



RESEARCH ARTICLE

# Construction of a One-Vector Multiplex CRISPR/Cas9 Editing System to Inhibit Nucleopolyhedrovirus Replication in Silkworms

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## Abstract

Recently the developed single guide (sg)RNA-guided clustered regularly interspaced short palindromic repeats/associated protein 9 nuclease (CRISPR/Cas9) technology has opened a new avenue for antiviral therapy. The CRISPR/Cas9 system uniquely allows targeting of multiple genome sites simultaneously. However, there are relatively few applications of CRISPR/Cas9 multigene editing to target insect viruses. To address the need for sustained delivery of a multiplex CRISPR/Cas9-based genome-editing vehicle against insect viruses, we developed a one-vector (pSL1180-Cas9-U6-sgRNA) system that expresses multiple sgRNA and Cas9 protein to excise *Bombyx mori* nucleopolyhedrovirus (BmNPV) in insect cells. We screened the immediate-early-1 gene (*ie-1*), the major envelope glycoprotein gene (*gp64*), and the late expression factor gene (*lef-11*), and identified multiple sgRNA editing sites through flow cytometry and viral DNA replication analysis. In addition, we constructed a multiplex editing vector (PSL1180-Cas9-sgIE1-sgLEF11-sgGP64, sgMultiple) to efficiently regulate multiplex gene-editing and inhibit BmNPV replication after viral infection. This is the first report of the application of a multiplex CRISPR/Cas9 system to inhibit insect virus replication. This multiplex system can significantly enhance the potential of CRISPR/Cas9-based multiplex genome engineering in insect virus.

**Keywords** *Bombyx mori* · *Bombyx mori* nucleopolyhedrovirus (BmNPV) · Antiviral therapeutic · CRISPR/Cas9 · Multigene editing

## Introduction

*Bombyx mori* sericulture for silk production is the best developed in China, India, Japan, and Southeast Asia. Grasserie disease, caused by *Bombyx mori*

nucleopolyhedrovirus (BmNPV), is the most common and serious disease in silkworms, accounting for 70%–80% of total losses (Jiang and Xia 2014). BmNPV belongs to the genus *Alphabaculovirus* in the family *Baculoviridae* (Harrison *et al.* 2018). It has a large circular, supercoiled, double-stranded DNA genome of 128,413 nucleotides containing 141 open reading frames (ORFs) packaged into rod-shaped virions (Gomi *et al.* 1999; Mikhailov 2003). The main measure to prevent and control BmNPV infection is breeding resistant silkworm varieties via genetic and transgenic breeding (Ponnuvel *et al.* 2003). However, overexpression of antiviral genes (genes encoding *Bmlispase-1*, *serine protease-2*, *Sprouty*, *BmAtlastin-n*, and *NADH-oxidoreductase-like protein*) or RNA interference (RNAi) of BmNPV key genes (e.g., immediate early-1 (*ie-1*), late expression factor 1 (*lef-1*), *lef-2*, *lef-3*, *lef-11*, oral infection factor *p74*, and *gp64*) by transgene technologies only partially inhibit viral replication and do not completely eliminate the viral genome (Isobe *et al.* 2004; Jiang and Xia 2014; Ponnuvel *et al.* 2003; Yao *et al.* 2008;

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Zhang *et al.* 2014a, b). Therefore, these technologies do not meet the needs of the sericulture industry.

A new strategy based on clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 nuclease (CRISPR/Cas9) genome editing has been widely used to eradicate pathogenic viruses, including human immunodeficiency virus 1 (HIV-1), human papillomavirus, hepatitis B virus (HBV), Epstein-Barr virus, plant viruses, and flaviviruses, in infected cells or animal models (Yin *et al.* 2015; Pellagatti *et al.* 2015). Over the past 4 years, CRISPR/Cas9 technology has been extensively utilized in basic science and transgenic breeding to treat genetic and infectious diseases in silkworms (Chen *et al.* 2017; Dong *et al.* 2018a, 2019). Our laboratory has employed CRISPR/Cas9 technology to develop a BmNPV-specific gene-editing system to excise the *ie-1* gene from the BmNPV genome in infected *B. mori* worms (Dong *et al.* 2016).

Since 2017, targeted viral genes, including *ie-0*, *ie-1*, *ie-2*, and *me53*, have been edited mainly through single-gene editing, double-gene editing, and large fragment deletion strategies in transgenic breeding (Dong *et al.* 2018a, 2019). We previously established a virus-inducible CRISPR/Cas9 system with reduced off-target mutagenesis and host toxicity compared to those of conventional CRISPR/Cas9 systems (Dong *et al.* 2018b). Our system allows not only direct editing of key BmNPV genes, but also editing of host factors upon which the virus depends to inhibit BmNPV multiplication and replication. The application of this technology has yielded remarkable antiviral effects; however, this technology is still inefficient and shows partial selectivity of viral gene editing for BmNPV genome removal (Chen *et al.* 2017; Dong *et al.* 2019). Thus, there is an urgent need to screen and identify BmNPV genes for potential use in a multigene-editing system to improve antiviral efficacy via multigene editing of the BmNPV genome.

To achieve highly specific BmNPV genome editing, we created an efficient multiplex CRISPR/Cas9 gene-editing technology to disrupt the BmNPV genome in infected silkworm cells. We first constructed a one-vector CRISPR/Cas9 gene-editing plasmid, pSL1180-Cas9-U6-single guide (sg)RNA, using BmNPV *lef-11* as a target gene. This plasmid can be used to quickly insert target genes and detect antiviral activity. In addition, we screened *ie-1* and *gp64* genes, which are important for viral DNA replication and systemic infection. More importantly, to completely eliminate the BmNPV genome from infected silkworm cells, we constructed an anti-BmNPV therapeutic multiplex (PSL1180-Cas9-sgIE1-sgLEF11-sgGP64) CRISPR/Cas9 system. We identified a series of highly active sgRNA candidates that would be optimal for therapeutic application. Using the multiplex CRISPR/Cas9 genome-editing system, we were able to inhibit BmNPV replication.

## Materials and Methods

### Cells and Viruses

The *B. mori* ovary cell line BmN-SWU1 was maintained in TC-100 medium supplemented with 10% (*v/v*) fetal bovine serum (FBS) (Thermo Fisher Scientific, MA, USA) and 10% (*v/v*) penicillin/streptomycin at 27 °C (Pan *et al.* 2010). An enhanced green fluorescent protein marker gene (*EGFP*) driven by the BmNPV 39K promoter (v39K<sup>pr<sup>m</sup></sup>-EGFP) was inserted into the polyhedrin locus of wild-type virus by Tn7-mediated transposition using the Bac-to-Bac system (Thermo Fisher Scientific, MA, USA) (Dong *et al.* 2014; Zhang *et al.* 2014a). Recombinant BmNPV particles were generated in BmN-SWU1 cells and viral titers were determined using the 50% tissue culture infective dose assay (TCID<sub>50</sub>) (Dong *et al.* 2014).

### sgRNA Design

SgRNA primer pairs targeting the amino-terminal regions of ORFs of key BmNPV genes (*ie-0*, *ie-1*, *ie-2*, *lef-1*, *lef-3*, *lef-11*, *gp64*, *vp39*, *poly*, *p143*, *p35*, and *DNApol*) were designed using the CRISPR website (<http://crispr.dbcls.jp/>) (Naito *et al.* 2015). To efficiently identify target genes, we scanning for GN<sub>19</sub>NGG sequences in the BmNPV genome, and identified anti-BmNPV sgRNA candidates that met target sequence requirements and had their own PAM recognition domains. All sgRNAs scaffold target sequences were inserted upstream and downstream of the *BbsI* site. In addition, we synthesized multiple sgRNAs at the Beijing Genomics Institute (BGI, Beijing, China). The primer sequences are listed in Supplementary Table S1.

### Plasmid Construction

Cloning of the *Cas9* gene and preparation of the expression cassette for sgRNA were conducted as described previously (Dong *et al.* 2016). Briefly, to generate a pSL1180-IE1-Cas9-Ser plasmid that encodes the target gene along with *Cas9* and a Flag epitope, the *Cas9* sequence was PCR-amplified from previously established vector construct and inserted behind the BmNPV *IE-1* promoter in the *B. mori* sericin polyA-based pSL1180 vector. The U6 promoter and sgRNA were ligated into the vector following single digestion of pSL1180-IE1-Cas9-Ser-PA with *EcoR* I. The resulting *Cas9* and sgRNA expression vector was named pSL1180-IE1-Cas9-Ser-U6-sgRNA. We linked the sgIE1-sgLEF11-sgGP64 expression cassette into the pSL1180-IE1-Cas9-Ser vector and used restriction enzymes to verify correct cloning. The final construct was named PSL1180-Cas9-sgIE1-sgLEF11-sgGP64 (sgMultiple). All clones

were verified by sequencing. Primers used are listed in Supplementary Table S2.

### Immunofluorescence

BmN-SWU1 cells ( $1 \times 10^5$ ) were seeded on cover slips (Thermo Fisher Scientific, MA, USA) in 24-well plates and cultured at 27 °C for 48 h such that they reached 70% confluence. After cell stabilization, the plasmid was transfected into the cells using the X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland). At 48 h post-transfection (h p.t.), the cells were infected with BmNPV at a multiplicity of infection (MOI) of 10. At 72 h post infection (h p.i.), the BmN-SWU1 cells were subjected to immunofluorescence. Briefly, at 72 h p.i. with BmNPV after transfection of the indicated plasmid, cells were collected and washed three times with phosphate-buffered saline (PBS). The cells were then fixed in cold 4% paraformaldehyde in PBS for 15 min and permeabilized with 1% Triton X-100 for 15 min. After blocking in 3% BSA and 10% sheep serum in PBS at 37 °C for 1 h, the cells were incubated with a monoclonal anti-Flag antibody diluted 1:200 in PBS at 37 °C for 1 h. Finally, after six washes for 5 min each in PBS, the cells were incubated with Alexa 555-conjugated goat anti-mouse IgG (1:500; Thermo Fisher Scientific, MA, USA) and DAPI (1:500; Thermo Fisher Scientific, MA, USA) for 1 h. They were then washed six times with PBS and observed and imaged under a confocal microscope (FV3000, Olympus, Tokyo, Japan).

### Sequence Analysis

Total DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Wisconsin, USA). The BmNPV genomic region surrounding the sgRNA target site of each gene was amplified by PCR for detection of cleavage of the *lef-11*, *ie-1*, and *gp64* genes. PCR products were purified and ligated into the pEASY-T5 vector (TransGen Biotech, Beijing, China) and sequenced with M13 primers (BGI, Beijing, China).

### Western Blotting

Transfected cells were lysed in western and immunoprecipitation buffer (pH 7.5) containing 20 mmol/L Tris-HCl, 150 mmol/L NaCl, and 1% Triton X-100 (Beyotime, Shanghai, China). The lysates were mixed with loading buffer (5×) and boiled for 5 min. The protein samples were then separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. After blocking with 5% skim milk, the membranes were incubated with an anti-

rabbit VP39 polyclonal antibody (1:2000) and an anti-rabbit tubulin polyclonal antibody (1:5000; Sigma, CA, USA) in 5% milk in Tris-buffered saline containing Tween-20 at 27 °C for 1 h. After washing, the blot was incubated with a horseradish peroxidase-labeled goat anti-rabbit IgG (1:20,000; Beyotime, Shanghai, China) at 37 °C for 1 h. Clarity Western ECL Substrate (Bio-Rad, CA, USA) was added and the blot was exposed to X-ray film for detection.

### Quantitative PCR (qPCR) for DNA Replication Assay

BmNPV genomic DNA was subjected to qPCR using SYBR Premix Ex Taq (TaKaRa, Dalian, China) following a previously described protocol and using reported gp41 specific primers (Dong *et al.* 2017). For quantification, a DNA standard curve was constructed based on Ct values to calculate gp41 gene copy number as previously described (Dong *et al.* 2014).

### Flow Cytometry

At 48 h post transfection, cells were infected with BmNPV at an MOI of 10. The cells were collected in PBS at the indicated time points after infection. The percentage of EGFP-positive cells was determined using a NovoCyte flow cytometer (ACEA Biosciences, MA, USA). The data were analyzed using the Novo Express software.

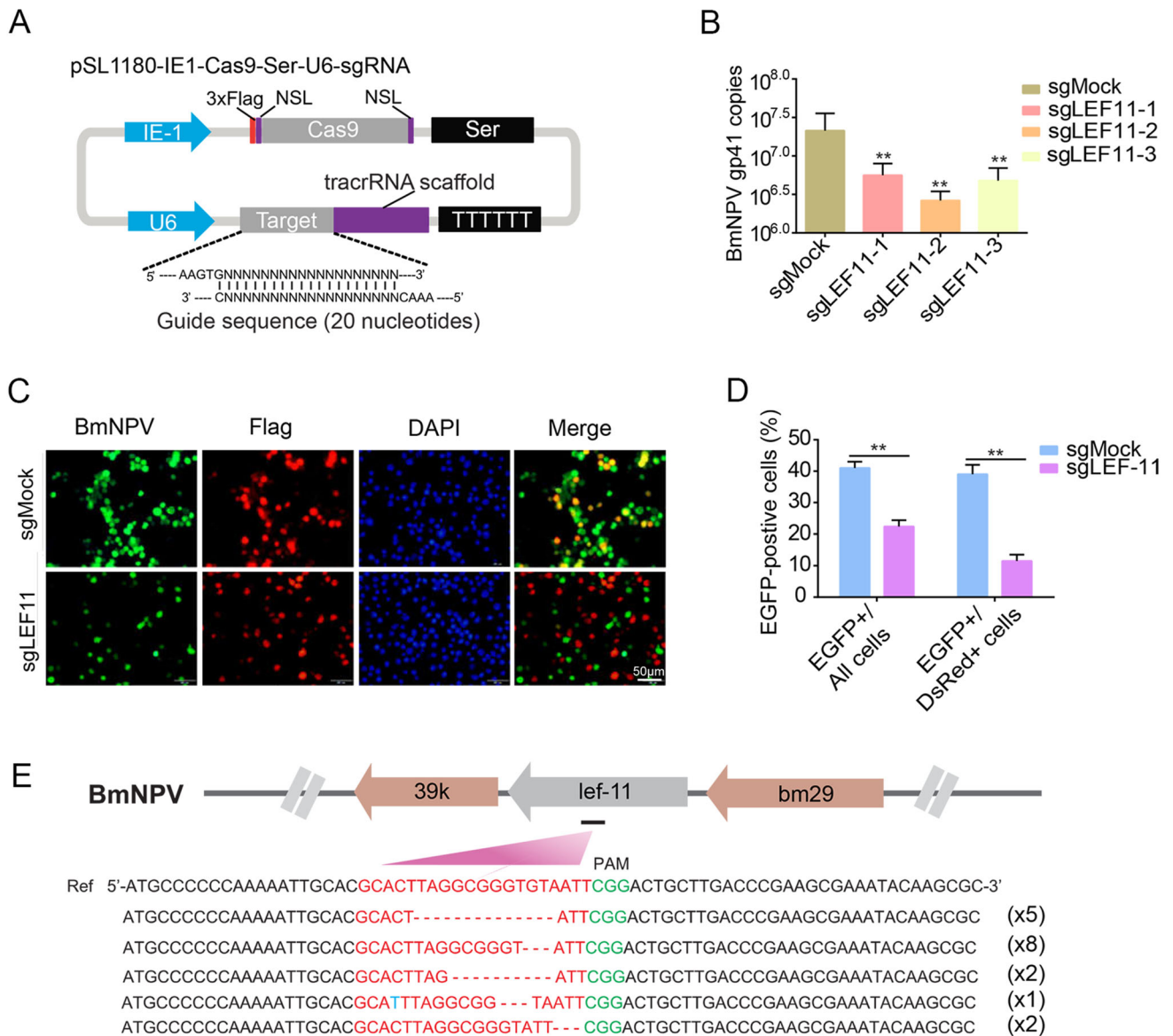
### Statistical Analysis

All data are presented as the mean  $\pm$  standard deviation. Unpaired, one-tailed Student's *t*-tests were used to compute differences between two independent groups in GraphPad Prism 6 (GraphPad Software, Inc., CA, USA).  $P < 0.01$  was considered statistically significant.

## Results

### Establishment of a One-vector CRISPR/Cas9 System for BmNPV Genome Excision

BmNPV contains 141 genes, most of which have not been studied previously (Ono *et al.* 2012). To evaluate the effect of knockout of many of these genes on BmNPV replication, we constructed a one-vector CRISPR/Cas9 system for rapid detection of BmNPV multiplication inhibition. We linked Cas9 to a U6-sgRNA expression cassette into the pSL1180 vector (Fig. 1A). We previously demonstrated that the BmNPV *lef-11* gene is necessary for virus replication (Dong *et al.* 2015). Here, we first evaluated gene



**Fig. 1** A one-vector CRISPR/Cas9 system for BmNPV genome excision. **A** Schematic representation of the one-vector CRISPR/Cas9 system constructed in this study. A single 20-bp sgRNA sequence was inserted downstream of the U6 promoter into pSL1180 using the *Bbs*I restriction site. **B** BmNPV *gp41* copy numbers in cells treated with sgLEF11-1, sgLEF11-2, and sgLEF11-3 as determined by qPCR. **C** Fluorescence microscopic images of BmN-SWU1 cells transfected with the one-vector CRISPR/Cas9 system followed by infection with BmNPV for 72 h. Cas9 protein was labeled with Alexa 555-conjugated goat anti-mouse IgG (red) and green

fluorescence represents BmNPV-infected cells. Scale bar = 50 μm (**D**) BmNPV infection rates were determined as EGFP+ cells in all cells and Cas9-expressing (DsRed+) cells counted in five fields. Three independent experiments were carried out in triplicate. **E** DNA sequence alignment of the CRISPR/Cas9 *lef-11* target sites. The wild-type reference sequence is shown at the top. The target sequences of *lef-11* are indicated in red. Deletions are indicated by dashes. The numbers in parentheses represent the number of occurrences of the different *lef-11* gene deletions.

editing and antiviral efficacy of the system by knocking out *lef-11* using three different target sites in *lef-11*. At 72 h post infection, the genome copy number of BmNPV was measured by qPCR. The cells treated with sgLEF11-1 (Targeting the 3' UTR region of LEF-11), sgLEF11-2 (Targeting the ORF region of LEF-11), and sgLEF11-3 (Targeting the 5' UTR region of LEF-11) had lower levels

of BmNPV DNA than cells transfected with sgMock. DNA levels in cells transfected with sgLEF11-2 were 10-fold lower than those in sgMock control cells (Fig. 1B).

Forty-eight hours after transfection with sgLEF11-2 (sgLEF11-2), BmN-SWU1 cells were infected with v39Kprm-EGFP for 72 h. Inhibition of virus replication was evaluated by immunofluorescence. The CRISPR/Cas9

system expressed the Cas9 protein with a Flag tag, and red fluorescence could be observed after the cells were stained using a monoclonal anti-Flag antibody and an Alexa 555-conjugated goat anti-mouse IgG antibody. Green fluorescence was observed from the recombinant virus harboring v39K<sup>pm</sup>-EGFP. Most cells transfected with sgLEF-11 did not have detectable EGFP levels, whereas EGFP signals were clearly detected in transfected sgMock cells (Fig. 1C). Quantitative analysis revealed that the number of EGFP+ cells decreased significantly compared with the sgMock from 41% to 20% upon transfection with sgLEF-11. There was a similar marked reduction in the number of EGFP+/Flag+ cells, from 40% to 12%, in cells transfected with sgLEF-11 (Fig. 1D). Sequence analysis of the *lef-11* region showed that EGFP expression was disrupted by various mutations, mostly 3–12-bp deletions near the putative target site (Fig. 1E). These results indicated that the one-vector CRISPR/Cas9 system can be used to rapidly identify key genes that inhibit BmNPV replication based on genome copy number and fluorescence of a reporter molecule.

### Screening of Key Genes Involved in BmNPV DNA Replication

The genes *ie-1*, *lef-3*, *p143*, *lef-1*, and *DNApol* are essential for BmNPV DNA replication, although the exact functions of most DNA replication-related genes in BmNPV are unknown (Vanarsdall *et al.* 2007). Based on the principle that early expressed genes in BmNPV have a greater influence on viral DNA replication, we selected *ie-1*, *gp64*, *lef-3*, *lef-11*, *ie-2*, *p35*, *p143*, *lef-1*, and *DNApol* genes as candidate target genes to use in our one-vector CRISPR/Cas9 system. To evaluate the efficacy of the selected sgRNAs in targeting BmNPV, we transfected the one-vector CRISPR/Cas9 system into BmN-SWU1 cells. At 72 h post virus infection, the cells were analyzed by immunofluorescence. As shown in Fig. 2A, sgIE-1, sgGP64, sgLEF-3, sgDNApol, and sgLEF-11 inhibited EGFP expression more effectively than sgLEF-1, sgP143, sgIE-2, sgP35, and sgMock. Quantitative analysis showed that the EGFP+ cells among total cells transfected with sgIE-1, sgGP64, sgLEF-3, sgDNApol, or sgLEF-11 were only 20%–30%, versus 50% for sgMock-transfected cells (Fig. 2B). Analysis of BmNPV DNA replication revealed that sgIE-1, sgGP64, and sgLEF-11 were the most effective at inhibiting viral DNA replication among all target genes. The copy numbers of BmNPV *gp41* in cells transfected with sgIE-1, sgGP64, or sgLEF-11 were 10-fold lower than those in cells transfected with sgMock (Fig. 2C). Flow cytometric analysis revealed that the numbers of EGFP+ cells among total cells transfected with sgIE-1, sgGP64, or sgLEF-11 were lower than that in sgMock cells (Fig. 2D).

The fraction of EGFP+ cells significantly decreased from 65.36% to 45.97%, 47.35%, and 46.07% after transfection with sgIE-1, sgGP64, or sgLEF-11, respectively (Fig. 2D).

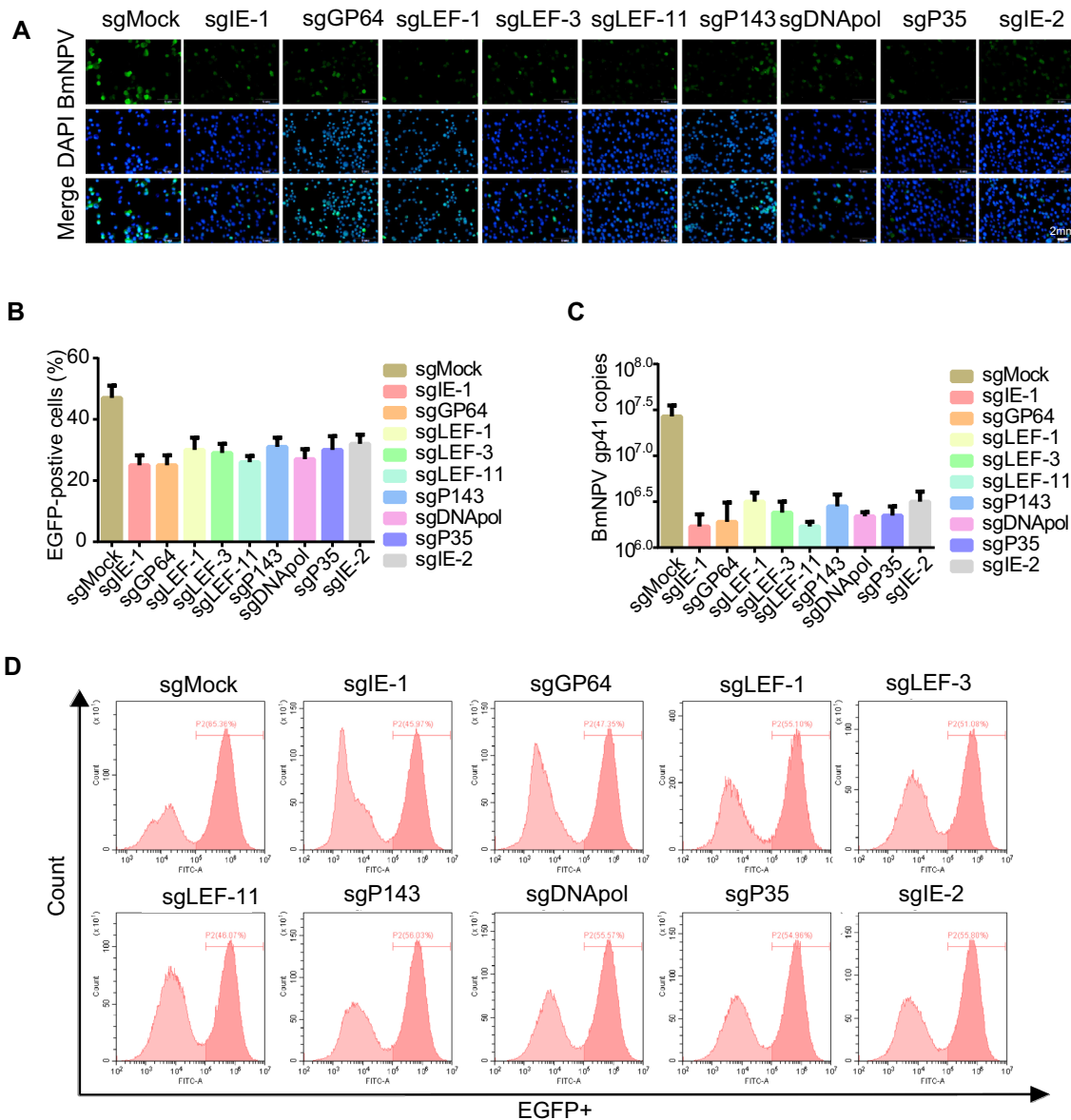
### CRISPR/Cas9-mediated Multiplex Gene Editing Impairs BmNPV Replication

Next, we tested whether a combination of two sgRNAs or multiple sgRNAs could more effectively inhibit viral replication. Thus, one CRISPR/Cas9 genome editing system contains multiple sgRNAs that the Cas9 protein edits simultaneously (Schiwon *et al.* 2018). First, we transduced cells with single and double sgRNAs via our one-vector system to disrupt BmNPV infection. Double sgRNAs inhibited viral infection more effectively than single sgRNAs (Supplementary Figure S1). Next, we selected *ie-1*, *gp64*, and *lef-11* as the target genes for multigene editing via a systematic screening combined with the baculovirus infection process. The sgRNA and Cas9 expression cassettes were inserted into a one-vector system named sgMultiple (Fig. 3A). Sequence analysis of the *ie-1*, *gp64*, and *lef-11* regions showed that BmNPV replication was disrupted due to various mutations and deletions near the putative target sites (Fig. 3A).

Fluorescence microscopy revealed that cells stably expressing sgMultiple (red) significantly inhibited virus replication compared with sgMock expressing cell with BmNPV (green) 72 h p.i. (Fig. 3B). Quantitative analysis revealed that the fractions of EGFP+ cells decreased significantly from 40%–45% to 18%–23% after transfection with sgMultiple. In addition, there was a marked reduction in the fraction of EGFP+/Flag+ cells from 40% to 3% in sgMultiple-transfected cells (Fig. 3C). Flow cytometry was used to further analyze the efficiency of BmNPV proliferation inhibition by sgMultiple. sgMultiple significantly inhibited BmNPV multiplication compared with sgMock at different time points post infection (Fig. 3D). Quantitative analysis revealed that the fraction of EGFP+ cells decreased significantly from 57.89% to 9.26% after transfection with sgMultiple (Fig. 3E). These results indicated that the simultaneous use of multiple sgRNAs can effectively increase the efficacy of viral replication inhibition.

### Antiviral Efficacy of the CRISPR/Cas9-mediated Multiplex Gene-editing System

We also analyzed viral DNA replication in sgMultiple-transfected cells. At 48 h p.i., the copy number of BmNPV was 75% lower in sgMultiple-transfected cells than in sgMock-transfected cells (Fig. 4A). To confirm that the multigene-editing system could effectively inhibit virus proliferation, we analyzed changes in the budded virus titer by TCID50 assays. After transfection with sgMultiple and



**Fig. 2** Screening of key genes involved in BmNPV DNA replication. **A** Antiviral efficiency of knockout of genes involved in BmNPV DNA replication. BmN-SWU1 cells were transfected with the indicated sgRNAs (sgMock, sgIE-1, sgLEF-1, sgLEF-3, sgLEF-11, sgP143, sgDNApol, sgP35, and sgIE-2) and infected with BmNPV at an MOI of 10. Light and fluorescence microscopic images were acquired after co-culturing the cells with BmNPV for 72 h. **B** EGFP-positive cell fractions among BmN-SWU1 cells transfected with *Cas9*

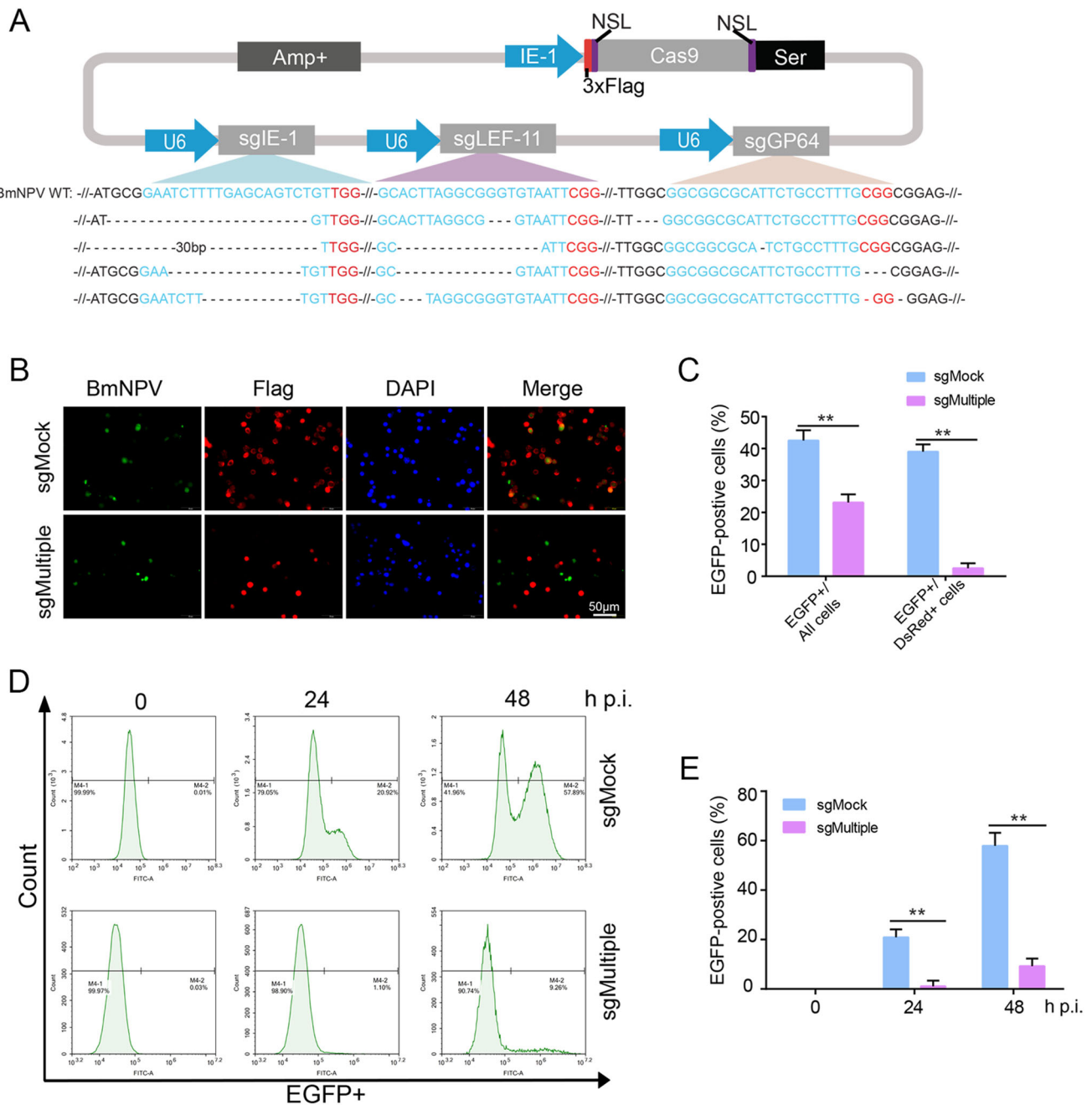
and the indicated sgRNA. **C** BmN-SWU1 cells were transfected with sgMock, sgIE-1, sgLEF-1, sgLEF-3, sgLEF-11, sgP143, sgDNApol, sgP35, or sgIE-2 and infected with BmNPV at an MOI of 10. At 72 h p.i., total DNA was extracted from each Cas9-transfected group and viral *gp41* copies were quantified by qPCR. **D** Flow cytometry analysis was used to quantify BmNPV replication at different time points in each set of cells transfected with sgRNA and infected with BmNPV.

sgMock, we measured the viral titer in the cells. The viral titer in sgMultiple-transfected cells was 100-fold lower than that in sgMock-transfected cells (Fig. 4B). Finally, we analyzed the effect of multigene editing on the expression of the BmNPV VP39 protein. VP39 expression was significantly lower in sgMultiple-transfected cells than in sgMock-transfected cells (Fig. 4C). These results suggested that the CRISPR/Cas9-mediated multigene-editing

system effectively impaired BmNPV replication and can be applied in antiviral research in transgenic silkworms.

## Discussion

CRISPR/Cas9-mediated gene editing has been widely used in insect pest control, sex regulation, silk protein synthesis, and infectious disease research (Ma *et al.* 2018).

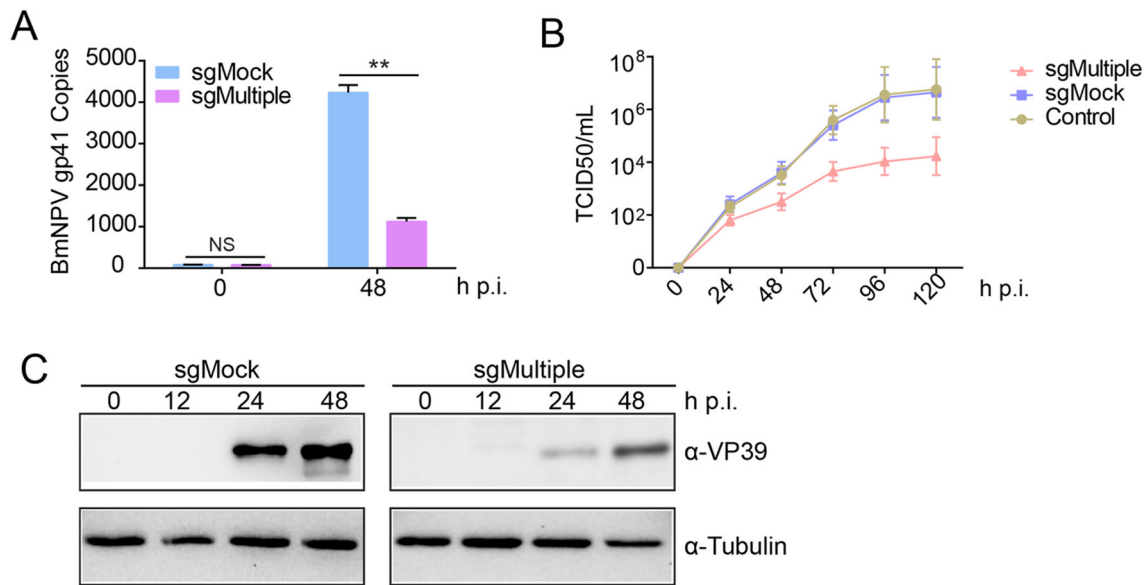


**Fig. 3** CRISPR/Cas9-mediated multigene editing impairs BmNPV replication. **A** Schematic representation of the CRISPR/Cas9 multi-gene-editing vector. DNA sequence alignment of the CRISPR/Cas9 target sites in the *ie-1*, *gp64*, and *lef-11* regions. The wild-type sequence of each region is shown on top, the target sequence is indicated in blue, and deletions are indicated by dashes. **B** BmN-SWU1 cells were transfected with multi-gene-editing vectors, infected with BmNPV, and examined by fluorescence microscopy at 72 h p.i. Cas9 protein was labeled with Alexa Fluor 555 (red), green fluorescence represents BmNPV-infected cells, and nuclei were stained with DAPI (blue). SgMock represents a non-target sequence,

and sgMultiple represents the four target sequences of this multiplex gene editing system. Scale bar = 50  $\mu$ m. **C** BmNPV infection rates determined based on the number of EGFP-positive cells in multiplex gene target cells and in non-target cells counted in five different fluorescent fields. All experiments were repeated three times.  $**P < 0.01$ . **D** BmNPV multiplication was measured in sgMock- and sgMultiple-transfected cells infected with BmNPV at different time points by flow-cytometric analysis. **(E)** EGFP+ cells among BmN-SWU1 cells transfected with sgMock or sgMultiple and infected with BmNPV.  $**P < 0.01$ .

Previously, we developed a series of transgenic silkworm lines with high antiviral ability via transgenic gene editing

(Dong *et al.* 2018a, b). Here, we successfully disrupted BmNPV replication by utilizing a newly established



**Fig. 4** Antiviral efficacy of the CRISPR/Cas9-mediated multigene-editing system **A** Viral DNA expression in sgMultiple cells infected with BmNPV at an MOI of 10. At 48 h p.i., total DNA was extracted from the transfected cells and viral *gp41* copies were quantified by qPCR. Data are the mean from three independent replicates.

\*\* $P < 0.01$ . NS, not significant. **B** Viral growth was determined by TCID50 assays. Fluorescence was measured after 24, 48, 72, 96, and 120 h p.i. **C** Antiviral activity of sgMultiple was monitored based on the protein levels of the late expression portion VP39 (top) measured by Western blotting. Tubulin (bottom) was used as a loading control.

multiplex CRISPR/Cas9 system. To the best of our knowledge, disruption of multiple steps of viral infection with the CRISPR/Cas9 gene-editing system has not yet been demonstrated in insect cells.

A single Cas9 protein can load multiple sgRNAs; therefore, the CRISPR/Cas9 system can effectively knock out multiple target genes (Jang *et al.* 2018; Zhang *et al.* 2016). This has allowed the identification of genetic traits of multiple genes and knockout of gene families (Jang *et al.* 2018; Schiml and Puchta 2016). Multigene-editing tools have been widely used to construct multigene-delivery systems to deliver multiple sgRNAs for the treatment of HIV, retrovirus, lentivirus, and HBV infections (Hu *et al.* 2017; Sakuma *et al.* 2016; Schiwon *et al.* 2018; Yin *et al.* 2018). Here, we constructed a one-vector CRISPR/Cas9 system that allows the screening and identification of key genes involved in BmNPV systemic infection as well as the verification of its antiviral efficacy (Fig. 4). This technology may be valuable in protecting silkworms by eliminating viral infection at multiple stages.

In 2004, the BmNPV *lef-1* gene was targeted by RNAi to inhibit viral replication (Isobe *et al.* 2004). Since then, multiple other genes involved in BmNPV systemic infection have been modulated, including microRNA, dsRNA, and shRNA (Zhang *et al.* 2014a, b; Zhou *et al.* 2014); however, the target genes used in these methods were different from those considered in our approach. Therefore, systematic comparison of the antiviral efficacy of the genes was not possible. Here, 11 genes with different functions in

different stages of BmNPV infection were targeted using the one-vector CRISPR/Cas9 system (Figs. 2 and 3). The antiviral ability of sgRNAs targeting these genes was systematically compared by assessing DNA replication and viral fluorescence. The screening yielded various reliable target genes for future development of transgenic antiviral silkworms. It also highlights future research directions to elucidate BmNPV gene functions. In addition to BmNPV, this method can be used to screen and edit target genes in other viruses, such as *Bombyx mori* cytovirus, *Bombyx mori* densovirus, and *Bombyx mori* flacherie virus (Cao *et al.* 2012; Li *et al.* 2001).

Other approaches can be used to generate transgenic silkworms that can resist bacterial diseases, fungal diseases, and *Nosema bombycis*, depending on the different characteristics of silkworm diseases. Recently, the CRISPR family gene-editing systems include CRISPR/CPF1 and CRISPR/Cas13, which can edit DNA, RNA, or both DNA and RNA sequences (Mahas and Mahfouz 2018; Tsukamoto *et al.* 2018). CRISPR/CPF1 and CRISPR/Cas13 systems have smaller nuclease proteins and have more RNA target sequences and lower off-target efficiency. Then these can be systematically applied to research on silkworm disease resistance breeding and the results may benefit the sericulture industry (Koneremann *et al.* 2018).

In conclusion, the multiplex CRISPR/Cas9 system constructed in this study has high gene editing efficiency and antiviral ability and may present a strategy for anti-BmNPV therapy. In future, the application of this



multiplex CRISPR/Cas9 genome-editing technology may provide new insights to improve the strategic inhibition of multiple silkworm pathogens simultaneously. CRISPR-mediated multigene editing can be used to breed transgenic antiviral lines and in insect pest control.

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**Author Contributions** ZD and ZH conceived and designed the experiments. LH, QQ, TT and XZ curated the Data. ZD, PC and QQ analyzed the data; ZD, CL and MP contributed to the writing of manuscript. All authors reviewed and approved the final manuscript for submission.

## Compliance with Ethical Standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Animal and Human Rights Statement** This article does not contain any studies with human or animal subjects performed by any of the authors.

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