Movement and transmission of banana bunchy top virus DNA component one in bananas

Gregory J. Hafner, Robert M. Harding and James L. Dale*

Centre for Molecular Biotechnology, Queensland University of Technology, GPO Box 2434, Brisbane, Queensland 4001, Australia

The systemic movement and replication of banana bunchy top virus (BBTV) DNA component one were investigated. Strand-specific RNA probes and PCR were used to indicate the presence of the virus in various parts of infected banana plants during infection on the basis of dsDNA replicative intermediates of BBTV. The strand-specific probes were not only able to detect the presence of the virus but also gave an indication of where the virus replicated. The results using both the virion sense and complementary to virion sense specific probes were essentially the same indicating that BBTV initially replicated for a short period at the site of inoculation, and subsequently moved down the pseudostem to the basal meristematic region and ultimately into the roots and newly formed leaves. The virus was detected in the leaves formed prior to inoculation after 21 days using PCR but was not detected by the RNA probes. This indicated that the virus had the ability to move into these leaves but may not have replicated or accumulated to significant levels. The appearance of multimeric forms of BBTV suggested that the virus may have replicated via a rolling circle mechanism. Additionally, BBTV DNA component one did not appear to replicate in its aphid vector, Pentalonia nigronervosa.

Introduction

Banana bunchy top disease (BBTD), caused by banana bunchy top virus (BBTV), is the most important viral disease of bananas (Musa spp.). BBTV, an 18–20 nm isometric virus, has a multicomponent genome consisting of at least six components of circular ssDNA ranging in size between 1018 and 1111 nucleotides (Burns, 1994; Burns et al., 1994). The sequence of only component one of BBTV has been published. This component has a single 858 nucleotide open reading frame (ORF) in the virion sense which encodes a putative replicase associated protein based on the presence of a conserved dNTP binding motif (Harding et al., 1993).

BBTV is transmitted by the banana aphid (Pentalonia nigronervosa) in a persistent manner (Magee, 1927). The virus is also transmitted through infected plant suckers and other plant components used in banana propagation but is not sap transmissible (Magee, 1927). The symptoms of BBTD include yellowing of leaf margins and the presence of dark green streaks on the petioles, pseudostem and leaf lamina. These later symptoms correlate internally with modification of the phloem and surrounding tissue of the vascular bundles. Based on this cytopathology and transmission characteristics, the virus is thought to be phloem associated (Magee, 1939).

Presently, the only described group of plant viruses with an ssDNA genome is the geminivirus group. However, geminiviruses have a number of important characteristics which differ from BBTV: geminiviruses have geminate virions (20 x 30 nm), their genome consists of one or two components of circular ssDNA approximately 2700 nucleotides in size which contain up to six ORFs, and their coat proteins are between 26 to 31 kDa. Furthermore, geminiviruses are transmitted by leafhoppers or whiteflies, and several are mechanically transmissible (reviewed by Harrison, 1985; Davies & Stanley, 1989; Lazarowitz, 1992). It therefore appears that BBTV is a member of a previously undescribed group of ssDNA plant viruses (Harding et al., 1993) which may include subterranean clover stunt virus (SCSV) (Chu & Helms, 1988), coconut foliar decay virus (CFDV) (Randles et al., 1987), faba bean necrotic yellows virus (FBNYV) (Katul et al., 1993) and milk vetch dwarf virus (MDV) (Sano et al., 1993).

There is little information on the replication of BBTV in either the plant or aphid vector. Magee (1940) first provided evidence of the circulative persistent manner in which the virus is transmitted by the aphid. It was demonstrated that the nymphal aphids could retain the virus through molts, and that the virus was retained by viruliferous aphids during daily transfers to fresh plants.

* Author for correspondence. Fax +61 7 864 1534. e-mail dalej@qut.edu.au
The virus was not transmitted by viruliferous aphids to as long as 13 days after removal from diseased plants. The virus was not transmitted by viruliferous aphids to their viviparous agamic (and only) progeny (Magee, 1940). These results provide some evidence to indicate that BBTV does not replicate within its aphid vector. However, since some viruses multiply in their vector and are not passed to progeny in transovarial passage (D'Arcy & Nault, 1982), it has yet to be conclusively demonstrated that BBTV does not replicate in the aphid vector.

Present attempts to control BBTV include regional and international distribution of virus free planting material, and the eradication of infected plants (Dale, 1987). Both strategies depend on the accurate detection of the virus. However, at present, there is little information available on the movement or accumulation of BBTV after initial viral infection. This information could have an important impact on the design and implementation of detection strategies for BBTV.

In this paper, we have studied the replication and systemic movement of BBTV DNA component one in infected plants and provide evidence that this BBTV component does not replicate in the aphid vector.

**Methods**

**Systemic spread of BBTV infection.** Nine pots each containing five healthy banana plantlets (*Musa sapientum* cv. Cavendish; three to five leaf stage) were inoculated with an Australian isolate of BBTV by introducing ten viruliferous aphids, isolated in cages, onto a small localized region on the outer edge of the second most recent expanded leaf. Aphids were caged on each plant for 3 days and then removed. Five pots each containing three healthy plantlets were used as uninoculated controls. All plantlets were grown in a controlled environment cabinet at 28 °C. Nucleic acids were extracted from several regions of each plant (Fig. 1) at different time intervals. BBTV inoculated plants were sampled after 12 and 24 h and 2, 4, 7, 14, 21, 31 and 41 days. Control plants were sampled after 0, 7, 21 and 41 days.

**Purification of BBTV.** BBTV was purified from banana midrib tissue as described by Harding et al. (1991).

**Nucleic acid extraction.** Total nucleic acids were extracted from plant tissue and aphids using a procedure adapted from Fang et al. (1992). Banana tissue was cut into fine pieces using a sterile scalpel and placed in a microcentrifuge tube. The tissue was frozen at -80 °C, and ground to a fine powder. The tissue was then resuspended in 1% SDS and allowed to thaw. The mixture was emulsified with an equal volume of phenol-chloroform (1:1), centrifuged, and the aqueous phase was re-extracted with an equal volume of chloroform. Nucleic acids were precipitated, washed in 70% ethanol, and resuspended in 50 µl of distilled water. Nucleic acids were extracted from aphids using the same procedure except that a second phenol-chloroform extraction was included.

**Nucleic acid electrophoresis and blotting.** Nucleic acids were electrophoresed through 1–2% agarose gels in Tris-acetate-EDTA buffer pH 7.6 (Sambrook et al., 1989) and visualized by ethidium bromide staining. Nucleic acids were transferred from agarose gels to Hybond-N (Amersham) using a PosiBlot pressure blotter (Stratagene) as per the manufacturer's instructions. Prior to blotting the nucleic acids were denatured in 0.5 M-NaOH, 1.5 M-NaCl for 30 min, followed by neutralization in 1 M-Tris–HCl pH 8.0, 1.5 M-NaCl for 30 min. Following transfer membranes were baked at 80 °C for 2 h.

**Construction of Riboprobe transcription plasmids.** The 980 bp fragment of BBTV DNA component one was excised from pBT338 (Harding et al., 1991) using PstI and EcoRI and ligated into PstI–EcoRI digested pGEM-3Zf(+) vector (Promega) to produce pGEMBT5. The nucleotide sequence and orientation of the insert were confirmed by sequence analysis. The pGEMBT5 vector contained the SP6 and T7 RNA polymerase promoter sequences to allow transcription of strand-specific probes to BBTV DNA component one.

**Transcription of strand-specific RNA probes.** pGEMBT5 was isolated by the alkaline lysis method (Sambrook et al., 1989) and in some instances further purified through CsCl gradients. Plasmid DNA was linearized with either EcoRI (SP6 transcript) or HindIII (T7 transcript) to produce transcription templates with protruding 5' termini. Plasmid DNA (20 µg) was treated with proteinase K (10 µg) for 1 h at 37 °C following linearization. Reactions were then extracted with phenol–chloroform (1:1) followed by chloroform. Samples were ethanol precipitated and dissolved in water to a final concentration of 1 µg/µl.

**Strand-specific probes were transcribed using 1–2 µg of linearized DNA template in a reaction containing 40 mM-Tris–HCl pH 7.5, 6 mM-MgCl₂, 2 mM-spermidine, 10 mM-dithiothreitol, 500 µM-rNTPs (A, C, G), 70–300 µCi [α-3²P]UTP, 0–240 µM-rUTP, 2 U/µl RNasin (Promega) and 15 U of either SP6 or T7 RNA polymerase (Promega).** Reactions were incubated at 37 °C for 90 min. Mixtures were treated with 1 U RQ1 RNase-free DNase (Promega) for 15 min at 37 °C to remove DNA templates after transcription. Reactions were extracted with 1 vol. of phenol–chloroform (1:1) followed by a chloroform extraction. Unincorporated nucleotides were removed by ethanol precipitation in the presence of ammonium acetate. The products of the transcription reactions were checked by electrophoresis in denaturing polyacrylamide gels (Miller, 1987).
Hybridization of RNA probes to plant extracts. Membranes were prehybridized in 5–10 ml of hybridization buffer (50 % formamide, 0.25 M-sodium phosphate buffer pH 7.2, 0.25 w NaCl, 1 mM-EDTA, 7 % SDS, 10 % polyethylene glycol) at 65 °C for 15 min. The solution was discarded and replaced with fresh hybridization buffer containing the labelled RNA probes and hybridized for 19 h at 65 °C. Membranes were then washed twice with 0.25 M-sodium phosphate pH 7.2, 2 % SDS, 1 mM-EDTA at 65 °C for 1 h and twice with 0.04 M-sodium phosphate pH 7.2, 1 % SDS, 1 mM-EDTA at 65 °C for 20 min. Membranes were then blotted dry and autoradiographed.

PCR detection of BBTV component one. From the nucleotide sequence of BBTV DNA component one (GenBank database, release 87.0: accession number S56276) two oligonucleotides (BT1F2.30, 5’ CGCCATGATATTTCTCCACCTCTGATGCCA 3’; BT1R13.30, 5’ TTCCACGGCGCACACCTTGAGAAACGAAAG 3’) were synthesized and were used as primers to hybridize to regions within the major ORF of BBTV DNA component one and to amplify a 524 bp product. DNA was amplified in 50 µl reactions containing 10 pmol of each primer, 50 mM-KCl, 1.5 mM-MgCl2, 10 mM-Tris-HCl pH 8.0, 200 µM-4NTP’s and 1 U AmpliTaq DNA polymerase (Boehringer). A 1 µl aliquot of each nucleic acid extract which had been diluted 100-fold, was used in the amplification reactions. Reactions were overlaid with 50 µl of paraffin oil and subjected to one initial cycle of heat denaturation at 94 °C for 5 min, followed by 32 cycles at 94 °C for 45 s, 55 °C for 30 s, and 72 °C for 30 s and a final extension step at 72 °C for 10 min. Following PCR amplification, 10 µl of the reaction was electrophoresed in a 2 % agarose gel and stained with ethidium bromide.

Included in the PCR amplification were a purified BBTV nucleic acid positive control, a healthy plant nucleic acid control, and a negative control containing no template.

Results

Detection of BBTV in nucleic acid extracts using strand-specific RNA probes

Strand-specific RNA probes for the detection of BBTV DNA component one were constructed so that the T7 RNA polymerase transcripts would hybridize with virion sense nucleic acids, while the SP6 RNA polymerase transcripts would hybridize with nucleic acids complementary to virion sense.

Nucleic acid extracts from healthy and infected banana tissue, viruliferous and non-viruliferous aphids, and from purified virus were electrophoresed in 1 % agarose gels, blotted to nylon membranes and hybridized with the RNA transcripts. The labelled T7 transcript hybridized strongly to purified viral nucleic acid and nucleic acids from infected banana plants and viruliferous aphids, but did not hybridize with extracts from either healthy banana plants or non-viruliferous aphids (Fig. 2A). The labelled SP6 transcript hybridized strongly with the infected plant extract but did not hybridize with extracts from healthy banana plants, viruliferous or non-viruliferous aphids or purified virus (Fig. 2B). The hybridization of only the T7 labelled transcript to nucleic acids within the aphid extracts was observed both when the aphids were removed directly from the diseased plants and extracted and also when the aphids were removed from the diseased plants and left 3 days prior to extraction (data not shown). Both the T7 and SP6 labelled transcripts hybridized to nucleic acids of different molecular masses (Fig. 2A, B: bands a, b, c, d, e, f and ss) within the infected plant extracts. In contrast, the T7 labelled transcript hybridized with only a single band (band ss) in extracts from purified virus and viruliferous aphid extracts.

Detection of BBTV DNA component one during systemic infection

Virion sense analysis. Nucleic acids were extracted from plants which had been aphid inoculated with BBTV. The nucleic acids were extracted on a tissue weight equivalence basis from different regions of each plant at various times after inoculation (Fig. 1), electrophoresed in agarose, posiblotted to nylon membrane and hybridized with the T7 transcript from pGEMBT5. Generally, the results at every time point were consistent between samples. However, five plants were inoculated for every time point, and it was apparent that not all plants became infected possibly due to inefficient viral transmission by the aphids. Therefore, results for those uninfected plants were disregarded in

Fig. 2. Detection of BBTV DNA component one using strand-specific RNA probes. Extracts from healthy banana plants (lane 1), 100 non-viruliferous aphids (lane 2), CsCl-gradient purified BBTV (lane 3), BBTV infected banana plants (lane 4), and 20 (lane 5), 50 (lane 6), and 100 (lane 7) BBTV viruliferous aphids were analysed on a 1 % agarose gel, blotted to Hybond-N membrane and hybridized with 32P-labelled transcripts. Panel A represents the virion sense detection of BBTV DNA component one using the T7 transcript of pGEMBT5, while panel B represents the complementary to virion sense detection using the SP6 transcript of pGEMBT5. The numbers to the right of the panel represents sizes of BstELI digested λ DNA. DNA bands indicated a, b, c, d, e, f and ss are described in the text.
Fig. 3. Quantitative analysis of BBTV systemic spread. BBTV inoculated plants were sampled after 12 (a) and 24 (b) h and 2 (c), 4 (d), 7 (e), 14 (f), 21 (g), 31 (h) and 41 (i) days. Nucleic acids were extracted from the inoculation site (I), lower region of the inoculated leaf (L), pseudostem (P), corm/meristem (C), roots (R), pre-inoculum formed leaf (O), and newest emerging leaf (N), electrophoresed through 1% agarose, transferred to Hybond-N membrane and probed with either the virion sense detecting (T7 transcript) or the complementary sense detecting (SP6 transcript) transcript of pGEMBT5. Results were scored on a 7 point scale based on reference to 20 ng of internal standard. Panel A represents the virion sense forms of BBTV DNA component one during systemic spread while panel B represents the complementary to virion sense forms.

The quantitative analysis. The results were analysed quantitatively by scoring extract hybridization intensity on a 7 point scale and correlating the results obtained between membranes by optical density estimations using 20 ng of pBT338 as an internal standard. The final values for each extract were averaged and are represented graphically (Fig. 3A).

Virion sense nucleic acids were first detected at 7 days after inoculation, at which time low levels were detected at the inoculation site and in the pseudostem. From 14 to 41 days after inoculation, virion sense nucleic acid was detected in the pseudostem, corm/meristem, roots and new leaf tissue. Virion sense nucleic acids were also detected at the inoculation site and the lower region of the inoculated leaf at 21 days but not at 14, 31 and 41 days. No hybridization was observed in extracts from leaves which had already developed prior to inoculation. The plants generally displayed the first symptoms of BBTV infection, dark-green streaks on the petioles, around 21 days following inoculation.

The values indicated that generally the virion sense BBTV DNA component one appeared to increase in concentration with time. The concentration of virus appeared to peak after approximately 31 days, especially in the pseudostem and corm/meristem regions but did not significantly decrease by 41 days.

Complementary to virion sense analysis. Complementary to virion sense nucleic acid of BBTV was detected using the labelled SP6 transcript of pGEMBT5.

Fig. 4. Detection of virion sense and complementary to virion sense BBTV DNA component one at 31 days following inoculation. Nucleic acids were extracted from the inoculation site (I), lower region of the inoculated leaf (L), pseudostem (P), corm/meristem (C), roots (R), pre-inoculum formed leaf (O), and newest emerging leaf (N), electrophoresed through 1% agarose, transferred to Hybond-N membrane and probed with either the virion sense detecting (T7 transcript) or the complementary sense detecting (SP6 transcript) transcript of pGEMBT5. Panel A illustrates the appearance of virion sense forms of BBTV while panel B illustrates the appearance of complementary to virion sense forms.

Since the extracts were the same as those used in the analysis of the virion sense BBTV DNA component one, it was again apparent that certain plants did not become infected. Those plants were again disregarded in the
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The results were again analysed quantitatively as described previously and the final values for each extract were averaged and represented graphically (Fig. 3B).

Low levels of complementary sense nucleic acids of BBTV were first detected 4 days after inoculation at the inoculation site and pseudostem. By day 7, the complementary sense nucleic acids were still present in very low concentrations in the inoculation site and pseudostem but were also present in some extracts from the corm/meristem region. As with the virion sense nucleic acids, the complementary to virion sense nucleic acids were easily detected in the pseudostem, corm/meristem, roots and new leaf at 14 days and remained detectable up to 41 days after inoculation.

Complementary sense nucleic acids were not detected in the lower region of the inoculated leaf, the leaves which had already developed prior to inoculation or in the inoculation site after 7 days.

The concentration of complementary to virion sense BBTV DNA component one also appeared to peak at around 31 days, similar to those results seen for the virion sense analysis.

All plant extracts in which BBTV DNA component one was detected showed the distinctive pattern whereby both the T7 and SP6 labelled transcripts hybridized to nucleic acids of different molecular masses (Fig. 4A, B).

PCR detection of BBTV component one

PCR was used in an attempt to detect very low concentrations of BBTV DNA component one. Several extracts which had previously tested negative for BBTV DNA component one by hybridization were retested using PCR. These extracts were from the inoculation site, pseudostem, corm/meristem, leaves which had already developed prior to inoculation and new leaves.

Using PCR, BBTV DNA component one was detected at the inoculation site 2 days after inoculation (Fig. 5). The virus was also detected in the pseudostem and new leaf 4 days after inoculation, and in the meristem/corm region 7 days after inoculation. Surprisingly, the virus was detected at 21 and 41 days post-inoculation in leaves which had already developed prior to inoculation.

Discussion

These results indicate that: (i) BBTV DNA component one replicated in infected plants; (ii) only the virion sense of BBTV DNA component one was encapsidated; and (iii) BBTV did not appear to replicate within its aphid vector. The virus–vector relationship of BBTV in the aphid vector, Pentalonia nigronervosa, therefore appears to be of a circulative (persistent) non-propagative type.
These results are not surprising since to date, of the plant viruses, only the aphid-transmitted Rhabdoviridae have been demonstrated to replicate in aphids (Harris, 1979) and the only recognized group of ssDNA viruses, the geminiviruses, do not replicate in their whitefly or leafhopper vectors (Harrison, 1985).

In plant extracts, the T7 and SP6 transcripts consistently hybridized to a number of different molecular mass nucleic acids (Fig. 2A, B: bands a, b, c, d, e, f and ss). The fastest migrating band appeared to represent the virion encapsidated ssDNA. The nucleic acids in bands a to f were not fully characterized but are postulated to be monomeric, dimeric and other multimeric forms of the genomic DNA in a variety of conformational forms, perhaps analogous to those seen with geminivirus infections (Slomka et al., 1988). However, further analysis is needed to identify each of the viral replicative forms present in BBTV infected plants. These multimeric forms were not detected in purified virions or aphids. The presence of multimeric forms of a viral genome is often indicative of a rolling circle type of replication (Stanley & Townsend, 1985; Slomka et al., 1988) suggesting that BBTV may replicate via this mechanism. This hypothesis is supported by BBTV (Harding et al., 1993), CFDV (Rohde et al., 1990) and the geminiviruses (Lazarowitz, 1992) containing a highly conserved loop sequence (5' TANTATTAC 3') located within a stem-loop structure. This conserved nonanucleotide sequence has been shown to be part of the viral strand origin of replication within the geminiviruses (Hofer et al., 1992; Revington et al., 1989; Schneider et al., 1992), which also appear to replicate by the rolling circle mechanism (Saunders et al., 1991; Stenger et al., 1991; Heyraud et al., 1993). This nonanucleotide sequence is also similar to the nicking site of protein A in φX174 phage which is also located within a stem-loop structure (van Mansfeld, 1979). The absence of the multimeric forms of BBTV in viruliferous aphid extracts when probed with the RNA transcript provides further evidence that BBTV does not replicate in aphids.

Using PCR, BBTV DNA component one was first detected at the site of inoculation after 2 days and in the pseudostem and the new leaf, but not the corm/meristem, after 4 days. Using RNA probes, BBTV DNA component one was detected at very low levels at the inoculation site at days 4 and 7 where it appeared to be transiently replicating based on the appearance of complementary to virion sense nucleic acids. Fourteen days after inoculation both virion and complementary to virion sense BBTV DNA component one were detected in the pseudostem, corm/meristem, roots and new leaf, indicating virus replication. After 21 days, the virus was detected using PCR at very low levels in the leaves which had already developed prior to inoculation but was not detected using the RNA probes indicating that the virus was present at low levels in this part of the plant in the latter stages of infection. It was possible, therefore, that the virus moved to this region but may not have replicated or accumulated to significant levels.

Thus, BBTV appeared to initially replicate transiently at the point of inoculation, then move through the pseudostem to the corm/meristem and subsequently into the roots and new leaves. It is possible that the virus moved with, rather than into, the newly formed leaves where it replicated to a high level, since it was first detected in the new leaf rather than the corm/meristem, and did not appear to replicate to high levels in the inoculation site or leaves formed prior to the inoculation.

There has been only one previous study on the movement of BBTV in banana plants. Raj et al. (1970) observed that BBTV had an incubation period of 5–15 days at the point of inoculation before migrating downward. The virus was observed to move to the lower regions of the plant at a very rapid rate, possibly a few hours or less, following the incubation period. These results were exclusively based on symptom development rather than virus detection. Furthermore, aphids were not confined to one site of inoculation and therefore the observed movement may have been complicated by aphid movement to other regions. These results do, however, correlate quite closely with those obtained in this study.

There have been few studies on the distribution of BBTV within plants. Wu & Su (1992) reported that BBTV was detected, using ELISA, in all young leaves of banana plants with symptoms but the virus was either absent or present in low concentrations in the symptomless older leaves of the infected plants. This correlated with the virus being first detectable in the fourth leaf unfurling, usually about 27 days after inoculation (Thomas & Dietzgen, 1991). Wu & Su (1992) also reported that BBTV was not detected in the corm (rhizome), and was either absent or present in low concentrations in the roots and the sheaths of the older symptomless leaves. In this study, however, relatively high levels of BBTV DNA were found in the roots of infected plants. It is possible, but unlikely, that this DNA was not encapsidated in the roots.

The work presented here indicates that: (i) there is a distinct pattern of viral replication and movement in banana plants; (ii) that BBTV does not replicate in its aphid vector; and (iii) that BBTV probably replicates by a rolling circle mechanism.

References
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