

# Suppression of Antiviral Silencing by Cucumber Mosaic Virus 2b Protein in *Arabidopsis* Is Associated with Drastically Reduced Accumulation of Three Classes of Viral Small Interfering RNAs <sup>W</sup><sup>OA</sup>

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We investigated the genetic pathway in *Arabidopsis thaliana* targeted during infection by cucumber mosaic virus (CMV) 2b protein, known to suppress non-cell-autonomous transgene silencing and salicylic acid (SA)-mediated virus resistance. We show that 2b expressed from the CMV genome drastically reduced the accumulation of 21-, 22-, and 24-nucleotide classes of viral small interfering RNAs (siRNAs) produced by *Dicer-like4* (*DCL4*), *DCL2*, and *DCL3*, respectively. The defect of a CMV 2b-deletion mutant (CMV- $\Delta$ 2b) in plant infection was efficiently rescued in *Arabidopsis* mutants producing neither 21- nor 22-nucleotide viral siRNAs. Since genetic analysis further identifies a unique antiviral role for *DCL3* upstream of *DCL4*, our data indicate that inhibition of the accumulation of distinct viral siRNAs plays a key role in 2b suppression of antiviral silencing. Strikingly, disease symptoms caused by CMV- $\Delta$ 2b in *Arabidopsis* mutants defective in antiviral silencing were as severe as those caused by CMV, demonstrating an indirect role for the silencing suppressor activity in virus virulence. We found that production of CMV siRNAs without 2b interference depended largely on *RNA-dependent RNA polymerase 1* (*RDR1*) inducible by SA. Given the known role of *RDR6*-dependent transgene siRNAs in non-cell-autonomous silencing, our results suggest a model in which 2b inhibits the production of *RDR1*-dependent viral siRNAs that confer SA-dependent virus resistance by directing non-cell-autonomous antiviral silencing.

## INTRODUCTION

RNA silencing refers to related gene-silencing mechanisms with specificity determined by small RNAs of 21 to 30 nucleotides in length (Meister and Tuschl, 2004; Baulcombe, 2005). In *Arabidopsis thaliana*, RNA silencing is mediated by microRNAs (miRNAs) and three size classes of small interfering RNAs (siRNAs) of 21, 22, and 24 nucleotides long (Herr, 2005). These small RNAs are the products of four Dicer-like (DCL), double-stranded (ds) RNA-specific ribonucleases encoded by the *Arabidopsis* genome. miRNAs are processed by DCL1 from stem-loop structures of primary nuclear transcripts, and only partial loss-of-function *dcl1* mutants are viable because of the essential roles of miRNAs in development (Chen, 2005). Both genetic and biochemical analyses have shown that the 21-, 22-, and 24-nucleotide classes of siRNAs are produced by DCL4, DCL2, and DCL3, respectively (Herr, 2005; Qi and Hannon, 2005). For example, *trans*-acting siRNAs mediating silencing of endogenous genes and secondary siRNAs required for cell-to-cell silencing spread

are 21 nucleotides long and *DCL4* dependent (Herr, 2005; Qi and Hannon, 2005; Voinnet, 2005). By contrast, the siRNAs derived from transposons, repeated sequences, and heterochromatin implicated in the methylation of DNA and chromatin are 24 nucleotides long and *DCL3* dependent (Matzke and Birchler, 2005). The precursors of these endogenous siRNAs are most likely dsRNAs, because of the requirement for a distinct RNA-dependent RNA polymerase gene (*RDR*) for the production of each siRNA class, *RDR6* and *RDR2* for 21- and 24-nucleotide classes of siRNAs, respectively (Herr, 2005; Qi and Hannon, 2005).

The first biological function established for RNA silencing was as a defense mechanism against viruses in plants (Voinnet, 2005). Virus-derived siRNAs (viRNAs) detected in infected plants and insects include both positive and negative polarities and cover the entire viral genome (Hamilton and Baulcombe, 1999; Li et al., 2002). In *Drosophila melanogaster*, infection with (+)-strand RNA viruses results in the production of the 22-nucleotide viRNAs by Dicer2, known to process exogenous dsRNA into siRNAs but dispensable for miRNA production (Wang et al., 2006). By contrast, R2D2, which acts downstream of Dicer2 to load siRNAs into the Argonaute2 (AGO2)-containing RNA-induced silencing complex, is essential for the activity but dispensable for the production of viRNAs (Wang et al., 2006). Recent genetic studies in *Arabidopsis* (Xie et al., 2004; Bouche et al., 2006; Deleris et al., 2006; Fusaro et al., 2006) showed that DCL4 acts as the primary virus sensor to produce 21-nucleotide siRNAs of potent antiviral activity. In the absence of DCL4,

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22- and 24-nucleotide viRNAs are produced by DCL2 and DCL3, respectively, although it appears that only the 22-nucleotide viRNAs are sufficient to mediate independent antiviral silencing (Deleris et al., 2006). Whether or not the biogenesis of viRNAs in *Arabidopsis* requires any additional host proteins is unknown.

Identification of potyviral helper component–proteinase (HC-Pro) and the cucumber mosaic virus (CMV) 2b protein as viral suppressors of RNA silencing/RNA interference (RNAi) (VSR) revealed a new virus counterdefensive strategy (Anandalakshmi et al., 1998; Beclin et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998). Since 1998, numerous VSRs encoded by both plant and animal viruses have been reported (Roth et al., 2004; Li and Ding, 2006). Extensive analyses in systems that assay for the suppression of transgene silencing have shown that HC-Pro and 2b, both required for long-distance virus movement and for disease symptom expression in infected plants, represent prototypes of two distinct groups of VSRs (Li and Ding, 2001; Roth et al., 2004). HC-Pro is a cytoplasmic protein that inhibits the processing of dsRNA into siRNAs without interfering with either RNA-directed DNA methylation (RdDM) or intercellular silencing spread mediated by the silencing signal (Llave et al., 2000; Mallory et al., 2001, 2002; Dunoyer et al., 2004). However, HC-Pro suppression of miRNA silencing is associated with an increased accumulation of miRNA duplexes, suggesting that HC-Pro may act downstream of the production of miRNAs (Mallory et al., 2002; Chapman et al., 2004). This is consistent with the recent observation that HC-Pro, similar to the tombusviral p19 (Silhavy et al., 2002; Lakatos et al., 2004), prevents the assembly of siRNAs into the active RNA-induced silencing complex by binding to and sequestering duplex siRNAs (Lakatos et al., 2006; Merai et al., 2006). By contrast, CMV 2b, which accumulates in the nucleus and cytoplasm of plant cells (Lucy et al., 2000; Mayers et al., 2000; Wang et al., 2004), is a potent inhibitor of both intercellular silencing spread and RdDM (Guo and Ding, 2002). Notably, CMV 2b also can block salicylic acid (SA)-mediated virus resistance (Ji and Ding, 2001). A model was proposed to account for the observed activities of 2b in which SA may induce virus resistance by potentiating antiviral silencing (Ji and Ding, 2001).

Here, we investigated the genetic basis of 2b suppression of antiviral silencing in *Arabidopsis* plants infected with CMV. CMV contains a (+)-strand RNA genome that encodes five proteins among three RNA molecules (Palukaitis and Garcia-Arenal, 2003). Two replicase subunits and the movement protein are translated directly from the genomic RNAs, whereas coat protein and 2b are each translated from RNAs 4 and 4A, both of which are subgenomic RNAs (Ding et al., 1994). We found that CMV infection resulted in the production of 21-, 22-, and 24-nucleotide classes of viRNAs in *Arabidopsis* by DCL4, DCL2, and DCL3, respectively, as reported recently (Bouche et al., 2006; Fusaro et al., 2006), and deletion of 2b from the CMV genome caused a characteristic defect in the systemic infection of *Arabidopsis* plants, as observed previously in other host species (Ding et al., 1995a; Ji and Ding, 2001). Examining virus infection and the accumulation of viRNAs in *dcl* and *rdm* mutants of *Arabidopsis* inoculated with CMV- $\Delta$ 2b revealed distinct roles for the three classes of viRNAs in antiviral silencing. These analyses indicate that 2b suppression of antiviral silencing depended on inhibition

of the accumulation of the three classes of viRNAs in infected *Arabidopsis* plants. Although the silencing suppressor activity of 2b was required to establish infection, it was dispensable in eliciting disease symptoms, indicating an indirect role for viral silencing suppressors in virus virulence. Notably, we demonstrate a specific recognition of CMV by *RDR1* and a key role for *RDR1* in the production of CMV siRNAs. A potential role of the *RDR1*-dependent secondary viRNAs in non-cell-autonomous antiviral silencing is discussed.

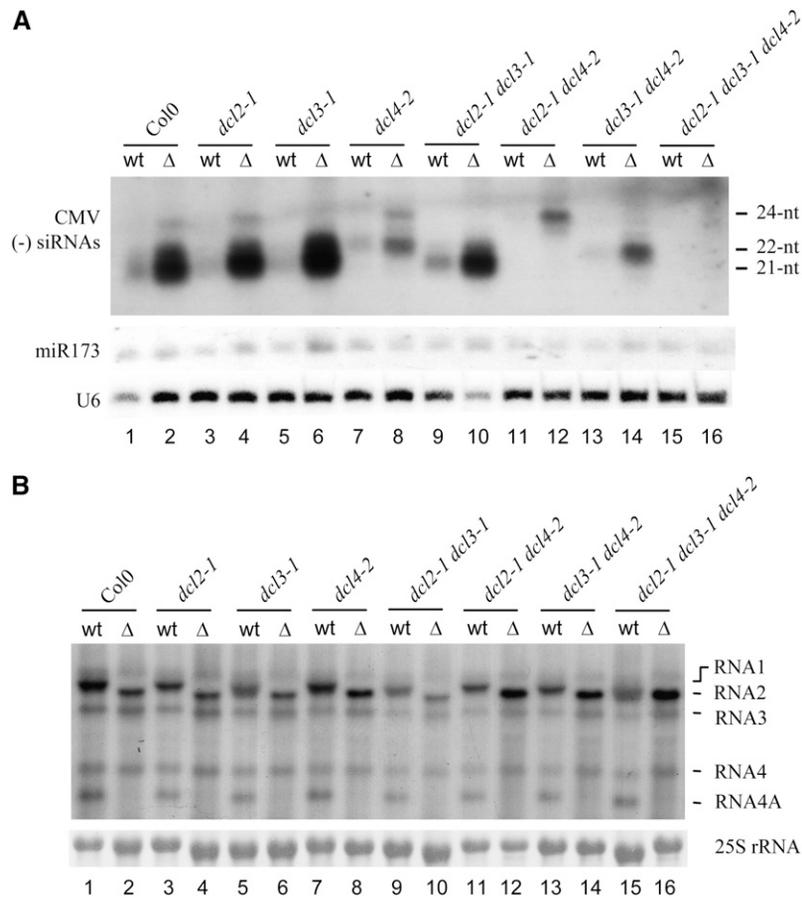
## RESULTS

### Expression of CMV 2b Inhibits the Accumulation of Three Classes of viRNAs

To investigate the genetic target of CMV 2b in infected plants, we inoculated wild-type and silencing-defective mutant *Arabidopsis* plants with purified virions of either wild-type CMV (CMV) or its 2b-deletion mutant (CMV- $\Delta$ 2b). Our analysis of CMV-derived (–)-strand siRNAs in single, double, and triple *dcl* mutants (Xie et al., 2005; Deleris et al., 2006; Henderson et al., 2006) revealed that CMV infection led to the accumulation of 21-, 22-, and 24-nucleotide viRNAs that required DCL4, DCL2, and DCL3, respectively (see Supplemental Figure 1 online). This result is consistent with the biogenesis of virus-derived siRNAs reported recently by several groups (Blevins et al., 2006; Bouche et al., 2006; Deleris et al., 2006; Fusaro et al., 2006).

We previously described CMV- $\Delta$ 2b, which contains a point mutation converting the fourth codon (UUG) of the 2b open reading frame to UAG (Ding et al., 1995b). Whereas CMV- $\Delta$ 2b is genetically stable in *Nicotiana* species (Ding et al., 1995a; Ji and Ding, 2001), the introduced stop codon UAG was rapidly reverted to UGG and the 2b open reading frame was restored after infection of *Arabidopsis*. Thus, we used a derivative of CMV- $\Delta$ 2b in which most of the 2b coding sequence was deleted (Li et al., 2002). Equal amounts of CMV and CMV- $\Delta$ 2b virions purified from *Nicotiana glutinosa* were inoculated onto the four fully extended leaves of each *Arabidopsis* plant at 40 ng/leaf. Five days after inoculation, total high and low molecular weight RNAs were extracted from the inoculated leaves pooled from 16 to 24 plants for the detection of virus and viRNAs. Although CMV- $\Delta$ 2b was defective in systemic infection (see below), the genomic and subgenomic RNAs of both CMV and CMV- $\Delta$ 2b accumulated to similar levels in the inoculated leaves of wild-type plants and the single *dcl* mutant *Arabidopsis* plants (Figure 1B, lanes 1 to 8). As expected, RNA 2 of CMV- $\Delta$ 2b migrated faster than that of CMV because of the deletion.

In contrast with the equivalent levels of virus genomic RNAs in the leaves inoculated with either CMV or CMV- $\Delta$ 2b, the three classes of viral (–)-strand siRNAs accumulated to drastically higher levels in the CMV- $\Delta$ 2b-inoculated leaves than in CMV-inoculated leaves (Figure 1A). For example, a significantly reduced accumulation of viral 21-nucleotide siRNAs was observed in wild-type, *dcl2*, *dcl3*, and *dcl2 dcl3* plants infected with CMV compared with those plants infected with CMV- $\Delta$ 2b. The levels of the 22-nucleotide siRNAs were drastically lower in wild-type, *dcl3*, and *dcl4* plants infected with CMV than in those plants infected with CMV- $\Delta$ 2b. Similarly, whereas the 24-nucleotide



**Figure 1.** Expression of 2b Inhibits the Accumulation of All Three Classes of viRNAs.

**(A)** Detection of (–)-strand CMV siRNAs in the inoculated leaves of wild-type Columbia (Col-0), *dcl2-1*, *dcl3-1*, *dcl4-2*, *dcl2-1 dcl3-1*, *dcl2-1 dcl4-2*, *dcl3-1 dcl4-2*, and *dcl2-1 dcl3-1 dcl4-2* plants at 5 d after infection with either CMV (wt) or CMV-Δ2b (Δ). The membrane was also probed for miR173 and U6 RNAs. Positions of the 21-, 22-, and 24-nucleotide (nt) siRNAs are indicated.

**(B)** Detection of CMV genomic and subgenomic RNAs (indicated at right) in the inoculated leaves of the same plants used in **(A)**. 25S rRNA served as a loading control.

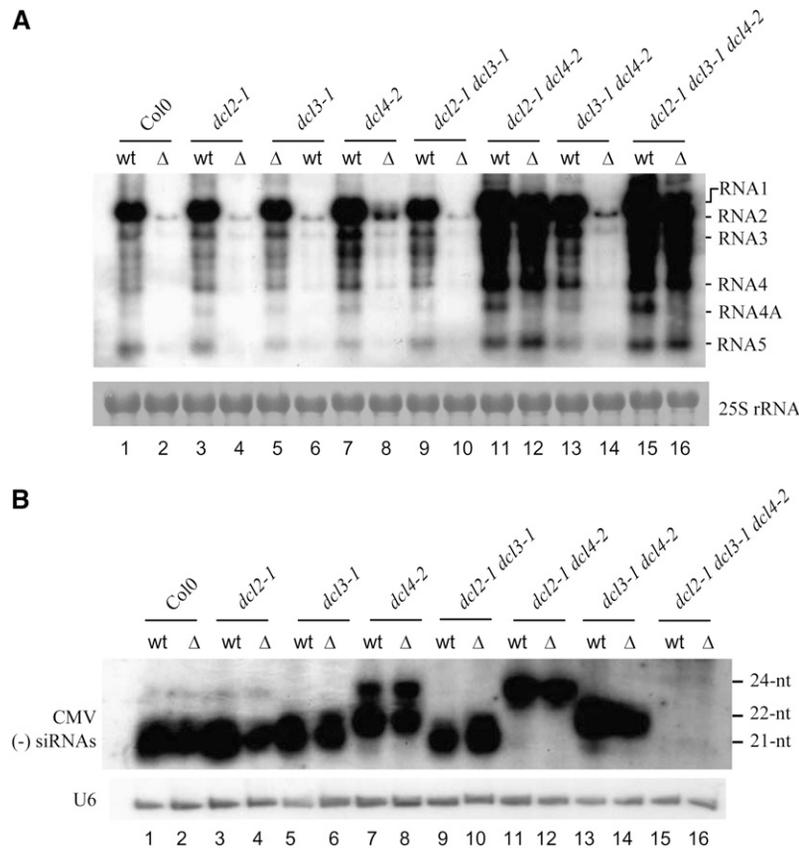
class of viRNAs was detected as a major band in wild-type, *dcl2*, *dcl4*, and *dcl2 dcl4* plants infected with CMV-Δ2b, it was undetectable in the same plants inoculated with the 2b-expressing wild-type CMV. However, no major difference in the accumulation of miR173 was detected following infection with either CMV or CMV-Δ2b (Figure 1A), supporting an earlier observation that CMV infection does not alter miRNA accumulation (Bouche et al., 2006). These results indicate that expression of 2b from CMV dramatically reduced the accumulation of all three classes of viRNAs in infected *Arabidopsis* plants.

#### DCL4- and DCL2-Mediated Pathways Are the Main Genetic Targets of CMV 2b

The above results suggest that CMV 2b may suppress the RNA-silencing antiviral immunity by inhibiting the accumulation of viRNAs in CMV-infected plants. Thus, we next investigated whether the defect of CMV-Δ2b in systemic infection was genetically rescued in *Arabidopsis* plants defective in the syn-

thesis of one or more classes of the viRNAs. The 21-nucleotide class of CMV-specific siRNAs was undetectable in the inoculated and the upper systemically infected leaves of all of the *dcl* mutants that contain the *dcl4-2* allele, including *dcl4-2*, *dcl2-1 dcl4-2*, *dcl3-1 dcl4-2*, and *dcl2-1 dcl3-1 dcl4-2* plants (Figures 1A and 2B). Similarly, the 22- and 24-nucleotide classes of CMV siRNAs were absent in the *dcl* mutants that contain *dcl2-1* and *dcl3-1*, respectively (Figures 1A and 2B). Thus, CMV infection did not lead to the accumulation of two classes of CMV siRNAs in double *dcl* mutants: 21- and 22-nucleotide siRNAs in *dcl2-1 dcl4-2* plants (Figures 1A and 2B, lanes 11 and 12), 21- and 24-nucleotide siRNAs in *dcl3-1 dcl4-2* plants (Figures 1A and 2B, lanes 13 and 14), and 22- and 24-nucleotide siRNAs in *dcl2-1 dcl3-1* plants (Figures 1A and 2A, lanes 9 and 10). Also as expected, none of the three classes of CMV siRNAs was detectable in *dcl2-1 dcl3-1 dcl4-2* plants (Figures 1A and 2B, lanes 15 and 16).

RNA gel blot analysis showed that CMV-Δ2b accumulated to much lower levels than CMV in the upper systemically infected



**Figure 2.** CMV- $\Delta$ 2b Is Rescued in *dcl2-1 dcl4-2* and *dcl2-1 dcl3-1 dcl4-2* Plants.

**(A)** Detection of CMV genomic/subgenomic RNAs from the upper systemically infected leaves of wild-type Col-0, *dcl2-1*, *dcl3-1*, and *dcl4-2* single mutants, *dcl2-1 dcl3-1*, *dcl2-1 dcl4-2*, and *dcl3-1 dcl4-2* double mutants, and the *dcl2-1 dcl3-1 dcl4-2* triple mutant at 14 d after infection with CMV (wt) or CMV- $\Delta$ 2b ( $\Delta$ ). 25S rRNA served as a loading control. The CMV species are indicated at right.

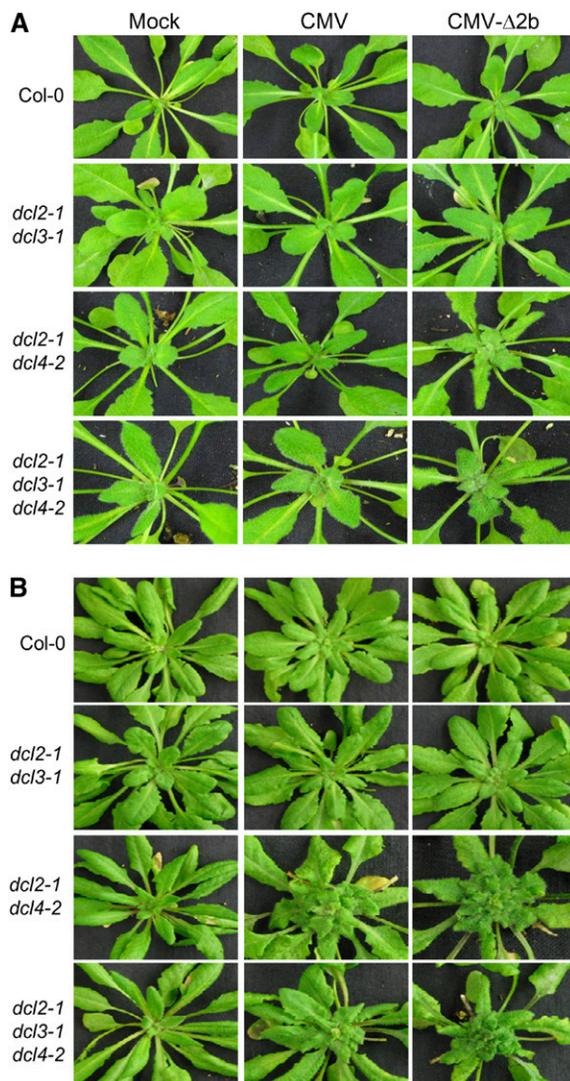
**(B)** Detection of (–)-strand CMV siRNAs in the upper systemically infected leaves of the same plants used in **(A)**. The membrane was also probed for U6 RNA. Positions of the 21-, 22-, and 24-nucleotide (nt) siRNAs are indicated.

leaves of wild-type *Arabidopsis* plants at 14 d after inoculation (DAI) with equal amounts of CMV and CMV- $\Delta$ 2b virions (Figure 2A, compare lanes 1 and 2). This recapitulated the previous observation made in other host species that CMV- $\Delta$ 2b exhibits a defect in the systemic virus spread (Ding et al., 1995a; Ji and Ding, 2001). CMV- $\Delta$ 2b remained defective in the systemic infection of *dcl2-1*, *dcl3-1*, *dcl4-2*, *dcl2-1 dcl3-1*, and *dcl3-1 dcl4-2* plants because CMV- $\Delta$ 2b accumulated to much lower levels than CMV did in the upper leaves (Figure 2A), similar to what occurred in wild-type plants. We noted that viRNAs accumulated to similarly high levels in the upper leaves of these plants infected with either CMV- $\Delta$ 2b or CMV (Figure 2B) in spite of their different accumulation levels of the viral genomic and subgenomic RNAs. Thus, low levels of CMV RNA accumulation were associated with high-level accumulation of CMV siRNAs in the absence of 2b expression, further supporting a role for 2b in inhibiting the accumulation of viRNAs. We found that both CMV and CMV- $\Delta$ 2b accumulated to similar levels in the upper leaves of *dcl2-1 dcl4-2* and *dcl2-1 dcl3-1 dcl4-2* plants (Figure 2A, compare lanes 11/12 and 15/16), indicating that systemic infection by CMV- $\Delta$ 2b was as efficient as that by CMV in these mutant plants.

These results showed that, first, either the 21- or 22-nucleotide class of CMV siRNAs alone, but not the 24-nucleotide class of viRNAs, was sufficient to repress the systemic spread of CMV- $\Delta$ 2b in *Arabidopsis* plants. Second, efficient systemic infection of CMV- $\Delta$ 2b occurred only when the host plants were defective in the synthesis of both the 21- and 22-nucleotide classes of CMV siRNAs, and eliminating the production of 24-nucleotide siRNAs did not further enhance CMV- $\Delta$ 2b infection when both 21- and 22-nucleotide siRNAs were absent. Third, our findings also showed that elimination of both the 21- and 22-nucleotide siRNA pathways effectively compensated for the loss of 2b expression and allowed robust systemic infection of CMV- $\Delta$ 2b. Thus, we conclude that the 21- and 22-nucleotide siRNA pathways are the key genetic targets of CMV 2b, although 2b inhibited the accumulation of all three classes of CMV siRNAs. In support of this conclusion, we found that a CMV 2b transgene integrated in wild-type *Arabidopsis* plants suppressed the silencing of potato virus X amplicon (Li, 2001) and rescued the systemic infection of the p38-deletion turnip crinkle virus (TCV) mutant (our unpublished data), known to be targeted by the 21- and 22-nucleotide siRNA pathways in *Arabidopsis* (Deleris et al., 2006).

### CMV 2b Is Dispensable in the Induction of Enhanced Disease Susceptibility

The Q strain of CMV used in this work is highly virulent in cucumber (*Cucumis sativus*) and *N. glutinosa* plants, but it causes only mild mottling in *Nicotiana tabacum* (Ding et al., 1995a; Ji and Ding, 2001) and very mild dwarfing in wild-type *Arabidopsis* (Figure 3B, top middle). Infection with CMV resulted in much higher levels of virus accumulation (Figure 2A, lanes 11 and 15) and induced the development of severe systemic disease symptoms (Figure 3B) in both *dcl2-1 dcl4-2* and *dcl2-1 dcl3-1 dcl4-2* plants compared with CMV infection in wild-type plants. Such enhanced disease susceptibility (EDS) phenotypes were not observed following CMV infection in *dcl2-1 dcl3-1* (Fig-



**Figure 3.** Expression of 2b Does Not Play a Direct Role in Eliciting Disease Symptoms.

Wild-type, *dcl2-1 dcl3-1*, *dcl2-1 dcl4-2*, and *dcl2-1 dcl3-1 dcl4-2* plants were photographed at 5 d (**A**) and 14 d (**B**) after infection with CMV, CMV- $\Delta$ 2b, or buffer (mock).

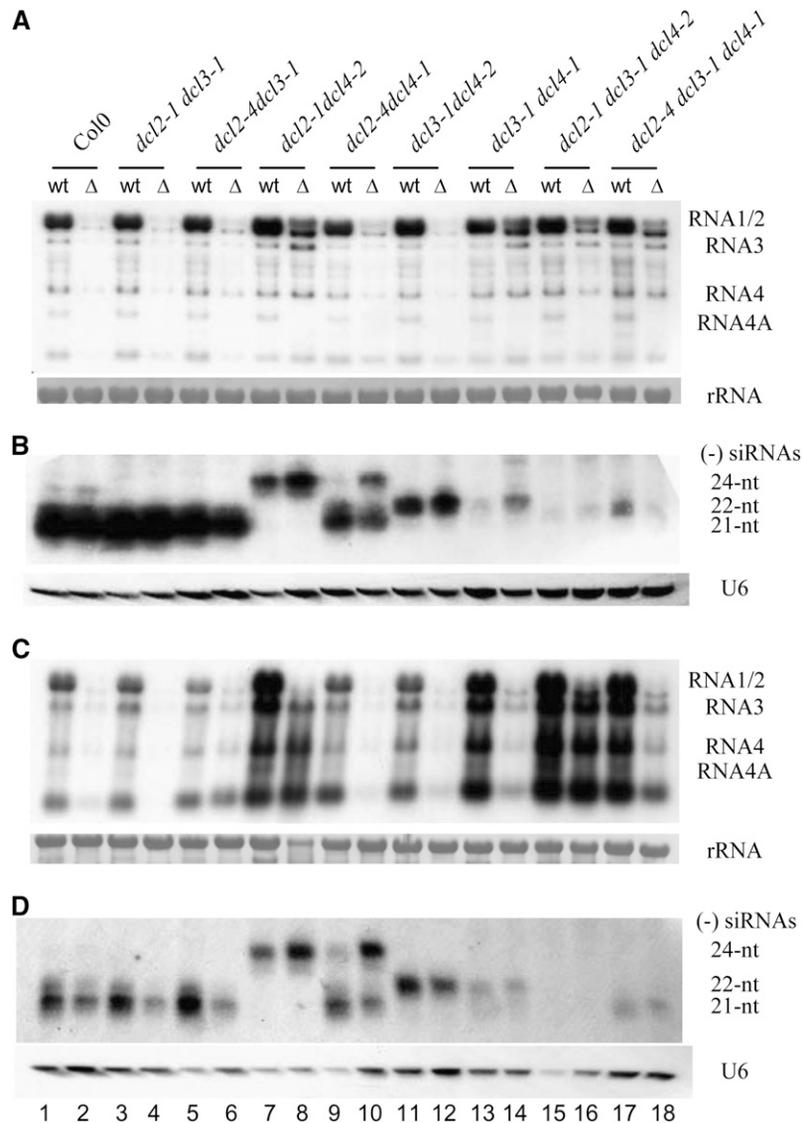
ures 2A, lane 9, and 3B) or any other single and double *dcl* mutants (data not shown). As indicated above, robust systemic infection by CMV- $\Delta$ 2b occurred only in *dcl2-1 dcl4-2* and *dcl2-1 dcl3-1 dcl4-2* plants. Notably, CMV- $\Delta$ 2b also accumulated to higher levels and induced severe disease symptoms in these plants compared with CMV infection in wild-type plants (Figures 2A and 3B). Thus, we conclude that the expression of 2b was not required for the induction of severe disease symptoms in these immunity-defective plants.

Intriguingly, we found that EDS development was much faster in *dcl2-1 dcl4-2* and *dcl2-1 dcl3-1 dcl4-2* plants infected with CMV- $\Delta$ 2b than in the same plants infected with CMV. The disease symptoms were clearly visible in the upper systemically infected leaves of *dcl2-1 dcl4-2* and *dcl2-1 dcl3-1 dcl4-2* plants at 5 DAI with CMV- $\Delta$ 2b, and the growth of the infected plants was arrested from this point (Figure 3A). By contrast, disease symptoms were invisible in the same mutant plants inoculated with CMV at 5 DAI (Figure 3A), and plant growth was not significantly affected until  $\sim$ 10 DAI. A 10-fold increase in CMV virion inoculum resulted in higher virus accumulation but failed to induce early disease symptoms in *dcl2-1 dcl4-2* and *dcl2-1 dcl3-1 dcl4-2* plants (data not shown). These data suggest that the expression of 2b delayed the development of severe disease symptoms in the early stages of infection.

### DCL3 Could Act Upstream of DCL4 to Enhance Antiviral Silencing

In contrast with the complete rescue of CMV- $\Delta$ 2b in *dcl2-1 dcl4-2* plants, we found that CMV- $\Delta$ 2b remained defective in systemic infection of *dcl2-4 dcl4-1* plants (Figures 4A and 4C, compare lanes 8 and 10). The accumulation of CMV- $\Delta$ 2b in the upper systemically infected leaves of *dcl2-4 dcl4-1* at either 5 or 14 DAI was as low as in wild-type plants (Figures 4A and 4C, compare lanes 2 and 10). *dcl4-2* was isolated directly from Col-0 and contains a T-DNA inserted in an exon of the *DCL4* gene, whereas T-DNA was inserted in an intron in *dcl4-1*, which was originally identified in the Wassilewskija ecotype and backcrossed twice to Col-0 (Gascioli et al., 2005; Xie et al., 2005). Since the accumulation of 21-nucleotide *trans*-acting siRNAs was reduced in *dcl4-1* plants but was undetectable in *dcl4-2* (Gascioli et al., 2005; Xie et al., 2005), we hypothesized that the resistance of *dcl2-4 dcl4-1* plants to CMV- $\Delta$ 2b might be mediated by the partially active *dcl4-1* allele. Indeed, although not as abundant as in plants carrying wild-type *DCL4* (Figures 4B and 4D, lanes 1 to 6), the 21-nucleotide CMV siRNAs reproducibly accumulated to high levels in *dcl2-4 dcl4-1* plants (Figures 4B and 4D, lanes 9 and 10). By contrast, 22-nucleotide CMV siRNAs remained undetectable in *dcl2-4 dcl4-1* plants, as in *dcl2-1 dcl4-2* plants (Figures 4B and 4D, lanes 7 to 10). These results show that, unlike *dcl4-2*, *dcl4-1* represented an incomplete loss-of-function allele in the production of viral 21-nucleotide siRNAs and that this allele was as effective as wild-type *DCL4* in repressing the systemic infection of CMV- $\Delta$ 2b in the absence of *DCL2*.

Notably, we found that the *dcl4-1* allele failed to effectively repress CMV- $\Delta$ 2b infection in *dcl3-1 dcl4-1* and *dcl2-4 dcl3-1 dcl4-1* plants in which *DCL3* was inactivated. Accumulation of



**Figure 4.** *DCL3* Enhances Antiviral Silencing by *dcl4-1*.

Detection of CMV genomic/subgenomic RNAs (**A**) and **(-)**-strand CMV siRNAs (**B**) and **(-)**-strand CMV siRNAs (**D**) from the upper systemically infected leaves of *Arabidopsis* seedlings of various genotypes as indicated at 5 d (**A**) and **(-)**-strand CMV siRNAs (**B**) and 14 d (**C**) and **(-)**-strand CMV siRNAs (**D**) after inoculation with CMV (wt) or CMV- $\Delta$ 2b ( $\Delta$ ). 25S rRNA served as a loading control. The membrane was also probed for U6 RNA. Positions of the 21-, 22-, and 24-nucleotide (nt) siRNAs are indicated.

CMV- $\Delta$ 2b in the upper leaves of both mutant plants (Figure 4A, lanes 14 and 8) was as high as in *dcl2-1 dcl4-2* plants at 5 DAI (Figure 4A, lane 8), indicating that *dcl3-1 dcl4-1* and *dcl2-4 dcl3-1 dcl4-1* mutants supported the efficient systemic infection of CMV- $\Delta$ 2b. By 14 DAI, the accumulation of CMV- $\Delta$ 2b in *dcl3-1 dcl4-1* and *dcl2-4 dcl3-1 dcl4-1* plants (Figure 4A, lanes 13 and 18) was higher than that in *dcl2-4 dcl4-1* plants but lower than that in *dcl2-1 dcl4-2* plants (Figure 4A, lane 8), suggesting more effective antiviral silencing in *dcl3-1 dcl4-1* and *dcl2-4 dcl3-1 dcl4-1* plants at 14 DAI (Figure 4C, lanes 14 and 18). These results showed that the antiviral activity of the *dcl4-1* allele depended on the function of *DCL3*, indicating a unique antiviral role of *DCL3* upstream of *DCL4*.

In contrast with the abundant accumulation of 21-nucleotide CMV siRNAs in *dcl2-4 dcl4-1* plants, the level of 21-nucleotide CMV siRNAs was extremely low in both *dcl3-1 dcl4-1* and *dcl2-4 dcl3-1 dcl4-1* plants, which contained the same *dcl4-1* allele (Figures 4B and 4D). This difference in the accumulation of 21-nucleotide CMV siRNAs explains, first, why *dcl3-1 dcl4-1* and *dcl2-4 dcl3-1 dcl4-1* plants, but not *dcl2-4 dcl4-1* plants, supported the efficient systemic infection of CMV- $\Delta$ 2b. Second, it also indicates that in *dcl3-1 dcl4-1* and *dcl2-4 dcl3-1 dcl4-1* plants, the *dcl3-1* allele not only eliminated production of the 24-nucleotide CMV siRNAs, as expected, but also potently inhibited production of the 21-nucleotide CMV siRNAs by the partially active *dcl4-1* allele. However, production of the

21-nucleotide viRNAs by wild-type *DCL4* was independent of DCL3, because the 21-nucleotide CMV siRNAs were as abundant in *dcl3-1* and *dcl2-4 dcl3-1* plants as in wild-type plants (Figure 1B, compare lanes 2 and 6; Figures 4B and 4D, compare lanes 1 and 5). Thus, these results indicate that DCL3 acts to amplify production of the 21-nucleotide viRNAs by the weak *dcl4-1* allele.

We noted that the accumulation levels of the 22-nucleotide CMV siRNAs were also very low in *dcl3-1 dcl4-1* plants (Figures 4B and 4D, lanes 13 and 14). However, the reduced accumulation of the 22-nucleotide viRNAs observed in *dcl3-1 dcl4-1* plants was associated with either wild-type or partially active DCL4 but not with the presence of the *dcl3-1* allele. This suggests that *dcl4-1* remained active in the observed hierarchical antiviral activities of DCL4 and DCL2, in which *DCL4* is dominant and inhibitory to *DCL2* (Deleris et al., 2006).

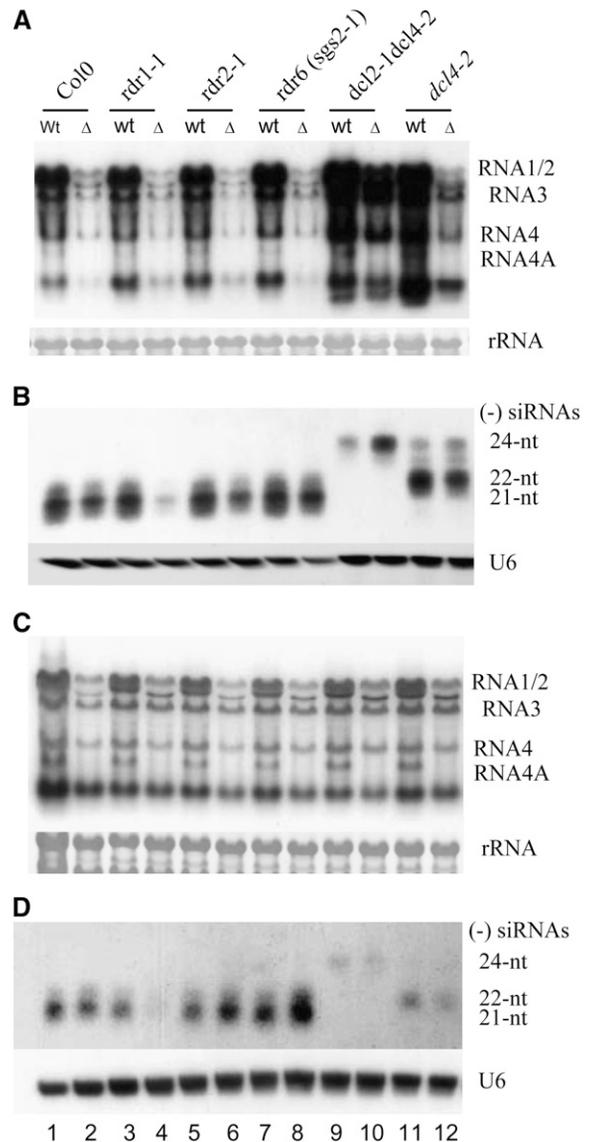
### Production of CMV siRNAs Is Largely RDR1 Dependent

We next investigated a potential role of cellular RDRs in the biogenesis of viRNAs by challenging wild-type and *rdr* mutant plants with equal amounts of CMV and CMV- $\Delta$ 2b virions. We found that the accumulation of viRNAs was significantly reduced in *rdr1-1* plants compared with wild-type, *rdr2-1*, or *rdr6 (sgs2-1)* plants following infection with CMV- $\Delta$ 2b (Figure 5B, compare lane 4 with lanes 2, 6, and 8). Similarly reduced accumulation of siRNAs was detected in the inoculated leaves at 5 DAI (Figure 5D) and in the upper systemically infected leaves at 14 DAI (Figure 5B), and this result was reproducible. Compared with infection with CMV, however, the accumulation of viral genomic and subgenomic RNAs remained as low in *rdr1-1* plants as in wild-type, *rdr2-1*, and *rdr6* plants following CMV- $\Delta$ 2b infection (Figure 5A), indicating that the *rdr1-1* mutation was insufficient to rescue the defect of CMV- $\Delta$ 2b in systemic infection. Nevertheless, our results strongly indicate that CMV was specifically recognized by RDR1 and that the production of CMV siRNAs in wild-type, *rdr2-1*, or *rdr6 (sgs2-1)* plants infected with CMV- $\Delta$ 2b was largely RDR1 dependent. It is unclear whether the low level of viRNAs detected in *rdr1-1* plants infected with CMV- $\Delta$ 2b was RDR1-dependent (e.g., primary siRNAs that are viral RdRP dependent) or dependent on one or more of the five remaining RDRs.

In contrast with infection with CMV- $\Delta$ 2b, we detected similar accumulation levels of viRNAs in wild-type, *rdr1-1*, *rdr2-1*, and *rdr6 (sgs2-1)* plants infected wild-type CMV (Figure 5B, lanes 1, 3, 5, and 7). Thus, the presence of wild-type *RDR1* in *rdr2-1*, *rdr6 (sgs2-1)*, and wild-type plants did not result in higher production of viRNAs compared with *rdr1-1* plants following CMV infection, suggesting a loss of the RDR1-dependent production of viRNAs when CMV 2b protein was expressed.

### DISCUSSION

The use of well-defined transgenic plant models based on silencing transgenes has greatly facilitated the identification and mechanistic analysis of VSRs (Roth et al., 2004). These systems assay for the suppression of RNA silencing of a transgene transcribed in the nucleus by a VSR often expressed from a non-targeted mRNA. However, VSRs target antiviral silencing induced



**Figure 5.** RDR1-Dependent Production of CMV siRNAs in *Arabidopsis*.

Detection of CMV genomic/subgenomic RNAs ([A] and [C]) and (–)-strand CMV siRNAs ([B] and [D]) from the inoculated ([C] and [D]) and upper systemically infected leaves ([A] and [B]) of *Arabidopsis* seedlings of various genotypes as indicated at 5 d ([A] and [B]) and 14 d ([C] and [D]), respectively, after inoculation with CMV (wt) or CMV- $\Delta$ 2b ( $\Delta$ ). 25S RNA served as a loading control. The CMV RNA species are indicated at right. The membrane was also probed for U6 RNA. Positions of the 21-, 22-, and 24-nucleotide (nt) siRNAs are indicated.

by a replicating and movement-competent virus in infected hosts by expression from the infecting virus genome, which is also under the control of the induced antiviral silencing. We previously identified the dsRNA-siRNA pathway of *Drosophila* and *Caenorhabditis elegans* as the genetic target of the flock house virus VSR, because genetic defects in the RNAi pathway rescued the accumulation defects of a B2-deficient FHV (Li et al., 2002; Lu et al., 2005; Wang et al., 2006). However, similar genetic rescue

of VSR-deficient mutant viruses has not been possible in plants until recently, because of the complex small RNA pathways controlled by four DCLs together with six RDRs. The recent availability of multiple *DCL* mutants of *Arabidopsis* allowed Voinnet and colleagues to demonstrate that p38 of TCV specifically inhibits the accumulation of the *DCL4*-dependent, 21-nucleotide class of viRNAs (Deleris et al., 2006).

In this study, we investigated the genetic mechanism involved in the induction and suppression of antiviral silencing during CMV infection in *Arabidopsis* plants. Our results demonstrate that 2b expressed from the CMV genome during infection potently inhibited the accumulation of the 21-, 22-, and 24-nucleotide CMV-specific siRNAs in *Arabidopsis*. We showed that the defect of CMV- $\Delta$ 2b in the systemic infection was efficiently rescued in *dcl2 dcl4* double mutant plants that are completely defective in the synthesis of both the 21- and 22-nucleotide viRNAs. These results indicate that the inhibitory effect of 2b on the accumulation of viRNAs, the 21- and 22-nucleotide classes in particular, plays a key role in both the suppression of antiviral silencing and the facilitation of systemic virus movement by 2b in infected plants. Recent studies have shown that *DCL4*-dependent, 21-nucleotide siRNAs and *DCL3*-dependent, 24-nucleotide siRNAs mediate non-cell-autonomous silencing and RdDM, respectively (Matzke and Birchler, 2005; Voinnet, 2005). Thus, the new activity of 2b in inhibiting the accumulation of three classes of siRNAs is consistent with previous observations that CMV 2b blocks both RdDM and the intercellular spread of RNA silencing in transgene-silencing models (Guo and Ding, 2002).

Analysis of both viral siRNA biogenesis and antiviral function showed that *dcl4-1* is a significantly weaker allele than *dcl4-2* in the production of the 21-nucleotide viRNAs, as found previously for the production of the 21-nucleotide *trans*-acting siRNAs (Gascioli et al., 2005; Xie et al., 2005; Yoshikawa et al., 2005). This is best illustrated by detection of abundant accumulation of 21-nucleotide siRNAs and potent antiviral silencing in *dcl2-4 dcl4-1* plants but not in *dcl2-1 dcl4-2* plants (Figure 4). Both *dcl4-1* and *dcl4-2* alleles contain a T-DNA inserted in the *DCL4* gene. T-DNA insertion in *dcl4-2* results in production of a chimeric mRNA with the sequence encoding the second dsRNA binding motif of *DCL4* replaced with a 192-nucleotide segment of T-DNA-derived sequence (Xie et al., 2005). By contrast, *dcl4-1* line contains a T-DNA inserted in an intron (Gascioli et al., 2005), which may not completely eliminate expression of wild-type *DCL4* protein.

We found that the incomplete loss-of-function allele of *DCL4* exhibits two interesting properties. First, like wild-type *DCL4* (Deleris et al., 2006), the weak *dcl4-1* allele remained dominant over *DCL2* because only very low levels of the 22-nucleotide viRNAs from wild-type *DCL2* were detected in the *dcl4-1* genetic background (Figures 4B and 4D, lanes 13 and 14). Second, the loss-of-function mutation in *DCL3* associated with the *dcl3-1* allele was severely inhibitory to both the production of the 21-nucleotide viRNAs and effective antiviral silencing mediated by the *dcl4-1* allele (Figure 4). Thus, our results confirm an antiviral role of *DCL3* against CMV suggested by previous studies (Bouche et al., 2006; Fusaro et al., 2006) and genetically place *DCL3* upstream of *DCL4* to enhance both the production of the 21-nucleotide viRNAs and antiviral silencing. This unique antiviral

activity of *DCL3*, readily detectable only in the *dcl4-1* background, may become critical in wild-type plants when *DCL4* function is compromised due to interference by physiological, environmental, or viral factors such as p38 of TCV (Deleris et al., 2006). It is possible that *DCL3* or the 24-nucleotide siRNAs produced by *DCL3* is involved in the perception of the non-cell-autonomous silencing signal, thereby potentiating subsequent antiviral silencing by *DCL4*, as proposed previously for RDR6 (Schwach et al., 2005).

Most of the RNAi pathway genes isolated from *Arabidopsis*, such as *RDR6*, *SDE3*, *SGS3*, and *AGO1*, are required for silencing induced by sense RNA transgenes but not by inverted repeat RNA transgenes, indicating a role for these genes in an RDR-dependent de novo synthesis of dsRNA (Dalmay et al., 2000; Mourrain et al., 2000; Beclin et al., 2002). Such de novo dsRNA synthesis mediated by the host RDR pathway may play a unique role in antiviral silencing against CMV, because *rd6*, *sde3*, *sgs3*, and *ago1* mutants exhibit enhanced disease susceptibility to CMV but not to other (+)-strand RNA viruses examined, such as TCV, *Tobacco rattle virus* (TRV), *Turnip mosaic virus*, and a tobamovirus (Voinnet, 2005; Vaucheret, 2006). Recent studies showed that loss-of-function mutations in *RDR1*, *RDR2*, or *RDR6* have no detectable impact on the production of viRNAs in *Arabidopsis* plants infected with TRV, TCV, and a tobamovirus (Blevins et al., 2006; Deleris et al., 2006). In this study, we also found no difference in the accumulation of viRNAs between the wild type and the three *rd6* mutants infected with wild-type CMV. However, the accumulation of viRNAs was markedly reduced in *rd1-1* plants compared with wild-type, *rd2-1*, and *rd6* plants following infection with CMV- $\Delta$ 2b. Thus, our results indicate that without interference of the 2b protein, most of the CMV siRNAs are RDR1 dependent, providing molecular evidence demonstrating a role for a host RDR in the biogenesis of virus-derived siRNAs. Recognition of CMV by the host RDR pathway is consistent with previous observations that *Arabidopsis* mutants defective for the host RDR pathway are hypersusceptible to CMV (Dalmay et al., 2000; Mourrain et al., 2000; Voinnet, 2005). It remained to be determined whether other RDRs, RDR6 in particular, contribute to the biogenesis of the viRNAs detected in *rd1-1* plants infected with CMV- $\Delta$ 2b and whether genetic rescue of CMV- $\Delta$ 2b infection occurs after the inactivation of additional RDRs.

Based on these findings, we propose that CMV infection results in the synthesis of CMV-specific dsRNA not only by the viral RdRP but also by the host RDR, leading to the production of primary and secondary viRNAs. RDR1 plays a key role in the production of viRNAs, probably because it is inducible by SA (Yu et al., 2003) and pathogen infection triggers SA production. It is possible that one or more of those genes, such as *SGS3* (Mourrain et al., 2000), *AGO1* (Morel et al., 2002), or *SDE3* (Dalmay et al., 2001), essential for RDR-dependent silencing amplification, also contributes to the biogenesis of CMV secondary siRNAs. These RDR-dependent viral secondary siRNAs may act cell-autonomously to destroy viral genomic and subgenomic RNAs in the infected cells where these siRNAs are made. More importantly, they may have potential to act non-cell-autonomously, as shown for the transgene-derived secondary siRNAs (Dunoyer and Voinnet, 2005), to spread outside of the

vasculature where CMV is unloaded to initiate replication in the upper systemically infected leaves (Havelda et al., 2003; Deleris et al., 2006). Application of exogenous SA enhances virus resistance possibly by amplifying the antiviral role of RDR1 and any other components in the antiviral silencing pathway that respond to SA (Ji and Ding, 2001; Yu et al., 2003).

We further propose that 2b is able to inhibit the RDR-dependent production of secondary CMV siRNAs, which are inducible by SA and have potential to act non-cell-autonomously. This hypothesis is based on our findings that in infected *Arabidopsis*, expression of 2b was associated with drastically reduced accumulation of CMV siRNAs (Figure 1A), most of the CMV siRNAs produced without 2b interference were RDR1 dependent (Figure 5B), and the RDR1-dependent production of CMV siRNAs was undetectable in the presence of 2b (Figure 5B). Our hypothesis also is consistent with a previous finding that 2b does not inhibit the accumulation of primary siRNAs derived from an inverted repeat RNA (Qi et al., 2004) and explains why 2b is a potent inhibitor of both non-cell-autonomous silencing and the SA-mediated virus resistance (Ji and Ding, 2001; Guo and Ding, 2002). Two possible biochemical mechanisms could explain how 2b inhibits the RDR-dependent production of secondary CMV siRNAs. First, the direct interaction between 2b and AGO1 observed recently was shown to inhibit the slicing activity of AGO1 (Zhang et al., 2006), which explains why 2b is able to suppress RNA silencing initiated by siRNAs (Qi et al., 2004) and why high levels of viRNAs inhibit the systemic infection of CMV- $\Delta$ 2b but not of CMV (Figure 2). Such a direct interaction may also inhibit the biogenesis of secondary siRNAs, because AGO1 is essential for RDR-dependent silencing amplification. Second, 2b may directly inhibit the production of secondary CMV siRNAs by binding to siRNAs and its dsRNA precursor, as shown for B2 of *Flock house virus* (Lu et al., 2005; Chao et al., 2005). The 2b protein encoded by *Tomato aspermy virus*, which belongs to the same *Cucumovirus* genus as CMV, indeed binds to dsRNA and siRNA, and the crystal structure of 2b in complex with siRNA was solved recently (J.B. Ma, F. Li, D.J. Patel, and S.W. Ding, unpublished data).

We found that CMV infection resulted in higher accumulation levels and induced more severe disease symptoms in both *dcl2-1 dcl4-2* and *dcl2-1 dcl3-1 dcl4-2* plants than in wild-type plants (Figures 2A and 3B), as reported recently for TCV, TRV, and other more virulent CMV isolates (Bouche et al., 2006; Deleris et al., 2006; Fusaro et al., 2006). However, CMV- $\Delta$ 2b induced similar EDS phenotypes in these *Arabidopsis* mutants that in fact occurred at least 1 week earlier than after CMV infection (Figures 2A and 3B). Therefore, our results show that, first, 2b was dispensable for eliciting disease symptoms. This finding argues against a direct role for VSRs in virus virulence (Mallory et al., 2002; Kasschau et al., 2003; Chen et al., 2004; Dunoyer et al., 2004; Mlotshwa et al., 2005; Zhang et al., 2006; Lewsey et al., 2007) but supports the hypothesis that symptom expression is complex and may result from interference of host physiological and developmental processes by virus replication and infection (Matthews, 1991; Poethig et al., 2006). Second, 2b exhibited a novel activity to repress disease symptom development at the early stages of infection. It is not clear at present whether 2b represses disease development in these mutant plants by a mechanism similar to or distinct from

silencing suppression. Nevertheless, if suppression of early disease development also occurs in CMV infection of wild-type plants, this new activity of 2b should facilitate virus propagation and dissemination, consistent with the notion that requisition of VSR genes represents an evolutionary adaptation of viruses to the host RNAi-mediated immunity (Li and Ding, 2006).

## METHODS

### Plant Materials

Mutant lines for *Arabidopsis thaliana* *rdr1-1*, *rdr2-1*, *rdr6* (*sgs2-1*), *dcl2-1*, *dcl3-1*, *dcl4-2*, *dcl2-1 dcl3-1*, *dcl2-4 dcl3-1*, *dcl2-1 dcl4-2*, *dc2-4 dcl4-1*, *dcl3-1 dcl4-2*, *dcl3-1 dcl4-1*, *dcl2-1 dcl3-1 dcl4-2*, and *dcl2-4 dcl3-1 dcl4-1* were described previously (Mourrain et al., 2000; Xie et al., 2004, 2005; Gascioli et al., 2005; Henderson et al., 2006). To ensure synchronous germination and development of *Arabidopsis*, seeds were first vernalized at 4°C in the dark for 5 d prior to transferring to a growth room for germination. Plants were maintained at 24°C and 10-h-light/14-h-dark cycles.

### Viruses and Infection Assays

The wild-type Q strain of CMV and the two 2b-deletion mutants used in this study were described previously (Ding et al., 1995b; Li et al., 2002). Virions were propagated in *Nicotiana glutinosa* plants and purified essentially by the method of (Peden and Symons, 1973). Wild-type and mutant *Arabidopsis* plants (~4 weeks old) were mock-inoculated or inoculated with CMV or CMV- $\Delta$ 2b at a concentration of 20  $\mu$ g/mL. Inoculated leaves were harvested at 5 DAI, and upper systemically infected leaves were harvested at both 5 and 14 DAI from pools of 16 to 24 plants.

### RNA Gel Blot Analyses

RNA gel blot analyses of low and high molecular weight RNAs were performed with 10 and 5  $\mu$ g of total RNA, respectively, as described previously (Guo and Ding, 2002). Small RNAs were separated by electrophoresis on 16% polyacrylamide gels and blotted on membranes. The blot hybridization was performed at 38°C for 12 to 16 h in PerfectHyb Plus buffer (Sigma-Aldrich), and the probes used were DNA oligonucleotides end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP by T4 polynucleotide kinase (New England Biolabs) and purified through MicroSpin G-25 columns (Amersham) according to the manufacturers' recommendations. The blot was washed once with 2 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.2% SDS for 30 min and twice with 0.2 $\times$  SSC and 0.1% SDS for 20 min at 48°C. For repeated hybridization, the membrane was stripped twice with 0.5% SDS and 20 mM EDTA for 30 min at 80°C. For detection of CMV siRNAs, a mixture of seven DNA oligonucleotides corresponding to the (+)-strand of CMV RNA3 (nucleotides 1 to 40, 241 to 280, 741 to 780, 1041 to 1080, 1341 to 1380, 1641 to 1680, and 2131 to 2170) was used. The same procedure was employed to detect U6 and miR173. High molecular weight RNA gel blots were probed with radiolabeled DNAs corresponding to the 3' terminal 340 nucleotides of CMV RNA 2 by random priming reactions (Promega) in the presence of [ $\alpha$ -<sup>32</sup>P]dCTP.

### Supplemental Data

The following material is available in the online version of this article.

**Supplemental Figure 1.** Genetic Requirements for DCLs in the Production of CMV siRNAs.

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