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Construction and biological activities of the first infectious cDNA clones of the genus *Foveavirus*

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ABSTRACT

Grapevine rupestris stem pitting-associated virus (GRSPaV, genus *Foveavirus*, family Betaflexiviridae) is one of the most prevalent viruses in grapevines and is associated with three distinct diseases: rupestris stem pitting, vein necrosis and Syrah decline. Little is known about the biology and pathological properties of GRSPaV. In this work, we engineered a full-length infectious cDNA clone for GRSPaV and a GFP-tagged variant, both under the transcriptional control of *Cauliflower mosaic virus* 35 S promoter. We demonstrated that these cDNA clones were infectious in grapevines and *Nicotiana benthamiana* through fluorescence microscopy, RT-PCR, Western blotting and immuno electron microscopy. Interestingly, GRSPaV does not cause systemic infection in four of the most commonly used herbaceous plants, even in the presence of the movement proteins of two other viruses which are known to complement numerous movement-defective viruses. These infectious clones are the first of members of *Foveavirus* which would allow further investigations into mechanisms governing different aspects of replication for GRSPaV and perhaps related viruses.

Introduction

Grapevine rupestris stem pitting-associated virus (GRSPaV) is a member of the genus *Foveavirus* (family *Betaflexiviridae*, order *Tymovirales*) (King et al., 2011). GRSPaV has a positive sense, single-stranded RNA (Martelli and Jelkmann, 1998) genome of 8725 nucleotides that contains five ORFs, encoding the replicase polyprotein, triple gene block (TGB) movement proteins, and the capsid protein (Fig. 1, Meng et al., 1998; Zhang et al., 1998; Meng and Gonsalves, 2007). *Betaflexiviridae* was a newly established family and contains viruses of the following genera: *Foveavirus*, *Carlavirus*, *Vitivirus*, *Capillovirus*, *Trichovirus*, and *Citrivirus* (King et al., 2011). A common feature for this family of viruses, except some members of *Carlavirus*, is that they naturally infect woody perennials. As with viruses infecting woody plants, grapevine viruses are much understudied compared to those infecting herbaceous plants due to difficulties in working with woody plants. First, woody plants take a long time to propagate. Second, it is more difficult to inoculate woody plants, with grafting and vector-mediated inoculation as the major means of virus inoculation. Third, virus titer is generally low and viruses are often unevenly distributed in an infected plant. Fourth, purification of viruses and viral components is more challenging due to the presence of inhibitory substances (such as polyphenols and polysaccharides) that abound in woody plants (Newbury and Possingham, 1997; Rowhani et al., 1997). Lastly, a single vine is often infected with multiple viruses and viral strains.

The translation product of GRSPaV ORF1 contains signature domains indicative of a typical replicase polyprotein of members of the alphavirus-like supergroup (Koonin and Dolja, 1993). These include a methyl-transferase (MTR), RNA helicase (HEL), and RNA-dependent RNA polymerase (POL) domain. An interesting feature that distinguish GRSPaV (and a few other related viruses) from the majority of plant RNA viruses is that the replicase polyprotein also contains two cysteine protease domains (the papain-like protease and the ovarian tumor protease) and an alkylation B (AlkB) domain. The AlkB domain has been recently identified through bioinformatics and is encoded by only a limited number of viruses infecting woody plants (Martelli et al., 2007). Although its biological function in these viruses is unknown, the AlkB domain is believed to be involved in safeguarding viral RNAs through restoration of nucleic acids methylated by RNA silencing machinery (Bratlie and Drablos, 2005). Clearly, GRSPaV represents a group of unique viruses that warrants further investigation.

GRSPaV is among the most prevalent viruses of grapevines (Meng et al., 2006; Nolasco et al., 2006; Terlizzi et al., 2010; Alabi et al., 2010).

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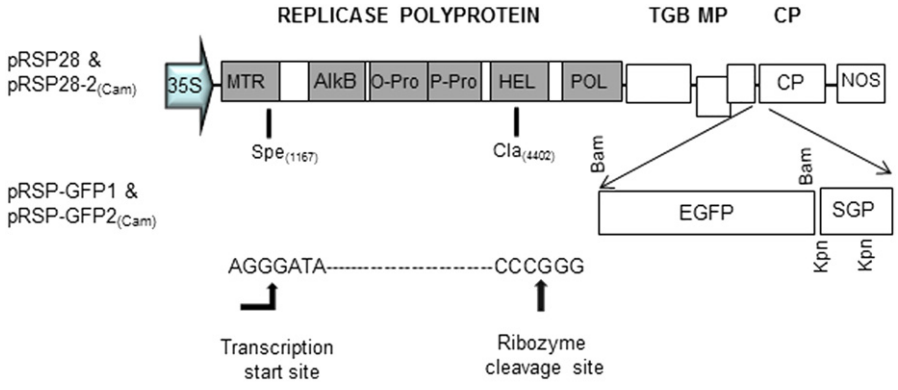

Fig. 1. Schematic representation of the key features of GRSPaV clones, pRSP28 and pRSP28-2$_{(Cam)}$, and its GFP-tagged variants, pRSP-GFP1 and pRSP-GFP2$_{(Cam)}$. These clones were under the control of CaMV 35S promoter and Nos terminator. Ribozyme sequence from *Hepatitis D virus* was included for cleavage of the transcribed RNA. EGFP sequence was inserted into the viral cDNA via BamHI while putative promoter for CP subgenomic RNA from strain GRSPaV-BS was inserted into the KpnI site. BamHI and KpnI sites were introduced into the viral cDNA through SDM. Also indicated are the transcription start site and the ribozyme cleavage site. Open rectangular boxes represent ORFs of the viral genome, solid boxes denote conserved domains of the replicase polyprotein. MTR: methyl transferase; HEL: RNA helicase; AlkB: alkylation b domain; O-Pro: Ovarian tumor cysteine protease; P-Pro: papain-like cysteine protease; POL: RNA-dependent RNA polymerase; TGB: triple gene block; CP: capsid protein; SGP: subgenomic promoter. For further information, refer to the text.

Although the pathological properties of GRSPaV infection remain unknown, it is consistently associated with "Rupestris stem pitting", a component of the important Rugose wood complex of diseases (Martelli, 1993; Zhang et al., 1998; Meng et al., 1999a; Nolasco et al., 2000; Nakaune et al., 2008). Recent evidence suggests that GRSPaV may also be involved in "Grapevine vein necrosis" (GVN) (Bouyahia et al., 2005; 2009; Morelli et al., 2011) and "Syrah decline" (Habili et al., 2006; Lima et al., 2006; Al Rwahnih et al., 2009). "Syrah decline" is a recently reported disease that is responsible for quick decline of newly planted vineyards in several countries including USA, Australia, and France.

GRSPaV exhibits extensive genetic diversity. Phylogenetic analyses of viral sequences derived from infected grapevines suggest that GRSPaV comprises a wide range of sequence variants (Meng et al., 1999b, 2006; Lima et al., 2006; Nolasco et al., 2006; Alabi et al., 2010; Terlizzi et al., 2010). Based on the most current information, seven groups of sequence variants have been detected (Terlizzi et al., 2011). The complete genomes of isolates representing five of these groups were sequenced, which include GRSPaV-1, -SG1, -BS, -SY, and -PN. It is possible that different GRSPaV strains may be responsible for different diseases. For example, Borgo et al. (2009) showed that GRSPaV-1 has a strong correlation with GVN, while GRSPaV-BS is closely associated with RSP. Similarly, Meng et al. (2005) demonstrated that GRSPaV-1 and GRSPaV-SG1 cause very mild or no symptoms on graft-inoculated 'St George' respectively. Furthermore, GRSPaV-SY has a close association with "Syrah decline" (Habili et al., 2006; Lima et al., 2006; Al Rwahnih et al., 2009). Given that grapevines are generally infected with a mixture of viruses and viral strains/variants, the ultimate resolution of this issue can only be achieved through inoculation of virus-free grapevine with infectious cDNA clones corresponding to different strains of GRSPaV.

As a recently discovered virus, molecular and cellular aspects of GRSPaV replication and infection await investigation. Using fluorescent protein tagging and ectopic expression, we recently investigated the subcellular localization of the proteins encoded by GRSPaV. We have found that TGBp1 has a cytoplasmic and nuclear localization and forms punctate structures as expected for an ATPase/helicase protein, that TGBp2 and TGBp3 are both associated with the ER (Rebelo et al., 2008), and that CP targets to the nucleus in tobacco protoplasts (Meng and Li, 2010). Because these findings were derived from plasmid-based ectopic expression, it would be necessary to verify these findings in the context of virus infection, which would also depend on the availability of viral infectious cDNA clones.

The objective of this investigation was to establish an infectious cDNA clone for GRSPaV. Infectious clones will be highly desirable in several ways, such as determination of the pathological properties of GRSPaV, the elucidation of mechanisms that govern various molecular and cellular aspects of the life cycle of GRSPaV, and development of GRSPaV into a vector for protein expression and/or virus-induced gene silencing (VIGS). We report here on the successful construction of full-length cDNA clones for GRSPaV and present compelling evidence that the viral clones are infectious in grapevine and *Nicotiana benthamiana*.

Results

Determination of the 5′ terminal genomic sequence of the viral isolate

The GRSPaV isolate used to make the full-length cDNA clone originated from rootstock variety 'Grande Glabre' (*Vitis riparia*). We designate this isolate "GRSPaV-GG". This isolate was chosen as the candidate for the construction of full-length cDNA clones based on two reasons. First, unlike most of the grapevine varieties infected with GRSPaV, this isolate is composed of relatively uniform population of sequence variants of GRSPaV (Meng et al., 2006; Meng, unpublished data), which would make the cloning of a single variant more straightforward. Second, this isolate appears to be mild as it induces few subtle symptoms in a grafting experiment (Meng et al., 2005). Since the exact 5′ terminal sequence is critical for the infectivity of the full-length clones for RNA viruses (Boyer and Haenni, 1994), we first sought to map the 5′ terminal genomic sequence of this isolate using polyadenylated dsRNAs as templates and 5′ RACE. We obtained six clones for sequencing and sequence analysis revealed that these six clones were 98.4–100% identical to one another. The first nucleotide of five clones was a guanine (data not shown). The only exception, clone GG5′-15, had a truncation corresponding to the first 6 nts of the viral genome. We concluded that the first nucleotide of the genome of GRSPaV-GG is guanine. This conclusion is in line with the observation that all of the other five sequenced GRSPaV isolates also have a G as the first nucleotide.

Construction of full-length cDNA clones of GRSPaV and GFP-tagged variants.

In the past, two approaches have been used to test infectivity of a given infectious viral clone. In the first approach, full-length cDNA corresponding to the RNA genome of a virus is cloned into

a vector under the transcriptional control of a bacteriophage promoter. Transcripts corresponding to the viral genome are produced using an in vitro transcription system and used as the inoculum to inoculate a plant host. In the second approach, a full-length viral cDNA is cloned in a vector so that it is flanked by CaMV 35S promoter and a transcription terminator. Plasmids containing the viral cDNA can be delivered into plants through rub-inoculation, biolistic bombardment, or indirectly through agro-infiltration . The 35S promoter would be recognized by the plant transcription machinery, producing transcripts that are equivalent to the viral genomic RNAs. Viral replication would hence commence.

In this study, we took the second approach to construct full-length cDNA clones for GRSPaV. Using a seven step strategy, the first full-length clone, pRSP28, was created (Figs. 1 and S1). The cDNA copy of the viral genome was inserted in $pHST_{40}$ in such a way that its transcription was controlled by the 35S promoter and the nopaline synthase terminator. The in vivo transcripts would undergo self cleavage to produce the exact viral genomic RNA, due to the activity of the ribozyme sequence that was included in $pHST_{40}$ (Scholthof, 1999). The second construct, pRSP-GFP1, was made so that the EGFP sequence was inserted into the viral cDNA in a position upstream of the CP ORF and therefore its transcription would be driven by the native promoter for the CP subgenomic RNA (Fig. 1). To ensure transcription of the CP mRNA and to avoid instability of the progeny recombinant virus, a putative subgenomic promoter sequence was obtained through PCR from a distinct isolate, GRSPaV-BS (Meng et al., 2005). The regions that contain the putative promoter sequence in these two isolates are 24% different (data not shown). Upon restriction digestion, the PCR products were inserted into the cDNA construct upstream of the CP ORF using the KpnI site that was earlier introduced via site-directed mutagenesis (Fig. 1). Thus, a recombinant viral RNA genome tagged with the GFP sequence would be transcribed in plant cells in a way similar to that of pRSP28. Both pRSP28 and pRSP-GFP1 were introduced into various plants through rub-inoculation . To enable more efficient delivery of viral constructs into plant tissue, we subcloned inserts in both constructs into a binary vector, $pCambia_{1390}$, resulting in pRSP28-2 $_{(Cam)}$ and pRSP-GFP2 $_{(Cam)}$ (Fig. 1. And see Materials and Methods). These new recombinant plasmids were then used to inoculate plants through agro-infiltration .

The accuracy of the viral cDNA sequences in all the full-length constructs was confirmed by DNA sequencing. The genome of this isolate (GenBank accession: JQ922417) is 98% identical to GRSPaV-1, 87% to GRSPaV-SG1, 84.2% to GRSPaV-BS, 77.1% to GRSPaV-SY, and 76.4% to GRSPaV-PN, with differences evenly distributed across the genome.

Infectivity assay in grapevine

To test the infectivity of the GRSPaV clones, we used plantlets of the grapevine varieties 'Thompson' and 'Prime', which were confirmed to be free of GRSPaV based on RT-PCR analyses. Ten plantlets for each grapevine variety were inoculated with pRSP-GFP2 $_{(Cam)}$ via agro-infection . GFP fluorescence was monitored as evidence of infectivity. At 3 weeks post-inoculation, none of the plants showed GFP fluorescence. One month after agro-inoculation, 8 of the inoculated 'Thompson' plantlets and 5 of the 'Prime' plantlets were positive for GFP (Fig. 2 and not shown). These positive plantlets mostly had weak GFP confined to clusters comprising a few cells. Plantlet no. 10 of 'Thompson' had strong GFP expression near the root tip. To confirm the presence of viral RNAs in these plantlets, total RNAs were isolated from roots of these 'Thomson' plantlets and subjected to DNase I treatment and RT-PCR analysis. Seven of the 9 plantlets (plant no. 8 was excluded from
RT-PCR analysis as RNA isolation from it was unsuccessful) were positive. The sequence of the PCR products was confirmed as GRSPaV sequence through DNA sequencing. The results correlated nicely with those from fluorescence microscopy (Fig. 2B). Collectively, these data demonstrated that the GFP-tagged clone, pRSP-GFP2 $_{(Cam)}$, was infectious in its natural host, grapevine.

The grapevine plantlets that tested positive for GRSPaV by RT-PCR were transferred to a greenhouse for further acclimatization. To test if systemic movement had taken place in these grapevine plants, total RNAs were isolated from upper leaves of the plants that were infected by the infectious clone six months post-inoculation . The RNA was subjected to RT followed by nested PCR. DNA of the expected size was obtained in one of the 10 'Thompson' vines. The PCR product was confirmed to be GRSPaV-specific through sequencing. Yet, examination by

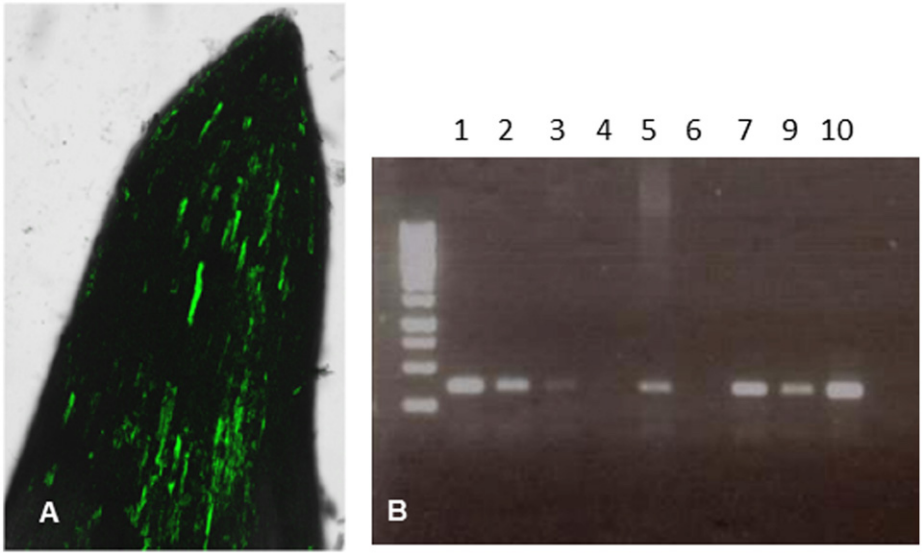

Fig. 2. Infectivity assays of pRSP-GFP2$_{(Cam)}$ in grapevine plantlets through CLSM and RT-PCR. GFP expression was observed in roots of cultivar 'Thompson' (A). Data on GFP expression matched nicely with RT-PCR results (B).

confocal fluorescence microscopy of this GRSPaV-positive plant reviewed no fluorescence due to GFP, suggesting extremely low virus titer or loss of the GFP sequence.

Infectivity assay in N. benthamiana through agro-infiltration

Because *N. benthamiana* is by far the most commonly used herbaceous host for many plant viruses (Goodin et al., 2008), we first attempted to infect *N. benthamiana* plants through rub-inoculation . However, repeated attempts were unsuccessful as judged by lack of symptoms and green fluorescence, as well as negative RT-PCR results (Meng et al., 2009 and data not shown). These data suggested that rub-inoculation was not suitable for initiating infection in *N. benthamiana* using cDNA clones of GRSPaV. To increase efficiency of inoculation, we then attempted inoculation through agro-infiltration . This method could achieve high levels of inoculation efficiency as agrobacteria can deliver T-DNA containing viral cDNA into a majority of plant cells. Again, upon agro-infiltration with pRSP28-2 $_{(Cam)}$, no symptoms were observed on either the infiltrated leaves or on the upper non-inoculated leaves of *N. benthamiana* up to three months post-inoculation .

Transmission electron microscopy was used to search for virions formed as a result of the infectivity of the viral clone. Extracts were obtained from infiltrated leaves as well as from upper, non-infiltrated leaves at 5 dpi, followed by EM observation. In total, 38 filamentous virions were observed on four grids. These virions range in length from 296 to 875 nm, with a majority (47%) of the virions falling in the size range between 700 and 799 nm (Fig. 3). This data is consistent with the distribution pattern of GRSPaV virions reported earlier from naturally infected grapevines (Petrovic et al., 2003). We found that addition of the fixative glutaraldehyde to the leaf extract appeared to stabilize the virions. However, no particles were ever observed in extracts made from upper, non-infiltrated leaves. To verify the identity of these virions, immunogold labeling using GRSPaV-specific antiserum, As7-276, was performed, followed by electron microscopy. As shown in Fig. 3B, these virions were clearly labeled with gold particles, confirming their identify as GRSPaV. Observation of

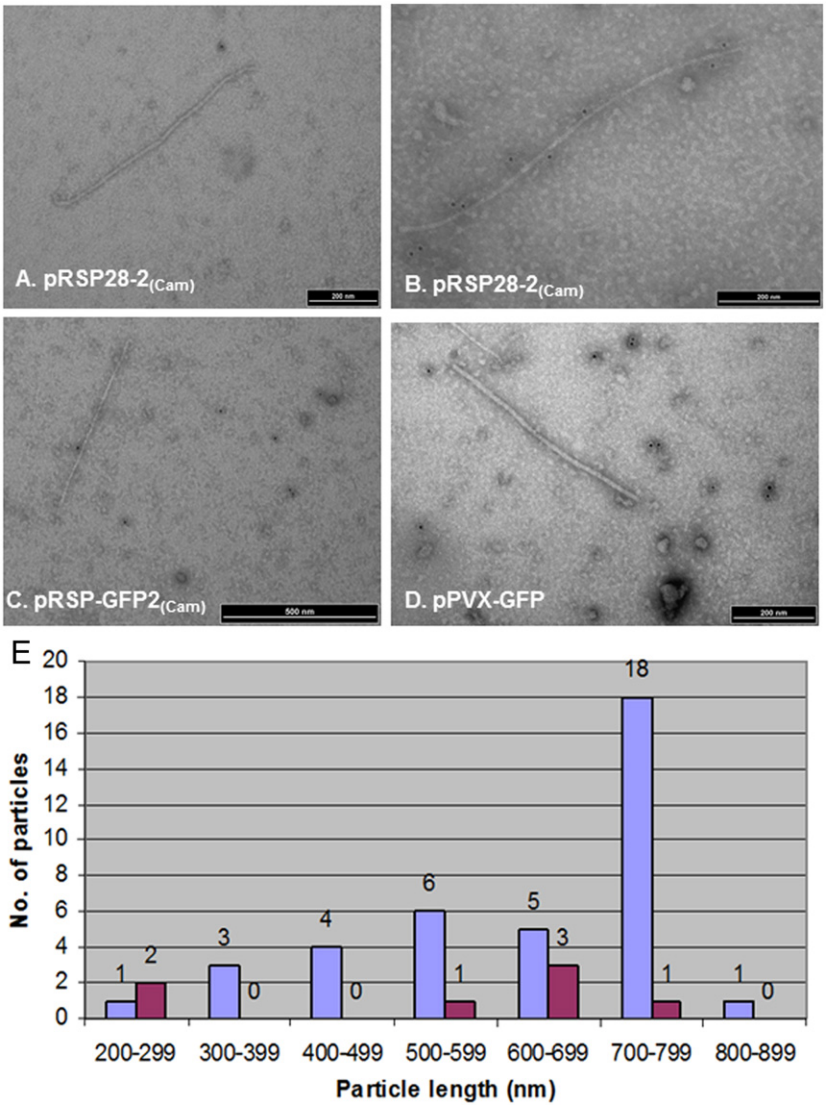

Fig. 3. Electron micrographs of virions observed in agro-infiltrated plants. Virions shown were from *N. benthamiana* plants infiltrated with pRSP28-2$_{(Cam)}$ (A-B), pRSP-GFP2$_{(Cam)}$ (C), or pPVX-GFP (D). Size distribution of virions generated by pRSP28-2$_{(Cam)}$ and pRSP-GFP2$_{(Cam)}$ is shown in E. Blue bars represent virions produced by pRSP28-2$_{(Cam)}$, while red bars denote virions produced by pRSP-GFP2$_{(Cam)}$. Numbers of virions in each size group is also given. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

GRSPaV virions demonstrates that this viral clone was indeed infectious, at least in the infiltrated leaves of *N. benthamiana*.

Electron microscopy of extracts from leaves infiltrated with pRSP-GFP2 $_{(Cam)}$ at 5 dpi also revealed the presence of virions indistinguishable from those produced by pRSP28-2 $_{(Cam)}$ (Fig. 3C). These data suggest that this GFP-tagged viral clone is also infectious in *N. benthamiana* leaves upon agro-infiltration . However, the number of virions produced by pRSP-GFP2 $_{(Cam)}$ was much lower than that from pRSP28-2 $_{(Cam)}$-infiltrated leaves. For example, we examined 4 grids for samples infiltrated with pRSP28-2 $_{(Cam)}$ and observed a total of 38 virions. In sharp contrast, we examined a total of eight grids and found only seven virions on three of the grids for samples infiltrated with pRSP-GFP2 $_{(Cam)}$. Reasons for this large discrepancy are not understood. The most likely possibility is that the heterologous subgenomic promoter for the coat protein inserted in pRSP-GFP2 $_{(Cam)}$ might be less effective than the native promoter. This putative promoter was derived from a distinct isolate, GRSPaV-BS, and differs by 24% from the

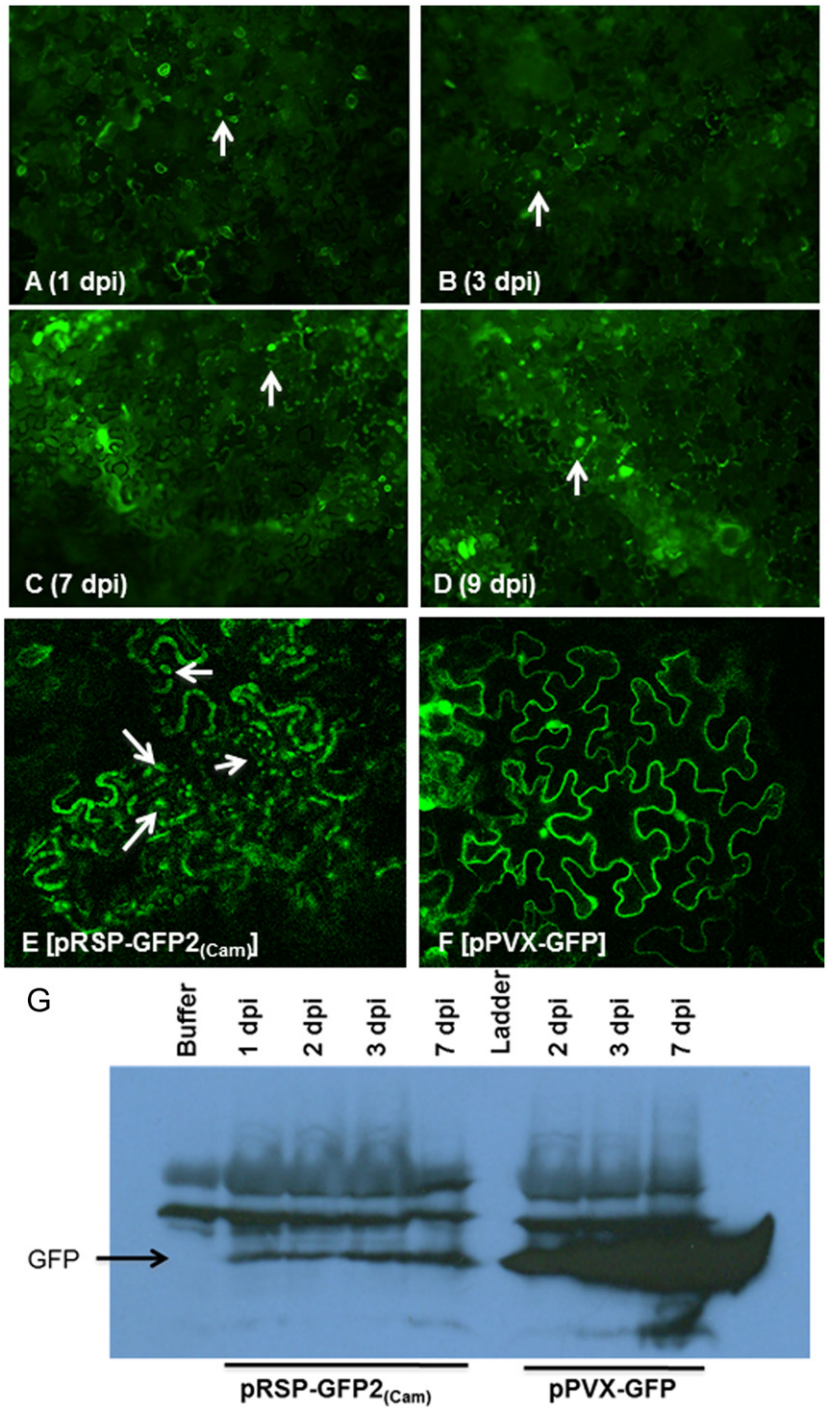

Fig. 4. Time-course analysis of infectivity of pRSP-GFP2$_{(Cam)}$ in *N. benthamiana* as judged by intensity of GFP fluorescence and Western blotting. Images representing GFP expression at different time points are shown in A-E. (A) 1 dpi; (B) 3 dpi; (C) 7 dpi; (D) 9 dpi. Confocal images of *N. benthamiana* infiltrated with pRSP-GFP2$_{(Cam)}$ (E) and pPVX-GFP (F), were captured at 8 dpi. GFP fluorescence was observed surrounding the cell periphery and the nuclei for pPVX-GFP (F). In contrast, GFP fluorescence due to pRSP-GFP2$_{(Cam)}$ was seen in cell periphery and in globular bodies (E). Result of Western blotting analysis using anti-GFP antibodies is shown in G. GFP was detected in *N. benthamiana* plants infiltrated with pRSP-GFP2$_{(Cam)}$ (left lanes) or pPVX-GFP (right lanes).

Table 1
Agro-infiltration of four commonly used herbaceous plant species for viral cDNA clones of GRSPaV. For each plant, 2 leaves (1 partially expanded and 1 fully expanded) were infiltrated with buffer or agrobacterial suspension containing either pRSP28-2$_{(Cam)}$ or pRSP-GFP2$_{(Cam)}$.

Plant species	Growth stage	Number of plants inoculated with			RT-PCR result	
		Buffer	pRSP28-2$_{(Cam)}$	pRSP-GFP2$_{(Cam)}$	PDS	GRSPaV
Nicotiana benthamiana	6	1	4	4	+	−
N. benthamiana-MP	6	1	4	4	+	−
N. occidentalis 37B	6–7	1	4	4	+	−
N. clevelandii	8–9	1	4	4	+	−

corresponding region in GRSPaV-GG . This sequence may not be readily recognized by the replicase enzymes encoded by GRSPaV-GG, leading to inefficient transcription of mRNA for CP. Alternatively, the inserted sequence may contain only part of the promoter sequence required for maximum transcription. It is also interestingly that only 1 virion fell in the size range of 700–799 and 3 virions fell in the size range of 600–699 (Fig. 3E).

To corroborate the EM results, plants infiltrated with pRSP-GFP2 $_{(Cam)}$ were monitored for GFP expression at different time points using fluorescence microscopy. Strong green fluorescence was observed in patches of epidermal cells of infiltrated leaves starting at 1 dpi (Fig. 4A). The green fluorescence was distributed mainly along the cell periphery (Fig. 4A–D). It is noteworthy that numerous fluorescent globular structures were also observed in these cells (Fig. 4A–D , arrows). Intensity of GFP signal increased with time, reached a peak at 7 dpi, and remained at similar intensity at 9 dpi (Fig. 4). To achieve a higher resolution, *N. benthamiana* leaves 7 dpi were observed under confocal laser scanning fluorescence microscope, which revealed the general GFP fluorescence as well as the globular structures (Fig. 4E). Similar to EM observations, GFP fluorescence was never observed in upper, non-infiltrated leaves throughout the entire course of observation. As a control, agrobacteria containing pPVX-GFP was also infiltrated into *N. benthamiana* leaves. Typical and uniform green fluorescence was observed in both the infiltrated and the upper non-infiltrated leaves (Fig. 4F).

To further verify the above findings, crude protein extracts were obtained from the leaves of *N. benthamiana* plants that were inoculated with pRSP-GFP2 $_{(Cam)}$ or pPVX-GFP and tested by Western blotting. As shown in Fig. 4G, protein bands corresponding to
GFP were clearly detected in *N. benthamiana* leaves infiltrated with pRSP-GFP2 $_{(Cam)}$ as early as 1 dpi. The intensity of GFP slowly increased with time and reached a maximum by 7 dpi (Fig. 4G). However, GFP expression levels of pRSP-GFP2 $_{(Cam)}$ were considerably lower compared to those of pPVX-GFP in the infiltrated leaves (Fig. 4G). As expected, GFP was not detected in control plants mock-infiltrated with buffer (Fig. 4G). These data further confirmed that pRSP-GFP2 $_{(Cam)}$ was capable of replication as GFP could only be expressed on a subgenomic RNA, which is an indicator of the biological activity of the viral clone.

Search for a herbaceous Host for systemic infection of GRSPaV

To search for a herbaceous host that would support systemic infection of GRSPaV, plants of four *Nicotiana* species were inoculated with clones pRSP28-2 $_{(Cam)}$ and pRSP-GFP2 $_{(Cam)}$ through agro-infiltration: *Nicotiana occidentalis* 37B, *Nicotiana clevelandii*, *N. benthamiana*, and transgenic *N. benthamiana*-MP expressing the movement protein of *Red clover necrotic mosaic virus* (RCNMV) (Vaewhongs and Lommel, 1995). These plants are among the most susceptible systemic hosts for a wide range of plant viruses with different origin, genome structure and expression strategies (Goodin et al., 2008). For example, *N. occidentalis* 37B can be infected through mechanical inoculation, albeit with difficulty, and serves as a systemic host for *Apple stem pitting virus*, the type member of the *Foveavirus* genus (King et al., 2011; Koganezawa and Yanase, 1990). Inclusion of transgenic *N. benthamiana* was based on the observation that RCNMV MP complemented movement of several diverse viruses which were rendered defective in movement, including PVX and *Tobacco mosaic virus* (Morozov et al., 1997; Giesman-Cookmeyer et al., 1995). In total, nine separate experiments were conducted involving some or all of these plant species in each experiment. Table 1 shows the result of one of these experiments in which all four *Nicotiana* species were tested in a single experiment.

Inoculated plants were monitored for development of possible symptoms and for GFP expression. No symptoms or GFP were ever detected on any of these plants. To test the possibility that GRSPaV may have moved to, and were present in, the upper non-inoculated leaves without showing symptoms or GFP fluorescence, total RNAs were isolated from a young leaf on top of each of the plants at 24 dpi and tested by RT-PCR using virus-specific primers RSP21 and RSP22. As internal control, primers PDS853F and PDS1352R that are specific to the plant gene phytoene desaturase (*pds*) were also used in RT-PCR . While RT-PCR using PDS-specific primers produced strong amplification of the expected DNA for all but one of the plants, GRSPaV-specific amplification was not detected in any of the samples (Table 1 and data not shown). These results demonstrated that viruses derived from pRSP28-2 $_{(Cam)}$ or pRSP-GFP2 $_{(Cam)}$ were unable to move systemically in any of these four *Nicotiana* species.

Co-infection with PVX does not enable GRSPaV to move systemically in N. benthamiana

Both GRSPaV and PVX genomes encode the TGB movement proteins, which are highly similar in modular structure and thus in biological function. Because PVX readily infects *N. benthamiana* both locally and systemically, we hoped that PVX would enable GRSPaV to move systemically in mixed infection. To test this possibility, agrobacteria containing pRSP28-2 $_{(Cam)}$ and pPVX-GFP were mixed, and infiltrated into *N. benthamiana* leaves. As controls, *N. benthamiana* plants were also infiltrated with buffer. Total RNAs were isolated from upper, non-infiltrated leaves at 9, 14, 27, and 150 dpi and subjected to RT-PCR using GRSPaV-specific (RSP21 and RSP22) and PVX-specific (PVX6440 and PVX6972) primers. While PVX RNA was consistently detected in all the samples tested, GRSPaV RNA was never detected in any of the samples (data not shown). This data suggested that GRSPaV systemic movement was not complemented by the movement function encoded by PVX in the context of co-infection .

Discussion

GRSPaV is a recently identified virus in the genus *Foveavirus* (family *Betaflexiviridae*) and is widespread in grapevines (Meng and

Gonsalves, 2007). Overall, information on the biology of members of the *Foveavirus* genus is scarce. In this study, we constructed a full-length clone for GRSPaV and its GFP-tagged variant. Through *Agrobacterium*-based inoculation, we have obtained compelling evidence that both viral clones are infectious in grapevine and *N. benthamiana*. GFP expression from pRSP-GFP2 $_{(Cam)}$ was clearly detected using fluorescence microscopy and Western blotting in both inoculated grapevine varieties and in *N. benthamiana* plants. RT-PCR detected GRSPaV-specific RNAs from grapevine plantlets inoculated with pRSP-GFP2 $_{(Cam)}$. Electron microscopy and immuno EM revealed GRSPaV virions in inoculated leaves of *N. benthamiana*. The availability of an infectious cDNA clone based on GRSPaV would be the first step for various in-depth investigations on the molecular and cellular mechanisms of different phases of the life cycle of GRSPaV and perhaps other foveaviruses.

One of our initial goals was to identify a herbaceous plant species that would support systemic infection of GRSPaV, which would serve as a productive experimental system for various studies on GRSPaV including genome replication and transcription, and cell-to-cell and long distance movement. Such a herbaceous host system would be advantageous over the natural host, grapevine, in that it has a much shorter life cycle and is amenable to inoculation and various assays. To this end, we attempted repeatedly to inoculate four of the most commonly used herbaceous hosts via rub-inoculation and agro-infiltration . Unfortunately, none of these plants could support systemic infection by this virus. Two possibilities exist for the lack of systemic infectivity of viruses derived from these viral cDNA clones in these herbaceous plants. The GRSPaV isolate we used to build the full-length clones does not cause systemic infection in these plant species in the first place. This can be true as not all plant RNA viruses systemically infect these plant species. If this is the case, the data would demonstrate the uniqueness of GRSPaV and reinforce the notion that GRSPaV may have been an ancient virus that has long co-existed with, and adapted only to, grapevine (Meng et al., 2006). Alternatively, the cDNA clones may contain detrimental mutations that impair the ability of the progeny viruses to move systemically in these herbaceous plant species. Such mutations could have originated from the initial source materials as RNA viruses are prone to mutations and exist as quasispecies in naturally infected plant hosts. It is interesting to note that *N. benthamiana* is by far the most widely used experimental host as it is susceptible to a larger number of diverse viruses, including several viruses that naturally infect woody perennials. For example, *Grapevine virus A* and *Grapevine virus B* (both members of *Vitivirus*, family *Betaflexiviridae*), potexviruses, and the closely related carlaviruses all cause systemic infections in *N. benthamiana*. Furthermore, *N. occidentalis* 37B is a systemic host for ASPV (Koganezawa and Yanase, 1990), the type member of *Foveavirus* to which GRSPaV also belongs. It will be logical to replace large genomic segments of this isolate with corresponding regions from other isolates. It may be also useful to test if GRSPaV infects other herbaceous plants that have been used as experimental hosts for plant RNA viruses.

The inability of GRSPaV to move systemically in *N. benthamiana* plants could not be complemented even by movement proteins of two other viruses which are known to complement other movement-defective viruses: (1) the *Red clover necrotic mosaic virus* MP expressed in transgenic *N. benthamiana* (Giesman-Cookmeyer et al., 1995); (2) PVX MP expressed in the form of mixed infections. If the lack of systemic movement is not due to mutations as described above, it would be interesting to identify the region of the GRSPaV genome that restricts its systemic movement in *N. benthamiana*. The first candidate is the TGB, which encode the movement proteins. This possibility could be tested by engineering a recombinant virus in which GRSPaV TGB is replaced with its counterpart from the closely related *Potato virus S* (genus *Carlavirus*, family *Betaflexiviridae*). We have recently constructed an infectious cDNA clone for PVS (Meng et al., unpublished data).

The availability of the infectious GRSPaV clones offers the possibility to address the long-standing question: what disease(s) is(are) caused by GRSPaV? A consistent association between GRSPaV and RSP has been established by different research groups (Meng et al., 1998; Zhang et al., 1998; Meng et al., 1999a, 1999b; Nolasco et al., 2000; Nakaune et al., 2008). Recent data points to the possibility that GRSPaV may also be involved in two other diseases, 'GVN' (Bouyahia et al., 2005, 2009) and 'Syrah decline' (Habili et al., 2006; Lima et al., 2006; Al Rwahnih et al., 2009). It is possible that infection with different strains of GRSPaV may manifest different symptoms. The availability of the infectious viral clones produced in this study as well as those for other GRSPaV strains (to be constructed using a similar approach) would provide a final resolution to this complex enigma. This can be achieved through inoculating virus-free plants derived from *V. rupestris* "St. George", "Richter 110R" and "Syrah" with cDNA clones corresponding to different GRSPaV strains, followed by monitoring for symptoms indicative of each of the diseases.

A major technical obstacle in studies of grapevine viruses is to develop highly effective methods for delivering infectious clones into the woody hosts. Until now, the only effective method is co-cultivation of micropropagated plantlets with agrobacteria carrying a viral clone in the form of a recombinant plasmid (Muruganantham et al., 2009; Kurth et al., 2012). While this method has proven effective, it takes a long time before the virus can be detected. Developing alternative strategies for more efficient delivery of infectious clones into grapevines will be crucial for various studies and practical applications such as developing GRSPaV into vectors for protein expression and VIGS. This line of research is currently being pursued in our laboratory and through collaboration with others.

In closing, we report here on the development of first infectious cDNA clones for GRSPaV or the genus *Foveavirus*. The availability of these viral clones enables studies on the mechanisms for replication and movement of GRSPaV. Information generated from GRSPaV would help advance the study of other members of the *Foveavirus* and perhaps of viruses that infect grapevines and other woody plants.

Materials and methods

Determination of 5′ terminal genomic sequence

DsRNAs were isolated from cambium scrapings of *Vitis riparia* cv. 'Grande Glabre', polyadenylated and subjected to 5′ RACE following Meng et al. (2005). Polyadenylated dsRNAs were reverse transcribed with SuperScript II and primer 28F3 (Table S1). Resulting cDNAs were PCR amplified by using primers dT and 28F3, positive clones were selected and sequenced using M13 primers.

Construction of full-length cDNA clone of GRSPaV and its GFP-tagged variant in pHST$_{40}$

Primers used for the construction of the full-length clone and its GFP-tagged variant are given in Table S1. dsRNAs were reverse transcribed using Superscript II (Invitrogen) and primer RCDNA3EN for 5 h at 42 °C. An outline for the construction of the first cDNA clone is shown in Fig. S1B. cDNA fragments corresponding to the 5′ half of the viral genome were amplified using AccuTaq LA polymerase (Sigma) with primers RSPV5ndpHST and 5halfCla. Resulting PCR products were first cloned in pGEM-T Easy to produce pRSP5′$_{(TV)}$, followed by subcloning into pBlueScript KS through XbaI and ClaI

sites, generating pRSP5′$_{(KS)}$. Similarly, cDNA fragments corresponding to the 3′ half of the viral genome were obtained using primers 3halfCla and NRSP3END. Resulting PCR products were first cloned into pGEM-T Easy to produce pRSP3′$_{(TV)}$. The insert in pRSP3′$_{(TV)}$ was released through double digest with ClaI and KpnI and subcloned into pRSP5′$_{(KS)}$, producing pRSP$_{(KS)}$ containing the full-length viral cDNA.

We first sought to subclone the full-length viral cDNA into pHST$_{40}$ for direct delivery into plants through rub-inoculation (Scholthof, 1999). pHST$_{40}$ contains CaMV 35S promoter, a multiple cloning site, followed by the ribozyme sequence from *Hepatitis D virus* and the Nos terminator. To achieve this, a cDNA fragment corresponding to the 5′-terminal 1420 nts of the viral genome was amplified with KOD Hot Start polymerase (Norgen Biotek Corp) using primers RSPV5′ and Bam1420. The PCR products were digested with BamHI and cloned into pHST$_{40}$ digested with StuI and BamHI, producing intermediate plasmid pRSP5′-58. Subsequently, the remaining 3′ region of the viral cDNA was released from pRSP$_{(KS)}$ via digestion with SpeI (located at nt position 1167 of the viral genome) and SmaI and cloned into pRSP5′-58, generating the full-length clone pRSP28 (Fig. S1).

Construction of a GFP-tagged viral clone in pHST$_{40}$

The KpnI site in pRSP3′$_{(TV)}$ (Fig. S1) was altered via site-directed mutagenesis (SDM) with primers DelKpnF and DelKpnR, resulting in pRSP3′T-DelK1. Subsequently, BamHI-AgeI-KpnI sites were introduced into pRSP3′T-DelK1 upstream of the CP ORF via SDM using primers RSP.BAK and RSP.KAB, resulting in plasmid pRSP3′T-BAK1. Putative subgenomic promoter sequence for the CP gene in isolate GRSPaV-BS was amplified with primers SGP-BSF and SGP-BSR . After KpnI digest, the PCR products were introduced into the KpnI site of pRSP3′T-BAK1, producing pRSP3′bsCP-16. Afterwards, EGFP sequence was amplified using primers GFP-BamF and GFP-BamR and inserted at the BamHI site of the above plasmid to produce pRSP3′GFP. Finally, cDNA fragment flanked by ClaI and SmaI in pRSP3′GFP was excised through restriction digest and used to replace the corresponding fragment in pRSP28, resulting in the GFP-tagged cDNA clone, pRSP-GFP1 .

Construction of viral clones in a binary vector

cDNA fragment corresponding to CaMV 35S promoter and the first 1420 nts of the viral genome was amplified from pRSP28 with primers 35S-Xba and Bam1420. After digestion with XbaI and SpeI, PCR product was cloned into plasmid 35S:mTalin-GFP (a derivative of pCambia$_{1390}$, Wang et al., 2004) resulting in intermediate plasmid pCamRSP5′-3. SmaI site was introduced into pCamRSP5′-3 via SDM using primers QCSmaF and QCSmaR, producing pRSP5′Sm-3$_{(Cam)}$ (not shown). Subsequently, the cDNA fragment between SpeI and SmaI was released from pRSP28 or pRSP-GFP1 through restriction digest and sub-cloned into pRSP5′Sm-3$_{(Cam)}$, producing full-length clones pRSP28-2 $_{(Cam)}$ and pRSP-GFP2 $_{(Cam)}$, respectively (Fig. 1).

Inoculation of N. benthamiana via rub-inoculation and agro-infiltration

For rub-inoculation, two young leaves of *N. benthamiana* were dusted with carborundum, followed by gently rubbing leaves with a finger using 15 μl of plasmid. Plants were kept in a growth chamber at 25 °C, monitored for symptoms, GFP fluorescence, and presence of viral RNA with RT-PCR . Agro-infiltration was performed using *Agrobacterium tumefaciens* strain EHA105 carrying either pRSP28-2 $_{(Cam)}$ or pRSP-GFP2 $_{(Cam)}$. Briefly, agrobacteria were cultured in LB medium containing rifampicin (25 μg/ml) and kanamycin (50 μg/ml) at 30 °C with shaking until reaching OD$_{600}$ of 1. Agrobacterial cells were pelleted at 5600 × *g* and washed in infiltration buffer. Agrobacterial cells were then resuspended in infiltration buffer containing 100 μM of acetosyringone and adjusted to OD$_{600}$ of 1. After induction for 3 h, agrobacteria were infiltrated into 2–3 leaves of *Nicotiana* plants at the 6 leaf stage. Agrobacteria containing pPVX-GFP constructed in the mini-binary vector pCB$_{301}$ (Xiang et al., 1999; Mann and Meng, unpublished data) was also used as a positive control.

Inoculation of grapevine plantlets via agro-infection .

Procedures for inoculating grapevine plantlets followed that of Muruganantham et al. (2009). *Vitis vinifera* plantlets were grown in Woody Plant Medium under sterile conditions and maintained at 26 °C with a 16 h photoperiod. Virus infections of micropropagated *V. vinifera* plantlets were achieved by inoculating the roots with *A. tumefaciens* EHA105 containing pRSP-GFP2 $_{(Cam)}$.

RNA isolation and RT-PCR .

Total RNAs of *Nicotiana* plants were isolated using the TRIzol reagent (Invitrogen) following the manufacturer's instructions. For RT-PCR, 1-5 μg of total RNA was denatured in the presence of oligo dT primer at 80 °C for 5 min, followed by reverse transcription using MMLV reverse transcriptase (Promega) at 42 °C for 2 h. Resulting cDNAs were amplified using primers specific for GRSPaV, PVX-GFP or the reference gene *pds* (Table S1). Total grapevine RNAs were extracted using methods of Chang et al. (1993). For RT-PCR, grapevine RNAs were first treated with DNAase I (Promega), followed by cDNA synthesis with random hexamers and PCR with primers GRSPV-F1 and GRSPV-R1 (Table S1).

Western blotting.

Infiltrated leaves were collected at different time points, ground in liquid nitrogen and then in 2.5 volumes of TMPDG buffer (Hayes and Buck, 1990) containing 2% Triton X-100, 0.5% NP40 and 7 M urea. Protein extracts were separated on 6–20 % gradient polyacrylamide gels and transferred to PVDF membrane. Extracts from *N. benthamiana* plants infiltrated with agrobacteria containing pPVX-GFP and buffer were used as positive and negative controls. Subsequent steps followed methods described in Meng et al. (2003). Equal loading was confirmed by Coomassie staining of the gel.

Fluorescence microscopy.

Small sections were taken from leaves of agro-infiltrated plants and placed up-side down on a glass slide for GFP observation using Leica DM4500B epifluorescence microscope (Leica Microsystems, Wetzlar, Germany). Confocal microscopy was performed using a Leica DM RE microscope in connection with Leica TCS SP2 system and software LCS version 2.61.

Electron microscopy and immunogold labeling.

Infiltrated *N. benthamiana* leaves were collected and macerated in 50 mM Tris–HCl buffer (pH7.5). A drop of the crude sap was placed on an EM grid, incubated for 5 min, and stained with 1% uranyl acetate. pPVX-GFP-infiltrated plants were used as positive control. Decoration was done using GRSPaV-specific As7-276 (Meng et al., 2003; Petrovic et al., 2003), followed by immuno-gold labeling. Briefly, extract was obtained from 2 infiltrated leaves with 750 μl of 0.1 M phosphate buffer (pH7.2) containing 2% PVP (Petrovic et al., 2003), followed by centrifugation for 10 min at 10,000 × *g*. Nickel grids covered with formvar

and carbon was placed, film side down, on top of a drop of the extract. After 5 min, grids were blotted and placed on a drop of blocking solution (0.3% nonfat milk in 0.1 M phosphate buffer) and incubated for 15 min. The grids were then placed on antibody As7-276 diluted 1:50 in blocking solution, and incubated for 30 min. After 3 washes with blocking solution, grids were incubated for 30 min with gold labeled anti-rabbit antibody diluted 20 ×, followed by 3 washes each with phosphate buffer and water. Grids were stained and viewed under a Philips CM-10 TEM.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2012.09.045.

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