Identification of a novel RNA silencing suppressor, NSs protein of *Tomato spotted wilt virus*

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Abstract RNA silencing or post-transcriptional gene silencing (PTGS) in plants is known as a defense system against virus infection. Several plant viruses have been shown to encode an RNA silencing suppressor. Using a green fluorescent protein-based transient suppression assay, we show that NSs protein of *Tomato spotted wilt virus* (TSWV) has RNA silencing suppressor activity. TSWV NSs protein suppressed sense transgene-induced PTGS but did not suppress inverted repeat transgene-induced PTGS. TSWV NSs protein is the first RNA silencing suppressor identified in negative-strand RNA viruses.

Key words: RNA silencing; Suppressor; *Tospovirus*; *Bunyaviridae*; NSs; p19

1. Introduction

Most eukaryotes, from fungi to animals, have an RNA silencing system [1]. In plants, RNA silencing is known as post-transcriptional gene silencing (PTGS) and it acts as a defense system against virus infection [2,3]. The RNA silencing mechanism recognizes 'aberrant' RNAs, which are derived from some transgenes, double-stranded RNAs (dsRNAs) and replication intermediates of viral RNAs, and degrades homologous RNA in the cytoplasm. The production of 21–25 nucleotide small interfering RNAs (siRNAs), corresponding to the target RNA, is the hallmark of RNA silencing [4]. When PTGS is induced at one site, systemic silencing signals spread and subsequently trigger PTGS in distant tissues.

Some plant viruses have suppressors to counteract PTGS [5]. The helper component-proteinase of *Tobacco etch virus* was identified as the first PTGS suppressor [6]. Subsequently, the 2b protein of *Cucumber mosaic virus*, the 19K protein (p19) of *Tomato bushy stunt virus* (TBSV) and several other viral proteins have been identified as suppressors [7–10]. These suppressors act at different steps in silencing pathways and are valuable for the dissection of PTGS pathways [5]. A recent study has suggested that plants have a branched pathway for transgene-induced PTGS [11]. One pathway is a sense transgene-induced PTGS (S-PTGS), which occurs via the action of dsRNAs produced by plant RNA-dependent RNA polymerase (RdRP). Another is an inverted repeat transgene-induced PTGS (IR-PTGS), which occurs via transgenes arranged as inverted repeats [11].

Tomato spotted wilt virus (TSWV), the type species of the genus *Tospovirus* within the family *Bunyaviridae*, infects many plant species. It is transmitted by several species of thrips and multiplies in insect cells [12]. The genome of TSWV consists of three negative or ambisense single-stranded RNAs designated as L (8.9 kb), M (4.8 kb) and S (2.9 kb). The L RNA is completely negative sense and contains a gene encoding the L protein (putative RdRP) [13]. The ambisense M RNA encodes the NSm protein (putative movement protein) and the structural proteins, G1 and G2 [14]. The ambisense S RNA encodes N (nucleocapsid) protein and NSs protein [15].

The NSs protein of TSWV mainly localizes and aggregates in paracrystalline arrays in the cytoplasm in plant and insect cells [16,17] and the amount of NSs protein is associated with the severity of symptoms in TSWV infected-plants [16]. However, the function of NSs protein is poorly understood. In this study we show, by utilizing a green fluorescent protein (GFP) reporter assay, that the NSs protein of TSWV has PTGS suppressor activity. The NSs inhibited the onset of S-PTGS, but not IR-PTGS. These results suggest that TSWV NSs interferes with a step(s) before the synthesis of dsRNA by the plant RdRP in the S-PTGS pathway.

2. Materials and methods

2.1. Plasmids

The 0.6-kb *Hin*dIII–*Sal*I fragment of pBICP35 [18] was cloned into a modified pUC19 vector in which the multi-cloning sites from *Eco*RI to *Bam*HI had been eliminated. The resulting plasmid, pUBP35, contains the 35S promoter sequence of *Cauliflower mosaic virus* (CaMV), *Stu*I, *Bam*HI, *Eco*RI, and *Kpn*I sites, and the CaMV terminator sequence. All primers for the following PCR contained a recognition sequence of the appropriate restriction enzyme for cloning. A PCR

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Abbreviations: dsRNA, double-stranded RNA; GFP, green fluorescent protein; IR-PTGS, inverted repeat transgene-induced post-transcriptional gene silencing; PTGS, post-transcriptional gene silencing; RdRP, RNA-dependent RNA polymerase; S-PTGS, sense transgene-induced post-transcriptional gene silencing; siRNAs, small interfering RNAs; TBSV, *Tomato bushy stunt virus*; TSWV, *Tomato spotted wilt virus*

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product containing the 5′ leader (5′-AAGGAGATATAAC-3′) and GFP gene sequences was amplified from CD3-326 [19] and cloned into the *Bam*HI site of pUBP35, creating pUBGFPBam. A PCR product, amplified from pYT2 [20] by using the primers Int8Eco5R (5′-GGTGAATTCAGGTACTTAGATTCATATATC-3′) and Int8-Eco3L (5′-GGTGAATTCACCTGCAATGAAAAGAGAC-3′), was cloned into the *Eco*RI site of pUBGFPBam to obtain pUBGFPInt. A PCR product containing the 5′ leader and GFP gene sequences was amplified from CD3-326 and cloned into the *Kpn*I site of pUBGFPInt to create pUBdsGFP. pUBGFPBam and pUBdsGFP were digested with *Stu*I and *Hin*dIII. The resulting 1.0- and 1.8-kb fragments were substituted for the small *Stu*I and *Hin*dIII fragments of pBICP35 to create pBICGFP and pBICdsGFP, respectively. pHST2 [21] contains the full-length cDNA sequence of TBSV with a point mutation in the p19 gene and we restored it to the wild type sequence. A PCR product containing the 5′ leader and p19 gene was amplified from the modified pHST2 and cloned into the *Bam*HI site of pBICP35, creating pBICp19. A PCR product containing the 5′ leader and the NSs gene was amplified from a partial cDNA of TSWV S RNA, including the NSs gene (Accession No. AB088385), and cloned into the *Bam*HI site of pBICP35 to obtain pBICNSs. pBICNSs was digested with *Spe*I, blunt-ended and self-ligated, to obtain pBICNSsfs. A PCR fragment containing the entire GFP sequence was amplified from TU#65 [22] and cloned into pGEM-T easy (Promega), which created pGEMGFP.

2.2. Agrobacterium infiltration

Transgenic *Nicotiana benthamiana* homozygous for the GFP transgene (line 16c) [23] and *Agrobacterium tumefaciens* strain MP90 were used for infiltration experiments. *Agrobacterium* cultures were prepared as described previously [24] using appropriate antibiotics. Before infiltration, *Agrobacterium* cells were suspended in infiltration medium [24] to 0.8 OD at 600 nm and mixed by combining equal volumes of individual cultures.

2.3. GFP imaging

GFP fluorescence was detected by using a 100-W long-wave UV lamp (Black Ray model B 100A, UV products). Leaves and whole plants were photographed with a Nikon Coolpix 950 digital camera through a Wratten filter number 8 and/or 58. These images were processed electronically with Adobe Photoshop software.

2.4. RNA analysis

Total RNA was isolated from the patches in the *Agrobacterium*-infiltrated leaves using TRIzoL (Invitrogen). For northern analysis of GFP mRNA and its siRNAs, 1 and 15 μg of total RNA were used, respectively. Specific RNA was detected using a ^{32}P-labeled antisense GFP probe transcribed from *Spe*I-linearized pGEMGFP as described [25]. Radioactive signals were detected by using a Fujix BAS2000 phosphoimager.

3. Results

The function of TSWV NSs protein is not well characterized [12]. Some RNA silencing suppressors have a common mechanism for effecting symptom expression in infected plants [8 and references therein]. A previous study has reported that the amount of NSs protein is associated with the severity of symptoms in TSWV-infected plants [16]. Thus, we speculated that TSWV NSs may function as an RNA silencing suppressor. To test this hypothesis, we have used a transient silencing suppression assay based on the *Agrobacterium* co-infiltration method, established in a GFP-expressing transgenic *N. benthamiana* (line 16c) [9]. If a suppressor blocks the onset of PTGS, GFP fluorescence is easily detected under UV light. The constructs used in this study are illustrated in Fig. 1.

3.1. NSs locally suppresses S-PTGS

We first tested whether NSs suppresses S-PTGS locally. We used an *Agrobacterium* strain containing pBICGFP as a PTGS inducer, together with a second *Agrobacterium* strain. A mixture of *Agrobacterium* carrying pBICNSs and *Agrobacterium* harboring pBICGFP was co-infiltrated into line 16c leaves. As controls, *Agrobacterium* harboring either pBICP35 or pBICp19 was co-infiltrated with pBICGFP. TBSV p19 has been shown to suppress PTGS in a similar assay [2]. For simplicity, we will refer to each *Agrobacterium* strain by the name of the plasmid it carries. The patches receiving pBICGFP+pBICP35 showed decreased green fluorescence (Fig. 2A) at 6 days post-infiltration (dpi). In contrast, the patches receiving pBICGFP plus either pBICNSs or pBICp19 showed bright green fluorescence (Fig. 2B,D), indicating that NSs has PTGS suppressor activity like p19. The GFP fluorescence in these patches remained bright during the examination period up to 25 dpi (data not shown). Infiltration with pBICGFP+pBICNSsfs, containing a frameshift version of the NSs gene, did not suppress PTGS in the patch (Fig. 2C).

To confirm the visual observations, we analyzed the accumulation of GFP mRNA in each infiltrated patch at 6 dpi. Northern analysis showed that GFP mRNA was abundant in the bright green fluorescent patches (Fig. 2E, lanes 2, 4), although the accumulation of GFP mRNA in the patches receiving pBICGFP plus either pBICP35 or pBICNSsfs (Fig. 2E, lanes 1, 3) was significantly lower than that in suppressor-infiltrated patches. The GFP-specific siRNAs in the patches receiving pBICGFP plus either pBICP35 or pBICNSsfs (Fig. 2F, lanes 1, 3) were readily detected. However, we detected significantly lower accumulation of siRNAs in the patches receiving pBICGFP plus either pBICNSs or pBICp19 (Fig. 2F, lanes 2, 4).

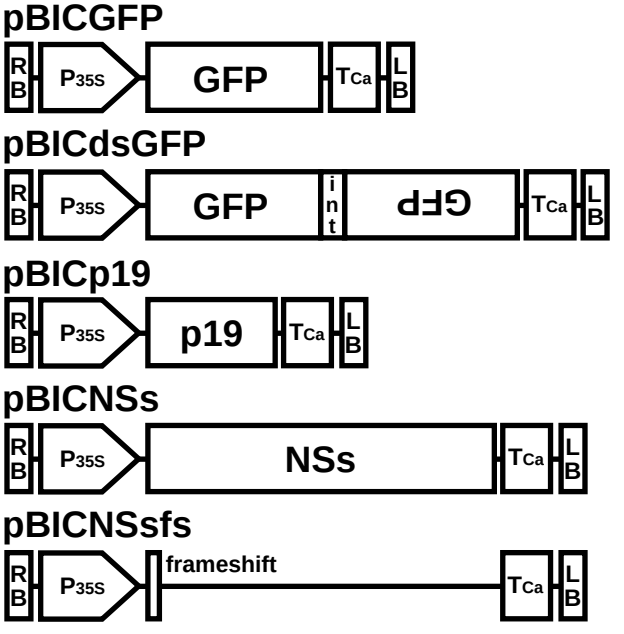

Fig. 1. Schematic representation of the transcription cassettes used in this study. The pentagon labeled P_{35S} and the box labeled T_{Ca} represent the 35S promoter and the terminator of CaMV, respectively. pBICGFP contains the GFP gene. pBICdsGFP contains both sense and antisense sequences of GFP separated by an intron. The box labeled int shows the intron sequence derived from *Arabidopsis thaliana TOM1* gene. pBICp19 and pBICNSs have the TBSV p19 and TSWV NSs gene, respectively. pBICNSsfs has a frameshift mutation in the 5′-terminal region of the NSs gene. RB and LB indicate right and left borders of the T-DNA region of pBICP35, respectively.

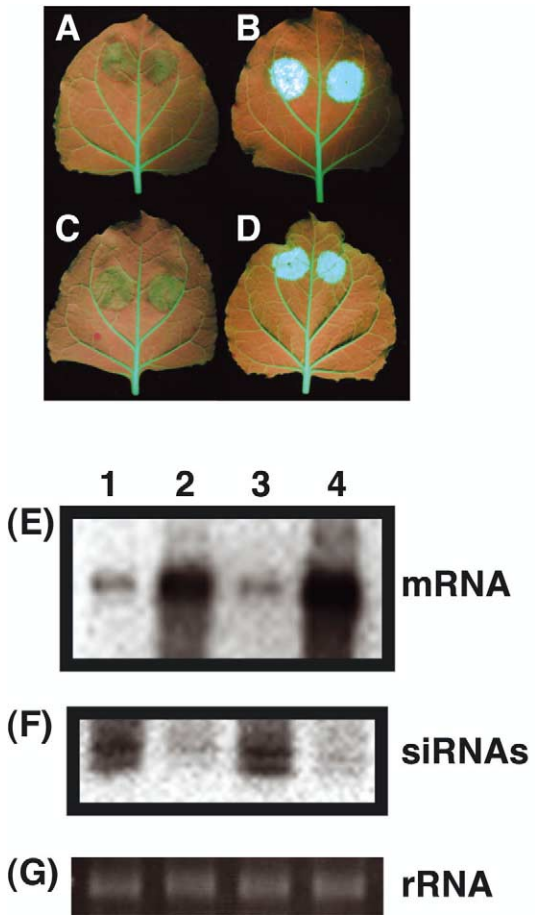

Fig. 2. TSWV NSs suppresses S-PTGS locally. A–D: Leaves of line 16c infiltrated with *Agrobacterium* carrying pBICGFP plus *Agrobacterium* harboring pBICP35 (A), pBICNSs (B), pBICNSsfs (C) or pBICp19 (D) were viewed under UV light at 6 dpi. E–G: Northern analysis of GFP mRNA (E) and GFP-specific siRNAs (F) extracted from the patches infiltrated with *Agrobacterium* carrying pBICGFP plus *Agrobacterium* harboring pBICP35 (lane 1), pBICNSs (lane 2), pBICNSsfs (lane 3) or pBICp19 (lane 4). G: rRNA stained with ethidium bromide indicates equal loading of samples in E and F.

3.2. NSs systemically suppresses S-PTGS

We then tested whether NSs systemically suppresses S-PTGS. All the mature leaves except the cotyledons of young 16c plants were infiltrated with two *Agrobacterium* strains and uninfiltrated upper leaves were observed under UV light at 14 dpi. We tested 15 plants in total for each infiltration in five independent experiments. The upper leaves of the plants receiving pBICGFP plus either pBICP35 or pBICNSsfs (Fig. 3A,C) showed systemic spread of silencing. In contrast, most plants receiving pBICGFP plus either pBICNSs or pBICp19 (Fig. 3B,D) did not display GFP silencing in the upper leaves, except for several plants in which vein-restricted systemic silencing was observed (data not shown). Thus, there were no significant differences between the activities of NSs and p19 in the S-PTGS suppression assay.

3.3. NSs does not suppress IR-PTGS

Finally, we tested whether NSs suppresses IR-PTGS locally, using an assay reported by Johansen and Carrington [26]. Previous studies have shown that inverted repeats are strong inducers of RNA silencing [26,27]. Young line 16c leaves were infiltrated with a mixture of three *Agrobacterium* strains, pBICGFP+pBICdsGFP plus either pBICP35 or pBICNSs. The infiltrated patches in these infiltrations had undergone silencing and were deep red at 6 dpi (Fig. 4A,B). Consistent with these observations, Northern blot analysis showed low levels of accumulation of GFP mRNA in these patches, whereas the amount of GFP mRNA was slightly, but significantly, increased by NSs protein (Fig. 4D, compare lane 2 with lane 1). In contrast, the patches receiving pBICGFP+pBICdsGFP+pBICp19 showed green fluorescence at 6 dpi (Fig. 4C) and a large amount of GFP mRNA was detected in the patches (Fig. 4D, lane 3). At 14 dpi, however, the infiltrated patches became deep red (data not shown), which suggests that the p19-directed suppression of local IR-PTGS was overcome. siRNAs accumulated even in the green fluorescent patches and the accumulation level was similar among the infiltrations (Fig. 4E). We further tested whether NSs systemically suppresses IR-PTGS. At 14 dpi, the upper leaves of line 16c receiving pBICGFP+pBICdsGFP+pBICNSs were deep red under UV light (Fig. 4G). On the other hand, the upper leaves with pBICGFP+pBICdsGFP+pBICp19 showed green fluorescence at 14 dpi (Fig. 4H) and were deep red at 25 dpi (data not shown).

4. Discussion

In this study, we have demonstrated that TSWV NSs functioned as a PTGS suppressor, using a patch co-infiltration assay. NSs is the first PTGS suppressor identified in negative-strand RNA viruses. NSs suppressed S-PTGS locally and systemically (Figs. 2B, 3B) and interfered with the accumulation of siRNAs (Fig. 2F). However, NSs did not suppress IR-PTGS (Fig. 4B,G). These results suggest that NSs mainly interferes with an initial step(s) that includes the synthesis of dsRNA by plant RdRP in the S-PTGS pathway (Fig. 5). In addition, NSs might weakly interfere with a later step(s) after the generation of dsRNA (Fig. 5) because NSs slightly,

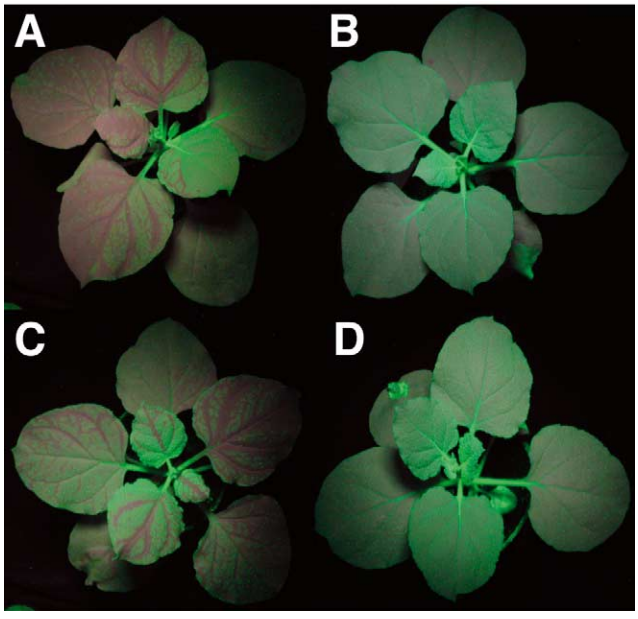

Fig. 3. TSWV NSs suppresses S-PTGS systemically. The upper leaves of line 16c were viewed under UV light at 14 dpi with *Agrobacterium* carrying pBICGFP plus *Agrobacterium* harboring pBICP35 (A), pBICNSs (B), pBICNSsfs (C) or pBICp19 (D).

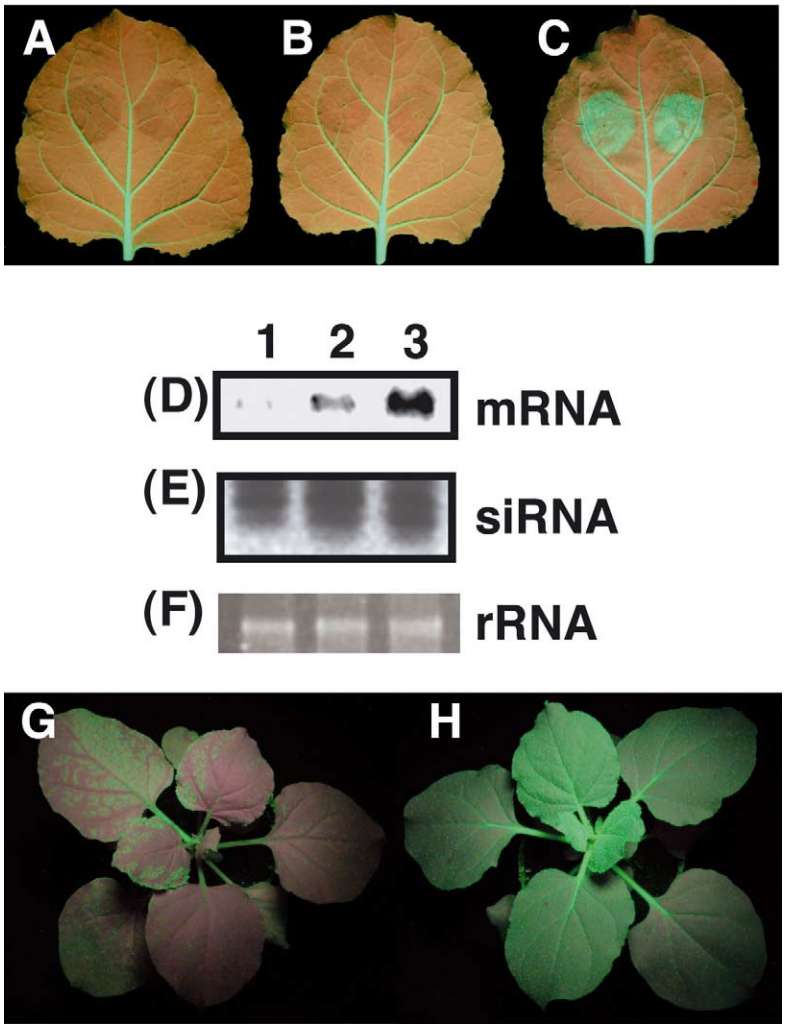

Fig. 4. TSWV NSs does not suppress IR-PTGS. A–C: Leaves of line 16c infiltrated with three strains of *Agrobacterium* carrying pBICGFP +pBICdsGFP plus either pBICdsGFP plus *Agrobacterium* harboring pBICP35 (A), pBICNSs (B) or pBICp19 (C) were viewed under UV light at 6 dpi. D–F: Northern analysis of GFP mRNA (D) and GFP specific siRNAs (E) extracted from the patches infiltrated with three strains of *Agrobacterium* carrying pBICGFP +pBICds GFP plus either pBICP35 (lane 1), pBICNSs (lane 2) or pBICp19 (lane 3). F: rRNA stained with ethidium bromide indicates equal loading of samples in D and E. G,H: The upper leaves of line 16c were viewed under UV light at 14 dpi with three strains of *Agrobacterium* carrying pBICGFP+pBICdsGFP plus either pBICNSs (G) or pBICp19 (H).

but significantly, increased GFP mRNA accumulation in the IR-PTGS assay (Fig. 4D, lanes 1, 2).

TBSV p19 was used as a PTGS suppressor control in this study. As reported previously [2], TBSV p19 suppressed S-PTGS locally (Fig. 2D). Moreover, TBSV p19 also suppressed S-PTGS systemically (Fig. 3D) as reported for the p19 of a related virus, *Cymbidium ringspot virus* (CymRSV) [28]. However, there has been no report on the suppressor activity of p19 against IR-PTGS. Here, our results show that TBSV p19 also suppresses IR-PTGS locally (Fig. 4C) and systemically (Fig. 4H) in a transient manner (data not shown). TBSV p19 would bind siRNA like a functional homolog, CymRSV p19 [28], and suppress IR-PTGS (Fig. 5). However, the suppression activity of TBSV p19 against IR-PTGS could be overcome by excess amounts of accumulated siRNAs. The large amount of siRNAs detected in the green fluorescent patches (Fig. 4E, lane 3) may be generated by the IR-transgene, as reported previously [26], and be involved in the delayed onset of PTGS observed in the IR-PTGS assay using the TBSV p19 construct.

In the family *Bunyaviridae*, viruses in the genus *Tospovirus* infect plants but those in the other genera do not. A recent study demonstrated that an animal virus, *Flock house virus* (FHV), also induces RNA silencing and the B2 protein of FHV functions as an RNA silencing suppressor [10]. The genetic organization and the ambisense-coding strategy of the S RNA of tospoviruses resemble those of viruses in the genus *Phlebovirus* belonging to the family *Bunyaviridae*. As suggested previously [16], it is likely that the NSs proteins of phleboviruses are functionally similar to TSWV NSs protein and may have an RNA silencing suppressor activity. A recent study showed that the NSs of *Rift Valley fever virus* (RVFV), belonging to the genus *Phlebovirus*, is an interferon (IFN) antagonist [29]. It is well known that IFNs are antiviral agents and the production of IFNs is induced by dsRNA (including replication intermediate of RNA viruses; reviewed in [30]). If the RVFV NSs has RNA silencing suppressor activity, the RNA silencing pathway may share some steps with the IFN-mediated virus inactivation pathway.

A reverse genetics system based on infectious cDNA clones

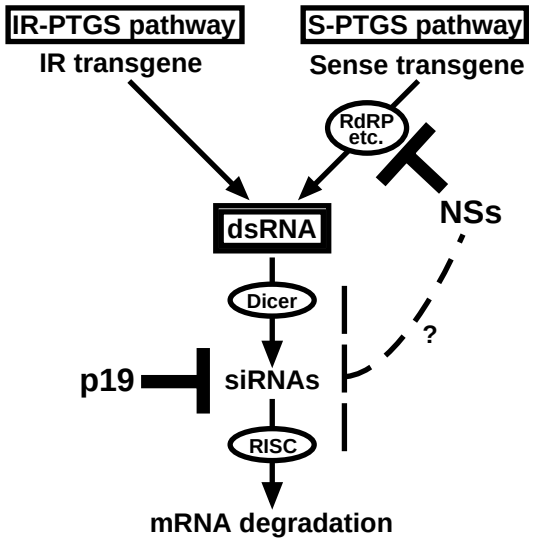

Fig. 5. A model for NSs-mediated PTGS suppression: the two-branched pathway, S-PTGS and IR-PTGS, of the transgene-induced PTGS in plants, as proposed previously [11]. In the S-PTGS pathway, plant RdRP and several proteins are required for dsRNA synthesis. The IR-transgene results in dsRNAs without these host components [11]. Dicer is an RNaseIII-like enzyme. siRNAs guide an RNA-induced silencing complex (RISC) to mRNA degradation. NSs could interfere with a step(s) for generating the dsRNA in the S-PTGS pathway. In addition, NSs might weakly interfere with a later step(s) after the generation of dsRNA. TBSV p19 would bind siRNAs and suppress S-PTGS and IR-PTGS, as proposed previously [28].

is not available for tospoviruses, whereas it has been established for some viruses in the *Bunyaviridae* [31,32]. The establishment of such systems will be of great benefit for investigations into the effects of mutations in the NSs gene on TSWV infection in plants and also on viral multiplication in insect vectors. Moreover, construction and analysis of chimeric viruses in terms of NSs gene between TSWV and the other animal-infecting bunyaviruses should provide information on the role of RNA silencing suppression in other virus infections and may shed light on the evolution of plant-intruding tospoviruses.

After we had submitted this paper, we noticed that R. Goldbach's group would be reporting in the 12th International Congress of Virology (abstract number 6-V-38) that TSWV NSs protein suppresses PTGS.

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