Aphid transmission studies using helper component proteins of Potato virus Y expressed from a vector derived from Potato virus X

Takahide Sasaya,† Lesley Torrance, Graham Cowan and Angelika Ziegler

Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK

The genes encoding the helper component (HC) proteins of two strains of Potato virus Y (PVY) were cloned and the proteins expressed from a vector derived from Potato virus X (PVX). The expressed HC contained six N-terminal histidine residues to facilitate purification by metal affinity chromatography. Approximately 2–4 µg/g of purified HC was obtained from leaves of Nicotiana benthamiana plants systemically infected by recombinant PVX. Preparations of the HC protein derived from PVY ordinary strain (PVYo) assisted aphid transmission of purified particles of PVYo and PVY strain C (PVYc; a naturally occurring non-aphid transmissible strain of PVY which contains a defective HC), as well as Potato aucuba mosaic virus. The HC derived from PVYc contained the Glu-Ile-Thr-Cys (EITC) motif, and mutation of Glu (E) to Lys (K) enabled the mutant PVX-expressed preparations to assist virus transmission by aphids. Expression of HC protein from the PVX vector produced biologically active protein. This approach should facilitate further studies to elucidate more precisely the molecular mechanism of virus transmission by aphids.

Introduction

Potato virus Y (PVY) is the type species of the genus Potyvirus, family Potyviridae. There are several naturally occurring strains of PVY that infect mainly solanaceous plants, causing substantial economic losses in crops such as potato, tobacco, tomato and pepper worldwide (de Bokx & Huttinga, 1981). PVY is spread between plants by aphids in a non-persistent manner and virus transmission involves interactions between virus particles, virus-encoded non-structural helper component (HC) protein and vector aphid styles (see review by Pirone & Blanc, 1996). For successful transmission, aphids must have access to HC before or at the same time as virus particles, which indicates that the HC forms a connection or ‘bridge’ between the virus particles and the surface of the aphid maxillary styles. Evidence is accumulating from studies of several potyviruses that there are key domains in the coat protein (CP) and HC proteins involved in these interactions. For example, experiments with mutants of infectious clones of Tobacco vein mottling virus (TVMV) and Tobacco etch virus (TEV). This work revealed that a single amino acid change from Lys (K) to Glu (E) in the N-terminal highly conserved Lys-Ile-Thr-Cys (KITC) motif of the HC protein abolished the capability of the HC protein to assist aphid transmission of virus particles (Atreya et al., 1992; Blanc et al., 1998). It has been predicted from sequence comparisons that the KITC motif is also important in transmission of PVY but it has not been directly demonstrated because the complete cDNA of PVY has been refractory to conventional cloning in Escherichia coli, despite efforts in several laboratories (Fakhfakh et al., 1996).

Blanc et al. (1997) showed specific binding between the HC protein and the CP or particles of aphid transmissible (containing the N-terminal DAG motif) but not aphid non-transmissible TVMV using a protein blotting-overlay assay. Also, mutation of the PTK motif to PAK in HC of Zucchini yellow mosaic virus (ZYMV) resulted in loss of ability to assist aphid transmission and abolished in vitro binding to particles of ZYMV (Peng et al., 1998). Recently, Blanc et al. (1998) have shown that preparations of HC protein from PVY that contain the N-terminal KITC motif were retained in aphid styles
whereas preparations of the HC of an aphid non-transmissible isolate containing the EITC motif were not. Furthermore, both PVY HC preparations, when immobilized on nitrocellulose membranes, bound to virus particles. Therefore, the KITC motif is thought to be involved in interactions with aphid styles whereas the PTK motif interacts with virus particles. However, additional differences have been noted in other N-terminal amino acids that correlate with defective HC in TEV, Turnip mosaic virus and PVY (Blanc et al., 1998; Nakashima et al., 1993; Canto et al., 1995).

On the other hand, pre-feeding aphids on preparations of the recombinant N-terminal polypeptide of the CP of Maize dwarf mosaic virus before access to virus-infected plants inhibited aphid transmission of virus particles (Salomon & Bernardi, 1995). This finding was interpreted as evidence to support the hypothesis that the interaction with HC induces a conformational change in the CP exposing the N-terminal residues and allowing them to interact with the aphid styles. However, similar experiments with the N-terminal polypeptide of TVMV did not inhibit transmission (Blanc et al., 1997).

Thus the molecular details favouring the interactions between the CP or virus particles and the HC protein may not be the same for all potyviruses. Attempts to investigate the nature of the PVY–HC interaction are hampered by the difficulty in obtaining preparations of functional HC protein by expression in E. coli or baculovirus systems (Thornbury et al., 1993). In this paper, we report the expression and purification of biologically active HC from a vector derived from Potato virus X, and the application of these preparations to studies on aphid transmission.

**Methods**

**Viruses strains.** Scottish isolates of PVY, an aphid transmissible ordinary strain, PVYo, and a non-aphid transmissible strain, PVYN, were obtained from the Scottish Agricultural Science Agency. They were maintained by mechanical inoculation to Nicotiana tabacum and N. debneyi, respectively, and virus particles were purified by the method of Govier & Kassas (1974).

A purified preparation of Potato aucuba mosaic virus (PAMV) was obtained from B. D. Harrison (SCRI): the coat protein of this isolate was previously shown to contain the DAG sequence motif (Baulcombe et al., 1993).

**Cloning and nucleotide sequencing of HC genes.** The cDNA fragments corresponding to the genes that encode the HC of PVYo or PVYN were obtained by reverse transcription of viral RNA with avian myeloblastosis virus reverse transcriptase followed by polymerase chain reaction (RT–PCR), using PVY-specific primers 5‘ CGCGGATCCC-GAATGCTGATAATTTTTGG 3’, identical to nucleotides 1031–1051, and 5‘ GCCTGTCGACCCACCAACCCTATAATG 3’, complementary to nucleotides 2384–2440 (Thornbury et al., 1990); the primers also contained BamHI and PstI sites (underlined). The PCR products were cloned into pGEM-T plasmid vector (Promega). The nucleotide sequence was determined in both directions by the dideoxynucleotide chain termination procedure, using an Applied Biosystems 377 DNA sequencer (Perkin-Elmer).

**Production of recombinant PVX.** The pGEM-T plasmid preparations containing HC cDNA were digested by BamHI and PstI and the cDNA fragments were cloned into BamHI/PstI-digested pQE-30 plasmid (Qiagen). Expression in this vector produces HC protein fused at the N terminus to six histidine residues. The pQE-30 plasmids containing HC cDNA were digested by EcoRI and PstI and the cDNA fragments were cloned into EcoRV-digested PVX-based vector pTXS.P3CS2 402 (Baulcombe et al., 1995) after treatment with Klenow fragment (BioLabs) to create blunt ends. PVX vector plasmids harbouring the HC gene in the desired orientation were linearized by digestion with SphI, and in vitro transcripts were synthesized with T7 RNA polymerase using the RiboMAX large scale RNA production system (Promega). Transcripts were mechanically inoculated to carorundum-dusted leaves of N. benthamiana plants.

**Mutagenesis of PVYN HC.** The cDNA fragment of the HC protein gene of PVYN was amplified by RT–PCR using the PVY-specific primers and cloned into pGEM-T vector plasmid. The E to K mutation was done by site-directed mutagenesis according to Sang et al. (1996) using two primers (5‘ CTGCCGTGTACAGAAATACTC 3’ and 5‘ AATG-GTGTGTCATCAATGCAGC 3’). After double digestion with BamHI and PstI, the cDNA fragment of the point mutant HC protein gene of PVYN was cloned into BamHI/PstI-digested pQE-30 plasmid (Qiagen). Construction of recombinant PVX-CK was done as described above.

**Purification of HC from PVX-infected tissue.** Leaves (10 g) showing fully developed symptoms were macerated in a blender in 50 ml of chilled 0.3 M potassium phosphate (KPB), pH 9.0, containing one protease inhibitor cocktail tablet (Boehringer Mannheim). The extract was separated from the plant debris by low-speed centrifugation at 8000 g for 10 min at 4 °C, and then high-speed centrifugation at 120000 g for 1 h at 4 °C. The supernatant was collected and mixed with 500 μl of a 50% slurry of nitritolriacet acid resin charged with nickel ions (Ni2+–NTA, Qiagen). The mixture was shaken for 1 h at 4 °C and then centrifuged for 3 min at 1000 g. The pellet was collected in a 2 ml tube and washed three times with chilled KPB. The proteins were eluted from the Ni2+–NTA resin with 1.5 ml of chilled KPB containing 200 mM imidazole or 400 mM EGTA. The eluate was filtered through an Ultrafree-MC filter (Millipore), and concentrated to 500 μl using a Centricon 30 concentrator (Amicon). The concentrated HC preparation was stored at –70 °C.

**Aphid transmissions.** Aphids (Myzus persicae) were reared on turnip and prior to each experiment were fasted for approx. 2 h. In preliminary experiments, aphids were allowed access to detached leaves systemically infected with wild-type or hybrid PVX for 5–10 min. Aphids seen to be feeding were transferred to either PVYN- or PVYN-infected leaves for a further 5–10 min, and then placed on healthy seedlings of N. tabacum or N. benthamiana for approx. 18 h. In experiments with purified HC, before each aphid transmission test the HC preparation was dialysed against TSM buffer (100 mM Tris–HCl, 20 mM MgCl2, pH 7.2) using Slide-A-Lyzer mini dialysis units (Pierce). Aphids were allowed a 20 min acquisition feed, through stretched Parafilm membranes, on a mixture of 100 μg/ml of purified virions and 40 μg/ml of Ni2+–NTA purified HC containing 20% sucrose, followed by an 18 h inoculation feed on seedlings of either N. clevelandii, or PVYN- or PVYN- pepper (Capsicum annuum) for PVAM. Ten to twenty aphids per plant were used in all experiments. Virus infection was confirmed by ELISA tests with monoclonal and polyclonal antibodies against PVY, or by the symptoms (systemic necrosis) induced by PAMV on pepper.
Results and Discussion

Preliminary work was done to establish the nucleotide sequences encoding the HC protein of the two PVY isolates used in these studies. The deduced amino acid sequences were 95.8% identical and most differences were in the N terminus (12 in the first 100 amino acids; Fig. 1). The amino acid sequence of the Scottish isolate of PVY\textsubscript{c} was 99.1% identical to the published PVY\textsubscript{c} (Thornbury \textit{et al.}, 1990); there were only four amino acid differences and none of these were in the N terminus.

Systemically infected leaves of plants infected with PVX transcripts expressing HC protein produced symptoms similar

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<tr>
<th>Virus</th>
<th>Source of HC\textsuperscript{*}</th>
<th>Transmission\textsuperscript{†}</th>
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<tbody>
<tr>
<td>PVY\textsubscript{o}</td>
<td>None</td>
<td>0/39</td>
</tr>
<tr>
<td>PVY\textsubscript{c}</td>
<td>None</td>
<td>0/36</td>
</tr>
<tr>
<td>PAMV</td>
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<td>0/36</td>
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<tr>
<td>PVY\textsubscript{o} HC-PVY\textsubscript{o}</td>
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</tr>
<tr>
<td>PVY\textsubscript{c} HC-PVY\textsubscript{o}</td>
<td>29/36</td>
<td></td>
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<tr>
<td>PAMV HC-PVY\textsubscript{o}</td>
<td>29/36</td>
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<tr>
<td>PVY\textsubscript{c} HC-PVY\textsubscript{c}</td>
<td>0/36</td>
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<tr>
<td>PVY\textsubscript{o} Mutated HC-PVY\textsubscript{o}</td>
<td>29/36</td>
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\textsuperscript{*} Aphids fed through membranes on purified particles of the respective virus preparation, or mixtures of virus and purified HC preparations.

\textsuperscript{†} No. of plants infected/no. tested. Totals from three independent experiments.

(100% similar) to the published PVY\textsubscript{c} (Thornbury \textit{et al.}, 1990); there were only four amino acid differences and none of these were in the N terminus.

![Fig. 1. Amino acid sequence of the N-terminal 100 residues of PVY\textsubscript{o} HC protein (Thornbury \textit{et al.}, 1990), and amino acids that differ in the sequences of the HCs of Scottish isolates (S) of PVY\textsubscript{o} shown above, and PVY\textsubscript{c} shown below. The conserved KITC motif is in bold typeface.](image)

![Fig. 2. Analysis of preparations of His-tagged HC protein expressed from recombinant PVX. (a) PAGE of preparations purified by metal affinity chromatography from \textit{N. benthamiana} leaves systemically infected with PVX expressing HC (arrowed) (lane 2), PVX (lane 3), non-infected leaves (lane 5), or from PVY-infected \textit{N. tabacum} leaves (lane 4). Lane 1 contains molecular size markers (Bio-Rad low range). (b) Immunoblot of a purified HC preparation, reaction with anti-HC antiserum.](image)
to those of PVX transcript alone (mosaic and veinal necrosis). HC was purified by metal affinity chromatography with yields of 2–4 µg of HC protein per g of leaf tissue, estimated by comparison of the intensity of the Coomassie blue-stained HC protein with known amounts of bovine serum albumin. Analysis of the preparations by SDS–PAGE revealed two major bands of approx. molecular mass 100 kDa and 50 kDa (Fig. 2a, lane 2). In addition, two minor bands of > 100 kDa were visible. The proteins in these bands reacted with PVY HC specific antiserum (Fig. 2b) and with anti-polystyridine monoclonal antibodies (data not shown). The upper bands (> 100 kDa) are probably dimers and oligomers of HC. None of these bands were seen in the eluted fractions prepared from plants inoculated with wild-type PVX vector, PVY-infected or healthy plants (Fig. 2a, lanes 3–5 respectively).

In preliminary aphid transmission tests, PVYc was transmitted by aphids that had previously fed on leaves systemically infected with PVX expressing HC (9/28 plants became infected) but not by aphids that had fed on the leaves of wild-type PVX (0/27) or healthy plants (0/10).

Transmission tests done with purified HC protein (Table 1) showed that purified particles of not only PVYc and PVYc but also PAMV were readily transmitted when the mixtures were supplemented with the HC protein of PVYc. These results show that the His-tagged HC protein expressed by the PVX vector is biologically active. On the other hand, the HC protein of PVYc (used at a similar concentration) did not mediate aphid transmission (Table 1).

A mutant of the PVYc HC was prepared that contained a K for E replacement in the EITC motif. It was expressed from PVX-CK, purified and tested for capability to assist transmission of PVYc particles. The mutation was shown to restore biological activity and assist aphid transmission (Table 1). The aphid transmission rate of the mutated HC protein was similar to that of PVX-expressed HC protein of PVYc. Thus these results show that only the K residue is essential to restore the capability to assist aphid transmission, and that the other amino acid differences at the N terminus do not play a role. It is also interesting to note that bands equivalent to dimers and other oligomers of the native and the mutant PVYc HC were present in stained SDS–polyacrylamide gels suggesting that dimerization occurred in both kinds of preparations (data not shown). Therefore, the presence of an E residue does not affect the capacity to form dimers as has been suggested (see review by Pirone & Blanc, 1996).

The work presented in this paper has demonstrated that biologically active HC can be expressed from the PVX vector, and readily purified by metal affinity chromatography. This system was used to confirm that the predicted K for E mutation restores the helper function of defective PVYc HC. Expression of HC from the PVX vector is a facile system that avoids problems of contamination of HC preparations with CP. Moreover, this system has the advantage that the effect of point mutations and deletions that might be lethal if using infectious clones can be studied. In future, purified recombinant HC may be used for in vitro binding studies, to dissect the molecular details of virus transmission, for PVY or other potyviruses.

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References


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