

Experiences with phytoplasma detection and identification by different methods

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Abstract

Several symptoms of yellowing/reddening, virescence, phyllody, sterility of flowers, flat stem, proliferation of axillary buds and generalized stunting have been observed in cultivated as well as wild plants growing in the Czech Republic. To detect and identify phytoplasmas, plants were subjected to biological assays, fluorescence microscopy, electron microscopy, PCR/RFLP DNA analysis and sequencing. Mostly, the results of all methods were consistent and phytoplasmas were identified as members of 16SrI-B, 16SrI-C, 16SrIII-B, 16SrX-A, 16SrX-B, 16SrX-C and 16SrXII-A ribosomal subgroups in single or mixed infection. In some cases, infection of phytoplasmas together with viruses was detected.

Key words: phytoplasma identification, symptoms, DAPI staining, electron microscopy, biological and molecular detection.

Introduction

Phytoplasmas (recently assigned to a novel genus '*Candidatus Phytoplasma*') are prokaryotic wall-less pathogens in the class *Mollicutes* that inhabit plant phloem and insects. They are associated with numerous diseases of economically important food, fibre, forage, fruit and ornamental plants as well as wild hosts. The inability of phytoplasma cultivation in pure cultures *in vitro*, their low concentration and uneven distribution in the host plant can make their detection difficult. Polymerase chain reaction with primers from sequencing of randomly cloned phytoplasma DNA, from 16S rRNA, from ribosomal protein gene sequences, from SecY and Tuf genes, and from membrane associated protein genes opened new paths for research on phytoplasma identification and classification. Restriction fragments length polymorphism (RFLP) analysis together with the sequencing of 16Sr phytoplasma genes was the first step on this way enabling the construction of phylogenetic trees of many micro-organisms especially in the *Mollicutes* taxon (Bertaccini, 2007).

A research program on occurrence and identification of phytoplasmas at the Department of Plant Virology (BC ASCR v.v.i. IPMB) began in 1992. Experiences with phytoplasma detection and identification using biological, microscopic and molecular methods are reported in this paper.

Materials and methods

Material and biological indexing

Samples were collected from about 2,000 plants of economically important crops (forage, fruit, vegetable,

oil-plants, medicinal and ornamental plants) as well as wild hosts. Preferentially, samples were collected from plants showing symptoms characteristic of phytoplasma infection; samples from nonsymptomatic plants were used as healthy controls.

Dodder plants (*Cuscuta* sp.) grown from seeds were connected to selected plant of strawberries, red clover, apple tree and lily, and stems of dodder were allowed to attach to periwinkle plants (*Catharanthus roseus*) raised from seeds for transmission of the infection agents. The dodder bridges were left about four months on experimental plants.

Double budding of pear trees was performed in RBIP Holovousy. Every tree of *Pyrus communis* Rogue Red, Beurré Bosc and Agata were examined five times on rootstock (*P. communis*) with indicator (*Pyronia veitchii*).

Fluorescence microscopy, scanning and transmission electron microscopy

Samples of branch and root tissues for fluorescent DAPI staining; branch, root, flowers and stem segments obtained from plants with and without symptoms for scanning (SEM) and transmission electron microscopy (TEM) were prepared according to the procedures described in Fránová *et al.* (2000).

Molecular methods

Total nucleic acids were extracted from fresh or frozen tissues by chloroform/phenol extraction method and used as template in PCR. Various universal and group specific ribosomal primer pairs (P1/P7, R16F2n/R2, R16F1/R0, F1/B6, 16R_{738F}/16R_{1232r}, R16fU5/rU3, R16(I)R1/F1) as well as primers for amplification of ribosomal protein S3, rp(I)F1(A)/rp(I)R1(A), for *Tuf*

gene (Tuf1f/r, fTufu/rTufu, fTufAy/rTufAy) and for putative helicase gene (Duduk and Bertaccini, 2006) (G35p/m) were used in direct, nested or second nested PCRs for phytoplasma detection. DNA of PCR amplicons were separately digested with restriction enzymes (*AluI*, *BfmI*, *HaeIII*, *HhaI*, *HpaI*, *HpaII*, *KpnI*, *MseI*, *RsaI*, *Sau3AI*, *TruI*, *TasI*). Amplified DNA fragments of representative samples were sequenced from both directions using a BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK). Sequencing was performed in an ABI PRISM 310 sequencer (PE Applied Biosystems, Foster City, CA, USA). The sequences were compared with sequences available in the GenBank using the www service BLAST 2.2.16 (<http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi>). The nucleotide sequences of the 16S rRNA were aligned for phylogenetic analysis.

Results

Cultivated and wild plants originating from South Bohemia, apple and pear cultivar collection and production plantations in the RBIP Holovously (East Bohemia) and red clover cultivated in the breeding station (North Moravia) has been observed for symptoms of phytoplasma infection during different seasons in years 1992 – 2007. The main symptoms observed were yellowing/reddening, virescence, phyllody with subsequent declining of herbaceous hosts, dwarf on cultivated clovers, proliferation on apple trees and premature leaf yellowing and reddening of pear trees.

Phytoplasmas were detected in different plant species including *Malus domestica*, *Pyrus communis*, *Prunus armeniaca*, *Daphne mezereum*, *Fragaria ananassa*, *F. vesca semperflorens*, *Brasica napus* var. *arvensis*, *Brasica oleracea* subsp. *botrytis*, *Lilium* sp., *Allium ampeloprasum* var. *porrum*, *Helichrysum bracteatum*, *Echinacea purpurea*, *Tagetes erecta*, *T. patula*, *Alliaria petiolata*, *Carum carvi*, *Armoniaca rustica*, *Matricaria chamomilla*, *Plantago lanceolata*, *Taraxacum officinale*, *Trifolium pratense*, *T. repens*, *T. hybridum*, *Callistephus chinensis*, *Gladiolus* sp. and *Verbascum densiflorum*. Phytoplasmas were identified by PCR/RFLP or sequencing as a member of 16SrI-B, 16SrI-C, 16SrIII-B, 16SrX-A, 16SrX-B, 16SrX-C and 16SrXII-A ribosomal subgroups in single or mixed infection, sometimes in co-infection with viruses.

Strawberry plants infected with phytoplasmas (16SrI-C ribosomal subgroup) were used for experimental transmission to *C. roseus*. Moreover, incidental transmission to *C. roseus* by unknown vector(s) of phytoplasmas belonging to 16SrI-B ribosomal subgroup was also successful. Double budding of pear trees in RBIP Holovously confirmed the phytoplasma presence in examined trees.

The DAPI staining, SEM and TEM usually revealed presence of fluorescent areas or of phytoplasma-like bodies. However, in some cases, none or sporadic occurrence of phytoplasma-like structures was observed by TEM. The use TEM distinguished also the presence of viruses, rickettsia and bacteria in some samples.

The PCR with primer pair combination P1/P7→F1/B6→R16F2n/R2 seems to be the most sensitive and reliable system for phytoplasma detection in our experiments. Using other primer pairs combination, especially system including primer pair 16R_{738F}/16R_{1232R}, forms amplification products also with sterile water and/or negative controls in nested or second nested PCR in our hands. The RFLP or sequencing of amplified fragments from these amplicons resulted in profiles characteristic of bacteria.

Discussion

DAPI staining and SEM are relatively cheap and quick methods, but they are not useful to distinguish the bodies of other micro-organisms or cell components (mitochondria, chloroplast) from phytoplasmas. For example, in the case of carrot and daphne, blue-white fluorescence areas were observed under fluorescence microscope in sieve tube cells of symptomatic plants, but TEM revealed rickettsia-like organisms and rhabdovirus particles presence in the sieve tube elements, respectively.

PCR provides the most sensitive, specific and quick detection system for phytoplasmas. Unfortunately, PCR also meets some difficulties: unspecific bands, false positives or negatives. Therefore, confirmation of PCR results by using different primer pairs combinations (generic and group-specific) with subsequent RFLP and/or sequencing of PCR amplicons seems to be the way for correct phytoplasma identification in the examined samples.

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