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Alfalfa dwarf cytorhabdovirus P protein is a local and systemic RNA silencing supressor which inhibits programmed RISC activity and prevents transitive amplification of RNA silencing

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ABSTRACT

Plants employ RNA silencing as an innate defense mechanism against viruses. As a counter-defense, plant viruses have evolved to express RNA silencing suppressor proteins (RSS), which target one or more steps of the silencing pathway. In this study, we show that the phosphoprotein (P) encoded by the negative-sense RNA virus alfalfa dwarf virus (ADV), a species of the genus *Cytorhabdovirus*, family *Rhabdoviridae*, is a suppressor of RNA silencing. ADV P has a relatively weak local RSS activity, and does not prevent siRNA accumulation. On the other hand, ADV P strongly suppresses systemic RNA silencing, but does not interfere with the short-distance spread of silencing, which is consistent with its lack of inhibition of siRNA accumulation. The mechanism of suppression appears to involve ADV P binding to RNA-induced silencing complex proteins AGO1 and AGO4 as shown in protein–protein interaction assays when ectopically expressed. *In planta*, we demonstrate that ADV P likely functions by inhibiting miRNA-guided AGO1 cleavage and prevents transitive amplification by repressing the production of secondary siRNAs. As recently described for lettuce necrotic yellows cytorhabdovirus P, but in contrast to other viral RSS known to disrupt AGO activity, ADV P sequence does not contain any recognizable GW/WG or F-box motifs, which suggests that cytorhabdovirus P proteins may use alternative motifs to bind to AGO proteins.

1. Introduction

Viruses are intracellular obligate parasites that exploit host cellular and molecular mechanisms to propagate in host cells causing many important plant diseases (Gergerich and Dolja, 2006). RNA silencing is a sequence-specific RNA-mediated degradation mechanism, a function of which is antiviral defense (Ding and Voinnet, 2007). RNA silencing is triggered by viral double-stranded RNA (dsRNA) (Voinnet, 2001) that, in the case of single-stranded RNA (ssRNA) viruses, can be derived from highly structured regions in the RNA genome or may be generated during replication (Molnar et al., 2005). DsRNA that accumulate during virus infection are processed into short dsRNAs of 21–24 nucleotide (nt), called small interfering RNAs (siRNAs), by members of the RNase III-like enzymes family, named DICER-like (DCL) proteins (Deleris et al., 2006). The siRNAs are loaded onto Argonaute (AGO) proteins, the effectors of the RNA-Induced Silencing Complex (RISC), to initate slicing or translational repression of target RNAs (Ding and Voinnet, 2007; Carbonell and Carrington, 2015). A family of ten AGO proteins was identified in *Arabidopsis thaliana* (Vaucheret, 2008), whereas nine AGO homologs were identified in the *Nicotiana benthamiana* transcriptome (Nakasugi et al., 2013). Antiviral activity is associated mainly with AGO1, the core component of RISC in the siRNAs-mediated silencing pathways (Baumberger and Baulcombe, 2005; Mallory and Vaucheret, 2010; Qu et al., 2008), AGO4 also has slicer activity (Qi et al., 2006), and an antiviral function of this protein was proposed during cucumber mosaic virus (CMV) infection in *N. benthamiana* (Ye et al., 2009) and other RNA viruses (Carbonell and Carrington, 2015). AGO2, AGO5 and AGO7 have also been shown to have a role in antiviral defense (Brosseau and Moffett, 2015; Garcia-Ruiz et al., 2015). Once RNA silencing is initiated in a plant cell, primary siRNAs can be amplified through a process referred to as transitive silencing, which involves generation of secondary siRNAs extending from the primary targeted regions of a transcript, which is thought to be responsible for the systemic or

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long-distance spread of the RNA silencing signal to target RNAs in distant tissues (Dunoyer et al., 2010; Himber et al., 2003; Moissiard et al., 2007). Silencing spread throughout a plant requires RDR6-mediated amplification of ssRNA into dsRNA, which is subsequently processed by DCL2 into transitive, secondary siRNAs (Himber et al., 2003; Mlotshwa et al., 2008). RDR6 activity is assisted by protein co-factors such as Suppressor of gene silencing 3 (SGS3) (Beclin et al., 2002), silencing defective 3 (SDE3) (Dalmay et al., 2001; Himber et al., 2003; Garcia et al., 2012) and SDE5 (Hernandez-Pinzon et al., 2007).

To counteract RNA silencing defenses, viruses encode RNA silencing suppressor (RSS) proteins (Ding and Voinnet, 2007). Plant viruses have evolved many different RSS, more than 70 to date have been identified (Csorba et al., 2015). Viral RSS have no obvious sequence similarity, are multifunctional and may target one or more steps in the silencing pathway, such as viral RNA recognition, dicing, RISC assembly, or RNA targeting and amplification, as reviewed by Burgyán and Havelda (2011), Csorba et al. (2015) and Incarbone and Dunoyer (2013). A central feature of the majority of known plant viral RSS is their ability to bind to and sequester long and/or short dsRNAs, in order to inhibit cleavage by DCL or RISC assembly, respectively (Csorba et al., 2007; Duan et al., 2012; Lakatos et al., 2006; Landeo-Ríos et al., 2016; Mérai et al., 2006; Perez-Canamas and Hernandez, 2015; Schnettler et al., 2010). The best-characterized RSS that employs this strategy is the tombusvirus P19 protein, which binds to both siRNAs and miRNAs (Lakatos et al., 2006). However, other RSS function by interacting with host proteins that are either important components of the silencing machinery or proposed regulators of the silencing pathway (Kong et al., 2014). This includes RSS that interact with AGO1 to block siRNA loading, inhibit slicing activity, or inhibit translational repression (Azevedo et al., 2010; Chiu et al., 2010; Csorba et al., 2010; Feng et al., 2013; Giner et al., 2010; Karran and Sanfacon, 2014; Kumar et al., 2015; Mann et al., 2016a; Zhang et al., 2006). In addition, tobacco rattle virus 16K and CMV 2b proteins interact with AGO4 to block RISC assembly and both *in vitro* slicing activity and RNA-directed DNA methylation, respectively (Fernandez-Calvino et al., 2016; Hamera et al., 2012). Other RSS have been shown to function by interacting with siRNA biogenesis machinery such as dsRNA-binding protein 4 (DRB4) leading to inhibition of dsRNA processing by DCL4 (Haas et al., 2008) or by interacting with SGS3 and RDR6 (Okano et al., 2014).

Rhabdoviruses are enveloped negative-sense, ssRNA viruses, which infect diverse animal and plant hosts (Jackson et al., 2005; Mann and Dietzgen, 2014). Like other plant viruses, plant rhabdoviruses are expected to encode protein(s) with RSS activity, but data on their identification and possible mode of action are limited. Among viruses of the genus *Nucleorhabdovirus*, sonchus yellow net virus (SYNV) P protein was shown to have local RSS activity but was not further characterized (Jackson et al., 2005), while rice yellow stunt virus (RYSV) P6 was identified as a systemic RSS and shown to prevent RNA silencing amplification by interacting with RDR6 (Guo et al., 2013). The RSS of only one cytorhabdovirus, lettuce necrotic yellows virus (LNYV), has been investigated thus far. LNYV P protein was shown to act as a weak local RSS that did not inhibit siRNA accumulation but temporarily delayed systemic RNA silencing in *N. benthamiana* (Mann et al., 2015). In addition, it was recently shown that LNYV P binds AGO1, AGO2, AGO4, RDR6 and SGS3 proteins. Targeting multiple components of the RNA silencing pathway LNYV P inhibited miRNA-guided AGO1 cleavage and translational repression, and RNA silencing amplification (Mann et al., 2016a). Given the importance of plant virus RSS function in the infection cycle and host interactions, we identified and examined the RSS of alfalfa dwarf virus (ADV), a virus that combines properties of both cytoplasmic and nuclear plant rhabdoviruses; ADV groups phylogenetically with cytorhabdoviruses, but its P protein accumulates in the cell nucleus (Bejerman et al., 2015). We also explored mechanisms of action of ADV RSS through study of targeted host components. The ADV genome consists of 14,491 nucleotides and encodes six proteins in the order 3′-N (nucleoprotein) – P (phosphoprotein) – P3 (movement protein) – M (matrix protein) – G (glycoprotein) – P6 (protein of unknown function) – L (polymerase) – 5′ (Bejerman et al., 2015; Mann et al., 2016b).

In this study, we examined the suppressor activity of the putative RSS ADV P and P6. We present evidence that ADV P acts as a weak local RSS, which does not inhibit siRNA accumulation, but acts as a strong systemic RSS. On the other hand, ADV P6 protein did neither suppress local nor systemic RNA silencing unlike nucleorhabdovirus P6 in RYSV. We also show that ADV P binds AGO1 and AGO 4 proteins, and inhibits miRNA-guided AGO1 cleavage and translational repression. Moreover, we show that ADV P prevents transitive amplification of RNA silencing by repressing the production of secondary siRNAs.

2. Material and methods

2.1. Plasmid construction

Gateway entry clones in pDONR221 for ADV N (used as negative control), P and P6 ORFs (Bejerman et al., 2015) (Life Technologies, California, USA) were recombined into plant expression destination vector pSITE-Flag-C1 (Chakrabarty et al., 2007) to yield N-terminal flag epitope-tagged proteins, following instructions described in the Gateway LR Clonase II Enzyme Mix kit (Life Technologies). For bimolecular fluorescence complementation (BiFC) protein–protein interaction assays, Gateway entry clones in pDONR221 for ADV P (Bejerman et al., 2015), AGO1b, AGO4a and RDR6 (kindly provided by Prof Peter Waterhouse, Queensland University of Technology; Nakasugi et al., 2013) and SGS3 were recombined into plant expression destination vectors pSITE-BiFC- nEYFP-C1 and pSITE-BiFC-cEYFP-C1 to allow expression of these proteins as C-terminal fusions to the amino- or carboxy-terminal halves of yellow fluorescent protein (YFP), using Gateway LR Clonase II Enzyme Mix kit instructions (Life Technologies). For co-localization studies ADV P was recombined into GFP-pSITE-C1, and AGO1b, AGO4a, RDR6 and SGS3 into RFP-pSITE-C1 (Chakrabarty et al., 2007). All p-SITE plant expressions vectors were kindly provided by A/Prof Michael Goodin, University of Kentucky, USA (See Table S1 for a list of plamids used).

Recombinant plasmids were individually transformed into *Agrobacterium tumefaciens* LBA 4404.

2.2. *Agroinfiltration of* Nicotiana benthamiana

A. tumefaciens LBA 4404 cultures were diluted in infiltration buffer (10 mM MES, 10 mM $MgCl_2$) to a final OD_{600} of 0.4-0.5 prior to agroinfiltration into the abaxial side of 4–5 week old GFP-expressing 16c *N. benthamiana* leaves (Ruiz et al., 1998) or wild type *N. benthamiana* as previously described (Lindbo, 2007). BiFC assays were carried out using transgenic *N. benthamiana* plants with cyan fluorescent nuclei (CFP-Histone 2B) (Martin et al., 2009). Agrobacteria harboring plasmids encoding ER-targeted GFP (mGFP5-ER; pBIN-mGFP5-ER) was used as reporter for both local and systemic RNA silencing assays, 171.1 GFP sensor (pUQC10193) and 171.2 GFP sensor (Mann et al., 2016a) for miRNA-AGO1 cleavage assays, sense GFP (pUQC214-GFP) and double-stranded hairpin GFP (dsGF; pUQC-251) for transitivity assays, and TBSV P19 (pUQC-10035) was used as positive RSS control in plants (Table S1). Local and systemic silencing assays both using mGFP5-ER (Table S1) were independently carried out at least three times. For local silencing assays, GFP expression was recorded at 4 days postinfiltration (dpi),

while for systemic silencing assays, GFP expression was recorded at 20 dpi and leaves were counted as systemically silenced if complete or partial development of red autofluorescence was observed in upper non-infiltrated leaves. Leaves were counted as not systemically silenced (i.e., silencing was suppressed) if the green GFP fluorescence in upper non-infiltrated leaves was unchanged and clearly detectable. For transitivity experiments, agrobacteria carrying dsGF plasmid were diluted to OD_{600} of 0.08 prior to mixing in equal volumes with both agrobacteria carrying sense GFP (OD_{600} 0.8) and viral protein (OD_{600} 0.8) expression constructs.

2.3. Protein analysis

Soluble protein extracted from agroinfiltrated leaf tissue (20 mg) was resuspended in sample buffer (Laemmli, 1970) and separated on an SDS-12% or 7% (for AGO1-Myc protein experiments) polyacrylamide gel. Separated proteins were transferred to PVDF membrane (Millipore, Massachusetts, USA) and detected as previously described (Mann et al., 2015). Protein sizes were estimated using the Page Ruler pre-stained protein ladder (Thermo Fisher Scientific, Massachusetts, USA).

2.4. RNA analysis

Total RNA was extracted from agroinfiltrated leaf tissue (1 g) using Tri Reagent (Sigma-Aldrich). For detection of *GFP* siRNAs and transitivity experiments total RNA was separated into high and low molecular weight (HMW/LMW) RNA as previously described (Mitter and Dietzgen, 2012). Total and HMW or LMW RNAs were transferred from denaturing 1.0% agarose or 17% polyacrylamide gels, respectively, to a BrightStar-Plus nylon membrane and were detected with BrightStar BioDetect Kit (Life Technologies) as previously described (Mann et al., 2015) or with the Chemiluminescent Nucleic Acid Detection Module Kit (Thermo Fisher Scientific). *GFP* mRNA from local and systemic silencing experiments was detected using 8 mg of total RNA. *GFP* sensor (171.1 and 171.2) and *GFP* mRNA from transitivity experiments were detected using 8 μg of total and HMW RNA, respectively. *AGO1*-Myc mRNA was detected using 14 μg of total RNA. *GFP* siRNAs were detected using 3–5 μg of LMW RNA. Unless otherwise stated, biotin-labeled *GFP* (720 nt; full-length), *AGO1* (992 nt; corresponding to nt position 330–1322) and "*GF*" (10–384 nt of *GFP*) and "*P*" (413–717 nt of *GFP*) DNA probes were generated as described by Mann et al., 2016a. Total RNA sizes were estimated using RiboRuler High Range RNA ladder (ThermoFisher Scientific) in conjunction with a linear regression analysis and siRNAs size by loading mixtures of *GFP*-specific short DNA oligonucleotides.

2.5. GFP fluorescence and confocal microscopy

GFP fluorescence in *N. benthamiana* leaves was observed using a long wavelength Black Ray model B 100AP UV lamp (UVP, California, USA) at 365 nm and images were captured using Canon EOS 450D camera equipped with a GFP filter. All microscopy was carried out on a Zeiss LSM-700 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) at 2 and 3 dpi using at least three leaves for each of three independent biological replicates (nine leaves total) for BiFC experiments. Images were processed using Zen 2012 Lite software.

3. Results

Initial results showed that ADV N, P3, M and G proteins were not able to suppress RNA silencing (data not shown). Therefore, we examined whether ADV phosphoprotein (P) and protein 6 (P6) had RNA silencing suppressor (RSS) activity as shown for LNYV P (Mann et al., 2015) and RYSV P6 (Guo et al., 2013). Since various suppressor proteins target different steps in the silencing pathways, the stage at which P and/or P6 may act to suppress RNA silencing was also investigated.

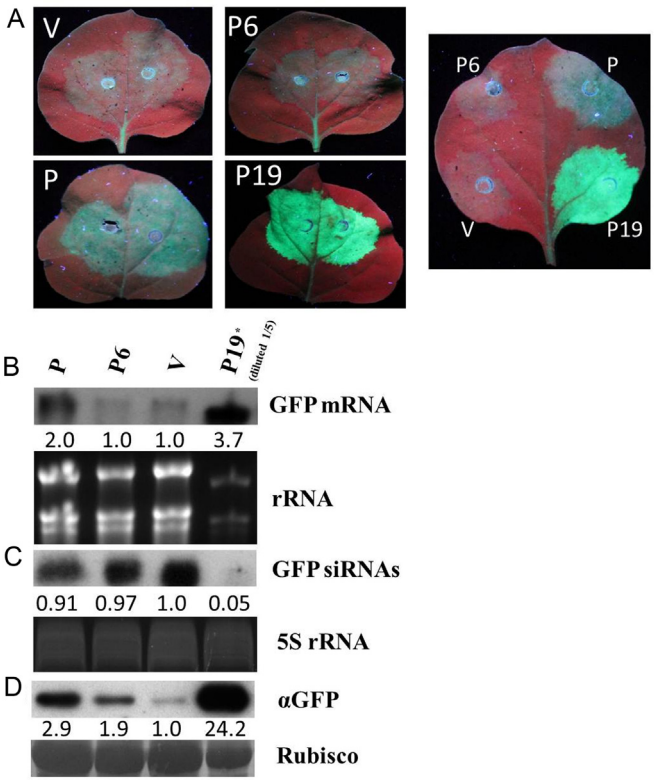

Fig. 1. Local GFP silencing suppression activity of ADV proteins. (A) *N. benthamiana* 16c leaves expressing mGFP5-ER were co-agroinfiltrated with mGFP5-ER plus either ADV P or P6. TBSV P19 or empty pSITE-Flag vector (V), were used as positive and negative controls, respectively. Leaves were photographed for GFP fluorescence under long wavelength UV light using a Canon EOS camera equipped with a GFP filter at 4 dpi. Infiltrated leaf patches were analyzed for accumulation of *GFP* mRNA (B) and *GFP* siRNA (C) using total and low molecular weight RNA, respectively. RNAs were detected using a full-length *GFP*-specific biotin-labeled probe. Using soluble protein fractions, GFP (D) was detected using an anti-GFP monoclonal antibody. Loading controls for *GFP* mRNA (ribosomal RNA; rRNA), siRNA (5SRNA) and protein (RuBisCO) are shown below respective blots. Note: all samples were loaded equally with the exception of TBSV P19 in the *GFP* mRNA blot, which was diluted by a factor of 5 to reduce high signal intensity. Relative accumulation levels in comparison to the empty vector reference (V) are shown below respective lanes.

3.1. ADV P but not P6 acts as a local silencing suppressor in N. benthamiana 16c

An *Agrobacterium* leaf patch assay using at least 20 green fluorescent protein (GFP)-transgenic *Nicotiana benthamiana* line 16c plants, which constitutively express mGFP5-ER (Ruiz et al., 1998), was carried out to investigate whether ADV P and P6 act as local RSS (see Table S1 for a list of plasmids used). Co-infiltrations of mGFP5-ER and empty expression vector or tomato bushy stunt virus (TBSV) P19 RSS were used as negative and positive controls, respectively. At 4 dpi, infiltrated leaves were examined using a hand-held UV lamp and photographed. Stronger green fluorescence was observed in the leaf patches expressing ADV P than in the patches expressing either ADV P6 or empty vector, although to a lesser extent than in those expressing TBSV P19 (Fig. 1A); however at 7 dpi no more silencing suppression was observed in those leaf patches expressing ADV P (Fig. S1 in the online version at DOI:http://dx.doi.org/10.1016/j.virusres.2016.08.008). Transient expression of flag-tagged ADV P and P6 proteins was confirmed by western blot analysis at 4

dpi (Fig. S2 in the online version at DOI:http://dx.doi.org/10.1016/j.virusres.2016.08.008). The visual results observed at 4 dpi were confirmed by western blot analysis of the GFP protein (Fig. 1D). Accumulation levels of GFP protein were significantly higher in leaf patches co-infiltrated with the *GFP* construct and TBSV P19 or ADV P constructs, than those expressing empty vector or ADV P6, which showed reduced GFP levels due to silencing of *GFP* mRNA. We observed a 8.3-fold lower GFP protein accumulation in the leaf patches expressing ADV P when compared to TBSV P19 (Fig. 1D). Consistent with the protein data, RNA blot analysis showed that the steady-state level of *GFP* mRNA in the patches expressing ADV P was higher than in those expressing either empty vector or ADV P6, but lower than in the patches expressing TBSV P19 (Fig. 1B). These observations suggest that ADV P acts as a RSS, but that its effect on local GFP silencing was considerably less efficient than that of TBSV P19. TBSV P19 RSS activity led to the accumulation of at least 5-fold more *GFP* RNA than the other constructs tested (Fig. 1B, note that 5 times less total RNA was loaded for the p19 sample in the northern blots). This finding is in agreement with the GFP fluorescence intensity observed in leaf patches and accumulation of GFP protein when comparing ADV P to TBSV P19 (Fig. 1B). Taken together, these data provide evidence that ADV P, but not ADV P6, functions as weak local RSS.

3.2. *ADV P protein does not prevent GFP siRNA accumulation in* N. benthamiana *16c*

We next examined the generation of GFP-derived siRNAs to determine whether ADV P affects their accumulation and to understand how this protein suppresses RNA silencing in plants. By 4 dpi, RNA blots showed that silencing of *GFP* mRNA (Fig. 1B) in tissue agroinfiltrated with GFP plus empty vector constructs or ADV P6 correlated with a strong accumulation of *GFP*-specific siRNAs (Fig. 1C). *GFP*-specific siRNA accumulation was not significantly altered in ADV P co-expressing patches, compared with those patches were the empty vector was co-expressed with mGFP5-ER (Fig. 1C). In contrast, accumulation of *GFP* siRNAs was negligible in the presence of TBSV P19, which is known to sequester siRNAs (Lakatos et al., 2006). These results indicate that ADV P, unlike TBSV P19, does not appear to prevent the accumulation of siRNAs and thus likely targets a step in the RNA silencing pathway downstream of primary siRNA biogenesis.

3.3. *ADV P does not effect the short distance movement of the silencing signal in* N. benthamiana *16c*

It has been reported that locally-induced RNA silencing can spread from initially silenced cells up to 10–15 neighboring cells through the cell-to-cell movement of siRNAs, which in leaves of *N. benthamiana* 16c line can be visualized under UV light as a narrow red halo surrounding the infiltrated area (Himber et al., 2003). To examine the effect of ADV P and P6 on the short-distance spread of *GFP* silencing, the formation of this red zone was monitored at 7 dpi in *N. benthamiana* 16c leaves infiltrated with mGFP5-ER plus ADV P, ADV P6, TBSV P19 or empty vector (see Table S1 for a list of plasmids used). Consistent with previous reports (Himber et al., 2003), the expression of TBSV P19 blocked the short-distance spread of the silencing signal, and thus, no red halo was observed around patches infiltrated with TBSV P19 (Fig. S1 in the online version at DOI:http://dx.doi.org/10.1016/j.virusres.2016.08.008). In contrast, a red zone was consistently observed in those patches expressing ADV P, ADV P6 or the empty vector (Fig. S1 in the online version at DOI:http://dx.doi.org/10.1016/j.virusres.2016.08.008). This data indicates that neither ADV P nor ADV P6 interfered with the short-distance spread of the silencing signal, which is consistent with its lack of inhibition of siRNA accumulation.

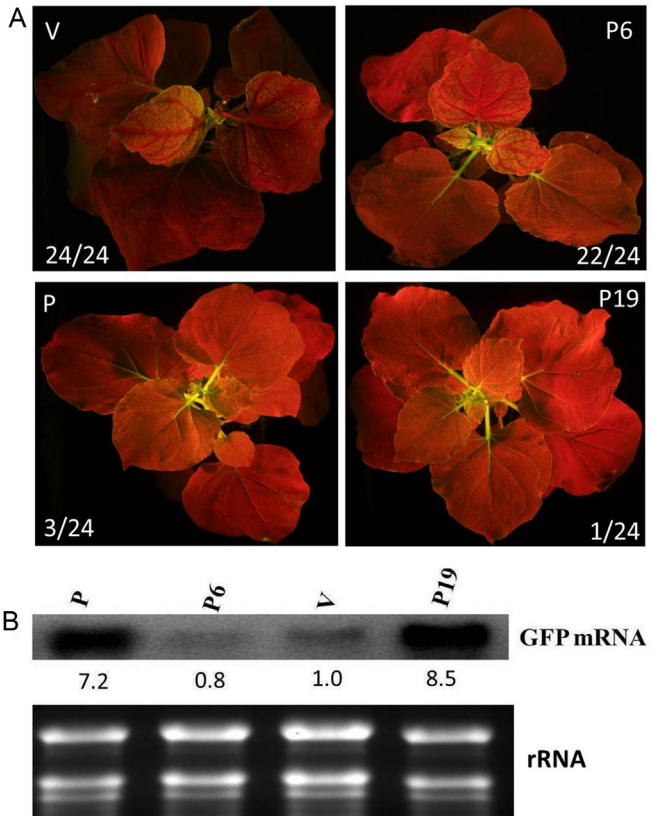

Fig. 2. Systemic GFP silencing suppression activity of ADV P and P6 proteins. (A) *N. benthamiana* 16c plants expressing mGFP5-ER were co-agroinfiltrated with mGFP5-ER plus either ADV P or P6. TBSV P19 or empty pSITE-Flag vector (V), were used as positive and negative controls, respectively. Infiltrated 16c plants were visually examined and scored for suppression of GFP silencing under UV light at 20 dpi. Systemic spread of silencing to distal leaves of infiltrated plants was observed as vein proximal GFP silencing. The number of plants that showed systemic silencing out of the total number of plants tested is indicated in each panel. All images were taken adaxially under long wavelength UV light using a Canon EOS camera equipped with a GFP filter. (B) Top fully expanded *N. benthamiana* line 16c leaves were analyzed for accumulation of *GFP* mRNA using a full-length mGFP5-ER-specific biotin-labeled probe. Ethidium bromide-stained rRNAs are shown as loading controls. Relative accumulation levels in comparison to the empty vector reference (V) are shown below respective lanes.

3.4. *ADV P but not P6 acts as a systemic silencing suppressor in* N. benthamiana *16c*

Once local RNA silencing is initiated in plants, the silencing signal can spread long distance and induce systemic silencing of homologous sequences in upper leaves (Himber et al., 2003), which in 16c plants is characterized by the vein proximal silencing of GFP in newly emerging leaves. To determine whether ADV P or P6 interferes with this process, GFP fluorescence was monitored at 20 dpi in upper leaves of *N. benthamiana* line 16c plants infiltrated with mGFP5-ER plus either ADV P or ADV P6. We used empty expression vector or TBSV P19 as negative and positive controls, respectively (see Table S1 for a list of plasmids used). All 16c plants agroinfiltrated in three independent experiments with mGFP5-ER and the empty vector (24/24) showed systemic GFP silencing (i.e., red auto-fluorescence in upper non-infiltrated leaves) (Fig. 2A). Similarily, all but two (22/24) 16c plants (92%) that were agroinfiltrated with mGFP5-ER and ADV P6 constructs showed systemic GFP silencing (Fig. 2A). In contrast, GFP silencing was observed in only 12.5% (3/24) of 16c plants that were agroinfiltrated with mGFP5-ER and ADV P constructs, while silencing was observed in just 4.2% (1/24) plants agroinfilitrated with mGFP5-ER and TBSV P19 constructs (Fig. 2A). Northern blot analysis indicated that in

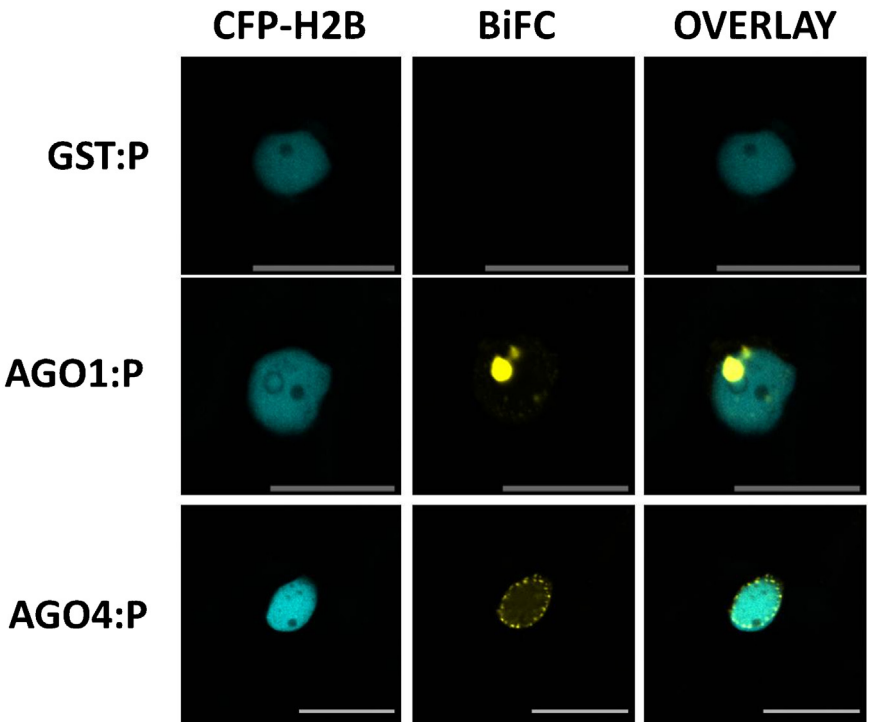

Fig. 3. Bimolecular fluorescence complementation (BiFC) of ADV P protein interactions with RNA silencing proteins AGO1 and AGO4. Protein–protein interaction assays were carried out in transgenic *N. benthamiana* expressing cyan fluorescent protein (CFP) fused to the nuclear marker histone 2B (CFP-H2B). Shown are the localization of CFP-H2B (nucleus, left column), interaction assay (BiFC, middle column) and a merge of both channels (Overlay, right column). Proteins listed first in the pair of interactors were expressed as fusions to the amino-terminal half of yellow fluorescent protein (YFP). Those listed second were expressed as fusions to the carboxy-terminal half of YFP. However, all pairwise combinations were tested but only a subset of those is shown here. Glutathione-S-transferase (GST) was used as a non-binding control to validate interactions for all ADV P interactors but only GST:P is shown here as a representative example. Live cell images of agroinfiltrated leaves were taken at 2 dpi using a confocal microscope at 40× magnification. The inset scale bar corresponds to 20 μm in all panels.

upper leaves of 16c plants that had been infiltrated 20 days earlier with GFP plus ADV P or TBSV P19 constructs, systemic GFP silencing was suppressed and significant levels of *GFP* mRNA had accumulated (Fig. 2B). The accumulation of *GFP* mRNA in upper leaves of plants infiltrated with GFP plus ADV P6 constructs was similar to the upper leaves of those plants co-infiltrated with the empty vector (Fig. 2B). Altogether, these data suggest that ADV P protein is a strong suppressor of systemic RNA silencing similar to TBSV P19, whereas ADV P6 protein does not appear to have systemic silencing suppressor activity.

3.5. ADV P interacts with NbAGO1 and NbAGO4 in planta

To shed further light on how ADV P mechanistically functions as an RSS, we examined its potential interactions with components downstream of siRNA biogenesis. We tested whether ADV P would co-localize and/or interact with RISC components AGO1 and AGO4 when transiently co-expressed in *N. benthamiana* leaves. *N. benthamiana* genes *AGO1* and *AGO4* (kindly provided by Dr. Peter Waterhouse, Queensland University of Technology) (Nakasugi et al., 2013) were cloned into pSITE-RFP-C1, and individually expressed as RFP fusions. At 2 dpi, confocal microscopy of agroinfiltrated *N. benthamiana* leaves showed that RFP-AGO1 fusions accumulated uniformly on the cell periphery and in the nucleus, while RFP-AGO4 was nuclear (Mann et al., 2016a). Next, we examined the localization of these RFP fusions when co-expressed with GFP-ADV P, which localizes to the nucleus (Bejerman et al., 2015). The partial cell periphery accumulation of RFP-AGO1 was no longer detectable when co-expressed with GFP-P, while the nuclear expression remained. On the other hand, AGO4 signal in the nucleus appeared to be mostly excluded from a large subnuclear region, where GFP-ADV P accumulated when RFP-AGO4 was co-expressed with GFP-P (Fig. S3 in the online version at DOI:http://dx.doi.org/10.1016/j.virusres.2016.08.008).

To determine if the observed re-localization effects by ADV P on AGO1 subcellular distribution involved heterotypic protein–protein interactions, BiFC assays were done. At 2 dpi, ADV P showed clear interactions with AGO1 and AGO4 within the cell nucleus as large and small aggregates, respectively (Fig. 3). As a control, ADV N was also tested but failed to show any detectable interaction with AGO1 or AGO4 (data not shown). None of the tested proteins interacted with GST negative control (Fig. 3). Collectivelly, these data indicate that ADV P can interact with AGO1 and AGO4 proteins in the nucleus, but this will need to be further validated with additional independent protein-protein interaction assays.

3.6. ADV P protein likely inhibits miRNA-mediated AGO1 cleavage and translational repression

Targeting of AGO proteins by RSS has been shown to cause inhibition of slicer activity (Azevedo et al., 2010; Fernandez-Calvino et al., 2016; Giner et al., 2010; Kumar et al., 2015; Mann et al., 2016a; Zhang et al., 2006). We hypothesized that ADV P may also interact with AGO1 and/or AGO4 to disrupt slicer activity of assembled RISC complexes. To test whether ADV P inhibits miRNA-loaded RISC complexes *in planta*, we co-expressed GFP sensor constructs with or without a viral suppressor (Csorba et al., 2010; Fernandez-Calvino et al., 2016; Giner et al., 2010; Lakatos et al., 2006; Mann et al., 2016a; Pantaleo et al., 2007; Parizotto et al., 2004). The GFP sensor construct, pUQC10193 (referred to henceforth as "171.1"), transiently expresses a *GFP* gene that contains a miR171 recognition site directly after the *GFP* open reading frame stop codon (Fig. 4A) (Christie et al., 2011). Expression of the GFP sensor construct in *N.*

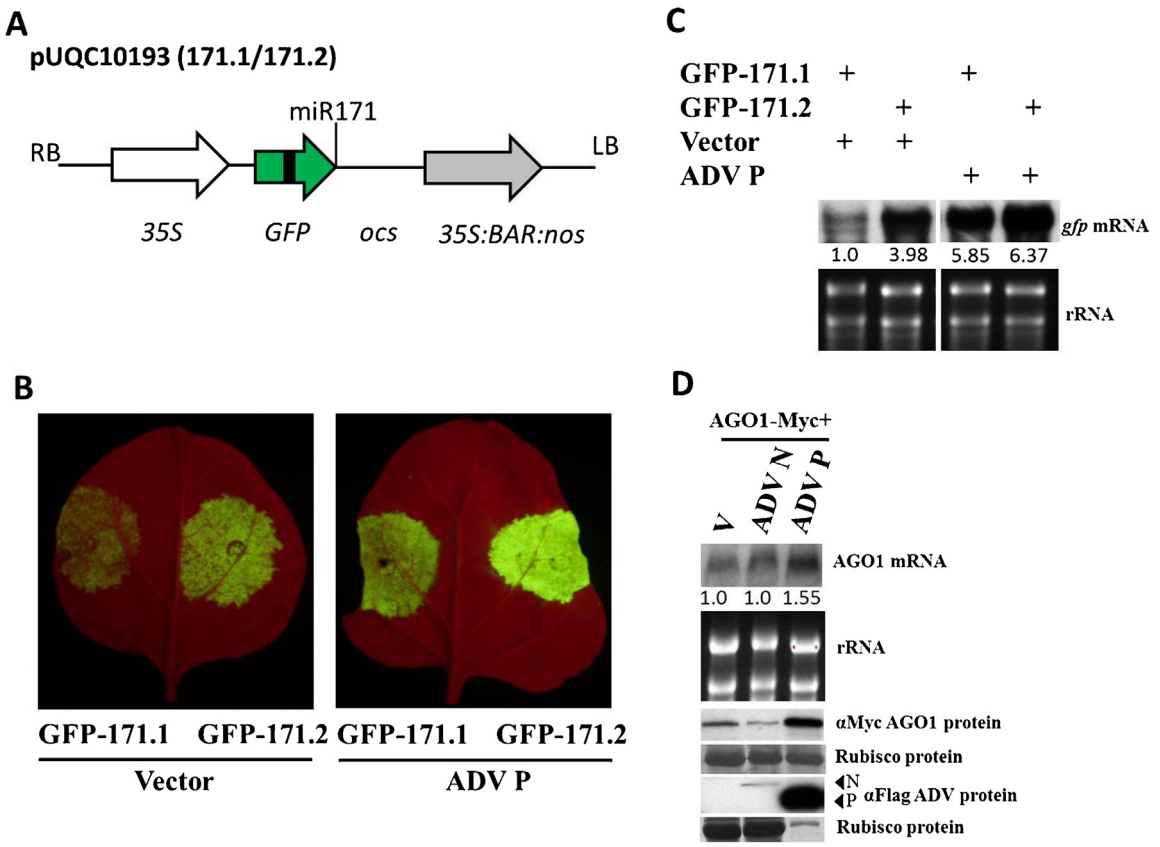

Fig. 4. miRNA-guided AGO1 slicer and AGO1 translational repression assays. *N. benthamiana* leaves were co-agroinfiltrated with (A) a *GFP* sensor construct (171.1–sliceable or 171.2–non sliceable) plus either empty vector or ADV P. (B) *GFP* sensor infiltrated leaves were photographed for GFP fluorescence and (C) analyzed for accumulation of *GFP* mRNA at 4 dpi. Leaf images were taken abaxially under long wavelength UV light using a Canon EOS camera equipped with a GFP filter. Relative accumulation levels of *GFP* mRNA in comparison to the empty vector reference (V) are shown below respective lanes. (D) *N. benthamiana* leaves were co-agroinfiltrated with AGO1-Myc plus either Flag-tagged empty vector (denoted "V"), ADV N or ADV P.Infiltrated patches were analyzed at 4 dpi for AGO1 mRNA and protein accumulation, and for expression of ADV proteins. Using total RNA, *GFP* sensor and AGO1 mRNA were detected using a full-length GFP-specific or *N. benthamiana* AGO1-specific (corresponding to nt position 330–1322) biotin-labeled probe, respectively. Using soluble protein fractions, AGO1-Myc and ADV-Flag tagged proteins were detected using anti-Myc or anti-Flag monoclonal antibodies, respectively. Loading controls for mRNA (ribosomal RNA; rRNA), and protein (RuBisCo) are shown below respective blots. All samples were loaded equally for both RNA and protein blots, with the exception of ADV P for only the protein blot, of which 10 times less protein was loaded to reduce high signal intensity. Relative accumulation levels of *AGO1* mRNA in comparison to the empty vector reference (V) are shown below respective lanes. RB/LB: right/left border; 35S: *cauliflower mosaic virus* 35S promoter; black bar in *GFP* gene indicates an intron; ocs: octopine synthase; BAR: glufosinate tolerance gene; nos: nos terminator.

benthamiana leaves triggers silencing of *GFP* mRNA by the slicer activity of AGO1 loaded with endogenous miR171 which can be observed by reduction of GFP fluorescence under UV light (Giner et al., 2010; Lakatos et al., 2006; Mann et al., 2016a; Parizotto et al., 2004). We also used a cleavage-resistant GFP sensor control construct, referred to as 171.2 (Mann et al., 2016a), which was obtained by mutating the 11th nucleotide in the miR171 recognition site of 171.1 as previously described (Parizotto et al., 2004). In a leaf patch agroinfiltration assay at 4 dpi, GFP fluorescence and mRNA levels were low for 171.1 but high for 171.2 when co-expressed with empty vector (Fig. 4B and C). This shows that the GFP sensor 171.1 was functional and could be cleaved, while the mutated control 171.2 was cleavage-resistant. Elevated GFP fluorescence and mRNA levels were reproducibly observed when either 171.1or 171.2 were co-expressed with ADV P (Fig. 4B and C). These results indicate that ADV P likely inhibits slicing activity of miR171-guided AGO1 RISC. We could not test if ADV P also inhibits siRNA-guided RISC due to lack of an infectious clone of ADV. Interestingly, we noted that ADV P protein sequence did not contain any GW/WG or F-box motifs (data not shown) which are typically present in viral RSS that disrupt AGO activity (Csorba et al., 2015; Incarbone and Dunoyer, 2013).

The above results provide evidence that ADV P protein interacts with AGO1 protein to likely inhibit slicer activity. To determine if ADV P protein interacts with AGO1 at the protein or RNA level, we co-expressed Flag-tagged ADV P and Myc- tagged AGO1 *in planta* and examined AGO1 mRNA and protein accumulation (Fig. 4D). At 4 dpi, northern blot analysis of *N. benthamiana* leaves agroinfiltrated with AGO1-Myc showed a slightly higher *AGO1* mRNA accumulation in those patches where ADV P was co-expressed compared with those patches where ADV N or empty vector were co-expressed (Fig. 4D). Protein blot analysis of the same samples showed that AGO1-Myc protein accumulation was significantly increased when co-expressed with ADV P, compared to co-expression with ADV N or empty vector (Fig. 4D). Taken together, our data provide evidence that ADV P protein stabilizes AGO1 protein in RISC without significantly affecting steady state levels of *AGO1* mRNA.

3.7. *ADV P protein inhibits amplification of RNA silencing*

To determine if ADV P inhibits the amplification of RNA silencing, we carried out GFP transitivity assays. In these assays, full-length sense *GFP* was co-expressed with a "GF"-specific dsRNA hairpin construct corresponding to nucleotides 9–400 of the 720 nt *GFP* coding sequence (Fig. 5A). Co-expression of these two vectors results in the generation of "P"-specific transitive siRNAs corresponding to sequences downstream of nucleotide 400 of the *GFP*

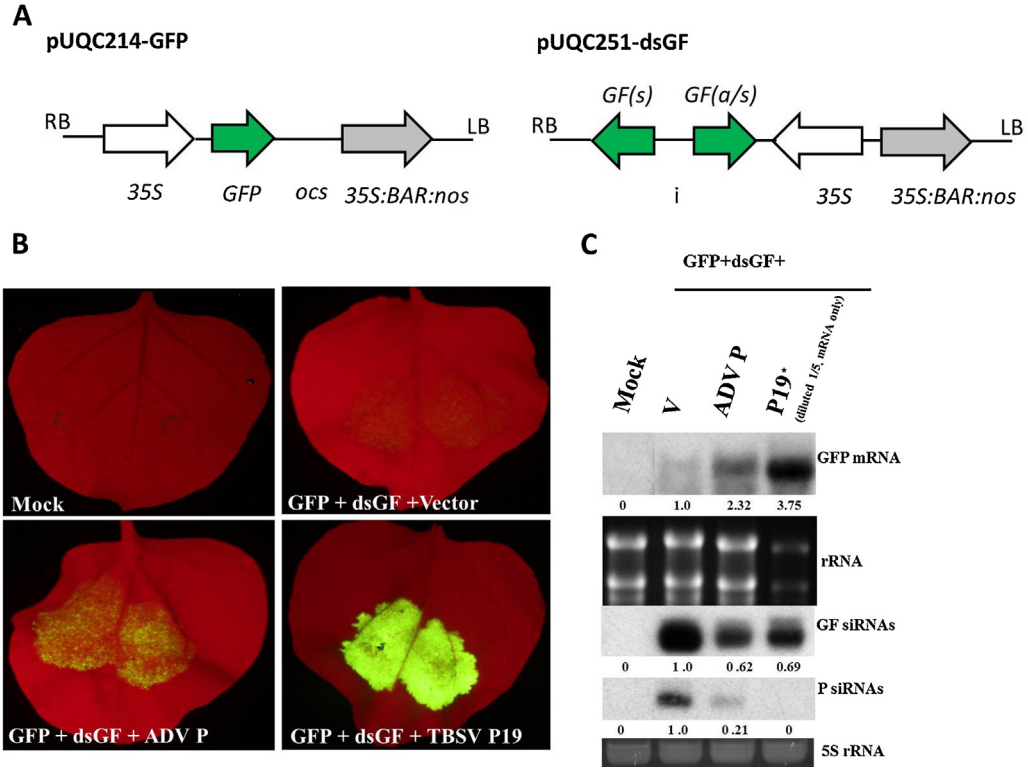

Fig. 5. RNA silencing transitivity assay. (A) T-DNA maps of two-component *GFP* reporter used for transitivity assay, pUQC214-GFP and pUQC251-dsGF. (B) *N. benthamiana* leaves were co-agroinfiltrated with GFP (pUQC214-GFP) and double-stranded GF-specific hairpin constructs (pUQC251-dsGF) plus either ADV P, empty pSITE-Flag vector ("Vector") or TBSV P19. Infiltrated leaves were photographed for GFP fluorescence at 4 dpi. All images were taken adaxially under long wavelength UV light using a Canon EOS camera equipped with a GFP filter. (C) Infiltrated leaf patches involving co-agroinfiltration for mock (buffer), ADV P, TBSV P19 ("P19") and empty vector ("V") were analyzed for accumulation of *GFP* mRNA, primary plus secondary GF-specific siRNAs ("GF siRNAs") and secondary transitive P specific siRNAs ("P siRNAs"). Total RNA was separated into high (mRNA) and low (siRNA) molecular weight fractions, and RNA gel blot analysis was done using a full-length GFP-specific or GF- and P-specific biotin probes, respectively. Loading controls for *GFP* mRNA (ribosomalRNA; rRNA), siRNA (5SRNA), and relative abundance of transcripts in comparison to the empty vector reference (V) are shown below respective lanes. ADV P and empty vector were loaded equally, whereas for the *GFP* mRNA blot only, the TBSV P19 positive control was diluted by a factor of five to reduce high signal intensity (denoted "*").

transcript (Himber et al., 2003). Due to the known high potency of GFP silencing induction by dsGF, we determined that diluting the solution of agrobacteria carrying the dsGFconstruct by a factor of ten (OD_{600} = 0.08) allowed for weaker RSS activities to be identified more reliably than in a standard assay (data not shown). At 4 dpi, GFP fluorescence was maintained only when GFP plus dsGF was co-expressed with ADV P (weak fluorescence) or TBSV P19 (strong fluorescence) but not for the empty vector (Fig. 5B). Consistent with the GFP fluorescence data, northern blot analysis of agroinfiltrated leaf patches showed that ADV P expression on average led to a 2-fold higher *GFP* mRNA accumulation, whereas TBSV P19 expression led to a 15–20-fold higher *GFP* mRNA accumulation (Fig. 5C). The level of primary "GF" siRNA in ADV P expression patches appeared lower than that in the vector expression patches (Fig. 5C). However, ADV P-expressing tissues showed almost no accumulation of secondary "P" siRNAs, whereas TBSV P19-expressing tissues did not accumulate any detectable secondary siRNAs. Since ADV P does not appear to prevent the accumulation of siRNAs (Fig. 1C), the above data suggests that ADV P likely prevents transitive amplification of RNA silencing by interfering with the production of secondary siRNAs.

3.8. *ADV P does not interact with RDR6 or SGS3 in planta*

The ability of ADV P protein to likely prevent transitive amplification of RNA silencing by suppressing the production of secondary siRNAs suggests its interference with components of amplification of RNA silencing, which involves the concerted activity of RDR6 and SGS3 (Glick et al., 2008; Guo et al., 2013). Therefore, we tested whether ADV P would co-localize and/or interact with RDR6 and SGS3 when transiently co-expressed in *N. benthamiana* leaves. *N. benthamiana* genes RDR6 and SGS3 were individually expressed as fusions to the C-terminus of RFP. At 2 dpi, confocal microscopy of agroinfiltrated *N. benthamiana* leaves showed that RFP-RDR6 accumulated on the cell periphery and in the nucleus (Fig. S4A in the online version at DOI:http://dx.doi.org/10.1016/j.virusres.2016.08.008), while RFP-SGS3 formed small aggregates on the cell periphery (Mann et al., 2016a). No major change in intracellular localization was observed for RFP-RDR6 or RFP-SGS3 when co-expressed with GFP-ADV P (Fig. S4B in the online version at DOI:http://dx.doi.org/10.1016/j.virusres.2016.08.008). When BiFC assays were performed ADV P failed to show any detectable interaction with RDR6 or SGS3 (Fig. S4C in the online version at DOI:http://dx.doi.org/10.1016/j.virusres.2016.08.008). Both plant proteins appeared to be correctly expressed, because we observed a cytoplasmic interaction between *N. benthamiana* RDR6 and SGS3 similar to that previously shown for *A. thaliana* counterparts (Kumakura et al., 2009). Taken together, our results appear to indicate that ADV P may be targeting different host factors other than RDR6 and SGS3, involved in secondary siRNA biogenesis, such as DCL2, SDE3 or SDE5.

4. Discussion

In this study, using an *in vivo* co-agroinfiltration approach, we present evidence that the phosprotein (P) encoded by the cytorhabdovirus ADV suppresses local RNA silencing without affecting

primary siRNA accumulation, suggesting effects on downstream steps of the pathway. We further provide evidence that ADV P local RSS functions by arresting RISC activity through binding to argonaute proteins and likely preventing RNA slicing. We also show that ADV P suppresses systemic silencing by inhibiting the production of secondary siRNAs. On the other hand, ADV P6 did not suppress either local or systemic silencing.

In agroinfiltration leaf patch assays, ADV P local GFP silencing suppression activity appeared visually weaker than that of TBSV P19, which was supported by mRNA accumulation levels (Fig. 1). ADV P weak local RSS activity was similar to that reported for the P protein of the cytorhabdovirus LNYV (Mann et al., 2015), and several other plant virus RSS including beet necrotic yellow vein virus p14, rice yellow mottle virus P1, Chinese wheat mosaic virus cysteine-rich protein (CRP), PVX P25 and potato virus M 12 kDa CRP, all of which also exhibit weak RSS activity in leaf patch assays (Andika et al., 2012; Fusaro et al., 2012; Senshu et al., 2009; Senshu et al., 2011; Sun et al., 2013). Interestingly, like ADV P, LNYV P and PVX P25 have been shown to target multiple components of the RNA silencing pathway by suppressing AGO1 and AGO4 activity and systemic silencing (Chiu et al., 2010; Mann et al., 2016a; Voinnet et al., 2000).

Unlike TBSV P19 and most of the known silencing supressors, which sequester siRNAs (Burgyán and Havelda, 2011; Csorba et al., 2015), ADV P does not prevent GFP siRNA accumulation (Fig. 1). This indicates that ADV P may exert its suppressor activity downstream of primary siRNA biogenesis, similar to LNYV P (Mann et al., 2015).

Although ADV P is a weak local RSS (Fig. 1) and is not able to block the cell-to-cell spread of the systemic silencing signal, consistent with its lack of sequestering siRNAs (Fig. S1 in the online version at DOI:http://dx.doi.org/10.1016/j.virusres.2016.08.008), this protein efficiently suppressed systemic silencing (Fig. 2), whereas LNYV P RSS delayed but did not prevent systemic silencing (Mann et al., 2015). ADV P6 does not noticibly suppress either local or systemic RNA silencing (Figs. 1 and 2), unlike its counterpart P6 of the nucleorhabdovirus RYSV, which was shown to suppress systemic RNA silencing efficiently by inhibiting RDR6-mediated secondary siRNA production (Guo et al., 2013). Apparently, RSS activity can differ even among analogous proteins encoded by viruses in the same genus or family. This is also evidenced by P0 of different poleroviruses (Kozlowska-Makulska et al., 2010), as well as the P25 of several potexviruses (Senshu et al., 2009) that show different levels of RSS activity. The function of ADV P6 remains unknown.

It was recently shown that at least the last 20 amino acids of LNYV P protein C-terminal domain is required for both local RNA silencing suppression and interaction with AGO1 and AGO4 (Mann et al., 2016a). Preliminary data suggest that C-terminal portion of ADV P is also likely to be involved in local RSS activity. Future studies using ADV P mutants may confirm this and will determine more generally if the C-terminal portions of cytorhabdovirus P proteins may represent the active domains of RSS activity.

AGO slicer protein in the RISC degrades target RNAs by direct cleavage or translational repression (Brodersen et al., 2008; Mallory and Vaucheret, 2010). RSS can be grouped based on whether they inhibit either RISC assembly or preassembled RISC complexes. ADV P does not appear to sequester siRNAs (Fig. 1), and therefore is unlikely to prevent siRNA loading into RISC. Our experimental evidence suggests that ADV P likely inhibits miRNA-guided AGO1 cleavage (Fig. 4) through direct protein–protein interaction (Fig. 3). Co-infections with an infectious ADV clone system, once available, could be used for siRNA-guided cleavage tests in the future. Nevertheless, it has been reported that other viral RSS that inhibit assembled AGO1-RISC activity impaired with similar efficiency siRNA or miRNA loaded AGO1-RISC (Schott et al., 2012), tempting us to speculate that ADV P may also inhibit siRNA-loaded RISC complexes *in planta*, as reported for SPMMV P1 (Giner et al., 2010).

It has been suggested that GW/WG motifs are essential for the RSS function of TCV P38 and SPMMV P1, which disrupt miRNA-mediated AGO1 cleavage (Azevedo et al., 2010; Giner et al., 2010). GW motifs are found in Argonaute binding proteins, such as Tas3, NRPE1 and RNA Pol V, and AGO-GW interactions have been shown to be important to initiate RISC assembly (El-Shami et al., 2007). However, *in silico* analysis of ADV P did not detect any sequence resembling the GW/WG motifs. Other AGO- inhibiting viral RSS, such as the P0 of poleroviruses and enamoviruses have been shown to encode F- box motifs, which are thought to be involved in the degradation of AGO1 via autophagy (Baumberger et al., 2007; Derrien et al., 2012; Fusaro et al., 2012). ADV P sequence does not contain any identifyable F-box motifs. Furthermore, ADV P appears to enhance rather than diminish AGO1 protein accumulation (Fig. 4), the opposite effect to that observed with P0 (Baumberger et al., 2007; Fusaro et al., 2012). Similarly, no recognizable GW/WG or F-box motifs were identified in LNYV P sequence (Mann et al., 2016a), suggesting that cytorhabdovirus P proteins may represent a novel family of AGO-binding proteins. Further studies using alanine-scanning mutagenesis could be used to determine the motifs linked with AGO binding and its RSS activity.

RNA silencing in plants is amplified with the help of host proteins such as RDR6, SGS3, SDE3, and others such as DCL2 and SDE5, in a process known as transitivity. Transitivity results in the synthesis of secondary siRNAs and is associated with promoting an effective RNA silencing response against viruses (Garcia et al., 2012; Hernandez-Pinzon et al., 2007; Himber et al., 2003; Mlotshwa et al., 2008; Moissiard et al., 2007; Parent et al., 2015). In this study, ADV P showed a strong and reproducibly detectable suppression activity of secondary siRNA synthesis (Fig. 5), whereas LNYV P showed a comparatively weaker activity (Mann et al., 2016a). LNYV P, similar to the potexvirus PlAMV P25, bound to both RDR6 and SGS3 (Mann et al., 2016a; Okano et al., 2014). The majority of RSS known to suppress RNA silencing amplification appear to target either RDR6 (Guo et al., 2013) or SGS3 (Glick et al., 2008; Rajamaki et al., 2014). However, ADV P failed to show any detectable interaction with RDR6 or SGS3 in BiFC assays. Okano et al. (2014) failed to detect an interaction between PlAMV P25 and RDR6 using BiFC but this interaction was detected using co-IP assays. These authors suggested that the reason that they did not detect the interaction using the BiFC assay might have been due to the low expression level of RDR6. However, Mann et al. (2016a) using a similar BiFC assay and the same RDR6 expression construct used here were able to detect an interaction between RDR6 and LNYV P. Alternatively, ADV P could be targeting a different host factor involved in secondary siRNA biogenesis, like DCL2, SDE3 or SDE5. Both SDE3 and DCL2 are localized in the nucleus (Garcia et al., 2012; Xie et al., 2004), where ADV P also accumulates (Bejerman et al., 2015).

AGO1-mediated cleavage would allow RDR6 recruitment to generate secondary siRNAs (Voinnet, 2008). Therefore, it is tempting to speculate that the inhibition of AGO1 by ADV P may be associated with the suppression of transitive silencing, leading to drastically reduced secondary siRNAs (Fig. 5). A similar scenario was proposed for the poleroviral P0 that shown to interfere with AGO1 activity (Fusaro et al., 2012).

The strong inhibition of transitivity by RYSV P6 has been proposed to be connected to its ability to strongly suppress systemic silencing (Guo et al., 2013). On the other hand, LNYV P suppresses transitivity weakly, which might be linked with its capability to delay but not prevent systemic silencing (Mann et al., 2016a). Therefore, it seems reasonable to speculate that the strong suppression of transitivity by ADV P may be linked to its strong suppression of systemic silencing (Fig. 2).

In summary, our data suggest that ADV P, like LNYV P (Mann et al., 2016a) targets multiple steps in the RNA silencing pathway, but unlike LNYV P, ADV P exhibits a strong systemic silencing

activity. We hypothesize that the P protein of cytorhabdoviruses has evolved to target multiple steps of the RNA silencing pathway, linked to their relative weak local RSS activity. Hence, further detailed studies should be conducted to probe the mechanism underlying the RSS activity of this protein, preferably using approaches that resemble the natural context of the virus-plant pathosystem, like the use of a reverse genetics system similar to the one recently developed for SYNV (Wang et al., 2015).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2016.08.008.

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