Document downloaded from:

http://hdl.handle.net/10251/161157

This paper must be cited as:

Carbonell, A.; Dujovny, G.; Garcia, JA.; Valli, A. (2012). The Cucumber vein yellowing virus silencing suppressor P1b can functionally replace HCPro in Plum pox virus infection in a host-specific manner. Molecular Plant-Microbe Interactions. 25(2):151-164. https://doi.org/10.1094/MPMI-08-11-0216

The final publication is available at

https://doi.org/10.1094/MPMI-08-11-0216

Copyright Scientific Societies

Additional Information

1 **The *Cucumber vein yellowing virus* silencing suppressor P1b can functionally replace HCPro in *Plum pox virus* infection in a host-specific manner**

3 Alberto Carbonell[1,2], Gabriela Dujovny[1], Juan Antonio García[1,*] and Adrian Valli[1,2,*]

4

5 [1]Centro Nacional de Biotecnología (CNB-CSIC), Campus Universidad Autónoma de Madrid, 28049 Madrid, Spain

7 [2]Present addresses: Donald Danforth Plant Science Center, St. Louis, Missouri 63132, USA (A.C.); Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, United Kingdom (A.V.)

10

11 [*] Corresponding authors: Juan Antonio García and Adrian Valli; E-mails: jagarcia@cnb.csic.es and aav30@cam.ac.uk

13

14

15

16

17

18

19

20

21

22

23

ABSTRACT

Plant viruses of the genera *Potyvirus* and *Ipomovirus* (*Potyviridae* family) use unrelated RNA silencing suppressors (RSSs) to counteract antiviral RNA silencing responses. HCPro is the RSS of potyviruses, 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 *N. benthamiana*. Testing the influence of RSSs in host specificity, we found that a P1b-expressing chimera infected poorly PPV's natural host *Prunus persica*. Conversely, P1b conferred PPV chimeras the ability to replicate locally in cucumber, CVYV's natural host. The deleterious effect of P1a on PPV infection is host-dependent, since 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 RSSs, and that particular RSSs and upstream P1-like proteins contribute to defining the virus host range.

1 The establishment of a viral infection in a particular host plant relies on the
2 availability of specific factors necessary for replication and spread of the virus, and in the
3 ability of the virus to escape or counteract a series of defence layers raised by the plant.
4 Among these antiviral barriers, innate immunity responses, which are also elicited by
5 other plant pathogens, are triggered by broadly conserved pathogen-associated molecular
6 patterns (PAMP) (Soosaar et al., 2005). Moreover, double stranded forms of viral RNA
7 are recognized as a special PAMP by the infected plant, which activates RNA silencing
8 pathways resulting in specific antiviral immunity (Ding, 2010). In order to circumvent
9 this defensive response, most plant viruses have evolved RNA silencing suppressors
10 (RSSs) (Moissiard and Voinnet, 2004; Roth et al., 2004; Burgyán, 2008; Valli et al.,
11 2009).

12 The genus *Potyvirus* of the family *Potyviridae* is the largest group of plant viruses
13 (López-Moya et al., 2009). The single-stranded RNA genome of potyviruses is translated
14 into a large polyprotein and a truncated frameshift product, which are processed by three
15 virus-encoded proteinases. One of these proteinases, the cysteine proteinase HCPro, was
16 the first viral product to be recognized as a RSS (Anandalakshmi et al., 1998; Brigneti et
17 al., 1998; Kasschau and Carrington, 1998), and its RNA silencing suppressor activity
18 appears to be essential for potyviral viability (Garcia-Ruiz et al., 2010). Indeed HCPro is
19 a multifunctional protein that acts in several steps of the potyviral infection cycle
20 including viral genome amplification (Kasschau et al., 1997), cell-to-cell and long-
21 distance spread (Cronin et al., 1995; Rojas et al., 1997) and aphid transmission (Berger et
22 al., 1989), and is also involved in symptom expression (Pruss et al., 1997; Gal-On and
23 Raccah, 2000; Sáenz et al., 2000). The overall genomic structure of potyviruses,

1 including HCPro-coding sequences, is also conserved in other monopartite genera of the
2 family *Potyviridae*. However, whereas an HCPro gene is included in the genome of
3 *Sweet potato mild mottle virus* (SPMMV), the type member of the genus *Ipomovirus*, this
4 gene is absent from the genome of other ipomoviruses such as *Cucumber vein yellowing*
5 *virus* (CVYV), *Squash vein yellowing virus* (SqVYV) and *Cassava brown streak virus*
6 (CBSV) (Janssen et al., 2005; Li et al., 2008; Mbanzibwa et al., 2009). In CVYV and
7 SqVYV, the RSS is the serine proteinase P1b that could have derived from a gene
8 duplication of the 5'-terminal gene that codes for the protein P1 (Valli et al., 2006;
9 Mbanzibwa et al., 2009). These P1b proteins, together with the single P1s of some
10 ipomoviruses, brambyviruses and poaceviruses (or susmoviruses), form a group of P1
11 homologs distinct from the typical P1 proteins of potyviruses and the first P1 copies,
12 named P1a, of CVYV and SqVYV (Valli et al., 2007; Susaimuthu et al., 2008; Fellers et
13 al., 2009; Tatineni et al., 2009). Interestingly, although the tritimoviruses and SPMMV
14 have HCPro, their silencing suppression activity is provided by their P1b-like P1 proteins
15 rather than by HCPro (Stenger et al., 2007; Giner et al., 2010). Moreover, whereas the
16 silencing suppression mechanism of potyviral HCPro and CVYV P1b appears to involve
17 siRNA sequestration (Lakatos et al., 2006; Valli et al., 2011), SPMMV P1 inhibits RNA
18 silencing by Argonaute binding (Giner et al., 2010). Thus, viruses of the family
19 *Potyviridae* can use RSSs with unrelated sequences and different mechanisms of action to
20 counteract antiviral silencing and facilitate viral infection.

21 In the present study, we have shown that, although RNA silencing suppression is an
22 essential activity for potyviruses, a particular potyviral infection does not depend on a

unique RSS. In addition, our data suggest that specific RSSs 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 RSSs used, respectively, by potyvirus and ipomovirus members 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-GFP, which also expresses the green fluorescent protein (GFP) to monitor the viral infection (Fernandez-Fernandez et al., 2001). For simplicity these clones are named here according the N-terminal regions of their polyproteins (P1HC, 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 1-2 days in the appearance of symptoms and GFP fluorescence in upper non-inoculated 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 they were notably less stunted than those infected with P1HC (Fig. 2).

1 Although visible symptoms were more prominent in leaves of P1P1b-infected plants, the
2 virus-derived GFP fluorescence was less intense in these plants than in those infected
3 with P1HC. Western blot analysis of leaf extracts showed high accumulation levels of
4 viral CP in P1P1b-infected *N. clevelandii* and *N. benthamiana* plants, but somewhat
5 lower than those detected in extracts from plants inoculated with P1HC (Fig. 2).
6 Immunoreactions with anti-P1b and anti-HCPro specific antibodies confirmed that each
7 virus expressed the expected RSS (Fig. 2). These results indicate that CVYV P1b can
8 functionally replace HCPro in a PPV infection.
9

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

11 Some reports suggest that the potyviral P1 protein enhances the activity of the RSS
12 HCPro (Pruss et al., 1997; Anandalakshmi et al., 1998; Kasschau and Carrington, 1998;
13 Rajamaki et al., 2005; Valli et al., 2006). We were interested in knowing whether P1a,
14 the homologous protein of P1 in CVYV, has a similar effect on P1b activity in the
15 context of a viral infection, and whether the natural CVYV P1a-P1b combination could
16 be more effective than the chimeric PPV P1-CVYV P1b one. With this aim, we
17 engineered the GFP-tagged recombinant viruses P1aP1b and P1b in which the P1-HC
18 sequence of PPV was replaced either by P1a-P1b or P1b from CVYV (Fig. 1). Leaves of
19 *N. benthamiana* plants were biolistically inoculated with the different constructs, and the
20 infection was monitored under visible and UV lights. P1b and P1aP1b chimeras showed
21 high infectivity levels similar to those of P1HC and P1P1b. However, whereas plants
22 infected with either P1P1b or P1b viruses showed similar disease symptoms, which
23 appeared with a small delay (1-2 days) with respect to P1HC-infected plants, plants

1 inoculated with P1aP1b showed no visible symptoms (data not shown). At three weeks
2 post inoculation, the spread of green fluorescence in upper non-inoculated leaves close to
3 the inoculated ones was more limited in the P1b-infected plants than in plants infected
4 with P1P1b (Fig. 3A). However, PPV CP accumulation in regions showing green
5 fluorescence was similar in plants infected with P1b or P1P1b, and only slightly lower
6 than in P1HC-infected plants (Fig. 3A). In contrast, at this time, green fluorescence was
7 very faint in the upper non-inoculated leaves of plants infected with P1aP1b, which
8 showed lower accumulation levels of viral CP compared with plants infected with the rest
9 of viruses (Fig. 3A).

10 At 21 dpi, the apical young leaves of plants infected with either P1b or P1aP1b viruses
11 did not show green fluorescence, and the green fluorescence of the corresponding leaves
12 of P1P1b-infected plants was much fainter than that observed in P1HC-infected plants
13 (Fig. 3A). This fact suggested that a recovery from infection could be starting in plants
14 infected with viruses lacking HCPro, mainly when P1 was also absent. To assess this
15 possibility, the infected plants were analysed at later stages (36 dpi). Whereas young
16 leaves of plants infected with P1HC or P1P1b showed viral symptoms, the apical leaves
17 of plants infected with P1b and P1aP1b appeared healthy (Fig. 3B). In agreement with
18 this observation, green fluorescence was apparent in young leaves of plants infected with
19 P1HC and, with less intensity, in those from P1P1b-infected plants, but it could not be
20 detected in young leaves of plants infected with P1b or P1aP1b (Fig. 3B). A Western blot
21 analysis showed that, in older (+5 and +6) and intermediate (+7 and +8) leaves (the +
22 sign indicates the position above the inoculated leaves), the CP accumulation levels of
23 plants infected with P1b or P1P1b were similar, and only slightly lower than those of

1 P1HC-infected plants, similarly than in the +2 and +3 leaves analysed at 21dpi (Fig. 3A).
2 In contrast, no viral CP was detected in young leaves (+9 and +10) of plants infected with
3 P1b, and CP accumulation was notably lower in the corresponding leaves of plants
4 infected with P1P1b than in those of P1HC-infected plants (Fig. 3B). At this time, viral
5 CP was detected only in the older leaves of P1aP1b-infected plants, and at extremely low
6 levels (Fig. 3B), reinforcing the idea that the combination of P1a and P1b supports PPV
7 infection very poorly, and plants recover easily from P1aP1b infection. We also
8 substituted PPV P1 by CVYV P1a in the wild type, HCPro-containing, PPV clone. The
9 resulting chimera, P1aHC, infected *N. benthamiana* as badly as P1aP1b (Supplementary
10 Fig. 1), demonstrating that P1a is also non functional when the virus is using HCPro as
11 RSS.

12 Together, these results indicate that despite P1 is dispensable to establish the infection
13 of a chimeric PPV carrying CVYV P1b instead of HCPro, P1 might be necessary to
14 prevent recovery from infection and CVYV P1a cannot replace PPV P1 in this task.
15

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

18 In order to assess the relevance of P1b-mediated silencing suppression in the context
19 of a potyviral infection, we constructed two variants of the P1P1b chimera containing
20 either the RK68,69AA or the C89A mutation, which affect the conserved LxKA motif or
21 a putative Zn finger, respectively, and abolish the RNA silencing suppression activity of
22 CVYV P1b (Valli et al., 2008). The C93A mutation, which does not affect the P1b
23 silencing suppression activity, was engineered in a control PPV P1P1b clone. In addition,

1 a PPV cDNA construct lacking HCPro (P1ΔHC) was also generated (Fig. 1). *N.*
2 *benthamiana* plants were biolistically inoculated with DNA of these PPV clones, and the
3 infection process was followed by monitoring GFP fluorescence and symptom
4 expression. Whereas systemic disease symptoms and green fluorescence were observed
5 in plants inoculated with viruses carrying an active RSS (P1HC, P1P1b and P1P1b
6 C93A), no visual signs of local or systemic infection were detected in those plants
7 inoculated with clones that do not encode a known RSS (P1ΔHC), or code for inactive
8 P1b mutants (P1P1b RK68,69AA or P1P1b C89A) (Fig. 4A).
9 Western blot analyses of protein extracts from inoculated and upper non-inoculated
10 leaves confirmed the visual observations, and showed no viral CP accumulation in plants
11 inoculated with P1P1b RK68,69AA, P1P1b C89A or P1ΔHC, while large amounts of
12 viral CP were detected in plants inoculated with clones expressing active RSSs (P1HC,
13 P1P1b and P1P1b C93A) (Fig. 4B). These results indicate that the RNA silencing
14 suppression activity, provided by either HCPro or P1b, is essential to support PPV
15 infection.
16
17 **CVYV P1a, but not PPV P1, interferes with the RNA silencing suppression activity**
18 **of CVYV P1b in agroinfiltration assays.**
19 To assess the contribution of differences in anti-silencing activities to the specific
20 biological features of the different PPV chimeras, silencing suppression assays were
21 conducted in an agroinfiltration system in *N. benthamiana*. cDNA fragments
22 corresponding to the 5' proximal region of the genomic RNAs of the different chimeras
23 were cloned in pBin19 under the control of the CaMV 35S promoter (Fig. 5A), and used

1 for transient expression in *N. benthamiana* plants by infiltration with *Agrobacterium*
2 *tumefaciens*. For simplicity, we refer to each *A. tumefaciens* strain by the plasmid it
3 carries.

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

19 We also analysed the silencing activity of the RSSs encoded by the different PPV
20 chimeras in an inverted repeat (IR)-triggered silencing assay. This is a more stringent
21 silencing procedure, since it does not depend on the activity of plant RNA-dependent
22 RNA polymerases, and could be more sensitive to small differences in the silencing
23 suppression activity of the different RSSs compared to the sense-triggering assay. We

1 expressed p35S:GFP as reporter and p35S:GF-IR, which codes for an IR of a 5' terminal
2 fragment (GF) of the GFP RNA, as silencing trigger (Fig. 5A). Fluorescence monitoring
3 and Northern blot analysis showed that GF-IR triggered a fast and strong silencing of the
4 reporter GFP mRNA, which was not counteracted by P1a-P1b expression (Fig. 5C). P1-
5 HC, P1-P1b and P1b were able to suppress the RNA silencing triggered by GF-IR, but
6 GFP fluorescence and GFP mRNA accumulation were higher in leaves expressing P1-
7 P1b or P1b than in those expressing P1-HC, suggesting that P1b could be a suppressor
8 stronger than HCPro in this assay (Fig. 5C). A Northern blot analysis of small RNAs
9 showed that the accumulation of primary GF siRNAs, which are expected to be produced
10 by DCL-mediated cleavage of GF-IR, was not affected by the expression of the RSSs
11 (Fig. 5C). In contrast, the levels of secondary (P) siRNAs, produced by transitive
12 silencing outside the GF trigger, were reduced in the presence of suppressors (Fig. 5C,
13 right). The decrease in the accumulation of secondary P siRNAs was observed, although
14 less pronounced than in leaves expressing other suppressor constructs, even in leaves
15 expressing P1a-P1b, which did not accumulate appreciable amounts of GFP mRNA (Fig.
16 5C).
17
18 **Incomplete self-cleavage activity of CVYV P1a in *N. benthamiana*.**
19 The results shown in Fig. 5 demonstrate that upstream P1a sequences drastically
20 interfere with the RNA silencing suppression activity of CVYV P1b. Similarly, the
21 silencing suppression activity of P1a-HC was much lower than that of P1-HC
22 (Supplementary Fig. 1). In contrast, P1 has been shown to enhance the silencing
23 suppression activity of PPV HCPro (Valli et al., 2006). However, P1 starts to have an

1 interfering effect when its self-cleavage activity is disturbed and P1HC remains
2 unprocessed (J.M. Viedma, A.V, J.A.G and C. Simon-Mateo, unpublished results). The
3 low silencing suppression activity of P1a-P1b does not lead to enough protein
4 accumulation to allow for a confident assessment of the self-cleavage activity of P1a in
5 the agroinfiltration assay. Thus, P1-P1b and P1a-P1b were expressed by agroinfiltration
6 together with the strong silencing suppressor P19 from *Tomato bushy stunt virus* (TBSV)
7 and GFP to easily monitor silencing suppression efficiency. Silencing suppression was
8 highly efficient in leaves expressing either P1-P1b or P1a-P1b, together with P19, as
9 revealed by similar strong green fluorescence and high GFP accumulation levels (Fig. 6).
10 In contrast, the levels of accumulation of P1b were more than ten times lower in leaves
11 expressing P1a-P1b than in those expressing P1-P1b. Moreover, in the leaves expressing
12 P1a-P1b, a faint band corresponding to a protein with the expected mobility of the
13 unprocessed product was clearly detected. An equivalent protein could not be detected in
14 the leaves expressing P1-P1b (Fig. 6).
15 This result suggests that CVYV P1a self-cleavage is incomplete, and its efficiency is
16 lower than that of PPV P1, in *N. benthamiana.*
17
18 **A PPV-derived virus expressing P1b instead of HCPro infects peach seedlings**
19 **poorly.**
20 To explore the contribution of RSSs in determining specific host ranges, we tested the
21 ability to infect *Prunus persica* (a natural host for PPV, but not for CVYV) of a PPV-
22 based chimeric virus expressing CVYV P1b, P1P1b-BD. P1P1b-BD was constructed by
23 replacing the HCPro sequence of P1HC-BD, a PPV chimera that infects GF-305 peach

1 seedlings efficiently (Salvador et al., 2008), with that of CVYV P1b (Fig. 1C). Peach
2 seedlings were biolistically inoculated with the chimeric cDNAs, and the progress of
3 infections was monitored under visible and UV illumination. Both P1HC-BD and P1P1b-
4 BD showed a 100% infectivity, however, while typical PPV symptoms of leaf curling
5 and chlorosis started at approximately 12 dpi in apical leaves of peach seedlings
6 inoculated with P1HC-BD, infection symptoms of P1P1b-BD were much milder,
7 consisting in small chlorotic areas, and only could be detected at approximately 21 dpi
8 (Fig. 7A). Moreover, strong and widely distributed green fluorescence was observed in
9 leaves of P1HC-BD-infected plants, whereas leaves of seedlings infected with P1P1b-BD
10 displayed very week fluorescence in small spots (Fig. 7B).
11 Western blot analyses detected noticeable levels of viral CP in the bombarded leaves
12 of almost all the plants inoculated with P1HC-BD, while no, or very low amount of CP
13 was detected in leaves inoculated with P1P1b-BD (Fig. 7C). Similar analyses showed
14 high levels of CP in upper non-inoculated leaves of plants infected with P1HC-BD,
15 contrasting with the very low amount of this protein that could be detected in the upper
16 leaves of plants infected with P1P1b-BD (Fig. 7C).
17
18 **CVYV P1b confers PPV the ability to infect cucumber locally.**
19 To further explore the contribution of RSSs to host specificity, we tested the
20 infectivity of HCPro- and P1b-expressing PPV variants in *Cucumis sativus*, a natural host
21 of CVYV, which has not been reported to be susceptible to PPV infection. Preliminary
22 experiments showed that cucumber leaves were particularly sensitive to the microparticle
23 bombardment method, as leaves were considerably damaged after the bombardment.

1 Therefore, we selected agroinfiltration as the inoculation procedure for the cucumber
2 experiments because it caused less damage to the inoculated leaf, and it is expected to
3 maintain local expression for longer periods. The full-length cDNA sequences of P1HC,
4 P1P1b, P1b and P1aP1b were engineered in pBin19-derived plasmids. *A. tumefaciens*
5 strains transformed with these plasmids were used to inoculate *C. sativus* and *N.*
6 *benthamiana* plants. The infection process was monitored by careful inspection of green
7 fluorescence under UV illumination. Cucumber leaves inoculated with either P1HC or
8 the empty vector displayed a similar yellow fluorescence as consequence of the leaf
9 damage caused by the infiltration procedure (Fig. 8A). However, although GFP
10 fluorescence could not be detected in P1HC-inoculated cucumber leaves, a strong GFP
11 expression was observed in *N. benthamiana* leaves inoculated with the same
12 *Agrobacterium* culture (Fig. 8A). In contrast, distinct green fluorescent foci were detected
13 around the damaged tissue displaying yellow fluorescence in all cucumber leaves
14 inoculated with the three PPV-derived chimeras expressing CVYV P1b (Fig. 8A).
15 Interestingly, the green fluorescence foci were quite similar in cucumber leaves
16 inoculated with either P1b, P1P1b or P1aP1b, contrasting with the much lower intensity
17 of the GFP signal of P1aP1b compared with P1P1b and P1b in *N. benthamiana* leaves
18 (Fig. 8A).

19 Western blot analyses of infiltrated leaves confirmed the results of GFP monitoring.
20 Hence, *N. benthamiana* leaves inoculated with P1HC accumulated PPV CP at high
21 levels, whereas this protein was either not or hardly detected in leaves of cucumber
22 inoculated with this virus (Fig. 8B). In contrast, PPV CP was detected in cucumber leaves
23 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 while 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 neither in the inoculated leaves outside the infiltration area, nor in upper non-inoculated 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 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 the RSS coding sequence conditions the phenotype of the P1b-expressing PPV chimeras.

Diverse engineered viruses lacking their RSSs have been constructed and well characterized, as the tombusviruses *Cymbidium ringspot virus* and TBSV lacking P19

(Qiu et al., 2002; Szittya et al., 2002; Omarov et al., 2006) and 2b-deletion mutants of the cucumovirus CMV (Diaz-Pendon et al., 2007; Ziebell et al., 2007). These mutants were able to initiate the infection process and reach upper non-inoculated 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 potyviruses 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, iv) the possibility that other viral factor(s) could provide some antiviral activity that partially compensates the absence of the main RSS in some viral systems 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 potyviruses, 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 (Anandalakshmi et

1 al., 2000; Guo et al., 2003; Jin et al., 2007a; Jin et al., 2007b; Cheng et al., 2008; Endres
2 et al., 2010; Ala-Poikela et al., 2011; Dielen et al., 2011). Our results also demonstrate
3 that, in spite of the lack of sequence similarity, CVYV P1b is able to supply all essential
4 functions of HCPro. However, the lower accumulation of P1P1b compared to P1HC
5 (wild type PPV) in *Nicotiana* species (Figs. 2 and 3) appears not to be due to an
6 unspecific weakness of the silencing suppression activity of CVYV P1b, since P1P1b
7 suppresses silencing even more strongly than P1HC in agroinfiltration assays (Fig. 5).
8 Thus, it is likely that either some specific coupling of the RNA silencing suppression of
9 HCPro with other viral processes, such as viral replication, cannot be exactly mimicked
10 by the CVYV P1b anti-silencing activity, or a silencing suppression-unrelated activity of
11 HCPro cannot be fully supplied by the heterologous protein. It is also interesting to
12 remark that, in spite of the lower virus accumulation, some disease symptoms are more
13 severe in plants infected with P1b-expressing viruses than in those infected with the wild
14 type virus (Fig. 2). A number of RSSs have been shown to be targets for plant defence
15 responses other than RNA silencing. For instance, HCPro of *Potato virus Y* (Moury et al.,
16 2011) and other RSSs, such as P38 of *Turnip crinkle virus* (Ren et al., 2000) and P126 of
17 *Tobacco mosaic virus* (Padgett et al., 1997) are elicitors of hypersensitive response (HR)
18 and viral resistance mediated by R genes, and P6 of *Cauliflower mosaic virus* (Kiraly et
19 al., 1999), 2b of *Tomato aspermy virus* (Li et al., 1999) and P19 of TBSV (Chu et al.,
20 2000) induce HR-like necrotic symptoms. Thus, a defensive reaction of the plants elicited
21 by P1b may be the cause of the strengthened symptoms caused by PPV P1P1b (rather
22 than a direct effect of virus multiplication), and could be one of the factors accounting for
23 the lower accumulation of the P1b-expressing chimera compared to wild type PPV.

1 The P1 protein has been shown to be an accessory factor that facilitates the genome
2 amplification of the potyvirus *Tobacco etch virus* (Verchot and Carrington, 1995). Some
3 evidence suggest that P1 could enhance the RNA silencing suppression activity of HCPro
4 (Pruss et al., 1997; Kasschau and Carrington, 1998; Rajamaki et al., 2005; Valli et al.,
5 2006). P1 is also a non-essential factor in PPV infection, but deletion of the P1 gene
6 notably debilitates PPV (unpublished results), as deletion of P1 in the chimera P1P1b
7 causes a drop in virus accumulation and movement (Fig. 3). P1 did not enhance the
8 silencing suppression activity of P1b in the agroinfiltration system; however, P1 deletion
9 facilitated plant recovery at late stages of infection (Fig. 3B), suggesting that P1 could be
10 contributing to efficient RNA silencing suppression. Surprisingly, CVYV P1a not only
11 does not reproduce the positive contribution of the natural P1 to PPV infection, but it is
12 clearly deleterious for the virus in *N. benthamiana*. PPV P1aP1b was able to infect *N.*
13 *benthamiana*, but much less efficiently than the P1-deficient PPV P1b virus, and the
14 P1aP1b-infected plant is cleared of virus a few weeks after inoculation (Fig. 3). Similarly,
15 PPV P1aHC also infects very mildly *N. benthamiana* (Supplementary Fig. 1). In contrast
16 with PPV P1, upstream CVYV P1a sequences drastically disturbed the RNA silencing
17 suppression activity of P1b (Fig. 5) and HCPro (Supplementary Fig. 1), and this
18 disturbance is likely the cause of the detrimental effect of P1a in PPV infection. Although
19 we do not know how P1a interferes with the silencing suppression activity of P1b,
20 transient expression experiments provided valuable clues. Agroinfiltration of bacteria
21 expressing P1a-P1b yielded much lower P1b protein levels than that expressing P1-P1b,
22 even though the strong RSS P19 was coexpressed to ensure P1b-independent silencing
23 suppression activity (Fig. 6), and unprocessed P1a-P1b product, but not P1-P1b, was

1 detected in the infiltrated leaves (Fig. 6). Although low P1b accumulation and inefficient
2 P1a self-cleavage might be unrelated, the previous observation that P1 self-cleavage
3 depends on plant proteins (Verchot et al., 1992) suggests an scenario in which proper
4 folding of the precursor polyprotein, assisted by specific host factors, results in
5 processing, whereas the incorrectly folded precursor is degraded. This would explain
6 why, in contrast with its detrimental effect in the *N. benthamiana* infection, P1a enhances
7 PPV amplification in cucumber (Fig. 8). The inefficiency of the agroinfiltration system in
8 cucumber has prevented us to test this hypothesis, but the fact that P1a-P1b polyprotein
9 precursor was not detected in *C. sativus* plants infected with either P1aP1b chimera (data
10 not shown), or wild type CVYV (Valli et al., 2008), supports this idea. In any case, these
11 results are in agreement with the important role of P1 proteins in host specificity of
12 potyviruses that has been previously suggested (Noa-Carrazana et al., 2006; Valli et al.,
13 2007; Salvador et al., 2008).

14 Previous reports have revealed the possibility that specific HCPro activities contribute
15 to virus adaptation to particular hosts (Sáenz et al., 2002). We show now that whereas
16 P1b-expressing PPV replicates efficiently in *N. benthamiana*, it infects very poorly the
17 natural PPV host *P. persica* (Fig. 7), suggesting that the function of P1b could be also
18 host-specific. In agreement with this assumption, expression of CVYV P1b enhanced
19 local PPV replication in leaves of *C. sativus* (Fig. 8). The suppression activity of HCPro
20 and P1b is thought to be mediated by binding to siRNAs, which are expected to be
21 similar in different plant species. Therefore, if the different behaviour of HCPro-
22 expressing and P1b-expressing PPV in different hosts is the consequence of host-specific
23 features of the RNA silencing suppression activities of HCPro and P1b, it would be

1 necessary to hypothesize that siRNA binding per se is not enough to suppress antiviral
2 RNA silencing, and host factors contribute to establish an effective silencing suppression
3 activity. This hypothesis needs to be tested in silencing suppression experimental
4 systems, which are unfortunately still not available for *P. persica* and *C. sativus*, and,
5 thus, we cannot rule out the possibility that RNA silencing-unrelated funcions of HCPro
6 and P1b could be involved in the host specificity shown by the different PPV variants.
7 Finally, P1aP1b PPV chimera only forms small foci inside the inoculated area of
8 cucumber leaves (Fig. 8), despite expressing P1a and P1b proteins from the cucumber-
9 infecting CVYV. This observation reveals the existence of specific virus genetic factors
10 required for cucumber infection outside the P1a and P1b coding sequences, in agreement
11 with earlier reports showing that host range determinants are extensively spread
12 throughout the PPV genome (Salvador et al., 2008). Plant-virus interactions affected by
13 these specific determinants are largely unknown and should be subject of further
14 research.
15
16 **MATERIALS AND METHODS**
17 **Plant hosts**.
18 Agroinfiltration assays were performed in *N. benthamiana* plants. Viral infectivity
19 assays were performed in *N. benthamiana*, *N. clevelandii*, *C. sativus* Albatroz RZ F1 and
20 *P. persica* GF305 plants. Plants were grown in a greenhouse maintained at 16 hours light
21 with supplementary illumination and 19-23 ºC
22 **Plasmids.**

1 A partial PPV clone (p35SeNOSB) carrying the cDNA corresponding to the 5' region
2 of the genome of the PPV-R isolate (nucleotides 1-3628 that correspond to the 5' UTR
3 and P1, HCPro, P3 and 6K1 cistrons) cloned between the CaMV 35S promoter and the
4 NOS terminator (López-Moya and García, 2000), in which the first AUG of the large
5 ORF was mutated and the second AUG was engineered to display an *Nco*I restriction site
6 (Simón-Buela et al., 1997), was used as backbone to generate intermediate cDNA clones
7 coding for different mutated PPV 5' genomic regions (p35S-P1ΔHC, p35S-P1P1b, p35S-
8 P1aP1b and p35S-P1b).

9 The gene splicing via overlap extension method (Horton et al., 1989) was used to
10 generate p35S-P1ΔHC and p35S-P1P1b. Primers and templates for PCRs used to
11 construct these clones are listed in Table S1 and S2 in the supplemental material. Hence,
12 p35S-P1ΔHC was obtained by replacing the *Nco*I-*Dra*III fragment of p35SeNOSB that
13 encodes the N-terminal region of the PPV polyprotein, with the corresponding fragment
14 from PCR3, which codes for PPV P1-P3 and lacks the HCPro coding sequence. p35S-
15 P1P1b was obtained by inserting a *Nco*I-*BamH*I fragment from PCR6 plus a *BamH*I-
16 *Dra*III fragment from PCR9, which together code for PPV P1-CVYV P1b-PPV P3, in
17 p35SeNOSB digested with *Nco*I and *Dra*III. p35S-P1P1b clones carrying RK68,69AA,
18 C89A or C93A mutant versions of CVYV P1b were obtained by replacing the *Nco*I-
19 *BamH*I fragment from p35S-P1P1b that codes for P1-P1b, with the corresponding
20 fragments from PCR11, PCR13 and PCR15, respectively. The CVYV P1a-P1b-coding
21 sequence lacking its internal *Nco*I site was obtained by using the mutagenesis method of
22 Herlitze and Koenen (Herlitze and Koenen, 1990). The resulting PCR product (PCR18),
23 digested with *Nco*I and *BamH*I, was used to substitute for the corresponding fragment of

1 p35S-P1P1b, yielding p35S-P1aP1b. p35S-P1b was obtained by replacing the *Nco*I-
2 *SexA*I fragment from p35SeNOSB with the corresponding fragment from PCR19, which
3 codes for CVYV P1b. Since *Sex*I cleaves into the PPV HCPro-coding sequence 9 nt away
4 from its 3' end, the CVYV P1b protein encoded by p35S-P1b is fused to the last 3 aa of
5 PPV HCPro.

6 P1ΔHC, P1P1b, P1b and P1aP1b full-length clones were obtained by substituting the
7 35S-P1ΔHC, p35S-P1P1b, p35S-P1b and p35S-P1aP1b *Xba*I-*Dra*III fragments that code
8 for the 5' region of the viral genome preceded by the CaMV 35S promoter, for the
9 corresponding fragment of pIC-PPV-NK-GFP (Fernandez-Fernandez et al., 2001) (Fig.
10 1).

11 The gene splicing via overlap extension method (Horton et al., 1989) was also used to
12 generate P1aHC full-length clone. Primers and templates for PCRs used to construct this
13 clone are listed in Table S1 and S2 in the supplemental material. It was obtained by
14 replacing the *Bsu36*I-*Dra*III fragment of P1aP1b infectious clone with the corresponding
15 fragment from PCR27, which codes for CVYV P1a and HCPro-P3 from PPV.

16 A partial PPV clone (p35S5'DNOS) carrying the cDNA corresponding to the 5'
17 region of the genome of the PPV-D isolate (nt 1-2923 that include the 5' UTR and P1,
18 HCPro and P3 cistrons) cloned between the CaMV 35S promoter and the NOS terminator
19 (Salvador, 2008) was used as backbone to generate an intermediate cDNA clone coding
20 for a chimeric PPV-CVYV 5' genomic region (p35S-P1DP1b). This chimeric clone was
21 constructed by using the gene splicing via overlap extension method (Horton et al.,
22 1989), with the PCR primers and templates listed in Table S1 and S2 in the supplemental
23 material. p35S-P1DP1b was obtained by inserting a *Cpo*I-*Vsp*I fragment from PCR21

1 plus a *Vsp*I-*Pst*I fragment from PCR24, which together code for PPV-D P1-CVYV P1b-
2 PPV-D P3, in p35S5'DNOS digested with *Cpo*I and *Pst*I. The P1P1b-BD full-length
3 clone (Fig. 1) was constructed by a triple ligation of the *Bpu*1102I-*Pst*I fragment that
4 encodes P1-P1b from p35S-P1DP1b, and *Sac*I-*Bpu*1102I and *Pst*I-*Sac*I fragments from
5 pICPPV-5'BD GFP (Salvador et al., 2008).

6 pBINPPV-NK-GFP, which contains a full-length cDNA copy of the PPV genome
7 cloned in pBin19 (Bevan, 1984) under the control of the CaMV 35S promoter (Lucini,
8 2004), was used as parental plasmid to generate infectious binary vectors for the different
9 recombinant viruses. These clones were constructed by replacing the *Sca*I-*Xho*I fragment
10 from pBINPPV-NK-GFP that includes the nt 1 to 6770 sequence of PPV preceded by the
11 CaMV 35S promoter, with the corresponding fragments from P1P1b, P1aP1b or P1b.

12 pBIN-P1HC, a pBin19-derivative carrying the cDNA corresponding to the 5' region
13 of the PPV-R genome (Delgadillo, MO, García, JA and Simón-Mateo, C, unpublished
14 results), was the parental plasmid in the construction of pBIN-P1P1b, pBIN-P1aP1b,
15 pBIN-P1b and pBIN-P1aHC, which were used in the agroinfiltration assays. pBIN-
16 P1P1b, pBIN-P1aP1b and pBIN-P1b were obtained by replacing the pBIN-P1HC *Sca*I-
17 *Xba*I fragment that codes for the 5' region of the viral genome preceded by the CaMV
18 35S promoter, with the corresponding fragments from p35S-P1P1b, p35S-P1aP1b or
19 p35S-P1b. pBIN-P1aHC was obtained by replacing the pBIN-P1HC *Xma*I-*SnaB*I
20 fragment that also codes for the 5' region of the viral genome preceded by the CaMV 35S
21 promoter, with the corresponding fragments from P1aHC full-length clone.

22 *A. tumefaciens* C58C1 strain carrying p35S:GFP (Haseloff et al., 1997) plus pCH32
23 (Hamilton et al., 1996), p35S:GF-IR (Schwach et al., 2005) and pBIN61:P19 (Voinnet et

al., 2003) were kindly provided by David 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 green fluorescent protein (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 using excitation and barrier filters at 425/60 nm and 480 nm respectively, and 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, SDS-PAGE 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 (HRP)-conjugated goat anti-rabbit IgG (Jackson) as secondary reagent. The immunostained proteins were visualized by enhanced chemiluminiscence detection with a LifeABlot kit (Euroclone). 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 *SacII*-linearized pGEMT-GF and pGEMT-P, respectively. These plasmids contain the nt 4 to 403 (GF) and 404 to 717 (P) of the GFP gene cloned in pGEM-T.

ACKNOWLEDGMENTS

We thank to Hernán García-Ruiz for critical reading of the manuscript, David Baulcombe for providing GFP and P19 expression vectors, and Rijk Zwaan Iberica (Almería, Spain) for supplying cucumber seeds. We are also grateful to Elvira Dominguez by technical assistance. This work was supported by grants BIO2010-18541from Spanish MICINN, SAL/0185/2006 from Comunidad de Madrid, and KBBE-204429 from European Union. A.V. was a recipient of an I3P fellowship from CSIC-Fondo Social Europeo.

LITERATURE CITED

Ala-Poikela, M., Goytia, E., Haikonen, T., Rajamaki, M.-L., and Valkonen, J.P.T. 2011. Helper component proteinase of genus *Potyvirus* is an interaction partner of

1 translation initiation factors eIF(iso)4E and eIF4E that contains a 4E binding
2 motif. J. Virol. 85:6784-6794.
3 Anandalakshmi, R., Pruss, G.J., Ge, X., Marathe, R., Mallory, A.C., Smith, T.H., and
4 Vance, V.B. 1998. A viral suppressor of gene silencing in plants. Proc. Natl.
5 Acad. Sci. USA 95:13079-13084.
6 Anandalakshmi, R., Marathe, R., Ge, X., Herr Jr., J.M., Mau, C., Mallory, A., Pruss, G.,
7 Bowman, L., and Vance, V.B. 2000. A calmodulin-related protein that suppresses
8 posttranscriptional gene silencing in plants. Science 290:142-144.
9 Berger, P.H., Hunt, A.G., Domier, G.M., Hellman, G.M., Stram, Y., Thornbury, D.W.,
10 and Pirone, T.P. 1989. Expression in transgenic plants of a viral gene product that
11 mediates insect transmission of potyviruses. Proc. Natl. Acad. Sci. USA 86:8402-
12 8406.
13 Bevan, M. 1984. Binary Agrobacterium vectors for plant transformation. Nucleic Acids
14 Res. 12:8711-8721.
15 Brigneti, G., Voinnet, O., Li, W.X., Ji, L.H., Ding, S.W., and Baulcombe, D.C. 1998.
16 Viral pathogenicity determinants are suppressors of transgene silencing in
17 *Nicotiana benthamiana*. EMBO J. 17:6739-6746.
18 Burgyán, J. 2008. Role of silencing suppressor proteins. Methods Mol. Biol. 451:69-79.
19 Cheng, Y.Q., Liu, Z.M., Xu, J., Zhou, T., Wang, M., Chen, Y.T., Li, H.F., and Fan, Z.F.
20 2008. HC-Pro protein of sugar cane mosaic virus interacts specifically with maize
21 ferredoxin-5 in vitro and in planta. J. Gen. Virol. 89:2046-2054.

1 Chu, M., Desvoyes, B., Turina, M., Noad, R., and Scholthof, H.B. 2000. Genetic
2 dissection of tomato bushy stunt virus p19-protein-mediated host-dependent
3 symptom induction and systemic invasion. Virology 266:79-87.
4 Cronin, S., Verchot, J., Haldeman-Cahill, R., Schaad, M.C., and Carrington, J.C. 1995.
5 Long-distance movement factor: A transport function of the potyvirus helper
6 component proteinase. Plant Cell 7:549-559.
7 Diaz-Pendon, J.A., Li, F., Li, W.X., and Ding, S.W. 2007. Suppression of antiviral
8 silencing by cucumber mosaic virus 2b protein in Arabidopsis is associated with
9 drastically reduced accumulation of three classes of viral small interfering RNAs.
10 Plant Cell 19:2053-2063.
11 Dielen, A.S., Sassaki, F.T., Walter, J., Michon, T., Menard, G., Pagny, G., Krause-
12 Sakate, R., Maia Ide, G., Badaoui, S., Le Gall, O., Candresse, T., and German-
13 Retana, S. 2011. The 20S proteasome alpha5 subunit of Arabidopsis thaliana
14 carries an RNase activity and interacts in planta with the lettuce mosaic potyvirus
15 HcPro protein. Mol. Plant Pathol. 12:137-150.
16 Ding, S.W. 2010. RNA-based antiviral immunity. Nat. Rev. Immunol. 10:632-644.
17 Endres, M.W., Gregory, B.D., Gao, Z., Foreman, A.W., Mlotshwa, S., Ge, X., Pruss,
18 G.J., Ecker, J.R., Bowman, L.H., and Vance, V. 2010. Two plant viral suppressors
19 of silencing require the ethylene-inducible host transcription factor RAV2 to
20 block RNA silencing. PLoS Pathog. 6:e1000729.
21 Fellers, J.P., Seifers, D., Ryba-White, M., and Martin, T.J. 2009. The complete genome
22 sequence of Triticum mosaic virus, a new wheat-infecting virus of the High
23 Plains. Arch. Virol. 154:1511-1515.

1 Fernandez-Fernandez, M.R., Mourino, M., Rivera, J., Rodriguez, F., Plana-Duran, J., and
2 Garcia, J.A. 2001. Protection of rabbits against rabbit hemorrhagic disease virus
3 by immunization with the VP60 protein expressed in plants with a potyvirus-
4 based vector. Virology 280:283-291.
5 Gal-On, A., and Raccah, B. 2000. A point mutation in the FRNK motif of the potyvirus
6 helper component-protease gene alters symptom expression in cucurbits and
7 elicits protection against the severe homologous virus. Phytopathology 90:467-
8 473.
9 Garcia-Ruiz, H., Takeda, A., Chapman, E.J., Sullivan, C.M., Fahlgren, N., Brempelis,
10 K.J., and Carrington, J.C. 2010. *Arabidopsis* RNA-dependent RNA polymerases
11 and dicer-like proteins in antiviral defense and small interfering RNA biogenesis
12 during *Turnip mosaic virus* infection. Plant Cell 22:481-496.
13 Giner, A., Lakatos, L., Garcia-Chapa, M., Lopez-Moya, J.J., and Burgyan, J. 2010. Viral
14 Protein Inhibits RISC Activity by Argonaute Binding through Conserved
15 WG/GW Motifs - art. no. e1000996. PLoS Pathog. 6:996-996.
16 Guo, D., Spetz, C., Saarma, M., and Valkonen, J.P. 2003. Two potato proteins, including
17 a novel RING finger protein (HIP1), interact with the potyviral multifunctional
18 protein HCpro. Mol. Plant-Microbe Interact. 16:405-410.
19 Hamilton, C.M., Frary, A., Lewis, C., and Tanksley, S.D. 1996. Stable transfer of intact
20 high molecular weight DNA into plant chromosomes. Proc. Natl. Acad. Sci. USA
21 93:9975-9979.
22 Haseloff, J., Siemering, K.R., Prasher, D.C., and Hodge, S. 1997. Removal of a cryptic
23 intron and subcellular localization of green fluorescent protein are required to

1 mark transgenic *Arabidopsis* plants brightly. Proc. Natl. Acad. Sci. USA 94:2122-
2 2127.
3 Herlitze, S., and Koenen, M. 1990. A general and rapid mutagenesis method using
4 polymerase chain reaction. Gene 91:143-147.
5 Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., and Pease, L.R. 1989. Engineering
6 hybrid genes without the use of restriction enzymes: gene splicing by overlap
7 extension. Gene 77:61-68.
8 Janssen, D., Martin, G., Velasco, L., Gomez, P., Segundo, E., Ruiz, L., and Cuadrado,
9 I.M. 2005. Absence of a coding region for the helper component-proteinase in the
10 genome of cucumber vein yellowing virus, a whitefly-transmitted member of the
11 Potyviridae. Arch. Virol. 150:1439-1447.
12 Jin, Y.S., Ma, D.Y., Dong, J.L., Jin, J.C., Li, D.F., Deng, C.W., and Wang, T. 2007a. HC-
13 Pro protein of *Potato Virus Y* can interact with three *Arabidopsis* 20S proteasome
14 subunits in planta. J. Virol. 81:12881-12888.
15 Jin, Y.S., Ma, D.Y., Dong, J.L., Li, D.F., Deng, C.W., Jin, J.C., and Wang, T. 2007b. The
16 HC-Pro protein of Potato virus Y interacts with NtMinD of tobacco. Mol. Plant-
17 Microbe Interact. 20:1505-1511.
18 Kasschau, K.D., and Carrington, J.C. 1998. A counterdefensive strategy of plant viruses:
19 Suppression of posttranscriptional gene silencing. Cell 95:461-470.
20 Kasschau, K.D., Cronin, S., and Carrington, J.C. 1997. Genome amplification and long-
21 distance movement functions associated with the central domain of tobacco etch
22 potyvirus helper component-proteinase. Virology 228:251-262.

1 Kiraly, L., Cole, A.B., Bourque, J.E., and Schoelz, J.E. 1999. Systemic cell death is
2 elicited by the interaction of a single gene in Nicotiana clevelandii and gene VI of
3 cauliflower mosaic virus. Mol. Plant-Microbe Interact. 12:919-925.
4 Lakatos, L., Csorba, T., Pantaleo, V., Chapman, E.J., Carrington, J.C., Liu, Y.P., Dolja,
5 V.V., Calvino, L.F., López-Moya, J.J., and Burgyán, J. 2006. Small RNA binding
6 is a common strategy to suppress RNA silencing by several viral suppressors.
7 EMBO J. 25:2768-2780.
8 Li, H.W., Lucy, A.P., Guo, H.S., Li, W.X., Ji, L.H., Wong, S.M., and Ding, S.W. 1999.
9 Strong host resistance targeted against a viral suppressor of the plant gene
10 silencing defence mechanism. EMBO J. 18:2683-2691.
11 Li, W.M., Hilf, M.E., Webb, S.E., Baker, C.A., and Adkins, S. 2008. Presence of P1b and
12 absence of HC-Pro in Squash vein yellowing virus suggests a general feature of
13 the genus *Ipomovirus* in the family *Potyviridae*. Virus Res. 135:213-219.
14 López-Moya, J.J., and García, J.A. 2000. Construction of a stable and highly infectious
15 intron-containing cDNA clone of plum pox potyvirus and its use to infect plants
16 by particle bombardment. Virus Res. 68:99-107.
17 López-Moya, J.J., Valli, A., and García, J.A. 2009. Potyviridae. Page http://www.els.net/
18 [DOI: 10.1002/9780470015902.a9780470000755.pub9780470015902] in:
19 Encyclopedia of Life Sciences (ELS)> N. S. Iacobellis, Collmer, A., Hutcheson,
20 S.W., Mansfield, J. W., Morris, C. E., Murillo J., Schaad, N. W., Stead,
21 D.E., Surico, G., and Ullrich, M, eds. Kluwer Academic John Wiley & Sons,
22 Ltds, Dordrecht, The Netherlands.

1 Lucini, C. (2004). Expresión de proteínas heterólogas en plantas por medio del virus de la
2 sharka (PPV). Universidad Politécnica de Madrid, Madrid.
3 Mbanzibwa, D.R., Tian, Y.P., Mukasa, S.B., and Valkonen, J.P.T. 2009. *Cassava brown*
4 *streak virus* (*Potyviridae*) encodes a putative Maf/HAM1 pyrophosphatase
5 implicated in reduction of mutations and a P1 proteinase that suppresses RNA
6 silencing but contains no HC-Pro. J. Virol. 83:6934-6940.
7 Moissiard, G., and Voinnet, O. 2004. Viral suppression of RNA silencing in plants. Mol.
8 Plant Pathol. 5:71-82.
9 Moury, B., Caromel, B., Johansen, E., Simon, V., Chauvin, L., Jacquot, E., Kerlan, C.,
10 and Lefebvre, V. 2011. The helper component proteinase cistron of *Potato virus Y*
11 induces hypersensitivity and resistance in potato genotypes carrying dominant
12 resistance genes on chromosome IV. Mol. Plant-Microbe Interact. 24:787-797.
13 Noa-Carrazana, J.C., González-de-León, D., and Silva-Rosales, L. 2006. Molecular
14 characterization of a severe isolate of papaya ringspot virus in Mexico and its
15 relationship with other isolates. Virus Genes 35:109-117.
16 Omarov, R., Sparks, K., Smith, L., Zindovic, J., and Scholthof, H.B. 2006. Biological
17 relevance of a stable biochemical interaction between the tombusvirus-encoded
18 P19 and short interfering RNAs. J.Virol. 80:3000-3008.
19 Padgett, H.S., Watanabe, Y., and Beachy, R.N. 1997. Identification of the TMV replicase
20 sequence that activates the N gene-mediated hypersensitive response. Mol. Plant-
21 Microbe Interact. 10:709-715.

1 Pruss, G., Ge, X., Shi, X.M., Carrington, J.C., and Vance, V.B. 1997. Plant viral
2 synergism: The potyviral genome encodes a broad-range pathogenicity enhancer
3 that transactivates replication of heterologous viruses. Plant Cell 9:859-868.
4 Qiu, W.P., Park, J.W., and Scholthof, H.B. 2002. Tombusvirus p19-mediated suppression
5 of virus-induced gene silencing is controlled by genetic and dosage features that
6 influence pathogenicity. Mol. Plant-Microbe Interact. 15:269-280.
7 Rajamaki, M.L., Kelloniemi, J., Alminaite, A., Kekarainen, T., Rabenstein, F., and
8 Valkonen, J.P. 2005. A novel insertion site inside the potyvirus P1 cistron allows
9 expression of heterologous proteins and suggests some P1 functions. Virology
10 342:88-101.
11 Ren, T., Qu, F., and Morris, T.J. 2000. *HRT* Gene Function Requires Interaction between
12 a NAC Protein and Viral Capsid Protein to Confer Resistance to Turnip Crinkle
13 Virus. Plant Cell 12:1917-1926.
14 Rojas, M.R., Zerbini, F.M., Allison, R.F., Gilbertson, R.L., and Lucas, W.J. 1997. Capsid
15 protein and helper component proteinase function as potyvirus cell-to-cell
16 movement proteins. Virology 237:283-295.
17 Roth, B.M., Pruss, G.J., and Vance, V.B. 2004. Plant viral suppressors of RNA silencing.
18 Virus Res. 102:97-108.
19 Sáenz, P., Salvador, B., Simón-Mateo, C., Kasschau, K.D., Carrington, J.C., and García,
20 J.A. 2002. Host-specific involvement of the HC protein in the long-distance
21 movement of potyviruses. J. Virol. 76:1922-1931.
22 Sáenz, P., Cervera, M.T., Dallot, S., Quiot, L., Quiot, J.B., Riechmann, J.L., and García,
23 J.A. 2000. Identification of a pathogenicity determinant of *Plum pox virus* in the

1 sequence encoding the C-terminal region of protein P3+6K$_1$. J. Gen. Virol.
2 81:557-566.
3 Salvador, B. (2008). Pathogenicity and host range determinants in plum pox virus
4 infection: A role for P1 protein. Universidad Autónoma de Madrid, Madrid.
5 Salvador, B., Delgadillo, M.O., Saénz, P., García, J.A., and Simón-Mateo, C. 2008.
6 Identification of *Plum pox virus* pathogenicity determinants in herbaceous and
7 woody hosts. Mol. Plant-Microbe Interact. 21:20-29.
8 Schwach, F., Vaistij, F.E., Jones, L., and Baulcombe, D.C. 2005. An RNA-dependent
9 RNA polymerase prevents meristem invasion by Potato Virus X and is required
10 for the activity but tot the production of a systemic silencing signal. Plant Physiol.
11 138:1842-1852.
12 Simón-Buela, L., Guo, H.S., and García, J.A. 1997. Cap-independent leaky scanning as
13 the mechanism of translation initiation of a plant viral genomic RNA. J. Gen.
14 Virol. 78:2691-2699.
15 Soosaar, J.L.M., Burch-Smith, T.M., and Dinesh-Kumar, S.P. 2005. Mechanisms of plant
16 resistance to viruses. Nat. Rev. Microbiol. 3:789-798.
17 Stenger, D.C., Young, B.A., Qu, F., Morris, T.J., and French, R. 2007. Wheat streak
18 mosaic virus Lacking Helper Component-Proteinase Is Competent to Produce
19 Disease Synergism in Double Infections with Maize chlorotic mottle virus.
20 Phytopathology 97:1213-1221.
21 Susaimuthu, J., Tzanetakis, I.E., Gergerich, R.C., and Martin, R.R. 2008. A member of a
22 new genus in the Potyviridae infects Rubus. Virus Res. 131:145-151.

1 Syller, J. 2005. The roles and mechanisms of helper component proteins encoded by
2 potyviruses and caulimoviruses. Physiol. Mol. Plant Pathol. 67:119-130.
3 Szittya, G., Molnar, A., Silhavy, D., Hornyik, C., and Burgyan, J. 2002. Short defective
4 interfering RNAs of tombusviruses are not targeted but trigger post-
5 transcriptional gene silencing against their helper virus. Plant Cell 14:359-372.
6 Tatineni, S., Ziems, A.D., Wegulo, S.N., and French, R. 2009. Triticum mosaic virus: a
7 distinct member of the family potyviridae with an unusually long leader sequence.
8 Phytopathology 99:943-950.
9 Valli, A., López-Moya, J.J., and García, J.A. 2007. Recombination and gene duplication
10 in the evolutionary diversification of P1 proteins in the family *Potyviridae*. J.
11 Gen. Virol. 88:1016-1028.
12 Valli, A., Dujovny, G., and García, J.A. 2008. Protease activity, self interaction, and
13 small interfering RNA binding of the silencing suppressor P1b from *Cucumber*
14 *vein yellowing ipomovirus*. J. Virol. 82:974-986.
15 Valli, A., López-Moya, J.J., and García, J.A. 2009. RNA silencing and its suppressors in
16 the plant-virus interplay. Page http://www.els.net/ [DOI:
17 10.1002/9780470015902.a9780470021261] in: Encyclopedia of Life Sciences
18 (ELS)> N. S. Iacobellis, Collmer, A., Hutcheson, S.W., Mansfield, J. W., Morris,
19 C. E., Murillo J., Schaad, N. W., Stead, D.E., Surico, G., and Ullrich, M, eds.
20 Kluwer Academic John Wiley & Sons, Ltds, Dordrecht, The Netherlands.
21 Valli, A., Martín-Hernández, A.M., López-Moya, J.J., and García, J.A. 2006. RNA
22 silencing suppression by a second copy of the P1 serine protease of *Cucumber*

1 *vein yellowing ipomovirus* (CVYV), a member of the family *Potyviridae* that
2 lacks the cysteine protease HCPro. J. Virol. 80:10055-10063.
3 Valli, A., Oliveros, J.C., Molnar, A., Baulcombe, D., and Garcia, J.A. 2011. The specific
4 binding to 21-nt double-stranded RNAs is crucial for the anti-silencing activity of
5 Cucumber vein yellowing virus P1b and perturbs endogenous small RNA
6 populations. RNA 17:1148-1158.
7 Verchot, J., and Carrington, J.C. 1995. Evidence that the potyvirus P1 proteinase
8 functions in trans as an accessory factor for genome amplification. J. Virol.
9 69:3668-3674.
10 Verchot, J., Herndon, K.L., and Carrington, J.C. 1992. Mutational analysis of the tobacco
11 etch potyviral 35-kDa proteinase: Identification of essential residues and
12 requirements for autoproteolysis. Virology 190:298-306.
13 Voinnet, O., Rivas, S., Mestre, P., and Baulcombe, D. 2003. An enhanced transient
14 expression system in plants based on suppression of gene silencing by the p19
15 protein of tomato bushy stunt virus. Plant J. 33:949-956.
16 Ziebell, H., Payne, T., Berry, J.O., Walsh, J.A., and Carr, J.P. 2007. A cucumber mosaic
17 virus mutant lacking the 2b counter-defence protein gene provides protection
18 against wild-type strains. J. Gen. Virol. 88:2862-2871.
19
20
21

1 **Legend to Figures**

2

3 **Figure 1**. Schematic representation of full-length cDNA clones derived from PPV and
4 CVYV. (A) Genome maps of the PPV R and D isolates and CVYV. (B) Chimeric full-
5 length cDNA clones derived from pICPPV-NK-GFP employed to infect herbaceous
6 hosts. (C) Chimeric cDNA clones derived from pICPPV5'BD-GFP employed to infect *P.*
7 *persica*. The coding sequence of the GFP protein inserted between the NIb and CP
8 cistrons is represented with a green rectangle.

9

10 **Figure 2**. A chimeric PPV in which HCPro was replaced by CVYV P1b (P1P1b) is able
11 to infect *N. clevelandii* and *N. benthamiana* plants. Top panels: symptoms and GFP
12 expression of plants infected with the P1P1b chimera or with the wild type PPV (P1HC).
13 Pictures of detached *N. clevelandii* upper non-inoculated leaves (A) and whole *N.*
14 *benthamiana* plants (B) taken under visible (upper row) or UV illumination with a hand
15 lamp (lower row) at 21 dpi. Bottom panels: Western blot analysis of systemically infected
16 leaves from two plants collected at 21 dpi. Polyclonal sera specific for CP and HCPro of
17 PPV, and CVYV P1b were used for the immunodetections. The membrane stained with
18 Ponceau red showing the Rubisco is included as a loading control.

19

20 **Figure 3**. Infection of PPV chimeric viruses expressing CVYV P1b in *N. benthamiana*.
21 (A) Patterns of infection at 21 dpi. GFP fluorescence pictures taken under a UV hand
22 lamp of whole infected plants, or under a epifluorescence microscope of the second (+2)
23 and third (+3) leaves above the inoculated one, and the most apical leaves are shown in

1 the left panels. A Western blot analysis of pools of tissue showing GFP expression of
2 systemically infected leaves (+2 and +3) from 2 plants is shown in the left panels. (B)
3 Patterns of infection at 36 dpi. Pictures showing GFP fluorescence under a UV hand lamp
4 of whole infected plants are shown in the top panels. A Western blot analysis of pools of
5 tissue showing GFP expression (or the equivalent tissue from plants not showing evident
6 green fluorescence) from young (+9, +10), intermediate (+7, +8) or old (+5, +6) upper
7 non-inoculated leaves of 2 plants is shown in the bottom panels. A polyclonal serum
8 specific for PPV CP was used for assessment of virus accumulation. Immunoreactions
9 with polyclonal sera specific for PPV HCPro and CVYV P1b confirmed the identity of
10 the infecting viruses. The membranes stained with Ponceau red showing the Rubisco are
11 included as loading controls.
12
13 **Figure 4**. PPV requires an active RSS to infect *N. benthamiana*. (A) Result of visual
14 inspection of *N. benthamiana* plants inoculated with PPV cDNA clones differing in their
15 encoded RSS. RNA silencing suppression activity was estimated from published
16 information (Valli et al., 2006; Valli et al., 2008). (B) Western blot analysis of leaf
17 extracts from pools of either inoculated (7 dpi) or upper (21 dpi) leaves of four *N.*
18 *benthamiana* plants inoculated with the indicated cDNA clones. A polyclonal serum
19 specific for PPV CP was used for the immunodetection. The membranes stained with
20 Ponceau red showing the Rubisco are included as loading controls.
21
22 **Figure 5**. RNA silencing suppression activity of the N-terminal regions of the genomic
23 polyproteins of PPV chimeras containing CVYV P1b. (A) Schematic representation of

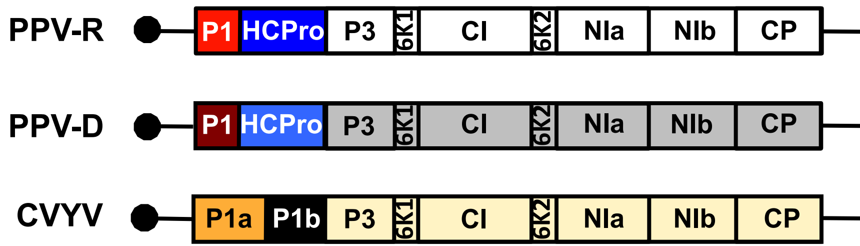
1 the GFP and viral-derived constructs used in the RNA silencing assays. Black arrows
2 indicated the auto-proteolitic processes mediated by P1 and HCPro from PPV, and P1a
3 and P1b from CVYV to produce the corresponding free proteins. (B) ssRNA-triggered
4 silencing assay. (C) dsRNA-triggered silencing assay. In both (B) and (C), left panels
5 show GFP fluorescence pictures of leaves from two independent plants taken under an
6 epifluorescence microscope at 6 dpi, and right panels show Northern blot analyses of
7 GFP mRNA and siRNA extracted at 6 dpi from leaf patches of two plants infiltrated with
8 agrobacteria carrying the plasmid indicated above each lane. Two different probes were
9 used for detection of GFP siRNAs: the GF probe (for primary plus secondary siRNAs)
10 corresponds to the GFP fragment included in the IR RNA encoded by the silencing
11 trigger plasmid p35S:GF-IR, and the P probe (specific for secondary siRNAs)
12 corresponds to the 3' terminal region of the GFP gene, which is not included in p35S:GF-
13 IR. EtBr-stained rRNA and 5S+tRNA are shown as loading controls for the blots of
14 mRNAs and siRNAs, respectively.

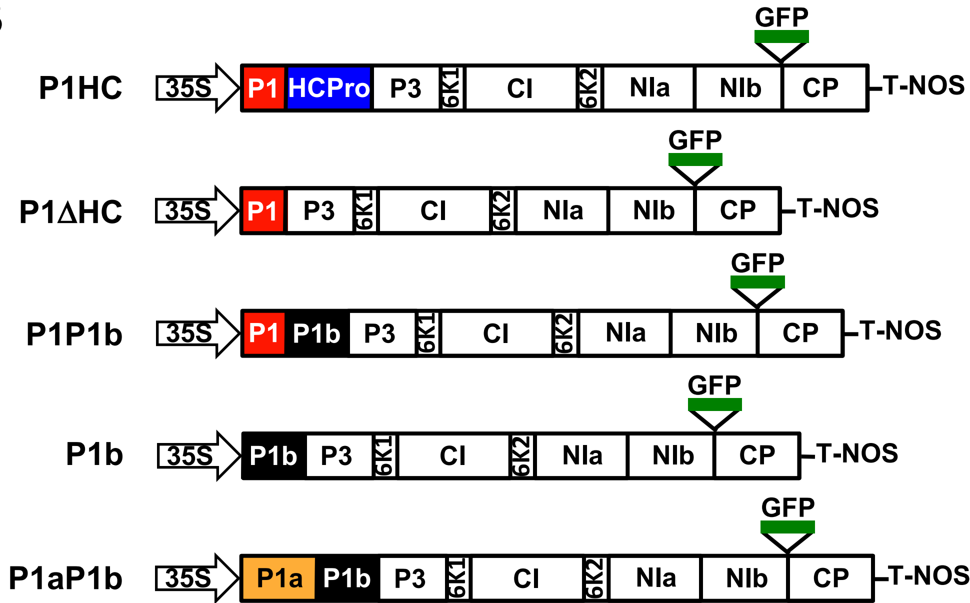
15

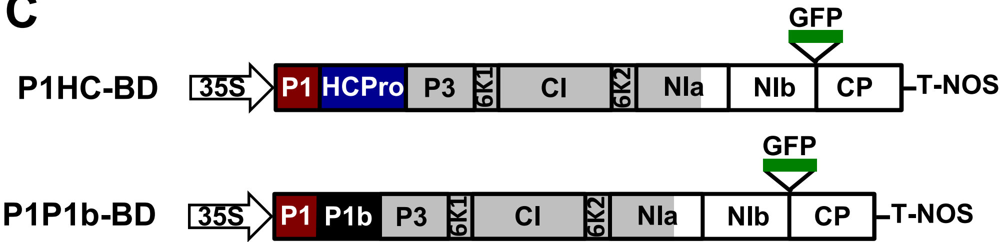
16 **Figure 6.** Analysis of the accumulation of self-processed P1b products and unprocessed
17 P1P1b or P1aP1b precursors in *N. benthamiana* leaves expressing P1P1b or P1aP1b
18 respectively. Leaves were agroinfiltrated with *A. tumefaciens* expressing GFP, TBSV P19
19 and either P1P1b or P1aP1b (see Fig. 5A). Leaves infiltrated with *A. tumefaciens*
20 expressing GFP and empty pBIN-19 (vector) were used as a control. Upper panels show
21 GFP fluorescence pictures of leaves from two independent plants taken under an
22 epifluorescence microscope at 6 dpi. Middle and bottom panels show western blot
23 analyses of *N. benthamiana* plants (two leaves per plant) infiltrated with agrobacteria

1 carrying the plasmids indicated above each lane. Ten times-diluted P1P1b extracts were
2 analysed in the bottom western. Immunoreactions were conducted with the indicated
3 antibodies. The positions of prestained molecular mass markers (New England Biolabs)
4 (in kilodaltons) run in the same gel are indicated to the right of an overexposed anti-P1b
5 immunoreaction. The position of the bands corresponding to unprocessed P1aP1b is
6 indicated by an asterisk. The membrane stained with Ponceau red showing the Rubisco is
7 included as a loading control.
8
9 **Figure 7**. Infection of GF-305 peach seedlings by a PPV-derived virus expressing CVYV
10 P1b. (A) Pictures taken under visible light at 24 dpi showing the symptoms caused by the
11 indicated viruses in GF-305 peach seedlings. Small chlorotic regions in a leaf of the
12 P1P1b-BD-infected plant are highlighted with white arrows. (B) Pictures taken under an
13 epifluorescence microscope at 21 dpi showing virus-derived GFP fluorescence in
14 detached leaves (from position +4 to +8 above the inoculated ones) of GF-305 peach
15 seedlings infected by the indicated viruses. (C) Western blot analysis of inoculated
16 (collected at 17 dpi) and upper non-inoculated (collected at 24 dpi) leaf tissue from two
17 GF-305 peach seedlings infected with the indicated viruses. A polyclonal serum specific
18 for PPV CP was used for assessment of virus accumulation. Immunoreactions with
19 polyclonal sera specific for HCPro of PPV and P1b of CVYV confirmed the identity of
20 the infecting viruses. The membranes stained with Ponceau red showing the Rubisco are
21 included as loading controls.
22

1 **Figure 8**. Accumulation of PPV-derived viruses expressing CVYV P1b in cucumber and
2 *N. benthamiana* leaves. (A) Pictures taken under an epifluorescence microscope at 7 dpi
3 showing virus-derived GFP fluorescence in leaves of *C. sativus* (upper row) or *N.*
4 *benthamiana* (lower row) infiltrated with Agrobacterium strains expressing the indicated
5 viral full-length cDNA clones. Regions with green fluorescence foci in cucumber leaves
6 are indicated with white arrows. (B) Western blot analysis of leaf patches of two plants
7 infiltrated with Agrobacterium strains expressing the indicated viral full-length cDNA
8 clones. Samples were collected at 7 dpi. A polyclonal serum specific for PPV CP was
9 used for assessment of virus accumulation. Immunoreactions with a polyclonal serum
10 specific for CVYV P1b confirmed the identity of the infecting viruses. The membranes
11 stained with Ponceau red showing the Rubisco are included as loading controls.

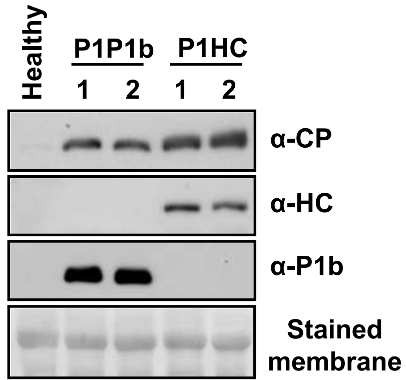

A
PPV-R
P1
HCPro
P3
6K1
CI
6K2
NIa
NIb
CP
PPV-D
CVYV
P1a
P1b


B
GFP
P1HC
35S
T-NOS
P1ΔHC
P1P1b
P1b
P1aP1b

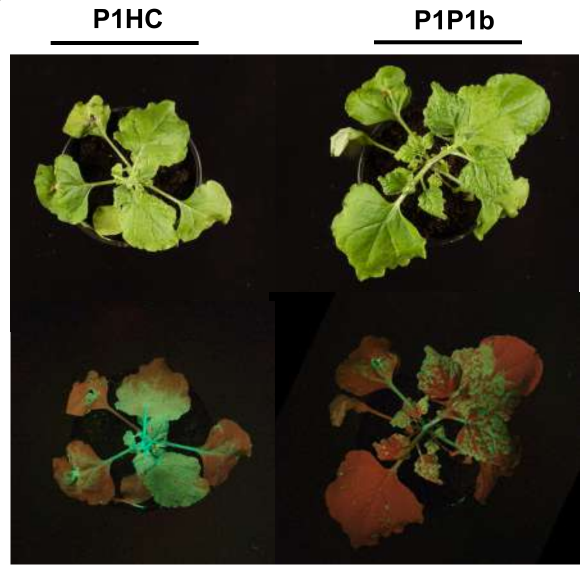

C
GFP
P1HC-BD
35S
T-NOS
P1P1b-BD

A

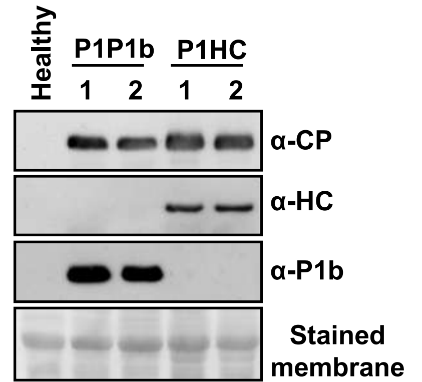
N. clevelandii

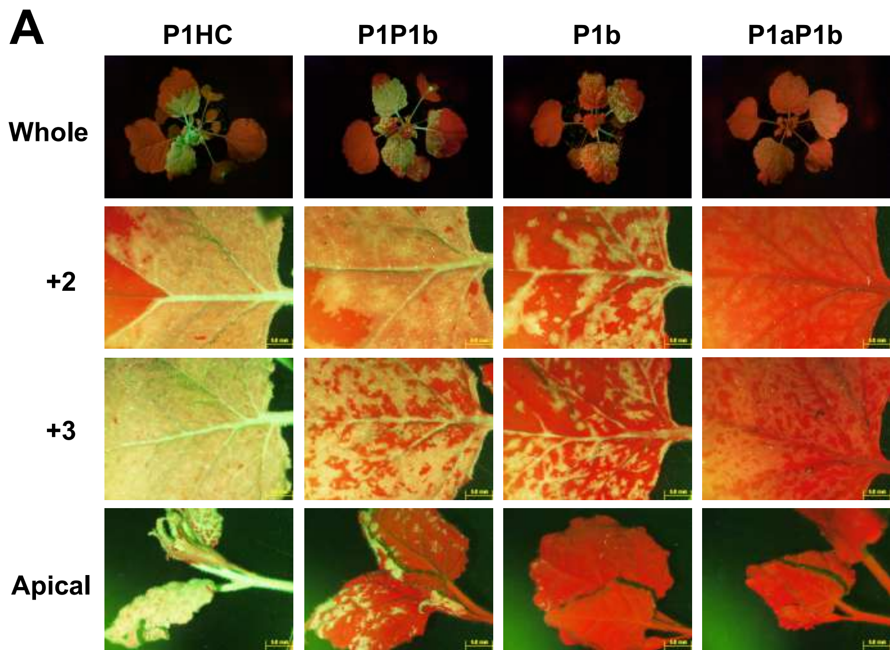


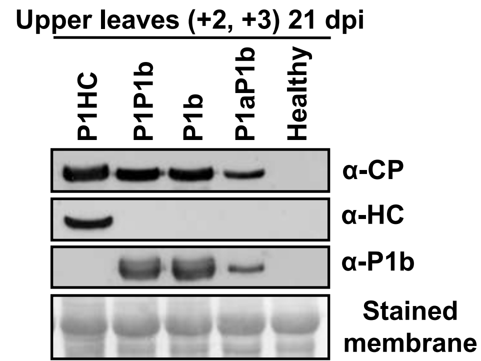
B

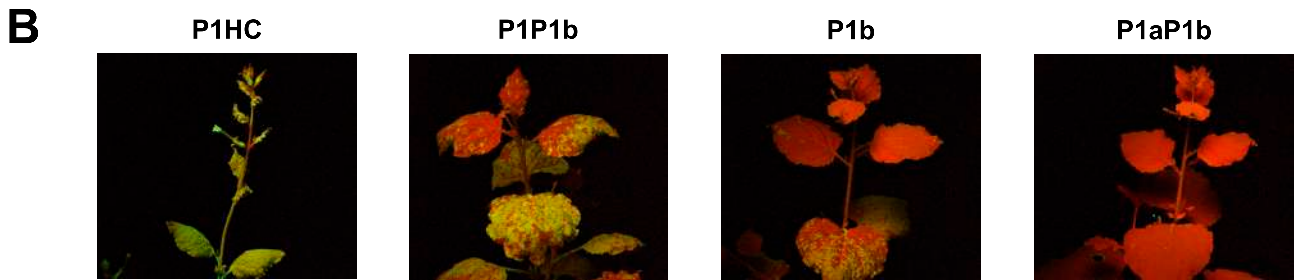


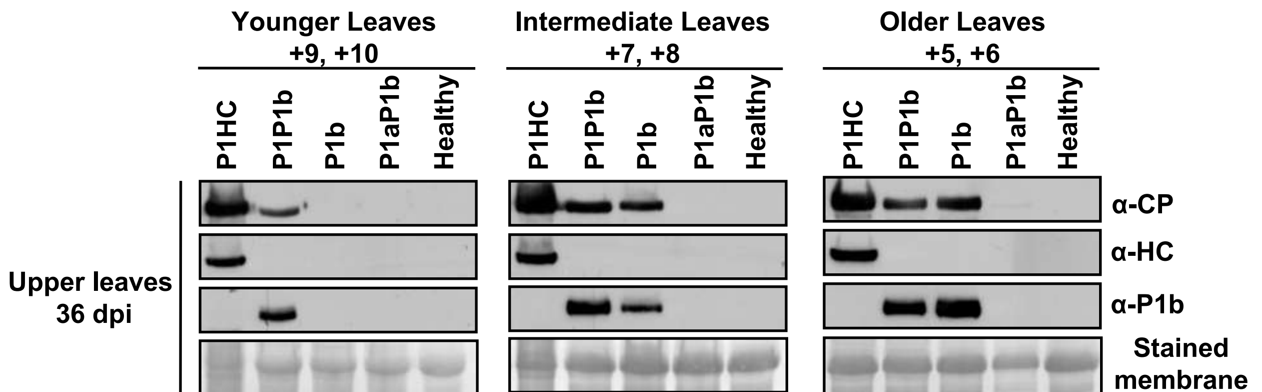
N. benthamiana




A
P1HC
P1P1b
P1b
P1aP1b
Whole
+2
+3
Apical


Upper leaves (+2, +3) 21 dpi
P1HC
P1P1b
P1b
P1aP1b
Healthy
α-CP
α-HC
α-P1b
Stained membrane

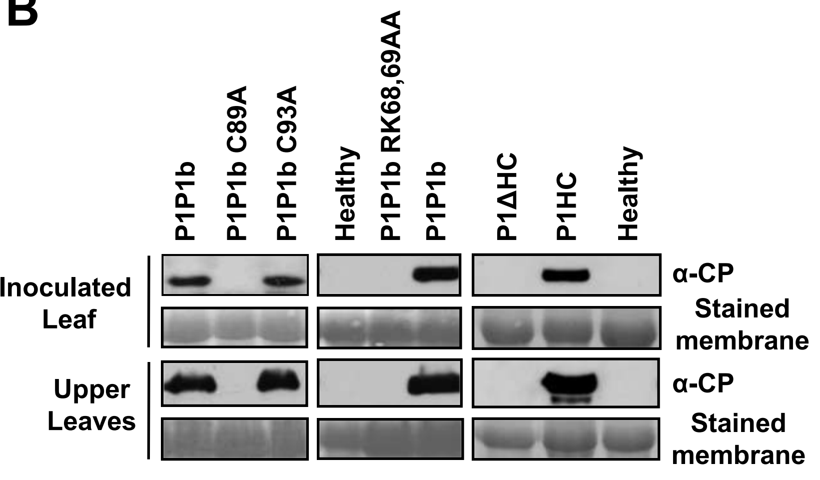

B
P1HC
P1P1b
P1b
P1aP1b

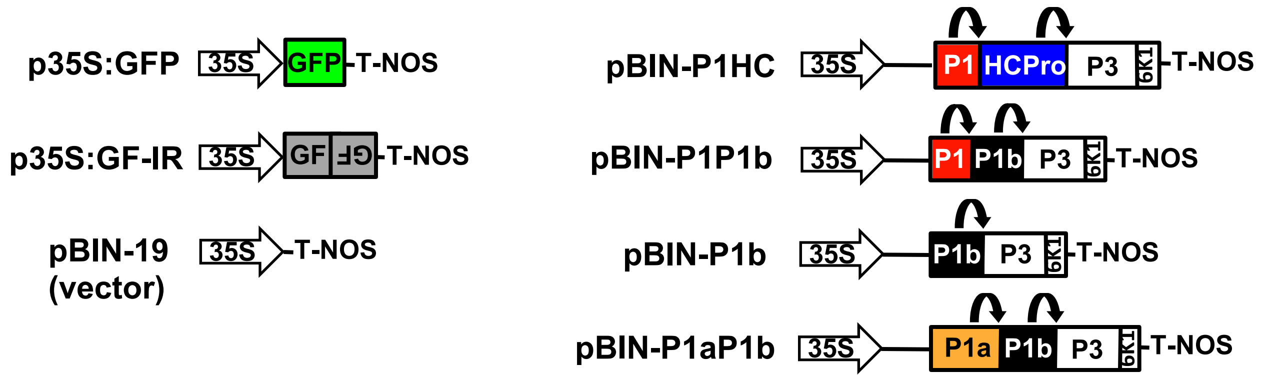

Younger Leaves +9, +10
Intermediate Leaves +7, +8
Older Leaves +5, +6
P1HC
P1P1b
P1b
P1aP1b
Healthy
Upper leaves 36 dpi
α-CP
α-HC
α-P1b
Stained membrane

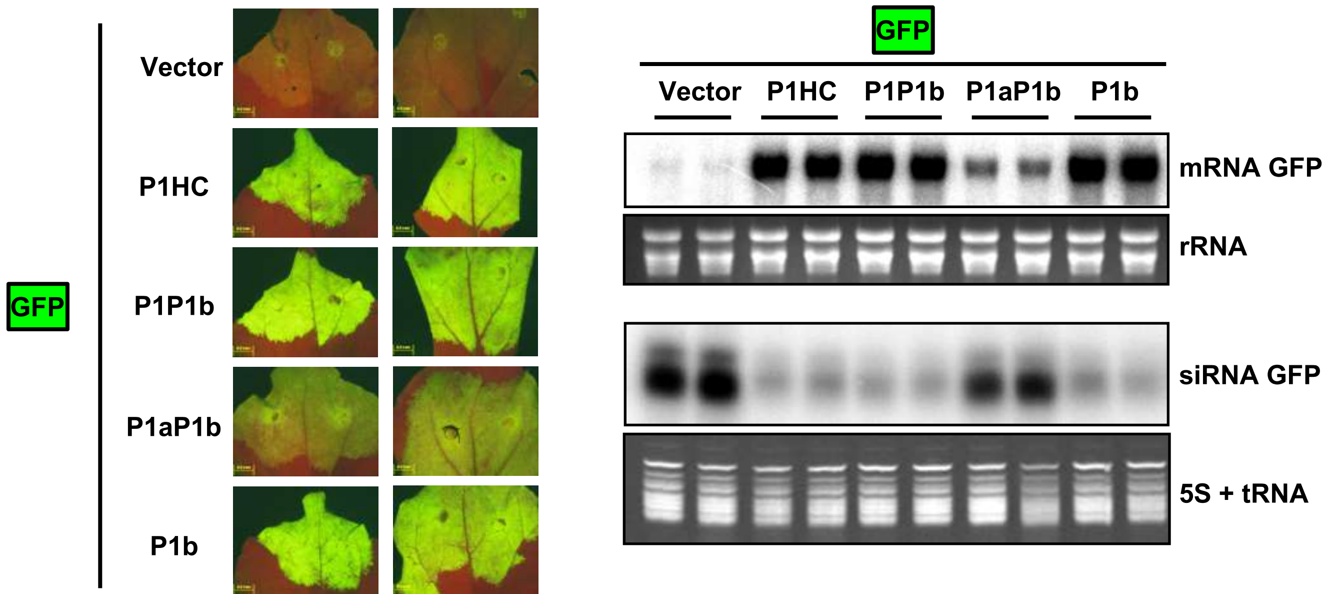
A

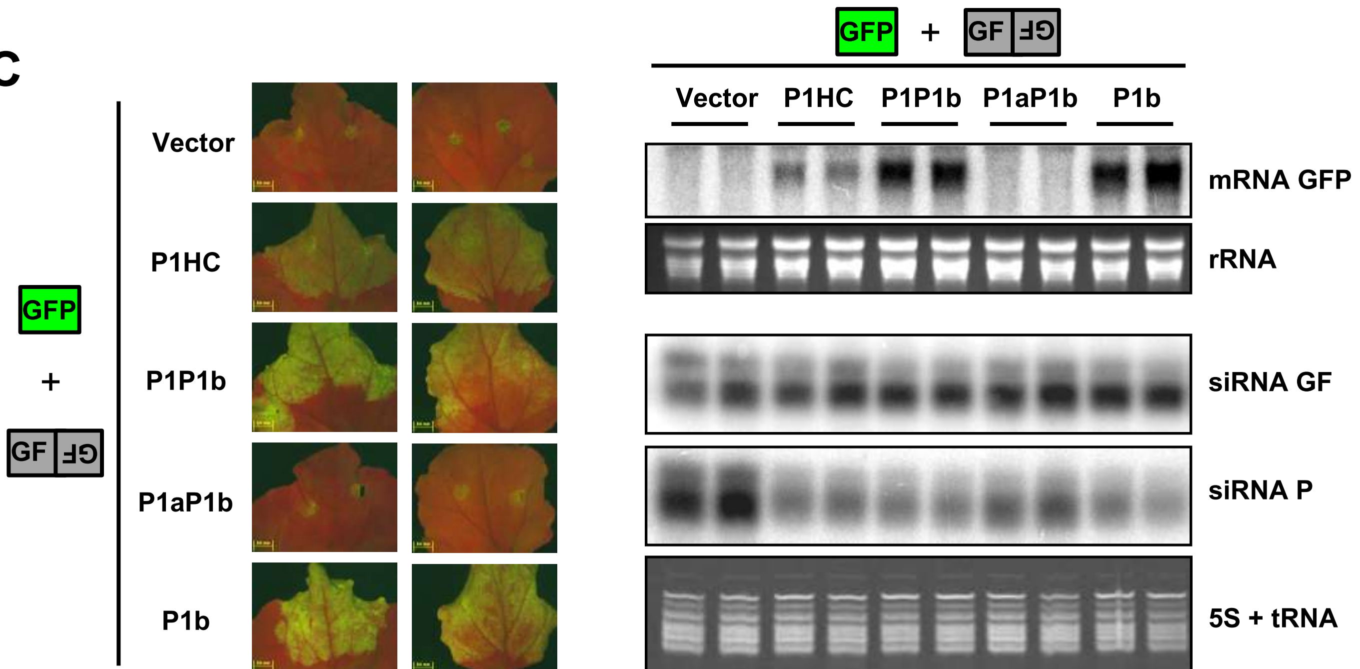
Construct	Suppressor protein	Suppressor Activity	Symptoms and GFP (%)
P1HC	HC-Pro	+	100
P1ΔHC	-	-	0
P1P1b	P1b	+	100
P1P1b RK68,69AA	P1b RK68,69AA	-	0
P1P1b C89A	P1b C89A	-	0
P1P1b C93A	P1b C93A	+	100

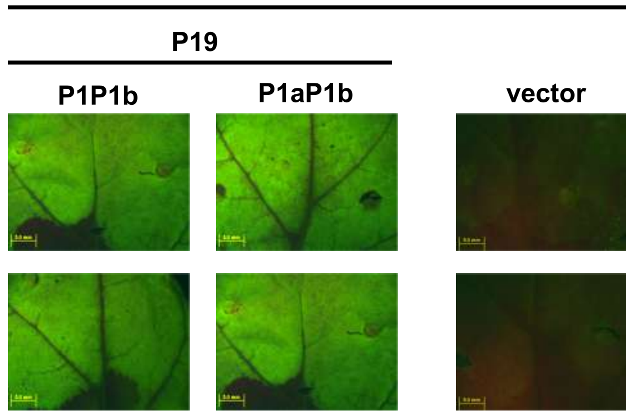
B

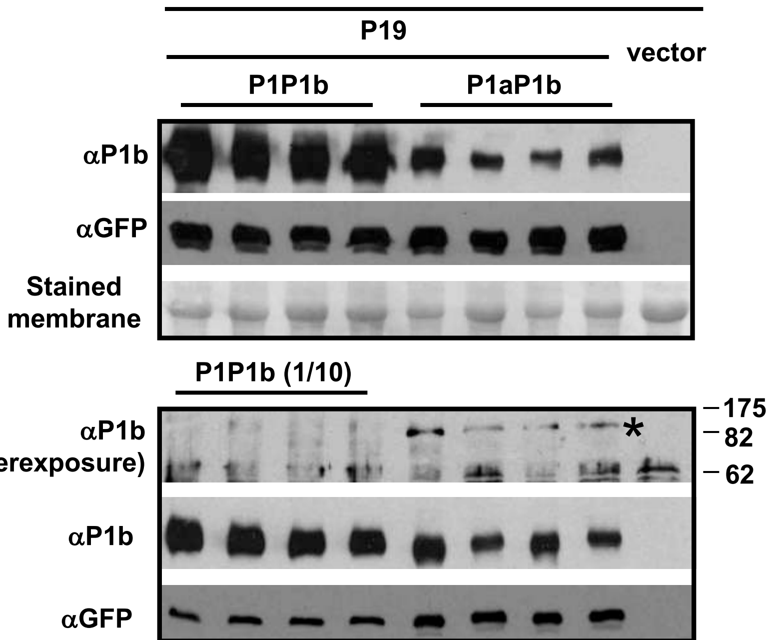



A
p35S:GFP
35S
GFP
T-NOS
p35S:GF-IR
GF
pBIN-19
(vector)
pBIN-P1HC
P1
HCPro
P3
6K1
pBIN-P1P1b
P1b
pBIN-P1b
pBIN-P1aP1b
P1a


B
GFP
Vector
P1HC
P1P1b
P1aP1b
P1b
mRNA GFP
rRNA
siRNA GFP
5S + tRNA

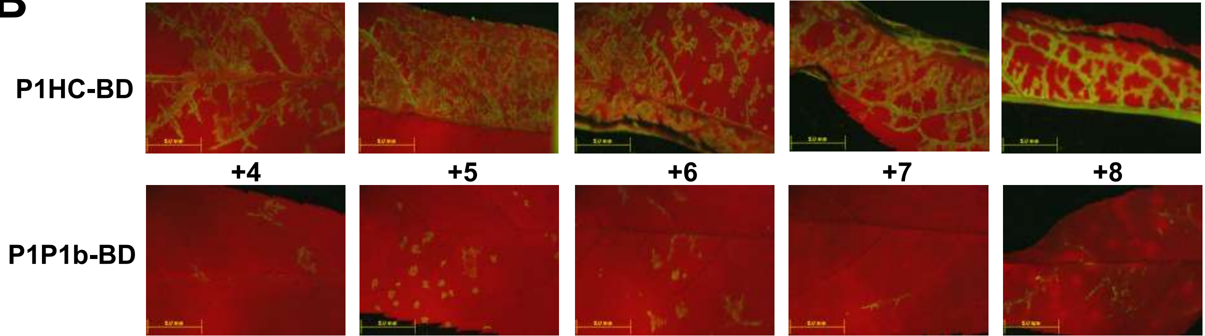

C
GFP
+
GF
Vector
P1HC
P1P1b
P1aP1b
P1b
mRNA GFP
rRNA
siRNA GF
siRNA P
5S + tRNA


GFP
P19
P1P1b
P1aP1b
vector

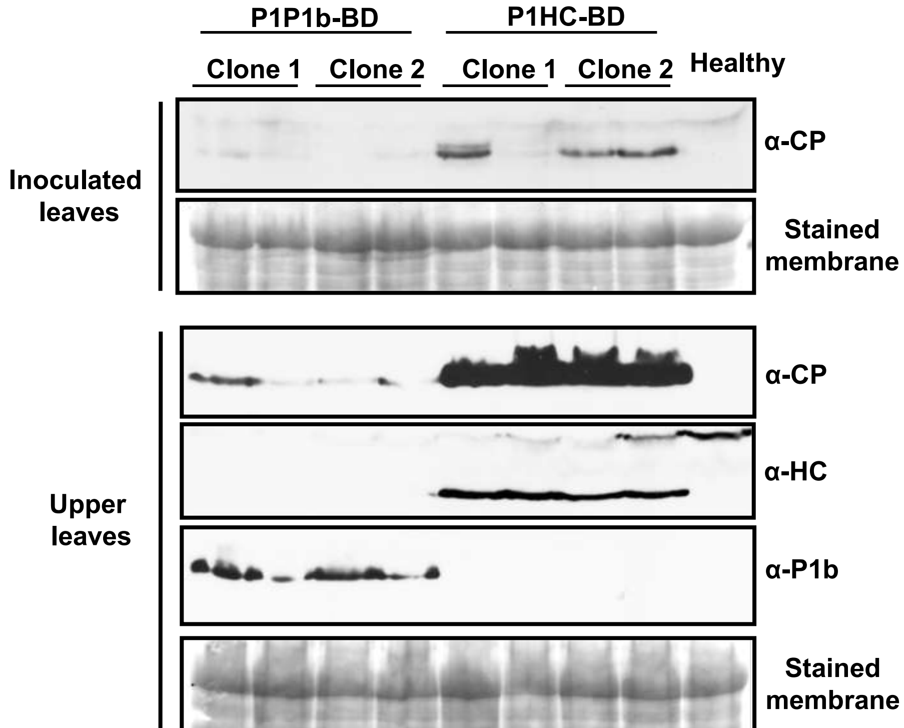

GFP
P19
vector
P1P1b
P1aP1b
αP1b
αGFP
Stained membrane
P1P1b (1/10)
αP1b (overexposure)
*
175
82
62
αP1b
αGFP

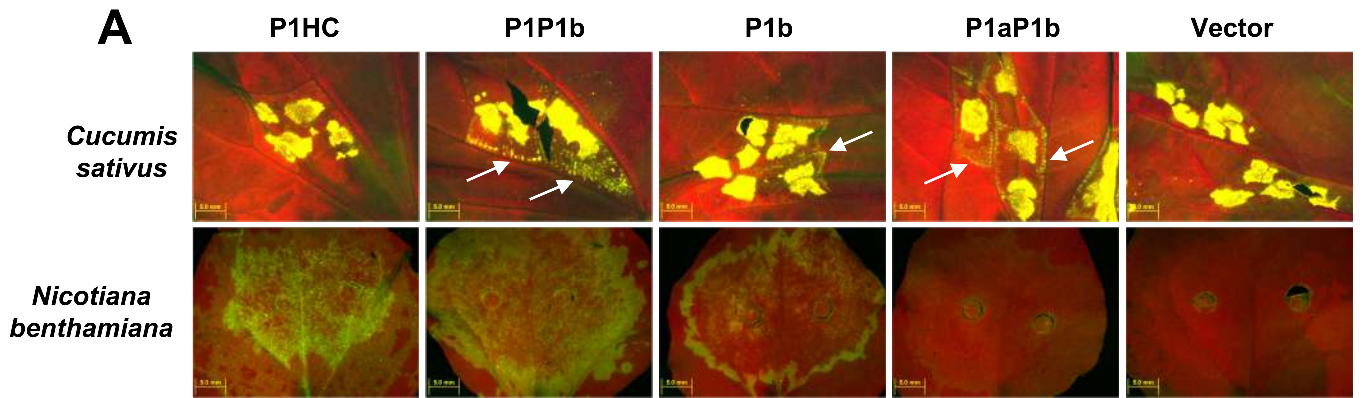
A

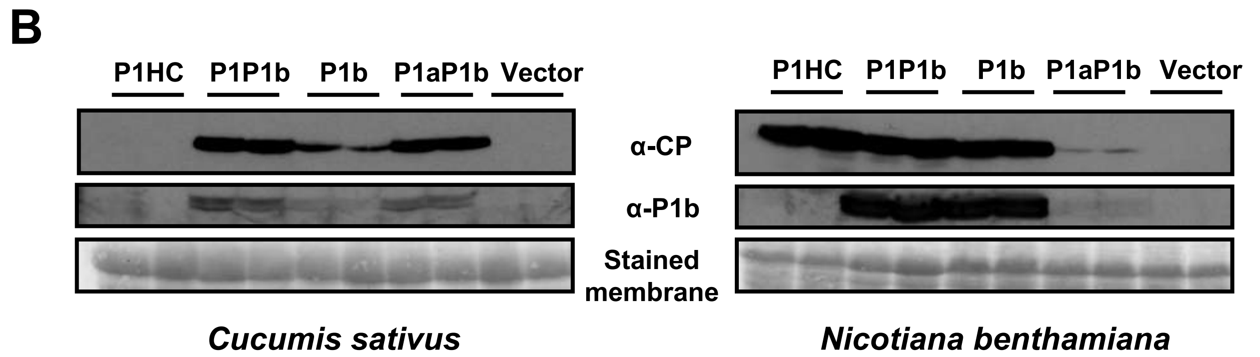
B

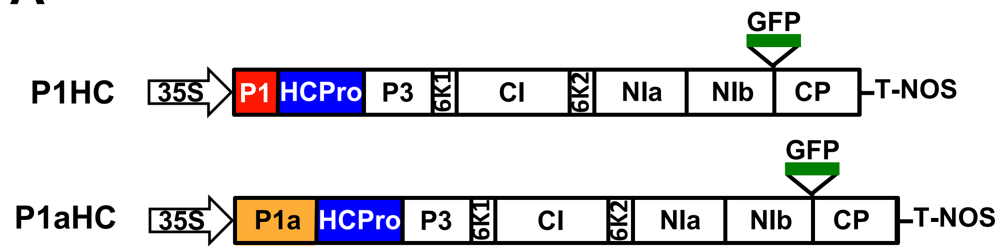


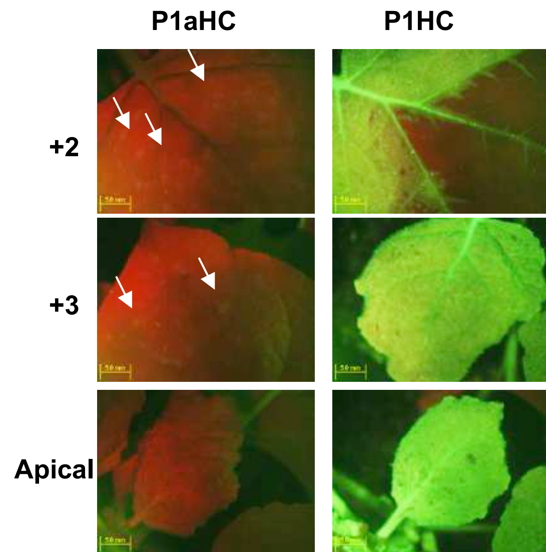
C

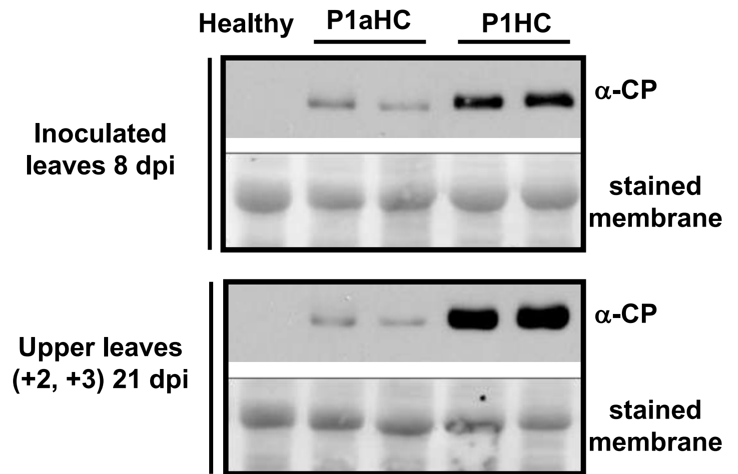


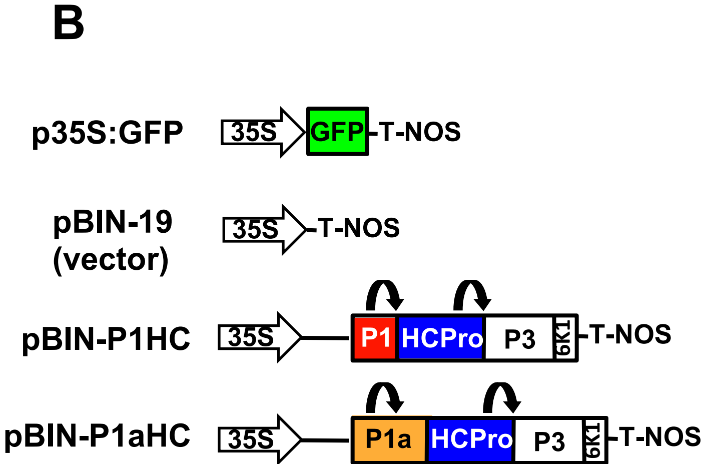

A
P1HC
P1P1b
P1b
P1aP1b
Vector
Cucumis sativus
Nicotiana benthamiana

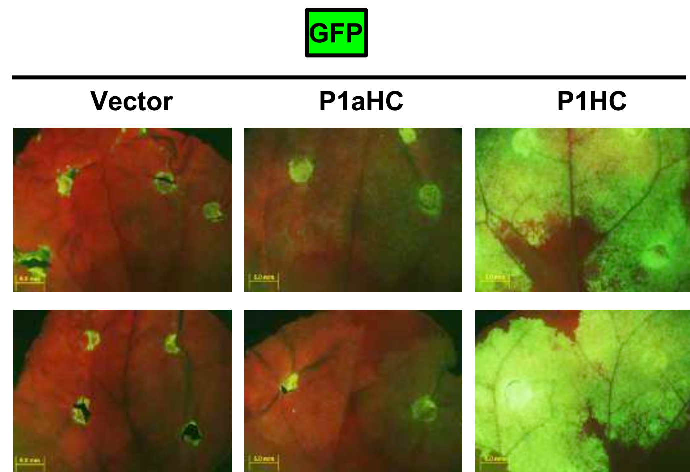

B
P1HC
P1P1b
P1b
P1aP1b
Vector
α-CP
α-P1b
Stained membrane
Cucumis sativus
Nicotiana benthamiana











Supplementary Fig. 1. Infection of the PPV chimeric virus P1aHC expressing CVYV P1a instead of P1, and silencing suppression activity of P1a-HC in *N. benthamiana*. The schematic representation of the full-length cDNA clones used in the infection experiment and the viral-derived constructs used in the RNA silencing assay are shown at the left of panels A and B. (A) Patterns of infection. GFP fluorescence pictures taken at 21 dpi under an epifluorescence microscope of the second (+2) and third (+3) leaves above the inoculated one, and the most apical leaves are shown in the middle panel. Faint fluorescent spots in P1aHC-infected leaves are indicated with white arrows. A Western blot analysis of pools of tissue showing GFP expression of the inoculated and systemically infected (+2 and +3) leaves from 2 plants is shown in the right panel. A polyclonal serum specific for PPV CP was used for assessment of virus accumulation. The membranes stained with Ponceau red showing the Rubisco are included as a loading control. (B) Leaves from two plants infiltrated with *A. tumefaciens* transformed with the indicated plasmids are shown. GFP fluorescence pictures were taken under an epifluorescence microscope at 6 dpi

Supplementary Table S1. List of primers and templates used for PCR reactions.

Plasmids	Forward [a]	Reverse [a]	Template
1- p35S-P1P3			
PCR1	#976	#1074	p35SeNOSB (López-Moya and García, 2000)
PCR2	#1075	#981	p35SeNOSB (López-Moya and García, 2000)
PCR3	#976	#981	PCR1 + PCR2
2- p35S-P1P1b			
PCR4	#976	#977	p35SeNOSB (López-Moya and García, 2000)
PCR5	#978	#979	pDONR-P1bcut (Valli et al., 2008)
PCR6	#976	#979	PCR4 + PCR5
PCR7	#980	#981	p35SeNOSB (López-Moya and García, 2000)
PCR8	#982	#983	pDONR-P1bcut
PCR9	#982	#981	PCR7 + PCR8
3- p35S-P1P1b			
RK68,69AA	#978	#979	pDONR-nonAUGP1b RK68,69AA (Valli et
PCR10	#976	#979	al., 2008)
PCR11			PCR4 + PCR10
4- p35S-P1P1b			
RK68,69AA	#978	#979	pDONR-nonAUGP1b C89A (Valli et al.,
PCR12	#976	#979	2008)
PCR13			PCR4 + PCR12
5- p35S-P1P1b			
RK68,69AA	#978	#979	pDONR-nonAUGP1b C93A (Valli et al.,
PCR14	#976	#979	2008)
PCR15			PCR4 + PCR14

6- p35S-P1aP1b			
PCR16	#984	#985	pUC-P1CVYV (Valli et al., 2006)
PCR17	#986	#987	pUC-P1CVYV (Valli et al., 2006)
PCR18	#984	#987	PCR16 + PCR17
7- p35S-P1b			
PCR19	#835	#979	pDONR-P1bcut (Valli et al., 2008)
8- p35S-			
P1DP1b	#279	#977	p35S'DNOS (Salvador, 2008)
PCR19	#978	#988	pDONR-P1bcut (Valli et al., 2008)
PCR20	#279	#988	PCR19 + PCR20
PCR21	#980	#441	p35S'DNOS (Salvador, 2008)
PCR22	#994	#983	pDONR-P1bcut (Valli et al., 2008)
PCR23	#994	#441	PCR22 + PCR23
PCR24			
9- P1aHC			
PCR25	#986	#1719	P1aP1b
PCR26	#1720	#271	p35SeNOSB (López-Moya and García, 2000)
PCR27	#986	#271	PCR25 + PCR26

[a] The sequences of primers are shown in the Supplementary Table S2.

LITERATURE CITED

López-Moya, J.J., and García, J.A. 2000. Construction of a stable and highly infectious intron-containing cDNA clone of plum pox potyvirus and its use to infect plants by particle bombardment. Virus Res. 68:99-107.

Salvador, B. (2008). Pathogenicity and host range determinants in plum pox virus infection: A role for P1 protein. Facultad de Ciencias (Universidad Autónoma de Madrid).

Valli, A., Dujovny, G., and García, J.A. 2008. Protease activity, self interaction, and small interfering RNA binding of the silencing suppressor P1b from *Cucumber vein yellowing ipomovirus*. J. Virol. 82:974-986.

Valli, A., Martín-Hernández, A.M., López-Moya, J.J., and García, J.A. 2006. RNA silencing suppression by a second copy of the P1 serine protease of *Cucumber vein yellowing ipomovirus* (CVYV), a member of the family *Potyviridae* that lacks the cysteine protease HCPro. J. Virol. 80:10055-10063.

Supplementary Table S2. Sequences of PCR primers used in the plasmid constructions.

Primer	Sequence (5'-3') [a]
#271	TGTCGTACCTGCCTCC
#279	CTCTGCAGGAGAGCGGCAAAAGTCTC
#441	TCCTGCAGATAACTTTTTTCAACC
#835	ggggacaagtttgtacaaaaaagcaggctccatggca*ACAATTCATGGATTGCATGCGTTC*
#976	gtcaccATGGCAACCATTG
#977	*CGCATGCAATCCATGAATTGT*GTCAGAGTAGTGGATTATCTC
#978	GAGATAATCCACTACTCTGAC*ACAATTCATGGATTGCATGCG*
#979	ccaaccaggt*AGAAGCAATAAAAGTCAATTTTATCTTTCTC*
#980	*GATAAAATTGACTTTTATTGCTTC*GGTCTTGAAGTGGATAAGTGTGACG
#981	CAAGCTTGCTCCAATTCCTGG
#982	*ACATTGAACCACATCTTTGGG*
#983	ACACTTATCCACTTCAAGACC*GAAGCAATAAAAGTCAATTTTATCTTTCTC*
#984	ggaacc*ATGGCCGAAGTTTATAGTTTCG*
#985	*CTTCTCTTCCAT**T**GCCACCCTGGAG*
#986	*CTCCAGGGTGGC**A**ATGGAAGAGAAG*
#987	*CTCTGGATCCATGTCATC*
#988	*GATCTCAGCGAC**A**GCAGGGTGCAC*
#994	*GTGCACCCTGC**T**GTCGCTGAGATCATGAAAGAAGC*
#1074	GTCAGAGTAGTGGATTATCTCATTGC
#1075	AGCAATGAGATAATCCACTACTCTGACGGTCTTGAAGTGGATAAGTG TGACG
#1719	TTGTTTGCCTGGGTCAGA*AATTGTGTAATTGCGAATCCTTC*
#1720	*ATTCGCAATTACACAATT*TCTGACCCAGGCAAACAATTTTGG

[a] Sequences corresponding to PPV are in upper case, sequences corresponding to CVYV are in upper italic case, restriction sites used for cloning are underlined, sequences as a tail are in lower case, and silent mutations abolishing restriction sites are indicated in bold.