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A silencing suppressor protein (NSs) of a tospovirus enhances baculovirus replication in permissive and semipermissive insect cell lines

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ABSTRACT

The nonstructural protein (NSs) of the *Tomato spotted wilt virus* (TSWV) has been identified as an RNAi suppressor in plant cells. A recombinant *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) designated vAcNSs, containing the *NSs* gene under the control of the viral polyhedrin (*polh*) gene promoter, was constructed and the effects of NSs in permissive, semipermissive and nonpermissive insect cells to vAcNSs infection were evaluated. vAcNSs produced more budded virus when compared to wild type in semipermissive cells. Co-infection of vAcNSs with wild type baculoviruses clearly enhanced polyhedra production in all host cells. Confocal microscopy analysis showed that NSs accumulated in abundance in the cytoplasm of permissive and semipermissive cells. In contrast, high amounts of NSs were detected in the nuclei of nonpermissive cells. Co-infection of vAcNSs with a recombinant AcMNPV containing the enhanced green fluorescent protein (*egfp*) gene, significantly increased EGFP expression in semipermissive cells and in *Anticarsia gemmatalis*-hemocytes. Absence of small RNA molecules of *egfp* transcripts in this cell line and in a permissive cell line indicates the suppression of gene silencing activity. On the other hand, vAcNSs was not able to suppress RNAi in a nonpermissive cell line. Our data showed that NSs protein of TSWV facilitates baculovirus replication in different lepidopteran cell lines, and these results indicate that NSs could play a similar role during TSWV-infection in its thrips vector.

1. Introduction

Baculoviruses comprise a very diverse group with double-stranded, circular DNA genomes, ranging from 80 to over 180 kb in size (Rohrmann, 2008). They have been applied as biological control agents against insect pests and have been used as vectors for high levels of heterologous protein expression in insect cells and insects (O'Reilly et al., 1992; Jarvis, 1997; Possee, 1997; Kamita et al., 2005). Their use in pest control is considered particularly safe due to their invertebrate-restricted infections (Granados and Federici, 1986; Ribeiro et al., 1998; Moscardi, 1999). Studies on baculovirus host specificity have shown that these viruses possess relatively narrow host ranges both *in vivo* and *in vitro* (Granados and Federici, 1986). Most investigations into the molecular basis of baculoviruses host range have been carried out with *Bombyx mori nucleopolyhedrovirus* (BmNPV), *Autographa californica nucleopolyhedrovirus* (AcMNPV) and *Lymantria dispar nucleopolyhedrovirus* (LdMNPV) (Rohrmann, 2008). Despite their genome similarities, significant differences in host range among these viruses have been demonstrated (Gomi et al., 1999; Katou et al., 2006). It has been demonstrated that the cell membrane does not act as a barrier against entry of AcMNPV viral particles in nonpermissive cells, since virus particles were shown to be internalized (Groener, 1986). Thus, the restriction in viral replication in nonpermissive insect cells occurs at the post entry level and maybe controlled or influenced by multiple cell line-specific factors (Miller and Lu, 1997).

Several attempts have been made to increase baculovirus virulence as a strategy for enhancing bioinsecticide action (Stewart et al., 1991; Tomalski and Miller, 1991; Kamita et al., 2005). These studies were based on viral genome manipulation by introduction of insect toxin genes and/or changing baculovirus infection cycle by deleting key virus genes for an efficient infection in insect cells (Stewart et al., 1991; Tomalski and Miller, 1991; O'Reilly and Miller, 1991; Pinedo et al., 2003). Efficient virus replication in host cells relies on the capacity of viruses to circumvent host defense mechanisms. Studies of host defense mechanisms against virus infection in different organisms have demonstrated that RNA silencing in plants or RNAi in animals has a well-established function as an antiviral defense mechanism (Lindbo and Dougherty, 1992; English et al., 1996; Fire et al., 1998; Wang et al., 2000). In response to these types of host antiviral defenses, many RNA viruses have acquired suppressor protein genes to counteract RNA silencing in

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plant (Carrington and Whitham, 1998; Li and Ding, 2001; Voinnet et al., 1999) or animal cells (Cullen, 2002; Gitlin et al., 2002).

Tomato spotted wilt virus (TSWV), the type species of the *Tospovirus* genus (De Haan et al., 1990; De Ávila et al., 1993), has a genome consisting of three single-stranded, linear RNA molecules (De Haan et al., 1990; Kormelink et al., 1993) coding for four structural proteins (L, N, G1, and G2) and two nonstructural proteins (NSm and NSs). The tospoviruses are the only plant-infecting members of the family *Bunyaviridae* (Bridgen et al., 2001), and replicate in their insect vector (thrips) (Wijkamp et al., 1993; Ullman et al., 2002). Nagata et al. (1999) showed that TSWV efficiently replicates and spreads into the thrips body, being able to establish virus infection in various tissues and reaching high virus titers in the salivary glands.

Similarly to the role of the *NSs* genes from related animal viruses (phleboviruses and orthobunyaviruses) in virulence (Elliott, 1990; Vialat et al., 2000; Bridgen et al., 2001; Billecocq et al., 2004; Le May et al., 2004; Ikegami et al., 2005; Blakqori et al., 2007), *NSs* of TSWV may also play an important role during virus infection in thrips due to the high accumulation of this protein in salivary glands of its insect vector (Wijkamp et al., 1993). The TSWV NSs protein has been shown to function as a strong suppressor of induced RNA silencing not only in plant cells (Takeda et al., 2002; Bucher et al., 2003), but also in tick cells (Garcia et al., 2006).

Based on these processes of gene expression regulation, and the action of NSs as a silencing suppressor in different cell systems, a recombinant baculovirus AcMNPV with the TSWV nonstructural *NSs* gene inserted into the viral genome was constructed and designated vAcNSs. In this report, the vAcNSs alone or coinfected with other baculovirus species, was able to efficiently enhance both its own gene expression and/or that in other baculoviruses. As a consequence, replication capacity was also enhanced in permissive *Trichoplusia ni* (BTI-Tn-5B1-4) and semipermissive *Anticarsia gemmatalis* (UFL-AG-286) insect cell lines. The possible roles of NSs protein during baculovirus infection and replication in the three different cell lines used in this work were discussed.

2. Materials and methods

2.1. Cells and viruses

Three cell lines established from lepidopteran insects were used: BTI-Tn-5B1-4 from *T. ni* (Granados et al., 1994); UFL-AG-286 from *A. gemmatalis* (Sieburth and Maruniak, 1988), and BM-5 from *B. mori* (Grace, 1967). BTI-Tn-5B1-4 and UFL-AG-286 cells were maintained at 27 °C in TC-100 medium (GIBCO-BRL Life Technologies) supplemented with 10% fetal bovine serum. BM-5 cells were maintained at 27 °C in Grace's medium (GIBCO-BRL Life Technologies), supplemented with 10% fetal bovine serum (TNM-FH medium).

The recombinant baculoviruses vHSGFP (Clarke and Clem, 2002), vAcNSs (this work), vAcCry4Aa and vSynNSm (B. Ribeiro, unpublished), and the wild type AcMNPV isolate L-1 (Lee and Miller, 1978) (Fig. 1) were propagated in BTI-Tn-5B1-4 cells (O'Reilly et al., 1992). AgMNPV 2D isolate (Johnson and Maruniak, 1989) was propagated in UFL-AG-286 cells. An isolate of BmNPV (BmNPV-I-01) was obtained from infected *B. mori* caterpillars of a Brazilian silk company (kindly provided by R.M.C. Brancalhão from Universidade Estadual do Oeste do Paraná, Cascavel, Brazil) was propagated in Bm-5 cells. The virus vHSGFP was derived from the AcMNPV L1 strain and has the gene for enhanced green fluorescent protein (EGFP) under the control of the *Drosophila melanogaster* constitutive promoter *hsp*70 at a site adjacent to the polyhedrin (*polh*) gene (Clarke and Clem, 2002). vSynNSm was derived from homologous recombination between the plasmid pSynNSm DNA and the vSynVI$^-$gal DNA (derived from AcMNPV, Wang et al., 1991) in insect cells. The plasmid pSynNSm was constructed inserting the TSWV's *NSm* gene into the transfer vector pSynXIVVI+X3 (Wang et al., 1991) where the gene is under the control of two promoters in tandem (P_{syn} and P_{XIV}) which have a similar very late expression activity to the *polh* promoter (Wang et al., 1991). The recombinant vAcCry4Aa has the *Bacillus thuringiensis cry4Aa* gene under the control of the *polh* promoter. Virus stocks were titered in the appropriate permissive cell lines by the $TCID_{50}$ method following the protocol described by O'Reilly et al. (1992).

2.2. Plasmids and recombinant virus construction

The *NSs* gene of TSWV was amplified by PCR from the pgR107-NSs plasmid vector (Lovato et al., 2008) using the specific oligonucleotides NSsF (5′-atgcaaaagcagggtgacaaa-3′) and NSsR (5′-agtagaaacaagggtgttttt-3′) (Integrated DNA Technologies) designed from the *NSs* gene sequence (GenBank access D13926). Amplification was carried out with Platinum Taq DNA Polymerase High Fidelity (Invitrogen) with one step at 95 °C for 2 min, 29 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min and a last step at 72 °C for 5 min. The amplified fragment was cloned into the pGEM®-T Easy cloning vector (Promega), using *Escherichia coli* DH5α cells as hosts, following the manufacturer's instructions. The DNA from the recombinant plasmid pGEM-NSs was digested with the *Eco*RI (Promega) enzyme and separated by electrophoresis in an agarose gel (0.8%). The DNA fragment of around 1.4 kb containing the entire *NSs* gene was purified from the gel using the GFX DNA extraction kit, according to the manufacturer's instructions (GE Healthcare Life Science) and cloned into the *Eco*RI site of the pFastBac^{TM}1 transfer vector (Invitrogen) under the control of the *polh* promoter (PH). The recombinant plasmid pFASTNSs was transformed into *E. coli* DH10Bac cells according to the manufacturer's instructions (Invitrogen). These cells contain the AcMNPV genome as a plasmid (bacmid) and after transformation, the gene cassette was transferred to the bacmid genome by site-specific transposition and the recombinant bacmid DNA was isolated following the manufacturer's instructions. All of the constructs were confirmed to have the expected sequences.

Bacmid DNA (2 μg) was transfected into BTI-Tn-5B1-4 cells (1.0×10^6 cells) using liposomes and following the manufacturer's instructions (Cellfectin, Invitrogen). The transfected insect cells were then incubated at 27 °C for seven days and the supernatant was collected and stored at 4 °C. Part of the supernatant was then used for the purification of viral DNA (budded virus form, BV) as described in O'Reilly et al. (1992).

To confirm the insertion of the *NSs* gene into the viral genome, 50 ng of the BV DNA were used in a PCR with the specific primers NSsF/NSsR as described above. The recombinant virus was named vAcNSs and was used to infect permissive (BTI-Tn-5B1-4), semipermissive (UFL-AG-286), and nonpermissive (BM-5) cell lines and *A. gemmatalis* larvae to monitor their cytopathic and pathogenic effects.

2.3. Western-blot analysis

The infected or co-infected cells were collected 48 h p.i. by centrifugation and the cell extracts were analyzed by SDS-PAGE and western-blot. The proteins were separated by electrophoresis in a 12% SDS-polyacrylamide gel using the Mini-PROTEAN Tetra electrophoresis system (Bio-Rad) and transferred onto nitrocellulose membranes (Immobilon-P Transfer Membrane, Millipore) by a Mini Trans-Blot Cell (Bio-Rad). After blocking with 1× TBS (Tris-buffered saline) containing 5% skim milk, the nitrocellulose membranes were incubated with a polyclonal antibody (1:1000 dilution in TBS plus 0.5% skim milk) raised in rabbit against NSs

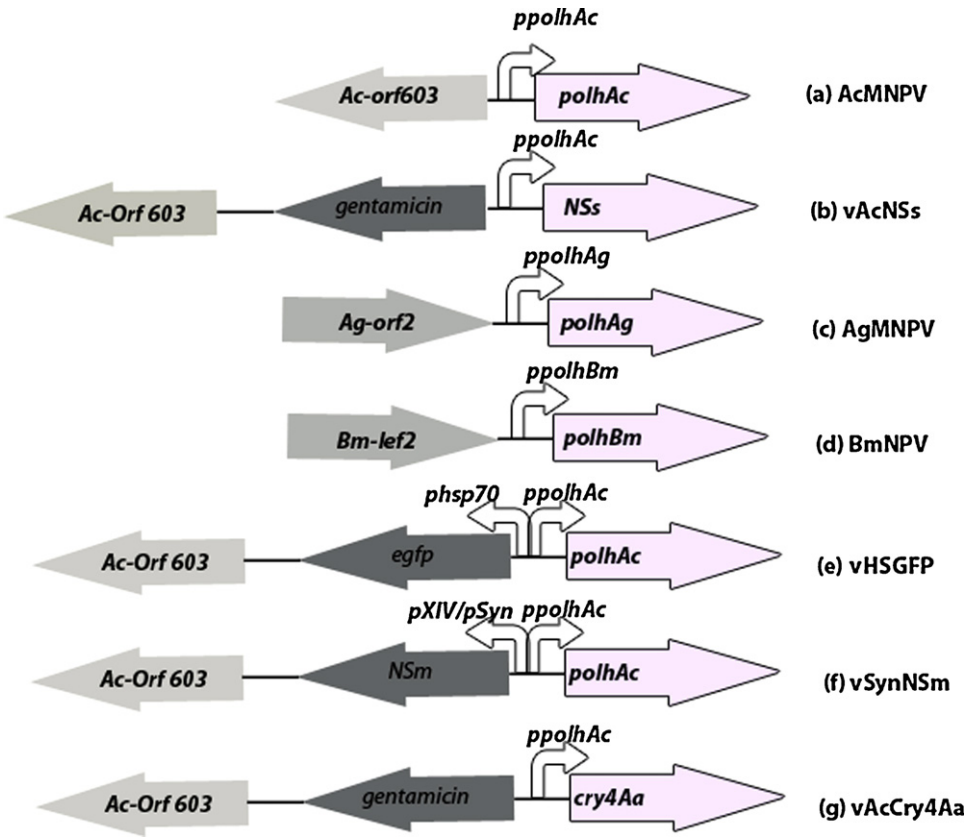

Fig. 1. Schematic representation showing the polyhedrin (*polh*) locus of different baculoviruses used in this study. (a) Wild type AcMNPV. (b) Recombinant vAcNSs; this virus has the *NSs* gene, under the control of the viral polyhedrin gene (*polh*) promoter. (c) and (d) Wild types AgMNPV and BmNPV, respectively, which show the *polyhedrin* gene promoter (*ppolh*) and the viral *polyedrin* gene (*polh*). (e) Reporter virus vHSGFP that contains the *egfp* gene under the control of the *Drosophila hsp*70 heat shock promoter at a site adjacent to the *polyhedrin* gene. (f) vSynNSm has the TSWV's *NSm* gene under the control of two promoter in tandem (P_{syn} and P_{XIV}). (g) Recombinant vAcCry4Aa; this virus has the *B. thuringiensis Cry4Aa* gene under the control of the *polh* promoter. The positions of gentamicin and viral genes (*Ac-orf*603, *Ag-orf*2 and *Bm-lef*2) on the left to the *polh* gene in the different viruses are also shown.

for 1 h at room temperature. Secondary anti-rabbit IgG conjugated with alkaline phosphatase was used for the enzymatic detection according to the manufacturer's instructions (Roche Diagnostics).

2.4. Titration of budded virus (BV)

BTI-Tn-5B1-4, UFL-AG-286 and BM-5 cells were individually seeded at a density of 4×10^6 cells per 25 cm × 25 cm tissue culture flask and after attachment, were separately infected at a MOI of 10 per cell with AcMNPV, AgMNPV, BmNPV and vAcNSs. After infection, the cells were incubated at 27 °C. Mock-infected cells were treated in the same manner but without addition of virus. After 48 h post-infection (p.i.), the supernatant was collected and titrated by the $TCID_{50}$ method described by O'Reilly et al. (1992). After 7 days of incubation under normal culture conditions, the occurrence of cytopathic effects was visualized by light microscopy (Axiovert 100, Zeiss) and documented using an AxioCam camera. Images were analyzed by the AxionVision Software (Zeiss). The experiment was repeated twice in three different tissue culture flasks for each virus analyzed.

2.5. Effect of NSs on production of wild type polyhedra inclusion bodies (PIBs)

BTI-Tn-5B1-4, UFL-AG-286 and BM-5 cells were seeded at a density of 4×10^6 cells per 25 cm × 25 cm tissue culture flask and after attachment, were infected separately with AcMNPV, AgMNPV, and BmNPV at a MOI of 10 or co-infected with vAcNSs. As an additional control, vHSGFP and a recombinant derived from AcMNPV carrying a TSWV's *NSm* gene under the control of two very late promoters in tandem, P_{syn} and P_{XIV} were also co-infected. Co-infections were performed using a MOI of 5 from each virus. After 48 h p.i., cells were harvested and pelleted (8000 g for 5 min). Each pellet was resuspended in 200 μl of cell lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1% Triton, 1 mM glycerol). The PIB number was determined by counting the number of polyhedra per milliliter of medium, using a hemocytometer and an inverted microscope (Axiovert 100, Zeiss). Counting was repeated twice in four different tissue culture flasks for each virus analyzed.

2.6. Immunofluorescent and confocal microscopy analysis

Immunofluorescent microscopy was used to localize the NSs in the different infected cell lines. For this, UFL-AG-286, BTI-Tn-5B1-4 and BM-5-infected and mock-infected cells were grown on sterile coverslips in six-well plates at 48 h p.i., were harvested and fixed (2% paraformaldehyde in PBS buffer) for 15 min. Following removal of the fixative, the cells on the coverslips were washed with 0.5% Triton X-100 in PBS for 10 min and then rinsed twice with PBS. Coverslips were blocked for 1 h in PBS–0.5% BSA and incubated for 1 h with the primary antibody (rabbit anti-NSs polyclonal antibody, as described above), in PBS–0.01% BSA diluted 1:1000. After washing, the coverslips were incubated for 1 h with anti-rabbit IgG conjugated with Alexa 594 (Molecular Probes, Carlsbad, CA, USA) diluted 1:500, in PBS–0.01% BSA. A cell line infected with vAcNSs, but not incubated with the primary antibody and mock infected cells were submitted to the same procedures and used as controls. Following these procedures, all cells were stained for 5 min with

4′,6-diamidino-2-phenylindole (DAPI) in PBS buffer at 0.1 μg/ml, rinsed again twice, and then mounted using an anti-fading (90% glycerol/10% PBS, including 0.1% n-propyl gallate) solution. Slides were viewed and photo-documented using a SP-5 Leica confocal microscope (40× oil objective) equipped with both UV laser and an Argon/Krypton laser, and prism spectrophotometer system to allow collection of the data at 488 and 594 nm. The software LAS AF (Leica Microsystems CMS GmbH) was used for analyzing two-color images and calculating three-dimensional reconstructions by 71–150 vertical projections of 0.1 μm sections of the cells.

2.7. EGFP expression in baculovirus-infected insect cells

Reporter gene expression was determined at 12, 24 and 48 h p.i. in BTI-Tn-5B1-4, UFL-AG-286 and BM-5 cell lines and hemocytes of *A. gemmatalis* larvae to evaluate the effect of the NSs protein on the infection of the recombinant vHSGFP (*egfp* containing virus).

Subconfluent cell monolayers grown in six-well plates were infected with vHSGFP alone (MOI of 10) or co-infected with vAcNSs (MOI of 5 for each virus). As AcMNPV does not replicate in BM-5 cells, the wild type BmNPV was used for infection and co-infection in these cells. As another control, the co-infection with vHSGFP and a recombinant AcMNPV carrying the *cry4Aa* gene from *B. thuringiensis* under the control of the *polh* promoter was also used. EGFP activity was monitored up to 3 days after infection. Cells were harvested, pelleted (8000 g for 5 min), and resuspended in 50 μl of 1% paraformaldehyde (PFA) buffer, 1 M NaOH, dissolved in dH_2O and 1× PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4). The EGFP fluorescence from the sample (150 μl) was analyzed with the software BD MultiSET cells in a FACSCalibur flow cytometer according to the manufacturer's instructions (BD Biosciences). Forward and side scatter parameters were used to eliminate dead cells and debris from the analysis.

The measurements of EGFP expression in vHSGFP-infected hemocytes alone or co-infected with vAcNSs were performed using hemocytes collected by bleeding the larvae at their last proleg. For this experiment, *A. gemmatalis* caterpillars were injected between the first and third abdominal segment of the caterpillar with 10 μl of viral inoculum directly into the hemocoel with a microsyringe. A viral dose of 1000 pfu was used for each virus. For each experiment cohorts of 30 caterpillars were examined at 12, 24, 48 and 72 h p.i. for EGFP fluorescence. Caterpillars were chilled for 10 min at 2 °C before hemolymph extraction. Hemolymph was collected by cutting an anal proleg and allowing the caterpillar to bleed. Fifty microliter of hemolymph was transferred into 50 μl of PFA buffer, and EGFP fluorescence was measured as described above.

2.8. RNAi reporter assay

Subconfluent cell monolayers (4×10^6 cells) of BTI-TN-5B1-4, UFL-AG-286, and BM-5 were separately infected with vHSGFP and vAcNSs or co-infected with both viruses as described above. At 48 h p.i., the cells were harvested (8000 g for 5 min) and RNA was isolated by extraction with Trizol (Invitrogen) following the manufacturer's instructions. Total RNA was analyzed by electrophoresis in 1% formaldehyde agarose gel (EDTA 0.1 M, MOPS 10× [MOPS 0.2 M, NA_2Ac 0.01 M, pH 5.5], H_2O 0.1% diethyl pyrocarbonate) and 1× Tris–borate–EDTA (TBE). The gel was stained with a 1× TBE containing ethidium bromide (0.5 μg/ml), and photo-documented to verify the RNA integrity. Small RNAs were precipitated with 5% polyethylene glycol 8000–0.5 M NaCl (Hamilton and Baulcombe, 1999). Low-molecular-weight RNAs were fractionated by 16% polyacrylamide–7 M urea gel electrophoresis, transferred to a Hybond-N+ membrane (Amersham Pharmacia Biotech), UV fixed and hybridized at 65 °C with a PCR amplified DNA probe corresponding to the *egfp* gene. The *egfp* PCR probe fragment was amplified using the primer pair EGFPF (5′-CCATGGTGAGCAAGGGGGA-3′)/EGFPR (5′-CCATGGGAATACTTGTAAGCTGGTC-3′) in a reaction containing 50 ng of viral vHSGFP genome DNA. Amplification was carried out with Platinum Taq DNA Polymerase High Fidelity (Invitrogen) with one step at 95 °C for 2 min, 29 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min and a last step at 72 °C for 5 min. The amplified fragment was labelled with 2.5 mM of digoxigenin-11-dUTP-peroxidase that was added to the reaction mixture described above (Roche Diagnostics). Hybridization and post-hybridization washes were conducted with SuperSignal West Pico Chemiluminescent Substrate kit according to the manufacturer's instructions (Thermo Scientific Life Science). A 24-nucleotide primer was used as a size marker in the RNAi gel analysis. A Typhoon 8600 imager (Molecular Dynamics) was used for photo-documentation.

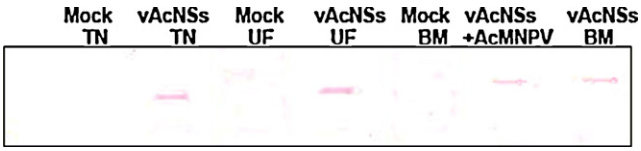

Fig. 2. Western blot analysis using polyclonal antibodies against NSs analysis of BTI-Tn-5B1-4, UFL-AG-286 and BM-5 cells infected with recombinant baculovirus vAcNSs or co-infected (vAcNSs + AcMNPV). Mock BTI-Tn-5B1-4 cells, BTI-Tn-5B1-4 cells infected with vAcNSs, mock UFL-AG-286, UFL-AG-286 cells infected with vAcNSs, mock BM-5, BM-5 cells co-infected with vAcNSs and AcMNPV, BM-5 cells infected with vAcNSs.

3. Results

3.1. Recombinant vAcNSs replication in different insect cells

Using the Bac-to-Bac Baculovirus Expression System a recombinant baculovirus containing the TSWV *NSs* gene was generated (Fig. 1b). vAcNSs infected cells were shown to efficiently express TSWV NSs protein when the gene was under regulatory control of the polyhedrin (*polh*) gene promoter in permissive, semipermissive and nonpermissive insect cells by immunodetection (Fig. 2).

BTI-Tn-5B1-4 (permissive), UFL-AG-286 (semi-permissive) and BM-5 (nonpermissive) cell lines were infected with the same amount of the recombinant vAcNSs and wild type baculoviruses (Fig. 1a, c and d) and virus titers were determined from the supernatants of infected insect cells at 48 h p.i. As shown in Table 1, vAcNSs accumulated to a higher titer when compared to wild type AcMNPV, in permissive and semipermissive cell lines. At 48 h p.i., vAcNSs reached titers of 1.02×10^9 pfu/ml in UFL-AG-286 cells, representing around 500-fold increase compared to wild type AcMNPV (Table 1). Even compared to AgMNPV titers in UFL-AG-286 cells

Table 1
Virus titer (pfu/ml) after infection of AcNSs, AcMNPV and AgMNPV with a MOI of 10 for each virus at 48 h postinfection in different insect cell lines.

Virus inoculum (MOI: 10)	Cell line[a]	Titer (pfu/ml)[b]	±SD[b]
vAcNSs	BTI-Tn-5B1-4	8.06×10^8	4.92×10^8
	UFL-AG-286	1.02×10^9	1.14×10^8
AcMNPV	BTI-Tn-5B1-4	2.63×10^7	3.55×10^6
	UFL-AG-286	2.31×10^6	2.21×10^5
AgMNPV	BTI-Tn-5B1-4	4.29×10^6	3.8×10^6
	UFL-AG-286	1.03×10^8	8.34×10^7

[a] Baculovirus replication in two insect cell lines. Infected cells' supernatants were used for virus titration by the $TCID_{50}$/ml method. AcMNPV, *A. californica nucleopolyhedrovirus*, AgMNPV, *A. gemmatalis nucleopolyhedrovirus*, vAcNSs, recombinant virus derived from AcMNPV containing the NSs gene from TSWV under the polyhedrin promoter control. BTI-Tn-5B-1-4, insect cell line derived from *T. ni* and UFL-AG-286, insect cell line derived from *A. gemmatalis*.

[b] Values correspond to media and standard deviation for three repetitions.

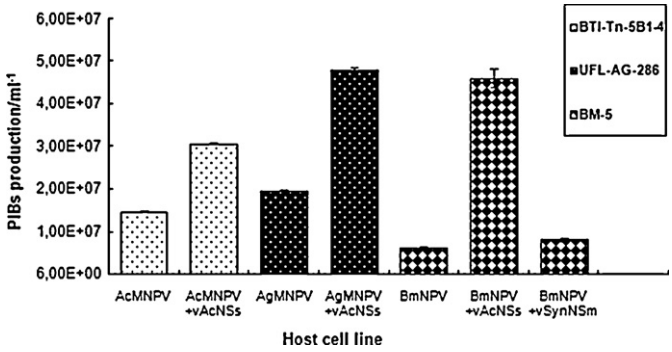

Fig. 3. PIB production in three insect cell lines infected with different baculoviruses. The TSWV NSs protein enhanced PIB production of the wild-types AcMNPV, AgMNPV and BmNPV in all host cell lines. BTI-Tn5B1-4, UFL-AG-286 and BM-5 cells were infected with AcMNPV, AgMNPV and BmNPV alone or in co-infections with vAcNSs (ooc$^-$ virus) and vSynNSm (ooc$^+$ virus) (in BM-5 cells), and the PIB formation was monitored microscopically at 48 h p.i.

that are permissive to this virus, vAcNSs titers were significantly higher in this cell line. Cytopathic effects were observed in BM-5 cells caused by the recombinant virus infection. However, no significant amount of BVs was detected in this cell line (data not shown). These results could be due to a toxic effect of the NSs protein present in the viral inoculums.

3.2. PIB production in permissive, semipermissive and nonpermissive cells

Single and mixed infections of the recombinant vAcNSs with the wild-types of AcMNPV, AgMNPV and BmNPV on their host cells were assayed to determine the effect of NSs in polyhedra inclusion body (PIB) production. Compared to the PIB production obtained with the wild type viruses, expression of NSs clearly enhanced PIB production of the wild type viruses in all host cells when co-infected with vAcNSs (Fig. 3). Notably when BmNPV was co-infected with vAcNSs in BM-5 cells, the PIB production was 7.5 times higher (4.61×10^7 PIBs/ml) than BmNPV alone (6.13×10^6 PIBs/ml). For comparison, BM-5 cells were also co-infected with BmNPV and another recombinant AcMNPV expressing the nonstructural protein NSm of TSWV (vSynNSm). No significant increase in polyhedra production was observed (8.11×10^6 PIBs/ml) compared with the BmNPV infection alone (Fig. 3). The co-infection of AcMNPV with vAcNSs, increased PIB number only twice as much in BTI-Tn-5B1-4 (3.07×10^7 PIBs/ml), when compared to AcMNPV alone (1.47×10^7 PIBs/ml). Similarly, wild type AgMNPV and vAcNSs co-infection produced PIB approximately twice as much (4.82×10^7 PIBs/ml) than AgMNPV alone (1.95×10^7 PIBs/ml) in UFL-AG-286 cells.

3.3. Localization of the NSs protein in baculovirus-infected insect cells

The NSs protein expressed by vAcNSs in three different insect cell lines was localized by immunofluorescence. BTI-Tn-5B1-4, UFL-AG-286 and BM-5 cells were grown on coverslips and infected with vAcNSs. Cells were collected at 48 h p.i. and incubated with a rabbit anti-NSs polyclonal antibody followed by incubation with an anti-rabbit IgG conjugated with Alexa 594. Confocal microscopy showed that NSs was present in all infected insect cell lines (Fig. 4a–c). The NSs protein was abundant in the cytoplasm of infected BTI-Tn-5B1-4 (permissive) and UFL-AG-286 (semipermissive) cells (Fig. 4d and f). Surprisingly, NSs was detected predominantly in the nuclei, especially in the perinuclear region of infected BM-5 (nonpermissive) cell line (Fig. 4h). This was clearly visible after

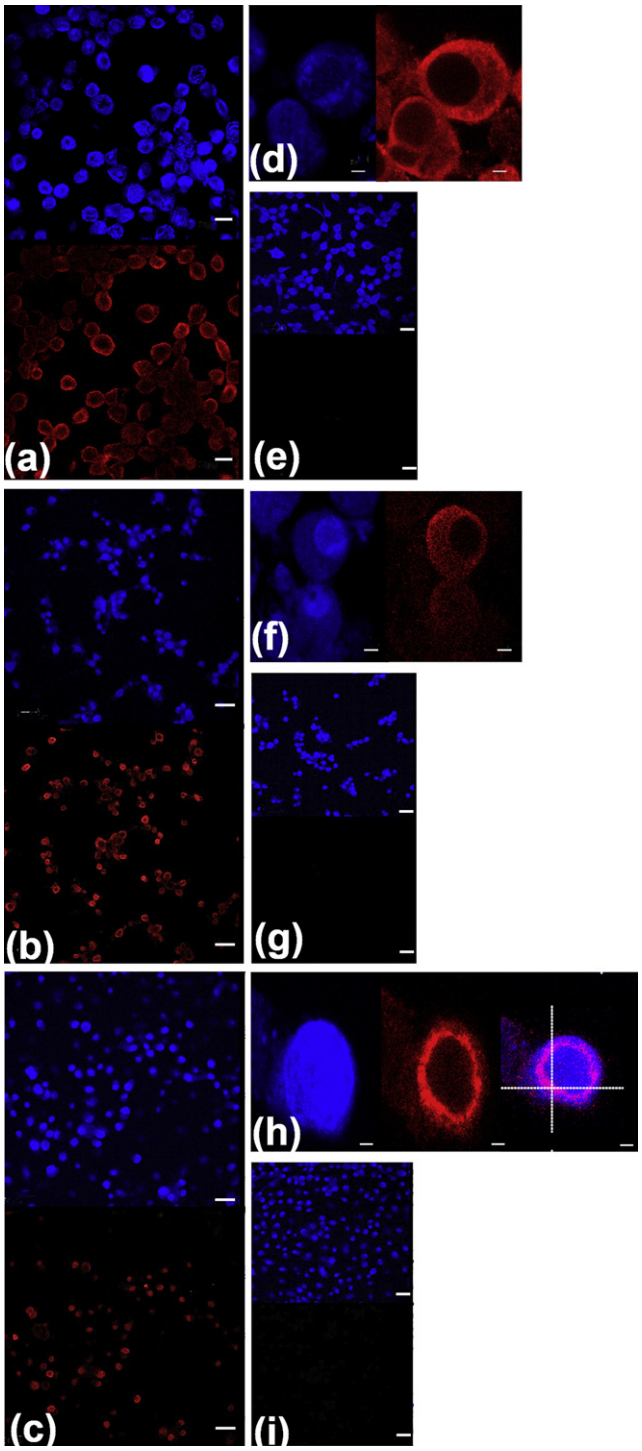

Fig. 4. Localization of recombinant TSWV NSs protein by immunofluorescence in the cytoplasm and nuclei of the three insect cell lines detected using rabbit polyclonal anti-NSs antibody conjugated to Alexa 594 (red) 48 h p.i. DAPI stains DNA in nuclei (blue). (a) BTI-Tn-5B1-4. (b) UFL-AG-286. (c) BM-5 cells. (d), (f) and (h) represent three-dimensional octagonal sections of the cells; in BTI-Tn-5B-1-4 (d) and UFL-AG-286 (f) lines, the NSs localization is confirmed predominant in the cytoplasm; in BM-5 cells (h), the NSs is detected mainly in the nuclei; merged images of DAPI and NSs are shown on far right of the panel (h). (e) BTI-Tn-5B1-4, mock infected without fluorescent anti-rabbit antibody. (g) UFL-AG-286 mock not infected stained with primary antibody and fluorescent anti-rabbit antibody. (i) BM-5 cells mock infected without primary antibody. Scale bars: (a) 20 μm, (b) and (c) 34 μm, (d) 2 μm, (f) 3 μm, (h) 2 μm, (e), (g) and (i) 31 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

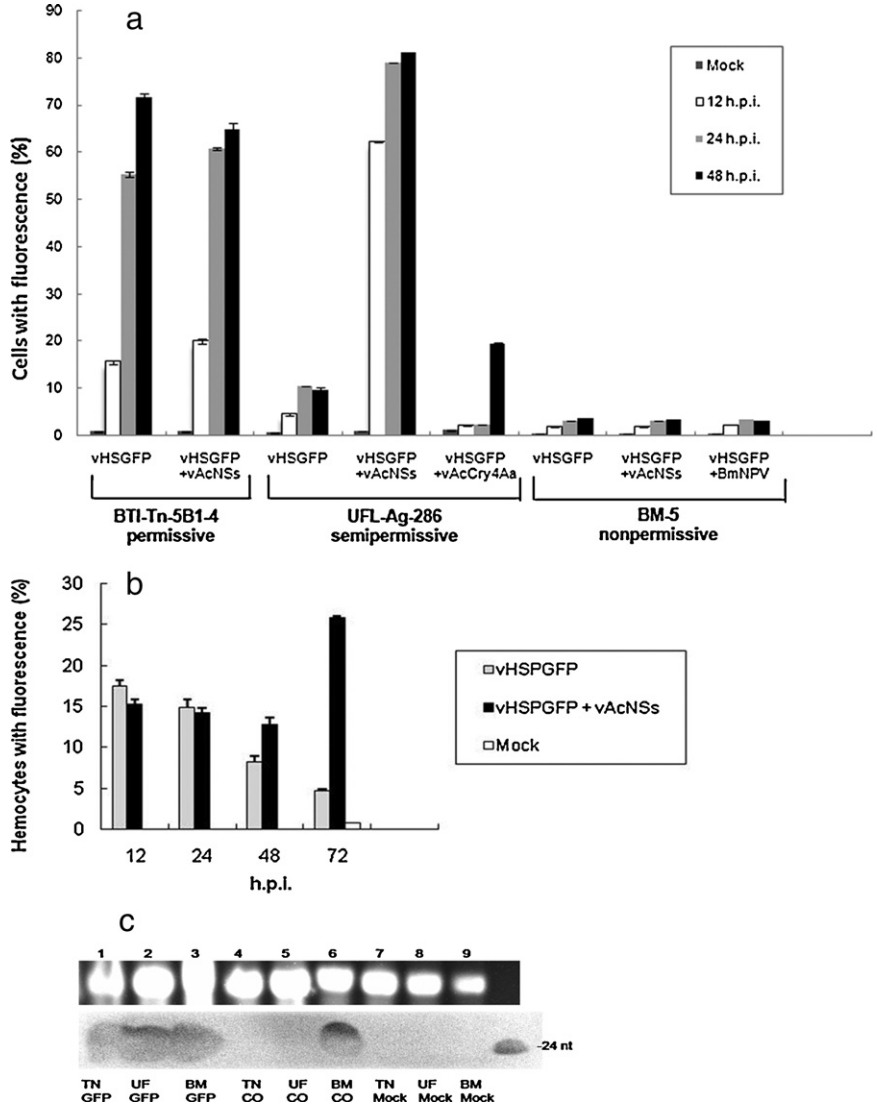

Fig. 5. (a) Effect of TSWV NSs on the infection of an *egfp*-containing virus in different insect cells. Insect cells (BTI-Tn5B1-4, UFL-Ag-286 and BM-5) were infected with the recombinant vHSGFP alone or in combination with vAcNSs or vAcCry4Aa (in UFL-AG-286 cells) or BmNPV (in BM-5 cells). At 12, 24, and 48 h p.i., cells were harvested and fixed. (b) Effect of TSWV NSs on the infection of an *egfp*-containing virus in *A. gemmatalis* hemocytes. Larvae were injected with vHSGFP alone or co-infected with vAcNSs and cohorts of 30 caterpillars were sacrificed at 12, 24, 48 and 72 h p.i. for hemolymph collection. The percentage of EGFP expressing cells and hemocytes was detected by a FACSCalibur flow cytometer. Mean values and standard deviations (SD) from three independent experiments are shown. (c) Northern blot analysis of small interfering (si)RNA isolated from vHSGFP-infected insect cells. siRNAs from BTI-Tn-5B-1-4, UFL-Ag-286 and BM-5 cells infected with only vHSGFP (lanes 1–3) or co-infected with vAcNSs (lanes 4–6) or mock infected (lanes 7–9) were harvested 48 h p.i., analyzed in a 15% denaturing polyacrylamide gel and last, hybridized with a *egfp*-specific probe. 18S rRNA serves as the loading control. The position of a primer sequence (24 nt) is indicated.

three-dimensional computer reconstruction (Fig. 4d, f and h). These analyses were carried out for all host cell lines. Control insect cells with and without primary and secondary antibody did not show any fluorescent signals with Alexa 594 (Fig. 4e, g and i).

3.4. EGFP expression in baculovirus-infected insect cells

The recombinant vHSGFP (Fig. 1e) was used with vAcNSs to evaluate the effect of NSs in virus infectivity in permissive, semi-permissive, and nonpermissive cells and in third instar larvae of *A. gemmatalis*. In the non-permissive BM-5 cells, vHSGFP was also co-infected with wild type BmNPV. As AcMNPV does not replicate in BM-5 cells, the wild type BmNPV was used for infection and co-infection in these cells. For comparison, another recombinant AcMNPV carrying the *cry4Aa* gene from *B. thuringiensis* (vAcCry4Aa) under the control of the *polh* promoter (P_H) was also co-infected with vHSGFP. EGFP fluorescence was monitored in infected cells and hemocytes at different times p.i. on a FACSCalibur flow cytometer.

When vHSGFP was infected alone in UFL-AG-286 cells (Fig. 5a) only 4.46%, 10.35% and 9.51% of cells showed EGFP expression at 12, 24 and 48 h p.i., respectively, indicating a limited infection. On the other hand, when the same cells were co-infected with vHSGFP and vAcNSs, a dramatic increase in the number of cells showing EGFP expression was observed from 12 (61.12%) to 48 h p.i. (80.97%). The co-infection with vHSGFP and vAcCry4Aa resulted in only 2.1% and 2.17% of EGFP expression at 12 and 24 h, respectively, and 19.41% at 48 h p.i. This indicated that this increase in EGFP expression was NSs dependent. However, this increase by vAcNSs was not observed in permissive and nonpermissive cells.

A similar result was observed in *A. gemmatalis* hemocytes co-infected with vHSGFP and vAcNSs (Fig. 5b). The percentage of hemocytes expressing EGFP decreased from little over 15% at 12 h p.i. to 4.71% at 72 h p.i. in vHSGFP infected hemocytes, whereas

when vHSGFP and vAcNSs were co-infected, the percentage of EGFP expressing cells increased from 15% at 12 h p.i. to 25.79% at 72 h p.i.

3.5. Detection of small RNAs in virus-infected insect cells

The ability of NSs to suppress the accumulation of small RNAs in permissive, semi-permissive and nonpermissive cells was tested using a recombinant baculovirus expressing GFP that was able to induce RNAi in these cells. For this purpose, vHSGFP and vAcNSs were used in co-infections of BTI-Tn-5B1-4 (permissive), UFL-AG-286 (semipermissive) and BM-5 (nonpermissive) insect cells, and the small RNAs of *egfp* gene were monitored by northern blot hybridization (Fig. 5c). The vHSGFP was able to trigger RNAi in BTI-Tn5B1-4, UFL-AG-286 and BM-5 cell lines (Fig. 5c, lines 1–3). However, when vHSGFP was co-infected with vAcNSs, *egfp*-derived small RNAs were not detected in BTI-Tn-5B1-4 and UFL-AG-286 cells. However, the formation of small RNA molecules in BM-5 cells was observed. These results indicate that TSWV-NSs seems to be able to act as a silencing suppressor in permissive and semi-permissive cells, but is not capable of suppressing the RNAi machinery in BM-5 nonpermissive cells.

4. Discussion

In this study, we provided evidence that TSWV NSs silencing suppressor protein enhanced gene expression and replication capacity of the baculovirus AcMNPV in permissive (BTI-Tn-5B1-4) and semipermissive (UFL-AG-286) insect cell lines. A recombinant AcMNPV carrying the *NSs* gene (vAcNSs) successfully replicated to higher titers than the wild-type virus in the two cell lines tested (Table 1). The effects of vAcNSs were more pronounced when PIB production was measured in insect cells co-infected with vAcNSs and different wild type baculoviruses. PIB production was enhanced in all three cell lines tested, including nonpermissive BM-5 cells (Fig. 3). These high levels of baculovirus infection could be attributed to NSs expression, since the recombinant vSynNSm carrying the nonstructural *NSm* gene of TSWV showed no increase in polyhedra production.

This ability of vAcNSs to infect nonpermissive host was confirmed by the detection of NSs protein by western-blot analysis (Fig. 2) and the cytopathic effects caused by the recombinant virus (data not shown). Recently, Woo et al. (2007) also detected very late gene expression by BmNPV in SF9 cell line via β-galactosidase expression under the control of the polyhedrin promoter. It has previously been shown that AcMNPV is able to infect a *B. mori* cell line (BmN), although resulting in an abortive infection, due to the failure of late and very late gene expression and production of BVs and PIBs (Rahman and Gopinathan, 2003). In the same way, we did not see PIB production in co-infections of vAcNSs and AcMNPV in BM-5 cells on light microscopy (data not shown). Using microarrays Iwanaga et al. (2004) described that the expression levels of the very late AcMNPV polyhedrin and p10 genes were dramatically reduced in BmN cells, which presumably, is one of the reasons for the inability of AcMNPV to form polyhedral inclusion bodies in these cells.

Our results showed that NSs accumulated differently in the virus-infected insect cells tested, either in the cytoplasm in case of permissive and semipermissive cells, or in the perinuclear space in nonpermissive cells (Fig. 4d, f and h). Intracellular localization of NSs of *Bunyaviridae* appears to vary according to viruses and host cells (Swanepoel and Blackburn, 1977; Smith and Pifat, 1982; Struthers and Swanepoel, 1982; Overton et al., 1987; Kormelink et al., 1991; Simons et al., 1992; Yadani et al., 1999). Our confocal microscopy analysis detected NSs dispersed mainly in the cytoplasm of vAcNSs-infected BTI-Tn-5B1-4 and UFL-AG-286 cells. The NSs cytoplasm localization can be related to the protein's role in suppression of gene silencing detected in these permissive and semipermissive cell lines (Fig. 5c), since the machinery of RNA silencing is present in the cytoplasm (Lindbo and Dougherty, 1992; English et al., 1996; Fire et al., 1998; Wang et al., 2000). In contrast, NSs was abundantly localized in the nuclei of vAcNSs-infected BM-5 cells. Since NSs lacks a nuclear localization signal, its presence in the nuclei might be due to the interaction either with a host and/or viral protein that is transported to the nucleus, as suggested by the work of Le May et al. (2004), where the NSs of RVFV is found in the nuclei of infected cells. They have demonstrated that the NSs of Rift Valley fever virus (RVFV) forms filamentous structures in the nuclei of RFVV-infected HeLa cells and blocks the assembly of the human TFIIH (a complex of basal transcription factors) and consequently shuts down cellular transcription. Similarly, TSWV NSs nuclear localization together with the capacity of vAcNSs to cause cytopathic effects in BM-5 cells and to enhance BmNPV PIB production in co-infections, suggests the possibility of TSWV NSs target transcription or other nuclear factors homologs in BM-5 cell line. In addition, NSs could play a role in the nuclear transcriptional gene silencing (TGS) mechanism as reported in plants by Wassenegger et al. (1994), and recently in other organisms from yeast to animals (Buhler and Moazed, 2007; Buhler et al., 2007).

We have reported in this work that a recombinant baculovirus expressing *egfp* gene under the *Drosophila* heat shock promoter 70 (hsp70) was able to trigger RNAi in insect cells (Fig. 5c), specially in the semipermissive cells in which the percentage of cells expressing EGFP was very low (about 9.5% at 48 h p.i.; Fig. 5a). In contrast, in the same cell line co-infected with the recombinant baculovirus (vAcNSs) encoding the NSs-TSWV RNAi suppressor, the fluorescence-positive cells was significantly increased up to 80% at 48 h p.i. (Fig. 5a). These results support that the expression levels of EGFP in these semipermissive insect cells and hemocytes co-infected with vHSGFP and vAcNSs are probably due to NSs suppression of *egfp* siRNA formation in permissive and semipermissive insect cells (Fig. 5c).

Our results corroborated the role of TSWV-NSs protein acting as a strong silencing suppressor in plants (Takeda et al., 2002) and its association with severity of symptoms in TSWV infected-plants (Kormelink et al., 1991). NSs is also efficient to suppress silencing in other cell systems crossing the plant kingdom. A recombinant Semliki Forest virus (SFV) expressing NSs of TSWV displayed suppressor activity in tick cells (Garcia et al., 2006). Similarly, Blakqori et al. (2007) using the same construct (SFVNSs-TSWV) with a luciferase reporter gene (SFVLuc), showed that the NSs protein rescued luciferase expression in *Aedes albopictus* U4.4 insect cells. The function of TSWV-NSs was similar to other known silencing suppressor proteins like the NS1 protein of influenza virus A that is active in insect cells as well as in plants (Bucher et al., 2004; Li et al., 2002). Recently, the NS3 protein of *Rice hoja blanca tenuivirus* (proposed as a new genus within the *Bunyaviridae* family), which is homologous protein of NSs of tospoviruses, suppressed RNA silencing in plants and insect hosts by efficiently binding both siRNAs and miRNAs (Hemmes et al., 2007).

Interestingly, NSs had no inhibitory effect on the formation of *egfp* siRNA in BM-5 cells, indicating a possible different role in vAcNSs in that cell line. No RNAi suppressor activity was observed in *A. albopictus* U4.4 cells infected with a recombinant SFV containing LACV's NSs gene (Blakqori et al., 2007). This difference in suppression activity in different insect cells could be due to the different cellular localizations of the TSWV's NSs protein. In this report, the TSWV's NSs protein was found in the perinuclear space of nonpermissive insect cells, suggesting a novel function of this protein allowing AcMNPV to induce structural alterations in BM-5 cells.

In summary, our results indicate that the TSWV-NSs protein could improve baculovirus-replication efficiency in permissive and semipermissive cells and strongly enhanced the production of foreign protein during insect cells infection with recombinant baculoviruses. Since tospoviruses replicate very efficient in its insect vector (Wijkamp et al., 1993; Nagata et al., 1999; Ullman et al., 2002), our data obtained in lepidopteran permissive and semipermissive cells indicates a similar role of NSs protein in thrips as an efficient silencing suppressor and/or acting on the enhancement of gene expression during tospovirus infection in its vectors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2010.10.019.

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