Lipid flippases promote antiviral silencing and the biogenesis of viral and host siRNAs in *Arabidopsis*

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Dicer-mediated processing of virus-specific dsRNA into short interfering RNAs (siRNAs) in plants and animals initiates a specific antiviral defense by RNA interference (RNAi). In this study, we developed a forward genetic screen for the identification of host factors required for antiviral RNAi in *Arabidopsis thaliana*. Using whole-genome sequencing and a computational pipeline, we identified aminophospholipid transporting ATPase 2 (*ALA2*) and the related *ALA1* in the type IV subfamily of P-type ATPases as key components of antiviral RNAi. ALA1 and ALA2 are flippases, which are transmembrane lipid transporter proteins that transport phospholipids across cellular membranes. We found that the *ala1/ala2* single- and double-mutant plants exhibited enhanced disease susceptibility to cucumber mosaic virus when the virus-encoded function to suppress RNAi was disrupted. Notably, the antiviral activity of both ALA1 and ALA2 was abolished by a single amino acid substitution known to inactivate the flippase activity. Genetic analysis revealed that ALA1 and ALA2 acted to enhance the amplification of the viral siRNAs by RNA-dependent RNA polymerase (RdRP) 1 (RDR1) and RDR6 and of the endogenous virus-activated siRNAs by RDR1. RNA virus replication by plant viral RdRPs occurs inside vesicle-like membrane invaginations induced by the recruitment of the viral RdRP and host factors to subcellular membrane microdomains enriched with specific phospholipids. Our results suggest that the phospholipid transporter activity of ALA1/ALA2 may be necessary for the formation of similar invaginations for the synthesis of dsRNA precursors of highly abundant viral and host siRNAs by the cellular RdRPs.

cucumber mosaic virus | RNA interference | lipid flippase | viral siRNAs | mutCAN

The genome of *Arabidopsis thaliana* plants encodes diverse classes of short interfering RNAs (siRNAs) in addition to microRNAs (1–3). Major classes of *Arabidopsis* endogenous siRNAs include heterochromatic siRNAs, transacting siRNAs, natural antisense siRNAs, phased siRNAs, double-strand break-induced siRNAs, epigenetically activated siRNAs, and virus-activated siRNAs (4–12). Much is known about the biogenesis of these siRNAs and their function in directing transcriptional and posttranscriptional gene silencing in *Arabidopsis* (1–3). *Arabidopsis* siRNAs are the products of dicer-like 3 (DCL3), DCL2, or DCL4, which are predominantly 24, 22, and 21 nt long, respectively (1–3). A common step in the biogenesis of *Arabidopsis* siRNAs is the synthesis of the precursor double-stranded RNA (dsRNA) by RNA-dependent RNA polymerase (RdRP) 1 (RDR1), RDR2, or RDR6 (1–3). RDR6-mediated dsRNA synthesis may occur in cytoplasmic siRNA bodies that also contain RNA-binding protein suppressor of gene silencing 3 (SGS3) (13, 14), which is dispensable for RDR1-dependent biogenesis of siRNAs (12).

Gene silencing by virus-derived siRNAs (vsiRNAs) directs a primary antiviral defense mechanism in plants (15, 16). In *A. thaliana*, DCL2, DCL3, and DCL4 all participate in the biogenesis of vsiRNAs, and an antiviral role has been demonstrated for 6 of the 10 ARGONAUTE proteins, including AGO1, AGO2, AGO4, AGO5, AGO7, and AGO10 (17–27). Moreover, antiviral silencing frequently depends on the amplification of vsiRNAs by RDR1, RDR2, and/or RDR6 (22, 28, 29). Several additional factors from *Arabidopsis* endogenous small RNA pathways such as SGS3, Hua enhancer 1 (HEN1), and dsRNA-binding 4 (DRB4) also contribute to antiviral silencing (13, 23, 30–33). These findings are consistent with the idea that small RNA silencing pathways may have evolved primarily as a defense mechanism against virus infection in addition to the control of transposons (1–3).

As a powerful unbiased approach for the genetic dissection of cellular pathways, forward genetics has led to the identification of several core RNA silencing pathway genes (13, 34–37). However, forward genetic screens have not been used widely to identify *Arabidopsis* genes required for antiviral silencing, probably because genetic mutations that abolish transgene silencing do not produce readily visible defects in antiviral silencing (36, 38). Plant viruses each encode at least one viral suppressor of RNA silencing (VSR), the expression of which is often sufficient to erase the difference in virus susceptibility between wild-type and silencing-defective mutant *Arabidopsis* plants (15, 16). Moreover, highly redundant or overlapping siRNA pathways control antiviral silencing in plants, so that genetic inactivation of a single pathway may not alter virus susceptibility (13, 36).

In this study, we developed a genetic screen in *A. thaliana* based on a mutant of cucumber mosaic virus (CMV) that does not express its VSR 2b protein (39) and is targeted for potent silencing primarily by an RDR6-dependent pathway (24). Our genetic screen identified a gene in the antiviral RNAi pathway that encodes a phospholipid flippase in the type IV subfamily of P-type ATPases (P4-ATPases). Further analysis revealed an antiviral function for a second member of the 12-gene *Arabidopsis*

Significance

Forward genetics as an unbiased approach for the genetic dissection of cellular pathways has not been widely used to identify host genes essential for antiviral RNAi. Here we report the identification of a phospholipid flippase as a component of antiviral RNAi from a forward genetic screen developed in *Arabidopsis thaliana*. We also describe a versatile computational pipeline capable of identifying different types of the causal mutation, including deletion, insertion, and single-nucleotide mutation, from whole-genome sequencing. We show that the identified flippase acted to enhance the amplification of viral siRNAs and the endogenous virus-activated siRNAs by host RNA-dependent RNA polymerases (RdRPs). Our findings suggest possible parallels in RNA synthesis by both the host and viral RdRPs.

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P4-ATPase family. We found that both of the flippases lost antiviral activity after substitution of a single invariable amino acid essential for flippase activity. Genetic characterization showed that the antiviral phospholipid transporters acted to enhance the amplification of both the viral siRNAs and the endogenous virus-activated siRNAs by RDR6 and/or RDR1. Our findings suggest that the synthesis of dsRNA precursors of the viral and host siRNAs by the cellular RDRs may require subcellular membrane enrichment of specific phospholipids known to be necessary for the formation of vesicle-like membrane invaginations during RNA replication of positive-strand RNA viruses (40, 41).

Results

Identification of a Genetic Mutation Defective in Host Antiviral Defense by Whole-Genome Sequencing and a Computational Pipeline. It is known that plant viruses encode VSRs to suppress antiviral silencing mediated by overlapping genetic pathways (15, 16). As a result, VSR-expressing wild-type viruses may cause similar disease symptoms in wild-type plants and mutant plants defective in a single pathway of antiviral silencing. To eliminate the interference of viral silencing suppression and avoid the induction of overlapping antiviral silencing pathways, we designed a forward genetic screen in *A. thaliana* based on a recently characterized VSR-deficient mutant of CMV, CMV-Δ2b (24). All 2b-deficient mutants of CMV replicate normally and spread systemically in host plants but accumulate to only low levels and frequently cause no visible disease symptoms in wild-type plants (42–44) because of antiviral silencing (22, 24, 28). However, CMV-Δ2b, which contains point mutations to prevent 2b expression, replicates to higher titers and causes virulent diseases in single-mutant plants such as *rdr6* and *sgs3* (24). Thus, we hypothesized that the screening of mutagenized *Arabidopsis* seedlings with CMV-Δ2b for disease induction would identify mutants enriched for those defective in antiviral silencing. To test the idea, we screened the mutants from the Salk homozygote T-DNA collection and identified several antiviral RNAi-defective (*avi*) mutants that exhibited disease symptoms after mechanical inoculation with CMV-Δ2b. Here we focused on *avi1*, which, whereas displaying no obvious developmental abnormity without infection, developed severe disease symptoms including chlorosis and dwarfing after infection with CMV-Δ2b (Fig. 1*A*). Consistently, Northern blot hybridization detected an increased virus accumulation in the upper, systemically infected leaves of *avi1* plants compared with wild-type *Col-0* plants (Fig. 1*B*).

Genetic analysis of F2 (second filial generation) progeny from *avi1* backcrossed to wild-type *Col-0* plants indicated that *avi1* segregated as a single recessive allele, which, unexpectedly, was not linked to a T-DNA insertion. To identify the causal mutation in *avi1*, we bulked F2 segregants from *avi1* backcrossed to *Col-0* into symptomatic and asymptomatic pools and sequenced the whole genome by Illumina HiSeq 2500. Because the types of the causal mutation in *avi1* were unpredictable, we developed a computational pipeline for mutation calling by coverage depth and nucleotide frequency (mutCAN; https://github.com/JinfengLu/mutCAN). Unlike the available pipeline (45), mutCAN compares the mapped paired-end reads from the two DNA libraries for both their coverage depth and nucleotide frequency at each genomic position (Fig. 1*C*). Our analysis identified two candidate single-nucleotide polymorphisms (SNPs) and 44 candidate deletions ranging from 1 to 25 bp but no candidate insertions except those also detected as candidate deletions. Neither of the identified SNPs was located in an exon of annotated genes, and 39 of the deletions were mapped within intergenic regions, annotated transposon fragments, or introns of annotated genes. Four of the remaining five candidate deletions were found to be false positive after close examination of all reads covering the candidate deletions. The final candidate was a deletion of 11 bp (5′-GGTTCAGTAAT-3′) in the 12th exon of the gene aminophospholipid transporting ATPase 2 (*ALA2*; At5g44240; Fig. 2*A*) characterized recently (46). The deletion caused a frameshift mutation that resulted in early translational

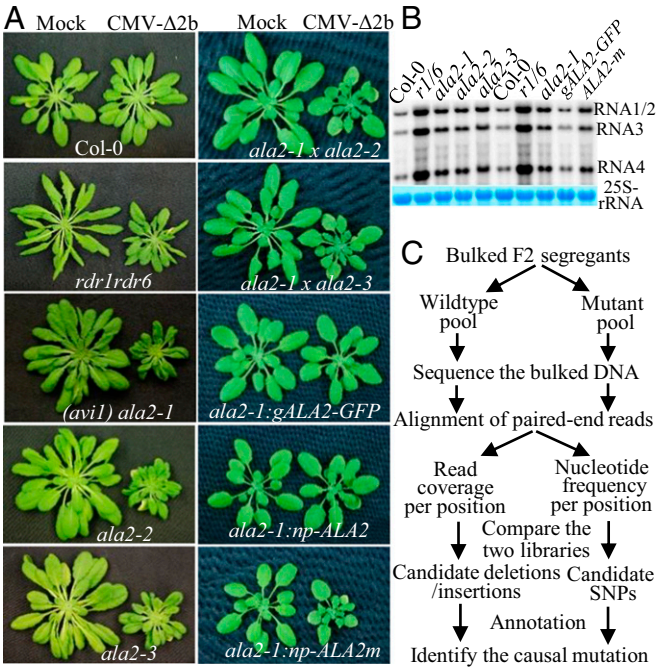

Fig. 1. Identification of *ALA2* by a genetic screen combined with mutCAN. (*A*) Wild-type *Col-0* and mutant plants were photographed 3 wk (*Left*) or 10 d (*Right*) after mock inoculation or infection with CMV-Δ2b. All of the mutant plants were homozygous for the mutant allele(s) except for *ala2-1* x *ala2-2* and *ala2-1* x *ala2-3*, which were F1 plants from crosses of the two homozygous mutant parents. Also shown are *ala2-1* (*avi1*) plants carrying a transgene for the genomic *ALA2* gene fused with GFP at the C terminus (*gALA2-GFP*), the wild-type *ALA2* cDNA (*np-ALA2*), or mutant cDNA with D381A substitution (*np-ALA2m*) driven by its native promoter (np). (*B*) Northern blot detection of CMV-Δ2b accumulation in the upper systemically infected leaves of plants of the indicated genotypes 2 wk after infection. *r1/6*, *rdr1 rdr6* double-mutant plants. The right two lanes were from *ala2-1* plants carrying the indicated transgene. 25S rRNA in the same membrane was stained to show equal loading. (*C*) Flowchart of the mutCAN pipeline for the identification of the causal mutation by whole-genome sequencing of symptomatic and asymptomatic F2 progeny plants from *ala2-1* after backcrossing with *Col-0* plants.

termination three codons after the deletion. A homozygous deletion of the same 11 bp in *ALA2* of *avi1* mutant plants was verified by Sanger sequencing. Thus, *avi1* was renamed *ala2-1*.

Lipid Flippases ALA1 and ALA2 Mediate Antiviral Defense in *Arabidopsis*. Three sets of findings confirmed the 11-bp deletion in *ALA2* as the causal mutation in the *ala2-1* mutant. We obtained and characterized two allelic T-DNA insertion mutants of *ALA2*, designated *ala2-2* and *ala2-3*, which contained T-DNA in the 7th and 22nd intron, respectively (Fig. 2*A*). We found that, similar to *ala2-1* plants, both *ala2-2* and *ala2-3* mutants developed severe disease symptoms after CMV-Δ2b infection (Fig. 1*A*), and the mutant virus replicated to higher levels in the mutant plants than in wild-type plants (Fig. 1*B*). F1 plants from crosses of *ala2-1* with either *ala2-2* or *ala2-3* also exhibited enhanced disease susceptibility to CMV-Δ2b (Fig. 1*A*). Moreover, we found that transgenic expression of the genomic *ALA2* (with GFP fused to the C terminus) under the control of its native promoter in *ala2-1* mutant plants rescued the host resistance to CMV-Δ2b infection, which was illustrated by the absence of disease symptoms (Fig. 1*A*) and the low levels of virus accumulation in the infected transgenic *ala2-1* plants (Fig. 1*B*).

ALA2 belongs to the type IV subfamily of P-type ATPases, which includes 12 members in *Arabidopsis* (47). We next examined the virus susceptibility of T-DNA insertional mutants targeting 10 additional *ALA* genes available in the Salk homozygote T-DNA collection. We found that among the 10 *ala* mutants examined,

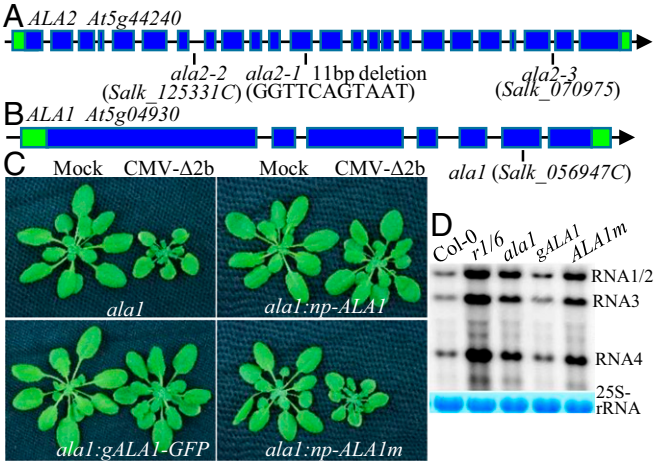

Fig. 2. Lipid flippases ALA1 and ALA2 mediate antiviral defense in *Arabidopsis*. (*A* and *B*) The positions of the mutant alleles of *ALA2* (*A*) and *ALA1* (*B*) are shown. (*C*) The *ala1* mutant exhibited enhanced disease susceptibility to CMV-Δ2b, and the defect was complemented by a transgene coding either for the genomic *ALA1* gene fused with GFP at the C terminus (*gALA1-GFP*) or *ALA1* cDNA driven by its native promoter (*np-ALA1*) but not by a transgene coding for the mutant *ALA1* cDNA with D451A substitution (*np-ALA1m*). The plants were photographed 10 d postinoculation. (*D*) Northern blot detection of CMV-Δ2b accumulation in wild-type and mutant plants 2 wk after infection. The right two lanes are from *ala1* plants carrying the indicated transgene. 25S rRNA in the same membrane was stained to show equal loading.

ala1 plants (salk_056947C), containing a verified T-DNA insertion in the sixth exon of *ALA1* (Fig. 2*B*), exhibited enhanced susceptibility to CMV-Δ2b as determined by both symptom expression (Fig. 2*C*) and Northern blot hybridization (Fig. 2*D*). The antiviral role of ALA1 was further confirmed by transgenic expression of either the genomic *ALA1* with GFP fused to the C terminus or native promoter-driven *ALA1* cDNA in *ala1* plants, as both types of transgenic *ala1* plants became as resistant as wild-type *Col-0* plants to CMV-Δ2b infection (Fig. 2 *C* and *D*). Interestingly, *ALA1*, but not *ALA2*, was induced by virus infection (Fig. S1).

P4-ATPases function as lipid flippases by using ATP to transport specific phospholipids from the luminal/exofacial to the cytofacial leaflet of cellular membranes (47). The flippase activity of ALA2 has been demonstrated in *Saccharomyces cerevisiae*, and alanine (A) substitution of the invariable aspartate (D) in the conserved sequence DKTG of P4-ATPases abolishes the flippase activity of ALA2 (46). To determine the role of the flippase activity in antiviral defense, we generated *ala2-1* transgenic plants expressing the wild-type *ALA2* cDNA under the control of its native promoter or a mutant *ALA2* cDNA carrying the same D381A substitution in ALA2. We found that *ala2* plants expressing the mutant ALA2 remained highly susceptible to CMV-Δ2b infection in contrast to the expression of the wild-type ALA2 (Fig. 1 *A* and *B*). Similarly, unlike *ala1* plants expressing the wild-type ALA1, CMV-Δ2b was highly virulent and replicated to higher levels in transgenic *ala1* plants expressing the mutant ALA1 carrying the D457A substitution at the conserved site (Fig. 2 *C* and *D*). These findings suggest that the lipid-transporting activity of both ALA1 and ALA2 is required for antiviral defense in *Arabidopsis* plants.

Redundant Roles of *ALA1* and *ALA2* in Plant Development and Antiviral Defense. The enhanced disease susceptibility of *ala1* and *ala2* mutants to CMV-Δ2b suggests independent genetic requirements for *ALA1* and *ALA2* in antiviral defense. To examine their genetic interactions, we generated an *ala1 ala2* double mutant by crossing *ala1* with *ala2-1*. Unlike *ala1* and *ala2* single mutants, *ala1 ala2* plants displayed obvious developmental defects, including small inflorescence, dark-green leaves, and the bending down of branches (Fig. 3 *A* and *B*). This suggested a redundant role for *ALA1* and *ALA2* in plant growth and development, which might explain why a modest increase in the accumulation of CMV-Δ2b in *ala1* and *ala2* single-mutant plants resulted in the development of severe disease symptoms (Figs. 1 and 2).

We found that, similar to the single mutants, *ala1 ala2* double-mutant plants also exhibited enhanced disease susceptibility to CMV-Δ2b (Fig. 3 *A* and *B*). By comparison, the infected *ala1 ala2* plants exhibited more severe dwarfing than the infected *ala1* and *ala2* single mutants, possibly because of the developmental defects associated with the double mutant.

We further examined the disease susceptibility of the double mutant to wild-type CMV and CMV-2aTΔ2b (43). CMV did not replicate to higher levels in *ala1 ala2*, *dcl2 dcl4*, or *rdr1 rdr6* double-mutant plants compared with *Col-0* plants (Fig. 3*C*). Thus, the antiviral activity of DCL2/4 and RDR1/6 as well as ALA1/2 was not detectable when the VSR 2b protein was expressed, demonstrating potent suppression of antiviral silencing by VSR 2b. CMV-2aTΔ2b contains a 295-nt deletion in the 2b coding sequence that also results in the C-terminal truncation of the viral RNA-dependent RNA polymerase encoded in an overlapping reading frame (43). However, the coding of the viral RdRP is not altered by the point substitutions introduced in CMV-Δ2b to prevent the expression of VSR 2b (24). Perhaps due to this difference, whereas CMV-Δ2b is highly virulent in *rdr6* single-mutant plants, CMV-2aTΔ2b induces strong amplification of vsiRNAs by both RDR1 and RDR6 so that it causes disease only in *rdr1 rdr6* double-mutant plants (22, 24, 28). We found that *ala1 ala2* double-mutant plants also developed disease symptoms after infection with CMV-2aTΔ2b and supported

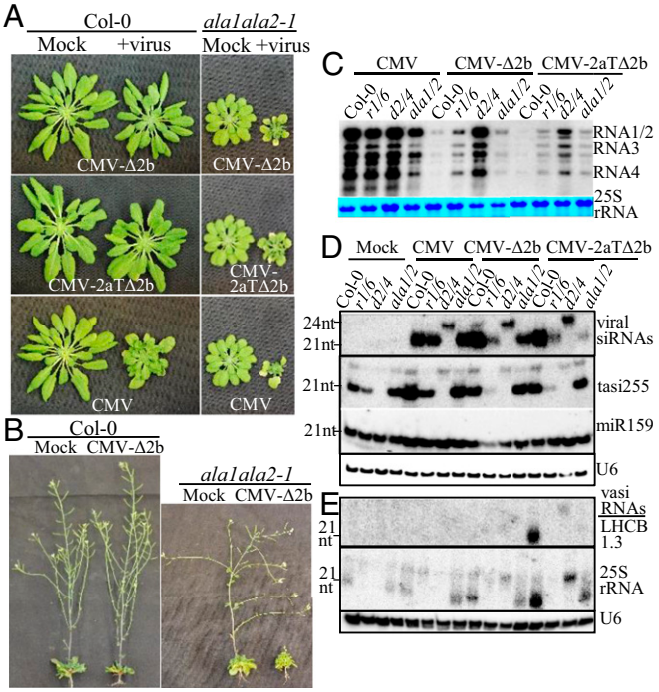

Fig. 3. ALA1 and ALA2 enhance the amplification of vsiRNAs and vasiRNAs in *Arabidopsis*. (*A* and *B*) *ala1 ala2-1* double-mutant plants displayed developmental defects at the seedling and adult plant stages and were susceptible to infection with CMV-Δ2b, CMV-2aTΔ2b, or CMV. The mock- and virus-inoculated plants were photographed 3 wk (*A*) or 2 mo (*B*) after inoculation. (*C*–*E*) Northern blot analysis to compare the accumulation of virus genomic/subgenomic RNAs (*C*), antisense primary and secondary vsiRNAs (*D*), and vasiRNAs targeting LHCB1.3 mRNA or 25S rRNA (*E*) in wild-type and double-mutant plants (a1/2, ala1 ala2-1; d2/4, dcl2 dcl4; *r1/6*, *rdr1 rdr6*) 2 wk after infection with CMV, CMV-Δ2b, or CMV-2aΔT2b. The same set of RNA samples was also probed for tasiRNA 255, miR159, and U6 RNA (Fig. S3).

higher levels of virus accumulation than wild-type *Col-0* plants (Fig. 3 *A* and *C*). However, both of the single-mutant plants remained highly resistant to CMV-2aTΔ2b (Fig. 4*A* and Fig. S2). These findings indicate that *ALA1* and *ALA2* act redundantly to inhibit both the accumulation of CMV-2aTΔ2b and the induced disease development in infected *Arabidopsis* plants.

Lipid Flippases ALA1 and ALA2 Mediate Antiviral Silencing by Enhancing the Amplification of Viral siRNAs. The identified lipid flippases may act in the antiviral silencing pathway, as *ala1* and *ala2* mutant plants exhibited enhanced disease susceptibility to the 2b-deficient CMV mutants defective in the suppression of antiviral silencing but not to wild-type CMV. However, it is known that the 2b-deficient mutants of CMV are also defective in the local virus movement and exhibit enhanced susceptibility to the resistance induced by salicylic acid (43, 44, 48, 49). Therefore, we investigated whether the antiviral flippases regulated the biogenesis of viral siRNAs by Northern blot hybridization, because vsiRNAs serve as both the specificity determinants and the molecular markers for the induction of antiviral silencing (15, 16). The vsiRNAs made by *Arabidopsis* plants are mostly the secondary vsiRNAs amplified by either RDR6 as in CMV-Δ2b–infected plants or both RDR1 and RDR6 as in CMV-2aTΔ2b–infected plants; as a result, the accumulation levels of vsiRNAs are extremely low in both the *rdr6* single-mutant plants infected with CMV-Δ2b and the *rdr1 rdr6* double-mutant plants infected with CMV-2aTΔ2b, even though the 2b-deficient mutants of CMV replicated to high levels in these mutant plants compared with wild-type plants (22, 24, 28). As described previously (24, 28), the DNA oligomer probes were selected according to previous genetic and bioinformatic analyses to detect the negative-strand vsiRNAs, including those that are dependent on or independent of the amplification by RDR1/6 (24, 28). Compared with *Col-0* plants, the accumulation of the 21- and 22-nt vsiRNAs, undetectable in *dcl2 dcl4* plants (Fig. 3*D*) as expected (24, 28), was drastically reduced in *rdr1 rdr6* mutant plants after infection with either CMV-Δ2b or CMV-2aTΔ2b but not with CMV (Fig. 3*D*). Consistent with the published data (22, 24, 28), therefore, the vsiRNAs detected in *Col-0* plants infected with the 2b-deficient mutants of CMV are predominantly RDR1/6-dependent, secondary vsiRNAs and their biogenesis is efficiently suppressed in CMV-infected plants by VSR 2b protein.

We first examined the RDR6-dependent amplification of vsiRNAs induced in plants infected with CMV-Δ2b (24). We found that compared with infection in *Col-0* plants, the accumulation of vsiRNAs was reduced approximately by fourfold in *ala1 ala2* double-mutant plants, which was similar to *rdr1 rdr6* double-mutant plants (Fig. 3*D* and Fig. S3, *Left*). Reduced accumulation of vsiRNAs was also detected in *ala1* and *ala2* single-mutant plants infected with CMV-Δ2b although, by comparison, the vsiRNAs accumulated to lower levels in *ala2* plants than in *ala1* plants (Fig. 4*B*). Because CMV-Δ2b replicated to higher levels in these single- and double-mutant plants than in *Col-0* plants (24), the reduced accumulation of the vsiRNAs in each of the three *Arabidopsis* mutants suggested an independent role for ALA1 and ALA2 in the RDR6-dependent amplification of vsiRNAs to silence CMV-Δ2b.

We further took a genetic approach to determine whether either *ALA1* or *ALA2* regulates antiviral defense independent of antiviral silencing mediated by vsiRNAs amplified by RDR1 and RDR6. To this end, we crossed the *rdr1 rdr6* double mutant with either the *ala1* or *ala2-2* mutant to generate homozygous *ala1 rdr1 rdr6* and *ala2 rdr1 rdr6* triple-mutant plants. We found that the loss-of-function mutation in neither *ALA1* nor *ALA2* enhanced the accumulation of CMV-Δ2b or altered the accumulation of vsiRNAs in the two triple mutants compared with the *rdr1 rdr6* double mutant (Fig. 4 *A* and *B*). Therefore, neither *ALA1* nor *ALA2* was able to restrict CMV-Δ2b infection or enhance the production of vsiRNAs in the absence of the vsiRNA amplification by RDR1 and RDR6. These findings together illustrate that both ALA1 and ALA2 act specifically in the antiviral silencing defense by enhancing the amplification of vsiRNAs and that neither P4-ATPase functions to directly inhibit the replication of CMV-Δ2b.

Consistently, we found that ALA1/2 were also required for the amplification of vsiRNAs to target CMV-2aTΔ2b, known to be mediated by RDR1 in addition to RDR6 (28). For example, although CMV-2aTΔ2b replicated to higher levels in *ala1 ala2* plants than in *Col-0* plants, the amounts of vsiRNAs detected in the double-mutant plants were as low as in *rdr1 rdr6* plants (Fig. 3*D* and Fig. S3, *Right*). Moreover, no major difference in virus accumulation and the amplification of vsiRNAs was detected in *ala1 rdr1 rdr6* and *ala2 rdr1 rdr6* triple-mutant plants compared with the *rdr1 rdr6* double-mutant plants after infection with CMV-2aTΔ2b (Fig. 4*A*). Unlike CMV-Δ2b infection, however, the amplification of vsiRNAs was not compromised in either *ala1* or *ala2* single-mutant plants after infection with CMV-2aTΔ2b (Fig. 4*A*), indicating a redundant role of ALA1 and ALA2 in the RDR1-dependent amplification of vsiRNAs.

Plants infected with wild-type CMV accumulate predominantly RDR-independent primary vsiRNAs due to 2b suppression of vsiRNA amplification (22, 24, 28). We found that *Col-0* and *ala1 ala2* as well as *rdr1 rdr6* plants accumulated similar amounts of vsiRNAs after infection with CMV (Fig. 3*D*), suggesting that ALA1 and ALA2 were dispensable for the biogenesis of the primary vsiRNAs.

Lipid Flippases ALA1 and ALA2 Are Necessary for the Biogenesis of Virus-Activated Host siRNAs. We next investigated whether the identified lipid flippases also played a role in the biogenesis of the endogenous RDR6-dependent transacting siRNAs (tasiRNAs) or RDR1-dependent virus-activated siRNAs (vasiRNAs), which accumulate to levels readily detectable by Northern blotting (5, 6, 12). We found that, similar to microRNA 159 (miR159), tasiRNA 255 accumulated to similar levels in *Col-0* and *ala1 ala2* double-mutant plants (Fig. 3*D*). We also detected no obvious difference in the accumulation of the host tasiRNA between *Col-0* and *ala1 ala2*

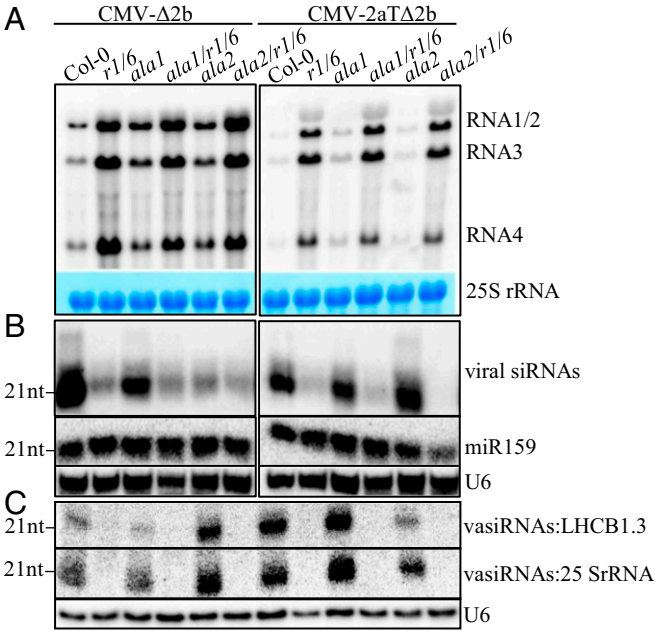

Fig. 4. ALA1 and ALA2 act in RDR1/6-dependent pathways. The accumulation of virus genomic/subgenomic RNAs (*A*), antisense primary and secondary vsiRNAs (*B*), and vasiRNAs targeting LHCB1.3 mRNA or 25S rRNA (*C*) in wild-type and mutant plants 2 wk after infection with CMV-Δ2b or CMV-2aΔT2b was compared by Northern blot analysis. 25S rRNA in the same membrane was stained to show equal loading (*A*), whereas the same set of small RNA samples was also probed for miR159 and/or U6 RNA.

plants after infection with CMV, CMV-Δ2b, or CMV-2aTΔ2b (Fig. 3*D*), suggesting that both ALA1 and ALA2 were dispensable for the biogenesis of tasiRNAs.

Infection with either CMV-2aTΔ2b or wild-type turnip mosaic virus activates RDR1-dependent biogenesis of 21-nt vasiRNAs made by DCL4 to target more than 1,000 protein-coding mRNAs such as photosystem II light-harvesting complex gene 1.3 (LHCB1.3) mRNA as well as 25S, 18S, and 5.8S rRNAs (12). After infection with CMV-2aTΔ2b, the vasiRNAs targeting either LHCB1.3 mRNA or 25S rRNA became highly abundant in *Col-0* plants as expected but remained hardly detectable in *ala1 ala2* plants, similar to the mock-infected *Col-0* plants (Fig. 3*E*), indicating that *ala1 ala2* plants were defective in the induction of vasiRNAs by CMV-2aTΔ2b. A markedly reduced accumulation of the vasiRNAs targeting LHCB1.3 mRNA and 25S rRNA was also detected in *ala2* mutant plants but not in *ala1* mutant plants (Fig. 4*E*). This result suggests a prominent role for ALA2 in the biogenesis of the vasiRNAs.

The biogenesis of the vasiRNAs is not induced in CMV-infected plants (12), in which the amplification of the vsiRNAs by both RDR1 and RDR6 is suppressed by the 2b protein (22, 24, 28). CMV-Δ2b also seemed to be a poor inducer of the vasiRNAs targeting either LHCB1.3 mRNA or 25S rRNA in *Col-0* plants (Fig. 3*E*), which was consistent with an early observation that this mutant virus does not induce RDR1-dependent antiviral RNAi (28). We found that the accumulation of both types of vasiRNAs remained low in *ala1 ala2* (Fig. 3*E*) and *ala1* plants (Fig. 4*C*) after infection with CMV-Δ2b. However, the biogenesis of vasiRNAs was modestly induced in *ala2-1* plants by CMV-Δ2b (Fig. 4*C*), suggesting a different role for ALA2 in the biogenesis of vasiRNAs in response to CMV-Δ2b infection.

Discussion

In this study, we developed a forward genetic screening system in *A. thaliana* for the genetic dissection of the antiviral silencing defense mechanism. Our system screens for gain-of-disease phenotypes in a mutagenized host population after infection with a silencing suppression-defective mutant virus (CMV-Δ2b), which, unlike its virulent wild-type virus, causes only symptomless infection in wild-type plants. We further developed a computational pipeline, mutCAN, for the identification of causal mutations by whole-genome resequencing. In contrast to the available pipeline (45), mutCAN can identify different types of causal mutation, including deletions and insertions as well as single-nucleotide mutations. Moreover, the genetic variations occurring frequently between the laboratory plants and the sequenced reference genome do not interfere with mutation calling by mutCAN. We generated and sequenced the two F2 pools derived from one of the *Arabidopsis avi* mutants identified from the genetic screen. Our analysis of the paired-end reads from the two libraries by mutCAN revealed a deletion of 11 bp in *ALA2* as the causal mutation, which was verified subsequently by independent approaches.

In addition to *ALA2*, an antiviral activity was also shown for *ALA1* from the *Arabidopsis* family of putative phospholipid flippases. We showed that the antiviral function of both ALA1 and ALA2 was abolished by a single amino acid substitution known to inactivate the flippase activity. Several lines of evidence support a specific role for ALA1 and ALA2 in the antiviral silencing defense mechanism, demonstrating the feasibility of using our forward genetic screen for the genetic dissection of antiviral silencing. Whereas both ALA1 and ALA2 restricted the infection of *Arabidopsis* plants with 2b-deficient mutants of CMV, neither was effective against wild-type CMV, indicating efficient inhibition of ALA1/2-mediated antiviral activity by a known suppressor of antiviral silencing (22, 39). We also showed that vsiRNAs accumulated to drastically reduced levels despite the enhanced virus accumulation in the *ala1 ala2* mutant plants compared with wild-type plants after infection with CMV-Δ2b or CMV-2aTΔ2b. Thus, *ala1 ala2* mutant plants exhibited a phenotype similar to that of *rdr1 rdr6* mutant plants known to be defective in the amplification of vsiRNAs (22, 24, 28). Moreover, no difference was detected in the accumulation of both the virus and vsiRNAs in *rdr1 rdr6 ala1* and *rdr1 rdr6 ala2* triple-mutant plants compared with *rdr1 rdr6* double-mutant plants after infection with CMV-Δ2b or CMV-2aTΔ2b, indicating that *ALA1* and *ALA2* restricted virus infection and enhanced the production of vsiRNAs only when there was active vsiRNA amplification by RDR1 and RDR6. These results provide strong genetic evidence that ALA1 and ALA2 act in the same pathway of vsiRNA amplification by RDR1 and RDR6 but do not act as negative regulators of the mutant virus replication (50). Our examination of the biogenesis of vsiRNAs induced by CMV-Δ2b, CMV-2aTΔ2b, or CMV in *ala1 ala2* single- and double-mutant plants further indicates that ALA1/2 enhanced the amplification of vsiRNAs by both RDR1 and RDR6 but had no obvious effect on the biogenesis of the primary vsiRNAs. Notably, we found that ALA1 and ALA2 also enhanced RDR1-dependent biogenesis of the vasiRNAs in the infected plants, a recently characterized class of endogenous siRNAs induced by viruses from two different families of positive-strand RNA viruses (12). These results together indicate that the lipid flippases identified from our genetic screen promote antiviral silencing by enhancing the RDR1/6-dependent biogenesis of vsiRNAs.

It is unknown how lipid flippases enhance the biogenesis of RDR1/6-dependent vsiRNAs and of RDR1-dependent vasiRNAs in the infected plants. Much is known about the mechanism of RNA synthesis by the RdRPs of plant and animal positive-strand RNA viruses (40, 41). For example, brome mosaic virus and tomato bushy stunt virus genomic RNAs replicate inside vesicle-like membrane invaginations (known as spherules) of the endoplasmic reticulum (ER) and peroxisomes, respectively (40, 41). The formation of these spherules is induced in the infected cell after the recruitment of the viral RdRP and host factors to the subcellular membrane microdomains and is correlated with the enrichment of specific phospholipids at the replication sites (51, 52). When expressed alone in tobacco cells, ALA1, ALA2, and ALA3 are all localized to the ER (46, 53). However, unlike ALA3, which can transport phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine (PS), ALA2 displays a narrow substrate specificity of phospholipid transport for PS only (46). An early study also suggested a substrate preference of ALA1 for PS (54). Thus, the specific lipid transporter activity of ALA1 and ALA2 may be necessary to induce PS enrichment at subcellular membrane sites for the formation of similar spherules to synthesize the dsRNA precursors of vsiRNAs and vasiRNAs by the cellular RDR6 and/or RDR1. Interestingly, we found that the function of both ALA1 and ALA2 was dispensable for RDR6-dependent biogenesis of tasiRNAs. This suggests that the synthesis of tasiRNA precursors may involve a distinct P4-ATPase. However, whereas *Arabidopsis* tasiRNAs are produced from only four loci (2, 3, 5, 6), up to 17% of the total endogenous 21-nt RNAs sequenced from the infected *Arabidopsis* plants correspond to the vasiRNAs that target >1,000 loci (12). Similar to vasiRNAs, vsiRNAs are also highly abundant and can amount to up to 30% of the total small RNAs in infected plants (15, 16). Thus, it is possible that only the synthesis of dsRNA precursors of the highly abundant vsiRNAs and vasiRNAs may require the extensive membrane reorganization to form spherules analogous to those for virus RNA replication (40, 41).

Materials and Methods

Plant Materials, Viruses, Infection Assays, and Northern Blot Analysis. Homozygote Salk line pools and the gene-specific T-DNA insertional mutants in Columbia-0 (Col-0) were obtained from the *Arabidopsis* Biological Resource Center (https://abrc.osu.edu). Additional plant and virus mutants used in this work, virus inoculation, and Northern blot detection of virus genomic and subgenomic RNAs and viral siRNAs (and vasiRNAs, tasiRNAs, and miRNAs) are described in *SI Materials and Methods*. Probes and primers used in the study are listed in Table S1.

Gene Cloning by Whole-Genome Sequencing and mutCAN. The F2 population of *ala2-1* generated after backcrossing with *Col-0* was infected with CMV-Δ2b, and ~80 plants either with disease symptoms (mutant pool) or without

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symptoms (wild-type pool) were pooled for genomic DNA extraction. Construction and sequencing of the two DNA libraries and subsequent identification of the causal mutation by mutCAN written in Perl language and available at https://github.com/JinfengLu/mutCAN are described in *SI Materials and Methods*.

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