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Heterologous RNA silencing suppressors from both plant- and animal-infecting viruses support *Plum pox virus* infection

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ABSTRACT

HCPro, the RNA silencing suppressor (RSS) of viruses belonging to the *Potyvirus* genus in the *Potyviridae* family, is a multifunctional protein presumably involved in all essential steps of the viral infection cycle. Recent studies have shown that *Plum pox potyvirus* (PPV) HCPro can be successfully replaced by *Cucumber vein yellowing ipomovirus* P1b, a sequence unrelated RSS from a virus of the same family. In order to gain insight into the requirement of a particular RSS to establish a successful potyviral infection, we tested the ability of different heterologous RSSs from both plant- and animal-infecting viruses to substitute HCPro. Making use of engineered PPV chimeras, we show that PPV HCPro can be functionally replaced by some, but not all, unrelated RSSs, including the NS1 protein of the mammalian-infecting Influenza A virus. Interestingly, the capacity of a particular RSS to replace HCPro does not strictly correlate with its RNA silencing suppression strength. Altogether, our results suggest that not all suppression strategies are equally suitable for an efficient escape of PPV from the RNA silencing machinery. The approach followed here based on using PPV chimeras in which an under-consideration RSS substitutes for HCPro could further help to study the function of diverse RSSs in a "highly-sensitive" RNA silencing context, such as that taking place in plant cells during the process of a viral infection.

INTRODUCTION

RNA silencing is a general term that refers to a complex set of RNA-guided gene regulatory mechanisms controlling crucial physiological processes, such as developmental patterning, responses to stress conditions and maintenance of genome stability, in a wide variety of eukaryotic organisms (Ambros & Chen, 2007; Baulcombe, 2005). Among its functions, RNA silencing plays a major role in natural antiviral immunity at least in plants, fungi and invertebrate animals, where infecting viruses induce the production of small-interfering (si)RNAs from viral double-stranded (ds)RNAs and/or secondary RNA structures by the action of RNase-III ribonuclease Dicer-like (DCL) proteins. These viral-derived (v)siRNAs are then incorporated into RNA-induced silencing complexes (RISCs), guiding them, by base pair complementarity, to viral RNAs for degradation. Intriguingly, it still remains controversial whether this mechanism is part of the innate antiviral response in vertebrate animals (for a complete review of RNA silencing-based viral immunity systems, see Ding, 2010).

In turn, to counteract this defensive response, viruses have evolved a variety of strategies, the most common of which is the expression of proteins that block the RNA silencing machinery of the host. These factors are called RNA silencing suppressors (RSSs) and show a large diversity in amino acid sequence and anti-silencing mechanisms (Burgyán & Havelda, 2011; Dunoyer & Voinnet, 2005; Roth *et al*., 2004; Valli *et al*., 2009). The helper component protease (HCPro) of plant potyviruses was the first RSS to be described (Anandalakshmi *et al*., 1998; Brigneti *et al*., 1998; Kasschau & Carrington, 1998). It is a potent suppressor that blocks RNA silencing by hijacking specifically 21-nt long vsiRNAs (Lakatos *et al*., 2006), and, possibly, by other mechanisms (Endres *et al*., 2010). HCPro is a cysteine protease (Carrington *et al*., 1989) defined as a multifunctional protein involved in all essential steps of the potyviral infection cycle (Maia *et al*., 1996; Syller, 2005), apparently, with host specificity (Carbonell *et al*., 2012; Sáenz *et al*., 2002). Nevertheless, whereas several HCPro functions may depend on its silencing suppression activity (Kasschau & Carrington, 2001), the relevance of specific silencing suppression mechanisms and the extent of dependence of plant viruses on their natural RSS(s) are largely unknown.

A large number of interactions with viral and host proteins have been attributed to HCPro. Thus, HCPro interacts with the viral coat protein (CP) (Blanc *et al*., 1997; Roudet-Tavert *et al*., 2002), cylindrical inclusion (CI) protein (Choi *et al*., 2000; Guo *et al*., 2001; Zilian &

Maiss, 2011), P1 (Merits *et al*., 1999), genome-linked protein (VPg) (Guo *et al*., 2001; Roudet-Tavert *et al*., 2007; Yambao *et al*., 2003) and its precursor, nuclear inclusion protein a (NIa) (Guo *et al*., 2001). As host factor partners, HCPro interacts with two potato RING-finger proteins, HIP1 and HIP2, (Guo *et al*., 2003), the maize ferredoxin-5 (Cheng *et al*., 2008), the NtMinD protein (Jin *et al*., 2007b), and the translation initiation factors eIF(iso)4E and eIF4E (Ala-Poikela *et al*., 2011) of tobacco, with unknown physiological consequences. HCPro has also been shown to interact with some subunits of the 20S proteasome, inhibiting its endonuclease activity (Ballut *et al*., 2005; Dielen *et al*., 2011; Jin *et al*., 2007a); and interactions of HCPro with a calmodulin related protein (rgs-CaM) (Anandalakshmi *et al*., 2000) and the ethylene-inducible transcription factor RAV2 (Endres *et al*., 2010) appear to regulate its silencing suppression activity. Although these interactions were identified in different heterologous systems, their biological relevance in the context of a natural potyviral infection has not been established yet.

Recent studies with viruses of the *Potyviridae* family have shown that, even though suppression of silencing is a pivotal potyviral function, a particular infection does not depend on a singular RSS. Hence, *Plum pox virus* (PPV, *Potyvirus* genus) HCPro can be successfully replaced by the sequence-unrelated RSS P1b from *Cucumber vein yellowing virus* (CVYV, *Ipomovirus* genus) (Carbonell *et al*., 2012). Although these two RSSs share no homology in their amino acid sequences, both are present in members of the *Potyviridae* family and use a similar mechanism to suppress the RNA silencing, which is based on siRNA sequestering (Valli *et al*., 2011). To gain further insight into how viruses evade the RNA silencing machinery of the host, we tested to what extent different RSSs can substitute HCPro without abolishing PPV infectivity. Our results show that PPV HCPro can be functionally replaced by some, but not all, unrelated RSSs, including the NS1 protein of Influenza A virus, and suggest the existence of some specific preferences in the silencing counteracting process.

RESULTS

Generation of a universal PPV intermediate cDNA clone lacking the HCPro silencing suppressor coding sequence

In order to easily generate PPV-based chimerical viruses by replacing HCPro with unrelated proteins, we first generated an intermediate clone in a pGEMT backbone carrying the PPV P1-P3 cistrons, but lacking the HCPro coding sequence (Fig. 1a). The amplified P1-P3

fragment was engineered to maintain the coding sequence of the two first amino acids of HCPro (SD) just downstream of P1 for ensuring an efficient P1 self cleavage. This sequence was followed by an EcoRV/SbfI cloning site (Fig. 1a). Since most of proteins aimed to replace the self-cleaving cysteine-protease HCPro lack a proteolytic activity that release themselves from the viral polyprotein once that the infection takes place, a DNA sequence coding for a PPV NIa protease cleavage site was added just upstream of the P3 coding sequence (Fig. 1a).

The coding sequence of a set of HCPro-unrelated proteins with suspected RNA silencing suppression activity (those fully described in this study are shown in Fig. 1b and Table 1) were cloned in the pGEMTp1p3 intermediate plasmid. Fragments containing the 5'-part of each chimerical viral genome were released from the pGEMTp1RSSp3 clones and subsequently used to replace the equivalent fragment from a full-length cDNA of a wild type PPV, which also contains the coding sequence of the green fluorescent protein (GFP) to further facilitate the monitoring of the infection process (Fig. 1b). For simplicity, each chimerical virus is termed here as PPV followed by the name of the protein that replaces HCPro (Fig. 1b). It is noteworthy that this universal PPV intermediate clone is useful for cloning any "under-study" protein in order to test its ability to replace the well-known suppressor HCPro in the context of a viral infection.

TBSV P19 functionally replaces PPV HCPro and efficiently prevents plant recovery

It has been previously described that PPV HCPro can be functionally replaced by the RSS P1b from the ipomovirus CVYV, another member of the family *Potyviridae* (Carbonell *et al*., 2012). P19 is a well-characterized RSS of viruses of the genus *Tombusvirus*, which suppresses silencing by sequestering double-stranded siRNAs (Silhavy *et al*., 2002; Vargason *et al*., 2003), the same mechanism that has been proposed for the potyviral HCPro and CVYV P1b (Lakatos *et al*., 2006; Valli *et al*., 2011). The infectivity of PPV-P19, in which the P19 coding sequence from *Tomato bushy stunt virus* (TBSV) replaces that of HCPro, was tested by biolistic inoculation in *N. benthamiana*. Previously described wild type PPV, HCPro deletion mutant (PPV-ΔHC) (P1ΔHC in Carbonell *et al*., 2012), and the chimerical virus PPV-P1b in which HCPro was replaced by CVYV P1b (P1P1b in Carbonell *et al*., 2012), were used as controls. As expected, just a few days post-inoculation (dpi), GFP foci were detected in all leaves inoculated with PPV and PPV-P1b, whereas no foci appeared in those leaves inoculated with P1ΔHC (Fig. 2a and Fig. S1). Interestingly, GFP foci also appeared in

all leaves inoculated with PPV-P19 (Fig. 2a and Fig. S1a). The size and number of PPV, PPV-P1b and PPV-P19 GFP foci were similar. Western blot analysis of extracts prepared from GFP foci-containing inoculated tissues showed high accumulation levels of viral CP in plants inoculated with PPV, PPV-P1b and PPV-P19 (Fig. 2b). No viral accumulation was detected in equivalent leaf areas of plants inoculated with PPV-ΔHC (Fig. 2b). Virus-like symptoms (chlorotic mottling) and GFP signals, equivalent to those of PPV and PPV-P1b, were observed in upper non-inoculated leaves of plants infected with PPV-P19 (Fig. 2c and Fig. S1b). Western blot analysis of extracts prepared from GFP expressing tissues of systemically infected leaves showed similar virus accumulation levels for PPV, PPV-P1b and PPV-P19 (Fig. 2d).

A previous report has shown that, whereas the replication of wild type PPV is maintained active at late times of infection in *N. benthamiana* plants, partial recovery occurs in new growing tissues of plants infected with the chimerical virus carrying P1b instead of HCPro, which is indicative of some defect in the silencing suppression machinery of the virus (Carbonell *et al.*, 2012). To test the capacity of a PPV chimerical virus with another heterologous RSS to escape from plant recovery, PPV-P19-infected plants were analyzed at later stages of the infection process (38 dpi) by testing viral accumulation in old and young leaves. As expected, whereas severe symptoms, intense green fluorescence and high CP levels were maintained in both types of tissue of plants infected with PPV, young leaves of plants infected with PPV-P1b showed a characteristic "recovery" phenotype with the new apical leaves appearing healthy, with much less intensity of green fluorescence, and lower accumulation of CP compared to the older leaves (Fig. 3). However, no recovery was observed in PPV-P19-infected plants, and young leaves still appeared heavily infected at 38 dpi (Fig. 3). Intriguingly, whereas the accumulation of wild type PPV was similar in old and young leaves, a decline in fluorescence intensity and CP levels was apparent in aging leaves of plants infected with PPV-P19 (Fig. 3).

Thus, our results indicate that CVYV P1b and TBSV P19 can functionally replace HCPro in a PPV infection, but only TBSV P19 prevents the recovery of plants from PPV infection in the absence of HCPro.

RSSs with different silencing suppression mechanisms are able to support PPV infection

Both CVYV P1b and TBSV P19 share with PPV HCPro the ability to specifically bind double-stranded siRNAs. In order to assess the ability of other proteins that suppress RNA silencing by different mechanisms to replace HCPro in a PPV infection, the HCPro coding sequence of PPV was replaced by sequences encoding the P1 N-terminal region of P1 ($P1^N$) from *Sweet potato mild mottle ipomovirus* (SPMMV), the P6 from *Cauliflower mosaic caulimovirus* (CaMV) and the protein NS1 from the animal-infecting orthomyxovirus Influenza A, which suppress the RNA silencing by targeting mature RISC complexes (Giner *et al*., 2010), impairing the DCL4-mediated processing of dsRNAs by interaction with DRB4 (Haas *et al*., 2008), and an unknown mechanism, respectively.

In the new experiment, wild type PPV and PPV-P19 efficiently infected *N. benthamiana* as previously shown. Local GFP foci were also detected in 6 out of 8 plants inoculated with PPV-NS1, but in smaller number and size than those of wild type PPV and PPV-P19 (Fig. 4a and Fig. S2a). No GFP foci were observed in leaves inoculated with PPV-$P1^N$ and PPV-P6 (Fig. 4a and Fig. S2a). Surprisingly, when leaf tissue around the inoculated area was assessed by western blot analysis, CP accumulation was detected, although at low levels, not only in leaves inoculated with PPV-NS1, but also in those inoculated with PPV-$P1^N$ (Fig. 4b). In contrast, no CP accumulation was observed in extracts prepared from plants inoculated with PPV-P6 (Fig. 4b).

No disease symptoms were observed in plants inoculated with PPV-NS1, -$P1^N$ and -P6. However, some GFP fluorescent spots were detected, with a long delay with respect to the wild type virus, in upper non-inoculated leaves of 2 plants infected with PPV-NS1 (Fig. 4c and Fig. S2b). In addition, a faint green fluorescence appeared, with a similar delay as the PPV-NS1 fluorescent spots, in the upper non-inoculated leaves of 2 plants infected with PPV-$P1^N$ (Fig. 4c and Fig. S2b). Western blot analysis of whole leaf extracts confirmed the systemic spread of PPV-$P1^N$ and PPV-NS1, although the CP accumulation levels of these viruses were very low compared to those of PPV-P19 and PPV wild type (Fig. 4d). Immunoreactions with available specific antibodies (anti-HCPro, anti-P19 and anti-NS1) (Fig. 4d) and sequencing of IC-RT-PCR-amplified products (Fig. S2c) confirmed the identity and the genetic stability of the infecting viruses. Neither GFP fluorescence nor viral CP accumulation was detected in upper leaves of plants infected with PPV-P6 (Fig. S2b and d).

These results indicate that an RSS with a different mechanism of action than HCPro, SPMMV P1, and a protein of an animal virus that suppresses silencing by a still uncharacterized

mechanism, the influenza A virus NS1, are able to support a limited systemic infection of a potyvirus in the absence of HCPro. However, not all the proteins with reported RNA silencing suppression activity were able to functionally replace HCPro in the PPV infection.

The ability to replace HCPro in the PPV infection process does not strictly correlate with the strength of the silencing suppressor

To assess the contribution of the RNA silencing suppression activity of each PPV chimera in its ability to infect *N. benthamiana* plants, each RSS was tested in a co-agroinfiltration assay. Therefore, we constructed *Agrobacterium* binary plasmids expressing the 5'-half-part of the different chimerical viruses (Fig. 5a), which were coagroinfiltrated with p35S:GFP (a plasmid expressing single-stranded GFP RNA that is here used as both trigger and reporter of silencing), and pMDC32-NIaPro (a plasmid expressing the protease domain of the PPV NIa protein) (Fig. 5a). For simplicity, in this part of the report, we will refer to each *A. tumefaciens* line by the plasmid it carries.

Whereas GFP fluorescence strongly declined at 4-5 days post agroinfiltration (dpa) in patches expressing p35S:GFP, pMDC32-NIaPro and the empty pBin19 empty vector, the green fluorescence remained strong at 6 dpa in patches co-agroinfiltrated with p35S:GFP and pMDC32NIaPro plus either pBIN-P1HCPro, pBIN-P1P19, pBIN-P1P1b or pBIN-P1NS1 (Fig. 5b), showing that PPV HCPro, TBSV P19, CVYV P1b and Influenza A virus NS1 suppress the RNA silencing with similar efficiency. In contrast, very weak fluorescence was observed in patches expressing p35S:GFP and pMDC32-NIaPro plus either pBIN-P1P1^{N} or pBINP1P6 indicating that, at least in this system, SPMMV P1^{N} and CaMV P6 have very weak silencing suppression activity (Fig. 5b).

Northern blot analysis confirmed the green fluorescence observations, showing that GFP mRNA accumulation was similarly high at 6 dpa in those leaves expressing TBSV P19, Influenza A virus NS1, PPV HCPro and CVYV P1b (Fig. 5c). The drop in GFP mRNA levels could not be prevented in leaves expressing any of the other tested proteins (Fig. 5c).

The genomic expression strategy of potyviruses through proteolytic processing of long polyprotein precursors results in the inevitable presence of extra amino acids at the end of the foreign sequences, which are necessary for the protease recognition in the polyprotein precursor (Fig. 1a). To assess the possibility that these extra amino acids incorporated at the termini of CaMV P6 could be disturbing the silencing suppression activity of this RSS, then

abolishing the infectivity of PPV-P6 chimera, we compared the anti-silencing activity of wild type protein with their counterparts as it was engineered in the PPV genome. Hence, we constructed binary plasmids expressing wild type CaMV P6 and a modified version of it carrying extra amino acids as they should be produced in the PPV context (SD-P6-QVVVHQ, where SD represents the amino acids +1 and +2 of P1 cleavage site, whereas QVVVHQ represents the amino acids -6 to -1 of the NIa cleavage site). Plasmids were co-agroinfiltrated together with p35S:GFP, and their ability to suppress the GFP silencing was assessed at 6 dpa by monitoring GFP fluorescence signal (Fig. S3). Intriguingly, wild type CaMV P6 was unable to suppress the RNA silencing in this co-agroinfiltration test, even though it was expressed in the wild type form, suggesting that a deficiency of P6 anti-silencing activity could be responsible for its inability to support PPV infection.

Altogether, these results indicate that a strong silencing suppression activity is required for an efficient potyviral infection, but that a week anti-silencing activity could be enough to support a limited viral infection. However, a strict correlation between HCPro replacement capacity and anti-silencing activity was not observed.

DISCUSSION

How specific is the dependence of potyviruses on HCPro for a successful infection?

Most plant viruses have been shown to produce RSSs that counteract antiviral defences mediated by RNA silencing (Burgyán & Havelda, 2011; Shimura & Pantaleo, 2011; Valli *et al*., 2009). However, RSSs are not always essential, and some RSS-defective viruses are able to develop restricted, but still productive, infections (for instance, Ding *et al*., 1995; Havelda *et al*., 2003). In turn, HCPro appears to be strictly required for infections caused by viruses of the genus *Potyvirus* (Fig. 2 and Carbonell *et al*., 2012; Garcia-Ruiz *et al*., 2010). Interestingly, viruses of other genera of the family *Potyviridae* do not depend on HCPro to infect their hosts, since their silencing suppression activity is provided by other viral proteins (Janssen *et al*., 2005; Stenger *et al*., 2005; Valli *et al*., 2006; Young *et al*., 2012), and one of these proteins, P1b from the ipomovirus CVYV can functionally replace the HCPro from the potyvirus PPV (Carbonell *et al*., 2012). In the present report we show that a heterologous RSS from an unrelated virus, the protein P19 from the tombusvirus TBSV, is also able to support an efficient PPV infection (Fig. 2). PPV-P19 appears to escape antiviral silencing with an efficiency similar to that of the wild type virus, since plants infected with this chimerical virus

do not show the recovery phenotype typical of silencing suppression-deficient viruses (Fig. 3), further supporting the conclusion that potyviruses do not depend on a specific silencing suppression provided by HCPro.

Do potyviruses depend on a specific RNA silencing suppression strategy?

Sequestering of siRNAs appears to be a very successful strategy to suppress silencing, which is used, among other RSSs, by HCPro, P1b and P19 (Lakatos *et al*., 2006; Valli *et al*., 2011). However, the lack of similarity, and the diverse dependence on specific features at the siRNA ends for efficient binding (Valli *et al*., 2011), support the idea that siRNA binding mechanisms of these RSSs are different. The high infectivity of PPV-P1b and PPV-P19 suggests that potyvirus infection does not require a specific strategy of siRNA sequestering, and, thus, different mechanisms of siRNA binding could be equally useful for the virus as long as they provide an effective silencing suppression.

To assess whether potyviruses could successfully use other silencing suppression mechanisms, a series of recombinant viruses in which PPV HCPro was replaced by RSSs with a broad range of anti-silencing strategies were constructed. We were unable to detect infection in the chimerical virus that express CaMV P6 (Fig. 4 and Fig. S2). Recombinant viruses expressing P0 from the polerovirus BWYV, RNase3 from the crinivirus SPCSV, Tat from the human retrovirus HIV and the ribonuclease XRN4 from *Arabidopsis thaliana*, for which silencing suppression activities in different experimental systems have been reported (Bennasser *et al*., 2005; Cuellar *et al*., 2009; Gazzani *et al*., 2004), appear to be also not infectious. Unfortunately, we were not able to detect silencing suppression activity for these proteins when they were expressed as part of the PPV polyprotein in a co-agroinfiltration assay (data not shown). In the case of BWYV P0, and perhaps in other cases, this is due to the extra amino acids added to its ends to facilitate its excision from the viral polyprotein (data not shown). For other proteins, such as CaMV P6, the deficiency could be genuine (Fig. S3). It should be noted that to our knowledge, for some of these RSSs, such as Tat and P6 viral factors and the *A. thaliana* XRN4 protein, anti-silencing activity using co-agroinfiltration assays has not been reported yet.

Two heterologous RSSs were also able to support PPV infection: the NS1 protein of Influenza A virus and an N-terminal fragment of the P1 protein from the ipomovirus SPMMV (Fig. 4). Although NS1 has shown potent RNA silencing suppression activity in different

experimental systems (Table 1, Fig. 5), PPV expressing NS1 instead of HCPro infects *N. benthamiana* very poorly (Fig. 4). NS1 is able to bind long dsRNA and ds-siRNAs, and both capacities are expected to contribute to its ability to suppress the RNA silencing (Bucher *et al*., 2004; Li *et al*., 2004). The poor infectivity of PPV-NS1 shows that even though RNA binding might provide strong silencing suppression activity, some additional RSS features seem to be required for supporting an efficient potyviral infection.

An N-terminal fragment of the P1 protein from SPMMV (aa 1-383) has been reported to be an efficient RSS (Giner *et al*., 2010). However, very little silencing suppression activity was observed here for a similar SPMMV P1 fragment ($P1^N$, aa 1-360) expressed as part of the PPV polyprotein (Fig. 5), which could be due to the extension of this particular deletion or to additional amino acids introduced at both ends of the protein to ensure the correct processing during the viral infection. In spite of that, PPV-$P1^N$ showed some infectivity (Fig. 4 and Fig. S2), indicating that very little RNA silencing suppression activity is enough for a limited potyviral systemic infection. The fact that the infection efficiencies of PPV-NS1 and PPV-$P1^N$ are very similar although the silencing suppression activity of NS1 is much stronger than that of $P1^N$, suggests that not all anti-silencing mechanisms are equally effective to support potyviral infection. P1 has been shown to suppress silencing by argonaute binding (Table 1), and this strategy appears to be more effective for PPV than the strategy, still to be characterized, used by NS1. It is interesting to remark that, although the infectivity of PPV-NS1 and PPV-$P1^N$ chimerical viruses is similar, their patterns of infection are quite different (Fig. 4), which suggest that specific infection features can be conditioned by the RSS used by the virus to counteract the antiviral silencing response of the host.

Only RNA silencing suppression or something else?

HCPro is a multifunctional protein, known to be involved in all essential steps of the viral infection, and it has been shown to interact with a large number of host factors (Ala-Poikela *et al*., 2011; Anandalakshmi *et al*., 2000; Ballut *et al*., 2005; Cheng *et al*., 2008; Dielen *et al*., 2011; Endres *et al*., 2010; Guo *et al*., 2003; Jin *et al*., 2007a; Jin *et al*., 2007b). Some of the functions of HCPro and several of its interactions with host factors appear to be closely related with its RNA silencing suppression activity (Anandalakshmi *et al*., 2000; Endres *et al*., 2010; Kasschau & Carrington, 2001; Kasschau *et al*., 2003). It is interesting to remark that, whereas the cysteine proteinase activity of HCPro is not necessary for RNA silencing

suppression, it appears to be required for genome amplification (Kasschau & Carrington, 1995), suggesting that potyviral infection could rely on silencing suppression-independent functions of HCPro. Our results do not support this hypothesis since TBSV P19, which lacks any proteolytic activity, is able to provide all HCPro activities essential for PPV infection. We cannot rule out the possibility that this heterologous protein provide a common hypothetical silencing suppression-independent function of HCPro, but given the large divergence between them, this possibility appears to be very unlikely. What does not seem improbable, however, is that HCPro has important, but not essential, functions in the potyviral infection not related with silencing suppression or with its first identified role as helper factor in aphid transmission, which would not be supplied by other heterologous RSSs.

Altogether, our findings demonstrate that the well-characterized RSS HCPro can be replaced by different heterologous RSSs from both plant- and animal-infecting viruses with dissimilar results, thus leading to the conclusion that, whereas PPV infection, and presumably potyviral infections in general, does not strictly rely on a particular RSS, specific silencing suppression mechanisms would fit better for a given virus. The approach followed here of exchanging the HCPro coding sequence of the PPV genome by other coding sequences producing heterologous proteins could contribute to identify novel RSSs and study their function in the process of a viral infection using a highly "silencing-sensitive" model like plants. Moreover, our particular viral system will be very helpful when the evaluation of the RNA silencing suppression activity of a given protein is not feasible in its original viral genome.

METHODS

Plant hosts. Agroinfiltration and viral infectivity assays were performed in *N. benthamiana* plants. All plants were grown in a greenhouse maintained at 16 hours light with supplementary illumination and a temperature range of 19-23 ºC.

Plasmids. A detailed description of plasmids used in this study can be found in the supplementary methods in the supplementary materials.

Biolistic inoculation. The Helios Gene Gun System (Bio-Rad, Hercules, CA, U.S.A.) was used for biolistic inoculation. Microcarrier cartridges were prepared from 2 different clones per construct, 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 bars was used for shooting plants. Each cartridge was shot twice onto two leaves of each plant.

Transient expression by agroinfiltration. *N. benthamiana* plants were infiltrated with *A. tumefaciens* C58C1 strain carrying the indicated plasmids as previously described (Valli *et al*., 2006). Appropriate *Agrobacterium* cultures were mixed after induction with acetosyringone. In the cases of pBIN-P1RSS plasmids expressing RSSs without self-cleaving activity, an *Agrobacterium* strain carrying a binary plasmid expressing the NIa protease domain (pMDC32-NIaPro) was also included in the infiltration mixtures.

Fluorescence imaging. The green fluorescent protein (GFP) fluorescence was monitored under long-wavelength UV light (Black Ray model B 100 AP). To capture pictures 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 inoculated leaves were harvested under UV light from GFP expressing foci or the whole inoculated area, whereas tissue of upper non-inoculated leaves was collected from areas showing green fluorescence or the indicated whole leaves. Control samples corresponding to 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, anti-P19 rabbit serum (kindly provided by Herman Scholthof, Texas A&M University, US), anti-NS1 rabbit serum (kindly provided by Ariel Rodriguez, Centro Nacional de Biotecnologia-CSIC, Madrid, Spain) or anti-CP rabbit serum, as primary antibodies, and horseradish peroxidase (HRP)-conjugated

goat anti-rabbit IgG (Jackson) as secondary reagent. The immunostained proteins were visualized by enhanced chemiluminescence detection with a LifeABlot kit (Euroclone). Ponceau red staining was used to check the global protein content of the samples.

IC-RT-PCR. Leaf extracts from infected *N. benthamiana* plants were homogenized in 5 mM sodium phosphate buffer, pH 7.5 (2 ml: 1g tissue) and incubated in tubes previously coated with anti-PPV IgGs overnight at 4ºC. The incubation was followed by two washing steps with PBS-Tween buffer (16 mM PBS, 0.1 M NaCl, 0.5 g/L Tween 20, pH 7.2). RT-PCR was performed using the Titan kit (Roche Molecular Biochemical) with primers targeting the 3' end of PPV P1 (#90: 5′-CGGACCCAATGCAAG-3′) and 5' end of PPV P3 (#317: 5′-TGAACCACTATTGAACAG-3′). For further sequence analysis, PCR fragments were purified using the MinElute PCR purification kit (QIAGEN).

RNA extraction and northern blot analysis. Samples containing messenger RNAs were prepared from agroinfiltrated leaf tissue and subjected to northern blot analysis as previously described (Valli *et al*., 2006).

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Legends to the figures

Figure 1. Schematic representations of viral constructs derived from *Plum pox virus* (PPV). (a) Intermediate plasmids created for cloning the coding sequence of different RNA silencing suppressors (RSSs). P1 and NIa cleavage sites engineered at the ends of the inserted RSSs are also indicated. (b) Chimerical full-length cDNA clones derived from PPV and expressing heterologous RSSs. The GFP reporter gene inserted between the NIb and CP coding sequences is represented with a green rectangle.

Figure 2. P19 from *Tobacco bushy stunt virus* functionally replaces HCPro from *Plum pox virus* (PPV). (a) Pictures of leaves inoculated with the indicated viruses, taken under an epifluorescence microscope at 9 days post-inoculation (dpi). White bar, 1 cm. (b) Western blot analysis of protein extracts prepared from inoculated leaves (2 plants per construct) collected at 9 dpi. (c) Pictures taken under an epifluorescence microscope at 21 dpi of the 4th leaves above the inoculated ones. White bar, 1 cm. (d) Western blot analysis of protein extracts prepared from the whole 3rd and 4th leaves above the inoculated ones collected at 21 dpi. A polyclonal antiserum specific for PPV CP was used for assessment of virus accumulation. Membranes stained with Ponceau red showing the Rubisco are included as loading controls.

Figure 3. A *Plum pox virus* (PPV) chimera expressing *Tobacco bushy stunt virus* P19 escapes from plant recovery. (a) Pictures of old and young upper non-inoculated leaves of plants infected with the indicated viruses taken under an epifluorescence microscope at 38 days post-inoculation (dpi). White bar, 1 cm. (b) Western blot analysis of protein extracts prepared from upper non-inoculated leaves (2 plants per construct) collected at 38 dpi. O: old tissues (leaves at position 6 to 9 above the inoculated ones); Y: young tissues, (leaves at position 10 to 13 above the inoculated ones). A polyclonal antiserum specific for PPV CP was used for assessment of virus accumulation. Membranes stained with Ponceau red showing the Rubisco are included as loading controls.

Figure 4. *Sweet potato mild mottle virus* $P1^N$ and Influenza A virus NS1 support *Plum pox virus* (PPV) infection. (a) Pictures of leaves inoculated with the indicated viruses taken under an epifluorescence microscope at 9 days post-inoculation (dpi). White bar, 1 cm. (b) Western blot analysis of protein extracts prepared from inoculated leaves (2 plants per construct) collected at 9 dpi. (c) Pictures taken under an epifluorescence microscope at 21 dpi of the 4th leaves above the inoculated ones. White bar, 1 cm. (d) Western blot analysis of protein extracts prepared from the whole 3rd and 4th leaves above the inoculated ones (2 plants per construct) collected at 21 dpi. A polyclonal antiserum specific for PPV CP was used for assessment of virus accumulation. Ten times more concentrated anti-CP serum was used as required. Specific antibodies recognizing different RSSs were also used, if available, for confirming viral identity. Membranes stained with Ponceau red showing the Rubisco are included as loading control.

Figure 5. Anti-silencing activity of RSSs expressed in different *Plum pox virus* (PPV)-based chimerical viruses. (a) Schematic representation of constructs used in the agro-infiltration tests. Black arrows indicate self-cleavages by the corresponding viral proteases, whereas the grey arrow indicates a cleavage *in trans* by the action of PPV NIaPro. (b) GFP fluorescence pictures of agroinfiltrated leaves expressing the indicated proteins, taken under

an epifluorescence microscope at 6 days post-agroinfiltration (dpa). White bar, 1 cm. (c) Northern blot analyses of GFP mRNA extracted at 6 dpa from leaf patches of two plants expressing the indicated proteins by agroinfiltration. EtBr-stained rRNAs are shown as loading control.

Table 1: List of RNA silencing suppressors used to replace PPV HCPro.

RNA silencing suppressor	Virus name	Tested by	Proposed RNA silencing suppression mechanism	References
P1b[a]	*Cucumber vein yellowing virus*	**Plants:** prevention of silencing in co-agroinfiltration assays, enhancement of PVX pathogenicity	Binding of ds-siRNAs	(Valli *et al*., 2006; Valli *et al*., 2011)
P19	*Tobacco bushy stunt virus*	**Plants:** prevention of silencing in co-agroinfiltration assays, enhancement of PVX pathogenicity, reversal of transgene silencing, inhibition of VIGS **Animals:** support of FHV infection in insect cells, enhancement of PFV-1 accumulation in mammalian cells, suppression of miRNA activity in co-transfected cells, rescue of gag mRNA translation in infected cells	Binding of ds-siRNAs	(Lecellier *et al*., 2005; Li *et al*., 2004; Qian *et al*., 2009; Qiu *et al*., 2002; Vargason *et al*., 2003; Voinnet *et al*., 1999)
NS1	*Influenza A virus*	**Plants:** prevention of GFP silencing in co-agroinfiltration assays, enhancement of PVX pathogenicity **Animals:** support of FHV infection in insect cells, interference with shRNA activity in co-transfected cells, complementation of Tat deficiency in HIV-1 infection	Binding of ds-siRNAs and long dsRNAs	(Bucher *et al*., 2004; Delgadillo *et al*., 2004; Haasnoot *et al*., 2007; Li *et al*., 2004)
P1, $P1_{1-383}$	*Sweet potato mild mottle virus*	**Plants:** prevention of silencing in co-agroinfiltration assays	AGO1 inhibition	(Giner *et al*., 2010)
P6	*Cauliflower mosaic virus*	**Plants:** reversion of amplicon silencing in transgenic plants	DRB4 inhibition	(Love *et al*., 2007)

[a] The chimerical virus that carries P1b instead of HCPro has been previously reported (Carbonell *et al*., 2012).

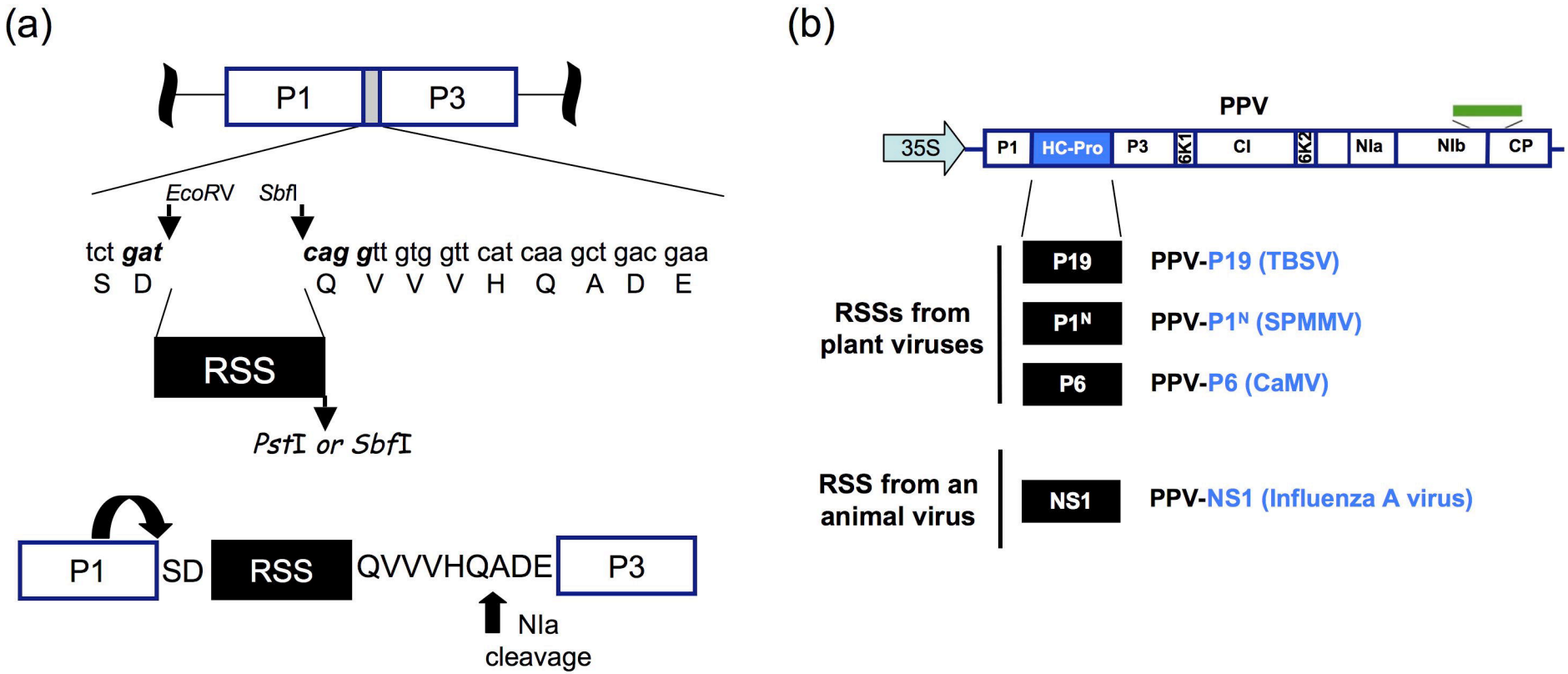

(a)
P1
P3
EcoRV
SbfI
tct gat
cag gtt gtg gtt cat caa gct gac gaa
S D
Q V V V H Q A D E
RSS
PstI or SbfI
P1
SD
RSS
QVVVHQADE
P3
NIa
cleavage
(b)
PPV
35S
P1
HC-Pro
P3
6K1
CI
6K2
NIa
NIb
CP
RSSs from plant viruses
P19
PPV-P19 (TBSV)
P1N
PPV-P1N (SPMMV)
P6
PPV-P6 (CaMV)
RSS from an animal virus
NS1
PPV-NS1 (Influenza A virus)

Figure 1

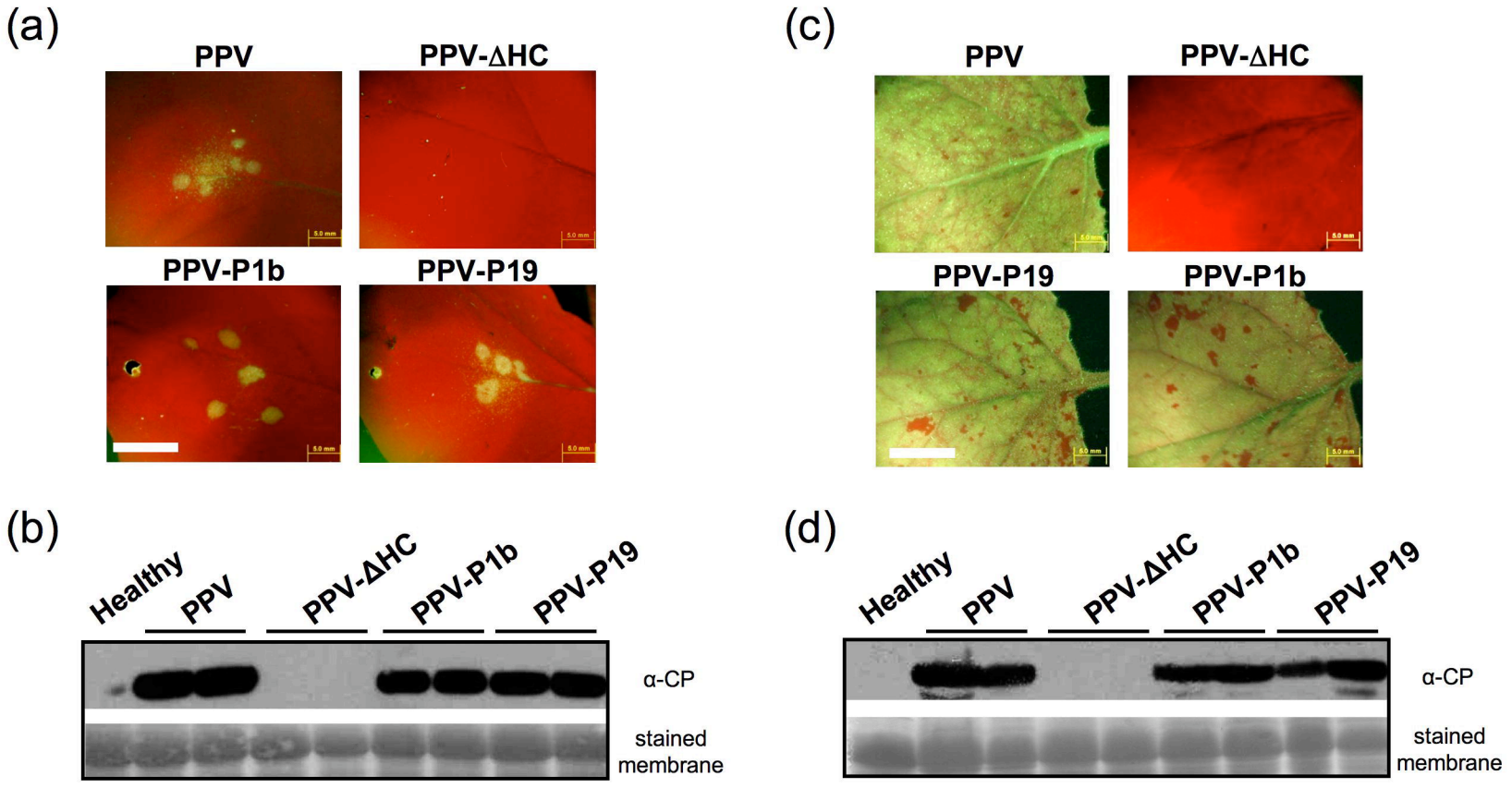

(a)
PPV
PPV-ΔHC
PPV-P1b
PPV-P19
(c)
PPV
PPV-ΔHC
PPV-P19
PPV-P1b
(b)
Healthy
PPV
PPV-ΔHC
PPV-P1b
PPV-P19
α-CP
stained membrane
(d)
Healthy
PPV
PPV-ΔHC
PPV-P1b
PPV-P19
α-CP
stained membrane

Figure 2

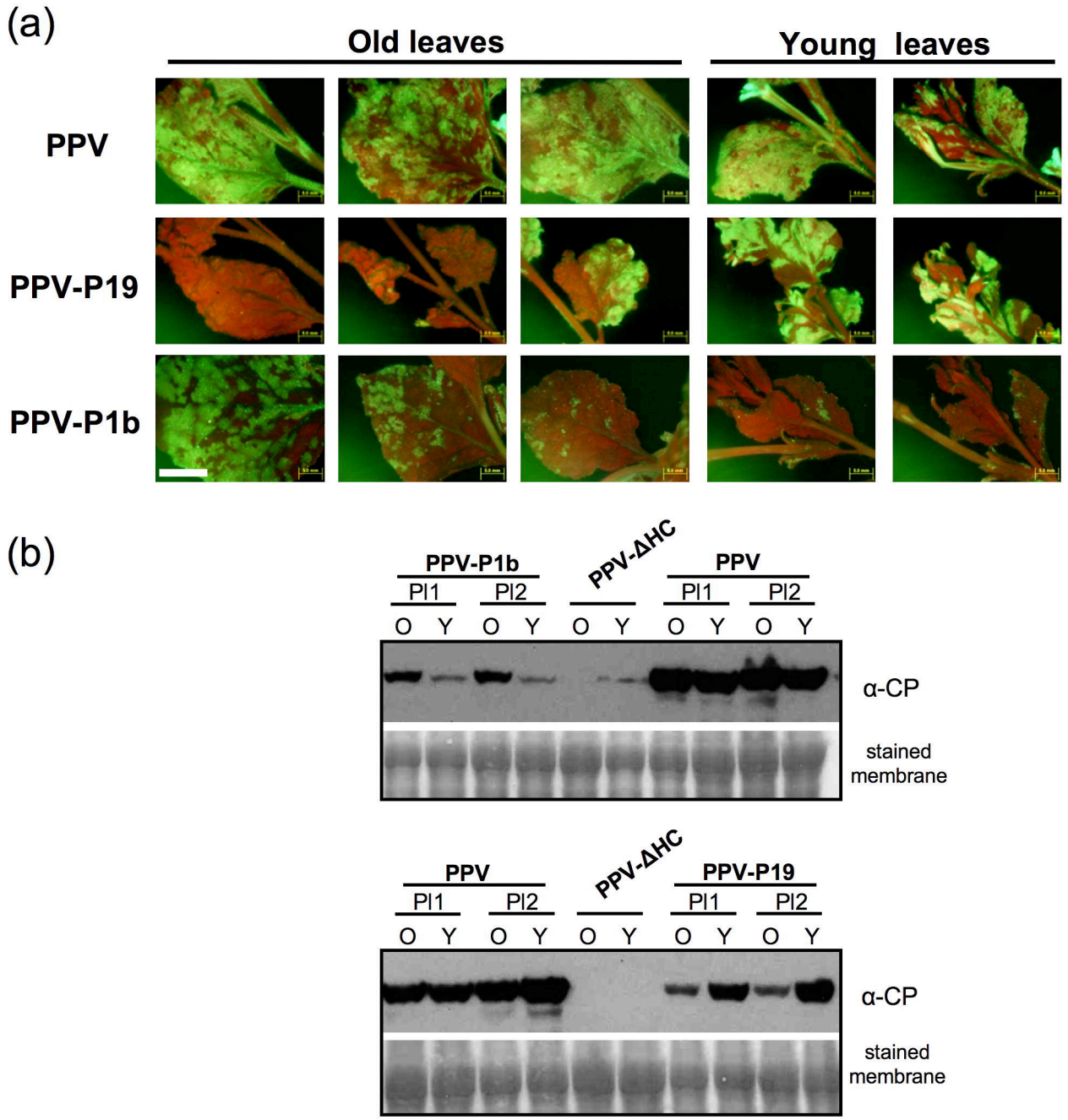

(a)
Old leaves
Young leaves
PPV
PPV-P19
PPV-P1b
(b)
PPV-P1b
PPV-ΔHC
PPV
PI1
PI2
O Y O Y O Y O Y O Y
α-CP
stained membrane
PPV
PPV-ΔHC
PPV-P19
PI1
PI2
O Y O Y O Y O Y O Y
α-CP
stained membrane

Figure 3

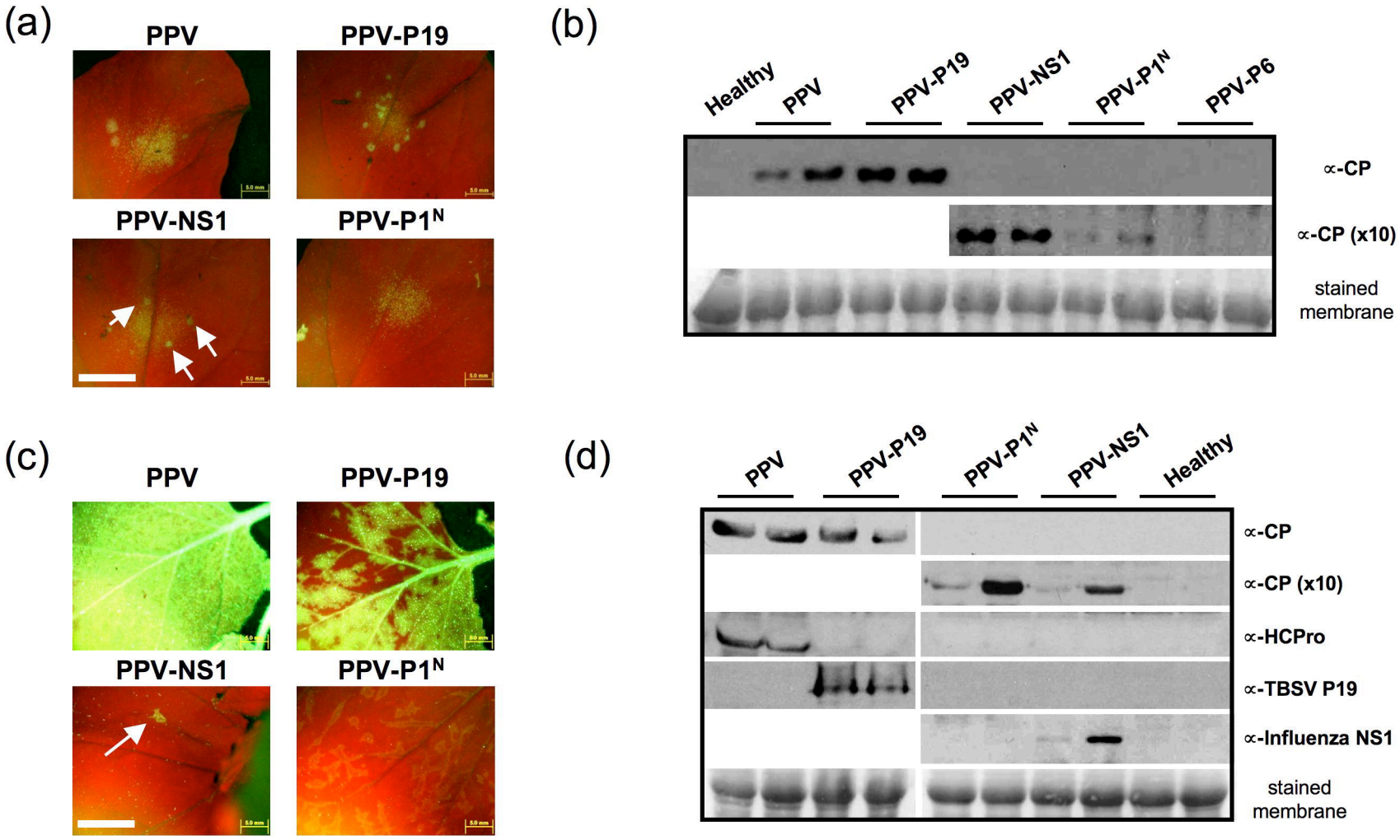
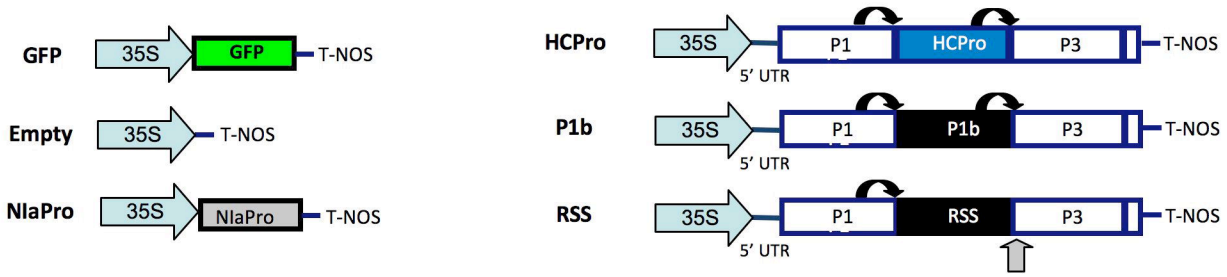

(a)
PPV
PPV-P19
PPV-NS1
PPV-P1N
(b)
Healthy
PPV
PPV-P19
PPV-NS1
PPV-P1N
PPV-P6
∝-CP
∝-CP (x10)
stained membrane
(c)
PPV
PPV-P19
PPV-NS1
PPV-P1N
(d)
PPV
PPV-P19
PPV-P1N
PPV-NS1
Healthy
∝-CP
∝-CP (x10)
∝-HCPro
∝-TBSV P19
∝-Influenza NS1
stained membrane

Figure 4

(a)


GFP
35S
GFP
T-NOS
Empty
35S
T-NOS
NIaPro
35S
NIaPro
T-NOS
HCPro
35S
P1
HCPro
P3
T-NOS
5′ UTR
P1b
35S
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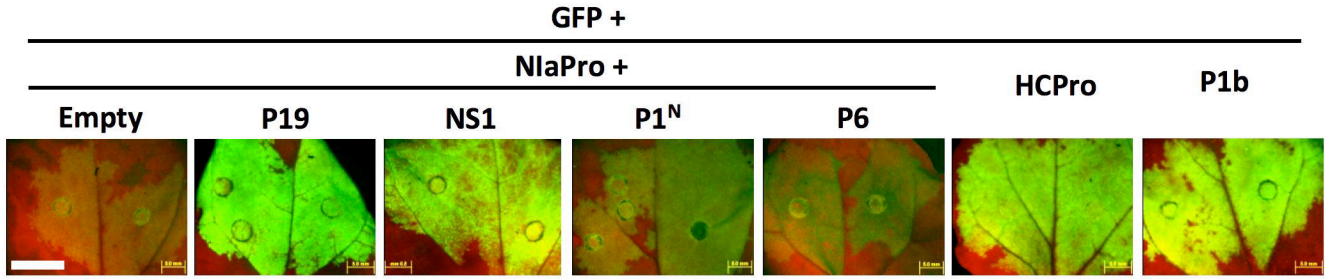
P1
RSS
P3
T-NOS
5′ UTR

(b)


GFP +
NIaPro +
Empty
P19
NS1
P1N
P6
HCPro
P1b

(c)

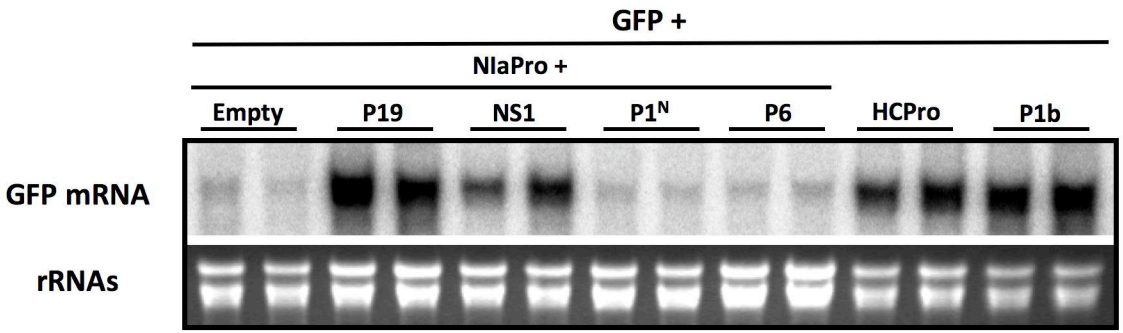

GFP +
NIaPro +
Empty
P19
NS1
P1N
P6
HCPro
P1b
GFP mRNA
rRNAs

Figure 5