

Banana bunchy top nanovirus DNA-1 encodes the ‘master’ replication initiation protein

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Banana bunchy top nanovirus has a multicomponent, circular single-stranded DNA genome comprising at least six integral components, BBTV DNA-1 to -6, which have been consistently associated with bunchy top disease worldwide. At least three other components, BBTV S1, S2 and Y, which have been isolated from Taiwanese BBTV isolates, do not appear to be integral components. We show here that both BBTV DNA-1 and S1, which encode replication initiation (Rep) proteins, were capable of self-replication when bombarded into banana embryogenic cell suspensions. However, only BBTV DNA-1 was capable of directing the replication of two other BBTV genomic components, namely BBTV DNA-3 which encodes the coat protein, and DNA-5 which encodes a retinoblastoma binding-like protein. These results indicate that (i) BBTV DNA-1 is the minimal replicative unit of BBTV and encodes the ‘master’ viral Rep and (ii) BBTV S1 is possibly a satellite DNA which is unable to replicate integral BBTV components.

Introduction

Banana bunchy top nanovirus (BBTV) has a genome comprising at least six components of circular single-stranded DNA (BBTV DNA-1 to -6) (Harding *et al.*, 1993; Burns *et al.*, 1995). The components are approximately 1 kb and share a common genome organization; all have a conserved major common region (CR-M) and stem-loop common region (CR-SL), a potential TATA box 3' of the stem-loop, at least one major gene in the virion sense and a polyadenylation signal associated with each gene (Burns *et al.*, 1995; Beetham *et al.*, 1997, 1999). The major gene of BBTV DNA-1 also contains a smaller internal gene in a +2 reading frame (Beetham *et al.*, 1997). There are two groups of BBTV, the South Pacific group (isolates from Australia, Burundi, Egypt, Fiji, India, Tonga and Western Samoa) and the Asian group (Vietnam, Philippines and Taiwan), based on sequence analysis of BBTV DNA-1, -3 and -6 (Karan *et al.*, 1994, 1997; Wanitchakorn *et al.*, 2000*b*). These two groups differ by an average of 9.6% (DNA-1), 11.86% (DNA-3) and 14.5% (DNA-6) over the entire nucleotide sequence with an average difference of 32% (DNA-

1), 38.6% (DNA-3) and 27% (DNA-6) within the CR-M (Karan *et al.*, 1994, 1997; Wanitchakorn *et al.*, 2000*b*).

Hafner *et al.* (1997*b*) have demonstrated that the major gene of DNA-1 encodes a replication initiation protein (Rep) while Wanitchakorn *et al.* (1997) have shown that DNA-3 encodes the viral coat protein. Recently, the BBTV DNA-5 gene product has been shown to contain an LXCXE motif and to have retinoblastoma protein (Rb)-binding activity (Wanitchakorn *et al.*, 2000*a*). Wanitchakorn *et al.* (2000*a*) have suggested that the gene product of BBTV DNA-5 is produced very early in the infection cycle and is responsible for switching the first infected cells to S-phase in preparation for virus replication. This is supported by the results of Hafner *et al.* (1997*a*) who showed that BBTV DNA-5 is the most efficiently self-primed of the BBTV DNA components. One component of another nanovirus, faba bean necrotic yellows virus (FBNYV) (FBNYV DNA-10), has been shown to encode a protein called Clink (cell cycle link) which is capable of binding human Rb and enhancing replication of FBNYV Rep proteins when co-infected (Aronson *et al.*, 2000). Since BBTV DNA-5 shares similar motifs with FBNYV DNA-10, it is likely that gene products of these components fulfil similar functions. BBTV DNA-4 and -6 appear to encode movement and nuclear shuttle proteins (Wanitchakorn *et al.*, 2000*a*), respectively, while the functions of the gene products of DNA-2 and the small internal gene of BBTV DNA-1 are unknown.

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BBTV DNA-1 to -6 have been consistently associated with BBTV worldwide, suggesting they are integral components of the BBTV genome (Karan *et al.*, 1997). Recently, two new BBTV-associated sequences, BBTV S1 and S2, have been characterized. Unlike BBTV DNA-1 to -6, S1 and S2 appear to vary in distribution with a high prevalence in Asian group isolates and a low prevalence or absence in South Pacific isolates (Horser *et al.*, 2000). These two sequences, like BBTV DNA-1, putatively encode Rep proteins but, unlike BBTV DNA-1, neither BBTV S1 nor S2 contain the small internal gene (Beetham *et al.*, 1997). Based on their restricted distribution and different genome organization, it is probable that BBTV S1 and S2 represent satellite DNAs and that the Rep proteins encoded by these components are not necessary for BBTV replication. Several additional Rep protein-encoding components have been associated with FBNYV but only one component, FBNYV DNA-2, is capable of self replication as well as initiating replication of the non-Rep protein-encoding components of this virus. A similar phenomenon has been observed for the two other nanoviruses, milk vetch dwarf virus (MDV) and subterranean clover stunt virus (SCSV), giving rise to the Master Rep concept (Timchenko *et al.*, 1999, 2000). In this study, we investigated whether the Rep proteins encoded by BBTV DNA-1 and S1 could direct self replication and/or replication of other BBTV components.

Methods

■ **Nucleic acid extraction.** Nucleic acid was extracted from Australian and Taiwanese BBTV-infected material and from banana embryogenic cell suspensions essentially as described (Stewart & Via, 1993).

■ **Generation of BBTV 1.1 mers.** The 1.1 mers of BBTV DNA-1, -3, -5 and S1 were generated by a PCR-based strategy using primers designed from the sequences of BBTV DNA-1, -3, -5 (Harding *et al.*, 1993; Burns *et al.*, 1995) and BBTV S1 (GenBank accession no. AF216221; Horser *et al.*, 2000) (Table 1). PCR mixes comprised 20 pmol of each primer, 10 mM dNTPs, 1 U *Taq* DNA polymerase (Roche) and 1 µl of total nucleic acid extract (diluted 1/10 or 1/100 in TE buffer, pH 8). The reaction mixes were denatured at 94 °C for 4 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min followed by 1 cycle of 72 °C for 10 min. Following amplification, PCR products were purified using a High Pure PCR Purification kit (Roche), and were digested at 37 °C for 2 h with *Ava*II, *Hin*fI, *Bgl*II and *Xba*I for BBTV DNA-1, -3, -5 and S1, respectively. The digested products were purified using High Pure columns (Roche) and were ligated into pGEM-T (Promega) at 4 °C overnight using 2 U T4 DNA ligase (Roche). The ligations were then electroporated into *E. coli* DH5. Selected clones were sequenced using automated sequencing and Big Dye Termination Cycle Sequencing Ready Reaction (PE Applied Biosystems). Primers used for sequencing were either universal sequencing primers (US Biochemical) or primers designed from published BBTV sequences.

■ **Microprojectile bombardment.** Bluggoe (*Musa* spp. ABB group) embryogenic suspension cultures were maintained and harvested essentially as described by Dugdale *et al.* (1998) except that cells were harvested 4 days post-subculture and were bombarded between 24 h and 4 days post-plating. Plasmid 1.1mer constructs for bombardment were purified using a Bresa-pure Maxi-prep Plasmid Purification kit (Geneworks). Bluggoe embryogenic cells were bombarded with various combinations of 1.1 mers using a particle inflow gun and gold microcarriers (Bio-Rad). Gold was prepared essentially as described by Mahon *et al.* (1996); a 40 µl suspension (containing 3 mg gold) was coated with 2 pmol of DNA and 4 µl of the suspension was used per bombardment. All gold preparations were supplemented with 'stuffer DNA' (comprising 900 bp of 5' untranslatable flanking sequence of the sugarcane actin gene) to ensure each treatment contained the same molar concentration of DNA. Each treatment was sampled at zero (i.e.

Table 1. Primers used in the construction of BBTV 1.1 mers

Bold letters indicate restriction sites within the primer sequences.

Primer	Sequence (5' to 3')	Component	Location (nt)
1-AvaII F1	TTATCGAAG GTCC CTTCGAGTTTGGTTC	DNA-1	360–385
1-AvaII R1	TT GGT CCTTCGATTCTTGATC		370–351
BT1.1F	ATGACTTTACAGCGCACGCTCCGACAAAAG		970–999
BT1.1R	GTAATGGAGAGGGGGGAGGTCTATTTATAG		80–51
3-Hin fI F	GCAG GAGT CGCAACAGGAACATCA	DNA-3	584–605
3-Hin fI R	TT GCGAGT CTGCTTCAAGTTCCC		594–570
BT3.1F	TAAGCATCACCCACCACCTTTAG		1016–1040
BT3.1R	GGGCCCTATATCCACAATCCATTA		72–49
C5-Bg I III F	GCGA AAGATCT CTATTGTGATGAGG	DNA-5	564–586
C5-Bg I III R	CGCA AAGATCT TGATATACTGAG		576–545
BT5.2F	AACGGAATAATATGAGCTGGCAAC		961–984
BT5.2R	TACTGCATTCTCACGTGCTGCTGT		81–57
S1T1F	G CTCTAGAT CTCCAGAACGCAT	S1	402–421
S1T1R	G CTCTAGAA ACCATCTCCGATA		407–388
S1.1F	ATTATGCACTATGACAATCGTACGCTATGA		930–959
S162R	ACTGATGACGTAGGGGCGAGGAAGA		62–38

immediately after bombardment), 4 and 8 days post-bombardment, and embryogenic cells were stored at -20°C until analysed.

■ Analysis of bombarded embryos

PCR. To determine whether the bombarded BBTV 1.1 mers had been excised from the plasmid vector and recircularized into double-stranded monomers, a PCR-based strategy employing immediately adjacent, outwardly extending component-specific primers was used. Cycling conditions were as previously outlined. The primers were BBTV DNA-1 (BT1-947F 5' GTTGGTTTCTTGCTGAACAAG 3'; 30merF3 5'GGAAGAAGCCTCATCTGCTTCAGAGAGC 3'), BBTV DNA-3 (3-HinfI and 3-HinfIR; Table 1), BBTV DNA-5 (BT5-726F 5' TGTAATATCCATTATCATCAATAA 3'; BT5-239R 5' TTCTCTCCGACGAGTGATTTCGGAAA 3'), BBTV S1 (S1T1F and S1T1R; Table 1).

Southern blotting and hybridization. Nucleic acid extracts (up to 60 μg) were electrophoresed through 1.5% agarose gels in TAE buffer, pH 7.8, and stained with ethidium bromide. Full-length or partial clones of BBTV DNA-1, -3, -5 and S1, as well as extracts from an Australian BBTV isolate, were used as positive controls. Nucleic acids were transferred to positively charged nylon membranes (Roche) as described by Southern (1985). Digoxigenin (DIG)-labelled component-specific probes were generated using PCR and DIG-11-dUTP (9:1) as per the manufacturer's protocol (Roche). Primers were designed to amplify the complete ORF of BBTV DNA-1 (ORF1 BamF 5' TTGGATCCATGGCGCGATTGTGGTATGCTGGATGTTT 3'; ORF1 SacR 5' TTTAAAGAGCTCTCAGCAAAACATTTTCGATC 3'), BBTV DNA-3 (BT3-13F 5' ATGTTACAGACAAGAAATGGCTAGG 3'; BT3-740R TCAACATGATATGTAATCTGTTCTGG 3'), BBTV DNA-5 (BT5-240F 5' ATGAGTTCTGGGAATCGTCTGCCATG 3'; BT5-725R 5' TTAGAGTAAATGTTACATAATCTG 3') and BBTV S1 (S163F 5' ATGCACTTTTAAATGGTGCTTCA 3'; S1ORF 5' TTAACAATAAATAATCTTTATTCTATCTTC 3'). Membranes were prehybridized in DIG Easy Hybe (Roche) for 1–2 h, and denatured probes were added directly to the prehybridization solution and hybridized for 12–16 h at 42°C . Membranes were then subjected to high stringency washes ($0.1 \times \text{SSC}$, 0.1% SDS) at 65°C prior to development as per the manufacturer's instructions (Roche).

Results

In vitro excision of BBTV 1.1 mers

Banana embryogenic cell suspensions were bombarded with BBTV 1.1 mers both individually and in various combinations. To detect the excision and recircularization of the 1.1 mers, DNA was extracted from the bombarded banana embryogenic cell suspensions and used in a PCR using immediately adjacent, outwardly extending component-specific primers. These primers were designed in such a way that amplification would only occur if the 1.1 mers had been nicked at each stem-loop and recircularized into a single monomeric unit. When analysed at day zero for DNA-1, -3, -5 and S1, no PCR products were amplified from any of the bombarded cells (Table 2). However, at 4 days post-bombardment, PCR products of the size expected for BBTV DNA-1 and S1 were amplified from embryogenic cells bombarded with 1.1 mers of BBTV DNA-1 and BBTV S1 alone, respectively, as was also the case for the embryogenic cells bombarded with these components in combination with BBTV DNA-3 and/or DNA-5. PCR products of the sizes expected

Table 2. PCR results for excision and recircularization of BBTV components

Bombarded components	Components detected*		
	Day 0	Day 4	Day 8
BBTV-1	–	1	1
BBTV-5	–	–	–
BBTV-1 and 5	–	1 and 5	1 and 5
BBTV-3	–	–	–
BBTV-1 and 3	–	1 and 3	1 and 3
BBTV-3 and 5	–	–	–
BBTV-1, 3 and 5	–	1, 3 and 5	1, 3 and 5
BBTV-S1	–	S1	S1
BBTV-S1 and 3	–	S1	S1
BBTV-S1, 3 and 5	–	S1	S1
BBTV-S1, 1, 3 and 5	–	S1, 1, 3 and 5	S1, 1, 3 and 5

* 1, 3, 5 and S1 represent a positive PCR result for BBTV DNA-1, -3, -5 and S1, respectively.

for BBTV DNA-3 and -5 were only amplified from embryogenic cells which had been co-bombarded with BBTV DNA-1; BBTV DNA-3 and -5 were not detected in embryogenic cells co-bombarded with BBTV S1, except for the combination of BBTV DNA-1, -3, -5 and S1, in which BBTV DNA-1 was also present. These results indicated that while both BBTV DNA-1 and S1 were capable of initiating self-replication, only BBTV DNA-1 was capable of initiating replication of BBTV DNA-3 and -5.

Replication of BBTV components

Initially, experiments were designed to obtain further evidence that BBTV DNA-1 encoded the viral Rep and to examine the effect of DNA-5, the Rb-binding-like protein, on replication levels. To determine whether replication of BBTV genomic components had occurred, DNA was extracted from the banana embryos at days 0, 4 and 8 post-bombardment and hybridized with probes specific for BBTV DNA-1 and -5. Replication was assessed by the presence of (i) different conformational forms of BBTV genomic DNA and/or (ii) multimeric forms of BBTV DNA produced during rolling circle replication.

When 1.1 mers of DNA-1 alone were bombarded into the embryogenic cells, no replication products were observed in any of the bombarded embryogenic cells at day 0, with hybridization only occurring with the higher molecular mass input plasmid DNA (Fig. 1). At day 4, however, BBTV DNA-1-specific bands of approximately 1 kbp, representing supercoiled, linear and open-circular forms of BBTV genomic DNA, were detected in embryogenic cells, indicating that replication

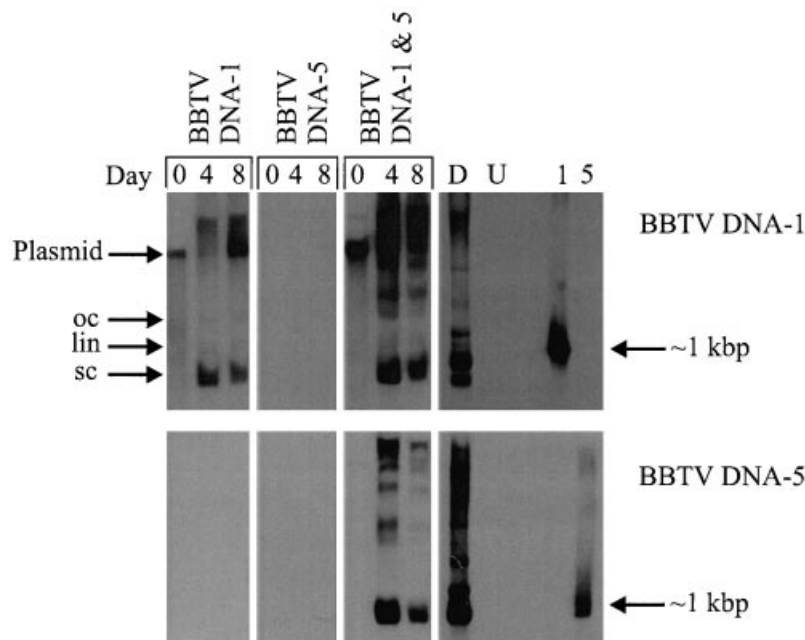


Fig. 1. Replication of BBTV DNA-1 and -5 in bombarded banana embryogenic cell suspensions. Cloned 1.1 mers of BBTV DNA-1 and/or DNA-5 were bombarded into banana embryogenic cell suspensions and replication was examined at days 0, 4 and 8 post-bombardment by Southern analysis using BBTV DNA-1- and DNA-5-specific probes as indicated on the right of each panel. Input plasmid as well as BBTV replicative intermediates [supercoiled (sc), linear (lin) and open circular (oc)] are indicated. D and U represent nucleic acids extracted from diseased and uninfected tissue, respectively, while 1 and 5 indicate component-specific controls for BBTV DNA-1 and -5, respectively.

of this component had occurred. While the intensity of the DNA-1-specific bands appeared to decrease slightly after 8 days, this observation was not consistent in all subsequent experiments. When 1.1 mers of DNA-5 alone were bombarded into the banana embryogenic cells, no replication products were observed at either 0, 4 or 8 days post-bombardment, indicating that this component was not able to self-replicate (Fig. 1). However, when 1.1 mers of DNA-1 and -5 were co-bombarded, BBTV DNA-5-specific bands were observed in the cells at days 4 and 8 post-bombardment, showing that this component was replicated in the presence of DNA-1.

Based on this result, we examined whether DNA-1 was able to replicate another BBTV genomic component, the coat protein-encoding DNA-3, and also to determine the replicative capability of BBTV S1, the Rep protein-encoding, putative satellite component. Using DNA-1 as a probe, replication products were detected in cells bombarded with DNA-1, -3 and -5, with or without BBTV S1, at both 4 and 8 days post-bombardment (Fig. 2). However, DNA-1 appeared to replicate at slightly lower levels in the presence of BBTV S1. When DNA-3 was used as a probe, this component was also shown to replicate in the presence of DNA-1 and -5 (Fig. 2). In contrast to DNA-1, however, no replication of DNA-3 was observed in cells co-bombarded with DNA-3 and BBTV S1. Further, no replication of DNA-3 occurred in the absence of DNA-1 (Fig. 2). The results obtained using DNA-5 as a probe were essentially similar to those with DNA-3; DNA-5 required the presence of DNA-1 for replication and was able to replicate in the presence of DNA-1 and -3 but not in the presence of BBTV S1 (Fig. 2). Finally, when BBTV S1 was used as a probe, self-replication of BBTV S1 was observed in bombarded cells (result not shown). Replication of BBTV S1 was also detected

in cells co-bombarded with DNA-3, while increased levels of replication were observed in cells co-bombarded with DNA-3 and -5 (Fig. 2). This level of replication was further increased in the presence of DNA-1 (Fig. 2).

Discussion

BBTV DNA-1 to -6 have been consistently associated with banana bunchy top disease worldwide in all geographical isolates tested (Karan *et al.*, 1994, 1997) although it has yet to be confirmed that these components represent the complete infectious unit of BBTV. We have now demonstrated that the minimal replicative unit of BBTV consists of BBTV DNA-1, which encodes the Rep protein and a 5 kDa protein of unknown function, since this component alone was able to self-replicate at low levels when bombarded into banana embryogenic cells and direct the replication of other BBTV DNA components. Furthermore, we have shown that BBTV DNA-1, but not BBTV S1, can direct the replication of a DNA component which has no obvious role in BBTV replication, namely the coat protein-encoding BBTV DNA-3. These results indicate that BBTV DNA-1 encodes the 'master' Rep protein.

Three possible satellite DNAs have been isolated from BBTV infections; BBTV S1 (Horser *et al.*, 2000), BBTV Y/W1 (Yeh *et al.*, 1994; Wu *et al.*, 1994) and BBTV S2/W2 (Horser *et al.*, 2000; Wu *et al.*, 1994). The genome organization of these DNA components resembles that of BBTV DNA-1 in that they putatively encode Rep proteins and have a stem-loop structure that is probably the origin of replication. The demonstration that BBTV S1 was capable of self-replication but was not capable of replicating integral components of the BBTV genome provides further evidence that BBTV S1, and therefore

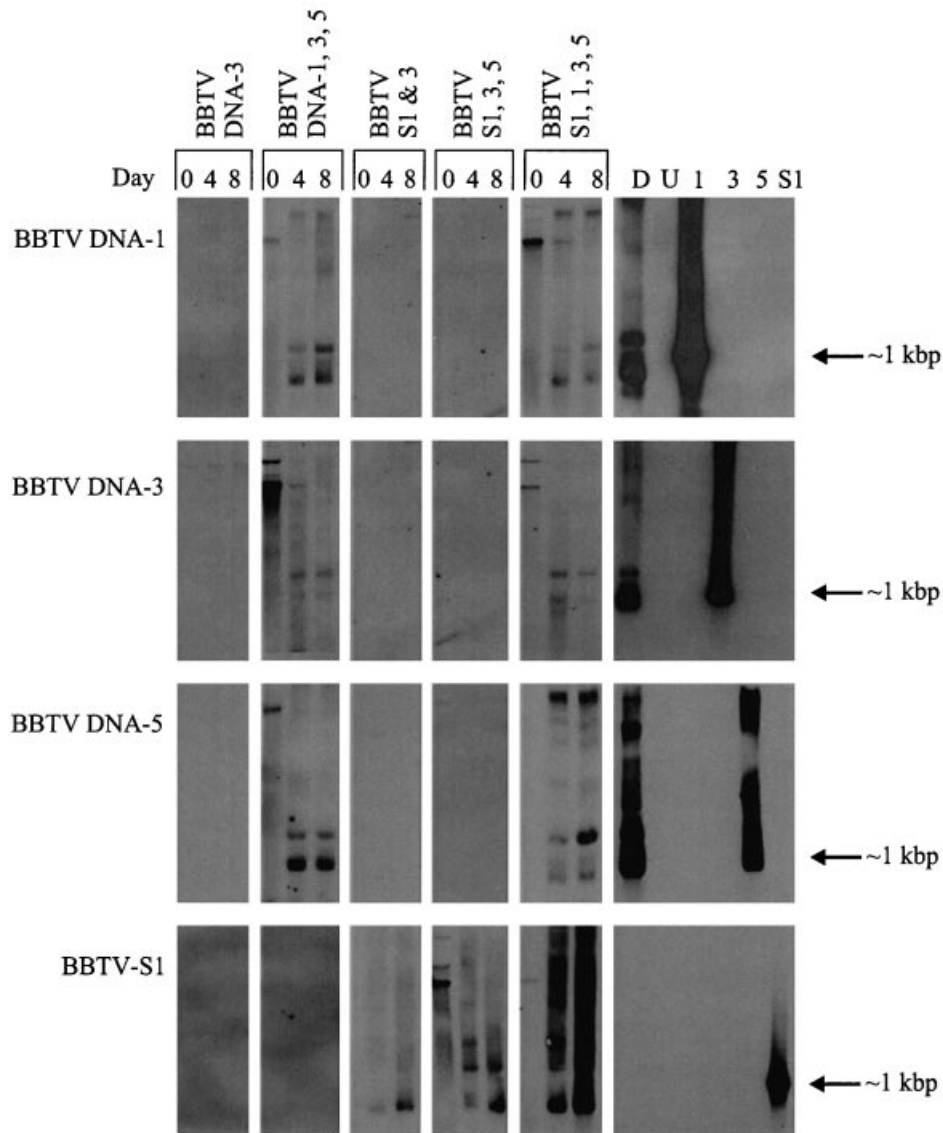


Fig. 2. Replication of BBTV DNA-1, -3, -5 and S1 in bombarded banana embryogenic cell suspensions. Cloned 1.1 mers of these components were bombarded into banana embryogenic cell suspensions in various combinations and replication was examined at 0, 4 and 8 days post-bombardment by Southern analysis using component-specific probes as indicated on the left of each panel. D and U represent nucleic acids extracted from diseased and uninfected tissue, respectively, while 1, 3, 5 and S1 indicate component-specific controls for BBTV DNA-1, -3, -5 and S1, respectively.

probably S2 and Y1, are novel satellite components of BBTV with equivalents in FBNYV, MDV and SCSV. In the characterization of FBNYV, MDV and SCSV, among the first components to be isolated were the Rep-encoding component equivalents of BBTV S1 (Boevink *et al.*, 1995; Katul *et al.*, 1995; Sano *et al.*, 1998). Further, Timchenko *et al.* (1999) reported the presence of multiple Rep proteins associated with the FBNYV genome. They demonstrated that, of the five potential Rep-encoding components associated with FBNYV, only one component (DNA-2) was capable of replicating itself as well as the other six non-Rep-encoding FBNYV DNAs. The isolation of these additional Rep-encoding components before the

'master' Rep-encoding component suggests that the putative satellite Rep-encoding components occur in higher concentrations than the integral genomic components of these viruses. This hypothesis is supported by our results which showed that BBTV S1 was replicated to a very high level in the presence of the BBTV DNA-1, -3 and -5. Thus, like FBNYV, MDV and SCSV, BBTV has a single component that encodes the viral 'master' Rep protein that is responsible for directing the replication of all other integral viral genomic components. In contrast, additional Rep-encoding components that are often associated with nanovirus (and sometimes even with geminivirus) infections are capable of self-replication only. It is thus

possible that these satellite Rep protein components depend on their helper virus (i) during early stages of infection through the expression of the virus-encoded Rb-binding-like protein, (ii) for nuclear shuttling, (iii) for cell-to-cell movement, (iv) for long-distance movement and (v) for plant-to-plant transmission.

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