



A transmembrane domain of *Andrias davidianus* ranavirus 13R is crucial for co-localization to endoplasmic reticulum and viromatrix

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Abstract

13R, a core gene of *Andrias davidianus* ranavirus (ADRV), encoded a protein containing a transmembrane domain (TMD) and a restriction endonuclease-like domain. However, the characterization and function of *13R* and the protein it encodes remain unclear. In this study, Chinese giant salamander thymus cell (GSTC) was used to investigate the function of *13R*. The results showed that the *13R* transcripts were detected first at 8 h post-infection (hpi) by RT-PCR and the protein was detected first at 24 hpi by western blot, but the transcription was inhibited by cycloheximide and cytosine arabinofuranoside, indicating that *13R* is a viral late gene. Subcellular localization showed that the 13R was co-localized with endoplasmic reticulum (ER) in the cytoplasm, while 13R deleting TMD (13R Δ TM) was distributed in cytoplasm and nucleus. During ADRV infection, 13R was observed first in the cytoplasm and nucleus, and later aggregated into the viromatrix, whereas 13R Δ TM remain dispersed in cytoplasm and nucleus. Western blot analysis suggested that 13R was a viral non-structural protein and its overexpression did not affect the viral titer in GSTC. All these indicated that the TMD of 13R is crucial for the co-localization into the ER and the viromatrix.

Keywords Ranavirus · *Andrias davidianus* ranavirus · Core gene · Transmembrane domain · Localization

Introduction

Members of the genus *Ranavirus* in the family *Iridoviridae* are large, double-stranded DNA containing virus, which infect fish, reptiles, and amphibians worldwide (Chinchar et al. 2017a, b; Zhang and Gui 2015). *Andrias davidianus* ranavirus (ADRV) was isolated from diseased Chinese giant salamanders (*A. davidianus*, CGS) in China and was identified as a member of a novel species in *Ranavirus* (Dong et al. 2011; Geng et al. 2011; Chen et al. 2013). In a previous study, the complete genome of ADRV has been sequenced and analyzed. Its genome contains 26 core genes, all of which have homologues in all sequenced iridovirids (Chen

et al. 2013). ADRV *13R*, a core gene of the family *Iridoviridae*, was identified containing a transmembrane domain (TMD) and a restriction endonuclease-like domain by bioinformatic analysis. However, the function of *13R* and the protein it encodes are still under investigation.

Membrane proteins account for one-third of all proteins in most genomes and carry out a wide range of essential cellular functions (Hong et al. 2012). Viral membrane proteins play important roles in the viral life cycle such as recognition of host receptors, membrane fusion, and virion assembly. Most of these viral membrane proteins have at least one TMD (Liu et al. 2018; Aguilar et al. 2006; Wang et al. 2009). Up to now, a large numbers of viral membrane proteins and their functions have been identified (Ouyang et al. 2018). However, the characteristics and functions of many potential viral membrane protein genes are still unknown. TMDs of some viral membrane proteins were reported as the localization signal, which can target the proteins to the endoplasmic reticulum (ER) (He et al. 2014), Golgi apparatus (Golgi) (Wang et al. 2016), mitochondria (Mito) (Yan et al. 2016), and the viromatrix (Zhao et al. 2008). The proper localization of viral membrane proteins in the cell membrane,

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intracellular spaces, or the viromatrix is required for the proteins to function.

The 468 bp open reading frame (ORF) of ADRV *13R* is predicted to encode a protein of 155 amino acids with molecular weight of 17.83 kDa. The subcellular localization of the 13R and the effect of TMD on the protein localization are not known yet. In this study, the transcription and expression patterns of the *13R* gene were studied for the first time. The intracellular localization of the 13R and 13R without TMD (13R Δ TM) was analyzed with or without ADRV infection. Furthermore, the impact of 13R on the viral titers was also investigated.

Materials and methods

Virus and cell

Andrias davidianus ranavirus (ADRV) was originally isolated from diseased Chinese giant salamanders (*A. davidianus*) in China (Chen et al. 2013). Chinese giant salamander thymus cell (GSTC) line was maintained at 25 °C in M199 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) for viral proliferation. The GSTC cell line was established by Yuan et al. (2015).

Sequences analysis of 13R and its homologues

Rana grylio virus (RGV) 101L (AFG73143.1), Frog virus 3 (FV3) 94L (YP_031673.1), Common midwife toad ranavirus (CMTV) 13R (AFA44917.1), Epizootic hematopoietic necrosis virus (EHNV) 11R (YP_009182010.1), European catfish ranavirus (ECV) 18R (YP_006347609.1), *Ambystoma tigrinum* virus (ATV) 11R (YP_003782.1), Tiger frog virus (TFV) 100L (AF389451.1), Bohle iridovirus (BIV) 96L (ANK58021.1), Singapore grouper iridovirus (SGIV) 98R (AAS18113.1), Invertebrate iridescent virus 3 (IIV-3) 33L (ABF82063.1), Invertebrate iridescent virus 6 (IIV-6) 307L (AAK82168.1), Infectious spleen and kidney necrosis virus (ISKNV) 86L (AAL98810.1), and Lymphocystis disease virus 1 (LCDV-1) 19R (NP_078641.1) with homology to the ADRV 13R (AGV20544.1) were retrieved from the National Center for Biotechnology Information (NCBI) for computer-assisted analysis. Conserved domains were predicted using the NCBI conserved domains search (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Multiple alignments of these protein sequences were performed using Clustal X 1.83 and edited by the GeneDoc program.

Drug inhibition assay of 13R gene transcription

Cycloheximide (CHX), a de novo protein synthesis inhibitor, and cytosine arabinofuranoside (AraC), a viral DNA

replication inhibitor, were used as inhibitors to characterize the transcription of the *13R* during viral infection. Briefly, GSTC monolayer was pre-treated with 50 μ g/ml CHX or 100 μ g/ml AraC for 1 h before ADRV infection at a multiplicity of infection (MOI) of 0.1. CHX-pretreated GSTC infected with the virus or with 1 \times PBS and non-treated GSTC infected with the virus were harvested at 6 h post-infection (hpi). Similarly, AraC-pretreated GSTC infected with the virus or with 1 \times PBS and non-treated GSTC infected with the virus were harvested at 48 hpi. Total RNA was isolated using Trizol reagent (Ambion, Life technologies) and reverse transcribed to the first strand cDNA by PrimeScriptTM RT reagent kit (Takara, Dalian, China). The *13R* transcription was analyzed by RT-PCR using the primers 13R-Er5/Sa3 (Table 1). The transcriptions of a delayed early ADRV gene, *dUTPase*, and an internal control gene β -actin were also analyzed using specific primers dUTPase-F/R and β -actin-F/R, respectively (Table 1).

Prokaryotic expression, purification, and antibody preparation of 13R

A 306 bp DNA fragment (+163 to +468 relative to translation initiation codon of *13R*) was cloned by PCR from the total DNA of ADRV-infected GSTC using the primers 13R Δ N54/13R-yXh3. The fragment digested with *EcoRI* and *XhoI* was cloned into a prokaryotic vector pET-32a (+) (Novagen) digested with the same enzymes to obtain the plasmid pET32a-13R Δ N54. The constructed plasmid was confirmed by restriction enzyme digestion and DNA sequencing.

Expression of the 13R Δ N54 fusion protein was induced by adding 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at 37 °C for 6 h. Fusion proteins were purified according to the protocol supplied with the HisBind purification kit (Novagen). To obtain antibody against 13R Δ N54, the fusion protein was mixed with equal volume of Freund's adjuvant (Sigma) to immunize mouse by subcutaneous injection at 7-day intervals. The anti-13R Δ N54 serum was collected at the fifth day after the fifth immunization. This experiment was carried out in strict accordance with the recommendations in the Regulations for the Administration of Affairs Concerning Experimental Animals of China. The protocol was approved by the Animal Center of Disease Control and Prevention of Hubei Province (approval ID: SCXK 2015-0018). All surgeries were performed under sodium pentobarbital anesthesia and all efforts were made to minimize suffering.

Temporal transcription and expression patterns of 13R

To examine the transcription pattern of *13R*, GSTC was infected with ADRV at a MOI of 0.1. Total RNA was

Table 1 Primers used in this study (enzyme cleavage sites underlined and enzymes indicated in parentheses)

Primers	Primers sequence (5'–3')	Usage
13R-Er5	CCGGA <u>ATTC</u> AATGGATCCAGAAGGAATGCTG (EcoR I)	RT-PCR, pEGFP-13R, pEGFP-13R Δ TM construction
13R-Sa3	CGCGT <u>CGACC</u> CAGCACCTTTCTCAGGTACCC (Sal I)	
13R Δ TM-gEr5	CCGGA <u>ATTC</u> AATGGATCCAGAACGTGAGAAGAAGAG CAAG (EcoR I)	
13R Δ N54	CCGGA <u>ATTC</u> GCCGCCTACAGGATAACGGGC (EcoR I)	pET32a-13R Δ N54 construction
13R-yXh3	CCGCT <u>CGAGT</u> CAGCACCTTTCTCAGGTAC (Xho I)	p50GFP-13R, p50GFP-13R Δ TM construction
P50GFP-F	GCTTAACCTCTGAGAAAAGCGCAAG	
P50GFP13r-R	CCTTCTGGATCCATCTTGTACAGCTCGTCC	
gfp13R-F	GGACGAGCTGTACAAGATGGATCCAGAAGG	
13R-R	TCACAGCACCTTTCTCAGGTACCC	
P50GFP13r Δ TM-R	CTTCTCACGTTCTGGATCCATCTTGTACAGCTCGTCC	
gfp13R Δ TM-F	GGACGAGCTGTACAAGATGGATCCAGAACGTGAGAAG	pcDNA3.1-13R construction
13R-wEr5	CCGGA <u>ATTC</u> AATGGATCCAGAAGGAATGCTG (EcoR I)	
13R-wXh3	CCGCT <u>CGAGT</u> CACAGCACCTTTCTCAGGTAC (Xho I)	
dUTPase-F	GCACGGAAACTCTCTGCAG	RT-PCR
dUTPase-R	GTGCCTCAGACAAAACCTCTCC	RT-PCR
β -Actin-F	CCACTGCTGCCTCCTCTT	
β -Actin-R	GCAATGCCTGGGTACATG	

isolated from virus-infected cells at 0, 2, 4, 8, 12, 16, 24, 36, and 48 hpi using Trizol reagent. The first strand cDNA of each sample was prepared as described above. PCR was conducted using the gene-specific primers 13R-Er5/Sa3. Detection of β -actin mRNA using primers β -actin-F/R was used as an internal control.

To examine the protein expression pattern of 13R, western blot was performed on the total protein from the ADRV-infected GSTC harvested at the same time points as described above. Equivalent amounts of cell extracts were electrophoresed in 12% SDS-PAGE and subsequently transferred to PVDF membrane (Millipore). The anti-13R Δ N54 serum was used as the primary antibody at a dilution of 1:500 and alkaline phosphatase-conjugated anti-mouse IgG (H+L) (Vector Laboratories, Inc.) was used as the secondary antibody at a dilution of 1:2500. Simultaneous internal control was performed by detecting β -actin protein with β -actin antibody (Boster; 1:2500).

Subcellular localization of 13R and 13R Δ TM

The designed primers 13R-Er5/Sa3 and 13R Δ TM-gEr5/13R-Sa3 were separately used to amplify the 13R and 13R Δ TM to introduce restriction enzyme sites and the fragments were cloned into eukaryotic vector pEGFP-N3 (Clontech, USA) to obtain the plasmids pEGFP-13R and pEGFP-13R Δ TM. GSTC cells were plated on coverslips in 6-well plates for 24 h and the plasmid pEGFP-13R or pEGFP-13R Δ TM was transfected into GSTC using the Lipofectamine 3000 transfection kit (Invitrogen, USA)

according to the manufacturer's instructions. At 48 h after transfection (hpt), the cells were rinsed with 1 \times PBS (pH 7.4) and fixed with 4% paraformaldehyde for 30 min. Subsequently, the cells were permeabilized with 0.2% Triton X-100 for 15 min and stained with hoechst 33,342 (1 μ g/mL) for 10 min. Finally, the cells were mounted with 50% glycerol and visualized under fluorescence microscope (OLYMPUS DP80).

To characterize the relationship between 13R protein and organelles such as endoplasmic reticulum (ER), mitochondria (Mito), and Golgi apparatus (Golgi), three organelle-specific markers pDsRed2-ER, pDsRed2-Mito, and pDsRed2-Golgi were used. Each organelle-specific marker was co-transfected with pEGFP-13R into GSTC using the Lipofectamine 3000 transfection kit. The cells were fixed at 48 hpt and observed by fluorescence microscopy as described above.

To further analyze the subcellular localization of 13R and 13R Δ TM during ADRV infection, two plasmids expressing EGFP-13R and EGFP-13R Δ TM fusion protein under the control of the promoter of RGV major capsid protein gene (*MCP*) were constructed (He et al. 2012). The 13R fragment was amplified from ADRV genomic DNA by primers gfp13R-F/13R-R, while *P50-EGFP* fragment containing the RGV *MCP* promoter and *EGFP* was amplified from plasmid pMD18T-P50-EGFP by primers P50GFP-F/P50GFP13r-R. The *P50-EGFP-13R* fragment was further obtained by overlap extension PCR by primers P50GFP-F/13R-R and cloned into plasmid pMD18-T to produce plasmid pMD-P50-EGFP-13R (p50GFP-13R). Similarly, the plasmid

pMD-P50-EGFP-13RΔTM (p50GFP-13RΔTM) was constructed using primers gfp13RΔTM-F/13R-R and P50GFP-F/P50GFP13rΔTM-R. GSTC cells were transfected with plasmid p50GFP-13R or p50GFP-13RΔTM using Lipofectamine 3000 transfection kit. At 24 hpt, the cells were infected with 0.5 MOI ADRV for 1 h, then fixed at 24 hpi and 48 hpi for fluorescence observation as described above.

Western blot analysis

To confirm whether 13R is a viral structural protein, the viral particle suspensions of ADRV were purified as described previously (He et al. 2012). Purified viral particles prior to and after sucrose gradient centrifugation were collected and analyzed by 12% SDS-PAGE followed by western blot analysis. The anti-13RΔN54 serum was used as the primary antibody at a dilution of 1:500 followed by detection with peroxidase-conjugated goat anti-mouse IgG (H + L) (OriGene) at a dilution of 1:2500. As controls, the known viral structural protein ADRV MCP and envelope protein ADRV 2L were analyzed under the same condition using anti-MCP (1:500) and anti-2L (1:500), respectively (He et al. 2012).

The effect of 13R overexpression on ADRV replication kinetics

To investigate the effect of 13R overexpression on ADRV titers in GSTC, an expression vector, pcDNA3.1-13R, was constructed using primers 13R-wEr5/wXh3. GSTC cells were transfected with pcDNA3.1-13R or pcDNA3.1-HA and selected by adding antibiotics G418 (Geneticin, Gibco). The pcDNA3.1-13R stable GSTC and pcDNA3.1-HA stable GSTC were termed as G/13R and G/Con, respectively. The ADRV replication kinetics were measured subsequently in G/13R and G/Con. Briefly, two stable cell lines were seeded in 24-well plates and infected with ADRV at an MOI of 0.1. The virus-infected cells were harvested at the indicated time points of 0, 4, 8, 16, 24, 36, 48, and 72 hpi and the viral titers were determined using a 50% tissue culture infectious dose (TCID₅₀) assay. Finally, the viral replication kinetics assay was performed and compared between ADRV-infected G/13R and ADRV-infected G/Con. Student’s t-test was used to evaluate the differences.

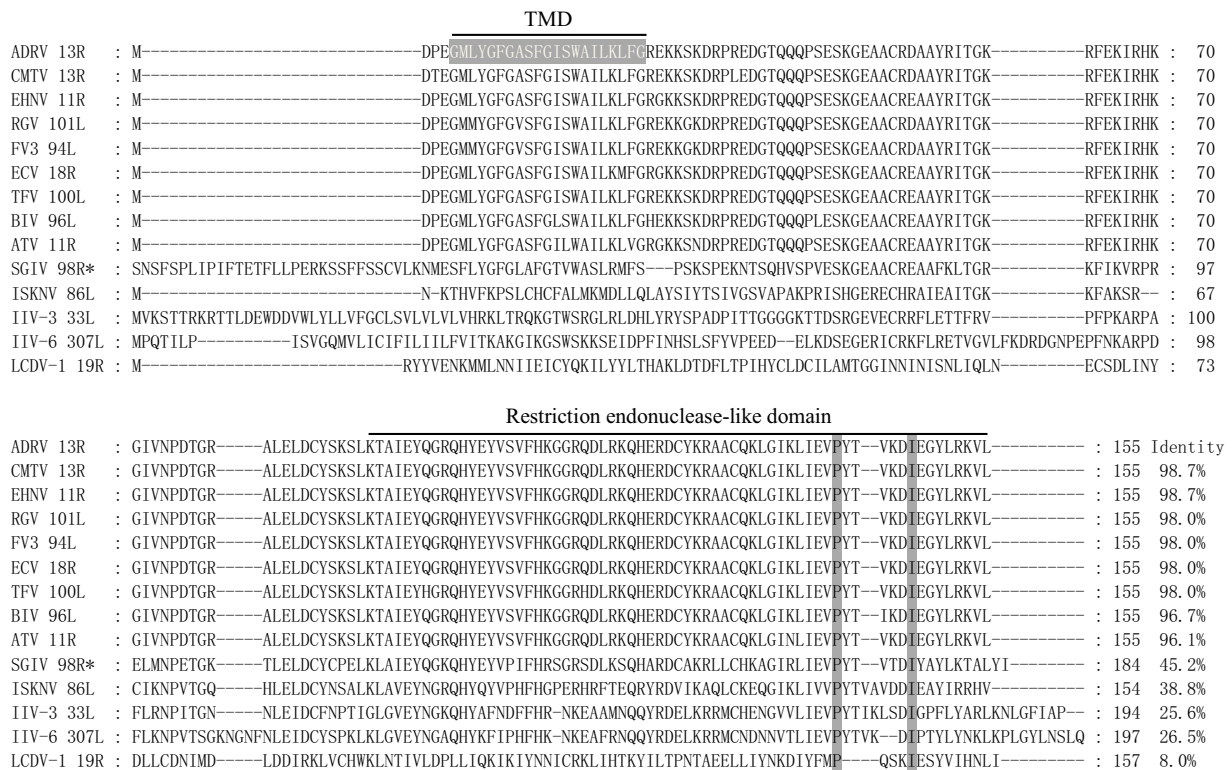


Fig. 1 Multiple alignment of amino acid sequences of 13R and its homologues in *Iridoviridae*. A transmembrane domain (TMD) and a restriction endonuclease-like domain are shown under the black lines.

SGIV_98R*: only the amino acids from 86 to 267 of this protein were used for this alignment

Results

Sequence analysis of ADRV 13R

The complete ORF of the ADRV 13R is 468 bp and the deduced protein contains 155 amino acids. Bioinformatic analysis indicated that the protein has a transmembrane domain (TMD) at the N-terminus and a restriction endonuclease-like domain at the C-terminus, which is conserved in most ranavirus (Fig. 1). It has been reported that ADRV 13R and its homologues may function in the maintenance of viral genomic DNA by participating in methylation-mediated mismatch repair (Kinch 2005; Marchlerbauer et al. 2017). When compared with the homologous proteins in the genus *Ranavirus*, ADRV 13R is highly similar to those of the CMTV 13R, EHNV 11R, RGV 101L, FV3 94L, ECV 18R, TFV 100L, BIV 96L, and ATV 11R with at least 96.1% sequence identities (Fig. 1).

Drug inhibition assay of 13R gene transcription

The RT-PCR showed that 13R transcripts were not detected in the CHX-treated sample (Fig. 2a, lane 2) or in the

AraC-treated sample (Fig. 2a, lane 5), but detected in the untreated sample at 48 hpi (Fig. 2a, lane 6). Meanwhile, ADRV *dUTPase*, a viral delayed early gene (Zhao et al. 2007), was not detected in the CHX-treated sample (Fig. 2a, lane 2), but detected in the AraC-treated sample (Fig. 2a, lane 5). Therefore, 13R transcription was blocked by both viral DNA replication inhibitor (AraC) and de novo protein synthesis inhibitor (CHX), indicating that 13R is a viral late (L) gene.

Transcription and expression patterns of 13R

RT-PCR was performed to detect the temporal transcription pattern of 13R during ADRV infection. As shown in Fig. 2b, the transcripts of 13R were detected as early as 8 hpi and the amount of the transcripts increased over time. The temporal protein expression pattern of 13R was also examined using the prepared anti-13R Δ N54 serum. Western blot analysis showed that a specific immunoreactive band of approximately 18 kDa was first detected at 24 hpi and the signal intensity increased by 48 hpi (Fig. 2c). These results indicated that 13R transcription started at 8 hpi, while the protein expression started 24 hpi.

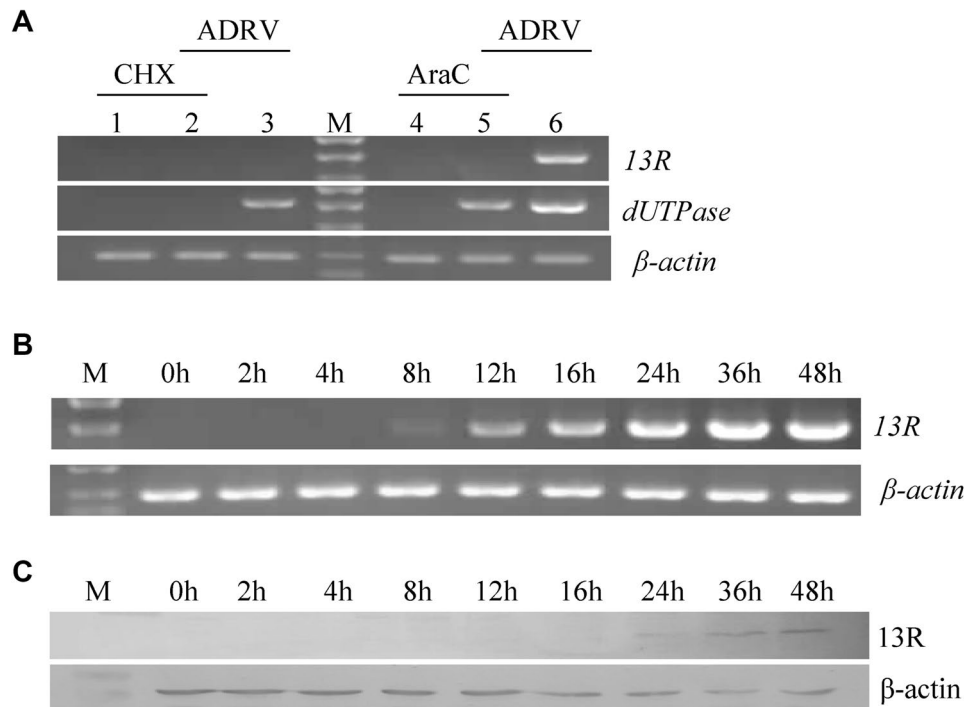


Fig. 2 Drug inhibition analysis of 13R transcription over time. Drug inhibition analysis of 13R transcription by CHX and AraC (**a**). Total RNA was isolated from CHX-treated cells (lane 1), CHX-treated cells infected with ADRV (lane 2), and untreated cells infected with ADRV (lane 3) at 6 hpi; AraC-treated cells (lane 4), AraC-treated cells infected with ADRV (lane 5), and untreated cells infected with

ADRV (lane 6) were collected at 48 hpi. Temporal RNA transcription (**b**) and protein expression (**c**) patterns of 13R after virus infection. GSTC cells were infected by 0.1 MOI ADRV and cells at different times (0, 2, 4, 8, 12, 16, 24, 36, and 48 hpi) were analyzed by RT-PCR and western blot. β -actin mRNA or its encoded protein was detected under the same condition as an internal control

The subcellular localization of 13R and 13R Δ TM

The subcellular localizations of 13R and 13R Δ TM were investigated in GSTC by transfection with different GFP-containing recombinant plasmids. The green fluorescence of 13R-EGFP was observed in the cytoplasm under a fluorescence microscope, showing that 13R protein was localized in the cytoplasm. However, the 13R Δ TM-EGFP fusion protein was observed in both the cytoplasm and the nucleus, indicating that the TMD affects the protein localization in the cell (Fig. 3). The vector expressing EGFP, as a control, was distributed in the cytoplasm and the nucleus. Therefore, the results indicated that the TMD is necessary for the exclusive localization of 13R in the cytoplasm.

The co-localization between 13R and organelles were further investigated. As shown in Fig. 4, 13R (green fluorescence signals) was localized in the cytoplasm, consistent with the results described above. The red fluorescence

signals of ER, Mito, and Golgi were distributed in cytoplasm at specific organelle in the GSTC cells. The merged images showed that 13R co-localizes mostly with ER (yellow fluorescence signals), but not with the Golgi or Mito. Therefore, the results indicated that 13R co-localizes mostly with the ER in cytoplasm. It is worthy to note that the distribution of 13R was wider than that of ER.

To analyze the subcellular localization of 13R and 13R Δ TM during ADRV infection, the 13R-EGFP or 13R Δ TM-EGFP fusion protein was expressed under the control of the promoter of RGV *MCP*. The green fluorescence of 13R-EGFP was distributed in cytoplasm and nucleus at 24 hpi. But at 48 hpi, the fluorescence was highly enriched and co-localized with viromatrix (Fig. 5a), suggesting that 13R co-localizes with the viromatrix. Interestingly, 13R Δ TM was distributed in cytoplasm and nucleus at 24 hpi and 48 hpi, but did not aggregate into the viromatrix (Fig. 5b), indicating that the TMD affects the

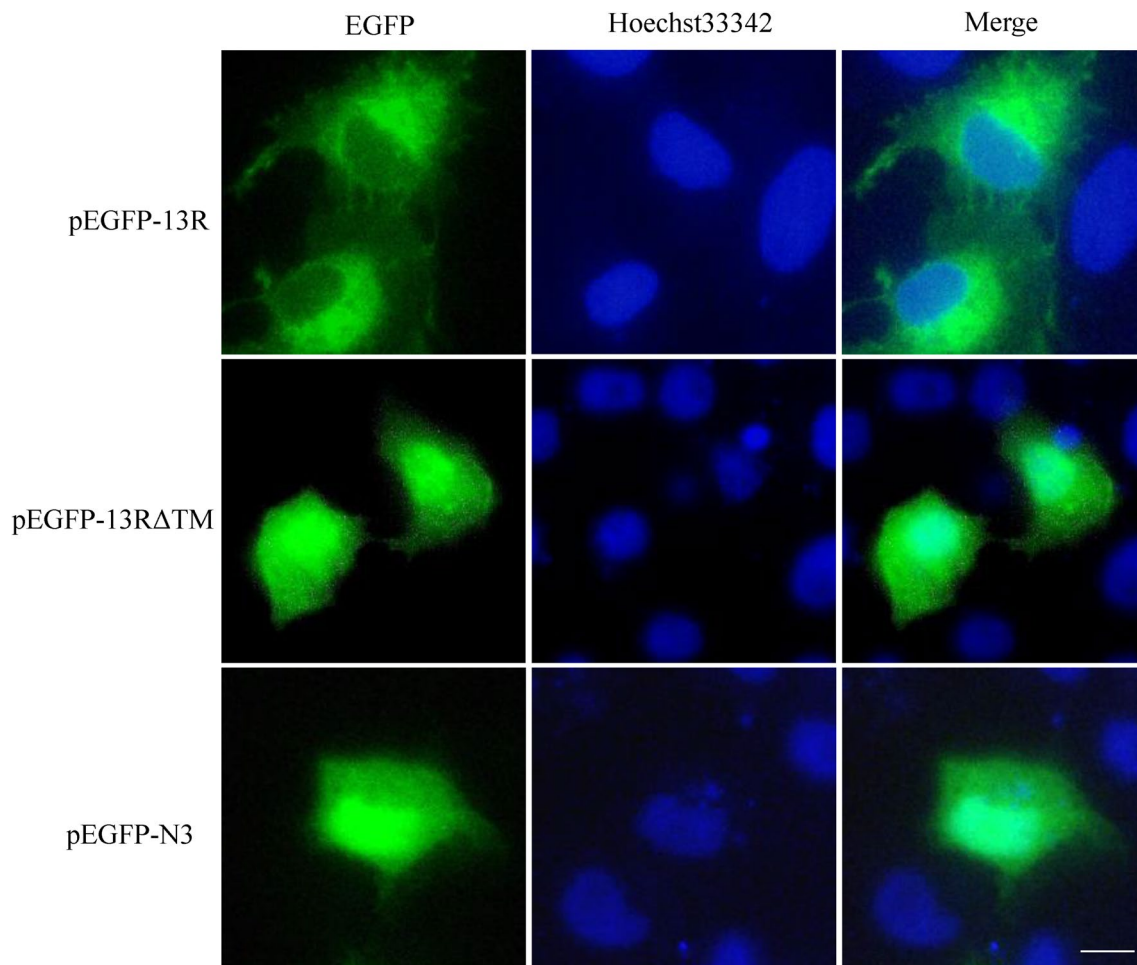


Fig. 3 The subcellular localization of 13R and 13R Δ TM in GSTC. GSTC cells transfected with plasmid pEGFP-13R, pEGFP-13R Δ TM or pEGFP-N3 were observed at 48 hpt. Green fluorescence signal

indicates 13R-EGFP, 13R Δ TM-EGFP or EGFP protein and blue fluorescence indicates the nuclei of GSTC. Scale bar, 5 μ m

13R co-localization with viromatrix. These results indicated that the TMD of 13R is crucial for co-localization into the viromatrix during viral infection.

13R is a viral non-structural protein and its overexpression did not contribute to virus titer

To identify whether 13R is a viral structural protein or not, the virions were purified with or without sucrose gradient centrifugation for western blot analysis. As shown in Fig. 6a, the 13R protein was not present in the purified virions, suggesting that 13R is a viral non-structural protein. As the controls, the viral structural protein ADRV MCP and the envelope protein ADRV 2L were detected in the virions. These results suggested that 13R is a viral non-structural protein.

ADRV replication kinetics was performed to study the role of 13R overexpression on virus titer in GSTC. As shown in Fig. 6b, although the ADRV titers in G/13R cells were

slightly higher than those of in the G/Con cells from 24 to 72 hpi, there was no significant difference ($P < 0.5$). The viral replication kinetics of the two groups from 0 to 16 hpi was no significant difference as well. Therefore, 13R overexpression did not contribute to the ADRV titers in GSTC.

Discussion

In large dsDNA virus, it is known that the transcription and expression of viral genes begin in the host cells after the viral infection and the immediate early (IE), delayed early (DE), and late (L) transcripts of the viral genes are synthesized in three temporal cascades in the viral life cycle (Ince et al. 2015; Jancovich et al. 2015; Majji et al. 2009). Generally, the products of viral L genes may function as structural proteins or membrane proteins. In our previous studies, numbers of structural protein genes and membrane protein genes were identified such as structural protein genes of RGV *MCP*, proliferating cell nuclear antigen protein gene

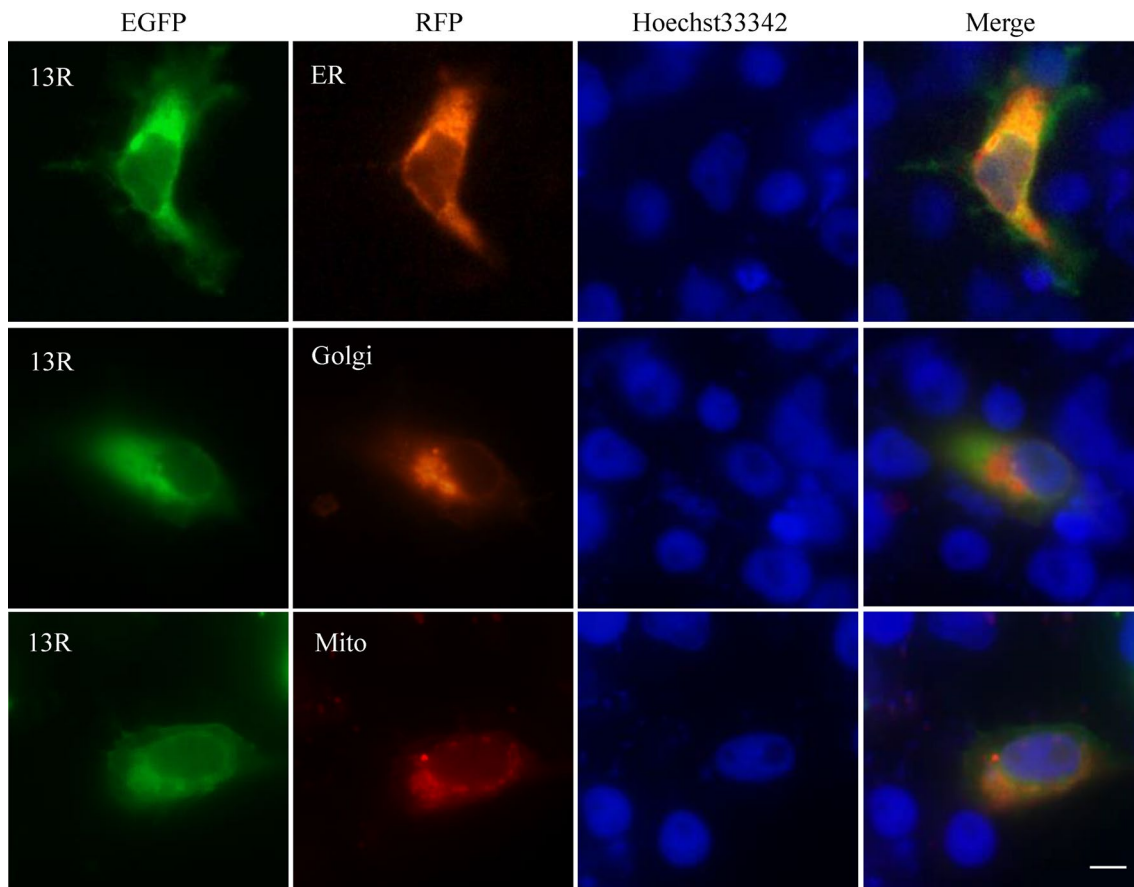


Fig. 4 Co-localization analysis of 13R with organelles in GSTC cells. The pEGFP-13R was co-transfected with plasmid pDsRed2-ER or pDsRed2-Mito or pDsRed2-Golgi, and subjected to fluorescence observation at 48 hpt. Green fluorescence showed the distribution

of fusion protein containing GFP; red fluorescence showed the distribution of cell organelles (ER, Mito, and Golgi apparatus) containing RFP; blue fluorescence showed the nucleus; yellow fluorescence showed the co-localization. Scale bar, 5 μm

(PCNA) and 50L (Lei et al. 2012), and membrane protein genes of RGV 43R and 53R (Zhao et al. 2008; Zeng et al. 2018). Bioinformatic analysis indicated that ADRV 13R contains a TMD and a restriction endonuclease-like domain, but the cellular localization and the function of the protein are still under investigation before this study.

Subcellular localization of the protein is demonstrated to be helpful to estimate the potential function (Cai et al. 2017). ADRV 13R co-localized with the ER in the cytoplasm, while

13R Δ TM was distributed in the cytoplasm and nucleus. However, the distribution patterns of 13R (aggregated in cytoplasm near the nucleus) and 13R Δ TM (uniform distribution in cytoplasm) in cytoplasm were different, implying that 13R may interact with protein in cytoplasm and its TMD affects this interaction. During viral infection, 13R was distributed in the cytoplasm and nucleus at 24 hpi, and western blot confirmed 13R expression began at this time. Subsequently, the protein aggregated into the viromatrix. However,

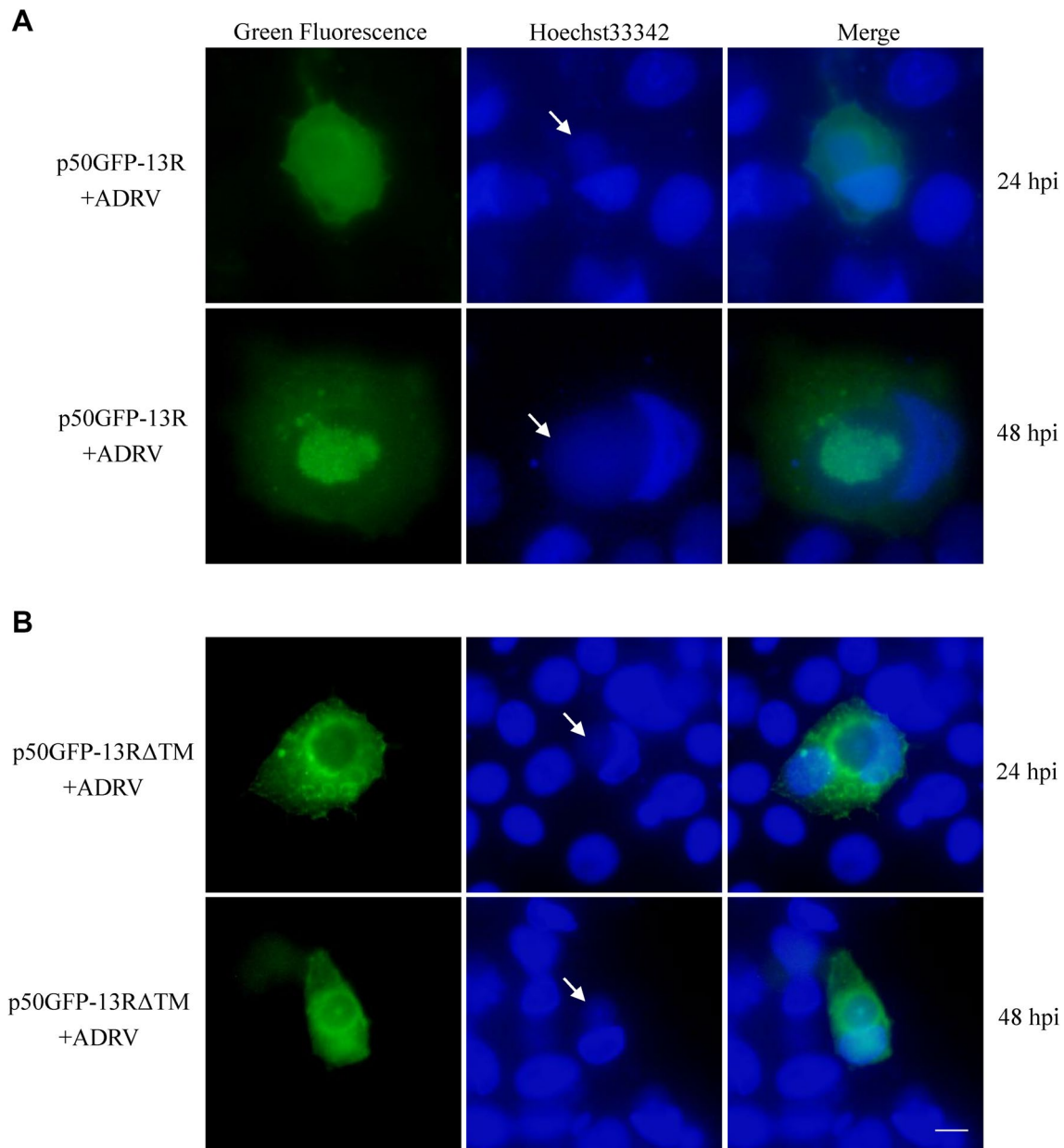


Fig. 5 The subcellular localization of 13R and 13R Δ TM in ADRV-infected GSTC. GSTC cells transfected with plasmid p50GFP-13R (**a**) or p50GFP-13R Δ TM (**b**) at 24 hpt were infected with ADRV for 24 hpi and 48 hpi, then the green fluorescence was observed.

13R-EGFP distributed initially in the cytoplasm and nucleus, and then aggregated into viromatrix (white arrows). However, 13R Δ TM-EGFP remained in cytoplasm and nucleus, but did not aggregate into viromatrix. Scale bar, 5 μ m

the 13R Δ TM remained in cytoplasm and nucleus, and did not co-localize with the viromatrix. These results indicated that 13R co-localization with the ER and viromatrix depends on its TMD. The ER is mainly involved in the synthesis of secreted protein and membrane protein, as well as the processes of protein glycosylation and acylation and so on (Braunger et al. 2018). Studies have shown that the viral protein co-localization with ER and viromatrix was related to the correct assembly of virions. For example, the E protein of yellow fever virus was modified in the ER and then involved in the proper formation of matured virions (Op De Beeck et al. 2004). Therefore, 13R may have a similar function, which is being processed and modified in the ER, and then involved in the correct formation of virions.

In this study, the purified virions were analyzed by western blot using the prepared 13R Δ N54 antibody, but no specific band of 13R was detected, indicating that the 13R is a viral non-structural protein. What are the functions of viral late non-structural proteins? RGV 95R is a late viral gene, presumably encoding a non-structural

protein with function of thiol oxidoreductase. Suppression of this protein expression did not affect the viral titer (Ke et al. 2009). *Helicoverpa armigera* nucleopolyhedrovirus (HearSNPV) ORF80 encoded a late viral non-structural protein that plays an important role in transporting viral proteins into the host nucleus (Wang and Zhang. 2007). The 13R containing the restriction endonuclease-like domain was predicted to maintain the fidelity of viral DNA molecules by participating in short fragment DNA repairing or methylation-mediated mismatches repairing. The core structure of this domain may have the function of enzymatic cleavage activity sites or the DNA-binding sites. Therefore, 13R may act as a catalytic enzyme during viral infection. The co-localization of this protein into the viromatrix provides a support for this bioinformatics prediction.

In summary, ADRV 13R encoded a viral late protein, which contains a TMD and a restriction endonuclease-like domain. Subcellular localization analyses indicated that the TMD of 13R is crucial for the co-localization into the ER and the viromatrix. Moreover, the 13R was identified as a viral non-structural protein and its overexpression did not affect the viral titers.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of financial or research interest.

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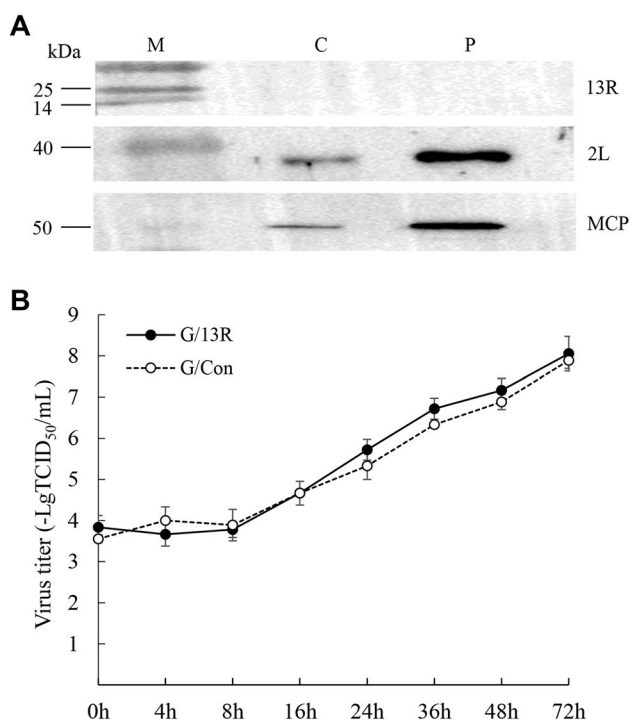


Fig. 6 Western blot detection of 13R and its effects on viral titers in GSTC. 13R was a viral non-structural protein by western blot detection (a). The purified virion suspensions prior to sucrose gradient centrifugation (lane C) and after sucrose gradient centrifugation (lane P) were collected. The envelope protein (ADRV 2L) and major capsid protein (ADRV MCP) were used as controls. Lane M, Trans Blue Plus II protein marker (14–120 kDa). ADRV replication kinetics on 13R expressed cells (G/13R) and vector expressed cells (G/Con) (b). The virus-infected cells were harvested at the indicated time points 0, 4, 8, 16, 24, 36, 48, and 72 hpi and the viral titers were determined using a TCID₅₀ assay

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