ARGONAUTE2 Mediates RNA-Silencing Antiviral Defenses against Potato virus X in Arabidopsis

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RNA-silencing mechanisms control many aspects of gene regulation including the detection and degradation of viral RNA through the action of, among others, Dicer-like and Argonaute (AGO) proteins. However, the extent to which RNA silencing restricts virus host range has been difficult to separate from other factors that can affect virus-plant compatibility. Here we show that Potato virus X (PVX) can infect Arabidopsis (Arabidopsis thaliana), which is normally a nonhost for PVX, if coinfected with a second virus, Pepper ringspot virus. Here we show that the pepper ringspot virus 12K protein functions as a suppressor of silencing that appears to enable PVX to infect Arabidopsis. We also show that PVX is able to infect Arabidopsis Dicer-like mutants, indicating that RNA silencing is responsible for Arabidopsis nonhost resistance to PVX. Furthermore, we find that restriction of PVX on Arabidopsis also depends on AGO2, suggesting that this AGO protein has evolved to specialize in antiviral defenses.

The outcome of a plant-virus interaction is determined by the balance between the ability of the virus to exploit host cellular mechanisms, and the multiple defense mechanisms employed against it by the host. As obligate parasites, viruses are unique in that their genetic material is transcribed, translated, and replicated entirely inside the host cell. As such, the inability of a virus to infect a given plant may be due to an incompatibility between viral proteins and the host translational machinery (Robaglia and Caranta, 2006), inhibition of viral replication (Ishibashi et al., 2007, 2009) and movement (Chisholm et al., 2000; Whitham et al., 2000; Cosson et al., 2010), or by detection and elimination by disease resistance (R) proteins (Moffett, 2009).

A major defense mechanism targeting foreign nucleic acids is based on RNA silencing (Ding and Voinnet, 2007). RNA silencing refers to a number of related cellular processes that employ small RNAs to regulate the expression of genetic material in a sequence-specific manner. Small RNAs are generated from dsRNA precursors by DICER-LIKE (DCL) RNase III-related enzymes and subsequently incorporated into RNA-induced silencing complexes (RISCs). The functions of RISCs are carried out in large part by the activity of RNAse H-like Argonaute (AGO) proteins. AGO proteins bind small RNAs, using them as guides to interact with homologous RNA molecules, and subsequently effect endonuclease activity, translational repression of mRNAs, or DNA methylation (Voinnet, 2009).

The highly structured or dsRNA of viruses is targeted by DCL proteins to generate virus-derived small interfering RNAs (vsiRNAs). These vsiRNAs can be incorporated into virus-induced RISC complexes that target viral RNAs, thus making RNA silencing a doubly effective antiviral mechanism (Ding and Voinnet, 2007; Omarov et al., 2007). Furthermore, small RNAs can serve as primers for host RNA-dependent RNA polymerases to generate additional dsRNA targets for DCL enzymes to amplify the silencing signal (Voinnet, 2008; Vaistij and Jones, 2009; Garcia-Ruiz et al., 2010).

In response to the pressure exerted by RNA silencing, plant viruses have evolved viral suppressors of RNA silencing (VSRs; Diaz-Pendón and Ding, 2008; Alvarado and Scholthof, 2009). Mixed infection by two viruses can extend the host range, increase virus accumulation, and exacerbate viral symptoms (Latham and Wilson, 2008). In certain cases, this synergistic effect has been shown to be due to the action, in trans, of the VSR of one of the coinfecting viruses, likely because the VSRs of different viruses can target different RNA-silencing components (Wu et al., 2010). An example of such a synergism is illustrated by the helper component protease from potyviruses that enhances the accumulation and systemic spreading of Potato virus X (PVX) in tobacco (Nicotiana tabacum) and sweet potato (Ipomoea batatas; Pruss et al., 1997; Sonoda et al., 2000).
ARGONAUTE2 and Antiviral Defense

The Arabidopsis (Arabidopsis thaliana) genome encodes four DCL and 10AGO proteins, suggesting a significant degree of diversification and specialization of RNA-silencing components (Vaucheret, 2008). DCL4 and DCL2 have been shown to play major, redundant but hierarchical, roles in silencing RNA viruses, while DCL3 also appears to play a minor role in antiviral defenses (Blevins et al., 2006; Bouche et al., 2006; Deleris et al., 2006; Fusaro et al., 2006; Dounaev et al., 2008; Qu et al., 2008; Garcia-Ruiz et al., 2010). As such, mutation of dcl2 and dcl4 allows VSR-defective viruses to establish systemic infection and increases susceptibility to Tobacco rattle virus (TRV; Deleris et al., 2006; Garcia-Ruiz et al., 2010). With respect to the Arabidopsis AGO genes, hypomorphic ago1 mutants were found to be somewhat more susceptible to Cucumber mosaic virus (CMV; Morel et al., 2002) whereas injection of ago2 mutants with Turnip crinkle virus (TCV) and CMV was shown to result in more severe symptoms, but only a small and transient effect on virus accumulation (Harvey et al., 2011). At the same time, injection of ago1 and ago7 mutants with VSR-defective derivatives of TCV indicates complementary roles for AGO1 and AGO7 in antiviral defenses (Qu et al., 2008; Azevedo et al., 2010). Additionally, Ago4-like proteins have been implicated in virus defense, but only upon induction of an R-gene-mediated response (Bhattacharjee et al., 2009).

RNA silencing may also be an important mechanism of nonhost resistance against viruses, whereby a plant is resistant to all known isolates of a given pathogen. However, the extent to which this may be true has not been investigated. PVX is the type member of the genus Potexvirus (Martelli et al., 2007) and infects several solanaceous hosts, but Arabidopsis is considered a nonhost for PVX. However, an Arabidopsis-infecting strain of PVX (PVX 2.7) has been described (Cooper et al., 2003) and used in microarray expression studies (Whitham et al., 2003). We show here that PVX 2.7 can infect Arabidopsis because of the presence of a second virus, Pepper ringspot virus (PepRSV). PepRSV is a tobavirus (MacFarlane, 1999, 2010) and as expected, it encodes a VSR, which we demonstrate to be the PepRSV 12K protein. To demonstrate that PVX restriction in Arabidopsis is due to RNA-silencing mechanisms we show that PVX is able to infect a dcl2 dcl3 dcl4 triple mutant. Finally, we show that mutation of AGO2 alters the ability of PVX to infect Arabidopsis. This report demonstrates that RNA silencing may represent an important mechanism that restricts wild-type, non-host-adapted viruses, and that the AGO2 protein plays an important role in antiviral defenses.

RESULTS

Identification of a Virus Allowing PVX Infection of Arabidopsis

Sap derived from PVX 2.7-infected Nicotiana clevelandii leaves (Cooper et al., 2003) was used to rub inoculate Arabidopsis Columbia-0 (Col-0) plants and systemic disease symptoms were observed, including plant stunting, necrosis of rosette leaves, and curved stems (Fig. 1A). In contrast, Arabidopsis Col-0 plants infected with PVX strain UK3 showed no obvious viral symptoms. Five days post inoculation, high levels of accumulation of PVX coat protein (CP) were detected in protein extracts from leaves inoculated with PVX 2.7, but not in leaves inoculated with PVX UK3 (Fig. 1B). Both PVX strains infected Nicotiana benthamiana with equal efficiency as judged by PVX CP accumulation in inoculated leaves (Fig. 1B). Furthermore, sap preparations of both strains also elicited similar numbers of local lesions on Rx2 transgenic plants (see below, Supplemental Fig. S2), indicating that the two

Figure 1. Infection of Arabidopsis by PVX 2.7. A, Symptoms induced in wild-type Arabidopsis (Col-0) by the PVX 2.7 strain. Three leaves per plant were rub inoculated with sap made from either PVX UK3-infected tobacco (PVX UK3) or PVX 2.7-infected N. clevelandii (PVX 2.7). Photographs were taken 3 weeks after inoculation. B, Representative immunoblots of PVX CP accumulation in Arabidopsis and N. benthamiana. Total protein extracts were prepared from inoculated Arabidopsis or N. benthamiana leaves 5 or 6 d, respectively, after inoculation, and subjected to SDS-PAGE followed by anti-CP immunoblotting. Coomassie (left) or Ponceau (right) staining of the same extracts is shown to demonstrate equal loading. Each lane corresponds to the pool of the three inoculated leaves from a single plant for Arabidopsis and a single leaf for N. benthamiana. Three plants per treatment were tested in each Arabidopsis experiment and the experiment was repeated five times for Arabidopsis and twice for N. benthamiana.
different inocula have similar viral infectious titres. At the same time however, rub inoculation of PVX 2.7 on Rx transgenic tobacco induced yellow ringspot lesions and vein necrosis whereas these plants are normally immune to PVX and show no visible lesions upon PVX infection (Supplemental Fig. S1; Bendahmane et al., 1999). Furthermore, in contrast to PVX UK3, PVX 2.7 induced necrotic lesions on cowpea (Vigna unguiculata; Supplemental Fig. S1), leading us to consider that PVX 2.7 might be contaminated with a second virus.

To determine if another virus was present in the PVX 2.7 sap, we used an expanded version of a macroarray developed to identify viruses from infected plants (Agindotan and Perry, 2007) that consists of 70-mer DNA oligonucleotides specific to over 100 known viruses along with ribosomal plant RNA hybridization controls (K.L. Perry, unpublished data). The presence of specific viruses can be detected by hybridization with a labeled preparation of randomly primed, reverse-transcribed, and amplified cDNAs derived from infected plant RNAs (Agindotan and Perry, 2008). As expected, labeled cDNA derived from PVX 2.7-infected plants hybridized to PVX sequences present on the macroarray (Fig. 2). At the same time, a robust signal was detected corresponding to PepRSV (Fig. 2). PepRSV is a bipartite single-stranded positive-sense RNA virus, and a member of the genus Tobravirus (MacFarlane, 2010). To confirm the identity of PepRSV, total RNA from a PVX 2.7-infected Arabidopsis leaf was subjected to reverse transcription (RT)-PCR using generic tobravirus PCR primers that amplified the open reading frames encoding the PepRSV-12K and -29K proteins (see below). Sequence analysis showed these to be 100% and 99% identical to corresponding sequences in the published PepRSV sequence (Supplemental Fig. S3). Given that sequence identity is low between tobravirus species (MacFarlane, 2008), we thus conclude that the contaminating virus in PVX 2.7 is indeed PepRSV.

To demonstrate that PepRSV is responsible for PVX accumulation in Arabidopsis, we inoculated plants with a mixture of PVX UK3- and PepRSV-containing saps. Five days post infection, inoculated leaves were collected and proteins extracted. Immunoblot analysis revealed high levels of PVX CP in the presence of PepRSV (Fig. 2). Moreover, although PepRSV by itself induces necrotic symptoms on Arabidopsis, coinfection of both viruses consistently induced more severe symptoms (Fig. 3), indicative of a synergistic interaction between the two viruses.

The PepRSV 12K Protein Possesses VSR Activity

The apparent synergistic relationship between PepRSV and PVX suggests that coinfection involves the inhibition of RNA silencing. The PepRSV 12K protein is homologous to the TRV 16K protein that possesses VSR activity (Ghazala et al., 2008; Martinez-Priego and Baulcombe, 2008; Martinez-Priego et al., 2008). To demonstrate VSR activity in the 12K protein we tested it in the standard Agrobacterium-mediated transient expression (agroinfiltration) assay that takes advantage of the fact that transiently expressed transgenes are subjected to RNA silencing, and that this silencing is suppressed by many VSRs.

Figure 2. Detection of PepRSV. Total RNA from PVX 2.7-inoculated Arabidopsis leaves was extracted 5 d after inoculation, converted to labeled cDNA, and hybridized to the macroarray. Black arrowheads indicate the locations of the columns and rows with the spotted control oligonucleotides specific to plant ribosomal RNAs. Arrows indicate the boxed columns with the spotted PepRSV- and PVX-specific oligonucleotide probes.
(Voinnet et al., 2003). Transient expression of 35S-GFP by agroinfiltration in N. benthamiana leaves results in bright fluorescence visible at 3 d post infiltration (dpi). However, by 5 dpi, GFP expression is silenced and fluorescence only faintly visible (Fig. 4A). Coexpression of GFP with either PepRSV 12K or the well-characterized VSR, P19 (Voinnet et al., 2003), resulted in strong GFP fluorescence at 5 dpi, whereas the PepRSV 29K movement protein had no effect on GFP persistence (Fig. 4A). PVX encodes three genes required for viral movement, known as the triple gene block (TGB), including P25, which also possesses VSR activity (Voinnet et al., 2000; Bayne et al., 2005). We expressed 12K with a version of PVX that expresses GFP but lacks the TGB (PVX-GFPΔTGB), to determine whether the 12K protein could substitute for a lack of P25 as a VSR. Indeed, GFP fluorescence derived from PVX-GFPΔTGB was significantly augmented by coexpression with 12K, to a degree that was consistently higher than that afforded by coexpression with P19 (Fig. 4B).

Role of RNA-Silencing Components in Arabidopsis Resistance to PVX

Given that PepRSV enables PVX to infect Arabidopsis, we hypothesized that this may be due to efficient elimination of PVX by RNA-silencing mechanisms rather than an intrinsic inability to replicate and move in this host. To test this, we infected an Arabidopsis triple dicer mutant, del2 del3 del4. Arabidopsis Dicer mutants allow enhanced accumulation of several Arabidopsis-infecting RNA viruses (Blevins et al., 2006; Bouche et al., 2006; Deleris et al., 2006; Fusaro et al., 2006; Donaire et al., 2008; Qu et al., 2008; Garcia-Ruiz et al., 2010), and the triple mutant has a dramatically reduced ability to produce vsiRNAs (Deleris et al., 2006). At 4 dpi we observed high levels of PVX CP accumulation in infected leaves and after 21 d.

Figure 3. Coinoculation of PVX and PepRSV on Arabidopsis. Three leaves per plant were rub inoculated with sap containing PVX UK3, sap containing PepRSV, or a mixture of both saps. A, Photographs were taken 3 weeks post infection. Enlargements of the singly or doubly infected rosettes are shown in the bottom section. B, Representative immunoblot analysis of the PVX CP accumulation in inoculated leaves. Total protein extracts were prepared from inoculated leaves 3 d after inoculation and subjected to SDS-PAGE followed by anti-CP immunoblotting. Coomassie staining of the same extracts is shown to demonstrate equal loading. Each lane corresponds to the pool of three inoculated leaves from the same plant. Three plants per treatment were tested in each experiment and the experiment was repeated five times.

Figure 4. VSR activity of PepRSV-12K. A, Persistence of fluorescence from GFP upon coexpression with PepRSV-12K. GFP was transiently expressed via agroinfiltration from the 35S promoter in N. benthamiana, together with either 35S:PepRSV-12K:HA, 35S:P19, 35S:PepRSV-29K:HA, or pBin61 empty vector (EV). GFP signal was monitored by UV illumination at 3 and 5 d after infiltration. B, PepRSV-12K enhances PVX-GFPΔTGB in N. benthamiana. A derivative of PVX expressing GFP but lacking the TGB (PVX-GFPΔTGB) was expressed from the 35S promoter by agroinfiltration, together with either 35S:PepRSV-12K:HA, 35S:TBSV-P19, 35S:PepRSV-29K:HA, or pBin61 empty vector (EV). GFP signal was monitored by UV illumination at 3 and 5 d after infiltration.
plants presented severe disease symptoms with systemic necrosis (Fig. 5).

Hypomorphic ago1 and null ago7 mutants are more susceptible to VSR-defective TCV (Qu et al., 2008; Azevedo et al., 2010). To determine whether wild-type PVX is targeted by similar AGO proteins, PVX was inoculated onto Arabidopsis lines possessing the hypomorphic ago1-27 allele, mutant lines with null alleles in the AGO2, AGO3, AGO4, AGO5, AGO6, AGO7, AGO8, AGO9, and AGO10 genes, as well as the dcl2 dcl3 dcl4 mutant as a control. Twenty-eight days after inoculation (at least 18 plants for each genotype), no visible virus symptoms were apparent in the systemic tissues in any of the 10 ago mutants (Fig. 6A). However at 5 dpi, PVX CP accumulation was detected in the inoculated leaves of ago2 mutant plants (Fig. 6B). Despite a lack of visible systemic symptoms following PVX inoculation, PVX CP was detected by immunoblotting in the systemic tissues of eight out of 18 PVX-inoculated ago2 plants (compared to zero out of similar numbers of all other ago mutant genotypes), albeit at lower levels than in the dcl2 dcl3 dcl4 mutants (Fig. 7A). In addition, sap collected from systemic tissues of PVX-infected ago2 mutants induced HR lesions on Rx2 plants (2/4 times), indicating the presence of PVX in these plants (Fig. 7B). In contrast no HR lesions were induced with sap from PVX-inoculated wild-type Arabidopsis or any of the other ago mutants (Fig. 7B). This result indicates that AGO2 plays a major role in the antiviral defense against PVX in Arabidopsis.

DISCUSSION

To our knowledge, these results provide the first demonstration that RNA silencing plays a major role in restricting a non-host-adapted virus. We show that synergy between PVX and PepRSV enable systemic infection of PVX in Arabidopsis. Both synergism and expansion of host range have been shown to occur upon virus coinfection and in certain cases VSRs, such as helper component proteinase, have been implicated in this phenomenon (Sonoda et al., 2000; Latham and Wilson, 2008). The PepRSV 12K Cys-rich protein is homologous to the TRV 16K protein, which has previously been shown to function as a VSR (Ghazala et al., 2008; Martin-Hernandez and Baulcombe, 2008; Martinez-Priego et al., 2008), and we find that PepRSV 12K functions efficiently as a VSR in N. benthamiana transient expression assays (Fig. 4).

We did not observe accumulation of a PVX-12K construct in either inoculated or systemic leaves of Arabidopsis (data not shown). This may not be surprising since, in addition to a cell-autonomous VSR function (Bayne et al., 2005), a critical property of the PVX VSR, the P25 protein, seems to be its ability to suppress a silencing signal, both locally and systematically (Voinnet et al., 2000; Himber et al., 2003; Bayne et al., 2005). The silencing signal involves the movement of siRNAs (Dunoyer et al., 2010; Molnar et al., 2010) and it is thought that such a mechanism allows vsiRNAs to accumulate in uninfected cells ahead of viral movement where they can target viral RNAs upon their entry into the cell. It is unclear how the P25 protein inhibits vsiRNA movement, although the use of this strategy may indicate that PVX is particularly sensitive to the effect of having mobile vsiRNAs present in uninfected cells prior to viral entry. If so, the presence of vsiRNAs in a cell prior to infection would be predicted to target PVX, via AGO2, before significant amounts of VSR protein can be expressed from the PVX genome. However, coinfection of Arabidopsis leaf cells by PepRSV appears to suppress silencing mechanisms, thereby allowing PVX to move into these cells. Likewise, a block in vsiRNA production in the triple dicer mutant, or a block in the appropriate vsiRNA effector AGO protein, would accomplish the same outcome.

The ability of PVX to infect the Arabidopsis triple dicer mutant suggests that the P25 protein may not fully function as an effective VSR in this host. Previous reports have shown that P25 is able to suppress silencing induced by RNA hairpins in both N. benthamiana and Arabidopsis (Dunoyer et al., 2004; Bayne et al., 2005). However the effect of P25 on the spread of silencing signals has not been investigated in Arabidopsis and the developmental phenotypes induced by

Figure 5. PVX infection of an Arabidopsis triple DICER mutant. A, Symptoms induced by PVX UK3 infection in wild type and dcl2 dcl3 dcl4 mutant Arabidopsis. Three leaves per plant were rub inoculated with sap containing PVX UK3. Photographs were taken 4 weeks after infection. B, Immunoblot analysis of PVX CP accumulation in inoculated leaves. Total protein extracts were prepared from inoculated leaves 6 d after inoculation and subjected to SDS-PAGE followed by anti-CP immunoblotting. Coomassie staining of the same extracts is shown to demonstrate equal loading. Each lane corresponds to a pool of the three inoculated leaves from a single plant. Four plants per treatment were tested in each experiment and the experiment was repeated five times.
transgenic expression of P25 in Arabidopsis are milder than for other VSRs (Dunoyer et al., 2004). Alternatively, the inability of PVX to infect Arabidopsis may be due to a combination of factors, with the inhibition or ablation of silencing allowing it to overcome a threshold required for systemic infection.

Several VSRs directly target AGO1 and either prevent it from loading siRNAs or induce its degradation (Zhang et al., 2006; Baumberger et al., 2007; Azevedo et al., 2010; Giner et al., 2010). The VSRs P1 and P38 exert their suppressor of silencing activity through direct interaction with AGO1 via their WG/GW motifs (Azevedo et al., 2010; Giner et al., 2010). Sequence analysis of tobamovirus 16K homologs indicates the presence of a conserved GW motif in the N termini of these proteins (Ghazala et al., 2008). However, attempts to coimmunoprecipitate multiple AGO proteins with 12K and 16K did not reveal any interactions (data not shown). Recently, it was reported that the PVX P25 protein is able to interact with AGO1, AGO2, AGO3, and AGO4, as well as destabilizing AGO1 (Chiu et al., 2010). Using comparable methodology, we are similarly unable to detect interactions between P25 and a number of AGO proteins, including Arabidopsis and tobacco AGO2 (data not shown).

It is generally assumed that degradation of viral RNA by Dicer proteins alone is not sufficient to effect virus resistance and that their vsiRNA products must eventually be loaded into RISC complexes to target viral RNAs. The AGO1, AGO2, and AGO5 proteins can bind vsiRNAs (Zhang et al., 2006; Takeda et al., 2008; Harvey et al., 2011) but, of the 10 Arabidopsis AGO proteins, genetic analysis has implicated only AGO1, AGO2, and possibly AGO7 in virus resistance. The ago1-27 hypomorphic mutant shows a 5-fold greater accumulation of CMV RNA and both an ago1 hypomorphic mutant and an ago7 null mutant allow increased accumulation of a VSR-defective version of TCV (Morel et al., 2002; Qu et al., 2008; Azevedo et al., 2010). Infection of ago2 mutants with CMV and TCV resulted in more severe viral symptoms, but little difference in virus accumulation (Harvey et al., 2011). Surprisingly, we did not observe any difference in PVX accumulation on either ago1-27 or ago7 mutants (Fig. 6). Instead, we observed PVX accumulation in inoculated and systemic tissues only in the ago2 mutant (Figs. 6 and 7), indicating that AGO2 is essential for curtailing PVX on this host. Furthermore, our results show that AGO2 activity can absolutely limit virus infection rather than simply playing a secondary role to AGO1, as previously suggested (Harvey et al., 2011). Interestingly, AGO2 is placed in the same phylogenetic clade as AGO7 (Vaucheret, 2008) but unlike ago7, which is involved in tasi-RNA biogenesis, ago2 mutants show no obvious phenotype (Vaucheret, 2008). This may indicate that AGO2 has evolved to specialize in antiviral defenses.

We do not formally rule out a role for AGO1 in resistance to PVX as the protein encoded by ago1-27 may possess sufficient activity to target PVX, but not TCV or CMV. Indeed, although we find that ago2 is the only single mutant capable of allowing PVX infection, this infection is not as efficient as in the dcl2 dcl3 dcl4 mutant (Figs. 5–7), suggesting that at least one other

![Figure 6. PVX infection in Arabidopsis ago mutants. A, Symptoms induced by PVX UK3 infection on wild type and ago1-27, ago2-1, ago3-2, ago4-2, ago5, ago6-3, ago7, ago8-1, ago9, and ago10 Arabidopsis mutants. Three leaves per plants were rub inoculated with sap made from PVX UK3-infected tobacco (PVX UK3). Photographs were taken 4 weeks after infection. B, Immunoblot analysis of PVX CP accumulation in inoculated leaves. Total protein extracts were prepared from inoculated leaves 5 d after inoculation and subjected to SDS-PAGE followed by anti-CP immunoblotting. Coomassie staining of the same extracts is shown to demonstrate equal loading. Each lane corresponds to a pool of the three inoculated leaves from a single plant. This result was obtained consistently in five experiments including at least three replicates for each genotype.](image-url)
AGO protein acts in PVX antiviral silencing. A supplemental or hierarchical role of another AGO protein (s) would not be surprising given the redundancy/ complementarity seen with members of the Dicer family (Deleris et al., 2006; Garcia-Ruiz et al., 2010). At the same time AGO2, which is regulated by an endogenous miRNA (Allen et al., 2005), is expressed at a 4.6-fold higher level in an ago1 hypomorphic mutant (Kurihara et al., 2009), and AGO2 protein levels are significantly up-regulated upon infection with TCV and CMV (Harvey et al., 2011). Thus, increased levels of AGO2 may in theory compensate for a lack of AGO1 with respect to PVX resistance.

A previous report has shown that the requirement for AGO1 versus AGO7 for resistance to a VSR-defective version of TCV differed depending on whether the virus contained a GFP insert (Qu et al., 2008). Likewise, although AGO1 has been shown to play an important role in defense against VSR-defective TCV in Arabidopsis (Qu et al., 2008; Azevedo et al., 2010), we find that down-regulation of NbAgo2 has a much greater effect than NbAgo1 on VSR-defective TBSV in N. benthamiana (Scholthof et al., 2011). This suggests that different AGO proteins may vary in their effectiveness in targeting different viral RNA genomes depending on as-yet-undefined characteristics of the viral RNA genome. Why certain AGO proteins might be more or less important for targeting different viruses is unclear. Recent reports have shown that the regions of a virus most effectively targeted by vsiRNAs do not necessarily correlate with those regions corresponding to the most abundant viRNAs (Siziyta et al., 2010) and that AGO proteins show bias in the siRNAs they bind (Mi et al., 2008; Takeda et al., 2008; Ho et al., 2010). Thus, it is tempting to speculate that targeting by a given AGO protein may depend on a combination of the availability of the viral genome to RNA-silencing components, determined by RNA structure, together with the nucleotide composition of such regions.

**CONCLUSION**

Our results show that in addition to its well-defined role in the interaction between viruses and their hosts, RNA silencing is also an important mechanism of nonhost resistance against non-host-adapted viruses. Furthermore, our results indicate an important role for the AGO2 protein in antiviral defenses that will lay the groundwork for future studies on the interplay between different AGOs, DCLs, vsiRNAs, and viruses.

**MATERIALS AND METHODS**

**Plant and Virus Materials**

*Nicotiana benthamiana*, *Nicotiana clevelandii*, and tobacco (*Nicotiana tabacum*) were germinated and grown in a glasshouse maintained at 21°C. Samples of PVX 2.7-infected *N. clevelandii* leaves were generously provided by Bret Cooper (Cooper et al., 2003) and were used to produce sap for further virus infections. PVX strains were propagated in tobacco (*N. clevelandii* UK3) and *N. clevelandii* (2.7), respectively. PepRSV was isolated by infecting *Rx2* transgenic *N. benthamiana* (Bhattacharjee et al., 2009) with PVX 2.7 sap and subsequently propagated in *N. clevelandii*. Virus saps were prepared by grinding infected or systemic tissue in 0.1 m potassium phosphate buffer, pH 7.5. The concentration of PVX-containing inocula was normalized according to the immunoblot signal of PVX CP. Arabidopsis (*Arabidopsis thaliana*) wild type and mutants are of the Col-0 ecotype and were cultivated in growth chambers under long-day period (16 h/8 h). All Arabidopsis mutant lines were of the Col-0 ecotype and have been previously described, including the triple dicer mutant *dcl2-1/dcl3-1/dcl4-2* (Deleris et al., 2006), *ago1-27* (Morel et al., 2002), *ago2-1, ago3-2, ago6-3, ago8-1 ago10-2* (Takeda et al., 2008), *ago5, ago7, ago9* (Katiyar-Agarwal et al., 2007), and *ago4-2* (Agorio and Vera, 2007). All virus infections of Arabidopsis were carried out by rubbing sap on carborundum-dusted leaves of 3-week-old plants with the side of pipette tip.

**Immunoblot Analysis**

Total proteins were extracted in 8 m urea solution, separated in a 10% SDS-polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane by electroblotting. Each sample analyzed corresponds to a pool of inoculated leaves or systemic tissue of a single plant. For each infection, at least three plants were tested in more than five different experiments. PVX CP was probed with
anti-PVX CP rabbit polyclonal antibodies (Agdia) and subsequently detected by a goat anti-rabbit Horseradish peroxidase-conjugated polyclonal antibody followed by chemiluminescence detection (ECL, GE Healthcare).

Cloning and Sequencing of PVX 2.7

Total RNA from PVX 2.7-infected Nicotiana glutinosa or Arabidopsis was extracted with Trizol reagent according to the manufacturer instructions (Invitrogen). cDNAs were obtained after RT using a primer targeting the 3′ untranslated region of PVX (5′-ATTTATATTCAATCAATCAAC-3′) and PCR (Expand Hi Fidelity Taq DNA polymerase, Roche) with primers targeting overlapping fragments of PVX, based on the PVX UK3 sequence. PCR products were cloned into pGEMT (Promega) and sequenced and overlap-PCR (Expand Hi Fidelity Taq DNA polymerase, Roche) with primers targeting #D (Voinnet et al., 2003). PVX-GFP of PepRSV was further confirmed by amplification of the #N primer targeting the 3′ end of the PepRSV genome and used to probe the macroarray membrane as previously described (Invitrogen). cDNAs were obtained after RT using polydT primers and PCR.

Macroarray Hybridizations and Identification of PepRSV

cDNA were generated from PVX 2.7-infected Arabidopsis RNA extraction and used to probe the macroarray membrane as previously described (Agindotan and Perry, 2007, 2008). The identity of PepRSV was confirmed by RT-PCR from total RNA isolated from a PVX 2.7-infected Arabidopsis plant using generic tobaviruses PCR primers Tobra-F3 and Tobra-R2, which amplify an approximately 988-bp fragment, as previously described (Jones et al., 2008). The latter PCR product was sequenced using the same primers and used to query sequence databases. The 668 bp that could be read from this sequencing had been deposited in GenBank as accession number JF906525. The identity of PepRSV was further confirmed by amplification of the PepRSV 12K and -29K genes from total Triloz-extracted (Invitrogen) RNA from PepRSV-infected N. benthamiana. cDNAs were obtained after RT using polydT primers and PCR (Expand Hi Fidelity Taq DNA polymerase, Roche) with the primer pairs PRSV12KXbaL.F (5′-CACCTCTAGAAGCTAGGATCTTGCTCTAC-3′) + PRSV12KXbaL.R (5′-CAGCAGATCCCACACTTAAACACAG-3′) or PRSV29KXbaL.F (5′-CACCTCTAGAAGCTAGGATCTTGCTCTAC-3′) + PRSV29KXbaL.R (5′-CAGCAGATCCCACACTTAAACACAG-3′). PRSV12K and PRSV29K PCR products described above were digested with XbaI/BamHI and inserted into the same sites of the pBin61 HA plasmid (Moffett et al., 2002).

Plasmid Construction

Plasmids 335-GFP, pBIN61, and 335-P19 have been previously described (Voinnet et al., 2003). PVX-GFP at TGB was constructed by overlapping PCR to delete the entire TGB from the binary vector pG208 (PVX-GFP, Peart et al., 2002). To clone the PepRSV 12K and -29K genes into expression vectors, the PepRSV12K and PRSV29K PCR products described above were digested with XbaI/BamHI and inserted into the same sites of the pBin61 HA plasmid (Moffett et al., 2002).

Transient Silencing Suppression Activity Assays

Wild-type N. benthamiana plants were infiltrated with Agrobacterium tumefaciens clones (agroinfiltration) as previously described (Moffett et al., 2002). Final concentrations for each transgene were: OD 600 = 0.2. GFP expression was monitored under UV light using a handheld lamp (UVP BP-100AP).

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