Visual monitoring of *Cucumber mosaic virus* infection in *Nicotiana benthamiana* following transmission by the aphid vector *Myzus persicae*

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The single-stranded, positive-sense and tripartite RNA virus *Cucumber mosaic virus* (CMV) was used in this study as a method for monitoring the initial stages of virus infection following aphid transmission. The RNA2 of CMV was modified to incorporate, in a variety of arrangements, an open reading frame (ORF) encoding an enhanced green fluorescent protein (eGFP). The phenotypes of five engineered RNA2s were tested in *Nicotiana tabacum*, *Nicotiana clevelandii* and *Nicotiana benthamiana*. Only one construct (F4), in which the 2b ORF was truncated at the 3′ end and fused in-frame with the eGFP ORF, was able to systemically infect *N. benthamiana* plants, express eGFP and be transmitted by the aphid *Myzus persicae*. The utility of this construct was demonstrated following infection as early as one day post-transmission (dpt) continuing through to systemic infection. Comparisons of the inoculation sites in different petiole sections one to three dpt clearly showed that the onset of infection and eGFP expression always occurred in the epidermal or collenchymatous tissue just below the epidermis; an observation consistent with the rapid time frame characteristic of the non-persistent mode of aphid transmission.

INTRODUCTION

Many plant viruses have evolved, not only with their host, but also the vector that transmits them. Understanding this three-way relationship is integral to developing control strategies. *Cucumber mosaic virus* (CMV) has a worldwide distribution with one of the broadest host ranges of any known species of virus (for a recent review see (Jacquemond, 2012)). CMV has a tripartite single-stranded RNA genome, with each genomic RNA packaged separately within icosahedral capsids. CMV genomic RNAs 1 and 2 encode the 1a and 2a proteins, respectively, which are involved in viral replication. RNA2 also encodes a small protein called 2b, which is a suppressor of RNA silencing (Pumplin & Voinnet, 2013; Wang *et al.*, 2012), and plays a role in promoting cell-to-cell movement (Soards *et al.*, 2002). RNA3 is also bicistronic, encoding the movement protein (MP) and the virion capsid protein (CP). Natural transmission of CMV is mediated by at least 75 species of aphid (Palukaitis *et al.*, 1992) in a non-persistent, non-circulative mode, which is characterized by short acquisition and inoculation periods (from seconds to minutes), with the aphid rapidly losing the ability to transmit.

Aphids are sap-sucking insects with a highly specialized needle-like stylet, which can penetrate and feed on plant cells. The typical feeding behaviour of an aphid includes initial exploratory probes, during which it is assumed the insect ‘tastes’ the plant to establish whether it is a host or non-host (Fereres & Moreno, 2009). Aphids need more than a minute to penetrate deeper than the epidermis (Pirone & Harris, 1977); the stylet advancing intercellularly before prolonged feeding is initiated by the puncturing of a cell membrane. This behaviour has been successfully monitored by measuring the changes in electric potential that occur during feeding. For non-persistent viruses, like CMV, an ingestion-salivation model for transmission is presently the most accepted, whereby the aphid is capable of acquiring and inoculating virus particles during the initial phases of probing (Martin *et al.*, 1997; Powell, 2005). Following the penetration of the stylet, the aphid secretes gel saliva which hardens, enclosing the entire stylet in a protective
Monitoring CMV infection post-transmission

The potential of CMV as a tool for heterologous expression was first demonstrated using green fluorescent protein (GFP) in a variety of arrangements within RNA3. For all constructs GFP expression was limited to a few cells around the inoculation site (Canto et al., 1997). In a similar study, a diploid RNA3 was engineered whereby in one RNA3 the MP was replaced by GFP, and in the other RNA3 the CP was replaced by a multiple cloning site for the insertion of foreign genes. Although functional, movement and gene expression were limited to the inoculated leaf, with recombination frequently restoring RNA3 to the wild-type (Zhao et al., 2000). In another system, dependency on the CP for cell-to-cell spread was circumvented by deleting the 33 amino acid C-terminal of the MP, thereby allowing the CP open reading frame (ORF) to be completely replaced by a heterologous protein (Fujiki et al., 2008). The above approaches all exploited the RNA4 subgenomic promoter located upstream of the CP. An alternative approach has been to use the other CMV subgenomic promoter (for subgenomic RNA4A) located upstream and driving transcription of an RNA encoding the 2b silencing suppressor. One construct was engineered to contain a cloning site immediately upstream of the 2b start codon thereby entirely deleting the 2b ORF and truncating the 2a ORF. This construct, named C2-H1, was used to efficiently express a human cytokine (Matsuo et al., 2007) and fluorescent proteins (Takeshita et al., 2012). Another construct, C2-A1, was used to insert both targets for gene silencing (Kanazawa et al., 2011; Otagaki et al., 2006) and fluorescent proteins (Kanazawa et al., 2011; Takeshita et al., 2012). It was also engineered to contain a cloning site, this time truncating the 2b ORF, but leaving the 2a ORF intact. The 2b ORF, although essential for normal functioning of the virus, can be truncated at the C terminus and still have a phenotype close to the wild-type (Lewsey et al., 2009); complete deletion of the 2b ORF (and the C-terminal region of the 2a ORF) results in impaired infection or movement and a significant attenuation of symptoms (Ding et al., 1995; Soards et al., 2002).

In this study our objective was to develop a means of identifying the initial sites of CMV inoculation by aphid feeding and to follow the spread of infection in the entire plant. To do this we developed and tested a number of heterologous infectious clones of CMV with an incorporated enhanced green fluorescent protein (eGFP) arranged in a variety of configurations in order to empirically test each one in planta for the desired phenotype (i.e. stability of the construct and eGFP expression during all phases of infection and transmission). We confirmed the amenability of CMV to stable fluorescent tagging of its 2b protein, both full-length and truncated, and demonstrated the biological stability of these constructs throughout infection, from aphid transmission and systemic spread in the plant to aphid acquisition. To our knowledge, this is the first demonstration of a phenotypically near wild-type, fluorescently expressing virus that is vector transmissible. Using this tool, we also provide evidence to support the hypothesis that the initial cells to be infected during aphid feeding are epidermal, an observation consistent with the rapid time frame of transmission for non-persistently transmitted viruses.

RESULTS

Infectivity and eGFP expression of F-constructs in Nicotiana spp.

In total, five different CMV RNA2 constructs with altered 2b regions were engineered and named F1 to F5 (Fig. 1). The F1 construct contained the complete 2b ORF fused in-frame with the eGFP ORF to produce an RNA2 of 3770 nt. The F2 construct fused the full-length 2a ORF in-frame with the eGFP ORF resulting in a C terminus (89 nt) truncation of the 2b ORF and a resulting RNA2 of 3679 nt. The F3 construct had the 2b ORF deleted and an eGFP ORF substituted and under the control of the 2b promoter; this resulted in a truncated 2a ORF, yielding an RNA2 of 3438 nt. The F4 construct, was similar to the F2 construct in that it contained a C terminus (90 nt) truncation of the 2b ORF and a full-length 2a ORF, but in contrast to F2 the truncated 2b ORF was fused in-frame with eGFP to give a full-length RNA2 of 3683 nt. Finally, the F5 construct organization corresponded to the F4 construct, but without eGFP. This latter construct was engineered as a reference for the effects of eGFP insertion in those constructs with a truncated 2b. Nicotiana tabacum, Nicotiana clevelandii and Nicotiana benthamiana plants were mechanically inoculated with capped transcripts derived from RNA1 (pFny109), RNA3 (pFny309), and either the wild-type RNA2 clone (pFny209) or one of the F-constructs; they were monitored for viral symptoms and local and systemic eGFP expression at 2, 7, 11, 14 and 21 days post-inoculation (days p.i.). Each F-construct was inoculated onto 3 plants (2 leaves each) and the experiment was repeated either three or four times (Table 1).

- N. tabacum. Three out of twelve (25%) of the plants inoculated with the F1 construct showed symptoms similar to CMV wild-type infection, but with no visible eGFP fluorescence in newly emerging leaves 7 days p.i. However, at 2 days p.i. F1 construct-inoculated leaves showed patches (approximately 10 cells in diameter) of eGFP fluorescence (data not shown), which did not expand over time. Half of the plants inoculated with the F5 construct showed symptoms similar to CMV wild-type infection. F2, F3 and F4 constructs did not systemically infect N. tabacum. (Table 1).

- N. clevelandii. The F5 construct was able to systemically infect, causing severe symptoms with necrosis. All other
Fig. 1. Schematic organization of CMV Fny RNA2 and derived F-constructs (F1 to F5). Open reading frames (ORFs) are indicated by shaded rectangles, primer annealing sites (for RT-PCR analysis by thin arrows, nucleotide length (nt) of individual RNA by numbers in parentheses and restriction sites of Ncol and PstI by dotted lines). F1 contains the complete 2b ORF fused in-frame with the eGFP ORF. F2 has the full-length 2a ORF in-frame fused with the eGFP ORF causing a C terminus truncation of the 2b ORF. F3 contains the 2b ORF deleted and an eGFP ORF substituted and under the control of the 2b promoter; this resulted in a truncated 2a ORF. F4 contains a full-length 2a ORF and a C terminus truncation of the 2b ORF fused in-frame with eGFP. F5 corresponds exactly to the F4 construct but without eGFP.

Table 1. Phenotypes of virus infection in N. tabacum, N. clevelandii and N. benthamiana plants mechanically inoculated with a mix of capped transcripts derived from wild type CMV Fny RNA2 (1–2), 2b (wild-type [WT] or one of the F-constructs with RNA1 and 3 [F1–5]), N. benthamiana. Fractions represent plants (out of a total of 9 or 12) showing systemic viral symptoms 7–14 days p.i. in three–four independent experiments. GFP, green fluorescent protein; I, inoculated leaves; S, systemic leaves; —, lack of GFP signal; NA, not applicable.

<table>
<thead>
<tr>
<th>Virus</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
<th>F5</th>
<th>Wt</th>
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<tr>
<td>N. tabacum</td>
<td>0/9</td>
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<td>0/9</td>
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<td>6/12</td>
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<tr>
<td>N. clevelandii</td>
<td>8/12</td>
<td>4/12</td>
<td>0/9</td>
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<td>9/9</td>
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<tr>
<td>N. benthamiana</td>
<td>8/12</td>
<td>7/12</td>
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*GFP signals were erratic in inoculated leaves and ranged from being absent (4 plants) to weak (3 plants) to strong (1 plant).
leaves showed patches (approximately 10 cells in diameter) of eGFP fluorescence at 2 days p.i., which subsequently expanded and in some cases moved along major veins (Fig. 2a–b). At 14 days p.i. F4 construct-inoculated plants showed systemic symptoms with eGFP expression in the upper leaves. Sap inoculations using F1-infected, eGFP-expressing leaf material as inoculum resulted in a systemic infection, but without eGFP expression in six out of six plants (total for two independent experiments). We hypothesized that an RNA2 with additional eGFP sequences may not be packaged due to its size or other limitations. To test this, we harvested eGFP expressing, systemically infected leaves at 7 days p.i. and analysed the RNAs extracted from purified virions versus total RNAs obtained directly from the leaves for the presence of the eGFP sequences. RT-PCR analysis of F4-infected leaves revealed that eGFP sequences could be recovered from virions and total RNA extracts (Fig. 3). By contrast, in F1-infected plants eGFP sequences could only be recovered from the total RNA extracts; eGFP sequences were not detected among the virion RNAs, suggesting that the F1 RNA2 is capable of systemic spread, but is not packaged into virions (Fig. 3).

F4 RNA can be stably transmitted by aphids

To test the transmissibility of the F1 and F4 RNAs, aphids were fed on eGFP expressing areas of systemically infected leaf tissue in order to acquire the virus, and then transferred to healthy N. benthamiana plants (3 plants per construct in three separate experiments). Local and systemic eGFP signals were checked at 2, 4 and 7 days post-transmission (dpt). None of the plants (zero out of nine) incubated with aphids that had fed on F1 construct infected plants developed eGFP signals, while all plants inoculated by aphids that had fed on F4 construct infected plants developed eGFP signals (nine of nine plants) in the inoculated area (Fig. 2c–d) and systemic (Fig. 2e–f) leaves. Repeated aphid transmission, as described above, every 14 days showed F4 RNA to be stably transmitted over the course of five passaging experiments.

Epidermal cells are the first to be infected upon transmission

N. benthamiana petioles (10 experiments, 2–3 petioles/experiment) and leaves (2 experiments, 1 plant/experiment...
Here we report on the development of a CMV reporter system and demonstrate its applicability for use in monitoring the initial stages of virus infection following vector transmission. The reported competency of CMV constructs exploiting the 4A subgenomic promoter for the expression of foreign genes (Kanazawa et al., 2011; Matsuo et al., 2007; Otagaki et al., 2006) in combination with the fact that the replication encoding RNAs will be the first to be translated upon infection (versus the structural proteins encoded on RNA3) led us to pursue a similar design.

Specifically, we engineered four eGFP-containing constructs in the RNA2 of Fny-CMV. Two of these, F3 and F4, were comparable to the GFP derivatives of the Y-CMV constructs, C2-H1 and C2-A1 (Kanazawa et al., 2011; Takeshita et al., 2012), respectively, except that the latter contained additionally engineered cloning sites. Of the four Fny-CMV constructs tested in this study, all were able to infect *N. benthamiana*, two (F1 and F4) expressed eGFP, and one (F4) was aphid transmissible. There was no difference in eGFP expression in local F1 or F4 inoculated leaves, with eGFP expression occurring throughout expanding leaves with a gradual decline in expression after 14 days p.i. The reason for the decrease in the F1 and F4 phenotypes expression of eGFP is most likely due to the absence of any selection pressure to maintain the eGFP ORF; RT-PCR analysis of inoculated and systemically infected tissue 21 days p.i. confirmed a loss in the integrity of the eGFP ORF in all constructs tested (Fig. 6). Such reversions due to recombination are a well-documented phenomenon in heterologous virus expression systems (Chapman et al., 1992; Culver, 1996; Dolja et al., 1993). Transmissibility or its absence could be explained by the physical limitations of virion assembly imposed by genome segment length. F4 RNA2 is 87 nt shorter than that of F1 and 633 nt longer than the wild-type RNA2. Full-length F1 RNA2 could be detected in eGFP-expressing islands in inoculated leaves, but not in virus particles isolated from those islands, whereas full-length F4 RNA2 was found in both eGFP islands and extracted virions (Fig. 3). We cannot exclude the possibility that virions from F1 and F4 infected plants were less stable and that the virion purification method employed here could be too harsh to isolate intact virus particles. However, the results of the aphid transmission experiments support the hypothesis that F1 RNA2 encapsidation is hindered, perhaps due to a size limitation, a phenomenon that has been demonstrated for plant RNA viruses both in vivo (Qu & Morris, 1997) and in vitro (Cadena-Nava et al., 2012).

The transmission phenotypes of F2 and F3 were not evaluated because eGFP expression was not consistently observed, despite the development of systemic infection in *N. benthamiana*. This was presumably due to loss of the integrity of the eGFP ORF by recombination early in infection, as was observed at later time points for all F genotypes (Fig. 6). Unlike the other constructs designed, both the F2 and F3 are predicted to have altered 2a proteins; the latter contained additionally engineered cloning sites. Of the four Fny-CMV constructs tested in this study, all were able to infect *N. benthamiana*, two (F1 and F4) expressed eGFP, and one (F4) was aphid transmissible. There was no difference in eGFP expression in local F1 or F4 inoculated leaves, with eGFP expression occurring throughout expanding leaves with a gradual decline in expression after 14 days p.i. The reason for the decrease in the F1 and F4 phenotypes expression of eGFP is most likely due to the absence of any selection pressure to maintain the eGFP ORF; RT-PCR analysis of inoculated and systemically infected tissue 21 days p.i. confirmed a loss in the integrity of the eGFP ORF in all constructs tested (Fig. 6). Such reversions due to recombination are a well-documented phenomenon in heterologous virus expression systems (Chapman et al., 1992; Culver, 1996; Dolja et al., 1993). Transmissibility or its absence could be explained by the physical limitations of virion assembly imposed by genome segment length. F4 RNA2 is 87 nt shorter than that of F1 and 633 nt longer than the wild-type RNA2. Full-length F1 RNA2 could be detected in eGFP-expressing islands in inoculated leaves, but not in virus particles isolated from those islands, whereas full-length F4 RNA2 was found in both eGFP islands and extracted virions (Fig. 3). We cannot exclude the possibility that virions from F1 and F4 infected plants were less stable and that the virion purification method employed here could be too harsh to isolate intact virus particles. However, the results of the aphid transmission experiments support the hypothesis that F1 RNA2 encapsidation is hindered, perhaps due to a size limitation, a phenomenon that has been demonstrated for plant RNA viruses both in vivo (Qu & Morris, 1997) and in vitro (Cadena-Nava et al., 2012).

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region of the 2a in F3 is truncated, an effect already shown to reduce virus accumulation and to attenuate symptoms in *N. glutinosa* (Du *et al.*, 2008). The F4 genotype is almost identical (apart from an extra 4 nt) to that of F2, except that the eGFP ORF is fused to the 2b instead, supporting the idea that the essential role of the 2a ORF in virus replication is encumbered when fused to eGFP (Margolin, 2000), in particular at the C terminus where a number of highly conserved polymerase motifs are located (Koonin, 1991).

Comparable phenotypes were observed in F1, F4 and wild-type infected plants, indicating that the presence of eGFP in F1 and F4 does not influence symptoms. In a detailed study of a CMV 2b GFP fusion protein there was no change observed in 2b functionality (González *et al.*, 2010). Functional mapping of the 2b ORF suggests that the 2b protein N terminus is critical for symptom induction; mutations of the first 17 N-terminal residues in this domain, two nuclear localization sequences or a putative phosphorylation sequence resulted in asymptomatic infections. However, a deletion of the 16 C-terminal residues of the 2b protein had little effect on symptoms in *Arabidopsis thaliana* when compared to the wild-type, but caused an increase in symptom severity in *N. benthamiana* (Lewsey *et al.*, 2009). A similar slight increase in symptom severity (leaf distortion) was observed in this study for those F-constructs with a truncated 2b (F1, F4 and F5) both in *N. benthamiana* and *N. tabacum*. This phenomenon was also demonstrated with engineered constructs in an analogous genotype of a CMV isolate from subgroup II (results not shown).

**Fig. 5.** Cellular localization of eGFP in petiole and leaf sections of F4-infected *N. benthamiana* plants 1–3 days after aphid transmission. Petiole sections in bright-field (bf) light are shown in (a), one dpt; (b), two dpt and (c), three dpt. (d)–(f), show eGFP expression in the corresponding petiole sections of (a)–(c). A magnification of the area delineated by the rectangle in (a) and (d) is shown in panels (g)–(i). Panels (g), (j) and (m) are in bf; (h), (k) and (n) show eGFP expression (a false red colour is shown for improved contrast); (i), (l) and (o) merged. Petioles in (a)–(l) were detached from the leaf during the inoculation experiment. Petioles and leaf in (j)–(o) were intact (attached to the plant) until harvested for processing. (g)–(o), One dpt; bar, 100 µm.
The use of fluorescent proteins to tag virus components and, therefore, track infection in the host has been extensively exploited both in animal and plant systems. The only previous case of a fluorescently tagged engineered virus that was stably transmitted via an insect vector is the phloem-limited Potato leafroll virus which was engineered to express GFP (Nurkiyanova et al., 2000). Aphids that fed on extracts from infected protoplasts were able to transmit the virus, but subsequent cell-to-cell movement from the inoculated cell was found to be defective; progeny that moved systemically were found to have de novo deletions annihilating the function of the GFP ORF (Nurkiyanova et al., 2000). The CMV (F4) genotype reported here provides a unique tool for the identification of the initial host cells inoculated by the insect vector upon transmission. Prior to the appearance of symptoms, systemic infection was observed as early as 4 dpt in the form of eGFP signals in major veins (Fig. 2a). However, because of the large surface area the use of whole plants for the identification of the primary inoculated cells proved impracticable. Therefore, in order to monitor the early onset of infection and to simplify the identification of the primary inoculated cells, detached petioles were used in aphid transmission experiments. Comparison of petioles with an infection site at 1, 2 and 3 dpt clearly showed that the onset of eGFP expression always occurred in the epidermal cells or collenchymatous cells just below the epidermis; observations in experiments with leaves were consistent with these results (Fig. 5m–o). Fluorescence was at times difficult to discriminate in epidermal cells (Fig. 4d–f and 5m–o) possibly due to the physical effects of hand-sectioning, which could slightly deform cells at the edges of the tissues. Nevertheless, in some sections fluorescence in the epidermis could be unambiguous (Fig. 5k–l) or present but notably weaker than in the underlying collenchyma (Fig. 5g–i). At these early stages, eGFP expression was never observed in the phloem or surrounding cells, although autofluorescence in xylem tissue was consistent in both infected and uninfected tissues. Therefore, our results indicate that aphid transmission of F4 occurs at the earliest time point of plant penetration by the aphid during a period of initial exploratory probing, as proposed previously by Martin et al., (1997).

**METHODS**

**Plant growth conditions.** *N. tabacum, N. clevelandii* and *N. benthamiana* seeds were grown in a greenhouse with a 16/8h (light/day−1) photoperiod at 22 ± 3 °C.

**F-constructs.** Restriction endonucleases and DNA-modifying enzymes were used as recommended by the manufacturers (New England Biolabs). Plasmids pFny 209 (Rizzo & Palukaitis, 1990) and pPRLVeGFP (Taliansky et al., 2004) were the templates for the F-constructs and enhanced GFP (eGFP), respectively. All F-constructs were engineered by PCR-mediated mutagenesis (Liu et al., 2002) involving the insertion of Pfu polymerase (Stratagene) generated PCR fusion products containing primer-generated mutations into pFny209 linearized by NcoI3 and PstI. For the F1 to F4 constructs, which were to contain eGFP, three separate PCR fragments (5′NcoI + virus; eGFP; virus + PstI3′) were initially generated and then fused stepwise. Each virus fragment (5′ and 3′) was first individually fused with eGFP (5′NcoI + virus + eGFP and eGFP + virus + PstI3′), and then both virus/eGFP fragments were joined into one final fusion fragment. For the F5 construct two PCR products (5′NcoI + virus; virus + PstI3′), which introduced the desired deletion in...
the 2b gene, were fused into a single fragment. All engineered constructs were verified by DNA sequencing (Genomics Facility, Institute of Biotechnology, Cornell University). Details of the primers used for each pre-fusion PCR fragment (‘5’ NcoI + virus) (eGFP) (‘5’ + PstI)) for each F-construct were as follows:

- **F1: (‘5’ NcoI + virus) – forward primer (L) (‘5’-TCGTCACATGG-CTGAGTTCGCT-3’) (corresponding to Fny CMV RNA2 (GenBank accession number D00355) nt 1846–1869) and the reverse primer (Rf1) (comparing to Fny 209 nt 2731–2748 and the first 17 nt of eGFP); (eGFP) – forward primer (complementary to f1) and reverse primer (eGFPstopRNA2) (‘5’-AGAACGTCTAAG-TAATGAAAACCTCCCTCGCATC-3’) (corresponding to the last 17 nt of eGFP, stop codon TGA and Fny RNA2 nt 2752–2768); (‘5’ + PstI3) – forward primer (complementary to the reverse primer (eGFPstopRNA2)) and the reverse primer (RNA2PstI) (‘5’-AGTCGACCTGAGTTCGCTTGGTTA-3’) (corresponding to Fny 209 nt 3036–3050 and sequence AGTCGACCTGACCGTCTTGGTTA-3’) as for F1.

- **F2: (‘5’ NcoI + virus) – forward primer (L) and the reverse primer (f2) (‘5’-TCGCGCTTCGCTACATGGTAACTCGCCA-3’) (corresponding to Fny 209 nt 2639–2657 and the first 17 nt of eGFP); (eGFP) – forward primer (complementary to the reverse primer f2) and reverse primer (eGFPstopRNA2); (‘5’ + PstI3) – as for F1.

- **F3: (‘5’ NcoI + virus) – forward primer (L) and the reverse primer (f3) (‘5’-TCGCCCTCAGTACATGGTAACTCGCCA-3’) (corresponding to Fny 209 nt 2403–2418, sequence GATAGA (introducing two stop codons so that the 2a gene will end at nt 2417) and the first 17 nt of eGFP; (eGFP) – forward primer (complementary to the reverse primer f3) and reverse primer (eGFPstopRNA2); (‘5’ + PstI3) – as for F1.

- **F4: (‘5’ NcoI + virus) – forward primer (L) and the reverse primer (f4) (‘5’-TCGCGCTTCGCTACAATCGTAACTCGCCA-3’) (corresponding to Fny 209 nt 2639–2661 and the first 17 nt of eGFP); (eGFP) – forward primer (complementary to the reverse primer f4) and reverse primer (eGFPstopRNA2); (‘5’ + PstI3) – as for F1.

- **F5: (‘5’ NcoI + virus) – forward primer (L) and the reverse primer (f5) (‘5’-ATGGCGAGAGGAGGTGCCACCTCCGACAAGCTGTTAACAAGTGTTCCA-3’) (corresponding to Fny 209 nt 2639–2661 and the first 20nt of RNA2 3’ UTR); (‘5’ + PstI3) – forward primer (complementary to the reverse primer f5) and reverse primer (RNA2PstI).

**Virion purification and RT-PCR analysis.** Virions were purified from infected *N. benthamiana* plants according to the methods of Lot et al., (1972) and resuspended in 5 mM sodium borate, 0.5 mM EDTA, pH 9.0. Concentrations were determined spectrophotometrically assuming an extinction coefficient for CMV of 5.0 nM mg⁻¹ cm⁻¹ at a wavelength of 260 nm (Franci et al., 1966). Total RNA was extracted using the RNeasy Plant Mini kit (Qiagen) and subjected to reverse-transcription PCR (RT-PCR) using M-MLV (Promega) and GoTaqDNA Polymerase (Promega), with primer sequences designed to amplify from upstream of the 2b coding region (forward 5’- CTATTATATCATGTCGTCGTC-3’) to the 3’ end of the eGFP ORF (reverse 5’- CTTGTACATCGTTCCT-ATGC-3’). The resulting amplification products were checked on a non-denaturing 1% agarose gel and sequenced.

**Aphid transmission.** Non-viruliferous and wingless adults of the aphid *Myzus persicae* were reared on healthy cucumber seedlings in a growth chamber with a 16:8h (light day⁻¹) photoperiod at 22 ± 3 °C. Aphids were placed in sealed plastic dishes and starved for 24 h at 4 °C. Before feeding the dishes were returned to room temperature for 1 h. Starved aphids were transferred to either petioles or leaves (detached and non-detached) of healthy *N. benthamiana* plants and left overnight. The following day the plants were sprayed with the insecticide Orthene (Valent) at a concentration of 0.4 g l⁻¹ and then returned to the greenhouse for another two weeks. For microscopic imaging, petioles were hand-sectioned with a razor blade and examined under a dissecting (Olympus SZH-ILLD) or fluorescence microscope (detailed below).

**Transcript and sap inoculations.** Infectious viral RNA from Fny CMV and the F-constricts (F1–F5) were reconstituted by mixing equal amounts of *in vitro* transcripts, synthesized using the T7 mMessage Machine kit (Ambion, Life Technologies), from full-length linearized cDNA clones (in parentheses) encoding RNA1 (pFny109) and RNA3 (pFny309) in combination with either RNA2 (pFny209) or one of the F-constricts. Equal volumes of transcripts were combined and gently rubbed onto corborundum-dusted leaves of plants at the three-to-five leaf stage. Mechanically inoculated leaves were examined for eGFP expression one week post-inoculation under an Olympus SZX-12 Stereo Microscope (Olympus Corporation). Virus symptoms were scored at 7 and 14 days post-inoculation (days p.i.). Younger leaves were analysed for systemic infection at two weeks p.i. Infection of the inoculated plants was confirmed serologically using CMV ImmunoStrip Tests (Agdia, Elkhart). All CMV RNA combinations were inoculated onto 3 plants (2 leaves each) in three or four separate experiments (Table 1).

Sap inoculations were carried out mechanically by grinding virus infected tissue in 0.1 M phosphate buffer (pH 7.0) and rubbing onto healthy *N. benthamiana* leaves dusted with corborundum. Mock inoculations using 0.1 M phosphate buffer (pH 7.0) alone were carried out. Inoculated plants were maintained in the greenhouse (as above) and inspected for symptom development and eGFP expression at 2, 7, 11, 14 and 21 days p.i. Infection of the inoculated plants was confirmed serologically.

**Microscopy.** GFP expression was investigated using either an Olympus SZX-12 Stereo Microscope equipped with DFLPLF 0.5X PF, DFPLAPO 1.2X and DFLPL 1.6X PF objectives coupled with 10 × eyepieces (Zoom range up to 108 ×) and two parfocal objectives (0.5 × and 1.6 ×) (filter Ex 470/40, Em LP 500) or a Leica DM5500 Epi-fluorescence Microscope (filter BP450/490; 500; BP500-530). (Leica Microsystems). Aphid stylet sheaths were stained with fuchsin as described by Brennan et al., (2001). Areas on the petioles where aphids had been feeding for 24 h were thinly hand-sectioned using a razor blade. Sections were fixed in 70 % (v/v) aqueous ethanol and then immersed in an acid fuchsin solution [10 parts 70 % ethanol and 1 part 0.2 % acid fuchsin in ethanol/glacial acetic acid (1 : 1, v/v)]. Stylet sheaths in these samples appeared bright orange under an epi-fluorescence microscope [Leica; DM5500 (filter Ex 515–560, DM 580)]. Images were processed using LAS-AF software (Leica). Samples were also visualized under UV light (filter Ex 340–380, DM 400) for optimal autofluorescence of cells around the stylet sheaths.

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**REFERENCES**


