Article 1

The Membrane Anchoring Region of the AcMNPV P74 Protein is Expendable or Interchangeable with Homologs from Other Species

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María Victoria Nugnes [1], Alexandra Marisa Targovnik [2,3], Adrià Mengual-Martí[4], María Victoria Miranda[2,3], Carolina Susana Cerrudo[1], Salvador Herrero[4, †] and Mariano Nicolás Belaich [1, †,*]

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[1] Laboratorio de Ingeniería Genética y Biología Celular y Molecular – Área Virosis de Insectos, Instituto de Microbiología Básica y Aplicada, Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Roque Sáenz Peña 352, B1876BXD, Bernal, Buenos Aires, Argentina; dcyt@unq.edu.ar

[2] Cátedra de Biotecnología, Departamento de Microbiología, Inmunología, Biotecnología y Genética, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, Sexto Piso, C1113AAD, Buenos Aires, Argentina.

[3] Instituto de Nanobiotecnología (NANOBIOTEC), Universidad de Buenos Aires (UBA) - Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Junín 956, Sexto Piso, C1113AAD, Buenos Aires, Argentina.

[4] Department of Genetics and University Institute of Biotechnology and Biomedicine-BIOTECMED, Universitat de València, Valencia, Spain.

* Correspondence: mbelaich@unq.edu.ar; Tel.: (+541143657100, ext 5635)

† These authors contributed equally to this work.

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Abstract: Baculoviruses are insect pathogens that are characterized by assembling the viral dsDNA into 2 different enveloped virions during an infective cycle: occluded virions (-ODV- immersed in a protein matrix known as occlusion body); and budded virions. ODVs are responsible for the primary infection in midgut cells of susceptible larvae thanks to the *per os* infectivity factor (PIF) complex, composed of at least 9 essential viral proteins. Among them, P74 is a crucial factor which activity has been identified as virus-specific. In this work, the *p74* gene from AcMNPV was pseudogenized using CRISPR/Cas9 technology, and then complemented with wild type alleles from SeMNPV and HearSNPV species, as well as chimeras combining the P74 amino- and carboxyl domains. The results on *Spodoptera exigua* and *Rachiplusia nu* larvae showed that an amino terminal sector of P74 (lacking two potential transmembrane regions but possessing a putative nuclear export signal) is sufficient to restore the virus infectivity by itself or fused to the P74 transmembrane regions of the other viral species evaluated. These results provide novel information about the functional role of P74 and delimits the region on which mutagenesis could be applied to enhance viral activity and thus produce better biopesticides.

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1. Introduction

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Baculoviridae family is composed by insect-specific rod-shaped viruses with large covalently closed circular double-stranded DNA (cccdsDNA), from 81,755 bp (NeleNPV) to 178,733 bp (XecnGV) in length, usually used as crop pest control agents among other biotechnological uses [1,2]. In nature, baculoviruses infect susceptible hosts in larval state and produce two genetically identical but morphologically distinct viral phenotypes: Occlusion-Derived Viruses (ODVs) and Budded Viruses (BVs) [3]. The first are the ones that initiate the infection in the midgut defining the host-range (primary infection), and are characterized by being embedded in a crystalline matrix denominated Occlusion Bodies

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(OBs) that physically protect virions against environmental stresses, while the BVs are responsible of cell-to-cell infections inside the larval body (secondary or systemic infection) [4].

Baculoviruses are classified in four genera according to their nucleotide sequence and phylogeny [5], permissive host species [6], and the type of main OB′s protein [7]: *Alphabaculovirus*, *Betabaculovirus*, *Gammabaculovirus*, and *Deltabaculovirus*. While alpha- and betabaculoviruses infect insects belonging to Lepidoptera order, gamma- and deltabaculoviruses infect members of Hymenoptera and Dyptera orders, respectively [3]. Besides, *Alphabaculovirus* genus is subclassified in two clades, "group I" and "group II", based on phylogeny inferences and the occurrence of different envelope fusion proteins in BVs [8]. GP64 protein is present in group I alphabaculoviruses [9], while for all the other ones, including those belonging to the group II, the F protein is the factor associated to cell-to-cell spreading [10–12].

Baculovirus infection starts with the ingestion of OB-contaminated food (*per os* route) by the larvae [13]. Then, OBs get dissolved in the alkaline larval midgut and release the ODVs into the gut lumen to infect the epithelial cells. Once ODVs pass through the peritrophic matrix, the viral envelope fuses with the cell membrane releasing the nucleocapsids. This process of recognition, binding and fusion determines the success of the infection and it is carried out by a group of virus proteins anchored in the ODV envelope, termed as *per os* infectivity factors (PIFs) [14–17]. So far ten PIFs have been identified: PIF0 (Ac138), originally called P74 because the molecular weight is 74 kDa [18,19]; PIF1 (Ac119) [20]; PIF2 (Ac22) [21,22]; PIF3 (Ac115) [23,24]; PIF4 (Ac96) [25,26]; PIF5 (ODV-E56 or Ac148) [27,28]; PIF6 (Ac68) [29]; PIF7 (Ac110) [30]; PIF8 (VP91 or Ac83) [31,32]; and PIF9 (Ac108) [33,34]. Interestingly, except for PIF9 which has no orthologs detected in *Deltabaculovirus* [35], all the genes that encode these factors are part of the 38 core genes described until now in *Baculoviridae* [31] and are also conserved in other invertebrate DNA viruses, like nudiviruses, hytrosaviruses, bracoviruses and nimaviruses [3,36]. In accordance with the information reported, all the PIF proteins but PIF5 are arranged in a complex, in which PIF1, 2, 3 and 4 form a stable core and PIF0 (P74), 6, 8 and 9 are more loosely associated [17,34,37]. When any of these factors is deleted from the baculoviral genome it results in the loss of oral infectivity by the inhibition of the primary infection [36,38]. But, the infective capacity of BVs does not seem diminish, since PIFs are not needed for cell to cell spreading. Consequently, *in vitro* propagation in insect cell culture of a PIF-deficient baculovirus is not affected and show wild type characteristics thus facilitating the study of these variants [20–22].

P74 was the first viral protein found to be essential for baculovirus oral infection and, more recently, has also been described to play a fundamental role in the ODV binding to larvae's midgut epithelium [16,39]. The N-terminus portion of the protein is directed outwards from the ODV-envelope, and unlike PIF1-4, 7 and 8, an inner nuclear membrane sorting motif (INM-SM) has not been described [38,40,41]. On the other hand, the C-terminus is believed to be anchored in the ODV envelope and whether it contains of two or three transmembrane (TM) domains is still under discussion [38,42]. It has been reported that P74 of Autographa californica nucleopolyhedrovirus (AcMNPV) undergoes two independent cleavages once the OB dissolves in the midgut lumen. The first one would be mediated by a host-derived protease and divides the protein into two fragments of approximately 35 and 40 kDa. The second cleavage would be executed by insect midgut trypsins releasing a 20-kDa fragment from the N-terminus, which still interacts with the rest of the PIF complex [43]. This process would be the crucial step to activate P74 [44]. Moreover, other studies have demonstrated that the infection of susceptible larvae with a *p74*-knockout AcMNPV could be rescued by co-feeding with the purified protein or even with a soluble truncated form without the C-terminal TM domain (1–580 aa) [42,45]. Nonetheless previous attempts to replace P74 with other homologs had failed, resulting in the completely loss of the oral infectivity. This highlights the highly specific interactions

among PIF proteins and between these and the cellular factors of the midgut epithelial cells to support the primary infection [46,47].

Manipulation trough viral genome engineering such as PIF deletions or modifications allow us to assess the protein function and its implications in the baculoviral infectivity. In recent years genome mutagenesis has become easier by the introduction of the clustered regularly interspaced short palindromic repeats-Cas9 or CRISPR/Cas9 technology [48–50]. The CRISPR/Cas9 system involves a single guide RNA (sgRNA) that interacts and directs the Cas9 endonuclease to a specific site to perform a double-strand DNA break (DSB) [51]. This triggers DNA repair by one of two major pathways, the error-prone non-homologous end joining (NHEJ) or the homology-directed repair (HDR) pathway. This last mechanism requires to provide a donor DNA with homology to the flanking sequence of the DSB. But in absence of this template, the endogenous cell repair machinery carry out the NHEJ leaving consequential insertions/deletions (indel) mutations as a scar near the site of the break [52].

In this work a variant of AcMNPV, the prototypic baculovirus most used for biotechnological purposes, was knocked out in the *p74* gene by CRISPR/Cas9 technology and NHEJ repair, and then complemented with sequences that encode P74 domains or P74 chimeras generated by combinations of domains from different baculovirus species (*Spodoptera exigua multiple nucleopolyhedrovirus* -SeMNPV- and *Helicoverpa armigera single nucleopolyhedrovirus* -HearSNPV). The characterization of P74 protein and the biological effects on susceptible larva add new evidence for the understanding of the baculovirus PIF complex.

2. Materials and Methods

2.1. In vitro cell culture and insect rearing

The *Spodoptera frugiperda* Sf21 insect cell line [53] was cultured on monolayers at 25°C in Grace's Medium (GIBCO BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL, Gaithersburg, MD, USA) and antibiotics and antimycotics (GIBCO BRL Gaithersburg, MD, USA). *Spodoptera exigua* larvae were derived from the stablished colony at the University of Valencia (Spain), and *Rachiplusia nu* larvae were obtained from AgIdea S.A. (Pergamino, Buenos Aires, Argentina). Insects were reared with artificial diet at 25°-27°C with 70% relative humidity and a photoperiod of 16/8 h (light/dark).

2.2. Bioinformatics studies

A non-redundant set of 76 P74 protein sequences were obtained from the reported genomes (22 group 1-alphabaculoviruses; 28 group II-alphabaculoviruses; 22 betabaculoviruses; 3 gammabaculoviruses; and 1 deltabaculovirus) (Supplementary Table 1) and aligned using ClustalX [54]. The consensus line was used to generate a sequence similarity plot employing a residue-by-residue sliding windows strategy (35 nucleotides-length), where arbitrary values were assigned to different symbols (+1 for identical residues; +0.5 and +0.25 for the different degree of conservative changes; and -0.2 for non-conservative changes). The algebraic sum was normalized by the window length and allotted to the window central position. Moreover, the evolutionary history was inferred with MEGA7 [55] using the Maximum Likelihood method based on the LG+G+I model [56] and also considering orthologs to P74 of nudiviruses, hytrosaviruses and nimaviruses (Supplementary Table 1). Initial trees for the heuristic search were obtained by Neighbor-Joining and BioNJ algorithms (the matrix of pairwise distances was estimated using a JTT model) and the topology with superior log likelihood value was selected. A discrete Gamma distribution was used to model the different evolutionary rates among sites [2 categories (+G, parameter = 1.1482)]. The weblogo server was used with default parameters (https://weblogo.berkeley.edu/logo.cgi) [57], and the hydropathy profiles were generated using a sliding overlapping window strategy (21 residues-length displaced from

one residue at a time). The putative transmembrane domains, the disulfide bonds, and the nuclear export signal (NES) predictions were performed using webservers (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0 [58]; http://disulfind.disi.unitn.it/ [59]; http://clavius.bc.edu/~clotelab/DiANNA/ [60]; https://services.healthtech.dtu.dk/service.php?NetNES-1.1 [61]) and standard parameters.

2.3. P74 gene knockout in AcMNPV-bacmid

The AcMNPV-bacmid bMON14272 [62] was depleted in *p74* gene using CRISPR/Cas9 technology. Briefly, the CRISPR RNA (crRNA, 5′ AACTGGCTTTCAG-CAAGCGC 3′) used to direct the *Streptococcus pyogenes* Cas9 nuclease (New England Biolabs, Ipswich, MA, USA) to the initial part of the *p74* open reading frame (ORF) was previously designed 207 bp downstream of ATG codon [50] using the CHOP-CHOP online platform (https://chopchop.rc.fas.harvard.edu) and synthesized (Integrated DNA Technologies, Leuven, Belgium). To generate the ribonucleoprotein (RNP) complex, 1 μL of the crRNA (1 μM) was incubated with 1 μL of tracrRNA (1 μM) and 98 μL of nuclease-free duplex buffer (both from Integrated DNA Technologies, Leuven, Belgium) for 5 min at 95°C. Then, 1 μL of the crRNA-tracrRNA mixture was combined with 6.2 μL of Cas9 (1 mM) and 2.8 μL of nuclease-free water to a final volume of 10 μL (incubation at 37°C for 5 min). The bacmid DNA was purified following the manufacturer's instructions of Bac-to-Bac system (GIBCO BRL Gaithersburg, MD, USA) and added to the previous mixture (1 μg), which was then transfected in Sf21 cells (70% confluent) using Cellfectin® II Reagent (Invitrogen, Carlsbad, CA Carlsbad, USA) in Grace's medium without FBS. After 5 hours of incubation, the medium was replaced by Grace's medium supplemented with 10% FBS and incubated for 72 hours at 25°C. Later, BVs were isolated from supernatants following the plaque assay protocol [63] and the individual clones were analyzed by amplicon Sanger sequencing using PrepMan® Ultra reagent (Applied Biosystems, Foster City, CA, USA) and the primers FwP74seq and RvP74seq (Table 1) [50] to detect indel mutations. Once P74 depleted bacmid (bMON14272Δp74) was identified, its BVs were replicated in Sf21 cells and the viral DNA purified using standard phenol/chloroform protocol [63] to be sequenced by Illumina (MiSeq System, Laboratorio de Secuenciación Servicio Central de Soporte a la Investigación-SCSIE, Universitat de València, Valencia, Spain). Finally, DH10b *Escherichia coli* competent cells were transformed with the Ac-MNPV-bacmidΔp74 DNA and the pMON7124 helper plasmid (GIBCO BRL Gaithersburg, MD, USA) to generate the DH10bacΔp74 strain for further Bac-to-Bac applications.

2.4. AcMNPV-bacmidΔp74 complementation

Accordingly with the bioinformatics analysis of the P74 protein, several baculovirus-transfer plasmids were constructed in pFastBac-Dual vector (GIBCO BRL Gaithersburg, MD, USA) carrying the green fluorescent protein (GFP) ORF under the *p10* promoter. Firstly, a synonym mutation (amino acid 458 -aspartic acid-; C_{1374} to T_{1374}) in the *p74* ORF from AcMNPV (NC_001623.1) was introduced by splicing by overlap-extension PCR using the primers pFwp74Ac, pRevp74Ac, pFwBamAc and pRevBamAc (Table 1) to add a BamHI recognition site between the amino (Nt) and carboxyl (Ct) protein domains. Then, the *p74* coding sequences of AcMNPV (mutated allele), SeMNPV and HearSNPV-G4 (NC_002169, NC_002654, respectively; both sequences naturally possessing the BamHI site in the desired position) were amplified by PCR using the corresponding primer pairs pFwp74Ac/pRevp74Ac or pFwP74Se/pRevP74Se or pFwP74Ha/pRevP74Ha (Table 1), and then cloned in phase with the 6xHis-tag of the pQe30 vector (Qiagen, Hilden, Germany) using BglII/BamHI (for insert or vector, respectively) and HindIII endonucleases, T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) and standard procedures [64]. These recombinant plasmids were used as source of complete, Nt or Ct P74 sequence domains to generate different ORF versions under the *polyhedrin* promoter in the pFastBac-

Dual-GFP vector [AcAc (Nt and Ct of *p74* from AcMNPV); SeSe (Nt and Ct of *p74* from SeMNPV); HaHa (Nt and Ct of *p74* from HearSNPV); AcSe (Nt of *p74* from AcMNPV and Ct from SeMNPV); AcHa (Nt of *p74* from AcMNPV and Ct from HearSNPV); SeAc (Nt of *p74* from SeMNPV and Ct from AcMNPV); Ac (only Nt of *p74* from AcMNPV); null (without *p74* ORF)] by the use of EcoRI, BamHI, HindIII, T4 DNA ligase and standard procedures [64]. These constructs (previously certified by Sanger sequencing; STAB VIDA, Caparica, Portugal; Macrogen, Republic of Korea) were used to generate recombinant baculovirus stocks (BacΔp74/GFP+p74AcAc; BacΔp74/GFP+p74SeSe; BacΔp74/GFP+p74HaHa; BacΔp74/GFP+p74AcSe; BacΔp74/GFP+p74AcHa; BacΔp74/GFP+p74SeAc; BacΔp74/GFP+p74Ac; BacΔp74/GFP) employing the DH10bacΔp74 strain and Bac-to-Bac protocols. Besides, a non *p74*-knockout version was produced (Bac/GFP) using the original bMON14272 bacmid. All virus stocks were stored at 4°C and tittered by end-point dilution assay [65,66].

Table 1. List of PCR primers**.**

Primer	Sequence (5′ to 3′)
FwP74seq	GGTTTTAACAGCCGTCGATTTA
RvP74seq	TAAGATGCATTTGTTGTCGAGC
pFwp74Ac	GA<u>AGATCT</u>ATGGCGGTTTTAACAG
pRevp74Ac	CCC<u>AAGCTT</u>AAAATAACAAATCAATTG
pRevBamAc	TAACCGAAC<u>GGATCC</u>CATAGCGC
pFwBamAc	GCGCTATG<u>GGATCC</u>GTTCGGTTA
pFwP74Se	ATC<u>AGATCT</u>GCTATGCTCACTTTTGTAGAC
pRvP74Se	ATC<u>AAGCTT</u>ATTCCGAATAGAGATTGTCGTAC
pFwP74Ha	ATC<u>AGATCT</u>TTCATGTCGAATATCATTTATATAC
pRvP74Ha	ATC<u>AAGCTT</u>ATGTGTATAAATTGTGGTACC
FwMe53	GCTTTGAAATGCACAACG

Restriction enzyme site sequences are indicated (underlined). AGATCT (BglII); AAGCTT (HindIII); GGATCC (BamHI).

2.5. OB generation for the recombinant baculoviruses

Both bMON14272 and bMON14272Δp74 do not express polyhedrin gene and therefore they do not generate OBs in cell culture. Due to this, a co-occlusion strategy was employed to produce infecting OBs using as helper a *p74* deficient virus that express polyhedrin. Thus, a recombinant baculovirus using bMON14272Δp74 and a pFastBac-Dual carrying mCherry ORF (under *p10* promoter) and AcMNPV polyhedrin ORF (under *polyhedrin* promoter) was generated (BacΔp74/Cherry+polH), multiplied and tittered as previously mentioned to produce BVs and OBs. All the OB productions were carried out by co-infecting Sf21 cells with each GFP-producing virus (providing variants of P74) with the mCherry-producing virus (providing polyhedrin) at a multiplicity of infection (MOI) 5 for each one. After 5 days of incubation at 25°C the cells were harvested, pelleted by centrifugation at 1,000xg for 5 min, and resuspended in 0.1% w/v SDS. OBs were separated from the cellular debris loading the lysate in a 40% w/v sucrose solution and centrifuging at 30,000xg for 30 min. The resulting pellets were washed with distilled water and centrifuged at 2,000xg for 5 min. Finally, after resuspending the OB pellets in distilled water they were quantified using a Neubauer chamber and an optical microscope thus generating AcMNPV-Δp74, AcMNPV-p74AcAc, AcMNPV-p74SeSe, AcMNPV-p74HaHa, Ac-MNPV-p74AcSe, AcMNPV-p74AcHa, AcMNPV-p74SeAc, AcMNPV-p74Ac, AcMNPV-p74wt. The presence of P74 protein in the OBs was verified by western blot, first resolving the viral proteomes in a 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and then using mouse anti-6xHIS as primary antibody (BD Biosciences, Franklin Lakes, NJ, USA) and a goat anti-mouse immunoglobulin conjugated with HRP as secondary antibody (Jackson ImmunoResearch Laboratories INC, West Grove, PA, USA). Protein bands were detected using an enhanced chemiluminescent substrate (ECL; Thermo Fisher

Scientific, Watham, MA, USA) and a C-Digit blot scanner (LI-COR, Bad Homburg, Germany).

2.6. Bioassays with recombinant AcMNPVs

Two methodologies were used to assess the infectivity of the recombinant viruses. On the one hand, 16 fourth instar *S. exigua* larvae were injected into the haemocoel with 5 µL of $5x10^4$ BVs of each different AcMNPV in MQ water (Grace's medium was used as negative control). The infections were confirmed by the visualization of fluorescence (Leica MZ10 F) and larvae were incubated at 26°C until death. On the other hand, to evaluate the oral infectivity of co-occluded recombinant viruses by the droplet feeding method [67], suspensions of OBs were diluted in a 10% v/v phenol red and 10% w/v sucrose solution to perform bioassays in early third instar *S. exigua* or *R. nu* larvae ($1x10^7$ OBs/mL). Exposed insects were maintained at 26°C and mortality was recorded each 12 h (*S. exigua*) or 24 h (*R. nu*) until all individuals died. The bioassays were performed side-by-side for all the viruses and repeated three times. Mortality curves were assessed using the Kaplan–Meier method and compared using the log-rank analysis (Mantel–Cox test) using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). The hemolymph was recovered from infected larvae to expose Sf21 cells growing in monolayers on 6-well plaques and was incubated for 4 days. The supernatants of infection recovered after this time were treated with PrepMan® Ultra reagent (Applied Biosystems, Waltham, MA, USA) to obtain the viral DNAs that was later used as PCR templates using the primers FwMe53 y RvP74seq (Table 1). These PCR products were sequenced by Sanger method (STAB VIDA, Caparica, Portugal) to confirm the genetic stability of the *p74* knockout.

3. Results

3.1. P74 phylogeny and protein domains

3.1.1. Phylogeny

The baculovirus PIF proteins are structural polypeptides associated with the ODV envelope and functionally essentials for the primary infection. P74 phylogeny reproduces the organization of *Baculoviridae* in the 4 recognized genera [6], showing an ancestral evolution pattern as the viral family diversified (**Figure 1**). Orthologs to this protein are found in other ancestrally related invertebrate virus families such as *Nudiviridae*, *Hytrosaviridae* and *Nimaviridae* [36], suggesting the existence of similar functional roles for this protein in those viruses..
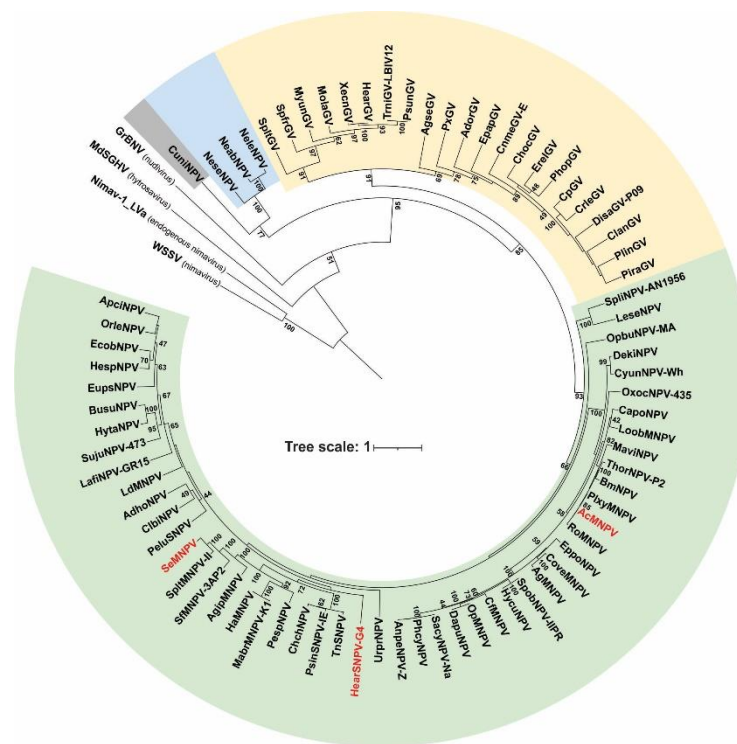
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Figure 1. Phylogenetic analysis of the P74 proteins. Evolutionary inference of the P74 protein con- 274
sidering species of the 4 baculovirus genera and representative species of other invertebrate viruses 275
(nudivirus, hytrosavirus and nimavirus). Bootstrapping values greater than 40 are shown. Al- 276
phabaculoviruses, betabaculoviruses, gammabaculoviruses, and deltabaculoviruses are shaded in 277
green, yellow, blue, and gray respectively. The 3 species of alphabaculoviruses considered in the 278
experimental studies are in red letters. 279

The P74 proteins considered in this work to evaluate their role in the AcMNPV PIF 280
complex belong to well-differentiated clades within alphabaculoviruses. Furthermore, 281
both AcMNPV and SeMNPV can infect *Spodoptera exigua* larvae (one of the models to be 282
evaluated with the P74 variants), while HearSNPV cannot. 283

3.1.2. Protein domains 284

A P74 similarity study considering baculoviruses from the 4 genera reveals the oc- 285
currence of few conserved motifs where it is possible to highlight 3 at the amino terminal- 286
region (Nt). Each of these 3 domains contain 2 cysteines (probably necessary for folding 287
by putative disulfide bonds), and 1 at the carboxyl-terminal region (Ct) containing a pu- 288
tative NES which could also contribute to the protein localization on the virion surface 289
(**Figure 2.A**). In this regard, 3 transmembrane (TM) regions can also be predicted (NES is 290
contained in TM-1) at the Ct, which would allow the polypeptide to be structured in the 291
ODV envelope exposing the Nt out of the virion (**Figure 2.B,C,D**) as previously reported 292
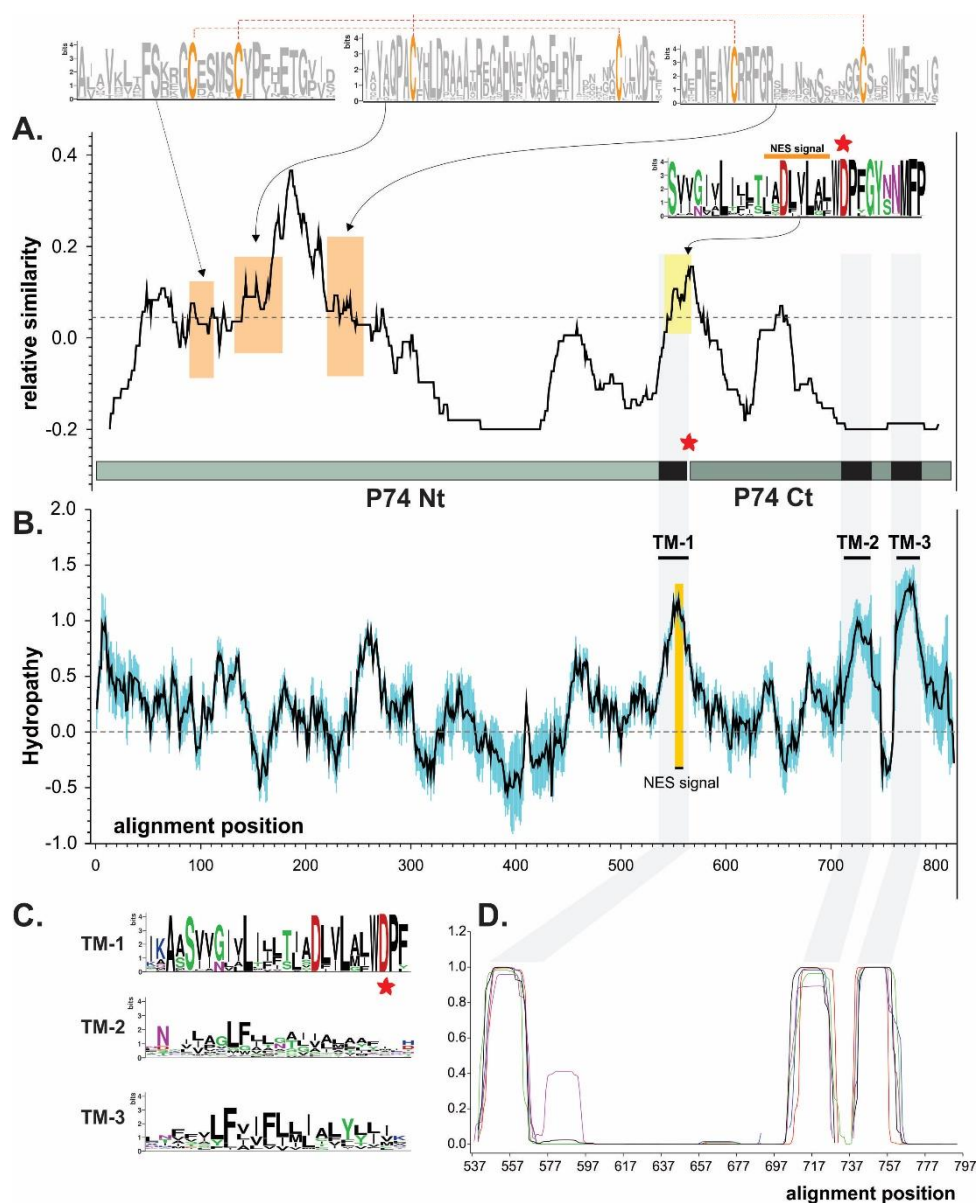[38]. 293

Figure 2. Domain architecture of P74 proteins. Bioinformatics studies for P74 protein consensus based on sequences from species of 4 baculovirus genera. **A**. P74 similarity plot. The dashed line indicates the average value. Sequence logos highlights the 6 cysteines perfectly conserved in all P74 (orange boxes). Dashed red lines shows the connectivity predicted with Disulfind server. The putative NES is also indicated (yellow box) and the point of separation between the Nt and Ct regions is marked with a red asterisk. The WDP triad contains a conserved BamHI site at the nucleotide sequence level (or the possibility of introducing it through a same-sense point mutation) further used on the construction of hybrid proteins. **B**. P74 hydropathy profile (black line). Variability was considered as the standard deviation (in blue). The 3 putative transmembrane regions (TM) are indicated, one of them (TM-1) containing a putative NES sequence. **C**. Sequence logos of each of the TM detected. **D**. TM predictions. These regions are highlighted in gray in all analyses.

This organization could suggest that the P74 protein has a sector with a structural role in the Ct (containing TMs), and another exposing a functional character in the Nt associated with the specific recognition for the host midgut cells. In fact, it is in the Nt where a protease-mediated activation process has been reported [43,44]. Interestingly, TM-2 and TM-3 differ from TM-1 in that they are in a region of minimal similarity; their physicochemical characteristics seem to be more important than the residues found there. By contrast, TM-1 is in one of the most conserved regions for P74 in *Baculoviridae*.

Immediately downstream of the NES (and at the end of the TM-1) appears a conserved amino acid triad consisting of tryptophan, aspartic acid, and proline (WDP). In this sense, the corresponding nucleotide sequence is "TGG GAY CCN" enabling the occurrence of a BamHI recognition site (--G GAT CC-). This is an opportunity to separate the ORF in 2 sectors (Nt of 458 vs Ct of 187 amino acids in AcMNPV) for any baculoviral *p74* (without affecting the amino acid sequence when in-phase chimerization are performed), thus assisting in a better analysis for this important factor.

3.2. AcMNPV knockout in p74 gene and complementation

3.2.1. CRISPR/Cas9 application

The AcMNPV bacmid was edited by CRISPR/Cas9 technology generating a knockout in the *p74* gene by NHEJ. To this, the Cas9/RNP complex was guided to the initial part of the *p74* ORF (207 bp downstream of ATG codon) and 3 mutant candidates were selected among 11 viral clones. Two of these affected viruses showed a deletion of 3 bp (nucleotides 205-207 and 203-205) and a third one presented a 4 bp deletion (nucleotides 207-210) in the *p74* ORF. Because the latter evidenced a frameshift that produce an early stop codon (**Figure 3**), it was the candidate of choice to continue the studies. After verifying the absence of off-target mutations by NGS whole genome sequencing, this mutagenized bacmid was then used to reconstitute the Bac-to-Bac system to carry out complementation assays with *p74* ORF variants. In this regard, 2 donor vectors were used to generate two types of viruses over the *p74* knockout backbone: one expressing P74 variants and GFP; and a second type expressing polyhedrin and mCherry. Thus, when cells are simultaneously infected by these recombinant bacmids orally infective particles could be generated (**Figure 1SM**).
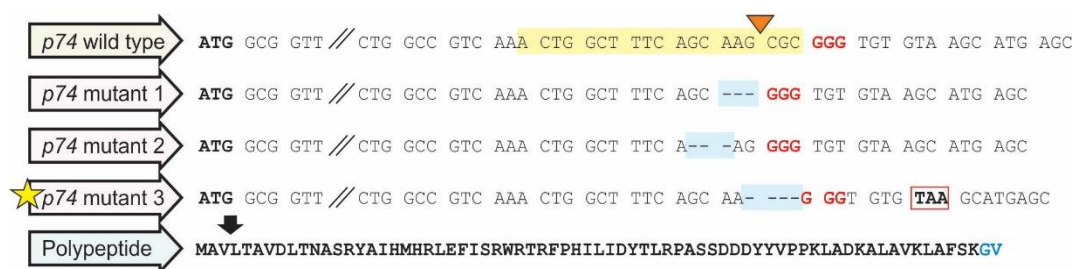

Figure 3. AcMNPV knockout in *p74* gene. Sequence result of the *p74*-knockout AcMNPV-bacmid. The cRNA recognition site is highlighted in yellow and the protospacer-adjacent motif (PAM) sequence is indicated in red. Dotted line shaded in light blue corresponds to the nucleotide deletion observed. The red box marks the early stop codon generated by the mutation, and the amino acids in blue are substitutions also consequence of the 4 bp deletion. Both initial and stop codons are in bold. The orange triangle shows where the DSB is expected to be generated. The mutant 3 was the selected one (highlighted with a yellow star).

3.2.2. AcMNPV OBs with P74 variants

Regarding the strict host specificity of P74, the corresponding ORFs from SeMNPV and HearSNPV (both species belonging to Group 2 *Alphabaculovirus* genus; **Figure 1**) were selected along with the Nt and Ct protein regions considering its separation at the natural BamHI site located in WDP, to generate *p74* chimeric alleles for their evaluation in the AcMNPV context (group 1 *Alphabaculovirus* genus; **Figure 1**). For the *p74* ORF from AcMNPV was first necessary to introduce a same-sense mutation to add a BamHI recognition site in the nucleotide sequence that translate in WDP. As the study of relative similarity among P74s of all baculoviral genera has shown (**Figure 2.A**), the protein of AcMNPV has a greater similarity in its Nt domain than in Ct with respect to its orthologs, although both with relatively low identity percentages (**Table 2**). Another striking fact is the difference in isoelectric point (PI) between the two sectors, being Ct predominantly acidic (**Table 2 SM**).

Table 2. P74 identity and similarity.   357

	P74	**AcMNPV**	**SeMNPV**	**HearSNPV**
Complete	**AcMNPV**	100	55	52
	SeMNPV	82	100	55
	HearSNPV	82	82	100
Nt	**AcMNPV**	100	59	57
	SeMNPV	85	100	59
	HearSNPV	83	84	100
Ct	**AcMNPV**	100	46	42
	SeMNPV	78	100	46
	HearSNPV	77	77	100

Values are expressed as percentages using whole numbers ("similarity" is shaded in light gray   358
and "identity" is indicated with uncolored background). "Complete" refers to the full protein   359
while "Nt" and "Ct" are respectively the amino and carboxyl terminal domains separated in the   360
amino acid triad WDP (containing at the nucleotide level a BamHI recognition site).   361

Different OB stocks containing variants of P74 (AcMNPV-p74AcAc, AcMNPV-   362
p74SeSe, AcMNPV-p74HaHa, AcMNPV-p74AcSe, AcMNPV-p74AcHa, AcMNPV-   363
p74SeAc, AcMNPV-p74Ac) and controls (AcMNPV-Δp74 and AcMNPV-p74wt) were   364
generated and produced in Sf21 cells using the knocked out-bacmid expressing poly-   365
hedrin and mCherry to co-occlude the viruses carrying the GFP-P74 versions (**Figure 1SM**   366
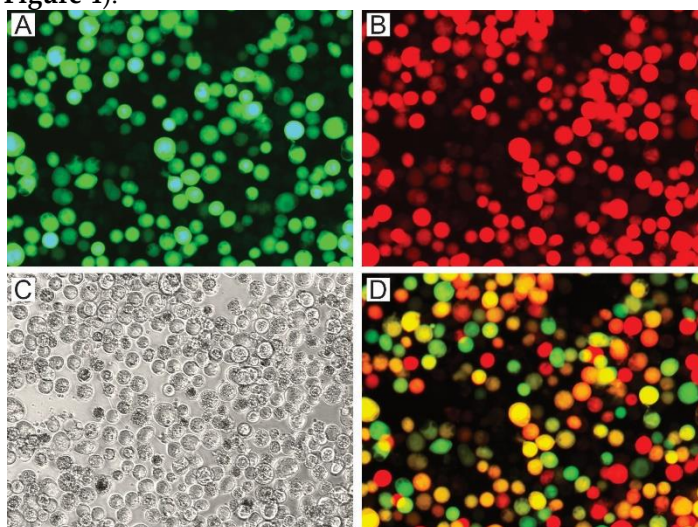**and Figure 4**).   367

368

Figure 4. Production of viral OBs with P74 variants. AcMNPV *per os* infective virions with P74   369
variants were generated in Sf21 cells by the co-infection of BVs expressing those proteins and GFP,   370
with BVs expressing polyhedrin and mCherry. Both BVs are based on the *p74*-knockout bacmid.   371
Representative microscopy photos (400X) are shown. **A**. Green fluorescent cells showing that the   372
infection induced GFP expression **B**. Red fluorescent cells showing that the infection induced   373
mCherry expression **C**. Bright field (the presence of OBs can be seen.). **D**. Merged image obtained   374
through the combination of the previous ones (A. and B.).   375

The green and red fluorescent cells, perceived as yellow and orange cells in the   376
merged image (**Figure 4.D**), shows that the co-infection occurred, and complemented OBs   377
could be generated. The presence of the P74 recombinant proteins in the OBs was then   378
confirmed by western blot using the 6xHIS-Tag added at the Nt (**Figure 5**).   379
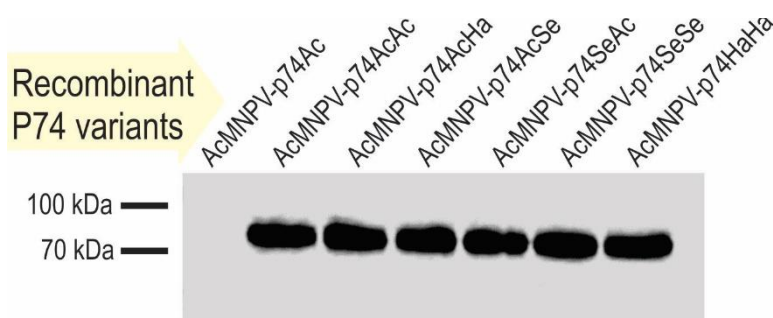
Figure 5. Recombinant P74 protein detection in OBs. Immunodetection of P74 variants in OBs by western blot using antibodies anti-6xHIS tag. The source of the OBs evaluated is indicated on each lane.

As indicated by the results of cell phenotype (**Figure 4**) and the immunoassay, all recombinant OBs could be adequately produced containing the different versions of the P74 protein. It was only not possible to detect the P74 Nt domain when not fused to any Ct, suggesting that it was probably found in a lower proportion, or that the tag was removed by some proteolytic activity.

3.3. Infectivity of AcMNPV variants

3.3.1. Infectivity of AcMNPV variants in S. exigua

The infectivity of all recombinant AcMNPVs was primarily validated by BV injections in *S. exigua* larvae (insect susceptible to oral and intrahemocelic AcMNPV infection) confirming that for all cases the secondary infection was not affected (**Figure 6 A, B**). In addition, to control the correct performance of the model under study, OBs of AcMNPV-p74AcAc were employed to *per os* infect the same invertebrate species (**Figure 6 C, D**).
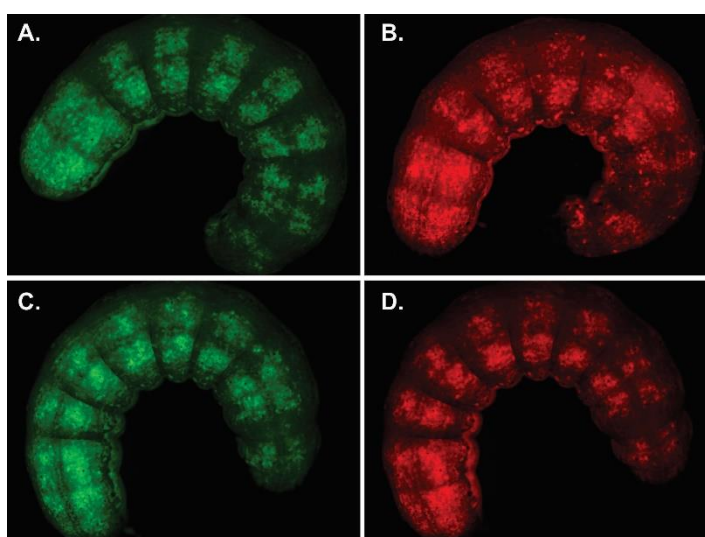
Figure 6. Infectivity of complemented AcMNPV *p74* knockout. *S. exigua* larvae exposed to virions derived from AcMNPV knocked out in *p74* and supplemented with the ORF variant of the same virus, AcMNPV-p74AcAc. (**A**.) Representative larva after BV injection showing GFP and (**B**.) mCherry expression. (**C**.) Representative larva after *per os* infection with OBs showing GFP and (**D**.) mCherry expression. All images (0.8X) were taken 120 hours after starting treatment.

These studies showed that cell-to-cell infection had not been affected, and that the 6xHIS-Tag (and the introduction of the single mutation to add the BamHI site in *p74* ORF from AcMNPV) did not prevent the success of the primary infection.

Comparative bioassays were then carried out to study the influence of the different P74 domains and variants on the viral specificity and pathogenicity (**Figure 7**).
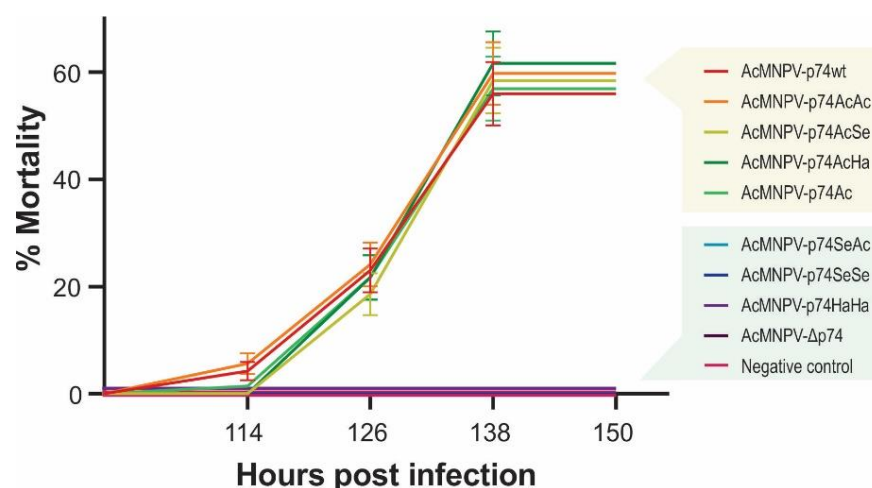
Figure 7. Virulence of the recombinant AcMNPVs in *S. exigua*. Mortality percentages of third instar larvae of *S. exigua* orally infected with the different variants of OBs. Results from 3 replicates are shown and standard deviations are included.

S. exigua larvae showed not to be susceptible to the *per os* infection with AcMNPV-Δp74, as expected since it does not express any P74, reconfirming its PIF nature as previously reported [18,45]. Thus, the remaining polypeptide of 71 amino acids resulting of NHEJ mutation in *p74* ORF (**Figure 3**) does not generate an infective phenotype, confirming the gene pseudogenization. In addition, the complementation with the P74 protein from SeMNPV (AcMNPV-p74SeSe) and HearSNPV (AcMNPV-p74HaHa) did not produce infectious OBs, regardless of whether this replacement is made from viruses for which *S. exigua* is naturally susceptible (SeMNPV) or not (HearSNPV). In the same direction, AcMNPV-p74SeAc also failed to infect the insects showing that the Ct domain would not contain the host-restricted functions. However, AcMNPV-p74AcAc, AcMNPV-p74AcSe, AcMNPV-p74AcHa and AcMNPV-p74Ac were infectious (as well as the positive control, AcMNPV-p74wt). Interestingly, not only AcMNPV-p74wt and AcMNPV-p74AcAc were equally infectious and lethal despite expressing P74 under different promoters (*p74* vs *polyhedrin* gene promoters, respectively), but also all of those that contained the P74-Nt domain from AcMNPV performed similarly (whether the motif was alone or combined with the Ct region of the other tested species), observing no significant differences in mortality among them. Although it was not possible to detect the Nt domain by western blot, this assay showed that it was present to rescue the virus infectivity. In all cases, the viral progeny produced in the larvae where the infection progressed were analyzed to certify the stability of the mutation in *p74* gene.

3.3.2. Infectivity of AcMNPV variants in R. nu

To analyze the influence of the host, similar assays were carried out in *R. nu* larvae (neither susceptible nor permissive for SeMNPV and HearSNPV, but orally and intrahemocelic infective for AcMNPV) obtaining equivalent results (**Figure 8; Figure 2SM**).
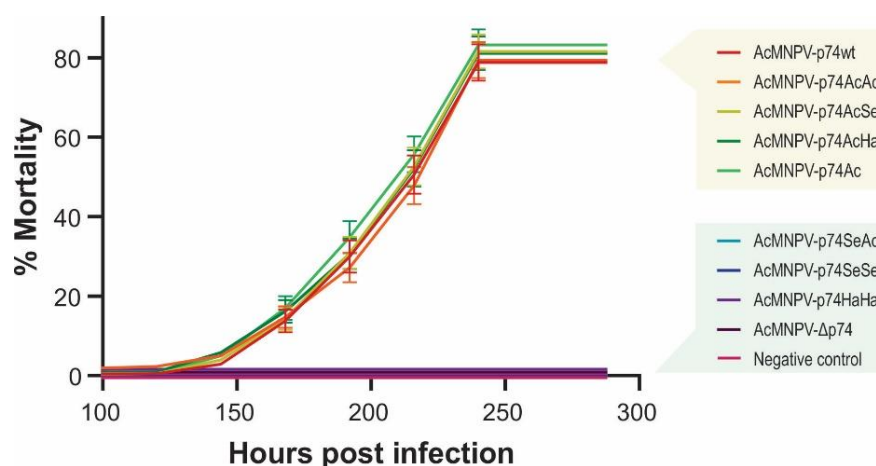
Figure 8. Virulence of the recombinant AcMNPVs in *R. nu*. Mortality percentages of third instar larvae of *R. nu* orally infected with the different variants of OBs. Results from 3 replicates are shown and standard deviations are included.

As observed in *S. exigua* larvae, AcMNPV-p74wt, AcMNPV-p74AcAc, Ac-MNPV-p74AcSe, AcMNPV-p74AcHa and AcMNPV-p74Ac were infective for *R. nu* and no significant differences were observed. Furthermore, signs of infection or mortality were neither detected for viruses that did not contain the AcMNPV P74 Nt domain, i.e., AcMNPV-p74SeAc, Ac-MNPV-p74SeSe, AcMNPV-p74HaHa and AcMNPV-Δp74.

4. Discussion

Previous studies failed to achieve infective baculoviruses when PIFs were replaced by orthologous sequences [36,68], except for some PIF3 replacements in Helicoverpa armigera nucleopolyhedrovirus (HearNPV) by the homologs from Spodoptera litura nucleopolyhedrovirus (SpltNPV) and Mamestra brassicae multiple nucleopolyhedrovirus (MbMNPV), which retained only partial oral infectivity [47,69], proving evidences of the strictness on the recognition process of the host in the primary infection. This may be due to the stability of the PIF complex in structural terms, and/or due to the need for specific interactions with host factors including cell receptors and activating enzymes such as proteases. This is the first study that analyze the expression of P74 chimeras (combining Nt and Ct domains from different species) during an infection cycle to try to find which region (if not the entire protein) is the one that carries such biological restrictions.

The non-expression of any PIF protein separately nullifies the infectivity of the ODVs in susceptible larvae. This would indicate that there are important structural and functional constraints where each intervening factor is required in an essential way. Three putative transmembrane domains were predicted in P74 protein consistent with previous reports [17,42], although other studies only identify the two closest (TM-2 and TM-3) to the carboxyl terminal end [36,38]. It is not clear whether TM-1 is a real transmembrane region, because truncated versions of P74 (only deleting TM-2 and TM-3 and replacing it with GFP) were soluble and apparently not anchored to the ODV membrane [45]. After being synthesized in the cytoplasm, PIFs must be imported into the nucleus for proper ODV assembly. Although most of the ODV envelope proteins contain an INM-SM sequence which tags them for nuclear import [38,40,70], no similar motif was reported in P74. Nonetheless, in this study the presence of a NES-like sequence was predicted at the end of TM-1, one of the most conserved regions for P74 in *Baculoviridae*. This kind of motifs are rich in hydrophobic residues (e.g., leucine), starts with an alpha-helix, and are usually involved in the transportation of proteins from the nucleus to the cytoplasm, binding with the export karyopherin known as chromosomal region maintenance 1 (CRM1 or exportin 1) [71]. Given the late appearance of the P74 protein during the infection cycle [18], it could be speculated that this region has the binding role needed for the conformation of the PIF

complex in association with the hypertrophied nucleus envelope of the infected cell. This could explain why truncated P74 versions lacking TM-1, TM-2 and TM-3 did not rescue oral infectivity, as demonstrated in previous works [45]. Our results showed that a truncated variant of P74 containing TM-1 was sufficient to rescue the infectivity of a P74 defective AcMNPV. This complementation was successful in the absence of TM-2 and TM-3, as well as when that region was replaced by orthologs from viral species of the same genus but different group, as SeMNPV and HearSNPV are. Remarkably, the bioassays also showed no significant differences in the infectivity or pathogenicity of these AcMNPV-P74 Ct domain replacement or missing variants, when compared to each other or even with AcMNPV-p74wt. This demonstrates that the acidic Ct region of 187 amino acids containing the most important TM regions (TM-2 and TM-3) seems to play a non-essential structural role and can be replaced by orthologous segments from other species (that naturally infect the same host or not) without compromising the P74 activity and the OB virulence.

The maintenance of infectivity does not occur when the Nt is replaced (regardless of whether it comes from a viral species infectious for the host), showing that this domain contains the functional restrictions. It is also interesting to note that the Nt supports peptide fusion without affecting its role, in accordance with previous evidence that shows that this region undergoes a proteolysis process for its activation [43,44]. These results together indicate that the region of the first 458 amino acids of P74 protein of AcMNPV is sufficient and essential to consolidate a functional PIF complex in the ODV envelopes and is responsible of the host-related restrictions.

It is important to highlight some methodological aspects associated with functional genomics studies which applies in this context. The introduction of knockout mutations is an indispensable tool for understanding gene functions. In baculoviruses, this type of research is traditionally performed by replacing viral sequences with marker or reporter genes [46,72]. Although this kind of strategy has made it possible to generate a lot of knowledge so far, it carries associated risks, such as the introduction of cryptic promoters or the elimination of sequences with other unknown important roles (e.g., miR genes, origins of replication, expression-modulators of neighboring genes, psi packaging like elements, etc.) that can impact on the viral cycle and on the virions that are produced (both morphologically and in their infectious capacities). Many of these functional sequences are usually contained within genes, so a knockout by replacement or deletion can affect several viral functions simultaneously [73–75]. Therefore, the application of CRISPR/Cas9 technology and NHEJ in baculovirus for these purposes appears as a better option due to its lower probability of multiple affectations and should now be the best tool for this type of genomic studies. In this sense, continuing with the work where the use of gene editing mediated by CRISPR/Cas9 is reported for the first time in baculovirus [50], the present study demonstrates its usefulness for baculovirus functional genomics research and proposes a methodological detail for its implementation henceforth.

5. Conclusions

The P74 protein is an essential factor to support primary baculovirus infection in a susceptible host, without affecting secondary infection. In this work it has been shown that the elimination of the transmembrane region of the terminal carboxyl end of P74 (an acidic part of low amino acid conservation but structural preservation) does not compromise its function, and that even this region can be replaced by orthologs of species that infect or do not infect the same host. However, considering our results and those of the literature, it seems that a central hydrophobic region (which can be hypothesized as TM and/or as NES) is essential for the activity and presumably necessary to associate this protein with the rest of the PIF complex and ODV envelope. This "hydrophobic core region" requires further research to elucidate its role because it is strikingly conserved in all baculoviral species. Furthermore, this study opens the door for future genetic editing mediated by CRISPR/Cas9 to improve/change the efficiency of the primary infection by

replacing regions of the *p74* gene in wild type baculovirus, thus increasing the biopesticide power in strategies of biologic control for crop pests.

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