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Identification of phytoplasma belonging to X-disease group in cherry in Chile

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Abstract

During summer 2006 sweet cherry (*Prunus avium* L.) trees, exhibiting disease symptoms suggestive of possible phytoplasma infection were observed in the Libertador General Bernardo O'Higgins region of Chile. Branches were collected from ten plants (five with and five without symptoms) and nucleic acid extracted from leaf midribs and phloem scrapings was used for phytoplasmas testing. Nested polymerase chain reaction (PCR) amplification allows the detection of phytoplasmas in samples from plants that showed symptoms, but not in those from asymptomatic ones. Restriction fragment length polymorphism, cloning, and sequencing allowed identification of phytoplasmas into ribosomal subgroup 16SrIII-J.

Key words: cherry, nested-PCR, phytoplasmas, RFLP, sequencing.

Introduction

Production of sweet cherry (*Prunus avium* L.) in Chile is concentrated in three regions: Libertador General Bernard O'Higgins (VI), Maule (VII) and Bío-Bío (VIII) and the fruit is for fresh consumption or for processing.

In one orchard located in the VI region a decay, low vigor and even death of plants in the variety Basler Langstieler (for processing), especially when grafted onto the rootstock Colt, were observed. The symptoms appear on plants of at least three years: the vigor reduction has been moderate to strong, and in most cases, removing the bark, phloem necrosis was observed in trunk or branches. No fungal or bacterial isolation was obtained.

Through the observation of symptoms phytoplasma presence was suspected and molecular analyses were carried out both in healthy and symptomatic plants to verify the presence of these prokaryotes.

Materials and methods

Samples were collected in summer 2006 from five symptomatic and five asymptomatic plants and consist of branches and leaves from which phloem and midribs respectively, were quickly separated, immediately frozen in liquid nitrogen and stored at -80°C.

Total nucleic acids were extracted from 1 g of mixture of main leaf midribs and phloem tissues (Prince *et al*., 1993), dissolved in Tris-EDTA pH 8.0 buffer, and maintained at 4°C; 20 ng/µl of nucleic acid were used for amplification.

After direct polymerase chain reaction (PCR) with primer pair P1/P7, nested PCR with R16F2n/R2 primers (Gundersen and Lee, 1996) was performed. PCR and nested PCR reactions were carried out following published protocol (Schaff *et al*., 1992). Identification of detected phytoplasma was done using restriction fragment length polymorphism (RFLP) analyses on ribosomal DNA amplified with primer pair R16F2n/R2, with *Tru*I and *Hha*I (Fermentas, Vilnius, Lithuania) restriction enzymes.

Selected R16F2n/R2 amplicons were purified using Concert Rapid PCR Purification System (Life Technologies, Gaithersburg, MD, USA). DNA fragments were ligated into T/A cloning vector pTZ57R/T following the instructions of the InsT/Aclone PCR Product Cloning Kit (Fermentas, Vilnius, Lithuania). Putative recombinant clones were analysed by colony-PCR, sometimes in combination with the insert-specific primers. Amplicons obtained from three colonies per cloned fragment were subjected to RFLP analyses, as described above. Selected fragments from cloned DNAs were sequenced in both directions using the BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK). The sequences were then aligned with BLAST engine for local alignment (version Blast N 2.2.12).

Results and discussion

Positive results were obtained only after nested amplification on P1/P7 amplicons. Cloned fragments of R16F2n/R2 amplicons were sequenced and subjected to RFLP analysis that allowed the assignment of the phytoplasma to the ribosomal subgroups 16SrIII-J (related to X-disease group) (figure 1). This phytoplasma was identified in all plants with symptoms, but not in the asymptomatic ones. In all cases there was no sequence difference among the three cloned R16F2n/R2 fragments from the same sample (1,244 bp each).

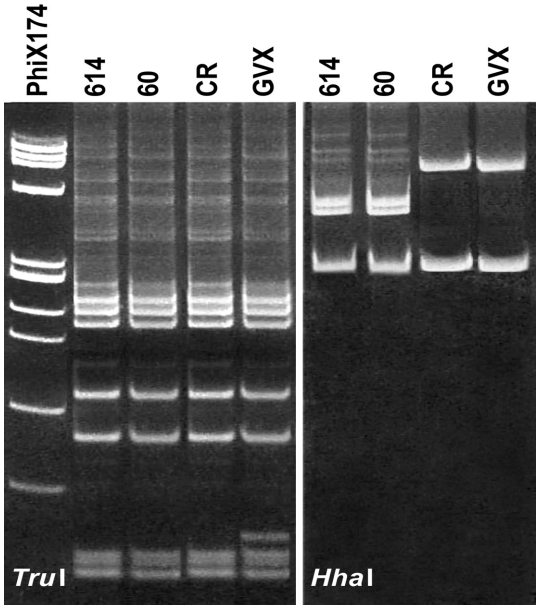

Figure 1. RFLP analysis of 16S rDNA amplified in nested-PCR with primer pair R16F2n/R2, from infected cherry sample 614, after digestion with *Tru*I and *Hha*I. Controls: 60, 16SrIII-J; CR, *Crepis biennis* yellows (16SrIII-B); GVX, Green Valley X-disease (16SrIII-A). PhiX174: marker ΦX174 *Hae*III digested.

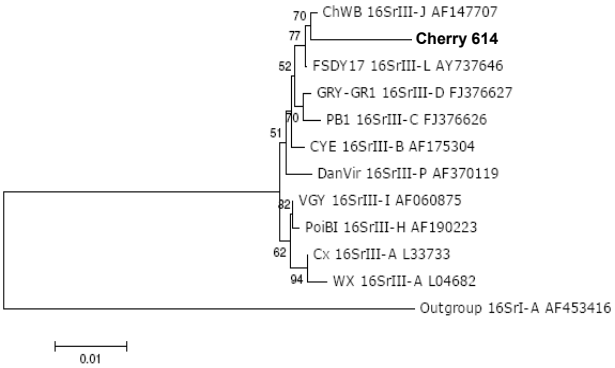

Figure 2. Phenetic tree constructed using neighbor-joining method with 16S rDNA region sequences from the strain # 614 from Chilean cherry and related phytoplasmas. Bar represents a phenetic distance of 1%.

The comparison of nucleotide sequences of 16S rDNA and the percentages of similarity showed the close correlation of Chilean isolates with those from X-disease group (figure 2). Phytoplasma 16S rRNA gene sequences were retrieved from the NCBI's nucleotide sequence database. The strain EF514210, corresponding to Delphinium phytoplasma from the UK, showed 99% similarity with the cherry strain #614 in the 16S rRNA gene. The same occurs with the strains AF147706 and AF147707, corresponding to chayote witches' broom phytoplasmas (16SrIII-J) from Brazil (Montano *et al*., 2000), and strain AF495657 chinaberry yellows phytoplasma from Bolivia (Harrison *et al.*, 2003).

Phytoplasmas belonging to ribosomal group 16SrIII are widespread in South America. Reports were made from Brazil, Colombia and Bolivia (Wheeler *et al.*, 2005). In Chile and Argentina phytoplasma of the 16SrIII ribosomal group had been detected in sugar beet (Castro *et al.*, 2000). This suggests that transmission to fruit trees in Chile may have occurred by polyphagous insect vector species. However it would be appropriate to verify whether cases of incompatibility scion/rootstock found often in Chile in cherry plants, whose production is for fresh consumption (Cruz, 2005), could be related to the presence of phytoplasma belonging to ribosomal subgroup 16SrIII-J.

The same samples used for phytoplasmas detection have been analyzed for cherry viruses (data not shown) and the same viruses (*Prunus necrotic ringspot virus* and *Prune dwarf virus*) were detected in symptomatic and asymptomatic plants. This suggests that the phytoplasmas belonging to the ribosomal subgroup 16SrIII-J are responsible for the described disease in the cherry variety Basler Langstieler in Chile.

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