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2 **Improvement of baculovirus as protein expression vector and as biopesticide by**

3 **CRISPR/Cas9 editing**

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23 **Abstract**

24 The CRISPR (Clustered Regularly Interspaced Short Palindromic repeats) system associated
25 Cas9 endonuclease is a molecular tool that enables specific sequence edition with high
26 efficiency. The edition using CRISPR/Cas9 system has been successfully reported in small and
27 large viral genomes. In this study, we have explored the use of CRISPR/Cas9 system for the
28 edition of the baculovirus genome. We have shown that the delivering of Cas9-sgRNA
29 ribonucleoprotein (RNP) complex with or without DNA repair template into Sf21 insect cells
30 through lipofection might be efficient to produce knocks-out as well as knocks-in into the
31 baculovirus. To evaluate potential application of our CRISPR/Cas9 method to improve
32 baculovirus as protein expression vector and as biopesticide, we attempted to knock-out several
33 genes from a recombinant AcMNPV form used in the baculovirus expression system as well as
34 in a natural occurring viral isolate from the same virus. We have additionally confirmed the
35 adaptation of this methodology for the generation of viral knocks-in specific regions of the viral
36 genome. Analysis of the generated mutants revealed that the edition efficiency and the type of
37 changes was variable but relatively high. Depending on the targeted gene, the rate of edition
38 ranged from 10% to 40%. This study established the first report revealing the potential of
39 CRISPR/Cas9 for the edition of baculovirus contributing to the engineering of baculovirus as
40 protein expression vector as well as a biological control agent.

41 **Keywords:** CRISPR/Cas9, baculovirus, AcMNPV, genome editing, knock-out, knock-in

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44 **INTRODUCTION**

45 Baculoviruses are rod-shaped DNA viruses with double stranded circular genome that infect
46 invertebrates, particularly insects of the order Lepidoptera (1). They are widely used as
47 biopesticide as well as versatile and powerful vector for recombinant protein expression (2). In
48 nature, the baculovirus mainly infects Lepidoptera larvae when they feed on plant contaminated
49 with the virus (3). During their viral biphasic life cycle, baculoviruses produce two distinct virion
50 phenotypes: occlusion derived viruses (ODV) and budded viruses (BV). While ODV are
51 involved in horizontal virus transmission from insect to insect through structures named
52 occlusion bodies (OB) which have the virus embedded within, BV are involved in spread of the
53 infection from cell to cell (4).

54 Autographa *californica multiple nucleopolyhedrovirus* (AcMNPV), the prototypic baculovirus
55 most common used for biotechnological purpose, has a circular genome of about 134 Kb that
56 contains 156 predicted open reading frames (ORF) (5). The baculovirus genomes, and specially
57 AcMNPV genome, have been genetically modified in order to enhance their pesticide potency
58 and increase the quality and quantity of the recombinant protein expressed in the system (6, 7).
59 These modifications include either the deletion of non-essential genes for virus survival or
60 infectivity and the insertion of foreign genes. For instance, in term of increase the insecticidal
61 activity of baculovirus, the cry1Ab gene from *Bacillus thuringensis* and neurotoxins from
62 scorpion venom have been incorporated into the baculoviru*s* genome (8, 9). In addition, the *egt*
63 gene (*Ac15*), involved in the inhibition of the molting, has been deleted from the virus
64 significantly improving the speed of killing of the virus (10). Moreover, different attempt has
65 been conducted to improve the heterologous protein expression in the baculovirus expression
66 system (BEVS). It has been reported that simultaneous deletion of non-essential genes for the *in*

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67 *vitro* replication of AcMNPV can enhance the expression of recombinant proteins (11, 12).
68 Another strategy implemented has been the addition of heterologous genes into de viral genome
69 with valuable properties for protein production (7).
70 The baculovirus expression vector system (BEVS) has become a powerful platform to produce
71 large quantity of recombinant proteins both in insect cells (Sf21, Sf9 and High Five™ cells) as in
72 Lepidoptera larvae. The recombinant virus was originally obtained by replacing the polyhedrin
73 gene from AcMNPV by co-transfection in insect cells with a viral genome and a donor plasmid
74 (13). Different improvements have been developed to optimize the method for the generation of
75 recombinant baculovirus. A remarkable success was the construction of the first bacmid that
76 contained the whole AcMNPV genome and could can be propagated in *Escherichia coli* cells
77 (14). The system, commercialized as Bac-to-Bac® system, is based on the introduction of a
78 heterologous gene from a shuttle vector to the bacmid by transposition, producing a 100%
79 recombinant progeny.
80 In the last years, multiple approaches for viral genome engineering have been develop. Among
81 the diverse genome editing technologies available, the clustered regularly interspaced short
82 palindromic repeats-Cas9 system (CRISPR/Cas9) appears as the most successful tool for editing
83 large viral genome (15). The system consists of a Cas9 endonuclease from *Streptococcus*
84 *pyogenes* that cleaves double-stranded DNA (dsDNA) and an RNA complex (sgRNA) that direct
85 sequence-specific dsDNA cleavage by Cas9. The double-strand break (DSB) are recognized and
86 repaired by the cell endogenous mechanisms through non-homologous end joining (NHEJ) that
87 produce insertions or deletions (indels), or homology-directed repair (HDR) in presence of a
88 donor template DNA with homology to the sequence flanking the DSB (16–18). Recent
89 developments in this technology have made possible to generated precise modifications into a

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90 wide variety of viral genomes (19). However, the CRISPR/Cas9 system has not been applied for
91 the edition of baculoviral genomes.
92 In this study, we developed for first time a CRISPR/Cas9-assisted method to edit AcMNPV
93 genome in multiple ways and with different purposes. We have shown that the delivering of
94 Cas9-sgRNA ribonucleoprotein (RNP) complex through lipofection in insect cells might be
95 efficient to generate gene knock-out and knock-in. To evaluate potential application of our
96 CRISPR/Cas9 method, we attempted to knock-out several genes from a recombinant baculovirus
97 used for the BEVS to study their effect on recombinant protein production as well as in a natural
98 occurring viral isolate to assess the potential of the methodology to improve baculovirus as a
99 pesticide. In addition, we have also show that our methodology can also be used for the efficient
100 introduction of foreign DNA on the genome of baculovirus.
101
102 **RESULTS**

103 **AcMNPV gene knock-out**
104 By direct transfection of the Cas9/RNP complex together to the viral genome of AcMNPV we
105 were able to introduce different mutations in the targeted genes. The procedure was reproduced
106 in several non-essential genes such as the *ODV-E26*, *F-Protein*, *p74* and *Ac18* with potential
107 application to increase the recombinant protein production using the BEVS. In addition, we also
108 attempted to edit *egt* gene to enhance the insecticidal properties of wild type (wt) AcMNPV.
109 The Cas9/sgRNA complexes were assembled *in vitro* for each target gene described in Table 1.
110 Individual viral clones were isolated from each edition by plaque assay and presence of indels in
111 each of the selected clones was assessed by PCR amplification of the targeted region and Sanger
112 sequencing.

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113 Sequencing analysis revealed the successful edition of several clones of each of the targeted
114 genes (Table 2). The edition efficiency and the type of changes was variable. Depending on the
115 targeted gene, the rate of edition ranged from 10% to 40% of the analyzed clones. All the edited
116 viruses had a deletion on the targeted gene, except for the *F-Protein*#1 that showed an insertion
117 of 125 nucleotides and *ODV-E26*#1 that contains a deletion of 4 nucleotides and an insertion of
118 16 nucleotides (Table 2). Except for one clone (*ODV-E26*#1), all the edited genes had a deletion
119 located at the expected region (adjacent to the PAM site). In agreement with the strand targeting
120 by the sgRNA (Table 2), all the deletion at the ODV-E26 gene were located down-stream from
121 the PAM sequence, while deletion for the other genes were located up-stream from the PAM
122 sequence. The effect of edition on the predicted protein was diverse. The different editions
123 introduced frameshift mutations producing early stop codons (*ODV-E26#2-4*, *F-Protein*#1,
124 *p74*#1, *Ac18*#1, and *egt*#2-4) or produce changes and deletion of few amino acids in the
125 predicted protein (*ODV-E26#1, Ac18*#2, and *egt*#1) (Table 2).
126
127 **Effect of mutations on viral replication and recombinant protein production**
128 To compare the performance of the individual viruses in cell culture we focused on the mutants
129 with a stop codon or a frameshift mutation derived from the edition of the recombinant virus
130 (derived from Bac-to-Bac® system) and expressing the GFP protein under the *pSeL* promoter.
131 No significant differences in the production of budded viruses was found among the different
132 viruses, suggesting that the produced knocks-out were not affecting the viral infectivity and
133 replication in cultured cells (Fig. 1). Next, we tested the recombinant protein production in Sf21
134 cell of the different viruses. For this purpose, Sf21 cells were infected with the AcMNPV
135 mutants at MOI 0.5 (Fig. 2A) and 5 (Fig. 2B). Three days post-infection, the cells were harvested

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136 and the GFP activity was measured. No statically significant differences between the control and
137 the mutants were found (p-value > 0.05) suggesting that knock-out of those genes does not have
138 major effect on the recombinant protein production in cell culture.
139 Insect larvae can be used as an inexpensive alternative to fermentative technologies for protein
140 production (7). We tested the effect of the previous mutations in protein production in *S. exigua*
141 larvae. Last instar larvae were infected by intrahemocelical injection with the AcMNPV mutants.
142 72 hours post-infection, GFP expression was estimated by measuring the fluorescence intensity
143 from the cellular extracts of the larvae (Fig. 3). No differences in GFP production were observed
144 for the F-protein, p74, and Ac18 knocks-out. Although, all the ODV-E26 mutants showed
145 protein expression values above the control viruses, only the values for the ODV-E26 #1 and
146 ODV-E26 #3 had statistically significant differences, with GFP production values of at least five
147 times higher than larvae infected with the control virus. Simultaneous quantification of the
148 different ODV-E26 clones together reported a significant increase of about 5-fold in protein
149 production (Fig.3). These results revealed that simple knock-out of *ODV-E26* using the
150 CRISPR/Cas9 can be used to increase the recombinant protein yield in insect larvae.
151

152 **Insecticidal activity of AcMNPV mutant**

153 We have additionally showed the generation of CRISPR/Cas9 knocks-out on the wild type
154 AcMNPV. Next, we tested if such viruses could become a recombinant free alternative to
155 improve the insecticidal properties of baculovirus. Bioassays were performed to evaluate the *egt*
156 mutants *in vivo.* Although we obtained different *egt* mutants (Table 2), the bioassay was
157 restricted to the mutant *egt*#2 (AcMNPV-Δ49egt), which introduced an early stop codon and a
158 frameshift mutation and to the wild-type (AcMNPV-WT) as control. At the tested concentration,

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159 no differences in mortality (pathogenicity) was observed between both viruses (Fig. 4), however,
160 as expected from previous EGT mutants (20, 21) the virulence (time needed to kill the insects)
161 was slightly higher for the AcMNPV-Δ49egt (Log-rank Mantel-Cox test; P<0.005). Under our
162 experimental conditions, the Median survival time values of AcMNPV-Δ49egt (120 h) was about
163 10% lower than the obtained with the wild-type AcMNPV (132 h).
164

165 **Gene Knock-in of wild type viruses**

166 The capacity of our procedure to introduce foreign DNA sequences into specific regions of the
167 AcMNPV genome was also tested. A fragment of DNA containing the *OpIE2* promoter and
168 terminator driving the expression of the e*GFP* gene and flanked by sequences from the
169 AcMNPV *chitinase* was used as a donor vector (Fig. 5A). The construction was then inserted
170 into the *chitinase* locus of a recombinant AcMNPV expressing polyhedrin under the *polh*
171 promoter (Bac-to-Bac system) using a similar procedure (see material and methods). Individual
172 viruses obtained after the transfection were isolated by plaque assay and the rate of
173 recombination was assessed by direct observation of eGFP production after infection of Sf21
174 cells (Fig. 5B). On average, 20% (4 out of 20) of the isolated clones were expressing GFP. In
175 addition, all the GFP positive clones were simultaneously expressing the recombinant *polyhedrin*
176 as reflected on the presence of viral OBs, indicating that CRISPR/Cas9-mediated edition does
177 not interfere with previous transgenes from the targeted virus.
178

179

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180 **DISCUSSION**

181 In this study, we have shown the potential of using the CRISPR/Cas9 technology for the edition
182 of baculovirus genomes to improve its use as protein expression system as well as insecticidal
183 agent. Gene edition using CRISPR/Cas9 system has been recently reported in other viral
184 genomes with biomedical implications such adenovirus (ADV) (22), type I herpes simplex virus
185 (HSV-1) (15, 23), HIV-1 provirus (24), Epstein-Barr virus (25) and Human Cytomegalovirus
186 (26). Our procedure has shown that baculovirus can also be edit with this system showing
187 relatively high efficiency in the generation of random deletions or insertions in all targeted genes
188 as well as specific gene insertions on the targeted loci.

189 Our procedure has shown efficient edition of all the genes that were initially targeted, though the
190 efficiency was variable and ranged from 10 to 40% depending of the targeted gene. Since the
191 transfection reactions were not systematically replicated, observed variability could be just
192 caused by the stochastic variation or other factors such as the targeted sequence and the selected
193 sgRNA (23). Although all the used sgRNA were validated *in vitro* and showed equivalent
194 efficiency, the *in vivo* efficiency could depend on other cellular factors associated to the viral
195 replication and the cellular repair after cleavage. It has been shown that *in vivo* efficiency can be
196 improved using dual or multiplex sgRNA strategy (27). It would be interesting to test if the
197 simultaneous use of multiple sgRNA targeting the gene of interest could increase the efficiency
198 in the edition of baculovirus.

199 One of the advantages of the CRISPR/Cas9 technique lies in the existence of many alternative
200 strategies for the delivering of the components into the nucleus (28). In our study, we transfected
201 the RNP and viral DNA complex together into cells and isolated the initial viral progeny. This
202 strategy has some advantages that could have contributed to the successful edition. First, RNP

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203 complex has easy access to the viral DNA due to simultaneous entry into the nucleus. Second,
204 the risk of generating off-target mutations is reduced because ribonucleoprotein complexes have
205 short half-life in the nucleus (29–31). In addition, in case of viral fitness-cost associated to the
206 introduced mutations, the isolation of the initial progeny prevents further dilutions of the
207 generated mutants with the non-edited genotypes.

208 In this study, we exploited the CRISPR/Cas9 system to engineering AcMNPV genome for
209 different applications. We knocked-out non-essential genes for viral survival or infectivity in cell
210 culture aiming to increase the production of recombinant proteins. We have shown that knock-
211 out of the *ODV-E26* gene, although does not enhances protein production in cell culture, had an
212 important increase in GFP expression in *S. exigua* larvae. The molecular function of the ODV-
213 E26 protein is unknown, but it has been shown to interacts with the IE0 and IE1 transcription
214 factors of AcMNPV (4, 32). These two factors are fundamental for the replication of the virus
215 since they are the main regulators and activators of transcription. It was described that the
216 binding sites for IE0 and IE1 in ODV-E26 are between amino acids 126-153 and 72-99
217 respectively. It was hypothesized that the function of ODV-E26 could be the regulation of these
218 2 factors, and as a consequence of ODV-E26 knock-out, the IE0 and IE1 levels increase,
219 contributing to the increase in expression of certain genes. In the present work the RNP produces
220 a cut-off between amino acid 23 and 24 and although some of the clones obtained had an early
221 stop codon or a frameshift mutation that affects the presence of the, the IE0 and IE1 binding
222 sites. However, ODV-E26#1 is not directly affected on the IE0 and IE1 binding sites and shows
223 a significant increase in protein production in the larvae.

224 The other knocked-out genes did not show effect on the protein expression in cells or larvae.
225 Nevertheless, our results have confirmed that their function is not essential for viral replication

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226 of the virus and open the possibility of using our procedure to test for simultaneous knocks-out
227 of these or additional non-essential genes for the enhancement in the protein production.
228 Previous studies have shown that single, as well as multiple deletion of certain viral genes, can
229 increase the production of recombinant protein without affecting the cell viability. Different
230 studies have shown that deletion of the *v-cath* and *chiA* genes, two enzymes responsible for the
231 liquefaction of infected larvae improve the expression and stability of the recombinant proteins
232 (11, 33–35). Latter improvements of these genomes were obtained by additional knocks-out of
233 other non-essential genes for *in vitro* replication as *p26, p10* and *p74* (12). Due to the simplicity
234 of the method reported here, procedures for systematic knock-out of all the AcMNPV predicted
235 loci could reveal additional know-outs with enhanced protein productions.

236 Alternative strategy for the enhancement of protein production with baculovirus have been
237 focused on the addition of certain genes into the viral genome. An example is the introduction of
238 *vankyrin* genes from an insect virus *Campoletis sonorensis ichnovirus* (Fath-Goodin et al. 2009)
239 that delays lysis of the infected cells, and an increased time for the expression of recombinant
240 proteins. In another hand, glycosylation performance has also been improved by the
241 simultaneous expression of glycosyltransferases (36, 37). Given the relatively high success rate
242 obtained the gene knock-in presented in this work, our procedure could be easily applied for the
243 simultaneous or serial insertion of those genes enhancing the expression, stability or properties of
244 the recombinant proteins.

245 We have additionally shown the application of our procedure for the edition of wild type viruses
246 and the generation of non-GM (genetically modified) viruses with potential applications in pest
247 control. As a prove of concept we have targeted the *egt* locus. Previous studies have revealed the
248 potential of truncation of this gene for the improvement of the insecticidal properties of

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249 baculovirus (10, 21, 38–40). Our *egt* knock-out have shown similar levels of improvement as
250 previous studies with other *egt* knock-out mutants generated by insertion on the *egt* locus of a
251 selecting marker (10, 21, 39) and consequently considered as GM virus. From an applied point of
252 view, and although certain countries are currently debating about the consideration of
253 CRISPR/Cas9 mutants as GM or not (41, 42), our results provide new tools for the generation of
254 GM-free virus with improved properties and potential use in the field. This methodology could
255 be extended to other viruses and other viral loci for the generation of new viral strains with
256 improved properties. For instance, two independent isolates from the *Cydia pomonella*
257 granulovirus (CpGV) that are able to overcome resistance in the codling moth to the classical
258 strains of CpGV have been recently isolated (43, 44). Although the mutations on these new
259 isolates responsible for the overcoming resistant phenotype are still unknown, gene edition using
260 CRISPR/Cas9 approaches could be used to engineering and improvement of new viral strains.
261 In summary, with this work we have shown how CRISPR/Cas9 methodology can also be applied
262 to the edition of baculovirus and could contribute to the engineering of baculovirus for the
263 increasing the protein yield and properties of the recombinant proteins expressed using the
264 BEVS as well as a to improve the biological properties of baculovirus as a pest control agent.
265

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266 **MATERIALS AND METHODS**

267 **Cell culture and insects**

268 The *Spodoptera frugiperda* (Sf21) insect cell line was cultured in Gibco® Grace's Medium (1X)
269 (Thermo Fisher Scientific, Waltham) supplemented with 10% heat-inactivated fetal bovine
270 serum (FBS) at 25°C.
271 *Spodoptera exigua* larvae were obtained from our laboratory colony. Larvae were reared on an
272 artificial diet at 25-27ºC with 70% relative humidity and a photoperiod of 16/8 hours (light/dark).
273

274 **Viruses and viral DNA**

275 Two types of AcMNPV genomes were targeted in this study. Wild type AcMNPV genome was
276 derived from the C6 strain. The virus was replicated in Sf21 cells under standard conditions and
277 the viral DNA was extracted using standard phenol/chloroform protocol (45). Briefly, Passage 1
278 (P1) viral stock was harvested and centrifuged at 500 × g for 5 min. BVs clarified were
279 concentrated by centrifugation at 40.000 x g for 1 hour. Pellet was resuspended in 200 µl Virus
280 Lysis Buffer (10 mM Tris-HCl pH 7.6, 10 mM EDTA, 0.5 % SDS) and 10 µl proteinase K (10
281 mg/ml) and incubated at 50 ºC for 1 hour with shaking. 200 µl of phenol/chloroform was added
282 and centrifugated at 14.000 × *g* for 10 min. The aqueous phase was transferred to another tube
283 and a 1/10 of volume of 3 M sodium acetate solution, 2 volumes of absolute ethanol, and 5 µl, of
284 glycogen (5 mg/ml) were added and kept overnight at -20 ºC. The solution was then centrifuged
285 at 14.000 x g for 15 minutes and the pellet was washed twice with ethanol 70 % by centrifuged at
286 8.000 x g for 5 minutes. The dry pellet was resuspended in 30 µl of TE (10 mM Tris-HCl pH 8.0,
287 1mM EDTA).

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288 In another hand, the bacmid DNA from recombinant AcMNPVs expressing eGFP under *pSeL*
289 promoter (46) or Polyhedrin under the *polh* promoter (47) were generated in previous studies of
290 the group using the Bac-to-Bac system (Thermo Fisher Scientific). Bacmid DNA was isolated
291 following the manufacturer's instructions.
292
293 **AcMNPV knock-out**
294 The following genes were targeted in the recombinant pSeL-GFP-AcMNPV baculovirus:
295 *ODV/E26* (NC_001623.1: 13092-13769), *F-Protein* (NC_001623.1:18513-20585), *p74*
296 (NC_001623.1:c121072-119135), and *Ac18* (NC_001623.1:c15459-14398). For the edition of
297 the wild type AcMNPV, *egt* gene (NC_001623.1: 11426-12946) was selected.
298 The ribonucleoprotein (RNP) complex consisting in the Alt-R *S. pyogenes* Cas9 nuclease in
299 complex with Alt-R CRISPR-Cas9 guide RNA (sgRNA) (IDT-Europe, Leuven) was generated
300 *in vitro*. The sgRNA was generated by combining of the crRNA (CRISPR RNA) with the
301 tracrRNA (trans-activating crRNA) in a 1:1 proportion, being the crRNAs the specific part of
302 each targeted genes. The crRNAs were designed using the CHOP-CHOP online platform
303 (https://chopchop.rc.fas.harvard.edu) and synthetized by Integrated DNA Technologies, Inc.
304 (IDT-Europe). Gene specific crRNAs forming sgRNAs with the highest on-target and lowest off-
305 target score were selected (Table 1). The targeted regions for each gene were limited to the 5'
306 region of the coding region (limited to the first 30 % nucleotides).
307 For the assembly of the sgRNA, 1 µl of each crRNA (1 µM) were incubated at 95 ºC for 5
308 minutes with 1 µl of Alt-R tracrRNA (1 µM) (IDT-Europe) and 98 µl of Nuclease-Free Duplex
309 Buffer (IDT-Europe). The RNP complex was then assembled in a final volume of 10 µl by
310 combining 1 µl of sgRNA (100 ng/µl), 6.2 µl of the Cas9 (1 mM), and 2.8 µl of nuclease free

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311 water. Finally, the mix was incubated at 37 ºC for 5 minutes and stored at 4 ºC. Efficacy of each
312 RNP complex was previously analyzed using *in vitro* Cas9 digestion (data not shown). One
313 microgram of purified viral DNA was co-transfected with 10 µl of the RNP complex *Sf21* cells
314 at 70% confluence in Grace's Medium without FBS by using Cellfectin® II Reagent
315 (Invitrogen). After 5 hours of incubation at 27 ºC, the medium was replaced by Grace's medium
316 supplemented with 10 % FBS and incubated for 72 hours at 27 ºC. The transfection efficiency
317 was confirmed by microscopic observation of GFP (for the pSeL-GFP-AcMNPV) or the
318 presence of viral occlusion bodies (for the wt-AcMNPV). The supernatant containing the virus
319 was cleaned by centrifugation at 500 x g and then stored at 4 ºC.
320 Individual viruses derived from each transfection event were isolated from the supernatants
321 (containing a mixture of edited and non-edited viruses) by plaque assay (O´Reilly et al, 1994).
322 The individual clones were amplified in *Sf21* cells and a fraction (50 µl) was treated with
323 PrepMan® Ultra reagent (Applied Biosystems™, Foster city) and used for PCR amplification of
324 the targeted region using specific primers (Table 3). PCR products were purified and sequenced
325 by Sanger sequencing to confirm the presence of mutations in the PAM-flaking region. Edited
326 baculoviruses were further amplified to high-titer stocks in Sf21 cells. Viral stock used in
327 subsequent studies were tittered by qPCR using specific primers for the viral *DNApol*
328 (Supplementary Table 1) as previously described (46). Non-edited baculovirus clones were also
329 included as a control.
330
331 **AcMNPV knock-in**
332 The pU57-Kan-Chi-eGFP donor plasmid (Fig. 5) was designed using SnapGene software (from
333 GSL Biotech; available at www.snapgene.com) and artificially synthesized by Genscript

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334 (Piscataway, NJ, USA). The length of upstream and downstream homology arms were 500 bp
335 long and targeted the *chitinase* gene from AcMNPV. The *eGFP* gene was cloned under the
336 control of the early viral promoter *OpIE-2*. The total length of the inserted sequence between the
337 two homology arms was 1539 bp.
338 The RNP complex was generated as described above. One microgram of Viral DNA (AcMNPV
339 expressing Polyhedrin under the *polh* promoter), 400 ng of the donor plasmid and 10 µl of the
340 RNP complex was co-transfect in *Sf21* cells at 70 % confluence in Grace's Medium without FBS
341 by using Cellfectin® II Reagent. For that, viral DNA and the donor plasmid were previously
342 mixed with the Cellfectin® II Reagent for 20 minutes, then the RNP was added and incubated
343 for additional 20 minutes. Seventy-two hours post-transfection, the supernatant containing the
344 virus was harvested and used to infect new Sf21 cells to confirm the presence of GFP foci
345 (representing knock-in viruses). Individual clones were further isolated by plaque assay and
346 amplified in *Sf21* cells. Correct location of the gene insertion on the chitinase locus was
347 confirmed by PCR amplification and sequencing of the flanking regions (Table 3).
348

349 **Infection assay in Sf21 cell culture and *S. exigua* larvae**

350 Sf21 cells were cultured in 24-well plate at a confluence of 70 %, then the cells were infected
351 with the different baculoviruses at multiplicity of infection (MOI) of 0.5 and 5 and incubated at
352 27 ºC. The cells were collected 72 hours post-infection by centrifugation at 500 x g for 5 minutes
353 and kept at -20 ºC until the quantification of GFP fluorescence expression.
354 *S. exigua* larvae were infected by intrahemocoelic injection with 5 µl of MQ-water containing 5
355 x 10^4 BVs of the different baculoviruses (15 larvae for each assay). Larvae were incubated at 25
356 ºC for 72 h, then were frozen at -20 ºC until its analysis.

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357

358 **Analysis of GFP expression**

359 Frozen cells were lysated in lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM DTT, 5
360 % glycerol) and centrifuged at 16000 x g for 1 minutes. The supernatants were used for the
361 determination of the expression of GFP by the determination of the emission of GFP
362 fluorescence in a microplate reader (TECAN infinite M200Pro) (λ excitation 485 nm and λ
363 emission 535 nm). Values were expressed as the relative GFP fluorescence intensity, taken as 1
364 of the value corresponding to the maximum intensity obtained with the control virus after
365 normalization to the total number of cells. For all of the experiments, the reported values
366 correspond to at least three independent replicates.

367 Frozen larvae were homogenised in group of 5 larvae in extraction buffer (0,01 % IGEPAL®
368 CA-630 (Sigma-Aldrich, Saint Louis), 1 mM PMSF, 25 mM DTT in PBS 1X). Homogenates
369 were centrifuged at 10,000 x g for 20 minutes at 4 ºC. Supernatants were diluted and used for the
370 determination of the expression of GFP as described above. The values were normalized
371 according to the total protein quantified by Bradford using the Quick Start™ Bradford reagent
372 (BioRad, Hercules, CA, USA). Values were expressed as the relative GFP fluorescence intensity,
373 taken as 1 of the value corresponding to the maximum intensity obtained with the larvae infected
374 with the control virus.

375 ***In vivo* evaluation of AcMNPV-Δegt mutant**

376 For the production of viral OBs, The *Sf21* cells culture ($6x10^6$ cells) were infected with wild-type
377 AcMNPV (strain C6) and *egt* edited virus at MOI 1 until most of cells were lysated. The
378 resulting OBs and remaining cells were collected by centrifugation at 1,000 x g for 5 minutes
379 and resuspension in 0,1 % SDS. The cell lysates containing the viral OBs were loaded over 40 %

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380 sucrose solution and centrifuged at 30,000 x g for 30 minutes, the pellet was washed with water
381 and centrifuged at 2,000 x g for 5 minutes. Finally, the pellet OBs were suspended in water and
382 quantified using a Neubauer chamber.

383 Bioassays were performed in third instar *S. exigua* using the droplet feeding method (48). Stock
384 suspensions of viral polyhedral ($2x10^8$ OBs/ml) were diluted, in a solution of 10 % sucrose and 1
385 % phenol red stain. Larvae were reared at 26 °C and mortality was recorded each 12 hours until
386 all larvae had died. The bioassay was performed side-by-side for the two viruses (AcMNPV-Δegt
387 and wt AcMNPV) and independently repeated three times. Mortality curves were assessed using
388 the Kaplan–Meier method, and compared using the log□rank analysis (Mantel–Cox test) using
389 GraphPad Prism software (GraphPad Software Inc., USA).

390 **Acknowledgments**

391 We thank Rosa Maria González□Martínez for her excellent help with insect rearing and
392 laboratory management. This work was partially supported by ERC Consolidator Grant 724519
393 Vis-a-Vis and the he Spanish Ministry of Economy, Industry and Competitiveness and by
394 European FEDER funds (grant AGL2014-57752-C2-2R).

395

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529 **Figures legend**
530
531 **Figure 1. Effect of mutations on viral replication.** Final viral titer of the different AcMNPV
532 mutants after 72 hours post infection in Sf21 cells infected at MOI 5. A non-edited virus was
533 included as a control.
534
535 **Figure 2. Effect of mutations on recombinant protein production in cell culture.** GFP
536 expression level in Sf21 cells infected with the different edited AcMNPV at MOI 0.5 (A) and 5
537 (B). The GFP expression was measured as relative fluorescence intensity at 72 hours post
538 infection. The results are expressed as the relative GFP fluorescence intensity, taken as 1 of the
539 value corresponding to the maximum intensity obtained with the control virus. The values are the
540 means of at least three independent assays. The error bars represent the standard error of the
541 mean. A non-edited virus was included as a control.
542
543 **Figure 3. Effect of mutations on recombinant protein production in larvae.** Analysis of GFP
544 expression level in *S. exigua* larvae infected with the different edited AcMNPV (A). The GFP
545 expression was measured as relative fluorescence intensity at 72 hours post infection. The results
546 are expressed as the relative GFP fluorescence intensity, taken as 1 of the value corresponding to
547 the maximum intensity obtained with the control virus. The values are the means of at least three
548 independent assays. The error bars represent the standard error of the mean. A non-edited virus
549 was included as a control. The average value obtained with the four different ODV-E26 mutants
550 is also reported (light grey bar). Representative image of *S. exigua* larvae infected with the
551 recombinant virus (72 hours post infection) (B).

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552 **Figure 4. Effect of *egt* deletion on AcMNPV virulence.** Time-mortality curves of the
553 AcMNPV-WT *vs* AcMNPV-Δ49egt viruses in *S. exigua* third instar larvae. The error bars
554 represent the standard error of the mean from 3 independent replicates.

555
556 **Table 1.** Summary of sgRNA characteristics
557

Target gene	cRNA Sequence (5'-3')	Localization[1]	Strand	GC (%)	Off-Target	Efficiency
ODV-E26	GTTCACAGAACCGACCGGCA	71	-	60	0	0.61
F-Protein	GATCTGGAATATGACGACAG	451	+	45	0	0.70
p74	AACTGGCTTTCAGCAAGCGC	192	+	55	0	0.72
Ac18	GCCGTAGTTTGCGAAAACGG	130	+	55	0	0.77
egt	GTTTGGTCACTTGTACGATC	461	+	45	0	0.52
chitinase	AGGCAATTTTGGACAGTTGA	720	+	40	MM3*	0.47

558 [1]Localization: from the sequence adjacent to the protospacer adjacent motif (PAM) to initial ATG; + Coding DNA Sequence; -
559 complementary DNA sequence. *Number of mismatches.
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Table 2. Summary of sequencing analysis of indels in target-site

TARGET GENE	SEQUENCE (5'-3')	INDELS	AMINO ACIDS	EFFICIENCY
ODV-E26	Wt TTGCCGTGCCGGTCGGTTCTGTGAACAGT #1 TTGCCGTGCAGTTTTTCTTTTTTCTCGGTTCTGTGAACAGT #2 TTGCCGTGC-----GGTTCTGTGAACAGT #3 TTGCCGTGC----------TGTGAACAGT #4 TTGCCGTGC--------TCTGTGAACAGT	 (Δ4, +16 pb) (Δ5 pb) (Δ10 pb) (Δ8 pb)	Wt LAVPV----GSVNSLTHTI #1 LAVQFFFFLGSVNSLTHTI #2 LAVRFCEQFDTHHHLHHRH #3 LAVL*TV*HTPSPPPPSPA #4 LAVLCEQFDTHHHLHHRHQ	40%
F-Protein	Wt CAAGATCTGGAATATGACGACAGCGGTGA #1 CAAGATCTGGAATATGACGA (+125 bp) CAGCGGTGA	 (+125 pb)	Wt EYDDSGEFDVYDEYEQPSHW #1 EYDENCAKISSLYVASAPVC	10%
p74	Wt TGGAACTGGCTTTCAGCAAGCGCGGGTGT #1 TGGAACTGGCTTTCA-----CGCGGGTGT	 (Δ5 pb)	Wt LAFSKRGCVSMSCYPFHETG #1 LAFTRV*KHELLSVSRNRRR	10%
Ac18	wt GAAGCCGTAGTTTGCGAAAACGGCGGTTT #1 GAAGCCGTAGTTTGCGAA--CGGCGGTTT #2 GAAGCCGTAGTTTGCGA------CGGTTT	 (Δ2 pb) (Δ6 pb)	wt VCENGGLFVLTGGAAVTCHI #1 VCERRFIRVNWRRSRDMPY* #2 VCD--GLFVLTGGAAVTCHI	20%
egt	wt AGTCGTCGTGGAAGCGTTTGCCGATTATGCGTTGGTGTTTGGTCACTTGTACGATCCGGCGC #1 AGTCGTCGTGGAAGCGTTTGCCGATTATGCGTTGGTGTTTG------------ATCCGGCGC #2 AGTG---ATCCGGCGC #3 AGTCGTCGTGGAAGCGTTTGCCGATTATGCGTTGGTGTTTGGTCACTTGTA--ATCCGGCGC #4 AGTCGTCGTGGAAGCGTTTGCCGATTATGCGT-----------------------------	 (Δ12 pb) (Δ49 pb, ,+1pb) (Δ2 pb) (Δ67 pb)	Wt LVVVEAFADYALVFGHLYDPAP #1 LVVVEAFADYALVFD---DPAP #2 LVIRRP*FKSRLATVWRKTLTR #3 LVVVEAFADYALVFGHL*SGAR #4 LVVVEAFADYA*TLTRSAPWRG	18%

Nucleic acid and amino acid sequence annealing of the targeting sites on the wild-type (wt) and the edited genes sequences. Bases in violet: protospacer adjacent motif (PAM); in blue: 20 nucleotides corresponding to the AltR cRNA recognition site; in red: insertion bases. Dotted line: each dot corresponding to a single base deletion. Amino acid in orange: amino acid from PAM site. Δ: number of bases deleted. +: insercions, * Stop codon.

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570 **Table 3.** Summary of Primers employed in the study
571

GENE	Orientation	Sequence (5'-3')
ODV-E26	F	CGTTTCCAGCGATCAACTAC
	R	TCTGTGCGTTGTCTTCTTCTGT
F-Protein	F	TATCATGGACGGTGTAAAGCTG
	R	GGATACAGTCATGTTGCTCCAA
p74	F	GGTTTTAACAGCCGTCGATTTA
	R	TAAGATGCATTTGTTGTCGAGC
Ac18	F	ATTGGCAAATAAAGTTGCAAGG
	R	GCACATTAACTGGTCGTTCAAA
EGT	F	CCAGTACAGTTATTCGGTTTGAAG
	R	GCTCTTTACAAGATGGATTCCTCC
DNA polymerase	F	GGGTCAGGCTCCTCTTTGC
	R	TTACGCAGCCATCACAAACAC
Chitinase insertion (left harm)	F	ACAACGCCGTACACAAACAA
	R	GTCCGGTCCGATAATGTGAT
Chitinase insertion (right harm)	F	CGACCACTACCAGCAGAACA
	R	CGCTGTCAAACGAAATCAAA

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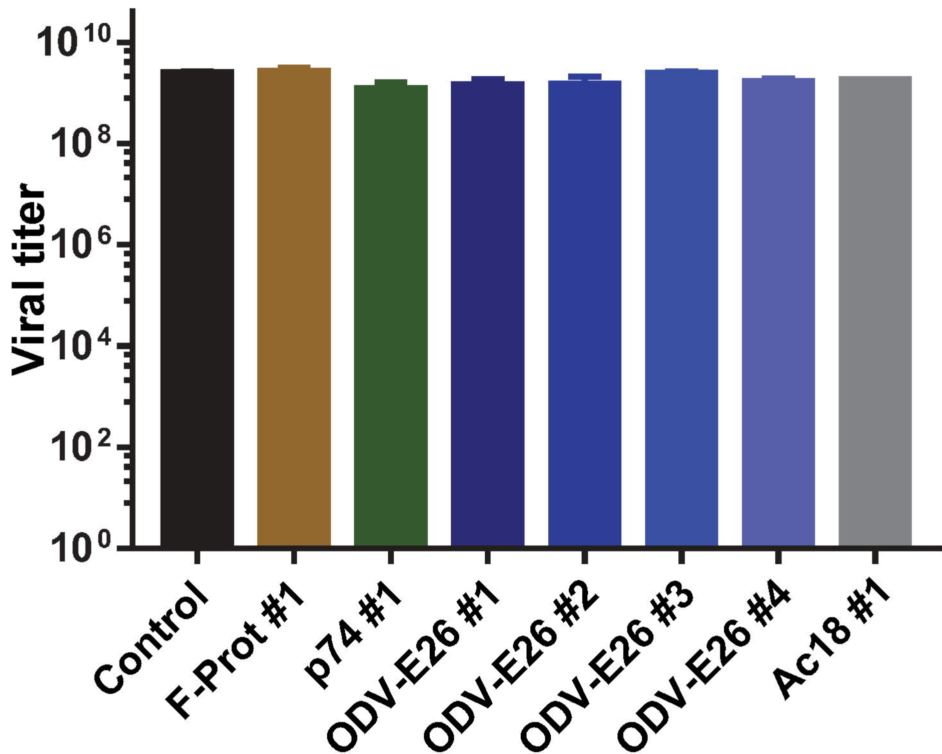

Viral titer
10^10
10^8
10^6
10^4
10^2
10^0
Control
F-Prot #1
p74 #1
ODV-E26 #1
ODV-E26 #2
ODV-E26 #3
ODV-E26 #4
Ac18 #1

A

Relative fluorescence intensity

1.5
1
0.5
0.0

F-Prot #1
p74 #1
ODV-E26 #1
ODV-E26 #2
ODV-E26 #3
ODV-E26 #4
Ac18 #1

B

Relative fluorescence intensity

1.5
1
0.5
0.0

F-Prot #1
p74 #1
ODV-E26 #1
ODV-E26 #2
ODV-E26 #3
ODV-E26 #4
Ac18 #1

A

Relative fluorescence intensity

7
6
5
4
3
2
1
0

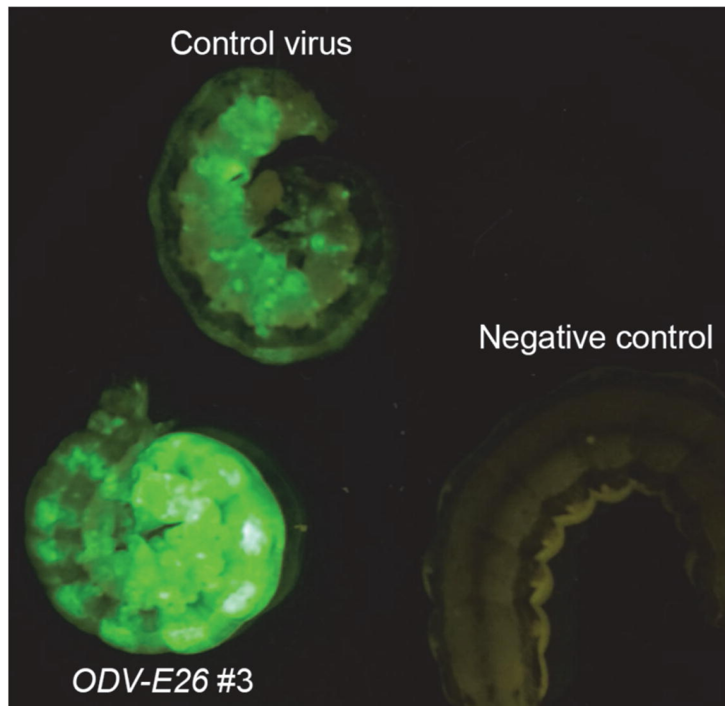
**

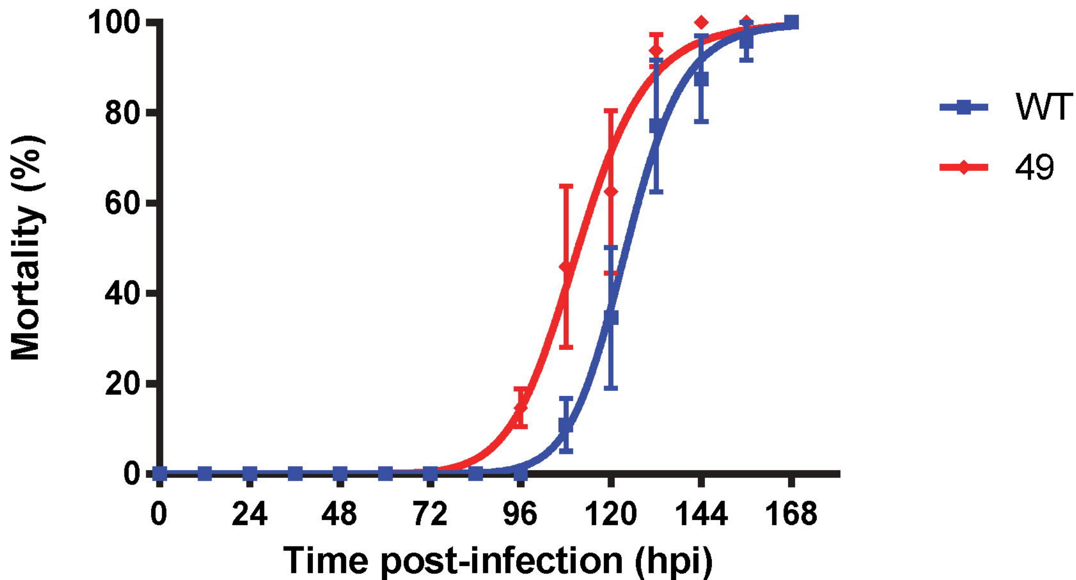
*

*

F-Prot #1
p74 #1
ODV-E26 #1
ODV-E26 #2
ODV-E26 #3
ODV-E26 #4
Ac18 #1
ODV-E26 All

B





100
80
60
40
20
0
Mortality (%)
0
24
48
72
96
120
144
168
Time post-infection (hpi)
WT
49

A

RNP Complex

Chitinase

CHI-left

OpIE2

eGFP

OpIE2-
pA

CHI-right

Donor DNA

Kan

HDR

OpIE2

eGFP

OpIE2-
pA

AcMNPV

B