Use of pentapeptide-insertion scanning mutagenesis for functional mapping of the plum pox virus helper component proteinase suppressor of gene silencing

Mark Varrelmann,1 Edgar Maiss,2 Ruth Pilot1 and Laszlo Palkovics3

Correspondence
Mark Varrelmann
mvarrel@gwdg.de
1Department of Crop Science, Section Plant Virology, University of Göttingen, Grisebachstraße 6, D-37077 Göttingen, Germany
2Institute of Plant Diseases and Plant Protection, University of Hannover, Herrenhaeuser Str. 2, D-30419 Hannover, Germany
3Department of Plant Pathology, Faculty of Horticultural Science, Corvinus University Budapest, H-1118 Budapest, Hungary

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Helper component proteinase (HC-Pro) of Plum pox virus is a multifunctional potyvirus protein that has been examined intensively. In addition to its involvement in aphid transmission, genome amplification and long-distance movement, it is also one of the better-studied plant virus suppressors of RNA silencing. The first systematic analysis using pentapeptide-insertion scanning mutagenesis of the silencing suppression function of a potyvirus HC-Pro is presented here. Sixty-three in-frame insertion mutants, each containing five extra amino acids inserted randomly within the HC-Pro protein, were analysed for their ability to suppress transgene-induced RNA silencing using Agrobacterium infiltration in transgenic Nicotiana benthamiana plants expressing green fluorescent protein. A functional map was obtained, consisting of clearly defined regions with different classes of silencing-suppression activity (wild-type, restricted and disabled). This map confirmed that the N-terminal part of the protein, which is indispensable for aphid transmission, is dispensable for silencing suppression and supports the involvement of the central region in silencing suppression, in addition to its role in maintenance of genome amplification and synergism with other viruses. Moreover, evidence is provided that the C-terminal part of the protein, previously known to be necessary mainly for proteolytic activity, also participates in silencing suppression. Pentapeptide-insertion scanning mutagenesis has been shown to be a fast and powerful tool to functionally characterize plant virus proteins.

INTRODUCTION

The helper component proteinase (HC-Pro) of potyviruses is a multifunctional plant virus protein that has been characterized in detail (Revers et al., 1999; Urcuqui-Inchima et al., 2001). HC-Pro intervenes at different steps of the virus replication cycle: maintenance of genome amplification, replication at the single-cell level (Kasschau et al., 1997), long-distance movement (Cronin et al., 1995; Kasschau et al., 1997; Klein et al., 1994), proteolytic processing (Carrington et al., 1989) and aphid transmission (Raccah et al., 2001). In addition, HC-Pro exhibits non-specific RNA-binding activity (Merits et al., 1998; Urcuqui-Inchima et al., 2000).

Over recent years, potyvirus HC-Pro has also been shown to be a highly effective suppressor of gene silencing in transient silencing-suppression assays (Llave et al., 2000; Johansen & Carrington, 2001; Hamilton et al., 2002; Silhavy & Burgyan, 2004) in transgenic plants (Anandalakshmi et al., 1998; Kasschau & Carrington, 1998; Llave et al., 2000) and upon expression from virus-based expression vectors (Brigneti et al., 1998). Potyvirus HC-Pro prevents and reverses already established RNA silencing. Moreover, expression of potyvirus P1–HC-Pro protein in Nicotiana tabacum and Arabidopsis thaliana alters microRNA (miRNA) accumulation, prevents cleavage of miRNA targets and induces developmental defects that partly resemble those of dcl-1 mutants (Mallory et al., 2002; Kasschau et al., 2003; Dunoyer et al., 2004). Transgenically expressed P1–HC-Pro derived from Potato virus Y (PVY) causes a significant reduction in levels of the 21 nt small interfering RNA (siRNA), but has a less pronounced effect on the 24 nt siRNA (Mallory et al., 2001; Dunoyer et al., 2004). Similar results have been obtained by transient expression of HC-Pro derived from PVY and Tobacco etch virus (TEV) (Hamilton et al., 2002; Qu et al.,...
2003). Different studies in tobacco and Arabidopsis (Mallory et al., 2002; Dunoyer et al., 2004) have shown that transgenically expressed P1–HC-Pro partially reduces double-stranded (ds) RNA processing by Dicer. In addition, P1–HC-Pro inhibits activity of the RNA-induced silencing complex. However, Dunoyer et al. (2004) found the reduction in dsRNA processing to be incomplete, detecting substantial residual levels of siRNA in a transgenic system, despite the strong prevention of mRNA degradation. Taken together, these findings indicate that the prevention of accumulation of siRNA by potyvirus HC-Pro depends on the experimental system and on the viral origin of the protein analysed.

Kasschau & Carrington (2001) related TEV HC-Pro silencing suppression to long-distance movement and genome amplification, associating these functions with the central domain of the protein by using a transient silencing-suppression assay with a set of nine alanine-scanning and five point mutants. From the effects of point mutations introduced into the active site of the proteinase, they concluded that the silencing-suppression ability of TEV HC-Pro is not dependent on the protein's proteolytic activity, but that the proteinase domain may be essential for silencing suppression. Moreover, the HC-Pro of several potyviruses is known to enhance RNA accumulation and for silencing suppression. Moreover, the HC-Pro of several potyviruses is known to enhance RNA accumulation and symptoms of heterologous viruses in double infections, i.e. potyviruses is known to enhance RNA accumulation and symptoms of heterologous viruses in double infections, i.e. with Potato virus X (PVX) (Vance, 1991; Vance et al., 1995; Pruss et al., 1997). Shi et al. (1997) allocated this function to the central domain of the TEV HC-Pro and showed that the N terminus was dispensable. In addition, Sáenz et al. (2001) found evidence that a single amino acid change at position 109 in the HC-Pro of Plum pox virus (PPV) influenced its synergism with PVX. González-Jara et al. (2005) pinpointed a link between silencing suppression by HC-Pro of PPV and its capacity to induce synergism with PVX in Nicotiana benthamiana by introducing one single amino acid mutation at position 134 in the central part of the protein. Finally, Yang & Ravelonandro (2002) demonstrated the involvement of the C-terminal PTK motif (aa 310–312) of PPV, previously known to be indispensable for aphid transmission, in synergism with PVX.

In this study, pentapeptide-insertion scanning mutagenesis (PSM) was chosen as an alternative approach to alanine scanning (Cunningham & Wells, 1989) to generate a dense scanning library of PPV HC-Pro mutants. PSM is the most widely used genetic tool for transposition-based generation of short peptide insertions by scanning-mutagenesis libraries (Hayes, 2003). In vitro DNA transposition by transposon Tn4430 (Tn3-based) (Hallet et al., 1997) and bacteriophage Mu (Haapa et al., 1999) recognizes 5 bp host sequences randomly, inserting an Entranceposon (Finnzymes) containing a selectable marker gene, thereby doubling the insertion site. After in vitro deletion of the Entranceposon and re-ligation, a 15 bp in-frame insertion remains within the target sequence. This results in 5 aa embedded as a fingerprint in the protein. As the insertion consists of transposon-derived 10 bp and a pentanucleotide duplication from the target gene, the pentapeptide varies in composition, depending on the insertion point in the reading frame. No additional amino acids in the target gene are mutated. Here, the effects of different pentapeptide scanning (PS) mutants on the silencing suppressor function of PPV HC-Pro were studied by using a transient silencing-suppression assay. It is demonstrated that the PPV HC-Pro can be dissected into regions that are essential for silencing-suppressor function and those that are not.

**METHODS**

**Plasmids.** The binary plant expression vector pBIN61S (Silhavy et al., 2002), a derivative of pBIN19, contains an enhanced cauliflower mosaic virus 35S promoter and pol(A)-terminator cassette. pBIN61S-gfp (35S–GFP) contains the mgfp4 variant cloned into the expression cassette (Haseloff et al., 1997). pBIN61S-P14 (35S–P14) encodes the P14 silencing-suppressor protein from Potyos latent (PoLV) (Mérai et al., 2005). All pBIN61S-derived plasmids were kindly provided by Dániel Silhavy, Agricultural Biotechnology Center, Gödöllő, Hungary.

**Plasmid constructs.** The HC-Pro ORF of PPV (nt 1072–2444; GenBank accession no. NC_001445) was PCR-amplified from p35PPV-NAT (Mai ss et al., 1992) introducing flanking restriction sites (SacI and BamHI), subcloned and the correctness of its sequence was verified. The HC-Pro ORF and the HC-Pro-PSM library, respectively, were finally inserted into the SacI–BamHI sites of the binary vector pBIN61S.

**Random-insertion scanning mutagenesis, mapping of the insertion and sequencing.** Generation of an HC-Pro PSM library was carried out by using the Mutation Generation system F701 MGS (Finnzymes) consisting of MuA transposase and Entranceposon (M1-KanR) following the manufacturer’s instructions. PS mutants in pBIN61S-HC-Pro were mapped by restriction digestion and the exact position of insertion of each mutation in the HC-Pro ORF was determined by sequencing. Sequencing reactions were carried out by MWG-Biotech.

**Plant material and Agrobacterium infiltration.** Transgenic N. benthamiana line 16c, which is homozygous for the green fluorescent protein (GFP) transgene, was described previously (Brigneti et al., 1998). Agrobacterium tumefaciens infiltration was carried out according to Voinnet et al. (2000). For co-infiltrations, equal volumes of the respective Agrobacterium cultures (OD600 of 1) were mixed before infiltration buffer. For single infiltrations, cultures were diluted by half before infiltration buffer.

**Transient silencing-suppression assay and detection of GFP.** Binary vectors containing the GFP ORF and HC-Pro PS mutants were transformed into A. tumefaciens C58C1 (pGV2260). The silencing-suppression assay was performed as described by Voinnet et al. (1999). GFP expression was detected visually by illumination of leaf surfaces with a hand-held long-wave UV lamp (Blak Ray model B 100 AP, 100 W; UV products). Pictures were taken with a digital camera.

**RNA gel-blot analysis.** Extraction of total RNA from 100 mg infiltrated leaf tissue of N. benthamiana 16c was carried out by using the RNA-extraction procedure described by Hamilton et al. (2002) and extracts were resuspended in 100 μl 50 % formamide. The same RNA extract was used for high- and low-molecular-mass RNA gel-blot analysis. For high-molecular-mass RNA gel-blot analysis, 9 μl was
separated by PAGE (8 % gel with 8.3 M urea and 0.5 M Tris/borate/EDTA). In the case of low-molecular-mass RNA gel-blot analysis, 24 µl was separated by PAGE (15 % gel). Relative quantification of total RNA was achieved by staining the 8 % gel in 0.5 x Tris/borate/EDTA and 50 µg ethidium bromide (ml solution) \(^{-1}\) for 15 min. RNA was electroblotted (45 min at 100 V) on Hybond-N+ membranes; the membrane was incubated for 5 min in 2 x SSC before fixation by cross-linking with UV light at 254 nm (0.07 J cm\(^{-2}\)). Digoxigenin (DIG)-labelled T7 in vitro transcripts corresponding to the antisense strand of GFP were generated by using plasmid pBluescript with the GFP ORF inserted in the antisense orientation into the T7 promoter. In vitro transcription was carried out after linearization of the plasmid by digestion with PstI using DIG–11-UTP (Roche), ribonucleotides and T7 RNA polymerase (Fermentas) following the manufacturer's instructions. Hybridization was carried out in DIG Easy Hyb solution (Roche) with approximately 100 ng probe (ml hybridization solution) \(^{-1}\). Hybridization conditions were 40 and 68°C for detection of GFP siRNA and mRNA, respectively. DIG detection was carried out by using anti-DIG–alkaline phosphatase (AP) Fab fragments and CDP-Star reagent (both Roche) following the manufacturer’s instructions (DIG Northern Starter kit; Roche). DIG chemiluminescence was detected by exposure of the blot to Lumi Film (Roche) for 5–10 min.

Protein extraction and immunoblot analysis. Total soluble plant proteins from agroinfiltrated N. benthamiana patches were obtained essentially as described by Loon (1975), separated by SDS-PAGE (12 % gel) and electrophoresed onto nitrocellulose filters (Protran; Schleicher & Schuell). PPV HC-Pro was probed with anti-HC-Pro rabbit polyclonal antiserum in a 1 : 1000 dilution (Riedel et al., 1998). For detecting antibody, an AP-conjugated goat anti-rabbit polyclonal antibody (Sigma) was used (1 : 10 000 dilution). Detection was performed by using the chromogenic substrates BCIP (5-bromo-4-chloro-3′-indoylphosphate p-toluidine salt) and NBT (nitro blue tetrazolium chloride) (Applichem).

RESULTS

Agroexpression of PPV HC-Pro inhibits transgene-induced RNA silencing

The PPV HC-Pro gene was used in a transient silencing-suppression assay (35S–HC-Pro + 35S–GFP) and GFP fluorescence was compared under UV light to infiltration with 35S–GFP and 35S–HC-Pro, respectively, in parallel at 3, 5 and 7 days post-infection (p.i.). Agro-infiltration of 35S–GFP led to an increase in GFP mRNA at 3 days p.i. and silencing of the GFP transgene was induced by agro-infiltration with 35S–GFP, leading to a reduction in green fluorescence at 5 days p.i. inside the infiltrated area and non-cell autonomous RNA silencing in the surrounding ring (Fig. 1a, see red ring labelled with an arrow). GFP-specific siRNAs were detectable at 3 days p.i. (Fig. 1b). At 5 days p.i., GFP mRNA accumulation in the infiltrated leaf area was reduced to background expression levels. At 7 days p.i., transgenic GFP mRNA was hardly detectable. 35S–HC-Pro induced no change in the transgenic GFP mRNA levels and no siRNA accumulation and thus served as a control to monitor GFP background expression (Fig. 1b; UV monitoring data are not shown). In contrast, co-infiltration of 35S–GFP and 35S–HC-Pro (Fig. 1a, b) increased GFP expression and a high GFP mRNA level remained more or less stable up to 7 days p.i. RNA gel-blot analysis of 21–25 bp GFP siRNAs confirmed that enhanced GFP expression was due to inhibition of transgene-induced silencing. Interestingly, at 5 days p.i., GFP siRNAs were detectable, indicating the onset of local RNA silencing despite HC-Pro expression. At 7 days p.i., siRNAs had

![Fig. 1. Effect of PPV HC-Pro on transgene-induced RNA silencing.](http://vir.sgmjournals.org)
reached a level similar to that for 35S–GFP infiltration alone. In summary, expression of PPV HC-Pro delayed transgene-induced cell autonomous and cell-to-cell RNA silencing for a period of 7 days, as well as siRNA accumulation.

**Construction of a transposon-mediated PS library of PPV HC-Pro and transient silencing-suppression assay of 63 independent mutants**

To map different parts of the PPV HC-Pro protein with respect to the silencing-suppression function, a library of random pentapeptide insertions into the subcloned PPV HC-Pro gene was constructed by *in vitro* Mu transposition. Several hundred independent transformants were obtained and pooled. After removal of the M1-KanR, the library was cloned in pBIN61S and DNA of 200 independent clones was subjected to restriction mapping in order to verify the Entrapponson footprint and to map the Entranceposon footprint site approximately. The HC-Pro ORF of 63 mutants, with insertions spread randomly over the HC-Pro ORF as estimated from restriction mapping, was subjected to sequencing to determine the exact position of the pentapeptide-encoding sequence insertion (Table 1). Depending on the reading frame, the 15 bp insertion can be translated into three different types of pentapeptide. Each possible reading frame results in two or three different invariant amino acids (CGR, RP and AAA) embedded in two to three variable amino acids translated from the duplicated target sequence. Sequence analysis showed that six PS mutants (118 : : AAAKD, 170 : : DAAAT, 256 : : MRPOQ, 277 : : NAAAS, 315 : : AAVAV and 353 : : AAAML) had been isolated twice from the library. To illustrate the reliability of the transient silencing-suppression assay, these mutants were included in further analysis. Moreover, in three cases, the 15 nt Entranceposon footprint in HC-Pro led to pentapeptide insertions at the same amino acid position with varying compositions (37 : : CGRSG and AAACG, 68 : : CGRKQ and DAAAQ, 117 : : CGRIK and DAAAK).

Subsequently, all 63 HC-Pro mutants were used in transient silencing-suppression assays and all assays were repeated three times using two plants each. UV monitoring was applied on days 3, 5 and 7 p.i. and GFP fluorescence in the infiltrated patch was compared with that in patches infiltrated in parallel with wild-type HC-Pro (35S–HC-Pro + 35S–GFP) and patches silenced by infiltration of the transgenic *N. benthamiana* containing the GFP gene controlled by the 35S promoter. Results of the UV monitoring in the three replications were confirmed for all mutants. HC-Pro mutants with no detectable silencing-suppression activity, i.e. strong reduction of GFP fluorescence in the infiltrated area at 5 days p.i. and appearance of the cell-to-cell silencing signal, were grouped into class III. Mutants displaying strong GFP fluorescence even at 7 days p.i. were assigned to the category 'function similar to wild type' (class I). HC-Pro PS mutants with increased GFP fluorescence compared with 35S–GFP fluorescence at 5 days p.i., but noticeably lower than the wild type, were assigned to the category 'function restricted' (class II) (Fig. 2).

**Effect of silencing suppression-disabling mutations on protein stability**

As there was a possibility that the introduced mutations influenced HC-Pro stability, thereby causing a silencing suppression-impaired phenotype, total soluble protein extracts from *N. benthamiana* tissue infiltrated with 35S–HC-Pro and different 35S–HC-Pro PS mutants, respectively, plus 35S–GFP were subjected to immunoblot analysis with anti-HC-Pro antiserum. When protein samples were taken at 5 days p.i., HC-Pro could only be detected in significant amounts when wild-type HC-Pro or a silencing suppression-active PS mutant was employed. The protein was scarcely detected in the case of a non-functional mutant (data not shown). This supported the hypothesis that mutant HC-Pro genes encoding the silencing suppression-defective proteins were targeted by RNA silencing. To demonstrate that the introduced pentapeptide was only affecting the silencing-suppression function and not interfering with protein stability, the expression of all class II and III silencing-suppression mutants of PPV HC-Pro was tested by inhibiting the induction of RNA silencing. The protein-stability assay was achieved by transient *Agrobacterium*-mediated co-expression of either HC-Pro or HC-Pro PS mutants (35S–HC-Pro) with a second functional plant virus silencing-suppressor protein (P14) encoded by PoLV (35S–P14) in *N. benthamiana* 16c leaves. Protein samples were taken at 5 days p.i. and used for HC-Pro immunodetection. Fig. 3 shows that mutant HC-Pro was detectable at 5 days p.i. in amounts comparable to wild-type HC-Pro when co-expressed under the same conditions.

**Effect of different PPV HC-Pro PS mutants on GFP mRNA accumulation**

Seventeen out of the total 63 mutants (PS insertion position HC-Pro aa 9 : : CGGRD, 30 : : AASD, 37 : : CGRSG, 37 : : AAACG, 68 : : CGRKQ, 77 : : VRPQT, 78 : : DAAAI, 87 : : AAAMD, 117 : : CGRIK, 145 : : CGRSG, 216 : : CGRNA, 256 : : MRPOQ, 285 : : CGRRG, 315 : : AAVAV, 341 : : VRPQA, 353 : : AAAML and 428 : : CGRTD) were chosen to be analysed for their influence on GFP mRNA accumulation. The different 35S–HC-Pro PS mutants and 35S–GFP were used for transient silencing-suppression assays and, at 5 days p.i., leaf samples from infiltrated areas were used for RNA gel-blot analysis with appropriate controls (35S–HC-Pro + 35S–GFP and 35S–GFP) (Fig. 4). The different levels of GFP mRNA derived from the selected mutants were compared with the assigned functional category following the results of UV monitoring. In all cases except for PS mutant 216 : : CGRNA, the results of both assays
were generally in agreement with the visual observation, despite some variability in GFP mRNA levels detected between mutants assigned to the same class following the visual observation of GFP expression. In the case of class I mutants, the detected GFP mRNA signal strength was comparable to or somewhat lower than that in the 35S–HC-Pro wild-type control. PS mutants in class II gave a stronger mRNA signal than the silencing control (35S–GFP), but the signal was not as strong as that of the 35S–HC-Pro wild type. Class III HC-Pro PS mutants gave a GFP mRNA level that was barely detectable, as observed with the 35S–GFP control. Mutant 216 : : CGRNA was placed into class I according to the visual observation of GFP expression, but showed less GFP mRNA accumulation in Northern blot and was comparable to other mutants with a restricted function. Repetition of the transient silencing-suppression assay confirmed these results.

Construction of a functional map of PPV HC-Pro in relation to silencing suppression

The correlation between GFP UV monitoring and mRNA analysis allowed us to combine the UV monitoring results of the remaining 46 mutants with the 17 mutants analysed more thoroughly above to construct a functional map of the HC-Pro ORF in relation to the influence of pentapeptide insertions on suppression of transgene-induced silencing (Fig. 5; Table 1). Of the 63 mutants analysed, 31 were class III, nine were class II and 23 were class I. The different phenotypes clustered into clearly delimited regions of the PPV HC-Pro protein.

In the first 88 N-terminal amino acids (PS mutants 1–16), only one non-functional (class III) PS mutant was found at the extreme N terminus (PS mutant 9). All other mutants analysed were class I or class II. The central phenotypes clustered into clearly delimited regions of the PPV HC-Pro protein.

The central PPV HC-Pro domain (aa 100–300) can be divided by our PSM analysis into regions with alternating functions. The region from HC-Pro aa 117–170, covered by

<table>
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Table 1. Effect of Entranceposon insertion mutations in PPV HC-Pro on the silencing-suppression ability of transgene-induced RNA silencing

Mutant class I, function similar to wild type; class II, function impaired; class III, poorly functional to non-functional. Amino acid position of HC-Pro is according to GenBank accession no. NC_001445.

Table 1. cont.

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<td>58</td>
<td>428</td>
<td>CRRTG</td>
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<tr>
<td>59</td>
<td>439</td>
<td>CRRNQ</td>
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<td>61</td>
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<td>62</td>
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<td>CGRNL</td>
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<td>63</td>
<td>458</td>
<td>AAAVG</td>
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PS mutants 17–28, contained only PSM class III mutants, whereas the region aa 189–215 contained only class I mutants (nos 29–33). This was followed by a larger region (aa 216–261) in which mutants did not show a uniform behaviour (nos 34–40). The last part of the central domain (aa 277–294; nos 41–44) seems to be involved in silencing suppression, as all of the pentapeptide insertions rendered the protein non-functional. Finally, the C terminus (aa 300–458) consists of four regions with varying functional importance for silencing suppression. Pentapeptide insertions in regions 310–332 and 353–357 did not influence silencing suppression, whereas insertions in aa 341–350, as well as in the approximate 80 C-terminal amino acids, inhibited the function of PPV HC-Pro in the assay.

Interestingly, all six PS mutants that had been isolated twice from the library displayed the same silencing-suppression phenotype, confirming the reliability of the assay. In addition, the three pentapeptide insertions at the same amino acid position with varying composition (37 : : CGRSG and AAACG, 68 : : CGRKQ and DAAAQ, 117 : : CGRIK and DAAAK) led in all three cases to the same phenotype in the transient silencing-suppression assay. This indicates that the different sequences of the additional five amino acid insertions have no influence beyond the phenotype caused by the insertion, as such at the above-mentioned positions of the protein. Taken together, these results show that different parts of PPV HC-Pro, which are clearly separable by mutation analysis, contribute to the silencing-suppression activity of the protein.

### DISCUSSION

Here, the ability of PPV HC-Pro to suppress GFP transgene-induced silencing in a different experimental system was verified, data were compared with the work of González-Jara et al. (2005) and the suitability of this assay to characterize different PPV HC-Pro mutants in relation to silencing-suppressor activity was demonstrated. The results of UV monitoring and RNA-blot analysis presented here demonstrate that HC-Pro of PPV works as a viral suppressor of gene silencing, as shown previously for HC-Pro proteins of other potyviruses, namely TEV (Anandalakshmi et al., 1998), PVY (Brigneti et al., 1998) and Turnip mosaic virus (Dunoyer et al., 2004).

In contrast to the data obtained by González-Jara et al. (2005), it has been shown here that the accumulation of GFP siRNAs is delayed by PPV HC-Pro in the transient assay only for approximately 5 days and not by up to 7 days, although comparable prevention of GFP mRNA degradation was found in both studies. This probably can be explained by differences in the experimental setup or virus isolate used. On the other hand, Lakatos et al. (2006) showed that potyvirus HC-Pro inhibits RNA silencing by sequestering siRNAs and this might explain our observation of siRNA accumulation at 7 days p.i. In our study, PSM was used for the first time as an alternative approach to conventional point-mutation analysis, like alanine scanning, to build a dense functional map of the silencing-suppression ability of a potyvirus HC-Pro. The resulting functional map of the PPV HC-Pro reflects the effect on
the silencing-suppression ability of 5 aa insertions at 54 different positions.

Remarkably, PSM in this study did not lead to instability of the target protein. All silencing-suppression class II and III HC-Pro mutants were expressed in detectable levels that were comparable to those of the wild-type HC-Pro. Other studies using PSM for functional characterization of proteins also found a very high percentage of stably expressed PS mutants in eukaryotic (Saccharomyces cerevisiae) and prokaryotic (Escherichia coli) experimental systems (Poussu et al., 2004; Fransen et al., 2005). The reasons for this remarkable finding can be complex and therefore remain speculative.

Dysfunction of a mutant protein may be due to several reasons. The mutation may either affect an active site consisting of one or a few amino acids, or alter the secondary or tertiary structure of the protein. In addition to deletion and insertion mutagenesis, where secondary structure is very likely to be affected, introduction of point mutations is mainly used to delimit functional domains,
**Fig. 4.** Effect of different PPV 35S–HC-Pro PS mutants on the GFP mRNA level in the transient silencing-suppression assay. Seventeen different mutants were co-infiltrated with 35S–GFP into leaves of GFP-silenced transgenic *N. benthamiana* 16c. Samples at 5 days p.i. were used for RNA gel-blot analysis of GFP mRNA. (a) Binary vector constructs in agrobacteria used for infiltration. (b) Silencing-suppression category according to Table 1. (c) GFP mRNA signal. (d) Ethidium bromide-stained gel (loading control). w.t., HC-Pro wild type; NA, not applicable.

**Fig. 5.** Functional map of the PPV HC-Pro. Regions involved in silencing suppression of transgene-induced silencing compared with information on structural domains, biological functions of potyvirus HC-Pro and mutations introduced into PPV HC-Pro in previous studies affecting silencing-suppressor function or viral synergism are shown. Amino acid numbering is according to PPV (GenBank accession no. NC_001445) (Maiss *et al.*, 1989). (a) PPV HC-Pro PS mutants created in this study displaying different degrees of silencing-suppressor function (classes I–III; descriptions in the text and Table 1). (b) Division of HC-Pro into three regions. (c) Biological functions of different HC-Pro regions and position of conserved motifs as cited in the text. (d) Mutations introduced into PPV HC-Pro in previous studies affecting virus synergism (1 Yang & Ravelonandro, 2002; 2 González-Jara *et al.*, 2005; 3 Saénz *et al.*, 2001).
regions or motifs. In PSM studies where protein secondary-structure information was already available from X-ray crystallography (XerD recombinase from *Salmonella enterica* serovar. *typhimurium* and β-lactamase from pBR322), Cao et al. (1997) and Hallet et al. (1997) have revealed that this technique can yield significant information on the structure–function relationship. Pentapeptide insertions close to the active site of a protein, as well as those that disrupt regions of secondary structure, were shown to have a stronger effect on protein functionality than insertions in linker regions or surface loops in both cases.

Our silencing-suppression map was compared with previous studies in which potyvirus HC-Pro silencing-suppression activity was modified by point mutations mainly by using alanine-scanning mutagenesis. As potyvirus HC-Pro activity, in synergism with other viruses, has been correlated positively with silencing suppression, studies dealing with characterization of virus synergism have also been considered. Mutational studies on the functional characterization of potyvirus HC-Pro are restricted to TEV (Dolja et al., 1993; Cronin et al., 1995; Shi et al., 1997; Kasschau et al., 1997; Kasschau & Carrington, 2001) and PPV (Sáenz et al., 2001; Yang & Ravelonandro, 2002; González-Jara et al., 2005). The results of the PPV studies with respect to PVX synergism are summarized in Fig. 5.

In our study, out of 16 mutants tested in the N-terminal 88 aa, only one was non-functional in silencing suppression (class III), eight showed intermediate silencing suppression (class II) and seven were similar to the wild type (class I). It is concluded that most of the N-terminal part of the PPV HC-Pro tolerates the 5 aa insertions without affecting the ability to suppress silencing significantly. This fits nicely with the results of previous studies on TEV (Shi et al., 1997), which showed that a 66 aa deletion of the HC-Pro protein did not influence synergism with PVX. The findings are also consistent with the results of Shi et al. (1997) and Yang & Ravelonandro (2002), who found no influence of N-terminal point mutations in the KITC motif (3 aa insertion V62-TMA and exchange of K52E) on TEV– and PPV–PVX synergism, respectively. Taken together, our results obtained with PSM support and strengthen previous findings that the N-terminal part of potyvirus HC-Pro is only involved in aphid transmission and is dispensable for silencing suppression and synergism with other viruses.

There are several lines of evidence that the central part (aa 100–300) of the potyvirus HC-Pro is indispensable for genome amplification, reflected by conserved motifs (IGN, CC/SC) that interfere strongly with genome amplification if mutated (Kasschau et al., 1997; Cronin et al., 1995). In PPV–PVX synergism, the amino acid mutation E109K (Sáenz et al., 2001) was shown to have a positive effect, whereas an L134H exchange (González-Jara et al., 2005) abolished silencing suppression as well as synergism. In addition, Shi et al. (1997) found that two mutations in the central region of TEV HC-Pro (Q122-TMA, D152-TMA) abolished TEV–PVX synergism. These findings are consistent with the PSM effect in the 117–170 region on silencing suppression. Our findings, however, do not confirm the effects of the TEV HC-Pro E154A and E155A mutations (Kasschau & Carrington, 2001), which did not influence silencing-suppression activity, but without knowledge of the three-dimensional structure of these two sequence variable proteins and derived mutants, the reasons for this inconsistency cannot be defined.

Comparing the effects of PSM insertions between aa 189 and 294 to previous work on PPV and TEV demonstrates that the reported lack of effect of S232G (Sáenz et al., 2001) on synergism is comparable to that of PS mutant 232, which did not influence silencing-suppression activity. The point mutation I272V analysed by González-Jara et al. (2005), which did not influence PVX synergism, cannot be mapped precisely onto the PPV HC-Pro sequence, but lies between PS mutant 261 (class I) and PS mutant 277 (class III). Comparing the map obtained in this study with the TEV work of Kasschau & Carrington (2001), it is shown that three of four mutants in the corresponding region (aa 235–300) had a strong or moderate influence on silencing suppression (class I, D235A and K236A; class III, D240A, K241A and H242A; class III, R247A and K248A; class II, E299A and D300A). Sixteen mutants in this region (aa 189–294) were analysed and nine belonged to class I, six to class II and one to class III. There is no exact concordance between the functional assignment of the PPV and TEV HC-Pro mutants, but, in both studies, mutants were detected that influenced silencing-suppression ability negatively. Therefore, it is suggested that there is no defined amino acid motif in this region that is responsible for silencing-suppression activity, but rather that the region presumably possesses a three-dimensional structure that is indispensable for this function. This structure may be destroyed by either insertion or exchange mutagenesis, as demonstrated here and in the different studies applying alanine-scanning mutagenesis.

There is strong evidence that potyvirus HC-Pro interferes with RNA silencing by siRNA binding (Lakatos et al., 2006). Possibly the PS mutations in the central part of the protein, influencing suppressor activity negatively, interfere with the RNA-binding ability of HC-Pro. Interestingly, Urcuqui-Inchima et al. (2000) have mapped the RNA-binding capacity of *Potato virus A* HC-Pro to two independent domains that are located in the central region, domain A (aa 89–230) and domain B (aa 234–321). Analysing the C-terminal 150 aa of PPV HC-Pro (aa 300–458) by using PSM detected four alternating regions with tolerance or intolerance to pentapeptide insertions (Table 1), whereas the extreme C terminus comprising aa 380–458 did not tolerate insertions. Using PPV, Yang & Ravelonandro (2002) detected a suppressor-abolishing effect of K312E (aphid transmission PTK motif) on PVX synergism. This PTK motif is localized in a region containing three PS mutations (310–332), which had no effect on silencing suppression in our study.
In previous studies, the C-terminal approximately 150 amino acids of TEV have been associated with the proteinase function of HC-Pro and active residues C345 and H418 have been identified (Oh & Carrington, 1989). Kasschau & Carrington (2001) demonstrated by single amino acid exchanges that the proteinase active sites are not involved in silencing-suppression ability. However, alanine-scanning mutants were found in the C-terminal part that diminished or abolished the activity of TEV HC-Pro. Alignment of the PPV and TEV HC-Pro revealed that PPV HC-Pro positions C344 and H417 correspond to the defined proteinase active sites of TEV HC-Pro and both amino acids are localized to a region that has been identified in our study as being non-tolerant to pentapeptide insertions. A possible higher sensitivity of the proteinase function to single amino acid exchanges compared with the silencing-suppressor function may explain this observation. Comparing our PSM map of the C-terminal 150 aa with the results obtained by Kasschau & Carrington (2001) using alanine scanning and conventional single amino acid exchanges reveals some concordant, but also some contradictory, observations. Thus, the E299A–D300A, S306T and E360A–D361A mutations are localized between two PSM insertions with different functionality. C390S in TEV HC-Pro had no influence on suppressor function, but corresponds to a non-functional region in PPV HC-Pro. The D411A–H412A mutation was shown to affect the TEV HC-Pro silencing-suppressor function, which is consistent with our finding that the PPV HC-Pro region 380–458 is non-tolerant for pentapeptide insertions, although, in contrast, the D411E and D413A–K414A mutations in TEV HC-Pro do not affect silencing suppression.

Some of the observed differences may be explained by sequence variability. HC-Pro of PPV (GenBank accession no. NC_001445) and TEV HC-Pro (NC_001555) possess 48.3 % amino acid similarity (CLUSTAL_X) (Thompson et al., 1997). Nevertheless, our findings provide additional evidence that the C-terminal part of HC-Pro, in addition to its proteinase function, may interact either directly or indirectly with the HC-Pro central domain involved in suppression of gene silencing.

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