

## Transcriptional regulation mechanism of sterol regulatory element binding proteins on $\Delta 6$ fatty acyl desaturase in razor clam *Simonovacula constricta*

Zhaoshou Ran<sup>1,2</sup>, Fei Kong<sup>1</sup>, Jilin Xu<sup>1,3\*</sup>, Kai Liao<sup>1</sup> and Xiaojun Yan<sup>2\*</sup>

<sup>1</sup>Key Laboratory of Applied Marine Biotechnology, Department of Marine Sciences, Ningbo University, Ministry of Education of China, Ningbo, Zhejiang 315211, People's Republic of China

<sup>2</sup>Collaborative Innovation Center for Zhejiang Marine High-efficiency and Healthy Aquaculture, Ningbo University, Ningbo, Zhejiang 315211, People's Republic of China

<sup>3</sup>Fujian Baozhi Aquatic Science and Technology Co. Ltd, Zbangzhou, Fujian 363503, People's Republic of China

(Submitted 20 October 2019 – Final revision received 29 April 2020 – Accepted 2 June 2020 – First published online 10 June 2020)

### Abstract

The razor clam, *Simonovacula constricta*, contains high levels of long-chain PUFA (LC-PUFA), which are critical for human health. In addition, *S. constricta* is the first marine mollusc demonstrated to possess  $\Delta 6$  fatty acyl desaturase (Fad) and complete LC-PUFA biosynthetic ability, providing a good representative to investigate the molecular mechanism of sterol regulatory element binding proteins (SREBP) in regulating  $\Delta 6$  Fad for LC-PUFA biosynthesis in marine molluscs. Herein, *S. constricta* SREBP and  $\Delta 6$  Fad promoter were cloned and characterised. Subsequently, dual luciferase and electrophoretic mobility shift assays were conducted to explore the SREBP binding elements in the core regulatory region of *S. constricta*  $\Delta 6$  Fad promoter. Results showed that *S. constricta* SREBP had a very conservative basic helix-loop-helix-leucine zipper motif, while *S. constricta*  $\Delta 6$  Fad promoter exhibited very poor identity with teleost Fads2 promoters, indicating their differentiation during evolution. A 454 bp region harbouring a core sequence in *S. constricta*  $\Delta 6$  Fad promoter was predicted to be essential for the transcriptional activation by SREBP. This was the first report on the regulatory mechanism of LC-PUFA biosynthesis in marine molluscs, which would facilitate optimising the LC-PUFA biosynthetic pathway of bivalves in further studies.

**Key words:** *Simonovacula constricta*: Sterol regulatory element binding proteins:  $\Delta 6$  Fatty acyl desaturase: Transcriptional regulation

Sterol regulatory element binding proteins (SREBP) are a family of membrane-bound transcription factors, which play a critical role in the maintenance of lipid homeostasis by activating genes encoding enzymes required for the biosynthesis of sterols and fatty acids<sup>(1)</sup>. To date, there are three kinds of SREBP, including SREBP-1a and -1c derived from utilisation of alternate promoters of one gene that differ in the first exon<sup>(2–4)</sup>, and SREBP-2 encoded by a separate gene<sup>(5)</sup>. Specifically, SREBP-1a and -1c preferentially activate transcription of genes required for fatty acids and TAG biosynthesis, while SREBP-2 mainly controls transcription of genes required for sterol biosynthesis<sup>(6–8)</sup>. SREBP typically contain a N-terminal transactivated domain possessing a basic helix-loop-helix-leucine zipper (bHLH-Zip) motif and a C-terminal regulatory domain, which are joined together by two membrane-binding regions interrupted by about thirty amino acids (aa)<sup>(1,9)</sup>. They are initially synthesised as inactive precursors that bind to membranes of the endoplasmic reticulum. To influence transcription, the SREBP must be proteolytically cleaved to release the N-

terminal transactivated domain so that they can enter the nucleus. Those segments are designed as nuclear (mature) SREBP, which further activate transcription of target genes by binding to the sterol regulatory element (SRE) or classic palindromic E-box within their promoters using bHLH-Zip motif<sup>(9)</sup>.

Long-chain PUFA (LC-PUFA), especially ARA (20 : 4*n*-6), EPA (20 : 5*n*-3) and DHA (22 : 6*n*-3), are critical in a number of physiological processes, such as cell membrane formation, neurological development, immune response and CVD treatments<sup>(10–13)</sup>. The endogenous biosynthesis of LC-PUFA from C<sub>18</sub> precursors (18 : 2*n*-6 and 18 : 3*n*-3) requires consecutive desaturation and elongation steps catalysed by fatty acyl desaturase (Fad) and elongase of very long-chain fatty acid (Elovl), respectively<sup>(14)</sup>. Among which,  $\Delta 6$  Fad not only catalyses the first desaturation step but also catalyses the rate-limiting step in DHA biosynthesis by conversion of 24 : 5*n*-3 to produce 24 : 6*n*-3, which is then partially  $\beta$ -oxidised to DHA via the Sprecher pathway<sup>(15)</sup>. Therefore,  $\Delta 6$  Fad has been considered as the key and rate-limiting enzyme

**Abbreviations:** aa, amino acid; cDNA, complementary DNA; EMSA, electrophoretic mobility shift assay; Fad, fatty acyl desaturase; LC-PUFA, long-chain PUFA; ORF, open reading frame; RACE, rapid amplification of cDNA ends; SRE, sterol regulatory element; SREBP, sterol regulatory element binding protein.

\* **Corresponding authors:** Jilin Xu, fax +86 574-87609570, email [xujilin@nbu.edu.cn](mailto:xujilin@nbu.edu.cn); Xiaojun Yan, fax +86 574-87600458, email [yanxiaojun@nbu.edu.cn](mailto:yanxiaojun@nbu.edu.cn)

in LC-PUFA biosynthesis. Meanwhile, SREBP as important transcriptional regulators of  $\Delta 6$  Fad have been well demonstrated or indicated by finding SRE within  $\Delta 6$  Fad promoters in vertebrates, that is, mammals<sup>(16,17)</sup> and teleosts<sup>(18–23)</sup>, but still not in invertebrates including marine molluscs.

The razor clam *Simonovacula constricta* is an economically and nutritionally important bivalve species that is widely distributed in the estuarine and intertidal zones along the coasts of the west Pacific Ocean, with a total production of over 823 000 tons and a value of US\$ 1.3 billion in 2016<sup>(24,25)</sup>. Particularly, it possesses high levels of EPA and DHA, each accounting for about 10% of the total fatty acids<sup>(26)</sup>. Therefore, *S. constricta* is an excellent LC-PUFA resource for human nutritional requirement. Importantly, *S. constricta* is demonstrated as the first marine mollusc to possess all Fad and Elovl activities required for LC-PUFA biosynthesis via the Sprecher pathway<sup>(27,28)</sup>. In addition, *S. constricta*  $\Delta 6$  Fad is also the first report of a  $\Delta 6$  Fad in a marine mollusc<sup>(27)</sup>. Therefore, *S. constricta* provides a favourable organism to investigate the regulatory mechanisms of LC-PUFA biosynthesis in marine molluscs.

In the present study, the characterisation of SREBP and its transcriptional regulation mechanism on  $\Delta 6$  Fad were investigated in *S. constricta*. In brief, the *S. constricta* SREBP was first cloned and its sequence was analysed. Second, the structure of *S. constricta*  $\Delta 6$  Fad promoter was characterised, including cloning of the promoter sequence, prediction of SRE by bioinformatic software and determination of the core promoter region by dual luciferase assay. Third, considering the SREBP activate transcription

independently by their N-terminal segments (nuclear SREBP) harbouring bHLH-Zip motif in cultured cells and intact animals<sup>(3–5,7,29)</sup>, the partial segment containing bHLH-Zip motif of *S. constricta* SREBP was subjected to a prokaryotic expression system. Followed by, the purified protein was used to interact with the potential SRE in *S. constricta*  $\Delta 6$  Fad promoter by electrophoretic mobility shift assay (EMSA). The results greatly increased our understanding on the regulatory mechanism of LC-PUFA biosynthesis in marine molluscs, which would facilitate optimising the LC-PUFA biosynthetic pathway of bivalves in further studies.

## Materials and methods

### Cloning and sequence analyses of *Simonovacula constricta* sterol regulatory element binding proteins

*S. constricta* RNA was extracted from fresh-mixed tissues of foot muscle, gill and gonad using a MiniBEST Universal RNA Extraction Kit (TaKaRa). The quality and concentration of the extracted RNA were determined on a 1% agarose gel and a NanoDrop® ND-1000 (NanoDrop), respectively. A quantity of 1  $\mu$ g of total RNA was reverse-transcribed into template complementary DNA (cDNA) using a PrimeScript™ RT-PCR Kit (TaKaRa).

The first fragment of *S. constricta* SREBP cDNA was obtained by searching against its transcriptome data, and only one gene with high homology to vertebrate SREBP was detected. To verify the target sequence, gene-specific primers (SREBP-V-F, V-R in

**Table 1.** Primers used for cloning of *Simonovacula constricta* sterol regulatory element binding proteins (SREBP) and  $\Delta 6$  fatty acyl desaturase (Fad) promoter, construction of reporter vector and express vector, prokaryotic expression of SREBP-D and electrophoretic mobility shift assay (EMSA) assay\*

Primer	Sequence (5' → 3')
SREBP-V-F	TCCAGATGGCATCCCCGT
SREBP-V-R	TCTAACTACAAGTGACAGTAGATGCAGC
SREBP-5'-GSP1	CGGCTGGGAGGATGGTTGGGG
SREBP-5'-GSP2	GATTGGGCTGGACACAGGCCACCAC
SREBP-3'-GSP1	CCGACAGTGAGGTCCCGGATAGGG
SREBP-3'-GSP2	CTGTCCAGAGCTGTCGTCCGACACTG
$\Delta 6$ Fad-promoter-V-F	CTCGTGCAGTCTTTTATATGCA
$\Delta 6$ Fad-promoter-V-R	ATTTACTTCTGCACGCCGGAT
pGL3- $\Delta 6$ Fad-1 F	GGGGTACCCTCGTGAGTTCTTTTATATGCA
pGL3- $\Delta 6$ Fad-2 F	GGGGTACCCTAGTACCGCTTATTTGGTATGAGA
pGL3- $\Delta 6$ Fad-3 F	GGGGTACCCTAGTCTTACCCGCTGTCCG
pGL3- $\Delta 6$ Fad-4 F	GGGGTACCCTTAAGTTGAAAACCCCAAATACATA
pGL3- $\Delta 6$ Fad-R	CCGCTCGAGATTTACTTCTGCACGCCGGAT
pCS-SREBP-ORF-F	CGGAATTCATGGCCGACCCGTTAAGTT
pCS-SREBP-ORF-R	CCGCTCGAGCTAACTACAAGTGACAGTAGATGCAGC
SREBP-D-F	CGGAATTCATGCAGCAGCACTCAACACC
SREBP-D-R	CCGCTCGAGCTATGGAGAGTTGGGTGGCG
W-F6-1F	AGGGAGGGTGGGGTAACTTAGTT
W-F6-1R	AACTAAGTTACCCACCCTCCCT
M-F6-1F	AGGGAAAGCGAGGCAGCTTAGTT
M-F6-1R	AACTAAGCTGCCTCGCTTTCCCT
W-F6-2F	GAGAAAGTAGGCCGGGAAGAC
W-F6-2R	GTCTTCCCGCTACTTTCTC
M-F6-2F	GAGAAAATAAACGAAAAGAC
M-F6-2R	GTCTTTCGTTATTTTCTC
W-F6-3F	ATATCATGTGGTTCTATGTCACGTAC
W-F6-3R	GTACGTGACATAGAACCACATGATAT
M-F6-3F	ATATCATGTGATTCTATGTCGTGCGC
M-F6-3R	GCGCACGACATAGAA TCACATGATAT

\* Restriction sites of *KpnI*, *XhoI* and *EcoRI* are underlined. The bolded letters in italics indicate the mutation sites.



Table 1) were designed using Primer 5 software. The PCR was carried out using LA Taq<sup>®</sup> Hot Star Version (TaKaRa). The resulting PCR products were purified, cloned into pMD<sup>™</sup> 18-T Vector (TaKaRa) and transformed into *E. coli* DH5 $\alpha$  competent cells. The recombinant single colonies successfully grown in Luria-Bertain (LB) plates containing ampicillin (50  $\mu$ g/ml) were selected, incubated and sequenced (BGI). Based on the verified fragment, gene-specific rapid amplification of cDNA ends (RACE) primers (GSP primers in Table 1) was designed to obtain the full length of *S. constricta* SREBP by two-round PCR using a SMARTer RACE 5'/3' kit (Clontech). The obtained PCR products were processed as described above. Finally, the full-length cDNA of *S. constricta* SREBP was obtained by aligning the first fragment with 5' and 3' RACE-PCR fragments.

Using the deduced aa of SREBP from *S. constricta* and representative mammals, fish and marine molluscs, multiple sequence alignment and phylogenetic tree were conducted using ClustalW 2.1<sup>(30)</sup> and MEGA 7<sup>(31)</sup>, respectively. The phylogenetic analysis was performed with the maximum-likelihood approach, and the confidence in the resulting phylogenetic tree branch topology was measured by bootstrapping through 1000 iterations.

#### Cloning and potential regulatory sites of *Sinonovacula constricta* $\Delta 6$ fatty acyl desaturase promoter

*S. constricta* genomic DNA was extracted from fresh foot muscle using a Marine Animal DNA Kit (CWBio) and used as a template for  $\Delta 6$  Fad promoter cloning. The 2000 bp upstream of  $\Delta 6$  Fad translation initiation codon (ATG) was obtained from the genomic sequencing data of *S. constricta*<sup>(32)</sup> by querying  $\Delta 6$  Fad cDNA (GenBank accession number MH220406). To further verify this promoter sequence, PCR was carried out by Mighty Amp<sup>™</sup> DNA Polymerase version 3 (TaKaRa) using specific primers ( $\Delta 6$  Fad promoter-V-F and -R in Table 1). Where necessary, the PCR products were subjected to the same processes as described above.

Online software JASPAR<sup>2020</sup> (<http://jaspar.genereg.net/>) and LASAGNA-Search 2.0 ([https://biogrid-lasagna.engr.uconn.edu/lasagna\\_search/](https://biogrid-lasagna.engr.uconn.edu/lasagna_search/)) were used to predict the potential transcription factor binding sites of SREBP on *S. constricta*  $\Delta 6$  Fad promoter. In addition, multiple-sequence alignment was performed with  $\Delta 6$  Fad promoters of *S. constricta* and some representative marine teleosts, including *Dicentrarchus labrax* (FP671139.1), *Epinephelus coioides*<sup>(22)</sup>, *Salmo salar* (AY736067.2), *Gadus morhua* (FJ859898.1) and *Siganus canaliculatus*<sup>(23)</sup>.

#### Construction of report vector and expression vector

To determine the core region of  $\Delta 6$  Fad promoter that interacts with SREBP, different forward primers harbouring restriction site *Kpn*I (underlined in Table 1) and a common reverse primer harbouring restriction site *Xho*I (underlined in Table 1) were designed using Primer 5 and used to obtain the full-length promoter fragment ( $\Delta 6$  Fad-2000) and truncated fragments ( $\Delta 6$  Fad-1389, -936, -463), respectively. The PCR was carried out using Mighty Amp<sup>™</sup> DNA Polymerase version 3 (TaKaRa). The resulting PCR products were purified, digested with the corresponding restriction endonucleases (New England BioLabs) and inserted into similarly digested firefly luciferase plasmids pGL3 basic (Promega). Subsequently, the recombinant plasmids pGL3- $\Delta 6$

Fad-2000, -1389, -936 and -463 were transformed into *E. coli* DH5 $\alpha$  competent cells, respectively. The recombinant single colonies successfully grown in LB plates containing ampicillin (50  $\mu$ g/ml) were selected, incubated and sequenced (BGI). The *E. coli* DH5 $\alpha$ -containing recombinant plasmids with correct sequence were further used to isolate the corresponding recombinant plasmids using Endo-free Plasmid Mini Kit I (Omega).

To obtain the expression vector, PCR fragments corresponding to the open reading frame of *S. constricta* SREBP were amplified from cDNA template by Mighty Amp<sup>™</sup> DNA Polymerase version 3 (TaKaRa) using specific primers harbouring restriction sites of *Eco*RI and *Xho*I (SREBP-ORF-F and -R in Table 1). The following operations were the same with the construction of the above reporter vector except that the vector was replaced with the expression vector of pCS2+ (Promega). Finally, the recombinant plasmids of pCS-SREBP were obtained.

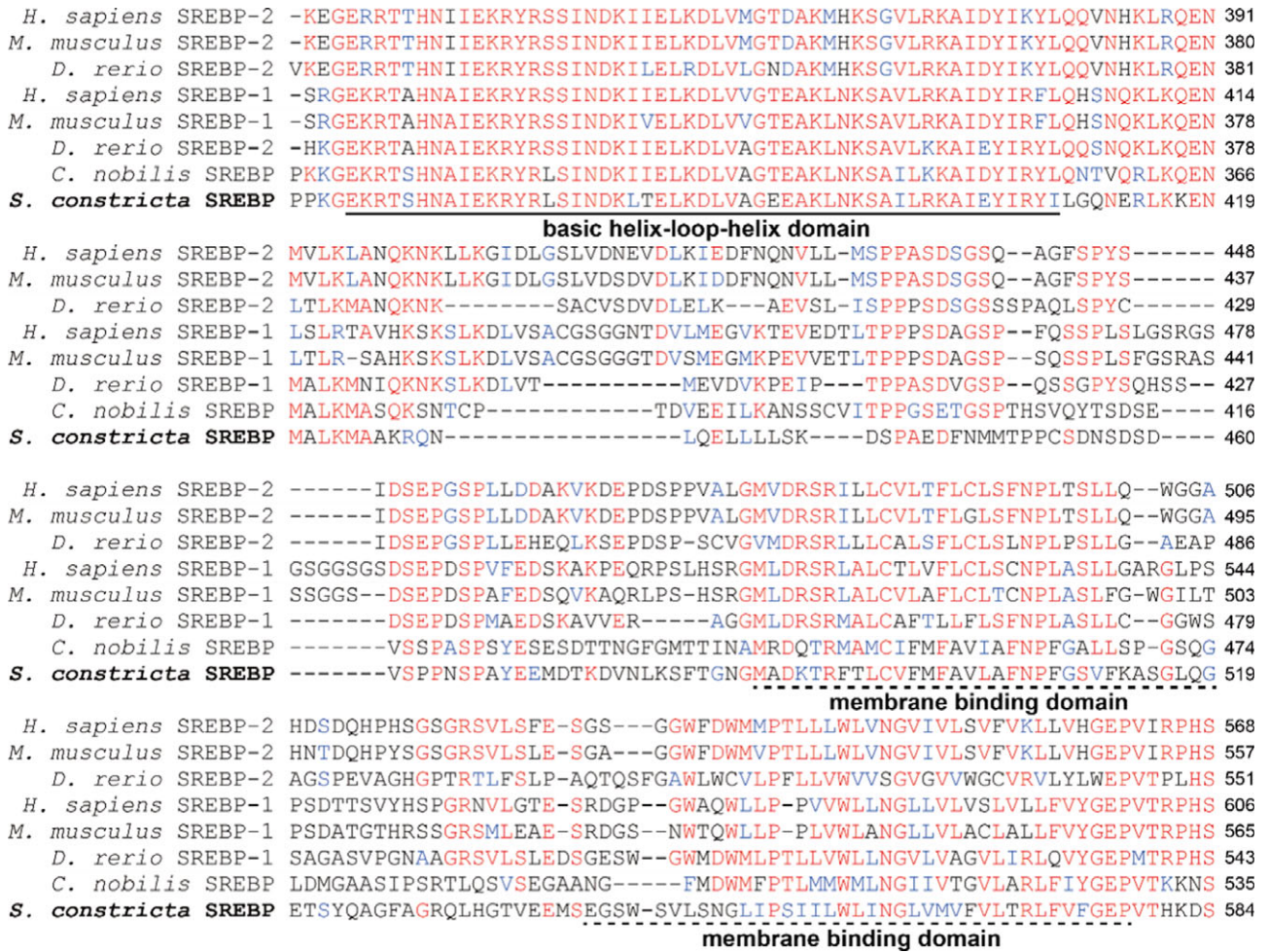
#### Dual luciferase assay

To confirm the core region of  $\Delta 6$  Fad promoter sufficient to initiate transcription by SREBP, dual luciferase assay was performed to detect the influence of the above reporter vectors on transcription activity. In brief, the human embryonic kidney cells (HEK 293 T) were seeded in ninety-six-well cell culture plates in 100  $\mu$ l high glucose Dulbecco's modified Eagle's medium (Transgen Biotech) with 10% fetal bovine serum (Transgen Biotech) per well at 37°C in a humidified incubator (ESCO). After growing 24 h to 90% confluent, the cultural medium was replaced with an Opti-MEM I Reduced Serum Medium (Gibco). Immediately, 50 ng of each reporter plasmids, 150 ng of expression plasmids and 5 ng of *Renilla luciferase* reporter plasmid PRL-CMV (Promega) in 10  $\mu$ l Opti-MEM I Reduced Serum Medium were co-transfected into cells using lipofectamine<sup>®</sup> 2000 Reagent (Invitrogen). Each trial was triplicated. At 6 h after transfection, the culture medium was replaced with fresh Dulbecco's modified Eagle's medium. At 24 h after transfection, the Firefly and *Renilla luciferase* activities were measured using Dual-Glo<sup>®</sup> Luciferase Assay System E2920 (Promega) by the variosk flash 3001 (Thermo). Finally, the relative luciferase activity was calculated using the ratio of firefly luciferase:*Renilla luciferase* and normalised by the control reporter.

#### Prokaryotic expression and purification of the truncated sterol regulatory element binding proteins

According to the function predication of *S. constricta* SREBP using online software of ExpASY-PROSITE (<https://prosite.expasy.org/>), the functional fragment of the SREBP N-terminal containing bHLH-Zip motif was identified (marked as SREBP-D) and used for prokaryotic expression. In brief, the corresponding cDNA sequence of SREBP-D was amplified by Mighty Amp<sup>™</sup> DNA Polymerase version 3 (TaKaRa) using specific primers harbouring restriction sites of *Eco*RI and *Xho*I (SREBP-D-F and -R in Table 1). The following operations were the same with the construction of the above reporter vector except that the vector was replaced with the expression vector of pET-28a (Novagen). The obtained recombinant plasmids of pET-SREBP-D were transformed into *Rosetta* (DE3) competent cells. Next, the recombinant cells were cultured in the LB medium containing ampicillin (50  $\mu$ g/ml) and chloramphenicol (34  $\mu$ g/ml) at 37°C.





**Fig. 1.** Partial peptide sequence alignment of sterol regulatory element binding proteins (SREBP) from *Sinovacula constricta* and representative organisms. Identical residues are shaded red, and similar residues are shaded blue. The solid line marked portion indicates the basic helix-loop-helix-leucine zipper structure, and the dotted line marked portion indicates the membrane binding domains. Representative species of SREBP include *Homo sapiens* SREBP-1 (NP\_001005291.1) and SREBP-2 (NP\_004590.2), *Mus musculus* SREBP-1 (NP\_035610.1) and SREBP-2 (NP\_150087.1), *Danio rerio* SREBP-1 (NP\_001098599.1) and SREBP-2 (NP\_001082935.1) and *Chlamydomonas reinhardtii* SREBP (AHB60716.1).

**Table 2.** Amino acid identity between *Sinovacula constricta* sterol regulatory element binding proteins (SREBP) and SREBP (-1/-2) of *Homo sapiens*, *Mus musculus*, *Danio rerio* or *Chlamydomonas reinhardtii* (Percentages)

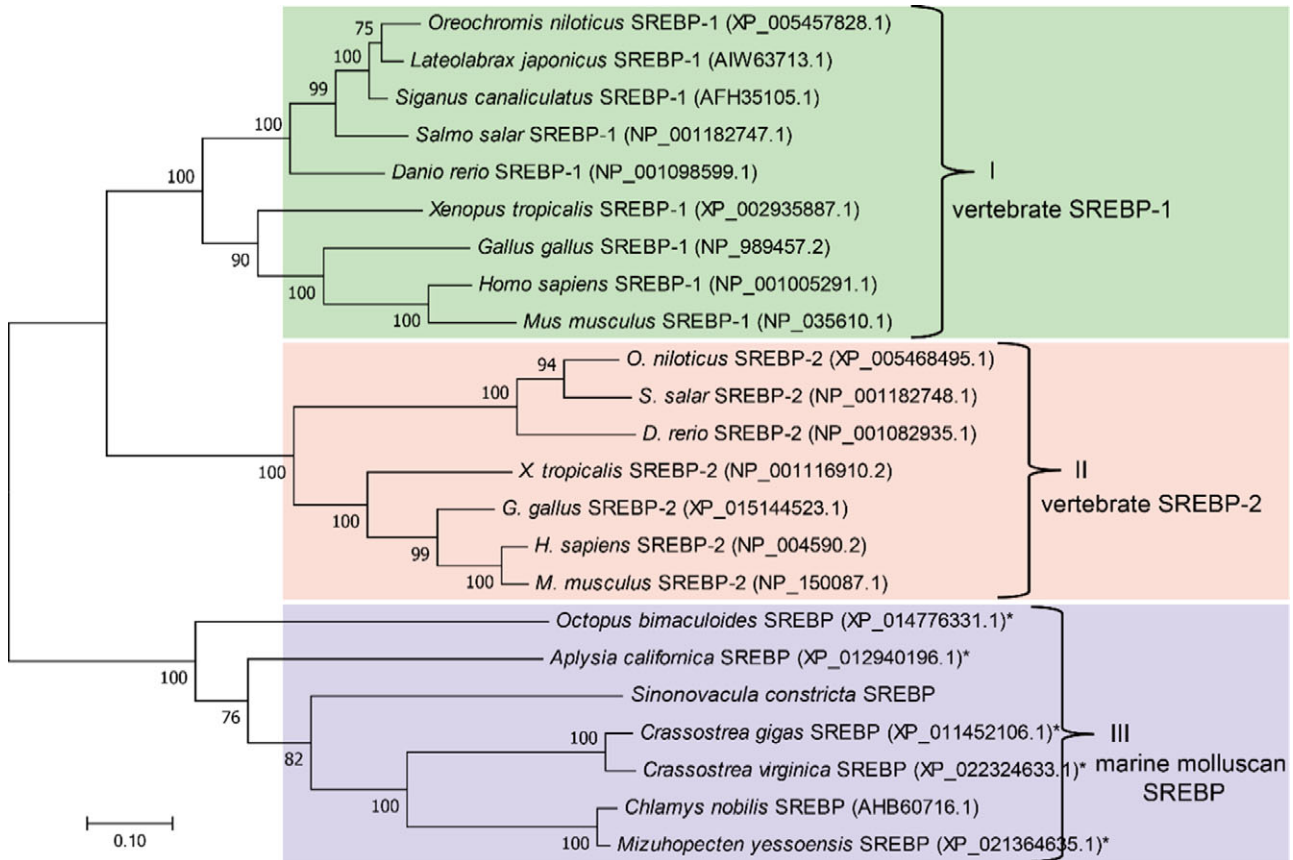
		Identity (%)
<i>S. constricta</i> SREBP	<i>H. sapiens</i> SREBP-1	38.63
	<i>H. sapiens</i> SREBP-2	36.24
	<i>M. musculus</i> SREBP-1	38.21
	<i>M. musculus</i> SREBP-2	35.60
	<i>D. rerio</i> SREBP-1	41.12
	<i>D. rerio</i> SREBP-2	33.16
	<i>C. nobilis</i> SREBP	49.83

When the bacteria grew to the mid-logarithmic phase, isopropyl thiogalactoside was added at a final concentration of 0.4 mM and incubation at 16°C for 12 h. Subsequently, the bacteria were collected by centrifugation at 8000 g at 4°C. The obtained cell pellet was sonicated and used for protein extraction using His60 Ni Gravity Columns (Clontech). The protein impurities and the

target protein were eluted with different concentrations of imidazole, and the eluted target protein was concentrated with an ultrafiltration centrifugal tube (UFC501096, Solarbio Science & Technology Co. Ltd). The concentrated SREBP-D was subjected to SDS-PAGE and confirmed by Western blot using Anti-6 × His rabbit polyclonal antibody (Sangon Biotech Co. Ltd), and its concentration was determined by the Enhanced BCA Protein Assay Kit (Beyotime Biotech Co. Ltd).

**Electrophoretic mobility shift assay**

The double-stranded 3'-biotinylated probes (50 nM) of the predicted binding sites (W primers in Table 1) were synthesised using an EMSA Probe Biotin Labeling Kit (Beyotime). Meanwhile, the corresponding unlabelled wild and site-direct mutant probes (10 μM) were synthesised (W and M primers in Table 1). Using Chemiluminescent EMSA Kit (Beyotime), the reaction mixture of above-mentioned SREBP-D (1.5 μg) and corresponding probes was incubated at 25°C for 30 min and then ran on a 6% non-denaturing polyacrylamide gel in an ice water



**Fig. 2.** Phylogenetic tree comparing the deduced amino acid sequences of sterol regulatory element binding proteins (SREBP) from *Sinonovacula constricta* (bold fonts) and representative organisms. The tree was constructed using the maximum-likelihood approach with MEGA 7. The horizontal branch length is proportional to amino acid substitution rate per site. The numbers represent the frequencies with which the tree topology presented was replicated after 1000 iterations. An asterisk indicates the predicted SREBP genes of *Crassostrea gigas* (genome ID: 10758), *Aplysia californica* (genome ID: 443), *Crassostrea virginica* (genome ID: 398), *Octopus bimaculoides* (genome ID: 41501) and *Mizuhopecten yessoensis* (genome ID: 12193) with complete genomes currently available.

bath. The target bands were then transferred to a positively charged nylon membrane (Beyotime). After being cross-linked by UV light and conjugated by Streptavidin-HRP, the results were detected by using BeyoECL Moon reagent (Beyotime).

### Statistical analyses

Statistical analyses of relative luciferase activity were conducted by one-way ANOVA, together with pairwise multiple comparisons by Newman-Keuls tests (SPSS 22.0). The values are represented as mean values and standard deviations, and  $P < 0.05$  was considered statistically significant.

## Results

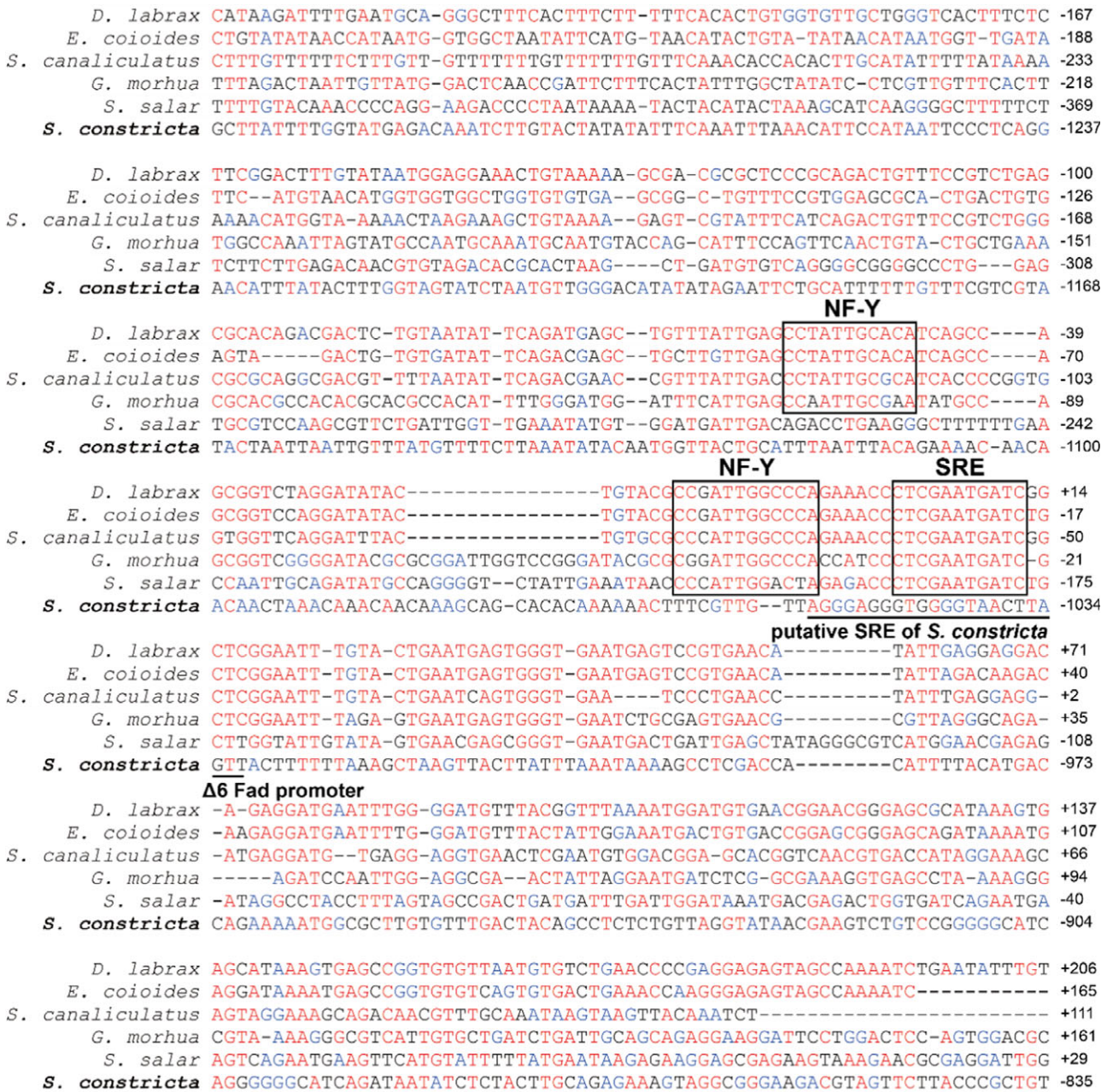
### Sequence and phylogenetics of *Sinonovacula constricta* sterol regulatory element binding proteins

The full length of mRNA transcript for *S. constricta* SREBP was 4222 bp, with a 5' untranslated region of 20 bp, an open reading frame (ORF) of 3438 bp and a 3' untranslated region of 764 bp. The ORF encoded a protein of 1146 aa, containing a basic bHLH-Zip motif and two membrane binding regions interrupted by 21 aa (Fig. 1). The detailed sequence information was

deposited in the GenBank database with accession number of MK584917. The aa sequence of *S. constricta* SREBP had the highest identity with that of *Chlamys nobilis* of 49.83%. Notably, compared with SREBP-2 (33.16–36.24%) of *Homo sapiens*, *Mus musculus* and *Danio rerio*, *S. constricta* SREBP protein exhibited relatively higher identities with their SREBP-1 (38.21–41.12%) (Table 2). The phylogenetic results showed that the vertebrate SREBP-1, SREBP-2 and marine molluscan SREBP were clustered into three separate groups, respectively (Fig. 2).

### Sequence and bioinformatics of *Sinonovacula constricta* $\Delta 6$ fatty acyl desaturase promoter

The detailed sequence information of the cloned 2000 bp of *S. constricta*  $\Delta 6$  Fad promoter was deposited in the GenBank database with accession number of MK584921. As shown in online Supplementary Fig. S1, three putative binding sites for SREBP on *S. constricta*  $\Delta 6$  Fad-2000 bp were detected by *in silico* analysis. Specifically, one binding site (5'-AGGGAGGGTGGGGTAAC TTAGTT-3') was aligned with the conserved SRE region of teleost Fads2 promoters (Fig. 3), while no feature was found in the distribution of the other two binding sites. In addition, no similar element with the two conserved NF-Y regions of teleost Fads2 promoters was predicted in the *S. constricta*  $\Delta 6$  Fad promoter (Fig. 3).



**Fig. 3.** Alignment of fatty acyl desaturase 2 (Fads2) promoter sequences from *Sinonovacula constricta* and representative marine teleosts including *Dicentrarchus labrax*, *Epinephelus coioides*, *Siganus canaliculatus*, *Gadus morhua* and *Salmo salar*. Identical residues are shaded red, and similar residues are shaded blue. The sequences are all numbered relative to the transcription start site. The conserved NF-Y and SRE regions of the teleost Fads2 promoters are shown in the frames, respectively. The putative SRE of *Sinonovacula constricta*  $\Delta 6$  Fad promoter is highlighted by a solid line.

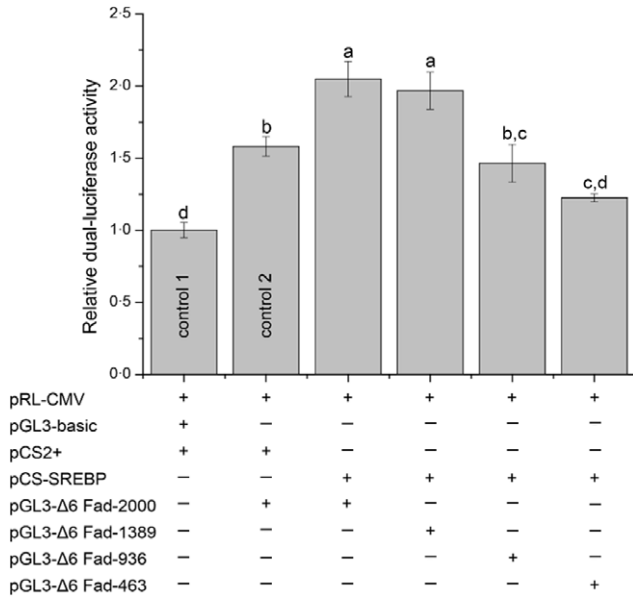
*Transactivation activities of 5'-truncated Sinonovacula constricta  $\Delta 6$  fatty acyl desaturase promoter by sterol regulatory element binding proteins*

When co-transfection with a report plasmid containing 5'-truncated *S. constricta*  $\Delta 6$  Fad promoter and an expression plasmid containing *S. constricta* SREBP in HEK 293 T cells, the maximal relative luciferase activity was detected in samples transfected with pGL3- $\Delta 6$  Fad-2000 or -1389 (Fig. 4). Transfection with pGL3- $\Delta 6$  Fad-936 significantly reduced the luciferase intensity. Transfection with additional deleted report vector of pGL3- $\Delta 6$

Fad-463 did not further reduce the transcriptional activity, which was comparable with the luciferase readout of the control transfected with empty report and expression plasmids.

*Binding activities of the truncated sterol regulatory element binding proteins on putative regulatory sites in  $\Delta 6$  fatty acyl desaturase promoter*

The truncated SREBP protein containing bHLH-Zip motif (Fig. 5(A)) was successfully expressed in *Rosetta* (DE3) cells and presented in the supernatant after ultrasonication (Fig. 5(B)). The purified



**Fig. 4.** Transcriptional activity of the segmentally truncated *Sinonovacula constricta*  $\Delta 6$  fatty acyl desaturase (Fad) promoter regulated by sterol regulatory element binding proteins (SREBP). pRL-CMV is the *Renilla luciferase* expression plasmid (internal reference); pGL3-basic, pGL3- $\Delta 6$  Fad-2000, -1389, -936 and -463 are the empty firefly luciferase reporter plasmid and the recombinant pGL3-basic plasmid inserted with 2000 bp, 1389 bp, 936 bp and 463 bp upstream of *S. constricta*  $\Delta 6$  Fad promoter, respectively. pCS2+ and pCS-SREBP are the empty protein expression vector plasmid and the recombinant pCS2+ plasmid inserted with the open reading frame (ORF) sequence of *S. constricta* SREBP, respectively. '+' and '-' indicate that the plasmid was transfected into the HEK 293T cells or not, respectively. Relative luciferase activity was expressed as mean values and standard deviations ( $n$  3), which was first calculated by the ratio of firefly luciferase activity: *Renilla luciferase* activity, and then normalised by the corresponding result of the control 1. Values sharing a common letter above the bar graph were not significantly different ( $P \geq 0.05$ ).

protein was further verified by Western blot (Fig. 5(C)) and subjected to EMSA analysis. Though three putative binding sites were predicted by bioinformatic analysis, only one sequence (5'-AGGGAGGGTGGGGTAACCTAGTT-3') was validated (Fig. 6). In brief, when only 3' biotin labelling sequence was added to the reaction, no blocked band was produced (Fig. 6 control). When 3' biotin labelling sequence was incubated with the truncated SREBP-D, an obviously band of DNA-protein complex was formed (Fig. 6 lane 1). The specificity of this binding was verified by adding wild or mutated sequences. Specifically, when incubated with the increasing amount of wild sequence, the intensity of this band was weakened and further disappeared (Fig. 6 lanes 2–4). In contrast, when incubated with excess mutated sequence, the intensity of this band was still very strong (Fig. 6 lanes 5–6) with minor damage, which probably due to excess mutations that hinder the binding of the labelling sequence to the protein.

## Discussion

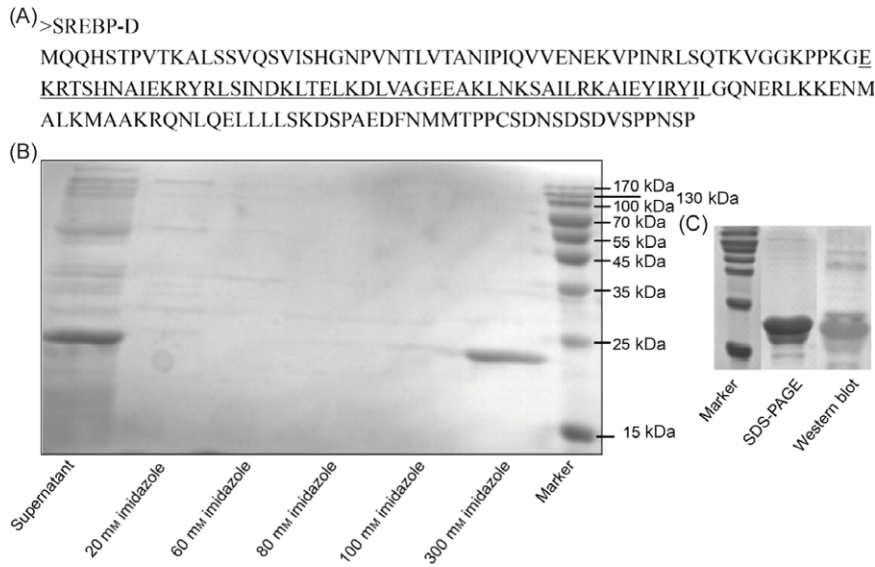
The newly cloned *S. constricta* SREBP contained a diagnostic bHLH-Zip domain and two interrupted membrane binding domains that conserved in typical vertebrate SREBP<sup>(1)</sup>, indicating that SREBP were highly conserved in functional regions during evolution. The phylogenetic tree analysis revealed that vertebrate

SREBP-1, SREBP-2 and marine molluscan SREBP were clearly clustered into three separate groups, indicating that they were significantly differentiated during species evolution. Interestingly, consistent with the finding that there was one SREBP in *C. nobilis*<sup>(33)</sup>, only one SREBP was detected in *S. constricta* and other five molluscs (*Crassostrea gigas*, *Aplysia californica*, *Crassostrea virginica*, *Octopus bimaculoides* and *Mizubopecten yessoensis*) by interrogating their genomes (Fig. 2). The result was different with the evidence of three SREBP (-1a, -1c and -2) in mammals<sup>(1)</sup> and two SREBP (-1 and -2) in teleosts<sup>(20,21,34)</sup>. This might be explained by that the invertebrates considered cannot biosynthesise sterol *de novo*, and the role of SREBP in regulating sterol metabolism might be subsequently acquired by gene duplication (producing SREBP-2) in chordates when diverged from arthropods and nematodes<sup>(35)</sup>. Consistently, Liu *et al.*<sup>(33)</sup> reported that *C. nobilis* SREBP shows more similarity with vertebrate SREBP-1 because its expression exhibits a positive correlation with the lipid content changes in ovary. However, in particular, the *de novo* sterol biosynthesis has been reported in some invertebrates, that is, echinoderms<sup>(36)</sup>. Therefore, whether the single SREBP found in invertebrates play both regulatory roles in sterol and fatty acid biosynthesis or not needs further investigation.

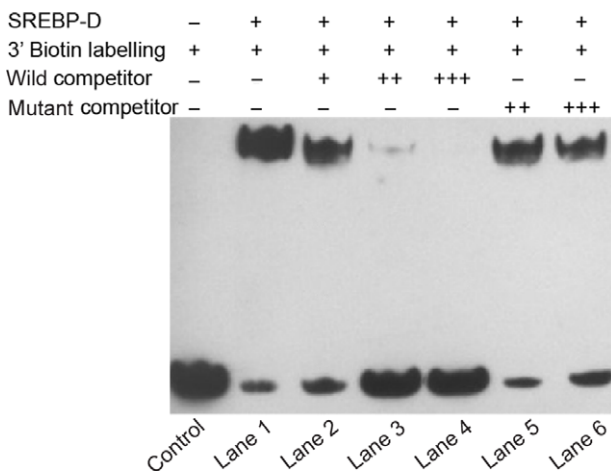
The transcription factor binding elements are usually distributed in the vicinity of gene promoter; thus, the 2000 bp upstream the translation initiation codon (ATG) of *S. constricta*  $\Delta 6$  Fad was cloned. Though very conservative binding sites of NF-Y and SRE exist in teleost Fads2 promoters, this was not the case with *S. constricta*  $\Delta 6$  Fad promoter. The result indicated that the DNA sequence of  $\Delta 6$  Fad promoters from teleosts and marine molluscs were differentiated during evolution, and the transcriptional regulation mechanism of SREBP on  $\Delta 6$  Fad might be very distinctive in *S. constricta*.

Overexpression of *S. constricta* SREBP in HEK 293 T cells induced a significantly higher promoter-driven luciferase activity, indicating that *S. constricta* SREBP was involved in the transcriptional regulation of  $\Delta 6$  Fad. Similar results had been found in vertebrates<sup>(17,20,21)</sup>. By 5'-deletion analysis of *S. constricta*  $\Delta 6$  Fad promoter, the region of 936–1389 bp upstream  $\Delta 6$  Fad promoter was predicted to be responsible for its sufficient transcriptional activation. Based on the *in silico* analysis of  $\Delta 6$  Fad promoter, a binding site was validated specifically to interact with SREBP-D protein by EMSA, which was exactly located in the core region identified above. Therefore, it was speculated that this binding site might be the potential element that acted by SREBP. Interestingly, though this site harbouring 5'-GTGGGGTAACT-3' was aligned in the same location with the conserved SRE (5'-CTCGAATGATC-3') of teleost Fads2 promoters, the base composition between them was obviously different excepted for the underlined four bases (T, G, T, A). It indicated that the conserved four bases might play a key role in the site recognition and binding on Fads2 promoter by SREBP. In addition, the predicted binding sequence was more identical to the classic SRE sequence (forward sequence 5'-ATCACCCAC-3', reverse sequence 5'-GTGGGGTGAT-3'), while the binding site of teleost Fads2 promoters was more similar with the modified SRE sequence of 5'-CTCACACGAG-3'<sup>(9,37)</sup>.





**Fig. 5.** Amino acid sequence (A), prokaryotic expression (B) and purified protein (C) of *Sinonovacula constricta* sterol regulatory element binding protein (SREBP)-D. The underlined portions are the bHLH-Zip motif of *S. constricta* SREBP.



**Fig. 6.** Binding activity of *Sinonovacula constricta* sterol regulatory element binding protein (SREBP)-D to the predicted regulatory site of  $\Delta 6$  fatty acyl desaturase (Fad) promoter. '+' and '-' indicate that the component was added or not, respectively. '+', '++' and '+++' mean that the amount of unlabelled wild or mutant competitor probe is 100-, 500- and 1000-fold, respectively, of that of the 3' biotin-labelling probe.

It indicated that the SRE sequence might be differentiated during species evolution.

Honestly, though no direct evidence of SREBP regulating  $\Delta 6$  Fad expression for LC-PUFA biosynthesis in *S. constricta* *in vivo*, some clues have been observed in our previous work. For example, the expressions of SREBP (not published) and  $\Delta 6$  Fad exhibit similar patterns in *S. constricta* larvae at early developmental stages and when fed with different microalgae of distinguished LC-PUFA composition<sup>(38)</sup>. Besides, an experiment of silencing SREBP in *S. constricta* has been in progress by our group, which should provide more direct evidence in the near future.

In conclusion, in the present study, the transcriptional activity of *S. constricta*  $\Delta 6$  Fad promoter was demonstrated to be

activated by SREBP, and the binding site of SREBP on the  $\Delta 6$  Fad promoter was predicted; thus, the potential molecular mechanism of SREBP regulating  $\Delta 6$  Fad expression in *S. constricta* was revealed. This was the first report on the regulatory mechanism of LC-PUFA biosynthesis in marine molluscs, which would facilitate optimising the LC-PUFA biosynthetic pathway of bivalves in further studies.

### Acknowledgements

This research was supported by the National Key Research and Development Program of China (2019YFD0900400), Zhejiang Major Science Project, China (2019C02057), Ningbo Science and Technology Research Projects, China (2019B10006), and the Earmarked Fund for Modern Agro-industry Technology Research System, China (CARS-49).

J. X., X. Y. and Z. R. designed the study. Z. R. and F. K. conducted the study and wrote the paper. Z. R. and K. L. analysed the data. All authors have read and approved the final manuscript.

The authors declare that there are no conflicts of interest.

### Supplementary material

For supplementary material referred to in this article, please visit <https://doi.org/10.1017/S0007114520002068>

### References

- Horton JD, Goldstein JL & Brown MS (2002) SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* **109**, 1125–1131.
- Yokoyama C, Wang X, Briggs MR, *et al.* (1993) SREBP-1, a basic helix-loop-helix leucine zipper protein that controls transcription of the LDL receptor gene. *Cell* **75**, 187–197.



3. Hua X, Wu J, Goldstein JL, *et al.* (1995) Structure of human gene encoding sterol regulatory element binding protein-1 (SREBF1) and localization of SREBF1 and SREBF2 to chromosomes 17p11.2 and 22q13. *Genomics* **25**, 667–673.
4. Shimano H, Horton JD, Shimomura I, *et al.* (1997) Isoform 1c of sterol regulatory element binding protein is less active than isoform 1a in livers of transgenic mice and in cultured cells. *J Clin Invest* **99**, 846–854.
5. Hua X, Yokoyama C, Wu J, *et al.* (1993) SREBP-2, a second basic-helix-loop-helix-leucine zipper protein that stimulates transcription by binding to a sterol regulatory element. *Proc Natl Acad Sci U S A* **90**, 11603–11607.
6. Pai JT, Guryev O, Brown MS, *et al.* (1998) Differential stimulation of cholesterol and unsaturated fatty acid biosynthesis in cells expressing individual nuclear sterol regulatory element-binding proteins. *J Biol Chem* **273**, 26138–26148.
7. Horton DJ, Shimomura L, Brown MS, *et al.* (1998) Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. *J Clin Invest* **101**, 2331–2339.
8. Horton JD, Shah NA, Warrington JA, *et al.* (2003) Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. *Proc Natl Acad Sci U S A* **100**, 12027–12032.
9. Shimano H (2001) Sterol regulatory element-binding proteins (SREBPs): transcriptional regulators of lipid synthetic genes. *Prog Lipid Res* **40**, 439–452.
10. McMurchie EJ (1988) Dietary lipids and the regulation of membrane fluidity and function. *Physiol Regul Membr Fluidity* **3**, 189–237.
11. Lauritzen L, Hansen HS, Jørgensen MH, *et al.* (2001) The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina. *Prog Lipid Res* **40**, 1–94.
12. Fritsche K (2006) Fatty acids as modulators of the immune response. *Annu Rev Nutr* **26**, 45–73.
13. Russo GL (2009) Dietary n-6 and n-3 polyunsaturated fatty acids: from biochemistry to clinical implications in cardiovascular prevention. *Biochem Pharmacol* **77**, 937–946.
14. Castro LFC, Tocher DR & Monroig O (2016) Long-chain polyunsaturated fatty acid biosynthesis in chordates: insights into the evolution of Fads and Elovl gene repertoire. *Prog Lipid Res* **62**, 25–40.
15. Voss A, Reinhart M, Sankar S, *et al.* (1991) The metabolism of 7, 10, 13, 16, 19-docosapentaenoic acid to 4, 7, 10, 13, 16, 19-docosahexaenoic acid in rat liver is independent of a 4-desaturase. *J Biol Chem* **266**, 19995–20000.
16. Matsuzaka T, Shimano H, Yahagi N, *et al.* (2002) Dual regulation of mouse  $\Delta 5$ - and  $\Delta 6$ -desaturase gene expression by SREBP-1 and PPAR $\alpha$ . *J Lipid Res* **43**, 107–114.
17. Nara TY, He WS, Tang C, *et al.* (2002) The E-box like sterol regulatory element mediates the suppression of human  $\Delta 6$  desaturase gene by highly unsaturated fatty acids. *Biochem Biophys Res Commun* **296**, 111–117.
18. Zheng XZ, Leaver MJ & Tocher DR (2009) Long-chain polyunsaturated fatty acid synthesis in fish: Comparative analysis of Atlantic salmon (*Salmo salar* L.) and Atlantic cod (*Gadus morhua* L.)  $\Delta 6$  fatty acyl desaturase gene promoters. *Comp Biochem Phys B Biochem Mol Biol* **154**, 255–263.
19. Geay F, Zambonino-Infante J, Reinhardt R, *et al.* (2012) Characteristics of fads2 gene expression and putative promoter in European sea bass (*Dicentrarchus labrax*): comparison with salmonid species and analysis of CpG methylation. *Mar Genomics* **5**, 7–13.
20. Dong XJ, Tan P, Cai ZN, *et al.* (2017) Regulation of FADS2 transcription by SREBP-1 and PPAR- $\alpha$  influences LC-PUFA biosynthesis in fish. *Sci Rep* **7**, 40024.
21. Tay SS, Kuah MK & Shu-Chien AC (2018) Transcriptional activation of zebrafish fads2 promoter and its transient transgene expression in yolk syncytial layer of zebrafish embryos. *Sci Rep* **8**, 3874.
22. Xie DZ, Fu ZX, Wang SQ, *et al.* (2018) Characteristics of the fads2 gene promoter in marine teleost *Epinephelus coioides* and role of Sp1-binding site in determining promoter activity. *Sci Rep* **8**, 5305.
23. Dong YW, Zhao JH, Chen JL, *et al.* (2018) Cloning and characterization of  $\Delta 6/\Delta 5$  fatty acyl desaturase (Fad) gene promoter in the marine teleost *Siganus canaliculatus*. *Gene* **647**, 174–180.
24. FAO (2018) *FAO Year Book. Fishery and Aquaculture Statistics, 2016*. Rome: Food and Agriculture Organization of the United Nations.
25. Ran ZS, Li S, Zhang RT, *et al.* (2017) Proximate, amino acid and lipid compositions in *Sinonovacula constricta* (Lamarck) reared at different salinities. *J Sci Food Agric* **97**, 4476–4483.
26. Ran ZS, Chen H, Ran Y, *et al.* (2017) Fatty acid and sterol changes in razor clam *Sinonovacula constricta* (Lamarck 1818) reared at different salinities. *Aquaculture* **473**, 493–500.
27. Ran ZS, Xu JL, Liao K, *et al.* (2018) Biosynthesis of polyunsaturated fatty acids in the razor clam *Sinonovacula constricta*: characterization of  $\Delta 5$  and  $\Delta 6$  fatty acid desaturases. *J Agric Food Chem* **66**, 4592–4601.
28. Ran ZS, Xu JL, Liao K, *et al.* (2019) Biosynthesis of long-chain polyunsaturated fatty acids in the razor clam *Sinonovacula constricta*: characterization of four fatty acyl elongases and a novel desaturase capacity. *Biochim Biophys Acta Mol Cell Biol Lipids* **1864**, 1083–1090.
29. Shimano H, Horton JD, Hammer RE, *et al.* (1996) Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. *J Clin Invest* **98**, 1575–1584.
30. Larkin MA, Blackshields G, Brown NP, *et al.* (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947–2948.
31. Kumar S, Stecher G & Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* **33**, 1870–1874.
32. Ran ZS, Li ZZ, Yan XJ, *et al.* (2019) Chromosome-level genome assembly of the razor clam *Sinonovacula constricta* (Lamarck, 1818). *Mol Ecol Resour* **19**, 1647–1658.
33. Liu HL, Zhang HK & Zheng HP (2018) Regulatory roles of sterol regulatory element-binding protein (SREBP) on lipid metabolism in marine invertebrate *Cblamys nobilis*. *Aquaculture* **493**, 251–257.
34. Minghetti M, Leaver MJ & Tocher DR (2011) Transcriptional control mechanisms of genes of lipid and fatty acid metabolism in the Atlantic salmon (*Salmo salar* L.) established cell line, SHK-1. *Biochim Biophys Acta Mol Cell Biol Lipids* **1811**, 194–202.
35. Rawson RB (2003) The SREBP pathway—insights from Insigns and insects. *Nat Rev Mol Cell Biol* **4**, 631–640.
36. Carson DD & Lennarz WJ (1979) Inhibition of polyisoprenoid and glycoprotein biosynthesis causes abnormal embryonic development. *Proc Natl Acad Sci U S A* **76**, 5709–5713.
37. Amemiya-Kudo M, Shimano H, Hasty AH, *et al.* (2002) Transcriptional activities of nuclear SREBP-1a, -1c, and -2 to different target promoters of lipogenic and cholesterogenic genes. *J Lipid Res* **43**, 1220–1235.
38. Ran ZS, Kong F, Xu JL, *et al.* (2020) Fad and Elovl expressions, fatty acid compositions, and feed effects of three representative microalgae in *Sinonovacula constricta* (Lamarck 1818) at early developmental stages. *Aquaculture* **521**, 735101.

