RNA Silencing Suppression by a Second Copy of the P1 Serine Protease of *Cucumber Vein Yellowing Ipomovirus*, a Member of the Family *Potyviridae* That Lacks the Cysteine Protease HCPro†

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The P1 protein of viruses of the family *Potyviridae* is a serine proteinase, which is highly variable in length and sequence, and its role in the virus infection cycle is not clear. One of the proposed activities of P1 is to assist HCPro, the product that viruses of the genus *Potyvirus* use to counteract antiviral defense mediated by RNA silencing. Indeed, an HCPro-coding region is present in all the genomes of members of the genera *Potyvirus*, *Rymovirus*, and *Tritimovirus* that have been sequenced. However, it was recently reported that a sequence coding for HCPro is lacking in the genome of *Cucumber vein yellowing virus* (CVYV), a member of the genus *Ipomovirus*, the fourth monopartite genus of the family. In this study, we provide further evidence that P1 enhances the activity of HCPro in members of the genus *Potyvirus* and show that it is duplicated in the ipomovirus CVYV. The two CVYV P1 copies are arranged in tandem, and the second copy (P1b) has RNA silencing suppression activity. CVYV P1b suppressed RNA silencing induced either by sense green fluorescent protein (GFP) mRNA or by a GFP inverted repeat RNA, indicating that CVYV P1b acts downstream of the formation of double-stranded RNA. CVYV P1b also suppressed local silencing in agroinfiltrated patches of transgenic *Nicotiana benthamiana* line 16c and delayed its propagation to the neighboring cells. However, neither the short-distance nor long-distance systemic spread of silencing of the GFP transgene was completely blocked by CVYV P1b. CVYV P1b and P1-HCPro from the potyvirus *Plum pox virus* showed very similar behaviors in all the assays carried out, suggesting that evolution has found a way to counteract RNA silencing by similar mechanisms using very different proteins in viruses of the same family.

The regulatory systems of gene expression mediated by sequence-specific RNA silencing are mechanisms conserved in a wide variety of eukaryotic organisms (50). These pathways are triggered by double-stranded RNA (dsRNA), which is recognized by Dicer-like RNases to produce short dsRNAs of 21 to 26 nucleotides (nt) in length (small interfering RNAs [siRNAs]) (3, 32). One strand of these small RNAs is then incorporated into different silencing effector complexes, guiding them, by sequence complementarity, to degrade mRNA, inhibit RNA translation, or interfere with transcription by chromatin rearrangements (47).

Different RNA silencing pathways have been identified, and one of them, active in the cytoplasm, has been shown to play an antiviral role in eukaryotes, where viruses generate dsRNA tracts in replicative intermediates, highly structured mRNAs, or other RNA molecules as a consequence of the action of cellular RNA-dependent RNA polymerases (10, 56). In plants, fungi, and *Caenorhabditis elegans* at least, this is a non-cell-autonomous process, which spreads to remote cells or tissues (57, 59). The systemic silencing signal has still not been identified, although the sequence specificity of this pathway suggests the involvement of a nucleic acid component (19, 34).

To counteract the RNA silencing-mediated defense response, many viruses express proteins with silencing suppression activity (7). These proteins do not show sequence homology, suggesting both independent and recent evolutionary origins (37, 42, 56, 59). Silencing suppressors also interfere with the RNA silencing pathway mediated by microRNAs (8, 13, 23, 31, 46). Although this interference has a notable impact on the development of disease symptoms, its possible relevance in antiviral defense is still unknown.

The multifunctional helper component protease (HCPro) of plant potyviruses was the first silencing suppressor identified (2, 5, 22). The mechanism of silencing suppression of HCPro has been debated for a long time (13, 30), but recent results strongly support the hypothesis that HCPro suppresses silencing by sequestering siRNAs (26).

*Cucumber vein yellowing virus* (CVYV) is a positive-sense single-stranded RNA virus member of the family *Potyviridae*. The genomic RNA of potyviruses is translated into a single polyprotein that is proteolytically processed by three virus-encoded proteases (40). CVYV was originally identified as a species of the genus *Ipomovirus* on the basis of partial sequence comparisons and biological properties, such as being whitefly transmissible (27). Recently, the full-length genome sequence of a CVYV isolate was determined, confirming its assignment to the genus *Ipomovirus*. However, CVYV differed from another ipomovirus, *Sweet potato mild mottle virus*

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(SPMMV) and from the remaining sequenced monopartite members of the family Potyviridae, in that it lacks a sequence coding for a putative HCPro. CVVV showed an exceptionally large P1 protein, with a C-terminal serine protease domain similar to those of the P1s of members of the genus Potyvirus (20). The putative cleavage site separating CVVV P1 and P3 was also similar to the P1-HCPro junction of potyviruses. The absence of HCPro has opened up debate about how CVVV can secure the multiple functions of this protein in viral replication and transmission.

P1 is the first protein in the potyviral polyprotein. It is a cis-acting serine protease that cleaves at its C terminus (55). The functions of P1 during the virus life cycle have not yet been elucidated, although some evidence suggests that, despite it not having RNA silencing suppressor activity per se, it might enhance HCPro-mediated suppression (22, 36, 39). Here, we show that the originally described long P1 protein of the isomovirus CVVV is really formed by two homologous proteins, P1a and P1b, and that P1b is able to suppress RNA silencing in a manner similar to that of HCPro from potyviruses, suggesting that P1b replacing HCPro at least in this function.

**MATERIALS AND METHODS**

**Plasmids.** GATEWAY technology (Invitrogen) was used to construct the plasmids expressing the proteins from the N-terminal region of the Plant parvovirus (PPV) and CVVV polypolypeptides. Primers used in cloning are listed in Table S1 in the supplemental material.

PPV sequences were amplified from pBC-PPV (28) using PPV 5′ and HCProPPV rev as primers for the 5′UTR-P1-PPVHP1-end, PPV 5′ and P1PPV rev for the 5′UTR-P1PPV-end, and HCProPPV and HCProrevPPV for the HCPro-end.

pDOSR-207 (Invitrogen) and pGW2R (a gift of Tatsuyoshi Nakagawa, University of Shimane) were used as donor and destination vectors, respectively, to generate the plasmids. The BP clonase reactions to introduce the PCR fragments 5′UTR-P1-PPVHP1-end, 5′UTR-P1PPV-end, and HCPro-end into entry vectors and the LR clonase reactions to transfer the DNA fragments from entry vectors to expression vectors (p35S-P1HCvPPV, p35S-P1PvPPV, and p35S-HCVPPV, respectively), were carried out according to the manufacturer’s instructions (Invitrogen).

The 5′ untranslated region (5′UTR), except for the first 6 nt, plus the 5′ coding sequence of CVVV were amplified by two reverse transcription-PCRs (RT-PCRs), using a crude nucleic acid extract from infected cucumber leaves (German Collection of Microorganisms and Cell Cultures, code PV-0724) as the template. Primers 7-CVVV and VVV-1667rev were used in one of the reactions to amplify a fragment including CVVV nt 7 to 1667 (numbering is according to the sequence published by Janssen et al. [20]). This fragment was cloned into pCRRI by the TOPO cloning system (Invitrogen) to create pCRRI-5′P1. The second RT-PCR used primers 1066-CVVV and VVV-2596rev to amplify the CVVV cDNA fragment from positions 1066 to 2596, which was digested with Sall and cloned into pUC19 digested with SmaI and SalI to create pUC-5′P1. pUC-5′P1-P1v, which includes the assembled CVVV 5′UTR-P1-coding sequence, was obtained by cloning into pUC19 digested with EcoRI and KpnI, the EcoRI-SalI and SalI/KpnI fragments from pCRRI-5′P1 and pUC-3′P1, respectively, including the partial CVVV sequences. 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RESULTS

P1 of PPV enhances the silencing suppression activity of HCPro. To gain insight into the role in silencing suppression of the potyviral P1 protein, we expressed the P1 protein from PPV as an independent protein and as part of the P1-HCPro polyprotein in N. benthamiana leaves by agroinfiltration (21). Thus, agrobacteria carrying the PPV constructs shown in Fig. 1A were coinfiltrated with agrobacteria carrying p35S:GFP as a silencing reporter. For simplicity, we will refer to each Agrobacterium strain by the name of the plasmid it carries. GFP fluorescence in leaves agroinfiltrated with p35S:GFP plus the empty vector pBin19 reached the highest intensity at 2 to 3 days postinfiltration (dpi) (not shown), dropping to hardly detectable levels by 6 dpi (Fig. 2A). In contrast, leaves infiltrated with p35S:GFP plus p35S-HCPPV, which express the well-known silencing suppressor HCPro, showed bright green fluorescence at 6 dpi (Fig. 2A) and later times (more than 9 dpi not shown). P1 alone did not increase the persistence of GFP expression of p35S:GFP, and no fluorescence was detected at 6 dpi in leaves infiltrated with p35S:GFP plus p35S-P1ppv (Fig. 2A). However, green fluorescence was notably more intense when GFP was expressed together with the P1-HCPro polyprotein than with HCPro alone (Fig. 2A). This stimulatory effect of P1 was not observed when P1 and HCPro were expressed in separate plasmids (Fig. 2A). Western blot analysis showed that free HCPro was produced in leaves infiltrated with p35S:GFP plus P1-HCPro and that the accumulated levels of this protein at 6 dpi were similar in leaves infiltrated with HCPro, P1-HCPro, and P1 plus HCPro (data not shown).

Northern blot analysis revealed that at 6 dpi the steady-state levels of GFP mRNA were very low in leaves expressing p35S:GFP plus either empty vector or p35S-P1ppv (Fig. 2B). In contrast, high GFP mRNA accumulation was detected in the three infiltration combinations expressing HCPro, with the highest level corresponding to leaves infiltrated with p35S:GFP plus p35S-P1HCPPV, and similar lower levels in those infiltrated with p35S:GFP plus either p35S-HCPPV or p35S-HCPPV plus p35S-P1ppv (Fig. 2B). Taken together, these results indicate that P1 of PPV is not a silencing suppressor by itself but enhances the activity of the silencing suppressor HCPro when it is supplied in cis.

The N-terminal region of the polyprotein of CVYV includes two P1-like serine proteases. In the paper reporting the full-length genome sequence of the ipomovirus CVYV Janssen et al. (20) noticed the presence of a P1-like serine protease domain characterized by a H746-D754-S789 catalytic triad, with the serine residue in a GXSG context, as well as a predicted cleavage site IDFY:C (amino acids [aa] 840 to 844) upstream of the P3 protein, which is in agreement with the consensus sequence for potyviral P1 cleavage sites. Preliminary results of agroinfiltration assays confirmed the protease activity of this CVYV protein region (A. Valli and J. A. García, unpublished results). Further sequence analysis revealed the presence of an additional P1-like serine protease domain (H442-D451-S484 catalytic triad) and a putative internal cleavage site IRNY:T (aa 522 to 526), which would split the P1 region into two proteins, P1a and P1b, showing 24% amino acid identity (sequences aligned by MAFFT v 5.860) (A. Valli, J. J. López-Moya, and J. A. García, submitted for publication). In order to verify the protease activity of the internal domain, we made two constructs to express by agroinfiltration either the complete P1 sequence from CVYV (p35S-P1cvyy-TAP) or its P1b fragment (p35S-P1b cvyy-TAP), fused to a TAP tag (Fig. 3A). Western blot analysis specific for the TAP tag showed the accumulation of a protein of ~60 kDa, the size of P1b-TAP, in N. benthamiana leaves infiltrated with p35S-P1b cvyy-TAP, at 3 and 6 dpi (Fig. 3B). A minor protein with the expected mobility of unprocessed P1-TAP (120 kDa) was detected at 3 dpi in leaves infiltrated with p35S-P1cvyy-TAP (Fig. 3B). However, the major protein at 3 dpi and the only one detected at 6 dpi in these leaves had the same electrophoretic mobility as that of P1b-TAP expressed from p35S-P1b cvyy-TAP.

FIG. 1. Schematic representations of the PPV-derived (A) and CVYV-derived (B) constructs used in the RNA silencing assays. Genome maps of the viruses are shown at the top of each panel. Stop codons introduced during cloning are indicated.

FIG. 2. Enhancement of silencing suppression activity of PPV HCPro by P1. N. benthamiana plants were coinfiltrated with A. tumefaciens carrying p35S:GFP and empty pBin19 (vector), p35S-P1ppv (P1), p35S-HCPPV (HC), p35S-P1HCPPV (P1HC), or p35S-P1ppv plus p35S-HCPPV (P1+HC). (A) GFP fluorescence pictures taken under a UV lamp at 6 dpi. (B) Northern blot analysis of GFP mRNA extracted at 6 dpi from leaf patches infiltrated with agrobacteria carrying the plasmid indicated above each lane. The bottom gel shows rRNA stained with ethidium bromide as a loading control.
TAP, strongly suggesting that P1-TAP was being processed at the predicted internal cleavage site (Fig. 3B).

CVYV P1b suppresses both sense RNA- and dsRNA-triggered RNA silencing. The lack of a sequence coding for the typical potyviral silencing suppressor HCPro in the CVYV genome (20) raised the possibility that the exceptionally large P1 sequence of this virus might contribute to counteract the antiviral defense mediated by RNA silencing. To assess this possibility, we constructed Agrobacterium plasmids expressing CVYV P1a, P1b, or the complete P1 protein (Fig. 1B), which were coagroinfiltrated with p35S:GFP (Fig. 4). The green fluorescence at 6 dpi remained as strong in patches coagroinfiltrated with p35S:GFP plus p35S-P1bCVYV as in those expressing p35S-P1HC PPV (Fig. 4A), suggesting that P1b from CVYV could suppress silencing as efficiently as PPV P1-HCPro. A similar GFP fluorescence decline was observed at 6 dpi in leaves infiltrated with p35S:GFP plus either empty pBin19 or p35S-P1aCVYV, indicating that P1a does not display silencing suppression activity (data not shown).

Accumulation of siRNAs is a universal feature associated with RNA silencing. As shown by Northern blot analysis, GFP mRNA accumulation at 3 dpi was similar in leaves infiltrated with p35S:GFP plus either pBin19, p35S-P1bCVYV, or p35S-P1HC PPV, and both CVYV P1b and PPV P1-HCPro were able to prevent, with similar efficiencies, the drop in GFP mRNA levels detected in leaves infiltrated with p35S:GFP plus the empty control plasmid at 6 dpi (Fig. 4B), confirming the silencing suppression activity of CVYV P1b.

A set of independent confirmatory experiments were performed with constructs derived from a second CVYV isolate, cloned in the pBIN61 vector. In these series, agroinfiltration with Tomato bushy stunt virus p19-expressing constructs was used as an internal control, and single-stranded GFP RNA was used as a trigger of silencing. P1a and the complete P1 protein were not able to suppress GFP silencing, whereas P1b had RNA silencing suppression activity comparable to that of Tomato bushy stunt virus p19 (data not shown).

Accumulation of siRNAs is a universal feature associated with RNA silencing. As expected, high levels of GFP siRNAs of ~21 to 24 nt were detected in leaves infiltrated with p35S:GFP plus pBin19, p35S-P1bCVYV, or p35S-P1HC PPV, and both CVYV P1b and PPV P1-HCPro were able to prevent, with similar efficiencies, the drop in GFP mRNA levels detected in leaves infiltrated with p35S:GFP plus the empty control plasmid at 6 dpi (Fig. 4B), confirming the silencing suppression activity of CVYV P1b.

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As shown by Northern blot analysis, GFP mRNA accumulation at 3 dpi was similar in leaves infiltrated with p35S:GFP plus pBin19, p35S-P1bCVYV, or p35S-P1HC PPV, and both CVYV P1b and PPV P1-HCPro were able to prevent, with similar efficiencies, the drop in GFP mRNA levels detected in leaves infiltrated with p35S:GFP plus the empty control plasmid at 6 dpi (Fig. 4B), confirming the silencing suppression activity of CVYV P1b.
To induce RNA silencing, the sense GFP RNA must first be converted to dsRNA. In order to assess whether CVYV P1b was targeting this first step or interfering with silencing downstream of dsRNA production, we carried out a dsRNA-triggered silencing assay. We agroinfiltrated leaves with p35S:GFP (GFP sense RNA), p35S:GF-IR (inverted repeat [IR] generating GFP dsRNA), and pBin19, p35S-P1bCVYV, or p35S-P1bPPV. IRs are strong inducers of RNA silencing, and thus, all infiltrated leaves showed weak green fluorescence under UV light at 3 dpi (data not shown), which dropped to undetectable levels at 6 dpi in patches infiltrated with p35S:GFP plus p35S:GF-IR plus pBin19, but fluorescence was maintained and even increased in patches expressing PPV P1-HCPro or CVYV P1b (Fig. 5A).

The results of Northern blot analysis corroborated the fluorescence observations. PPV P1-HCPro and, more efficiently, CVYV P1b enhanced GFP mRNA accumulation, both at 3 dpi and 6 dpi (Fig. 5B), supporting the hypothesis that CVYV P1b, as the potyviral HCPro, was able to interfere with dsRNA-triggered RNA silencing. GFP-specific siRNA accumulation was detected at 3 dpi, indicating that RNA silencing was already induced at this time, but the much higher siRNA levels at 6 dpi indicated a progressive strengthening of the silencing response (Fig. 5B). Suppression of dsRNA-triggered silencing by PPV P1-HCPro or CVYV P1b, like that of the sense RNA-triggered one commented above, did not abolish siRNA accumulation, which appeared to be even greater in leaves expressing the PPV P1-HCPro silencing suppressor (Fig. 5B).

CVYV P1b suppresses local transgene silencing but does not prevent cell-to-cell or long-distance spread of RNA silencing in GFP-transformed N. benthamiana line 16c. In order to verify whether CVYV P1b could suppress not only the RNA silencing induced by transient expression of sense RNA or dsRNA but also that involving transgene RNA and to assess the ability of this protein to prevent silencing spread, we agroinfiltrated N. benthamiana line 16c, which actively expresses its GFP transgene, with p35S:GFP and plasmids expressing CVYV P1b or PPV P1-HCPro (Fig. 6). Enhanced green fluorescence was observed in the infiltrated patches at 2 to 3 dpi regardless of the expression of silencing suppressors (data not shown) but later declined until it was hardly detectable at 7 dpi in leaves coinfected with p35S:GFP plus the empty vector (data not shown). In contrast, the green fluorescence remained strong in patches coinfected with p35S:GFP plus either p35S-P1bCVYV or p35S-P1bPPV for more than 13 dpi (Fig. 6A).

Northern blot analysis showed similar GFP mRNA levels at 3 dpi in the patches infiltrated with any of the bacterial combinations. At 7 dpi, the steady-state level of GFP mRNA was much higher in patches infiltrated with p35S:GFP plus either p35S-P1bCVYV or p35S-P1bPPV than in those infiltrated with p35S:GFP plus the control plasmid (Fig. 6B). In contrast with the results obtained for wild-type N. benthamiana, suppression of RNA silencing by PPV P1-HCPro and CVYV P1b in N. benthamiana line 16c caused a drastic reduction in GFP-specific siRNA levels; the reduction was especially marked for CVYV P1b (Fig. 6B).

It has been reported that RNA silencing can spread from cell to cell from agroinfiltrated patches. In GFP-transgenic lines, this spread provokes shutting down of GFP expression in the neighboring cells, which is manifest by a narrow red ring around the infiltrated spot (58). These red rings were observed around all patches infiltrated with p35S:GFP plus pBin19 by 6 or 7 dpi (not shown). Expression of PPV P1-HCPro or CVYV P1b appeared to cause a delay in short-distance spread of silencing, and patches with bright green fluorescence surrounded by red borders were observed in only some leaves infiltrated with p35S:GFP plus p35S-P1bPPV at 7 dpi (not shown). However, the patches of all leaves expressing PPV P1-HCPro showed red rings at 13 dpi (Fig. 6B). At this time, only 1 out of 50 patches of leaves infiltrated with p35S:GFP plus p35S-P1bCVYV showed the red line (Fig. 6B), but this proportion increased to ~50% at later times (24 out of 48 patches at 23 dpi). Thus, neither PPV P1-HCPro nor CVYV P1b was able to completely block short-distance spread of RNA silencing.

Monitoring of upper noninfiltrated leaves of the agroinfiltrated plants at 13 dpi showed that they were starting to lose GFP fluorescence around major veins, regardless of the fact that the infiltrated patches were silenced or were expressing high levels of GFP owing to the local silencing suppression activity of PPV P1-HCPro or CVYV P1b (Fig. 6C).
onstrated that PPV P1-HCPro and CVYV P1b also fail to block the long-distance spread of the systemic silencing signal.

**DISCUSSION**

Although alternative strategies for escaping RNA silencing have been proposed (28, 44, 51), most plant RNA viruses appear to depend on virus-encoded suppressor proteins to counteract this antiviral defense mechanism (37, 42, 56, 59). In some cases, the virus has more than one silencing suppressor (29) or produces auxiliary proteins that enhance the activity of the viral silencing suppressor (24). Until now, only one silencing suppressor, HCPro, had been identified in members of the family *Potyviridae* (2, 5, 22), although another protein, P1, had been suggested to enhance the silencing suppression activity of HCPro (22, 36, 39). In this study, we show that P1 proteins from different members of the family *Potyviridae* may play different roles in silencing suppression, either enhancing the activity of the silencing suppressor HCPro or suppressing silencing by itself.

**PPV P1 as a HCPro assistant protein.** Although the contribution of P1 to the synergistic interaction between *Potato virus X* and *Tobacco etch virus* (TEV) was well established some years ago (36) and the suggestion that P1 and HCPro could act cooperatively in suppressing RNA silencing had already been raised when HCPro was identified as a silencing suppressor (22), few studies have investigated how P1 enhances the silencing suppression activity of HCPro (39). We have demonstrated that PPV P1-HCPro suppresses silencing more efficiently than PPV HCPro alone (Fig. 2), although the activity of PPV HCPro appears to rely on the concomitant expression of P1 less than the HCPro protein from *Potato virus A* (39), suggesting that the extent of the cooperation between P1 and HCPro might be virus specific.

Since both P1 and HCPro are proteases that cleave at their C-terminal ends (6, 55), it is possible to envisage that coexpression of P1 plus HCPro could have a similar effect in silencing suppression as the single expression of the P1-HCPro polypeptide. However, this possibility has not been experimentally approached until now. Our results show that, although HCPro accumulation levels were similar in leaves expressing P1-HCPro, P1 plus HCPro, and HCPro alone, silencing suppression was more efficient in leaves expressing P1-HCPro than in the other leaves (Fig. 2). This indicates that P1 enhances HCPro activity only when both proteins are expressed in cis, mimicking the expression of the N-terminal region of the viral polypeptide. The simplest explanation for this fact is that P1 enhances silencing suppression only indirectly by producing the “natural” HCPro N terminus (36). However, this is not in agreement with the fact that TEV deletion mutants lacking a large fragment from the HCPro N-terminal region efficiently infected *N. tabacum* (11). A more exciting possibility is that a
physical interaction between P1 and HCPo (33) might stabilize a hypothetical silencing suppression complex (39). In this scenario, the interaction would take place only when P1 and HCPo are derived from the same polyprotein. We cannot rule out other possibilities either, such as the fact that unprocessed P1-HCPo polyprotein could play a specific role in silencing suppression before being split into P1 and HCPo proteins or that the expression of P1 from the same molecule targets HCPo to a different cellular compartment than constructs where HCPo is expressed in the first place without a leading product.

Two serine proteases in the CVYV P1 region. P1, together with the N-terminal region of the capsid protein, is the most variable potyviral protein, both in length and amino acid sequence (1). The sizes of P1 proteins of viruses of the genera Potyvirus, Rymovirus, and Tritimovirus range between 211 and 664 aa, with an average of approximately 340 aa. However, the size of the putative P1 protein of the ipomovirus CVYV, 884 aa, largely exceeded the upper limit (20). Sequence alignment analyses have revealed the existence of two serine protease motifs in the P1 region of CVYV, probably derived from a sequence duplication (Valli et al., submitted). Our results showing that transient expression of the complete CVYV P1 region gives rise to a polypeptide with the size expected for processing at the predicted internal cleavage site strongly suggest that CVYV produces two P1 proteases, P1a and P1b, although a definitive conclusion should await mutagenesis data on the residues of the predicted active sites and characterization of the P1-related products present in CVYV-infected plants.

Silencing suppression activity of CVYV P1b. All monopartite members of the family Potyviridae characterized until now, except for CVYV, concur in having an HCPo-like cysteine protease at the second position of the viral polyprotein (20). This raises many questions about how CVYV can supply the activities of this multifunctional protein. The presence of an extra P1 protein at the N-terminal region of the CVYV polyprotein, together with the cooperation of P1 and HCPo of members of the genus Potyvirus in silencing suppression, suggest that one of the P1 proteins, or both, might replace HCPo in CVYV infection. Our data demonstrate that the second P1 (P1b), the protein that occupies the position of HCPo in the CVYV polyprotein, has RNA silencing suppression activity (Fig. 4 to 6). Viral silencing suppressors largely differ in amino acid sequence, and the rather scarce data available suggest that they also have quite different mechanisms of action (42, 45, 56, 59). CVYV P1b and PPV HCPo show a complete sequence disparity; however, these silencing suppressors behaved in a very similar manner in the three experimental systems that we used, although CVYV P1b appeared to be more efficient than PPV P1-HCPo. The mechanism of silencing suppression of the potyviral P1-HCPo is not well established, but it clearly acts downstream of the formation of dsRNA, since it is able to suppress both sense RNA- and dsRNA-induced silencing. The same is true for CVYV P1b (Fig. 4 and 5). However, there are conflicting results about the effect of P1-HCPo in siRNA accumulation and in systemic propagation of silencing, which, at least in part, can be due to the use of different experimental approaches (discussed in reference 42). Our results also show that PPV P1-HCPo and CVYV P1b have different effects on siRNA accumulation in wild-type N. benthamiana (Fig. 4 and 5) and transgenic N. benthamiana 16c (Fig. 6) plants. However, although PPV P1-HCPo and CVYV P1b caused a clear reduction in siRNA accumulation in the N. benthamiana 16c plants while siRNA accumulation levels were very similar in the absence and presence of these silencing suppressors in wild-type plants, we always observed a drastic reduction in the siRNA/mRNA ratio when the silencing suppressors were expressed. Since target mRNA is the substrate for the production of secondary siRNAs, our results support the hypothesis that the potyviral HCPo and the ipomoviral P1b interfere in some way with the metabolism of siRNAs, although this interference is not complete. A recent report has demonstrated that TEV HCPo, as well as tombusviral p19 and closteroviral p2l, acts by binding double-stranded siRNAs and preventing loading onto the RNA-induced silencing complex (26). Our results are fully compatible with that conclusion for both PPV HCPo and CVYV P1b, although direct binding experiments will be needed to confirm this point.

Neither PPV P1-HCPo nor CVYV P1b was able to block the systemic silencing of the GFP transgene of N. benthamiana 16c plants (Fig. 6). This is in agreement with the suggestion that the systemic silencing signals are siRNAs (19), which are not abolished by these silencing suppressors (Fig. 4 to 6). Both silencing suppressors seemed to enhance to some extent the appearance of silenced areas around veins in upper noninfected leaves (data not shown). This could be due to the fact that the very active GFP expression in the infiltrated tissue, which is facilitated by the silencing suppressors, increases the production of the systemic silencing signal, which is not efficiently blocked by them. In contrast, PPV P1-HCPo and especially CVYV P1b, although unable to completely block the short-distance silencing spread, clearly interfered with it (Fig. 6). The most probable interpretation of our results, together with those previously reported for the HCPo proteins from other potyviruses, is that in plants expressing PPV P1-HCPo or CVYV P1b, there are two opposite effects, (i) Local silencing suppression enhances mRNA accumulation and, as a consequence, facilitates the production of systemic silencing signal. (ii) The silencing suppressors interfere with the synthesis or movement of the systemic silencing signal. In this scenario, the actual balance of the two effects and the specific requirement of the silencing signal determine the efficiency of the short- and long-distance silencing spread. However, we cannot rule out the possibility that the short- and long-distance systemic silencing signals are different and have different susceptibilities to the silencing suppressors.

P1s, HCPo, and silencing suppression in the family Potyviridae. Sequence alignment analyses showed that although CVYV P1a and P1b and the potyviral P1s appear to be homologous, the potyviral P1s are more closely related to CVYV P1a than to CVYV P1b (Valli et al., submitted). Thus, the N-terminal ends of the polyproteins of CVYV (P1a-P1b) and of members of the genus Potyvirus (P1-HCPo) could be equivalent. However, in contrast with the enhancement of HCPo silencing suppression activity by P1 (Fig. 1) (39), the silencing suppression activity of P1a-P1b is much lower than that of P1b. Although there is still no clear explanation for this hypothetical disagreement, it is probably due to differences in the stability of the transcription and translation products of the constructs
used in the transient-expression experiments. In this regard, it is interesting to note that whereas HCPo accumulation was similar in leaves expressing P1-HC-Pro or HC-Pro alone (data not shown), the accumulation of P1b-TAP was much lower in leaves expressing P1a-P1b-TAP than in those expressing P1b-TAP alone (Fig. 2).

Sequence analysis of the P1 region of the other ipomovirus from which its genomic sequence is available, SPMMV, revealed evidence of a gene duplication similar to that found in CVYV. However, the serine protease domain of P1a is missing from SPMMV (9; Valli et al., submitted). Moreover, whereas CVYV P1a and the N-terminal portion of P1 of SPMMV resemble the P1 proteins of members of the genus Potyvirus, P1b of CVYV and the C-terminal part of SPMMV P1 are more closely related to the P1 proteins of tritiviruses (Valli et al., submitted). Although SPMMV and tritiviruses conserve the HCPro region, a mutant of the tritivirus Wheat streak mosaic virus lacking the complete HCPro was viable for systemic infection (49). It would, therefore, be very interesting to know whether the P1-like proteins of SPMMV and tritiviruses have silencing suppression activity. It is interesting to remark that similar duplications of leader proteinases have been proposed as a mechanism of evolving viral genomes in nidoviruses (15) and closteroviruses (12, 35).

HCPro is a multifunctional protein, and probably not all its functions are related to RNA silencing. Are RNA silencing suppression and enhancement of RNA silencing the only functions of the P1 proteins of the family Potyviridae? We do not yet know for certain the answer to this question, but it is probably no. A potyviral P1 protein has been shown to function in trans as an accessory factor for genome amplification (54), and we have shown that it is unable to enhance the silencing suppression activity of HCPro in trans (Fig. 2), suggesting that P1 could have at least two independent functions. Apparently, evolution has provided plant viruses with RNA silencing suppression activity very recently, adapting very different viral proteins to the novel job (56). Even within the same virus family, alternative silencing suppressors could be chosen, which is the case for p19 and the capsid protein in the family Tombusviridae (38) or AC2 and AC4 in the family Geminiviridae (53). HCPro and the P1-related proteins do not share any apparent sequence similarity, but HCPro is a protease (6, 55) and a RNA binding protein (52), and according to previously published results (48, 55) and data reported here, the P1-related proteins also appear to have these activities. It will be interesting to assess whether having these activities in common is relevant for the recruitment of HCPro and P1-related proteins as principal or accessory factors in RNA silencing suppression and to ascertain the degree of overlap of their functions. Moreover, it will also be fascinating to try to elucidate the evolutionary history of the acquisition of silencing suppression functions by members of the family Potyviridae.

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