MicroRNA-binding viral protein interferes with *Arabidopsis* development

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MicroRNAs (miRNAs) are small (≈21 nt), noncoding RNAs that negatively regulate target mRNAs at the posttranscriptional level that are involved in development. In plants, virus-induced disease symptoms often result in developmental abnormalities resembling perturbation of miRNA-mediated function. Here, we report that expression in transgenic plants of a geminivirus-encoded AC4 protein from African cassava mosaic virus Cameroon Strain (ACMV), a suppressor of posttranscriptional gene silencing, was correlated with decreased accumulation of host miRNAs and increased development abnormalities in *Arabidopsis*. Down-regulation of miRNA correlated with an up-regulation of target mRNA level. *In vitro* binding assays revealed the ability of AC4 of ACMV (A-AC4) but not East African cassava mosaic Cameroon virus AC2 to bind single-stranded forms of miRNAs and short interfering RNAs but not double-stranded RNA forms. Normally, a labile intermediate during the miRNA biogenesis/RNA-induced silencing complex assembly, miRNA*, was below the level of detection, indicating that AC4 might interfere at a point downstream of the miRNA duplex unwinding process. The association of AC4 with miRNA was demonstrated by the association of A-AC4–GFP fusion protein, extracted from *Arabidopsis* protoplasts, with 2′-*O*-methyl-oligonucleotide complementary to miR159 (miR159*) and by the presence of miRNA with the A-AC4–GFP fusion protein after immunoprecipitation with antibody against GFP. In both assays, A-AC4 protein and miRNA complexes were copurified. These results provide direct evidence that AC4 is a unique virus-encoded posttranscriptional gene-silencing suppressor protein that binds to and presumably inactivates mature miRNAs and thus blocks the normal miRNA-mediated regulation of target mRNAs, resulting in developmental defects in *Arabidopsis*.

geminivirus | short interfering RNA | posttranscriptional gene silencing suppressor | RNA interference

MicroRNAs (miRNAs) are endogenous, small, noncoding RNAs of ≈21 nt in length that negatively regulate target mRNAs at the posttranscriptional level in many eukaryotic organisms (1). The biogenesis of mature miRNA occurs by multistep process involving the activity of Dicer-Like 1 in *Arabidopsis* (2, 3). However, miRNA accumulation also requires the activity of HEN1 and HYL1, the nuclear double-stranded RNA-binding proteins (4, 5), and depends on ARGONAUTE1 for the miRNA-mediated target mRNA cleavage (6). In plants, miRNAs target a wide range of mRNAs encoding transcription factors required for development. These include factors required for meristem identity and maintenance, cell division, hormone signaling, and developmental timing (7). In *Arabidopsis,* loss of miRNA biogenesis or activity results in pleiotropic defects during embryonic, vegetative, and reproductive development (8).

RNA silencing in plants is an antiviral defense response, and as a counterdefense strategy, viruses have evolved with distinct RNA-silencing suppressor proteins. These symptom phenotypes often are associated with virus-encoded pathogenicity factors, many of which are suppressors of RNA silencing (9). Systemic infection by plant viruses frequently results in symptoms in the newly developed leaves that resemble developmental defects because of loss of proper control of gene expression that regulates growth. Although short interfering RNAs (siRNAs), the intermediates of posttranscriptional gene silencing (PTGS), and miRNAs are chemically similar and guide RNA silencing by means of RNA-induced silencing complex (RISC) through strand-specific selection process, siRNAs are processed from perfectly base-paired double-stranded RNA, whereas miRNAs are processed from imperfect hairpin RNAs. However, in plants, siRNAs are stable and can be purified as double-stranded RNA molecules, whereas miRNAs exist in single-stranded RNA forms. The short RNA duplex intermediates (miRNA·miRNA* or siRNA) are likely unwound in a complex similar to the RISC-loading complex before incorporation into RISC (10). Strand asymmetry depends on the strength of base-pair interactions at each end of the duplex, and the molecule at the 5′ end participating in the weakest interaction is used preferentially (11, 12). The nonselected (sense) strand of siRNA or miRNA* is rapidly degraded (7). Among the different silencing suppressor proteins reported, a detailed analysis revealed that the p19 of tombusviruses and p21 of beet yellows virus bind to duplex forms of both siRNAs and miRNAs, indicating that these two suppressors might interfere at the RISC-assembly step of the silencing pathway (13, 14). Although the potyvirus P1/HC-Pro inhibits miRNA-mediated cleavage of target mRNAs, the exact mode of action of this protein in the silencing process still remains unknown (15).

Geminiviruses have single-stranded DNA genomes and encounter no double-stranded RNA phase in their replication cycle; however, they trigger PTGS possibly by the occurrence of overlapping transcripts with the production of virus-derived siRNAs in infected plants (16, 17). In a previous study, we identified two types of virus-encoded PTGS-suppressor proteins: (*i*) the AC4 of African cassava mosaic virus Cameroon Strain (ACMV) and of Sri Lankan cassava mosaic virus (SLCMV) and (*ii*) the AC2 of East African cassava mosaic Cameroon virus (EACMCV) and of Indian cassava mosaic virus (ICMV). It is worth noting that not all AC4 proteins are PTGS suppressors (AC4+), and that, for example, the AC4 of EACMCV (E-AC4) and ICMV are not PTGS suppressors (AC4−) (18). In addition, the AC2 of ACMV-Kenyan strain (A-AC2) and mungbean yellow mosaic virus and the C2 of tomato yellow leaf curl China virus were reported to possess silencing-suppression activity (19–21). However, the role of geminivirus-encoded silencing suppressors on the miRNA pathway in the context of disease development is lacking. In this study, we analyzed the role of the A-AC4 and E-AC2, representing each of the two types of geminivirus-encoded silencing suppressors, on the miRNA pathway. ACMV-infected plants show severe developmental abnormalities,

Abbreviations: miRNA, microRNA; ACMV, African cassava mosaic virus Cameroon Strain; EACMCV, East African cassava mosaic Cameroon virus; A-AC2, AC2 of ACMV; A-AC4, AC4 of ACMV; E-AC4, AC4 of EACMCV; siRNA, short interfering RNA; PTGS, posttranscriptional gene silencing; RISC, RNA-induced silencing complex; 35S, cauliflower mosaic virus 35S promoter; *Pp*-luc, *Photinus pyralis* luciferase.

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whereas EACMCV infection causes mild symptoms in host plants (16). Transgenic expression of A-AC4 but not E-AC2 induced disease-like developmental defects in *Arabidopsis* and decreased the levels of host miRNA accumulation, indicating that A-AC4 does interfere with the miRNA pathway. The down-regulation of miRNA correlated with increased accumulation of the corresponding mRNA expression. Our *in vitro* binding assays revealed that A-AC4 binds to single-stranded forms of miRNAs. Further, the A-AC4–miRNA association *in vivo* was demonstrated by affinity purification using tethered miR159-complementary 2′-*O*-methyloligonucleotide and by immunoprecipitation assays. In addition, the ability of A-AC4 to bind to single-stranded siRNAs explains that it blocks PTGS at a step downstream of siRNA production.

Materials and Methods

Gene Constructs, Virus Infectivity, and Transgenic Plants. Coding sequences of AC2 and AC4 genes from ACMV and EACMCV were fused to the cauliflower mosaic virus (CaMV) 35S promoter (35S) in a binary vector and introduced into *Agrobacterium tumefaciens* strain GV3101 as described in ref. 18. Transformation of *Arabidopsis thaliana* ecotype Columbia plants (Col-0) was performed by the floral dipping method (22). Seeds from transformants were selected for kanamycin resistance (50 mg/liter). Virus inoculation was performed as described in ref. 16.

Northern Blot Analysis. Total RNA (20 μg) was isolated from leaf tissue collected from infected plants and transgenic *Arabidopsis* by using an RNA isolation kit (Qiagen). The A-AC4 and MYB sequences were used as probes. Hybridization and quantification of the signals were done as described in ref. 23.

Detection of miRNAs. Low-molecular-mass RNA was isolated as described in ref. 16. Oligonucleotide sequences complementary to miR159, miR165/166, and miR171 (24) 5′-labeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase (NEB, Beverly, MA) were used separately as probes. Hybridization and washing were done as described in ref. 16. Synthetic miR159 (24) and miRJAW (25) were used as molecular size markers (Dharmacon Research, Lafayette, CO).

***In Vitro* Binding Assay.** The AC4 and AC2 genes were PCR-amplified with specific primers integrated with BamHI and XhoI restriction sites by using *Pfx* DNA polymerase (Invitrogen) and were cloned into pET41a (Novagen). Proteins were expressed in *Escherichia coli* strain BL21 and purified by using Ni-NTA resin (Qiagen) according to the manufacturer's recommendations. The RNA oligonucleotides miR159 from *Arabidopsis*; miR159* (5′-GAGCUCCUUAAAGUUCAAACA-3′); miR-lin4 from *Caenorhabditis elegans* (5′-UCCCUGAGACCUCAAGUGUGA-3′); miR-lin4* (5′-ACACCUGGGCUCUCCGGGUAC-3′); siGFP-sense, siGFP-antisense, and double-stranded siGFP (23); and miRNA duplexes (miR159·miR159* and miR-lin4·miR-lin4*) were 5′-labeled by using T4 polynucleotide kinase. Single-stranded small RNAs were annealed to their complementary strands to form duplexes as described in ref. 23 and were confirmed by gel electrophoresis for double-stranded siRNA and miRNA duplexes before labeling. Labeled probe was purified by using spun columns (Amersham Pharmacia) and quantified by scintillation counting, and 10,000 cpm of probe was used for each binding reaction with 500 ng of purified A-AC2, A-AC4, E-AC2, and E-AC4 proteins. Binding buffer contained 20 mM Tris·Cl (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 25 mM NaCl, 2.5 mM DTT, and 10% glycerol. Reactions were incubated at 22°C for 15 min, and the complexes were resolved on 8% polyacrylamide gels in 0.5× TBE buffer. For competition assay, 10-, 50-, and 100-fold excesses of unlabeled oligonucleotides were used. Gels were exposed to image screens and scanned by using a PhosphorImager (Molecular Dynamics).

Immobilized 2′-*O*-Methyloligonucleotide-Affinity Capture of AC4. Synthetic 2′-*O*-methyloligonucleotides of miR159* (complementary sequence of miR159: 5′-Bio-GAGCUCCUUAAAGUUCAAACA-3′) and the antisense siRNA strand targeting the firefly (*Photinus pyralis*) luciferase (*Pp*-luc) mRNA (5′-Bio-CGUACGCGGAAUACUUCGAAA-3′), in which Bio indicates biotin attached at the 5′ end through a six-carbon spacer arm (26), were obtained from Integrated DNA Technologies (Coralville, IA).

Biotinylated 2′-*O*-methyloligonucleotides, miR159*, and unrelated *Pp*-luc (300 pM) were incubated for 1 h on ice in a binding buffer (40 mM Tris·Cl, pH 7.4/1 mM EDTA/200 mM NaCl) with 50 μl of streptavidin-coated Dynabeads M280 (Dynal, Brown Deer, WI) to immobilize the oligonucleotides on the beads. Extracts from *A. thaliana* (ecotype Col-0) leaf-derived protoplasts were used for *in vivo* binding assays. A-AC4 gene was PCR-amplified (*Pfx* DNA polymerase) and fused in-frame to the N terminus of 35S–GFP. Protoplasts were inoculated with the 35S–AC4–GFP fusion construct by using PEG-4000 (Sigma) and incubated at 28°C in the dark. Cells were harvested at 24 h after transfection and were homogenized in ice-cold buffer [50 mM Tris·Cl, pH 7.4/100 mM KCl/2.5 mM $MgCl_2$/0.1% (vol/vol) Triton X-100 and complete mini protease inhibitor mixture (1 tablet per 10 ml of solution; Roche)]. The extract was clarified by centrifugation at 12,000 × *g* for 10 min at 4°C. Extracts from control and protoplasts transfected with GFP-plasmid or A-AC4–GFP construct were incubated with immobilized 2′-*O*-methyloligonucleotides (miR159* or *Pp*-luc) tethered by a 5′-biotin to streptavidin-conjugated magnetic beads for 1 h at 25°C. The beads were collected by using a magnetic stand (Dynal), and the unbound supernatant was analyzed for the depletion of miR159 on Northern blots.

To isolate miR159-associated protein, extracts from control and protoplasts expressing GFP or A-AC4–GFP fusion protein were separately incubated with magnetic beads immobilized with miR159*-2′-*O*-methyloligonucleotide. After washing, the beads were boiled for 10 min in 20 μl of SDS loading buffer. Proteins were fractionated on SDS/12% PAGE, transferred to nitrocellulose membrane (Bio-Rad), and detected on Western blots by using anti-GFP monoclonal antibody (Clontech) as described in ref. 27.

Immunocapture of AC4 and Associated miRNAs. Immunoprecipitation of A-AC4–GFP protein complexes was performed by treating the total protoplast extract with 50 μg/ml protein-A-agarose beads (Roche) for 30 min at 4°C. The cleared extract was then incubated with anti-GFP monoclonal antibody at a concentration of 1:2,000 (Clontech) for 1 h at 4°C, followed by treatment with 150 μg/ml protein-A-agarose beads, and incubation was continued for 3 h at 4°C. The agarose beads were washed three times with ice-cold homogenization buffer.

RNA was eluted from the immune complexes by digestion with 1 mg/ml proteinase K [200 mM Tris·Cl, pH 7.4/25 mM EDTA/300 mM NaCl/2% (wt/vol) SDS] at 50°C for 30 min, followed by extraction with phenol/chloroform, and was recovered by precipitation with ethanol (26). The recovered RNA was resuspended in 20 μl of formamide-containing loading buffer, and the RNA was detected by using a 5′-labeled complementary sequence of miR159 probe as described in ref. 16. The presence of A-AC4–GFP fusion protein in the immune complexes was analyzed by Western blotting using anti-GFP monoclonal antibody (26).

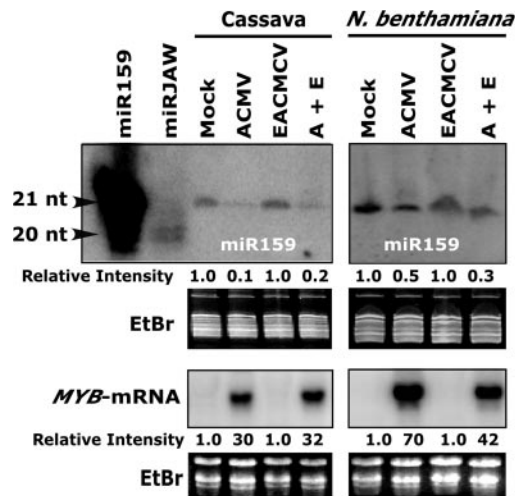

Fig. 1. Expression levels of miRNA and their target mRNA in virus-infected plants. Low-molecular-mass RNAs (20 μg) isolated from mock-inoculated control plant (mock), ACMV alone, EACMCV alone, or ACMV plus EACMCV (A + E)-infected cassava and *N. benthamiana* were electrophoresed, blotted, and probed with synthetic oligonucleotide sequence complementary to miR159 (miR159*) of *Arabidopsis*. As positive controls, synthetic miR159 (21 nt) and miRJAW (20 nt) RNA oligonucleotides were loaded as size markers. miR159 probe cross-hybridized with miRJAW because of partial sequence homology. Total RNA (20 μg) isolated from the virus-infected plants (as mentioned above) was blotted and probed with labeled *MYB* DNA. The relative level of mRNA accumulation was calculated with reference to the level accumulated in the mock-inoculated control plant. Ethidium bromide (EtBr)-stained gels serve as loading controls.

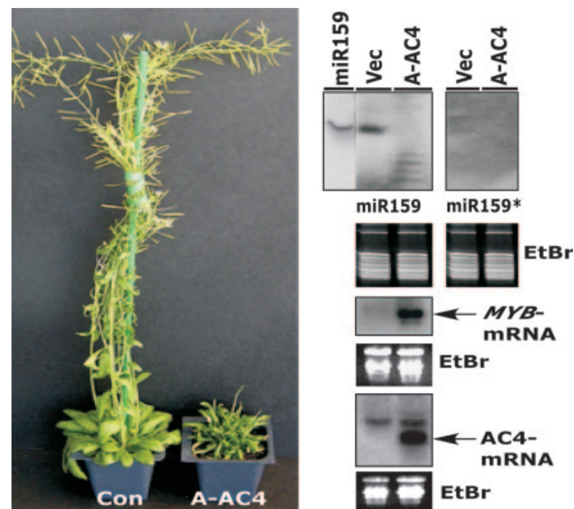

Fig. 2. Transgenic expression of ACMV-AC4 in *Arabidopsis*. (*Left*) Vector-transformed control plant (Con) and A-AC4 transformed *Arabidopsis*. (*Right*) Levels of miR159, miR159* (*Top*), and *MYB* mRNA (*Middle*) accumulation in vector-transformed control (Vec) and in A-AC4 transgenic plants. Labeled synthetic miR159 was used as a positive control. (*Bottom*) Level of A-AC4 mRNA expression in A-AC4-transgenic *Arabidopsis*. Some level of cross-hybridization (top band) was encountered in both control and A-AC4 transgenic plants. Ethidium bromide (EtBr)-stained gels serve as loading controls.

Results

Developmental Abnormalities Induced by Expressing ACMV-Encoded RNA Silencing Suppressor in *Arabidopsis*. miRNAs play a major role in development by regulating host gene expression at the post-transcriptional level. We have previously identified the A-AC4 and the E-AC2 as RNA silencing suppressors (18). Here, we analyzed whether induction of geminivirus-induced developmental phenotypes is due to interference of miRNA-mediated regulatory functions by the viruses and virus-encoded silencing suppressors. Infection by ACMV resulted in severe developmental abnormalities in host plants *Nicotiana benthamiana* and cassava (*Manihot esculenta*) (18). By contrast, a related virus, EACMCV, caused mild symptoms (16). In ACMV-infected but not in EACMCV-infected host plants, we found a reduction in levels of three tested miRNAs: miR159 (in both *N. benthamiana* and cassava), miR165/166, and miR171 (cassava) that regulate several transcription factors affecting leaf development, axial meristem initiation, radial patterning in roots, and hormone signaling (7) (Fig. 1 and Fig. 5*A*, which is published as supporting information on the PNAS web site). This result indicates the presence of *Arabidopsis* miRNA (miR159, miR165/166, and miR171) homologues in cassava, despite the considerable evolutionary distance between *Arabidopsis* and cassava. To determine whether induction of developmental defects is a general property of silencing suppressors (14), the AC2 and AC4 genes from ACMV and EACMCV were introduced separately as transgenes into *Arabidopsis* plants. Transgenic expression of A-AC4 in *Arabidopsis* resulted in stunted plants with severe developmental defects such as narrow rosette leaves and lack of reproductive tissue growth (Fig. 2). Growth and developmental phenotypes were analyzed by using primary transformants. We tested the level of miR159 in A-AC4 transgenic *Arabidopsis* plants because it is one of the most abundant miRNAs present in *Arabidopsis* leaves (28) and found a reduction in miR159 accumulation, which was accompanied by several degraded polynucleotides that hybridized to the complementary sequence of miR159 termed miR159* (Fig. 2 *Top Left*). The fact that we could detect degraded shorter polynucleotides of miR159 suggests that A-AC4 promotes miRNA degradation probably by interacting with host factor(s). In theory, if A-AC4 interacted with duplex forms of miRNA·miRNA*, then we should be able to detect the complementary strand of miRNA in A-AC4 transgenic but not in vector-transformed control plants, because the nonselected strand (miRNA*) of the duplex is highly labile. Northern blot analysis revealed that the miR159* accumulation was below the level of detection, similar to vector-transformed control plants, indicating that A-AC4 has no effect on the unwinding process of miRNA·miRNA* (Fig. 2 *Top Right*). On the other hand, transgenic expression of E-AC2 and A-AC2 as well as E-AC4 elicited no measurable phenotypes but resembled the vector-transformed control plants (data not shown). Our results revealed reduced levels of miRNA accumulation in both ACMV-infected plants and A-AC4-expressing transgenic *Arabidopsis* plants but not in EACMCV-infected plants (Figs. 1 and 2). Overall, ACMV-encoded AC4 is a unique protein in the sense that it decreased host miRNA accumulation, a property that is different from other reported RNA virus-encoded silencing suppressors such as P1/HC-Pro, p19, and p21. These data support the hypothesis that pathogenicity associated with ACMV-AC4 involves the nonavailability of miRNAs to facilitate miRNA-mediated control of gene expression, and as a consequence, A-AC4 transgenic plants showed developmental abnormalities (Fig. 2). These data also indicate that each silencing suppressor is distinct in exerting its function in relation to disease induction.

Interference of ACMV-AC4 with miRNA-Guided Target mRNA Cleavage of Host Gene. To determine the effect of A-AC4 on miRNA-guided inhibition of host mRNA expression, we chose miR159, which represents one of the most abundant miRNAs in leaf tissue (28), and measured the level of *MYB* gene expression, which is normally under negative regulation by miR159 (25, 29). Northern blot hybridization revealed a severalfold (≈20-fold)

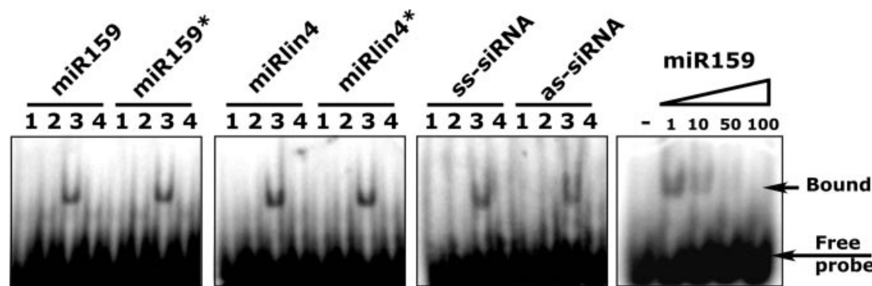

Fig. 3. *In vitro* binding studies. Electrophoretic mobility-shift assays using purified A-AC4 and E-AC2 GST fusion proteins, and synthetic miR159, miR159*, miR-lin4, miR-lin4*, single-stranded sense (ss-siRNA) and antisense-strands (as-siRNA) of siGFP as indicated at the top of each blot. The protein–RNA complexes were analyzed by native PAGE. Lanes: 1, control (5′-labeled corresponding oligoribonucleotide); 2, GST protein; 3, A-AC4–GST fusion protein; and 4, E-AC2–GST fusion protein. High-affinity binding of A-AC4 with miR159 in competition-binding assay using an excess of unlabeled miR159 as shown under the triangle (10-, 50-, and 100-fold) is shown on the right.

increase of *MYB* mRNA accumulation in A-AC4-expressing transgenic *Arabidopsis* (Fig. 2) relative to the level accumulated in vector-transformed control plants (Fig. 2 *Right Middle*). This result was confirmed by quantitative real-time RT-PCR (data not shown; see *Supporting Methods*, which is published as supporting information on the PNAS web site). A similar result of an increase in *MYB* mRNA accumulation was observed in ACMV-infected *N. benthamiana* and cassava plants (Fig. 1 *Lower*). To confirm that down-regulation of miRNAs and up-regulation of target mRNAs by A-AC4 was not an artifact of the transgenic system, the levels of miRNA and target mRNA were measured by transiently expressing A-AC4 on *N. benthamiana* leaves by agroinfiltration (*Agrobacterium*-based infiltration). The RNA isolated from infiltrated leaf patches was subjected to Northern blot analysis. We observed a reduction in miR159 accumulation (Fig. 5*B*), an effect similar to that detected in transgenic systems and in infected host plants. Among other reported suppressors, A-AC4 is distinct in the sense that it down-regulated the miRNAs levels consistently; therefore, it is conceivable that there would be an up-regulation of target mRNAs because of the lack of miRNAs to control the level of mRNA expression. As a consequence, the normal developmental processes would be perturbed, leading to developmental abnormalities.

Interaction of ACMV-AC4 with Small RNAs *in Vitro*. Previously, the A-AC4 and E-AC2, both RNA-silencing suppressor proteins, were shown to inhibit the accumulation of GFP siRNAs in a transient agroinfiltration assay using GFP-transgenic *N. benthamiana* (18). In this study, we found that A-AC4 but not E-AC2 inhibited the accumulation of host miRNAs, suggesting that these two proteins might target different steps of the RNA-silencing pathway. Toward that goal, we tested the ability of the AC4 and AC2 proteins from ACMV and EACMCV, respectively, to bind synthetic small RNAs [the sense and antisense strands of siRNAs designed to target GFP (siGFP), miR159, and miR159*] *in vitro* by using electrophoretic mobility-shift assays. For these assays, we used purified viral proteins (AC2 and AC4 proteins from ACMV and EACMCV expressed in *E. coli*), synthetic oligoribonucleotides miR159 and miR159*, and sense and antisense strands of siGFP. The single-stranded forms of miRNAs and siRNAs were annealed to obtain duplex molecules as described in ref. 23 and were tested on a gel before use in binding assays. The results of our binding assays revealed that A-AC4 binds to single-stranded miR159 and miR159* and single-stranded sense and antisense forms of siGFP (Fig. 3) but does not bind to duplex forms of miRNA159·miRNA159* or to siGFP (Fig. 6*A*, which is published as supporting information on the PNAS web site). By contrast, the E-AC4 and the A-AC2 (Fig. 6*B*) and E-AC2 did not show any binding activity with either single-stranded or duplex forms of both miRNAs and siRNAs (Fig. 3 and Fig. 6*A*). In addition, the high-affinity binding of A-AC4 to single-stranded miRNA (miR159) was evidenced in a competition-binding assay when an excess of unlabeled competitor (miR159) was added with the labeled probes (Fig. 3). As expected, the amount of signal due to miRNA–AC4 protein complex formation decreased with an increasing amount of unlabeled miR159 as the competitor, indicating the high-affinity binding nature of AC4-protein to miRNA.

Furthermore, to determine whether miRNA binding of AC4 protein is a general property, we tested the ability of purified A-AC4 protein to interact with miR-lin4, an miRNA that is involved in regulating developmental timing in *C. elegans* (1). Consistent with the previous results, A-AC4 protein binds to synthetic miR-lin4 and miR-lin4* (Fig. 3) but not to miR-lin4·miR-lin4* duplex molecules (Fig. 6*A*), suggesting the general miRNA-binding nature of A-AC4. To date, A-AC4 is the only RNA-silencing suppressor protein that binds to single-stranded forms of miRNA and siRNA, suggesting that A-AC4 is likely to interfere downstream of the miRNA·miRNA duplex unwinding process either by interfering at a step when single-stranded forms of miRNA and siRNA are being transferred to RISC or with RISC activity rather than RISC assembly, because it had no effect on the unwinding process because it did not retain the complementary strand of miRNA (Fig. 2 *Top Right*).

Isolation of AC4 Protein–miRNA Complex by Using a Tethered 2′-*O*-Methyloligonucleotide. To determine the ability of A-AC4 protein to bind miRNA *in vivo*, the A-AC4 was expressed as a GFP fusion protein in *Arabidopsis*-leaf derived protoplasts, and the A-AC4–miRNA complexes were assessed by affinity-binding assay using a tethered 2′-*O*-methyloligonucleotide complementary to a miRNA. Recently, 2′-*O*-methyloligonucleotide has been successfully used as an RNA bait to isolate RNA-associated proteins in *Drosophila* (26). In this study, the interaction of A-AC4 protein with miRNAs *in vivo* was determined by treating the extract from *Arabidopsis* protoplasts expressing the A-AC4–GFP fusion protein with a 2′-*O*-methyloligonucleotide complementary to miR159 (miR159*), which is tethered to streptavidin-coated magnetic bead through a 5′-biotin linkage. As a control, a 2′-*O*-methyloligonucleotide corresponding to the complementary strand of siRNA targeting the luciferase gene (*Pp*-luc) was used. Protoplasts were transfected with either 35S–GFP alone or 35S–A-AC4–GFP fusion construct, and 24 h later, extracts from protoplasts were prepared as mentioned in *Materials and Methods*. The ability of 2′-*O*-methyl-miR159* oligonucleotide to retain the miR159 was revealed by a reduction in the level of miR159 from the extracts obtained from non-transfected control protoplasts, and protoplasts transfected with 35S–GFP alone or 35S–A-AC4–GFP fusion construct as assayed

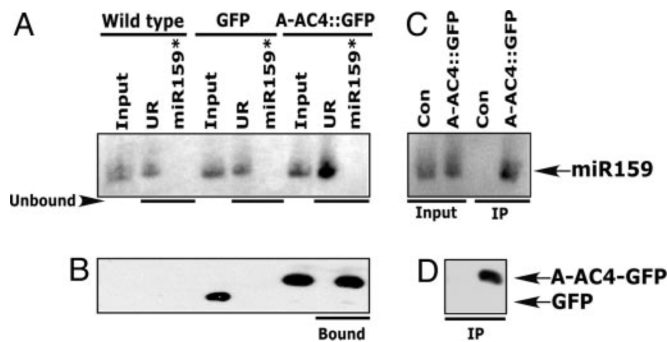

Fig. 4. Isolation of miRNA-associated ACMV-AC4 protein by affinity-binding and coimmunoprecipitation assays. (*A*) Detection of miR159 in the input and in the supernatant of *Arabidopsis* protoplasts, wild type or expressing GFP or A-AC4-GFP, after treatment with tethered 2′-*O*-methyloligonucleotides. UR and miR159* refer to an unrelated (*Pp*-luc) and complementary sequence of miR159, respectively. The blot was probed with 5′-labeled miR159*. (*B*) Detection of GFP-tagged A-AC4 protein that copurified with miR159. The upper band represents the A-AC4–GFP fusion protein, and the lower band represents the GFP protein. Extracts are from wild-type protoplasts or protoplasts expressing GFP or A-AC4–GFP after treatment with tethered 2′-*O*-methyloligonucleotide (miR159* or UR). The bound proteins were fractionated on SDS/12% PAGE and probed by using anti-GFP monoclonal antibody. (*C*) Detection of miR159 associated with A-AC4. Extracts from protoplasts nontransfected (Con) or expressing A-AC4–GFP were immunoprecipitated by using anti-GFP antibodies. The presence of miR159 in the input and in the immunoprecipitate (IP) was analyzed in a Northern blot using 5′-labeled synthetic miR159* as the probe. (*D*) The presence of A-AC4–GFP protein in the IP was detected by Western blotting using anti-GFP antibodies.

in Northern blots (Fig. 4*A*). On the other hand, no reduction was observed in the samples treated with an unrelated 2′-*O*-methyloligonucleotide complementary to the *Pp*-luc siRNA and in the input extracts (Fig. 4*A*). These results indicate that 2′-*O*-methyloligonucleotide complementary to miR159 (miR159*) efficiently binds to miR159 from the protoplast extracts. Having the methodology worked out in a plant system, next we examined the association of A-AC4 protein with miRNA by using 2′-*O*-methyl-miR159* oligonucleotide as an RNA bait. The extracts obtained from nontransfected control protoplasts and protoplasts transfected with either 35S–GFP or 35S–A-AC4–GFP constructs were treated with the immobilized 2′-*O*-methyl-miR159* oligonucleotide on magnetic beads, followed by Western blotting using anti-GFP monoclonal antibodies. The GFP-tagged A-AC4-protein–miR159 complex copurified with the tethered 2′-*O*-methyl-miR159* oligonucleotide but not with the unrelated *Pp*-luc oligonucleotide (Fig. 4*B*), indicating the association of A-AC4 protein with miRNAs. As expected, the expression of the GFP protein and A-AC4–GFP fusion proteins was detected in the input samples obtained from protoplasts transfected with the respective constructs (Fig. 4*B*). These data revealed that 2′-*O*-methyl-miRNA oligonucleotides can be used as an RNA bait to isolate miRNA-binding proteins in plant systems.

Furthermore, the association of A-AC4-protein–miRNA was evaluated by immunoprecipitation using 35S–A-AC4–GFP fusion construct in *Arabidopsis* protoplasts and anti-GFP monoclonal antibodies. Extracts from nontransfected control protoplasts and protoplasts expressing A-AC4–GFP fusion protein were incubated with protein-A-agarose and anti-GFP monoclonal antibodies, washed, and fractionated by SDS/PAGE. Western blot analysis of the input samples and immunoprecipitated fractions revealed the presence of A-AC4–GFP fusion protein in the immune complex obtained from protoplasts transfected with A-AC4–GFP expression construct but not in nontransfected control protoplasts (Fig. 4*D*). Then, the presence of associated miRNA with the A-AC4 protein in the immune complex was analyzed by using the recovered RNAs from the immunoprecipitates in Northern blot hybridization using 5′-labeled miR159* sequence as the probe. RNA from the immunoprecipitates was recovered by proteinase-K treatment followed by ethanol precipitation. The presence of miR159 was detected in the input samples and in the immune complexes obtained from protoplasts transfected with A-AC4–GFP fusion construct but not in the immune complexes obtained from the nontransfected control protoplasts (Fig. 4*C*). In conclusion, from these results, it is evident that A-AC4 protein has the inherent capacity to form complexes with miRNAs. This technology can be widely used in plant systems to isolate small RNA-associated protein complexes.

Discussion

Our results show that the ACMV-encoded AC4, an RNA-silencing suppressor protein, binds to single-stranded miRNAs both *in vitro* and *in vivo* and inhibits miRNA-mediated negative regulation of gene expression in plants. miRNAs are processed from endogenous noncoding RNAs by Dicer-Like 1 and have been shown to negatively regulate target mRNAs that are involved in development at the posttranscriptional level, directing either mRNA cleavage or translational suppression. Mutations in Dicer-Like 1 cause severe morphological defects in *Arabidopsis*, suggesting a role for miRNA in development (2). Indeed, 80% of the predicted or verified miRNA targets encode transcription factors that regulate development (7). To date, ≈29 RNA-silencing suppressors have been identified in plant viruses (30), and three from animal viruses (31); however, these proteins are structurally and functionally diverse, and they likely target distinct steps of the RNA-silencing processes (30). In a previous study, we identified A-AC4 and E-AC2 as RNA-silencing suppressors and found that the level of local silencing suppression by these proteins correlated with reduced levels of GFP-derived siRNA accumulation in agroinfiltration assays using GFP-transgenic *N. benthamiana* (18).

In this study, we examined the role of miRNAs in the developmental abnormalities induced in the leaves of ACMV-infected but not EACMCV-infected host plants. The symptoms elicited by many viruses include developmental abnormalities, and recent study has shown a link among miRNAs, their targets, and virus-induced disease symptoms (15). The fact that we found a reduction in the levels of miRNAs (miR159, miR165/166, and miR171) in ACMV-infected but not EACMCV-infected host plants *N. benthamiana* and cassava indicates that ACMV-infection interfered with the miRNA pathway. In addition, these results revealed the presence of the above-mentioned *Arabidopsis* miRNA homologous in cassava, implying that miRNAs are evolutionarily conserved across plant species. Although A-AC4 and E-AC2 were identified as suppressors of RNA silencing, we encountered a different situation in which transgenic expression of the A-AC4 but not E-AC2 resulted in developmental abnormalities associated with leaf morphology and arrest of flower development. In addition, in A-AC4 transgenic plants, we found a reduction in the level of the tested miR159, suggesting that the A-AC4 sequestered the host miRNAs that are involved in regulating plant development gene expression. Nevertheless, transgenic expression of E-AC2, A-AC2, and E-AC4 induced no measurable phenotypes, compared with vector-transformed control plants. Although the AC2 of EACMCV was previously identified as a silencing suppressor, it had no effect on the miRNAs, indicating that it resembles the 2b protein of cucumber mosaic virus and the capsid protein of turnip crinkle virus with respect to miRNA-mediated functions. Earlier studies have established the effect of the expression of silencing suppressors: P1/HC-Pro, p19, and p21 induced elevated levels of miRNA accumulation and inhibited the miRNA-mediated cleavage of target mRNAs, resulting in a range of phenotypes in *Arabidopsis*

(14, 15). In addition, p69 of turnip yellow mosaic virus was found to up-regulate miRNAs and also to promote miRNA-mediated regulation of target mRNAs (32).

Single-stranded forms of miRNAs are the final products of miRNA biogenesis, which are being selectively incorporated into the RISC complex, and the complementary strand is rapidly degraded in the cell. Interestingly, we found that the A-AC4 (PTGS suppressor) but not A-AC2 (mild PTGS suppressor), E-AC2 (PTGS suppressor), and E-AC4 (non-PTGS suppressor) binds to single-stranded forms of miRNAs and siRNAs *in vitro* by using purified proteins. In fact, we followed different approaches to ensure the single-stranded small RNA-binding property of A-AC4. Modified 2′-*O*-methyloligonucleotides are highly resistant to nucleases and were shown as potent inhibitors of sequence-specific small RNA-mediated functions in *Drosophila* (26). In addition, miRNA let-7-complementary 2′-*O*-methyloligonucleotide has been used to isolate let-7-associated ALG1 and ALG2 miRNA-associated proteins in *Drosophila* (26). From our results, it is evident that tethered miR159-complementary 2′-*O*-methyloligonucleotide has the capacity to deplete miRNA in the protoplast extracts, suggesting that it can be used as an RNA bait to isolate miRNA-associated proteins in plant systems. By using tethered mi159-complementary 2′-*O*-methyloligonucleotide, we have successfully isolated miR159-associated A-AC4 protein, which was detected in Western blots using anti-GFP monoclonal antibodies. The miRNA associated with A-AC4 was recovered from the immunocomplexes obtained from A-AC4–GFP fusion protein-expressing protoplasts by using miR159-complementary sequence as the probe. Previous studies have shown the ability of RNA virus-encoded silencing suppressor proteins such as p19 and p21 that bind to siRNAs and duplex forms of miRNAs and have been speculated to target a step, most likely to interfere in the unwinding process (the RISC assembly) of the silencing pathway (Fig. 7, which is published as supporting information on the PNAS web site) (13, 14). Assembly of RISC-containing miRNA requires unwinding of the miRNA·miRNA* duplex intermediate, followed by incorporation of miRNA into RISC and degradation of miRNA* (11, 12). RISC in plants is comprised mainly of ARGONAUTE proteins having helicase and RNase activities (6). If the A-AC4 inhibited the RISC assembly at the point of unwinding miRNA·miRNA* duplex, then it is expected that miRNA* species would have accumulated in plants expressing A-AC4, because the complementary strand of mature miRNA is labile and hard to detect under normal circumstances. Our data showed that detection of miR159* was below the level of detection, similar to the level in the vector-transformed control plant, suggesting that A-AC4 is likely to act at a point downstream of RISC assembly (Fig. 7). Recently, a RISC-loading complex involved in unwinding of double-stranded siRNAs and transferring single-stranded siRNAs selectively to RISC has been identified in *Drosophila* (10). An equivalent of RISC-loading complex in plants is yet to be unveiled. In conclusion, based on the ability of A-AC4 to bind to mature miRNAs, we predict that A-AC4 recruits the miRNAs by interacting with one or more cellular factors that are associated with RISC-loading complex or RISC. This information will provide another dimension to how viruses have evolved with distinct mechanisms to manipulate the cell system and develop diseases in plants. Finally, we envisage that identification of host counterpart(s) that interact with the A-AC4 will provide insight in unveiling components associated with the miRNA pathway in plants.

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