Virology 476 (2015) 264–270

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

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

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ARTICLE INFO

Article history:
Received 31 October 2014
Returned to author for revisions
24 November 2014
Accepted 7 December 2014
Available online 3 January 2015

Keywords:
P1 proteases
RNA silencing suppression
Potyviridae
Plum pox virus
Cucumber vein yellowing virus
Host range

ABSTRACT

The P1a protein of the ipomovirus *Cucumber vein yellowing virus* is one of the self-cleavage serine proteases present in *Potyviridae* family members. P1a is located at the N-terminal end of the viral polyprotein, and is closely related to potyviral P1 protease. For its proteolytic activity, P1a requires a still unknown host factor; this might be linked to involvement in host specificity. Here we built a series of constructs and chimeric viruses to help elucidate the role of P1a cleavage in host range definition. We demonstrate that host-dependent separation of P1a from the remainder of the polyprotein is essential for suppressing RNA silencing defenses and for efficient viral infection. These findings support the role of viral proteases as important determinants in host adaptation.

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Introduction

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

Plum pox virus (PPV) is the causative agent of sharka disease, which affects stone-fruit production worldwide (Cambra et al., 2006; Herrera, 2013). 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 HCPro coding sequence in its genome and instead has a tandem of P1-like proteins, P1a and P1b (Janssen et al., 2005; Valli et al., 2006). The latter is an RNA silencing suppressor (RSS) that can functionally replace HCPro in potyviral infections (Carbonell et al., 2012; Maliogka et al., 2012a). CVYV P1a is phylogenetically related to the P1 sequence of the potyviruses *Papaya ringspot virus* (Valli et al., 2007) and *Zucchini tigré mosaic virus* (Romay et al., 2014). Its function is not known, although some of its roles might resemble those of P1 in PPV (Rodamilans et al., 2013).

Experiments with PPV-based constructs, in which P1 and HCPro were replaced by P1a and P1b of CVYV, highlighted the relevance of

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http://dx.doi.org/10.1016/j.virol.2014.12.013
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the leader serine protease activity on virus infection (Carbonell et al., 2012). Whereas replacement of PPV HCPro alone by P1b has only a minor effect on PPV chimera infectivity in *Nicotiana benthamiana* plants, substitution of the PPV P1-HCPro cistron by CVYV P1a-P1b considerably reduces PPV accumulation. This P1a interference appears to be absent when the chimeric virus replicates in the CVYV natural host, *Cucumis sativus* (Carbonell et al., 2012).

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

Results

N. benthamiana: cis-supply of CVYV P1a impairs HCPro silencing suppression activity which is restored by NIa 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 and B) and confirmed that an 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 NIa protease (P1aNIa construct; Fig. 1A). In all cases, the PPV NIa 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 (dpa; Fig. 1B). At this time, only the co-delivery of functional suppressors could alleviate the host RNA silencing activation and allow high levels of transient reporter expression (Voinnet et al., 2003).

As anticipated, the P1 construct released a functional HCPro RSS and allowed high levels of GFP accumulation, which were significantly higher than those observed for the P1a protease mutant construct (P1a*; Fig. 1B and C). The wild-type P1a construct did not sustain reporter protein accumulation and behaved like the negative control. In contrast, inclusion of an NIa cleavage site between wild-type P1a and HCPro (P1aNIa) significantly increased GFP accumulation, as shown by fluorescence intensity levels similar to those of the positive control (Fig. 1B and C). The results were supported by GFP immunoblot analysis of protein extracts from the agro-infiltrated leaves (6 dpa; Fig. 1D). There were no significant differences ($p > 0.05$) between P1a constructs when the NIa protease-expressing strain was not included in agro-infiltration mixes; neither P1aNIa nor P1a plasmids enhanced GFP accumulation (Supplementary Fig. 2).

N. benthamiana: CVYV P1a shows incomplete catalytic activity and the p19 silencing suppressor complements P1a defects

P1a processing from the different P1a-HCPro precursors was tested by co-infiltrating *N. benthamiana* plants with the strong RSS p19 from *Tomato bushy stunt virus*, to provide an even silencing suppression effect across samples and increase recombinant protein

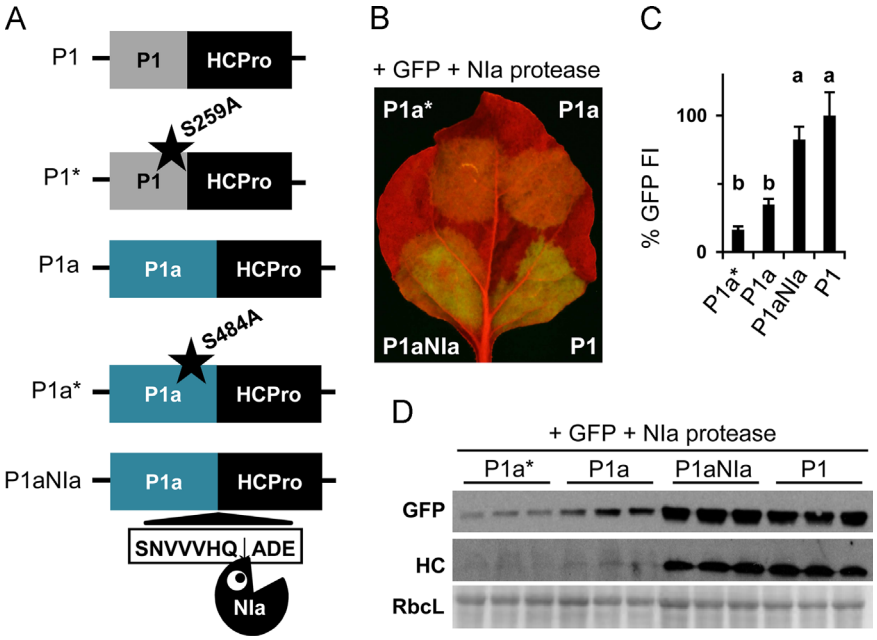

Fig. 1. Effect of upstream CVYV P1a on HCPro silencing suppressor activity in *Nicotiana benthamiana*. (A) Scheme of the plasmids used in transient agro-infiltration experiments. Stars mark serine-to-alanine mutations in the protease catalytic domains. (B) GFP fluorescence in a single leaf agro-infiltrated 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 ± 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.

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