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Full Length Research Paper

Molecular diagnostics and phylogenetic analysis of '*Candidatus* phytoplasma asteris' (16SrI- Aster yellow group) infecting banana (*Musa* spp.)

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The presence of phytoplasma in diseased banana plants exhibiting acute stunting symptoms was detected by the polymerase chain reaction using a primer set specific for the 16SrRNA gene of phytoplasma. The amplified DNA fragments of 1500 bp were cloned in pGEMT-Easy plasmid cloning vector and sequenced. The BLASTN and phylogenetic analyses revealed the infecting agents to be the closely related members of the '*Candidatus* phytoplasma asteris' (16Sr I-Aster yellow) group.

Key words: Phytoplasma, polymerase chain reaction, cloning, 16SrDNA, banana, phylogenetic analysis.

INTRODUCTION

Banana (*Musa* spp.) is one of the world's most important subsistence and cash crops in subtropical and tropical regions (http://www.croptrust/org/main). This crop is severely affected by a variety of diseases caused by viruses (Banana bunchy top disease, Streak disease, Bract mosaic disease), fungus (Panama disease) and bacteria (Moko disease), causing serious economic losses. Amongst them, the banana bunchy top disease, (BBTD) caused by Banana bunchy top virus (BBTV) is considered to be the most important (Dale, 1987; Harish et al., 2008). The symptoms of BBTD infected plants are yellowing, dark green streaks on midrib, petioles, extending down into pseudostem, dark green dots and dashes along the minor leaf veins, stunted growth with bunchy top appearance (Magee, 1940; Vetten et al., 2005). It is a major constraint to banana production in many areas of Southeast Asia and the Pacific (Kagy et al., 2001). The diseased banana plants found in field in the Aligarh district (Uttar Pradesh), located at 27^0 29' to 28^0 11'N latitude and 77^0 29' to 78^0 38' longitude, in Northern India, exhibiting stunted growth with symptoms similar to BBTD excluding the bunchy top formation and an earlier report of phytoplasma association with BBTD (Li et al., 1999) has prompted us for *a priori* model study on molecular diagnostics and validation of phytoplasma association with symptomatic plants. Thus, the total genomic DNA from leave tissue of normal and diseased banana plants were extracted for (i) detecting phytoplasma as a causative agent for stunted plant growth, (ii) comparative analysis of 16SrDNA sequence, PCR amplified from the infected plant DNA, with the known phytoplasma genetic sequences for assessment of genetic variability and (iii) phylogenetic analysis to ascertain their genetic relatedness using bioinformatic tools, in order to understand the phytopathological implications of phytoplasma in growth stunting disorder in banana crop.

MATERIALS AND METHODS

Sources of healthy and diseased banana plants

Symptomatic and healthy leaves were collected from different normal and naturally infected banana plants from field in Aligarh district, located at 27^0 29' to 28^0 11'N latitude and 77^0 29' to 78^0 38' longitude, in Northern India, exhibiting stunted growth with symptoms

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similar to BBTD excluding the bunchy top formation.

PCR amplification of phytoplasma and BBTV DNA

Total genomic DNA was extracted from leaf tissues using the procedure of Ahrens and Seemüller (1992). In brief, 500 mg leaf tissues collected from naturally infected as well as healthy plants of banana were minced and grounded in liquid nitrogen. The nucleic acids in the cell free extracts were precipitated with isopropanol and resuspended in 50 µl of TE buffer, pH 8.0. The extracted DNA was analyzed for the presence of BBTV and phytoplasma by specific amplification through polymerase chain reaction (PCR) using primer sets (BBT1/BBT2) (Thomson and Dietzgen, 1995) and (P1/P6) (Deng and Hiruki, 1991) specific for BBTV and phytoplasma 16Sr RNA gene, respectively. Genomic DNA (~50 ng) isolated from healthy and symptomatic banana leaf tissues were used as templates. A total of 50 µl PCR mixture contained 0.20 mM each dNTPs, 25 pmole each primer, 5 µl 10X Taq polymerase buffer, 2.0 mM $MgCl_2$ and 1.5U Taq DNA polymerase. The PCR parameters for BBTV and phytoplasma consisted of 30 cycles of denaturation at 94°C for 1 min, annealing temperature (54°C for BBTV and 58°C for phytoplasma) for 1 min and extension at 72°C for 1 min with an initial denaturation and final extension for 5 and 10 min at 94 and 72°C, respectively. Amplification was carried out in Perkin Elmer DNA thermocycler 9700. An aliquot (5 µl) of each PCR product was analysed by electrophoresis on 1% agarose gel containing ethidium bromide and visualized through UV transillumination and photographed on UVP gel documentation system.

Southern hybridization of banana phytoplasma 16SrRNA gene

The 16S rRNA gene amplicons obtained from diseased banana plants were validated through southern hybridization using a cloned 16SrRNA gene of *Catharanthus roseus* phytoplasma (16SrI-Aster yellow group) (EF015464.1 GI:116490061). The 16S rRNA gene amplicons from banana phytoplasma were transferred to Hybond N membrane (Amersham Biosciences) following the capillary method (Sambrook et al., 1989). The DNA bound membrane was then UV cross-linked for 7 min. The hybridization probe consisting of 16S rRNA gene *C. roseus* labeled with α-^{32}P dCTP was prepared following the Random Priming Extension method (Fienberg and Vogelstein, 1983), using Random Primer Labeling Kit (Genie, Bangalore). The membrane was subjected to prehybridization at 42°C for 1 h following hybridization with the radiolabelled probe (0.5 x 10^6 dpm/ml) at 65°C overnight in an hybridization oven. The membrane was washed twice each in 2X SSC, 0.1% SDS and 1X SSC, 0.1% SDS for 5 and 15 min, respectively, followed by another wash in 1 X SSC, 0.1% SDS for 15 min. All the washing steps were carried out at room temperature. Auto-radiographs were obtained on Fuji X-ray film and the blot was phosphoimaged using Fluor-S ™ Multi-Imager (Bio-Rad).

Cloning and sequencing of phytoplasma 16SrRNA gene

The amplified 16SrRNA gene fragments obtained from the symptomatic samples were purified from the gel using the QIAEX II agarose gel extraction Kit (Qiagen, USA) as per manufacturer's instructions and sub-cloned into pGEMT-Easy plasmid cloning vector at EcoR I site (Promega, USA). Following transformation of *Escherichia coli* strain DH5α cells, the white colonies on LB plate containing appropriate amount of ampicillin antibiotic, X-gal (5-bromo 4-chloro-3 indolyl-β-D-galactoside) and IPTG (isopropyl β-D-thiogalactopyranoside) were selected. Clones from master plates were sub-cultured in presence of 100 µg/ml ampicillin and recombinant plasmid DNA isolated using the method of Birnboim and Doly (1979). The gene insert in the recombinant plasmids of the clones were confirmed by the dot blot hybridization. The positive clones harboring 16SrRNA gene were selected after primary screening and sequenced using an automated ABI-Prism 377 DNA Sequencer (Applied Biosystems) at DNA Sequencing Facility, Department of Biochemistry, South Campus, University of Delhi, India. The sequencing reactions were carried out with Ampli Taq™ DNA Polymerase FS dye terminator cycle sequencing chemistry using the 'ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit' (Applied Biosystems,) according to the manufacturer's protocol.

Multiple sequence alignments and phylogenetic analysis

The 16SrRNA gene sequence was compared with other phytoplasma gene sequences retrieved from GenBank using the Blastn (Altschul et al., 1997). Multiple sequence analysis with the sequences of different phytoplasma groups was performed by using CLUSTALW with default parameters (Thompson et al., 1994). A phylogenetic tree was constructed by the neighbour-joining (NJ) method (Saitou and Nei, 1987) with nucleotide pair-wise genetic distances corrected by Kimura two-parameter method (Kimura, 1980) using TreeCon tool. The reliability of tree topology was subjected to a bootstrap test and numbers at nodes indicate bootstrap support values as a percentage of 1000 replications. All branches with < 60% bootstrap support were judged as inconclusive and were collapsed and branch lengths for all trees were normalized to 0.02% divergence.

RESULTS AND DISCUSSION

Phytoplasmas are the cell-wall-less prokaryotic pathogens reported to cause devastating diseases in a wide range of plant hosts. There presence in the phloem of infected plants, low concentration in host plants and non-culturability in artificial media has hampered their detection. In this study, a phytoplasma has been detected using molecular methods in diseased banana plants showing foliar yellowing, necrosis and stunted growth without any bunchy top appearance (Figure 1). The molecular diagnostics based on DNA sequencing, BLASTN sequence analysis and southern hybridization of cloned PCR amplicons of phytoplasma 16S rRNA gene revealed the presence of a member of *Candidatus* phytoplasma asteris (16S I-Aster yellow) group in infected plants. Representative symptomatic banana plants subjected to PCR amplification yielded the DNA fragments of the expected size (*ca.*1500 bp) using the phytoplasma group-specific primers, from both the symptomatic samples (Figure 2, Lanes 1 and 2) but not from the asymptomatic samples (Figure 2, lane 5). Furthermore, no PCR amplification of DNA occurred in same symptomatic samples when the BBTV specific primers were used (Figure 2, lanes 3 and 4). The validity of PCR-amplified 16S rRNA gene DNA fragments from diseased banana plants has been ascertained through southern hybridization using α-^{32}P-labelled DNA probes prepared from hetrologous cloned 16S rRNA gene of related phytoplasma harbouring *C. roseus*. The results revealed the hybridization of phytoplasma 16S rRNA gene from diseased plant with

Figure 1. Panel depicts the naturally infected banana plant samples exhibiting symptoms of stunted growth.

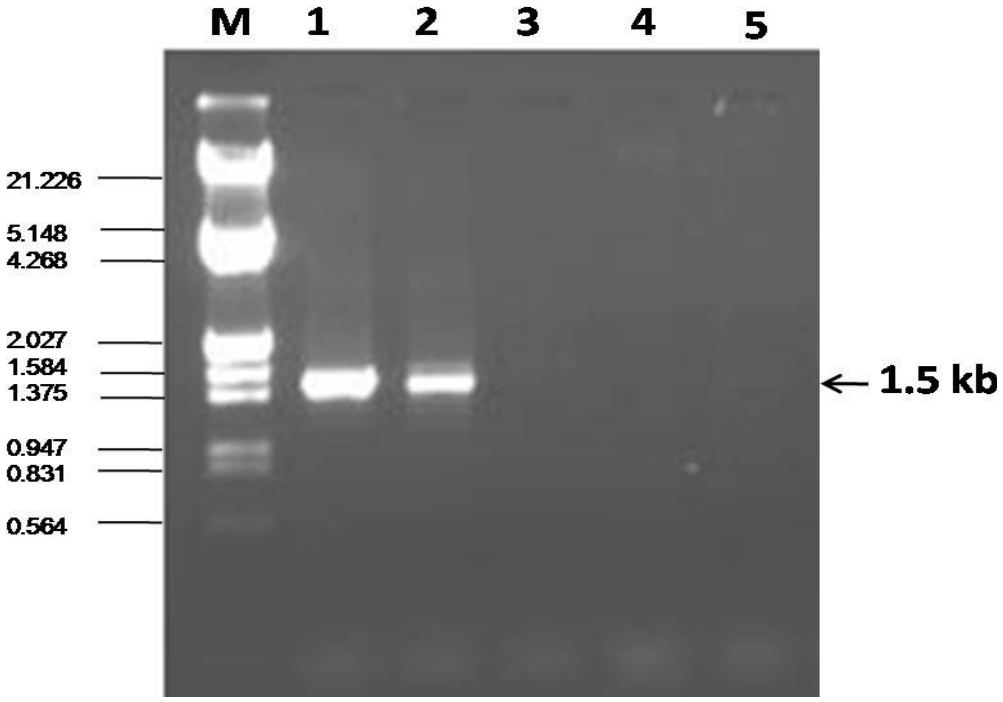

Figure 2. PCR testing of banana plants using the phytoplasma group-specific primers and *Banana bunchy top virus*-specific primers. Lane M: The molecular weight DNA marker (λ phage DNA digested with *Eco*RI and *Hind*III); Lanes 1 and 2: diseased banana plants tested for phytoplasma infection; Lanes 3 - 4, diseased banana plants tested for BBTV infection; Lane 5: healthy (asymptomatic) plant tested for phytoplasma.

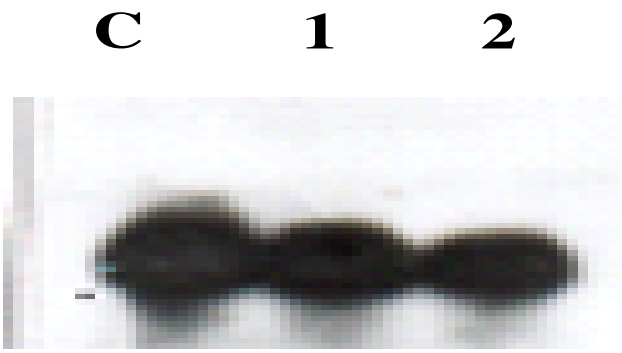

Figure 3. Southern hybridization of 16S rRNA gene PCR amplicon from infected banana plants probed with α-^{32}P-labelled 16S rRNA gene of *Catharanthus roseus* phytoplasma (16SrI-Aster yellow group) (EF015464.1 GI: 116490061). Lane C; Control (16SrRNA gene amplicon of *C. roseus* phytoplasma). Lanes 1 and 2; Phytoplasma 16SrRNA gene amplicons from infected banana plants.

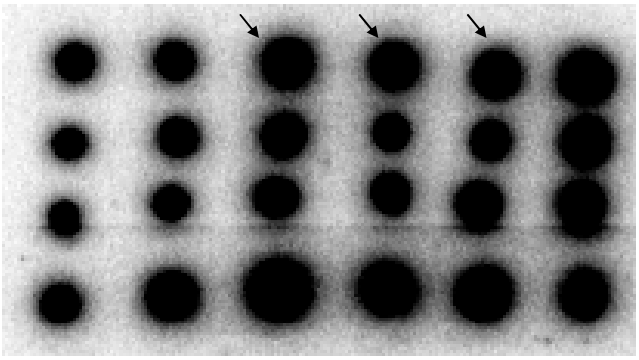

Figure 4. Dot blot hybridization of recombinant plasmids (Banana infecting phytoplasma 16SrRNA gene inserts) obtained from different clones, probed with the α-^{32}P-labelled PCR amplified 16SrRNA gene of diseased banana plant. Arrows indicate the clones selected for DNA sequence analysis.

C. roseus phytoplasma. The pattern of southern hybridization exhibiting positive signals with the banana infecting phytoplasma 16S rRNA gene is shown in Figure 3. The amplified DNA fragments obtained from the symptomatic samples were purified from the gel and subcloned into pGEMT-Easy plasmid cloning vector (Promega, USA). The positive clones containing the desired 16S rRNA gene insert were selected by the dot blot hybridization (Figure 4). The clones were subjected to DNA sequence analysis and the final sequence obtained has been deposited in NCBI GenBank (gi: 224593167; Accession No. FJ688007). Blastn analysis of the 16S rRNA partial sequence exhibited the highest identity (99%) to members of the *Candidatus* phytoplasma asteris (16S I-Aster yellow) group. The current classification of phytoplasmas is based on nucleotide sequence and RFLP (restriction fragment length polymorphism) of the 16S rRNA gene. This gene is present in all the prokaryotes and its conserved and variable regions makes it ideal for phylogenetic classification (Lee et al., 1998). PCR amplification of 16SrDNA of phytoplasmas has significantly contributed to the identification and characterization of unidentified phytoplasmas (Harrison et al., 2003). It is considered to be more sensitive than microscopic, serological and hybridization methods.

Multiple sequence analysis with the sequences of different phytoplasma groups has been performed and a phylogenetic tree constructed by the neighbour-joining (NJ) method with nucleotide pair-wise genetic distances corrected by Kimura two-parameter method using Tree Con tool. The reliability of tree topology was subjected to a bootstrap test and numbers at nodes indicate bootstrap support values as a percentage of 1000 replications. The tree topology also revealed the closest relationship of the banana stunting phytoplasma (gi: 224593167) with members under the sub-clade consisting of *Candidatus* phytoplasma asteris sequences (Aster yellows watercress (gi: 50364083), Eucalyptus yellows and witches'-broom (gi: 51512422), *Capsicum annuum* leaf (gi: 209967516), Barley deformation (gi: 56785865) and Bamboo witches-broom (gi: 226903421) (Figure 5). Thus, the sequence homology and phylogenetic analysis validated the occurrence and association of phytoplasma (gi: 224593167) belonging to *Candidatus* phytoplasma asteris (16S I-Aster yellow group) with symptomatic banana, whereas no infection of phytoplasma was detected in healthy plants. Indeed, several isolates of *Candidatus* phytoplasma asteris (16S I-Aster yellow group) infecting the various crops from the same geographical region (Uttar Pradesh, India) have been reported in recent years (Khan and Raj 2006; Khan et al., 2007; Raj et al., 2006).

Li et al. (1999) have also reported the amplification of the BBT phytoplasma 16S rDNA fragment by nested PCR using DNA extracted from banana plants with banana bunchy top disease. Based on RFLP pattern, sequence data and phylogenetic tree, the authors have demonstrated that the phytoplasma belonged to Group I (Phytoplasma asteri). Moreover, comparative analysis of the homologous regions of the BBT phytoplasma (Li et al., 1999) and phytoplasma (gi: 224593167) reported in this study revealed some sequence variability with two A-G transitions at positions 19 and 401 as well as an insertion at position 154 of amplified DNA fragments. However, it has not been specified as to whether any change in the disease symptoms has been noticed or the presence of BBTV was assessed in the same set of samples. Also, the phylogenetic analyses in earlier report has not been extensive and the multiple sequence alignment was done only with few sequences. On the contrary, a comprehensive multiple sequence analyses has been performed in this study using at least eighteen representative members from different phytoplasma groups, in order to ascertain the phylogenetic position of

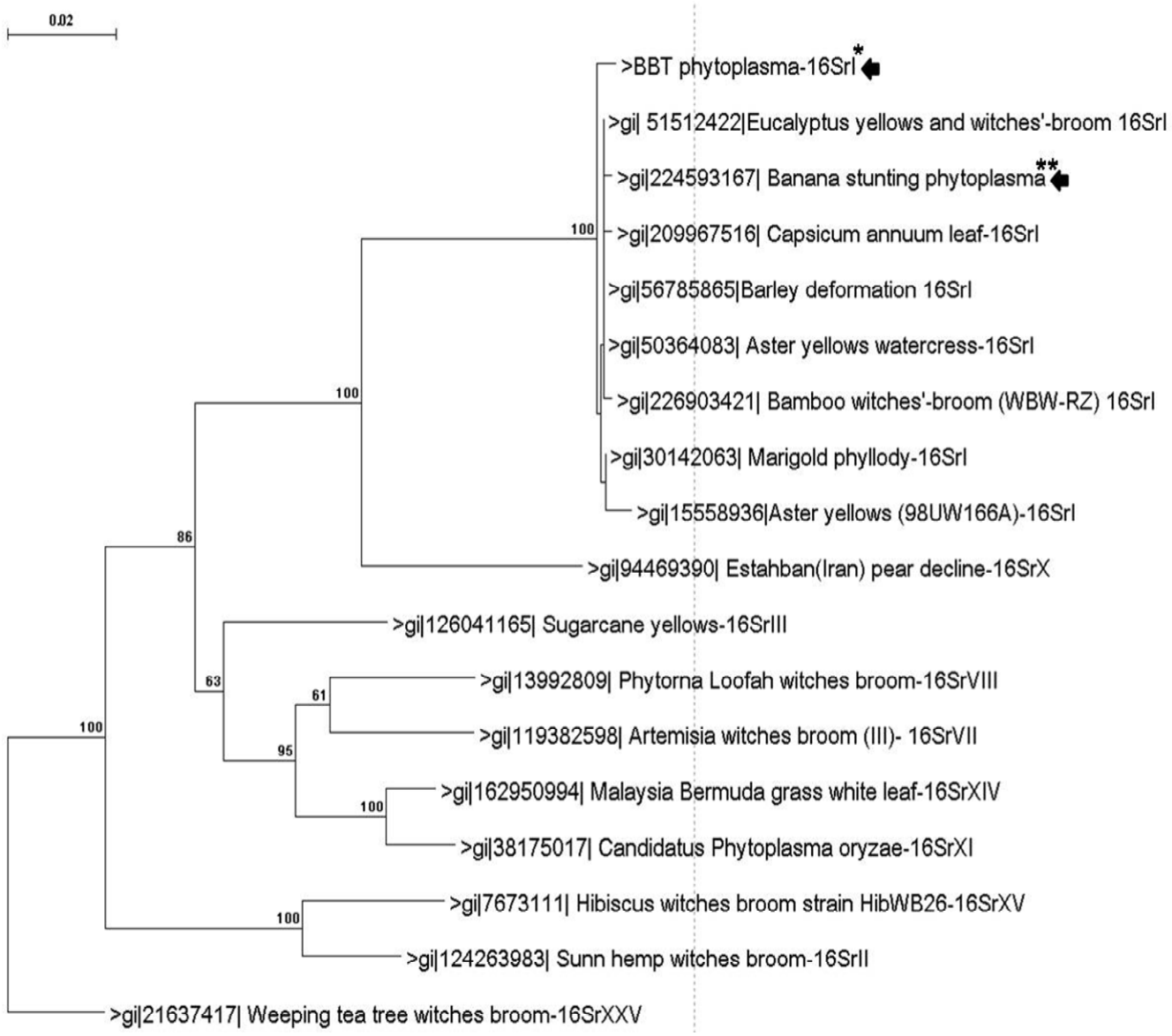

Figure 5. Phylogenetic relationships between the banana associated phytoplasma and reference phytoplasma sequences retrieved from NCBI GenBank, based on 16S rRNA gene nucleotide sequences. All bootstrap values of 60% or greater are indicated on the tree. The tree rooting was forced with the out-group (Weeping tea tree witches broom phytoplasma- 16SrXXV). The arrows represent the status of *Chinese BBT phytoplasma and **Banana stunting phytoplasma from this study in phylogenetic tree. The scale bar indicates the numbers of nucleotide substitutions per site.

the banana infecting phytoplasma. It is concluded that this is the first report from India on the infection of '*Candidatus* Phytoplasma asteris' in banana with stunted growth. Furthermore, the association of phytoplasma with banana may have implications in germplasm exchange and warrants careful diagnostic indexing for healthy cultivation of this major horticultural crop.

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