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Development of a reverse genetics system for snakehead vesiculovirus (SHVV)

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ABSTRACT

Snakehead vesiculovirus (SHVV) is a new rhabdovirus isolated from diseased hybrid snakehead fish (*Channa maculate* ♀ x *Channa argus* ♂) and has caused serious economic losses in snakehead fish culture in China. To better understand the pathogenicity of SHVV, we developed a reverse genetics system for SHVV by using human and fish cells. In detail, human 293T cells were co-transfected with four plasmids encoding the full-length SHVV antigenomic RNA or the supporting proteins including nucleoprotein (N), phosphoprotein (P), and large polymerase (L), followed by the cultivation in Channel catfish ovary (CCO) cells. We also rescued a recombinant SHVV expressing enhanced green fluorescent protein (EGFP), which was inserted into the 3′ non-coding region (NCR) of the glycoprotein (G) gene of SHVV. Our study provides a potential tool for unveiling the pathogenicity of SHVV and a template for the rescue of other fish viruses by using both human 293T and fish cells.

1. Introduction

In 2014, a serious disease with high mortality outbroke in cultured hybrid snakehead fish (*Channa maculate* ♀ x *Channa argus* ♂) in Guangdong province of China. From the diseased hybrid snakehead fish, we isolated a virus that was further identified as a new rhabdovirus called snakehead vesiculovirus (SHVV) via genome sequencing (Liu et al., 2015). The genome of SHVV consists of a non-segmented negative-sense RNA, which encodes five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase or called large polymerase (L). Since the first outbreaks of SHVV, it has caused serious economic losses in snakehead fish culture in China. However, little is known about the pathogenicity of SHVV and the available vaccine against SHVV is currently lacking.

The availability of reverse genetics systems have expanded the range of studies that can be conducted with rhabdoviruses (Fang et al., 2015; Nakagawa et al., 2017; Okada et al., 2016; Schnell et al., 1994; Stanifer et al., 2011). The most extensively studied rhabdoviruses are rabies virus (RV) and vesicular stomatitis virus (VSV), which belong to *Lyssavirus* or *Vesiculovirus* genus, respectively. Similar to the mammalian rhabdoviruses RV and VSV, reverse genetics has also been developed for fish rhabdoviruses that belong to *Novirhabdovirus* genus, including infectious hematopoietic necrosis virus (IHNV) and viral haemorrhagic septicaemia virus (VHSV)(Ammayappan et al., 2011; Ammayappan et al., 2010). However, no reverse genetics has been established for fish rhabdoviruses that belong to *Sprivivirus* or *Perhabdovirus* genus, such as spring viraemia of carp virus (SVCV), Siniperca chuatsi rhabdovirus (SCRV), Monopterus albus rhabdovirus (MARV), and SHVV. Thereby, it is necessary to generate reverse genetics system for fish rhabdovirus SHVV, which will help understanding the virus pathogenicity.

In this study, we developed a reverse genetics system for SHVV. To our knowledge, it is the first time to rescue fish viruses using both human and fish cells. We also generated a recombinant SHVV expressing a fluorescent reporter protein EGFP by inserting EGFP gene into the viral genome. This study builts an appropriate tool that will be useful for future studies on understanding the molecular mechanism of SHVV pathogenecity.

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2. Results and discussion

Reverse genetics system allows for precise manipulation of viral genomes to study viral pathogenecity, to screen antiviral compounds, and to develop vaccines. Since the first revovery of rabies virus from cDNA in 1994 (Schnell et al., 1994), the reverse genetics has been established for rhabdoviruses by co-transfecting the supporting plasmids encoding N, P, and L proteins together with a plasmid encoding the full-length viral antigenome into permissive cells (Biacchesi, 2011). Initially, all the plasmids used in the reverse genetics system were under control of T7 polymerase promoter. Thereby, the permissive cells were either infected with a recombinant vaccinia virus to provide T7 RNA polymerase or constitutively expressing T7 RNA polymerase (Alonso et al., 2004; Fuerst et al., 1986). Later, the cDNA encoding N, P, and L proteins and the viral antigenome were cloned into plasmids under control of Cytomegalovirus (CMV) promotor, which was driven by cellular RNA polymerase II (Ammayappan et al., 2011; Ammayappan et al., 2010; Huang et al., 2010). The aim of this study is to establish the reverse genetics system of SHVV by using plasmids under control of CMV promoter. The whole genome sequencing of SHVV has been previously performed (Liu et al., 2015). Using this genome sequence (KP876483.1), the entire SHVV antigenome flanked with the self-cleaving hammerhead ribozyme (HamRz) and hepatitis delta virus ribozyme (HdvRz) sequences at its 5′- and 3′-end was cloned step by step into vector pCDNA3.1 (+) (Fig. 1A). Ribozyme sequences were used to guarantee the expression of an antigenome RNA having precise 5′- and 3′-end without any additional nucleotides (Biacchesi, 2011). The constructed plasmid pCDNA-SHVV was about 17 Kb in length and confirmed by restriction digestion (Fig. 1B). In addition, for the rescue of SHVV, we also constructed three supporting plasmids pCDNA-N, pCDNA-P, and pCDNA-L expressing N, P, or L protein of SHVV, respectively (data not shown).

To rescue the recombinant SHVV (rSHVV), we initially tried several fish cells including Channel catfish ovary (CCO) cells, striped snakehead (SSN-1) cells, and fathhead monnow (FHM) cells using different transfection reagents and electrotransfection. Probaly because of the low transfection efficiency of fish cells, the rescue of SHVV was not successful. Human 293T cells have been extensively used for the rescue of influenza viruses due to its high transfection efficiency (Crescenzo-Chaigne and van der Werf, 2007; Tu et al., 2011; Zhao et al., 2014). In this study, we tried to rescue fish rhabdovirus SHVV using human 293T cells based on its high transfection efficiency and fish CCO cells based on its high efficiency for SHVV replication. 293T cells were transfected with the plasmid pCDNA-SHVV and supporting plasmids pCDNA-N, pCDNA-P, pCDNA-L. For the negative control, 293T cells were only transfected with plasmid pCDNA-SHVV. The cytopathic effect (CPE) was observed daily for 3 days. As shown in Fig. 2A, CPE that cells became rounded and floated could be observed in partial 293T cells transfected with the full-length plasmid and supporting plasmids at 3 days post of transfection (Fig. 2A right), but not in the negative control (Fig. 2A left). To determine whetehr the CPE was caused by the generated virus, we detected viral G mRNA using RT-PCR. The results

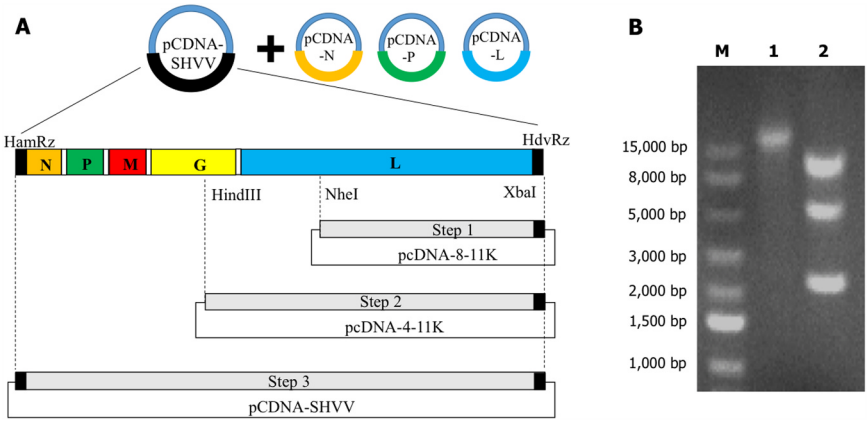

Fig. 1. Construction of the plasmids used for the rescue of SHVV. (A) Schematic representation of the plasmid pCDNA-SHVV encoding the full-length SHVV antigenome and the supporting plasmids pCDNA-N, pCDNA-P, pCDNA-L. (B) Confirmation of the constructed plasmid pCDNA-SHVV by restriction digestion with enzyme *Sal*I. M: DNA marker; Lane 1: the plasmid of pCDNA-SHVV; Lane2: restriction digestion of pCDNA-SHVV with enzyme SalI, the predicted products are 9760, 5069, and 2187 bp.

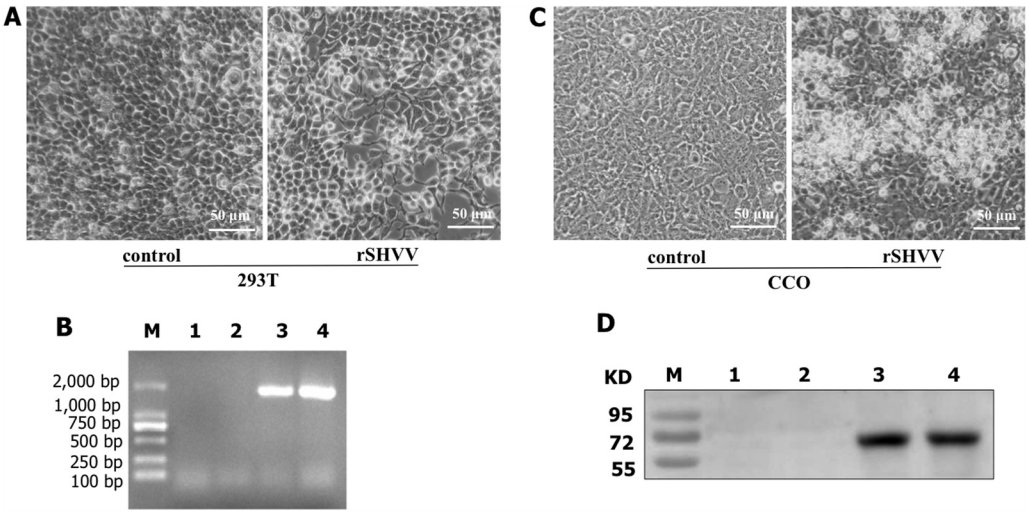

Fig. 2. The rescue of SHVV from cDNA. (A) 293T cells in 6-well plates were transfected with plasmids pCDNA-SHVV, pCDNA-N, pCDNA-P, and pCDNA-L (right) or only pCDNA-SHVV as control (left) for 3 days. (B) SHVV G gene was detected by RT-PCR for the cells collected from (A), M: DNA marker; lane 1: CCO cells without SHVV infection was used as negative control; lane 2: sample form the left of (A); lane 3: sample from the right of (A); lane 4: CCO cells with SHVV infection was used as positive control. (C) The supernatants collected from (A) were incubated with fresh CCO cells and the CPE was observed daily. (D) The SHVV G protein was detected by western blot for the cells collected from (C), M: protein marker; lane 1: CCO cells without SHVV infection was used as negative control; lane 2: sample form the left of (C); lane 3: sample from the right of (C); lane 4: CCO cells with SHVV infection was used as positive control.

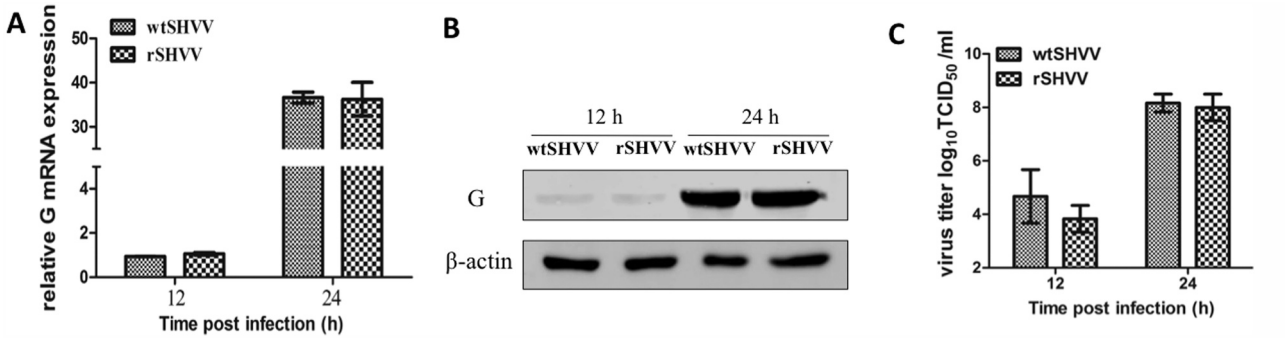

Fig. 3. Comparison of the growth ability of rSHVV and wtSHVV. CCO cells were infected with 1 MOI of wtSHVV or rSHVV, at 12 and 24 h poi, the cells and supernatants were collected for viral detection. (A) The viral G mRNA was measured using qRT-PCR. β-actin was used as the internal control. (B) The viral G protein was detected by western blot, β-actin was used as the internal control. (C) The SHVV titers in the supernatants were measured using $TCID_{50}$.

showed that SHVV G mRNA could be detected in 293T cells transfected with the four plasmids, but not for the negative control, indicating that SHVV was succesfully rescued in 293T cells (Fig. 2B). The 293T cells and supernatants were collected together and then freeze-thrawed for three times. After centifugated at 10,000 ×*g* for 5 min, the supernatants were incubated with fish CCO cells. During the observation period, CPE was not observed in the negative control (Fig. 2C left). However, in CCO cells incubated with 293T cells sample transfected with the four plasmids, the CPE was observed in most of the cells at 36 h poi (Fig. 2C right). The CCO cells with or without CPE were then subjected to viral detection by western bolt. Viral G protein could be detected in CCO cells with CPE, demonstrating that this reverse genetics system was highly efficient for SHVV rescue (Fig. 2D).

The growth ability of rSHVV was subsequently compared with wild-type SHVV (wtSHVV). CCO cells were infected with wtSHVV or rSHVV at a multiplicity of infection (MOI) of 1, the cells and supernatants were collected at 12 and 24 h post of infection (poi) for viral detection via quantitative real-time PCR (qRT-PCR), western blot, or 50% tissue culture infectious dose ($TCID_{50}$). The results showed that rSHVV grew equally well as wtSHVV without any attenuation (Fig. 3), further demonstrating the succsessful rescue of rSHVV. To our knowledge, this is the first time to successfully generate fish virus using both human and fish cells, and our data has proved that this is an efficient way for fish virus rescue.

After confirming the successful rescue of rSHVV, we engineered a recombinant virus expressing a fluoresent reporter protein. Usually, a reporter gene was inserted into the intergenic regions of viral genome or fused to one of the viral genes (Albarino et al., 2013; Lo et al., 2014; Schudt et al., 2013; Uebelhoer et al., 2014). In this study, we tried a different way by inserting the open reading frame (ORF) of EGFP into the 3′ non-coding region (NCR) region of SHVV G gene. In this way, the EGFP was translated from the same mRNA with G protein. To generate this recombinant virus, an Sse8387 I restriction site was engineered into the 3′NCR of G gene, which permitted insertion of EGFP coding sequence (Fig. 4A). Using human 293T and fish CCO cells, we successfully rescued the recombinant virus rSHVV-EGFP (Figs. 4B and 4C), which could efficiently express EGFP in cells (Figs. 4B, 4C and 4D). However, viral titer detection showed that the rescue efficiency of rSHVV-EGFP was lower than that of rSHVV, indicating that the growth ability of rSHVV-EGFP was probably impaired (Fig. 5A). We then compared the

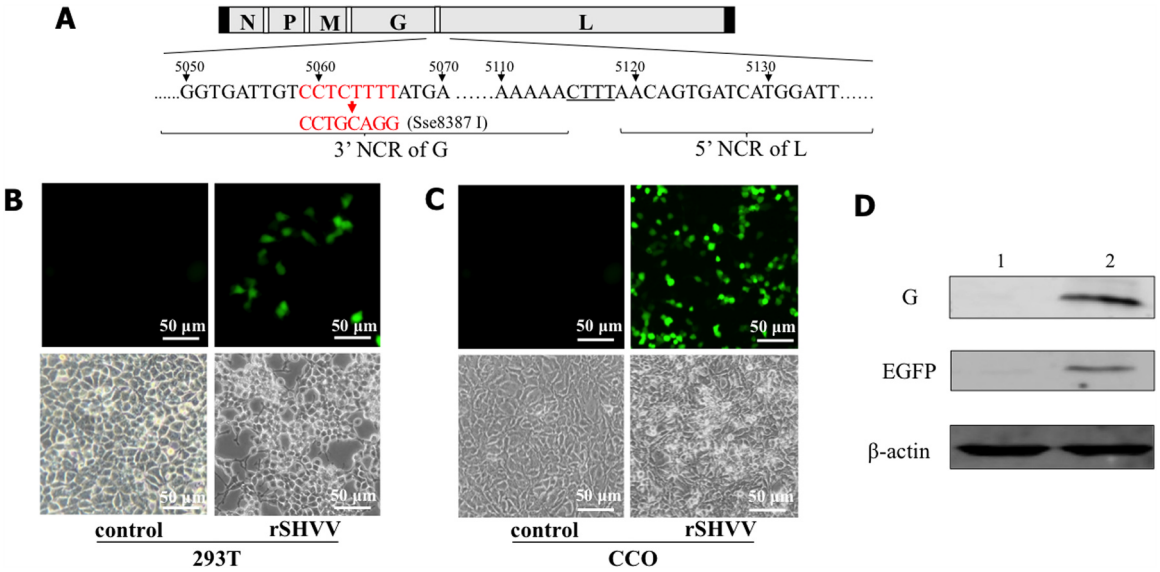

Fig. 4. The rescue of recombinant SHVV expressing EGFP. (A) Schematic representation of SHVV antigenome with an enzyme Sse8387 I insertion by mutating CCTCTTTT to CCTGCAGG at 3′NCR of G gene around the position of 5060. (B) 293T cells in 6-well plates were transfected with plasmids pCDNA-SHVV-EGFP, pCDNA-N, pCDNA-P, and pCDNA-L (right) or only pCDNA-SHVV-EGFP as control (left) for 3 days. (C) The supernatants collected from (B) were incubated with fresh CCO cells and the CPE was observed daily. (D) The SHVV G protein and EGFP of the cells was detected by western blot, lane 1: sample from the left of (C); lane 2: sample from the right of (C). β-actin was used as the internal control.

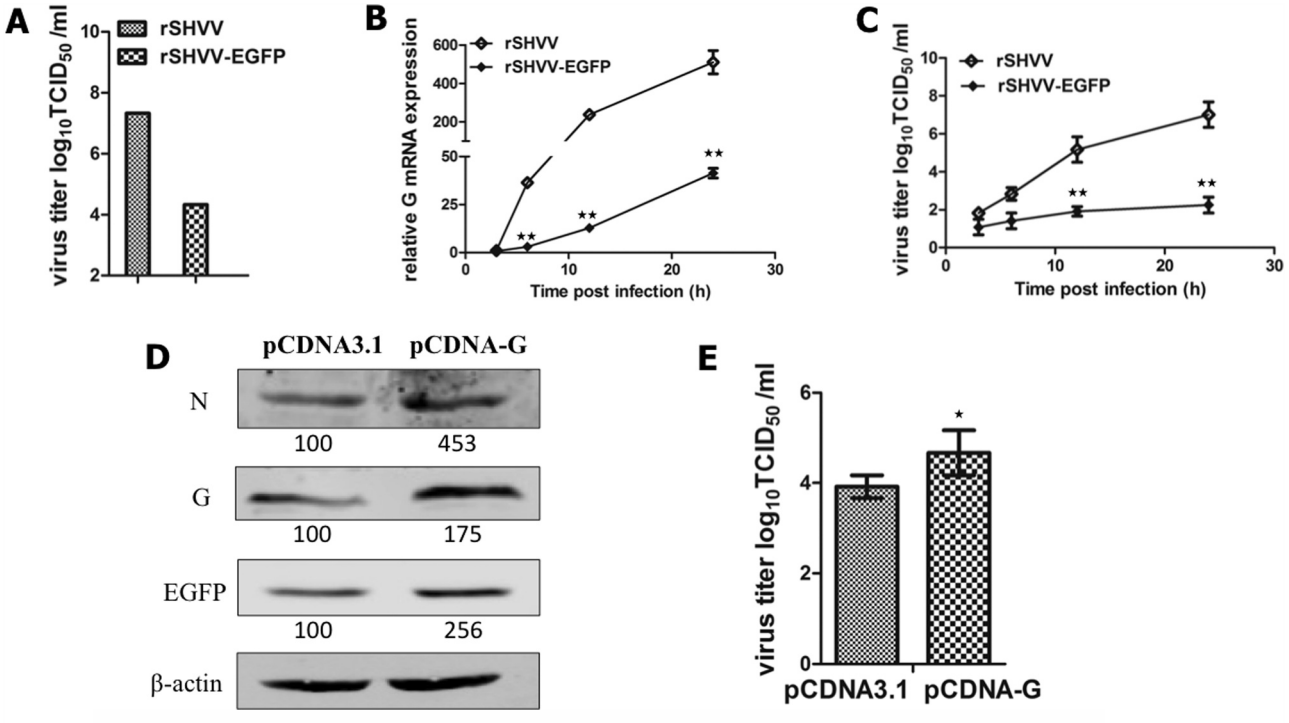

Fig. 5. Comparison of the growth ability of rSHVV and rSHVV-EGFP. (A) Comparison of the rescue efficiency of rSHVV and rSHVV-EGFP by measuring the viral titers in the supernatants of the samples from Fig. 2C (right) and Fig. 4C (right) using $TCID_{50}$. (B) CCO cells were infected with 1 MOI of rSHVV or rSHVV-EGFP, at 3, 6, 12, and 24 h poi, the cells and supernatants were collected for viral detection. The viral G mRNA was measured using qRT-PCR. β-actin was used as the internal control. (C) The SHVV titers in the supernatants from (B) were measured using $TCID_{50}$. (D) CCO cells were transfected with 2 μg of pCDNA3.1 or pCDNA-G for 24 h, followed by the incubation with the supernatants from Fig. 4B (right). The viral N, G, and EGFP proteins were detected by western blot, β-actin was used as the internal control. The integrated optical densities of the protein bands were measured using Image-Pro Plus 6.0. The value of viral N, G and EGFP protein bands were normalized to that of β-actin. The value of the viral N, G and EGFP protein bands in pCDNA3.1 transfected cells were set as 100, respectively. (E) The SHVV titers in the supernatants from (D) were measured using $TCID_{50}$. All the data are performed in triplicate (mean ± SD). The * and ** respectively indicate statistically significant differences (*, $p < 0.05$; **, $p < 0.01$).

growth kinetics of rSHVV and rSHVV-EGFP. CCO cells were infected with 1MOI of rSHVV or rSHVV-EGFP, the cells and supernatants were collected at 3, 6, 12 and 24 h poi for viral detection via qRT-PCR or $TCID_{50}$. The results showed that rSHVV-EGFP grew slower than rSHVV (Figs. 5B and 5C). At 24 h poi, the viral titer of rSHVV-EGFP was about 5 log less than that of rSHVV (Fig. 5C). These data demonstrated that insertion of a foreigner gene EGFP into the 3′NCR of viral G gene impaired the growth ability of rSHVV-EGFP.

It was hypothesized that the insertion of EGFP into the 3′NCR of viral G gene probably affected the expression of viral G protein, thus impaired the virus growth. To determine whether additionally providing G protein in cells will improve the growth ability of rSHVV-EGFP, CCO cells were transfected with pCDNA3.1 or pCDNA-G, followed by the infection of rSHVV-EGFP. As shown in Figs. 5D and 5E, additionally providing G protein in cells significantly increased expression of EGFP and viral proteins. Moreover, the viral titer was also increased by providing additional G protein in cells although it was still lower than that of rSHVV (Figs. 5A and 5E). These data suggested that insertion of EGFP into the 3′NCR of G gene impaired the growth of SHVV via affecting G protein expression, while additionally providing G protein could improve its growth ability.

3. Conclusions

In this study, we describe a highly efficient reverse genetics system to generate fish rhabdovirus SHVV using human 293T and fish CCO cells. This human-fish cell combined way might also be used for the rescue of other fish viruses. We also report the successful rescue of a recombinant virus rSHVV-EGFP expressing a fluorescent report protein EGFP. However, the insertion of EGFP into the 3′NCR of SHVV G gene suppresses the rescue efficiency and growth ability of the rSHVV-EGFP. Further studies are needed to generate more suitable recombinant virus rSHVV-EGFP for screening of antiviral drugs.

4. Materials and methods

4.1. Cells and viruses

Human 293T cells were cultured at 37 °C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, New Zealand) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, New Zealand), penicillin (100 μg/ml), and streptomycin (100 μg/ml). CCO cells were cultured at 25 °C in minimum essential medium (MEM) (Gibco, New Zealand) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, New Zealand), penicillin (100 μg/ml), and streptomycin (100 μg/ml). SHVV was isolated from diseased hybrid snakehead fish and stored at − 80 °C.

4.2. Antibodies

The antibody against G and N proteins of SHVV was produced by immunizing rabbits with G or N proteins expressed in *E. coli*. The antibody against β-actin and EGFP was purchased from Bioss Biotechnology Co., LTD. (Beijing, China). The secondary anti-rabbit or anti-mouse IgG antibody was purchased from Gene Co., LTD. (Shanghai, China).

Table 1
Primers used in this study.

Application	Primer	Sequence (5′ – 3′)
Full-length plasmid	8K-FW	CGGCTAGCGTTTTGGAAGGTGGTCTAC
	Hdv-BW	GCTCTAGATGGCTCTCCCTTAGCCATCCGAGTGGACGTGCGTCCTCCTTCGGATGCCCAGG TCGGACCGCGAGGAGGTGGAGATGCCATGCCGACCCACGAGAAAAACAAACACAGTCTCTGATTAGC
	4K-FW	CGGCTAGCAAACACACAGTATCTCTGCG
	8K-BW	CGGAATTCGGCCAAAAATCTTGGAAATA
	Ham-FW	TCACTATAGGGAGACCCAAGCTGGACTGATGAGTCCGTGAGGACGAAACTATAGGAAAGGAATTCCT ATAGTCACGAGAAAAAAAGAAACCAA
	4K-BW	ACCCTTGCATAGGTAGCCTTGGAAGCTTGC
qRT-PCR	SHVV-G-FW	ACACCATACATGCCAGAGGC
	SHVV-G-BW	GCCTCGCTGGGTATCCAAAT
	β-actin-FW	CACTGTGCCCATCTACGAG
	β-actin-BW	CCATCTCCTGCTCGAAGTC
Supporting plasmids	PCDNA-N-FW	GGGGTACCAACAGATATCATATCTGAAT
	PCDNA-N-BW	GCTCTAGATTTTTTTCATATCCCATCAC
	PCDNA-P-FW	GGGGTACCAACAGATATCATGGCAAAAC
	PCDNA-P-BW	GCTCTAGATTTTTTTCATATCCTCCTGA
	PCDNA-L-FW	GGGGTACCATGGATTATTCTCAGGAATA
	PCDNA-L-BW	GCCGGGCCCTTATTCCGTCCACGCAGCTT
Mutant plasmids	*Sse*8387I-FW	CTTAAATGGGTGATTGTCCTGCAGGATGATATATTG
	*Sse*8387I-BW	CCTGCAGGACAATCACCCATTTAAGAGCTTGTAATAG
	EGFP-*Sse*8387I-FW	GCTCTTAAATGGGTGATTGTCCTGCAATGGTGAGCAAGGGCGAGGA
	EGFP-*Sse*8387I-BW	TCATCGAGACAATATATCATCCTGCATTACTTGTACAGCTCGTCCATG

4.3. Plasmids

The plasmid pCDNA-SHVV containing the full-length of SHVV antigenome was constructed as shown in Fig. 1A using primers listed in Table 1. In step 1, *Nhe*I and *Xba*I were used to clone ~8–11 K of SHVV antigenome with HdvRz at its 3′-end into pCDNA3.1. In step 2 and 3, the ~4–8 K and ~1–4 K of SHVV antigenome with HamRz at its 5′-end were cloned into pCDNA-8–11K by *Nhe*I or *Hin*dIII respectively using pEASY-Uni Seamless Cloning and Assembly Kit (TransGen, China). The supporting plasmids pCDNA-N, pCDNA-P, and pCDNA-L were constructed by amplifying the ORF of corresponding genes into vector pCDNA3.1 using primers listed in Table 1. The mutant plasmid pCDNA-SHVV-*Sse*8387I was constructed by PCR-mediated insertion of Sse8387 I enzyme site into the 3′NCR of G gene in the plasmid pCDNA-SHVV using primers listed in Table 1. The plasmid pCDNA-SHVV-EGFP was constructed by the insertion of EGFP into the Sse8387 I site of the plasmid pCDNA-SHVV-*Sse*8387I using primers listed in Table 1.

4.4. Reverse genetics

Human 293T cells were co-transfected with the supporting plasmids pCDNA-N (1.6 μg), pCDNA-P (1.6 μg), and pCDNA-L (0.8 μg) together with the full-length plasmid pCDNA-SHVV (3.2 μg) or the mutant plasmid pCDNA-SHVV-EGFP (3.2 μg) using TransIntroTM EL Transfection Reagent (TransGen Biotech, China). At 6 h post of transfection, the medium was replaced by 1 ml of DMEM with 10% FBS and continued incubation at at 37 °C with 5% CO_2 for 3 days. The cells were freeze-thawed for three times and then centrifuged at 10,000 × *g* for 5 min. The supernatant was subsequently incubated with fresh CCO cells and the CPE was observed daily.

4.5. Virus infection and titration

CCO cells were incubated with wtSHVV, rSHVV, or rSHVV-EGFP at a MOI of 1. After 2-h adsorption at 25 °C, the inoculum was removed and the cells were washed twice with PBS followed by adding MEM with 5% FBS. At different time point poi, the supernatants were collected for virus titration by $TCID_{50}$, and the cells were harvested for the detection of viral proteins by western blot or viral mRNAs by qRT-PCR with primers listed in Table 1.

4.6. Quantitative RT-PCR

Total RNAs were extracted from cells with TRIzol reagent (Invitrogen) according to manufacturer's instructions as described previously (Zhang et al., 2017a; Zhang et al., 2017b). For the detection of SHVV G mRNA, 1 μg of RNA was mixed with 1 μl Oligo (dT), 4 μl 4 × gDNA wiper Mix, and RNase free H_2O to a total volume of 16 μl. After incubated at 42 °C for 2 min, 4 μl 5x select qRT supermix II was added and incubated at 50 °C for 15 min and 85 °C for 2 min. The PCR reactions were conducted in 20 μl volumes containing 10 μl mix, 1 μl cDNA template, 1 μl of SHVV-G forward primer, 1 μl of SHVV-G backward primer, and 7 μl ddH_2O with the following cycling conditions: 95 °C for 5 min, 30 cycles at 94 °C for 1 min, 57 °C for 30 s, and 72 °C for 90 s, and ended with a 72 °C at 7 min. The quantitative PCR reactions were conducted in 20 μl volumes containing 10 μl AceQ qPCR SYBR Green Master mix, 1 μl cDNA template, 0.4 μl of forward primer, 0.4 μl of backward primer, and 8.2 μl ddH_2O with the following cycling conditions: 95 °C for 5 min, 45 cycles at 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 15 s, and ended with a 95 °C at 5 °C/s calefactive velocity to make the melt curve. Data were normalized to the level of β-actin in each sample using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

4.7. Western blot

The extracted proteins from CCO cells were extracted by cell lysis buffer, separated by SDS-PAGE gel and transferred onto a nitrocellulose membrane (Biosharp, China). Membranes were blocked with 5% skim milk in tris-buffered saline with tween 20 (TBST) at 4 °C overnight, followed by incubation with the anti-G (1:1000), anti-N (1:1000), anti-EGFP (1:3000), or anti-β-actin (1:1000) antibodies for 2 h at room temperature. The membranes were then washed three times by TBST and incubated with IRDye 800CW conjugated anti-rabbit antibody (for G and N proteins, 1:10,000) or anti-mouse antibody (for EGFP protein, 1:10,000) for 1 h at room temperature. The signal intensity was then determined using Odyssey CLx (LI-COR, USA).

4.8. Statistical analysis

All statistical analyses were performed using Graphpad Prism 5.0 (GraphPad Software, CA). The statistical significance of the data was

determined by Student *t*-test, and $P < 0.05$ was considered statistically significant.

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