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Short communication

Interference in plant defense and development by non-structural protein NSs of *Groundnut bud necrosis virus*

Suneha Goswami[a], Nandita Sahana[a], Vanita Pandey[a], Paula Doblas[b], R.K. Jain[c], Peter Palukaitis[d], Tomas Canto[b,*], Shelly Praveen[c,**]

[a] Division of Biochemistry, Indian Agricultural Research Institute, New Delhi-110012, India
[b] Centro de Investigaciones Biologicas, CIB,CSIC, Ramiro de Maeztu 9, 28040 Madrid, Spain
[c] Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi-110012, India
[d] Department of Horticultural Science, Seoul Women's University, Seoul 139-774, South Korea

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ABSTRACT

Groundnut bud necrosis virus (GBNV) infects a large number of leguminous and solanaceous plants. To elucidate the biological function of the non-structural protein encoded by the S RNA of GBNV (NSs), we studied its role in RNA silencing suppression and in viral pathogenesis. Our results demonstrated that GBNV NSs functions as a suppressor of RNA silencing using the agroinfiltration patch assay. An *in silico* analysis suggested the presence of pro-apoptotic protein Reaper-like sequences in the GBNV NSs, which were known to be present in animal infecting bunyaviruses. Utilizing NSs mutants, we demonstrated that a Leu-rich domain was required for RNA silencing suppression activity, but not the non-overlapping Trp/GH3 motif of the Reaper-like sequence. To investigate the role of NSs in symptom development we generated transgenic tomato expressing the GBNV NSs and showed that the expression of NSs in tomato mimics symptoms induced by infection with GBNV, such as leaf senescence and necrosis. As leaf senescence is controlled by miR319 regulation of the transcription factor TCP1, we assessed the accumulation of both RNAs in transgenic NSs-expressing and GBNV-infected tomato plants. In both types of plants the levels of miR319 decreased, while the levels of TCP1 transcripts increased. We propose that GBNV-NSs affects miRNA biogenesis through its RNA silencing suppressor activity and interferes with TCP1-regulated leaf developmental pathways.

Groundnut bud necrosis virus (GBNV), also known as *Peanut bud necrosis virus*, is a member of the genus *Tospovirus* in the family of arthropod-borne *Bunyaviridae*. In contrast to all other mammal-infecting members of the family *Bunyaviridae*, tospoviruses specifically infect plants and are transmitted in a propagative manner by thrips (Prins and Goldbach, 1998; Medeiros et al., 2004). The tospoviral non-structural protein (NSs) is encoded by the small RNA segment (S) of the tripartite RNA genome (Satyanarayana et al., 1996) and mainly localizes and aggregates in paracrystalline arrays in the cytoplasm within plant and insect cells (Ullman et al., 1993). In the case of the type member of the genus *Tospovirus*, *Tomato spotted wilt virus* (TSWV), the RNA silencing suppressive activity of the NSs protein was established utilizing a green fluorescent protein-based suppression assay (Takeda et al., 2002; Bucher et al., 2003); however, the RNA silencing suppressor activity of GBNV NSs protein had not been elucidated, to date.

To establish whether the GBNV NSs protein had RNA silencing suppressor activity, the ability of the GBNV NSs protein to interfere with the silencing of the gene encoding the green fluorescent protein (GFP) was examined using an agropatch assay (Canto et al., 2002). The GBNV NSs gene was amplified by RT-PCR from an Indian mungbean isolate of GBNV (Saritha and Jain, 2007) using the forward primer 5′-GGG CCC ATG TCG ACC GCA AGG AGT-3′ and reverse primer 5′-CTC GAG CTG CAG TTA CTC TGG CTT CAC AAT GA-3′, based on the sequence of GBNV NSs gene (Accession Number AY871098). The amplicon (1320 bp), representing the full ORF of NSs, was subsequently ligated into the pGEM-T vector (Promega) and transformed in *Escherichia coli* DH5α cells. The identity of the cloned NSs gene then was confirmed by sequencing. The amplicon also was cloned in pUC118 vector in the sense orientation under the control of cauliflower mosaic virus 35S RNA promoter and transcriptional termination sequences. A cassette of ~2 kbp containing the 1320-bp NSs gene and the flanking 35S RNA promoter and terminator sequences was ligated into the binary vector pCAMBIA 2301 between the *Xba*I and *Hin*dIII sites. The

* Corresponding author. Tel.: +34 918373112x4223; fax: +34 915360432.
** Corresponding author. Tel.: +91 11 25843474; fax: +91 11 25840772.
E-mail addresses: tomas.canto@cib.csic.es (T. Canto), shellypraveen@iari.res.in, shellypraveen@hotmail.com (S. Praveen).

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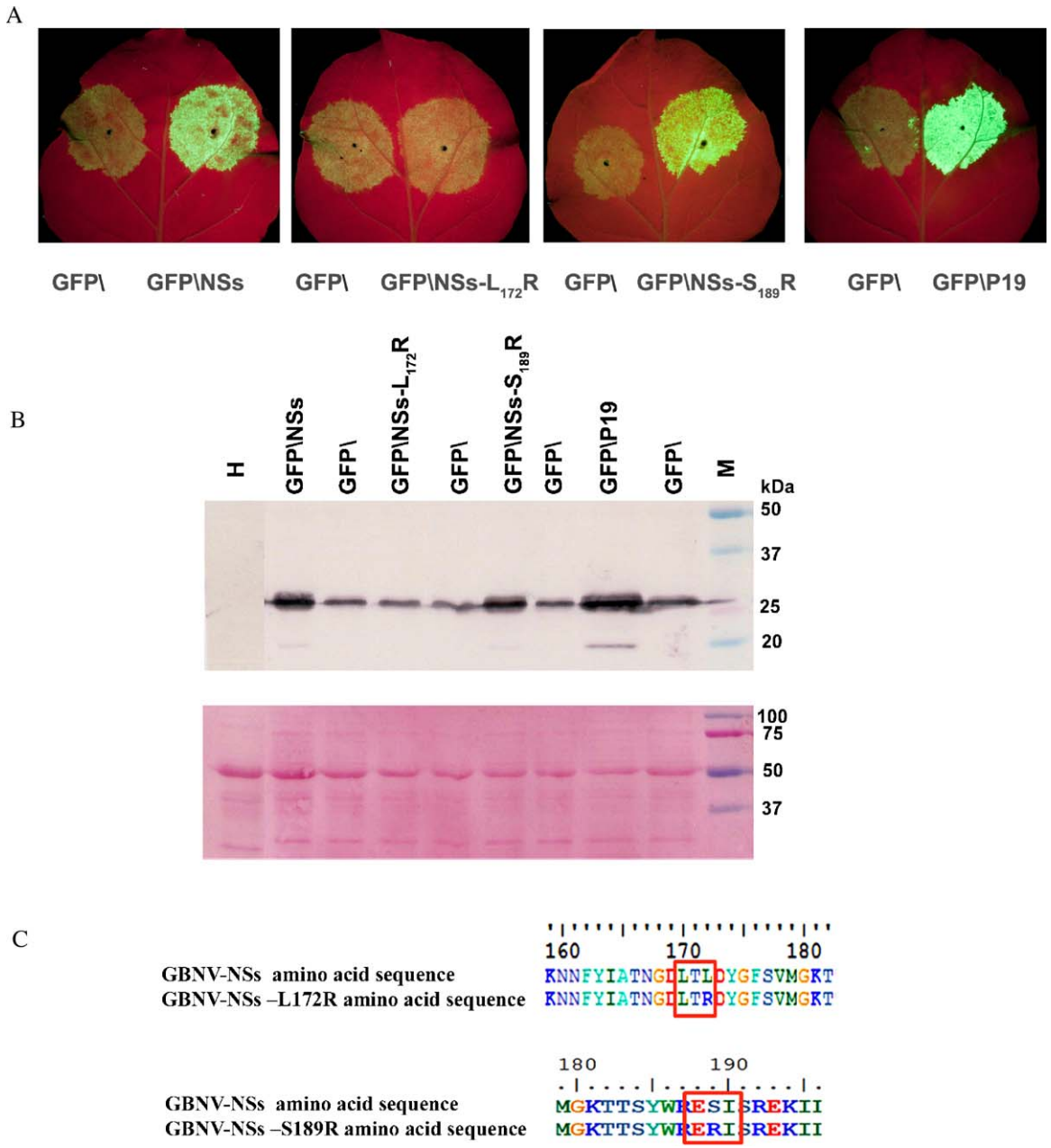

Fig. 1. Characterisation of the RNA silencing suppression activity of GBNV NSs protein and of two NSs mutants (NSs-L_{172}R; NSs-S_{189}R): (A) patch assay of GBNV-NSs and its mutants for RNA silencing activity. In the left side of each *Nicothiana benthamiana* leaf a binary vector expressing green fluorescent protein (GFP) under the control of the 35S promoter was agroinfiltrated, together with an empty binary vector. In the right side of the leaf, the same binary vector expressing GFP was agroinfiltrated together with a binary vector expressing NSs protein, intact or mutant as described (Canto et al., 2002). The suppressor of silencing activity of the *Tomato bushy stunt* P19 protein is shown for comparison. (B) Quantification by western blotting of the steady-state level of accumulation of GFP in the infiltrated patch using a GFP rabbit polyclonal antiserum. Lower panel shows a ponceau S stained membrane. (C) Location of mutation 1 and of mutation 2 in the NSs gene.

recombinant binary vector pCAMBIA 2301 (35S:NSs) was mobilized into the *Agrobacterium tumefaciens* disarmed strain C58C1 by the freeze–thaw method (Hofgen and Willmitzer, 1988). When *A. tumefaciens* cells harboring a plasmid expressing the gene encoding the GFP (Canto et al., 2002) were co-infiltrated with cells containing the binary vector expressing the native NSs protein, the level of green fluorescence was enhanced markedly, as a result of the suppression of the silencing of the ectopic GFP gene (Fig. 1A). The enhancement of fluorescence observed in the presence of the suppressor correlated with enhanced levels of GFP as detected by immunoblot analysis using a GFP antibody (Fig. 1B). This demonstrates that the GBNV NSs is also an RNA silencing suppressor, as was shown for the TSWV NSs (Takeda et al., 2002; Bucher et al., 2003).

Previous studies suggested that NSs protein of GBNV is a bifunctional enzyme, exhibiting RNA-stimulated NTPase activity along with ATP-independent RNA/DNA phosphatase activity (Lokesh et al., 2010). These activities are represented by amino acid sequences of the Walker A (GXXXXGKT) and Walker B (DEXX) motifs, respectively, present in the NSs protein of GBNV and many other tospoviruses (Lokesh et al., 2010). In addition to the Walker motifs, NSs proteins from animal-infecting serogroups of the family *Buniyaviridae* possess sequence similarity to Reaper, a proapoptotic protein from *Drosophila* (Thomenius and Kornbluth, 2006). That sequence analysis further revealed the presence of a conserved Trp/GH3 motif in this Reaper-like sequence, which is known to be critical for the proteins ability to induce programmed cell death