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A new approach to apple proliferation detection: a highly sensitive real-time PCR assay

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Abstract

The present paper describes a new approach for diagnosis of apple proliferation (AP) phytoplasma in plant material using a multiplex real-time PCR assay simultaneously amplifying a fragment of the pathogen 16S rRNA gene and the host, *Malus domestica*, chloroplast gene coding for tRNA leucine. For the first time, such an approach, with an internal analytical control, is described in a diagnostic procedure for plant pathogenic phytoplasmas enabling distinction between uninfected plant material and false-negative results caused by PCR inhibition. Pathogen detection is based on the highly conserved 16S rRNA gene to ensure amplification of different AP phytoplasma strains. The newly designed primer/probe set allows specific detection of all examined AP strains, without amplifying other fruit tree phytoplasmas or more distantly related phytoplasma strains. Apart from its specificity, real-time PCR with serial dilutions of initial template DNA ranging over almost five orders of magnitude (undiluted to 80,000-fold diluted) demonstrated linear amplification over the whole range, while conventional PCR showed a reliable detection only up to 500-fold or 10,000-fold dilutions, respectively. Compared to existing analytical diagnostic procedures for phytoplasmas, a rapid, highly specific and highly sensitive diagnostic method becomes now available.

Keywords: Apple proliferation; *Malus domestica*; Phytoplasma; Plant disease; Diagnosis; Real-time PCR; Multiplex assay

1. Introduction

Phytoplasmas are the smallest known plant-pathogenic bacteria that are not culturable in vitro in cell-free media and are associated with diseases of several hundred plant species. The apple proliferation (AP) phytoplasma causes an important apple tree disease occurring in many European apple growing areas and potentially causing considerable economic losses by decreasing size, quality and overall yield of fruit (Frisinghelli et al., 2000; Loi et al., 1995; Seemüller et al., 1998a). This cell wall-less and phloem-restricted pathogen has been shown to be transmitted in propagation material and by sap-sucking insect vectors of the genus *Cacopsylla* (Frisinghelli et al., 2000; Tedeschi et al., 2002). Phylogenetic analyses of the 16S rRNA gene and ribosomal protein sequences showed that phytoplasmas form a monophyletic clade within the class Mollicutes, which is related more to *Acholeplasma* species than to the genus *Mycoplasma* associated with animal hosts. The causal agent of AP is closely related to other fruit tree phytoplasmas clustering in a very homogenous phylogenetic sub-

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clade (overall genetic divergence: 1.6%) together with the pear decline (PD), European stone fruit yellows (ESFY) and peach yellow leaf roll (PYLR) phytoplasmas (Lee et al., 2000; Seemüller et al., 1998c).

The major concern in controlling apple proliferation is to prevent spread by planting disease-free material and controlling vectors, since infected trees cannot be cured. Although AP phytoplasma is classified as a quarantine organism in Europe and North America, a rapid, specific and sensitive diagnostic method for large-scale analyses is still lacking. A simple and cost-efficient method suitable for large-scale utilization is ELISA, but its limited sensitivity can lead to inaccurate diagnosis if pathogen titer in the plant is low (Loi et al., 2002). Hence, PCR-based assays provide so far the most sensitive method for phytoplasma detection. The majority of PCR protocols employ oligonucleotide primers that amplify a fragment of the 16S ribosomal RNA gene and the 16S–23S intergenic spacer region (Heinrich et al., 2001; Lee et al., 1995; Lorenz et al., 1995; Schneider et al., 1995; Smart et al., 1996). These 16S ribosomal DNA primers allow either universal amplification of phytoplasmas or specific detection of certain phytoplasma groups, such as fruit tree phytoplasmas of the AP group, but not unique identification of the AP agent (Lorenz et al., 1995). Furthermore, it was shown that some primer combinations are prone to cross-reactions with plastid DNA of the host plant or bacteria colonizing the surface of plant material (Ahrens and Seemüller, 1992; Heinrich et al., 2001; Skrzeczkowski et al., 2001; Baric and Dalla Via, in preparation). For that reason, post-amplification steps like digestion with restriction endonucleases, hybridization with specific probes or immunoenzymatic determination of amplified products (PCR–ELISA) are recommended to confirm the identity of a PCR amplificate and to obtain an accurate diagnosis (Lorenz et al., 1995; Poggi Pollini et al., 1997; Seemüller et al., 1998b). One possibility to circumvent these labor-intensive and time-consuming post-PCR manipulations is the use of non-ribosomal AP-specific primers (Jarausch et al., 1994, 2000). However, despite their high specificity (eliminating the possibility of cross-reactions with the closely related PD and ESFY phytoplasmas), it must be considered that the detection sensitivity of primers amplifying a chromosomal gene might be lower than that of primers amplifying ribosomal genes present in two copies in the phytoplasma genome (Schneider and Seemüller, 1994).

The aim of the present study was to develop a diagnostic procedure for the detection of AP phytoplasma combining high sensitivity and high specificity while, at the same time, being suitable for high-throughput testing. Our method of choice was real-time PCR which has gained wide acceptance in molecular diagnostics and is broadly used in clinical, food and veterinary microbiology (Ginzinger, 2002; Mackay et al., 2002; Walker, 2002). Although it is well known that DNA isolated from plant material might contain substances inhibitory to PCR (Green et al., 1999; Heinrich et al., 2001), this is the first phytoplasma detection method employing an endogenous reference serving as an internal positive control. By simultaneous amplification and individual detection of pathogen and host DNA, it is not only possible to detect the AP agent but also to distinguish between uninfected plant material and PCR inhibition.

2. Materials and methods

2.1. Sources of phytoplasmas and DNA isolation

The present study includes phytoplasma isolates from naturally and experimentally infected apple trees showing typical AP symptoms (enlarged stipulae, witches' broom and bronze reddish discoloration of leaves), pears infected with PD and a Japanese plum with ESFY. The following reference phytoplasma strains maintained on periwinkle (*Catharanthus roseus*) were used: AT and AP15 (apple proliferation), ESFY (European stone fruit yellows), ALY (alder yellows), ULW (elm yellows), and CH1 and STOL (Stolbur). Periwinkle is widely used to culture phytoplasmas since these pathogens cannot be cultured on media and the culture in plants represents an important resource to maintain different strains in the laboratory. Field samples of 12 nonsymptomatic *Malus domestica* Borkh. trees and 81 apple plants from the greenhouse served as negative controls. A detailed overview of sample numbers and sampling locations is given in Table 1. Samples from naturally infected apple trees were collected in summer/autumn 2002.

Total nucleic acid was isolated from freshly prepared phloem tissue of fruit tree roots (about 500 mg)

Table 1
Phytoplasmas and host plants from different localities examined with conventional PCR and the real-time PCR assay

	Sampling locality	N° of samples examined	N° of samples tested positive with			
			conventional PCR		real-time PCR	
			fU5-rU4	fO1-rO1	qAP-16S	qMd-cpLeu
Apple *(Malus domestica)*						
AP, natural infection	Brixen (N-Italy)	4	4	4	4	4
AP, natural infection	Chioggia (N-Italy)	3	3	3	3	3
AP, natural infection	Glurns (N-Italy)	8	6	6	8	8[x]
AP, natural infection	Laimburg (N-Italy)	1	1	1	1	1
AP, grafted	Laimburg (N-Italy)	3	3	3	3	3
AP, natural infection	Latsch (N-Italy)	2	2	2	2	2
AP, natural infection	Ritten (N-Italy)	11	11	11	11	11
AP, sub-strain AP[a]	Neustadt (Germany)	1	1	1	1	1
AP, sub-strain AT-1[a]	Neustadt (Germany)	1	1	1	1	1
AP, sub-strain AT-2[a]	Neustadt (Germany)	1	1	1	1	1
Non symptomatic for AP	Ritten (N-Italy)	12	0	0	0	12
Non symptomatic for AP	Laimburg (N-Italy)[b]	81	0	0	0	81
Pear *(Pyrus communis)*						
PD, natural infection[c]	Bologna (Italy)	5	5	5	0	5
PD, in vitro plant[d]	England	1	1	1	0	1
Japanese plum *(Prunus salicina)*						
ESFY, natural infection[e]	Verona (Italy)	1	1	1	0	1
Periwinkle *(Catharanthus roseus)*[f]						
AP, strain AP15	Italy	1	1	1	1	0[y]
AP, strain AT	Germany	1	1	1	1	0
ALY	Italy	1	1	0	0	0
CH-1	Italy	1	1	0	0	0
GSFY-1	Germany	1	1	1	0	0
STOL	Eastern Europe	1	1	0	0	0
ULW	France	1	1	0	0	0

[a]DNA extracts from naturally infected *M. domestica* provided by W. Jarausch, Neustadt, Germany.
[b]Maintained in the greenhouse.
[c]Root samples supplied by C. Poggi Pollini, Bologna, Italy.
[d]DNA extract provided by W. Jarausch, Neustadt, Germany.
[e]DNA extract supplied by C. Poggi Pollini, Bologna, Italy.
[f]*C. roseus* is used to main different phytoplasma strains; all plants provided by E. Seemüller, Dossenheim, Germany.
[x]Highlighted row includes two samples with low total DNA concentration; the pathogen could be detected by real-time PCR but not by conventional PCR.
[y]The qMd-cpLeu set did not amplify DNA from periwinkle due to its distant phylogenetic relationship to apple.
ALY = alder yellows; AP = apple proliferation; CH-1 = Stolbur; ESFY and GSFY = European stone fruit yellows; PD = pear decline; STOL = Stolbur; ULW = elm yellows.

or young shoots and leaves of periwinkle following a previously described phytoplasma enrichment protocol (Ahrens and Seemüller, 1992). Quantification of DNA was done by spectrophotometry at 260/280 nm.

2.2. Design of probes and primers for multiplex PCR

Primers and probes were designed using the 'Primer Express' software (Version 2.0; Applied Biosystems, Foster City, CA, USA). The design of the primer/probe set for detecting AP phytoplasma was based on an alignment of the 16S rRNA gene including all major phytoplasma groups and four different AP isolates from Spain, France, Italy and Germany (GenBank accession numbers: X76426, AJ542542, AJ542541 and X68375; alignment not shown; Seemüller et al., 1998c). The highly conserved and well-studied 16S rRNA gene was chosen to ensure amplification of all AP strains, since it is known that primers derived from non-ribosomal

Table 2
Primer pairs and reaction conditions employed for conventional PCR

Primer pair		Specificity	T_A (°C)	N_C	Fragment size (bp)
B49317/A49855	(Taberlet et al., 1991)	Plants in general	50	38	~ 500
fU5/rU4	(Ahrens and Seemüller, 1992)	Phytoplasmas in general	55	38	942
fO1/rO1	(Lorenz et al., 1995)	Fruit tree phytoplasmas	54	38	1057
AP5/AP4	(Jarausch et al., 2000)	AP phytoplasma	50	40	483

T_A = annealing temperature.
N_C = number of PCR cycles.

sequences might be specific for certain isolates (Jarausch et al., 2000). In addition to the primers and probe for detecting AP phytoplasma, a second primer/probe combination was designed as an internal positive control for multiplex PCR. The slowly evolving chloroplast gene of *M. domestica* coding for tRNA leucine was selected to assure amplification of host DNA even when diverse apple cultivars or different *Malus* species are surveyed (Taberlet et al., 1991). PCR and sequencing of this gene were performed using primers B49317 and A49855 (Taberlet et al., 1991). PCR was carried out on the "Mastercycler gradient" (Eppendorf, Hamburg, Germany) in 20-μl reaction mixtures containing 1 μM of each primer, 200 μM dNTP, 1.5 mM $MgCl_2$, 0.5 U BioTherm DNA polymerase (GeneCraft, Münster, Germany) and approximately 50 ng total DNA isolated from *M. domestica* cultivar 'Golden Delicious'. PCR conditions are summarized in Table 2. PCR products were purified with Montage PCR Centrifugal Filter Devices (Millipore, Bedford, USA). Cycle sequencing reactions with 50–70 ng of the 500-bp product were performed in both 5′ to 3′ and 3′ to 5′ reactions using the CEQ DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) and 32 cycles of 20 s at 96 °C, 20 s at 50 °C and 4 min at 60 °C. Sequencing products were separated and visualized on the CEQ 8000 Genetic Analysis System (Beckman Coulter). The sequence was deposited in GenBank under accession number AY 510067.

The probes and primers for a simultaneous detection of AP phytoplasma and its host plant in a real-time PCR assay were obtained from Applied Biosystems (Table 3). The probe for detecting AP phytoplasma was conjugated with the reporter dye FAM at its 5′ end while the probe for apple chloroplast DNA amplification was conjugated with the reporter dye VIC. Both probes were attached with a Minor Groove Binder (MGB) and a non-fluorescent quencher dye (NFQ) at their 3′ ends (Applied Biosystems).

2.3. Multiplex real-time PCR assay

For each target, the optimal primer concentrations were determined in separate tubes by running a matrix of forward and reverse primer concentrations according to Applied Biosystems user bulletin no. 5 (P/N 4306236B; http://www.appliedbiosystems.com). In a subsequent multiplex optimization experiment, the primer concentration of qMd-cpLeu was limited

Table 3
Primer and probe sequences for simultaneous detection of the AP phytoplasma 16S rRNA gene and the *M. domestica* chloroplast gene for tRNA leucine

Name	Specificity	Sequence 5′ → 3′
qAP-16S-F	AP phytoplasma	CGA ACG GGT GAG TAA CAC GTA A
qAP-16S-R	AP phytoplasma	CCA GTC TTA GCA GTC GTT TCC A
qAP-16S	AP phytoplasma	**FAM**-TAA CCT GCC TCT TAG ACG
qMd-cpLeu-F	*M. domestica*	CCT TCA TCC TTT CTG AAG TTT CG
qMd-cpLeu-R	*M. domestica*	AAC AAA TGG AGT TGG CTG CAT
qMd-cpLeu	*M. domestica*	**VIC**-TGG AAG GAT TCC TTT ACT AAC

while the concentration of both qAP-16S primers was kept constant at 900 nM. The adjustment of primer concentrations in the multiplex real-time PCR assay was necessary to avoid competition between the two systems and obtain two independent reactions in a single tube. Since chloroplast DNA was expected to be more abundant in total DNA extracts, amplification of chloroplast DNA was assumed to use up the common reagents and impair amplification of phytoplasmal DNA. Therefore, primer concentrations of the internal positive control were limited so that primers would be exhausted soon after obtaining C_T values. In this way, sufficient amounts of reagents would be available to amplify pathogen DNA and ensure reliable diagnosis.

Multiplex reactions were carried out in a total volume of 20 μl containing 10–100 ng of template DNA, 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM of both qAP-16S primers, 100 nM of both qMd-cpLeu primers and 200 nM of each probe. Amplification and detection were performed using an automated ABI PRISM 7000 Sequence Detection System (Applied Biosystems) in MicroAmp optical 96-well plates. PCR was initiated with two incubation steps: 2 min at 50 °C to activate AmpErase UNG (Applied Biosystems), which prevents reamplification of carry-over PCR products, 10 min at 95 °C to activate AmpliTaq Gold DNA polymerase (Applied Biosystems), followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Each reaction included at least two negative controls without template. Threshold levels were set to intersect the amplification curves in the linear region of the semilog plot amounting 0.16 for qAP-16S and 0.1 for qMd-cpLeu. Threshold cycles for each PCR reaction were calculated with ABI Prism 7000 SDS Software (version 1.0).

2.4. Validation of multiplex real-time PCR efficiency

To evaluate the performance of multiplex real-time PCR in comparison to separate reactions, 12 apple proliferation-positive samples were chosen to prepare tenfold serial dilutions ranging from an average of 80 ng to 80 pg total DNA. Every sample was amplified in duplicate in one multiplex reaction and two single PCR reactions in which primer/probe sets qAP-16S and qMd-cpLeu were used separately, maintaining the same primer concentrations as in multiplex PCR. Cycle thresholds obtained for each of the two targets in multiplex and separate reactions were plotted against the log of initial DNA concentration. After calculating linear regression equations, an equivalence test was implemented (Schuirmann, 1987) to compare the differences between slopes obtained with multiplex and single real-time PCR. The slope of a resulting trend line is a function of PCR efficiency and should amount to −3.32 if PCR reaction is 100% efficient. Confidence intervals (90%) were calculated and placed around the mean values of the slopes. Equivalence was claimed if the two confidence intervals were smaller than the boundary limit which was set to 10% around −3.32. Deviations from optimal PCR efficiency were calculated by including the value for the slope (S) in the equation: PCR efficiency=$(10^{(1/-S)})-1$ (Ginzinger, 2002).

Eighty-six different DNA extracts were amplified using solely primers and probe qAP-16S, solely primers and probe qMd-cpLeu, and by combining these two systems. This was done to examine the reproducibility and accuracy of C_T values inferred in singleplex and multiplex real-time PCR assays. A paired t test was performed to determine whether threshold cycles obtained in separate reactions differ from that obtained in multiplex PCR.

2.5. Evaluation of multiplex real-time PCR specificity

The specificity of the real-time PCR assay developed for the detection of AP phytoplasma was tested in multiplex reactions in samples infected with AP as well as other phytoplasma strains (Table 1). Eighty-one nonsymptomatic apple trees were included to examine the performance of the internal analytical positive control. All samples tested with the real-time PCR assay were also tested with conventional PCR on the Mastercycler gradient (Eppendorf) using primer combinations fU5/rU4 and fO1/rO1 (Table 2; Ahrens and Seemüller, 1992; Lorenz et al., 1995). Each 20-μl reaction contained 10–100 ng of total DNA, 1 μM of each primer, 200 μM dNTP, 1.5 mM $MgCl_2$ and 0.5 U BioTherm DNA polymerase (GeneCraft, Germany). PCR amplification products were separated on ethidium bromide stained 1.5% agarose gels and visualized on a UV transilluminator.

2.6. Detection sensitivity of multiplex real-time PCR

Detection sensitivity of multiplex real-time PCR in comparison to conventional PCR with primer pairs fU5/rU4 and AP5/AP4 (Jarausch et al., 1994, 2000) was investigated by preparing a serial dilution of eight field-collected samples infected with AP phytoplasma covering the following range: undiluted (from 60 to 100 ng total DNA), 10-, 100-, 500-, 1,000-, 5,000-, 10,000-, 20,000-, 40,000- and 80,000-fold dilution (reaction conditions in Table 2).

3. Results

The primers and the probe for detection of AP phytoplasma matched sequences in a variable region of the 16S rRNA gene starting at position 82 and ending at position 157. There was no variability within this region among sequences of the four AP strains from Spain, France, Italy and Germany downloaded from GenBank (see Section 2.2). However, the qAP-16S probe differs by two mismatches from the sequence of ESFY and three mismatches from that of PD, and shows even more point mutations to sequences of other phytoplasma strains (Fig. 1), promising a specific and exclusive detection of AP phytoplasma. The second primer/probe set was designed to be a compatible internal positive control for amplification of a 69-bp fragment from the *M. domestica* chloroplast DNA tRNA leucine gene.

Performance of the multiplex real-time PCR assay was evaluated in comparison to singleplex reactions after primer concentrations for qAP-16S and qMd-cpLeu were adjusted. C_T values according to four different initial DNA concentrations were obtained for each of the twelve samples amplified in single and multiplex reactions. Slopes of the regression lines, as a function of PCR efficiency, ranged from -3.633 to -3.211 (mean -3.518 ± 0.117 S.D.) for the AP phytoplasma target DNA run in multiplex reactions, and from -3.546 to -3.277 (mean -3.340 ± 0.083 S.D.) for the same target run in single reactions. For chloroplast DNA of the host plant, slopes between -3.748 and -3.274 (mean -3.524 ± 0.147 S.D.) were obtained for multiplex and between -3.694 and -3.216 (mean -3.454 ± 0.139 S.D.) for singleplex reactions. Correlation coefficients for all regression lines were between 0.983 and 0.999. Mean calculated PCR efficieney for qAP-16S run in multiplex was 0.926 (± 0.045 S.D.) compared to 0.970 (± 0.0325 S.D.) in single reactions. For qMd-cpLeu mean PCR efficiency in multiplex reactions was 0.925 (± 0.053 S.D.), in singleplex reactions 0.950 (± 0.052 S.D.). The test of equivalency showed that 90% confidence intervals of the slopes obtained in multiplex and singleplex reactions with

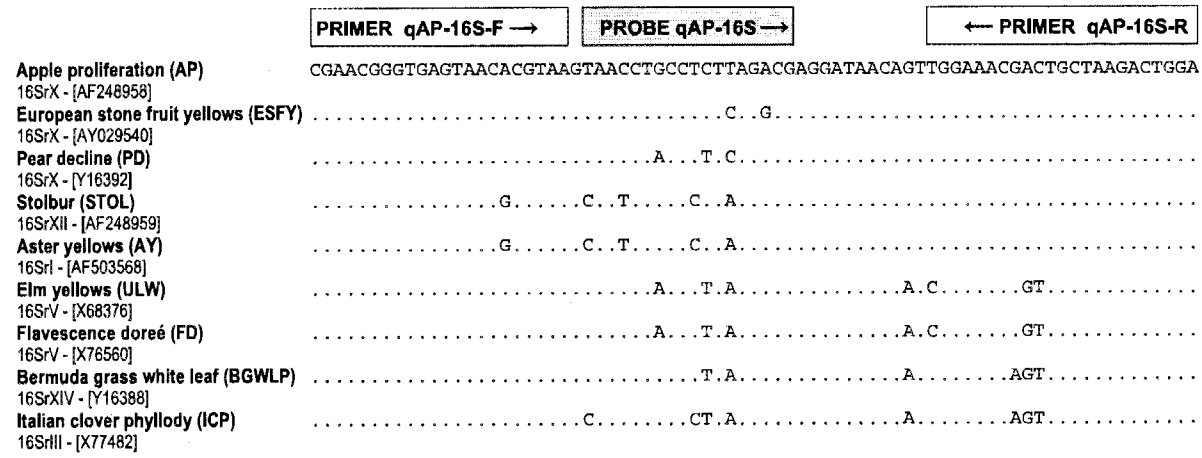

Fig. 1. Sequence alignment of the region of the 16S rRNA gene (starting at position 82) used to design the primer/probe set for detecting AP phytoplasma by real-time PCR. The nine different phytoplasma strains belong to six distinct 16S rRNA subclades indicated under each name. GenBank accession numbers are given in brackets.

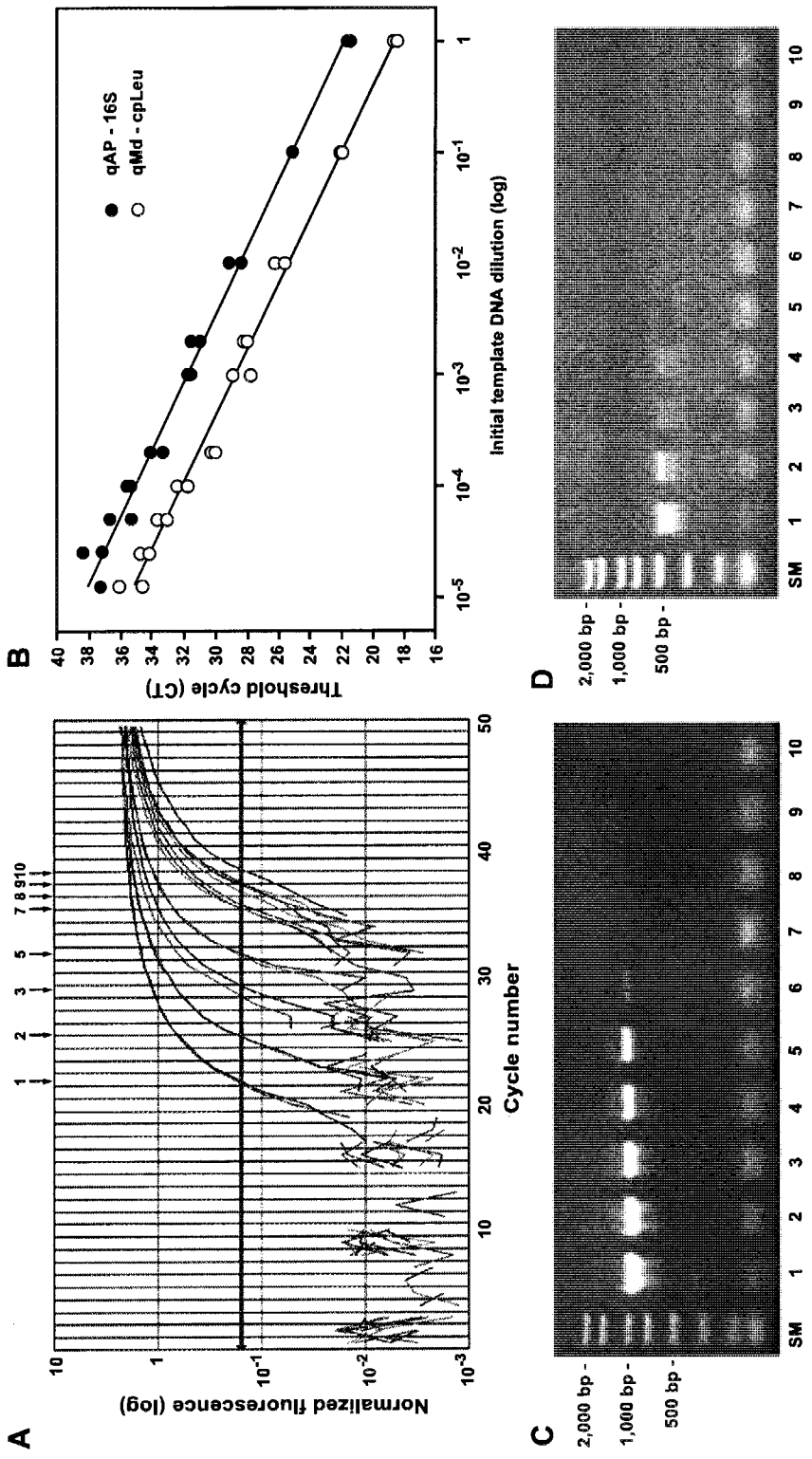

Fig. 2. Sensitivity validation for real-time PCR in comparison to conventional PCR by amplifying a dilution series of a DNA extract from one field sample. (A) Amplification plot of a dilution series with primer/probe set qAP-16S, numbers on the top of the graph correspond to dilutions given below. (B) Dynamic range of a multiplex real-time PCR with primer/probe set qAP-16S (black circles; slope = − 3.3135, $R^2 = 0.9881$) and qMd-cpLeu (white circles; slope = − 3.3688, $R^2 = 0.9885$). (C) Agarose gel (1.5%) showing amplification products obtained with primer fU5/rU4. (D) Agarose gel (2.0%) showing amplification products obtained with primer AP5/AP4. Dilution series: (1) undiluted, (2) 10-, (3) 100-, (4) 500-, (5) 1000-, (6) 5000-, (7) 10,000-, (8) 20,000-, (9) 40,000-, (10) 80,000-fold dilution. SM = size marker.

both real-time PCR systems lie within the specified boundary limit of 10%, while a t test pointed to a difference in amplification efficiency of multiplex and singleplex reactions using qAP-16S ($N=12$, $P=0.0086$) and no difference between multiplex and singleplex reactions with qMd-cpLeu ($N=12$, $P=0.242$).

Comparison of threshold cycles did not result in significant difference between values obtained in multiplex and single reactions for any of the targets (qAP-16S: $N=55$, $P=0.23$; qMd-cpLeu: $N=86$, $P=0.26$; t test), indicating that primer concentrations were adjusted so that the two reactions do not compete in a multiplex assay.

The primer/probe set qAP-16S yielded products from all nucleic acid samples prepared from apple trees infected with AP phytoplasma and also from both AP phytoplasma strains maintained in periwinkle (Table 1). Target DNAs of all three subtypes of apple proliferation phytoplasma, AP, AT-1 and AT-2 (Jarausch et al., 2000) were successfully amplified with qAP-16S. No typical amplification plots were obtained in reactions employing the closely related phytoplasma strains PD and ESFY as template. Although in some reactions, a slight linear increase of fluorescence was present, this signal remained lower than the defined threshold. The qAP-16S set did neither amplify DNA from distantly related phytoplasma strains nor from healthy apple trees.

The real-time PCR system qMd-cpLeu amplified DNA isolated from all tested apple trees, pear and Japanese plum. No amplification products were obtained with periwinkle (Table 1) due to its distant phylogenetic relationship to apple.

Conventional PCR with primer pairs fU5/rU4 and fO1/rO1 amplified the DNA from all field samples of apple showing typical symptoms of apple proliferation, except two samples collected at one location (Glurns, N-Italy, evidenced in Table 1). Although DNA extraction was performed twice independently, overall yield of isolated DNA remained very low (5 ng/μl). Repeated amplification reactions with conventional PCR primers delivered no products that could be visualized on an agarose gel, but real-time PCR generated reproducible amplification plots. The primer pair fU5/rU4 yielded products with all phytoplasma strains included in the present study, while fO1/rO1 amplified fruit tree phytoplasmas AP, PD and ESFY (Lorenz et al., 1995).

Real-time PCR yielded amplification products for the complete serial dilution of initial template DNA. Amplification of the apple proliferation target was linear over almost five orders of magnitude (average $R^2=0.988 \pm 0.004$ S.D.) and a mean PCR efficiency of 0.986 (± 0.027 S.D.) was achieved (Fig. 2A and B). Similar values were gained for the internal analytical positive control. By applying conventional PCR with the primer pair fU5/rU4 weak amplification products could be visualized for samples where initial template DNA was diluted 5000-fold, whereas weak bands were apparent in only three out of eight samples where initial template DNA was diluted 10,000-fold. No amplification products were obtained with higher template dilutions (Fig. 2C). Using AP-specific primers AP5/AP4 PCR products could be visualized only up to a 500-fold dilution (Fig. 2D) of the same dilution series.

4. Discussion

Accurate and rapid detection of apple proliferation phytoplasma is a major prerequisite to control the disease and may also contribute to obtain a deeper insight into the biological cycle of the pathogen. So far, several PCR-based methods for diagnosis of AP phytoplasma have been proposed (Heinrich et al., 2001; Jarausch et al., 1994, 2000; Lorenz et al., 1995; Poggi Pollini et al., 1997; Seemüller et al., 1998b), but all of them involve time-consuming post-amplification steps and are not really suitable for high-throughput testing. Moreover, multiple PCR product handling steps bear the risk to spread the amplicon in the laboratory and generate carry-over contaminations.

Only recently, real-time PCR has been employed to develop specific, accurate and highly sensitive assays to be used in plant pathogen detection and quantification (Cullen et al., 2001; Winton et al., 2003; Zhang et al., 1999). In real-time PCR, products are detected during the exponential phase of the reaction using primers, probes or amplicons labeled with fluorogenic molecules in combination with an automatic sequence detection system. The possibility to use different

reporter dyes enables the development of multiplex reactions where host and pathogen DNA can be amplified simultaneously.

In the present study, a real-time PCR assay was used for the first time to detect plant pathogenic phytoplasmas. The advantages of the new approach for the detection of apple proliferation phytoplasma compared to conventional methods are its high specificity, enhanced sensitivity, high throughput and rapid testing time, as well as the processing in a closed system involving no post-PCR manipulations, thus minimizing the risk of transmitting contamination. The most commonly applied detection method of AP phytoplasma targets unique sequences in the highly conserved 16S rRNA gene to assure amplification of all AP strains (Jarausch et al., 2000; Kison et al., 1994). Because of their conserved nature it was not possible to find oligonucleotide primers specific for AP phytoplasma without amplifying the closely related PD and ESFY phytoplasmas (Lorenz et al., 1995; Seemüller et al., 1998b) to be used in conventional PCR on this gene. This problem was overcome with real-time PCR which employs a primer pair and a fluorogenic probe. The probe was designed to be used at high annealing temperatures and to be unique to the target sequence of AP phytoplasma, showing a minimum of two mismatches to other phytoplasma strains. Another consideration in favor of using a ribosomal gene is its presence in two copies in the phytoplasma genome (Schneider and Seemüller, 1994), thus increasing the sensitivity of a diagnostic assay. This fact might be important in situations where pathogen titer in plant material is low.

Specificity of the newly developed primer/probe set qAP-16S for detecting apple proliferation phytoplasma was demonstrated by amplification experiments using template DNAs of different phytoplasma strains. AP phytoplasma was detected in all naturally infected apple trees sampled at several localities in northern Italy. Furthermore, amplification products were obtained from the three subtypes of apple proliferation phytoplasma sampled in Germany, AP, AT-1 and AT-2, which are also common in other European orchards (Jarausch et al., 2000), as well as from the AP strains maintained in periwinkle stemming from two distinct European regions. This finding shows the suitability of the real-time PCR assay for general diagnosis of AP phytoplasma. Furthermore, the primer/probe set qAP-16S did not amplify phytoplasma strains other than AP, demonstrating the high specificity of the new approach. In contrast to real-time PCR, the use of both, universal phytoplasma primers fU5/rU4 and AP group-specific primers fO1/rO1 for conventional PCR (Ahrens and Seemüller, 1992; Lorenz et al., 1995) in several cases resulted in weak, unspecific bands of similar size as the expected product. At least in one case, there is strong evidence that these primers can amplify the 16S rRNA gene of uncultured soil bacteria (Skrzeczkowski et al., 2001; Baric and Dalla Via, in preparation).

Although direct comparisons of the specificity and the sensitivity between conventional and real-time PCR are exceedingly difficult because of differing reaction conditions (e.g., sequences, primer positions, amplicon length, PCR conditions and detection techniques), the parallel application of these methods to the same samples demonstrates a much higher detection sensitivity of the multiplex real-time PCR assay covering a range of almost five orders of magnitude. While the real-time PCR assay was capable of reliably detecting AP phytoplasma in DNA extracts diluted 80,000-fold (corresponding to 1 pg of initial total DNA; Fig. 2A), the detection limit of conventional PCR was reached at a 10,000-fold or 500-fold dilution of the original DNA extract for fU5/rU4 and AP5/AP4 (Fig. 2). Although sensitivity of conventional PCR could be increased by performing a nested PCR, this would require much more handling time and raise the risk of carry-over contamination resulting in false-positive results. The high detection sensitivity of real-time PCR could also be demonstrated in two field samples showing typical AP symptoms (evidenced location Glurns in Table 1). Because of the low yield of isolated DNA, conventional PCR failed to detect the pathogens while real-time PCR gave reproducible positive results. This finding clearly shows that real-time PCR can be particularly useful at low infection levels.

The approach described herein to detect apple proliferation is not only the first using real-time PCR but also the first employing an internal positive control in a diagnostic procedure for plant pathogenic phytoplasmas. The possibility to distinguish between uninfected plant material and false-negative results caused by inhibitors is essential for accurate diagnosis, since infected plant material might contain high concentrations of substances inhibitory to PCR (Heinrich et al.,

2001). The authors' unpublished results strongly indicate that inhibition problems might also occur with cultivated apples and that these problems can be overcome by diluting original DNA extracts. However, the dilution of initial template DNA might have major impacts on detection sensitivity of conventional PCR. The development of the multiplex real-time PCR assay to simultaneously detect a gene of the pathogen and the host plant required adjustment of primer concentrations to prevent competitive interference between these systems. Although direct comparisons of amplification efficiency in singleplex and multiplex reactions indicated that multiplexing might slightly affect the qAP-16S system, this seems to be negligible since reproducible C_T values were obtained in both procedures and detection sensitivity in the multiplex assay was given over a wide range of template dilution.

The real-time PCR assay promises to be a fast, highly sensitive and highly specific method in research and, in addition, useful for routine testing of numerous samples. Apart from its applicability in diagnostic procedures, the new approach opens the possibility of a challenging aspect of research—the quantitative measurement of pathogen colonization. This might contribute considerably to a better understanding of the biological cycle of apple proliferation phytoplasmas as well as giving insight into how these pathogens become associated with their host plants and their natural vectors.

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