Production of plum pox virus HC-Pro functionally active for aphid transmission in a transient-expression system

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Potyviruses are non-persistently transmitted by aphid vectors with the assistance of a viral accessory factor known as helper component (HC-Pro), a multifunctional protein that is also involved in many other essential processes during the virus infection cycle. A transient Agrobacterium-mediated expression system was used to produce Plum pox virus (PPV) HC-Pro in Nicotiana benthamiana leaves from constructs that incorporated the 5' region of the genome, yielding high levels of HC-Pro in agroinfiltrated leaves. The expressed PPV HC-Pro was able to assist aphid transmission of purified virus particles in a sequential feeding assay, and to complement transmission-defective variants of the virus. Also, HC-Pro of a second potyvirus, Tobacco etch virus (TEV), was expressed and found to be functional for aphid transmission. These results show that this transient system can be useful for production of functionally active HC-Pro in potyviruses, and the possible uses of this approach to study the mechanism of transmission are discussed.

INTRODUCTION

Aphid transmission is the main method of spread for plant viruses belonging to the genus Potyvirus, family Potyviridae. This genus comprises a large number of members, including some important plant pathogens, such as Plum pox virus (PPV), a virus responsible for severe losses in stone fruit production worldwide. PPV is transmitted by different aphid species in a non-persistent manner. An attractive means of control for PPV and other potyviruses could be to stop effective vector transmission, and numerous research efforts are being devoted to understand the molecular mechanisms governing this process.

Pioneering studies on aphid transmission of potyviruses demonstrated that the process requires a helper factor in addition to virion particles (Govier & Kassanis, 1974a, b). It was later demonstrated that this factor is a non-structural virus-encoded helper protein (HC-Pro) (Thornbury et al., 1985). During transmission, aphids must have access to HC-Pro before or at the same time as virus particles. This fact and other evidence suggested that HC-Pro might act as a ‘bridge’ between aphid mouthparts and virion particles (reviewed by Pirone & Blanc, 1996).

Analysis of naturally occurring non-transmissible variants of potyviruses and site-directed mutagenesis studies have been used to investigate the domains of HC-Pro and coat protein (CP) involved in aphid transmission. Two conserved motifs in HC-Pro and one in CP were found. The N-terminal part of HC-Pro, including the conserved motif KITC, is apparently involved in binding to aphid mouthparts (Blanc et al., 1998). The second motif in HC-Pro, PTK, is implicated in binding to CP (Peng et al., 1998). In addition, an HC-Pro-binding domain, DAG, was found at the N terminus of CP (Blanc et al., 1997; Lopez-Moya et al., 1999). In spite of these important advances in our understanding of the phenomenon, mutational analyses have some limitations. First, a full-length clone of the studied virus must be available. Furthermore, it is frequently the case that non-infectious variants arise when mutations are introduced into a full-length clone, a common problem when dealing with multifunctional proteins such as HC-Pro (Urcuqui-Inchima et al., 2001). In the case of Tobacco etch virus (TEV) for which an infectious clone is available (Dolja et al., 1992), the 89 N-terminal residues of HC-Pro were found to be dispensable for systemic infection, although its loss impaired aphid transmission (Dolja et al., 1993). This fact allowed the generation of mutants and analysis of their transmissibility (Llave et al., 2002). However, the available infectious full-length PPV clones seem not to tolerate deletions in this region (Guo et al., 1998).

These limitations have prompted a search for expression systems in which the multifunctional HC-Pro could be...
produced uncoupled from virus infection, and therefore aphid transmission could be studied independently from other functions. Several HC-Pro expression systems have been assayed, including transgenesis (Berger et al., 1989), viral vectors (Sasaya et al., 2000) and heterologous expression in baculovirus (Thornbury et al., 1993) or yeast (Ruiz-Ferrer et al., 2004). Transgenic plants exhibited low levels of expression and testing of its activity during transmission required semi-purification and concentration of the protein (Berger et al., 1989). In the case of PPV HC-Pro, expression in transgenic lines was below detection limits (Barajas et al., 2004). Expression of Potato virus Y (PVY) HC-Pro using a Potato virus X (PVX)-based vector proved to be functional for aphid transmission (Sasaya et al., 2000) but, as commonly occurs in viral vectors, the stability of the insert is compromised and, furthermore, the presence of HC-Pro might trigger synergistic reactions, as shown in the equivalent PVX-based construct expressing PPV HC-Pro, causing severe systemic necrosis (Gonzalez-Jara et al., 2005). Regarding expression in heterologous systems, baculovirus-expressed Tobacco vein mottling virus HC-Pro was not functional for transmission (Thornbury et al., 1993). The Pichia pastoris-based system allowed the recovery of TEV HC-Pro that was functional in transmission (Ruiz-Ferrer et al., 2004). However, this system required the protein to be concentrated for testing its biological activity and the efficiency of transmission was low compared to that obtained with protein purified from infected leaves (Blanc et al., 1999). To date, purifying tagged HC-Pro from infected plants has been the most successful system for obtaining transmission-active HC-Pro (Blanc et al., 1999; Plisson et al., 2003). Unfortunately, the use of this strategy for PPV HC-Pro renders a protein that is not functional in transmission (unpublished data).

The discovery of the RNA silencing suppression activity of HC-Pro from potyviruses (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau & Carrington, 1998) has boosted research efforts to further study this product. One of the tools that has been demonstrated to be useful in the study of post-transcriptional gene silencing is the transient expression of viral suppressors using Agrobacterium tumefaciens (Voinnet et al., 1999). Since then, the system has been used in numerous studies in which the expression of diverse potyvirus HC-Pro was used as a positive control for suppression (reviewed by Voinnet, 2005). In the case of PPV, the functionality of HC-Pro in standard silencing suppression assays has been demonstrated recently (Gonzalez-Jara et al., 2005).

In the present work we searched for a new method of expression of transmission-active PPV HC-Pro, outside the context of a virus infection. Transient expression using an A. tumefaciens-based system was chosen and tested in Nicotiana benthamiana, a systemic host of PPV, and in Nicotiana tabacum, a related species not supporting systemic movement (Saenz et al., 2002). Transmission efficiency was tested in both membrane artificial-feeding and plant-to-plant assays. The system was also tested successfully with another potyvirus.

**METHODS**

**Virus variants, host plants, bacterial strains and aphid vectors.** *N. benthamiana* plants were inoculated with PPV using the full-length infectious clone pICPPV-5.15. This clone is a derivative of pICPPV (Lopez-Moya & Garcia, 2000) which contains a full-length copy of the PPV Rankovic isolate with replacement of the HC-Pro region with the corresponding region from the aphid-transmissible PPV isolate 5.15 (Lopez-Moya et al., 1995), to ensure its transmissibility. Three derivatives of pICPPV-5.15 were also used (Fig. 1a), pICPPV-CPNAT reproduced the deletion of PPV isolates NAT and 3.3, affecting the third position of the conserved DAG motif at the N terminus of the CP coding region (Maiss et al., 1989; Lopez-Moya et al., 1995). pICPPV-EITC contains a single-point mutation at the KITC motif (EITC) and produces a fully infectious virus variant. This modification was done by recombinant PCR using primers 5'-GAGAAATCATGCTGAGGCTGTCAAAG-3’ and 5’-ACACTGAGACATGTAATTCTTCGCAAGGG-3’, corresponding to positions 1222–1254 and complementary to 1214–1243, respectively, in the PPV genome (Lain et al., 1989). These primers incorporated a lysine to glutamic acid change (bold) and a silent mutation introducing a restriction site for PstI (underlined) at position 1236. pICPPV-HCPVY is a chimaeric virus constructed by replacement of most of the PPV HC-Pro sequence with the corresponding sequence from the PVY isolate OAT (Canto et al., 1995), able to systematically infected *N. benthamiana* plants with symptoms similar to PPV (Martinez-Garcia, 2000). In this chimaeric construct, the first three amino acids in the HC-Pro N terminus were replaced from the original PVY sequence (Canto et al., 1995) to the corresponding ones in the PPV sequence, representing two residue changes (SNA to SDP).

PPV virion particles were purified according to Lain et al. (1998) from systemically infected *N. benthamiana* plants.

The clone pTEV-HCH10, containing a full-length copy of the TEV genome (Blanc et al., 1999), was used to inoculate *N. tabacum* cv. Xanthi nc plants. Virions were purified according to Murphy et al. (1990) from systemically infected plants.

*N. benthamiana* and *N. tabacum* plants were grown in chambers at 23–20 °C with a 16 h light period. Plants were mechanically inoculated using carborundum as abrasive and infectious DNA (for PPV) or RNA (for TEV). *N. benthamiana* seedlings were used as test plants for aphid transmission experiments.

DNA manipulations, cloning and plasmid production were performed using *Escherichia coli* DH5α and standard procedures (Sambrook et al., 1989). *A. tumefaciens* GV2260 was used for infiltration experiments on plant tissues.

A clone of *Myzus persicae* Sulzer aphids, kindly provided by Dr A. Fereses (CCMA, CSIC, Madrid, Spain) was used for transmission studies.

**Construction of PPV HC-Pro and TEV HC-Pro expression vectors.** The commercial plasmid pCAMBIA2300 (CAMBIA) was used to clone the 5′ regions of the PPV and TEV genomes, generating plasmids pTRANS3′PPV and pTRANS5′TEV, respectively (Fig. 1b). pTRANS5′PPV incorporates a viral fragment of 2924 nt, including the 5′ UTR, P1, HC-Pro and part of the P3 coding region. The cauliflower mosaic virus (CaMV) 35S promoter and viral sequences were extracted from pICPPV-5.15 using *XmnI* and PstI, and ligated to a derivative of pCAMBIA2300 in which the 3′ terminator region of the nopaline synthase gene (NOS) was inserted between PstI and HindIII sites.
To generate pTRANS5' TEV, a fragment of plasmid pTEVH10 was amplified by PCR with Expand High Fidelity Taq Polymerase (Roche) using primers 5'-AAATAACAAATCTCAACACAC-3' and 5'-CGCTGACAGGTCAGCATACAGCCACC-3', corresponding to positions 1–22 and complementary to 2849–2871, respectively, in the TEV genome (Allison et al., 1986). The antisense primer contained a Pst I restriction site (underlined). The amplified DNA fragment was subcloned into Stu I- and Pst I-digested plasmid p35SNOS-B, a previously described plasmid containing the CaMV 35S promoter (Lopez-Moya & Garcia, 2000). Promoter and viral sequences were extracted using Xma I and Pst I, and introduced into the same pCAMBIA2300 derivative containing NOSt.

RNA analysis. Total RNA was extracted from infiltrated leaf tissue with Trizol reagent (Invitrogen), according to the manufacturer's instructions. To isolate low- and high-molecular-mass fractions, the DNA/RNA Midi kit (Qiagen) was used. For Northern analysis, 5 µg purified high-molecular-mass fraction RNA was loaded into 1.5% agarose-formaldehyde gels (Sambrook et al., 1989) and transferred to a positively charged nylon membrane. In the case of the low-molecular-mass fraction, 20 µg purified RNA was electrophoresed and blotted as described by Llave et al. (2000). Membranes were cross-linked and hybridized overnight with an HC-Pro-specific 32P-labelled probe produced by random priming. Hybridizations were conducted at 42 or 38 °C for the high- and low-molecular-mass fractions, respectively. In both cases, ethidium bromide staining was used to confirm equal loading of samples.

Transformation of A. tumefaciens and agroinfiltration experiments. The binary plant vectors were introduced into A. tumefaciens cells by heat-shock transformation and the infiltration of plant tissues was performed essentially as described by English et al. (1997).
Protein expression analysis. Production of HC-Pro in *N. benthamiana* or *N. tabacum* leaves was analysed by Western blotting. Leaf tissue (50 mg) was collected and ground in 200 μl extraction buffer (50 mM Tris/HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.05% bromophenol blue, 10% glycerol). Homogenized samples were boiled for 5 min and clarified by centrifugation. Ten to 20 μl were separated by electrophoresis in a 10% SDS-PAGE gel. Proteins were transferred to PVDF membranes. Blotted membranes were probed with rabbit polyclonal antiserum against PPV HC-Pro (Martinez-Garcia, 2000) and then with goat anti-rabbit antibody conjugated with horseradish peroxidase. Detection was carried out with the ECL system (Amersham Biosciences). Equal protein loading was confirmed in a duplicated gel stained with Coomassie blue.

Aphid transmission assays. Transmissibility of the virus variants was tested in standard plant-to-plant transmission assays as described by Atreya *et al.* (1990). To test the ability of *Agrobacterium*-expressed products to help aphid transmission, two types of experiments were performed (Fig. 2): (i) sequential feeding in agroinfiltrated leaves followed by membrane acquisition of purified virions; and (ii) simultaneous acquisition of HC-Pro and virions from agroinfiltrated leaves of a previously virus-infected plant. For sequential feeding, aphids were released first onto an *N. benthamiana* agroinfiltrated leaf at 4 days post-agroinfiltration (p.a.) for HC-Pro acquisition over a 5 min period, and then transferred to cages covered with stretched Parafilm membranes to acquire the virus for another 5 min on a solution containing 20% sucrose and purified...
virions at 0.2 mg ml\(^{-1}\). Groups of 10 aphids were finally placed on N. benthamiana test plants for inoculation and allowed to feed overnight before spraying with pirimicarb at 0.05% (w/v). For the single-feeding assay, leaves from plants systemically infected with either PPV-5.15, the mutant PPV-EITC, or the chimaeric variant PPV-HCPVY at 8 days post-inoculation (p.i.) were also agroinfiltrated with the corresponding A. tumefaciens cultures and used to feed aphids at 4 days p.a. After 5 min of acquisition, groups of 10 aphids were transferred to N. benthamiana test plants for inoculation as described above.

Virus detection and identification of virus variants. PPV was detected in N. benthamiana plants by ELISA using PPV-specific antibodies (Durviz). The identity of PPV variants was tested by immunocapture RT-PCR (IC-RT-PCR), essentially as described by Fernandez-Fernandez et al. (2002). For PPV-HCPVY, primers 5'-AGCAAGGCCATCTCAAG-3' and 5'-TTTTGAATTGGTCACACTTATC-3' were used, corresponding to positions 1411–1427 from the PVY sequence (Lain et al., 1989) and complementary to 2457–2478 from the PPV sequence (Robaglia et al., 1989). In the case of PPV-EITC, the identity was also tested by IC-RT-PCR with primers 5'-AACCGTCGGATGCAAT-3' and 5'-GCATAGCAAGGAAAA-TG-3', corresponding to positions 500–516 and complementary to 2127–2110 from the PPV sequence, followed by restriction of the PsI site present only in the EITC variant.

**RESULTS**

Characterization of transmissibility of PPV variants and the chimaera PPV-HCPVY

Results of plant-to-plant transmission experiments with the PPV variants used in this study are shown in Table 1. While PPV-5.15 was readily transmitted by aphids, the modification of the DAG motif in PPV-CPNAT, or the alteration of the KITC motif to EITC in PPV-EITC (Fig. 1) abolished aphid transmissibility (Table 1). The substitution of the DAG motif in PPV-CPNAT, or the alteration of the KITC motif to EITC in PPV-EITC (Fig. 1) abolished aphid transmissibility (Table 1). The substitution of the KITC motif to EITC in PPV-EITC (Fig. 1) abolished aphid transmissibility (Table 1). The substitution of the KITC motif to EITC in PPV-EITC (Fig. 1) abolished aphid transmissibility (Table 1). The substitution of the KITC motif to EITC in PPV-EITC (Fig. 1) abolished aphid transmissibility (Table 1).

**Table 1. Aphid transmission of PPV variants**

<table>
<thead>
<tr>
<th>Acquisition*</th>
<th>Transmission†</th>
<th>Experiments</th>
<th>Total</th>
<th>Percentage‡</th>
<th>Range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPV-CPNAT</td>
<td></td>
<td>4</td>
<td>0/96</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>PPV-EITC</td>
<td></td>
<td>5</td>
<td>0/149</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>PPV-HCPVY</td>
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<td>9</td>
<td>0/135</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>PPV-5.15</td>
<td></td>
<td>5</td>
<td>42/116</td>
<td>36</td>
<td>21–46</td>
</tr>
</tbody>
</table>

* N. benthamiana plants were infected with the indicated PPV variant. Infected leaves showing clear symptoms were used to feed aphids (M. persicae).
†Plant-to-plant transmission experiments were performed using 10 aphids per plant. The number of independent experiments is indicated in the first column. Pooled transmission rates are indicated as number of infected plants/number of test plants.
‡Percentages of pooled values are shown. Fisher’s exact tests indicated significant differences (P < 0.001) between the last row (PPV-5.15) and all other rows.

**Transient expression of PPV HC-Pro in agroinfiltrated leaf tissue of N. benthamiana**

The Agrobacterium-mediated delivery system was used to express the pTRANS5’PPV construct in N. benthamiana leaf tissues. As shown in Fig. 3, expressed PPV HC-Pro could be detected by Western blot analysis using a specific polyclonal antibody (Martinez-Garcia, 2000). A time-course analysis of protein accumulation was performed, with samples collected at 2, 4 and 6 days p.a. The result of the Western blot analysis (Fig. 3) showed that HC-Pro accumulated in the agroinfiltrated tissue to a level that was comparable or even higher to that observed in leaves from a plant systemically infected by PPV at 12 days p.i.

To test whether the Agrobacterium-mediated expression of HC-Pro is effective in plant species other than N. benthamiana, leaves of N. tabacum plants were agroinfiltrated with the pTRANS5’PPV construct and analysed by Western blotting as described above. Interestingly, our analysis repeatedly failed to detect PPV HC-Pro in agroinfiltrated N. tabacum leaves, and only occasional faint bands were observed after long exposures (not shown). In contrast, HC-Pro was easily detected in N. benthamiana leaves agroinfiltrated with the same cultures.

To find out if this reduced protein expression in N. tabacum was associated with low accumulation of transiently expressed mRNA, a Northern hybridization time-course analysis was performed, sampling tissue at 0, 2, 4 and 6 days.
p.a., using an HC-Pro-specific probe. Although mRNAs were produced in both hosts, *N. tabacum* consistently accumulated lower amounts than *N. benthamiana*. The peak of accumulation varied between 2 and 4 days p.a., and a decrease in mRNA levels was observed at 6 days p.a. in both species (Fig. 4a, b). Repeated experiments showed that HC-Pro levels in *N. tabacum* were rather variable, although they were consistently lower than those produced in *N. benthamiana*.

A similar time-course analysis was performed in both hosts to detect the accumulation of small interfering RNAs (siRNAs) derived from HC-Pro. In the case of *N. benthamiana*, the detection of HC-Pro siRNAs showed progressive accumulation at 4, 6 and 8 days p.a., which correlated well with the observed reduction in mRNA levels. However, in most cases we were unable to detect siRNAs in the infiltrated leaves of *N. tabacum* (Fig. 4c), suggesting that lower levels of mRNA in *N. tabacum* were not a consequence of a stronger silencing phenomenon.

**Functionality of transiently expressed PPV HC-Pro in sequential aphid transmission**

Since HC-Pro was efficiently expressed in *N. benthamiana*, we used this plant species to investigate whether transiently expressed HC-Pro was competent to assist aphid transmission. Our previous results showed that HC-Pro accumulated at the highest level at 4 days p.a. and, therefore, this day was selected for transmissions.

Sequential feeding allowed aphids to acquire HC-Pro first and virions later (Fig. 2a). Our experiments demonstrated that aphids fed on leaves agroinfiltrated with a construct that expressed HC-Pro were able to transmit purified virus acquired in a second feeding (Table 2). The non-transmissible variant PPV-CPNAT was used as a source for functional HC-Pro in control experiments. This virus variant has a deletion in the CP (Maiss et al., 1989; Lopez-Moya et al., 1995) that prevents active HC-Pro from interacting with virion particles and, consequently, effective aphid transmission. The results of these bioassays showed that PPV HC-Pro produced by agroinfiltration was active for aphid transmission (31-8% transmission), while aphids fed on the negative control agroinfiltrated with empty vector failed to assist transmission, as expected. Aphid acquisition of HC-Pro from plants infected with PPV-CPNAT also assisted transmission (28-1%) of purified virions, with no significant differences when compared to the transmission rate obtained with HC-Pro produced by agroinfiltration.

To extend our results to other viruses, a second potyvirus, TEV, was chosen. The equivalent construct, pTRANS5’TEV was tested for transmission in a similar way by agroinfiltration and sequential feeding. It was observed that
Agrobacterium-expressed TEV HC-Pro was functional in assisting transmission of purified TEV virion particles (Table 3).

Functionality of the transiently expressed PPV HC-Pro to complement transmission-defective variants in a single-feeding plant-to-plant transmission test

To determine if the transiently expressed HC-Pro can complement transmission when acquired along with virions in a single feeding, leaves showing systemic symptoms of the non-transmissible PPV-EITC and PPV-HCPVY virus variants were agroinfiltrated with pTRANS5′PPV. This system allows aphids to feed only on one plant, reducing the stress caused by manipulation of insects during the experiment (Fig. 2b).

Leaves of PPV-EITC- and PPV-HCPVY-infected N. benthamiana plants showing systemic symptoms (8 days p.i.) were agroinfiltrated with A. tumefaciens cultures transformed with pTRANS5′PPV or empty vector. After 4 days (12 days p.i. and 4 days p.a.), leaves were used to feed aphids on regular plant-to-plant transmission tests. The results (Table 4) showed that transiently expressed PPV HC-Pro was able to assist transmission of the EITC mutant (22-8 % transmission rate) or the chimaeric virus (22 % transmission rate), while aphids fed on infected plants non-agroinfiltrated or infiltrated with empty vector failed to transmit the virus, as expected. The transmission rates were lower than those obtained in parallel transmission

Table 2. Transmission of purified PPV in sequential feeding experiments performed with aphids that had previous access to agroinfiltrated plant tissue as a source of PPV HC-Pro

<table>
<thead>
<tr>
<th>Acquisition</th>
<th>Transmission*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiments</td>
</tr>
<tr>
<td>Agroinfiltrated with empty vector</td>
<td>Purified PPV</td>
</tr>
<tr>
<td>Agroinfiltrated with pTRANS5′PPV</td>
<td>Purified PPV</td>
</tr>
<tr>
<td>PPV-CPNAT-infected</td>
<td>Purified PPV</td>
</tr>
</tbody>
</table>

$\dagger$Transmission experiments were performed with 10 aphids per plant. The number of independent experiments (with at least 20 plants per experiment) is indicated in the first column. Pooled transmission rates are indicated as number of infected plants/number of test plants.

$\dagger$N. benthamiana leaves were agroinfiltrated with empty vector or pTRANS5′PPV and used after 4 days p.a. to feed aphids (M. persicae). In the third group, N. benthamiana leaves showing systemic infection of PPV-HC5.15-CPNAT after 12 days p.i. were used to feed aphids.

$\dagger$Aphids pre-fed as indicated in the previous column were transferred to membrane-covered cages for a second feeding in solutions containing 20% sucrose and purified PPV virion particles at a concentration of 0·2 mg ml$^{-1}$.

$\dagger$Percentages of pooled values are shown. Fisher’s exact tests indicated significant differences ($P < 0·0001$) between the first row (negative control, empty vector) and each of the other two rows, while no significant differences were found between the last two rows.

Table 3. Transmission of purified TEV in sequential feeding experiments performed with aphids that had previous access to agroinfiltrated plant tissue as a source of TEV HC-Pro

<table>
<thead>
<tr>
<th>Acquisition</th>
<th>Transmission*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiments</td>
</tr>
<tr>
<td>Agroinfiltrated with empty vector</td>
<td>Purified TEV</td>
</tr>
<tr>
<td>Agroinfiltrated with pTRANS5′TEV</td>
<td>Purified TEV</td>
</tr>
</tbody>
</table>

$\dagger$Transmission experiments were performed with 10 aphids per plant. The number of independent experiments (with at least 20 plants per experiment) is indicated in the first column. Pooled transmission rates are indicated as number of infected plants/number of test plants.

$\dagger$N. benthamiana leaves were agroinfiltrated with empty vector or pTRANS5′TEV constructions and used after 4 days p.a. to feed aphids (M. persicae).

$\dagger$Aphids pre-fed as indicated in the previous column were transferred to membrane-covered cages for a second feeding in solutions containing 20% sucrose and purified TEV virion particles at a concentration of 0·2 mg ml$^{-1}$.

$\dagger$Percentages of pooled values are shown. Fisher’s exact tests indicated significant differences ($P < 0·0001$) between the first row (negative control, empty vector) and the second row.
Table 4. Aphid transmission of PPV derivatives (PPV-EITC and PPV-HCPVY) assisted with transiently expressed PPV HC-Pro provided by agroinfiltration in infected plant tissues

<table>
<thead>
<tr>
<th>Virus†</th>
<th>Acquisition</th>
<th>Transmission*</th>
<th>Experiments</th>
<th>Total</th>
<th>Percentage‡</th>
<th>Range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPV-EITC</td>
<td>Empty vector</td>
<td>1</td>
<td>0/24</td>
<td>0</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>PPV-EITC</td>
<td>pTRANS5’PPV</td>
<td>5</td>
<td>32/140</td>
<td>22.8</td>
<td>0–45.8</td>
<td></td>
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<tr>
<td>PPV-HCPVY</td>
<td>Empty vector</td>
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<td>0/47</td>
<td>0</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>PPV-HCPVY</td>
<td>pTRANS5’PPV</td>
<td>8</td>
<td>41/186</td>
<td>22</td>
<td>4.3–50</td>
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<tr>
<td>PPV-5.15</td>
<td>Empty vector</td>
<td>7</td>
<td>63/153</td>
<td>41</td>
<td>20–60</td>
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</table>

*Plant-to-plant transmission experiments were performed, using 10 aphids per plant. The number of independent experiments (with at least 20 plants per experiment) is indicated in the first column. Pooled transmission rates are indicated as number of infected plants/number of test plants.
†N. benthamiana plants were infected with the indicated PPV-derived virus.
‡Infected leaves showing clear symptoms at 8 days p.i. were agroinfiltrated with the indicated constructs, and used after 4 days p.a. to feed aphids (M. persicae). Where no agroinfiltration occurred, N. benthamiana leaves were used directly for transmission after 12 days p.i.
§Percentages of pooled values are shown. Fisher’s exact tests indicated significant differences (P<0.001) between negative controls where empty vector was agroinfiltrated and cases where non-transmissible PPV-EITC and PPV-HCPVY variants where assisted in transmission, or between negative controls and the positive control of transmissible PPV-5.15 agroinfiltrated with empty vector (last row). The latter case was significantly different (P<0.002) to both of the PPV-EITC and PPV-HCPVY cases assisted by pTRANS5’PPV.

experiments performed with acquisition from regular infection with the transmissible PPV-5.15 variant, agroinfiltrated with empty vector (41% transmission).

As pTRANS5’PPV and the non-transmissible PPV variants share sequences (all except the point mutation in PPV-EITC and the sequences flanking the replaced HC-Pro in PPV-HCPVY), and as they might be expressed simultaneously in cells, the possibility of recombination exists, and in such a case restoration of an aphid-transmissible variant might occur. To check if any of the viruses transmitted in our experiments could be derived from a restored transmissible recombinant, IC-RT-PCR was done to test plants after transmission, confirming that all the infected test plants carried the expected sequence without recombination.

DISCUSSION

Transient expression of proteins mediated by A. tumefaciens in plants has become a widely used technique (Kapila et al., 1997; Fischer et al., 1999). It has been exploited for the analysis of suppressors of post-transcriptional gene silencing (Johansen & Carrington, 2001; Voinnet et al., 2003) and has also been shown to be an excellent tool for plant biotechnology and functional genetics (Earley et al., 2006; Orzaez et al., 2006).

Our results demonstrated that transient expression of PPV HC-Pro by means of A. tumefaciens on N. benthamiana plant tissues resulted in the production of a transmission-active protein. Furthermore, HC-Pro of a second potyvirus (TEV) was expressed and tested for transmission with positive results.

One of the most successful systems for expression of HC-Pro in aphid transmission studies is based on tagging HC-Pro in a full-length infectious clone and further purification (Blanc et al., 1999). However, this system has important drawbacks because HC-Pro is a multifunctional protein involved in crucial steps during systemic infection (Carrington et al., 1989; Cronin et al., 1995; Kasschau & Carrington, 1995; Kasschau et al., 1997) and mutational analysis is limited to those mutations that preserve infectivity. Moreover, attempts to use this method on PPV HC-Pro rendered the protein non-functional for transmission (unpublished observations).

Our purpose was to overcome the limitations of site-directed mutational analyses in full-length clones in order to study the activity of HC-Pro on aphid transmission. Efficient expression systems providing transmission-active HC-Pro are required, but until now only a few systems have been described (Berger et al., 1989; Thornbury et al., 1993; Sasaya et al., 2000; Ruiz-Ferrer et al., 2004), and all have shown different problems that prevent their extensive use. Our expression system was designed to take advantage of the translation-enhancing properties of PPV 5’ UTR sequences (Simon-Buela et al., 1997) and to use the proteolytic processing activities of P1 and HC-Pro to produce unmodified HC-Pro. This strategy allowed a high level of expression of HC-Pro to levels comparable to those observed in infected plants. We performed our initial experiments in N. benthamiana plants, a systemic host for PPV. Interestingly, when tobacco plants were used, we found lower accumulation of PPV HC-Pro, which might reflect host specificity of the construct used for expression. To further address this point, we performed comparisons of HC-Pro mRNA and siRNA accumulation in both
agroinfiltrated hosts. siRNA accumulation is considered a universal feature associated with RNA silencing, and HC-Pro is a potent silencing suppressor. However, the low level of mRNA accumulation in tobacco apparently was not correlated with a strong silencing phenomenon, because siRNAs remained almost undetectable. In addition, high levels of siRNAs were found in *N. benthamiana* along with a high level of expression of the protein, a result that agrees with the recent discovery of the molecular mechanism of suppression by HC-Pro acting through duplex siRNA binding (Lakatos *et al.*, 2006). All together, these results point to a complex scenario in which host factors and perhaps the presence of P1 could strongly influence protein accumulation. It is known that P1 contributes to synergistic responses in combination with HC-Pro (Pruss *et al.*, 1997), and recent reports have suggested that P1 is indeed involved in essential processes during the virus infection cycle (Rajamaki *et al.*, 2005). It can be speculated that if proteolytic processing by P1 of PPV is host-dependent, a low level of activity of this protein in tobacco might lead to reduced levels of HC-Pro, thus having a negative impact on the accumulation of its own transiently expressed mRNA, as observed with suppression-defective HC-Pro mutants (Gonzalez-Jara *et al.*, 2005). On the other hand, we cannot rule out the possibility that unknown peculiarities of *N. benthamiana* were responsible for the improved expression observed, independent of the construct used. Lower expression levels in *N. tabacum* than in *N. benthamiana* might be a general phenomenon for transient expression assays, as this has been observed elsewhere (Marillonnet *et al.*, 2004; Andrews & Curtis, 2005). Further studies are needed to solve these issues, although our observations suggest that for each particular virus a selection of suitable hosts for expression might be required.

The PPV HC-Pro expressed transiently was biologically functional for transmission in two different bioassays. Sequential feeding assays showed that the protein was fully active, with transmission rates similar to those obtained in experiments where HC-Pro was provided by a viral infection with a CP-defective virus variant. In this kind of experiment, aphids need to be moved twice during the assay, being forced to probe first in a plant and later through an artificial membrane. This might result in an overall reduction of the transmission efficiency. In an attempt to reduce the experimental stress on aphids, we designed a single-feeding assay in which aphids only need to probe on a single source of agroinfiltrated HC-Pro and virions, using PPV variants with non-functional HC-Pro, including a single point mutation in the KITC motif (PPV-EITC), and a chimaeric construct (PPV-HCPVY) that proved to be non-aphid-transmissible for unknown reasons after the majority of the HC-Pro region was replaced by the corresponding one from PVY. With this system we demonstrated again that transiently expressed HC-Pro was able to assist transmission. However, in this case we found a different rate of transmission between the HC-Pro-deficient mutants supplemented with the agroinfiltrated HC-Pro and the transmissible virus control. A possible explanation for this divergence could be interference from the inactive HC-Pro that could somehow alter the interaction between the functional agroinfiltrated HC-Pro and the virus particles. Another possible reason might be putative differential accumulation inside the infected cell of virions and the transiently expressed HC-Pro, making the acquisition of both products less likely to happen simultaneously. Interestingly, a recent study has shown that HC-Pro attaches to one of the termini of purified viral particles of two potyviruses (Torrance *et al.*, 2005) and the authors hypothesized that this might facilitate the acquisition of both HC-Pro and virus by the aphid in a single step in the form of a preformed complex.

To summarize, our results showed that potyvirus HC-Pro, biologically active for transmission, can be obtained via transient expression mediated by *A. tumefaciens*. PPV HC-Pro was expressed at high levels in agroinfiltrated leaves of *N. benthamiana* and the protein was capable of assisting aphid transmission in two different assays. The system also worked for HC-Pro of another potyvirus, TEV. Potentially, the transient expression of HC-Pro might serve to further dissect the molecular mechanisms of aphid transmission in potyviruses, and it could be extended to study the transmission mechanisms in other viruses where helper proteins are involved.

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