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The 21-Nucleotide, but Not 22-Nucleotide, Viral Secondary Small Interfering RNAs Direct Potent Antiviral Defense by Two Cooperative Argonautes in *Arabidopsis thaliana* [W] [OA]

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Arabidopsis thaliana **defense against distinct positive-strand RNA viruses requires production of virus-derived secondary small interfering RNAs (siRNAs) by multiple RNA-dependent RNA polymerases. However, little is known about the biogenesis pathway and effector mechanism of viral secondary siRNAs. Here, we describe a mutant of *Cucumber mosaic virus* (CMV-Δ2b) that is silenced predominantly by the RNA-DEPENDENT RNA POLYMERASE6 (RDR6)-dependent viral secondary siRNA pathway. We show that production of the viral secondary siRNAs targeting CMV-Δ2b requires *SUPPRESSOR OF GENE SILENCING3* and *DICER-LIKE4* (*DCL4*) in addition to *RDR6*. Examination of 25 single, double, and triple mutants impaired in nine *ARGONAUTE* (*AGO*) genes combined with coimmunoprecipitation and deep sequencing identifies an essential function for AGO1 and AGO2 in defense against CMV-Δ2b, which act downstream the biogenesis of viral secondary siRNAs in a nonredundant and cooperative manner. Our findings also illustrate that dicing of the viral RNA precursors of primary and secondary siRNA is insufficient to confer virus resistance. Notably, although DCL2 is able to produce abundant viral secondary siRNAs in the absence of DCL4, the resultant 22-nucleotide viral siRNAs alone do not guide efficient silencing of CMV-Δ2b. Possible mechanisms for the observed qualitative difference in RNA silencing between 21- and 22-nucleotide secondary siRNAs are discussed.**

INTRODUCTION

RNA silencing provides protection against diverse RNA viruses in many eukaryotic organisms (Ding, 2010; Llave, 2010; Qu, 2010). In this antiviral defense, small interfering RNAs (siRNAs) are processed from a viral RNA precursor and used to guide specific silencing of the cognate viral RNAs in an Argonaute-containing effector complex. In fruit flies, nematodes, and fungi, double-stranded RNA (dsRNA) replicative intermediates of viral RNA genomes are recognized and processed into virus-derived siRNAs by a single host Dicer (Schott et al., 2005; Wilkins et al., 2005; Galiana-Arnoux et al., 2006; Wang et al., 2006; Aliyari et al., 2008; Zhang et al., 2008; Lu et al., 2009; Wu et al., 2010). However, production of viral siRNAs in plants involves multiple Dicer-like proteins (DCLs) (Blevins et al., 2006; Bouché et al., 2006; Deleris et al., 2006; Fusaro et al., 2006; Moissiard and Voinnet, 2006; Diaz-Pendon et al., 2007).

Viral siRNAs detected in *Arabidopsis thaliana* plants infected with positive-strand RNA viruses are predominantly 21 nucleotides in length made by DCL4, which also produces endogenous trans-acting siRNAs (ta-siRNAs) and siRNAs targeting transgenes (Ding and Voinnet, 2007; Voinnet, 2008). The 22-nucleotide viral siRNAs produced by DCL2 in the presence of DCL4 often constitute <20% of the total viral small RNA population (Ding, 2010; Llave, 2010). However, elimination of both DCL4 and DCL2 is required to dramatically increase disease susceptibility to distinct RNA viruses or to restore pathogenicity of mutant viruses defective in silencing suppression (Bouché et al., 2006; Deleris et al., 2006; Diaz-Pendon et al., 2007), indicating that effective virus resistance is initiated by either DCL4 or DCL2. DCL3-dependent 24-nucleotide repeat-associated siRNAs target transposons, repeat elements, and transcriptionally silenced transgenes (Matzke et al., 2009). Infection with DNA viruses induces more abundant production of 24-nucleotide viral siRNAs than 21- and 22-nucleotide siRNAs in plants (Blevins et al., 2006; Moissiard and Voinnet, 2006). However, 24-nucleotide siRNAs targeting positive-strand RNA viruses are difficult to detect in wild-type plants, unable to guide antiviral silencing independently, and enhance virus resistance only in certain conditions, such as a compromised DCL4 function (Bouché et al., 2006; Deleris et al., 2006; Fusaro et al., 2006; Diaz-Pendon et al., 2007).

Less is known about the downstream components in RNA-based antiviral immunity (Ding, 2010; Llave, 2010; Qu, 2010). The function of ARGONAUTE (AGO) proteins is clearly essential since

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genetic inactivation of a single AGO without altering dicing enhances virus susceptibility in fungal, insect, nematode, and plant hosts (Li et al., 2002; Morel et al., 2002; Lu et al., 2005, 2009; Schott et al., 2005; Wilkins et al., 2005; van Rij et al., 2006; Zambon et al., 2006; Qu et al., 2008; Sun et al., 2009). In *Arabidopsis*, hypomorphic *ago1* and null *ago7* mutants accumulate higher levels of viral RNA, and AGO1, AGO2, and AGO5 bind to viral siRNAs in the infected cells, suggesting an antiviral role for these AGOs (Morel et al., 2002; Zhang et al., 2006; Qu et al., 2008; Takeda et al., 2008; Azevedo et al., 2010). Loss-of-function mutation in several additional *Arabidopsis* genes, including *RDR6*, *SGS3*, and *HEN1*, also leads to enhanced disease susceptibility to RNA viruses (Mourrain et al., 2000; Boutet et al., 2003; Ding and Voinnet, 2007; Ding, 2010; Llave, 2010; Qu, 2010).

Recent studies have demonstrated production of viral secondary siRNAs by multiple host RDR pathways in *Arabidopsis* infected by distinct positive-strand RNA viruses (Diaz-Pendon et al., 2007; Donaire et al., 2008; Garcia-Ruiz et al., 2010; Wang et al., 2010). RDR recruitment and synthesis of secondary siRNAs in *Caenorhabditis elegans* occur after cleavages of RNA transcripts targeted by primary siRNAs loaded in a specific AGO. *Arabidopsis* may encode six RDRs, of which RDR2 and RDR6 participate in the biogenesis of repeat-associated siRNAs and transgene siRNAs, respectively (Mourrain et al., 2000; Boutet et al., 2003; Xie et al., 2003). Plant ta-siRNAs resemble secondary siRNAs because production of ta-siRNAs requires AGO1/AGO7-mediated cleavages of transcripts targeted by microRNAs (miRNAs), followed by SGS3/RDR6-dependent production of dsRNAs and DCL4-dependent processing (Voinnet, 2008). However, unlike *C. elegans* secondary siRNAs that are Dicer independent, plant RDR-dependent siRNAs are Dicer products and are thus structurally indistinguishable from primary siRNAs (Voinnet, 2008).

Infection of *Arabidopsis rdr* mutants by virus mutants deficient in silencing suppression has recently revealed that resistance to distinct positive-strand RNA viruses depends on the production of viral secondary siRNAs by RDR1, RDR2, or RDR6 (Donaire et al., 2008; Garcia-Ruiz et al., 2010; Wang et al., 2010). These studies support early findings that plant lines compromised for the function of either RDR1 or RDR6 exhibit increased susceptibility to some of the RNA viruses examined (Mourrain et al., 2000; Xie et al., 2001; Yang et al., 2004; Qu et al., 2005; Schwach et al., 2005; Donaire et al., 2008). In this study, we investigated the biogenesis pathway of the viral secondary siRNAs following identification of a cucumber mosaic virus (CMV) mutant that was targeted for silencing predominantly by the RDR6-dependent viral secondary siRNA pathway. We also determined if the mutant virus was silenced by the destruction of the virus RNAs during the production of viral secondary siRNAs or by the activity of the resultant viral secondary siRNAs. Examination of 25 single, double, and triple *ago* mutants involving nine of the 10 *Arabidopsis AGO* genes revealed the role of AGO1 and AGO2 in the defense against the mutant virus. Notably, our studies revealed a qualitative difference in silencing virus RNAs between 21- and 22-nucleotide siRNAs produced by DCL4 and DCL2, respectively, which might be mechanistically related to the distinct activities of 21- and 22-nucleotide miRNAs in triggering the biogenesis of ta-siRNAs reported recently (Chen et al., 2010; Cuperus et al., 2010).

RESULTS

A Mutant CMV Uncouples RDR1- and RDR6-Dependent Virus Resistance

We have shown previously that disease resistance to a mutant of CMV deficient in silencing suppression is mediated by viral secondary siRNAs produced by both the RDR1 and RDR6 pathway (Wang et al., 2010). Here, we constructed a different mutant of CMV in which the start codon and two closely spaced AUG codons of the 2b open reading frame were mutated by point mutations so that the 2a sequence coding for the viral RDR in the overlapping reading frame was not altered (Figure 1A). This mutant was designated CMV-Δ2b and the mutant used in the previous study (Wang et al., 2010) renamed as CMV-2aTΔ2b since the 295-nucleotide deletion introduced in CMV RNA2 also led to early termination of the 2a protein (Figure 1A). As expected, expression of the 2b protein was detected in plants infected with CMV but not in those infected with either CMV-Δ2b or CMV-2aTΔ2b (see Supplemental Figure 1A online).

Inoculation of wild-type *Arabidopsis* plants with CMV-Δ2b induced no phenotypic change compared with mock inoculation (Figure 1B, top right) as found in previous studies (Ding et al., 1995; Soards et al., 2002; Lewsey et al., 2009). However, we found that CMV-Δ2b caused severe disease symptoms in all of the *Arabidopsis* mutants that contained a loss-of-function mutation in *RDR6*, including *rdr6*, *rdr1 rdr6*, *rdr2 rdr6*, and *rdr1 rdr2 rdr6* mutants (Figure 1B; see Supplemental Figure 1B online). By contrast, *rdr1*, *rdr2*, and *rdr1 rdr2* mutants that were wild type for *RDR6* were as resistant as wild-type plants to CMV-Δ2b (Figure 1B; see Supplemental Figure 1B online). Thus, CMV-Δ2b induced severe disease symptoms following genetic inactivation of the single host gene *RDR6* and therefore was distinct from CMV-2aTΔ2b, which became highly virulent only in mutant plants where neither *RDR6* nor *RDR1* was functional (Wang et al., 2010) (Figure 1B, compare middle and right columns). Moreover, we found that CMV-Δ2b, but not CMV-2aTΔ2b, was highly virulent in plants carrying a loss-of-function allele in *SGS3*, which is required for RDR6-dependent biogenesis of transgene/ta-siRNAs (Voinnet, 2008). RNA gel blot hybridizations detected greatly increased levels of CMV-Δ2b genomic and subgenomic RNAs in *rdr6*, *rdr1 rdr6*, and *sgs3* plants that exhibited severe disease symptoms compared with wild-type and *rdr1* plants that displayed no visible symptoms (Figures 1B and 1C). These results show that the RDR6/SGS3 pathway mediates the *Arabidopsis* resistance to CMV-Δ2b in an RDR1-independent manner.

Characterization of the Genetic Pathway for Viral Secondary siRNA Biogenesis

RNA gel blot hybridizations were used to compare the accumulation of viral siRNAs in wild-type and mutant plants inoculated with CMV-Δ2b. CMV-Δ2b replicated to lower levels and was expected to produce less dsRNA replicative intermediates for

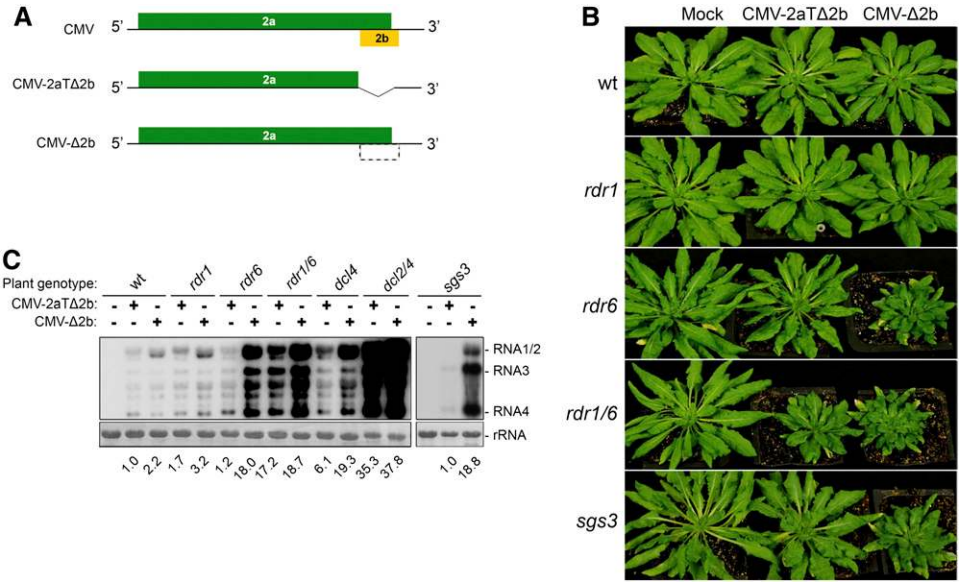


Figure 1. RNA Silencing of Distinct CMV Mutants by Single and Double RDR Pathways.

(A) Genome organization of RNA2 from wild-type and mutant CMV isolates. Wild-type RNA2 encodes the viral RdRp 2a protein and the 2b protein in overlapping reading frames. The 295-nucleotide deletion introduced into RNA2 of CMV-2aTΔ2b abolished expression of the 2b protein and also resulted in the truncation of the 2a protein. By contrast, CMV-Δ2b contained point mutations that inactivated the expression of 2b but did not alter coding in the 2a reading frame.

(B) Pathogenetic responses of CMV-2aTΔ2b and CMV-Δ2b in wild-type (wt) and mutant plants. Infected plants were photographed 3 weeks after inoculation.

(C) Accumulation of CMV-Δ2b and CMV-2aTΔ2b genomic RNAs (RNAs 1-3) and the subgenomic RNA (RNA4) in wild-type and mutant plants 3 weeks after inoculation detected by a probe specific for the 3′-untranslated region conserved in all of the four CMV RNAs. 25S rRNA was used as the loading control. Values at the bottom of this panel refer to the averages of the relative hybridization signal intensity of RNA1, 2, and 3 in three independent experiments with the accumulation levels of CMV-2aTΔ2b in wild-type plants (left panel) or *sgs3* plants (right panel) set as 1. Note that the hybridization signal intensity readings were from measuring both a short exposure (for *rdr6*, *rdr1/6*, *sgs3*, *dcl4*, and *dcl2 dcl4* samples) and a long exposure (for wild-type, *rdr1*, *rdr6*, *rdr1/6*, *sgs3*, and *dcl4* samples). The image shown was from a longer exposure to detect the accumulation of mutant viruses in wild-type and *rdr1* plants.

dicing into viral primary siRNAs in wild-type plants than in *rdr6* plants. However, we found that viral siRNAs in fact accumulated to higher levels in wild-type plants than in *rdr6* plants (Figure 2A). This indicates that most of the viral siRNAs detected in CMV-Δ2b–challenged wild-type plants are viral secondary siRNAs amplified by an RDR6-dependent pathway. Amplification of viral siRNAs was also detected in *rdr1* plants but not in *rdr1 rdr6* and *sgs3* plants (Figure 2A). These findings show that the RDR6/SGS3 pathway produces viral secondary siRNAs to confer resistance to CMV-Δ2b in an RDR1-independent manner. We noted that CMV-Δ2b accumulated to higher levels in the *dcl2 dcl4* mutant than in *rdr6* and *sgs3* plants (Figure 2A; see below). This indicates that production of RDR-independent viral primary siRNAs in *rdr6* and *sgs3* plants was associated with silencing of CMV-Δ2b but was insufficient to confer resistance to CMV-Δ2b, which was similar to that reported previously (Wang et al., 2010).

To verify RDR6-dependent production of viral secondary siRNAs targeting CMV-Δ2b, total small RNAs were extracted from wild-type and *rdr6* plants 3 weeks after CMV-Δ2b inoculation and sequenced by the Illumina Analyzer II as described previously (Wang et al., 2010). Small RNAs sequenced from both wild-type and *rdr6* plants that were 100% identical or complementary in sequence to the three genomic RNAs of CMV-Δ2b were predominantly 21 nucleotides in length (see Supplemental Figure 2 online). Approximately 10% of CMV-Δ2b–derived small RNAs were 22 nucleotides in length. These results are consistent with previous observations that DCL4 has a dominant role over DCL2 in the production of siRNAs targeting positive-strand RNA viruses (Ding, 2010; Llave, 2010).

Mapping of the perfectly matched siRNAs to the three viral genomic RNAs revealed shared features in the production of viral siRNAs between CMV-Δ2b–challenged wild-type plants (Figure 2B) and CMV-2aTΔ2b–challenged *rdr1* mutant plants (Wang et al., 2010) because amplification of viral siRNAs only relies on the RDR6 pathway in both cases (GSM498721-6). Compared with wild-type plants, the profile of viral siRNAs in *rdr1* plants is characterized with a reduced density of RDR1-dependent siRNAs targeting the 5′-terminal region of the viral RNAs 1 and 2 and an increased production of RDR6-dependent siRNAs targeting the 4th region of the viral RNA3 as specified in Figure 2C (Wang et al., 2010). Low density of the 5′-terminal viral siRNAs and high density of siRNAs targeting the same region of RNA3 were both detected in CMV-Δ2b–challenged wild-type plants (Figure 2B, top; see Supplemental Figure 3 online). Furthermore,

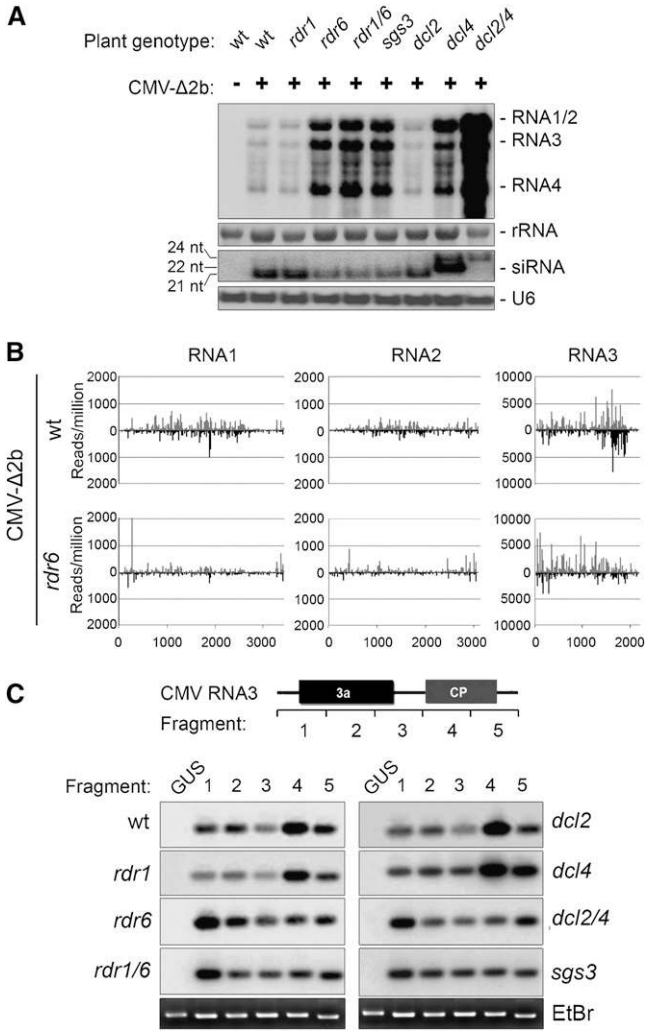


Figure 2. The Biogenesis Pathway for the Viral Secondary siRNAs Targeting CMV-Δ2b.

(A) Accumulation of CMV-Δ2b RNAs and siRNAs in wild-type (wt) and mutant plants 3 weeks after inoculation. 25S rRNA and U6 RNA were used as the high and low molecular RNA loading controls, respectively. nt, nucleotide.

(B) Mapping of perfect match 18- to 25-nucleotide virus-derived small RNAs (reads per million of total reads) sequenced by Illumina from wild-type and *rdr6* mutant plants challenged by CMV-Δ2b. Positive- and negative-strand reads are shown in gray and black, respectively. Note that the scale for RNA3 is 5 times that of RNAs 1 and 2.

(C) Mapping of siRNA hot spots targeting RNA3 by gel blot hybridization. Equal amount of DNA fragments specific to RNA3 (five fragments, 450 bp each) or β-glucuronidase (GUS; as negative control) was fractionated and hybridized to the labeled total small RNAs (18- to 30-nucleotide) harvested and gel purified from wild-type and mutant plants challenged by CMV-Δ2b.

the density of siRNAs targeting the RNA3 region was markedly reduced in *rdr6* plants (Figure 2B, bottom; see Supplemental Figure 3 online) compared with wild-type plants, indicating that amplification of siRNAs targeting this region of RNA3 is RDR6 dependent. Moreover, we noted an overall reduction in the density of siRNAs targeting both RNA1 and RNA2 in *rdr6* plants compared with wild-type plants (Figure 2B). These results from deep sequencing support the genetic analysis and show that siRNAs targeting CMV-Δ2b in *Arabidopsis* plants are amplified by the RDR6-dependent pathway in an RDR1-independent manner.

Gel blot hybridization approaches have been used previously to map hot spots of siRNAs targeting distinct genomic regions of CMV and other viruses (Szittya et al., 2010; Wang et al., 2010). As described previously (Wang et al., 2010), RNA3 of CMV was first divided evenly into five regions for DNA synthesis by PCR. Equal amount of DNA fragments corresponding to each region was fractionated and hybridized to the ^{32}P-labeled total small RNAs harvested and gel purified from wild-type and *rdr6* plants 3 weeks after CMV-Δ2b inoculation (Figure 2C). We found that hybridization with the small RNA probe from CMV-Δ2b–challenged wild-type plants produced much stronger signal to the 4th region of RNA3 than the neighboring regions. However, probing the same panel of DNA fragments with the small RNA probe from CMV-Δ2b–infected *rdr6* plants failed to detect a stronger signal to the 4th region of RNA3 compared with the neighboring regions (Figure 2C). This region corresponds to the 5′ half of RNA4, which is the most abundantly expressed viral RNA to act as mRNA for the viral coat protein encoded by RNA3. Thus, both deep sequencing and the gel blot hybridization approach demonstrate RDR6-dependent production of the hot spots of siRNAs targeting the 4th region of CMV-Δ2b RNA3 in the challenged *Arabidopsis* plants.

We then used the diagnostic hot spots of siRNAs targeting the 4th region of CMV-Δ2b RNA3 as a molecular marker to follow RDR6-dependent production of viral secondary siRNAs in *rdr1*, *rdr1 rdr6*, and *sgs3* plants (Figure 2C). We found that the strong signal hybridizing to the 4th region of RNA3 was detected in *rdr1* plants but not in *rdr1 rdr6* and *sgs3* plants (Figure 2C). Therefore, these findings together show that challenge by CMV-Δ2b induces production of viral secondary siRNAs in a pathway dependent on RDR6 and SGS3 but independent of RDR1.

Potent Antiviral Silencing Requires Production of Viral Secondary siRNAs by DCL4 but Not by DCL2

Regardless of RDR6-dependent amplification, viral siRNAs were predominantly 21 nucleotides in length in all of the CMV-Δ2b-inoculated plants where DCL4 was functional (Figure 2A). These included wild-type plants and *rdr1*, *rdr6*, *rdr1 rdr6*, *sgs3*, and *dcl2* mutant plants. By contrast, DCL2-dependent 22-nucleotide viral siRNAs accumulated to readily detectable levels only in the *dcl4* mutant where DCL4-mediated biogenesis of 21-nucleotide siRNAs was eliminated (Figure 2A). Notably, the diagnostic hot spots of siRNAs targeting the 4th region of CMV-Δ2b RNA3 were detected in both *dcl2* and *dcl4* single mutant plants but not in the *dcl2 dcl4* mutant in which both DCL1 and DCL3 are functional (Figure 2C). In addition, *Arabidopsis* plants infected with CMV-Δ2b produced high density of 21- and 22-nucleotide viral siRNAs targeting this region of RNA3 in an *RDR6*-dependent manner (see Supplemental Figure 3 online). These results show that either DCL4 or DCL2 alone can recognize and process the

dsRNA products of the RDR6/SGS3 pathway, leading to abundant accumulation of viral secondary siRNAs of 21 nucleotides in length in the *dcl2* mutant and of 22 nucleotides in length in the *dcl4* mutant, respectively (Figure 2A). By contrast, neither DCL1 nor DCL3 has access to the dsRNA precursor of viral secondary siRNAs, although DCL3 is able to produce viral primary siRNAs.

We found that, despite the absence of 22-nucleotide viral siRNAs, the *dcl2* mutant was as resistant to CMV-Δ2b as wild-type plants and potently inhibited the replication of CMV-Δ2b as efficiently as wild-type plants (Figure 2A). By contrast, the *dcl4* mutant was as susceptible to CMV-Δ2b as *rdr6* and *sgs3* mutants and supported high levels of CMV-Δ2b replication (Figure 2A). It should be pointed out that the accumulation of CMV-Δ2b was higher in *dcl2-dcl4* plants than in *dcl4*, *rdr6*, and *sgs3* plants (Figure 2A, see below), indicating that silencing of CMV-Δ2b occurs in *dcl4*, *rdr6*, and *sgs3* mutants, likely through primary viral siRNAs. Together, these results show that production of 21-nucleotide viral siRNAs by DCL4 is sufficient to confer resistance to CMV-Δ2b in the absence of the 22-nucleotide viral siRNAs produced by DCL2, whereas 22-nucleotide viral siRNAs produced by DCL2 do not guide efficient silencing of CMV-Δ2b in the absence of the 21-nucleotide viral siRNAs produced by DCL4. Therefore, secondary siRNA-dependent silencing of CMV-Δ2b relies on DCL4-dependent 21-nucleotide siRNAs but not DCL2-dependent 22-nucleotide siRNAs, revealing a difference between 21- and 22-nucleotide viral siRNAs in antiviral defense.

AGO1 and AGO2 Have Independent Function in Defense against CMV-Δ2b

Viral secondary siRNAs may guide specific silencing of viral RNAs in an AGO-containing effector complex as shown in previous studies that did not distinguish between viral primary and secondary siRNAs (Omarov et al., 2007; Pantaleo et al., 2007; Azevedo et al., 2010). However, the step of dicing to produce viral secondary siRNAs may be sufficient to confer virus resistance because it would destroy the viral RNAs targeted for dsRNA synthesis by the RDR6 pathway. To investigate these possibilities, we systematically probed the role of AGOs in defense against CMV-Δ2b, which was highly susceptible to silencing by RDR6-dependent viral secondary siRNAs. *Arabidopsis* encodes 10 AGOs that are divided into three clades (Vaucheret, 2008). One AGO clade contains AGO1, AGO5, and AGO10 (Vaucheret, 2008). We found that CMV-Δ2b induced disease symptoms in hypomorphic *ago1* mutants but not in *ago5* or *ago10* mutants (Figure 3). Both RNA gel blot hybridizations and real-time PCR detection of the viral positive and negative strand RNA2 showed that CMV-Δ2b accumulated to ~9-fold higher levels in *ago1* mutant plants compared with wild-type plants (Figures 4B and 5B; P values < 0.005). The accumulation levels of CMV-Δ2b were similar among *ago1*, *rdr6*, and *sgs3* mutant plants but were ~10-fold lower than those in *dcl2-dcl4* plants (Figures 2A, 4B, and 5B), suggesting that AGO1 is required for antiviral silencing by viral secondary siRNAs. We observed no major differences in disease symptoms and accumulation levels of CMV-Δ2b in the *ago1 ago5*, *ago1 ago10*, and *ago1 ago5 ago10* combinations compared with *ago1* (Figure 4B). These results indicate that AGO1 plays a key role in *Arabidopsis* resistance to CMV-Δ2b, whereas AGO5 and AGO10 do not have an independent or additive role in wild-type plants, even though a previous study detected loading of CMV siRNAs in plants overexpressing AGO5 (Takeda et al., 2008).

The second AGO clade contains AGO2, AGO3, and AGO7, among which AGO2 and AGO3 have high sequence similarity to each other and their respective genes are found directly in tandem in the genome (Vaucheret, 2008). We found that CMV-Δ2b induced mild dwarfing in the *ago2* mutant (Figure 3) and accumulated to ~3-fold higher levels in this mutant compared with wild-type, *ago3*, and *ago7* plants (Figures 4C and 5B; P values < 0.005). The *ago2 ago7* double mutant exhibited a response to CMV-Δ2b similar to that of the *ago2* single mutant, and the *ago3 ago7* double mutant was as resistant to CMV-Δ2b as wild-type, *ago3*, and *ago7* plants. These findings reveal an antiviral function for AGO2 that is independent of AGO1, whereas AGO3 and AGO7 do not have an independent or additive role.

The last AGO clade contains three full-length proteins, AGO4, AGO6, and AGO9, and a truncated protein, AGO8, which lacks the catalytic DDH motif of the PIWI domain because of incorrect splicing (Takeda et al., 2008). We found that none of the *ago4*, *ago6*, *ago9*, *ago4 ago6*, *ago4 ago9*, *ago6 ago9*, and *ago4 ago6 ago9* mutants was detectably compromised in resistance to CMV-Δ2b (Figure 4A). This result suggests that AGO4, AGO6, and AGO9 do not play a critical role in the silencing of CMV-Δ2b by the viral secondary siRNAs.

AGO1 and AGO2 Act Cooperatively Downstream of Viral Secondary siRNA Biogenesis

Six additional double and triple *ago* mutants were constructed between members of the AGO1 and AGO2 clades and examined for susceptibility to CMV-Δ2b. We found that pyramiding *ago3* and *ago7* mutations either alone or simultaneously over the *ago1* allele did not further increase *Arabidopsis* susceptibility to CMV-Δ2b (Figures 3 and 4D). By contrast, CMV-Δ2b caused more severe disease symptoms (Figure 3) and accumulated at higher levels in the *ago1 ago2* double mutant compared with either *ago1* or *ago2* single mutant (Figures 5A and 5B), indicating cooperative activities of AGO1 and AGO2. Adding either the *ago10* mutation or the *ago7* mutation over the *ago1 ago2* double mutant did not further increase *Arabidopsis* susceptibility to CMV-Δ2b (Figures 3 and 5A), indicating that AGO7 and AGO10 do not have additive roles.

In contrast with the reduced accumulation of viral siRNAs in *rdr6* and *sgs3* plants (Figure 2A), we found that viral siRNAs were more abundant in *ago1* and *ago2* single mutants as well as in *ago1 ago2*, *ago1 ago2 ago7*, and *ago1 ago2 ago10* mutants compared with wild-type plants following CMV-Δ2b challenges (Figure 5A). We further determined if the abundant viral siRNAs accumulated in mutant plants carrying *ago1* and/or *ago2* alleles included RDR6-dependent viral secondary siRNAs using the gel blot hybridization approach described above (Figure 5C). The characteristic strong hybridization signal corresponding to the 4th region of CMV-Δ2b RNA3 was detected in *ago1*, *ago2*, and *ago1 ago2* mutant plants (Figure 5C). These findings show that AGO2 and possibly AGO1 are dispensable for the biogenesis of

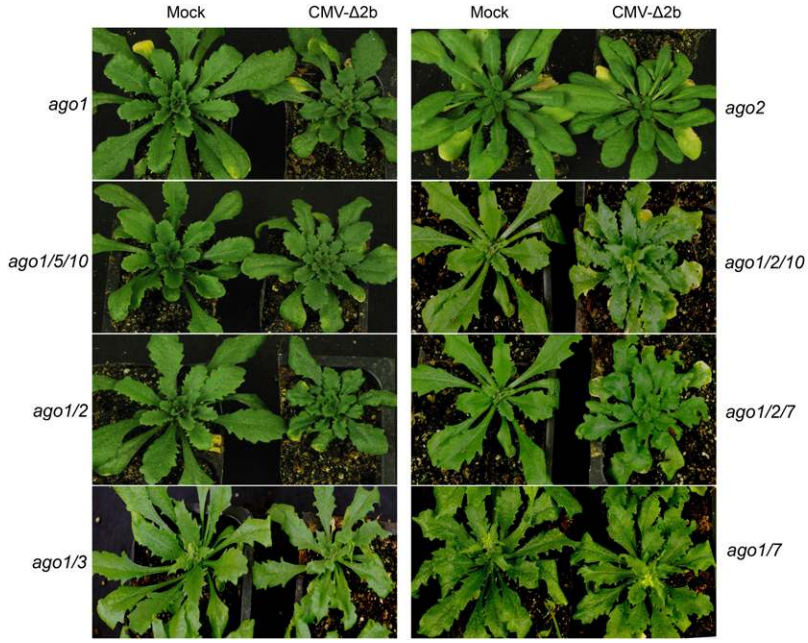


Figure 3. CMV-Δ2b Caused Enhanced Symptom Severity in the Double and Triple Mutants Containing Both *ago1 ago2* Alleles Compared with *ago1* and *ago2* Single Mutants.

Plants were photographed 3 weeks after inoculation.

viral secondary siRNAs. We therefore conclude that the defect of *ago1*, *ago2*, and *ago1 ago2* mutant plants in resistance to CMV-Δ2b is not caused by loss of production of viral secondary siRNAs and that AGO1 and AGO2 act cooperatively downstream the biogenesis of viral secondary siRNAs. Our results also showed that active dicing of the viral siRNA precursors, which occurred in the *ago1 ago2* mutant highly susceptible to CMV-Δ2b, was insufficient to confer virus resistance.

By comparison, CMV-Δ2b accumulated to significantly higher levels in the mutant plants containing both the *ago1* and *ago2* alleles than in *rdr6* and *sgs3* mutants (Figures 5A and 5B). As described above, *rdr6* and *sgs3* mutants were defective in the biogenesis of RDR6-dependent viral secondary siRNAs, and silencing of CMV-Δ2b by viral primary siRNAs was insufficient to confer resistance to CMV-Δ2b. Therefore, our results indicate that AGO1 and AGO2 are required for the antiviral activities of viral primary and secondary siRNAs. We noted that the accumulation of CMV-Δ2b in *ago1 ago2* plants was ~2-fold lower than those in *dcl2 dcl4* plants (Figures 5A and 5B; P values < 0.01), probably because the *ago1* allele used in this study is a hypomorphic allele. Indeed, only hypomorphic *ago1* mutants that retain partial AGO1 activity develop enough to be challenged by viruses (Morel et al., 2002).

Strong Positive-Strand Bias of Viral siRNAs Loaded in AGO1 but Not in AGO2

To characterize the viral siRNA populations loaded in AGO1 and AGO2, we constructed and sequenced small RNA libraries from CMV-Δ2b–infected plants expressing tagged AGO1 or AGO2 both before and after immunoprecipitation. Both AGO1 and AGO2 complexes contained viral siRNAs in addition to the known classes of endogenous small RNAs (Figure 6) as reported previously (Baumberger and Baulcombe, 2005; Zhang et al., 2006; Mi et al., 2008; Montgomery et al., 2008; Takeda et al., 2008). Comparisons between the small RNA populations obtained before and after immunoprecipitation revealed several notable differences (Figure 6). The virus-derived siRNAs were highly enriched following coimmunoprecipitation with AGO2 in two biological repeats (Figure 6A). However, we observed enrichment of viral siRNAs after AGO1 coimmunoprecipitation only in one of the two biological repeats due to variations of the endogenous small RNA contents. We found that sorting of viral siRNAs into AGO1 and AGO2 depends on 5′-terminal U and A, respectively, as found previously for endogenous small RNAs (Mi et al., 2008; Montgomery et al., 2008; Takeda et al., 2008). Approximately equal ratios of the total viral siRNAs produced in the CMV-Δ2b–infected plants were mapped to the positive and negative strands of the viral genomic RNAs, respectively (Figures 6B). Viral siRNAs loaded in AGO2 also contained approximately equal ratios of the plus and minus strands. By contrast, viral siRNAs loaded in AGO1 were predominantly from positive strands (Figures 6B). In addition, we noted that the viral siRNAs loaded in AGO2 contained a lower ratio of 22- and 21-nucleotide species than those loaded in AGO1. Nevertheless, we detected the characteristic strong hybridization signal corresponding to the 4th region of CMV-Δ2b RNA3 using as the probe the total small RNAs coimmunoprecipitated with either AGO1 or AGO1 (Figure 6C; see Supplemental Figure 4 online), indicating that both AGO1 and AGO2 have the ability to bind viral secondary siRNAs.

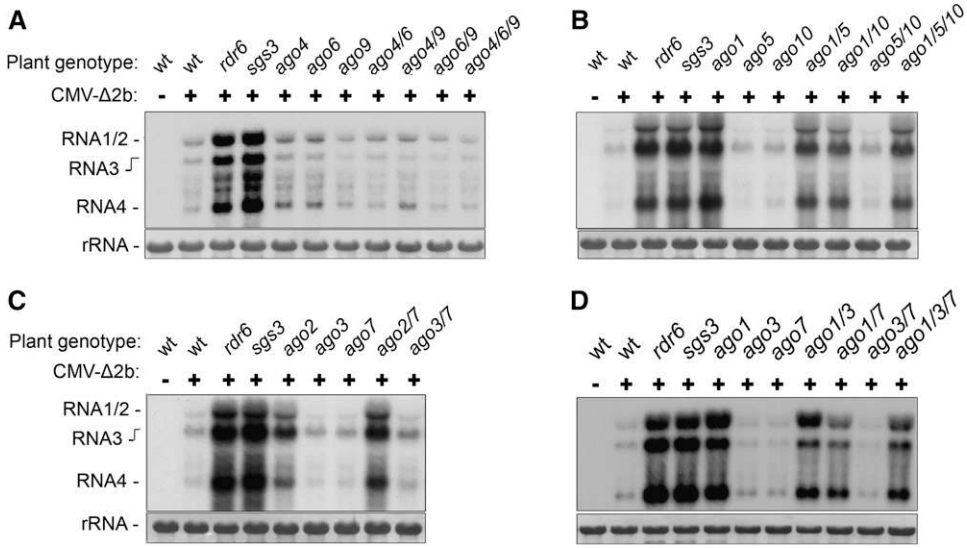


Figure 4. CMV-Δ2b Accumulated to Higher Levels in *ago1* and *ago2* Single Mutants Than in Wild-Type Plants and Other Single/Double/Triple *ago* Mutants That Did Not Contain Either Allele.

(A) to **(C)** All available single, double, and triple mutants within the AGO4 clade **(A)**, the AGO1 clade **(B)**, or the AGO2 clade **(C)** were challenged by CMV-Δ2b, and the accumulation of virus RNAs was analyzed 3 weeks after inoculation by RNA gel blot hybridization. wt, wild type.
(D) No additive effect was detected between the *ago1* allele and the *ago3* and *ago7* alleles from the AGO2 clade.

DISCUSSION

Silencing of Distinct Mutant Viruses Depends on Amplification of Viral siRNAs by Single or Double RDR-Dependent Pathways

As described previously (Wang et al., 2010), CMV-2aTΔ2b challenges induce amplification of viral siRNAs by both RDR1- and RDR6-dependent pathways with diagnostic hot spots of siRNAs targeting the 5′-terminal regions of RNA1/RNA2 and a region near the 3′ terminus of RNA3, respectively. Importantly, production of viral secondary siRNAs from either genetic pathway in *rdr1* and *rdr6* single mutants is sufficient to confer resistance so that CMV-2aTΔ2b causes diseases and replicates to high levels only in plants carrying both *rdr1* and *rdr6* mutant alleles (Wang et al., 2010). Here, we show that *Arabidopsis* resistance to CMV-Δ2b is associated with amplification of viral siRNAs predominantly by the RDR6-dependent pathway (Figure 2). Notably, CMV-Δ2b became highly virulent in *rdr6* and *sgs3* mutants (Figures 1 and 2) and accumulated to ~9-fold higher levels than in wild-type plants (Figure 5B; P values < 0.025), demonstrating that effective defense against CMV-Δ2b requires production of viral secondary siRNAs. However, eliminating both primary and secondary siRNAs in the *dcl2 dcl4* mutant further increased accumulation of CMV-Δ2b compared with that in *rdr6* and *sgs3* mutants defective only in the production of secondary siRNAs (Figure 5B). Therefore, production of RDR-independent viral primary siRNAs in *rdr6* and *sgs3* mutants is associated with silencing of CMV-Δ2b but is insufficient to confer virus resistance in the absence of viral secondary siRNAs (Figure 1), which is consistent with previous studies (Garcia-Ruiz et al., 2010; Wang et al., 2010).

Differential induction and/or suppression of RDR1- and RDR6-dependent amplification of viral siRNAs by distinct viruses may explain why RDR6-deficient *Arabidopsis* and *Nicotiana benthamiana* plants are more susceptible than wild-type plants to only some of the RNA viruses examined (Dalmay et al., 2000; Mourrain et al., 2000; Qu et al., 2005; Schwach et al., 2005; Vaistij and Jones, 2009). To account for the specific induction of RDR1- and RDR6-dependent amplification of viral siRNAs, we hypothesize that RDR6 and RDR1 are recruited to viral positive-strand RNAs acting as mRNA for translation and as template for RNA replication, respectively. In this model, recognition and silencing of viral mRNAs is analogous to the silencing of sense transgenes by the RDR6/SGS3 pathway (Voinnet, 2008). This is consistent with the observed preferential targeting of the more abundant RNAs of CMV, RNA3/RNA4, in plants challenged by either CMV-Δ2b or CMV-2aTΔ2b. On the other hand, production of the 5′-terminal siRNAs targeting a viral positive-strand RNA genome in *Drosophila melanogaster* is triggered by the synthesis of progeny positive-strand viral RNAs from the negative-strand RNA template (Aliyari et al., 2008). Similarly produced 5′-terminal viral primary siRNAs in plants may facilitate recruiting RDR1 to target the nascent products synthesized by the truncated viral RdRp of CMV-2aTΔ2b (Figure 1A). However, viral replication complexes assembled from the full-length viral RdRp encoded by CMV-Δ2b may prevent RDR1 access to the nascent replication products, leading to the absence of RDR1-dependent production of viral secondary siRNAs. The accumulation of CMV-2aTΔ2b was slightly lower than that of CMV-Δ2b in wild-type, *rdr1*, and *rdr1 rdr6* plants (Figure 1C), indicating that the truncated viral RdRp encoded by CMV-2aTΔ2b may indeed be partially defective to support replication.

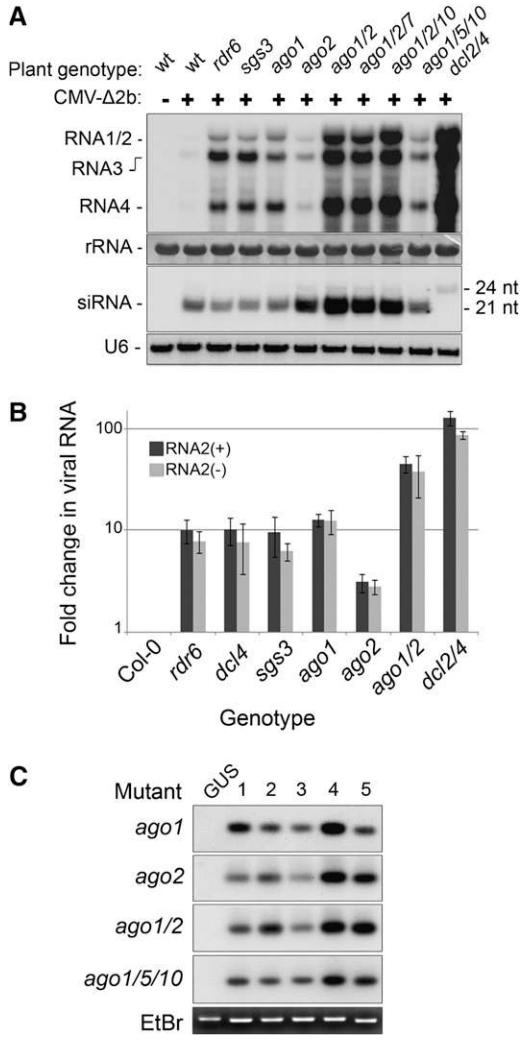


Figure 5. AGO1 and AGO2 Act Downstream in the Biogenesis of Viral Secondary siRNAs in a Nonredundant and Cooperative Manner.

(A) Accumulation of CMV-Δ2b RNAs and siRNAs in wild-type (wt) and mutant plants 3 weeks after inoculation. nt, nucleotide.

(B) The relative accumulation levels for the positive and negative strands of viral genomic RNA2 were measured by real-time PCR in three independent experiments. The accumulation level of RNA2 in wild-type Col-0 plants was set as 1. The data represented mean value of triplicates, and the error bars indicated SD.

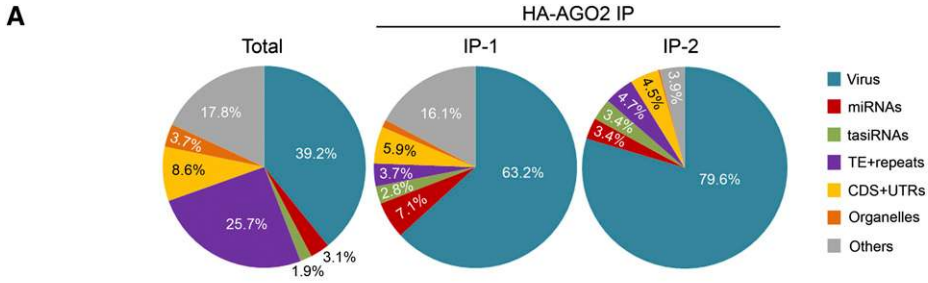
(C) Mapping of siRNA hot spots targeting RNA3 by gel blot hybridization.

Effector Mechanisms of Antiviral Silencing in Plants

Among *ago1*, *ago2*, *ago3*, *ago4*, *ago5*, *ago6*, *ago7*, *ago9*, and *ago10* single mutants, only *ago1* and *ago2* mutants exhibited enhanced disease susceptibility to CMV-Δ2b (Figures 3 to 5), demonstrating independent function of AGO1 and AGO2 in defense against CMV-Δ2b. However, viral siRNAs including viral secondary siRNAs accumulated to high levels in *ago1*, *ago2*, and *ago1 ago2* mutants infected with CMV-Δ2b (Figures 5A and 5C). These findings indicate that dicing of the viral precursor RNAs of siRNAs in absence of AGO1, AGO2, or both is insufficient to confer resistance and that AGO1 and AGO2 are essential for the antiviral activities of viral siRNAs downstream siRNA biogenesis. Coimmunoprecipitation with AGO1- and AGO2-specific antibodies followed by Illumina sequencing and gel blot hybridizations (Figure 6) revealed that in wild-type plants, both AGOs bind in vivo viral siRNAs, including viral secondary siRNAs. Further analysis showed that viral siRNAs with 5′ terminal uridine and adenosine are recruited preferentially by AGO1 and AGO2, respectively (Figure 6), as found previously for endogenous small RNAs (Mi et al., 2008; Montgomery et al., 2008; Takeda et al., 2008). Interestingly, whereas approximately equal ratios of the positive- and negative-strand viral siRNAs are detected in the AGO2 complex and in the total viral siRNAs, viral siRNAs loaded in AGO1 exhibited strong bias for the positive strands (Figure 6). It is known that *ago1* mutants are hypersensitive to virus infection and AGO1 binds to viral siRNAs in the infected cells (Morel et al., 2002; Zhang et al., 2006; Qu et al., 2008). A previous study detected loading of viral siRNAs in plants overexpressing AGO2 but did not demonstrate an antiviral function for AGO2 in wild-type plants (Takeda et al., 2008). During revision of our manuscript, Harvey et al. (2011) reported similar enhanced disease phenotypes of the single *ago2* mutant plants to wild-type viruses and AGO2 loading of viral siRNAs.

Further examination of 16 double and triple mutants generated both within and between the three clades of AGOs revealed additive effects of *ago1* and *ago2* in enhancing the symptom severity and virus accumulation (Figures 3 to 5) but not between *ago3*, *ago4*, *ago5*, *ago6*, *ago7*, *ago9*, and *ago10* or in combination with *ago1* and *ago2*. In particular, *ago1 ago5* mutants behaved like *ago1* toward CMV-Δ2b (Figure 4), and *ago1 ago7*, *ago2 ago7*, and *ago1 ago2 ago7* mutants behaved like *ago1*, *ago2* single, or double mutants (Figures 4 and 5). These results suggest that AGO5 and AGO7 have no detectable antiviral role against CMV-Δ2b in wild-type plants even through previous studies detected the binding of CMV siRNAs to AGO5 overexpressed in plants and an enhanced accumulation of turnip crinkle virus in both *ago1* and *ago7* single mutants (Qu et al., 2008; Takeda et al., 2008). Notably, CMV-Δ2b accumulated to significantly higher levels in the *ago1 ago2* mutant than in *rdr6* and *sgs3* mutants (Figure 5), suggesting that AGO1 and AGO2 mediate antiviral activities by both viral primary and secondary siRNAs.

Among the 10 AGOs, only AGO1 and AGO2 appear to be regulated by small RNAs. Indeed, miR168 and miR403 target *AGO1* and *AGO2* mRNA, respectively, and these regulations occur through AGO1, which associates to miR168 and miR403 (Rhoades et al., 2002; Vaucheret et al., 2004; Allen et al., 2005). Induction of *AGO1* mRNA accumulation is a general response to viral infection (Zhang et al., 2006; Csorba et al., 2007; Havelda et al., 2008). However, this induction is generally not accompanied by an increase in AGO1 activity because most viruses encode VSR (viral suppressors of RNA silencing) proteins that counteract either small RNA production, small RNA stability, small RNA loading on AGO1, *AGO1* mRNA translation, AGO1 protein stability, or AGO1 activity (Chapman et al., 2004; Lakatos et al., 2006; Zhang et al., 2006; Baumberger et al., 2007; Bortolamiol et al., 2007; Csorba et al., 2007; Azevedo et al.,



B

		Total reads*	Virus reads: Total (21-nt reads)	Plus/minus strand ratio	5′ nucleotide (%) A	C	G	U	22nt/21nt ratio
FLAG-AGO1	Total	4,991,301	(1,514,929)	0.84	28.1	18.9	37.0	16.0	0.17
	IP-1	739,625	(482,811)	2.85	11.7	9.3	1.7	77.3	0.10
	IP-2	7,949,837	(2,364,996)	2.64	2.7	3.5	0.4	93.4	0.17
HA-AGO2	Total	194,906	(58,535)	0.85	29.7	21.8	28.7	19.8	0.17
	IP-1	558,418	(332,081)	1.27	97.2	0.1	0.5	2.2	0.04
	IP-2	1,227,191	(920,850)	1.14	99.5	0.1	0.1	0.3	0.05

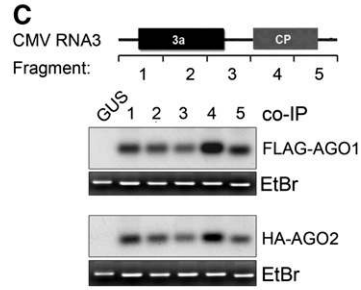


Figure 6. Features of Viral siRNAs Loaded in AGO1 and AGO2.

(A) Relative abundance of different categories of small RNAs obtained from plants infected with CMV-Δ2b before (left) and after coimmunoprecipitation (IP) with AGO2 (middle and right). CDS, coding sequences; TE, transposable elements; tasiRNAs, trans-acting siRNAs; UTR, untranslated region.

(B) Properties of viral siRNAs loaded in AGO1 and AGO2 sequenced from plants infected with CMV-Δ2b. Three independent libraries were sequenced each from the FLAG-tagged AGO1 and HA-tagged AGO2 plants infected with CMV-Δ2b: one before coimmunoprecipitation and two after coimmunoprecipitation. *Total reads analyzed include only those reads that perfectly matched either the *Arabidopsis* genome or the CMV genome. nt, nucleotide.

(C) Mapping of siRNA hot spots targeting RNA3 by gel blot hybridization as described in Figures 2C and 5C except that small RNAs coimmunoprecipitated (coIP) with AGO1 or AGO2 from CMV-Δ2b–infected plants were used as probes. CP, coat protein.

2010; Chiu et al., 2010; Giner et al., 2010; Várallyay et al., 2010). Induction of *AGO2* mRNA and AGO2 protein accumulation in response to viral infection has been reported (Lewsey et al., 2010; Endres et al., 2010; Harvey et al., 2011), likely because AGO1 association with miR403 or AGO1/miR403-mediated regulation of *AGO2* mRNA is impaired by VSR proteins. Whether AGO2 activity follows the increase in AGO2 protein level or is affected by VSR proteins remains to be determined.

RDR-dependent RNA silencing in *C. elegans* and *Arabidopsis* sometimes requires sequential activities of AGOs to bind primary and secondary siRNAs, respectively (Yigit et al., 2006; Montgomery et al., 2008). For example, AGO7 associates with miR390 to target *TAS3* single-stranded RNA that subsequently produce ta-siRNAs that associate with AGO1. As a result, *TAS3* ta-siRNAs are not detectable in *Arabidopsis ago1* or *ago7* null alleles (Adenot et al., 2006; Montgomery et al., 2008). However, abundant CMV siRNAs were detected in both the single and double *ago1/ago2* mutants, suggesting that AGO1 and AGO2 may not act sequentially in the biogenesis of viral secondary siRNAs. It should be pointed out that a hypomorphic *ago1* allele was used in this work because *ago1* null mutants exhibit pleiotropic developmental defects that make virus inoculation difficult (Morel et al., 2002). This hypomorphic *ago1* mutant retains partial AGO1 activity (Baumberger and Baulcombe, 2005), which could target CMV RNAs and is also expected to overaccumulate AGO2 because of reduced regulation of *AGO2* mRNA by miR403. Thus, an essential role for AGO1 in the biogenesis of viral secondary siRNAs cannot be ruled out. Alternatively, other AGOs, such as AGO5 (Takeda et al., 2008), may act redundantly in the biogenesis of viral secondary siRNAs without being able to target virus RNAs for silencing.

Future studies are necessary to understand why AGO1 and AGO2 act in a nonredundant and cooperative manner in antiviral defense. Nevertheless, it could be related to one or more of the following different properties of AGO1 and AGO2. First, AGO1 and AGO2 associate to different sets of viral siRNAs with distinct 5-terminal nucleotides (Figure 6B). This distinction is important at least for the activities of miRNAs because replacing the 5′ terminal uridine of a miRNA by an adenosine redirects the modified miRNA from AGO1 to AGO2 and impairs the regulation of its target (Mi et al., 2008). Whether AGO2/siRNA complexes can execute mRNA cleavage or translational repression like AGO1/siRNA complexes remains to be determined. Second, AGO1 and AGO2 appear to exhibit different affinities to positive- to

negative-strand viral siRNAs (Figure 6B). Thus, AGO1 and AGO2 may have different antiviral activities because negative-strand siRNAs are more active than positive-strand siRNAs in antiviral silencing against positive-strand RNA viruses (Pantaleo et al., 2007; Schubert et al., 2007). Third, different ratios of 22- to 21-nucleotide viral siRNAs associate with AGO1 and AGO2, which could influence the overall activity of AGO1/siRNA and AGO2/siRNA complexes (see below).

22-Nucleotide Viral Secondary siRNAs Produced by DCL2 Do Not Mediate Efficient Antiviral Defense

Several lines of evidence indicate that *Arabidopsis* resistance to CMV-Δ2b is conferred by the viral secondary siRNAs made by DCL4. The 21-nucleotide viral siRNAs were the most dominant species of viral siRNAs detected in the wild-type, *rdr1*, and *dcl2* plants, which were resistant to CMV-Δ2b and supported production of viral secondary siRNAs targeting CMV-Δ2b (Figure 2). By comparison, the accumulation of 21-nucleotide viral siRNAs was markedly reduced in the highly susceptible plants, such as *rdr6*, *rdr1 rdr6*, and *sgs3* mutants, none of which supported production of viral secondary siRNAs targeting CMV-Δ2b (Figure 2). Importantly, loss of 21-nucleotide viral siRNAs in the *dcl4* mutant abolished resistance to CMV-Δ2b, whereas preventing production of 22-nucleotide viral siRNAs in the *dcl2* mutant did not decrease resistance to CMV-Δ2b (Figure 2).

RNA gel blot hybridizations showed that 22-nucleotide viral siRNAs in fact accumulated to higher levels in the *dcl4* mutant than 21-nucleotide viral siRNAs in the *dcl2* mutant (Figure 2A). Importantly, the *dcl4* mutant supported production of the RDR6-dependent hot spots of siRNAs targeting the 4th region of RNA3, indicating that when DCL4 was not functional, DCL2 was able to recognize and process the RDR6-dependent dsRNA precursor into secondary siRNAs. As a result, viral secondary siRNAs were undetectable only in the *dcl2 dcl4* mutant (Figure 2C). Therefore, abundant production of viral primary and secondary 22-nucleotide siRNAs by DCL2 in the *dcl4* mutant failed to confer effective antiviral defense in absence of the DCL4-processed 21-nucleotide siRNAs. Nevertheless, CMV-Δ2b reproducibly accumulated to significantly higher levels in the *dcl2-dcl4* mutant than in *dcl4*, *sgs3*, and *rdr6* mutants (Figures 2 and 5; P values < 0.001). This suggests that DCL2-processed 22-nucleotide viral siRNAs direct silencing of CMV-Δ2b in the *dcl4* mutant with similar efficiency to the silencing of CMV-Δ2b by the RDR6-independent viral primary siRNAs found in *rdr6* and *sgs3* mutants.

A previous study found that a VSR-deficient mutant of turnip crinkle virus is partially rescued in the *dcl4* mutant but not in the *dcl2* mutant and that increasing the inoculum strength could bypass the antiviral effects of DCL2 but not those of DCL4 (Deleris et al., 2006). Although the study did not differentiate viral primary and secondary siRNAs, the results are consistent with the qualitative difference in antiviral defense observed in this work between 21- and 22-nucleotide viral secondary siRNAs processed by DCL4 and DCL2, respectively. The same qualitative difference is also known for siRNAs that mediate non-cell-autonomous transgene silencing (Dunoyer et al., 2005). Indeed, when dsRNAs are specifically expressed in the phloem, spreading of RNA silencing in the adjacent cells depends on DCL4-processed 21-nucleotide siRNAs, whereas 22-nucleotide siRNAs processed by DCL2 in the *dcl4* mutant are ineffective. By contrast, both DCL4- and DCL2-processed secondary siRNAs direct effective RNA silencing when inducing transgenes are expressed constitutively (Fusaro et al., 2006; Moissiard et al., 2007; Mallory and Vaucheret, 2009). Therefore, the efficiency of siRNAs may depend on their site of production and/or their route for spreading. Alternatively, the qualitative difference between 21- and 22-nucleotide secondary siRNAs in RNA silencing could be mechanistically related to the distinct properties of 21- and 22-nucleotide miRNAs loaded in AGO1. Indeed, although both species of miRNAs guide AGO1 to cleave the target transcripts, only 22-nucleotide miRNAs trigger RDR6-dependent synthesis of the dsRNA precursor of ta-siRNAs (Chen et al., 2010; Cuperus et al., 2010). It is possible that efficient and multiple rounds of RNA cleavage are directed only by AGO1/AGO2 complexes loaded with 21-nucleotide siRNAs/miRNAs (Hutvágner and Zamore, 2002). By contrast, AGO1/AGO2 complexes loaded with 22-nucleotide siRNAs/miRNAs either direct inefficient cleavages or fail to efficiently release the cleaved products of the target transcripts, leading to the recruitment of RDR6 and SGS3.

METHODS

Plant Materials

Arabidopsis thaliana mutant lines in Columbia (Col) ecotype for *rdr1-1*(SAIL_672F11), *rdr2-1*(SAIL_1277H08), *rdr6-15* (SAIL_617_H07), *dcl2-1* (SALK_064627), *dcl4-2* (GABI_160G05), *sgs3-1*, *ago1-27*, *ago2-1* (SALK_003380), *ago3-1* (SM_3_31520), *ago7-1* (SALK_037458), *ago10-3* (SALK_019738), *ago4-4* (FLAG_216G02), and *ago6-2* (SALK_031553) were described previously (Mourrain et al., 2000; Morel et al., 2002; Allen et al., 2004; Vazquez et al., 2004; Xie et al., 2004, 2005; Elmayan et al., 2005; Lobbes et al., 2006; Zheng et al., 2007; Mallory et al., 2009). *ago5* and *ago9* mutants were new T-DNA insertion mutants in the Wassilewskija ecotype (H. Vaucheret, unpublished data). The double and triple *ago* mutants were obtained by crossing single *ago* mutants. The *ago5* and *ago9* single mutants used in this study were backcrossed twice to Col and confirmed by sequencing to ensure a comparable genetic background to the other double and triple *ago* mutants. Transgenic expression of HA-tagged AGO2 in the wild-type background and FLAG-tagged AGO1 in the *ago1-36* mutant background driven by their native promoters were described previously (Baumberger and Baulcombe, 2005; Montgomery et al., 2008). After vernalization in the dark at 4°C, the seeds were transferred into a growth room programmed for 10 h in light and 14 h in dark at 24°C.

Viruses and Infection Assays

The mutant virus CMV-2aTΔ2b was described as CMVf-Δ2b previously (Wang et al., 2010). CMV-Δ2b was obtained by introducing three missense point mutations that abolished three ATG codons of 2b open reading frame at nucleotides 2420 (the start codon of 2b), 2441, and 2471. Purified virions propagated in *Nicotiana clevelandii* were used as the inocula at the concentration of 20 μg/mL.

RNA Gel Blotting and Immunoblotting Analyses

The systemically infected leaves of 20 to 30 plants were pooled for RNA extraction three weeks after inoculation. As described previously, high (5 μg) and low (15 μg) molecular RNAs were used to detect viral RNAs

and siRNAs, respectively (Wang et al., 2010). The probe for viral RNA detection was the randomly labeled cDNA corresponding to the 3′ terminal 240 nucleotides of RNA2. The small RNAs were detected by the labeled DNA oligonucleotides corresponding to the genomic RNA3 (nucleotides 241 to 280, 741 to 780, 1041 to 1080, 1341 to 1380, 1600 to 1640, 1681 to 1710, and 1731 to 1770). The gel blot hybridization assay to map the hot spots of viral siRNAs was performed as described previously (Wang et al., 2010). Briefly, an equal amount of each of the DNA fragments specific to RNA3 (divided into five fragments of 450 bp each) was fractionated and hybridized to the [γ-^{32}P]ATP-labeled total small RNAs harvested and gel purified from wild-type Col-0 and mutant *Arabidopsis* plants infected with CMV-Δ2b. The blot signals were detected by phosphor imager and multiple film exposures. The systemically infected leaves were used for immunoblot detection of the 2b protein using the polyclonal anti-2b of the Fny strain of CMV as described previously (Zhang et al., 2006). Cloning, sequencing, and analysis of viral siRNAs were performed as described (Wang et al., 2010).

Real-Time PCR

Real-time quantitative PCR was used to measure the accumulation of the positive and negative strands of the viral RNA2 in infected leaves using *Arabidopsis* Actin2 mRNA as the control for normalization. Briefly, 1 μg of total RNA extracted from the upper systemically infected leaves was used for reverse transcription using Super Script III reverse transcriptase (Invitrogen) and subsequent real-time quantitative PCR reactions using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. Three independent repeats were performed for each experiment. The data analysis of relative expression was conducted using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Construction, Sequencing, and Analyses of Small RNA Libraries

Construction, Illumina sequencing, and analyses of small RNA libraries from the CMV infected plants were performed as described (Wang et al., 2010). To analyze the small RNA populations loaded in AGO1 and AGO2, libraries were constructed from total small RNAs purified from the leaves of *FLAG-AGO1/ago1-36* and *HA-AGO2/AGO2* plants systemically infected with CMV-Δ2b both before and after coimmunoprecipitation with FLAG- and HA-specific antibodies as described (Baumberger and Baulcombe, 2005). After sequencing, adapters' sequences were removed and the downstream analyses were performed with in-house pipelines. Perfect matches to the *Arabidopsis* and CMV genomes were extracted with Bowtie (Langmead et al., 2009) and considered as the total reads in each library. The *Arabidopsis*-specific reads were further classified into miRNAs, ta-siRNAs, transposable elements and repeats, coding regions and untranslated regions, and organellar (mitochondria and chloroplast) small RNAs. The fraction of small RNAs that did not match any of the above-mentioned categories was assigned to the "others" category, such that the sum of all categories equals the total number of reads in each library. The following databases were used: TAIR10 blast_datasets (ftp://ftp.Arabidopsis.org/home/tair/Sequences/), Repbase (http://www.girinst.org), ASBP (http://asrp.cgrb.oregonstate.edu), and miRBase (http://microrna.sanger.ac.uk/sequences).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: Actin2 mRNA, AY096381.1; CMV-RNA1, NC_002034.1; CMV-RNA2, NC_002035.1; CMV-RNA3, NC_001440.1; small RNA libraries, GSE27477.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Morphology of Wild-Type and Mutant Plants Inoculated with Two Different 2b-Deficient Mutant Viruses, CMV-2aTΔ2b or CMV-Δ2b.

Supplemental Figure 2. The Profiles of Virus-Derived siRNAs Sequenced by Illumina from Wild-Type and *rdr6* Plants Infected with CMV-Δ2b.

Supplemental Figure 3. Detection of RDR6-Dependent Hot Spots of Both 21- and 22-Nucleotide Viral siRNAs in Wild-Type Plants Challenged by CMV-Δ2b.

Supplemental Figure 4. Mapping of Perfect Match 18- to 25-Nucleotide Virus-Derived Small RNAs Sequenced by Illumina from FLAG-AGO1 or HA-AGO2 Plants Challenged by CMV-Δ2b.

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