Identification of positive and negative regulators of antiviral RNA interference in *Arabidopsis thaliana*

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Virus-host coevolution often drives virus immune escape. However, it remains unknown whether natural variations of plant virus resistance are enriched in genes of RNA interference (RNAi) pathway known to confer essential antiviral defense in plants. Here, we report two genome-wide association study screens to interrogate natural variation among wild-collected *Arabidopsis thaliana* accessions in quantitative resistance to the endemic cucumber mosaic virus (CMV). We demonstrate that the highest-ranked gene significantly associated with resistance from both screens acts to regulate antiviral RNAi in ecotype Columbia-0. One gene, corresponding to *Reduced Dormancy 5* (*RDO5*), enhances resistance by promoting amplification of the virus-derived small interfering RNAs (vsiRNAs). Interestingly, the second gene, designated *Antiviral RNAi Regulator 1* (*VIR1*), dampens antiviral RNAi so its genetic inactivation by CRISPR/Cas9 editing enhances both vsiRNA production and CMV resistance. Our findings identify positive and negative regulators of the antiviral RNAi defense that may play important roles in virus-host coevolution.

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Diverse antiviral defense mechanisms have been documented in higher plants. Several lines of evidence show that the RNA interference (RNAi) pathway mediates an essential antiviral immunity mechanism in plants. In antiviral RNAi, host cells process viral long dsRNA into small interfering RNAs (siRNAs) by a Dicer nuclease to trigger specific virus clearance in RNA-induced silencing complex (RISC) by an Argonaute (AGO) protein. Both Dicer and AGO gene families appear to have expanded in plants for the control of virus infection. In the model plant species *Arabidopsis thaliana*, for example, 3 of the 4 Dicer-like genes (DCLs) and 7 of the 10 AGO genes participate in the RNAi-mediated antiviral immunity. Moreover, among the 6 RNA-dependent RNA polymerase (RdRP) genes of *A. thaliana*, RdRP 1 (RDR1), RDR2 and RDR6 have all been shown to direct viral siRNA amplification. Interestingly, the 19-member AGO family of rice plants includes a hormone-inducible AGO18 that promotes antiviral RNAi by enhancing the expression of AGO1, which is necessary for vRNA-RISC assembly. Notably, viral suppressors of RNAi (VSRs) are essential virulence proteins of plant RNA and DNA viruses. Studies on the origins and variability of VSR genes have shown that antiviral RNAi exerts selection pressure on plant virus genomes.

Viruses with an RNA genome possess extraordinary adaptive abilities because of their error-prone replication mechanisms. Less is known about the natural variation that wild host plants in ecosystems with little human intervention accumulate and select to inherit in their progeny in response to virus infection. Recently, whole genome information has become available for *A. thaliana* to inherit in their progeny in response to virus infection 1–3. CMV is transmissible by mechanical inoculation of *A. thaliana* seedlings. We first assessed natural variation among the fully re-sequenced *A. thaliana* accessions in quantitative resistance to CMV-Δ2b, a previously characterized mutant of the highly virulent subgroup I CMV strain Fny that is rendered susceptible to antiviral RNAi by introducing nucleotide substitutions to prevent VSR-2b expression. Virus accumulation levels in the upper non-inoculated leaves were measured by enzyme-linked immunosorbent assay (ELISA) of the viral coat protein (CP) two weeks after mechanical inoculation of *A. thaliana* seedlings.

The virus titers in 496 accessions displayed a nearly normal distribution after log-transformation (Fig. 1a and Supplementary Data 1). Analyzing the data with the easyGWAS pipeline found that none of the SNPs significantly associated with quantitative virus resistance mapped to any of the known antiviral RNAi genes. The most significantly associated SNP resided in a region of chromosome 4 that codes for the gene At4g11040 (Fig. 1b, c and Supplementary Fig. 1a), corresponding to the previously reported Reduced Dormancy 5 (RDO5)/Delay of Germination 18 (DOG18) 18–19. We found that CMV-Δ2b titers were significantly different between the accessions classified as haplotypes T and G according to the SNP (Supplementary Fig. 1b). Northern blot analysis further verified that CMV-Δ2b replicated to lower levels in 4 selected accessions of haplotype T including Col-0 than the 3 accessions of haplotype G (Supplementary Fig. 1c). We then compared CMV-Δ2b infection in accession Antwerpen-1 (An-1) carrying a single base-pair (bp) frameshifting deletion in *rdo5* with accession Columbia-0 (Col-0), classified in the resistant haplotype along with 380 additional accessions among those examined (Fig. 1c and Supplementary Fig. 1b). Western blot analysis of the viral CP showed that CMV-Δ2b accumulated to much higher levels in An-1 plants than either Col-0 plants or the two independent lines of An-1 transgenically complemented with the *RDO5* gene from Col-0 (Fig. 1d). These results suggest suppression of CMV-Δ2b accumulation by *RDO5* from Col-0 plants.

To verify the role of *RDO5* in Col-0 plants, we obtained two Col-0 mutants carrying a T-DNA insertion at different positions in the second exon of *RDO5*, designated *rdo5-4* and *rdo5-5* (Fig. 1c). We also generated two independent transgene complementation lines of *rdo5-4* with the same *RDO5* gene driven by its native promoter described above. ELISA detection of the viral CP revealed significantly enhanced accumulation of CMV-Δ2b in both *rdo5-4* and *rdo5-5* mutant plants compared to either wild-type Col-0 plants or either of the two *RDO5*-complemented lines of *rdo5-4* plants (Fig. 1e). Moreover, both Western blotting detection of CP and Northern blotting detection of the viral genomic RNAs showed that CMV-Δ2b accumulated to higher levels in *rdo5-4* and *rdo5-5* mutant plants than wild-type Col-0 plants and the complemented lines of *rdo5-4* mutant (Fig. 1e, f). By comparison, CMV-Δ2b replicated to higher levels in *rd6* mutant plants than *rdo5-4* and *rdo5-5* mutant plants (Fig. 1e, f), which may explain the absence of clear symptomatic differences between the infected Col-0 and *rdo5* mutant plants (Supplementary Fig. 2a).

To further verify the role of *RDO5* by an independent approach, we performed gene knockout via CRISPR/Cas9 in Col-0 plants and obtained another homozygous *rdo5* mutant containing a deletion of 344 bp starting from the 12th codon of *RDO5*, designated *cr15* (Fig. 1c and Supplementary Fig. 2b). We found that *cr15* plants also supported significantly enhanced replication of CMV-Δ2b compared to its wild-type Col-0 and the two transgene complemented lines of *rdo5-4* mutant (Fig. 1e, f). Together, our findings show that the highest-ranked gene in the GWAS screen confers qualitative virus resistance in Col-0 accession.

**Results**

**Mapping host natural variation identifies a quantitative virus resistance gene.** We first assessed natural variation among the fully re-sequenced *A. thaliana* accessions in quantitative resistance to CMV-Δ2b, a previously characterized mutant of the highly virulent subgroup I CMV strain Fny that is rendered susceptible to antiviral RNAi by introducing nucleotide substitutions to prevent VSR-2b expression. Virus accumulation levels in the upper non-inoculated leaves were measured by enzyme-linked immunosorbent assay (ELISA) of the viral coat protein (CP) two weeks after mechanical inoculation of *A. thaliana* seedlings.

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**RDO5 enhances antiviral RNAi by promoting viral siRNA amplification.** The seed-specific *RDO5* codes for a nuclear pseudophosphatase that promotes seed dormancy in a manner independent of the biosynthesis of phytohormone abscisic acid 37–39. Consistently, *rdo5-4*, *rdo5-5* and *cr15* mutants exhibited...
Fig. 1 RDO5 confers quantitative virus resistance. a A nearly normal distribution of CMV-Δ2b accumulation levels in 496 Arabidopsis accessions after ELISA readings at 2 weeks post-infection were log-transformed. b Manhattan plot for GWAS mapping of the quantitative resistance phenotype to CMV-Δ2b infection. Five A. thaliana chromosomes were depicted in different colors. The horizontal dash-dot line corresponds to the significance threshold ($p = 5.48 \times 10^{-7}$). The black tringle above the threshold indicates the most significantly associated locus. c Regional Manhattan plot (from 6745 kb to 6747 kb), structure of RDO5, and positions of T-DNA insertion alleles (rdo5-4 and rdo5-5), CRISPR/Cas9 deletion allele (cr15) and the single base pair deletion in accession An-1. d Western detection of viral coat protein (CP) accumulation in An-1 and two RDO5-complemented lines. Detection of tubulin alpha chain was shown as loading control. e, f ELISA and Western detection (e) of viral CP accumulation with the titer in Col-0 set as 1 and Northern detection (f) of CMV-Δ2b genomic RNAs 1-3 (gRNAs), subgenomic RNA4 and vsiRNAs as well as endogenous miRNA 173 (miR173) and trans-acting siRNA 255 (tasi255) in wild-type (Col-0), rdo5 mutants and complemented lines. 25 S rRNA was stained and U6 RNA probed on the same membrane as loading controls. g Ratios of vsiRNAs/gRNAs were calculated from Phosphor-imager readings of Northern hybridization signals in (f) with the ratio in Col-0 set as 1. The experiments in (d) and (f) were repeated three times independently with similar results. Data presented are means ± SEM from three replicates (e) or independent experiments (g), letters indicate significant differences (one-way ANOVA, Duncan, $p < 0.05$) and black dots represent the individual values. The source data underlying blots in (d), (e) and (f), ELISA data in (e) and ratio data in (g) are provided as a Source Data file.

RDO5-dependent mainly on the DCL4 and RDR6 pathway, supplementary Fig. 2d). RDO5 expression of RDO5 in vegetative tissues in response to virus infection (Supplementary Fig. 2c), providing evidence for functional rescue by an additional RDO5 wild-type deletion in accession An-1. Western detection of viral coat protein (CP) accumulation in An-1 and two RDO5-complemented lines. Detection of tubulin alpha chain was shown as loading control. ELISA readings at 2 weeks post-infection were log-transformed. The horizontal dash-dot line corresponds to the significance threshold ($p = 5.48 \times 10^{-7}$). The black tringle above the threshold indicates the most significantly associated locus. Regional Manhattan plot (from 6745 kb to 6747 kb), structure of RDO5, and positions of T-DNA insertion alleles (rdo5-4 and rdo5-5), CRISPR/Cas9 deletion allele (cr15) and the single base pair deletion in accession An-1. Western detection of viral coat protein (CP) accumulation in An-1 and two RDO5-complemented lines. Detection of tubulin alpha chain was shown as loading control. ELISA and Western detection (e) of viral CP accumulation with the titer in Col-0 set as 1 and Northern detection (f) of CMV-Δ2b genomic RNAs 1-3 (gRNAs), subgenomic RNA4 and vsiRNAs as well as endogenous miRNA 173 (miR173) and trans-acting siRNA 255 (tasi255) in wild-type (Col-0), rdo5 mutants and complemented lines. 25 S rRNA was stained and U6 RNA probed on the same membrane as loading controls. Ratios of vsiRNAs/gRNAs were calculated from Phosphor-imager readings of Northern hybridization signals in (f) with the ratio in Col-0 set as 1. The experiments in (d) and (f) were repeated three times independently with similar results. Data presented are means ± SEM from three replicates (e) or independent experiments (g), letters indicate significant differences (one-way ANOVA, Duncan, $p < 0.05$) and black dots represent the individual values. The source data underlying blots in (d), (e) and (f), ELISA data in (e) and ratio data in (g) are provided as a Source Data file.

A. thaliana resistance to CMV-Δ2b is mediated by antiviral RNAi dependent mainly on the DCL4 and RDR6 pathway. To investigate the mechanism of RDO5, we first compared the accumulation levels of the vsiRNAs in the same panel of wild-type and mutant infected plants used above for determining viral accumulation by Northern blot analysis (Fig. 1f). We found that the vsiRNAs accumulated to similar levels in wild-type Col-0 plants and the three rdo5 mutants (rdo5-4, rdo5-5 and cr15) despite the fact that CMV-Δ2b replicated to significantly higher levels in the rdo5 mutant plants (Fig. 1e, f) and were thus expected to generate more abundant vsiRNA precursors for Dicer processing, suggesting deficiency of the mutant plants in the vsiRNA biogenesis. No marked differences were found in the accumulation of endogenous siRNA or microRNAs between wild-type and rdo5 mutant plants with or without CMV-Δ2b infection (Fig. 1f and Supplementary Fig. 2e). In A. thaliana plants infected with 2b-deficient mutants of CMV, the vsiRNAs are amplified by the host RDR1 and/or RDR6 so that loss of vsiRNA amplification in mutant plants leads to decreased ratios of vsiRNAs and viral genomic RNAs (gRNAs) although viral gRNAs accumulate to higher levels compared to the wild-type plants. Thus, we measured the ratios of vsiRNAs and viral gRNAs detected in the infected wild-type and mutant plants. As expected, loss of RDR6-dependent vsiRNA amplification in the control rdr6 mutant plants was associated with ~7-fold decrease of the vsiRNAs/gRNAs ratio compared to the resistant Col-0 plants (Fig. 1g). We observed significantly decreased vsiRNAs/gRNAs ratios in rdo5-4, rdo5-5 and cr15 mutant plants than those in Col-0 plants and the two RDO5-complemented lines of rdo5-4 plants (Fig. 1g), suggesting a role of RDO5 in the amplification of vsiRNAs.

To further investigate the mechanism of RDO5, we generated homozygous double or triple mutant plants by genetic crosses of rdo5-5 plants with rdr1 and rdr6 single or double mutants.
Consistent with a predominantly RDR6-dependent antiviral RNAi against CMV-Δ2b described previously, CMV-Δ2b replicated to significantly lower levels in both wild-type Col-0 and rdr1 plants than rdr6 or rdr1 rdr6 plants (Fig. 2a, b). In contrast to defective vsiRNA amplification in rdr6 or rdr1 rdr6 plants (Fig. 2b, c), we found that CMV-Δ2b accumulation was significantly enhanced in rdr6 double mutant plants compared to rdr1 single mutant plants (Fig. 2a–c). By contrast, no significant differences in either CMV-Δ2b accumulation or the vsiRNAs/gRNAs ratio were observed between rdr6 and rdr5 rdr6 plants (Fig. 2a–c). Moreover, neither CMV-Δ2b accumulation nor the vsiRNAs/gRNAs ratio was significantly different between rdr1 rdr6 double mutant plants and rdr5 rdr1 rdr6 triple mutant plants (Fig. 2a–c). These findings indicate that RDO5 acts specifically in the antiviral RNAi defense mechanism by enhancing vsiRNA amplification in an RDR6-dependent pathway.

We performed an additional set of infection experiments to verify the proposed RDR6-dependent antiviral activity of RDO5 using CMV-2aTΔ2b, which contains a 295-nt deletion in RNA 2 of Fny-CMV resulting in both the loss of VSR-2b expression and a C-terminal truncation of the viral RdRP protein. Unlike CMV-Δ2b, efficient CMV-2aTΔ2b infection occurs only in rdr1 rdr6 double mutant plants because it triggers potent amplification of vsiRNAs by both RDR1 and RDR6 pathways. ELISA and Western blotting detection of the viral CP as well as Northern blotting detection of the viral RNAs found no significant differences in the accumulation of CMV-2aTΔ2b and the vsiRNAs/gRNAs ratios between either wild-type plants (Col-0) or rdr6 mutant plants with rdr6 mutant plants (rdr5-4, rdr5-5 and cr15) or the two complemented lines of rdr5-4 plants (Supplementary Fig. 3). These results indicate that RDO5 is dispensable for RDR1-dependent amplification of the vsiRNAs. Consistently, enhanced accumulation of CMV-2aTΔ2b and decreased vsiRNAs/gRNAs ratio were observed in rdr6 rdr1 plants compared to rdr1 plants, but no significant differences in either CMV-2aTΔ2b accumulation or the vsiRNAs/gRNAs ratio were observed between rdr6 and rdr5 rdr6 plants (Fig. 2a, c, d). Moreover, we detected no significant differences in either CMV-2aTΔ2b accumulation or the vsiRNAs/gRNAs ratio between rdr1

Fig. 2 RDO5 promotes antiviral RNAi. a–h Accumulation of CMV-Δ2b (a, b, e, f) or CMV-2aTΔ2b (a, d, g, h) detected in wild-type (Col-0), single, double or triple mutant plants as indicated at 2 weeks post-infection by ELISA (a, e, g) and Western blot analysis of the viral coat protein (CP) or Northern blot analysis of the viral RNAs 1-4 (b, d, f, h). Detection of the vsiRNAs and plant endogenous small RNAs (b, d), and the calculation of vsiRNAs/gRNAs ratios (c) were as described in the legend to Fig. 1. Note the reduced sample loading (to ½) for total proteins and total RNAs (b, d, f, h). Letters indicate groups with significant differences (one-way ANOVA, Duncan, p < 0.05) and black dots represent the individual values. The experiments in (b), (d), (f) and (h) were repeated three times independently with similar results. The source data underlying blots in (b), (d), (f) and (h), ELISA data in (a), (e) and (g), and ratio data in (c) are provided as a Source Data file.
Natural variation identifies a host gene that inhibits antiviral defense. In a parallel GWAS screen, we used a wild-type isolate of subgroup II CMV strain Q (Q-CMV) that is not modified in counter-defense against antiviral RNAi, but causes much weaker disease symptoms than Fny-CMV in Col-0 plants. We measured the accumulation levels of Q-CMV in 500 accessions of Arabidopsis thaliana by detecting the viral genomic RNA3 with quantitative reverse transcription-polymerase chain reaction (RT-qPCR) (Supplementary Fig. 4a and Supplementary Data 1). Using the easyGWAS pipeline34, we did not identify SNPs significantly associated with quantitative resistance in any of the known antiviral RNAi genes as found in the above GWAS screen. The SNP associated most significantly with quantitative resistance against Q-CMV resided in a region of chromosome 5 between genes At5g05130 and At5g05140 (Fig. 3a, b and Supplementary Fig. 4b), which respectively contained an insertion and deletion of single cytosine nucleotide (C) in the 15th codon of VIR1 protein (Supplementary Fig. 4c). Northern blot analysis further confirmed that Q-CMV accumulation levels as measured by RT-qPCR were significantly different between the accessions classified as haplotypes A and G according to the SNP (Supplementary Fig. 4c). Northern blot analysis further confirmed that Q-CMV replicated to lower levels in 3 selected accessions of haplotype G than the 4 accessions of haplotype A including Col-0 (Supplementary Fig. 4d).

Functional studies in Col-0 plants identified At5g05140 as a regulator of antiviral RNAi, designated Antiviral RNAi Regulator 1 (VIR1). Firstly, we found that expression of VIR1, but not At5g05130, was induced in Col-0 plants by infection with Q-CMV, Q-CMV-Δ2b or CMV-2aTa2b (Supplementary Fig. 5a). VIR1 expression levels were also significantly higher in the selected accessions of haplotype A than those from haplotype G after Q-CMV infection (Supplementary Fig. 5b). As shown in Fig. 3b, we obtained VIR1 and At5g05130 knockout mutants of Col-0 plants. vir1-1 mutant contained a T-DNA insertion in the eighth exon of VIR1. cr5 mutant was generated by CRISPR/Cas9 genome editing to delete a 215-bp fragment in the first exon of At5g05130. Neither vir1-1 nor cr5 exhibited visible developmental defects (Supplementary Fig. 6a). RT-qPCR analysis showed that Q-CMV accumulated to significantly lower levels in vir1-1 mutant plants than Col-0 (Fig. 3c). In contrast, no statistically significant differences in Q-CMV accumulation were found between Col-0 and At5g05130-cr5 mutant plants or two independent VIR1-transgene complementation lines of vir1-1 (Fig. 3c). Moreover, vir1-1 plants, but not cr5 plants or the transgene-complemented lines of vir1-1, supported significantly reduced replication of Q-CMV-Δ2b (Fig. 3c), a 2b-deletion mutant of Q-CMV defective in suppressing antiviral RNAi initiated by either DCL2 or DCL4 shown by a previous study33. Notably, whereas no significant differences were detected in virus accumulation between the accessions classified as haplotypes A and G in double mutant plants compared to Col-0 plants, cr5 mutant plants or the two VIR1-complemented lines of vir1-1 (Fig. 3d–f).

To further verify the role of VIR1, we generated two additional vir1 mutants by CRISPR/Cas9 genome editing, designated cr6-14 and cr6-31, which respectively contained an insertion and deletion of single cytosine nucleotide (C) in the 15th codon of VIR1 (Supplementary Fig. 6b). Similar to vir1-1, both cr6-14 and cr6-31 mutants exhibited enhanced resistance to either Q-CMV or Q-CMV-Δ2b since both viruses accumulated to lower levels and triggered production of more abundant vsiRNAs in cr6-14 and cr6-31 mutants than Col-0 plants (Fig. 3c–f). However, we observed no obvious differences in the accumulation of endogenous siRNA and miRNAs between wild-type and vir1 mutant plants either with or without CMV infection (Fig. 3d, f and Supplementary Fig. 6c). These findings indicate that the highest-ranked gene in the independent GWAS screen dampens antiviral RNAi in Col-0 accession against both Q-CMV and Q-CMV-Δ2b by inhibiting production of the vsiRNAs.

VIR1 is a negative regulator of antiviral RNAi. The N-terminal region of VIR1 protein shares strong homology with Arabidopsis transcript elongation factor II (AtTFIIS) conserved broadly in eukaryotes (Supplementary Fig. 5c). However, most of the critical residues in the C-terminal domain of TFIIS essential to facilitate mRNA synthesis by RNA polymerase II complex are not conserved in VIR1 protein31. Similar to rdo5 mutant plants, rdo2 mutant plants that lack AtTFIIS display clearly reduced seed dormancy.42 We found that vir1-1 mutant plants exhibited significantly enhanced seed dormancy and that VIR1 expression was detectable in seeds (Supplementary Fig. 6d, e), suggesting VIR1 as a negative regulator of seed dormancy.

To determine whether VIR1 interferes with vsiRNA biogenesis, we constructed double and triple mutants by genetic crosses of vir1-1 plants with dcl2-1 and/or dcl4-2 mutant plants characterized previously33. We challenged this panel of mutant plants with CMV-2aTa2b and CMV-Δ2b derived from Fny-CMV since our previous studies have defined the genetic pathways in the biogenesis of vsiRNAs triggered by these mutant viruses13,19. RT-qPCR and Northern blotting analysis showed that CMV-2aTa2b replicated to significantly lower levels in vir1-1 mutant plants than Col-0 plants (Fig. 4a, b), as did Q-CMV and Q-CMV-Δ2b (Fig. 3c–f). Notably, whereas no significant differences were detected in virus accumulation between dcl2 and vir1 dcl2 mutant plants, CMV-2aTa2b replicated to significantly higher levels in vir1 dcl4 double mutant plants than dcl4 single mutant plants (Fig. 4a, b). Consistent with the known dominant role of DCL4 over DCL2 in the biogenesis of vsiRNAs9,11,19,33, 21-nt vsiRNAs made by DCL4 were undetectable whereas 22-nt vsiRNAs by DCL2 accumulated to high levels in either dcl4 plants or vir1 dcl4 plants (Fig. 4b). By comparison, however, 22-nt vsiRNAs accumulated to markedly lower levels in vir1 dcl4 plants than...
The role of DCL2 mutant plants (Fig. 4a, b). This result indicates that although reported previously33, neither of the double and triple mutant do so in enhanced antiviral defense in VIR1

The vsiRNAs and plant endogenous small RNAs were also detected by Northern blotting (d, f). The viSRNA/gRNA ratios were calculated as described in the legend to Fig. 1. Data in (c) and (e) are means ± SEM from three independent experiments, letters indicate groups with significant differences (one-way ANOVA; Duncan, p < 0.05) and black dots represent the individual values. The source data underlying blots in (d) and (f), qRT-PCR data in (c) and ratio data in (e) are provided as a Source Data file.

dcl4 plants (Fig. 4b), indicating that activation of DCL2-dependent antiviral RNAi by 22-nt vsiRNAs in the absence of DCL4 is upregulated by VIR1.

RT-qPCR analysis revealed significant suppression of DCL4 induction by CMV-2aTΔ2b in Col-0 plants compared to vir1-1 mutant plants (Fig. 4e). By contrast, DCL2 expression was induced by CMV-2aTΔ2b in Δ2b single mutant plants, but not in vir1-1Δ2b double mutant plants or any other wild-type and mutant plants examined (Fig. 4e). Reduced induction of DCL4 in Col-0 plants and strong induction of DCL2 in Δ2b mutant plants were both observed after infection with Q-CMV (Supplementary Fig. 7). These findings together suggest a model in which VIR1 negatively regulates antiviral RNAi in wild-type plants by restricting transcriptional induction of DCL4, but upregulates 22-nt vsiRNA-directed antiviral RNAi by transcriptional induction of DCL2 in the absence of DCL4.

We observed no statistically significant differences in CMV-2aTΔ2b accumulation between Δ2b and vir1 Δ2b Δ4 mutant plants (Fig. 4a, b). This result indicates that although VIR1 enhanced antiviral defense in Δ4 single mutant, it failed to do so in Δ2b Δ4 double mutant, consistent with the proposed role of DCL2 in the upregulation of antiviral RNAi by VIR1. As reported previously33, neither of the double and triple mutant plants produced 21- or 22-nt vsiRNAs. The results from CMV-Δ2b infection in the same panel of mutants (Fig. 4c, d) essentially reproduced those from CMV-2aTΔ2b infection (Fig. 4a, b). For example, genetic inactivation of VIR1 enhanced the resistance to CMV-Δ2b in Col-0 plants, but promoted virus susceptibility and reduced DCL2-dependent production of 22-nt vsiRNAs in dcl4 plants (Fig. 4b).

Discussion

In this work, we searched for the natural variation among wild A. thaliana populations that is most significantly associated with quantitative virus resistance to an endemic RNA virus. We conducted independent GWAS screens with wild-type Q-CMV and the VSR-deficient CMV-Δ2b, respectively. Unlike Fny-CMV, from which CMV-Δ2b was derived, Q-CMV replicates to higher levels in Δ2b Δ4 mutant plants than wild-type Col-0 plants33,36, indicating incomplete suppression of antiviral RNAi by Q-CMV. Surprisingly, none of the SNPs significantly associated with quantitative virus resistance from both of our GWAS screens and an additional GWAS screen reported recently by others43 mapped to any of the known antiviral RNAi genes, including AGO2 shown recently to exhibit natural variation in non-host virus resistance44. However, genetic studies show that both of the highest-ranked gene significantly associated with quantitative virus resistance identified from each of our GWAS screens...
function in antiviral RNAi. Further studies on the two identified genes evolved in Columbia-0 accession demonstrate opposing roles in antiviral RNAi. Our findings provide direct evidence to support antiviral RNAi as a dominant defense mechanism in virus-host coevolution, which is consistent with previous genetic studies that identify antiviral RNAi as an essential antiviral defense in plants1,2,5,6,8,18,19,45.

Most of the antiviral RNAi pathway genes characterized to date have been identified by their known activity in experimentally induced RNAi to target mRNAs transcribed in the nucleus2,7,8,18,19,45–49. Recently, mutant Arabidopsis thaliana and Caenorhabditis elegans defective in antiviral RNAi have been isolated by sensitized genetic screens using viruses or viral RNA replicons rendered inactive in RNAi suppression36,50–52. In this work, we showed that antiviral RNAi pathway genes can be identified by GWAS mapping of natural variation in quantitative virus resistance among wild-collected A. thaliana accessions. Our findings illustrated the technical feasibility of using GWAS to identify antiviral RNAi pathway genes.

Whereas VIR1 has not been characterized before this work, RDO5 has a known function to promote seed dormancy with the mechanism yet to be defined. Our mechanistic studies demonstrate that RDO5 specifically enhances antiviral RNAi by promoting amplification of the vsiRNAs in a pathway dependent on RDR6, but independent of RDR1. By contrast, VIR1 dampens antiviral RNAi by restricting production of the vsiRNAs and suppresses seed dormancy in wild-type Col-0 plants. Our results suggest that VIR1 may act by blocking viral induction of DCL4 in a manner similar to the dominant negative mutant version of AtTFIIS shown recently to modify the transcriptome in tfiIS mutant plants41. Interestingly, VIR1 is necessary for the transcriptional induction of DCL2 and the upregulation of DCL2-dependent antiviral RNAi by 22-nt vsiRNAs in the absence of DCL4, which may explain at least in part the dominant role of DCL4 over DCL2 in the biogenesis of vsiRNAs known since 20069–11,19,33. However, it is unknown whether VIR1 modulates the functional roles of DCL2 that are active in the presence or absence of DCL4 in uninfected plants9,35–58. Notably, we show that VIR1 inactivation by CRISPR/Cas9 genome editing confers resistance to CMV either active or defective in RNAi suppression, providing a strategy to generate transgene-free virus resistant plants. In summary, our findings indicate that RDO5 and VIR1
have opposing roles in both antiviral RNAi and seed dormancy. We propose that a shared mechanism is under natural selection to regulate antiviral RNAi and seed dormancy.

Methods

Viruses and plant materials. Mutant viruses CMV-Δ2b and CMV-2aTΔ2b were derived from the subgroup 1 strain Finy-CMV isolated and cloned in New York from a muskmelon farm. In CMV-Δ2b, three A/G codons at the first (start) codon, 8th, and 18th positions of RDRP 2a protein were replaced by T/C codons. In the subgroup 1 strain Finy-CMV, two ORFs of Finy-CMV were deleted to mutate to ACG so that the amino acids encoded in the +1 overlapping 2a ORF were not altered. CMV-2aTΔ2b contained a 295-nl deletion in the 2b coding sequence, which also removed the C-terminal 80 amino acids of the viral RDRP 2a protein. The CMV-Finy strain of Nicotiana tabacum was molecularly cloned in 1995 following passages since 1964 in laboratory host plants. In Q-CMV, 2b, the coding sequence of RNA2 was deleted and replaced with CCCGGG, which also removed the C-terminal 68 amino acids of the 2a protein. All viruses were purified after propagation in Nicotiana clevelandii. Virion concentration used for mechanical inoculation of A. thaliana was 10 µg/ml for both CMV-Δ2b and Q-CMV and 20 µg/ml for CMV-2aTΔ2b. The virus titer in each sample was calculated using the ANOVA approach, the heritability was 0.9727 for CMV-Δ2b, 0.976, 0.970 and 0.972 in CMV-Δ2b screen and, 0.909, 0.905 and 0.925 in Q-CMV screen. The mapping trait was the mean value of three replicates of the qPCR on the viral RNA3 for Q-CMV screen and the log-transformed mean value of three replicates of ELISA on the viral CP for CMV-Δ2b screen. We applied a p value threshold of 1/m to GWAS mapping, where m is the number of SNPs used for the analysis. The R program “qman” was used to create Manhattan plots and quantile-quantile plots based on the results of the GWAS mapping. Two-sample t-test was performed to compare virus titers of different haplotypes using GraphPad Prism 7, with p = 0.05 as the significance level.

Characterization of virus infection. To characterize virus infection, upper systematically infected leaves from 16 plants were harvested at two weeks post-inoculation and pooled for total RNA and protein extraction. Five and 20 µg total RNAs were loaded each lane for Northern detection of the viral genomic and vRNAiS by a-32P- and γ-32P-labelled probes, respectively. Hybridization signals were detected by phosphor imager Typhoon 9410 and analyzed by ImageQuant TL 7.0 (GE Healthcare). Ratios of vRNAiS vs viral genomic RNAs (gRNAs) were calculated from Phosphor-imager readings of Northern hybridization signals. With the primer pairs listed in Supplementary Data 2 and EFla mRNA as the internal reference, RT-qPCR was used to detect the accumulation of RDO5, VIR1, DCL1, DCL2, DCL3 or DCL4 mRNA in upper non-inoculated leaves of plants one week after mock or virus inoculation. For Western blotting, total proteins were separated on 12% polyacrylamide gel before transferred to 0.45 µm nitrocellulose membrane (GE Healthcare). Monoclonal antibody (1: 5000 dilution) specific to Fny-CMV CP was used for viral CP detection and probing with A. thaliana tubulin alpha chain specific antibody (Agriera, AS52 4483, 1:5000 dilution) as loading control, Goat anti-mouse IgG (H + L) (Invitrogen, G-21040, 1:2000 dilution) was used as secondary antibody. All experiments were biologically repeated at least three times. We examined the expression pattern of VIR1 in A. thaliana from the Arabidopsis RNA-Seq Database (http://ipf.sueltich.edu/pub/athENA/) using 960 libraries generated from Col-0.

Seed dormancy assay. For seed dormancy assays, 50 newly harvested seeds were sown on wet filter paper in 9 cm diameter culture dishes, and incubated in growth room with 16 h light - 8 h dark cycle at 23°C. Seed germination rates for each of the genotypes were determined 7 days after incubation with 4 independent repeats.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Data supporting the findings of this work are available within the paper and its Supplementary Information files. A reporting summary for this Article is available as a Supplementary Information file. Source data are provided with this paper.

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**Author contributions**

S.L. designed and performed experiments, analyzed data, and wrote the paper. S.-W.D. conceived, designed, and supervised the study, and wrote the paper. Z.J. designed and supervised the GWAS screens and statistical analysis, and wrote the paper. M.C., R.L., W.-X.L. performed experiments or analyzed data. A.G.-O. provided intellectual input to the study. All authors revised and provided feedback for the final version of the paper.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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