The passage of Potato leafroll virus through Myzus persicae gut membrane regulates transmission efficiency

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Potato leafroll virus (PLRV) is transmitted by aphids in a persistent manner. Although virus circulation within the aphid leading to transmission has been well characterized, the mechanisms involved in virus recognition at aphid membranes are still poorly understood. One isolate in our collection (PLRV-14.2) has been shown to be non- or only poorly transmitted by some clones of aphids belonging to the Myzus persicae complex. To determine where the transmission process was blocked within the aphid, three virus transmission procedures were used. PLRV-14.2 could not be transmitted, or was only very poorly transmitted, after acquisition from infected plants or from purified preparations. In contrast, it could be transmitted with more than 70% efficiency when microinjected. Therefore, it is concluded that the gut membrane was a barrier regulating passage of PLRV particles from the gut lumen into the haemocoel of M. persicae. Comparison of coat protein (CP) and readthrough protein (RTP) sequences between poorly and readily transmissible isolates showed that PLRV-14.2 differed from other PLRV isolates by amino acid changes in both of these proteins. It is hypothesized that at least some of the changes found in CP and/or RTP reduced virus recognition by aphid gut receptors, resulting in reduced acquisition and subsequent transmission of PLRV-14.2.

Introduction

Potato leafroll virus (PLRV) is the type member of the genus Polerovirus (family Luteoviridae) (Mayo & d’Arcy, 1999). It is obligately transmitted by aphids in a persistent manner. This means that aphid vectors ingest virus particles with plant sap when feeding in phloem tissues of an infected plant. Then, virions move from the gut lumen into the haemolymph. Once there, they may be protected from proteolytic breakdown by associating non-specifically with symbionin, a chaperon protein produced by Buchnera endosymbionts (van den Heuvel et al., 1994). At the level of the accessory salivary gland (ASG), virus particles must cross the ASG basal lamina and plasmalemma membrane before being released into the salivary canal, from where they can be inoculated (reviewed by Gildow, 1999). Along this route, three barriers have been shown to regulate virus transmission, with various degrees of specificity.

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vector and their interactions are involved in regulating the virus transmission (Terradot et al., 1999). Although few studies have been done with PLRV, virus particles have also been observed specifically attached to ASG membrane, suggesting that virus–vector interactions occur at this site for PLRV as well (Gildow, 1982).

Luteo- and polerovirus particles contain two structural proteins, the coat protein (CP) and the minor capsid read-through protein (RTP) (Bahner et al., 1990). Most results suggest that the CP alone allows transport through the gut membrane (van den Heuvel et al., 1993; Chay et al., 1996; Gildow, 1999), whereas the role of the RTP remains much less clear. RTP has been shown to be necessary for BYDV transport through ASG membrane (Chay et al., 1996) but more recent results suggested that it was also involved in the passage of Beet western yellows virus (BWYV; genus Polerovirus, family Luteoviridae) through the gut membrane (Brault et al., 2000). For BWYV, the ability of RTP to mediate transmission has been associated with the conserved N-terminal half of the protein (Bruyère et al., 1997). In contrast, PLRV isolates that had lost aphid transmissibility were shown to harbour amino acid changes in the non-conserved C-terminal domain of RTP (Jolly & Mayo, 1994). Moreover, PLRV-like particles devoid of RTP were able to complete their route in M. persicae, from the gut lumen to the accessory salivary gland canal (Gildow, 1999).

Several aphid species have been shown to transmit PLRV with various efficiencies, the more efficient one being M. persicae (Kennedy et al., 1962). Among other parameters, transmission depends largely on aphid species, clone, morph and instar (Björling & Ossiannilsson, 1958; Upreti & Nagaich, 1971; Hinz, 1966; Robert & Maury, 1970; Robert, 1971) and virus isolate (Tamada et al., 1984; Jolly & Mayo, 1994). However, Bourdin et al. (1998) showed that, among clones of the M. persicae complex which were efficient at transmitting the PLRV-HAT (Highly Aphid Transmissible) isolate PLRV-CU87, most transmitted the PLRV-PAT (Poorly Aphid Transmissible) isolate PLRV-14.2 with a low efficiency (0–25%), although two clones could transmit it at up to 80% efficiency. Interestingly, most of the poor vector clones belonged to the Myzus antirrhini taxon within the M. persicae complex, showing that aphid genotype variation can affect virus transmission (Terradot et al., 1999). Overall, these results provide evidence that both the properties of the virus and the vector and their interactions are involved in regulating the transmission process.

In this paper, we report that the efficiency with which PLRV particles cross M. persicae gut membrane determines the success of transmission. Comparison of CP and RTP sequences between PLRV-HAT and -PAT isolates revealed several amino acid changes that are associated with inefficient passage of virus particles across this membrane.

**Methods**

**Virus isolates and aphid clones.** A collection of PLRV isolates originating from different countries has been maintained for years on *Physalis floridana* (Rydby) by vegetative propagation (cuttings). Two isolates collected in the north of France in 1985 (PLRV-14.2) and in Cuba in 1987 (PLRV-CU87) were chosen for this study. PLRV-CU87 is highly aphid transmissible by all the clones of the M. persicae complex that we have tested, whereas PLRV-14.2 is poorly aphid transmissible by most clones (Bourdin et al., 1998).

Nonviruliferous aphids were reared under controlled conditions (16 h light/8 h dark; 20 °C) on Chinese cabbage (*Brassica campestris* Linné var. *pekinesis*) to keep them free of PLRV (Chuquillanqui & Jones, 1980). Clone Mp3, used in this study, has been recently characterized as belonging to the *Myzus antirrhini* taxon within the *M. persicae* complex (Terradot et al., 1999). Although it is efficient at transmitting several PLRV isolates, it is a very poor vector of PLRV-14.2 (less than 10% transmission) (Bourdin et al., 1998).

**Virus purification.** Virus isolates were purified following the method described by Tamada & Harrison (1980) and modified as followed. About 300 g frozen infected *P. floridana* leaves was homogenized with 3 vols 0.1 M sodium citrate, pH 6, containing 0.5% β-mercaptoethanol and 5% Cellulost (Novo Nordisk). The homogenate was incubated at room temperature for 2–3 h, filtered through muslin and the pH adjusted to 7. The virus preparation was emulsified with 1/3 vol. chloroform and 1/3 vol. butanol and centrifuged for 15 min at 5000 r.p.m. (rotor JA14, Beckman). Polyelectrolyte glycol (PEG) 6000 and NaCl were added to the aqueous phase to 8% (w/v) and 0.2 M, respectively. After overnight incubation at 4 °C, the suspension was centrifuged for 15 min at 10000 r.p.m. (rotor JA14, Beckman) and the pellets were resuspended in phosphate buffer (0.01 M, pH 7) containing 1% Triton X-100. Further purification was performed by ultra-centrifugation through a 30% sucrose cushion followed by sucrose density-gradient centrifugation. The peak-containing fractions of the gradients were pooled and the virus was pelleted by ultracentrifugation. Virus concentration was calculated by measuring the optical density of the virus preparation at 260 nm and using ε₉₀₀ = 8.6 (Takanami & Kubo, 1979). Yields were 300–400 µg/kg leaf.

**Virion transmission experiments.** Three virus acquisition procedures were designed. Virus-free young apterous adults were employed in all cases.

**Plant-to-plant transmission experiments.** Batches of aphids were allowed to feed on PLRV-14.2- or -CU87-infected cuttings of *P. floridana* for a 3 day acquisition access period (AAP) under controlled conditions (16 h light/8 h dark, 20 °C).

**Membrane feeding.** Aphids were fed for a 24 h AAP, through a stretched Parafilm membrane, on purified virus suspensions of PLRV-14.2 or -CU87 containing 100 µg/ml of virus.

**Microinjection of purified virus.** Purified PLRV-14.2 or -CU87 (10–20 nl containing 100 µg/ml of virus) was microinjected into the haemocoele of immobilized aphids using a pantograph micromanipulator (Micro Instruments Ltd) and an Inject-Matic apparatus regulated by an electronic programme (A. Gabay, Geneva, Switzerland).
In each procedure, following the AAP, three aphids were then transferred to each of 20 healthy *P. floridana* seedlings for a 3 day inoculation access period (IAP). Three replicates were performed and in the first test, purified virus from the same preparation was used in both the membrane feeding and microinjection procedures. At the end of the IAP, aphids were killed with an insecticide spray (Pirimicarb).

Virus infection was assessed through symptom expression 2–3 weeks after inoculation and confirmed using DAS-ELISA (Clark & Adams, 1977) 2–3 weeks later. Test plants were considered infected with PLRV when the DAS-ELISA absorbance values were greater than twice the average values of healthy *P. floridana*.

Back inoculations were performed from plants that had been inoculated by microinjected aphids. Two plants were inoculated following two different replicates were chosen. Twenty test plants were inoculated with three aphids following a 3 day AAP on each source plant. Transmission efficiency was assessed as described above.

For each procedure, mean transmission rates obtained with PLRV-14.2 and -CU87 were transformed using angular transformation and analysed by one- or two-way ANOVA (analysis of variance), using the GLM (General Linear Model) procedure of the SAS (Statistical Analysis Software) package before being compared using Duncan's multiple range test (SAS Institute Inc., 1995).

**Sequencing of CP and RTP genes.** The ORFs corresponding to the CP and the RTP were sequenced for both PLRV-14.2 and -CU87. All the primers were designed based on the sequence of the Canadian isolate, PLRVC (Keese et al., 1990). Total RNAs from infected plant tissues were extracted using the RNeasy plant Mini Kit (Qiagen), following the manufacturer's instructions. For cDNA synthesis, 10 µl of total RNAs was used. Reverse transcription was primed with an oligonucleotide complementary to nucleotides 5863–5882 (PLRV1), and a PCR product of about 2.5 kb was synthesized with oligonucleotides PLRV1 and PLRV2 (complementary to nucleotides 3382–3401). PCR products were purified (Concert rapid PCR purification system; Gibco-BRL) and approximately 100 ng of DNA was used as matrix for sequencing reactions (ABI Prism Big Dye dRhodamine terminator cycle sequencing ready reaction kit; Perkin-Elmer). For each isolate, purified PCR products obtained in at least three independent PCR reactions were sequenced as overlapping fragments, using 10 internal forward primers, with an ABI 310 automated DNA sequencer (Perkin-Elmer). Sequences were assembled and analysed using the program BioEdit (Hall, 1999), available at http://www.mbio.ncsu.edu/RNaseP/info/programs/BIOEDIT/bioedit.html.

### Results

#### Identification of a specificity barrier in *M. persicae*

PLRV-14.2 has been described previously as poorly transmissible by several clones of aphids belonging to the *M. persicae* complex (Bourdin et al., 1998). In this study, these results were confirmed in transmission experiments with Mp3 aphids. PLRV-CU87 was efficiently transmitted (98%) from infected *P. floridana* cuttings to healthy test plants whereas, under the same conditions, mean transmission of PLRV-14.2 was 3% (Table 1). Similarly, when Mp3 aphids acquired purified virus from artificial diets adjusted to contain the same virus concentration, the mean transmission rates of PLRV-CU87 and -14.2 were 55 and 6%, respectively (Table 1).

**Table 1. Transmission rates of PLRV-14.2 and -CU87 by Mp3 aphids after acquisition on infected plants, membrane feeding or microinjection of purified virus**

<table>
<thead>
<tr>
<th>Replicate</th>
<th>PLRV-CU87</th>
<th>PLRV-14.2</th>
<th>PLRV-CU87</th>
<th>PLRV-14.2</th>
<th>PLRV-CU87</th>
<th>PLRV-14.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>0</td>
<td>60</td>
<td>8</td>
<td>80</td>
<td>73</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>10</td>
<td>50</td>
<td>4</td>
<td>85</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>95</td>
<td>0</td>
<td>NT</td>
<td>NT</td>
<td>70</td>
<td>65</td>
</tr>
<tr>
<td>Mean†</td>
<td>98*</td>
<td>3*</td>
<td>55*</td>
<td>6*</td>
<td>78*</td>
<td>63*</td>
</tr>
</tbody>
</table>

* For each replicate 20 test plants were inoculated with three aphids.

† Within one procedure, mean transmission rates followed by the same letter did not differ significantly (*P* < 0.05).

**Table 2. Back inoculations using Mp3 aphids from plants inoculated by aphids microinjected with either PLRV-CU87 or PLRV-14.2**

<table>
<thead>
<tr>
<th>Replicate</th>
<th>% Transmission from plants infected with:*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLRV-CU87</td>
<td>PLRV-14.2</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
</tr>
</tbody>
</table>

* For each replicate 20 test plants were inoculated with three aphids.
When Mp3 aphids were microinjected with 1–2 ng of purified PLRV-CU87, at least 70% of test plants were infected in each replicate (Table 1). These results were similar to those obtained in plant-to-plant transmission and membrane-feeding experiments. In contrast, when PLRV-14.2 was microinjected, transmission rates ranged from 50 to 73%, compared to the zero or very low transmission rates observed after acquisition on infected plants or on artificial diets (Table 1). Under these conditions, transmission of PLRV-14.2 did not differ statistically from that of PLRV-CU87 (P > 0.05).

When back inoculations were performed from infected plants that had been inoculated by microinjected aphids, PLRV-14.2 was still poorly transmitted whereas PLRV-CU87 was transmitted up to 100% (Table 2). These observations confirmed that the virus material used in microinjection had not become contaminated with a HAT isolate.

**Amino acid sequence of PLRV-14.2 and -CU87 CP and RTP**

The nucleotide sequence corresponding to the CP and RTP ORFs of PLRV-14.2 and -CU87 were determined and the deduced amino acid sequences were aligned to locate differences that might correlate with differences in transmissibility. Published sequences are also included for the isolates PLRV-V and PLRV-C which are, respectively, poorly and well transmitted by most *M. persicae* clones. PLRV-V in particular is poorly transmitted by Mp3 [Jolly & Mayo (1994) and unpublished data; the transmissibility of PLRV-C by Mp3 has not been tested]. Fig. 1 shows the positions in the PLRV-14.2 sequence where amino acids differed from those of other isolates. Overall, PLRV-14.2 showed a high percentage similarity with the other isolates, but it differed more from PLRV-CU87 than from the two Scottish ones. PLRV-14.2 differed from the other three isolates at 13 sites within CP and RTP sequences. With two exceptions in the CP (positions 14–15 and 160), the changes were located in the RTP at positions 271–272, 385, 402, 439, 554–555, 564, 612, 661, 665, 679 and 695. However, the lysine residue found at position 564 has also been reported for a Dutch isolate (van der Wilk et al., 1989) and the valine and tyrosine residues at positions 661 and 695, respectively, have been reported for a Polish isolate (Palucha et al., 1994). While not conclusive, the presence of the PLRV-14.2 residues at these positions in other presumably HAT PLRV isolates suggests that the aforesaid positions are not critical for transmission.

Amino acid changes at positions 611–612 (from SL in
PLRV-C to SP in PLRV-V) and 707 (from I to M in PLRV-C and -V, respectively) have been described previously as potentially responsible for the poor transmissibility of PLRV-V (Jolly & Mayo, 1994). At these positions PLRV-14.2 had the amino acids LS and I, respectively. The LS motif has also been found for another isolate in our collection that is efficiently transmitted by Mp3 (data not shown). The significance for transmission of the alterations at positions 611–612 and 707 of PLRV-V remains to be determined.

Discussion

In this paper, we have shown that PLRV-14.2 can be successfully transmitted when microinjected into a very poor vector clone of the *M. persicae* complex and that this isolate differed from other PLRV isolates by changes in the amino acid sequence of the CP and RTP. These results demonstrate that the gut membrane is a barrier regulating the passage of virus particles from the gut lumen into the haemocoel and suggest that some of the amino acid changes found in the CP and/or RTP can reduce virus recognition at this membrane.

Plant-to-plant transmission experiments first showed that, although Mp3 aphids were very poor vectors of PLRV-14.2 and PLRV-V, they efficiently transmitted PLRV-CU87 and other PLRV isolates (Table 1; Bourdin et al., 1998 and unpublished data). This finding establishes that Mp3 aphids are able to feed properly on PLRV-infected *P. floridana* and that their intrinsic behavioural properties cannot account for the observed poor transmissibility. Nor is low transmissibility of PLRV-14.2 linked to lower virus availability from infected source plants since, when PLRV-14.2 was provided at the same concentration as PLRV-CU87 using the membrane-feeding protocol, it was still poorly transmitted (Table 1). Therefore, although we cannot rule out the possibility that the distribution of PLRV-14.2 particles in phloem tissues of infected *P. floridana* is uneven (van den Heuvel et al., 1995), such a distribution, if it exists, is not responsible for the differences in acquisition by aphids in our experiments.

After microinjection, Mp3 aphids transmitted PLRV-14.2 with 50 to 73% efficiency, showing that the ASG membrane was easily crossed by this isolate and arguing that virus particles passed through the gut membrane with a very low efficiency when naturally ingested (Table 1). This is in contrast with most previous results which indicated that the ASG basal lamina and/or basal plasmalemma are responsible for vector specificity of several viruses in the *Luteoviridae* family (reviewed by Gildow, 1999). For example, when Rochow (1969) microinjected purified BYDV isolates to non-vector aphid species, virus particles were not transmitted, suggesting that the gut membrane played no role in the observed specificity. Rochow et al. (1975) later confirmed that the ASG regulated BYDV transmission specificity. In only one case has the gut membrane previously been shown to be responsible for transmission specificity: CYDV-RPV particles could not reach the haemolymph of the non-vector *Metopolophium dirhodum* (Wilk) and were never observed attached to the gut apical plasmalemma of this aphid species (Gildow, 1993). This led the author to conclude that *M. dirhodum* lacked the receptors to recognize CYDV-RPV (Gildow, 1999). However, our results suggest that, for PLRV at least, such specificity is not controlled in an all-or-nothing fashion. Although Mp3 aphids were shown to be non-vectors of PLRV-14.2 in most cases, they did transmit PLRV-14.2 very poorly in some experiments. Moreover, they could transmit other isolates very efficiently. This suggests that these aphids possess at their gut membrane the receptor(s) needed for efficient transcytosis and that PLRV-14.2 particle transport through the midgut is more probably impeded by low affinity between virus particles and their receptor(s) in the aphids rather than by an absence of the appropriate receptor(s).

Sequence comparisons have revealed a number of alterations in the PLRV-14.2 CP and RTP with regard to the other isolates (Fig. 1). One or more of these changes presumably account for the poor transmissibility of PLRV-14.2. Two of the changes found in the RTP seem of particular interest. The first change (QN to RS) at amino acids 271–272 is very close to the strictly conserved ED sequence found at position 267–268. When the ED motif was replaced by alanine residues in a BWYV infectious cDNA clone, the resulting progeny was unable to cross *M. persicae* gut membrane (Brault et al., 2000). The second change was the substitution of amino acids KA at position 554–555 by amino acids ET and was located in the ‘*Myzus* homology domain’ i.e. a sequence that is highly conserved among poleroviruses transmitted by *M. persicae* (Mayo & Ziegler-Graff, 1996). More recent studies, however, have cast doubt on the importance of the ‘*Myzus* homology domain’ on transmission of BWYV (Bruyère et al., 1997).

Evidently, more information is needed to determine the effect of the substitutions found in PLRV-14.2 CP and RTP on transmission efficiency. However, the similar transmission rates of PLRV-14.2 and -CU87 after microinjection suggest that, although the observed amino acid changes can affect the passage of PLRV-14.2 through the gut membrane, they had little or no effect on transport through ASG basal lamina and basal plasmalemma. Moreover, it is of interest, that none of the changes previously reported to affect PLRV-V transmission (Jolly & Mayo, 1994) were found in PLRV-14.2. This suggests that different sequence modifications can have similar effects on transmission and supports the hypothesis of structural redundancy within the RTP (Brault et al., 2000).

The respective roles of virus structural proteins in virus recognition within the aphid are at present unsettled because of the conflicting results obtained by different authors (Brault et al., 1995; Chay et al., 1996; Bruyère et al., 1997; Gildow, 1999). The PLRV-14.2/Mp3 model will undoubtedly help to clarify these questions.

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References


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