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Differential requirement of ribosomal protein S6 by plant RNA viruses with different translation initiation strategies

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ABSTRACT

Potyvirus infection has been reported to cause an increase in the mRNA transcripts of many plant ribosomal proteins (r-proteins). In this study, increased expression of r-protein mRNA transcripts was determined to occur in *Nicotiana benthamiana* during infection by potyviruses as well as a tobamovirus demonstrating that this response is not unique to potyviruses. Five r-protein genes, *RPS6*, *RPL19*, *RPL13*, *RPL7*, and *RPS2*, were silenced in *N. benthamiana* to test their roles in viral infection. The accumulation of both *Turnip mosaic virus* (TuMV), a potyvirus, and *Tobacco mosaic virus* (TMV), a tobamovirus, was dependent on *RPL19*, *RPL13*, *RPL7*, and *RPS2*. However, TMV was able to accumulate in *RPS6*-silenced plants while accumulation of TuMV and *Tomato bushy stunt virus* (TBSV) was abolished. These results demonstrate that cap-independent TuMV and TBSV require RPS6 for their accumulation, whereas accumulation of TMV is independent of RPS6.

Introduction

Viruses are obligate intracellular pathogens that must parasitize their host's translational machinery to produce structural and non-structural proteins from their genomic and/or messenger RNAs (mRNAs). Plant cells possess three distinct sets of ribosomes in the cytoplasm, chloroplasts, and mitochondria, and it is the cytosolic ribosomes on which viral RNAs are translated. The cytosolic ribosomes of the model plant *Arabidopsis thaliana* comprise four ribosomal RNA molecules (rRNAs) and 80 unique ribosomal proteins (r-proteins) encoded by 251 genes, and thus, many plant r-proteins are encoded by small gene families (Barakat et al., 2001; Chang et al., 2005; Nakao et al., 2004). Among the 80 unique r-proteins, 32 are predicted to be present in the small 40S ribosomal subunit and the other 48 in the large 60S subunit. This localization has been validated for 79 of the r-proteins by proteomic studies of purified *A. thaliana* ribosomes (Carroll et al., 2008; Chang et al., 2005). In general, the r-proteins of cytosolic ribosomes in eukaryotes are highly conserved (Lecompte et al., 2002; Wilson and Nierhaus, 2005). For example, the homologous r-proteins from *A. thaliana* and rat share 66% amino acid identity on average (Barakat et al., 2001).

R-proteins have been identified and named by their association with the small or large ribosomal subunit and their order of appearance on 2-D protein gels (Kaltschmidt and Wittmann, 1970). R-proteins are considered to be distinct from translation initiation factors, because they are not found free in the cytosol and are present in a stoichiometric ratio with one another and ribosomes. One general function ascribed to r-proteins is RNA chaperone activity thought to assist in proper folding of specific domains of the rRNA subunits (Semrad et al., 2004). However, multiple lines of evidence demonstrate that r-proteins are not merely scaffolds needed to maintain the structure of mature ribosomes. Some r-proteins appear to possess regulatory functions in fundamental processes related to the cell cycle, apoptosis, development, and oncogenesis (Bee et al., 2006; Campagnoli et al., 2008; Chen and Ioannou, 1999; Choesmel et al., 2008; Farrar et al., 2008; Gazda et al., 2008; Jeon et al., 2008; Kobayashi et al., 2006; Lindstrom, 2009; Neumann and Krawinkel, 1997; Panic et al., 2007; Sasaki et al., 2000).

Currently, limited information is available on the functional relationship between r-proteins and virus infection. A genome-wide screen of *Drosophila melanogaster* genes demonstrated the critical role of r-proteins in virus accumulation (Cherry et al., 2005). RNA silencing of 66 *D. melanogaster* r-protein genes, including *R-protein S6* (*Rps6*) and *Rpl19*, had deleterious effects on *Drosophila C virus* (DCV) accumulation, and silencing *Rps6* and *Rpl19* in human cells affected the accumulation of poliovirus. DCV and poliovirus utilize cap-independent translation initiation strategies that are mediated by internal ribosome entry sites (IRES). However, the depletion of Rps6 or Rpl19 did not block the replication of *Vesicular stomatitis virus* (VSV), which produces mRNAs with a 7-methyl guanosine (m^7Gppp) cap (Cherry et al., 2005). These results suggest that animal viruses using cap-independent translation initiation mechanisms are more dependent on the ribosomal proteins.

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In studies related to plant viruses, the P6 protein of the plant DNA virus *Cauliflower mosaic virus* (CaMV) was found in a complex with ribosomal proteins. CaMV P6 interacts with about a dozen proteins from a ribosomal fraction, including r-proteins RPL18, RPL24, and RPL13, as well as the translation initiation factor eIF3 (Bureau et al., 2004). It was proposed that these interactions may be involved in the re-initiation of translation of polycistronic CaMV RNAs although the functional significance is yet to be determined. In the case of geminiviruses, RPL10A and RPL18AB were found to interact with nuclear shuttle protein interacting kinase (NIK), and subsequently RPL10A was shown to be phosphorylated by NIK, which is a virulence target of the begomovirus nuclear shuttle protein (Rocha et al., 2008). *rpl10A* loss-of-function mutants were more susceptible to an attenuated begomovirus suggesting that RPL10A is a component of the antiviral defense pathway mediated by NIK. In regard to plant RNA viruses, an interesting phenomenon was observed recently when the mRNA transcripts of large groups of r-protein genes were found to be up-regulated in response to the potyviruses *Turnip mosaic virus* (TuMV) in *A. thaliana* (Yang et al., 2007) and *Plum pox virus* (PPV) in *Nicotiana benthamiana* (Dardick, 2007). The increased expression of suites of r-protein genes in response to virus infection is consistent with their co-regulated expression across a variety of other conditions (Jen et al., 2006), and it suggests potential roles for these proteins in modulating potyvirus infection.

The induction of r-protein genes in response to potyvirus infection coupled with the RNA silencing studies in animal cells and the high conservation of eukaryotic r-proteins led us to further investigate the expression and necessity of r-proteins in plant RNA virus interactions. The data presented here demonstrate a specific role of the r-protein, RPS6, in the accumulation of cap-independent potyviruses and tombusviruses. This result is consistent with the role of RPS6 reported in animal host–virus interactions. In contrast to the animal host–virus systems, the other four r-proteins that were tested, including RPL19, were required for infection by plant viruses regardless of their translation strategies.

Results

RPS6 and RPL19 loss-of-function mutants in A. thaliana

Since plant viruses utilize cytosolic ribosomes for protein synthesis and mRNA transcript levels of specific r-protein genes are induced, we decided to determine if *RPS6* and/or *RPL19* were required for plant–virus infection. There are two *RPS6* paralogs in *A. thaliana* named *RPS6A* and *RPS6B* and three *RPL19* paralogs named *RPL19A*, *RPL19B,* and *RPL19C* (AGI locus names are provided in Table 1). To test the functions of *RPS6* and *RPL19* in TuMV infection, we obtained lines carrying T-DNA insertions in the coding and/or intron sequences of each of the five genes (Table 1). Homozygous T-DNA insertion mutants were recovered for *RPS6B*, *RPL19B*, and *RPL19C*, but not for *RPS6A* and *RPL19A* suggesting that these two genes are essential. The rosette leaves of *rps6B*, *rpl19B*, and *rpl19C* mutants were rub-inoculated just prior to bolting with TuMV-GFP and observed daily until 14 days post inoculation (dpi), but no difference in the distribution of GFP fluorescence or symptom severity was observed when compared to wild type Col-0 plants (Fig. 1S). This experiment was repeated twice with four to five plants per genotype in each replicate. The ability of the *rps6B*, *rpl19B*, and *rpl19C* mutants to support TuMV accumulation might be explained by functional redundancy with *RPS6A* and *RPL19A*.

Table 1
A. thaliana T-DNA lines and corresponding oligonucleotide primers used to determine zygosity.

T-DNA line	Locus	Gene	Insertion	Oligonucleotide sequence
SALK_061539	At4g31700	*RPS6A*	Exon	F:AAAATCTTACAGTTTCTGGTCATCG R:TCCGTGATTGAAACAGGTTGCTTA
SALK_048825	At4g31700	*RPS6A*	Intron	F:GGCATCCAGTAGTTGGATTCGCA R:CGATTCCGTGATTGAAACAGGTTG
SALK_012147	At5g10360	*RPS6B*	Exon	F:AGGCTCTCTCAGGAAGTTAGCG R:GCCTGCACATGGAAGCCTCATA
SALK_099890	At1g02780	*RPL19A*	Intron	F:CCGTCGCAGACAAACACAGGACA R:ACAATTCTCATTGGGTTGAAACATTAT
SALK_013042	At3g16780	*RPL19B*	Exon	F:AGGGTCGTCACTCTGGATACGG R:TTGTTGAGGTGCGCCAGCAGGA
SALK_042253	At4g02230	*RPL19C*	Exon	F:ACGTCCCTTTCTCTTGGCTATATT R:CTCGGTCTCGTGCTTGTTTTGG

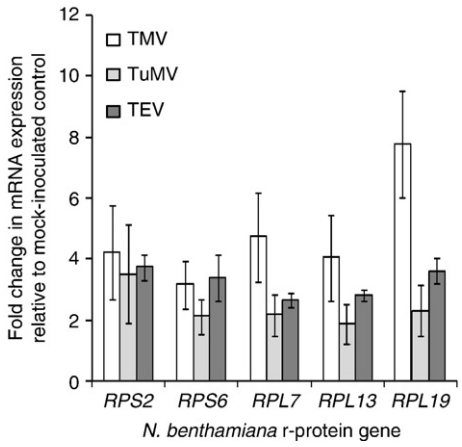

Fig. 1. Fold change in expression of *N. benthamiana* r-protein mRNAs in response to the potyviruses, TuMV and TEV, and the tobamovirus, TMV. The average fold change in r-protein mRNA expression was determined in systemic leaves sampled at 5 dpi by qRT-PCR from three independent biological replicates. The average normalized expression level of r-protein mRNAs in leaf samples treated with each virus was divided by the average normalized expression level for the mock-inoculated control. The standard error is indicated by the vertical bars.

Expression of r-protein genes in N. benthamiana in response to potyviruses and the tobamovirus, TMV

Because we did not succeed in generating a complete set of heritable loss-of-function mutations for the *RPS6* and *RPL19* isoforms, we decided to use the transient loss-of-function approach of virus-induced gene silencing (VIGS) in *N. benthamiana*. The rationale for using VIGS is that this method is transient, allowing essential genes to be targeted, and it is effective against very similar paralogous sequences. Before VIGS experiments were initiated in *N. benthamiana*, we determined if TuMV infection induced r-protein mRNA expression. *Tobacco etch virus*, another potyvirus, and TMV, a tobamovirus, were also used to test whether this was a potyvirus-specific response. *N. benthamiana* plants were rub-inoculated with infectious leaf sap or mock treated with non-infectious leaf sap. Five genes, *RPS2*, *RPS6*, *RPL7*, *RPL13*, and *RPL19* were tested by quantitative real-time PCR (qRT-PCR), and all genes were induced in response to the three viruses in systemic leaves at 5 dpi (Fig. 1). These data demonstrate that both potyviruses and the tobamovirus can induce the expression of r-protein genes in *N. benthamiana*.

Characterization of RPS6- and RPL19-silenced N. benthamiana plants

To test if *RPS6* or *RPL19* is required for TuMV and TMV infection, these genes were silenced in *N. benthamiana* by using the *Tobacco rattle virus* (TRV) VIGS vector (Dinesh-Kumar et al., 2003; Liu et al., 2002). *N. benthamiana* plants in which *RPS6* or *RPL19* were silenced by VIGS were characterized by chlorotic leaves and completely arrested growth. The plants remained viable up to 45 dpi although necrosis was observed sporadically and especially on the oldest leaves of plants infected by TRV::RPS6 (Fig. 2C). These phenotypes are in contrast to infection by the

TRV empty vector (TRV::00) that causes mild mosaic symptoms and reduced growth from which the plants recover to resemble non-inoculated control plants (Fig. 2C; side view at 45 dpi). All subsequent studies used only the third (+3), fourth (+4), and fifth (+5) leaf above the TRV inoculated leaves, which were the first and second fully expanded leaves on each plant. For the TRV empty vector control and wild type control, the leaves corresponding to the same developmental positions were collected for assays or inoculated. Furthermore, VIGS plants were not used if they had visible areas of necrosis on the +3, +4, or +5 leaves at the time of assays and inoculations.

To confirm that the VIGS constructs effectively silenced the r-protein genes, Northern blot and immunoblot analyses were performed at 14 dpi. Northern blot analysis showed that the mRNA transcripts of *RPS6* and *RPL19* were reduced in the VIGS plants when compared to non-treated or TRV::00 plants (Fig. 2A). Immunoblot analysis using the maize RPS6 antibody demonstrated that RPS6 protein was reduced to undetectable levels by TRV::RPS6 (Fig. 2B). *RPL19* silencing also resulted in decreased RPS6 protein levels (Fig. 2B). A pair-wise comparison of the nucleic acid sequences of *RPL19* and *RPS6* showed that these genes do not share sufficient stretches of sequence identity that could support simultaneous VIGS of both genes (data not shown). In support of the sequence comparison, qRT-PCR analyses showed that *RPL19* and *RPS6* mRNA transcripts were not cross silenced. The mRNA transcripts of *RPS6* were induced 2.20 fold when *RPL19* was silenced and the mRNA transcripts of *RPL19* were induced 3.18 fold when *RPS6* was silenced (Table 2). Therefore, the reduced accumulation of RPS6 protein in *RPL19*-silenced leaves was due to some post-transcriptional cause.

To determine if overall ribosome content was depleted in *N. benthamiana* leaves in which *RPS6* or *RPL19* had been silenced, total ribosomes were purified from the leaves of six non-infected plants or six plants from each of the TRV:00, TRV::RPS6, and TRV::RPL19 treatments. The average, normalized yields from two replications of this experiment were determined in non-infected (97 ± 11 ng ribosomes/mg fresh wt), TRV::00 (158 ± 96 ng ribosomes/mg fresh wt), TRV::RPS6 (157 ± 3 ng ribosomes/mg fresh wt), and TRV::RPL19 (95 ± 59 ng ribosomes/mg fresh wt) leaves. These results indicate that ribosome concentrations are not severely depleted in *RPS6*- and *RPL19*-silenced plants as compared to the controls.

The effects of RPS6 and RPL19 silencing on TuMV, TMV, and TBSV infection in N. benthamiana

To determine the effect of *RPS6* or *RPL19* depletion on virus accumulation, silenced plants as well as TRV::00 and mock-inoculated control plants were infected with TuMV-GFP. TuMV-GFP infection foci were abundant in the inoculated leaves of wild type plants or plants that were previously inoculated with the empty TRV vector after 5 dpi (Fig. 2D, panels a, b, e, f). In contrast, few or no TuMV-GFP infection foci were present in inoculated leaves of *RPS6*-silenced plants (Fig. 2D, panels c, g). These data demonstrate that RPS6 is necessary for TuMV accumulation. It is possible that *RPS6* silencing leads to general defects in host translation that directly influence early virus infection by preventing viral genes from being translated. To further test this possibility, *RPS6*-silenced plants were inoculated with TMV-GFP. Surprisingly, green fluorescent TMV-GFP infection foci developed in *RPS6*-silenced leaves with timing and intensity similar to non-silenced control plants (Fig. 2D, panels k, o). The average numbers of TuMV and TMV infection foci per cm^2 were determined on *RPS6*-silenced plants (Table 3). These data were collected from three independent replicates, and each replicate with a total of 9 to 12 leaves for each virus. For TuMV, a 99% decrease in foci was observed on *RPS6*-silenced leaves as compared to the TRV::00 empty vector control. In contrast, the number of TMV infection foci per cm^2 decreased only 12% in the *RPS6*-silenced leaves when compared to the TRV::00 empty vector control (Table 3). These results demonstrate that TuMV and TMV have different requirements for RPS6. Further, it demonstrated that certain RNA species can be effectively translated in host plant cells deficient in RPS6. We also challenged *RPS6*-silenced plants with TBSV-GFP, a tombusvirus whose RNA has no cap or VPg at the 5′ end and no 3′ poly (A) tail. Similar to TuMV-GFP, TBSV-GFP foci were reduced 97% in *RPS6*-silenced plants demonstrating that TBSV also requires RPS6 for infection (Table 3).

RPL19-silenced plants were inoculated with TuMV-GFP, TBSV-GFP, or TMV-GFP, and few or no GFP infection foci were visible in the inoculated leaves, which was in contrast to the wild type and TRV:00 empty vector controls (Fig. 2D, panels d, h, l, p; Table 3). These data showed that *RPL19* is required for the accumulation of all three viruses tested. Because the silencing of both *RPS6* and *RPL19* prevented the accumulation of TuMV and TBSV, we conclude that both r-proteins are essential for TuMV and TBSV infection. However, TMV accumulation is dependent on RPL19 but not RPS6.

The plants were monitored up to 35 dpi for signs of systemic infection characterized by GFP fluorescence in systemic tissues. Systemic TuMV-GFP fluorescence was observed in only one *RPS6*-silenced plant at 33 dpi in the first replication of the experiment. Systemic TMV-GFP fluorescence was observed at 11 dpi for one plant in the first replicate and at 34 dpi for two plants in the third replicate but not in the second despite the presence of numerous TMV-GFP infection foci on the inoculated leaves in *RPS6*-silenced plants. Systemic GFP fluorescence was not accompanied by additional symptoms, which is not unexpected given the severe phenotype caused by r-protein silencing. No systemic infection was observed for either virus in the *RPL19*-silenced plants. Systemic movement of TBSV-GFP was not evaluated, because it can only establish a local infection (H. Scholthof, personal communication).

Agrobacterium infiltration of TuMV and TMV into RPS6- and RPL19-silenced leaves

The experiments above used infectious leaf sap containing virions as inoculum for TuMV-GFP and TMV-GFP. The interpretation of these data could be complicated by the co-translational disassembly of virions (Wilson, 1984), which could be blocked in *RPS6* and/or *RPL19*-silenced plants. This potential complication could be overcome by using infectious viral RNA as an inoculum source as was done for TBSV-GFP. To determine if the inoculum source could account for the different requirement for RPS6, *N. benthamiana* plants were first inoculated with TRV::00, TRV::RPS6, and TRV::RPL19, and 14 days later we inoculated systemic leaves with TuMV-GFP and TMV-GFP by *Agrobacterium* infiltration, which results in the production of infectious RNA transcripts in the recipient cells. In agreement with the sap inoculation experiments, TMV-GFP fluorescence in *RPS6*-silenced leaves developed similarly to non-silenced control plants, and much weaker green fluorescence were observed on *RPL19*-silenced plants at 8 dpi (Fig. 3A). Very weak to no GFP fluorescence was detected after *Agrobacterium* infiltration of TuMV-GFP in the *RPS6* and *RPL19*-silenced plants, whereas intense green fluorescence was observed in the non-silenced control plants (Fig. 3B). As with TMV-GFP, the TuMV-GFP results mirrored those from the sap inoculation experiments. This experiment demonstrated that bypassing virion uncoating still resulted in attenuation of TuMV infection in *RPS6*- or *RPL19*-silenced plants and TMV infection in *RPL19*-silenced plants, and therefore, prevention of uncoating was not the mechanism through which virus accumulation was abolished.

Silencing of RPS2, RPL13 and RPL7

To investigate the roles of other r-proteins in host–virus interactions, we designed three additional TRV VIGS constructs to silence *RPS2*, *RPL13* and *RPL7*. We also note here that RNA silencing of the orthologs of these three genes in *D. melanogaster* demonstrated that they are essential for DCV replication (Cherry et al., 2005). Silencing of

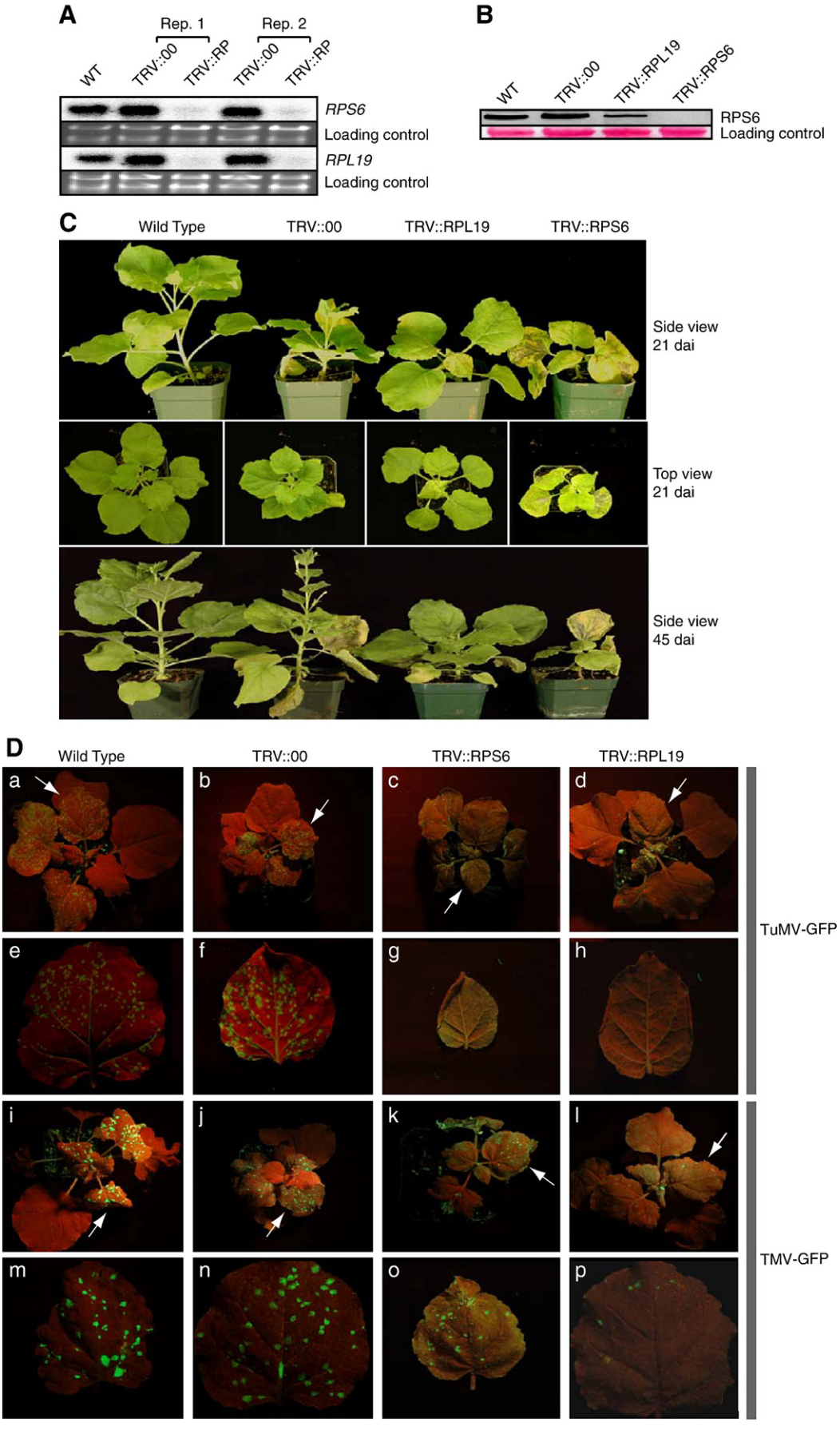
A
Rep. 1
Rep. 2
WT
TRV::00
TRV::RP
TRV::00
TRV::RP
RPS6
Loading control
RPL19
Loading control
B
WT
TRV::00
TRV::RPL19
TRV::RPS6
RPS6
Loading control
C
Wild Type
TRV::00
TRV::RPL19
TRV::RPS6
Side view
21 dai
Top view
21 dai
Side view
45 dai
D
Wild Type
TRV::00
TRV::RPS6
TRV::RPL19
a
b
c
d
e
f
g
h
TuMV-GFP
i
j
k
l
m
n
o
p
TMV-GFP

Table 2
Fold change in r-protein mRNA expression at 14 dpi with the empty TRV vector and TRV VIGS constructs.

TRV VIGS Construct	Fold change in mRNA expression relative to mock-inoculated plants				
	RPS2	RPS6	RPL7	RPL13	RPL19
TRV::00	1.09 ± 0.12	0.82 ± 0.28	1.01 ± 0.46	1.10 ± 0.46	1.19 ± 0.56
TRV::RPS2[a]	–	2.51 ± 0.93	3.09 ± 1.54	3.18 ± 1.46	2.58 ± 0.59
TRV::RPS6[a]	3.07 ± 1.75	–	3.50 ± 1.21	4.60 ± 1.31	3.18 ± 1.98
TRV::RPL7[a]	4.95 ± 1.17	3.56 ± 1.11	–	9.01 ± 2.08	4.91 ± 1.89
TRV::RPL13[a]	4.68 ± 2.32	3.81 ± 0.99	4.30 ± 1.32	–	7.79 ± 3.88
TRV::RPL19[a]	5.91 ± 1.26	2.20 ± 1.29	3.09 ± 1.49	4.95 ± 1.75	–

[a] All fold changes in mRNA expression values are significant when each of the TRV VIGS silencing constructs is compared to the TRV::00 empty vector control ($p<0.05$, *t*-test).

RPS2, *RPL7*, and *RPL13* was confirmed by Northern blot analysis (Fig. 4A), and plant growth was completely arrested as observed for *RPS6*- and *RPL19*-silenced plants (Figs. 4C and 2D). However, we could distinguish two distinct classes of phenotypes among the five target genes. In 5 independent repeats, each with 9 plants, silencing of *RPS6* and *RPL7* resulted in completely stunted growth, more intense chlorosis and spontaneous necrosis on the oldest leaves, and the newest leaves were planar. In contrast, the newest leaves of *RPL19*-, *RPL13*- and *RPS2*-silenced plants were epinastic and had mosaic patches of chlorosis in addition to the completely stunted growth. These observations suggest that depleting the r-proteins may restrict plant growth and development by different mechanisms. qRT-PCR analyses showed that silencing each of the r-proteins did not result in cross silencing of the other r-proteins of interest. In general, the silencing of any individual r-protein led to an increase in mRNA expression of the others by 2 to 9 fold (Table 2). An immunoblot analysis was also performed to detect the RPS6 protein level in *RPL13*-, *RPL7*-, and *RPS2*-silenced plants. Interestingly, RPS6 was reduced in *RPL13*- and *RPL7*-silenced plants while silencing of *RPS2* did not reduce RPS6 accumulation when compared to control plants at 14 days after silencing (Fig. 4B).

RPS2-, *RPL7*-, and *RPL13*-silenced plants were infected with TuMV-GFP and TMV-GFP and the numbers of infection foci were quantified in inoculated leaves. Silencing of all three genes resulted in a dramatic reduction of both TuMV and TMV infection foci (Fig. 4D, Table 3). These data demonstrate that RPS2, RPL7, and RPL13 are required for accumulation of both TuMV and TMV as was RPL19. Systemic fluorescence was observed in only one replication of this experiment for TuMV-GFP and TMV-GFP in *RPL13*-silenced plants at 15 dpi.

Expression of PR-1 mRNA in TRV empty vector and r-protein-silenced plants

The appearance of spontaneous necrosis on some of the VIGS-treated plants led us to investigate if a defense-like response was activated that could potentially suppress the infection of the three different viruses that were tested. The *PR-1* gene is a useful marker for local and systemic acquired resistance responses mediated by salicylic acid (Malamy et al., 1990; Metraux et al., 1990). qRT-PCR showed that *PR-1* was induced an average of 48.3 fold by the empty TRV vector when compared to the corresponding leaves on mock-inoculated plants (Fig. 5). The induction of *PR-1* by TRV is not unexpected, because many viruses are known to induce weak defense-like responses during compatible interactions (Whitham et al., 2006). *PR-1* mRNA was also induced in the *RPS6*, *RPL7*, *RPL13*, and *RPL19*-silenced plants (Fig. 5), but the observed fold changes were not significantly different from the empty vector ($p>0.10$, *t*-test). The comparable or reduced levels of *PR-1* gene expression demonstrate that a local or systemic acquired resistance response does not explain the lack of virus accumulation in the r-protein silenced plants.

Discussion

RPS6 is necessary for TuMV and TBSV accumulation but not TMV accumulation in N. benthamiana inoculated leaves

The induction of r-protein mRNA expression occurs in response to potyviruses in *N. benthamiana* and *A. thaliana* (Dardick, 2007; Yang et al., 2007). Here, we showed that the induction of r-protein mRNA expression is not unique to potyviruses, because the tobamovirus TMV was also able to induce this response. These expression analyses led us to determine if depletion of *N. benthamiana* r-proteins would affect viral infection, and we found that TuMV, TMV, and TBSV infection was typically abrogated. The necessity of a particular r-protein is not correlated with the ribosomal subunit in which it resides. Silencing *RPS2* from the small ribosomal subunit or *RPL7, RPL13,* or *RPL19* from the large ribosomal subunit prevented the accumulation of both TuMV and TMV. Most interestingly, *RPS6*-silenced plants supported TMV accumulation in the inoculated leaves but not TuMV or TBSV demonstrating the differential viral requirement for RPS6 — a host translational component that is critically involved in potyvirus and tombusvirus infections but not in tobamovirus infections.

The silencing of ribosomal proteins has pleiotropic effects on plant cells and the viruses themselves encode activities that could potentially affect the interpretation of the results presented here. Some VIGS plants developed spontaneous necrosis, which could potentially lead to the establishment of systemic acquired resistance as has been observed for lesion mimic mutants (Lorrain et al., 2003). However, expression of *PR-1* mRNA, a marker for systemic acquired resistance, was not significantly greater in the r-protein silenced lines when compared to infection by the empty TRV vector. This result demonstrated that establishment of systemic acquired resistance does not underlie the inability of the VIGS plants to support viral infection. Another possible explanation for the differential viral responses on RPS6-silenced plants is that TMV is more efficient at using fully intact ribosomes that might remain in the silenced cells compared to TuMV or TBSV. However, this explanation is expected to be applicable when any of the r-protein genes are silenced, and thus, similar numbers of TMV-GFP infection foci should have been observed in the other r-protein-silenced plants as well. Alternatively, the overall ribosome content of the r-protein-silenced plants might have been dramatically reduced in r-protein-silenced plants, which might be tolerated better by TMV. Quantification of ribosomes indicated that there were at least as many ribosomes in *RPL19*- and *RPS6*-silenced plants as in mock-treated controls providing further evidence that differences in virus infection were due to loss of specific r-protein genes as opposed to an overall decrease in cellular ribosome concentrations.

On the virus side of the equation, TuMV encodes HC-Pro, which is a strong suppressor of both established and nascent gene silencing (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998). The tobamoviruses encode a silencing suppressor

Fig. 2. VIGS of *RPS6* and *RPL19* in *N. benthamiana*. (A) Northern blot analysis of *RPS6* and *RPL19* mRNA transcript levels in wild type and TRV::00 control plants and TRV::RPS6 or TRV::RPL19 silenced plants at 14 dpi. Data for two independent replicates are shown. WT indicates wild type *N. benthamiana* plants that were not infected with any TRV vector. TRV::00 indicates *N. benthamiana* plants that were infected with the empty TRV vector. (B) Immunoblot analysis of RPS6 protein levels among the control and r-protein-silenced plants. In each lane, 20 μg of total protein were separated by SDS-PAGE and a maize RPS6 polyclonal antibody was used to detect the *N. benthamiana* RPS6 accumulation (Williams et al., 2003). Membranes were stained with Ponceau S to detect total proteins, and the region of rubisco is shown as a loading control. (C) The phenotypes of *RPL19*- and *RPS6*-silenced plants at 21 and 45 dpi with TRV::00 (empty vector control) or TRV carrying a fragment of the indicated r-protein gene. (D) TuMV-GFP and TMV-GFP infection foci in representative leaves at 5 dpi in *RPL19*-, and *RPS6*-silenced and control plants. This time point corresponds to 19 days after TRV agroinoculation. The white arrows indicate the leaves that are shown in the corresponding close-up photographs.

Table 3
TuMV-GFP, TMV-GFP, TBSV-GFP infection foci per cm^2 in r-protein-silenced plants.

Virus	TRV VIGS constructs											
	–[a]	TRV::00[a]	TRV::RPS6[b]		TRV::RPL19[b]		TRV::RPS2[b]		TRV::RPL7[b]		TRV::RPL13[b]	
	Foci/ cm^2	Foci/ cm^2	Foci/ cm^2	Decrease %[c]	Foci/ cm^2	Decrease %[c]	Foci/ cm^2	Decrease %[c]	Foci/ cm^2	Decrease %[c]	Foci/ cm^2	Decrease %[c]
TuMV	2.15	1.32	0.011	99	0.032	98	0.19	86	0.012	99	0.097	93
TMV	1.83	1.22	1.07	12	0.12	90	0.25	80	0.012	99	0.118	90
TBSV	0.54	0.47	0.0135	97	0.107	77	–	–	–	–	–	–

–No test was conducted.
[a] The infection foci per cm^2 is the average from twelve leaves.
[b] The infection foci per cm^2 is the average from nine leaves.
[c] The percentage of decrease is compared to foci per cm^2 of TRV empty vector control plants.

that can prevent nascent silencing but has not been reported to suppress established gene silencing as does HC-Pro (Kubota et al., 2003). Thus, the accumulation of TMV-GFP in *RPS6*-silenced plants cannot be explained by the possibility that TMV is a better suppressor of established VIGS than TuMV. These well-characterized abilities of the viral silencing suppressors demonstrate that differential phenotypes observed for TuMV and TMV infection foci in inoculated leaves are not artifacts of the RNA silencing suppressor activities of each virus. Furthermore, it would be unexpected if TMV could specifically suppress the silencing of *RPS6* but not the other r-protein genes. Therefore, the most likely conclusion is that TMV does not have a specific requirement for RPS6 but TuMV and TBSV do.

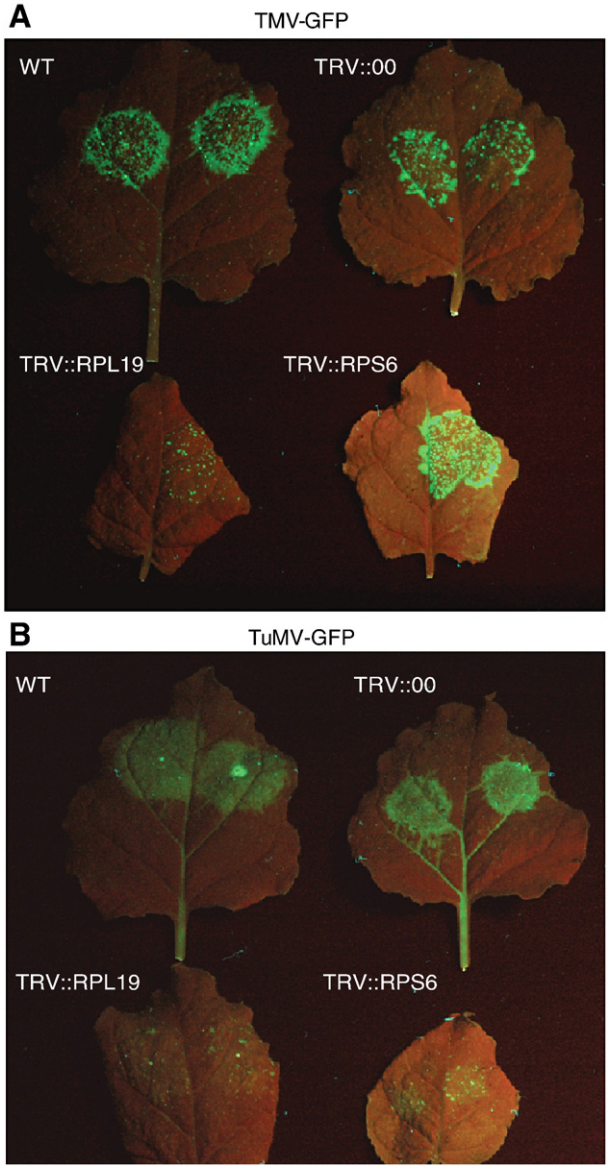

Fig. 3. Effects of silencing *RP* genes on TuMV and TMV accumulation in agroinoculated leaves. (A) TMV-GFP infection in *RPS6*- and *RPL19*-silenced and control plants at 8 days after agroinoculation. (B) TuMV-GFP infection in RPS6- and RPL19-silenced and control plants at 8 days after agroinoculation. This time point corresponds to 21 days after TRV agroinoculation.

The differential effects of RPS6 depletion on tobamoviruses versus potyviruses and tombusviruses may be related to the distinct mechanisms by which these viruses initiate translation. TuMV is a potyvirus that utilizes a cap-independent translation mechanism. Potyviruses lack a 5′ m^7Gppp cap, and instead possess a covalently linked 5′ genome-associated viral protein (VPg). Using TEV as a model potyvirus, it has been shown that cap-independent translation is mediated by the 143 bases in the 5′ leader that have properties consistent with an IRES (Gallie, 2001; Niepel and Gallie, 1999). The IRES functions have been assigned to one of two pseudoknots in the TEV leader and appear to be mediated through interactions with the translation initiation factor eIF4G (Ray et al., 2006; Zeenko and Gallie, 2005). There is also evidence indicating that an IRES mediates the translation of TuMV RNA (Basso et al., 1994). TBSV also lacks a 5′ m^7Gppp cap and utilizes a cap-independent translation mechanism that is not associated with an IRES (Wu and White, 1999). In contrast, TMV genomic and subgenomic RNAs all possess 5′ m^7Gppp caps similar to cellular mRNAs (Guilley et al., 1979; Richards et al., 1978). Another distinction is that the 5′ untranslated region of TMV contains a translation enhancer element known as the Ω leader (Sleat et al., 1987). Because TMV translation is probably facilitated by both the m^7Gppp cap and the Ω leader as well as other factors, we cannot reach the absolute conclusion that the dependence upon RPS6 is related to whether viral RNAs are capped or not. This will be an interesting hypothesis to test in future experiments.

The results obtained for RPS6 in plants are consistent with those from *D. melanogaster* and human cells. In these cells, Rps6 is required for DCV and poliovirus, which both utilize cap-independent translation that is initiated by IRESs in their 5′ untranslated regions (Cherry et al., 2005). The depletion of Rps6 from *D. melanogaster* cells did not block the replication of the unrelated VSV, which produces mRNAs with an m^7Gppp cap. The depletion of several r-proteins, including Rpl19, showed this differential effect on DCV versus VSV replication when silenced in *D. melanogaster* cells. However, our study showed that plant viruses with both capped and uncapped RNAs generally require r-proteins for their accumulation. *RPS6*-silenced plants were an exception in which the accumulation of TuMV was abolished whereas formation of TMV infection foci on inoculated leaves was only slightly reduced when compared to non-silenced TRV control plants.

Of the five r-proteins that were silenced, RPS6 is the most likely to participate in translation initiation (Helps et al., 1995; Lee et al., 1983; Noll et al., 1978; Nygard et al., 1987; Todokoro et al., 1981). RPS6 is located in the small head region of the cytosolic 40S ribosomal subunit, and there is evidence that it can interact with mRNAs, tRNAs, translation initiation factor (eIF2), and the 28S rRNA suggesting that it might be involved in initiation of translation (Nygard and Nilsson,

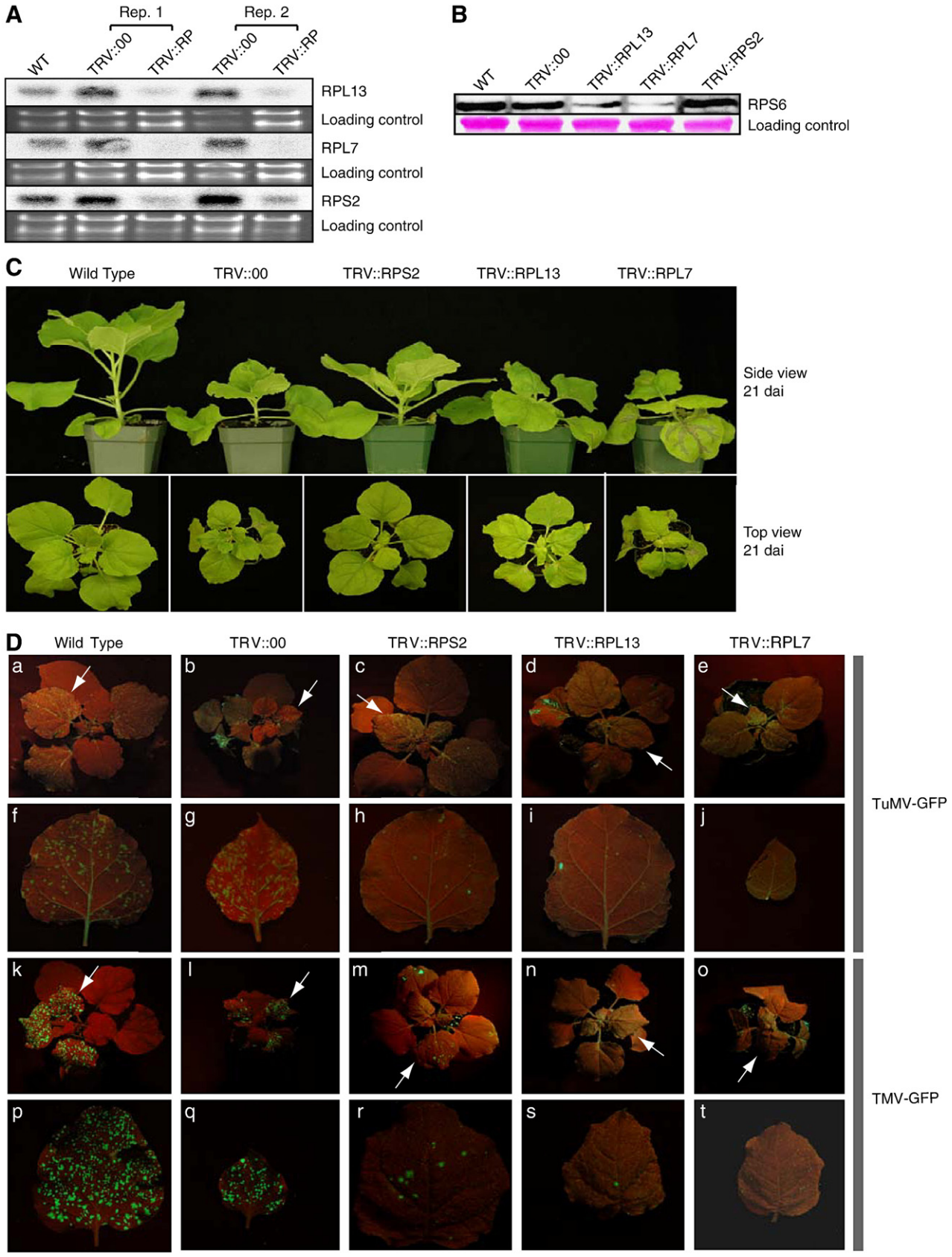

Fig. 4. VIGS of *RPL13*, *RPL7*, and *RPS2* in *N. benthamiana*. (A) Northern blot analysis of *RPL13*, *RPL7*, and *RPS2* mRNA transcripts levels in wild type and TRV::00 control plants and TRV::RPL13, TRV::RPL7, or TRV::RPS2 silenced plants at 14 dpi. Data for two independent replicates are shown. WT indicates wild type *N. benthamiana* plants that were not infected with any TRV vector. TRV::00 indicates *N. benthamiana* plants that were infected with the empty TRV vector. (B) Immunoblot analysis of RPS6 protein levels in the control and r-protein-silenced plants. (C) The phenotypes of *RPL13*-, *RPL7*-, and *RPS2*-silenced plants at 21 dpi with TRV::00 or TRV carrying fragments of the indicated r-proteins. (D) TuMV-GFP and TMV-GFP infection foci in representative leaves at 5 dpi in *RPS2*-, *RPL13*- and *RPL7*-silenced and control plants. This time point corresponds to 19 days after TRV agroinoculation. The white arrows indicate the leaves that are shown in the corresponding close-up photographs.

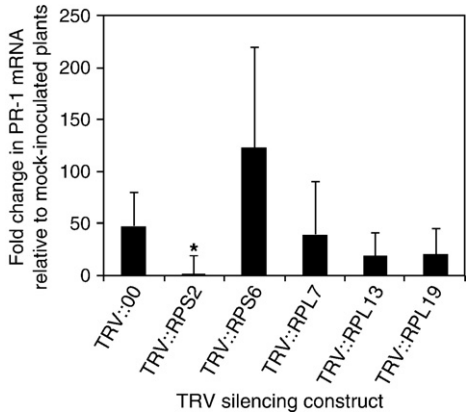

Fig. 5. Expression of *PR-1* mRNA in response to the TRV::00 empty vector and the r-protein silencing constructs. The average fold change in *PR-1* mRNA expression was determined by qRT-PCR from three independent biological replicates. The average normalized expression level of *PR-1* mRNA in leaf samples treated with each TRV construct was divided by the average normalized expression level for the mock-inoculated control. The error bars display the standard deviation for the three replicates. *PR-1* expression was quantified in the same biological replicates that were used to generate data presented in Table 2. * indicates a statistically significant difference from TRV::00 ($p<0.05$, *t*-test).

1990). RPS6 is one of the targets of phosphorylation by r-protein S6 kinase (S6K), a serine/threonine kinase, which is regulated by a variety of environmental and developmental stimuli including infection by numerous DNA and RNA viruses (Holz et al., 2005; Holz and Blenis, 2005; Jeon et al., 2008; Mahfouz et al., 2006; Meyuhas, 2008; Turck et al., 2004). Interestingly, alphaviruses were recently shown to both cause and benefit from diminished phosphorylation of Rps6 in human embryonic kidney cells (Montgomery et al., 2006). The relative positions of the phosphorylatable sites are conserved and clustered in the C-terminus of RPS6 (Meyuhas, 2008). In plants, RPS6 phosphorylation is altered under conditions such as cold stress, heat shock, and oxygen deprivation (Williams et al., 2003). It is currently unknown whether plant viruses can influence the phosphorylation state of RPS6.

Effects of r-protein silencing on plant phenotype were not correlated with effect on viral infection

Because some r-proteins are essential, the TRV VIGS system was used to transiently silence the expression of *RPS6*, *RPL19*, *RPL13*, *RPL7* and *RPS2*, which led to severe developmental defects in *N. benthamiana*. However, the plants remained viable and did not die up through 45 dpi with the TRV silencing constructs. These features of the plant system parallel *D. melanogaster*, in which mutations in r-protein genes cause reduced growth and cell division rates, characterized by a reduced body size and short, thin bristles (Lambertsson, 1998). In addition, *D. melanogaster* cells in which r-proteins were silenced remained viable although with reduced physiological capacities (Cherry et al., 2005).

The loss-of-function of each of the five r-proteins fell into one of two phenotypic classes. *RPS6* and *RPL7* silencing resulted in completely arrested growth with chlorosis, frequent spontaneous necrosis on the oldest fully expanded leaves, and the blades of the newest leaves were planar. *RPL19*, *RPL13*, and *RPS2* silenced plants had the epinastic appearance of the newest leaves, a mosaic pattern of chlorosis, and completely arrested growth. These phenotypic classes did not correlate with the ribosomal subunit that each protein is localized to, and we also noted that the severity of the silencing phenotype did not necessarily correspond to inhibition of virus accumulation. For example, the phenotypes of *RPL19*-plants were less severe than *RPS6*-silenced plants, but *RPL19*-silenced plants were nearly immune to both TuMV and TMV infection. *RPS6*- and *RPL7*-silenced plants had very severe and indistinguishable phenotypes, but *RPS6*-silenced plants supported TMV infection, whereas *RPL7*-silenced plants did not. These observations demonstrate that general defects in translation machinery and the corresponding effects on plant development did not necessarily cause the inhibition of virus accumulation. Future experiments that address the specificity of r-proteins in virus lifecycles are expected to provide new insight into the ways in which plant viruses interact with plant ribosomes to facilitate gene expression and replication as well as cell-to-cell and systemic movement.

Systemic infection, when it occurred, was extensively delayed and was not accompanied by additional symptoms probably because the plants already had severe developmental defects and growth had ceased. We do not interpret this result as an indication that each r-protein is required for systemic movement. Factors that make interpretation of systemic movement difficult are that VIGS is not homogeneous or completely null. Thus, if the virus is allowed to replicate and move locally in cells in which the VIGS is less effective it could establish an infection focus, but if it then encountered cells where silencing was effective, it could be blocked giving the appearance of inhibited systemic infection. The viral silencing suppressors may influence r-protein silencing if the virus is allowed to replicate, move to the vasculature, and gain access to distal cells in which the VIGS is less effective. In the case of TMV-GFP in *RPS6*-silenced plants, there were many foci per leaf and so other factors should be considered. TMV and most other viruses depend on transport through phloem for rapid systemic infection (Carrington et al., 1996; Scholthof, 2005). We expect that the upper-uninoculated leaves of the VIGS plants had ceased growing and were no longer strong sinks, and the inoculated leaves were not strong sources due to the chlorotic phenotypes that would suppress accumulation of photoassimilates. Additional clarification of the roles of r-proteins in systemic infection will require viable stable mutants (probably not based on RNA silencing), assessment of source–sink relationships, detailed microscopic analyses of the infection kinetics, and grafting studies if possible.

Materials and methods

Identification of homozygous T-DNA lines

Seeds of SALK T-DNA lines were obtained from the Arabidopsis Biological Resource Center (Ohio State University), germinated, and the seedlings were tested by PCR for the presence of the T-DNA insertion and wild type alleles (Table 1). All PCR reactions were conducted using the following thermal cycle profile: 95 °C, 30 s; 60 °C, 30 s; and 72 °C, 40 s for 30 cycles. Plants homozygous for each T-DNA

Table 4
Sequence and annealing temperature of primer pairs used for quantitative RT-PCR or synthesis of probes for Northern blot analyses.

Genes	Forward primer	Reverse primer	Temp (°C)
qRT-PCR primers			
NbActin	CCCAGGTATTGCCGATAGAA	CATCTGTTGGAAGGTGCTGA	60
NbRPS2	AAGATTGGGAAGCCACACAC	GCAGGGACCATTCTCACAGT	60
NbRPS6	TATTCAGCTGCCTCCGACTT	GGCTTGTGACACCCTTGACT	60
NbRPL7a	GCCCTATGCAGAAAGATGGA	AACACAGGGCTGAAGCAGTT	60
NbRPL13	GGATGGTCATTCCTGATGCT	TTGTCCTCGAGCTCCTTGAT	60
NbRPL19	CAGGGGATCGAAGAAGTCAA	AAGGACAAAAAGTAGGCGTAGC	60
NbPR1A	TGAGATGTGGGTCGATGAGA	CCTAGCACATCCAACACGAA	60
PCR primers for Northern blot probes			
RPS6	CGAGCTTTCTTTGACAAGAGGA	CTGACAATGCATCCACGGACA	50
RPL19	CAAAGGCTGAGAAGGCTAGAGA	TGCTCACTTCTTTGACTTCTT	50
RPL13	ATCAAGAGGATGGTCATTCCT	TCGATAACTGCAAGTTGAGGA	50
RPL7	ATGGCTCCGAAGAAGGGTGT	TCAAGTGTCTTGGTGAACTGGT	50
RPS2	GAGGAGGATTCGGCCGTGGAT	GCTTCTGAACCGGCATGATTTTCA	50

insertion were expected to have a product from forward primer LBe (5′-GGAACAACACTCAACCCTATCTCG-3′) and the reverse primers (Table 1), and no PCR product from the forward and reverse primer pairs (Table 1). Heterozygous or homozygous wild type plants yielded PCR products from forward and reverse primer sets.

Plasmid construction

The DNA sequences of *N. benthamiana RPS6*, *RPL19*, *RPL13*, *RPL7*, and *RPS2* used for TRV VIGS were amplified from cDNA using HiFi Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and the following oligonucleotide primers: NbRPS6F (5′-ACAAGTTTGTACAAAAAAG-CAGGCTGACCCACCAAATAGCAGGAA-3′) and NbRPS6R (5′-ACCACTTTGTACAAGAAAGCTGGGTCGTGAAAAAGGGTGAGAAGG-3′); NbRPL19F (5′-GGATGGTTTCATCATCAGGAA-3′) and NbRPL19R (5′-CTAGCCTTCTCAGCCTTTG-3′); NbRPL13F (5′-GATGCTCGTCACCA-TATGCT-3′) and NbRPL13R (5′-GACCATCCTCTTGATCTTGTC-3′); NbRPL7F (5′-GCTCTTAACCAGTTCACCAA-3′) and NbRPL7R (5′-CCTCA-TACTTGTCATTGAAGTT-3′); and NbRPS2F (5′-TCATGCCTGTTCAGAAAC-3′) and NbRPS2R (5′-AAGACATCATCAATACCAGC-3′). Because the *N. benthamiana* sequence for these genes was not available, sequences from *Capsicum annuum* (RPS2 (AY486481.1), RPL7a (AY496113.1), RPL13A (AY498745.1), RPL19 (AY489023.1)) or *N. tabacum* (RPS6, X68050) were used to design the primers for VIGS constructs and for qRT-PCR analyses (Table 4). The *NbRPS6* PCR product was recombined into the pDONR vector containing the attP1 and attP2 recombination sites using BP CLONASE (Invitrogen) to obtain pDONR-NbRPS6. Then pDONR-NbRPS6 was recombined into the pTRV2-attR1-attR2 destination vector using the LR CLONASE enzyme (Invitrogen; Liu et al., 2002). All other PCR products were first TOPO-cloned into the pGATE vector (Invitrogen) containing the attL1 and attL2 recombination sites prior to recombination into pTRV2-attR2-attR1.

Plant material and Agrobacterium inoculation (agroinoculation)

N. benthamiana plants were grown in 4 inch pots at 23 °C in a growth chamber with a 16 h light/8 h dark cycle. For the VIGS assay, the TRV VIGS system (Dinesh-Kumar et al., 2003) was used. Briefly, pTRV1 or pTRV2 and its derivatives were introduced into *Agrobacterium tumefaciens* strain GV2260. Overnight cultures of *Agrobacterium* were grown at 29 °C in LB medium containing antibiotics (50 mg/L kanamycin, 100 mg/L carbenicillin and 25 mg/L rifampicin), 10 mM MES, and 20 mM acetosyringone. *Agrobacterium* cells were pelleted and resuspended in infiltration media (10 mM $MgCl_2$, 10 mM MES, 200 mM acetosyringone), adjusted to 0.8 OD_{600}, and incubated at room temperature for at least 3 h. *Agrobacterium* carrying pTRV1 was mixed in a 1:1 ratio with pTRV2 or its derivatives and infiltrated into *N. benthamiana* leaves. pCB-TuMV-GFP was used for agroinoculation of TuMV-GFP. An *Agrobacterium* strain carrying the TMV-GFP cDNA under control of the 35S promoter was used for agroinoculation of TMV-GFP.

RNA isolation, RT-PCR, and Northern blots

Total RNA was isolated from *A. thaliana* or *N. benthamiana* leaves as previously described (Huang et al., 2005) and treated with RNase-free DNaseI (Invitrogen). First-strand cDNA was synthesized using 1 μg of total RNA, oligo $(dT)_{24}$ primer, and Superscript III reverse transcriptase (Invitrogen). Primers and annealing temperature used for semi-quantitative RT-PCR with 28 cycles are shown in Table 4.

Northern blot hybridizations were performed as previously described (Zhang and Ghabrial, 2006). The hybridization probes for the silenced target genes were PCR amplified from *N. benthamiana* cDNA using the oligonucleotide primers listed in Table 4. The sequences used for hybridization probes did not overlap with the VIGS inserts. Probes were labeled with α^{32}P-dCTP using the Prime-a-Gene labeling system (Promega, Madison, WI, USA), and hybridization was detected by phosphorimager (PharosFX Plus, Bio-Rad, Hercules, CA, USA) and analyzed with ImageQuant v5.2 software (Amersham, Piscataway, NJ, USA).

qRT-PCR analyses

Expression levels of r-protein genes in three biological replicates were quantified by qRT-PCR analysis using iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad, Hercules, CA) and the iQ Real-Time PCR Detection System (Bio-Rad). Ten nanograms of RNA was used in the following RT-PCR program: cDNA synthesis for 10 min at 50 °C, iScript reverse transcription inactivation for 5 min at 95 °C, PCR cycling at 95 °C for 10 s and data collection for 30 s at the extension temperature given in Table 4, and ending with a melt curve analysis. The reactions were performed in triplicate. Relative quantification was performed using the standard curve method, and transcript accumulation of each gene was normalized to the quantity of a *N. benthamiana* actin gene (AY594294). The fold change was calculated by dividing the relative expression level of the r-protein gene silenced sample by the corresponding TRV empty vector-inoculated sample.

Western blot analysis

Protein was extracted as described (Moffett et al., 2002), separated by polyacrylamide gel electrophoresis (PAGE) using 12% stacking and 15% resolving polyacrylamide with SDS running buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 1% SDS), and then transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). Protein transfer and equal loading was estimated by staining the membranes with Ponceau S solution (0.1% w/v in 5% acetic acid v/v). For immunoblot assay, the membranes were incubated in 5% non-fat dry milk/PBST blocking buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH to 7.4 with HCl, 0.1% Tween-20) for 3 h, then incubated overnight at 4 °C with antiserum of maize RPS6 diluted 1:1250 (Williams et al., 2003). The membranes were incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:10,000 dilution; Amersham, Piscataway, NJ, USA) for 1 h at room temperature. The membrane blot images were developed using an enhanced chemiluminescence system (ECL, Amersham).

Quantification of ribosomes

Total ribosomes were purified as previously described (Hollingsworth et al., 1998). *N. benthamiana* leaves were weighed, snap-frozen in liquid nitrogen, and ground into a fine powder. Each gram of tissue was mixed with 2 ml of extraction buffer (200 mM Tris–HCl pH 8.5, 200 mM KCL, 10 mM EGTA, 30 mM $MgCl_2$, 200 mM sucrose, 2.5 mM DTT, 5 μg/ml proteinase K, 50 μg/ml cycloheximide, 100 μg/ml chloramphenicol, and 0.5 mg/ml heparin), and the homogenate was filtered through two layers of Miracloth (Calbiochem). The filtrate was centrifuged at 16,000 g for 10 min. The supernatant was layered onto a 1.75 M sucrose cushion (1.75 M sucrose, 40 mM Tris–HCl, pH 9.0, 200 mM KCl, 5 mM EGTA, and 30 mM $MgCl_2$) and centrifuged at 48,000 g for 21 to 24 h. The pellets were washed twice with resuspension buffer (200 mM Tris–HCl pH 8.5, 60 mM KCl, 30 mM $MgCl_2$, 50 μg/ml cycloheximide, 100 μg/ml chloramphenicol), and then resuspended in resuspension buffer. The ribosome yield was determined based on absorption at 260 nm (1 A_{260} unit = 12.5 μg/ml (Brady and Scott, 1977)). The average yield of ribosomes from two replications of this experiment was normalized to the milligrams of fresh weight of starting leaf material.

Inoculum preparation, virus infection and GFP imaging

TuMV-GFP inoculum was prepared from leaves of *N. benthamiana* plants that had been inoculated with p35STuMV-GFP (Lellis et al.,

2002). TMV-GFP inoculum was prepared from leaves of *N. benthamiana* plants that were infiltrated with *A. tumefaciens* strain GV3101 carrying the infectious TMV-GFP genome. The TMV-GFP-infected leaves were stored in aliquots at −80 °C, and for inoculation, they were diluted 1:5 w/v in 20 mM potassium phosphate buffer pH 7.2. The TBSV-GFP cDNA clone was linearized with SmaI, and *in vitro* RNA transcripts were synthesized with the mMESSAGE mMACHINE® T7 Kit (Ambion, Austin, TX, USA). At 14 days after agroinoculation with TRV constructs, leaves of *N. benthamiana* plants with obvious VIGS loss-of-function phenotypes for *RPS6*, *RPL13*, *RPL19*, *RPL7*, or *RPS2* were dusted with carborundum and rub-inoculated with leaf sap for TuMV-GFP and TMV-GFP or with infectious RNA transcripts for TBSV-GFP. The +3, +4, and +5 leaves above the TRV agroinoculated leaves were used for all virus inoculation experiments. In each replication of these experiments, the corresponding leaves of control plants that were either not treated or infected with the TRV empty vector (TRV::00) were included. GFP was visualized by UV illumination (100-W Blak-Ray longwave UV lamp; UVP, Upland, CA, USA) and photographs were taken using a Nikon D70 digital camera fitted with a yellow Y2 filter.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.05.018.

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