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Electron microscopy and molecular identification of phytoplasmas associated with strawberry green petals in the Czech Republic

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Abstract

The presence of phytoplasma in *Fragaria ananassa* × Duch cv Senga Sengana showing strawberry green petals symptoms was observed by electron microscopy of phloem tissue. No phytoplasmas were found in asymptomatic strawberry plants used as controls. Nucleic acids extracted from these plants were used in nested-PCR assays with primers amplifying 16S rRNA sequences specific for phytoplasmas. Bands of 1.2 kb were obtained and the subsequent nested-PCR with specific primers and RFLP analyses allowed to classify the detected phytoplasmas in the aster yellows group (16SrI). They belonged to the subgroup I-C of which type strain is clover phyllody phytoplasma.

Introduction

Strawberry green petals (SGP) is a disease of strawberry transmitted by leafhoppers; the pathogen is known to be related to the phytoplasma associated with clover phyllody (Frazier and Posnette, 1957). SGP is characterized by small and red-leaves, the infected plants bear abnormal fruits and frequently show diagnostic symptoms of virescence on flowers (Converse et al., 1988).

In the Czech Republic a frequent occurrence of green petals symptoms was reported in *Fragaria ananassa* × Duch, cvs Madame Moutot and Surprise de Halle in 1953–1958 (Blattný and Blattný, 1959). This symptomatological study concluded that the agent was identical or closely related to green petals disease described by Posnette (1953) in England. Symptoms of SGP on plants of *F. vesca semperflorens* cv Rujana were then described by Miculka (1974), but no further investigations were made on SGP in the Czech Republic till now.

Plants of *F. ananassa* × Duch. cv Senga Sengana with SGP symptoms were found in the Czech Republic during surveys carried out in 1991–1995. Electron microscopy was used to verify the presence of phytoplasmas in the sieve tubes of symptomatic plants and molecular methods for analysis of 16S rRNA gene sequence were applied to identify the pathogen(s).

Material and methods

Samples

Three years old strawberry plants of *F. ananassa* × Duch cv Senga Sengana were taken from a garden in Třísov (south Bohemia) in July 1994. The plants, showing small flowers with green petals, were stunted and had dark green leaves. Flowers were slightly twisted and the petal edges were curled. New leaves were asymmetrical, pale green with chlorotic margins and shorter petioles. The few runners formed did not produce new plants. Samples from seed propagated *Fragaria vesca semperflorens* cv Alpine were used as healthy controls.

Electron microscopy

The material examined included samples of green petals and pedicels from symptomatic plants and from healthy control. Pieces of tissues of about 2 × 2 mm were fixed in 0.1 M potassium phosphate buffer pH 7.3 with 5% glutaraldehyde and 4% sucrose for 20 mins at room temperature under mild vacuum. The samples were postfixed in 1% osmium tetraoxide, dehydrated by washing in solutions with increasing ethanol content and embedded in Durcupan (Fluka, Buchs, Switzerland) resin (Honetšlegrová et al., 1994). Thin sections were double-stained with uranyl acetate in 70% ethanol and lead citrate and examined in Jeol 100 MB and Philips 420 electron microscopes.

Nucleic acid extraction and phytoplasma identification

Nucleic acid samples from seven symptomatic strawberries and two healthy controls, prepared from freshly cut leaf midribs and pedicels, were extracted according to Lee et al. (1991) and used in polymerase chain reaction (PCR) experiments and restriction fragment length polymorphism (RFLP) analyses to identify phytoplasmas. The reference phytoplasma strains used, maintained in periwinkle, were: Tomato big bud (BB) (group 16SrI-A), Maryland aster yellows (AY) (group 16SrI-B), Clover phyllody (CPh) (group 16SrI-C) (Lee et al., 1993) and Italian periwinkle virescence (IPVR) (group 16SrI-G) (Vibio et al., 1996). Nucleic acid samples diluted in TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)] to give a final concentration of 20 ng/μl were employed in direct PCR reactions as previously described (Schaff et al., 1992). Reaction mixtures contain 0.5 μl of nucleic acid, 200 μM each dNTP and 0.4 μM primer in a total of 25μl volume. Thirty-five PCR cycles were conducted in an automated thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). Tubes with the reaction mixture devoid of DNA templates or containing DNA from healthy periwinkle and asymptomatic strawberry were included in each experiment as negative controls. The universal primer pairs R16F1/R0 was used in direct PCR while the R16F2/R2 and R16(I)F1/R1 (Lee et al., 1994; 1995) were employed in nested assays. In these nested-PCR assays the products obtained with the universal primer pairs R16F1/R0 were diluted (1:40) with sterile deionized water and used as template for a second PCR run using the primer pair R16F2/R2. Primer pair specific for phytoplasma group 16SrI [R16(I)F1/R1] was further employed in nested-PCR using R16F2/R2-amplified products as templates.

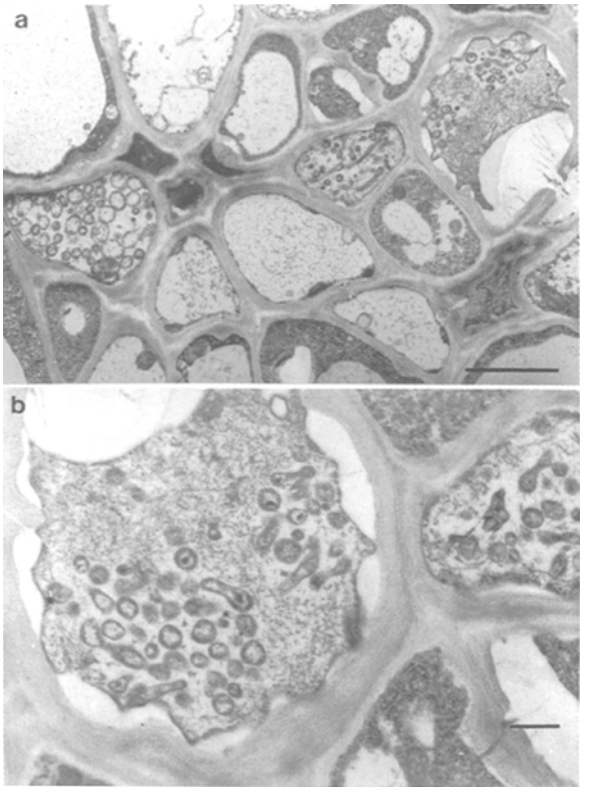

Figure 1. a. Ultrathin section of sieve cells of *Fragaria ananassa* × Duch cv Senga Sengana with strawberry green petals symptoms (bar = 2 μm); b. Phloem cell filled with phytoplasmas of different sizes and shapes (bar = 500nm).

The 16S rDNA sequences (5 μl of PCR products) amplified with primers R16F2/R2 and R16(I)F1/R1 were analyzed with the restriction endonucleases *Alu*I, *Hha*I, *Mse*I, and *Kpn*I and the collective RFLP patterns obtained were compared with those of control phytoplasma strains after electrophoresis through a 5% polyacrylamide gel.

Results

Ultrathin sections of strawberry phloem tissue from green petals and pedicels showed the presence of pleomorphic micro-organisms resembling phytoplasmas. Their size varied from 70 to 550 nm in diameter (average 400 nm) (Figure 1). There were spherical and ovoid bodies with visible unit membrane but without cell wall (Figure 2). They contained granules mainly peripheral (ribosomes) and centrally located net-like structures (DNA). Beaded filamentous forms with var-

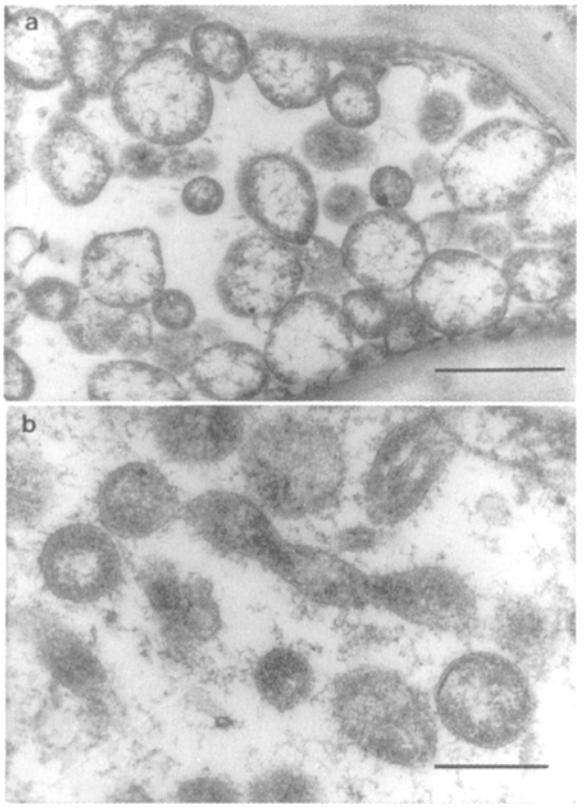

Figure 2. a. Spherical and filamentous phytoplasmas with various degree of constriction (bar = 500 nm); b. close up of some phytoplasmas (bars 200 nm).

ious degrees of constrictions contained areas more or less densely stained. Some phloem cells were almost completely filled with phytoplasmas and contained little or no cytoplasmic residues.

No phytoplasmas were observed in healthy plants of *F. vesca sempreflorens* cv Alpine.

When the primer pair R16F1/R0 was used in direct PCR assays, no visible amplification products were obtained with any of the strawberry templates (data not shown), but a DNA fragment of 1.2 kb was produced from symptomatic strawberries when the invisible PCR products were reamplified with the primer pair R16F2/R2 in nested-PCR assays (Figure 3). A specific DNA band of 1.1 kb was then obtained when the primers R16(I)F1/R1 were employed in nested-PCR after amplification with R16F2/R2 from symptomatic strawberries (Figure 3). DNA fragments of the expected sizes were amplified from positive controls, AY, BB, CPh and IPVR, when generic (R16F2/R2) and phytoplasma group I-specific primer pairs [R16(I)F1/R1] were used. No amplification products were observed

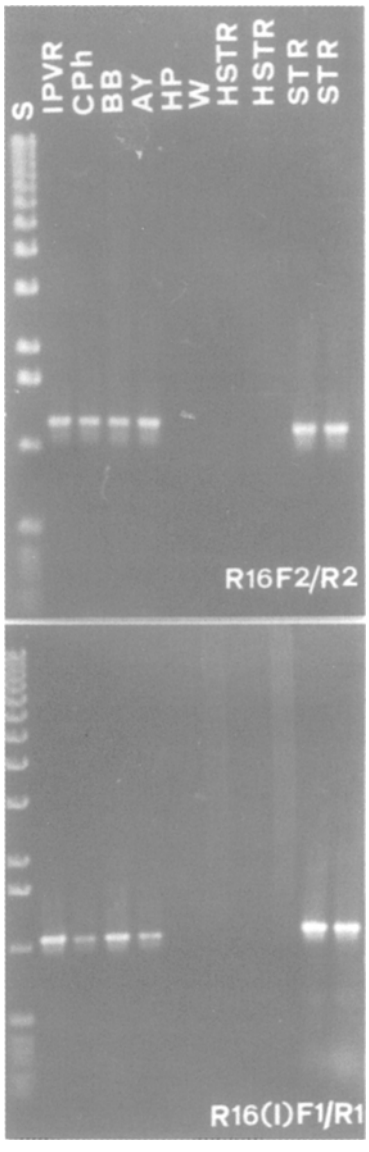

Figure 3. Results in agarose gel of nested-polymerase chain reaction (PCR) amplification of phytoplasma 16SrDNA sequences from strawberry and phytoplasma control strains using the primer pair R16F2/R2 after the direct PCR with R16F1/R0 and using phytoplasma group I-specific primer pair R16(I)F1/R1 after nested-PCR with R16F2/R2. S, 1 kb lambda DNA fragment size standard from top to bottom 12.2, 11.1, 10.1, 9.1, 8.1,. 7.1, 6.1, 5.0, 4.0, 3.0, 2.0, 1.6, 1.0, 0.5, 0.3, 0.2, e 0.1 kb. W, water control; HP, healthy periwinkle; HSTR, asymptomatic strawberry; STR, strawberry with SGP symptoms; reference strains IPVR, CPh, BB and AY are in periwinkle as described in the text.

in the negative control devoid of template DNA or in samples containing DNA from asymptomatic strawberry and healthy periwinkle with any of the primers used.

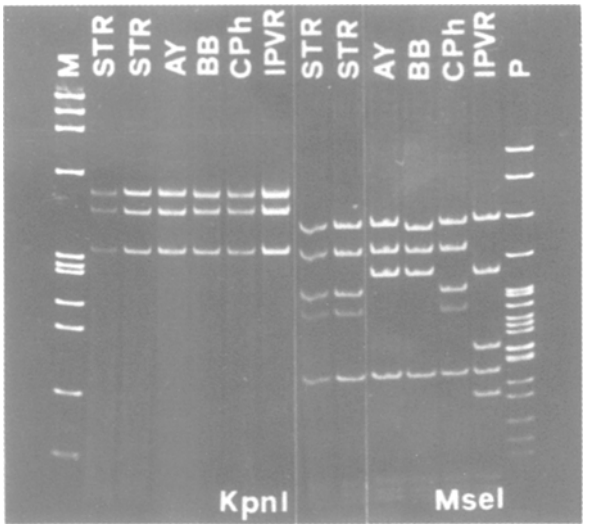

Figure 4. Results in polyacrylamide gel of restriction fragment length polymorphisms (RFLP) analysis of phytoplasma 16S rDNA sequences amplified with primers R16F2/R2 from strawberry with SGP and control phytoplasmas strains. Samples abbreviations are as in Fig. 3. M, øX174 RF I DNA *Hae*III digest; P, pBR322 DNA *Msp*I digest, enzymes used are indicated at the bottom of the figure.

Restriction fragment length polymorphism (RFLP) analysis with different enzymes of the DNA sequences amplified by nested-PCR with primers R16F1/R0 followed by R16F2/R2 showed that strawberry phytoplasma isolates from Czech Republic had a pattern identical to each others and to the control CPh phytoplasma (Figure 4). The phytoplasma identification was confirmed by the RFLP patterns of DNA bands amplified with primers R16(I)F1/R1 (Figure 5).

Discussion

The present study is in agreement with previous reports based on symptomatological and electron microscopic observations confirming that strawberry green petals disease is associated with phytoplasma presence (Beakbane et al., 1971, Posnette and Chiykowski, 1987). The simultaneous presence of small and large particles in the same phloem cell suggests different section planes in phytoplasma ovoid cells or the presence of filamentous branches in accordance with phytoplasma morphological descriptions in Ploaie (1981).

Molecular tests confirm phytoplasma presence after the first nested-PCR run with universal primers (Lee et al., 1993). The phytoplasma identification was obtained with the second nested-PCR carried out with primers R16(I)F1/R1, that specifically amplify DNA from phytoplasmas belonging to the aster yellows group (16SrI) (Lee et al., 1993; 1994). The positive results obtained with all the symptomatic strawberry samples indicate that phytoplasmas associated with Czech SGP disease belong to this group. RFLP analysis of the DNA fragments amplified with both primers confirms this result and shows that these phytoplasmas belong to subgroup 16SrI-C.

These results are in agreement with biological data, based on dodder transmission experiments, reporting that strawberry disease, known as virescence, is induced by clover phyllody phytoplasma (Frazier and Posnette, 1957). The molecular data confirm also pioneering work demonstating that antibodies raised against an European strain of CPh reacted strongly with SGP affected plants in ELISA and showing that the two phytoplasmas are closely related (Clark et al., 1983). Recently molecular analyses showed that also a Canadian isolate of SGP could be classified in the subgroup 16SrI-C of the aster yellows group (Gundersen et al., 1996). In this latter report it is also shown that not only the 16S RFLP pattern of SGP and American CPh (the same strain used in the present work) are identical but also RFLP pattern of a segment of the ribosomal protein gene operon, that is able to distinguish further phytoplasmas subgroups, are indistinguishable. It is however necessary to outline that identical RFLP profiles of some genetic regions (16Sr DNA or chromosomal DNA) could be referred to biologically diverse phytoplasma isolates (Vibio et al., 1996).

Although strawberry plantations in Southern (Planá near České Budějovice, Lhenice, Třísov, Křemže), Central (Jesenice near Prague) and Eastern Bohemia (Breeding station Turnov) were surveyed from 1991 to 1995, it is not possible to confirm the frequent occurrence of SGP in the Czech Republic reported more than thirty years ago by Blattný sen and Blattný jun (1959). The finding of strawberry with SGP symptoms is very rare indicating a low incidence of this disease in the Czech Republic during 1991–1995; this may be the positive consequence of long term use of meristem-derived strawberry seedlings by Czech Republic growers.

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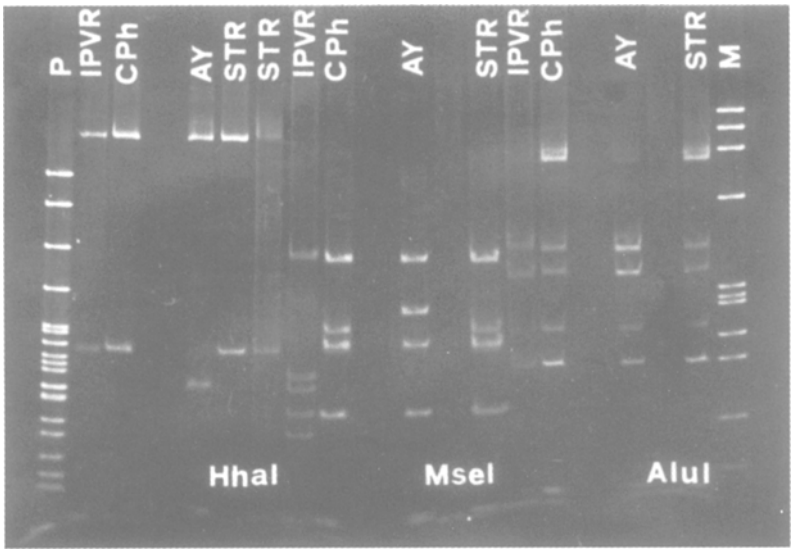

Figure 5. Results in polyacrylamide gel of restriction fragment length polymorphisms (RFLP) analysis of phytoplasma 16S rDNA sequences amplified with group I (aster yellows) specific primers R16(I)F1/R1 from strawberry with SGP and controls phytoplasmas strains. Samples abbreviations are as in Figure 3, markers are as in Figure 4, enzymes used are indicated at the bottom of the figure.

Beltsville, MD, USA for providing phytoplasma control strains AY, BB and CPh used in this work.

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