Small deletions in the potato leafroll virus readthrough protein affect particle morphology, aphid transmission, virus movement and accumulation

Kari A. Peter,1,2 Delin Liang,1,2 Peter Palukaitis3 and Stewart M. Gray1,2

1USDA/ARS, Biological Integrated Pest Management Research Unit, Ithaca, NY 14853, USA
2Department of Plant Pathology, Cornell University, Ithaca, NY 14853, USA
3Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK

INTRODUCTION

Potato leafroll virus (PLRV) is the type member of the genus Polerovirus in the family Luteoviridae. The host range includes members of the family Solanaceae, with the primary host being potato. PLRV is phloem-restricted and the virus is spread from plant to plant only by colonizing aphid species, the most efficient being Myzus persicae (Harrison, 1999). Following acquisition, the virus must circulate through the aphid before it can be transmitted; the virus persists but does not replicate in the aphid. The virus must pass through two barriers in the aphid, the posterior midgut (Garret et al., 1993; Reinbold et al., 2001) and the accessory salivary glands, via receptor-mediated endocytosis and exocytosis (Garret et al., 1993; Reinbold et al., 2001).

The icosahedral PLRV particle is 24 nm in diameter and contains a 6 kb positive-sense, single-stranded RNA genome. PLRV virions are assembled mainly from the 23 kDa coat protein (CP), but contain minor amounts of a readthrough protein (RTP) translated when the stop codon of the CP is suppressed. The 80 kDa RTP contains the 23 kDa CP and the 57 kDa readthrough domain (RTD) (Mayo & Miller, 1999). The RTP can substitute for a 23 kDa CP monomer when assembling into the virion. The CP portion of the RTP assembles into the icosahedral virion, while the RTD is predicted to be exposed on the surface of the particle. The ratio of RTP to CP for PLRV is unknown and appears to differ markedly between luteovirids, ranging from 1:4 to 1:100 (Bahner et al., 1990; Filichkin et al., 1994). Within the RTD there is a highly conserved N-terminal region and a variable C-terminal region. The full-length RTP can be detected readily in infected tissue, but in purified virus preparations a significant portion of the C terminus of the RTD is proteolytically processed yielding a 51–58 kDa RTP (Brault et al., 1995; Filichkin et al., 1994; Jolly & Mayo, 1994; Wang et al., 1995). This phenomenon has been seen among other members of the family Luteoviridae and despite such truncations, the virus is still aphid transmissible (Bruyere et al., 1997; Wang et al., 1995).

The RTD contains several identifiable domains. Adjacent to the CP termination codon is a cysteine-rich sequence encoding an alternating tract of proline residues, which has often been referred to as the proline hinge and may act as a tether for anchoring the RTD into the virion by joining it to the CP moiety (Guilley et al., 1994). Following this sequence is the N-terminal domain, encompassing approximately 210 aa that are highly conserved among all
Luteoviruses, with about 50% of the amino acids specifically conserved among members of the genus Polerovirus (Guilley et al., 1994) (Fig. 1). In addition, in barley yellow dwarf virus-PAV (BYDV-PAV), it has been shown that the cysteine-rich element proximal to the CP stop codon and a distal element spanning the N- and C-terminal junction act as translational enhancer elements for the translation of RTP (Brown et al., 1996). Homologous sequences may also be present in poleroviruses (Bruyere et al., 1997).

Luteovirids move systemically in host plants and through their aphid vectors as intact virions. CP is essential for the encapsidation of the genome, whereas RTP is not required for virion formation and a particle devoid of the RTP is still infectious but is not aphid transmissible (Bruyere et al., 2000; Bruyere et al., 1997; Chay et al., 1996). The RTD N-terminus has been shown to be responsible for aphid transmission and aphid endosymbiont interaction, whereas the C terminus was dispensable for aphid transmission (Bruyere et al., 1997; van den Heuvel et al., 1997; Wang et al., 1995). The C terminus does contain a homologous sequence among those poleroviruses that are transmitted by M. persicae, referred to as the ‘Myzus persicae homology domain’ (Guilley et al., 1994); however, mutation of this domain had little effect on the transmission efficiency of beet western yellows virus (BWYV) (Bruyere et al., 1997).

The N- and C-terminal regions of the RTD have been shown to be involved in viral movement and accumulation in plant hosts, suggesting that both are critical for whole-plant infection (Bruyere et al., 2000; Bruyere et al., 1997; Chay et al., 1996; Mutterer et al., 1999). BWYV and BYDV-PAV RTD mutants have been shown to accumulate to a lower titre in infected plants than wild-type (WT) virus (Bruyere et al., 1995; Chay et al., 1996). In subsequent plant tissue immunolocalization experiments, the BWYV RTD mutants had reduced virus movement to new infection sites in Nicotiana clevelandii plants, indicating that RTD affected the efficiency with which the virus could move systemically (Mutterer et al., 1999).

The focus of this study was to examine the biological role of the PLRV RTD. Deletion mutants were generated in order to understand better the determinants involved in virion formation, virus transmission and persistence in aphids, as well as local and systemic movement in different plant hosts.

**METHODS**

**Generation of recombinant cDNA PLRV RTD mutant constructs.** Fourteen three-amino-acid deletions were made in the N-terminal half of the RTD mutants (Fig. 1) as described previously by Liang et al. (2004). Deletions targeted amino acids conserved among the poleroviruses.

**Agrobacterium-mediated infection and analysis of infected tissue.** Using *Agrobacterium tumefaciens* cultures containing the full-length cDNA of the PLRV WT or one of the mutant viruses, agroinfiltration into mesophyll cells and agroinjection into vascular cells were done as described previously by Lee et al. (2005) and Kaplan et al. (2007). Translation and accumulation of RTP was measured in agroinfiltrated tissue samples by Western blot analysis as described previously in Lee et al. (2005) and Kaplan et al. (2007). *Nicotiana benthamiana*, *N. clevelandii*, *Physalis floridana* and *Solanum sarrachoides* plants were agroinjected and analysed for systemic infection as described previously by Lee et al. (2002) and Kaplan et al. (2007).

To identify if mutant forms of the RTP were incorporated into assembled virions, virus was purified by using a modified version of the protocol of Hammond et al. (1983). Virus was purified from 20–30 g infiltrated *N. benthamiana* tissue. Following agroinoculation, plants were kept in a growth room (20 °C, continuous light) for 6 days, after which the tissue was harvested and kept at −80 °C. Frozen tissue was homogenized in a blender for three 30 s intervals in 0.1 M sodium citrate buffer (pH adjusted to 6.5 using 0.5 M NaH₂PO₄) containing 0.5% 2-mercaptoethanol, at 5 ml g⁻¹ tissue. All steps were done at 4 °C. Homogenized tissue was filtered using cheesecloth, 1/4 volume of 2:1 chloroform: N-amylov alcohol was added, and the mixture stirred for 25 min then centrifuged for 10 min at 6614 × g in a JA14 rotor (Beckman Coulter). The supernatant was recovered by aspiration and adjusted to 0.2 M NaCl and 8% polyethylene glycol 8000. The mixture was stirred for 2 h and then centrifuged for 20 min at 6614 × g in a JA14 rotor. The supernatant was discarded and the pellet resuspended in 1/10 the original volume of 0.1 M phosphate buffer, pH 7. The solution was centrifuged for 10 min at 1960 × g in a JA20 rotor. The supernatant was layered onto a 30% sucrose (buffered in 0.1 M phosphate buffer, pH 7) pad (1:4 sucrose: supernatant) and centrifuged for 2 h at 145,421 × g in a Ti50.2 rotor (Beckman Coulter). The supernatant was discarded and the pellet was resuspended in 0.5 ml 0.1 M phosphate buffer, pH 7, transferred to a 15 ml Corex tube, then centrifuged for 7 min at 1960 × g in a JA20 rotor (Beckman Coulter). The supernatant was layered on top of a 10–40% linear sucrose gradient and centrifuged for 2.5 h at 111,132 × g in a SW41 swinging bucket rotor (Beckman Coulter). Gradients were fractionated using a density-gradient fractionator (Teledyne-ISCO) and the virus fractions were concentrated by centrifuging for 1.5 h at 117,734 × g in a Ti70 rotor (Beckman Coulter). The supernatant was discarded and the pellet was resuspended in 0.1 ml 0.1 M phosphate buffer, pH 7. Virus concentration was determined by reading the A₉₀₀, A₂₆₀ and A₃₂₀ and using the following calculation: [(A₂₆₀−A₃₂₀) × dilution factor] / 8.0. Purified virus was aliquotted and stored at −80 °C. CP and RTP in 100 ng purified virus were analysed by Western blotting as described previously by Kaplan et al. (2007).

**Fig. 1.** Genome organization of PLRV and the position of the 14 three-amino-acid deletions. Numbers 0–7 refer to ORFs. RTP consists of the sequences encoding ORF3, which is CP, and ORF 5, which is the RTD. The 14 three-amino-acid deletions are indicated by underlined bold type.
Sequencing progeny virus in infected tissue. The progeny viruses from systemically infected plants were analysed by RT-PCR and direct sequencing of the PCR products. Total RNA was extracted and RT-PCR was performed as described previously by Kaplan et al. (2007). RT-PCR analysis was done with primers PLRV 5′ p4159 (5′-GATCCGGCAGGATCTTGAG-3′) and PLRV 3′ p4941 (5′-GCCGAGCCATATAGTGGCCCT-3′). Primers amplified a 782 bp fragment, which included a portion of the 3′ end of the CP and the N-terminal region of the RTD. The RT-PCR parameters used, as per manufacturer’s instructions (Invitrogen), included a reverse transcription step at 50 °C for 30 min and 94 °C for 2 min, followed by PCR, 30 cycles of 94 °C for 15 s, 58 °C for 30 s and 72 °C for 1 min, followed by one cycle of 72 °C for 10 min. The amplified fragments were sequenced as described previously by Kaplan et al. (2007).

Aphid transmission assays. Systemically infected P. floridana or N. clevelandi plants were used as virus source tissue in aphid transmission assays. The tests were performed as described previously by Lee et al. (2005) with the exception that five aphids were placed onto each plant. A 48 h acquisition access period was followed by a 4–5 day inoculation access period. The fumigated plants were placed in a greenhouse and observed for symptom expression. At 3–4 weeks post-inoculation, plants were tested for systemic infection by double antibody sandwich (DAS)-ELISA. For plants testing positive for infection, the infecting virus was sequenced using the same primers as described above.

An additional method for testing aphid transmission was to bypass feeding by injecting 0.1 μl 50 ng purified virus ml⁻¹ into an aphid’s haemocoel (Mueller & Rochow, 1961). Five injected aphids were placed onto each of 4–6 plants per sample per experiment and feeding was terminated after 5 days. The plants were tested for infection by DAS-ELISA 3–4 weeks post-inoculation and open reading frame (ORF) 5 of the progeny viruses from infected plants was sequenced.

Virus detection in aphids. Healthy M. persicae were allowed a 72 h acquisition access period on tissue systemically infected with WT PLRV, an RTP-incorporating mutant, or an RTP-non-incorporating mutant. Nine aphids were removed, divided randomly into groups of three and stored at −80 °C in 25 μl nuclease-free water in an RNase-free microcentrifuge tube. The remaining aphids were transferred to 3-week-old healthy plants. Aphids were similarly collected and transferred to healthy plants at 3, 6 and 9 days. Total RNA was isolated by adding 200 μl Tri-Reagent (Molecular Research Center) to each tube, and homogenizing the aphids using RNase-free micro-pestles, while keeping the samples on ice. The samples were incubated at room temperature for 5 min. A 40 μl volume of chloroform was added and the mixture was vortexed, and incubated at room temperature for 2–3 min. The samples were centrifuged at 16 110 g for 15 min at 4 °C. A 100 μl aliquot from the aqueous layer was removed to a new tube, and 100 μl 2-propanol and 3 μl glycogen were added and the samples were vortexed. Samples were stored overnight at −20 °C and then centrifuged for 30 min at 16 110 g at 4 °C. The supernatant was removed and the pellet was washed with cold 70% ethanol and dried. The dried pellet was resuspended in 25 μl nuclease-free water and was DNase treated using the DNA-free kit (Ambion). After DNase treatment, 5 μl was used immediately in an RT-PCR as described above. Primers PLRV 5′ p3580 (5′-CCTAAAGATTCTCCTCCACGTGCG-3′) and PLRV 3′ p4240 (5′-GGATGGGGTTGTTGTGGGCCT-3′) were used to amplify a 660 bp fragment, which included the CP, to indicate the presence of virus. A control for total RNA isolation, primers were used to amplify a 303 bp fragment of 18S rRNA simultaneously during the RT-PCR reactions; forward primer (5′-CTGGCGACGGCATCATGTGG-3′) and reverse primer (5′-GAATTACCCGGCGCTGCT-3′).

RESULTS AND DISCUSSION

Triple amino acid deletions in the N-terminal domain of RTD and their effects on virus infection in inoculated cells

The 14 three-amino-acid-deletion mutants were generated in the conserved N terminus of the RTD (Fig. 1) to ensure stable mutants, since single and double amino acid deletions have been observed to revert or to generate compensatory mutations (Braught et al., 2000). Also, large deletions in the conserved region of the RTD either reduced translation of the modified RTP or resulted in the RTP no longer being incorporated into the virion (Bruyere et al., 1997). Initially, conserved amino acids (in bold type) were targeted for deletion (RFI, EDE, KGQ, IAY, GHP, ERD, GPA, YNY, SYG and LDE). When preliminary data suggested that the virions did not contain RTP, four additional three-amino-acid-deletion mutants targeted non-conserved amino acids (PML, QSS, SST and DRD). Upon sequencing, two mutants differed from the intended deletion. The GHP mutant contained a 12 nt deletion, resulting in a deletion of the GHPE sequence. The SYG mutant, in addition to its 9 nt deletion, had an additional single nt deletion 14 nt downstream from the SYG deletion, resulting in a frameshift mutation that created a stop codon 17 nt downstream from the second mutation. This mutant was expected to translate a truncated RTP that was 413 aa long, whereas the wild-type length of RTP is 817 aa.

N. benthamiana leaves were infiltrated with Agrobacterium containing infectious cDNA clones of the WT and the RTD mutants, which led to a nearly synchronous infection of the mesophyll cells in the infiltrated areas. Since virus infection was not limited to the phloem, this facilitated the evaluation of virus replication and protein translation, as well as virion assembly. All of the mutants accumulated within the infiltrated areas of the leaf to levels not significantly different from those for WT virus as determined by DAS-ELISA (data not shown), indicating the deletions did not affect virus accumulation and, presumably, replication. The CP and RTP were detected by Western blot analysis in total protein extracts from infiltrated leaves (Fig. 2) by using an antibody, SCR3, that recognizes the N terminus of the CP (Torrance, 1992). The 23 kDa CP and 80 kDa RTP for WT and 13 of 14 RTD mutants were similarly detected in infected tissue. The one exception was the SYG mutant. As expected, this mutant translated a truncated version of the RTP, of approximately 48 kDa and smaller than the truncated RTP associated with purified virus. The coding sequence for the RTD N-terminal region was sequenced for each mutant and the introduced triple amino acid deletions were maintained and no other changes were observed within this region.

In order to examine if any of the mutants contained RTP incorporated into the virion, virus was purified from infiltrated tissue and the structural viral proteins were evaluated by Western blot analysis (Fig. 2). All the purified
viruses had similar sedimentation coefficients in sucrose gradients (data not shown). As expected, all of the RTD mutant viruses contained the 23 kDa CP. When CP detected in the plant tissue versus the purified virion were examined in a side-by-side comparison, there was a slight decrease in size of the protein found in purified virus (Fig. 2). The CP detected in infected tissue could be post-translationally modified, such as being phosphorylated or glycosylated (Seddas & Boissinot, 2006), which would result in the protein being larger than predicted. It is doubtful that this reduction in size is due to proteolytic degradation of the N terminus of the CP since the SCR3 antibody binds to the extreme N terminus (Torrance, 1992). The truncated RTP was detectable in virions of only three viruses (RFI, EDE and SST). These viruses were purified several times and the level of RTP detection for SST was similar to that of the WT, whereas RFI and EDE had a consistently lower level of RTP. In previous studies where RTD mutants of the polerovirus BWYV were examined, point mutations did not interfere with RTP incorporation, but additional mutations often occurred as a result of those alterations (Brault et al., 2000). It is uncertain if BWYV RTP incorporation was associated with the original mutant or resulted from the additional mutations. Overall, based on the results presented here and those in prior studies, the inability to easily generate virions with incorporated mutant RTP suggest that the N terminus of the RTD is sensitive to manipulation. Deletions in the C terminus of the RTD do not appear to affect incorporation (Bruyere et al., 1997), but this is the first report of stable N-terminal mutants that incorporate mutant RTP into virions and they provide an opportunity to examine the domains of the N terminus of the RTD that are important for aphid transmission and host plant infection.

Effects of deletions in the RTD on systemic movement in different hosts

Since the RTP has been shown to be involved in virus movement and accumulation in plants (Brault et al., 1995; Bruyere et al., 1997; Mutterer et al., 1999), RTD mutants were examined in N. benthamiana, N. clevelandii, P. floridana (a weed host of PLRV), and hairy nightshade, S. sarrachoides, another weed host that is a "potential infective bridge" for PLRV spread in the field and a preferred host for M. persicae, a major vector for PLRV (Alvarez & Srinivasan, 2005). Multiple hosts were tested because host-specific effects have been reported for mutations in both the PLRV 17 kDa movement protein (P17) (Lee et al., 2002) and CP (Kaplan et al., 2007; Lee et al., 2005). The P17 movement protein was not required for infection in the Nicotiana species, but it was required for infection in P. floridana and Solanum tuberosum (Lee et al., 2002). Virion formation is essential for virus movement in the Nicotiana species, P. floridana and S. tuberosum, but mutations in the CP demonstrated that infection efficiencies varied among host plants (Kaplan et al., 2007; Lee et al., 2005).

In Agrobacterium-infiltrated tissue, large numbers of mesophyll cells are infected. This is in contrast to the natural phloem-limited infections, and usually agroinfiltration does not lead to systemic infection. In order to initiate a normal, phloem-limited infection, A. tumefaciens containing the cDNA clones of the RTD mutants were injected directly into the petiole of the leaf. This often results in a phloem-limited systemic infection, presumably since some bacteria are introduced into phloem-associated cells. This facilitated the study of virus movement, host specificity and aphid transmission. Several RTD mutants were chosen for further analysis of systemic infection in multiple hosts. In addition to the RTP-incorporating mutants (RFI, EDE and SST), four non-incorporating mutants (QSS, GHPE, ERD and SYG) were chosen because of their location in the sequence. In addition, the ΔRTP mutant, described previously (Liang et al., 2004) and which does not translate RTP, was tested for its ability to cause a systemic infection in different hosts. The RTP non-incorporating mutants were selected not only to study how non-incorporated RTP virions moved in different hosts, but also to observe if the deletions affected virus movement. The RTD mutants RFI, EDE, SST, QSS, GHPE and ERD were able to systemically infect all four hosts; however, infection was not equal among the different hosts (Table 1). The Nicotiana species and S. sarrachoides were easily systemically infected with WT, RFI, EDE, SST, QSS, GHPE and ERD, whereas P. floridana was a more difficult host to infect. In the Nicotiana species and S. sarrachoides, infection was established similarly in the WT and the
RTP-incorporating mutants, whereas RTP-non-incorporating mutants (QSS, GHPE and ERD) were delayed by approximately 1–2 weeks in reaching WT virus levels of infection as measured by DAS-ELISA. In the case of S. sarrachoides, the symptoms mirrored virus presence, i.e. only symptomatic leaves contained detectable levels of virus. This was different for the Nicotiana species, in which asymptomatic leaves often had detectable levels of virus. For each infected plant, the RTD-coding region was sequenced for each mutant virus and the three-amino-acid deletions were maintained and no additional changes in the region were detected.

The development of the infection was different for SYG and ΔRTP mutants. The SYG mutant, which translated a truncated RTP, was not detected in the Nicotiana species and P. floridana until approximately 7 weeks post-inoculation, and in S. sarrachoides it was not detected until 10 weeks post-inoculation. WT virus was detected by 3 weeks post-inoculation. After sequencing, the SYG deletion was maintained with no additional changes in the infected Nicotiana species and S. sarrachoides plants. Interestingly, in the three SYG-infected P. floridana plants, the SYG deletion was maintained, but the second mutation downstream was filled in, such that a frameshift did not occur and the truncation of the RTP did not result. Perhaps this additional mutation, which should have restored the synthesis of a full-length RTP, allowed the movement of the SYG mutant in P. floridana. The low virus titre and the antibodies do not allow Western blot detection of the RTP in systemically infected leaves; data that would allow us to verify our hypothesis.

It was difficult to infect P. floridana with the ΔRTP mutant (Table 1). S. sarrachoides was more easily infected and virus was detected as early as 6 weeks, but more typically at 8–10 weeks. The ΔRTP viruses in the infected plants maintained their deletions, and no additional changes were detected. The most interesting result was related to symptom development in S. sarrachoides infected with SYG and ΔRTP (Fig. 3). In plants infected with viruses producing full-length RTP (WT, RFI, EDE, SST, QSS, GHPE and ERD), inter-veinal chlorosis was observed in the mature leaves, suggesting that phloem loading was being affected and the symptoms progressed to the entire plant over time (Fig. 3a–c). In plants infected with ΔRTP and SYG, symptoms were first observed in the youngest tissue 6–10 weeks post-inoculation and, in contrast to the intercostal chlorosis observed with WT virus, the chlorosis was confined to small local-lesion-type areas (Fig. 3d and e). Virus was not detected by DAS-ELISA in mature leaves and they remained asymptomatic. In the Nicotiana species and P. floridana, infection with all viruses, regardless of RTP production, produced similar symptoms, which were chlorotic leaves and decreased growth. Although systemic movement of the non-incorporating RTP mutants was slowed in the first few weeks of infection, by 4 weeks post-infection there were no differences between the WT and the RTP mutants with respect to virus distribution (measured by symptom expression) or virus detection (measured by DAS-ELISA). This suggests that the role of RTP in movement can be host-specific and that it functions as a non-structural protein although incorporation into the virion may facilitate movement early on. Further studies are under way in order to examine this hypothesis further.

### Aphid transmission of the RTD mutants via tissue feeding and injection of purified virus into the aphid haemocoel

To study the aphid transmissibility of our RTD mutants, RFI, EDE and SST were chosen for further analysis since these mutants incorporated RTP into the virion. Four additional deletion mutants not incorporating RTP into the virion were also chosen based on their position in the N-terminal sequence (QSS, GHPE, ERD and SYG). M. persicae aphids fed on P. floridana or N. clevelandii plants systemically infected with the RTD mutants, and were transferred to healthy P. floridana plants. Virus presence in the source tissue was determined by ELISA. Titres were not always equal to those of the WT, but 48 h acquisition access periods would have minimized the effect of titre on transmission efficiency (Gray et al., 1991). The results indicated that regardless of whether the RTD mutant incorporated RTP into the virion (RFI, EDE and SST) or not (QSS, GHPE, ERD and SYG), none of the RTD mutants were aphid transmissible (Table 2). Incorporated RTP, as well as surface loops of the CP, have been shown to

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<th>PLRV</th>
<th>No. plants infected/no. plants agroinjected</th>
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<tr>
<td></td>
<td>N. benthamiana*</td>
</tr>
<tr>
<td>WT</td>
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</tr>
<tr>
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</tr>
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<td>EDE</td>
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</tr>
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<td>GHPE</td>
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<td>ERD</td>
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<tr>
<td>SYG†</td>
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<tr>
<td>ΔRTP</td>
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*Plants were tested by DAS-ELISA 3–4 weeks post-inoculation unless otherwise noted.
†Results indicate the number of plants with detectable levels of SYG in Nicotiana species or P. floridana plants at 7 weeks post-inoculation and in S. sarrachoides at 10 weeks post-inoculation. All plants had detectable levels of SYG at 4 months post-inoculation.
‡ΔRTP plants had detectable levels of virus starting at 6 weeks post-inoculation; when tested at 4 months post-inoculation all plants had detectable levels of virus.
be required for aphid transmission of poleroviruses (Brault et al., 2003; Bruyere et al., 1997; Jolly & Mayo, 1994; Kaplan et al., 2007; Lee et al., 2005). The results reported here showing that non-incorporating RTP mutants were not aphid transmissible are consistent with previous reports that RTP is required for aphid transmission. However, RTP incorporation into the virion did not ensure aphid transmission. These results imply that determinants within the N terminus of the RTD are critical for passage of the virion through the aphid and the small change in the mutant incorporated RTP was significant enough to hinder transmission. In order to examine where the mutants were encountering problems within the aphid, we needed to consider the barriers the virion must overcome in order to be successfully transmissible.

When PLRV virions circulate through the aphid, they must be transmitted across the midgut epithelial cells, survive in the haemocoel, and be transported through the accessory salivary gland cells. To determine if the midgut was a barrier and prevented transmission of the RTD mutants, which produce the full-length RTP. Note that typical symptoms are inter-veinal chlorosis that develops throughout the plant. (d) ΔRTP symptoms at 6 weeks post-inoculation. (e) SYG symptoms at 10 weeks post-inoculation. In ΔRTP- and SYG-infected plants, symptoms are slow to develop and appear as chlorotic spots or local lesions only on the youngest leaves. Mature tissues present on the plant when symptoms begin developing are never affected.

Fig. 3. S. sarrachoides infected with PLRV. (a–c) Symptoms at 3, 6 and 10 weeks post-inoculation, respectively, for WT and RTD mutants, which produce the full-length RTP. Note that typical symptoms are inter-veinal chlorosis that develops throughout the plant. (d) ΔRTP symptoms at 6 weeks post-inoculation. (e) SYG symptoms at 10 weeks post-inoculation. In ΔRTP- and SYG-infected plants, symptoms are slow to develop and appear as chlorotic spots or local lesions only on the youngest leaves. Mature tissues present on the plant when symptoms begin developing are never affected.

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have shown that many RTD mutants assemble normal looking virions and are stable under many conditions including within an aphid (Brault et al., 1995; Gildow et al., 2000; Kaplan et al., 2007). These results suggest that the midgut is not the primary barrier, although it may reduce efficiency of virus movement (Reinbold et al., 2001). This was not surprising considering that previous work had shown that virions of BYDV devoid of the RTP were able to cross the gut barrier, indicating that the CP possessed the determinants for mediating gut uptake (Chay et al., 1996). However, RTP can affect the efficiency of virus transport across the gut (Brault et al., 2000, 2007; Reinbold et al., 2001) and RTP can determine intestinal tropism when mediating acquisition (Brault et al., 2005).

Determining the persistence of virus in aphids over time after feeding on systemically infected tissue for 72 h

Since the midgut was not the primary barrier for aphid transmission of the RTP-incorporating mutant viruses, further experiments were conducted to observe how these viruses persisted in aphids. M. persicae were fed on S. sarrachoides plants systemically infected with WT, SST (RTP-incorporating mutant), or ERD (RTP-non-incorporating mutant) for 3 days, then transferred to a different healthy plant every 3 days for 9 days. Virus was detected for up to 9 days in all samples of aphids fed on the WT and SST mutant (Fig. 4). In contrast, there was a variable amount of virus detected among the aphids fed on
Table 2. Transmission of PLRV RTD mutants by *M. persicae*
that either fed on systemically infected *P. floridana* or *N. clevelandii*, or received injections of purified virus

For plant-to-plant transmission aphids were allowed a 48 h acquisition access period. Five aphids were transferred to each of 5–6 plants per experiment and allowed a 4–5 day inoculation access period on *P. floridana* seedlings.

<table>
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<th>PLRV mutant</th>
<th>No. of plants infected with virus/no. infested with aphids</th>
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<td>NT</td>
<td>0/15</td>
<td>0/12</td>
</tr>
<tr>
<td>SST</td>
<td>0/15</td>
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<tr>
<td>GHPE</td>
<td>0/16</td>
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<tr>
<td>ERD</td>
<td>0/31</td>
<td>NT</td>
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<tr>
<td>SYG</td>
<td>0/31</td>
<td>NT</td>
<td>0/13</td>
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</tbody>
</table>

*Aphids received injections of approximately 0.1 μl purified virus (50 ng ml⁻¹) and were transferred to four to six healthy *P. floridana* seedlings, five aphids per seedling, for a 4–5 day inoculation access period. Results are a summary of two to four experiments. NT, Not tested.

ERD-infected tissue (Fig. 4). Virions lacking RTP are capable of crossing the midgut membrane (Reinbold et al., 2001); however, they do so less efficiently. Thus, there is likely to be aphid-to-aphid variability in how much virus will pass through the digestive system and out of the aphid. The variability also could be directly associated with RTP incorporated into the virion. Although we could not detect RTP incorporated into virion for ERD (Fig. 2), there is a possibility that some virions may have contained an undetectable amount of RTP and that those virions were able to survive in the aphid. Virus that does not contain RTP and moves into the haemocoel is likely to be sequestered or degraded more rapidly (van den Heuvel et al., 1997). Another reason could be acquisition differences among the aphids; not all aphids will feed at the same rate or ingest the same amount of virus due to non-uniform distribution in the leaves. Despite the variability between the RTD mutants, the virus persisted in the aphids for a period sufficient for virus to be transmitted so rapid degradation does not appear to be the reason for the lack of transmission.

The cause of the lack of transmission is still uncertain, but additional possibilities exist. The aphid’s immune system may attack virions present in the haemocoel. The virions with missing or modified RTP could be sequestered in the haemocoel, making them unavailable to interact with the accessory salivary gland, or these virions could be degraded in the haemocoel. Such a scenario is possible since the N-terminal region of the RTD of PLRV and BWYV has been shown to bind to symbionin, a protein produced by aphid endosymbionts existing in mycetocytes in the haemocoel (Hogenhout et al., 2000; van den Heuvel et al., 1997). Symbionin is not secreted into the aphid haemocoel, but is suggested to be present in the haemocoel due to the degradation of the endosymbionts and mycetocytes as the aphids mature (Baumann et al., 1995; Fukatsu & Ishikawa, 1992). There is a possibility that the attachment of symbionin to the virus protects the virus from the aphid’s immune system and the amino acids deleted from the PLRV RTD-incorporating mutants may be critical for this binding. A lack of protection may result in sequestration of the virus rather than degradation. Sequestered viral RNA may be detected by RT-PCR, but may not be detected by serological assays that were used by researchers who speculated that virus unable to bind symbionin is degraded (van den Heuvel et al., 1997). Also, lack of transmission could be attributed to the RTD mutant virus not possessing the proper recognition motifs for specific transport across the membranes of the accessory salivary gland. It is unclear at this point if the reason the RTD mutants’ failure to be transmitted was due to a disruption of a direct interaction with the sequence or changes in the sequence affecting the structure of the protein.
We have shown that the conserved N-terminal region of the PLRV RTD is extremely sensitive to perturbations of the amino acid sequence and that most changes eliminate or significantly reduce the incorporation of the RTP into the assembled virion. These properties will complicate efforts to identify critical residues or domains within the RTD N terminus that regulate virus transport through aphids. The free RTP does, however, function as a non-structural movement protein in a somewhat host-specific manner. Domains in the N-terminal region can have minor effects on systemic movement and accumulation, but the most dramatic alterations in long distance movement appear to be regulated by the C-terminal domain of the RTD.

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REFERENCES


