Aphid Transmission and *Buchnera* sp. GroEL Affinity of a *Potato leafroll virus* RTD Deficient Mutant

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ABSTRACT

Potato leafroll virus (PLRV), genus *Polerovirus*, family *Luteoviridae*, is transmitted by aphids in a persistent and circulative manner. Members of the family *Luteoviridae* associate with a GroEL homologue produced by the primary endosymbiont (*Buchnera* sp.) of aphids to avoid degradation in the hemolymph. Purified luteovirus particles contain two types of proteins: a major capsid protein (CP) of ~22 kDa and a minor capsid component of 54 kDa, which is a truncated form of a translation read-through protein of the CP gene termination codon. The read-through domain (RTD) contains determinants responsible for virus transmission. An infectious full-length cDNA clone of PLRV and a mutant devoid of the RTD were used to analyze the molecular interactions between this luteovirus and its aphid vector *Myzus persicae*. The PLRV mutant virions, lacking the entire RTD protein, were not transmissible by *M. persicae* and did not bind to *Buchnera* sp. GroEL. Furthermore, this mutant was less persistent in the aphid's hemolymph than in the wild type virus.

Additional keywords: *Luteoviridae*, *Myzus persicae*, *Polerovirus*, read-through protein.

RESUMO

Transmissão por afídeos e afinidade a *Buchnera* sp. GroEL de um mutante deletério da proteína de RTD do *Potato leafroll virus*

O *Potato leafroll virus* (PLRV), gênero *Polerovirus*, família *Luteoviridae*, é transmitido por afídeos de um modo persistente e circulativo. Membros da família *Luteoviridae* associam-se a um homólogo de GroEL produzido pelo endosimbionte primário (*Buchnera* sp.) de afídeos para evitar a degradação na hemolinfa. Partículas purificadas de luteovirus contêm dois tipos de proteínas: a capa protéica (CP) de ~22 kDa e um componente "capsidial" de 54 kDa, o qual é uma forma truncada de uma proteína de "transleitura" a partir do códon de terminação do gene da CP. O domínio de transleitura (RTD) contém determinantes responsáveis pela transmissão do vírus. Um clone de cDNA infeccioso do PLRV e um mutante deletério da RTD foram usados para analisar as interações entre esse luteovirus e seu afídeo vetor *Myzus persicae*. As partículas mutantes do PLRV, deficientes da proteína RTD inteira, não foram transmissíveis por *M. persicae* e não se ligaram a *Buchnera* GroEL. Adicionalmente, esse mutante foi menos persistente na hemolinfa do afídeo do que o vírus selvagem.

Palavras-chave adicionais: *Luteoviridae*, *Myzus persicae*, *Polerovirus*, proteina de transleitura.

INTRODUCTION

Potato leafroll virus (PLRV) is the type member of the genus *Polerovirus*, family *Luteoviridae* (Mayo & D'arcy, 1999). The PLRV virions are particles of 25 nm diameter harbouring a positive-strand RNA of ~6 kb with a viral protein genome-linked (VPg) at the 5' end (van der Wilk *et al.*, 1997). The genomic RNA encodes six large open reading frames (ORFs) (van der Wilk *et al.*, 1989) arranged into 5' end (ORF0, ORF1, and ORF2) and 3' end (ORF3, ORF4, and ORF 5) gene clusters expressed by different mechanisms (Miller *et al.*, 1995). The 3' end proximal ORFs are expressed via a subgenomic RNA synthesized in host cells during the infection process. The ORF3 encodes the major capsid protein (CP) of about 23 kDa and ORF4 encodes a 17 kDa putative movement protein. The ORF5 encodes the carboxy terminal region of the minor capsid component expressed by translational readthrough of the ORF 3 amber stop codon. The resulting full-length protein has a MW of about 74 kDa but, in preparations of purified virus, it is present in a C-terminally truncated form of about 54 kDa (Bahner *et al.*, 1990).

Members of the family *Luteoviridae*, "luteovirids" (Smith *et al.*, 2000) replicate mainly in the phloem tissue of

their host plants and are transmitted by aphids in a persistent and circulative, non-propagative manner (Gildow, 1999). The process of acquisition and transmission of these viruses by their specific vectors has been the object of extensive studies in the last decade. After ingestion with the phloem sap during aphid feeding on an infected plant, the virus is actively transported through the epithelial cells to the hemocoel by receptor-mediated endocytosis and exocytosis (Garret *et al.*, 1993, Reinbold *et al*., 2001). The virus particles are retained in an infective form in the hemolymph of the aphid; upon contacting the basal lamina of the accessory salivary gland (ASG), they may be transported through the underlying plasmalemma into the salivary canal, from where they are excreted with the saliva while the aphid feeds. The high degree of specificity among luteovirids and the aphid species at this level determines the successful transport across the ASG basal lamina and plasmalemma, and consequent virus transmission (Gildow & Gray, 1993, Pfeiffer *et al.*, 1997).

Symbionin, a GroEL homologue synthesized by the primary endosymbiotic bacteria (*Buchnera* sp) of aphids is abundant in the hemolymph and is essential for persistence of luteovirids in the insect's body (van den Heuvel *et al.*, 1994, 1997). In ligand binding assays, a large number of luteovirids and the geminivirus *Tomato yellow leaf curl virus* (TYLCV) showed a high specific affinity for GroEL homologues of vector and non-vector species (van den Heuvel *et al.*, 1994, 1997; Filichkin *et al.*, 1997; Morin *et al.*, 1999). Antibiotic treatments decreased *Buchnera* sp. GroEL levels in the aphid's body, caused loss of capsid integrity and inhibited transmissibility of these viruses. This suggests that the association of this protein retards luteovirids and geminiviruses proteolytic breakdown (van den Heuvel *et al.*, 1994, 1997; Morin *et al.*, 1999).

The role of the viral CP and readthrough domain (RTD) in determining the efficiency of luteovirid transmission by aphids has been an important object of research (Brault *el al*., 2003; Gray & Gildow, 2003; Taliansky *et al.,* 2003). Previous studies on the role of the viral capsid-associated proteins of luteoviruses have shown that the BWYV RTD protein is indispensable for transmission by *Myzus persicae* (Sulz.), and harbour determinants which mediate circulation of the virus in the aphid (Brault *et al.*, 1995). The recognition and movement of BWYV into the aphid vector hemocoel is greatly reduced in RTD deficient mutants, suggesting an important role for this protein in virus recognition and the trans-cellular transport process (Reinbold *et al.*, 2001). It is known that BWYV and BYDV virions lacking the RTD protein are stable in the aphid's intestinal lumen and able to cross the gut epithelium (Brault *et al.*, 1995). Nevertheless, these particles are not able to cross the ASG basal lamina, and therefore are not transmissible by the aphid (van den Heuvel *et al.*, 1997). On the other hand, RTD-deficient PLRV virus-like particles (VLPs), which were produced in a baculovirus-insect cell expression system, were found in the same organs and locations of the vector as observed for the wild-type PLRV (Gildow *et al.*, 2000). In this case, the absence of the RTD protein did not greatly affect the ability of virions to penetrate the ASG basal lamina.

Several subdomains can be identified within the readthrough domain of luteovirids genome (Mayo & Ziegler-Graff, 1996). Located in the N-terminal half of the RTD, downstream from the coat protein suppressible termination codon, is a cytidine-rich sequence encoding a tract of alternating proline residues. This proline tract is followed by a region of 200 amino acids which displays considerable homology throughout the family *Luteoviridae* (Mayo & Ziegler-Graff, 1996; Bruyère *et al.*, 1997; Brault *et al.*, 2000). The C-terminal half of the RTD is poorly conserved, although it contains a region of homology unique to luteovirids efficiently transmitted by *M. persicae* (Guilley *et al.*, 1994). The presence of the N-terminal region of RTD in BWYV virions determines the ability of the virus to bind with the GroEL homologue of the endosymbiont bacteria *Buchnera* sp. and the persistence of the virus in the aphid's hemolymph (van den Heuvel *et al.*, 1997). Brault *et al.* (2000) have identified specific amino acids in the conserved part of the RTD involved in virus accumulation in plants and in transmission of BWYV by *M. persicae*. Successful aphid transmission of BWYV RTD mutants accompanied by compensatory mutations in this protein suggests that the RTD possesses structural redundancy that is involved in transmission by *M. persicae*.

In this study we have used an infectious cDNA clone of a Dutch isolate of PLRV to show the role of the RTD protein of PLRV in the transmission by *M. persicae*. For this purpose, an infectious construct was used that contained a mutation eliminating RTD expression and the result was followed in microinjected aphids and throughout the transmission process.

MATERIALS AND METHODS

Virus strain and aphid colony

The PLRV-Wageningen (van der Wilk *et al*., 1989) used in this study was maintained in *Physalis floridana* Rydb. The virus was purified from frozen *P. floridana* leaves using an enzyme-assisted procedure described by van den Heuvel *et al.* (1997). Purified virus was stored at –80 °C in 0.1M sodium citrate (pH 6.0) containing 20% sucrose. Non-viruliferous *M. persicae* aphids reared on cabbage [*Brassica napus* subsp. *oleifera* (Moench) Metzg] at 20 ± 3 °C under a photoperiod of 16 hours were used throughout these studies.

PLRV full-length clone and RTD-deficient mutant

The clone of the wild-type and a deletion mutant of a full-length cDNA clone of PLRV were used to generate infectious transcripts. The clones were placed under the control of the bacteriophage T7 promoter by cloning the PLRV sequence directly after the promoter sequence. At the 3'-end a *ScaI* restriction site was created enabling linearisation of the construct. Two different transcription vectors were used, a construct identical to the wild-type PLRV sequence (van der Wilk *et al*., 1989), pT7PLRV, and a construct harbouring

a deletion of a G residue at position 4705 of the PLRV genome, T7PLRV△4705 (Figure 1). This deletion was located in the RTD gene sequence 491nt downstream of the amber stop codon separating ORF4 and ORF5. Capped T7 RNA transcripts of the constructs were synthesized using the T7 Cap-Scribe kit (Roche, Germany) according to the manufacturer's instructions.

Infection of protoplasts and virus purification

Cowpea (*Vigna unguiculata* L.) protoplasts were inoculated with viral RNA transcripts according to van Bokhoven *et al*. (1993) with minor modifications. One million protoplasts plus 2 µg transcripts were precipitated with 40% PEG 6000 and 0.1 M $Ca(NO_3)_2$ in MES/Mannitol buffer (0.5 M Mannitol, 15 mM $MgCl_2$ and 0.1% MES, pH 5.6). Protoplasts were incubated for 20 min at room temperature and centrifuged for 3 min at 600 rpm. After washing with 0.5 M Mannitol and 10 mM $CaCl_2$, protoplasts were collected by centrifugation for 3 min at 600 rpm and the pellet resuspended in Rottier medium (Rottier *et al*., 1979). Protoplasts were then incubated for 48 h at 24 °C under weak light and centrifuged for 2 min at 1,000 rpm. Pellets were stored at –80 °C.

Virions of the wild-type T7PLRV and of the mutant T7PLRV△4705 were purified from infected protoplasts by resuspending 1 x 10^6 protoplasts in 1 ml of 0.1 M sodium citrate, pH 6.0, containing 0.5% cellulase, 0.5% macerozyme, 1 µl of thioglycolic acid and 5 µl of 100% ethanol. After incubation with gentle agitation for 2 h at room temperature, 0.5 ml of a mixture of chloroform:butanol (1:1) was added, mixed, and centrifuged for 5 min at 1,000 rpm. Ten microliters of Triton X-100 were added at the aqueous phase, which was incubated and agitated for 30 min at room temperature. The solution was loaded onto a 20 to 50% linear sucrose gradient and centrifuged in a Beckman SW41 rotor at 35,000 rpm for 5 h. Fractions of 0.5 ml were collected in an automatic fraction collector and peaks at 254 nm were tested for the presence of viral antigen by antigen-coated plate enzyme-linked immunosorbent assay (ACP-ELISA) with a polyclonal antiserum raised against PLRV. Virus fractions were combined, diluted in 0.1 M sodium citrate, pH 6.0, and centrifuged for 3 h at 40,000 rpm in a Beckman SW55 rotor. The pellet was resuspended in 0.1 M sodium citrate, pH 6.0, containing 20% sucrose. Virus concentration was determined by ACP-ELISA, and purified virus was stored at -80 °C.

Analysis of viral RNA and capsid proteins

Infection of protoplasts by RNA transcripts synthesized from full-length cDNA clones was assessed by transmission electron microscopy (TEM). Protoplasts were sonicated for 1 min and stained with 2% uranyl acetate before examination by TEM.

Viral structural proteins of the virions purified from protoplasts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), in 10% gels, as previously described (Brault *et al.*, 1995) and detected by Western blot analysis using a specific antiserum raised against PLRV.

Purification of *Buchnera* sp. GroEL and GroEL ligand assay

Endosymbiotic GroEL homologue of *M. persicae* was purified from six-day-old aphids as described previously (van den Heuvel *et al.*, 1997). GroEL samples collected from bands of a 10 to 50% linear sucrose gradient were diluted (1:10) in SYM buffer (50 mM Tris-HCl, pH 7.6 containing 35 mM KCl,

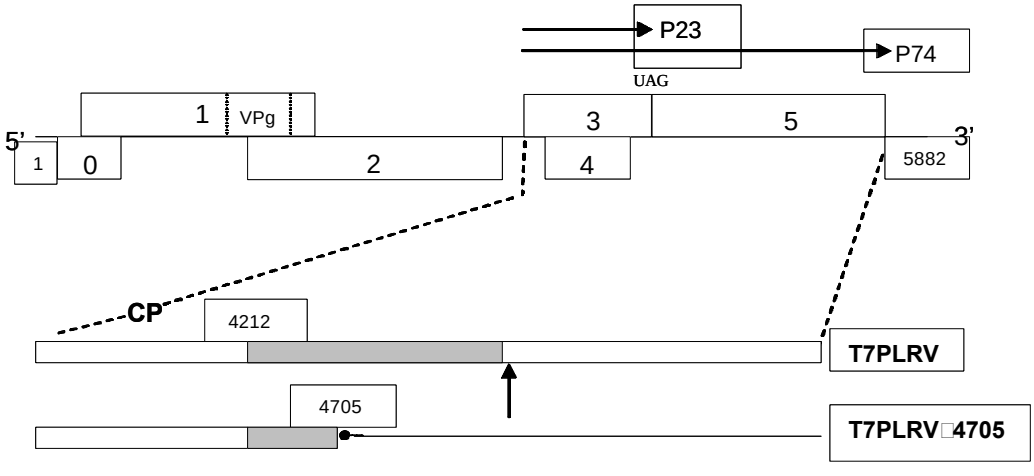

FIG. 1 - Genomic organization of *Potato leafroll virus* (PLRV) and structure of the readthrough protein (RTD) of wild-type T7PLRV and RTD deletion mutant T7PLRV△4705. Open reading frames (ORFs) are represented by numbered boxes and linked protein (VPg) coding sequence is represented between dashed lines in ORF 1. The conserved N-terminal half of the RTD is shaded; the predicted site of cleavage of P74 to generate the C-terminally truncated form of the RTD is indicated by an arrow. The deletion in mutant T7PLRV△4705 is indicated by a dot and numbering refers to nucleotide positions in the PLRV genome. The deletion of a single nucleotide in the mutant caused a frameshift in the ORF5 reading frame and a premature truncation of the RTD, represented by a thick line.

25 mM NH_4Cl, 10 mM magnesium acetate and 1 mM DTT). Concentration of GroEL in the sucrose gradient fractions containing the protein was estimated using the Coomassie Plus Protein Assay (Pierce, Rockford, IL, USA) with a protein dilution series ranging from 2000 to 10 µg/ml. Optical density was measured at 630 nm with a Bio-Kinetics reader EL 312 (Biotech Instruments Inc. Winooski, Vt, USA), and purified GroEL was stored at –80 °C.

The affinity binding of T7PLRV and T7PLRV△4705 virions for *Buchnera* sp. GroEL ("GroEL ligand assay") was determined using immunoplates as described by van den Heuvel *et al.* (1997) with minor modifications. Plates were coated with 100 µl of 6 µg/ml purified GroEL in 0.05 M sodium carbonate, pH 9.6 (coating buffer), and incubated overnight at 4 °C. Samples, consisting of 100 µl of 40 to 80 ng/ml of purified virus from protoplasts in sample buffer (0.14 M NaCl, 2 mM KCl, 2 mM KH_2PO_4, 8 mM $Na_2HPO_4.2H_2O$, 0.05% Tween 20, 2% PVP 44,000, 0.2% ovalbumin, 0.1% Na-azide and 0.5% BSA, pH 7.4), were incubated overnight at 4 °C. Subsequent steps were followed as previously described (van den Heuvel *et al.*, 1997).

Aphid transmission assays and microinjection

Six-day-old non-viruliferous *M. persicae* were microinjected with approximately 7 ng of purified virus from protoplasts using calibrated glass capillaries prepared with a needle puller (Gabay Instruments, Geneva, Switzerland). Positive controls consisted of aphids microinjected with 16 ng of PLRV purified from plants. Fifteen to 20 microinjected aphids were transferred to healthy *P. floridana* or cabbage (*Brassica oleracea* L.) plants maintained at 20 °C under a photoperiod of 16 h. After a 96 h inoculation access period (IAP) aphids were removed from the *P. floridana* plants and symptom expression was monitored. Plants were analyzed for virus infection by double antibody enzyme-linked immunosorbent assay (DAS-ELISA), three weeks after inoculation. Samples of two aphids were collected from cabbage plants 0, 24, 72, and 96 h after initiation of IAP and stored at –80 °C for further analysis by triple antibody sandwich (TAS)-ELISA.

Detection of virus in microinjected aphids

For TAS-ELISA analysis, immunoplates were first coated with 100 µl per well of 1 µg/ml anti-PLRV IgG in coating buffer for 3 h at 37 °C. Samples of two aphids collected at different times after microinjection, were ground in 110 µl of SEB and incubated overnight at 4 °C. Intact virus particles were detected with 100 µl of 2 µg/ml monoclonal antibody WAU-B9 (van den Heuvel *et al.*, 1990) in SEB by incubation for 3 h at 37 °C. Immunoplates were then incubated for 3 h at 37 °C with 100 µl goat anti-mouse IgG linked to alkaline phosphatase (Sigma, St. Louis, Mo., USA) in SEB buffer following the manufacturer's instructions. Bound enzyme was detected by addition of 1 mM p-nitrophenyl phosphate in 10% diethanolamide (pH 9.8). Readings at 405 nm determined the amount of immobilized enzyme.

RESULTS

Biological activity in protoplasts

To investigate the role of the RTD in the transmission of PLRV by *M. persicae*, a full-length PLRV cDNA clone was constructed using existing cDNA clones and newly generated RT-PCR fragments. The cDNA clone was placed under the control of the T7 promoter by cloning the PLRV sequence directly behind the promoter sequence. The T7 RNA transcripts of this construct were shown to replicate normally in protoplasts and to produce virus particles, as revealed by TEM examination (Figure 2). However, Western blot analysis showed that the particles were devoid of the RTD. Sequence analysis revealed that the clone named pT7PLRV△4705 contained a deletion of a G-residue at position 4705. The deletion was repaired by site-directed mutagenesis to generate the wild-type clone named T7PLRV.

Western blot analysis detected the two PLRV structural proteins in the wild-type virus purified from infected *P. floridana* and in T7PLRV purified from infected cowpea protoplasts (Figure 3). Only the major CP, about 23 kDa,

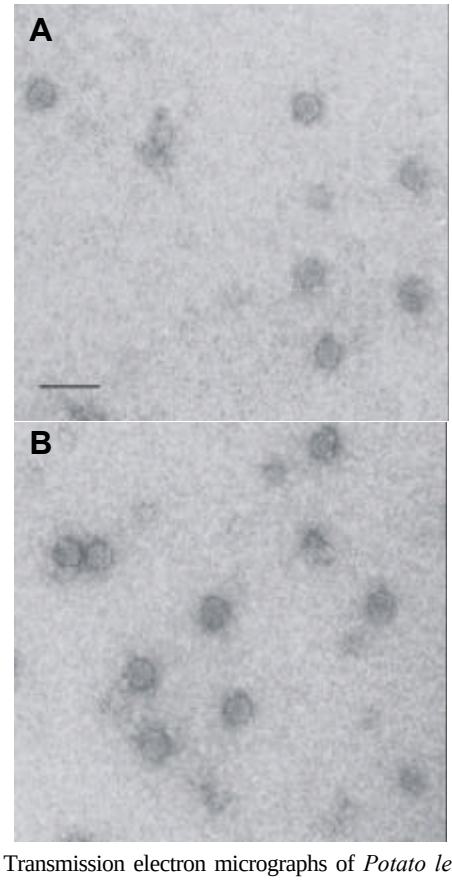

FIG. 2 - Transmission electron micrographs of *Potato leafroll virus* (PLRV) particles purified from infected protoplasts. Particles were stained with 2% uranyl acetate. A: virus particles from protoplasts infected with wild type full-length clone T7PLRV; B: virus particles from protoplasts infected with RTD deletion mutant T7PLRV△4705. Scale bar = 100 nm.

encoded by ORF 3, was detected in protoplasts infected with the T7PLRV△4705. The RTD proteins shown are present in a truncated form of about 54 kDa, lacking the C-terminal region, which is characteristic of purified virus particles (Bahner *et al.*, 1990). The deletion in T7PLRV△4705 sequence resulted in a frameshift in the ORF5 and a premature truncation of the RTD. The deduced molecular weight of the truncated RTD was approximately 45 kDa. However, the Western blot analysis revealed that neither the truncated nor any other form of the RTD was present in purified T7PLR△4705 virus particles (Figure 3). Moreover, the truncated RTD could not be detected in infected protoplasts (data not shown). These results suggest that the truncated RTD may be unstable and prone to degradation in the cell.

Although the absence of the entire RTD protein in the mutant T7PLRV△4705 did not inhibit viral RNA replication and the synthesis of virus particles in transcript-infected cowpea protoplasts, it had significant effects in subsequent steps of the virus life cycle as discussed below.

Transmission by microinjected aphids

In order to elucidate the essential role of the RTD protein in mediating the transmission of PRLV by *M. persicae*, purified T7PLRV and RTD-deficient mutant T7PLRV△4705 virions were microinjected into the aphid hemocoel and their ability to be transmitted to *P. floridana* was tested. Non-viruliferous aphids and aphids microinjected with wild-type PLRV were used respectively as negative and positive controls. Virions derived from clone T7PLRV were efficiently transmitted by aphids to all tested plants (3/3 test plants with 15 to 20 aphids/plant), whereas virions derived from clone T7PLRV△4705 were not transmitted (0/3 test plants with 15 to 20 aphids/plant). Symptom expression and the results of DAS-ELISA, with an antiserum raised against PLRV, are shown in Table 1.

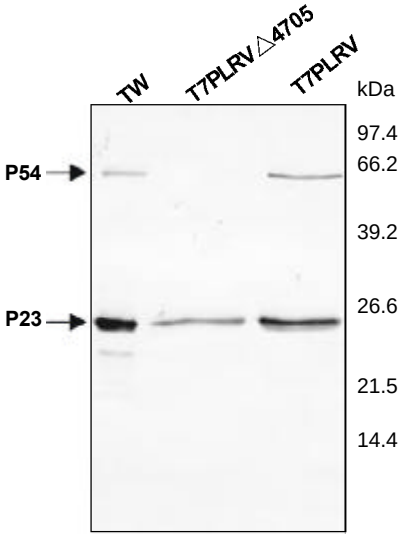

FIG 3 - Western blot analysis of *Potato leafroll virus* (PLRV) structural proteins CP (23 kDa) and read-through domain (RTD) (74 kDa) in virus purified from transcript-infected *Vigna unguiculata* protoplasts (T7PLRV and T7PLRV△4705) and in virus purified from frozen PLRV (WT) infected *Physalis floridana* leaves. Proteins were separated by SDS-PAGE, blotted onto nitrocellulose and probed with a specific antiserum raised against PLRV. The positions of the major capsid protein (P23) and the C-terminally truncated form of the RTD protein (P54) are indicated to the left. Position of molecular markers are shown to the right.

Affinity binding to *Buchnera* sp. GroEL

Western blot analysis of GroEL protein isolated from endosymbiotic bacteria of *M. persicae* with the antiserum against *Buchnera* sp. GroEL revealed a single band of ~65 kDa (data not shown). The concentration of purified GroEL was estimated to be 60 µg/ml. The results of the GroEL ligand assay showed that T7PLRV bound efficiently to *Buchnera* sp. GroEL depending on the virus concentration in the samples (Figure 4). On the other hand, the mutant T7PLRV△4705 that does not incorporate the RTD protein did not bind to GroEL.

Stability in microinjected aphids

Acquired PLRV particles are retained in an infective form in the hemolymph of *M. persicae* for the lifespan of the aphid (Eskandari *et al*., 1979). One of the characteristics that determine the persistent nature of PLRV in its vector body is its association with *Buchnera* sp. GroEL. This protein is involved in preventing proteolytic degradation of virions in the hemolymph. Microinjected aphids were used in this study to check the stability of wild type T7PLRV and RTD-deficient mutant in the aphid's hemolymph. Results of TAS-ELISA 24, 72 and 96 h after injection showed that mutant T7PLRV△4705 particles were less stable than the T7PLRV particles (Figure 5). Degradation was faster in the first 24 h post-injection, after which virus titer remained at similar levels until 96 h post-injection.

TABLE 1 - Symptom expression in *Physalis floridana* plants and relative amount of viral antigen given by DAS-ELISA three weeks after a 96-h inoculation access period with microinjected *Myzus persicae* nymphs with *Potato leafroll virus* (PLRV)

VIRUS SOURCE	SYMPTOM SEVERITY[1]	ELISA OD 405 nm[2]
Wild-type PLRV[3]	++++	0.957 ± 0.195
T7PLRV	+++	0.430 ± 0.023
T7PLRVΔ4705	-	0.008 ± 0.003
Positive control[4]	++++	0.840
Negative control[5]	-	0.006

[1]Scale varies from – (negative) to ++++ (maximum intensity) with three plants per test
[2] Mean of three plants ± SD
[3] Purified from leaf material
[4] Wild-type PLRV
[5] Mock inoculated

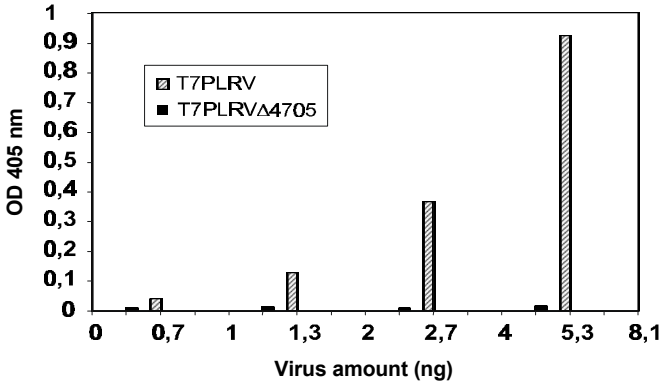

FIG. 4 - Affinity binding of T7PLRV and read-through domain (RTD) deficient mutant PLRV, T7PLRV△4705, to *Buchnera* sp. GroEL purified from *Myzus persicae*. Microtitre plates were coated with 100 µl of 6 µg/ml of purified GroEL in coating buffer and incubated overnight at 4 ºC. Each virus sample was tested with three different dilutions in sample buffer. Other steps were performed following GroEL ligand assay (see text). Absorbance measurements at 405 nm were performed after 15 min of addition of the substrate. Negative controls (sample buffer) showed absorbance values of less than 0.015.

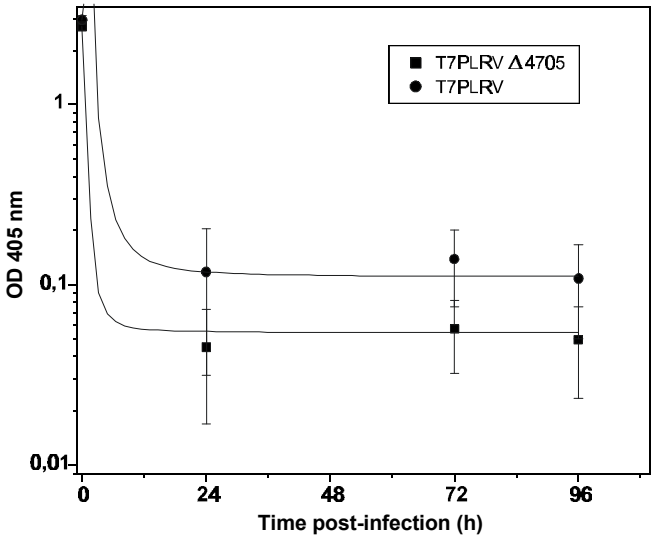

FIG. 5 - Stability of T7PLRV and T7PLRV△4705 read-through domain (RTD) deletion mutant in microinjected *Myzus persicae*. Aphids were analysed by ELISA (A_{405}) after the indicated times post-injection. Each point represents the mean (± standard error) of three repetitions from two microinjected aphids. Negative controls (non-viruliferous aphids) gave $A_{405} \approx 0.003$.

DISCUSSION

In the present study, we have demonstrated some of the roles of the RTD protein in the transmission process of PLRV by its main vector. Earlier reports have already postulated that the luteoviral RTD is important and indispensable for aphid transmission (Rottier *et al.*, 1979; Brault *et al.*, 1995; Bruyère *et al.*, 1997; Brault *et al.*, 2000). However, the molecular aspects of luteovirids-vector interactions and the involvement of RTD in the transmission process are essentially based on work with BYDV and BWYV. Most of the studies with PLRV are related to the interaction of the particles with *Buchnera* GroEL (van den Heuvel *et al.*, 1994; Hogenhout *et al.*, 1998, 2000). Perhaps primaly because of the difficulty in constructing infectious transcripts from cloned cDNA of PLRV until recently.

In an attempt to overcome this limitation, Gildow *et al.* (2000) worked with PLRV VLPs produced in a baculovirus system to elucidate the role of the RTD protein in PLRV transmission by *M. persicae*. However, these VLPs were not infectious to plants and the work focused on electron microscopy studies of RTD deficient VLPs acquisition and circulation through aphid tissues. These authors reported that the absence of the RTD did not greatly affect the stability of VLPs in the hemolymph or their ability to cross the accessory salivary gland basal lamina, considered a specific barrier for transmission by aphids (Pfeiffer *et al.*, 1997; Gildow, 1999). Furthermore, no structural evidence was encountered for GroEL binding to either PLRV or to VLP. In our experiments, the RTD deficient mutant was less stable in the hemocoel and was not transmitted by *M. persicae*. The decreased stability of the mutant in the hemolymph, compared to the wild-type-like virus T7PLRV, may be explained by its inability to bind to GroEL (as shown in Figure 4), leading to its degradation in this hostile environment. The differences observed between our results and those with PLRV VLPs can be explained by the possibility that the histidine tag fused at the N-terminus of the CP could be exposed on the surface of the VLPs and mimic the function of the RTD, interacting with *Buchnera* sp. GroEL. As suggested by Gildow *et al.* (2000), "the addition of this N-terminal sequence on the CP may have influenced particle recognition and survival in the aphid hemocoel".

The fact that the truncated RTD in the mutant used in our experiments could not be detected in transcript-infected protoplasts and was not present in purified T7PLRV△4705 virus particles suggests that this form of the protein cannot fold in the same structural conformation as the wild-type RTD and, therefore, cannot be incorporated into virions, as similarly described for BWYV RTD deficient mutants (Bruyère *et al.*, 1997). We assumed that the absence of this protein in the viral particle was responsible for the inability of the mutant to be transmitted. However, our results can not rule out the possibility that deletion in the conserved region of the RTD may be directly involved in virus particle recognition, interactions with *Buchnera* sp. GroEL, and consequently, in its persistence in the vector hemolymph. Amino acid replacement studies with BWYV RTD have already shown that the determinants in luteovirus vector interactions reside in the N-terminal region of this protein (Bruyère *et al.*, 1997; Brault *et al.*, 2000).

The role of the RTD in luteovirids-vector interactions seems to cover different steps of the transmission process.

Amino acid changes in the conserved region of the RTD of a PLRV isolate account for its poor transmissibility (Rouzé-Jouan *et al.*, 2001). In this case, the barrier for no or only poor transmission was the gut epithelium that regulates the passage of virus particles from the gut into the hemocoel in *M. persicae*. Moreover, when microinjected in the hemolymph of aphids, these particles crossed the ASG basal lamina and were transmitted by the vector. Similar observations were reported for a BWYV cDNA infectious clone, in which mutations at amino acids 267-268 in the conserved region of the RTD resulted in loss of ability to cross the gut membrane of *M. persicae* (Brault *et al*., 2000). However, very low rates of transmission were observed when the mutant was acquired from agro-infected plants, and efficient transmission was observed when these particles were microinjected in the hemocoel, indicating that these mutations in the RTD interfere with the initial step of the transmission circuit, probably the passage through the intestinal epithelial cell barrier. We have shown in recent work that the RTD of BWYV is not strictly required for recognition at the gut level and transport of virions into the aphid hemocoel, but its presence greatly enhances the efficiency of these processes (Reinbold *et al.*, 2001). On the other hand, the RTD was absolutely necessary for maintenance of BWYV particles in the hemolymph and/or their passage through the ASG (Reinbold *et al.*, 2001).

In spite of the great volume of information generated, contrasting results have been obtained in recent years on the specific roles of the luteovirid RTD. As a consequence, several questions remain unclarified. In summary, our results employing a RTD deficient mutant of PLRV establish this protein as indispensable for virus transmission, association with *Buchnera* sp. GroEL and stability of particles in the hemolymph, corroborating earlier findings on the role of this protein for BWYV and BYDV transmission by its vectors.

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