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Genetics

Defective viral DNA ameliorates symptoms of geminivirus infection in transgenic plants

(African cassava mosaic virus/crop protection/defective interfering DNA/plant virus)

JOHN STANLEY*, THOMAS FRISCHMUTH, AND SUSAN ELLWOOD

Department of Virus Research, John Innes Institute, John Innes Centre for Plant Science Research, Colney Lane, Norwich NR4 7UH, United Kingdom

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ABSTRACT **_Nicotiana benthamiana_ was transformed with a single copy of a tandem repeat of subgenomic DNA B isolated from plants infected with a Kenyan isolate of the bipartite geminivirus African cassava mosaic virus. Symptoms in transformed plants were less severe than in nontransformed controls when challenged with virus or cloned DNA of Kenyan or Nigerian isolates. Symptom amelioration was associated with the mobilization and amplification of the subgenomic DNA, producing a comparable reduction in the amount of DNA specific to each genomic component. The disproportionate reduction in the levels of full-length components (DNA A, 20%; DNA B, 70%) indicates that the episomally replicating subgenomic DNA has been amplified at the expense of full-length DNA B to three times the level of the latter. Serial infection of transformants resulted in a further decrease in symptom severity and viral DNA levels. No differences were observed in the severity of symptoms or levels of viral DNA when transformants and controls were challenged with the related geminiviruses beet curly top virus and tomato golden mosaic virus, demonstrating the specific nature of the interaction. Analysis of infected tissue showed that tomato golden mosaic virus was unable to amplify the subgenomic DNA. However, since the production of subgenomic DNA is possibly a common feature of the bipartite geminiviruses, this approach might contribute to the production of plants showing increased tolerance to a number of economically important viral diseases.**

Cassava (*Manihot esculenta*) is grown widely throughout the tropics and provides the greatest tonnage of all African food crops. Cassava mosaic disease (CMD) is widespread throughout the African continent and is considered to be the most devastating disease of this crop. Most cassava cultivars are susceptible to the disease and its incidence exceeds 80% in many parts of Africa where it can cause 60–80% decrease in tuber yield when propagated vegetatively (1). The causal agent of CMD is African cassava mosaic virus (ACMV; synonym, cassava latent virus) (2, 3), a single-stranded DNA (ssDNA) virus belonging to the geminivirus group. The genome of ACMV comprises two components of similar size, both of which are required for infection (4, 5). DNA A encodes the coat protein (6) as well as products required for replication (7), implicating the two essential genes encoded by DNA B (8) in spread of the virus throughout the plant.

Infection with the native Kenyan isolate of ACMV is associated with the production of subgenomic DNA B, occurring as single-stranded and double-stranded forms, the ssDNA being associated with virions (5, 9). The subgenomic DNA comprises a family of closely related DNA B molecules of approximately half the size of the full-length component in which both genes have been disrupted. This defective DNA was shown to interfere with ACMV proliferation in *Nicotiana benthamiana*, manifested as a delay in symptom development and symptom amelioration (9). Thus, the encapsidated subgenomic ssDNA was considered to behave similarly to defective interfering (DI) particles (reviewed in ref. 10), which serve to modulate virus levels in natural infections and so prevent the development of severe symptoms that might otherwise ultimately have an adverse effect on the virus population.

In this article we explore the potential of using integrated copies of the subgenomic DNA to protect plants against viral infection. Integrated multimeric copies of DNA B can escape from the host genome and replicate episomally when the plant is challenged with DNA A (11). Here we demonstrate that the defective genomic component is similarly mobilized and amplified in infected plants and that the episomally replicating DNA has a marked effect on virus proliferation and symptom development.

MATERIALS AND METHODS

Virus Stocks and Clone Construction. The construction of infectious clones of Kenyan and Nigerian isolates of ACMV and beet curly top virus (BCTV) and tandem or partial repeats of the genomic components of ACMV and BCTV for the purpose of agroinoculation has been described (5, 12–15). The construction and agroinoculation of partial repeats of the tomato golden mosaic virus (TGMV; common strain) genome will be described elsewhere. Subgenomic DNA B from the Kenyan isolate of ACMV, containing an identical deletion to that in clone pDEF004 (9) but as a *Sal* I fragment (clone pDEF012), was cloned as a head-to-tail dimer into pBin19 (16) to produce pBin2DI. ACMV was isolated and purified as described (17) from plants that had been inoculated with cloned DNA.

Plant Transformation. Clone pBin2DI was mobilized into *Agrobacterium tumefaciens* LBA4404 (18) by triparental mating (19), and transconjugants were selected for their resistance to kanamycin and rifampicin. The integrity of the ACMV DNA in *A. tumefaciens* was verified by Southern blot analysis (20). *N. benthamiana* was transformed using a leaf disc method (21, 22) and transformants were selected for resistance to 0.1 mg of kanamycin per ml. The copy number of integrated DNA was determined by Southern blot analysis of genomic DNA, isolated as described (23), using copy number standards based on a 2C nuclear DNA weight of 6.4 pg (24). Following self-fertilization, F_1 and F_2 progeny were tested for antibiotic sensitivity by germinating seeds on 0.5 mg of kanamycin per ml.

Plant Inoculation. Plants were maintained in accordance with the requirements of the Advisory Committee on Genetic Manipulation, in an insect-free glasshouse at 25°C (reduced to

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Abbreviations: ACMV, African cassava mosaic virus; BCTV, beet curly top virus; scDNA, supercoiled DNA; ssDNA, single-stranded DNA; TGMV, tomato golden mosaic virus; DI, defective interfering.
*To whom reprint requests should be addressed.

20°C at night) with supplementary lighting to give a 16-hr photoperiod. Seeds were treated with 0.5 mg of gibberellic acid per ml overnight at room temperature before germination. Approximately 3 weeks after germination, plants were challenged with either purified ACMV (Kenyan isolate) or cloned genomic components of ACMV (Kenyan and Nigerian isolates) (5, 12) by mechanical inoculation. ACMV (Kenyan isolate), BCTV, and TGMV were also introduced into plants by stem agroinoculation (14, 15) using *A. tumefaciens* strains LBA4404 and C58 (18, 25). ACMV and TGMV were mechanically transmitted from extracts of plants infected using cloned DNA components.

Characterization of Viral DNA. Total cellular nucleic acids were extracted as described (26) and 5 μg samples were run on agarose gels in 45 mM Tris-borate/1.25 mM EDTA, pH 8.0. Viral DNA was analyzed by Southern blotting following sample depurination (27). Blots were hybridized to ACMV component-specific probes (14) or to probes comprising full-length copies of the TGMV genome. The relative amounts of genomic and subgenomic components were estimated by dot blot analysis of pooled ssDNA and supercoiled (scDNA) eluted from agarose gels. Radioactivity of individual samples was monitored following hybridization to ACMV component-specific probes. Results were the average of duplicate samples from four control and four transformed plants.

RESULTS

Plant Transformation. *N. benthamiana* was transformed using *A. tumefaciens* carrying a tandem repeat of subgenomic DNA B from a Kenyan isolate of ACMV between the T DNA borders of the binary vector pBin19. A kanamycin-resistant transformant (DEF1) containing a single integrated copy of the T DNA, as judged by Southern blot analysis of genomic DNA, was selected and self-fertilized. Resistance to kanamycin at 0.5 mg/ml segregated in the ratio of 3:1 in F_1 plants (73 resistant, 23 sensitive plants). F_2 lines from self-fertilized F_1 plants were either completely resistant or sensitive to kanamycin or resistance again segregated in a 3:1 ratio (75 resistant, 25 sensitive plants in two lines tested), consistent with the presence of a single integrated copy in line DEF1. F_1 lines producing kanamycin-resistant (DEF1.1) and -sensitive (DEF1.4) F_2 lines, referred to as transformants and control plants, respectively, were selected for further experiments.

Plant Inoculation and Symptom Development. To facilitate description, symptoms of ACMV infection in *N. benthamiana* were graded as follows: type 1, occasional mild curling of leaves, infrequent chlorotic lesions, no stunting; type 2, more pronounced leaf curling, frequent chlorotic lesions, some stunting due to stem deformation; type 3, severe leaf curling, extensive chlorosis often confluent on newly emerging leaves, marked stunting, plants often recover; type 4, as for type 3 but more severe, plants seldom recover.

Plants were challenged with the Kenyan isolate of ACMV either by mechanical inoculation of cloned DNA or virus or by agroinoculation (Table 1). Infection of control plants, irrespective of the inoculation route, routinely produced severe symptoms (types 3 and 4) within 2 weeks. Following the mechanical inoculation of cloned DNA, chlorotic lesions appeared on the inoculated leaves at the same time (≈5 days after inoculation) on control and transformed plants. However, systemic infection took longer to become established in the transformants, which were, as a consequence, invariably much less severely affected (symptom types 1 and 2). On occasions, symptoms in transformants were limited to just one or two chlorotic lesions on the inoculated leaves. Symptom amelioration also occurred when cloned DNA was introduced into plants by agroinoculation. Typical examples of agroinoculated transformed and control plants, exhibiting symptom types 2 and 4, respectively, are shown in Fig. 1. Inoculation using purified virus generally produced the most severe symptoms in control and transformed plants, although fewer transformants became infected and symptoms in the latter (types 2 and 3) were clearly attenuated in comparison with those in control plants (predominantly type 4). A 25-fold reduction in the amounts of cloned DNA components or virus in the inoculum, to 40 ng and 4 ng, respectively, had little effect on the numbers of plants infected or on the severity of symptoms.

Table 1. Infectivity of geminiviruses and symptom production in control and transformed *N. benthamiana*

Source	Amount, μg	Control plants*					Transformants*				
		I	II	III	IV	Symptoms†	I	II	III	IV	Symptoms†
ACMV (Kenyan)											
Agroinoculation		—	5/5	—	5/5	3/4	—	5/5	—	5/5	2/3
Cloned DNA	1.0	5/5	4/4	5/5	5/5	4	5/5	4/4	5/5	4/5	1/2
	0.3	5/5	4/4	—	—	4	5/5	3/4	—	—	2
	0.2	—	—	5/5	—	3/4	—	—	5/5	—	2
	0.1	5/5	4/4	—	—	3/4	4/5	4/4	—	—	1/2
	0.04	—	—	4/5	—	3/4	—	—	4/5	—	2
Purified virus	0.1	4/5	4/4	5/5	—	4	2/5	0/4	5/5	—	3
	0.02	—	—	5/5	—	4	—	—	4/5	—	2
	0.01	4/5	1/4	—	—	4	0/5	0/4	—	—	—
	0.004	—	—	5/5	—	3/4	—	—	4/5	—	2/3
Transformant extract		—	—	5/5	—	3/4	—	—	4/5	—	1
Control extract		—	—	5/5	4/5	3/4	—	—	4/5	4/5	1/2
ACMV (Nigerian)											
Cloned DNA	1.0	—	—	—	5/5	4	—	—	—	4/5	1/2
Plant extract		—	—	—	4/4	4	—	—	—	5/5	3
TGMV											
Agroinoculation		—	5/5	—	3/4	Severe	—	5/5	—	5/5	Severe
Plant extract		—	—	4/5	—	Severe	—	—	4/5	—	Severe
BCTV											
Agroinoculation		—	5/5	—	—	Severe	—	5/5	—	—	Severe

*Numbers of plants infected/inoculated are shown for four separate experiments (I–IV). Plants were judged to be infected by the appearance of symptoms.

†ACMV symptoms are graded 1–4 in order of increasing severity as described in the text.

FIG. 1. Control plant (*Left*) and transformant (*Center*) agroinoculated with ACMV, exhibiting symptom types 4 and 2, respectively, and a healthy transformant (*Right*). Plants were photographed 3 weeks after inoculation.

Analysis of Viral DNA Forms. Total nucleic acids were extracted from individual leaves and the viral DNA forms were investigated by Southern blot analysis. Full-length ssDNA and scDNA forms of both genomic components were present in control plants mechanically inoculated with either cloned DNA (Fig. 2, lanes 2 and 7) or purified virus (lanes 3 and 8). Additional faster migrating DNA B forms were specifically associated with infection of transformed plants, in both systemically infected leaves (lanes 9 and 10) and inoculated leaves (lane 11). These DNA forms were invariably present in ACMV-infected transformants irrespective of the inoculation route. No extrachromosomal virus-specific DNA was observed in healthy transformants (lanes 1 and 6). The scDNA form of the episomally replicating subgenomic DNA was identified by digestion with the single-cutting restriction enzymes *Eco*RV and *Sal* I (lanes 15 and 16) to give

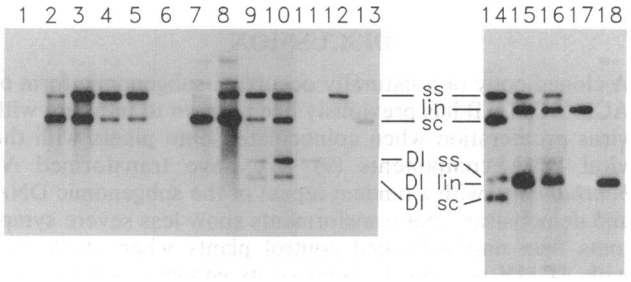

FIG. 2. Southern blot analysis of total nucleic acids extracted from individual systemically infected leaves, harvested 3 weeks after inoculation of plants with 1 μg of cloned ACMV DNA (controls, lanes 2 and 7; transformants, lanes 4, 9, and 14–16) and 0.1 μg of purified ACMV (controls, lanes 3 and 8; transformants, lanes 5 and 10) and from a healthy transformant (lanes 1 and 6). Extracts in lanes 15 and 16 have been treated with *Eco*RV and *Sal* I, respectively. Lane 11, extract of the inoculated leaf of a transformant mechanically inoculated using cloned DNA; lane 12, ACMV ssDNA; lane 13, ACMV scDNA; lane 17, *Pst* I-digested pJS094 (full-length DNA B clone insert); lane 18, *Sal* I-digested pDEF012 (subgenomic DNA B clone insert). Blots were probed specifically for DNA A (lanes 1–5) or DNA B (lanes 6–18). Positions of single-stranded (ss), linear (lin), and supercoiled (sc) DNAs and their subgenomic counterparts (DI prefix) are indicated.

fragments that comigrated with the subgenomic DNA insert of clone pDEF012 (lane 18). Under these conditions, full-length scDNA B was also linearized to give fragments that comigrated with the full-length DNA B insert of clone pJS094 (lane 17). Digestion with S1 nuclease selectively removed the single-stranded forms of the genomic and subgenomic DNAs and converted much of the scDNA to an open-circular form (data not shown).

The relative amounts of each DNA form in pooled systemically infected leaves of individual plants infected using cloned DNA were estimated. The levels of component-specific DNA in transformed plants exhibiting type 2 symptoms were reduced by approximately 20% and 30% for DNAs A and B, respectively, when compared with the levels in control plants with type 4 symptoms. Approximately 60% of the DNA B-specific material in transformants was in the form of subgenomic DNA.

Plant-to-Plant Transmission of ACMV. Chlorotic lesions developed on inoculated leaves at approximately the same time for all inoculum/host combinations. Two weeks after virus transmission from infected to healthy control plants, symptoms were typically severe (type 3 or 4, Table 1). When virus was transmitted from transformants to control plants, symptom development was delayed by 3–4 days, although equally severe symptoms eventually developed. In both cases, the levels of the genomic components were similar (Fig. 3, compare lanes 1–4 and 9–12) and free copies of the subgenomic DNA were present in three of the four plants examined following inoculation using extracts from infected transformants (lanes 9–12). However, the level of subgenomic DNA relative to full-length DNA B was reduced in comparison with the relative level in transformants (compare with lanes 5–8).

When transmitted from control to transformed plants, the virus produced ameliorated symptoms (types 1 and 2) associated with a decrease in the level of viral DNA similar to transformants inoculated using cloned DNA (described above). Again, the majority of the DNA B-specific material was in the form of subgenomic DNA (lanes 5, 7, and 8). In this instance, one transformant remained asymptomatic and contained no detectable viral DNA (lane 6), possibly due to inefficient inoculation. When virus was transmitted between

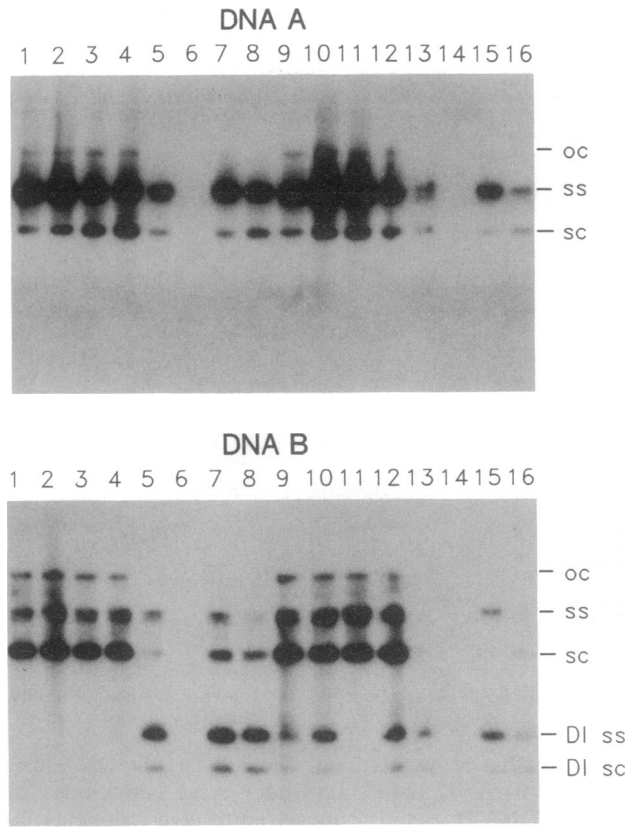

FIG. 3. Southern blot analysis of total nucleic acids extracted from whole control plants (lanes 1–4 and 9–12) and transformants (lanes 5–8 and 13–16) infected by transmitting ACMV from extracts of control plants (lanes 1–8) and transformants (lanes 9–16). Plant material was harvested 3 weeks after inoculation. Blots were probed specifically for DNA A or B as indicated. The position of open-circular (oc) DNA is indicated in addition to the viral DNA forms described in the legend to Fig. 2.

transformants, symptoms were extremely mild (type 1) and infection was associated with reduced levels of both genomic components, in particular full-length DNA B (lanes 13, 15, and 16). Again, one transformant remained asymptomatic and contained no detectable viral DNA in this experiment (lane 14).

Plant Inoculation Using Related Geminiviruses. The Nigerian isolate of ACMV behaved in the same way as the Kenyan isolate, producing severe symptoms (type 4) in control plants and attenuated symptoms (types 1 and 2) in transformants when challenged with cloned DNA (Table 1). More severe symptoms (type 3) developed in transformants following transmission of the virus from extracts of infected plants. Infection of transformants was associated with the amplification of episomally replicating subgenomic DNA to levels similar to those found in plants infected with the Kenyan isolate. In contrast, there was no difference in the time of appearance or severity of symptoms produced in control and transformed plants following agroinoculation of TGMV using *A. tumefaciens* strain LBA4404 or C58 or BCTV using strain C58, and following transmission of TGMV from infected plant extracts.

Analysis of viral DNA associated with TGMV infection (Fig. 4) showed variable levels of both genomic components in transformed (lanes 3–6) and control plants (lanes 7 and 8) and the absence of episomally replicating ACMV subgenomic DNA in transformants, although this DNA form was readily detected in ACMV-infected transformants (lane 2) under the hybridization conditions. Furthermore, no *de novo*-

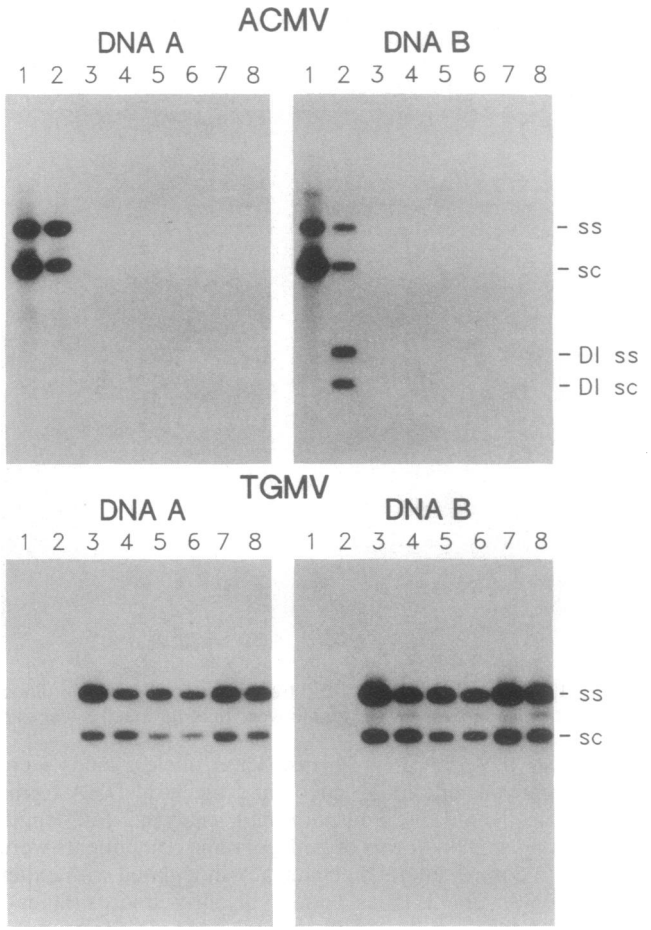

FIG. 4. Southern blot analysis of total nucleic acids extracted from individual systemically infected leaves from plants agroinoculated with ACMV (control, lane 1; transformant, lane 2) or TGMV (controls, lanes 7 and 8; transformants, lanes 3–6). TGMV-infected plant material was harvested 2 weeks after inoculation. Blots were probed for ACMV components, stripped of label, and reprobed for TGMV component counterparts as indicated.

synthesized TGMV subgenomic DNA was detected in either control or transformed plants.

DISCUSSION

A cloned copy of a naturally occurring subgenomic form of ACMV DNA B has previously been shown to interfere with virus proliferation when coinoculated onto plants with the viral DNA components (9). We have transformed *N. benthamiana* with a tandem repeat of the subgenomic DNA and demonstrate that transformants show less severe symptoms than untransformed control plants when challenged with ACMV or cloned copies of its genomic components. Symptom amelioration is further enhanced when virus is transmitted between transformants and is associated with a progressive reduction in the level of viral DNA.

Unlike other *in vitro* transformation strategies used to control plant virus infections, such as the use of the coat protein to mimic cross-protection, antisense RNA to sequester mRNA and satellite RNAs to interfere with helper-virus proliferation, the strategy described here does not require transcription of, or gene expression from, the integrated DNA. In healthy transformants, the construct will remain integrated, and not until the cell becomes infected will the subgenomic DNA become excised and amplified. Amplification occurs at the expense of full-length DNA B production, the episomally replicating subgenomic DNA comprising

≈60% of the component-specific material in infected transformed plants. As this DNA is just under half the size of the full-length genomic component (1343 and 2724 nucleotides, respectively), it is approximately three times more abundant than the latter. In contrast, the relative abundance of the subgenomic DNA in *N. benthamiana* infected with the native Kenyan isolate is extremely low (5, 9), balanced by the competing effects of dependence on, and interference of, the helper virus. To maintain the high levels of subgenomic DNA in the transformants, escape of the integrated DNA must frequently occur during systemic invasion of the plant, possibly in every infected cell. Consequently, when the virus is reintroduced into nontransformed plants, the high levels of the subgenomic DNA are not maintained, virus proliferation increases, and plants once again become severely affected. The selective amplification of the subgenomic DNA is not simply a result of competition between the DNA components for limited gene products required for replication since the levels of full-length DNAs A and B are affected to different extents. The data suggest a regulatory influence of one genomic component over the other whereby the replication of DNA A is coupled to that of the pool of full-length and subgenomic DNA B.

The subgenomic DNA selected for this series of experiments is representative of the most abundant form isolated from infected tissue (9), retaining the potential to encode only the amino-terminal two-thirds (181 amino acids) of the product of gene BC1 fused to an additional fortuitous 79 amino acids that encroach into the common region. It is conceivable that enhanced expression of this modified gene effects symptom development by, for example, nonproductive binding to virus or cell receptors. However, if the unmodified gene is involved in virus spread as suggested, it might be expected to exert a similar influence on both genomic components. Therefore, symptom amelioration is probably a direct consequence of the decrease in the abundance of full-length DNA B, and hence DNA B-encoded products responsible for virus spread, brought about by the competitive nature of the subgenomic DNA.

It might be possible to enhance the effect of the subgenomic DNA by further increasing its copy number. This might be achieved by reducing the size of the DNA while ensuring that cis-acting sequences responsible for replication remain intact. Alternatively, if DNA replication and transcription are controlled from adjacent or overlapping sequences, possibly within the common region, and assuming that gene expression from the subgenomic DNA is not responsible for symptom amelioration, elimination of competitive transcriptional activity from the DNA might serve to confer an additional replicative advantage over the full-length genomic component. In a similar way, competition of replication and transcription factors for available templates has been invoked to explain the different interference behavior of related DI particles of the negative-strand RNA virus vesicular stomatitis virus (28).

In contrast to the behavior of ACMV isolates, symptom severity and viral DNA levels are unchanged when transformants are challenged with BCTV and TGMV. This was anticipated for BCTV since this monopartite virus has no DNA B counterpart (13). TGMV infection of transformants is not affected because the virus is unable to amplify the ACMV subgenomic DNA. This serves to emphasize that ACMV symptom amelioration in transformed plants is a direct result of subgenomic DNA amplification and not a response peculiar to this particular plant line. The fact that the specificity of the interference phenomenon might be limited to closely related virus isolates does not preclude the adaptation of this approach to other geminiviruses. Subgenomic DNAs are associated with TGMV, potato yellow mosaic virus, and tomato yellow leaf curl virus infection (29–31), suggesting that they may be a common feature of bipartite geminiviruses. Therefore, this approach might find application in the control of economically important diseases caused by these and other whitefly-transmitted geminiviruses.

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