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Enhancement of exogenous protein stability in AcMNPV by overexpressing *lef5* gene during passaging

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Abstract

The baculovirus insect cell expression system is pivotal for exogenous protein expression. However, serial passages of baculovirus in insect cells often result in defective virus generation and a rapid decline in exogenous protein expression, limiting its wider application. Previous studies have shown that the expression of the late expression factor 5 (*lef5*) from other baculoviruses can enhance the stability of exogenous genes in *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV). In this study, we engineered diverse recombinant strains of AcMNPV to express the enhanced green fluorescent protein (eGFP) and enterovirus 71 virus-like particles (EV71-VLPs), co-expressed with the P1 and 3CD proteins of EV71. We investigated the influence of *lef5* overexpression, regulated by various promoters, on the stability of exogenous genes and their protein expressions. Notably, *lef5* overexpression significantly improved the stability of *eGFP* and *EV71-P1* gene and protein expressions during serial passages in Sf9 cells. Specifically, the *lef5* overexpression driven by the op166 promoter was more beneficial for enhancing the expression stability of complex exogenous proteins, such as EV71-VLPs. This study underscores the importance of *lef5* overexpression in maintaining the stability of exogenous gene expression in baculovirus systems, particularly for producing complex proteins such as VLPs of enterovirus.

Key points

- *The overexpression of lef5 stabilizes exogenous gene expression in the baculovirus system.*
- *Promoter choice affects lef5-mediated gene expression stability.*
- *The overexpression of lef5 is crucial for producing complex proteins like EV71-VLPs.*

Keywords Baculovirus · Serial passage · *lef5* gene · Gene expression stability · eGFP · EV71-VLPs

Introduction

The baculovirus expression vector system (BEVS) has emerged as a versatile platform for vaccine development and production in insect-cultured cells (Fan et al. 2022; Poodts et al. 2022), offering advantages such as high safety, rapid production, flexible design, and scalability (Hong et al. 2023). This technology allows for efficient protein expression in insect cells with proper eukaryotic post-translational modifications (Hu et al. 2008) and has been successfully applied to produce subunit vaccines, including virus-like particles, for both human and veterinary use. Regulatory acceptance of BEVS-derived vaccines, exemplified by the approval of Cervarix™ in 2007, has paved the way for broader adoption of this technology in vaccine manufacturing (Mena and Kamen 2011). To date, several BEVS-derived vaccine products have been approved for use, including four human vaccines (Cervarix against cervical cancer caused by *human papillomavirus* (HPV), Flublok and Flublok Quadrivalent against seasonal influenza, Nuvaxovid/Covovax against COVID-19) and five veterinary vaccines (Porcilis Pesti, BAYOVAC CSF E2, Circumvent PCV, Ingelvac CircoFLEX and Porcilis PCV). As research progresses, BEVS is expected to play an increasingly important role in future vaccine development and production.

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Despite its advantages, the BEVS system faces challenges, including unstable expression and reduced levels of protein glycosylation (Hong et al. 2022; van Oers 2011). One common issue is the formation of defective interfering viruses (DIs) due to baculovirus gene deletions during passage in insect cells (Willemsen and Zwart 2019). These DIs can rapidly emerge and negatively impact heterologous protein expression (Pijlman et al. 2001). Although deletion of the non-homologous region (hr) origin of replication can enhance genetic stability, spontaneous excision of the entire bacterial artificial chromosome (BAC) vector may still occur (Pijlman et al. 2003; Yu et al. 2023). The utilization of baculovirus for expressing adeno-associated virus (AAV) involves the expression of multiple genes, with stability issues being the most typical challenges encountered (Destro et al. 2023). Different strategies have been tried to improve the genetic stability and production of recombinant AAV with the baculovirus system (Jacob et al. 2021; Joshi et al. 2021; Negrete et al. 2007). Though the novel bifunctional recombinant baculovirus expression vector showed good stability, the yield of rAAV remained high level only in the 1^{st} – 5^{th} generation (Wu et al. 2018, 2019).

The baculovirus late expression factor-5 (*lef5*) gene plays a crucial role in late gene expression and productive infection of *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV). The *lef5* interacts with itself and contains a zinc ribbon domain homologous to elongation factor TFIIS, which is essential for maximal late transcription activity (Harwood et al. 1998). Studies have shown that a *lef5* knockout virus results in severe impairment of late gene expression and progeny virus production, while early gene expression and DNA replication remain unaffected (Su et al. 2011). Interestingly, overexpression of the *lef5* from *Spodoptera exigua multiple nucleopolyhedrovirus* (SeMNPV) in recombinant AcMNPV increases the stability of the baculovirus and transgenes during serial passages in insect cell culture (Martinez-Solis et al. 2017).

In the present study, we aim to investigate the impact of overexpressing the *lef5* of AcMNPV in recombinant AcMNPVs. Specifically, we will assess the stability of genes and proteins, including the simple enhanced green fluorescent protein (eGFP) and the more complex enterovirus 71 virus-like particles (EV71-VLPs, co-expressing the P1 and 3CD of EV71) (Li et al. 2013; Zhao et al. 2017), when expressed using baculovirus systems. By overexpressing the *lef5*, we hope to gain a better understanding of its role in enhancing the stability of recombinant AcMNPVs during in vitro propagation, ultimately facilitating more efficient production of heterologous proteins in insect cell cultures for vaccine development.

Materials and methods

Cells, viral strains and plasmids

The Sf9 insect cell line, originally obtained from the fall armyworm *Spodoptera frugiperda*, was cultured at 27 ℃ in IB serum-free cell culture medium (YSK BIO, Zhejiang, China). AcMNPV was used as the parent virus in this study. The modified Bac-to-bac baculovirus expression system was obtained from Prof. Zhihong Hu of the Wuhan Institute of Virology, Chinese Academy of Sciences. The chemically competent *E. coli* AcBac-syn carries the helper plasmid and the AcMNPV bacmid (shuttle vector). The donor plasmid pBacT contains three different promoters vp39, p6.9 and op166.

Preparation of recombinant baculoviruses

The recombinant AcMNPVs were generated by following the manufacturer's instructions for the Bac-to-Bac® baculovirus expression system. The pBacT was used as the backbone to construct the donor vectors. The bacmids were then purified and used to transfect one million Sf9 cells in the 12-well plate by using Cellfectin II Reagent (Thermo Fisher Scientific). After a 4-day incubation at 27 ℃, the cell culture supernatant was collected and centrifuged at 500 × *g* for 10 min to obtain the passage zero (P0) recombinant AcMNPVs.

Virus titer determination and gene copy number determination

For virus titer determination, we targeted the DNA polymerase (*DNApol*) gene (a conserved and abundant baculoviral marker), along with two exogenous genes (*eGFP* and *EV71-P1*), using qPCR with specific primers. The standard curve for qPCR was established using serial dilutions of known concentrations of Bacmid containing the *DNApol, eGFP,* or *EV71-P1* gene. Cycle threshold (Ct) values obtained from the qPCR reactions were compared to the standard curve to calculate the gene copy numbers. The ratio of the *eGFP* gene or *EV71-P1* gene relative to the *DNApol* gene in each passage of the virus was further calculated. Briefly, Total viral DNA was prepared from the supernatants of each passage using the FastPure Viral DNA/RNA Mini Kit V2 (Vazyme, Nanjing, China). The qPCR was conducted with ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). The primers designed for *DNApol, eGFP and EV71-P1* were as follows: DNApol-F, 5'-CAATGCCTAGCCACCGTAAT-3';

DNApol-R, 5'-AATCGTTGACCGACTACAGC-3'; eGFP-F, 5'-TATCATGGCCGACAAGCAGA-3'; eGFP-R, 5'-ATGCCGAGAGTGATCCCG-3'; EV71-F, 5'-GGACCTTGAATACGGAGCCT-3'; EV71-R, 5'-GAGTCGTGATGGCAGTTCG-3'.

Gene expression detection

Expression levels of *DNApol* and *lef5* genes of the recombinant viruses were analyzed at days 1,2 and 3 post-infection. The total RNA was purified by FastPure Cell/Tissue Total RNA Isolation Kit (Vazyme, Nanjing, China). Reverse transcription was then conducted with the HiScript II Q RT SuperMix for qPCR (Vazyme, Nanjing, China). SYBR Green-based qPCR was performed using Pro Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). Tubulin gene expression from Sf9 cells was used as a reference. The primers designed for *tubulin*, *DNApol* and *lef5* were as follows: tubulin-F, 5'-GGGCATGGACGAGATGGA-3'; tubulin-R, 5'-GGACACCAGGTCGTTCATGTT-3'; DNApol-F, 5'-CAATGCCTAGCCACCGTAAT-3'; DNApol-R, 5'-AATCGTTGACCGACTACAGC-3'; lef5-F, 5'-CCATTTGCTTTGAAGCGAGG-3'; lef5-R, 5'-GCGCTCTTTCACCAAATCAC-3'. Gene expression was calculated relative to tubulin using the formula $2^{[Ct\,(\beta-tubulin)-Ct\,(DNApol/lef5)]}$. The relative expression of *lef5* to *DNApol w*as further calculated.

Cell infection and serial passages

The titers of the P0 generation viruses were determined using qPCR to ensure successful rescue and nearly identical titers of each recombinant AcMNPV. Sf9 cells were seeded in 6-well plates at a density of 2×10^6 cells per well. In the first experimental setup, cells were infected with recombinant viruses using a volume ratio of 0.5%. After incubation at 27 °C for 4 days, samples were collected. The medium containing multiplied viruses was centrifuged at 3000 rpm for 5 min to remove cells. Budded viruses (BVs) in the supernatant were stored at 4 °C for viral quantification and subsequent infection passages. A total of 15 infection passages were completed for each recombinant AcMNPV, using two independently rescued viruses per recombinant. An additional experiment was conducted using a volume ratio of 2.5%, following the same methodology.

Immunofluorescence Assay (IFA)

Sf9 cells were cultured in 24-well plates and inoculated with recombinant AcMNPVs. For immunofluorescence assay, cells were fixed with 4% paraformaldehyde in PBS for 30 min. Subsequently, cells were washed with PBS and permeated with 0.2% (wt/vol) Triton X-100-PBS solution for 10 min at room temperature. Then, cells were washed and blocked with 3% bovine serum albumin (BSA) in PBS at 37℃ for 30 min. After washing, cells were incubated with rabbit anti-EV71 polyclonal antibody for 2 h at 37℃, followed by Alexa Fluor 594-conjugated donkey anti-rabbit IgG H+L for 1 h at 37°C.

Western blotting

Cells were harvested and lysed in SDS lysis buffer for 30 min on ice, followed by centrifugation at 14,000 × *g* for 15 min at 4°C. The supernatant was boiled in 1 × loading buffer at 100°C for 10 min and separated through a 4 to 12% gradient gel sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto a nitrocellulose membrane and incubated with rabbit anti-EV71 polyclonal antibody or mouse anti-GP64 monoclonal antibody after blocking with 5% BSA in PBST. Proteins were detected using horseradish peroxidase-conjugated secondary antibodies and chemiluminescence.

Statistical analysis

Graphs were plotted and analyzed by using GraphPad Prism 8.0 software (GraphPad, CA, USA). Experimental data are presented as the mean ± standard deviation (SD) for a minimum of three biological replicates. The study utilized an unpaired *t*-test to examine the significant differences between each group. Differences were denoted by *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$.

Results

Generation of the recombinant AcMNPVs expressing eGFP or EV71-VLPs

To explore the impact of overexpressing the *lef5* of AcMNPV on the stability of exogenous genes and protein expression, a series of recombinant AcMNPVs were constructed. First, the *eGFP* gene was cloned into the pBacT vector under the control of the vp39 promoter, resulting in the pBacT-eGFP plasmid. Subsequently, the *lef5* gene of AcMNPV was cloned into pBacT-eGFP under the control of the op166 promoter (a modified early promoter from OpMNPV) to create the pBacT-eGFP-op166-lef5 plasmid. The op166 promoter was then replaced with the late p10 promoter to generate the pBacT-eGFP-p10-lef5 plasmid (Fig. 1a). These plasmids were used to produce the corresponding bacmids, which were transfected into Sf9 cells to obtain the AcGFP, AcGFP-op166-lef5 and AcGFP-p10-lef5, respectively.

For the production of EV71-VLPs, both the *3CD* and *P1* genes were cloned into the pBacT vector. The *3CD* gene was placed under the control of the p6.9 promoter, and the *P1* gene was placed under the control of the vp39 promoter, resulting

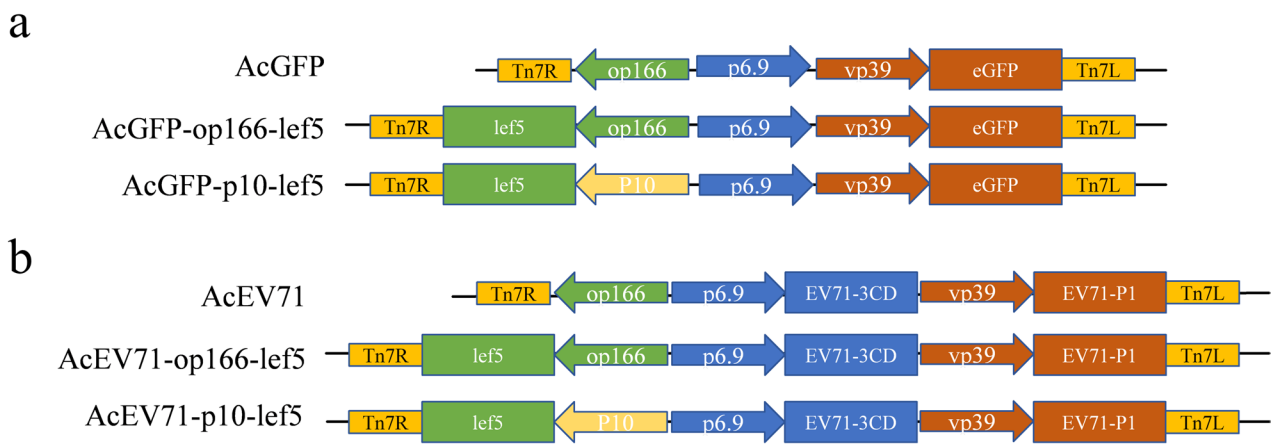

Fig. 1 Schematic representation of recombinant AcMNPVs. **a** Schematic diagram of the recombinant AcMNPVs expressing eGFP. The *eGFP* gene was cloned under the control of the vp39 promoter, with the lef5 gene further introduced under control of the op166 promoter or p10 promoter. **b** Schematic diagram of the recombinant AcMNPVs expressing EV71-VLPs. The *3CD* gene and *P1* gene were cloned under the control of the p6.9 and vp39 promoters, respectively. Similarly, the *lef5* gene was introduced, controlled by either the op166 or p10 promoter. These plasmids were used to produce the corresponding bacmids, which were transfected into Sf9 cells to obtain the recombinant AcMNPVs

in the pBacT-EV71-VLPs plasmid. The *lef5* gene was then introduced into this plasmid as described above to create the pBacT-EV71-op166-lef5 and pBacT-EV71-p10-lef5 plasmids (Fig. 1b). These plasmids were used to produce the corresponding bacmids, which were transfected into Sf9 cells to obtain the AcEV71, AcEV71-op166-lef5 and AcEV71-p10-lef5, respectively.

Effects of different promoters on enhancing *lef5* gene transcription in recombinant AcMNPVs expressing eGFP and EV71-VLPs

For the recombinant AcMNPVs designed to express eGFP and EV71-VLPs, we evaluated the effect of incorporating either the op166 or p10 promoter to drive the *lef5* gene. Our results showed that the ratios of *lef5* gene transcription to *DNApol* gene transcription (used as a reference for viral replication activity) increased with infection time (Fig. 2a and b). Notably, the op166 promoter initiated *lef5* gene transcription earlier compared to the p10 promoter. On the first day post-infection, the *lef5* transcription level driven by the op166 promoter was significantly enhanced in both recombinant AcMNPVs expressing eGFP and EV71-VLPs. However, starting from the second day post-infection, the *lef5* transcription level initiated by the p10 promoter was significantly higher than that in the group without any additional *lef5* introduction or the group with *lef5* driven by the op166 promoter. For the recombinant AcMNPVs expressing eGFP, the *lef5* transcription level in the AcGFP-p10-lef5 group remained significantly higher than that in the other

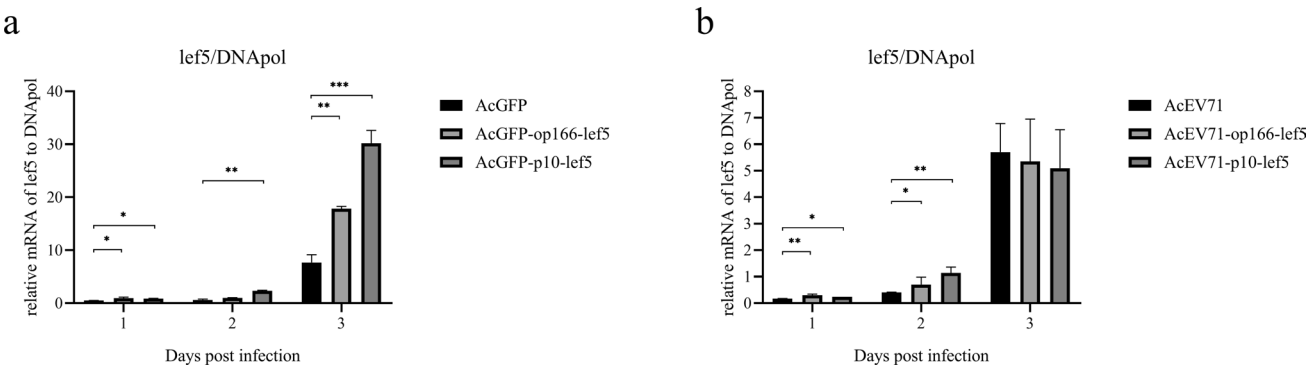

Fig. 2 Relative transcription levels of the *lef5* compared to *DNApol*. **a** Ratio of *lef5* to *DNApol* gene transcription levels in Sf9 cells infected with recombinant AcMNPVs expressing eGFP. **b** Ratio of *lef5* to *DNApol* gene transcription levels in Sf9 cells infected with recombinant AcMNPVs expressing EV71-VLPs. Error bars represent the mean ± SD, and the data are averages from three biological replicates, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

two groups on the third day post-infection. In contrast, for the recombinant AcMNPVs expressing EV71-VLPs, no significant differences were observed among the groups in terms of *lef5* transcription levels. These results suggest that the effect of the promoters on *lef5* gene transcription may vary depending on the specific recombinant baculovirus and the target protein being expressed.

Enhanced stability of *eGFP* gene and protein expression in recombinant AcMNPVs with *lef5* introduction

The impact of *lef5* introduction on the stability of the *eGFP* gene and protein expression was evaluated during the continuous passage of recombinant AcMNPVs in Sf9 cells. To assess the effect of *lef5* on the *eGFP* gene stability, passages were conducted at two different inoculation ratios (0.5% and 2.5%). The copy numbers of both the *DNApol* gene and the *eGFP* gene were determined for each passage (Fig. 3a and d). Throughout the passages, the copy number of the *DNApol* gene remained consistently high, whereas a significant decline in the copy number of the eGFP gene in AcGFP was observed from the 11th passage onwards. A further comparison of the *eGFP* gene copy number to the *DNApol* gene copy number (Fig. 3b and e) revealed that the *eGFP* gene loss was more severe during passages with a lower inoculation ratio compared to a higher ratio. Specifically, the *eGFP* gene ratio of AcGFP decreased to 10^{-6} (0.5% inoculation ratio) (Fig. 3b) and 10^{-3} (2.5% inoculation ratio) (Fig. 3e), respectively. The introduction of the *lef5* was found to maintain the stability of the *eGFP* gene during passages under both the promoters p10 and op166.

To corroborate these findings, the expression of the eGFP protein was also examined (Fig. 3c and f). The reduction green fluorescent protein expression of AcGFP was consistent with the loss of the *eGFP* gene copies. Each passage was inoculated into Sf9 cells at the same ratio as previously, and green fluorescent protein expression was observed on the fourth day post-infection (with comparable cell densities across all tested wells). At the lower inoculation ratio, the loss of green fluorescent protein expression was more severe without the introduction of *lef5* (Fig. 3c). With a significant decrease in fluorescence intensity by the 5th passage, followed by sporadic expression by the 10th passage and no detectable expression by the 15th passage. In contrast, with a higher inoculation ratio (Fig. 3f), the

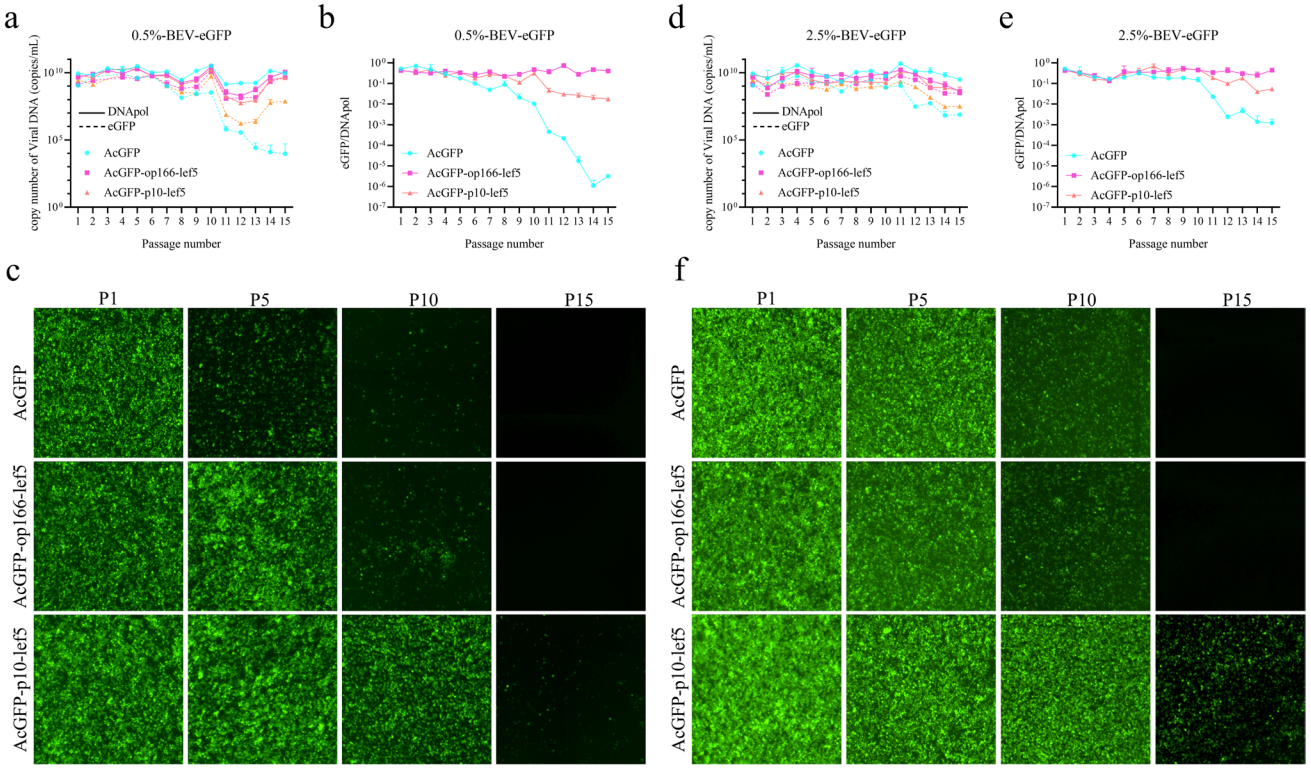

Fig. 3 Stability of recombinant AcMNPVs expressing eGFP during continuous passaging. Copy numbers of *DNApol* and *eGFP* genes in consecutive passages with a 0.5% inoculation ratio (**a**) and 2.5% inoculation ratio (**d**). Ratio of eGFP to *DNApol* gene copy numbers in consecutive passages with a 0.5% inoculation ratio (**b**) and 2.5% inoculation ratio (**e**). Detection of eGFP expression in Sf9 cells in consecutive passages with a 0.5% inoculation ratio (**c**) and 2.5% inoculation ratio (**f**), photographed on day 4 post-infection (Consistent cell density across all passages)

expression of green fluorescent protein decreased to some extent by the 10th passage without *lef5*, but sporadic fluorescent spots were still visible by the 15th passage. The introduction of *lef5* significantly delayed the attenuation of the eGFP protein expression. Notably, the recombinant AcGFP-p10-lef5 exhibited the slowest attenuation of the eGFP protein expression during continuous passages compared to the other two viruses. Our results reveal that the *eGFP* gene gradually decreases over successive passages, particularly at lower inoculation ratios, leading to a corresponding reduction in the eGFP protein expression. The introduction of the *lef5* significantly enhances the stability of the *eGFP* gene and protein expression in recombinant AcMNPVs, particularly the use of the p10 promoter to drive *lef5* expression.

Enhanced stability of *EV71-P1* gene expression and protein processing in recombinant AcMNPVs with *lef5* introduction

Further, the impact of *lef5* introduction on the stability of EV71-VLPs was evaluated during the continuous passage of recombinant AcMNPVs in Sf9 cells. Given that a higher inoculation ratio favored the stability of exogenous genes during baculovirus propagation; a 2.5% inoculation ratio was employed for the serial passage of baculoviruses expressing EV71-VLPs. During the serial passage process, the copy numbers of both the *DNApol* gene and the *EV71-P1* gene were monitored (Fig. 4a). Additionally, the ratio of the *EV71- P1* gene copy number to the *DNApol* gene copy number was compared (Fig. 4b). Similar to

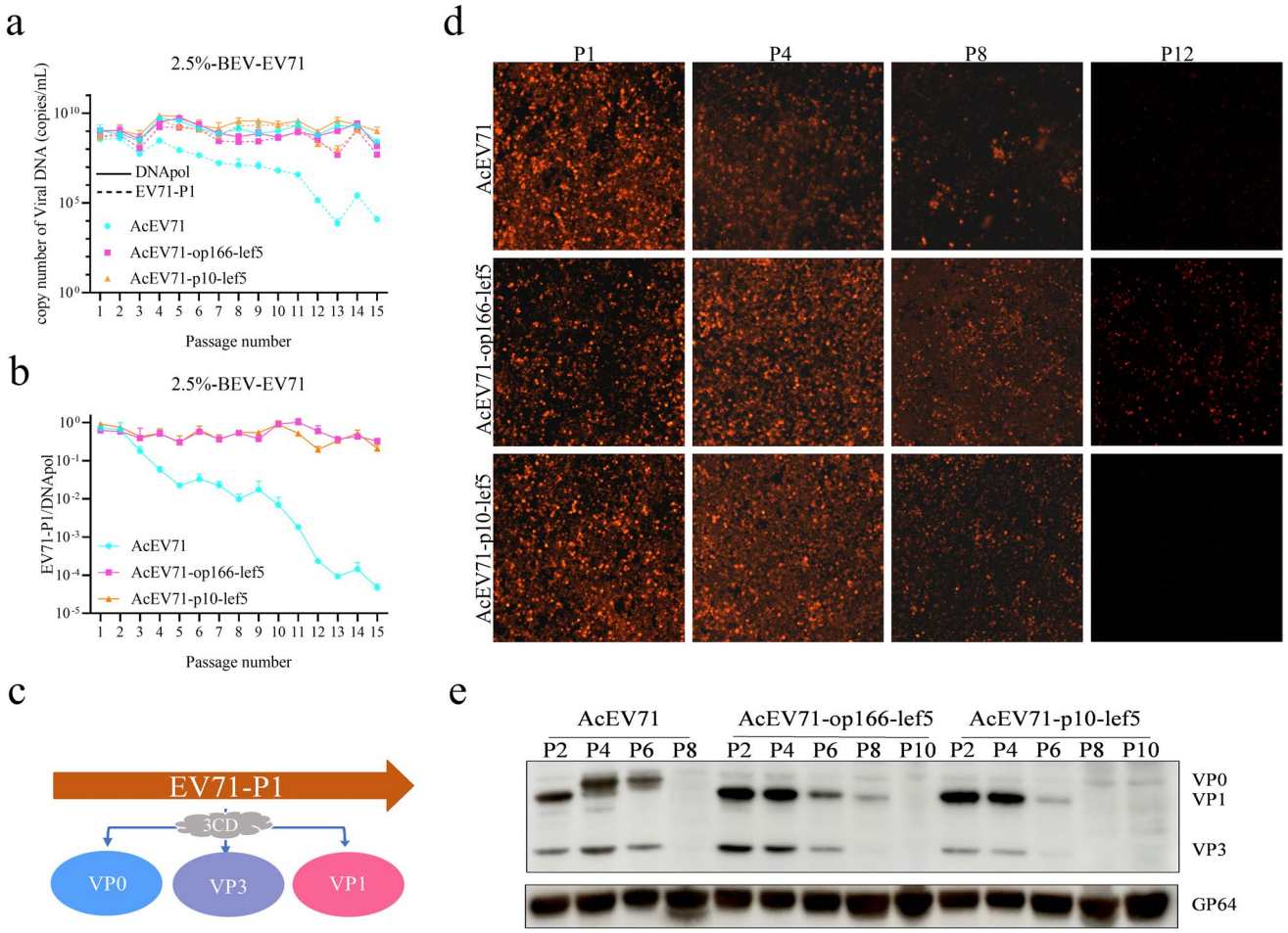

Fig. 4 Stability of recombinant AcMNPVs expressing EV71-VLPs during continuous passaging. **a** Copy numbers of *DNApol* and *EV71-P1* genes in consecutive passages with a 2.5% inoculation ratio. **b** Ratio of *EV71-P1* to *DNApol* gene copy numbers in consecutive passages with a 2.5% inoculation ratio. **c** Detection of structural proteins of EV71 by indirect immunofluorescence (IFA) in Sf9 cells in consecutive passages with a 2.5% inoculation ratio. **d** Schematic representation of EV71-P1 protein cleavage into VP0, VP3, and VP1. **e** Western blot analysis of cleaved structural proteins of EV71 in consecutive passages

the AcGFP, the AcEV71 exhibited loss of the *P1* gene of EV71 during the continuous passage, and this loss was more severe compared to that observed with eGFP. However, the introduction of the *lef5* significantly improved the stability of the *EV71- P1* gene during serial passages.

Analysis by indirect immunofluorescence (IFA) was also conducted to assess the expression of the P1 protein (Fig. 4c). Notably, the EV71 structural proteins failed to be detected at passage 12 (P12) in Sf9 cells infected with AcEV71. The EV71 structural proteins were still detectable at P12 in Sf9 cells infected with AcEV71-op166-lef5. The formation of enterovirus VLPs requires not only the expression of the P1 protein but also the assistance of the 3CD protein to cleave P1 into VP0, VP3, and VP1 (Chung et al. 2006) (Fig. 4d). To evaluate the cleavage of the P1 protein, Western blot analysis was performed. In Sf9 cells infected with AcEV71, VP1 was undetectable at the 4th passage. However, with the extra introduction of lef5, a high level of cleaved VP1 protein was detected at the 4th passage in Sf9 cells infected with both AcEV71-op166-lef5 and AcEV71-p10-lef5. For the AcEV71-p10-lef5, VP1 was undetectable by P8, whereas for the AcEV71-op166-lef5, VP1 remained detectable until P8 (Fig. 4e). These findings demonstrate that the introduction of the *lef5* enhances the stability of *EV71- P1* gene expression and facilitates the sustained production of cleaved P1 protein with the protease activity of 3CD protein.

Discussion

The baculovirus expression vector system (BEVS) has emerged as a cornerstone in the realm of heterologous protein expression, showcasing its versatility and efficacy across various applications (Kost and Kemp 2016; Mishra 2020). Its commercialization has further propelled its utility, establishing it as a reliable platform for biotechnological product production (Mena and Kamen 2011). However, despite these advancements, challenges remain, particularly regarding virus stability during serial passages in insect cells (Hong et al. 2022). Notably, defective viruses can accumulate rapidly over consecutive passages (Pijlman et al. 2001), posing a significant threat to BEVS applications, especially in vaccine development where stability is paramount. The accumulation of defective viruses leads to a drastic decline in the expression levels of the exogenous protein, undermining the system's efficiency and reliability for high-quality vaccine and other stability-sensitive product production (Pijlman et al. 2003).

Despite the lack of definitive studies demonstrating a complete elimination of defective virus generation and the subsequent decline in exogenous protein expression during serial passages of baculoviruses, several research endeavors have yielded promising results in delaying these detrimental effects (Giri et al. 2012; Pijlman et al. 2003). Notably, a study reported that the *lef5* expression from SeMNPV enhances the stability of a recombinant AcMNPV expressing green fluorescent protein (GFP) (Martinez-Solis et al. 2017). Given the pivotal role of the *lef5* in the late gene expression cascade of AcMNPV (Guarino et al. 2002; Harwood et al. 1998; Su et al. 2011). Given these insights, it is reasonable to speculate on the potential impact of overexpressing the endogenous *lef5 f*rom AcMNPV on the stability of various exogenous proteins within a recombinant AcMNPV-based expression system.

Gratifyingly, the results aligned with our expectations, demonstrating that *lef5* overexpression significantly enhances both genetic stability and protein expression stability of exogenous genes such as *eGFP* and *EV71-P1*. Notably, the expression level of the *lef5* is significantly influenced by the promoter used. We compared the regulatory effects of the op166 and p10 promoters o*n lef5* transcription. The op166 promoter initiates *lef5* transcription earlier, while the p10 promoter exhibits a stronger initiation effect. This difference in promoters further impacts the enhancement of exogenous protein expression stability. Specifically, for the simple exogenous protein eGFP, *lef5* overexpression driven by the p10 promoter more effectively increases protein expression stability. In the expression of complex exogenous genes involving multiple large fragments, such as those in EV71-VLPs, particularly the expression of 3CD and P1 proteins, *lef5* transcription initiated earlier by the op166 promoter not only significantly extends P1 protein expression but also ensures the effective cleavage of P1 protein, which requires the integrity of 3CD protease function to ensure the formation of VLPs (Chung et al. 2006). Our study not only confirms the potential of *lef5* in enhancing the stability of exogenous protein expression but also reveals the importance of promoter selection in optimizing *lef5* expression for exogenous protein production. These findings provide valuable strategic considerations for further optimizing baculovirus expression systems and improving the efficiency and stability of exogenous protein production.

During the serial passage experiments, an intriguing observation emerged concerning the accumulation of gene-deleted viruses under different inoculation conditions. Specifically, a more rapid accumulation of gene-deleted recombinant AcMNPV expressing eGFP was noted when the inoculation ratio was lower. This finding aligns with previous research conducted by Martinez-Solis, which similarly reported an increased incidence of gene deletions at lower multiplicity of infection (MOI) (Martinez-Solis et al. 2017). It is plausible to hypothesize that the higher number of replication cycles at lower

MOI likely subjects the viral genome to greater stress and replicative pressure, increasing the potential for errors or deletions to arise and accumulate. However, despite this observed trend, the impact of the *lef5* on viral replication stability remains independent of the inoculation ratio.

In summary, our study reveals the significant potential of *lef5* as a key factor in enhancing the long-term expression stability of exogenous proteins within the baculovirus expression vector system (BEVS). Notably, this effect is particularly evident in the production of complex proteins, such as virus-like particles (VLPs). By overexpressing the *lef5*, we observed substantial improvements in both genetic stability and protein expression levels of exogenous proteins, exemplified by eGFP and EV71-VLPs. These findings not only deepen our understanding of the mechanisms governing exogenous protein expression stability in BEVS but also provide valuable strategic insights for optimizing this system, ultimately enhancing the efficiency and reliability of biotechnological product production.

Author contributions JG, JP and RLL contributed to experiment design. JG, JP, RLL, WL and SSQ contributed to the conceptualization, data curation, formal analysis, visualization, and writing. JG, JP, RLL, JG, JY, SLM and SS contributed to the data curation. JG, JP and RLL contributed to experiment development. All authors approved the final manuscript.

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Data availability All of the data generated or analyzed during this study are included in this published article.

Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of interest The authors declare no conflict of interest.

Informed consent Not applicable.

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