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Temperature-Dependent Survival of *Turnip Crinkle Virus*-Infected *Arabidopsis* Plants Relies on an RNA Silencing-Based Defense That Requires DCL2, AGO2, and HEN1

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While RNA silencing is a potent antiviral defense in plants, well-adapted plant viruses are known to encode suppressors of RNA silencing (VSR) that can neutralize the effectiveness of RNA silencing. As a result, most plant genes involved in antiviral silencing were identified by using debilitated viruses lacking silencing suppression capabilities. Therefore, it remains to be resolved whether RNA silencing plays a significant part in defending plants against wild-type viruses. We report here that, at a higher plant growth temperature (26°C) that permits rigorous replication of *Turnip crinkle virus* (TCV) in *Arabidopsis*, plants containing loss-of-function mutations within the *Dicer-like 2* (*DCL2*), *Argonaute 2* (*AGO2*), and *HEN1* RNA methyltransferase genes died of TCV infection, whereas the wild-type Col-0 plants survived to produce viable seeds. To account for the critical role of DCL2 in ensuring the survival of wild-type plants, we established that higher temperature upregulates the activity of DCL2 to produce viral 22-nucleotide (nt) small interfering RNAs (vsRNAs). We further demonstrated that DCL2-produced 22-nt vsRNAs were fully capable of silencing target genes, but that this activity was suppressed by the TCV VSR. Finally, we provide additional evidence supporting the notion that TCV VSR suppresses RNA silencing through directly interacting with AGO2. Together, these results have revealed a specialized RNA silencing pathway involving DCL2, AGO2, and HEN1 that provides the host plants with a competitive edge against adapted viruses under environmental conditions that facilitates robust virus reproduction.

The plant RNA silencing-based defense enlists a complex set of proteins to combat intracellular parasites including viruses, retrotransposons, and other highly repetitive genome elements (11). This defense cascade is commonly triggered by the intracellular occurrence of double-stranded RNA (dsRNA) or partially double-stranded stem-loop RNA, which are processed by Dicer-like (DCL) nucleases into small RNAs of discrete sizes (21 to 25 nucleotides [nt]) referred to as small interfering RNAs (siRNAs). A characteristic feature of RNA silencing is that siRNAs are not the end product of the cascade. Rather, they become the sequence specificity determinant of RNA-induced silencing complexes (RISC), directing Argonaute (AGO) proteins in the complexes to cellular RNA or DNA complementary to the siRNAs produced, silencing corresponding genes or genetic elements through targeted cleavage, translational repression, or DNA methylation (4, 7).

The model plant *Arabidopsis* encodes four DCLs and 10 AGOs, which participate in multiple RNA silencing pathways with distinct, yet partially overlapping defense roles. To add to this complexity, at least two additional protein families are essential players of the RNA silencing process. One of them is the dsRNA-binding (DRB) protein family with five known members. Two of the DRBs, HYL1 and DRB4, have been found to be required for the proper function of DCL1 and DCL4, respectively (12, 14). The other family is the RNA-dependent RNA polymerase (RDR) family, which contain six RDRs, three of them (RDR1, -2, and -6) shown to be critical for a number of RNA silencing pathways (22, 30, 31). Other plant proteins, among them RNA methyltransferase HEN1, nuclear DNA-dependent RNA polymerase IV and V (NRPD and NRPE), and exonuclease XRN4, have also been implicated in siRNA-mediated silencing processes (17, 20, 27, 35).

Plant viruses, especially those with RNA genomes, are primary targets of RNA silencing since they produce dsRNA replication intermediates during the multiplication process. To counteract RNA silencing-based defenses, well-adapted plant viruses have generally evolved the capacity to encode viral suppressors of RNA silencing (VSRs) (3). VSRs neutralize the action of RNA silencing, making its defense role against wild-type viruses difficult to discern. Consequently, attenuated viruses with mutated VSRs had to be used to identify most of the plant factors involved in antiviral silencing, including DCL2, DCL4, AGO1, AGO2, DRB4, RDR1, RDR6, and HEN1 (9, 10, 15, 25, 30, 31). Indeed, only DCL2 and AGO2 have been implicated in the frequently transient defenses against wild-type viruses (16, 18, 32). Thus, it is unclear to which extent RNA silencing provides meaningful protection in plants infected by adapted or wild-type viruses.

Turnip crinkle virus (TCV) is a small virus with a single-stranded, positive sense RNA genome that counts *Arabidopsis* as one of its adapted hosts (21). TCV encodes a strong VSR, which is also the viral capsid protein (CP), that functions to inhibit the dsRNA-processing activities of both DCL3 and DCL4 of *Arabidopsis* (1, 9, 23). As a result, DCL2-produced 22-nt viral siRNAs (vsRNAs) are the predominant vsRNA class in plants infected with wild-type TCV (9, 25, 32). Furthermore, despite of the presence of 22-nt vsRNAs, neither DCL2, DCL3, nor DCL4, contrib-

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utes discernibly to the containment of wild-type TCV under conditions commonly used to grow *Arabidopsis* (5, 9, 25, 32). Nevertheless, each of these three DCLs was found to contribute to the clearance of mutant TCV RNAs encoding defective VSRs, with DCL4 as the primary dicer (5, 9, 25). Together, these earlier studies suggest that the anti-TCV silencing of these three DCLs is almost completely suppressed by the TCV-coded VSR. More recently, it was proposed that TCV CP suppresses RNA silencing by binding to and interfering with the function of AGO1, a key component of the antiviral RISC (1). Inactivation of AGO1 then additionally causes the loss of DCL3 and DCL4 activities through a complex feedback loop (1).

In this report, we attempted to determine whether RNA silencing plays a significant defense role against wild-type, adapted viruses. To this end, we have subjected *Arabidopsis* plants with defects in silencing pathway components to infections with wild-type TCV and observed their responses under different environmental conditions. Our results revealed an alternative RNA silencing pathway that functions to ensure the survival of virus-infected plants under conditions that favor rigorous virus multiplications. This finding suggests that, in addition to its prominent role in clearing viruses encoding nonadapted VSRs (18), RNA silencing can be crucial in establishing a balanced state in virus-infected plants to allow for the reproduction of both plants and viruses.

MATERIALS AND METHODS

Constructs. The pTCV (previously known as T1d1) construct has been described (23). The CPB and CPC constructs have been generated in a previous study (5). CPB was further modified by changing the AT dinucleotide at nucleotides (nt) 3807 to 3808 of TCV genome to CC, creating CPB-CC with a new KpnI site immediately downstream of TCV CP coding region. A 90-nt fragment derived from *Arabidopsis* phytoene desaturase gene (PDS) was then inserted into CPB-CC to produce CPB-CC-PDS. Similar changes were also incorporated into pTCV to create TCV-CC and TCV-CC-PDS, respectively (see Fig. 3A for diagrams of these constructs).

For bimolecular fluorescence complementation (BiFC) experiments, a set of four vectors assembled by J. Lindbo were used to accommodate cDNAs of various viral and plant proteins (33). These vectors are referred to as YN1, YC1, 2YN, and 2YC, respectively, with YN and YC standing for the N-terminal 159 and C-terminal 81 amino acid (aa) residues of the yellow fluorescent protein (YFP). In addition, a T7 and a hemagglutinin (HA) epitope tag (denoted as 1 and 2, respectively) were included in the vectors to facilitate the detection of the fusion proteins. The T7 and HA epitope tags are always sandwiched by YFP fragments and cDNAs of genes of interest, so that YN1 and YC1 are always at the N-terminal side of the cDNAs to be examined, whereas 2YN and 2YC are always at the C-terminal ends. Together, these four vectors permit the in-frame fusion of genes-of-interest in all four possible configurations. Additional details of these vectors are available upon request.

Plant materials. The sources of Col-0, *dcl* mutant plants, *drb4*, *ago1-11*, *ago7*, and *rdr6* mutants have been described previously (25). The *hyl1-2* and *hen1-1* mutants and double and triple mutants of *rdr1*, *rdr2*, and *rdr6* were kindly provided by J. C. Carrington. The *ago1-27* and *ago2* mutants, as well as *ago1-27 ago2-1* double mutants, were kind gifts from Shou-wei Ding. The *xrn4-5* and *sgs3-12* mutants were generously provided by Pam Green and Scott Poethig, respectively. The *drb2*, *drb3*, and *drb5* mutants were obtained from *Arabidopsis* Biological Resource Center. Uninfected *Arabidopsis* plants were reared in a growth room set at 20°C, with 14 h of daylight. After inoculation with *in vitro* transcripts of various TCV derivatives, they were divided into two equal sets and moved into two identical Adaptis A1000 growth chambers (Conviron, Winnipeg, Manitoba, Canada), with temperatures set at 18 and 26°C, respectively, to reveal temperature-dependent symptoms. Leaves samples were collected at 4 days postinoculation (dpi) for inoculated leaves (IL) and at 6 to 7 dpi for upper young leaves (UL) for analysis. Leaves from six different plants, one leaf per plant, were pooled before RNA or protein extractions to minimize the sampling errors.

BiFC assays were carried out by transforming the assembled constructs into agrobacteria (strain C58C1), which were then used to infiltrate leaves of *N. benthamiana* plants. The infiltrated leaves were examined using epifluorescence microscopy and laser scanning confocal microscopy at 24 to 28 h after infiltration.

RNA blot analysis. Total RNAs were extracted from infected plants and subjected to RNA blot analysis to detect TCV viral RNAs, or vsRNAs using protocols outlined in previous reports (23, 25). Different amounts of *Arabidopsis* RNA were used to detect viral RNA (1 μg) and vsRNAs (5 μg). The probes were a mixture of five different oligonucleotides end labeled with ^{32}P.

dsRNA enrichment. Total RNA samples were mixed with 1/2 volumes of a 7.5 M lithium chloride (LiCl) solution to achieve a final concentration of 2.5 M LiCl. The mixtures were kept on ice for at least 2 h to precipitate single-stranded RNAs longer than 200 nt. After a centrifugation of 14,000 rpm at 4°C for 30 min, the supernatants were transferred to a fresh tube and mixed with 2.5 volumes of 100% ethanol to precipitate the dsRNA and smaller RNAs (tRNAs, 5S rRNA, etc.). The pellets were then dissolved in a small volume (approximately 1/10 of the starting RNA sample) of water. The concentration of RNAs (dsRNA plus smaller ssRNA) was determined with NanoDrop. An equal amount was withdrawn from each sample and analyzed with a 0.5× TBE (Tris-borate-EDTA) and 1.2% agarose gel.

RESULTS

Higher temperatures facilitate more rigorous replication of wild-type (wt) TCV in *Arabidopsis*. Previous studies suggested that virus-infected plants generally are less symptomatic at higher temperature due to enhanced RNA silencing-mediated antiviral defense (6, 24, 28, 29). To determine the underlying mechanism for this phenomenon, we decided to use the well-characterized TCV-*Arabidopsis* model system to gauge how differences in temperature affect the accumulation levels of TCV genomic RNA (gRNA), subgenomic RNA (sgRNA), and viral siRNA (vsRNA). The *Arabidopsis* Col-0 plants, together with *dcl2*, *dcl3*, and *dcl4* knockout mutants, as well as *dcl2 dcl3* double knockouts, were infected with *in vitro*-transcribed infectious RNA of TCV and kept at 18 and 26°C after inoculation. Inoculated leaves (IL) and upper uninoculated leaves (UL) were collected from the plants at 4 and 7 dpi, respectively, and subjected to RNA extraction and Northern blot hybridization with TCV-specific probes.

As shown in Fig. 1A, TCV viral RNA levels were similar in the five types of infected plants when they were kept at the same temperature (18 or 26°C), indicating that the antiviral activity of these three DCLs were effectively suppressed by TCV CP, the TCV-encoded VSR (9, 25). However, when plants grown at the two different temperatures were compared, TCV gRNA accumulation at 26°C was noticeably higher than at 18°C, in both IL and UL (Fig. 1A, top panel, compare lanes 1 to 5 to lanes 6 to 10 [IL data not shown]). Therefore, in contrast to other examples of virus-host pairs reported earlier (6, 24, 28), our new results indicate that higher temperature stimulates the multiplication of TCV.

The activity of DCL2 is likewise upregulated by a higher temperature. We and others reported earlier that TCV-infected *Arabidopsis* plants contained large amounts of virus-specific 22-nt vsRNAs, suggesting that DCL2, which generates 22-nt vsRNAs, is

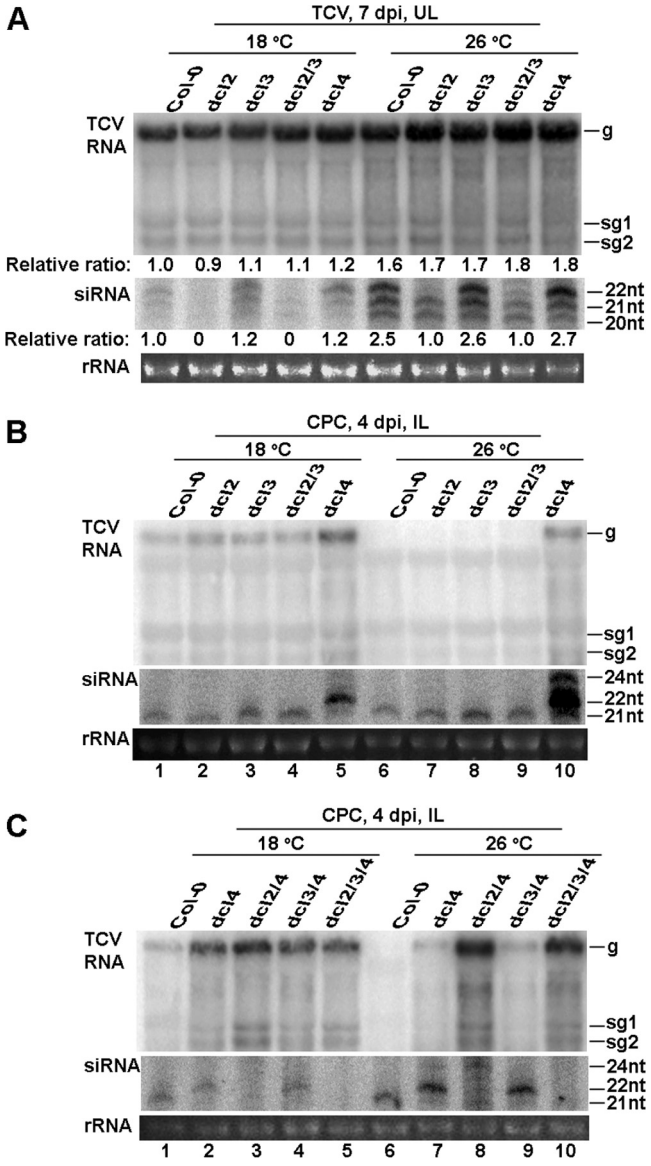

FIG 1 DCL2 targets wild-type TCV in a temperature-dependent manner. (A) Detection of TCV gRNA and sgRNAs, as well as vsRNAs, by Northern blot hybridizations in samples of Col-0, *dcl2*, *dcl3*, *dcl2 dcl3*, and *dcl4* plants kept at two different temperatures as indicated above the panels. The relative accumulation levels of TCV gRNA and 22-nt vsRNA were estimated by averaging the relative signal strength of each treatment in three independent blots, with the value of TCV-infected Col-0 plants at 18°C set as 1. (B) Detection of CPC gRNA, sgRNAs, and vsRNAs by Northern blot hybridizations in the inoculated leaves of the same set of plants kept at two different temperatures as indicated above the panels. (C) Detection of CPC gRNA, sgRNAs, and vsRNAs by Northern blot hybridizations in the inoculated leaves of Col-0, *dcl4*, *dcl2 dcl4*, *dcl3 dcl4*, and *dcl2 dcl3 dcl4* plants kept at two different temperatures as indicated above the panels. (D) RNA samples shown in panel A were enriched for dsRNA and run on a native agarose gel to reveal the accumulation levels of TCV-specific dsRNA. The size marker (M) on the left is a DNA ladder.

able to use some form of TCV-specific dsRNA to produce vsRNAs (9, 25, 32). To determine whether biogenesis of 22-nt vsRNAs by DCL2 was influenced by temperature, we then examined the levels of TCV vsRNAs in the infected plants. As shown on Fig. 1A, middle panel, levels of TCV vsRNAs, especially the 22-nt vsRNAs, were indeed elevated at higher temperature in plants with functional *DCL2* (lanes 1, 3, and 5 versus lanes 6, 8, and 10). Furthermore, it appeared that the temperature-dependent elevation of 22-nt vsRNA levels was more pronounced than that of viral gRNA. This observation was confirmed by quantitatively analyzing the intensities of TCV gRNA signals, as well as 22-nt vsRNAs, on three sets of blots bearing samples from independent experiments: while viral gRNA increased an average of 1.5-fold, vsRNAs increased ~2.5-fold. These data suggested to us that even though higher TCV gRNA levels could have provided more substrate for 22-nt vsRNA production, they could not have been responsible for all of the temperature-dependent changes in 22-nt vsRNAs. Thus, the activity of DCL2 is probably also stimulated by a higher temperature.

To further assess the temperature dependence of DCL2 activity independent of increased viral RNA levels, we then subjected the same set of plants to CPC infection. CPC is a TCV mutant in which the VSR function of CP is completely eliminated by a point mutation within the CP coding sequence (5). Consequently, CPC replication is restricted in the IL of wt *Arabidopsis* as viral RNAs are quickly cleared by RNA silencing. In contrast with wt TCV, CPC RNA accumulated to lower levels when the temperature was high, regardless of the genetic backgrounds of the plants examined (Fig. 1B, top panel). This result hinted that RNA silencing-based antiviral defense is indeed more active at higher temperature when the virus-encoded silencing suppressor was absent. Nevertheless, disruption of DCL4 function allowed for slightly higher CPC RNA accumulation at both temperatures (Fig. 1B, top panel, lanes 5 and 10), indicating that DCL4 contributed to CPC RNA clearance. Notably, the accumulation of 22-nt vsRNAs, which was the dominant vsRNA species in *dcl4* mutant plants, was significantly enhanced at higher temperature despite of the lower levels of CPC viral RNA (Fig. 1B, top and middle panels, lanes 5 and 10). This observation is highly consistent with the conclusion that the 22-nt vsRNA biogenesis by DCL2 is upregulated by higher plant growth temperature.

We then attempted to confirm this conclusion by subjecting other double and triple *dcl* mutants (*dcl2 dcl4*, *dcl3 dcl4*, and *dcl2 dcl3 dcl4*) to CPC infections. The results presented in Fig. 1C demonstrated that *dcl3 dcl4* plants shared similar viral RNA and vsRNA accumulation patterns with *dcl4*, namely, lower viral RNA levels coupled with higher 22-nt vsRNA levels at higher temperature (lanes 2 and 4 and lanes 7 and 9), further supporting a primary role of DCL2 in enhancing antiviral silencing in response to elevated temperature.

Results presented in Fig. 1C also corroborated the stimulative effect of higher temperature on TCV RNA replication with the CPC mutant, even though this mutant lacked the capability to suppress RNA silencing (5). CPC replicated to similar levels in all mutants containing the *dcl4* lesion at 18°C (Fig. 1C, top panel, lanes 2 to 5). In contrast, disrupting both *DCL2* and *DCL4* (in both *dcl2 dcl4* and *dcl2 dcl3 dcl4* mutants) allowed it to replicate to higher levels at 26°C than at 18°C (Fig. 1C, top panel, compare lanes 3 and 5 to lanes 8 and 10). These results indicate that in the absence of active antiviral silencing, replication of TCV mutants devoid of silencing suppressors was likewise stimulated by a higher temperature.

Functional DCL2 is indispensable for the survival of TCV-infected plants at a higher temperature. The results presented above established that a higher plant growth temperature upregu-

lated the activity of DCL2 but also the replication of TCV RNA. As a result, viral RNA levels were higher at 26°C than at 18°C. Thus, it was unclear whether the elevated DCL2 activity at higher temperature provided any protection to TCV-infected plants. To address this question, we decided to monitor the infected plants for signs of differences in symptoms. While at 18°C all infected plants showed similar symptoms (Fig. 2A, top), at 26°C *dcl2* or double/triple *dcl* mutants containing the *dcl2* allele were drastically more severe, leading to death of infected plants within 2 weeks (Fig. 2A, bottom, and data not shown). Notably, other mutants, including *dcl4*, were indistinguishable to Col-0, suggesting that DCL4 played a more limited role under this condition. This is likely due to the effective suppression of DCL4 function by the strong VSR encoded by TCV (9, 23, 25). In conclusion, the temperature-dependent antiviral defense conferred by DCL2 is primarily responsible for the survival of TCV-infected *Arabidopsis* plants at a higher temperature, which correlates with higher TCV RNA accumulations.

AGO2 and HEN1 participate in the DCL2-mediated antiviral defense to ensure the survival of TCV-infected plants at 26°C. Having shown that disruption of *DCL2* gene resulted in the death of TCV-infected plants at higher temperature (26°C), we then sought to determine whether disruption of other *Arabidopsis* genes of the RNA silencing machinery would have similar effects. To this end, we subjected a large array of mutant plants to similar treatments. These included a number of *ago* mutants (*ago1-11*, *ago1-27*, *ago2-1*, *ago1-27 ago2-1*, *ago3-1*, *ago4-1*, *ago5-1*, *ago7-1*, *ago10-4*, *drb* mutants (*hyl1-2*, *drb2-1*, *drb3-1*, *drb4-1*, and *drb5-1*) and *rdr* mutants (*rdr1*, *rdr2*, and *rdr6*), as well as *hen1-1*, *xrn4-5*, and *sgs3-12* mutants. At 18°C, none of the TCV-infected *ago* mutant plants were significantly different from their wild-type control plants judging from both symptoms, and accumulation levels of viral RNA and vsRNAs (data not shown). At 26°C however, TCV infections led to the death of both *ago2-1* single knockout and the *ago1-27 ago2-1* double mutant within 2 weeks of TCV inoculations (Fig. 2B). All other *ago* mutants, including *ago1-11* and *ago1-27*, survived the infection at this temperature (data not shown). The fact that knocking out AGO2 alone was enough to thwart DCL2-dependent survival of TCV-infected plant at higher temperature strongly suggests that this survival requires AGO2-based RISC, programmed by DCL2-produced 22-nt vsRNAs.

Among other mutants, *hen1-1* also showed more pronounced symptoms at 26°C, leading to the death of TCV-infected plants within 2 weeks (Fig. 2C). However, the antiviral role of HEN1 appears to be less temperature dependent, since TCV-infected *hen1-1* plants kept at 18°C also succumbed to death after a longer time period (4 weeks). Interestingly, TCV RNA actually accumulated to lower levels in *hen1-1* mutants under both temperature conditions (Fig. 2D, top panel). This could be due to the pleiotropic effects of the *hen1-1* mutation, which may interfere with the replication of viral RNA. Nevertheless, the reduction in vsRNA levels in *hen1-1* mutant was more pronounced at 26°C (Fig. 2D, lower panel), confirming the role of HEN1 in the stabilization of vsRNAs through 3′-end methylation (34, 35). Together these results identified three RNA silencing genes required for the survival of TCV-infected *Arabidopsis* at higher temperature, thus establishing an unequivocal role for RNA silencing in maintain the robustness of antiviral defense under adverse environmental conditions.

The DCL2-produced 22-nt vsRNAs are capable of silencing a complementary mRNA target. The data presented thus far suggest that DCL2 activity is elevated at 26°C, and this elevated DCL2 activity is needed for keeping TCV-infected plants alive at this temperature. The additional requirement of AGO2 in this process further supports a role of RNA silencing. However, the effective-

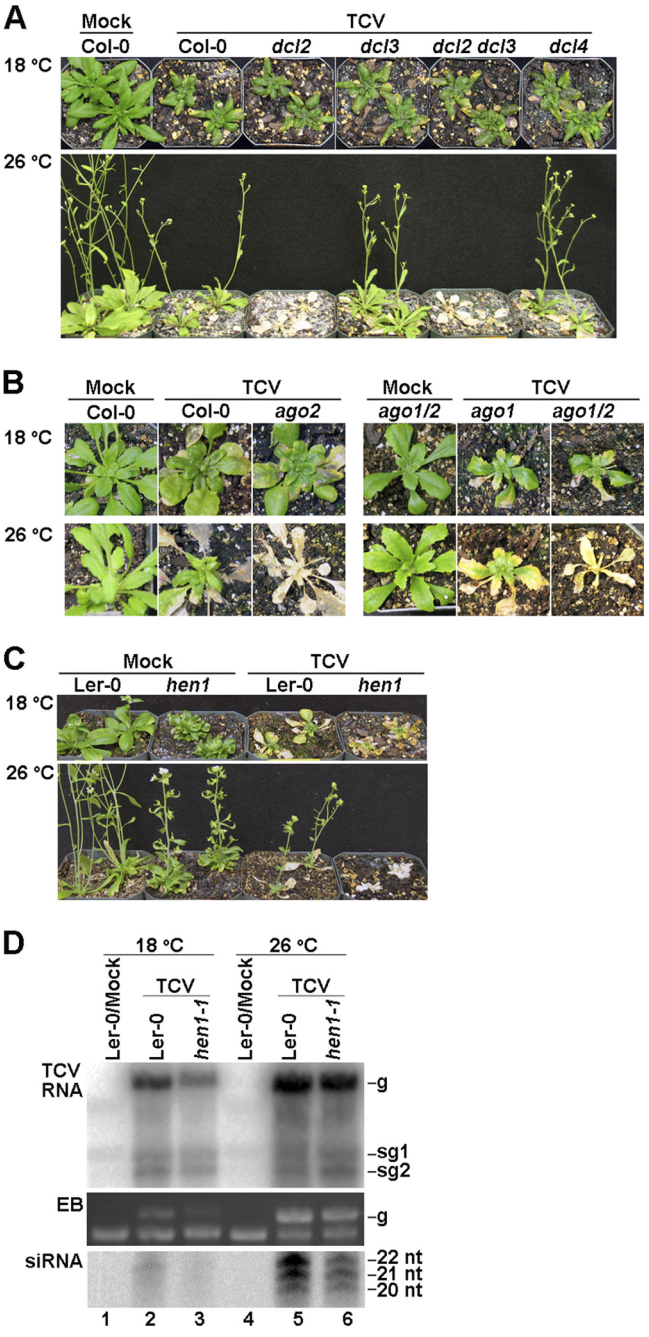

FIG 2 Temperature-dependent survival of TCV-infected *dcl2*, *ago2*, and *hen1* plants. (A). Col-0, *dcl2*, *dcl3*, *dcl2 dcl3*, and *dcl4* plants were infected with TCV and placed in two growth chambers set at different temperatures (18°C versus 26°C), and the infected plants were photographed at 28 dpi. (B) Col-0, *ago1* (1–27 allele), *ago2*, and *ago1 ago2* plants were likewise infected with TCV and subjected to different temperature conditions, except that the images were taken 1 week earlier, at 21 dpi. (C) Ler-0 (the parental ecotype of *hen1-1*) and *hen1-1* plants were subjected to the treatments described in panels A and B and photographed at 28 dpi. (D) Accumulation levels of viral RNAs and vsRNAs in Ler-0 and *hen1-1* plants at 7 dpi as detected with Northern blot hybridizations.

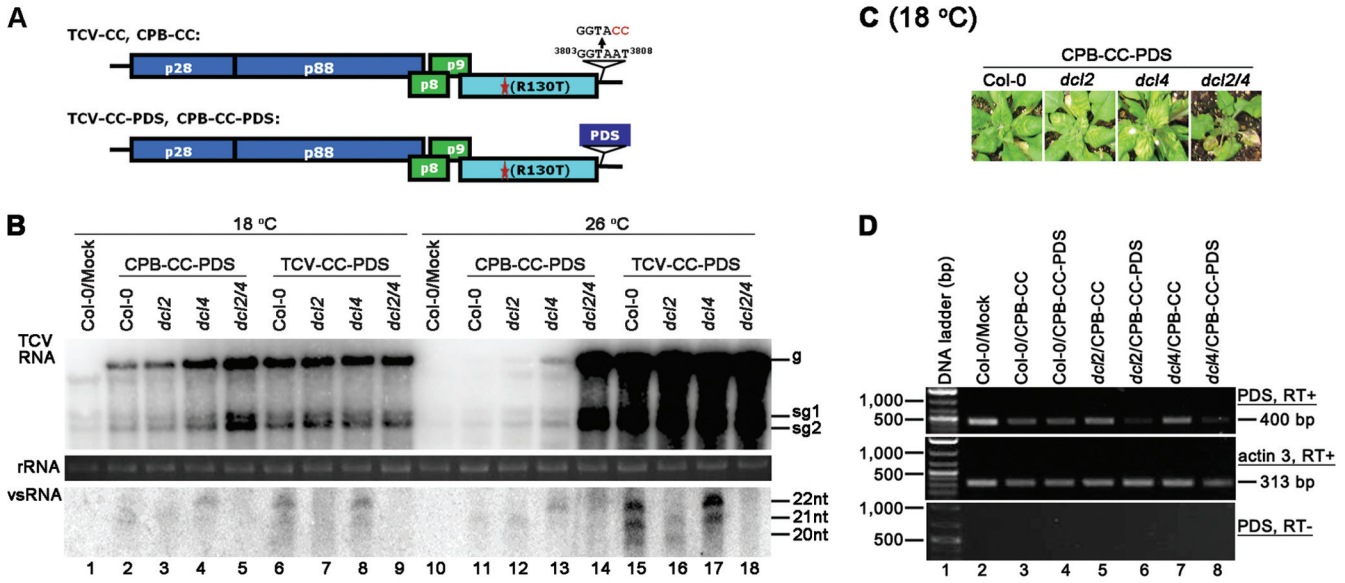

FIG 3 DCL2-produced 22-nt vsRNAs are effective inducers of target gene silencing. (A) Diagrams of TCV-CC, CPB-CC, TCV-CC-PDS, and CPB-CC-PDS constructs, with the R130T mutation of CPB denoted by a red star. The TCV-CC and CPB-CC constructs were produced by changing the AT dinucleotides to CC at nt 3807 to 3808, within the 3′UTR of TCV, resulting in a new KpnI site (GGTACC). A 90-nt PDS fragment was then inserted into the KpnI site to create TCV-CC-PDS and CPB-CC-PDS. (B) The viral RNA and vsRNA accumulation levels of CPB-CC-PDS and TCV-CC-PDS in Col-0, *dcl2*, *dcl4*, and *dcl2 dcl4* plants kept at two different temperatures as detected by Northern blot hybridization. (C) PDS silencing induced by CPB-CC-PDS in Col-0, *dcl2*, *dcl4*, and *dcl2 dcl4* plants. Images were recorded at 18 dpi. (D) Downregulation of PDS mRNA levels by CPB-CC-PDS in *dcl2* and *dcl4* plants as determined with semiquantitative RT-PCR. The samples were collected at 7 dpi, before photobleaching becoming apparent. Actin 3 mRNA was used as a control to ensure that similar amounts of RNA were used in all reactions. Omission of reverse transcriptase led to the failure to obtain the PDS PCR fragment (bottom panel), proving that the PCR products in top panel were dependent on the presence of PDS mRNA.

ness of this alternative RNA silencing pathway remained to be evaluated as plants without *dcl2* or *ago2* mutant allele still developed serious symptoms upon TCV infection. To resolve this question, we first determined whether the 22-nt vsRNAs generated by DCL2 are capable of silencing cRNA. For this purpose, we created two new constructs, TCV-CC-PDS and CPB-CC-PDS, by inserting a 90-nt fragment of the *Arabidopsis* phytoene desaturase (PDS) gene into the 3′-untranslated regions (3′UTRs) of TCV and CPB, respectively (Fig. 3A; see also Materials and Methods for details of the constructs). CPB is another mutant of TCV containing a single amino acid mutation within the CP gene. Unlike CPC, CPB retains a low level of silencing suppression activity and is capable of limited systemic movement in infected plants (5).

Both TCV-CC-PDS and CPB-CC-PDS were inoculated into Col-0, *dcl2*, *dcl4*, and *dcl2 dcl4* plants, and the infected plants were kept at 18 and 26°C. At 26°C, TCV-CC-PDS caused slightly milder symptoms than TCV but nevertheless resulted in the death of *dcl2* and *dcl2 dcl4* plants (data not shown). In contrast, CPB-CC-PDS-infected plants kept at this temperature were asymptomatic except for *dcl2 dcl4* double knockouts. RNA blot hybridization with both TCV and PDS-specific oligonucleotide probe yielded similar results, suggesting that both constructs retained the PDS inserts (Fig. 3B, lanes 10 to 18; and data not shown). As expected, CPB-CC-PDS RNA levels in Col-0, *dcl2*, and *dcl4* plants were much lower at 26°C than at 18°C, reflecting the combined effects of enhanced host RNA silencing activity and the reduced CPB VSR activity (Fig. 3B, top panel, compare lanes 2 to 4 to lanes 11 to 13). Notably, none of the infected plants kept at 26°C showed photobleaching, indicating that neither TCV-CC-PDS nor CPB-CC-PDS silenced the expression of PDS at this temperature (data not shown). This is particularly noteworthy in Col-0 and *dcl4* plants infected with TCV-CC-PDS, in which viral RNAs, as well as 22-nt, PDS-specific vsRNAs, accumulated to very high levels. These results seemed to suggest that 22-nt vsRNAs might not be competent at silencing complementary mRNA.

However, CPB-CC-PDS, but not TCV-CC-PDS, was able to induce PDS silencing in Col-0, *dcl2*, and *dcl4* plants kept at 18°C (Fig. 3C). The extent of PDS silencing as reflected by photobleaching on systemically infected leaves was weakest in Col-0 plants and strongest in *dcl4* plants (Fig. 3C). Consistent with the photobleaching phenotype, PDS mRNA levels as detected by semiquantitative reverse transcription-PCR (RT-PCR) were significantly lower in both *dcl2* and *dcl4* plants infected with CPB-CC-PDS (compared to the same plants infected with the CPB-CC virus that did not contain the PDS insert; Fig. 3D, top panel). Since *dcl2 dcl4* double mutant plants did not show any photobleaching despite the higher levels of viral RNA, we reason that photobleaching on *dcl4* plants depended on the 22-nt vsRNAs produced by DCL2. These results demonstrated that the 22-nt vsRNAs produced by DCL2 from CPB-CC-PDS-associated dsRNA were capable of silencing complementary PDS mRNA. Consequently, the inability of similar vsRNAs to silence PDS in TCV-CC-PDS-infected plants at either temperature strongly suggests that the TCV-encoded VSR (CP) blocked the function of 22-nt vsRNAs. To summarize, the experiments thus far confirmed that DCL2-produced 22-nt vsRNAs were effective at silencing complementary targets in the absence of TCV VSR, but their functionality was debilitated in plants infected with wt TCV.

The activity of *Arabidopsis* AGO2 is likely targeted by the TCV-coded silencing suppressor. We next tried to resolve how

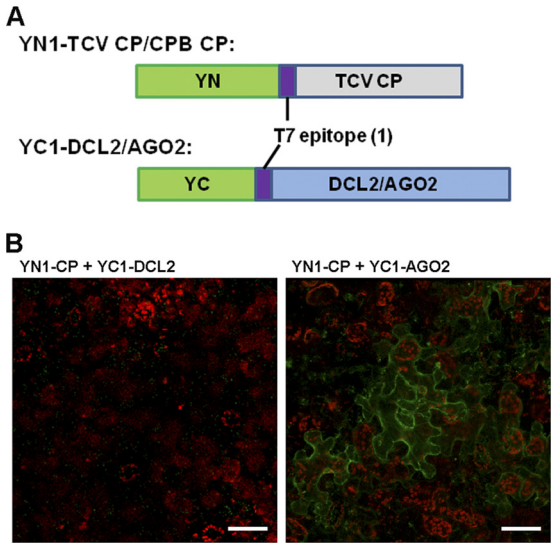

FIG 4 TCV CP and AGO2 interact to restore YFP fluorescence in a BIFC assay. (A) Illustrations of the BIFC constructs used in these experiments. (B) Laser scanning confocal images of *N. benthamiana* leaves transiently expressing the indicated constructs. Bar, 50 μm.

the TCV CP silencing suppressor interferes with the function of 22-nt vsRNAs. Since TCV CP was recently shown to suppress RNA silencing by interacting with AGO1 (1), we hypothesized that it might also interfere with the functionality of AGO2 through direct interaction. To test this possibility, we determined whether TCV CP interacted with AGO2 *in vivo* using a BiFC approach. As shown in Fig. 4, TCV CP was fused to the C terminus of YN1 consisting of the YFP N-terminus fragment (YN, 159 aa) and a T7 epitope tag to produce YN1-TCV CP. Conversely, DCL2 and AGO2 were fused to the C terminus of YC1 consisting of the C-terminal fragment of YFP (YC, 81 aa) and the T7 tag to produce YC1-DCL2 or YC1-AGO2. All constructs were fully sequenced to confirm their identities. Fusion constructs of other configurations (for example, TCV CP-2YN, in which TCV CP was fused to the N terminus of YN, separated by an HA epitope tag) were also produced but excluded from further experiments due to high levels of nonspecific fluorescence (data not shown). Coexpression of YN1-CP and YC1-DCL2 did not result in the restoration of YFP fluorescence, suggesting that CP and DCL2 did not interact directly in coinfiltrated *N. benthamiana* cells. This lack of interaction also served as the negative control of our BiFC experiments. In contrast, YFP fluorescent cells were easily identifiable in leaves coinfiltrated with YN1-CP and YC1-AGO2, indicating that CP and AGO2 interacted with each other in the coexpressed cells. As an additional control, we also subjected the CP of the CPB mutant to similar experiments and found that it no longer interacted with AGO2 (data not shown). Since the mutated amino acid in CPB (R130T) is not part of the two GW/WG motifs previously shown to mediate CP-AGO1 interaction (aa 32 to 33 and aa 289 to 290, respectively [1]), we speculate that R130 also contributes to the CP-AGO interactions, possibly through the stabilization of the complex. Together, these observations indicate that TCV CP may interact with multiple AGOs to achieve maximal silencing suppression (1, 16).

DISCUSSION

While RNA silencing plays a critical role in defending plant hosts against virus infections, well-adapted viruses have evolved to encode VSRs that obscure the action of RNA silencing. As a result, most plant genes involved in antiviral silencing were identified using debilitated viruses lacking silencing suppression capabilities (9, 15, 25, 30, 31). Therefore, it remains to be resolved whether RNA silencing plays a significant part in defending plants against adapted, wild-type viruses. In the present study, we sought to clarify this question by subjecting TCV-infected *Arabidopsis* plants to two different growth conditions that differ only in temperatures (18 versus 26°C). Although the growth pace of uninfected plants were accelerated at 26°C compared to that at 18°C, both conditions are within the range of ambient temperature that supports healthy growth of *Arabidopis*, leading to the production of large amounts of healthy seeds. To further control for the potential variations in virus susceptibility caused by temperature-dependent developmental differences, plants were transferred to the two temperature environments only after virus inoculation. Furthermore, tissues were harvested early, at 4 and 7 dpi for IL and UL analyses, respectively, thus helping to minimize the differences in plant development. Admittedly, some general temperature-dependent physiological effects are almost impossible to discount, however, through targeted mutational analyses, we were able to clearly demonstrate that the temperature-dependent phenotypes of our infected plants were directly related to the plant antiviral defense system and corresponding viral activities. In summary, the results of our experiments maximally reflected the effect of temperature on virus multiplication and plant antiviral defense.

The first notable observation is that TCV with an intact VSR actually produced higher levels of viral RNAs at 26°C than at 18°C, suggesting that TCV RNA replication is stimulated by higher temperature. This result was further corroborated by the fact that a TCV mutant lacking VSR activity (CPC) also replicated to higher levels at 26°C when the host RNA silencing machinery was disrupted (in *dcl2 dcl4* and *dcl2 dcl3 dcl4* mutants). This conclusion appears to contradict previous reports with other virus-host systems showing that virus symptoms were generally milder, and the virus titer lower, at higher plant growth temperatures (24, 26). This discrepancy could have been caused by the fact that VSRs encoded by different viruses may interfere with the RNA silencing pathway at different steps and exert different extents of suppression, allowing for a varying degree of RNA silencing activity in the host plants. Indeed, our results also showed that the host RNA silencing activity is likewise stimulated by higher temperatures, thus adding another layer of complexity in accounting for the final outcome (see below). Finally, other defense mechanisms may also affect the disease progression and host health.

Although all infected plants initially displayed much more severe symptoms at 26°C than at 18°C, wild-type plants were able to recover and produce younger leaves with fewer symptoms, allowing the plants to survive and bear seeds. In contrast, both *dcl2* plants and *ago2* and *hen1* plants kept at 26°C died of TCV infection within 2 weeks of virus inoculation. These results demonstrated for the first time that, although the role of RNA silencing in countering TCV infection at optimal *Arabidopsis* growth conditions (21 to 23°C) is difficult to discern, it becomes crucial for the survival of the host plants under conditions that favors more rigorous virus multiplications. Although our results unambiguously

implicated DCL2, AGO2, and HEN1 in this defense pathway, the participation of other silencing factors cannot be ruled out, especially when the plants are attacked by other viruses, since TCV is known to preferentially suppress the function of DCL4 and AGO1 (1, 9, 25).

We further revealed that the TCV mutants defective in silencing suppression (the CPB and CPC mutants) accumulated lower levels of viral RNA at 26°C, thus supporting the notion that RNA silencing activity is also stimulated by higher temperature. Importantly, we observed that in *dcl4* knockout mutants, the DCL2-specific 22-nt vsRNAs of CPC origin were at higher levels at 26°C despite of lower viral RNA levels. This result established that the dicing activity of DCL2 is enhanced by higher temperatures. What could account for this enhancement of DCL2 activity? It is probably not transcriptional since our extensive RT-PCR survey failed to identify any RNA silencing pathway genes that transcribed detectably more mRNA at higher temperature (X. Zhang and F. Qu, data not shown). However, temperature could also influence the translation of DCL2 proteins or the enzymatic activity of DCL2. In addition, the various alternative splice-forms of *DCL2* transcript might also play a role in the temperature dependence of DCL2 (19).

Previous studies have implicated DCL2 in the production of 22-nt vsRNAs, but it seemed to play only a minor or transient role in defending the plant hosts against various viruses (1, 2, 9, 25, 32). However, most of those observations were based on defective viruses with compromised silencing suppression capabilities. In contrast, data presented in the present report revealed a unique antiviral role of DCL2 under environmental conditions that permit rigorous virus replication. This role of DCL2 is apparently not shared by DCL4 or DCL3 since knocking out *DCL2* alone was enough to cause the death of TCV-infected plants. Similarly, AGO2 was recently found to confer antiviral defense to suppressor-less mutants of *Cucumber mosaic virus* (30) and *Tomato bushy stunt virus* (26), as well as to a nonadapted virus (*Potato virus X* in *Arabidopsis* [18]). It has also been shown to act to transiently suppress TCV-induced symptoms in *Arabidopsis* grown under commonly used conditions (16). Our study further corroborates the importance of AGO2 in antiviral defense since it is the sole AGO required to maintain the viability of TCV-infected plants under the temperature condition that allowed for rigorous viral replication.

It has been recently reported that DCL2 was the primary dicer for a specific class of 22-nt endogenous (endo) siRNAs derived from two endogenous inverted repeat loci (13). Although the accumulation levels of these endo-siRNAs were not examined here, it is possible that some of these siRNAs may function to target certain plant genes to regulate cellular responses to changing temperature and virus susceptibility. Evaluation of the potential antiviral role of the endogenous siRNAs could reveal additional new insights in the cross talk between endogenous and antiviral RNA silencing pathways.

Interestingly, we were unable to implicate any of the RDR proteins in this RNA silencing-based defense process. TCV inoculations of all of single-, double-, or triple-knockout mutants of *RDR1*, *RDR2*, and *RDR6* produced infection symptoms that were indistinguishable from wild-type plants at both temperatures (Zhang and Qu, unpublished). Viral RNA levels, as well as vsRNA levels, in these plants were likewise similar to those of wild-type plants. Furthermore, inoculations with the CPB mutant (with partial silencing suppression activity) also failed to yield discernible elevation of viral RNA levels in the *rdr* mutants. Finally, the CPC mutant (no VSR activity) failed to move systemically in the triple *rdr* knockouts, despite the fact that it was able to move systemically in *dcl2 dcl4* double knockouts (5; Zhang and Qu, unpublished). Our findings are consistent with the study by Dalmay et al. (8) showing that *rdr6* mutant plants failed to become more susceptible to TCV. Together these data suggest that unlike *Cucumber mosaic virus* and *Turnip mosaic virus* (TuMV), siRNA amplification through RDRs does not significantly contribute to the silencing-based defense against TCV (15, 22, 30, 31). These findings further suggest that viruses with different replication strategies and intracellular accumulation levels may have been subject to different coevolutionary adaptation with the RNA silencing machinery of the host plants.

In conclusion, we propose that a surrogate RNA silencing pathway comprised of at least DCL2, AGO2, and HEN1 functions to partially attenuate the stress caused by rigorously replicating TCV at higher temperature, thus preventing the death of infected *Arabidopsis* plants. Due to the presence of the strong VSR encoded by TCV, this defense process is unable to clear all viral RNAs from the infected cells. Rather, it manages to achieve a modest competitive advantage that allows for the completion of the reproductive cycle of the plants. This advantage appears to be attributable to increased DCL2 activity at higher temperature, although the activities of AGO2 and HEN1 could also be upregulated by temperature. Our observations establish yet another layer of arms race between a virus pathogen and its plant host, highlighting the relevance of maintaining an intermediate state that permits the survival of both organisms.

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REFERENCES

1. **Azevedo J, et al.** 2010. Argonaute quenching and global changes in Dicer homeostasis caused by a pathogen-encoded GW repeat protein. Genes Dev. **24**:904–915.
2. **Blevins T, et al.** 2006. Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. Nucleic Acids Res. **34**:6233–6246.
3. **Burgyán J, Havelda Z.** 2011. Viral suppressors of RNA silencing. Trends Plant Sci. **16**:265–272.
4. **Calarco JP, Martienssen RA.** 2011. Genome reprogramming and small interfering RNA in the *Arabidopsis* germline. Curr. Opin. Genet. Dev. **21**:134–139.
5. **Cao M, et al.** 2010. The Capsid protein of Turnip crinkle virus overcomes two separate defense barriers to facilitate systemic movement of the virus in *Arabidopsis*. J. Virol. **84**:7793–7802.
6. **Chellappan P, Vanitharani R, Ogbe F, Fauquet CM.** 2005. Effect of temperature on geminivirus-induced RNA silencing in plants. Plant Physiol. **138**:1828–1841.
7. **Chen X.** 2010. Small RNAs: secrets and surprises of the genome. Plant J. **61**:941–958.
8. **Dalmay T, Hamilton A, Rudd S, Angell S, Baulcombe DC.** 2000. An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. Cell **101**:543–553.

9. **Deleris A, et al.** 2006. Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. Science **313**:68–71.
10. **Diaz-Pendon JA, Li F, Li W-X, Ding S-W.** 2007. 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. Plant Cell **19**:2053–2063.
11. **Ding S-W, Voinnet O.** 2007. Antiviral immunity directed by small RNAs. Cell **130**:413–426.
12. **Dong Z, Han M-H, Fedoroff N.** 2008. The RNA-binding proteins HYL1 and SE promote accurate in vitro processing of pri-miRNA by DCL1. Proc. Natl. Acad. of Sci. U. S. A. **105**:9970–9975.
13. **Dunoyer P, et al.** 2010. An endogenous, systemic RNAi pathway in plants. EMBO J. **29**:1699–1712.
14. **Fukudome A, et al.** 2011. Specific requirement of DRB4, a dsRNA-binding protein, for the in vitro dsRNA-cleaving activity of *Arabidopsis* Dicer-like 4. RNA **17**:750–760.
15. **Garcia-Ruiz H, et al.** 2010. *Arabidopsis* RNA-dependent RNA polymerases and Dicer-like proteins in antiviral defense and small interfering RNA biogenesis during *Turnip mosaic virus* infection. Plant Cell **22**:481–496.
16. **Harvey JJW, et al.** 2011. An antiviral defense role of AGO2 in plants. PLoS One **6**:e14639. doi:10.1371/journal.pone.0014639.
17. **Herr AJ, Jensen MB, Dalmay T, Baulcombe DC.** 2005. RNA polymerase IV directs silencing of endogenous DNA. Science **308**:118–120.
18. **Jaubert M, Bhattacharjee S, Mello AFS, Perry KL, Moffett P.** 2011. ARGONAUTE2 mediates RNA-silencing antiviral defenses against *Potato virus X* in *Arabidopsis*. Plant Physiol. **156**:1556–1564.
19. **Margis R, et al.** 2006. The evolution and diversification of Dicers in plants. FEBS Lett. **580**:2442–2450.
20. **Onodera Y, et al.** 2005. Plant nuclear RNA polymerase IV mediates siRNA and DNA methylation-dependent heterochromatin formation. Cell **120**:613–622.
21. **Pagan I, et al.** 2010. *Arabidopsis thaliana* as a model for the study of plant-virus co-evolution. Philos. Trans. R. Soc. B **365**:1983–1995.
22. **Qu F.** 2010. Antiviral role of plant-encoded RNA-dependent RNA polymerases revisited with deep sequencing of small interfering RNAs of virus origin. Mol. Plant-Microbe Interact. **23**:1248–1252.
23. **Qu F, Ren T, Morris TJ.** 2003. The coat protein of Turnip crinkle virus suppresses posttranscriptional gene silencing at an early initiation step. J. Virol. **77**:511–522.
24. **Qu F, et al.** 2005. RDR6 has a broad-spectrum but temperature-dependent antiviral defense role in *Nicotiana benthamiana*. J. Virol. **79**:15209–15217.
25. **Qu F, Ye X, Morris TJ.** 2008. *Arabidopsis* DRB4, AGO1, AGO7, and RDR6 participate in a DCL4-initiated antiviral RNA silencing pathway negatively regulated by DCL1. Proc. Natl. Acad. Sci. U. S. A. **105**:14732–14737.
26. **Scholthof HB, et al.** 2011. Identification of an ARGONAUTE for antiviral RNA silencing in *Nicotiana benthamiana*. Plant Physiol. **156**:1548–1555.
27. **Souret FF, Kastenmayer JP, Green PJ.** 2004. AtXRN4 degrades mRNA in *Arabidopsis* and its substrates include selected miRNA targets. Mol. Cell **15**:173–183.
28. **Szittya G, et al.** 2003. Low temperature inhibits RNA silencing-mediated defense by the control of siRNA generation. EMBO J. **22**:633–640.
29. **Tuttle JR, Idris AM, Brown JK, Haigler CH, Robertson D.** 2008. Geminivirus-mediated gene silencing from Cotton leaf crumple virus is enhanced by low temperature in cotton. Plant Physiol. **148**:41–50.
30. **Wang X-B, et al.** 2011. The 21-nucleotide, but Not 22-nucleotide, viral secondary small interfering RNAs direct potent antiviral defense by two cooperative Argonautes in *Arabidopsis thaliana*. Plant Cell **23**:1625–1638.
31. **Wang X-B, et al.** 2010. RNAi-mediated viral immunity requires amplification of virus-derived siRNAs in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. U. S. A. **107**:484–489.
32. **Xie Z, et al.** 2004. Genetic and functional diversification of small RNA pathways in plants. PLoS Biol. **2**:e104. doi:10.1371/journal.pbio.0020104.
33. **Yang X, et al.** 2007. Functional modulation of the Geminivirus AL2 transcription factor and silencing suppressor by self-interaction. J. Virol. **81**:11972–11981.
34. **Yu B, et al.** 2010. siRNAs compete with miRNAs for methylation by HEN1 in *Arabidopsis*. Nucleic Acids Res. **38**:5844–5850.
35. **Yu B, et al.** 2005. Methylation as a crucial step in plant microRNA biogenesis. Science **307**:932–935.