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Research Article

AMV153 IS ESSENTIAL FOR REPLICATION OF *AMSACTA MOOREI ENTOMOPOXVIRUS* (AMEV), AND PROTEIN KINASE ENCODED BY *AMV197* CAN NOT COMPLETE ITS FUNCTION

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ABSTRACT. The *Amsacta moorei entomopoxvirus* (AMEV) belongs to the *Entomopoxvirinae* subfamily of poxviruses. Although entomopoxviruses are very similar to poxviruses of humans, they have no potential risk for humans because they only infect insects. Intensive studies have been carried out on this virus because it can potentially be used as gene therapy and gene expression vectors. The AMEV genome has 256 open reading frames and two of them, *AMV153* and *AMV197*, are putative protein kinases. Protein kinases of some vertebrate poxviruses have some functions in virus morphogenesis, regulation of the cell cycle, and apoptosis of the host. In previous studies, the molecular structure of *AMV197* protein kinase was elucidated, its transcriptomic properties in cell culture and its function in progeny virus production were determined, and an *AMV197*-defective virus (AmΔPK/*gfp*) was produced. However, the function of *AMV153* in this scenario remained as unclear, and it is crucial to know its function to better understand the role of protein kinases in the replication of *AMEV*. The *AMV153* was first deleted from the genome of AmΔPK/*gfp* and subsequently from the genome of AMEV, creating two separate recombinant viruses. The homologous recombination method was used to replace *AMV153* ORF with *mCherry* gene that produces red fluorescent protein. Mutant viruses in which the *AMV153* gene was replaced with *mCherry* were identified by fluorescence microscopy but could not be propagated separately from the AmΔPK/*gfp* or wild-type viruses in insect cells. Unsuccessful attempts to isolate the mutant viruses with the *AMV153* gene deletion first in AmΔPK/*gfp* virus and second in wild type AMEV structure, suggested that the protein kinase encoded by *AMV197* cannot substitute the function of *AMV153* and the *AMV153* protein is essential for virus replication.

Keywords: *Amsacta moorei entomopoxvirus (AMEV), protein kinases, AMV197, AMV153.*

INTRODUCTION

Entomopoxviruses (EPVs) are insect pathogens with double-stranded DNA genomes that replicate in the cytoplasm of the cells [1]. EPVs are viruses belonging to the poxvirus family, which includes vaccinia and variola viruses. *Amsacta moorei entomopoxvirus* (AMEV), a type species of entomopoxvirus, is also one of the insect viruses that can be produced in insect cell culture [2]. The name AMEV derives from the name of the insect (*Amsacta moorei*) which is its natural host [3].

The general feature of EPVs is the formation of large intracellular structures known as inclusion structures (spheroid). These structures form in the late stage of viral infection.

Virions are embedded within these spheroids and thus, the virions are protected from extreme environmental factors. The spheroids of EPVs are composed of a 115 kDa protein known as spheroidin [4]. In earlier studies, the *spheroidin* (*sph*) gene coding spheroidin was deleted from the virus genome and showed that this gene is not essential for the replication of the virus in cell culture [5]. Additionally, it was determined in this study that the coding sequence of the *sph* gene could be replaced to ensure the expression of foreign genes. Thanks to this feature, entomopoxviruses have a potential to be used as a good gene transfer and gene therapy vector [6-8]. On the other hand, these insect viruses are important in biotechnology because they can be used as gene expression vectors to produce medically, agriculturally, and industrially important proteins [7, 9].

AMEV has always attracted the attention of researchers because it is an insect virus related to the vaccinia virus that infects vertebrates, and thus its genome analysis was performed [10]. AMEV has a double-stranded DNA genome (232,392 bp) that replicates in the cytoplasm of the infected cells. Bioinformatic analysis of the genome showed that there are 292 open reading frames (ORFs) encoding polypeptides larger than approximately 60 aa. Subsequently, according to a recent bioinformatic analysis performed by Guo and Yu [11], 38 of 292 AMEV ORFs were determined to be non-protein coding. Various studies have been conducted on the 254 protein-coding genes of AMEV [12-16]. In one of the studies carried out in this direction, it was shown that the recombinant virus was expressed in vertebrate cells [8]. In the AMEV genome, the ORFs encoded by *AMV153* and *AMV197* are known to be like genes encoding proteins with serine/threonine protein kinase function [10].

Protein kinases are a large group of enzymes that transport a phosphate in ATP to serine, threonine, or tyrosine amino acid of the target substrate protein. These enzymes, together with protein phosphatases, have a key role in the regulation of cellular signalling pathways. Phosphorylation is one of the most general and widespread protein modifications that regulate cellular or viral activity [17]. The protein modification regulated by kinases is effective in cellular events such as transcription, translation, cell division cycle, protein degradation, intercellular transport, and apoptosis [17]. It has been reported that viruses use the same mechanisms. For example, in vaccinia viruses, a protein kinase enzyme known as B1R is known to be important for the phosphorylation of the protein and packaging into the virion [18-20]. This enzyme has been shown to phosphorylate serine and threonine amino acids in both viral and non-viral proteins [21]. The best substrates of recombinant B1R are the S2 and Sa proteins of the 40S ribosomal subunit [22]. The F10L product, the other protein kinase in Vaccinia virus, has been shown to be essential for viability [23]. Also, the studies provide evidence for an important catalytic role of F10 kinase in vaccinia virus morphogenesis [24].

The protein kinases of insect viruses, as well as the protein kinases of other viruses, have been the subject of many studies (*Choristoneura fumiferana granulovirus* (ChfuGV) [25], *Lymantria dispar nucleopolyhedrovirus* (LdNPV) [26] and *Autographa californica nucleopolyhedrovirus* (AcNPV) [27]), and in these studies, the related genes were sequenced, their transcription times were determined, and the range of substrates they phosphorylate were determined. The first study on the protein kinase of the Entomopoxvirus family was on the transcriptional analysis of the *AMV197* open reading frame [28]. In another study, the *AMV197* was deleted from the AMEV genome by homologous recombination, and a recombinant virus (AmΔPK/*gfp*) producing green fluorescent protein (GFP) was created and it was shown that the concentration of virus produced from the generated AmΔPK/*gfp* decreased approximately 3-fold compared to

the control virus [29].

Gene inactivation is the best way to define the biological role of a protein [30]. Various studies have been conducted on viruses in which gene knockout was achieved through homologous recombination [29, 31, 32]. In this study, the homologous recombination technique was successfully used to inactivate the *AMV153* protein kinase gene, and the red fluorescent protein gene (*mCherry*) was added to the virus genome. Although *AMV197* deficiency does not cause any vital problems for AMEV, *AMV153*, the other protein kinase of AMEV, is essential for virus replication.

MATERIALS AND METHODS

Cells, Media, and Viruses

In this work, the cell lineLd652, derived from the *Lymantria dispar*, and *Amsacta moorei entomopoxvirus* (AMEV, GenBank accession number: NC_002520) were used. The cells were grown in 45% Grace's Insect Medium (GIBCO) supplemented with 10% fetal bovine serum (Sigma, USA) at 28°C. Cells were sub-cultured twice a week, transferred to a fresh medium, and allowed to grow in the incubator.

AMEV and recombinant virus Am∆*PK*/*gfp* were propagated in the Ld652 cells. The titer of the virus suspension harvested at 5 days post-infection was determined by the EPDA method using 60-well cell culture dishes and the titer was calculated according to Reed and Muench [33].

AMV153 Gene Structure and Conserved Motif Analysis

The coding region of *AMV153* was carefully and bioinformatically analysed to avoid disturbing neighbouring genes or nucleotides during knocked-out. Additionally, the *AMV153* protein sequence was investigated for its protein kinase motif using the Expert Protein Analysis System (ExPAsy, https://prosite.expasy.org/).

Construction of the Transfer Vector to Create an AMV153 Knocked-out Recombinant Virus

Primers used to construct the transfer vector are shown in Table 1. Using AMEV DNA as a template and primer pair specific for the upstream (AMV153Up, 861 bp) and downstream (AMV153Down, 1,114 bp) regions of *AMV153*, the respective fragments were amplified by PCR. Additionally, the fusion PCR was used to add the *sph* gene promoter upstream of the *mCherry* DNA sequence (*sph20mCherry*, 925 bp). Then, the amplified flanking sequences and *sph20mCherry* were cloned between the *Not* I and *Kpn* I restriction sites of the pBlueScript SK(-) vector, respectively, and the recombinant plasmid was named pBS-∆*AMV153*/*mCherry* (Fig. 1).

Table 1. *Primers used in the study. Underlined sequences are enzyme-target sequence*

Names of Primers	Sequences of Primers
AMV153UpFw	5'-gggcggccgccatattaaatggtttagcacgc-3'
AMV153UpRv	5'-ggggatccgaaccagctataaatagtacttcc-3'
AMV153DownFw	5'-ggctcgaggaatgatatttttttcacaatgatataaaaccc-3'
AMV153DownRv	5'-ggggtaccgtttgaatatgacaataattcgaatagtataaatg-3'
sphProFw	5'-cgaggatcctactgtcgaaacatctaacg-3'
mCherryRv	5'-gagctcgagttacagatcttcttcagaaataagtttttgttc-3'

Virus Infection and Transfection of pBS-ΔAMV153/mCherry Vector

Ld652 cell line was used for infection and transfection of pBS-Δ*AMV153*/*mCherry* vector. Firstly, Ld652 cells in the concentration of 9.4×10^5 were added in 6-well tissue culture plates. Cells were infected with AmΔ*PK*/*gfp* at a multiplicity of infection (MOI) 5 to produce double protein kinase deletion (Fig. 1). At 2 hours post-infection, 5 µg of pBS-Δ*AMV153*/*mCherry* DNA was transfected to cells by 15 µl of lipofectin (Invitrogen, 18292037), and incubated at 28 °C for 5 hours. At 5 hours post-infection, the old medium was removed, 2 ml of fresh medium with FBS was added, and incubation was continued for additional 4 days. The formation of red colour, that is, the appearance of mCherry protein, in the cells was investigated by observing under Axiovert fluorescent inverted microscope (Zeiss, Germany).

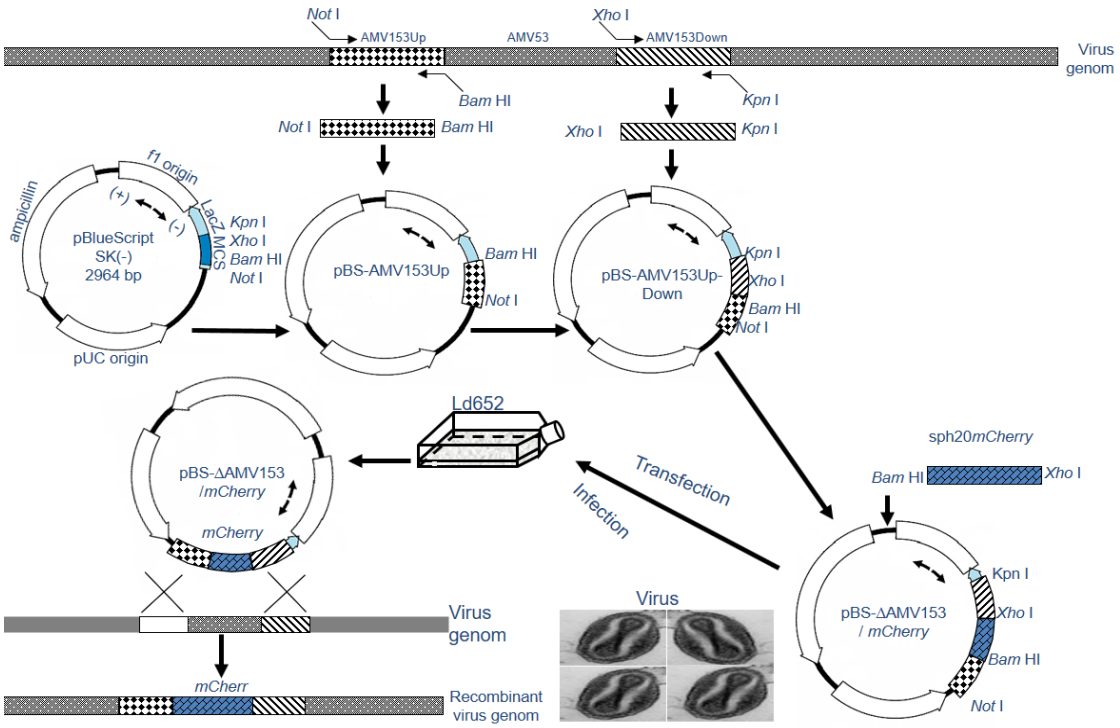

Fig. 1. *Schematic representation of the recombinant virus generation*

Plaque Purification of the Recombinant Virus

At 4 days of post-transfection, it was estimated that the sufficient recombinant virus was produced based on the abundance of *mCherry* protein in the medium. Cells were harvested with medium and centrifuged at 1,000×g for 5 min to harvest the ancestral and recombinant virus mixtures. The recombinant virus was named as AmΔ153-PK/*mCherry-gfp*. The new virus included both *AMV197* and *AMV153* deletions, and green and red fluorescent protein genes respectively. The resulting virus suspension was stored at 4 °C and in the dark, and the solution was used in the plaque assay [34] to isolate the new recombinant AmΔ153-PK/*mCherry-gfp* from the first recombinant AmΔ*PK*/*gfp*.

For the plaque assay, Ld652 cells in the concentration of 9.4×10^5 were placed in 6-well tissue culture plates. Dilutions of the virus mixture (10^{-1}, 10^{-3}, 10^{-5}) were prepared using un-supplemented Grace's Insect Medium. Each dilution was dropped onto cells in a different well, allowing the viruses to adhere to the cells. After 2 hours adhesion period, the infected cells were covered with a 1:1 mixture of 2.5% sea plaque agarose and medium and incubated at 28 °C. Plaque formation was checked by observing with an Axiovert fluorescent inverted microscope. Approximately, on the third day, plaques containing mCherry protein were observed. One week later, the viruses from red mCherry protein-containing plaques were amplified and the next cycle was performed using the amplified virus suspension. The plaque assays were repeated consecutively to purify AmΔ153-PK/*mCherry-gfp*. Virus suspensions were stored at 4 °C and in the dark for further studies.

RESULTS AND DISCUSSION

Concentration of Viruses

The concentrations of the viruses were determined as 7.76×10^8 pfu/ml for AMEV and 2.04×10^7 pfu/ml for AmΔ*PK*/*gfp*, respectively.

AMV153 Gene Structure and Conserved Motif Analysis

AMV153, found between 134,987 and 136,393 nucleotide positions in the AMEV genome, was found to be included 1,407 bases. *AMV154* and *AMV155* ORFs were within *AMV153* gene and in the opposite direction to *AMV153* [10] (Fig. 2). While constructing the transfer vector to delete *AMV153*, the fragment between 135,051-135,954 bases, marked as red in Figure 2, was targeted to be removed from the virus genome.

Serine/threonine protein kinase active-site signature, placing between 326-338 aa positions and has FfHnDIKpnNILV protein kinase motif, was determined on the protein sequence of 468 aa (Fig. 3A) (ExPAsy, https://www.expasy.org/). Furthermore, the amino acid sequence similarity of the *AMV153* and the protein kinase motif was determined by Clustal W (https://www.genome.jp/tools-bin/clustalw) (Fig. 3B).

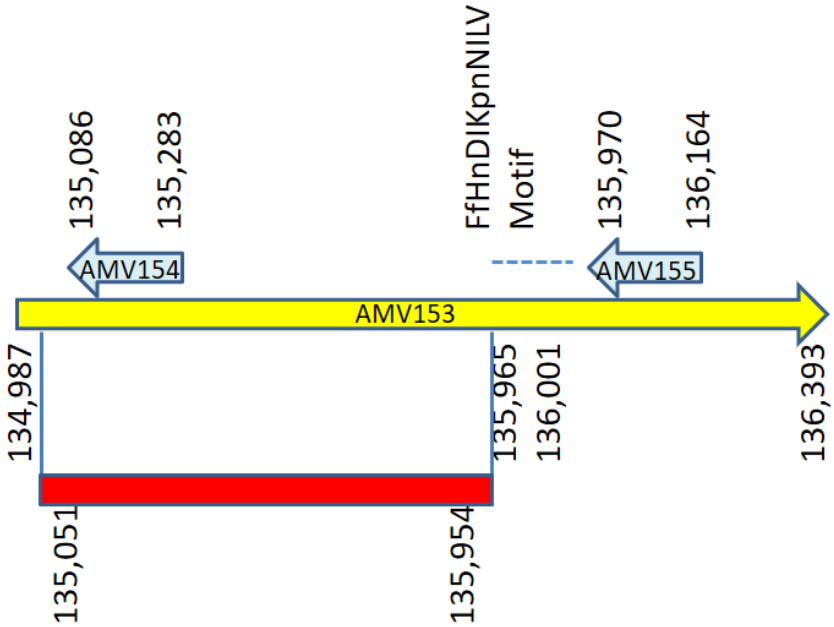

Fig. 2. *Location of AMV153 in the AMEV genome. The numbers in Figure represent the starts and ends of the AMEV ORFs (AMV153, AMV154, AMV155). The red line marks the genomic region where the deletion was performed.*

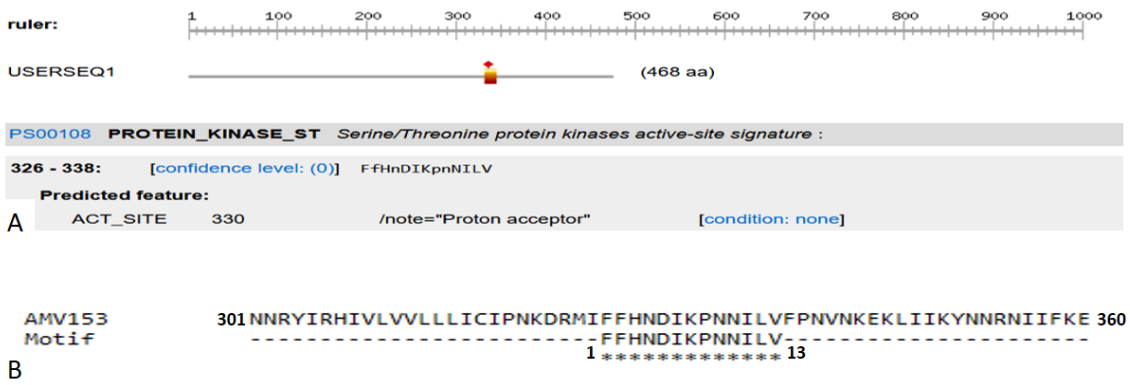

Fig. 3*. AMV153 gene structure and conserved serine/threonine protein kinase motif. The presence of the protein kinase motif was demonstrated by ExPAsy (A) and the amino acid similarity was shown with* Clustal W *(B) programs.*

According to the other bioinformatic study conducted by Guo and Yu [11], *AMV154* cannot be translated into any protein. When deleting *AMV153*, the *AMV155* function should not be disrupted. Therefore, the transfer vector was created by removing part of *AMV153*, and the *AMV154* region as well. The region deleted from the genome was shown as red in Fig. 2.

Construction of the Transfer Vector to Create a Recombinant Virus with Deletion AMV153

A homologous recombination technique was used to produce a recombinant virus lacking *AMV153*. Firstly, DNAs of AMV153Up, AMV153Down, and *sph20mCherry* (with *sph* promoter upstream of *mCherry*) were amplified by PCR using specific primers and run on agarose gel with the DNA ladder (Fig. 4). Then, 861 bp, 1,114 bp and 925 bp bands confirmed the amplification of respective fragments. DNA sequence analysis to confirm the amplified sequences was performed by Macrogen (Netherlands).

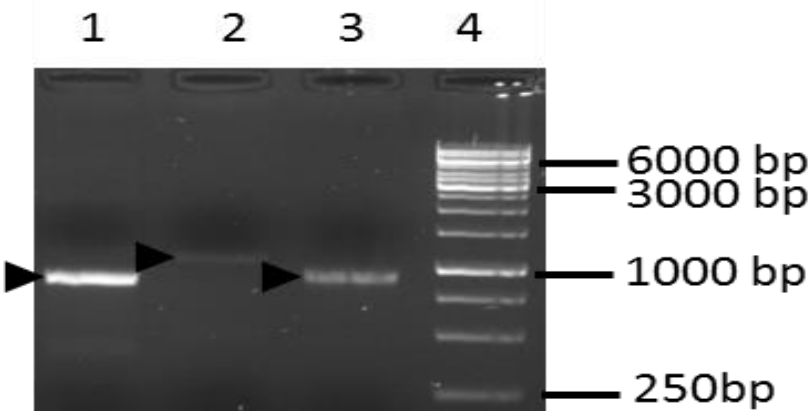

Fig. 4. *Image of AMV153Up, AMV153Down, and sph20mCherry PCR products. 1. AMV153Up, 2. AMV153Down, 3. sph20mCherry, 4. 1 kb Marker (Thermo Scientific, SM0311)*

The transfer vector was constructed to include the upstream and downstream of *AMV153* to create homologous regions with the virus genome, and then the gene encoding the marker *mCheery* gene (GenBank accession number: KY563059.1) under the promoter of the spheroid was inserted between flanking regions. And then, the vector containing the AMV153Up, *sph20mCherry*, and AMV153Down parts named as pBS-Δ*AMV153*/*mCherry* was used for transfection.

Virus Infection and Transfection of pBS-ΔAMV153/mCherry Vector

After 4 days from infection of AmΔ*PK/gfp* and transfection of pBS-Δ*AMV153*/*mCherry* vector, the transfected cells were investigated by fluorescent microscope. The presence of red-coloured proteins was then detected, indicating the success of homologous recombination (Fig. 5C).

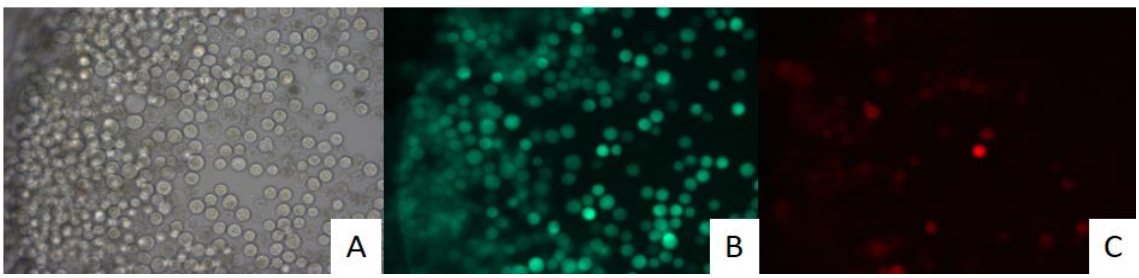

Fig. 5*. Ld652 cells infected with AmΔPK/gfp and transfected with pBS-ΔAMV153/mCherry vector. Under halogen lamp (A) and fluorescent lamp (B and C, with different filters).*

Plaque Purification of the Recombinant Virus

The *AMV153*-defective viruses generated from *AMV197*-defective AmΔ*PK*/*gfp* were produced by homologous recombination. The resulting transfection fluid was used for reinfecting Ld652 cells, and the infection in the cell line was observed by fluorescence microscopy (Fig. 6). And then the infection fluid was used in the plaque assay to distinguish the new recombinant AmΔ153-PK/*mCherry-gfp* from the first recombinant AmΔ*PK*/*gfp*.

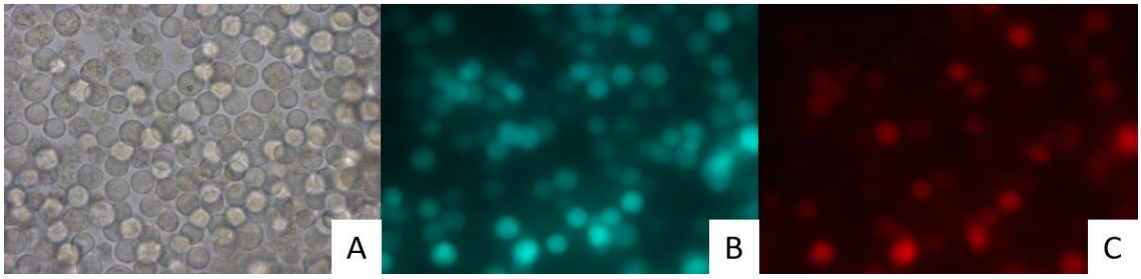

Fig. 6. *Reinfection of Ld652 cells with recombinant AmΔ153-PK/mCherry-gfp, produced after transfection. Under halogen lamp (A) and fluorescent lamp (B and C, with different filters).*

In a general deletion study, 4-6 consecutive plaque assay repeats are sufficient to obtain pure recombinant viruses. Although both transfection and the first infection cycles were successful (Fig. 6C), after several plaque assay cycles, the amount of AmΔ*PK*/*gfp* virus was decreased significantly and the presence of mCherry protein, which is a sign of recombination, was no longer detected. In this case, the plaque assay cycle was restarted using the virus suspension from the previous plaque containing mCherry protein. However, although 10-15 plaque cycles were performed, the amount of AmΔ*PK*/*gfp* virus decreased for each time and the AmΔ153-PK/*mCherry-gfp* virus also decreased.

The reason for not being able to isolate both *AMV197* and *AMV153* protein kinases-deleted virus was that an *AMV197*-defective virus could not tolerate the deficiency of the second protein kinase. Therefore, the wild type AMEV and pBS-Δ*AMV153*/*mCherry* transfer vector were used this time to create only the *AMV153*-defective virus, AmΔ153/*mCherry*. Besides, although both transfection of the pBS-Δ*AMV153*/*mCherry* transfer vector with AMEV virus into insect cells and initial infection cycles seems successful (Fig. 7C), after plaque assay studies, the AmΔ153/*mCherry* recombinant virus could not be purified, as in the creation of AmΔ153-PK/*mCherry-gfp*.

Fig. 7. *Images of AmΔ153/mCherry recombinant viruses infection after transfection of the pBS-ΔAMV153/mCherry transfer vector. Under halogen lamp (A) and fluorescent lamp (B and C, with different filters).*

In this study, the protein kinase *AMV153* was separately deleted from both the genome of AMEV and the AmΔPK/*gfp*, creating two separate recombinant viruses. Since these viruses could not be purified during consecutive plaque assays, it was concluded that *AMV153* is required for AMEV replication, and the protein kinase encoded by *AMV197* cannot complete its function. When there were still ancestral viruses (AMEV or AmΔPK/*gfp*) in the culture, the recombinant virus deleted *AMV153* provides the necessary proteins from the ancestral viruses and thus can express the mCherry protein. However, when the amount of ancestral virus in the medium decreases, unsuccessful replication occurs in cell culture because the deficiency of this gene cannot be compensated by AmΔ153/*mCherry* or AmΔ153-PK/*mCherry-gfp*. Some studies in the literature [35, 36] also confirm this assumption. Considering this information, this study has scientifically revealed for the first time that, unlike the *AMV197* protein kinase of the virus, the *AMV153* protein kinase is an essential gene for the virus to multiply in cell culture.

Similarly, the studies of protein kinase gene deletions, which belong to other viruses, are present in the literature. In one study, a Human cytomegalovirus line with a defect in the protein kinase (UL97) was generated and was found that this gene is not necessary for virus replication [37]. However, the same study also concluded that the function of this gene was required to create the virus phenotype. Additionally, the pathogenicity of the US3 protein kinase deficiency mutant (L1 BR1) of Herpes simplex virus type 2 (HSV-2) was investigated in 4-week-old ICR mice to define the role of the viral protein kinase in the virus-host interaction and the study is one of the examples of the use of deletion mutants of viruses in protein kinase studies [38].

CONCLUSION

This study is the first to investigate the significance of *AMV153*, in viral replication. Although *AMV153* was successfully deleted from the genomes of both the recombinant AmΔPK/*gfp* and wild-type AMEV, new recombinant viruses could not be obtained pure, as they were unable to be separated from the ancestral viruses. The *AMV153* protein from the parent virus (AmΔPK/*gfp* or AMEV) was lost when new recombinant viruses lacking the *AMV153* gene (AmΔ153-PK/*mCherry-gfp* or AmΔ153/*mCherry*) were constructed. Thus, it was concluded that *AMV153* and its product are essential for AMEV replication. Further detailed studies are necessary to confirm these findings.

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Conflict of Interest. The authors declared that there is no conflict of interest.

Authorship Contributions. Concept: HM, Design: HM, Data Collection or Processing: HM, Analysis or Interpretation: HM, Literature Search: HM, Writing: HM.

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