

DNA Sequences Essential for Replication of the B Genome Component of Tomato Golden Mosaic Virus

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The genome of the geminivirus tomato golden mosaic virus (TGMV) is divided between two DNA components, designated A and B, which differ in sequence except for a 230-nucleotide common region. The A genome component is known to encode viral functions necessary for viral DNA replication, while the B genome component specifies functions necessary for spread of the virus through the infected plant. To identify *cis*-acting sequences required for viral DNA replication, several mutants were constructed by the introduction of small insertions into TGMV B at selected sites within and just outside the common region. Other mutants had the common region inverted or deleted. All of the mutants were tested for their effects on infectivity and DNA replication in whole plants and leaf discs. Our results indicate that the common region in its correct orientation is required for infectivity and for replication of TGMV B. Furthermore, the conserved hairpin loop sequence located within the TGMV common region and found in all geminiviruses is necessary for DNA replication, and may be part of the viral replication origin.

INTRODUCTION

Tomato golden mosaic virus (TGMV) is a whitefly-transmitted member of the geminivirus group which, like all group members, has a single-stranded circular DNA genome (for review see Davies, Townsend, and Stanley, 1987; Lazarowitz, 1987). The TGMV genome is divided between two DNA components, referred to as A and B, both of which are required for infectivity (Bisaro et al., 1982; Hamilton et al., 1983, 1984). The two genomic DNAs differ considerably in nucleotide sequence, with the exception of a highly conserved common region of approximately 230 nucleotides and two smaller regions of homology about 13 and 33 nucleotides in length.

The 2588-nucleotide TGMV A genome component contains all viral information necessary for the replication and encapsidation of viral DNA (Rogers et al., 1986; Sunter et al., 1987). Four open reading frames (ORFs) with the potential to encode a protein greater than 10 kD reside on the A genome component. These ORFs diverge from the common region and are transcribed in a bidirectional fashion from a double-stranded DNA intermediate (Sunter, Gardiner, and Bisaro, 1989). Viral ORFs are defined according to genome component (A versus B) and orientation relative to the common region (Hamilton et al., 1984). Rightward transcription spans ORF AR1, which encodes the 28.7-kD viral capsid protein (Kallender et al., 1988). Leftward transcription spans ORFs AL1 (40.3 kD), AL2

(14.9 kD), and AL3 (15.7 kD). Mutational analysis of AL1 has demonstrated that it is essential for viral DNA replication (Elmer et al., 1988a). However, the capsid protein, as well as the products of the AL2 and AL3 ORFs, are not necessary for viral DNA synthesis (Elmer et al., 1988a; Gardiner et al., 1988).

Genome component B (2522 nucleotides; MacDowell, Coutts, and Buck, 1986) is believed to encode information needed for the spread of virus and/or viral DNA between cells (Rogers et al., 1986). TGMV B has two coding regions, BR1 (29.3 kD) and BL1 (26.4 kD), both of which are necessary for infectivity (Brough et al., 1988). The BR1 and BL1 ORFs also diverge from the common region and are transcribed in opposite directions (Sunter, Gardiner, and Bisaro, 1989).

TGMV accumulates in the nuclei of infected plants, from which unit-length and concatameric single- and double-stranded viral DNA forms can be recovered (Hamilton, Bisaro, and Buck, 1982; Rushing et al., 1987; Slomka, Buck, and Coutts, 1988). The presence of such double-stranded DNAs suggests that TGMV and other geminiviruses employ a multiplication strategy that is similar in some respects to the one used by single-stranded DNA-containing coliphages (e.g., ϕ X174). In these phages, host functions convert the infectious single-stranded DNA to a double-stranded form that is amplified and later serves as template for both viral transcription and single-stranded DNA synthesis.

TGMV A can replicate in the absence of TGMV B, but the latter is not capable of replicating alone (Rogers et al.,

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1986). Therefore, the A component must supply in *trans* a factor(s) required for TGMV B replication. Since this factor also is required for replication of TGMV A itself, it is reasonable to suppose that *cis*-acting sequences that might interact with this factor, or with host coded proteins, are held in common between the two viral genome components. There is some circumstantial evidence that suggests that the 230-nucleotide common region contains such *cis*-acting sequences, which formally may constitute the viral replication origin: (1) Subgenomic TGMV B molecules recovered from virions possess a variety of different deletions, yet the common region is retained in all subgenomic molecules (MacDowell et al., 1986). (2) The efficiency with which certain tandemly repeated one and one-half TGMV-A constructs produce unit-length, replicating viral DNA appears to depend on whether the repeat contains one or two common regions (Elmer et al., 1988b). (3) A 33-nucleotide sequence that has the potential to form a stable hairpin is found within the common region of TGMV (Hamilton et al., 1984; Sunter, Buck, and Coutts, 1985). This potential hairpin structure is conserved in an analogous genome position in all geminiviruses. A similar hairpin exists at the site where second-strand DNA synthesis is initiated on single-stranded ϕ X174 DNA (Arai and Kornberg, 1981), and the A + T-rich consensus sequence in the geminivirus hairpin loop bears a striking resemblance to the sequence at the gene A protein cleavage site in the ϕ X174 replicative form (Heidekamp, Baas, and Jansz, 1982; Rogers et al., 1986). The presence of inverted repeats and an adjacent A + T-rich region also is a feature of SV40 and polyomavirus replication origins (Bergsma et al., 1982; Li et al., 1986; Dean et al., 1987).

We have undertaken an investigation of the common region within the B genome component in an attempt to define *cis*-acting sequences involved in TGMV replication. We have found that mutations within a 30-nucleotide sequence near the conserved hairpin compromise both infectivity and replication of TGMV B. Other mutations in different parts of the common region had no noticeable effect on B DNA replication. However, a mutation just outside the common region reduced the time of symptom appearance in inoculated plants by about 3 days. These results provide the first direct evidence for the presence within the common region of DNA sequences necessary for replication.

RESULTS

TGMV B Common Region Mutants and Their Assay

Because the common region of TGMV is likely to harbor the viral replication origin, the effect on viral replication of mutations within and surrounding this sequence was investigated. The B component was chosen as the focus of

this study because mutations in the A DNA could affect replication either by disrupting *cis*-acting sequences or by interfering with the expression of the *trans*-acting replication factor. Mutations in B DNA should permit identification of *cis*-acting sequences involved in replication.

As shown in Figure 1, the 413-bp PstI fragment containing the common region of TGMV B was altered by inversion (CR-1), by deletion (CR-2), or by insertion of a short sequence (CR-3, CR-4, CR-5, and CR-6). Mutation CR-3 was created by insertion of an XhoI linker (underlined in the following sequence) into the SspI site (nucleotide 136) found in the loop sequence of the putative hairpin structure: 5'-AATCCTCGAGGATT. Mutation CR-4 contains a 3-bp insertion in the SauI site at nucleotide 106: 5'-CCTAATAAGG. Mutation CR-5 was constructed by insertion of a HindIII linker into the HpaI site, which lies at

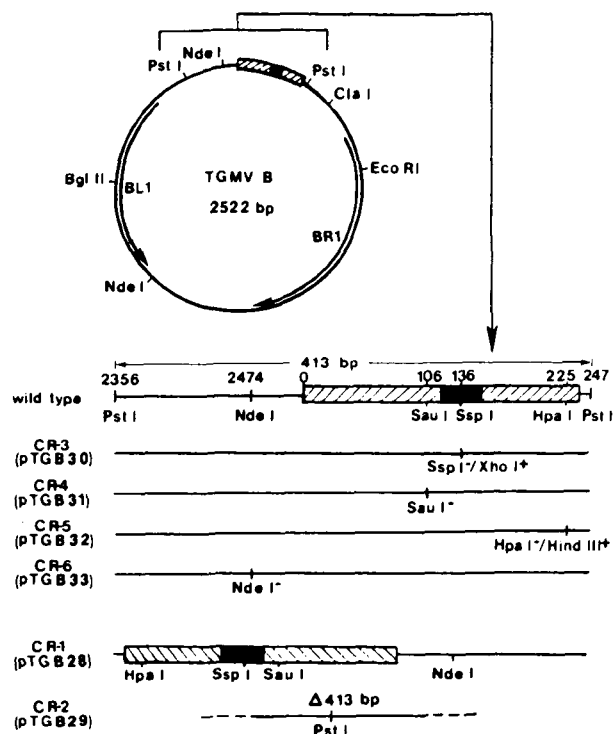


Figure 1. Diagrams of TGMV B Common Region Mutants.

The common region (cross-hatched box) is a sequence of 230 bp that is nearly identical in the A and B genome components, and the shaded box within the common region indicates the position of the putative hairpin structure that is found in all geminiviruses. The arrows show the position of ORFs BR1 and BL1. The expanded region represents the 413-bp PstI fragment, and restriction sites where alterations within this fragment were constructed for each CR mutant are noted. CR-1 and CR-2 contain an inversion and a deletion of the PstI fragment, respectively. A detailed description of these mutants appears in the text.

nucleotide 225: 5'-GTTCTGAAGCTTCGAAC. Mutation CR-6 contains a 2-bp insertion in the NdeI site at nucleotide 2474: 5'-CATATATG. The CR-mutant TGMV B molecules were inserted in tandem with a portion of wild-type TGMV B (1.6-kb BglII to ClaI fragment lacking the common region) in the binary Ti plasmid vector pMON330. Tandem copies of TGMV DNAs in the plasmid are necessary to ensure an uninterrupted linear genome sequence. The Ti plasmid constructs were transferred to *Agrobacterium* and co-inoculated onto plants with *Agrobacterium* containing TGMV A DNA in the binary vector pMON337 (Elmer et al., 1988b). The agroinoculation (agroinfection) procedure, in which *Agrobacterium* is used to deliver viral genomes into susceptible plants, was chosen because of its efficiency and ease of use (Grimsley and Bisaro, 1987; Grimsley et al., 1987; Elmer et al., 1988b).

Effects of CR Mutations on Infectivity

Characteristic symptoms of TGMV infection (vein clearing, mosaic pattern on the leaves, and curling of new leaves) were observed within 21 days in tobacco plants co-inoculated with pMON337 (wild-type A) and pMON392 (wild-type B) as well as with mutants CR-5 and CR-6 (Table 1). However, plants inoculated with CR-4 displayed less severe symptoms; infected plants did not show leaf curling, but flecks of chlorosis were visible on the youngest leaves. No symptoms were observed after 21 days in tobacco plants co-inoculated with pMON337 and CR-1, CR-2, and CR-3, or in plants inoculated with pMON337 or pMON392 alone.

The time of symptom appearance in plants inoculated with wild-type B is compared with that in plants inoculated with the infectious CR mutants in Figure 2. The average time of symptom appearance in plants inoculated with the CR-4 or CR-5 mutants was similar to that of plants infected

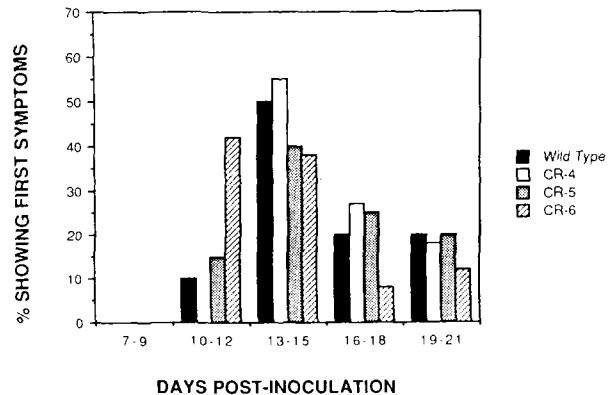


Figure 2. Time of Symptom Appearance in Plants Inoculated with TGMV A and CR Mutants.

N. benthamiana plants were inoculated with TGMV A and wild-type TGMV B or mutants CR-4, CR-5, and CR-6. Plants were scored as positive when leaf curl or chlorosis became evident.

with wild-type TGMV B (average = 16 days). Surprisingly, symptoms appeared an average of 3 days earlier in plants infected with mutant CR-6.

Analysis of TGMV DNA in Infected Plants

The presence and severity of symptoms correlates with the presence and amount of B DNA detected by DNA gel blot analysis of total DNA extracts from inoculated tobacco plants. The results shown in Figure 3 are derived from one experiment, but are typical of the results seen in all seven experiments conducted. Circular forms of both A and B DNA were present in plants co-inoculated with TGMV A and wild-type B or mutants CR-4, CR-5, or CR-6 (Figure 3, lanes 1, 5, 6, and 7). The amounts of B DNA detected in plants inoculated with CR-5 and CR-6 were approximately the same as in wild-type B-inoculated plants. However, the amount of B DNA detected in the weakly symptomatic CR-4-inoculated plants was markedly less. Plants that did not display symptoms did not contain detectable levels of B DNA under the blot hybridization conditions employed.

Although TGMV A DNA was not detected in plants inoculated with A DNA alone (presumably because it cannot spread from cell to cell), a small amount was detected in some asymptomatic plants inoculated with CR-1 and CR-3 (Figure 3, lanes 2 and 4; other data not shown). This suggests that a small amount of B DNA, below the level of detection in DNA gel blots, may be present and may provide A with the factor(s) it needs to move between cells.

Table 1. Infection of *N. benthamiana* Plants Inoculated with Wild-Type or CR-Mutant TGMV B

Mutation	Inoculum ^a	No. Infected/ No. Inoculated ^b	Days to Symptoms	
			Range	Average
Wild-Type	pMON392	15/40	10-21	15.9
CR-1	pTGB28	0/30	-	-
CR-2	pTGB29	0/25	-	-
CR-3	pTGB30	0/38	-	-
CR-4	pTGB31	13/44	13-21	16.3
CR-5	pTGB32	23/44	10-21	15.7
CR-6	pTGB33	29/46	10-21	13.3

^a Mixed cultures of *Agrobacterium* carrying pMON337 (wild-type TGMV A) and the TGMV B derivatives indicated were used as inoculum.

^b Totals obtained from seven independent experiments.

Analysis of DNA Replication in Leaf Discs

The lack of infectivity observed with mutants CR-1, CR-2, and CR-3 may result from the disruption of replication or from interference with the expression of a movement function encoded by TGMV B. To determine whether these mutant DNAs are capable of being replicated, a similar co-inoculation experiment was conducted using tobacco leaf

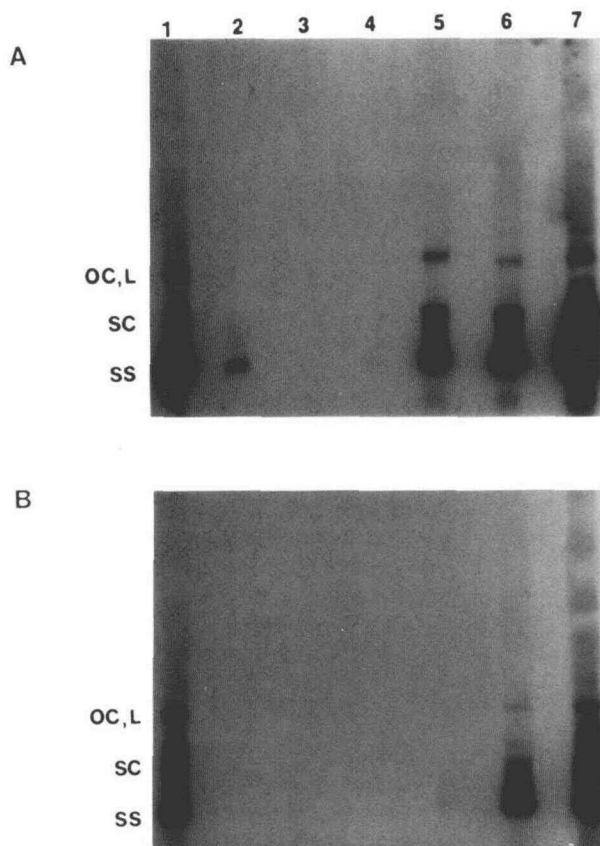


Figure 3. DNA Gel Blot Analysis of DNA Isolated from Plants Inoculated with TGMV A and CR Mutants.

DNA samples were run on agarose gels and hybridized with ^{32}P -labeled probes as described in Methods. DNA preparations from plants inoculated with the same DNAs were pooled, and samples from plants inoculated with TGMV A and the following B components are shown: Lane 1, wild-type TGMV B (3 μg); lane 2, CR-1 (10 μg); lane 3, CR-2 (10 μg); lane 4, CR-3 (10 μg); lane 5, CR-4 (10 μg); lane 6, CR-5 (3 μg); lane 7, CR-6 (5 μg). The DNA forms identified are open circular (OC), linear (L), and supercoiled (SC) double-stranded TGMV DNA. The position of single-stranded TGMV is also noted (SS). Other bands that appear between SS and OC,L as well as just above OC,L represent subgenomic or dimeric supercoiled, open circular, and linear forms of viral DNA.

(A) TGMV A-specific probe.

(B) TGMV B-specific probe.

discs, which provide a convenient assay for viral DNA replication that is independent of virus movement (Elmer et al., 1988a).

The relative amount of B DNA detected in leaf discs co-inoculated with wild-type TGMV A and B, or the TGMV B CR mutants, reflects what is observed in whole plants (Figure 4). No unit-length B DNA was detected in leaf discs inoculated with A DNA alone or in leaf discs co-inoculated with TGMV A and mutants CR-1, CR-2, or CR-3 (Figure 4, lanes 2, 3, and 4). A small amount of B DNA was replicated in CR-4-inoculated leaf discs (Figure 4, lane 5), while levels of B DNA comparable to the wild-type TGMV B control were seen in discs inoculated with CR-5 or CR-6 (Figure 4, lanes 6, 7, and 8). As expected, A DNA replication in leaf discs was unaffected by the presence or absence of B DNA.

Restriction endonuclease analysis of the DNA recovered from plants or leaf discs inoculated with CR-4, CR-5, and CR-6 confirmed that these mutants had not reverted to wild-type (Figure 5). The high molecular weight DNA detected in leaf disc DNA samples is residual *Agrobacterium* vector DNA, and the amount of this can be reduced but not eliminated by surface sterilization of the discs prior to DNA extraction.

DISCUSSION

The results of this study are consistent with the TGMV B replication origin residing within the common region. Removal of the common region by a 413-nucleotide deletion (CR-2) abolishes replication of B DNA in tobacco plants and leaf discs. Inversion of the same 413-nucleotide sequence (CR-1) or insertion of 8 nucleotides into the loop of the conserved hairpin structure (CR-3) likewise reduces replication to a level below the limit of detection in our assay system. It is possible for the inversion mutation (CR-1) to affect viral DNA replication if initiation is strand-specific or if *cis*-acting sequences that enhance or are required for the process are critically spaced within and outside of the inverted region. Furthermore, the endpoints of the inversion might lie within a sequence that is required for the normal binding of replication factors.

The insertion of the octamer XhoI linker into the SspI site within the putative hairpin loop (mutation CR-3) alters this sequence so that it has the potential to form a more stable structure ($G = -42.6$ versus -31.6 kcal/mol). In the hypothetical mutant structure, 8 of the 12 nucleotides that form the loop in the wild-type configuration are base-paired (Figure 6). As the hairpin structure and its loop sequence are highly conserved in all geminiviruses, our observation corroborates the hypothesis that it plays an important role in viral DNA replication. A recent study by Fenoll, Black, and Howell (1988) also suggests that this structure is part of an upstream activating sequence that is necessary for

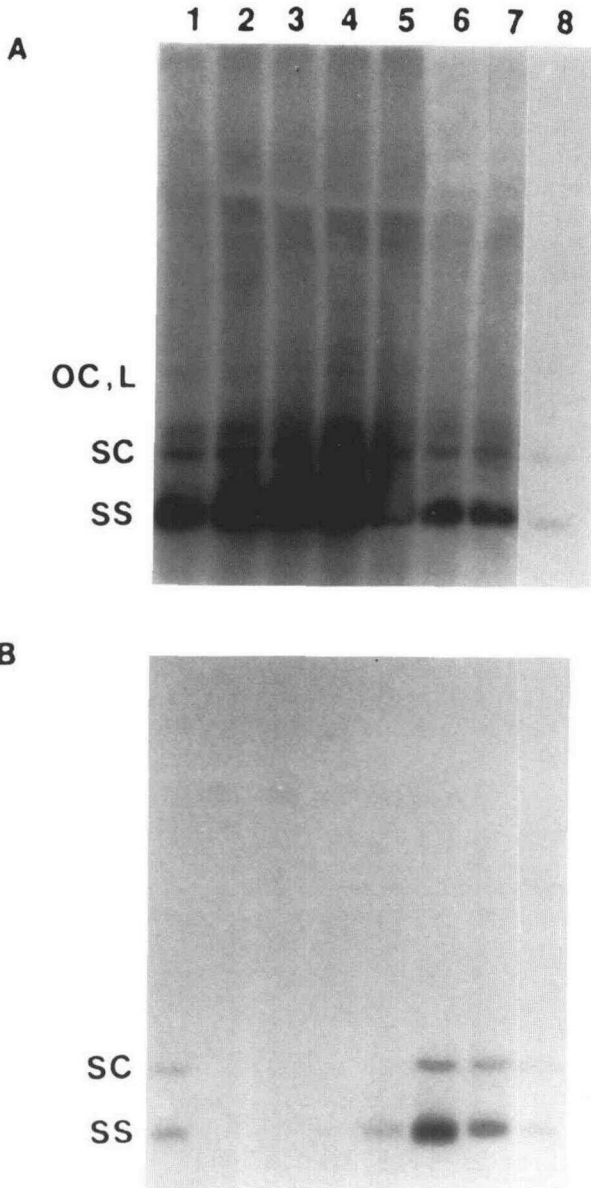


Figure 4. DNA Gel Blot Analysis of DNA Isolated from Tobacco Leaf Discs Inoculated with TGMV A and CR Mutants.

DNA hybridizations were performed as described in Methods. Each lane contains 5 μ g of DNA from discs inoculated with TGMV A and the following B components: Lane 1, wild-type TGMV B; lane 2, CR-1; lane 3, CR-2; lane 4, CR-3; lane 5, CR-4; lane 6, CR-5; lane 7, CR-6. Lane 8 contains marker TGMV DNA. The positions of single-stranded (SS) and double-stranded open circular (OC), linear (L), and supercoiled (SC) forms of TGMV DNA are given.

(A) TGMV A-specific probe.

(B) TGMV B-specific probe.

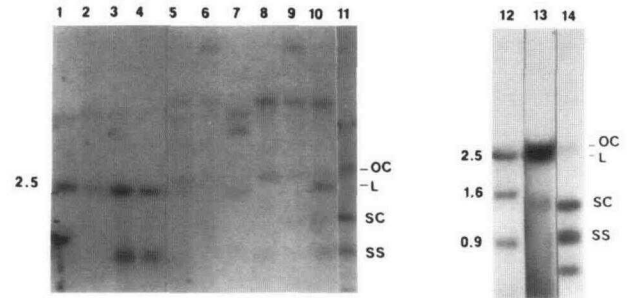


Figure 5. Restriction Analysis of TGMV B DNA Isolated from Plants and Leaf Discs Inoculated with TGMV A and CR Mutants.

DNA samples were run on agarose gels following digestion with the appropriate enzyme and hybridized with a probe specific for TGMV B. Lanes 1 to 10 contain DNA from leaf discs and lanes 12 and 13 contain DNA from plants inoculated with TGMV A and the following B components: Lane 1, wild-type B/Clal; lane 2, CR-4/Clal; lane 3, CR-5/Clal; lane 4, CR-6/Clal; lane 5, wild-type B/Saul; lane 6, CR-4/Saul; lane 7, wild-type B/Hpal; lane 8, CR-5/Hpal; lane 9, wild-type B/HindIII; lane 10, CR-5/HindIII; lane 12, wild-type B/Ndel; lane 13, CR-6/Ndel. Lanes 11 and 14 contain marker TGMV DNA. Approximate sizes (in kilobases) of linear restriction fragments are noted. The positions of single-stranded (SS) and double-stranded open circular (OC), linear (L), and supercoiled (SC) forms of TGMV DNA are also given. Single-stranded, subgenomic B DNA is present below SS DNA in lane 14. High molecular weight DNA species in lanes 1 to 10 are residual *Agrobacterium* vector DNAs.

efficient rightward transcription of maize streak virus. It is conceivable that it is important for leftward transcription as well.

The insertion of 3 nucleotides within the Saul site (CR-4) reduces the amount of B DNA replicated. This sequence, which lies about 30 nucleotides upstream from the hairpin loop, must therefore also be important for the efficient replication of TGMV B. The insertion of a 10-nucleotide HindIII linker into the Hpal site (CR-5) about 90 nucleotides downstream of the hairpin loop, or the insertion of 2 nucleotides into the Ndel site 180 nucleotides upstream of it (CR-6), had no detectable effect on the ability of TGMV B to replicate.

As the common region sequence is nearly identical in TGMV A and B, it probably serves similar functions in both components. Therefore, it is likely that the sequences we have identified as necessary or important for the replication of B DNA should also be important for the replication of A DNA. Experiments are in progress to further define the replication origin of TGMV B, and to identify a minimal sequence that will confer replication competence to a heterologous molecule in the presence of TGMV A.

TGMV A DNA is not found in plants inoculated with A DNA alone, and it is believed that TGMV A cannot spread throughout the plant without movement functions provided

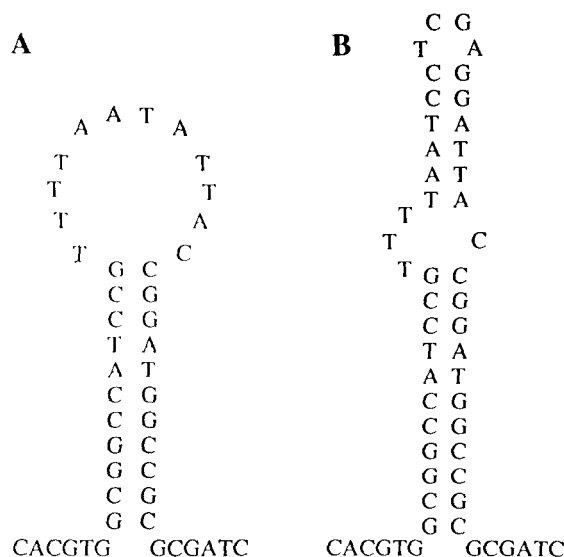


Figure 6. Putative Secondary Structures of TGMV B and Mutant CR-3 Hairpins.

Proposed structures surrounding the conserved geminivirus sequence at nucleotide 336 were drawn and free energies calculated according to the principles of Tinoco et al. (1973).

(A) Wild-type TGMV B sequence.

(B) CR-3 mutant sequence.

by TGMV B (Rogers et al., 1986). The presence of undetectable amounts of TGMV B in plants inoculated with CR-1 or CR-3, and in other asymptomatic plants, may be inferred by the small amount of A DNA that is detected in plants co-inoculated with TGMV A and these mutants. This evidence suggests that movement factors provided by TGMV B are required in very small amounts, and raises the possibility that the factors themselves may be transported between cells.

In general, the severity of symptoms seen in inoculated plants is proportional to the amount of B DNA present. Although this might imply that disease determinants reside on TGMV B, it is also possible that disease determinants reside on the A DNA and that their expression is indirectly dependent on B-encoded movement factors. It is reasonable to suppose that the virus must spread to a sufficient number of contiguous cells in order for a lesion to be visible.

The time of symptom appearance in plants co-inoculated with TGMV A and wild-type TGMV B or the CR-4 and CR-5 mutants is about 16 days. It is remarkable that the NdeI mutation outside of the common region (CR-6) resulted in the appearance of symptoms within 13 days on average, 3 days sooner than the wild-type control. Because this mutation does not appear to affect the replication of B DNA, it is possible that rapid symptom appearance is due

to elevated expression of the BL1 ORF. It will be interesting to pursue this observation in the future.

Due to its relatively small genome size and ability to amplify itself to high copy number, TGMV lends itself as a potential model for the study of plant DNA replication. Definition of the viral replication origin should aid the identification of *trans*-acting factors that support this process. A defined origin of replication also might be useful as a component of plasmid vectors designed to replicate and express foreign genes in plant cells.

METHODS

DNA Techniques

The map locations of the TGMV B restriction endonuclease sites cited here refer to the published TGMV DNA sequence of Hamilton et al. (1984) as modified by MacDowell, Coutts, and Buck (1986). All restriction endonucleases and DNA modifying enzymes were used as recommended by the manufacturers. Other techniques were performed according to Maniatis, Fritsch, and Sambrook (1982) unless otherwise stated. Sequence alterations were confirmed by restriction endonuclease analysis and by sequencing double-stranded plasmids (Chen and Seeburg, 1985).

Construction and Molecular Description of Mutations Created in or near the TGMV B Common Region

Plasmid pMON330 was constructed by inserting the 1.6-kb BglII-ClaI TGMV B fragment lacking the common region into the binary T-DNA vector pMON806 (Rogers et al., 1987). Altered, full-length TGMV B molecules (initially obtained from pBH602; Hamilton et al., 1983) were inserted into the ClaI site of pMON330 so that the resulting derivatives contained a tandemly repeated one and one-half TGMV B sequence. The unaltered TGMV B sequence was inserted into pMON330 and used as the wild-type control (pMON392; J. S. Elmer and S. G. Rogers, unpublished). The pMON330 derivatives were transferred to *Agrobacterium tumefaciens* strain GV3111 carrying the disarmed pTiB6S3-SE plasmid using the triparental mating procedure of Horsch and Klee (1986).

A diagram of TGMV B common region mutants is presented in Figure 1. Mutation CR-1 was constructed by inverting the 413-bp common region-containing PstI fragment (nucleotides 2356 to 247) in TGMV B. The plasmid obtained following insertion of the altered TGMV B into pMON330 was designated pTGB28.

Mutation CR-2 was created by deleting 413 bp between the PstI sites that flank the common region in TGMV B. The remaining 2.1 kb of the B component was recovered and inserted into the ClaI site of pMON330. The resulting plasmid was designated pTGB29.

For construction of the remaining mutants, the 413-bp PstI fragment containing the common region was inserted into the unique PstI site of an SspI⁻ derivative of pAT153. Plasmid pAT153 is a deletion derivative of pBR322 lacking the 705-bp HaeII fragment (Twigg and Sherratt, 1980). After the specified alterations to the 413-bp PstI fragment were made, the modified fragment was excised and inserted into pTGB3. pTGB3 is a pUC8

derivative that lacks a PstI site and contains, at its EcoRI site, a TGMV B component with its 413-bp PstI fragment deleted. The resulting full-length TGMV B derivatives carrying mutations within their 413-bp PstI fragments were recovered by digestion with EcoRI, self-ligated, digested with ClaI, and inserted into pMON330.

Mutation CR-3 consists of an alteration at the SspI site (nucleotide 136) in the loop of the hairpin structure. The sequence was cleaved with SspI and blunt-end-ligated with an XhoI linker (5'-CCTCGAGG), resulting in an 8-bp insertion. The plasmid obtained following insertion of TGMV B containing an XhoI site into pMON330 was designated pTGB30.

Mutation CR-4 was constructed at the Saul site (nucleotide 106), which was cleaved, filled in using the Klenow fragment of DNA polymerase I in the presence of the four deoxyribonucleotide triphosphates, and re-ligated. The plasmid resulting from the insertion of TGMV B lacking an Saul site into pMON330 was designated pTGB31.

Mutation CR-5 was made at the HpaI site (nucleotide 225). The site was replaced by the ligation of HindIII linkers (5'-CGAAGCTTCG) to the blunt ends created by digestion with HpaI, resulting in a 10-bp insertion. The plasmid obtained by insertion of TGMV B containing a HindIII site into pMON330 was designated pTGB32.

Mutation CR-6 contains an alteration outside the common region. The NdeI site (nucleotide 2454) was abolished by digestion with NdeI, followed by end-filling and blunt-end ligation. The net effect was a disruption of the NdeI site by a 2-bp insertion. The plasmid obtained after insertion of TGMV lacking the NdeI site into pMON330 was designated pTGB33.

Assay for Symptom Production

Healthy *Nicotiana benthamiana* plants at the six-leaf to eight-leaf stage were inoculated at the shoot tip with *Agrobacterium* cultures (agroinoculation). Briefly, shoot tips were excised with a scalpel, and bacterial cells were applied directly to the cut stem. Cells carrying either wild-type TGMV B (pMON392) or the CR mutants (pMON330 derivatives) were co-inoculated with cells containing wild-type TGMV A (pMON337; Elmer et al., 1988b). pMON337 contains one and one-half copies of the wild-type TGMV A sequence in binary transformation vector pMON505. The *Agrobacterium* cultures were grown separately and mixed immediately prior to inoculation. Inoculated plants were placed in a growth chamber (24°C, 14 hr light) and observed for 21 days.

Analysis of TGMV Replication in Infected Plants

Twenty-one days post-inoculation, DNA was extracted from all plants as described previously (Stein, Coutts, and Buck, 1983). The presence or absence of replicated A and B DNA was detected by DNA gel blot analysis using ³²P-labeled A-specific or B-specific riboprobes prepared from TGMV subclones in pGEM plasmids (Promega, Madison, WI). The subclones consisted of the 1460-bp EcoRI to Scal fragment of TGMV A (pTGA18), a full-length EcoRI clone of TGMV A (pTGA6), a 2109-bp clone of TGMV B with the common region-containing 413-bp PstI fragment deleted (pTGB34), and the 720-bp EcoRI to NdeI fragment of TGMV B (pTGB17). The templates were cleaved with appropriate restriction endonucleases prior to transcription in vitro with SP6 or T7

polymerase. All probes were constructed such that they lacked common region sequences.

Analysis of Replication in Leaf Discs

Leaf discs were cut from surface-sterilized *N. benthamiana* leaves as described previously (Elmer et al., 1988a), placed on MS104 medium, and incubated in a growth chamber (24°C, 14 hr light) for 2 days. At this time, 10 to 20 discs were immersed for 3 min in suspensions of equal volumes (500 µL) of *Agrobacterium* carrying a mutant B binary vector (see above) and pMON337 (A DNA). The infected discs were placed on MS104 medium for 2 days, then transferred to MS104 containing carbenicillin (500 µg/mL), and incubated an additional 3 days under the same conditions. The discs were then surface-sterilized (10% Chlorox, 0.5% Tween 20) to remove residual *Agrobacterium* cells. DNA was extracted from the leaf discs using the method of Dellaporta, Wood, and Hicks (1983), and replication of A and B DNA was assessed by DNA gel blot hybridization. A-specific and B-specific probes were the same as those used in DNA gel blot hybridizations with DNA from infected plants.

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