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A functional polymorphism in the promoter of RhoB is associated with susceptibility to *Vibrio anguillarum* in turbot (*Scophthalmus maximus*)

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ABSTRACT

As an isoform of Rho family GTPases, RhoB plays a pivotal role in cytoskeletal organization, cell proliferation, apoptosis and immune response. However, the regulatory mechanisms of RhoB expression in aquatic animals are still unknown. In the present study, we first construct *Vibrio anguillarum* infection model in *S. maximus*, including susceptible and resistant individuals. Then the temporal expression of RhoB was detected after *V. anguillarum* challenge using qRT-PCR and found that RhoB transcripts were significantly induced in the liver, gill and blood despite of differential expression levels and responsive time points. In addition, the mRNA levels of RhoB in resistant individuals were significantly higher than in susceptible ones. The length of 2083 bp sequences of RhoB promoter was cloned and characterized. Moreover, DNA methylation of the RhoB promoter was measured by bisulfite sequencing (BSP) and hypo-methylated was detected in the CpG islands. Three SNPs (−1590, −1575 and −1449) and two haplotypes in the promoter region of RhoB were identified to be associated with *V. anguillarum* resistance in turbot by association analysis in group 17-R and 17-S. Deletion analysis indicated that these SNPs could negatively mediate the activity of RhoB promoter. Site-directed mutagenesis and qRT-PCR of individuals with different genotypes demonstrated that −1575 T/A polymorphism affected promoter activity. Further study showed that this mutation altered the binding site of the transcription factor CREB. Co-transfection of *Sm*CREB and RhoB promoter was performed in HEK293T cells which confirmed the −1575 allelic differences on transcriptional activity, with the susceptibility allele showing reduced activity. Taken together, our findings implicate that losing of binding of CREB to *Sm*RhoB promoter due to −1575T/A polymorphisms enhances *Sm*RhoB expression in resistant turbot, which provide insights into the effect of *Sm*RhoB expression in response to *V. anguillarum* infection.

1. Introduction

As a member of the Rho subfamily of small GTPases, RhoB functions as a molecular switch in varieties of signal transduction pathways cycling between the active (GTP-bound form) and inactive (GDP-bound form) states. Although sharing an identity of 85% with other two members of Rho subfamily (RhoA and RhoC), they each play unique biological functions [1,2]. Unlike RhoA and RhoC distributing in the cytoplasm and cell membrane, RhoB expressed on endosomes, the plasma membrane and in the nucleus [3,4]. It has been well-established in many studies that expressions of RhoA and RhoC are up-regulated in malignant cells, which were involved in the aggressive development and metastasis of tumors [5]. In contrast, RhoB was found to be a negative regulator in suppressing the proliferation and inducing apoptosis of tumor cells [6,7]. Recently, increasing evidence suggested that RhoB plays a pivotal role in the immune and inflammatory responses. Significant changes were observed in cell shape as well as adhesive and migratory capacities after knock down of RhoB from mice macrophages [8]. RhoB also regulates the expression of intercellular adhesion molecule 1 (ICAM-1), interleukin 6 (IL-6) and interleukin 8 (IL-8), to mediate the trafficking of tumor necrosis factor alpha (TNF-α) receptor and the TNF-α-triggered proinflammatory response of vascular endothelial cells [9]. However, little is known about the expression response mechanism of RhoB in teleost after being challenged by various pathogens.

Expressions of genes are regulated at different stages ranging from transcription to post-translational modification of protein [10]. It contains the interaction of transcription factors with promoters (SNP or

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methylation), which influences accessibility of promoter sequences [11]. Miyamoto [12] demonstrated the polymorphism of SNP +104T/C in 5′UTR of growth differentiation factor 5 (GDF5) accounted for the differences on transcriptional activity in chondrogenic cells. Studies on functional analysis of RhoB promoter have been reported in mammals. Activating transcription factor 2 (ATF2) acted in concert with CCAAT-binding factors NF-YA in RhoB promoter region elevated RhoB expression upon genotoxic stress in NIH 3T3 fibroblast cells [13]. Therefore, it is worthwhile to investigate the regulation pattern of RhoB in the teleost innate immune response after pathogens infection.

Turbot (*Scophthalmus maximus*) has become the predominant aquaculture species since it was first introduced from Europe to China in 1990s. However, due to serious diseases under intensive cultivation, the turbot industry has been declining in recent years. Among these diseases, the vibriosis caused by *Vibrio anguillarum*, a Gram-negative bacterium, leads to high mortality and enormous economic losses [14,15]. Thus, it is very important to investigate the molecular mechanisms for transcriptional or post-transcriptional regulation of genes associated with *V. anguillarum* resistance. In this study, we examine the nucleotide polymorphisms and DNA methylation in the promoter region of turbot RhoB gene. Our results reveal that in *S. maximus* RhoB expression was not regulated by DNA methylation, but by a mutation in the promoter region that alters a cAMP-response element binding protein (CREB) binding site.

2. Materials and methods

2.1. Experimental animals

In 2017, 220 turbot (136 ± 17 g) were obtained from Haiyang Yellow Sea Aquatic Product CO., Ltd, Shandong, China. Fish were maintained at 19 °C for three days and then challenged in 2000 L aquaria with control group containing 10 individuals. Water was replaced once daily with continuous aeration.

All experiments in this study were conducted in accordance with the Institutional Animal and Use Committee of the Ocean University of China and the China Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training (State Science and Technology Commission of the People's Republic of China for No. 2, October 31, 1988. http://www.gov.cn/gongbao/content/2011/content_1860757.htm).

2.2. Vibrio infections and sample collection

A *V. anguillarum* strain LMG 4437(T), isolated from turbot and characterized to be pathogenic to *S. maximus*, was use in the challenge test. Before challenge, bacteria were inoculated in 2216E broth and incubated in a shaker (200 rpm) at 28 °C overnight, centrifuged and resuspended to a final concentration of 3×10^8 CFU (half lethal concentration) with Ringer's solution for marine teleost. During challenge, the treatment group was injected intraperitoneally with *V. anguillarum* suspension by weight (0.1 ml/100 g), whereas the control group were injected with Ringer's solution in same way. During 24 h after injection, all dying fish with typical *V. anguillarum* clinic signs were collected as susceptible fish. After seven days of the challenge, all surviving fish were collected as resistant fish. Control group was also collected at the same time. All 220 individuals were used for SNP genotyping. Among which, 192 individuals were divided into the resistant group (17-R, n = 96) and the susceptible group (17-S, n = 96) according to their survived time. Rest of the individuals were used for sampling at different hours after *V. anguillarum* challenge.

Tissues of dorsal muscle from each individual were sampled for genomic DNA preparation through phenol/chloroform procedure [16]. Nine tissues, including heart, liver, spleen, kidney, brain, gill, muscle, intestine and blood, were collected from three uninfected fish and the same tissues were homogenized in TRIzol reagent (Invitrogen, USA) to obtain the total RNA following the manufacturer's instructions. In addition, the liver, gill and blood samples were isolated from three injected fish at 0–72 h post infection (hpi) to analyze the response of RhoB gene to *V. anguillarum* infection. For gene expression comparison in resistant and susceptible group, the liver, spleen, kidney, gill, intestine and blood samples were collected from three resistant individuals and three susceptible individuals, respectively.

2.3. Single nucleotide polymorphism analysis

SNPs were predicted based on whole genomic sequencing data [17]. According to annotations, 5 SNPs located in the promoter region of *S. maximus* RhoB (*Sm*RhoB) were selected for association analysis and activity analysis. The selected SNPs were genotyped in 220 individuals using Fluidigm EP1 KASP (The Kompetitive Allele Specific PCR genotyping) system. Data were collected by software Fluidigm SNP Genotyping Analysis.

2.4. Cloning of RhoB promoter sequence and prediction of potential transcription factor binding sites (TFBS)

The promoter of *Sm*RhoB was amplified from turbot genomic DNA. A pair of primers (Table S1) was designed to amplify a 2.2-kb DNA fragment containing the region from −2083 to +152 bp. PCR products were subcloned into a pMD-19T vector (TaKaRa, China) and sequenced. The putative transcription factor binding sites in RhoB promoter were predicted with the online program MatInspector (http://www.genomatix.de/).

2.5. Bisulfite genomic sequencing

Genomic DNA was extracted from 8 susceptible and 8 resistant individuals, respectively. Equal amounts of DNA from each individual were mixed and then measured by NanoPhotometer Pearl for quality and concentration. Online software (http://www.urogene.org/methprimer/index1.html) was used to predict the CpG island of *Sm*RhoB promoter and primers were designed using MethPrimer. The mixed DNA was modified using the EZ DNA Methylation-Gold Kit (Zymo Research) and amplified by methylation-specific PCR with the two sets of primers shown in Table S1. Eight positive clones were sequenced for each group.

2.6. Plasmids construction

To examine the promoter activity of RhoB, different 5′ deletions of RhoB promoter sequence containing *Xho* I and *Hand* III restriction sites were amplified and inserted into the promoter-less vector pGL3-basic (Promega, USA). First, recombinant plasmid pGL3-2083 (fragment from −2083 to +152 bp far from the start codon "ATG") including complete RhoB potential promoter sequence was constructed and sequenced. Next, five truncated RhoB promoters (−1588/+152 bp, −1173/+152 bp, −798/+152 bp, −398/+152 bp, and −189/+152 bp) were generated by PCR using forward primers (which contain a *Xho* I site) Del 1, Del 2, Del 3, Del 4, Del 5 and reverse primer Del_rv (which contains a *Hand* III site) (Table S1), using pGL3-2083 as template. The recombinant constructs were named pGL3-1588, pGL3-1173, pGL3-798, pGL3-398 and pGL3-189, respectively.

To examine the function of CREB in regulating RhoB promoter, a *Sm*CREB expression plasmid was constructed. After Blast and sequencing alignment, the ORF of *S. maximus* CREB (*Sm*CREB) was cloned by PCR using specific primers *Sm*CREB-pEGFP-N1 Fw and *Sm*CREB-pEGFP-N1 Rv. Next, the PCR products were inserted into the expression vector pEGFP-N1 (Invitrogen, USA) by restriction enzyme *Xho* I and *Hand* III.

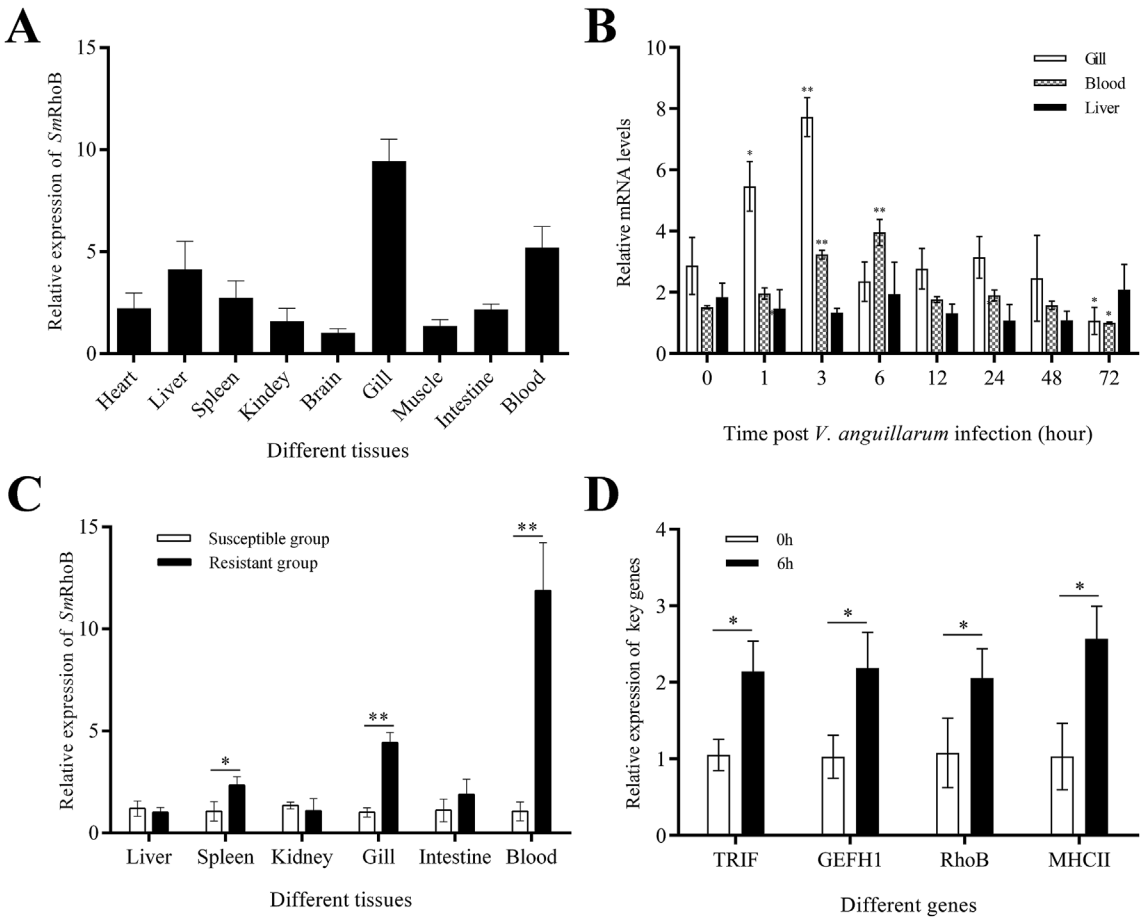

Fig. 1. Expression profiles of *Sm*RhoB transcription. (A) Distribution of *Sm*RhoB in different tissues. (B) Expression patterns of *Sm*RhoB mRNA after *V. anguillarum* infection in gill, blood and liver. (C) Relative mRNA levels of *Sm*RhoB in susceptible and resistant groups upon *V. anguillarum* challenge. (D) Temporal expression of key genes in TRIF-GEFH1-RhoB pathways after challenge with *V. anguillarum*. *β*-actin gene was used as an internal control. The variation of this expression was represented as a ratio (the amount of *Sm*RhoB mRNA normalized to the corresponding reference gene value). Data are shown as mean ± SD (n = 3). Asterisks '**' and '*' indicate statistical significance of $P < 0.01$ and $P < 0.05$, respectively.

2.7. Transfection and dual-luciferase assay

Since the deficiency of available cell lines for *S. maximus*, HEK293T cell was used to perform luciferase reporter assays. Approximately 2×10^5 cells were seeded onto 24-well plates (NEST Biotechnology) and cultured to more than 70% confluence, followed by plasmid transfection using LipoGene™ 2000 PLus Transfection Reagent (US Everbright® Inc., Suzhou, China) according to the manufacturer's instructions.

For luciferase reporter assays, pRL-TK vector (20 ng/μl) was transiently co-transfected with 5′ deletion plasmids (500 ng/well) into HEK293T cells to normalize the variation of transfection efficiency. At 48 h after transfection, luciferase activity of total cell lysates was measured using Modulus™ single tube multimode reader (TurnerBioSystems, USA) according to the manufacturer's instructions. Each experiment was repeated at least six times and the average value was calculated.

2.8. Site-directed mutagenesis

Mutations on SNP locations were introduced with the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies) into the wild-type pGL3-2083 constructs. Transcription factor binding sites predicted to be changed by SNPs were chosen for site-directed mutagenesis. Online software Agilent QuickChange primer design program (http://www.genomics.agilent.com/en/home.jsp) was used to design specific mutagenic primers. After sequence verification, mutant constructs were transferred into HEK293T cells and luciferase activities were measured as described above.

2.9. Overexpression of SmCREB

To investigate whether or not *Sm*CREB regulated the *Sm*RhoB promoter, wildtype (WT-pGL3-1575) and mutant construct (MU-pGL3-1575) was co-transfected with pEGFP-N1-CREB into HEK293T cells, respectively. The protocols for cell culture, transient transfection and dual-luciferase reporter assay are as described in section 2.7.

2.10. Quantitative real-time PCR (qRT-PCR) to measure RhoB mRNA

Total RNA was reverse transcribed using the Reverse Transcriptase M-MLV System (TaKaRa, China) according to the manufacturer's protocol. *β*-actin served as an internal reference gene. qRT-PCR was conducted on Roche LightCycler 480 using the 10 μl SYBR qPCR SuperMix (Novoprotein, Shanghai, China). Three technical replicates were set for each sample and the relative expression level of RhoB and *β*-actin was calculated with formula $2^{-\Delta\Delta Ct}$ [18].

2.11. Statistical analysis

The data of luciferase assays and qRT-PCR were statistically analyzed using one-way ANOVA followed by SPSS 20.0. Significance was

set at $P < 0.05$. All data were expressed as the mean ± standard deviation.

Allele frequencies of each SNP locus from stock 17 were calculated. *V. anguillarum*-resistant association analysis was conducted by the Chi-square using SPSS 20.0. *P*-value < 0.05 was regarded as significant. Haploview software package 4.2 was used to calculate linkage disequilibrium (LD) pattern and haplotype structure of *Sm*RhoB. Haplotypes and their frequencies were established and analyzed by the same software.

3. Results

3.1. SmRhoB expression in response to V. anguillarum challenge

The distribution of *S. maximus* RhoB mRNA in different tissues was investigated using qRT-PCR. The transcripts of *Sm*RhoB were ubiquitously observed in nine tested tissues including heart, liver, spleen, kidney, brain, gill, muscle, intestine and blood. As shown in Fig. 1A, *Sm*RhoB was highly expressed in gill and blood, followed by the expression in liver, spleen, heart and intestine. The lowest expression level was detected in brain.

The expression patterns of *Sm*RhoB in three immune tissues (gill, blood and liver) were investigated at 0, 1, 3, 6, 12, 24, 48 and 72 hpi. In Fig. 1B, a gradual increase of gill *Sm*RhoB mRNA expression was observed from 1 h to 3 hpi, and peaked at 3 hpi with the highest value of 2.69-fold greater than that of the control ($P < 0.05$), followed by a sharp decrease from 6 hpi to 72 hpi. In blood, the up-regulation of *Sm*RhoB mRNA expression reached a peak level at 6 hpi with the highest value of 2.61-fold greater than that of the control ($P < 0.05$), and decreased significantly from 12 hpi to 72 hpi. In contrast, no significant difference in *Sm*RhoB expression level was observed in liver.

Moreover, the mRNA expression profiles of *Sm*RhoB in different organs of susceptible and resistant individuals were detected. As shown in Fig. 1C, compared with susceptible individuals, *Sm*RhoB transcripts were significantly higher in blood, gill and spleen in resistant individuals ($P < 0.05$). In blood, difference of *Sm*RhoB between the two groups reached upon to 11.10-fold. These results indicated that *Sm*RhoB was up-regulated in immune organs in resistant group after *V. anguillarum* challenge.

At last, the expression level of key genes in TRIF-GEFH1-RhoB pathway were also examined. Similar to the results of Hokuto [19], temporal expressions of TRIF, GEFH1, RhoB and MHCIIα were highly induced after 6 h of *V. anguillarum* infection ($P < 0.05$).

3.2. Identification of SNPs in the SmRhoB promoter and association analysis against V. anguillarum

In this study, 5 SNPs were discovered in the DNA sequence spanned 2083 bp of the *Sm*RhoB promoter. These SNPs were genotyped in 220 individuals of population 17 by KASP system. After general statistical analysis and Chi-square test, all the five SNPs were common (MAF > 5%) and applied to association analysis after HWE analysis (Table 1). The location of each SNP was named relative to the start codon (ATG).

Table 1
Polymorphism of SNPs in promoter of *Sm*RhoB in population 17 of *S. maximus*.

Code	Location	SNP	Number of individuals	MAF	Test for HWE Chi-Square	DF	Pr > ChiSq
1	−1929	A > T	218	0.357	2.538	1	0.111
2	−1590	T > C	217	0.384	2.769	1	0.096
3	−1575	T > A	217	0.462	3.748	1	0.053
4	−1449	G > A	218	0.407	2.359	1	0.125
5	−1318	A > C	217	0.475	2.127	1	0.145

MAF: minor allele frequency; HWE: HardyeWeinberg equilibrium.

In total, 192 individuals from susceptible and resistant group (17-S: n = 96; 17-R: n = 96) were used for association analysis with selected SNPs. As shown in Table 2, SNPs −1575 and −1449 were associated with *V. anguillarum* resistance both in genotypes and alleles. While, SNP -1590 was only associated in alleles. Data in linkage disequilibrium (LD) pattern suggested that SNP -1575 and −1449 were in strong LD (D' = 0.93) (Fig. 2). Three haplotypes developed from the two significant SNPs with a cumulative frequency of 100%. The association between the haplotypes and *V. anguillarum* resistance was also investigated (Table 3). The results revealed that Hap1 (TG) was significantly underrepresented in the 17-S group and could be protective, the haplotype specific score value was 7.833 ($P = 0.005$). In contrast, Hap2 (AA) was significantly underrepresented in the 17-R group and could increase the risk of *V. anguillarum* infection, with a haplotype-specific score of 3.912 ($P = 0.048$).

3.3. Identification of the SmRhoB core promoter region

A partial *Sm*RhoB gene fragment was amplified and confirmed by Sanger sequencing. To investigate the core region of *Sm*RhoB promoter, 5 deletion plasmids were constructed and their activity was reflected by dual-luciferase assay (Fig. 3). The deletion fragment vector pGL3-1173 had the highest relative luciferase activity, whereas deletion of the sequences from −398 to −189 bp significantly decreased the relative luciferase activity. The deletion of the sequences from −2083 to −1173 bp significantly enhanced the relative luciferase activity, suggesting that all 5 SNPs were in negative transcriptional regulatory region.

3.4. Comparison of SmRhoB promoter methylation between susceptible and resistant turbot

A CpG island located in the −1252 to −1383 region was predicted in the *Sm*RhoB promoter which included 12 CpG sites (Fig. 4). The methylation levels in the *Sm*RhoB promoter of susceptible and resistant turbot infected with *V. anguillarum* were detected by bisulfite DNA sequencing. No significant differences of methylation in *Sm*RhoB promoter was observed in the susceptible and resistant individuals indicating that the *Sm*RhoB promoter was hypomethylated. Therefore, promoter methylation was not involved in the effect of *Sm*RhoB expression during *V. anguillarum* challenge in *S. maximus*.

3.5. Transcription factors binding to the SNP loci on SmRhoB promoter

A number of potential cis-acting regulatory elements at SNP loci was identified in *Sm*RhoB promoter using Genomatix. Among them, we found that two allelic variations (−1929A/T and −1575T/A) could alter transcription factor Jun-B and CREB binding site, respectively (Fig. 5A/B). Then, site-mutation analysis of SNP -1929 and −1575 in *Sm*RhoB promoter was performed. Compared with the wild type pGl3-2083/+152 of RhoB vectors, the relative luciferase activities were significantly reduced after the mutation of A-1929T and T-1575A, respectively (Fig. 5C). After double mutations of both sites, the relative luciferase activity of mutant vector was almost lost and showed no significant difference with the control vector pGL3-Basic. The expression levels of RhoB in turbot with different genotypes at loci −1929 and −1575 were detected through qRT-PCR (Fig. 5D/E). Significant differences were found in three genotypes at loci −1575. Contrarily, no obvious difference was observed at loci −1929. These results suggested that the promoter polymorphism of SNP -1575 regulates RhoB expression by altering the transcription factor binding site. The mechanism of SNP -1929 affecting the promoter activity remains to be further confirmed.

Next, to further investigate the effect of *Sm*CREB over-expression on RhoB transcriptional activity, the *Sm*CREB constructs were co-

Table 2
Association analysis of single SNPs associated with *V. anguillarum*-resistance.

Code	SNP	Genotype	Count (frequence)		ChiSq genotype	ChiSq Allele	*P* value	
	position		17-S	17-R			ProbGenotype	ProbAllele
1	−1929	AA	18 (0.188)	25 (0.26)	1.476	1.071	0.478	0.301
		AT	39 (0.406)	35 (0.365)				
		TT	39 (0.406)	36 (0.375)				
2	−1590	TT	29 (0.305)	39 (0.411)	4.434	0.109	4.738	0.03*
		TC	40 (0.421)	41 (0.432)				
		CC	26 (0.274)	15 (0.158)				
3	−1575	TT	21 (0.223)	35 (0.368)	6.092	6.096	0.048*	0.014*
		TA	48 (0.511)	45 (0.474)				
		AA	25 (0.266)	15 (0.158)				
4	−1449	GG	12 (0.126)	24 (0.255)	7.046	7.935	0.03*	0.005**
		GA	38 (0.4)	40 (0.426)				
		AA	45 (0.474)	30 (0.319)				
5	−1318	AA	28 (0.295)	27 (0.284)	2.971	1.275	0.226	0.259
		AC	47 (0.495)	38 (0.4)				
		CC	20 (0.211)	30 (0.316)				

Superscript (*) and (**) indicate significant difference at the $P < 0.05$ and $P < 0.01$ level, respectively.

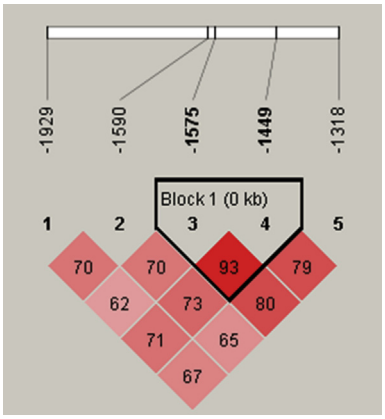

Fig. 2. LD structure among the 5 *Sm*RhoB SNPs in 17-R and 17-S groups of *S. maximus*. One LD blocks are circled, which were derived using Haploview 4.2 software. The numbers in red boxes represent the values of the D'. The D′ is a measure of LD which is the correlation coefficient between pairs of SNP loci and the number is absent when the value equals 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

transfected with wildtype and mutant vector of loci −1575, respectively. The results showed that over-expression of mutant allele −1575A of the transcription factor *Sm*CREB could decrease the promoter activities of RhoB gene (Fig. 6). However, no change was detected in the promoter activities of RhoB gene with the wildtype allele −1575T. The results thus suggest that *Sm*CREB could suppress RhoB gene expression through the promoter polymorphism.

4. Discussion

Although the association with *V. anguillarum* resistance of a SNP in the promoter region of *S. maximus* RhoB gene were detected and confirmed in our previous study [17], the underlying transcriptional regulatory mechanisms of RhoB promoter were still unknown. As a continuation of our study series involved in the SNP detection and functional analysis of RhoB gene in turbot, the present study cloned and characterized the promoter sequences of *Sm*RhoB and explored its functions.

4.1. Expression profiles of SmRhoB

Previously studies on vertebrate demonstrated that RhoB was ubiquitously expressed in various organs, including the liver, muscle, kidney, spleen and gill [20,21]. Our qRT-PCR results showed that *Sm*RhoB was broadly expressed in all examined tissues (Fig. 1A), indicating that RhoB is a widely distributed Rho GTPases in teleost. Interestingly, RhoB exhibited the highest expression level in gill, blood and liver which were all immunological organ that might be involved in the host's defensive response against *V. anguillarum*. Studies by Tan et al. [20] suggested that RhoB expression was higher in the gill and skin of channel catfish (*Ictalurus punctatus*). As is all known, the fish mucosal surfaces, constituted by gill, skin and intestine, serve as the first line of immune defence response to various pathogen infections in aquatic environments [22]. Thus, the high expression of *Sm*RhoB in immune related organs suggests that *Sm*RhoB may play an important role in the innate immune system of turbot.

An *in vivo* experiment was performed to characterize the innate immune responses of *Sm*RhoB in *S. maximus* after *V. anguillarum* infection. Temporal expression analysis showed an induction of the RhoB transcripts in response to *V. anguillarum* challenge, despite the different mRNA expression levels and time points of response in gill and blood

Table 3
Association between *Sm*RhoB haplotypes and *V. anguillarum*-resistance in 17-R and 17-S[a].

Variables	Haplotype	Frequency			Chi square values	*P* value	OR (95%CI)
		All	17-S	17-R			
Hap1	TG	0.499	79 (0.429)	108 (0.574)	7.833	0.005**	1.794 (1.190, 2.706)
Hap2	AA	0.28	60 (0.326)	44 (0.234)	3.912	0.048*	0.631 (0.400, 0.997)
Hap 3	AG	0.221	45 (0.245)	36 (0.191)	1.538	0.215	0.732 (0.446, 1.200)

[a] Haplotypes with frequencies < 0.05 were not included in the table; OR: odds ratio; CI: 95% percent confidence interval; Superscript (*) and (**) indicate significant difference at the $P < 0.05$ and $P < 0.01$ level, respectively.

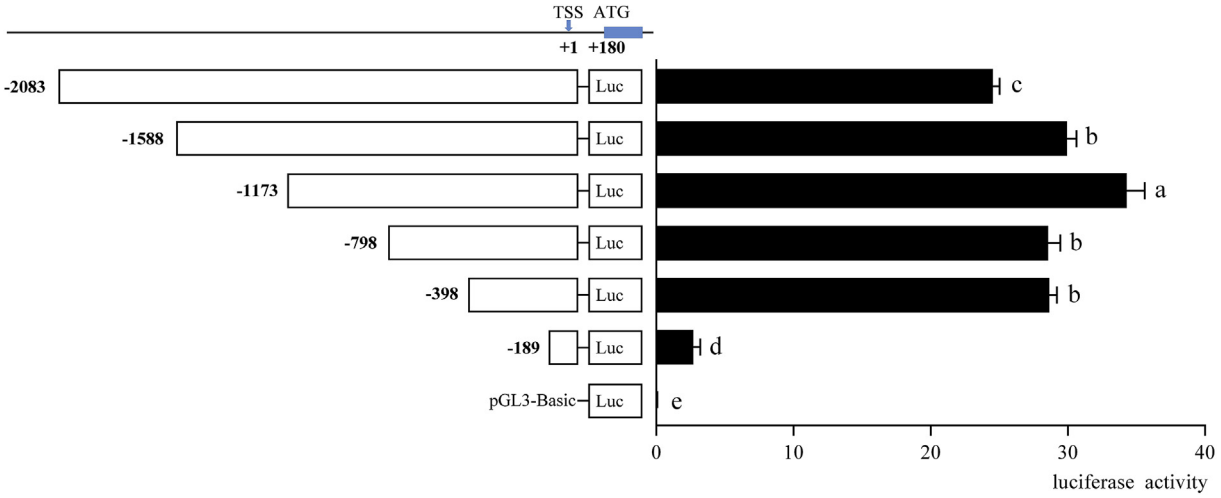

Fig. 3. The results of 5′ unidirectional deletion analysis of the *Sm*RhoB promoter region for *S. maximus*. Schematic diagram of truncated promoters is shown in the left panel. Positions of the transcription start site (TSS) (+1 bp) and the start codon "ATG" are depicted. A series of plasmids containing 5′ unidirectional deletions of the RhoB promoter region (pGl3-2083, −1588, −1173, −798, −398, −189 and pGl3-basic) and the internal control pRL-TK were co-transfected into HEK293T cells. Luciferase activities were assayed 48 h after transfection and the ratio between firefly and Renilla luciferase activities were calculated. Data are shown as mean ± SEM of six biological replicates. Values with different letters indicate statistical significance ($P < 0.05$).

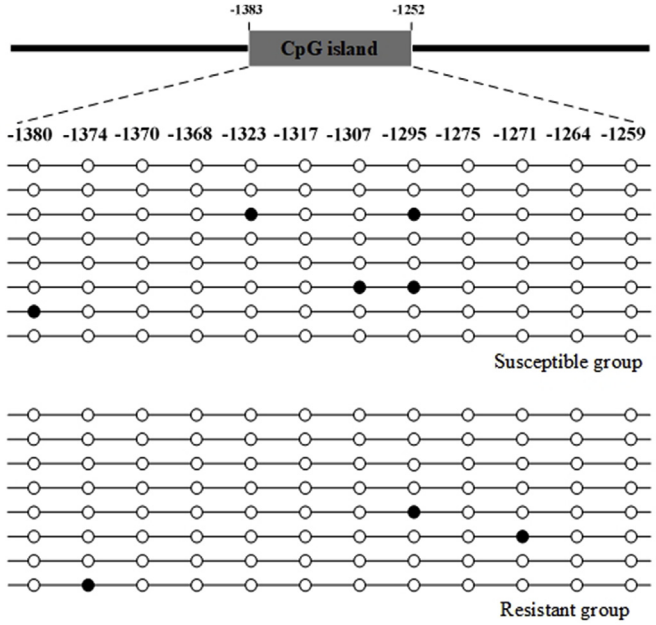

Fig. 4. Methylation levels of the *Sm*RhoB promoter in turbot challenged with *V. anguillarum*. Bisulfite sequencing analysis was performed to determine the DNA methylation profile of individual CpG sites. Open and filled circles represent unmethylated or methylated positions, respectively. Eight independent clones were sequenced in each group and each row of circles is the data from a single clone.

(Fig. 1B). This observation was in consistence with the results of RNA-Seq analysis in channel catfish gill tissue, in which RhoB was predominantly elevated at 4 h post-infection with *Flavobacterium columnare* [20]. In mammals, RhoB was significantly up-regulated after 4 h in human colonic CaCo-2 cells after the treatment with exotoxin of *Clostridium difficile* [23]. RhoB is the key mediators of diverse processes in regulating cell migration, proliferation and inflammation [24]. Studies conducted by Huang et al. [25] demonstrated that RhoB promoted the production of proinflammatory cytokines, including IL-1β, IL-6 and TNF-α, through activate NF-κB signaling and enhance macrophage adhesion which sustained the inflammation. Another study in TLR-stimulated macrophages found that RhoB could activate NF-κB signaling by interact with the tyrosine kinase Btk at early time of response [26]. In turbot, RhoB is intensely activated in gill at 1 hpi and in blood at 3 hpi, revealing that immune inflammatory response occurs at the early stage of *V. anguillarum* stimulation. Moreover, the mRNA level of RhoB in resistant group was extremely higher than in susceptible group in blood, gill and spleen after *V. anguillarum* challenge (Fig. 1C). Similar results were observed in the expression profile of c-type lysozyme in resistant stock and susceptible stock of Japanese flounder (*Paralichthys olivaceus*) upon *Listonella anguillarum* stimulation [27]. The c-type lysozyme was certified to have anti-bacteria activity in fish and shrimp [28–30]. It has been indicated that the activation of TRIF-GEFH1-RhoB pathway of LPS in dendritic cells will rise the activation of $CD4^+$ T cells in immune responses [19]. After detecting the transcripts of key genes in TRIF-GEFH1-RhoB pathway at 6 h upon *V. anguillarum* infection, significant inductions were observed in TRIF, GEFH1, RhoB and MHCIIα. All these findings suggested that RhoB gene in *S. maximus* was involved in immune response challenged with *V. anguillarum*.

4.2. SmRhoB is not regulated by methylation

Studies have shown that methylation in promoter region can significantly suppress the expression of target genes [31]. In this study, one CpG islands were predicted to be in the RhoB promoter from −1432 to −1563. However, no methylation changes was identified in the RhoB promoter region between susceptible and resistant group, implying that DNA methylation in promoter is not involved in the regulation of RhoB expression.

4.3. SNPs associated with V. anguillarum resistance

Mutations in promoter region can regulate mRNA transcription by the binding of transcription factors. It is reported that SNPs in promoter region were associated with diseases in mammals [32,33]. Evidences were also found in aquatic animals. The −404 indel in the promoter of serine protease inhibitor caused increased transcription and confers *Perkinsus marinus* resistance in the eastern oyster (*Crassostrea virginica*) [34]. In *Chlamys farreri*, the polymorphism of two SNP, −753 and −391, in the promoter of lysozyme gene were related with resistance to *L. anguillarum* [35]. In the present study, based on groups with different levels of *V. anguillarum* resistance, five SNPs were found in the promoter

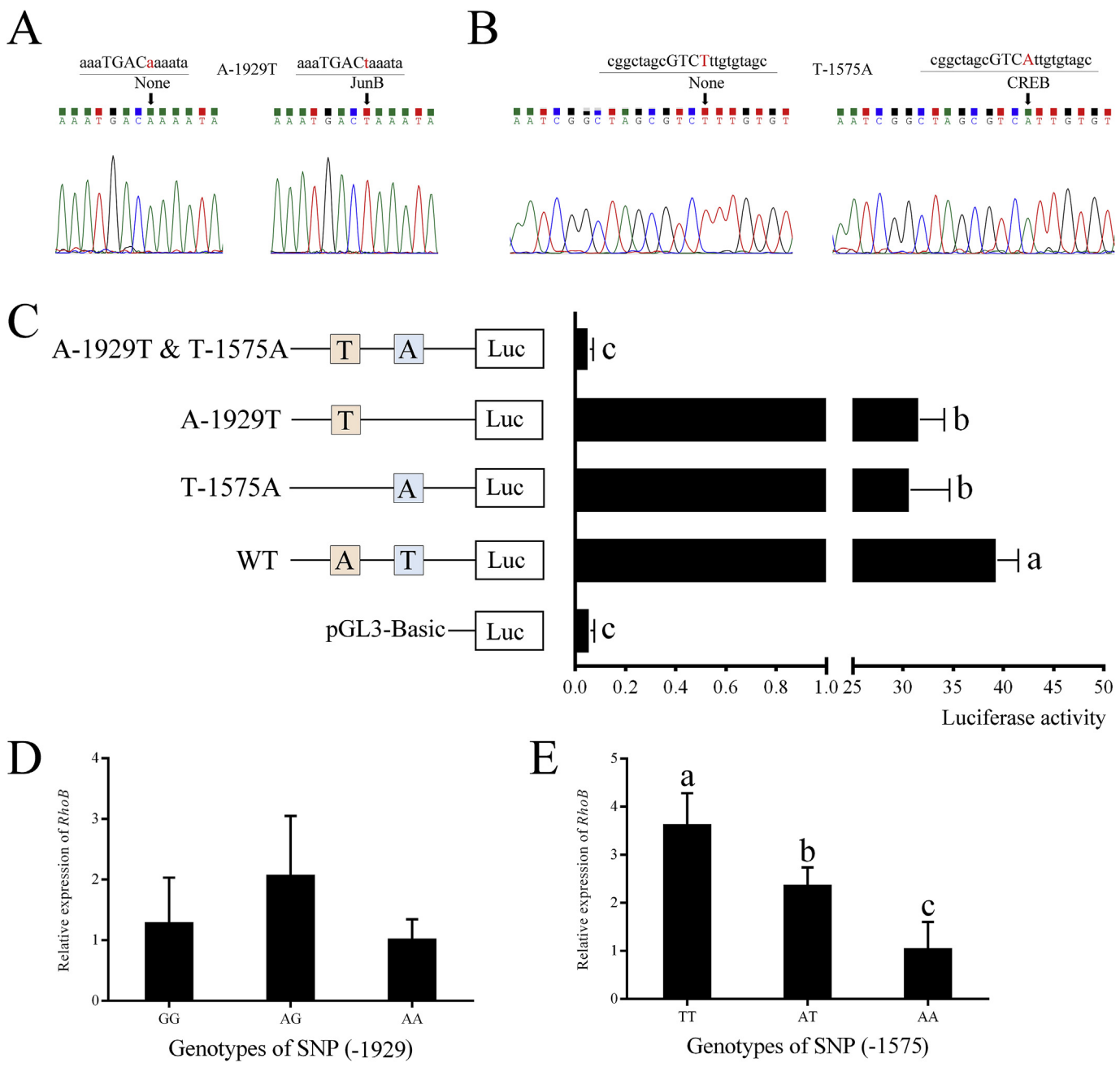

Fig. 5. Site-directed mutagenesis analysis of *Sm*RhoB promoter at locus -1929 and -1575. (A, B) Allelic variations −1929A/T and −1575T/A alter the JunB and CREB binding sites, respectively. The mutant allele −1929T and −1575A have JunB and CREB transcription factor binding sites, respectively. (C) Activity analysis of RhoB promoter containing either the WT or MU nucleotide at locus −1929A/T and −1575T/A. Each value represents the mean ± SEM of six replicates. (D, E) The expression pattern of RhoB with different genotypes at locus −1929 and −1575 in *S. maximus* upon *V. anguillarum* challenge. Data are shown as mean ± SD (n = 3). Different letters indicate statistical significance ($P < 0.05$).

region of *Sm*RhoB and three were identified as *V. anguillarum* resistance-related SNPs. Among these five SNPs, SNP -1929 and −1575 were located in the transcription factor binding sites that affect the transcription of *Sm*RhoB. Interestingly, SNP -1575 was the only SNP that both showed *V. anguillarum* resistance and affection of transcription factor CREB binding site, implying its crucial roles in the expression of RhoB upon *V. anguillarum* challenge. Furthermore, SNP -1575 was found to have similar variation tendency with SNP -1449 which was confirmed to be associated with *V. anguillarum* resistance of turbot in our previous study [17]. Moreover, it is an effective way to identify disease-associated genes by determination of haplotypes [36]. The haplotypes were extensively existed in vertebrates and invertebrates in disease resistance associations [27,37,38]. In our study, a protective haplotype (Hap1) and a risk haplotype (Hap2) tightly associated with *V. anguillarum* resistance were identified. However, further confirmation of these two haplotypes is needed due to the small number of samples used in this study.

4.4. *SmRhoB is regulated by SNP polymorphism*

Because SNP -1929 and −1575 lies in a region that is likely to affect promoter activity, we hypothesized that these SNPs yields allelic differences in RhoB transcription. In HEK293T cells, the deletion experiment indicated that both of the SNPs were located in the region negatively regulated RhoB promoter activity (Fig. 3). We next examined allelic differences in promoter activity of SNP -1929 and −1575 by site-

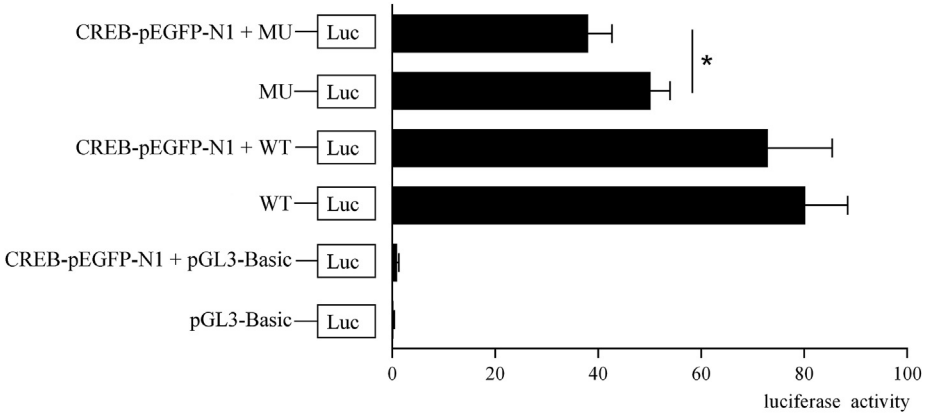

Fig. 6. Effects of *Sm*CREB on the promoter activity of WT and MU *Sm*RhoB promoter plasmid at locus -1575 in HEK293T cells. Each value represents the mean ± SEM (n = 6). The ratio between firefly and Renilla luciferase activities were calculated after 48 h co-transfection. Asterisks "*" indicate statistical significance ($P < 0.05$).

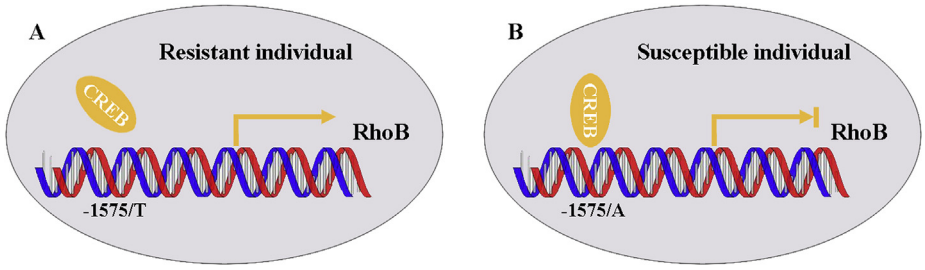

Fig. 7. Plausible mechanism for *Sm*CREB regulating *Sm*RhoB expression. (A) The CREB putative transcription factor binding site is absent in the RhoB promoter at locus −1575 when the wild-type T nucleotide is present, which will accelerate the RhoB expression. (B) The CREB putative transcription factor binding site is exposed in the RhoB promoter at locus −1575 when the mutant A nucleotide is present, which will suppress the RhoB expression.

directed mutagenesis. Significant allelic differences were observed in promoter activity in the 2.2-kb promoter construct (Fig. 5C). Wherein, the promoter activity was almost lost after double mutagenesis at these two sites. However, SNP -1929 was not associated with *V. anguillarum* resistance of *S. maximus*. Therefore, we speculate that SNP -1575 may works synergistically with SNP -1929 and plays a more important role in regulating the RhoB expression in turbot after *V. anguillarum* infection. Temporal expression of RhoB in different genotypes of SNP -1929 and −1575 confirmed this hypothesis (Fig. 5D and E). More evidence is necessary to verify this hypothesis in future study.

Further, overexpression of transcription factor demonstrated that CREB bound to the −1575 mutant *Sm*RhoB promoter and weakened its activity (Fig. 6). CREB is a stimulus-induced transcription factor and involved in apoptosis [39], oncogenesis [40] and immune responses [41]. Evidence showed that CREB could be induced by a series of stimuli, including hormones, inflammatory cytokines and bacteria, and turns on the transcription of target genes [42–44]. However, Liu et al. [45] found that CREB negatively regulated the transcription of tumor necrosis factor alpha (TNF-α)-induced protein 1 (TNFAIP1) by binding to the cAMP response element (CRE). In the present study, the −1575A allele, which is overrepresented in susceptible group, increased the frequency of CREB binding site and suppressed RhoB promoter activity. This mode was also observed in transcriptional activity regulation of duck FTH1 gene via promoter polymorphisms in response to DHV-1 challenge [46]. Hence, CREB worked as a negative transcriptional regulation factor to modify RhoB expression in *S. maximus*. Together with the previous studies, we speculate that low RhoB expression is due to the high frequency of the CREB binding site in susceptible group (−1575A) (Fig. 7).

5. Conclusions

In summary, the results showed that RhoB expression was associated with promoter polymorphisms upon *V. anguillarum* challenge rather than DNA methylation. Our findings will open a new window for the study of RhoB gene expression during *V. anguillarum* challenge.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.024.

Conflicts of interest

The authors declare that they have no competing interests.

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