

# Molecular Variation of Satellite DNA $\beta$ Molecules Associated with *Malvastrum yellow vein virus* and Their Role in Pathogenicity<sup>†</sup>

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Previous studies have found that the diversity of begomovirus-associated DNA $\beta$  satellites is related to host and geographical origin. In this study, we have cloned and sequenced 20 different isolates of DNA $\beta$  molecules associated with *Malvastrum yellow vein virus* (MYVV) isolated from *Malvastrum coromandelianum* plants in different geographical locations of Yunnan Province, China. Analyses of their molecular variation indicate that the satellites are clustered together according to their geographical location but that they have only limited sequence diversity. Infectivity tests using infectious clones of MYVV and its associated DNA $\beta$  molecule indicate that MYVV DNA $\beta$  is indispensable for symptom induction in *Nicotiana benthamiana*, *N. glutinosa*, *Petunia hybrida*, and *M. coromandelianum* plants. Furthermore, we showed that MYVV interacts functionally with heterologous DNA $\beta$  molecules in *N. benthamiana* plants.

Geminiviruses are a group of plant viruses characterized by a circular single-stranded DNA genome that is encapsidated by the geminate-shaped particles that range in size from 18 nm to 20 nm. Four genera (*Mastrevirus*, *Topocivirus*, *Curtovirus*, and *Begomovirus*) of geminiviruses are recognized, but begomoviruses are the most numerous and geographically widespread. Most begomoviruses have a bipartite genome consisting of two similarly sized DNA components, referred to as DNA-A and DNA-B, both of which are essential for infectivity. A smaller number of begomoviruses have only one monopartite genome, equivalent to the DNA-A of bipartite geminiviruses, and appear to lack DNA-B. In recent years, some monopartite begomoviruses such as *Ageratum yellow vein virus*, *Cotton leaf curl Multan virus*, and *Tomato yellow leaf curl China virus* (TYLCCNV) have been shown to be associated with a novel satellite DNA $\beta$  molecule. Without the satellite, these begomoviruses are either poorly infectious or not infectious on the hosts from which they were initially isolated (3, 5, 15).

Previous analyses of the diversity of begomovirus-associated DNA $\beta$  satellites focused mainly on satellites originating from different hosts (1, 4). In this study, 20 different isolates of DNA $\beta$  molecules associated with *Malvastrum yellow vein virus* (MYVV) isolated from *Malvastrum coromandelianum* plants in different geographical locations in Yunnan Province, China, were cloned and sequenced. In addition, infectious clones of MYVV and its associated DNA $\beta$  molecule were constructed, and we found that the DNA $\beta$  molecule is indispensable for symptom induction in *Nicotiana benthamiana*, *N. glutinosa*, *Petunia hybrida*, and *M. coromandelianum* plants. Furthermore, we demonstrated that MYVV interacts functionally with heterologous DNA $\beta$  molecules in *N. benthamiana* plants.

## MATERIALS AND METHODS

**Virus sources, DNA extraction, and virus detection.** *M. coromandelianum* plants showing typical yellow vein symptoms were collected in Chuxiong, Yuxi, and Honghe districts, Yunnan Province, China, between 2003 and 2004 (see Fig. S1 in the supplemental material), and total nucleic acids were extracted from leaf tissue as described previously (21). The MYVV-specific primers Y47F (5'-GAATTCCTTATAGCTGCTAT-3') and Y47R (5'-GAATTCCTCGACGAGAAA GA-3') (EcoRI is underlined), designed based on the complete nucleotide sequence of MYVV isolate Y47 (AJ457827), were used to test for the presence of MYVV by PCR. PCR products were purified, cloned, and sequenced.

**PCR and sequence determination.** Based on the conserved nucleotide sequence reported for DNA $\beta$ , abutting primers  $\beta$ 01 (5'-GGTACCACCTACGCTACGCAGCAGCC-3') and  $\beta$ 02 (5'-GGTACCCTACCCTCCAGGGGTACAC-3') were designed and used to amplify full-length DNA $\beta$  as described previously (2, 23). PCR products were recovered, purified, and cloned into a pGEM-T Easy vector (Promega, Madison, WI). Sequences were determined using the automated model 3730 DNA sequencing system (Perkin-Elmer, Foster City, CA).

**Sequence and phylogenetic analysis.** Nucleotide sequences were edited using EditSeq (Lasergene package of programs from DNASTar, Inc.) and aligned with the aid of the multiple alignment program Clustal X 1.81 (18). The levels of variation among these sequences were evaluated by two parameters ( $\pi$  and  $\theta$ ) using DnaSP version 4.10.3 (14). Nucleotide sequence diversity ( $\pi$ ) was the average number of nucleotide differences per site between two sequences (17), and the population mutation parameter  $\theta$  was based on the number of segregating sites  $S$  (20). A phylogenetic tree from multiple alignments of the 21 sequences was constructed by using the neighbor-joining method calculated with MEGA 3.1 (9). The DNA $\beta$  sequence associated with *Malvastrum yellow vein Yunnan virus* (MYVYNV $\beta$ ; AJ786712) was used as a root for the comparisons. Bootstrap values were produced from 1,000 replicates.

**Construction of infectious clones of MYVV and its associated DNA $\beta$ .** The primers Y47F and Y47R were used to amplify the complete nucleotide sequence of MYVV isolate Y47, and the PCR products were cloned into a pGEM-T vector (Promega) to yield the clone T-Y47A-FL. A SalI-EcoRI-digested fragment containing the entire intergenic region was released from T-Y47A-FL and introduced into a PLH9000 binary plasmid, which was kindly provided by Jörg Schubert, Federal Centre for Breeding Research on Cultivated Plants, Germany, to produce PLH9000-Y47A-PL. Then, T-Y47A-FL was digested with EcoRI to obtain a full-length unit of MYVV and inserted into the EcoRI site of PLH9000-Y47A-PL to produce clone PLH9000-Y47-1.9A containing a 1.9-mer partial tandem repeat of MYVV.

The complete monomeric sequence of MYVV DNA $\beta$  was amplified using primers  $\beta$ 01 and  $\beta$ 02, and the fragment was inserted into a pGEM-T Easy vector (Promega) to produce the clone T-Y47 $\beta$ -FL. Subsequently, another copy of complete MYVV DNA $\beta$  sequence was amplified using primers  $\beta$ 05 (5'-GAAA CCACTACGCTACGCAGCAGCC-3') and  $\beta$ 02 to produce the clone T-Y47 $\beta$ -PL. The T-Y47 $\beta$ -FL clone was digested with KpnI and inserted into the unique KpnI site of T-Y47 $\beta$ -PL to produce T-Y47-2 $\beta$ . Then T-Y47-2 $\beta$  was digested with

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EcoRI and inserted into the binary vector pBinPLUS (19) to produce clone pBinPLUS-Y47-2 $\beta$ , which contains a tandem dimeric repeat of MYVV DNA $\beta$  molecules. All the amplified fragments used for construction of infectious clones were sequenced with an automated model 3730 DNA sequencer (Perkin-Elmer), and sequence analysis indicated that no mutations were introduced into the clones.

**Agroinoculation of plants.** *Agrobacterium tumefaciens* strain EHA105 was transformed with PLH9000-Y47-1.9A or pBinPLUS-Y47-2 $\beta$  by electrotransformation, and the transconjugants were selected on kanamycin (50  $\mu$ g/ml) and streptomycin (50  $\mu$ g/ml). *A. tumefaciens* cultures were grown at 28°C for 48 h, after which a 1-ml fine syringe was used to inject 0.2 ml of the culture into the stem or petioles of plants at the six-leaf stage. *N. benthamiana*, *N. glutinosa*, and *P. hybrida* plants were agroinoculated with PLH9000-Y47-1.9A either alone or as a mixture with pBinPLUS-Y47-2 $\beta$ . To test the interaction between MYVV and DNA $\beta$  molecules of different origins, *N. benthamiana* plants were agroinoculated with MYVV and either TYLCCNV DNA $\beta$  or *Tomato yellow leaf curl Thailand virus* (TYLCTHV) DNA $\beta$  previously constructed by our laboratory (23; X. P. Zhou, unpublished data). After inoculation, plants were grown in an insect-free greenhouse with supplementary light corresponding to a 16-h day length.

**Whitefly transmission.** *N. glutinosa* plants infected with MYVV alone or in combination with MYVV DNA $\beta$  were used as sources of inocula for whitefly transmission. In these experiments, *Bemisia tabaci* whiteflies were fed on the source plants in closed cages within an insect-free cabinet for an acquisition access period of 3 days. Then, 20 viruliferous adult whiteflies were transferred to each healthy *M. coromandelianum* plant for a minimum 48-h period of transmission access as described previously (7). After inoculation, insects were removed and plants were sprayed twice weekly with an insecticide and maintained in an insect-free cabinet. Four weeks later, virus infections were evaluated by symptom observation and by PCR using the MYVV-specific primers Y47F and Y47R.

**Assay of viral DNA.** Nucleic acids were isolated from young leaves of *N. benthamiana* and *N. glutinosa* plants at 35 days postinoculation as previously described (24), fractionated by 1% agarose gel electrophoresis in TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.3), and then transferred to Hybond-N<sup>+</sup> membranes (Amersham Pharmacia, Little Chalfont, Buckinghamshire, England). After alkali denaturation and neutralization, hybridization was detected with randomly labeled probes using a random primer DNA labeling kit (Promega) according to the manufacturer's instructions. A DNA probe specific for MYVV was produced with BamHI digestion of T-Y47A-FL. The MYVV DNA $\beta$  probe was amplified from the satellite conserved region (SCR) of MYVV DNA $\beta$  using  $\beta$ SCR1 and  $\beta$ SCR2 primers as described previously (22). Hybridization signals were detected by phosphorimaging using a Typhoon 9200 imager (Amersham Pharmacia).

**Nucleotide sequence accession numbers.** The complete nucleotide sequences of DNA $\beta$  molecules associated with the Yunnan isolates have been submitted to GenBank (accession numbers AJ971459-63, AJ971695-700, and AJ971702-10).

## RESULTS AND DISCUSSION

**Genomic organization and variation of DNA $\beta$  molecules.** PCR analysis using Y47F/Y47R primers specific for MYVV yielded approximately 2.7-kb bands from all 20 samples collected in Yunnan, indicating that they were infected with MYVV. The complete nucleotide sequences of DNA $\beta$  molecules associated with these isolates were determined and ranged from 1,339 to 1,358 nucleotides in length. The molecules have common structural features of DNA $\beta$  detailed in previous studies (1, 23), including the SCR, which contains a predicted hairpin structure with the loop sequence TAATAT T $\downarrow$ AC. Comparisons of the SCRs, which spans 115 bp from 100 bp upstream to 15 bp downstream of the stem-loop, revealed between 97.4% and 100% sequence identity among these isolates. The A-rich regions, which contained 58.3% and 60.5% adenylic acid and ranged from 204 to 206 nucleotides in length, were located between positions 765 and 970 on the virion-sense DNA. These regions have repeated blocks of up to nine consecutive adenylic acid residues. A single conserved  $\beta$ C1 open reading frame encoding a predicted 118-amino-acid product was also located on the complementary-sense strand

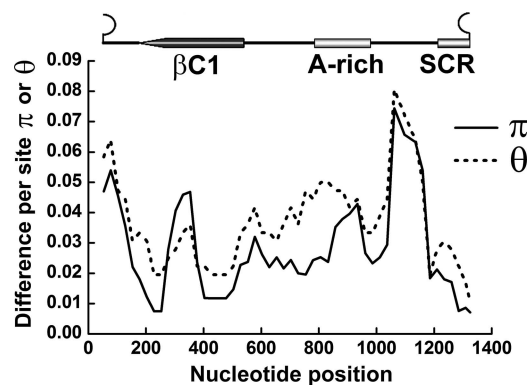


FIG. 1. Sliding window plot showing the distribution of genetic variation estimated by nucleotide diversity ( $\pi$ ) and Watterson's parameter ( $\theta$ ) for MYVV DNA $\beta$  molecules. The window size is 100 sites wide, with sliding by 50-site increments. The relative positions of the  $\beta$ C1 gene, A-rich region, and SCR of the DNA $\beta$  genome are illustrated above the plot in linear DNA format.

of viral satellite DNA. These predicted proteins share 92.4% to 100% amino acid sequence identity with each other.

The complete nucleotide sequences of the 20 DNA $\beta$  molecules share 93.0% to 99.8% sequence identity. The sequences were most closely related to MYVV DNA $\beta$  with the sequence identities ranging from 93.2% to 97.3%, further indicating that these samples were infected by MYVV. The MYVV DNA $\beta$  molecules had much less variation than those of TYLCCNV DNA $\beta$  (72 to 99%) or *Tobacco curly shoot virus* DNA $\beta$  molecules (83 to 98%) collected in Yunnan Province (23). This is probably due to the fact that TYLCCNV/DNA $\beta$  and tobacco curly shoot virus/DNA $\beta$  complexes infect a wide variety of hosts, whereas MYVV/DNA $\beta$  complexes infect mainly *M. coromandelianum*.

To evaluate the extent of DNA $\beta$  variation, statistical analyses regarding nucleotide sequence diversity were carried using the versatile program DnaSP version 4.10.3. The level of intraspecific variation of MYVV DNA $\beta$  molecules, including the 20 DNA $\beta$  molecules and the DNA $\beta$  sequence from MYVV isolate Y47, was estimated by the Watterson parameter  $\theta$  and the mean pairwise diversity  $\pi$  at all sites along the genome. Using the sliding windows option of DnaSP 4.10.3 software (window length, 100; step size, 25), a noteworthy observation is that there are two regions with a high level of nucleotide diversity located within the  $\beta$ C1 coding region adjacent to the C terminus and the noncoding region between the SCR and the A-rich region (Fig. 1).

A rooted phylogenetic tree of DNA $\beta$  molecules was created by the neighbor-joining method using MEGA version 3.1 (9). The tree delineated two subpopulations: subgroup 1 isolates from Chuxiong and Yuxi districts and subgroup 2 isolates from the Honghe district (Fig. 2). However, there is an exception in the tree where DNA $\beta$  molecules from Y300 (Y300 $\beta$ ) and Y301 (Y301 $\beta$ ) should cluster into subgroup 1 according to geographical location but instead form a relatively independent cluster. Sequence comparison among DNA $\beta$  molecules from Y300, Y301, Y302, and Y303, which were taken from the same area in Chuxiong, shows that the major sequence differences are point mutations.

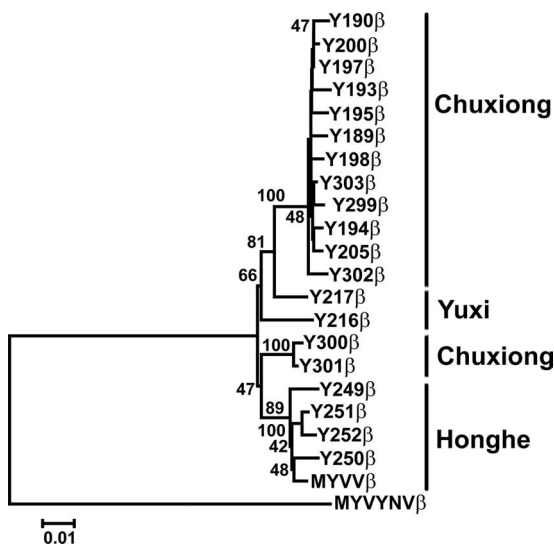


FIG. 2. Rooted neighbor-joining tree constructed based on the full-length DNA $\beta$  sequences. Sequences of the DNA $\beta$ s obtained from the above samples are named according to their associated begomovirus; thus, Y189 $\beta$  refers to DNA $\beta$  from isolate Y189. The sequence of MYVYNV DNA $\beta$  (MYVYNV $\beta$ ) was used as the root. The statistical significance of the branches was calculated by bootstrap analysis with 1,000 replicates of the data. Numbers at the nodes indicate the percentages of identical branches obtained by bootstrapping. Only bootstrap values greater than 40% are indicated.

**Infectivity and symptoms induced by MYVV and its associated DNA $\beta$ .** To investigate the biological role of DNA $\beta$ , MYVV (PLH9000-Y47-1.9A) was agroinoculated into plants either alone or together with MYVV DNA $\beta$  (pBinPLUS-Y47-2 $\beta$ ). MYVV alone was systemically infectious in *N. benthamiana*, *N. glutinosa*, and *P. hybrida* plants, but no symptoms were visible in these plants (Fig. 3C, E, and G). In contrast, when these plants were infected with MYVV and MYVV DNA $\beta$ , the coinoculated plants developed downward curling of the leaves associated with yellow vein and leaf crinkling (Fig. 3D, F, and H). Agroinoculation of either MYVV alone or together

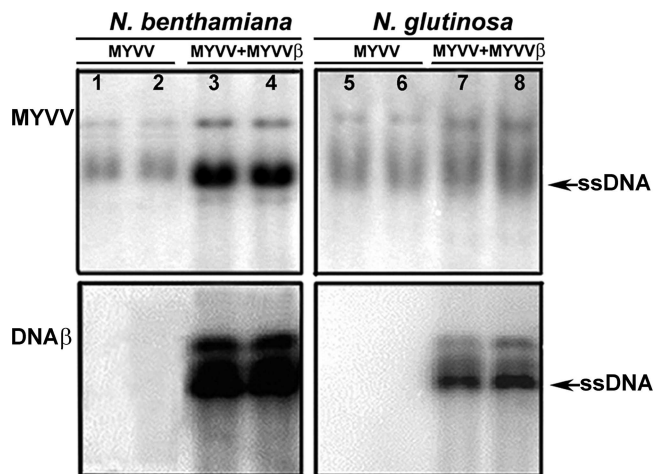


FIG. 4. Southern blot analysis of nucleic acids extracted from infected *N. benthamiana* and *N. glutinosa* plants agroinoculated with MYVV alone (lanes 1, 2, 5, and 6) or in the presence of MYVV DNA $\beta$  (MYVV $\beta$ ) (lanes 3, 4, 7, and 8) at 35 days after inoculation. Equal amounts of nucleic acid (20  $\mu$ g) were loaded in each lane. Blots were probed with either MYVV (top) or MYVV DNA $\beta$  (bottom). The positions of single-stranded DNA (ssDNA) forms are indicated.

with MYVV DNA $\beta$  was not successful in *M. coromandelianum* plants, and so whitefly transmission assays were carried out to fulfill Koch's postulates. In these transmission tests, *M. coromandelianum* plants infected with MYVV and MYVV DNA $\beta$  displayed yellow vein symptoms similar to those observed in the field (Fig. 3B). However, plants exposed to whiteflies that had fed only on MYVV-infected plants displayed no symptoms, although MYVV could be detected by PCR with MYVV-specific primers (Fig. 3A).

Southern hybridization analysis of *N. benthamiana* and *N. glutinosa* plants agroinoculated with both MYVV and MYVV DNA $\beta$  showed that both MYVV and MYVV DNA $\beta$  were present (Fig. 4, lanes 3, 4, 7, and 8). *N. benthamiana* and *N. glutinosa* plants coinfecting with MYVV and MYVV DNA $\beta$  accumulated more viral DNA than did those infected with

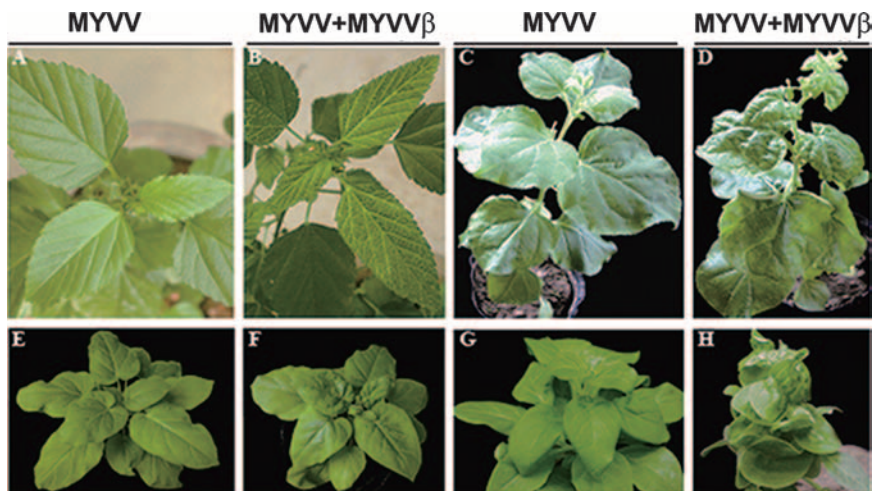


FIG. 3. Symptoms induced by MYVV alone or in the presence of MYVV DNA $\beta$  (MYVV $\beta$ ) in *M. coromandelianum* at 45 days after whitefly transmission (A and B) and in *N. benthamiana* (C and D), *N. glutinosa* (E and F), or *P. hybrida* (G and H) at 35 days after agroinoculation.

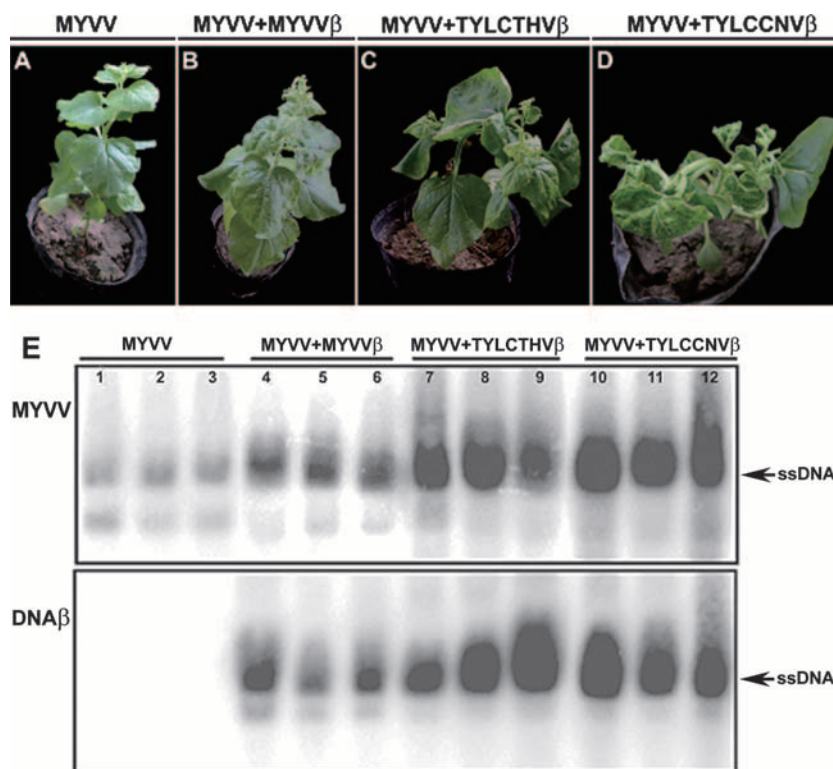


FIG. 5. Symptoms and Southern blot analyses in *N. benthamiana* plants induced by MYVV and different DNA $\beta$  molecules at 35 days postinoculation. (A to D) Plants were agroinoculated with MYVV alone (A) or in the presence of MYVV DNA $\beta$  (B), TYLCTHV DNA $\beta$  (C), or TYLCCNV DNA $\beta$  (D). (E) Southern blot analyses of nucleic acids extracted from agroinoculated *N. benthamiana* plants with MYVV alone (lanes 1 to 3) or in the presence of MYVV DNA $\beta$  (MYVV $\beta$ , lanes 4 to 6), TYLCTHV DNA $\beta$  (TYLCTHV $\beta$ , lanes 7 to 9), or TYLCCNV DNA $\beta$  (TYLCCNV $\beta$ , lanes 10 to 12). Equal amounts of nucleic acid (20  $\mu$ g) were loaded in each lane. Blots were probed with either MYVV (top) or MYVV DNA $\beta$  (bottom). The positions of the single-stranded DNA (ssDNA) forms are indicated to the right of the blot.

MYVV alone (Fig. 4). Also, *N. benthamiana* has much more intense bands than does *N. glutinosa* whether the plants were infected with MYVV alone or with combinations of MYVV DNA $\beta$  (Fig. 4).

The results above confirmed that MYVV is a monopartite begomovirus associated with a satellite DNA $\beta$ , which is necessary for symptom induction, and that DNA $\beta$  can enhance the accumulation level of viral DNA in infected plants.

**Interaction between MYVV and other DNA $\beta$  molecules.** The ability of MYVV to act as a helper virus to replicate heterologous DNA $\beta$  molecules was evaluated by agrobacterium-mediated coinoculation of *N. benthamiana* plants with MYVV and TYLCTHV DNA $\beta$  or TYLCCNV DNA $\beta$ . *N. benthamiana* seedlings systemically infected with MYVV and either TYLCTHV DNA $\beta$  or TYLCCNV DNA $\beta$  developed typical systemic symptoms that were distinguishable from those induced by MYVV and MYVV DNA $\beta$  (Fig. 5A to D). *N. benthamiana* plants coinoculated with MYVV and TYLCTHV DNA $\beta$  showed downward leaf curling and stem distortion (Fig. 5C). However, when agroinoculated with MYVV and TYLCCNV DNA $\beta$ , *N. benthamiana* plants developed more accentuated downward leaf curling and stem distortion symptoms accompanied by enations on the undersides of the leaves (Fig. 5D). These symptoms were substantially more severe than those induced by MYVV and MYVV DNA $\beta$  (Fig. 5B).

The relative amounts of MYVV and of the homologous and

heterologous DNA $\beta$ s in the MYVV-infected and -coinoculated *N. benthamiana* plants were compared by Southern hybridization analysis. All of the heterologous DNA $\beta$  isolates could be detected in systemically symptomatic leaves, indicating that they can replicate and spread systemically in association with MYVV. However, viral DNA concentrations in the plants were variable. Viral DNA levels in *N. benthamiana* plants coinoculated with MYVV and TYLCCNV DNA $\beta$  were similar to those in plants coinoculated with MYVV and TYLCTHV DNA $\beta$  (Fig. 5E, lanes 5 and 7 to 12). In contrast, the relative concentrations of viral DNA in *N. benthamiana* plants coinoculated with MYVV and MYVV DNA $\beta$  were nearly an order of magnitude lower than concentrations in those inoculated with the heterologous DNA $\beta$  molecules (Fig. 5E). These results indicate that MYVV can interact functionally with homologous and heterologous DNA $\beta$  and that the variation of viral DNA amounts in infected plants correlates well with the severity of symptoms.

Pseudorecombination is one of the most important sources of viral genetic variation. In bipartite begomoviruses, the two components share a common region containing repeat sequences (iteron). When the replication-associated protein (Rep) initiates transreplication of DNA-B, Rep specifically recognizes the iteron. This recognition mechanism is extremely sequence specific (6) and is usually confined to a particular begomovirus species, although interspecific replication can oc-

cur if the iterons are conserved (16). Our results further indicate that begomoviruses can support heterologous DNA $\beta$  replication. The replication of DNA-A and DNA-B genomic components of different bipartite begomoviruses is highly isolate and sequence specific, compared to the interactions between DNA $\beta$  and its helper virus (11, 13).

So far, three types of begomoviruses have been identified that are distinguishable by their genome composition. One group consists of bipartite begomoviruses that contain DNA-A and DNA-B components; a second group is composed of monopartite begomoviruses, which lack a component equivalent to DNA-B; and the third group is represented by monopartite begomoviruses that harbor a satellite DNA $\beta$  molecule in infected plants (1, 4, 12). The monopartite begomoviruses associated with DNA $\beta$  molecules can be divided into two types according to whether or not their DNA $\beta$  molecules are indispensable for induction of symptoms. In the majority of these begomoviruses, DNA $\beta$  molecules are required for induction of typical disease symptoms. The viral DNA components are either poorly infectious or not infectious without DNA $\beta$  in the host from which they were initially isolated, although cloned viral DNA genomes have been shown to be infectious in the experimental host *N. benthamiana* (3, 8, 15). In the remaining minority, the associated DNA $\beta$  molecules do not appear to play a key role in symptom induction and their relationship with the helper virus is less well understood. These classes of monopartite begomoviruses may represent intermediates between the majority type and true monopartite begomoviruses that have a single genomic component similar to the DNA-A component of bipartite begomoviruses (10). Based on our results, it is evident that MYVV belongs to the majority type.

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