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Additional Information

The *Potyviridae* P1a leader protease contributes to host range specificity

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Abstract

1
2 The P1a protein of the ipomovirus *Cucumber vein yellowing virus* is one of the
3 self-cleavage serine proteases present in *Potyviridae* family members. P1a is
4 located at the N-terminal end of the viral polyprotein, and is closely related to
5 potyviral P1 protease. For its proteolytic activity, P1a requires a still unknown host
6 factor; this might be linked to involvement in host specificity. Here we built a series
7 of constructs and chimeric viruses to help elucidate the role of P1a cleavage in host
8 range definition. We demonstrate that host-dependent separation of P1a from the
9 remainder of the polyprotein is essential for suppressing RNA silencing defenses
10 and for efficient viral infection. Inter-genus replacement of leader proteases could
11 broaden host range definition, as shown by the local infection capacity of viral
12 chimeras. These findings support the role of viral proteases as important
13 determinants in host adaptation.
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Keywords

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36 P1 proteases, RNA silencing suppression, *Potyviridae*, *Plum pox virus*, *Cucumber*
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39 *vein yellowing virus*, host range
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Introduction

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2 The *Potyviridae* family is a major group of plant viruses, with 177 assigned
3 species distributed in seven monopartite and one bipartite genera (Adams et al.,
4 2012). In the monopartite *Potyvirus* and *Ipomovirus* genera, translation of viral
5 genomic RNA gives rise to a polyprotein that is cleaved into at least 10 different
6 products (López-Moya et al., 2009). A frameshift in the P3 cistron originates an
7 additional polyprotein product, P3N-PIPO (Chung et al., 2008). The polyprotein is
8 processed by leader self-cleaving proteases such as P1 or HCPro, and by the
9 protease domain of NIa, a protein with self- and *trans*-cleaving proteolytic activity
10 found in all *Potyviridae* (Adams et al., 2005). HCPro has protease activity which is
11 independent of host factors (Carrington et al., 1989), and acts as a suppressor of
12 host antiviral RNA silencing defenses, among other functions (Brigneti et al., 1998;
13 Kasschau and Carrington, 1998; Ivanov et al., 2014). Full-length P1 protein needs a
14 still unidentified host factor for its proteolytic activity and has been linked to viral host
15 specificity (Verchot et al., 1992; Salvador et al., 2008; Maliogka et al., 2012b;
16 Rodamilans et al., 2013). Defects in P1 protease activity impair the HCPro silencing
17 suppressor activity and preclude viral infectivity (Pasin et al., 2014b). Infectivity is
18 restored in RNA silencing-defective hosts, as well as when an NIa target site or a 2A
19 “self-cleaving” peptide are inserted between a cleavage-deficient P1 and HCPro
20 (Verchot and Carrington, 1995a; Pasin et al., 2014b).

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Plum pox virus (PPV) is the causative agent of sharka disease, which affects
stone-fruit production worldwide (Cambra et al., 2006). PPV is a representative
member of the genus *Potyvirus* and codes for P1 and HCPro at the N-terminal end
of the viral polyprotein (Šubr and Glasa, 2012; García et al., 2014). In contrast,
Cucumber vein yellowing virus (CVYV; genus *Ipomovirus*) does not include an

1 HCPPro coding sequence in its genome and instead has a tandem of P1-like proteins,
2 P1a and P1b (Janssen et al., 2005; Valli et al., 2006). The latter is an RNA silencing
3 suppressor (RSS) that can functionally replace HCPPro in potyviral infections
4 (Carbonell et al., 2012; Maliogka et al., 2012a). CVYV P1a is phylogenetically
5 related to the P1 sequence of the potyviruses *Papaya ringspot virus* (Valli et al.,
6 2007) and *Zucchini tigré mosaic virus* (Romay et al., 2014). Its function is not
7 known, although some of its roles might resemble those of P1 in PPV (Rodamilans
8 et al., 2013).

9 Experiments with PPV-based constructs, in which P1 and HCPPro were replaced
10 by P1a and P1b of CVYV, highlighted the relevance of the leader serine protease
11 activity on virus infection (Carbonell et al., 2012). Whereas replacement of PPV
12 HCPPro alone by P1b has only a minor effect on PPV chimera infectivity in *Nicotiana*
13 *benthamiana* plants, substitution of the PPV P1-HCPPro cistron by CVYV P1a-P1b
14 considerably reduces PPV accumulation. This P1a interference appears to be
15 absent when the chimeric virus replicates in the CVYV natural host, *Cucumis sativus*
16 (Carbonell et al., 2012).

17 In this study, we generated several protein-expressing constructs and PPV-based
18 chimeras. We used these constructs to analyze the effect of P1a protein processing
19 on viral silencing suppressor activity and on viral infections. The experimental host
20 *N. benthamiana* and the CVYV natural host *C. sativus* were used to assess the
21 relevance of P1 proteins as host range determinants.

Materials and methods

Plant agro-infiltration

Nicotiana benthamiana and *Cucumis sativus* cv. Cadiz (Rijk Zwaan) plants were grown in a greenhouse maintained at a 16 h light/8 h dark photoperiod and a temperature range of 19-23°C. Transient expression and viral cDNA vectors were delivered to plants by agro-infiltration as described (Maliogka et al., 2012a). In each experiment, two independent *Agrobacterium tumefaciens* cultures containing the same set of constructs was delivered in parallel. For fluorescence intensity and protein accumulation measurements, bacterial mixtures were infiltrated into individual plants and samples collected at the indicated time points.

DNA plasmids and constructs

A full-length cDNA copy of a PPV isolate adapted to *Nicotiana*, tagged with sGFP(S65T) and inserted into the pSN-PPV binary plasmid, has been reported (Pasin et al., 2014b), as was the PPV-based vector in which the P1-HCPro sequence was replaced by that of CVYV P1a-P1b (Carbonell et al., 2012). The remaining viral clones were generated by Gibson assembly (Gibson et al., 2009), using SfaAI-linearized pSN-ccdB plasmid as a backbone (Pasin et al., 2014b). Newly generated viral clones are described below.

To generate pSN-PPV P1a, the PPV P1 coding sequence was replaced by those of CVYV P1a, five extra CVYV P1b residues and a GlySerGly linker. To generate pSN-PPV P1a*, the PPV P1 coding sequence was replaced by that of CVYV P1a into which the Ser484 codon AGC was mutated to alanine GCC. To generate pSN-PPV P1aN1a, a sequence coding for the PPV N1a protease cleavage site SNVVVHQ↓ADE was inserted upstream of the PPV HCPro coding sequence in pSN-PPV P1a.

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Transient expression binary vectors pSN.5 PPV (expressing the PPV P1-HCPro sequence), pSN.5 P1-S (expressing the PPV P1 S259A-HCPro sequence) and the intermediate clone pSN2-ccdB have been described (Pasin et al., 2014b). The pSN2-ccdB backbone was digested with XbaI/BstBI, and ligated to SpeI/BstBI fragments from pSN-PPV P1a, pSN-PPV P1a* and pSN-PPV P1aNla to obtain pSN.5 P1a, pSN.5 P1a* and pSN.5 P1aNla, respectively. The pMDC32-NlaPro plasmid that expresses the protease domain of the PPV Nla protein was described (Maliogka et al., 2012a). p35S:GFP and pBIN61-P19 binary vectors expressing GFP and p19, respectively, were reported (Voinnet et al., 2003).

***In vitro* cleavage assay**

The *in vitro* cleavage assay was performed as reported (Pasin et al., 2014b). Briefly, RNA templates were synthesized using the T7-Scribe Standard RNA IVT kit (Cellscript) and translated in the presence of a mixture of L-[³⁵S]methionine and L-[³⁵S]cysteine (PerkinElmer) using the wheat germ extract system (Promega). Samples were resolved in 12% tricine-SDS-PAGE (Schägger and von Jagow, 1987) and the signal detected by phosphorimaging.

GFP fluorescence imaging and quantification

Leaves were placed on a blue light transilluminator (Safe Imager, Invitrogen) and photographed with a Nikon D3X digital camera. Alternatively, images were acquired under an epifluorescence microscope (MZ FLIII, GFP3 filter; Leica) or by confocal laser scanning microscopy (TCS SP5 system, excitation laser at 488 nm and emission bandwidth of 505-549 nm; Leica). GFP fluorescence intensity in agro-infiltrated leaf discs was quantified in a 96-well plate reader as described (Pasin et al., 2014a).

Immunoblot assays

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Liquid nitrogen-frozen plant tissue was ground in a TissueLyzer bead mill (Qiagen). Total proteins were extracted, separated by glycine-SDS-PAGE and electroblotted onto nitrocellulose membrane as reported (Pasin et al., 2014b). Proteins were detected using anti-GFP monoclonal antibody (clones 7.1 and 13.1, Roche), anti-PPV coat protein (CP) and -PPV HCPro rabbit sera as primary antibodies; horseradish peroxidase-conjugated sheep anti-mouse IgG (GE Healthcare) or goat anti-rabbit IgG (Jackson) were used as secondary antibody. For signal quantification, chemiluminescence was acquired in a ChemiDoc XRS imager (BioRad) and analyzed with ImageJ software (Schneider et al., 2012).

Results

***N. benthamiana*: Cis-supply of CVYV P1a impairs HCPro silencing suppression activity which is restored by Nla protease-mediated cleavage**

CVYV P1a interferes with the RNA silencing suppression activity of CVYV P1b in *N. benthamiana* agro-infiltration assays (Carbonell et al., 2012). Here, as HCPro is the reference potyviral RNA silencing suppressor (RSS) (Brigneti et al., 1998; Kasschau and Carrington, 1998), we designed a set of constructs to test the effect of P1a processing on HCPro silencing suppression activity when P1a was located upstream of the PPV HCPro sequence (Fig. 1A). Transient silencing assays that use a GFP construct as both silencing trigger and reporter were performed in *N. benthamiana*, as described (Voinnet et al., 2003).

In PPV, replacement of the P1 catalytic serine (S259) by an alanine (Fig. 1A) impairs P1 processing from the P1-HCPro cistron and silencing suppression activity (Pasin et al., 2014b). We identified the corresponding catalytic residue in CVYV P1a by sequence analysis (Supplementary Fig. 1A, B) and confirmed that a S484A mutation in P1a abrogates its protease activity (Supplementary Fig. 1C). The serine protease mutant of P1a fused to HCPro, plasmid P1a*, was therefore used as negative control in a transient RNA silencing assay; the P1 plasmid bearing the coding sequences of P1 and HCPro from PPV was used as positive control (Fig. 1A). The wild-type P1a sequence cloned upstream of HCPro was evaluated alone (P1a construct) or with the insertion of an extra cleavage site recognized by the PPV Nla protease (P1aNla construct; Fig. 1A). In all cases, the PPV Nla protease domain was co-expressed to provide *trans*-cleavage activity (Maliogka et al., 2012a). Constructs were delivered to *N. benthamiana*, and accumulation of green fluorescence protein (GFP) was measured at six days post-agro-infiltration

1 (dpa; Fig. 1B). At this time, only the co-delivery of functional suppressors could
2 alleviate the host RNA silencing activation and allow high levels of transient reporter
3 expression (Voinnet et al., 2003).
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7 As anticipated, the P1 construct released a functional HCPro RSS and allowed
8 high levels of GFP accumulation, which were significantly higher than those
9 observed for the P1a protease mutant construct (P1a*; Fig. 1B, C). The wild-type
10 P1a construct did not sustain reporter protein accumulation and behaved like the
11 negative control. In contrast, inclusion of an Nla cleavage site between wild-type
12 P1a and HCPro (P1aNla) significantly increased GFP accumulation, as shown by
13 fluorescence intensity levels similar to those of the positive control (Fig. 1B, C). The
14 results were supported by GFP immunoblot analysis of protein extracts from the
15 agro-infiltrated leaves (6 dpa; Fig. 1D). There were no significant differences (p
16 >0.05) between P1a constructs when the Nla protease-expressing strain was not
17 included in agro-infiltration mixes; neither P1aNla nor P1a plasmids enhanced GFP
18 accumulation (Supplementary Fig. 2).
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36 ***N. benthamiana*: CVYV P1a shows incomplete catalytic activity and the p19** 37 **silencing suppressor complements P1a defects** 38 39

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41 P1a processing from the different P1a-HCPro precursors was tested by co-
42 infiltrating *N. benthamiana* plants with the strong RSS p19 from *Tomato bushy stunt*
43 *virus*, to provide an even silencing suppression effect across samples. GFP was
44 included as suppressor activity reporter, and fluorescence was detected at 6 dpa
45 (Fig. 2A). Anti-HCPro polyclonal antibody was used in immunoblot analysis to detect
46 mature HCPro as well as uncleaved protein precursors in the protein extracts (6 dpa;
47 Fig. 2B). We included a construct encoding a cleavage-deficient P1 mutant (P1*
48 construct, Fig. 1A) as background control. Anti-HCPro immunoblotting of P1*
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1 samples showed a major band whose electrophoretic mobility was consistent with
2 the predicted size of the unprocessed P1-HCPro fusion product (87.5 kDa). In the
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4 case of P1 and P1aNIa construct samples, we detected an intense band
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6 corresponding to the size of free HCPro (52.2 kDa), with faint bands fitting the
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8 polyprotein precursors (Fig. 2B). In extracts from leaves expressing the P1a
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10 construct that lacks the NIa cleavage site, we observed bands corresponding to
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12 HCPro (52.2 kDa) and to the P1a-HCPro unprocessed product (113.6 kDa) (Fig. 2B).
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14 These results indicate that both PPV P1 and NIa proteases outperform CVYV P1a
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16 processivity in *N. benthamiana*, in agreement with previous results (Carbonell et al.,
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18 2012).
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24 As in protease-deficient P1 mutants (Pasin et al., 2014b), incomplete self-
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26 cleavage of P1a in *N. benthamiana* might affect activity of a downstream RSS and
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28 infectivity of P1a-containing viral clones. In a transient infection assay, supply of p19
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30 RSS allowed propagation of a suppressor-deficient *Turnip mosaic virus* clone
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32 (Garcia-Ruiz et al., 2010). To test whether P1a self-cleavage defects are attenuated
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34 by a heterologous suppressor supply, we generated a chimeric PPV cDNA clone
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36 (PPV-P1a*) that expresses the protease-deficient P1a S484A mutant rather than P1.
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38 This viral construct was co-delivered to *N. benthamiana* with the p19 or an empty
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40 vector (Φ ; Fig. 2C, D). The PPV-P1a* clone was non-infectious, as shown by
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42 absence of GFP fluorescence and PPV coat protein (CP) accumulation in leaves co-
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44 infiltrated with the empty vector (Fig. 2C, D). As predicted and consistent with RSS
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46 defects, p19 co-expression rescued infectivity of the PPV-P1a* clone, since GFP
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48 fluorescence intensity and CP levels in PPV-P1a* samples co-infiltrated with p19
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50 were significantly higher than those of samples co-infiltrated with the empty vector
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52 control (Fig. 2C, D).
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***N. benthamiana*: Viral accumulation of chimeric PPV bearing P1a is increased in the presence of an extra Nla cleavage site between P1a and HCPro**

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5 In the previous experiment, the effectiveness of p19 supply to support PPV-P1a*
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7 amplification was restricted to agro-infiltrated tissue that expresses the RSS
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9 (Fig. 2C). As Nla protease is coded by the PPV genome, an engineered Nla
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11 cleavage site might rescue P1a self-cleavage defects and allow continuous HCPro
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13 release during viral infections. To test this hypothesis, we built several PPV-based
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15 chimeric constructs equivalent to the P1a-HCPro constructs used in the transient
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17 expression assays (Fig. 1, 2). In this experiment (and below), we used a PPV-
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19 P1aP1b chimeric virus (Carbonell et al., 2012) in which the P1-HCPro cistron from
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21 PPV was replaced by the P1aP1b cistron from CVYV as control. All viral chimeras
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23 were delivered to *N. benthamiana* plants by agro-inoculation and systemic spread
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25 was monitored by GFP fluorescence (Fig. 3A). Viral CP and mature HCPro
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27 accumulations in inoculated and upper tissue (at 10 and 14 dpa, respectively) were
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29 assessed by anti-CP and anti-HCPro immunoblot analyses (Fig. 3B, C). In
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31 accordance with results shown in Fig. 2, plants challenged with the PPV cDNA clone
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33 carrying the P1a protease mutant showed no sign of local or systemic infection
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35 (PPV-P1a*; Fig. 3). Replacement of the P1 sequence with wild-type CVYV P1a
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37 protein (PPV-P1a construct) severely impaired viral accumulation, and only a faint
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39 CP signal was detected by immunoblot analysis of upper leaves (Fig. 3C). Inclusion
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41 of an Nla cleavage site between wild-type P1a and HCPro significantly enhanced
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43 viral accumulation (Fig. 3B, C). Finally, the PPV-P1aP1b chimera accumulated at
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45 lower levels compared to the wild-type PPV clone, as reported (Carbonell et al.,
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C. sativus: Cis-supply of CVYV P1a effectively sustains HCPro silencing suppressor activity and boosts PPV-based chimera accumulation in local infections

CVYV P1a proteolytic activity might require one or more still-unidentified host factors (Rodamilans et al., 2013). The natural host of CVYV is cucumber, which might supply co-factor(s) necessary for optimal P1a self-cleavage. Our results showed that inefficient P1a processing impairs HCPro silencing suppressor activity in *N. benthamiana* (Fig. 1, 2). To test P1a effects on suppression activity in a CVYV natural host, we performed a transient silencing assay in *C. sativus*. GFP was transiently co-expressed with P1a-HCPro (P1a construct) in cucumber leaves; the p19 and empty (Φ) vectors were used as positive and negative controls, respectively. Immunoblot analysis of agro-infiltrated tissue at 6 dpa showed that P1a co-expression sustained GFP accumulation to levels significantly higher than those observed in leaves containing the empty vector (Fig. 4A). Similar GFP levels were detected when the reporter was co-expressed with the P1a construct or the p19 RSS positive control (Fig. 4A). As an additional control, the experiment was performed in parallel in *N. benthamiana* plants using the same agro-infiltration cultures. In this case, as in *C. sativus*, p19 co-expression sustained high levels of GFP protein in the agro-infiltrated leaves (Fig. 4B). In accordance with the earlier results (Fig. 1), we found little GFP accumulation in leaves expressing the P1a construct, which did not differ significantly from that of the empty vector samples (Fig. 4B).

We showed that incomplete P1a self-cleavage precludes the efficient amplification of PPV P1a in *N. benthamiana* (Fig. 2, 3). Based on cucumber silencing suppression assay results, we anticipated that deleterious CVYV P1a effects on viral infections would be attenuated in a CVYV natural host. The PPV-based chimeras

1 used to agro-inoculate *N. benthamiana* plants were therefore tested in cucumber
2 (Fig. 4C). To assess and compare the infectivity of the distinct constructs, the agro-
3 inoculated leaves were analyzed by GFP fluorescence monitoring and anti-PPV CP
4 immunoblotting at 8 dpa (Fig. 4C). In contrast to results in the *N. benthamiana*
5 infection assays (Fig. 3), cucumber leaves agro-infiltrated with the wild-type PPV
6 construct showed no appreciable GFP fluorescence or CP signals, similar to the
7 negative control samples agro-infiltrated with the PPV-P1a* clone (Fig. 4C). Leaves
8 inoculated with chimeric viruses bearing the P1a and HCPro sequences (PPV-P1a
9 and PPV-P1aN1a) showed GFP fluorescence and accumulated relevant amounts of
10 CP, regardless of the inclusion of an N1a cleavage site between P1a and HCPro
11 (Fig. 4C). In agreement with previous results (Carbonell et al., 2012), leaves
12 agroinfiltrated with the PPV-P1aP1b construct appeared to accumulate more CP
13 (Fig. 4C). In our experimental conditions, cucumber infections with PPV-based
14 chimeras could only be detected locally, suggesting that host-specific viral
15 movement determinants are present elsewhere in potyviral genomes.
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Discussion

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2 Some viruses of the genus *Ipomovirus*, such as CVYV and SqVYV (*Squash vein*
3 *yellowing virus*), lack HCPro and have two P1-like proteins, P1a and P1b (Valli et al.,
4 2007; Li et al., 2008). The specific function of P1a is not known, but the replacement
5 of PPV P1-HCPro cistron by CVYV P1a-P1b disturbs PPV infections in the PPV host
6 *N. benthamiana* (Carbonell et al., 2012). In this host, levels of P1b expressed from a
7 P1a-P1b construct were much lower than those of P1b expressed alone. The
8 authors hypothesized that P1a self-cleavage activity is suboptimal in *N.*
9 *benthamiana*, and that inefficient P1a self-release impairs accumulation and function
10 of a downstream RSS (Carbonell et al., 2012).
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24 Using HCPro, the reference potyviral RSS, we show that engineering an artificial
25 protease cleavage site between P1a and HCPro enables efficient P1a release from
26 HCPro and restores HCPro suppressor activity in *N. benthamiana* (Fig. 1). To
27 further elucidate P1a-dependent defects, we agro-infiltrated *N. benthamiana* plants
28 in the presence of p19 to overcome the reduced suppressor activity observed in
29 some of the constructs tested. Indeed, co-expression of this RSS with each
30 construct permitted detection of fusion products that could not otherwise be
31 observed (Fig. 2B). This indicates that free HCPro amounts released by P1a are
32 insufficient to counteract the *N. benthamiana* silencing response, or that non-
33 functional P1a-HCPro fusion products might have dominant negative effects. Such
34 effects appear to be unlikely, however, since transgenic plants that express the P1-
35 HCPro cistron can restore viability of *Tobacco etch potyvirus* (TEV) clones altered by
36 mutations in the P1 protease (Verchot and Carrington, 1995b).
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56 In the context of viral infection, lack of P1a-HCPro separation by a protease-
57 inactivating mutation precludes viability of a PPV chimera. Co-expression of a
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1 heterologous suppressor is reported to sustain accumulation of viral clones with non-
2 functional suppressor proteins (Garcia-Ruiz et al., 2010). We show that in a
3 transient infection assay, co-delivery of p19 rescued infectivity of the PPV chimera
4 that expresses a P1a self-cleavage mutant (Fig. 2). These and above results link
5 P1a self-cleavage defects to silencing suppressor failures.
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11 The inclusion of an Nla cleavage site sequence between a protease-deficient P1
12 mutant and HCPro restores infectivity of a TEV clone (Verchot and Carrington,
13 1995a). Our results demonstrate that a similar Nla-based strategy significantly
14 enhances infection efficiency of the PPV-P1a chimera, which codes for a wild-type
15 leader protease. Although the P1a sequence included in our clones has two amino
16 acid differences (Glu293Asp, Arg432Cys) compared to the sequence variability of
17 CVYV genomic accessions (GenBank: YP_224077.1 and AEP81236.1), its self-
18 cleavage activity was demonstrated (Valli et al., 2006; Rodamilans et al., 2013).
19 Moreover, transient RSS assays in cucumber (the CVYV natural host) showed that
20 the P1a-HCPro construct behaves similarly to the strong suppressor p19, and
21 sustains accumulation of the GFP reporter product. This observation is consistent
22 with accumulation of the PPV-P1a chimera in this host, which was greater than that
23 of wild-type PPV and somewhat independent of the addition of a Nla cleavage site
24 between P1a and HCPro. In *N. benthamiana*, PPV-P1a chimera accumulated
25 poorly, however, indicating that P1a is released efficiently from HCPro in cucumber
26 but not in *N. benthamiana*. These findings suggest that cucumber provides the co-
27 factor(s) needed for optimal P1a self-cleavage, and directly implicates P1a and
28 P1-like proteases in host range definition, as proposed (Valli et al., 2007; Salvador et
29 al., 2008; Rodamilans et al., 2013). Replacement of HCPro by P1b in PPV-based
30 chimeras appears to favor viral accumulation in *C. sativus* (Fig. 4C and Carbonell et
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al. 2012). Since HCPro suppressor activity is maintained in *C. sativus* (Fig. 4A), it is possible that an unknown P1b function (other than RSS) is responsible for the increased infectivity in this host.

These data support the idea that to ensure correct viral amplification, CVYV P1a must be effectively separated from either P1b or HCPro, whether mediated by its own protease domain or by a protease that cleaves *in trans*. P1a self-cleavage efficiency is host-dependent, and lack of processing would impair downstream RSS activity and viral accumulation. The host-dependent regulation of protease efficiency was previously suggested to control viral virulence and defense responses (Lackner et al., 2004; Pasin et al., 2014b), and a gene swapping approach of phylogenetically related leader proteases revealed a marked degree of functional specialization (Peng et al., 2001). Sequence evolution of an NIa protease recognition site is reported to be critical for PPV alternative adaptation to cherry and *N. benthamiana* (Calvo et al., 2014). Here we show that leader proteases and polyprotein processing are important determinants in viral speciation and host-range definition. Future identification of the P1a and P1 activating effector(s) will help to better understand potyviral biology and ultimately, to design more effective antiviral strategies.

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Figure legends

Fig. 1. Effect of upstream CVYV P1a on HCPro silencing suppressor activity in *Nicotiana benthamiana*. (A) Scheme of the plasmids used in transient agro-infiltration experiments. Stars mark serine-to-alanine mutations in the protease catalytic domains. (B) GFP fluorescence in a single leaf agro-infiltrated with four different constructs. Picture taken on a blue light transilluminator at 6 days post-agro-infiltration (dpa). (C) GFP fluorescence intensity (FI) of agro-infiltrated leaves was quantified in a 96-well plate reader, at 6 dpa. Relative FI was plotted using P1 mean value equal to 100. Bar graph shows mean \pm SEM ($n = 16$); the difference between the results marked with different letters is statistically significant, $p < 0.01$, one-way Anova and Tukey's HSD test. (D) Anti-GFP and -HCPro immunoblot of protein extracts from agro-infiltrated tissue collected at 6 dpa. Each lane corresponds to a sample pool from one or two agro-infiltrated plants; a Ponceau red-stained blot (Rbcl) is shown as loading control of protein extracts.

Fig. 2. Proteolytic processing of P1/P1a-HCPro products and cleavage-defect complementation by a heterologous silencing suppressor in *N. benthamiana*.

(A, B) *N. benthamiana* plants were co-infiltrated with plasmids expressing GFP, the N1a protease domain and p19 RSS plus the corresponding experimental constructs, and analyzed at 6 dpa. (A) Pictures of agro-infiltrated leaves taken on a blue light transilluminator. (B) Anti-HCPro immunoblotting of protein extracts from the agro-infiltrated tissue. On the left, scheme of free HCPro protein (52.2 kDa) and unprocessed products (P1-HCPro, 87.5 kDa; P1a-HCPro, 113.6 kDa); molecular weight markers are indicated (right; in kDa). Each lane corresponds to a sample pool from two agro-infiltrated plants. (C, D) The p19 or an empty vector (Φ) were co-expressed by agro-infiltration with PPV-P1a*, a PPV cDNA clone in which P1 was

replaced by the P1a S484A cleavage-deficient mutant. (C) Images of transient infection taken under an epifluorescence microscope at 6 dpa. GFP FI was quantified in a 96-well plate reader. Relative GFP signal intensities are indicated using p19 mean value equal to 100; bar graph shows mean \pm SEM ($n = 8$). The difference between the values is statistically significant ($p < 0.001$, Student's t -test). (D) Viral accumulation was assessed by anti-PPV coat protein (CP) immunoblotting of protein extracts from agroinfiltrated tissue at 6 dpa. Each lane corresponds to a sample pool from a single agro-infiltrated plant; Ponceau red-stained blot (RbcL) is shown as loading control of protein extracts.

Fig. 3. Viral accumulation of PPV-based chimeras expressing CVYV P1a in *N. benthamiana*. PPV-based chimeras were delivered to *N. benthamiana* plants by agro-inoculation, and samples were analyzed at 10 dpa (LOCAL, infiltrated leaves) and 14 dpa (SYSTEMIC, upper non-inoculated leaves). (A) Pictures taken on a blue light transilluminator. (B, C) Anti-CP and -HCPro immunoblot analyses of protein extracts from infiltrated or upper non-inoculated leaves; the difference in CP signal intensity between the indicated samples is statistically significant ($p < 0.01$, Student's t -test). Each lane corresponds to a sample pool from one or two agro-infiltrated plants; Ponceau red-stained blots (RbcL) are shown as loading control of protein extracts.

Fig. 4. Effects of CVYV P1a on HCPro silencing suppression activity and viral accumulation in cucumber leaves. (A, B) Transient expression assays were performed in *Cucumis sativus* (A) and, in a parallel experiment, in *N. benthamiana* leaves (B). A GFP-expressing construct was co-infiltrated with the empty vector (Φ) or the P1a-HC construct (P1a); the p19 construct was used as positive control. Samples were analyzed at 6 dpa. Images of *C. sativus* samples were taken under a

1 confocal microscope; scale bars, 200 μ m. GFP accumulation was assessed by anti-
2 GFP immunoblot; the difference in GFP signal intensity between the indicated
3 samples is statistically significant ($p < 0.05$) or not significant (n.s.), by Student's
4 *t*-test. (C) PPV-based chimeras were delivered to cucumber plants by agro-
5 inoculation. At 8 dpa, confocal microscopy pictures were taken (scale bars, 50 μ m)
6 and local inoculated tissue was collected. Viral accumulation was determined by
7 anti-CP immunoblotting. In all panels, each lane corresponds to a sample pool from
8 a single agroinfiltrated plant; Ponceau red-stained blots (RbcL) are shown as loading
9 control of protein extracts.

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22 **Supplementary Fig. 1. Sequence analysis of P1-like proteins from several**
23 ***Potyviridae*, and *in vitro* cleavage activity of CVYV P1a.** (A, B) CVYV P1a
24 sequence (GenBank: YP_224077.1) was aligned with serine protease sequences
25 from several members of the *Potyviridae* family: SqVYV_P1a, *Squash vein yellowing*
26 *ipomovirus* P1a (GenBank: YP_001788992.1); PRSV_P1, *Papaya ringspot potyvirus*
27 P1 (GenBank: NP_734233.1); ZTMV_P1, *Zucchini tigré mosaic potyvirus* P1
28 (GenBank: YP_008992245.1); PPV_P1, *Plum pox potyvirus* P1 reference sequence
29 characterized in (Pasin et al., 2014b). A phylogenetic tree is shown in (A). Partial
30 alignment of protease domains is shown in (B); in each row, the positions of the first
31 and last amino acids of the corresponding accession are indicated, and the CVYV
32 P1a catalytic serine 484 is marked with a star. (C) CVYV P1a self-cleavage was
33 evaluated by *in vitro* translation assay using the wheat germ extract system.
34 Labeled translation products were resolved by tricine-SDS-PAGE and the 35 S signal
35 detected; molecular weight markers are indicated (left; in kDa). In the wild-type P1a
36 sample (WT), polyprotein self-processing releases a \approx 60 kDa mature P1a protein
37 and a \approx 20 kDa C-terminal peptide. Alanine replacement of the catalytic S484
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residue (S484A) precludes processing of the ≈ 80 kDa polyprotein precursor.

Supplementary Fig. 2. HCPPro silencing suppressor activity in P1a-containing constructs in the absence of Nla co-expression. (A) GFP fluorescence in a single leaf agro-infiltrated with four different constructs. Picture taken on a blue light transilluminator, at 6 dpa. (B) GFP FI of the agro-infiltrated leaf patches at 6 dpa quantified in a 96-well plate reader. Relative FI was plotted using P1 mean value equal to 100. Bar graph shows mean \pm SEM ($n = 16$); the difference between the results marked with different letters is statistically significant, $p < 0.01$, one-way Anova and Tukey's HSD test. (C) Anti-GFP and -HCPPro immunoblotting of agro-infiltrated tissue at 6 dpa. Each lane corresponds to a sample pool from one or two agro-infiltrated plants; a Ponceau red-stained blot (RbcL) is shown as loading control of protein extracts.

References

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- Adams, M.J., Antoniw, J.F., Beaudoin, F., 2005. Overview and analysis of the polyprotein cleavage sites in the family *Potyviridae*. *Mol. Plant Pathol.* 6, 471–487. doi:10.1111/j.1364-3703.2005.00296.x
- Adams, M.J., Zerbini, F.M., French, R., Rabenstein, F., Stenger, D.C., Valkonen, J.P.T., 2012. Family *Potyviridae*, in: *Virus Taxonomy*. Elsevier, San Diego, CA, USA. pp. 1069–1089.
- Brigneti, G., Voinnet, O., Li, W.-X., Ji, L.-H., Ding, S.-W., Baulcombe, D.C., 1998. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO J.* 17, 6739–6746. doi:10.1093/emboj/17.22.6739
- Calvo, M., Malinowski, T., García, J.A., 2014. Single amino acid changes in the 6K1-CI region can promote the alternative adaptation of *Prunus*- and *Nicotiana*-propagated *Plum pox virus* C isolates to either host. *Mol. Plant. Microbe Interact.* 27, 136–149. doi:10.1094/MPMI-08-13-0242-R
- Cambra, M., Capote, N., Myrta, A., Llácer, G., 2006. *Plum pox virus* and the estimated costs associated with sharka disease. *EPPO Bull.* 36, 202–204. doi:10.1111/j.1365-2338.2006.01027.x
- Carbonell, A., Dujovny, G., García, J.A., 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. *Mol. Plant. Microbe Interact.* 25, 151–164. doi:10.1094/MPMI-08-11-0216
- Carrington, J.C., Freed, D.D., Sanders, T.C., 1989. Autocatalytic processing of the potyvirus helper component proteinase in *Escherichia coli* and in vitro. *J. Virol.* 63, 4459–4463.
- Chung, B.Y.-W., Miller, W.A., Atkins, J.F., Firth, A.E., 2008. An overlapping essential gene in the Potyviridae. *Proc. Natl. Acad. Sci. U. S. A.* 105, 5897–5902. doi:10.1073/pnas.0800468105
- García, J.A., Glasa, M., Cambra, M., Candresse, T., 2014. *Plum pox virus* and sharka: a model potyvirus and a major disease. *Mol. Plant Pathol.* 15, 226–241. doi:10.1111/mps.12083
- García-Ruiz, H., Takeda, A., Chapman, E.J., Sullivan, C.M., Fahlgren, N., Brempelis, K.J., Carrington, J.C., 2010. *Arabidopsis* RNA-dependent RNA polymerases and Dicer-like proteins in antiviral defense and small interfering RNA biogenesis during *Turnip mosaic virus* infection. *Plant Cell* 22, 481–496. doi:10.1105/tpc.109.073056
- Gibson, D.G., Young, L., Chuang, R.-Y., Venter, J.C., Hutchison III, C.A., Smith, H.O., 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* 6, 343–345. doi:10.1038/nmeth.1318
- Ivanov, K.I., Eskelin, K., Löhmus, A., Mäkinen, K., 2014. Molecular and cellular mechanisms underlying potyvirus infection. *J. Gen. Virol.* 95, 1415–1429. doi:10.1099/vir.0.064220-0
- Janssen, D., Martín, G., Velasco, L., Gómez, P., Segundo, E., Ruiz, L., Cuadrado, I.M., 2005. Absence of a coding region for the helper component-proteinase in the genome of cucumber vein yellowing virus, a whitefly-transmitted member of the *Potyviridae*. *Arch. Virol.* 150, 1439–1447. doi:10.1007/s00705-005-0515-z

- 1 Kasschau, K.D., Carrington, J.C., 1998. A counterdefensive strategy of plant viruses:
2 suppression of posttranscriptional gene silencing. *Cell* 95, 461–470.
3 doi:10.1016/S0092-8674(00)81614-1
- 4 Lackner, T., Müller, A., Pankraz, A., Becher, P., Thiel, H.-J., Gorbalenya, A.E.,
5 Tautz, N., 2004. Temporal modulation of an autoprotease is crucial for
6 replication and pathogenicity of an RNA virus. *J. Virol.* 78, 10765–10775.
7 doi:10.1128/JVI.78.19.10765-10775.2004
- 8 Li, W., Hilf, M.E., Webb, S.E., Baker, C.A., Adkins, S., 2008. Presence of P1b and
9 absence of HC-Pro in Squash vein yellowing virus suggests a general feature
10 of the genus *Ipomovirus* in the family *Potyviridae*. *Virus Res.* 135, 213–219.
- 11 López-Moya, J.J., Valli, A., García, J.A., 2009. *Potyviridae*, in: *Encyclopedia of Life*
12 *Sciences*. John Wiley & Sons, Ltd, pp. 1–10.
- 13 Maliogka, V.I., Calvo, M., Carbonell, A., García, J.A., Valli, A., 2012a. Heterologous
14 RNA-silencing suppressors from both plant- and animal-infecting viruses
15 support plum pox virus infection. *J. Gen. Virol.* 93, 1601–1611.
16 doi:10.1099/vir.0.042168-0
- 17 Maliogka, V.I., Salvador, B., Carbonell, A., Sáenz, P., San León, D., Oliveros, J.C.,
18 Delgadillo, M.O., García, J.A., Simón-Mateo, C., 2012b. Virus variants with
19 differences in the P1 protein coexist in a *Plum pox virus* population and
20 display particular host-dependent pathogenicity features. *Mol. Plant Pathol.*
21 13, 877–886. doi:10.1111/j.1364-3703.2012.00796.x
- 22 Pasin, F., Kulasekaran, S., Natale, P., Simón-Mateo, C., García, J.A., 2014a. Rapid
23 fluorescent reporter quantification by leaf disc analysis and its application in
24 plant-virus studies. *Plant Methods* 10, 22. doi:10.1186/1746-4811-10-22
- 25 Pasin, F., Simón-Mateo, C., García, J.A., 2014b. The hypervariable amino-terminus
26 of P1 protease modulates potyviral replication and host defense responses.
27 *PLoS Pathog.* 10, e1003985. doi:10.1371/journal.ppat.1003985
- 28 Peng, C.-W., Peremyslov, V.V., Mushegian, A.R., Dawson, W.O., Dolja, V.V., 2001.
29 Functional specialization and evolution of leader proteinases in the family
30 *Closteroviridae*. *J. Virol.* 75, 12153–12160. doi:10.1128/JVI.75.24.12153-
31 12160.2001
- 32 Rodamilans, B., Valli, A., García, J.A., 2013. Mechanistic divergence between P1
33 proteases of the family *Potyviridae*. *J. Gen. Virol.* 94, 1407–1414.
34 doi:10.1099/vir.0.050781-0
- 35 Romay, G., Lecoq, H., Desbiez, C., 2014. Zucchini tigré mosaic virus is a distinct
36 potyvirus in the papaya ringspot virus cluster: molecular and biological
37 insights. *Arch. Virol.* 159, 277–289. doi:10.1007/s00705-013-1798-0
- 38 Salvador, B., Saézn, P., Yangüez, E., Quiot, J.B., Quiot, L., Delgadillo, M.O., García,
39 J.A., Simón-Mateo, C., 2008. Host-specific effect of P1 exchange between
40 two potyviruses. *Mol. Plant Pathol.* 9, 147–155. doi:10.1111/j.1364-
41 3703.2007.00450.x
- 42 Schägger, H., von Jagow, G., 1987. Tricine-sodium dodecyl sulfate-polyacrylamide
43 gel electrophoresis for the separation of proteins in the range from 1 to 100
44 kDa. *Anal. Biochem.* 166, 368–379. doi:10.1016/0003-2697(87)90587-2
- 45 Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25
46 years of image analysis. *Nat. Methods* 9, 671–675. doi:10.1038/nmeth.2089
- 47 Šubr, Z., Glasa, M., 2012. Unfolding the secrets of plum pox virus: from
48 epidemiology to genomics. *Acta Virol.* 57, 217–228.
49 doi:10.4149/av_2013_02_217
- 50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1 Valli, A., López-Moya, J.J., García, J.A., 2007. Recombination and gene duplication
2 in the evolutionary diversification of P1 proteins in the family *Potyviridae*. *J.*
3 *Gen. Virol.* 88, 1016–1028. doi:10.1099/vir.0.82402-0
- 4 Valli, A., Martín-Hernández, A.M., López-Moya, J.J., García, J.A., 2006. RNA
5 silencing suppression by a second copy of the P1 serine protease of
6 *Cucumber vein yellowing ipomovirus*, a member of the family *Potyviridae* that
7 lacks the cysteine protease HCPPro. *J. Virol.* 80, 10055–10063.
8 doi:10.1128/JVI.00985-06
- 9 Verchot, J., Carrington, J.C., 1995a. Debilitation of plant potyvirus infectivity by P1
10 proteinase-inactivating mutations and restoration by second-site
11 modifications. *J. Virol.* 69, 1582–1590.
- 12 Verchot, J., Carrington, J.C., 1995b. Evidence that the potyvirus P1 proteinase
13 functions in *trans* as an accessory factor for genome amplification. *J. Virol.* 69,
14 3668–3674.
- 15 Verchot, J., Herndon, K.L., Carrington, J.C., 1992. Mutational analysis of the tobacco
16 etch potyviral 35-kDa proteinase: identification of essential residues and
17 requirements for autoproteolysis. *Virology* 190, 298–306. doi:10.1016/0042-
18 6822(92)91216-H
- 19 Voinnet, O., Rivas, S., Mestre, P., Baulcombe, D., 2003. An enhanced transient
20 expression system in plants based on suppression of gene silencing by the
21 p19 protein of tomato bushy stunt virus. *Plant J.* 33, 949–956.
22 doi:10.1046/j.1365-313X.2003.01676.x
- 23
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Figure 1
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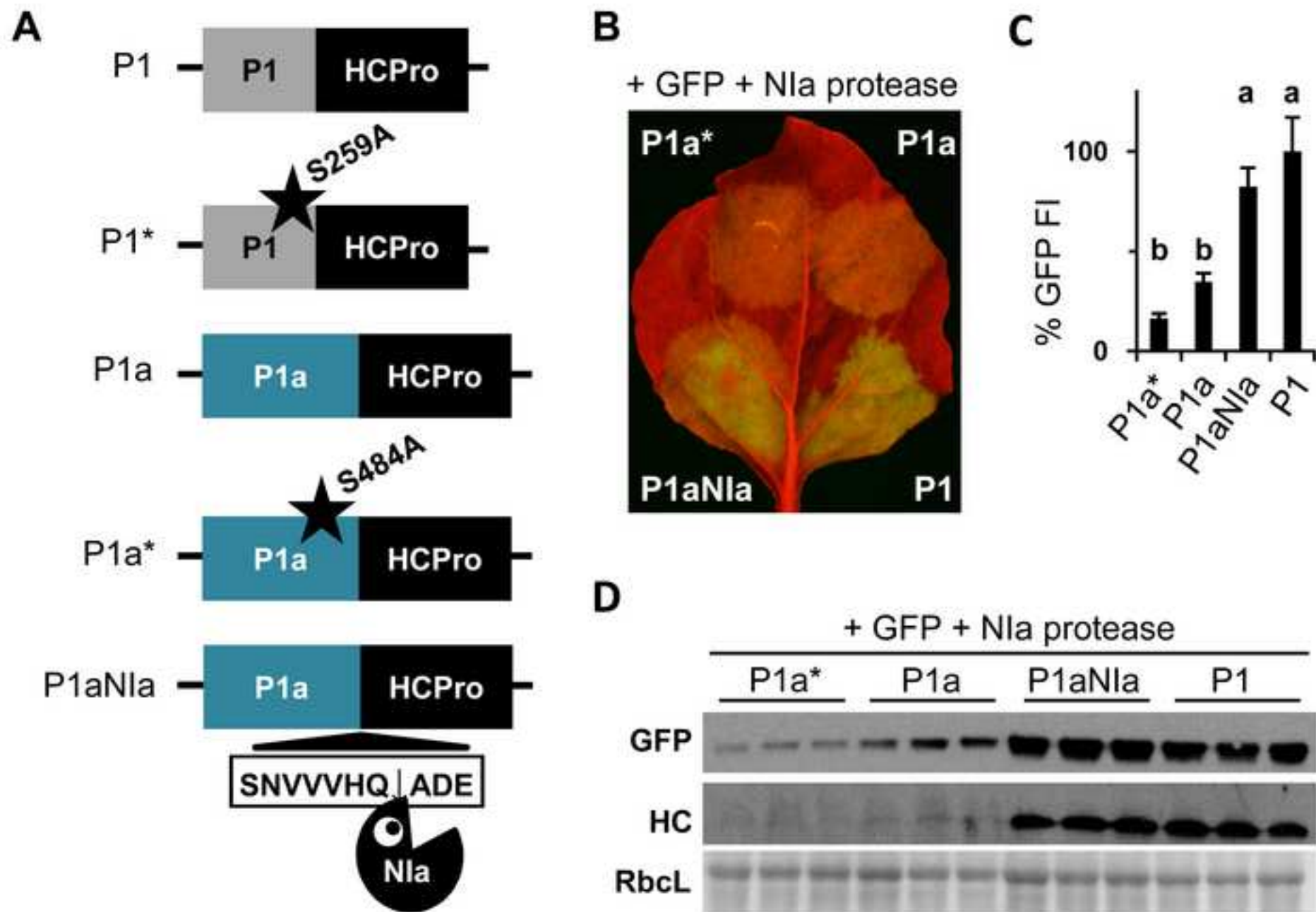


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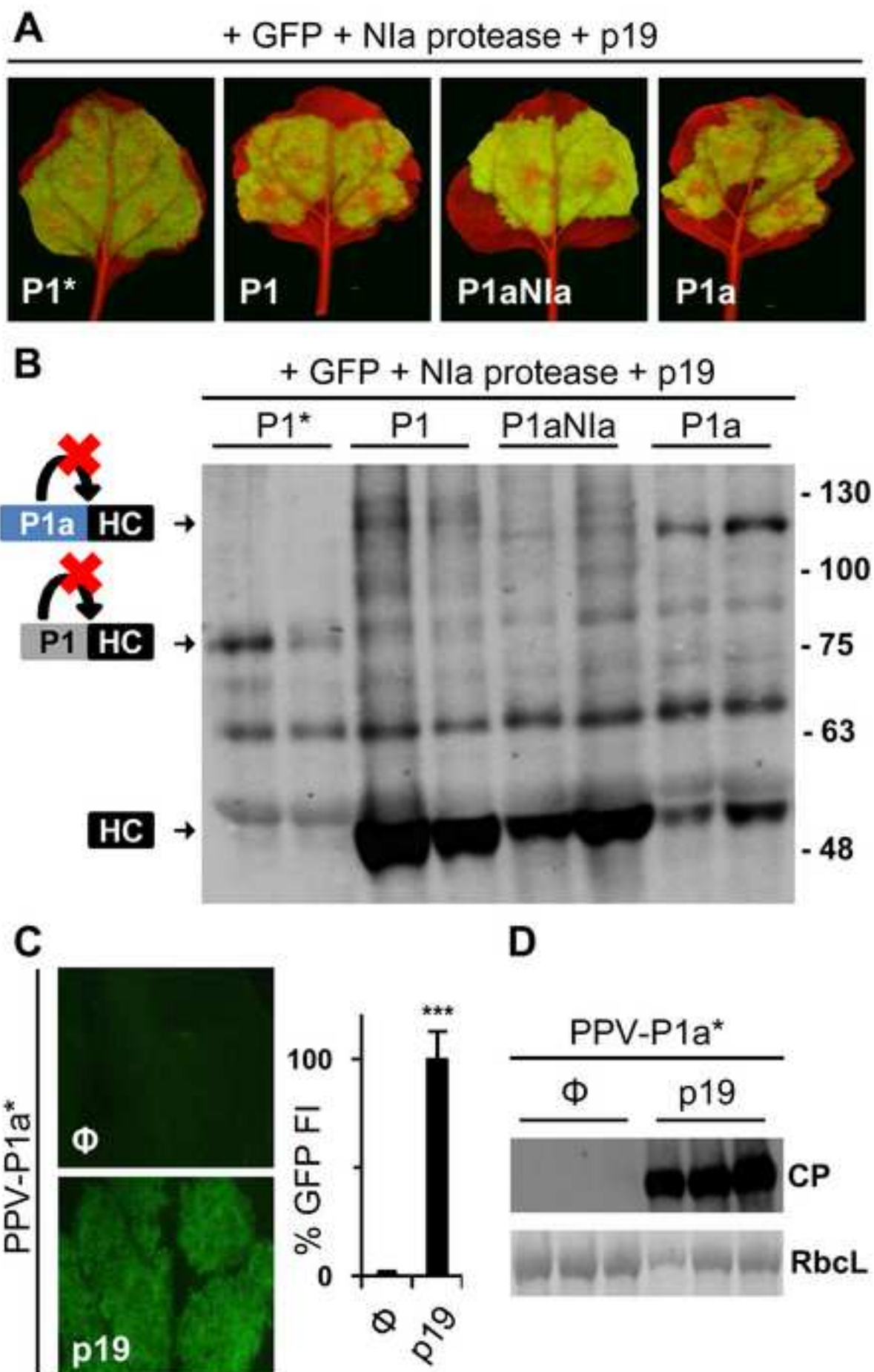


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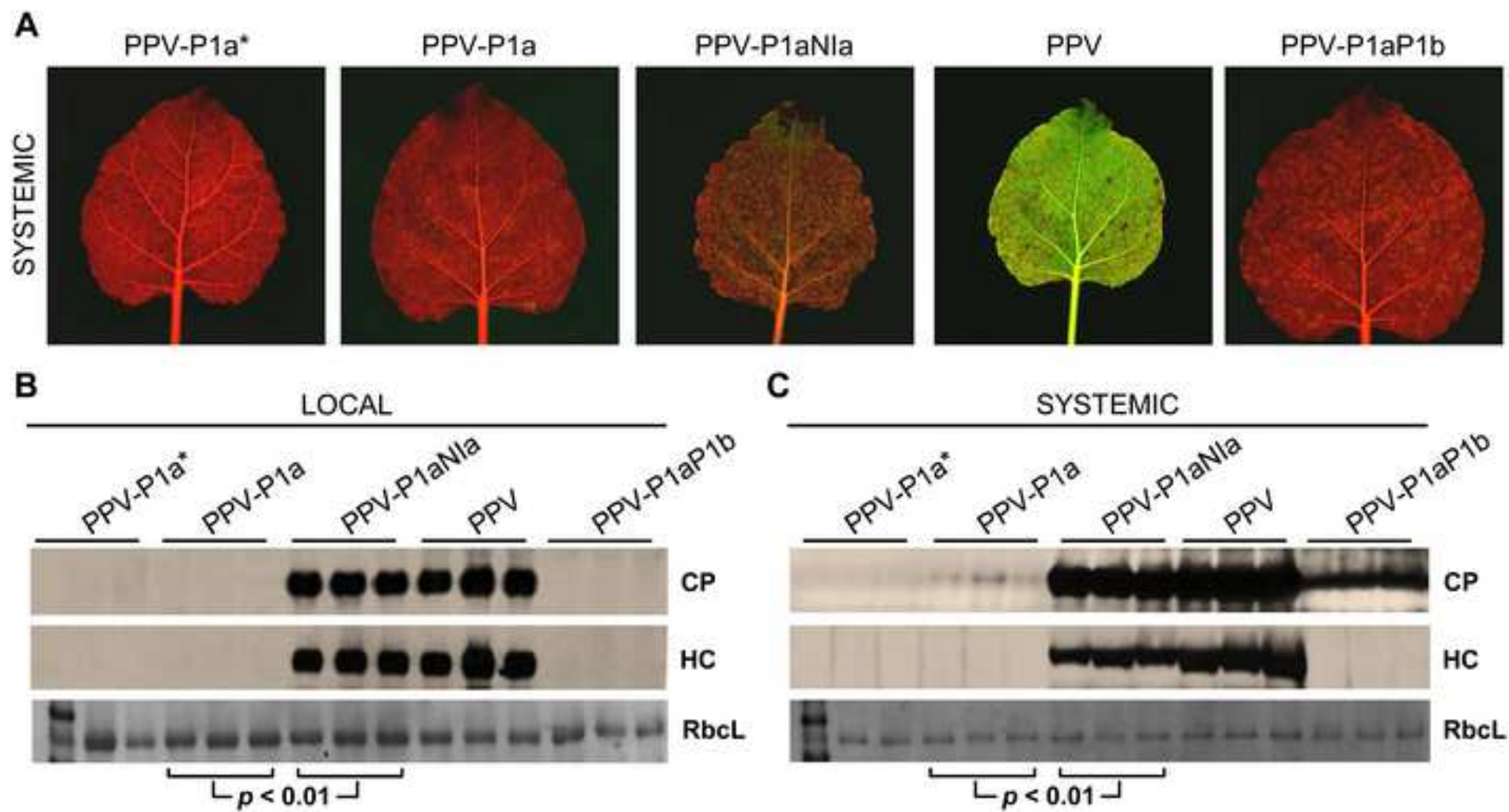
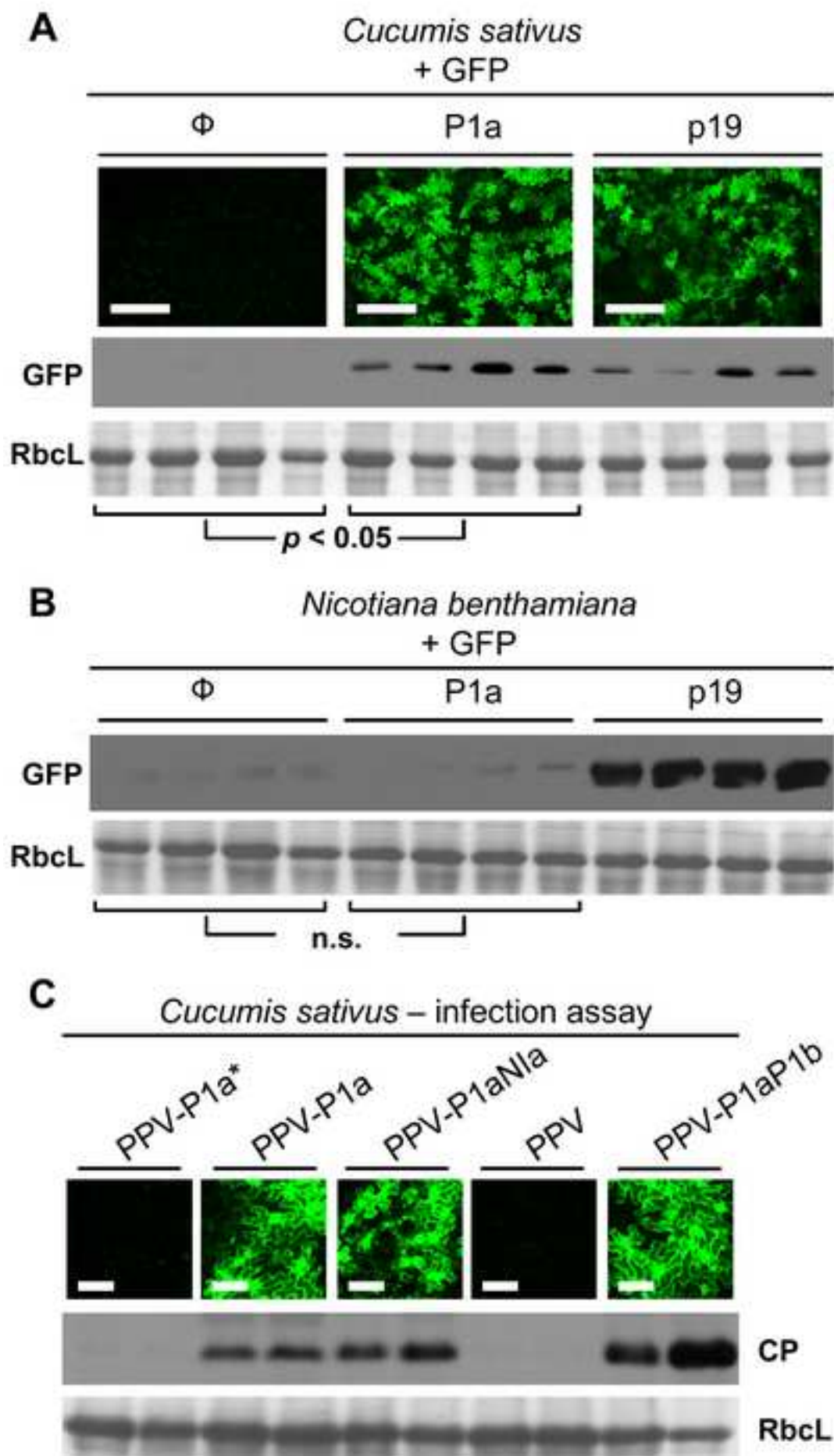
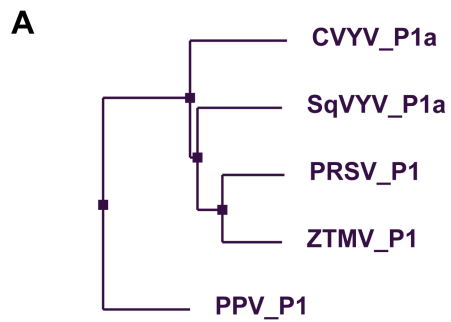


Figure 4
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CVYV_P1a	481	P	G	T	S	G	L	I	487
SqVYV_P1a	495	R	G	D	S	G	S	I	501
PRSV_P1	496	F	G	S	S	G	L	I	502
ZTMV_P1	492	K	G	S	S	G	L	I	498
PPV_P1	256	P	G	M	S	G	F	V	262

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