Tagging *Potato leafroll virus* with the jellyfish green fluorescent protein gene

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A full-length cDNA corresponding to the RNA genome of *Potato leafroll virus* (PLRV) was modified by inserting cDNA that encoded the jellyfish green fluorescent protein (GFP) into the P5 gene near its 3′ end. *Nicotiana benthamiana* protoplasts electroporated with plasmid DNA containing this cDNA behind the 35S RNA promoter of *Cauliflower mosaic virus* became infected with the recombinant virus (PLRV-GFP). Up to 5% of transfected protoplasts showed GFP-specific fluorescence. Progeny virus particles were morphologically indistinguishable from those of wild-type PLRV but, unlike PLRV particles, they bound to grids coated with antibodies to GFP. Aphids fed on extracts of these protoplasts transmitted PLRV-GFP to test plants, as shown by specific fluorescence in some vascular tissue and epidermal cells and subsequent systemic infection. In plants agroinfected with PLRV-GFP cDNA in pBIN19, some cells became fluorescent and systemic infections developed. However, after either type of inoculation, fluorescence was mostly restricted to single cells and the only PLRV genome detected in systemically infected tissues lacked some or all of the inserted GFP cDNA, apparently because of naturally occurring deletions. Thus, intact PLRV-GFP was unable to move from cell to cell. Nevertheless, PLRV-GFP has novel potential for exploring the initial stages of PLRV infection.

Introduction

*Potato leafroll virus* (PLRV; genus Polerovirus, family Luteoviridae), like all other luteoviruses, has a monopartite, single-stranded RNA genome. PLRV is transmitted by aphids in a circulative non-propagative manner and is mainly restricted to phloem tissues of infected plants (see for review Mayo & Ziegler-Graff, 1996). PLRV forms 25 to 30 nm diameter isometric particles that encapsidate genomic RNA of about 5.9 kb that contains six large open reading frames (ORF) (Fig. 1). A 5′-located gene cluster contains three ORFs that code for a polypeptide of 28 kDa with unknown functions (ORF0) and two proteins of 70 kDa (ORF1) and 108 kDa protein (ORF1/2) that appear to be replication-associated proteins (Mayo & Ziegler-Graff, 1996). Within the 3′-located gene cluster, ORF3 encodes the 23 kDa coat protein (CP), ORF4, which encodes a 17 kDa product (P4), is contained within the CP gene, but in a different reading frame. P4 is thought to be a movement protein (Tacke *et al*., 1993). PLRV ORF5 is separated from the upstream CP gene by a single amber termination codon. During infection, ORF5 is expressed by occasional translational readthrough of this codon, presumably by a plant-encoded suppressor tRNA, to give a ‘readthrough’ product of 79 kDa (P5) (Bahner *et al*., 1990). In addition to the major CP, PLRV particles contain small amounts of P5 (Bahner *et al*., 1990). Two other ORFs (ORF6 and ORF7) were detected recently near the 3′-end of the genome (Ashoub *et al*., 1998) but their functions are not understood.

The major CP component of PLRV particles is believed to carry the determinants that interact with specific cellular receptors in aphids and allow transmission to occur (Gildow, 1999). The minor P5 component is not absolutely required for particle assembly but luteovirus particles containing only the
Fig. 1. Schematic representation of cDNA constructs of wild-type and chimeric PLRV RNA flanked by the CaMV 35S RNA promoter (35S) and polyadenylation signal [poly(A) signal] that were inserted into pUC18 and pBIN19. The scale is marked in nucleotides. Boxes represent open reading frames 0, 1, 2, 3, 4 and 5; lines represent untranslated sequences. CP, coat protein; RT, readthrough portion of P5. cDNA encoding the GFP was inserted into the XhoI (nt 5487) restriction site as indicated to give PLRV-GFP; nucleotide positions for the GFP gene are indicated in parentheses. Arrows above the construct maps represent the major CP and wild-type PLRV P5 and chimeric PLRV-GFP P5–GFP fusion. Positions of the GFP-specific primers [primer set 1; nt (1) and nt (717) and PLRV ORF5-specific primers (nt 5205 and nt 5742; primer set 2) used in PCR assays are indicated.

CP were not transmissible by aphids to plants (Brault et al., 1995; Chay et al., 1996). This led to the assumption that the P5 component was essential for aphid transmission. Moreover, circumstantial evidence from sequence comparison studies among readily transmissible and poorly transmissible strains of PLRV suggested that at least some transmission determinants were in the P5 sequence (Jolly & Mayo, 1994). However, PLRV-like particles that lacked P5 were acquired by aphids and were found to cross the gut and accessory salivary gland barriers to accumulate in the salivary duct (Gildow et al., 1997; Gildow, 1999). These results suggest that P5 is involved in some other stages of virus transmission and/or initial stages of virus infection in plants. It has been suggested, for example, that P5 functions by interacting with symbionin, a protein produced in aphids by symbiont bacteria (Van den Heuvel et al., 1997).

Luteovirus particles or RNA cannot infect plants when mechanically inoculated, but this barrier can be overcome by Agrobacterium tumefaciens-mediated infection (agroinfection) (Leiser et al., 1992; Commandeur & Martin, 1993). Agroinfection experiments with mutants of Beet western yellow virus (BWYV) have established that deletion or truncation of portions of P5 does not result in diminished replication in protoplasts but impedes virus accumulation in systemically infected plants (Brault et al., 1995; Ziegler-Graff et al., 1996). Similar deletions in the genome of another luteovirus, Barley yellow dwarf virus-PAV (BYDV-PAV), have recently been shown to have the same effect (Chay et al., 1996). These observations, and immuno-localization studies (Mutterer et al., 1999), indicate that P5 may be involved in efficient systemic virus movement, but its role in this process is obscure.

To gain a better understanding of the mechanisms of luteovirus infection, we have attempted to use a strategy that has been exploited in a number of fields of biology, including plant virology. This is the use of the jellyfish green fluorescent protein (GFP) as a molecular reporter (Baulcombe et al., 1995; Oparka et al., 1995, 1996). The GFP gene has been incorporated into plant virus genomes to monitor virus infections and to study (sub)cellular locations of virus proteins fused to GFP. Here we describe the successful insertion of cDNA encoding GFP into PLRV cDNA and test the utility of the recombinant virus for studies of aphid transmission and systemic movement of PLRV. In particular, this new experimental system has allowed the first direct visualization of the sites of establishment of luteovirus infection, and thus by inference their sites of entry into a host plant.

**Methods**

- **Plasmids and generation of recombinant cDNA constructs.** Plasmid pBNUP110 contained a full-length cDNA copy of PLRV (Canadian isolate) between the 35S RNA promoter of Cauliflower mosaic virus (CaMV) and the corresponding transcription termination signal.
sequence (Commandeur & Martin, 1993; Franco-Lara et al., 1999). The SphI fragment of pBNUP110 containing the 35S promoter, a full-length cDNA copy of PLRV and the termination signal sequence was inserted into the SphI site of pUC18 to give pUC.PLRV (Fig. 1). Plasmid pTX.GFP (Baulcombe et al., 1995) was used for amplification of the GFP gene sequences. The fragment containing the GFP gene flanked by XhoI restriction sites was amplified using oligonucleotides (A) 5' GTCACTCGAGATGAGTAAAGGAGAAGAA 3' and (B) 5' GTCACTCGAGTTATTTGTATAGTTCATC 3' as the forward and reverse primers, respectively. The amplified fragment was cloned into the unique XhoI restriction site (nt 5487 in the PLRV RNA sequence) of pUC.PLRV to give pUC.PLRV-G (Fig. 1). The SpeI (nt 1070 in the PLRV RNA sequence)–BsaI (nt 5822 in the PLRV RNA sequence) fragment of pUC.PLRV-G was used to replace the SpeI–BsaI fragment of pBNUP110 to give pBIN.PLRV-G, which contains chimeric full-length PLRV sequence tagged with the GFP gene, between a 35S promoter and a polyadenylation signal. Standard DNA manipulation techniques (Sambrook et al., 1989) were used for the generation of the constructs.

Inoculation of protoplasts. Mesophyll protoplasts were prepared from leaves of N. benthamiana as described by Power & Chapman (1985). Approximately 10^6 protoplasts were electroporated with 10–20 µg of plasmid DNA as described by Gal-On et al. (1994). Samples were collected after 72 h of incubation for detection of GFP fluorescence, ISEM, immunocapture-RT–PCR, ELISA and immunoblot analysis.

Aphid transmission assay. Virus particles in extracts of 2 × 10^5 N. benthamiana protoplasts infected with PLRV-GFP were used as a virus source in aphid transmission tests. Non-viruliferous M. persicae nymphs were allowed to feed on these extracts, prepared as described by Bruyère et al. (1997), through Parafilm membranes for 24 h. The aphids were then transferred to healthy N. clevelandii plants for 24 h inoculation access periods and then killed by fumigation. The presence of fluorescent cells was assayed by confocal laser scanning microscopy starting from the fifth day after transmission.

Agrobacterium-mediated infection of plants. Agrobacterium tumefaciens (strain LBA 4404) carrying pBNUP110 or pBIN.PLRV-G was grown in YB broth with kanamycin (25 µg/ml) and rifampicin (100 µg/ml) for 48 h. The culture was centrifuged and resuspended in 1/10 to 1/20 vols of water and used for agroinfection. N. benthamiana and N. clevelandii plants at the three-to-four leaf stage were used for agroinfiltration (English et al., 1997) or agroinoculation (Leiser et al., 1992) as described above.

RNA and protein analysis. CP accumulation was detected in samples of 10^6 protoplasts or 0.2 g of leaf tissues disrupted in 1 ml of PBS.
(pH 7.2) by ELISA, essentially as described by Barker & Solomon (1980). ELISA A$_{405}$ values were obtained after 1 or 2 h incubation with substrate; values for both uninfected plants or protoplasts (negative control) were between 0.01 and 0.09. For immunoblot analysis, samples of protoplasts ($5 \times 10^4$) or plant tissues (30 mg) were disrupted in 50 mM Tris–HCl, pH 6.8 containing 10% (v/v) glycerol, 1% 2-mercaptoethanol and 2% SDS and kept at 95 °C for 5 min. Samples were then separated by electrophoresis in 7 or 12.5% SDS–polyacrylamide gels (Sambrook et al., 1989). Proteins were transferred to nitrocellulose membrane Protran BA85 (Schleicher & Schuell) using a Trans-Blot Cell (Bio-Rad), and blots were treated with MAb SCR3 (diluted 1:1000), prepared against PLRV, and then with goat anti-mouse antibody conjugated to alkaline phosphatase (Sigma). For immunocapture of virus particles, extracts from infected protoplasts were incubated on ELISA plates pre-coated with polyclonal antibodies against PLRV CP. Virus RNA was isolated using the RNeasy Plant Mini Kit (Qiagen). RT–PCR analysis was done by using random hexamers to prime first cDNA strand synthesis and two sets of primers for subsequent PCR amplification. PCR primers were (A) and (B) (described above), which are specific for sequences at each end of the GFP gene (set 1), or (C) 5’ GATCAAGCTTTAGTTTCTCCCTTGGAAATG 3’ and (D) 5’ GATCGGATCCGACCTAGAGTTTCCG 3’, which are specific for ORF5 sequences between nt 5205 and 5742 (set 2). The amplified fragments were sequenced using an ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer).

**Immunosorbent electron microscopy and immunogold labelling of virions.** Virus particles extracted from PLRV-infected or PLRV-GFP-infected protoplasts were concentrated ten times using Microcon centrifugal filter devices (Millipore). Immunosorbent electron microscopy (ISEM) was done essentially as described by Roberts (1986), using a polyclonal antiserum prepared to PLRV or GFP. Immunogold labelling was optimized and performed as described by Pereira et al. (1994). Virus particles were treated for 16 h at 4 °C with GFP antibodies conjugated (by I. M. Roberts, Scottish Crop Research Institute) to colloidal gold beads of 10 nm diameter (GC-10, British BioCell International). After immunogold labelling, the particles were trapped by grids coated with antibodies against PLRV, stained using 2% uranyl
acetate or 2% sodium phosphotungstate and photographed in a Philips CM 10 transmission electron microscope.

**Detection of green fluorescence.** GFP fluorescence in protoplasts or plant tissues was viewed with a Bio-Rad MRC 1000 confocal laser scanning microscope, using methods that were described previously (Baulcombe *et al.*, 1995; Opara *et al.*, 1995). The conditions used for confocal microscopy, excitation at 488 nm using a krypton–argon laser and 522 nm emission filter, allowed detection of GFP-mediated fluorescence with no significant autofluorescence.

**Results**

**Inserting the GFP gene into the PLRV genome**

A DNA fragment comprising the CaMV 35S RNA promoter, a cDNA corresponding to the full-length sequence of PLRV RNA (Canadian isolate) and CaMV polyadenylation signal (Fig. 1) was re-cloned from plasmid pBNUP110 (Commandeur & Martin, 1993; Franco-Lara *et al.*, 1999) into plasmid pUC18 to give pUC.PLRV. When the plasmid was electroporated into *N. benthamiana* protoplasts, ELISA and electron microscopy confirmed that pUC.PLRV had initiated infection of the protoplasts and had produced PLRV CP (a typical ELISA yielded $A_{405} = 1.75$; Fig. 2a). GFP cDNA was inserted in-frame with P5 at the unique *Xho* I restriction site located in PLRV ORF5 approximately 300 nt from its 3'-end (nt 5487 in the PLRV genome) (Fig. 1). The native amber termination codon of the inserted GFP gene was predicted to prevent translation of the carboxyl-terminal part of P5 (approximately 100 amino acid residues). Thus, the GFP was expected to be produced as a C-terminal part of the fusion with the truncated P5 (Fig. 1). The construct obtained (pUC.PLRV-G) was infectious when electroporated into *N. benthamiana* protoplasts (a typical ELISA yielded $A_{405} = 0.62$; Fig. 2b, c). Virus derived from such infections is subsequently referred to as PLRV-GFP.

**Replication of PLRV-GFP in protoplasts and analysis of the progeny**

In confocal laser scanning microscopy, up to 5% of *N. benthamiana* protoplasts electroporated with pUC.PLRV-G displayed a green fluorescence characteristic of GFP 72 h post-inoculation (Fig. 3a, b) indicating that the chimeric PLRV-GFP had replicated and that the GFP gene was expressed in infected protoplasts. The fluorescence was distributed in cytoplasm of the infected protoplasts diffusely and sometimes was present in small granules (Fig. 3a, b). The position of the GFP gene in the genome of PLRV-GFP (Fig. 1) suggests that the GFP was translated as a fusion with the amino-terminal part of P5 by the same translational readthrough mechanism as ‘normal’ P5. Immunoblot analysis of extracts from protoplasts infected with PLRV-GFP detected two protein products that reacted with MAbs against PLRV CP, one of which corresponded to the normal CP (Fig. 4, lane 3). The other protein presumably corresponded to the fusion of the P5 truncated at the *Xho* site, and GFP (P5–GFP) with a molecular mass of approximately 97 kDa (Fig. 1; Fig. 4, lane 3).

ISEM of extracts from the protoplasts infected with PLRV-GFP on grids coated with polyclonal antiserum against PLRV or GFP revealed the presence of isometric particles that resembled those of PLRV (Fig. 2a, c). In control experiments, particles that had accumulated in protoplasts electroporated with pUC.PLRV were detected only on the grids coated with antiserum against PLRV (Fig. 2a). To visualize the presumed association between GFP (GFP fusion with P5 protein) and virions, particles of PLRV were decorated with GFP-specific antibodies conjugated to colloidal gold beads of 10 nm diameter and then trapped on grids coated with PLRV-specific antibodies. About 40% of the virions from protoplasts infected with PLRV-GFP displayed immunogold labelling (Fig. 2d, e), whereas virions trapped from PLRV-infected protoplasts were...
the left.

**Fig. 5.** Immunocapture–RT–PCR analysis of viral RNA progeny accumulated in protoplasts infected with PLRV or PLRV-GFP. Mock-inoculated protoplasts were used as a negative control (indicated). Extracts from protoplasts prepared 72 h post-electroporation were incubated in ELISA plates pre-coated with polyclonal antibodies against PLRV CP. RNA samples isolated from immunocaptured virus particles were analysed by RT–PCR using random primers for reverse transcription, and two sets of primers for PCR amplification consisting of either GFP sequence-specific primers (GFP primers) or ORF5-specific primers (ORF5 primers) as indicated. The GFP primers encompass the full GFP gene sequence (nt 1–717), the ORF5 primers encompass nt 5205–5742 sequence of the PLRV ORF5 (see Fig. 1). Electrophoresis was in 1% agarose gel. Positions of DNA markers and their sizes in nucleotides are indicated on the left.

not labelled. This indicates that the GFP, presumably in the form of a fusion with the P5, had associated with virus particles and was accessible on the surface of at least a portion of the virions produced in PLRV-GFP-infected protoplasts. To confirm that the particles from PLRV-GFP-infected protoplasts contained RNA with an insert corresponding to the GFP gene, RNA was extracted from virions trapped by PLRV-specific antibodies and subjected to RT–PCR analysis. RT–PCR using primers which were specific for the GFP gene (primer set 1; Fig. 1) yielded the expected product that corresponded to that of the GFP gene (717 bp) from protoplasts infected with PLRV-GFP but no product was obtained with extracts of protoplasts infected with wild-type PLRV (Fig. 5). RT–PCR using primers specific for PLRV RNA sequences between nt 5205 and 5742 in ORF5 (i.e. either side of the XhoI site; primer set 2) (Fig. 1) amplified the expected 537 and 1260 bp products from extracts of PLRV-infected and PLRV-GFP-infected protoplasts, respectively (Fig. 5).

**Aphid transmission of PLRV-GFP from infected protoplasts**

Confocal laser scanning microscopy showed that aphids fed on extracts of protoplasts infected with PLRV-GFP transmitted infection to significant number (typically about 50%) of test plants. Similar proportions of test plants became infected following transmission from extracts made by aphids fed on PLRV-GFP or wild-type PLRV (Table 1). In plants inoculated by aphids carrying PLRV-GFP, up to five fluorescent foci were found in each of inoculated leaves of these plants. No fluorescence was detected in leaves of uninoculated plants. Most foci were single fluorescent cells; only occasionally were groups of two or three neighbouring fluorescent cells detected. Foci were detected in elongated cells of vascular tissues (approx. 75% of all fluorescent foci were in phloem-associated cells), although some (approx. 25%) foci were in epidermal, mesophyll or trichome cells (Fig. 3 c, d, e). The green fluorescence was present throughout the cytoplasm including transvacuolar strands and granules of various sizes and intensity; highly fluorescent bodies, some of which appear to be associated with nucleus (Fig. 3 c, e), were also observed. The fluorescence did not spread from cell to cell with time, which suggests that all foci were primary sites of inoculation. In spite of the absence of fluorescence outside primary infection sites, ELISAs done 3 weeks post-inoculation detected PLRV antigen in non-inoculated leaf tissues of infected plants (typical $A_{405} = 1.73$; see also Table 1).

The nature of the progeny viral RNA in systemically infected tissue was investigated by RT–PCR using primer set 2 [specific for the region of ORF5 (nt 5205–5742) encompassing the site of GFP gene insertion (nt 5487)]. Analysis of RT–PCR products prepared from RNA extracted from systemically infected tissue of three different plants inoculated by aphids carrying PLRV-GFP showed that the progeny RNA molecules contained deletions. Two of these plants ac-

### Table 1. Development of systemic infection in plants inoculated with PLRV-GFP

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<tr>
<th>Mode of inoculation/inoculum</th>
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<td>Aphid transmission*</td>
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<td>PLRV-GFP</td>
<td>3/6† 5/11</td>
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<td>PLRV</td>
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<td>Agroinfiltration†</td>
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<td>PLRV-GFP</td>
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<td>PLRV</td>
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* Aphids were fed through Parafilm membranes on media containing extracts from *N. benthamiana* protoplasts electroporated 3 days previously with pUC.PLRV-G or pUC.PLRV. Groups of five aphids were transferred to individual *N. clevelandii* plants.
† *N. benthamiana* or *N. clevelandii* plants were agroinfiltrated with *A. tumefaciens* carrying pBIN.PLRV-G or pBNUP110, expressing wild-type PLRV.
‡ Total no. of systemically infected plants/total no. of test plants (determined by ELISA).
Fig. 6. Analysis of naturally occurring mutants of PLRV-GFP accumulated in systemically infected leaves of *N. clevelandii* (Mut-1, Mut-2, Mut-3A, Mut-3B and Mut-4) and *N. benthamiana* (Mut-5 and Mut-6) plants. The plants were inoculated by aphids fed on extracts from protoplasts infected with PLRV-GFP (Mut-1, Mut-2, Mut-3A and Mut-3B) or infiltrated with *A. tumefaciens* carrying pBIN.PLRV-G (Mut-4, Mut-5 and Mut-6). Total RNA was isolated from systemically infected leaves of six separate plants 3 weeks post-inoculation and analysed by RT–PCR (a) using primer set two indicated by arrows in (b). One of the plants gave two major PCR products (Mut-3A and Mut-3B) while the rest gave single products. PLRV and PLRV-GFP RNA accumulated in infected protoplasts were used for RT–PCR as size standards (indicated). An uninfected *N. benthamiana* plant was used as negative control (indicated). Positions of DNA markers and their sizes in nucleotides are shown on the left. The PCR products were directly sequenced and the sequences of the corresponding mutants are presented schematically (b). Translated sequences in-frame with the P5 gene and GFP gene are indicated by empty and shadowed boxes, respectively. Straight lines represent untranslated RNA sequences. Deletions are indicated by broken lines. Black boxes correspond to new translated sequences that appeared as a result of frame shift due to deletions. Numerals above maps indicate positions in nucleotides in the PLRV genome; numerals in parentheses correspond to nucleotide positions in the GFP gene.

cumulated single mutated RNA species (Mut-1 and Mut-2) while the third plant contained at least two major mutants (Mut-3A and Mut-3B). The sizes of the deletions differed, and some (Mut-2, Mut-3A and Mut-3B) had resulted in the loss of more than the GFP gene (Fig. 6a), indicating that some ORF5 sequence was deleted. Sequencing of the RT–PCR products confirmed this and showed that in all four mutated RNA species all or most of the GFP gene, and sometimes some ORF5 sequences, were deleted (Fig. 6b). The longest deletion detected (nt 5388–5686; Mut-2; Fig. 6b), which was expected to truncate the readthrough protein at nt 5388, did not prevent the virus from accumulating and spreading.
**Agroinfection experiments**

*N. clevelandii* and *N. benthamiana* plants were agroinfected with PLRV-GFP in two ways. For agroinfiltration, two or three small holes were made on the underside of the leaf using a razor blade, and a syringe (without needle) was used to inject the *Agrobacterium* culture into the leaf (English et al., 1997). For agroinoculation, an *Agrobacterium* culture was injected using a Hamilton syringe with a needle into about ten sites in the stems, leaf midribs and petioles (Leiser et al., 1992). Agroinoculation initiated infection in a greater proportion of plants than agroinfiltration, but the sites of inoculation were clearer in agroinfected leaves. Therefore the latter were used in the experiments reported here.

Agroinfiltration of both *N. clevelandii* and *N. benthamiana* plants with pBIN.PLRV-G resulted in the development of fluorescent foci in the infiltrated areas adjacent to the sites of infiltration. The foci were clearly visible by confocal laser scanning microscopy on or after the fifth day post-inoculation (Fig. 3f, g, h). The fluorescent foci comprised different types of single cells or occasionally small groups of cells surrounding the infiltration sites. Fluorescence was detected in epidermal, mesophyll (approximately 80% of the cells were in epidermis and mesophyll) and phloem-associated cells (10% of the cells) (Fig. 3f, g, h). However, in agroinoculated plants, most of the fluorescent cells (90%) were in phloem tissues, and the remainder were in mesophyll and epidermis. The intracellular distribution of the fluorescence was similar to that observed in plants infected by aphids. As in the aphid transmission experiments, the initially formed fluorescent foci did not increase in size. Moreover, fluorescent cells were only seen in the infiltrated areas, indicating that agroinfiltrated PLRV-GFP, as well as that transmitted by aphids, was unable to spread systemically. ISEM of the extracts obtained from leaf tissues around infiltration sites 1 week post-inoculation detected virus particles that were indistinguishable in appearance from those that accumulated in protoplasts infected with PLRV-GFP. The particles were trapped with antibodies against both PLRV or GFP, indicating that these particles contained GFP epitope(s) on their surfaces (data not shown). Immunoblot analysis of tissues in zones of primary infection detected two protein products that reacted with MAb against PLRV CP. These corresponded to the major CP and readthrough protein with a molecular mass of approximately 97 kDa as predicted for the GFP gene fused with the readthrough portion of the P5 gene at the Xhol site (Fig. 1 and Fig. 4, lane 4).

Thus, these data confirm the suggestion from the results of aphid transmission experiments (see above) that the GFP-tagged PLRV is able to replicate, to form virus particles and to express the GFP gene in primarily infected cells, but that it cannot spread systemically. However, as in the aphid transmission experiments, PLRV antigen, but not GFP fluorescence, was detected by ELISA in non-infiltrated leaves of some *N. benthamiana* (typical *A*<sub>405</sub> = 0·49) and *N. clevelandii* (typical *A*<sub>405</sub> = 0·69) plants 3 weeks after agroinfiltration with PLRV-GFP (Table 1). RT–PCR analysis and sequencing of the progeny viral RNA from systemically infected leaves detected only virus mutants of PLRV-GFP (Mut-4, Mut-5 and Mut-6) that had lost most of the GFP gene sequence (Fig. 6).

**Discussion**

In contrast to viruses with rigid rod-shaped or filamentous particles that have no theoretical restriction on virion size because protein subunits pack in a helical array, viruses with icosahedral symmetry would appear to have a structural limitation on the size of viral RNA that can be encapsidated into virus particles that have a fixed number of protein subunits. However, in this work we show that the insertion of the 717 nt that constitute the GFP gene into the genome of PLRV did not prevent the modified genomic RNA from being encapsidated to form isometric virions that were essentially similar to wild-type PLRV particles (Fig. 2). This allowed us to use the GFP gene fused in-frame with P5 as a molecular reporter to study aphid transmission and systemic spread of PLRV.

The chimeric PLRV-GFP was able not only to replicate and express the GFP, but also to form virus particles in protoplasts, or primarily infected cells, that could be transmitted by aphids. Immunoblot analysis of PLRV-GFP-infected protoplasts using antibodies against the PLRV CP detected two protein products that corresponded to the major CP and the P5–GFP fusion of approximately 97 kDa (Fig. 4, lane 3). Although our attempts to confirm these results using available antibodies against the GFP or P5 were unsuccessful, probably because of low antibody titres and/or the small amounts of the proteins that accumulated, these results indicate that the fusion protein (or at least, most of it) was not cleaved *in vivo*. Taken together with the ISEM and immunogold labelling data, these observations suggest that the P5 molecules are contained in the capsids of virus particles only (or mostly) in the form of a fusion with GFP. These observations add to earlier findings with other luteoviruses (Wang et al., 1995; Bruyère et al., 1997) that a carboxyl-terminal part of P5, which was not expressed in PLRV-GFP, is not essential for the aphid transmission. Moreover, they indicate that the fusion of P5 with GFP does not affect the ability of the aphids to transmit the virus particles, suggesting that GFP does not functionally mask putative P5 domain(s) involved in aphid transmission.

The findings that PLRV-GFP is transmitted by the aphid *M. persicae*, and that it replicates in primarily inoculated cells and expresses the GFP but cannot spread, show that PLRV-GFP could be used to visualize the cells initially infected during aphid transmission. This approach allowed us to detect the primarily inoculated cells in the vascular tissue (Fig. 2c) and occasionally elsewhere, for example in trichomes (Fig. 2d) or epidermal cells (Fig. 2e). These results provide direct evidence that different types of leaf cells can be inoculated by aphids.
However, we cannot rule out completely the possibility that these cells were infected by virus contaminating the aphid stylets rather than by circulating virus particles. Confocal laser scanning microscopy did not allow precise identification of the types of elongated phloem-associated cells (such as bundle sheath, vascular parenchyma, companion cells or sieve elements) that were infected. Nevertheless, these data represent first direct visualization of putative primary infection sites and demonstrate the possibility of studying factors involved in modulating the susceptibility of hosts or the efficacy of aphid vectors.

Aphid transmission and agroinfection experiments showed that, regardless of the mode of virus inoculation, the GFP-tagged PLRV described in this work was unable to spread from initially infected sites. Due to the phloem-limited character of PLRV infection, it was not surprising that PLRV-GFP did not spread from initial infection sites in mesophyll and epidermis. Previously, it was shown that another luteovirus, Tobacco necrotic dwarf virus, was able to replicate in single epidermal cells inoculated mechanically but could not move out of them (Imaiizumi & Kubo, 1980). The restriction of fluorescence to primary infection sites in phloem tissues apparently reflects the inability of the GFP-tagged PLRV to move even in those tissues that are sites of wild-type PLRV infection. It might be suggested that PLRV-GFP was delivered into ‘incompetent’ phloem-associated cells unable to initiate a systemic infection. However, this is unlikely because the naturally occurring deletion mutants of PLRV-GFP spread systemically from the same primarily infected cells (Fig. 6, Table 1). The failure of PLRV-GFP to spread systemically suggests that the movement functions themselves may be diminished. P5 has been shown to play a role in the development of systemic infection (Brault et al., 1995; Ziegler-Graff et al., 1996; Chay et al., 1996), facilitating intercellular luteovirus movement in vascular tissues (Mutterer et al., 1999). Our results confirm this conclusion and suggest that some putative ‘transport’ domain(s) in the P5 may be functionally disrupted by the fused GFP. If so, it can be assumed that different functional domains are involved in aphid transmission and systemic spread of PLRV. Another possible explanation is that P5 mediates virus movement indirectly by enhancing the stability of virions (Ziegler-Graff et al., 1996) and that the P5–GFP fusion protein does not possess this stabilizing activity. It is also possible that the increased size or changed secondary structure of the chimeric PLRV-GFP RNA itself inhibits systemic movement of the virus, for example by decreasing the stability of virus particles.

Although replication of the GFP-tagged PLRV was strictly limited to primary infection sites, naturally occurring deletion mutants of PLRV-GFP were detected outside these sites. No deletion mutants were detected in leaf zones (up to 3 cm in diameter) into which the infiltrated agrobacteria had spread, 6 days post-inoculation (Fig. 4, lane 4), presumably because such mutations were rare and/or mutant sequences present in primary infected cells were in very low concentration. However, once such mutants formed during replication in primarily infected cells, they would probably acquire the ability to move systemically and then would accumulate throughout the plants. Thus, restoration of an ability to move would have an overwhelming selective advantage and any such mutants would rapidly out-compete the non-moving PLRV-GFP. All sequenced mutants lacked a significant part of or all of the GFP gene, and some also lacked part of ORF5 indicating that the deleted sequences (Fig. 6) are not essential for the systemic movement of PLRV. With other systems, it has also been found that some foreign sequences tend to be eliminated from virus genomes by deletion (Chapman et al., 1992; Culver et al., 1996). Interestingly, infections with recombinant Tobacco etch virus (TEV) expressing foreign genes as fusions with nucleotide sequences encoding helper component-proteinase (HC-Pro) resulted in appearance of spontaneous deletion mutants (Dolja et al., 1993, 1997) which were similar to the PLRV-GFP mutants described in this work. Some of the TEV mutants lacked not only foreign nucleotide sequences but also parts of HC-Pro gene.

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References


Determinants of barley yellow dwarf luteovirus-PAV are contained in the coat protein readthrough domain and 17-kDa protein, respectively.


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