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Identification of A functional region in *Bombyx mori* nucleopolyhedrovirus VP39 that is essential for nuclear actin polymerization

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ABSTRACT

Nuclear actin polymerization plays an indispensable role in the nuclear assembly of baculovirus nucleocapsid, but the underlying viral infection-mediated mechanism remains unclear. VP39 is the major protein in baculovirus capsid, which builds the skeleton of the capsid tubular structure. VP39 is suggested in previous studies to interact with cellular actin and mediate actin polymerization. However, it is unclear about the role of VP39 in mediating nuclear actin polymerization. Results in this study indicated that *vp39* deletion abolished nuclear actin polymerization, which was recovered after *vp39* repair, revealing the essential part of VP39 in nuclear actin polymerization. Furthermore, a series of mutants with *vp39* deletions were constructed to analyze the important region responsible for nuclear actin polymerization. In addition, intracellular localization analysis demonstrated that the amino acids 192–286 in VP39 C-terminal are responsible for nuclear actin polymerization.

1. Introduction

The Baculoviridae is a family of enveloped viruses that contain the circular double-stranded DNA genomes with a size of 80–180 kb (Herniou et al., 2003). After the virus enters a susceptible cell via receptor mediated endocytosis, the nucleocapsid is released from the endocytic vesicle and transported into the nucleus where it is disassembled followed by virus gene transcription, DNA replication, and nucleocapsid assembly. Progeny nucleocapsids are either transported to the nuclear periphery where they bud out of the cell membrane (budded virus - BV) spreading the infection to other cells, or later in infection remain in nuclei where they are occluded (occlusion derived virus - ODV) (Keddie et al., 1989). The polymerization of actin from globular (g) to the filamentous (f) form is involved in the transport of the nucleocapsids through the cytoplasm to nuclear pores where it propels them into the nuclei (Ohkawa et al., 2010; Jan Mueller et al., 2014). It is also involved in the disruption of the nuclear envelope during egress (Ohkawa and Welch, 2018) and drives baculovirus transit to the cell surface, resulting in transmission to neighboring cells (Ohkawa et al., 2010).

The key host factors involved in cellular actin polymerization include G-actin, the actin-related protein 2/3 complex (Arp2/3), and the nucleation promoting factors (NPFs) (Wang et al., 2007a; Rohatgi et al., 1999). The arp2/3 complex is composed of two actin related proteins and 5 other proteins (Goley and Welch, 2006; Machesky and Gould, 1999). The NPFs are members of the WASP protein superfamily (Machesky et al., 1999; Yarar et al., 1999; Rohatgi et al., 1999). A conserved baculovirus protein (pp78/83, AcMNPV orf 9, BmNPV orf 2) that is located at one end of baculovirus nucleocapsids (Vialard and Richardson, 1993) (Russell et al., 1997) is a WASP ortholog and has been shown to be capable of stimulating actin polymerization (Ohkawa et al., 2010). In addition, other viral factors also participate in actin polymerization. Of them, VP39 has been identified to colocalize and interact with F-actin (Charlton and Volkman, 1991) (Lanier and Volkman, 1998). In addition, VP39 of *Heliothus armigera* NPV(HaNPV) binds to and mediates its polymerization of actin *in vitro* (Lu et al., 2004).

Because viral VP39 can interact with cellular actin and mediate its polymerization we wanted to determine if it mediates nuclear actin polymerization. Therefore, in this study, we described the effect of VP39 on nuclear actin polymerization using knockout and mutagenesis technology. Our results indicated that nuclear actin polymerization is dependent on VP39, and the region within amino acids 192–286 is indispensable for nuclear actin polymerization. (**Response to comment 1**, **Reviewer #2**).

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Abbreviation

B.mori	Bombyx mori
BmNPV	*Bombyx mori* nucleopolyhedrovirus
ODV	occlusion-derived virus
BV	budded virus
HaNPV	*Helicoverpa armigera* nucleopolyhedrovirus
AcMNPV	*Autographa californica* multiple nucleopolyhedrovirus
E.coli	Escherichia coli
PKIP	Protein kinase interacting protein
E25	ODV-E25

2. Materials and methods

2.1. Virus and cell lines

BmNPV *vp39* and *gp64* were deleted by the λ-*Red* homologous recombination system. The bacmid extracted from *E. coli* strain BW25113 (λ-*Red* homologous recombination system) (Wang et al., 2007b) was used as the PCR template. The truncated *vp39* fragments were transposed into the *vp39* knockout (KO) bacmid to form recombinant bacmid constructs (Bac-to-Bac Baculovirus Expression System, Invitrogen). The virus was generated from BmN cells transfected with bacmid. The BmN cell line derived from *Bombyx mori* (Zhou, 2000) was cultured at 27 °C in Gibco Sf-900™ II SFM (Thermo Fisher Scientific - CN) supplemented with 3% Gibco fetal bovine serum.

2.2. Bioinformatic analysis

To obtain the evolutionary relationships and conservation of VP39 among baculoviruses, the amino acid sequences of VP39 protein homologs from 54 baculoviruses were aligned with BLAST. (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). A phylogenetic tree was generated by MEGA 7.0.26. The conserved amino acid regions of VP39s were analyzed by Geneious 9.1.4. All the possible alpha-helix and beta-strand secondary structures formed in the conserved regions of VP39s were predicted with Ali2D (http://toolkit.tuebingen.mpg.de/sections/secstruct).

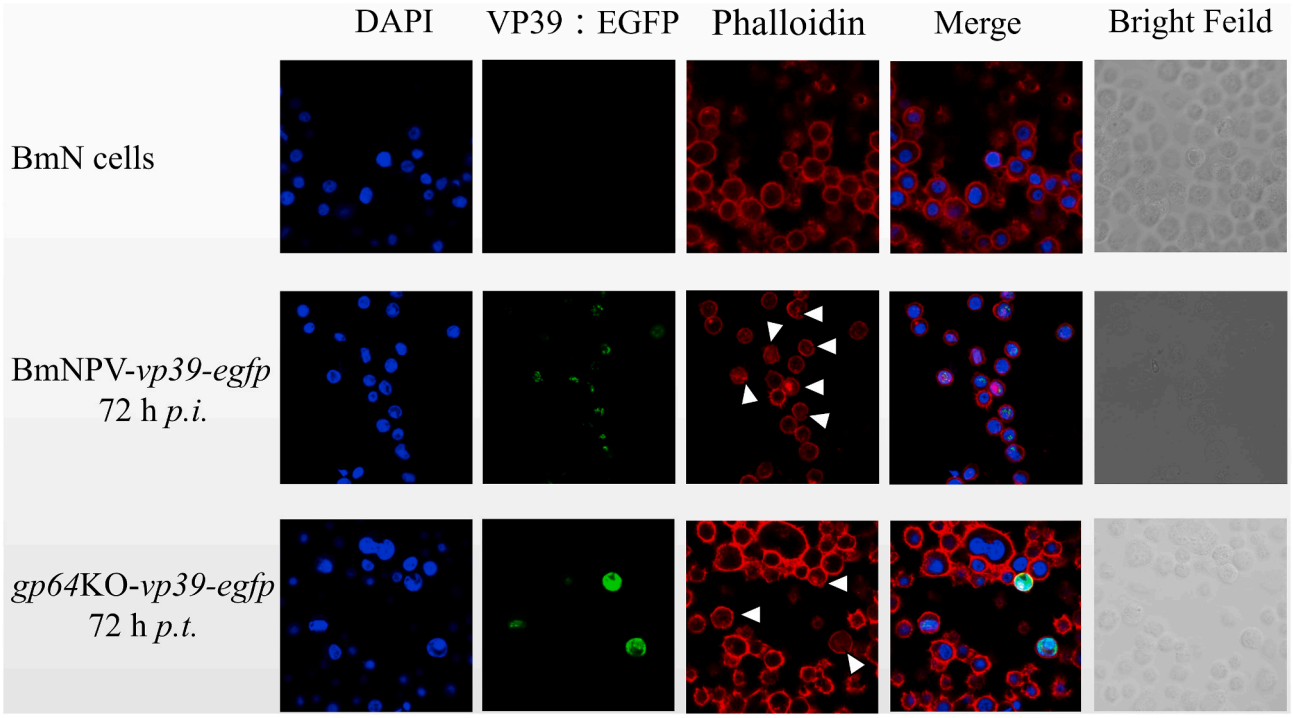

Fig. 1. Subcellular localization of VP39 and F-actin in BmN cells. Subcellular localization of VP39 and F-actin in BmN cells, cells were infected with BmNPV-*vp39-egfp* at 72 h *p.i.* and transfected with *gp64*KO-*vp39-egfp* at 72 h *p.t.* Cells were fixed and stained with DAPI and Phalloidin. The arrows indicated the actin polymerization in the nucleus.

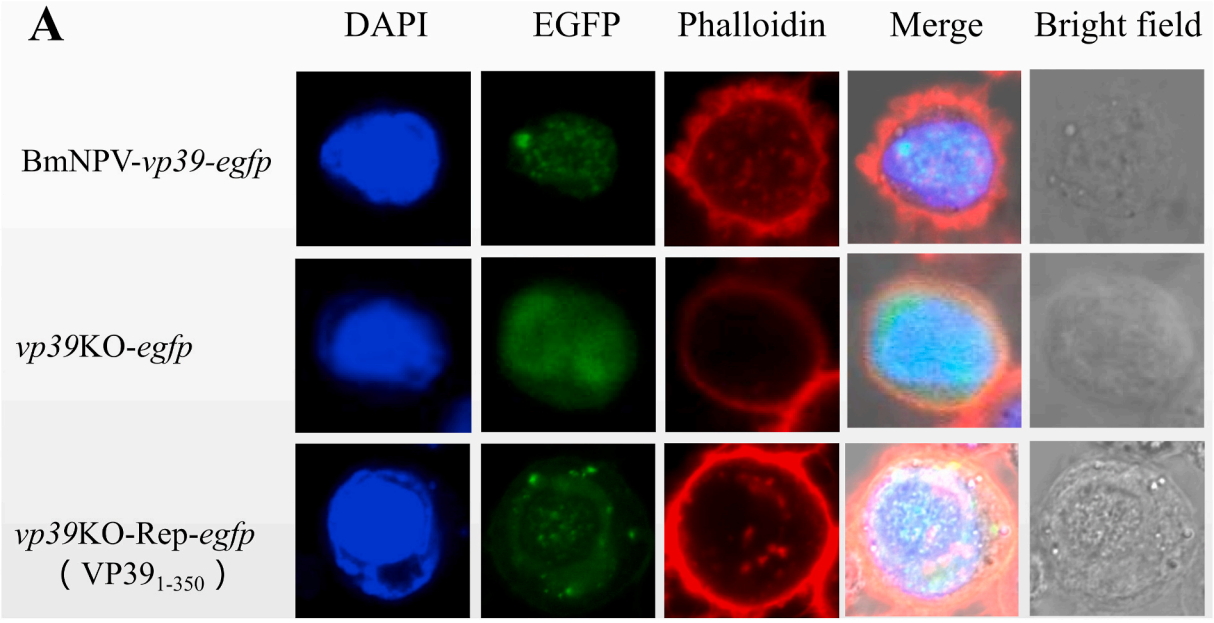

Fig. 2. Nuclear actin polymerization in BmN cells transfected with bacmids with or without *vp39,* and in cells transfected with *pIZ-mCherry-vp39*. Cells were fixed and stained with DAPI and Phalloidin. (A) Nuclear actin polymerization in cells transfected with BmNPV-*vp39-egfp*, *vp39*KO-*egfp*, *vp39KO-Rep-egfp* (1-350aa) at 72 h post-transfection. (B) Nuclear actin polymerization in cells transfected with bacmids containing *pkip* as a control for VP39. The two upper rows showed the localization of PKIP protein. The following three rows indicated nuclear actin polymerization in cells transfected with Bm-*pkip-egfp, vp39KO-pkip-egfp, gp64KO-pkip-egfp* at 72 h post-transfection. (C) Nuclear actin polymerization in cells transfected with bacmids containing *e25* as a control for VP39. The two upper rows showed the localization of E25 protein. The following three rows indicated nuclear actin polymerization in cells transfected with Bm-*e25-egfp, vp39KO-e25-egfp, gp64KO-e25-egfp* at 72 h post-transfection. (D) Nuclear actin polymerization in cells transfected with *pIZ-mCherry-vp39* at 48 h and 72 h post-transfection, and in cells transfected with *pIZ-mCherry-vp39* and infected by wild type BmNPV at 48 h post-infection.

2.3. Generation of EGFP-fused VP39-truncated BmNPV bacmids, control bacmids

Enhanced green fluorescence protein (*egfp*) was amplified from plasmid pUC18-*egfp* by using primers *egfp*-F (gCgCCATgggATCCggAATTCATggTgAgCAAgggC; the *Eco*RI site is indicated by underline) and *egfp*-R (CTTCTCgACAAgCTTggTACCCT

TgTACAgCTCgTC; the *Kpn*I site is indicated by underline) and cloned into pFastHTb using *Eco*RI and *Kpn*I, generating pFast-*egfp*. The *vp39* promoter was amplified from the BmNPV bacmid using the primers *vp39*pro-F (ATCGACCATggTTggTAAACgTACACTTT; the *Nco*I site is indicated by underline) and *vp39*pro-R (TACTGggATCCATTgTTgCCg TTATAAAT; the *Bam*HI site is indicated by underline) and cloned into pFastHTb-*egfp* using *Nco*I and *Bam*HI, generating pFastHTb-*pro-egfp*. The *vp39* truncated fragments were amplified from BmNPV bacmid by using primers listed in Supplementary Table S1. The fragments were then cloned into pFastHTb-*pro-egfp* using *Bam*HI and *Eco*RI between the *vp39* promoter and the *egfp* gene. A full-length *vp39* was also cloned (pFast-*pro-vp39-egfp*) as described above. The mid-deletion fragments were amplified with overlap PCR (Heckman and Pease, 2007). All cloned plasmids were confirmed by sequencing (Sangon Biotech). These plasmids were transposed into *vp39* knockout (KO) bacmid as described above. The full-length pFast-*pro-vp39-egfp* plasmid was transposed into wild type BmNPV bacmid to form BmNPV-*vp39-egfp*. The pFastHTb-*pro-egfp* plasmid was transposed into *vp39* knockout (KO) bacmid and wild type BmNPV bacmid to form *vp39*KO-*egfp* and BmNPV-*egfp*, respectively. *pkip and e25* were amplified from the BmNPV bacmid and cloned into the above pFast-*egfp* plasmid using *Bam*HI and *Eco*RI. Then these two clones were transposed into wild type BmNPV bacmid to form Bm-*pkip/e25-egfp*, or *vp39* knockout (KO) bacmid to form *vp39*KO-*pkip/e25-egfp*, or *gp64* knockout (KO) bacmid to form *gp64*KO-*pkip/e25-egfp*. The pFast-*pro-vp39-egfp* plasmid was transposed into *gp64* knockout (KO) bacmid to form *gp64*KO- *vp39-egfp*. The full length of *vp39* was amplified from the BmNPV bacmid using the primers *vp39*-F (ATATAggATCCATggCgCTAATgCC; the *BamH*I site is in italics) and *vp39*-R (CTCACCATgAATTCggCggCTACACCTC; the *Eco*RI site is in italics) and cloned into pIZ-*mCherry* using *BamH*I and *Eco*RI, generating pIZ-*mCherry-vp39*. The pIZ-*mCherry* (with *Hind*III and *BamH*I) plasmid was saved in our lab.

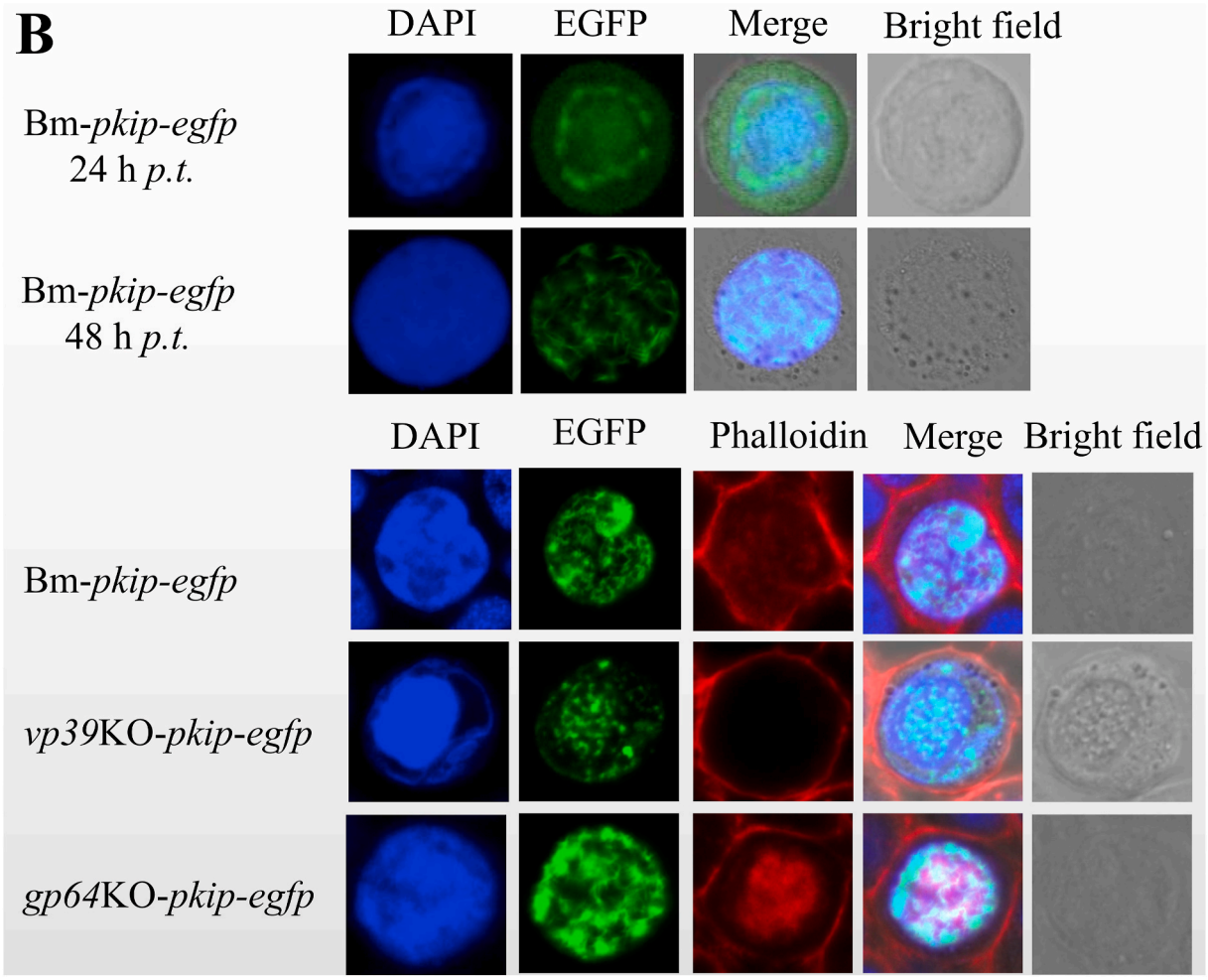

Fig. 2. (*continued*)

2.4. Transfection

Recombinant bacmid DNA was purified using the alkali extraction method (Bac-to-Bac Baculovirus Expression System, Invitrogen). The pIZ-*mCherry-vp39* plasmid was purified by kit (Beijing Zoman Biotechnology Co., Ltd.). 9×10^5 BmN cells were seeded at 1×10^6 cells/35-mm plates overnight and transfected with 5 μg of the bacmid DNA by using 8 μl lipoInsect (Beyotime) according to the manufacturer's protocol. After incubation for 5 h, the transfection mixture was removed and replaced with fresh Gibco Sf-900™ II SFM supplemented with 3% fetal bovine serum, and subsequently incubated at 27 ˚C.

2.5. Immunofluorescence and confocal microscopy

BmN cells seeded on coverslips overnight were transfected with each of the recombinant bacmids. At the indicated time points, cells were first washed three times by PBS for 15 min, then fixed with 4% paraformaldehyde (Beyotime) for 10min, washed three times for 5 min each time with PBS, permeabilized in 0.1% TritonX-100 (Beyotime) for 10 min, rinsed 3 times in 1 ml PBS for 5 min each time, incubated with 0.8 ml DAPI (Beyotime), washed three times for 5 min each time with PBS. F-actin was stained by YF594-Phalloidin (red) and YF488-Phalloidin (green) (Hangzhou Baimaihang Technology Co., Ltd.) for 20 min, then cells were washed three times for 5 min each time with PBS. The modified cells were examined with a ZEISS LSM 780/880 confocal laser scanning microscopy. For VP39: GFP, excitation was at 488 nm and the acquisition was between 500 and 523 nm for GFP. For F-actin: Phalloidin, excitation was at 587 nm and the acquisition was 610 nm for Phalloidin.

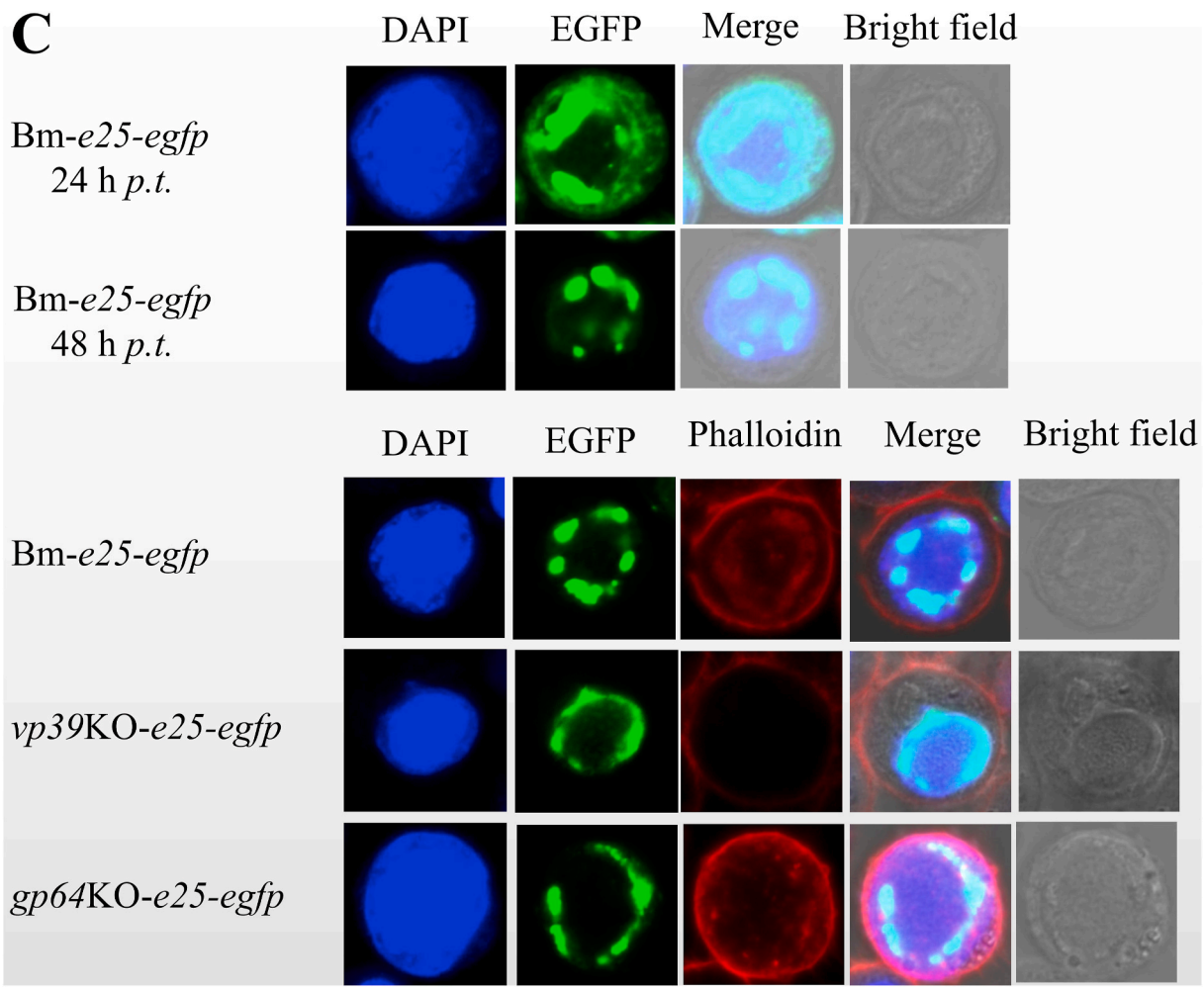

Fig. 2. (*continued*)

3. Results

3.1. Nuclear actin polymerization was mediated by a viral infection or bacmid transfection

In this study, only bacmid DNA transfection was carried out, as VP39 deletion abolished viral production (Ono et al., 2012). To identify whether nuclear actin polymerization was affected by bacmid transfection, nuclear actin polymerization in cells infected with BmNPV-*vp39-egfp* virus or transfected with *gp64*KO-*vp39-egfp* bacmids (in which virus was not produced) was observed by confocal microscopy. To obtain enough successfully transfected cells for observation, the 72-h *p.t.* time point was adopted to analyze nuclear actin polymerization. According to our results, nuclear actin polymerization was detected in cells infected with BmNPV-*vp39-egfp* virus and transfected with *gp64*KO-*vp39-egfp* bacmids, but not in cells without any infection or transfection treatment (Fig. 1). Such observations indicated that nuclear actin polymerization was not affected by bacmid transfection.

3.2. Nuclear actin polymerization was dependent on VP39

As suggested by the previous study, VP39 interacts with cellular actin and mediates its polymerization. To reveal whether nuclear actin polymerization was mediated by VP39, actin localization in cells transfected with *vp39*KO-repair bacmids and *gp64*KO-repair bacmids was observed in this study. As shown in Fig. 2A, by 72 h *p.t.*, nuclear actin polymerization was observed in cells transfected with BmNPV-*vp39-egfp*, which was not detected in cells transfected with *vp39*KO-*egfp* and was identified when *vp39* was repaired (transfection with *vp39*KO-Rep-*egfp*, $VP39_{1\text{-}350}$). Besides, actin and VP39 showed colocalization in

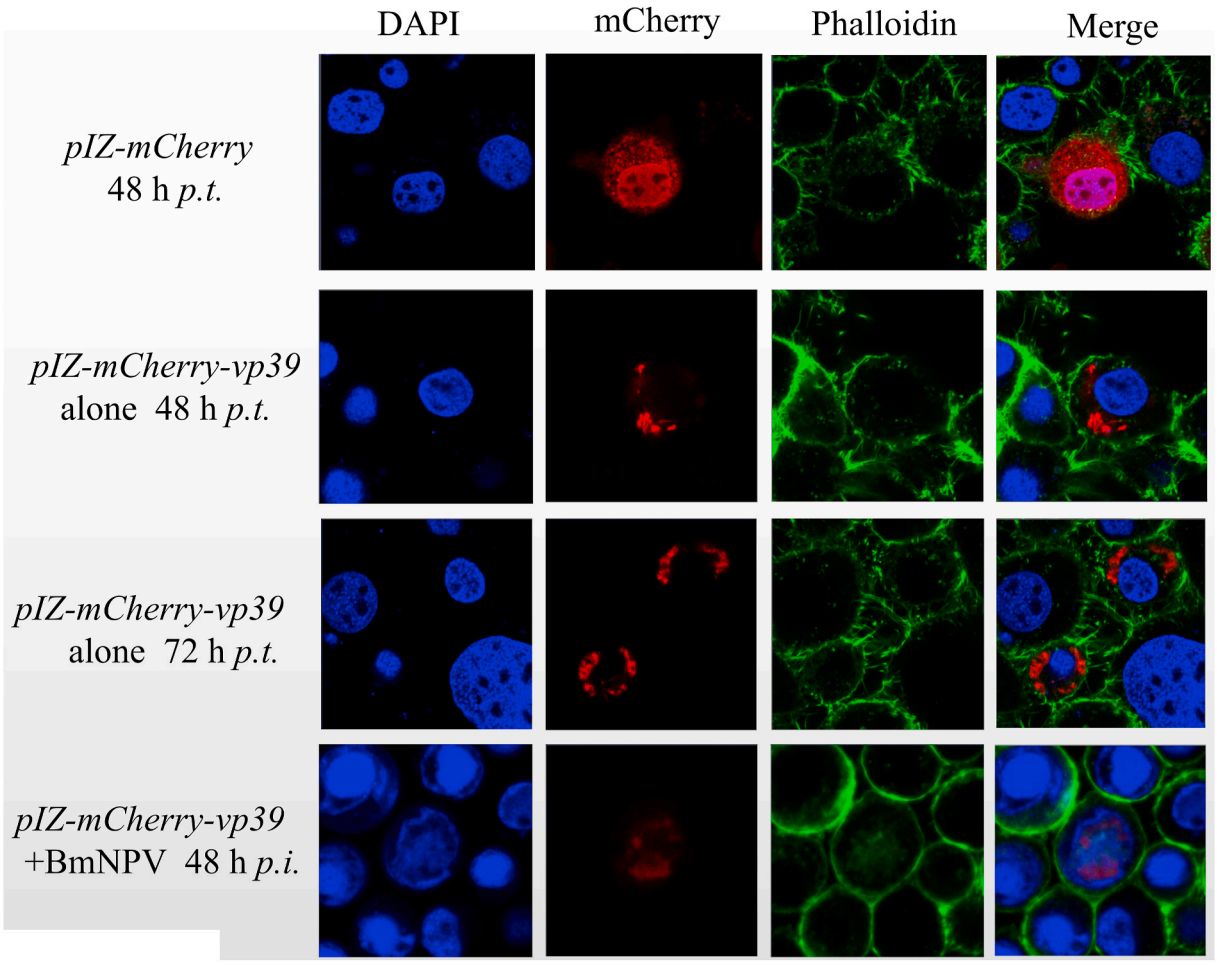

Fig. 2. (*continued*)

the nucleus of the cells transfected with BmNPV-*vp39-egfp* or *vp39*KO-Rep-*egfp* (1-350aa) (Fig. 2A). EGFP was dispersed in the whole-cell in the case of *vp39* deletion (Fig. 2A), in this regard, a viral protein that was able to enter the nucleus at the time of *vp39* deletion was required as the indicator protein (a control protein). To confirm whether VP39 was responsible for nuclear actin polymerization, *gp64* was knocked out as another control. In addition, PKIP and E25, the two BmNPV proteins showing nuclear translocation, were fused with an *egfp* at the C-terminal and used as two controls. Further, Bm-*pkip/e25-egfp* bacmid and *gp64*KO- *pkip/e25-egfp* bacmid were used as the negative and positive controls for *vp39*KO-*pkip/e25-egfp* bacmid, respectively. According to Fig. 2B and C, PKIP and E25 completely entered the nucleus at 48 h *p.t.*. By 72 h *p.t.*, nuclear actin polymerization was observed in phalloidin-stained F-actin within cells transfected with Bm-*pkip/e25-egfp* and *gp64*KO-*pkip/e25-egfp*, while that was not detected in cells transfected with *vp39*KO-*pkip/e25-egfp*. Such observations verified that *vp39* knockout abolished nuclear actin polymerization.

To further confirm that VP39 is required for nuclear actin polymerization, only pIZ-*mCherry-vp39* was transfected. In Fig. 2D, as a negative control, mCherry fluorescence diffused in the whole-cell when only pIZ-*mCherry* was transfected (48 h *p.t.*). Besides, VP39 was localized to the cytoplasm, and the cellular actin is not localized to the nucleus when only pIZ-*mCherry-vp39* was transfected (48 h *p.t.* and 72 h *p.t.*). Such observations indicated that VP39 cannot enter into the nucleus by itself and actin is not able to polymerize in the nucleus without VP39 nuclear import. It informed us that although VP39 express alone normally, the cellular actin still cannot polymerize in the nucleus without VP39 nuclear translocation. However, actin can polymerize in the nucleus and colocalize with VP39 when pIZ-*mCherry-vp39* was transfected into the cells infected by wild type BmNPV (48 h *p.i.*). This result indicated that VP39 can enter into the nucleus with the assistance of other viral proteins, and nuclear actin polymerization was indeed

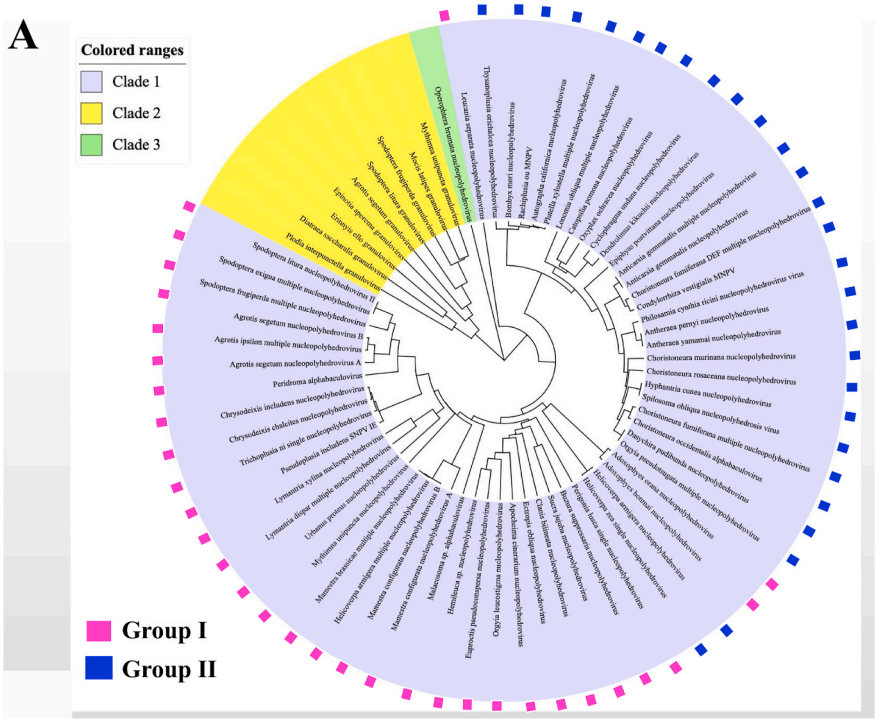

Fig. 3. Bioinformatic analysis of VP39s from 54 baculoviruses. (A) The phylogenetic tree of VP39 proteins. Clades 1, 2, and 3 are indicated by different colors. Virus from Group I and Group II are indicated by red and blue rectangles, respectively. (B) BmNPV VP39 amino acid sequences were divided into seven domains based on the conservative analysis of 54 baculovirus (by Geneious), including $VP39_{1\text{-}42}$, $VP39_{43\text{-}91}$, $VP39_{92\text{-}139}$, $VP39_{140\text{-}191}$, $VP39_{192\text{-}245}$, $VP39_{246\text{-}286}$, $VP39_{287\text{-}350}$. VP39s from the three groups (Group I, Group II and GV) share three highly conserved regions, including aa 1–140, aa 166–220, aa 228–338. $VP39_{192\text{-}286}$ were indicated by a purple cylinder.

dependent on VP39 nuclear import. Taken together, the above-mentioned results indicated that nuclear actin polymerization was dependent on VP39.

3.3. Seven conserved regions of BmNPV VP39 were predicted

Nuclear actin polymerization was confirmed to be dependent on VP39 as mentioned above, thus, we attempted to identify the region responsible for this event. To obtain the functional regions of VP39, the conservative domains were divided. Firstly, the evolutionary relationships of VP39 homologs from 54 the baculoviruses were analyzed, and thereby a phylogenetic tree was generated (Fig. 3A). Specifically, these VP39 homologs were categorized as three clades, among which, all the VP39s from Group I and Group II alphabaculoviruses belonged to clade 1, while those from betabaculoviruseses (GV) belonged to clade 2, and only one baculovirus was in clade 3. Thereafter, VP39s from the three groups (Group I, Group II and GV) share three highly conserved regions, *i.e.* aa 1–140, aa 166–220, aa 228–338 (Fig. 3B). These results indicated that VP39s from group I and group II alphabaculoviruses were more distant from betabaculoviruses VP39s. Later, seven domains (BmNPV VP39) were divided based on the identities of amino acid sequences in VP39s from 54 baculoviruses, including $VP39_{1\text{-}42}$, $VP39_{43\text{-}91}$, $VP39_{92\text{-}139}$, $VP39_{140\text{-}191}$, $VP39_{192\text{-}245}$, $VP39_{246\text{-}286}$, and $VP39_{287\text{-}350}$ e predicted secondary structures of BmNPV VP39 were shown in Fig. 4.

3.4. Preliminary mapping of sequence in VP39 required for nuclear actin polymerization

To identify the region in VP39 that is responsible for nuclear actin polymerization, a series of VP39 mutants were constructed based on those seven conserved domains. The deletion of *vp39* and the repair of its mutants are displayed in Fig. 5A and Fig. 5B, respectively. Meanwhile, bacmid transfection was carried out as mentioned above. By 72 h *p.t.*, it was observed from Fig. 6A and Fig. 6B that nuclear actin polymerization was not detected in cells transfected with partial *vp39*KOrep-VP39 mutants, including the C-terminal truncated mutants ($VP39_{1\text{-}42}$, $VP39_{1\text{-}91}$, $VP39_{1\text{-}139}$, $VP39_{1\text{-}191}$, and $VP39_{1\text{-}245}$, Fig. 6A), and the N-terminal truncated mutants ($VP39_{246\text{-}350}$ and $VP39_{287\text{-}350}$, Fig. 6B). Nonetheless, nuclear actin polymerization was detected in cells transfected with the rest *vp39*KOrep-VP39 mutants, namely, the C-

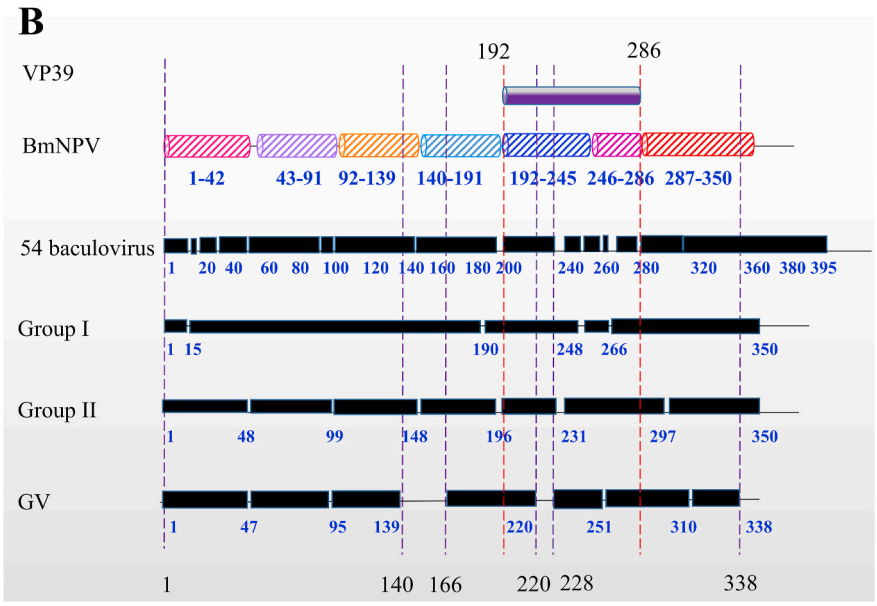

Fig. 3. (*continued*)

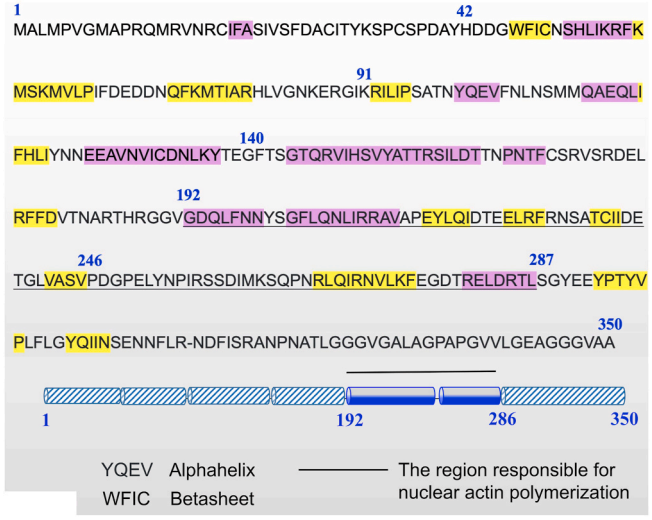

Fig. 4. The predicted secondary structures of BmNPV VP39. The alpha-helix was indicated by purple, and the beta-sheet was indicated by yellow. The region responsible for nuclear actin polymerization is indicated by underline.

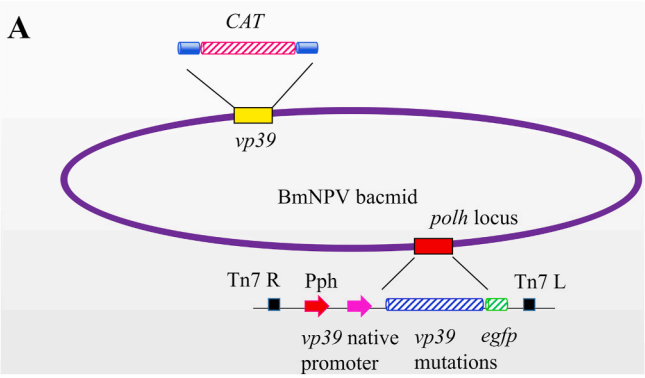

Fig. 5. Schematics of VP39 bacmid constructions. (A) Schematic diagram of *vp39* knockout and mutants repair. (B) Cloning schematic diagram of VP39 truncations using the BmNPV *vp39* knockout bacmid as the backbone and schematics of the VP39 mutants that could mediate nuclear actin polymerization (**Response to comment 2**, **Reviewer #2**). A GFP tag (green stick) was fused to the C terminus of VP39 mutants (purple stick) to follow VP39 localization.

terminal truncated mutants ($VP39_{1\text{-}286}$ and $VP39_{1\text{-}350}$, Fig. 6A), and the N-terminal truncated mutants ($VP39_{43\text{-}350}$, $VP39_{92\text{-}350}$, $VP39_{140\text{-}350}$ and $VP39_{192\text{-}350}$, Fig. 6B). Such results implied that partial VP39 mutants, including the C-terminal truncated mutants ($VP39_{1\text{-}286}$ and $VP39_{1\text{-}350}$), and the N-terminal truncated mutants ($VP39_{43\text{-}350}$, $VP39_{92\text{-}350}$, $VP39_{140\text{-}350}$, and $VP39_{192\text{-}350}$), repaired the *vp39* deletion-induced abolishment of nuclear actin polymerization. Fig. 5B (**Response to comment 2**, **Reviewer #2**) shows the mutants that mediate nuclear actin polymerization. Besides, the amino acid sequence 1–191 did not mediate nuclear actin polymerization, which suggested that the four domains in the VP39 N-terminal were not required for nuclear actin

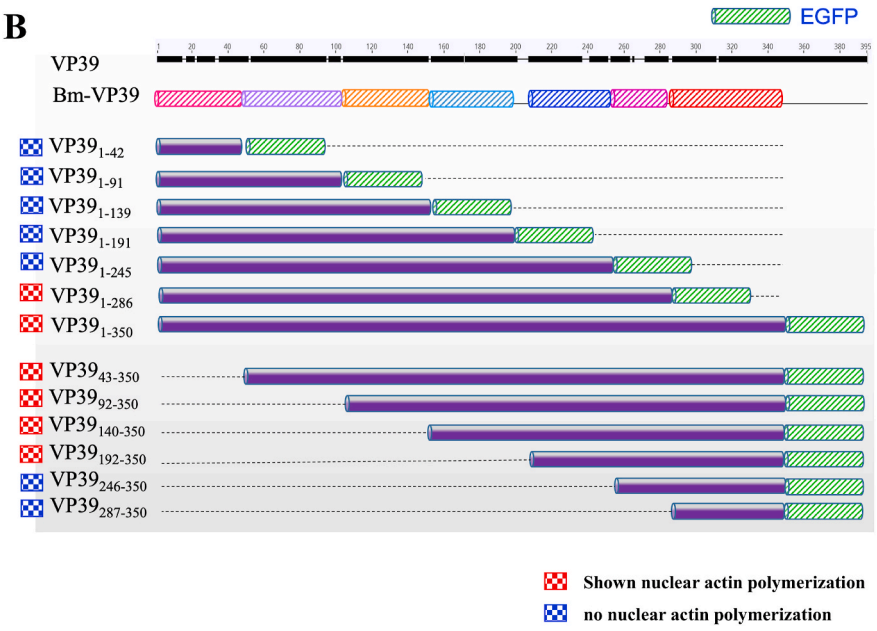

Fig. 5. (*continued*)

polymerization.

3.5. Fine mapping of sequence in VP39 required for nuclear actin polymerization

To more accurately define the region in VP39 that is responsible for the nuclear actin polymerization, further truncations and internal deletions of the VP39 C-terminal were constructed, including $VP39_{192-286}$, $VP39_{192-245}$, $VP39_{246-286}$, and $VP39_{192-245+287-350}$ (Fig. 7A). As shown in Fig. 7B, nuclear actin polymerization was found in cells transfected with the mutant containing the aa 192 to 286, but not in those transfected with the mutant containing its corresponding truncation $VP39_{287-250}$ (Fig. 7B). Besides, nuclear actin polymerization was not detected in cells transfected with the mutants containing the aa 192 to 245 (Fig. 7B) or its corresponding truncation $VP39_{246-350}$ (Fig. 7B). Similarly, nuclear actin polymerization was not discovered in cells transfected with mutants containing aa 246 to 286 or its corresponding internal deletion $VP39_{192-245+287-350}$ (Fig. 7B). These results revealed that nuclear actin polymerization was only detected when both aa 192 to 245 and aa 246 to 286 were repaired. In other words, the amino acid sequence 192–286 was the key region in VP39 responsible for nuclear actin polymerization (Fig. 7C). To further confirm this speculation, its corresponding internal deletion $VP39_{1-191\ +\ 287-350}$ was also constructed and transfected. Results in Fig. 7D demonstrated that actin in cells transfected with this mutant showed only cytoplasmic membrane localization. Therefore, it was confirmed that the amino acid sequence 192–286 in VP39 was responsible for nuclear actin polymerization.

4. Discussion

Actin is the cytoplasmic skeleton involved in intracellular transport, and actin polymerization is a universal biological process in eukaryotes. Host factors, including G-actin, the actin-related protein 2/3 complex (Arp2/3) and the nucleation promoting factors (NPFs), are related to actin polymerization. Under steady-state, these factors are predominantly localized in the cytoplasm. However, increasing evidence suggests that these contributing factors of actin polymerization are not only present in the cytoplasm but also the nucleus, and that they play vital roles ranging from chromatin remodeling to transcription regulation (Kapoor and Shen, 2013; Grosse and Vartiainen, 2013). It is reported that, some intracellular pathogens, such as Listeria monocytogenes (Welch et al., 1997), Rickettsia spp. (Gouin et al., 2004), vaccinia virus (Cudmore et al., 1995), alpha-herpesvirus (Feierbach et al., 2006), human immunodeficiency virus (Spear et al., 2014), and Burkholderia thailandensis (Stevens et al., 2005), utilize the host actin polymerization to achieve their reproduction. (Welch and Way, 2013, Gouin et al., 2005; Cossart, 2000; Spear and Wu, 2014). Furthermore, baculovirus can manipulate its host in nuclear actin polymerization, thus accomplishing the eventual viral reproduction. Notably, the actin cytoskeleton in the cytoplasm of host cells will rearrange shortly after a viral infection, followed by actin polymerization in the nucleus mediated by p78/83 (a viral protein) and the Arp2/3 complex (Goley et al., 2006). Moreover, Ac102 was identified as crucial for nuclear actin polymerization. (Hepp et al., 2018). In addition to p78/83 and Ac102, VP39 may also participate in this process.

VP39 is a late gene product that builds the basic skeleton of

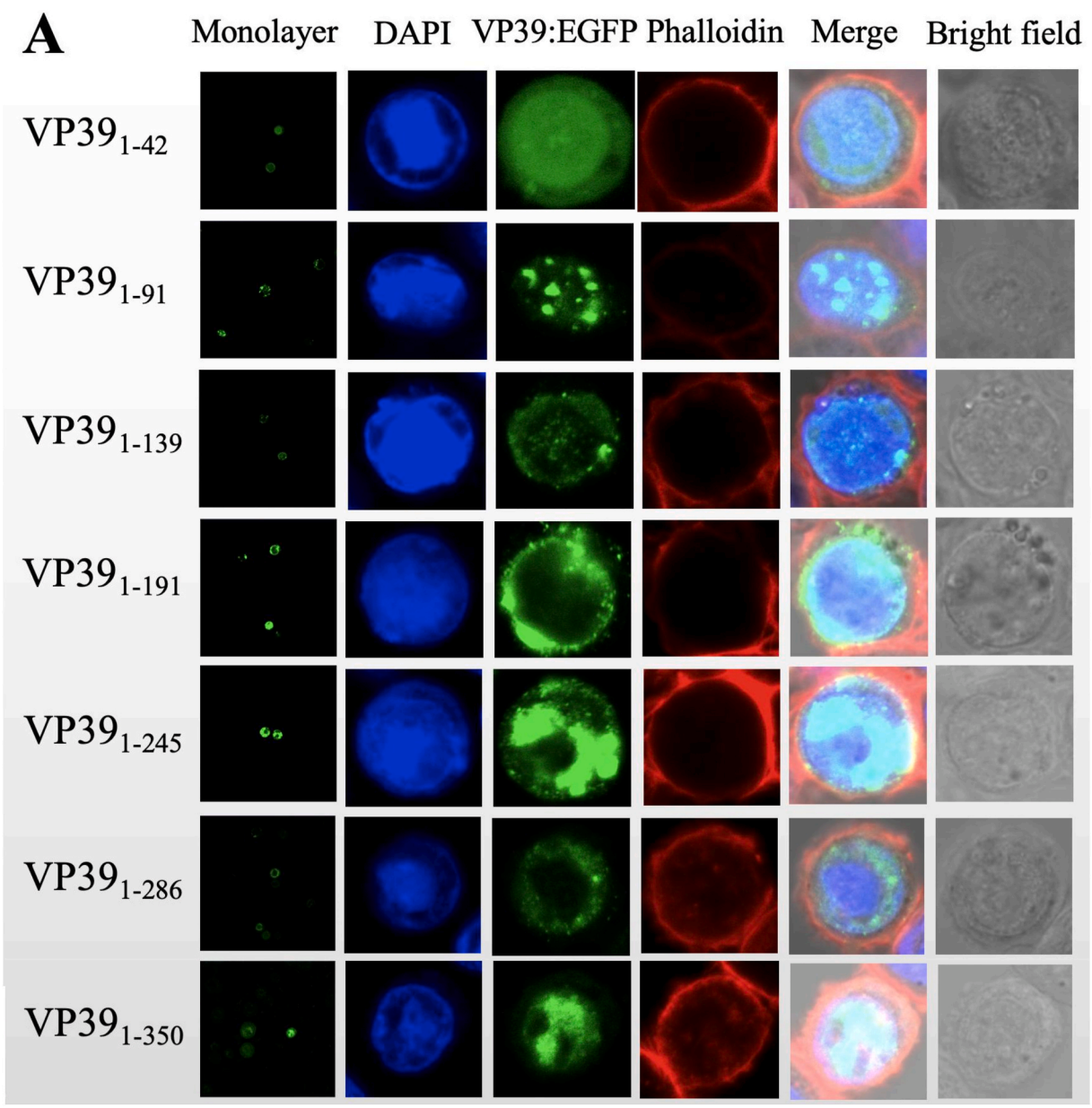

Fig. 6. Nuclear actin polymerization in BmN cells transfected with *vp39*KOrep-VP39 truncated mutants at 72 h post-transfection. Cells were fixed and stained with DAPI and Phalloidin. (A) Nuclear actin polymerization in cells transfected with *vp39*KOrep-VP39 N-terminal mutants. (B) Nuclear actin polymerization in cells transfected with *vp39*KOrep-VP39 C-terminal mutants.

baculovirus capsid, which is not only a structural protein but also a functional protein implicated in a series of viral activities. As indicated in previous studies, VP39 interacts with cellular actin and mediates its polymerization (Charlton and Volkman, 1991; Lu et al., 2004; Lanier and Volkman, 1998). The binding of VP39 and actin is revealed by an isothermal titration calorimeter. Typically, the ΔH and binding constants (K) detected strongly suggest that the first binding of VP39 to actin mediates the formation of a hexamer complex of actin. Afterward, the actin cable structures are twisted from filaments formed by the interconnected hexamers (Lu et al., 2004). However, it remains

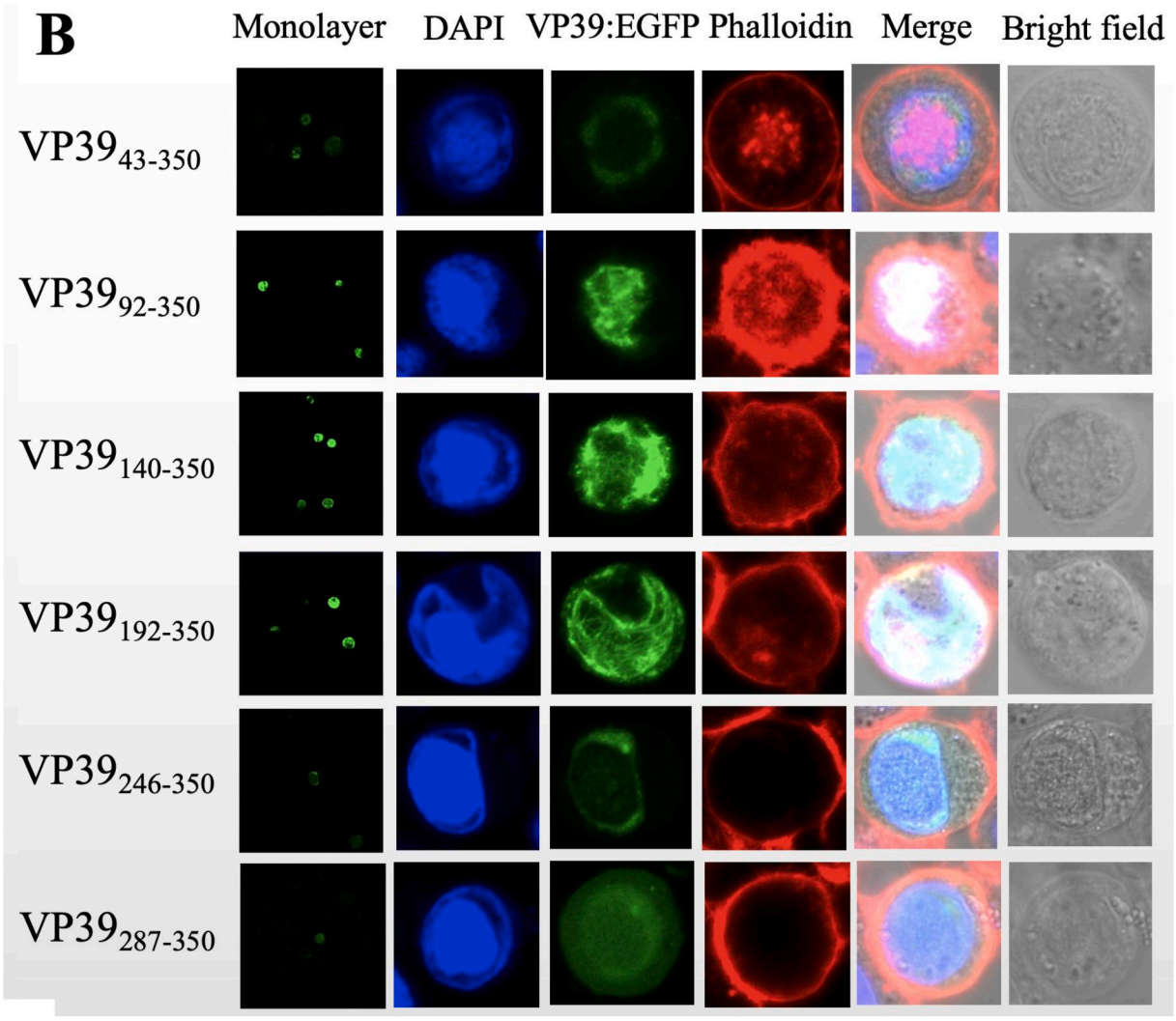

Fig. 6. (*continued*)

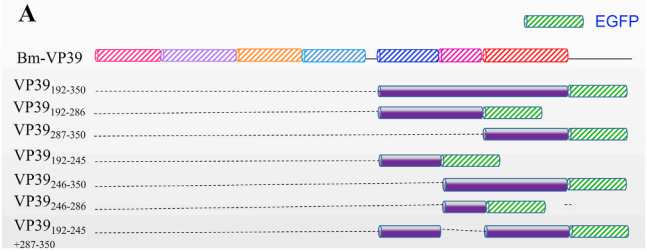

Fig. 7. Nuclear actin polymerization in BmN cells transfected with *vp39*KOrep-VP39 internal deleted mutants at 72 h post-transfection. (A) Cloning schematic diagram of VP39 internal deletions using the BmNPV *vp39* knockout bacmid as the backbone. A GFP tag (green stick) was fused to the C terminus of VP39 mutants (purple stick) to follow VP39 localization. (B) Nuclear actin polymerization in cells transfected with *vp39*KOrep-VP39 internal deleted mutants. (C) Schematics of the VP39 mutants that can mediate nuclear actin polymerization and the region in VP39 that is responsible for nuclear actin polymerization. (D) Nuclear actin polymerization in cells transfected with mutants that contain aa 192 to 286 and aa 1–191 + 287–350, respectively.

unknown whether VP39 mediates nuclear actin polymerization. To answer this question, (**Response to comment 3**, **Reviewer #2**) *vp39* was deleted and repaired in this study, and nuclear actin polymerization was not observed when *vp39* was knocked out, whereas it was detected after *vp39* was repaired. These results indicated that VP39 may be responsible for nuclear actin polymerization. However, the formation of viral capsids was also abolished when *vp39* deletion, indicating a second possibility that nuclear actin polymerization may be triggered by capsids' formation. In this regard, it is reported that the formation of normal capsids and the assembly of nucleocapsids were disrupted when nuclear actin polymerization was abolished (Volkman et al., 1987; Volkman, 1988; Charlton and Volkman, 1991), which indicated that nuclear actin polymerization is an upstream event for capsids' formation, Therefore, the second possibility was ruled out. Furthermore, to further confirm that VP39 triggered the nuclear actin polymerization, only pIZ-*mCherry-vp39* was transfected. It is observed that F-actin cannot polymerize in the nucleus when VP39 was expressed alone and localized in the cytoplasm, while it was able to polymerize in the nucleus and colocalized with VP39 after VP39 translocated into the

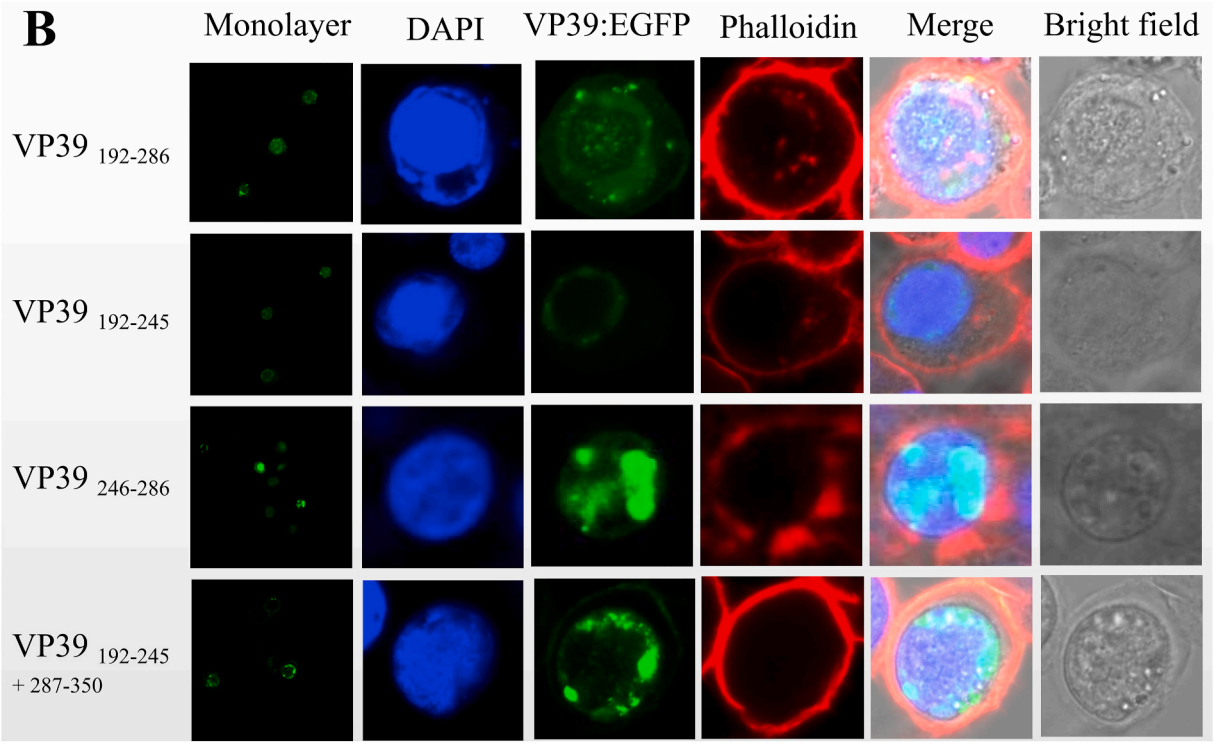

Fig. 7. (*continued*)

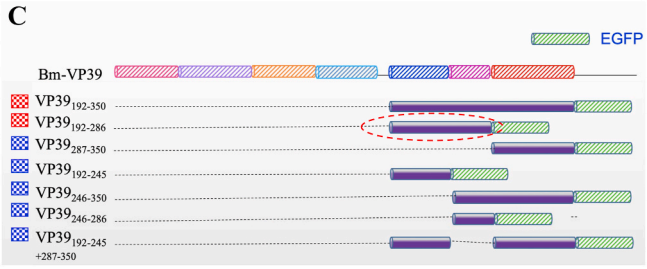

Fig. 7. (*continued*)

nucleus with the assistance of other viral protein. Taken together, all the observations suggested that nuclear actin polymerization was dependent on VP39. The actin polymer, which is referred to as F-actin, is polymerized by the cellular G-actin (monomer). Several early gene products of virus, including IE-1, Pe38, He65, Ac004, Ac102, and Ac152, stimulate the nuclear accumulation of G-actin monomers. Notably, VP39 may participate in this process when it is transported to the nucleus. In this study, VP39 was confirmed to be colocalized with actin in the nucleus. These results verified that VP39 was responsible for nuclear actin polymerization. Further, a series of VP39 mutants were also constructed based on the seven functional domains that were divided according to the amino acid conservation in VP39 from 54 baculovirus. The amino acid sequence 192–286 in the VP39 C-terminal was identified as the key region responsible for nuclear actin polymerization. Notably, the conserved glycine (residue 276) required for proper functioning of VP39 is also located in the region 192–286 (Katsuma et al., 2017), indicating the important function of this region. Moreover, three alpha-helixes and five beta-sheets were predicted within this region of BmNPV VP39 (Fig. 4). These structures were highly conservative in VP39s from all alphabaculovirus and betabaculovirus (Fig. 3B). Besides, that the two highly conserved regions of alpha- and betabaculovirus, aa 166–220 and aa 228–338, containing this region also indicated that this region has an important function.

In conclusion, baculovirus VP39 is involved in the nuclear actin polymerization, and the amino acid sequence 192–286 of VP39 is the key region for this event.

CRediT authorship contribution statement

Jianjia Zhang: Conceptualization, Methodology, Software, Investigation, Writing - original draft, Validation, Formal analysis, Visualization, Resources. **Yang Li:** Validation, Software, Resources, Writing - review & editing, Supervision, Data curation. **Shudi Zhao:** Resources, Writing - review & editing, Supervision. **Xiaofeng Wu:** Conceptualization, Methodology, Writing - review & editing, Supervision.

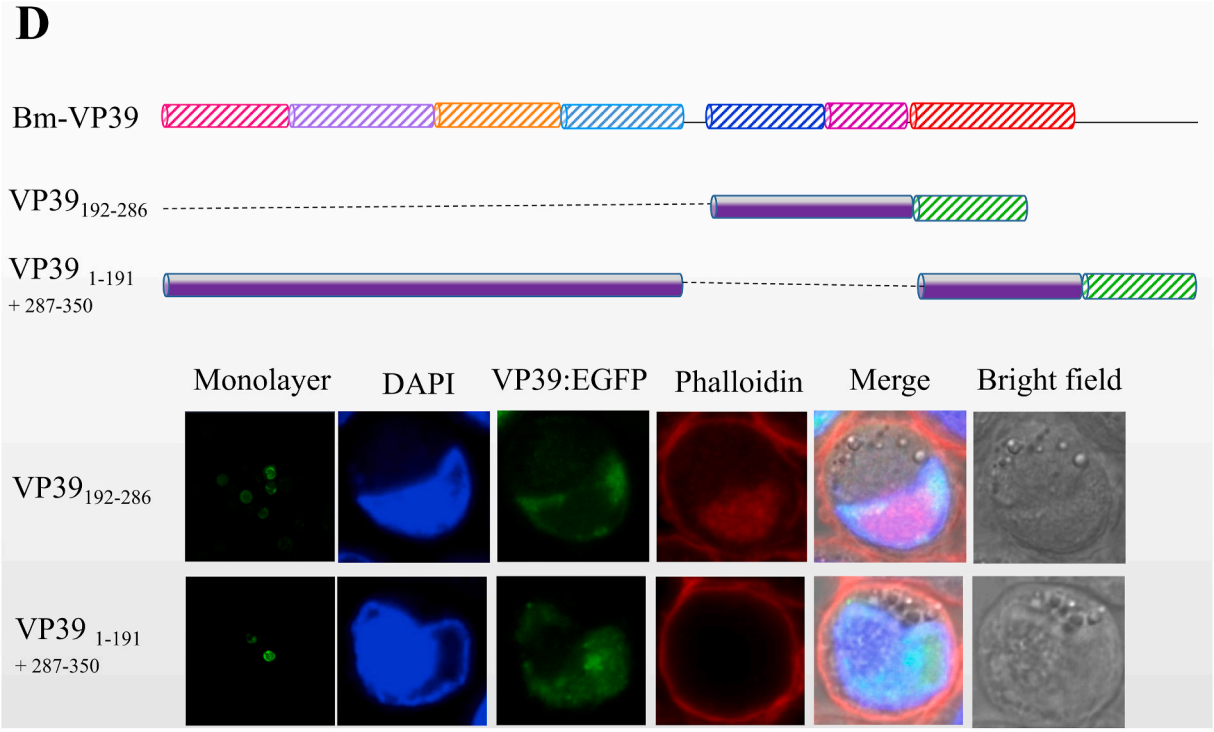

Fig. 7. (*continued*)

Declaration of competing interest

No conflict of interest exits in the submission of this manuscript, and the manuscript is approved by all authors for publication. The work described is original research and has not been published previously, and not under review at any other publication, in whole or in part.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.virol.2020.06.015.

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