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Detection and characterization of phytoplasmas infecting apple trees in Czech Republic during 2010

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Abstract

Apple trees showing proliferation disease symptoms were found at different locations in Czech Republic during 2010. Direct and nested PCR with primers specific for phytoplasmas, and the subsequent RFLP analyses were used for phytoplasma detection and in order to differentiate ribosomal subgroups and apple proliferation phytoplasma subtypes. Out of 74 apple trees examined, 63 plants revealed positive reaction for phytoplasma presence. The majority of apple trees was infected by phytoplasmas belonging to apple proliferation ribosomal group (16SrX). In the 16S plus spacer region two apple proliferation phytoplasma profiles P-I and P-II were determined as well as the presence of two phytoplasma genetic lineages designated as pattern '1' and '2'. Apple proliferation phytoplasmas belonging to ribosomal protein rpX-A subgroup were detected in the majority of apple trees, while phytoplasmas belonging to subgroup rpX-B were detected sporadically. Apple proliferation phytoplasma subtypes AP, AT-1 and AT-2 singly or in different combination were also found in samples collected from apple trees.

Key words: phytoplasma, apple proliferation, PCR/RFLP, subgroups differentiation.

Introduction

Phytoplasmas are prokaryote organisms of the *Mollicutes* class. In apple trees, phytoplasmas belonging to different ribosomal groups and subgroups have been described till this time. However, apple proliferation phytoplasma (ribosomal subgroup 16SrX-A, '*Candidatus* Phytoplasma mali') seems to be the most frequent phytoplasmas associated with apple proliferation disease. As revealed by RFLP analysis, '*Ca.* P. mali' is homogenous at the level of 16S rRNA gene: however, differences were observed when more specific tools were used for characterization (Casati *et al.*, 2010; Martini *et al*., 2008; Paltrinieri *et al.*, 2010). The results presented here confirm that phytoplasmas classified in different ribosomal subgroups affect apple trees in Czech Republic, and, especially, showed a high degree of genetic diversity among '*Ca.* P. mali' population in samples collected from apple trees all over the country during 2010.

Materials and methods

Apple trees showing shoot proliferation, enlarged stipules, reduced growth habit, abnormal growth of shoots in the autumn and fruit malformation were found at different locations in Czech Republic during 2010.

DNA was extracted from phloem tissues of 82 samples from 74 apple trees. The polymerase chain reaction (PCR) was carried out to amplify region that includes the 16S-23S rRNA gene of the phytoplasma genome. The primer pairs P1/P7 and P1A/P7A were used in direct PCR. PCR products were diluted with sterile distilled water (1:29) prior to amplification by nested PCR using P1A/P7A, F1/B6, R16F2n/R2 and F1/B6, F2n/R2 primer pairs, respectively. Double nested PCR was carried out by two ways with subsequent primer pairs combinations: P1/P7 – P1A/P7A – F2n/R2 and P1/P7 – F1/B6 – F2n/R2. About 20 ng of each DNA preparation in water were added to the PCR mix (Schaff *et al*., 1992) in a final reaction mixture volume of 25µl. Approximately 200 ng of DNA of each positive PCR product were separately digested from R16F2n/R2, F1/B6 and P1A/P7A amplicons. Digestions were carried out with 2.5 U of *Mse*I and *Hpa*II restriction enzymes after R16F2n/R2 and F1/B6, P1A/P7A amplification, respectively. Restriction patterns obtained were compared with those described in the literature (Paltrinieri *et al.*, 2010; Casati *et al*., 2010) after electrophoresis in 8% polyacrylamide gel in 1x TBE buffer followed by staining with GelRed and visualization under an UV transiluminator.

Non-ribosomal region (nitroreductase-like gene) was amplified using primer pair AP13/AP10, followed by primer pair AP14/AP15. PCR reactions were carried out as previously described by Casati *et al.* (2010). Products obtained by means of primer pair AP14/AP15 were separately digested with *Hinc*II and *Pag*I endonucleases, according to the manufacturerś instruction. The restriction patterns were then observed and compared with literature as described above.

For PCR amplification based on *rpl22* and *rps3* genes of S10 ribosomal protein (rp) operon primer pair rpAP15f/rpAP15r was used in direct PCR according to Martini *et al.* (2008). PCR products were digested using *Alu*I restriction enzyme.

Results

Although all 74 apple trees examined showed apple proliferation disease symptoms, 63 plants (e.g. 85%) were positive for phytoplasma infection using at least one of the PCR/RFLP methods. The best results were obtained using nested PCR with primer combination P1A/P7A – R16F2n/R2 (32 samples were positive out of 36 samples

tested, e.g. 88.9%), nested PCR using primers AP13/AP10 – AP14/AP15 (59 samples were positive out of 82 tested, e.g. 72.0%) and direct PCR by primers rpAP15f/rpAP15r (57 samples positive out of 82 examined, e.g. 69.5%). RFLP with *Mse*I endonuclease of the DNA sequences amplified by PCR with primers R16F2n/R2 showed that apple phytoplasma strains from 51 trees had a pattern identical to each other and to the apple proliferation phytoplasma (ribosomal group 16SrX, AP). Mixed infection of AP and phytoplasmas belonging to 16SrI-C subgroup (aster yellows group) was detected in five plants. Phytoplasmas belonging to 16SrI-C subgroup (which type strain is clover phyllody) alone were detected in two apple trees.

Primers F1/B6 amplified DNA out of 50 trees (67.6%) in nested PCR assay. RFLP analyses (*Hpa*II) on these amplicons distinguished AP phytoplasma profile P-I in 39 plants, P-II in four plants and both P-I and P-II profiles were observed in six trees. Moreover, unusual pattern, previously unpublished, was observed in one sample originated from AP infected tree.

Primers P1A/P7A amplified DNA out of 42 plants (56.8%) in direct and/or nested PCR assay. RFLP analyses of 1,759 bp fragment, revealed the presence of two phytoplasma genetic lineages designated as pattern '1' in five apple trees, pattern '2' in 32 trees and both together in five plants.

In parallel, the PCR/RFLP method based on ribosomal protein gene sequences as well as on a non-ribosomal DNA fragment recently established were used in order to differentiate ribosomal subgroups (rpX-A, -B, -C, -D) and AP phytoplasma subtypes AP, AT-1 and AT-2, respectively. In the majority of apple trees examined (53 trees out of 74 tested), revealed the presence of AP phytoplasmas belonging to subgroups rpX-A, while phytoplasmas of rpX-B subgroup were detected in three plants. All three phytoplasma subtypes (AP: 18 plants, AT-1: 7 plants, AT-2: 19 plants) singly (44 plants) or in different combination (10 plants) were found in examined samples.

Discussion

The first biological proof of apple proliferation disease was carried out in Czech Republic by Seidl (1965). Subsequently phytoplasma bodies were observed on ultra-thin sections of roots and abnormal underground sprouts of apple trees artificially infected with the proliferation disease (Brčák *et al.*, 1972). Using molecular tools, phytoplasmas belonging to different ribosomal groups and subgroups were described in limited number of apple and pear trees (Bertaccini *et al*., 2001; Fránová, 2005) in our country. Firstly, we report here the genetic diversity of '*Ca.* P. mali' population in Czech Republic. The majority of apple trees examined was infected by '*Ca*. P. mali' belonging to ribosomal subgroup rpX-A, subtypes AP and AT-2. Diversity in 16S-23S rDNA was also observed. Most frequently, pattern designated as '2' by Casati *et al*. (2010) and profile P-I according to Paltrinieri *et al.* (2010) were determined in specimens collected from apple trees during 2010. Our data demonstrated high degree of genetic diversity of AP in Czech Republic. Sequencing of different strains on the various genes is in progress.

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