MOLECULAR DETECTION AND ANALYSIS OF ASIAN STRAIN SPECIFIC LAS FRAGMENT OF HUANGLONGBING BACTERIUM (Candidatus Liberibacter asiaticus) VECTORED BY Diaphorina citri Kuwayama (HEMIPTERA:PSYLLIDAE) ASSOCIATED WITH DECLINE OF COORG MANDARIN (Citrus reticulata Blanco).

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ABSTRACT: Coorg mandarins have been declining in a condition called as “greening”. Phloem limited Huanglongbing bacterium vectored by Diaphorina citri Kuwayama has been implicated as a major cause. PCR detection specific for asian strains using leaf mid vein tissues of Budlings adult psylla was done. The primer pair F-Las and R-Las amplified a 226 bp fragment specific for L. asiaticus (asian strain) both from infected plants and also from single infective Psylla. This fragment was analyzed and secondary structure of the proteins was predicted. The sequence is lacking dimers or complex regions of similarity with 7 ORF’s. The protein sequences FLAR_1F_4 had homology with RpoB putative 50S ribosomal subunit, Ribosomal protein L1 and UDP-3-O-3-hydroxymyristoyl glucosamine N-acyltransferase of Candidatus L. asiaticus and Candidatus L. africans. The secondary structure was of alpha helices, extended strands, and random coils. The method is sensitive for detection and is time saving.

Key Words: Citrus Greening Bacterium, Coorg mandarins, decline, Diaphorina citri, greenning, Liberibacter

INTRODUCTION
Kodagu is situated on the Western ghats of India, between 11° 15’ and 20° 50’ North Latitudes and 72° 25’ and 76° 14’ East Longitudes. The yearly rainfall is between 3,000 to 3,800mm with an average temperature of 15 °C. Coffee is the main crop, in the coffee plantations Coorg mandarin is grown as a shade and inter crop. Coorg mandarin plants have been showing symptoms of decline (Photo 1) commonly called as “Greening” (da Graca, 1991). Affected plants show yellow green mottling on the leaves, leading to a general decline and a steep fall in fruit set (Das et al., 2004, 2007). In the humid tropical ecosystem where these plants are grown the decline syndrome has been associated to fungal and nutrient stress. Huanglongbing bacterium (Candidatus Liberibacter asiaticus) (Ahlawat, 1997, Bové, 2006) which is a phloem limited
Photo 1. Symptoms of Coorg mandarin decline

member of the sub-division of the proteobacteria (Jagoueix, et al., 1994) vectored by Diaphorina citri Kuwayama (Caoop et al., 1967, Albert and Manjunath, 2004,) has been implicated as a major cause of greening.

MATERIALS AND METHODS

To develop a sensitive polymerase chain reaction (PCR) detection protocol specific for asian strain of greening experiments were conducted. Leaf mid vein tissues of budlings of Coorg mandarin and adult psylla fed on PCR positive greening plants were used. DNA was extracted and PCR was done as described by (Hung et al., 2004) The 226 bp amplified fragment was sequenced (Bioserve Biotechnologies, Hyderabad) and was analyzed using the following programs described in the Appendix. Clustalw 1.81 (Cluster Alignment on Windows) (Jeanmougin et al., 1998), Treeview (Page, 1996), BioEdit (Hall, 1999) and EMBOSS (The European Molecular Biology Open Software Suite) - dotup, dotmatcher, plotorf, remap, pepnet and pepwheel . (Rice et al., 2000). The nucleic acids were BLAST-P (The Basic Local Alignment Search Tool – Protein) matched and the structure of similar enzyme was visualized using the program cn3d (www.ncbi.nlm.nih.gov/ Structure/CN3D/cn3d.shtml). The secondary structure prediction of the proteins was done using the software PREDATOR (Incorporator of non-local interactions in protein secondary structure prediction from the amino acid sequence) (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_predo.html) pepnet and pepcoil programs of EMBOSS were used to draw the transmembrane structure.

RESULTS AND DISCUSSION

The primer pair F-Las and R-Las amplified a 226 bp fragment specific for L. asiaticus (asian strain) as reported by Hung et al., 2004. This fragment was consistently amplified both from the infected plants and also from single infective psylla (Photo 2 and 3), dotup and dotmatcher analysis of the nucleotide sequence revealed that this sequence is lacking dimers or complex regions of similarity. (Fig 1 and 2) plotorf revealed the presence of 7 ORF’s in the amplicon. In strand 2F one continuous ORF of 120 nt, and in strands R1, R2 and R3 strands interrupted ORF’s of (50,7), (20, 30) and (15,15) nts. (fig 3)
remap revealed that more than 600 restriction enzymes (REs) and Isoschizomers cut once, 13 REs cut twice, 264 REs do not cut within the sequence. (fig 4). BLAST-P matching revealed that the translated protein sequences FLAR_1F_4 of citrus greening bacterium in Chettalli had homology with *RpoB* putative 50S ribosomal subunit, Ribosomal protein L1 of *Candidatus L. asiaticus* (fig 4) the visualization of the 3 dimensional structure of the enzyme with cn3d correlated to the protein prediction by PREDATOR. Protein sequences FLAR-1R.fasta4, FLAR-1R.fasta5, FLAR-1R.fasta6 of Citrus Greening Bacterium in Chettalli had homology with putative UDP-3-O-3-hydroxymyristoyl glucosamine N-acyltransferase of both (*Candidatus L. asiaticus*) and (*Candidatus L. africanus*). Protein sequences FLAR-1F-4, FLAR-1R.fasta4, FLAR-1R.fasta5, FLAR-1R.fasta6 of citrus greening bacterium in Chettalli had homology with gene bank protein sequences gi|55380592, gi|55380586 and gi|55380588. The secondary structure prediction by PREDATOR of the proteins indicated that the proteins coded by this amplicon were made up of alpha helices, extended strands, and random coils (Fig. 6).

**Photo 3.** Left adult male Psylla (*Diaphorina citri kuwayama*) feeding on Coorg Mandarin. Right Lane 1 - Marker (100 bp Fermentas), Lane 2 - PCR of 3 adults, Lane 3 - PCR of 1 adult, Lane 4 - Control

**Fig. 1.** Research Flow Diagram
Peptide structure prediction done using the EMBOSS program pepnet which displayed proteins as a helical net and pepwheel which displayed the peptide sequences in a helical representation in which the side chains were placed on the outside of the circle, staggered in a fashion of 3.6 amino acids per turn of the helix and gave a view down the axis of the helix highlighting amphipathicity and other properties of residues around the helix. The residues of the protein were displayed in a simple 3,4,3,4 repeating pattern which emulated the arrangement of residues around an alpha helix. In both the programs the aliphatic residues ILVM were marked with squares, the residues DENQST with diamonds and the positively charged residues with octagons revealed that in proteins coded by FLAR-I were arranged. pepnet display (Fig.7) revealed that blue colored alpha helices predominated with a few red colored extended strands, pepwheel display (Fig.8) was also similar and showed an unequal distribution of red colored non-polar residues relating to the hydrophilic nature of the protein (Rachamandran et al.,1968)

Coorg mandarin decline needs to be reversed at the earliest as the native Kodavas have a high affinity to the fruits and value it as a part of their culture. Decline is a physical condition and can be seen and felt as the crop would become unproductive, but the real cause of decline has to be diagnosed, as greening bacteria are few and are unequally distributed in the plant, the test needs to be very sensitive. Rejuvenation programs demand accurate diagnosis. The developed PCR method detects the presence of the asian strain of Huanglongbing bacterium in Coorg. The results were similar to Huang et al.,(2006). The bacterium was also detected from single viruliferous psylla confirming it as a vector (Albert and Manjunath 2004). However in a mixed mountain cropping ecosystem, the role of other pathogens like Phytophthora sp.,(Antonio et al.,2002) Fusarium sp. (Rensburg et al.2002), Aspergillus sp.,(Varma and Verma, 1987) nematodes (Duncan, 2005) and nutrient stress (Srivastava and Singh,2006) cannot be ruled out as causal agents of decline. The PCR method of diagnosis helps to detect the infection of CGB in a situation where similar
Ribosomal protein L1 is the largest protein from the large ribosomal subunit and binds to the 23S rRNA. The L1 protein is a component of the 50S subunit of the ribosome and also functions in the post-transcriptional regulation of the ribosomal protein genes encoded in the L11 operon. Ribosome's lacking L1 show a lower translation activity than wild type (Subramanian and Dabbs, 1980). Hence this amplicon can be used in further studies for development of drug targets (Greer et al., 1994) or for preparation of rDNA antibodies (Bastianel et al., 2005). As the presence of Asian strain of greening and its vector psylla have been identified in Coorg, it can be concluded that they are a cause for decline. Hence appropriate budwood certification programs need to be strengthened for establishment of disease free bud blocks

ACKNOWLEDGEMENTS

The funding provided by the Government of Karnataka, Department of Horticulture under the project Diagnostic Approaches and Development of Management Strategies for Rejuvenation of Coorg Mandarin in Kodagu District and the technical services rendered by Ms.P.B.Swathy, Lab Tech T-4, and Shri.B.N.Krishnappa, Tech Officer T5 are acknowledged.

APPENDIX

Additional image files relating to this paper is available on the website http://dksamuel.110mb.com (CLUSTAL-W alignment, TREEVIEW dendrograms, related sequences, pdf of paper, etc)

REFERENCES


