

Two mRNAs are transcribed from banana bunchy top virus DNA-1

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We have mapped the mRNA transcripts of banana bunchy top virus (BBTV) DNA-1. Northern hybridization and 3' RACE analysis identified two polyadenylated RNAs associated with BBTV DNA-1. Previously, one major ORF in the virion sense of DNA-1 had been identified, which encoded a putative replication protein (Rep). An mRNA was identified in BBTV infected bananas that was clearly transcribed from this Rep ORF. Further, a second transcript was identified which mapped to an ORF completely within the Rep ORF. This encoded a putative 5 kDa protein of unknown function. Both these transcripts were also identified in a tobacco plant that had been transformed with *Agrobacterium tumefaciens* harbouring a binary construct containing the Rep ORF from BBTV DNA-1. This Rep ORF

was inserted 3' of a cauliflower mosaic virus 35S promoter and 5' of a vegetable storage protein terminator. The transcripts mapped from these tobacco plants were identical at the 3' end to the transcripts from BBTV infected banana plants. The site of polyadenylation for the Rep ORF was at base 963 immediately 3' of the translational stop codon confirming that the polyadenylation signals for this transcript were all within the ORF. However, the internal ORF had a large untranslated region of 272 bases with its site of polyadenylation at nucleotide 803 and a polyadenylation signal 3' of the translational stop codon. A possible upstream termination signal (A/TTGTAA) was identified and was conserved within BBTV DNA-1 sequences from different international isolates.

Introduction

Banana bunchy top virus (BBTV) is an ssDNA plant virus with small isometric virions, 18–20 nm in diameter and it is persistently transmitted by the aphid *Pentalonia nigronervosa* (Harding *et al.*, 1991). Its genome consists of at least six components of circular ssDNA each of about 1 kb (Harding *et al.*, 1993; Burns *et al.*, 1995; Xie & Hu, 1995). Each DNA component contains two conserved regions, the stem-loop common region (CR-SL) and the major common region (CR-M) (Burns *et al.*, 1995). The CR-SL includes a sequence capable of forming a stem-loop structure, the loop sequence of which has the highly conserved nonanucleotide sequence similar to all geminiviruses (Lazarowitz, 1992). Each of the six components also contains one large open reading frame (ORF) in the virion sense (Burns *et al.*, 1995; Xie & Hu, 1995; P. R. Beetham, R. M. Harding & J. L. Dale unpublished data) with a

potential TATA box 3' of the CR-SL and with associated polyadenylation signals. On the basis of its virion morphology, genome structure and organization, and mode of transmission, BBTV is clearly not a geminivirus, the only recognized group of ssDNA plant viruses. It is more likely that BBTV is a member of a new group of plant viruses that potentially includes subterranean clover stunt virus (SCSV) (Chu & Helms, 1988; Boevink *et al.*, 1995), faba bean necrotic yellows virus (FBNYV) (Katul *et al.*, 1995), milk vetch dwarf virus (MVDV) (Sano *et al.*, 1993) and possibly the planthopper borne coconut foliar decay virus (CFDV) (Rohde *et al.*, 1990).

The large ORF (856 nt) of BBTV component 1 (DNA-1) potentially encodes a replication protein (Rep) of 33·6 kDa, as this putative protein contains a dNTP binding motif, GGEGKT (Harding *et al.*, 1993; Gorbalenya *et al.*, 1990). Karan *et al.* (1994) have demonstrated that this BBTV DNA-1 is present in all BBTV infections from isolates tested from 11 countries. Interestingly, Yeh *et al.* (1994) and Wu *et al.* (1994) have isolated additional DNA components associated with BBTV infections in Taiwan that encode putative Reps and these Reps

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are different to the putative Rep of BBTV DNA-1. Further, these components are different in their organization to BBTV DNA-1 to 6 of Burns *et al.* (1995).

None of the ORFs of BBTV DNA-1 to 6, or of the additional components, has been shown to be transcribed. In this paper, we report the analysis of mRNA transcripts from BBTV DNA-1 in BBTV infected bananas and in tobacco transformed with BBTV DNA-1 Rep encoding ORF under the control of the cauliflower mosaic caulimovirus (CaMV) 35S promoter.

Methods

■ **RNA extractions.** Bananas were infected with an Australian (Nambour, Queensland) isolate of BBTV by aphid transmission using viruliferous *Pentalonia nigronervosa*. RNA was extracted from plants showing characteristic symptoms 30–40 days after aphid inoculation using a method developed for isolating RNA from pine trees (Chang *et al.*, 1993). Two to three grams of infected or healthy leaf tissue from equivalent aged plants was used for all extractions. RNA quality was assessed spectrophotometrically and by denaturing gel electrophoresis.

■ **RNA electrophoresis and blotting.** Denaturing agarose formaldehyde gels were run as described in Sambrook *et al.* (1989). The size of RNAs was estimated using Pharmacia ssRNA markers (0.16–1.77 kb). RNAs were transferred from the agarose to Hybond-N+ (Amersham) using a PosiBlot pressure blotter (Stratagene) and 7.5 mM NaOH, as per the manufacturer's instructions.

■ **Transcription of strand-specific RNA probes and hybridization.** Primers E (5' ATGGCGCGATATGTGG 3') and A (5' AGCAAGAAACCAACTTT 3') (Fig. 1), designed to amplify the major Rep ORF sequence of BBTV DNA-1, were used to amplify an 882 bp fragment which was cloned directly into pGEM-T (Promega) to produce the plasmid pBT1T. The orientation of the insert in pBT1T was confirmed by sequence analysis. DNA was prepared for transcription as described in Hafner *et al.* (1995) except that pBT1T was linearized with *NcoI* for transcription with SP6 RNA polymerase. RNA was transcribed as described in the Promega Riboprobe kit except that [α -³²P]UTP was used instead of [α -³²P]CTP and the subsequent Northern hybridization performed as described in Hafner *et al.* (1995). The membranes were then

washed as described in the Promega Riboprobe kit at 65 °C. The membranes were post-treated with RNase A (1 µg/ml) to remove any non-specific hybridization as described in the Promega Riboprobe kit. Membranes were visualized by autoradiography.

■ **3' Rapid amplification of cDNA ends (3' RACE).** Following Northern analysis of RNAs associated with BBTV DNA-1, 3' RACE was used to map the 3' ends of the transcripts. Prior to the 3' RACE, the same preparation of RNA as used in the Northern analysis was tested using PCR to ensure all DNA had been removed during the extraction procedure. Primers A and B (5' GAAGGAAAGACAACGTATGC 3') (Fig. 1) specific to BBTV DNA-1 were used and the possible suppression of PCR amplification by high levels of RNA was taken into account (Pikaart *et al.*, 1993). The 3' RACE primers were primer B for the major Rep ORF transcript, primer C (5' TACAGGATATGCGTGAA 3') (Fig. 1) specific for the potential transcript associated with an internal ORF and primer D [5' CGCCTAGG(T)₁₇ 3'] for both ORFs. The cDNA was synthesized using a Pharmacia first-strand synthesis kit in a 33 µl reaction using 5 µg of total RNA from healthy or infected banana tissue and primer D [oligo(dT)]. The 3' RACE PCR was performed using 50 µl reactions containing 20 pmol of primer D and 30 pmol of either primer B or C with 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl pH 8.0, 200 µM dNTPs, 0.125 units of Perkin Elmer AmpliTaq polymerase, 5 µl of each cDNA reaction or 1 µl of a control BBTV genomic DNA. These reactions were overlaid with paraffin oil and subjected to the following cycling; 94 °C for 5 min, 40 cycles of 94 °C for 45 s, 42 °C for 45 s, 72 °C for 60 s and a final extension step of 72 °C for 10 min. DNA products were electrophoresed in 1% or 3% agarose gels and stained with ethidium bromide.

■ **Analysis of 3' RACE products from BBTV infected banana tissue.** PCR products electrophoresed in either 1% or 3% agarose gels were transferred to Hybond-N+ (Amersham) using a PosiBlot pressure blotter (Stratagene) and 0.4 M NaOH. These membranes were probed with a 980 bp fragment of BBTV DNA-1 excised from pBT338 (Fig. 1) (Harding *et al.*, 1991) labelled with either [α -³²P]dCTP or with digoxigenin. The [α -³²P]dCTP probes were labelled using the Pharmacia Ready-To-Go kit and the digoxigenin probes were labelled, hybridized and washed as described in the manufacturer's instructions (Boehringer Mannheim). Pre-hybridization, hybridization and washes for [α -³²P]dCTP labelled probes were as described in the Hybond-N+ (Amersham) protocol. The bands were visualized using autoradiography for [α -³²P]dCTP labelled probes and chemiluminescence for digoxigenin

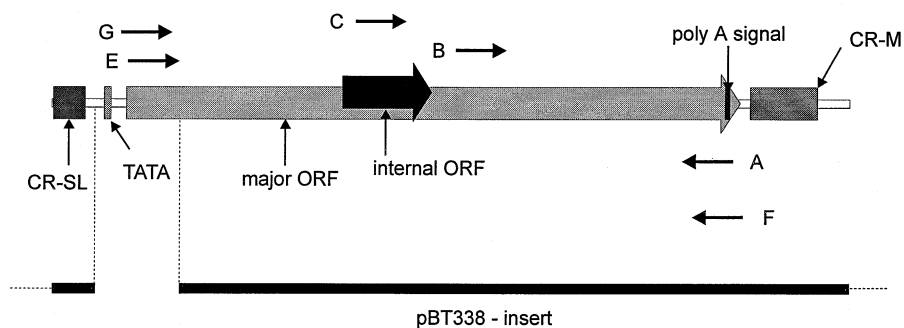


Fig. 1. Linear representation of BBTV DNA-1 and positions of primers A, B, C, E, F and G. The general genome organization includes the stem-loop common region (CR-SL), the TATA box, the major ORF, the internal ORF, the polyadenylation signal (poly A signal) and the major common region (CR-M). The location within BBTV DNA-1 of the 980 bp insert excised from pBT338 is represented by the pBT338-insert.

probes. The 3' RACE PCR products were gel purified, cloned directly into pGEM-T (Promega) and sequenced. All sequences were analysed using a range of programs and databases within the Australian National Genomic Information Service (ANGIS), University of Sydney, Australia. The sequences were all aligned and compared with the original BBTV DNA-1 sequence (Harding *et al.*, 1993), GenBank accession no. S56276. However, we used a different numbering convention such that nucleotide 1 of BBTV DNA-1 was the first nucleotide of the putative stem-loop structure (Karan *et al.*, 1994).

■ Analysis of 3' RACE products from tobacco plants transformed with the BBTV DNA-1 Rep-encoding ORF. Tobacco plants were transformed using *Agrobacterium tumefaciens*, LBA4404 strain, harbouring a binary plasmid containing the sequence of BBTV DNA-1 Rep ORF 3' of the CaMV 35S promoter, using a standard leaf-disk transformation-regeneration method (Horsch *et al.*, 1985). The plasmid was constructed using PCR and a two step sub-cloning. The BBTV DNA-1 Rep ORF was amplified using primers F (5' GCGAGCTCAGC-AAGAAACCAACT 3') and G (5' GCGGATCCATGGCGCGATA-TGTGGT 3') (Fig. 1) and cloned into pGEM-T (Promega) to produce pBR501. Using the *Bam*HI and *Sac*I restriction sites within the primers, the ORF fragment was ligated into a *Bam*HI/*Sac*I cut binary vector, pIBT102 (supplied by Texas A&M University, Texas, USA) 3' of the CaMV 35S promoter and 5' of a vegetable storage protein terminator sequence (Mason *et al.*, 1993). Two tobacco plant transformants were screened using 3' RACE. The RNA extraction, cDNA, PCR and analysis were as described above for banana plants.

Results

Extraction of RNA

The total RNA extraction method of Chang *et al.* (1993) was the only method attempted that yielded RNA from banana suitable for both 3' RACE and Northern analysis. When 5 µg of RNA was electrophoresed in a denaturing formaldehyde gel, ribosomal RNAs were visible and smaller RNA bands were visible. To confirm that there was no contaminating genomic BBTV DNA, the RNA samples were subjected to PCR at different RNA concentrations of 1 µg and 5 µg per reaction tube using primers that would amplify BBTV genomic DNA. In both experiments, no amplification was observed. Extractions from the transgenic tobacco plants using the same method also resulted in RNA suitable for cDNA synthesis and 3' RACE.

Northern analysis

Six different plasmid preparations each containing inserts from BBTV DNA-1 to 6 respectively were spotted onto the membrane to ensure that the labelled probe was specific for BBTV DNA-1. There was strong hybridization with the BBTV DNA-1 but not with the other BBTV DNAs (Fig. 2a). When RNA extracted from BBTV infected bananas was electrophoresed and Northern blotted, BBTV DNA-1 mRNAs were only detected using RNA probes rather than DNA probes. BBTV is known to be phloem-associated and probably only replicates in companion cells associated with this phloem

tissue, suggesting that the mRNAs from BBTV would be relatively rare transcripts. The labelled RNA probes hybridized non-specifically with both healthy and infected banana tissue RNAs but after RNase A post-treatment, all non-specific hybridization was eliminated and hybridization to two diffuse RNA bands with the RNA probe transcribed from BBTV DNA-1 was clearly evident (Fig. 2b). There was no specific hybridization to any RNAs extracted from healthy tissue. The sizes of the two diffuse bands in the BBTV infected samples were estimated to be 900 nt and 350 nt. It had previously been assumed that BBTV DNA-1 contained a single gene (in the virion sense) of 856 nt which probably corresponded to the 900 nt band. The 350 nt band was therefore unexpected.

3' RACE and Southern detection of products

Banana samples. When RNA aliquots used for Northern analysis were subjected to cDNA synthesis with primer D and 3' RACE primers D and B, diffuse PCR products were visualized in a 3% agarose gel. There was more amplification of DNA from the cDNA produced using RNA from BBTV infected tissue than from healthy tissue. The PCR products were transferred onto nylon membrane and hybridized to a BBTV DNA-1 specific DNA probe which resulted in hybridization with two bands. The estimated size of the larger band corresponded to the expected size of DNA amplified from the transcript of the major Rep ORF in BBTV DNA-1. The estimated size of the smaller band corresponded to the expected size of DNA amplified from a transcript of an ORF within the major Rep ORF. These PCR products were cloned and inserts from three clones corresponding to the size expected for both large and small bands were sequenced. The 3' RACE reaction was then repeated using newly synthesized cDNA and primer C. The sequence of primer C was located within the potential internal ORF. The PCR products were electrophoresed in a 1% agarose gel, transferred to a nylon membrane and hybridized to a digoxigenin labelled BBTV DNA-1 specific probe. The resulting autoradiograph again revealed two bands associated with the expected size for both the major Rep ORF and the internal ORF (Fig. 3a). Again these products were cloned and sequenced.

Transgenic tobacco samples. RNA samples were extracted from transgenic tobacco plants transformed with the major Rep ORF from BBTV DNA-1. This RNA was subjected to cDNA synthesis and 3' RACE and products were visualized after electrophoresis in a 3% agarose gel. The results were very similar to the results with banana RNA. At least two diffuse bands were identified, one of which was of the expected size for the major Rep ORF transcript. Compared with 3' RACE from the banana samples, the tobacco 3' RACE products were more distinct from the healthy controls when visualized after electrophoresis. The PCR products were transferred onto nylon membrane and hybridized to a BBTV DNA-1 specific

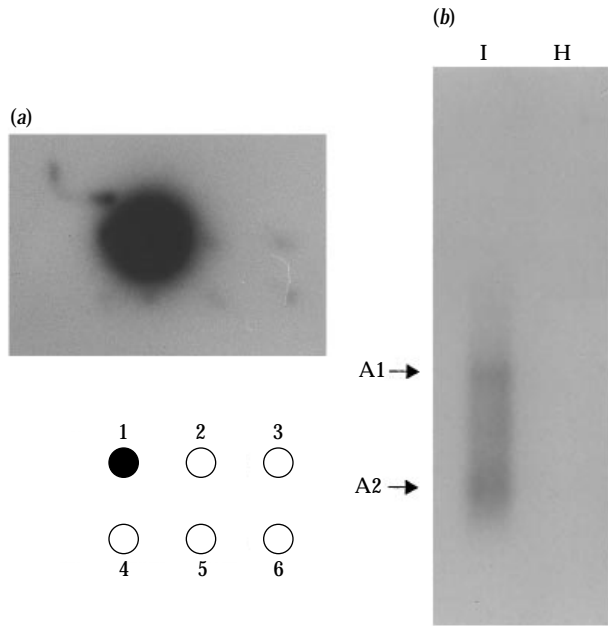


Fig. 2

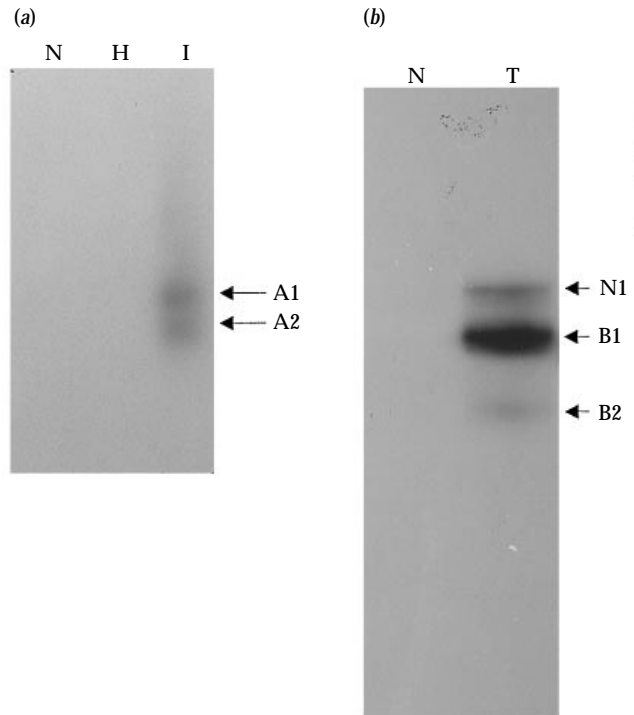


Fig. 3

Fig. 2. (a) Hybridization of the BBTV DNA-1 specific RNA probe. PCR products of BBTV DNA-1 to 6 were spotted onto Hybond-N+ membrane and probed with BBTV DNA-1 specific probe. (b) Northern hybridization panel showing healthy (H) and BBTV infected (I) banana RNA probed with BBTV DNA-1 specific RNA probe. The band marked as A1 corresponds to a band of about 900 bases and A2 corresponds to a band of 350 bases.

Fig. 3. (a) Southern hybridization of 3' RACE products from BBTV infected banana tissue (lane I), healthy banana tissue (lane H) and a PCR negative (lane N) probed with a BBTV DNA-1 specific digoxigenin labelled probe and visualized by chemiluminescence. Band A1 corresponds to a product of 600 bp and A2 corresponds to a product of 400 bp. (b) Southern hybridization of 3' RACE products from transgenic tobacco tissue (lane T) and non-transformed tobacco tissue (lane N) probed with a BBTV specific ³²P labelled probe and visualized by autoradiography. Band N1 corresponds to a 450 bp product, B1 corresponds to a product of 350 bp and B2 corresponds to a product of 200 bp.

DNA probe. The probe hybridized with three bands (Fig. 3b) of which two, B1 and B2, were of the expected sizes for the major and internal ORF respectively. The third band, N1, was approximately 100 bp larger than B1. These PCR products were also cloned and selected clones were sequenced.

Analysis of the 3' sequences of BBTV DNA-1 transcripts

The 3' RACE clones derived from banana mRNA using primer sets B and D or C and D were sequenced and aligned with the sequence of BBTV DNA-1. The larger 3' RACE product using primers B and D was 308 nt excluding the poly(A) tail which varied in length, but in the clones sequenced was not greater than 45 nt. The larger 3' RACE product using primers C and D was 538 nt excluding the poly(A) tail which also varied in length up to 35 nt. Both PCR products were clearly derived from the RNA transcript of the major Rep ORF of BBTV DNA-1 identified by Harding *et al.* (1993) (Fig. 4a).

Interestingly, there was no 3' untranslated region as polyadenylation began one nucleotide 3' of the translational stop codon. This site of polyadenylation suggests polyadenylation was controlled by the conserved poly(A) signal sequence and GT rich region within the coding region (Fig. 4a).

The smaller 3' RACE product using primers B and D was 148 nt excluding the poly(A) tail which varied in length, but in the clones sequenced was not greater than 38 nt. This product appeared to be derived from a transcript of the small ORF completely internal to the major Rep ORF of BBTV DNA-1 in a +2 coding frame (Fig. 4a). The sequence of this small clone only contained untranslatable sequence. However, when 3' RACE was repeated with primers C and D, the smaller 3' RACE product was 352 nt excluding the poly(A) tail which varied in length up to 28 nt. Again this product was derived from a transcript from the small internal ORF (Fig. 4a). The cloned sequence included 80 nt of coding region and 272 nt of untranslated region (Fig. 4a). It was assumed that the ATG start codon for this internal ORF was at nucleotide 403 (Karan,

polyadenylation of transcripts in CaMV, figwort mosaic caulimovirus (FMV) and CFDV, a BBTV-like DNA virus (Rothnie *et al.*, 1994; Sanfaçon, 1994; Merits *et al.*, 1995).

The 3' RACE products derived from transgenic tobacco mRNA were identical to the sequences of the two 3' RACE products derived from BBTV infected bananas and had identical sites of polyadenylation, which suggested that the viral polyadenylation signals had been utilized in tobacco. The clones sequenced were selected on size based on the size of the two bands B1 and B2. No clones sequenced corresponded to the N1 band (Fig. 3*b*).

Discussion

In this study, we have used Northern hybridization and 3' RACE to map the RNA transcripts of BBTV DNA-1 and have determined that: (i) the major Rep ORF, identified by Harding *et al.* (1993), is transcribed in BBTV infected bananas, (ii) this major Rep ORF has the commonly identified eukaryotic termination signals (Proudfoot, 1991) within the ORF, (iii) there is a small ORF within the major Rep ORF which is in a +2 coding frame with respect to the Rep ORF and (iv) that this internal ORF also has associated eukaryotic termination signals and has a comparatively large 3' untranslated region.

The site of polyadenylation at base 963 was identified for the major Rep ORF for BBTV DNA-1. This site immediately 3' of the translational stop codon for the major Rep ORF would most likely be controlled by the polyadenylation signal and associated GT rich region identified by Harding *et al.* (1993) and Burns *et al.* (1995). The polyadenylation signal AATAAA with a GT rich region has been well characterized in eukaryotes (Joshi, 1987; Jackson & Standart, 1990; Proudfoot, 1991) and was conserved within all isolates of BBTV examined from south-east Asia and the South Pacific (Karan *et al.*, 1994). Both the AATAAA (941–946) and a GT rich region (948–958) were within the major Rep ORF and the GT rich region had two TTG trinucleotide sequences identified by Burns *et al.* (1995). Burns *et al.* identified these termination signals in sequences of BBTV DNA-1 to 6. In addition to these termination signals, this study has identified another termination signal reported to be associated with polyadenylation. The consensus sequence was T(A/T)TGTA, which has also been found as an upstream element in CaMV (Rothnie *et al.*, 1994), FMV (Sanfaçon, 1994) and CFDV (Merits *et al.*, 1995). Mutational studies with CaMV, FMV and CFDV indicated that this upstream element affected both the site of polyadenylation and its processing efficiency (Rothnie *et al.*, 1994; Sanfaçon, 1994; Merits *et al.*, 1995). This upstream element was also found in the BBTV DNA-1 major Rep ORF of all BBTV isolates sequenced by Karan *et al.* (1994) and in all cases was 49 bases 5' of the polyadenylation signal and was conserved as (A/T)TGTA. It is clear that these termination signals are important since the transcripts from the transformed tobacco had the same site of polyadenylation. Although the transformation binary con-

struct was designed with a reportedly strong vegetable storage protein (VSP) termination sequence (Mason *et al.*, 1993), the transcripts mapped did not appear to use the VSP signals for polyadenylation. The larger band N1 (Fig. 3*b*) was most likely a 3' RACE product amplified from a transcript of the major Rep ORF terminated with the VSP terminator sequence. Sequence comparisons of the termination signals for SCSV DNA-6 and FBNYV (both potentially encoding Reps) also contain the conserved (A/T)TGTA, AATAAA and a GT rich region all 5' of their translational stop codons (Katul *et al.*, 1995; Boevink *et al.*, 1995). SCSV DNA-2 (also potentially encoding a Rep) contains the conserved signals, but the AATAAA is immediately 3' of the translational stop codon (Boevink *et al.*, 1995).

The mapping of the second smaller transcript has shown that it is novel in that it is internal to the BBTV DNA-1 major Rep ORF and that it has a long untranslated region relative to its coding region. This internal ORF was identified by Karan (1995) as it was conserved between sequences of BBTV DNA-1 from BBTV isolates sequenced from south-east Asia and the South Pacific. The site of polyadenylation at base 803 in the mRNA both from BBTV infected banana and from the transformed tobacco suggests that in the transgenic tobacco the transcript was processed using 5' and 3' signals contained within the major Rep ORF. The termination signals possibly associated with this BBTV internal ORF are most likely associated with the polyadenylation signal, AATAAA, and the many potential GT rich regions in the untranslated sequence of the internal ORF. The only report of a plant DNA virus containing a gene within the gene of a replication protein was a report for tomato golden mosaic geminivirus (TGMV) (Thömmes & Buck, 1994). *In vitro* translation studies produced a 10 kDa protein associated with a transcript designated AL4; further studies have shown this protein contributes to regulation of the AL1 gene, which encodes a known replication protein (Gröning *et al.*, 1994; Elmer *et al.*, 1988). Therefore, it is possible the protein encoded by the internal ORF of BBTV DNA-1 is involved with regulation of the Rep encoded by the major Rep ORF. However, amino acid sequence comparisons of the TGMV and BBTV internal ORFs did not reveal any reported conserved motifs.

Yeh *et al.* (1994), Wu *et al.* (1994) and Karan (1995) have isolated additional DNA components associated with BBTV infections in Taiwan. Each of these components potentially encoded Reps that were significantly different to the putative Rep encoded by BBTV DNA-1 (Karan, 1995). Interestingly, none of these additional components contained an ORF equivalent to the small internal ORF identified in BBTV DNA-1. Further, it would appear that the ORFs putatively encoding Reps in components of SCSV (Boevink *et al.*, 1995), CFDV (Rohde *et al.*, 1990) and FBNYV (Katul *et al.*, 1995) also do not contain an equivalent small internal ORF.

Rohde *et al.* (1990) identified all the potential ORFs in CFDV which included two ORFs (ORF2 and ORF3) internal to

the large ORF1, a putative Rep encoding ORF. Analysis of the CFDV sequence suggested that the ORF3 (52 amino acids, 6.4 kDa) coded for a positively charged arginine rich polypeptide and had homology with the human T-lymphotropic virus III *art* gene product, which is involved in post-transcriptional regulation of viral gene expression (Rohde *et al.*, 1990). However, the BBTV DNA-I internal ORF is not arginine rich and comparisons of the amino acid sequence revealed no homology between the CFDV ORF3 and the BBTV internal ORF.

It is now important to determine the function of the protein expressed from the small internal ORF, particularly whether it has a regulatory function in replication, and to investigate the significance of its absence in the additional potential Rep encoding components associated with BBTV infections in Taiwan.

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