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Detection of 'C*andidatus* Phytoplasma brasiliense' in a new geographic region and existence of two genetically distinct *dna*K genotypes

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Abstract

In September 2007, a peach tree (*Prunus persica*) displaying yellowing symptoms indicative of phytoplasma infection was sampled in Quba region of Azerbaijan. A phytoplasma was detected in the diseased peach tree by nested PCR amplification of its 16Sr DNA with universal primers for phytoplasmas. Phylogenetic analyses of the amplified 16S rDNA showed that the phytoplasma infecting peach tree corresponded to '*Candidatus* Phytoplasma brasiliense', a phytoplasma not previously reported in the Euro-Mediterranean area. To set up a detection assay, cloning of a '*Ca.* P. brasiliense' DNA fragment was undertaken by comparative RAPD. The amplified *dna*K-*dna*J genetic locus was used to design a nested PCR assay able to amplify all '*Ca.* P. brasiliense' strains of the subgroup 16SrXV-A without amplifying the related members of the group 16SrII. The use of this assay also confirmed detection for the first time of '*Ca.* P. brasiliense' in diseased basil collected in south Lebanon.

Key words: '*Ca.* P. brasiliense', hibiscus witches' broom disease, *dna*K gene, molecular characterization.

Introduction

Phytoplasmas are plant pathogenic bacteria belonging to the class *Mollicutes*, a group of wall-less microorganisms having low G+C content, Gram-positive bacteria. They cause hundreds of diseases worldwide and are transmitted from plant to plant by sap-feeding hemiteran insects (Lee *et al*., 2000; Weintraub and Beanland, 2006). Phytoplasmas have been classified into 30 phylogenetic groups and 28 *'Candidatus* Phytoplasma*'* species according to 16S rDNA phylogeny and RFLP profiles (Zhao *et al*., 2010). Among these, '*Ca*. P. brasiliense' has been described as the agent of hibiscus witches' broom in Brazil. During a survey of temperate fruit tree orchards of the North of Azerbaijan, a phytoplasma could be detected by 16S rDNA PCR of a chlorotic peach tree (*Prunus persica*). We report in this paper its identification as a strain of '*Ca.* P. brasiliense' and the development of a specific PCR detection test developed from a '*Ca.* P. brasiliense' sequence cloned after comparative RAPD analyses.

Materials and methods

Yellowing peach tree (*Prunus persica*) samples indicative of phytoplasma infection were collected in September 2007 in Quba region. The DNAs were extracted following the CTAB extraction protocol of Maixner *et al*. (1995). Detection of phytoplasma infection was performed by nested PCR with the 16S rDNA universal primers for phytoplasmas as described by Gundersen and Lee (1996). The PCR product obtained from one of the peach trees (PEACH19) was sequenced. The raw sequences were assembled and edited using GAP4 and the consensus sequence deposited at EMBL (FR717540). ClustalW multiple alignments and maximum of parsimony phylogenetic analyses were performed by MEGA 4 (Tamura *et al.*, 2007).

To investigate genetic variability of the detected phytoplasmas non ribosomal *dna*K isolated by comparative RAPDs, was amplified by group specific primers recently developed (Balakishiyeva *et al.*, in press) Nested PCR products were digested with *Taq*I (Promega) according to the manufacturer's instructions.

Results

The 16S rRNA sequence of the PCR product obtained from PEACH19 phytoplasma shared 100% identity with the 16S rRNA sequence of '*Ca.* P. brasiliense' group 16SrXV (hibiscus witches' broom) (Montano *et al.*, 2001). Both sequences clustered together on the same phylogenetic branch supported by a bootstrap value of 100 (data not shown) indicating phytoplasma affiliation to subgroup 16SrXV-A, '*Ca.* P. brasiliense'.

No specific detection tool was available for '*Ca.* P. brasiliense' mainly because except for the 16S rRNA gene, no '*Ca.* P. brasiliense' gene had been sequenced. Therefore, the characterization of a '*Ca.* P. brasiliense' non ribosomal genetic locus was undertaken using random-PCR that allowed the *dna*F-*dna*J locus to be cloned and sequenced. A PCR test amplifying the non ribosomal *dna*K gene was developed with the aim of specifically detecting phytoplasmas of subgroup 16SrXV-A ('*Ca.* P. brasiliense') (Balakishiyeva *et al.*, unpublished). To verify the specificity of the *dnaK* gene primers (Bra-dnaKF1/R1 and Bra-dnaKF2/R2), nested-PCR

was performed on DNAs extracted from diseased peach, healthy peach, Suriname virescence infected periwinkle, '*Ca.* P. brasiliense'-infected basil (Choueiri *et al.*, unpublished), three '*Ca.* P. brasiliense'-infected *Hibiscus rosa-sinensis* plants, and ten phytoplasma strains belonging to the 16SrII group. The Bra-dnaK nested-PCR amplified phytoplasmas of the subgroup 16SrXV-A but did not amplify the related phytoplasmas of the group 16SrII. For further characterization of phytoplasmas of group 16SrXV-A, the PCR products were submitted to restriction fragment length polymorphism (RFLP) analysis with restriction enzyme *Taq*I. Results of RFLP analysis (figure 1A) showed that the restriction of the amplicons using *Taq*I revealed two restriction profiles among the different '*Ca.* P. brasiliense' strains. All hibiscus witches' broom phytoplasmas gave the same pattern characterized by a DNA band at 0.45 kbp, whereas PEACH19, Suriname virescence and basil (figure 1A) had no 0.45 kbp DNA band but an additional band at 0.3 kbp and a brighter band at 0.15 kbp. These results indicate the existence of two genetically different strains of '*Ca.* P. brasiliense'. This was confirmed by twp sequence types after sequencing the six amplicons. The first sequence type (accession number FR775800), that of hibiscus witches' broom 121, 122 and CB2 exhibited 19 mutations when compared to the sequence of Suriname virescence, basil and PEACH19 which were totally identical and constitute a second sequence type (accession number FR717541). A guanine to thymine mutation at position 689 (figure 1B) in the bra-dnaK PCR product of hibiscus witches' broom strains eliminated a *Taq*I restriction site, responsible for the difference in restriction profiles with the Suriname virescence, basil and PEACH19 strains.

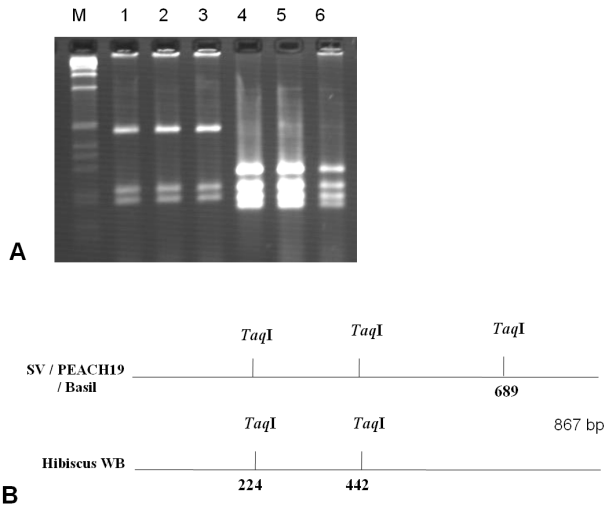

Figure 1. A: RFLP analysis with *Taq*I. Digested DNAs were analyzed on 3% agarose gel in 1X TBE. B: *Taq*I restriction map of bradnaK PCR products amplified from '*Ca.* P. brasiliense' strains. Lane M, 1kb DNA ladder (Invitrogen), lane 1-Hib121, lane2-Hib122, lane3-Hib CB02, lane 4-SV, Suriname virescence infected periwinkle, lane 5- Bas, Basil from Lebanon, lane 6-PEACH19, diseased peach tree from Azerbaijan.

Discussion

Reported geographical distribution and host range of '*Ca*. P. brasiliense' include *Sida rhombifolia* in Brazil (Eckstein *et al*., 2011), *Guazuma ulmifolia* in Costa Rica (Villalobos *et al*., 2010), and *Gliricidia sepium* in Ethiopia (unpublished GenBank accession AF361018). This phytoplasma, new for the old world was detected in a peach tree in the Quba region of Azerbaijan and in basel from Lebanon. Its presence not only in a woody host but also in an annual host (basil) indicates its possible local transmission by insects.

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