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Protocols

Improved Detection Methods for Fruit Tree Phytoplasmas

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Abstract. Phytoplasmas infecting fruit trees are considered quarantine organisms in Europe and North America. Detection often is hampered by their extremely irregular distribution in host plants. A sensitive, specific and quick diagnostic test would be highly desirable for routine detection, mainly to avoid using infected planting material. PCR methods require tedious preparation of DNA; also, the available primers are highly specific and exhibit some homology to chloroplast and plastid DNA. To address these problems, we compared several DNA preparation protocols for purity of DNA, cost and time required. We also developed new primers using rDNA sequence information from an Austrian isolate of European Stone Fruit Yellows (ESFY). These primers operate at high annealing temperatures and, thus, increase the specificity and decrease the risk of false positives. The primers could reliably detect the European phytoplasmas (AP, ESFY and PD) within a collection of isolates maintained in micropropagated periwinkle. Thus, they are suitable as general primers for phytoplasma detection. The primers also can be used for strain identification by direct PCR followed by RFLP analysis as demonstrated with micropropagated fruit tree material. Finally, an IC-PCR method that uses the primers for AP detection was found very sensitive and suitable for large-scale testing of apple material *in vivo* and *in vitro*.

Key words: apple, apricot, fruit trees, *in vitro* cultures, pathogen detection, phytoplasmas

Abbreviations: ACLSV, apple chlorotic leafspot virus; A-AY, American aster yellows; AP, apple proliferation; ASGV, apple stem grooving virus; ASHY, ash yellows; ASPV, apple stem pitting virus; AT, apple proliferation strain; BVK, phytoplasma from *Psammotettix cephalotes*; CHRY, chrysanthemum yellows; DAS-ELISA, double antibody sandwich-enzyme linked immunosorbent assay; ESFY, European stone fruit yellows; EY-C, elm yellows; FBPSA, faba bean phyllody; GVX, Green Valley X disease; GSFY, German stone fruit yellows; IC-PCR, immuno capture-polymerase chain reaction; KVM, clover phyllody; LNS, plum leptonecrosis; LUM, Lucerne virescence; MOL, Molière disease;

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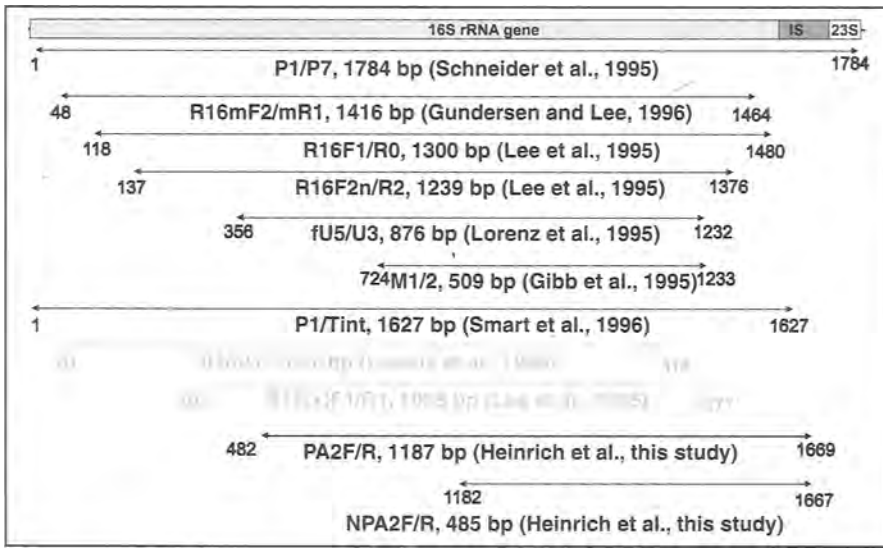

Figure 1. Genomic location of several general (in black) and fruit tree specific primers (in grey as printed (in green in electronic version)) commonly used for phytoplasma detection.

NAX, Naxos virescence; PD, pear decline; PRIVA, primula yellows; RFLP, restriction fragment length polymorphism; RT-PCR, reverse transcription-polymerase chain reaction.

Introduction

Phytoplasmas associated with diseases of perennial fruit crops in Europe, America and Australia are spreading rapidly because of transmission by insects and grafting. The poor taste and small size of fruits and the decline of infected trees makes these diseases a major economical threat to orchards. The most important fruit tree phytoplasmas in Europe are the closely related apple proliferation (AP), European stone fruit yellows (ESFY) and pear decline (PD) (Jarausch et al., 2000b; Lorenz et al., 1995; Lee et al., 1995; Kison et al., 1997).

PCR provides the most sensitive, specific and quick detection system for phytoplasma. General and specific primers, located in the 16S rDNA, intergenic spacer (IS) and the 23S rDNA region of the phytoplasma genome, are presently used (Figure 1). Unfortunately, some primers can induce dimers or unspecific bands. They also have sequence homology in the 16S-spacer region to chloroplasts and plastids increasing the risk of false positives. To address this problem, we identified new general primers from the 16S rDNA of an Austrian isolate of ESFY.

Another problem with PCR detection is the presence of putative inhibitors in phytoplasma-infected plant material (Musetti et al., 2000; Lepka et al., 1999). To study the inhibitory effects of a typical plant DNA preparation from infected material, we performed spike PCR. With this technique, increasing volumes of sample preparations are added to a PCR mix containing purified plasmid DNA representing the sequence to be amplified. Amplification will not occur if the

DNA preparation contains PCR-inhibiting substances. We also compared DNA isolation procedures for cost and time required. Finally, we established an IC-PCR method for AP using our primers and a newly available antiserum (Loi et al., 1998).

Materials and Methods

Isolates of phytoplasmas

We used an established *in vitro* collection of phytoplasma-infected fruit tree cultures, i.e. apple, pear and apricot (http://www.boku.ac.at/iam/pbiotech/phytopath/col.html) as source of phytoplasma DNA. The DNA of ESFY was isolated from a symptomatic apricot tree in Austria (Laimer da Câmara Machado et al., 2001) and the 16S DNA and spacer region were sequenced. Newly developed primers (Figure 1) also were used to amplify the corresponding region for sequencing from AP and PD isolates in infected tissue cultures of *Malus domestica* and *Pyrus sp.* (kindly provided by W. Jarausch [INRA, Bordeaux, F] [Jarausch et al., 1996] and D.L. Davies [HRI, East Malling, UK]), respectively. Phytoplasma isolates maintained in a micropropagated collection of periwinkle (Bertaccini et al., 2000) were used as controls: CHRY (16SrI-A); PRIVA (16SrI-B); KVM (16SrI-C); A-AY (16SrI-F); FBPSA (16SrII); GVX (16SrIII-A); EY-C (16SrV-A); LUM (16SrVI); ASHY (16SrVII); NAX (16SrIX); AT (16SrX-A); AP-15 (16SrX-A); GSFY-1 (16SrX-B); GSFY-2 (16SrX-B); LNS1 (16SrX-B); LNS2 (16SrX-B); PD (16SrX-C); BVK (16SrXI-C); MOL (16SrXII-A) (phytoplasma classification according to Lee et al., 1998).

DNA extraction

A) After Kobayashi et al. (1998)

DNA extraction for spike experiments from symptomatic field samples of *Prunus armeniaca* was performed essentially as described by Kobayashi et al. (1998). For direct PCR, 100 ng of DNA was used.

B) After Bertheau et al. (1998)

DNA extraction described by Bertheau et al. (1998) was modified as follows: 100 mg of leaf material was powdered in liquid nitrogen and dissolved in 1.8 mL PBS-Tween buffer supplemented with 2% (w/v) PVP (K25, Fluka) and 20 mM Diethylcarbamic Acid (DIECA). Tubes were spun for 10 min at high speed and 20 µL of 10% SDS was added to 200 µL of supernatant. Further steps were carried out as described by Bertheau et al. (1998). Samples were stored at -20°C. For direct PCR , 3 µL were used as template.

C) With the Extract–N-Amp^{TM} Plant PCR kit

DNA extraction was performed with the Extract–N-Amp^{TM} Plant PCR kit (XNAP) (Sigma) according to the manufacturer's instructions. For direct PCR, 1 µL of extraction solution was used.

Sequence comparisons as prerequisite for primer design

A BLAST search of the NCBI database was carried out to identify homology with phytoplasma-specific sequences. For nonhomology of primer sequences with

chloroplast or plastid 16S rDNA sequences, a BLAST search at the TAIR (The Arabidopsis Information Resource) database was performed choosing the "All Higher Plant Sequences" (All Viridiplantae sequences from GenBank) selection mode. PA2F/R and NPA2F/R primers were designed based on the sequence derived from an isolate found in Austria and using the PrimerSelect program from the Lasergene99 software package (DNASTAR Inc., WI, USA).

PCR amplification

PCR was carried out in a volume of 20 µL as follows: 0.5 µL of dNTP (10 mM each), 0.5 µL of the forward primer and 0.5 µL of the reverse primer (20 pmol/µL), 2.0 µL 10x PCR buffer (Promega, Madison, WI, USA), 1.4 µL $MgCl_2$ (25 mM, Promega), 0.1 µL *Taq* polymerase (5 units/µL, Promega), 12 µL sterile water and 3 µL of template isolated after Bertheau et al. (1998); 100 ng of DNA isolated after Kobayashi et al. (1998) and 1 µL of DNA sap extracted with the Extract-N-Amp™ Plant PCR kit were used for direct PCR. PA2F/R PCR products were diluted 1:40 and 1 µL was used for nested PCR. Tubes without nucleic acid were employed as negative PCR controls.

The following thermal cycling programs were used for PA2F/R PCR: 35 cycles of 30 s at 94°C, 1 min 15 s at 60°C and 1 min 30 s at 72°C, with a final elongation step of 10 min at 72°C. For NPA2F/R PCR, the following (shorter) PCR program was used: 35 cycles of 30 s at 94°C, 30 s at 60°C and 45 s at 72°C, with a final elongation of 10 min at 72°C. Direct and nested PCR reactions also were performed with Polymed (Florence, Italy) reagents adjusting the annealing temperatures to 62°C for direct and 65°C for nested PCR. PCR products were analysed on 1.2% agarose gels and photographed under UV illumination.

IC-PCR

A monoclonal antibody against AP (151712, Bioreba AG, Switzerland) was diluted 1:200 in coating buffer (pH 9.6) (100 mM $NaHCO_3$, 37 mM Na_2CO_3). Fifty microlitres of diluted antibody was incubated in a 0.5 mL PCR Eppendorf tube for 3 h at 37°C. Leaf material was homogenised in liquid nitrogen and 10 µL extraction buffer (1x PBS pH 7.4; 2% PVP; 0.05% Tween 20) was added per mg of leaf material. The mixture was vortexed and centrifuged at 4000 *g*-force for 7 min. Tubes pretreated with the antibody were washed twice with washing buffer (1x PBS [pH 7.4], 0.05% Tween 20) and 100 µL of the supernatant from the leaf extraction was added. The tubes were incubated at 4°C overnight and washed three times with washing buffer; 20 µL of the above PA2F/R PCR mix then was added. PCR was performed using the above cycle conditions. The amplification product from direct PCR was diluted 1:30 and 1 µL was used for nested PCR with NPA2F/R primers.

Inhibition studies

Plant sap especially from woody species may contain inhibitors of PCR. These inhibitors sometimes can be reduced or circumvented by dilution. A field isolate of ESFY was chosen, reflecting the most difficult sample situation; the method of Kobayashi et al. (1998) was used because it yields the highest DNA purity.

Decreasing amounts (400, 200, 150 and 100 ng) of a genomic DNA preparation were used for inhibition studies. One hundred nanograms of a DNA preparation from an ESFY-infected micropropagated apricot and 100 ng DNA extracted from a healthy plant were used as controls. PA2F/R PCR products were diluted 1:40 and 1 µL was used as a template for subsequent NPA2F/R PCR.

Spike PCR

Spike PCR was performed with four concentrations of the above DNA preparation. Genomic DNA in the amounts of 400, 200, 150 and 100 ng was added to 400 pg of plasmid DNA (plasmid PA2kb contains a subcloned region of ESFY) to a direct PCR mix with primers PA2F/R. To study the affect of primer on the inhibiting effects from the plant sap, nested PCR (1 µL of a 1:40 dilution) was performed with primers M1/M2 (Gibb et al., 1995) and NPA2F/R.

Restriction analysis of PCR products

The PA2F/R PCR products (200 ng) were digested with 2.5 units of either *Tai* I, *Tps509*I (MBI, Vilnius, Lithuania) or *Taq* I (Promega) at 65 °C for 16 h according to the manufacturer's instructions. Fragments were analysed on a 5% polyacrylamide gel stained in ethidium bromide and the bands were photographed under UV illumination. Restriction patterns for NPA2F/R amplified products for enzymes *Bsp*6 II, *Hin*8 II, *Cje* I, *Mae* II and *Ttm* I were predicted and compared using MapDraw software from the Lasergene99 software package (DNASTAR Inc., WI, USA).

Results

Sequence analysis of genomic rRNA from an ESFY isolate

A 1784 bp region between primers P1 and P7 (corresponding to the16S rRNA gene), 16/23S spacer region and partial 23S rRNA gene region of an ESFY isolate (PA2kb) from an infected field sample of *Prunus armeniaca* from Austria has been sequenced (GenBank accession number AY029540). Sequence comparisons with published databases revealed highest similarity to an ESFY isolate from the Czech Republic (Y11933).

Primer design

Based on the ESFY sequence, PA2kb primers were designed for direct and nested PCR. PA2F/R are the primers for direct PCR, amplifying a product of 1187 bp between nt positions 482 and 1669. Nested NPA2F/R primers amplify a product ranging from nt positions 1182-1667. Sequences of primers for the first PCR were as follows: PA2F: 5′-GCC CCG GCT AAC TAT GTG C-3′, PA2R: 5′-TTG GTG GGC CTA AAT GGA CTC-3′. Primers for the nested PCR were NPA2F: 5′-ATG ACC TGG GCT ACA AAC GTG A-3′, NPA2R: 5′-GGT GGG CCT AAA TGG ACT CG-3′. Sequence alignment using the GenBank Higher Plant sequence database showed no significant homology between the primer sequences and chloroplast sequences, plastid 16S sequences or to any published plant sequences. Database searches in the NCBI Blast Search showed full homology to 16 phytoplasma sequences.

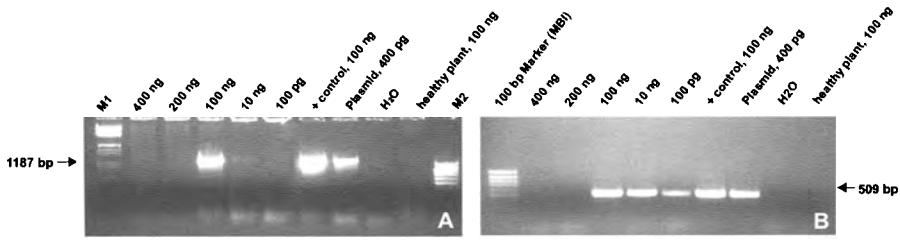

Figure 2. Direct PCR with PA2F/R (A) and nested M1/M2 (B) of an ESFY isolate from the field was performed using decreasing amounts of purified DNA (lanes 2-6). DNA preparations of 400 and 200 ng inhibit both direct and nested PCR. (A) 100 ng allowed a strong amplification signal, 10 ng DNA produced a weak band on the agarose gel, while 100 pg DNA produced no visible product after direct PCR. (B) Nested PCR using M1/M2 even allows detection of 100 pg of genomic DNA from the field sample. Lane 7, positive plant control: 100 ng of a DNA preparation from an ESFY infected micropropagated apricot. Lane 8, positive plasmid control: 400 pg of a DNA preparation from PA2kb plasmid. Lane 9 and 10, healthy plant and water negative controls. M1: 500 ng Lambda *Eco*R I / *Hin*d III Marker (MBI); M2: 500 ng 100 bp Marker (MBI).

Comparison of DNA extraction methods

Three methods for DNA isolation for phytoplasma detection (Kobayashi et al., 1998; Bertheau et al., 1998; Extract–N-Amp™ Plant PCR kit) were evaluated using the newly developed primers. The extraction procedure described by Kobayashi et al. requires only inexpensive chemicals and low materials cost, but labour costs potentially are high. For example, 35 samples require about one-and-a-half days.

Using direct PCR, the new primers PA2F/R and the isolation protocol of Bertheau et al. (1998), it is possible to detect phytoplasmas in tissue cultures of apple, pear and apricot infected with AP, PD and ESFY respectively (data not shown). For detection of phytoplasmas in field samples, a nested PCR is required because of low concentration and uneven distribution of the pathogens. However, if the titre is sufficiently high in the appropriate season, field material can also be assayed directly. Inhibition studies demonstrate that supraoptimal amounts of DNA template can completely impede even nested PCR. These findings are supported by reports of considerably higher levels of likely PCR inhibitors in phytoplasma-infected plant material (Musetti et al., 2000; Lepka et al., 1999).

Even 150 ng of genomic DNA (1.5 µL) resulted in a total inhibition of direct and nested PCR, indicating that this volume of template contains enough substances to inhibit PCR; 1 µL of the genomic DNA solution yielded an amplification product comparable in intensity to 100 ng of a DNA preparation from an ESFY-infected micropropagated apricot (Figure 2).

For spike PCR, different amounts of DNA from phytoplasma-infected plant material were added to the PA2F/R PCR mix together with 400 pg plasmid DNA containing the template sequence. The addition of 150, 200 and 400 ng DNA inhibited direct PCR completely (Figure 3). Nested PCR comparing the 2 primers, M1/M2 and NPA2F/R, clearly showed that primers NPA2F/R are less sensitive to

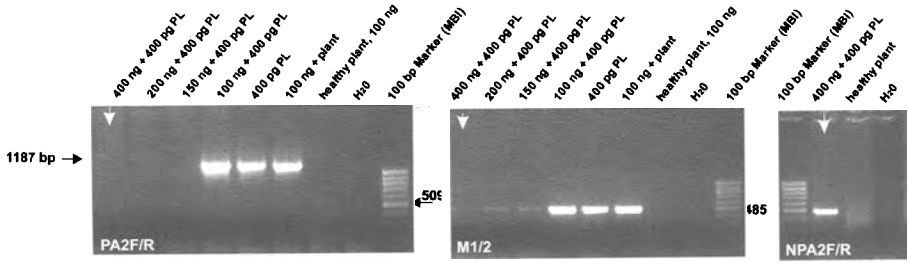

Figure 3. Spike PCR with primers PA2F/R in direct PCR (left) and nested PCR with primers M1/M2 (middle) and NPA2F/R (right). 400 pg plasmid (PL) DNA were combined with decreasing volumes of field sample DNA preparations (lanes 1-4). Amplification occurring from 100 ng DNA mixed with healthy plant sap (lane 6) strongly indicates that the supraoptimal presence of template in lanes 1-3 is responsible for the inhibition. 400 ng PL DNA were used as positive control (lane 5). A healthy control plant (100 ng DNA) and water were used as negative controls (lanes 7-8).

PCR inhibitors, by giving an amplification signal even at 400 ng DNA. In contrast, 150 ng DNA solution was the upper limit before inhibition of PCR occurred using primers M1/M2 (Figure 3).

DNA extraction with the Sigma kit yields DNA suitable for PCR in only 15 min. The supplied PCR mix improves PCR performance considerably by yielding intense positive signals with only 1 µL of template in direct PCR; 4, 3 and 2 µL of template again gave less intense bands (data not shown). It is possible to detect phytoplasmas in direct PCR in most infected samples, and after nested PCR in the remainder (data not shown). The achieved detection limit is higher than a 1:2000 dilution for direct PCR.

RFLP

RFLP analyses using PA2F/R as general primers were carried out on 19 different isolates from a micropropagated reference collection (Bertaccini et al., 2000). With three restriction enzymes a polymorphism between fruit tree phytoplasma strains AP, PD and ESFY could be assigned (Figure 4a, b and c), i.e. *Tai* I distinguishes ESFY from AP/PD; *Tsp509* I digestion reveals a polymorphism between AP/ESFY and PD and *Taq* I differentiates AP from ESFY and PD.

Moreover, additional restriction polymorphisms between AP, AP*, PD and ESFY were predicted for fragments created with primers NPA2F/R for *Bsp*6 I, *Hin*8 I, *Mae* II and *Ttm* I (Table 1). The published AP* sequence contains specific restriction patterns differing from those of the AP sequence obtained in our laboratory (Table 1).

IC-PCR

An IC-PCR test method for AP phytoplasma was established based on an immuno capture step combined with PCR applying PA2F/R and, subsequently, NPA2F/R primers. This assay is very sensitive and detects AP *in vitro* in infected plant

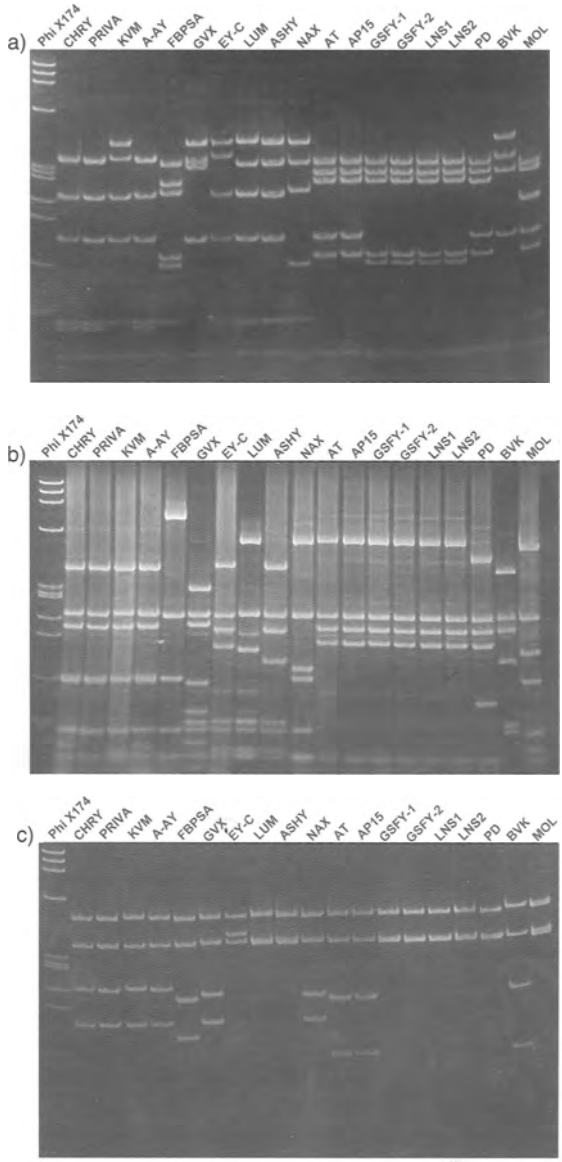

Figure 4. RFLP analyses of PCR products obtained with PA2F/R on control strains of the majority of known phytoplasma groups identified (Lee et al., 1998). a) *Tai* I differentiates AP/PD subgroups from ESFY subgroups. b) *Tsp509*I shows differences between AP/ESFY and PD subgroups. c) *Taq* I differentiates AP from ESFY/PD subgroups. PhiX174, *Hae* III digested marker; fragment sizes in base pairs from top to bottom are 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72. Strains are chrysanthemum yellows (CHRY), primula yellows (PRIVA), clover phyllody (KVM), American aster yellows (A-AY), faba bean phyllody (FBPSA), Green Valley X disease (GVX), elm yellows (EY-C), Lucerne virescence (LUM), ash yellows (ASHY), Naxos virescence (NAX), apple proliferation strain (AT), German stone fruit yellows (AP15, GSFY-1 and GSFY-2), plum leptonecrosis (LNS1 and LNS2), pear decline (PD), phytoplasma from *Psammotettix cephalotes* (BVK), Molière disease (MOL).

Table 1. Restriction sites of NPA2F/R PCR product sequences (485 bp) derived for strains AP* [X92869], AP (a strain sequenced in our laboratory), PD [Y16392] and ESFY [Y11933].

***Tai* I**				***Taq* I**				***Bsp6* I**			
AP*/ESFY≠AP/PD				AP*≠AP≠PD/ESFY				AP*/ESFY≠AP/PD			
AP*	AP	PD	ESFY	AP*	AP	PD	ESFY	AP*	AP	PD	ESFY
20	23	20	20	110	113	110	110		49	46	
55			55	460	222	461	460	349	352	349	349
169	172	169	169		271	469	468				
***Hin* 8 I**				***Mae* II**				***Ttm* I**			
AP*/ESFY≠AP/PD				AP*/ESFY≠AP/PD				AP*/ESFY≠AP/PD			
AP*	AP	PD	ESFY	AP*	AP	PD	ESFY	AP*	AP	PD	ESFY
116	119	116	116	17	20	17	17	13	19	16	16
150	153	150	150	52			52	51			51
	203	200		166	169	166	166	165	168	165	165

material up to a dilution of 1:1600 (data not shown). The IC-PCR test method is rather inexpensive.

Discussion

We present two new approaches for the detection of fruit tree phytoplasmas, IC-PCR and nested PCR. These can be used with the primers PA2F/R and NPA2F/R to substantially shorten detection time and reduce the risk of false positive results.

The new primers PA2F/R work as general primers detecting 19 different phytoplasma strains from various phylogenetic groups. This agrees with sequence analysis predictions from database searches. The new primers have the advantages of high annealing temperatures and the lack of significant sequence homology with chloroplast sequences.

Levels of soluble carbohydrates in leaves of tobacco plants infected with AP strain AT recently were shown to be significantly higher than those of healthy plants (Lepka et al., 1999). These findings confirm earlier studies with woody plants such as apple, pear and elm trees, showing that phytoplasma infections lead to the accumulation of carbohydrates in mature leaves (Batjer and Schneider, 1960; Braun and Sinclair, 1978; Catlin et al., 1975; Kartte and Seemüller, 1991). Musetti et al. (2000) reported a significantly higher polyphenol content in phytoplasma-diseased plants, particularly in plum leaves infected with ESFY. We have shown that some primers used for detecting phytoplasmas of fruit trees are sensitive to inhibitors present in the plant sap. Our inhibition and spike studies strongly implicate the higher concentration of inhibitors in infected material as the cause of problems with PCR. Therefore, the value of the newly developed primers is evident.

The use of group-specific primers for the detection of phytoplasmas AP and ESFY has been recommended for trees (Jarausch et al., 2000a, b). In contrast, a general detection of phytoplasma infection is a better strategy since a host plant can be infected by more than one phytoplasma strain (Lee et al., 1994, 1995).

Until we cannot exclude a latent infection with a phytoplasma strain not recognised by group specific primers, we prefer general primers for phytoplasma detection and a subsequent identification by RFLP analysis.

IC-PCR proved to be more sensitive and reliable than DAS-ELISA. When compared to ELISA tests or DNA extraction procedures and subsequent PCR detection, IC-PCR, for its simplicity, sensitivity and reproducibility, had some advantage; therefore, IC-PCR can be regarded as a valuable alternative for large-scale testing of apple trees. The major disadvantage is that antisera are not yet available for all known phytoplasmas infecting fruit trees.

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