Studies on the role of the minor capsid protein in transport of Beet western yellows virus through Myzus persicae

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Beet western yellows virus (BWYV), family Luteoviridae, is an icosahedral plant virus which is strictly transmitted by aphids in a persistent and circulative manner. Virions cross two cellular barriers in the aphid by receptor-based mechanisms involving endocytosis and exocytosis. Particles are first transported across intestinal cells into the haemolymph and then across accessory salivary gland cells for delivery to the plant via saliva. We identified the midgut part of the digestive tract as the site of intestinal passage by BWYV virions. To analyse the role in transmission of the minor capsid component, the readthrough (RT) protein, the fate of a BWYV RT-deficient non-transmissible mutant was followed by transmission electron microscopy in the vector Myzus persicae. This mutant was observed in the gut lumen but was never found inside midgut cells. However, virion aggregates were detected in the basal lamina of midgut cells when BWYV antisera was microinjected into the haemolymph. The presence of virions in the haemolymph was confirmed by a sensitive molecular technique for detecting viral RNA. Thus, transport of the mutant virions through intestinal cells occurred but at a low frequency. Even when microinjected into the haemolymph, the RT protein mutant was never detected near or in the accessory salivary gland cells. We conclude that the RT protein is not strictly required for the transport of virus particles through midgut cells, but is necessary for the maintenance of virions in the haemolymph and their passage through accessory salivary gland cells.

Introduction

Beet western yellows virus (BWYV) is a member of the genus Polerovirus in the family Luteoviridae (Mayo & D’Arcy, 1999). Virions of BWYV are icosahedral particles of 25 nm diameter containing a monopartite RNA of 5–6 kb. Typically, luteoviruses are limited to phloem cells of their host plants and are obligately transmitted by aphids (Homoptera, Aphididae). Their transmission mode is persistent and circulative. The route of virions in the vector’s body includes, successively, ingestion with phloem sap during aphid feeding on an infected plant, transport across the gut wall to the haemocoel and then transport from there into the accessory salivary glands (ASG). Finally, the particles are delivered into the salivary canal from where they are injected into a plant with saliva during a subsequent feed (Gildow, 1999).

Earlier studies on Luteoviridae, especially on the barley yellow dwarf complex, composed mainly of Barley yellow dwarf virus-PAV and -MAV (BYDV-PAV and -MAV), and Cereal yellow dwarf virus-RPV (CYDV-RPV), revealed that digestive and salivary epithelia have differential specificity to luteovirus particles (Rochow, 1969; Gildow, 1999). Particles of many luteoviruses, with some exceptions (Gildow, 1999), can be transported across the gut wall of different aphid species, whether efficient vectors or non-vectors. Therefore, the ability of luteoviruses to cross the gut wall is a trait generally unrelated to vector-specificity. However, the gut wall may
affect transmission efficiency (Rouzé-Jouan et al., 2001). The salivary gland barrier, on the other hand, appears to be virus species-specific and accounts for most of the transmission specificity between vector and virus species. Moreover, the salivary gland barrier has been shown recently to function at two different levels: the basal lamina, which can be penetrated by some luteoviruses with certain non-efficient vector species, and the basal plasmalemma, whose permeability ultimately dictates vector specificity (Pfeiffer et al., 1997). In addition to these two specificity barriers, efficient retention and transmission of luteoviruses require the virions to associate with *Buchnera* GroEL, produced by endosymbiotic bacteria (*Buchnera* spp.) of aphids and released into their haemolymph (van den Heuvel et al., 1994; 1997; van den Heuvel, 1999).

Transmission electron microscopy (TEM) is a powerful approach to analyse virus–vector relationships (Ammar, 1991). Ultrastructural studies aimed at the localization of luteovirus particles in organs and cells of vector and non-vector aphids (Gildow, 1999) revealed the site of passage (acquisition) of particles across the digestive tract. Virions of BYDV-PAV and -MAV (*Luteovirus*) and CYDV-RPV (*Polerovirus*) are acquired across the hindgut of their vectors, which are several species of cereal aphids (Gildow, 1999). However, *Potato leafroll virus* (PLRV; *Polerovirus*) is known to be acquired in the midgut of *Myzus persicae* (Garret et al., 1993; Gildow et al., 2000b), whereas *Soybean dwarf virus* (SbDV; unassigned member of the *Luteoviridae*) is acquired through the hindgut of the same species (Gildow et al., 2000a). Furthermore, *Poa enation mosaic virus*-1 (PEMV-1; *Enamovirus*) is acquired through the midgut of *Acrithosiphon pisum* (Harris & Bath, 1972). The site of passage of BWYV in *M. persicae* has not yet been determined.

Extensive TEM observations led Gildow (1987, 1999) to propose a model of receptor-mediated endocytosis and exocytosis to explain the transport of luteovirus particles through the different barriers in the aphid. These processes rely on the presence of putative receptors in the different cell membranes. The narrow specificity of luteovirus transmission is thought to be regulated by interactions of viral capsid components with specific receptors, whose nature and localization remain to be determined (van den Heuvel et al., 1999). The capsid of *Luteoviridae* members is composed of two proteins, the major coat protein (CP), encoded by ORF 3, and the minor readthrough (RT) protein, encoded by ORF 3 and the downstream ORF 5, which is expressed by a stop codon readthrough mechanism. The RT component is known to play a role in virus accumulation in planta (Brault et al., 1995; Chay et al., 1996; Bruyère et al., 1997). Moreover, the RT protein is important for virus–vector interactions, as demonstrated by experiments with BWYV or BYDV-PAV mutants carrying no RT protein or a mutated version thereof (Brault et al., 1995; Chay et al., 1996; Bruyère et al., 1997; Brault et al., 2000). Thus, a BWYV mutant carrying a deletion of ORF 5 (BWYV-6.4) was found to be non-transmissible when acquired either by feeding or by microinjection of purified virions into the haemocoel (Brault et al., 1995). Moreover, this mutant was unable to bind to *Buchnera* GroEL in vitro and was rapidly degraded in the haemolymph when microinjected into aphids (van den Heuvel et al., 1997). These findings suggest that the RT protein is required for stability of virions in the haemolymph and/or their transport to the ASG (Brault et al., 1995). In the case of BYDV-PAV, even though the RT protein was required for efficient transmission, it was not necessary for virus uptake in the haemolymph. The RT protein, however, was required for transport of virions through the ASG cells (Chay et al., 1996). More recently, however, the role of the RT protein in transmission has been called into question by experiments with virus-like particles (VLP) obtained through baculovirus expressing the CP gene of PLRV fused to a histidine tag (Gildow et al., 2000b). Such VLPs, containing no RT protein, were shown to circulate in the vector *M. persicae* in a way similar to wild-type virions; moreover, these VLPs could readily be observed in the salivary canal. These observations suggested that RT protein may not be required for passage of virions through both gut and salivary cells, or for their diffusion in the haemocoel. Aiming to better understand the role of RT protein in the transmission process, TEM observations were undertaken to localize particles of the mutant BWYYV-6.4 in both the gut cells and the ASG of *M. persicae*. Nucleic acid sequence-based amplification (NASBA; Kieviets et al., 1991) was used to assess whether this mutant was present in the haemolymph.

## Methods

### Virus acquisition by aphids.

Virus-free aphid colonies (*Myzus persicae* (Salzer), clone Colmar) were reared on caged pepper seedlings (*Capsicum annuum*) in a growth chamber at 20 °C with a 16 h photoperiod. All transmission experiments were done at room temperature. Third or fourth instar nymphs or adults were given a 24 or 72 h acquisition access period (AAP) on a purified virus suspension through a stretched Parafilm membrane. Virus suspensions were prepared in 20% sucrose in the artificial diet MP148 (Harrewijn, 1983). Unless otherwise noted, aphids were immediately fixed and embedded after the AAP. In some experiments, aphids were transferred after the AAP onto healthy *Montia perfoliata* seedlings to assess their capacity to transmit the virus. Five aphids were placed on each test plant for a 4 day inoculation access period (IAP) before being fixed and embedded. The test plants were assayed for virus infection 4 weeks later by an enzyme-linked immunosorbent assay (ELISA).

Microinjection of purified virus at approximately 100 µg/ml for wild-type BWYV (BWYYV-WT) and BWYYV-6.4 was performed as previously described (Bruyère et al., 1997). After a 72 h IAP on *M. perfoliata*, microinjected aphids were prepared for ultrastructural TEM examination of the ASG. The plants were tested for virus infection 4 weeks later by ELISA.

### Sample preparation.

For ultrastructural TEM examination, aphids were fixed and embedded as previously described (Gildow & Gray, 1993) with some modifications. Aphids were bisected in the middle of the thorax into an anterior part containing the foregut and the salivary glands, and a posterior part containing the midgut joined to the hindgut.
Table 1. Specific primers for NASBA amplification and molecular beacons for detection of BWYV-WT and BWYV-6.4

<table>
<thead>
<tr>
<th>Primer/molecular beacon</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Antisense CP primer – ’P1-CP’</td>
<td>5’TATCAGACTGGCCGGCATTCT3’</td>
</tr>
<tr>
<td>Sense CP primer – ’P2-CP’</td>
<td>5’TATCAGACTGGCCGGCATTCT3’</td>
</tr>
<tr>
<td>Antisense RTD primer – ’P1-RTD’</td>
<td>5’TATCAGACTGGCCGGCATTCT3’</td>
</tr>
<tr>
<td>Sense RTD primer – ’P2-RTD’</td>
<td>5’TATCAGACTGGCCGGCATTCT3’</td>
</tr>
<tr>
<td>CP molecular beacon – ’MB-CP’</td>
<td>5’FAMGCA.CCT.ATC.TCG.CGG.GAA.GTT.CCA.AGG.TGC.DABCYL 3’</td>
</tr>
<tr>
<td>RTD molecular beacon – ’MB-RTD’</td>
<td>5’FAMGCA.CCT.ATC.TCG.CGG.GAA.GTT.CCA.AGG.TGC.DABCYL 3’</td>
</tr>
</tbody>
</table>

This operation was carried out in the primary fixative (5% glutaraldehyde, 2% formaldehyde, 0.01% CaCl₂, 0.05% Na₂HPO₃ in 0.02 M sodium cacodylate, pH 7.4). After an overnight fixation at 4 °C, the tissues were post-fixed in 1% osmium tetroxide, transferred to 2% aqueous uranyl acetate, and finally dehydrated in an acetone series before being embedded in Epon–Araldite plastic. Ultrathin sections (60–80 nm) were stained by two successive incubations in 2% uranyl acetate–isobutanol (9:1) for 45 min and in 0.04% lead citrate for 15 min.

In order to facilitate visualization of virions at the basal pole of cells, some aphids were microinjected with a non-diluted BWYV polyclonal antiserum (Bio-Rad/Sanofi) or BWYV anti-P19 (Reutenauer et al., 1993) polyclonal antiserum, after a 72 h AAP on purified virus, as previously described (Gildow, 1982, 1993). These aphids were fixed and embedded 3 h after microinjection. All observations were made with a Philips EM 208 transmission electron microscope operating at 80 kV.

■ Virus purification and detection. BWYV-WT and BWYV-6.4 were purified from agro-infected Nicotiana clevelandii (Braught et al., 1995) according to the method of van den Heuvel et al. (1991). Purified virions were stored at −80 °C in citrate buffer (0.1 M sodium citrate, pH 6.0) containing 25% sucrose. Virus infection was assayed by double antibody sandwich ELISA (Clark & Adams, 1977) using an anti-BWYV polyclonal antiserum (Bio-Rad/Sanofi).

■ Virus stability. To assess stability of the particles in the aphid diet, RNA was extracted from purified virus in the presence of Tris–saturated phenol and 100 mM Tris–HCl pH 8.0, 50 mM NaCl, 10 mM EDTA, 1% SDS and 2 mg/ml proteinase K (Euromedex). After incubation at 60 °C for 10 min, samples were extracted with phenol–chloroform and ethanol-precipitated. Viral RNA was detected by Northern blot analysis (Veidt et al., 1992).

Viral RNA was also extracted from whole aphids or haemolymph samples as described by Chay et al. (1996). Haemolymph was collected by removing a mesothoracic leg with fine forceps and applying gentle pressure on the abdomen to force haemolymph droplets from the wound. The samples were collected using a fine glass capillary.

■ Detection of viral RNA by AmpliDet RNA. To detect BWYV RNA in haemolymph samples of M. persicae, AmpliDet RNA (Leone et al., 1998) was used. This method combines the NASBA technique (Kievits et al., 1991) with molecular beacon probes (Tyagi & Kramer, 1996; Eun & Wong, 2000).

Two different primer sets (Table 1) were designed to amplify viral RNA, based on the nucleotide sequence of the CP (P1-CP, P2-CP) and the readthrough domain (RTD expressed from ORF 5) (P1-RTD, P2-RTD). The P1 primers contain both a target complementary sequence and a 5′-T7 polymerase recognition site sequence. To detect the amplicons, two molecular beacons, MB-CP and MB-RTD, complementary to the target sequences were used (Table 1). Each beacon carried a target-specific sequence and 5′ and 3′ arm sequences of 6 nucleotides, tagged either with a fluorophore (6-carboxyfluorescein; FAM) or a quencher (DABCYL). These arm sequences are complementary to each other and form a double-stranded structure if no target is present. In this conformational state fluorescence is quenched. Upon hybridization of the molecular beacon to its target, the double-stranded structure is broken and the fluorophore is no longer quenched. The sensitivity of the molecular beacons was estimated as 50 and 5 fg of purified virus, for MB-CP and MB-RTD, respectively. The fluorescence of FAM was measured at 538 nm using a Fluorocam FL instrument.

Results

Identification of aphid tissues associated with BWYV-WT acquisition

The digestive tract of M. persicae can be divided into three parts: the foregut, the midgut and the hindgut. Our observations were focused on the midgut and the hindgut because these are known to be associated with the acquisition of luteovirus particles in other aphid–luteovirus systems (Garret et al., 1993; Gildow, 1993). Different segments of the midgut are easily recognized based on the structural morphology of the dilated anterior midgut, referred to as the stomach, and the narrow tubular posterior midgut, referred to as the intestine (Forbes, 1964). The hindgut is easily recognized by the occurrence of an array of 25 nm diameter tubules lining the lumen (Gildow, 1993). The chitin-lined foregut is not known to be involved in luteovirus acquisition and was not observed.

Aphid acquisition of BWYV-WT occurred following membrane feeding on a purified virus suspension. Efficiency of virus acquisition was tested by transferring some of the membrane-fed aphids onto test plants for transmission tests. Subsequent ELISA measurements revealed that all 10 test plants fed on by aphids that had been previously acquisition-fed with BWYV-WT were infected, indicating that a high number of aphids had acquired and transmitted the virus. The transmission test also demonstrated that the purified BWYV-WT used in our experiments retained transmissibility and infectivity following purification and storage at −80 °C. None of the 10 plants fed on by buffer-fed aphids (aphid control) became infected, confirming that the aphid culture was non-viruliferous.
Table 2. Electron microscopy localization of BWYV-WT and BWYV-6.4 in the gut of M. persicae

The localization of virions in the gut lumen (Lu), cell cytoplasm (Cyto) or basal lamina (BL) of the posterior midgut and hindgut in *M. persicae* was determined following an acquisition feeding on membranes containing virus suspended in 20% sucrose.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Virus</th>
<th>Midgut</th>
<th>Hindgut</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lu</td>
<td>Cyto</td>
</tr>
<tr>
<td>Membrane acquisition*</td>
<td>WT</td>
<td>28/28</td>
<td>24/28</td>
</tr>
<tr>
<td>Membrane acquisition + anti-BWYV injection†</td>
<td>WT</td>
<td>11/11</td>
<td>6/11</td>
</tr>
<tr>
<td>Membrane acquisition + anti-P19 injection‡</td>
<td>WT</td>
<td>7/8</td>
<td>5/8</td>
</tr>
<tr>
<td>Membrane acquisition</td>
<td>6.4</td>
<td>10/26</td>
<td>0/26</td>
</tr>
<tr>
<td>Membrane acquisition + anti-BWYV injection</td>
<td>6.4</td>
<td>18/42</td>
<td>0/42</td>
</tr>
<tr>
<td>Aphid control§</td>
<td>No virus</td>
<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

* Aphids were given a 72 h acquisition feeding on approximately 100 μg virus/ml in MP148 artificial diet (Harrewijn, 1983) through stretched Parafilm membranes.
† To enhance the probability of virus visualization, aphids were given a 72 h acquisition feeding on membranes, followed by microinjection of polyclonal anti-BWYV antibody into the haemocoel.
‡ Aphids treated as in (†), but injected with antiserum to the P19 nonstructural protein of BWYV.
§ Feeding in the control treatment was on MP148 diet without virus.
¶ No. of aphids in which virus was observed/total no. of aphids observed.
§ Virus–antibody aggregates were observed between the basal plasmalemma and basal lamina in one aphid. Only a few single particles were observed in each of the other five aphids.

In the anterior midgut (stomach), BWYV-WT particles were frequently observed in the lumen mixed with other gut contents, but particles were never observed attached to the apical membranes of stomach cells. Particles were never observed to occur within the stomach cell cytoplasm in structures similar to those described below for the posterior midgut. Therefore, there is no evidence to support the idea that the stomach is involved in BWYV acquisition by *M. persicae*.

BWYV-WT particles were consistently observed in the lumen of the posterior midgut and the hindgut (Table 2) following membrane feeding. This observation demonstrates that aphids were ingesting the virus in detectable amounts. However, BWYV-WT particles were observed only within cells of the midgut and not in the hindgut cells, indicating that BWYV-WT was acquired specifically through the midgut. In the posterior midgut (intestine), virions freely suspended in the lumen were only occasionally observed, but particles were frequently observed immediately adjacent to or in contact with the midgut cell membranes lining the lumen. Virus particles appeared to accumulate in close proximity to intestinal cell membranes. Individual particles associated with the midgut apical plasmalemma accumulated at the tips of microvilli of cells (Fig. 1a), or within apical plasmalemma invaginations forming narrow channels into midgut cells (Fig. 1b).

Within the intestinal cells, numerous virus particles were frequently observed concentrated within a variety of membranous structures including coated vesicles (Fig. 2a), and tubular vesicles (Fig. 2c). Particles were also observed in large multilamellar vesicles and in multivesicular bodies (Fig. 2b). Virions in some of the larger vesicles were apparently attached to the membrane around the perimeter of the vesicle in a manner similar to that previously described for receptosomes or endosomes (Gildow, 1993; Gildow *et al.*, 2000a). Virions escaping in membranes from larger vesicles appeared to be captured in the process of forming tubular vesicles (Fig. 2a). Although virus particles were readily observed inside midgut cells (86% of observations), virions were only rarely observed having been released from these cells (14% of observations) and penetrating through the extracellular basal lamina surrounding the midgut (Table 2). Tubular vesicles containing virions fused with the basal plasmalemma and apparently releasing particles into the haemocoel were never observed. Individual virions were, however, observed between the basal plasmalemma and the basal lamina or embedded in the basal lamina (Fig. 3a), suggesting that exocytosis had occurred. Attempts to detect virions in the haemocoel by TEM remained unsuccessful, suggesting that once released into the haemocoel the particles are quickly dispersed. Furthermore, free particles may have been washed away during the fixation and dehydration protocol prior to embedding.

To facilitate observation of particles, aphids, which had been acquisition-fed for 72 h on purified BWYV-WT were injected with polyclonal antiserum specific for BWYV structural proteins (coat proteins), and fixed for TEM observations 3 h later. Antibody injections have been shown to cause accumulation of luteoviruses in viruliferous aphids at specific sites and increase the probability of virus detection by TEM (Gildow, 1982, 1993). When anti-BWYV antiserum was
TEM localization of BWYV virions in aphids

(a) Virions close to microvilli (mv). (b) Virions in invaginations of the apical plasmalemma (apl). lu, lumen; r, ribosomes. Unlabelled arrows indicate virions. Bars represent 200 µm.

Fig. 1. BWYV-WT virions in midgut lumen. (a) Virions close to microvilli (mv). (b) Virions in invaginations of the apical plasmalemma (apl). lu, lumen; r, ribosomes. Unlabelled arrows indicate virions. Bars represent 200 µm.

Acquisition of the non-transmissible BWYV-6.4 RT-minus mutant

The fate of the non-transmissible mutant BWYV-6.4, which lacks the RT protein, was first analysed in membrane-fed M. persicae. To test for transmissibility of BWYV-6.4, 10 Montia perfoliata seedlings were given a 3 day inoculation feeding with aphids previously membrane-fed for 72 h on purified BWYV-6.4 virions. None of these plants became infected after 4 weeks, confirming the reported inability of M. persicae to transmit this mutant (Brault et al., 1995).

When aphids were acquisition-fed for 72 h on BWYV-6.4, virions were present as free particles suspended in the lumen of the midgut and the hindgut in 10 of 26 aphids observed (38% vs 100% for BWYV-WT) and 5 of 15 (33% vs 82% for BWYV-WT), respectively (Table 2). This observation confirms that aphids were ingesting virus during feeding and that

microinjected into the haemocoel of viruliferous aphids, virions were observed associated within posterior midgut cells in membrane vesicles such as those described above (Table 2). In addition, virions were observed as virus–antibody aggregates accumulating between the basal lamina and the basal plasmalemma of intestine cells (Fig. 3b). Apparently, the antibody molecules suspended in the haemolymph attach to and bind together virus particles after their release from midgut cells into the haemocoel. Similar accumulations of particles were never observed associated with stomach or hindgut cells of antibody-injected aphids, further illustrating that BWYV was not acquired through these tissues (Table 2).

As a control, membrane-fed aphids from the same experiment were injected with an antiserum directed against a non-structural protein of BWYV (P19, putative movement protein). In these aphids, isolated particles were observed in the basal lamina (three of eight aphids examined; Table 2) but virus aggregates were never observed, providing further evidence that the virus-like particles observed after injection of anti-BWYV antiserum were indeed BWYV particles aggregated after exocytosis from midgut cells.

Virions were occasionally observed suspended in the hindgut lumen, but virus was not observed attached to the hindgut apical cell membrane. Particles were never observed within the cytoplasm of hindgut cells in membrane structures, or embedded in the basal lamina surrounding the hindgut as described above (Table 2). These observations provided no evidence that the hindgut is involved in BWYV acquisition. Virions observed in the hindgut lumen are presumed to be passing out of the aphid through the alimentary canal.
Fig. 2. BWYV-WT virions in the cytoplasm of a midgut cell. (a) Virions in coated vesicles (cv) and in multilamellar vesicles (mlv). (b) Virions in mlv and multivesicular bodies (mvb). (c) Tubular vesicles containing virions (tv). r, ribosomes. Bars represent 200 µm.
detectable amounts of the virus remained intact during the 
72 h membrane feeding. The virus particles were dispersed in 
low concentrations in the lumen (Fig. 4a), but crystal-like 
arrays of particles were sometimes also found in the midgut 
(Fig. 4b) and the hindgut lumen (Fig. 4c). No virus particles 
were visualized in the cytoplasm of intestine or hindgut cells in 
any of the aphids examined (Table 2). This indicated that 
BWYV-6.4 was not being acquired into cells in amounts 
detectable by TEM.

To test the hypothesis that BWYV-6.4 might be acquired, 
but at undetectable levels, anti-BWYV antiserum was injected 
into the haemolymph of M. persicae fed for 72 h on purified 
BWYV-6.4, as previously described. This method was 
expected to aggregate any BWYV-6.4 particles being exo-
cytosed from midgut cells and enhance their detection. In 
aphids microinjected with anti-BWYV antiserum, BWYV-6.4 
particles were visualized suspended in the midgut lumen in 18 
of 42 (40%) aphids examined (Table 2). However, no virion 
was detected in the midgut cell cytoplasm. Virions were 
observed aggregated adjacent to the midgut basal lamina in 1 
of 42 aphids (Fig. 4d, e); however, in 5 additional aphids only 
an occasional isolated virus-like particle was observed em-
bedded in the midgut basal lamina. No virus particles were 
seen in the same location at stomach or hindgut cells, and no 
virus was ever observed aggregated in the haemolymph. Thus, 
visual evidence for midgut acquisition of BWYV-6.4 was 
somewhat equivocal and suggested that only low amounts of 
particles were moving into the haemolymph in a small percentage 
of the aphids tested.

Association of BWYV-WT and BWYV-6.4 with the aphid 
accessory salivary glands

Localization of BWYV-WT in the ASG was analysed 
following purified virus acquisition either by membrane 
feeding or by microinjection into the haemolymph (Table 3). 
The ability of M. persicae to transmit BWYV-WT following 
microinjection of purified virus was tested by allowing injected 
aphids a 3 day inoculation feeding on Montia perfoliata 
seedlings, followed by ELISA testing for infection. All 10 test 
plants fed on by the BWYV-WT-injected aphids were infected, 
demonstrating that the aphids were viruliferous and that the 
purified virus was infectious. None of 5 test plants became 
infected when fed on by non-injected aphids. The ASG of M.
Fig. 4. For legend see facing page.
Table 3. Localization of BWYV-WT and BWYV 6.4 in the ASG of M. persicae

Localization was determined following virus acquisition by feeding on stretched Parafilm membranes or microinjection of purified virus into the aphid haemocoel.

<table>
<thead>
<tr>
<th>Treatment/virus†</th>
<th>Site of virus accumulation or visualization*</th>
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<tbody>
<tr>
<td></td>
<td>BL</td>
</tr>
<tr>
<td>Membrane acquisition</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>4/4</td>
</tr>
<tr>
<td>6.4</td>
<td>0/4</td>
</tr>
<tr>
<td>Microinjection</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>5/5</td>
</tr>
<tr>
<td>6.4</td>
<td>0/9</td>
</tr>
</tbody>
</table>

* Cellular sites in the ASG examined for virus localization or accumulation: basal lamina (BL), basal plasmalemma membrane invaginations (INV), cytoplasmic tubular vesicles (TV), coated vesicles (CV), salivary canal (SC).
† Acquisition of virus was performed either by a 48 h membrane feeding on a suspension of virus at 100 µg/ml prepared in 20% sucrose or by microinjection of purified virus at approximately 100 µg/ml. Aphids were fixed and embedded after a 72 h inoculation access period on the test plant.
‡ No. of aphids containing virus at indicated site/total no. observed.

M. persicae consists of four secretory cells connected at their apical poles to the salivary ducts through a branched network of microvilli-lined secretory canals (Gildow, 1987). The entire ASG is surrounded by a complex matrix consisting of collagen and laminin glycoproteins assembled into a network containing several other components, and is capable of preventing luteovirus transmission (Peiffer et al., 1997). When purified BWYV-WT was membrane-fed or injected into aphids, virions were consistently observed embedded within the basal lamina (Fig. 5a) surrounding the ASG in all 9 aphids examined (Table 3). Therefore, the ASG basal lamina does not present a barrier to BWYV-WT transmission in M. persicae. BWYV-WT particles were seen in invaginations of the basal plasmalemma and occasionally in coated or tubular vesicles within the cytoplasm (Table 3), indicative of virus uptake into the ASG. Particles were present in the salivary canal in several aphids.

When purified BWYV-6.4 was membrane-fed or microinjected into the haemocoel, particles were never found in the basal lamina surrounding the ASG cells, in the cytoplasm of these cells or in the salivary canals in any of 13 aphids observed (Table 3). When aphids from the same treatment were transferred onto M. perforata for an inoculation feeding, none of 15 plants tested became infected.

Structural stability of BWYV-6.4 virions during acquisition

Rapid degradation of BWYV-6.4 particles in the aphid diet could account for the low number of particles visualized in the aphid gut and ASG. That this was not the case was shown by analysis of RNA extracted from the diet containing either BWYV-WT or BWYV-6.4, 0 and 72 h after the beginning of acquisition. Both BWYV-WT and BWYV-6.4 genomic RNA were detected in the samples (Fig. 6). The bands were less intense when RNA were extracted 72 h after acquisition, indicating that some particle breakdown and RNA degradation occurs in the diet. This degradation, however, occurred to similar extents for BWYV-WT and BWYV-6.4, indicating that there is no particular instability of BWYV-6.4 virions. Stability of BWYV-6.4 particles was also confirmed by TEM visualization of equivalent amounts of each type of particle (BWYV-WT or BWYV-6.4) in samples of the diet following the 72 h acquisition feeding (data not shown). Furthermore, observations of BWYV-WT and BWYV-6.4 particles from artificial diet immuno-captured onto anti-BWYV-coated grids revealed that the virions remained structurally intact for up to 7 days at room temperature. Thus, loss of structural integrity does not seem to explain the reduced ability to visualize BWYV-6.4 in membrane-fed aphids or the inability of aphids to transmit this mutant.

AmpliDet RNA detection of BWYV-6.4 in the aphid haemocoel

TEM observations suggest that, at least in some aphids, small amounts of BWYV-6.4 may be passing through midgut cells into the haemocoel after oral acquisition. To provide further evidence of this hypothesis, a more sensitive virus detection method was employed. Haemolymph was collected from M. persicae nymphs following a 72 h AAP on purified BWYV-6.4. Aphids feeding on virus-free or BWYV-WT-containing diets served as controls. To differentiate between BWYV-6.4 and BWYV-WT by AmpliDet RNA, two sets of primers and two molecular beacons were used. One set specifically amplified a region in the CP, and the other a region in the readthrough domain (RTD, ORF 5). In haemolymph samples containing BWYV-6.4, only the CP-specific detection set should give a positive result since this mutant lacks the entire RTD region, whereas both sets should react with...
BWYV-WT. AmpliDet RNA revealed that after a 72 h AAP on virus suspension, CP RNA was detected in the haemolymph of all aphids tested (6/6 for BWYV-WT and 6/6 for BWYV-6.4), whereas none of the aphids fed a virus-free diet responded positively. RTD-specific sequences were amplified in all 6 BWYV-WT-fed aphids, and in 0 of 6 BWYV-6.4-fed aphids, indicating that the CP RNA amplification was not due to cross-contamination with BWYV-WT during RNA extraction or amplification. These results confirm those obtained by TEM observations and indicate that at least small amounts of BWYV-6.4 can be acquired in the haemolymph.

**Discussion**

In this paper, we have attempted to elucidate the role of the RT protein in the transmission process in both the aphid midgut and ASG cells by comparing the tissue localization in *M. persicae* of an RT-deficient BWYV mutant (BWYV-6.4) with that of BWYV-WT. Preliminary studies of BWYV-WT trans-
port through *M. persicae* were carried out to ascertain the route of BWYV through aphid vectors during the transmission process. Our observations indicated that BWYV is specifically acquired through the midgut of *M. persicae*, in a manner similar to that described for PLRV (Garret et al., 1993). Being of endodermal origin, the midgut is the only part of the alimentary canal of most insects that is not lined by impermeable chitin, a feature that would facilitate virus attachment to midgut cell membranes and penetration. Probably for this reason, the midgut is the major acquisition site of many arthropod-transmitted viruses, including geminiviruses by whiteflies (Hunter et al., 1998), sobemoviruses by beetles (Wang et al., 1994) and tospoviruses by thrips (Ullman et al., 1992). Furthermore, the midgut is the primary site for infection of arboviruses in their Dipteran vectors (Leake, 1992; Fu et al., 1999; Mellor, 2000). Unlike many insects, however, aphids lack a chitin lining of the hindgut and some luteoviruses, including BYDV, CYDV and SbDV, have evolved to utilize the hindgut as the specific site for virus ingress into the aphid vector (Gildow, 1999). As expected, transmission of BWYV-WT was associated with the ASG in a manner previously described (Gildow, 1982). Virions of BWYV-WT were easily observed embedded in the ASG basal lamina in all viruliferous aphids examined. Observations of virions in membrane invaginations, coated vesicles, tubular transport vesicles and in the process of release into the salivary canal provided ultrastructural evidence of the endocytotic and exocytotic cellular processes involved in BWYV transmission (Gildow, 1999).

The BWYV-6.4 mutant has been shown to be non-transmissible by membrane-fed or microinjected aphids (Brault et al., 1995). Our results show that in membrane-fed aphids BWYV-6.4 virions were only occasionally observed in the gut lumen. Instability of viral particles in the aphids’ diet was not responsible for the infrequent presence of BWYV-6.4, as shown by TEM observations of intact virus in the diet and the detection of viral genomic RNA extracted from the diet following membrane feeding. The low amount of BWYV-6.4 particles visualized in the gut lumen could result from a lack of adsorption on the apical plasmalemma leading to a rapid elimination of the particles in the honeydew. However, the crystals of BWYV-6.4 observed in the gut lumen could also reduce the number of free particles seen in the lumen. Apart from the intestine lumen, BWYV-6.4 virions were also observed between the basal plasmalemma and basal lamina, in the basal lamina or between two basal laminae of adjacent intestine cells, but only in aphids microinjected with anti-BWYV antibodies. BWYV-6.4 particles, however, were never detected in the cytoplasm of intestine cells. We assume that intracellular BWYV-6.4 virions were present but simply too rare to be detected by our observations. Even if large amounts of BWYV-6.4 particles are aggregated in the crystals found in the lumen, we can assume that this arrangement of particles cannot be endocytosed in intestinal cells. The release of BWYV-6.4 virions in the haemocoel was also demonstrated by our detection of viral RNA in the haemolymph using the AmpliDet RNA technique. This mutant, which is unable to bind *Buchnera* GroEL (van den Heuvel et al., 1997), is probably degraded in the haemolymph before attaining the ASG. Mutant virions were not detected near or within ASG cells, even when purified BWYV-6.4 was microinjected directly into the haemocoel. These results confirm those reported by Chay et al. (1996), who showed that a BVDV RT protein-deficient mutant was detected by reverse transcription–PCR in the haemolymph of the vector, but that particles were never observed in the ASG basal lamina. On the other hand, contrasting results have been obtained with PLRV VLPs produced in a baculovirus system (Gildow et al., 2000a). In this work, RT protein-deficient VLPs were found in the vector at the same locations as observed for the wild-type virions. It cannot be ruled out, however, that the histidine-tag fused at the N terminus of the CP, potentially exposed on the surface of the VLPs, could stabilize the capsid of these VLPs by interacting with *Buchnera* GroEL and, thus, functionally substitute for the RT protein.

Based on our results, the RT protein of BWYV does not seem to be strictly required for the transport of virions through intestinal cells, although the presence of RT protein greatly enhances the efficiency of this process. Based upon these findings, at least two models can be proposed to describe virus uptake by intestinal cells. In the first model, putative receptors on the midgut apical plasmalemma could recognize a protruding or easily accessible domain of the viral capsid consisting of both CP and RT motifs. Deletion of the RT protein, as in BWYV-6.4, would not abolish virus recognition by the receptor, but this process would occur with lower efficiency. The second model postulates that luteovirus endocytosis in aphids may involve multiple receptors. This situation could occur at the inner surface of the gut, where virions must first be captured from the food bolus before being endocytosed. Involvement of multiple receptor binding events has already been described for entry into human cells by adenoviruses (Nemerow, 2000), which have been shown to associate first with an attachment receptor by means of a fibre protruding from the viral capsid. Internalization of the virus is triggered by interaction of the second receptor (integrin) with the base of the fibre. Applied to the BWYV–aphid system, such a model would hypothesize that the first type of receptor, thought to be frequent along the gut epithelium, would recognize motifs of the RT protein. The second type of receptor, which would be less common and would recognize motifs of the CP, would intervene in the slower process of endocytosis. The uneven distribution of receptors and their different specificities could account for the differential efficiency of transcellular transport between wild-type virions and the RT protein-deficient mutant.

In summary, we have detected specific association of BWYV with the aphid midgut tissue during the virus...
acquisition process and presented evidence indicating that the RT protein of BWYV and presumably other members of the Luteoviridae is not absolutely required for recognition and acquisition into the aphid vector haemocoel. However, efficiency of acquisition was greatly reduced for the RT-minus mutant BWYV-6.4, suggesting an important role for the readthrough domain in the virus recognition and transcellular transport process. BWYV-6.4 was never detected at the ASG and was not transmitted to plants, indicating that it was either acquired at concentrations too low for such phenomena to be detected by the methods employed here or the mutant virus was inactivated in the haemocoel due to its inability to associate with protective GroEL homologues.

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References


TEM localization of BWYV virions in aphids


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