Identification of an RNA-silencing suppressor in the genome of Grapevine virus A

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INTRODUCTION

RNA silencing, a sequence-specific RNA degradation process discovered in different eukaryotic organisms, is an ancient defence mechanism that protects them from invading RNA molecules (Ratcliff et al., 1997; Vance & Vaucheret, 2001; Voinnet, 2001; Ding et al., 2004). The RNA silencing pathway is triggered by dsRNAs originated by exogenous or endogenous transgenes, transposons or infecting viral RNAs (Fire et al., 1998; Hannon, 2002). A key role in the silencing process is played by small interfering RNAs (siRNAs), i.e. dsRNAs of 21–25 nt, generated by the cleavage of larger dsRNA molecules by RNase III-like enzymes which, in Arabidopsis thaliana, are called DCL-1 to -4 (Hamilton & Baulcombe, 1999; Schauer et al., 2004; Xie et al., 2004). These siRNAs are incorporated into an RNA-induced silencing complex (RISC) that operates the sequence-specific degradation of target RNAs. Their presence is a marker of post-transcriptional gene silencing (PTGS) (Frederick, 2000; Elbashir et al., 2001). Once locally induced in a plant, PTGS generates mobile signals that move from cell to cell through plasmodesmata and systemically via the vascular system to cleave target RNA. Although the nature of the silencing signal is unknown, it must have an RNA component that accounts for its sequence specificity (Voinnet, 2005a).

Plant viruses are both inducers and targets of PTGS (Voinnet, 2005b). Their replication in plant cells generates a complex of defence and counterdefence reactions that can result in the reduction of viral titre, disappearance of symptoms (recovery), or immunity of the upper non-inoculated leaves. To counteract plant PTGS, a number of viruses have evolved a strategy based on silencing suppressor proteins, which interfere at different stages with the RNA silencing pathway (Roth et al., 2004). Thus, the p25 product expressed by ORF2 of Potato virus X (PVX) is supposed to interfere with the assembly of siRNAs in RISC (Voinnet et al., 2000), whereas tombusvirus p19 (Lakatos et al., 2004) and aureusvirus p14 (Merai et al., 2005) bind siRNAs, thus making them unavailable for RISC. Several of these proteins have been shown to interfere with the steady-state levels of microRNAs (miRNAs) (Chapman et al., 2004; Chellappan et al., 2005), a further class of small non-coding RNAs that regulate mRNAs involved in development at the post-transcriptional level.

Members of the genus Vitivirus (Martelli et al., 1997) have monopartite ssRNA genomes encoding five proteins which, in the case of Grapevine virus A (GVA), the type species of the genus, are expressed through a series of nested subgenomic RNAs (Galiakparov et al., 2003a). Functional analysis of the GVA genome has shown that ORF1 encodes replication-associated protein ORF3 for the movement protein (MP) and ORF4 for the viral coat protein (CP). The
function of ORF2 remains undetermined, whereas the 10 kDa product of ORF5 (p10) is a nucleic acid (NA)-binding protein. ORF5 suppression in a GVA infectious transcript results in marked attenuation of symptoms and restriction of systemic viral movement, but has no effect on replication (Galiakparov et al., 2003b). It has also been shown that the NA-binding property of p10 is non-specific with regard to the type of nucleic acid, and requires a stretch of basic amino acids located at its amino terminus (Galiakparov et al., 2003b).

In this study, we show that GVA p10 is a suppressor of viral and transgene-induced RNA silencing, and that its potential mechanism of action is based on sequestering siRNAs.

METHODS

Plasmid constructs. Each single GVA ORF was amplified by reverse transcription-PCR (RT-PCR) with 5′- and 3′-specific primers containing suitable restriction sites from total RNA (TNA) extracts from GVA strain Is151-infected Nicotiana benthamiana plants, and cloned into plasmid pDrive (Qiagen). Individual sequences were digested with XhoI/BamHI (pDrive-MP, pDrive-CP and pDrive-ORF5) or SpeI/BamHI (pDrive-ORF2) from each pDrive-ORF and cloned into similarly restricted binary plasmid pBI121 to generate cassettes driven by the 35S RNA promoter (35S). An ORF5 mutant (ORF5mutFS) lacking the initial ATG and potentially generating a frame-shifted 20 aa polypeptide (Fig. 1), was obtained by RT-PCR with primers 5′GTCGACATTAATTTCTACGAGG-3′ and 3′ORF5 (5′GGATGACCCATAATTTCTACGAGG-3′), and was used as above to produce plasmid pBI-ORF5mutFS. Each of the five binary constructs (pBI-ORF2, pBI-MP, pBI-CP, pBI-ORF5 and pBI-ORF5mutFS) was transformed into Agrobacterium tumefaciens (strain C58C1 by electroporation and selected in LB medium containing 50 μg kanamycin ml−1. Plasmid pBin61-GFP in Agrobacterium strain C58C1 and plasmid pCB302/P1/HcPro containing the sequence of the P1/HcPro silencing suppressor of Potato virus Y (PVY) in Agrobacterium strain GV2260 were kindly provided by Dr D. Baulcombe and was kept throughout the assays at 22–24 °C in a growth chamber. Agrobacterium infiltration was done as described by Hamilton et al. (2002), resuspending the final bacterial cultures at an OD600 of 1. In coinfiltration experiments, 0.4 vols of an Agrobacterium culture containing 35S-GFP was mixed with 0.6 vols of each individual Agrobacterium bearing 35S-ORFs immediately before infiltration. GFP fluorescence was observed under long-wavelength UV light (Black Ray model B 100A; UV products) and photographed using a Nikon D70 digital camera with an opticlight 52 yellow filter.

RNA isolation and analysis. N. benthamiana tissues were ground to a fine powder in liquid nitrogen and TNAs were extracted with Trí-Reagent (Sigma), according to the manufacturer’s instructions. The same TNA extract was used for gel blot analysis of high- and low-molecular-mass RNAs. TNAs (10 μg) from agroinfiltrated or virus-infected tissues were separated on a 1 % formaldehyde agarose gel and transferred to Hybond-N+ membranes (Amersham Biosciences) for Northern blot analysis. Membranes were hybridized with digoxigenin (Dig)-labelled complementary RNA probes, corresponding to the GFP sequence, a fragment of the PVX CP sequence or the full-length GVA ORF5 sequence. Hybridization conditions and detection were according to the manufacturer’s instructions (Roche Diagnostics). Northern blot analysis of siRNAs was done after separation of TNAs (15 μg) on denaturing high- and low-molecular-mass RNAs. TNAs (10 μg) from agroinfiltrated or virus-infected tissues were separated on a 1 % formaldehyde agarose gel and transferred to Hybond-N+ membranes with a Bio-Rad semi-dry apparatus (Johansen & Carrington, 2001). Hybridization and detection of siRNAs was done with Dig-RNA probes as described by Canizares et al. (2004). The RNA probe corresponding to the GFP sequence (about 0.7 kb) was degraded under alkaline conditions (200 mM carbonate buffer, pH 11) to fragments of about 0.1 kb.

In vitro RNA transcription and plant inoculation. Transcription of pP2C2s-GFP, pP2C2S-ORF5 and pP2C2S-ORF5mutTR Spel-linearized plasmids (1 μg) was made with the mMESSAGE mMACHINE T7 (Ambion) using T7 RNA polymerase. Approximately 100 ng transcribed RNAs, resuspended in a celite-containing buffer (1 % celite, 1 % bentonite, 50 mM glycine, 30 mM K2HPO4, pH 9), were inoculated onto leaves of normal or transgenic (line 16c) N. benthamiana plants.

E. coli expression of GVA ORF5 and electrophoretic mobility shift assays. ORF5 sequence was excised by BamHI/HindIII digestion of the previously constructed pDrive-ORF5 plasmid, ligated to pP2C2S and used to transform Escherichia coli Top10 cells. The untranslatable ORF5 mutant pP2C2S-ORF5mutTR was obtained by PCR mutagenesis using complementary primers sST1 (5′-ATCGATGGATGACCCATAATTTCTACGAGG-3′) and antisST1 (5′-ACCTACCGTGAGAAATTATGGGTCAATCGTG-3′), which changed the fifth UCG codon into UAA, thus introducing a translational stop. Mutagenesis was done with the Quick change mutantesis kit (Stratagene, following the manufacturer’s instructions). All described plasmids were verified by nucleotide sequencing.

Plant materials and agroinfiltration. Transgenic N. benthamiana, constitutively expressing the GFP transgene (line 16c) was kindly provided by Dr D. Baulcombe and was kept throughout the assays at 22–24 °C in a growth chamber. Agrobacterium infiltration was done as described by Hamilton et al. (2002), resuspending the final bacterial cultures at an OD600 of 1. In coinfiltration experiments, 0.4 vols of an Agrobacterium culture containing 35S-GFP was mixed with 0.6 vols of each individual Agrobacterium bearing 35S-ORFs immediately before infiltration. GFP fluorescence was observed under long-wavelength UV light (Black Ray model B 100A; UV products) and photographed using a Nikon D70 digital camera with an opticlight 52 yellow filter.
a similarly digested pQE30 plasmid (Qiagen) and the construct was introduced into E. coli strain M15(pREP4). The N-terminal histidine-tagged protein was expressed and purified under native conditions with NiNTA resin (Qiagen), following the manufacturer’s instructions. Synthetic siRNAs (Chellappan et al., 2005) were synthesized by end-labelling RNA oligonucleotides miRNA159 (5’-UUUGGA-UUAAAGGGGACUCUA-3’), siRNA GFP (5’-GCUGACCCUGA-GUUCACUCU-3’/5’-GAUGCACUGAGGGUGACCUU-3’) and miRNA159/159* (5’-UUUGGAUUGAGGGGACUCUA-3’/5’-GAG-CUCUUAAAGUGCAAAA-3’) (Invitrogen) in a 10 μl reaction containing [32-P]ATP, RNase inhibitor and 7 units T4 polyribonucleotide kinase. RNA duplexes were checked by 15 % PAGE in native conditions. ORF5 complexes were formed in a 20 μl reaction containing 5 μM protein, 0.04 μM oligonucleotide, 20 mM HEPES-KOH, pH 7-9, 60 mM KCl, 12% glycerol, 2 mM EDTA, 2 mM DTT (Sorger & Pelham, 1987) and 8 units RNase inhibitor, and incubated at 22°C for 20 min. Complexes were resolved on 8 % native polyacrylamide gels which were then dried and exposed for autoradiography with an intensifying screen.

RESULTS

Screening for RNA silencing suppressors in the GVA genome

Initial screening of the GVA genome for the expression of a potential protein suppressor of RNA silencing was carried out by reversal assay (Roth et al., 2004) of post-transcriptionally silenced GFP transgenic N. benthamiana 16c plants (Brigneti et al., 1998). Plants completely silenced by infiltration with an Agrobacterium strain carrying the homologous GFP transgene under the control of a 35S promoter (35S-GFP) were successively infected with GVA or PVY, which expresses the P1/HcPro silencing suppressor. GVA infection did not suppress silencing in inoculated or upper non-inoculated leaves (Fig. 2a). By contrast, in PVY-infected control plants green fluorescence appeared 20 days post-inoculation (d.p.i.) in newly emerging leaves (Fig. 2b), thus reversing the established silencing.

ORF5 p10 suppresses local RNA silencing

Individual GVA proteins were screened to identify suppressors by the Agrobacterium coinfiltration assay on GFP transgenic N. benthamiana 16c plants. Sequences encoding ORF2p20, MP, CP and ORF5p10 proteins were cloned into a binary vector under the control of a 35S promoter and expressed in plant tissues by infiltration with A. tumefaciens. Mixed cultures of Agrobacterium carrying individual ORFs together with another Agrobacterium strain expressing the silencing inducer 35S-GFP cassette, were co-infiltrated in N. benthamiana 16c leaves and the progression of silencing was observed over time. Examination of infiltrated leaves 6 days post-infiltration (d.p.i.f.) showed that in tissues infiltrated with 35S-GFP plus the empty vector, green fluorescence decreased (Fig. 3a, −), while a characteristic silenced red ring bordered the infiltrated area (Himber et al., 2003). By contrast, in tissues infiltrated with the silencing suppressor P1/HcPro, fluorescence clearly increased (Fig. 3a, P1). Among the four GVA coding sequences tested, only ORF5 (Fig. 3a, 5) showed a persistence of fluorescence up to 12 d.p.i.f., whereas P1/HcPro suppression was visible up to 20 d.p.i.f. ORF5 expression also suppressed the cell-to-cell spread of the silencing signal as shown by the lack of a silenced ring of cells bordering agroinfiltrated tissues (Himber et al., 2003). An ORF5 mutant (ORF5mutFS) that does not contain the arginine-rich motif (Galiakparov et al., 2003b and Fig. 1) did not suppress local or cell-to-cell silencing (Fig. 3a, 5mut). These experiments were repeated no less than three times by infiltrating each ORF on 10 different plants. Northern blot analysis of TNAs from infiltrated tissues confirmed visual observations since, similar to the positive control P1/HcPro (Fig. 3b, lane 4), steady-state levels of GFP mRNAs increased with ORF5 expression (Fig. 3b, lanes 5, 7), with respect to the silenced control (Fig. 3b, lane 2). None of the other GVA ORFs (Fig. 3b, lanes 6, 8, 9) nor the ORF5 mutant (Fig. 3b, lane 3) displayed such a suppressor activity in the patch assay.

A time-course analysis showed that ORF5-induced suppression was detected as early as 3 d.p.i.f., although GFP mRNA levels were lower than those induced by the P1/HcPro suppressor (not shown). When 21–25 siRNAs levels were analysed at 3 and 6 d.p.i.f., the decrease in GFP mRNA accumulation in tissues agroinfiltrated with 35S-GFP and ORF5mutFS was accompanied by the appearance of GFP-specific 21–25 siRNAs (Fig. 3c, lanes − and 5mut). By contrast, siRNAs were much reduced with P1/HcPro suppression.
Similar transient assays were carried out with normal N. benthamiana plants by infiltration with Agrobacterium carrying 35S-GFP with or without 35S-ORF5. As found by Johansen & Carrington (2001), GFP fluorescence faded 6 d.p.i.f. in infiltrated patches because of the silencing of exogenous GFP mRNA carried by Agrobacterium (Fig. 4a, −). Co-expression of P1/HcPro (Fig. 4a, P1) or ORF5 (Fig. 4a, 5) suppressed silencing. In fact, in patches infiltrated with ORF5 or P1/HcPro, levels of GFP mRNAs were increased (Fig. 4b, lanes 5 and P1) with respect to the control (Fig. 4b, lane −). Concurrently, tissues infiltrated with ORF5 showed strongly reduced accumulation of silencing-associated 21–25 siRNAs (Fig. 4b, lane 5), with respect to GFP-infiltrated patches (Fig. 4b, lane −).

**Fig. 3.** ORF5 p10 suppresses ssRNA-induced RNA silencing. (a) Leaves of GFP transgenic N. benthamiana were infiltrated with mixtures of Agrobacterium cultures containing 35S-GFP plus the empty vector (−), 35S-MP (MP), 35S-CP (CP), 35S-ORF2 (2), 35S-ORF5 (5), 35S-P1/HcPro (P1) and 35S-ORF5mutFS (5mut). Photos were taken 6 d.p.i.f. (b) Northern blot analysis of total RNAs extracted from agroinfiltrated patches in (a) or from a non-transgenic plant (Hty). GFP mRNAs were detected by hybridization with a Dig-RNA probe complementary to the sense GFP mRNA. rRNAs are shown for equal loading. (c) Detection of GFP-associated siRNAs in leaves of GFP transgenic N. benthamiana infiltrated with mixtures of Agrobacterium cultures containing 35S-GFP plus the empty vector (−), 35S-ORF5 (5), 35S-P1/HcPro (P1) and 35S-ORF5mutFS (5mut). Northern blots of total RNA extracts from agroinfiltrated patches hybridized with a degraded Dig-RNA probe complementary to sense GFP mRNA are shown. rRNAs are shown for equal loading. A GFP-related 26-mer oligonucleotide (oligo) is included as control. Hty, total RNAs from healthy N. benthamiana tissues.

**ORF5 p10 suppresses systemic RNA silencing and interferes with the spread of the silencing signal**

To study the effect of p10 on systemic silencing, N. benthamiana 16c leaves were infiltrated and the progress of silencing was monitored on the upper leaves for 25 days (Table 1). In plants infiltrated with ORF5, systemic RNA silencing, ascertained by the loss of fluorescence along the veins of the upper leaves, was delayed and took place in a small number of plants (6 out of 20 at the most), similar to what was observed in plants infiltrated with the strong suppressor P1/HcPro. By contrast, systemic silencing developed quickly and in a larger number of plants infiltrated with 35S-GFP or ORF5mutFS. Further analysis of the involvement of ORF5 in the evolution of systemic silencing was sought with an assay described by Guo & Ding (2002),
in which the 2b protein of *Cucumber mosaic virus* was locally expressed along the presumed path of spread of the mobile signal of RNA silencing. Thus, 35S-GFP and ORF5 were infiltrated, respectively, at the tip and the base of two basal leaves of *N. benthamiana* 16c plants. Since the rationale of the assay was to evaluate the interference of the locally expressed ORF5 with the movement of the tip-generated mobile silencing signal in a source/sink direction, control plants were infiltrated with both *Agrobacterium* constructs in the opposite orientation. Systemic silencing of upper non-infiltrated leaves developed 5 d.p.i. only in control plants (i.e. 35S-GFP-infiltrated at the base of the leaves), 6 of which out of 8 were silenced 11 d.p.i., whereas, at the same time, none of the 8 plants infiltrated with ORF5 at the base of the leaves showed systemic silencing (Table 2). Northern blot analysis of the GFP mRNA steady-state levels carried out on systemic leaves confirmed visual observations of GFP fluorescence (data not shown). These findings indicate that GVA ORF5 can inhibit the onset of local silencing and reduces or delays the progress of systemic silencing triggered by a GFP ssRNA.

**ORF5 p10 is a symptom determinant and is active in virus-induced gene silencing (VIGS)**

Silencing suppressors enhance symptom severity when expressed by a heterologous virus, as shown for P1/HcPro and *Cucumber mosaic virus* 2b (Pruss et al., 1997; Ding, 2000). GVA ORF5 was therefore inserted into the PVX-based vector pP2C2S (PVX-ORF5), and transcripts were inoculated into *N. benthamiana*, *Nicotiana occidentalis* and *Nicotiana glutinosa*. In all hosts, ORF5 expression induced stunting and necrosis 8–10 d.p.i., as opposed to the mild mosaic elicited by wild-type PVX (Fig. 5A, e, g). In particular, *N. benthamiana* and *N. occidentalis* reacted with necrosis of the upper leaves, often followed by death of the plant (Fig. 5A, b, c, d and f). Northern blotting of TNAs extracted from symptomatic leaves using an ORF5 probe showed that the ORF5 sequence was retained in the progeny PVX RNA up to the third passage (not shown). Plants inoculated with PVX-ORF5mutTR developed symptoms as mild as those of the wild-type PVX in all *Nicotiana* species. Taken together, these findings indicate that expression of GVA ORF5 enhances the pathogenicity of the unrelated PVX, and that this is probably due to the p10 protein.

Transcripts from PVX-ORF5 were inoculated into silenced 16c *N. benthamiana* plants, to evaluate the ability of p10 to reverse the established silencing when ectopically expressed from the strong PVX subgenomic promoter. The protein failed to suppress the silencing in this system as well (not shown), thus demonstrating that this inability is due to intrinsic properties rather than poor expression from the GVA genome.

Infection of *N. benthamiana* 16c plants with a PVX vector expressing GFP-coding sequences (PVX-GFP) gives rise to complete transgene silencing, the phenomenon known as VIGS (Ruiz et al., 1998). Simultaneous expression of a silencing suppressor (P1/HcPro) by the same viral vector in a mixed infection with PVX-GFP, inhibits the silencing process (Anandalakshmi et al., 1998). VIGS of the GFP transgene was therefore monitored in single- (PVX-GFP) or double- (PVX-GFP + PVX-ORF5) infected *N. benthamiana* 16c plants for 30 d.p.i. Plants infected with PVX-GFP showed a progressive silencing of the GFP transgene that induced an almost complete loss of fluorescence of the upper leaves at 25 d.p.i. (Fig. 5B; leaf C.Si., complete silencing). Concurrently, leaves of plants double-infected with PVX-GFP and PVX-ORF5 displayed different phenotypes, ranging from intermediate (Fig. 5B; leaf I.Su., fluorescence limited to the veins) to complete (Fig. 5B; leaf C.Su., extensive fluorescence of the upper leaves) suppression of silencing. Visual observations were confirmed by Northern blotting, since in PVX-GFP-infected plants neither GFP mRNA (not shown) nor PVX genomic RNA (Fig. 5C, lane PVX-GFP) were detected. By contrast, viral RNAs were readily detected in the upper leaves of double-infected

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**Fig. 4.** p10 suppresses ssRNA-induced RNA silencing in *N. benthamiana*. (a) Leaves were infiltrated with mixtures of *Agrobacterium* cultures containing 35S-GFP plus the empty vector (−), 35S-ORF5 (5) and 35S-P1/HcPro (P1). Photos were taken 6 d.p.i. Northern blot detection of GFP mRNAs (b) or siRNAs from agroinfiltrated patches in (a) or from a non-agroinfiltrated plant (Hty) is shown. rRNAs are shown for equal loading.
Fig. 5. ORF5 enhances symptoms and suppresses VIGS. (A) Symptoms of PVX-ORF5 in *N. benthamiana* and *N. occidentalis* 10 d.p.i. (a) Mock-inoculated *N. benthamiana*; (b, c, d) systemic leaves of PVX-ORF5 infected *N. benthamiana* and (f) *N. occidentalis*; (e) PVX-infected *N. benthamiana* and (g) *N. occidentalis*. Arrows point to necrotic lesions. (B) Systemic leaves of GFP transgenic *N. benthamiana* infected with PVX-GFP or a mixture of PVX-GFP plus PVX-ORF5 observed under UV 25 d.p.i. (C) Northern blot detection of PVX (left) or ORF5 (right) related sequences in total RNAs from leaves photographed in (b). C.Si., Complete silencing; I.Su., intermediate suppression; C.Su., complete suppression. Arrowheads point to PVX genomic (top) and larger subgenomic RNAs (bottom).
plants, showing inhibition of silencing (Fig. 5C, lanes PVX-GFP + PVX-ORF5) as well as GFP mRNA levels (not shown). Hybridization with an ORF5-specific probe indicated that ORF5 sequences were retained in the viral progeny (Fig. 5C, lanes PVX-GFP + PVX-ORF5). These findings suggest that expression of GVA ORF5 interferes with the establishment of VIGS, and strongly support the role of the p10 as a silencing suppressor.

**ORF5 p10 binds small RNAs**

Galiakparov et al. (2003b) showed that the GVA ORF5 expression product was able to bind long ssRNAs and dsDNAs, due to the presence of a basic arginine-rich domain located at the amino terminus of p10. This prompted us to investigate the ability of this protein to bind small RNAs (siRNAs and miRNAs) as well, since expression of p10 decreases GFP siRNA levels in co-agroinfiltrated tissues (Fig. 3c). Because suppression by tombusviral p19, Beet yellow virus p21 and African cassava mosaic virus AC4 occurs through binding of siRNAs or miRNAs (Vargason et al., 2003; Chapman et al., 2004; Chellappan et al., 2005), this was also investigated for p10 by electrophoretic mobility shift assays. When purified recombinant p10 was mixed with synthetic siRNA duplexes, miRNA159/miRNA159* duplexes and miRNA159 oligonucleotides, slower migrating complexes were observed both with small RNA duplexes (siRNAs and miRNA159/miRNA159*) and single-stranded miRNA159 (Fig. 6, lanes 3, 6 and 9, respectively). No complexes formed in the control consisting of *Grapevine leafroll-associated virus 2* (GLRaV-2) CP, expressed and

### Table 1. Results of systemic silencing progression in infiltrated *N. benthamiana* 16c plants

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<th>Agroinfiltration*</th>
<th>No. of plants with systemic silencing/total agroinfiltrated plants</th>
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<tr>
<td></td>
<td>5 d.p.i.f.</td>
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<tr>
<td>ORF5mutFS</td>
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<tr>
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<td>0/20</td>
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<tr>
<td>P1/HcPro</td>
<td>0/12</td>
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*Basal leaves were infiltrated with mixed cultures of *Agrobacterium* carrying individual ORFs (ORF5mutFS, ORF5 and P1/HcPro) together with another *Agrobacterium* strain expressing the silencing inducer 35S-GFP cassette. Control plants (GFP) were infiltrated with 35S-GFP plus the empty vector. The progression of silencing was observed on the upper leaves for 25 days.

### Table 2. Results of systemic silencing progression in *N. benthamiana* 16c plants with leaves infiltrated at two sites (tip and base)

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<th>Infiltration type*</th>
<th>No. of plants with systemic silencing/total agroinfiltrated plants</th>
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<td>ORF5 (tip)/GFP (base)</td>
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*Agrobacterium* cells carrying 35S-GFP or 35S-ORF5 were simultaneously but separately infiltrated into the base or tip portion of the same basal leaf (three leaves per plant) and the progression of silencing was monitored on the upper leaves for 18 days.

†Silencing along the veins.

### Fig. 6. p10 binds small RNAs

Electrophoretic mobility shift assays of recombinant p10 (lanes 3, 6 and 9) and GLRaV-2 CP (lanes 2, 5, 8) incubated with synthetic 21 nt RNAs (lanes 1, 2 and 3), 21 nt duplex-siRNAs (lanes 4, 5 and 6) or 21 nt duplex miRNA159/miRNA159* (lanes 7, 8 and 9). Lanes 1, 4 and 7 contain free oligonucleotides. Free probes (F) and protein-complexed probes (C) are shown. Polyacrylamide gels were dried and exposed to autoradiography.
purified as above (Fig. 6, lanes 2, 5 and 8). These results are taken as evidence that p10 non-specifically binds small RNA molecules of the type involved in RNA silencing.

**DISCUSSION**

In this study, the GVA genome was screened for the presence of a gene potentially able to suppress RNA silencing. Co-infiltration assays of GFP-transformed *N. benthamiana* 16c plants, showed that of four proteins tested, only p10 was able to inhibit local RNA silencing, and that this effect was determined by the protein itself rather than its mRNA. This protein was shown to prevent systemic GFP silencing induced by GFP ssRNA using two diverse approaches, i.e. co-infiltration of leaf tissues at a site and concurrent infiltration at the tip and the base of a leaf. ORF5 p10 suppressor activity was further confirmed by agroinfiltration at the tip and the base of a leaf. ORF5 p10 was shown to inhibit local RNA silencing and that this effect was determined by the protein itself rather than its mRNA. This protein was shown to prevent systemic GFP silencing induced by GFP ssRNA using two diverse approaches, i.e. co-infiltration of leaf tissues at a site and concurrent infiltration at the tip and the base of a leaf. ORF5 p10 suppressor activity was further confirmed by agroinfiltration assays in normal *N. benthamiana* plants and, more convincingly, following expression from a PVX vector in VIGS inhibition tests. In these experiments, ORF5 expression from the PVX vector had a dramatic effect on the phenotype of three different *Nicotiana* species, thus acting as a pathogenicity determinant, in agreement with data reported by Galiakparov et al. (2003b). In addition, ectopic expression of ORF5 in a strongly silenced context, represented by PVX-GFP-infected 16c plants, was able to counteract the VIGS reaction. In a paper that appeared after our investigation was completed, Chiba et al. (2006), using a *Beet yellows virus* replicon system, suggested that GVA p10 may be a suppressor of RNA silencing, but pointed out that additional experiments were desirable to confirm this finding. Using an experimental approach different to that of Chiba et al. (2006), we have provided more extensive evidence of the suppressive property of p10, as it suppresses viral and transgene-induced RNA silencing, reduces or abolishes the accumulation of silencing-associated sRNAs in *Agrobacterium* co-infiltration experiments, and is able to bind non-specifically to single-stranded sRNAs and miRNAs and their duplexes in *in vitro* reactions. Based on this, it seems plausible to conclude that p10 is a non-specific RNA binder that suppresses silencing by sequestering sRNAs.

GVA p10 has been shown to belong to a new family of suppressors (Chiba et al., 2006), containing a basic amino acid region followed by a Zn-ribbon domain, whose members have been found in three different viral genera (Mintra et al., 1994; Chiba et al., 2006). Of these proteins, p11 of *Potato virus M* had already been shown to be a non-specific NA-binding protein (Gramstat et al., 1990), similar to GVA p10. Interestingly, p11 is supposed to be translated from the CP subgenomic RNA, possibly through a (−1) frameshift, yielding a CP/p11 fusion protein (Gramstat et al., 1990). GVA ORF5 could also be hypothetically expressed as a CP/p10 translational fusion from the sequence CAGUAU/AAGUA (ORF5 start codon in bold type), since a tRNA1by recognizing the CP UAG readthrough codon (Fig. 1, italic type) has already been identified in several viruses (Miller et al., 1988).

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**REFERENCES**


