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DETECTION OF BUNCHY TOP VIRUS IN MICROPROPAGATED PLANTS USING A PCR GENERATED DNA PROBE

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ABSTRACT: Polymerase Chain Reaction was used to amplify genome sequence of Banana bunchy top virus in the total nucleic acid extract of petiole tissues collected from BBTV infected banana plants. An amplified fragment of 1.00 kb was isolated, labeled with digoxigenin and used as a probe to detect BBTV viral sequences in banana sap-smears made on nylon membranes. In tissue culture facilities this probe will be useful for virus detection.

Key Words: Banana Bunchy Top Virus (BBTV), DNA probe, Micropropagation, PCR

INTRODUCTION

Banana (*Musa* sp) is a major fruit in India. Bunchy top disease (BBTD) caused by Bunchy top virus (BBTV) is the most important viral disease affecting bananas in India and other countries (Dale, 1987). BBTV is vectored in a persistent manner by the banana aphid *Pentalonia nigronervosa*. Coq. This virus has a multi-component genome consisting of at least six circular single stranded DNA (Wanitchakoru *et al.*, 1997) and shares similarity with geminiviruses which have single stranded (ss) DNA (Harding *et al.*, 1993; ICTV, 1995). Management of BBTV in the main field is difficult due to secondary spread by suckers vector. Use of insecticides to manage the vector is not very successful. Banana has been micropropagated with great success world over and today ranks as the number one fruit crop of the Indian micropropagation industry. Of late farmers in India have started using micropropagated plants to meet their planting needs. BBTV can remain asymptomatic in such planting material. Thus, if the source is not effectively screened for BBTV infection, chances are high for the virus disease to find its way into the main field through the planting material (Drew *et al.*, 1989). Moreover Wu and Su (1992) had detected BBTV in symptomless plants using monoclonal antibodies. Thomas *et al.*, (1995) reported that BBTV was readily transmitted through tissue culture in the banana cultivar Lady's finger (AAB) and that the rate of multiplication of lines derived from infected and healthy cultivars were the same. Berg and Bustamante (1974) first reported the technique of meristem culture as a means of producing BBTV free plants from infected material. Later, Doreswamy and Sahijram (1991) reported that shoot meristem dissected from BBTV infected plant (encompassing the apical

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dome measuring 0.1mm and the first pair of leaf primordia) produced BBTV free plants, I but, Drew *et al* (1992) reported that from a portion of BBTV infected plants multiplied in vitro, 73% of the plants progressively developed symptoms over a 6 month period. Thus, detection of BBTV in micropropagated plants before its supply to the farmers is a pre-requisite. Wu and Su (1991) reported that heat therapy was ineffective in eliminating BBTV from infected banana plantlets and that exposure in nutrient culture at 40°C for 16 h each day tended to reduce the growth of tissue and plantlets. The method also failed to eliminate BBTV. This finding proves that BBTV cannot be eliminated from micropropagated material during the *in vitro* culture period. The Food and Fertilizer Technology Centre, Taiwan (FFTC, I 1989) cautioned that even the micropropagated healthy plants of banana must be indexed using molecular methods to prevent BBTV dissemination. Ontiz (1997) reported that *invitro* sucker propagules i.e. pre-basic and basic propagules, need to be indexed for all known viruses using recommended protocols (FAO, 1996) before the germplasm is exchanged or distributed.Hence, it can be concluded that micropropagated plants supplied from infected mother plants cannot be diagnosed by visual symptoms alone. The use of indexed and virus free micropropagated plantlets is a good method of control of BBTV. But, as thousands of micropropagated banana plants are produced every day in a tissue culture facility, there is an urgent need for an effective and rapid assay for detecting BBTV in micropropagated plantlets. In this paper we describe the generation of a specific DNA probe for BBTV and its use.

MATERIALS AND METHODS

i. Micropropagation of BBTV infected plants

Banana plants of cv. *Robusta* showing typical symptoms of BBTV (Jeger *et al.,* 1995) were maintained in the glasshouse in pots. Shoot apices of 0.5-cm length were micropropagated on MS medium (Murashige and Skoog, 1962) containing 30 g sucrose and 8g agar with benzyl adenine at 2 mg/l and were subcultured in the same medium. Micropropagated, healthy plants served as the control.

ii. PCR based amplification of BBTV sequences

Leaves from micropropagated diseased and healthy banana plants were collected and the total nucleic acid extracted from the petiole as follows: 1 g of banana tissue was macerated in liquid nitrogen to a fine powder and was transferred to a sterile 30 ml centrifuge tube containing 15 ml of extraction buffer (0.1M Tris-HCl, 0.5M NaCl, 0.05M EDTA and 0.01M (mercaptoethanol, pH 8.0) and 2 ml of 10% SDS. The contents were incubated in a waterbath at 65°C for 20 minutes. Later, 5 ml of 5M potassium acetate was added to the tube and it was placed on ice for 30 minutes. The extract was centrifuged at 15,000x g for.30 minutes at 5°C. The supernatant was transferred to another tube and the nucleic acids were precipitated by adding 1/2 volume of icecold isopropanol and the tube was incubated at -20°C for 1h and centrifuged at 15,000 x g for 15 minutes at 5°C to sediment the nucleic acids. The pellet was resuspended in minimum volume of water and treated with RNAse A (15µg/ml) and proteinase K (140 µg/ml) followed by extraction with phenol: chloroform. DNA was precipitated with 0.3M sodium acetate and 2 volumes of ice cold ethanol. Precipitated DNA was centrifuged at 15,000 x g for 25 minutes at 5°C. The DNA pellet was washed with 70% ethanol and air-dried. The dried DNA pellet was dissolved in 100 µl of sterile distilled water and used in PCR reactions. The PCR primers used were synthesized (Bangalore Genei, Bangalore) as per Wu *et al* (1994). The primer BB-1 was the forward primer and had a sequence of CATGGTCTATCGAGGCAAG, while, the reverse primer BB-3 had a sequence of GCAGATTCAATTGACGGA. The reaction-mix of 50µl contained the template DNA 5µl; dNTP mix 250µM; primers forward and reverse 2µM

each; Tris-HCl (pH 8.8) 10µM, KCl 50µM, $MgCl_2$ 2.5µM and Taq DNA Polymerase 1.5U. Amplification was performed in a PTC-100 thermal cycler (MJ Research Inc, USA) with the following cycling parameters: Initial melting of template DNA (as replicate forms can be expected in plant tissue) at 94°C for 5 minutes followed by 40 cycles at 94°C for 1 minute; 50°C for 2 minutes; 72°C for 2 minutes. A final primer extension step of 72°C for 8 minutes was given. The amplified fragments were analyzed using agarose (1.2-%) gel electrophoresis. The amplified fragment of 1.0kb was excised and electroeluted into a dialysis bag and was precipitated with ethanol as per standard procedures (Sambrock *et al.*, 1989). The redisolved fragment was labeled using the Wizard Dig DNA labeling kit (Boehringer Mannheim) as per manufacturers instructions briefly described as follows: The amplified fragment was reamplified by PCR using the same protocol as described above but the last primer extension step was reduced to 1 minute. Digoxigenin-dUTP was added to the PCR reaction mix at a concentration of 100µm (dig UTP: UTP ratio was 1:1.5)

Southern blot analysis of the probe for specificity was done as follows: Total nucleic acid extracts from BBTV infected and healthy banana tissue were electrophoresed through 1% agarose gel, denatured and blotted onto a polyvinyl difluride (PVDF) Immobilon® (r) membrane (Millipore Corporation, USA) as per standard protocols (Sambrock *et al.* 1989). The membrane was probed with the dig-labeled 1.0kb fragment as per manufacturers' instructions (Boehringer Mannheim) briefly described as follows: The membrane was incubated in prehybridisation solution at 68°C for 2 h followed by incubation in the hybridization solution containing 5 µl of labeled probe at 68°C for 3h. The filter was washed twice for 15 minutes with 25ml of 2x sodium chloride-sodium citrate (SSC) and 0.1% sodium dodecyl sulphate (SDS) at room temperature (RT) and twice for 15minutes at 68°C with 0.1x SSC containing 0.1% SDS. On completion of the washings the blot was developed using anti-Dig antibodies and anti-species antibodies with BCIP/NBT as substrates as per the instructions of the manufacturer.

iii. Detection of BBTV in sap smears

A PVDF - based membrane (Millipore Corporation, USA) of size 3 x 2 cm was cut and pre-treated in 20x SSC for 30 minutes and excess SSC was drained from the membrane. Tissue cultured *in vitro* plants derived from BBTV infected banana plants were cut across their psuedostems using a sterile blade. The cut areas were pressed firmly on the membrane so as to make an impression with the sap. Also, a small piece of the petiole (5mm dia) was cut with a sterile blade and was placed on the membrane and a small plastic sheet was placed over the cut piece. Using a pestle the petiole was crushed on the membrane. Similarly cut surfaces from tissue cultured *in vitro* plants derived from healthy banana plants were imprinted on the membrane to serve as the control. The membrane was air-dried and baked in vacuum for 2hrs at 80°C. The sap imprints were probed with the Dig labeled 1.0 kb fragment as described above.

RESULTS AND DISCUSSION

Tissue cultured banana plants from both healthy and diseased plants did not show any difference in morphology and growth. Drew *et al.* (1989) reported similar observations. The PCR primers BB-1 and BB-3 amplified a total of 4 fragments of size 1.0, 0.7, 0.5 and 0.3 kb only when the nucleic acid extract from the diseased plants was used as the template (Fig. 1). This agrees with the report of Wu *et al.* (1994) who reported 3 discrete bands when the same pair of primers were used to amplify BBTV sequences from diseased plants. The multiplicity of bands could be due to amplification of segments from different single stranded DNA genomes that might share regions of homology. Similarly, Wu *et al.*, (1994) attribute the multiple bands in PCR to the multiple genomes of BBTV. In our studies, we isolated the 1 kb, PCR amplified fragment and

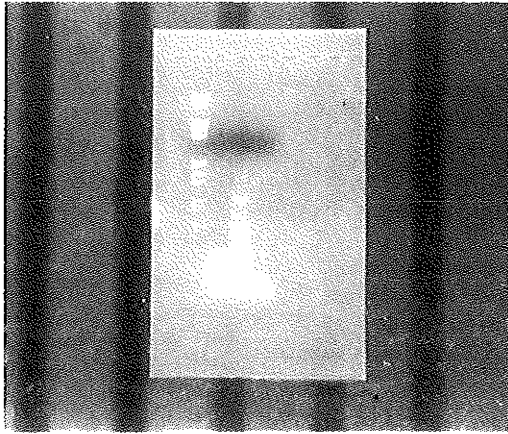

Fig.1. PCR amplified fragments separated on 1.2% agarose gel
Lane 1. Marker (l/Eco RI/ Hind III fragments 21,226, 5143/4973, 4277, 3550, 2027/1904, 1584, 1330, 983/831, 548, 561 bp).
Lane 2. Nucleic acid extract from healthy banana plant as template
Lanes 3 & 4. Nucleic acid extract from BBTV infected plant.

used as a probe in view of the reported genome size of BBTV to be of 1 kb, by Yeh *et al.* (1994) and the primers used were expected to amplify the entire genomic length of BBTV (Wu *et al.*, 1994). The labeled probe hybridized with a 1 kb fragment in the Southern blot of nucleic acid extract of diseased banana plants and not healthy plants (data not shown). This confirmed that the PCR generated fragments of 1 kb were indeed the BBTV sequences. The specificity of the probe was proved by its hybridization with a 1kb fragment present only in the total nucleic acid extract from the infected plant when a Southern blot of total nucleic acid extracts from infected and healthy banana plants was probed. Wu (1994) also reported that a Dig labeled probe for BBTV hybridized only with a 1kb fragment. In the sap smears the Dig labeled probe hybridized only with the smears made by the infected plants in all three replicates. (Fig.2). The probe was able to detect BBTV in sap smears irrespective of whether the smear was made by pressing the cut surface of the psuedostem or by squashing the petiole on the membrane. However, squashing the petiole on the membrane has the advantage of being both quicker and easier apart from being non-destructive in nature as the plant can be marketed after testing. The probe was sensitive enough to detect presence of BBTV in the smears made by just pressing the cut surface on the membrane without any sample pre-treatment. Similar results about Dig probes that detected a variety of viruses in plant tissues by dot blot hybridization with a very high degree of specificity have been reported (Harper and Creamer, 1995). In the abscence of resistant varieties, Integrated methods are the only way to manage BBTV. While *Pentalonia* can spread BBTV in the field, use of disease free planting material will greatly reduce the virus load and consequently the infection. Hence, it is imperative that the planting materials be tested for BBTV by such non-radioactive nucleic acid probes or at the minimum the source material should be tested for BBTV infestation and the micropropagated plants should be grown under aphid proof conditions before the micropropagated banana plants are supplied to farmers.

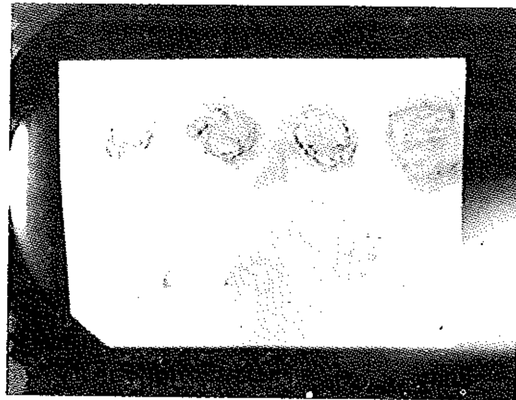

Fig. 2. Sap Imprints of Banana plantlets on PVDF - based membrane, probed with digoxigenin labelled PCR - amplified fragments of 1.0 kb size.
Top Row : Psuedostem imprints of micropropagated Banana plantlets derived from BBTV diseased source plants. (Imprint at extreme is of a crushed petiole on the membrane)
Bottom Row : Psuedostem imprints of micropropagated Banana plantlets derived from healthy plants.

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