (44 bp) from 5'-RACE and the other (60 bp) from 3'-RACE. The full-length cDNA of *AjSto* was 2085 bp, with an 837-bp open reading frame (ORF) encoding a 278-amino acid polypeptide with a predicted molecular mass of 3.5 kDa and a theoretical pI of 5.25. In addition, the gene also contained 131 bp of 5' untraslated region (UTR) and 1117 bp of 3' UTR (Fig. 1). No signal peptide was found in the amino acid sequence, but SignalP 4.0 detected a discriminating signal peptide within the transmembrane region. The transmembrane region comprised 29-51 amino acids, and it was connected

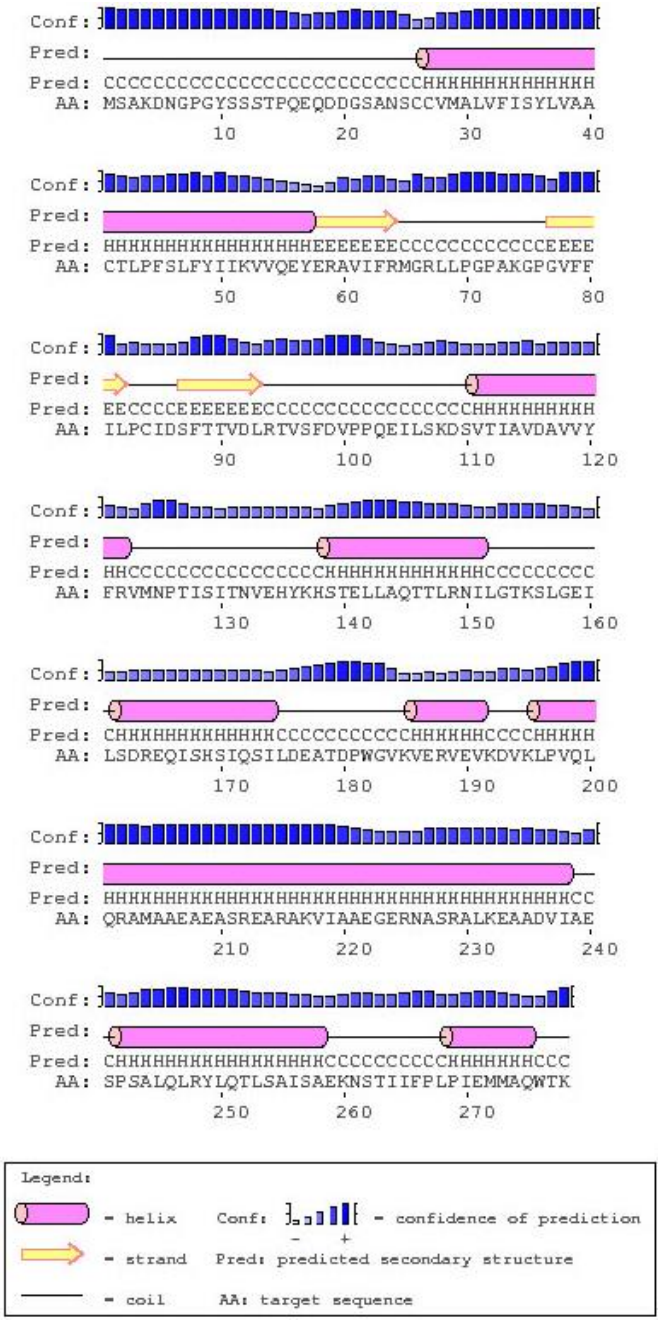


Fig. 2 Secondary structure of *AjSto* protein. Black lines (C), yellow arrows (E) pink cylinders (H) and blue column chart represent coils, strands, helices and confidence of prediction, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article)

by an intracellular N-terminus (52-278 amino acid) and an extracellular C-terminus (1-28 amino acid). A typically representative SPFH domain of band 7 family (residues of 52-225) was found in polypeptide sequence of *AjSto*. The secondary structure of *AjSto* protein analysis showed that 8 helices and 3 strands were located among amino acid positions 27-275 (Fig. 2). The result is different from *Pyrococcus horikoshii* stomatin that included 7 helices and 9 strands, which were regarded as the major contributors to dimeric interaction in N-terminal of *P. horikoshii* stomain (Yokoyama *et al*., 2006). Blastp analysis showed that the amino acid of *AjSto* had 79 %, 77 %, 74 % and 62 % sequence identity with the sequences of stomatin from *Strongylocentrotus purpuratus* (XP_780332.3), *Branchiostoma.belcheri* (XP_019618408.1), *Crassostrea gigas* (XP_011417141.1) and *Homo sapiens* (EAW87494.1), respectively. Band 7 domain was found in all aligned orthologs, illustrating the conservation of stomatin family proteins in both vertebrates and invertebrates.

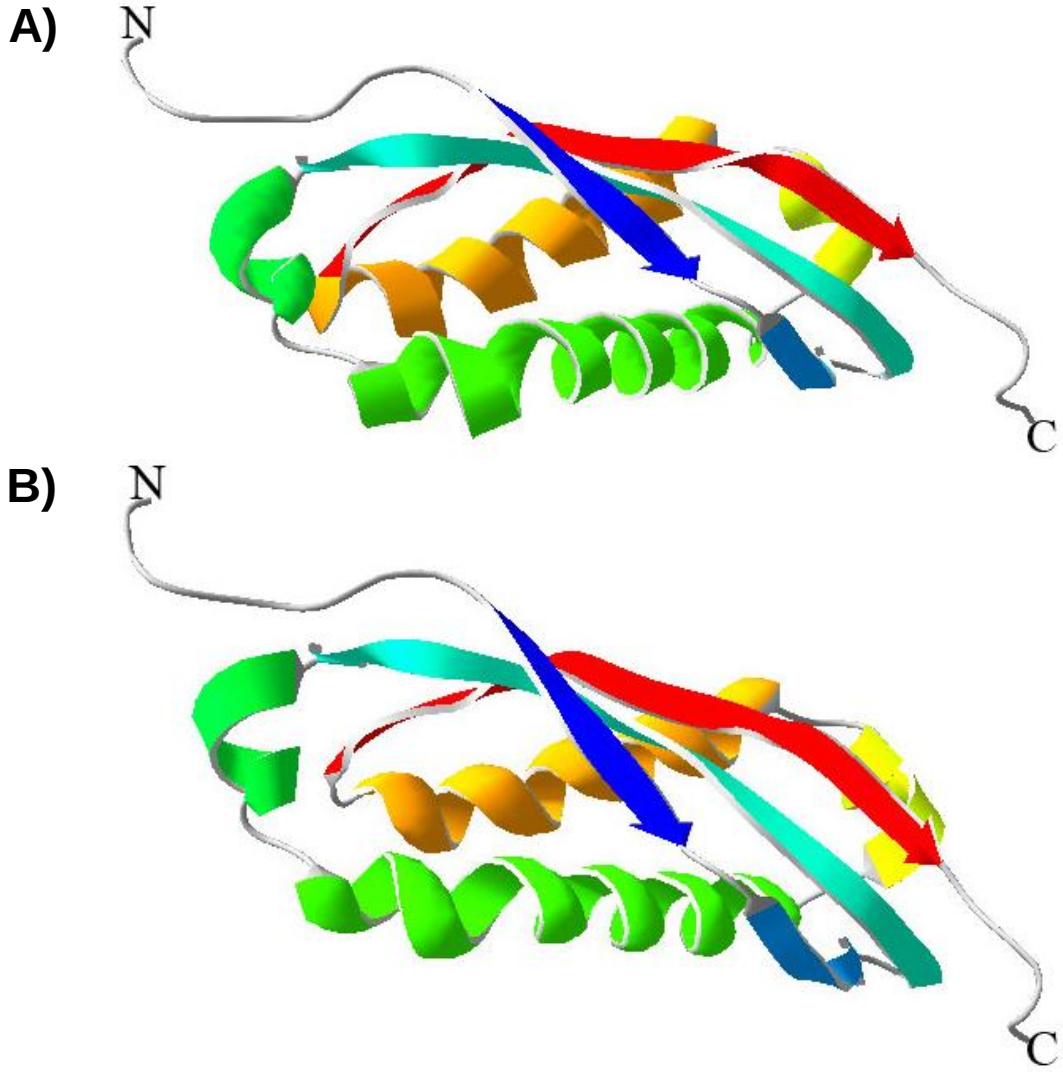


Fig. 3 3D structure prediction of *AjSto* protein (A) and mouse stomatin protein (B). N- and C-termini are marked.

3D molecular modeling and phylogenetic analysis

3D molecular modeling of *AjSto* was generated using mouse stomatin (PDB accession No. 4fvf) as the template (Fig. 3). The rate of consistency on the basis of sequence identity between *AjSto* and the template was 69.53 %. According to the result of *AjSto* protein 3D molecular modeling analysis, the core of the sea cucumber stomatin domain is very similar to that of mouse stomatin. N- and C-termini are located at opposing sides of the molecular (Brand *et al*., 2012). Phylogenetic analysis of the various stomatin sequences assigned *AjSto* to the stomatin sub-family, with *AjSto* and *S. purpuratus* stomatin grouped into the same branch of the phylogenetic tree (Fig. 4). The result demonstrated that *AjSto* exhibited a closer relationship with *S. purpuratus* stomatin, corresponding to the result of traditional taxonomy. Taken together, *AjSto* is highly conserved as compared to those from other vertebrate and invertebrate species. Therefore, we named this novel gene as *AjSto*.

Tissues distribution of AjSto

The spatial expression pattern of the *AjSto* gene obtained from *q*RT-PCR showed that *AjSto* may be a ubiquitous gene. The order of relative *AjSto* expression levels in the various *A. japonicas* tissues from high to low was respiratory tree > body wall > intestine > celomocytes > tube feet > longitudinal muscle (Fig. 5). For comparison purpose, the transcript level of *AjSto* in intestine (Taken as 1) was compared with the transcript levels in the other tissues. The highest expression level was detected in the respiratory tree, which accounted for 2.4-fold, while the lowest expression, 0.06 fold, occurred in the longitudinal muscle. There has been no reported study on the stomatin protein in deuterostome, but SLP-2 from the turbot *Scopthalmus maximus* has been studied and shown to be involved in host immune defense against bacterial and viral pathogens (Chi and Hu, 2016). However, in human, stomatin participates in immunoreaction and its immunoreactivity has been explored in the ciliated

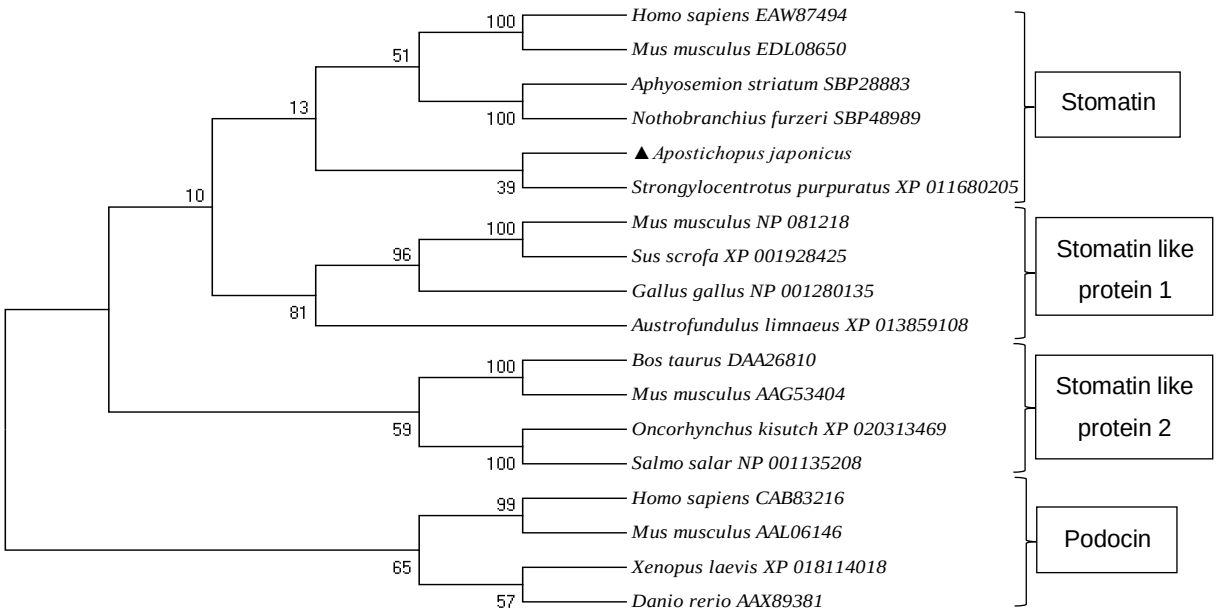


Fig. 4 Consensus neighbor-joining tree based on the amino acid sequences of SPFH superfamily members from other species. The phylogenetic tree was constructed by the Neighbor-joining method using MEGA 7 software. The numbers at the forks indicate the numbers of bootstraps. The acronyms including Sto, SLP-1, SLP-2 and Pod represent stomatin, stomatin-like protein 1, stomatin-like protein 2 and podocin, respectively. The complete name of the species and their GenBank accession numbers are the following: *H. sapiens* Sto (*Homo sapiens* EAW87494), *M. musculus* Sto (*Mus musculus* EDL08650), *A. striatum* Sto (*Aphyosemion striatum* SBP28883), *N. furzeri* Sto (*Nothobranchius furzeri* SBP48989), ▲*A. japonicus* Sto (*Apostichopus japonicus* MG209701), *S. purpuratus* Sto (*Strongylocentrotus purpuratus* XP_011680205), *M. musculus* SLP-1 (*Mus musculus* NP_081218), *S. scrofa* SLP-1 (*Sus scrofa* XP_001928425), *G. gallus* SLP-1 (*Gallus gallus* NP_001280135), *A. limnaeus* SLP-1 (*Austrofundulus limnaeus* XP_013859108), *B. taurus* SLP-2 (*Bos taurus* DAA26810), *M. musculus* SLP-2 (*Mus musculus* AAG53404), *O. kisutch* SLP-2 (*Oncorhynchus kisutch* XP_020313469), *S. salar* SLP-2 (*Salmo salar* NP_001135208), *H. sapiens* Pod (*Homo sapiens* CAB83216), *M. musculus* Pod (*Mus musculus* AAL06146), *X. laevis* Pod (*Xenopus laevis* XP_018114018) and *D. rerio* Pod (*Danio rerio* AAX89381).

cells of human airway epithelin (Fricke *et al*., 2003). Based on currently available data, *AjSto* may be associated with immunologic function in *A. japonicus*.

Temporal expression pattern of AjSto *in celomocytes after bacterial challenge*

Vibrio splendidus is a gram-negative bacterium and the main pathogen of skin ulceration disease in *A. japonicas* (Zhang *et al*., 2006). One hundred and seven immune-related genes have been characterized from *A. japonicus* celomocytes after bacterial challenge (Dong *et al*., 2014; Zhang *et al*., 2014). Although the differential types distribution of cell in different animals can modify the gene expression and influence the results, celomocytes are essential cells for exploring immune related genes because they are a fundamental component of the innate immune system in echinoderm animals. Therefore, to verify the immune function of *AjSto*, we further tested the expression profile of *AjSto* in the celomocytes of *A. japonicus* in response to bacterial challenge. *AjSto* in the celomocytes was found to display a dynamic expression profile in response *V. splendidus* challenge at six time points (0, 6, 12, 24,48 and 72 h) following exposure to the bacteria (Fig. 6). *AjSto* expression was significantly depressed 6 h after exposure to *V. spendidus*, resulting in a 0.18-fold decrease compared to the control. However, *AjSto* expression was up-regulated 72 h after exposure to the bacteria, yielding a 2.16-fold increase.

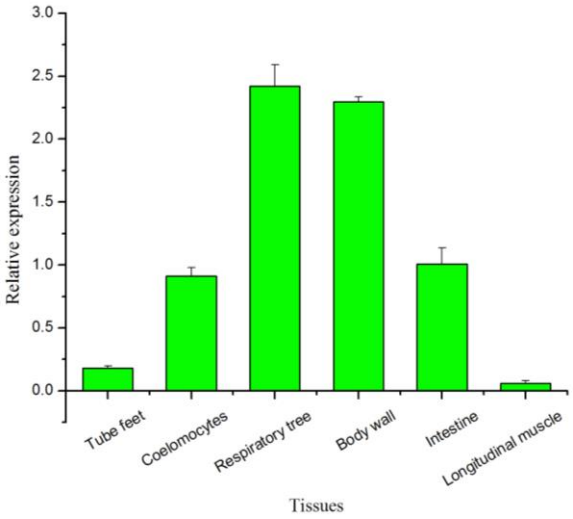
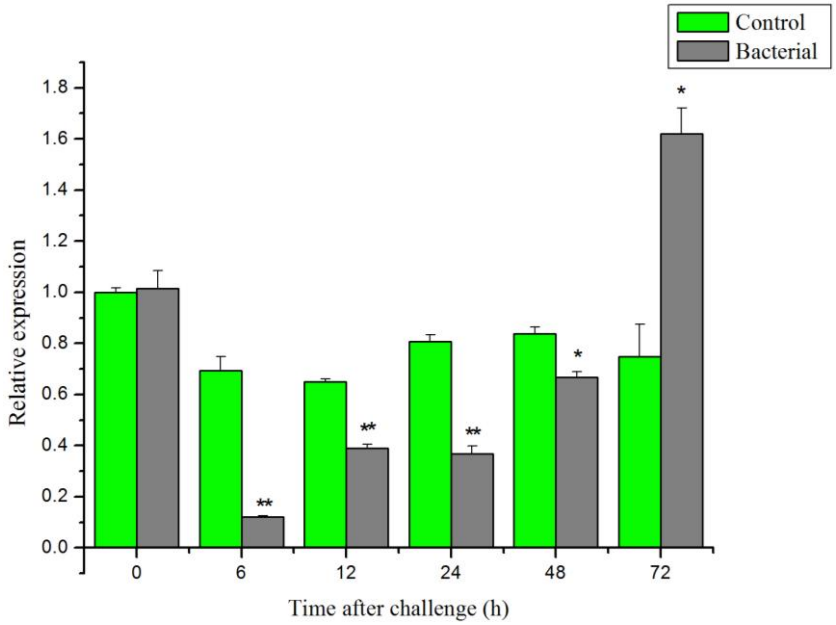


Fig. 5 Relative expression of *AjSto* in different tissues. Each vertical bar represents the mean ± SD (n = 3).



Obviously, all data in this study suggested that *AjSto* might involve in innate immune response of *A. japonicus* against bacterial infection. The presence of the highly conserved SPFH domain in *AjSto* further suggested that stomatin might have the same function in both vertebrates and invertebrates. Our study was the first to describe the *stomatin* gene and its corresponding protein in a marine organism. Temporal expression levels of *AjSto* also have been emerged in the organism in response to bacterial infection. Stomatin has been widely studied in human cancer and tumor, where its expression was found to decrease in non-small cell lung cancer and breast cancer (Chen *et al*., 2012; Arkhipova *et al*., 2014). A possible explanation is the decrease in stomatin expression an favorable factor for bacterial challenge.

In summary, a full-length of cDNA sequence of a stomatin gene from *A. japonicas* was cloned and characterized. The encoded protein shared a number of conserved structural features characteristic of the stomatin family proteins. Although the gene was expressed in all the *A. japonicas* tissues examined, the pattern of expression did vary to some extent, suggesting a preference in certain tissue type, the respiratory tree in this case. Further analysis of its transcript level following bacterial challenge revealed expressional changes that were dictated by infection time, suggesting that the stomatin gene characterized in *A. japonicus* may well be linked to immune response. Studying the stomatin gene of *A. japonicus* could fill the vacancy in marine organism research. Further study will seek to clarify the immune pathway of stomatin and the specific mechanism governing its regulation of the innate immunity in *A. japonicus.*

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