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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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The first barrier is the gut membrane, which the virus appears to traverse by an endocytosis–exocytosis mechanism, presumably involving specific recognition between virus particles and aphid components. For PLRV, Garret *et al.* (1993) observed that in *Myzus persicae* (Sulz.) the site of this passage is the midgut. In contrast, this same aphid species acquires *Soybean dwarf virus* (SbDV; family *Luteoviridae*) through the hindgut (Gildow *et al.*, 1994). *Rhopalosiphum padi* (L.) and *Sitobion avenae* (Fab.) also acquire *Cereal yellow dwarf virus*-RPV (CYDV-RPV; genus *Polerovirus*, family *Luteoviridae*) and *Barley yellow dwarf virus*-PAV (BYDV-PAV; genus *Luteovirus*, family *Luteoviridae*) respectively, through their hindgut (Gildow, 1993). However, in spite of the high degree of tissue specificity within and between vector species, the gut membrane does not seem to be very selective. In most cases, luteo- and poleroviruses were shown to be able to cross the gut membrane of both efficient and poor vector species (reviewed by Gildow, 1999).

Two more selective barriers have been distinguished for BYDV and CYDV, the ASG basal lamina and basal plasmalemma. The mechanisms allowing virus particles to penetrate the ASG basal lamina are unknown. However, BYDV-PAV or

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CYDV-RPV particles were shown to attach specifically to ASG basal lamina and different types of interaction were described, depending on the aphid species (Peiffer *et al.*, 1997). At the ASG basal plasmalemma, a receptor-mediated endocytosis–exocytosis process similar to that at the midgut is apparently involved. Therefore, once the basal lamina has been crossed, virus transmission is probably still dependent on specific interactions that determine virus passage through the basal plasmalemma (Gildow & Rochow, 1980; Gildow & Gray, 1993). 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

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 infected with each isolate and originating from 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
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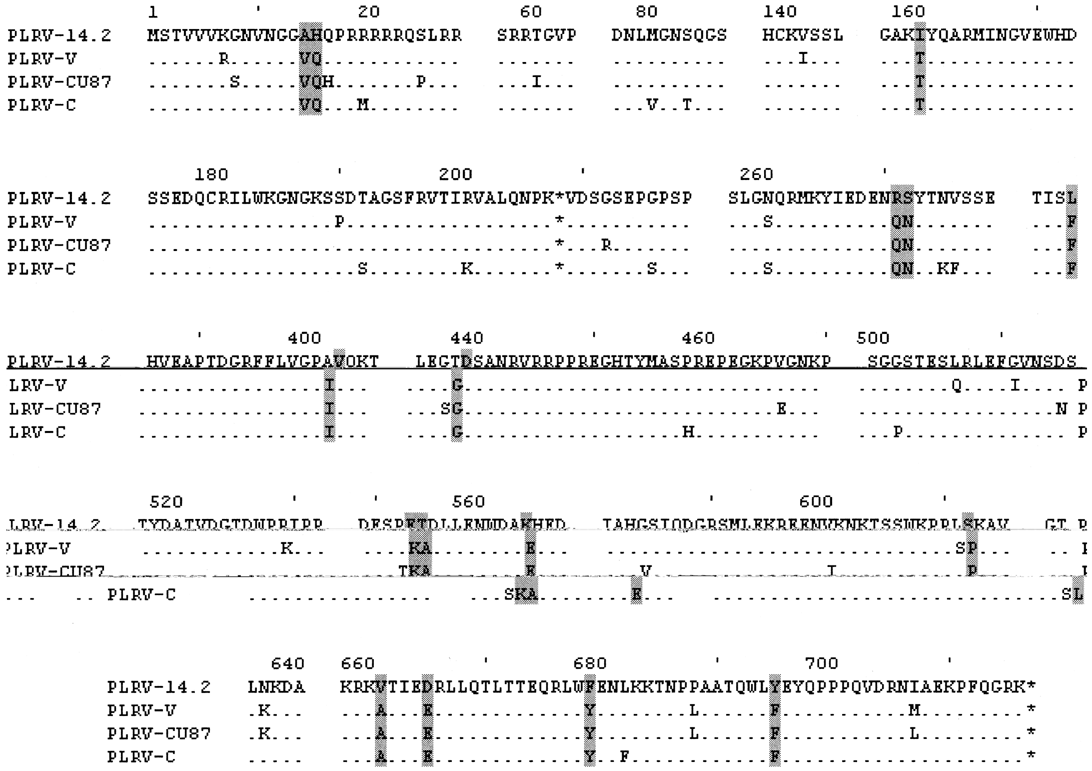

Fig. 1. Comparison of amino acid sequence of the CP and RTP between PLRV-14.2 and other PLRV isolates either poorly (PLRV-V) or readily (PLRV-CU87 and -C) transmissible by aphid clones belonging to the *M. persicae* complex. Amino acids are indicated in regions where sequences differ between two or more isolates and dots indicate where sequences are identical to PLRV-14.2 (used as a reference). Gaps correspond to regions where amino acids were identical between all isolates. Shaded boxes show the 13 sites where PLRV-14.2 differed from the three other PLRV isolates. Amino acids are numbered from the beginning of the CP. Asterisks mark the end of the CP and RTP.

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 ($P < 0{\cdot}05$).

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* (Wlk.) 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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