Detection of ‘Candidatus Phytoplasma brasiilense’ in a new geographic region and existence of two genetically distinct dnaK genotypes

Gulnara BALAKISHIYEVA1,2,3, Madat QURBANOY4, Alamdar MAMMADOV3, Shahniyar BAYRAMOV3, Xavier FOISSAC1,2

1INRA, UMR1332 Fruit Biology and Pathology (BFP), Villenave d’Ornon, France
2University of Bordeaux, UMR1332 Fruit Biology and Pathology (BFP), Villenave d’Ornon, France
3National Academy of Science, Institute of Botany, Baku, Azerbaijan
4Institute of Horticulture and subtropical crops, Quba, Azerbaijan

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 brasiilense’, a phytoplasma not previously reported in the Euro-Mediterranean area. To set up a detection assay, cloning of a ‘Ca. P. brasiilense’ DNA fragment was undertaken by comparative RAPD. The amplified dnaK-dnaJ genetic locus was used to design a nested PCR assay able to amplify all ‘Ca. P. brasiilense’ 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. brasiilense’ in diseased basil collected in south Lebanon.

Key words: ‘Ca. P. brasiilense’, hibiscus witches’ broom disease, dnaK 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 hemipteran 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. brasiilense’ has been described as the agent of hibiscus witches’ broom disease in Brazil. During a survey of temperate fruit 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. brasiilense’ and the development of a specific PCR detection test developed from a ‘Ca. P. brasiilense’ 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 dnaK isolated by comparative RAPDs, was amplified by group specific primers recently developed (Balakishiyeva et al., in press) Nested PCR products were digested with TaqI (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. brasiilense’ 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. brasiilense’.

No specific detection tool was available for ‘Ca. P. brasiilense’ mainly because except for the 16S rRNA gene, no ‘Ca. P. brasiilense’ gene had been sequenced. Therefore, the characterization of a ‘Ca. P. brasiilense’ non ribosomal genetic locus was undertaken using random-PCR that allowed the dnaF-dnaJ locus to be cloned and sequenced. A PCR test amplifying the non ribosomal dnaK gene was developed with the aim of specifically detecting phytoplasmas of subgroup 16SrXV-A (‘Ca. P. brasiilense’) (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. brasilienise’-infected basil (Choueiri et al., unpublished), three ‘Ca. P. brasilienise’-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 TaqI. Results of RFLP analysis (figure 1A) showed that the restriction of the amplicons using TaqI revealed two restriction profiles among the different ‘Ca. P. brasilienise’ strains. All hibiscus witches’ broom phytoplasms 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. brasilienise’. 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 FR771541). A guanine to thymine mutation at position 689 (figure 1B) in the bra-dnaK PCR product of hibiscus witches’ broom strains eliminated a TaqI restriction site, responsible for the difference in restriction profiles with the Suriname virescence, basil and PEACH19 strains.

**Figure 1.** A: RFLP analysis with TaqI. Digested DNAs were analyzed on 3% agarose gel in 1X TBE. B: TaqI restriction map of bra-dnaK PCR products amplified from ‘Ca. P. brasilienise’ 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. brasilienise’ 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 basil from Lebanon. Its presence not only in a woody host but also in an annual host (basil) indicates its possible local transmission by insects.

**Acknowledgements**

We greatly acknowledge the French Embassy in Baku and the Federation of European Biochemical Societies (FEBS) for their financial support. We thank Ivan Paulo Bedendo for kindly providing phytoplasma strains from hibiscus from Brazil and Elia Choueiri for providing phytoplasma infected basil from Lebanon.

**References**


**Corresponding author:** Gulnara BALAKISHIYEVA (e-mail: gbalkishiyeva@yahoo.com), Institute of Botany, Azerbaijan National Academy of Sciences, Patamdar shosse 40, AZ-1073, Baku, Azerbaijan.