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PCR detection of *Candidatus* Liberibacter asiaticus, the agent of Huanglongbin or greening disease in citrus

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ABSTRACT: The causal agent of Huanglongbing (HLB) or citrus greening disease (CGD) was detected from total DNA isolated from midrib of leaves of citrus trees from Delhi, Jammu and Maharashtra showing symptoms of yellowing or yellow mottling by PCR amplification of ribosomal protein gene. The amplicon was cloned in pGEM-T easy vector and sequenced. The clone was 703 nucleotide long and showed 100 % nucleotide sequence identity with part of β operon gene of Asian species of greening bacterium i.e. *Candidatus* Liberibacter asiaticus but differed from African species of greening bacterium i.e. *Candidatus* Liberibacter africanus. Our studies indicated that β operon gene of Asian species of greening bacterium is highly conserved and can be used for quick detection of greening bacterium in citrus plant for phytosanitary and certification programme.

Key words: PCR detection, greening bacterium, *Candidatus* Liberibacter asiaticus, citrus greening disease

Citrus greening disease (CGD) is an important disease of citrus and affects the production of citrus fruits in several parts of India. The disease has been reported from several Asian and African countries (Garnier and Bove, 1996). It was first reported by Reinking in 1919 by the name Huanglongbin meaning yellow shoot (Garnier and Bove, 1993). Among the commercial cultivars, group of sweet oranges [(*Citrus sinensis* (L.) Osbeck] is more sensitive to the greening bacterium than the groups of lemons [*C. limon* (L.) Burm.f.] and limes [(*C. aurantifolia* (Christm.) Swingle] (Ahlawat, 1997). Though the disease has non-specific foliar symptoms, symptoms like chlorotic interveinal chlorosis and various types of chlorotic mottling are often associated with disease. The symptoms are often confused with nutritional deficiencies and other stress related factors. Therefore, it is essential that a reliable assay procedure be available to distinguish between nutritional or stress related symptoms and CGD.

Confirmation that a citrus tree is affected by greening has up to now relied on the electron microscopical identification of the bacteria. However, several indirect approaches such as biological indexing, monoclonal antibodies and DNA probes have been used for diagnosis. Biological indexing though advantageous over immunoresponse indexing, which is titer dependent, is basically a time consuming procedure. It requires a well-equipped glass house and long term maintenance of indicator host. The use of monoclonal antibodies for field diagnosis has proven unsatisfactory (Korsten *et al.*, 1993; Varma *et al.*, 1993). The study by Hocquellet *et al.*, (2000) and Ahlawat *et al.*, (2003) have shown that amplification of β operon ribosomal protein gene is a sensitive and promising technique for the detection and differentiation of greening bacterium of Asian and African origin. We tested this procedure on field samples. Cloning and sequence analysis were done for two samples of mandarin collected from Delhi, India and compared with that of isolates from Pune (India), Bhutan and African countries.

MATERIALS AND METHODS

Leaf samples were collected from citrus plants in IARI orchards (Mandarin oranage- *C. reticulata*

Blanco and sweet orange 'mosambi') and other locations viz. Nagpur and Jalna in Maharashtra (sweet orange 'mosambi') and Jammu (grapefruit - *C. Paradisi* Macf). Samples from symptomatic citrus trees were collected from each location. Total DNA was extracted using DNeasy Plant Mini Kit (Qiagen Gmbh, Hilden, Germany) from 200 mg midrib of citrus leaves showing yellowing / yellow mottling symptom as well as from healthy citrus leaves. Primers designed previously from conserved region of ribosomal protein genes in β - operon (5' TATAAAGGTTGACCTTTCGAGTTT and 5'' ACAAAAGCAGAAATAGCACGAACAA) were obtained from Qiagen Operon, Germany. PCR amplification was performed in a thermal cycler (Biometra, Germany) in 50 µl reaction mixture using 1 µM of each primer, 200-µM of each dNTPs, 0.05 unit / µl of Taq DNA polymerase (Fermentas, Lithuania), 1 x reaction buffer, 1.5 mM of $MgCl_2$ and 5 µl of DNA template isolated from healthy and symptomatic citrus plants. The temperature regimes were 94 °C for 4min, followed by 30 cycles of 94 °C for 45 sec, 58 °C for 45 sec, 72°C for 60 sec, followed by a final extension of 10 min at 72 °C. PCR amplification of samples were done at least twice.

PCR products were analysed on 1% agarose gel in Tris Acetate EDTA buffer containing ethidium bromide. DNA was visualized and photographed using a gel documentation apparatus. The PCR product from Delhi mandarin was excised and eluted from the gel using QIAquick gel extraction kit (Qiagen Gmbh, Hilden, Germany). The gel eluted DNA was ligated into pGEM-T easy vector (Promega, Madison, USA) and competent *Escherichia coli* strain DH5α were transformed by standandard molecular biology protocol (Sambrook and Russel, 2001). Restriction endonuclease digestion and colony PCR identified recombinant clones. Selected clones were sequenced at automated DNA sequencing facility at Department of Biochemistry, University of Delhi, South Campus. BLAST programme was used to compare the homology with sequences available in the GenBank database [M94319 (Liberibacter asiaticus, Pune India), U09675 (L. africanus nelspruit, S. Africa) and AF2484 (L. africanus subsp capensis, S. Africa) and with sequence from sample of greening disease of Bhutan. Sequences were aligned using CLUSTAL W and the sequence identity matrix for pair wise combination of aligned sequences was calculated with Bio Edit Sequence Alignment Editor (Hall, 1999).

RESULTS AND DISCUSSION

Approximately ~700 bp amplicon was obtained from 3 samples out of 4 citrus plants showing symptoms of yellowing and mottling (Fig. 1). These positive samples were from sweet orange and mandarin of IARI (lane 2 and 3) and sweet orange 'mosambi' of Nagpur (lane 5). The sweet orange samples from Jalna (lane 4) did not produce any amplicon. Healthy plant of mosambi was used as negative control and did not produce any amplified

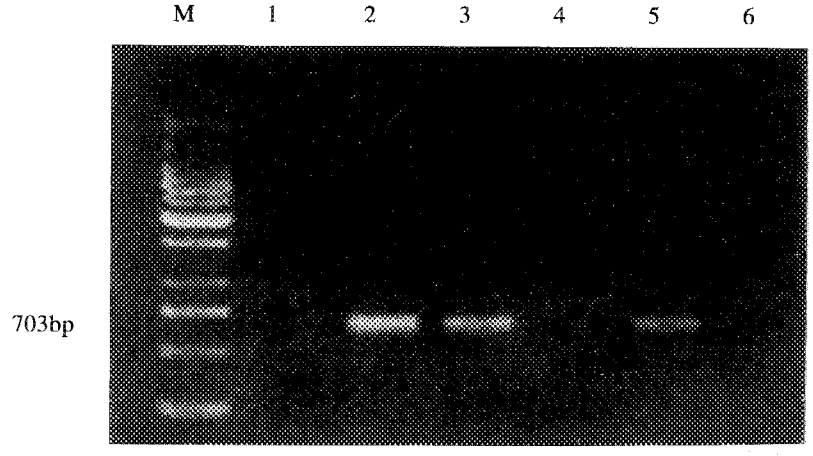

Fig. 1. PCR detection of *Candidatus* Liberibacter asiaticus beta - operon ribosomal protein genes.1 Kb DNA ladder (M), DNA extracted from healthy (1, 6) and symptomatic Delhi sweet orange (2), Delhi mandarin (3) Jalna sweet orange (4) and Nagpur mosambi (5)

product (lane 1 and 6). Out of total 6 samples from Delhi, PCR amplification was observed in 5 samples while amplification was observed only in 3 out of 5 symptomatic samples from Nagpur. PCR amplification of greening bacterium was also observed in three out of 4 samples of Grapefruit from Jammu (Not shown). The non-amplification of bacterial gene in symptomatic plants from Jalna (Maharashtra) and other regions may be because the yellowing symptoms are not caused by greening bacterium.

The sequence analysis showed that the cloned DNA from both the samples of mandarin orange from Delhi were 703 nt long (GenBank accession no. AY266352) having 100% sequence identity with the corresponding nucleotide sequence of ribosomal protein gene of *Candidatus* Liberibacter asiaticus (GenBank accession no. M94319) (Fig 2). A similar result was obtained from samples of mandarin orange showing the symptoms of CGD in Bhutan (Ahlawat *et al.*, 2003). The same pair of primer produces an amplicon of around 650 bp from citrus plants infected with *Candidatus* L. africanus strain nelspruit (Hocquellet *et al.*, 2000). The specificity of the amplification with primers from β operon gene was earlier evaluated by Hocquellet *et al.*, (2000) and amplification products of expected size were obtained from *Candidatus* Liberibacter asiaticus

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Poona   TATAAAGGTTGACCTTTCGAGTTTCTCTGTTTAATAC-TCTTGTTTAGTTGTTTTTTGTGTGGATTCCTT 69
Delhi   ...................................................................... 69
Bhutan  ...................................................................... 69
A-nel   ........................TG..........A.....A..GG...A..G...A..A--...T... 67
Af-su   ........................T........G..AA..C.T...G.A....A.......--...T... 68

Poona   TTTCGCTATCGGATCGCTTCTTTTTTGTAAGGGATGCGTTAGGATTTTTGTTCTTCTTCGAAATCAAG-A 138
Delhi   ...................................................................... 138
Bhutan  ...................................................................... 138
A-nel   AGC-.A.G..TCTAT.A..T.-----------------------------.G..T..A..GG.ATTC.G 105
Af-su   G.C........TT.T.T..T.C..G.A-.......T...AGA.G-------.G..T..T..GG....... 129

Poona   TATGAAAATATTTTCTTGGTATAGATATAGGAAAA--GGAATGGGTATATTTGT--CATCTGGAGATGAA 204
Delhi   ...................................................................... 204
Bhutan  ...................................................................... 204
A-nel   ..GA..TG.T....-...-.G.....G...A.G..C...TT.AAT.G.....A.......G......... 170
Af-su   ...AG.T.......-...A....A.........G.T...TT.AAT.G...G.A.......G....N.AG. 195

Poona   AGTTGAATAGACAAGGAAAGAGCGTAGAAATTTCTGAATTAAGTAAGATTTTTTCTTCTTCTGGATCAAT 274
Delhi   ...................................................................... 274
Bhutan  ...................................................................... 274
A-nel   ..........G....A......T..G...........................................G. 240
Af-su   ...............A...A..T.....G........G..G...........G...........G...G. 265

Poona   TGTTGTTGCACATTATAAGGGAATTAGTGTTGCGCAAATTAAAGATCTTCGGAAAAAGATGCG--GGAAG 342
Delhi   ...................................................................... 342
Bhutan  ...................................................................... 342
A-nel   .........G..C...................A.....G..A............A..G..AG......A.... 308
Af-su   ...C.....G..C.....A...............A..G..C.GGA--.....A..G...G.NNNCGA.... 333

Poona   CTGGTGGAGGTGTAAAAGTTGCCAAAAATCGTCTCGTCAAGATTGCTATCC--GTGATACTAGTATTAGA 410
Delhi   ...................................................................... 410
Bhutan  ...................................................................... 410
A-nel   ................G.....G...........T..T........CG..A...C.........T.G.AG 376
Af-su   .............G..G..NNNNN.......C..T..T........CG.T.............CG...AG 401

Poona   GGAATATCTGATCTTTTCGTTGGGCAG---TCTCTAATTGTCTATTCGGATAGTCCTGTTATTGCTCCTA 477
Delhi   ...................................................................... 477
Bhutan  ...................................................................... 477
A-nel   ..TG.T..A........T........A.....AT.G.....T.......T.GAC...A..G......... 443
Af-su   .A.G.T.....C.....T........A.....A..G.....T.....A.T.GA....A..G......... 468
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Poona    AAATTTCGGTTAGCTTTTCAAATGACAATAATGAATTTAGAGTTCTTGGTGGGGTTGTAGAGAAGGGCGT 547
Delhi    ............................................................................ 547
Bhutan   ............................................................................ 547
A-nel    .G.....T..G......G.G.....T.....AC.G...GTG.........C..TA..T.G.....A.ATA. 513
Af-su    .G.....T..A.A...CG.......T..C..GC.....GT............A...T....A..T.AT.. 538

Poona    CCTTAATCAAGATTCTATCAAGCAAATTGCTTCTTTACCCGATCTTGAGGGTATTCGAGCTGGTATCATA 617
Delhi    ............................................................................ 617
Bhutan   ............................................................................ 617
A-nel    T...G.C..............A.GG........G..G..TA..A....T.........TT..ATG.....T 583
Af-su    T...G.....TG......A..A..............G..AA..A....T.....C..T...AT....... 608

Poona    AGTGCTATCCAATCTAATGCAACTAGATTGGTTAGACTTCTTGGTACGCCACAGACTCAAGTTGTTCGTG 687
Delhi    ............................................................................ 687
Bhutan   ............................................................................ 687
A-nel    ........T....T....T.G...........A.AT......AA.G.A..T......A..A......... 653
Af-su    ........T....T......G....A....C.C..G......A..G....T...G.A.............. 678

Poona    CTATTTCTGCTTTTGT 703
Delhi    ................ 703
Bhutan   ................ 703
A-nel    ................ 669
Af-su    ..C............. 694
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Fig. 2. Sequence alignment of a portion of the bacterial ribosomal protein gene from citrus greening infected sample from Delhi (AY 266352) and Bhutan with published sequences from Poona, India (M94319) and South Africa [A-nel (U09675), Af-su (AF2484). ----- indicates deletions, ... indicates similarity

and *Candidatus* L. africanus only and not from other citrus associated bacteria. The sequence analysis showed that ribosomal protein gene sequence from *Candidatus* Liberibacter asiaticus from India (Poona and Delhi) and Bhutan were completely identical with each other and showed 89% and 79% identity with L. africanus nelspruit and L. africanus subsp capensis respectively. This study demonstrated that the primers from ribosomal protein genes in β - operon can be used for specific detection of *Candidatus* Liberibacter asiaticus, the agent for citrus greening disease and should be highly useful in phytosanitary assay and bud wood certification.

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