EXPERIMENTAL TRANSMISSION OF PHYTOPLASMAS FROM DISEASED MAGNOLIAS TO CATHARANTHUS ROSEUS TEST PLANTS BY GRAFTING

H. Śliwa and M. Kamińska

Abstract

Phytoplasmas classified as aster yellows and apple proliferation phytoplasmas were transmitted from naturally infected magnolias with stunting disease symptoms to periwinkle (Catharanthus roseus) by grafting. The identity of the graft-transmitted phytoplasmas was confirmed by RFLP analysis of polymerase chain reaction (PCR) amplified 16S rDNA fragments.

Key words: magnolia, stunting disease, phytoplasma, PCR, RFLP, graft transmission

Introduction

Magnolias are relatively free of pest and disease problems, however a new severe disease was recorded recently in Magnolia spp. plants (Kamińska et al. 2001b). The symptoms included stunted growth, leaf necrosis and malformation, apical shoot necrosis and abnormal production of secondary shoots. The disease occurs in some gardens and nurseries in Poland and in the imported plants. Despite negative results of electron microscopy examination, the data obtained by PCR amplification of 16S rDNA and RFLP analysis revealed for the first time that incidence of the symptoms in magnolia plants was associated with infection of the aster yellows phytoplasma (16SrI) and a phytoplasma related to apple proliferation phytoplasma group (16SrX) (Kamińska and Śliwa 2003). The authors’ study showed also that antibiotic treatments temporary promoted shoot growth and the development of asymptomatic leaves and flower buds of naturally infected magnolias, thereby supporting the idea that magnolia stunt is caused by phytoplasmas.
Phytoplasmas are known to cause diseases in several hundred plant species (Plant diseases... 1989, Seemüller et al. 1998 a). Many of them, including aster yellows phytoplasma group (AY; 16SrI) and apple proliferation phytoplasma group (AP; 16SrX) are of considerable economic importance. The phytoplasmas called aster yellows are associated with a range of diseases in Europe and America and they are transmitted by leafhoppers (Lee et al. 1993, Víbio et al. 1996). In Poland, they were identified in several ornamental crops (Kamińska and Korbin 2002, Kamińska et al. 1997, 2001 a, b).

According to the current molecular classification scheme (Lee et al. 1998, Seemüller et al. 1998 a), the phytoplasmas responsible for apple proliferation (AP; 16SrX-A), European stone fruit yellows (ESFY; 16SrX-B) and pear decline diseases (PD; 16SrX-C) of fruit trees in Europe, are genetically related and they belong to the phylogenetic group of apple proliferation. RFLP profiles corresponding to AP proliferation phytoplasma were found in European hazel (Marcone et al. 1996), Celtis australis L. (Bertaccini et al. 1996), Spartium junceum L., Sarothamnus scoparius (L.) Winm. (Marcone et al. 1997 b, c) and peach trees in USA (Scott and Zimmerman 2001). Very recent publications provided evidence that a mixed infection of 16SrI and 16SrX was detected in fruit trees (Lee et al. 1995, Paltrinieri et al. 2001, Bertaccini et al. 2001) and in red and white currant with full blossom symptoms in Czech Republic (Navrátil et al. 2001). Phytoplasmas belonging to AP group are transmitted by psyllids, which overwinter as infectious adults (Carraro et al. 2001, Frisinghelli et al. 2000). In Poland, apple proliferation disease has been known from more than 30 years (Kamińska and Zawadzka 1970) and pear decline was first identified in 1996 (Malinowski et al. 1996).

Work with phytoplasmas of woody plants is difficult due to low titre and seasonal fluctuation of these pathogens. Some of these problems can be solved by transmission phytoplasmas to periwinkle (Catharanthus roseus L.G. Don). Periwinkle, commonly used as a source plant to maintain phytoplasma isolates, can harbour the majority of known phytoplasmas, and greatly facilitate the research. Several phytoplasmas causing important diseases of trees, e.g. white ash declining (Hiben and Wolanski 1971) and alder yellows (Marcone et al. 1997 a) have been transmitted to periwinkle using Cuscuta spp. plant, while apple proliferation phytoplasma – by psyllids (Refatti et al. 1986) and by dodder (Carraro et al. 1988, Loi et al. 1995, Marcone et al. 1999). Some aster yellows phytoplasma isolates have been transmitted to periwinkle by grafting (Kamińska and Korbin 1999, Kamińska et al. 1997, 2001 a).

The main objectives of this work were to confirm the association of an AY and AP phytoplasmas with magnolia stunting disease by PCR with universal and group specific phytoplasma primers and RFLP analyses, and to study the transmission rate of phytoplasmas through grafting with buds collected from diseased magnolias to C. roseus test plants.
Materials and methods

Plant material

Nine two years old Magnolia liliiflora L. cv. ‘Nigra’ plants were collected in a commercial nursery in central Poland. In the nursery they were propagated by woody cuttings in summer and cultivated in a greenhouse. All plants showed stunted growth, leaf necrosis and malformation, shoot proliferation and dieback.

Graft-transmission

Seven or eight periwinkle seedlings, in the age of three–four months, were grafted each with two bud chips from each selected donor plant. Buds for grafting were collected in June or August 2001. For comparison five plants were left not grafted. The experimentally inoculated plants were maintained in an insect-proof greenhouse and observed for the development of symptoms such as leaf discoloration and diminishing, poor growth, secondary shoot production and flower abnormalities for two growing seasons. In order to verify that the disease symptoms were due to phytoplasmal infection, about half of the plants were examined for the presence of the phytoplasma by PCR.

Plant samples and the reference strains

For each of the magnolia plant samples of tissue from the growing shoots were taken in May and August 2001 or October 2002. Leaf samples were collected from 29 experimentally inoculated by grafting C. roseus plants. The first samples were collected three–four months after inoculation; the final testing was made in November 2002.

For comparison leaf samples from plants inoculated with the reference strain of aster yellows phytoplasma (AY1, 16SrI-B, kindly supplied by Dr. I.-M. Lee, Beltsville, USA) and apple proliferation phytoplasma (AP; 16SrX-A, kindly supplied by Dr. A. Bertaccini, Bologna, Italy) were included.

Phytoplasma detection and identification

DNA was isolated from approximately 1 g of fresh tissue (leaves or phloem stem) using the phytoplasma enrichment procedure described by Ahrens and Seemüller (1992).

The PCR amplification was performed using nested PCR, primed by P1/P7 (Schneider et al. 1995, expected size of PCR product – approximately 1800 bp) and followed by the phytoplasma universal primer pairs: fA/rA (Ahrens and Seemüller 1992, approximately 560 bp) and R16F2n/R16R2 (Lee et al. 1998, approximately 1250 bp) or primers specific for the aster yellows group – R16(I)F1/R16(I)16 (Lee et al. 1994, approximately 1100 bp) or apple proliferation group – fAT/rAS (Smart et al. 1996, approximately 500 bp).
Each PCR mixture (50 µl) contained: 1 µl of total nucleic acid extraction from plant tissue, 1.25 µl of 10 mM solution of four dNTPs, 0.1 µl of 100 µM forward and reverse primers, 1 × DNA polymerase buffer and 1 U of HotStarTaq DNA polymerase (Qiagen, Syngen Biotech, Wrocław, Poland). The following parameters were used: for direct PCR with primers P1/P7 and nested PCR with primers R16F2n/R16R2 – 35 cycles: 1 min at 94°C, 2 min at 60°C (for P1/P7) and 50°C (for R16F2n/R16R2), 3 min at 72°C; for nested PCR with primers R16(I)F1/R16(I)R1 – 35 cycles: 45 s at 94°C, 1 min at 55°C, 2 min at 72°C; for nested PCR with primers fA/rA and fAT/rAS – 25 cycles: 1 min at 95°C, 1 min at 55°C, 1 min at 72°C. During the last cycle, elongation time was extended to 9 min.

PCR products (5 µl) were analyzed by 1% agarose gel electrophoresis in 0.5 X TBE buffer followed by staining with ethidium bromide (0.5 µg/ml) and visualized with UV transilluminator.

RFLP analyses were performed after digestion of 10 µl of PCR products (primed by R16F2n/R16R2 primer pair) with single restriction endonucleases AluI, MseI, HpaII (Gibco BRL, Life Technologies, Warsaw, Poland), SspI and HhaI (Roche Diagnostics, Warsaw, Poland) according to the manufacture’s instructions. The digested DNA was resolved by electrophoresis through 6% polyacrylamide gel, stained with ethidium bromide and observed under UV light. The lengths of DNA fragments were estimated by comparison of the position of DNA bands with those of molecular weight markers (φX174 DNA/HinfI, Promega Symbios, Gdańsk, Poland). Phytoplasma 16S rRNA group designations were based on RFLP analysis following the system of Lee et al. (1998).

Results

Phytoplasma detection and identification

The results of our experiments, presented in Tables 1 and 2, demonstrate that detection of phytoplasmas using PCR technique in affected magnolias depends mainly upon time of testing and primer pairs applied. After amplification of DNA isolated from the tested plants with universal primer pair P1/P7, specific DNA band (~1800 bp) was obtained only from samples of reference strains AY1 and AP. No visible product was amplified by direct PCR from samples obtained from magnolias and experimentally inoculated or control periwinkle plants.

Nested PCR with universal fA/rA and group 16SrI specific primer pair – R16(I)F1/R16(I)R1 amplified the target sequences from all samples collected from the magnolias in August. In samples collected in October no amplification was observed using the primers fA/rA. However, the primer pairs R16(I)F1/R16(I)R1 or F16F2n/R16R2 amplified the target sequences from samples collected in October. After amplification of DNA with group 16SrX specific primer pair – fAT/rAS, specific DNA bands (~ 500 bp) were obtained. These
bands were observed in samples collected in August from five out of six affected magnolia plants but not in samples collected in October (Table 1).

Phytoplasmas were detected in most of the examined samples collected from experimentally grafted periwinkles by amplification in nested PCRs with universal fA/rA or R16F2n/R16R2 and groups 16SrX (Phot. 1a) or 16SrI (Phot. 1b) specific primer pairs – R16(I)F1/R16(I)R1 and fAT/rAS (Table 2).

### Table 1

Detection of phytoplasmas by the nested PCR in *Magnolia liliiflora* cv. ‘Nigra’ source plants

<table>
<thead>
<tr>
<th>Phytoplasma source plants</th>
<th>12 May 2001</th>
<th>14 August 2001</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fA/rA R16(I)F1/R16(I)R1</td>
<td>fA/rA R16F2n/R16R2</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>11</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>18</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>20</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>196</td>
<td>nd</td>
<td>–</td>
</tr>
<tr>
<td>214</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>235</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

nd – not determined.

### Table 2

Experimental transmission of phytoplasmas from diseased magnolias to *Catharanthus roseus* test plants by grafting

<table>
<thead>
<tr>
<th>Source plants</th>
<th>Plants with symptoms/plants inoculated</th>
<th>Phytoplasma detection by the nested PCR with primer pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>fA/rA R16F2n/R16R2</td>
</tr>
<tr>
<td>2**</td>
<td>6/8</td>
<td>3/3</td>
</tr>
<tr>
<td>3**</td>
<td>1/8</td>
<td>0/1</td>
</tr>
<tr>
<td>4**</td>
<td>3/8</td>
<td>1/3</td>
</tr>
<tr>
<td>18**</td>
<td>3/8</td>
<td>3/5</td>
</tr>
<tr>
<td>196*</td>
<td>2/7</td>
<td>3/5</td>
</tr>
<tr>
<td>214*</td>
<td>4/7</td>
<td>3/5</td>
</tr>
<tr>
<td>235*</td>
<td>6/8</td>
<td>6/7</td>
</tr>
<tr>
<td>Total</td>
<td>25/54</td>
<td>19/29</td>
</tr>
</tbody>
</table>


**Grafted on 23 August 2001.

nd – not determined.
No amplification was observed in nucleic acid extracts in samples collected from the diseased magnolia plants in May and from the asymptomatic magnolias.

Products of PCR primed by R16F2n/R16R2 primer pair from naturally infected magnolias and inoculated periwinkles were subjected to RFLP analyses using five restriction enzymes (Phot. 2). The restriction patterns obtained after enzymatic digestion indicated that the tested plants were infected with two distinct phytomonas: a phytomonas belonging to subgroup 16SrI-B was detected in all PCR-positive magnolias and experimentally infected periwinkle plants, whereas five magnolias and eight tested periwinkles were double infected with aster yellows and apple proliferation (16SrV-A) phytomonas.
Experimental transmission of phytoplasmas...  

Phytoplasma transmission

Evaluation of symptoms developed in experimentally grafted periwinkles revealed that AY and AP phytoplasmas could be transmitted in summer from all donors tested. Three to four months after grafting retarded growth and leaf chlorosis were observed on single periwinkle plants. Within one year after inoculation, these symptoms were followed by leaf chlorosis and diminishing as well as increased production of the secondary shoots. During winter some of the inoculated plants developed reduced number of small and pale flowers; in summer they did not produce flower symptoms. The most pronounced symptoms developed periwinkles inoculated with buds from magnolia No. 235. About 50% of the inoculated periwinkle plants did not develop symptoms within 24 months of observation (Table 2).

Examination of selected recipient periwinkles with PCR showed the presence of AY or AY and AP phytoplasmas in symptomatic plants, thus confirming the results of visual disease evaluation (Table 2). In a few cases, phytoplasmas were also detected in plants, which did not show clear symptoms. None of the uninoculated periwinkles developed symptoms.

Discussion

The results obtained in this work indicate that phytoplasmas, responsible for magnolia stunting can be detected in phloem shoots of affected plants using nested
PCR. Analyses of samples collected in the middle of August or October showed that tested magnolias contained mostly phytoplasmas belonging to group 16SrI, subgroup B. Phytoplasmas belonging to the apple proliferation group 16SrX, subgroup A, were found in smaller number of samples collected in August but not in October, in plants infected by both phytoplasmas from groups 16SrI and 16SrX. For differentiation of the magnolia phytoplasmas of the AY and AP groups and subgroups, restriction site analysis using endonuclease HhaI, SspI proved to be highly suitable (Alma et al. 1996, Marcone et al. 1997 b, Lorenz et al. 1995).

Detectable amount of phytoplasmal DNA was obtained by nested PCR from majority of magnolia samples collected at the end of summer and in the autumn but not in the spring. This difficulty in phytoplasma 16SrX detection in late spring seems to be common to both pome (Schaper and Seemüller 1984) and stone (Jarausch et al. 1999, Seemüller et al. 1998 b) fruit species. Jarausch et al. (1999) indicated, that in case of ESFY the sampling has to be done between July and September. Jarausch et al. (1999) explained these difficulties of phytoplasma detection in Prunus in terms of the limitation of movement from old into new phloem since the rapid degeneration of old phloem would result in elimination of a considerable amount of phytoplasmas.

In our work, efficient detection methods were employed to examine both donor magnolias and recipient periwinkle plants for the presence of phytoplasmas. The use of bud wood from carefully selected donor plants appeared to be responsible for the high transmission rates achieved in our experiments. Based on symptomology and PCR-RFLP analyses we stated that AY and AP phytoplasmas have been successfully transmitted by grafting, although the transmission rate of AY was higher than AP. Studies by several authors have shown that phytoplasmas can be propagated through grafting with phytoplasma infected buds (Seemüller et al. 1998 a, Errea et al. 2002). However, this is the first report of AP and AY phytoplasmas transmitted by grafting to C. roseus test plants. Previously, phytoplasmas belonging to 16SrX were transmitted to C. roseus test plants by psyllids (Refatti et al. 1986) and by dodder (Loi et al. 1995, Marcone et al. 1999). Very recently, we recorded AY phytoplasma transmission from magnolia to periwinkle via dodder (Kamińska et al. 2001 b) and by grafting from lily and rose plants (Kamińska and Korbin 1999, Kamińska et al. 2001 a).

The experimentally infected periwinkle plants with phytoplasmas from magnolia showed a reduced vigor, leaf yellowing and diminishing, and flower malformation and discoloration but no virescence or phyllody. Similar symptoms have been reported to occur in C. roseus plants experimentally infected with AY phytoplasma from diseased magnolia (Kamińska et al. 2001 b). The same type of symptoms showed periwinkles infected with PD and ESFY phytoplasmas from fruit trees (Marcone et al. 1999), with alder yellows phytoplasma from naturally infected Alnus glutinosa (L.) Gaertn. tree (Marcone et al. 1997 a) or with AY phytoplasma from lily (Kamińska and Korbin 1999) and rose plants (Kamińska et al. 2001 a).

Although in the present study a correlation of symptom expression with the presence of phytoplasma in experimentally infected C. roseus plants is difficult to establish, the results confirm these from previous study of dodder inoculation and
strongly suggest that typical symptoms like leaf chlorosis and diminishing and shoot proliferation are highly correlated to the presence of AY and AP phytoplasmas.

The results obtained in this study provide good basis for the development of sampling protocols for routine diagnosis of AY and AP phytoplasmas in Magnolia species by PCR. Indications from these and other unpublished results of the authors suggest that in order to obtain reliable results sampling has to be done in August–October. The insect vectors of AY and AP phytoplasmas in Magnolia spp. plants are unknown. It seems likely that the efficient acquisition occurs at the end of summer when the phytoplasma titres are high.

**Summary**

Phytoplasmas detected in magnolia plants with stunting symptoms were classified as aster yellows phytoplasma (16SrI-B) and apple proliferation phytoplasma (16SrX-A) on the basis of restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rDNA phytoplasma fragments. The phytoplasmas were successfully transmitted to Catharanthus roseus test plants by grafting, and the infection was confirmed by symptom expression and phytoplasma specific PCR and RFLP analysis.

**Streszczenie**

EKSPERYMENTALNE PRZENIESIENIE FITOPLAZM Z CHORYCH MAGNOLII NA ROŚLINĘ TESTOWĄ CATHARANTHUS ROSEUS PRZEZ SZCZEPENIE

Fitoplazmy występujące w roślinach magnoli z objawami karłowatości zostały zaklasyfikowane jako fitoplazma żółtaczki astra (16SrI-B) i fitoplazma proliferacji jabłoni (16SrX-A). Identyfikację dokonano za pomocą analizy polimorfizmu długości fragmentów restrykcyjnych (RFLP) produktów łańcuchowej reakcji polimerazy (PCR) ze starterami umożliwiającymi amplifikację fragmentu genu 16S rDNA fitoplazm. Fitoplazmy przeniesiono z chorych magnoli na siewki rośliny testowej kataranta różowego (Catharanthus roseus) techniką szczepienia. Porażenie roślin barwinka przez fitoplazmy zostało potwierdzone występowaniem charakterystycznych objawów chorobowych oraz wynikami analizy PCR-RFLP.

**Literature**


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