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

Hongying Shan [1], Fabio Pasin [*,1], Adrián Valli [2], Carla Castillo [3], Charukesi Rajulu, Alberto Carbonell [4], Carmen Simón-Mateo, Juan Antonio García [*], Bernardo Rodamilans [*]

Departamento de Genética Molecular de Plantas, Centro Nacional de Biotecnología (CNB-CSIC), Darwin 3, 28049 Madrid, Spain

ARTICLE INFO

Article history:
Received 31 October 2014
Returned to author for revisions
24 November 2014
Accepted 7 December 2014

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.

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

* Corresponding authors. Tel.: +34 91 585 4535; fax: +34 915854506.
E-mail addresses: fpasin@cnb.csic.es (F. Pasin), jagarcia@cnb.csic.es (J.A. García), brodamilans@cnb.csic.es (B. Rodamilans).
[1] These two authors equally contributed to this work.
[2] Present address: Department of Plant Sciences, University of Cambridge, Cambridge CB2 3EA, UK.
[3] Present address: Laboratorio de Biotecnología y Desarrollo, Instituto Nacional de Higiene "Rafael Rangel", Caracas, Venezuela.
[4] Present address: Donald Danforth Plant Science Center, St. Louis, MO 63132, USA.

http://dx.doi.org/10.1016/j.virol.2014.12.013
0042-6822/

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

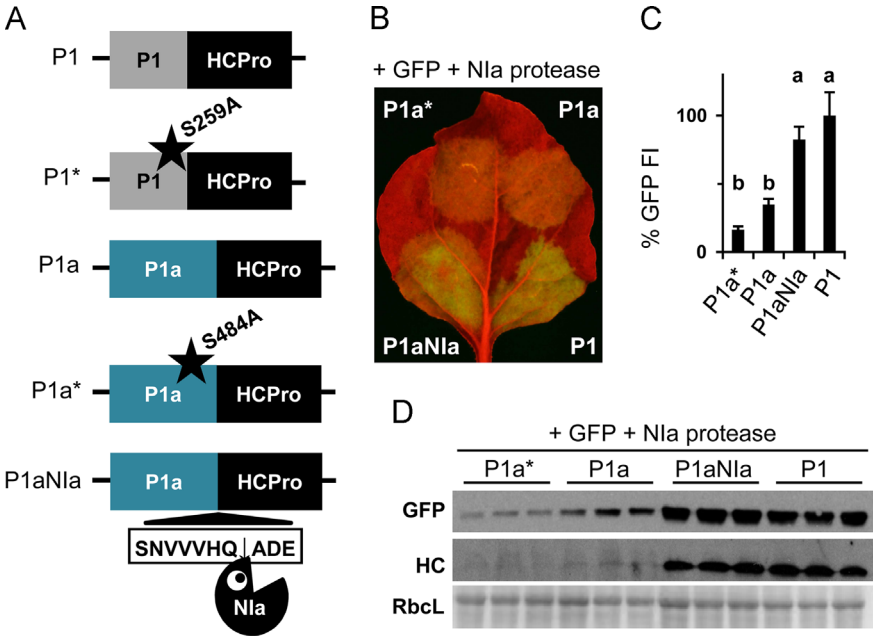

Fig. 1. Effect of upstream CVYV P1a on HCPro silencing suppressor activity in *Nicotiana benthamiana*. (A) Scheme of the plasmids used in transient agro-infiltration experiments. Stars mark serine-to-alanine mutations in the protease catalytic domains. (B) GFP fluorescence in a single leaf agro-infiltrated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

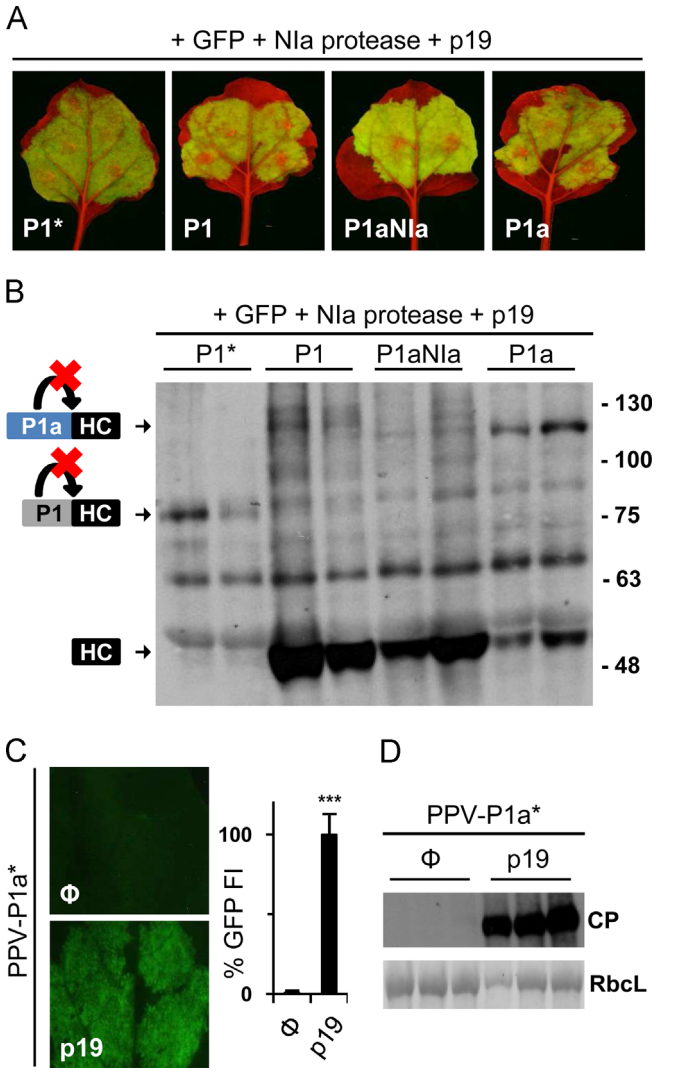

Fig. 2. Proteolytic processing of P1/P1a-HCPro products and cleavage-defect complementation by a heterologous silencing suppressor in *N. benthamiana*. (A and B) *N. benthamiana* plants were co-infiltrated with plasmids expressing GFP, the NIa protease domain and p19 RSS plus the corresponding experimental 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 and 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 ± 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.

yields. GFP was included as suppressor activity reporter, and fluorescence was detected at 6 dpa (Fig. 2A). Anti-HCPro polyclonal antibody was used in immunoblot analysis to detect mature HCPro as well as uncleaved protein precursors in the protein extracts (6 dpa; Fig. 2B). We included a construct encoding a cleavage-deficient P1 mutant (P1* construct, Fig. 1A) as background control. Anti-HCPro immunoblotting of P1* samples showed a major band whose electrophoretic mobility was consistent with the predicted size of the unprocessed P1-HCPro fusion product (87.5 kDa). In the case of P1 and P1aNIa construct samples, we detected an intense band corresponding to the size of free HCPro (52.2 kDa), with faint bands fitting the polyprotein precursors (Fig. 2B). In extracts from leaves expressing the P1a construct that lacks the NIa cleavage site, we observed bands corresponding to HCPro (52.2 kDa) and to the P1a-HCPro unprocessed product (113.6 kDa) (Fig. 2B). These results indicate that both PPV P1 and NIa proteases outperform CVYV P1a processivity in *N. benthamiana*, in agreement with previous results (Carbonell et al., 2012).

As in protease-deficient P1 mutants (Pasin et al., 2014b), incomplete self-cleavage of P1a in *N. benthamiana* might affect activity of a downstream RSS and infectivity of P1a-containing viral clones. In a transient infection assay, supply of p19 RSS allowed propagation of a suppressor-deficient *Turnip mosaic virus* clone (Garcia-Ruiz et al., 2010). To test whether P1a self-cleavage defects are attenuated by a heterologous suppressor supply, we generated a chimeric PPV cDNA clone (PPV-P1a*) that expresses the protease-deficient P1a S484A mutant rather than P1. This viral construct was co-delivered to *N. benthamiana* with the p19 or an empty vector (Φ; Fig. 2C and D). The PPV-P1a* clone was non-infectious, as shown by absence of GFP fluorescence and PPV coat protein (CP) accumulation in leaves co-infiltrated with the empty vector (Fig. 2C and D). As predicted and consistent with RSS defects, p19 co-expression rescued infectivity of the PPV-P1a* clone, since GFP fluorescence intensity and CP levels in PPV-P1a* samples co-infiltrated with p19 were significantly higher than those of samples co-infiltrated with the empty vector control (Fig. 2C and D).

N. benthamiana: viral accumulation of chimeric PPV bearing P1a is increased in the presence of an extra NIa cleavage site between P1a and HCPro

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

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

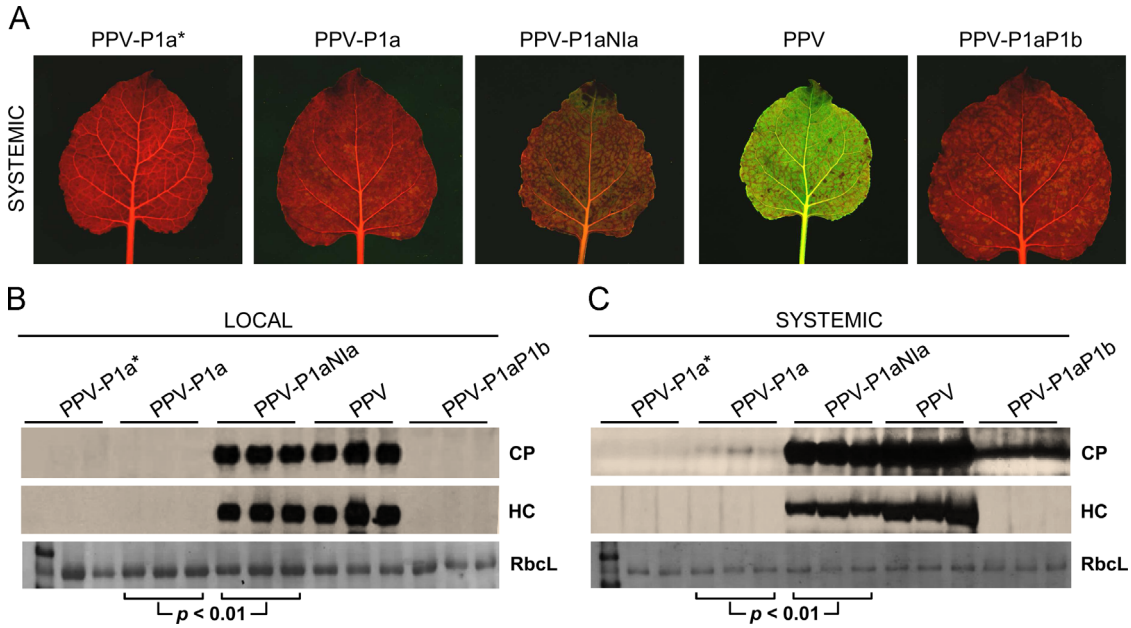

Fig. 3. Viral accumulation of PPV-based chimeras expressing CVYV P1a in *N. benthamiana*. PPV-based chimeras were delivered to *N. benthamiana* plants by agro-inoculation, and samples were analyzed at 10 dpa (LOCAL, infiltrated leaves) and 14 dpa (SYSTEMIC, upper non-inoculated leaves). (A) Pictures 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.

that inefficient P1a processing impairs HCPro silencing suppressor activity in *N. benthamiana* (Figs. 1 and 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* (Figs. 2 and 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 used to agro-inoculate *N. benthamiana* plants were therefore tested in cucumber (Fig. 4C). To assess and compare the infectivity of the distinct constructs, the agro-inoculated leaves were analyzed by GFP fluorescence monitoring and anti-PPV CP immunoblotting at 8 dpa (Fig. 4C). In contrast to results in the *N. benthamiana* infection assays (Fig. 3), cucumber leaves agro-infiltrated with the wild-type PPV construct showed no appreciable GFP fluorescence or CP signals, similar to the negative control samples agro-infiltrated with the PPV-P1a* clone (Fig. 4C). Leaves inoculated with chimeric viruses bearing the P1a and HCPro sequences (PPV-P1a and PPV-P1aNIa) showed GFP fluorescence and accumulated relevant amounts of CP, regardless of the inclusion of an NIa cleavage site between P1a and HCPro (Fig. 4C). In agreement with previous results (Carbonell et al., 2012), leaves agroinfiltrated with the PPV-P1aP1b construct appeared to accumulate more CP (Fig. 4C). Under our experimental conditions, cucumber infections with PPV-based chimeras could only be detected locally, suggesting that host-specific viral movement determinants are present elsewhere in potyviral genomes.

Discussion

Some viruses of the genus *Ipomovirus*, such as CVYV and SqVYV (*Squash vein yellowing virus*), lack HCPro and have two P1-like proteins, P1a and P1b (Valli et al., 2007; Li et al., 2008). The specific function of P1a is not known, but the replacement of PPV P1-HCPro cistron by CVYV P1a-P1b disturbs PPV infections in the PPV host *N. benthamiana* (Carbonell et al., 2012). In this host, levels of P1b expressed from a P1a-P1b construct were much lower than those of P1b expressed alone. The authors hypothesized that P1a self-cleavage activity is suboptimal in *N. benthamiana*, and that inefficient P1a self-release impairs accumulation and function of a downstream RSS (Carbonell et al., 2012).

Using HCPro, the reference potyviral RSS, we show that engineering an artificial protease cleavage site between P1a and HCPro enables efficient P1a release from HCPro and restores HCPro suppressor activity in *N. benthamiana* (Fig. 1). To further elucidate P1a-dependent defects, we agro-infiltrated *N. benthamiana* plants in the presence of p19 to overcome the reduced suppressor activity observed in some of the constructs tested. Indeed, co-expression of this RSS with each construct permitted detection of fusion products that could not otherwise be observed (Fig. 2B). This indicates that free HCPro amounts released by P1a are insufficient to counteract the *N. benthamiana* silencing response, or that non-functional P1a-HCPro fusion products might have dominant negative effects. Such effects appear to be unlikely, however, since transgenic plants that express the P1-HCPro cistron can restore viability of *Tobacco etch virus* (TEV) clones altered by mutations in the P1 protease (Verchot and Carrington, 1995b).

In the context of viral infection, lack of P1a-HCPro separation by a protease-inactivating mutation precludes viability of a PPV

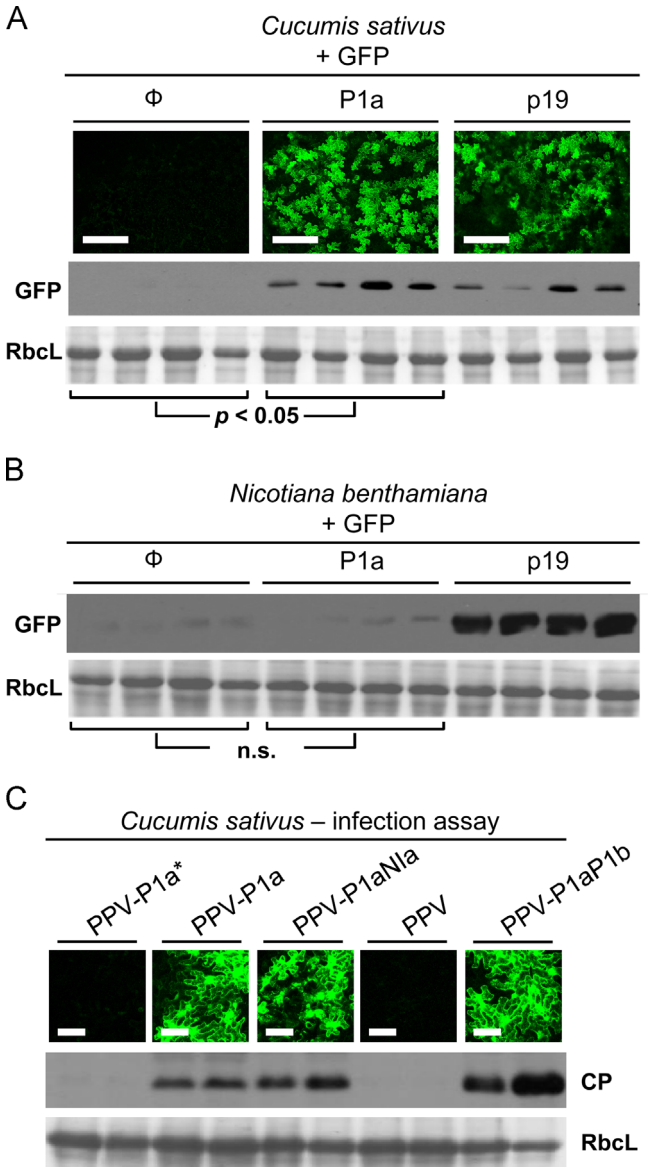

Fig. 4. Effects of CVYV P1a on HCPro silencing suppression activity and viral accumulation in cucumber leaves. (A and B) Transient expression assays were performed in *Cucumis sativus* (A) and, in a parallel experiment, in *N. benthamiana* leaves (B). A GFP-expressing construct was co-infiltrated with 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 confocal microscope; scale bars, 200 μm. GFP accumulation was assessed by anti-GFP immunoblot; the difference in GFP signal intensity between the indicated samples is statistically significant ($p < 0.05$) or not significant (n.s.), by Student's *t*-test. (C) PPV-based chimeras were delivered to cucumber plants by agro-inoculation. At 8 dpa, confocal microscopy pictures were taken (scale bars, 50 μm) and local inoculated tissue was collected. Viral accumulation was determined by anti-CP immunoblotting. In all panels, each lane corresponds to a sample pool from a single agroinfiltrated plant; Ponceau red-stained blots (RbcL) are shown as loading control of protein extracts.

chimera. Co-expression of a heterologous suppressor is reported to sustain accumulation of viral clones with non-functional suppressor proteins (Garcia-Ruiz et al., 2010). We show that in a transient infection assay, co-delivery of p19 rescued infectivity of the PPV chimera that expresses a P1a self-cleavage mutant (Fig. 2). These and above results strongly link P1a self-cleavage defects to silencing suppressor failures. Although RSS malfunction seems to be the major infectivity constraint of the PPV-P1a chimeras, absence of specific P1 function(s) cannot be ruled out as contributor to this effect.

The inclusion of an NIa cleavage site sequence between a protease-deficient P1 mutant and HCPro restores infectivity of a TEV clone (Verchot and Carrington, 1995a). Our results demonstrate that a similar NIa-based strategy significantly enhances infection efficiency of the PPV-P1a chimera, which codes for a wild-type leader protease. Although the P1a sequence included in our clones has two amino acid differences (Glu293Asp, Arg432Cys; GenBank: DQ496114) compared to the sequence variability of CVYV genomic accessions (GenBank: YP_224077.1 and AEP81236.1), its self-cleavage activity was demonstrated (Valli et al., 2006; Rodamilans et al., 2013). Moreover, transient RSS assays in cucumber (the CVYV natural host) showed that the P1a-HCPro construct behaves similarly to the strong suppressor p19, and sustains accumulation of the GFP reporter product. This observation is consistent with accumulation of the PPV-P1a chimera in this host, which was greater than that of wild-type PPV and somewhat independent of the addition of an NIa cleavage site between P1a and HCPro. In *N. benthamiana*, PPV-P1a chimera accumulated poorly, however, indicating that P1a is released efficiently from HCPro in cucumber but not in *N. benthamiana*. These findings suggest that cucumber provides the co-factor(s) needed for optimal P1a self-cleavage, and directly implicates P1a and P1-like proteases in host range definition, as proposed (Valli et al., 2007; Salvador et al., 2008; Rodamilans et al., 2013). P1 was recently suggested to participate in viral polyprotein translation and host defense responses (Martínez and Daròs, 2014; Pasin et al., 2014b), it would be of interest to determine whether P1a has similar positive roles during cucumber infections.

Replacement of HCPro by P1b in PPV-based chimeras appears to favor viral accumulation in *C. sativus* (Fig. 4C and Carbonell et 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.

Materials and methods

Plant agro-infiltration

N. benthamiana and *C. 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). CVYV P1a sequence was amplified from pDONR207-CVYV-P1stop plasmid (Valli et al., 2006; GenBank: DQ496114). 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 P1aNIa, a sequence coding for the PPV NIa protease cleavage site SNVVVHQ↓ADE was inserted upstream of the PPV HCPro coding sequence in pSN-PPV P1a.

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 P1aNIa to obtain pSN.5 P1a, pSN.5 P1a* and pSN.5 P1aNIa, respectively. The pMDC32-NIaPro plasmid that expresses the protease domain of the PPV NIa 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-[^{35}S]methionine and L-[^{35}S]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

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).

Acknowledgments

We are grateful to B. García for technical assistance, to D. Baulcombe for supply of p35S:GFP and pBIN61-P19 plasmids, and to the Rijk Zwaan company for providing cucumber seeds. We acknowledge C. Mark for editorial assistance.

H.S. is supported by the China Scholarship Council; F.P. and C.R. were financed by La Caixa PhD fellowships, and C.C. by a scholarship from the Spanish government (MAEC-AECID). This work was funded by Grants BIO2013-49053-R and Plant-KBBE PCIN-2013-056 from the Spanish Ministerio de Economía y Competitividad.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.12.013.

References

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. http://dx.doi.org/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, 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. http://dx.doi.org/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. http://dx.doi.org/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. http://dx.doi.org/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. http://dx.doi.org/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. USA 105, 5897–5902. http://dx.doi.org/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. http://dx.doi.org/10.1111/mpp.12083.

Garcia-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. http://dx.doi.org/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. http://dx.doi.org/10.1038/nmeth.1318.

Herrera, G., 2013. Investigations of the *Plum pox virus* in Chile in the past 20 years. Chil. J. Agric. Res. 73, 60–65. http://dx.doi.org/10.4067/S0718-58392013000100009.

Ivanov, K.I., Eskelin, K., Lõhmus, A., Mäkinen, K., 2014. Molecular and cellular mechanisms underlying potyvirus infection. J. Gen. Virol. 95, 1415–1429. http://dx.doi.org/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. http://dx.doi.org/10.1007/s00705-005-0515-z.

Kasschau, K.D., Carrington, J.C., 1998. A counterdefensive strategy of plant viruses: suppression of posttranscriptional gene silencing. Cell 95, 461–470. http://dx.doi.org/10.1016/S0092-8674(00)81614-1.

Lackner, T., Müller, A., Pankraz, A., Becher, P., Thiel, H.-J., Gorbalenya, A.E., Tautz, N., 2004. Temporal modulation of an autoprotease is crucial for replication and pathogenicity of an RNA virus. J. Virol. 78, 10765–10775. http://dx.doi.org/10.1128/JVI.78.19.10765-10775.2004.

Li, W., Hilf, M.E., Webb, S.E., Baker, C.A., Adkins, S., 2008. Presence of P1b and absence of HC-Pro in Squash vein yellowing virus suggests a general feature of the genus *Ipomovirus* in the family *Potyviridae*. Virus Res. 135, 213–219.

López-Moya, J.J., Valli, A., García, J.A., 2009. Potyviridae, Encyclopedia of Life Sciences. John Wiley & Sons, Ltd, Chichester, pp. 1–10.

Maliogka, V.I., Calvo, M., Carbonell, A., García, J.A., Valli, A., 2012a. Heterologous RNA-silencing suppressors from both plant- and animal-infecting viruses support plum pox virus infection. J. Gen. Virol. 93, 1601–1611. http://dx.doi.org/10.1099/vir.0.042168-0.

Maliogka, V.I., Salvador, B., Carbonell, A., Sáenz, P., San León, D., Oliveros, J.C., Delgadillo, M.O., García, J.A., Simón-Mateo, C., 2012b. Virus variants with differences in the P1 protein coexist in a Plum pox virus population and display particular host-dependent pathogenicity features. Mol. Plant Pathol. 13, 877–886. http://dx.doi.org/10.1111/j.1364-3703.2012.00796.x.

Martínez, F., Daròs, J.-A., 2014. *Tobacco etch virus* protein P1 traffics to the nucleolus and associates with the host 60S ribosomal subunits during infection. J. Virol. 88, 10725–10737. http://dx.doi.org/10.1128/JVI.00928-14.

Pasin, F., Kulasekaran, S., Natale, P., Simón-Mateo, C., García, J.A., 2014a. Rapid fluorescent reporter quantification by leaf disc analysis and its application in plant-virus studies. Plant Methods 10, 22. http://dx.doi.org/10.1186/1746-4811-10-22.

Pasin, F., Simón-Mateo, C., García, J.A., 2014b. The hypervariable amino-terminus of P1 protease modulates potyviral replication and host defense responses. PLoS Pathog. 10, e1003985. http://dx.doi.org/10.1371/journal.ppat.1003985.

Peng, C.-W., Peremyslov, V.V., Mushegian, A.R., Dawson, W.O., Dolja, V.V., 2001. Functional specialization and evolution of leader proteinases in the family *Closteroviridae*. J. Virol. 75, 12153–12160. http://dx.doi.org/10.1128/JVI.75.24.12153-12160.2001.

Rodamilans, B., Valli, A., García, J.A., 2013. Mechanistic divergence between P1 proteases of the family *Potyviridae*. J. Gen. Virol. 94, 1407–1414. http://dx.doi.org/10.1099/vir.0.050781-0.

Romay, G., Lecoq, H., Desbiez, C., 2014. Zucchini tigré mosaic virus is a distinct potyvirus in the papaya ringspot virus cluster: molecular and biological insights. Arch. Virol. 159, 277–289. http://dx.doi.org/10.1007/s00705-013-1798-0.

Salvador, B., Saénz, P., Yangüez, E., Quiot, J.B., Quiot, L., Delgadillo, M.O., García, J.A., Simón-Mateo, C., 2008. Host-specific effect of P1 exchange between two potyviruses. Mol. Plant Pathol. 9, 147–155. http://dx.doi.org/10.1111/j.1364-3703.2007.00450.x.

Schägger, H., von Jagow, G., 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. 166, 368–379. http://dx.doi.org/10.1016/0003-2697(87)90587-2.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675. http://dx.doi.org/10.1038/nmeth.2089.

Šubr, Z., Glasa, M., 2012. Unfolding the secrets of plum pox virus: from epidemiology to genomics. Acta Virol. 57, 217–228. http://dx.doi.org/10.4149/av_2013_02_217.

Valli, A., López-Moya, J.J., García, J.A., 2007. Recombination and gene duplication in the evolutionary diversification of P1 proteins in the family *Potyviridae*. J. Gen. Virol. 88, 1016–1028. http://dx.doi.org/10.1099/vir.0.82402-0.

Valli, A., Martín-Hernández, A.M., López-Moya, J.J., García, J.A., 2006. RNA silencing suppression by a second copy of the P1 serine protease of *Cucumber vein yellowing ipomovirus*, a member of the family *Potyviridae* that lacks the cysteine protease HCPro. J. Virol. 80, 10055–10063. http://dx.doi.org/10.1128/JVI.00985-06.

Verchot, J., Carrington, J.C., 1995a. Debilitation of plant potyvirus infectivity by P1 proteinase-inactivating mutations and restoration by second-site modifications. J. Virol. 69, 1582–1590.

Verchot, J., Carrington, J.C., 1995b. Evidence that the potyvirus P1 proteinase functions in trans as an accessory factor for genome amplification. J. Virol. 69, 3668–3674.

Verchot, J., Herndon, K.L., Carrington, J.C., 1992. Mutational analysis of the tobacco etch potyviral 35-kDa proteinase: identification of essential residues and requirements for autoproteolysis. Virology 190, 298–306. http://dx.doi.org/10.1016/0042-6822(92)91216-H.

Voinnet, O., Rivas, S., Mestre, P., Baulcombe, D., 2003. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J. 33, 949–956. http://dx.doi.org/10.1046/j.1365-313X.2003.01676.x.