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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.
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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 vAc-Cry4Aa 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™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

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