The epigenetic factor FVE orchestrates cytoplasmic SGS3-DRB4-DCL4 activities to promote transgene silencing in Arabidopsis

Di Sun1†, Yanjun Li1†, Zeyang Ma1, Xingxing Yan1, Niankui Li1, Baoshuan Shang1, Xiaomei Hu1, Kai Cui1,2, Hisashi Koiwa3, Xiuren Zhang1*

Posttranscriptional gene silencing (PTGS) is a regulatory mechanism to suppress undesired transcripts. Here, we identified Flowering locus VE (FVE), a well-known epigenetic component, as a new player in cytoplasmic PTGS. Loss-of-function fve mutations substantially reduced the accumulation of transgene-derived small interfering RNAs (siRNAs). FVE interacts with suppressor of gene silencing 3 (SGS3), a master component in PTGS. FVE promotes SGS3 homodimerization that is essential for its function. FVE can bind to single-stranded RNA and double-stranded RNA (dsRNA) with moderate affinities, while its truncated form FVE-8 has a significantly increased binding affinity to dsRNA. These affinities affect the association and channeling of SGS3-RNA to downstream dsRNA binding protein 4 (DRB4)/Dicer-like protein 2/4 (DCL2/4) complexes. Hence, FVE, but not FVE-8, biochemically enhances the DRB4/DCL2/4 activity in vitro. We surmise that FVE promotes production of transgene-derived siRNAs through concertedly tuning SGS3-DRB4/DCL2/4 functions. Thus, this study revealed a noncanonical role of FVE in PTGS.

INTRODUCTION

Posttranscriptional gene silencing (PTGS) is a regulatory mechanism that suppresses the expression of endogenous genes while also degrading invasive RNAs such as transgene-derived and viral transcripts. PTGS is guided through microRNAs (miRNAs) and small interfering RNAs (siRNAs) (1–3). miRNAs originate from primary transcripts (pri-miRNAs) through sequential cleavage by microprocessor that comprises Dicer-like protein 1 (DCL1), double-stranded RNA (dsRNA) binding protein 1 (DRB1/HYL1) and Serrate (SE) (4, 5). Subsequently, miRNAs are loaded into Argonaute proteins (AGOs) to form RNA-induced silencing complexes (RISCs) that repress gene expression through target cleavage and/or translational repression (3). The mode of action of siRNAs is similar to that of miRNAs. However, a prerequisite step for initiating siRNA-mediated RNA silencing in plants is the conversion of single-stranded RNA (ssRNA) substrates to dsRNAs, which is accomplished by RNA-dependent RNA polymerase 6 (RDR6) and suppressor of gene silencing 3 (SGS3) (6, 7). SGS3 functions as a homodimer (8) and has been reported to stabilize the primary small RNA (sRNA) cleavage product for dsRNA synthesis (9, 10). In turn, dsRNAs are processed by DCL2 or DCL4 together with its partner DRB4 to 21- to 22-nucleotide (nt) siRNAs, which are eventually loaded into AGO1 to destroy target RNAs (10–12). As a special situation, inverted-repeat or hairpin PTGS is triggered by self-folded dsRNA from ssRNA substrates that can circumvent the RDR6/SGS3 activity. However, the inverted-repeat/hairpin-derived siRNAs could, in turn, extend beyond the inverted-repeat region to produce secondary siRNAs, and this process entails RDR6/SGS3 function (13–15).

Notwithstanding, the biochemical partners and functional bridges of RDR6/SGS3-DCL4/DRB4 are far less understood compared with microprocessor.

Retinoblastoma-associated protein 48 (RbAp48) functions through multiple epigenetic complexes to affect tumorigenesis, cytoskeletal organization, age-related memory loss, and cardiomyocyte hypertrophy (16–18). A plant homolog, FLOWERING LOCUS VE (FVE), has been known to regulate flowering timing and cold response sensitivity (19, 20). Similar to RbAp48, FVE acts as a structural scaffold for the assembly of large complexes exemplified by histone deacetylation and demethylation machinery in epigenetic silencing of protein-coding genes (21). In addition, FVE also contributes to transcriptional silencing of transposons as AtMu1 and AtSN1 through RNA-dependent DNA methylation mechanisms (22). Despite the well-characterized functions at the epigenetic level, the involvement and mechanistic function of FVE/RbAp48 in PTGS are unclear.

In this study, a new allele of fve (fve-8) was identified in a forward genetic screen that is compromised in transgene-PTGS pathway. Loss-of-function mutants of fve have reduced siRNA production from sense and inverted-repeat transgenes. FVE is localized in the nucleus and cytoplasm, but the cytoplasmic portion of FVE fully rescued the fve defect in PTGS. We found that FVE, but not its truncated mutant FVE-8, could interact with SGS3 and promote its homodimerization. Unexpectedly, FVE binds to ssRNAs and dsRNAs with moderate affinities, while FVE-8 shows a significant increase in dsRNA binding activity. These features affect the association and routing of SGS3/RNA to DRB4/DCL2/4 complexes. In turn, FVE promotes DRB4/DCL2/4 activity in generating siRNAs, whereas FVE-8 suppresses the same reaction in vitro. We concluded that FVE synchronizes the RDR6/SGS3-DRB4/DCL2/4 channel to promote siRNA production, whereas FVE-8 hijacks dsRNA substrates to prevent the downstream processing. Thus, this study reveals a noncanonical role of the epigenetic factor FVE in PTGS and sheds light on a new regulatory layer in transgene silencing.
RESULTS
A new mutant of attenuated RNA silencing (ars) is identified from a forward genetic screen
We previously reported that AGO10 antagonizes AGO1 silencing activity by sequestering miR165/166 to regulate shoot apical meristem development (23, 24). To identify new genes that regulate miR165/166 activity, we designed a reporter system containing a section of genomic PHABULOSA (PHB) (exon4, intron4, and exon5), which harbors the miR165/166 complementary sequence after splice. The miR165/166 target site is flanked by Green Fluorescent Protein (GFP) and Luciferase (LUC) genes and driven by the native AGO10 promoter (PAGO10-GFP-PHB-LUC) (Fig. 1A). The construct was transformed into Arabidopsis thaliana Landsberg (Ler) et al., Sci. Adv. 2021; 7 : eabf3898     4 August 2021
Green Fluorescent Protein
The miR165/166 target site is flanked by Green Fluorescent Protein (GFP) and Luciferase (LUC) genes and driven by the native AGO10 promoter (PAGO10-GFP-PHB-LUC) (Fig. 1A). The construct was transformed into Arabidopsis thaliana Landsberg (Ler) et al., Sci. Adv. 2021; 7 : eabf3898     4 August 2021. The miR165/166 activity by sequestering miR165/166 to regulate shoot apical meristem development (23, 24). To identify new genes that regulate miR165/166 activity, we designed a reporter system containing a section of genomic PHABULOSA (PHB) (exon4, intron4, and exon5), which harbors the miR165/166 complementary sequence after splice. The miR165/166 target site is flanked by Green Fluorescent Protein (GFP) and Luciferase (LUC) genes and driven by the native AGO10 promoter (PAGO10-GFP-PHB-LUC) (Fig. 1A). The construct was transformed into Arabidopsis thaliana Landsberg (Ler) et al., Sci. Adv. 2021; 7 : eabf3898     4 August 2021.

We previously reported that AGO10 antagonizes AGO1 silencing activity by sequestering miR165/166 to regulate shoot apical meristem development (23, 24). To identify new genes that regulate miR165/166 activity, we designed a reporter system containing a section of genomic PHABULOSA (PHB) (exon4, intron4, and exon5), which harbors the miR165/166 complementary sequence after splice. The miR165/166 target site is flanked by Green Fluorescent Protein (GFP) and Luciferase (LUC) genes and driven by the native AGO10 promoter (PAGO10-GFP-PHB-LUC) (Fig. 1A). The construct was transformed into Arabidopsis thaliana Landsberg (Ler) et al., Sci. Adv. 2021; 7 : eabf3898     4 August 2021.

To test whether fve-8 up-regulates LUC expression through a TGS pathway, we first treated fve-8 with 5-aza-2′-deoxycytidine (aza-dc), a chemical that can abolish DNA cytosine methylation. The transposon element AtMu1 served as a positive control, as its expression is suppressed by FVE at the TGS level (25, 26). AtMu1 displayed significantly increased expression in fve-8 compared with E5-4, but this increase was abolished by the aza-dc treatment. However, this scenario was not observed with LUC signal and LUC transcripts, inferring that the increased LUC expression in fve-8 may not be through suppression of the TGS pathway (Fig. 2, A and B). We also performed an histone 3 lysine 27 tri-methylation (H3K27me3)–chromatin immunoprecipitation (ChIP) assay with FLC and EF1α as positive and negative controls, respectively (27). We did not observe a noticeable difference of H3K27me3 signal in the LUC gene body between fve-8 and E5-4 lines (Fig. 2C). A marginally reduced H3K27me3 ChIP signal in the AGO10 promoter and 5′ untranslated region was detected in fve-8, suggesting that FVE might have a repressive effect on the expression of endogenous AGO10 and arguably transgene LUC expression at certain levels. This result is in agreement with a previous report that endogenous AGO10 promoter locus contains relatively lower levels of CHG and CHH (where H is any base except G) DNA methylation in fve compared with WT plants (28). Notwithstanding, the AGO10 protein level was not affected (fig. S2A), indicating that the enhanced LUC signal in fve-8 did not result from the possible change of AGO10 ability to decay miR165/166 (23, 24).

To further investigate whether FVE regulated the LUC transgene through TGS, we conducted RNA polymerase II ChIP quantitative reverse transcription polymerase chain reaction (qRT–PCR) assays. We consistently observed that the occupancy of polymerase II at LUC gene body was not significantly altered in fve-8 compared with that of E5-4 (Fig. 2D). Furthermore, we isolated nuclear RNA and quantified the amount of nascent LUC transcript. In contrast to FLC, RT–PCR analysis did not reveal an obvious difference of LUC transcript levels in the nucleus of fve-8 relative to E5-4 (Fig. 2E). These results further indicated that fve-8 does not affect the LUC reporter expression through the nuclear TGS pathway.

Because the above results suggested that FVE has an additional non-nuclear function, we examined the subcellular localization of FVE protein. FVE protein not only was highly enriched in the nucleus but also was distributed in the cytoplasm (Fig. 3A and fig. S2C). Computational analysis predicted the presence of a nuclear localization signal (NLS) at the N terminus of FVE (GPKKRGRK) (19, 29). To test ex-nuclear function of FVE, we replaced the FVE NLS with a nuclear export signal (NES) (fig. S2B) (30) and transfected 35S-YFP-cFVE (the coding sequence of FVE) and 35S-YFP-cFVENES constructs into Nicotiana benthamiana leaves and Arabidopsis protoplasts. The confocal microscopy imaging showed that unlike yellow fluorescent protein (YFP)–FVE, YFP-FVENES was exclusively accumulated in the cytoplasm (Fig. 3A and fig. S2C). To further validate this result, we generated stable fve-8;35S-Flag-4Myc (FM)–cFVE and fve-8;35S-FM-cFVENES transgenic lines. Cell fractionation assays showed that FVE was present in both nuclei and cytoplasm of fve-8;35S-FM-cFVE lines, whereas the protein was only detected in the cytoplasm of fve-8;35S-FM-cFVENES lines (fig. S2, D and E). Notably, YFP-FVE amount in the nucleus appeared higher in confocal imaging than in cell fractionation experiments, likely because the fluorescent signal was concentrated in the nucleus but diluted in the cytoplasm in the confocal imaging. The late-flowering phenotype in fve-8 was only recovered by fve-8;35S-FM-cFVE lines but not fve-8;35S-FM-cFVENES lines despite the fact that FVE protein levels were abundant in the two types of transgenic lines (Fig. 3, B and C). However, both constructs could rescue the LUC signal in fve-8 to a normal level in E5-4. These results indicated that FVE has separate functions in the nucleus and cytoplasm, and
Fig. 1. Isolation of fve-8 as a new mutant with attenuated RNA silencing through a genetic screening. (A) Schematic construct of E5-4 used for the EMS screening in this study. E4 and E5, the fourth and fifth exons of PHB, respectively; \( P_{AGO10} \) the native promoter of AGO10 locus; Nos Ter, nopaline synthase terminator. (B) The established mutations in miRNA and siRNA pathways increased LUC luminescence. Five-day seedlings of Ec5-4/se-2, Ec5-4/hyl1-2, and Ec5-4/rdr6-11 were photographed in bright field (top), under charge-coupled device (CCD) camera for LUC signal (middle), and under regular camera (bottom). The crossing lines of M17-1 with sgs3-1 displayed similar patterns (see also fig. S1A). Scale bars, 0.5 cm. (C) Mutation in FVE caused increased LUC luminescence. Five-day seedlings of ars1-1 (fve-8), E5-4, and fve-8; gFVE were photographed in bright field (top) and under CCD camera for LUC signal (middle). The signals were displayed by LightField software. Scale bars, 1 cm. Bottom: Six-week-old ars1-1/fve-8 and complementation lines displayed later and normal flowering phenotypes, respectively. Scale bars, 1 cm. (D) RNA blot analysis showed LUC transcript accumulated in ars1-1/fve-8 and complementation lines displayed later and normal flowering phenotypes, respectively. (E) Next-generation mapping (NGM) analysis of \( F_2 \) mapping population delineated eight candidates in 1.3 Mb of chromosome 2. \( \text{bp} \), base pairs. (F) Top: The gene structure of FVE includes untranslated regions (gray boxes), exons (black boxes), and introns (lines). The fve-8 mutation (triangle) is labeled. Bottom: The protein schematic shows a low complexity region (light gray boxes), 6 × WD40 domains (dark gray boxes), and putative nuclear localization signal (NLS; black box).
there must be a new function of FVE NES in cytoplasmic RNA silencing. To further validate this concept, we also generated FVE native promoter–driven complementation lines (fve-8; P_FVE-cFVE and fve-8; P_FVE-cFVE NES) and determined LUC luminescence in parallel. Again, a majority of transformants of the two constructs could recover the LUC signal in fve-8 to the level in E5-4 (Fig. 3D). In agreement with the changes of the LUC signal, LUC transcripts were significantly reduced in the two kinds of complementation lines (Fig. 3E). This result indicated that cytoplasmic FVE is functionally sufficient to silence LUC transgene. Notably, we observed that LUC expression was marginally reduced in fve-8; P_FVE-cFVE NES compared with that in fve-8; P_FVE-cFVE, which was likely due to variations of FVE expression in independent lines (Fig. 3E).

To test whether FVE NES had a similar impact on endogenous AGO10, we examined the expression of AGO10, the assumed proxy of the LUC transgene at an epigenetic status. AGO10 level was approximately twofold higher in fve-8 compared with E5-4 and was fully rescued in fve-8; P_FVE-cFVE. However, the AGO10
Fig. 3. FVE regulates LUC expression through a PTGS pathway. (A) Confocal microscope imaging showed subcellular localizations of YFP-FVE and YFP-FVENES. The proteins were expressed in leaves of N. benthamiana. Scale bars, 50 μm. (B) Western blot analysis of FM-cFVE and FM-cFVENES accumulation in overexpression lines using an anti-Myc antibody. Coomassie brilliant blue (CBB)–stained ribulose-1,5-bisphosphate carboxylase-oxygenase (rubisco) is an internal control. (C) FVENES different from FVE, could efficiently rescue LUC luminescence but not the late-flowering phenotype of fve-8 to the parental level. Five-day-old seedlings in (B) were photographed in bright field (top) and under CCD camera for LUC signal (middle). Scale bars, 1 cm. The signals were displayed by LightField software. Bottom: Six-week-old plants were photographed. Scale bars, 1 cm. (D) FVENES could efficiently rescue LUC luminescence in fve-8 to the parental level. Five-day-old seedlings or T1 transformants of the indicated constructs were photographed under CCD camera. Scale bars, 2 cm. (E) qRT-PCR analysis showed that LUC transgene and endogenous AGO10 were regulated differently by FVE and FVENES. Five-day-old seedlings of fve-8, ES-4, and indicated transgene lines were used for the assay. EF-1α is an internal control. The asterisks indicate significant difference between the mutants and ES-4 with ±SD (n = 3) biologically independent replicates. **P < 0.01; ***P < 0.001; t-test.
level was increased approximately three- or fourfold in fve-8; P_{FVE- cFVE\_NES} transformants (Fig. 3E). These results indicated that the nuclear-localized FVE, but not the cytoplasmic FVE, repressed the transcription of AGO10, but not that of LUC transgene at an epigenetic level. This observation also suggested that the slightly reduced H3K27me3 mark in fve-8 would be likely from the promoter of endogenous AGO10 rather than the one for LUC transgene (Fig. 2C).

This result further suggested that FVE affects native genes and transgenes differently at the epigenetic level. One plausible hypothesis for the enhanced AGO10 expression in the FVE\_NES complementation lines is that FVE\_NES might hijack nuclear FVE cofactors from the nucleus to the cytoplasm and thereby partially releasing the epigenetic suppression at the native AGO10 promoter. Together, we concluded that FVE has a noncanonical role in regulating LUC expression at a PTGS level in the cytoplasm, in addition to its classic roles in epigenetic regulation of native genes in the nucleus.

**fve-8 has a negligible impact on biogenesis of endogenous sRNAs**

Given that cytoplasmic FVE could promote PTGS, we evaluated the impact of fve-8 mutations on the miRNA and trans-acting siRNA (ta-siRNA) pathways. sRNA sequencing (sRNA-seq) did not reveal significant changes in the accumulation of most miRNAs and ta-siRNAs in fve-8 compared with E5-4 plants (Fig. 4A). The results were conclusively validated by sRNA blot assays (Fig. 4, B and C). Similarly, there were no significant changes in the accumulation levels of most pri-miRNAs (Fig. 4D) and miRNA targets (Fig. 4E).

We next examined whether the attenuated LUC signal was due to miR166-mediated translation repression. Isogenic fve-8 (−/−) and heterozygous (+/−) lines containing 35S-PHB-Myc, 35S-PHBm-Myc, or P_{MIR166\_CNA-Myc} were generated. The accumulation of transgenic proteins was similar between fve-8 (−/−) and fve-8 (+/−) (Fig. 4F). These results indicated that the enhanced LUC signal in fve did not result from defects of miR165/166 activity. In addition, the expression levels of key components in miRNA and RNA decay pathways were not affected except for noticeable increases in XRN2 and ESP3 levels (Fig. 5, A and B). Thus, it is unlikely that LUC activity in fve-8 was caused by indirect effect on the miRNA and RNA decay pathways.

**FVE promotes the accumulation of transgene-derived siRNA**

We investigated whether fve-8 affects the transgene-PTGS pathway. In the sRNA-seq dataset, we found that the 21- to 22-nt sRNAs derived from GFP-PHB-LUC transgene were clearly reduced in fve-8 compared with E5-4 (Fig. 5, A and B). This result suggested that FVE decreases LUC accumulation through regulating transgene-derived siRNAs. To further test this, we crossed fve-8 with L1 line, which is an established model system for a sense transgene-PTGS pathway with a silenced β-glucuronidase (GUS) transgene reporter (7). Genotyping was performed for the F2 population with a pair of derived cleaved amplified polymorphic sequences (dCAPS) primers designed to distinguish fve-8 from WT FVE (Fig. 5C). An sRNA blot showed that the amount of 21/22-nt-GUS loci-derived siRNAs in fve-8 (−/−) decreased compared with that in heterozygotes (+/−) or WT background (+/+) (Fig. 5D). Consistently, GUS activity was higher in fve-8 (−/−) than that in fve-8 heterozygotes (+/−) or WT (+/+) lines (Fig. 5E). Thus, these results indicated that FVE is indeed engaged in the transgene-PTGS pathway.

To test whether FVE affects the silencing triggered by inverted-repeat transgene, we used CRISPR-Cas9 technology to knock out FVE in a well-known hpCHS (hairpin chalcone synthase) reporter line that lacks flavonoid pigments due to the silencing of CHS (Fig. 5F) (31). At least two biallelic homozygous mutants were obtained (Fig. 5G). The CHS siRNA level in the cas9-fve background was remarkably lower than that in FVE (+/+ ) background (Fig. 5H and fig. S3C). Correspondingly, the leaf color of cas9-fve mutants became darker than that of the parental line due to the release of the silenced CHS (Fig. 5I). Similarly, the seed coat of cas9-fve mutants turned brown, indicative of flavonoid production, whereas the parental line produced light yellow seed coat pigmentation (Fig. 5I). Both leaf and seed phenotypes indicated that the endogenous CHS expression level was increased in the fve mutants compared with the parental line.

In conclusion, FVE participates in the silencing pathway triggered by both sense and inverted-repeat transgenes and reduces transgene siRNA production. One possibility for recovering the fve mutant using the miRNA-targeted readout is likely because the transgene construct harbors a large section of foreign transgenes (GFP-LUC) that is highly sensitive to transgene silencing. Another likely reason is that we focused on the mutants with relatively normal morphology and avoided the lines with severe developmental defects (i.e., ago1, hy11, and se-like mutants), which are often controlled through the miRNA pathway.

**FVE interacts with SGS3 and promotes its homodimerization**

Next, we hypothesized that FVE might target key components in the transgene-PTGS pathway. To test this, we conducted a yeast two-hybrid (Y2H) screening of more than a dozen candidates involved in RNA silencing (Fig. 6A and fig. S4A). We found that FVE specifically interacted with SGS3. We then tested several truncated forms of SGS3 with the N-terminal region, the zinc finger (ZF) domain, the rice gene X and SGS3 (XS) domain, and the coiled-coil (CC) domain. Y2H assays showed that FVE interacted with 219 to 450 amino acid residues of SGS3, which harbors the ZF and XS domains (fig. S4B). To further validate the FVE-SGS3 interaction, we conducted Co-IP assays using fve-8; FVE\_FM-gFVE transgenic lines. We immunopurified FVE complexes using an anti-Flag antibody and readily recovered SGS3 protein in the immunoprecipitates. The addition of ribonuclease A (RNase A) did not disrupt the interaction of FVE and SGS3, indicating that their interaction is RNA independent (Fig. 6B). We also conducted bimolecular fluorescence complementation (BiFC) and LUC complementation imaging (LCI) assays (Fig. 6C and fig. S4C). For the BiFC, we cotransfected N-terminal fragment of YFP (nYFP)-FVE and C-terminal fragment of YFP (cYFP)-SGS3 into Arabidopsis protoplasts with nYFP-SGS3 and cYFP-SGS3 as a positive control. Again, we observed YFP fluorescence complementation between FVE and SGS3, but not for other combinations (Fig. 6C). In the LCI, FVE displayed LUC complementation with SGS3, as did the positive control of AGO1 and cucumber mosaic virus (CMV) 2b (32), but to a lesser extent (fig. S4C). Collectively, these results indicated that FVE interacts with SGS3 and is targeted to the cytoplasmic granules. FVE-8, a truncated form of FVE encoding the N-terminal 413 amino acid residues and missing the last two WD40 (trp-asp dipeptide) domains (FVE\_5+6), lost its interaction with SGS3 (Fig. 6A), suggesting that only a full-length FVE can interact with SGS3. We also noticed that FVE is monomeric, whereas FVE-8 could form a dimer or oligomer (Fig. 6A). Furthermore, FVE-8 could not interact with FVE (Fig. 6A), suggesting that the C-terminal part of FVE might harbor an autoinhibitory domain, preventing self-interaction under normal conditions.
We examined whether the fve-8 mutation altered SGS3 protein levels, but this possibility was disproved by Western blots (fig. S4D). Next, we investigated whether fve-8 affected SGS3 cellular compartmentation. The 35S-driven YFP-SGS3 accumulated in granule-like foci in the transfected protoplasts of fve-8, E5-4, and fve-8; gFVE lines (fig. S5, A and B), reminiscent of the SGS3 foci reported previously (8, 33). The numbers of foci per cell were comparable in those lines, indicating that the fve-8 mutation might not change the cellular localization of SGS3 (fig. S5A). However, a BiFC assay for SGS3 homodimerization (8) revealed that the number of foci for SGS3 homodimers per protoplast was significantly reduced in fve-8 compared with either E5-4 or fve-8; gFVE (Fig. 6D). These results indicated that fve-8 affects SGS3 homodimerization and, consequently, SGS3-mediated function in vivo. To further investigate this, we performed yeast three-hybrid (Y3H) and evaluated the growth rate of transfected colonies as a proxy of SGS3-SGS3 homodimer level. In

Fig. 4. FVE has a negligible impact on the endogenous PTGS pathway. (A) sRNA-seq analysis showed sRNA distributions in fve-8 and E5-4 with relatively stable (0.67 ≤ fve-8/E5-4 ≤ 1.5, gray), increased (fve-8/E5-4 > 1.5, orange), and decreased (fve-8/E5-4 < 0.67, green) expression. The x and y axes indicate the logarithms of normalized expression levels of sRNAs. The top-left pie in the top left indicates the numbers of different categories of sRNAs. See also table. S2. (B and C) sRNA blot analyses of selected miRNAs (B) and ta-siRNAs (C) in the fve mutants and WT plants. U6 is a control. In (B) and (C), the experiments were independently repeated with similar results. In (C), statistics of three replicates (R) was shown at the bottom. (D and E) RNA-seq analysis showed that the expression of most of MIR genes (D) and sRNA target genes (E) was not affected in fve-8. The x and y axes indicate the logarithms of the normalized gene expression levels in E5-4 and fve-8, respectively. The pie in the top left indicates the numbers of different categories of MIR genes (D) and sRNA target genes (E). See also table. S2. (F) Western blot analyses showed that protein levels of several miRNA targets were not changed in F2 of segregated FVE (+/−) and FVE (−/−) using an anti-Myc antibody. CBB staining of rubisco is a control.
this system, the addition of methionine is used to suppress FVE expression driven by a promoter Met23. We observed that the inclusion of FVE could promote colony growth in the combinations of SGS3-FVE-SGS3 relative to SGS3-SGS3 in the absence of methionine. However, the addition of methionine eliminated the growth advantage of the SGS3-FVE-SGS3 cells (Fig. 6E and fig. S5C). This result further validated that FVE could facilitate SGS3-SGS3 homodimerization. This conclusion is also supported by the in vitro pull-down assay in which the application of FVE [but not FVE-8 or small ubiquitin-like modifier (SUMO) protein] enhanced the interaction between maltose binding protein (MBP)–SGS3 and glutathione S-transferase (GST)–SGS3 (Fig. 6F and fig. S5D).

**FVE does not affect RDR6 activity in vitro**

SGS3 and RDR6 interact in specific cytoplasmic granules and form SGS3/RDR6 bodies (33). To investigate whether FVE influences polymerase activity of RDR6, we used recombinant RDR6, SGS3, FVE, and FVE-8 from either *Escherichia coli* or a baculovirus-insect

---

**Fig. 5. Transgene-derived siRNA is decreased in fve-8 compared to WT.** (A) The Integrative Genomics Viewer (IGV) graphs showed the reads distribution of 21- to 22-nt siRNAs in the GFP-PHB-LUC gene body. Two independent biological repeats were sequenced for each material. (B) Box plot showed reduced 21- to 22-nt siRNA (reads per million) mapped to the GFP-PHB-LUC in fve-8. Lines in the middle of boxes indicate mean values from two replicates. (C) Genotyping of different lines of L1 × fve-8 F2 materials by PCR with dCAPS primers, followed by digestion by Bam HI. (D) siRNA blot assay of GUS-mapped siRNAs in 10-day-old F2 seedlings genotyped in (C). U6 is a loading control. This assay was performed with three groups of isolated FVE (+/) and FVE (−/−) F2 materials. (E) GUS staining assay with 10-day-old seedlings analyzed in (D). Scale bars, 1 cm. At least three independent crossed lines were tested and showed similar results. (F) Diagram shows the guide RNA–targeted sites in FVE transcript using the CRISPR-Cas9 system. (G) Sequence results of two independent 35S-hpCHS; cas9-fve mutants. The edited DNA sequences were shown. Dashes in alleles and red nucleotides indicate deletion and insertion, respectively. (H) siRNA blot assay showed that transgenic CHS-mapped siRNAs were reduced in the fve mutants in F2 plants genotyped in (G). U6 is a loading control. Three biological repeats were conducted with similar results (see also fig. S3C). (I) CHS loss-of-function specific color phenotypes of 45-day-old plants (top) and seeds (bottom) from the lines used in (H). Scale bars, 0.2 cm.
Fig. 6. FVE interacts with SGS3 and promotes its homodimerization. (A) Y2H screening showed the FVE-SGS3 interaction. SE and ARABIDOPSIS TRITHORAX-RELATED PROTEIN 5 (ATXR5) were used as positive control. AD, galactose-responsive transcription factor (GAL4) activation domain; BD, DNA binding domain; FVE 5+6, truncated FVE with WD40 domains #5 and #6. (B) Co-IP validated the FVE-SGS3 interaction. IP was performed with an anti-Flag antibody. Western blot assay was performed with the indicated antibodies. The experiment was independently repeated with similar results. (C) Confocal imaging showed the interaction between FVE and SGS3. cYFP and nYFP without fusion proteins serve as negative controls. nYFP-SGS3 and cYFP-SGS3 serve as a positive control. Scale bars, 10 μm. (D) BiFC of YFP assays showed that FVE promotes homodimerization of SGS3. Box plot showed numbers of SGS3 dimer foci per cell. The line in the middle of the box refers to the median. The whiskers are drawn from minimum to maximum percentiles. Individual data points are shown. *P < 0.05; ***P < 0.001; t-test. (E) Y3H assay showed that FVE promoted the interaction between pBridge-SGS3 and AD-SGS3. The colonies of pBridge-SGS3-FVE-AD-SGS3, but not those of pBridge-AD-SGS3, grew in the dilution of 10⁻² without Met. SE and DCL1 serve as positive control. See also fig. S5B and Materials and Methods. (F) In vitro pull-down assay showed that FVE promoted SGS3 homodimerization. GST-ATXR5, His-SUMO, and FVE-8 serve as negative controls. Left: Input. Top right: Bead-recovered MBP-SGS3 by CBB staining. Bottom right: Western blot analysis using an anti-GST antibody (see also fig. S5C).
cell system and established an in vitro RDR6/SGS3 reconstitution system (Fig. 7, A and B). After the reaction, the samples were digested with proteinase K and then vigorously washed by phenol and chloroform before fractionation in a urea–polyacrylamide gel electrophoresis (PAGE) gel. Michaelis-Menten kinetics analysis showed that the RDR6/SGS3 activity was not significantly affected by FVE. In contrast, FVE-8 appears to have some marginally inhibitory effect on the RDR6/SGS3 activity in vitro (Fig. 7, C to E). We next hypothesized that the RDR6/SGS3 complex entails some endogenous cellular cofactors for optimized function. To test this, we immunoprecipitated...
hemagglutinin (HA)–tagged RDR6 using N. benthamiana based on previous reports (Fig. 7F) (34, 35) and recondoned the assay. This time, the samples were directly denatured and fractionated in the urea-PAGE without prior protein digestion. Again, we did not observe an enhanced effect of FVE on RDR6/SGS3 performance in vitro (Fig. 7G). Unexpectedly, an unusually strong signal was detected in the reaction mixture containing FVE-8 but no other proteins. A closer examination of the urea-PAGE gel revealed that the shorter RNA products disappeared in the FVE-8–containing reaction (Fig. 7G), suggesting that FVE-8 might retain RNA transcripts produced by RDR6/SGS3.

**FVE and FVE-8 bind to RNA**

To further determine whether FVE and FVE-8 associated with RNA in vitro, we performed electrophoretic mobility shift assays (EMSA). EMSAs showed that FVE could bind to ssRNA with a dissociation constant ($K_d$) of 145.5 ± 13.5 nM (Fig. 8, A and B, and fig. S6, A and B). FVE could also bind to dsRNA with an apparent $K_d$ of 143.5 ± 4.0 nM (Fig. 8, C and D, and fig. S7, A and B). These results were unexpected, as RbAp48 has not been previously reported to bind to RNA. To further confirm this observation, we performed ribonucleoprotein IP (RIP) experiments in which we cotransfected 35S-FM-FVE with 35S-LUC or 35S-hpLUC constructs in N. benthamiana, respectively. The RIP result showed that $K_d$ was recovered in the FVE immunoprecipitates, but not in the control, suggesting that FVE is indeed an RNA binding protein in vivo (Fig. 8E).

Similar to FVE, FVE-8 bound to ssRNA with a moderate binding affinity ($K_d$ = 149.3 ± 76.0 nM) (Fig. 8, F and G, and fig. S6, C and D). Different from a single-shifting band of FVE-ssRNA binding, there were two shifting bands of FVE-8–ssRNA in the EMSA, which is reminiscent of the association of dimerized HYL1 with RNA (5). FVE-8 displayed a substantially increased binding affinity to dsRNA with an apparent dissociation constant app$K_d$ = 5.3 ± 0.3 nM and $h$ = 1.997 (Fig. 8, H and I, and fig. S7, C and D). The $h$ value and the sigmoidal FVE-8–dsRNA binding curves suggested cooperativity between multiple nucleic acid binding sites in FVE-8 in substrate binding. This result was consistent with the fact that FVE-8 could form dimer or oligomers in vitro and in vivo.

A previous study showed that SGS3 bound to dsRNA but not ssRNA (36). We revisited this assay and found that SGS3 could indeed bind to dsRNA with a binding affinity of app$K_d$ = 30.8 ± 1.4 nM (Fig. 8, J and K, and fig. S7, E and F). For ssRNA binding, SGS3 showed a complicated scenario. A subtle shift was observed, but the bound ribonucleoprotein complexes were quickly dissociated and could not reach a complete shift (Fig. 8L and fig. S6, E and F), suggesting that SGS3 has a very low binding affinity for ssRNA.

Given that FVE interacts with SGS3, we examined whether these constituents could synergistically cooperate in binding to RNA. EMSAs showed that coincubation of FVE and SGS3 with ssRNA resulted in a shifting pattern different from the mobility of either FVE-ssRNA or SGS3-ssRNA complexes (Fig. 8M and fig. S6, G and H). These results suggested that FVE-SSG3-ssRNA can form a new stable complex with distinct electrophoretic mobility. In contrast, coincubation of FVE-8 and SGS3 with ssRNA displayed a similar mobility-shifting pattern to those of either SGS3-ssRNA or FVE-8–ssRNA complexes (Fig. 8M and fig. S6, G and H). Notably, the addition of FVE-8 to FVE-ssRNA or FVE-SSG3-ssRNA complex did not alter the mobility shift patterns. These results suggested that FVE (but not FVE-8) could promote SGS3-ssRNA binding and further implicate that FVE could facilitate and strengthen SGS3 association to the transgene transcripts to produce dsRNA substrates in vivo.

For the binding to dsRNA, FVE and SGS3 appeared to be in the same ribonucleoprotein complex. However, regardless of which protein was co-incubated with FVE-8, the shifting pattern of the mobility was always identical to that of FVE-8–dsRNA alone, which clearly relates to its predominant strong affinity to dsRNA compared with other proteins (Fig. 8N and fig. S7G). Although the differential RNA binding patterns by the combinations of the proteins above do not appear to affect dsRNA synthesis in the RDR6/SGS3 assays in vitro, they likely affect their function in vivo and the downstream step of siRNA synthesis.

**FVE promotes while FVE-8 impedes DCL2/4 activity in vitro**

The dsRNA produced by RDR6/SGS3 are routed to DRB4/DCL2/4 complexes for further processing. DRB4 serves as an auxiliary factor of DCL4 (37), similar to DRB1 with DCL1 in producing miRNAs (4, 5). Given that FVE could interact with DRB2, we next hypothesized that FVE might interact with DRB4, a homolog of DRB2 (38), to regulate DRB4/DCL2/4 activity. Both FVE and FVE-8 showed interaction with DRB4 in the LCI assay (Fig. 9A and fig. S8A). The interaction between FVE and DRB4 was further confirmed by the Co-IP assay with protein extracts from transfected N. benthamiana leaves (Fig. 9B) (39). Y2H and LCI assays for SGS3 and DRB4 also detected their interaction in vivo (Fig. 9, C and D, and fig. S8B), which suggested that the FVE/SGS3/dsRNA ribonucleoprotein complex could be channeled to DRB4/DCL2/4 machinery through FVE/SGS3/DRB4 interaction. This result is consistent with the previously reported partial colocalization of SGS3 with DCL4 complexes (40).

Last, we investigated the impact of FVE and FVE-8 on DRB4/DCL2/4 activity. 3HA-DCL2 and 3HA-DCL4 were immunoprecipitated from N. benthamiana leaves that were transfected with corresponding constructs, respectively (fig. S8, C and D). DRB4 was purified from E. coli (fig. S8E). We performed in vitro DCL2/4 assays by incubating IP products and dsRNA substrates with different combinations of FVE or FVE-8. The incubation with FVE consistently enhanced the DCL2 and DCL4 activity, while the incubation with FVE-8 remarkably quenched the activities of DCLs (Fig. 9, E to H, and fig. S8, F and G). Our results suggested that FVE could promote DCL4 activity likely through interaction with DRB4, whereas FVE-8 hijacked dsRNA and inhibited its processing despite its interaction with DRB4. Thus, the decrease of LUC and GUS transgenic siRNAs could result from the compromised DRB4/DCL2/4 activity in fve-8 (Fig. 5, A, B, and D). The result also explained that the reduction of transgenic siRNAs in fve-cas9-hpCHS mutants should attribute to the compromised DRB4/DCL2/4 activity in the absence of FVE (Fig. 5H and fig. S3C).

**DISCUSSION**

Here, we report a newly developed dual-reporter system for monitoring both miRNA- and siRNA-mediated RNA silencing. Using the system, we found FVE, a classical epigenetic component, as a new player in the transgene-PTGS pathway. We propose that FVE coordinates with SGS3 and DCL4/DRB4 function to promote the biogenesis of transgene-derived siRNAs (Fig. 9I). Several independent forms of evidence support this assertion. First, loss-of-function mutations of FVE compromised siRNA production from transgenic transcripts in several independent reporter lines (Fig. 5). Second,
Fig. 8. EMSA showed that FVE, FVE-8, and SGS3 display different binding affinities to ssRNA and dsRNA. (A, F, and L) The mobility pattern of FVE (A), FVE-8 (F), and SGS3 (L) with homogenous ssRNA. Protein concentrations were shown above. Arrowheads indicate the mobility of protein-RNA complexes or free RNA. Additional repeats were shown in fig. S5. (B and G) The binding affinities ($K_d$ and apparent $K_d$) of protein-ssRNA were calculated from quantification of EMSA images from (A) and (F) and additional repeats in fig. S6 with ±SD (n = 3). (C, H, and J) The mobility pattern of FVE (C), FVE-8 (H), and SGS3 (J) with dsRNA (see also fig. S6). (D, I, and K) The binding affinities ($K_d$ and apparent $K_d$) of protein-dsRNA were calculated from quantification of EMSA images from (C, H, and J) and additional repeats in fig. S7 with ±SD (n = 3). (E) RIP assay showed that FVE binds to RNA in vivo. IP was performed with an anti-Flag antibody with the protein extracts from infiltrated N. benthamiana leaves. Western blot assay was performed with indicated antibodies. No RT samples served as negative controls. (M and N) EMSA showed mobility patterns of different combinations of protein-ssRNA complexes (M) and of protein-dsRNA complexes (N). His-sumo is a negative control (see also figs. S6 and S7).
Fig. 9. In vitro reconstitution assay showed that FVE promotes whereas FVE-8 inhibits activities of DCL2/4-DRB4 complexes. (A) LCI assay showed that FVE (top) and FVE-8 (bottom) interact with DRB4 in N. benthamiana. PAG1-nLUC/cLUC-SE is a positive control (39). See also fig. S7. (B) Co-IP validated the FVE-DRB4 interaction. IP was performed with an anti-Flag antibody with the protein extracts from transfected N. benthamiana. Western blot assay was performed with indicated antibodies. (C) Y2H assay validated the DRB4-SGS3 interaction. SE/DCL1 is a positive control. (D) LCI assay showed that SGS3 interacts with DRB4 in N. benthamiana. See also fig. S7. (E and F) DCL2 (E) and DCL4 (F) dicing activity with and without FVE/FVE-8 at the indicated time points. The positions of intact substrates, processed products, and RNA markers are shown. At least three independent repeats were conducted with similar results (see also fig. S7). (G and H) Statistics of image quantitation of DCL2 (G) and DCL4 (H) activity. Individual data points are shown. \( * P < 0.05; ** P < 0.001; \) t-test. (I) A proposed model for FVE function in transgene silencing. FVE promotes dimerization and launching of SGS3 on ssRNA substrate, which, in turn, recruits RDR6 to produce dsRNA in vivo. Subsequently, FVE/SGS3/dsRNA is translocated to DCL4/DRB4 complexes for siRNA production. By contrast, FVE-8 does not interact with SGS3 and inefficiently promotes RDR6/SGS3 association with ssRNA substrates. Furthermore, FVE promotes DCL4/DRB4 activity, while FVE-8 sequesters dsRNA to prevent DCL4/DRB4 function in producing siRNAs.
FVE protein accumulated in the cytoplasm despite being previously thought to be exclusively in nucleus. Moreover, cytoplasmic FVE fully rescued the defect of *fve-8* in transgene silencing, indicating that the subcellular location for this function is indeed in the cytoplasm where PTGS typically occurs (Fig. 3). Third, FVE directly interacted with SG3 and promoted its homodimerization, which represents the prerequisite for SG3 function in PTGS (Figs. 6 and figs. S4 and S5) (8). Fourth, FVE bound to ssRNA substrate, whereas SG3 barely. In this scenario, FVE might bind to ssRNA while associating with SG3, which, in turn, recruits RDR6 for dsRNA synthesis in vivo, although this function was unnecessary in vitro. On the other hand, FVE-8 still binds to ssRNA but fails to interact with SG3 to promote its dimerization, leading to inefficient launching of SG3-RDR6 to ssRNA substrate in vivo (Fig. 8 and fig. S6). Fifth, given that both FVE and SG3 interacted with DRB4, dsRNA, once generated, could be channeled from FVE/SG3 into DRB4/DCL4 complexes for further processing to produce siRNAs. Last, FVE protein itself could directly promote the activity of DCL4/DRB4 to produce siRNAs. However, FVE-8 forms a homodimer and is granted a neo function to bind to dsRNA (Figs. 6A and 8, H and I, and fig. S7, C and D). Because FVE-8 binds to dsRNA with a significantly higher affinity than SG3, the protein could compete with SG3, hijack the dsRNA product, and prevent the downstream dsRNA processing by DRB4/DCL2/4 complex (Fig. 9, E to H, and fig. S8, F and G). Thus, we propose that FVE directly coordinates the two consecutive processes of transgene silencing and promotes siRNA production through its interaction with SG3 and DRB4 as well as its RNA binding activity.

How has FVE evolved a new function in PTGS? One possibility is that FVE harbors six-tandem repeated WD40 domains. WD40 domains are widely involved in protein-protein interaction and serve as scaffolds for the assembly of large protein complexes (41, 42). The presence of six WD40 domains allows FVE to partner with different targets or to shuffle between macromolecular complexes to fulfill various functions. Here, we also found two new features of FVE as a WD40 domain protein. First, WT FVE protein does not typically form oligomers, so it could create a platform or scaffold for other partners. However, the truncated variants such as FVE-8 are self-associating; correspondingly, FVE-8 homodimerization or oligomerization could block the interaction interface and, subsequently, the assembly of macromolecular complexes for proper functioning. Thus, the integrity of WD40 domains is critical for the biological roles of FVE. Second, the WD40 domain is one of the most abundant and conserved domains in eukaryotes (41, 42), and emerging evidence shows that mammalian WD40 domain proteins can specifically bind to Sm site [A(U)4-6G] and m7GpppG cap of pre–small nuclear RNAs (43, 44). In addition, it has been implicated (but not confirmed) that RbAp48 may also have the potential to bind to RNA (45). Here, we determined that FVE binds to both ssRNA and dsRNA in a sequence-independent manner. This discovery would largely expand the spectrum of potential functions for WD40 domain–enriched proteins. Thus, the evolution of FVE to bind to RNA would allow the protein to directly modulate the functions of ribonucleoprotein complexes from SG3/DRR6 to DRB4/DCL4.

SG3 also exhibits several unique features that facilitate targeting by FVE. SG3 harbors a CC domain that is essential for protein-protein interaction and homodimerization and an XS domain that is critical for RNA binding activity (36, 46). SG3 also contains a highly disordered N-terminal region with a Prion-like character (fig. S8H) (47, 48). The presence of these features infers that SG3 would have different partners and functions through various ribonucleoprotein complexes. Here, FVE promoted SG3 dimerization, which is essential in the formation of cytoplasmic granule-like foci, called siRNA bodies (8). Since SG3 and DCL4 complexes are partially colocalized (40), SG3 could translocate FVE (or vice versa) to the siRNA bodies to modulate the downstream siRNA production. Notably, SG3 has been recently reported to contribute to translocating proteasome subunits [e.g., the regulatory particle AAA-ATPase 2A (RPT2a)] into the proximity of the siRNA bodies to repress RNA quality control and to promote PTGS (49). Given these observations, SG3/FVE might translocate additional presently unidentified targets to siRNA bodies for fine-tuning PTGS activities.

Many components are shared in siRNA biogenesis for endogenous RNA and foreign transcripts in PTGS. Here, SG3 and DCL2/4 also contribute to the production of ta-siRNAs. It appears that FVE has an impact on transgene silencing but barely on endogenous RNA silencing. This scenario is also reminiscent of genes such as *JMJ14* and RPT2a that appear to only affect transgene siRNA production, but not the abundance of endogenous sRNAs (49, 50). One plausible explanation is that the expression of transgenes, but not endogenous loci, might often lead to production of aberrant transcripts that reach the threshold of the RNA quality control machinery. Abundant transgene transcripts could be easily captured by RNA binding proteins such as FVE. Another possibility is that production of ta-siRNAs entails initial recruitment of AG01/miR173- or AG07/miR390-centered RISCs to TAS transcripts (51). If the identified components do not function at the interface of RDR6/SG3-RISC, then they might not affect the production of endogenous ta-siRNAs. Of course, there might be other unknown mechanisms that would be revealed by future investigations.

In summary, we have provided conclusive evidence that FVE targets the SG3-DCL4/DRB4 metabolism channel to promote PTGS of transgenes. An earlier report showed that the mutants of *FCA*/*FPA* and *FLD*, which all partner with FVE to regulate flowering time, can partially rescue the RNA interference (RNAi) phenotype of transgenic lines expressing *SUCROSE-PROTON SYMPORTER 2* promoter-driven hairpin transcripts of *PHYTOENE DESATURASE (SUC-hpPDS)*. The mechanism for *fca/fpa* in rescuing *SUC-hpPDS* phenotype was interpreted through the TGS pathway (26). Given that FVE directly targets the SG3/DRB4/DCL2/4 channel, it might be hypothesized that these FVE partners also directly influence SG3/DCL4/DRB4 function. In addition, FVE and its mammalian homologs such as RbAp48 are well conserved through the eukaryotes, and whether mammalian FVE plays a similar role in RNA biology would be another exciting topic of investigation in the future.

**MATERIALS AND METHODS**

**Experimental design**

**Vector construction and transgenic plants**

Most of the constructs were generated by a Gateway cloning system. To obtain pENTR-GFP-PHB-LUC, a truncation form of *PHB* containing exon 4, intron 4, and exon 5 was amplified from Ler genomic DNA by *Thermococcus kodakaraensis* (KOD) DNA polymerase (Novagen). The *PHB* fragment was fused with GFP, LUC, and pENTR by DNA fragment assembly [New England Biolabs (NEB) HiFi DNA Assembly Master Mix] to generate pENTR-GFP-PHB-LUC. Then, pENTR-GFP-PHB-LUC was transferred into the Gateway compatible binary vector of pBA002a–multicloning sites (MCS)–DC by LR
Clonase (Invitrogen).AGO10 promoter was amplified with primers PAGO10 For and PAGO10 Xho I Rev (table S1). The PCR products were digested with Xma I and Xho I (NEB) as insert. Meanwhile, pBA002a-MCS-GFP-PHB-LUC was digested with the same enzymes as backbones. The two parts were ligated by T4 DNA ligase (NEB) and amplified by DH5α. To obtain pENTR-GFP-cPHB-LUC, a truncation form of cPHB containing exon 4 and exon 5 was amplified from WT cDNA by KOD DNA polymerase. After digestion with Bam HI and Kpn I, pENTR-GFP-PHB-LUC and cPHB fragment was ligated to get pENTR-GFP-cPHB-LUC. pENTR-GFP-cPHB-LUC was transferred into the Gateway compatible binary vector of pK-35S-FM-DC by LR Clonase. The final vectors were transformed into Agrobacterium tumefaciens strain ABI and then transferred to Ler Arabidopsis by flower dipping (52). Matured seeds were collected, and positive transgenic lines were selected on standard Murashige and Skoog (MS) medium containing phosphoinotrinic (10 mg/liter; for pBA vector; Sigma-Aldrich) or kanamycin (50 mg/liter; for pK vector; Sigma-Aldrich) together with carbenicillin (100 mg/liter; Sigma-Aldrich). Single-copy homozygous transformants were selected according to the Mendelian segregation ratio.

To obtain pK-DC-FM, pK-FM-DC was digested with Xba I and Pac I, and the resultant fragments were used with KOD DNA polymerase to synthesize the intermediate vector and pBA002a-DC-FM were digested with Sma I/ Xho I. The ends were blunted using DNA Polymerase I Large (Klenow). Single-copy homozygous transformants were selected by the standard MS medium containing carbenicillin (40 mg/liter; Sigma-Aldrich) together with carbenicillin (100 mg/liter; Sigma-Aldrich). Mutation in positive plants was confirmed by dCAPS primers shown in table S1. The WT PCR fragment can be digested by Bam HI while that of the mutants cannot.

For CRISPR-Cas9–FVE constructs, two guide sequences targetting FVE were designed on the basis of (54). The guide sequences without protospacer adjacent motif (PAM) sequence, and its corresponding complementary strand sequences were designed to be annealed with a Bsa I–digested sticky end added at the 5′ and 3′ ends. The annealed duplexes were ligated to a BsI–digested AtU6-26–sgRNA (single-guide RNA) vector by T4 ligase, respectively. After sequencing confirmation, AtU6-26–sgRNA1 was digested by Spe I, and a U6-sgRNA1 fragment was recovered and ligated to fragments of AtU6-26–sgRNA2 digested by Spe I and Nhe I. The tandem sgRNA sequences were extracted from AtU6-26–sgRNA1-U6-26–sgRNA2 digestion with Spe I and Nhe I and ligated to Spe I–digested pCambia1300-pYAO-cas9-MCS. Digestion confirmed that pCambia1300-pYAO-cas9-U6-6-sgRNA1-U6-6-sgRNA2 was transferred to GV3101 and then to L1 line and 35S-hpCHS line. T0 plants were selected by the standard MS medium containing hygromycin B (40 mg/liter; Sigma-Aldrich) together with carbenicillin (100 mg/liter; Sigma-Aldrich) Mutation in positive plants was confirmed by sequencing.

For split-YFP constructs, full-length CDSs of FVE, FVE-8, DRB4, and SGS3 were cloned into pBA-35S-nYFP-DC and pBA-35S-cYFP-DC by the LR reaction. For split-LUC constructs, those CDSs were cloned into pCambia1300–Myc-CNA, pCambia-35S-Myc-PHB, and pCambia-35S-Myc-PHB (m), and pCambia-35S-Myc-PHB (m) were transformed into A. tumefaciens strain ABI and then transferred to Ler Arabidopsis by flower dipping (52). Matured seeds were collected, and positive transgenic lines were selected on standard Murashige and Skoog (MS) medium containing phosphoinotrinic (10 mg/liter; for pBA vector; Sigma-Aldrich) or kanamycin (50 mg/liter; for pK vector; Sigma-Aldrich) together with carbenicillin (100 mg/liter; Sigma-Aldrich). Single-copy homozygous transformants were selected according to the Mendelian segregation ratio.
amplified from corresponding pENTR-FVE/FVE-8/DRB4 and ligated to pET28a-Avi-6×His-SUMO vector by Bam HI/Xho I. Sequence-confirmed plasmids were transferred to E. coli BL21 (DE3) cells for protein induction. For MBP-SGS3, SGS3 was cloned into vector pMAL-DC by LR recombination. For GST-6×His-SGS3 and GST-6×His-RDR6, SGS3 and RDR6 were obtained from pENTR-SGS3 and pENTR-RDR6, respectively, by digestion with Not I/Asc I. Two nucleotides were depleted between Nco I and Not I in pAcGHLT-C (BD Biosciences) to make sure the genes ligated were in frame with the GST tag. Digestion-confirmed plasmids were transfected to sf9 insect cells with baculovirus.

For cloning of 3HA-RDR6, 3HA-DCL2, and 3HA-DCL4, cDNAs of RDR6, DCL2, and DCL4 were recombined into pBA-3HA-DC to yield pBA-3HA-RDR6, pBA-3HA-DCL2, and pBA-3HA-DCL4 by LR Clonase, accordingly. Digestion-confirmed plasmids were transferred to GV3101 for transient infection in N. benthamiana leaves as described (55). Primers are listed in table S1.

Plant materials and growth conditions

A. thaliana ecotype Landsberg (Ler), Columbia (Col-0), E5-4 (Ler; PAGO10-GFP-PHB-LUC), M17-1 (Ler; 35S-GFP-pPHB-LUC), se-2 (SAIL_44_G12), hy1-2 (SALK_064863), sg3-1, rdr6-11 (CS24285), ago10-3 (SALK_519738), and pnh2 (CS3853) were used for this study. Plants were grown on soil at 22°C or MS plates in 12-hour light/12-hour dark.

E5-4 was crossed with Col-0 for seven generations to get Col-0 background E5-4 (Ec5-4). Ec5-4 in se-2, hy1-2, and rdr6-11 background plants were obtained by crossing Ec5-4 with the mutants, respectively. M17-1 in sg3-1 background was obtained by crossing M17-1 with sg3-1, respectively. In the F2 generation, homozygous rdr6-11 and sg3-1 mutants were identified by PCR using primers listed in table S1.

EMS mutagenesis, mutant screen, and LUC assays

EMS mutagenesis was performed as described with minor modifications (56). EMS (0.4%) was used to treat 20,000 seeds of E5-4 for 8 hours at room temperature. About 7000 M1 plants survived. Approximately 56,000 5-day-old M2 seedlings were assayed for LUC activity with an electron-multiplying charge-coupled device (CCD) camera (Princeton) and WinView 32 and LightField, of which showing enhanced LUC luminescent were picked. M2 seedlings were out-crossed with Col-0. Genomic DNA was extracted with hexadecyltrimethylammonium bromide. Traditional mapping markers were designed on the basis of an Arabidopsis mapping platform. The DNA sequence library was constructed according to the manufacturer’s (NEB) instructions with modifications. Next-generation sequencing was performed by Illumina, and sequence data were analyzed with SHOREmap version 2.0 (57) and NGM (58).

Aza-dc treatment

Aza-dc was dissolved in water and added to MS medium to a final concentration of 4 μM (59). Seeds were germinated on MS medium with or without aza for 5 days before LUC activity assay. After LUC activity was recorded, seedlings were harvested for RT-PCR.

RT-PCR and qRT-PCR

Total RNA was extracted using TRIzol (Sigma-Aldrich) from 5-day-old seedlings. Total RNA was treated with deoxyribonuclease I (DNase I; Sigma-Aldrich, AMPD1) and reverse-transcribed with SuperScript III reverse transcriptase (Invitrogen, catalog no. 1808093) primed by oligo(dT) according to the manufacturer’s instructions. Actin, EF1a, or UBQ10 genes were used as internal controls. For analysis using regular PCR, the PCR products were fractioned on agarose gels. qRT-PCR was performed using CFX384 Touch Real-Time PCR Detection System (Bio-Rad). Primers are listed in table S1.

RNA-seq and sRNA-seq

Total RNA was prepared from 5-day-old seedlings grown on MS medium using TRIzol. Construction of RNA and sRNA libraries, Illumina sequencing, and bioinformatic analysis were performed as described (35, 60). The differential gene expressions analyzed in Fig. 4 (A, D, and E) and fig. S3 (A and B) are listed in table S2.

RNA blot and sRNA blot

Total RNA was extracted using TRIzol from 5-day-old seedlings (for GUS-sRNA blot in Fig. 5D, 10-day-old seedlings were used). RNA blot hybridizations of low–molecular weight RNAs (sRNA blot) and high–molecular weight RNAs (Northern blot) were performed as described previously (32). For miRNAs and ta-siRNA blot, the probes, which are 21-nt DNA oligo complementary to the corresponding sRNAs (the probes are listed in table S1), were labeled using [γ-32P]adenosine 5′-triphosphate (ATP) (PerkinElmer) with T4 polynucleotide kinase (PNK) (NEB). For Northern blot and transgenic sRNA blot, the probes were PCR products amplified with primers listed in table S1. Then, they were labeled using [α-32P]dATP. Digestion-confirmed plasmids were transfected to sf9 insect cells with baculovirus.

Western blot

Western blot analyses were performed as described (23). Blots were detected with antibodies against Myc (Sigma-Aldrich, C 9596), YFP (Roche and Agrisera, AS15 2987), actin (Sigma-Aldrich, A0480), histone 3 (Agrisera, AS10 710), GST (Sigma-Aldrich, G-7781), AGO10 (Agrisera, AS15 3071), SGS3 (Agrisera, AS15 3099), Flag (Sigma-Aldrich, F1804), and HA (Sigma-Aldrich, H9658). Secondary antibodies were goat-developed anti-rabbit (GE Healthcare, catalog no. NA934) and anti-mouse immunoglobulin G (GE Healthcare, catalog no. NA931). Western blot membranes were developed with ECL+, and signals were detected with ChemiDoc XRS+ and captured with the Image Lab software (Bio-Rad) as per the manufacturer’s instruction.

Chromatin immunoprecipitation–polymerase chain reaction

ChIP assay was performed as described (61). Five-day-old seedlings were harvested from MS medium. IP was developed with an anti-H3K27me3 antibody (Millipore, catalog no. 07-449) and anti-AtRPB1 (62). PCR primers are listed in table S1.

Nuclei RNA extraction

Well-ground powder (0.5 g) of 6-day-old seedlings of E5-4 and five-8 was homogenized in 5 ml of nuclei isolation buffer [0.25 M sucrose, 15 mM Pipes (pH 6.8), 1 mM MgCl2, 60 mM KCl, 15 mM NaCl, 1 mM CaCl2, 0.9% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), RNase In (100 U/ml), and TURBO DNase (10 U/ml)] prepared in RNase-free water for 10 min on ice. The slurry was filtered through a layer Miracloth to a new tube. After centrifuging at 11,000 g for 10 min at 4°C, the pellet was washed once with nuclei isolation buffer. The pellet was kept for RNA extraction using TRIzol as the nuclear RNA fraction.

Cellular localization assay and BiFC assay of YFP

Large-scale plasmid purification and protoplast transformation were performed as described (35). Leaves of 4-week-old plants of Col-0, E5-4, five-8, and five-8; gFVE were used as resources of protoplasts. Eighteen hours after transfection, fluorescence signals in the protoplast were visualized by a Leica SP8 confocal microscope (39). Samples...
were excited by a 514-nm argon laser with an emission of 527 nm for YFP and 633 nm for chlorophyll autofluorescence, respectively. At least 10 individual protoplasts were observed with similar results for each transformation.

**Nuclear-cytoplasmic fractionation assay**

Five-day-old seedlings on MS medium were harvested for nuclear-cytoplasmic separation as described previously (63). Rubisco stained with Ponceau S and histone 3 protein level were used for fractionation quality validation and internal control.

**GUS staining**

F₂ seedlings were harvested and developed with GUS staining as described (5).

**Y2H and Y3H assays**

Y2H and Y3H assays were performed as previously described (5). In the Y3H system, pBridge plasmid contains a met-suppressed promoter (Met23 promoter) to regulate the third gene compared with Y2H system. The growing speed of transfected colonies serves as a proxy of SGS3-SGS3 homodimer amount. The constructs were cotransformed and selected on the medium lacking leucine (Leu), tryptophan (Trp), histidine (His), and methionine (Met) but supplemented with 5 mM 3-amino-1,2,4-triazole for Y3H assays. Different amounts of Met (0, 1, and 2 mM) were added to the medium to regulate the transcription activity of Met23 promoter. Note that yeast colonies tend to grow faster in the medium with the nutrient Met than the one without Met (Fig. 6E and fig. S5C). At least 10 yeast colonies tend to grow faster in the medium with the nutrient Met than the one without Met (Fig. 6E and fig. S5C). At least 10 independent colonies were tested for each combination, and they all showed similar results.

**LCI assay**

LCI assays were performed as previously described (61). For each LCI assay, at least three individual leaves were observed with similar results.

**Comimonoprecipitation**

For Co-IP in Fig. 6B, 10-day-old Ler and fve-8; P_{FVE-PM-CFVE} seedlings were harvested and ground well in liquid nitrogen, respectively. Powder (0.5 g) was homogenized in 2.5 ml of IP buffer [50 mM tris-HCl (pH 7.6), 150 mM NaCl, 5 mM MgCl₂, 0.1% Triton X-100, 1% glycerol, 2 mM PMSF, 5 mM dithiothreitol (DTT), one pellet per 15 ml of IP buffer EDTA-free proteinase inhibitor (Roche), and 50 µM MG132] and mixed well for 10 min on ice. The total protein extracts were centrifuged at 15,000 rpm for 15 min at 4°C twice. After total proteins were extracted, anti-Flag M2 magnetic beads (Sigma-Aldrich, M8823) were added to extracts and incubated at 4°C for 2 hours. For RNase A treatment, RNase A (0.05 mg/ml) was added to the IP buffer before incubation. After incubation, beads were washed four times with wash buffer [20 mM tris-HCl (pH 7.6), 100 mM NaCl, 0.05% Tween 20, 2 mM DTT, and one pellet per 15 ml of buffer EDTA-free proteinase inhibitor (Roche)] at 4°C for 5 min. The beads were boiled in 2× SDS protein loading buffer for 10 min before Western blot analysis. An anti-Flag antibody was used to detect IP products, and an anti-YFP antibody was used to detect coimmonoprecipitates.

**Expression and purification of recombinant proteins**

Protein expression and purification were performed as described in (5) with modifications. 6×His-SUMO-FVE, 6×His-SUMO-FVE-8, MBP-SGS3, and 6×His-SUMO-DRB4 were expressed in E. coli BL21 (DE3) cells. GST-6×His-SGS3, GST-6×His-RDR6, and GST-6×His-DCL4 were expressed in a baculovirus/insect cell expression system. For recombinant proteins expressed in E. coli, transformed BL21 cells were grown in LB at 37°C until the optical density at 600 nm is equal to 0.6. Then, the expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside at 37°C for 3 hours.

For purification of FVE, the induced bacterial cells were collected and resuspended in lysis buffer [40 mM tris-HCl buffer (pH 8.0), 300 mM KCl, 2% glycerol, 1 mM β-mercaptoethanol, 1 mM PMSF, and 0.1% Triton X-100]. Cells were disrupted using LM20 Digital Microfluidizer Processor at 25,000 psi for three cycles. The disrupted cells were centrifuged at 18,000 rpm for 15 min at 4°C. The supernatant was filtered with a 0.45-µm filter and made up to 20 mM imidazole and 1% Triton X-100. The cleared lysate was loaded onto a HisTrap HP column (GE Healthcare, catalog no. 17-5248-02). The column was washed with 25 ml of wash buffer [40 mM tris-HCl (pH 8.0), 300 mM KCl, 2% glycerol, 1 mM β-mercaptoethanol, 1 mM PMSF, and 80 mM imidazole] and eluted with gradient elution buffer from 80 to 300 mM imidazole [40 mM tris-HCl (pH 8.0), 300 mM KCl, 2% glycerol, 1 mM β-mercaptoethanol, and 1 mM PMSF]. The peak fractions of the recombinant protein were pooled and dialyzed in dialysis tubing with 10-kDa–molecular weight cutoff (MWCO) stirring in dialysis buffer [20 mM tris-HCl (pH 7.5), 60 mM KCl, 2% glycerol, 2 mM β-mercaptoethanol, 2 mM DTT, and 140 mM SUMO protease] at 4°C overnight. The uncut recombinant protein was removed by Ni beads. The fractions were concentrated by a 30-kDa MWCO Centricron (Millipore, catalog no. Z71785) and loaded onto a HiLoad 16/600 Superdex 200 pg column (GE Healthcare). The gel filtration buffer contained 20 mM tris-HCl (pH 7.5), 60 mM KCl, 2 mM β-mercaptoethanol, and 2 mM DTT. The peak fractions containing FVE were collected and dialyzed in dialysis tubing with 10-kDa-MWCO stirring in dialysis buffer [20 mM tris-HCl (pH 7.5), 60 mM KCl, 2 mM β-mercaptoethanol, and 50% glycerol] at 4°C overnight. The final dialysate was aliquoted and frozen by liquid nitrogen and stored at −80°C. The purifications of FVE-8 and DRB4 are similar to that of FVE. MBP-SGS3 is purified as described in (61).

For purification of recombinant protein in insect cells, pAcGHLT-GST-6×His vectors were transfected with BaculoGold baculovirus DNA system (BD Biosciences, catalog no. 554740) into sf9 insect cells (BD Biosciences, catalog no. 554738; authenticated by the vendor BD Biosciences) to generate recombinant baculovirus. After two rounds
of viruses were amplified. P3 virus was collected for large-scale protein expression (5).

For GST-6xHis-SGS3, the P3 virus was added to 2.5 × 10^6 sf9 insect cells/ml for propagation, and the insect cells were collected 65 hours later. Insect cells were resuspended in lysis buffer [40 mM tris-HCl (pH 8.0), 500 mM NaCl, 5% glycerol, 1 mM β-mercaptoethanol, 1 mM PMSF, and 1 mM PMSE] and eluted with gradient elution buffer from 0 to 300 mM imidazole [40 mM tris-HCl (pH 8.0), 500 mM NaCl, 5% glycerol, 1 mM β-mercaptoethanol, and 1 mM PMSF]. The peak fractions of recombinant protein were pooled and dialyzed in dialysis buffer [40 mM tris-HCl (pH 8.0), 500 mM NaCl, 5% glycerol, 1 mM β-mercaptoethanol, and 1 mM PMSF]. The substrate of ssRNA for EMSA is G3A44 synthesized by T7 (Sigma-Aldrich) and disrupted with LM20 Digital Microfluidizer Processor at 5000 psi for 2 cycles. Disrupted cells were centrifuged at 20,000 rpm for 30 min at 4°C. The supernatant was filtered with a 0.45-μm filter and made up to 20 mM imidazole and 1% Triton X-100. The cleared lysate was loaded onto a HisTrap HP column (GE Healthcare). The column was washed with 25 ml of wash buffer [40 mM tris-HCl (pH 8.0), 500 mM NaCl, 5% glycerol, 1 mM β-mercaptoethanol, and 1 mM DTT] and eluted with gradient elution buffer from 0 to 15 mM reduced glutathione [40 mM tris-HCl (pH 8.0), 500 mM NaCl, 5% glycerol, 1 mM β-mercaptoethanol, and 1 mM DTT]. The mixture was incubated on ice for 30 min before labeled RNA was added. For dsRNA EMSA, RNA was denatured at 95°C for 3 min in buffer [100 mM KCl and 50 mM tris-HCl (pH 7.5)] and slowly cooled down to room temperature. The mixtures were incubated at room temperature for 30 min. Bound complexes were resolved on a native agarose gel. The gel was incubated in fixing buffer at 65°C over-night. Samples were digested with proteinase K for 30 min at 37°C. Samples were purified with phenol-chloroform and fractionated on 6% urea-polyacrylamide gel. The RDR6 product was visualized by radiography. The experiments were repeated three times for statistical analysis. The semi–in vitro RdRP reconstitution assay was performed as described (34, 35) with modifications. Reaction mixtures (20 μl) containing 50 mM Hepes-KOH (pH 7.6), 20 mM NH4OAc, 2% (w/v) PEG4000, 16 mM MgCl2, 0.1 mM EDTA, 1 mM ATP, 1 mM CTP, 1 mM GTP, 0.1 mM UTP, 0.5 μM [α-32P]UTP (3000 Ci/mmol), SUPERaseIn (1 U/μl), and 2 pmol of each recombinant protein except RDR6 were mixed as indicated and preincubated on ice for 10 min. ssRNA template (10 pmol) was added to each reaction and incubated for 30 min. RDR6 (1 pmol) was added to each sample and to initiate the reaction at room temperature for 20 min, respectively. The samples were digested with proteinase K for 30 min at 37°C. Samples were purified with phenol-chloroform and fractionated on 6% urea-polyacrylamide gel. The RDR6 product was visualized by radiography. The experiments were repeated three times for statistical analysis. The semi–in vitro RdRP reconstitution assay was performed as described (34, 35) with modifications. Reaction mixtures (20 μl) containing 50 mM Hepes-KOH (pH 7.6), 20 mM NH4OAc, 2% (w/v) PEG4000, 16 mM MgCl2, 0.1 mM EDTA, 1 mM ATP, 1 mM CTP, 1 mM GTP, 0.1 mM UTP, 0.5 μM [α-32P]UTP (3000 Ci/mmol), 10 pmol of ssRNA template, SUPERaseIn (1 U/μl), and recombinant proteins were mixed and preincubated on ice for 30 min. Then, 3 μl of HA beads containing HA-RDR6 were added to each sample and to initiate reaction at room temperature for 0.5, 1, and 1.5 hours, respectively. Samples were boiled with urea loading buffer and fractionated on 6% urea-polyacrylamide gel. The final result was visualized by radiography. EMSA was performed as described with modifications (5). Recombinant proteins were mixed in the EMSA buffer [10 mM tris-HCl (pH 7.5), 4% glycerol, 1 mM MgCl2, 0.2 mM EDTA, and 0.5 mM DTT]. The mixture was incubated on ice for 30 min before labeled RNA was added. For dsRNA EMSA, RNA was denatured at 95°C for 3 min in buffer [100 mM KCl and 30 mM tris-HCl (pH 7.5)] and slowly cooled down to room temperature. The mixtures were incubated at room temperature for 30 min. Bound complexes were resolved on a native agarose gel. The gel was incubated in fixing buffer (40% ethanol, 10% acetic acid, and 5% glycerol) for 15 min and dried at 80°C for 2 hours and then visualized by radiography. The Kd and appKd were calculated using Prism GraphPad 8 software fit with a Hill slope model.

**Electrophoretic mobility shift assays**

EMSAs were performed as described with modifications (5). Recombinant proteins were mixed in the EMSA buffer [10 mM tris-HCl (pH 7.5), 4% glycerol, 1 mM MgCl2, 0.2 mM EDTA, and 0.5 mM DTT]. The mixture was incubated on ice for 30 min before labeled RNA was added. For dsRNA EMSA, RNA was denatured at 95°C for 3 min in buffer [100 mM KCl and 30 mM tris-HCl (pH 7.5)] and slowly cooled down to room temperature. The mixtures were incubated at room temperature for 30 min. Bound complexes were resolved on a native agarose gel. The gel was incubated in fixing buffer (40% ethanol, 10% acetic acid, and 5% glycerol) for 15 min and dried at 80°C for 2 hours and then visualized by radiography. The Kd and appKd were calculated using Prism GraphPad 8 software fit with a Hill slope model.

**Ribonucleoprotein complex IP (RIP) RT-PCR**

For RIP, after 48 hours of infection, infiltrated N. benthamiana leaves were harvested and cross-linked as described in the “Coimmunoprecipitation” section. Powder (0.5 g) was homogenized in 2.5 ml of RIP buffer [40 mM tris-HCl (pH 7.5), 100 mM KCl, 5 mM MgCl2, 0.2% Triton X-100, 0.1% glycerol, 1 mM PMSF, 5 mM DTT, one pellet per 15 ml of IP buffer EDTA-free proteinase inhibitor (Roche), 15 μM MG132, RNase In (100 U/ml), and TURBO DNase (10 U/ml)] prepared in RNase-free water for 10 min on ice. The total protein extracts were centrifuged at 15,000 rpm for 15 min at 4°C twice. After proteins were extracted, balanced anti-Flag M2 magnetic beads were added to the extracts for Flag tag enrichment at 4°C for 2 hours. After incubation, the beads were washed twice with RIP buffer and twice with high-salt buffer [40 mM tris-HCl (pH 7.5), 500 mM KCl, 5 mM MgCl2, 5 mM DTT, 0.2% Triton X-100, 2% glycerol, 1 mM PMSF, 25 μM MG132, and one pellet per 10 ml of cComplete RTA-free protease inhibitor] at 4°C for 5 min, followed by one-time wash with proteinase K buffer [50 mM tris-HCl (pH 7.5), 200 mM NaCl, 10 mM EDTA, and 1% SDS]. The beads were treated with proteinase K (2 mg/ml) in 150 μl of proteinase K buffer at 65°C over-night. One share of beads was boiled in 2× SDS protein loading buffer for 10 min before Western blot. Anti-Flag antibody was used to detect...
IP. The other share of beads was used for RNA extraction. After TURBO DNase digestion, reverse transcription was performed as described in the “RT-PCR and qRT-PCR” section except primed with random primers. Primers used for PCR are listed in table S1.

**In vitro DCL2/4 assay**

HA-DCL2/4 IP assay was performed with a modified protocol from DCL1 IP (4). After 48 hours of infection, infiltrated *N. benthamiana* leaves and mock leaves were harvested and ground well in liquid nitrogen, respectively. Powder (0.5 g) was homogenized in 2.5 ml of IP buffer [40 mM tris-HCl (pH 7.5), 300 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA (pH 8.0), 5 mM DTT, 0.2% Triton X-100, one pellet per 15 ml of IP buffer EDTA-free proteinase inhibitor (Roche), 1 mM PMSF, and 2% glycerol] for 10 min on ice. After total proteins were extracted, anti-HA agarose beads (Sigma-Aldrich, A2095) were used for HA tag enrichment at 4°C for 2 hours. After incubation, the beads were washed four times with wash buffer [20 mM tris-HCl (pH 7.5), 1 mM DTT, 4 mM MgCl₂, and 100 mM KCl] at 4°C for 5 min. The final beads were stored with 7 volumes of DCL storage buffer [20 mM tris-HCl (pH 7.5), 1 mM DTT, 4 mM MgCl₂, and 20% glycerol] and stored at −20°C before use. 1 pmol of FVE, 1 pmol of FVE-8, and 1 pmol of SGS3 (for DCL4 assay, 1 pmol of DRB4 was added) were added to a 20-μl reaction system, respectively, as also added) were added to a 20-μl reaction system, respectively, as needed, containing 1000 counts per minute of 32P-labeled dsRNA substrates, 20 mM tris-HCl (pH 7.5), 4 mM MgCl₂, 1 mM DTT, 5 mM ATP, 1 mM GTP, and SUPERaseIn RNase Inhibitor (1 U/μl; Invitrogen). The mixture above was homogenized on ice for 30 min. The assay was initiated by adding 3.5 μl of HA-IP products as indicated and tumbling at 25°C at 1000 rpm in a thermomixer. HA-IP product from mock *N. benthamiana* serves as a negative control. The reactions were stopped by 1% SDS and proteinase K (2 mg/ml) digestion at 37°C for 30 min. After phenol-chloroform purification, DCL-processed products were fractionated using 12% denaturing polyacrylamide gel and detected with a phosphor imaging plate (GE Healthcare).

**Statistical analysis**

For RNA-seq and sRNA-seq, an edgeR (version 3.3) package was used to normalize gene expression levels with trimmed mean of M-values according to the false discovery rate (60). The cutoff for significance was 0.05.

Quantification of Northern blot, Western blot, EMSAs, RdRP assays, and DCL2/4 assays was performed by measuring the intensity of the bands by ImageJ 1.52 software. For EMSAs and RdRP assays, the curves were fitted, and the values were calculated using GraphPad prism 8.

For qRT-PCR and confocal foci statistics, the data were presented as means of at least three replicates ±SD. For Figs. 2 (B and E) and 3D, the relative expression of tested genes was initially normalized to the expression of *EF-1α* (Figs. 2B and 3D) and *UBQ10* (Fig. 2E) and then to WT (E5-4) where the ratio was arbitrarily assigned a value of 1 with ±SD (n = 3) biologically independent replicates. For DCL2/4 assays, the histogram was presented as the mean of three or four replicates ±SEM.

Unpaired two-tailed Student’s t test was performed to calculate the P value. The cutoff for significance was 0.05. *P < 0.05, **P < 0.01, and ***P < 0.001.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/12/eabf3898/DC1

View request a protocol for this paper from Bio-protocol.
26. I. Barle, L. Smith, D. C. Baulcombe, C. Dean, Widespread role for the flowering-time regulators FCA and FPA in RNA-mediated chromatin silencing. Science 318, 109–112 (2007).

27. M. Fazhounhandeh, J. Molinier, A. Berr, P. Genschik, MS4A/FVE interacts with CUL4-DDB1 and a PR2C-like complex to control epigenetic regulation of flowering time in Arabidopsis. Proc. Natl. Acad. Sci. U.S.A. 108, 3430–3435 (2011).

28. H. Streud, M. V. C. Greenberg, S. Feng, Y. V. Bernatavichute, S. E. Jacobsen, Comprehensive analysis of silencing mutants reveals complex regulation of the Arabidopsis methylome. Cell 152, 352–364 (2013).

29. M. Brameier, A. Krings, R. M. MacCallum, NucPred—Predicting nuclear localization of proteins. Bioinformatics 23, 1159–1160 (2007).

30. R. Ye, W. Wang, T. Ili, C. Liu, Y. Wu, M. Ishikawa, X. Zhou, Y. Qi, Cytoplasmic assembly and selective nuclear import of Arabidopsis Argonaute4/siRNA complexes. Mol. Cell 46, 859–870 (2012).

31. S. V. Wesley, C. A. Hellinwell, N. A. Smith, M. B. Wang, D. T. Rouse, Q. Liu, P. S. Gooding, S. P. Singh, D. Abbott, P. A. Stoutjesdijk, S. P. Robinson, A. P. Gloave, A. G. Green, M. P. Waterhouse, Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J. 27, 581–590 (2001).

32. X. Zhang, Y.-R. Tuan, Y. Pei, S.-S. Lin, T. Tuschl, D. J. Patel, N.-H. Chua, Cucumber mosaic virus-encoded 2b suppressor inhibits Arabidopsis Argonaute4 cleavage activity to counter plant defense. Genes Dev. 20, 3255–3268 (2006).

33. N. Kumakura, A. Takeda, Y. Fujioka, H. Motose, R. Takano, Y. Watanabe, SGS3 and RDR6 interact and colocalize in cytoplasmic SG3/SG3-RD6 bodies. FEBS Lett. 583, 1261–1266 (2009).

34. J. Curaba, X. Chen, Biochemical activities of Arabidopsis RNA-dependent RNA polymerase 6. J. Biol. Chem. 283, 3059–3066 (2008).

35. Z. Ma, C. Castillo-González, Z. Wang, D. Sun, X. Hu, X. Shen, M. E. Potok, X. Zhang, Arabidopsis Serrate coordinates histone methyltransferases ATXR5/6 and RNA processing and antiviral RNA silencing pathways in plants. Mol. Biol. Cell 8, 3430–3435 (2011).

36. H. Liu, Y. Ding, Y. Zhou, W. Jin, X. Chen, L. Chen, CRISPR-Cas9 tool for genome editing in plants. Mol. Plant 10, 530–532 (2017).

37. Z. Zhang, F. Hu, M. W. Sung, C. Shu, C. Castillo-González, H. Koiwa, G. Tang, M. Dickman, P. Li, X. Zhang, RISC-interacting cleaving 3’-exoribonucleases (RICEs) degrade uridylated cleavage fragments to maintain functional RISC in Arabidopsis thaliana. eLife 6, e24666 (2017).

38. W. Wang, R. Ye, Y. Xin, X. Fang, C. Li, H. Shi, X. Zhou, Y. Qi, An importin β protein negatively regulates microRNA activity in Arabidopsis. Plant Cell 23, 3565–3576 (2011).

39. K. Schneeberger, S. Ossowski, C. Lanz, T. Jau, A. H. Petersen, K. N. Nielsen, J. J. Jorgensen, D. Weigel, S. U. Andersen, SHOREmap: Simultaneous mapping and mutation identification by deep sequencing. Nat. Methods 6, 550–551 (2009).

40. H. Li, J. Ruan, R. Durbin, Mapping short DNA sequencing reads and calling variants using mapping quality scores. Genome Res. 18, 1851–1858 (2008).

41. O. Mathieu, J. Reinders, M. Caïkowski, C. Smathajitt, J. Paszkowski, Transgenerational stability of the Arabidopsis epigenome is coordinated by CG methylation. Cell 130, 851–862 (2007).

42. D. Sun, Z. Ma, J. Zhu, X. Zhang, Identification and quantification of small RNAs, in Arabidopsis Protocols (Springer, 2021), pp. 225–254.

43. C. Castillo-González, X. Liu, C. Huang, C. Zhao, Z. Ma, T. Hu, F. Sun, Y. Zhou, X. Zhou, X.-J. Wang, X. Zhang, Geminivirus-encoded TrAP suppressor inhibits the histone methyltransferase SUVH4/KYP to counter host defense. eLife 4, e06671 (2015).

44. A. Fukudome, D. Sun, X. Zhang, H. Koiwa, Salt stress and CTD PHOSPHATASE-LIKE4 mediate the switch between production of small nuclear RNAs and miRNAs. Plant Cell 29, 3214–3233 (2017).

45. Z. Zhang, X. Guo, C. Ge, Z. Ma, M. Jiang, T. Li, H. Koiwa, S. W. Yang, X. Zhang, KET1 imports HYL1 to nucleus for miRNA biogenesis in Arabidopsis. Proc. Natl. Acad. Sci. U.S.A. 114, 4011–4016 (2017).

Acknowledgments: We thank X. Chen for pCambia-35S-PHB/PHBm-Myc plasmids, R. Klein and S. Rodgers for careful editing, and Zhang Lab members for discussion and proofreading of this manuscript. Funding: The work was supported by grants from NIH R01GM127742 and NSF (MCB-1716243) to X.Z. D.S. and X.Y. were partially supported by the China Scholar Council Fellowship. Author contributions: X.Z. conceived the project. D.S., Z.M., and X.Z. designed the study. D.S. and Y.L. performed the experiments. Z.M. conducted bioinformatics analysis. X.Y. conducted protein purification. N.L. verified the EMS-responder system of M17-1. H.K. guided initial CCD-based screening, provided RPB1 antibody, and edited the manuscript. B.S. performed confocal experiments. X.H. conducted part of siRNA blot assays. K.C. did part of genotyping. D.S. and Y.L. analyzed the data. D.S. and X.Z. wrote the paper. Competing interests: The authors declare that they have no competing interests. Data and materials availability: The data generated during this study have been deposited in GEO under the series reference of GSE171316. All other data needed to evaluate the conclusions in the paper are present in the paper and/or in the Supplementary Materials. Additional data related to this paper may be requested from the authors. Submitted 22 October 2020 Accepted 15 June 2021 Published 4 August 2021 10.1126/sciadv.aatf3989

Citation: D. Sun, Y. Li, Z. Ma, H. Yan, N. Li, B. Shang, X. Hu, K. Cui, H. Koiwa, X. Zhang. The epigenetic factor FVE orchestrates cytoplasmic SG3-DRD4-DCL4 activities to promote transgene silencing in Arabidopsis. Sci. Adv. 7, eabf3898 (2021).