Quantitative parameters determining whitefly (Bemisia tabaci) transmission of Lettuce infectious yellows virus and an engineered defective RNA

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In this study, quantitative parameters affecting in vitro acquisition and whitefly (Bemisia tabaci) transmission of Lettuce infectious yellows virus (LIYV) were examined and B. tabaci transmission of an engineered defective RNA (D-RNA) was demonstrated. Virions purified from virus- and virion RNA-inoculated Chenopodium murale plants and protoplasts of Nicotiana tabacum, respectively, were consistently transmitted to plants by B. tabaci when virion concentrations were 0-1 ng µl⁻¹ or greater. Transmission efficiency increased with increasing virion concentration and number of whiteflies used for inoculation. When in vitro-derived transcripts of the M5gfp D-RNA (engineered to express the green fluorescent protein, GFP) were co-inoculated to protoplasts with wild-type LIYV virion RNAs, the resulting virions were transmissible to plants. LIYV and the M5gfp D-RNA systemically invaded inoculated plants; however, GFP expression was not detected in these plants. Unlike LIYV, the M5gfp D-RNA was not subsequently transmitted by B. tabaci from the initially infected plants, but, when high concentrations of virions from plants infected by LIYV and the M5gfp D-RNA were used for in vitro acquisition by whiteflies, both were transmitted to plants. Quantitative and qualitative analyses showed that, although the M5gfp D-RNA replicated within and systemically invaded plants along with LIYV, compared with LIYV RNA 2 it was not as abundant in plants or in the resulting virions, and concentration of encapsidated RNAs is an important factor affecting transmission efficiency.

INTRODUCTION

Lettuce infectious yellows virus (LIYV) is the type species of the genus Crinivirus (family Closteroviridae) (Martelli et al., 2002). The LIYV genome consists of two single-stranded positive-sense RNAs. RNA 1 (8117 nt) contains genes encoding proteins associated with the replication of the virus genomic RNAs (Karasev, 2000). RNA 2 (7193 nt) has seven open reading frames and contains the five-gene ‘hallmark closterovirus gene array’ that encodes the proteins p5 (a small hydrophobic protein), HSP70h (a heat-shock protein 70 homologue), p59 (a protein of unknown function), CP (the major coat protein) and CPm (the minor coat protein). This gene module is shared by all viruses of the family Closteroviridae (Martelli et al., 2002). The LIYV genomic RNAs are individually encapsidated in flexuous filamentous particles formed by the assembly of the CP along most of the length of the virion, similar to that of other helical viruses. However, in addition to the CP, another protein, the CPm, is located at the tip of one end of the virion (Tian et al., 1999). This morphological arrangement gives the virus a ‘rattlesnake’ structure unique to members of Closteroviridae (Agranovsky et al., 1995; Feibes et al., 1996; Tian et al., 1999; Zinovkin et al., 1999). In addition to the CP and CPm, LIYV virions contain at least two additional LIYV-encoded proteins: HSP70h and p59 (Tian et al., 1999). Other viruses in this family that have so far been studied [Beet yellows virus (BYV) and Citrus tristeza virus (CTV)] also have virion-associated HSP70h and p59-like proteins (Napuli et al., 2003; Satyanarayana et al., 2004), and recently BYV virions have been shown also to contain one other virus-encoded protein, p20 (Dolja, 2003). The genomes and virion composition of viruses in the family Closteroviridae are more complex than those found in the majority of positive-sense ssRNA plant viruses.

Specific insect vectors transmit LIYV and several other members of the Closteroviridae from plant to plant in a non-circulative, semi-persistent manner (Karasev, 2000). Various whiteflies are vectors for LIYV and other viruses of the genus Crinivirus, while aphids are the most common vectors for BYV, CTV and viruses of the genus Closterovirus and mealy bugs appear to be vectors of at least some of the viruses in the genus Ampelovirus (Martelli et al., 2002). As yet, the virus-encoded genetic determinants that facilitate vector-mediated transmission are not known for any virus in the Closteroviridae, thus the molecular mechanism(s) of vector transmission remains elusive. Also not known is whether vector transmission results in dissemination of defective RNAs (D-RNAs). D-RNAs are commonly associated with...
infections for some viruses of the family Clusteroviridae, and have been well characterized, at least for CTV and LIYV (Ayllon et al., 1999; Mawassi et al., 1995b; Rubio et al., 2000). One reliable approach that has greatly facilitated the study of insect vector-mediated transmission of many other plant viruses is by allowing insect vectors to acquire virus via feeding or probing in vitro through artificial membranes containing virus-infected plant sap, or purified or partially purified virions and, if needed, accessory vector transmission proteins (helper components). This approach has greatly facilitated identification of both qualitative (mutants) and quantitative determinants of virus transmission for viruses of the Potyviridae (Atreya et al., 1992; Atreya & Pirone, 1993; Peng et al., 1998; Wang et al., 1996), Caulimoviridae (Blanc et al., 1993; Leh et al., 1999) and Luteoviridae (Gildow, 1999). Recently, we successfully developed an in vitro acquisition system for LIYV, the only such system so far reported for a member of the Clusteroviridae. Virions alone are sufficient for acquisition and transmission, demonstrating that accessory proteins are not required. We also used antisera to neutralize Bemisia tabaci transmission of purified LIYV virions prepared from infected Chenopodium murale plants and demonstrated that the virion protein CPM is a potential determinant of whitefly transmission (Tian et al., 1999).

Here we utilized this system to extend our knowledge of quantitative parameters affecting B. tabaci transmission of LIYV by identifying minimum concentrations of virions required for efficient transmission. In addition, we used an engineered D-RNA to demonstrate that LIYV D-RNAs can be transmitted by B. tabaci to plants and that transmission efficiency is correlated with concentration of encapsidated RNAs in the acquisition source.

**METHODS**

**Virus maintenance and virion purification from plants.** LIYV was maintained in C. murale and Lactuca sativa (lettuce) plants by transmission using the whitefly B. tabaci. LIYV virions were purified from C. murale plants essentially according to the methods of Klaassen et al. (1994) and Tian et al. (1999). In some cases virions were purified from 1–10 g LIYV-infected Nicotiana benthamiana and Capsella bursa-pastoris plants by the same procedure, except that only one round of ultracentrifugation was performed.

**Protoplast manipulation, LIYV inocula and virion purification from protoplasts.** Protoplasts were derived from cultured suspension cells of Nicotiana tabacum var. Xanthi maintained in Murashige and Skoog organics medium (Gibco-BRL) supplemented with 0.9 μM 2,4-dichlorophenoxyacetic acid, 0.4 μM kinetin, 1.5 μM thiamin hydrochloride (Passmore et al., 1993), 4.1 μM nicotinic acid and 2.4 μM pyridoxine hydrochloride. The extraction of virion RNAs and synthesis of capped transcripts of pM5gfp were done as described previously (Klaassen et al., 1994, 1996; Yeh et al., 2001). Protoplasts were inoculated with virion RNAs and capped pM5gfp transcripts as described by Lindbo et al. (1993) and Yeh et al. (2001). LIYV virions were purified from N. tabacum var. Xanthi protoplasts using a scaled-down version of the methods of Klaassen et al. (1994). Protoplasts (4 × 10⁶) were harvested at 72 h post-inoculation (p.i.). Protoplasts were subjected to a brief (10 min) centrifugation (2300 g) in a Beckman SA600 rotor. Pellets were resuspended in 10 ml extraction buffer (0.1 M Tris/HCl, pH 7.4, 0.5% w/v sodium sulfate, 0.5% w/v 2-mercaptoethanol), stirred on ice for 2 h, with Triton X-100 added to a final concentration of 2% (v/v), and subjected to centrifugation as previously described (Klaassen et al., 1994). The resulting supernatant was subjected to ultracentrifugation as previously described (Klaassen et al., 1994) and the final pellets were each resuspended overnight in 10–20 μl of either 1 × TE (0.01 M Tris/HCl, 1 mM EDTA, pH 7.4) or artificial diet solution (1 × TE, 15% w/v sucrose, 1% BSA). Virions were disrupted by boiling and proteins were separated in a 12% SDS-polyacrylamide gel (Laemmli, 1970). Virion concentrations were estimated by densitometry (Scion Corporation) of stained gels or proteins detected by immunoblot analysis (Tian et al., 1999) were compared with known amounts of co-electrophoresed purified virion proteins.

**In vitro LIYV acquisition and whitefly transmission.** Non-viruliferous whiteflies (B. tabaci), reared on lima beans (Phaseolus limensis) (Perring et al., 1993; Tian et al., 1999), were collected and starved for 15–18 h in a room maintained at 22 °C prior to in vitro virion acquisition. To assess the effects of virion concentration on transmission efficiency, virion samples were serially diluted and 5 μl in artificial diet solution was used as the acquisition source. Following an acquisition access period (AAP) of 6–7 h, whiteflies were transferred onto target plants as described previously (Tian et al., 1999). Percentage transmission was determined by the number of infected target plants over the total number of plants tested, and the results were analysed using non-linear regression analysis. To assess the effects of whitefly numbers on transmission efficiency, whiteflies were briefly anaesthetized using CO₂ following in vitro acquisition and 10, 50 or 100 whiteflies were transferred onto the leaves of each target plant. Parallel plant-to-plant transmissions were performed using 1 and 10 CO₂-anesthetized whiteflies after an overnight AAP on LIYV-infected lettuce plants. Virions purified from infected protoplasts were directly resuspended in artificial diet solution and used for in vitro acquisition. Comparisons of virus transmission efficiency were made using estimates of the probability of transmission by a single whitefly (Gibbs & Gower, 1960; Ng & Perry, 1999).

**RT-PCR, nucleotide sequence and Northern blot hybridization analyses.** Virion RNAs were extracted essentially according to Klaassen et al. (1994). Total RNA extraction was performed using TRI Reagent (MRO) according to the manufacturer’s recommendations. AMV reverse transcriptase (Promega) and the oligonucleotide P26-r2 (5′-ACTATCATGTTATTCGCAACT-3′; Fig. 1a), complementary to nucleotides 6516–6537 of the LIYV RNA 2 p26 coding region, were used for synthesis of the first-strand cDNA to LIYV RNA 2 or the M5gfp D-RNA (Fig. 1a). Second-strand cDNA synthesis was primed with the oligonucleotide P26-F (5′-GACCACAGCCTTTGGACAGGT-3′), corresponding to nucleotides 6235–6255, 64 nt upstream of the LIYV RNA 2 p26 start codon, and PCR-amplified to yield a 303 nt fragment. To detect the M5gfp D-RNA, second-strand cDNA synthesis was primed with the oligonucleotide GFP-F (5′-GATCATATGAAAGCGGACG-3′), corresponding to nucleotides 289–309 of the green fluorescent protein (GFP)-coding sequence (Yeh et al., 2001), and PCR-amplified to yield a 675 nt fragment. PCR-amplified products were separated on an agarose gel and visualized by ethidium bromide staining.

To ensure that the M5gfp D-RNA in plants was the same as that used for inoculation, the GFP-coding region was specifically amplified by RT-PCR and the products cloned and sequenced. The reverse primer GFP-P26-r2 (5′-GTTCCTGGTGATCTATCTCGAC-3′; Fig. 1a) was used for synthesis of the first-strand cDNA to M5gfp D-RNA in the total RNA extracted from infected plants. The first 17 nt of GFP-P26-r2 were complementary to nucleotides 6375–6391 of the p26 coding
region; this was followed by six nucleotides that were complementary to nucleotides 1787–1792 of M5gfp D-RNA, a region just downstream of the stop codon (Fig. 1a). The forward primer P26-F2 (5′-AGAATTGACGGAGGCTGAGAC-3′; Fig. 1a), was complementary to nucleotides 6217–6237, 82 nt upstream of the p26 start codon. PCR using GFP-P26-r2 and P26-F2 yielded an 889 nt fragment that was blunt-end cloned into the pCR-Blunt II-TOPO vector (Invitrogen).

Three to six cDNA clones of M5gfp D-RNA from each infected host-plant species were sequenced in both directions.

For Northern hybridization analysis, RNAs were denatured with glyoxal, separated by electrophoresis in a 1 % agarose gel and transferred to Hybond-NX membrane (Amersham) as described previously (Yeh et al., 2000). Probes for Northern blot analysis were DIG-labelled transcripts from (i) pSKL16, a plasmid containing the cDNA corresponding to nucleotides 6685–7193 of LIYV RNA 2 (Yeh et al., 2001) (Fig. 1a), and (ii) AT1gfp, a plasmid containing the cDNA corresponding to nucleotides 62–792 of the GFP-coding region (Fig. 1a). Hybridization and detection procedures were as described previously (Yeh et al., 2000).

**RESULTS**

**Quantitative parameters that determine LIYV transmission following in vitro acquisition**

Although we previously showed that purified LIYV virions could be acquired in vitro and transmitted to plants by B. tabaci (Tian et al., 1999), in those experiments we used only high concentrations of virions (approx. 25–50 ng ml⁻¹) purified from infected plants. To assess the effects of virion concentration on transmission efficiency, we first quantified the virion yield from LIYV-infected plants and then prepared serial dilutions for use in transmission experiments. We consistently obtained a few micrograms of LIYV virions per g LIYV-infected C. murale plants. SDS-PAGE analysis showed the four LIYV-encoded virion-associated proteins (Fig. 1b).

Fluorescence microscopy was used to visualize N. tabacum var. Xanthi cells for GFP expression at 72 h p.i. (Yeh et al., 2001). To detect GFP in whole plants, fresh tissue sections (75 μm) were prepared on a model TC-2 Sorvall microtome (DuPont) and analysed using a Leica TCS-SP laser scanning confocal microscope (Leica Microsystems). Fluorescence associated with GFP was detected using a krypton/argon laser with an excitation wavelength of 488 nm and an emission filter (500–510 nm).
proteins, even by Coomassie blue staining, allowing us easily to quantify virions by densitometry of the major capsid protein (Fig. 1b). Virion concentrations ranging from 120 to 0.012 ng ml⁻¹ were presented to whiteflies for in vitro acquisition and transmission to plants, using approximately 100 whiteflies per target plant. Transmission efficiency was greater than 50% in eight of nine instances when the virion concentration in the acquisition source was ≥1 ng ml⁻¹ (Fig. 2). Transmission was still observed in three of four instances when virion concentrations were within the 0.1 and 1 ng ml⁻¹ range, although at reduced efficiency (Fig. 2). Using a defined number of whiteflies (10, 50 and 100) and virion concentrations ranging from 1000 to 0.1 ng ml⁻¹ in the acquisition source, the probability (P*; Gibbs & Gower, 1960) for a single B. tabaci to transmit LIYV was estimated to be 1–4% (Table 1).

**Fig. 2.** Quantitative determinants of the efficiency of LIYV transmission by B. tabaci (y-axis) using serially diluted preparations of LIYV virions. Approximately 100 whiteflies were used per plant. In most instances at least 10 plants were used per concentration (x-axis) per experiment. Data were collected from four experiments and analysed using non-linear regression.

**Transmissibility of virions from LIYV-infected protoplasts**

The above data allowed us to identify minimum concentrations of LIYV virions required for efficient in vitro acquisition and transmission of LIYV to plants by B. tabaci. This is important as LIYV infections are phloem-limited within plants, and LIYV is not mechanically transmissible (Duffus et al., 1986; Hoefert et al., 1988; Medina et al., 2003). Furthermore, although we have generated infectious cloned cDNAs for the LIYV genomic and several D-RNAs, we have so far been able to evaluate them only in protoplasts (Klaassen et al., 1996; Yeh et al., 2000). Thus, an efficient means to transfer LIYV from protoplasts to plants would greatly facilitate quantitative and qualitative analysis of specific LIYV and D-RNA constructs with regard to their transmissibility by B. tabaci and their ability to establish whole-plant infections. Therefore we used LIYV virion RNAs to inoculate protoplasts to determine whether sufficient quantities of virions could be obtained from protoplasts for use in in vitro acquisition and, if so, whether virions could be acquired and transmitted to plants. Yields of virions from protoplasts were low relative to yields from plants. Typically we obtained between approximately 2 and 40 ng LIYV virions per 10⁶ protoplasts, requiring immunoblot analysis to quantify virions (Fig. 1c). When virions were diluted in the artificial diet solution, we were able to evaluate their transmissibility using concentrations ranging between 0.3 and 5.2 ng ml⁻¹. These protoplast-purified virions, in five separate experiments, gave an overall transmission score of 17 infected of 24 target plants (71%; Table 2). These data also confirmed our quantitative threshold estimates using virions purified from plants: as long as LIYV virion concentrations in the acquisition source were above a threshold concentration of approx. 0.1 ng ml⁻¹, this was sufficient for in vitro acquisition and plant inoculation whether virions were obtained from whole plants or single cells (protoplasts).

**Table 1.** Quantitative determinants of LIYV transmission by B. tabaci: probability of transmission by a single B. tabaci

Tenfold serial dilutions of purified LIYV virions were used for in vitro acquisition and inoculations to plants with the number of whiteflies indicated. Transmission was scored as number of infected target plants over total number of plants tested. Experiments were repeated three times using three or four target plants per experiment; data are shown as a cumulative total. NT, Not tested; P*, estimated probability of transmission by a single whitefly (Gibbs & Gower, 1960).

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<thead>
<tr>
<th>Virion concentration (ng ml⁻¹)</th>
<th>Whitefly transmission (whiteflies per plant)</th>
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<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>1000</td>
<td>3/9 (P*=0.04)</td>
</tr>
<tr>
<td>100</td>
<td>2/10 (P*=0.022)</td>
</tr>
<tr>
<td>1</td>
<td>1/10 (P*=0.01)</td>
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<tr>
<td>0.1</td>
<td>NT</td>
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†Because the use of a P* value of 1 (9/9) would be a biased overestimate, a more conservative underestimate is given, assuming eight of nine plants infected.
**Transmissibility of a GFP gene-marked LIYV-defective RNA**

The above data suggested that we could probably use *B. tabaci* and *in vitro* acquisition to transfer specific engineered LIYV constructs from protoplasts into whole plants. We previously constructed a genetically marked LIYV RNA 2 D-RNA (M5gfp D-RNA) engineered to contain and express the GFP gene (Yeh et al., 2001). Replication of M5gfp D-RNA in protoplasts was dependent on its helper virus, LIYV, and resulted in GFP expression in protoplasts. Although D-RNAs are common among LIYV and some other members of the *Closteroviridae*, it is not known whether they are vector-transmitted and therefore common in nature with their helper viruses or whether they arise continuously *de novo* (Albiach-Martí et al., 2000; Mawassi et al., 1995a, b; Rubio et al., 2002). Therefore we reasoned that using *in vitro* acquisition of virions purified from protoplasts co-infected by LIYV and the M5gfp D-RNA was an opportunity to assess whether a specific D-RNA could be vector (whitely)-transmitted to and maintained in plants along with the helper virus LIYV.

We co-inoculated *in vitro* transcripts of the M5gfp D-RNA and LIYV virion RNAs to *N. tabacum* protoplasts. At 60–70 h p.i., GFP fluorescence was typically observed in 8–12% of inoculated cells, indicating LIYV and M5gfp D-RNA replication. Virions were then purified and used for *in vitro* acquisition and transmission by *B. tabaci* to plants of three different species (Table 3). At 2 weeks p.i., total RNAs were extracted from the upper, non-inoculated leaves of target plants, and analysis for LIYV genomic RNA 2 and the M5gfp D-RNA was performed by RT-PCR. By using primers specific for LIYV RNA 2 and specific for the GFP-coding sequence (Fig. 1a), we identified LIYV RNA 2 and the M5gfp D-RNA in plants (data not shown). LIYV and the M5gfp D-RNA were transmitted to plants of all three species tested: *L. sativa*, *C. bursa-pastoris* and *N. benthamiana* (Table 3, preparations 2–7). However, not all LIYV-infected plants also contained the M5gfp D-RNA. Of seven *L. sativa* plants analysed, all were infected by LIYV, while only 16 of 20 *C. bursa-pastoris* plants and 11 of 14 *N. benthamiana* plants were LIYV-positive (Table 3, virion preparations 2–7). The M5gfp D-RNA was detected in only three of seven, 12 of 16 and four of 11 of the LIYV-infected *L. sativa*, *C. bursa-pastoris* and *N. benthamiana* plants, respectively (Table 3, virion preparations 2–7). These data showed that an engineered D-RNA could be vector-transmitted, but its transmission was not as efficient as that of the helper virus.

In previous experiments using protoplasts, the M5gfp RNA always replicated to relatively high levels compared with LIYV RNA 2 (Yeh et al., 2001). As the RT-PCR analyses were not quantitative, Northern blot hybridization analysis was used to confirm the presence of LIYV genomic RNA 2 and M5gfp D-RNAs in the same plants and to assess the relative titres of each (plants are those shown in Table 3, preparations 2, 4 and 6). When total RNAs from *C. bursa-pastoris* and *N. benthamiana* plants were probed using a DIG-labelled minus-sense RNA probe corresponding to the LIYV RNA 2 p26 region, both the LIYV genomic RNA 2 and the M5gfp D-RNA were detected [Fig. 3, lanes 2–4 and 7; the M5gfp D-RNA contains the p26 region (Yeh et al., 2001); and see Fig. 1a]. By comparing the hybridization signals for the LIYV genomic RNA 2 and the M5gfp D-RNA in the same RNA samples, it is clear that the M5gfp D-RNA accumulated to levels almost comparable to LIYV RNA 2 in some, but not all of the infected plants (Fig. 4, lanes 2–4 and 7), while LIYV RNA 2 was relatively abundant in all plants. These RNAs were also analysed by using a DIG-labelled minus-sense RNA probe corresponding to the GFP-coding region and, as expected, the M5gfp D-RNA, but not LIYV RNA 2 was detected [Fig. 3, lanes 10–12 and 15; and see Fig. 1a].

Because we could readily detect LIYV replication-driven GFP expression from the M5gfp D-RNA in protoplasts, and the M5gfp D-RNA was transmitted to plants, we used

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**Table 2. Transmission of LIYV by *B. tabaci* after *in vitro* acquisition of virions derived from *N. tabacum* protoplasts**

Virions were purified from LIYV-infected protoplasts of *N. tabacum* var. Xanthi 72 h p.i. with LIYV virion RNA. Virion concentrations were estimated by immunoblot analysis and densitometry of viral coat proteins using known amounts of virions as standards. Transmission was performed using approximately 100 caged *B. tabaci* per target plant (lettuce) and scored as number of infected target plants over total number of plants tested.

<table>
<thead>
<tr>
<th>Virus preparation</th>
<th>Yield (ng per 10⁶ cells)</th>
<th>Virion concentration (ng µl⁻¹)</th>
<th>Whitely transmission</th>
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<tr>
<td>1</td>
<td>36·8</td>
<td>2·4</td>
<td>2/4</td>
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<tr>
<td>2</td>
<td>2·2</td>
<td>0·3</td>
<td>1/4</td>
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<td>3</td>
<td>20·8</td>
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<tr>
<td>4</td>
<td>24·7</td>
<td>3·9</td>
<td>5/5</td>
</tr>
<tr>
<td>5</td>
<td>37·6</td>
<td>5·2</td>
<td>3/4</td>
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confocal laser scanning microscopy in attempts to determine if GFP expression was detectable in plants infected by LIYV and the M5gfp D-RNA. We examined petiole and leaf sections of plants of all three species, but surprisingly were unable conclusively to detect GFP expression in any of these plants (data not shown). Leaf sections of plants mechanically inoculated with the \textit{in vitro}-derived transcripts of a recombinant construct of \textit{Tobacco mosaic virus} (TMV) [\textit{pTMV(30B)-GFP}] engineered to express GFP (Rabindran \\& Dawson, 2001) consistently produced GFP fluorescence (data not shown). Because D-RNAs are presumably generated by recombination and rearrangement events during replication, it seemed possible that the lack of detectable fluorescence in plants might be due to mutations accumulating in the M5gfp D-RNA GFP-coding sequence. Therefore we specifically cloned and sequenced RT-PCR-amplified fragments corresponding to the GFP-coding region from infected plants (one \textit{L. sativa} plant, one \textit{N. benthamiana} plant, two \textit{C. bursa-pastoris} plants). Eighteen clones from these plants showed no stop codon or deletion mutations (data not shown). Synonymous changes were observed in four clones, and two others showed a single non-synonymous change, both arising from single nucleotide substitutions. Thus, while GFP fluorescence was not observed in the plants, the GFP gene-coding sequence was still intact and the lack of fluorescence was not due to mutations.

### Effects of LIYV and M5gfp D-RNA concentration on whitefly transmission

Although we did not see obvious GFP expression in the above plants, because it contained a unique, non-LIYV sequence, the M5gfp D-RNA was still a good genetic marker for use in assessing D-RNA transmissibility. Our Northern hybridization and RT-PCR analyses showed that the M5gfp D-RNA was detected in leaves distal from the inoculation site; thus, it invaded infected plants systemically along with the LIYV helper virus. Therefore we assessed whether or not the M5gfp D-RNA could be stably maintained along with LIYV by whitefly transmission from plant to plant. We performed plant-to-plant transmission experiments using as source plants \textit{C. bursa-pastoris} that tested positive for LIYV and the M5gfp D-RNA (Table 3, preparations 2 and 4). RT-PCR amplification of total RNAs extracted from \textit{C. bursa-pastoris} target plants indicated that 63 of 69 plants were infected by LIYV, but none contained detectable levels of the M5gfp D-RNA (Table 4, experiments 1–3). Northern blot analysis of total RNA extracts of these plants also showed only LIYV genomic RNA 2, not the M5gfp D-RNA (Fig. 3, lanes 5 and 13, 6 and 14).

Because our data indicated that the M5gfp D-RNA was not transmitted as efficiently as LIYV, we hypothesized that one possibility for its reduced transmission efficiency could be a lowered concentration relative to LIYV within acquisition
Fig. 3. Northern blot hybridization analysis of LIYV RNA 2 and the M5gfp D-RNA. Total RNAs were extracted from individual plants following whitefly transmission and subjected to denaturing electrophoresis in a 1% agarose gel. Hybridizations were performed using DIG-labelled RNA probes corresponding to LIYV RNA 2 (lanes 1–8; Fig. 1a) and the M5gfp D-RNA (lanes 9–16; Fig. 1a). Total RNAs were prepared from the following plants: C. bursa-pastoris non-inoculated control (lanes 1 and 9); C. bursa-pastoris inoculated with LIYV and the M5gfp D-RNA after in vitro acquisition (lanes 2 and 10, 3 and 11, 4 and 12); C. bursa-pastoris inoculated by whiteflies that previously fed on the plant represented in lane 2 (lanes 5 and 13) and 3 (lanes 6 and 14); and N. benthamiana inoculated with LIYV and the M5gfp D-RNA after in vitro acquisition (lanes 7 and 15). In vitro transcripts of pR6, the cDNA clone of LIYV RNA 2 (lane 8), and pM5gfp, the cDNA clone of the M5gfp D-RNA (lane 16), were included as positive controls. Arrows on the right denote the positions of LIYV genomic RNA 2 and the M5gfp D-RNA. The positions of two RNA size (kb) markers are indicated on the left.

Sources. In order to test this possibility, we purified virions from C. bursa-pastoris and N. benthamiana plants shown by Northern hybridization and RT-PCR to contain both LIYV and M5gfp D-RNA (plants from experiment 7, Table 3). A low but sufficient yield of virions was obtained from the infected C. bursa-pastoris plant, while a much greater yield was obtained from the N. benthamiana plants. These virions were then used at approx. 8.1 and approx. 300 ng μl⁻¹, respectively, for in vitro acquisition and transmission by B. tabaci (Table 4, experiments 4 and 5). As expected, LIYV was efficiently transmitted from both virion preparations; however, the M5gfp D-RNA was transmitted only when virion concentration in the acquisition source was very high, 300 ng μl⁻¹ (Table 4, experiment 5; Fig. 4b, lane 10), a concentration much higher than was needed from the initially inoculated protoplasts (Table 3). We next compared the relative abundance of LIYV RNA 2 and the M5gfp D-RNA in virions, protoplasts and plants to see whether there was a correlation between M5gfp D-RNA concentration and whitefly transmission. Very large amounts of the M5gfp D-RNA relative to RNA 2 were always detected in virion preparations purified from initially inoculated protoplasts, and in total RNAs extracted from these protoplasts (Fig. 4, lanes 1 and 12). The ratio of the hybridization signals for the M5gfp D-RNA to those for LIYV RNA 2 in these samples was estimated by densitometry of the exposed X-ray film and was approx. 30 : 1 and 10 : 1 (Fig. 4, lanes 1 and 12, respectively). As shown in Table 3, both RNA species were consistently detected in target plants following in vitro acquisition and whitefly transmission. However, the opposite was seen when total RNAs and virion RNAs from infected plants were analysed. Faint hybridization signals were seen for the M5gfp D-RNA, while plants gave strong signals for LIYV RNA 2 (Fig. 4, lanes 8–10). When the virions were purified from these plants and virion RNAs were analysed similarly, the ratio of hybridization signal for the M5gfp D-RNA to LIYV RNA 2 (approx. 1 : 8; Fig. 4, lane 3) was much less than that seen for virion RNAs obtained from protoplasts (compare lanes 1 and 3, Fig. 4). The reduced concentration of M5gfp D-RNA relative to that of LIYV RNA 2 in both total RNAs and virion RNAs obtained from infected plants correlated with the transmission phenotype of the respective RNA species – the sustained transmission of LIYV RNA 2 rather than M5gfp D-RNA following plant-to-plant whitefly transfers. To further ensure that the RNAs detected in these virion preparations reflected encapsidated RNAs, we used RNase A (100 ng μl⁻¹) treatment of purified virions to eliminate possible contaminating, unencapsidated RNAs. Lane 4 shows RNAs recovered from RNase-treated virions, and again both LIYV RNA 2 and the M5gfp D-RNA were detected, but the ratio of hybridization signals for the M5gfp D-RNA relative to that for LIYV RNA 2 was approx. 1 : 10, further demonstrating that encapsidated LIYV RNA 2 is much more abundant in the infected plants than is encapsidated M5gfp D-RNA.

**DISCUSSION**

Viruses of the *Closteroviridae* have large, complex virions and the largest genomes of the positive-sense ssRNA plant viruses (Martelli *et al.*, 2002). Although various viruses of this family are transmitted from plant to plant by different types of homopteran vectors, the aphid and whitefly transmission of viruses of the genera *Closterovirus* and *Crinivirus*, respectively, is described as non-circulative and semi-persistent (Karasev, 2000; Martelli *et al.*, 2002). Molecular determinants that facilitate vector transmission of these viruses remain to be identified, and so far LIYV is the only member of the *Closteroviridae* for which in vitro acquisition has proven to be a successful means for transmitting virus to plants.

Data accumulated so far on vector-mediated transmission of plant viruses have established that encapsidation/virion assembly is a prerequisite for transmission to occur. One of the earliest attempts to examine aphid transmission of several different viruses mechanistically showed that infectious genomic RNAs and DNAs were not aphid-transmissible, while appropriately prepared virion preparations were (Pirone & Megahed, 1966). Virions (sometimes requiring accessory helper proteins) are acquired and
transmitted to plants by insect, nematode and fungal vectors of many taxonomically diverse plant viruses (Campbell, 1996; Gray & Gildow, 2003; MacFarlane, 2003; Pirone & Blanc, 1996; Rochow, 1970). Because LIYV virions can be acquired in vitro and then transmitted to plants by

\[ B.\ tabaci, \] we were able to use specific numbers of whiteflies along with known virion concentrations and to estimate transmission efficiency at limiting thresholds for each. Although LIYV transmission was seen over a wide range of the virion concentrations tested here, the transmission

![Fig. 4](image-url)

**Fig. 4.** Relative levels of LIYV genomic RNA 2 and the M5gfp D-RNA in protoplasts, plants and virions. Northern blot analysis was performed using virion RNAs and total RNAs prepared from protoplasts and individual plants infected with LIYV and the M5gfp D-RNA. A DIG-labelled RNA probe corresponding to LIYV RNA 2 (p26 region; Fig. 1a) was used for hybridization in (a) (lanes 1–13); a probe corresponding to the M5gfp D-RNA (GFP region only; Fig. 1a) was used in (b) (inset) for samples in lanes 8–11. (a1) Extended exposure for the respective lanes (1–4). Virion RNA samples were prepared from infected \( N.\ tabacum \) protoplasts (lane 1) and \( N.\ benthamiana \) plants [virion RNAs were not treated with RNase A (lane 3) or treated with 100 ng RNase A mL⁻¹ (lane 4)]. Total RNA samples were prepared from uninfected \( C.\ bursa-pastoris \) plants (lane 5), \( N.\ benthamiana \) plants (lane 6) and \( N.\ tabacum \) protoplasts (lane 7); from infected \( N.\ benthamiana \) plants (lane 8), \( C.\ bursa-pastoris \) plants (lane 9) and \( N.\ tabacum \) protoplasts (lane 12); and from plants inoculated by whiteflies that previously fed on virions purified from the plants represented in lanes 8 (lane 10) and 9 (lane 11), respectively. In vitro transcripts of pR6, the cDNA clone of LIYV RNA 2 (lane 13), were included as a positive control. Lane 2 contains a no-RNA negative control. Arrows denote the positions of LIYV genomic RNA 2 and the M5gfp D-RNA. Positions of two RNA size (kb) markers are indicated.

**Table 4.** \( B.\ tabaci \) transmission of LIYV and the engineered LIYV M5gfp D-RNA

In three independent experiments, \( C.\ bursa-pastoris \) plants that tested positive for the presence of LIYV and M5gfp D-RNA were used as source plants for whitefly transmission approximately 3 (experiment 1), 6 (experiment 2) and 10 (experiment 3) weeks following inoculation. In experiments 4 and 5, a \( C.\ bursa-pastoris \) plant and \( N.\ benthamiana \) plants, respectively, that tested positive for the presence of the LIYV and M5gfp D-RNA were used as the source for virion purification. Purified virions were used at the concentration shown for in vitro acquisition and whitefly transmission. Experiments were performed using approximately 100 caged \( B.\ tabaci \) per target plant; 3–4 weeks post-transmission, total RNAs were extracted from target plants and detection of the LIYV RNA 2 and the M5gfp D-RNAs was done using RT-PCR and, in some cases, Northern blot analysis.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Virion concentration (ng mL⁻¹)</th>
<th>Target plant</th>
<th>Transmission</th>
<th>Plants tested (n)</th>
<th>LIYV</th>
<th>M5gfp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>–</td>
<td>( C.\ bursa-pastoris )</td>
<td>30</td>
<td>26</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>( C.\ bursa-pastoris )</td>
<td>19</td>
<td>19</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>( C.\ bursa-pastoris )</td>
<td>20</td>
<td>18</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>8·1</td>
<td>( N.\ benthamiana )</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>300</td>
<td>( C.\ bursa-pastoris )</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>
frequency decreased with increasing dilutions of LIYV virions in the acquisition source. However, in all experiments here, the estimated minimum threshold virion concentration was quite consistent, between 0·01 and 0·1 ng μl⁻¹. It is remarkable, given the biological variability inherent among vector transmission experiments, that this virion concentration range was so consistent in our experiments. The transmission efficiencies using specific whitefly numbers, as reported in Table 1, support the results from Fig. 2 and go so far as to estimate the probability for a single whitefly transmitting LIYV when specific virion concentrations are present in the acquisition source. As might be expected, and is now demonstrated by our data, whitefly numbers and virion concentration both play important roles in mediating LIYV transmission to plants.

One of our goals in elucidating the mechanism(s) associated with B. tabaci transmission of LIYV is to examine the transmission of wild-type and engineered LIYV mutants. Such analyses have been made possible in the study of several other non-mechanically transmissible plant viruses (such as the circulative, non-propagatively transmitted phloem-limited poleroviruses and luteoviruses) by combining protoplast infection and in vitro acquisition of virions by their aphid vectors (Brault et al., 1995, 2000; Bruyere et al., 1997; Chay et al., 1996; Rouze-Jouan et al., 2001; Sanger et al., 1994). In those studies, aphids readily transmitted virus when virions in the acquisition source ranged from 10 to 100 ng μl⁻¹ and, as for LIYV, the quality and concentration (quantity) of virions in the acquisition source were important. The best way to control virion quantity and quality in the acquisition source, and then accurately to compare relative transmission efficiencies, is by using in vitro acquisition with virions of defined concentration and quality (e.g. wild-type or mutant constructs).

Although we have not demonstrated whitely transmission of specific LIYV mutants, here we demonstrated unequivocal transmission of an engineered LIYV D-RNA. We showed that the M5gfp D-RNA was transmissible by B. tabaci to plants by using in vitro acquisition of virions purified from infected protoplasts. These protoplasts always showed high levels of M5gfp D-RNA replication, and virions purified from them also contained large amounts of the M5gfp D-RNA relative to LIYV genomic RNA 2 (Fig. 4). In contrast, although plants of three species contained the M5gfp D-RNA, it was not transmissible from these source plants to target plants unless virions were first concentrated and used for in vitro acquisition. Thus it seems that, although the M5gfp D-RNA replicated and systemically invaded plants along with the helper virus LIYV, it may not be competitive in some aspects of the infection cycle so as to ensure its subsequent transmission to new plant hosts. Most plants had higher levels of LIYV RNA 2 relative to the M5gfp D-RNA, and virions from plants analysed here contained greater amounts of LIYV RNA 2 relative to the M5gfp D-RNA (Fig. 4). Whether this was due to competition during replication, whole-plant invasion or encapsidation or a combination of these or other critical events in maintaining the infection is not known. Similarly, engineered D-RNAs for TMV and CTV have also been transmitted to plants along with the corresponding helper viruses, and shown not always to be competitive (Knapp et al., 2001; Yang et al., 1997).

Although the M5gfp D-RNA was present in plants, we did not see GFP fluorescence. One possibility is that the observed reduced amount of the M5gfp D-RNA in plants versus protoplasts resulted in GFP concentrations lower than those required to allow observable discrimination of GFP fluorescence versus xylem autofluorescence. A low-level GFP fluorescence might still be detectable if the M5gfp D-RNA and the LIYV helper virus were present in tissues where autofluorescence was less abundant (e.g. the mesophyll), but because of the phloem-limited nature of LIYV this has not been possible. Still, by using the GFP-coding sequence as a marker in the M5gfp D-RNA, our study has shown that virions containing the M5gfp D-RNA could be transmitted by whiteflies, albeit only when high concentrations of the encapsidated RNA were present in the acquisition source.

This study clearly shows that the in vitro acquisition system, and knowledge of its characteristics in facilitating the delivery of LIYV and engineered RNAs to plants, should allow us to identify further LIYV-encoded determinants of transmission by B. tabaci. It remains to be seen whether in vitro acquisition will prove universally useful for other members of the Closteroviridae, as it seems to be for viruses of the Luteoviridae and of the genus Potyvirus. We have so far attempted to use in vitro acquisition for transmission of two other members of the genus Closterovirus: BYV using Myzus persicae and CTV using Aphis gossypii. So far we have been unsuccessful in transmitting these two viruses to plants after in vitro acquisition (unpublished). Thus, for the Closteroviridae, it appears that, like their virions and genomes, the molecular mechanisms facilitating vector transmission of viruses in this family may be complex and quite different for the different viruses.

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