A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Functional characterization of gene knockout mutants of *Autographa californica* multiple nucleopolyhedrovirus

Autographa californica mutiple nucleopolyhedrovirus의
유전자 knockout을 통한 기능적 특성 연구

By

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ABSTRACT

The baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), which has a large double-stranded DNA genome of approximately 134 kb and comprises 154 open reading frames (ORFs), highly pathogenic to a number of lepidopteran insects and widely used to transduce various cells for exogenous gene expression. The function of AcMNPV genes in viral replication has been studied using gene knock-out technology, however, the function of more than one-third of viral genes which including some highly conserved genes are still unknown. In this study, to investigate the function of AcMNPV genes, a novel AcMNPV genome that can be maintained in *Escherichia coli* as a plasmid and infect susceptible lepidopteran insect cells was generated. This engineered AcMNPV, named Ac-MK, contains an *E. coli* origin of replication (mini-F replicon) and a

kanamycin resistance gene (*Kan*). Using a convenient Tn7 transposon-based plasmid capture system, pPCS-S, which contains a pUC origin and an ampicillin resistance gene (*Amp*), 54 single ORF-knockout AcMNPV mutants were generated by random insertion into Ac-MK genome. Subsequently, the growth properties of these ORF-knockout mutant viruses in *Spodoptera frugiperda* 9 (Sf9) cells, and the gene knockout-specific effects on the production of infectious progeny were analyzed. Three of these mutants, Ac11KO, Ac43KO and Ac78KO, of which ORF11 (*ac11*), ORF43 (*ac43*), and ORF78 (*ac78*) were knocked-out respectively, were selected and subjected to further study since *ac11*, *ac43* and *ac78* are highly conserved genes in baculovirus which suggest that they may play important roles in the baculovirus life cycle.

The result of quantitative PCR (qPCR) analysis revealed that *ac11* is an early gene in the viral life cycle. Microscopy, titration assays, and Western blot analysis revealed that budded viruses (BVs) were not produced in the Ac11KO-transfected Sf9 cells. However, qPCR analysis demonstrated that deletion of *ac11* did not affect viral DNA replication. Furthermore, electron microscopy revealed that there was no nucleocapsid observed in cytoplasm or plasma membrane in Ac11KO-transfected cells, which demonstrated that the defection of BV production from the Ac11KO-transfected cells was due to inefficient egress of nucleocapsids from the nucleus to the cytoplasm. In addition, electron microscopy showed that the nucleocapsids in the nucleus were not enveloped to form occlusion-derived virus (ODV), and their subsequent embedding into the occlusion bodies (OBs) was also blocked in the Ac11KO-transfected cells demonstrated that *ac11* is required for ODV envelopment. These results therefore demonstrated that *ac11* is an essential gene in the viral life cycle.

The function of *ac43* gene during viral replication was also investigated. After transfection into Sf9 cells, Ac43KO produced polyhedra much larger in size than those of wild-type AcMNPV. Interestingly, some of the nucleocapsids were singly enveloped in the polyhedrin matrix while the nucleocapsids of AcMNPV are known to be multiply enveloped. Furthermore, Ac43KO led to a defect in the transcription and expression of *polyhedrin*, which resulted in reduced OB production. However, Ac43KO did not affect production of BV as there was no remarkable difference in budded virus titer. These results suggest that *ac43* plays an important role in the expression of *polyhedrin*, the morphogenesis of OB, and the assembly of virions occluded in OBs.

Quantitative PCR analysis revealed that *ac78* is a late gene in the viral life cycle. In the transfected Sf9 cells, Ac78KO produced a single-cell infection phenotype, indicating that no infectious BVs were produced. The defect in BV production was also confirmed by both viral titration and Western blotting. However, viral DNA replication was unaffected, and occlusion bodies were formed. An analysis of BVs and ODVs revealed that Ac78 is associated with both forms of the virions and is an envelope structural protein. Electron microscopy revealed that Ac78 also plays an important role in the embedding of ODV into the occlusion body. The results of this study demonstrated that Ac78 is a late virion-associated protein and is essential for the viral life cycle.

Key words: *Autographa californica* multiple nucleopolyhedrovirus, *ac11*, *ac43*, *ac78*, BV production, ODV envelopment, virus morphology

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INTRODUCTION

The Baculovirudae is a family of arthropod-specific dsDNA viruses. Viruses from this family are characterized by rod-shaped, enveloped nucleocapsids with circular, covalently closed, double-stranded DNA genomes of 80-180 kb (Ayres *et al.*, 1994; Jakubowska *et al.*, 2006). To date, more than 600 baculoviruses have been described to infect insect specious in the orders lepidoptera, diptera, and hymenoptera, and it is likely that baculoviruses represent the largest and most diverse family of DNA viruses (Herniou *et al.*, 2003; Martignoni & Iwai, 1981; Murphy *et al.*, 1995). Recently, a proposed reclassification has expanded the family to include four genera, Alpha-, Beta-, Gamma- and Delta-baculovirus. While Alpha- and Beta-baculoviruses infect lepidoptera larvae, Gamma- and Delta-baculoviruses infect hymenoptera and diptera larvae, respectively (Jehle *et al.*, 2006). The Alpha-baculoviruses are further divided into group I and group II NPVs on the basis of phylogenetic analysis (Herniou & Jehle, 2007) and the type of envelope fusion protein (GP64 or F, respectively) (Pearson & Rohrmann, 2002).

The infection cycle of baculoviruses includes two distinct viral phenotypes, budded virus (BV) and occlusion-derived virus (ODV). Both BVs and ODVs are identical in nucleocapsid structure and genetic information, but the compositions of their envelopes

are different to accommodate their respective functions in the infection cycle (Braunagel & Summers, 1994). The ODVs, which are enveloped within the nucleus, initiate a primary infection of the insect by infecting epithelial cells in the midgut when the virions are released by the alkaline pH of the gut. The BVs are produced from the infected cells and can initiate a secondary infection (Federici, 1997). The ODVs are embedded within a paracrystalline matrix made of a protein called polyhedrin forming OBs, which are responsible for protecting the virions from UV radiation and desiccation, allowing infectious virions to survive for long periods of time in harsh environments before a susceptible host is infected.

A baculovirus infection progresses in three phases, early, late, and very late, in cultured insect cells. A complex cascade of transcription and protein synthesis occurs within the first 6-8 h following viral infection (Friesen, 1997). The progression from early to late stages of infection coincides with the initiation of viral DNA replication between 6 and 12 h post-infection (pi) (Blissard, 1996). Newly replicated viral DNA is condensed and packaged into capsid structures within the nucleus to form nucleocapsids (Lu *et al.*, 1997). Nucleocapsids initially egress from the nucleus, migrate through the cytoplasm, and bud through a modified plasma membrane to acquire an envelope to form BVs (Monsma *et al.*, 1996). During the late phase of infection beginning at approximately 20 hpi, there is a

switchover from BV to ODV production by some unknown mechanism. Nucleocapsids then remain within the nucleus and develop an envelope *de novo* to form ODVs, which become incorporated into the matrix of the OB (Williams & Faulkner, 1997).

Autographa californica multiple nucleopolyhedrovirus (AcMNPV) is the type species of the Alphabaculovirus genus. AcMNPV has a double-stranded DNA genome of approximately 134 kb which contains 154 predicted open reading frames (ORFs) of predicted polypeptides of 50 amino acids or more using the criterion that an ORF is a single, contiguous, non-overlapping coding region (Ayres *et al.*, 1994). It has been reported that there are 37 core genes which are conserved in the 58 completely sequenced and comparatively analyzed baculovirus genomes, and these genes are likely to serve essential roles in the baculovirus life cycle (Garavaglia *et al.*, 2012; Rohrmann, 2011; Yuan *et al.*, 2011). The function of many AcMNPV genes in viral replication has been studied, however, the function of more than one-third of viral genes including some highly conserved genes are still unknown (Rohrmann, 2011).

In this study, to investigate the function of AcMNPV genes, an engineered AcMNPV genome, Ac-MK, was constructed. Ac-MK contains a kanamycin resistance gene (*Kan*) and a mini-F replicon (MiniF) which makes it possible to be maintained in *Escherichia coli* (*E. coli*) as a plasmid. Using a convenient Tn7 transposon-based system, pPCS-S, 54

single ORF-knockout AcMNPV mutants were generated. Furthermore, the phenotype of the gene knockout mutants were identified by introducing the 54 selected bacmids into Sf9 cells and analyzing of the OB production. The *ac43* gene knocked-out mutant (Ac43KO) which produced lower viral infectivity and the *ac11* gene knocked-out mutant (Ac11KO) and *ac78* gene knocked-out mutant (Ac78KO) which produced OBs but not produced infectious viruses were selected and subjected to further analyses.

LITERATURE REVIEW

1. Baculoviruses

Baculoviruses are a family of arthropod-specific viruses found ubiquitously in the environment and have been isolated from more than 600 host insect species including the orders lepidoptera, hymenoptera, diptera, orthoptera, coleoptera, neuroptera, thysanura, and trichoptera (Herniou *et al.*, 2003; Larsson, 1984; Slack & Arif, 2007). The viruses of Lepidoptera are divided into Alpha- and Beta-baculoviruses encompassing the nucleopolyhedroviruses (NPVs) and granuloviruses (GVs), respectively, and those infecting hymenoptera and diptera would be named Gamma- and Delta-baculoviruses, respectively (Jehle *et al.*, 2006). The lepidopteran-specific NPVs are further divided into group I and group II NPVs on the basis of phylogenetic analysis (Herniou & Jehle, 2007) and the type of envelope fusion protein (GP64 or F, respectively) (Pearson & Rohrmann, 2002). Most baculovirus species have been isolated from lepidoptera and the majority of non-lepidopteran isolates have not been well characterized. They are DNA viruses with closed, circular, double-stranded DNA genomes ranging from 80 to 180 kb in size (Ayres *et al.*, 1994; Herniou *et al.*, 2003; Jakubowska *et al.*, 2006). The genomes are packaged in

bacillus-shaped nucleocapsids, and the name “baculovirus” is in reference to the nucleocapsid shape.

One common feature of all baculoviruses is the production of occlusion bodies (OBs). The OBs of baculoviruses are large enough to be observed by light microscopy and range from 500 to 2000 nm in diameter (Adams & McClintock, 1991; Bilimoria, 1991; Boucias & Pendland, 1998) depending on the virus. The OBs of the NPV and GV are termed polyhedron and granule, respectively. Each granule occludes a single virion, whereas the polyhedron occludes multiple virions. Additionally, the NPV virion can contain a single (S morpho-type) or multiple (M morpho-type) nucleocapsids. Occlusion is an adaptation to permit baculoviruses to remain in a dormant but viable state in the environment for decades and perhaps even centuries (Bergold, 1963).

Baculoviruses play a major ecological role regulating the size of insect populations (Odindo, 1983). For many decades, baculoviruses have been applied as targeted biocontrol agents against forestry and agriculture pests. Baculovirus insecticides have been effective against insect pests such as velvetbean caterpillar (*Anticarsia gemmatalis*) (Moscardi, 1999), cotton bollworm (*Helicoverpa zea*), and gypsy moth (*Lymantria dispar*) (Cook *et al.*, 2003). Baculovirus-based biocontrol applications have been restricted to lepidopteran and hymenopteran (sawflies) pests. Mosquito-specific baculoviruses have

been characterized (Moser *et al.*, 2001) with the potential to develop them for biocontrol of mosquitoes.

2. The baculovirus life cycle

1) Two virion phenotypes

The baculovirus produces two types of progeny in the life cycle, the budded virus (BV) and the occlusion-derived virus (ODV). Both BVs and ODVs are identical in nucleocapsid structure and genetic information, but the compositions of their envelopes are different to accommodate their respective functions in the infection cycle (Braunagel & Summers, 1994). The ODVs, which are enveloped within the nucleus, initiate a primary infection of the insect by infecting epithelial cells in the midgut when the virions are released by the alkaline pH of the gut. The BVs are produced from the infected cells and can initiate a secondary infection (Federici, 1997). The ODVs are embedded within a paracrystalline matrix made of a protein called polyhedrin forming OBs, which are responsible for protecting the virions from UV radiation and desiccation, allowing infectious virions to survive for long periods in harsh environments before a susceptible host is infected.

2) Replication cycle

The baculovirus replication cycle begins almost immediately after the nucleocapsid delivers the viral genome into host cell nucleus. Viral immediate early genes are expressed within 30 minutes post infection (Chisholm & Henner, 1988) and their protein products along with virion-associated proteins begin to manipulate the host cell to become competent for DNA replication. The structure of the nucleus is modified resulting in its expansion, so called nuclear hypertrophy. An electron dense, irregular-shaped, granular region begins to form in the center of the nucleus (Harrap, 1972; Young *et al.*, 1993). This region is called the virogenic stroma in which the viral RNA transcription, DNA replication, and nucleocapsid assembly are progressed.

Late-phase infection is divided into two stages, late and very late, which extend from around 6 to 76 hpi. Between 12 and 20 hpi, the BV virion phenotype is produced. Newly replicated viral DNA is condensed and packaged into capsid structure within the nucleus to form nucleocapsids (Lu *et al.*, 1997). The earliest made nucleocapsids in the baculovirus infection migrate out of the virogenic stroma, across the ring zone and to the nuclear membrane. Nucleocapsids are transported through the nuclear membrane and migrate across the cytosol to the cell membrane where they bud out. In the process of budding, nucleocapsids are enveloped in the host membrane and acquire virus-encoded proteins in their envelopes (Monsma *et al.*, 1996). During the late phase of infection

beginning at approximately 20 hpi, there is a switchover from BV to ODV production by some unknown mechanism. Nucleocapsids remain within the nucleus and develop an envelope *de novo* to form ODVs, which then become incorporated into the matrix of the OBs (Williams & Faulkner, 1997).

3. Baculovirus structure

1) Nucleocapsids proteins

The nucleocapsids of the ODV and BV have many similarities as they both contain complete viral genomes and have major proteins in common. Baculovirus nucleocapsids are 40–70 nm in diameter and 250–400 nm in length (Boucias & Pendland, 1998). The size of the viral genome determines the length of the nucleocapsid. Nucleocapsids are polar, with a claw or base on one end and a nipple or apical cap on the other end (Federici, 1986; Fraser, 1986).

VP39 (Ac89) is the major capsid protein which is one of the three most abundant proteins found by proteomic analysis of BV (Wang *et al.*, 2010). VP39 formed monomers arranged in stacked rings around the nucleoprotein core (Federici, 1986).

P6.9 (Ac100) is a DNA binding protein and one of the three most abundant proteins found in proteomic analysis of BV (Wang *et al.*, 2010). It is a small (55aa) arginine/serine/threonine rich protein (Wilson, 1987) which are involved in the

production of highly condensed DNA. P6.9 localizes to the nuclear matrix during infection (Wilson & Price, 1988). Once viral DNA has been delivered to the nucleus, P6.9 is phosphorylated resulting in both DNA and P6.9 being negatively charged. It is thought that this causes the removal of P6.9 from the viral DNA thereby allowing access to transcription factors (Wilson & Consigli, 1985).

In addition to the major capsid protein, there are number of other minor but important capsid-associated proteins. The PP78/83 (Ac9) is a phosphorylated protein that is located at one end of nucleocapsids (Russell *et al.*, 1997; Vialard & Richardson, 1993). It is a Wiskott-Aldrich syndrome protein (WASP)-like protein. Such proteins are involved in nuclear actin assembly, and it has been demonstrated that pp78/83 serves this function during AcMNPV infection (Goley *et al.*, 2006; Ohkawa *et al.*, 2010).

BV/ODV-C42 (Ac101) encodes a capsid-associated protein. Homologs have been identified in all sequenced baculovirus genomes with the exception of the virus pathogenic for the dipteran, *Culex nigripalpus* (CuNiNPV) (Afonso *et al.*, 2001). BV/ODV-C42 was shown by yeast two-hybrid analysis to interact directly with PP78/83 (Braunagel *et al.*, 2001) and is required for its transport into nuclei (Wang *et al.*, 2008b).

The very late factor, VLF-1 (Ac77), is a member of the lambda integrase (Int) family of proteins and was originally identified because it influences the hyper expression of

very late genes (McLachlin & Miller, 1994). VLF-1 appears to be a structural protein present in both BV and ODV (Yang & Miller, 1998) and is clearly required for the production of nucleocapsids.

38K (Ac98) is encoded by all baculoviruses and is associated with both BV and ODV nucleocapsids. By yeast two-hybrid assays it interacted with VP1054, VP39, VP80, and itself (Wu *et al.*, 2008b). When deleted, tube-like structures devoid of DNA but those stain with VP39 antibody are produced (Wu *et al.*, 2006).

EXON0 (Ac141) is conserved in all lepidopteran baculoviruses and is associated with both BV and ODV nucleocapsids (Dai *et al.*, 2004; Wang *et al.*, 2010) and interacts with BV/ODV-C42 and FP25 (Ac61) (Fang *et al.*, 2008). It appears to be required for the efficient transport of nucleocapsids from nuclei through the cytoplasm (Dai *et al.*, 2004). It has been suggested that the interaction of EXON0 with microtubules might be important in the egress of BV (Fang *et al.*, 2009d).

P49 (Ac142) is associated with both BV and ODV virions. Deletion of P49 appeared to affect nucleocapsid formation but did not appear to affect DNA synthesis (Vanarsdall *et al.*, 2007b). Homologs have been identified in all sequenced baculovirus genomes.

2) BV envelope proteins

The source and content of the envelope are the major distinguishing features between BV and ODV. Envelope fusion protein of Group I baculoviruses, GP64 (Ac128), is the most well characterized baculovirus structural protein. It is a fatty acid acylated glycoprotein (Roberts & Faulkner, 1989) and a low pH activated envelope fusion protein (Hohmann & Faulkner, 1983; Volkman, 1986; Whitford *et al.*, 1989) that is one of the three most abundant proteins found associated with AcMNPV budded virions (Wang *et al.*, 2010). The presence of the *gp64* gene is one of the major distinguishing features of the Group I viruses. Deletion of AcMNPV *gp64* is lethal and results in viruses that replicate in a single cell but cannot bud out and infect surrounding cells (Oomens & Blissard, 1999). It is thought that all Group I viruses use GP64 for the entry of BV into cells, whereas all other baculoviruses lack a *gp64* homolog and appear to use the F (Ac23 homolog) protein as their envelope fusion protein except for hymenopteran NPVs, which lack both genes.

Although fusion protein-F (Ac23) is not an active envelope fusion protein in AcMNPV, in Group II NPVs, GVs and the dipteran virus (CuniNPV) orthologs of F are likely used as the fusion protein, because all these viruses lack homologs of *gp64*. F proteins of Group II NPVs function as low-pH envelope fusion proteins (Pearson *et al.*, 2000; W.F.J., 2000) and can also rescue AcMNPV lacking *gp64* (Lung *et al.*, 2002).

BV/ODV-E26 (Ac16) is a BV and ODV envelopes protein in AcMNPV (Beniya *et al.*, 1998; Braunagel *et al.*, 2003; Wang *et al.*, 2010). BV/ODV-E26 interacts with FP25 (Ac61), forms a complex with cellular actin (Beniya *et al.*, 1998). The cells infected an *ac16*-deleted virus showed a delay in BV production (D.A., 2010).

3) ODV envelope proteins

In contrast to BV, where one or two virus-encoded proteins have been identified as envelope associated, the ODV envelope is much more complex. ODV envelope proteins are playing biological roles in ODV occlusion and interaction with the midgut. There may be five or more such proteins categorized as envelope proteins and another group of proteins called *per os* infectivity factors (PIFs) that are likely envelope components.

ODV-E66 (Ac46) is a component of ODV envelopes (Hong *et al.*, 1994). When the N-terminal 23 amino acids of ODV-E66 are fused to a reporter gene, it is targeted to the nucleus (Hong *et al.*, 1997). Such a sequence would be necessary since the protein must be localized to the nucleus for ODV morphogenesis. Evidence suggests that Ac46 encodes an enzyme, hyaluronan lyase, which is capable of digesting hyaluronan, a polysaccharide that is a major component of the extracellular matrix (S. *et al.*, 1999). The extracellular matrix is a tissue component that provides structural support for cells.

ODV-E25 (Ac94) has also been shown to be associated with ODV of AcMNPV and *H. armigera* NPV (HearNPV) by proteomic analysis (Braunagel *et al.*, 2003; Deng *et al.*, 2007) and with AcMNPV BV (Wang *et al.*, 2010). Similar to ODV-E66, the hydrophobic N-terminal 24 aa of AcMNPV ODV-E25 appears to contain a nuclear targeting signal (Hong *et al.*, 1997).

ODV-EC43 (Ac109) is ODV-associated in AcMNPV (Braunagel *et al.*, 2003). Although deletion of Ac109 did not appear to affect DNA replication or the appearance of BV, the virions were non-infectious (Fang *et al.*, 2009b).

An antibody generated against Ac143-GST fusion reacted with a protein of 18 kDa in the ODV envelope fraction, so that Ac143 was named ODV-E18 (Braunagel *et al.*, 1996b). Homologs of ODV-E18 are present in all baculoviruses and it is an essential gene (McCarthy & Theilmann, 2008). Deletion of the ODV-E18 gene results in single cell infections that produce polyhedra and therefore appears to be essential for BV production (McCarthy & Theilmann, 2008). In addition to its presence in ODV envelopes, it is also BV associated (Wang *et al.*, 2010).

Per os infectivity factors were originally identified because they were required for infection of insects, but dispensable for infection of cultured cells (Kikhno *et al.*, 2002). Six *pif* genes have been identified in AcMNPV. They include, *p74-pif* (*Ac138*) (Kuzio *et*

al., 1989), *Ac 22* (*pif-2*) (Pijlman *et al.*, 2003) , *Ac115* (*pif-3*) (Ohkawa *et al.*, 2005), *Ac96* (*pif-4*) (Fang *et al.*, 2009c), *Ac119* (*pif-1*) (Kikhno *et al.*, 2002), *Ac148* (*ODV-E56*, *pif-5*) (Harrison *et al.*, 2010; Xiang *et al.*, 2011a).

4. Baculovirus genomes

Baculovirus genomes are covalently closed circles of double stranded-DNA varying in size between 80 and 180 kb that encode between 90 and 180 genes (Theilmann *et al.*, 2005). To date, 58 baculovirus genomes have been completely sequenced (Garavaglia *et al.*, 2012; Rohrmann, 2011; Yuan *et al.*, 2011). The majority of sequenced baculoviruses are pathogenic to lepidopteran hosts (van Oers & Vlak, 2007). Analyses of these genome sequences have been published in the scientific literature, with this information, general patterns of genome structure and gene content became apparent.

All baculovirus genomes consist of a single, circular double stranded DNA molecule that varies widely in size and nucleotide distribution from baculovirus to baculovirus (van Oers & Vlak, 2007). Baculovirus open reading frames are tightly packed with minimal intergenic regions and the coding sequences are almost equally distributed over both strands. Baculovirus genes form single transcription units, with early and late transcribed ORFs scattered along the genome. Comparative analyses have reported that 37 core genes are conserved in all genomes (Garavaglia *et al.*, 2012; Rohrmann, 2011; Yuan *et al.*,

2011). Most baculovirus genomes contain multiple homologous regions with repeated sequences and often palindromic motifs, which play a crucial role as enhancers of early transcription and most likely in viral DNA replication. Baculovirus genomes have a certain degree of plasticity, as evidenced from the genomic variations within virus isolates from the field. Recombination events and transposon insertions appear to play a role in the uptake of new genes from co-infecting viruses or from the insect host (van Oers & Vlak, 2007).

MATERIALS AND METHODS

1. Bacterial strains and bacmid DNA

Escherichia coli strains TOP10 and DH10B (Invitrogen, USA) were used throughout the experiments. All restriction endonucleases and modifying enzymes were from Roche Applied Science (Germany). All recombinant bacmids used in this study were propagated in *E. coli* strain DH10B.

2. Viruses, insect cells and transfection

The *Spodoptera frugiperda* IPLB-Sf21-AE clonal isolate 9 (Sf9) insect cells were cultured at 27°C in TC-100 medium (WelGene, Korea) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (WelGene, Korea) and sub-cultured every 3-4 days. Wild-type AcMNPV C6 strain and all recombinant AcMNPVs used in this study were propagated in Sf9 cells maintained in TC-100 medium. Transfection was performed using the CellfectinTM reagent (Invitrogen, USA) according to the manufacturer's instructions.

3. Construction of recombinant AcMNPV bacmid, Ac-MK

To construct Ac-MK bacmid, the baculovirus transfer vector pBacMKPol, which the *E. coli* origin of replication (Mini-F replicon) coupled with *polyhedrin* gene and kanamycin resistance gene (*Kan*) was cloned into a modified pBacPAK transfer vector (Clontech, USA), was inserted into the locus between *orf603* and *polh* of the AcMNPV sequence (Fig. 1). The novel recombinant bacmid, Ac-MK, was generated via homologous recombination of pBacMKPol and AcMNPV genomic DNA in co-transfected Sf9 cells, and successful recombinants were selected in *E. coli* plated on nutrient agar plates containing kanamycin (50 μg/ml).

4. Generation of gene knockout mutants via Tn7-mediated transposition

The transposition procedure was carried out as described previously (Choi *et al.*, 2005) with minor modification. For the transposition reaction, 12 ng of *Hin*dIII and *Sph*I-digested pPCS-S (donor-S) plasmid was mixed with 200 ng of Ac-MK bacmid DNA. TnsABC* transposase (New England BioLabs, USA) was added to the transposition reaction, and the mixture was incubated at 37°C for 10 min. Next, 1 μl of start solution was added to the mixture, which was then incubated at 30°C for at least 2 h. Finally, the transposition reaction was stopped by heat at 75°C for 10 min. The reacted DNA was transformed into competent *E. coli* DH10B cells (Invitrogen, USA), and the transformed

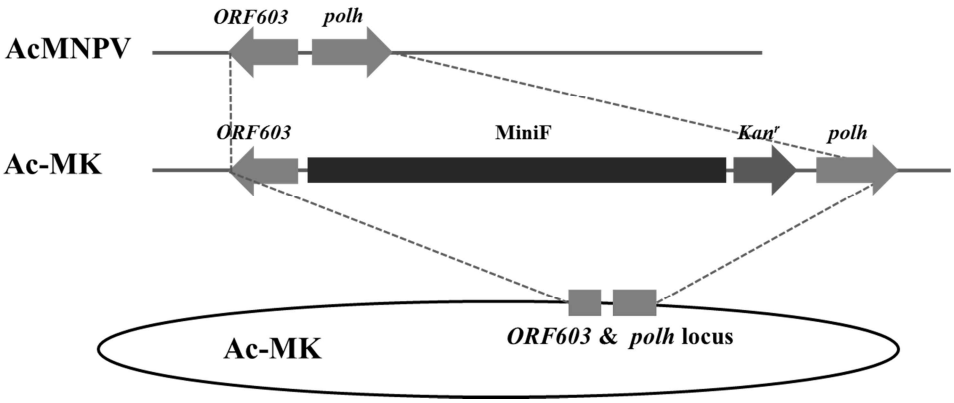


Fig.1. Construction map of Ac-MK. The *E. coli* origin of replication (Mini-F replicon) and kanamycin resistance gene (*Kan*) was cloned into the locus between *ORF603* and *polyhedrin* of AcMNPV via homologous recombination of pBacMKPol and AcMNPV genomic DNA.

cells were subsequently plated onto nutrient agar plates containing kanamycin (50 μg/ml) and ampicillin (50 μg/ml). The plates were incubated at 37°C for 2 days. The colonies resistant to both kanamycin and ampicillin were selected, and successful transposition was verified by end sequence analysis using the transprimers, primerN and primerS (New England Biolabs).

5. Verification knockout mutants by PCR

PCR was performed using an *AccuPower*® PCR Premix (Bioneer, Korea) in a 20-μl volume according to the manufacturer's instructions. The specific oligonucleotides were designed and used to amplify each knocked-out gene in the genome. The thermal cycling profile used for PCR was as follows: 33 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec.

6. Construction of *ac11* repair and *ac78* repair bacmids

To generate the *ac11* and *ac78* knockout-repaired bacmids, firstly, a transfer vectors pUC19-MCP was constructed using double-joint PCR (DJ-PCR) coupled with In-Fusion cloning (Takara, Japan) as follows. Double-Joint PCR was performed as previously described (Kim *et al.*, 2009) with modification. Briefly, in the first round of PCR, the chloramphenicol resistance gene with its promoter, and an *Xho*I restriction enzyme site

(double underlined) were amplified with primers Cm r-Fw and Cm r-Re using the plasmid, pDEST-32 as a template. The Mini-F replicon region and the *polyhedrin* region were amplified with primers MiniF-Fw and MiniF-Re, Polh-Fw and Polh-Re respectively, using the genomic DNA of Ac-MK as a template. MiniF-Re and Polh-Fw contain 20-bp homologous to upstream and downstream regions of *Cm* (underlined), respectively. For DJ-PCR, the three PCR products (Mini-F replicon fragment, the *Cm* gene, and the *polyhedrin* gene) were purified using a PCR-purification kit (Qiagen, Germany), and then 40 ng of each purified PCR product were combined together. In the second round of PCR, the cassette containing a Mini-F replicon, *Cm* gene and *polyhedrin* gene was PCR-amplified using primer MCP-Fw and MCP-Re with 5 μl of combined templates. To perform the In-Fusion cloning, the cassette which named MCP, containing the Mini-F replicon 3'-flanking-region, chloramphenicol resistance gene and *polyhedrin* 5'-flanking region was PCR-amplified with primer MCP-In-Fusion-Fw and MCP-In-Fusion-Re using 2 μl of the second-round PCR product as template. MCP-In-Fusion-Fw and MCP-In-Fusion-Re contain 15-bp homologous to upstream and downstream regions of the pUC-19 *EcoR*I digestion site (underlined), respectively. All PCR amplifications were performed using Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). The In-Fusion cloning procedure was carried out using an In-Fusion HD cloning kit (Takara,

Japan) according to the manufacturer's instructions. After 100 ng of the purified PCR product was mixed with 50 ng *EcoR*I-digested pUC19 DNA (Takara, Japan), 2 μl of 5× In-Fusion HD Enzyme Premix was added to the reaction and the mixture was pre-incubated at 50°C for 15 min. The resulting DNA was transformed into Stellar competent *E. coli* cells (Invitrogen, USA) according to the manufacturer's instructions, and the transformed cells were subsequently spread onto nutrient agar containing chloramphenicol (50 μg/ml) and ampicillin (50 μg/ml). The plates were incubated overnight at 37°C, and colonies resistant to kanamycin and ampicillin were selected and verified by restriction enzyme digestion and sequence analysis. The resulting plasmid, pUC19-MCP (Fig. 2), was then digested with *Xho*I for use.

To construct the repair transfer vectors, the *ac11* gene and *ac78* gene were PCR-amplified with primers Ac11-In-Fusion-Fw and Ac11-In-Fusion-Re, Ac78-In-Fusion-Fw and Ac78-In-Fusion-Re, respectively, using Ac-MK DNA as template. Ac11-In-Fusion-Fw, Ac11-In-Fusion-Re, Ac78-In-Fusion-Fw and Ac11-In-Fusion-Re contain 15-bp homologous to upstream and downstream regions of the pUC-19 *Xho*I digestion site (underlined), respectively. The PCR-amplified *ac11* and *ac78* DNAs was then mixed with the *Xho*I digested pUC19-MCP, respectively, to generate the repair transfer vectors, pUC19-MCP-Ac11 (Fig. 3) and pUC19-MCP-Ac78

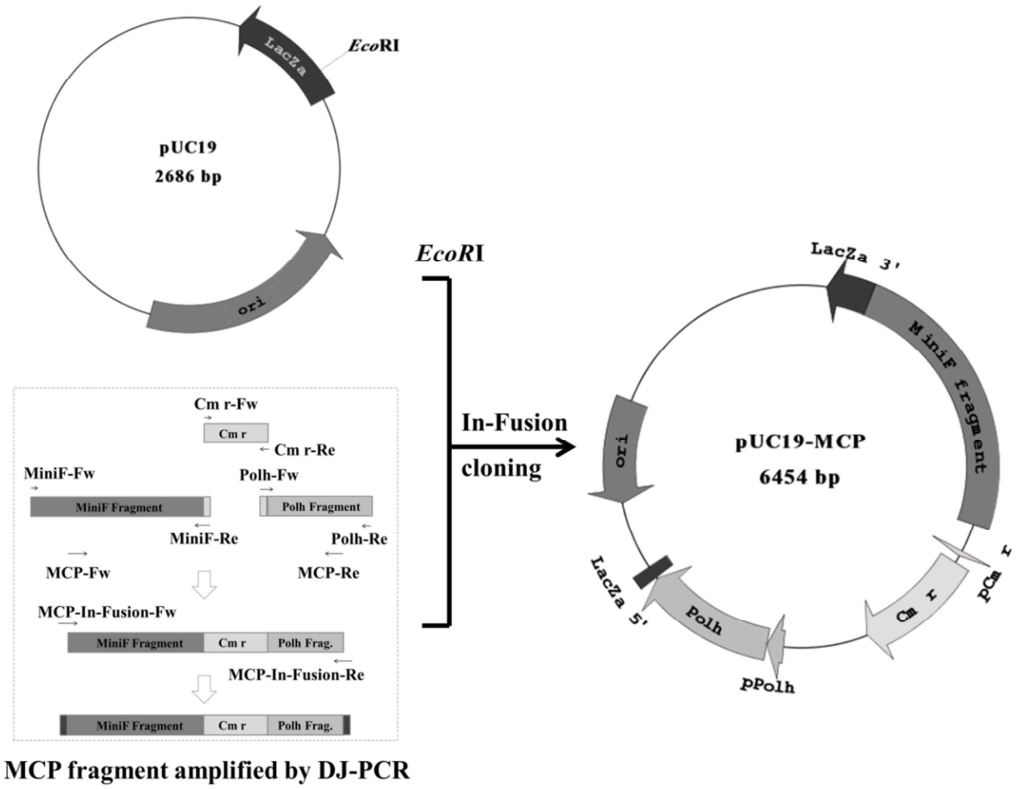


Fig. 2. Construction map of transfer vector, pUC19-MCP. The PCR-amplified MCP cassette, which contains the Mini-F replicon, chloramphenicol resistance gene and *polyhedrin* gene, was inserted into *EcoR*I-digested pUC19 by In-Fusion cloning to get pUC19-MCP.

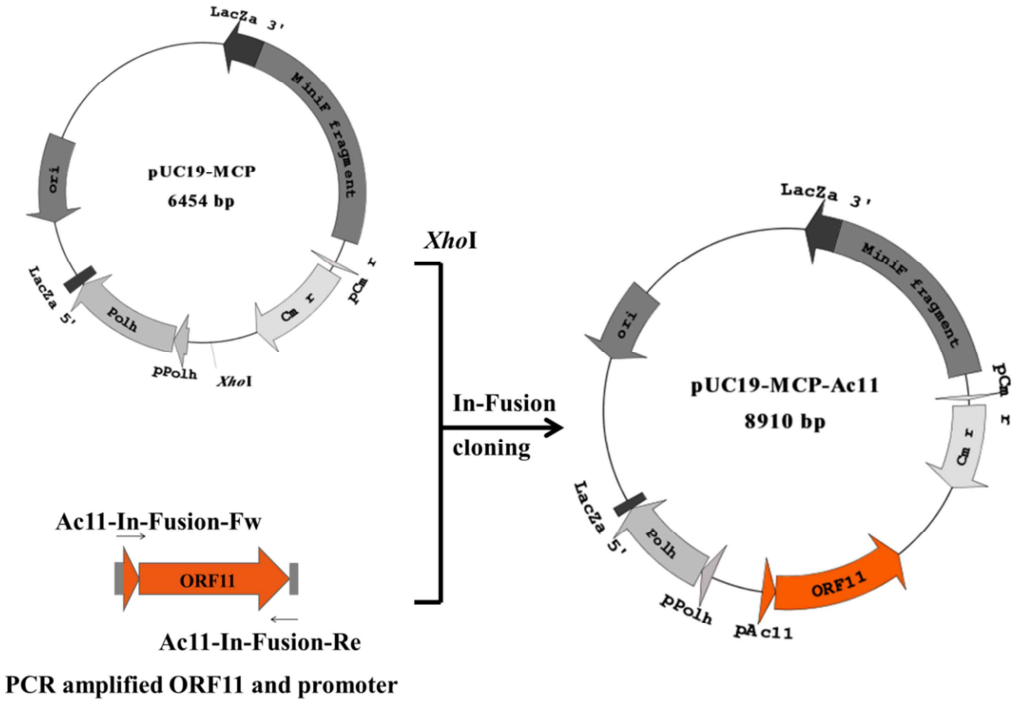


Fig. 3. Construction map of *ac11* repair transfer vector, pUC19-MCP-Ac11. The PCR-amplified *ac11* gene was inserted into *Xho*I digested pUC19-MCP by In-Fusion cloning to construct pUC19-MCP-Ac11.

(Fig. 4), using the In-Fusion HD cloning kit described as above.

The knocked-out *ac11* repair bacmid (Ac11Re) was generated via homologous recombination of pUC19-MCP-Ac11 and Ac11KO genomic DNA, and *ac78* repair bacmid (Ac78Re) was generated via homologous recombination of pUC19-MCP-Ac78 and Ac78KO genomic DNA in co-transfected Sf9 cells, respectively, and selected in *E. coli* plated on nutrient agar plate containing chloramphenicol (50 μg/ml) and ampicillin (50 μg/ml). All primers used above were listed in Table 1.

7. RNA and reverse transcription PCR (RT-PCR)

Sf9 cells (1×10^6 cells/35 mm-diameter six-well plate) were transfected with viruses, and total RNA was isolated from transfected Sf9 cells at 72 hours post-transfection (hpt) using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. After the RNA samples were treated with RNAse-free DNAse I (Takara, Japan), RT-PCR was performed using an *AccuPower*® RT/PCR Premix (Bioneer, Korea) in a 20-μl volume according to the manufacturer's instructions. To analysis the transcription of genes in Ac-MK, Ac11KO, Ac43KO, Ac78KO, and GP64KO transfected Sf9 cells, and the oligonucleotides, Ac11-RealTime-Fw and Ac11-RealTime-Re, Ac43-F and Ac43-R, Ac78-RT-Fw and Ac78-RT-Re were used which are specific to *ac11*, *ac43*, and *ac78*,

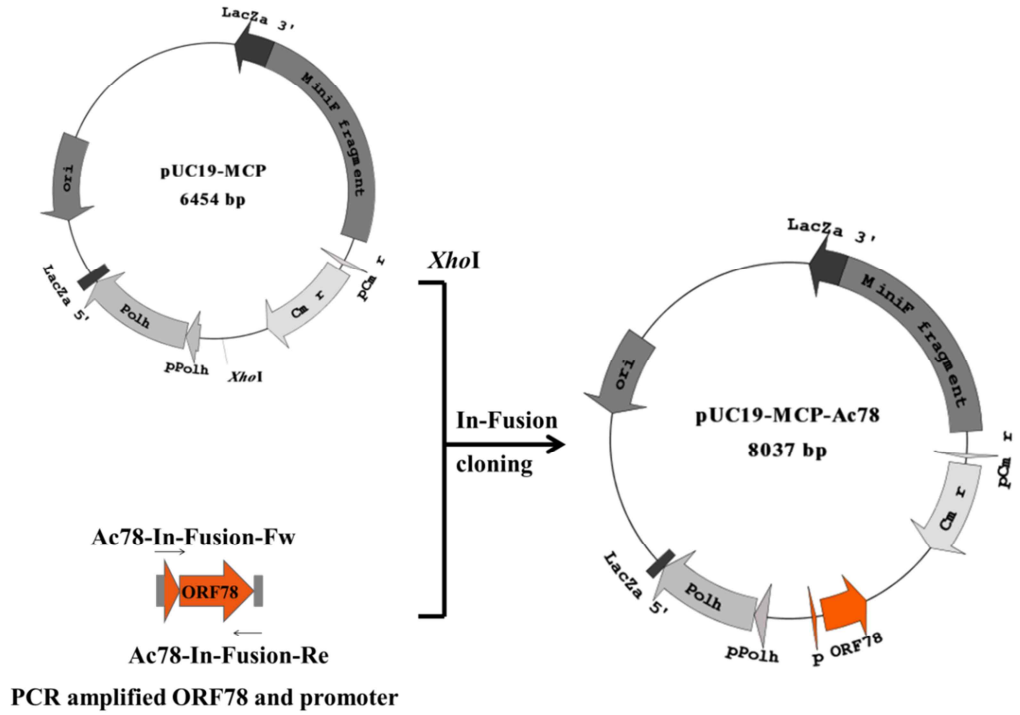


Fig. 4. Construction map of *ac78* repair transfer vector, pUC19-MCP-Ac78. The PCR-amplified *ac78* gene was inserted into *Xho*I digested pUC19-MCP by In-Fusion cloning to construct pUC19-MCP-Ac78.

Table. 1 Primers used for *ac11* repair and *ac78* repair bacmids construction.

Primer Name	**Primer Sequence (5'-3')**
Cm r-Fw	TGAGTCAGCATCACCCGACG
Cm r-Re	CACCAGCCCCTGTTCTCGAGTCAGC
MiniF-Fw	GATCTTAAAGGGTTCGAGCCTG
MiniF-Re	CGTCGGGTGATGCTGACTCAAACGTGCCGGCACGGCCT
Polh-Fw	CTCGAGAACAGGGGCTGGTGCAGCCATTGTAATGAGACGCAC
Polh-Re	CAATTGCTTACATTGAGCGGTTG
MCP-Fw	GAAGTGCTCCGGGGTGATAG
MCP-Re	CCATTAGTAGATTTGCCGTCTG
MCP-In-Fusion-Fw	GACGGCCAGTGAATTGAAGTGCTCCGGGGTGATAG
MCP-In-Fusion-Re	TACCGAGCTCGAATTCCATTAGTAGATTTGCCGTCTG
Ac11-In-Fusion-Fw	CTTTTGCTGACTCGAAGCGAAGACGAAATGTTGGAC
Ac11-In-Fusion-Re	AGCCCCTGTTCTCGAGCTTGTTATTTGCACGTCTGTC
Ac78-In-Fusion-Fw	CTTTTGCTGACTCGAGAGTACGACATGTCTTCCAGGT
Ac78-In-Fusion-Re	AGCCCCTGTTCTCGACACGGGCATCACGAGCAATC

respectively. AcGP64-F and AcGP64-R specific to gp64 were used. The primer sequences are listed in Table 2.

8. Quantitative real-time PCR (qPCR)

To identify the transcription phase of *ac11*, *ac43*, and *ac78* in AcMNPV life cycle and the expression level of *polyhedrin* gene in Ac-MK and Ac43KO, Sf9 cells were infected with Ac-MK and Ac43KO at a multiplicity of infection (MOI) of 10 followed by gentle rocking for 1 h. The virus-containing culture medium was removed and fresh medium was added after washing two times with incomplete TC-100 medium. The total RNA extracted from infected Sf9 cells at 0, 0.5, 1, 2, 6, 12, 24, 48, 72, and 96 hpi was treated with DNAse I prior to cDNA synthesis. Single-strand cDNA was synthesized from the total RNA using the SuperScript™ III First-Synthesis System for RT-PCR (Invitrogen, USA) according to the manufacturer's instructions. The qPCR was conducted with a 2× DyNAmo™ HS SYBR® Green qPCR Kit (Finnzymes, Finland) and CFX96™ Real-Time System (BIO-RAD, USA). The cycling profile used for qPCR was as follows: a preheating step for enzyme activation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 58°C for 15 sec and 72°C for 30 sec. The relative transcription level was calculated using the $2^{-\Delta Ct}$ method (Pfaffl, 2001). The 28S rRNA was used as a reference

gene (Xue *et al.*, 2010). Oligonucleotides Ac11-RealTime-Fw and Ac11-RealTime-Re specific to *ac11*, ORF43-RTF and ORF43-RTR and specific to *ac43*, Polh-RTF and Polh-RTR specific to *polyhedrin*, and Ac78-RealTime-Fw and Ac78-RealTime-Re specific to *ac78*, were used for qPCR. The 28S rRNA was used as a reference gene.

To investigate the expression of *vlf-1* gene in Ac78KO transfected Sf9 cells, 1×10^6 Sf9 cells were transfected with 1 μg of Ac78KO bacmid DNA. At the designated time points, total RNA was extracted from the Ac78KO-transfected Sf9 cells and treated with DNAse I. Single-strand cDNA was synthesized and subjected to qPCR analysis using *vlf-1*-specific primer vlf-1-RT-Fw and vlf-1-RT-Re. The 28S rRNA was used as a reference gene. The primer sequences are listed in Table 3.

9. Titration of BV in Ac-MK and Ac43KO infected Sf9 cells

Sf9 cells were infected with Ac-MK and Ac43KO at an MOI of 1.0. The collected media containing BVs were harvested at 24, 48, 72, and 96 hpi, and cell debris were removed by centrifugation at 5,000×g for 5 min. The extracellular BV titers in the collected media were determined using the end-point dilution method in triplicate as previously described (O'Reilly, et al., 1992).

Table. 2 Primers used for reverse transcription PCR (RT-PCR).

Primer Name	**Primer Sequence (5'-3')**
Ac11-RealTime-Fw	TACATTTCGGCGATTACACG
Ac11-RealTime-Re	GAATTGGTGCCTCGTTTGT
Ac43-F	GGGGGATCCCCAAGACAAAAAGATTGC
Ac43-R	CCCCTCGAGCACCGTGAACACCTTGC
Ac78-RT-Fw	GTCGTGTTGTCATAGCCCAC
Ac78-RT-Re	GAATTTGGACGTGCCCTAC
AcGP64-F	ATATGTGCTTTTGGCGG
AcGP64-R	TTTGGCGCGTGGTGAAC
gp64-PCR-Fw	CTTCTTTGTAGATGCTGTTGTTG
gp64-PCR-Re	CGTTAGAGCCAAGTACACAG

Table. 3 Primers used for Quantitative real-time PCR (qPCR).

Primer Name	**Primer Sequence (5'-3')**
Ac11-RealTime-Fw	TACATTTCGGCGATTACACG
Ac11-RealTime-Re	GAATTGGTGCCTCGTTTGT
ORF43-RTF	GCTATGTTTGCGACGAGTTGGTG
ORF43-RTR	TGGGCACAGCACGATACCG
Polh-RTF	ACCCAACCGTTGTTACAAATTCCT
Polh-RTR	GCCGCCGCCCTTCTTAGC
Ac78-RealTime-Fw	TCGGTGTCAATACTATCCGAA
Ac78-RealTime-Re	GTGGGCTATGACAACACGAC
vlf-1-RT-Fw	CAACAACGATTCTCCCGAC
vlf-1-RT-Re	CTTGAAGAGTCGGGCGTC

10. BV production analysis of Ac11KO and Ac78KO transfected Sf9 cells

Sf9 cells (1×10^6 cells/35-mm-diameter six-well plate) were transfected with 1 μg of each bacmid (Ac-MK, Ac11KO, Ac11Re, Ac78KO, and Ac78Re). At various hpt, the supernatant containing BV was harvested and cell debris was removed by centrifugation at 8,000×g for 5 min. The extracellular BV titer in the harvested culture supernatant was determined using the end-point dilution method in triplicate. For titration of BV using qPCR, 1 ml of the above supernatant containing BV was centrifuged at 80,000×g for 2 h at 4°C, and the pellets were re-suspended with 200 μl of lysis buffer (10 mM Tris-Cl pH 7.5, 10 mM EDTA, 0.25% SDS, 20 μg/ml RNase A and 80 μg/ml Proteinase K). After overnight incubation at 65°C, the viral DNA was extracted by phenol extraction and alcohol precipitation. To perform qPCR, 2 μl of diluted DNA was used along with a 2× DyNAmo™ HS SYBR® Green qPCR Kit (Finnzymes, Finland) and the primers IE1-RTF (5'-ACCATCGCCCAGTTCTGCTTATC-3') and IE1-RTR (5'-GCTTCCGTTTAGTTCCAGTTGCC-3'), which amplify a 100-bp fragment of the *ie-1* gene. A stock of wild-type Ac-MK (4.25×10^8 pfu/ml) that was previously titered by end-point dilution was serially diluted and used to develop a standard curve (Fig. 5).

The samples were analyzed using a CFX96TM Real-Time System (BIO-RAD, USA) under the following conditions: a preheating step for enzyme activation at 95°C for 15

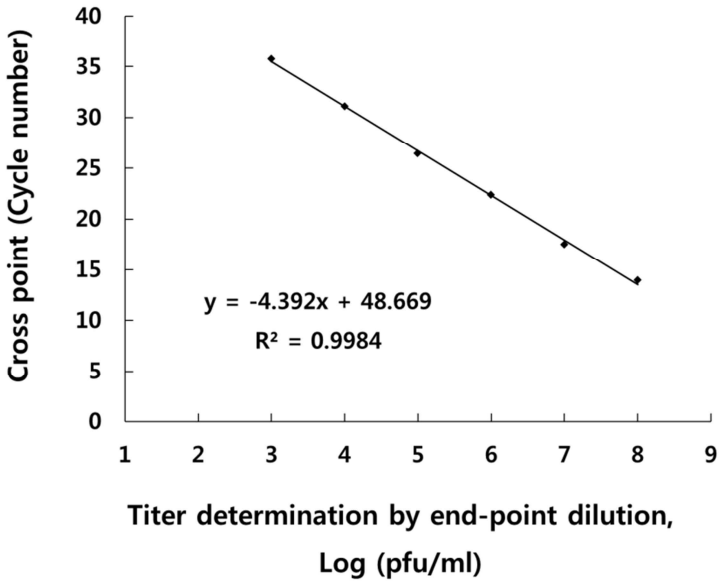


Fig. 5. Standard curve of qPCR for BV titration. A stock virus solution of wild-type Ac-MK (4.25×10^8 pfu/ml) was 10-fold diluted, and the titer of each dilution was determined and compared by Q-PCR and end-point dilution assay. The cross points were determined by qPCR with *ie-1* primers, and the titers determined by end-point dilutions were based on the formation of occlusion bodies.

min, followed by 45 cycles of 95°C for 30 sec, 60°C for 20 sec and 72°C for 20 sec.

11. Quantification of viral DNA replication

To assess viral DNA replication, a qPCR assay was performed as previously described (Vanarsdall *et al.*, 2005) with slight modifications. To prepare viral DNA for analysis, Sf9 cells (1×10^6 cells/35-mm-diameter well of a 6-well plate) were transfected with 1 μg of bacmid DNA (Ac-MK, Ac11KO, Ac11Re, Ac78KO, Ac78Re, and GP64KO), and the transfected cells were washed once with 1× phosphate-buffered saline (Sigma, USA) at the designated hpt and centrifuged at 8,000×g for 5 min. The harvested cell pellets were incubated for 30 min at 37°C in 250 μl of lysis buffer (10 mM Tris-Cl pH 7.5, 10 mM EDTA, 0.25% SDS, 20 μg/ml RNAase A) and then incubated overnight at 65°C after the addition of 80 μg/ml proteinase K. Viral DNA was extracted with 250 μl of phenol:chloroform and 250 μl of chloroform, and the aqueous layer containing the viral DNA was carefully harvested. Prior to the PCR, 2 μl of total DNA from each time point was digested with 40 U of *Dpn*I restriction enzyme (New England Biolabs, USA) overnight in a 20-μl total reaction volume. To perform qPCR, 2 μl of the digested DNA was used along with a 2× DyNAmo™ HS SYBR® Green qPCR Kit (Finnzymes, Finland) and the primers gp41-Fw (5'-CGTAGTGGTAGTAATCGCCGC-3') and gp41-Re (5'-AGTCGAGTCGCGTCGCTTT-3'). The samples were analyzed in a CFX96TM

Real-Time System (BIO-RAD, USA) under the following conditions: a preheating step for enzyme activation at 95°C for 15 min, followed by 45 cycles of 95°C for 30 sec, 60°C for 20 sec and 72°C for 20 sec.

12. BV partial purification and concentration

Sf9 cells (1×10^6 cells/35-mm-diameter well of a 6-well plate) were transfected with 1 μg of bacmid DNA (Ac-MK, Ac11KO, Ac11Re, Ac78KO and Ac78Re). At 120 hpt, the supernatant containing BV was harvested and BVs were purified as previously described (Oomens & Blissard, 1999)**.** Briefly, the medium was harvested and cell debris was removed by centrifugation at 2,000×g for 20 min. Supernatant (3 ml) was loaded onto a 25% sucrose cushion and centrifuged at 80,000×g for 90 min at 4 °C. BV pellets were resuspended in 25 μl of 50 mM Tris–Cl (pH 7.5). An equal volume of 2× protein sample buffer (Sigma, USA) was added and the samples were placed at 100 °C for 10 min. 10μl the Ac-MK, Ac11Re and Ac78Re samples and the totality of the Ac11KO or Ac78KO samples were analyzed by 12% SDS-PAGE and Western blotting.

13. Purification of BV and ODV

Sf9 cells were infected with Ac-MK at an MOI of 0.1 and harvested at 5 days post infection (dpi) by centrifugation at 1,800×g for 10 min. The resulting supernatant was

used for BV purification, and the pellet was used for ODV purification. The purification of BV, ODV and the fractionation of envelope and nucleocapsid of BV and ODV were performed as previously described (Braunagel & Summers, 1994; Wu *et al.*, 2008a).

14. Preparation of antibody

For production of polyclonal antibody against Ac11 and Ac78, PCR-amplified *ac11* gene and *ac78* genes were introduced into *Escherichia coli* expression vector pET30a(+) (Novagen, Germany) to obtain pET30a(+)-Ac11 (Fig. 6) and pET30a(+)-Ac78 (Fig. 7), which was cloned into *Escherichia coli* BL21 (DE3), respectively. Ac11 and Ac78 were expressed by IPTG induction, and then purified using a Ni-NTA Superflow Cartridges (QIAGEN, Germany) and FPLC (GE Healthcare, USA). The purified proteins were immunized into ICR female rabbits by intervenous injection. The first immunization of 200 μg/rabbit in incomplete Freund's Adjuvant (Sigma Co., USA) was followed at 7 days interval by a series of 200 μg/rabbit injection in incomplete Freund's Adjuvant (Sigma Co., USA). The rabbits were bled 3 days after the last injection and antisera were separated after storage of total bloods overnight at 4°C.

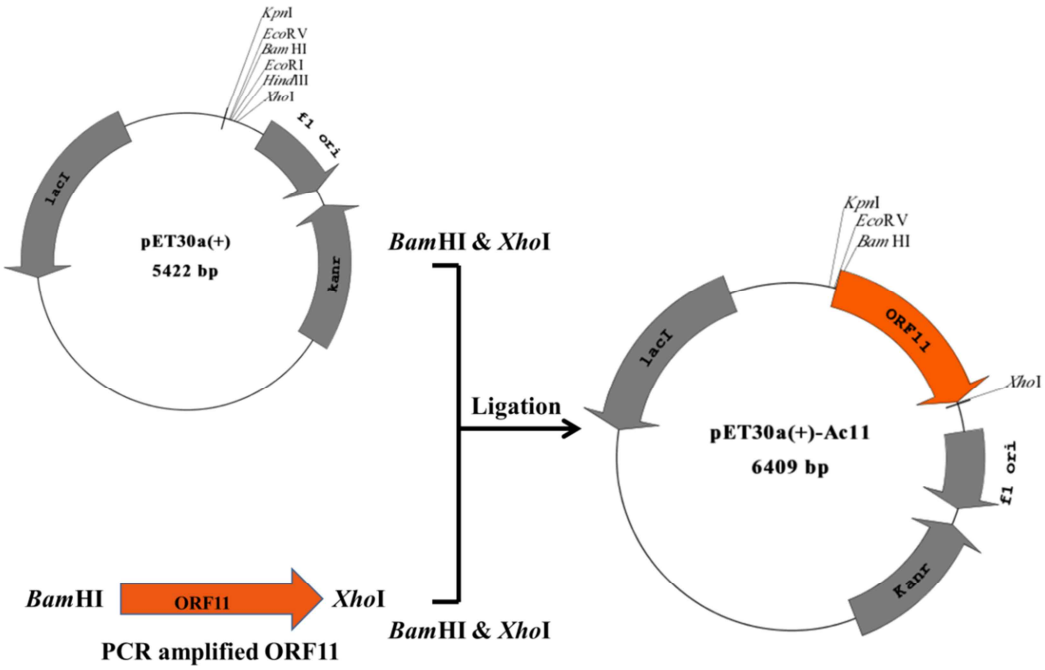


Fig. 6. Construction map of *E. coli* expression vector, pET30a(+)-Ac11. The PCR-amplified *ac11* gene was inserted into *Bam*HI and *Xho*I digested pET30a(+) to obtain the expression vector pET30a(+)-Ac11.

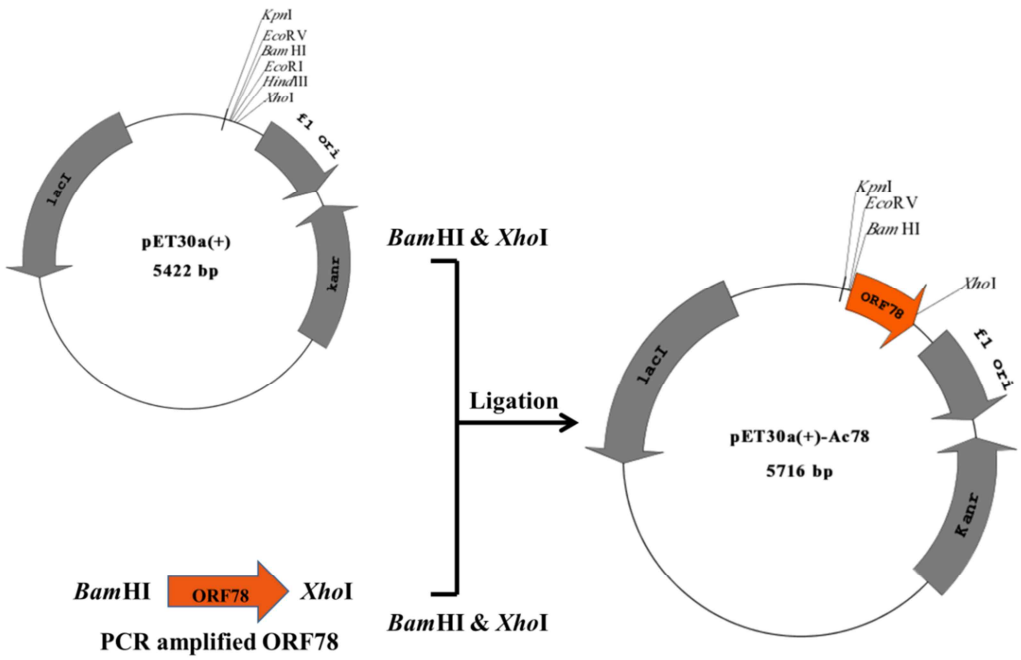


Fig. 7. Construction map of *E. coli* expression vector, pET30a(+)-Ac78. The PCR-amplified *ac11* gene was inserted into *Bam*HI and *Xho*I digested pET30a(+) to obtain the expression vector pET30a(+)-Ac78.

15. Western blot analysis

The purified BV and ODV, and the fractions of BV and ODV envelope and nucleocapsid were mixed with equal volumes of 2× protein sample buffer (Sigma, USA) and boiled at 100°C for 10 min. These protein samples were resolved in 12% SDS-PAGE, transferred onto a hydrophobic polyvinylidene difluoride (PVDF) membrane (GE healthcare, USA), and probed with primary antibodies, rabbit polyclonal Ac11 antiserum (1:5,000), rabbit polyclonal Ac78 antiserum (1:5,000), rabbit polyclonal VP39 antiserum (1:5,000) (Provided by Professor Kai Yang in Sun Yat-Sen University), or mouse monoclonal GP64 AcV5 antibody (1:5,000) (eBioscience, USA). Peroxidase conjugated goat anti-rabbit antibody (1:50,000) (ABM, Canada) or horseradish peroxidase-conjugated sheep anti-mouse antibody (1:10,000) (Amersham biosciences, USA) were used as the secondary antibodies. The signals were detected with Western Blotting Luminol Reagent (Santa Cruz Biotechnology, USA).

16. Quantification of OB

Sf9 cells were infected with viruses at an MOI of 10. Infected cells were harvested at 96 hpi by centrifugation at 5,000×g for 5 min. The resulting pellet was resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 0.4% SDS, 10 mM EDTA, and 5% 2-mercaptoethanol), and OBs were pelleted by centrifugation at 5,000×g for 5 min and

then resuspended in 0.5 M NaCl. To calculate the number of OBs, OB stocks were serially diluted and counted in triplicate using a hemocytometer.

17. Electron microscopy

For transmission electron microscopy (TEM), Sf9 cells (1×10^6 cells/35-mm-diameter well of a 6-well plate) were transfected with 1 μg of each bacmid DNA. At designated time points post transfection, the cells were harvested by centrifugation at 5,000×g for 5 min, and fixed for 4 h at 4°C with 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2). After post-fixation by 1% OsO_4 in the same buffer, the samples were dehydrated in ethanol/propylene oxide series and embedded in an Epon-Araldite mixture. Ultra-thin sections were obtained with a RMC MT-X ultramicrotome and subsequently stained with a mixture of 2% uranyl acetate and Sato's lead. The transmission electron microscope JEM-1010 (JEOL, Japan) was used. For scanning electron microscopy (SEM), infected cells were fixed and dehydrated as described above for TEM, followed by drying in hexamethyldisilazane and mounting on metal stubs. Then the samples were gold coated and observed under the scanning electron microscope JSM-5410LV (JEOL, Japan).

For scanning electron microscopy, the purified OBs were dried at the critical point in

CO_2. The samples were sputtered with gold in a sputter coater SC502 (Polaron, USA) and observed using field emission scanning electron microscope JSM-6700F (JEOL, Japan).

For transmission electron microscopy, the purified OBs were fixed for 2 h by 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2). After post-fixation by 1% OsO_4 in the same buffer, the samples were dehydrated in ethanol/propylene oxide series and embedded in Epon-Araldite mixture. Ultra-thin sections were prepared with a RMC MT-X ultramicrotome and photographed under the transmission electron microscope JEM-1010 (JEOL, Japan).

RESULTS

1. Generation of AcMNPV gene knockout mutants

1. 1. Generation of a novel AcMNPV virus, Ac-MK

The novel recombinant bacmid, bAc-MK, was generated via homologous recombination of pBacMKPol and AcMNPV genomic DNA in co-transfected Sf9 cells, and successful recombinants were selected in *E. coli* plated on nutrient agar plates containing kanamycin. The genomic structure of Ac-MK was verified by nucleotide sequence analysis (bAc-MK, GenBank accession No. KF022001) and endonuclease digestion, and the insertion of Mini-F and Kan into the bacmid bAc-MK yielded *Eco*R I 21.1 kb and 8.4 kb, and *Hin*d III 15.8 kb fragments (Fig. 8).

1. 2. Generation and identification of AcMNPV gene knockout mutants

Tn7-mediated transposition between the Ac-MK and the donor-S of plasmid capture system was carried out to generate random recombination of AcMNPV genome. Through the transposition, about 1100 *E. coli* colonies, which were resistant to both kanamycin and ampicillin, were selected and 262 clones were end-sequenced using the transprimers, primerN and primerS. The insertion sites and sequences of each clone were analyzed (Fig.

9). In summary, among the total of 262 clones, 205 mutants contained mutations inside coding region and 15 mutants in non-translated regions in Ac-MK genome, respectively. Since the insertion sites of some mutants existed in the same ORF, total of 54 groups of single gene knockout AcMNPV mutants were confirmed, and one each of representative mutants were selected from the each group for further studies (Table. 4).

To define the phenotype of the gene knockout mutants, the 54 selected bacmids were introduced into Sf9 cells respectively, and the polyhedral production was analyzed. In addition, at 120 hpt, the culture medium was collected and inoculated into a new cell layer in order to determine whether the infectious virion was produced or not. The results showed that 38 mutants produced infectious viruses after transfection and infection, and 10 of them resulted in lower infectivity compared with wild type virus. The remaining 16 mutants did not produce infectious viruses, in which 10 of them produced polyhedron in the bacmid transfected cells but not spreading of the infection, and the remained mutants neither produced polyhedral or production of infectious viruses (Table. 4 and 5). These observations suggested that these 26 genes may play important roles in the morphogenesis of AcMNPV.

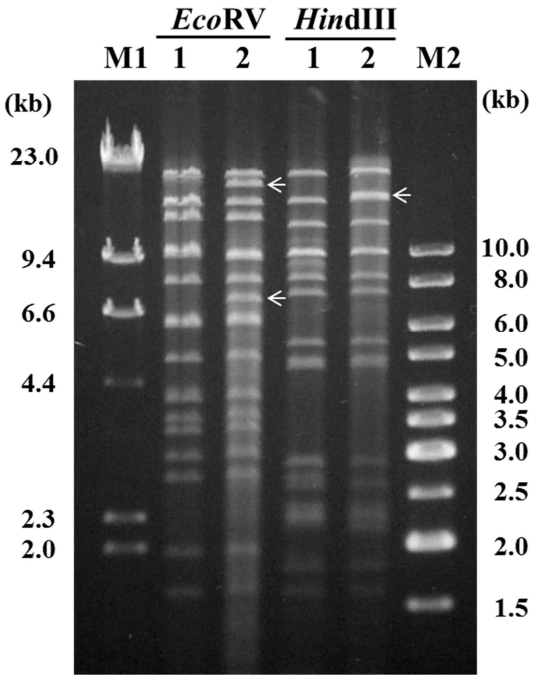
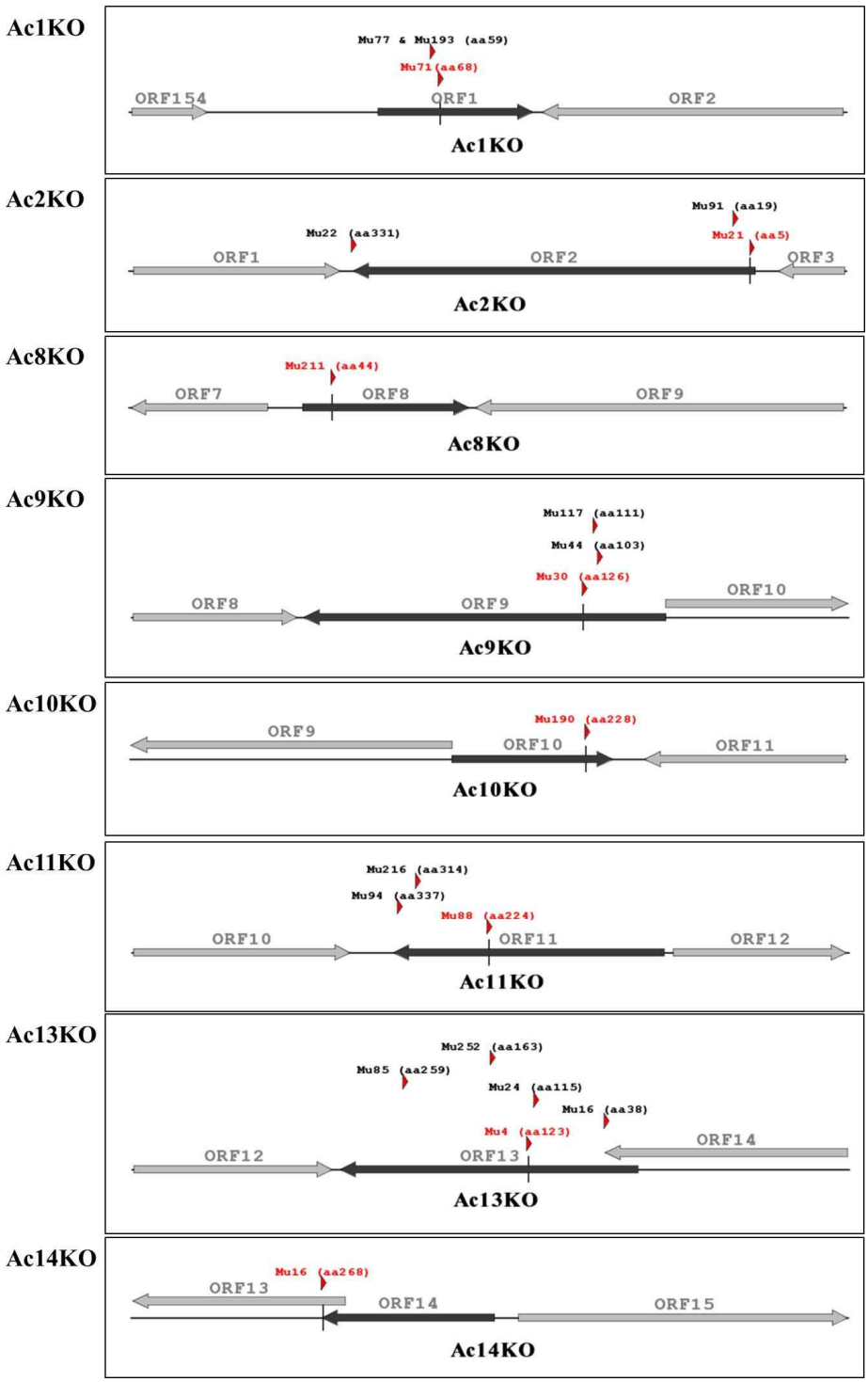


Fig. 8. Restriction endonuclease digestion patterns of the bAc-MK. Lanes: M1, λ DNA digested with *Hin*d III; 1, AcMNPV; 2, bAc-MK; M2, 1 Kb Ladder. The insertion of Mini-F and *Kan* into the bacmid bAc-MK yielded *Eco*R I 21.1 kb and 8.4 kb, and *Hin*d III 15.8 kb fragments (indicated with arrows).

Fig. 9. Schematic diagram of gene knocked-out mutants. The insertion sites of the mutants were indicated as red arrows, and the numbers in parenthesis indicate the mutation positions of each mutant in each AcMNPV genes. Mutants used for experiments were marked in red.


Ac1KO
Mu77 & Mu193 (aa59)
Mu71 (aa68)
ORF154
ORF1
ORF2
Ac1KO
Ac2KO
Mu91 (aa19)
Mu22 (aa331)
Mu21 (aa5)
ORF1
ORF2
ORF3
Ac2KO
Ac8KO
Mu211 (aa44)
ORF7
ORF8
ORF9
Ac8KO
Ac9KO
Mu117 (aa111)
Mu44 (aa103)
Mu30 (aa126)
ORF8
ORF9
ORF10
Ac9KO
Ac10KO
Mu190 (aa228)
ORF9
ORF10
ORF11
Ac10KO
Ac11KO
Mu216 (aa314)
Mu94 (aa337)
Mu88 (aa224)
ORF10
ORF11
ORF12
Ac11KO
Ac13KO
Mu252 (aa163)
Mu85 (aa259)
Mu24 (aa115)
Mu16 (aa38)
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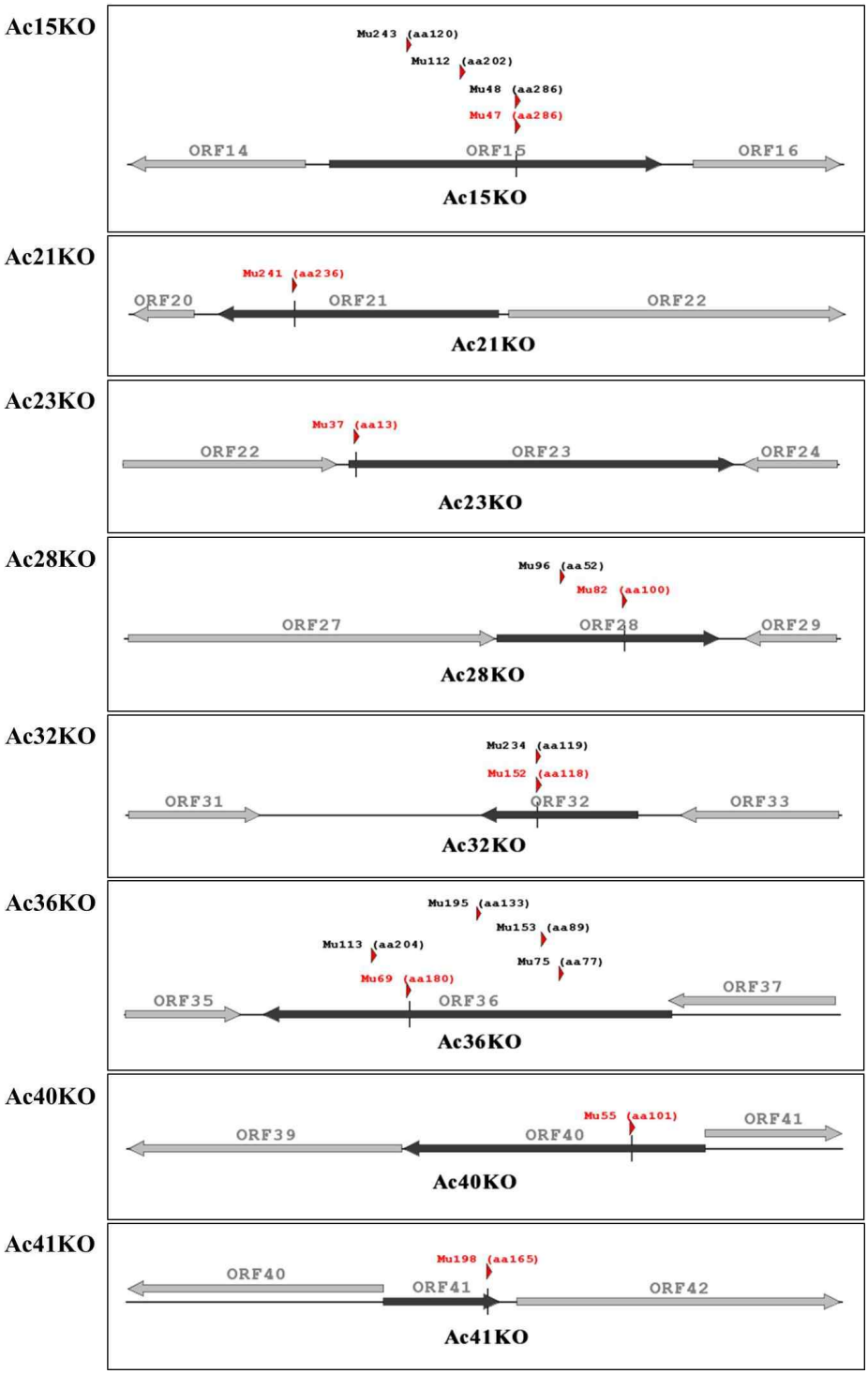
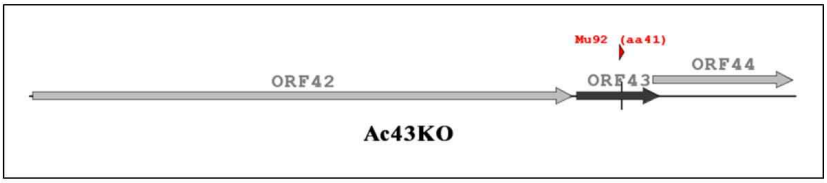
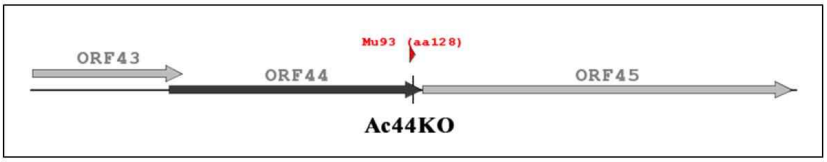
Mu16 (aa268)
ORF13
ORF14
ORF15
Ac14KO


Ac15KO
Mu243 (aa120)
Mu112 (aa202)
Mu48 (aa286)
Mu47 (aa286)
ORF14
ORF15
ORF16
Ac15KO
Ac21KO
Mu241 (aa236)
ORF20
ORF21
ORF22
Ac21KO
Ac23KO
Mu37 (aa13)
ORF22
ORF23
ORF24
Ac23KO
Ac28KO
Mu96 (aa52)
Mu82 (aa100)
ORF27
ORF28
ORF29
Ac28KO
Ac32KO
Mu234 (aa119)
Mu152 (aa118)
ORF31
ORF32
ORF33
Ac32KO
Ac36KO
Mu195 (aa133)
Mu153 (aa89)
Mu113 (aa204)
Mu75 (aa77)
Mu69 (aa180)
ORF37
ORF35
ORF36
Ac36KO
Ac40KO
Mu55 (aa101)
ORF41
ORF39
ORF40
Ac40KO
Ac41KO
Mu198 (aa165)
ORF40
ORF41
ORF42
Ac41KO

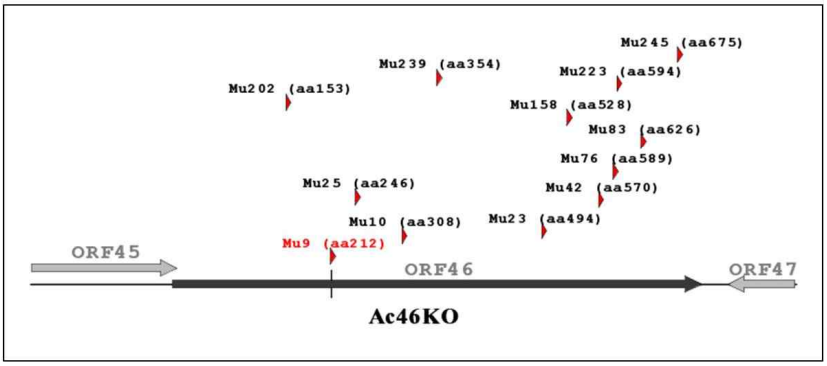
Ac43KO



Ac44KO



Ac46KO



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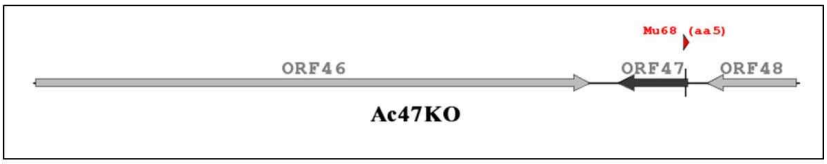

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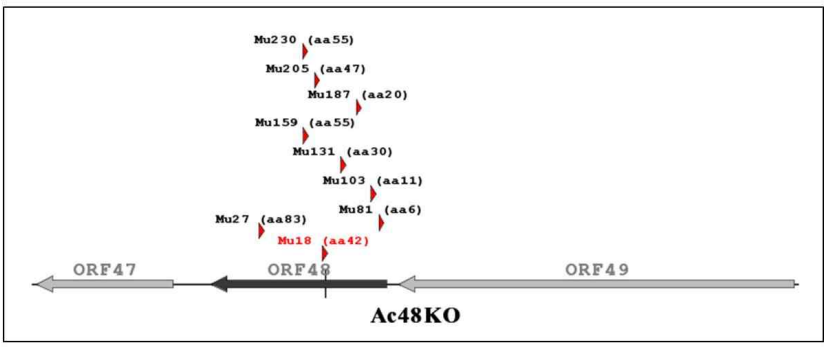

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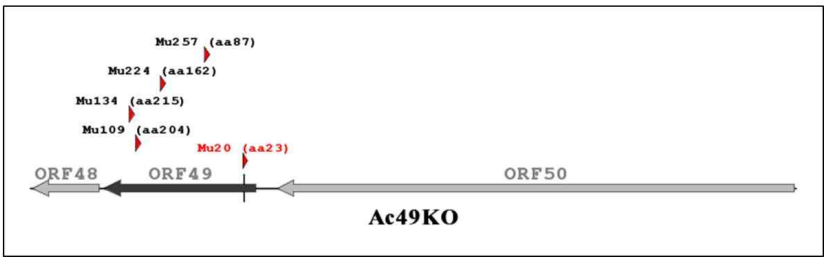

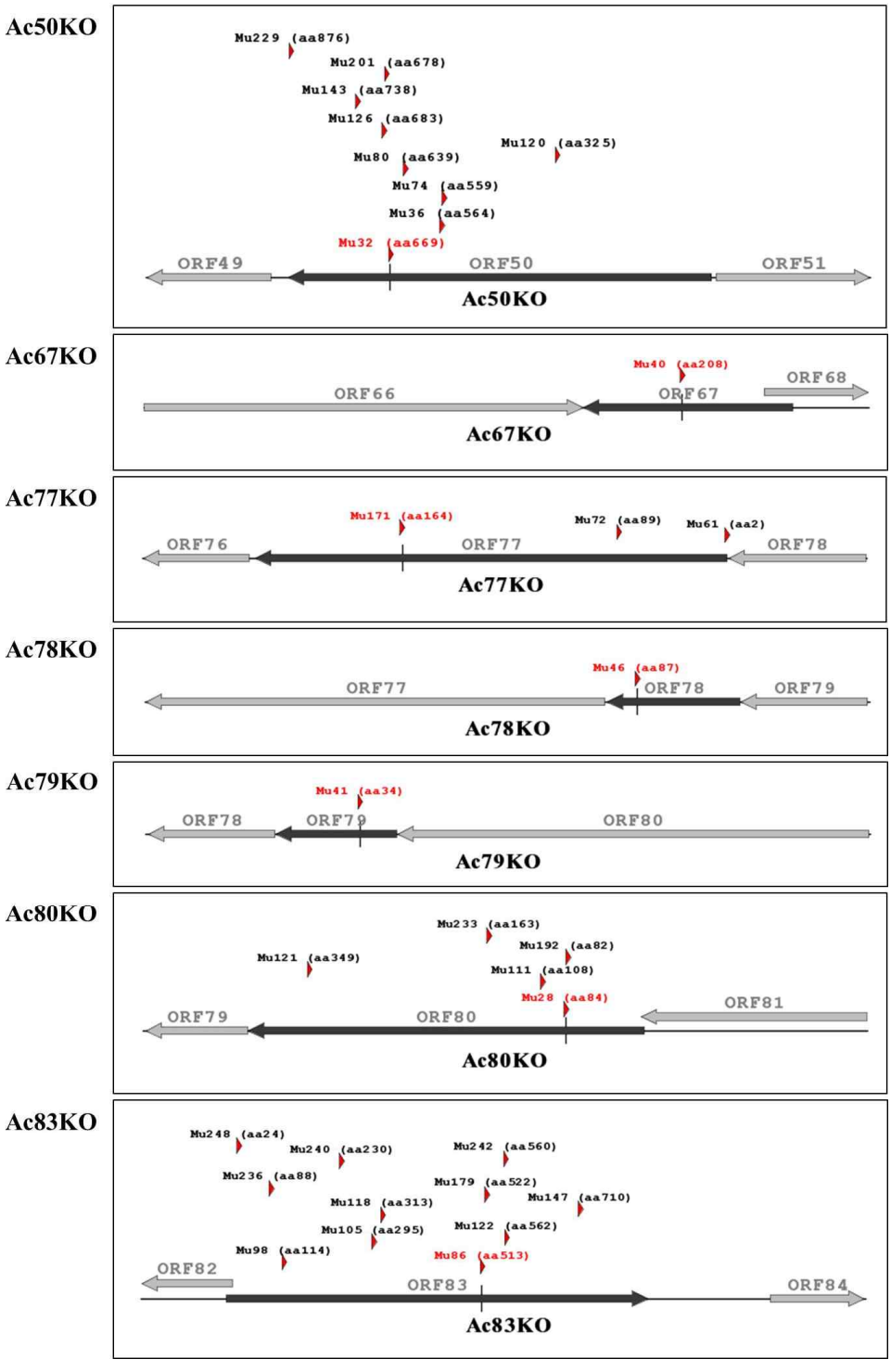
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Mu201 (aa678)
Mu143 (aa738)
Mu126 (aa683)
Mu120 (aa325)
Mu80 (aa639)
Mu74 (aa559)
Mu36 (aa564)
Mu32 (aa669)
ORF49
ORF50
ORF51
Ac50KO
Ac67KO
Mu40 (aa208)
ORF66
ORF67
ORF68
Ac67KO
Ac77KO
Mu171 (aa164)
Mu72 (aa89)
Mu61 (aa2)
ORF76
ORF77
ORF78
Ac77KO
Ac78KO
Mu46 (aa87)
ORF77
ORF78
ORF79
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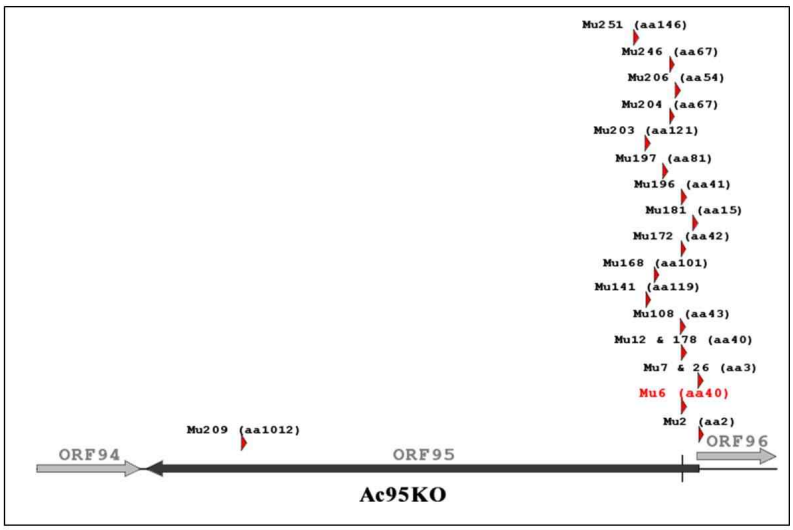
Ac79KO
Mu41 (aa34)
ORF78
ORF79
ORF80
Ac79KO
Ac80KO
Mu233 (aa163)
Mu192 (aa82)
Mu121 (aa349)
Mu111 (aa108)
Mu28 (aa84)
ORF79
ORF80
ORF81
Ac80KO
Ac83KO
Mu248 (aa24)
Mu240 (aa230)
Mu242 (aa560)
Mu236 (aa88)
Mu179 (aa522)
Mu147 (aa710)
Mu118 (aa313)
Mu105 (aa295)
Mu122 (aa562)
Mu98 (aa114)
Mu86 (aa513)
ORF82
ORF83
ORF84
Ac83KO

Ac95KO



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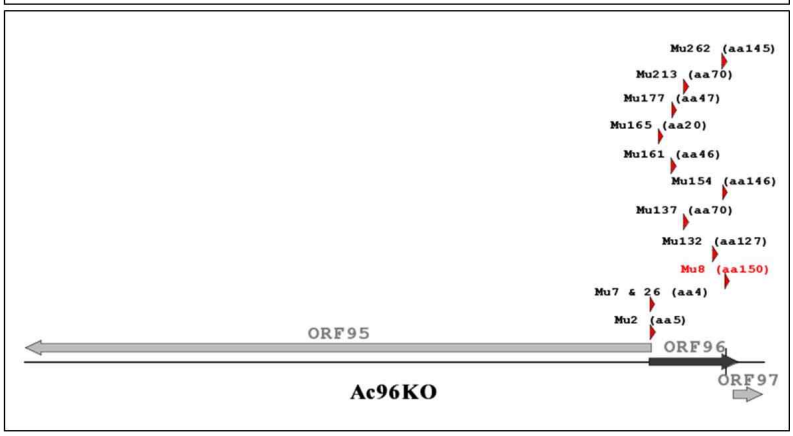

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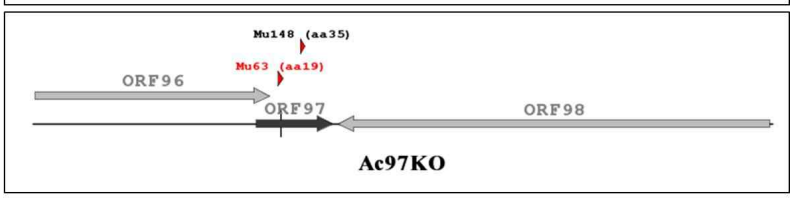

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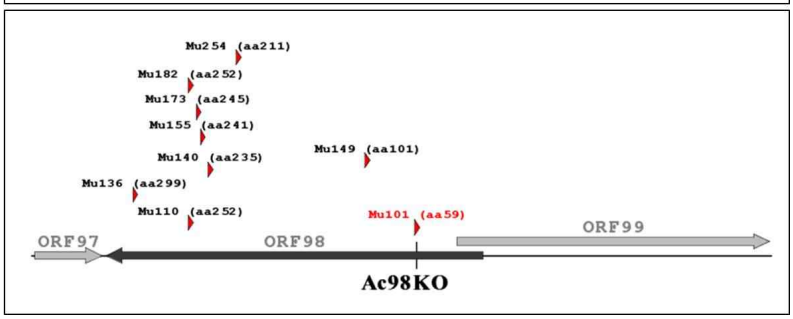

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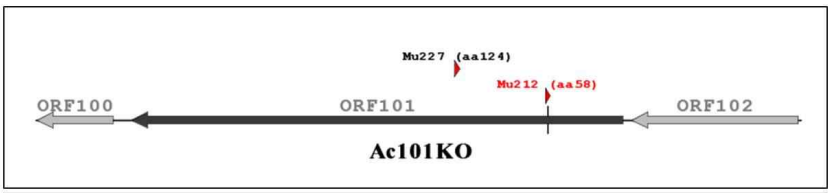

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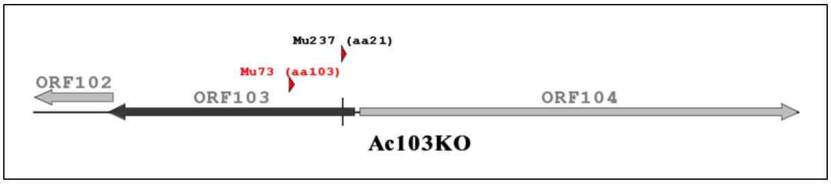

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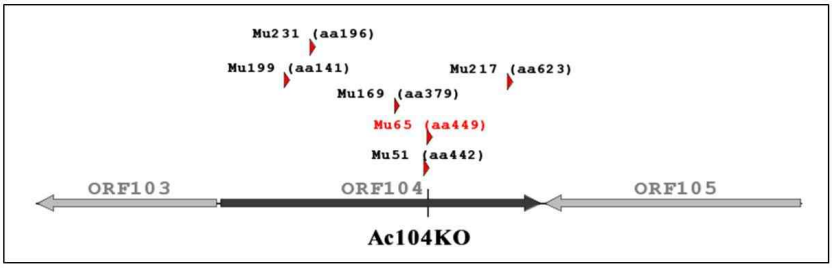
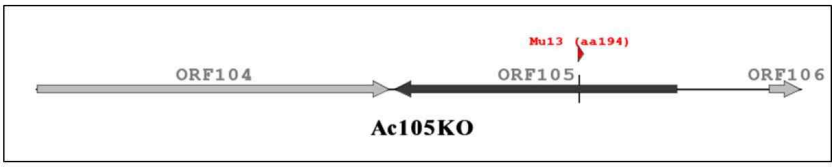
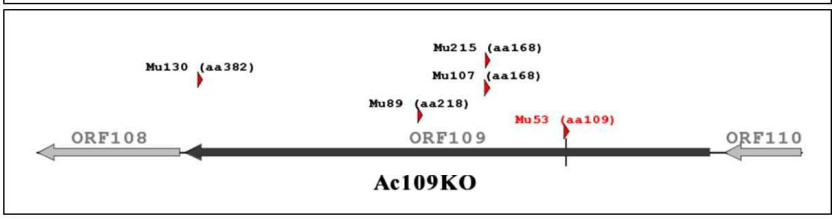


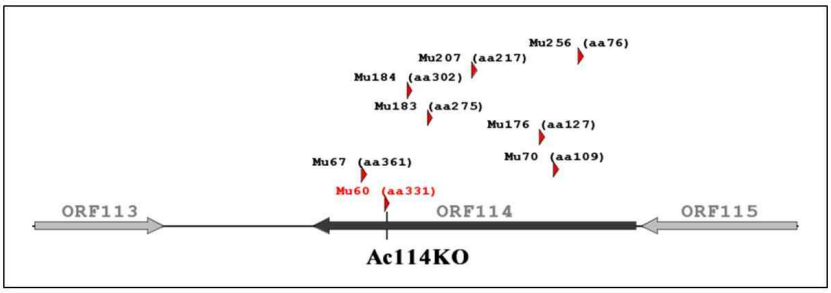
Ac105KO



Ac109KO



Ac114KO



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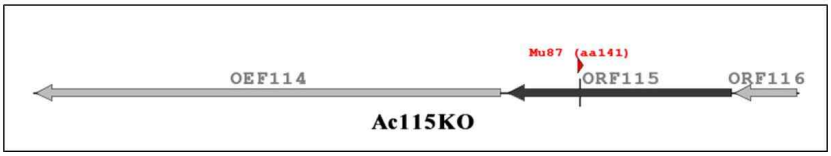

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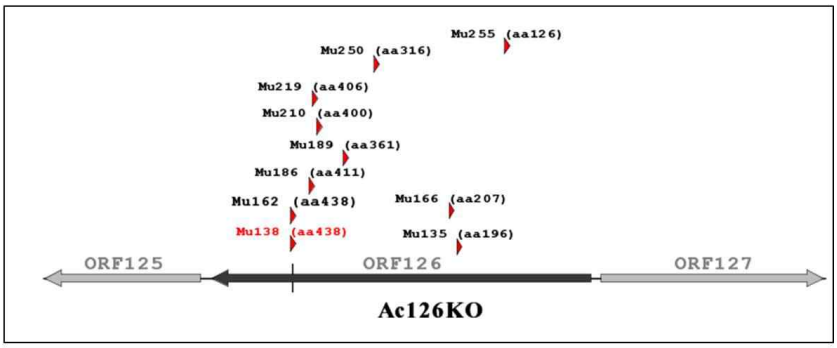

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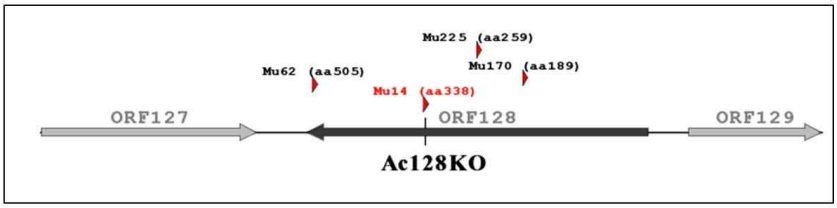

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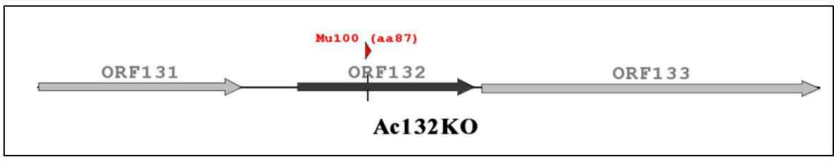

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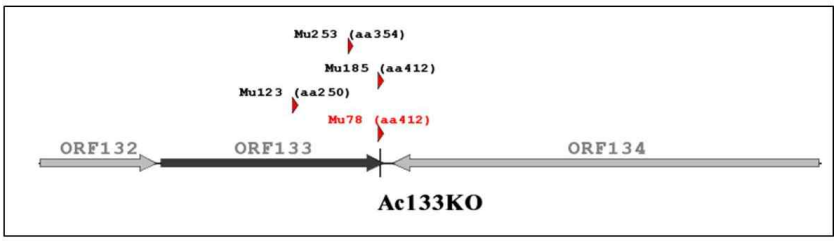

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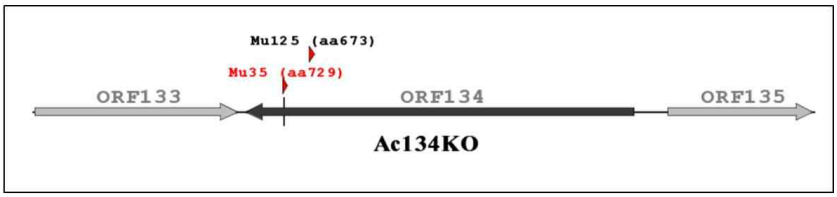

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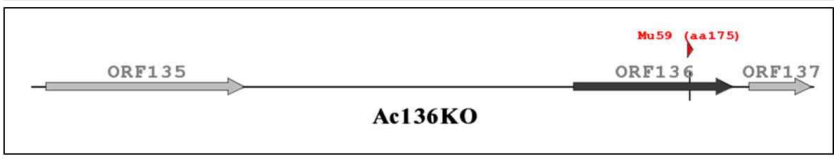

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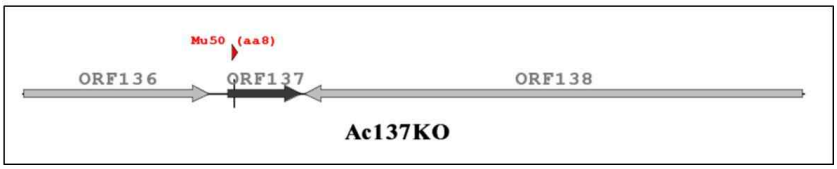

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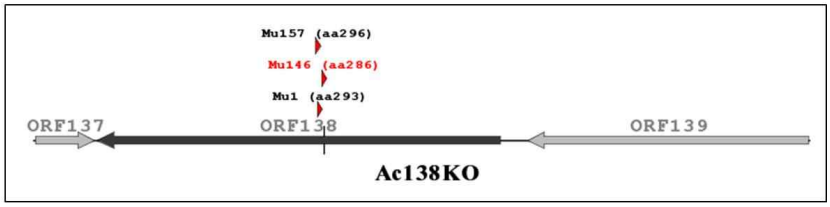

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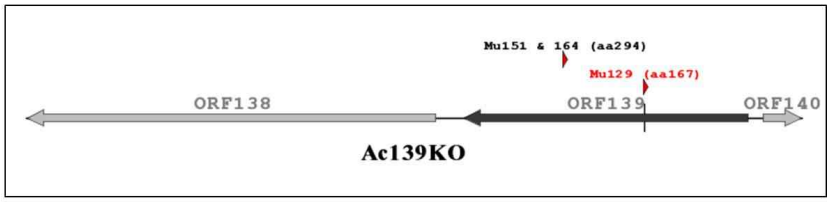
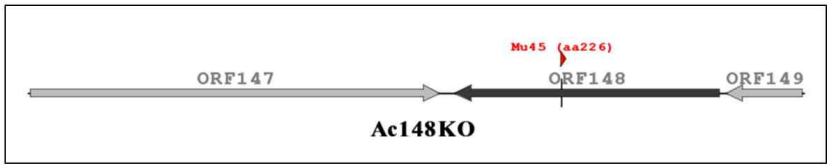
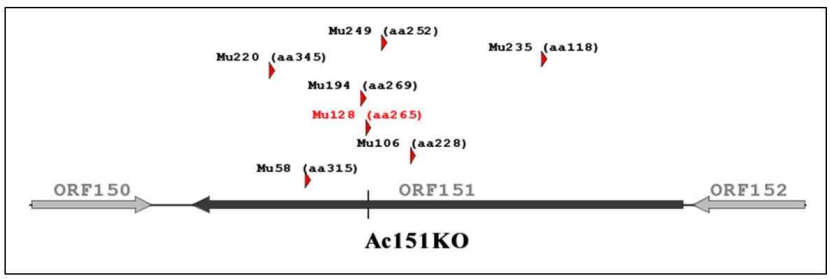


Ac148KO



Ac151KO



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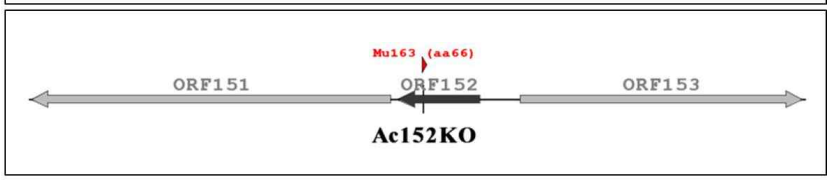

Ac153KO

Ac154KO

Table 4. Summary of AcMNPV gene knockout mutants.

Location on AcMNPV C6	Gene annotation	Serial number of mutants	Mutants amount	Transfection (Occ+)	Infection (Occ+)	Phase of transcription (motif)[a]
AcOrf-1	*ptp*	**71**, 77, 193	3	***	***	EL
AcOrf-2	*bro*	**21**, 22, 91	3	***	***	L
AcOrf-8	*PH*	**211**	1	-	-	CL
AcOrf-9	*ORF1629*	**30**, 44, 117	3	*	*	C
AcOrf-10	*pk-1*	**190**	1	-	-	CL
AcOrf-11	*orf11*	**88**, 94, 216	3	*	-	EC
AcOrf-13	*orf13*	**4**, 16, 24, 85, 252	5	*	**	ECL
AcOrf-14	*lef1*	**16**	1	**	**	C
AcOrf-15	*egt*	**47**, 48, 112, 243	4	***	***	C
AcOrf-21	*arif-1*	**241**	1	**	***	EC
AcOrf-23	*Env-prot*	**37**	1	***	***	CL
AcOrf-28	*lef6*	**82**, 96	2	**	*	EC
AcOrf-32	*fgf*	**152**, 234	2	***	***	C
AcOrf-36	*39K/pp31*	**69**, 75, 113, 153, 195	5	**	**	C
AcOrf-40	*p47*	**55**	1	-	-	E
AcOrf-41	*lef12*	**198**	1	***	***	C
AcOrf-43	*orf43*	**92**	1	**	**	L
AcOrf-44	*orf44*	**93**	1	***	***	E
AcOrf-46	*odv-e66*	**9**, 10, 23, 25, 42, 76, 83, 158, 202, 223, 239, 245	12	***	***	L
AcOrf-47	*TRAX-like*	**68**	1	***	***	E
AcOrf-48	*orf48*	**18**, 27, 81, 103, 131, 159, 187, 205, 230	9	***	***	EC
AcOrf-49	*pcna*	**20**, 109, 134, 224, 257	5	***	***	E

Table 4. Continued.

Location on AcMNPV C6	Gene annotation	Serial number of mutants	Mutants amount	Transfection (Occ+)	Infection (Occ+)	Phase of transcription (motif)[a]
AcOrf-50	*lef8*	**32**, 36, 74, 80, 120, 126, 143, 201, 229	9	*	***	C
AcOrf-67	*lef3*	**40**	1	*	-	EC
AcOrf-77	*vlf-1*	61, 72, **171**	3	-	-	L
AcOrf-78	*orf78*	**46**	1	*	-	L
AcOrf-79	*orf79*	**41**	1	***	**	
AcOrf-80	*gp41*	**28**, 111, 121, 192, 233	5	*	-	EL
AcOrf-83	*orf83*	**86**, 98, 105, 118, 122, 147, 179, 236, 240, 242, 248	11	***	***	L
AcOrf-95	*helicase*	2, **6**, 7, 12, 26, 108, 141, 168, 172, 178, 181, 196, 197, 203, 204, 206, 209, 246, 251	19	-	-	EL
AcOrf-96	*orf96*	2, 7, **8**, 26, 132, 137, 154, 161, 165, 177, 213, 262	12	***	***	
AcOrf-97	*orf97*	**63**, 148	2	***	***	EC
AcOrf-98	*38K*	**101**, 110, 136, 140, 149, 155, 173, 182, 254	9	*	-	ECL
AcOrf-101	*BV/ODV-C42*	**212**, 227	2	**	-	ECL
AcOrf-103	*p48*	**73**, 237	2	**	-	ECL
AcOrf-104	*vp80*	51, **65**, 169, 199, 217, 231	6	**	-	L
AcOrf-105	*HE65*	**13**	1	***	***	CL
AcOrf-109	*orf109*	**53**, 89, 107, 130, 215	5	**	-	EL
AcOrf-114	*orf114*	**60**, 67, 70, 176, 183, 184, 207, 256	8	***	***	EL
AcOrf-115	*pif-3*	**87**	1	***	***	ECL
AcOrf-126	*chitinase*	135, **138**, 162, 166, 186, 189, 210, 219, 250, 255	10	**	***	L
AcOrf-128	*gp64*	**14**, 62, 170, 225	4	**	-	EL

Table 4. Continued.

Location on AcMNPV C6	Gene annotation	Serial number of mutants	Mutants amount	Transfection (Occ+) [a]	Infection (Occ+) [a]	Phase of transcription (motif)[b]
AcOrf-132	*orf132*	**100**	1	*	*	EL
AcOrf-133	*alk-exo*	**78**, 123, 185, 253	4	***	***	CL
AcOrf-134	*94K*	**35**, 125	2	***	***	E
AcOrf-136	*p26*	**59**	1	***	***	
AcOrf-137	*p10*	**50**	1	***	***	L
AcOrf-138	*p74*	1, **146**, 157	3	***	***	EL
AcOrf-139	*ME53*	**129**, 151, 164	3	-	-	EC
AcOrf-148	*odv-e56*	**45**	2	***	***	CL
AcOrf-151	*IE-2*	58, 106, **128**, 194, 220, 235, 249	7	**	**	
AcOrf-152	*orf152*	**163**	1	**	***	ECL
AcOrf-153	*PE38*	**33**, 64	2	**	*	ECL
AcOrf-154	*orf154*	**102**	1	***	***	L
No translational region		34, 49, 52, 54, 66, 84, 104, 114, 144, 214, 228, 232, 247, 259, 260	15			

[a] Occlusion body production after transfection and infection: * * *, much; * *, medium; *, little; -, none. Transfection, 1ug of each bacmid DNA was introduced into 1×10^6 Sf9 cells respectively, and the polyhedral production was analyzed. Infection, at 120 hpt, the culture medium from transfected sample was collected and inoculated into a new cell layer, and the polyhedral production was analyzed.

[b] Phase of transcription indicates if at least one early, TATA, CGTGC, CAGT (E) or CATG (C) motif is present the 160 nucleotides upstream of an ORF, or a late promoter motif, TAAG (L), within 80 nucleotides upstream of an ATG. The mutants used for transfection and infection were marked in bold.

Table 5. Grouping of AcMNPV gene knockout mutants. Group 1, mutants produced infectious viruses with normal infectivity; Group 2, mutants produced infectious viruses with lower infectivity; Group 3, mutants produced non-infectious viruses and polyhedron; Group 4, mutants produced non-infectious viruses but no polyhedron.

Group	**Mutants**
Group 1	AcOrf-1, AcOrf-2, AcOrf-15, AcOrf-21, AcOrf-23, AcOrf-32, AcOrf-41, AcOrf-44, AcOrf-46, AcOrf-47, AcOrf-48, AcOrf-49, AcOrf-50, AcOrf-83, AcOrf-96, AcOrf-97, AcOrf-105, AcOrf-114, AcOrf-115, AcOrf-126, AcOrf-133, AcOrf-134, AcOrf-136, AcOrf-137, AcOrf-138, AcOrf-148, AcOrf-152, AcOrf-154
Group 2	AcOrf-9, AcOrf-13, AcOrf14, AcOrf-28, AcOrf-36, AcOrf-43, AcOrf-79, AcOrf-132, AcOrf-151, AcOrf-153
Group 3	AcOrf-11, AcOrf-67, AcOrf-78, AcOrf-80, AcOrf-98, AcOrf-101, AcOrf-103, AcOrf-104, AcOrf-109, AcOrf-128,
Group 4	AcOrf-8, AcOrf-10, AcOrf-40, AcOrf-77, AcOrf-95, AcOrf-139

2. The ORF11 is essential for BV production and ODV envelopment

2. 1. Verification of *ac11* knockout, repair and control bacmids

The recombinant bacmid, Ac11KO, which the *ac11* gene was interrupted with a pUC origin (pUC ori) and an ampicillin resistance gene (*Amp*) 670-bp downstream of the predicted translational start site was generated (Fig. 10).

To confirm the phenotypes resulted from knockout of the *ac11*, a repaired bacmid, Ac11Re, in which *ac11* gene was inserted upstream of the *polyhedrin* in Ac11KO and expressed by its own promoter was also generated (Fig. 10). The internal genomic structure of the bacmids, Ac-MK, Ac11KO and Ac11Re, were verified by nucleotide sequence analysis and PCR using primers Ac11-RealTime-Fw and Ac11-RealTime-Re specific to the *ac11* gene (Fig. 11). And the nucleotide sequence analysis result confirmed that *ac11* was disrupted at the amino acid position 224 in the middle of the ORF.

RT-PCR analysis was performed to confirm the lack of *ac11* expression in Ac11KO-transfected Sf9 cells. RT-PCR using a *gp64*-specific primer set, gp64-PCR-Fw and gp64-PCR-Re, successfully amplified the corresponding gene from the cDNA of Sf9 cells transfected with Ac-MK or Ac11KO. While the single RT-PCR product of expected size was obtained from the Sf9 cells transfected with Ac-MK, no product was amplified from Ac11KO-transfected cells using *ac11*-specific primers, Ac11-RealTime-Fw and Ac11-RealTime-Re (Fig. 12). These results demonstrated that the *ac11* gene was successfully knocked-out in Ac11KO.

In addition, a *gp64* knockout bacmid, GP64KO, of which *gp64* gene was interrupted with pUC ori and *Amp* at 1013 bp downstream of the predicted start site was used as a control (Fig. 10). The internal genomic structure of GP64KO was verified by PCR

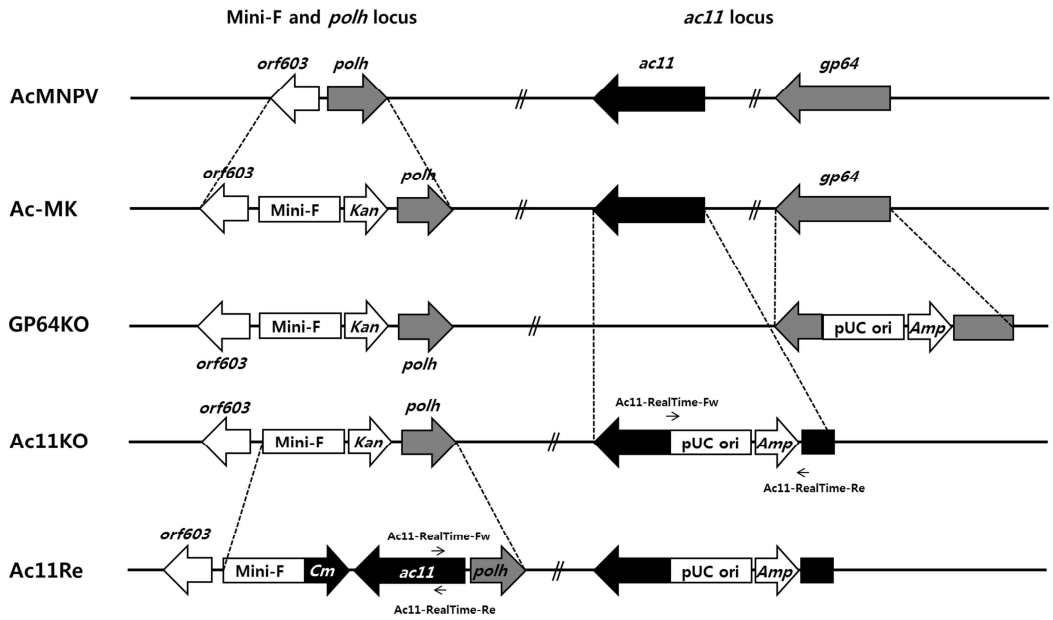

Fig. 10. Schematic diagram of Ac-MK, Ac11KO and Ac11Re viruses. The *ac11* gene was knocked-out by insertion of pUC ori and *Amp* into amino acid 224 of ac11 via Tn7-mediated transposition. The deletion of *ac11* was repaired by replacement of *Kan* with *ac11* and *Cm* via homologous recombination between pUC-19-MCP-Ac11 and Ac11KO. The *ac11* gene inserted into Ac11Re was expressed by its own promoter.

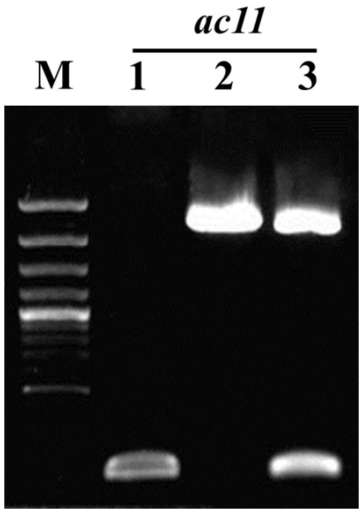

Fig. 11. PCR verification of Ac11KO and Ac11Re bacmids. Lanes: M, 100 bp Ladder; 1, Ac-MK; 2, Ac11KO; 3, Ac11Re.

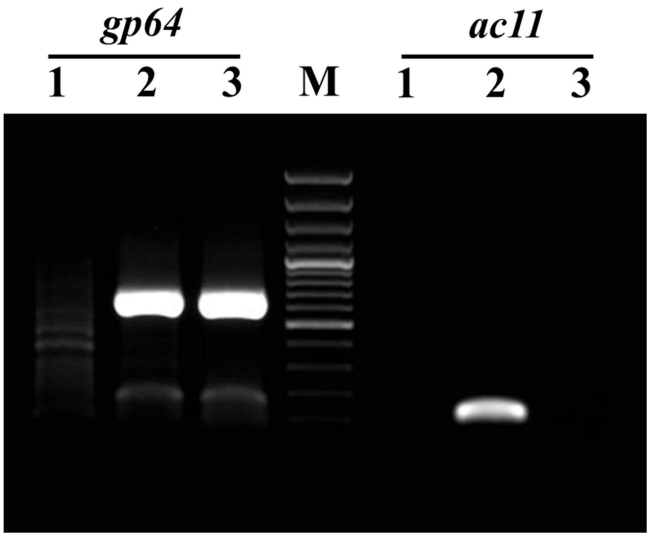

Fig. 12. RT-PCR analysis of *ac11* transcription. Total RNA was extracted from transfected Sf9 cells at 72 hpt. Lanes: M, 100-bp Ladder; 1, mock-transfected Sf9 cells; 2, Ac-MK-transfected Sf9 cells; 3, Ac11KO-transfected Sf9 cells.

analysis by using *gp64*-specific primers, gp64-PCR-Fw and gp64-PCR-Re (Fig. 13). And the nucleotide sequence analysis result confirmed that *gp64* was disrupted at the amino acid position 338 in the middle of the ORF.

RT-PCR analysis was performed to confirm the lack of *gp64* gene expression in GP64KO-transfected Sf9 cells. RT-PCR using a vp39-specific primer set, AcVP39-F (5'-CTGCATTTTCGCGTCCA-3') and AcVP39-R (5'-TGGTCACGTCAAAGAAA-3'), successfully amplified the corresponding gene from the cDNA of Sf9 cells transfected with Ac-MK or GP64KO. While the single RT-PCR product of expected size was obtained from the Sf9 cells transfected with Ac-MK, no product was amplified from GP64KO-transfected cells using a *gp64*-specific primer (Fig. 14). These results demonstrated that the *gp64* gene was successfully knocked-out in GP64KO.

2. 2. Transcriptional analysis of *ac11*

Temporal transcription of the *ac11* during viral replication was investigated using qPCR (Fig. 15). Transcript of the *ac11* was detected as early as 2 hpi in Ac-MK infected Sf9 cells, accumulated to its maximum level until 48 hpi and remained detectable up to 72 hpi. This result suggested that *ac11* is an early gene which coincides with the fact that the *ac11* possesses CAGT early promoter motifs at its putative promoter region. In contrast, no *ac11* transcript was detected at any time point in Sf9 cells infected with Ac11KO, which resulted from the knockout of the corresponding gene in Ac11KO.

2. 3. BV production was defeated by the deletion of *ac11*

To determine the effect of *ac11* deletion on virus replication, Sf9 cells were separately transfected and infected with Ac-MK, Ac11KO or Ac11Re respectively.

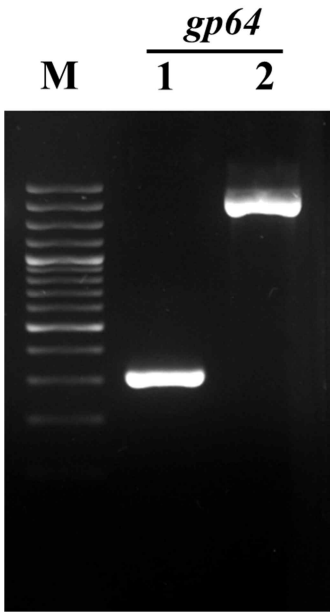

Fig. 13. PCR verification of GP64KO. Lanes: M, 100 bp Ladder; 1, Ac-MK; 2, GP64KO. The *gp64*-specific primers gp64-PCR-Fw and gp64-PCR-Re were used.

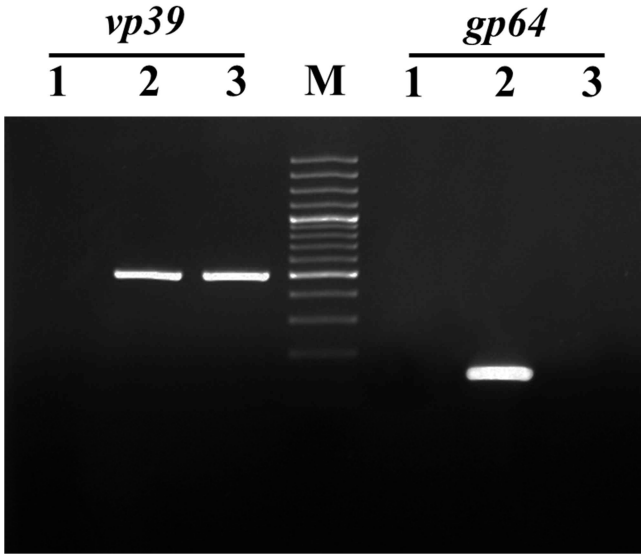

Fig. 14. RT-PCR analysis of *gp64* transcription. Total RNA was extracted from transfected Sf9 cells at 72 hpt. Lanes: M, 100-bp Ladder; 1, mock-transfected Sf9 cells; 2, Ac-MK-transfected Sf9 cells; 3, GP64KO-transfected Sf9 cells.

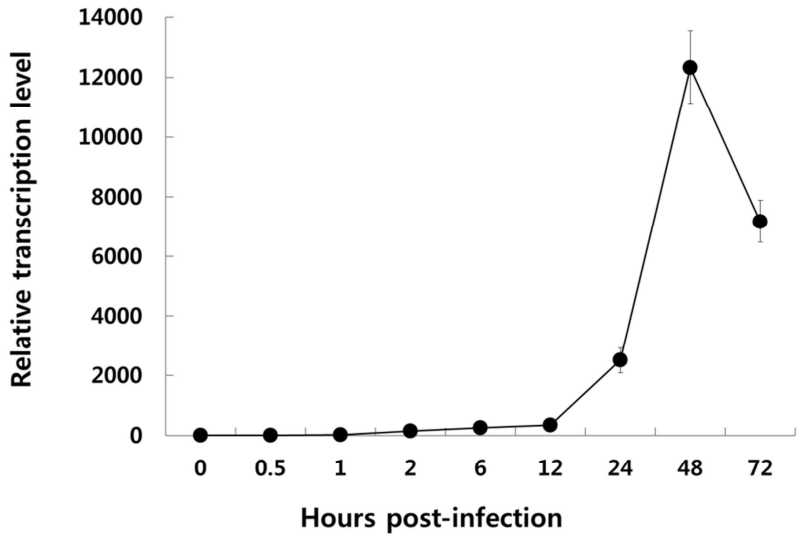

Fig. 15. Transcription of *ac11* in Ac-MK infected Sf9 cells. Total RNA was extracted from Sf9 cells infected with Ac-MK at 0, 0.5, 1, 2, 6, 12, 24, 48 and 72 hpi and subjected to qPCR analysis using *ac11*-specific primer set.

Microscopy analysis revealed no difference among the three viruses at 24 hpt. At 48 hpt, only a small proportion of the cells contained OBs. By 72 hpt, significant differences were observed between Ac-MK- and Ac11Re-transfected cells and Ac11KO-transfected cells. While a large proportion of the Ac-MK- and Ac11Re-transfected Sf9 cells contained OBs, the number of the Ac11KO-transfected cells containing OBs did not increase. Furthermore there was no any Ac11KO-infected cell containing OBs until 72 hpi (Fig. 16). These results suggested that the deletion of *ac11* leads to a defect in the production of infectious BV progeny in Sf9 cells.

To better define the effect of lacking *ac11* on virus replication and investigate the replication kinetics of the virus constructs, BV levels were analyzed using a 50% tissue culture infective dose ($TCID_{50}$) and qPCR. For these, Sf9 cells were transfected with the Ac-MK, Ac11KO or Ac11Re bacmid DNA respectively, and the BV titers were determined by $TCID_{50}$ end-point dilution at selected time points. While Sf9 cells transfected with Ac-MK or Ac11Re displayed a normal increase in BV production that reached equivalent titers, no BV was detectable for Ac11KO-transfected cells at any time point tested, indicating no infectious virus was produced (Fig. 17). These results suggested that *ac11* is required for infectious BV production in Sf9 cells.

The $TCID_{50}$ end-point dilution assay only determines the production of infectious BV and cannot detect if any non-infectious BVs are produced. To address this, the BV titers were also assayed by qPCR, which determines BV titers by detecting viral genomes regardless of infectivity (Fig. 18), whereas Ac-MK- and Ac11Re-transfected cells revealed a steady increase in BV production, only a background level of viral genomes resulting from initially transfected bacmid DNA was detected up to 120 hpt in Ac11KO-

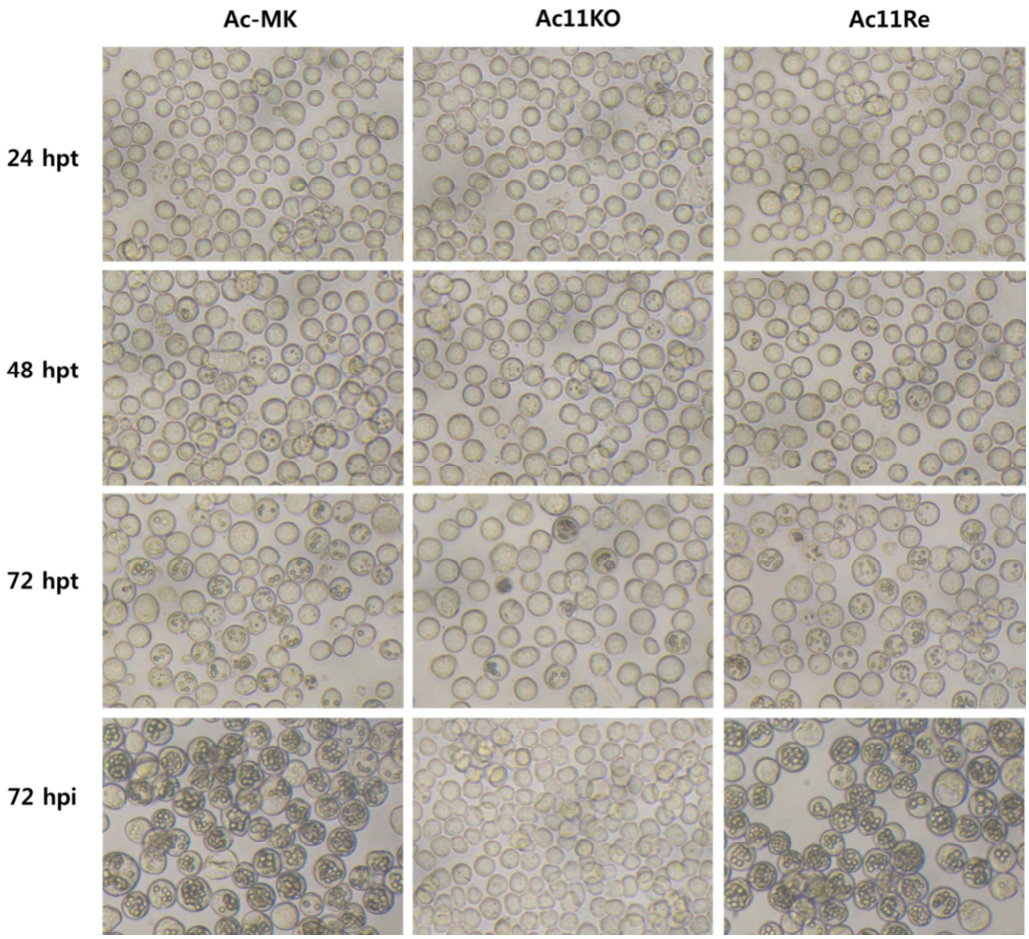

Fig. 16. Light microscopy of Sf9 cells transfected or infected with Ac-MK, Ac11KO or Ac11Re respectively. 1×10^6 Sf9 cells were transfected with 1 μg of Ac-MK, Ac11KO, or Ac11Re bacmid DNA. 500 μl supernatants from transfected Sf9 cells were used to infect 1×10^6 Sf9 cells. At the designated time points, transfected and infected Sf9 cells were observed under light microscope.

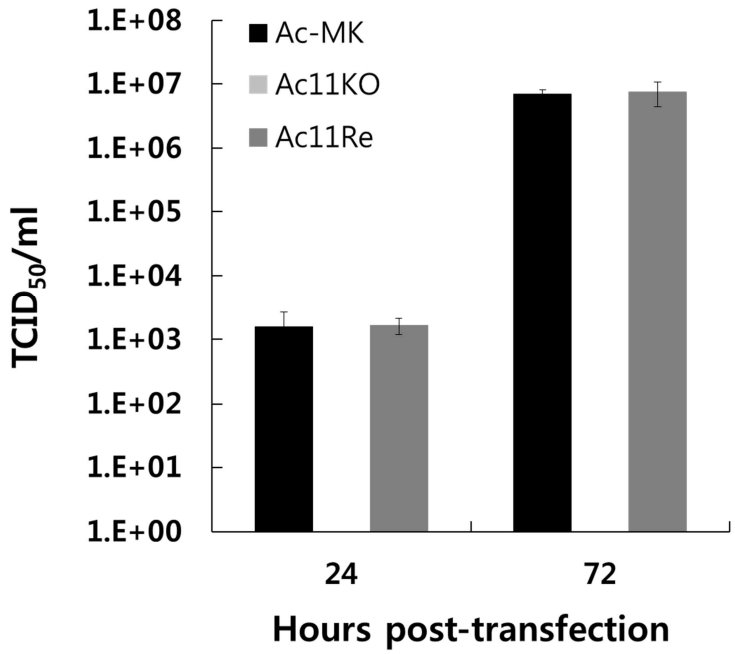

Fig. 17. BV production analysis of Ac11KO transfected Sf9 cells by $TCID_{50}$ end-point dilution assay. The extracellular BV titers at 24 and 72 hpi were determined by $TCID_{50}$ end-point dilution from Sf9 cells separately transfected with Ac-MK, Ac11KO and Ac11Re. Each point represents the average titer derived from three independent $TCID_{50}$ assays. Error bars represent standard deviation.

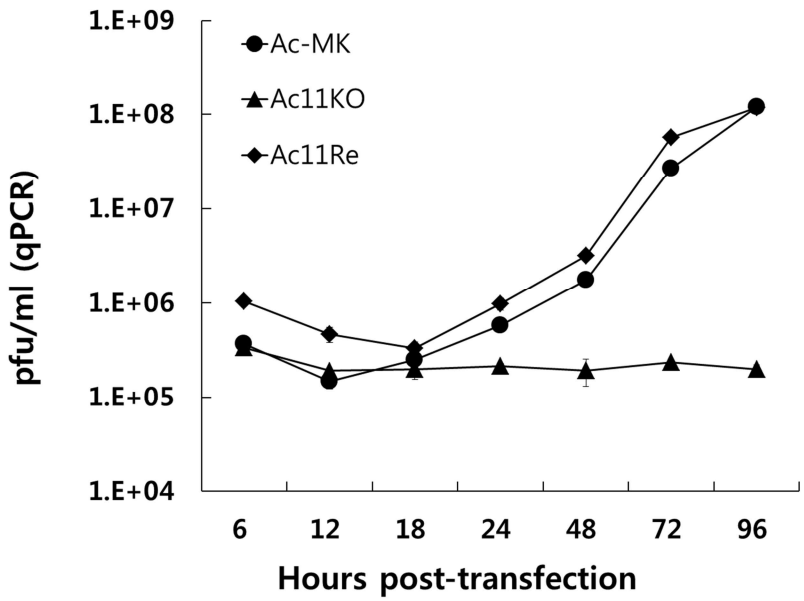

Fig. 18. BV production analysis of Ac11KO transfected Sf9 cells by qPCR. The virus titer, regardless virion infectivity, was determined by qPCR analysis of supernatants of Sf9 cells transfected with Ac-MK, Ac11KO or Ac11Re at the designated time points. Results are from three separate transfections and the error bars represent the standard deviation.

transfected cells.

An additional assay was performed to confirm the absence of BV production in Ac11KO-transfected cells. Western blotting using the BVs purified from Ac-MK-, Ac11KO- and Ac11Re-transfected cell supernatants were performed to compare the levels of nucleocapsid protein VP39 (Fig. 19). Whereas the VP39 was detected in the Ac-MK- and Ac11Re-transfected cells supernatants, this nucleocapsid protein was detected in the Ac11KO-transfected cells supernatants. These results suggested that the deletion of *ac11* results in a viral phenotype incapable of producing BV.

2. 4. Viral DNA replication was not affected by the deletion of *ac11*

To determine whether *ac11* is required for viral DNA replication, a qPCR analysis was performed to investigate the level of viral DNA replication in Ac-MK, Ac11KO, and Ac11Re-transfected cells (Fig. 20). A *gp64* gene knocked-out bacmid, GP64KO, used as a non-infectious control as the deletion of *gp64* results in a virus unable to propagate infection from cell to cell and could provide a more accurate comparison (Oomens & Blissard, 1999; Vanarsdall *et al.*, 2006). Equal amounts of transfected cells were collected at designated time points, and total DNA was extracted from the cell lysates and subjected to qPCR. The results showed that both Ac11KO and GP64KO could synthesize similar levels of nascent DNA compared with Ac-MK up to 18 hpt, suggesting that the onset and level of viral DNA replication in the initially transfected cells are not affected by deletion of *ac11*. However,in spite of the levels of DNA replication of Ac-MK and Ac78Re increased until 24 hpt correlating with the spread of the infection from the

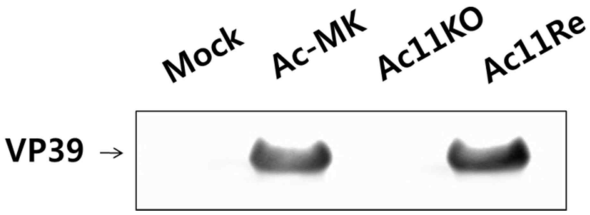

Fig. 19. Western blot analysis of BV from Ac11KO transfected Sf9 cells. BV isolated from Sf9 cells transfected with Ac-MK, Ac11KO or Ac11Re at 72 hpt. The blots were probed with a monoclonal antibody specific for VP39 nucleocapsid protein.

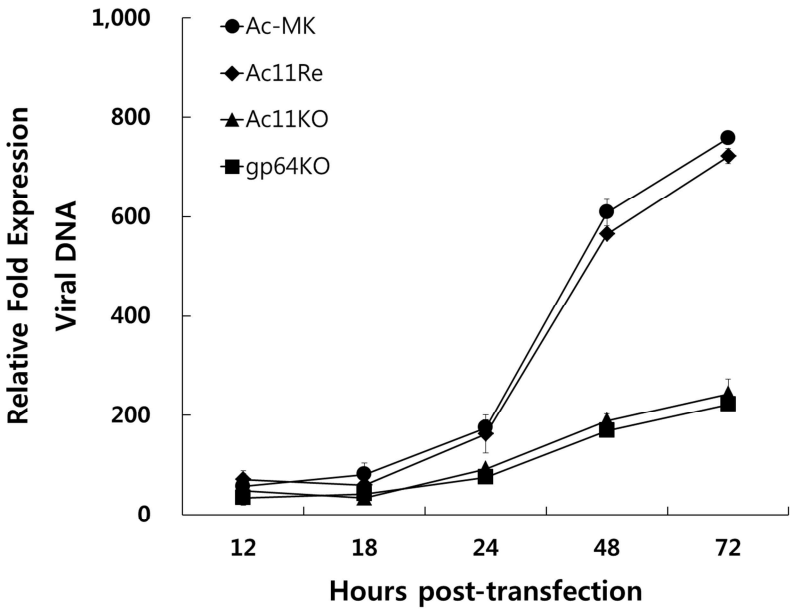

Fig. 20. Effect of ac11 on viral DNA replication. 1×10^6 Sf9 cells were transfected with 1 μg of Ac-MK, Ac11KO, Ac11Re, or GP64KO bacmids. At the designated time points, total cellular DNA was isolated from each virus bacmid DNA-transfected Sf9 cells, digested with the restriction enzyme *Dpn*I to eliminate input bacmid, and analyzed by qPCR. The results are from three separate transfections and the error bars represent the standard deviation.

production of BV, the DNA replication levels of Ac11KO and GP64KO did not increase until 72 hpt which correlates with the absence of BV production in the transfected cells.

2. 5. Substructural localization of Ac11 in purified BV and ODV

To confirm whether Ac11 is an ODV or BV structural protein, BV and ODV were purified from Sf9 cells infected with Ac-MK and analyzed by Western blot (Fig. 21). In addition, the biochemically fractionated nucleocapsid and envelope fractions of the BV and ODV particles were also analyzed by Western blot. The results of the analyses indicated that Ac11 is not a structural protein of BV or ODV either. As a control to confirm the efficiency of the BV fractionation, the nucleocapsid protein VP39 and the BV envelope-specific protein GP64 were analyzed by Western blotting. Both proteins were observed in the expected fractions.

2. 6. Electron microscopy analysis of Ac-MK, Ac11KO or Ac11Re-transfected cells

To further analyze whether the deletion of *ac11* has any effect on virus morphogenesis, electron microscopic analysis was performed with thin sections generated from cells transfected with Ac-MK, Ac11KO or Ac11Re (Fig. 22 and 23). At 24 hpt, cells transfected with Ac-MK, Ac11KO or Ac11Re exhibited the typical baculovirus infection symptoms, including enlarged nuclei, a typically reorganized electron-dense virogenic stroma (Fig. 22a, b and c) and rod-shaped nucleocapsids associated with the electron-dense edges of the virogenic stroma. The virogenic stroma is the active site for viral DNA replication, condensation, and packaging into capsids (Fraser, 1986; Young *et al.*, 1993). In Ac-MK- and Ac11Re-transfected cells, most nucleocapsids were mainly

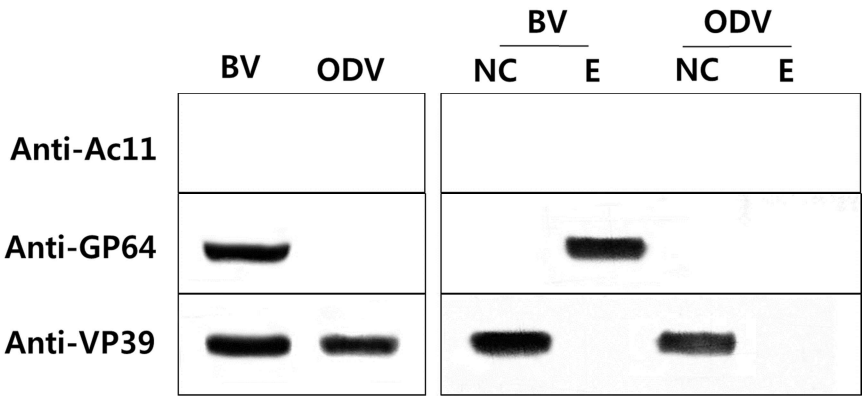

Fig. 21. Western blot analysis of Ac11 in purified and fractionated virions. BV and ODV were purified using a sucrose gradient ultracentrifugation and analyzed by SDS-PAGE and Western blotting. The blots were probed individually with an anti-Ac11 antibody to detect Ac11, AcV5 monoclonal antibody to detect the BV envelope protein GP64, and anti-VP39 to detect the nucleocapsid protein VP39. BV, budded virus; ODV, occlusion-derived virus; NC, nucleocapsid fraction; E, envelope fraction. GP64 and VP39 were analyzed to confirm correct fractionation of the BV particle in envelope and nucleocapsid fractions, respectively.

concentrated in the nucleus, but a significant number of nucleocapsids were observed in the cytoplasm and budding at the plasma membrane (Fig. 22d, and f). In contrast, the nucleocapsids were consistently observed only in the nucleus in the Ac11KO transfected cells observed up to 72 hpt (Fig. 22e). In addition, no nucleocapsid was observed in cytoplasm or budding at the plasma membrane (Fig. 22e). At 72 hpt, the nucleocapsids associated with edges of the virogenic stroma, forming bundles, aligning with *de novo* developed nuclear envelopes and acquiring envelopes, were observed in both Ac-MK- or Ac11Re-transfected cells. These enveloped virions contain multiple nucleocapsids prior to occlusion in the protein crystalline matrix of the developing OBs (Fig. 23a and c), and OBs containing numerous enveloped virions were observed in the ring zone (Fig. 23d and f). While bundles of nucleocapsids and masses of electron-lucent tubular structures were also appeared at the electron-dense edges of the stroma in Ac11KO-transfected cells, none of them were enveloped to form ODVs (Fig. 23b).

In addition, ODVs were not embedded in the OBs, although their shape and the size were similar to those of Ac-MK and Ac78Re (Fig. 23e). These observations indicated that deletion of *ac11* did not abolish nucleocapsid or OB morphogenesis, but affected the formation of normal BV and ODV and subsequent embedding of ODVs into OBs.

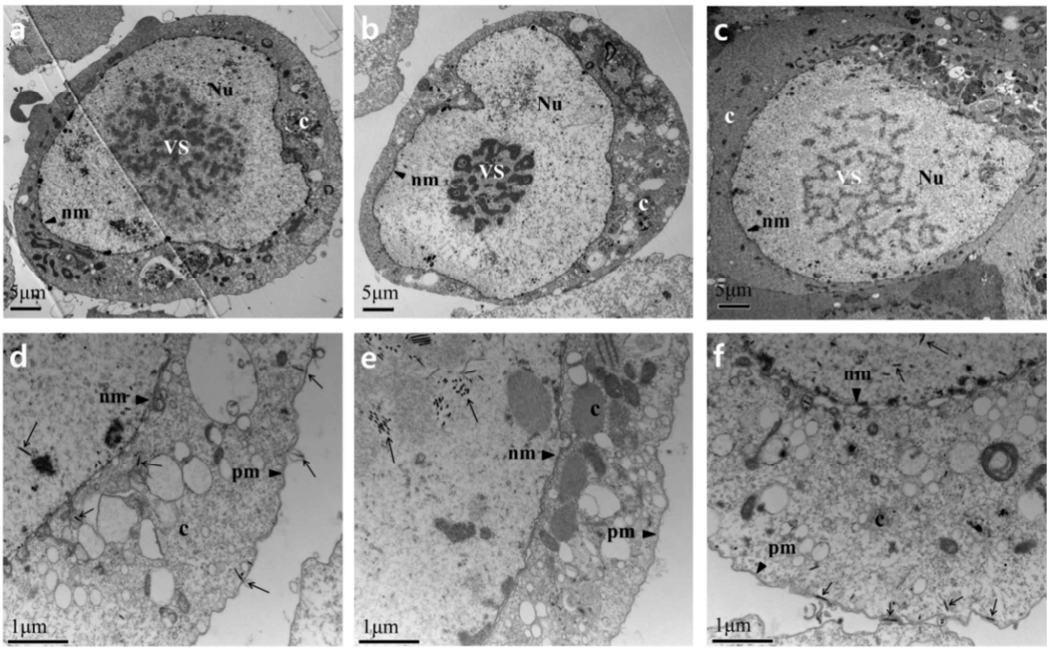

Fig. 22. Transmission electron microscopy analysis of Ac11KO transfected Sf9 cells. Sf9 cells transfected with Ac-MK (a, d), Ac11KO (b, e) or Ac11Re (c, f) at 24 hpt. Image showing enlarged nucleus (Nu) and virogenic stroma (VS) in the Ac-MK-, Ac11KO-, or Ac11Re-transfected cells (a, b, c). The higher magnification micrographs of Ac-MK- or Ac11Re-transfected cells showed normal nucleocapsids residing in the cytoplasm (c) and budding from the plasma membrane (pm) (arrows) (d and f). In Ac11KO-transfected cells, nucleocapsids (arrows) were observed in the nucleus, but no nucleocapsids were observed in the cytoplasm (e).

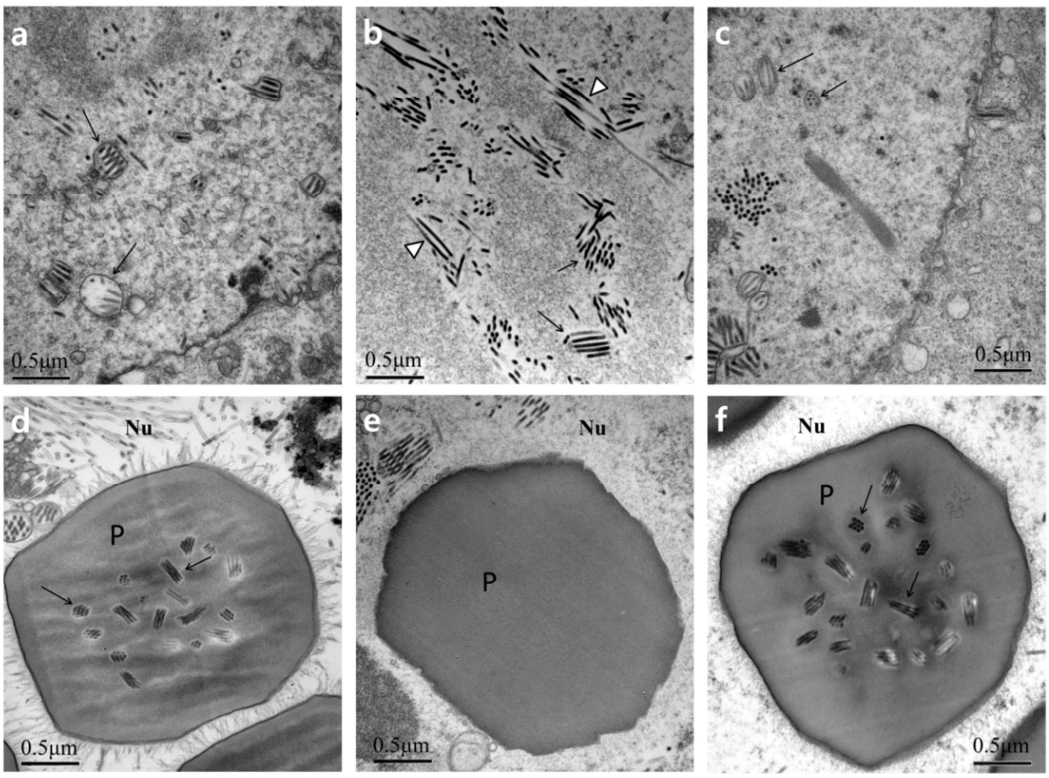

Fig. 23. Nucleocapsids envelopment and OB morphogenesis of Ac11KO transfected Sf9 cells. In Ac-MK- or Ac11Re-transfected cells, normally enveloped virions containing multiple nucleocapsids (arrows) (a and c) were embedded within the OBs (P) (arrows) (d and f). In Ac11KO-transfected cells, the bundles of nucleocapsids (arrows) and masses of electron-lucent tubular structures (white triangle) appeared at the electron-dense edges of the stroma (b), and no normal virions were embedded in the OBs (e).

3. Functional characterization of ORF43 and phenotypic changes of ORF43-knockout mutant

3. 1. Generation of *ac43* knockout and complementary virus

In the recombinant bacmid, Ac43KO, the *ac43* gene was interrupted with pUC ori and *Amp* (Fig. 24). The genomic structure of the Ac43KO bacmid was verified by PCR using *ac43*-specific primers ORF43-RTF and ORF43-RTR (Fig. 25), and nucleotide sequence analysis result confirmed that *ac43* was disrupted at the amino acid position 41 in the middle of the ORF. In addition, a complementary bacmid, AcPolhKO in which the *polyhedrin* gene was knocked-out by insertion of pUC ori-*Amp* donor cassette into *polyhedrin* locus, was used as a complementary virus (Fig. 24).

RT-PCR analysis was performed to confirm the nullified *ac43* expression in Ac43KO-infected Sf9 cells (Fig. 26). RT-PCR using the *gp64*-specific primer pair successfully amplified the corresponding gene from cDNAs of Sf9 cells infected with Ac-MK or Ac43KO, indicating that both of the viruses replicated in Sf9 cells. While a single RT-PCR product of the expected size was obtained from the Sf9 cells infected with Ac-MK, no product was amplified from Ac43KO-infected cells using the *ac43*-specific primer set. These results demonstrated that the *ac43* gene was successfully knocked-out from the Ac43KO.

3. 2. Transcriptional analysis of *ac43*

Temporal expression of the *ac43* gene during viral replication was investigated using qPCR (Fig. 27). In the Sf9 cells infected with Ac-MK, the transcription of *ac43* started at 6 hpi, continued to increase until 48 hpi and then slightly declined by 72 hpi time point,

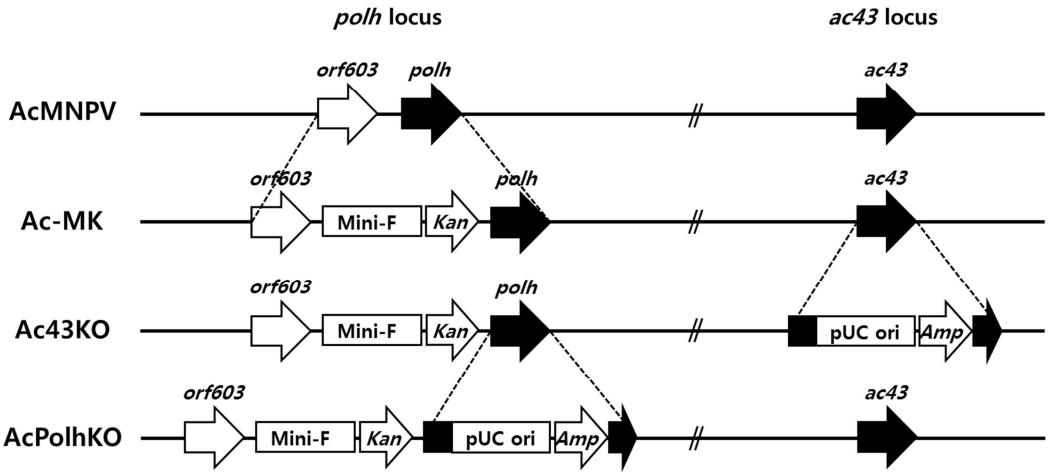

Fig. 24. Schematic diagram of Ac43KO and AcPolhKO bacmids. The pUC ori and *Amp* were inserted at amino acid 41 of *ac43* gene.

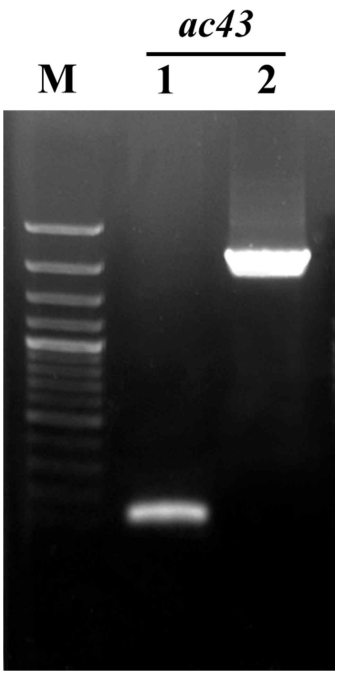

Fig. 25. Verification of *ac43* knockout virus by PCR. Lanes: M, 100 bp Ladder; 1, Ac-MK; 2, Ac43KO. The *ac43*-specific primers ORF43-RTF and ORF43-RTR were used.

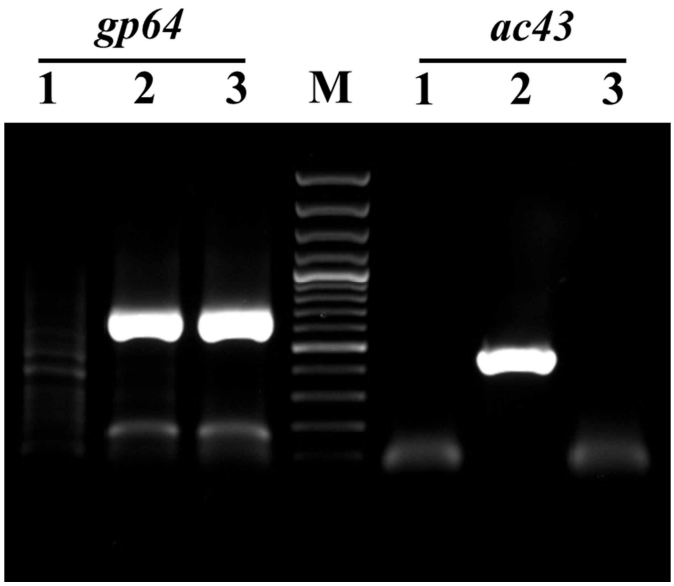

Fig. 26. RT-PCR analysis of *ac43* transcription. Sf9 cells were infected at an MOI of 10, and total RNA was extracted at 72 hpi. Lanes: M, 100 bp Ladder; 1, mock-infected Sf9 cells; 2, Ac-MK-infected Sf9 cells; 3, Ac43KO-infected Sf9 cells.

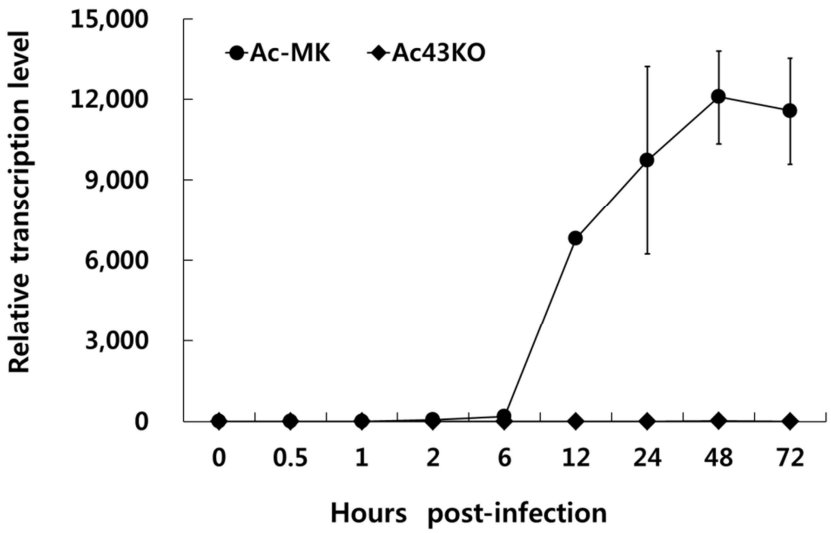

Fig. 27. Transcription of *ac43* in Ac-MK and Ac43KO infected Sf9 cells. Total RNA was extracted from Sf9 cells infected with Ac-MK or Ac43KO at 0, 0.5, 1, 2, 6, 12, 24, 48, and 72 hpi and subjected to qPCR analysis using an *ac43*-specific primer set.

indicating that ac43 belongs to the late gene category. In contrast, no ac43 transcripti on was detected at any time points in the Sf9 cells infected with Ac43KO as no fun ctional copy of this gene was present on this bacmid.

3. 3. Effect of *ac43* deletion on BV and OB production

To examine the effects of *ac43* deletion on BV production, Sf9 cells were infected with Ac-MK or Ac43KO at an MOI of 1.0, and the resulting cell culture supernatant at selected time points were subjected to extra-cellular BV titration (Fig. 28). The result revealed that there were no significant differences in BV production between Ac-MK and Ac43KO indicating that *ac43*-knockout virus is capable of infectious progeny virus production, and *ac43* does not play a role in viral replication in infected cells.

The effect of *ac43* deletion on OB production in Sf9 cells was investigated and the result showed that OB production was markedly reduced in Ac43KO-infected Sf9 cells (Fig. 29). Because *polyhedrin* is the major component of OBs, the effect of *ac43* deletion on *polyhedrin* gene expression was also characterized. The qPCR analysis showed that the reduced production of OB was caused by the down-regulation of *polyhedrin* expression (Fig. 30). While the expression of *polyhedrin* started at around 12 hpi and kept increasing until 48 hpi in both Ac-MK- and Ac43KO-infected Sf9 cells, the expression level of polyhedrin was reduced by approximately 50% in Ac43KO-infected Sf9 cells than that of Ac-MK-infected Sf9 cells at all time-points evaluated. These results suggest that the deletion of *ac43* causes a reduction in the level of *polyhedrin* expression and thereby also reduces OB production in Sf9 cells.

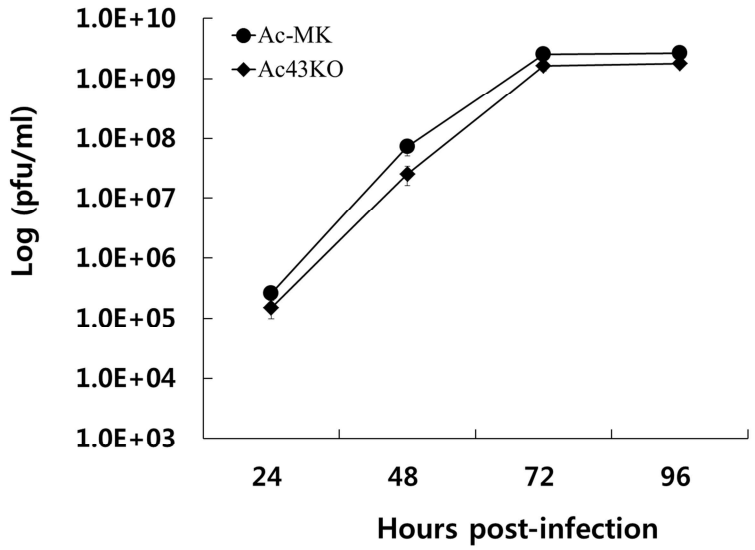

Fig. 28. BV production analysis of Ac-MK and Ac43KO transfected Sf9 cells by $TCID_{50}$ end-point dilution assay. 1×10^6 Sf9 cells were infected with Ac-MK or Ac43KO at an MOI of 1.0. The extracellular BV titers at 24, 48, 72, and 96 hpi were determined by end-point dilution assay. The error bars indicate one standard deviation (n=3).

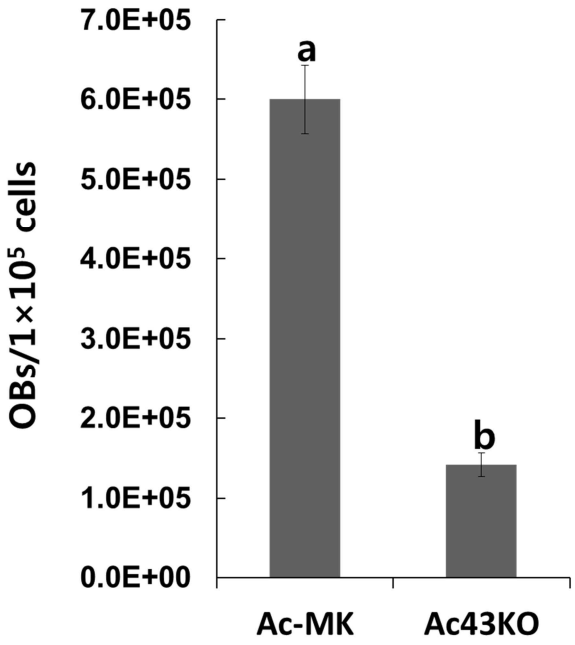

Fig. 29. OB production in Ac-MK and Ac43KO infected Sf9 cells OB production. 1×10^5 Sf9 cells were infected with the virus at an MOI of 10. Infected cells were harvested at 96 hpi and lysed, and three independent polyhedra counts were made using a hemocytometer. Different letters above the error bars (indicating standard deviation, n=3) indicate significant difference by Duncan's multiple range test ($p<0.05$).

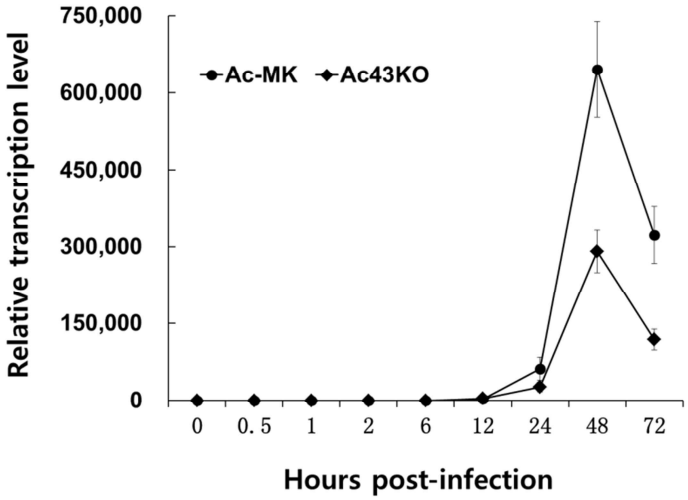

Fig. 30. Effect of *ac43* on polyhedrin expression in Sf9 cells. Total RNA was extracted from Sf9 cells infected with Ac-MK or Ac43KO at 0, 0.5, 1, 2, 6, 12, 24, 48, and 72 hpi, and subjected to qPCR analysis using a polyhedrin-specific primer set.

3. 4. Effect of *ac43* deletion on OB morphogenesis

The Sf9 cells infected with Ac-MK or Ac43KO were examined by microscopy to detect any effect of the *ac43* deletion on viral morphogenesis. Surprisingly, Sf9 cells infected with Ac43KO produced OBs were much larger in size (3.9 ± 0.1 μm) than those from Sf9 cells infected with Ac-MK (2.7 ± 0.1 μm) (Fig. 31). There were no other differences in the external morphology between OBs from Sf9 cells infected with Ac-MK and Ac43KO. When 2.5×10^5 OBs were analyzed by SDS-PAGE, a much larger amount of polyhedrin protein from OBs of Ac43KO was present compared with OBs of Ac-MK (Fig. 32). These results indicated that the size of Ac43KO-derived OBs were significantly larger than Ac-MK-derived OBs.

To further study whether the deletion of *ac43* affects assembly and occlusion of ODVs into OBs, the internal structure of OBs produced from Sf9 cells infected with Ac-MK or Ac43KO was investigated using transmission electron microscopy. Whereas ODVs occluded in Ac-MK-derived OBs showed the normal multiple nucleocapsids phenotype, a comparatively larger number of ODVs with single nucleocapsid phenotype were occluded in the Ac43KO-derived OBs, although ODVs with multiple nucleocapsids were also occluded (Fig. 33A and B). When the deletion of *ac43* was complemented by co-infection of Ac43KO with AcPolhKO, only ODVs with multiple nucleocapsids were occluded in OBs (Fig. 33C). These results suggest that *ac43* is involved in multiple nucleocapsid packaging in ODV assembly.

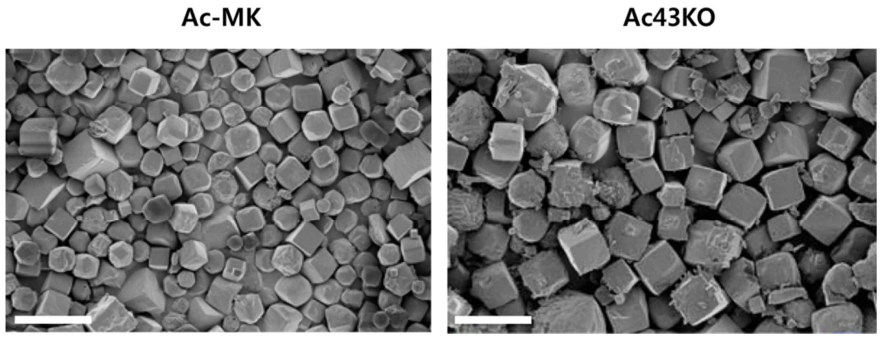

Fig. 31. Effect of *ac43* on OB morphogenesis. Scanning electron microscopy of OBs produced from Sf9 cells infected with Ac-MK or Ac43KO (scalebar = 10 μm). The average polyhedra sizes of Ac-MK and Ac43KO were 2.7 ± 0.1 μm and 3.9 ± 0.1 μm, respectively.

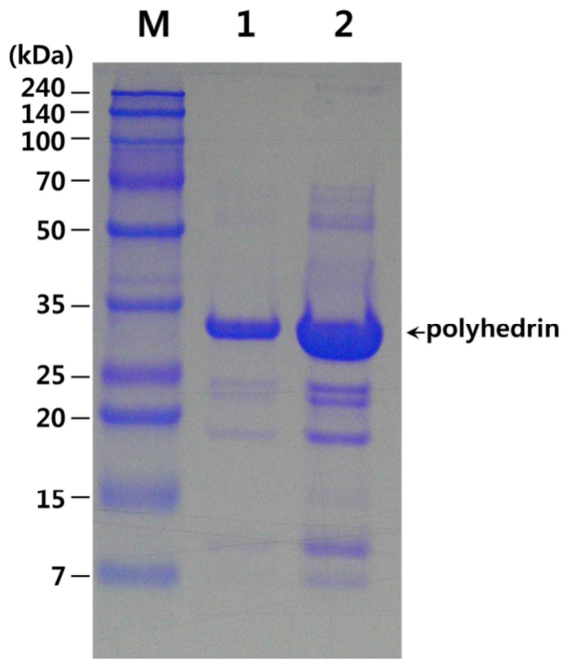

Fig. 32. SDS-PAGE analysis of OBs produced from Ac-MK and Ac43KO infected cells. 2.5×10^5 OBs purified from Sf9 cells infected with Ac-MK or Ac43KO. Lanes: M, protein molecular weight marker; 1, Ac-MK-derived OBs; 2, Ac43KO-derived OBs.

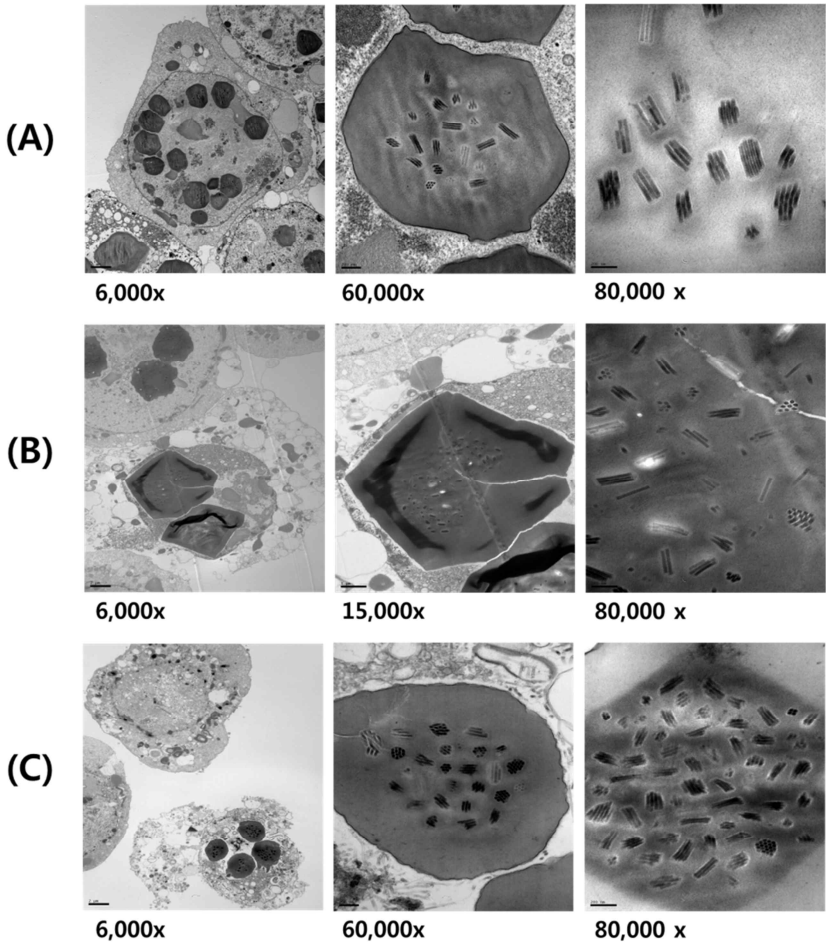

Fig. 33. Effect of *ac43* on ODV assembly. (A) Transmission electron microscopy of Sf9 cells infected with Ac-MK. (B) Transmission electron microscopy of Sf9 cells infected with Ac43KO. (C) Transmission electron microscopy of Sf9 cells co-infected with Ac43KO and AcPolhKO.

4. The ORF78 is essential for BV production and general occlusion body formation

4. 1. Verification of *ac78* knockout and repair bacmids

In the recombinant bacmid, Ac78KO, the *ac78* gene was interrupted with pUC ori and *Amp* at 261-bp downstream of the predicted translational start site (Fig. 34). The genomic structure of the Ac78KO bacmid was verified by PCR using *ac78*-specific primers (Fig. 35), and nucleotide sequence analysis result confirmed that *ac78* was disrupted at the amino acid position 87 in the middle of the ORF.

RT-PCR analysis was performed to confirm the lack of *ac78* expression in GP64KO-transfected Sf9 cells (Fig. 36). RT-PCR using a *gp64*-specific primer set successfully amplified the corresponding gene from the cDNA of Sf9 cells transfected with Ac-MK or Ac78KO. While the single RT-PCR product of expected size was obtained from the Sf9 cells transfected with Ac-MK, no product was amplified from Ac78KO-transfected cells using an *ac78*-specific primer set.

To confirm the phenotypes resulted from knockout of the *ac78*, a repaired bacmid, Ac78Re, was used. In this bacmid, *ac78*, expressed by its own promoter, was inserted upstream of the *polyhedrin* in Ac78KO (Fig. 34). The internal genomic structure of Ac78Re was verified by PCR and nucleotide sequence analysis.

4. 2. Transcriptional analysis of *ac78*

The consensus sequence of the baculovirus late gene promoter motif, TAAG, was found at 8 nt and 89 nt upstream of the translation initiating codon, ATG, of the *ac78*, indicating that this gene might belong to late gene category. Temporal expression of the *ac78* during viral replication was investigated using qPCR (Fig. 37). In Sf9 cells infected

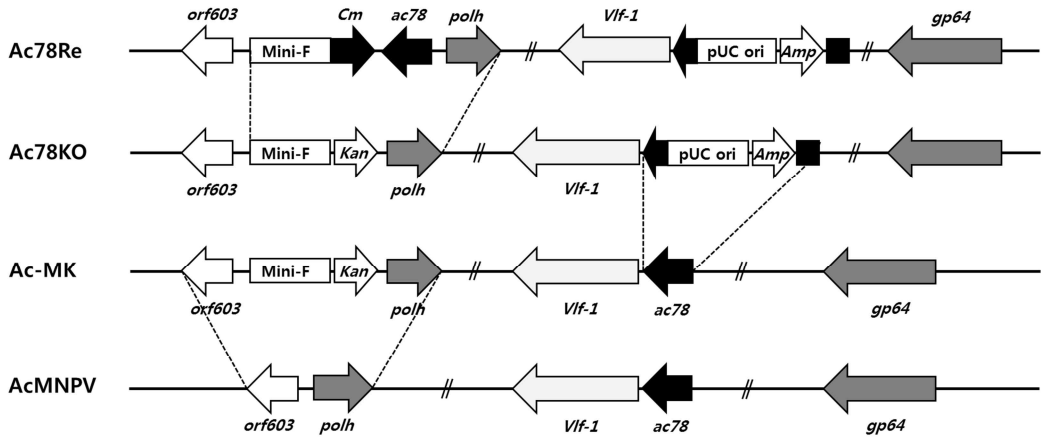

Fig. 34. Schematic diagram of Ac78KO, and Ac78Re bacmids. The *ac78* gene was knocked-out by insertion of pUC ori and *Amp* into amino acid 87 of AC78 via Tn7-mediated transposition. The deletion of *ac78* was repaired by replacement of *Kan* with *ac78* and *Cm* via homologous recombination between pUC-19-MCP-Ac78 and Ac78KO. The *ac78* gene was inserted into Ac78Re is driven by its own promoter.

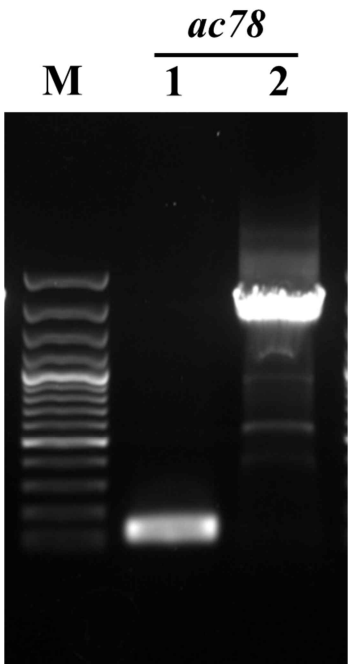

Fig. 35. PCR analysis of *ac78* knockout virus. Lanes: M, 100 bp Ladder; 1, Ac-MK; 2, Ac78KO. The *ac78*-specific primers were used.

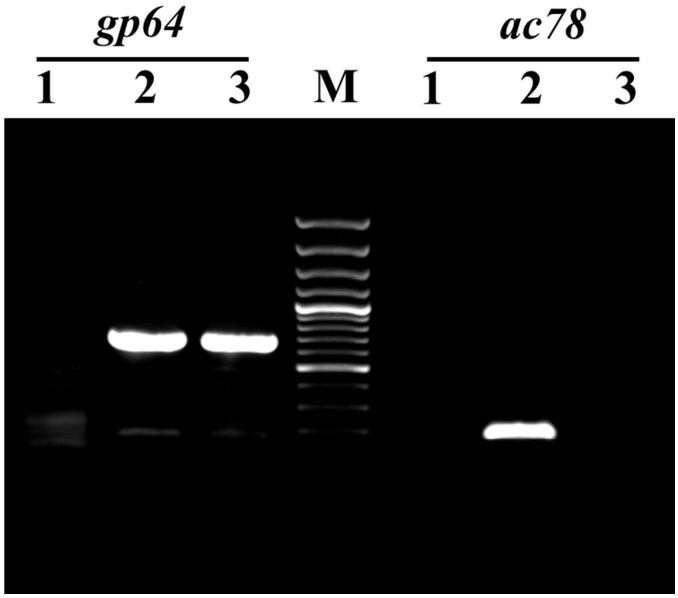

Fig. 36. RT-PCR analysis of *ac78* transcription. Total RNA was extracted from transfected Sf9 cells at 72 hpt. Lanes: M, 100-bp Ladder; 1, mock-transfected Sf9 cells; 2, Ac-MK-transfected Sf9 cells; 3, Ac78KO-transfected Sf9 cells.

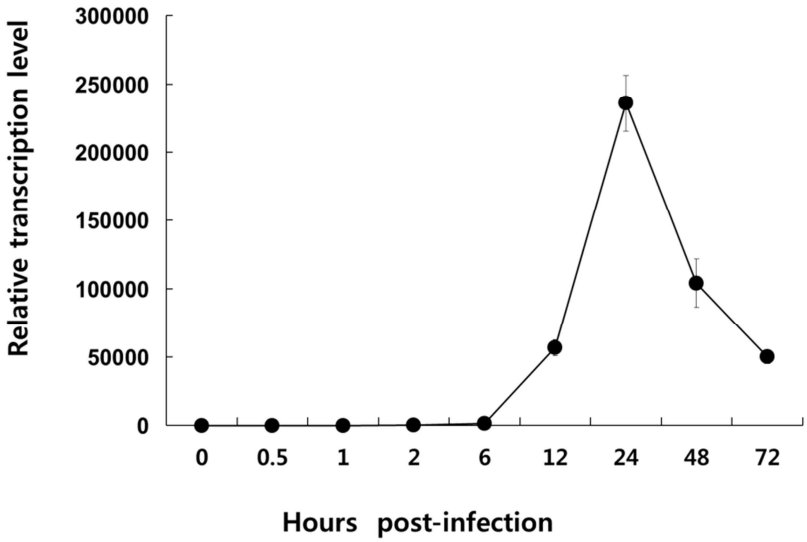

Fig. 37. Transcription of *ac78* in Ac-MK infected Sf9 cells. Total RNA was extracted from Sf9 cells infected with Ac-MK at 0, 0.5, 1, 2, 6, 12, 24, 48 and 72 hpi and subjected to qPCR analysis using *ac78*-specific primer set. The 28S rRNA was used as a reference gene.

with the Ac-MK, transcription of *ac78* started at 6 hpi. Transcription of *ac78* continued to increase until 24 hpi and then slightly declined up to 72 hpi. In contrast, no *ac78* transcript was detected at any time point in Sf9 cells infected with Ac78KO, which resulted from the knockout of the corresponding gene in Ac78KO.

4. 3. Effect of *ac78* deletion on viral replication in transfected Sf9 cells

To determine the effect of *ac78* deletion on viral replication, Sf9 cells were separately transfected with Ac-MK, Ac78KO or Ac78Re. Light microscopy analysis revealed no difference among these three viruses at 24 hpt. At 48 hpt, only a small proportion of the cells contained occlusion bodies (OBs), and the number of transfected cells that contained OBs was not significantly different among the three viruses (Fig. 38). By 96 hpt, significant differences were observed between Ac-MK- and Ac78Re-transfected cells and Ac78KO-transfected cells. A large proportion of the Ac-MK- and Ac78Re-transfected Sf9 cells contained OBs, whereas the number of the Ac78KO-transfected cells containing OBs did not increase (Fig. 38).

To better define the effect of lacking *ac78* on virus replication and investigate the replication kinetics of the virus constructs, BV levels were analyzed using $TCID_{50}$ and qPCR. Sf9 cells were transfected with the Ac-MK, Ac78KO or Ac78Re bacmid DNA, separately, and the BV titers were determined by $TCID_{50}$ end-point dilution at selected time points. Whereas Sf9 cells transfected with Ac-MK or Ac78Re displayed a normal increase in BV production that reached equivalent titers, no BV was detectable for Ac78KO-transfected cells at any time point tested, indicating no infectious virus was produced (Fig. 39).

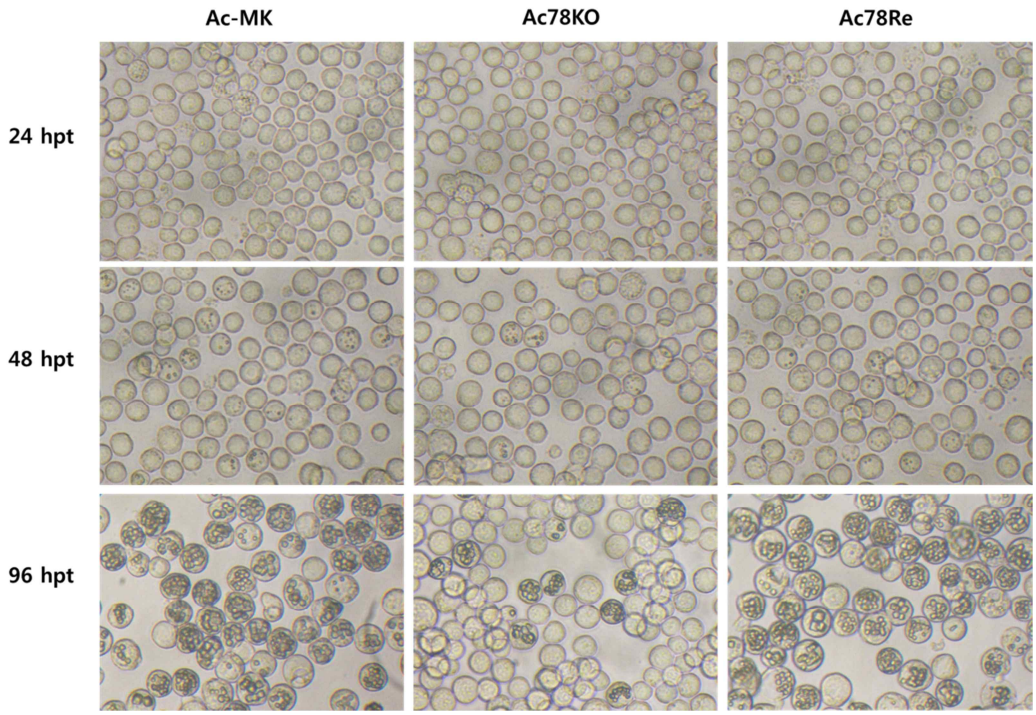

Fig. 38. Light microscopy of Ac-MK, Ac78KO or Ac78Re-transfected Sf9 cells. 1×10^6 Sf9 cells were transfected with 1 μg of Ac-MK, Ac78KO, or Ac78Re bacmid DNA. At the designated time points, transfected Sf9 cells were observed under light microscope.

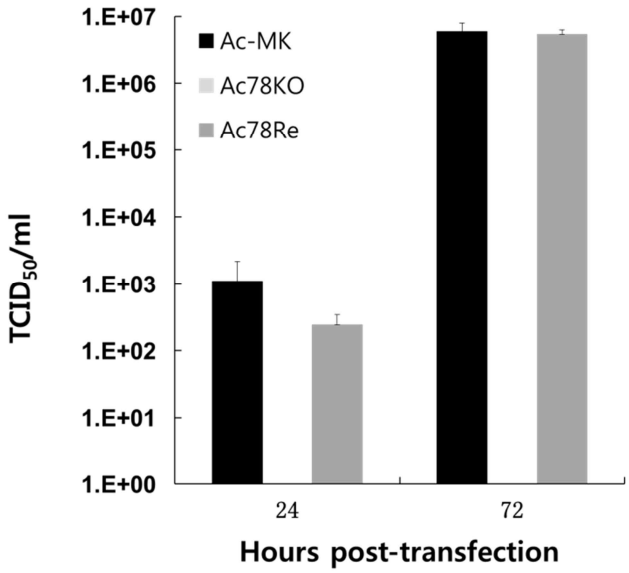

Fig. 39. BV production analysis of Ac78KO transfected Sf9 cells by $TCID_{50}$ end-point dilution assay. The extracellular BV titers at 24 and 72 hpt were determined by $TCID_{50}$ end-point dilution from Sf9 cells separately transfected with Ac-MK, Ac78KO and Ac78Re. Each point represents the average titer derived from three independent $TCID_{50}$ assays. Error bars represent standard deviation.

Because the end-point dilution assay cannot detect the production of non-infectious BVs, the BV titers were also assayed by qPCR, which determines BV titers by detecting viral genomes regardless of infectivity (Fig. 40). Although there was a background of viral genomes detected at every time point due to the initially transfected bacmid DNA, Sf9 cells transfected with Ac-MK or Ac78Re revealed a steady increase in BV production, whereas no increase in BV production was observed above background level up to 120 hpt for Ac78KO-transfected cells.

To additionally confirm the absence of BV production in Ac78KO-transfected cells, Western blotting using the BVs purified from Ac-MK, Ac78KO and Ac78Re-transfected cell supernatants was performed to compare the levels of nucleocapsid protein VP39 (Fig. 41). Whereas the VP39 was detected in the Ac-MK and Ac78Re-transfected cells supernatants, this nucleocapsid protein was not detected in the Ac78KO-transfected cells supernatants. These results suggested that the deletion of *ac78* results in a viral phenotype incapable of producing BV.

4. 4. Effect of *ac78* deletion on *vlf-1* expression in Sf9 cells

Previous study has reported that insertion anywhere within or downstream of the promoter motif disrupted expression of *vlf-1* (Yang & Miller, 1998), of which knockout was previously shown to affect BV production and nucleocapsid assembly (Vanarsdall *et al.*, 2004). To investigate whether the deletion of *ac78* also disrupted the *vlf-1* gene, the expression of *vlf-1* gene in Ac78KO transfected Sf9 cells was characterized by qPCR (Fig. 42). The result revealed that *vlf-1* expression was not disrupted in Ac78KO. Therefore, the defection of BV production in the Ac78KO-transfected Sf9 cells was not resulted

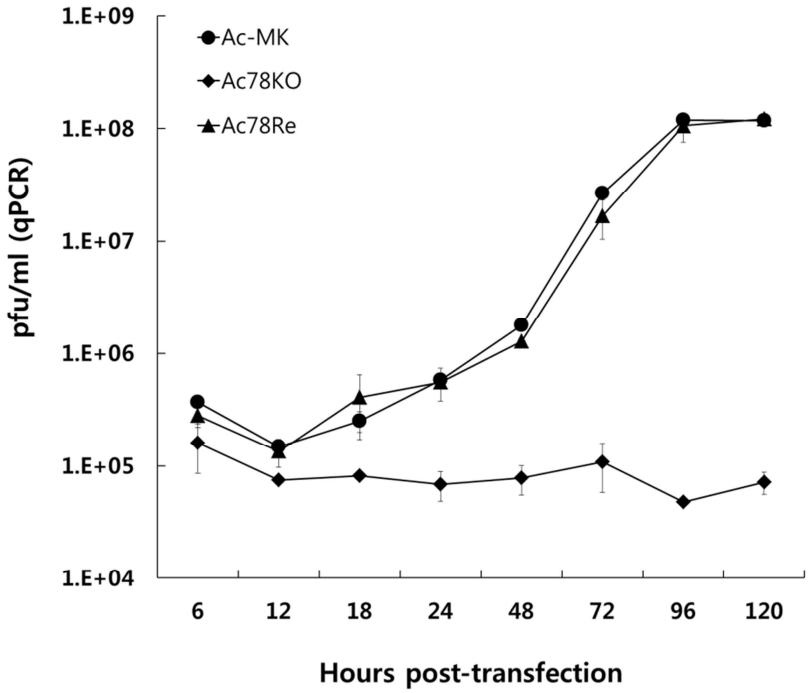

Fig. 40. BV production analysis of Ac78KO transfected Sf9 cells by qPCR. The virus titer, regardless of virion infectivity, was determined by qPCR analysis of supernatants of Sf9 cells transfected with Ac-MK, Ac78KO or Ac78Re at the designated time points.

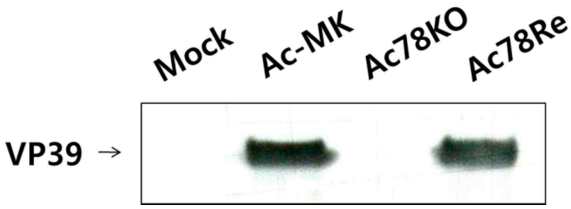

Fig. 41. Western blot analysis of BV from Ac78KO transfected Sf9 cells. Sf9 cells were transfected with Ac-MK, Ac78KO or Ac78Re at 72 hpt. The blots were probed with a monoclonal antibody specific for VP39 nucleocapsid protein.

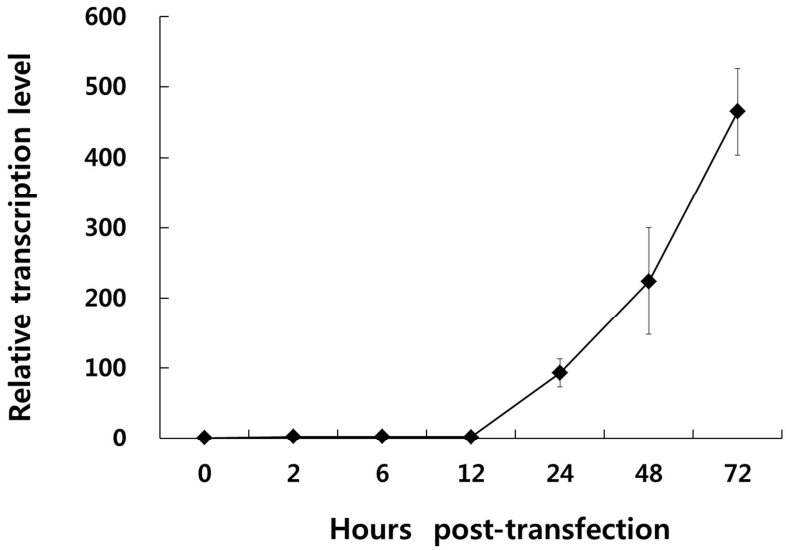

Fig. 42. qPCR analysis of *vlf-1* in Ac78KO transfected Sf9 cells. 1×10^6 Sf9 cells were transfected with 1 μg of Ac78KO bacmid DNA. At the designated time points, total RNA was extracted from Ac78KO-transfected Sf9 cells and treated with DNAse I. Single-strand cDNA was synthesized and subjected to qPCR analysis using *vlf-1*-specific primer. The 28S rRNA was used as a reference gene.

from the disruption of *vlf-1* expression but from the disruption of *ac78* gene.

4. 5. Effect of *ac78* deletion on viral DNA replication in Sf9 cells

To determine whether *ac78* is required for viral DNA replication, a qPCR analysis was performed to investigate the level of viral DNA replication in Ac-MK, Ac78KO, Ac78Re, and GP64KO-transfected cells (Fig. 43). Equal amounts of transfected cells were collected at designated time points, and total DNA was extracted from the cell lysates and subjected to qPCR. The results of this analysis showed that cells transfected with Ac78KO was able to synthesize similar levels of nascent DNA compared with Ac-MK up to 18 hpt and had similar levels of viral DNA replication to the *gp64* knockout bacmid up to 72 hpt. This result indicated that the rates and levels of viral DNA synthesis in Ac78KO and GP64KO-transfected cells are similar and the deletion of *ac78* does not affect viral DNA replication in Sf9 cells.

4. 6. Substructural localization of Ac78 in purified BV and ODV

Two proteomic studies of AcMNPV ODV (Braunagel *et al.*, 2003) and BV (Wang *et al.*, 2010) in an attempt to identify all the proteins associated with each virion phenotype, neither study identified Ac78 as an ODV or BV protein. However, another proteomic analysis demonstrated that the ortholog of Ac78 is an ODV structural protein in CuniNPV (Perera *et al.*, 2007). Since other known ODV-associated proteins were also missing from the AcMNPV proteomic studies and it was suggested that their absence may be due to the masking of low-abundance proteins by highly expressed proteins. To confirm whether Ac78 is an ODV or BV structural protein, BV and ODV were purified from Sf9 cells

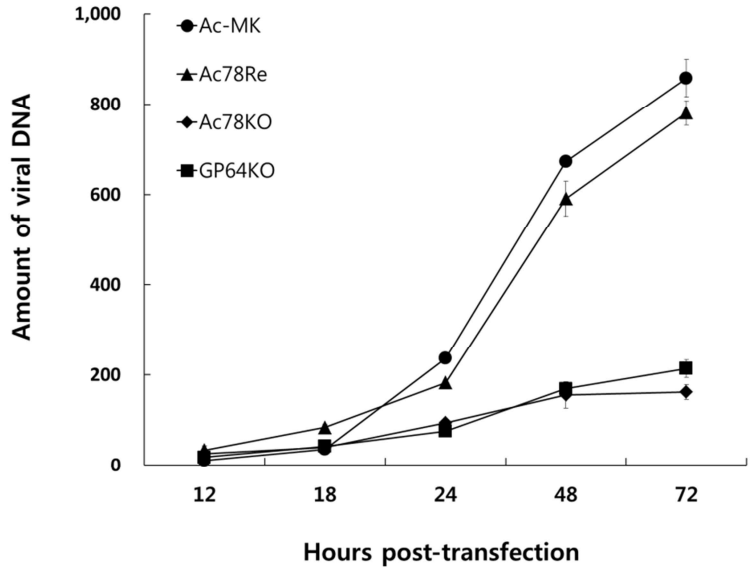

Fig. 43. Effect of *ac78* on viral DNA replication. In total, 1×10^6 Sf9 cells were transfected with 1 μg of Ac-MK or Ac78KO bacmids. At the designated time points, total cellular DNA was isolated from each bacmid DNA-transfected Sf9 cells, digested with the restriction enzyme *Dpn*I to eliminate input bacmid, and analyzed by qPCR. The results are from three separate transfections, and the error bars represent the standard deviation.

infected with Ac-MK and analyzed by Western blot. In addition, the biochemically fractionated nucleocapsid and envelope fractions of the BV and ODV particles were also analyzed by Western blot. The results of the analyses indicated that Ac78 is associated with both BV and ODV and localizes to the envelope fraction (Fig. 44). As a control to confirm the efficiency of the BV fractionation, the nucleocapsid protein VP39 and the BV envelope-specific protein GP64 were analyzed by Western blotting. Both proteins were observed in the expected fractions (Fig. 44).

4. 7. Electron microscopy of Ac-MK, Ac78KO, or Ac78Re-transfected cells

As evidenced by the presence of OBs in the nuclei of transfected Sf9 cells, the viral infection of Ac78KO can progress to very late phases. $TCID_{50}$ end-point dilution and qPCR results indicated that *ac78* is essential for BV production. Western blot analysis indicated that the Ac78 is associated with the envelope of both BV and ODV. To further analyze whether the deletion of *ac78* has any effect on viral morphogenesis, electron microscopic analysis was performed (Fig. 45 and 46). At 24 hpt, cells transfected with Ac-MK, Ac78KO or Ac78Re exhibited the typical baculovirus infection symptoms, including enlarged nuclei and a typically reorganized electron-dense virogenic stroma and rod-shaped nucleocapsids associating with the electron-dense edges of the virogenic stroma. The virogenic stroma is the active site for viral DNA replication and nucleocapsid assembly. In Ac-MK or Ac78Re-transfected cells, most nucleocapsids were mainly concentrated in the nucleus, but a significant number of nucleocapsids were observed in the cytoplasm and budding at the plasma membrane (Fig. 45D and F). In contrast, in

Ac78KO-transfected cells observed up to 72 hpt, the nucleocapsids were consistently observed only in the nucleus (Fig. 45E). At 48 and 72 hpt, the nucleocapsids associated with edges of the virogenic stroma, forming bundles, aligning with *de novo* developed nuclear envelopes and acquiring envelopes, were observed in both Ac-MK and Ac78Re-transfected cells. These enveloped virions contain multiple nucleocapsids prior to occlusion in the protein crystalline matrix of the developing OBs (Fig. 46A and C), and OBs containing numerous enveloped virions were observed in the ring zone (Fig. 46D and F). In Ac78KO-transfected cells, while bundles of nucleocapsids were also observed, only few of them were enveloped, and the enveloped virions contained fewer nucleocapsids compared than Ac-MK or Ac78Re (Fig. 46B). In addition, ODVs were not embedded in the OBs, although their shape and the size were similar to those of Ac-MK and Ac78Re (Fig. 46E). These observations indicated that deletion of *ac78* affects not only BV production but also ODV morphogenesis and subsequent embedding of ODVs into OBs.

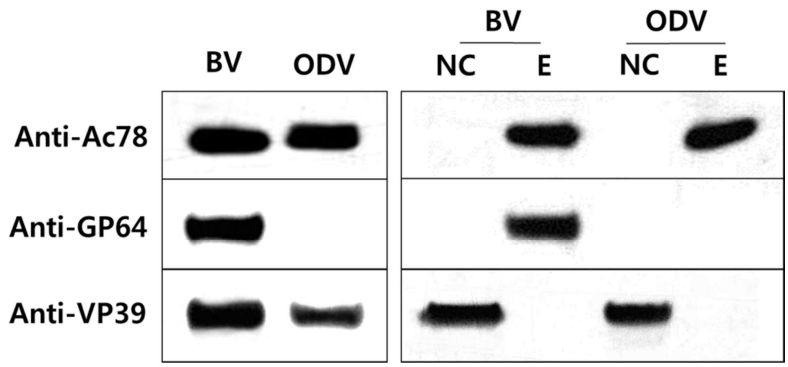

Fig. 44. Western blot analysis of Ac78 in purified and fractionated virions. BV and ODV were purified using a sucrose gradient and analyzed by SDS-PAGE and Western blotting. The blots were probed individually with an anti-Ac78 antibody to detect Ac78, AcV5 monoclonal antibody to detect the BV envelope protein GP64, and anti-VP39 to detect the nucleocapsid protein VP39. BV, budded virus; ODV, occlusion-derived virus; NC, nucleocapsid fraction; E, envelope fraction. GP64 and VP39 were analyzed to confirm correct fractionation of the BV particle in envelope and nucleocapsid fractions, respectively.

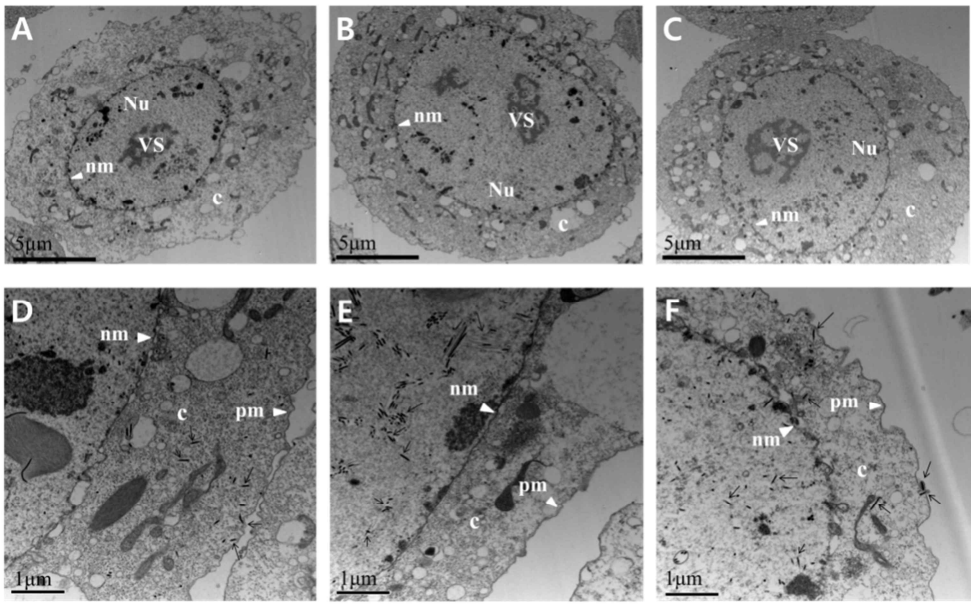

Fig. 45. Transmission electron microscopy analysis of Ac78KO transfected Sf9 cells. Sf9 cells were transfected with Ac-MK (A, D), Ac78KO (B, E) or Ac78Re (C, F) at 24 hpt. Image showing enlarged nucleus (Nu) and virogenic stroma (VS) in the Ac-MK, Ac78KO, or Ac78Re-transfected cells (A, B, C). The higher magnification micrographs of Ac-MK or Ac78Re-transfected cells displayed normal nucleocapsids residing in the cytoplasm (c) and budding from the plasma membrane (pm) (arrows) (D and F). In Ac78KO-transfected cells, nucleocapsids and masses of electron-lucent tubular structures (arrows) were observed in the nucleus, but no nucleocapsids were observed in the cytoplasm (E).

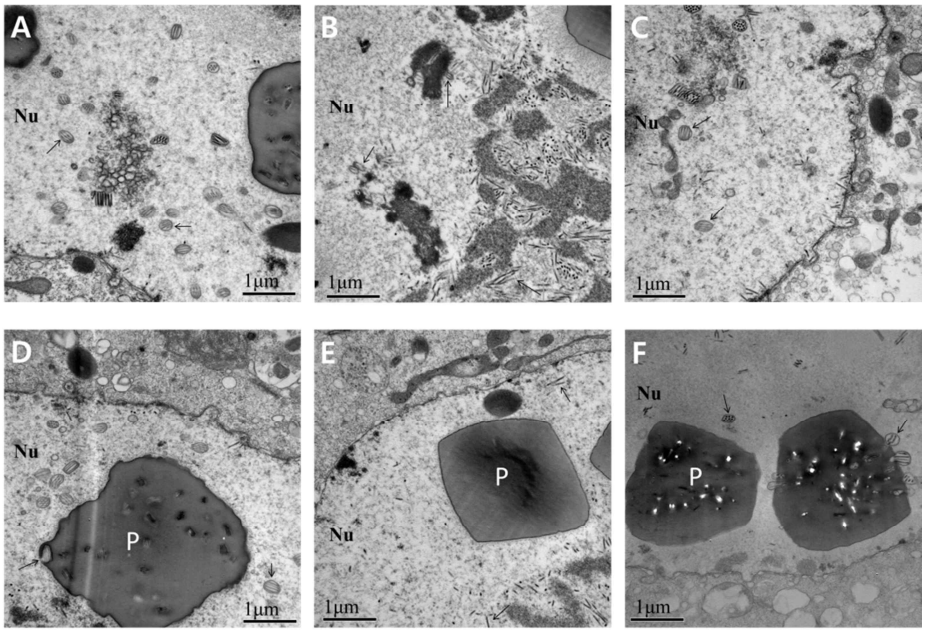

Fig. 46. Nucleocapsid envelopment and OB morphogenesis. In Ac-MK or Ac78Re-transfected cells, normally enveloped virions containing multiple nucleocapsids (arrows) (A and C) were embedded within the polyhedra (P) (arrows) (D and F). In Ac78KO-transfected cells, the nucleocapsids and masses of electron-lucent tubular structures (arrows) appeared at the electron-dense edges of the stroma, enveloped virions contained few nucleocapsids (arrows) (B), and no normal virions were embedded in the polyhedra (E).

DISCUSSION

NPV is one of the largest DNA viruses with genome contains over 100 genes, which are divided into early, late and very late phases (Friesen & Miller, 1986). The function of baculovirus genes in replication has been studied vigorously by using different gene knock-out methods including temperature-sensitive mutation, deletion or insertion into a wild-type viral genome or bacmid in baculoviruses such as AcMNPV and BmNPV (Rohrmann, 2011). In the 1980s, the gene knockout viruses were obtained by conventional homologous recombination in insect cells which is difficult to isolate the recombinant virus when the essential gene was knocked out. The AcMNPV bacmid system solved this problem (Luckow et al., 1993) since the bacmid DNAs were able to replicate in *E. coli*. AcMNPV, the type species of Alphabaculovirus genus, has about 154 genes; thus far, the functions of more than one third of viral genes are still unknown.

In this study, to investigate the function of AcMNPV genes, a novel AcMNPV genome, Ac-MK was generated in which a MiniF replicon and a *Kan* selection marker were inserted into the location of ORF603 and *polyhedrin* in AcMNPV genome, making it can be replicated in *E. coli* as the other bacmids. Moreover, a Tn7 transposon-based plasmids capture system, pPCS-S was used for mutations. The pPCS-S has a donor that containing an *E. coli* origin of replication (pUC ori.) for amplification and an ampicillin resistance gene (*Amp*) for selection between Tn7 left and right end, which can be inserted into target circular DNA by transposition reaction using TnsABC* transposase (Choi *et al.*, 2005). Due to the target immunity, there is only one insertion per target molecule of transposition reaction when this system was used. Therefore, this system produces a

population of target DNA molecules containing the Transprimer element at a different position by the *in vitro* transposition, and useful for a knockout mutagenesis. Using the Tn7-mediated transposition, 220 recombinant viruses were generated via the random insertion of donor of pPCS-S into the Ac-MK genome, and 54 genes of AcMNPV were knocked-out in there recombinant viruses.

The phenotype of the gene knockout mutants were identified by introducing the 54 selected bacmids into Sf9 cells and analyzing of the OB production. The results showed that a group of 10 mutants resulted in lower viral infectivity compared with wild type virus though they produced OBs. And Ac43KO was included in this group which exhibited a distinct phenotype that showed much larger size of OBs, this indicated *ac43* gene may play an important role in the morphogenesis of AcMNPV. In addition, other 16 mutants did not produce infectious viruses, which showed two phenotypes: 6 mutants that neither produced OBs or production of infectious viruses, and 10 mutants that produced OBs in the bacmid transfected cells but not spreading of the infection, in which Ac11KO and Ac78KO were included. This suggested that *ac11* and *ac78* may also play important roles in the AcMNPV life cycle.

It has been reported that *ac11*, *ac43*, and *ac78* are all highly conserved genes in baculovirus genomes. The homologs of *ac11* have been found in all lepidopteran Group I NPV and in one Group II (LdMNPV) genomes, homologues of *ac43* are found in genomes of all group I and most of group II NPVs, and AcMNPV *ac78* has been observed in all the baculovirus genomes sequenced thus far and is one of 37 core genes (Garavaglia *et al.*, 2012), which agree with the phenotype showed in this study and suggests they may perform key functions in the baculovirus life cycle. In the present

study, the roles of *ac11*, *ac43* and *ac78* in the viral life cycle were investigated.

The infection of Sf9 cells with Ac43KO showed that the deletion of *ac43* did not affect BV production but led to a defect in OB production. In addition, deletion of *ac43* resulted in markedly down-regulated transcription and expression of the polyhedrin gene. These results were consistent with the previous report that *Bm34*, the *ac43* homolog of BmNPV, was required for efficient OB production and polyhedrin expression. Furthermore, *Bm34* played an important role in the transcription of *vlf-1* and *fp25K* (Katsuma & Shimada, 2009) which indirectly control the expression of late and very late genes. In this study, reduced expression of polyhedrin in Ac43KO-infected cells might also result from the down-regulation of *vlf-1* and *fp25K* expression.

It has been reported that the *Bm34* gene product is mainly located in the nucleus and is not a component of either BV or ODV. Interestingly, whereas OBs from BmN cells infected with *Bm34*-knockout virus showed normal shapes and sizes (Katsuma & Shimada, 2009), OBs from Sf9 cells infected with Ac43KO were significantly larger in sizes compared with those from AcMNPV-infected cells. Furthermore, these OBs contained a much larger amount of ODVs of the single nucleocapsid phenotype. The processes of virion occlusion in the OBs and the genes that regulate this occlusion process have not been well identified yet. Previous analysis has suggested that OB formation depends on amino acid sequence and the secondary structure of polyhedrin (Carstens *et al.*, 1992). Four spontaneous mutant viruses with altered OB morphology have been isolated and characterized in AcMNPV. In each case, a single amino acid substitution in the polyhedrin sequence was responsible for the abnormal phenotype of the OBs. One of those mutants, bearing a substitution at position 118, produced large

amounts of small particles instead of crystalline OBs (Ribeiro *et al.*, 2009). The other reported mutants, which had a substitution at residue 58 (Carstens *et al.*, 1986), 25 (Lin *et al.*, 2000) or 130 (Lopez *et al.*, 2011), produced a single large cubic OB in the nuclei of the infected cells, and only a small portion of these OBs contained nucleocapsids in them. These OB-deficient mutants have been classified as class I and II based on their different characteristics (Ji *et al.*, 2010).

In contrast, several studies have demonstrated that OB formation is determined by the interactions between polyhedrin and other proteins present on the viral envelope (Wang *et al.*, 2009; Woo *et al.*, 1998). The co-infection of AcMNPV and BmNPV generated a recombinant virus that produced OBs varying in morphology when infecting different host cell types. The polyhedrin gene of this recombinant virus showed 100% identity with the polyhedrin gene of the BmNPV, suggesting that the size and morphology of the OBs might be affected by host cell factors as well as viral genes or regulatory sequences other than polyhedrin (Woo *et al.*, 1998). Other results also indicated that some viral or host factors may play a role in the process of virion occlusion (Hu *et al.*, 1999). No virions were detected in the OBs of an AcMNPV mutant in which the *p26*, *p10*, and *p74* genes were deleted, demonstrating that *p26*, *p10*, and *p74* are all required for proper virion occlusion into the OBs of AcMNPV (Wang *et al.*, 2009). In this study, deletion of *ac43* not only altered the size of OBs but also affected the virion occlusion in the polyhedra. The *ac43* gene is the first reported viral gene other than the polyhedrin gene that determines the size of OBs.

For AcMNPV, the development of two viral morphotypes (i.e., BV and ODV) and the multiple nucleocapsid packaging of ODV are major evolutionary developments that

contribute to its success as a pathogen of lepidopteran larvae (Washburn *et al.*, 2003). In this study, deletion of *ac43* resulted in the production of ODVs with singly packaged nucleocapsids. The evolutionary history and host ranges of the NPVs suggest that the MNPV has evolved from the SNPV and, therefore, may offer selective advantages (Washburn *et al.*, 1999). At least two hypotheses have been proposed to explain the multiple nucleocapsid packaging strategy of the MNPVs. First, it is well documented that viral occlusions rapidly lose viability when exposed to sunlight, presumably because UV radiation damages viral DNA. By infecting primary target cells with multiple copies of the viral genome, it is possible that gene complementation compensates for the damaged DNA and facilitates productive infections (Washburn *et al.*, 1999). The second hypothesis postulates that infection of primary target cells with multiple copies of the viral genome increases infection efficiency *in vivo* by accelerating the onset of secondary infection (Blissard, 1996; Volkman, 1997).

Although the biological basis for multiple nucleocapsid envelopment has not been determined, *ac142* was recently shown to be essential for ODV nucleocapsid envelopment (McCarthy *et al.*, 2008). In addition, deletion of *Sf29* from *Spodoptera frugiperda* MNPV (SfMNPV) reduced the number of ODVs occluded in OBs but had no apparent effect on ODV nucleocapsid content (Simon *et al.*, 2008). Another gene, *ac23*, homologous to the F proteins of group II NPVs, is also involved in ODV formation. Like the *ac43*-knockout virus in this study, a greater percentage of ODVs with singly-enveloped nucleocapsids are occluded in OBs of the *ac23*-null mutant virus compared with that of wild-type virus, suggesting that *ac23* somehow facilitates multiple nucleocapsid envelopment (Yu *et al.*, 2009). However, OBs of *ac23*-null mutant and

wild-type virus do not differ significantly in size or the number of ODVs occluded (Yu *et al.*, 2009). However, OBs produced by the *ac92*-knockout mutant lacked ODVs with multiply-enveloped nucleocapsids, but singly-enveloped nucleocapsids were detected (Wu & Passarelli, 2010). The mutant virus which the *ac92* C155XXC158 amino acids (important for sulfhydryl oxidase activity) were mutated to A155XXA158, exhibited a similar phenotype to the *ac92*-knockout virus, suggesting that the C-X-X-C motif was responsible for the altered ODV phenotype (Wu & Passarelli, 2010).

In the present study, the results showed that *ac43* is a novel late gene required for efficient expression of the *polyhedrin* gene and production of OBs. Additionally, *ac43* appears to play an important role in the process of OB formation and multiple nucleocapsid packaging of ODVs. Further studies to address whether *ac43* interacts with other viral sequences, viral proteins, or even other factors from the host cells would provide greater insight into the processes of OB morphogenesis and nucleocapsid packaging in the AcMNPV life cycle.

Compared with *ac43* gene, *ac11* and *ac78* shared some similar phenotype when the investigation was carried out using gene knockout viruses, Ac11KO and Ac78KO. The results revealed that BV production was shown to be blocked by deletion of *ac11* or *ac78*. The presence of OBs in the Ac11KO- or Ac78KO-transfected Sf9 cells indicated that the infection can be initiated without *ac11* or *ac78*, but the infection was restricted to the initially transfected cells. This phenotype was further confirmed by BV titration and Western blot assay. To investigate whether the defected of BV production was due to blocking of DNA replication in Ac11KO- or Ac78KO-transfected Sf9 cells, qPCR analyses were performed by using the *gp64* gene knockout mutant, GP64KO, as control.

The results revealed that both Ac11KO and Ac78KO showed similar replication level with the GP64KO, which indicating that both *ac11* and *ac78* are not essential for viral DNA replication.

Previously, many AcMNPV genes have also been deleted to study their specific function in the viral life cycle. Among them, there is a group of genes shown to be essential for BV production which shown as follows: *dbp* (*ac25*) encodes a single DNA binding protein which is essential for production of nucleocapsids and virogenic stroma (Vanarsdall *et al.*, 2007a). *ac76* (Hu *et al.*, 2010) expresses a protein localized to the ring zone late in infection. One of the core gene, *gp41* (*ac80*) (Olszewski & Miller, 1997b) encoding a tegument protein located between the virion envelope and capsid (Whitford & Faulkner, 1992a; b), is required for the egress of nucleocapsids from nucleus. And *ac93* (Yuan *et al.*, 2011) encodes a BV and ODV associated nucleocapsids protein which also present in BV envelope fraction. In addition, *p78/83* (*ac9*) (Russell *et al.*, 1997), *vp1054* (*ac54*) (Olszewski & Miller, 1997a), *vlf-1* (*ac77*) (Li *et al.*, 2005), *38k* (*ac98*) (Wu *et al.*, 2008b), *ac142* (McCarthy *et al.*, 2008), and *ac146* (Dickison *et al.*, 2012) are also essential genes for BV production, which have shown to be encoding nucleocapsids proteins located both in BV and ODV. Interestingly, all the genes above have shown to be not essential for DNA replication as *ac11* and *ac78* in this study.

Observation by electron microscopy indicated that abundant nucleocapsids with a normal appearance were observed in the intrastromal space of the virogenic stroma of Ac11KO- or Ac78KO-transfected cells and were morphologically indistinguishable from those in cells transfected with either Ac-MK or repair bacmids, indicating that nucleocapsid assembly was not affected by the deletion of *ac11* or *ac78*. The

nucleocapsids of Ac11KO and Ac78KO also revealed that new viral DNAs were incorporated into the capsids. This is consistent with the qPCR analysis which demonstrated that *ac11* and *ac78* are not involved in viral DNA synthesis. However, no nucleocapsid budding through the nuclear membrane was observed in the cytoplasm of Ac11KO- or Ac78KO-transfected cells, indicating that the inefficient BV production from the Ac11KO- or Ac78KO-transfected cells were due to inefficient egress of nucleocapsids from the nucleus to the cytoplasm. These results demonstrated that both *ac11* and *ac78* are not required for nucleocapsids formation but essential for nucleocapsids egress from nucleus to form BV.

Furthermore, electron microscopy showed that deletion of *ac11* and *ac78* resulted in producing OBs that were completely devoid of ODV. The nucleocapsids could normally bundle together in the nuclear ring zone of Ac11KO- and Ac78KO-transfected cells, however, they were never enveloped to form ODVs and incorporated into the developing OBs in Ac11KO-transfected cells, and only few of the nucleocapsids bundles were envelopment forming ODVs but not embedded into OBs in Ac78KO-transfected cells. Several morphogenetic processes, such as nucleocapsid bundling, envelopment of ODVs, and embedding of ODVs into OB have been reported to occur in the ring zone (Williams & Faulkner, 1997). Therefore, the results suggested that *ac11* and *ac78* execute their functions after nucleocapsid assembly, during nucleocapsid envelopment and ODVs embedding. Previous studies have also reported that OBs without embedded virions can be formed in cells transfected with knockout viruses lacking *38k* (*ac98*) (Wu *et al.*, 2006), *ac142* (McCarthy *et al.*, 2008), *p48* (*ac103*) (Yuan *et al.*, 2008), *ac53* (Liu *et al.*, 2008), *ac76* (Hu *et al.*, 2010), *ac94* (Chen *et al.*, 2012) and *ac109* (Alfonso *et al.*, 2012; Lehiy *et*

al., 2012). Deletion of *38k* or *ac53* leads to defects in nucleocapsid assembly, whereas knockouts of *ac142*, *p48*, *ac76*, or *ac109* interfere with nucleocapsid envelopment, which is similar to that of for *ac11* and *ac78* knockouts in this study, and demonstrated that ODVs embedding was not essential for formation of OBs.

It has been reported that most baculovirus morphogenesis related genes encode viral structural proteins. Western blot analysis demonstrated that Ac78 is an envelope component of both BV and ODV. Though Ac11 was not detected in either BV or ODV fraction in western blot, since *ac11* belonging early gene category, it is possible that disruption of *ac11* affect the expression of some late genes associated with ODV, consequently inhibiting ODV envelopment and formation of normal OBs. To date, only a small number of proteins have been reported to be associated with both the BV and ODV envelope, including BV/ODV-E26 (Ac16) (Beniya *et al.*, 1998), F-like (Ac23) (Braunagel *et al.*, 2003; Wang *et al.*, 2010), GP37 (Ac64) (Li *et al.*, 2003), ODV-E25 (Ac94) (Russell & Rohrmann, 1993; Wang *et al.*, 2010), Ac96 (Fang *et al.*, 2009a), and ODV-E18 (Ac143) (Wang *et al.*, 2010). BV/ODV-E26 is a peripheral membrane protein that may interact with other proteins to play an important role in trafficking ODV envelope proteins (Beniya *et al.*, 1998; Burks *et al.*, 2007). F-like is important for BV infectivity (Wang *et al.*, 2008a), and GP37 is a chitin-binding protein (Li *et al.*, 2003). Both of these proteins are not essential for viral replication (Lung *et al.*, 2003; X. W. Cheng, 2001). In addition, ODV-E25 and Ac96 are not essential for viral DNA replication or BV production (Chen *et al.*, 2012; Fang *et al.*, 2009a). ODV-E25 is an integral ODV envelope protein with an envelope-anchored N-terminus (Hong *et al.*, 1997; Russell & Rohrmann, 1993) that is required for the formation of intranuclear microvesicles and ODV (Chen *et al.*, 2012).

Ac96 is a *per os* infectivity factor (PIF), which is not related to BV production or viral DNA replication (Fang *et al.*, 2009a). ODV-E18 is an integral ODV envelope protein, and all homologues have N-terminal transmembrane motif regions (Braunagel *et al.*, 1996b), which is essential for BV production (McCarthy & Theilmann, 2008).

In addition, there are two envelope proteins that present only in BV, Ac35 (V-ubiquitin) and Ac128 (GP64). Ac35 (V-ubiquitin) is not essential for viral replication, but viruses with frame shift mutations of *v-ubiquitin* have reduced BV production (LINDA M. REILLYa, 1996). GP64 is the major envelope protein of BV responsible for membrane fusion and is important for efficient BV budding (Blissard & Wenz, 1992).

ODV envelope proteins are much more complex in composition compared with those of BV envelopes. ODV envelope proteins are composed of ODV-E66 (Ac46), GP41 (Ac80) (Liu & Maruniak, 1999), ODV-E56 (Ac148) and a group of PIFs including P74 (PIF-0) (Faulkner *et al.*, 1997; Kuzio *et al.*, 1989; Yao *et al.*, 2004), PIF-1 (Ac119) , PIF-2 (Ac22) (Pijlman *et al.*, 2003) , PIF-3 (Ac115) (Ohkawa *et al.*, 2005), and PIF-4 (Ac96) (Fang *et al.*, 2009a). ODV-E66 is an integral ODV envelope protein with an envelope-anchored N-terminus (Hong *et al.*, 1997) that is not required for virion morphogenesis or occlusion assembly but is required for oral infectivity (Xiang *et al.*, 2011b). GP41 is a tegument protein modified with O-linked N-acetylglucosamine, located between the virion envelope and capsid (Whitford & Faulkner, 1992a; b), that is required for the egress of nucleocapsids from the nucleus forming BV (Olszewski & Miller, 1997b). ODV-E56 has a transmembrane motif on its C-terminus and is not required for BV production, ODV assembly or occlusion (Braunagel *et al.*, 1996a; Theilmann *et al.*, 1996).

In conclusion, 54 AcMNPV genes were knocked-out, and the gene knockout bacmids were introduced into Sf9 cells to analysis the phenotype of OBs and infectious virus production. Three of the knockout mutants were selected and functional investigated, and it was revealed that they all play important roles in the baculovirus life cycle as the phenotype presented. Although the exact roles of these genes in the processes are still unclear, these results will be helpful in the functional analysis of viral genes although other experiments are still required to evaluate the knockouts that showed an effect in detail.

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Autographa californica mutiple nucleopolyhedrovirus의 유전자 knockout을 통한 기능적 특성 연구

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초 록

AcMNPV는 약 134kb의 이중가닥 DNA genome과 154 ORF를 가지고 있다. 다수의 나비목 곤충에 고병원성을 나타내며 외부유전자의 발현을 위한 세포 형질도입에 널리 이용되고 있다. 바이러스의 복제에 있어서 AcMNPV의 기능을 알아내기 위해 gene knock-out 기술을 이용한 연구가 진행되었으나 몇몇 conserved gene을 포함한 바이러스 유전자의 1/3 이상에 대해서는 아직도 그 기능이 밝혀지지 않은 실정이다. 본 연구에서는, AcMNPV 유전자의 기능을 밝혀내기 위하여 플라스미드 형태로 *Escherichia coli*에서 유지될 수 있고, 감수성인 나비목 곤충의 세포를 감염시킬 수 있는 새로운 형태의

AcMNPV를 제작하였다. 새롭게 제작된 AcMNPV는 *E. coli* origin (mini-F replicon)과 kanamycin 저항성 유전자(*Kan*)을 포함하고, Ac-MK라 명명하였다. pUC origin과 ampicillin 저항성 유전자를 포함하고 Tn7 transposon을 기반으로 한 plasmid capture system인 pPCS-S을 이용하여 Ac-MK genome에 무작위로 삽입하는 방식을 통해 총 54개의 단일 ORF-knockout AcMNPV mutants를 제작하였다. 그 후, 이 ORF-knockout mutant 바이러스들의 *Spodoptera frugiperda* 9 (Sf9) 세포에서의 growth properties와 감염된 후손의 생성에 미치는 gene knockout-specific effects를 관찰하고 분석하였다. 이들 중에서 각각 ORF11 (*ac11*), ORF43 (*ac43*), ORF78 (*ac78*)가 knocked-out 되어 진 Ac11KO, Ac43KO, Ac78KO 세 개의 mutant를 선발하였다. *ac11*, *ac43*, *ac78*은 베큘로바이러스에서 매우 잘 보존되어 있는 유전자들로 베큘로바이러스의 생활사에서 중요한 역할을 할 것이라 추정된다.

qPCR 분석을 통해 *ac11*은 바이러스 생활사에서 초기발현유전자인 것이 밝혀졌고, 현미경, 적정 검정, Western blot을 수행한 결과, Ac11KO이 형질감염된 sf9 세포에서는 BVs(budded viruses)가 생성되지 않는 것이 관찰되었다. 그러나 *ac11*의 부재는 바이러스 DNA의 복제에는 영향을 미치지 않는다는 사실이 qPCR을 통해 입증되었다. 또한, 전자현미경 관찰 결과 Ac11KO가 형질감염된 세포의 세포질이나 세포막에서 뉴클레오캡시드가 관찰되지 않는 것으로 보아 Ac11KO가 형질감염된 세포에서의 BV 생성결함은 뉴클레오캡시드의 핵에서 세포질로의 비효율적인 방출이 원인이라는 사실이

입증되었다. 게다가 Ac11KO가 형질감염된 세포에서는 핵 내의 뉴클레오캡시드는 ODV(occlusion-derived virus) 형성을 위한 enveloped 과정이 이루어지지 않고, 그에 따른 OB(occlusion body)로의 embedding도 저해되는 현상이 전자현미경 상에서 관찰되었다. 이것은 *ac11*이 ODV envelopment에 필요한 유전자라는 사실을 시사한다. 이러한 결과들을 토대로 *ac11*은 바이러스 생활사에서 필수적인 유전자라는 결론을 도출해내었다.

바이러스 복제 과정 중 *ac43* 유전자의 기능에 대한 연구도 수행되었는데, sf9 세포로의 트랜스펙션 결과, Ac43KO는 wild-type AcMNPV에 비하여 훨씬 큰 사이즈의 다각체를 생성하였다. 흥미롭게도, AcMNPV가 multiply enveloped 되는 것으로 알려진 데 반하여 몇몇 뉴클레오캡시드는 singly enveloped 되는 현상도 관찰되었다. 또한, Ac43KO는 다각체의 전사와 발현에서 결함을 유도함으로써 OB의 생성을 감소시키는 결과를 초래하였다. 그러나 출아된 바이러스 역가에서 주목할 만한 차이점을 보이지 않은 것으로 보아 Ac43KO는 BV의 생성에는 영향을 미치지 않을 것이라 생각된다. 이러한 결과들을 토대로 OB에 봉입되는 비리온의 조합, OB의 형태형성, 다각체의 발현에 있어서 *ac43* 유전자는 중요한 역할을 할 것이라 추정된다.

ac78 유전자에 대한 qPCR을 수행한 결과, *ac78*은 바이러스 생활사에서 후기유전자라는 것이 밝혀졌다. Ac78KO는 단일세포감염의 표현형을 생산하였는데 이는 감염성 BV를 생성하지 않는다는 것을 의미한다. BV 생성에서의 결함은 바이러스 적정과 웨스턴 블로팅 모두에서도 재확인되었다.

그러나 바이러스 DNA의 복제에는 영향을 미치지 않았고, 봉입체는 형성되는 것을 관찰하였다. BV와 ODV 분석 결과 Ac78은 envelope structural protein이며, 비리온의 형태에 연관된 유전자라는 사실이 밝혀졌다. 또한 Ac78은 봉입체 내로의 ODV embedding에서 중요한 역할을 한다는 사실도 전자현미경 상에서 확인되었다. 본 연구 결과 Ac78은 late virion-associated protein이며 바이러스 생활사에서 필수적인 유전자라는 사실이 입증되었다.

검색어: *Autographa californica* multiple nucleopolyhedrovirus, *ac11*, *ac43*, *ac78*, BV production, ODV envelopment, virus morphology

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