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Construction and Co-expression of Grass Carp Reovirus VP6 Protein and Enhanced Green Fluorescence Protein in the Insect Cells*

Qin FANG[1**], Eng Khuan Seng[2], Wen DAI[1#] and Lan-lan ZHANG[1,3]

(1. State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China; 2. School of Chemical & Life Sciences, Nanyang Polytechnic, 180 Ang Mo Kio Ave 8, 569830, Singapore; 3.Graduate school of the Chinese Academy of Sciences,Beijing 100039,China)

Abstract: Grass carp reovirus (GCRV), a disaster agent to aquatic animals, belongs to Genus *Aquareovirus* of family *Reoviridea.* Sequence analysis revealed GCRV genome segment 8 (s8) was 1 296 bp nucleotides in length encoding an inner capsid protein VP6 of about 43kDa. To obtain *in vitro* non-fusion expression of a GCRV VP6 protein containing a molecular of fluorescence reporter, the recombinant baculovirus, which contained the GCRVs8 and eGFP (enhanced green fluorescence protein) genes, was constructed by using the Bac-to-Bac insect expression system. In this study, the whole GCRVs8 and eGFP genes, amplified by PCR, were constructed into a pFastBacDual vector under polyhedron (PH) and p10 promoters, respectively. The constructed dual recombinant plasmid (pFbDGCRVs8/eGFP) was transformed into DH10Bac cells to obtain recombinant Bacmid (AcGCRVs8/eGFP) by transposition. Finally, the recombinant bacluovirus (vAcGCRVs8/eGFP) was obtained from transfected Sf9 insect cells. The green fluorescence that was expressed by transfected Sf9 cells was initially observed 3 days post transfection, and gradually enhanced and extended around 5 days culture in P1(Passage1) stock. The stable high level expression of recombinant protein was observed in P2 and subsequent passage budding virus (BV) stock. Additionally, PCR amplification from P1 and amplified P2 BV stock further confirmed the validity of the dual-recombinant baculovirus. Our results provide a foundation for expression and assembly of the GCRV structural protein in vitro.

Key Word: Grass carp reovirus (GCRV); Inner capsid protein VP6; Enhanced green fluorescence protein (eGFP); Baculovirus expression system; Co-expression

Pathogens which are members the family *Reoviridae* can infect a wide variety of organisms, including vertebrates, invertebrates, and plants. Genus *Aqureovirus*, the one of the members of the family *Reoviridae*, mainly cause infection in aquatic animals like bony fish and shellfish (28). Specifically, some of

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Undergraduate training student from College of Life Sciences, Wuhan University.

** Corresponding author. Tel: +86-27-87198551, Fax: +86- 27-87198072, E-mail : qfang@wh.iov.cn

aquareoviruse isolates can cause severe epidemic diseases in fish, such as hemorrhagic disease and pancreatitis, but the majority of isolated have been obtained during regular examination of seemingly healthy finfish and shellfish (19). Among all the aquareovirus isolates, Grass carp reovirus (GCRV), which was identified from a disastrous outbreak haemorrhage disease in fingerling and yearling grass carp from southern China, is considered to be the most pathogenic agent (24). In this regard, GCRV represents an ideal model for the study of the replication and pathogenesis of *Aquareovirus*.

As a member of the family *Reoviridae*, GCRV is a non-envelope multilayered spherical particle of icosahedral symmetry with a observed diameter of about 80 nm from results obtained with negative stained electron microscopy (20). The genome of GCRV enclosed in the inner core is composed of eleven segments of double stranded RNA (dsRNA). There are six or seven established sub-genogroup (*Aquareovirus* A-F and/or G) identified among the aquareovirus isolates, which is mainly on the basis of dsRNA genome electrophoretype and correlated RNA hybridization as well as analysis of their antigenic properties (20, 24). To date, more than 50 aquareovi ruses have been isolated throughout the world, but only a few isolates have been investigated in detail, such as GCRV and SBRV (striped bass reovirus), which belong to different species in the genus (1, 20). Recent genome sequences and phylogenic analyses of *Aquareovirus* showed that there was a common evolutionary origin with that of the mammalian orthreovirus since they shared a high level of sequence homology. Moreover, the virions of SBRV and GCRV, which were analyzed by electron cryomicroscopy (cryoEM) and three-dimensional (3D) single particle reconstruction, also showed many similarities with that of Mammalian reovirus (MRV) (8, 22, 27). Based on above similarities on both structure resembling and genome identities, there is an argument raised on taxonomy classification or evolution origin between *Orthoreoviruses* and *Aquareoviruses* (1, 15). *Orthoreoviruses* and *Aquareoviruses* have been attributed to a distinct genus in family *Reoviridae*, but the similarities and differences between the two genera in both molecular divergence and structure assembly/package in their infected cells remain a great mystery. So, a detailed study on GCRV structural protein may expedite our understanding of the mechanism involved in the assembly of dsRNA viruses.

While a significant amount of structural and biochemical information is available about several members of the family such as Orthoreovirus (4, 13，23, 26, 29, 30, 31), Rotavirus (5), Cypovirus (32) and Orbivirus (9), little is known about the aquareovirus at the molecular level. In an attempt to understand the infection and assembly mechanism of GCRV, it is indispensable to establish a stable system for the molecular genetic analysis of GCRV particles *in vitro*. In this paper, we report the construction and co-expression of Grass carp reovirus (GCRV) inner capsid non-fusion protein VP6 and enhanced green fluorescence protein (eGFP) in the insect cells for the first time, as a step moving towards an understanding of the structural basis of GCRV and its pathogenesis. Our results provide a reliable system for further *in vitro* expression and assembly GCRV particle by using baculovirus expression system.

MATERIALS AND METHODS

Cell and virus

Spodoptera frugiperda Sf9 insect cells (Invitrogen, Carlsbad, USA) were grown in Grace medium (Gibco BRL, Rockville, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Rockville, MD，USA) ,100 U/ml of penicillin and 100 mg of streptomycin (Sigma, St. Louis, USA). GCRV 873 strain, the third-passage infected CIK cell lysate stocks of plaque-purified suspension as described elsewhere (7), were stored in author's laboratory (14). The baculovirus expression system was from Invitrogen (Invitrogen, Carlsbad, USA). Plaque assays to determine the infectivity of the recombinant viruses were performed as described above (14).

Amplification and cloning of interest gene

The extraction of GCRV dsRNA genome was described elsewhere (6). RT-PCR of GCRV dsRNA segment 8 (GCRV s8), The PCR of eGFP amplification and cloning of interest gene segments were followed by previous report (6). Primers of GCRVs8 designated based on GenBank sequence (AF403394). The primers used for eGFP gene amplification was designed according to the vector of pEGFP-C1 (Clonetech, USA). The sense and antisense primers used for GCRV-S8 segment amplification is: S8-S(Sense)：5'-GCT***GAATCC***GTGATGGCACAGCGT-3'; S8-AS(Antisense): 5'-CAT***CTGCAG***AGCAGCC CGTCTCAG-3'. The primer pair used for GFP segment amplification is: eGFP-S: 5'-CT***CCCGGG***C GCCACCATGGTGAG-3', eGFP-AS: 5'- AC***CTCGA G***TTATGATCAGTTACT-3'. All the PCR reactions were carried out using 2.5 U of *Taq* polymerase (Gibco BRL, Rockville, USA) and 0.5 μmol/L of each primer. Thermal cycling parameters were as follows: one cycle of denaturation (94℃, 3min) followed by 38 cycles of denaturation (94℃, 30 sec), annealing (55℃, 60 sec) and extension (70℃, 2 min). The cycling program was ended by an extension step at 70℃ for 10 min. The amplicons were analyzed by agarose gel electrophoresis, then ligated into the PGEM-T cloning vector (Promega, Madison, USA). The recombinant vector was transformed into competent DH5α *E. coli* and the selected recombinant plasmids that contain the gene of interest were sequenced by Invitrogen Biotechnology Inc. (Shang- hai, China).

Construction of recombinant bacmid

To generate a recombinant baculovirus expressing both GCRV VP6 and eGFP, the GCRVs8 and eGFP genes were cut from a constructed PGEM-T cloning vector and then cloned into the pFastBacDual vector (Invitrogen，Carlsbad, USA), using the *Eco*R I and *Pst* I sites for GCRVs8 and the *Sma* I and *Xho* I sites for eGFP to generate pFbDGCRVs8/eGFP plasmid. This cloning strategy positioned the GCRVs8 and eGFP genes for transcription from the baculovirus polyhedrin (ph) and p10 promoters, respectively. The dual clone was then used to generate a recombinant baculovirus through the Bac-to-Bac system (Invitrogen, Carlsbad, USA). Once a pFbDGCRV-s8/ eGFP recombinant construction was generated, it was ready to transform the purified plasmid DNA into DH10Bac *E.coli*. The white positive colonies containing the recombinant bacmid were obtained by using kanamycin, gentamicin and tetracycline antibiotic selection. The recombinant bacmids (AcGCRVs8/ eGFP) were further identified by M13 primer based PCR amplification. The thermal cycling parameters were chosen based on segment size of amplification.

Sf9 cell transfection and Identification of recombinant virus

Sf9 cell and the cationic liquid Cellfectin reagent (Invitrogen, Carlsbad, USA) were used for AcGCRVs-8/eGFP recombinant bacmid transfection. The detailed transfection experiment was performed using the Bac to Bac expression kit according to the manufacturer's instructions. The empty Bacmid is used for transfection control. The analysis of transfected cell and protein expression was observed by utilizing inversed phase fluorescence microscopy. For further recombinant virus P1 (Passage1) and amplified stock identification, both GCRvs8/eGFP and M13 based primer PCR were used to confirm the correct insertion from the recombinant vAcGCRVs8/ eGFP budding virus (BV) extraction.

Budding viral amplification and titer determination

To produce stable vAcGCRVs8/eGFP budding virus in large amounts, Sf9 cells were infected with second-passage virus stocks at an MOI of 5 to 10 PFU/cell, and cells were harvested at 48 to 72h post infection. For viral titer determination, cells were grown in monolayers at 27°C in 24-well plates (Corning, USA). Confluent monolayers of cells were infected with 10-fold serially diluted of budding virus stock, each with 3 repeats. The infectious virus titer was determined by using 50% tissue culture infectious dose ($TCID_{50}$ /mL) assay with endpoints calculated by the methods of Reed & Muench (25).

RESULTS

Amplification of interest genes

To obtain the GCRVs8 and eGFP genes, a pair of 1.3kb GCRV S8 and 0.75kb GFP genes were amplified by both RT-TCR and regular PCR, respectively. The PCR amplified fragments are shown in Fig.1, and correspond closely to the predicted value.

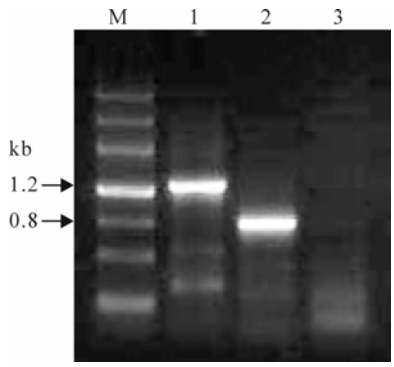

Fig. 1. The PCR amplifications of GCRV s8 and eGFP. M, DNA markerⅢ; 1, Positive amplification by using GCRVs8 as template (~1.3kb); 2, Positive amplification by using pEGFP-C1 as template (~ 0.75kb); 3, Negative amplification using ddH_2O as template.

Construction of donor plasmid

To construct co-expression vector, the amplified GCRVs8 and eGFP genes were cut from the constructed PGEM-T vector and inserted into pFast-BacDual vector downstream from the baculovirus polyhedron (PH) and p10 promoter, respectively. Positive recombinant plasmid pFbDGCRVs8/eGFP was identified by restriction enzyme digestion. Fig.2 showed the electrophoresis image of restriction enzyme digestion, which corresponded well to the predicted GCRVs8 and GFP gene size. Further sequence analysis confirmed the validity of the inserted

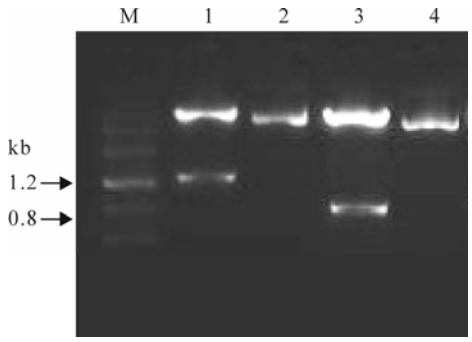

Fig. 2. Identification of pFbDGCRVs8/eGFP recombinant plasmid. M, DNA markerⅢ; 1, pFbDGCRVs8/eGFP digested with *Eco*R I and *Pst* I ; 2, pFbDGCRVs8/eGFP digested with *Eco*R I only; 3, pFbDGCRVs8/eGFP digested with S*ma* I and X*ho* I; 4, pFbDGCRVs8/eGFP digested with *Sma* I only.

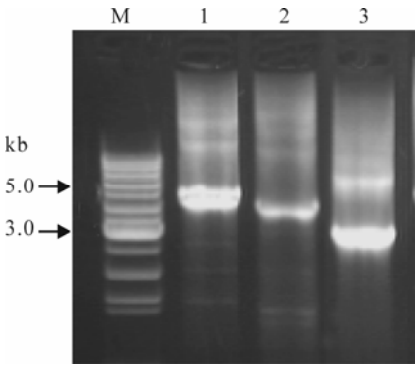

Fig. 3. The PCR analysis for recombinants AcGCRVs8/eGFP Bacmid. M, 1kbDNA Generuler; 1-3, Positive amplification by using M13-S+M13-AS(~4.6kb), GCRVs8-AS(~4.3kb), eGFP-AS (~2.8 kb) primer pairs, respectively.

segments (data not shown).

Identification of recombinant AcMNPV Bacmid

After obtaining recombinant pFbDGCRVs8/eGFP plasmid, the purified recombinant plasmid was transformed into DH10Bac competent cells for transposition into the Bacmid. The positive recombinant Bacmid (AcGCRVs8/eGFP) was screened by utilizing 3 antibiotics and white/blue colonies selection. Fig.3 showed the successful transposition by using M13 primer based PCR analysis.

Observation of transfected Sf9 cell

Sf9 cells were used to transfect recombinant Bacmid AcGCRVs8/eGFP to produce recombinant baculovirus. Green fluorescence was initially observed in ~20% of transfected recombinant Bacmid Sf9 cells that co-expressed GCRV VP6 and eGFP under inversed phase fluorescence microscopy 3 days post transfection (Fig.4 A,B). After another 2 days, More than 50% cells presented green fluorescence (Fig.4 C,D). To further amplify the recombinant budding virus, the clarified P1 viral stock was used to generate a high-titer P2 baculoviral stock by infecting Sf9 cells. Fig.4 F shows that more than 80% of Sf9 cells producing green fluorescence under uv light 48-72h post infection, indicating high-titer P2 recom- binant baculoviral stock (vAc-GCRV/eGFP) was obtained. But there is no green fluorescence observed in Sf9 cells by transfecting empty Bacmid as control (Fig.4 E).

Identification of recombinant Baculovirus

As described above, a high level co-expression recombinant baculoviral stock (vAcGCRV/eGFP) was obtained. To further identify whether GCRV-s8 and eGFP, the genes of interest, were included in the recombinant virus, the specific GCRVs8 and eGFP gene primers combined with M13 primer based PCR was conducted by using extracted budding virus genome DNA from transfected cell supernatant as template. Our data (Fig.5) showed the special seg-

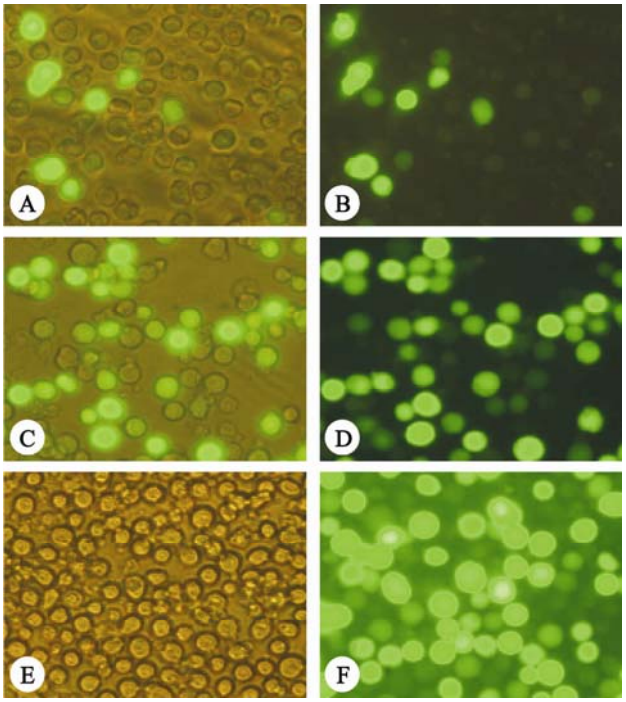

Fig. 4. Recombinant vAcGCRVs8/GFP virus expressed in Sf9 cells. **A/C**, Transfected Sf9 cells observed 3 days and 5 days post transfection under both visible and UV light; **B/D**: Corresponding image to A ,and C, only under UV light observation; **E:** Control cells, transfected empty Bacmid under normal light; **F**: Amplified P2 viral stock, observed under UV light.

ments by PCR amplification are corresponding to predicted size, which further confirmed the validity of our identified vAcGCRVs8/eGFP recombinant virus. The amplified P2 viral stock can be used for further scale up culture and protein characterization.

Virus titer determination

To determine the titer of recombinant viral stock for further scale-up proliferation, Sf9 cells were infected with second-passage viral stocks at an MOI of 5 to 10 PFU/cell, and cells were harvested at 48 to 72 h post infection. It is showed from Fig.6 that initial titer of the recombinant viral stock (P1) was around 6 log, an increase in viral titer (about 100 fold) was detected after amplified passage culture. The recombinant viral titer was in a stable status following P3 culture, indicating the high titer recombinant virus stock was obtained.

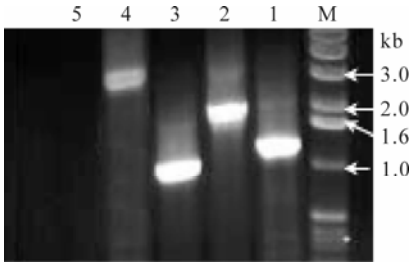

Fig. 5. Identification of recombinant vAcGCRVs8/eGFP virue by PCR. M, 1kb DNA ladder; 1-4, positive amplification by using GCRVs8-S+s8-AS (1.3kb), M13-AS (1.8kb) primer pairs, and eGFP-S+eGFP-AS(0.75kb), M13-S+eGFP-AS (2.8kb) primer pairs; 5, Negative control, transfected empty Bacmid cell extract as template, amplification by using M13-S+eGFP-AS primers.

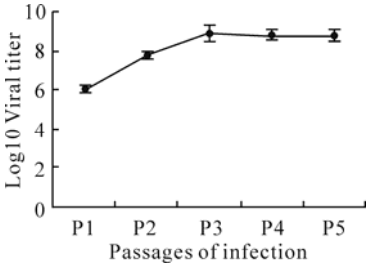

Fig. 6. Titers of recombinant vAcGCRVs8/eGFP virus determined from Passage 1 to Passage 5. Experiments were performed in triplicate, and the results are presented as the mean (± standard deviation).

DISCUSSION

The GCRV mature particle is composed of 7 proteins, VP1-VP7. Among the 7 structural proteins, there are 5 protein components (VP1, VP2, VP3, VP4 and VP6) involved in forming the viral core, and the remaining two proteins VP5 and VP7 comprise the outer capsid shell. Based on the comparative study between GCRV and MRV sequence and 3D structural images, the location and function of core component of GCRV VP1, VP2, VP3, VP4 and VP6 presented corresponded to λ2, λ3, λ1, μ2 and σ2 of MRV, respectively. The Protein VP5 and VP7 may relate to u1 and σ3 of MRV and play a role during virus infection (2-4, 18).

A recent report showed GCRV protein VP6, which is encoded by the GCRV s8 gene, is recognised as the counterpart of protein σ2 of MRV and occupied similar positions, exhibiting as the nodules on the surface of inner capsid (Cheng *et al*, unpublished data). Similar to the GCRV core, some reoviruses in the genus of cypovirus, which have been studied by cryoEM, have nodule proteins at positions the same as those of Aquareovirus, serving as clamps required for capsid assembly (12, 32). The core structures of Aquareovirus, Orthoreovirus and Cypovirus, named as the "turreted viruses", are distinguished from some other reovirus, such as Bluetongue virus (BTV) (11,21), and Rotavirus (5,17) by sharing additional features such as turrets and nodules. In contrast, the inner layers of Rotavirus, BTV, the so-called "smooth" viruses, don't have any decorated element on the core shell. The existence of the two kinds of core

structural organizations reflects the divergence of evolution within the family Reoviridae. It is reported the viruses in the "smooth virus" group lack the GCRV VP6-like protein, the proteins that constitute the innermost shell (VP2 and VP3 in virus and orbivirus, respectively) and which can self-assemble into icosahedral particles (9, 17, 21). But for "turreted viruses", such as well defined MRV, when reovirus λ1 is expressed in insect cells, however, no icosahedral particles form unless σ2 is also expressed (16, 26). This indicates that protein σ2 of MRV or VP6 of in "turrent virus" is indispensable for core shell assembly.

The Bac-to-Bac Baculovirus Expression System provides a rapid and efficient method to generate recombinant baculoviruses (10). pFastBacdual vector is a non-fusion vector, which contains two multiple cloning sites to allow simultaneous expression of two proteins; one controlled by the polyhedrin (PH) promoter and the other by the p10 promoter. In order to obtain an active and non-fusion expression of GCRV VP6 that combined a direct report of expressed protein from transfected cells, we use the strategy of inserting the eGFP gene downstream of a p10 promoter from the pFastBacDual vector, while the GCRVs8 gene is inset under PH promoter. We chose this method, rather than constructing a eGFPGCRV VP6 fusion Protein, because we wish to obtain the natural expression of GCRV VP6 protein and the long fusion tag of eGFP may affect natural properties of GCRV VP6 and the protein folding. In fact, using a dual vector to allow expression of two heterologous genes not only provides an independent expression of the two protein, but also performs a direct report of fluorescence to confirm recombinant expression from transfected cells.

We successfully constructed a co-expression vector and obtained high level co-expression of GCRV VP6 and eGFP in a baculovirus expression system. The non-fusional co-expression of GCRV VP6 and eGFP will not only provide useful evidence for establishing a stable system for further structural protein of GCRV expression, but also indicate the feasibility of co-expressing the GCRV inner core proteins VP3 and VP6 or other structural proteins to further finding out the basis of inner capsid or infectious virion assembly. The next work will be focused on characterization of GCRV VP6 and co-expression of other GCRV structural proteins *in vitro*.

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