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Molecular characterization and expression analysis of protein enhancer of sevenless 2B from *Artemia sinica* at early embryonic development and during immune response to bacterial stimulation

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ABSTRACT

Protein enhancer of sevenless 2B, E(sev)2B, is a key adapter protein in the Ras/MAPK signaling pathway which has been reported to be involved in innate immunity. In this study, the gene that encodes *As*E(sev)2B was isolated from *A. sinica*. It was found to contain a 636 bp open reading frame encoding 211 amino acids with a calculated molecular mass of 24.357 kDa and a predicted isoelectric point of 5.39. The predicted protein contains a N-terminal Src homology 3 domain (SH3), a central Src homology 2 domain (SH2), and a C-terminal Src homology 3 domain (SH3). Homology analysis revealed that *As*E(sev)2B shares 49%–95% identity with E(sev)2B homologs from other species. In this study, the expression pattern and location of *As*E(sev)2B during different stages of embryonic development and bacterial challenge were investigated by means of real-time qPCR, Western blotting and immunohistochemistry. Results showed that the highest expression level of *As*E(sev)2B was at 0 h. After challenged by Gram-positive bacteria and Gram-negative bacteria, *As*E(sev)2B was remarkably upregulated at 10^6 cellsL^{-1} bacterial concentrations. These results suggested that *As*E(sev)2B plays a vital role during early embryonic development and in immune responses against bacterial challenge.

1. Introduction

The protein enhancer of sevenless 2B, E(sev)2B, is a vital adapter protein involved in signal transduction of various cells via regulation of upstream receptor tyrosine kinases (RTK) [1,2]. Homologs of the adaptor protein include Growth factor receptor bound protein 2 (Grb2) in mammal, Sex muscle abnormal protein 5 (Sem-5) in *Caenorhabditis elegans*, and Downstream of receptor kinase (Drk) in *Drosophila melanogaster*. They are composed of one Src homology 2 domain (SH2) flanked by a N- and a C-terminal Src homology 3 domains (SH3) [3]. Binding of the SH2 domain to the phosphotyrosine motifs on RTK, along with interaction between the SH3 domains and the proline-rich domain of Ras guanine nucleotide exchange factor Sos, converts Ras to the active GTP-bound state, which allows Ras activation of raf and ultimately leads to MAP kinase activation [4–6].

E(sev)2B protein is an important mediator in the Ras/MAPK signal transduction pathway, which is a highly conserved module involved in various cellular functions, including cell proliferation, differentiation and migration [7]. Additionally, it has been reported that MAP kinases are involved in all aspects of immune responses, from the initiation phase of innate immunity, to activation of adaptive immunity in mammals [8]. Moreover, E(sev)2B was found to be essential for embryonic development in higher animals [9–11] and involved in many cancers [12,13].

Artemia sinica is a small aquatic crustacean that lives in the hyperosmotic environment of salt pools and salt lakes in china [14,15]. As an important economic species, *A. sinica* has been used as the main commercial food resource for feeding newborn fish and shrimp in aquaculture due to its high protein and unsaturated fatty acid content in the nauplii. In addition, its resistance to high salinity, low temperature, and other adverse environmental stress has consolidated the potential use of *Artemia* as a research model organism in various fields, ranging from developmental biology to evolution and ecology [16]. Nevertheless, the innate immune defense mechanisms of *A. sinica* are still poorly understood. Therefore, identification of immune genes will provide new insights into the innate immune defense mechanisms of *A. sinica*.

To our knowledge, many previous studies of E(sev)2B were focused on mammals or model animals. However, the expression pattern, distribution and functional roles of *As*E(sev)2B from *A. sinica* remain unknown. In this study, we first cloned the open reading frame cDNA of *As*E(sev)2B to investigate its expression profile in different stages of embryonic development and in response to bacterial challenge by real-

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time qPCR and Western blotting. Subsequently, whole-mount immunohistochemistry was performed to identify the localization of *As*E(sev)2B proteins.

2. Materials and methods

2.1. Animal preparation

A. sinica cysts were harvested from the salt lake of Yuncheng in Shanxi Province, China during the summer of 2010 and stored at −20 °C in the dark. The cysts were hatched in saline water (salinity 28‰) under laboratory conditions: 28 °C and illumination intensity of 1000 Lux. The samples were collected at different time points during development (0, 5, 10, 15, 20, 40 h and 3, 5, 7, 10 d) for subsequent experiments. In the bacteria stimulation assay, pseudo-adult stage *A. sinica* (7 d) cultured in axenic seawater for 24 h were used as the control group and pseudo-adult stage *A. sinica* (7 d) maintained in seawater with halophilic Gram-negative bacteria *Vibrio harveyi* and Gram-positive bacteria *Micrococcus lysodeikticus* for 24 h were used as the experimental groups, respectively. The bacterial concentrations were 10^4 cells L^{-1}, 10^5 cells L^{-1} and 10^6 cells L^{-1}.

2.2. Cloning of AsE(sev)2B cDNA

Total RNA was extracted from adult stage *A. sinica* using RNAiso Plus (Takara, Dalian, China) according to the user manual. The total RNA was subsequently reverse transcribed into cDNA. The Primer Premier 5.0 was used to design the specific primers (E(sev)2B-F, E(sev)2B-R, Table 1) based on the transcriptomic database of *A. sinica* sequenced by Novegene (Tianjin, China). All primers were synthesized by Genewiz (Suzhou, China). Amplifications were performed with an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of denaturation (94 °C, 30 s), annealing (56 °C, 30 s), extension (72 °C, 1 min) and a final extension step (72 °C, 10 min). The PCR products were detected using electrophoresis with 1.0% agarose/TAE gels, and then sequenced by Genewiz (Suzhou, China). The 3′ and 5’ terminal fragments were spliced together using DNAMAN to yield the complete ORF of *As*E(sev)2B.

2.3. Bioinformatic analysis

Sequence analysis of *As*E(sev)2B was performed using the National Center for Biotechnology Information (NCBI) online Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The open reading frame and the deduced amino acid sequences were identified by the ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The ProtParam tool of ExPASy (http://web.expasy.org/protparam) was used to predict the molecular weight and theoretical isoelectric point of the protein. A conserved domain search tool (http://www.ncbi.nlm.nih.Gov/Structure/cdd/wrpsb.cgi) was used to predict the functional domains and protein structure. SignalP 4.1 program (http://www.cbs.dtu.dk/services/SignalP) and TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM/) were used to predict the signal peptide and transmembrane regions of *As*E(sev)2B. Protscale (http://web.expasy.org/protscale/) analyzed the hydrophobicity and hydrophilicity of the protein. Multiple sequence alignments were performed for the amino acid sequence of *As*E(sev)2B and those from other species using the DNAMAN programs. Clustalx1.81 program and MEGA6.06 software were used to generate the phylogenetic tree of E(sev)2B protein using the neighbor-joining (NJ) method. Statistical significance of groups within the phylogenetic tree was evaluated using the bootstrap method with 1000 replications.

Table 1
Oligonucleotide primers used in this study.

Primer	Sequence (5'-3')	Application
E(sev)2B -F	AATCCCTGAAACGAAAC	cDNA cloning
E(sev)2B -R	AAGCAAAGGTGCAAGTC	cDNA cloning
qE(sev)2B -F	CTGTCAAATGTGGGGATGG	RT-qPCR
qE(sev)2B -R	CCTTGAAACGGAGGTGGAT	RT-qPCR
β-actinF	GTGTGACGATGATGTTGCGG	internal control
β-actinR	GCTGTCCTTTTGACCCATTCC	internal control
exE(sev)2B -F	GGGGTACCGAAGCCATTGCCAAACA	prokaryotic expression
exE(sev)2B -R	ATTTGCGGCCGCTTAAATTTCCCCTGTCCACC	prokaryotic expression

2.4. Expression analysis of AsE(sev)2B by real-time qPCR

2.4.1. Expression of AsE(sev)2B during different developmental stages

Total RNA was extracted from *A. sinica* at different stages of development (0, 5, 10, 15, 20 and 40 h; 3, 5, 7, and 10 d). RNA concentration was measured and used as templates for cDNA synthesis with a Two-step Reverse Transcription Kit (Takara, Dalian, China). β-actin primers (β-actinF, β-actinR, Table 1) were used as normalization control for target gene expression [17]. A pair of primers specific (qE(sev)2B-F, qE(sev)2B-R, Table 1) for qPCR were designed in Primer Premier 5.0. Real-time qPCR was performed in triplicate for every sample in a parallel design using the LightCycler 96 System (Roche, Mannheim, Germany). Each PCR was performed in a 10 μl reaction volume containing 5 μl 2 × SYBR Premix Ex Taq (Takara, Dalian, China), 1 μl diluted cDNA template, 0.4 μl of each primer (10 mM), and 3.2 μl sterile distilled H_2O. The amplification programs included an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 51 °C for 30 s, melting curve was generated at the end of the qPCR reaction followed by 95 °C for 10 s and 58 °C for 30 s. The comparative CT method ($2^{-\Delta\Delta Ct}$) was used to calculate the relative quantification [18]. P-value < 0.05 was used as the statistically significance threshold for determining differences between treatment and control groups.

2.4.2. Expression of AsE(sev)2B in response to bacterial stimulation

Gram-negative bacteria *Vibrio harveyi* and Gram-positive bacteria *Micrococcus lysodeikticus* were cultivated in 5 ml 2216E and LB liquid medium separately with shaking for 12 h and the bacterial cells were counted using a blood cell counter plate. Each bacterial culture was diluted to 10^4 cellsL^{-1}, 10^5 cellsL^{-1} and 10^6 cellsL^{-1} and then used to challenge the pseudo-adult stage *A. sinica* (7 d). Pseudo-adult stage *A. sinica* (7 d) not subjected to bacterial stimulation was used as control. Total mRNA was extracted from *A. sinica* and reverse transcribed into cDNA. Real-time qPCR was performed on the samples under reaction conditions detailed in Section 2.4.1.

2.5. Vector construction, expression and purification of the recombinant protein

The 579 bp cDNA sequences of *As*E(sev)2B were amplified with primer exE(sev)2B-F and exE(sev)2B-R (Table 1, *Kpn* I and *Not* I sites were incorporated to the 5’- end of primers, respectively). The obtained PCR product was cloned into the vector pET-30a to form pET-30a-*As*E(sev)2B recombinant plasmid using the T4 DNA ligase enzyme (TaKaRa) at 16 °C overnight. Subsequently, the recombinant plasmids were transformed into *E. coli* Trans5α competent cell (TransGen Biotech, Beijing, China) and the positive clones were identified by colony PCR and Sanger sequencing. The recombinant plasmids were extracted using EasyPure Plasmid MiniPrep Kit (TransGen) and transformed into *E. coli* BL21 (DE3) (TransGen) for expression by heat shocking at 42 °C for 90 s followed by ice bath for 2 min. Finally, bacteria were incubated in LB medium containing kanamycin at 37 °C with shaking at 200 rpm until the culture reached an OD600 of 0.6–0.8. Induction condition was as follows: 0.1 mM IPTG for 5 h at 37 °C. Cells were collected by high-speed centrifugation and the precipitation was suspended with 1x PBS

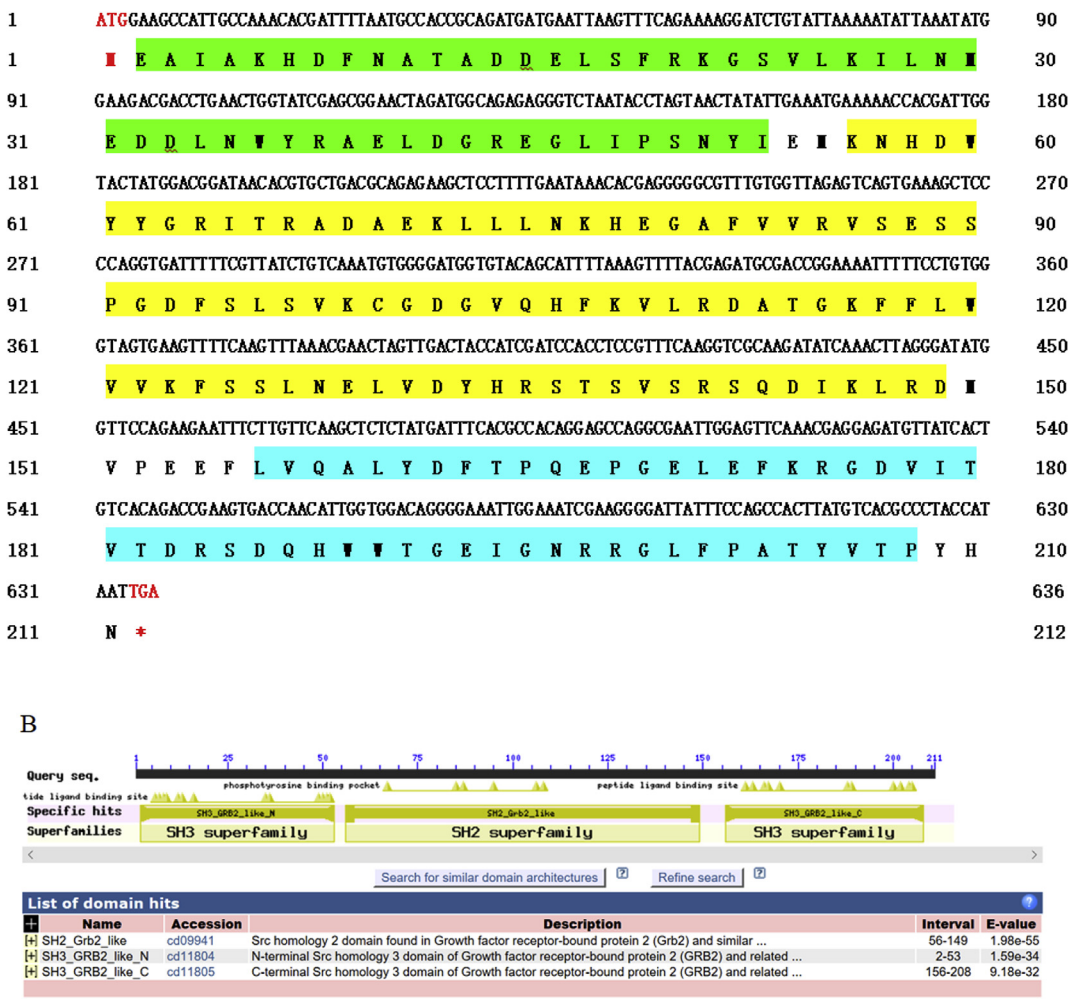

Fig. 1. (A) Nucleotide sequences and deduced amino acid sequences of AsE(sev)2B. The red letters represent the start codon and end codon; the green area and blue area represent the two SH3 domains; the yellow area represents the SH2 domain. (B) Domain analysis of the putative *AsE*(sev)2B protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and then broken up by ultrasound. The recombinant protein was purified using Ni-NTA Resin (GenScript, Nanjing, China) according to the supplier's protocol. Protein was examined by SDS-PAGE on a 12% gel and visualized with Coomassie brilliant blue R250. The concentration of purified protein was around 1.4 mg/ml as quantified by BCA Protein Assay Kit (Solarbio, Beijing, China).

2.6. Preparation of polyclonal antibodies

The purified E(sev)2B fusion protein was used to generate antibodies by Beijing Protein Innovation (Beijing, China). The specificity of the antibody for the recombinant protein was determined using Western blotting. The polyclonal antibodies were used for subsequent Western blot and immunohistochemistry assay.

2.7. Western blotting

2.7.1. Protein production of AsE(sev)2B at different stages of early embryo development

Total proteins were extracted from each sample (15, 20 and 40 h; 3, 5, 7, and 10 d) using RIPA lysis buffer and quantified by the BCA method. Each protein sample (100 mg) was fractionated by SDS-PAGE and transferred to NC membranes. The membrane was blocked in non-fat powdered milk for 3 h at room temperature. Rabbit anti-*As*E(sev)2B polyclonal antibodies was diluted to 1:100 and beta actin mouse monoclonal antibodies (Proteintech) were diluted to 1:10000 with TBST. The membranes were incubated with the antibodies overnight at 4 °C. Then, the membrane was washed with TBST (3 × 10 min), and incubated with HRP-conjugated goat anti-rabbit and goat anti-mouse IgG (Transgen, Beijing, China) for 3 h at 37 °C, followed by three washes with TBST (10 min each). The reactive protein bands on the membrane were visualized using ECL reagent (Transgen, Beijing, China) and exposed to an x-ray film in darkroom. The intensities of expressed *As*E(sev)2B specific bands were normalized against the β-actin bands.

2.7.2. Protein production analysis of AsE(sev)2B in response to bacterial stimulation

The pseudo-adult stage *A. sinica* (7 d) was treated with the halophilic Gram-negative bacteria *V. harveyi* and Gram-positive bacteria *M. lysodeikticus* at different densities separately for 24 h. Each of the two types of bacteria were diluted to 10^4 cells L^{-1}, 10^5 cells L^{-1} and 10^6 cells L^{-1}. Total proteins were extracted from each sample and quantified. Western blot was performed to detect the expression trend of *As*E(sev)2B protein in response to increasing bacteria concentration as described in Section 2.7.1.

2.8. Whole mount immunohistochemistry assay of AsE(sev)2B

For whole mount immunohistochemical studies, polypides were introduced into tubes containing *A. sinica* at different developmental

stages (polypides from 0, 5 and 10 h were completely dechlorinated with 50% NaClO for 3 min). Heptane and PEM-FA (PIPES 100 mM, $MgSO_4$ 1.0 mM, EGTA 2.0 mM, formalde hyde 10%) fixatives were added to the tubes, which were then shaken slowly for 30 min. Polypides were then held on methanol at −20 °C. Subsequently, the polypides were suspended in PBT (1 × PBS, Triton X-100 10%) and sonicated to expose the antigens. The sonicated polypides were incubated with 0.5% H_2O_2 for 30 min to eliminate the endogenous substance reactions, followed by washes with PBT and then blocked with 5% BSA blocking buffer (Solarbio) for 30 min. The Rabbit anti-*As*E(sev)2B polyclonal antibodies (diluted to 1:100 with PBT) was then added and incubated with the polypides overnight at 4 °C. After several washes (3 × 30 min) with PBT, the samples were further incubated with HRP-conjugated goat anti-rabbit IgG (Transgen, Beijing, China) at a dilution of 1:10000 with PBT for 2 h at 37 °C. After two washes with PBT (30 min per wash), followed by addition of DAB reagent (Solarbio), the samples were incubated for 8 min in dark and then washed with distilled water to terminate the reaction. The polypides were observed using a Leica camera and images of polypides were handled by Photoshop CS6 software.

3. Results

3.1. Cloning and bioinformatics analysis of AsE(sev)2B

Based on the transcriptomic database, a 636 bp open reading frame (ORF) of *As*E(sev)2B was obtained (GenBank accession number: MH579718). The putative *As*E(sev)2B protein is encoded by 211 amino acids (aa) with a calculated molecular mass of 24.357 kDa and a pI of 5.39. Prediction of protein domains revealed three conserved domains: an N-terminal Src homology 3 domain (SH3), a central Src homology 2 domain (SH2), and a C-terminal Src homology 3 domain (SH3) (Fig. 1A and B). SignalP 4.1 showed that *As*E(sev)2B lacks an N-terminal signal peptide, indicating that it is a non-secretory protein. Analysis by TMHMM Server 2.0 showed that *As*E(sev)2B has no transmembrane domains. Analysis of hydrophobicity and hydrophilicity demonstrated that *As*E(sev)2B is a hydrophilic protein. Multiple protein sequence alignment revealed the conserved amino acid sequences among different species, especially in the N-terminal SH3 domain (2-53 aa), the central SH2 domain (56-149 aa) and the C-terminal SH3 domain (156-208aa) (Fig. 2). Homology analysis revealed that *As*E(sev)2B exhibits the highest homology (95%) to that of *Daphnia magna,* and shares 49%–90% similarity to the other E(sev)2B homologs (Table 2). Analysis of the phylogenetic tree showed two main clusters: invertebrates (insects *Ceratosolen solmsi marchali*, *Diachasma alloeum*, *Apis mellifera*, *Bemisia tabaci*, *Diaphorina citri*, *Bombyx mori*, *Drosophila melanogaster* and *Drosophila simulans*; crustacean *Artemia sinica*, *Daphnia magna*, *Hyalella azteca*, *Hirondellea gigas*, *Eurytemora affinis* and *Lepeophtheirus salmonis*), and vertebrates (mammalian *Homo sapiens* and *Pan paniscus*; pisces *Oryzias latipes*, *Nothobranchius furzeri)*, The two nematodes *Brugia malayi* and *Loa loa* were set as outgroups (Fig. 3).

Table 2
Amino acid identity comparison of *As*E(sev)2B protein and those from other species from GenBank.

Classification	Species	Accession No.	Identity (%)
Insect	*Ceratosolen solmsi marchali*	XP_011497742	89%
	Diachasma alloeum	XP_015124285	89%
	Apis mellifera	XP_006560290	88%
	Bemisia tabaci	XP_018899061	90%
	Diaphorina citri	XP_008472352	89%
	Bombyx mori	NP_001243920	85%
	Drosophila melanogaster	NP_476858	86%
	Drosophila simulans	XP_016027399	86%
Crustacea	*Artemia sinica*	QAX26144	100%
	Daphnia magna	KZS14957	95%
	Hyalella azteca	XP_018012221	88%
	Hirondellea gigas	LAB70899	89%
	Eurytemora affinis	XP_023329434	90%
	Lepeophtheirus salmonis	ADD38444	80%
Mammalia	*Homo sapiens*	NP_002077	66%
	Pan paniscus	XP_003813338	66%
Pisces	*Oryzias latipes*	XP_023813750	65%
	Nothobranchius furzeri	XP_015800741	66%
Nematoda	*Brugia malayi*	XP_001899435	49%
	Loa loa	XP_003141535	62%

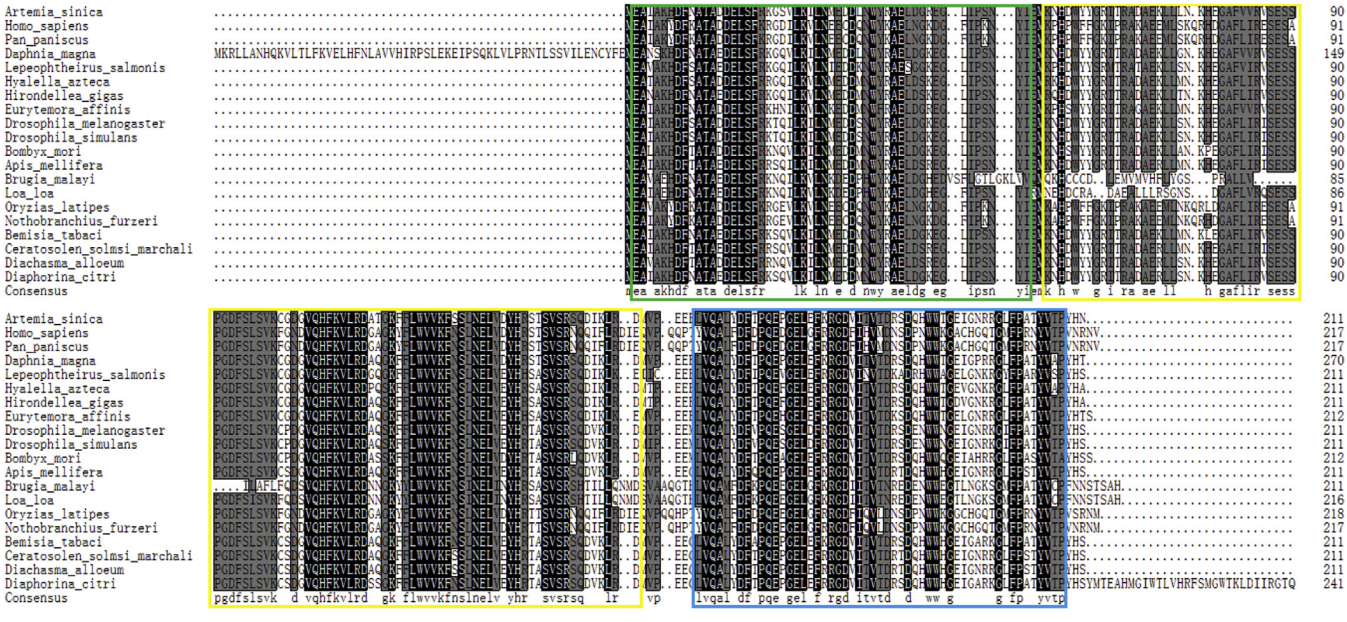

Fig. 2. Protein sequences alignment of *As*E(sev)2B and E(sev)2B proteins of 19 other species from GenBank. The sequences and their accession numbers are as Table 2. The green and blue boxes indicate two SH3 domains (2-53aa and 156-208aa), respectively; the yellow box indicates the SH2 domain (56-149aa). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

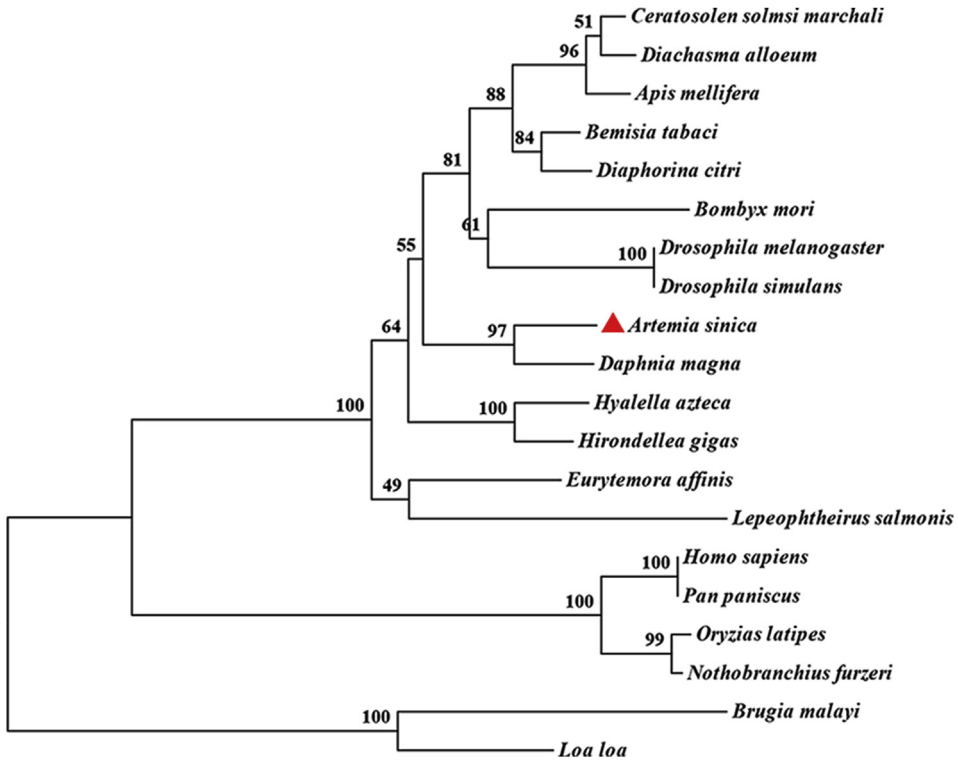

Fig. 3. The phylogenetic tree of E(sev)2B homologues was constructed based on the amino acid sequence of *As*E(sev)2B and others obtained from GenBank. The sequences and their accession numbers are same as Table 2. A triangle ( ) indicates *As*E(sev)2B protein. Bootstrap = 1000.

3.2. Expression analysis of AsE(sev)2B by real-time qPCR at different development stages and under bacterial challenge

The qPCR results indicated that *As*E(sev)2B was expressed at all developmental stages. The expression level of *As*E(sev)2B was highest at 0 h, and then remarkably decreased and remained at a low level throughout the rest of the development (Fig. 4). When challenged by Gram-positive bacteria, *As*E(sev)2B was expressed at a noticeably higher level at bacterial concentration of 10^6 cellsL^{-1}, but the expression level was close to that in the control when challenged with low bacterial concentrations (10^5cellsL^{-1}and 10^4 cellsL^{-1}). When stimulated by Gram-negative bacteria, the relative expression level of *As*E(sev)2B showed a strikingly up-regulated trend as the bacterial concentration increased from 10^4 cellsL^{-1} to 10^6 cellsL^{-1}(Fig. 5A and B).

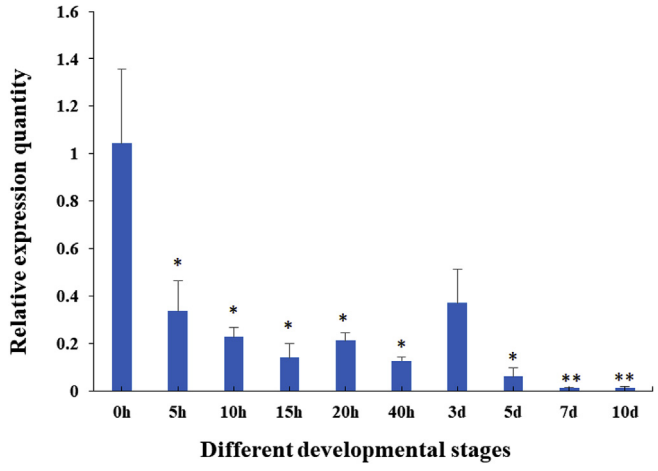

Fig. 4. The expression of *As*E(sev)2B in different development stages. The control group was set at 0 h stage. Data are the means ± SD of triplicate experiments. The statistically significant differences relative to the control group are indicated with asterisks (*) ($P < 0.05$) or asterisks (**) ($P < 0.01$).

3.3. Expression and purification of AsE(sev)2B recombinant protein

The expression sequences were cloned by specific primers and digested with restriction enzymes *Kpn* I and *Not* I, and then cloned into a vector for fusion protein expression. The recombinant plasmid pET30a-*As*E(sev)2B was confirmed by sequencing. The SDS-PAGE results showed that the 27KD of the protein was expressed in inclusion bodies. Finally, a relatively pure protein was obtained after purification and condensation (Fig. 6).

3.4. Expression pattern of AsE(sev)2B protein at different developmental stages and under bacterial stimulation

The expression pattern of *As*E(sev)2B protein at different developmental stages and under two bacterial stimulation were similar to the qPCR results (Fig. 7, Fig. 8A and B).

3.5. Expression location analysis of AsE(sev)2B

Whole mount immunohistochemistry assay was performed to detect the location of *As*E(sev)2B expression at different developmental stages in *A. sinica* (Fig. 9). Results revealed that *As*E(sev)2B was expressed in almost the whole embryo of *A. sinica* at 0 h (Fig. 9A2). When *A. sinica* entered the umbrella stage from 5 h to 10 h, the oolemma was ruptured by the head of the embryo and *As*E(sev)2B was mainly expressed at the upper side, especially in the head of the embryo (Fig. 9B2 and C2). When the embryo was completely out of the cyst shell at 15 h (nauplius stage), *As*E(sev)2B was detected in the entire body (Fig. 9D2). At 20 h, *As*E(sev)2B was mainly expressed in the head and chest, while weak expression was detected in other areas (Fig. 9E2). As *A. sinica* developed into metanauplius after 40 h, the internal tissue and external appendages began to develop and strong, *As*E(sev)2B signals were detected

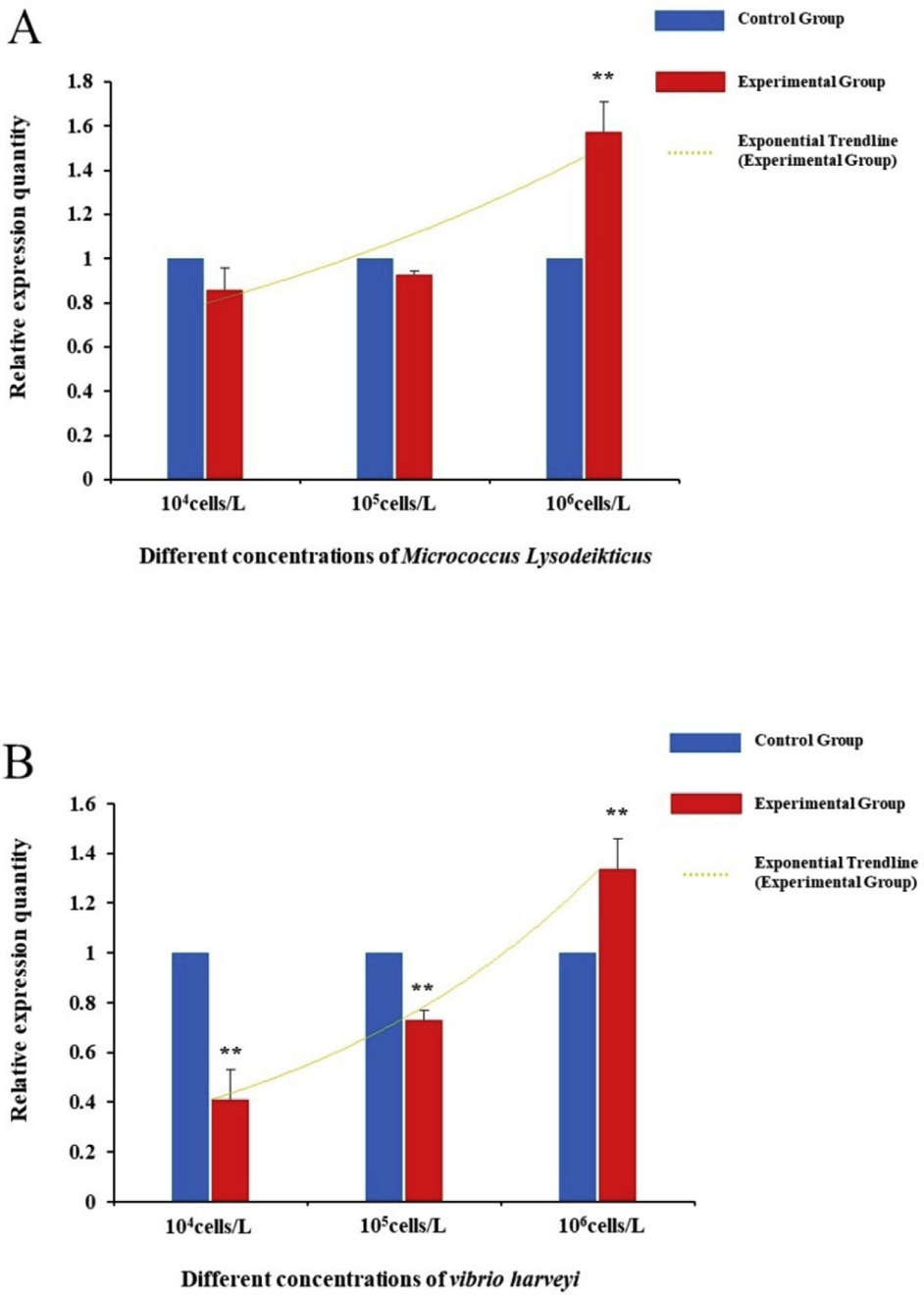

Fig. 5. (A) The relative expression level of *As*E(sev)2B stimulated by gram-positive bacteria. (B) The relative expression level of *As*E(sev)2B stimulated by gram-negative bacteria. The expression level at sterile seawater was set as the control group. Data are the means ± SD of triplicate experiments. Significant differences between experimental and control groups are indicated with asterisks (*) ($P < 0.05$) or (**) ($P < 0.01$).

from head to tail (Fig. 9F2). At 3 d, *As*E(sev)2B expression was observed in the cephalothorax and slight expression was found in the tail (Fig. 9G2). As *A. sinica* developed into pseudo-adult after 5 d, the abdomen began to form sub-sections and gradually approached the final shape, as *As*E(sev)2B was mainly expressed in the head and chest (Fig. 9H2). From 7 d to 10 d, as *A. sinica* developed into adulthood, the expression of *As*E(sev)2B was significantly different from those in other periods, and positive signals were mainly detected in the head (Fig. 9I2 and J2).

4. Discussion and conclusion

In this study, a 636 bp ORF cDNA sequence encoding a polypeptide of 211 amino acids with a calculated molecular mass of 24.357 kDa of *As*E(sev)2B was obtained from *A. sinica*. Sequence analysis revealed that the *As*E(sev)2B protein contains three domains: SH3-SH2-SH3 domain, which was consistent with previously reported homologs: Grb2 in humans [9], Sem-5 in *Caenorhabditis elegans* [19], and Drk in *Drosophila melanogaster* [20], suggesting that it is a member of the evolutionarily conserved adaptor protein. For these reasons, *As*E(sev)2B is predicted to play an essential role in the Ras signal pathway in *A. sinica,* which regulates cell growth, proliferation and differentiation [21,22]. Similar to the other adaptor proteins involved in the activation of the Ras/MAPK pathways, E(sev)2B, via its SH2 domain, binds to the phosphorylated tyrosine kinase receptor while its SH3 domain interacts with the proline-rich domain of Ras guanine nucleotide releasing

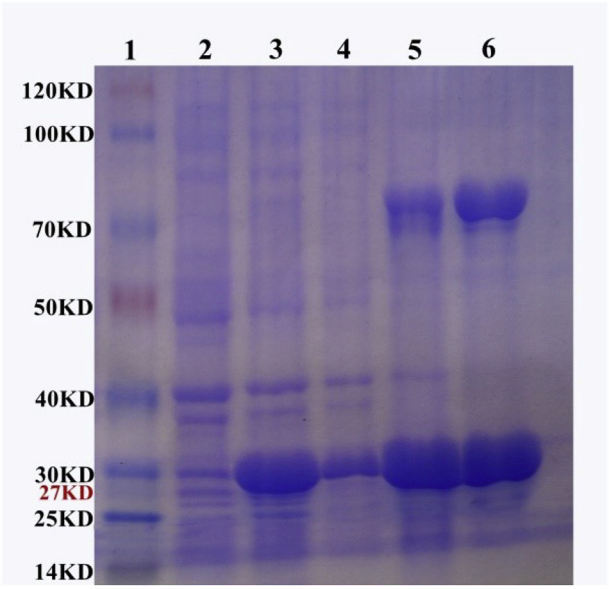

Fig. 6. The expression of *As*E(sev)2B recombinant protein in *E. coli* BL21 (DE3). 1: protein markers. 2: The recombinant product of pET-30a-*As*E(sev)2B without inducing. 3: Induced recombinant product. 4: Soluble fraction of the lysate from induced cells. 5: Precipitate fraction. 6: Purified recombinant protein. The red letter indicates calculated molecular mass of recombinant protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

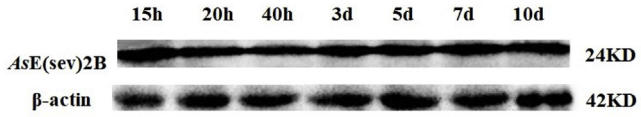

Fig. 7. Relative expression of *As*E(sev)2B at different developmental stages of *A. sinica* detected by Western blotting. The intensities of *As*E(sev)2B protein bands were normalized against that of β-actin.

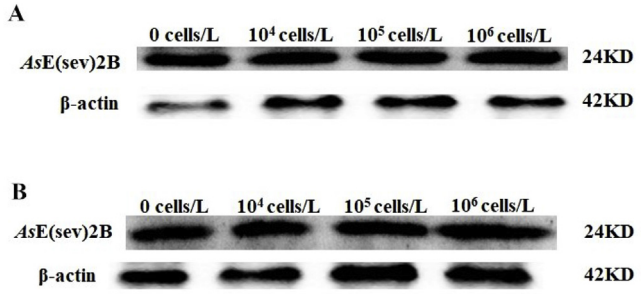

Fig. 8. (A) Western blotting analysis of *As*E(sev)2B stimulated by gram-positive bacteria. The band intensities for *As*E(sev)2B were normalized against that of β-actin. (B) Western blotting analysis of *As*E(sev)2B challenged by gram-negative bacteria. The band intensities for *As*E(sev)2B were normalized against that of β-actin.

protein Sos [3]. Besides, E(sev)2B can also interact with non-RTK proteins such as focal adhesion kinase [23]. Additionally, transmembrane analysis shows that *As*E(sev)2B is a non-membrane protein, which corresponds to its function. In fact, E(sev)2B is a soluble cytoplasmic protein, which is present downstream of the transmembrane proteins tyrosine kinase receptors in the Ras pathway [6].

Phylogenetic tree analysis revealed similar results to the homology comparison, indicating that the evolutionary relationship between *As*E(sev)2B and E(sev)2B homologs in crustacea and insect is closely linked (80%–95%), while it is distant from nematode, fish and mammal

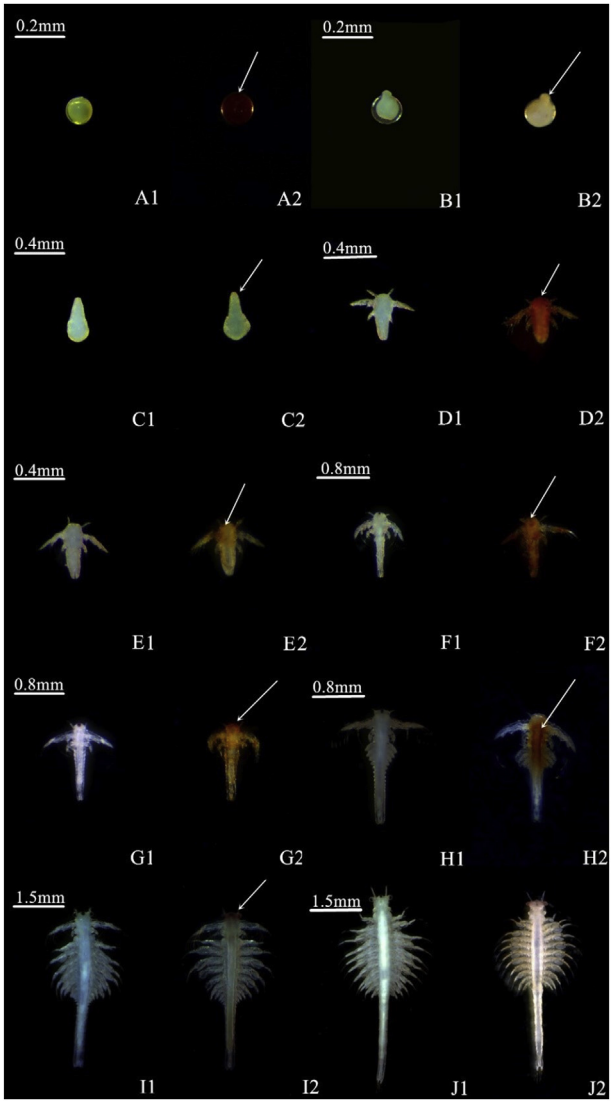

Fig. 9. Immunohistochemistry staining at different developmental stages of *A. sinica*. A1–J1: control groups, A2–J2: experimental groups. (A) 0 h, the gastrula stage; (B and C) 5 h and 10 h, umbrella stage; (D and E) 15 h and 20 h, nauplius stage; (F and G) 40 h and 3 d, Metanauplius stage; (H and I) 5 d and 7 d, pseudoadult stage; (J) 10 d, adult stage. The white arrows indicate positive signal regions.

(49%–66%). Based on the above analysis, *As*E(sev)2B contains conserved domains and shares high sequence identity with other species, which further confirms that *As*E(sev)2B is a homologous protein of Grb2/Sem-5/drk. Taken together, our results suggest that *As*E(sev)2B might have similar biological function as that of corresponding proteins in other species. It is worth noting that E(sev)2B proteins in vertebrate and invertebrate exhibit clear clade division, indicating different evolutionary direction.

To date, our study is the first to detect a relationship between E(sev)2B expression and innate immunity of *A. sinica* by analyzing the relative expression levels of *As*E(sev)2B at different developmental stages and under Gram-negative and Gram-positive bacterial challenge. The development of *A. sinica* consists of five main stages: gastrula stage (0 h), umbrella stage (5–10 h), nauplius stage (15–20 h), metanauplius stage (40 h-3 d), pseudoadult stage (5–7 d) and adult stage (10 d) [24]. The results of real-time qPCR at different development stages of *A. sinica* suggested that the expression level of *As*E(sev)2B was highest at 0 h. This period corresponds to the gastrulation stage, indicating that *As*E(sev)2B was already present in the embryo before the cysts began to

incubate. Since E(sev)2B is a crucial adaptor protein in multiple signal transduction pathways [25–29], *As*E(sev)2B would have to maintain high expression level during early embryonic development. Many studies have also indicated that low expression of Grb2 gene in mammal can lead to abnormal embryonic development [30,31]. At the later stages of embryonic development, the larvae are surrounded by the external environment and then exposed to other microorganisms. At these later stages, the expression levels of *As*E(sev)2B decreased significantly and maintained at a stable but low level. The relative stability of their environment may be reflected in the subtle changes in *As*E(sev)2B expression.

When *A. sinica* was stimulated by Gram-positive bacteria, the expression levels of *As*E(sev)2B increased noticeably at 10^6 cellsL^{-1} bacteria concentration, while the levels were similar to that of control at 10^5 cellsL^{-1}and 10^4 cellsL^{-1}. This phenomenon could potentially be attributed to that the immune system of *A. sinica* is not fully developed until the pseudoadult stage, therefore, low concentration of bacteria is inadequate to induce high expression of *As*E(sev)2B. When challenged by Gram-negative bacteria, the expression level of *As*E(sev)2B was similar to that of *A. sinica* stimulated with Gram-positive bacteria in 10^6 cellsL^{-1}. However, *As*E(sev)2B expression gradually declined and was found to be lower than that of control at 10^5cellsL^{-1} and 10^4 cellsL^{-1}. We speculate that the low bacterial concentrations might negatively regulate *As*E(sev)2B transcription and translation.

Immunohistochemistry results showed that *As*E(sev)2B signals were detected at all development stages in *A. sinica*, which is consistent with the previous report that Grb2 exhibits constitutive, non-specific expression [32]. From 0 h to 5 d, the *As*E(sev)2B signal gradually expands with the development of *A. sinica*, especially after the digestive gland is developed. This phenomenon suggested that *As*E(sev)2B plays an important role in early embryonic development. As *A. sinica* enters into the adult stage at 7–10 d, the distribution of weak signals was mainly observed in the head. We hypothesize that the expression of *As*E(sev)2B may be localized to the head after the pseudo-adult stage.

Taken together, we conclude that *As*E(sev)2B may be involved in the early embryonic development and the immune defense response against *V. harveyi* and *M. lysodeikticus*. Our findings provide important insights into the innate immune defense mechanisms of *A. sinica*. Further studies are necessary to fully elucidate the function of E(sev)2B in *A. sinica*.

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