Purification, characterization and serological detection of virus-like particles associated with banana bunchy top disease in Australia

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Isometric virus-like particles, 18 nm in diameter, have been isolated from banana (*Musa* spp.) affected by bunchy top disease in Australia. Banana bunchy top disease-associated virus-like particles (BBTV) banded as a single component with buoyant density of 1·28 to 1·29 g/ml in Cs_2SO_4 and sedimented at about 46S in isokinetic sucrose density gradients. The A_{260}/A_{280} of purified preparations was about 1·33. A single coat protein of M_r 20500 was identified with antibodies to BBTV particles from Australia. Single-stranded DNA of about 1 kb as well as ssRNA smaller than 0·45 kb was also associated with the particles. A polyclonal antiserum to BBTV, suitable for use in ELISA, was prepared. Stability and antigenicity of purified BBTV was impaired by storage at pH $\geqslant$ 8·5 and freezing at −20 °C without protectants. BBTV was detected by double antibody sandwich–ELISA with monoclonal and polyclonal antibodies, in field-infected banana plants, single aphids from an infective colony, and in experimentally aphid-inoculated banana plants. After transmission of BBTV particles by aphids from a banana bunchy top disease-affected to an uninfected banana plant, the disease was induced and BBTV was detected by ELISA in symptomatic leaves only. BBTV isolates from Australia, Taiwan, People's Republic of China, Tonga, Western Samoa and Hawaii were found to be serologically related, which suggests a common aetiology for the disease.

Introduction

Banana bunchy top disease (BBTD) occurs in the Asian-Pacific region and Africa and is considered the most serious virus-like disease of bananas (*Musa* spp.) worldwide (reviewed by Dale, 1987). In the 1920s it almost completely destroyed the banana growing industry in Australia. The pathogen is transmitted in the persistent manner by the banana aphid (*Pentalonia nigronervosa*), causes cytopathological effects in the banana phloem tissue and yellowing symptoms on the leaves (Magee, 1927, 1940) and has therefore been considered to be a possible luteovirus (Matthews, 1982). This assumption was supported by the work of Dale *et al.* (1986), who isolated BBTD-specific dsRNAs which resembled in size dsRNA isolated from plants infected with barley yellow dwarf and beet western yellows luteoviruses.

Control of BBTD involves the use of uninfected planting material and intensive roguing schemes which currently rely on the detection of infected plants by visual symptoms alone. As banana is predominantly a vegetatively propagated crop, the inadvertent spread of the disease by the use of infected planting material, including micropropagated plants (Drew *et al.*, 1989), is a constant risk. The lack of a sensitive detection assay has been a serious constraint on the distribution of banana germplasm especially to areas such as Central and South America, and southern Africa, where the disease has not been recorded.

The purification of virus-like particles (hereafter called BBTV) from BBTD-affected banana plants has been independently reported recently by two research groups. Iskra *et al.* (1989) detected 28 nm isometric particles in BBTD-affected plants from Gabon whereas Su & Wu (1988, 1989) isolated 20 to 22 nm diameter particles from BBTD-affected bananas in Taiwan. Physicochemical analysis of Taiwanese BBTV revealed a coat protein of M_r 21 000 and an ssRNA of M_r $2{\cdot}0 \times 10^6$ (Wu & Su, 1990*a*). Monoclonal antibodies (MAbs) to Taiwanese BBTV were used in ELISA and in immunofluorescence assays to detect BBTV and provide for the first time a diagnostic tool for the disease (Wu & Su, 1990*b*). However, neither group has demonstrated infectivity of the purified particles and so the exact role of these particles in BBTD remains unknown at this stage.

In this paper we report the purification of Australian isolates of BBTV. The physicochemical properties of these particles are discussed in relation to BBTV from

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Taiwan and Africa. The production and use of a BBTV-specific polyclonal antiserum and the evaluation of Australian and Taiwanese MAbs for the detection of BBTV from Australia, People's Republic of China, Tonga, Western Samoa and Hawaii is presented.

Methods

Virus isolates. Australian BBTV field isolates were collected from south-east Queensland in banana (*Musa* spp.) cultivars Cavendish (AAA genotype) and Lady finger (AAB genotype). Non-infective banana aphids were collected from a healthy banana plant and maintained in an insectary at 25 °C. Infective banana aphids were collected from a single BBTV-infected plant and were maintained under the same conditions. This latter virus isolate was used for all subsequent transmission tests and as a source of infective aphids for ELISA tests. Field samples with typical BBTD symptoms were imported to Australia as dried leaf material from Hawaii (N. M. Nagata), Tonga and Western Samoa (M. K. Smith). Field samples were also collected in Kunming Province in the People's Republic of China (Maoling Sun & J. E. Thomas, unpublished results) and tested there as fresh tissue. For comparative purposes, the following viruses were used: cucumber mosaic virus (silverbeet isolate, CMV, authors), sunnhemp mosaic virus (J. L. Dale) and tobacco ringspot virus (TobRV, R. I. B. Francki).

Purification of BBTV. Banana midrib and petiole tissue was processed according to a modification of the method of Su & Wu (1989). Tissue was diced into pieces less than 1 cm in diameter, immediately placed into liquid nitrogen, then pulverized in a commercial coffee grinder (Climax Industries) and processed immediately or stored at −70 °C for up to 12 months. The powder was thawed in 2 vol. extraction buffer (0·2 M-potassium phosphate pH 7·4, containing 0·5% w/v Na_2SO_3) and then stirred for 30 min at 5 °C. The extract was filtered through cheesecloth and the fibres were re-extracted with one volume of extraction buffer, using a mortar and pestle and acid-washed sand. Chloroform : butanol (1 : 1, 0·1 vol.) was added gradually to the combined filtrates; the mixture was stirred for 1 h at 5 °C and then centrifuged at 8000 *g* for 10 min. The aqueous phase was centrifuged at 170000 *g* for 90 min. The pellets were resuspended in 0·07 M-sodium phosphate pH 7·2 (PB) (at least 1 ml/5 g original tissue), stirred for 2 days and then allowed to stand for a further 2 days at 5 °C. The extract was then clarified by centrifugation at 8000 *g* for 10 min, prior to ultracentrifugation at 270000 *g* for 60 min. Pellets were resuspended in about 1 ml PB/100 g original tissue, layered onto 10 to 40% sucrose density gradients in PB and centrifuged at 70000 *g* for 4 h. The zone containing virus particles was located and removed using an Isco density gradient fractionator and u.v. monitor, diluted in PB and concentrated by ultracentrifugation. Virus particles were further purified by equilibrium centrifugation in Cs_2SO_4 (3 ml gradient of initial density of 1·325 g/ml, overlayered with paraffin oil) at 35000 r.p.m. (Beckman SW41 Ti rotor) for about 20 h. The virus zone was located with a vertical beam of light and removed by puncturing the side of the tube with a needle. The virus preparation was diluted in PB and concentrated by ultracentrifugation and the final pellet was resuspended in a small volume of PB. Alternatively, after sucrose density gradient centrifugation, some preparations were centrifuged in gradients of 30 to 60% (w/v) Nycodenz (Nycomed) at 55000 r.p.m. (Beckman TLS 55 rotor) for 3 h at 15 °C. The gradients were fractionated by carefully removing 100 µl aliquots from the top. Virus concentrations were estimated assuming $E_{260} = 3{\cdot}6$, based on that of subterranean clover stunt virus (SCSV) (Chu & Helms, 1988).

Antisera. An antiserum to BBTV was produced in a New Zealand White rabbit following two intramuscular injections (150 µg in 0·3 ml on day 0 and 10 µg in 0·3 ml on day 21) each emulsified with an equal volume of Freund's adjuvant. Blood taken 20 days after the last injection was used throughout. The antiserum titre was determined by gel diffusion (Thomas, 1984). Immunoglobulin (Ig) and IgG purification and preparation of alkaline phosphatase conjugates was essentially as described by Clark & Adams (1977). The sources of other antisera were as follows: Taiwanese BBTV MAb 3D12 and 2H6 and 3D12–alkaline phosphatase conjugate (Wu & Su, 1990*b*), Australian BBTV MAb F10 (J. E. Thomas & R. G. Dietzgen, unpublished results).

ELISA. Two forms of ELISA were used with either Nunc Maxisorb or Dynatech M129B microtitre plates. The double antibody sandwich (DAS) form of direct ELISA was essentially as described by Clark & Adams (1977). Coating was with polyclonal Ig at 3 µg/ml or MAb 3D12 at 0·5 µg/ml. Banana leaf tissue and aphids were prepared in purification extraction buffer while purified virus preparations were diluted in extraction buffer or PB. For routine detection, banana tissue was extracted at the rate of 1 g/4 ml. BBTV polyclonal Ig–alkaline phosphatase conjugates (1 : 500) or MAb 3D12 conjugate (1 : 4000) were incubated in 0·01 M-phosphate-buffered 0·8% (w/v) saline pH 7·4 (PBS) containing 0·05% (w/v) Tween 20 (PBST), 2% (w/v) polyvinylpyrrolidone (PVP) and 1% (w/v) skim milk powder. In the plate-trapped antigen (PTA) form of indirect ELISA, antigen was bound to plates in the presence of 0·05 M-carbonate buffer pH 9·6. Bound antigen was detected with 1 to 10 µg/ml of BBTV MAb 2H6 in PBST–PVP. Rabbit anti-mouse IgG conjugated with alkaline phosphatase (ICN) in PBST–PVP was used at a dilution of 1 : 1000 to 1 : 2000. ELISA reactions were considered positive when the A_{405} value was greater than the mean plus two standard deviations of appropriate control samples.

Aphid transmission. Micropropagated banana plants (cv. Cavendish), all originally derived from the one source and shown to be free of BBTV by DAS–ELISA, were used in these studies. Groups of 20 aphids from either infective or non-infective colonies were caged on individual plants for an inoculation access period of 48 h. The aphids were then removed from the plants and assayed for BBTV by DAS–ELISA (coating with polyclonal Ig, MAb 3D12–alkaline phosphatase conjugate). After inoculation, as each new leaf emerged it was checked for BBTD symptoms and then the apical half was sampled and stored at −70 °C prior to testing by DAS–ELISA (coating with polyclonal Ig, polyclonal IgG and MAb 3D12–alkaline phosphatase conjugates).

Electron microscopy. Virus preparations were negatively stained with 1% (w/v) ammonium molybdate pH 5·8, and viewed in a Hitachi H7000 electron microscope. Grid preparation, immunosorbent electron microscopy (ISEM) and particle size measurements were essentially as described by Thomas (1986).

Gel electrophoresis of proteins and nucleic acids. Purified BBTV preparations were denatured in 3% (w/v) SDS and 5% (v/v) 2-mercaptoethanol and analysed in discontinuous SDS–12% polyacrylamide gels as described by Laemmli (1970). After electrophoresis, the separated proteins were either visualized by staining with silver nitrate (Wedrychowski *et al.*, 1986) or transferred electrophoretically to nitrocellulose membrane (0·45 µm pore size, Bio-Rad) after which the blot was incubated in PBS overnight at 37 °C. Skim milk (5% w/v) in rinse buffer was used for blocking. Immunoblots were probed with BBTV MAb F10 (1 : 5 diluted culture supernatant) or polyclonal antiserum (1 : 1000) and processed as described by Dietzgen & Francki (1988) using nitroblue tetrazolium–phenazine methosulphate–5-bromo-4-chloro-3-indolyl phosphate as an alkaline phosphatase substrate (Ey & Ashman, 1986).

Nucleic acids were extracted as described by Francki & Randles (1973), fractionated by electrophoresis in horizontal 1% (w/v) agarose

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gels in Tris–borate–EDTA buffer pH 8·3 (Maniatis *et al.*, 1982) and visualized by staining with ethidium bromide. CMV RNAs and a 0·24 to 9·5 kb RNA ladder (BRL) were used as markers. Total RNA was extracted from healthy banana midrib tissue as described by Rezaian *et al.* (1983). Digestions with 1 µg/ml of boiled ribonuclease A (Sigma) or 100 µg/ml DNase I (Promega Biotec RQ1 DNase) were carried out in 0·04 M-Tris–HCl pH 7·6 containing 0·01 M-NaCl, 0·006 M-$MgCl_2$, 0·01 M-$CaCl_2$ and 100 µg/ml proteinase K (Sigma) for 30 min at 37 °C (Tullis & Rubin, 1980). Digestion with 50 units/ml of S1 nuclease (Boehringer Mannheim) was in 0·033 M-sodium acetate pH 4·5, containing 0·3 M-NaCl and 0·001 M-$ZnSO_4$, for 30 min at 37 °C. Subsequently, samples were extracted with phenol:chloroform (1:1) and the nucleic acid was precipitated from the aqueous phase with 2 vol. of ethanol and 1/20 vol. of 4 M-sodium acetate pH 6·0.

Results

Purification of BBTV

Virus-like particles 18 nm in diameter were consistently associated with BBTD-affected banana plants (Fig. 1). When infected tissue was processed using the purification method of Su & Wu (1989), only relatively impure preparations of Australian BBTV were obtained. The modification of several steps in this procedure improved the purity of BBTV preparations to a state suitable for biochemical analysis and antibody production. The method finally adopted is described in Methods. We obtained similar BBTV yields with the extraction buffers from either procedure, as assessed by DAS–ELISA. However, the use of potassium phosphate buffer containing Na_2SO_3 reduced the amount of copurified host material as judged by electron microscopy and sucrose density gradient profiles and avoided the use of 2-mercaptoethanol. We recovered similar amounts of BBTV from infected banana tissue after the initial extraction and after re-extraction of the fibres by grinding with acid-washed sand. However, BBTV was not recovered when chloroform–butanol-clarified extracts were precipitated with 8% (w/v) polyethylene glycol 6000 in 0·4 M-NaCl. Furthermore, when Celluclast (Novo Industri), a crude cellulase enzyme preparation, in 0·1 M-sodium citrate pH 6·0 containing 0·2% (v/v) thioglycerol was used for extraction, virus yields were reduced by a factor of about three.

BBTV particles were associated with a u.v.-absorbing zone in sucrose density gradients (Fig. 2). The presence of virus-like particles in gradient fractions was confirmed by ELISA, because BBTV could not always be easily detected by electron microscopy due to the presence of host contaminants. The particles banded with purple-coloured host components in Nycodenz, but could be separated from the contaminants by using caesium sulphate isopycnic density gradients. BBTV was purified from freshly harvested infected tissue, tissue stored at 5 °C for up to 2 weeks or tissue frozen at −70 °C for up to 12 months. Yields were typically less than 300 µg/kg.

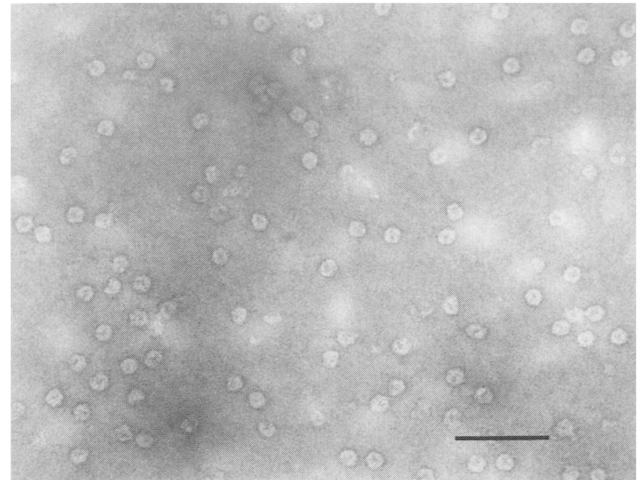

Fig. 1. Purified particles, associated with BBTD, after centrifugation in a Cs_2SO_4 gradient, stained with 1% ammonium molybdate. Bar represents 100 nm.

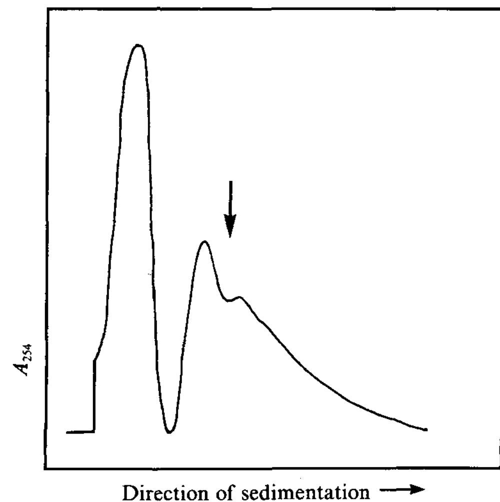

Fig. 2. Sedimentation profile of a sucrose density gradient after centrifugation of a BBTV-infected banana leaf extract. Arrow indicates the zone containing virus particles.

Properties of BBTV

Particles of BBTV were isometric and averaged 17·6 nm (standard deviation, 0·8 nm) in diameter (100 particles measured) when mounted in 1% (w/v) ammonium molybdate pH 5·8. They often had electron-dense cores and sometimes a hexagonal profile (Fig. 1) and were also stable in 1% (w/v) aqueous uranyl acetate.

In gradients of caesium sulphate, BBTV particles formed a diffuse zone at a density of approximately 1·28 to 1·29 g/ml. In Nycodenz gradients the particles were identified by ELISA and electron microscopy in a broad zone of density 1·18 to 1·23 g/ml, with a peak at about 1·20 g/ml. In isokinetic sucrose density gradients, BBTV particles sedimented in a zone of about 30S to 60S by comparison with the top, middle and bottom components of TobRV. Highest ELISA readings were recorded at a position equivalent to 46S.

Two preparations of BBTV from caesium sulphate gradients, apparently pure and homogeneous as assessed by electron microscopy (Fig. 1), were analysed by u.v. spectrophotometry. The preparations had u.v. absorption spectra characteristic of nucleoprotein with an A_{260}/A_{280} ratio of approx. 1·33, an A_{max} at 258 nm and an A_{min} at 245 nm.

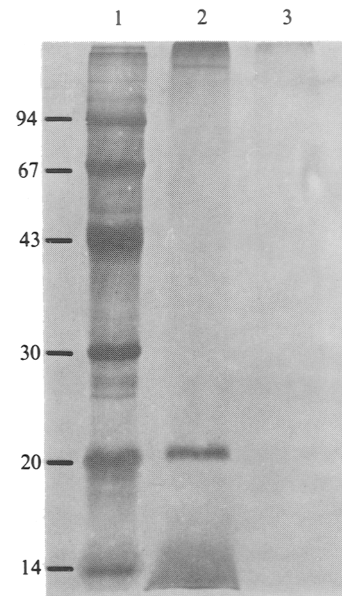

Fig. 3. Analysis of protein associated with BBTV. Protein was resolved on SDS–12% polyacrylamide gels and stained with silver. Lane 1, protein markers (phosphorylase b, M_r 94000; albumin, M_r 67000; ovalbumin, M_r 43000; carbonic anhydrase, M_r 30000; trypsin inhibitor, M_r 20100; α-lactalbumin, M_r 14400); lane 2, dissociated BBTV; lane 3, mock virus preparation from uninfected banana. The numbers at the left refer to the M_r values ($\times 10^{-3}$) of marker proteins.

Identification of coat protein and nucleic acid of BBTV

BBTV particles contained one major polypeptide of M_r 20500 which migrated in 12% SDS–PAGE slightly slower than soybean trypsin inhibitor. This protein was not detected in mock virus preparations from uninfected bananas (Fig. 3) and it reacted specifically with polyclonal and monoclonal F10 antibodies to BBTV in immunoblots (data not shown). Nucleic acid extracted from purified BBTV preparations migrated as a discrete band of about 1·0 kb when compared to ssRNA size markers (Fig. 4, lanes 3 and 7). This band was sensitive to digestion by DNase I (Fig. 4, lane 9) and S1 nuclease (data not shown) but not to RNase A (Fig. 4, lane 8). An additional broad zone, which in some preparations contained several discrete bands, migrating below this DNA and faster than banana 16S ribosomal RNA (Fig. 4, lane 10) and a 0·45 kb marker RNA (Fig. 4, lanes 4 and 5) was also consistently present. This material was ssRNA as judged by its sensitivity to RNase A (Fig. 4, lane 8) and S1 nuclease (data not shown) but not to DNase I (Fig. 4, lane 9).

Effect of various treatments on particle stability and antigenicity

The effect of various sample buffers on particle stability was examined by ISEM. Virus particles remained well preserved after incubation for 2 h or overnight at pH 7·4 in PBS or 0·1 M-potassium phosphate, prior to ISEM examination. Some particle damage was detected after 2 h incubation at pH 8·5 in 0·1 M-Tris–HCl or 0·1 M-borate. After overnight incubation, many particles were damaged in the Tris–HCl, and virtually no particles were detected in the borate. Carbonate (0·05 M, pH 9·6) almost completely disrupted BBTV particles after 2 h incubation, while some indistinct and poorly contrasted particles were still detected even after overnight incubation in 0·05 M-glycine–NaOH pH 9·6.

BBTV particles appeared to be very prone to antigenic changes. The ability to detect BBTV particles or coat protein with specific antibodies in dot blots or immunoblots was severely reduced or eliminated by replacing methanol with ethanol during electrophoretic transfer. The freezing of ELISA plates at −20 °C after polyclonal antibody precoating, virus particle trapping and milk blocking resulted in reduced reactions as compared to freshly prepared plates. A_{405} values of 0·54 compared to 1·29 were obtained from frozen and fresh plates respectively when polyclonal antibody enzyme conjugate was used. When the MAb–enzyme conjugate was used, the corresponding A_{405} values were 0·18 and 1·85, respectively. Healthy control samples had A_{405} values of 0·01 in both tests. In a similar experiment the effect of various storage conditions on polyclonal antibody precoated plates containing BBTV was analysed. Comparable readings were obtained with plates stored at 5 °C or −70 °C for 3 days, but readings were greatly reduced by storage for 3 days at −20 °C or at 5 °C after dessication. For all storage conditions, A_{405} values were substantially reduced when milk blocking was omitted prior to storage.

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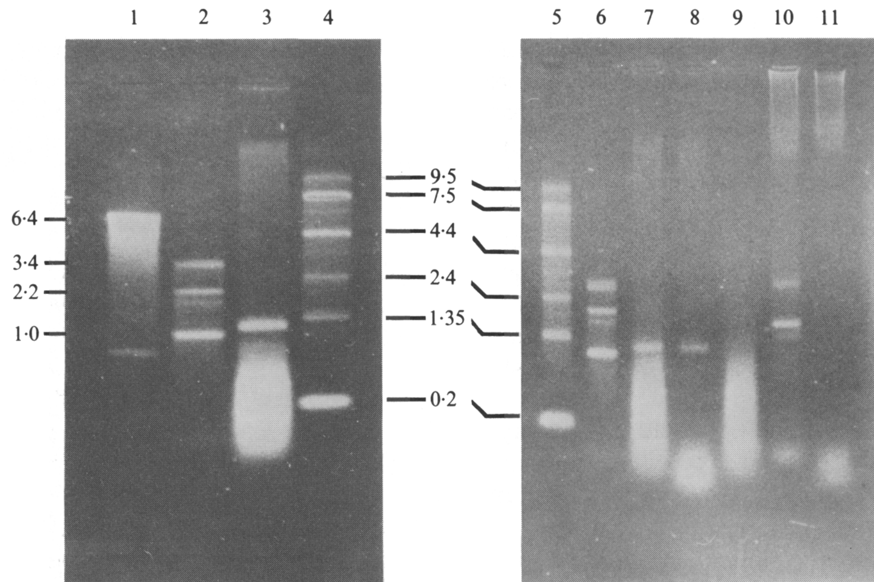

Fig. 4. Analysis of nucleic acids associated with BBTV. Nucleic acid extracts were separated by electrophoresis in 1% agarose gels in TBE buffer and stained with ethidium bromide. Lane 1, sunnhemp mosaic virus RNA; lanes 2 and 6, CMV RNAs; lanes 3 and 7, untreated BBTV; lanes 4 and 5, 0·24 to 9·5 kb RNA ladder (BRL); lane 8, BBTV after RNase A digestion; lane 9, BBTV after DNase I digestion; lanes 10 and 11, total RNA extracted from healthy banana midrib tissue, untreated and after digestion with RNase A, respectively. Sizes of RNAs are indicated (in kb).

Serological detection of BBTV

Polyclonal rabbit antiserum to Australian BBTV had a virus-specific titre of at least 1/256 and a host-specific titre of 1/2 and was successfully used in a DAS–ELISA system. Virus-specific reactions were enhanced when the enzyme conjugate was applied in PBST–PVP containing 1:20 diluted healthy banana sap or 1% (w/v) skim milk powder, and to a lesser extent with bovine serum albumin (data not shown).

When using Australian polyclonal Ig for both coating and enzyme conjugation in DAS–ELISA, positive reactions were obtained from over 30 plants with typical BBTD symptoms but not from banana plants infected with CMV. MAbs to a Taiwanese isolate of BBTV (Wu & Su, 1990*b*) reacted with Australian BBTV in ELISA and ISEM. The MAbs were used to detect field isolates of Australian BBTV from over 20 different infected banana plants, from banana plants experimentally aphid-inoculated and from infective banana aphids in a variety of assays. In no case, with Australian or Taiwanese antibodies, did we fail to obtain positive ELISA reactions with symptomatic plants, and all known non-infected plants gave negative reactions in these tests. Using MAb 2H6 in PTA–ELISA, positive reactions ($A_{405} \geqslant 0{\cdot}1$) were obtained from cv. Cavendish and cv. Lady finger plants with BBTD symptoms when extracts were concentrated at least 10-fold, but no BBTV was detected in extracts diluted fourfold. Using ISEM with MAbs 2H6 or 3D12, large numbers of isometric virus-like particles were detected in partially purified preparations.

The detection limit for BBTV in the sap of 'Cavendish' banana was 1/128 when polyclonal Ig was used for coating and MAb 3D12 as enzyme conjugate in DAS–ELISA. However the limits were only 1/32 and 1/16 when MAb 3D12 and polyclonal Ig respectively were used for both coating and enzyme conjugate. Extracts of midrib tissue consistently resulted in higher A_{405} values in DAS–ELISA (1·3- to 4·6-fold) than did extracts from the leaf lamina. BBTV was also detected by DAS–ELISA in experimentally aphid-inoculated plants of *M. velutina* and ornamental brown spotted banana plants which showed typical symptoms of BBTD.

We detected BBTV in six out of seven adult aphids, but only one out of eight early instar nymphs from an infective glasshouse stock colony, using DAS–ELISA with MAb 3D12 (Table 1). When aphids from an infective colony were stored in 95% ethanol at 5 °C for 2 days prior to DAS–ELISA with MAb 3D12, A_{405} values were greatly reduced as compared to freshly prepared aphids (data not shown).

Time course of BBTV detection and symptom development after aphid inoculation

Leaves were sampled from each plant sequentially as they unfurled, the first leaf (leaf 1) immediately prior to

inoculation (day 0), subsequent leaves (2 to 8) up to 62 days after inoculation. Of three plants inoculated with infective aphids, one developed typical BBTD symptoms, first evident in leaf 4 which unfurled 27 days after inoculation. BBTV was first detected by DAS–ELISA in this leaf at day 27, though characteristic but barely discernible 'dot-dash' symptoms were not apparent in this leaf until day 34. All subsequent leaves from this plant had strong, characteristic BBTD symptoms and the virus was readily detected in them. Virus was not detected in the two BBTV-inoculated plants which did not develop symptoms, but was detected in all three groups of infective aphids. No symptoms were detected on any leaves of three mock-inoculated plants and BBTV was not detected in these plants nor in the non-infective aphids used for the mock inoculation.

Table 1. *Detection of BBTV in nymphs and adult banana aphids by DAS–ELISA with MAbs*

Treatment*	Sample	A_{405} value†
Aphids from infective colony		
Group of five nymphs	a	0·08‡
Single nymphs	a	0·00
	b	0·00
	c	0·00
	d	0·00
	e	0·00
	f	0·09‡
	g	0·00
	h	0·02
Group of five adults	a	0·91‡
	b	1·75‡
Single adults	a	0·25‡
	b	0·51‡
	c	0·30‡
	d	1·44‡
	e	0·37‡
	f	0·03
	g	1·08‡
Aphids from non-infective colony		
Group of five nymphs	a	0·00
	b	0·02
Single nymphs	a	0·00
	b	0·00
Group of five adults	a	0·02
Single adults	a	0·02
	b	0·00

* Samples were individually prepared in 70 µl extraction buffer using a Dounce homogenizer. Fifty µl of this extract was transferred to a single ELISA plate well.

† Coating with MAb 3D12 at 0·5 µg/ml; enzyme conjugate dilution of 1:4000. A_{405} values were read after 3 h substrate incubation.

‡ Positive reactions.

Serological relationships between BBTV isolates from different countries

Both Taiwanese MAbs reacted positively in both forms of ELISA with all tested samples of Australian banana with bunchy top disease symptoms. This was also the case when Australian BBTV polyclonal Ig was used for coating and Taiwanese MAbs were used for detecting. DAS–ELISA using Taiwanese MAb–enzyme conjugate was done with field specimens of dessert bananas (Cavendish types) with typical BBTD symptoms from Yunnan Province, People's Republic of China. We obtained strong positive reactions using either Taiwanese MAb 3D12 or Australian polyclonal Ig for coating. Extracts from two air-dried leaf samples of Cavendish bananas with typical BBTD symptoms from the island of Oahu, Hawaii gave positive, but weak reactions in DAS–ELISA with MAb 3D12. Using Taiwanese MAb a total of 21 Hawaiian BBTD specimens were positive when tested by the State of Hawaii Department of Agriculture (N. M. Nagata, personal communication). Extracts from dried leaf samples of BBTV from Western Samoa and Tonga were tested by DAS–ELISA using polyclonal Ig

Table 2. *Comparison of properties of BBTV with luteoviruses and other plant viruses with small isometric particles*

	Particle diameter (nm)	Sedimentation coefficient	Density in Cs_2SO_4 (g/ml)	Particle protein size ($M_r \times 10^{-3}$)	Nucleic acid type and size	A_{260}/A_{280}
Luteovirus group*	25–28	104–118S	1·32–1·34	23–26	ssRNA, 6 kb	1·8–1·9
BBTV-Gabon	28	–†	–	–	–	–
BBTV-Taiwan	20–22	–	–	21	ssRNA, 6 kb	1·46
BBTV-Australia	18	46S	1·28–1·29	20·5	ssDNA, 1 kb‡ ssRNA, <0·45 kb	1·33
SCSV	17–19	–	1·24	19	ssDNA, 0·9 kb§	1·35
CFDV	20	<75S	–	–	ssDNA, 1·3 kb	–

* Source of data: luteovirus group (Waterhouse *et al.*, 1988), BBTV-Gabon (Iskra *et al.*, 1989), BBTV-Taiwan (Su & Wu, 1989), BBTV-Australia (present work), SCSV (Chu & Helms, 1988) CFDV (Randles & Hanold, 1989).

† (–), Not determined.

‡ Both DNA and RNA detected in a purified BBTV preparation.

§ Multiple species of ssDNA, each approximately 0·9 kb.

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coating and either polyclonal Ig or MAb 3D12 enzyme conjugate. Three samples (including two different cultivars) were tested from each country and all gave positive reactions in both DAS–ELISA combinations.

Discussion

We consistently isolated 18 nm diameter virus-like particles from BBTD-affected bananas from Australia. These particles had typical nucleoprotein u.v. absorbance properties and were similar in size to BBTV from Taiwan (Su & Wu, 1989; Wu & Su, 1990*a*). Highest yields were obtained from the midrib and petiole tissue. For convenience the powdered tissue was stored at −70 °C until needed. In our hands the modified purification method yielded purer BBTV preparations than the original protocol (Su & Wu, 1989; Wu & Su, 1990*a*). We found it essential to use Cs_2SO_4 gradients to separate BBTV particles from impurities that cosedimented in sucrose density gradients.

Under some conditions and treatments, purified BBTV particles appear to be fairly unstable, which is in contrast to the stability reported for luteovirus particles. These conditions include storage in 95% ethanol, in buffers at pH $\geqslant$ 8·5 and freezing at −20 °C without protectants. ELISA parameters were optimized taking into account the apparent instability of BBTV. Skim milk played some protective role to prevent denaturation of virus particles during freezing and incubation with detecting antibodies. MAbs and polyclonal antibodies were used successfully in ELISA to detect BBTV in infected banana tissue and infective aphids. Nymphs of *P. nigronervosa* are known to be more efficient vectors of BBTV than adult aphids (Magee, 1940). It is therefore surprising that we detected BBTV in only one of eight nymphs from an infective colony. It is possible that the ELISA lacked sufficient sensitivity to detect BBTV in these nymphs. However, we did not test these individual nymphs for the ability to transmit BBTV. It is possible that feeding behaviour, rather than the total amount of virus ingested, determines the efficiency of nymphs as vectors.

Although the causal agent of BBTD is regarded as a possible luteovirus (Matthews, 1982; Dale, 1987), typical luteovirus purification steps like cellulase digestion of plant tissue and polyethylene glycol precipitation were unsuccessful with Australian isolates of BBTV. We did not detect typical luteovirus-like particles (25 to 28 nm) in extracts prepared from BBTD-affected bananas using either our standard BBTV purification method or typical luteovirus purification methods (D'Arcy *et al.*, 1989). The physical and chemical properties of purified Australian BBTV also seem inconsistent with classification in the luteovirus group, and indeed BBTV particles share several properties with the recently described SCSV and coconut foliar decay virus (CFDV) (Table 2). However, BBTV and SCSV are both transmitted by aphids in the persistent manner, whereas CFDV is transmitted by planthoppers (Randles & Hanold, 1989).

BBTV preparations used to analyse the protein and nucleic acid composition of the particles appeared relatively pure and homogeneous when assessed by electron microscopy and SDS–PAGE. A major protein of M_r 20500 was detected in purified preparations of BBTV particles but was absent from mock virus preparations from uninfected banana tissue. We assume that this protein is the viral coat protein because it reacts specifically with MAbs and polyclonal antibodies to BBTV in immunoblots, and particles were decorated by the polyclonal antibodies in ISEM tests.

We found both ssDNA of about 1 kb and ssRNA associated with purified BBTV. Just prior to and independently from our experiments Harding *et al.* (1991) also identified an ssDNA associated with partially purified BBTV preparations. A cloned cDNA probe hybridized specifically and consistently with this 1 kb ssDNA from purified BBTV and in nucleic acid extracts of BBTD-affected, but not healthy bananas. These results suggest that this 1 kb ssDNA constitutes the genome of Australian BBTV.

The 1 kb ssDNA component is similar in size to the ssDNA associated with SCSV and CFDV (Table 2). It is not known whether the ssDNA associated with BBTV particles consists of one or multiple DNA species of similar electrophoretic mobility or whether the DNA is linear or circular. SCSV has a multicomponent genome of at least four circular ssDNA molecules (Chu & Helms, 1988) and the DNA associated with CFDV has recently been shown to be circular (Rohde *et al.*, 1990). If the ssDNA associated with BBTV is also circular, our size estimate obtained using linear ssRNA markers would have to be revised. The RNA component may be a host contaminant, though it differs in size from RNAs extracted from virus-free bananas. Were the RNA component due to the breakdown of a larger RNA molecule (e.g. a 6 kb luteovirus genomic RNA), some evidence of this larger band and a continuous smear below this band would be expected in gels. Its size of less than 0·45 kb is similar to that of satellite RNAs (Francki, 1985), but at this stage its identity and function are unclear.

Particle properties for the most part agree with those reported for Taiwanese BBTV (Su & Wu, 1989; Wu & Su, 1990*a*). However, a discrepancy concerns the type of nucleic acid found. Wu & Su (1990*a*) described an ssRNA of M_r $2{\cdot}0 \times 10^6$ associated with purified particles, which is consistent with the characteristics of a

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luteovirus, despite other conflicting particle properties. At this stage we have no explanation for these differences. Dale *et al.* (1986) detected dsRNA suggestive of a luteovirus from Australian BBTD-affected bananas. However, they did not associate any virus particles with these infected bananas. Therefore, the possibility cannot be excluded that the BBTD syndrome is caused by a complex of viruses, one of which is an 18 to 20 nm ssDNA virus, the other a possible luteovirus. In this context, it is interesting that Iskra *et al.* (1989) reported 28 nm luteovirus-like particles isolated from BBTD-affected bananas in Gabon. It would be useful to directly compare these particles, and BBTD-affected plants with the Australian and Taiwanese BBTV and their respective antisera. We were unable to demonstrate infectivity for the 18 nm BBTV particles in a preliminary aphid transmission test of a purified BBTV preparation. Many luteoviruses are known to play a helper role in the aphid transmission of unrelated viruses (Waterhouse *et al.*, 1988) and it is conceivable that a similar situation may occur with BBTD.

The aetiology of BBTD is far from clear at this stage. However, the 18 nm particles appear to be consistently and intimately associated with BBTD. The particles were present in infective but not non-infective aphids and were found consistently in affected banana plants in the field. They were also detected by ELISA after a latent period in the plant of 27 days following aphid inoculation and were present exclusively in symptomatic leaves formed after inoculation. The polyclonal antiserum prepared against Australian BBTV and the Taiwanese MAbs reacted with BBTD samples from Australia, People's Republic of China, Hawaii, Tonga, and Western Samoa indicating a probable common aetiology for the disease in all these countries.

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