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Identification and functional analysis of the GTPV bidirectional promoter region

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Abstract The goat pox chick embryo-attenuated virus (GTPV) has been developed as an effective vaccine that can elicit protective immune responses. It possesses a large genome and a robust ability to express exogenous genes. Thus, this virus is an ideal vector for recombinant live vaccines for infectious diseases in ruminant animals. In this study, we identified a novel bidirectional promoter region of GTPV through screening named PbVV($\pm$). PbVV($\pm$) is located between ETF-l and VITF-3, which are transcribed in opposite directions. A new recombinant goat pox virus (rGTPV) was constructed, in which duplicate PbVV($+$) was used as a promoter element to enhance *Brucella* OMP31 expression, and duplicate PbVV($-$) was used as a promoter element to regulate enhanced green fluorescent protein (EGFP) at the same time as the selection marker. PbVV($-$) promoter activity was compared to that of the P7.5 promoter of vaccinia virus, as measured by EGFP expression; the fluorescence intensity of EGFP expressed in cells was confirmed by fluorescence microscopy and flow cytometry. PbVV($+$) promoter activity was measured by *Brucella* OMP31 expression. Interaction with the anti-*Brucella*-OMP31 monoclonal antibody was confirmed by western blotting, and OMP31 mRNA expression was assessed by qRT-PCR. The results of this study will be useful for the further study of effective multivalent vaccines based on rGTPV. This study also provides a theoretical basis for overcoming the problem of low expression of exogenous genes.

Keywords Goat pox virus · Bidirectional promoter · Vector · Omp31

Introduction

Goat pox is a disease of goats that is caused by goat pox virus (GTPV) (Venkatesan et al. 2012). The virus belongs to the Capripoxvirus (CaPV) genus of the Poxviridae family and is closely related to sheep pox virus (SPPV) and lumpy skin disease virus (LSDV) (Tulman et al. 2002). Both GTPV and SPPV are responsible for some of the most economically significant diseases of domestic ruminants in Central and Northern Africa, the Middle East, the Indian subcontinent, Central Asia, and parts of China (Zhou et al. 2012). CaPVs are generally considered to be host-specific because disease outbreaks tend to occur preferentially, and virus isolates usually cause disease in one host species (Tulman et al. 2002). The most recent outbreaks include instances in Mongolia in 2008 and 2009, an isolated outbreak in Greece in 2008, and occurrences in Kazakhstan and Azerbaijan in 2009. Goat pox has been established in Vietnam since 2005. The first goat pox outbreak in Chinese Taipei occurred in 2008, and in 2010 the disease recurred and was declared endemic. In 2012, the first report of an outbreak of sheep pox associated with GTPV was made in

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Gansu Province, China (Yan et al. 2012). Goat pox carries a high economic burden in developing countries.

The GTPV genome is 150 kb of double-stranded DNA, which encodes at least 147 open reading frames (ORFs), including conserved replicative and structural genes and other genes that are likely involved in virulence and host range (Zhao et al. 2012). GTPV has many advantages: (1) it can provide strong protection against virulent strains of goat pox; (2) its large genome allows it to carry exogenous genes; and (3) it does not infect humans and has a good biosafety profile in humans health. This virus is an ideal vector for recombinant live vaccines against infectious diseases in ruminant animals.

Currently, the expression of exogenous genes is dependent on promoters in recombinant pox viruses. The promoters of pox virus (Cochran et al. 1985; Weir and Moss 1987; Davison and Moss 1989a, b; Baldick et al. 1992) and fowl pox virus (Kumar and Boyle 1990a, b; Zantinge et al. 1996) have advantages for expressing the exogenous genes used for vaccines. However, the regulatory elements in GTPV have rarely been studied, and the bidirectional promoter of GTPV has not yet been investigated. Researchers have proposed that recombinant GTPV can be used to take advantage of the strong vaccine virus promoter to express exogenous genes (Romero et al. 1993, 1994, 1995; Wade-Evans et al. 1996). The expression of target genes is generally higher with a homologous recombinant virus promoter compared with a heterologous recombinant virus promoter (Boyle 1992). Therefore, understanding the GTPV promoter is required to construct an efficient recombinant GTPV vaccine vector.

The aim of the current study was to conduct a screen to identify a bidirectional promoter for goat pox virus that has a high capacity to express exogenous genes. A novel bidirectional promoter, PbVV(±), was found between the early transcription factor, VETE-1, and the middle transcription factor, VITF-3, in the GTPV genome. A novel approach based on duplicate PbVV(±) was developed in which rGTPV was constructed by fusing the promoter fragment with the *egfp* and *Brucella omp*31 genes; the expression levels of those genes were then detected.

Materials and methods

Cells, viruses, plasmids, and antibody

The GTPV-permissive Vero cell line was obtained from Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, Rockville, MD, USA) supplemented with 10 % fetal bovine serum (FBS, Gibco Life Technologies, Rockville, MD,

USA) at 37 °C with 5 % CO_2 (vol./vol.). The GTPV-attenuated vaccine AV41, an attenuated live vaccine, was provided by Xinjiang Tecon Animal Husbandry Biotechnology Co. (Urumqi, China). rGTPV-TK-P7.5-EGFP and rGTPV-TK-P11-Omp31 were constructed in our laboratory. pUC119 and pMD18-T simple were provided by TaKaRa Biotechnology (Dalian) Co., Ltd (Dalian China). The PEGFP-N1 plasmid vector was maintained by the Key Laboratory of Xinjiang Endemic and Ethnic Disease (Shihezi, China). The anti-Omp31, anti-Bp26, and anti-Omp19 monoclonal antibodies (MAbs) were kindly provided by Dr. Wenjing Wang (Department of Transfusion Medicine, Southern Medical University, Guangzhou, China).

Construction of the transfer vector pUC119-GTPV-TK

GTPV genomic DNA was extracted from infected Vero cells, and PCR was performed using customized primers (Table 1) to amplify the attachment gene of GTPV. A volume of 2 µL of extracted DNA was used as a template in a 20-µL reaction mixture system containing 0.2 µL of each primer (TK-F and TK-R, 0.4 µM final concentration of each primer), 10 µL 2 × Ex Taq Mastermix DNA polymerase (CWBIO, China, Cat#CW0690) and 7.6 µL ddH₂O. PCR was performed using a SensoQuest LabCycler standard plus (SensoQuest GmbH, Goettingen, Germany) with an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 50 s and extension at 72 °C for 4 min. There was a final extension at 72 °C for 10 min. The PCR amplification products were analyzed using 1.0 % agarose gel electrophoresis. The desired PCR product was purified using a multifunction DNA purification kit (BioTeke Corporation, Cat#DP1502).

The thymidine kinase (TK) products were ligated into the pMD18-T simple vector to generate pMD-TK, which was then transformed into *E. coli* DH5α competent cells. Positive clones were screened by PCR using the primers TK-F and TK-R. The recombinant plasmid was extracted from positive clones using a high-purification plasmid mini-preparation kit (BioTeke Corporation, Cat#DP1002). Then, the pMD-TK plasmid and pUC119 vector were treated with *Xba*I and *Sac*I and ligated into *Xba*I/*Sac*I-digested pUC119 to generate pUC119-GTPV-TK. The *Kpn*I restriction enzyme was used to digest sites flanking the TK gene in pUC119-GTPV-TK.

Construction of the bidirectional promoter clone vector pMD-PbVV(±)-EGFP-Omp31

The recombinant transfer vector, pMD-PbVV(±)-EGFP-Omp31, was constructed by fusion PCR. The EGFP fragment was amplified by PCR from the PEGFP-N1 plasmid

using the primer pair PbVV(−)-EGFP-F and PbVV(−)-EGFP-R (Table 1). The *omp*31 fragment was amplified by PCR from heat-killed *Brucella* using the primer pair PbVV(+)-Omp31-F and PbVV(+)-Omp31-R (Table 1). A volume of 1 µL of extracted DNA was used as the template in a 20-µL reaction mixture containing 2.0 µL 10 × Pfu Buffer (TIANGEN Biotech Co., Ltd, Beijing, China, Cat# EP101-01), 0.2 µL of each primer (EGFP-F and EGFP-R, 0.4 µM final concentrations of each primer), 1.4 µL of deoxynucleoside triphosphates (dNTPs, 10 mM), 2.5 µL of dye, 0.5 µL of Pfu DNA polymerase (2.5 U/µL, TIANGEN Biotech Co., Ltd, Beijing, China, Cat# EP101-01), and 13.2 µL of ddH$_2$O. PCR was performed using a SensoQuest LabCycler standard plus (SensoQuest GmbH, Goettingen, Germany) with 5 min of denaturation at 95 °C, followed by 30 cycles of denaturation at 94 °C for 50 s, annealing at 57 °C for 35 s and extension at 72 °C for 1 min and then a final extension at 72 °C for 10 min. The PCR amplification products were analyzed using 1.5 % agarose gel electrophoresis. The desired PCR product was extracted using a multifunction DNA extraction kit (CWBIO, China, Cat#CW0511).

The EGFP DNA fragment and *omp*31 DNA fragment were mixed at a ratio of 1:1. The PbVV(±)-EGFP-Omp31 fragment was amplified by PCR using the primer pair PbVV(−)-EGFP-F and PbVV(+)-Omp31-R (Table 1). A

volume of 5 µL of extracted DNA was used as a template in a 25-µL reaction mixture system containing 0.4 µL of each primer (PbVV(−)-EGFP-F and PbVV(+)-Omp31-R, 0.4 µM final concentrations of each primer), 12.5 µL 2 × Ex Taq Mastermix DNA polymerase (CWBIO, China, Cat#CW0690) and 6.7 µL ddH$_2$O. PCR was performed using a SensoQuest LabCycler standard plus (SensoQuest GmbH, Goettingen, Germany) with an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C for 40 s and extension at 72 °C for 2 min. There was a final extension at 72 °C for 10 min. The PCR amplification products were analyzed using 1.0 % agarose gel electrophoresis. The PCR products were extracted using a multifunction DNA extraction kit (CWBIO, China, Cat#CW0511).

The PbVV(±)-EGFP-Omp31 products were ligated into the pMD18-T simple vector to generate pMD-PbVV(±)-EGFP-Omp31, which was then transformed into *E. coli* DH5α competent cells. Positive clones were screened by PCR using the primers PbVV(−)-EGFP-F and PbVV(+)-Omp31-R. The recombinant plasmid was extracted from positive clones using a high-purification plasmid mini-preparation kit (BioTeke Corporation, Cat#DP1002).

Construction of the bidirectional promoter transfer vector pUC119-PbVV(±)-EGFP-Omp31

The pMD-PbVV(±)-EGFP-Omp31 plasmid and pUC119-GTPV-TK vector were treated with *Kpn*I and ligated into *Kpn*I-digested pUC119-GTPV-TK to generate pUC119-PbVV(±)-EGFP-Omp31, which was then transformed into *E. coli* DH5α competent cells. Positive clones were screened by PCR using the primers TK-F and TK-R. The recombinant plasmid was extracted from positive clones using a high-purification plasmid mini-preparation kit (BioTeke Corporation, Cat#DP1002).

Screening for recombinant virus

The transfer vector pUC119-PbVV(±)-EGFP-Omp31 was transfected using Lipofectamine 2000 (Invitrogen, CA, USA) into Vero cells that had been infected with the GTPV vaccine AV41 strain to generate rGTPV-EGFP-Omp31. The infected cells were observed at 1, 3, 4 5, and 6 days post-infection using an LSM510 confocal microscope (ZEISS, Germany) for GFP expression in Vero cells and cell lesions.

Confirmation of genetic stability

rGTPV-EGFP-Omp31 was passaged for ten generations, and the viral genome DNA was extracted every two generations. PCR was performed using the primer pair Omp31-F and Omp31-R (Table 1) to amplify the rGTPV attachment gene.

Table 1 Primers used in this study

Primers	Sequence (5′ → 3′)
TK12-F	<u>TCTAGA</u>ATTGGTAGTAACTGTTCTTTTGCGT (*Xba*I)
TK12-R	<u>GAGCTC</u>TCGTCTTCCTCGTTATTGTCTGC (*Sac*I)
PbVV(−)-EGFP-F	<u>GGTACC</u>TTACTTGTACAGCTCGTCCATGCCGA (*Kpn*I)
PbVV(−)-EGFP-R	AATAACAAAAGTTTATGTAATAGGTATAGAATAACAAAAGTTTATGTAATAGGTATAGAGAATTTCACTTTATACAGTATAAATAAAAGAATTTCACTTTATACAGTATAAATAAA
PbVV(+)-Omp31-F	TTTATTTATACTGTATAAAGTGAAATTCTTTTATTTATACTGTATAAAGTGAAATTCTCTATACCTATTACATAAACTTTTGTTATTCTATACCTATTACATAAACTTTTGTTATTATGAAATCCGTAATTTTGGCG
PbVV(+)-Omp31-R	<u>GGTACC</u>TTAGAACTTGTAGTTCAGACCGAC (*Kpn*I)
Omp31-F	ATGAAATCCGTAATTTTGGCG
Omp31-R	TTAGAACTTGTAGTTCAGACCGAC
Omp31-qRT-F	GGCTTCGTCGGTGGTGTTCAGG
Omp31-qRT-R	TCGACCTTGGTTTCAGCTTTGC
β-actin-qRT-F	ATTGTCACCAACTGGGACGATA
β-actin-qRT-R	TCTGGGTCATCTTTTCACGGTT

Measurements of GFP

Vero cells were infected by rGTPV-EGFP-Omp31 and rGTPV-TK-P7.5-EGFP. At 24 h post-infection, infected cells were observed using an LSM510 confocal microscope (ZEISS, Germany) to visualize GFP expression. Additionally, at 6, 12, 18, and 24 h post-infection, the activity of the cells was evaluated by flow cytometry. The rGTPV-TK-P7.5-EGFP-infected group was used as a control.

Analysis of *omp*31 expression

Vero cells were infected with rGTPV-EGFP-Omp31 and rGTPV-TK-P11-Omp31. At 6, 12, 18, and 24 h post-infection, cell samples were collected, and total RNA was isolated using a commercial kit (Omega Bio-Tek, USA), according to the manufacturer's instructions. Then, 2 μg of total RNA was reverse transcribed to create cDNA using the Omniscript Reverse Transcription kit (Takara), according to the manufacturer's instructions. The relative expression of *omp*31 was detected by quantitative real-time PCR (qRT-PCR) performed in 20 μL reaction containing 10 μL 2 × SYBR Green I Master Mix (Roche, Basel Switzerland), each primer at a final concentration of 100 μM and 2 μL of cDNA. The thermocycling conditions consisted of an initial incubation at 95 °C for 5 min, followed by 45 cycles of amplification (95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s). The primers used for qRT-PCR are listed in Table 1. Relative gene expression levels were determined by the $2^{-\Delta\Delta Ct}$ method, as described previously (Cui et al. 2013). β-actin was used as a reference gene to normalize the expression data for the target gene, and the rGTPV-TK-P11-Omp31-infected Vero cells were used as the control group.

The rGTPV-EGFP-Omp31- and rGTPV-TK-P11-Omp31-infected Vero cells were cultured for 3–4 days. Then, 100 μL of lysis buffer (60 mmol/L Tris-HCl, pH 7.1; 1 mmol/L MgCl$_2$; 0.05 % NP-40 and 20 μg/mL DNAse) was added to the cell culture medium after centrifugation at 12,000 rpm for 30 min, and the supernatants were collected. The cell lysates and purified Omp31 and Bp26 proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose (NC) membrane with a Mini Trans-Blot Cell (Bio-Rad, Hercules, CA, USA) at 200 mA for 1 h. Then, the membrane was blocked for 1 h with membrane blocking solution (Sangon Biotech, Shanghai, China, Cat#AA17DA0001) at 37 °C; the membranes were washed three times with TBST buffer (100 mM Tris-HCl, 150 mM NaCl, and 0.05 % Tween 20, pH 7.2) and incubated with anti-Omp31, anti-Bp26, or anti-Omp19 MAbs for 1 h at 37 °C. The membranes were then washed three times

with TBST buffer and incubated with rabbit anti-mouse IgG (peroxidase conjugated) for 1 h at 37 °C. After three washes, the bound conjugate was visualized with a DAB substrate kit (TIANGEN Biotech Co., Ltd, Beijing, China).

Results

rGTPV AV41 strain transfer vector

PCR amplification revealed that the predicted 2400 bp fragments of the TK gene were successfully amplified from GTPV genomic DNA. The TK gene was cloned into the pMD18-T simple vector and verified by sequencing and digestion using *Xba*I and *Sac*I restriction endonucleases before cloning into the pUC119 vector. The *Kpn*I restriction enzyme cleaved the digestion sites of the TK gene in pUC119-GTPV-TK, which divided the TK gene and its homologous arms (Fig. 1).

The PbVV(−)-EGFP and PbVV(+)-Omp31 sequences were successfully amplified from the PEGFP-N1 plasmid and heat-killed *Brucella* genomic DNA by PCR. PbVV(±)-EGFP-Omp31 sequences were also successfully amplified by fusion PCR. The PbVV(±)-EGFP-Omp31 fragments were cloned into the pMD18-T simple vector and verified by sequencing and digestion using *Kpn*I restriction endonucleases. The pUC119-GTPV-TK vector was also digested using *Kpn*I restriction endonucleases. PbVV(±)-EGFP-Omp31 was sub-cloned into the pUC119-GTPV-TK vector. The rGTPV AV41 strain transfer vector pUC119-PbVV(±)-EGFP-Omp31 was successfully constructed (Fig. 1).

Construction, screening, and characterization of the viral stability of rGTPV-PbVV(±)-EGFP-Omp31

The recombinant vectors pUC119-PbVV(±)-EGFP-Omp31 and GTPV were screened in Vero cells. After being cultured in screening medium for 24 h, the cells were observed daily for 6 days by microscopy. As the infection time increased, the cell lesions become more and more obvious (Fig. 2). The recombinant virus was passaged for ten generations, and the genetic stability of rGTPV was confirmed by PCR using customized primers to yield amplified fragments with sizes of 723 bp (Fig. 3).

Evaluation of GFP protein expression

Vero cells were infected by rGTPV-PbVV(±)-EGFP-Omp31 and rGTPV-TK-P7.5-EGFP. After 24 h, the infected cells were observed using an LSM510 confocal microscope to assess GFP expression in infected Vero cells. Our results

Fig. 1 Construction of the bidirectional promoter recombinant vector pUC119-PbVV(±)-EGFP-Omp31. Schematic representations of the insertion sites in the genomes of the recombinant vector (**a**). The position of the bidirectional promoter in the GTPV genome (**b**)
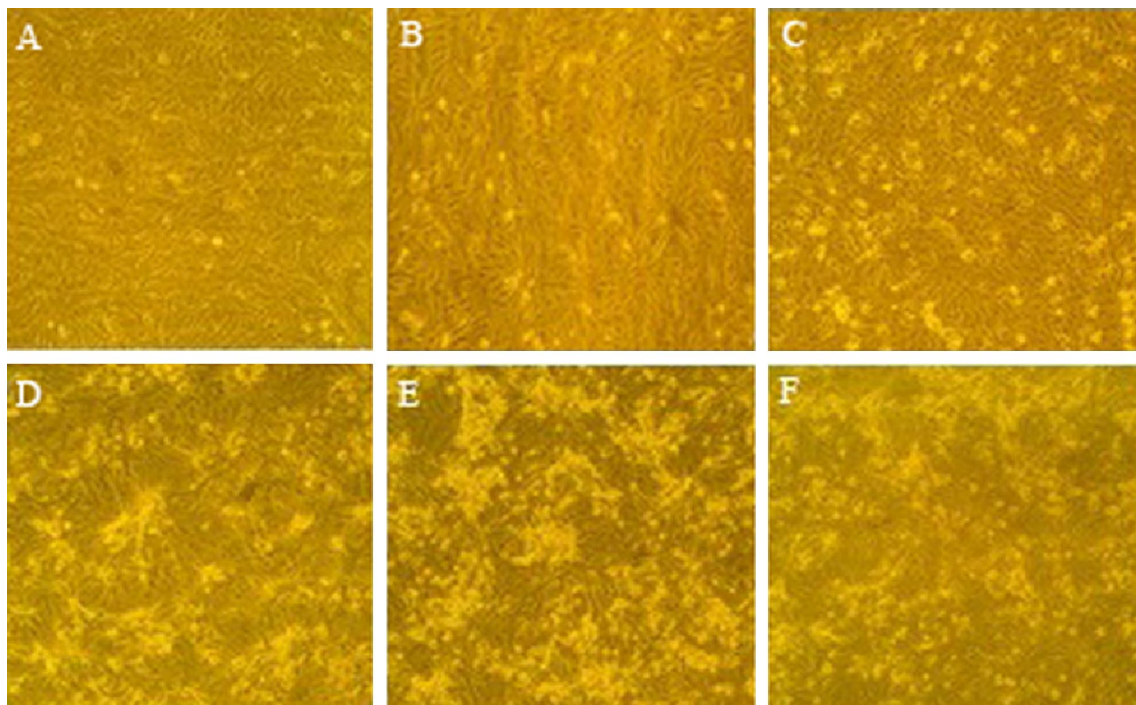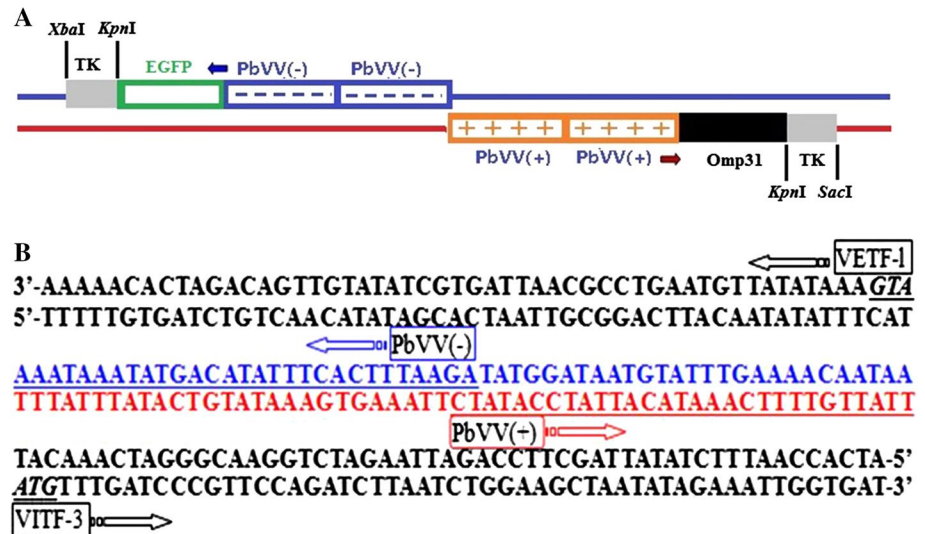
Fig. 2 rGTPV-induced cytopathic effect. The recombinant vector pUC119-PbVV(±)-EGFP-Omp31 was transfected into Vero cells that had been infected with the GTPV vaccine strain. After being cultured in screening medium for 24 h, the cells were inoculated with the transfection product and cultured at 37 °C under 5 % CO_2 for 6 days. Post-infection day 0 results are shown in (**a**). Vero cells appeared to exhibit lesions after culture for 1 (**b**), 3 (**c**), 4 (**d**), 5 (**e**), and 6 days (**e**)

showed that both the PbVV(−) promoter and the P7.5 promoter could regulate EGFP expression (Fig. 4a).

The cell samples were harvested, and the average fluorescence intensity of individual cells was evaluated by flow cytometry at 6, 12, 18, and 24 h post-infection. Our results showed that the transcriptional activity of the PbVV(−) promoter was as strong as that of the P7.5 promoter (Fig. 4b).

OMP31 expression in cells infected with rGTPV

To further characterize the expression levels of *omp*31 in cells infected with rGTPV, the cells were collected at 6, 12, 18, and 24 h post-transfection for qRT-PCR analysis. The qRT-PCR results showed that the expression of the *omp*31 gene under the PbVV(+) promoter was higher than that under the P11 promoter at various time points (Fig. 5a).

Finally, OMP31 expression in cells infected with rGTPV was detected by Western blot. The results showed that the OMP31 protein expressed under the PbVV(+) promoter interacted with the anti-*Brucella*-OMP31 antibody (Fig. 5b).
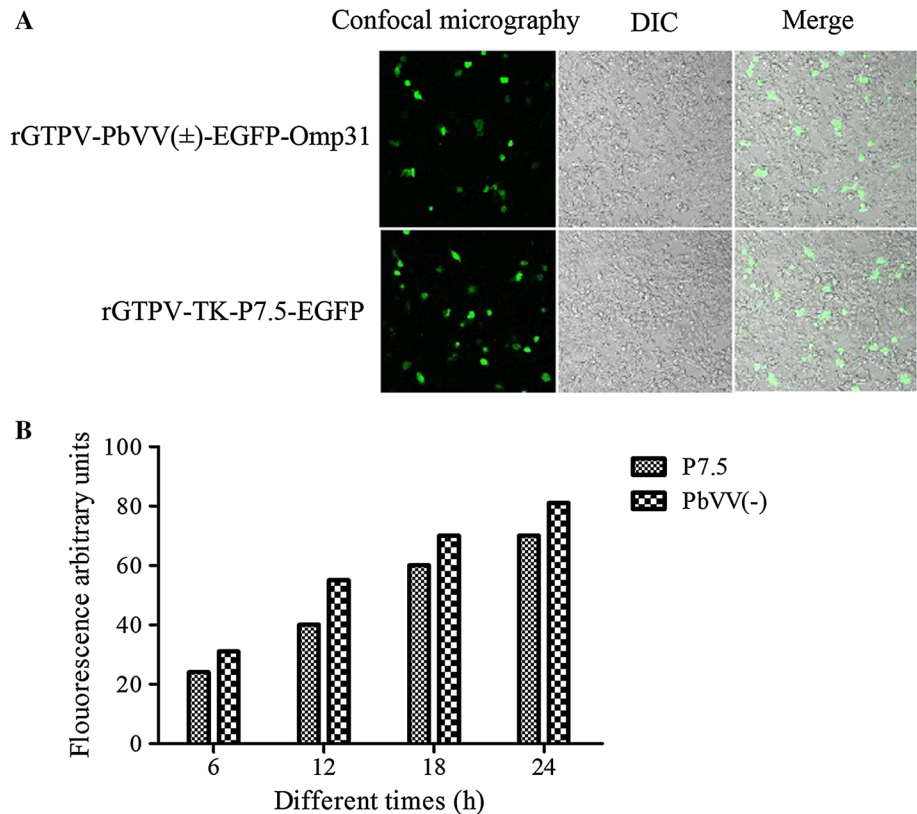
Discussion

Goat pox and sheep pox cause similar lesions and pathologies in goats and sheep. Clinically, both diseases are
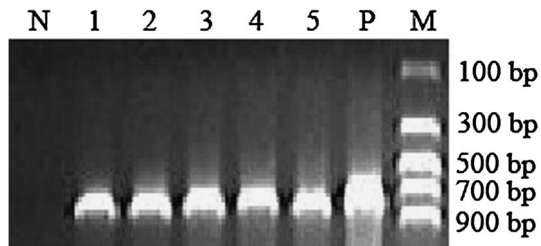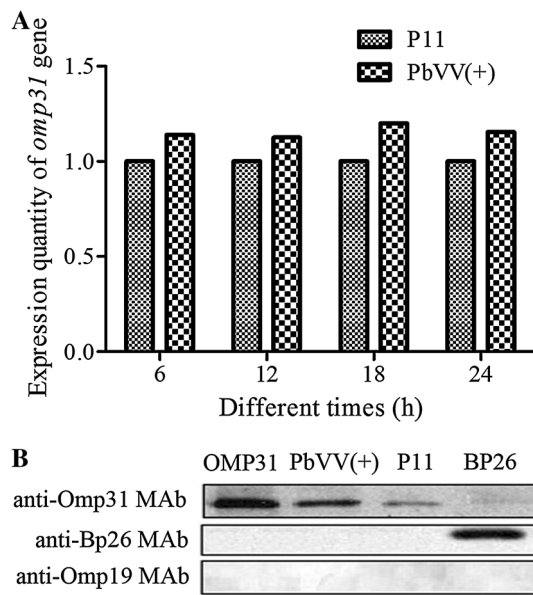

Fig. 3 Evaluating the stability of rGTPV-PbVV(±)-EGFP-Omp31 by PCR. The recombinant vector was passaged for ten generations, and viral genomic DNA was extracted every two generations. PCR was performed using custom primers to amplify the attachment gene of rGTPV. *Lane N* Negative control. *Lane 1* A sample of the second passage; *Lane 2* A sample of the fourth passage; *Lane 3* A sample of the sixth passage; *Lane 4* A sample of the eighth passage; *Lane 5* A sample of the tenth passage; *Lane P* Positive control. *Lane M* DNA marker

characterized by fever and generalized pock lesions (Venkatesan et al. 2012). These two viruses belong to the CaPV genus of the Poxviridae family. CaPVs usually do not have a single specific host, as they infect both sheep and goats (Rao and Bandyopadhyay 2000). Therefore, these viruses have been extensively studied for their potential use as vectors in recombinant vaccine production because of their narrow host range specificity. However, sheep pox and goat pox are considered different entities in India (Hosamani et al. 2004), and recently this designation has also been made in other countries. Currently, attenuated vaccines are already available to control CaPV infection.

Goat pox virus vaccine vectors have a large genome structure that can safely accommodate large exogenous genes; furthermore, the virus can replicate by itself and express an exogenous gene after establishing infection (Tulman et al. 2002). Therefore, GTPV is an ideal poxvirus vector for the development of recombinant multivalent vaccines to enable the delivery of immunogenic genes from other ruminant pathogens. Recently, the use of GTPV as a vaccine vector was reported in China and abroad. Additionally, many ruminant mammal protective antigens have been characterized that could be expressed in a GTPV vaccine vector expression system, so this system holds great promise (Mackett et al. 1992; Diallo et al. 2002; Berhe et al. 2003; Perrin et al. 2007). Brucellosis is a global epidemic zoonosis that can lead to substantial economic losses and

Fig. 4 Observation and detection of EGFP expression. Fluorescent detection of the expression of EGFP in Vero cells after co-transfection with GTPV AV41 and a recombinant vector. The EGFP expression level was measured in Vero cells co-transfected with pUC119-PbVV(±)-EGFP-Omp31 vector or pUC-TK-P7.5-EGFP vector. The recombinant vector was transfected into Vero cells that had been infected with the GTPV vaccine strain. At 24 h post-infection, infected cells were observed using a LSM510 confocal microscope to detect GFP expression (**a**). Flow cytometry analysis of co-transfection of EGFP and a corresponding expression vector at 6, 12, 18, and 24 h post-transfection (**b**)

Fig. 5 Analysis of OMP31. Analysis of *omp*31 mRNA transcript levels by qRT-PCR (**a**). Vero cells were transfected with GTPV and then with a pUC119-PbVV(±)-EGFP-Omp31 vector or pUC-TK-P11-Omp31 vector. At 6, 12, 18, and 24 h post-transfection, total RNA was extracted for qRT-PCR analysis. The mRNA expression levels of *omp*31 under the promoter PbVV(+) were higher than those under the P11 promoter at all time points. The levels of OMP31 in rGTPV infected cells were analyzed by Western blot (**b**). Equal amounts of cell lysates and purified OMP31 or BP26 proteins were then separated by SDS-PAGE, transferred to NC membranes, and probed with anti-Omp31, anti-Bp26, and anti-Omp19 (loading control) antibodies. After incubation with peroxidase-conjugated anti-mouse antibodies, immune complexes were detected with a DAB substrate kit. The protein expression levels of OMP31 under the promoter PbVV(+) were higher than those under the P11 promoter

serious public health problems. Traditional *Brucella* vaccines, especially the attenuated vaccine, play an important role in controlling the disease, but their shortcomings, which include potential problems with safety and other side effects, remain a major concern. Nevertheless, brucellosis is still not yet eradicated, and vaccination remains the primary and most economical mode of preventative measures (Heald and Berg 2014). Most of the current license vaccines have several limitations, such as residual virulence. Thus, no efficient vaccines have been developed for the prevention and control of GTPV.

Outer membrane proteins are common to bacteria; they maintain the stability of the bacterial outer membrane structure and play an important role in host interactions. Many protective bacterial antigens are located on the bacterial surface because most surface proteins and antigens are recognized by the immune system and can produce antibodies or cause an immune response. This same principle applies to the outer membrane proteins of *Brucella*, and OMP31 is an important *Brucella* outer membrane protein.

Studies have shown that OMP31 plays an important role in maintaining the structural stability of the bacterium. Additionally, OMP31 is related to *Brucella's* virulence (Estein et al. 2003; Caro-Hernández et al. 2007).

Conclusions

In this study, we screened and identified a novel effective bidirectional promoter region of GTPV, named PbVV(±), between ETF-l and VITF-3 whose expression pulls in the opposite direction. We constructed rGPTV, in which duplicate PbVV(+) was used as a promoter element to enhance effectively *Brucella* OMP31 expression, and duplicate PbVV(−) as another promoter element to regulate EGFP. The rGTPV has high ability to express exogenous genes. These results will be useful resources for the further study of effective multivalent vaccines based on rGTPV.

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