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Molecular detection of phytoplasmas infecting apple trees in Poland

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Abstract

In 2010 more than 120 apple trees from different regions of Poland were tested for phytoplasma presence. Shoots from symptomatic and asymptomatic plants were collected and total DNA was extracted from phloem tissue. DNA was subjected to a nested-polymerase chain reaction (PCR) for amplification of the 23S ribosomal RNA (rRNA) sequences using universal primers and primers specific for '*Candidatus* Phytoplasma mali' and '*Ca.* P. asteris'. Restriction fragment length polymorphism (RFLP) nucleotide sequence analysis of phytoplasmas' 16S-23S rDNA fragment analyses of PCR products made possible to identify '*Ca*. P. mali' in seven apple trees and '*Ca.* P. asteris' in two other trees.

Key words: apple proliferation, aster yellows, PCR/RFLP, sequencing, phylogenetic analysis.

Introduction

'*Candidatus* Phytoplasma mali', the agent associated with apple proliferation is one of the most economically important phytoplasmas in Europe. The disease was reported in all countries of Central and Southern Europe associated with serious economic losses in apple production. Symptoms include witches' brooms of affected shoots and enlarged stipules. Phytoplasmas of the aster yellows (AY) group (16SrI) occurred incidentally in apple orchards. The trees infected by '*Ca*. P. asteris' expressed different symptoms including leaf yellowing, shoot proliferation, branch twisting and rubbery wood (Bertaccini *et al*., 1998; Fránová, 2005; Jomantiene and Davis, 2005).

The aim of this study was to determine the possible association of the phytoplasmas with symptoms of shoot proliferation and leaf deformation in apple trees in Poland.

Materials and methods

A hundred twenty seven symptomatic and asymptomatic apple trees of different cultivars grown in commercial orchards and home gardens were tested for phytoplasmas. Some of the trees showed shoot proliferation, witches' broom, leaf deformation, and enlarged stipules.

Total DNA was extracted from shoot phloem tissue using DNeasy Plant Mini Kit (Qiagen). DNA was subjected to PCR with P1/P7 followed by nested PCR with phytoplasma-universal primers R16F2n/R16R2 (Gundersen and Lee, 1996), as well as primers R16(I)F1/R1 and fAT/rAS specific to 16SrI, 16SrII, 16SrXII and 16SrXV groups, and apple proliferation phytoplasma (16SrX) groups, respectively. The fragments of the 16S rRNA gene amplified with R16F2n/R16R2 primers were digested by *Mse*I, *Hha*I, *Ssp*I, and *Rsa*I enzymes (Fermentas, Vilnius, Lithuania). The generated restriction patterns were analyzed by electrophoresis in 8% polyacrylamide gels and compared with the reference strains (Lee *et al*., 1998).

The partial nucleotide sequences of the 16S rDNA amplified with primers R16F2n/R16R2 were compared with sequences available in GenBank using the BLAST algorithm (http://ncbi.nlm.nih.gov/BLAST/). Multiple alignment was made using CLUSTALW of the DNASTAR's Lasergene software. Phylogenetic and molecular evolutionary analyses were carried out by the neighbor-joining method implemented in CLUSTALW of the genetic analysis software MEGA, 4.0 (Tamura *et al*., 2007).

Results

PCR products (~1.2 kb) amplified with R16F2n/R16R2 primers were obtained from nine apple tree samples ('Shampion', 'Elstar', 'Golden Delicious', 'Yellow Transparent', 'Cox's Orange Pippin', 'Pinova', 'Evelina', and two trees of unknown cultivars). Products of the nested PCR with fAT/rAS specific for '*Ca.* P. mali' (apple proliferation, 16SrX group) were obtained for 16S rDNA fragments of phytoplasma strains from seven apple trees. Phytoplasma rDNA fragments from 'Pinova' and 'Evelina' trees were amplified by nested PCR with primer pair R16(I)F1/R1, but not with apple proliferation-specific primers fAT/rAS.

The resulting RFLP patterns of rDNA digested singly with *Mse*I, *Hha*I, *Ssp*I, and *Rsa*I enzymes revealed differences among phytoplasma strains and indicated that the apple trees were infected by phytoplasmas belonging to apple proliferation group, subgroup A ('*Ca*. P. mali'-related strains) and aster yellows group 16SrI, subgroup B ('*Ca.* P. asteris'-related strains).

Sequence analysis of the 16S rRNA gene fragment confirmed the presence of two different phytoplasmas in infected apple trees. Multiple alignments revealed that the phytoplasma detected in seven apple trees ('Shampion', 'Elstar', 'Golden Delicious', Yellow Transparent', 'Cox's Orange Pippin', and two trees of unknown

cultivars) shared a 100% nucleotide sequence identity with that of the 16S rDNA of '*Ca*. P. mali' strain AT from Germany (GenBank ID: X68375). The phylogenetic analysis placed detected phytoplasma close to the other members of the apple proliferation group, subgroup A. In turn, '*Ca*. P. asteris' strains from 'Pinova' and 'Evelina' were closely related with OAY isolate (GenBank ID: M30790), a reference strain of 16SrI-B group, '*Ca*. P. asteris'.

Discussion

Results of PCR/RFLP and sequences analyses showed the presence of '*Ca*. P. mali' and '*Ca*. P. asteris' in apple trees in Poland. Witches' broom and leaf deformation were observed on shoots of the trees positively tested for '*Ca*. P. asteris', while some trees infected by '*Ca*. P. mali' showed shoot proliferation and incidentally enlarge stipules ('Golden Delicious'). Although the symptoms induced by '*Ca*. P. mali' and '*Ca*. P. asteris' differed, their association with these phytoplasmas was ambiguous.

Apple proliferation phytoplasma was reported in most of the European countries and its negative impact on apple production was evidenced. There are only few reports concerning an occurrence of phytoplasmas classified to aster yellows group in apple trees. Phytoplasma classified to 16SrI-B subgroup ('*Ca.* P. asteris') was detected in apple trees with leaf yellowing, shoot proliferation, and symptom 'sessile leaf' in Lithuania (Jomantiene and Davis, 2005). PCR/RFLP results confirmed the presence of phytoplasmas from the aster yellows (AY) group, subgroup B, (16SrI-B); in apple tree with rubbery wood symptom grown in the Czech Republic (Bertaccini *et al*., 1998). The trees found in Czech Republic and United Kingdom showed diverse symptoms, and yielded positive results for phytoplasmas of subgroups 16SrI-B and 16SrI-C of AY group (Bertaccini *et al*., 2001).

Occurrence of the phytoplasmas belonging to the same subgroups were also reported in apple trees with branch twisting symptoms in Czech Republic (Fránová, 2005). The relevance of phytoplasmas from aster yellows group in fruit production is unclear. This is the first report of the natural occurrence of '*Ca.* P. asteris' in *Malus* sp. in Poland.

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