Ageratum yellow vein China virus Is a Distinct Begomovirus Species Associated with a DNA β Molecule

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ABSTRACT

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Ageratum conyzoides plants exhibiting yellow vein symptoms, collected near Haikou, Hainan Province, China, contained begomoviral DNA-A-like molecules. The complete sequences of the molecules from two samples, Hn2 and Hn2-19, were shown to consist of 2,768 and 2,748 nucleotides (nt), respectively. These sequences have more than 97% nucleotide sequence identity, but less than 86% identity with other reported begomovirus sequences. In line with the taxonomic convention for begomoviruses, Hn2 and Hn2-19 are therefore considered to represent isolates of a distinct begomovirus species, for which the name Ageratum yellow vein China virus (AYVCNV) is proposed. Sequence alignment shows AYVCNV has arisen by recombination among viruses related to *Ageratum yellow vein virus*, *Papaya leaf curl China virus*, and an unidentified begomovirus. Southern blot analyses revealed that all plants

sampled contained molecules resembling DNA β . DNA β molecules from three samples were 1,323 or 1,324 nt long and had >98% sequence identity but <81% identity with previously reported DNA β sequences. Infectious clones of Hn2 and its associated DNA β were constructed and agroinoculated to plants. Hn2 alone caused sporadic asymptomatic systemic infection of *Nicotiana benthamiana*, *N. glutinosa*, *Lycopersicon esculentum*, *Petunia hybrida*, and *A. conyzoides* but its accumulation was much enhanced in plants co-inoculated with DNA β . The co-inoculated *N. benthamiana*, *N. glutinosa*, *P. hybrida*, and *L. esculentum* plants developed leaf curling or leaf crinkling symptom; those in *A. conyzoides* were typical of ageratum yellow vein disease. When the DNA β molecules associated with four other Chinese begomoviruses were coinoculated with Hn2 to *N. benthamiana* and *N. glutinosa*, the DNA β molecules were replicated, and the plants developed systemic symptoms of types that were specific for each DNA β . This illustrates that there is less specific interaction between monopartite begomovirus and DNA β than between the DNA-A and DNA-B of begomoviruses with bipartite genomes.

Geminiviruses are a family of small, circular, single-stranded DNA (ssDNA) viruses that cause severe diseases in major crop plants worldwide (5,18). Viruses in the family *Geminiviridae* have been classified into four genera based on host range, type of insect vector, and genome organization (27). *Begomovirus* is the largest of these genera and contains whitefly-transmitted geminiviruses that infect dicotyledonous plants. Many begomoviral species have a bipartite genome: a DNA-A component encodes all the proteins necessary for virus replication, transcription, and encapsidation, and a DNA-B component encodes proteins required for movement functions. Typically, both components are essential for systemic virus infection. However, some begomoviruses lack a DNA-B, and have a genome that consists of only one component, which resembles DNA-A of bipartite begomoviruses (13,35).

Recently, a novel type of satellite DNA, known as DNA β , was found to be associated with some monopartite begomoviruses, including *Ageratum yellow vein virus* (AYVV), *Cotton leaf curl Multan virus* (CLCuMV), *Bhendi yellow vein mosaic virus* (BYVMV), and *Eupatorium yellow vein virus* (EpYVV). The DNA β molecules typically enhance the symptoms induced by the begomovirus alone, and are essential for producing the full range of systemic symptoms that are found in naturally infected plants (1,12,24–26). DNA β molecules consist of circular ssDNA of about 1,350 nucleotides (nt), and so are about half the size of their respective helper begomovirus DNA molecules (1,3,12,24,31,38). Apart from the sequence TAATATTAC, which is conserved in all begomoviruses and contains the initiation site of rolling circle

replication, DNA β shows little sequence similarity to begomoviral DNA-A or DNA-B (38). Analysis of DNA β sequences revealed a highly conserved organization consisting of a single open-reading frame designated β C1, a region of high sequence similarity referred to as the satellite conserved region (SCR), and an adenine (A)-rich region (1,38).

In China, DNA β molecules are associated with begomoviruses obtained from tobacco, tomato, and weeds (31,34,38). In the present study, we describe the molecular characterization of a new begomovirus infecting *Ageratum conyzoides* in Hainan, China, and of its associated DNA β . We also show that the DNA β is necessary for induction of typical symptoms in *A. conyzoides* and experimental hosts, and that the virus can interact functionally in *Nicotiana benthamiana* and *N. glutinosa* plants with DNA β components that are associated with other begomoviruses in China.

MATERIALS AND METHODS

Viral isolates. Between 2001 and 2004, 14 *A. conyzoides* plants (Hn2, Hn2-19, Hn9-Hn14, Hn17, Hn19, Hn20, and Hn22-Hn24) exhibiting yellow vein symptoms were collected in fields near Haikou, Hainan Province, China, taken to Hangzhou, Zhejiang Province, kept in an insect-proof greenhouse at 20 to 30°C, and used as virus sources.

DNA extraction, polymerase chain reaction (PCR), and sequence determination. Total DNA was extracted from tissues of naturally infected symptomatic plants as described previously (39). The degenerate primer pair PA and PB was used to amplify an approximately 500-bp fragment covering parts of the intergenic region (IR) and the AV2 (CP) gene of the DNA-A-like component as described (39). PCR products were sequenced, and primers Hn2F (5'-GTGGGATCCTCTTTTGAACGAG-3') and

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Hn2R (5'-AGAGGATCCCACATTGCTTT-3') (each containing an *EcoRI* site), based on these sequences, were used to amplify the full length DNA-A-like molecule from infected *A. conyzoides* plants. Comparison of previously reported DNA β sequences allowed the degenerate primer β CR/R (5'-GACGATCARATACAVCAVCA-3', R = A and G; V = A, C, and G) to be designed and used in combination with β O1 (2) to amplify the putative DNA β component. Additional primers HindIII β F (5'-GAAAGCTTGGGCCGTTGAGTT-3') and HindIII β R (5'-CAAGCTTTCGGCCACACACGTT-3'), designed on the basis of the determined sequences, were used to amplify full-length DNA β . PCR was performed as described previously (33). The PCR products were recovered, purified and cloned into pGEM-T vector (Promega, Madison, WI), and then sequenced using the automated model 377 DNA sequencing system (Perkin Elmer, Wellesley, MA).

Sequence analysis. Sequence data were assembled and analyzed with the aid of DNA Star Software (DNASTar Inc., Madison, WI). Multiple sequence alignments were performed using DNAMAN Version 4.0 for Windows (Lynnon Biosoft, Quebec). Phylogenetic trees were constructed by the neighbor-joining method using DNAMAN with 1,000 bootstrap repeats. The published sequences used in comparisons were obtained from GenBank (Table 1).

Construction of infectious clones of Hn2 and its associated DNA β . A partial tandem repeat of Hn2 was made in three cloning steps. First, the full-length genome of isolate Hn2 was amplified from a Hn2-infected plant using primers Hn2F and Hn2R, overlapping at a unique *EcoRI* site, and cloned into pGEM-T vector (Promega) to produce clone pGEMT-Hn2. After confirming the sequences of this clone, a 1.3-kb fragment encompassing the IR was released from pGEMT-Hn2 with *EcoRI* and *Bam*HI and subcloned into *EcoRI*/*Bam*HI-digested pBINPLUS (28) to produce clone pBin-Hn2-0.4A. In the third

cloning step, a full-length genome unit of Hn2 was excised from pGEMT-Hn2 with *EcoRI* and was subsequently introduced into the unique *EcoRI* site of clone pBin-Hn2-0.4A to yield clone pBin-Hn2-1.4A.

DNA β from a plant infected with isolate Hn2 was used for construction of an infectious clone of DNA β . A complete genome unit of Hn2 β was amplified using HindIII β F and β R (5'-CATTCGGCCACACACGTT-3') or HindIII β F and HindIII β R, respectively, and the fragment inserted into a pGEM-T vector to produce clone pGEMT- β or pGEMT- β -1, respectively. After confirming the sequences of these clones, a full-length unit of DNA β was digested from pGEMT- β with *Hind*III and *Sal*I and introduced into the binary vector pBINPLUS to yield clone pBin-Hn β and another full-length copy of DNA β was excised with *Hind*III from pGEMT- β -1 and inserted into the unique *Hind*III site of pBin-Hn β to produce pBin-Hn2 β , containing a tandem repeat of Hn2 DNA β . Infectious clones of DNA β s of *Tomato yellow leaf curl China virus* (TYLCCNV), *Tobacco curly shoot virus* (TbCSV), *Malvastrum yellow vein virus* (MYVV), and *Sida yellow mosaic China virus* (SiYMCNV) used in this study were those constructed previously (14,38).

Agroinoculation of plants. The two binary vectors, pBinHn2-1.4A and pBin-Hn2 β , were moved into *Agrobacterium* strain GV3850 (36) by triparental mating (6) and the transconjugants were selected on kanamycin (50 μ g/ml) and rifampicin (10 μ g/ml). *A. tumefaciens* cultures were grown at 28°C for 48 h, and were then agroinoculated by injecting 0.2 ml of culture fluid through a fine needle into stems or petioles of *N. benthamiana*, *N. glutinosa*, *N. tabacum* cv. Samsun, *Lycopersicon esculentum*, *Petunia hybrida*, and *A. conyzoides* plants at the four-to-six leaf stage (38). Inocula contained either pBinHn2-1.4A separately or in combination with pBin-Hn2 β . To test the interactions between Hn2 and DNA β molecules of different origins, Hn2 was co-inoculated with DNA β of TYLCCNV, TbCSV, MYVV, or

TABLE 1. GenBank accession numbers of representative begomovirus and satellite DNA sequences used in comparisons

Begomovirus or satellite	Abbreviation	Accession number
Begomovirus sequences		
<i>Ageratum yellow vein virus</i>	AYVV	X74516
<i>Ageratum yellow vein Sri Lanka virus</i>	AYVSLV	AF314144
<i>Bhendi yellow vein mosaic virus</i>	BYVMV	AJ002453
<i>Chilli leaf curl virus</i>	ChiLCuV	AF336806
<i>Cotton leaf curl Multan virus</i>	CLCuMV	AJ002447
<i>Eupatorium yellow vein virus</i>	EpYVV	AB007990
<i>Euphorbia leaf curl virus</i>	EuLCV	AJ558121
<i>Malvastrum yellow vein virus</i>	MYVV	AJ457824
<i>Papaya leaf curl China virus</i>	PaLCuCNV	AJ558125
<i>Pepper leaf curl virus</i>	PeplCV	AF414287
<i>Sida yellow mosaic China virus</i>	SiYMCNV	AJ810096
<i>Soybean crinkle leaf virus</i>	SbCLV	AB050781
<i>Squash leaf curl Yunnan virus</i>	SLCYNV	AJ420319
<i>Stachytarpheta leaf curl virus</i>	StaLCV	AJ495814
<i>Tobacco curly shoot virus</i>	TbCSV	AF240675
<i>Tobacco leaf curl Yunnan virus</i>	TbLCYNV	AF240674
<i>Tomato leaf curl Karnataka virus</i>	ToLCKV	U38239
<i>Tomato leaf curl Vietnam virus</i>	ToLCVV	AF264063
<i>Tomato yellow leaf curl China virus</i>	TYLCCNV	AJ319675
DNAβ sequences		
<i>Ageratum yellow vein virus</i> DNA β	AYVV β	AJ252072
<i>Bhendi yellow vein virus</i> DNA β	BYVV β	AJ308425
<i>Chilli leaf curl disease</i> DNA β	ChiLCuD β	AJ316032
<i>Cotton leaf curl Multan virus</i> DNA β	CLCuMV β	AJ292769
<i>Eupatorium yellow vein virus</i> DNA β	EpYVV β	AJ438938
<i>Malvastrum yellow vein virus</i> DNA β	MYVV β	AJ421482
<i>Okra leaf curl disease</i> DNA β	OLCD β	AJ316029
<i>Sida yellow mosaic China virus</i> DNA β	SiYMCNV β	AJ810093
<i>Tobacco leaf curl disease</i> DNA β	TbLCD β	AJ316033
<i>Tobacco curly shoot virus</i> DNA β	TbCSV β	AJ421484
<i>Tomato leaf curl disease</i> DNA β	ToLCD β	AJ316035
<i>Tomato yellow leaf curl China virus</i> DNA β	TYLCCNV β	AJ421621
<i>Tomato leaf curl Thailand virus</i> DNA β	TYLCTHV β	AJ566746

SiYMCNV. Following agroinoculation, the plants were kept in the insect-free cabinet with supplementary lighting corresponding to a 16-h day length. Asymptomatic infection was confirmed by PCR using primers Hn2F and Hn2R.

Suppression assays of RNA silencing. In order to test whether Hn2 DNA β is able to suppress RNA silencing, transgenic *N. benthamiana* plants (line 16c) carrying a highly expressed green fluorescence protein (GFP) transgene were agroinfiltrated at the two- to three-leaf stage, with infectious clones of Hn2 (pBin-Hn2-1.4A) separately or in combination with Hn2 β (pBin-Hn2 β). By 20 days postinoculation (dpi), leaf curling symptom was detected and the seedlings were induced for GFP silencing via agroinfiltration with a strain of *A. tumefaciens* harboring the same functional GFP in a binary Ti plasmid (35S:GFP). Visual detection of GFP fluorescence in whole plants was performed as described (4). 16c transgenic *N. benthamiana* plants and the binary plasmid 35S:GFP were provided by D. C. Baulcome, John Innes Center, Norwich, UK.

Detection of viral and satellite DNAs in inoculated plants. Total nucleic acids were isolated from systemically infected plant tissue 28 or 30 dpi as previously described (33), fractionated by 1% agarose gel electrophoresis in Tris-borate-EDTA buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.3), and then transferred onto Hybond-N+ membranes after denaturation (Amersham Pharmacia, Buckinghamshire, England). Duplicate blots were prepared and probed with 32 P-labeled DNA hybridizing to either viral genomic DNA or DNA β sequences (21). A probe specific for Hn2 was produced by *Eco*RI-*Bam*HI digestion of pGEMT-Hn2. A probe specific for DNA β was produced by PCR-mediated amplification of an approximately 115-nt SCR of TYLCCNV DNA β (38) using primers β SCR1 (5'-GTCGACATTGAGGG-CCTTCCGGTCATC-3') and β SCR2 (5'-TCTAGATGTGGGAC-CCACCTCAACG-3'). Hybridization signals were detected by phosphorimaging with a Typhoon 9200 imager (Amersham Pharmacia).

RESULTS

Genomic organization of viral DNA and affinities to other geminiviruses. Fragments of 500 bp were amplified, using the degenerate primer pair PA/PB, from all the *A. conyzoides* plants collected, and these fragments were cloned and sequenced (AJ849900-11). Alignment of the 500-bp sequences revealed that nucleotide sequence identities ranged from 90.1 to 99.8% among these samples. Hn2 and Hn2-19 were selected for complete sequencing. Sequence analysis showed that Hn2 (AJ495813) is 2,768 nt and Hn2-19 (AJ564744) is 2,748 nt in length. Open reading frames in both Hn2 and Hn2-19 are organized similarly to those of other begomoviruses, with two (AV1, AV2) in the virion-sense DNA and four (AC1 to AC4) in the complementary-sense DNA, separated by an IR. The IR sequence contains several features characteristic of begomoviruses: a stem-loop structure with the nonanucleotide sequence TAATATTAC, which is common to all geminiviruses identified so far (10); two TATA motifs at nucleotides 2760 to 2764 and 100 to 104 (Hn2), or 2740 to 2744 and 98 to 102 (Hn2-19); a repeat sequence AATTGGTGTCT at nucleotides 2641 to 2652 and 2669 to 2680 (Hn2), or 2621 to 2632 and 2649 to 2660 (Hn2-19). The two viral DNA molecules (Hn2 and Hn2-19) had 97.7% nucleotide sequence identity and 82.8 to 100% amino acid sequence identity for the predicted AC1 to AC4 and AV2 proteins, demonstrating that they represent isolates of a single begomovirus species.

Sequence similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) algorithm available in GenBank, and the results showed that Hn2 had higher sequence similarities with begomoviruses found in Asia than those found in other regions of the world. So the Hn2 sequence was then compared with the sequences of other begomoviruses from Asia

with the aid of DNA Star Software. The nucleotide sequences of viral genomic DNA or those of the IR, and the amino acid sequences of predicted proteins were aligned. Hn2 was most closely related to AYVV (X74516), *Papaya leaf curl China virus* (PaLCuCNV, AJ558125), and *Soybean crinkle leaf virus* (SbCLV, AB059781), with 85.6, 83.3, and 80.6% nucleotide sequence identity, respectively. The IR of Hn2 has the highest sequence identity (68.9%) with PaLCuCNV. When individual encoded proteins were compared, Hn2 proteins had the highest amino acid sequence identity with those of AYVV for AV1 (95.3%) and AV2 (95.3%), and those of PaLCuCNV for AC1 (95.0%), AC2 (94.8%), AC3 (99.3%), and AC4 (88.7%). Phylogenetic analyses of the two new complete nucleotide sequences and those of other representative begomovirus species indicated that the two isolates (Hn2 and Hn2-19) cluster together and form a branch with PaLCuCNV AYVV, SbCLV, and *Tobacco leaf curl Yunnan virus* (TbLCYNV, AF240674) (Fig. 1A).

Hn2 was most closely related to AYVV (85.6%) in terms of nucleotide sequence, but Hn2 proteins had the highest amino acid sequence identity with PaLCuCNV for AC1, AC2, AC3, and AC4. Alignments were then made among Hn2, AYVV, and PaLCuCNV. Closer inspection of the alignments shows that the Hn2 can be divided into three parts: from 274 to 1,113 nt, which includes the

Fig. 1. Phylogenetic trees obtained using the neighbor-joining method in DNAMAN (Lynnon Biosoft, Quebec), based on total nucleotide sequences of **A**, Hn2 or **B**, Hn2 DNA β and other representative begomoviruses and their associated DNA β components (Table 1). Vertical branch lengths are arbitrary; horizontal branch lengths are proportional to calculated mutation distances. Bar length represents 5% sequence difference. The numbers at each node indicate the percentage of 1,000 bootstrap repeats that support the branching at that node. Bootstrap scores exceeding 90% are shown at major nodes. *African cassava mosaic virus* (ACMV) (AF112352) was used as an outgroup.

majority of AV2 and the whole of AV1; from 1,134 to 2,382 nt, which includes the majority of AC1, AC3, and AC4, and the whole of AC2; and the rest of the genome (2,383 to 273 nt), which includes the IR and part of AV2, AC1, and AC4. The percentage identities of these three parts between Hn2 and AYVV are 96, 90, and 74, respectively, whereas, when compared with PaLCuCNV, they are 81, 98, and 78, respectively (Table 2). These data are strong evidence that Hn2 has arisen by recombination among viruses related to AYVV, PaLCuCNV, and an unidentified begomovirus.

Structural features of Hn2 DNA β and affinities to other DNA β molecules. When primers beta01 and beta02 (2) were used to amplify the possible DNA β molecules in the field-collected samples, no specific band was amplified, but Southern blot analyses using the SCR of TYLCCNV DNA β as a probe revealed that all those samples contained DNA β -like molecules (data not shown). So a degenerate primer (β CR/R) was designed and used in combination with beta01 to amplify the putative DNA β component, and a specific band of approximately 0.7 kb was

TABLE 2. Percentages of nucleotide sequence identities of the three regions of Hn2 with those of *Ageratum yellow vein virus* (AYVV) and *Papaya leaf curl China virus* (PaLCuCNV)

Isolate compared	Region of DNA-A (number of nucleotides)		
	273-1112	1113-2382	2383-272
AYVV	96	90	74
PaLCuCNV	81	98	78

TABLE 3. Percentages of nucleotide or amino acid sequence identities between Hn2 β and other DNA β molecules

Virus ^a	DNA β ^b	β C1 ^c	SCR ^b
Hn9 β	99.7	98.3	100.0
Hn12 β	98.7	96.6	100.0
AYVV β	80.9	97.5	84.1
TYLCCNV β	49.1	60.2	85.0
TbCSV β	45.0	57.6	83.2
SiYMCNV β	42.8	57.6	82.3
MYVV β	35.8	27.1	79.6

^a DNA β Hn9 β was isolated from *Ageratum conyzoides* Hn9 plants exhibiting symptoms of yellow vein virus; Hn12 β was isolated from *A. conyzoides* Hn12 plants exhibiting symptoms of yellow vein virus; AYVV β was isolated from plants exhibiting symptoms of *A. yellow vein virus*; TYLCCNV β was isolated from plants exhibiting symptoms of *Tomato leaf curl China virus*; TbCSV β was isolated from plants exhibiting symptoms of *Tobacco curly shoot virus*; SiYMCNV β was isolated from plants exhibiting symptoms of *Sida yellow China mosaic virus*; and MYVV β was isolated from plants exhibiting symptoms of *Malvastrum yellow vein virus*.

^b Nucleotide.

^c Amino acid.

consistently amplified from all samples. Sequence analyses revealed that it is a partial DNA β molecule. DNA β sequences were then amplified by PCR with abutting primers HindIII β F/HindIII β R designed based on determined sequences from the 0.7-kb DNA fragment. A fragment approximately 1.4 kb in size was consistently amplified from all samples. Among these DNA β components, DNA β of Hn2 (Hn2 β), Hn9 (Hn9 β), and Hn12 (Hn12 β) were chosen for sequencing. The complete nucleotide sequences were 1,323, 1,323, and 1,324 nt in size (AM048834 to AM04048836), respectively. These three DNA β molecules all contain an A-rich region (approximately 64%) at nucleotides 721 to 939, an SCR of 115 nt from nucleotides 1224/1225 to 15, and an ORF (β C1), located in the complementary-sense strand, that has a coding capacity of 13.8 kDa.

Hn2 β , Hn9 β , and Hn12 β have >98.6% nucleotide sequence identity but less than 50% identities with other reported DNA β molecules except AYVV β (AJ252072, 81%) (Table 3). The β C1-encoded protein in Hn2 β , Hn9 β , and Hn12 β has 96.6 to 97.5% amino acid sequence identity with AYVV β β C1 protein, and the SCR nucleotide sequences of the three isolates have 84.1% sequence identity with AYVV β . Thus, Hn2 β , Hn9 β , and Hn12 β are the closest relatives of AYVV β . A phylogenetic tree based on alignment of DNA β nucleotide sequences also shows that Hn2 β , Hn9 β , and Hn12 β cluster with AYVV β and form a separate branch (Fig. 1B).

In an attempt to detect a DNA-B component in virus-infected *A. conyzoides* plants, total DNA extracts were used in PCR with the degenerate primers for DNA-B described by Rojas et al. (22) and Fondong et al. (9). In addition, PCR products amplified with a pair of primers based on the nucleotide sequence of the IR of DNA-A (and therefore also that of a putative DNA-B) were subjected to restriction fragment analysis. No evidence was obtained of the existence of a DNA-B component (data not shown).

Infectivity and symptoms induced. To test the effect of DNA β on infectivity and symptoms, Hn2 (pBin-Hn2-1.4A) was agroinoculated to plants either separately or in combination with Hn2 β (pBin-Hn2 β). Hn2 alone produced an asymptomatic infection in *N. benthamiana*, *N. glutinosa*, *P. hybrida*, *L. esculentum*, and *A. conyzoides*. The symptoms induced by Hn2 and DNA β combined were dramatically different. *N. benthamiana* seedlings inoculated with Hn2 and DNA β developed systemic symptoms consisting of severe downward leaf curl accompanied by leaf crinkle and stunting, with vein swelling on the undersides of leaves (Fig. 2A, C). Similarly, *N. glutinosa*, *P. hybrida*, and *L. esculentum* plants infected by co-inoculation with Hn2 and DNA β developed leaf curling or leaf distortion (Table 4). *A. conyzoides* seedlings co-agroinoculated with Hn2 and DNA β developed yellow vein symptoms that were indistinguishable from those in naturally infected plants and were clearly evident 14 dpi

TABLE 4. Effects of different DNA β components on infection and symptoms induced by Hn2

Plant	Inoculum				
	Hn2+Hn2 β	Hn2+TYLCCNV β	Hn2+TbCSV β	Hn2+MYVV β	Hn2+SiYMCNV
<i>Nicotiana benthamiana</i>	10/10 (3) ^a LC, CR, VT, St ^b	10/10 (3) LC, CR, VT, YV, Et	10/10 (3) LC, CR, VT, St	10/10 (3) LC, CR, YV	10/10 (3) LC, CR, VT, YV, St
<i>N. glutinosa</i>	10/10 (3) LC, CR, VT, St	10/10 (3) LC, CR, VT, YV, Et	10/10 (3) LC, CR, VT, St	7/10 (3) LC, CR	10/10 (3) LC, CR, YV, St
<i>Lycopersicon esculentum</i>	5/20 (2) LD, CR	1/15 (3) LC, VT, YV, St	—	—	—
<i>Petunia hybrida</i>	7/9 (2) LC	8/8 (3) LC, CR	9/9 (3) CR	7/7 (3) MCR	8/8 (2) LC, CR
<i>Ageratum conyzoides</i>	8/10 (3) YV	—	—	—	—

^a Number of plants infected/number of plants inoculated. Numbers in parentheses are the number of times the experiment was repeated.

^b Symptoms: LC, leaf curling; CR, leaf crinkling; Et, enation; LD, leaf distortion; MCR, mild leaf crinkling; St, stunting; YV, vein yellowing; VT, vein thickening; and —, not infected.

(Fig. 2B and D). Agroinoculation of DNA β alone did not produce a local or systemic infection (data not shown).

We have previously demonstrated that TYLCCNV DNA β could function as gene silencing suppressor (4). In order to determine whether Hn2 infection can interfere with the initiation of GFP silencing, GFP-expressing 16c seedlings were agroinoculated either with Hn2 separately or with Hn2 in combination with its associated DNA β . By 20 dpi, these infected 16c seedlings were induced for GFP silencing via agroinfiltration.

Three weeks after agroinfiltration, plants co-inoculated with Hn2 plus DNA β exhibited a strong green fluorescence in the emerging leaves. Incomplete suppression appeared in the intermediate leaves. However, no green fluorescence or only minor flecks of fluorescence were observed in the seedlings inoculated with Hn2 alone (Fig. 2E). These results indicate that Hn2 DNA β could prevent establishment of RNA silencing in infected plants.

To complement information on symptoms, Southern blot hybridization analysis was used to assess the relative concentrations of Hn2 and DNA β in systemically infected leaves of inoculated plants. Hn2 DNA was readily detected in *N. benthamiana* plants inoculated with pBin-Hn2-1.4A but was only barely detected in *A. conyzoides*. In both species its concentration was much increased in plants co-inoculated with Hn2 and DNA β . DNA β was readily detected in co-inoculated plants of both species (Fig. 3). In addition to its effect on symptoms, DNA β also enhanced the accumulation of viral DNA in systemically infected leaves, which was most obvious in *A. conyzoides*.

Interaction between Hn2 and heterologous DNA β components. To test the ability of Hn2 to act as a helper virus for replicating DNA β molecules found in association with other begomoviruses in China, *N. benthamiana* and *N. glutinosa* plants were inoculated with Hn2 with or without DNA β of TYLCCNV, TbCSV, MYVV, or SiYMCNV (Fig. 4). In *N. benthamiana*, downward leaf curling, crinkling of leaves, and vein yellowing developed in plants co-inoculated with either Hn2 and TYLCCNV DNA β , Hn2 and MYVV DNA β , or Hn2 and SiYMCNV DNA β . In the presence of TYLCCNV DNA β , enations were produced on the underside of leaves. Co-inoculation of Hn2 and TbCSV DNA β induced only leaf crinkling and downward leaf curling (Fig. 4A). Inoculated *N. glutinosa* plants developed different symptoms when different DNA β components were used. The leaf curl symptoms displayed by *N. glutinosa* plants inoculated with Hn2 and either DNA β of TYLCCNV, TbCSV, or SiYMCNV are reminiscent of those in *N. benthamiana* infected with the same complexes. In contrast, *N. glutinosa* plants co-inoculated with Hn2 and MYVV DNA β did not develop vein yellowing symptoms, and the symptoms were milder than those induced by co-inoculation with Hn2 and MYVV DNA β in *N. benthamiana* (Fig. 4B).

The relative amounts of Hn2 and of the heterologous DNA β s in the Hn2-inoculated and co-inoculated *N. benthamiana* plants were compared by Southern blot analysis. All the heterologous DNA β molecules were detected in the newly emerging leaves of co-inoculated plants, showing that transreplication and transmovement of DNA β by Hn2 had occurred in all the complexes. A DNA β specific probe was used by PCR-mediated amplification of the SCR of TYLCCNV DNA β (38). The SCR has high sequence identities among DNA β molecules (Table 3). So, it is expected to cross-hybridize between distinct DNA β molecules. Nevertheless, DNA β concentrations in plants co-inoculated with Hn2 and either MYVV DNA β or SiYMCNV DNA β were consistently lower than those in plants co-inoculated with Hn2 and either TYLCCNV DNA β or TbCSV DNA β (Fig. 5). In contrast, the concentration of Hn2 in plants inoculated with Hn2 alone was similar to that in plants co-inoculated with Hn2 and heterologous DNA β molecules. Taken together, these results

indicate that Hn2 can interact functionally with each of the heterologous DNA β components but that the interactions with the DNA β of MYVV and SiYMCNV are the least effective for replicating DNA β .

DISCUSSION

In this study we report that *A. conyzoides* plants showing yellow vein symptoms are infected by a previously undescribed begomovirus. The complete nucleotide sequences of two isolates, Hn2 and Hn2-19, have >97% sequence identity but <86%

Fig. 2. A and C show symptoms on *Nicotiana benthamiana*; B and D show symptoms on *Ageratum conyzoides* induced by (left) Hn2 alone or by (right) Hn2 together with Hn2 DNA β at 25 days postinoculation (dpi); and E shows prevention of the establishment of green fluorescence protein (GFP) silencing by begomovirus infection. Those line 16c seedlings that expressed GFP were agroinoculated with Hn2 alone or with Hn2 plus its DNA β . By 20 dpi the lower leaves of the infected plants were infiltrated with 35S:GFP and evaluated for the ability to induce silencing 3 weeks later.

Fig. 3. Detection of viral DNAs in *Nicotiana benthamiana* (lanes 1 to 6) and *Ageratum conyzoides* (lanes 7 to 12) plants inoculated with Hn2 (lanes 1 to 3 and 7 to 9) or Hn2 and Hn2 DNA β (lanes 4 to 6 and 10 to 12) at 28 days postinoculation. The blots were probed for Hn2 (above), or for DNA β (below). Approximately equal amounts (15 μ g) of nucleic acid were loaded in each lane. The position of single-stranded (SS) DNA is shown.

sequence identity with other reported begomoviruses. In line with the taxonomy and nomenclature adopted for begomoviruses, which are considered to be distinct species if their viral DNA sequences have <89% identity (7,8,27), Hn2 and Hn2-19 are considered to represent isolates of a distinct begomovirus species, for which the name *Ageratum yellow vein China virus* (AYVCNV) is proposed.

Recombination involving begomoviruses has occurred between strains and between species, and possibly also at the generic level (11,37). We have reported that several new begomovirus species found in China are considered to have arisen by recombination (16,32,33). Genomic data indicate that AYVCNV consists of the AV1 and AV2 regions derived from a virus related to AYVV, and that the AC1, AC2, AC3, and AC4 regions derived from a virus related to PaLCuCNV.

In previous work, DNA β components were found only in association with begomoviruses that occur in Asia or Africa and lack a DNA-B component (1,17). Our discovery of DNA β and failure to find a DNA-B component in infected *A. conyzoides* plants from Hainan Province, China, strongly suggests that these plants, too, are infected with a begomovirus that has a monopartite genome. Agroinoculation studies showed that although AYVCNV can replicate on its own and invades *N. benthamiana* and *A. conyzoides* plants systemically, it is unable to induce the symptoms typically displayed by naturally infected *A. conyzoides*. The occurrence of DNA β in *A. conyzoides*

was obvious when DNA extracts from naturally infected plants were analyzed by Southern blot hybridization using probes specific for the SCR, which is almost identical among DNA β components (38). Agroinoculation of *A. conyzoides* plants with AYVCNV or with AYVCNV plus DNA β confirmed that severe yellow vein symptoms developed only when plants were inoculated with both components. The presence of both components in symptomatic plants was confirmed by blot hybridization (Fig. 3). Yellow vein disease of *A. conyzoides* in Hainan Province is therefore the result of co-infection by AYVCNV and DNA β .

DNA β components represent a kind of satellite molecule that depends upon its helper virus for replication, encapsidation, insect transmission, and movement in plants (3,24). However, in our tests, AYVCNV attained greater concentrations in systemically infected leaves in the presence of DNA β . This phenomenon resembles the behavior of AYVV and CLCuMV in their natural hosts (3,24). It was therefore suggested that DNA β may have a direct effect on viral DNA replication, possibly by providing a proper cellular environment (15,19,29), or may facilitate the systemic movement of viral DNA in the plant, thereby enhancing the level of viral DNA that accumulates (20,23). More recent work with TYLCCNV has shown that the β C1 protein encoded by DNA β , accumulates in nuclei, binds to DNA without sequence specificity and acts as a suppressor of RNA silencing activity in *N. benthamiana*, and that its nuclear localization is required for it to affect symptoms (4). AYVCNV DNA β could prevent the establishment of RNA silencing, indicating similar activities of its β C1 protein.

Different DNA β components can be transreplicated by a single begomovirus DNA: for example, heterologous DNA β components were capable of transreplication by AYVV (1). An extension of this phenomenon is our finding that all DNA β s of TYLCCNV, TbCSV, MYVV, and SiYMCNV can interact functionally with AYVCNV in *N. benthamiana* plants, but with different degrees of effectiveness, so that different amounts of DNA β accumulate. Varying levels of DNA β accumulation have also reported for the interaction of heterologous DNA β components with *Stachytarpheta leaf curl virus* (33) and *TbLCYNV* (30). This suggests different degrees of adaptation of DNA β components to associate with a single viral DNA, and scope for competitive advantage among DNA β components for such an association.

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Fig. 4. Symptoms induced by Hn2 alone or in the presence of different heterologous DNA β components in **A**, *Nicotiana benthamiana* or **B**, *N. glutinosa* at 28 days postinoculation. From left to right, plants were infected with Hn2 alone or with Hn2 plus different heterologous DNA β components from *Tomato yellow leaf curl China virus* (TYLCCNV β), *Tobacco curly shoot virus* (TbCSV β), *Malvastrum yellow vein virus* (MYVV β), or *Sida yellow mosaic China virus* (SiYMCNV β).

Fig. 5. Southern blot analysis of nucleic acids extracted from infected *Nicotiana benthamiana* plants agroinoculated at 30 days postinoculation with Hn2 (lanes 1 to 3), Hn2 and *Tomato yellow leaf curl China virus* (TYLCCNV β) (lanes 4 to 6), Hn2 and *Tobacco curly shoot virus* (TbCSV β) (lanes 7 to 9), Hn2 and *Malvastrum yellow vein virus* (MYVV β) (lanes 10 to 12), or Hn2 and *Sida yellow mosaic China virus* (SiYMCNV β) (lanes 13 to 15). The blots were probed either for Hn2 (above) or for DNA β (below). A DNA β -specific fragment of the satellite conserved region (SCR) was used as probe, so it is expected to cross-hybridize between distinct DNA β molecules. Approximately equal amounts (15 μ g) of nucleic acid were loaded in each lane. The position of single-stranded DNA (ssDNA) is indicated.

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