ISSN 1672-9145 Acta Biochimica et Biophysica Sinica 2005, **37**(1): 25–31 **CN** 31-1940/Q

Interaction between a Nanovirus-like Component and the *Tobacco Curly Shoot Virus*/Satellite Complex

Pei-Jun WU and Xue-Ping ZHOU*

Institute of Biotechnology, Zhejiang University, Hangzhou 310029, China

Abstract The biological role of DNA1, a nanovirus-like component shown to be associated with the begomovirus/satellite complex, has not yet been identified. Here, we demonstrated that DNA1 of *Tobacco curly shoot virus* isolate Y35 (TbCSV-Y35) attenuated leaf-curling symptoms induced by TbCSV-Y35 or TbCSV-Y35 plus Y35 DNAβ in the early stage of symptom development and induced leaf cluster at a later stage of symptom development in *Nicotiana benthamiana* plants. The leaf disc assay demonstrated that TbCSV-Y35 DNA1 replicated autonomously. Southern blot analysis revealed that TbCSV-Y35 DNA1 reduced viral DNA accumulation. Viral DNA accumulation was not reduced when plants were co-inoculated with TbCSV-Y35 DNAβ, but the TbCSV-Y35 DNAβ level was dramatically reduced in the presence of TbCSV-Y35 DNA1. To determine whether the interaction between TbCSV/satellite complex and DNA1 had isolate specificity, DNA1 of TbCSV isolate Y132 was cloned and sequenced. It was found to have 75% nucleotide sequence identity with TbCSV-Y35 DNA1. Infectivity tests showed that TbCSV-Y132 DNA1 had no effect on the symptoms induced by TbCSV-Y35 or TbCSV-Y35 and Y35 DNAβ in *N. benthamiana* plants, although Y132 DNA1 could replicate in these plants.

Key words *Tobacco curly shoot virus*; DNA1; DNAβ; symptom; replication; interaction

The viruses in the family *Geminiviridae* are divided into four genera according to genome organization, host range and vector transmission [1]. Most geminiviruses belong to the genus *Begomovirus*, which are transmitted by *Bemisia tabaci* and can infect dicotyledonous plants. The majority of begomoviruses have two components, referred to as DNA-A and DNA-B, both of which are essential for systemic infection. Some begomoviruses have only a single genomic component (resembling DNA-A) which is sufficient to induce typical disease in plants [2,3]. However, for some monopartite begomoviruses, such as *Ageratum yellow vein virus* (AYVV), *Cotton leaf curl Multan virus* (CLCuMV) and *Tomato yellow leaf curl China virus* (TYLCCNV), a satellite molecule named DNAβ is required to induce typical disease symptoms in the plant species from which they are isolated [4–6]. DNAβ depends on a helper virus for replication, movement and encapsidation. The begomovirus/satellite DNA molecule complexes have a widespread distribution and pose a serious threat to tropical and subtropical agro-ecosystems worldwide [7]. In addition to the DNAβ component, a nanovirus-like DNA component, referred to as DNA1, has been also found to be associated with the begomovirus/satellite complex [7–11]. DNA1 contains a single open reading frame (ORF) for replication and depends on the helper begomoviruses for spreading in plants and insect transmission. However, its role in the development of disease is not clear.

Tobacco curly shoot virus isolate Y35 (TbCSV-Y35) was first isolated in Yunnan, China, and was found to be associated with DNAβ [6]. It is also associated with DNA1 [11]. Here, we report that the interaction between DNA1 and the TbCSV/satellite complex can modulate viral symptoms and reduce viral DNA accumulation.

Received: September 24, 2004 Accepted: November 18, 2004

This work was supported by the grants from the National Out standing Youth Foundation (No. 30125032) and the National Natural Science Foundation of China (No. 30370927)

*Corresponding author: Tel, 86-571-86971680; Fax, 86-571-86971498; E-mail, zzhou@zju.edu.cn

©Institute of Biochemistry and Cell Biology, SIBS, CAS

Downloaded from https://academic.oup.com/abbs/article/37/1/25/151 by guest on 18 April 2024

Materials and Methods

Virus isolates

TbCSV-Y35 was collected previously [6]. TbCSV isolate Y132 (TbCSV-Y132), showing curly shoot, stunting, leaf curl and vein-darkening symptoms, was collected from a tobacco field in Baoshan, Yunnan Province, China.

Construction of infectious DNA clones

A full-length copy of the TbCSV-Y35 DNA1 was amplified using the primers Y35DNA1F1 (5′-GTCGACCAGGGTACTGTAGCTG-3′; a *Sal*I site was introduced) and Y35DNA1R (5′-GAATTCTTCCTCCGCAAGCTTAG-3′; an *Eco*RI site was introduced), and cloned into the pGEM T-Vector (Promega, Madison, USA) to produce pGEMT-Y35DNA1-SE. The pGEMT-Y35DNA1-SE was digested by *Sal*I and *Eco*RI, and inserted into the binary vector pBinPLUS [12] to produce pBinPLUS-Y35DNA1-SE. Another full-length copy of the TbCSV-Y35 DNA1 genome was amplified using the primers Y35DNA1F (5′-GAATTCCAGGGTACTGTAGCTG-3′, an *Eco*RI site was introduced) and Y35DNA1R, and cloned into the pGEM T-Vector to create pGEMT-Y35DNA1-E. pGEMT-Y35DNA1-E was digested with *Eco*RI and the digested fragment was inserted into pBinPLUS-Y35DNA1-SE to produce pBinPLUS-2-mer-Y35DNA1, containing the dimer of full-length Y35 DNA1. pBinPLUS-2-mer-Y35DNA1 was introduced into *Agrobacterium tumefaciens* strain EHA105 by direct transformation or by triparental mating. The infectious clone of Y132DNA1 was constructed by the same technique. The infectious clone of TbCSV-Y35 and associated DNAβ were constructed in this lab previously (data not shown).

Agro-inoculation of plants

A. tumefaciens cultures were grown at 28 °C for 48 h (A_{550}=1). For co-inoculation, a mixture containing equal volumes of separate bacterial cultures (TbCSV-Y35+Y35 DNA1, TbCSV-Y35+Y35 DNAβ, or TbCSV-Y35+Y35 DNA1+Y35 DNAβ) was used. A 21-gauge needle was used to inject 0.2 ml of the bacterial culture mixture into the stem or petiole of plants at the 4–6 leaf stage. Inoculated plants were grown in an insect-free cabinet with 16 h of light per day.

Leaf disc assay for DNA1 replication

The replication of DNA1 was investigated in *N. benthamiana* leaf discs as described by Klinkenberg *et al*. [13]. Leaf discs of 6 mm in diameter were incubated on pre-callusing medium plates for 24 h at 25 °C under continuous lighting, then dipped into an overnight culture of the appropriate transconjugant and returned to the plates for a further 48 h. Leaf discs were then transferred to a selective medium containing 100 μg/ml kanamycin and 500 μg/ml carbenicillin (Sigma, St. Louis, USA), incubated at 25 °C, and then collected for isolation of viral DNA at 2, 4 and 6 days post-inoculation (dpi).

Isolation and characterization of viral DNA forms in plants

The total DNA was isolated from young leaves of systemically infected *N. benthamiana* plants as described by Zhou *et al.* [14]. Nucleic acids were fractionated by 0.8% agarose gel electrophoresis in TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.3), and transferred to Hybond-N^+ membranes (Amersham Biosciences, Buckinghamshire, England) after alkali denaturation and neutralization. The membranes were then hybridized to [α-^{32}P]dCTP-labeled probes specifically for TbCSV-Y35, Y35 DNAβ and Y35 DNA1.

Cloning and sequencing of TbCSV-Y132 DNA1

The total nucleic acid of TbCSV-Y132 was extracted as described by Xie *et al*. [15]. A full-length copy of the Y132 DNA1 was amplified using primers UNA101 (AAGCTTGCGACTATTGTATGAAAGAGG) and UNA102 (AAGCTTCGTCTGTCTTACGAGCTCGCTG) [11], and the amplified fragment was cloned into the pGEM-T vector and sequenced using the automated Model 377 DNA sequencing system (Perkin Elmer Inc., Wellesley, USA). Sequence data was assembled and analyzed with the aid of the DNASTAR program (DNASTAR Inc., Madison, USA).

Results

Effect of TbCSV-Y35 DNA1 on viral symptoms

To identify the biological role of TbCSV-Y35 DNA1, *N. benthamiana* plants were agro-inoculated with TbCSV-Y35, TbCSV-Y35 plus Y35 DNA1, TbCSV-Y35 plus Y35 DNAβ and Y35 DNA1. The *N. benthamiana* plants inoculated with only TbCSV-Y35 began to develop upward leaf-curling symptoms at 7 dpi, while those co-inoculated with TbCSV-Y35 plus Y35 DNA1 began to develop similar symptoms at 9 dpi. The upward curling symptoms of the newly emerging leaves inoculated with TbCSV-Y35 plus Y35

©Institute of Biochemistry and Cell Biology, SIBS, CAS

Downloaded from https://academic.oup.com/abbs/article/37/1/25/151 by guest on 18 April 2024

DNA1 were milder than those induced with only TbCSV-Y35 at the early stage of symptom development (9–30 dpi) [**Fig. 1(A)**]. At the late stage of symptom development (60–90 dpi), the top leaves of the plants inoculated with TbCSV-Y35 plus Y35 DNA1 formed a cluster, while the plants inoculated with only TbCSV-Y35 kept on growing upward and the top leaves never formed a cluster [**Fig. 1(B)**].

The *N. benthamiana* plants co-inoculated with TbCSV-Y35 plus Y35 DNAβ and Y35 DNA1 developed downward curling symptoms, which were indistinguishable from those co-inoculated with TbCSV-Y35 plus Y35 DNAβ at the early stage of symptom development [**Fig. 1(C)**]. However, at the late stage of symptom development, the top leaves of the plants inoculated with TbCSV-Y35 plus Y35 DNAβ and Y35 DNA1 formed a cluster. The cluster symptoms were not observed in the plants inoculated with TbCSV-Y35 plus Y35 DNAβ [**Fig. 1(D)**].

Autonomous replication of TbCSV-Y35 DNA1 in plants

PCR detection showed that DNA1 alone could not infect *N. benthamiana* plants systemically (data not shown).

Downloaded from https://academic.oup.com/abbs/article/37/1/25/151 by guest on 18 April 2024

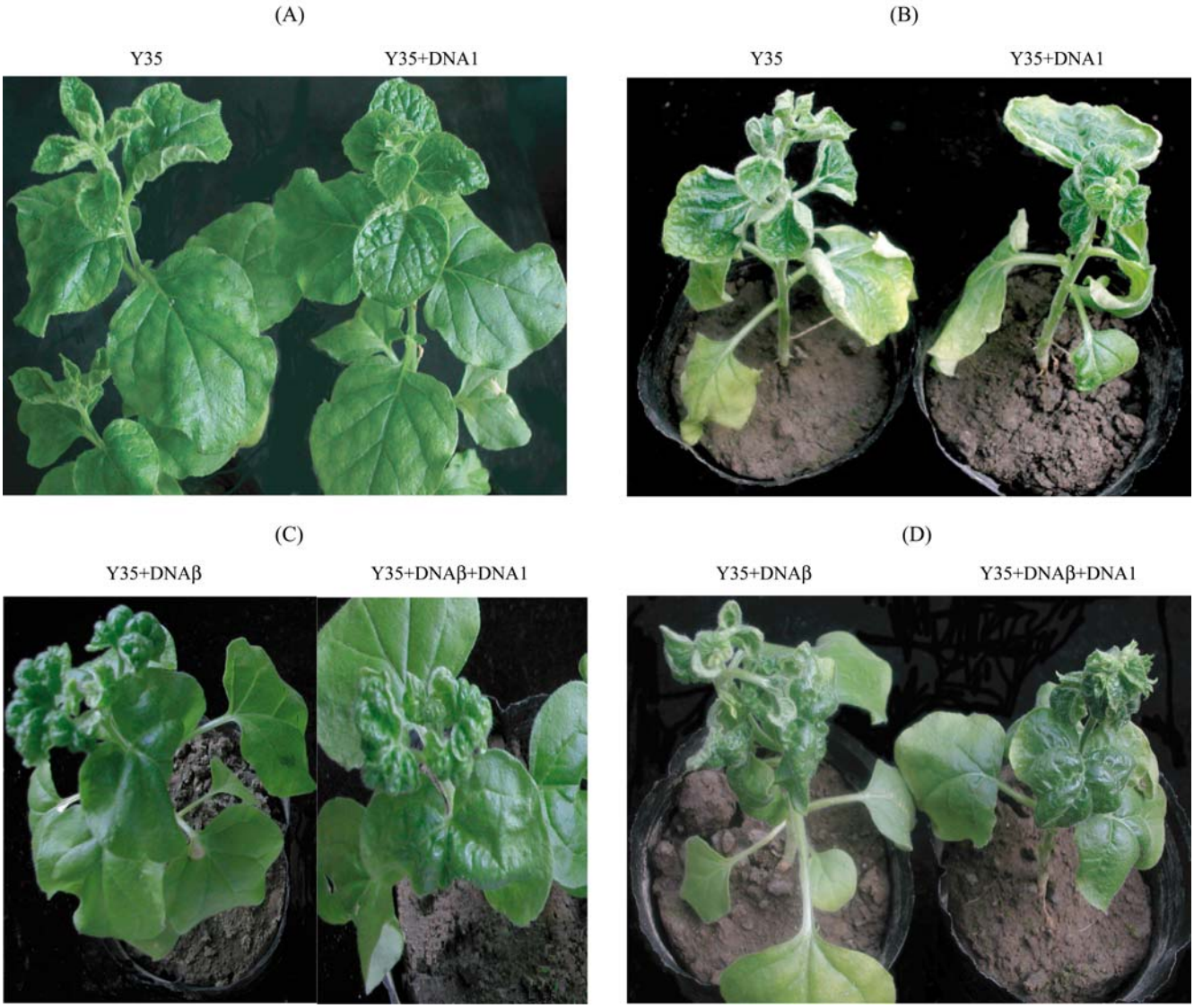

Fig. 1 Symptoms of *N. benthamiana* plants inoculated

(A) TbCSV-Y35 only and TbCSV-Y35 plus Y35 DNA1 at 30 dpi. (B) TbCSV-Y35 only and TbCSV-Y35 plus Y35 DNA1 at 90 dpi. (C) TbCSV-Y35 plus Y35 DNAβ and TbCSV-Y35 plus Y35 DNAβ and Y35 DNA1 at 30 dpi. (D) TbCSV-Y35 plus Y35 DNAβ and TbCSV-Y35 plus Y35 DNAβ and Y35 DNA1 at 90 dpi.

In order to determine whether DNA1 could replicate autonomously, an *N. benthamiana* leaf disc assay was carried out. Southern blot analysis showed that the leaf discs infected with only TbCSV-Y35 DNA1 produced typical single-stranded DNA forms, indicating that it can replicate autonomously. A dramatic increase in the DNA1 accumulation levels was found in the leaf discs co-inoculated with TbCSV-35 plus Y35 DNA1 or with TbCSV-35 plus Y35 DNA1 and Y35 DNAβ (**Fig. 2**).

Effects of TbCSV-Y35 DNA1 on the accumulation of TbCSV-Y35 and Y35 DNAβ

To determine the effects of TbCSV-Y35 DNA1 on the accumulation of TbCSV-Y35 and Y35 DNAβ in infected plants, nucleic acids were extracted from systemically infected *N. benthamiana* plants at 30 dpi and analyzed by Southern blot with probes specifically for TbCSV-Y35, Y35 DNAβ and Y35 DNA1. In the plants co-inoculated with TbCSV-Y35 and Y35 DNA1, the single-stranded DNA form of DNA1 was predominantly found in infected tissues (**Fig. 3**). The viral DNA in the plants co-inoculated with TbCSV-Y35 and Y35 DNA1 accumulated at obviously lower levels than those in the plants inoculated with TbCSV-Y35 alone. In the plants co-inoculated with TbCSV-Y35 plus Y35 DNAβ and Y35 DNA1, the viral DNA level was similar to that observed in plants co-inoculated with TbCSV-Y35 plus Y35 DNAβ. However, the level of DNAβ was significantly reduced in the presence of TbCSV-Y35 DNA1. These results suggest that DNA1 is capable of reducing the accumulation of both DNA-A and DNAβ in plants.

Interaction between TbCSV-Y35 and DNA1 of a different TbCSV isolate

To find out whether the interaction between TbCSV/satellite complex and DNA1 is isolate-specific, DNA1 of TbCSV-Y132 was cloned and sequenced. The complete sequence of Y132 DNA1 consists of 1375 nucleotides (nt) and encodes a conserved ORF that resembles Rep of nanoviruses (accession No. AJ579349). Sequence analysis revealed that Y132 DNA1 had 75% nucleotide sequence identity with Y35 DNA1, while the Rep protein they encoded had 91% amino acid sequence identity.

The infectious clone of Y132DNA1 was constructed and Y132 DNA1 was then co-inoculated with TbCSV-Y35 or with TbCSV-Y35 and Y35 DNAβ in *N. benthamiana* plants. The plants agro-inoculated with TbCSV-Y35 and Y132 DNA1 developed upward leaf-curling symptoms which were indistinguishable from those inoculated with TbCSV-Y35 alone. The plants co-inoculated with TbCSV-Y35 plus Y35 DNAβ and TbCSV-Y132 DNA1 developed downward leaf-curling symptoms resembling those induced by TbCSV-Y35 and Y35 DNAβ, indicating that TbCSV-Y132 DNA1 has no effect on the symptoms induced by TbCSV-Y35 or TbCSV-Y35 and Y35 DNAβ. Southern blot analysis showed that TbCSV-Y132 DNA1 could be easily detected systemically in leaves inoculated with TbCSV-Y35 and Y132 DNA1 or TbCSV-Y35, Y35 DNAβ and Y132 DNA1, indicating that the systemic movement of TbCSV DNA1 from a different isolate can be supported by TbCSV-Y35 (**Fig. 4**).

Discussion

In recent years, a satellite DNAβ component has been found to be associated with monopartite begomoviruses and shown to be required for the induction of typical disease symptoms. In addition to DNAβ, a nanovirus-like component has also been found to be associated with

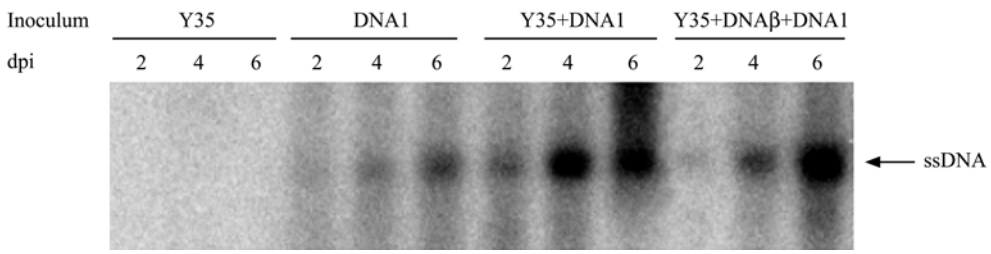

Fig. 2 Southern blot analysis of the replication of TbCSV-Y35 DNA1 in *N. benthamiana* leaf discs

Samples were extracted from leaf discs agro-inoculated with TbCSV-Y35, TbCSV-Y35 DNA1, TbCSV-Y35 and Y35 DNA1, or TbCSV-Y35 plus Y35 DNAβ and Y35 DNA1 and harvested at 2, 4 or 6 dpi. Equal amounts of nucleic acid (10 μg) were loaded in each lane. The blot was probed with the full-length sequence of Y35 DNA1. The position of single-stranded DNA (ssDNA) forms is indicated.

©Institute of Biochemistry and Cell Biology, SIBS, CAS

Downloaded from https://academic.oup.com/abbs/article/37/1/25/151 by guest on 18 April 2024

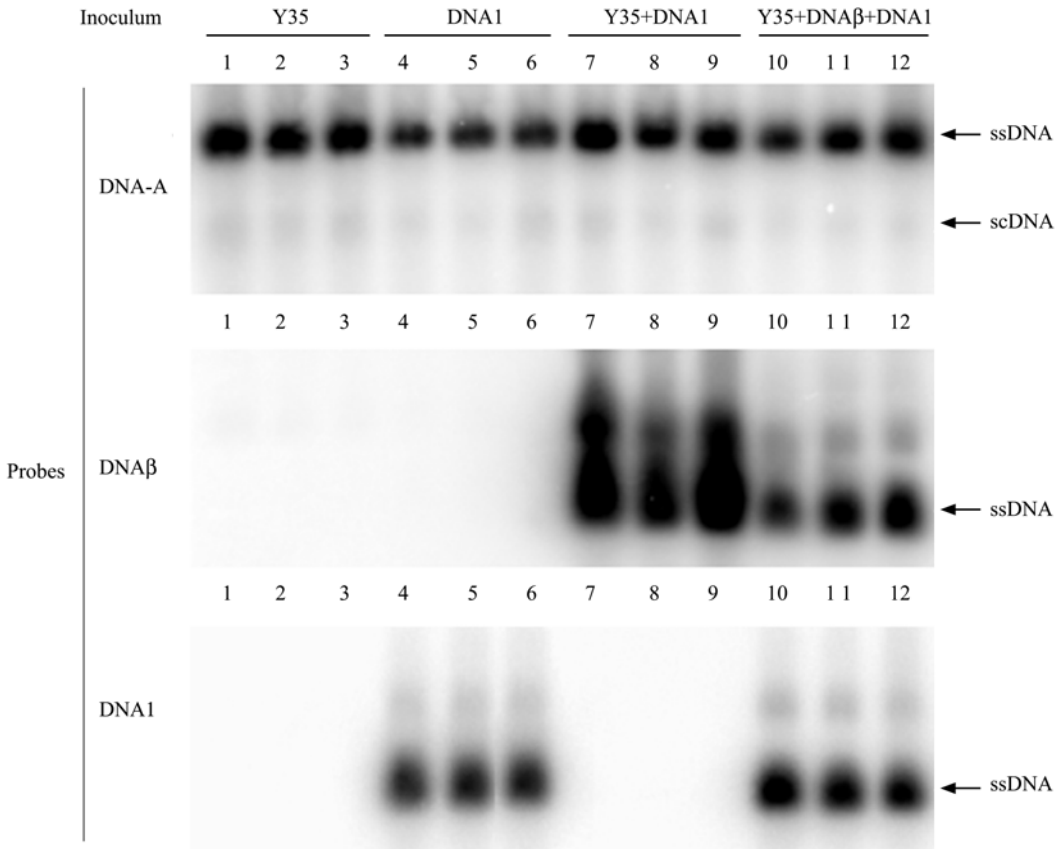

Fig. 3 Detection of viral DNAs in *N. benthamiana* plants

Samples were extracted from individual plants agro-inoculated with TbCSV-Y35 (lanes 1–3), TbCSV-Y35 plus Y35 DNA1 (lanes 4–6), TbCSV-Y35 plus Y35 DNAβ (lanes 7–9), and a mixture of TbCSV-Y35, Y35 DNAβ and Y35 DNA1 (lanes 10–12). Equal amounts of nucleic acid (10 μg) were loaded in each lane. Blots were probed with part of TbCSV-Y35 (top), the full-length sequence of Y35 DNAβ (middle) or Y35 DNA1 (bottom). The positions of single-stranded DNA (ssDNA) and supercoiled DNA (scDNA) forms are indicated.

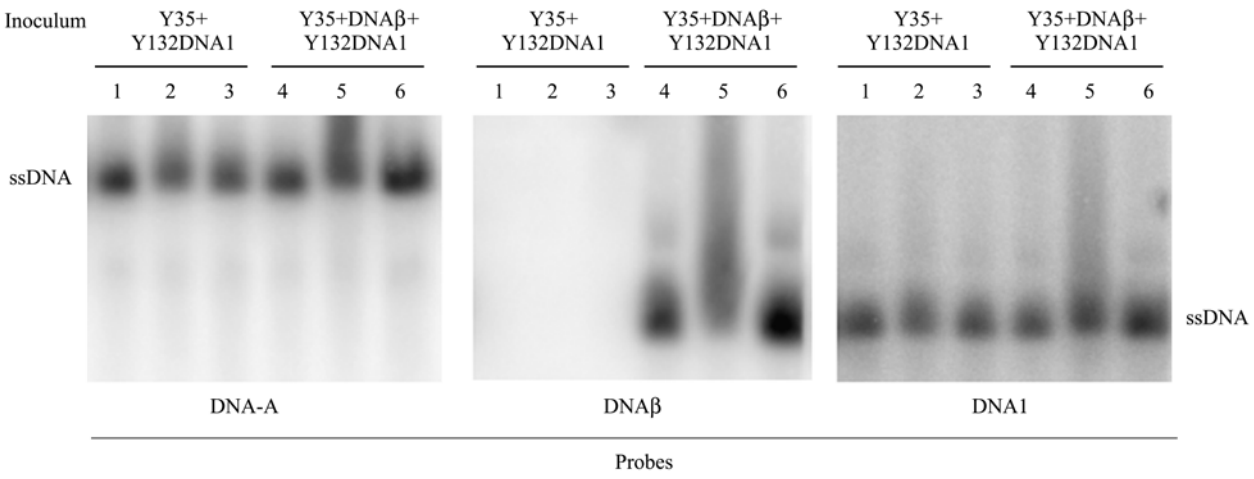

Fig. 4 Detection of viral DNAs in *N. benthamiana* plants

Samples were extracted from individual plants agro-inoculated with TbCSV-Y35 plus Y132 DNA1 (lanes 1–3) and a mixture of TbCSV-Y35, Y35 DNAβ and Y132 DNA1 (lanes 4–6). Equal amounts of nucleic acid (10 μg) were loaded in each lane. Blots were probed with part of TbCSV-Y35 (left), the full-length sequence of Y35 DNAβ (middle) or Y132 DNA1 (right). The positions of single-stranded DNA (ssDNA) forms are indicated.

Downloaded from https://academic.oup.com/abbs/article/37/1/25/151 by guest on 18 April 2024

begomovirus/DNAβ complexes [8]. In China, many begomoviruses obtained from tobacco, tomato, *Malvastrum coromandelianum* and *Siegesbeckia orientalis* have been found to be associated with DNAβ molecules [6,16]. Phylogenetic analysis of DNAβ molecules and their cognate begomoviruses suggest that DNAβ has co-evolved with their cognate helper viruses [6]. DNA1 is also reported to be associated with the TbCSV/DNAβ complex [11]. TbCSV DNA1 is approximately half the size of TbCSV and it depends on TbCSV for encapsidation [11]. Our results show that TbCSV DNA1 alone is unable to systemically infect *N. benthamiana* plants, with its systemic movement being mediated by TbCSV. The leaf disc assay shows that TbCSV DNA1 can replicate autonomously; this is probably attributable to the Rep protein encoded by DNA1. The accumulation level of the single-stranded form of TbCSV DNA1 is much higher in the presence of TbCSV or the TbCSV/satellite complex, implying a direct effect of TbCSV or the TbCSV/satellite complex on DNA1 replication. Similar findings are also reported for AYVV and CLCuMV [8,10].

We have demonstrated that DNA1 not only affects the symptoms but also modulates the accumulation of TbCSV and DNAβ. DNAβ cannot auto-replicate and depends on a helper virus for replication, movement and encapsidation. There is an open reading frame (*βC1*) on the complementary strand, which is a key determinant of symptom induction in many monopartite geminiviruses such as AYVV, CLCuMV and TYLCCV [4–6]. Although TbCSV can induce severe symptoms without DNAβ, DNAβ can enhance the symptoms induced by TbCSV (Li and Zhou, data not shown). When the plants are co-inoculated with TbCSV, DNA1 and its satellite, a competition for replication between DNA1 and DNAβ may exist, as both of the molecules depend on the helper virus for movement and encapsidation. DNA1 can auto-replicate and it may have the priority to replicate and use the helper virus for movement and encapsidation. This may explain why the accumulation of DNAβ is dramatically reduced and the symptoms are attenuated in the presence of DNA1. Modulation of the DNA levels of begomoviruses and DNAβ components by DNA1 is also reported for AYVV and CLCuMV, but the DNA1 associated with AYVV and CLCuMV plays no direct role in the disease etiology [8,10]. These results suggest that TbCSV-Y35 DNA1 may functionally interact with TbCSV-Y35 or the TbCSV-Y35/DNAβ complex, resulting in symptom alteration.

In contrast to TbCSV-Y35 DNA1, TbCSV-Y132 DNA1 has no effect on the symptoms induced by the TbCSV-Y35/DNAβ complex, implying that the interaction between DNA1 and the TbCSV/DNAβ complex may have isolate specificity. However, Southern blot analysis reveals that the systemic movement of TbCSV-Y132 DNA1 can be mediated by TbCSV-Y35. Saunders *et al.* [10,17] have demonstrated that DNA1 of AYVV can accumulate in plants when co-inoculated with the old- and new-world bipartite begomoviruses, ACMV and TGMV, and with the curtovirus BCTV. These results suggest that DNA1 is facilitated by different helper viruses in its cell-to-cell and systemic movement.

Our study shows that DNA1 is associated with the TbCSV isolates containing DNAβ. With the exception of two isolates from the Far East, Briddon *et al*. [9] have shown that all begomoviruses-DNAβ complexes investigated are associated with a DNA1 component. The near-ubiquitous association of DNA1 components with complexes suggests that DNA1 may have a role to play. It has been hypothesized that DNA1 may moderate infection by "mopping up" cellular resources [9]. The reduction in the level of viral DNA and DNAβ in infected plants in the presence of DNA1 supports this suggestion. More studies are required to elucidate the specific role played by DNA1 in disease development or in the virus life cycle.

References

1 Fauquet CM, Bisaro DM, Briddon RW, Brown JK, Harrison BD, Rybicki EP, Stenger DC *et al*. Revision of taxonomic criteria for species demarcation in the family *Geminiviridae*, and an updated list of begomovirus species. Arch Virol, 2003, 148(2): 405–421

2 Czosnek H, Laterrot H. A worldwide survey of tomato yellow leaf curl viruses. Arch Virol, 1997, 142(7): 1391–1406

3 Navot N, Pichersky E, Zeidan M, Zamir D, Czosnek H. *Tomato yellow leaf curl virus*: A whitefly-transmitted geminivirus with a single genomic component. Virology, 1991, 185(1): 151–161

4 Briddon RW, Mansoor S, Bedford ID, Pinner MS, Saunders K, Stanley J, Zafar Y *et al*. Identification of DNA components required for induction of cotton leaf curl disease. Virology, 2001, 285(2): 234–243

5 Jose J, Usha R. Bhendi yellow vein mosaic disease in India is caused by association of a DNAβ satellite with a begomovirus. Virology, 2003, 305(2): 310–317

6 Zhou XP, Xie Y, Tao XR, Zhang ZK, Li ZH, Fauquet CM. Characterization of DNAβ associated with begomoviruses in China and evidence for co-evolution with their cognate viral DNA-A. J Gen Virol, 2003, 84(1): 237–247

7 Mansoor S, Briddon RW, Zafar Y, Stanley J. Geminivirus disease complexes: an emerging threat. Trends Plant Sci, 2003, 8(3): 128–134

8 Mansoor S, Khan SH, Bashir A, Saeed M, Zafar Y, Malik KA, Briddon R *et al*. Identification of a novel circular single-stranded DNA associated with cotton leaf curl disease in Pakistan. Virology, 1999, 259(1): 190–199

9 Briddon RW, Bull SE, Amin I, Mansoor S, Bedford ID, Rishi N, Siwatch SS *et al*. Diversity of DNA1: A satellite-like molecule associated with monopartite begomovirus-DNAβ complexes. Virology, 2004, 324(2): 462–474

©Institute of Biochemistry and Cell Biology, SIBS, CAS

Downloaded from https://academic.oup.com/abbs/article/37/1/25/151 by guest on 18 April 2024

10 Saunders K, Stanley J. A nanovirus-like DNA component associated with yellow vein disease of *Ageratum conyzoides*: Evidence for interfamilial recombination between plant DNA viruses. Virology, 1999, 264(1): 142–152
11 Xie Y, Wu PJ, Tao XR, Zhou XP. Identification of a nanovirus-like DNA molecule associated with *Tobacco curly shoot virus* isolates containing satellite DNA. Prog Nat Sci, 2004, 14(8): 689–693
12 van Engelen FA, Molthoff JW, Conner AJ, Nap JP, Pereira A, Stiekema WJ. pBINPLUS: An improved plant transformation vector based on pBIN19. Transgenic Res, 1995, 4(4): 288–290
13 Klinkenberg FA, Ellwood S, Stanley J. Fate of African cassava mosaic virus coat protein deletion mutants after agroinoculation. J Gen Virol, 1989, 70: 1837–1844
14 Cui XF, Tao XR, Xie Y, Fauquet CM, Zhou XP. A DNAβ associated with *Tomato yellow leaf curl China virus* is required for symptom induction in hosts. J Virol, 2004, 78(24): 13966–13974
15 Xie Y, Zhou XP. *Tobacco curly shoot virus* isolated in Yunnan is a distinct species of *Begomovirus*. Chin Sci Bull, 2002, 47(3): 197–200
16 Zhou XP, Xie Y, Peng Y, Zhang ZK. *Malvastrum yellow vein virus*, a new *Begomovirus* species associated with satellite DNA molecule. Chin Sci Bull, 2003, 48 (20): 2205–2209
17 Saunders K, Bedford ID, Stanley J. Adaptation from whitefly to leafhopper transmission of an autonomously replicating nanovirus-like DNA component associated with ageratum yellow vein disease. J Gen Virol, 2002, 83(4): 907–913

Edited by
Zu-Xun GONG

Downloaded from https://academic.oup.com/abbs/article/37/1/25/151 by guest on 18 April 2024