

# Key Importance of Small RNA Binding for the Activity of a Glycine-Tryptophan (GW) Motif-containing Viral Suppressor of RNA Silencing\*

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**Background:** GW/WG motif-containing viral suppressors of RNA silencing (VSRs) have been proposed to act through interaction with Argonaute (AGO) proteins.

**Results:** The activity of a GW motif-containing VSR was found to rely on small RNA binding capability rather than on AGO interaction.

**Conclusion:** Overlapping signals in VSRs may lead to misinterpretation of relevant molecular traits.

**Significance:** Knowing primary target(s) of VSRs is critical for better understanding of the host-virus arms race.

Viruses express viral suppressors of RNA silencing (VSRs) to counteract RNA silencing-based host defenses. Although virtually all stages of the antiviral silencing pathway can be inhibited by VSRs, small RNAs (sRNAs) and Argonaute (AGO) proteins seem to be the most frequent targets. Recently, GW/WG motifs of some VSRs have been proposed to dictate their suppressor function by mediating interaction with AGO(s). Here we have studied the VSR encoded by *Pelargonium* line pattern virus (family Tombusviridae). The results show that p37, the viral coat protein, blocks RNA silencing. Site-directed mutagenesis of some p37 sequence traits, including a conserved GW motif, allowed generation of suppressor-competent and -incompetent molecules and uncoupling of the VSR and particle assembly capacities. The engineered mutants were used to assess the importance of p37 functions for viral infection and the relative contribution of diverse molecular interactions to suppressor activity. Two main conclusions can be drawn: (i) the silencing suppression and encapsidation functions of p37 are both required for systemic *Pelargonium* line pattern virus infection, and (ii) the suppressor activity of p37 relies on the ability to bind sRNAs rather than on interaction with AGOs. The data also caution against potential misinterpretations of results due to overlap of sequence signals related to distinct protein properties. This is well illustrated by mutation of the GW motif in p37 that concurrently affects nucleolar localization, efficient interaction with AGO1, and sRNA binding capability. These concomitant effects could have been overlooked in other GW motif-containing suppressors, as we exemplify with the orthologous p38 of turnip crinkle virus.

These pathways are conserved in most eukaryotes and play an essential role in developmental programs, stress responses, and maintenance of genome integrity (1, 2). In plants, as well as in invertebrates and, to a lesser extent, in fungi and probably in vertebrates (3–8), RNA silencing acts as a potent antiviral system. This system is triggered by double-stranded RNAs (dsRNAs)<sup>3</sup> that, in the case of single-stranded RNA (ssRNA) viruses, may correspond to dsRNA replicative intermediates, self-complementary regions of the viral genome, or products of the action of host RNA-dependent RNA polymerases on viral templates (9). The dsRNA triggers are cleaved by Dicer-like (DCL) RNase III-related enzymes into small RNAs (sRNAs) of 21–24 nt (4). These small duplexes are unbound by an RNA helicase, and one strand is incorporated into an RNA-induced silencing complex (RISC), whose core component is an Argonaute (AGO) protein that is endowed with RNA slicer activity (10–12). After integration into the RISC, the sRNA strand guides annealing and endonucleolytic cleavage of complementary RNA molecules (1). The sRNAs that bind to their cognate RNAs do not only trigger RNA cleavage, but they may also serve as primers for RNA-dependent RNA polymerase activity, which generates additional dsRNA targets for DCL enzymes. This results in the production of a new wave of sRNAs, thus leading to amplification of the silencing signal (13–15). In plants, studies with the model organism *Arabidopsis thaliana* indicate that DCL4 and DCL2 are the most important DCLs involved in virus-induced RNA silencing and that AGO1 is the major antiviral effector molecule, although other AGOs might also participate in viral clearance (16–22).

RNA silencing refers to several mechanistically related pathways that result in sequence-specific regulation of gene expres-

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<sup>3</sup> The abbreviations used are: dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; DCL, Dicer-like; GFP, green fluorescent protein; sRNA, small RNA; nt, nucleotide(s); RISC, RNA-induced silencing complex; AGO, Argonaute; VSR, viral suppressor of RNA silencing; TCV, turnip crinkle virus; PLPV, *Pelargonium* line pattern virus; gRNA, genomic RNA; sgRNA, subgenomic RNA; CP, coat protein; MP, movement protein; GFP-IR, GFP inverted repeat; BiFC, bimolecular fluorescence complementation; mRFP, monomeric red fluorescent protein; sYFP, superyellow fluorescent protein; dpif, days postinfiltration; vsRNA, virus-derived sRNA.

In order to counteract RNA silencing-mediated host defenses, viruses have evolved a vast array of proteins called viral suppressors of RNA silencing (VSRs) (23, 24). VSRs are very diverse in sequence and additional function(s), and no correlation among VSR types and viral phylogeny can be established (25). Moreover, differences in VSR activity have been found among viruses of the same genus (26–28) or even among isolates of the same virus species (29, 30). Although many questions remain to be answered, the growing knowledge about the mechanism of action of several VSRs reveals that virtually all stages of the antiviral silencing pathway can be targeted by these viral products and that a given VSR may inhibit or inactivate one or more components of the silencing machinery (31, 32). One of the best studied VSRs is the tombusvirus p19 protein that binds sRNAs, the key mediators of RNA silencing, preventing their incorporation into RISC (33, 34). Distinct unrelated VSRs have adopted the same strategy to block RNA silencing (35–38), and some of them have been reported to bind dsRNAs in a size-independent manner (38–40). Inhibition of the 2'-O-methylation that protects sRNAs from degradation or blockage of biogenesis of 21-nt sRNAs would be other examples of the mode of action of VSRs (16, 41). Besides RNAs, protein components of the silencing pathways may be also affected by VSRs. For instance, several VSRs seem to target AGO proteins, such as the 2b protein of cucumber mosaic virus, which was proposed to inhibit RISC activity via physical interaction with the PAZ domain of AGO1 (42). In addition, the polerovirus-encoded F-box protein P0 targets AGO proteins for ubiquitination and subsequent degradation, thereby precluding RISC assembly (43–45). Moreover, ipomovirus P1 protein and protein p38 of turnip crinkle virus (TCV; genus *Carmovirus*, family Tombusviridae) were recently shown to bind directly and specifically AGO1, affecting the activity of this RISC component (46, 47). In the latter cases, such binding was proposed to be achieved through mimicry of host-encoded GW/WG motif-containing proteins normally required for RISC assembly/function in diverse organisms (48).

*Pelargonium* line pattern virus (PLPV) belongs to the large and diverse family Tombusviridae and harbors a monopartite, positive-sense ssRNA genome that is encapsidated into isometric viral particles of about 30 nm in diameter (49). The 3,883-nt genomic RNA (gRNA) lacks a 5' cap structure and a 3' poly(A) tail and contains five open reading frames (ORFs) (Fig. 1A). The two 5'-proximal ORFs encode two proteins involved in replication, the essential accessory factor p27 and its read-through product p87, which corresponds to the viral RNA-dependent RNA polymerase. Two centrally located, small ORFs encode the movement proteins p7 (MP1) and p9.7 (MP2), and the 3'-proximal ORF encodes a coat protein (CP or p37) (50, 51). PLPV genomic organization and derived proteins closely resemble those of members of the genus *Carmovirus*. However, whereas carmoviruses generate two subgenomic RNAs (sgRNAs) that serve as mRNAs for expression of the MPs and the CP, respectively, PLPV produces only one. In addition, PLPV lacks any AUG codon between the MP1 AUG and CP AUG initiation codons, whereas carmoviruses have 1–8 AUGs in the equivalent region. Moreover, the PLPV MP2 gene contains a non-AUG initiation codon contrary to the canonical

AUG present in the MP2 gene of carmoviruses (50, 51). These distinguishing characteristics are shared by other related, poorly characterized, small isometric ssRNA viruses, which has prompted the proposal of their inclusion into a new genus, tentatively named *Pelarspovirus*, within the family Tombusviridae (50–52).

So far, VSR activity has been identified in four of the 12 genera currently included in the family Tombusviridae, particularly *Aureusvirus*, *Carmovirus*, *Dianthovirus*, and *Tombusvirus*. Despite their phylogenetic closeness, the available information indicates that different genera employ distinct proteins and/or strategies to inhibit RNA silencing. Whereas the CPs of several carmoviruses have been reported to act as VSRs (27, 53, 54), tombusviruses and aureusviruses encode small, unrelated proteins (p19 and p14, respectively) that perform such specific tasks (40, 55). The dianthoviral suppressor seems unique in that it consists of multiple components, including the replication proteins (p27 and p88) and the viral RNA templates (56). Moreover, the dianthoviral MP probably functions as a secondary VSR (57). To further explore the diversity of silencing suppression within Tombusviridae, our first goal in the present work was to identify the PLPV VSR, a role that was found to be played by p37, the viral CP. Investigations to understand the molecular bases of the suppressor function of p37 were subsequently undertaken. Through site-directed mutagenesis of distinct sequence motifs in the protein, including a conserved GW motif at the N terminus, a series of suppressor-competent and -incompetent forms of p37 was generated, some of which retained the particle assembly capacity, whereas others did not. This series was used to analyze the relevance of p37 functions for viral infection and the relative contribution of diverse protein properties (binding to dsRNAs, virion formation, subcellular localization, self-interaction, and interaction with certain AGO proteins) to the suppressor activity. The results have provided valuable insights into the mode of action of p37 that seems to rely on the ability to bind sRNAs rather than on interaction with AGOs, despite the protein having an apparently *bona fide* GW motif. Moreover, the obtained data reveal considerable overlap of sequence signals in p37 related to distinct protein properties. This situation can be present in other GW motif-containing VSRs, as we illustrate with the orthologous TCV p38, and can lead to misinterpretations of results or to deficient understanding of the molecular bases of VSR activity.

## EXPERIMENTAL PROCEDURES

**DNA Constructs**—Individual PLPV ORFs (p27, p87, p7, p9.7, and p37) were PCR-amplified from a plasmid containing a full-length PLPV infectious cDNA (58) using specific primer pairs and the Expand High Fidelity PCR System (Roche Applied Science). The amplification of ORF p87 was performed from plasmid p27tyr, a full-length PLPV clone in which the amber stop codon of ORF1 was mutated to a tyrosine codon (51). The amplification products, containing proper restriction sites at the 5' ends, were inserted between the cauliflower mosaic virus 35S promoter and the terminator sequence of the *Solanum tuberosum* proteinase inhibitor II gene (PoPit) and cloned into the binary vector pMOG800 (59).

# Main Relevance of sRNA Binding in a GW Motif-containing VSR

**TABLE 1**

Primers used in this work

Primer	Position <sup>a</sup>	Sequence <sup>b</sup>	Restriction site	Constructs <sup>c</sup>
CH358	7–17 (S)	5'–GCGGATCCATGGCACACTATTTTGG–3'	BamHI	35S:p27
CH192	710–728 (AS)	5'–CCGGATCCATTTGACGAACCTCGAAAAG–3'	BamHI	
CH358	7–17 (S)	5'–GCGGATCCATGGCACACTATTTTGG–3'	BamHI	35S:p87
CH359	2287–2304 (AS)	5'–CCCTGCAGTTAGATTGGAATCTTTTG–3'	PstI	
CH360	2274–2292 (S)	5'–CCGGATCCATGGATATTTCAATCAAAG–3'	BamHI	35S:p7
CH361	2448–2465 (AS)	5'–CCCTGCAGTCAGAAATGAGATGGAC–3'	PstI	
CH362	2621–2647 (S)	5'–GTGGATCCATGGCGGCCAAGGATAATC–3'	BamHI	35S:p37
CH363	3618–3637 (AS)	5'–GGCTGCAGTCACAGCTTGTGTGATGTAAG–3'	PstI	
CH364	2371–2389 (S)	5'–CCGGATCCATGGAGTATCCAAGGGTAC–3'	BamHI	35S:p9.7
CH365	2618–2637 (AS)	5'–GTCTGCAGTTATCCTTGGCCGCCATTTTC–3'	PstI	
CH377	2690–2715 (S)	5'–CAATCCAAGGGTgcgGGCTCTTTGTC–3'		35S:p37-W <sub>28</sub>
CH378	2690–2715 (AS)	5'–GACAAAGAGCCgCgCACCCTGGATTG–3'		pgPLPV (p37-W <sub>28</sub> )
CH404	2652–2679 (S)	5'–CAGCCGTGGCgCgCgGGAACAGTGGGC–3'		35S:p37–2R <sub>15</sub>
CH405	2652–2679 (AS)	5'–GCCCCACTGTTCCgCgCgTGCCACGGCTG–3'		pgPLPV (p37–2R <sub>15</sub> )
CH406	2683–2711 (S)	5'–CAAGCTCCAATCCgCgGGGTGGGGCTCTT–3'		35S:p37-K <sub>26</sub>
CH407	2683–2711 (AS)	5'–AAGAGCCCCAACCCgCgGATTGGAGCTTG–3'		pgPLPV (p37-K <sub>26</sub> )
CH408	2806–2843 (S)	5'–TCCCACAAATGCGcGgCgGGCGcGGGAGAGCCGGTA–3'		35S:p37–3R <sub>67</sub>
CH409	2806–2843 (AS)	5'–TACCCGGCTTCCCGcCGCCgCgCgCGCATTTGTGGGA–3'		pgPLPV (p37–3R <sub>67</sub> )
CH362	2621–2647 (S)	5'–GTGGATCCATGGCGGCCAAGGATAATC–3'	BamHI	35S:p37-HA (WT and mutants)
CH410	3625–3634 (AS)	5'–GGCTGCAGAGCGTAATCTGGAACATCGTATGGGTATGCCAGCTTGTG–3'	PstI	
CH518	2807–2832 (S)	5'–CAGAAGAAGGGCGGTCAACCCTAAC–3'		35S:p38-W <sub>26</sub>
CH519	2807–2832 (AS)	5'–GTTAGGGTTGACGCGCCCTTCTTCTG–3'		
CH520	3552–3576 (S)	5'–GGACAGCTGGGGCGGAGCAGATTG–3'		35S:p38-W <sub>283</sub>
CH521	3552–3576 (AS)	5'–CAATCGTGCTCCGCCAGCTGTCC–3'		
CH362	2621–2647 (S)	5'–GTGGATCCATGGCGGCCAAGGATAATC–3'	BamHI	sYFPN:p37 (WT and mutants)
CH381	3618–3637 (AS)	5'–GGGAGCTCTCACAGCTTGTGTGATGTAAG–3'	SacI	sYFPC:p37 (WT and mutants)
CH362	2621–2647 (S)	5'–GTGGATCCATGGCGGCCAAGGATAATC–3'	BamHI	35S:p37 WT:GFP
CH385	3615–3634 (AS)	5'–CCGGATCCAGCTTGTGATGTAAGCTC–3'	BamHI	(WT and mutants)

<sup>a</sup> Positions of the PLPV or the TCV genome (CH518, CH519, CH520, and CH521) covered by the primers. S and AS, sense and antisense, respectively.

<sup>b</sup> Restriction sites introduced for cloning purposes are underlined; lowercase type indicates nucleotide substitutions to PLPV and TCV WT sequence, and sequence encoding an HA tag is in italic type.

<sup>c</sup> pgPLPV refers to both pUC18-based and pMOG800-based constructs bearing full-length PLPV cDNAs.

Distinct mutations were introduced by PCR with the QuikChange site-directed mutagenesis kit (Stratagene) into the p37 gene of (i) a PLPV infectious cDNA inserted into pUC18 just downstream from a T7 RNA polymerase promoter (58) and (ii) the same PLPV infectious cDNA flanked by the cauliflower mosaic virus 35S promoter and the PoPit and cloned into pMOG800 (51). Such mutations led to the replacement by Ala of the following p37 amino acid residues: Trp at position 28 (mutant p37-W<sub>28</sub>), two Arg residues at positions 15 and 16 (mutant p37–2R<sub>15</sub>), Lys at position 26 (mutant p37-K<sub>26</sub>), or three Arg residues at positions 67, 68, and 70 (mutant p37–3R<sub>67</sub>). The mutagenized full-length PLPV clones were used as templates for PCR amplification of the mutant p37 genes that were cloned individually into pMOG800 as described above for the wild-type (WT) p37 gene. In addition, similar pMOG800-based constructs, which allowed expression of WT and mutant p37 molecules tagged at their C termini with the hemagglutinin (HA) peptide, were generated by standard PCR and cloning procedures (60). Binary constructs carrying a green fluorescent protein (GFP) inverted repeat (GFP-IR) or allowing expression of GFP, tombusviral p19, or TCV p38 have been described earlier (27, 61). Mutations in sequences encoding the GW motifs of TCV p38 were introduced with the QuikChange site-directed mutagenesis kit and proper oligonucleotides.

To analyze protein subcellular localization, WT p37 gene and mutant versions were PCR-amplified, and the amplification products, bearing proper restriction sites at the ends, were fused in frame to the 5' end or the 3' end of the GFP gene. Such fusions were inserted between the cauliflower mosaic virus 35S promoter and the PoPit and cloned into plasmid pMOG800.

For bimolecular fluorescence complementation (BiFC) assays, WT and mutant p37 genes were PCR-amplified using oligonucleotides with appropriate restriction sites and cloned into vectors pROK-sYFPN and pROK-sYFPC to allow expression of the p37 protein fused to the yellow fluorescent protein (YFP) N- and C-terminal halves (amino acids 1–154 and 155–238, respectively) (62). In addition, pROK2-based constructs that allowed expression of AGO1 and AGO4 tagged with sYFP halves were used in some BiFC experiments (62). A pROK2-based construct for expression of fibrillarlin fused to monomeric red fluorescent protein (mRFP) has been described elsewhere (63).

All constructs were routinely sequenced with an ABI PRISM DNA sequencer 377 (PerkinElmer Life Sciences) to avoid unwanted modifications. The primers used to generate the distinct recombinant plasmids are listed in Table 1.

*Agrobacterium-mediated Transient Gene Expression and Virus Inoculation*—All binary plasmid constructs were transformed into *Agrobacterium tumefaciens* strain C58C1 CH32 by electroporation. *A. tumefaciens* infiltration was performed on the abaxial side of leaves from 2-week-old *Nicotiana benthamiana* plants (27). In co-infiltration assays, equal volumes of the corresponding bacterial cultures (OD<sub>660</sub> = 0.5) were mixed before infiltration.

PLPV was agroinoculated to plants using the pMOG800-based constructs with full-length PLPV cDNAs and the procedure described above. Alternatively, virus mechanical inoculation was performed. With this aim, RNA transcripts were synthesized *in vitro* from the pUC18-derived full-length PLPV clones with T7 RNA polymerase (Thermo Scientific) following

digestion of plasmids with *Sma*I. The resulting transcripts were gently rubbed onto carborundum-dusted leaves as reported before (51). Plants were maintained under greenhouse conditions (16-h day at 24 °C and 8-h night at 20 °C) until leaf samples were harvested.

**Fluorescence Imaging and Microscopy**—In assays aimed at identification of suppressor activity, visual detection of GFP fluorescence in agroinfiltrated leaf patches was performed using a fluorescence stereomicroscope (MZZ16F Leica). Pictures were taken with a digital camera DFC300 FX Leica. In BiFC and subcellular localization assays, GFP, mRFP, or reconstituted sYFP fluorophores of tagged proteins were monitored in epidermal cells of *N. benthamiana*-infiltrated tissue at 72 h postinfiltration using a Leica TCS SL confocal microscope with an HCX PL APO  $\times 40/1.25$ –0.75 oil CS objective. GFP and sYFP fluorescence was recorded by excitation with a 488-nm argon laser line with emission being collected through a band pass filter from 505 to 550 nm. In the case of mRFP, excitation was performed by means of a 543-nm green neon laser line, and fluorescence emission was collected at 610–630 nm.

**RNA Extraction, Virion Purification, and Northern Blot Analysis**—Total RNA was extracted from plant tissue with buffer-saturated phenol and then fractionated with 2 M LiCl (64). Virions were purified by pelleting through a sucrose cushion (65). For detection of GFP mRNA or PLPV RNAs, Northern blot analyses were performed as reported previously (50), using radioactive DNA probes corresponding to the GFP gene or to nt 3095–3883 of the PLPV genome, respectively. Virion preparations were resolved in non-denaturing agarose gels and subjected to Northern blot analysis with the PLPV probe. In order to check the presence of GFP-specific sRNAs, the RNA fraction soluble in 2 M LiCl was electrophoresed in denaturing polyacrylamide gels (20%), electroblotted to Hybond-XL membranes (GE Healthcare), and subjected to hybridization with a radioactive GFP-specific riboprobe (27). Hybridization signals were visualized by autoradiography or with a PhosphorImager (Fujifilm FLA-5100, GE Healthcare).

**Electrophoretic Mobility Shift Assay (EMSA)**—Preparation of crude protein extracts from agroinfiltrated tissue, labeling of synthetic double-stranded sRNAs or of long dsRNAs, binding reactions, and electrophoresis in polyacrylamide gels were performed as described earlier (27, 38). For generation of long dsRNAs, transcripts of opposite polarity produced with T7 and T3 RNA polymerase, respectively, from a pBluescript KS(+)-based plasmid with a 250-bp DNA insert were used. Detection of HA-tagged proteins was done through Western blot (66), using 10  $\mu$ l of each protein extract and an anti-HA antibody (Roche Applied Science). Signals from EMSAs or Western blots were recorded by autoradiography or with a PhosphorImager (Fujifilm FLA-5100, GE Healthcare).

## RESULTS

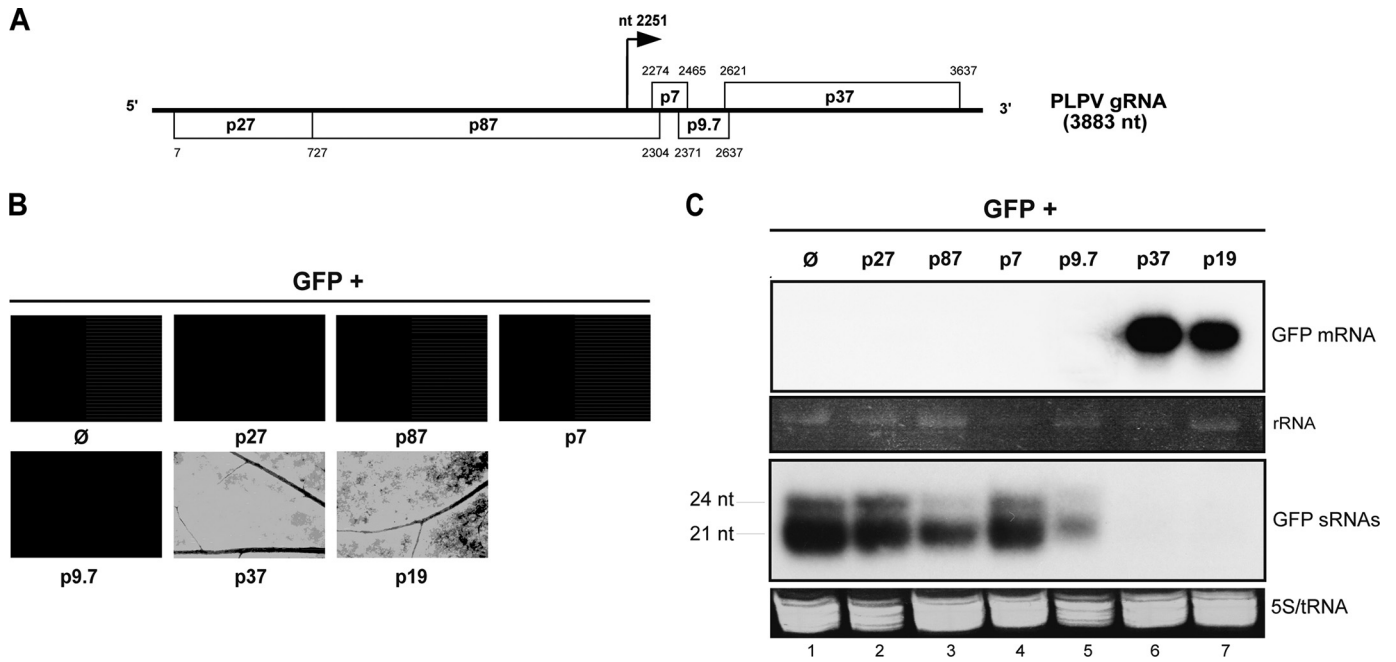
**PLPV p37 Efficiently Inhibits RNA Silencing**—To identify potential PLPV-encoded suppressor(s), we tested the ability of the distinct PLPV proteins to block the silencing of a GFP reporter gene transiently expressed from a binary T-DNA vector agroinfiltrated in *N. benthamiana*. This type of assay has been widely employed to recognize both plant and animal sup-

pressors (55, 67). In the absence of a functional VSR, GFP expression from the Ti-plasmid is recognized as exogenous by the host and is silenced within 3 days postinfiltration (dpif). If the VSR is operative, the expression level of GFP is stabilized for a considerably larger period. Leaf patches infiltrated with the GFP plasmid in combination with an empty vector or with constructs allowing expression of PLPV replication (p27 and p87) or movement (p7 and p9.7) proteins showed high expression of GFP at 2 dpif (data not shown), which was almost completely silenced at 5 dpif according to observations of GFP fluorescence (Fig. 1B). In contrast, co-expression of GFP with PLPV p37 maintained high levels of fluorescence at 5 dpif and beyond, similar to that found with the tombusviral p19 that was used as a positive control of suppressor activity in parallel assays (Fig. 1B).

To confirm the visual observations, Northern blot analysis of RNA extracted from the infiltrated tissue was carried out with a GFP probe. The results corroborated that the fluorescence patterns reflected changes in the steady state levels of GFP mRNA. Similar GFP mRNA accumulation was detected at 2 dpif in the patches infiltrated with any of the plasmid combinations (data not shown). However, at 5 dpif, GFP mRNA accumulation was very low in patches expressing GFP alone or in combination with p27, p87, p7, and p9.7, whereas GFP mRNA levels in patches co-expressing GFP and p37 were high and comparable with those found with p19 (Fig. 1C, top). As expected from a silencing process, the reduced levels of GFP mRNA observed at 5 dpif in tissue expressing GFP either alone or together with any PLPV protein but p37 correlated with elevated levels of GFP sRNAs. Conversely, GFP sRNAs were hardly detected in the patches co-expressing GFP and p37 or p19 (Fig. 1C, bottom). Collectively, the results allowed identification of p37 as the VSR encoded by PLPV.

**Mutations in a GW or an Arginine-rich Motif at the N Terminus of p37 Abolish the Suppressor Function of the Protein**—As reported in distinct studies, one or few point mutations may severely impair the suppressor function of a VSR (27, 46, 68). In order to obtain suppressor-competent and -incompetent forms of p37 that would allow us to investigate properties of the protein critical for its role as VSR, several mutant versions were generated, and their ability to inhibit RNA silencing was subsequently evaluated. One of the mutants harbored a Trp to Ala change that affected a GW motif present at the N terminus of the protein (mutant p37-W<sub>28</sub>; Fig. 2A). Such a motif is conserved in the homologous proteins of pelarspoviruses and of several carmoviruses (Fig. 2A, inset), and its relevance for the suppressor function of TCV p38 (which possesses another relevant, non-conserved GW motif at its C terminus) has been recently highlighted (46). Three additional mutant proteins bearing Ala replacements in basic residues at the N terminus were also engineered: mutant p37-2R<sub>15</sub>, mutant p37-K<sub>26</sub>, and mutant p37-3R<sub>67</sub> (Fig. 2A). Co-expression of the mutant proteins with GFP in transient assays revealed that p37-2R<sub>15</sub> and p37-K<sub>26</sub> maintained a suppressor activity comparable with that of the WT protein, whereas such activity was abolished by the Ala replacements present in p37-W<sub>28</sub> and p37-3R<sub>67</sub> (Fig. 2B). These results revealed a pivotal role of the N-terminal GW motif and of an arginine-rich motif for the silencing suppressor function of p37.

## Main Relevance of sRNA Binding in a GW Motif-containing VSR



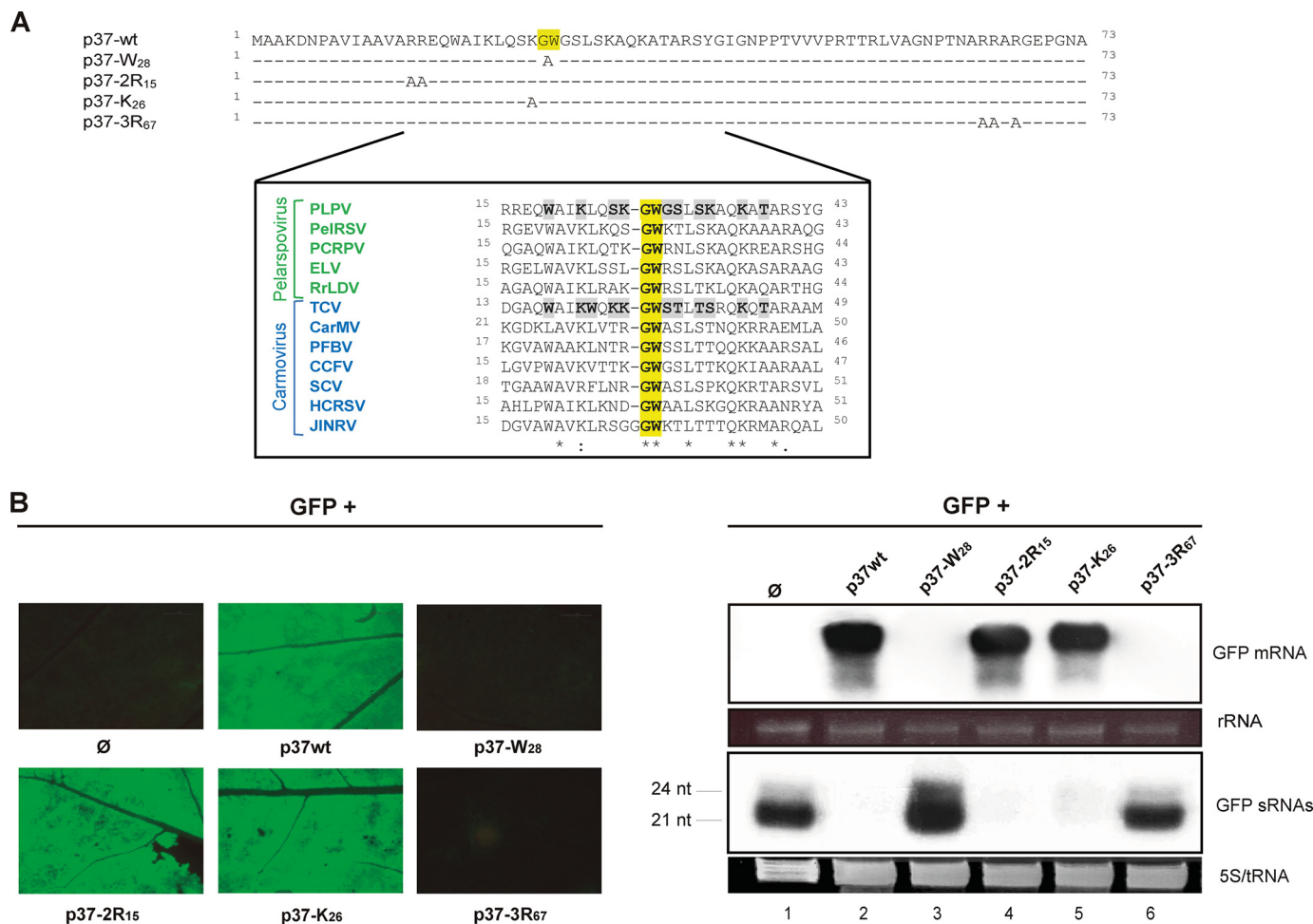
**FIGURE 1. Evaluation of suppressor activity of PLPV proteins.** *N. benthamiana* plants were agroinfiltrated with constructs for expression of GFP either alone ( $\emptyset$ ) or in combination with PLPV p27, p87, p7, p9.7, p37, or the tombusviral p19. **A**, schematic representation of the PLPV genome. *Open boxes*, ORFs; *arrow*, transcription start site of the sgRNA. **B**, GFP fluorescence at 5 dpif in infiltrated leaf patches. **C**, Northern blot hybridization for detection of the GFP mRNA or derived sRNAs in infiltrated tissues harvested at 5 dpif. Ethidium bromide staining of RNA is shown as loading control.

*Uncoupling of the VSR and the Encapsidation Functions of p37 Reveals That Both Are Required for Effective Systemic Spread of the Virus*—In order to test whether abolishing the suppression function had effects on PLPV infectivity, the above p37 mutations were introduced into the viral genome context. *N. benthamiana* plants were inoculated mechanically using transcripts derived from pUC18-based (WT and mutant) PLPV constructs. Local and systemic leaves were harvested at 7 and 30 days postinoculation, respectively. Northern blot analysis revealed the presence of viral RNAs in local samples of plants inoculated with WT PLPV and with PLPV mutants p37-2R<sub>15</sub> and p37-K<sub>26</sub>, whereas the accumulation level of such RNAs in samples from plants inoculated with PLPV mutants p37-W<sub>28</sub> and p37-3R<sub>67</sub> was negligible (Fig. 3A, left). Equivalent results were obtained when *N. benthamiana* plants were virus-inoculated through infiltration of *A. tumefaciens* strains carrying the corresponding pMOG800-based (WT and mutant) full-length PLPV constructs (data not shown). These results indicated that the suppressor function is critical for viral infectivity. In accordance with the lack of local infection, the suppressor-deficient PLPV mutants p37-W<sub>28</sub> and p37-3R<sub>67</sub> were not detected in systemic leaves (Fig. 3A, right). Remarkably, whereas the suppressor-competent virus PLPV p37-K<sub>26</sub> became systemic, the likewise suppressor-competent PLPV p37-2R<sub>15</sub> did not. This suggested that p37 amino acid replacements in the latter mutant affected protein function(s) other than RNA silencing suppression that could be crucial for systemic viral dispersion. One possibility was that the encapsidation function was impaired in mutant p37-2R<sub>15</sub> because particle assembly has been reported to be required and dispensable, respectively, for the systemic and local spread of distinct Tombusviridae in different hosts (69, 70). To test this possibility, an attempt to purify viral particles from all of the assayed mutants was done. To this

end, *N. benthamiana* leaves agroinoculated with the distinct PLPV constructs were harvested at 3 days postinoculation. We reasoned that, at this early time, the triggering of RNA silencing would be just getting started, and, thus, suppressor-deficient mutants could still accumulate at detectable levels. Northern blot hybridization confirmed this assumption (Fig. 3B, left), and the infected tissues were employed for virion purification. Viral particle preparations could be readily obtained from tissue infected with the WT virus, PLPV p37-K<sub>26</sub> (suppressor-competent and able to systemically infect), or PLPV p37-W<sub>28</sub> (suppressor-incompetent and unable to systemically infect), whereas attempts to purify viral particles from plant tissue infected with mutant PLPV p37-2R<sub>15</sub> (suppressor-competent and unable to systemically infect) or p37-3R<sub>67</sub> (suppressor-incompetent and unable to systemically infect) were unsuccessful (Fig. 3B, right). Altogether, the results indicated that the p37 VSR function is required but not sufficient for effective PLPV systemic infection and that the encapsidation function must be also preserved. In addition, we cannot discard the possibility that particle formation, although not strictly required for local infection, somehow promotes stability and/or cell to cell movement of the virus because the accumulation level of PLPV mutant p37-2R<sub>15</sub> in inoculated leaves was usually lower than that observed for WT PLPV or for mutant p37-K<sub>26</sub> (Fig. 3A, left). It should also be mentioned that local and systemic infections established by PLPV p37-K<sub>26</sub> were generally delayed with respect to that of the WT virus (compare accumulation levels of mutant p37-K<sub>26</sub> versus WT in Fig. 3A), suggesting that the engineered mutation has slight detrimental effects on virus fitness.

*PLPV p37 Is Able to Bind sRNAs and This Ability Correlates with Its Suppressor Function*—As indicated in the Introduction, the ability to bind sRNA duplexes seems to be crucial for the function of distinct VSRs (33, 37, 38, 40, 71). To assess whether

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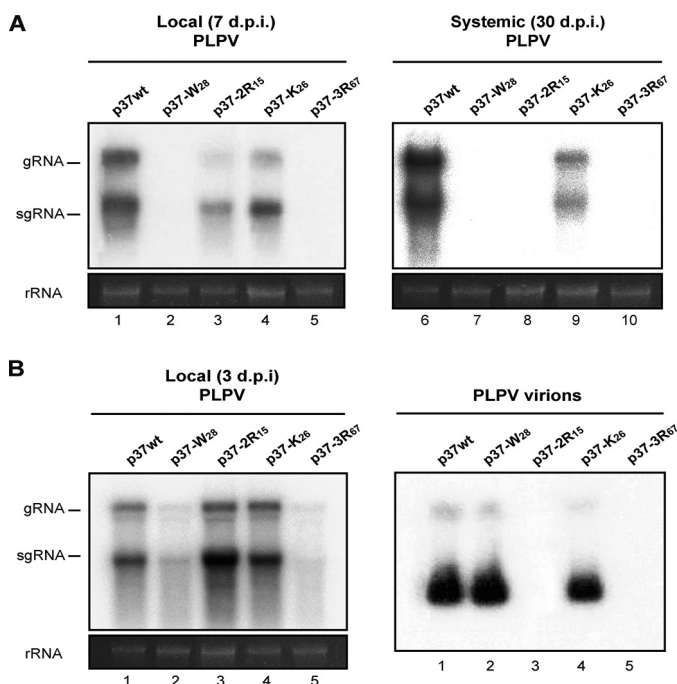
**FIGURE 2. Effect of amino acid replacements in PLPV p37.** *A*, scheme of PLPV p37 mutants. The amino acid sequence of the N terminus of WT p37 is shown at the top, and the amino acid substitutions introduced in each mutant are specified below. Dashes indicate identical residues. Inset, partial alignment of PLPV p37 with homologous proteins of pelarspoviruses (green) and carmoviruses (blue) to illustrate conservation of the N-terminal GW motif. Amino acid residues typically found surrounding GW motifs of AGO-binding proteins (Gly, Trp, Ser, Thr, and charged amino acids, prominently Asp, Lys, and Glu) have been boxed in the context of GW motifs of PLPV and TCV proteins. *PelRSV*, *Pelargonium* ringspot virus; *PCRPV*, *Pelargonium* chlorotic ringspot virus; *ELV*, elderberry latent virus; *RrLDV*, *Rosa rugosa* leaf distortion virus; *CarMV*, carnation mottle virus; *PFBV*, *Pelargonium* flower break virus; *CCFV*, cardamine chlorotic fleck virus; *SCV*, saguaro cactus virus; *HCRSV*, hibiscus chlorotic ringspot virus; *JINRV*, Japanese iris necrotic ring virus. *B*, evaluation of suppressor activity of p37 molecules. *N. benthamiana* plants were agroinfiltrated with constructs for expression of GFP either alone (∅) or in combination with WT (positive control) or mutated p37 forms. GFP fluorescence and Northern blot hybridization for detection of the GFP mRNA or derived sRNAs at 5 dpif are shown at the left and right, respectively. Ethidium bromide staining of RNA is shown as a loading control in the Northern blot assay.

PLPV p37 shares this ability, first, the WT and mutant versions of the protein were fused to an HA tag and transiently co-expressed with GFP in *N. benthamiana* leaves. The HA-tagged proteins exhibited exactly the same behavior as the unfused partners in these suppressor assays: the WT and mutant proteins p37-2R<sub>15</sub> and p37-K<sub>26</sub> showed equivalent, high suppressor activity, whereas mutants p37-W<sub>28</sub> and p37-3R<sub>67</sub> were incapable of preventing silencing (data not shown). Next, *N. benthamiana* leaves were separately agroinfiltrated with the constructs of the HA-tagged proteins to prepare crude protein extracts that, at 3 dpif, showed comparable accumulation levels of the corresponding p37 molecules (Fig. 4A), indicating that the engineered mutations did not affect protein stability. These crude extracts were employed to perform EMSAs with sRNAs. A shift in sRNA mobility was observed when extracts from leaves expressing HA-tagged p37 WT were used (Fig. 4B), indicating that the protein is able to bind sRNAs. Interestingly, extracts containing p37-2R<sub>15</sub> and p37-K<sub>26</sub>, both functional in

silencing suppression, also showed sRNA binding activity, whereas those containing p37-W<sub>28</sub> and p37-3R<sub>67</sub>, which had no detectable suppressor function, failed to form complexes with sRNAs (Fig. 4B). Subtle differences in the mobility of the protein-sRNA complexes were detected among suppressor-competent p37 variants, suggesting small conformational alterations probably caused by the introduced mutations.

Some VSRs have been reported to bind dsRNAs irrespective of their size (5, 38–40). Such VSRs may compromise DCL-mediated processing of long dsRNAs (38). To further explore dsRNA binding capabilities of PLPV p37, we thus tested whether this protein was also able to bind large dsRNAs. In contrast with the results with double-stranded sRNAs (Fig. 4B), no shift in long dsRNA mobility was observed when p37 WT extracts were used (Fig. 4C). Indeed, the outcome of such an EMSA was identical to that observed with extracts from tissue agroinfiltrated with an empty vector (negative control), whereas a clear shift was detected when extracts from tissue

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**FIGURE 3. Evaluation of infectivity and virion formation of PLPV mutants.** *A*, detection of PLPV by Northern blot hybridization in total RNAs from local (7 dpif) and systemic tissue (30 days postinoculation (*d.p.i.*)) of *N. benthamiana* plants mechanically inoculated with WT and mutant PLPV transcripts. *B*, detection of PLPV by Northern blot hybridization in local leaves of *N. benthamiana* plants agroinoculated with WT and mutant constructs and collected at 3 dpif. Detection was performed on total RNA extracts (*left*) or on virion preparations (*right*). Virus titers, estimated from the autoradiogram on the *left*, were considered to normalize the loading of the virion preparations. In all panels, the positions of the genomic (*g*) and subgenomic (*sg*) PLPV RNAs are indicated at the *left*. Ethidium bromide staining of RNA was used as a loading control of total RNA samples.

expressing TCV p38, a VSR that is able to bind dsRNAs in a size-independent manner (38), were employed (Fig. 4C). To rule out the possibility that the *in vitro* conditions for dsRNA binding could substantially differ from those found *in vivo*, we tested whether PLPV p37 was able to prevent sRNA generation from hairpin transcripts transiently expressed in plants. To this end, *N. benthamiana* leaves were agroinfiltrated with GFP-IR (72) together with the constructs that allowed expression of WT or mutant p37 molecules. TCV p38 and tombusviral p19 (able and unable, respectively, to bind long dsRNAs) (33, 34, 38) were used as controls in parallel assays (Fig. 4D). As expected, hairpin transcripts were detected in GFP-IR- and TCV p38-coinfiltrated leaves, whereas sRNAs could not be found. Conversely, hairpin transcripts could not be detected in GFP-IR- and p19-coinfiltrated samples, whereas sRNAs accumulated to high levels. The latter situation was observed in GFP-IR- and PLPV (WT or mutant) p37-coinfiltrated leaves, thus confirming that the PLPV VSR lacks the ability to bind long dsRNAs. Globally, the results indicated that p37 is able to bind small, but not long, dsRNAs and supported a direct relationship between the sRNA binding capability of the protein and its ability to inhibit RNA silencing.

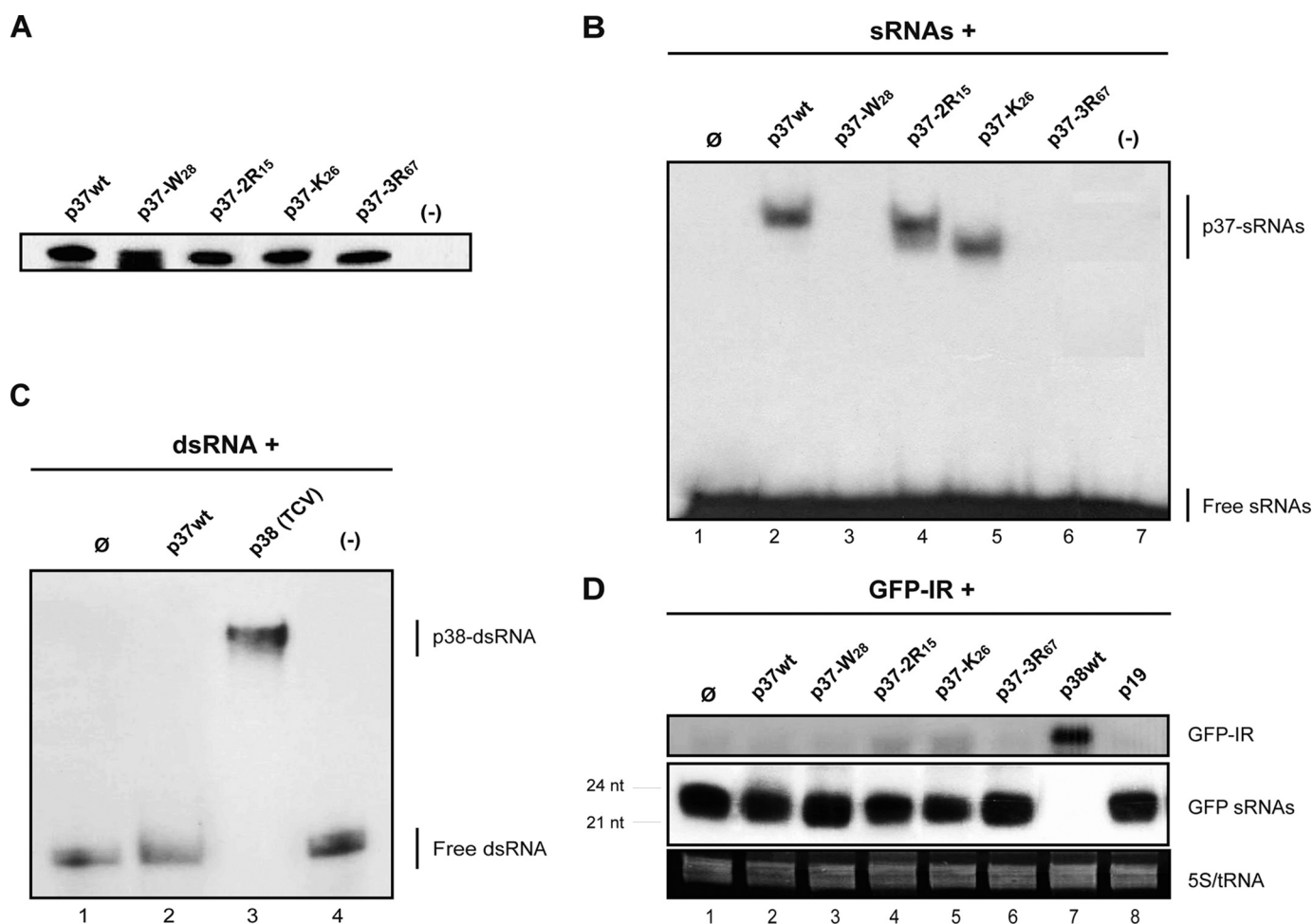
*Investigations on p37 Subcellular Localization, Self-interaction, and Interaction with AGO Proteins in Plants; Assessment of the Potential Contribution of These Protein Properties to Suppressor Activity*—Although binding to sRNAs seems fundamental for the activity of many VSRs, other properties, such as

dimerization, specific subcellular localization, or interaction with AGO proteins, have been proposed to be important in some instances (34, 42, 46, 47, 73–75). Thus, experiments were conducted to assess the potential relevance of the latter features in p37 suppressor function.

To investigate the subcellular localization of p37 in plant cells, this PLPV product was tagged at its N or C terminus with GFP and transiently expressed in *N. benthamiana* leaves via agroinfiltration. The green fluorescence derived from the p37 WT:GFP (or GFP:p37 WT; data not shown) fusion was evenly distributed through the cytoplasm and the nucleus (Fig. 5, *A panels*). Within the latter organelle, a central region showed a bright fluorescence, which corresponded to the nucleolus, as confirmed by the use of an mRFP-tagged fibrillarin as nucleolar marker (Fig. 5, *A2–A4*). The distribution pattern of p37 tagged with GFP differed from that of an unfused GFP, used as control, which showed the typical cytoplasmic and nuclear distribution but was excluded from the nucleolus (Fig. 5, *F panels*). The same approach was employed to analyze the subcellular localization of p37 mutants. Intracellular distribution of mutants p37-K<sub>26</sub> and p37-3R<sub>67</sub> was essentially identical to that of the WT protein (Fig. 5, *D and E panels*), whereas mutants p37-W<sub>28</sub> and p37-2R<sub>15</sub> maintained the cytoplasmic and nuclear localization but had lost the nucleolar one (Fig. 5, *B and C panels*). These results indicated that targeting of PLPV p37 to the nucleolus is apparently dispensable for its suppressor function, because mutant p37-2R<sub>15</sub>, which is capable of inhibiting RNA silencing, does not retain nucleolar localization.

Next, the potential of p37 to self-interact was analyzed through BiFC assays. To this end, constructs allowing transient expression of p37 fused to the N- or C-terminal part of the sYFP protein were generated. Co-expression of the two resulting fusion proteins, sYFPN-p37 WT and sYFPC-p37 WT, in *N. benthamiana* leaves gave rise to clear YFP-derived fluorescence within the plant cells, indicating reconstitution of the YFP and, thus, demonstrating that p37 is able to dimerize (Fig. 6, *A panels*). The same approach was employed to evaluate the dimerization capability of p37 mutants, and the results showed that, like the WT protein, all of them were able to interact with themselves (Fig. 6, *B–E*). For most p37 molecules, the distribution of fluorescence in these assays was essentially identical to that displayed by the corresponding GFP-tagged protein, the p37-K<sub>26</sub> self-interaction being the only exception, with no nucleolar signal. Control experiments in which the distinct fusion proteins were co-expressed with unfused sYFP halves (*e.g.* in Fig. 6F) did not yield significant fluorescence signals, substantiating the reliability of the fluorescence observations.

BiFC assays were also used to assess potential interactions of p37 with some AGO proteins, particularly AGO1 and AGO4, two RISC components whose interaction with other VSRs has been reported previously (42, 46, 62, 76). Co-expression of sYFPN-p37 WT and sYFPC-AGO1 (Fig. 7A) or the reverse combination sYFPC-p37 WT and sYFPN-AGO1 (data not shown) led to equivalent results and supported interaction of the two assayed proteins, with the resulting reconstituted fluorescence being detected through the cytoplasm and nucleus of the agroinfiltrated *N. benthamiana* cells. Similarly, co-expression of sYFPN-p37 WT and sYFPC-AGO4 (or sYFPC-p37 WT



**FIGURE 4. Analysis of the ability of p37 to bind dsRNAs.** *N. benthamiana* leaves were agroinfiltrated with an empty vector (negative control) or with constructs for expression of HA-tagged PLPV p37, p37-W<sub>28</sub>, p37-2R<sub>15</sub>, p37-K<sub>26</sub>, or p37-3R<sub>67</sub>. Samples were collected at 3 dpif to prepare crude protein extracts. **A**, Western blot analysis of protein extracts using an anti-HA antibody. **B**, EMSA performed with <sup>32</sup>P-labeled synthetic 21-nt sRNA (19-nt duplex region and 2-nt 3' overhangs) and no extract (∅) or extracts from leaves agroinfiltrated with an empty vector (-) or expressing distinct HA-tagged PLPV p37 molecules. The positions of free sRNAs and of p37-sRNA complexes are indicated on the right. **C**, EMSA performed with a <sup>32</sup>P-labeled long (250-bp) dsRNA and no extract (∅) or extracts from leaves agroinfiltrated with an empty vector (-) or expressing HA-tagged PLPV p37 variants. In this experiment, an extract from leaves expressing TCV p38 was used as a positive control. The positions of free long dsRNA and of TCV p38-long dsRNA complex are indicated on the right. **D**, Northern blot analyses to evaluate the effect of PLPV p37 molecules on the accumulation of hairpin transcripts and hairpin-derived sRNAs. *N. benthamiana* leaves were agroinfiltrated with GFP-IR (-) or with GFP-IR together with WT and mutant p37 forms. TCV p38 and tombusviral p19 were used in parallel assays as control VSRs able and unable, respectively, to bind hairpin transcripts. Samples were taken at 4 dpif.

and sYFPN-AGO4; data not shown) indicated that p37 is also able to associate with AGO4 (Fig. 7G), although, in this case, fluorescence was mainly detected in the cell nucleus, suggesting that the interaction essentially takes place in this cellular organelle. Next, we investigated whether p37 mutants maintained the capability of interacting with AGO1 and AGO4 as the WT protein. The results of the corresponding BiFC assays showed that all mutants were able to interact with both AGO1 (Fig. 7, B–E) and AGO4 (Fig. 7, H–K), although the intensity of the reconstituted fluorescence was considerably lower in the case of mutant p37-W<sub>28</sub> and AGO1 (Fig. 7B). No fluorescence was observed in control experiments in which the distinct YFPN-tagged proteins were co-expressed with unfused YFPN or *vice versa* (e.g. in Fig. 7, F and L), thus supporting that the fluorescence signals detected corresponded to true interactions.

Collectively, the results of the BiFC assays showed, on one hand, that p37 is able to self-associate. However, such a property is not sufficient by itself to sustain the suppressor function

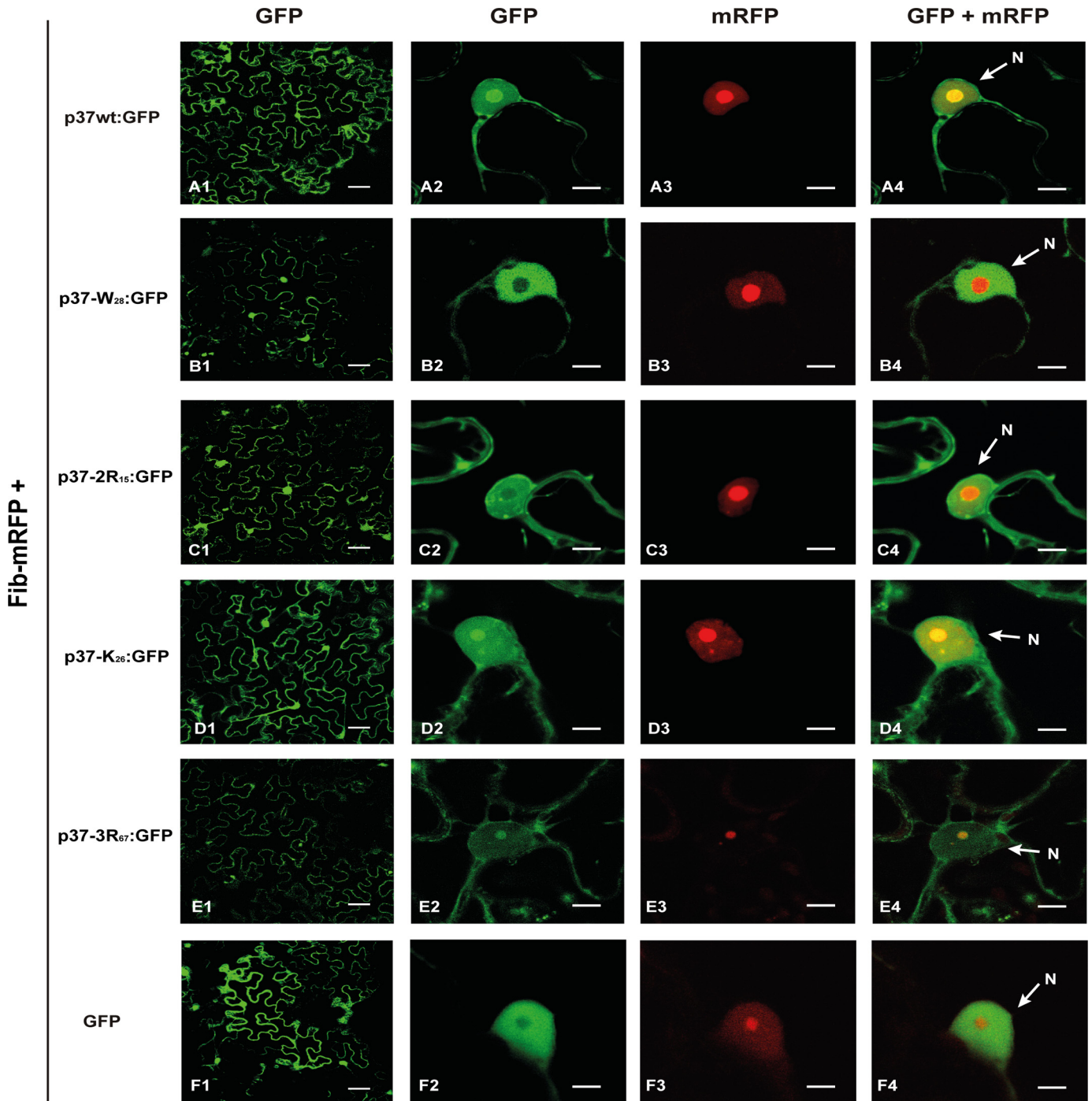
because dimerization of mutants p37-W<sub>28</sub> and p37-3R<sub>67</sub> was detected despite the fact that these p37 molecules do not block RNA silencing. On the other hand, the obtained data indicated that p37 can interact with AGO1 and AGO4 proteins. Nevertheless, no clear correlation among this capability and the silencing inhibition function of the protein could be established because the observed interactions were fully (p37-3R<sub>67</sub> with any AGO) or partially (p37-W<sub>28</sub> with AGO1) preserved in suppressor-incompetent p37 molecules.

## DISCUSSION

Distinct genera in the family Tombusviridae use different proteins as VSRs. In this study, the existence of suppressor(s) of RNA silencing encoded by PLPV, a member of the proposed new genus *Pelarspovirus*, has been investigated. As reported for several species of the related genus *Carmovirus* (27, 53, 54, 77), none of the PLPV non-structural proteins exhibited suppressor activity in co-infiltration assays of *N. benthamiana* plants,



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**FIGURE 5. Subcellular distribution of p37 molecules with a C-terminal GFP tag.** *N. benthamiana* leaves were agroinfiltrated with constructs that allowed transient expression of unfused GFP or GFP-tagged p37 proteins together with mRFP-tagged fibrilarin (*Fib-mRFP*, nucleolar marker). The tombusviral p19 was also included in these co-infiltration experiments to compensate for the lack of suppressor activity of some p37 molecules. Confocal laser-scanning microscopy was used for observation of fluorescence at 3 dpif. Micrographs of the *first column* (A–F, panels 1; starting from the left) show a general view of GFP-derived fluorescence in epidermal cells expressing the distinct protein combinations. Micrographs of the *second and third columns* (A–F, panels 2 and 3) show a *close-up view* of GFP- and mRFP-derived fluorescence, respectively, in individual cells, and micrographs of the *fourth column* show merged images of GFP and mRFP signals in such individual cells. The nucleus (N) is marked by an arrow in the *fourth column panels* (A–F, panels 4). The *inset scale bar* corresponds to 40  $\mu\text{m}$  in the *first column panels* and to 8  $\mu\text{m}$  in all remaining panels.

whereas p37, the viral CP, strongly inhibited RNA silencing of a GFP reporter gene.

The available information on VSRs indicates that such viral products may inhibit one or more of the silencing machinery actors, although the primary or most relevant target of a given

VSR is often unclear. Distinct analyses performed with engineered p37 variants have allowed the establishment of a direct correlation among the ability of the protein to bind sRNAs and its competence to inhibit RNA silencing (summary in Table 2), suggesting that, as proposed for other VSRs (33, 34, 37, 38, 78),

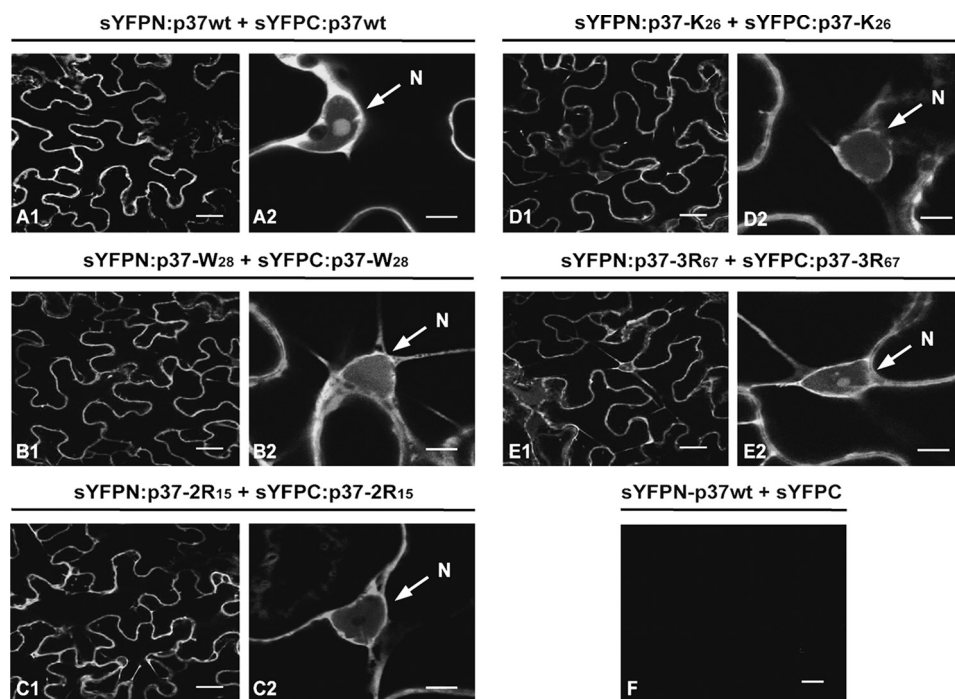


FIGURE 6. **In vivo analysis of p37 self-interaction.** WT (A) and mutant p37 molecules (B) were tagged at their N terminus with YFP halves (*sYFPN* and *sYFPC*) and transiently co-expressed in *N. benthamiana* leaves to study self-interaction through a BiFC assay. The tombusviral p19 was also included in these co-infiltration experiments to compensate for the lack of suppressor activity of some p37 molecules. Confocal laser-scanning microscopy was used for the observation of fluorescence at 3 dpif. For each protein combination, the micrograph at the left shows a general view of YFP-derived fluorescence in epidermal cells (inset scale bar, 20  $\mu$ m), and the micrograph at the right shows a close-up view of individual cells (inset scale bar, 8  $\mu$ m) with the nucleus (N) marked by an arrow. A negative control combination (*sYFPN-p37 WT* + *sYFPC*) is displayed in F.

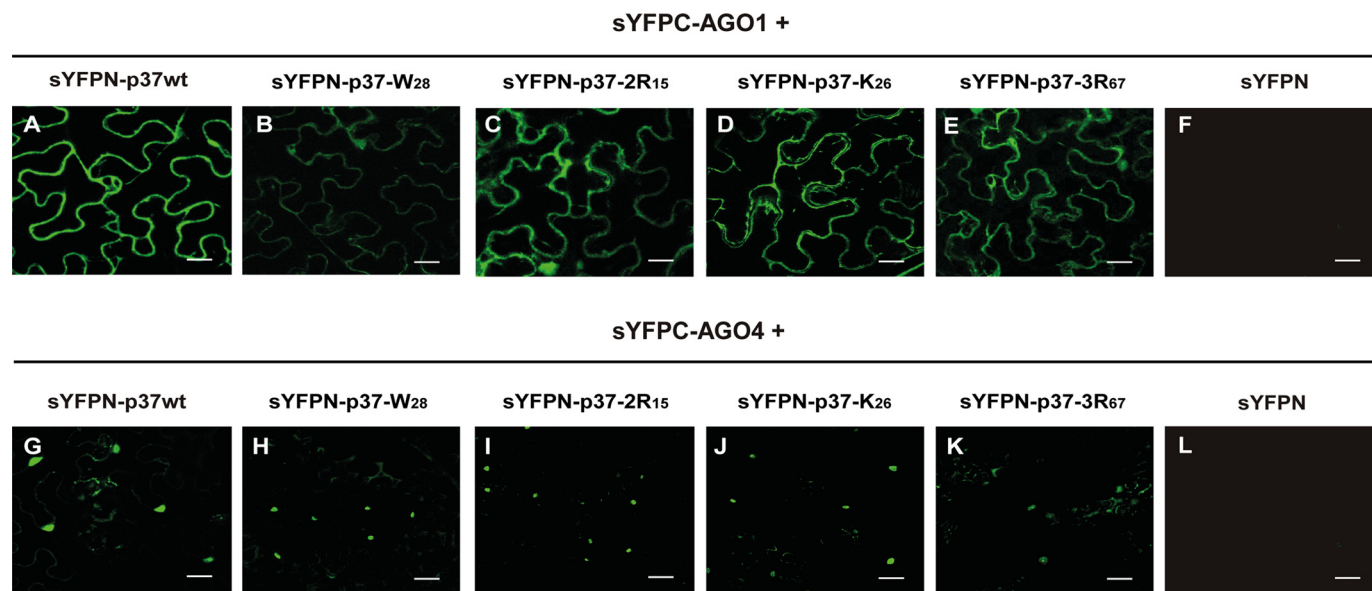


FIGURE 7. **In vivo analysis of p37 interaction with AGO proteins.** WT and mutant p37 molecules and Argonaute proteins (AGO1 and AGO4) were tagged at their N terminus with YFP halves (*sYFPN* and *sYFPC*) and transiently co-expressed in *N. benthamiana* leaves to study protein-protein interactions through a BiFC assay. Confocal laser-scanning microscopy was used for the observation of fluorescence at 3 dpif. The top and bottom micrographs show a general view of YFP-derived fluorescence in epidermal cells expressing *sYFPN-AGO1* and *sYFPN-AGO4*, respectively, in combination with *sYFPC*-tagged p37 molecules. Equivalent images were obtained with reverse combinations (*sYFPC-AGO1* or *sYFPC-AGO4* co-expressed with *sYFPN*-tagged p37 molecules) (data not shown). A negative control combination is displayed in the top (*sYFPC-AGO1* + *sYFPN*) and bottom (*sYFPC-AGO4* + *sYFPN*) raw. Inset scale bar, 20  $\mu$ m in all panels.

sequestering of sRNAs is a hallmark feature of the p37 suppressor function. This functional feature most likely precludes slicing of target RNAs by preventing sRNA loading into RISC. In addition, it may hinder amplification of the silencing signal, as suggested by the negligible detection of sRNAs in samples in which sense RNA-triggered RNA silencing was inhibited by

suppressor-competent p37 molecules (as by p19) (Fig. 2). The contribution of other properties, such as nucleolar localization, dimerization, or interaction with AGOs, to the suppressor activity of p37 might be disparate (Table 2). Sorting to the nucleolus has been considered as a relevant trait in some VSRs (62, 73, 74), but this supposition has become questionable after

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**TABLE 2**  
Properties of p37 variants

p37 variant	RNA silencing suppression	dsRNA binding		Nucleolar localization	Dimerization	Interaction with		
		sRNAs	Long dsRNA			AGO1	AGO4	Encapsidation
p37 WT	+	+	–	+	+	+++	+	+
p37-W <sub>28</sub>	–	–	NA <sup>a</sup>	–	+	+	+	+
p37-2R <sub>15</sub>	+	+	NA	–	+	+++	+	–
p37-K <sub>26</sub>	+	+	NA	+	+	+++	+	+
p37-3R <sub>67</sub>	–	–	NA	+	+	+++	+	–

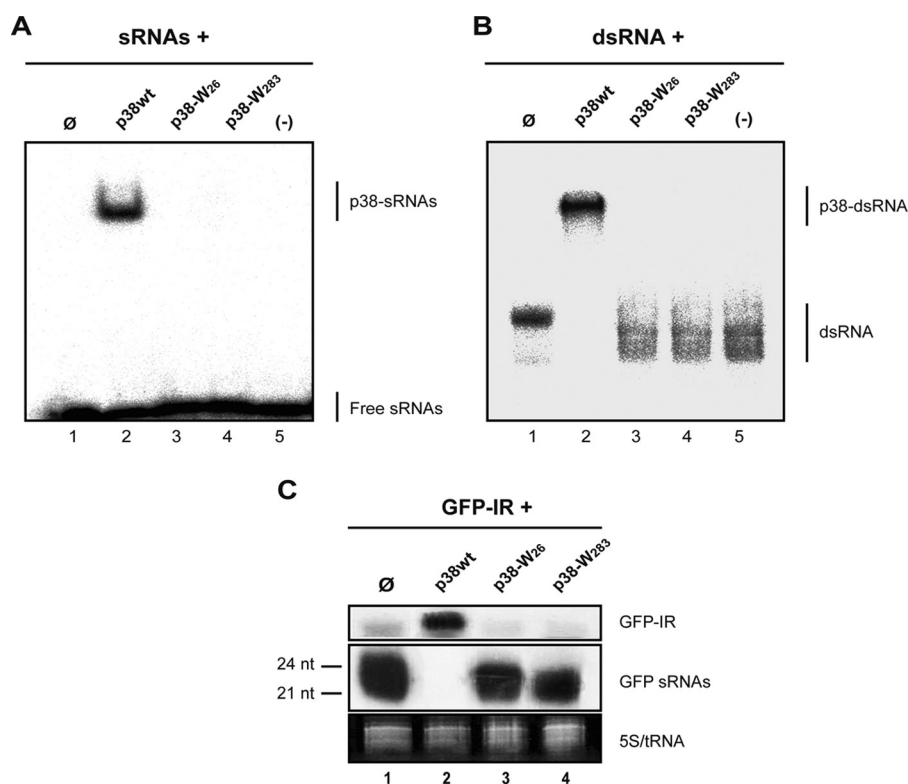
<sup>a</sup> NA, not applicable.

broader evaluations (78–80). The observation that p37, besides being targeted to the cytoplasm and nucleus, is targeted to the nucleolus was unexpected. Programs for subcellular localization prediction did not recognize any conventional nucleolar localization signal (or even nuclear localization signal) in the p37 molecule (data not shown), and, to our best knowledge, nucleolar localization has not been reported previously for any p37 ortholog. In any case, such subcellular localization seems to be dispensable for suppressor activity, as deduced from results with mutant p37-2R<sub>15</sub> that is able to inhibit RNA silencing despite being excluded from the nucleolus. On the other hand, dimerization has been shown to be a requirement for distinct VSRs, including the well characterized tombusviral p19 (33, 39, 71, 75, 81). Although we have shown here that PLPV p37 is able to interact with itself, all of the assayed protein mutants retained this capacity, and, thus, we could not critically assess whether this property is a prerequisite for the suppressor function. The relevance of dimer formation for the VSR role of some p37 relatives has been anticipated (27, 46), although, as for p37, formal demonstration has yet to be achieved. Finally, we cannot rule out the possibility that the detected interactions of p37 with AGO1 and AGO4 contribute to its suppressor function. However, such interactions by themselves seem to be insufficient to sustain the VSR activity of p37 because the interaction with AGO4 was maintained by all the assayed p37 variants, and the interaction with AGO1, although notably diminished regarding p37-W<sub>28</sub>, was retained at WT levels in the case of the loss-of-function form p37-3R<sub>67</sub>. The great importance of the sRNA binding capability over other protein properties is in agreement with recent reports on other VSRs, such as cucumber mosaic virus 2b, which, like p37, exhibits nucleolar localization, self-interaction, and interaction with AGO1 and AGO4 proteins (42, 62, 75, 78, 82).

Distinct studies with variant experimental systems led to different models for the mechanism of silencing suppression of TCV p38, a VSR structurally and phylogenetically related to PLPV p37. For instance, TCV p38 has been postulated to exert its action through binding and sequestering of sRNAs (38), inhibition of sRNA methylation (83), interference with DCL4 (16, 38), or induction of stress and defense pathways (84). In a recent study, the N-terminal and C-terminal GW motifs of TCV p38 have been implicated in interaction with AGO1 which, in turn, has been proposed to dictate the suppressor activity of the protein (46). Mutation of the unique, conserved N-terminal GW motif of PLPV p37 notably reduced (suggesting that it is a *bona fide* GW protein) but did not completely abolish its interaction with AGO1 despite the fact that the engineered amino acid replacement was equivalent to one reported

to abrogate TCV p38-AGO1 interaction. These discrepancies probably reflect distinct characteristics of the corresponding proteins. Nevertheless, they could also be due to the fact that the interaction of TCV p38 with AGO1 was tested *in vitro* using an AGO1 antibody to direct immunoprecipitation of peptides containing either of the two GW motifs of the VSR, whereas we have used an *in vivo*, BiFC assay with the entire PLPV p37 protein. Although both approaches have limitations, the former one might be more prone to inaccuracies because it obviates the influence of the protein conformation and protein compartmentalization in the tested molecular interaction. In any case, the dsRNA binding capability of TCV p38 molecules bearing mutations in the GW motifs was not analyzed, and an effect of the mutations on this capacity of the protein, similar to that observed for PLPV p37, could not be discarded. Indeed, we have performed EMSAs with the TCV p38-GW mutant forms and have corroborated that such mutants have lost the ability to bind to both small and long dsRNAs (Fig. 8). The lack of sRNA binding capability could actually provide an alternative or complementary explanation to the recovery of infectivity of TCV carrying p38-GW mutations in *dcl2-dcl4* plants (46). The knockdown of the two main anti-TCV DCLs (16) in these plants precludes the generation of virus-derived sRNAs (vsRNAs), and, thus, hijacking of vsRNAs by the VSR to prevent viral RNA degradation would be dispensable. Similarly, the TCV p38-GW mutants could show infection recovery in *ago1-27* plants (46) because the absence of AGO1 effector protein would make the vsRNAs useless for antiviral defense. Whether the loss of sRNA binding capability provoked by mutation of GW motif(s) contributes, to any extent, to impairment of the suppressor function of p38 has yet to be explored.

In the context of PLPV infection, the need to counteract RNA silencing for effective viral multiplication and dispersion was underlined by viruses bearing mutations that abolished the p37 suppressor activity not being viable. Remarkably, some of the engineered mutations successfully uncoupled the VSR function of p37 from its particle assembly function, and such genetic uncoupling has allowed us to find out that both are strictly required for PLPV systemic spread in *N. benthamiana*. The requirement of assembled virions for long distance movement seems to be variable in distinct Tombusviridae and hosts (69, 85–90). A recent report on the TCV-*A. thaliana* pathosystem has demonstrated the key importance of virus particles for egress of the TCV genome from the vasculature of systemic leaves (70). Although this question has not been specifically tackled in this work, a similar scenario seems likely for PLPV in *N. benthamiana*.



**FIGURE 8. Analysis of the ability of TCV p38 to bind dsRNAs.** *N. benthamiana* leaves were agroinfiltrated with an empty vector (negative control) or with constructs for the expression of TCV p38 either in its WT version or with mutations in the N-terminal (p38-W<sub>26</sub>) or C-terminal (p38-W<sub>283</sub>) GW motif, as described previously (46). The agroinfiltrated tissue was collected at 3 dpif to prepare crude protein extracts. **A**, EMSA performed using <sup>32</sup>P-labeled synthetic 21-nt sRNA (19-nt duplex region and 2-nt 3' overhangs) and no extract (∅) or extracts from leaves agroinfiltrated with an empty vector (-) or expressing TCV p38 variants. The positions of free sRNAs and of p38-sRNAs complexes are indicated on the right. **B**, EMSA performed with a <sup>32</sup>P-labeled long (250 bp) dsRNA and extracts from leaves agroinfiltrated with an empty vector (-) or expressing TCV p38 variants. **C**, Northern blot analyses to evaluate the effect of TCV p38 molecules on the accumulation of hairpin transcripts and hairpin-derived sRNAs. *N. benthamiana* leaves were agroinfiltrated with GFP-IR (-) or with GFP-IR together with WT and mutant TCV p38 forms. Samples were taken at 4 dpif.

The expression of VSRs during viral infections often results in severe damage of the host. Indeed, many VSRs were initially identified as viral pathogenicity determinants (55, 91). The molecular bases of such an effect might be diverse and seem mostly to be related with viral accumulation enhancement and/or interference with silencing pathways affecting regulation of host gene expression (55, 92, 93). Strikingly, PLPV usually causes asymptomatic infections in their natural hosts (*Pelargonium* spp.) as well as in experimental hosts, such as *N. benthamiana* or *Nicotiana clevelandii* (58, 94). Moreover, PLPV titers are considerably low in infected tissue (65), which could suggest that either the virus lacks an efficient VSR or the expression/activity of such a VSR is modulated during infection. We have shown that PLPV p37 behaves as a strong VSR, at least in transient expression assays. Thus, it seems more plausible that the virus has evolved strategies to minimize the impact that its infection may have on the host because such an impact may adversely affect virus reproduction and/or transmission. One of these strategies may be related to the production of a single, tricistronic sgRNA for the expression of internal and 3'-proximal genes. As we have previously reported (51), the translation of p7, p9.7, and p37 proteins from the sole PLPV sgRNA is accomplished through leaky scanning processes that are facilitated by specific genomic traits (weak context of the AUG initiation codon of the MP1 gene, non-AUG initiation codon of the MP2 gene, and lack of AUG codons in any frame

between the initiation codons and the MP1 and CP gene). However, the expression of the 3'-terminal gene, which encodes p37, is probably more inefficient from the tricistronic mRNA than, for instance, the monocistronic sgRNA that typically directs translation of CP in carmoviruses. Down-regulation of p37 expression through this mechanism might contribute to alleviate potential side effects caused by the VSR on host expression.

In conclusion, we have shown here that the PLPV p37 protein inhibits RNA silencing and that this GW motif-containing VSR probably exerts its action mainly through sequestration of sRNAs. The obtained data also caution against potential misinterpretations of results due to overlap of sequence signals related to distinct protein properties. This is well illustrated by the GW motif mutation in p37 that concurrently affects its nucleolar localization, its efficient interaction with AGO1, and its sRNA binding capability. These concomitant effects could have been overlooked in other GW motif-containing suppressors, as we have exemplified with the orthologous p38 protein of TCV. Some other interesting questions derived from the results remain to be investigated. One of them refers to the biological meaning of the nuclear/nucleolar localization of p37, a protein whose known functions (RNA silencing suppression and encapsidation) are expected to be executed essentially in the cellular cytosol. None of these functions is apparently affected by defective nucleolar localization, as indicated by the

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suppressor-incompetent, virion assembly-competent mutant p37- $W_{28}$  or the suppressor-competent, virion assembly-incompetent mutant p37- $2R_{15}$ . Such subcellular targeting could be related to still uncovered functions of p37. On the other hand, we have found that distinct p37 variants exhibit similar interactions with AGO1 or AGO4, irrespective of their suppressor capability. This observation raises doubts about the significance of such interactions for the suppressor function of p37, although the involvement of other AGO proteins (not assayed in the present study) in anti-PLPV defense cannot be ruled out. Further studies are being designed in an attempt to shed new light on these outstanding issues.

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