Trans-Replication of, and Competition Between, DNA β Satellites in Plants Inoculated with *Tomato yellow leaf curl China virus* and *Tobacco curly shoot virus*

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ABSTRACT

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Tomato yellow leaf curl China virus (TYLCCNV) Y10 isolate (Y10) and *Tobacco curly shoot virus* (TbCSV) Y35 isolate (Y35), both obtained from Yunnan Province, were each found to be associated with a distinct species of satellite DNA (DNA β). Here, we demonstrate that both Y10 DNA β (Y10 β) and Y35 DNA β (Y35 β) were stably trans-replicated by the noncognate *Begomovirus*, although the noncognate DNA β accumulated in plants at a lower level than did the cognate DNA β . In

Nicotiana benthamiana and *N. glutinosa* plants agroinoculated with Y10+Y10 β +Y35 β or with Y35+Y35 β +Y10 β , all components accumulated in the early stages of infection but, at later stages, the noncognate DNA β decreased in relative concentration and was undetectable at 80 to 120 days after inoculation. The helper viruses and cognate DNA β species persisted at higher levels throughout the experiments. When the initiation codon of the β C1 gene of the cognate DNA β was mutated, the dominance of the cognate over the noncognate DNA β in mixed infections was unimpaired. These results imply that the cognate DNA β competes with the noncognate DNA β and that the ability for selective maintenance of DNA β is not controlled by the β C1 protein.

Geminiviruses are a group of DNA plant viruses which have a circular, single-stranded DNA (ssDNA) genome that is encapsidated in twinned quasi-icosahedral particles that are $\approx$ 18 to 20 by 30 nm. Geminiviruses belong to the genus *Begomovirus*, family *Geminiviridae*, infect dicotyledonous plants, and pose a serious threat in tropical and subtropical agroecosystems worldwide due to their widespread distribution, coupled with the explosion of populations of their whitefly vectors (*Bemisia* spp.) (18). Many *Begomovirus* spp. have a genome consisting of two components, referred to as DNA A and DNA B, while others have a monopartite genome, which are homologous to the DNA A component of bipartite *Begomovirus* spp. A satellite DNA, designated DNA β , was found to be associated with many of these monopartite-genome *Begomovirus* spp., including *Ageratum yellow vein virus* (AYVV), *Bhendi yellow vein mosaic virus* (BYVMV), *Cotton leaf curl Multan virus* (CLCuMV), and *Eupatorium yellow vein virus* (EpYVV). DNA β are circular, ssDNA molecules of $\approx$ 1,350 nucleotides that are required for induction symptoms in the respective natural hosts of these helper viruses (6,17,28,29). They depend on their helper *Begomovirus* sp. for its replication, encapsidation, insect transmission, and movement in plants, and they have no appreciable sequence identity to their helper *Begomovirus* sp., except for a conserved hairpin structure that includes a TAATATTAC loop sequence necessary for its replication (7,22,23,33).

In China, DNA β molecules were found to be associated with many *Begomovirus* spp. obtained from tobacco, tomato, and weed species (19,34–37). *Tomato yellow leaf curl China virus* (TYLCCNV) and *Tobacco curly shoot virus* (TbCSV), both iso-

lated from Yunnan Province, were found to be associated with DNA β molecules. TYLCCNV alone caused symptomless systemic infections in several plant species, and co-infection with DNA β was required for induction of leaf curl symptoms in tobacco and tomato. In contrast, TbCSV DNA β was not essential for symptom induction but it intensified symptoms in a host-dependent manner (11,19). In this report, we studied the replication specificity of the DNA β species of TYLCCNV and TbCSV with their helper viruses. We show that both TYLCCNV DNA β and TbCSV DNA β can be trans-replicated stably by the noncognate *Begomovirus* sp. When either TYLCCNV or TbCSV was associated with both DNA β species simultaneously, the cognate DNA β became dominant and the noncognate DNA β became undetectable at late stages of infection.

MATERIALS AND METHODS

Plant materials, virus sources, and agroinoculation. *Nicotiana benthamiana* and *N. glutinosa* plants used in this study were grown in an insect-proof greenhouse as described previously (37). The infectious clones of TYLCCNV isolate Y10 (pBinPLUS-1.7A) (Y10) and its DNA β (pBinPLUS-2 β) (Y10 β), TbCSV isolate Y35 (pBinY35A-1.9) (Y35) and its DNA β (pBinY35 β -2.0) (Y35 β), and also the β C1 mutants of Y10 β (pBinPLUS-C1M-S β) (Y10 β -M) and Y35 β (pBinY35 β m-2.0) (Y35 β -M), were constructed previously (19,37). The two unmutated DNA β species have only 56% sequence identity and belong to different species of the satellites; in the mutants, the ATG start codon of the β C1 gene is mutated (19,37). In each experiment, 2-week-old *Nicotiana* seedlings were agroinoculated (37) with a selection of the following mixtures, which each contained equal volumes of the separate bacterial cultures: Y10, Y35, Y10+Y10 β , Y35+Y35 β , Y10+Y35 β , Y35+Y10 β , Y10+Y10 β +Y35 β , Y35+Y10 β +Y35 β , Y10+Y10 β -M+Y35 β , and Y35+Y35 β -M+Y10 β . Mock inocu-

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lation was performed by inoculating the tested plants with *Agrobacterium tumefaciens* strain EHA105 containing pBinPLUS. All inoculated plants were grown in an insect-free greenhouse at a constant temperature of 25°C with supplementary lighting corresponding to a 16-h day length. Experiments were replicated three times.

DNA extraction. Leaf tissues of *Nicotiana* plants showing typical systemic symptoms were harvested at different days post inoculation. Total nucleic acids were extracted from young leaf tissues as described previously (36). The DNA-containing extract was stored at -20°C and used for polymerase chain reaction (PCR) and Southern blot analysis.

PCR. Primers designed on the basis of the nucleotide sequences of Y10 (AJ319675), Y35 (AJ421621), Y10β (AJ421621), and Y35β (AJ421485) are listed in Table 1. PCR amplification was carried out as described by Zhou et al. (37). The resulting products were separated by electrophoresis on 1% (wt/vol) agarose gels, stained in ethidium bromide, and viewed with a UV transilluminator.

Southern blot analysis. Nucleic acid (10 µg) extracted from leaf tissue of *Nicotiana* plants was fractionated by electrophoresis in 1% (wt/vol) agarose gels and transferred to nylon membranes following alkali denaturation and neutralization as described (10). The DNA fragments, produced by PCR amplification using specific primer pairs Y10F2/Y10R2 for Y10, Y35F2/Y35R2 for Y35, Y35βF/Y35βR for Y35β, Y10βF/Y10βR for Y10β, and β01/β02 for both Y10β and Y35β (Table 1), were labeled with α-[³²P]-dCTP and used in hybridization. After prehybridization and hybridization, the blots were washed at high stringency (0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 65°C). Hybridization signals were detected by phosphorimaging using a Typhoon 9200 imager (Amersham Pharmacia, Piscataway, NJ).

RESULTS

DNA β can stably maintained by a noncognate *Begomovirus* sp. To determine whether Y35β and Y10β molecules could be *trans*-replicated stably by their noncognate *Begomovirus* spp., *N. benthamiana* and *N. glutinosa* plants were agroinoculated with the following combinations of the infectious clones of viral and satellite DNA mixed in equal proportions: (i) Y10, (ii) Y35, (iii) Y10+Y10β, (iv) Y10+Y35β, (v) Y35+Y35β, and (vi) Y35+Y10β. PCR with the specific primer pairs Y10β/β02 for Y10β and Y35β/β02 for Y35β was then used to detect the presence of Y10β and Y35β in systemically infected tissue of agroinoculated plants. Using these primer pairs, amplification from the plants inoculated

with Y10+Y35β or Y35+Y35β always yielded an ≈1.2-kb band specific for Y35β, whereas an ≈0.9-kb Y10β-specific band was amplified from the plants agroinoculated with Y10+Y10β or Y35+Y10β. These specific bands could be produced for more than 120 days after agroinoculation and until the plants died, indicating that DNA β can be stably maintained by a noncognate *Begomovirus* sp.

Southern blot analysis was performed to analyze the *trans*-replication of DNA β in the *N. benthamiana* plants (Fig. 1). The Southern blots showed that Y10β could be *trans*-replicated by Y35 but accumulated in plants at a lower level than Y35β, although the plants contained similar concentrations of Y35 (Fig. 1A and B). Similarly, Y35β accumulated at a lower level in plants infected by Y10+Y35β than did Y10β in plants infected by Y10+Y10β, although similar concentrations of Y10 were detected in both sets of plants (Fig. 1C and D). The results indicate that both Y10β and Y35β could be *trans*-replicated by their noncognate helper viruses but that the noncognate DNA β accumulated at a lower level than did the cognate DNA β. Also, whereas neither satellite DNA increased the accumulation of Y35, both increased the accumulation of Y10.

The noncognate DNA β is not maintained in the presence of the cognate DNA β. Although Y10 DNA β and Y35 DNA β could

Fig. 1. Southern blot analysis for viral (top) or satellite (bottom) DNA accumulation in 13 *Nicotiana benthamiana* plants agroinoculated with *Tomato yellow leaf curl China virus*-Y10 (Y10) or *Tobacco curly shoot virus*-Y35 (Y35) with or without cognate or noncognate DNA β (Y10 β, Y35 β). Total nucleic acids (10 µg) were extracted at 30 days postinoculation. Blots were hybridized with A, Y35; C, Y10; or B and D, DNA β probes, respectively. The positions of single-stranded (ss)DNA forms are indicated.

TABLE 1. Nucleotide sequences of primers

Primer ^a	Sequence (5'–3')	Position in DNA A or DNA β (nucleotides)
Specific detection ^b		
β02	GGTACCTACCCTCCAGGGGTACAC	1,265–1,289
Y35β	GCCAAAAAGGATTACTGGGTAT	67–88
Y10β	CGGCATTATTTTGAGGCAGT	345–364
Y35F1	CGTAGGCCTGTGGATAAACCTCAAGAT	720–746
Y10F1	CGTCACCAGAAGACAAATGT	406–428
Y35+10R	GGAAGCCAGTTCAAATTTAAAGG	1,738–1,717
Southern blot ^c		
β01	GTAGGTACCACTACGCTACGCAGCAGCC	1,290–1,314
Y35F2	GACCAAGAATCACACTAAC	674–692
Y35R2	TAGAGCACCAGAACCCTCC	1,371–1,353
Y10F2	GAAGAACCATAACAAACACCG	671–690
Y10R2	TTCCTGAGGAACACGACGT	1,345–1,326
Y35βF	TTACTGGGTATGCAATTTG	78–96
Y35βR	GTACTAACGTCTAACTACAC	767–748
Y10βF	GGTAATGCAATTGGGCCG	71–88
Y10βR	CTTTTACACATAATAATCCAG	768–747

^a F: viral sense; R: complementary sense.

^b Primers used for specific detection of DNA A and DNA β.

^c Additional primers used for Southern blot analysis.

be *trans*-replicated by both Y10 and Y35, respectively, no co-infections of DNA β species were found in samples from plants naturally infected in the field (data not shown). To study the possible competition between DNA β species, *N. benthamiana* and *N. glutinosa* plants were agroinoculated with Y10+Y10 β +Y35 β or Y35+Y35 β +Y10 β . PCR using primer pairs Y10F1/Y35+10R, Y35F1/Y35+10R, Y35 β / β 02, or Y10 β / β 02 specific for Y10, Y35, Y35 β , and Y10 β , respectively, was conducted to detect these DNA molecules in the test plants at different times after inoculation. At 15 days postinoculation (dpi), both species of DNA β were detected in all agroinoculated *N. benthamiana* and all but one of the *N. glutinosa* plants (Table 2), indicating that both satellites could be *trans*-replicated simultaneously by either Y10 or Y35. Interestingly, at 50 dpi, the DNA band corresponding to Y10 β was detected from only 15 of 25 *N. benthamiana* plants inoculated with Y35+Y35 β +Y10 β . Similar results were obtained with *N. glutinosa* plants. Subsequently, the numbers of plants of both *Nicotiana* spp. containing Y10 β decreased as the time after inoculation increased. At 120 dpi, Y10 β was not detected by PCR in any of these plants, suggesting that it was absent or occurred at undetectable levels. Y35 β was always present in all agroinoculated plants (Table 2). Similarly, when *N. benthamiana* or *N. glutinosa* plants were inoculated with Y10+Y10 β +Y35 β , the numbers of plants of both *Nicotiana* spp. that contained Y35 β decreased as the interval after inoculation increased. At 80 dpi, no band corresponding to the noncognate Y35 β was obtained from any of the inoculated plants, whereas Y10 β remained present in all plants in association with its cognate helper virus. The bands specific for either Y10 or Y35 could be amplified from these inoculated plants throughout the experiment (Table 2). No band corresponding to Y10, Y35, Y35 β , and Y10 β was obtained in mock-inoculated plants (data not shown).

Southern blot analysis was then performed to analyze the *trans*-replication of DNA β in these plants, and the results showed that the noncognate DNA β , either Y10 β (Fig. 2, top, lanes 2 and 6) or Y35 β (Fig. 2, bottom, lanes 4 and 8), was eventually lost in all the plants co-inoculated with one *Begomovirus* sp. and two DNA β species. In contrast, cognate Y10 β or Y35 β was always present at the later stages of infection (Fig. 2).

The β C1 gene is not involved in dominance of the cognate DNA β . Previous work showed that the DNA β C1 gene has various functions (10,11,19). To test whether it controls the *trans*-replication dominance of the cognate DNA β , *N. benthamiana* and *N. glutinosa* plants were agroinoculated with combinations of wild-type noncognate DNA β and the cognate DNA β mutants (Y10 β -M or Y35 β -M). PCR using specific primer pairs Y10F1/Y35+10R, Y35F1/Y35+10R, Y35 β / β 02, and Y10 β / β 02 corresponding to Y10, Y35, Y35 β or Y35 β -M, and Y10 β or Y10 β -M,

respectively, was done to detect these DNA molecules in plants. Although both species of noncognate DNA β could be detected at earlier stages of infection of plants inoculated with Y10+Y10 β -M+Y35 β or Y35+Y35 β -M+Y10 β , the number of plants containing the noncognate DNA β decreased as the interval after inoculation increased. No band corresponding to the noncognate Y10 β at 120 dpi or noncognate Y35 β at 80 dpi was obtained by PCR from any of the plants (Table 3). In contrast, cognate DNA β containing the mutant β C1 gene was always present in the same plants. For *N. benthamiana* plants without noncognate DNA β , sequence analyses of 12 clones from 12 *N. benthamiana* plants inoculated with Y10+Y10 β -M+Y35 β and 10 clones from 10 *N. benthamiana* plants inoculated with Y35+Y35 β -M+Y10 β plants confirmed that each cognate DNA β mutant (Y10 β -M or Y35 β -M) was maintained in these plants and no noncognate DNA β was identified (data not shown). The band specific for either Y10 or Y35 was obtained from all the test plants throughout the experi-

Fig. 2. Southern blot analysis of *trans*-replication of cognate and noncognate satellites in *Nicotiana benthamiana* (lanes 1 to 4) and *N. glutinosa* (lanes 5 to 8) plants agroinoculated with Y35+Y35 β +Y10 β or Y10+Y10 β +Y35 β . Total nucleic acids (10 μ g) were extracted at 30, 80, or 100 days postinoculation (pi) from plants showing systemic symptoms. Blots were hybridized with either Y10 β (above) or Y35 β (bottom) probes. The positions of single-stranded (ss)DNA forms are indicated.

TABLE 2. Polymerase chain reaction detection of *trans*-replication of DNA β satellites in plants inoculated with two satellites and one helper virus

Inoculum, dpi ^a	Detection of viral or satellite DNA ^b							
	<i>Nicotiana benthamiana</i> ^c				<i>N. glutinosa</i>			
	Y35	Y10	Y35 β	Y10 β	Y35	Y10	Y35 β	Y10 β
Y35+Y35 β +Y10 β								
15	25/25	—	25/25	25/25	8/8	—	8/8	8/8
50	25/25	—	25/25	15/25	8/8	—	8/8	4/8
80	25/25	—	25/25	7/25	8/8	—	8/8	1/8
120	25/25	—	25/25	0/25	8/8	—	8/8	0/8
Y10+Y10 β +Y35 β								
15	—	27/27	27/27	27/27	—	8/8	7/8	8/8
30	—	27/27	11/27	27/27	—	8/8	2/8	8/8
50	—	27/27	4/27	27/27	—	8/8	1/8	8/8
80	—	27/27	0/27	27/27	—	8/8	0/8	8/8

^a Y35 = Tobacco curly shoot virus (TbCSV)-Y35; Y35 β = TbCSV-Y35 DNA β ; Y10 = Tomato yellow leaf curl China virus (TYLCCNV)-Y10; Y10 β = TYLCCNV-Y10 DNA β ; dpi = days postinoculation.

^b Number of plants containing specified DNA/number of plants inoculated; — = no viral DNA was obtained.

^c Total of three independent trials.

ment (Table 3). No band corresponding to Y10, Y35, Y35 β or Y35 β -M, and Y10 β or Y10 β -M was obtained in mock-inoculated plants (data not shown). This result indicates that dominance of the cognate DNA β does not require expression of the wild-type β CI gene.

DISCUSSION

The Rep-binding motifs of *Begomovirus* spp. are composed of short reiterated sequences (iterons), which typically differ between *Begomovirus* spp. and are located immediately upstream of a highly conserved stem-loop structure. Rep–iteron interaction is considered to be a key step in the initiation of begomoviral DNA replication (16). Rep–DNA interaction is usually highly specific for each *Begomovirus* spp.; *trans*-replication of a *Begomovirus* sp. by a noncognate Rep is usually impossible (2,20). DNA β satellite species are found to be associated with many *Begomovirus* spp. (4,6–8,13,17,19,22,23,28,29,33,37). Like the satellite molecules associated with a number of RNA viruses (26), these DNA β satellites have little sequence identity to either the DNA A or DNA B genomic molecules of *Begomovirus* spp. but nevertheless rely completely on their helper viruses for replication, movement within plants, encapsidation, and whitefly transmission between plants (7,22,23,33). DNA β molecules depend on the helper virus-encoded Rep for replication, although they do not possess the iteron sequences of the helper *Begomovirus* spp. Moreover, DNA β species were reported to be *trans*-replicated by noncognate *Begomovirus* spp. For example, *Tomato leaf curl virus* (ToLCV) sat-DNA, which is a defective non-symptom-modulating molecule derived from DNA β , can be *trans*-replicated by ToLCV, by *African cassava mosaic virus* (ACMV), and even by a *Curtovirus* sp. (*Beet curly top virus*) (13,20). ToLCV, which occurs in Australia, can interact with the DNA β associated with CLCuMV, which occurs in the Indian subcontinent (1). Indeed, both AYVV DNA β and CLCuMV DNA β can be *trans*-replicated by noncognate helper *Begomovirus* spp. that have different Rep-binding specificities needed for replication (5,21,32). These findings suggest that the interaction between *Begomovirus* spp. and DNA β molecules is not highly specific (4). In this report, our results also show that both Y10 β and Y35 β can be *trans*-replicated stably by a noncognate helper virus (Y35 or Y10), although the level of *trans*-replication of noncognate DNA β clearly was lower than that of cognate DNA β .

Infections of individual plants with mixtures of two or more *Begomovirus* spp. are common (15,24,25). In our tests, we found that two distinct DNA β species can be *trans*-replicated simultaneously by the same helper *Begomovirus* sp. When only a noncognate DNA β was present in the virus culture, it was maintained

indefinitely; but, when both cognate and noncognate satellites were present, the cognate DNA β became dominant and the noncognate DNA β was eventually lost. Thus, DNA β species compete for *trans*-replication, with the cognate species having an advantage. This may explain why there are no reports of co-infection DNA β species in field-infected plant samples except for the two tobacco samples in Yunnan, China (12).

Begomovirus–DNA β disease complexes fall into two general types that are distinguishable by the necessity of their DNA β molecules in pathogenicity. In the majority of these disease complexes, DNA β is responsible for the induction of typical symptoms and all the virus isolates are always associated with DNA β . In the remaining minority, the interaction of DNA β with its helper virus is much less stringent (19). Y10 β belongs to the majority type and Y35 β belongs to the minority type. We are not clear whether the competition between Y10 β and Y35 β is an oddity of the particular virus–satellite–host system or if it is true of all other DNA β molecules as well. These questions will be answered by further studies with more virus–satellite–host systems.

Previous work showed that DNA β is a symptom and pathogenicity determinant, as well as a suppressor of gene silencing (3,9–11,27,31), and that these effects depend on its β CI gene. In our tests, mutating this gene in a cognate DNA β did not alter the dominance of cognate over noncognate satellite species, suggesting that the dominance is not a function of β CI protein. How DNA β is recognized by helper viral Rep, and the molecular mechanism underlying the promiscuity of DNA β species, remain obscure. Deletion mutagenesis of AYVV DNA β localizes the origin of replication to $\approx$ 360 nucleotides, encompassing the ubiquitous nonanucleotide/stem-loop structure, satellite-conserved region (SCR), and part of the intergenic region immediately upstream of the SCR (30), while real-time PCR demonstrates that the G-box motif (CACGTG), located 143 nucleotides upstream of the β CI start codon, is required for efficient replication of CLCuMV DNA β (14). The sequences of G-box motif between Y10 β and Y35 β are quite different. It would be interesting to elucidate whether this G-box motif is involved in selective maintenance of DNA β , and further studies will be continued to unravel the molecular basis for this selective maintenance of DNA β .

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TABLE 3. Polymerase chain reaction detection of *trans*-replication of two DNA β satellites in mixed infections when one satellite is mutated

Inoculum, dpi ^a	Detection of viral or satellite DNA ^b							
	<i>Nicotiana benthamiana</i> ^c				<i>N. glutinosa</i>			
	Y35	Y10	Y35 β	Y10 β	Y35	Y10	Y35 β	Y10 β
Y35+Y35 β -M+Y10 β								
15	19/19	–	19/19	19/19	6/6	–	6/6	6/6
40	19/19	–	19/19	14/19	6/6	–	6/6	3/6
80	19/19	–	19/19	6/19	6/6	–	6/6	2/6
120	19/19	–	19/19	0/19	6/6	–	6/6	0/6
Y10+Y10 β -M+Y35 β								
15	–	20/20	20/20	20/20	–	6/6	6/6	6/6
30	–	20/20	9/20	20/20	–	6/6	3/6	6/6
50	–	20/20	4/20	20/20	–	6/6	0/6	6/6
80	–	20/20	0/20	20/20	–	6/6	0/6	6/6

^a Y35 = *Tobacco curly shoot virus* (TbCSV)-Y35; Y35 β = TbCSV-Y35 DNA β ; Y35 β -M = β CI mutant of TbCSV-Y35 DNA β ; Y10 = *Tomato yellow leaf curl China virus* (TYLCCNV)-Y10; Y10 β = TYLCCNV-Y10 DNA β ; Y10 β -M = β CI mutant of TYLCCNV-Y10 DNA β ; dpi = days postinoculation.

^b Number of plants containing specified DNA/number of plants inoculated; – = no viral DNA was obtained.

^c Total of three independent trials.

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