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The *Cucumber vein yellowing virus* Silencing Suppressor P1b Can Functionally Replace HCPro in *Plum pox virus* Infection in a Host-Specific Manner

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Plant viruses of the genera *Potyvirus* and *Ipomovirus* (*Potyviridae* family) use unrelated RNA silencing suppressors (RSS) to counteract antiviral RNA silencing responses. HCPro is the RSS of *Potyvirus* spp., and its activity is enhanced by the upstream P1 protein. Distinctively, the ipomovirus *Cucumber vein yellowing virus* (CVYV) lacks HCPro but contains two P1 copies in tandem (P1aP1b), the second of which functions as RSS. Using chimeras based on the potyvirus *Plum pox virus* (PPV), we found that P1b can functionally replace HCPro in potyviral infections of *Nicotiana* plants. Interestingly, P1a, the CVYV protein homologous to potyviral P1, disrupted the silencing suppression activity of P1b and reduced the infection efficiency of PPV in *Nicotiana benthamiana*. Testing the influence of RSS in host specificity, we found that a P1b-expressing chimera poorly infected PPV's natural host, *Prunus persica*. Conversely, P1b conferred on PPV chimeras the ability to replicate locally in cucumber, CVYV's natural host. The deleterious effect of P1a on PPV infection is host dependent, because the P1aP1b-expressing PPV chimera accumulated in cucumber to higher levels than PPV expressing P1b alone. These results demonstrate that a potyvirus can use different RSS, and that particular RSS and upstream P1-like proteins contribute to defining the virus host range.

The establishment of a viral infection in a particular host plant relies on the availability of specific factors necessary for replication and spread of the virus, and on the ability of the virus to escape or counteract a series of defense layers raised by the plant. Among these antiviral barriers, innate immunity responses, which are also elicited by other plant pathogens, are triggered by broadly conserved pathogen-associated molecular patterns (PAMPs) (Soosaar et al. 2005). Moreover, double-stranded forms of viral RNA are recognized as a special PAMP by the infected plant, which activates RNA silencing pathways resulting in specific antiviral immunity (Ding 2010). In order to circumvent this defensive response, most plant viruses have evolved RNA silencing suppressors (RSS) (Burgyán 2008; Moissiard and Voinnet 2004; Roth et al. 2004; Valli et al. 2009).

The genus *Potyvirus* of the family *Potyviridae* is the largest group of plant viruses (López-Moya et al. 2009). The single-stranded RNA genome of *Potyvirus* spp. is translated into a large polyprotein and a truncated frameshift product, which are processed by three virus-encoded proteinases. One of these proteinases, the cysteine proteinase HCPro, was the first viral product to be recognized as an RSS (Anandalakshmi et al. 1998; Brigneti et al. 1998; Kasschau and Carrington 1998), and its RNA silencing suppression activity appears to be essential for potyviral viability (Garcia-Ruiz et al. 2010). Indeed, HCPro is a multifunctional protein that acts in several steps of the potyviral infection cycle, including viral genome amplification (Kasschau et al. 1997), cell-to-cell and long-distance spread (Cronin et al. 1995; Rojas et al. 1997), and aphid transmission (Berger et al. 1989), and is also involved in symptom expression (Gal-On and Raccah 2000; Pruss et al. 1997; Sáenz et al. 2000). The overall genomic structure of *Potyvirus* spp., including HCPro-coding sequences, is also conserved in other monopartite genera of the family *Potyviridae*. However, whereas an HCPro gene is included in the genome of *Sweet potato mild mottle virus* (SPMMV), the type member of the genus *Ipomovirus*, this gene is absent from the genome of other *Ipomovirus* spp. such as *Cucumber vein yellowing virus* (CVYV), *Squash vein yellowing virus* (SqVYV), and *Cassava brown streak virus* (CBSV) (Janssen et al. 2005; Li et al. 2008; Mbanzibwa et al. 2009). In CVYV and SqVYV, the RSS is the serine proteinase P1b that could have derived from a gene duplication of the 5′-terminal gene that codes for the protein P1 (Mbanzibwa et al. 2009; Valli et al. 2006). These P1b proteins, together with the single P1s of some *Ipomovirus, Brambyvirus*, and *Poacevirus* (or *Susmovirus*) spp., form a group of P1 homologs distinct from the typical P1 proteins of *Potyvirus* spp. and the first P1 copies, named P1a, of CVYV and SqVYV (Fellers et al. 2009; Susaimuthu et al. 2008; Tatineni et al. 2009; Valli et al. 2007). Interestingly, although the *Tritimovirus* spp. and SPMMV have HCPro, their silencing suppression activity is provided by their P1b-like P1 proteins rather than by HCPro (Giner et al. 2010; Stenger et al. 2007). Moreover, whereas the silencing suppression mechanism of potyviral HCPro and CVYV P1b appears to involve small interfering (si)RNA sequestration (Lakatos et al. 2006; Valli et al. 2011), SPMMV P1 inhibits RNA silencing by Argonaute binding (Giner et al. 2010). Thus, viruses of the family *Potyviridae* can use RSS with unrelated sequences and different mechanisms of action to counteract antiviral silencing and facilitate viral infection.

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In the present study, we have shown that, although RNA silencing suppression is an essential activity for *Potyvirus* spp., a particular potyviral infection does not depend on a unique RSS. In addition, our data suggest that specific RSS play a significant role in potyviral host range determination.

RESULTS

P1b from CVYV can functionally replace HCPro in a PPV infection.

HCPro and P1b are sequence-unrelated RSS used by *Potyvirus* spp. and some *Ipomovirus* spp., respectively, of the family *Potyviridae*. To test the ability of CVYV P1b to support a potyviral infection, the HCPro coding sequence of the potyvirus *Plum pox virus* (PPV) was replaced by that of CVYV P1b in the infectious cDNA clone pICPPV-NK-GFPn, which also expresses the green fluorescent protein (GFP) to monitor the viral infection. For simplicity, these clones are named here according the N-terminal regions of their polyproteins (P1HC and P1P1b) (Fig. 1).

Nicotiana benthamiana and *N. clevelandii* plants were biolistically inoculated with the different cDNA clones, and the infection was tracked by monitoring the inoculated plants under visible and UV light. The chimeric PPV expressing P1b showed in both plant species a high infectivity rate (usually 100%), similar to that of wild-type PPV expressing HCPro. However, there was a small delay of 1 to 2 days in the appearance of symptoms and GFP fluorescence in upper noninoculated leaves of plants infected with the P1b-expressing P1P1b virus with respect to plants infected with wild-type P1HC. Both P1P1b and P1HC caused systemic chlorotic mottling in *N. clevelandii* and *N. benthamiana* but these symptoms were more intense in plants infected with the P1b-expressing virus. In addition, *N. benthamiana* plants infected with P1P1b showed striking leaf distortion and edge curling but were notably less stunted than those infected with P1HC (Fig. 2). Although visi-

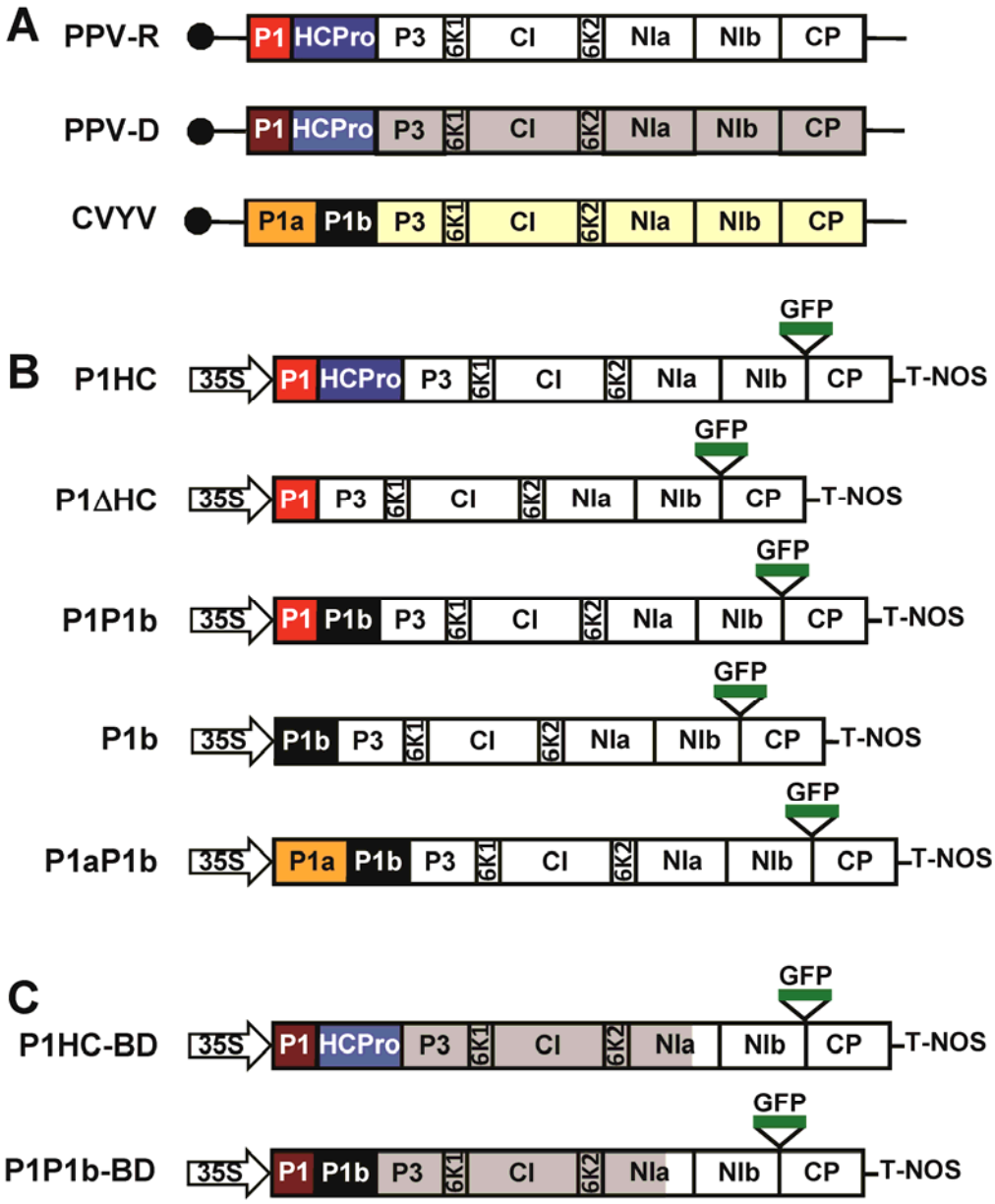

Fig. 1. Schematic representation of full-length cDNA clones derived from *Plum pox virus* (PPV) and *Cucumber vein yellowing virus* (CVYV). **A,** Genome maps of the PPV R and D isolates and CVYV. **B,** Chimeric full-length cDNA clones derived from pICPPV-NK-green fluorescent protein (GFP) employed to infect herbaceous hosts. **C,** Chimeric cDNA clones derived from pICPPV5′BD-GFP employed to infect *Prunus persica*. The coding sequence of the GFP protein inserted between the NIb and coat protein (CP) cistrons is represented with a green rectangle.

ble symptoms were more prominent in leaves of P1P1b-infected plants, the virus-derived GFP fluorescence was less intense in these plants than in those infected with P1HC. Western blot analysis of leaf extracts showed high accumulation levels of viral coat protein (CP) in P1P1b-infected *N. clevelandii* and *N. benthamiana* plants but somewhat lower levels than those detected in extracts from plants inoculated with P1HC (Fig. 2). Immunoreactions with anti-P1b- and anti-HCPro-specific antibodies confirmed that each virus expressed the expected RSS (Fig. 2). These results indicate that CVYV P1b can functionally replace HCPro in a PPV infection.

Relevance of P1 and P1a proteins in P1b-expressing PPV chimeras.

Some reports suggest that the potyviral P1 protein enhances the activity of the RSS HCPro (Anandalakshmi et al. 1998; Kasschau and Carrington 1998; Pruss et al. 1997; Rajamaki et al. 2005; Valli et al. 2006). We were interested in knowing whether P1a, the homologous protein of P1 in CVYV, has a similar effect on P1b activity in the context of a viral infection, and whether the natural CVYV P1a-P1b combination could be more effective than the chimeric PPV P1-CVYV P1b one. With this aim, we engineered the GFP-tagged recombinant viruses P1aP1b and P1b, in which the P1-HC sequence of PPV was replaced by either P1a-P1b or P1b from CVYV (Fig. 1). Leaves of *N. benthamiana* plants were biolistically inoculated with the different constructs, and the infection was monitored under visible and UV lights. P1b and P1aP1b chimeras showed high infectivity levels similar to those of P1HC and P1P1b. However, whereas plants infected with either P1P1b or P1b viruses showed similar disease symptoms, which appeared with a small delay (1 to 2 days) with respect to P1HC-infected plants, plants inoculated with P1aP1b showed no visible symptoms (data not shown). At 3 weeks postinoculation, the spread of green fluorescence in upper noninoculated leaves close to the inoculated ones was more limited in the P1b-infected plants than in plants infected with P1P1b (Fig. 3A). However, PPV CP accumulation in regions showing green fluorescence was similar in plants infected with P1b or P1P1b, and only slightly lower than in P1HC-infected plants (Fig. 3A). In contrast, at this time, green fluorescence was very faint in the upper noninoculated leaves of plants infected with P1aP1b, which showed

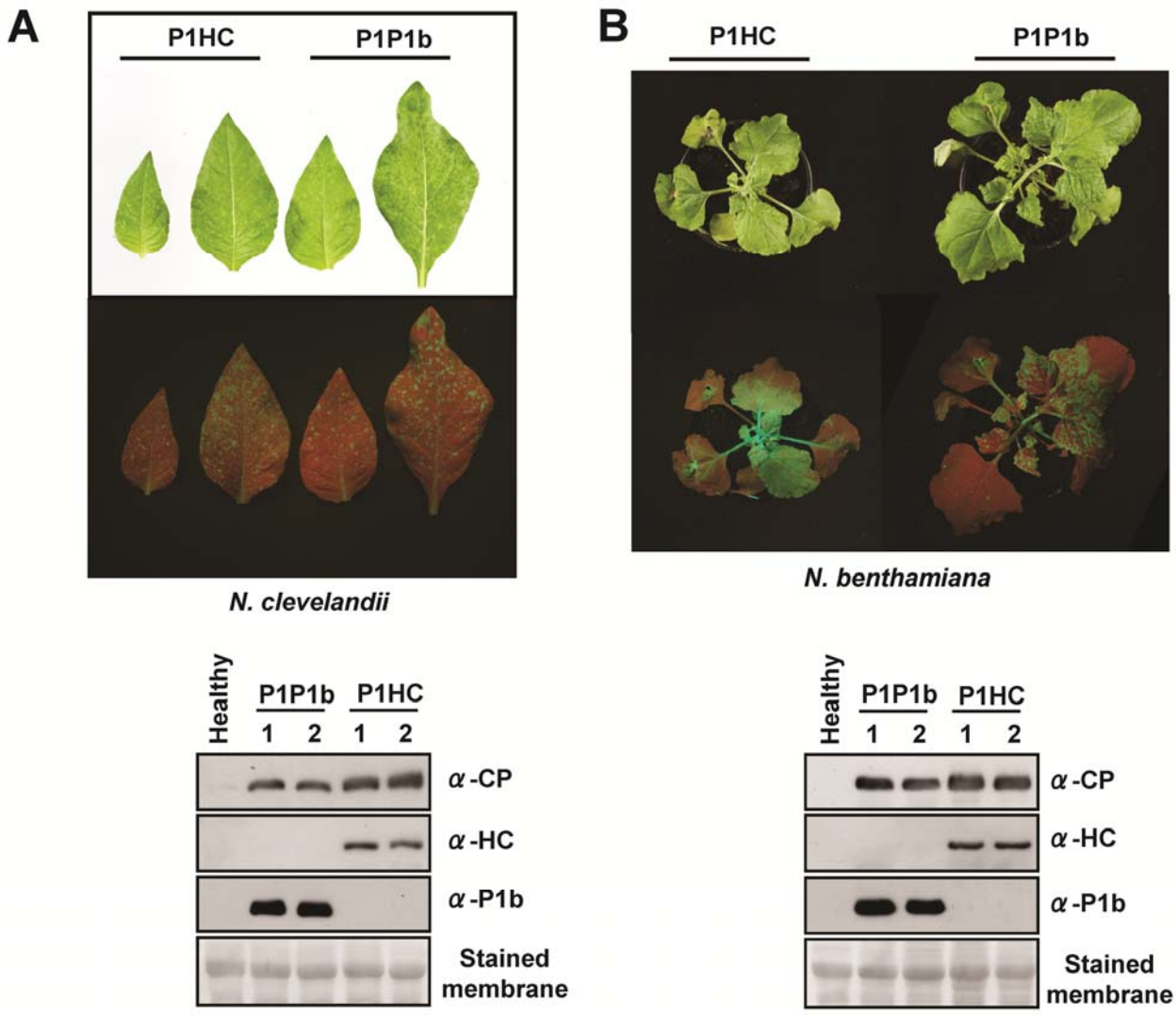

Fig. 2. P1P1b, a chimerical *Plum pox virus* (PPV) in which HCPro was replaced by *Cucumber vein yellowing virus* (CVYV) P1b, is able to infect *Nicotiana clevelandii* and *N. benthamiana* plants. Top panels: symptoms and green fluorescent protein (GFP) expression of plants infected with the P1P1b chimera or with the wild-type PPV (P1HC). Pictures of **A,** detached *N. clevelandii* upper noninoculated leaves and **B,** whole *N. benthamiana* plants taken under visible (upper row) or UV illumination with a hand lamp (lower row) at 21 days postinoculation (dpi). Bottom panels: Western blot analysis of systemically infected leaves from two plants collected at 21 dpi. Polyclonal sera specific for coat protein (CP) and HCPro of PPV, and CVYV P1b, were used for the immunodetections. The membrane stained with Ponceau red showing the Rubisco is included as a loading control.

lower accumulation levels of viral CP compared with plants infected with the rest of viruses (Fig. 3A).

At 21 days postinoculation (dpi), the apical young leaves of plants infected with either P1b or P1aP1b viruses did not show green fluorescence, and the green fluorescence of the corresponding leaves of P1P1b-infected plants was much fainter than that observed in P1HC-infected plants (Fig. 3A). This fact suggested that a recovery from infection could be starting in plants infected with viruses lacking HCPro, mainly when P1 was also absent. To assess this possibility, the infected plants were analyzed at later stages (36 dpi). Whereas young leaves of plants infected with P1HC or P1P1b showed viral symptoms, the apical leaves of plants infected with P1b and P1aP1b appeared healthy (Fig. 3B). In agreement with this observation, green fluorescence was apparent in young leaves of plants infected with P1HC and, with less intensity, in those from P1P1b-infected plants, but it could not be detected in young leaves of plants infected with P1b or P1aP1b (Fig. 3B). A Western blot analysis showed that, in older (+5 and +6) and intermediate (+7 and +8) leaves (where "+" indicates the position above the inoculated

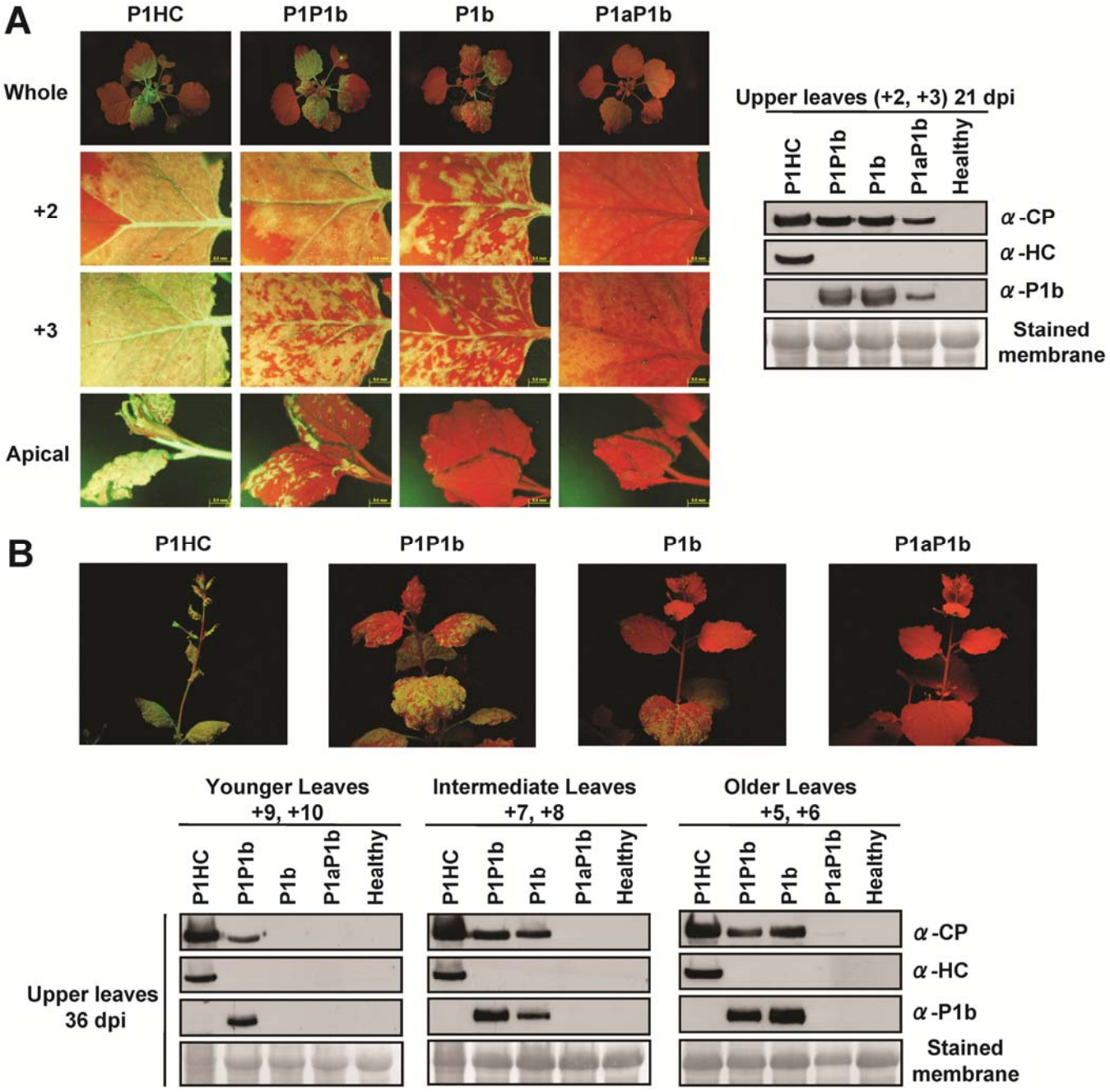

Fig. 3. Infection of *Plum pox virus* (PPV) chimeric viruses expressing *Cucumber vein yellowing virus* (CVYV) P1b in *Nicotiana benthamiana*. **A,** Patterns of infection at 21 days postinoculation (dpi). Green fluorescent protein (GFP) fluorescence pictures taken under a UV hand lamp of whole infected plants, or under a epifluorescence microscope of the second (+2) and third (+3) leaves above the inoculated one, and the most apical leaves are shown in the left panels. Western blot analysis of pools of tissue showing GFP expression of systemically infected leaves (+2 and +3) from two plants is shown in the left panels. **B,** Patterns of infection at 36 dpi. Pictures showing GFP fluorescence under a UV hand lamp of whole infected plants are shown in the top panels. Western blot analysis of pools of tissue showing GFP expression (or the equivalent tissue from plants not showing evident green fluorescence) from young (+9 and +10), intermediate (+7 and +8), or old (+5 and +6) upper noninoculated leaves of two plants is shown in the bottom panels. A polyclonal serum specific for PPV coat protein (CP) was used for assessment of virus accumulation. Immunoreactions with polyclonal sera specific for PPV HCPro and CVYV P1b confirmed the identity of the infecting viruses. Membranes stained with Ponceau red showing the Rubisco are included as loading controls.

leaves), the CP accumulation levels of plants infected with P1b or P1P1b were similar and only slightly lower than those of P1HC-infected plants, similar to the +2 and +3 leaves analyzed at 21 dpi (Fig. 3A). In contrast, no viral CP was detected in young leaves (+9 and +10) of plants infected with P1b, and CP accumulation was notably lower in the corresponding leaves of plants infected with P1P1b than in those of P1HC-infected plants (Fig. 3B). At this time, viral CP was detected only in the older leaves of P1aP1b-infected plants and at extremely low levels (Fig. 3B), reinforcing the idea that the combination of P1a and P1b supports PPV infection very poorly, and plants recover easily from P1aP1b infection. We also substituted PPV P1 by CVYV P1a in the wild-type, HCPro-containing, PPV clone. The resulting chimera, P1aHC, infected *N. benthamiana* as badly as P1aP1b (Supplementary Fig. S1), demonstrating that P1a is also nonfunctional when the virus is using HCPro as RSS.

Together, these results indicate that, despite P1 being dispensable to establish the infection of a chimeric PPV carrying CVYV P1b instead of HCPro, P1 might be necessary to prevent recovery from infection, and CVYV P1a cannot replace PPV P1 in this task.

The silencing suppression activity of P1b is essential for its ability to replace HCPro in the PPV infection.

In order to assess the relevance of P1b-mediated silencing suppression in the context of a potyviral infection, we constructed two variants of the P1P1b chimera containing either the RK68,69AA or the C89A mutation, which affect the conserved LxKA motif or a putative Zn finger, respectively, and abolish the RNA silencing suppression activity of CVYV P1b (Valli et al. 2008). The C93A mutation, which does not affect the P1b silencing suppression activity, was engineered in a control PPV P1P1b clone. In addition, a PPV cDNA construct lacking HCPro (P1ΔHC) was also generated (Fig. 1). *N. benthamiana* plants were biolistically inoculated with DNA of these PPV clones, and the infection process was followed by monitoring GFP fluorescence and symptom expression. Whereas systemic disease symptoms and green fluorescence were observed in plants inoculated with viruses carrying an active RSS (P1HC, P1P1b, and P1P1b C93A), no visual signs of local or systemic infection were detected in those plants inoculated with clones that do not encode a known RSS (P1ΔHC), or code for inactive P1b mutants (P1P1b RK68,69AA or P1P1b C89A) (Fig. 4A).

Western blot analyses of protein extracts from inoculated and upper noninoculated leaves confirmed the visual observations, and showed no viral CP accumulation in plants inoculated with P1P1b RK68,69AA, P1P1b C89A, or P1ΔHC, whereas large amounts of viral CP were detected in plants inoculated with clones expressing active RSS (P1HC, P1P1b, and P1P1b C93A) (Fig. 4B). These results indicate that the RNA silencing suppression activity provided by either HCPro or P1b is essential to support PPV infection.

CVYV P1a but not PPV P1 interferes with the RNA silencing suppression activity of CVYV P1b in agroinfiltration assays.

To assess the contribution of differences in antisilencing activities to the specific biological features of the different PPV

A

Construct	Suppressor protein	Suppressor Activity	Symptoms and GFP (%)
P1HC	HC-Pro	+	100
P1ΔHC	-	-	0
P1P1b	P1b	+	100
P1P1b RK68,69AA	P1b RK68,69AA	-	0
P1P1b C89A	P1b C89A	-	0
P1P1b C93A	P1b C93A	+	100

B

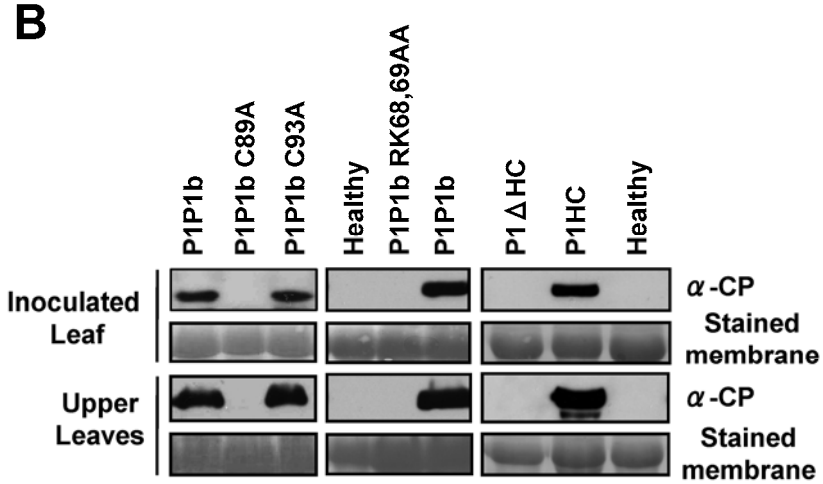

Fig. 4. *Plum pox virus* (PPV) requires an active RNA silencing suppressor (RSS) to infect *Nicotiana benthamiana*. **A,** Result of visual inspection of *N. benthamiana* plants inoculated with PPV cDNA clones differing in their encoded RSS. RNA silencing suppression activity was estimated from published information (Valli et al. 2006, 2008). **B,** Western blot analysis of leaf extracts from pools of either inoculated (7 days postinoculation [dpi]) or upper (21 dpi) leaves of four *N. benthamiana* plants inoculated with the indicated cDNA clones. A polyclonal serum specific for PPV coat protein (CP) was used for the immunodetection. Membranes stained with Ponceau red showing the Rubisco are included as loading controls.

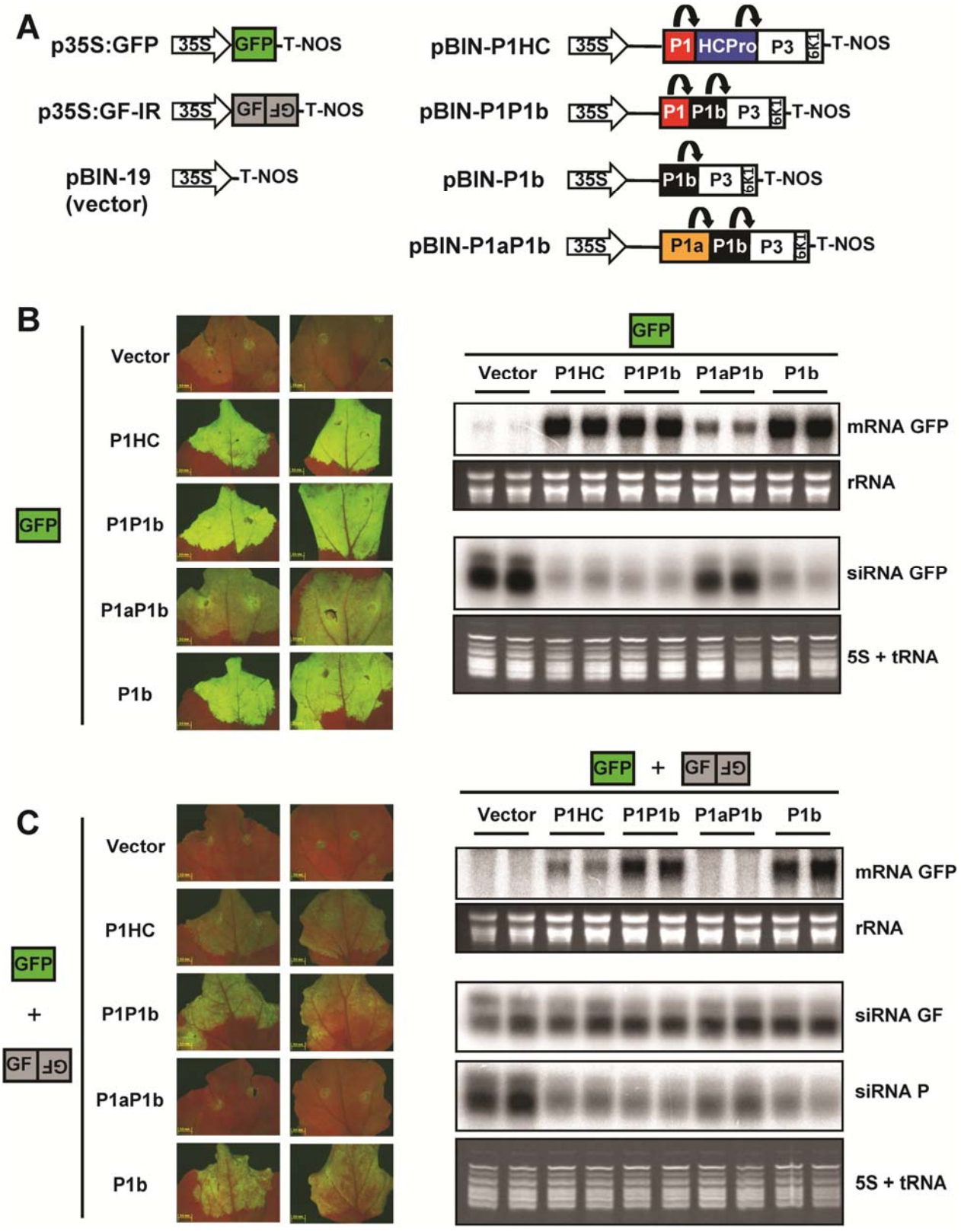

Fig. 5. RNA silencing suppression activity of the N-terminal regions of the genomic polyproteins of *Plum pox virus* (PPV) chimeras containing *Cucumber vein yellowing virus* (CVYV) P1b. **A,** Schematic representation of the green fluorescent protein (GFP) and viral-derived constructs used in the RNA silencing assays. Black arrows indicated the auto-proteolitic processes mediated by P1 and HCPro from PPV, and P1a and P1b from CVYV, to produce the corresponding free proteins. **B,** Single-stranded RNA-triggered silencing assay. **C,** Double-stranded RNA-triggered silencing assay. **B** and **C,** Left panels show GFP fluorescence pictures of leaves from two independent plants taken under an epifluorescence microscope at 6 days post-agroinfiltration (dpa), and right panels show Northern blot analyses of GFP mRNA and small interfering (si)RNA extracted at 6 dpa from leaf patches of two plants infiltrated with agrobacteria carrying the plasmid indicated above each lane. Two different probes were used for detection of GFP siRNAs: the GF probe (for primary plus secondary siRNAs) corresponds to the GFP fragment included in the inverted repeat (IR) RNA encoded by the silencing trigger plasmid p35S:GF-IR, and the P probe (specific for secondary siRNAs) corresponds to the 3′ terminal region of the GFP gene, which is not included in p35S:GF-IR. EtBr-stained rRNA and 5S+tRNA are shown as loading controls for the blots of mRNAs and siRNAs, respectively.

chimeras, silencing suppression assays were conducted in an agroinfiltration system in *N. benthamiana*. cDNA fragments corresponding to the 5′ proximal region of the genomic RNAs of the different chimeras were cloned in pBin19 under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter (Fig. 5A), and used for transient expression in *N. benthamiana* plants by infiltration with *Agrobacterium tumefaciens*. For simplicity, we refer to each *A. tumefaciens* strain by the plasmid it carries.

A sense RNA-triggered silencing assay was performed by agroinfiltration of p35S:GFP, expressing GFP mRNA as both silencing trigger and reporter (Fig. 5A). Very weak green fluorescence was observed at 6 days post-agroinfiltration (dpa) in leaf patches co-infiltrated with p35S:GFP plus an empty control plasmid as a consequence of RNA silencing induction (Fig. 5B). Co-infiltration with p35S:GFP plus plasmids expressing either P1-HC, P1-P1b, or P1b prevented the induction of silencing, and green fluorescence remained strong at 6 dpa (Fig. 5B). Patches co-infiltrated with pBIN-P1aP1b also displayed green fluorescence at 6 dpa but with less intensity than patches expressing P1-HC, P1-P1b, or P1b (Fig. 5B), suggesting that the presence of P1a disturbed the antisilencing activity of P1b in this assay. Consistent with these observations, Northern blot analyses showed high levels of GFP mRNA in leaves expressing P1-HC, P1-P1b, or P1b, which were much reduced in leaves expressing P1a-P1b and extremely reduced in leaves not expressing an RSS (Fig. 5B). Concomitantly, the large amounts of GFP siRNAs caused by RNA silencing were only slightly affected by P1a-P1b expression but markedly reduced by the expression of P1-HC, P1-P1b, or P1b (Fig. 5B).

We also analyzed the anti-silencing activity of the RSS encoded by the different PPV chimeras in an inverted repeat (IR)-triggered silencing assay. This is a more stringent silencing procedure, because it does not depend on the activity of plant RNA-dependent RNA polymerases, and could be more sensitive to small differences in the silencing suppression activity of the different RSS compared with the sense-triggering assay. We expressed p35S:GFP as reporter and p35S:GF-IR, which codes for an IR of a 5′-terminal fragment (GF) of the GFP RNA, as silencing trigger (Fig. 5A). Fluorescence monitoring and Northern blot analysis showed that GF-IR triggered a fast and strong silencing of the reporter GFP mRNA, which was not counteracted by P1a-P1b expression (Fig. 5C). P1-HC, P1-P1b, and P1b were able to suppress the RNA silencing triggered by GF-IR but GFP fluorescence and GFP mRNA accumulation were higher in leaves expressing P1-P1b or P1b than in those expressing P1-HC, suggesting that P1b could be a suppressor stronger than HCPro in this assay (Fig. 5C). A Northern blot analysis of small RNAs showed that the accumulation of primary GF siRNAs, which are expected to be produced by DCL-mediated cleavage of GF-IR, was not affected by the expression of the RSS (Fig. 5C). In contrast, the levels of secondary (P) siRNAs, produced by transitive silencing outside the GF trigger, were reduced in the presence of suppressors (Fig. 5C, right). The decrease in the accumulation of secondary P siRNAs was observed, although less pronounced than in leaves expressing other suppressor constructs, even in leaves expressing P1a-P1b, which did not accumulate appreciable amounts of GFP mRNA (Fig. 5C).

Incomplete self-cleavage activity of CVYV P1a in *N. benthamiana*.

The results shown in Figure 5 demonstrate that upstream P1a sequences drastically interfere with the RNA silencing suppression activity of CVYV P1b. Similarly, the silencing suppression activity of P1a-HC was much lower than that of P1-HC. In contrast, P1 has been shown to enhance the silencing suppression activity of PPV HCPro (Valli et al. 2006). However, P1 starts to have an interfering effect when its self-cleavage activity is disturbed and P1HC remains unprocessed (J. M. Viedma, A. Valli, J. A. García, and C. Simon-Mateo, *unpublished results*). The low silencing suppression activity of P1a-P1b does not lead to enough protein accumulation to allow for a confident assessment of the self-cleavage activity of P1a in the agroinfiltration assay. Thus, P1-P1b and P1a-P1b were expressed by agroinfiltration together with the strong silencing suppressor P19 from *Tomato bushy stunt virus* (TBSV) and GFP to easily monitor silencing suppression efficiency. Silencing suppression was highly efficient in leaves expressing either P1-P1b or P1a-P1b, together with P19, as revealed by similar strong green fluorescence and high GFP accumulation levels (Fig. 6). In contrast, the levels of accumu-

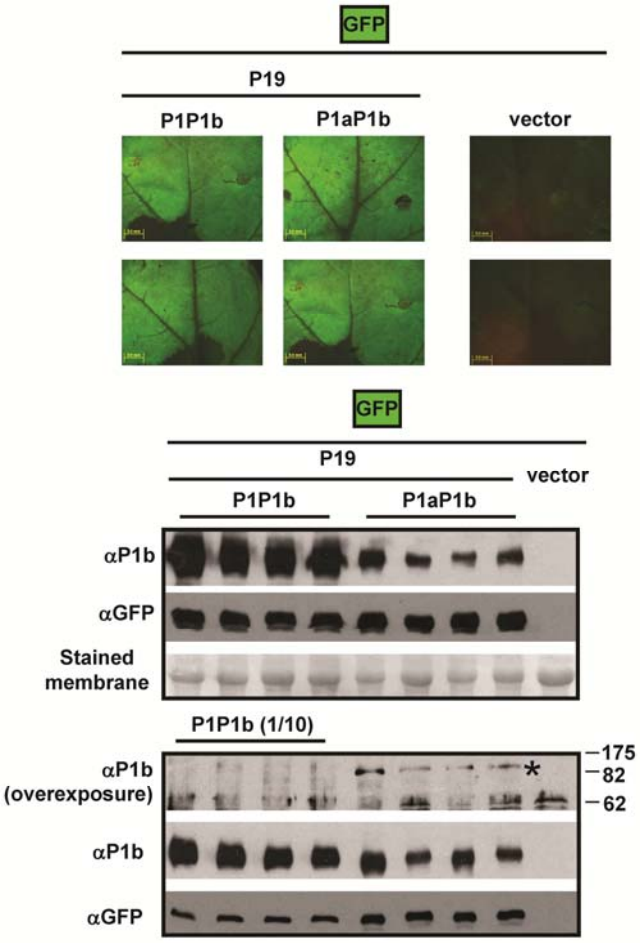

Fig. 6. Analysis of the accumulation of self-processed P1b products and unprocessed P1P1b or P1aP1b precursors in *Nicotiana benthamiana* leaves expressing P1P1b or P1aP1b, respectively. Leaves were agroinfiltrated with *Agrobacterium tumefaciens* expressing green fluorescent protein (GFP), *Tomato bushy stunt virus* P19, and either P1P1b or P1aP1b. Leaves infiltrated with *A. tumefaciens* expressing GFP and empty pBIN-19 (vector) were used as a control. Upper panels show GFP fluorescence pictures of leaves from two independent plants taken under an epifluorescence microscope at 6 days post-agroinfiltration. Middle and bottom panels show Western blot analyses of *N. benthamiana* plants (two leaves per plant) infiltrated with agrobacteria carrying the plasmids indicated above each lane. Ten-times-diluted P1P1b extracts were analyzed in the bottom Western blot. Immunoreactions were conducted with the indicated antibodies. Positions of prestained molecular mass markers (New England Biolabs) (in kilodaltons) run in the same gel are indicated to the right of an overexposed anti-P1b immunoreaction. The position of the bands corresponding to unprocessed P1aP1b is indicated by an asterisk. The membrane stained with Ponceau red showing the Rubisco is included as a loading control.

lation of P1b were more than 10 times lower in leaves expressing P1a-P1b than in those expressing P1-P1b. Moreover, in the leaves expressing P1a-P1b, a faint band corresponding to a protein with the expected mobility of the unprocessed product was clearly detected. An equivalent protein could not be detected in the leaves expressing P1-P1b (Fig. 6).

This result suggests that CVYV P1a self-cleavage is incomplete, and its efficiency is lower than that of PPV P1, in *N. benthamiana*.

A PPV-derived virus expressing P1b instead of HCPro infects peach seedlings poorly.

To explore the contribution of RSS in determining specific host ranges, we tested the ability to infect *Prunus persica* (a natural host for PPV but not for CVYV) of a PPV-based chimeric virus expressing CVYV P1b, P1P1b-BD. P1P1b-BD was constructed by replacing the HCPro sequence of P1HC-BD, a PPV chimera that infects GF-305 peach seedlings efficiently (Salvador et al. 2008), with that of CVYV P1b (Fig. 1C).

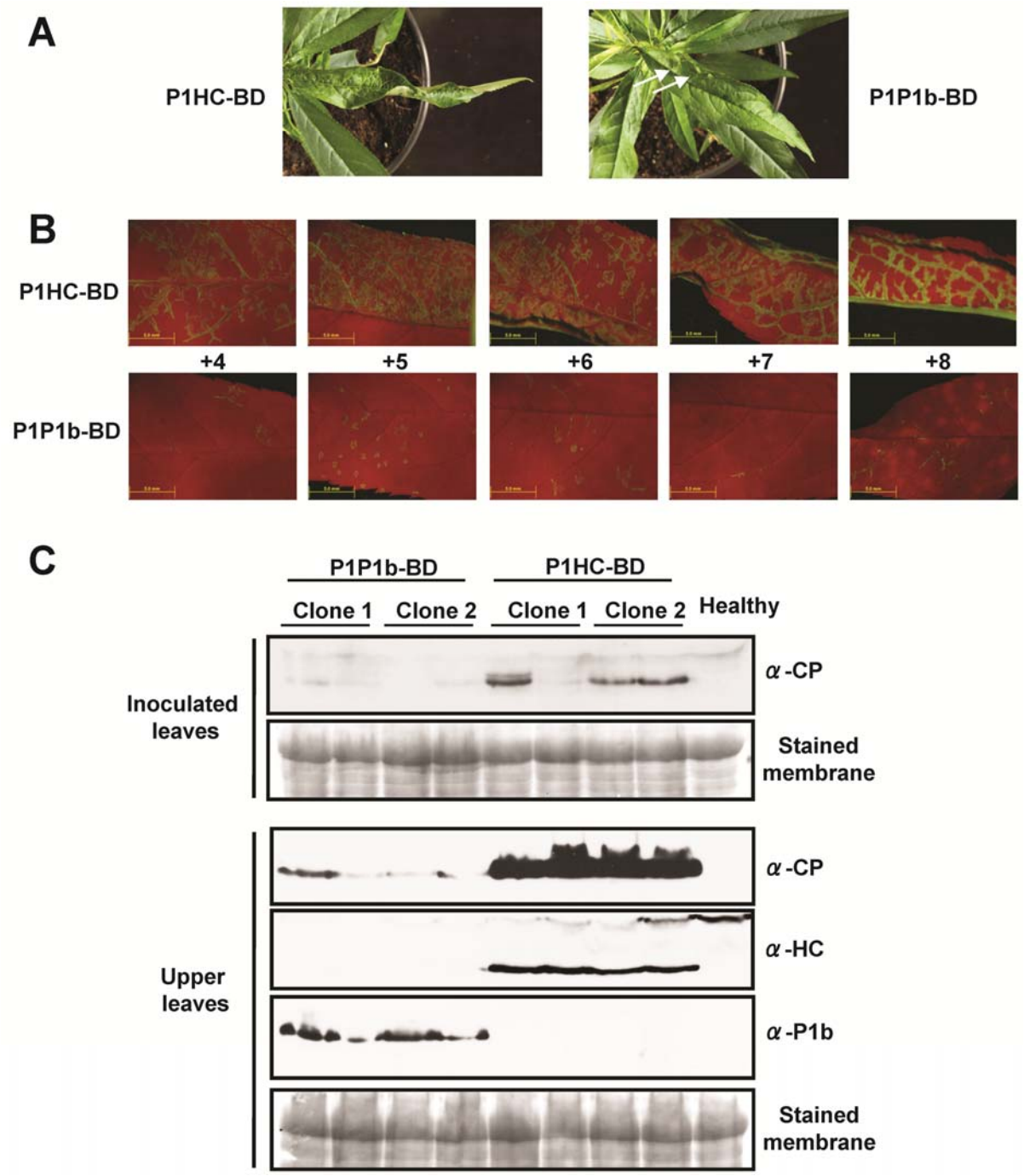

Fig. 7. Infection of GF-305 peach seedlings by a *Plum pox virus* (PPV)-derived virus expressing *Cucumber vein yellowing virus* (CVYV) P1b. **A,** Pictures taken under visible light at 24 days postinoculation (dpi) showing the symptoms caused by the indicated viruses in GF-305 peach seedlings. Small chlorotic regions in a leaf of the P1P1b-BD-infected plant are highlighted with white arrows. **B,** Pictures taken under an epifluorescence microscope at 21 dpi showing virus-derived green fluorescent protein (GFP) fluorescence in detached leaves (from position +4 to +8 above the inoculated ones) of GF-305 peach seedlings infected by the indicated viruses. **C,** Western blot analysis of inoculated (collected at 17 dpi) and upper noninoculated (collected at 24 dpi) leaf tissue from two GF-305 peach seedlings infected with the indicated viruses. A polyclonal serum specific for PPV coat protein (CP) was used for assessment of virus accumulation. Immunoreactions with polyclonal sera specific for HCPro of PPV and P1b of CVYV confirmed the identity of the infecting viruses. Membranes stained with Ponceau red showing the Rubisco are included as loading controls.

Peach seedlings were biolistically inoculated with the chimeric cDNAs, and the progress of infections was monitored under visible and UV illumination. Both P1HC-BD and P1P1b-BD showed 100% infectivity; however, whereas typical PPV symptoms of leaf curling and chlorosis started at approximately 12 dpi in apical leaves of peach seedlings inoculated with P1HC-BD, infection symptoms of P1P1b-BD were much milder, consisting of small chlorotic areas, and could be detected only at approximately 21 dpi (Fig. 7A). Moreover, strong and widely distributed green fluorescence was observed in leaves of P1HC-BD-infected plants, whereas leaves of seedlings infected with P1P1b-BD displayed very weak fluorescence in small spots (Fig. 7B).

Western blot analyses detected noticeable levels of viral CP in the bombarded leaves of almost all the plants inoculated with P1HC-BD, whereas no or a very low amount of CP was detected in leaves inoculated with P1P1b-BD (Fig. 7C). Similar analyses showed high levels of CP in upper noninoculated leaves of plants infected with P1HC-BD, contrasting with the very low amount of this protein that could be detected in the upper leaves of plants infected with P1P1b-BD (Fig. 7C).

CVYV P1b confers on PPV the ability to infect cucumber locally.

To further explore the contribution of RSS to host specificity, we tested the infectivity of HCPro- and P1b-expressing PPV variants in *Cucumis sativus*, a natural host of CVYV, which has not been reported to be susceptible to PPV infection. Preliminary experiments showed that cucumber leaves were particularly sensitive to the microparticle bombardment method, because leaves were considerably damaged after the bombardment. Therefore, we selected agroinfiltration as the inoculation procedure for the cucumber experiments because it caused less damage to the inoculated leaf, and it is expected to maintain local expression for longer periods. The full-length cDNA sequences of P1HC, P1P1b, P1b, and P1aP1b were engineered in pBin19-derived plasmids. *A. tumefaciens* strains transformed with these plasmids were used to inoculate *C. sativus* and *N. benthamiana* plants. The infection process was monitored by careful inspection of green fluorescence. Cucumber leaves inoculated with either P1HC or the empty vector displayed a similar yellow fluorescence as a consequence of the leaf damage caused by the infiltration procedure (Fig. 8A). However, although GFP fluorescence could not be detected in P1HC-inoculated cucumber leaves, a strong GFP expression was observed in *N. benthamiana* leaves inoculated with the same *Agrobacterium* culture (Fig. 8A). In contrast, distinct green fluorescent foci were detected around the damaged tissue displaying yellow fluorescence in all cucumber leaves inoculated with the three PPV-derived chimeras expressing CVYV P1b (Fig. 8A). Interestingly, the green fluorescence foci were quite similar in cucumber leaves inoculated with either P1b, P1P1b, or P1aP1b, contrasting with the much lower intensity of the GFP signal of P1aP1b compared with P1P1b and P1b in *N. benthamiana* leaves (Fig. 8A).

Western blot analyses of infiltrated leaves confirmed the results of GFP monitoring. Hence, *N. benthamiana* leaves inoculated with P1HC accumulated PPV CP at high levels, whereas this protein was either not or hardly detected in leaves of cucumber inoculated with this virus (Fig. 8B). In contrast,

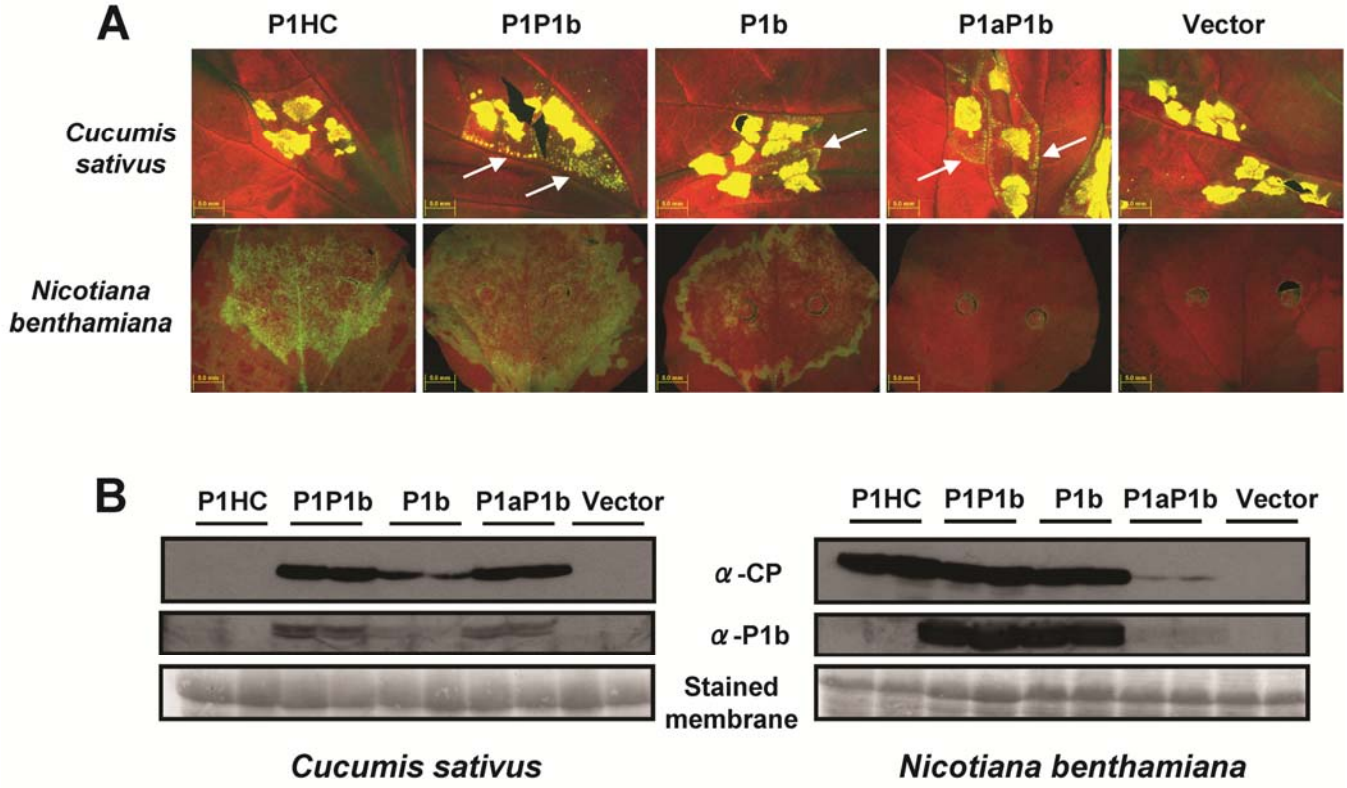

Fig. 8. Accumulation of *Plum pox virus* (PPV)-derived viruses expressing *Cucumber vein yellowing virus* (CVYV) P1b in cucumber and *Nicotiana benthamiana* leaves. **A,** Pictures taken under an epifluorescence microscope at 7 days post-agroinfiltration (dpa) showing virus-derived green fluorescent protein (GFP) fluorescence in leaves of *Cucumis sativus* (upper row) or *N. benthamiana* (lower row) infiltrated with *Agrobacterium* strains expressing the indicated viral full-length cDNA clones. Regions with green fluorescence foci in cucumber leaves are indicated with white arrows. **B,** Western blot analysis of leaf patches of two plants infiltrated with *Agrobacterium* strains expressing the indicated viral full-length cDNA clones. Samples were collected at 7 dpa. A polyclonal serum specific for PPV coat protein (CP) was used for assessment of virus accumulation. Immunoreactions with a polyclonal serum specific for CVYV P1b confirmed the identity of the infecting viruses. Membranes stained with Ponceau red showing the Rubisco are included as loading controls.

PPV CP was detected in cucumber leaves inoculated with P1b, P1P1b, or P1aP1b, which differ in producing high (P1b and P1P1b) or very low (P1aP1b) amounts of viral CP in *N. benthamiana* (Fig. 8B). It is also noteworthy that, in cucumber leaves, the CP of both P1P1b and P1aP1b viruses appear to accumulate at a higher level than that of the P1b virus. Together, these results indicate that the replacement of PPV HCPro by CVYV P1b is crucial for proper PPV replication in cucumber but not in *N. benthamiana* and that, although the presence of CVYV P1a has a strong negative effect on PPV infection in *N. benthamiana*, both this protein and PPV P1 could facilitate PPV replication in cucumber.

On the other hand, whereas all PPV variants agroinoculated into *N. benthamiana* plants were able to establish a systemic infection, GFP fluorescence and CP accumulation were detected in neither the inoculated leaves outside the infiltration area nor the upper noninoculated leaves of cucumber plants agroinoculated with HCPro- or P1b-expressing PPV chimeras (data not shown), indicating that CVYV P1b alone is not enough to confer on PPV the ability to spread away from the initial infection foci in cucumber.

DISCUSSION

Previous studies have demonstrated that viruses of the family *Potyviridae* use different proteins to counteract antiviral RNA silencing. Here, we show that a heterologous RSS, CVYV P1b, can functionally replace HCPro in the infection of the potyvirus PPV, affecting its host specificity and symptomatology. We also show that the presence in the viral genome of different P1 genes upstream from the RSS coding sequence conditions the phenotype of the P1b-expressing PPV chimeras.

Diverse engineered viruses lacking their RSS have been constructed and well characterized, such as the *Tombusvirus* spp. *Cymbidium ringspot virus* and TBSV lacking P19 (Omarov et al. 2006; Qiu et al. 2002; Szittya et al. 2002) and 2b-deletion mutants of the cucumovirus *Cucumber mosaic virus* (Diaz-Pendon et al. 2007; Ziebell et al. 2007). These mutants were able to initiate the infection process and reach upper noninoculated leaves but resulted in attenuated infections characterized by milder symptoms and recovery phenotypes caused by antiviral RNA silencing responses. In contrast, PPV P1ΔHC was completely unable to initiate an infection process in the susceptible host *N. benthamiana* (Fig. 4), demonstrating the key relevance of an active RSS for potyviral viability. This strict requirement was also recently reported for an RSS-deficient *Turnip mosaic virus* expressing an inactive HCPro, which only infected *N. benthamiana* when the tombusviral RSS P19 was exogenously supplied (Garcia-Ruiz et al. 2010). Several reasons might explain the different degrees of requirement for RNA silencing suppression activity of *Potyvirus* spp. and other viruses: i) higher rates of replication and movement could allow some viruses to escape antiviral silencing more easily; ii) although all the compared viruses replicate in the cytoplasm, their genomic RNAs could differ in the accessibility by the silencing effector machinery; iii) the larger size of the genomic potyviral RNAs could make them more susceptible to the RNA silencing action; and iv) the possibility that, in the absence of the main RSS in some viral systems, other viral factors could help the virus to evade plant antiviral defenses cannot be ruled out.

The ability of PPV P1P1b, in which HCPro has been replaced by CVYV P1b, to infect very efficiently two plant species of the *Nicotiana* genus (Fig. 2) demonstrates that, in spite of the strict requirement for RNA silencing suppression of *Potyvirus* spp., these viruses do not depend on the specific activity of a particular RSS. HCPro is a multifunctional protein (Syller 2005), which interacts with a number of host proteins (Ala-Poikela et al. 2011; Anandalakshmi et al. 2000; Cheng et al. 2008; Dielen et al. 2011; Endres et al. 2010; Guo et al. 2003; Jin et al. 2007a and b). Our results also demonstrate that, in spite of the lack of sequence similarity, CVYV P1b is able to supply all essential functions of HCPro. However, the lower accumulation of P1P1b compared with P1HC (wild-type PPV) in *Nicotiana* spp. (Figs. 2 and 3) appears not to be due to an unspecific weakness of the silencing suppression activity of CVYV P1b, because P1P1b suppresses silencing even more strongly than P1HC in agroinfiltration assays (Fig. 5). Thus, it is likely that either some specific coupling of the RNA silencing suppression of HCPro with other viral processes, such as viral replication, cannot be exactly mimicked by the CVYV P1b antisilencing activity, or a silencing suppression-unrelated activity of HCPro cannot be fully supplied by the heterologous protein. It is also interesting to remark that, in spite of the lower virus accumulation, some disease symptoms are more severe in plants infected with P1b-expressing viruses than in those infected with the wild-type virus (Fig. 2). A number of RSS have been shown to be targets for plant defense responses other than RNA silencing. For instance, HCPro of *Potato virus Y* (Moury et al. 2011) and other RSS, such as P38 of *Turnip crinkle virus* (Ren et al. 2000) and P126 of *Tobacco mosaic virus* (Padgett et al. 1997), are elicitors of hypersensitive response (HR) and viral resistance mediated by resistance genes; and P6 of CaMV (Kiraly et al. 1999), 2b of *Tomato aspermy virus* (Li et al. 1999), and P19 of TBSV (Chu et al. 2000) induce HR-like necrotic symptoms. Thus, a defensive reaction of the plants elicited by P1b may be the cause of the strengthened symptoms caused by PPV P1P1b (rather than a direct effect of virus multiplication), and could be one of the factors accounting for the lower accumulation of the P1b-expressing chimera compared with wild-type PPV.

The P1 protein has been shown to be an accessory factor that facilitates the genome amplification of the potyvirus *Tobacco etch virus* (Verchot and Carrington 1995). Some evidence suggest that P1 could enhance the RNA silencing suppression activity of HCPro (Kasschau and Carrington 1998; Pruss et al. 1997; Rajamaki et al. 2005; Valli et al. 2006). P1 is also a nonessential factor in PPV infection but deletion of the P1 gene notably debilitates PPV (*unpublished results*), because deletion of P1 in the chimera P1P1b causes a drop in virus accumulation and movement (Fig. 3). P1 did not enhance the silencing suppression activity of P1b in the agroinfiltration system; however, P1 deletion facilitated plant recovery at late stages of infection (Fig. 3B), suggesting that P1 could be contributing to efficient RNA silencing suppression. Surprisingly, CVYV P1a not only does not reproduce the positive contribution of the natural P1 to PPV infection but it is clearly deleterious for the virus in *N. benthamiana*. PPV P1aP1b was able to infect *N. benthamiana*, although much less efficiently than the P1-deficient PPV P1b virus, and the P1aP1b-infected plant is cleared of virus a few weeks after inoculation (Fig. 3). Similarly, PPV P1aHC also infects, very mildly, *N. benthamiana*. In contrast with PPV P1, upstream CVYV P1a sequences drastically disturbed the RNA silencing suppression activity of P1b (Fig. 5) and HCPro, and this disturbance is likely the cause of the detrimental effect of P1a in PPV infection. Although we do not know how P1a interferes with the silencing suppression activity of P1b, transient expression experiments provided valuable clues. Agroinfiltration of bacteria expressing P1a-P1b yielded much lower P1b protein levels than that expressing P1-P1b, even though the strong RSS P19 was coexpressed to ensure P1b-independent silencing suppression activity (Fig. 6), and unprocessed P1a-P1b product, but not P1-P1b, was detected in the infiltrated leaves (Fig. 6). Although low P1b accumulation

and inefficient P1a self-cleavage might be unrelated, the previous observation that P1 self-cleavage depends on plant proteins (Verchot et al. 1992) suggests a scenario in which proper folding of the precursor polyprotein, assisted by specific host factors, results in processing, whereas the incorrectly folded precursor is degraded. This would explain why, in contrast with its detrimental effect in the *N. benthamiana* infection, P1a enhances PPV amplification in cucumber (Fig. 8). The inefficiency of the agroinfiltration system in cucumber has prevented us from testing this hypothesis; however, the fact that P1a-P1b polyprotein precursor was not detected in *C. sativus* plants infected with either P1aP1b chimera (data not shown) or wild-type CVYV (Valli et al. 2008) supports this idea. In any case, these results are in agreement with the important role of P1 proteins in host specificity of *Potyvirus* spp. that has been previously suggested (Noa-Carrazana et al. 2006; Salvador et al. 2008; Valli et al. 2007).

Previous reports have revealed the possibility that specific HCPro activities contribute to virus adaptation to particular hosts (Sáenz et al. 2002). We show now that, whereas P1b-expressing PPV replicates efficiently in *N. benthamiana*, it infects very poorly the natural PPV host *P. persica* (Fig. 7), suggesting that the function of P1b could also be host specific. In agreement with this assumption, expression of CVYV P1b enhanced local PPV replication in leaves of *C. sativus* (Fig. 8). The suppression activity of HCPro and P1b is thought to be mediated by binding to siRNAs, which are expected to be similar in different plant species. Therefore, if the different behavior of HCPro-expressing and P1b-expressing PPV in different hosts is the consequence of host-specific features of the RNA silencing suppression activities of HCPro and P1b, it would be necessary to hypothesize that siRNA binding per se is not enough to suppress antiviral RNA silencing, and host factors contribute to establish an effective silencing suppression activity. This hypothesis needs to be tested in silencing suppression experimental systems which, unfortunately, are still not available for *P. persica* and *C. sativus*; thus, we cannot rule out the possibility that RNA silencing-unrelated functions of HCPro and P1b could be involved in the host specificity shown by the different PPV variants.

Finally, the P1aP1b PPV chimera only forms small foci inside the inoculated area of cucumber leaves (Fig. 8), despite expressing P1a and P1b proteins from the cucumber-infecting CVYV. This observation reveals the existence of specific virus genetic factors required for cucumber infection outside the P1a and P1b coding sequences, in agreement with earlier reports showing that host range determinants are extensively spread throughout the PPV genome (Salvador et al. 2008). Plant–virus interactions affected by these specific determinants are largely unknown and should be the subject of further research.

MATERIALS AND METHODS

Plant hosts.

Agroinfiltration assays were performed in *N. benthamiana* plants. Viral infectivity assays were performed in *N. benthamiana*, *N. clevelandii*, *C. sativus* Albatroz RZ F1, and *P. persica* GF305 plants. Plants were grown in a greenhouse maintained under 16 h of light with supplementary illumination at 19 to 23°C.

Plasmids.

A partial PPV clone (p35SeNOSB) carrying the cDNA corresponding to the 5′ region of the genome of the PPV-R isolate (nucleotides 1 to 3,628 that correspond to the 5′ untranslated region [UTR] and P1, HCPro, P3, and 6K1 cistrons) cloned between the CaMV 35S promoter and the NOS terminator (López-Moya and García 2000), in which the first AUG of the large open reading frame was mutated and the second AUG was engineered to display an *Nco*I restriction site (Simón-Buela et al. 1997), was used as backbone to generate intermediate cDNA clones coding for different mutated PPV 5′ genomic regions (p35S-P1ΔHC, p35S-P1P1b, p35S-P1aP1b, and p35S-P1b).

The gene splicing via overlap extension method (Horton et al. 1989) was used to generate p35S-P1ΔHC and p35S-P1P1b. Primers and templates for polymerase chain reactions (PCR) used to construct these clones are listed in Supplementary Tables S1 and S2. Hence, p35S-P1ΔHC was obtained by replacing the *Nco*I-*Dra*III fragment of p35SeNOSB that encodes the N-terminal region of the PPV polyprotein, with the corresponding fragment from PCR3, which codes for PPV P1-P3 and lacks the HCPro coding sequence. p35S-P1P1b was obtained by inserting an *Nco*I-*Bam*HI fragment from PCR6 plus a *Bam*HI-*Dra*III fragment from PCR9, which together code for PPV P1-CVYV P1b-PPV P3, in p35SeNOSB digested with *Nco*I and *Dra*III. p35S-P1P1b clones carrying RK68,69AA, C89A, or C93A mutant versions of CVYV P1b were obtained by replacing the *Nco*I-*Bam*HI fragment from p35S-P1P1b that codes for P1-P1b with the corresponding fragments from PCR11, PCR13, and PCR15, respectively. The CVYV P1a-P1b-coding sequence lacking its internal *Nco*I site was obtained by using the mutagenesis method of Herlitze and Koenen (1990). The resulting PCR product (PCR18), digested with *Nco*I and *Bam*HI, was used to substitute for the corresponding fragment of p35S-P1P1b, yielding p35S-P1aP1b. p35S-P1b was obtained by replacing the *Nco*I-*Sex*AI fragment from p35SeNOSB with the corresponding fragment from PCR19, which codes for CVYV P1b. Because *Sex*I cleaves into the PPV HCPro-coding sequence 9 nucleotides away from its 3′ end, the CVYV P1b protein encoded by p35S-P1b is fused to the last 3 amino acids of PPV HCPro.

P1ΔHC, P1P1b, P1b, and P1aP1b full-length clones were obtained by substituting the 35S-P1ΔHC, p35S-P1P1b, p35S-P1b, and p35S-P1aP1b *Xba*I-*Dra*III fragments that code for the 5′ region of the viral genome preceded by the CaMV 35S promoter for the corresponding fragment of pICPPV-NK-GFPn (P. Sáenz, M. R. Fernández-Fernández, and J. A. García, *unpublished results*), a plasmid derived from pICPPV-NK-GFP (Fernandez-Fernandez et al. 2001) in which the enhanced GFP had been replaced by the wild-type GFP from *Aequorea victoria* (Fig. 1).

The gene splicing via overlap extension method (Horton et al. 1989) was also used to generate a P1aHC full-length clone. It was obtained by replacing the *Bsu*36I-*Dra*III fragment of P1aP1b infectious clone with the corresponding fragment from PCR27, which codes for CVYV P1a and HCPro-P3 from PPV.

A partial PPV clone (p35S5′DNOS) carrying the cDNA corresponding to the 5′ region of the genome of the PPV-D isolate (nucleotides 1 to 2,923 that include the 5′ UTR and P1, HCPro, and P3 cistrons) cloned between the CaMV 35S promoter and the NOS terminator (Salvador 2008) was used as backbone to generate an intermediate cDNA clone coding for a chimeric PPV-CVYV 5′ genomic region (p35S-P1DP1b). This chimeric clone was constructed by using the gene splicing via overlap extension method (Horton et al. 1989). p35S-P1DP1b was obtained by inserting a *Cpo*I-*Vsp*I fragment from PCR21 plus a *Vsp*I-*Pst*I fragment from PCR24 which, together, code for PPV-D P1-CVYV P1b-PPV-D P3, in p35S5′DNOS digested with *Cpo*I and *Pst*I. The P1P1b-BD full-length clone (Fig. 1) was constructed by a triple ligation of the *Bpu*1102I-*Pst*I fragment that encodes P1-P1b from p35S-P1DP1b, and *Sac*I-*Bpu*1102I and *Pst*I-*Sac*I fragments from pICPPV-5′BD GFP (Salvador et al. 2008).

pBINPPV-NK-GFP, which contains a full-length cDNA copy of the PPV genome cloned in pBin19 (Bevan 1984) under the control of the CaMV 35S promoter (Lucini 2004), was used as parental plasmid to generate infectious binary vectors for the different recombinant viruses. These clones were constructed by replacing the *Sca*I-*Xho*I fragment from pBINPPV-NK-GFP that includes the nucleotide 1 to 6,770 sequence of PPV preceded by the CaMV 35S promoter, with the corresponding fragments from P1P1b, P1aP1b, or P1b.

pBIN-P1HC, a pBin19-derivative carrying the cDNA corresponding to the 5′ region of the PPV-R genome (M. O. Delgadillo, J. A. García, and C. Simón-Mateo, *unpublished results*), was the parental plasmid in the construction of pBIN-P1P1b, pBIN-P1aP1b, pBIN-P1b, and pBIN-P1aHC, which were used in the agroinfiltration assays. pBIN-P1P1b, pBIN-P1aP1b, and pBIN-P1b were obtained by replacing the pBIN-P1HC *Sca*I-*Xba*I fragment that codes for the 5′ region of the viral genome preceded by the CaMV 35S promoter, with the corresponding fragments from p35S-P1P1b, p35S-P1aP1b, or p35S-P1b. pBIN-P1aHC was obtained by replacing the pBIN-P1HC *Xma*I-*Sna*BI fragment that also codes for the 5′ region of the viral genome preceded by the CaMV 35S promoter, with the corresponding fragments from the P1aHC full-length clone.

A. tumefaciens C58C1 strain carrying p35S:GFP (Haseloff et al. 1997) plus pCH32 (Hamilton et al. 1996), p35S:GF-IR (Schwach et al. 2005), and pBIN61:P19 (Voinnet et al. 2003) were kindly provided by D. Baulcombe (University of Cambridge, United Kingdom).

Biolistic inoculation.

The Helios Gene Gun System (Bio-Rad, Hercules, CA, U.S.A.) was used for biolistic inoculation. Microcarrier cartridges were prepared with 1.0-µm gold particles coated with the different plasmids at a DNA loading ratio of 2 µg/mg of gold and a microcarrier loading of 0.5 mg/shooting. Helium pressure of 7 and 10 bars were used for shooting *Nicotiana* and *P. persica* plants, respectively.

Agroinfiltration and GFP imaging.

N. benthamiana and *C. sativus* plants were infiltrated with *A. tumefaciens* strain C58C1 carrying the indicated plasmids, as previously described (Valli et al. 2006). The GFP fluorescence was monitored under long-wavelength UV light (Black Ray model B 100 AP) and photographed with a Nikon D1X digital camera equipped with a 62E 022 filter. For amplified visualization of fluorescent areas, leaves were examined with a Leica MZ FLIII epifluorescence microscope equipped with a filter that had an excitation window at 425/460 nm and a barrier filter at 480 nm and were photographed with an Olympus DP70 digital camera.

Western blot assays.

Tissue samples of infected leaves were harvested under UV light from GFP-expressing areas, while tissue samples of non-infected leaves were taken from equivalent areas. Preparation of protein samples, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and electroblotting were done as previously described (Valli et al. 2006). Specific proteins were detected using anti-HCPro rabbit serum, anti-P1b rabbit serum, or anti-CP rabbit serum as primary antibody, and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Jackson ImmunoResearch, West Grove, PA, U.S.A.) as secondary reagent. The immunostained proteins were visualized by enhanced chemiluminiscence detection with a LifeABlot kit (Euroclone S.p.A., Siziano, Italy). Ponceau red staining was used to check the global protein content of the samples.

RNA extraction and Northern blot analysis.

Samples of large and small RNAs were prepared from agroinfiltrated leaf tissue and subjected to Northern blot analysis as previously described (Valli et al. 2006). GFP siRNAs were detected with ^{32}P-labeled GF and P riboprobes, which were prepared by transcription with SP6 RNA polymerase from *Sac*II-linearized pGEMT-GF and pGEMT-P, respectively. These plasmids contain the nucleotides 4 to 403 (GF) and 404 to 717 (P) of the GFP gene cloned in pGEM-T.

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