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Functional and Genetic Analysis Identify a Role for Arabidopsis ARGONAUTE5 in Antiviral RNA Silencing

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RNA silencing functions as an antiviral defense through the action of DICER-like (DCL) and ARGONAUTE (AGO) proteins. In turn, plant viruses have evolved strategies to counteract this defense mechanism, including the expression of suppressors of RNA silencing. *Potato virus X* (PVX) does not systemically infect *Arabidopsis thaliana* Columbia-0, but is able to do so effectively in mutants lacking at least two of the four Arabidopsis DCL proteins. PVX can also infect *Arabidopsis ago2* mutants, albeit less effectively than double DCL mutants, suggesting that additional AGO proteins may mediate anti-viral defenses. Here we show, using functional assays, that all Arabidopsis AGO proteins have the potential to target PVX lacking its viral suppressor of RNA silencing (VSR), P25, but that only AGO2 and AGO5 are able to target wild-type PVX. However, P25 directly affects only a small subset of AGO proteins, and we present evidence indicating that its protective effect is mediated by precluding AGO proteins from accessing viral RNA, as well as by directly inhibiting the RNA silencing machinery. In agreement with functional assays, we show that Potexvirus infection induces *AGO5* expression and that both AGO2 and AGO5 are required for full restriction of PVX infection in systemic tissues of Arabidopsis.

INTRODUCTION

Plants employ multiple defense mechanisms to restrict virus replication and movement (Incarbone and Dunoyer, 2013). RNA silencing is utilized by plants to counteract invading nucleic acids, including viruses, and is conserved in most eukaryotic organisms (Ding and Voinnet, 2007). RNA silencing refers collectively to diverse RNA-based processes that are triggered by the presence of double-stranded RNA (dsRNA). In the case of defense against single-stranded RNA (ssRNA) viruses, dsRNA arises from replication intermediates as well as highly structured ssRNA. This viral dsRNA is recognized and cleaved into small interfering RNAs (siRNAs), or viral small interfering RNAs (vsiRNAs), by DICER-like (DCL) proteins. After incorporation of these vsiRNAs duplexes into an RNA-induced silencing complex (RISC), one strand of the duplex is then used as a sequence-specific guide to suppress gene expression of complementarity ssRNA either by cleavage or translational repression (Baulcombe, 2004; Voinnet, 2009). Although the exact composition of RISC complexes is still unclear, the core components of these complexes are the RNase H-like ARGONAUTE (AGO) proteins (Iwakawa and Tomari, 2013).

Plant viruses are able to counteract RNA silencing defense mechanisms by expressing viral suppressors of RNA silencing (VSRs). VSRs have been identified in almost all plant virus genera and are highly diverse. VSRs may affect different steps of RNA silencing by sequestering sRNAs or by inhibiting RISC assembly, sRNA methylation, or silencing-signal amplification (Pumplin and Voinnet, 2013). In addition, multiple VSR proteins have been shown to directly target AGO proteins, by either destabilizing AGO proteins directly or by inhibiting RISC formation (Csorba et al., 2015). The triple gene block protein 1 (TGB1) of *Potato virus X* (PVX), also known as P25, suppresses movement of a systemic silencing signal (Voinnet et al., 1999, 2000).

Arabidopsis thaliana encodes 10 AGO proteins that are thought to be specialized to function in different RNA silencing-related mechanisms (Mallory and Vaucheret, 2010), some of which have been implicated in antiviral defense. Hypomorphic *ago1* mutants have increased susceptibility to *Brome mosaic virus* and *Cucumber mosaic virus* (CMV) and to VSR-defective CMV and *Turnip crinkle virus* (TCV) (Morel et al., 2002; Qu et al., 2008; Azevedo et al., 2010; Dzianott et al., 2012). However, an *ago1* mutant is less susceptible than the wild type to *Tobacco rattle virus* (TRV), while AGO1 is required for virus-induced gene silencing (Ma et al., 2015). In addition, an *ago7* mutant has increased susceptibility to specific derivatives of VSR-defective TCV (Qu et al., 2008), and *ago4* mutants are more susceptible to TRV (Ma et al., 2015). By contrast, AGO2 appears to be broadly required for antiviral defenses, having been shown to be involved in defense against a wide range of viruses, including CMV, TCV, TRV, PVX, *Turnip mosaic virus* (TuMV), and *Tomato bushy stunt virus* (Harvey et al., 2011; Jaubert et al., 2011; Scholthof et al., 2011; Wang et al., 2011; Carbonell et al., 2012; Zhang et al., 2012; Ma et al., 2015). RISC complexes containing AGO1, 2, 3, or 5 act on viruses in in vitro Tombuvirus replication assays, and AGO1, 2, 3, 4, 5, and 9 can all bind to sRNAs derived from viruses or viroids (Takeda et al., 2008; Wang et al., 2011; Schuck et al., 2013; Minoia et al., 2014). These observations suggest that multiple AGO proteins may have the intrinsic ability to bind vsiRNA and target viral RNAs but raise the questions of which AGO proteins have antiviral functions in a biological context and whether different AGO proteins may have cooperative or redundant functions.

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PVX is the type member of the Potexvirus family of viruses (Verchot-Lubicz et al., 2007). Although the PVX VSR, P25, destabilizes Arabidopsis AGO1, this virus does not normally systemically infect Arabidopsis Columbia-0 (Col-0) (Chiu et al., 2010; Jaubert et al., 2011). However, PVX can effectively infect Arabidopsis if a VSR is supplied in trans from a second virus or if RNA silencing is attenuated by mutation of DCL-encoding genes or *AGO2* (Jaubert et al., 2011; Andika et al., 2015). At the same time, PVX accumulates to a lesser extent in the *ago2* mutant than it does in a triple *dicer* (*dcl2 dcl3 dcl4*) mutant (Jaubert et al., 2011), suggesting that at least one other AGO protein may function in resistance to PVX in Arabidopsis in concert with AGO2. The PVX/Arabidopsis system is one of the only examples where infection by a wild-type virus has been shown to be dramatically restricted due to an inability of its VSR to overcome the RNA silencing mechanisms of a specific host. The use of wild-type viruses to study RNA silencing may be important, as VSRs are often multifunctional proteins. For example, the PVX P25 protein is essential for cell-to-cell movement, as well as for the formation of replication-associated X-bodies, the latter of which may physically protect viral genomes from host RNA silencing machinery (Tilsner et al., 2012). However, the role of virus-induced subcellular structures in defense against RNA silencing has not been investigated.

Here, we have systematically investigated the involvement of different AGO proteins in defense against PVX in Arabidopsis using functional and genetic analysis. In functional assays in *Nicotiana benthamiana*, we found that Arabidopsis AGO1, 2, 3, 4, 5, 6, 7, 9, and 10 all have the ability to target PVX lacking its VSR, P25. By contrast, only Arabidopsis AGO2 and AGO5 had the ability to target wild-type PVX in *N. benthamiana*. In agreement with our functional analysis, expression of *AGO5* was induced in virus-infected plants and a double *ago2 ago5* mutant showed greater susceptibility to PVX than either single mutant. However, this cooperation appears to have a temporal/spatial aspect in that AGO5 was important in curtailing systemic PVX infection only once AGO2 was overcome in initially infected leaves.

RESULTS

Arabidopsis AGO2 and AGO5 Act Synergistically to Compromise PVX Accumulation in *N. benthamiana*

As no other single *ago* mutant allows for PVX accumulation in Arabidopsis, we undertook a functional approach by transiently overexpressing Arabidopsis AGO proteins together with PVX-GFP (green fluorescent protein) in *N. benthamiana* leaves. This included two versions of AGO1 (1S and 1L, which differ by a two amino acid deletion in AGO1S), AGO2 to 7, AGO9, and AGO10, but not AGO8, as it is thought to be a pseudogene (Takeda et al., 2008). Consistent with genetic analyses, transient expression of AGO2 resulted in a lower accumulation of PVX-derived GFP, as determined visually and by immunoblotting (Figures 1A and 1B). Although all AGO proteins tested were expressed, only AGO5 caused a similar effect (Figures 1A and 1B). Coexpression of both AGO2 and AGO5 induced an even greater decrease in virus-derived GFP (Figures 1C and 1D; Supplemental Figures 1C and 1D). In a parallel set of experiments, we coexpressed AGO2 and/or AGO5 with PVX-expressing luciferase (PVX-LUC) along with 35S:R-LUC for normalization. Quantification of luciferase assays confirmed that only AGO2 and AGO5 reduced luciferase activity significantly (40 and 35%, respectively), with AGO1 inducing only a minor (6%) decrease (Supplemental Figures 1A and 1B). Furthermore, AGO2 and AGO5 had an additive effect in reducing accumulation of virally encoded protein (Supplemental Figures 1C and 1D). Although coimmunoprecipitation experiments did not detect physical interaction between AGO2 and AGO5 (Figure 1D), these results suggest that both AGO2 and AGO5 act nonredundantly to restrict PVX accumulation in Arabidopsis.

All Arabidopsis AGO Proteins Can Target Viral RNA

Several studies have shown that the presence of VSR can modulate plant-virus interactions and the effectiveness of RNA silencing. For this reason, many studies on RNA silencing have used viruses lacking their VSR in order to identify which AGO protein is implicated in defense against viruses. To test this, we transiently expressed the same ten Arabidopsis AGO proteins in *N. benthamiana* leaves with a version of PVX lacking the three triple gene block proteins (PVX-GFPΔTGB). Surprisingly, overexpression of all Arabidopsis AGO proteins tested compromised accumulation of PVX-GFPΔTGB-expressed GFP in *N. benthamiana*, as assessed visually and by immunoblotting, albeit to different degrees (Figures 2A and 2B). Similar results were seen with a PVX mutant lacking only P25 (Supplemental Figure 2). Importantly, none of the AGO proteins affected GFP accumulation expressed from a 35S:GFP construct (Supplemental Figure 3). These results suggest that all Arabidopsis AGO proteins can specifically recognize and target viral RNAs.

A previous study has shown that PVX P25 may counteract RNA silencing by destabilizing AGO1 (Chiu et al., 2010). To better understand the importance of P25 in the modulation of RNA silencing efficiency, we monitored AGO protein accumulation in the presence of P25. Immunoblot analysis showed that only AGO1 and AGO7 showed lower levels of accumulation in the presence of P25 (Figure 2C). Furthermore, complementation assays performed by coexpressing PVX-GFPΔTGB and P25 in trans restored the ability of the virus to accumulate (as inferred by GFP accumulation) in the presence of AGO1 and AGO7, but not AGO2, 5, or 9 (Supplemental Figure 4). Together, these results suggest that although P25 may counteract AGO1 and AGO7 action, its lack of effect on AGO2 and AGO5 explains the inability of PVX to infect Arabidopsis.

Catalytic Residues Are Required for Effective PVX Antiviral Activity of AGO2 and AGO5 but Are Dispensable for Viral RNA Binding

AGO-mediated repression of target RNAs may occur through direct cleavage, destabilization, or translational repression. However, some AGO proteins could have an indirect effect through association with endogenous microRNAs (Zhang et al., 2011; Seo et al., 2013). We therefore tested if the AGO2 and AGO5 proteins

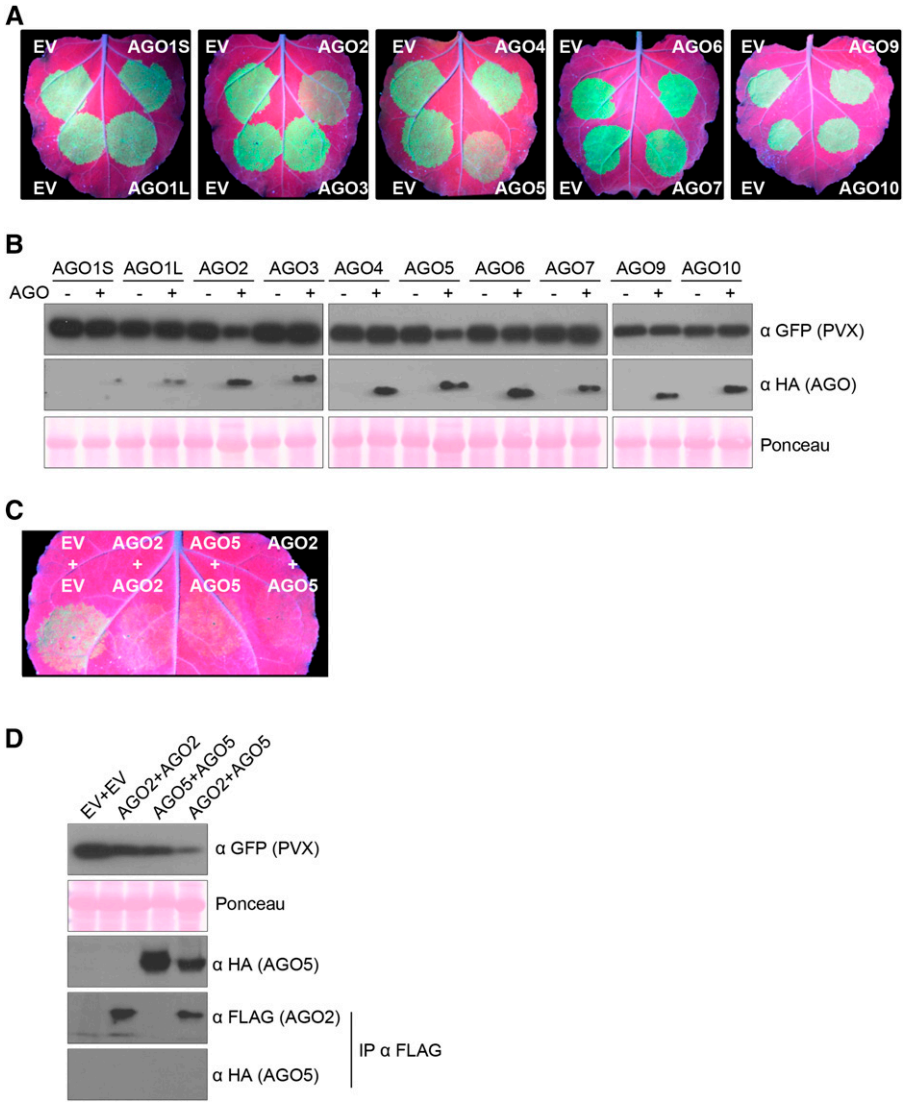

Figure 1. AGO2 and AGO5 Act Synergistically to Counteract PVX Accumulation in *N. benthamiana*.

(A) *N. benthamiana* leaves were agroinfiltrated with PVX-GFP along with 10 different HA-tagged Arabidopsis AGO proteins or empty vector (EV), as indicated. Leaves were photographed under UV illumination 4 dpi.

(B) Total protein extracts were prepared from *N. benthamiana* leaves agroinfiltrated 4 dpi as in **(A)** and subjected to SDS-PAGE, followed by anti-GFP immunoblotting (top panel). The "+" indicates the presence of the indicated AGO protein and "−" indicates EV. HA-tagged AGO proteins were immunoprecipitated from the same extracts and subjected to anti-HA immunoblotting (middle panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading.

(C) PVX-GFP was agroinfiltrated along with EV, FLAG-AGO2, or HA-AGO5 alone or in combination, as indicated. Leaves were photographed under UV illumination 4 dpi.

(D) Total protein extracts prepared from leaves agroinfiltrated as in **(B)** and subjected to anti-GFP immunoblotting (top panel). Aliquots from the same extracts were also subjected to anti-HA and anti-FLAG immunoprecipitation (IP) followed by anti-FLAG and/or anti-HA and immunoblotting. Ponceau staining of the same extracts is shown to demonstrate equal loading. Representative pictures are shown of experiments performed eight times with at least three plants per treatment.

could bind PVX RNA. Because a previous study demonstrated that catalytically inactive AGO proteins associate more stably with target RNAs, the second key residues of the catalytic triads of AGO2 and AGO5 were mutated to alanine (Carbonell et al., 2012). HA epitope-tagged wild type and catalytically dead mutants were coexpressed with PVX-LUC or PVX-GFP in *N. benthamiana* leaves. Three days after agroinfiltration, protein extracts were subjected to anti-HA immunoprecipitation. AGO/RNA interaction was assessed by extracting RNA from the immunoprecipitates, followed by RT-PCR using PVX-specific primers. Interestingly, both wild-type and mutant variants of AGO2 and AGO5 pulled down PVX RNAs with similar efficiency. By contrast, AGO9, which

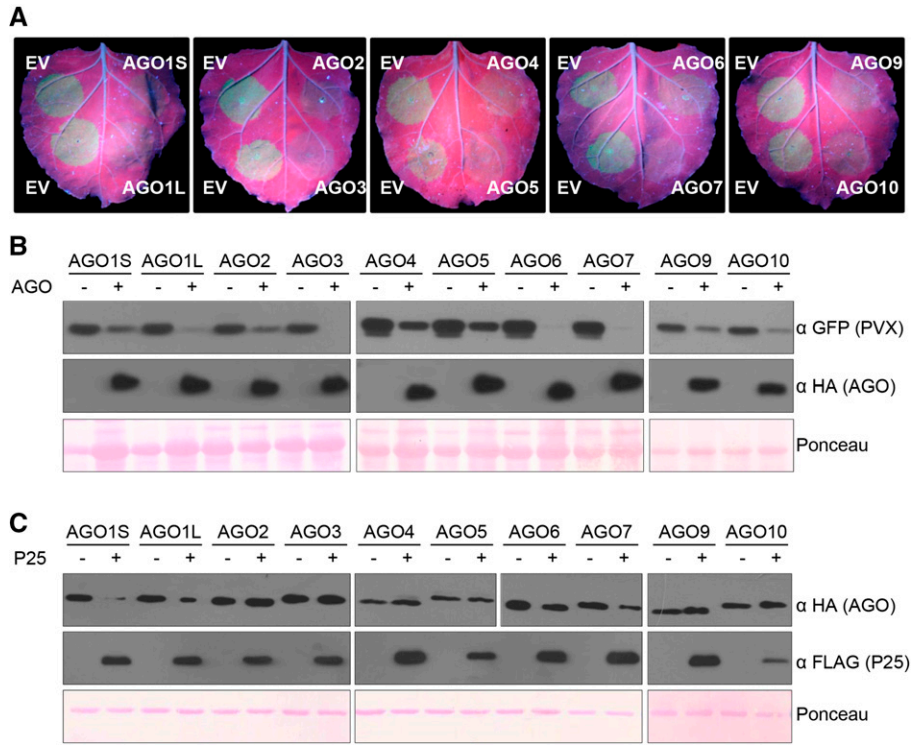

Figure 2. All Arabidopsis Argonautes Can Target Viral RNA.

(A) *N. benthamiana* leaves were agroinfiltrated with PVX-GFPΔTGB along with either empty vector (EV) or HA-tagged Arabidopsis AGO proteins, as indicated. Leaves were photographed under UV illumination 4 dpi. Representative pictures are shown of experiments performed six times with at least three plants per treatment.

(B) Total protein extracts were prepared from *N. benthamiana* leaves agroinfiltrated 4 dpi as in **(A)** and subjected to SDS-PAGE, followed by anti-GFP immunoblotting (top panel). The "+" indicates the presence of the indicated AGO protein and "−" indicates EV. HA-tagged AGO proteins were immunoprecipitated from the same extracts and subjected to anti-HA immunoblotting (middle panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading.

(C) HA-tagged AGO proteins were coexpressed by agroinfiltration in *N. benthamiana* leaves with either FLAG-tagged P25 (+) or with empty vector (−). Total proteins were extracted and subjected to anti-FLAG immunoblotting. HA-tagged AGO proteins were immunoprecipitated and subjected to anti-HA immunoblotting. Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading. Experiments have been performed three times with similar results.

affects PVX-GFPΔTGB but not wild-type PVX (Figures 1A and 2A), was found to associate only with the mutant version of PVX (Figure 3A). To confirm association between AGO proteins and PVX RNA, we inserted a streptavidin aptamer sequence (Srisawat and Engelke, 2001) into the PVX genome (PVX-S1). This allowed us to pull down PVX RNA and verify the presence of specific proteins bound to viral RNAs by immunoblot analysis (Supplemental Methods). No AGO proteins were detected in samples where we expressed PVX-GFP lacking the aptamer sequence (Supplemental Figure 5, second panel). However, upon pull-down of PVX-S1 after coexpression with AGO2, AGO5, or AGO9, we detected AGO2 and AGO5, but not AGO9 in the pulled-down fraction (Supplemental Figure 5).

Because slicer-competent AGOs were found to associate with PVX RNAs, we hypothesized that these AGOs might repress PVX-derived protein accumulation by translational repression wherein catalytic activity is dispensable. To investigate this possibility, we monitored luciferase activity from PVX-LUC in the presence of both variants of the AGO proteins. Mutation in the active site compromised the antiviral activity of both AGO2 and AGO5. Moreover, we observed a significantly higher accumulation of luciferase when PVX-LUC was when coexpressed with a slicer-defective variant of AGO2 (Figures 3B and 3C). Similar results were seen with PVX-GFP (Supplemental Figure 6). These results suggest that the mutant AGO2 protein might act as a dominant negative by inhibiting endogenous AGO2, similar to previously described results with TuMV (Carbonell et al., 2012). Together, these results suggest that AGO2 and AGO5 directly target PVX RNA by cleavage. Given the fact that both the wild type and catalytically dead mutants bind PVX RNAs, it also suggests that AGO2 and AGO5 catalytic residues are not essential for passenger strand clearance from siRNA duplexes in this context.

Antiviral Activity of AGO2 and AGO5 Requires Small RNAs

A number of reports have characterized the production of vsiRNAs upon infection by RNA viruses (Donaire et al., 2008;

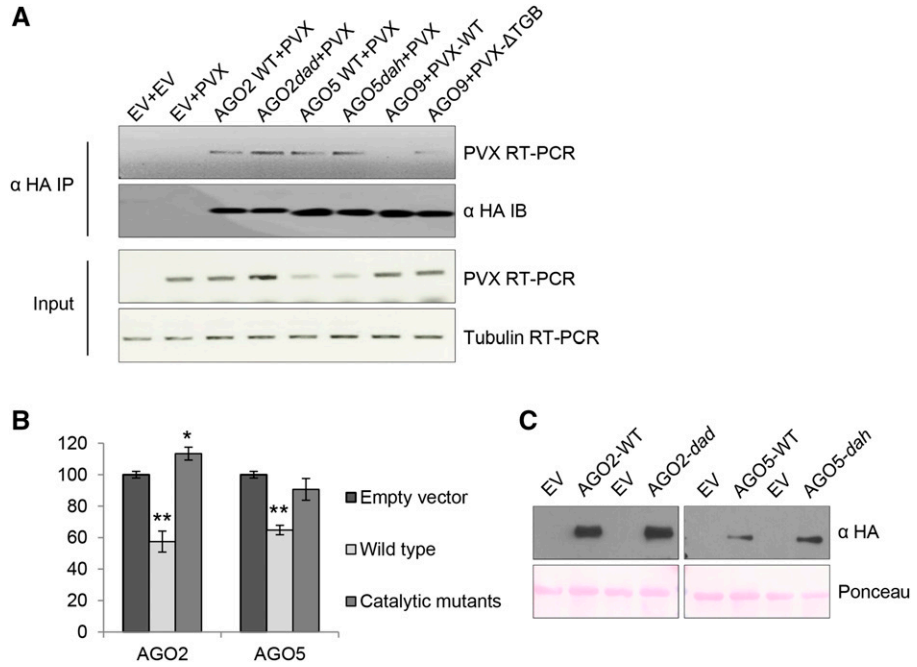

Figure 3. Catalytic Activity of AGO2 and AGO5 Is Required for Efficient Defense against PVX but Is Dispensable for Viral RNA Binding.

(A) *N. benthamiana* leaves were agroinfiltrated with empty vector (EV) or with PVX-GFP together with either HA-tagged AGO2 or AGO5 or their catalytically dead mutant derivatives *dad* and *dah*, respectively, as indicated. HA-AGO proteins were immunoprecipitated (IP) from total protein extracts prepared 3 dpi. RNAs from input and from HA-immunoprecipitated fractions were extracted and subjected to RT-PCR analysis with PVX-specific primers. Tubulin RT-PCR was used as a control. Similar treatments were performed by coexpressing HA-AGO9 with PVX-GFP or PVX-GFPΔTGB as indicated. This experiment was performed three times with similar results.

(B) *N. benthamiana* leaves were agroinfiltrated with PVX-F-LUC and 35S:R-LUC together with either EV or the wild type or catalytically inactive AGO2 or AGO5. At 4 dpi, firefly and renilla luciferase activities were measured from total protein extracts prepared from infiltrated tissues. Bar plots represents F-LUC activity normalized to R-LUC activity. Values represent the means ± SE from three independent experiments ($n = 9$). Data sets marked with one or two asterisks are significantly different from EV-infiltrated leaves as assessed by Student's t test at P values < 0.05 and 0.0001, respectively.

(C) HA-tagged AGO proteins were immunoprecipitated from total extracts from **(B)** and subjected to anti-HA immunoblotting (top panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading.

Takeda et al., 2008; Wang et al., 2011). These studies have shown that the 21-nucleotide vsiRNAs are the most abundant in infected plants, followed by 22-nucleotide vsiRNAs. However, as previously noted in the case of TuMV, vsiRNA abundance is a poor indicator of antiviral silencing activity (Garcia-Ruiz et al., 2010). To investigate which class of vsiRNAs is required for the antiviral activities of AGO2 and AGO5 against PVX, we performed overexpression assays in the presence of several VSRs. These included VSRs that sequester different sized siRNAs, including P14 (which sequesters siRNAs of all sizes) as well as P15, P21, and P19 (which sequester only siRNAs of 21 nucleotides) (Mérai et al., 2006), all of which were functional in a separate silencing suppression assay (Supplemental Figure 7). Coexpression of P14 with PVX-LUC completely compromised the antiviral activities of both AGO2 and AGO5. By contrast, the 21-nucleotide-sequestering proteins P19, P15, and P21 partially attenuated the activity of AGO2 but completely inhibited that of AGO5 (Figure 4A). PVX-derived luciferase accumulation was reduced by ~15% in the presence of VSRs sequestering 21-nucleotide sRNAs compared with a reduction around 35% without any VSR (Figure 4A). None of the VSRs affected the accumulation of AGO2 or AGO5, and coexpression of P14 resulted in inhibition of AGO2 and AGO5 association with PVX, as assessed by RNA immunoprecipitation (Figures 4B and 4C). These results suggest that optimal AGO2 antiviral activity is mediated through both 21-nucleotide and longer small RNAs. By contrast, AGO5 activity is completely abolished by all VSRs tested, suggesting that AGO5 activity against PVX depends mainly on 21-nucleotide sRNAs.

To further characterize the requirement of small RNAs for optimal antiviral defense response, we challenged single, double, and triple *dcl* mutant plants with PVX and followed the accumulation of PVX in both local and systemic tissues at 5 and 21 d postinoculation (dpi), respectively. Mutation of both *dcl2* and *dcl4* allowed for PVX accumulation in inoculated leaves, although *dcl4* had a much greater effect than *dcl2* (Figure 5A). In systemic tissues, no significant difference in PVX accumulation was observed between these two single mutants, whereas the *dcl2 dcl4* double mutant allowed higher accumulation of PVX than either single mutant (Figure 5B).

An *ago2 ago5* Double Mutant Displays Increased Susceptibility to PVX Compared with Single Mutants

To validate functional results showing that AGO2 and AGO5 may act synergistically to restrict PVX (Figures 1A to 1D;

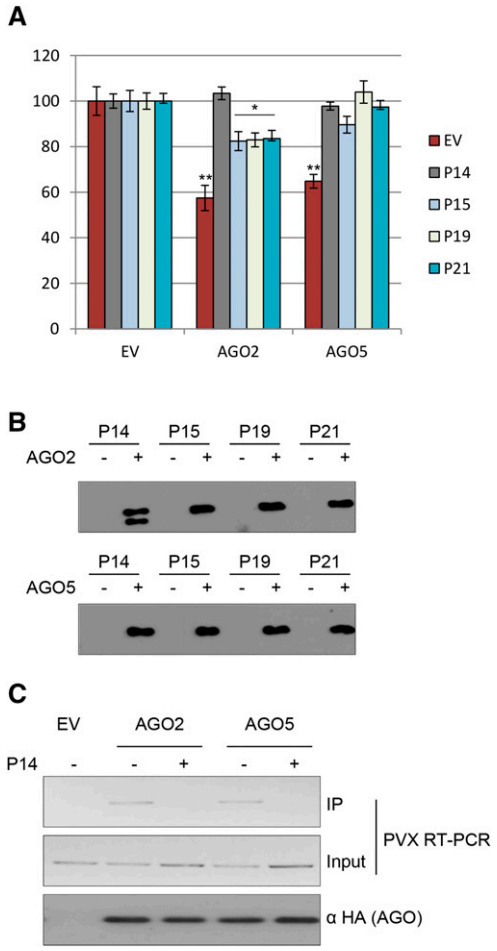

Figure 4. Antiviral Activities of AGO2 and AGO5 Depend Mainly on 21-Nucleotide Small Interfering RNAs.

(A) *N. benthamiana* leaves were agroinfiltrated with PVX-LUC and 35S:R-LUC, together with combinations of the VSRs P14, P15, P19, and P21 as well as EV, AGO2, or AGO5, as indicated. Luciferase activities were measured from protein extracts prepared from infiltrated tissues 4 dpi. Bar plots represent F-LUC activity normalized to R-LUC activity. Values represent means ± SE from three independent experiments ($n = 9$). Data sets marked with one or two asterisks are significantly different from EV-infiltrated leaves as assessed by Student's t test at P value < 0.05 or 0.005, respectively.
(B) Total protein extracts were prepared from *N. benthamiana* leaves agroinfiltrated 4 dpi as in **(A)**. HA-tagged AGO2 and AGO5 proteins were immunoprecipitated and subjected to SDS-PAGE followed by anti-HA immunoblotting.
(C) *N. benthamiana* leaves were agroinfiltrated with PVX-GFP, together with P14 and EV, AGO2, or AGO5. At 4 dpi, total proteins were extracted and subjected to immunoprecipitation (IP) with anti-HA antibodies. Subsequently, RNA from input and IP fractions were extracted and subjected to RT-PCR with PVX-specific primers. This experiment was performed three times with similar results.

Supplemental Figures 1A to 1D), we created two independent *ago2 ago5* double mutant lines by crossing the *ago2-1* and *ago5-1* lines and challenged them with PVX. In agreement with our previous report (Jaubert et al., 2011), the *ago5* single mutant behaved the same as the wild type (Figure 5C), whereas the double mutant plants showed somewhat higher levels of accumulation of PVX in inoculated leaves than the single *ago2* (Figure 5C). Interestingly, this effect appears to have a more pronounced effect in systemic leaves (Figure 5D), suggesting that AGO5 is more important in systemic tissues. Similar results were observed with an independent double mutant made by crossing the *ago2-1* and an additional *ago5* knock out (*ago5-5*; SALK_037270C) line (Supplemental Figures 8A and 8B).

Because luciferase assays indicated a possible antiviral activity of AGO1 and AGO7, we also challenged double and triple mutants: *ago1 ago2*, *ago2 ago7*, and *ago1 ago2 ago7*. None of these mutants was more susceptible to PVX than the single *ago2* mutant at either the local or systemic level and *ago1 ago2* mutants even appeared somewhat less susceptible to PVX (Figures 5C and 5D; Supplemental Figures 8C and 8D). These results confirm that AGO1 and AGO7 do not play important roles in defense against PVX in Arabidopsis and are consistent with *ago1* mutants being more resistant to TRV (Ma et al., 2015).

AGO5 Expression Is Induced upon PVX Infection

Transcriptional analysis monitoring *AGO* gene expression in Arabidopsis indicated that *AGO5* is poorly, if at all, expressed in Arabidopsis leaves in unchallenged conditions as well as under biotic and abiotic stresses (Schmid et al., 2005; Borges et al., 2011; Thieme et al., 2012; AtGenExpress Visualization Tool). We therefore monitored *AGO5* expression in uninfected and PVX-inoculated plants (wild type, triple *dicer*, *ago2*, and *ago2 ago5*) in both local and systemic tissues. Interestingly, we observed, by RT-PCR, a low level of expression of *AGO5* in the *ago2* mutant in the absence of PVX. In the other genotypes, we observed expression of *AGO5* mRNA only in PVX-infected plants and only in the systemic tissues of those genotypes that allow PVX accumulation, with the exception of the *ago2 ago5* mutant (Figure 6A). A similar induction was seen by immunoblotting with AGO5-specific antibodies, with AGO5 protein detected only in the systemic tissues of PVX-infected plants. Furthermore, AGO5 protein accumulation appeared consistent with level of PVX accumulation in these genotypes; the triple *dcl* mutant showed higher accumulation of AGO5 protein compared with the *ago2* mutant, and no AGO5 protein was detected in wild-type plants. These observations were also confirmed with a transgenic Arabidopsis line (P_{AGO5}:GFP-AGO5) expressing GFP-AGO5 from the *AGO5* promoter (McCue et al., 2012). Although some AGO5-GFP expression was detected in unchallenged conditions in these plants, expression was significantly enhanced in systemic leaves by PVX infection. Likewise, upon PVX infection, GFP fluorescence could be detected by microscopy in systemically infected leaves, particularly in the cytoplasm of guard cells (Supplemental Figure 9).

To determine if other Potexviruses might induce AGO5 accumulation and to rule out an effect of the mutant backgrounds used above, we challenged Arabidopsis Col-0 plants with *Plantago asiatica mosaic virus* expressing GFP (PlAMV-GFP) (Yamaji et al., 2012). Similar to the results observed with PVX, *AGO5* induction was observed by both RT-PCR and immunoblot analysis in systemic tissues upon PlAMV-GFP infection (Figures 6C and 6D). Taken together, these results indicate that

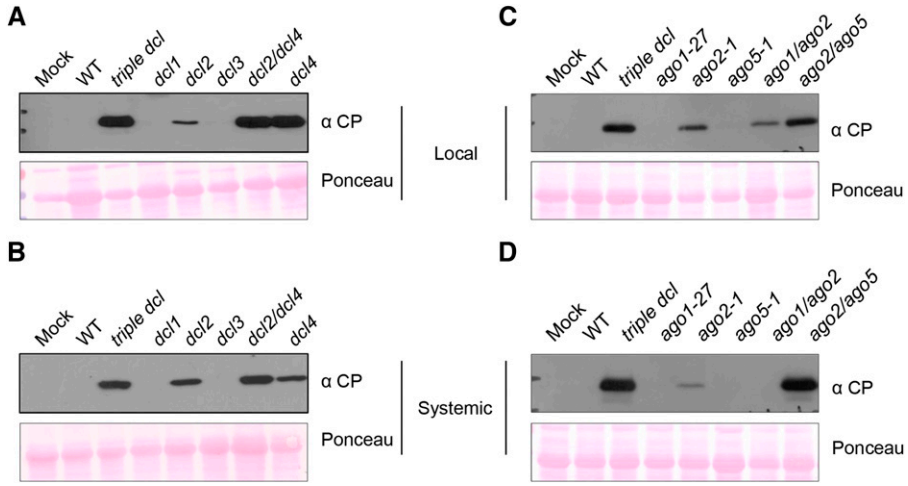

Figure 5. Arabidopsis Mutants Reveal Additive Effects of DCL2 and DCL4 and of AGO2 and AGO5 in Systemic PVX Infection.

Wild-type Col-0 Arabidopsis as well as single, double, or triple mutant *dcl* and *ago* mutant lines, as indicated, were infected with PVX. At 5 dpi (**[A]** and **[C]**) and 21 dpi (**[B]** and **[D]**), total protein extracts were prepared from inoculated and systemic leaves, respectively, and subjected to SDS-PAGE followed by anti-PVX CP immunoblotting. Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading. Three plants per genotype were tested in each experiment and the experiment was repeated three and eight times for **(A)** and **(B)** and **(C)** and **(D)**, respectively.

AGO5 expression is induced in response to Potexvirus infection and is consistent with the higher susceptibility to PVX observed in the *ago2 ago5* double mutant.

DISCUSSION

The investigation of RNA silencing in antiviral resistance presents a challenge in that most viruses are already able to overcome this defense response in their host plant. A number of studies have shown that RNA silencing components in antiviral defense can be studied using viruses lacking their cognate VSR (Deleris et al., 2006; Diaz-Pendon et al., 2007; Qu et al., 2008; Garcia-Ruiz et al., 2010; Scholthof et al., 2011; Wang et al., 2011). However, VSRs are often multifunctional proteins involved not only in suppressing RNA silencing (Incarbone and Dunoyer, 2013). As such, their deletion may result in viruses that do not reflect a normal infection

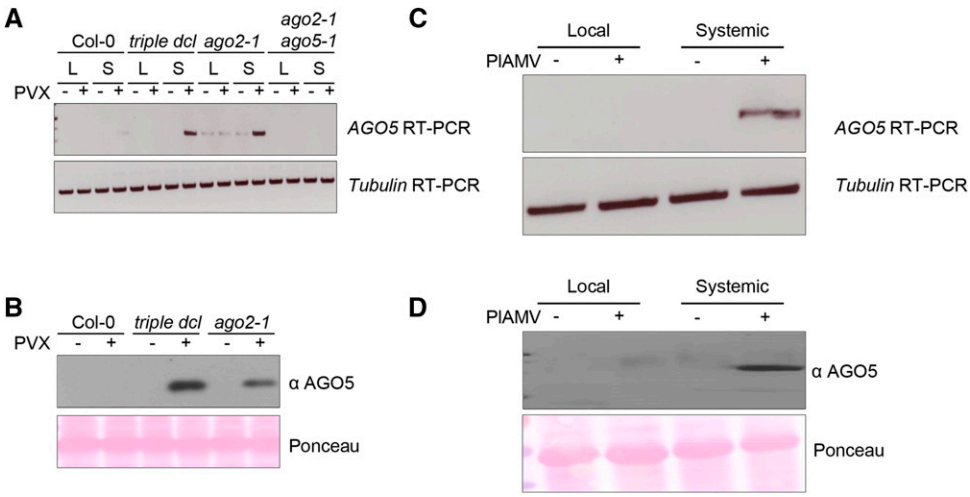

Figure 6. AGO5 Expression Is Induced in Arabidopsis Systemic Leaves upon Potexvirus Infection.

Arabidopsis plants of the indicated genotypes were either mock inoculated or inoculated with PVX (**[A]** and **[B]**) or PlAMV (**[C]** and **[D]**). At 7 dpi, total RNA was extracted from inoculated and systemic leaves and subjected to RT-PCR with *AGO5*- or *tubulin*-specific primers, as indicated (**[A]** and **[C]**). Total protein extracts were prepared from inoculated or systemic leaves and subjected to SDS-PAGE followed by anti-AGO5 immunoblotting and Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading (**[B]** and **[D]**). Data shown are representative of three independent experiments in which at least three separate plants of each genotype were tested.

and may alter which defenses are effective against it. Indeed, our study showed that there are striking differences between the AGO proteins that can target PVX deletion mutants compared with the wild-type virus. Thus, in using a combination of wild-type and mutant PVX along with a combination of compatible and incompatible host plants (*N. benthamiana* and Arabidopsis, respectively), we have shown the importance of viral structures in antiviral defenses. Furthermore, we demonstrated that AGO5 plays an essential role in curtailing PVX infection in systemic tissues in Arabidopsis.

Which AGOs Are Antiviral?

Previously, genetic analysis of *AGO* genes in Arabidopsis identified AGO1, AGO2, AGO4, and AGO7 as being required for defense against different RNA viruses, leading to the hypothesis that only a subset of AGO proteins are specialized in recognition and restriction of RNA viruses. Of the AGO proteins tested (AGOs 1, 2, 5, and 7), AGO1, AGO2, and AGO5 have been shown to bind to vsiRNAs by immunoprecipitation analysis in Arabidopsis (Takeda et al., 2008; Azevedo et al., 2010). At the same time, studies in *N. benthamiana* have shown that AGOs 1, 2, 3, 4, 5, and 9 are associated with sRNAs derived from the viroid PSTVd, which to the RNA silencing machinery may appear much like a virus lacking a VSR (Minoia et al., 2014). In vitro experiments also demonstrated the ability of several AGO proteins to target viral transcript in presence of artificial siRNA (Schuck et al., 2013). These observations strongly suggest that the majority, if not all, AGO proteins possess the intrinsic capacity to target viral RNAs. Our results observed upon overexpression of Arabidopsis AGO proteins with PVX-GFPΔTGB and PVX-GFPΔP25 (Figure 2; Supplemental Figure 2) are in agreement with this hypothesis. The latter results are not likely to be due to overexpression as wild-type PVX is affected only by those AGOs that also show a phenotype in Arabidopsis (Figures 1 and 5; Supplemental Figures 1 and 8). Furthermore, this activity was specific to viral RNA, as none of the AGO proteins tested affected the accumulation of GFP derived from a 35S:mGFP5 construct (Supplemental Figure 3).

The simplest explanation for the difference between wild-type and mutant PVX in their sensitivities to different AGOs would be that P25 inhibits all AGO proteins except AGO2 and AGO5. However, in our complementation assays of PVX-GFPΔTGB with P25 expressed in trans, we observed that P25 affected the antiviral activity of only AGO proteins that are targeted by P25 (Supplemental Figure 4), namely, AGO1 and AGO7 (Figure 2). By contrast, all other AGO proteins tested, which are not destabilized in the presence of P25, are still efficient in restricting PVX-GFPΔTGB in the presence of P25 (Figure 2; Supplemental Figure 4). We observed similar results with PVX-GFPΔP25 (data not shown). This lack of complementation in trans may be because a certain stoichiometry between TGB1 (P25) and TGB2/TGB3 is required for the formation of certain structures (Verchot et al., 1998). TGB proteins are required for cell-to-cell movement and for the formation of virus replication factories known as X-bodies, wherein P25 is required to remodel host actin and endomembranes and to recruit TGB2 and TGB3 to the perinuclear X-body (Tilsner et al., 2012). TGB proteins are not required for viral replication, although PVX replication is enhanced by X-bodies, presumably as they serve as scaffolds and protective compartments for virus replication and assembly (Verchot et al., 1998; Morozov and Solovyev, 2003; Tilsner et al., 2012). The lack of X-body formation can be complemented by coexpression of a VSR (Tilsner et al., 2012). Together, these observations suggest that viruses may be protected by VSRs but also by virus-induced subcellular structures that shield them from the RNA silencing machinery. Thus, we propose that to target viruses efficiently, not only must AGO proteins not be inhibited by the virus VSR, but they must also be able to access viral RNA. Indeed, both AGO2 and AGO5 are able to bind wild-type PVX RNA, but AGO9, which can target only "naked" (i.e., unprotected by TGB proteins) viral RNA, does not (Figure 3A; Supplemental Figure 5). The reasons why only certain AGO proteins would be able to access viral RNA remain to be elucidated, but it is likely that it depends both on the types of subcellular structures formed by individual viruses and by the localization properties of different AGO proteins. We speculate that in some cases, for example, when a virus possesses a weak VSR, VRC/virus lifestyle may be as important as the VSR activity by itself. Indeed, the P25 protein by itself is a relatively weak VSR in non-virus-based VSR assays in *N. benthamiana*, but nonetheless appears to be quite effective in the context of a viral infection in this host (Senshu et al., 2009).

Previous reports have shown a requirement for AGO1 and/or AGO7 in defense against attenuated CMV and TCV (Morel et al., 2002; Qu et al., 2008). However, these two proteins appear to be dispensable for defense against TRV (Ma et al., 2015), and AGO2 is more important than AGO1 in defense against wild-type TCV (Zhang et al., 2012). Likewise, even though AGO1 and AGO7 are destabilized by the PVX VSR, our genetic and functional analyses suggest that they are not major determinants of PVX infection. This finding reinforces the importance of using wild-type virus in studying plant-virus interactions. At the same time, it may also indicate that different viruses may be affected by different AGO proteins, depending on factors such as replication strategies, VSR mode of action, and accessibility of viral RNA to different AGO proteins. For example, a recent report has shown that AGO5 plays only a minor role in defense against TuMV (Garcia-Ruiz et al., 2015). As such, different *ago* mutants may confer divergent phenotypes when infected with various viruses.

Redundant Functions for DCL2 and DCL4 in PVX-Arabidopsis Interaction

Multiple studies have shown that both DCL2 and DCL4 are involved in curtailing systemic infection by TuMV, TRV, TCV, and CMV (Bouché et al., 2006; Deleris et al., 2006; Diaz-Pendon et al., 2007; Dunoyer et al., 2007; Qu et al., 2008; Garcia-Ruiz et al., 2010). It has been proposed that DCL4 and DCL2 have hierarchical antiviral activities where DCL4 is the primary sensor of viral RNAs. This is based on the observation that in wild-type plants, VSR-defective TCV RNA appears to be mainly processed by DCL4 but that in its absence, DCL2 is sufficient for antiviral defense (Deleris et al., 2006). TuMV is likewise preferentially targeted by DCL4, although the *dcl2 dcl4* double mutant is more

susceptible to TuMV than a *dcl4* single mutant (Garcia-Ruiz et al., 2010). In this study, we found that both *dcl2* and *dcl4* single mutants allow PVX to produce a similar degree of systemic infection, although to a lesser extent than in *dcl2 dcl4* double mutant (Figures 5A and 5B). This result suggests that both DCL2 and DCL4 are required in local and systemic leaves to restrict PVX although DCL2 appear to be less important in local leaves. Our results are similar to a recent report showing that the *dcl2 dcl4* mutant is susceptible to PVX, although they did not find that *dcl2* and *dcl4* single mutants were systemically susceptible to PVX (Andika et al., 2015). This may be due to inoculation method or dosage as DCL2 activity may be overcome by increasing inoculum (Deleris et al., 2006) or different growth conditions, which can alter RNA silencing efficiency (Zhang et al., 2012; Ma et al., 2015). Nonetheless, our functional data in *N. benthamiana* are in agreement with our genetic data in Arabidopsis. That is, in the presence of VSRs that sequester 21-nucleotide siRNAs, AGO2 still retains a degree of antiviral activity suggesting that AGO2 can utilize both DCL4- and DCL2-derived siRNAs to target viruses (Figure 4). This is in agreement with a report showing that the survival of TCV-inoculated Arabidopsis requires AGO2 programmed by DCL2-produced 22-nucleotide vsiRNAs (Zhang et al., 2012). Thus, given that AGO2 binds 21- and 22-nucleotide but not 24-nucleotide sRNAs (Mi et al., 2008; Takeda et al., 2008; Minoia et al., 2014), it would appear that DCL2 and DCL4 have an additive effect in the PVX-Arabidopsis interaction.

AGO2 and AGO5 Act Cooperatively to Counteract PVX Infection

AGO2 antiviral activity has been demonstrated for several positive-sense ssRNA viruses (Garcia-Ruiz et al., 2010; Harvey et al., 2011; Jaubert et al., 2011; Scholthof et al., 2011; Zhang et al., 2012; Carbonell et al., 2012; Ma et al., 2015) but a major antiviral role for AGO5 has not previously been shown. Although AGO5 has been shown to bind CMV- and PSTVd-derived siRNAs, such an observation is not informative as most AGO proteins tested bind such sRNAs (Takeda et al., 2008; Minoia et al., 2014). In this report, we find that the *ago5* single mutant shows no increased susceptibility to PVX. However, in inoculated leaves, the *ago2 ago5* double mutant is more susceptible to PVX than the *ago2* single mutant (Figures 5C and 5D; Supplemental Figures 8A and 8B). This effect is even more pronounced in systemic tissues where the *ago2 ago5* double mutant showed a much greater susceptibility to PVX than the *ago2* single mutant, similar to that of the triple *dicer* mutant (Figures 5C and 5D; Supplemental Figures 8A and 8B). We suggest therefore that AGO2 is most important in inoculated leaves, while AGO5 is more important in systemic tissues. Thus, unless AGO2 is absent, PVX cannot move beyond the inoculated leaves, explaining the lack of phenotype in the *ago5* single mutant. Our finding underlines the utility of studying AGO proteins using functional assays, which can then be followed up with genetic analysis.

A lesser role for AGO5 in inoculated tissues is further supported by the expression profile of *AGO5*, which in uninfected plants has been shown to be highly specific to reproductive tissues (Schmid et al., 2005; Borges et al., 2011). However, we found that *AGO5* expression is induced in systemic, but not inoculated, tissues after PVX infection. This induction correlated with the degree of susceptibility, being undetectable in Col-0 plants, but detectable in triple *dicer* and *ago2* mutants, suggesting that a certain threshold of PVX accumulation is necessary to induce *AGO5* expression. This induction is not dependant on the latter mutations as PlAMV, which infects Arabidopsis (Yamaji et al., 2012), induced *AGO5* expression in wild-type Col-0 plants. Interestingly, it has recently been reported that AGO18, a member of a monocot-specific AGO protein clade, mediates virus resistance and is induced in virus-infected tissues (Wu et al., 2015). Whether systemic induction of *AGO5* expression is induced by the presence of viral RNA itself, secondary vsiRNAs, or some other endogenous signal, and whether it shares similarities with *AGO18* induction, remain to be elucidated. However, it is curious that *AGO5* mRNA was consistently, albeit faintly, detectable in the leaves of *ago2* mutant plants (Figure 6; Supplemental Figure 9), which could indicate that if AGO2 is overcome in inoculated leaves the plant induces AGO5 to counteract a systemic infection.

AGO2 has been shown to bind 21- and 22-nucleotide sRNAs, with a preference for 21 nucleotides, while AGO5 binds 21-, 22-, and 24-nucleotide sRNAs equally (Mi et al., 2008; Takeda et al., 2008; Wang et al., 2011; Minoia et al., 2014). Transient assays with VSRs demonstrated that AGO2 and AGO5 require vsiRNAs for their antiviral function. However, when expressed with P19, which affects only 21-nucleotide sRNAs, AGO2 still possess some antiviral activity, indicating that in the absence of 21-nucleotide sRNAs, it can make use of 22-nucleotide sRNAs, consistent with the additive effects of DCL2 and DCL4 in the PVX-Arabidopsis interaction (Figures 4, 5A, and 5B). However, 21-nucleotide binding VSRs inhibited the antiviral activity of AGO5 (Figure 4), suggesting that AGO5 utilizes 21-nucleotide vsiRNAs to target viruses.

AGO2 and AGO5 Associate with PVX RNAs

In this study, both wild-type and catalytically inactive versions of AGO2 and AGO5 were found to be associated with PVX RNAs using two different methods. Although we do not rule out a possible indirect interaction, given the known modes of action of AGO proteins, we suggest that AGO2 and AGO5 bind directly to viral RNAs. Given the requirement for siRNAs for this association (Figure 4C), this binding likely involves an active RISC and may involve additional proteins. The ability of a functional AGO to bind an RNA stably enough for detection may seem counterintuitive, as it would be predicted to cleave the substrate. However, several studies have shown that product release after cleavage by human RISC complexes is the limiting step (Haley and Zamore, 2004; Martinez and Tuschl, 2004; Ameres et al., 2007; Parker, 2010). Thus, despite being active, it is still possible to immunoprecipitate AGO-RNA complexes (Schwarz et al., 2004; Wang et al., 2009, 2010).

Although catalytic residues are dispensable for viral RNA binding, they are nonetheless essential for antiviral activity against PVX, suggesting that binding viral RNAs is not sufficient to compromise virus accumulation. In vitro catalytic assays have

shown that AGO2 and AGO5 possess a strong slicer activity against *Tomato bushy stunt virus* transcripts (Schuck et al., 2013). This is in contrast with AGO18, which does not appear to directly target viral RNA but rather functions by sequestering an endogenous microRNA targeting AGO1 (Wu et al., 2015). However, our results are in agreement with a previous study showing that the catalytic activity of AGO2 is required for defense against TuMV (Carbonell et al., 2012). Furthermore, both in this and in a previous study (Carbonell et al., 2012), the presence of catalytically inactive AGO2 increased viral titer, suggesting it may compete with wild-type AGO2 for binding to protein partners and/or RNA (Carbonell et al., 2012) (Figure 3).

A Model for AGO2 and AGO5 in Antiviral Defenses

We propose a model for defense against Potexviruses wherein highly structured or dsRNA is processed by DCL2, 3, and 4 in initially infected cells. DCL4-produced vsiRNAs appear to be most important in these leaves and are likely bound by AGO2, which in turn targets viral RNAs for slicing. If, however, the virus is able to overcome AGO2 in inoculated leaves, this appears to initiate the production of an as yet unknown signal that induces the expression of AGO5 in systemic tissues. The reason for the induction of AGO5 in systemic tissues may be because it is involved in utilizing the systemic signal, presumed to include vsiRNAs, to target viruses (Mourrain et al., 2000; Voinnet et al., 2000; Parent et al., 2015). However, DCL2 and DCL4 appear to play equally important roles in systemic infections, and it remains to be seen if this is due to their roles in the systemic tissues or in the production of the systemic signal. If this signal is able to move more quickly than the virus into systemic tissues, then we speculate that it may be incorporated into AGO5-containing RISC complexes in uninfected tissues. Thus, this second line of defense could target incoming viral RNAs before they are able to establish an infection in systemic tissues. Differences in the requirement for AGO5 in defense against specific viruses, such as TuMV (Garcia-Ruiz et al., 2015), could be due to differences in the production of, or susceptibility to, the systemic silencing signal. The importance of systemic signals in PVX infection is underlined by the fact that the PVX VSR, P25, functions by inhibiting the systemic movement of the silencing signal in *N. benthamiana* (Voinnet et al., 2000). The PVX P25 protein has not been demonstrated to inhibit this aspect of RNA silencing in Arabidopsis, whereas the P25 protein of PlAMV is thought to do so (Okano et al., 2014). Given that AGO2 seems to be involved in most plant-virus systems tested, this model is likely to extend to other viruses. However, the involvement of AGO5 may vary between viruses depending on the mode of action of their VSRs and their movement strategies.

METHODS

Plant Material and Growth Conditions

Nicotiana benthamiana and *Arabidopsis thaliana* plants were grown on soil (BM6, Berger and Agromix, Fafard, respectively) in growth chambers with 16-h-light/8-h-dark photoperiod at 23°C and 21°C, respectively. Except for the pAGO5:AGO5-GFP transgenic line, which is in Landsberg *erecta* background (McCue et al., 2012), all Arabidopsis mutant lines were of the Col-0 ecotype and have been previously described, including the *ago1-27* (Morel et al., 2002), *ago2-1* (Takeda et al., 2008), *ago5*-1 (Katiyar-Agarwal et al., 2007; Mi et al., 2008), *ago1 ago2*, *ago1 ago2 ago7*, and *ago2 ago7* (Wang et al., 2011), triple *dicer* (Deleris et al., 2006), *dcl1-9* (Jacobsen et al., 1999), *dcl2-1* and *dcl3-1* (Xie et al., 2004), and *dcl4-2* and *dcl2 dcl4* (Xie et al., 2005).

The *ago2 ago5* mutant lines were generated by standard genetic crosses between homozygous *ago2-1* mutants (Salk_003380) and either the *ago5-1* mutant (Salk_ 063806) or the *ago5-5* mutant (Salk_037270C). Homozygous double mutant genotypes were confirmed by allele-specific PCR at the second generation.

Plasmid Construction

Construction of all pBIC-HA-AtAGO constructs has been described previously (Takeda et al., 2008). For the generation of pBIN61-FLAG-AGO2, pBIC-HA-AGO constructs were used as templates for PCR amplification using KOD high-fidelity DNA polymerase (Novagen). Primer sequences are listed in Supplemental Table 1. PCR fragments were then A-tailed with Taq DNA polymerase (New England Biolabs) and cloned into pGEM-T easy vector (Promega) for sequencing. Inserts were then digested and cloned into the *Xba*I and *Bam*HI sites of pBIN61-FLAG. 35S:HA-AGO2 and 35S:HA-AGO5 slicer-defective variants were generated by PCR mutagenesis using pBIC-HA-AGO2 and pBIC-HA-AGO5 as templates with primers listed in Supplemental Table 1.

PVX, PVX-GFP, and PVX-GFPΔTGB binary constructs (Peart et al., 2002; Bhattacharjee et al., 2009), PlAMV-GFP (Yamaji et al., 2012), 35S:P14, 35S:P21 (Mérai et al., 2006), 35S:P15 (Dunoyer et al., 2002), and 35:P19 (Voinnet et al., 1999) have been previously described.

pBIN61-P25:HA was generated by RT-PCR from PVX-infected plants using primers (Supplemental Table 1) to introduce *Xba*I and *Bam*HI sites at the 5′ and 3′ ends of the P25 open reading frame, respectively. The resulting PCR fragment was the cloned into the same sites of pBIN61:FLAG (Moffett et al., 2002) and verified by sequencing.

Firefly luciferase was amplified from Luciferase T7 Control DNA (Promega) with specific primers (Supplemental Table 1) and cloned into the *Asc*I and *Sal*I sites of pGR106 (Jones et al., 1999). To generate the 35S:R-LUC-expressing construct, the pGreenII61 MCS (including the 35S expression cassette) was first subcloned into the *Asc*I and *Stu*I sites of pEAQ-SelectK (Sainsbury et al., 2009) to produce the pEAQ-SE expression vector (Ali et al., 2015). Subsequently, *Renilla luciferase* was excised from pRL-SV40 (Promega) with *Nhe*I and *Xba*I and cloned into the *Xba*I site of pEAQ-SE. Insert orientation was verified by sequencing.

Virus Inoculation

Infections of 3-week-old Arabidopsis plants were performed by rub inoculation as previously described (Jaubert et al., 2011). Briefly, saps were produced from PVX-infected or PlAMV-GFP-infected *N. benthamiana* plants by grinding infected tissue in in 0.1 M phosphate buffer, pH 7.0 (2 mL/g of infected tissues). Mock infections were performed with sap produced from uninfected *N. benthamiana* plants (2 mL/g healthy tissues).

Transient Expression Assays

Agrobacterium tumefaciens-mediated transient expression assays in *N. benthamiana* were performed as previously described (Moffett, 2011). Briefly, binary expression constructs were transformed into the C58C1 Agrobacterium strain carrying pCH32 virulence plasmid. For virus agroinoculation, GV3101 Agrobacterium strain carrying the pSoup helper plasmid was transformed with pGr106/pGr107/pGr208 derivatives constructs (PVX-GFP WT, PVX-GFPΔTBG, PVX-GFPΔP25, PVX-LUC, PVX-LUCΔTBG,

and PVX-S1). Agrobacterium cultures were centrifuged at 3724*g* for 10 min and resuspended in 10 mM $MgCl_2$ to a final OD_{600} = 0.2 and 0.1 for protein expression and virus vectors, respectively.

Protein Extraction and Analysis

For AGO expression analysis, 1 g fresh tissue was ground in 2 mL RISC buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, and 0.5% Nonidet P-40) supplemented with protease inhibitor cocktail. Total protein extract was centrifuged at 16,000*g* for 10 min at 4°C. A fraction of total protein extract was kept for detection of GFP. Immunoprecipitation was performed with 1.4 mL supernatant and 25 μL HA-agarose beads (Sigma-Aldrich) or FLAG-agarose beads (Sigma-Aldrich) for 2 h at 4°C on a rotatory shaker. Beads were washed four times with RISC buffer. After centrifugation, beads were resuspended in 50 μL 1.5× of Laemmli loading buffer. Proteins were separated by SDS-PAGE on 7.5 or 10.5% acrylamide gels for AGO or P25, GFP and CP detection, respectively, and transferred to polyvinylidene fluoride membranes (Bio-Rad) by electroblotting. HA-AGO proteins were probed with anti-HA-horseradish peroxidase (HRP) conjugated antibodies (Sigma-Aldrich; 1:3000 dilution). FLAG-tagged proteins were probed with anti-FLAG-HRP antibodies (Sigma-Aldrich; 1:5000 dilution). Detection of GFP was performed by probing membranes with anti-GFP-HRP antibodies (Santa Cruz; 1:3000 dilution). Anti-PVX-CP rabbit polyclonal antibodies (Agdia; 1:3000 dilution) were used to detect PVX in Arabidopsis followed by donkey anti-IgG rabbit-HRP polyclonal antibodies (BioLegend; 1:10,000 dilution). Detection of Arabidopsis AGO5 was performed by probing membranes with anti-AGO5 antibody (Agrisera; 1:3000 dilution) and subsequently with donkey anti-IgG rabbit-HRP polyclonal antibodies (BioLegend; 1:10,000 dilution). Equal loading of proteins was confirmed with polyclonal antibody phosphoenol pyruvate carboxylase (anti-PEPC, Rockland; 1:10,000 dilution).

RNA Immunoprecipitation

Analyses of AGO-RNA interactions were performed as described previously (Carbonell et al., 2012), with some modifications. Briefly, Agrobacterium-infiltrated *N. benthamiana* leaves were ground with mortar and pestle in cold extraction buffer (2 mL/g tissue; 50 mM Tris-HCl, pH 7.4, 2.5 mM $MgCl_2$, 100 mM KCl, and 0.1% Nonidet P-40) supplemented with protease inhibitor cocktail and 40 units/mL Ribolock (Thermo-Scientific) followed by centrifugation at 16,000*g* for 10 min at 4°C. Total RNA (aliquots of 250 μL) was used for input controls. A preclearing step was performed on 5 mL supernatant by adding 35 μL nonspecific IgG-agarose beads (Rockland Immunochemicals) for 30 min at 4°C on a rotatory shaker. After spinning down beads, supernatant was incubated with 75 μL anti-HA agarose beads (Sigma-Aldrich) for 2 h at 4°C. Beads were washed eight times with 5 mL extraction buffer. AGO-associated RNAs and proteins were isolated from beads with Trizol, per the manufacturer's instructions (Ambion).

Dual-Luciferase Reporter Assay

Four days after agroinoculation, three leaf discs were ground in 100 μL passive lysis buffer (Promega). After centrifugation, 20 μL protein extract of each sample was distributed in triplicate in 96-well plates and dual-luciferase reporter assay was performed in accordance with the manufacturer's protocol (Promega).

Supplemental Data

Supplemental Figure 1. AGO2 and AGO5 act synergistically to counteract PVX accumulation in *N. benthamiana.*

Supplemental Figure 2. All Arabidopsis Argonaute proteins can target viral RNA.

Supplemental Figure 3. Overexpression of Arabidopsis AGO does not compromise GFP accumulation from a 35S:GFP construct.

Supplemental Figure 4. Expression in trans of P25 compromises antiviral activity of AGO1 and AGO7.

Supplemental Figure 5. AGO2 and AGO5 bind PVX RNAs.

Supplemental Figure 6. Catalytic residues of AGO2 and AGO5 are required to target PVX-GFP.

Supplemental Figure 7. Validation of VSR expression: viral suppressors of RNA silencing compromise silencing in a transient assay.

Supplemental Figure 8. PVX infection in an additional *ago5* mutant line and various double mutant lines.

Supplemental Figure 9. Expression of AGO5 is induced by PVX infection in P_{AGO5}:GFP-AGO5 transgenic plants.

Supplemental Table 1. Oligonucleotides used in this study.

Supplemental Methods. PVX-aptamer purification and microscopy.

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AUTHOR CONTRIBUTIONS

C.B. and P.M. designed the research, analyzed the data, and wrote the article. C.B. performed the experiments. Both authors read, helped to edit, and approved the final version of the article.

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