Etiology of Ageratum Yellow Vein Diseases in South China

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Abstract

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Ageratum conyzoides is a common weed in agricultural regions in Asia. *A. conyzoides* plants exhibiting yellow vein symptoms were collected from Yunnan and Guangxi provinces of China. Polymerase chain reaction detection and sequence analysis showed that samples collected from Yunnan were mainly infected by *Tobacco curly shoot virus* (TbCSV) associated with *Ageratum yellow vein China betasatellite* (AYVCNB), while samples from Guangxi were mostly infected by *Papaya leaf curl China virus* (PaLCuCNV) and AYVCNB, or by *Ageratum yellow vein China virus* (AYVCNV) and AYVCNB, with a few

exhibiting dual infections by PaLCuCNV, AYVCNV, and AYVCNB. *Agrobacterium*-mediated inoculation of infectious clones showed that both TbCSV and AYVCNB or PaLCuCNV and AYVCNB produced typical yellow vein symptoms in *A. conyzoides*. Consequently, Ageratum yellow vein diseases in Yunnan and Guangxi provinces were caused by TbCSV/AYVCNB, PaLCuCNV/AYVCNB, or AYVCNV/AYVCNB. The implications of these results in relation to the prevalence of begomoviruses in cultivated plants are discussed.

Geminiviruses are plant-pathogenic viruses with single-stranded DNA (ssDNA) genomes encapsidated in twinned quasi-isometric particles. They cause severe diseases in major crop plants worldwide (11). Based on insect vectors, host ranges, and genome structures, the family *Geminiviridae* has been divided into four genera, *Mastrevirus*, *Curtovirus*, *Topocuvirus*, and *Begomovirus* (4). The viruses of the genus *Begomovirus*, which account for the largest number of species in the family, infect dicotyledonous plants. Most begomoviruses have bipartite genomes, designated as DNA-A and DNA-B components, while many species have only a single genomic component which is homologous to DNA-A of bipartite begomoviruses (3,11).

Within the last few years, many monopartite begomoviruses have been found to be associated with betasatellites (formerly known as DNA β). The representative betasatellites are *Ageratum yellow vein betasatellite* (AYVB) and *Cotton leaf curl Multan betasatellite* (CLCuMB), which are essential for typical disease symptoms of Ageratum yellow vein disease (AYVD) and Cotton leaf curl disease (CLCuD), respectively (1,17). Betasatellites are approximately half the size of the viral genome and have little sequence identity with viral genomic DNA; they depend on the helper viruses for replication, encapsidation, movement in plants, and transmission between plants. The β C1 protein encoded by the complementary-sense strand of betasatellites has been demonstrated to be a symptom determinant (6).

The study presented here shows that the *Ageratum yellow vein China betasatellite* (AYVCNB) is necessary for inducing typical yellow vein disease symptoms when co-infected with *Tobacco curly shoot virus* (TbCSV), *Papaya leaf curl China virus* (PaLCuCNV), or *Ageratum yellow vein China virus* (AYVCNV) in *Ageratum conyzoides*.

Materials and Methods

1. Viral isolates. *A. conyzoides* plants exhibiting yellow vein disease symptoms were collected in fields from the cities of Baoshan, Dehong, Tengchong, and Xishuangbanna in Yunnan Province, and Nanning in Guangxi Province, China from 2002 to 2012 (Table 1).

2. DNA extraction and sequence analysis. Total nucleic acids were extracted from field-collected leaf samples as described (20). The degenerate primer pair PA and PB was used to amplify a fragment approximately 500 bp in size, which partially covered the intergenic region (IR) and the VI (CP) gene of the genome (24). The specific primer pairs AYVCNVF/AYVCNVR for AYVCNV, Y1F1/Y6R2 for TbCSV, HeNfullF/HeNfullR for PaLCuCNV, and TbLCYNV-F/TbLCYNV-R for *Tobacco leaf curl Yunnan virus* (TbLCYNV) were designed to amplify the corresponding begomoviruses, respectively. The primer pairs Hn2F/Hn2R, Y35F/Y35R, and HeNfullF/HeNfullR were used for amplification of the full length of AYVCNV, TbCSV, and PaLCuCNV, respectively. Betasatellites were amplified with the universal primer pair beta01/beta02 (23), while a specific primer pair AYVCNBF/AYVCNBR was used for AYVCNB. The polymerase chain reaction (PCR) products were cloned and sequenced by an automated ABI sequencer model 3730 (Perkin Elmer, Wellesley, MA, USA). The sequences of primers used for PCR detection are shown in Table 2. Sequences were assembled with the aid of Lasergene (DNASTAR Inc., Madison, WI, USA). Multiple sequence alignments were performed using MegAlign in Lasergene.

3. Infectious clones and agroinoculation of plants. The infectious clones of TbCSV (pBinplus-Y35-1.9A), PaLCuCNV (pBinplus-HeNZM1-1.4A), AYVCNV (pBinplus-Hn2-1.4A), TbLCYNV (pBinplus-Y143-1.5A), AYVCNB (pBinplus-Hn19-2 β), and *Tobacco curly shoot betasatellite* (TbCSB) (pBinplus-Y35 β -2.0) used in this study were reported previously (13,19,21,22). The overnight *Agrobacterium tumefaciens* cultures containing the corresponding constructs were pelleted and resuspended in infiltration buffer containing 10 mM MES (pH 5.5), 10 mM MgCl₂, and 100 μ M acetosyringone and adjusted to an OD₆₀₀ volume of 1.0. *Nicotiana benthamiana* and *A. conyzoides* seedlings in the four- to six-leaf stages were inoculated with viruses by injecting on the stems using a 1-ml syringe. The inoculated plants were placed in an insect-free growth chamber at 25°C with supplementary lighting to provide a 16-h photoperiod.

4. Southern blot hybridization. Total nucleic acids were extracted from young leaves of *N. benthamiana* and *A. conyzoides*

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*The e-Xtra logo stands for "electronic extra" and indicates that Figures 1 and 2 appear in color online.

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Table 1. Information on *Ageratum conyzoides* collection locations, years, and detection of begomoviruses and betasatellites

Location	Year	Isolate ^a	Virus (accession number)	Betasatellite (accession number)
Baoshan	2004	Y271	TbCSV (AJ971510)	AYVCNB
		Y272	TbCSV (AJ971511)	AYVCNB
		Y273	TbCSV	AYVCNB
			TbLCYNV (AJ971506)	
		Y274	TbCSV (AJ971512)	AYVCNB (HF569263)
		Y275	TbCSV	AYVCNB
			TbLCYNV (AJ971507)	
		Y276	TbCSV	AYVCNB
			TbLCYNV (AJ971508)	
		Y282	TbCSV (AJ971513)	AYVCNB (HF569264)
		Y283	TbCSV	AYVCNB
			TbLCYNV (AJ971509)	
	2005	Y309	TbCSV (HF569269)	AYVCNB
			TbLCYNV (AM261657)	
		Y310	TbCSV	AYVCNB
			TbLCYNV (AM261658)	ToLCGxB (HF569268)
		Y311	TbCSV	AYVCNB
			TbLCYNV (AM261659)	
		Y314	TbCSV	AYVCNB
			TbLCYNV (AM261660)	ToLCGxB (HF569265)
		Y315	TbCSV (AM261667)	AYVCNB
				MYVYNB (HF569266)
		Y316	TbCSV	AYVCNB
			TbLCYNV (AM261661)	
		Y317	TbCSV	AYVCNB
			TbLCYNV(AM261662)	TbCSB (AM260736)
Dehong	2008	Y342	TbCSV (HF569270)	AYVCNB (HF569261)
				MYVYNB (HF569267)
		Y343	TbCSV (HF569271)	AYVCNB
		Y344	TbCSV (HF569272)	AYVCNB
		Y345	TbCSV (HF569279)	AYVCNB
		Y346	TbCSV (HF569273)	AYVCNB (HF569262)
		Y347	TbCSV (HF569280)	AYVCNB
		Y348	TbCSV (HF569274)	AYVCNB
		Y349	TbCSV (HF569275)	AYVCNB
		Y350	TbCSV (HF569276)	AYVCNB
		Y351	TbCSV (HF569277)	AYVCNB
		Y352	TbCSV (HF569278)	AYVCNB
	2012	Y-R1	TbCSV (HF569282)	AYVCNB
		Y-R2	TbCSV (HF569283)	AYVCNB
		Y-R8	TbCSV (HF569284)	AYVCNB
Tengchong	2012	Y-T7	TbCSV (HF569285)	AYVCNB
		Y-T8	TbCSV (HG003650) ^b	AYVCNB
Xishuangbanna	2012	Y-M7	TbCSV (HF569281)	AYVCNB
Nanning	2002	G8	PaLCuCNV (AJ558124)	AYVCNB
		G9	PaLCuCNV (AJ811913)	AYVCNB
		G10	PaLCuCNV (AJ558125)	AYVCNB
	2003	G47	PaLCuCNV (AJ849937)	AYVCNB
		G48	PaLCuCNV (AJ849938)	AYVCNB (HF569260)
		G49	PaLCuCNV	AYVCNB
		G50	PaLCuCNV (AJ849939)	AYVCNB (HF569259)
		G51	PaLCuCNV (AJ849940)	AYVCNB
		G52	PaLCuCNV	AYVCNB
		G66	AYVCNV (AJ849912)	AYVCNB
		G67	AYVCNV (AJ849913)	AYVCNB
		G68	AYVCNV (AJ849916)	AYVCNB
		G69	AYVCNV (AJ849914)	AYVCNB (AJ971258)
	2004	G86	AYVCNV (AJ849915)	AYVCNB (AJ971259)
		G95	AYVCNV (AJ971253)	AYVCNB (AJ971260)
		G96	PaLCuCNV (HF569288)	AYVCNB
			AYVCNV (AJ971254)	
		G97	PaLCuCNV (HF569287)	AYVCNB
			AYVCNV (HF569291)	
		G98	PaLCuCNV (AJ971255)	AYVCNB (AJ971262)
		G99	PaLCuCNV (AJ971256)	AYVCNB
			AYVCNV	
	2012	G111	PaLCuCNV (HG003651) ^b	AYVCNB (HF569258)
		G113	PaLCuCNV (HF569290)	AYVCNB
		G115	AYVCNV (HG003652) ^b	AYVCNB

^a Isolates beginning with Y and G were collected from Yunnan Province and Guangxi Province, respectively.^b Accession numbers for the full-length genome.

plants using the CTAB method as described (20). Nucleic acids were fractionated on 1% agarose gel in TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.3) and transferred to Hybond-N⁺ membranes (Amersham Biosciences, Buckinghamshire, England) and hybridized with Digoxin-labeled probes specific for AYVCNV, TbCSV, PaLCuCNV, or AYVCNB using a DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Diagnostics, Indianapolis IN, USA).

Results

Detection of begomoviruses from samples collected in South China. Using the degenerate primer pair PA/PB, a fragment of about 500 bp was amplified from all the collected *A. conyzoides* plant samples. The amplified fragments were cloned and then sequenced. Sequence similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) algorithm available in GenBank, and the results showed that most of the *A. conyzoides* samples isolated from Yunnan (Y271-272, Y274, Y282, Y315, Y342-Y352, Y-R1-R2, Y-T7-T8, Y-R8, Y-M7) had the highest sequence similarities (more than 93.0%) with TbCSV, while some samples (Y273, Y275-276, Y283, Y309-311, Y314, Y316-317) had more than 96.2% sequence identities with TbLCYNV (Table 1). The TbCSV-specific primer pair Y1F1/Y6R2 and TbLCYNV-specific primer pair TbLCYNV-F/TbLCYNV-R were then used to amplify the putative product from the DNA extracted from all the 32 Yunnan samples. The specific 1.0-kb fragment of TbCSV was generated from all *A. conyzoides* samples collected in Yunnan, including samples shown to be infected with TbLCYNV, while the specific 1.0-kb fragment of TbLCYNV was only detected in Y273, Y275-276, Y283, Y309-311, Y314, Y316, and Y317 samples, suggesting that all *A. conyzoides* samples collected in Yunnan were infected by TbCSV, and that some samples were dually infected by both TbCSV and TbLCYNV (Table 1). Y-T8 was randomly selected for the purpose of amplifying its entire genome using Y35F/Y35R primers, and fully sequenced, which revealed that it shared 96.1% sequence identity with TbCSV.

Amplified fragments from *A. conyzoides* plant samples in Guangxi Province were also sequenced. Sequence analyses showed that *A. conyzoides* samples collected in Guangxi could be clearly classified into two groups. G8-10, G47-52, G97-99, G111, and G113 were most closely related to PaLCuCNV with sequence identity exceeding 94.0%, while G66-69, G86, G95-96, and G115 each exhibited more than 90.8% identity with AYVCNV. These findings were successfully validated by using the specific primers HeNfullF/HeNfullR for PaLCuCNV and AYVCNVF/AYVCNVR for AYVCNV to detect the respective viruses in all 22 samples

isolated from Guangxi. A specific band of PaLCuCNV approximately 2.7 kb in size was amplified from the samples G8-10, G47-52, G96-99, G111, and G113. G111 was then chosen for the full genome cloning and sequencing, which revealed that it shared 93.4% sequence identity with PaLCuCNV. A 500-bp fragment of AYVCNV was obtained from the samples G66-69, G86, G95-97, G99, and G115. G115 was amplified with the primers Hn2F/Hn2R by PCR, and sequence analysis showed that its full-length DNA sequence shared 90.1% identity with that of AYVCNV. Additionally, both AYVCNV and PaLCuCNV were detected in G96-97 and G99.

Detection of betasatellites from samples collected in South China. Betasatellites were detected in all *A. conyzoides* plants by PCR using the abutting primer pair beta01/beta02. Only five of the 32 samples from Yunnan yielded the specific 1.3-kb fragment. The PCR fragments were cloned and then sequenced, and sequence analyses showed that *Tomato leaf curl Guangxi betasatellite* (ToLCGxB) existed in samples Y310 and Y314, *Malvastrum yellow vein Yunnan betasatellite* (MYVYNB) was detected in samples Y315 and Y342, and TbCSB was found in sample Y317 (Table 1). A previous study demonstrated that betasatellites are essential for the induction of typical yellow vein symptoms for AYVD (17) and that severe yellow vein symptoms were only produced after coinoculation with both AYVCNV and AYVCNB (21). Accordingly, the specific primer pair AYVCNBF/AYVCNBR for detection of AYVCNB was used to screen all the Yunnan samples. As a result, a specific band of approximately 1.3 kb was consistently amplified from all *A. conyzoides* plants. The amplified PCR fragments from isolates of Y274, Y282, Y342, and Y346 were randomly selected for cloning and sequencing, and sequence analyses revealed that the nucleotide sequences of betasatellites from these isolates were most closely related to AYVCNB (>86.2% nucleotide sequence identity). These data suggested that the predominant betasatellite associated with *A. conyzoides* plants in Yunnan was AYVCNB.

In an attempt to detect betasatellite components from *A. conyzoides* plant samples in Guangxi, the specific primers AYVCNBF/AYVCNBR for AYVCNB and the abutting primers beta01/beta02 were used to amplify the potential betasatellites. PCR revealed that all the samples from Guangxi were associated with AYVCNB (Table 1). The amplified PCR fragments from isolates G48, G50, G69, G86, G95, G98, and G111 were randomly selected for cloning and sequencing; sequence analyses revealed that the full sequences of betasatellites obtained from these isolates shared more than 94% nucleotide sequence identity with that of AYVCNB. The above results show that plants from Guangxi were mostly infected by PaLCuCNV/AYVCNB, and seven plants were infected by the AYVCNV/AYVCNB complex, while three plants

Table 2. Primers used in polymerase chain reaction (PCR) analysis of samples for virus detection

Primer	Sequence (5'→3') ^a	Position in genome
For virus detection		
PA	5'-TAATATTACCKGWKGVCSC-3'	2762-13
PB	5'-TGGACYTTRCAWGGBCCTTCACA-3'	544-522
AYVCNVF	5'-AAGTGGTCCCCACCACTAAC-3'	28-47
AYVCNVR	5'-ACATGGGATATGTCGTGCCTCTG-3'	555-577
Y1F1	5'-CGTAGGCCTGTGGATAAACCTCAAGAT-3'	720-746
Y6R2	5'-GGAAGCCAGTTCAAATTAAGG-3'	1736-1715
HeNfullF	5'-GGGATCCTTTACTAAACGAGTTTC-3'	141-165
HeNfullR	5'-AGGATCCCACATGTTTGACGTG-3'	148-127
TbLCYNV-F	5'-GATTGGTACCTGAGTACAATG-3'	1566-1586
TbLCYNV-R	5'-TTATCCACAGTGCTCTCTCAC-3'	2554-2534
Hn2F	5'-GTGGGATCCTCTTTTGAACGAG-3'	139-160
Hn2R	5'-AGAGGATCCCACATGTTTGATTAG-3'	150-131
Y35F	5'-CTGGATCCATTAGTAAACGAGTTTC-3'	139-161
Y35R	5'-TAGGATCCCACATAGTGGGAGTG-3'	144-122
For betasatellite detection		
beta 01	5'-GTAGGTACCACTACGCTACGCAGCAGCC-3'	1274-1298
beta 02	5'-AGTGGTACCTACCCTCCAGGGGTACAC-3'	1279-1255
AYVCNBF	5'-CCGATAGGTAATGAGACACCGATAC-3'	1037-1062
AYVCNBR	5'-GGTGTCCAATTATTTACCTTGTGAGG-3'	1013-1038

^a B = C, T, or G; K = G or T; R = A or G; S = C or G; V = A, C, or G; W = A or T; Y = C or T.

were dually infected by both PaLCuCNV and the AYVCNV/AYVCNB complex.

Infectivity and symptoms associated with either the begomovirus or the begomovirus/betasatellite complex. To ascertain the etiology of AYVD in Yunnan and Guangxi, the infectious clones AYVCNV (pBin-Hn2-1.4A), TbCSV (pBinplus-Y35-1.9A), PaLCuCNV (pBinplus-HeNZM1-1.4A), and TbLCYNV (pBinPLUS-Y143-1.5A) were agroinoculated into *N. benthamiana* and *A. conyzoides* plants either alone or in association with AYVCNB (pBinplus-Hn19-2β) or TbCSB (pBinplus-Y35β-2.0). Using *Agrobacterium*-mediated inoculation into *N. benthamiana*, AYVCNV alone was found to be incapable of inducing any symptoms, although it could be detected in inoculated plants; but when co-inoculated with AYVCNB, the severe downward leaf curling and vein thickening symptoms were observed (Fig. 1A and B). When

Fig. 1. Symptoms displayed by *Nicotiana benthamiana* plants induced with **A**, *Ageratum* yellow vein China virus (AYVCNV) alone, **B**, AYVCNV and *Ageratum* yellow vein China betasatellite (AYVCNB), **C**, Tobacco curly shoot virus (TbCSV) alone, **D**, TbCSV and AYVCNB, **E**, Papaya leaf curl China virus (PaLCuCNV) alone, **F**, PaLCuCNV and AYVCNB, **G**, TbLCYNV alone, or **H**, TbLCYNV and AYVCNB at 30 dpi.

agroinoculated with TbCSV alone, *N. benthamiana* plants produced upward leaf curling, vein thickening, and stunting symptoms; whereas severe downward leaf curling, vein thickening, and stunting symptoms were induced when co-inoculated with AYVCNB (Fig. 1C and D). PaLCuCNV alone could induce severe upward leaf curling and vein thickening symptoms, while severe downward leaf curling and vein thickening symptoms developed after 7 days postinoculation (dpi) when co-inoculated with AYVCNB (Fig. 1E and F). All the infectious clones were highly pathogenic to *N. benthamiana* plants with 100% infection rates (Table 3).

In *A. conyzoides*, AYVCNV, TbCSV, or PaLCuCNV alone (Fig. 2A, D, and G), or TbCSV and TbCSB together produced asymptomatic infections. However, *A. conyzoides* plants co-inoculated with AYVCNB and one of these begomoviruses developed yellow vein symptoms that were indistinguishable from those of naturally infected plants (Fig. 2B, E, and H). More severe yellow vein disease symptoms developed within 70 dpi (Fig. 2C, F, and I). In contrast, TbLCYNV alone or in co-inoculation with AYVCNB could not induce symptoms in *A. conyzoides*, while it induced upward leaf curling and vein thickening symptoms in *N. benthamiana* (Table 3; Fig. 1G and H).

Southern blot hybridization analysis was used to assess the accumulation of TbCSV, PaLCuCNV, AYVCNV, and AYVCNB in systemically infected leaves of inoculated plants. The accumulation of viral DNA of TbCSV and PaLCuCNV was readily detected in *N. benthamiana* plants inoculated with TbCSV or PaLCuCNV alone or during co-inoculation with AYVCNB. In *A. conyzoides* plants, the accumulation of viral DNA was detected at low levels after inoculations with TbCSV or PaLCuCNV alone, but was significantly higher in plants co-inoculated with AYVCNB. The accumulation of AYVCNB was easily detected in co-inoculated plants of both *N. benthamiana* and *A. conyzoides* (Fig. 3). AYVCNB enhanced the accumulation of viral DNA of TbCSV and PaLCuCNV in systemically infected leaves in *A. conyzoides*. The DNA accumulation levels of TbCSV and PaLCuCNV in systemically infected *N. benthamiana* plants were similar with or without AYVCNB. These results indicate that TbCSV or PaLCuCNV requires the presence of AYVCNB to induce typical yellow vein disease symptoms in *A. conyzoides* plants.

Discussion

Weeds are important intermediate hosts for geminiviruses and play a significant role in virus epidemiology in the field due to

Table 3. Infectivity and symptoms induced by different combinations of begomoviruses and betasatellites

Plant species	Inoculum	Plants infected/ plants inoculated	Symptoms ^a
<i>Nicotiana benthamiana</i>			
	AYVCNV	4/4	NS
	AYVCNV + AYVCNB	6/6	DLC, VT
	TbCSV	6/6	ULC, VT, ST
	TbCSV + TbCSB	6/6	DLC, VT, ST
	TbCSV + AYVCNB	6/6	DLC, VT, ST
	PaLCuCNV	6/6	ULC, VT
	PaLCuCNV + AYVCNB	6/6	DLC, VT
	TbLCYNV	6/6	ULC, VT
	TbLCYNV + AYVCNB	6/6	ULC, VT
<i>Ageratum conyzoides</i>			
	AYVCNV	6/6	NS
	AYVCNV + AYVCNB	26/26	YV
	TbCSV	24/26	NS
	TbCSV + TbCSB	24/25	NS
	TbCSV + AYVCNB	26/26	YV
	PaLCuCNV	25/26	NS
	PaLCuCNV + AYVCNB	26/26	YV
	TbLCYNV	0/25	NS
	TbLCYNV + AYVCNB	0/25	NS

^a DLC, downward leaf curling; VT, vein thickening; ULC, upward leaf curling; ST, stunting; YV, yellow vein; NS, no symptoms.

their survival ability and wide distribution. *A. conyzoides* is a widespread weed acting as a reservoir host for economically significant viral diseases (8,9,12). In addition to AYVB associated with AYVB, which occurs widely in *A. conyzoides* throughout south-eastern Asia (15), other begomoviruses are also progressively identified from *A. conyzoides* each year, such as the *Ageratum yellow vein Hualian virus* (AYVHuV) and the *Ageratum yellow vein Sri Lanka virus* (AYVSLV) (7). Recently, *Tomato leaf curl Java virus* (ToLCJV) and AYVV-[Java] recombinant virus named ToLCJV-Ageratum, and the associated betasatellite ToLCJV-Ageratum DNA β 03, were isolated from *A. conyzoides* in Indonesia (9). In Nepal, Pakistan, and India, *A. conyzoides* has exhibited infections by *Ageratum enation virus* (AEV) (10). In Cameroon, *A. conyzoides* was found to be infected with *Ageratum leaf curl Cameroon virus* (ALCCMV) accompanied by *Ageratum leaf curl Cameroon betasatellite* (ALCCMB) and *Ageratum leaf curl Cameroon alphasatellite* (ALCCMA) (12). AYVD is widespread in South China. AYVCNV accompanied by AYVCNB was observed in *A. conyzoides* plants in Haikou, Hainan Province (21). In addition to AYVCNV, *Ageratum leaf curl virus* (ALCV) was isolated from *A. conyzoides* in Guangxi Province (8). While begomoviruses alone have no ability to cause AYVD in *A. conyzoides* plants, only some betasatellites like AYVB, AYVCNB, or ToLCJV-Ageratum

DNA β 03 are required for the induction of yellow vein disease symptoms in *A. conyzoides* plants (9,15,19).

In this paper, 54 field-grown *A. conyzoides* plants showing yellow vein symptoms were collected in Yunnan and Guangxi provinces from 2002 to 2012, and the sequence analyses showed that they were mostly infected by TbCSV/AYVCNB or PaLCuCNV/AYVCNB complexes, rather than by an AYVCNV/AYVCNB complex. To fulfill Koch's postulates, the infectious clone assays were performed, which showed that the heterogeneous begomovirus complexes, TbCSV/AYVCNB or PaLCuCNV/AYVCNB, could induce the typically severe symptoms associated with yellow vein disease in *A. conyzoides*. PCR and Southern blot analyses confirmed that AYVCNB could interact successfully with TbCSV or PaLCuCNV in *A. conyzoides*. Therefore TbCSV/AYVCNB and PaLCuCNV/AYVCNB were the predominant complexes found in *A. conyzoides* plants collected in Yunnan and Guangxi provinces, respectively. Given its unique microclimate, Yunnan possesses the greatest biodiversity among the provinces in China. This may explain the predominance of the two begomoviruses we observed in adjacent provinces. The abutting primers beta01/beta02 were used for amplification of betasatellites in *A. conyzoides* plants, and only a few samples yielded the specific 1.3-kb fragment. When the specific primer pair AYVCNBF/AYVCNBR was used, AYVCNB

Fig. 2. Symptoms exhibited by *Ageratum conyzoides* plants induced by **A**, *Ageratum yellow vein China virus* (AYVCNV), **D**, *Tobacco curly shoot virus* (TbCSV), and **G**, *Papaya leaf curl China virus* (PaLCuCNV) alone at 50 dpi, or AYVCNV and *Ageratum yellow vein China betasatellite* (AYVCNB) at **B**, 50 dpi and **C**, 70 dpi, or TbCSV and AYVCNB at **E**, 50 dpi and **F**, 70 dpi, or PaLCuCNV and AYVCNB at **H**, 50 dpi and **I**, 70 dpi.

Fig. 3. Southern blot analysis of nucleic acid extracted from infected *Nicotiana benthamiana* and *Ageratum conyzoides* plants agroinoculated with *Ageratum yellow vein China virus* (AYVCNV), *Tobacco curly shoot virus* (TbCSV), or *Papaya leaf curl China virus* (PaLCuCNV) alone (-) or together with *Ageratum yellow vein China betasatellite* (AYVCNB) (+). Blots were probed with AYVCNV, TbCSV, and PaLCuCNV (upper) or AYVCNB (lower). Equal amount of total DNA extracts (approximately 30 µg) were loaded in each lane. Expected positions of single-stranded, supercoiled, and subgenomic DNA forms are labeled as SS, SC, and Sg, respectively.

was detected in all samples. Sequence analysis of all the betasatellites indicated that the sequence of the beta02 primer is not present in the AYVCNB genome; this can explain why the abutting primers beta01 and beta02 are not suitable for PCR amplification of AYVCNB.

Surveys in China have found that there are three types of monopartite begomoviruses. Most monopartite begomoviruses, such as TbLCYNV and PaLCCNV, do not need betasatellites to infect host plants (19,22). Many monopartite begomoviruses, such as *Tomato yellow leaf curl China virus* (TYLCCNV) and AYVCNV, are associated with betasatellites, and are completely dependent on the corresponding betasatellites for efficient infection of host plants and for symptom development (5,21). Some monopartite begomoviruses like TbCSV are associated with betasatellites, while TbCSV is capable of infecting host plants and inducing disease symptoms in the presence or absence of a betasatellite (13). Betasatellite components have a relaxed specificity for begomoviruses during their replication (14). AYVV can support the replication and systemic spread of betasatellites associated with CLCuMV, *Eupatorium yellow vein virus* (EpYVV), and *Honeysuckle yellow vein virus* (HYVV) (16). AYVCNV can interact functionally with the heterologous betasatellite components associated with TYLCCNV, TbCSV, *Malvastrum yellow vein virus* (MYVV), and *Sida yellow mosaic China virus* (SiYMCNV) (21). Hence, begomoviruses trans-replicating heterologous betasatellites is a common phenomenon in nature. AYVCNV and other similar begomoviruses have the ability to trans-replicate several heterologous betasatellites but were unable to induce AYVD with these other betasatellites in *A. conyzoides* plants in our study. This is because of unique defensive mechanisms present in *A. conyzoides*.

A. conyzoides can be infected by begomoviruses and serves as a reservoir of viruses that are also pathogenic to cultivated plant species. The AYVV/AYVB complex was reported to be an emerging disease in cultivated tomato plants, causing tomato yellow leaf curl disease (TYLCD) in Japan (2). AYVB has the ability to

associate with *Tomato yellow leaf curl virus* (TYLCV) to produce severe TYLCD, suggesting that reassortant viruses like TYLCV/AYVB can potentially cause severe diseases in vegetables and ornamental crops (18). Our data showed that AYVCNB is necessary to induce severe yellow vein disease symptoms in *A. conyzoides* and could be trans-replicated by heterogeneous TbCSV or PaLCuCNV. TbCSV, a monopartite begomovirus associated with TbCSB, which is not necessary for causing infections in host plants, is widely distributed on tobacco and tomato plants in Yunnan Province, South China (13). On the other hand, PaLCuCNV, a monopartite begomovirus lacking betasatellite DNA, occurs widely on tobacco and tomato plants in Guangxi and Henan provinces in South China (22). TbCSV and PaLCuCNV are capable of acquiring the betasatellite AYVCNB from other begomovirus to form new reassortments, and thus expand their host range and cause prevalent yellow vein disease in *A. conyzoides* plants. These new reassortments in *A. conyzoides* plants might represent original inoculum with the potential to infect cultivated tobacco and tomato crops, thereby aggravating diseases caused by TbCSV and PaLCCNV in these crops. Therefore, to control these diseases, it is more important to eliminate any wild *A. conyzoides* plants growing near cultivated plants.

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