Introduction

RNA silencing is an innate cellular response involved in antiviral defense. Arabidopsis calmodulin-like protein 39 (At-rgsCaM) is related to known regulators of RNA silencing in tomato and Nicotiana tabacum. Geminivirus AL2 protein functions to suppress post-transcriptional and transcriptional gene silencing, possibly through induction of an endogenous regulator. In support of this, the At-rgsCaM promoter responds to Tomato golden mosaic virus (TGMV) AL2 in protoplasts and geminivirus infection increases rgsCaM expression in Arabidopsis and Nicotiana benthamiana. Further, over-expression of rgsCaM leads to increased susceptibility to infection, as a consequence of increased viral DNA loads. It has been shown that rgsCaM may target silencing suppressors of RNA viruses for degradation via the autophagy pathway. This interaction occurs within the cytoplasm, but AL2 interacts with rgsCaM in the nucleus. It is tempting to speculate that AL2 may act to sequester rgsCaM in the nucleus to prevent targeting of AL2 for degradation.
dependent- and -independent mechanisms (van Wezel et al., 2002; Wang et al., 2005). Results from transcriptional profiling in Arabidopsis protoplasts transfected with African cassava mosaic virus (ACMV) or Mungbean yellow mosaic virus (MYMV) AL2 suggest that this protein can suppress silencing by activating expression of a cellular protein (Werner exonuclease-like 1 protein) that may function as an endogenous negative regulator of the system (Trinks et al., 2005). AL2 has also been shown to interact with adenosine kinase (ADK), which plays an important role in sustaining the methyl cycle and consequently S-adenosyl-methionine-nine-dependent methyltransferases (Hao et al., 2003). A link between silencing suppression by AL2, ADK and methylation is supported by two lines of evidence: (1) ADK activity is reduced in virus-infected tissue and in transgenic plants expressing AL2 (Wang et al., 2003; 2005), and (2) ADK-deficient plants display silencing defects (Moffatt et al., 2002). The silencing pathways are not necessarily mutually exclusive and it would be novel to discover if AL2 proteins target all three.

Geminivirus AL2 protein also functions as a transcription factor to activate expression of the viral coat protein and BR1 movement protein genes (Sunter and Bisaro, 1991, 1992; 2003; Berger and Sunter, 2013). However, AL2 does not specifically bind dsDNA and appears to be targeted to promoters by interaction with a host factor, TIFY4B, which recognizes sequences within AL2 responsive promoters (Lacatus and Sunter, 2009; Berger and Sunter, 2013). This implies that AL2 influences the expression of host genes through this interaction(s). This is supported by results demonstrating that AL2 can interact with responsive promoters integrated into the plant chromosome (Sunter and Bisaro, 1997; Lacatus and Sunter, 2008; Berger and Sunter, 2013).

Despite the key role played by AL2 in suppression of RNA silencing, we still have a limited understanding of how AL2 interacts with the host to suppress this important defense pathway. A recent study has demonstrated that the activity of βC1, the VSR encoded by the satellite DNA of China virus (TYLCCNV), is dependent on the Nicotiana benthamiana calmodulin-like protein Nb-rgsCaM, a homolog of Nt-rgsCaM. Induction of Nb-rgsCaM by βC1 resulted in suppression of RNA silencing most likely through a reduction in RNA-Dependent RNA polymerase 6 (RDR6) mRNA levels (Li et al., 2014). These findings illustrate that suppression of PTGS by βC1 is mediated through a host endogenous suppressor of RNA silencing (ESR).

In this study we extend our analysis of the geminivirus AL2 protein and its effects on host gene regulation. Expression of a calmodulin-related protein, similar to rgsCaM from N. tabacum (Anandalakshmi et al., 2000), increases in response to exogenous geminivirus AL2 protein and during geminivirus infection, and the promoter for Arabidopsis rgsCaM is responsive to geminivirus AL2 in protoplasts. Localization of rgsCaM is altered in response to AL2, providing further evidence that AL2 influences genes involved in host defense responses, specifically RNA silencing.

Results

Expression of rgsCaM is induced in response to virus infection

Based on studies that indicate the silencing suppressor HC-Pro induces expression of Nt-rgsCaM and that AL2 can suppress silencing in a transcription-dependent manner (Trinks et al., 2005), we investigated the response of rgsCaM to geminivirus infection. To perform these experiments we took several approaches. First we measured levels of RNA specific to rgsCaM in Arabidopsis plants infected with Cabbage leaf curl virus (CabrLCuV) and Beet curly top virus-A (BCTV-A) (formerly known as Spinach curly top virus), two geminiviruses that cause an infection in Arabidopsis. The protein sequence of At-rgsCaM (AT1g76640: CML39) was identified by comparison to the amino acid sequence for rgsCaM from N. benthamiana (Anandalakshmi et al., 2000). Using quantitative real time PCR (qPCR), levels of At-rgsCaM increased approximately 2- and 4-fold, at 48 h post-infection, in the presence of CabLCuV and BCTV-A respectively (data not shown). We next tested whether geminiviruses would also induce expression of rgsCaM in N. benthamiana. To do this we first identified a homolog in the N. benthamiana genome (Nb-rgsCaM) (JX402081), using a BLAST alignment with the Arabidopsis sequence. Based on the sequence we identified designed primers to amplify a specific region of the Nb-rgsCaM sequence, which was confirmed by sequencing (data not shown). Leaves of N. benthamiana plants were infused with Agrobacterium cultures containing either TGMV DNA A, CabLCuV DNA A, BCTV-A DNA or vector alone (pMON521). CabLCuV and TGMV were used in the absence of the cognate DNA B to eliminate the influence of cell-to-cell movement of the virus from the site of infection. For the monopartite curtovirus, BCTV-A, we could not eliminate movement of the virus from the site of infection as all viral genes are located on a single DNA. Total RNA was isolated one and two days post-infection (dpi) and levels of Nb-rgsCaM mRNA assessed by qPCR. For CabLCuV, the level of Nb-rgsCaM mRNA was induced between 2 and 3-fold after 1 or 2 dpi, respectively (Table 1). Levels of Nb-rgsCaM mRNA did increase at 1 dpi in the presence of both TGMV and BCTV-A DNA, but to a lesser degree than CabLCuV, and then decreased to control levels after 2 dpi (Table 1). The reason why we observed this decrease in TGMV and BCTV-A, but not CabLCuV, is unknown, but may reflect different kinetics of viral replication, and therefore timing of viral gene expression.

Expression of rgsCaM is induced early in response to virus infection

The experiments above indicate small increases in expression of Nb-rgsCaM in the presence of two begomoviruses and one curtovirus, between 1 and 2 dpi. However, as AL2 is known to activate expression of the TGMV CP gene between 18 and 24 h (Brough et al., 1992), expression of AL2 is likely prior to that time. Based on our hypothesis that AL2 is involved in inducing the expression of rgsCaM, we measured levels of Nb-rgsCaM mRNA in leaves at 4, 8, 12, 18 and 24 h after infection with TGMV DNA A, CabLCuV DNA A, BCTV-A DNA or vector alone (pMON521) as described above. Increases in Nb-rgsCaM expression were detected in the presence of all three viruses at 8 h post-infusion.

<table>
<thead>
<tr>
<th>Inoculum&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Time&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Fold-change&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock (pMON521)</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.00</td>
</tr>
<tr>
<td>CabLCuV DNA A</td>
<td>1</td>
<td>2.05 ± 0.65</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.69 ± 0.87</td>
</tr>
<tr>
<td>TGMV DNA A</td>
<td>1</td>
<td>1.66 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.10 ± 0.25</td>
</tr>
<tr>
<td>BCTV-A DNA A</td>
<td>1</td>
<td>1.20 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.97 ± 0.19</td>
</tr>
</tbody>
</table>

<sup>a</sup> Inoculum consisted of Agrobacterium (OD<sub>600nm</sub> = 1.0) containing Ti plasmids harboring 1.5 copies of wild type TGMV DNA A [F1E5-902] (Wu et al., 2012), CabLCuV DNA A (U65529) [Abouzid et al., 1992], BCTV-A (AY548948) DNA (formerly Spinach curly top virus) or empty vector (pMON521).

<sup>b</sup> Time of RNA isolation, in days, after infusion of N. benthamiana leaves with Agrobacterium containing the inoculum indicated.

<sup>c</sup> The fold-change represents an average calculated from ΔΔCt values using three technical replicates from each of two independent experiments using quantitative real time RT-PCR. ΔCt values were calculated relative to an endogenous control (EF1α). The standard error of the mean is given.
as compared to a vector control at the same time (Fig. 1). Expression continued to increase to approximately 3-fold over mock controls, between 8 and 24 h. Interestingly, for all viruses an initial reduction in rgsCaM expression of approximately 2-fold was detected at 4 h post-infusion. The findings suggest that expression of rgsCaM increases in response to the presence of both begomovirus and curtoviruses.

**AL2 induces expression of Nb-rgsCaM**

The data above is consistent with the hypothesis that geminiviruses induce the expression of an endogenous silencing suppressor. Previous work has shown that AL2 can suppress RNA silencing by both transcription dependent- and independent mechanisms (van Wezel et al., 2002; Wang et al., 2005). We therefore tested whether AL2 would specifically induce expression of Nb-rgsCaM. *N. benthamiana* leaves were infused with *Agrobacterium* containing a binary vector capable of expressing TGMV AL2 from the *Cauliflower mosaic virus* (CaMV) 35S promoter (pTGA89) (Lacatus and Sunter, 2008). Total RNA was isolated 4, 8, 12, 18 and 24 h post-infusion and levels of Nb-rgsCaM mRNA assessed by qPCR. A 2-fold increase in expression of Nb-rgsCaM was also detectable as early as 8 h post-infusion as compared to a vector control at the same time (Fig. 1). Expression continued to increase over mock controls until 12 h after which expression appears to remain constant through 24 h. Again, an initial decrease in expression was detectable 4 h post-infusion. The findings are consistent with the hypothesis that AL2 is capable of increasing steady state levels of rgsCaM mRNA in *N. benthamiana*.

**The rgsCaM promoter is responsive to AL2-mediated activation in protoplasts**

Experiments in which steady state levels of Nb-rgsCaM are shown to increase in response to AL2 could be a consequence of AL2 interaction with the promoter to increase transcription, or be due to the silencing suppression activity of the AL2 protein itself (Wang et al., 2005). We therefore decided to examine the ability of AL2 to activate the promoter for *At-rgsCaM*. As the promoter for the *At-rgsCaM* gene has not been identified, we generated a series of 5′-truncated promoters with 5′-end-points at −922, −615 and −333 bp linked to the β-glucuronidase (GUS) reporter in a translational fusion to the N-terminal 12 amino acids of the rgsCaM protein. The resulting constructs were transfected into protoplasts prepared from *N. benthamiana* suspension culture cells with either a negative control plasmid (pUC) to determine the basal activity or with DNA capable of constitutively expressing TGMV AL2 from CaMV 35S promoter (pTGA89) (Lacatus and Sunter, 2008). Total cell extracts were prepared and fluorometric GUS assays performed 3 days post-transfection as described (Sunter and Bisaro, 2003). An increase of 2 to 3-fold in GUS activity over basal levels was observed in protoplasts co-transfected with a TGMV 35S-AL2 expression plasmid and constructs containing promoter 5′-end points at −922 and −615
predominantly associated with phloem cells early in infection (Wege et al., 2001). However, our data is consistent with distribution during late stages of infection where TGMV is detected in spongy and palisade parenchyma layers of the mesophyll. In addition, eGFP containing cells occur in patches of what appear to be contiguous cells, with several non-fluorescing cells in-between, which are presumably non-infected as this was also observed in TGMV-infected tissue (Wege et al., 2001). These results suggest that increases in rgsCaM expression may be localized to tissue containing replicating virus, and not detectable in all cells. This data is also consistent with results obtained in leaf infusion assays, and demonstrates that the At-rgsCaM promoter exhibits a response to infection by TGMV, most likely a consequence of AL2 activation (Fig. 2). In contrast, no eGFP fluorescence was detectable in plants inoculated with the curtovirus BCTV-H (Table 2). To confirm these observations we isolated RNA from the transgenic At-rgsCaM promoter-reporter plants at 12 dpi and performed qPCR. As shown (Fig. 4), a small increase in promoter activity, as measured by eGFP mRNA levels, was detectable in plants infected with TGMV, as compared to uninfected plants or plants infected with a vector control (pBIN19). However, we could not detect any increase in promoter activity in response to BCTV-H (Fig. 4), confirming the results obtained by microscopy. Although the increase in expression observed with TGMV was not statistically significant, it did illustrate an upward trend, which is supported by the microscopy. These results are not necessarily surprising given the small number of cells that apparently replicate the virus (Wege et al., 2001) and that we did not enrich for eGFP containing cells.

As the silencing suppressor HC-Pro from TEV has been shown to induce expression of rgsCaM upon viral infection (Anandalakshmi et al., 1998) we also tested whether the At-rgsCaM promoter could be induced upon infection by additional RNA viruses using agroinoculation. A higher level of eGFP fluorescence was detectable in leaves infected with Potato virus X (PVX) although the signal did not appear to be localized to a specific region in the plant (Table 2). However, the promoter did not appear to be activated by Tobacco mosaic virus (TMV) (Table 2). Again, qPCR analysis confirmed that eGFP mRNA levels increased in response to infection by PVX, but no increase was detectable in plants infected with TMV (Fig. 4). Thus, induction of rgsCaM expression may be a more general response to virus infection, but does not appear to respond to all viruses tested.

Overexpression of rgsCaM leads to increased susceptibility to virus infection

To examine the biological relevance of rgsCaM to geminivirus infection, we generated transgenic N. benthamiana plants constitutively expressing either At-rgsCaM or Nt-rgsCaM from the CaMV 35S promoter. Examination of each line by RT-PCR indicated that mRNA specific to either At-rgsCaM or Nt-rgsCaM was present in 5 independent lines for each construct (data not shown). Transgenic N. benthamiana plants were agroinoculated with wild type TGMV A in the presence of TGMV DNA B, using a standard inoculum dose (OD_{500 nm} = 1.0) (Sunter et al., 2001). Characteristic symptoms typical of a TGMV infection were detected on both wild type and transgenic N. benthamiana plants (data not shown), indicating the presence of rgsCaM had no impact on symptom severity. The presence of rgsCaM also had no effect on the percentage of plants that became infected, with nearly 100% infection observed in all transgenic lines (Table 3). Plants were scored for the time to first appearance of symptoms (mean latent period) typical of a TGMV infection. In general, N. benthamiana plants transgenic for either At-rgsCaM or Nt-rgsCaM were more susceptible to infection with TGMV than non-transgenic plants as judged by a reduction in the time to first appearance of symptoms...
Under the conditions of the test, wild type plants exhibited a mean latent period of 15.3 days (Table 3). In contrast, the mean latent period for transgenic *N. benthamiana* plants expressing rgsCaM ranged from 10 to 14.3 days, depending on the transgenic line. Thus, transgenic plants inoculated with TGMV exhibited symptoms 1 to 5 days earlier than wild type plants. Analysis of the data by one-way analysis of variance (ANOVA) and Dunnett’s post-hoc test confirmed that the reductions were statistically significant in four *N. benthamiana* lines expressing At-rgsCaM and two lines expressing Nt-rgsCaM (Table 3). The evidence from these experiments strongly suggests that expression of rgsCaM, in transgenic *N. benthamiana*, results in increased virulence/pathogenicity, as measured by a decrease in mean latent period as compared to wild type plants.

**Table 2**

<table>
<thead>
<tr>
<th>Virus&lt;sup&gt;a&lt;/sup&gt;</th>
<th>rgsCaM promoter activity</th>
<th>eGFP signal&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-inoculated</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>Mock (pBIN)</td>
<td>No</td>
<td>very weak signal</td>
</tr>
<tr>
<td>TGMV</td>
<td>Yes</td>
<td>localized regions; P, V</td>
</tr>
<tr>
<td>BCTV-H</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>TMV</td>
<td>No</td>
<td>very weak signal</td>
</tr>
<tr>
<td>PVX</td>
<td>Yes</td>
<td>strong signal, no specific cells</td>
</tr>
</tbody>
</table>

<sup>a</sup> Viruses were introduced by Agrobacterium-mediated delivery as described in Materials and methods.<br><sup>b</sup> Activation of the promoter is observed by the presence of eGFP fluorescence.<br><sup>c</sup> A description of the fluorescence signal observed.

Fig. 3. Activation of the rgsCaM promoter in transgenic plants infected with TGMV. *N. benthamiana* plants transgenic for the promoter-reporter construct containing a 5′ end-point at −922 (rgsCaM ‐ 922)-GFP were inoculated with Agrobacterium containing TGMV DNA A and DNA B or mock inoculated with empty vector (pMON521). Tissue from systemic leaves exhibiting symptoms typical of a TGMV infection (bottom two rows) and mock inoculated (top row) was examined under bright-field illumination using differential interference contrast optics (DIC). GFP fluorescence was detected using a GFP narrow band filter set. A DIC ‐ GFP merged image is shown in the third column. Images in the top two rows are thick cross sections of a leaf made by hand, and images in the bottom row are leaves viewed from the top onto the upper epidermis (UE). The different cell types that exhibit eGFP fluorescence are indicated: PP = palisade parenchyma; SP = spongy parenchyma. Scale bars in the top two rows represent 20 μm and in the bottom row represents 50 μm.

Fig. 4. The At-rgsCaM promoter is activated in response to different viruses. Columns represent relative levels of eGFP mRNA in infected as compared to uninfected control leaves (Un), which was arbitrarily assigned a value of 1. Values were determined by qPCR analysis of RNA isolated from *N. benthamiana* leaves infused with Agrobacterium containing a binary vector capable of expressing TCMV, BCTV-H, PVX, TMV or empty vector (pBIN). The fold change was calculated from the mean ΔΔCt values from three independent qPCR experiments using RNA isolated 14 days post-infusion. Error bars represent the Standard Error of the mean. A Student’s t-test failed to detect any significance difference between the ΔCt values for the three experiments.

(mean latent period). Under the conditions of the test, wild type plants exhibited a mean latent period of 15.3 days (Table 3). In contrast, the mean latent period for transgenic *N. benthamiana* plants expressing rgsCaM ranged from 10 to 14.3 days, depending on the transgenic line. Thus, transgenic plants inoculated with TGMV exhibited symptoms 1 to 5 days earlier than wild type plants. Analysis of the data by one-way analysis of variance (ANOVA) and Dunnett’s post-hoc test confirmed that the reductions were statistically significant in four *N. benthamiana* lines expressing At-rgsCaM and two lines expressing Nt-rgsCaM (Table 3). The evidence from these experiments strongly suggests that expression of rgsCaM, in transgenic *N. benthamiana*, results in increased virulence/pathogenicity, as measured by a decrease in mean latent period as compared to wild type plants.
Table 3
Mean latent period for TGMV inoculated to N. benthamiana plants expressing rgsCaM or control plants.

<table>
<thead>
<tr>
<th>N. benthamiana line</th>
<th>Mean latent period</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>15.3 ± 1.1 (16/18)</td>
<td>NA</td>
</tr>
<tr>
<td>At-rgs 2</td>
<td>13.6 ± 1.2 (13/18)</td>
<td>NS</td>
</tr>
<tr>
<td>At-rgs 4</td>
<td>11.0 ± 0.8 (18/18)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>At-rgs 5</td>
<td>10.4 ± 0.4 (17/18)</td>
<td>NS</td>
</tr>
<tr>
<td>At-rgs 7</td>
<td>10.4 ± 0.5 (17/18)</td>
<td>NS</td>
</tr>
<tr>
<td>At-rgs 8</td>
<td>12.3 ± 1.2 (17/18)</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>SALK_078400C 1</td>
<td>12.9 ± 2.0 (17/18)</td>
<td>NS</td>
</tr>
<tr>
<td>Nt-rgs 2</td>
<td>10.0 ± 0.9 (17/18)</td>
<td>NS</td>
</tr>
<tr>
<td>Nt-rgs 5</td>
<td>14.0 ± 1.1 (17/18)</td>
<td>NS</td>
</tr>
<tr>
<td>Nt-rgs 7</td>
<td>10.4 ± 0.3 (18/18)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>At-rgs 8</td>
<td>14.3 ± 1.5 (15/18)</td>
<td>NS</td>
</tr>
</tbody>
</table>

* N. benthamiana plants used in the analysis were either wild type or transgenic for Arabidopsis (At-rgs) or N. tabacum (Nt-rgs) rgsCaM. Numbers represent independent transgenic lines.

# Values represent the mean latent period (± SE) of plants exhibiting symptoms typical of a TGMV infection, calculated using data from plants inoculated with Agrobacterium at an inoculum dose of OD₆₅₀ = 1.0. Numbers in parentheses indicate infectivity (number of plants infected/number plants inoculated) from a single experiment for each line.

* The significance of mean latent period differences observed was confirmed by one-way ANOVA followed by Dunnett's post-hoc test. NS = not significant. NA = not applicable.

In agreement with a recent study where N. benthamiana plants silenced for rgsCaM exhibited symptoms of a TYLCV infection nine to 10 days later than in wild type plants (Li et al. 2014), this result is also consistent with our results where N. benthamiana plants over-expressing rgsCaM were more susceptible to infection (Table 3).

Overexpression of rgsCaM leads to an increase in viral DNA loads

To further assess the effect of overexpression of rgsCaM on virus infection we analyzed replication kinetics of TGMV and a second begomovirus, CabLCuV, in transgenic as compared to wild type, non-transgenic plants. For these experiments we chose to use two N. benthamiana lines expressing rgsCaM that exhibited symptoms typical of a TGMV infection ~5 days earlier than in wild type plants (At-rgs 7 and Nt-rgs 7) (Table 3). Wild type N. benthamiana plants or plants expressing either At-rgsCaM or Nt-rgsCaM were infected with Agrobacterium cultures containing either TGMV DNA A or CabLCuV DNA A. Again, DNA A components were used to ensure lack of cell to cell movement of the virus from the site of infection. Total DNA was isolated 1 to 3 dpi and presence of viral DNA assessed by DNA gel blot hybridization and phosphorimager analysis (Fig. 5). As can be seen, TGMV and CabLCuV DNA were barely detectable after 1 dpi, but was clearly detectable after 2 and 3 dpi in both wild type and transgenic plants. Total viral DNA content was significantly higher in transgenic plants as compared to wild type plants.

At-rgsCaM knockout lines exhibit decreased susceptibility to geminivirus infection

To assess the biological significance of rgsCaM to geminivirus infection we identified a T-DNA insertion mutant (SALK_078400C) in Arabidopsis and inoculated mutant and wild-type Col-0 lines with CabLCuV DNA-A and DNA-B or BCTV-A. Both lines exhibited symptoms typical of either CabLCuV or BCTV-A infection (data not shown). In general, plants containing the T-DNA knockout for rgsCaM were less susceptible to infection with CabLCuV and BCTV-A (Table 4) than wild type plants as judged by an increase in the time to first appearance of symptoms (mean latent period). Under the conditions of the test, wild type plants exhibited a mean latent period of 13.6 to 14.9 days (Table 3). In contrast, the mean latent period for rgsCaM mutant plants ranged from 15.7 to 19.4 days, depending on the virus. Although the differences in mean latent period were not statistically significant as determined by ANOVA and Student Newman Keuls test post-hoc test, the trend is that plants lacking rgsCaM are less susceptible to infection. This is

Table 4
Mean latent period for CabLCuV and BCTV-A inoculated to wild type Arabidopsis Col-0 plants or At-rgsCaM knockout Arabidopsis plants.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Arabidopsis line</th>
<th>Mean latent period</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>CabLCuV</td>
<td>Wild type Col-0</td>
<td>13.6 ± 1.1 (9/30)</td>
<td>NA</td>
</tr>
<tr>
<td>SALK_078400C</td>
<td>15.7 ± 1.9 (9/29)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>BCTV-A</td>
<td>Wild type Col-0</td>
<td>14.9 ± 1.7 (10/30)</td>
<td>NA</td>
</tr>
<tr>
<td>SALK_078400C</td>
<td>19.4 ± 1.6 (8/30)</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

* Viruses were introduced by Agrobacterium-mediated delivery as described in Materials and Methods.

* Arabidopsis plants used in the analysis were either wild type (Col-0) or contained a T-DNA insertion in the At-rgsCaM gene (SALK_078400C).

* Values represent the mean latent period (± SE) of plants exhibiting symptoms typical of an infection, calculated using data from plants inoculated with Agrobacterium at an inoculum dose of OD₆₅₀ = 1.0. Numbers in parentheses indicate infectivity (number of plants infected/number plants inoculated) from a single experiment for each line.

* The significance of mean latent period differences observed was confirmed by one-way ANOVA followed by Student Newman Keuls post-hoc test. NS = not significant. NA = not applicable.

Fig. 5. Expression of At-rgsCaM affects accumulation of viral nucleic acid in N. benthamiana. Leaves from wild type N. benthamiana plants or plants transgenic for At-rgsCaM were inoculated with Agrobacterium cultures containing wild type TGMV or CabLCuV DNA. Total DNA was isolated 1 to 3 days post-infection from the infusion sites of 9 leaves from 3 individual plants and pooled. The images illustrate phosphorimager analysis (Fig. 5). As can be seen, TGMV and CabLCuV DNA were barely detectable after 1 dpi, but was clearly detectable after 2 and 3 dpi in both wild type and transgenic plants. Total viral DNA content was significantly higher in transgenic plants as compared to wild type plants.
Fig. 6. Bimolecular fluorescence complementation (BiFC) analysis of AL2-rgsCaM complexes in *N. benthamiana* epidermal cells. Constructs expressing full length AL2 protein and At-rgsCaM fused to the N- or C-terminal portion of YFP were delivered to *N. benthamiana* leaves by agroinfiltration. Images were examined for fluorescence with a 40x objective using FITC (cEFP signal) and Rhodamine (RFP-Histone signal) filter sets. Photographs represent merged images of from the two filter sets. H4-RFP localizes to the nucleus and is identified by red fluorescence (N), while reconstituted YFP is identified by green fluorescence either in the cytoplasm (C) for the At-rgs::At-rgs interaction (first two panels) or in the nucleus (N) for the At-rgs::AL2 interaction (last two panels). Note the presence of punctate spots (ps) within the nuclei of cells where the At-rgs::AL2 interaction takes place. Protein combinations are indicated on the left. Images in the top row illustrate leaves viewed from the top onto the upper epidermis (UE), showing individual cells. Insets in the bottom row represent a higher magnification of nuclei shown in images from the top row. Scale bars in the top row of images represent 25 μm and in the bottom row represents 10 μm.

Geminivirus AL2/C2 proteins interact with At-rgsCaM

Recent evidence indicates that Nt-rgsCaM interacts with a number of viral silencing suppressors, and inhibits their activity, through direct binding to an arginine-rich region within the suppressor proteins (Nakahara et al., 2012). Given that the N-terminus of the TGMV AL2 protein also contains an arginine-rich region we tested whether geminivirus AL2 proteins are also capable of interacting with rgsCaM. Using yeast two hybrid, we detected an interaction between TGMV AL2 and both At-rgsCaM and Nb-rgsCaM (Table S1 in Supplementary data). The interactions between TGMV AL2 and rgsCaM from *Arabidopsis* (Fig. 6) and *N. benthamiana* (not shown) were then confirmed using Bimolecular Fluorescence Complementation (BiFC). DNA encoding full length AL2 was cloned into the pSITe-nEYFP-N1 and pSITe-cEYFP-C1 binary vectors (nYFP-AL2, cYFP-AL2)(Chung and Sunter, personal communication). The full length At-rgsCaM and Nt-rgsCaM coding sequences were cloned into pSITe-nEYFP-N1 and pSITe-cEYFP-C1, generating constructs containing the N- and C-terminal halves of eYFP respectively fused to the N-terminus of each protein (nYFP-At-rgsCaM, cYFP-At-rgsCaM, nYFP-Nt-rgsCaM, cYFP-Nt-rgsCaM). Each construct was introduced into Agrobacterium and used to infuse *N. benthamiana* leaves. Analysis of infused leaves by fluorescence microscopy shows that TGMV AL2 interacts with At-rgsCaM in the nucleus (Fig. 6), based on an overlapping signal with a nuclear marker (histone H4-RFP) (Martin et al., 2009), which exhibits red fluorescence. TGMV AL2 also interacted with Nb-rgsCaM in the nucleus (data not shown). This is consistent with previous reports that Nt-rgsCaM interacts with the viral silencing suppressors of *Turnip mosaic virus* (HC-Pro) and *Cucumber mosaic virus* (2b protein) (Nakahara et al., 2012). In these cases the interaction appeared to take place in the cytoplasm, although this was not explicitly mentioned. For TGMV AL2, the interaction was localized within the nucleus as defined spots (Fig. 6), rather than a diffuse signal encompassing the entire nucleus that is typically observed with an AL2 self-interaction (Yang et al., 2007). To reconcile these differences we examined whether rgsCaM could self-interact. Although both At-rgsCaM and Nb-rgsCaM were able to self-interact, these interactions occurred in the cytoplasm, with fluorescence observed throughout the cell, enveloping, but not within the nucleus (Fig. 6). This suggests that AL2 may act to sequester rgsCaM within the nucleus, possibly in specific structures, through a direct interaction.

Discussion

RNA silencing (quelling in fungi and RNA interference in animals) is an innate cellular response involved in antiviral defense, histone/DNA methylation, establishment of heterochromatin, and gene regulation via microRNAs (Vance and Vaucheret, 2001; Baulcombe, 2004). Current evidence suggests two of these pathways impact geminivirus replication, post-transcriptional gene silencing (PTGS) mediated by small interfering RNAs (siRNAs) (Lucioli et al., 2003; Bisaro, 2006), and transcriptional gene silencing (TGS) mediated by siRNA-directed methylation of histones and DNA (Buchmann et al., 2009; Raja et al., 2008; Raja et al., 2010; Seemannpillai et al., 2003). Several studies have demonstrated that AL2 proteins from different begomoviruses function as viral suppressors of RNA silencing (VSRs), as a robust response...
to counter RNA silencing (Incarbone and Dunoyer, 2013). However, none of the begomovirus AL2 proteins have been shown to bind siRNA, excluding the possibility that AL2 might interfere with silencing pathways at this step. Studies using MYMV AL2 suggest that the transcriptional activation function of AL2 is required for suppression of silencing, and several genes were identified that are potential candidates for a role in RNA silencing. One gene encodes a Werner exonuclease-like 1 protein, which was able to suppress RNA silencing in N. benthamiana 16c plants expressing GFP (Trinks et al., 2005). However, there is also a transcription-independent mechanism for suppressing silencing by AL2, which involves suppression of TGS via the methylation pathway (Raja et al., 2008).

Previous work has shown the silencing suppressor HC-Pro, encoded by TEV, interacts with a regulator of gene silencing-calmodulin-like protein (rgsCaM) from N. tabacum (Nt-rgsCaM) (Anandalakshmi et al., 1998). It also appears that expression of rgsCaM is induced upon viral infection with TEV and with over-expression of HC-Pro, suggesting that expression of rgsCaM may be controlled by HC-Pro either directly or indirectly (Anandalakshmi et al., 1998). In addition, a comparison of transcriptome changes induced in N. tabacum in response to the VSRS HC-Pro, P25 from Potato virus X (PVX) and AC2 of African cassava mosaic virus (ACMV), found that Nt-rgsCaM was up-regulated (Jada et al., 2012). This is again consistent with the involvement of endogenous silencing suppressors (ESRs) in the mechanism of action of VSRS (Jada et al., 2012). Our investigation into the role of rgsCaM in suppression of gene silencing suggests that the ability of AL2 to inhibit the silencing pathway may also be mediated through this endogenous cellular silencing suppressor. Our results indicate that rgsCaM in N. benthamiana is induced early in the response to geminivirus replication (Fig. 1), and with evidence that rgsCaM suppresses RNA silencing (Anandalakshmi et al., 2000), this could indicate that suppressor activity of geminivirus AL2 protein is also mediated through rgsCaM. This is supported by the use of promoter-reporter constructs in protoplasts, where At-rgsCaM promoter activity increases in response to TGMV AL2 (Fig. 2). A second line of evidence is provided by infecting plants transgenic for an At-rgsCaM promoter:eGFP transgene. These plants exhibited no marked eGFP fluorescence until infection by TGMV. Cells within symptomatic tissue exhibited fluorescence, an indication of increased promoter activity. However, there were a very small number of cells infected, suggesting the response was limited to cells containing the virus, and not a generalized systemic response. It has been suggested that rgsCaM expression may be strongly induced by virus infection or viral silencing suppressor expression (Nakahara et al., 2012). We also observed increases in response to several additional viruses, including PVX (Fig. 4; Table 2), suggesting the plants may respond to the presence of viruses by increasing expression of rgsCaM. Additional evidence that the PVX-derived VSR (P25), and the VSR (βC1) encoded by the betasatellite of TYLCNV increase rgsCaM mRNA accumulation in N. tabacum (Jada et al., 2013) and N. benthamiana (Li et al., 2014), respectively, supports a role for rgsCaM in the silencing suppression mechanism. The 2 to 3-fold increases in expression that we observed upon either viral infection, or AL2 expression, could be relevant biologically given that rgsCaM appears to be degraded by autophagosomes (Nakahara et al., 2012). Thus, as suggested, in uninfected tissue rgsCaM might be unstable and a small increase in expression could lead to suppression of the RNA silencing pathway.

If rgsCaM does function as an endogenous silencing suppressor, then we would expect that AL2-mediated induction of rgsCaM expression would lead to an increase in suppressor activity. In turn this would be predicted to lead to plants that are more susceptible to infection. This is supported by data demonstrating that the mean latent period of infection is decreased by up to 5 days in plants over-expressing either At-rgsCaM or Nt-rgsCaM (Table 3). The decrease in mean latent period appears to be correlated with increases in viral DNA loads (Fig. 4), where plants over-expressing Nt-rgsCaM accumulated approximately 3-fold higher TGMV viral DNA than in wild type plants after 2 dpi (Fig. 5). When these plants were infected with CabLCuV, viral DNA levels increased between 8 and 577-fold higher in N. benthamiana plants transgenic for Nt-rgsCaM as compared to wild type plants (Fig. 5). Similar results were observed for N. benthamiana plants transgenic for At-rgsCaM. This supports the conclusion that geminivirus infection induces expression of rgsCaM, which in turn acts as an endogenous silencing suppressor. This would ultimately result in increased viral titer, since the silencing system is unable to control viral replication. In turn this would increase susceptibility of plants to infection, as seen by a decrease in mean latent period (Table 3). This conclusion is also supported by results that indicate that Arabidopsis plants lacking rgsCaM are less susceptible to infection as suggested by an increase in mean latent period (Table 4). These results are consistent with recent data that shows an earlier onset of symptoms typical of a TYLCNNV+-betasatellite infection in transgenic N. benthamiana plants over-expressing Nb-rgsCaM (Li et al. 2014). In contrast, in plants silenced for rgsCaM, symptoms appearance was delayed 9 to 10 days (Li et al. 2014). Our results differ somewhat to those reported recently (Li et al. 2014) where plants over-expressing Nb-rgsCaM exhibited symptoms that phenocopied transgenic lines over-expressing βC1. While we do not know the reason for this difference it may be a consequence of our use of At-rgsCaM and Nt-rgsCaM. Alternatively, expression levels of the transgene may be considerably different, although we cannot confirm this.

There is however, a recent study, which shows that Nt-rgsCaM actually bound to a number of viral silencing suppressors and inhibited their activity (Nakahara et al., 2012). This was mediated through an arginine-rich region within the silencing suppressor. This data does not support the conclusions that rgsCaM is an endogenous silencing suppressor, and it was in fact reported that no suppressor activity was detectable in protoplasts or using conventional agroinfiltration (Nakahara et al., 2012). In addition, overexpression of rgsCaM resulted in plants that were less susceptible to viral infection. This is again in contrast to our results where overexpression of rgsCaM from either tobacco or Arabidopsis led to an increase in susceptibility (Table 3), and to results with TYLCNNV (Li et al. 2014). While we cannot explain this apparent discrepancy, it could reflect a difference between the viruses used in the recent study (Nakahara et al., 2012) and geminiviruses, which have DNA genomes. This could also be reflected in the observation that viral silencing suppressors from RNA viruses, including Turnip mosaic virus (TuMV) HC-Pro and Cucumber mosaic virus 2b, interact with rgsCaM, which may sequester the viral proteins and inhibit their ability to bind siRNAs and thus to suppress silencing (Nakahara et al., 2012). As an example, Tomato bushy stunt virus (TBSV) P19 suppresses silencing induced by sense RNA (S-PTGS) and inverted repeat (IR-PTGS) or hairpin RNA triggers, consistent with the ability of P19 to sequester 21–nt siRNA (Vargason et al., 2003). In contrast, geminivirus βC1 only suppresses silencing induced by S-PTGS and not by IR-PTGS (Li et al., 2014). Given that TGMV AL2 does not bind siRNA, even under conditions that support binding by P19 (Wang et al., 2005), this supports a different model of suppression than observed for HC-Pro, 2b and P19.

Despite these observable differences, we did demonstrate an interaction between AL2 from TGMV and rgsCaM from both Arabidopsis and tobacco within the nucleus (Fig. 6). This interaction appeared to be localized within specific structures, in contrast to the reports using HC-Pro and 2b where the interaction was localized to the perinuclear region (Nakahara et al., 2012). This may not be surprising given that we showed both At-rgsCaM and Nb-rgsCaM exhibited the ability to self-interact, but in the cytoplasm (Fig. 6). Together this supports a different mechanism for
AL2 silencing suppression, possibly mediated by induction of rgsCaM. It is tempting to speculate that AL2 may act to sequester rgsCaM in the nucleus, possibly in defined structures, to prevent targeting of AL2 by rgsCaM for degradation via the autophagy pathway (Nakahara et al., 2012). At this time we do not know whether relocation of rgsCaM to structures within the nucleus has any role in silencing suppression. However, it is interesting to note that Cajal bodies in plants are associated with the nucleolus and are possible sites for siRNA and miRNA biogenesis (Pontes and Pikaard, 2008). Given the role of AL2 in suppressing TGS, and that Cajal bodies may play a role in guiding the RISC complexes associated with TGS to the chromatin (Pontes and Pikaard, 2008), it is exciting to speculate that the interaction between AL2 and rgsCaM may play a role in this.

Materials and methods

Cloning of promoter-reporter gene constructs

The constructs pTGAl26 (TGMV DNA A) and pTGA79 (35S-AL2) have been previously described (Sunter and Bisaro, 1991, 1992; Sunter et al., 1994). A series of truncated rgsCaM promoters were generated by replacing the CaMV 35S promoter in pBI221 (Jefferson, 1987) with different amounts of 5′ flanking sequences of the Arabidopsis rgsCaM gene (At1g76640). These 5′-truncated promoter constructs were linked to the β-glucuronidase (GUS) reporter in a translational fusion consisting of the N-terminal 12 amino acids of the rgsCaM protein and flanked at the 3′-end by the nopaline synthase (nos 3′) polyadenylation signal. Using Arabidopsis total DNA as template and different forward primers with a common reverse primer (Table S2 in Supplementary data), DNA fragments of 922 bp (RGS-922+RGSR), 615 bp (RGS-615+RGSR) and 337 bp (RGS-337+RGSR) were amplified. The DNA fragments were restricted with HindIII and BamHI and cloned into similarly digested pBI221 to generate At-rgsCaM[–922]-GUS, At-rgsCaM[–615]-GUS and At-rgsCaM[–337]-GUS, respectively.

Cloned DNA capable of constitutively expressing eGFP from the CaMV 35S promoter was generated. The eGFP coding sequence was amplified by PCR using pEFP plasmid DNA (BD Biosciences, Palo Alto, CA) as template with forward (eGFPF) and reverse (eGPRR) primers (Table S2 in Supplementary data). The resulting 720 bp PCR product was cleaved with BamHI and EcoRI (underlined) and used to replace the GUS coding sequence of pBI221 to generate pG287, which contains a CaMV 35S-eGFP-nos cassette. The HindIII and BamHI DNA fragment of pG287 containing the CaMV 35S promoter was replaced with the 922 bp DNA fragment of At-rgsCaM[–922]-GUS to generate At-rgsCaM[–922]-GFP.

Binary plasmids containing DNA capable of constitutively expressing N. tabacum or Arabidopsis rgsCaM from the CaMV 35S promoter were generated. Using gene specific primers (Table S2 in Supplementary data) the coding region of rgsCaM from N. tabacum (Nt-rgsSF + NT-rgsSR) and Arabidopsis (At-rgsSF + At-rgsSR) were amplified by RT-PCR with total RNA from N. tabacum or Arabidopsis as template. The PCR products were restricted with BamHI-HindIII and cloned into the binary vector pMON530 (Rogers et al., 1987) restricted with BglII-HindIII, generating 35S-Nt-rgsCaM and 35S-At-rgsCaM respectively as template. Entry clones containing the TGMV AL2 coding region have been previously described (Chung and Sunter, personnel communication).

Gateway cloning into destination vectors

LR recombination reactions using Clonassell were performed according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Entry clones containing At-rgsCaM, Nb-rgsCaM or TGMV AL2 were introduced into the Gateway compatible yeast two-hybrid vectors pDEST32 and pDEST22 (Proquest Two-Hybrid System, Invitrogen, Carlsbad, CA). The resulting constructs comprised the Gal4 DNA binding (pDEST32) or activation (pDEST22) domains fused to the N-terminus of each protein (GAD At-rgsCaM, GBD Nb-rgsCaM, GBD-TGMV AL2, GAD At-rgsCaM, GAD Nb-rgsCaM, GAD-TGMV AL2). Selected colonies were isolated and the clones verified through sequence analysis.

Entry clones containing At-rgsCaM or Nb-rgsCaM were introduced into the Gateway compatible BiFC binary vectors, pSITE-eYFP-C1 or pSITE-cEYFP-C1 (Martin et al., 2009). The resulting constructs comprised the N- and C-terminal halves of eYFP respectively fused to the N-terminus of each protein (nYFP-At-rgsCaM, cYFP-Nb-rgsCaM; cYFP-At-rgsCaM, cYFP-Nb-rgsCaM). BiFC constructs containing the TGMV AL2 coding region have been previously described (Chung and Sunter, personnel communication).

Yeast two-hybrid analysis

To assess the interaction of geminivirus AL2 with rgsCaM Bait and Prey constructs were introduced into yeast strain Mal203 and tested according to the manufacturers protocol (Proquest Two-Hybrid System).

Protoplast transfection and analysis

Protoplasts were isolated from an N. benthamiana suspension culture cell line, transfected with various DNAs and fluorometric GUS assays performed after 3 days as described (Sunter and Bisaro, 2003). Three to 5 independent experiments were performed and statistical analysis using Student’s t-test carried out to determine significant differences in expression.

Generation of transgenic plants

A binary plasmid construct containing DNA capable of expressing eGFP from the At-rgsCaM promoter was generated. The At-rgsCaM promoter-eGFP cassette was excised from At-rgsCaM[–922]-GFP by restriction with HindIII and EcoRI. The 1979 bp DNA fragment was cloned into similarly digested pMON521 and the resulting binary Ti plasmid vector mobilized into Agrobacterium tumefaciens strain GV3110Se as described (Sunter et al., 2001). Agrobacterium cultures were used to transform N. benthamiana leaf discs and transformants selected and grown as described previously (Sunter et al., 2001). From kanamycin resistant T1 transformants, 5 to 7 plants were selected and self-pollinated to generate T2 plants. Three to 5 individual plants from each T2 line were chosen for subsequent analysis. The presence of the transgene was confirmed by PCR (data not shown).

Plant inoculation and microscopic analysis of GFP fluorescence

Healthy non-transformed or transgenic N. benthamiana plants expressing eGFP from the At-rgsCaM promoter were agroinoculated with TGMV DNA A (Wyant et al., 2012), BCTV-H (formerly BCTV Logan) (Stenger et al., 1990), PVX (Buchmann et al., 2009), TMV (Lindbo, 2007) or mock-inoculated as described (Sunter et al., 2003). Three to 5 independent experiments were performed and statistical analysis using Student’s t-test carried out to determine significant differences in expression.
were used in the analysis. For each biological sample we used 2010). Independent biological samples from 2 to 3 experiments Biosystems, Foster city, CA) as described previously (Baliji et al., analysis performed using a 7500 Real-time PCR system (Applied described above was reverse transcribed using a high-capacity Biosystems, CA), treated with DNaseI (Ambion, Austin, TX) and puri

data) was used to assess differences in expression of genes in

for all replicates within the control or treatment groups as

Hercules, CA). Levels of EF1

for viral DNA levels ensuring equivalent amounts of DNA were

Upper asymptomatic leaf tissue from mock-inoculated plants was

examined using a fluorescence microscope (Axioskop; Carl Zeiss, Jena, Germany). Samples were imaged using either a 20× or 40× objective under bright field using differential interference contrast (DIC). eGFP fluorescence was detected using a filter set for excitation at 485 nm and emission at 515 nm.

Leaf infusion assays

Agrobacterium-mediated transient expression in N. benthamiana leaves was performed using leaf infusion assays as described (Rao and Sunter, 2012). Typically, discs were cut from the infusion sites of 9 leaves, from 3 different plants, and pooled for extraction 1 to 3 days post-inoculation, depending on the experiment.

RNA isolation and quantitative real-time PCR analysis

Total RNA was isolated from infiltrated leaves using Plant RNA Reagent as described by the manufacturer (Invitrogen, Carlsbad, CA), treated with DNasel (Ambion, Austin, TX) and purified as described (Rao and Sunter, 2012). Real time quantitative RT-PCR (qRT-PCR) with gene-specific primers (Table S2 in Supplementary data) was used to assess differences in expression of genes in response to TGMV DNA A, CabLCuV DNA A (U65529), or BCTV-A (AY548948; formerly Spinach curly top virus) infection by comparison to a vector treated control. Total RNA (1 μg) isolated as described above was reverse transcribed using a high-capacity cDNA kit (Applied Biosystems, Foster city, CA) and qPCR analysis performed using a 7500 Real-time PCR system (Applied Biosystems, Foster city, CA) as described previously (Baliji et al., 2010). Independent biological samples from 2 to 3 experiments were used in the analysis. For each biological sample we used 3 replicates. Differences in gene expression between the target gene and endogenous control (ΔΔCt) for each replicate were calculated and used to measure differences between treatments (ΔΔCt) as described (Baliji et al., 2010). A parametric Student’s t-test was used to test for statistical variation between ΔCt values for all replicates within the control or treatment groups as described (Baliji et al., 2010).

DNA isolation and southern blot hybridization

DNA was isolated from virus-infected N. benthamiana leaves and the presence of viral DNA assessed by DNA gel blot hybridization to 32P-labeled probes specific for TGMV or CabLCuV DNA A as described (Baliji et al., 2007). Hybridization signals were quantified by phosphorimager analysis (Molecular Imager FX, Bio-Rad, Hercules, CA). Levels of EF1α were measured by qPCR using the absolute quantification technique with gene-specific primers. This value was then used to adjust the phosphorimager values obtained for viral DNA levels ensuring equivalent amounts of DNA were used (Table S3 in Supplementary data).

Binuclear fluorescence complementation (BIFC) assays

For microscopic observations of fluorescence, discs from infused leaves were mounted on microscope slides and analyzed for YFP, RFP or eGFP expression using a fluorescent microscope (Axioskop, Carl Zeiss) as described previously (Baliji et al., 2010). Samples were imaged with a 40X objective under bright field using differential interference contrast (DIC). FITC fluorescence was detected using a filter set for excitation at 485 nm and emission at 515 nm. RFP fluorescence was detected using a filter set for excitation at 545 nm and emission at 620 nm.

Acknowledgments

This material is based upon work supported by the National Science Foundation under Grant number IOS-0948669. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation. We thank Janet Sunter for maintenance and generation of N. benthamiana plants and microscopy.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.04.034.

References


