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REDUCTION OF BACULOVIRUS REPLICATION IN INSECT CELLS BY INFECTION AND/OR COINFECTION WITH A RECOMBINANT BACULOVIRUS CONTAINING THE NS1 PROTEIN OF INFLUENZA A VIRUS

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Abstract: This study aimed to evaluate the effect of the NS1 protein from *Influenza A* virus in stablished insect cells lines from different hosts (BM5 cells from *Bombyx mori*, UFL-AG-286 cells from *Anticarsia gemmatalis*, IPLB-SF21cells from *Spodoptera frugiperda* and BTI-Tn-5B1-4 from *Trichoplusia ni*) and with different virus susceptibility by coinfection of these cells with a recombinant baculovirus (vAcNS1) derived from the Autographa californica multiple nucleopolyhedrovirus isolate L-1 (AcMNPV L-1) and wild-type baculoviruses (AcMNPV L-1, Anticarsia gemmatalis multiple nucleopolyhedrovirus, isolate AgMNPV-2D, and Bombyx mori nucleopolyhedrovirus isolate, BmNPV-I-01). Polyhedra inclusion bodies (PIB's) produced by wild type baculoviruses in coinfections with vAcNS1 was significantly reduced in all hosts insect cell lines tested. The production of budded virus was also decreased when the recombinant vAcNS1 was coinfected with all wild type baculoviruses. These results suggest that the NS1 protein expressed by vAcNS1 caused mostly adverse effects on the replication of baculoviruses in different cell lines.
Keywords: NS1 protein, RNAi, insect cells, coinfection.

Baculoviruses are insect viruses belonging to the *Baculoviridae* family that infect *Lepidoptera*, *Himenoptera* and *Diptera* [10, 12]. They have a supercoiled double-stranded DNA genome with an approximate size of 80-180 kbp [12, 16]. Baculoviruses have been used as protein expression vectors for more than 30 years and represent a well-established platform for the production of new biological products [1, 5, 24].

The ability of a baculovirus to efficiently replicate in the host cell depends on its capacity to bypass cellular defense mechanisms [20]. Insects, as well as other eukaryotic organisms, such as plants, fungi and other animals, have developed an RNA silencing system that limits viral replication [14, 17]. In insects, the RNA interference (RNAi) pathway plays a major role in antiviral responses to many RNA viruses. The mechanism includes double-stranded RNA genome cleavage or intermediates, produced during replication, in short viral interference RNAs (siRNAs) [3, 11, 28]. The nonstructural protein 1 (NS1) of *Influenza A virus* has been related to the inhibition of the antiviral defense mediated by interferon, the regulation of viral translation and inhibition of host mRNA by RNAi suppression mechanisms and other cellular functions [2, 15, 18, 23, 25, 26, 27].

This work aimed to evaluate the effects of the expression of an RNAi-suppressor from an animal RNA virus using a recombinant baculovirus containing the *NS1* gene of the *Influenza A virus*, in different insect cell lines.

BTI-Tn-5B1-4 from *Trichoplusia ni* [8], UFL-AG-286 from *Anticarsia gemmatalis* [24], BM5 from *Bombyx mori* [6] and IPLB-SF21 from *Spodoptera frugiperda* cell lines were used in this work. BTI-Tn-5B1-4 and UFL-AG-286 cells were maintained at 27°C in TC-100 medium (Vitrocell, Brazil) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, USA). BM5 and IPLB-SF21 cells were maintained at 27°C in TNM-FH medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, USA).

The wild type AcMNPV L-1 [22] and the AcMNPV-derived recombinant virus (vAcNS1) were propagated in BTI-Tn-5B1-4 cells (Fig. 1). BmNPV-I-01 (Fig. 1) isolate (kindly provided by R.M.C. Brancalhão from State University of Parana West, Cascavel, Brazil) was propagated in BM5 cells. AgMNPV-2D isolate (Fig. 1) [13] was propagated in UFL-AG-286 cell line.

Virus stocks were titrated in the appropriate permissive cell lines by the $TCID_{50}$ method following the protocol described by O'Reilly et al. [22].

The *NS1* gene of Influenza A virus was amplified by PCR using the specific oligonucleotides from a cloning vector based on the pBluescript SK (+) (Stratagene La Jolla, CA, USA) plasmid [19]. Amplification and vAcNS1 recombinant virus construction were carried out as described previously [19, 20]. To detection of the NS1 protein in infections, BTI-Tn-5B1-4, UFL-AG-286 and BM5 cells were seeded at a density of 4×106 cells per twelve-wells plate and after attachment, were infected separately with vAcNS1 at a multiplicity of infection (MOI) of 10. After 48 h post infection (p.i.), cells were harvested and pelleted (8000 x g for 5 min) and submitted to western blot [19].

To test the effect of NS1 on wild type baculoviruses, PIB's production on BTI-Tn-5B1-4, UFL-AG-286, BM5 and IPLB-SF21 cells, the infection, coinfections and counting of the PIBs was determined as described in Oliveira et al. [20]. Counting was repeated thrice in four different tissue culture plates for each virus analyzed.

To analyze budded virus production (BVs) of wild type baculovirus in mixed infections with vAcNS1, BTI-Tn-5B1-4, UFL-AG-286, BM5 and IPLB-SF21 cells, part of the supernatant of infected cells [20] were collected and used for virus titration using the $TCID_{50}$ method described by O'Reilly et al. [22]. The experiment was repeated thrice for each virus.

Statistical analyses were performed using BioEstat software and the results were subjected to analysis of variance (ANOVA) between two cells lines group and means compared by t-test, adopting a significance level of 5% ($p < 0.05$).

Recombinant vAcNS1 infected cells were shown to efficiently express the Influenza A NS1 protein in permissive, semipermissive and nonpermissive insect cells (Fig. 1 b).

The co-infection results showed a significant reduction in the yield of PIBs produced by wild-type baculovirus in cells coinfected with vAcNS1 in all tested host cell lines. (Fig. 1 c). The most significant result was observed in coinfection with vAcNS1 + AgMNPV-2D in UFL-AG-286 cells, followed by coinfection of BmNPV-I-01 + vAcNS1 in BM5 cells. The coinfection AgMNPV-2D + vAcNS1 in UFL-AG-286 cells, which are semipermissive to AcMNPV, resulted in the production of 3.71×10^5 PIBs/mL^{-1}, a reduction of 4.8-fold in PIBs number in relation to the infection with AgMNPV-2D (1.77×10^6 PIBs/mL^{-1}). Coinfection of BmNPV-I-1 + vAcNS1 in AcMNPV-nonpermissive BM5 cells resulted in the production of 6.87×10^5 PIBs/mL^{-1}, a reduction of 3.1-fold in PIB's production in relation to the wild type BmNPV-I-1 (2.20×10^6 PIBs/ml^{-1}). When AcMNPV L-1 was coinfected with vAcNS1 in AcMNPV-permissive IPLB-SF21 and BTI-Tn-5B1-4 cells, the PIB number reduced 1.8-fold and 1.5-fold, respectively in relation to AcMNPV L-1 alone. The amount of PIB's produced in IPLB-SF21 cells with AcMNPV L-1 infection was 2.11×10^6 PIBs/mL^{-1}, while the coinfection with vAcNS1 significantly resulted in to $1.19x10^6$ PIBs/mL^{-1}. In BTI-Tn-5B1-4 cells, the infection with AcMNPV L-1 produced $3.35x10^6$ PIBs/mL^{-1}, but coinfection with vAcNS1 caused a significant reduction in the polyhedra number to 2.21×10^6 PIBs/ml^{-1} in this permissive cell line.

The production of BV's was also reduced when the recombinant vAcNS1 was coinfected with the wild type BmNPV-I-1 in BM5 cell lines. The BV titer was reduced 1.9-fold compared to infection BmNPV-I -1 alone. BmNPV-I-1 produced a viral titer of 1.21×10^7 pfu/ml^{-1}, and the coinfection with

vAcNS1 showed a titer of 6.26×10^6 pfu/ml^{-1}. The same effect was shown in coinfections with the wild type baculoviruses and vAcNS1 in BTI-Tn-5B1-4, IPLB-SF21 and UFL-AG-286 cells but was less evident. In these cell lines, the reduction on BVs titers were of 1.6-fold, 1.1-fold and 1.2-fold, respectively in relation to the wild type baculoviruses alone (Table 1).

Previous research has indicated that the NS1 protein of Influenza A is known for its ability to suppress the RNA interference pathway in plants [2]. In this work, an AcMNPV recombinant virus carrying the Influenza A NS1 gene was shown to interfere with wild type baculoviruses replication during coinfections in different insect host cells that are AcMNPV permissive (BTI-Tn-5B1-4); semipermissive (UFL-AG-286) and nonpermissive (BM5).

The recombinant vAcNS1 influenced negatively the late viral expression of the wild type baculoviruses on infections and coinfections with AcMNPV L-1, AgMNPV-2D and BmNPV-I-01, in BTI-Tn-5B1-4 and IPLB-SF21, UFL-AG-286 and BM5 insect cells, respectively. In these infections, all wild type baculoviruses presented significantly decrease for PIBs production, even when the cell line was non-permissive to the recombinant virus (Fig. 1 c). When vAcNS1 was coinfected with the wild type baculovirus AgMNPV-2D in BTI-Tn-5B1-4 cell line, which is permissive to both wild type AgMNPV and vAcNS1, the PIBs production by wild type baculovirus was also reduced significantly (Fig. 1 c). On the other hand, the NSs gene from Tomato spotted wilt virus (TSWV), another gene silencing suppressor gene, was also inserted in a baculovirus genome and the recombinant virus (vAcNSs) was shown to enhance the replication capacity, heterologous protein expression and cytopathogenicity of the wild type baculoviruses AcMNPV, AgMNPV and BmNPV in different host cell lines [20].

Besides the reduction on wild type PIB's production, the budded virus (BVs) phenotype production was also affected by coinfection with vAcNS1. The amount of budded virus (BVs) was reduced when the cells were coinfected with wild type baculoviruses and vAcNS1, with the most significant difference found when BM5 cells were coinfected with BmNPV-I-1 and vAcNS1 (Table 1).

These results showed that the NS1 protein expression reduce baculovirus viral replication in permissive insect cells suggesting that NS1 could interference with essential factors necessary for viral replication. Other fact that endorses this disadvantage on replication showed by recombinant vAcNS1 is that the *NS1* gene is derived from a human virus. Insect cells have many pathogen defense mechanisms involving physical, chemical or biological barriers. Some baculovirus infections in non-permissive cells induce the shut off of cellular and viral proteins suppressing viral replication [7, 9].

On the other hand, the NS1 protein could interfere with gene expression of the baculovirus itself which are important for viral replication, virus particle assembly and progeny virus formation. The interruption of the Open Reading Frame 41 (ORF41) of Bombyx mori NPV showed reduced of viral replication and affected the PIB formation [25]. NS1 protein could also be able to induce cell death in insect cells infected with vAcNS1. Previous studies have shown that in infections by Influenza A virus, the NS1 protein is sufficient to induce apoptosis in MDCK and HeLa cells [23]. Influenza virus infection also induced a decrease in the expression of the anti-apoptotic protein Bcl-2 in A549 human lung carcinoma cells [26].

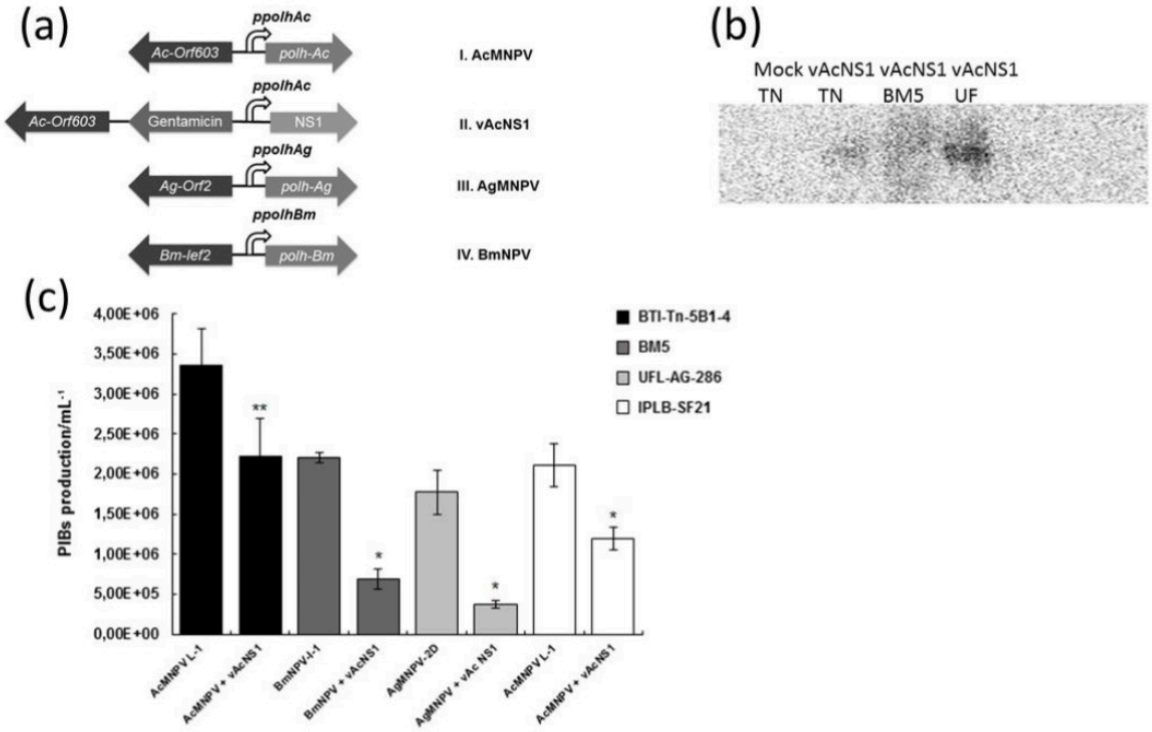

Fig. 1. **a)** Schematic representation showing the polyhedrin (*polh*) locus of different baculoviruses used in this study. (I) Wild type AcMNPV. (II) Recombinant vAcNS1; this virus has the *NS1* gene, under the control of the viral polyhedrin gene (*polh*) promoter (ppolhAc). (III) and (IV) wild types AgMNPV and BmNPV, respectively, which show the polyhedrin gene promoter (ppolhAg/ppolhBm) and the viral polyedrin gene (*polh*). The positions of gentamicin and viral genes (Ac-orf603, Ag-orf2 and Bm-lef2) on the left to the *polh* gene in the different viruses are also shown. **b)** Detection of NS1 by chemiluminescent western blotting immunodetection in BTI-Tn-5B1-4, BM-5 and UFL-AG-286 cells infected with recombinant baculovirus vAcNS1. Mock BTI-Tn-5B1-4 cells, BTI-Tn-5B1-4 cells infected with vAcNS1, BM-5 cells infected with vAcNS1, UFL-AG-286 cells infected with vAcNS1. **c)** PIBs production in insect cell lines infected with different baculoviruses. IPLB-SF21, BTI-Tn-5B1-4, UFL-AG-286 and BM5 cells were infected with wild type virus AcMNPV L-1, AgMNPV-2D and BmNPV-I-1, respectively, or were coinfected with recombinant vAcNS1. The PIB formation was monitored microscopically at 48 h p.i. Values correspond to media and standard deviation of the repetitions. *p<0,01 e **p<0.05 for Student's t-test between cells in the same group.

Cell lines	Virus inoculum (MOI:10)	Titer (pfu/mL^{-1})*	±SD*
BM5	BmNPV-I-1	1.21×10^{7}	1.18×10^{6}
	BmNPV-I-1 + vAcNS1	6.26×10^{6}	4.37×10^{5} **
BTI-Tn-5B1-4	AcMNPV L-1	9.77×10^{6}	1.79×10^{6}
	AcMNPV L-1 + vAcNS1	6.23×10^{6}	1.81×10^{6}
IPLB-SF21	AcMNPV L-1	1.01×10^{7}	1.02×10^{6}
	AcMNPV L-1 + vAcNS1	9.02×10^{6}	6.90×10^{5}
UFL-AG-286	AgMNPV-2D	8.44×10^{6}	1.40×10^{6}
	AgMNPV-2D + vAcNS1	6.91×10^{6}	1.05×10^{6}

Table 1: Virus titer (pfu/mL^{-1}) of the wild type baculovirus in host cells and co-infections with vAcNS1.

IPLB-SF21, BTI-Tn-5B1-4, UFL-AG-286 and BM5 cells were infected with wild type virus AcMNPV L-1, AgMNPV-2D and BmNPV-I-1, respectively, or were coinfected with recombinant vAcNS1. After 7 days p.i. infected cells' supernatants were used for virus titration by the $TCID_{50}$/ml method. *pfu* = plaque forming unit. * Values correspond to media and standard deviation for three repetitions. **p<0.05 for Student's t-test.

The NS1 protein probably do not suppress RNAi in AcMNPV permissive insect cells.

In summary, all findings in this work indicate a distinct function to the NS1 protein in lepidopteran insect cells and open possibilities to study the action of this recombinant baculovirus (vAcNS1) or the NS1 gene in others systems.

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ETHICAL STATEMENT

This research did not involve any animal and/or human participants.

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