Loss of potyvirus transmissibility and helper-component activity correlate with non-retention of virions in aphid stylets


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The hypothesis that loss of aphid transmissibility of potyvirus mutants is due to non-retention of virions in the mouthparts was tested by feeding aphids through membranes on purified virions of aphid transmissible (AT or HAT) and non-aphid-transmissible (NAT) tobacco vein mottling virus (TVMV) or tobacco etch virus (TEV), in the presence of functional [potato virus Y (PVY) HC or TVMV HC] or non-functional (PVC HC) helper component (HC). TVMV virions were detected, by electron microscopic examination of immunogold-labelled thin sections, in the food canal or cibarium of 57% of 28 aphids fed on the transmissible combination of TVMV-AT and functional HC, while no virions were found in these structures in 25 aphids fed on the non-transmissible combinations: TVMV-NAT and PVY HC, or TVMV-AT and PVC HC. Autoradiography of intact stylets allowed the examination of much larger numbers of aphids, fed on 125I-labelled TEV; 48% of 523 aphids fed on the TEV-HAT and PVY HC combination retained label in the stylets; this correlated well with the percentage transmission in bioassays. In contrast, in non-transmissible combinations, label was found in the stylets of 0.77% of 389 aphids fed on TEV-NAT and PVY HC, and 1.35% of 223 aphids fed on TEV-HAT and PVC HC. No differences were found in the overall amount of label in the bodies of aphids fed on the transmissible and non-transmissible combinations. There was a strong tendency for virions to be retained in the distal third of the stylets; 56% of aphids positive for TVMV, and 82% of those positive for TEV, had label in this area. These data support the concept that virions retained within the stylets are those that are primarily involved in potyvirus transmission.

Introduction

Transmission of potyviruses by aphids requires virions with an appropriate sequence near the N terminus of the coat protein, as well as active helper component (HC) protein, which effects transmission of the virus (Pirone, 1991). Mutations in either the coat protein or the helper component protein can result in loss of aphid transmissibility of a potyvirus (Atreya et al., 1990, 1991, 1992a).

Our laboratory has characterized mutants of tobacco vein mottling virus (TVMV) and potato virus Y (PVY) that have lost virion transmissibility and HC activity, respectively. The non-transmissibility of the TVMV mutant, TVMV-NAT, resulted from a change in the highly conserved DAG sequence near the coat protein N terminus to DAE (Atreya et al., 1990), while the loss of HC activity in the PVY variant, PVC, was attributed to a lysine to glutamic acid mutation in the highly conserved KITC sequence in the HC-Pro region (Atreya et al., 1992a). The reason for lack of virion transmissibility and HC activity has not been determined, however.

Previous studies, using light microscopic autoradiography, showed that transmissible tobacco etch virus (TEV) virions were retained in the mouthparts only if active HC was present in the feeding mixture (Berger & Pirone, 1986). More recently, using transmission electron microscopy (TEM) and immunogold labelling of thin sectioned aphids, TVMV-AT virions were found to be associated with TVMV HC in the maxillary food canal and foregut, while virions were not found there when ingested in the absence of HC (Ammar et al., 1994). Both studies thus suggested that the ability of a potyvirus to be transmitted by aphids is dependent upon retention of virions in the mouthparts, and that HC functions by facilitating such retention. We thus hypothesized that TVMV-NAT virions were not transmitted because they were not retained, and that PVC HC was non-functional because it did not effect retention, even of the intrinsically transmissible TVMV-AT.

While the initial focus of our study compared the retention of TVMV-AT and TVMV-NAT using TEM of...
immunogold-labelled ultrathin sections, it became apparent that an alternative approach that did not require sectioning for TEM was needed to generate extensive comparative data. An autoradiographic-light microscopy technique was thus developed, which in turn required the production of a TEV-NAT mutant for comparison with TEV-HAT (highly aphid transmissible), since TVMV virions could not be sufficiently labelled with $^{125}$I to allow detection.

**Methods**

**Viruses and HC.** The origin and properties of TVMV-AT and TVMV-NAT (DAE mutant) have been described (Atreya et al., 1990). The TEV-HAT isolate was that used in previous studies (Pirone, 1981; Pirone & Thornbury, 1983). The mutant TEV-NAT was created using plasmid pTEV 7DA, provided by J. C. Carrington (Carrington et al., 1993), which contains a full-length cDNA copy of TEV-HAT. Details of the construction of TEV-NAT are described below.

The properties of PVY HC and TVMV HC, both of which are capable of effecting the transmission of TVMV-AT and TEV-HAT, have been described (Thornbury et al., 1985, 1990), as have the properties of the transmission-defective PVC HC (Thornbury et al., 1990).

Virus purification was by the method of Murphy et al. (1990), and HC purification, through the sucrose gradient step, followed our previously described procedures (Thornbury et al., 1985, 1990). The intrinsic infectivity of virus preparations was tested by mechanical inoculation; TVMV preparations were assayed on tobacco (Nicotiana tabacum cv. Ky 14) plants by dilution assay (Atreya et al., 1991), while TEV preparations were tested by local lesion assay on Chenopodium amaranticolor.

The activity of TVMV HC and PVY HC preparations was quantified by our standard aphid bioassay (Pirone, 1981). The amount of PVC HC used in the experiments was made equivalent to that of PVY HC, based upon quantitative Western blotting (Atreya & Pirone, 1993). The lack of transmission-effecting ability of PVC HC was verified in parallel aphid transmission tests.

**Construction and bioassay of TEV-NAT.** Our previous studies with TVMV showed that substitution of a variety of amino acids for glutamic acid in the highly conserved DAG sequence near the coat protein (CP) N-terminus resulted in loss of aphid transmissibility (Atreya et al., 1995). The N-terminal sequences of TVMV-AT (SRTVDAG) and TEV-HAT (SGTVDAG) are similar. Plasmid pTEV 7DA (Carrington et al., 1993), which contains a full-length cDNA copy of TEV-HAT RNA, and is able to produce infectious in vitro transcripts, was thus used to create TEV-NAT, containing an aspartic acid to lysine mutation. Manipulations were performed in a subclone designated pTEV SB, obtained by insertion of a SalI (position 7165, according to numbering by Allison et al. (1986))-BgIII [located after the poly(A) tail] fragment into pBluescript II KS (+) (Stratagene). A PCR strategy (Tao & Lee, 1994) was used to create the aspartic acid to lysine mutation. Briefly, two separate PCR reactions were performed with appropriate primers to create overlapping DNA fragments containing the mutation and a new KpnI site in the dipeptide motif GT (Atreya et al., 1992b). After restriction, both fragments were ligated and inserted into pTEV-SB. Recombinant plasmids containing the mutation were identified, and the SalI-BgIII restriction fragment was inserted into the genomic-length plasmid pTEV-7DA to create pTEV-NAT. The mutation, resulting in the N-terminal sequence SGTVKAG, was confirmed by sequencing with Sequenase version 2.0 (United States Biochemical).

pTEV-NAT was linearized with BgIII, and transcripts were synthesized in vitro using SP6 RNA polymerase (Gibco-BRL) in the presence of mGpppG gap analogue (New England Biolabs). Tobacco plants were mechanically inoculated with transcripts, and the presence of the mutation in progeny virus was confirmed by RT-PCR of viral RNA and partial direct sequencing using the CircumVent sequencing kit (New England Biolabs). Plants infected with TEV-NAT were used for virus purification, and the specific infectivity of TEV-NAT was found to be identical to that of TEV-HAT in mechanical inoculation assays on C. amaranticolor. The non-aphid-transmissibility of TEV-NAT was verified in aphid transmission tests from tobacco plants, and with purified virus in combination with PVY HC.

**Virus iodination.** Virus was radioiodinated using Iodogen (Pierce) according to the manufacturer's instructions. Two-hundred μl of purified virus (3.2-4.5 mg/ml) was reacted for 20 min with 1 mCi Na$^{125}$I. All reactions were done at 4 °C. Labelled virus was separated from unreacted Na$^{125}$I by gel filtration on a Sephadex G-25 spin column equilibrated with 20 mm-Tris-HCl buffer containing 1 mm-EDTA, pH 7.5. The concentration of labelled virus was determined spectrophotometrically. The specific activity of labelled virus was 2300-5400 d.p.m./ng virus as determined in a single-well gamma counter (Bioscan). Labelled virus was assayed for infectivity on C. amaranticolor, and for transmissibility by aphid transmission to tobacco seedlings.

**Virus acquisition and transmission by aphids.** Myzus persicae were reared and handled as previously described (Raccah & Pirone, 1984); apterae were collected and kept in glass vials for 2-3 h of preacquisition fasting in all experiments. Acquisition of purified virus through Parafilm membranes and subsequent processing for detection were as described below for each type of experiment.

Procedures for, and results of, transmission assays for purified TVMV were described previously (Ammar et al., 1994). For purified TEV, after acquisition access (Pirone, 1981; Ammar et al., 1994), a single aphid was placed on each test plant for the transmission-positive combination (TEV-HAT and PVY HC), whereas for the transmission-negative combinations (TEV-NAT and PVY HC, or TEV-HAT and PVY HC), ten aphids were placed on each test plant. Aphids were allowed to remain on the test plants overnight (14-18 h); plants were then sprayed with an insecticide and placed in a growth room for symptom development.

**Processing of aphids for immunogold labelling and TEM.** For these experiments, done with TVMV, five aphids were placed in each feeding cage, and probing was monitored under a stereomicroscope. Aphids that probed for 1–2 min were removed, immediately anaesthetized with CO$_2$, and processed for TEM as described by Ammar et al. (1994). The head and prothorax were dissected, fixed in 25% glutaraldehyde containing 1% paraformaldehyde, cold embedded in LR Gold, and sectioned and processed for immunogold labelling. From 40–60 sections of the styliets and/or cibarium of each aphid were examined.

**Autoradiographic detection of TEV in aphid styliets.** About 20 aphids were placed in each feeding chamber for a 10 min acquisition access to solutions which contained 320-450 μg/ml 5'-labeled TEV-HAT or TEV-NAT, and either PVY HC or PVY HC. Those aphids that were on the membrane at the end of the acquisition access period were selected for further processing. Aphids were placed in polyethylene specimen-processing holders, 14 mm in diameter and 18 mm high, perforated at the bottom (Electron Microscopy Sciences), and immediately dipped into liquid nitrogen. They were then thawed at room temperature and transferred onto a piece of double stick tape on a glass slide (20–30 aphids/slide) each oriented ventral side up.

The styliets of each aphid were carefully separated from the styliets groove of the proboscis and laid flat on the tape using a finely sharpened insect pin. The slides were then coated with liquid nuclear
track emulsion (type NTB2, Eastman Kodak) according to the manufacturer’s instructions, dried thoroughly, and stored at 4 °C in the dark. Based on preliminary trials, incubation for 4 weeks allowed detection of label in aphids that acquired from solutions containing from 10–500 μg/ml TEV, and this period was thus used for the experiments. After incubation, slides were developed following the manufacturer’s instructions. Results were determined using a Zeiss photomicroscope III.

Liquid scintillation counting for virus uptake by aphids. Approximately 30 uniform-sized aphids were placed in each feeding chamber for a 10 min acquisition access to solutions containing 300–450 μg/ml 125I-labelled TEV-HAT and TEV-NAT, with PVY HC, PVC HC or without HC. Ten aphids that were still on the feeding membrane at the end of the acquisition access period were transferred to a liquid scintillation counting vial. They were then thoroughly crushed with a wooden applicator stick; the part of the stick that touched the aphids was broken off and left in the vial. Water (0.5 ml) and 4·5 ml of counting cocktail (Bio-Safe II, Research Products International) were added and mixed. Radioactivity was measured in a liquid scintillation counter (TRI-CARB Liquid Scintillation Analyzer model 2200CA, Packard). Aphids that acquired unlabelled TEV-HAT plus PVY HC were used as background controls. Five vials were counted for each treatment, and experiments were repeated twice. Data were subjected to analysis of variance, and means were separated by Duncan’s new multiple range test (SAS Institute Inc.).

Results

Comparative retention as determined by TEM

Gold-labelled virions were found to be associated with the cuticle of the food canal or cibarium only in aphids that had acquired the transmissible combinations (TVMV-AT with TVMV HC or PVY HC). Labelled virions were found in 16 of 28 aphids fed on the transmissible combinations; a typical section is shown in Fig. 1(a). Virions were detected most often in the distal part of the food canal and least often in the cibarium. No virions or label were found in 25 aphids fed on the non-transmissible combinations (Fig. 1b). The data are presented in Table 1.

Comparative retention as determined with radiolabelled TEV

When 125I-labelled TEV-HAT was acquired in combination with PVY HC, label was found in the stylets of 253 of 523 aphids, whereas after acquisition of TEV-HAT in the presence of PVC HC, only 2 of 181 aphids had label in the stylets. Similar results were obtained with the other non-transmissible combination, TEV-NAT and PVY HC, for which 3 of 339 aphids retained label in the stylets (Table 2). No label was found in the stylets of 72 control aphids not given access to labelled virus. There was a good correlation between aphid transmission of TEV-HAT acquired with PVY HC and virus retention in the stylets. The data for retention and transmission are summarized in Table 2.

Fig. 1. (a) Cross-section of the stylets of an aphid fed on TVMV-AT and TVMV HC showing gold-labelling of TVMV-AT particles which are primarily associated with the epicuticle lining the maxillary food canal. (b) Cross-section typical of those of stylets from aphids fed on the non-transmissible combinations of virions and HC. Bars represent 125 nm.

Comparative virus uptake by aphids

To determine whether differences in the numbers of aphids containing label in the stylets could be a reflection of differential uptake, entire aphids given acquisition
Table 1. Occurrence of cuticle-associated virions in thin sections of the stylets and foregut of aphids given acquisition access to aphid-transmissible (AT) or non-transmissible (NAT) TVMV virions in the presence of functional (TVMV HC or PVY HC) or non-functional (PVC HC) helper component

<table>
<thead>
<tr>
<th>Combination of virus and HC in feeding solution</th>
<th>No. of aphids sectioned</th>
<th>Distal food canal</th>
<th>Proximal food canal</th>
<th>Cibarium</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transmissible</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TVMV-AT + TVMV HC</td>
<td>15</td>
<td>8/13</td>
<td>6/14</td>
<td>2/11</td>
</tr>
<tr>
<td>TVMV-AT + PVY HC</td>
<td>13</td>
<td>6/12</td>
<td>3/12</td>
<td>0/6</td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>14/25</td>
<td>9/26</td>
<td>2/17</td>
</tr>
<tr>
<td>(56.0%)</td>
<td>(34.6%)</td>
<td>(12%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Non-transmissible</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TVMV-NAT + TVMV HC</td>
<td>8</td>
<td>0/6</td>
<td>0/6</td>
<td>0/5</td>
</tr>
<tr>
<td>TVMV-NAT + PVY HC</td>
<td>9</td>
<td>0/8</td>
<td>0/9</td>
<td></td>
</tr>
<tr>
<td>TVMV-AT + PVC HC</td>
<td>8</td>
<td>0/7</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>0/21</td>
<td>0/23</td>
<td>0/5</td>
</tr>
</tbody>
</table>

* No. of aphids in which virions were detected/no. examined. From 40–60 sections were examined for each aphid. Not all aphids could be sectioned for the three indicated regions due to difficulties in sectioning and/or orientation.

Table 2. Occurrence of label in the stylets of aphids given acquisition access to 125I-labelled transmissible (TEV-HAT) or non-transmissible (TEV-NAT) virions in the presence of functional (PVY HC) or non-functional (PVC HC) helper component and its correlation with virus transmission

Data are pooled results of four experiments.

<table>
<thead>
<tr>
<th>Assay method</th>
<th>Virus</th>
<th>PVY HC</th>
<th>PVC HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoradiography</td>
<td>TEV-HAT</td>
<td>253/523*</td>
<td>3/223*</td>
</tr>
<tr>
<td></td>
<td>TEV-NAT</td>
<td>3/389*</td>
<td>0/8</td>
</tr>
<tr>
<td>Aphid transmission</td>
<td>TEV-HAT</td>
<td>47/150†</td>
<td>0/60†</td>
</tr>
<tr>
<td></td>
<td>TEV-HAT$</td>
<td>52/80†</td>
<td>650</td>
</tr>
<tr>
<td></td>
<td>TEV-NAT</td>
<td>0/30†</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>TEV-NAT$</td>
<td>0/50†</td>
<td>0</td>
</tr>
</tbody>
</table>

* No. of aphid stylets positive for label/total no. of stylets examined.
† No. of plants infected/total no. of test plants; one aphid/test plant.
‡ No. of plants infected/total no. of test plants; ten aphids/test plant.
§ Unlabelled virus.

Results are means ± standard errors from two trials, each with five replicates. Numbers followed by the same letter are not significantly different at the 0.01 level according to Duncan's new multiple range test.

Virus distribution in the stylets

The availability of large numbers of stylets containing label acquired with the transmissible TEV-HAT and PVY HC combination allowed a detailed analysis of the pattern of distribution. The stylets were arbitrarily divided into thirds: the distal (farthest from the aphid body), the central, and the proximal. Representative patterns of distribution are shown in Fig. 2. The data, summarized in Table 4, show that label was present in the distal third in 82% of the stylets, either solely or in combination with the other regions. Only three of the 253 aphids had labelled virus evenly distributed throughout the stylets. Occasionally, one or both mandibular stylets became separated from the maxillary stylets, and in such cases label remained associated with the maxillary...
Fig. 2. Autoradiographs of stylets of *Myzus persicae* depicting the classes of distribution in Table 4. (a) Stylets of a control aphid; (b-f) label in stylets of aphids fed on ^125^I-labelled TEV-HAT with PVY HC; (b) label only in the distal third of the stylets; (c) label in both anterior and central thirds of the stylets; (d) label in anterior central and posterior thirds of the stylets; (e) label evenly distributed in the stylets; (f) label only on maxillary stylets; L, leg; MA, mandibular stylets; MX, maxillary stylets; P, proboscis; S, stylets. All magnifications × 440.
There was no apparent difference in the distribution of label between the transmissible and non-transmissible combinations, although the number of positives for the latter was too low to allow meaningful comparison.

**Discussion**

Loss of aphid transmissibility of potyviruses can result from mutations in either the CP or the HC protein (Pirone, 1991). Although the molecular basis of these dysfunctions has been established in some cases, the actual step(s) in the transmission process (acquisition, retention, inoculation) which is disrupted has not been determined. The results reported here indicate that mutations, in either of these proteins, that lead to loss of aphid transmissibility do so because they affect retention of virions in the food canal of the stylets.

HC was initially proposed to function by ‘binding’ to sites in the food canal and to virus particles (Govier & Kassanis, 1974), and evidence was later provided to support this (Berger & Pirone, 1986). The CP DAG motif has been proposed to function in binding of the virion to the HC (Pirone, 1991). The inability of virions with an altered motif to be retained in the stylets, even in the presence of functional HC, whereas virions with the DAG motif are retained, supports a role for DAG in an HC–virion interaction that allows retention. The results of the present study suggest that PVC HC does not function because it is not retained in the food canal, or does not function in retention of virions, or both. Further experiments are needed to discriminate among these possibilities.

The concept that virus carried in (or on) the stylets is of relevance in non-persistent transmission was first advanced by Hoggan (1933), but it was the classical experiments of Bradley (1964) that first provided evidence to support this hypothesis. Bradley found that chemical and physical treatments of the tips of the stylets of viruliferous aphids resulted in a decrease in (or loss of) ability to transmit PVY. It was largely due to this evidence that Kennedy et al. (1962) proposed the term ‘stylet-borne’ to describe the route of transport of non-persistently transmitted viruses. Based upon evidence obtained with the same experimental protocols, Bradley (1966) concluded that transmissible PVY was carried near the tips of the maxillary stylets. Bradley’s experiments did not, however, distinguish whether transmissible virus was carried in or on the stylets. The finding, by Taylor & Robertson (1974) of TEV-like particles in the distal part of the food canal supported an internal site, although the relevance of this virus to the transmission process could not be established.

Although Bradley’s conclusions were widely accepted at the time, it was later pointed out that such treatments might have affected aphid behaviour and hence transmission (Harris, 1977). This, coupled with evidence that aphids can egest previously imbibed materials (Harris & Bath, 1973), led to the concept that transmissible virus might be that retained as far back as the anterior alimentary canal (i.e. foregut or oesophagus; Harris & Bath, 1973; Harris, 1977). As a consequence, the term stylet-borne has been largely supplanted by the less restrictive terms non-circulative or foregut-borne to describe the vector relationship of non-persistently transmitted viruses (e.g. Matthews, 1991). The results of our TEM study show a strong bias towards retention of TVMV in the food canal rather than the cibarium (Table 1), while the 125I-labelled virus experiments, which were pertinent only for the stylets, showed a strong bias toward retention in the distal portion (Table 4). Taken together, these data strongly suggest that it is primarily stylet-retained virus that is ultimately transmitted.

Given the concept that the virus particles of relevance to the transmission process are those retained in the food canal, the question remains as to how, and under what circumstances, they are released for the process of inoculation. The combination of labelled virions and autoradiography of the stylets should serve as a useful tool to study these as well as other questions such as vector efficiency and specificity with potyviruses as well as other non-circulatively transmitted viruses.

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**References**


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