

Enhanced production of porcine circovirus type 2 (PCV2) virus-like particles in Sf9 cells by translational enhancers

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Abstract

Objective To investigate the effect of three translational enhancers for enhancing transgene expression in baculovirus expression vector system using GFP as a reporter gene and selected translational enhancers to increase porcine circovirus type 2 (PCV2) VLPs production.

Results P10UTR (the 3'-untranslated region from the baculovirus p10 gene), Syn21 (a synthetic AT-rich 21-bp sequence) and P10UTR/Syn21 increased the GFP yield by 1.4-, 4- and 4.8-fold, respectively. While IVS (intron from *Drosophila* myosin heavy chain gene) decreased the GFP yield by 65 %. Moreover, the

synergy of P10UTR/Syn21 increased the yield of PCV2 VLPs by 4.1 fold (45 µg/10⁶ cells) compared with standard baculovirus vector.

Conclusion The synergy of P10UTR/Syn21 is a potential strategy to improve the recombinant vaccine production besides PCV2 VLPs in BEVS.

Keywords Baculovirus · Porcine circovirus type 2 · Sf9 cell · Translational enhancers · Virus-like particles

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Introduction

Porcine circovirus type 2 (PCV2) is the primary pathogen of porcine circovirus associated-diseases that cause huge economic loss in swine industry worldwide (Segales et al. 2005). Commercial PCV2 subunit vaccines are based on the viral capsid protein (Cap), which has the intrinsic ability to self-assemble into highly immunogenic virus-like particles (VLPs) (Fan et al. 2007; Kim et al. 2002). The baculovirus expression vector system (BEVS) has been used as one of the preferred platforms for the production of VLPs (Summers 2006). However, the relatively low expression yield of BEVS limits the development of current PCV2 VLPs vaccine.

In light of this limitation, we focused on the translational enhancers, which have the potential to increase protein production in the BEVS (Ezure et al.

2010; Iizuka et al. 2008; Royall et al. 2004). Vectors containing the 3'-UTR from the *Autographa californica* nucleopolyhedrovirus (AcNPV) p10 gene (P10UTR) increased the gene expression efficiency compared with the vectors containing the simian virus 40 (SV40) 3'-UTR in *Drosophila* and P10 promoter-based BEVS (van Oers et al. 1999, 2001; Pfeiffer et al. 2012). However, none of the current polh promoter-based BEVS employs P10UTR for protein expression. Moreover, Syn21 and IVS methods have also increased protein expression levels in *Drosophila* (Pfeiffer et al. 2010, 2012) but whether these enhancers would function in BEVS is still unknown. Because the influence of P10UTR, Syn21, and IVS for the expression of recombinant protein driven by polh promoter has not been evaluated, the current study firstly aimed to investigate their influence on gene expression in polh-based baculovirus vectors.

Three translational enhancers have been investigated to enhance transgene expression in Sf9 cells using green fluorescence protein (GFP) as reporter. Syn21 and P10UTR were selected as a translation-enhancer combination for enhancing the production of the PCV2 VLPs– and synergistically increased the yield of PCV2 VLPs by 4.1-fold compared with that production using a control baculovirus vector. This procedure is a hitherto unreported novel approach to improve the production of VLPs in Sf9 cells.

Materials and methods

Construction of expression vectors containing different translational enhancers

Three translational enhancers (IVS, Syn21 and P10UTR) were employed to construct eight expression cassettes for GFP in *Spodoptera frugiperda* Sf9 cells. All primers used in this study are listed in Supplementary Table 1. The assembly of pFBDM-control-GFP and pFBDM-Syn21-GFP are used as examples below to illustrate the procedure for the vectors construction. The GFP and Syn21-GFP genes were amplified from pEGFP-N1 plasmid (Clontech) using primer pairs GFP-F/R and Syn21-GFP-F/GFP-R, respectively. The amplified gene fragments were introduced into the EcoRI and SalI sites of pFBDM (Invitrogen) to generate pFBDM-control-GFP and pFBDM-Syn21-GFP, respectively. The translational enhancer P10UTR was amplified from genomic

DNA of AcNPV with primers P10UTR-F and P10UTR-R. A 67 bp IVS sequence was amplified from the *Drosophila* myosin heavy chain gene with primers IVS-F and IVS-R. Then pFBDM-P10UTR-GFP (P10UTR), pFBDM-Syn21-GFP (Syn21), pFBDM-I/S-GFP (IVS and Syn21), pFBDM-I/P-GFP (IVS and P10UTR), pFBDM-S/P-GFP (Syn21 and P10UTR), and pFBDM-I/S/P-GFP (IVS, Syn21, and P10UTR) were constructed on the basis of pFBDM-control-GFP, as described previously. The resulting plasmids were sequenced transformed into DH10Bac competent cells to obtain recombinant bacmids. The recombinant bacmids were then extracted and transfected into Sf9 cells using Cellfectin II Reagent (Invitrogen) as directed by the manufacturer. The P1 viruses were collected after incubation of the transfected cells at 28 °C for 7 days.

Observation of expression of GFP from recombinant baculoviruses

Sf9 cells were cultured in 6-well cell culture plates (1×10^6 cells/well). Recombinant baculoviruses were used to infect the cells at multiplicity of infection (MOI) of 10. Fluorescence images of infected cells expressing GFP were obtained by fluorescence microscopy; GFP fluorescence was measured (excitation 485 nm; emission 510 nm) by using a Varioskan Flash spectral scanning multimode reader (Thermo Scientific) at 96 h post-infection (hpi). Then the infected cells were collected and incubated with RIPA buffer (50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 % NP-40) for 30 min on ice. The cell lysate was mixed with protein sample buffer and then boiled. The protein samples were subjected to Western blotting. Mouse anti-GFP antibody (Abcam, Cambridge, MA, USA) was used as primary antibody. Horseradish peroxidase (HRP)-coupled goat anti-mouse IgG antibody (Abcam, Cambridge, MA, USA) was used as secondary antibody. The bound antibody was detected using a cECL Western blot kit (ComWin Biotech, Beijing, China) according to the manufacturer's instructions. Mouse anti- β -actin antibody (Abcam, Cambridge, MA, USA) was used as the internal control.

Recombinant viruses carrying PCV2 Cap genes

Two expression vectors, namely pFBDM-control-Cap and pFBDM-S/P-Cap (see Fig. 2a below) were constructed to determine whether Syn21 and P10UTR

could synergistically increase the expression of the PCV2 Cap protein. Briefly, the Cap gene from PCV2 with 6 × His tagged at its C-terminus was amplified with primers Cap-F and Cap-R. In addition, the Syn21-Cap gene was amplified with primers Syn21-Cap-F and Cap-R. After digestion with *Bam*HI and *Sal*II, the PCR products were inserted into plasmids pFBDM and pFBDM-P10UTR-GFP. The result transfer vectors were named pFBDM-control-Cap and pFBDM-S/P-Cap. Recombinant baculoviruses BV-control-Cap and BV-S/P-Cap were generated in the same way as described earlier.

Western blotting

The recombinant viruses containing PCV2 Cap genes were expressed as C-terminally 6 × His-tagged fusion proteins in Sf9 cells, which subsequently were homogenized in phosphate-buffered saline (PBS, pH 7.4) at 96 hpi. The fusion protein samples were subjected to Western blotting analysis after SDS-PAGE. Rabbit anti-Cap antibody (made in our laboratory) was used as primary antibody and HRP-coupled goat anti-rabbit IgG antibody (Abcam, Cambridge, MA, USA) was used as secondary antibody, a cECL Western blot kit was used to visualize signals.

Immunofluorescence

Sf9 cells were seeded in 24-well cell culture plates (2×10^5 cells/well) and infected with recombinant baculoviruses. Cells were fixed with 4 % (v/v) paraformaldehyde at 48 hpi and washed three times with PBS. Immunostaining was performed with mouse anti-His antibody (Abcam, Cambridge, MA, USA) as primary antibody and Alexa Fluor 594 donkey anti-mouse IgG antibody (Invitrogen, China) as secondary antibody. Cells were washed five times with PBS after incubation with each antibody. Digital images were captured using fluorescence microscopy.

Purification and quantification of baculovirus expressed PCV2 VLPs

Sf9 cells, 10^8 , were infected with recombinant baculoviruses at MOI of 1. The cells were harvested at 72 hpi and lysed by sonication. Subsequently, the lysate was centrifugated at $12,000 \times g$ for 10 min at 4 °C and the supernatant was collected for Cap

purification according to the handbook of Ni-NTA columns. The VLPs formed from Cap self-assembly was dialyzed against PBS and further confirmed by transmission electron microscopy (TEM). The yield of PCV2 VLPs was quantified by Bradford method (see Noble and Bailey 2009).

Transmission electron microscopy

VLPs were applied onto carbon coated grids and stained with 2 % (w/v) phosphotungstic acid. Grids were viewed using a transmission electron microscope operating at 200 kV.

Statistical analysis

Data analysis was performed with Prism 5 software (GraphPad). Student's *t* test and One-way ANOVA followed by Tukey's post hoc test were used to determine significant difference. Data were presented as mean \pm standard deviations (SD) from three independent experiments. A *p* value < 0.05 was considered statistically significant.

Results and discussion

Effect of different translational enhancers on GFP expression

Previous studies have shown that the three translational enhancers (Syn21, P10UTR and IVS) can enhance protein production under the control of Hsp70 promoter in *Drosophila* and P10 promoter in BEVS. However, it is still unknown whether these translational enhancers could be applied to enhance transgene expression under the control of most widely used promoter of polyhedron (polh) in BEVS (Ayres et al. 1994; Smith et al. 1985). Thus, the present study investigated whether these known translational enhancers would function in enhancing the transgene expression under the control of polh promoter for the first time.

To evaluate the effect of the translational enhancers on the expression of the GFP reporter, eight expression cassettes with different translational enhancers were constructed as shown in Fig. 1a. Recombinant baculoviruses generated from these plasmids were used to infect Sf9 cells and the emission of green fluorescence

in the cells was observed at 96 hpi (Fig. 1b). Then the GFP expression level was further confirmed using western blot analysis of the total cellular extract with Mouse anti-GFP antibodies (Fig. 1c).

The expression level of GFP was increased considerably by the translational enhancer Syn21. To provide further evidence supporting the results described above, a quantitative analysis was performed to compare the production yield of GFP by using a Varioskan Flash spectral scanning multimode reader. As shown in Fig. 1d, Syn21 and P10UTR increased the expression of GFP by factors of 4.04 ± 0.12 times ($n = 3$) and 1.35 ± 0.02 times ($n = 3$), respectively, whereas IVS decreased the

expression by a factor of 0.65 ± 0.02 times ($n = 3$). The results also showed that Syn21 and P10UTR have synergistic effects and increased the GFP yield by 4.8 ± 0.08 fold ($n = 3$). As a result, it could be concluded that the combination use of Syn21 and P10UTR is the most effective candidate for foreign protein production among the selected three translation enhancers.

Syn21/P10UTR synergistically enhanced the expression of the PCV2 Cap protein

Based on the results above, we asked whether the combination of Syn21 and P10UTR would have

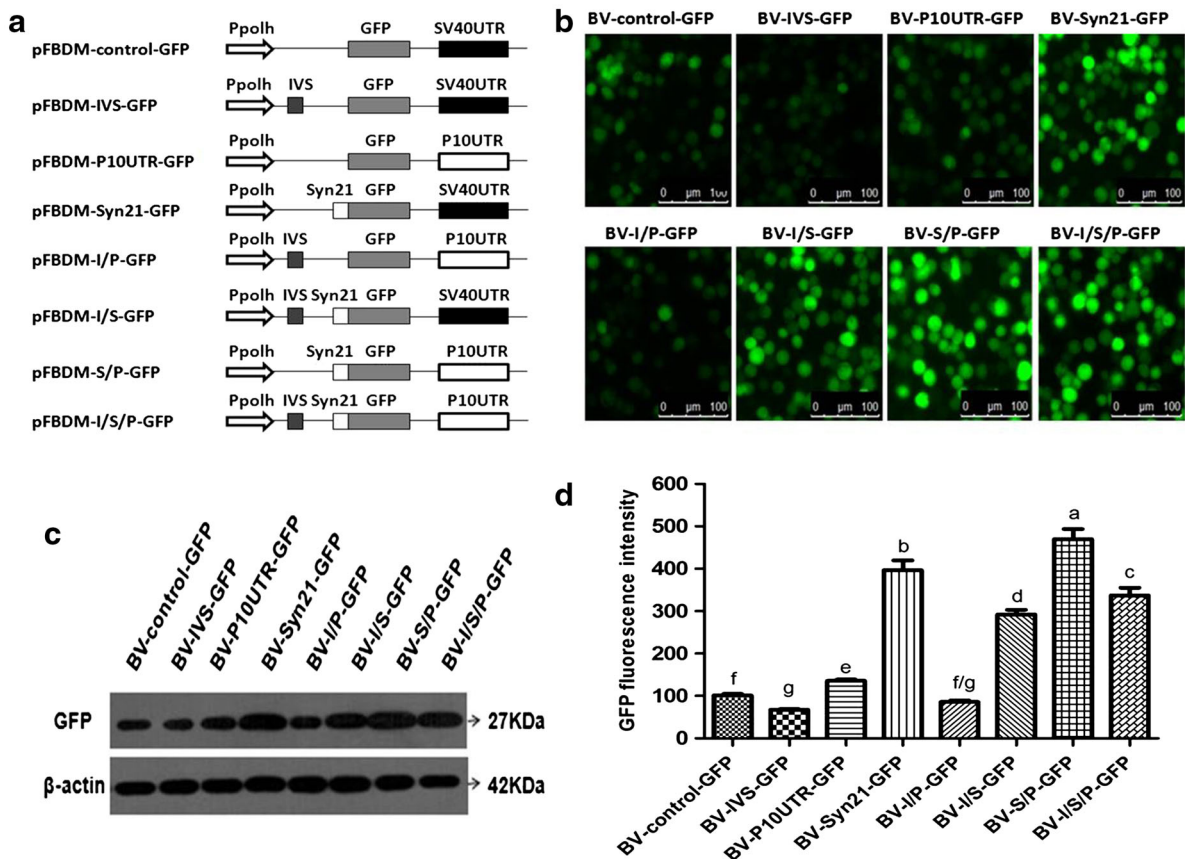


Fig. 1 Effect of translational enhancers on GFP expression. **a** Schematic diagrams of the expression plasmids used in this study. **b** Fluorescence microscopy of SF9 cells showing the expression of GFP at 96 hpi. **c** GFP expression was determined in cell lysates by western blotting. Mouse anti-GFP antibody was used as primary antibody and HRP-coupled goat anti-mouse IgG antibody was used as secondary antibody. β -Actin was used as the internal control. **d** The GFP expression level analyzed

with Varioskan Flash spectral scanning multimode reader. Data are shown as mean \pm SD from three independent experiments. One-way ANOVA followed by Tukey's post hoc test was used to determine significant difference between groups. When two sets of data are labeled with superscripts of *different letters*, it indicates that these sets of data are statistically different ($P < 0.05$)

synergistic effects on gene expression of PCV2 Cap. To examine this hypothesis, we constructed baculovirus vectors that included both Syn21 and P10UTR (pFBDM-S/P-Cap), and compared the Cap expression levels relative to Cap vectors lacking the Syn21 and P10UTR (pFBDM-control-Cap) (Fig. 2a. The expression of Cap in baculoviruses-infected cells was then determined by western blotting. We observed that Cap expression level in BV-S/P-Cap infected cells was significantly higher than that of BV-control-Cap infected cells (Fig. 2b. We also analysed the Cap expression in Sf9 cells infected with different baculoviruses by immunofluorescence. Consistent with the result of Western blotting, the stronger red fluorescent intensity was appeared in BV-S/P-Cap infected Sf9 cells than BV-control-Cap infected Sf9 cells (Fig. 2c. These results indicated that Syn21 and P10UTR could synergistically enhance the expression efficiency of PCV2 Cap.

Combined use of Syn21 and P10UTR enhances the yield of PCV2 VLPs

As recombinant Cap expressed in insect cells could self-assemble into VLPs (Nawagitgul et al. 2000), the purified Cap was analyzed by TEM to verify the proper assembly of PCV2 VLPs post BV-S/P-Cap infection. As shown in Fig. 3a, the purified Cap protein assembled into VLPs with diameters ranging from 15 to 20 nm. To further confirm whether simply different expression levels of the Cap protein correlate with the improved VLP yield, the purified PCV2 VLPs were quantified using a Bradford protein assay kit. The yield of PCV2 VLPs in cells infected with BV-S/P-Cap was estimated to be $45 \mu\text{g}/10^6$ cells, which has been increased by 4.1-fold compared with that of BV-control-Cap infected cells (Fig. 3b). These results demonstrate that combined use of Syn21 and P10UTR can effectively increase the yield of PCV2 VLPs.

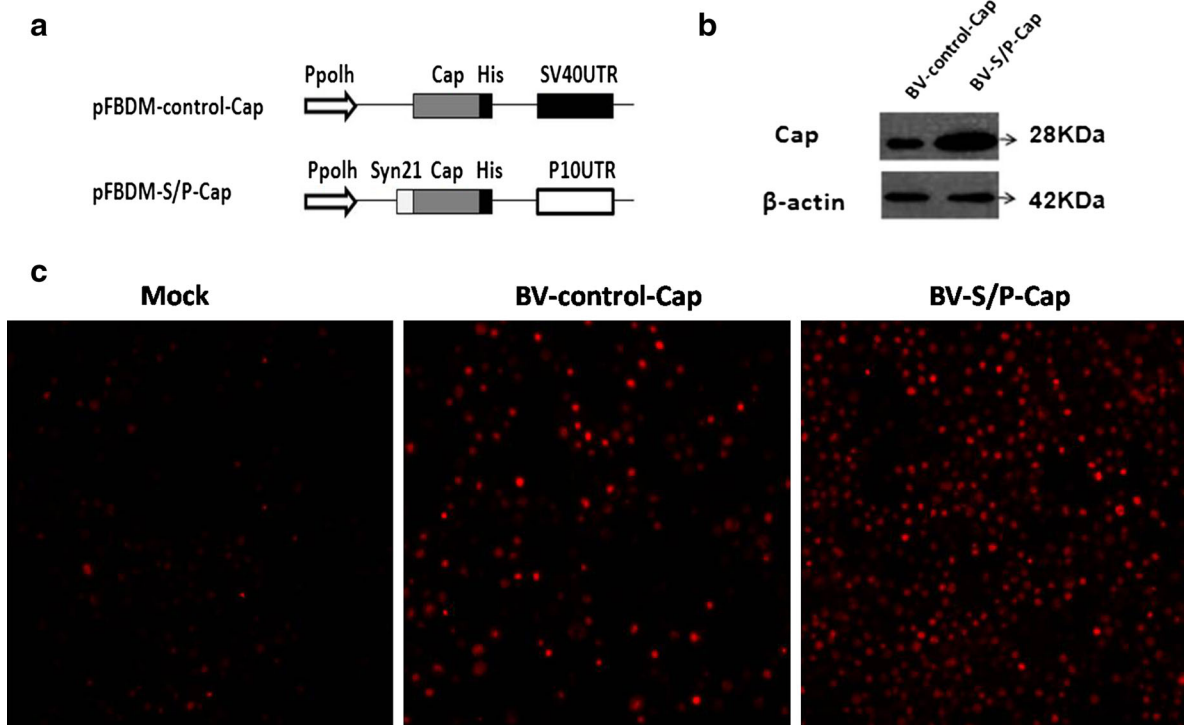


Fig. 2 Increased expression of the PCV2 Cap protein by the combination use of Syn21 and P10UTR. **a** Construction of recombinant baculovirus vectors. **b** Western blot analysis of the Cap protein expression in Sf9 cells at 96 hpi. Rabbit anti-Cap antibody was used as primary antibody and HRP-coupled goat

anti-rabbit IgG antibody was used as secondary antibody. β -Actin was used as the internal control. **c** Detection of PCV2 Cap in infected insect cells by immunofluorescence with Mouse anti-His antibody

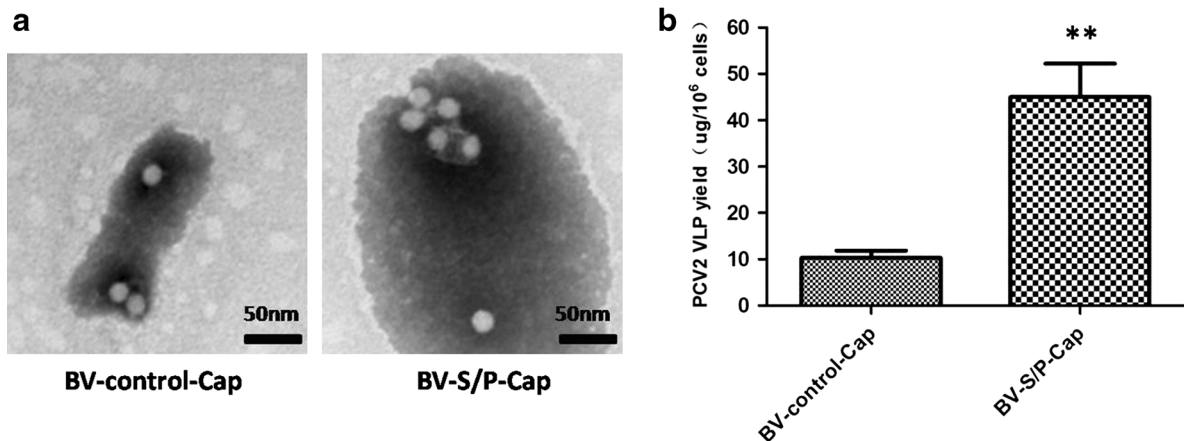


Fig. 3 Improved yield of PCV2 VLPs by the combination use of Syn21 and P10UTR. **a** Electron microscope images of Cap protein VLPs of PCV2. Scale bars indicates 50 nm. **b** The amount of VLPs in the baculoviruses-infected cells after

purification was quantified by Bradford method. Data are mean \pm SD from three independent experiments. Columns with an asterisk were significantly different as calculated with Student's *t*-test (***P* value < 0.05)

Conclusion

To screen robust translation enhancer/enhancers to increase the yield of PCV2 VLPs, we studied the effect of three translational enhancers on increase the expression level of heterologous protein in Sf9 cells using GFP gene as a reporter. Syn21 and P10UTR had synergistic effects to enhance the expression efficiency of GFP reporter. Then we confirmed that P10UTR and Syn21 could synergistically enhance the production of PCV2 VLPs as for GFP. And the yield of PCV2 VLPs was increased by 4.1-fold compared with a standard baculovirus vector. Therefore, we concluded that P10UTR and Syn21 was an effective translational-enhancer combination to improve PCV2 VLPs vaccines production and has great potential as universal strategy to improve the production of other VLPs and subunit vaccines.

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Supporting information Supplementary Table 1—Primers used in this study.

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