JOURNAL OF VIROLOGY, Sept. 2004, p. 9487–9498 Vol. 78, No. 17
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.17.9487–9498.2004

Differential Roles of AC2 and AC4 of Cassava Geminiviruses in Mediating Synergism and Suppression of Posttranscriptional Gene Silencing

Ramachandran Vanitharani, Padmanabhan Chellappan, Justin S. Pita, and Claude M. Fauquet*

International Laboratory for Tropical Agricultural Biotechnology, Donald Danforth Plant Science Center, St. Louis, Missouri

Received 22 January 2004/Accepted 21 April 2004

Posttranscriptional gene silencing (PTGS) in plants is a natural defense mechanism against virus infection. In mixed infections, virus synergism is proposed to result from suppression of the host defense mechanism by the viruses. Synergistic severe mosaic disease caused by simultaneous infection with isolates of the Cameroon strain of *African cassava mosaic virus* (ACMV-[CM]) and *East African cassava mosaic Cameroon virus* (EACMCV) in cassava and tobacco is characterized by a dramatic increase in symptom severity and a severalfold increase in viral-DNA accumulation by both viruses compared to that in singly infected plants. Here, we report that synergism between ACMV-[CM] and EACMCV is a two-way process, as the presence of the DNA-A component of ACMV-[CM] or EACMCV in *trans* enhanced the accumulation of viral DNA of EACMCV and ACMV-[CM], respectively, in tobacco BY-2 protoplasts. Furthermore, transient expression of ACMV-[CM] AC4 driven by the *Cauliflower mosaic virus* 35S promoter (p35S-AC4) enhanced EACMCV DNA accumulation by ~8-fold in protoplasts, while p35S-AC2 of EACMCV enhanced ACMV-[CM] DNA accumulation, also by ~8-fold. An *Agrobacterium*-based leaf infiltration assay determined that ACMV-[CM] AC4 and EACMCV AC2, the putative synergistic genes, were able to suppress PTGS induced by green fluorescent protein (GFP) and eliminated the short interfering RNAs associated with PTGS, with a correlated increase in GFP mRNA accumulation. In addition, we have identified AC4 of *Sri Lankan cassava mosaic virus* and AC2 of *Indian cassava mosaic virus* as suppressors of PTGS, indicating that geminiviruses evolved differently in regard to interaction with the host. The specific and different roles played by these AC2 and AC4 proteins of cassava geminiviruses in regulating anti-PTGS activity and their relation to synergism are discussed.

Posttranscriptional gene silencing (PTGS) involves the degradation of viral and cellular mRNAs in a homology-dependent manner, and it is conserved in diverse eukaryotes (15, 25). In plants, PTGS functions as a natural antiviral defense because plant viruses are both initiators and targets of PTGS (47). PTGS was first discovered in plants (30); however, a mechanistically similar phenomenon was later described in other organisms: it is called quelling in fungi (8) and RNA interference in *Caenorhabditis elegans* (11) and in *Drosophila melanogaster* (16). Recent studies at the molecular level revealed that all of these can be considered to be manifestations of an RNA-targeting pathway. Even though the mechanism by which a virus infection triggers PTGS in plants is not fully understood, double-stranded RNA (dsRNA) has been found to be a strong inducer of PTGS (57). Such a form is produced during replication of an RNA virus or conversion of aberrant single-stranded RNAs into dsRNA in the cell by host-encoded RNA-directed RNA polymerase. These dsRNAs are first processed into 21- to 26-nt short interfering RNAs (siRNAs) by an RNase DICER enzyme and subsequently serve as guides by forming an active multicomplex RNA-induced silencing complex, which cleaves homologous RNA molecules (5). In plants, gene silencing generates an unknown mobile signal that can trigger PTGS in distant tissues and across a graft union (32).

In recent years, RNA silencing-inhibiting proteins that counter antiviral RNA silencing have been identified in several plant viruses (47) and in an insect virus (25). These identified silencing suppressor proteins may act at different steps in the PTGS pathway. Three distinct phases have been identified in the RNA-silencing process: initiation, maintenance, and systemic signaling. Thus, (i) the potyvirus helper component proteinase (HC-Pro) interferes with the initiation and maintenance of silencing at a step coincident with or upstream of siRNA production, because it did not prevent the silencing signal from becoming systemic (1, 22, 26); (ii) the 2b protein of *Cucumber mosaic virus* (CMV) prevents the initiation of PTGS in newly emerging tissues by inhibiting long-range PTGS-signaling activity (6, 13); and (iii) p25 of *Potato virus X* (PVX) suppresses the production or accumulation of the mobile silencing signal (54). Recently, the p19 protein of tombusviruses was implicated in inhibiting RNA silencing by physically interacting with siRNAs and thus providing another mechanism to interfere with RNA silencing (43). In geminiviruses, AC2, encoding the transcriptional activator protein (TrAP) of the Kenyan strain of *African cassava mosaic virus* (ACMV-[KE]), and the product of C2, a positional homologue of AC2 in the monopartite *Tomato yellow leaf curl China virus* (TYLCCNV) have both been identified as suppressors of PTGS (52, 55).

In nature, mixed viral infections occur in the same plant,

* Corresponding author. Mailing address: International Laboratory for Tropical Agricultural Biotechnology, Donald Danforth Plant Science Center, 975 North Warson Rd., St. Louis, MO 63132. Phone: (314) 587-1241. Fax: (314) 587-1956. E-mail: ILTAB@danforthcenter.org.

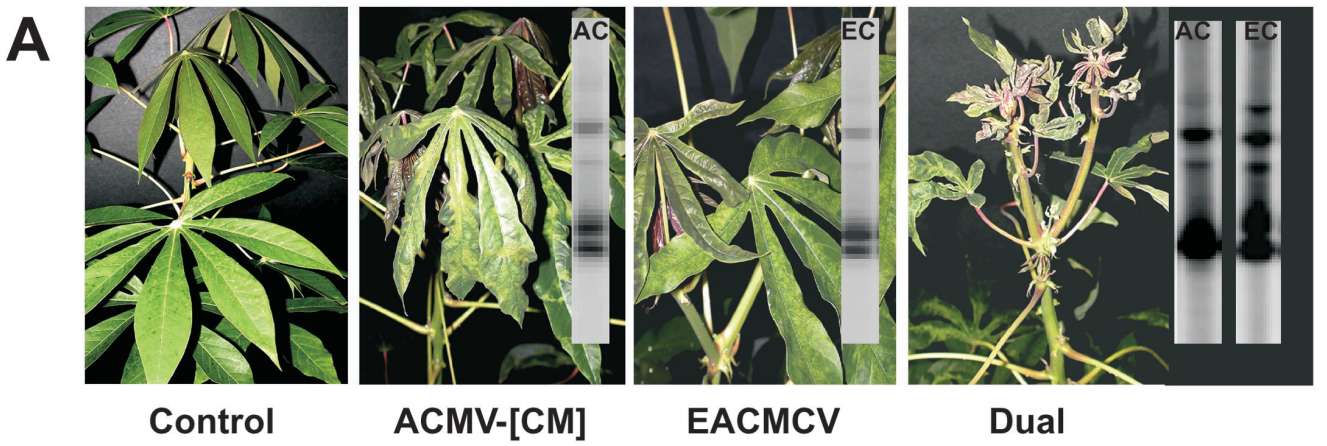
with biological and epidemiological implications. In many cases, mixed infection results in an increase in the titer of one or both viruses and elicits disease symptoms that are more severe than the sum of those induced in either single infection (40, 41, 48). The best-studied synergistic pair is the interaction between an isolate of *Potato virus Y* (PVY; genus *Potyvirus*) and an isolate of PVX (genus *Potexvirus*) in tobacco plants (*Nicotiana tabacum* L.), in which the level of PVX was enhanced from 3- to 10-fold compared with that in singly infected plants (48). Most of the reported synergistic viral diseases involve a potyvirus as one of the synergistic pair; for example, PVX synergizes with tobacco vein mottle potyvirus, tobacco etch potyvirus, and pepper mottle potyvirus (49); cucumber mosaic cucumovirus synergizes with blackeye cowpea mosaic potyvirus (2); cowpea mosaic and bean pod mottle comoviruses are synergistic with soybean mosaic potyvirus (3); maize chlorotic mottle machlomovirus synergizes with maize dwarf mosaic potyvirus; and wheat streak mosaic rymovirus synergizes with sugarcane mosaic potyvirus (41). In addition, most of the time it is a pair of viruses belonging to two different genera or families that synergize, implying a very different nature and origin of the involvement of viral proteins. Cassava mosaic disease is a highly complex disease associated with eight different species of geminiviruses, all belonging to the genus *Begomovirus* of the family *Geminiviridae* (10). Geminiviruses are characterized by small geminate particles containing single-stranded circular DNA molecules. These viruses amplify their genomes in the nuclei of host cells by a rolling-circle replication mechanism that uses dsDNA intermediates as the replication and transcription templates (17). Cassava-infecting geminiviruses are transmitted by whiteflies (*Bemisia tabaci*) and also spread through infected cuttings, which is the usual mode of cassava propagation. The genome of these viruses is divided into two components defined as DNA-A and DNA-B, and both are required for infectivity in plants (44). Previously, it was reported that severe mosaic disease in cassava in the field is associated with the presence of double infection with ACMV and the Ugandan strain of *East African cassava mosaic virus* (EACMV-[UG]) (19), and it was found that these two viruses interact synergistically in causing severe symptoms and a severalfold increase in viral-DNA accumulation (12, 33). This is the first example in which the occurrence of synergism was shown to be due to the presence of viruses belonging to the same genus, *Begomovirus*, of the family *Geminiviridae*. However, the viral gene(s) responsible for this interaction has not been determined.

Virus-virus interaction in mixed infections has been suggested to result from suppression of the host defense mechanism by one of the interacting viruses (42). In PVY and PVX synergism, HC-Pro of PVY, the determinant of the synergistic effect, was found to be a suppressor of gene silencing, permitting viruses to accumulate beyond the normal host-mediated limits (1, 35, 49). Synergism at the level of replication between cymbidium mosaic potexvirus and odontoglossum ringspot tobamovirus has been studied using protoplasts as tools (20). In this paper, we report that the severe cassava mosaic disease associated with synergism between the Cameroon strain of ACMV (ACMV-[CM]) and *East African cassava mosaic Cameroon virus* (EACMCV) is a two-way process involving the DNA-A components of both viruses in a double infection. In addition, we have determined that AC4 of ACMV-[CM] and AC2 of EACMCV, the molecular determinants responsible for the severe synergistic cassava mosaic disease, are able to suppress the locally induced PTGS, indicating that synergism between the two viruses is due to dual suppression of the host defense mechanism. However, the capacities of some AC2 and AC4 proteins to suppress PTGS indicates that each virus is different and thus explains why the observed synergism is very rare, despite the fact that multiple geminivirus infections are frequent (29). The different roles played by AC2 and AC4 of ACMV-[CM] and EACMCV in terms of their abilities to suppress PTGS and thereby mediate synergistic severe disease are discussed.

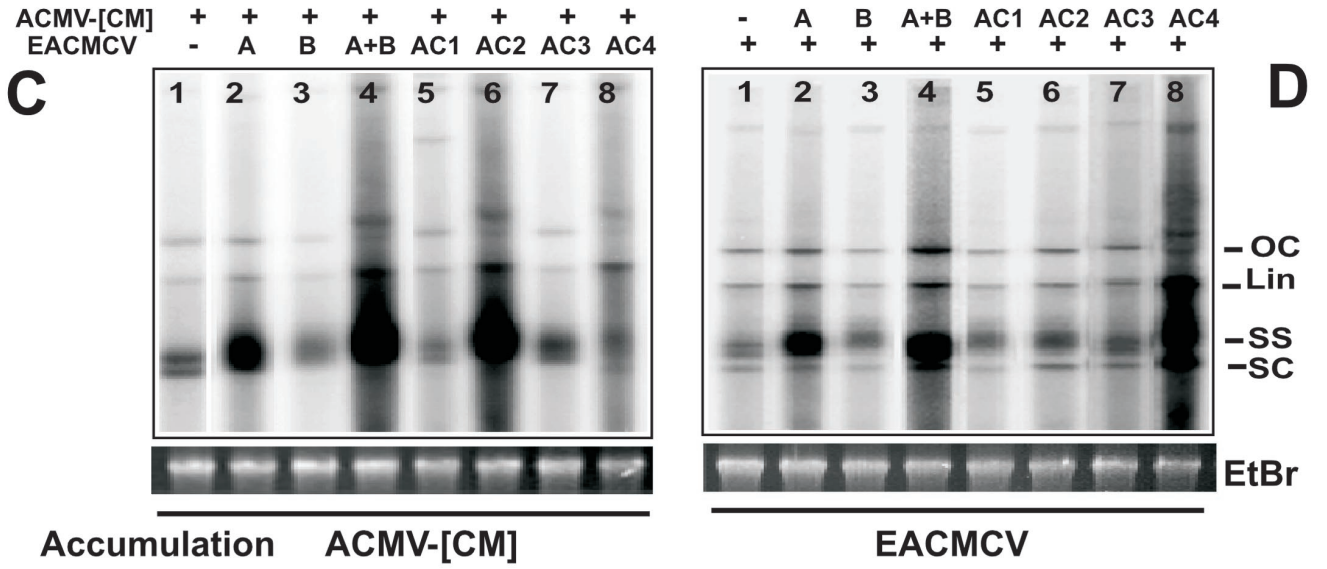
MATERIALS AND METHODS

Viruses and constructs. Construction of the infectious clones of DNA-A and DNA-B of ACMV-[CM] and EACMCV has been described previously (12). Infectious clones of DNA-A and DNA-B of *Sri Lankan cassava mosaic virus* (SLCMV) and *Indian cassava mosaic virus* (ICMV) were kindly provided by J. Stanley, John Innes Centre, Norwich, United Kingdom (39). The genes AC1, AC2, AC3, AC4, AV1, AV2, BV1, and BC1 of ACMV-[CM]; AC1, AC2, AC3, and AC4 of EACMCV; and AC2 and AC4 from SLCMV and ICMV were PCR amplified with *Platinum pfx* DNA polymerase (Invitrogen) by using specific primers with restriction enzyme sites. These PCR products, after cleavage with appropriate restriction enzymes, were introduced separately between the *Cauliflower mosaic virus* (CaMV) 35S promoter and nopaline synthase terminator sequences in a pUC18-based plant expression vector without affecting the 5′ untranslated region. All inserts were sequenced to make sure no sequence errors were introduced by PCR. The whole expression cassette was excised as a HindIII-EcoRI fragment and subcloned into pCAMBIA2300 (AF234315), an *Agrobacterium tumefaciens* binary vector (Fig. 1B). The resulting clones in the binary vector were introduced into *A. tumefaciens* strain GV3101 by the freeze-thaw method and selected on Luria-Bertani agar containing kanamycin (50 μg per ml). An *A. tumefaciens* strain harboring pBin-P1/HC-Pro from *Tobacco etch virus* (TEV) was kindly provided by Vicki Vance, University of South Carolina

FIG. 1. Symptom severity and levels of viral DNA accumulation associated with cassava mosaic disease induced by ACMV-[CM] and EACMCV. (A) From left to right, cassava plants mock inoculated (Control), inoculation with ACMV-[CM] alone and EACMCV alone, and dual inoculation with ACMV-[CM] and EACMCV together. The Southern blot strips next to the inoculated plants indicate the relative amounts of viral-DNA accumulation (ACMV-[CM] DNA accumulation in the ACMV-[CM]-infected plant, EACMCV DNA accumulation in the EACMCV-infected plant, and ACMV-[CM] and EACMCV DNA accumulation in the doubly infected plant). (B) Schematic representation of gene constructs used in this study. ORF, open reading frame. (C to F) Southern blot analysis of relative levels of viral-DNA accumulation in transfected BY-2 protoplasts. Total DNA extracted from protoplasts 48 h after cotransfection with (+) different combinations (Table 1) of infectious clones of DNA-A and DNA-B of ACMV-[CM], along with or without (−) different gene constructs derived from EACMCV (C and E), or with the infectious clones of DNA-A and DNA-B of EACMCV, along with or without gene constructs derived from ACMV-[CM] (D and F). The blots were probed for accumulation of ACMV-[CM] (C) and EACMCV (D) using [α-^{32}P]dCTP-labeled specific probes as described in Materials and Methods. A, DNA-A; B, DNA-B; AC1, replication-associated protein; AC2, transcriptional activator protein; AC3, replication enhancer protein; AC4, function not attributed. The different forms of viral DNA are labeled as follows: SC, supercoiled; SS, single stranded; Lin, linear; and OC, open circular. Ethidium bromide (EtBr)-stained genomic DNA served as the loading control.


A
AC
EC
AC
EC
Control
ACMV-[CM]
EACMCV
Dual


B
5'
RB
35S-CaMV
ORF
NOS-T
LB
3'


ACMV-[CM] + + + + + + + +
EACMCV - A B A+B AC1 AC2 AC3 AC4
C
1 2 3 4 5 6 7 8
- A B A+B AC1 AC2 AC3 AC4
+ + + + + + + +
D
1 2 3 4 5 6 7 8
OC
Lin
SS
SC
EtBr
Accumulation
ACMV-[CM]
EACMCV

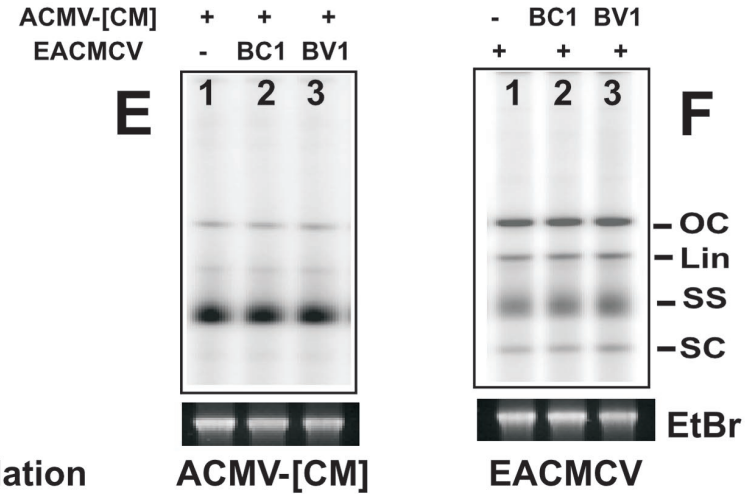

ACMV-[CM] + + +
EACMCV - BC1 BV1
E
1 2 3
- BC1 BV1
+ + +
F
1 2 3
OC
Lin
SS
SC
EtBr
Accumulation
ACMV-[CM]
EACMCV

TABLE 1. Combinations used for protoplast transfection

ACMV-[CM]		EACMCV	
Combination[a]	Accumulation (*n*-fold)[b]	Combination[a]	Accumulation (*n*-fold)[b]
AA + AB	1	EA + EB	1
AA + AB + EA	7.1 ± 1.0	EA + EB + AA	7.0 ± 0.9
AA + AB + EB	0.9 ± 0.1	EA + EB + AB	0.9 ± 0.1
AA + AB + EA + EB	7.7 ± 0.9	EA + EB + AA + AB	7.6 ± 1.1
AA + AB + EAC1	1.0 ± 0.1	EA + EB + AAC1	0.9 ± 0.1
AA + AB + EAC2	8.5 ± 0.9	EA + EB + AAC2	1.0 ± 0.1
AA + AB + EAC3	1.1 ± 0.2	EA + EB + AAC3	1.0 ± 0.1
AA + AB + EAC4	1.1 ± 0.2	EA + EB + AAC4	8.6 ± 1.1
AA + AB + SAC2	1.1 ± 0.1	EA + EB + SAC2	1.1 ± 0.1
AA + AB + SAC4	0.9 ± 0.1	EA + EB + SAC4	4.2 ± 0.7
AA + AB + IAC2	5.3 ± 0.8	EA + EB + IAC2	0.9 ± 0.1
AA + AB + IAC4	1.0 ± 0.1	EA + EB + IAC4	1.0 ± 0.1

[a] AA and AB refer to DNA-A and DNA-B of ACMV-[CM]. EA and EB refer to DNA-A and DNA-B of EACMCV. For AC1, AC2, AC3, and AC4, the prefix A refers to ACMV-[CM]; E refers to EACMCV, S refers to SLCMV, and I refers to ICMV.

[b] The number 1 refers to the amount (1-fold = 100%) of viral DNA accumulation in protoplasts electroporated with combination AA plus AB or EA plus EB and it is the 100% reference for each column. The intensities of bands representing viral-DNA accumulation on Southern blots of three independent experiments for each combination were quantified (± standard deviation) using a PhosphorImager (Molecular Dynamics).

(36). The plasmid pBin containing the 2b gene from CMV (28), kindly provided by Shou-Wei Ding, University of California, Riverside, was introduced into *A. tumefaciens* strain GV3101.

Protoplast transfection and DNA analysis. Protoplasts were isolated from 3-day-old tobacco BY-2 suspension cells derived from *N. tabacum* L. cv. Bright Yellow-2 (50). For transfection, 1.5×10^6 protoplasts were electroporated (using a Bio-Rad electroporater) at 250 V and 500 μF with 4 μg each of the DNA-A and DNA-B components of ACMV-[CM] or EACMCV, along with 20 μg of sheared herring sperm DNA, with or without a gene construct driven from the CaMV 35S promoter (Table 1). Two days later, total DNA was isolated from protoplasts, and Southern blot hybridization was performed essentially as described previously (50). Four micrograms of total DNA isolated from protoplasts was separated by electrophoresis in a 1% agarose gel in 1× Tris-borate-EDTA and transferred to a Hybond-N+ membrane (Amersham). For making an ACMV DNA-A-specific probe, a 794-bp EcoRI fragment (coordinates, nt 1789 to 2583) of ACMV-[UG] DNA-A was used; for an EACMCV DNA-A-specific probe, a 944-bp EcoRI fragment (coordinates, nt 1821 to 2765) of EACMV-[UG2] DNA-A was used; for an ACMV DNA-B-specific probe, a 997-bp EcoRV-HindIII fragment of ACMV-[CM] DNA-B was used; and for EACMCV DNA-B, a 500-bp PCR-amplified DNA fragment from EACMCV DNA-B was used (33). These DNA fragments were gel purified and labeled with [α-^{32}P]dCTP using a random-primer labeling kit (Prime II kit; Stratagene). Hybridization was carried out at 65°C overnight, and posthybridization washes (each for 30 min at 65°C) were done sequentially with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate), 0.5× SSC, and 0.2× SSC, along with 0.1% sodium dodecyl sulfate. The blots were scanned and quantified using a PhosphorImager (Molecular Dynamics) and IQMac version 1.2 software (Amersham).

Virus inoculation and agroinfiltration. The green fluorescent protein (GFP) transgene of *Nicotiana benthamiana* plant line 16C was silenced by agroinfiltration using an *A. tumefaciens* strain harboring the same functional GFP in a binary Ti plasmid (kindly provided by D. C. Baulcombe, John Innes Center). Two weeks later, the silenced plant was inoculated with the infectious clones of DNA-A and DNA-B of ACMV-[CM]. For virus inoculation, gold particles (0.6-μm diameter) were coated separately with a mixture of 10 ng each of DNA-A and DNA-B from infectious clones of ACMV-[CM] (7). Plants were inoculated using a particle delivery system at 1,100 lb/in^2 (model PDS-1000/He; Bio-Rad). The plants were kept in a greenhouse free of whiteflies and monitored for symptom severity on a regular basis. GFP silencing or suppression of GFP silencing was monitored using a handheld 125-W UV lamp (UV Products, Upland, Calif.).

For the agroinfiltration patch assay, *A. tumefaciens* strains harboring the different constructs of ACMV-[CM], EACMCV, SLCMV, and ICMV and the GFP homologous to the GFP transgene in *N. benthamiana* plant line 16C were infiltrated onto the fully expanded leaves of a 3-week-old GFP-transgenic *N. benthamiana*, plant line 16C, as described previously using a 3-ml needleless syringe (26). For coagroinfiltration, each *A. tumefaciens* strain was grown to an optical density at 600 nm of 1 and mixed in equal volumes prior to infiltration. The plants were subsequently monitored for GFP reactivation by using a handheld 125-W UV lamp. The infiltrated plants were kept in a greenhouse at 28°C with a 16-h photoperiod. Images were documented using a fluorescence dissecting microscope (Olympus).

High- and low-molecular-weight RNA isolation and Northern blot analysis. Total high-molecular-weight RNA (10 μg) extracted from the infiltrated leaf patches using an RNeasy kit (Qiagen) were separated in a 1.5% denaturing agarose gel and transferred to Hybond-N+ (Amersham). Low-molecular-weight RNA was isolated as described previously (14) with slight modifications (7). A pool of infiltrated leaf patches collected from five plants was used as the starting material for both types of RNA isolations. RNA samples were preheated to 65°C for 5 min before being loaded onto a 15% polyacrylamide (19:1) gel containing 7 M urea in 0.5× Tris-borate-EDTA. The samples were electrophoresed until the bromophenol blue reached the bottom of the gel, blotted to a Hybond-N+ membrane, and UV cross-linked. Hybridizations were carried out at 42°C with 50% formamide, and posthybridization washes (each for 30 min) were done sequentially with 2× SSC, 0.5× SSC, and 0.2× SSC, along with 0.1% sodium dodecyl sulfate. The blots were scanned and quantified using a PhosphorImager and IQMac version 1.2 software.

RESULTS

Gene(s) involved in synergism between ACMV and EACMCV at the level of viral-DNA accumulation in tobacco protoplasts. Cassava and *N. benthamiana* are excellent hosts for both ACMV and EACMCV, and these viruses produce distinct reproducible symptoms in both plant species. In the field, cassava plants simultaneously infected with ACMV and EACMV display severe symptoms well correlated with higher levels of accumulation of viral DNA from both viruses compared to single infections with either virus (12, 33). A similar scenario has been reproduced under laboratory conditions using infectious clones of these viruses in both *N. benthamiana* (12) and cassava hosts (Fig. 1A).

To elucidate the viral gene(s) involved in the occurrence of synergism between ACMV-[CM] and EACMCV, we used tobacco BY-2 protoplasts as a tool to measure the synergistic effect at the level of viral-DNA accumulation. Cassava-infecting geminiviruses contain a bipartite genome defined as DNA-A and DNA-B, and both components are required for infectivity (44). Initially, in order to determine whether synergistic interaction between the two viruses is a one-way or a two-way process, protoplasts were inoculated with various combinations of the two components of both viruses as shown in Table 1.

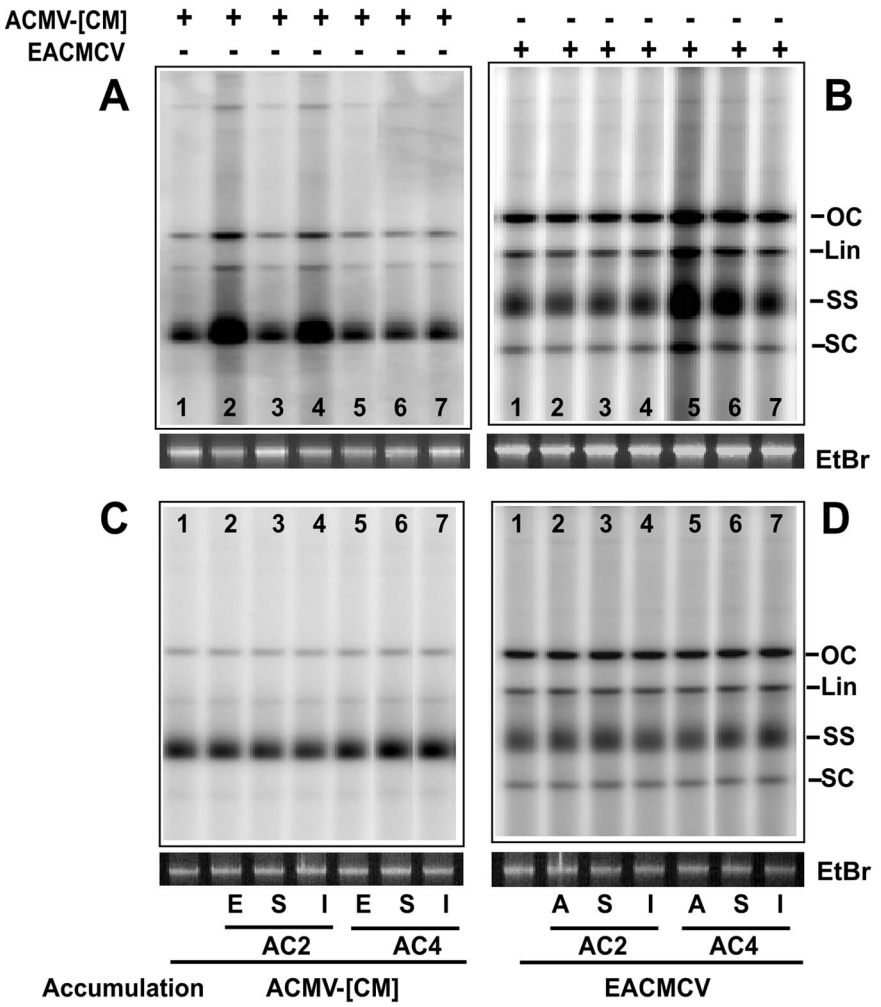


FIG. 2. Relative levels of ACMV-[CM] and EACMCV DNA accumulation upon transfection with AC2 and AC4 of cassava geminiviruses in BY-2 protoplasts. Total DNAs extracted from protoplasts 48 h after cotransfection with (+) the infectious clones of DNA-A and DNA-B of ACMV-[CM] or EACMCV, along with the wild type (A and B) and ATG frameshift mutants (C and D) of AC2 and AC4 gene constructs, as marked at the bottoms of the blots (A, ACMV-[CM]; E, EACMCV; S, SLCMV; I, ICMV) and described in Table 1. The blots were probed for accumulation of ACMV-[CM] (A and C) and EACMCV (B and D) using [α-^{32}P]dCTP-labeled specific probes, as described in Materials and Methods. The different forms of viral DNA are labeled as follows: SC, supercoiled; SS, single stranded, Lin, linear; and OC, open circular. Ethidium bromide (EtBr)-stained genomic DNA served as the loading control.

Two days later, total DNAs isolated from inoculated protoplasts were subjected to Southern blot analysis using virus-specific probes to evaluate the levels of viral-DNA accumulation. The results revealed that DNA-A of EACMCV, when cotransfected with DNA-A and DNA-B of ACMV-[CM], enhanced ACMV-[CM] DNA accumulation by ~7-fold in protoplasts compared with a control that had only two components of ACMV-[CM] (Fig. 1C, lane 2). Similarly, DNA-A of ACMV-[CM], upon cotransfection with EACMCV DNA-A and DNA-B, increased EACMCV DNA accumulation by ~7-fold compared to a control that had only both components of EACMCV (Fig. 1D, lane 2). These results clearly indicate that synergistic interaction between the two viruses is a two-way process. The presence of the DNA-B component of ACMV-[CM] or EACMCV in *trans* by itself did not alter viral-DNA accumulation of the primary virus or vice versa (Fig. 1C and D, lane 3). However, a slight enhancement (0.4- to 0.6-fold) in the single-stranded-DNA form of the heterologous virus has been noticed when the two components of both viruses are present together, but it is not statistically significant and is therefore probably due to experimental differences (Table 1).

The DNA-A components of ACMV-[CM] and EACMCV consist of six genes: AC1 codes for a replication-associated protein (Rep), AC2 codes for a transcriptional activator protein (TrAP), AC3 codes for a replication enhancer protein (REn), AV1 codes for the coat protein (CP), AV2 codes for the precoat, and AC4 codes for a protein to which no function has yet been attributed (17, 31, 45). The DNA-B components of these viruses comprise two genes, BC1 and BV1, that are involved in systemic movement and symptom development of the virus (38). Codelivering the two components of either of the viruses in combination with a plant expression vector harboring one of the genes of the second virus in *trans* would identify the particular gene(s) involved in synergistic interaction between the two viruses at the level of viral-DNA accumulation in protoplasts (Fig. 1B and Table 1). Southern blot

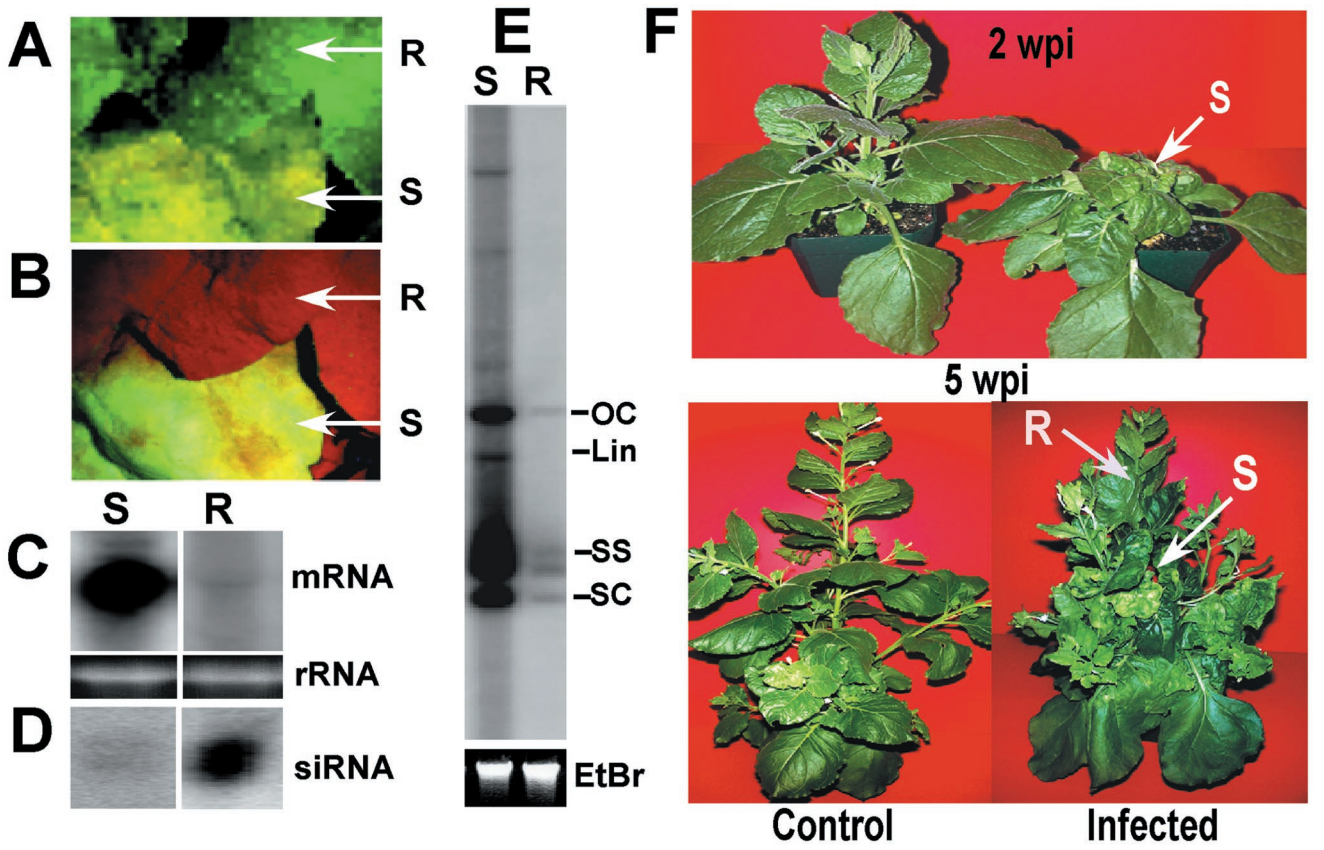


FIG. 3. ACMV-[CM]-infected GFP-transgenic *N. benthamiana* silenced for GFP showed recovery from symptoms. GFP-transgenic *N. benthamiana* line 16C was infiltrated with an *A. tumefaciens* strain harboring the cognate GFP. A plant silenced for GFP was bombarded with the infectious clones of DNA-A and DNA-B of ACMV-[CM]. (A and B) Plant photographed under normal light (A) and using a UV filter (B). Symptom-free recovered leaves appeared red under UV light. (C and D) Northern blots containing 20 μg of RNA were probed for the accumulation of GFP mRNA (C) and GFP-specific siRNA (D) in the lower symptomatic (S) and the upper recovered (R) silenced leaves, using an [α-^{32}P]dCTP-labeled GFP-specific probe. (E) Southern blot analysis of total DNA (10 μg) extracted from lower symptomatic leaves (S) and upper symptom-free leaves (R) to detect levels of viral-DNA accumulation. Ethidium bromide (EtBr)-stained rRNA and genomic DNA served as loading controls. (F) Full view of ACMV-[CM]-inoculated *N. benthamiana* plants with symptoms (2 weeks p.i. [wpi]) and symptom-free recovered plants (5 wpi). S and R indicate symptomatic and recovered leaves, respectively.

analyses of the total DNAs isolated from inoculated protoplasts revealed that EACMCV AC2 expressed in *trans* under a 35S promoter enhanced ACMV-[CM] DNA accumulation by ~8-fold compared to that of protoplasts transfected with ACMV-[CM] alone (Fig. 1C, lane 6, and 2A, lane 2). On the other hand, ACMV-[CM] AC4 expressed in *trans* from the 35S promoter increased EACMCV DNA accumulation by ~8-fold compared to control transfection with EACMCV alone (Fig. 1D, lane 8, and 2B, lane 5). Nevertheless, ACMV-[CM] AC2 (Fig. 1D, lane 6) or EACMCV AC4 (Fig. 1C, lane 8) independently did not significantly alter the accumulation of viral DNA in either way. Codelivery of either BC1 or BV1 of DNA-B of ACMV-[CM] or EACMCV independently, in combination with the two components of EACMCV or ACMV-[CM], respectively, did not alter DNA accumulation of the primary virus (Fig. 1E and F). These results indicate that synergism between the two viruses is possibly mediated by AC2 of EACMCV and AC4 of ACMV-[CM] by enhancing viral-DNA accumulation, implying that the presence of a higher virus titer is responsible for causing severe disease. In addition, our cotransfection experiments revealed that ICMV AC2 in *trans* enhanced ACMV-[CM] DNA accumulation by ~5-fold (Fig. 2A, lane 4), similarly to EACMCV AC2. On the other hand, SLCMV AC4 in *trans* increased the rate of EACMCV DNA accumulation by ~4-fold (Fig. 2B, lane 6), indicating behavior similar to that of ACMV-[CM] AC4. The presence of translated products of these genes to enhance heterologous viral-DNA accumulation was confirmed by using ATG frameshift mutants of the genes. We found that neither the mutated EACMCV AC2 or ICMV AC2 enhanced ACMV-[CM] DNA accumulation (Fig. 2C), nor did ACMV-[CM] AC4 or SLCMV AC4 increase EACMCV DNA accumulation (Fig. 2D). In all cases, we have shown the level of DNA-A accumulation for each virus; however, the effect of this gene(s) on the level of DNA-B accumulation of each virus was similar to that on DNA-A (data not shown). Variations at the level of viral-DNA accumulation in blots between experiments were not significant (Table 1), indicating only experimental differences.

Suppression of gene silencing by ACMV-[CM] and EACMCV cassava geminiviruses. To investigate the virulence capacities of ACMV-[CM] and EACMCV in relation to their abilities to suppress gene silencing, the infectious clones of these viruses

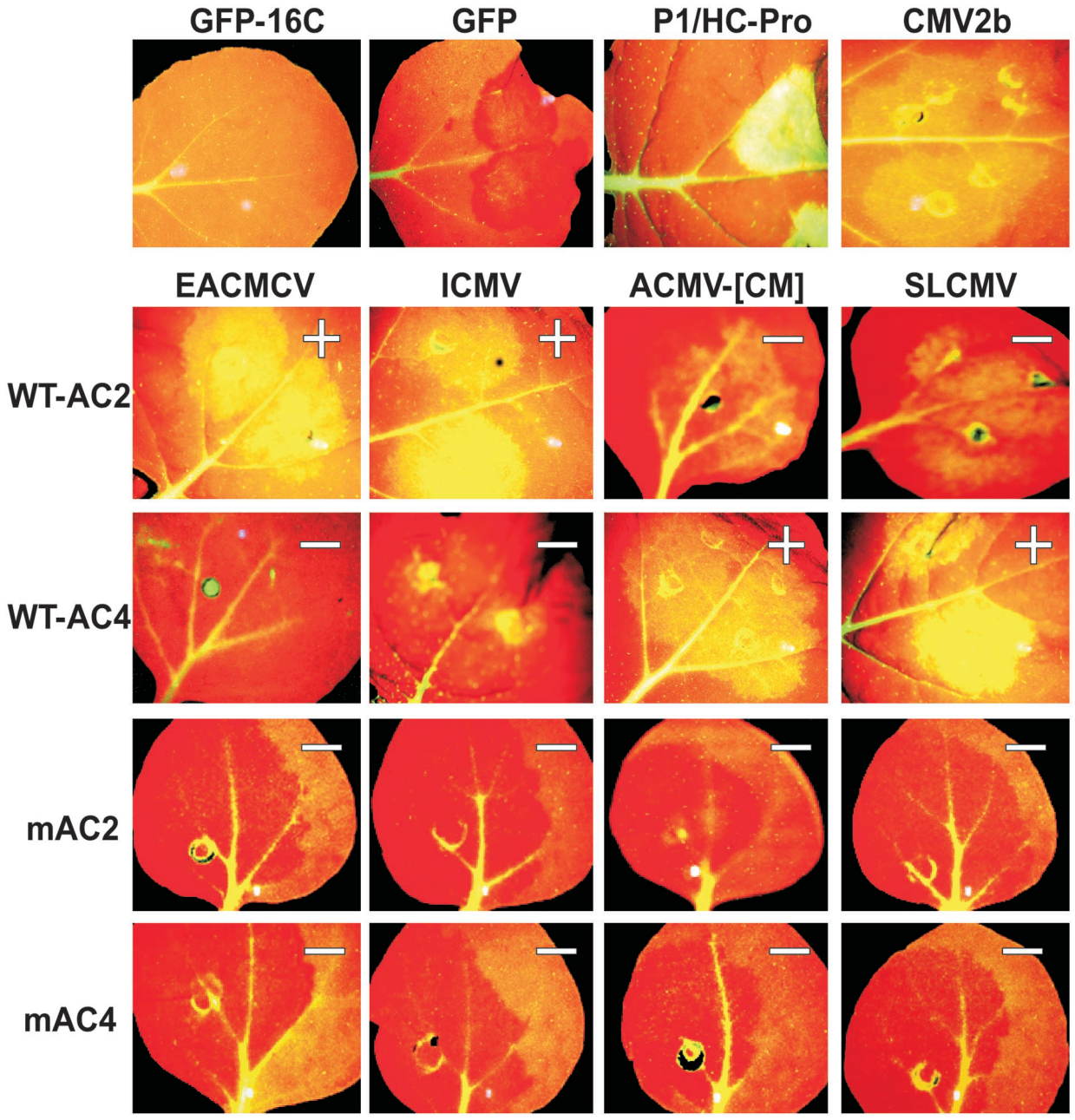


FIG. 4. RNA-silencing suppression activity of AC2 and AC4 genes of cassava-infecting geminiviruses. Shown are leaves of an *N. benthaminana* line 16C plant infiltrated with an *A. tumefaciens* strain harboring pBin-GFP alone (GFP) or coinfiltrated along with P1/HC-Pro of TEV (P1/HC-Pro), 2b of CMV (CMV2b), or wild-type (WT) and ATG frameshift mutants (m) of AC2 and AC4 of ACMV-[CM], EACMCV, SLCMV, and ICMV. The leaves were photographed 7 days after infiltration. The PTGS suppression levels were positive (+) or negative (−). GFP-16C is a noninfiltrated leaf of a 16C plant.

were inoculated onto *N. benthamiana* plants of line 16C, in which the GFP transgene was silenced. Infiltrating the lower leaf of the seedling with *A. tumefaciens* harboring a binary plasmid containing a GFP transcription cassette induced silencing of the GFP transgene. PTGS induced in the infiltrated patch was followed by systemic spread throughout the plant. Tissue that has undergone silencing appears red under UV light due to the fluorescence of the chlorophyll. Infection of GFP-silenced 16C plants with ACMV-[CM] showed a complete reversal of GFP silencing, and the infected tissue became highly GFP fluorescent, concomitant with the occurrence of virus symptoms from 3 to 21 days postinoculation (p.i.) (Fig. 3A, B, and F). Eventually, however, after 21 days p.i., GFP silencing was not blocked in the subsequent newly emerged leaves; as a result, these leaves appeared red under UV illumination (Fig. 3B), indicating the nonavailability of suppressor protein to suppress the host defense system in order to continue the infection. ACMV-[CM]-infected *N. benthamiana* showed severe symptoms at 2 weeks p.i. (Fig. 3F, top), but after 3 weeks p.i., the newly emerging leaves recovered from the

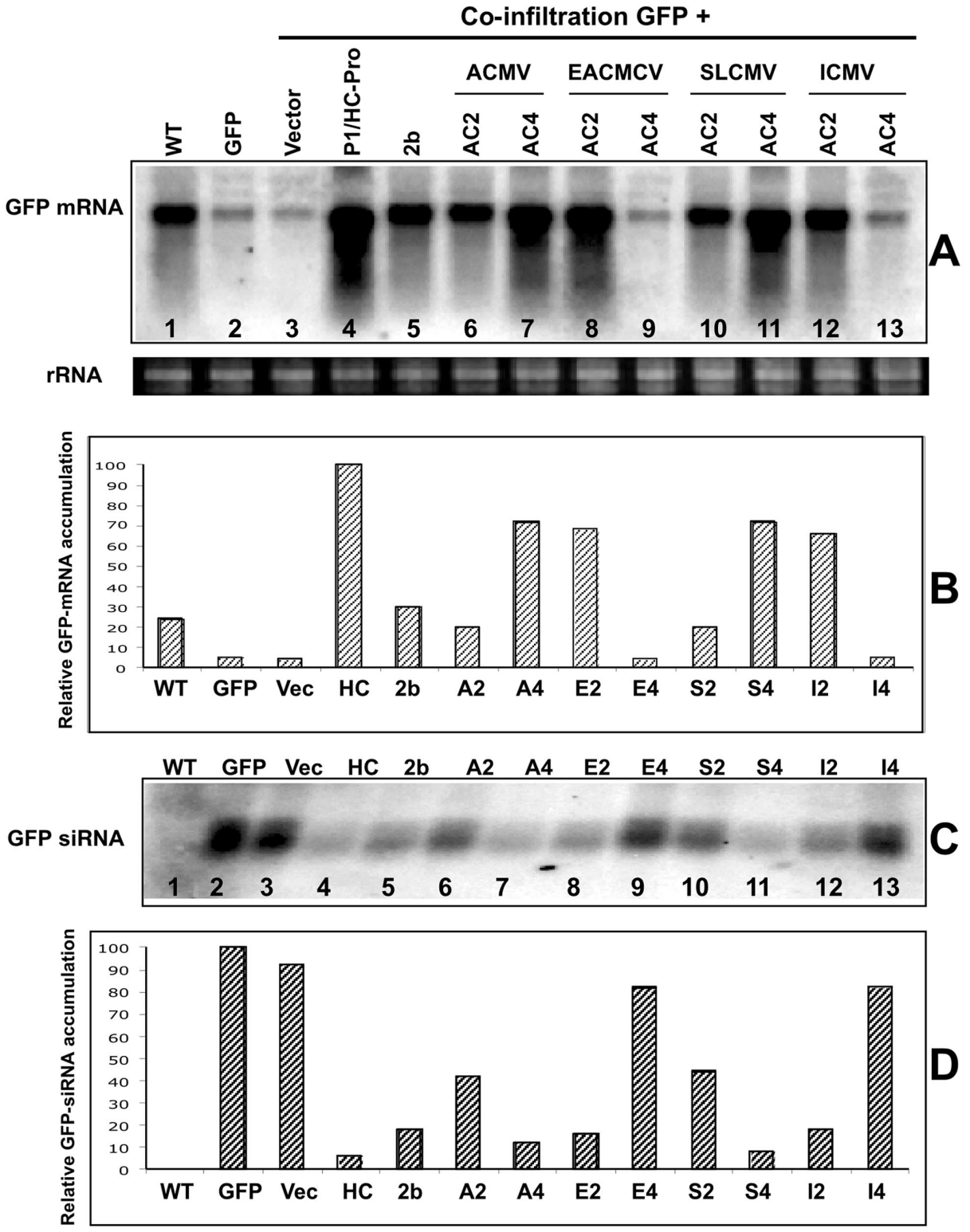


FIG. 5. Northern blot analysis of GFP mRNA and siRNAs extracted from patches of *N. benthamiana* 16C leaves infiltrated with *Agrobacterium* suspensions carrying different constructs. (A) Total RNA was isolated from a pool of leaf patches obtained from five plants for each combination. Lane WT, RNA from a noninfiltrated *N. benthamiana* line 16C plant serving as a control; lane GFP, RNA from the leaf sample of a line 16C plant infiltrated with pBin-GFP alone. The remaining lanes carried RNAs extracted from leaf patches coinfiltrated with pBin-GFP along with empty vector (pCAMBIA2300), P1/HC-Pro of TEV, 2b of CMV, and AC2 and AC4 from ACMV-[CM], EACMCV, SLCMV, and ICMV. Ethidium bromide staining of the same gel served as the loading control. (B) Intensities of signals obtained for GFP mRNA expression as percentages from 0 to 100 (where the highest level of mRNA expression by P1/HC-Pro was scored as 100%), using ImageQuant (IqMac version 1.2) software for further comparison. I2, ICMV AC2; I4, ICMV AC4. (C) Analysis of siRNAs isolated from noninfiltrated leaf of line 16C plant

symptoms (Fig. 3F, bottom). This was further supported by the fact that the newly developed symptom-free recovered leaves had only a low level of viral-DNA accumulation compared with the symptomatic leaves (Fig. 3E). GFP silencing was not reversed in these leaves, which correlated well with larger amounts of GFP-specific siRNAs (Fig. 3D) and a low level of GFP mRNA accumulation (Fig. 3C). In addition, viral suppression of PTGS correlated strongly with the physical presence of the virus in the symptomatic leaves (Fig. 3A and B) compared to the presence of a low level of viral DNA in the recovered upper leaves, in which GFP silencing persisted (Fig. 3E). GFP expression in mock-inoculated control plants remained silenced, indicating that the observed fluorescence was due to the reversal of GFP silencing. Our results clearly show that ACMV-[CM] encodes a protein that suppresses PTGS and that is active locally in the virus-infected leaves.

In contrast, EACMCV-infected plants started to show symptoms ~14 days p.i., but the symptoms persisted until senescence and the plants never recovered. A similar experiment inoculating GFP-silenced plants with EACMCV revealed the silencing suppression capability of the virus, indicating that EACMCV encodes a protein that blocks the gene silencing as well. A strict direct correlation between symptom severity and viral-DNA accumulation (J. S. Pita et al., unpublished data) and a reverse correlation between viral-DNA accumulation and virus-derived siRNA accumulation (7) were observed, indicating a very strong relationship between the molecular behavior of each virus and the PTGS system of the host plant.

Synergistic genes are characterized as viral suppressors of gene silencing. To test the abilities of the identified synergistic genes (AC2 of EACMCV and AC4 of ACMV-[CM] at the level of viral-DNA accumulation in protoplasts) to suppress gene silencing, an *Agrobacterium*-based leaf infiltration assay was conducted. In each experiment, two *Agrobacterium* strains, one carrying the binary plasmid harboring either AC2 or AC4 of ACMV-[CM] and EACMCV driven by a 35S CaMV promoter and a second strain containing the pBin-GFP gene to initiate and enhance RNA silencing in GFP-transgenic *N. benthamiana*, were coinfiltrated into the leaves of 3-week-old GFP-transgenic *N. benthamiana* plants. Suppression of GFP silencing was monitored during the following days, and the leaves were photographed 7 days after infiltration. Leaves infiltrated with GFP construct alone showed a marked increase in GFP fluorescence 2 days after infiltration due to transient GFP expression but then started to decrease at 4 days postinfiltration and had almost disappeared from the leaf areas between the veins by 6 days postinfiltration (Fig. 4). This result is consistent with previous reports demonstrating that transient expression of GFP mRNA at high levels rapidly triggered PTGS. When leaves were infiltrated with a mixture of suspensions carrying GFP and a construct harboring either EACMCV AC2 or ACMV-[CM] AC4, fluorescence was initially much stronger than in leaves infiltrated with GFP alone. The fluorescence continued to increase to a very high level at 5 days p.i. and remained at this level for ~8 to 9 days (Fig. 4). Leaves that received ACMV-[CM] AC2 also showed GFP fluorescence at the beginning, but later it was very mild compared with that of AC4 of the same virus (Fig. 4). Patches that had received pBin-GFP plus EACMCV AC4 or an empty vector (pCAMBIA2300) had undergone silencing of the GFP signal similar to that of patches infiltrated with pBin-GFP alone and consequently appeared red (Fig. 4). The requirement for translated products of these silencing suppressors was confirmed by using ATG frameshift mutants of the constructs. Upon coinfiltration along with GFP, the frameshift constructs were unable to suppress the GFP-induced silencing, and as a result, the infiltrated leaves appeared red in UV light (Fig. 4). As a positive control, we used P1/HC-Pro of TEV and 2b of CMV and found that the level of enhanced GFP fluorescence in leaves infiltrated with a mixture of GFP and P1/HC-Pro was very marked (Fig. 4); we also observed mild suppression with the 2b protein of CMV (Fig. 4). The possibility of viral genes other than AC2 and AC4 suppressing gene silencing was investigated by dissecting individual genes (AC1, AC3, AV1, AV2, BV1, and BC1) of ACMV-[CM], one of the viruses tested in this study. *Agrobacterium* leaf patch assays revealed that none of these constructs could suppress GFP silencing (data not shown).

Northern blot analysis of GFP mRNA transcript levels confirmed the visual observations of the AC2- and AC4-mediated silencing suppression. Marked reduction in the level of GFP mRNA was found in leaves in which silencing had been induced by infiltration with GFP (Fig. 5A, lane 2). As reported earlier, GFP transcript was abundant in the RNAs of patches that had received pBin-GFP plus pBin-P1/HC-Pro (Fig. 5A, lane 4), which was considered to be 100% for further comparison with the levels of mRNA accumulation by other suppressor proteins (Fig. 5B). GFP reversion with 2b of CMV was mild, with a relative GFP mRNA accumulation of 30% (Fig. 5A, lane 5). GFP transcript accumulations were 68 to 72% in the RNAs of patches that had received EACMCV AC2 or ACMV-[CM] AC4 along with pBin-GFP (Fig. 5A, lanes 7 and 8), but they were only 20% in the RNAs of patches treated with ACMV-[CM] AC2 plus pBin-GFP, which correlated well with a mild level of silencing suppression exhibited by AC2 of ACMV-[CM] in the infiltrated leaves (Fig. 5A, lane 6). The GFP mRNA level was only 4% in the RNAs of patches that had received pBin-GFP plus EACMCV-AC4, which is similar to the level accumulated by patches infiltrated with pBin-GFP alone (Fig. 5A, lanes 9). Consistent with the fluorescence results, the level of GFP mRNA accumulation was very low (5%) in the leaves infiltrated with either GFP alone (5%) or GFP plus empty vector (4%).

In addition to GFP mRNA levels, we also assessed the levels of the GFP-specific siRNAs as a way of confirming that the reduced levels of mRNA were indeed the result of PTGS.

as a control (WT; lane 1) and patches that received either pBin-GFP alone (GFP; lane 2) or leaf patches coinfiltrated with pBin-GFP along with the empty vector pCAMBIA2300 (Vec; lane 3), P1/HC-Pro of TEV (HC; lane 4), 2b of CMV (2b; lane 5), or ACMV-[CM] AC2 (A2; lane 6) and AC4 (A4; lane 7), EACMCV AC2 (E2; lane 8) and AC4 (E4; lane 9), SLCMV AC2 (S2; lane 10) and AC4 (S4; lane 11), or ICMV AC2 (I2; lane 12) and AC4 (I4; lane 13). (D) Intensities of signals obtained for levels of GFP siRNA accumulation as percentages from 0 to 100 (where the greatest amount of signal obtained for pBin-GFP infiltrated alone was scored as 100%), using IqMac version 1.2 software.

Low-molecular-weight RNA was extracted from *Agrobacterium*-infiltrated leaf patches of GFP-transgenic *N. benthamiana*. GFP-specific siRNAs were readily detected in a transgenic plant infiltrated with GFP alone, which was considered to be 100% for further comparison with other combinations in the infiltration assays. Accumulation of GFP-specific siRNAs was abundant (92%) in leaf patches that had received pBin-GFP plus the empty vector (Fig. 5C and D) but was less abundant in the patches which had received pBin-GFP along with constructs that could suppress GFP silencing, i.e., EACMCV AC2 accumulated only 16% and ACMV-[CM] AC4 had only 12%. The GFP-specific siRNAs were abundant (82%) in the leaves that received EACMCV AC4, in which silencing suppression was not effective (Fig. 5C and D). ACMV-[CM] AC2, a mild suppressor, accumulated a larger amount of GFP-specific siRNAs (42%) (Fig. 5C, lane 6). As reported previously, siRNA accumulation was very low (6%) when GFP was coinfiltrated with P1/HC-Pro, and we also observed a reduction (18%) with 2b of CMV (Fig. 5C and D). Based on the silencing suppression abilities of the AC2 and AC4 genes of ACMV-[CM] and EACMCV that were tested in this study, and for the sake of simplicity of discussion, we categorized ACMV-[CM] AC4 and EACMCV AC2 as positive and ACMV-[CM] AC2 and EACMCV AC4 as negative.

Silencing suppression activities of AC2 and AC4 from other cassava geminiviruses. In addition, we tested the abilities of AC2 and AC4 of other cassava-infecting geminiviruses, such as SLCMV and ICMV from the Indian subcontinent, to suppress gene silencing. Among bipartite geminiviruses, AC2 is phylogenetically conserved, similarly to all the other viral proteins, while AC4 is the most divergent protein (Fauquet, unpublished data). Constructs expressing AC2 or AC4 of SLCMV and ICMV were created in an *Agrobacterium*-based binary vector. *Agrobacterium* leaf infiltration assays revealed that AC2 of ICMV is a strong suppressor of gene silencing while SLCMV AC2 is a weak suppressor (Fig. 4). In contrast, SLCMV AC4 is a strong suppressor of gene silencing while ICMV AC4 is not a suppressor of gene silencing (Fig. 4). Northern blot analysis of GFP mRNA accumulation confirmed the visually observed results that SLCMV AC4 and ICMV AC2 suppressed GFP silencing, with the production of high levels of GFP mRNA (66 to 72%), while the level of GFP mRNA accumulation was relatively low (21%) with SLCMV AC2 (Fig. 5A and B). Low-molecular-weight RNA analysis further confirmed the results showing that ICMV AC2 and SLCMV AC4, with accumulations of only 18 and 8%, respectively, and to a certain extent (44%) SLCMV AC2, are able to block the GFP-derived siRNAs (Fig. 5C and D). However, ICMV AC4 did not block PTGS of GFP (Fig. 5C), and as a consequence, the infiltrated leaves accumulated 82% of GFP siRNAs. SLCMV AC2 is also considered to be negative, similarly to ACMV-[CM] AC2, based on its much lower capacity to accumulate GFP mRNA in infiltrated leaves.

DISCUSSION

Under field conditions, the simultaneous occurrence of ACMV and EACMCV in cassava plants results in unusually severe synergistic disease (12, 19, 33). ACMV-[CM] is a rapidly developing virus that elicits symptoms within 5 days p.i. both in *N. benthamiana* and in cassava plants, but the infected plants recover from symptoms within 3 weeks p.i., while EACMCV is a slowly developing virus which elicits symptoms ~14 days p.i. from which the infected plants never recover (Pita et al., unpublished data). However, in dual infections, symptom severity correlated well with a larger amount of viral-DNA accumulation for both viruses (12, 33). The work presented here, using tobacco BY-2 protoplasts, has identified the genes in ACMV-[CM] and EACMCV that are capable of mediating enhanced viral-DNA accumulation. Our results, based on viral-DNA accumulation in protoplasts, revealed that synergism between ACMV-[CM] and EACMCV is a two-way process, because on one hand the DNA-A of ACMV-[CM] enhanced EACMCV DNA accumulation by ~7-fold, and on the other hand, the DNA-A of EACMCV enhanced ACMV-[CM] DNA accumulation by ~7-fold. ACMV and EACMCV are bipartite geminiviruses with a divided genome of DNA-A, containing six genes (AC1, AC2, AC3, AC4, AV1, and AV2), and DNA-B with two genes (BC1 and BV1) (44). Our results, based on viral-DNA accumulation in protoplasts, revealed that EACMCV AC2 in *trans* increased ACMV-[CM] DNA accumulation by ~8-fold, while ACMV-[CM] AC2 did not alter EACMCV DNA accumulation. In similar experiments, we found that ACMV-[CM] AC4 enhanced EACMCV DNA accumulation by ~8-fold, whereas EACMCV AC4 failed to increase ACMV-[CM] DNA accumulation. The AC2 and the AC4 proteins in the two viruses show 56 and 30% amino acid sequence homology, respectively. The transcriptional-activation function of AC2 on AV1 and BV1 genes is highly conserved in bipartite geminiviruses (45). In contrast, AC4 is highly variable among different geminiviruses, and so far, no definitive and clear function has been attributed to this gene product. However, in monopartite geminiviruses, C4, a positional homologue of AC4, is a major determinant of pathogenesis. C4 of TYLCCNV was found to counter the replication-associated protein-induced hypersensitive response in *N. benthamiana* (51), expression of the C4 gene of *Tomato leaf curl virus* produced virus-like symptoms in transgenic plants (23, 37), C4 of TYLCV was shown to be involved in virus movement (21), and C4 of *Beet curly top virus* was shown to enhance cell elongation and cell division as a means to favor virus multiplication (24). The involvement of the DNA-B components of both ACMV-[CM] and EACMCV was ruled out because neither the BC1 nor the BV1 gene independently interfered with primary viral-DNA accumulation.

Mixed infection leading to synergistic viral diseases in plants has been known for a long time, but only recently has it been implicated in the suppression of PTGS, a host surveillance mechanism of the interacting viruses, suggesting that the two phenomena may be linked. An illustrative example of such a phenomenon is the synergistic effect of HC-Pro of TEV on PVX replication, where HC-Pro was a strong suppressor of RNA silencing (1, 35, 40, 49). In addition, variation in the capacity of the similar protein to suppress silencing has been reported for the 2b protein of cucumoviruses, such as CMV and *Tomato aspermy virus* (TAV). In fact, TAV 2b, but not CMV 2b, induced a synergistic disease and also triggered typical hypersensitive virus resistance with the transcriptional induction of pathogenesis-related protein genes in related host species, indicating that TAV 2b is a different type of suppressor

than 2b of CMV. In our case, using an *Agrobacterium* leaf infiltration assay, we demonstrated that two genes, EACMCV AC2 and ACMV-[CM] AC4, that were involved in enhanced viral-DNA accumulation in protoplasts were able to suppress locally induced PTGS. ACMV-[CM] AC2 in *trans* did not alter EACMCV DNA accumulation, but it could act as a mild suppressor of PTGS, as shown for AC2 of ACMV-[KE] (14, 55). The AC2 genes of ACMV-[CM] and EACMCV show 56% homology and contain conserved zinc finger, transactivator, and DNA-binding domains; however, these genes act differently. In monopartite geminiviruses, it has been demonstrated recently that the C2 protein of TYLCCNV requires the DNA-binding domain, the zinc finger domain, and the nuclear localization signal for anti-PTGS activity (9, 52, 53). C2 is a positional homologue of AC2 in bipartite geminiviruses, but it varies functionally in that it does not facilitate the transcriptional activation of coat protein, as in the case of AC2 of bipartite geminiviruses. Therefore, we propose that the different functions of the AC2 proteins that we noticed could be due to differences at the level of point mutations, as indicated by sequence comparisons (data not shown). In addition, AC2 of *Tomato golden mosaic virus* and C2 of *Beet curly top virus* have been shown to manipulate host metabolism by interfering with SNF1 kinase and adenosine kinase to provide enhanced susceptibility to virus infection (18, 46, 56); however, their roles in relation to PTGS have not been established. All these data support the fact that these tiny proteins are multifunctional in nature.

We extended our studies by testing the AC2s and AC4s of two other cassava-infecting geminiviruses, namely, SLCMV and ICMV. Our results revealed that ICMV AC2 is a strong suppressor of PTGS while SLCMV AC2 is a weak suppressor. On the other hand, SLCMV AC4 is capable of suppressing PTGS, but ICMV AC4 is unable to suppress the locally induced PTGS. These results further indicate that AC2s and AC4s of different viruses behave differently. Moreover, the ability of AC4 of ACMV-[CM] and SLCMV to suppress PTGS coincides with their rapid symptom-inducible natures in the hosts, a common feature of ACMV-[CM] and SLCMV. In addition, these AC4 proteins contain a stretch of amino acid sequence that is different in the EACMCV and ICMV AC4 sequences. The role of this domain in the functions of these AC4 genes is under investigation. Similarly, the anti-PTGS activity of AC2 of EACMCV and ICMV coincides with the slowly infecting nature of these viruses (7). We are looking at the synergistic interaction of SLCMV and ICMV with ACMV-[CM] and EACMCV. The possible involvement of other gene products of ACMV-[CM], such as AC1, AC3, AV1, AV2, BV1, and BC1, in suppression of PTGS in the GFP-transgenic *N. benthamiana* revealed that none of the tested genes were able to suppress the locally induced PTGS.

RNA silencing is an ancient cellular defense mechanism conserved among different kingdoms of organisms (34). It is therefore understandable that viruses utilizing RNA during replication and gene expression would need to develop strategies to evade this defense system. Many RNA viruses have evolved an active mechanism to counteract silencing by encoding suppressor proteins that interfere with the process (47). Geminiviruses do not have a dsRNA phase in their life cycles. Nevertheless, these viruses are able to trigger the host's PTGS system with the production of virus-derived siRNAs, as reported recently for the monopartite geminivirus *Tomato yellow leaf curl Sardinia virus* (27) and also in bipartite cassava-infecting geminiviruses (7). When inoculated into a host plant containing a posttranscriptionally silenced transgene, a virus that possesses anti-PTGS activity can reverse silencing that is already established and/or prevent its onset in the new growth (4, 6). Upon infection, ACMV-[CM] and EACMCV reversed the established GFP silencing in *N. benthamiana* plants, indicating that RNA silencing is suppressed in the infected plant cells. However, a difference in the strengths of anti-PTGS activities in ACMV-[CM]- and EACMCV-infected plants was observed. ACMV-[CM] reversed the established GFP silencing in transgenic *N. benthamiana* for a period of ~2 weeks; later, GFP silencing persisted in the newly emerged leaves, which correlated well with a lower viral load and greater amounts of GFP-derived siRNA accumulation, indicating that the physical presence of the virus is essential to suppress RNA silencing. Moreover, it appears that the suppressor protein in ACMV-[CM] is active only for a certain time. Therefore, ACMV-[CM]-infected plants showed recovery from symptoms at a later stage of the infection cycle. In the case of EACMCV, symptoms appear ~14 days p.i. but last until plant senescence. We speculate that the EACMCV-encoded suppressor may be stronger and longer lasting than the one encoded by ACMV-[CM]. In summary, we postulate that ACMV-[CM] AC4 suppresses the antiviral defense system in plants at the beginning of infection, and as a result, plants develop symptoms rapidly after inoculation. In contrast, the inability of EACMCV AC4 to suppress gene silencing might account for the delay in the onset of symptoms, although AC2 of this virus is a suppressor of PTGS, which becomes active perhaps 2 weeks after infection in plants. Therefore, we propose that the different roles played by AC2 and AC4 of ACMV-[CM] and EACMCV might target different steps in the RNA-silencing pathway in a temporal and spatial manner, permitting the other interacting viruses to accumulate beyond the normal host-mediated limits and leading to severe disease in plants.

ACKNOWLEDGMENTS

We thank David Baulcombe for GFP-transgenic *N. benthamiana* line 16C seed and the cognate pBin-GFP plasmid, Vicki Vance for P1/HC-Pro of TEV, and Shou-Wei Ding for the pBin-2b plasmid. We gratefully acknowledge John Stanley for providing SLCMV and ICMV. We thank Masaaki Fujiki of Danforth Center for the pUC18-based plant expression vector.

This research was funded by the Donald Danforth Plant Science Center.

REFERENCES

1. **Anandalakshmi, R., G. J. Pruss, X. Ge, R. Marathe, A. C. Mallory, T. H. Smith, and V. B. Vance.** 1998. A viral suppressor of gene silencing in plants. Proc. Natl. Acad. Sci. USA **95:**13079–13084.
2. **Anderson, E. J., A. S. Kline, T. E. Morelock, and R. W. McNew.** 1996. Tolerance to blackeye cowpea mosaic potyvirus not correlated with decreased virus accumulation or protection from cowpea stunt disease. Phytopathology **80:**847–852.
3. **Anjos, J. R., U. Jarlfors, and S. A. Ghabrial.** 1992. Soybean mosaic potyvirus enhances the titer of two comoviruses in dually infected soybean plants. Phytopathology **82:**1022–1027.
4. **Beclin, C., R. Berthome, J. C. Palauqui, M. Tepfer, and H. Vaucheret.** 1998. Infection of tobacco or Arabidopsis plants by CMV counteracts systemic post-transcriptional silencing of nonviral (trans)genes. Virology **252:**313–317.
5. **Bernstein, E., A. A. Caudy, S. M. Hammond, and G. J. Hannon.** 2001. Role

for a bidentate ribonuclease in the initiation step of RNA interference. Nature **409:**363–366.
6. **Brigneti, G., O. Voinnet, W. Li, L. Ji, S. Ding, and D. C. Baulcombe.** 1998. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. EMBO J. **17:**6739–6746.
7. **Chellappan, P., R. Vanitharani, and C. M. Fauquet.** 2004. siRNA accumulation correlates with host recovery in DNA virus infected hosts and gene silencing targets specific viral sequences. J. Virol. **78:**7465–7477.
8. **Cogoni, C., and G. Macino.** 1997. Isolation of quelling-defective (qde) mutants impaired in posttranscriptional transgene-induced gene silencing in *Neurospora crassa*. Proc. Natl. Acad. Sci. USA **94:**10233–10238.
9. **Dong, X., R. van Wezel, J. Stanley, and Y. Hong.** 2003. Functional characterization of the nuclear localization signal for a suppressor of posttranscriptional gene silencing. J. Virol. **77:**7026–7033.
10. **Fauquet, C. M., and J. Stanley.** 2003. Geminivirus classification and nomenclature: progress and problems. Ann. Appl. Biol. **142:**165–189.
11. **Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello.** 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature **391:**806–811.
12. **Fondong, V. N., J. S. Pita, M. E. Rey, A. de Kochko, R. N. Beachy, and C. M. Fauquet.** 2000. Evidence of synergism between African cassava mosaic virus and a new double-recombinant geminivirus infecting cassava in Cameroon. J. Gen. Virol. **81:**287–297.
13. **Guo, H., and S. Ding.** 2002. A viral protein inhibits the long range signaling activity of the gene silencing signal. EMBO J. **21:**398–407.
14. **Hamilton, A., O. Voinnet, L. Chappell, and D. Baulcombe.** 2002. Two classes of short interfering RNA in RNA silencing. EMBO J. **21:**4671–4679.
15. **Hamilton, A. J., and D. C. Baulcombe.** 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science **286:**950–952.
16. **Hammond, S. M., E. Bernstein, D. Beach, and G. J. Hannon.** 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature **404:**293–296.
17. **Hanley-Bowdoin, L., S. B. Settlage, B. M. Orozco, S. Nagar, and D. Robertson.** 1999. Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. Crit. Rev. Plant Sci. **18:**71–106.
18. **Hao, L., H. Wang, G. Sunter, and D. M. Bisaro.** 2003. Geminivirus AL2 and L2 proteins interact with and inactivate SNF1 kinase. Plant Cell **15:**1034–1048.
19. **Harrison, B. D., X. Zhou, G. W. Otim-Nape, Y. Liu, and D. J. Robinson.** 1997. Role of a novel type of double infection in the geminivirus-induced epidemic of severe cassava mosaic in Uganda. Ann. Appl. Biol. **131:**437–448.
20. **Hu, W. W., S. M. Wong, C. J. Goh, and C. S. Loh.** 1998. Synergism in replication of cymbidium mosaic potexvirus (CymMV) and odontoglossum ringspot tobamovirus (ORSV) RNA in orchid protoplasts. Arch. Virol. **143:**1265–1275.
21. **Jupin, I., F. De Kouchkovsky, F. Jouanneau, and B. Gronenborn.** 1994. Movement of tomato yellow leaf curl geminivirus (TYLCV): involvement of the protein encoded by ORF C4. Virology **204:**82–90.
22. **Kasschau, K. D., and J. C. Carrington.** 1998. A counterdefensive strategy of plant viruses: suppression of posttranscriptional gene silencing. Cell **95:**461–470.
23. **Krake, L. R., M. A. Rezaian, and I. B. Dry.** 1998. Expression of the tomato leaf curl geminivirus C4 gene produces virus like symptoms in transgenic plants. Mol. Plant-Microbe Interact. **11:**413–417.
24. **Latham, J. R., K. Saunders, M. S. Pinner, and J. Stanley.** 1997. Induction of plant cell division by beet curly top virus gene C4. Plant J. **11:**1273–1283.
25. **Li, H., W. X. Li, and S. W. Ding.** 2002. Induction and suppression of RNA silencing by an animal virus. Science **296:**1319–1321.
26. **Llave, C., K. D. Kasschau, and J. C. Carrington.** 2000. Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. Proc. Natl. Acad. Sci. USA **97:**13401–13406.
27. **Lucioli, A., E. Noris, A. Brunetti, R. Tavazza, V. Ruzza, A. G. Castillo, E. R. Bejarano, G. P. Accotto, and M. Tavazza.** 2003. Tomato yellow leaf curl Sardinia virus rep-derived resistance to homologous and heterologous geminiviruses occurs by different mechanisms and is overcome if virus-mediated transgene silencing is activated. J. Virol. **77:**6785–6798.
28. **Lucy, A. P., H. Guo, W. Li, and S. Ding.** 2000. Suppression of post-transcriptional gene silencing by a plant viral protein localized in the nucleus. EMBO J. **19:**1672–1680.
29. **Méndez-Lozano, J., I. Torres-Pacheco, C. M. Fauquet, and R. F. Rivera-Bustamante.** 2003. Interactions between geminiviruses in a naturally occurring mixture: pepper huasteco virus and pepper golden mosaic virus. Phytopathology **93:**270–277.
30. **Napoli, C., C. Lemieux, and R. Jorgensen.** 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in *trans*. Plant Cell **2:**279–289.
31. **Padidam, M., R. N. Beachy, and C. M. Fauquet.** 1996. The role of AV2 ("precoat") and coat protein in viral replication and movement in tomato leaf curl geminivirus. Virology **224:**390–404.
32. **Palauqui, J. C., T. Elmayan, J. M. Pollien, and H. Vaucheret.** 1997. Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. EMBO J. **16:**4738–4745.
33. **Pita, J. S., V. N. Fondong, A. Sangare, G. W. Otim-Nape, S. Ogwal, and C. M. Fauquet.** 2001. Recombination, pseudorecombination and synergism of geminiviruses are determinant keys to the epidemic of severe cassava mosaic disease in Uganda. J. Gen. Virol. **82:**655–665.
34. **Plasterk, R. H.** 2002. RNA silencing: the genome's immune system. Science **296:**1263–1265.
35. **Pruss, G., X. Ge, X. M. Shi, J. C. Carrington, and V. B. Vance.** 1997. Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. Plant Cell **9:**859–868.
36. **Restrepo, M. A., D. D. Freed, and J. C. Carrington.** 1990. Nuclear transport of plant potyviral proteins. Plant Cell **2:**987–998.
37. **Rigden, J. E., L. R. Krake, M. A. Rezaian, and I. B. Dry.** 1994. ORF C4 of tomato leaf curl geminivirus is a determinant of symptom severity. Virology **204:**847–850.
38. **Sanderfoot, A. A., and S. G. Lazarowitz.** 1995. Cooperation in viral movement: the geminivirus BL1 movement protein interacts with BR1 and redirects it from the nucleus to the cell periphery. Plant Cell **7:**1185–1194.
39. **Saunders, K., N. Salim, V. R. Mali, V. G. Malathi, R. Briddon, P. G. Markham, and J. Stanley.** 2002. Characterisation of Sri Lankan cassava mosaic virus and Indian cassava mosaic virus: evidence for acquisition of a DNA B component by a monopartite begomovirus. Virology **293:**63–74.
40. **Savenkov, E. I., and J. P. Valkonen.** 2001. Potyviral helper-component proteinase expressed in transgenic plants enhances titers of Potato leaf roll virus but does not alleviate its phloem limitation. Virology **283:**285–293.
41. **Scheets, K.** 1998. Maize chlorotic mottle machlomovirus and wheat streak mosaic rymovirus concentrations increase in the synergistic disease corn lethal necrosis. Virology **242:**28–38.
42. **Sijen, T., and J. M. Kooter.** 2000. Post-transcriptional gene-silencing: RNAs on the attack or on the defense. Bioessays **22:**520–531.
43. **Silhavy, D., A. Molnar, A. Lucioli, G. Szittya, C. Hornyik, M. Tavazza, and J. Burgyan.** 2002. A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. EMBO J. **21:**3070–3080.
44. **Stanley, J.** 1983. Infectivity of the cloned geminivirus genome requires sequences from both DNAs. Nature **305:**643–645.
45. **Sunter, G., and D. M. Bisaro.** 1992. Transactivation of geminivirus AR1 and BR1 gene expression by the viral AL2 gene product occurs at the level of transcription. Plant Cell **4:**1321–1331.
46. **Sunter, G., J. L. Sunter, and D. M. Bisaro.** 2001. Plants expressing tomato golden mosaic virus AL2 or beet curly top virus L2 transgenes show enhanced susceptibility to infection by DNA and RNA viruses. Virology **285:**59–70.
47. **Vance, V., and H. Vaucheret.** 2001. RNA silencing in plants—defense and counterdefense. Science **292:**2277–2280.
48. **Vance, V. B.** 1991. Replication of potato virus X RNA is altered in coinfections with potato virus Y. Virology **182:**486–494.
49. **Vance, V. B., P. H. Berger, J. C. Carrington, A. G. Hunt, and X. M. Shi.** 1995. 5′ proximal potyviral sequences mediate potato virus X/potyviral synergistic disease in transgenic tobacco. Virology **206:**583–590.
50. **Vanitharani, R., P. Chellappan, and C. M. Fauquet.** 2003. Short interfering RNA-mediated interference of gene expression and viral DNA accumulation in cultured plant cells. Proc. Natl. Acad. Sci. USA **100:**9632–9636.
51. **Van Wezel, R., X. Dong, P. Blake, J. Stanley, and Y. Hong.** 2002. Differential roles of geminivirus Rep and AC4 (C4) in the induction of necrosis in *Nicotiana benthamiana*. Mol. Plant Pathol. **3:**461–471.
52. **Van Wezel, R., X. Dong, H. Liu, P. Tien, J. Stanley, and Y. Hong.** 2002. Mutation of three cysteine residues in Tomato yellow leaf curl virus-China C2 protein causes dysfunction in pathogenesis and posttranscriptional gene-silencing suppression. Mol. Plant-Microbe Interact. **15:**203–208.
53. **Van Wezel, R., H. Liu, Z. Wu, J. Stanley, and Y. Hong.** 2003. Contribution of the zinc finger to zinc and DNA binding by a suppressor of posttranscriptional gene silencing. J. Virol. **77:**696–700.
54. **Voinnet, O., C. Lederer, and D. C. Baulcombe.** 2000. A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana*. Cell **103:**157–167.
55. **Voinnet, O., Y. M. Pinto, and D. C. Baulcombe.** 1999. Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. Proc. Natl. Acad. Sci. USA **96:**14147–14152.
56. **Wang, H., L. Hao, C. Y. Shung, G. Sunter, and D. M. Bisaro.** 2003. Adenosine kinase is inactivated by geminivirus AL2 and L2 proteins. Plant Cell **15:**3020–3032.
57. **Waterhouse, P. M., M. Wang, and T. Lough.** 2001. Gene silencing as an adaptive defence against viruses. Nature **411:**834–842.