European Journal of Plant Pathology (2006) 115: 369–375
DOI 10.1007/s10658-006-9021-8

Agroinoculation shows *Tobacco leaf curl Yunnan virus* is a monopartite begomovirus

Yan Xie, Tong Jiang, and Xueping Zhou*
*Institute of Biotechnology, Zhejiang University, Hangzhou, 310029, P.R. China *Author for Correspondence (Fax: +86-571-86971680; E-mail: zzhou@zju.edu.cn)*

Accepted 1 May 2006

Key words: begomovirus, tobacco leaf curl Yunnan virus, infectivity

Abstract

We demonstrated that only 2 out of 15 isolates of *Tobacco leaf curl Yunnan virus* (TbLCYNV) were associated with the satellite DNAβ molecules. To investigate the infectivity of this virus, an infectious clone of TbLCYNV isolate Y143 (TbLCYNV-Y143) was agroinoculated or whitefly transmitted into *Nicotiana benthamiana*, *N. glutinasa*, *Petunia hybrida* and *N. tabacum*. TbLCYNV-Y143 alone was able to induce severe upward leaf curling, vein thickening or stunt symptoms in these plants. Co-inoculation of TbLCYNV-Y143 with DNAβ molecules associated with other begomoviruses induced similar symptom types on these plants. This indicates that TbLCYNV is a monopartite begomovirus. The relevance of results that only two isolates of TbLCYNV were associated with DNAβ molecules is discussed.

Introduction

Geminiviruses (family *Geminiviridae*) are a large and diverse group of plant viruses characterized by their unique twinned particles. They have circular, single-stranded DNA genomes that replicate via double-stranded DNA intermediates in plant cell nuclei (Hanley-Bowdoin et al., 1999). The majority of the economically important geminiviruses belong to the genus *Begomovirus*, which cause a number of serious diseases of crop plants in tropical and subtropical regions of at least 39 countries (Moffat, 1999; Harrison and Robinson, 1999). Begomoviruses are transmitted by the whitefly *Bemisia tabaci*, and typically have a bipartite genome, referred to as DNA-A and DNA-B, both of which are required for systemic infection. However, a small number of begomoviruses, such as *Tomato leaf curl virus* (ToLCV, Dry et al., 1993) and *Tomato yellow leaf curl virus* (TYLCV, Navot et al., 1991), have only a single genomic component, resembling the DNA-A of the bipartite begomoviruses. The single genomic DNA of these begomoviruses is sufficient for maintenance of the disease. Recently, some monopartite begomoviruses were found to be associated with a novel single-stranded DNA satellite, termed DNAβ (Saunders et al., 2001, 2003; Briddon et al., 2001; Zhou et al., 2003). Without satellite DNAβ, these begomoviruses are either not infectious or poorly infectious to their natural hosts.

In recent years, several begomoviruses infecting squash, tobacco and tomato crops have been reported in China (Zhou et al., 2001, 2003;Yin et al., 2001; Xie et al., 2002b; Xie and Zhou, 2003), and whitefly-transmitted begomovirus disease outbreaks have occurred in tobacco, tomato and papaya in Yunnan and Guangxi provinces (Zhou et al., 2003). *Tobacco leaf curl Yunnan virus* (TbLCYNV) was first isolated in Yunnan, China, and it was suggested to be a monopartite begomoviruses without DNA-B (Zhou et al., 2001). Two TbLCYNV isolates were found to be associated with two types of DNAβ molecules (Cui et al., 2004). In the present study, we confirmed that only two isolates were associated with

DNAβ molecules among 15 TbLCYNV-infected samples. TbLCYNV alone was sufficient for symptom induction, and co-inoculation with the DNAβ molecule induced similar symptom types.

Materials and methods

Virus sources and DNA extraction

From 1999 to 2003, 215 naturally infected *Nicotiana tabacum* and *Lycopersicon esculentum* plants, showing leaf curling, curly shoot, vein thickening and stunt symptoms, were collected from fields in different districts of Yunnan province, China. Nucleic acid samples were obtained from plant leaves by method A of Harrison et al. (1997).

*Detection of TbLCYNV and DNA*β

Specific primers for PCR-mediated detection of TbLCYNV and DNAβ were designed based on the previously published sequences of these components (Zhou et al., 2001; Xie et al., 2002a), TbLCYNV specific primers (TbLCYNVF, 5′- AA TAACCGCGCAGCGGCATGCCTT-3′; TbLCY NVR, 5′-GTRYACGGTTTVGRGAACTC-3′, R = A or G, Y = C or T, V = A, C or G) and DNAβ specific primers beta01 (5′-GGTACCAC-TACGCTACGCAGCAGCC-3′) and beta02 (5′-GGTACCTACCCTCCCAGGGGTACAC-3′) were designed to amplify fragments about 0.9 kb and 1.3 kb in size, respectively. PCR reactions were done as described by Li et al., (2005).

Sequence determination and analysis

The complete nucleotide sequences of TbLCYNV isolates Y136 and Y143 were determined as described (Zhou et al., 2001). Sequence data were assembled and analyzed with the aid of DNAStar (DNASTAR Inc., Madison, USA) and DNAMAN software packages (Lynnon Biosoft, Quebec, Canada).

Construction of infectious clones

Based on the determined complete nucleotide sequence of TbLCYNV isolate Y143 (TbLCYNV-Y143), a full-length copy of the genome was amplified using specific primers Y143F (5′-TCA GGTACCTGAGTACAATG-3′) and Y143R (5′-TCTGGTACCAATCAAAGTGC-3′) which overlap at a unique *Kpn*I (underlined) site, and the 2.7 kb PCR product was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) to produce pGEM-1A. After confirmation by sequencing with the automated DNA sequencing system (Model 377, Perkins Elmer, Wellesley, MA, USA), a *Bam*HI-*Kpn*I digested fragment of pGEM-1A including the intergenic region was introduced into the binary vector pBinPLUS (Van Engelen et al., 1995) to produce pBinPLUS-0.5A. The full-length *Kpn*I-digested fragment of pGEM-1A was inserted into the unique *Kpn*I site of pBinPLUS-0.5A to produce pBinPLUS-1.5A, yielding a 1.5-mer tandem repeat of TbLCYNV-Y143. The infectious clones of DNAβ associated with *Tomato yellow leaf curl Thailand virus* (TYLCTHV-Y72β) were produced using the method described by Zhou et al. (2003). The infectious clones of DNAβ associated with *Tomato yellow leaf Curl China virus* (TYLCCNV-Y10β), *Tobacco curly shoot virus* (TbCSV-Y35β) or *Malvastrum yellow vein virus* (MYVV-Y47β) were previously constructed in our lab (Zhou et al., 2003; Li et al., 2005; Xiong et al., 2005). These binary vectors were introduced into *Agrobacterium tumefaciens* stain EHA105 by triparental mating.

Agroinoculation of plants

Agrobacterium tumefaciens cultures were grown at 28 °C for 48 h (OD_{550} = 1). *N. benthamiana*, *N. glutinosa*, *N. tabacum* Samsum and *Petunia hybrida* plants were agro-inoculated either with TbLCYNV-Y143 alone or together with other DNAβ molecules. A 21-gauge needle was used to inject 0.2 ml of bacterial cultures into stems or petioles of plants at 4–6 leaf stages. Inoculated plants were grown in an insect-free cabinet with supplementary lighting to give a 16 h day.

Whitefly transmission assay

Whitefly transmission was tested as described by Nateshan et al. (1996).

Analysis of viral DNA in inoculated plants

Total DNA was isolated from young leaves of systemically infected *N. benthamiana* plants using

the CTAB method (Reichardt and Roger, 1995). Nucleic acids were fractionated by 1% agarose gel electrophoresis in TBE buffer (90 mM Tris–borate, 2 mM EDTA, pH8.3), and transferred to Hybond-N+ membranes (Amersham Biosciences, Buckinghamshire, England) after alkali denaturation and neutralization, and then hybridized to [α^{32}P] dCTP-labelled probes. A probe specific for TbLCYNV-Y143 was produced by *Bam*HI–*Kpn*I digestion of pGEM-1A and labelling the fragment using Prime-a-Gene Labelling kit (Promega). A DNAβ specific probe was produced by PCR amplification of an approximately 150 nucleotide fragment from the satellite conserved region (SCR) of TYLCCNV-Y10β (Zhou et al., 2003) using primers βSCR1 (5′-GTCGACATTGAGGGCCT TCCGGTCATC-3′) and βSCR2 (5′-TCTAGAT GTGGGACCCACCTCAACG-3′).

Results

DNAβ is only associated with two TbLCYNV isolates

TbLCYNV isolates Y136 and Y143 were found to be associated with two types of DNAβ molecules (Cui et al., 2004). To determine if other TbLCYNV isolates were also associated with DNAβ molecules, TbLCYNV was screened by PCR with specific primers TbLCYNVF and TbLCYNVR among 215 DNA samples from tobacco and tomato plants showing begomovirus-like symptoms collected from Yunnan province. Ten DNA samples from tobacco including isolates Y136 and Y143 and 5 from tomato were found to be infected by TbLCYNV (Table 1). PCR-mediated amplification using the specific DNAβ primer pair beta01 and beta02 showed none of these TbLCYNV isolates was associated with DNAβ except for isolates Y136 and Y143. The absence of DNAβ in most TbLCYNV isolates was also confirmed by Southern blot analysis with a probe of SCR of DNAβ (data not shown). The results show that all the TbLCYNV samples except for isolates Y136 and Y143 were not associated with DNAβ molecules.

Infectivity of TbLCYNV

Sequence analysis showed the complete sequence of Y136 and Y143 were both 2750 nucleotides (nts) (AJ512761-62), having the typical genome organization of *Begomovirus*. The complete nucleotide sequences of Y136 and Y143 had the highest identity (98–99%) with TbLCYNV isolate Y3 (Zhou et al., 2001), and shared less than 82% identity with other begomoviruses. To investigate infectivity of this virus, an infectious clone of TbLCYNV-Y143 was constructed and its infectivity was tested. In inoculated *N. benthamiana* plants, upward leaf curling symptoms developed at 16 days post inoculation (dpi), followed by severe upward leaf curling, and vein thickening symptoms (Figure 1A). In *P. hybrida* plants, leaves

Table 1. PCR detection of TbLCYNV and DNAβ in naturally infected plants collected from Yunnan province, China

Samples	Plant species	Symptoms	Time	Location	TbLCYNV	DNAβ
Y3	Tobacco	LC, VT, ST	1999.3	Baoshan	+	−
Y4	Tobacco	LC, VT, ST	1999.3	Baoshan	+	−
Y118	Tobacco	CS	2002.1	Baoshan	+	−
Y120	Tobacco	CS, ST	2002.1	Baoshan	+	−
Y123	Tobacco	LC, VT	2002.1	Baoshan	+	−
Y125	Tobacco	LC, VT	2002.1	Baoshan	+	−
Y131	Tobacco	CS	2002.1	Baoshan	+	−
Y133	Tobacco	CS, LC, VT	2002.1	Baoshan	+	−
Y136	Tobacco	CS, LC	2002.1	Baoshan	+	+
Y143	Tobacco	LC, ST	2002.1	Baoshan	+	+
Y161	Tomato	LC, VT, ST	2003.3	Baoshan	+	−
Y211	Tomato	YLC	2003.11	Yuxi	+	−
Y212	Tomato	YLC	2003.11	Yuxi	+	−
Y213	Tomato	YLC	2003.11	Yuxi	+	−
Y215	Tomato	YLC	2003.11	Yuxi	+	−

CS, LC, ST, VT and YLC were curly shoot, leaf curl, stunt, vein thickening and yellow leaf curl, respectively.

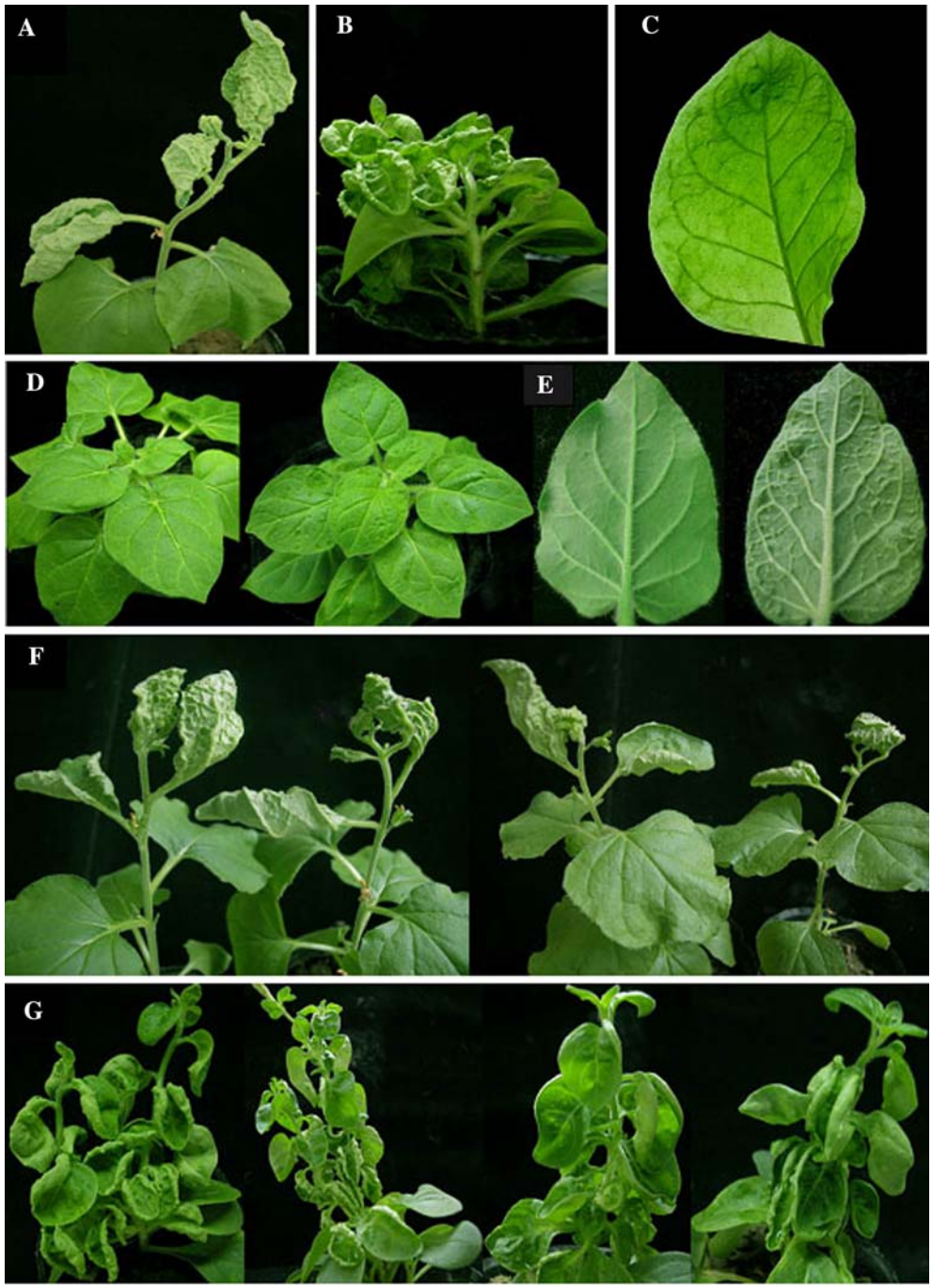

Figure 1. Symptoms induced by TbLCYNV-Y143 alone or in the presence of distinct DNAβ molecules. (A, B) Symptoms in *N. benthamiana* (A) or *P. hybrida* (B) by TbLCYNV-Y143 at 30 days after agro-inoculation. (C) Symptoms in *N. tabacum* Samsum by TbLCYNV-Y143 at 40 days after *Bemisia tabaci* transmission. (D, E) Healthy (left) or TbLCYNV-Y143 infected (right) plant (D) or single leaf (E) of *N. glutinosa* at 30 days after agro-inoculation. (F, G) Symptoms induced in *N. benthamiana* (F) or *P. hybrida* (G) at 30 days after agro-inoculation. From left to right, plants were infected with TbLCYNV-Y143 and TYLCCNV-Y10β, TYLCTHV-Y72β, TbCSV-Y35β, or MYVV-Y47β, respectively.

exhibited severe upward leaf curling and stunt symptoms (Figure 1B). In *N. glutinosa*, only slight leaf curling was observed in inoculated plants, but vein thickening symptoms were easily observed on the underside of the leaves at 21 dpi (Figure 1, D and E). All the inoculated *N. benthamiana*, *N. glutinosa* and *P. hybrida* plants produced symptoms. In *N. tabacum*, TbLCYNV-Y143 did not exhibit any symptoms and the virus was not detected by PCR using primers TbLCYNVF and TbLCYNVR, indicating it could not infect this plant by agroinoculation. Whitefly transmission tests were then done using agroinoculated *N. glutinasa* plants as the virus source. Vein thickening and leaf curling symptoms were observed 3 weeks after whitefly inoculation (Figure. 1C).

Interaction of TbLCYNV and DNAβ

Two types of DNAβ molecules, related to TYLCCNV and TYLCTHV, were found to be associated with TbLCYNV-Y143. Therefore the infectious clone of TYLCCNV DNAβ (TYLCCNV-Y10β) or TYLCTHV DNAβ (TYLCTHV-Y72β) was co-inoculated with TbLCYNV-Y143 by agroinoculation. The symptoms induced by co-inoculation of TbLCYNV-Y143 with TYLCCNV-Y10β or TYLCTHV-Y72β were nearly identical to those induced by TbLCYNV-Y143 alone in *N. benthamiana*, *N. glutinosa* and *P. hybrida* plants, but symptoms appeared 3–4 days earlier in the presence of DNAβ (Figure 1F and G). These results indicated that DNAβ had no effect on symptom induction by TbLCYNV-Y143, although it was found to be associated with these two types of DNAβ molecules in nature.

To determine if other DNAβ molecules had an effect on symptom induction by TbLCYNV-Y143, infectious clones of DNAβ associated with TbCSV (TbCSV-Y35β) or MYVV (MYVV-Y47β) were co-inoculated with TbLCYNV-Y143. All the co-inoculated *N. benthamiana*, *N. glutinosa* and *P. hybrida* plants developed similar symptoms to those induced by TbLCYNV-Y143 alone (Figure 1, F and G).

The relative amounts of TbLCYNV-Y143 and DNAβ in inoculated plants were compared by Southern blot hybridization. In co-inoculated *N. benthaminana* plants, all the DNAβ molecules could be detected in systemically symptomatic leaves (Figure 2). However, the DNAβ concentration in plants co-inoculated with TbLCYNV-Y143 and MYVV DNAβ was far higher than that in plants co-inoculated with the other three DNAβ molecules, indicating that the efficiency for replication of MYVV DNAβ by TbLCYNV-Y143 was higher than that of other DNAβ molecules. The concentration of TbLCYNV-Y143 in plants

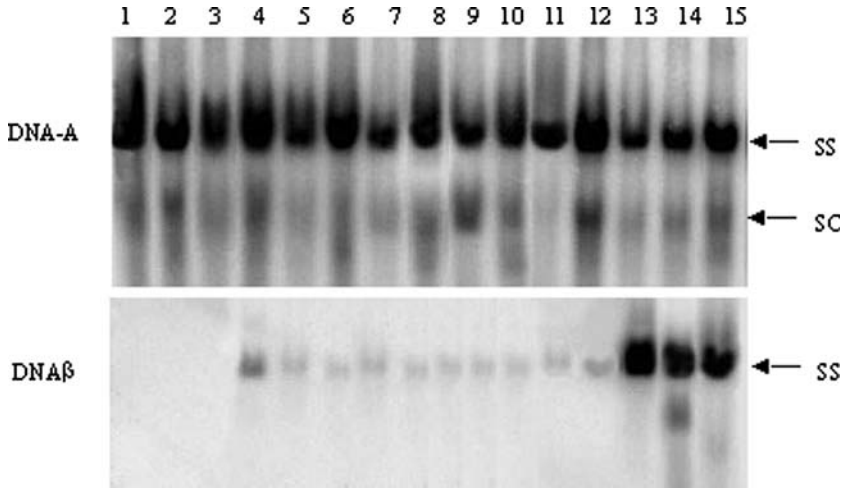

Figure 2. Southern blot analysis of nucleic acids extracted from infected *N. benthamiana* plants agro-inoculated with TbLCYNV-Y143 (lanes 1–3), TbLCYNV-Y143 and TYLCCNV-Y10β (lanes 4–6), TbLCYNV-Y143 and TYLCTHV-Y72β (lanes 7–9), TbLCYNV-Y143 and TbCSV-Y35β (lanes 10–12), TbLCYNV-Y143 and MYVV-Y47β (lanes 13–15) at 35 days after inoculation. The blots were probed with either viral DNA (upper) or DNA β (lower). Approximately equal amounts (10 μg) of nucleic acids were loaded in each lane. The positions of single-stranded (ss) and supercoiled (sc) DNA forms are indicated.

inoculated with TbLCYNV-Y143 alone was similar to that co-inoculated with TbLCYNV-Y143 and DNAβ suggesting that viral DNA accumulation is unaffected by the presence or absence of DNAβ.

Discussion

In our previous study, TbLCYNV isolates Y136 and Y143 were both shown to contain DNAβ molecules and the two isolates were associated with two types of DNAβ molecules (Cui et al., 2004). Meanwhile, TbLCYNV isolates Y3 and Y161 were not associated with DNAβ molecules (Li et al., 2004). Here we further demonstrate that all the TbLCYNV samples except for isolates Y136 and Y143 are not associated with DNAβ molecules, suggesting that TbLCYNV is not associated with DNAβ molecules in nature. In order to elucidate whether TbLCYNV requires DNAβ molecules for induction of typical symptoms in host plants, an infectious clone of TbLCYNV isolate Y143 was constructed. Agro-inoculation or whitefly transmissions showed that TbLCYNV-Y143 alone was able to induce severe upward leaf curling, vein thickening or stunt symptoms in *N. benthamiana*, *N. glutinasa*, *P. hybrida* and *N. tabacum* plants and co-inoculation of TbLCYNV-Y143 with DNAβ molecules associated with other begomoviruses induced similar symptom types on these plants. These results indicate that TbLCYNV is a truly monopartite begomovirus and that the previously reported association with DNAβ molecules might have occurred by chance. This probably arose from mixed infections of the samples with other monopartite begomovirus DNAβ molecules by whitefly transmission.

Since the discovery of DNAβ associated with the monopartite begomoviruses AYVV (*Ageratum yellow vein virus*, AYVV) from Singapore and CLCuMV (*Cotton leaf curl Multan virus*, CLCuMV) from Pakistan (Saunders et al., 2000; Briddon et al., 2001), an increasing number of such begomovirus/DNAβ disease complexes have been found to infect numerous economically important plants in different geographical regions (Mansoor et al., 2003). In China, three categories of monopartite begomoviruses have been characterized. In the first category, exemplified by TYLCCNV, all virus isolates were associated with DNAβ molecules. In the second category, represented by TbCSV, only some isolates were associated with DNAβ molecules. In the third category, exemplified by *Stachytarpheta leaf curl virus* (StaLCV) none of the isolates were found associated with DNAβ molecules (Xiong et al., 2005). TbLCYNV should be classified into the third type of monopartite begomoviruses. To rule out the possibility of a false association of the DNAβ molecule with a begomovirus as demonstrated here, we propose that in future studies it will be necessary to test more virus samples, and conduct infectivity tests with infectious clones of the begomovirus.

DNAβ could not replicate autonomously and it depends on its helper begomoviruses for replication, transmission and spread. It has been demonstrated that there is a relaxed relationship between begomoviruses and DNAβ molecules. Similar to the interaction between distinct DNAβ molecules and StaLCV (Xiong et al., 2005), the DNAβ molecules associated with TYLCCNV, TbCSV, TYLCTHV and MYVV were capable of trans-replication and maintenance in *N. benthamiana*, *N. glutinasa* and *P. hybrida* plants by TbLCYNV. However, variable concentrations of DNAβ in *N. benthamiana* plants were found upon co-inoculation with TbLCYNV and DNAβ molecules associated with other begomoviruses. Further studies are necessary to elucidate the replication interactions between the helper virus species and the satellite DNAβ molecules.

The GenBank accession number of the sequences reported in this paper is AJ512761-62.

Acknowledgements

This work was supported by National Natural Science Foundation of China (Grant No. 30530520 and 30400015) and the Post-Doctoral Science Foundation of China (Grant No. X90401).

References

Briddon RW, Mansoor S, Bedford ID, Pinner MS, Saunders K, Stanley J, Zafar Y, Malik KA and Markham PG (2001) Identification of DNA components required for induction of cotton leaf curl disease. Virology 285: 234–243.

Cui XF, Xie Y and Zhou XP (2004) Molecular characterization of DNAβ molecules associated with *Tobacco leaf curl Yunnan virus*. Journal of Phytopathology 152: 647–650.

Dry IB, Rigden JE, Krake LR, Mullineaux PM and Rezaian MA (1993) Nucleotide sequence and genome organization of tomato leaf curl geminivirus. Journal of Genemal Virology 74: 147–151.

Hanley-Bowdoin L, Settlage SB, Orozco BM, Nagar S and Robertson D (1999) Geminiviruses: Models for plant DNA replication, transcription, and cell cycle regulation. Critical Reviews in Plant Science 18: 71–106.

Harrison BD, Liu YL, Khalid S, Hameed S and Otim-Nape GW (1997) Detection and relationships of cotton leaf curl virus and allied whitefly-transmitted geminiviruses occurring in Pakistan. Annals of Applied Biology 130: 61–75.

Harrison BD and Robinson DJ (1999) Natural genomic and antigenic variation in whitefly-transmitted geminiviruses (Begomoviruses). Annual Review of Phytopathology 37: 369–398.

Li ZH, Xie Y and Zhou XP (2005) *Tobacco curly shoot virus* DNAβ is not essential for symptom induction and intensifies symptoms in a host-dependent manner. Phytopathology 95: 902–908.

Li ZH, Zhou XP, Zhang X and Xie Y (2004) Molecular characterization of tomato-infecting begomoviruses in Yunnan, China. Archives of Virology 149: 1721–1732.

Mansoor S, Briddon RW, Zafar Y and Stanley J (2003) Geminivirus disease complexes: an emerging threat. Trends in Plant Science 8: 128–134.

Moffat AS (1999) Plant pathology: geminivirus emerges as a serious crop threat. Science 286: 1835.

Nateshan HM, Muniyappa V and Swanson MM (1996) Host range, vector relations and serological relationships of cotton leaf curl virus from southern India. Annals of Applied Biology 128: 233–244.

Navot N, Pichersky E, Zeidan M, Zamir D and Czosnek H (1991) *Tomato yellow leaf curl virus* - A whitefly-transmitted geminivirus with a single genomic component. Virology 185: 151–161.

Reichardt M and Roger S (1995) Preparation of plant DNA using CTAB. In: Ausubel FM, Brent R, Kinston RE, Moore DD, Seidman JG, Smith JA and Struhl K (eds.) Current Protocol in Molecular Biology, Vol. 1 (pp. 233–237) John Wiley and Sons, Inc, New York.

Saunders K, Bedford ID, Briddon RW, Markham PG, Wong SM and Stanley J (2000) A unique virus complex causes Ageratum yellow vein disease. Proceedings of the National Academy of Sciences of the USA 97: 6890–6895.

Saunders K, Bedford ID and Stanley J (2001) Pathogenicity of a natural recombinant associated with Ageratum yellow vein disease: Implications for geminivirus evolution and disease aetiology. Virology 282: 38–47.

Saunders K, Bedford ID, Yahara T and Stanley J (2003) The earliest recorded plant virus disease. Nature 422: 831.

Van Engelen FA, Molthoff JW, Conner AJ, Nap JP, Pereira A and Stiekema WJ (1995) pBINPLUS: an improved plant transformation vector based on pBIN19. Transgenic Research 4: 288–290.

Xie Y and Zhou XP (2003) Molecular characterization of *Squash leaf curl Yunnan virus*, a new begomovirus and evidence for recombination. Archives of Virology 148: 2047–2054.

Xie Y, Zhou XP, Li ZH, Zhang ZK and Li GX (2002a) Identification of a novel DNA molecule associated with *Tobacco leaf curl virus*. Chinese Science Bulletin 47: 1273–1276.

Xie Y, Zhou XP, Zhang ZK and Qi YJ (2002b) *Tobacco curly shoot viru*s isolated in Yunnan is a distinct species of *Begomovirus*. Chinese Science Bulletin 47: 197–200.

Xiong Q, Fan SW, Guo XJ and Zhou XP (2005) *Stachytarpheta leaf curl virus* is a novel monopartite begomovirus species. Archives of Virology 150: 2257–2270.

Yin QY, Yang HY, Gong QH, Wang H, Liu Y, Hong Y and Tian P (2001) Tomato yellow leaf curl China virus: monopartite genome organization and agroinfection of plants. Virus Research 81: 69–76.

Zhou X, Xie Y, Tao X, Zhang Z, Li Z and Fauquet CM (2003) Characterization of DNAβ associated with begomoviruses in China and evidence for co-evolution with their cognate viral DNA-A. Journal of General Virology 84: 237–247.

Zhou XP, Xie Y and Zhang ZK (2001) Molecular characterization of a distinct begomovirus infecting tobacco in Yunnan, China. Archives of Virology 146: 1599–1606.