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Malvastrum yellow vein Yunnan virus is a monopartite begomovirus

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Summary. – Nine samples of diseased *Malvastrum coromandelianum* plants collected from the fields in Yunnan province of China were found to be infected with Malvastrum yellow vein Yunnan virus (MYVYNV), when tested by PCR using specific primers. The results of PCR and Southern blot analysis showed that only 4 samples out of 9 were associated with the satellite DNAβ molecules. Their sequence analysis indicated that DNAβ molecules share 97.8%–99.4% of nucleotide sequence identities with DNAβ associated with MYVYNV isolate Y160 and less than 82.0% with those associated with other begomoviruses. Two infectious clones of MYVYNV (isolates Y160 and Y277) produced yellow vein, vein thickening and upward leaf curl symptoms in *Nicotiana benthamiana* plants. In the presence of its cognate DNAβ, the symptoms changed to downward leaf curl and crinkle. Southern blot analysis showed that DNAβ could increase accumulation of its cognate virus in the infected *N. benthamiana* plants. The above results indicated that MYVYNV is a monopartite begomovirus and its association with DNAβ is not necessary for the infection of plants, but is able to intensify symptoms specific for the disease.

Keyword: begomovirus; Malvastrum yellow vein Yunnan virus; DNAβ; satellite DNA

Introduction

Members of the genus *Begomovirus* in the family *Geminiviridae* are circular single-stranded DNA viruses that infect dicotyledonous plants. They consist of two ssDNA components designated as DNA-A and DNA-B each of about 2.7 kb (Fauquet *et al*., 2005, 2008). However, a small number of begomoviruses are monopartite lacking the component equivalent to DNA-B with all viral functions encoded by a single component homologous to DNA-A (Navot *et al*., 1991; Dry *et al*., 1993). Some monopartite begomoviruses are associated with a novel single-stranded satellite DNA molecule (DNAβ) that depends on the helper begomovirus for replication, encapsidation and movement within and between plants (Dry *et al*., 1997; Zhou *et al*., 2003a; Nawaz-ul-Rehman and Fauquet, 2009). So far, the precise function of DNAβ in the disease process has remained obscure, although evidence showed that DNAβ was required for the symptom modulation and some plant genes involved in the DNAβ-induced viral symptoms were identified (Cui *et al*., 2004; Yang *et al*., 2008; Ding *et al*., 2009).

In recent years, we have reported that several begomoviruses infected some crops and weeds in Yunnan, Guangxi and Hainan provinces (Xie *et al*., 2002b, 2003; Xie and Zhou, 2003; Zhou *et al*., 2003b; Xiong *et al*., 2005). Two begomoviruses, MYVYNV and Malvastrum yellow vein virus (MYVV) were found in *M. coromandelianum* plants in Yunnan province showing yellow vein symptoms. All MYVV isolates were found to be associated with DNAβ (Zhou *et al*., 2003b; Jiang and Zhou, 2004, 2005).

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Abbreviations: AYVV = Ageratum yellow vein virus; BYVMVV = Bhendi yellow vein mosaic virus; CLCuMV = Cotton leaf curl Multan virus; MYVV = Malvastrum yellow vein virus; MYVYNV = Malvastrum yellow vein Yunnan virus; OLCD = Okra leaf curl disease; SiLCV = Sida leaf curl virus; TbCSV = Tobacco curly shoot virus; ToLCCV = Tomato leaf curl China virus; TYLCCNV = Tomato yellow leaf curl China virus; TYLCTHV = Tomato yellow leaf curl Thailand virus

Table 1. Date of isolation and Acc. Nos. of 9 MYVYNV isolates

Isolate	Date of isolation	Acc. Nos. in GenBank	
		Partial viral sequence	DNAβ sequence
Y277	August, 2004	AJ971501	
Y278	August, 2004	AJ971502	AJ971701
Y279	August, 2004	AJ971503	
Y280	August, 2004	AJ971504	
Y304	August, 2005	AM236771	AM236776
Y305	August, 2005	AM236772	
Y306	August, 2005	AM236773	
Y307	August, 2005	AM236774	AM236777
Y308	August, 2005	AM236775	AM236778

In order to investigate molecular characterization of MYVYNV, we analyzed samples of diseased *M. coromandelianum* plants collected from the fields by using PCR and southern blot and showed that only some MYVYNV isolates were associated with DNAβ. By using agroinoculation, we also confirmed that MYVYNV was a monopartite begomovirus and its associated DNAβ was not necessary for infection, but intensified the symptoms.

Materials and Methods

Virus isolates and DNA extraction. Nine virus isolates were collected from *M. coromandelianum* plants displaying yellow vein symptoms in Baoshan, Yunnan province in 2004 and 2005 (Table 1). Total nucleic acid was extracted from symptomatic leaves (Xie *et al*., 2002b).

Detection of MYVYNV and MYVYNV DNAβ. Degenerate primers PA and PB (Table 2) were designed to amplify a part of the intergenic region and a coat protein gene of begomovirus (Xie *et al*., 2002a). The MYVYNV-specific primers (MYVYNVF, Y6R2) used in PCR-mediated detection of MYVYNV are listed in Table 2.

Abutting primers β01 and β02 were used to amplify the full-length DNAβ by PCR (Cui *et al*., 2004). The PCR products were cloned into pMD 18-T vector (Takara) and sequenced using the automated 3730 DNA sequencing system (Perkin Elmer).

Sequence analysis. Sequences were assembled and analyzed by DNAStar (DNASTAR Inc.) and DNAMAN Version 5.22 software (Lynnon Biosoft). Acc. Nos. of begomovirus DNAβ sequences in the GenBank were: Ageratum yellow vein virus (AYVVβ; AJ252072), Bhendi yellow vein mosaic virus (BYVMVVβ; AJ308425), Okra leaf curl disease (OLCDβ01-PAK; AJ316029), Cotton leaf curl Multan virus (CLCuMVβ01; AJ292769), MYVV-[Y250] (MYVVβ-[Y250]; AJ971704), MYVVβ-[Y206] (AJ744882), MYVVβ-[Y189] (AJ971459), MYVYNV-[Y160] (MYVYNVβ-[Y160]; AJ786712), Sida leaf curl virus (SiLCVβ; AM050733), Tobacco curly shoot virus (TbCSVβ; AJ457822), Tomato leaf curl China virus (ToLCCVβ; AJ704615), Tomato yellow leaf curl China virus (TYLCCNVβ; AJ971334), Tomato yellow leaf curl Thailand virus (TYLCTHVβ; AJ566746).

Construction of infectious clones of MYVYNV and DNAβ. The full-length genomic DNA of MYVYNV isolate Y160 (MYVYNV-[Y160]) was amplified from a MYVYNV-infected *M. coromandelianum* plant using primers Y160SacF and Y160SacR that overlapped at the unique *Sac*I site and cloned into the pGEM-T easy vector (Promega) to produce pGEMT-Y160-1A. The pGEMT-Y160-1A plasmid was digested with *Sac*I and *Xba*I and the resulting 2.2 kb fragment (0.8 copy of

Table 2. Sequence of used primers

Primer	Nucleotide sequence (5'-3')[a]	Position in viral DNA or DNAβ[b]
Detection and amplification of MYVYNV		
PA	TAATATTACCKGWKGVCCSC	2738–13
PB	TGGACYTTRCAWGGBCCTTCACA	518–494
MYVYNVF	CACTAACTACTGTCTGCCA	35–53
Y6R2	GGAAGCCAGTTCAAATTAAAGG	1738–1717
Construction of infectious clone of MYVYNV		
Y160SacF	GAGCTCTCCGAAGGTTGTAGTTG	1285–1307
Y160SacR	GAGCTCTGGGAGTGCACAAGTGT	1290–1268
Y277EcoRF	GAATTCTTTATAACTGCTGTTGG	1654–1676
Y277EcorR	GAATTCCTCGACGAGGACAAG	1659–1639
Amplification of fragment as probe for MYVYNV		
Y160P/F	TTCTATTTTGCGTTCCAGGA	252–271
Y160P/R	AAGTACAGCACAGGGAAGCG	1548–1529
Cloning and sequencing of MYVYNV DNAβ		
β01	GTAGGTACCACTACGCTACGCAGCAGCC	1290–1314
β02	AGTGGTACCTACCCTCCCAGGGGTACAC	1289–1265

[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; [b]nucleotide 1 is defined as the eighth nucleotide in the conserved TAATATTAC sequence, present at the origin of replication of all geminiviruses.

full-length virus genomic DNA, 0.8A) was introduced into the binary vector pBinPLUS to produce pBinPLUS-Y160-0.8A (van Engelen *et al.*, 1995). The product of the pGEMT-Y160-1A plasmid digested with *Sac*I (one copy of the full-length virus genomic DNA, 1A) was inserted into pBinPLUS-Y160-0.8A that was also digested with *Sac*I to produce pBinPLUS-Y160-1.8A. Based on same strategy, we constructed infectious clone of MYVYNV isolate Y277 (MYVYNV-[Y277]). Using Y277EcoRF and Y277EcoRR that overlapped at the unique *Eco*RI site, we obtained the full-length genomic DNA of MYVYNV isolate Y277 and cloned it into the pGEM-T easy vector to produce pGEMT-Y277-1A. The pGEMT-Y277-1A plasmid was double-digested with *Sac*I and *Eco*RI and single-digested with *Eco*RI and the resulting 2.3 kb fragment (0.8A) and the full-length genomic DNA were introduced into pBinPLUS tandem to produce pBinPLUS-Y277-1.8A. Infectious clone of MYVYNV-[Y160] DNAβ (pBinPLUS-Y160-2β) was constructed as described (Zhou *et al.*, 2003a). The fragment 0.8A was inserted into pBinPLUS-Y160-2β that was digested with *Sac*I and *Xba*I to produce pBinPLUS-Y160-0.8A-2β. A product of pGEMT-1A digested with *Sac*I (1A) was inserted into pBinPLUS-Y160-0.8A-2β to obtain pBinPLUS-Y160-1.8A-2β.

Agroinoculation of plants. Clones pBinPLUS-Y160-1.8A, pBinPLUS-Y277-1.8A, pBinPLUS-Y160-2β, and pBinPLUS-Y160-1.8A-2β were conjugated into *Agrobacterium tumefaciens* strain EHA105 by triparental mating. *A. tumefaciens* cultures were grown at 28°C for 48 hrs and inoculated with a syringe into the stems or petioles of *N. benthamiana, N. tabacum*, *N. glutinosa*, *Solanum lycopersicum*, *Petunia hybrida,* and *M. coromandelianum* plants at the stage of four to six leaves. After agroinoculation, the plants were kept in an insect-free chamber maintained at 25°C with supplementary lighting to give a 16 hrs photoperiod.

Southern blot analysis of viral DNA in inoculated plant. Total nucleic acids were extracted from leaves of systemically infected plant of *N. benthamiana* 30 days post-inoculation by the cetyltrimethylammonium bromide (CTAB) method and electrophoresed on 1% agarose gels in TBE buffer (90 mmol/l Tris-borate, 2 mmol/l EDTA) (Zhou *et al.*, 2001). DNA was transferred to the nylon membranes Hybond-N+ (Amersham Biosciences) by capillary blotting and hybridized to the labeled probes as described previously (Huang *et al.*, 2009). Membranes were hybridized to digoxigenin-labeled probes specific for MYVYNV-[Y160] produced by PCR-mediated amplification using primers Y160P/F and Y160P/R or MYVYNV-[Y160] DNAβ amplified by β01 and β02 with a Prime-a-Gene® labeling system kit according to the manufacturer's instructions (Promega).

Results and Discussion

DNAβ was not associated with all MYVYNV isolates

PCR-mediated detection using primers MYVYNVF and Y6R2 revealed that 9 virus isolates (Y277, Y278, Y279, Y280, Y304, Y305, Y306, Y307, and Y308) collected from *M. coromandelianum* plants were infected with MYVYNV. Partial DNA-A sequences of 500 bp fragments amplified by PCR using the degenerate primer pair PA/PB were determined

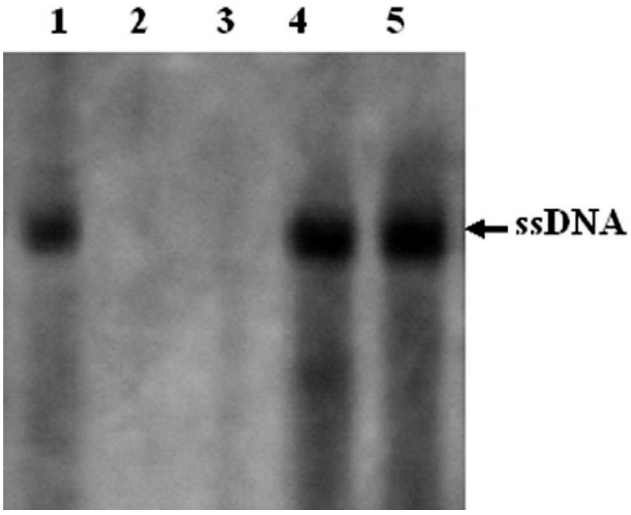

Fig. 1

Detection of DNAβ in selected MYVYNV isolates by Southern blot analysis

DNA was extracted from isolates Y304 (lane 1), Y305 (lane 2), Y306 (lane 3), Y307 (lane 4), and Y308 (lane 5). The position of single-stranded (ssDNA) is indicated.

(Table 1). Comparison of 500 bp fragments showed that viral DNA of 9 isolates had a higher nucleotide (nt) sequence identity (94.4% to 99.6%) with MYVYNV-[Y160] (Acc. No. AJ971500) than with other begomoviruses (less than 88.0%). These results indicated that 9 examined samples were infected with MYVYNV. When the samples were PCR-amplified with primers β01 and β02, a band of approximately 1.3 kb was consistently amplified from 4 samples (Y278, Y304, Y307, and Y308), but not from the remaining 5 samples (Y277, Y279, Y280, Y305, and Y306). Southern blot hybridization was also used for the detection of DNAβ presence in the examined samples. The results revealed that isolates Y304, Y307, and Y308 were associated with DNAβ molecule, but the isolates Y305 and Y306 were not (Fig. 1). Both methods, e.g. Southern blot analysis and PCR demonstrated that only some isolates of MYVYNV contained DNAβ.

The complete nucleotide sequences of DNAβ molecules from 4 MYVYNV isolates showed that they were 1350–1355 nt in length (Table 1). The 4 DNAβ molecules were similar to MYVYNVβ-[Y160], contained an A-rich region (about 56.7%) and satellite conserved region of 115 nt which contained conserved nonanucleotide sequence TAATATTAC in the loop of a putative stem-loop structure shared by all begomoviruses. Moreover, all of the 4 DNAβ molecules had a potential to encode βC1 protein (118 aa) from the complementary-sense DNA located between nts 802–1158. Comparison of the 4 DNAβ sequences of isolates Y278,

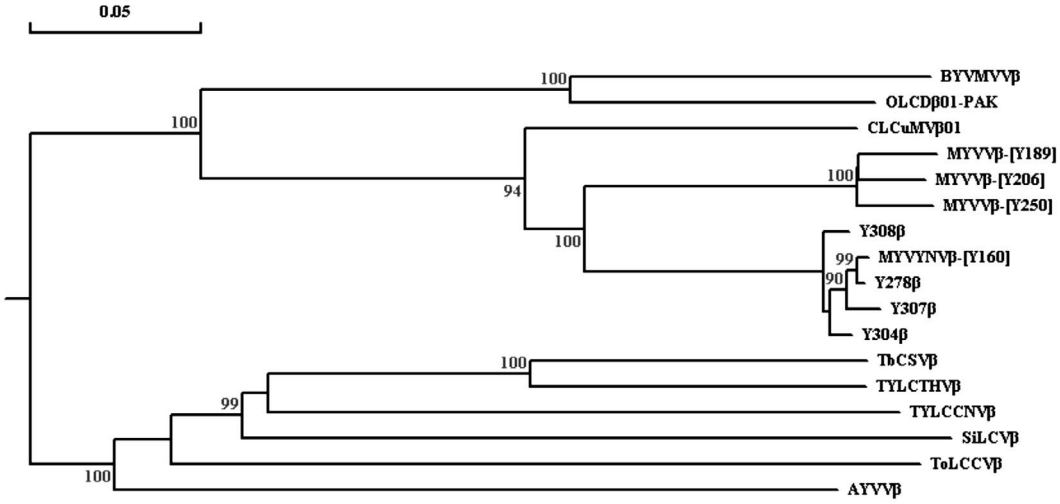

Fig. 2

Phylogenetic tree of DNAβ molecules based on an alignment of full-length sequences

The tree was generated using neighbor-joining method. Horizontal distances are proportional to the sequence distances (see scale bar), vertical distances are arbitrary. The numbers at each branch indicate bootstrap percentage value.

Y304, Y307, and Y308 with other begomoviruses showed that they had higher sequence identities (97.8%–99.4%) with MYVYNVβ-[Y160] than other begomovirus-associated DNAβs. The βC1 protein in 4 isolates (Y278, Y304, Y307, and Y308) also shared high amino acid (aa) sequence identities (95.3%–95.8%) with MYVYNVβ-[Y160], while less than 78.0% identity was found with other begomoviruses. The phylogenetic analysis was consistent with the idea that 4 DNAβs were satellite molecules associated with MYVYNV (Fig. 2).

Infectivity of MYVYNV and its associated DNAβ

In the plants *N. benthamiana* inoculated by *Agrobacterium*-MYVYNV-[Y160] was found upward leaf curl, yellow vein and vein thickening symptoms (Fig. 3b,c,d) in comparison with healthy plants (Fig. 3a). Co-inoculation of *N. benthamiana* with MYVYNV-[Y160] and MYVYNV-[Y160] DNAβ produced downward leaf curl and crinkle symptoms, while no symptoms were induced in *N. tabacum*, *N. glutinosa*, *S. lycopersicum*, *P. hybrida,* and *M. coromandelianum* plants (Fig. 3e,f). Obviously, MYVYNV-[Y160] and MYVYNV-[Y160] DNAβ were detected by PCR using primer pairs Y160P/F and Y160P/R or β01 and β02 in *N. benthamiana,* but not in *N. tabacum*, *N. glutinosa*, *S. lycopersicum*, *P. hybrida*, and *M. coromandelianum* plants (data not shown). When MYVYNV-[Y160] and DNAβ were present on the same binary vector pBinPLUS (pBinPLUS-Y160-1.8A-2β) with the purpose to keep a ratio of viral DNA and DNAβ identical in all infected cells, disease symptoms were not found in *N. tabacum*, *N. glutinosa*, *S. lycopersicum*, *P. hybrida*, and *M. coromandelianum* plants. Nevertheless, PCR detection confirmed that these plants were not infected by MYVYNV-[Y160].

In order to determine an effect of different MYVYNV isolates on the induction of symptoms, we constructed the infectious clone of MYVYNV isolate Y277 that was not associated with DNAβ in the field. Agroinoculation of Y277 infectious clone showed that MYVYNV-[Y277] like as MYVYNV-[Y160] was able to infect *N. benthamiana* plant and induce upward leaf curl, yellow vein and vein thickening symptoms, but was not able to infect *N. tabacum*, *N. glutinosa*, *S. lycopersicum*, *P. hybrida*, and *M. coromandelianum* (Fig. 3g,h). Co-inoculation of MYVYNV-[Y277] and MYVYNV-[Y160] DNAβ induced downward leaf curl and crinkle symptoms in *N. benthamiana* (Fig. 3i), while any symptoms in *N. tabacum*, *N. glutinosa*, *S. lycopersicum*, *P. hybrida*, and *M. coromandelianum* were induced. MYVYNV-[Y277] and MYVYNV-[Y160] DNAβ were detected by PCR using primer pairs Y277EcoRF and Y277EcoRR or β01 and β02 in *N. benthamiana,* but not in *N. tabacum*, *N. glutinosa*, *S. lycopersicum*, *P. hybrida*, and *M. coromandelianum* plants (data not shown).

Thirty days after agroinoculation with MYVYNV-[Y160] alone or simultaneously with MYVYNV-[Y160] DNAβ, Southern blot hybridization analysis was used to detect nucleic acids extracted from the infected *N. benthamiana* leaves. The MYVYNV probe detected viral DNA in the plants

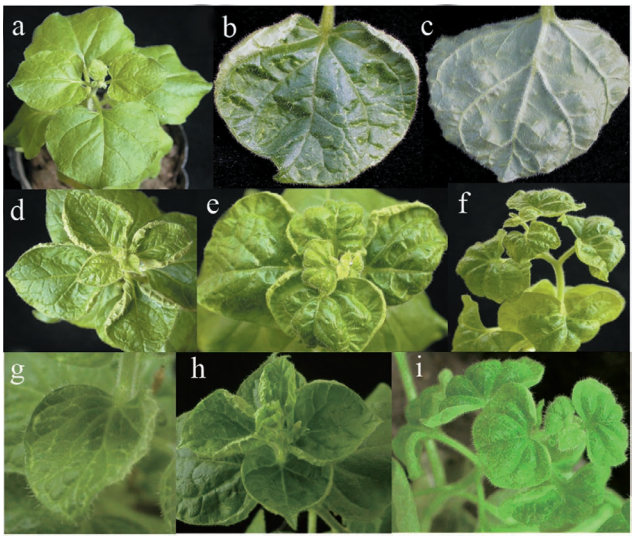

Fig. 3

Symptoms induced by MYVYNV-[Y160], MYVYNV-[Y277], and MYVYNV-[Y160] DNAβ on *N. benthamiana* plants

Healthy plant (a), yellow vein, vein thickening and upward leaf curl symptoms induced by MYVYNV-[Y160] alone (b,c,d), downward leaf curl and crinkle symptoms induced by MYVYNV-[Y160] and MYVYNV-[Y160] DNAβ (e,f), yellow vein and upward leaf curl symptoms induced by MYVYNV-[Y277] alone (g,h), downward leaf curl and crinkle symptoms induced by MYVYNV-[Y277] and MYVYNV-[Y160] DNAβ (i).

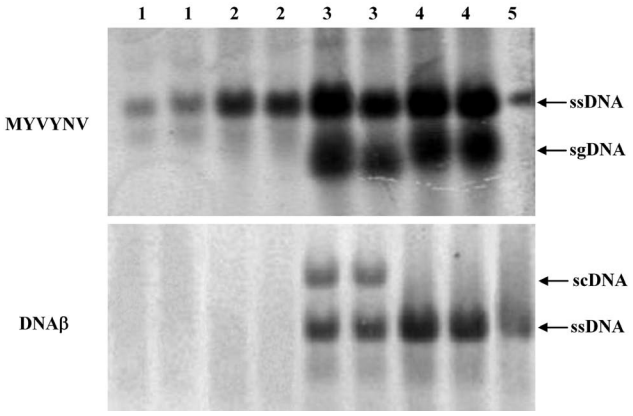

Fig. 4

Detection of viral DNA in *N. benthamiana* plants agroinoculated with MYVYNV-[Y160] alone (lanes 1, 2) or together with DNAβ (lanes 3, 4)

Template DNA (lane 5). ssDNA = single-stranded DNA, sg DNA = subgenomic DNA, scDNA = supercoiled DNA.

agroinoculated with MYVYNV-[Y160] alone confirming that MYVYNV-[Y160] was able to infect *N. benthamiana* systemically. Both MYVYNV and DNAβ were detected in *N. benthamiana* plants co-inoculated with MYVYNV-[Y160] and MYVYNV-[Y160] DNAβ (Fig. 4). Comparison of MYVYNV accumulation level indicated that *N. benthamiana* plants co-inoculated with MYVYNV-[Y160] and DNAβ accumulated more MYVYNV than those infected with MYVYNV-[Y160] alone indicating that MYVYNV-[Y160] DNAβ was able to increase the accumulation of MYVYNV-[Y160] (Fig. 4).

MYVYNV was reported to be most likely a monopartite begomovirus (Jiang and Zhou, 2005). Here, we demonstrated that only some of the MYVYNV isolates were associated with DNAβ. Previously, we demonstrated that TbCSV was associated with DNAβ in some strains (Li *et al.*, 2005). Currently, MYVYNV is the second example of such observation. Agroinoculation of two infectious clones of MYVYNV (isolate Y160 and Y277) showed that MYVYNV alone was able to induce a yellow vein, vein thickening and upward leaf curl symptom in *N. benthamiana*. In the presence of MYVYNV DNAβ, the symptoms changed to downward leaf curl and crinkle indicating that DNAβ associated with MYVYNV was not necessary for infection, but was able to intensify the symptoms. A similar observation was reported for TbCSV that showed upward leaf curling in *N. benthamiana* and co-inoculation of TbCSV with cognate DNAβ produced severe downward curling of the leaves (Li *et al.*, 2005). MYVYNV in the absence of DNAβ was capable of the induction of disease symptoms and virus accumulation in the plants, at least in *N. benthamiana*. This conclusion was supported by the field data that only a small proportion of MYVYNV isolates contained DNAβ molecules.

In China, three types of monopartite begomoviruses were characterized by their mode of association with cognate DNAβ. For TYLCCNV, all tested virus isolates were associated with DNAβ molecules (Cui *et al.*, 2004). For the second type of monopartite begomovirus such as TbCSV, DNAβ molecules were associated only with some isolates (Li *et al.*, 2005). The third type of monopartite begomoviruses exampled by Stachytarpheta leaf curl virus was not associated with DNAβ molecule (Xiong *et al.*, 2005). Definitely, MYVYNV could be classified into the second group together with the isolates that were not always associated with a satellite component.

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