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RESEARCH ARTICLE

Universal and group-specific real-time PCR diagnosis of flavescence dorée (16Sr-V), bois noir (16Sr-XII) and apple proliferation (16Sr-X) phytoplasmas from field-collected plant hosts and insect vectors

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Abstract

Three real-time PCR–based assays for the specific diagnosis of flavescence dorée (FD), bois noir (BN) and apple proliferation (AP) phytoplasmas and a universal one for the detection of phytoplasmas belonging to groups 16Sr-V, 16Sr-X and 16Sr-XII have been developed. Ribosomal-based primers CYS2Fw/Rv and TaqMan probe CYS2 were used for universal diagnosis in real-time PCR. For group-specific detection of FD phytoplasma, ribosomal-based primers fAY/rEY, specific for 16Sr-V phytoplasmas, were chosen. For diagnosis of BN and AP phytoplasmas, specific primers were designed on non-ribosomal and nitroreductase DNA sequences, respectively. SYBR® Green I detection coupled with melting curve analysis was used in each group-specific protocol. Field-collected grapevines infected with FD and BN phytoplasmas and apple trees infected with AP phytoplasma, together with *Scaphoideus titanus*, *Hyalesthes obsoletus* and *Cacopsylla melanoneura* adults, captured in the same vineyards and orchards, were used as templates in real-time PCR assays. The diagnostic efficiency of each group-specific protocol was compared with well-established detection procedures, based on conventional nested PCR. Universal amplification was obtained in real-time PCR from DNAs of European aster yellows (16Sr-I), elm yellows (16Sr-V), stolbur (16Sr-XII) and AP phytoplasma reference isolates maintained in periwinkles. The same assay detected phytoplasma DNA in all test plants and test insect vectors infected with FD, BN and AP phytoplasmas. Our group-specific assays detected FD, BN, and AP phytoplasmas with high efficiencies, similar to those obtained with nested PCR and did not amplify phytoplasma DNA of other taxonomic groups. Melting curve analysis was necessary for the correct identification of the specific amplicons generated in the presence of very low target concentrations. Our work shows that real-time PCR methods can sensitively and rapidly detect phytoplasmas at the universal or group-specific level. This should be useful in developing defence strategies and for quantitative studies of phytoplasma–plant–vector interactions.

Introduction

Flavescence dorée (FD), bois noir (BN) and apple proliferation (AP) phytoplasmas cause serious diseases in two economically important woody species in Europe: grapevine (*Vitis vinifera* (L.)) and apple (*Malus* × *domestica*). Phytoplasmas are wall-less and phloem-restricted pathogens transmitted in a persistent manner by leafhoppers and planthoppers (Homoptera: Auchenorrhyncha) and psyllids (Homoptera: Sternorrhyncha) (Lee *et al.*, 2000).

Flavescence dorée, one of the most economically important diseases of grapevine in southern France, northern Italy and Spain (Boudon-Padieu, 2003), is caused by *Candidatus* (*Ca.*) Phytoplasma ulmi (The IRPCM, 2004), subgroups 16Sr-VC and 16Sr-VD (Angelini *et al.*, 2001). The monophagous grapevine-limited leafhopper *Scaphoideus titanus* Ball is the natural vector of FD (Schvester, *et al.*, 1963).

Bois noir is the most widespread grapevine yellows (GY) disease in Europe and the Mediterranean basin and is caused by phytoplasmas belonging to the stolbur taxon (*Ca*. Phytoplasma solani; The IRPCM, 2004) (16Sr-XII), which also infect a wide range of wild and cultivated herbaceous hosts besides grapevines (Marzachì *et al.*, 1999; Langer & Maixner, 2004). BN is vectored by the cixiid *Hyalesthes obsoletus* Signoret to grapevine (Maixner *et al.*, 1995) and by *Pentastiridius beierii* Wagner to other herbaceous hosts (Gatineau *et al.*, 2001), although the spread of the disease in areas where these vectors do not occur suggests the existence of other vectors (Battle *et al.*, 2000).

Apple proliferation, an important disease of apple trees in Europe, is an economic threat in areas where the disease is present (Loi *et al.*, 1995; Seemüller *et al.*, 1998). It is caused by a phytoplasma related to pear decline (PD), European stone fruit yellows (ESFY) and peach yellows leafroll phytoplasmas (Lee *et al.*, 2000) but for which a separate species has recently been proposed (*Ca.* Phytoplasma mali, 16Sr-X; Seemüller & Schneider, 2004). AP is vectored by the psyllids *Cacopsylla melanoneura* Förster and *Cacopsylla costalis* Flor in northern Italy and in Germany (Frisinghelli *et al.*, 2000; Jarausch *et al.*, 2003).

Control of phytoplasma-associated diseases is based on spraying insecticides to reduce vector populations and use of healthy replanting material. FD and AP phytoplasmas are classified as quarantine organisms in Europe (AP phytoplasma is a quarantine organism also in North America). There is thus a need for more rapid and sensitive phytoplasma detection and identification. Enzyme-linked immunosorbent assay is easy and inexpensive for large-scale screening but lacks sensitivity especially when the pathogen titre is low (Loi *et al.*, 2002). To overcome this problem, diagnosis of phytoplasmas relies on nested PCR assays. Most of these protocols use primers designed on sequences in the phytoplasma 16S rDNA, the spacer region between the 16S and 23S rDNA genes and the 5′ part of the 23S rDNA gene (Lee *et al.*, 2000). These primers allow universal identification of phytoplasmas in direct PCR assay, followed by identification of specific taxonomic groups in nested assays. Restriction fragment length polymorphism (RFLP) analysis of amplicons is then required to identify FD, BN or AP phytoplasmas. The complete procedure is laborious and requires time-consuming postamplification steps. To overcome these problems, group-specific primers have been designed on ribosomal genes or other genomic sequences for the detection of specific phytoplasmas in direct PCR, as in the case of BN (Maixner *et al.*, 1995; Daire *et al.*, 1997; Marzachì *et al.*, 2000), FD (Marcone *et al.*, 1996; Daire *et al.*, 1997; Marzachì *et al.*, 2001) and AP phytoplasmas (Jarausch *et al.*, 2000; Heinrich *et al.*, 2001). Primers based on non-ribosomal genes usually have lower sensitivity than those designed on ribosomal genes, which are present in double copy in the phytoplasma genome (Schneider & Seemüller, 1994). Moreover, conventional PCR always requires a further gel electrophoresis step.

Real-time PCR has been proposed to improve diagnosis of plant pathogens, and recently, protocols have been published for (a) the identification of FD and AP phytoplasmas in infected field-collected samples (Baric & Dalla-Via, 2004; Bianco *et al.*, 2004; Jarausch *et al.*, 2004), (b) the identification of *Ca.* Phytoplasma asteris (16Sr-I) in herbaceous hosts (Wei *et al.*, 2004; Marzachì & Bosco, 2005) and (c) universal amplification of phytoplasma DNA (Christensen *et al.*, 2004).

This work aimed to develop real-time PCR assays specific for FD, BN and AP phytoplasmas using SYBR Green I detection and a universal assay to detect phytoplasmas belonging to 16Sr-V, 16Sr-X and 16Sr-XII groups using TaqMan probe technology.

Materials and methods

Plant and insect material, reference phytoplasmas and DNA isolation

Leaf samples were collected from 23, 17, 64 and 105 GY-symptomatic grapevines from several commercial vineyards in Piemonte region, north-west Italy, during June–August 2001, 2002, 2003 and 2004, respectively. Leaf samples were collected from 5 and 15 naturally infected apple trees showing typical AP symptoms in commercial orchards in the same area during September–October 2003 and 2004, respectively. Samples from glasshouse-grown seedlings were used as negative controls.

Isolates of European aster yellows (EAY), American elm yellows (EY), and AP and Serbian stolbur pepper isolate (EAY, 16Sr-IB; EY-1, 16Sr-VA; AP, 16Sr-XA, strain AP and Stol, 16Sr-XIIA) from the collection of Istituto di Virologia Vegetale, CNR, propagated and maintained in periwinkle (*Catharanthus roseus* (L.) G. Don), were used for comparison. Total DNAs extracted from periwinkle infected with AP, ESFY and PD phytoplasmas (AP, 16Sr-XA, strain AT; ESFY, 16Sr-XB and PD, 16Sr-XC) and from five field-collected, PD-phytoplasma-infected pear trees (*Pyrus communis* (L.)) and three ESFY-phytoplasma-infected

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plum trees (*Prunus domestica* (L.)) were also used. DNAs were extracted as detailed by Marzachì *et al.* (1999) from ~1500 mg of leaf midribs. Final products were suspended in 100 μL sterile double distilled water (SDW).

Total DNAs were extracted using the method of Marzachì *et al.* (1998) from 18 adults of *S. titanus*, 31 of *H. obsoletus* and 22 of *C. melanoneura* sweep captured in orchards infected with FD, BN and AP phytoplasmas during 2003 and 2004. DNAs from the same insect species captured in unaffected orchards were used as negative controls.

Two microlitres of DNAs from each extraction were used in conventional PCR experiments, while 5 μL of 1:5 (plant DNA), 1:2.5 (*H. obsoletus* and *S. titanus* DNAs) and 1:10 *C. melanoneura* DNA dilutions in SDW were used in real-time PCR assays.

Conventional PCR assay, restriction fragment length polymorphism and dot blot analysis

The universal primers P1/P7 (Schneider *et al.*, 1995) were used in conventional direct PCR assays for diagnosis of FD and AP. Irrespective of the results, reaction products were diluted 1 : 40 in SDW and used as templates in nested reactions driven by primers R16(V)F1/R1 (Lee *et al.*, 1994) and fO1/rO1 (Lorenz *et al.*, 1995) for specific detection of group 16Sr-V and 16Sr-X phytoplasmas, respectively. Reaction and cycling conditions were as detailed in the original papers.

Nested-amplified fragments obtained with primers R16(V)F1/R1 (Lee *et al.*, 1994) were separately digested (2 μL aliquots), according to the manufacturer's recommendations, for 2 h with 1 unit of *Taq*I (Invitrogen, Carlsbad, CA, USA) at 65°C. Nested-amplified fragments obtained with primers fO1/rO1 (Lorenz *et al.*, 1995) were separately digested (2 μL aliquots), according to the manufacturer's recommendations, for 1 h with 1 unit of *Ssp*I (Gibco BRL, Paisley, UK) at 37°C. Digestion products were run in 5% polyacrylamide gels buffered in 1× TBE (Tris-Borate-EDTA) along with a 1-kb plus DNA size marker (Gibco BRL) and visualised by UV light after staining with ethidium bromide.

Undiluted grapevine DNAs were directly amplified with Stol-specific primers M1/P8 (Marzachì *et al.*, 2000) for conventional diagnosis of BN phytoplasma. Reactions and cycling conditions were as detailed in the original paper.

Products of M1/P8 PCR reactions that did not yield visible bands were further hybridised by dot blot with digoxigenin-labelled riboprobe Ts_1224, as detailed in Marzachì *et al.* (2000).

Design of real-time PCR primers and standards preparation

Ribosomal-based primers CYS2Fw/Rv and TaqMan probe CYS2 (Marzachì & Bosco, 2005) were used for universal diagnosis. Three different concentrations (400 pg μL^{-1}, 400 fg μL^{-1} and 400 ag μL^{-1}) of the plasmid pOP74, containing the ribosomal DNA sequence from chrysanthemum yellows (CY) phytoplasma (16Sr-I) recognised by CYS2Fw/Rv and the CYS2 probe, were used as positive controls.

Ribosomal-based primers fAY/rEY (Marcone *et al.*, 1996; Marzachì *et al.*, 2001) were chosen for FD phytoplasma diagnosis. The 1800-bp ribosomal fragment amplified with phytoplasma-specific primers P1/P7 (Schneider *et al.*, 1995) from total DNA of a field-collected, FD-phytoplasma-infected grapevine was isolated from agarose gel with Gel Extraction Kit (Qiagen, Hilden, Germany), cloned into pGemT plasmid (EasyVector System, Promega, Madison, WI, USA) and named pFD81-03. Three different concentrations (400 pg μL^{-1}, 400 fg μL^{-1} and 4 fg μL^{-1}) of pFD81-03 were used in each FD phytoplasma test as positive controls.

Primers StolFw/Rev (Table 1) were designed on a non-ribosomal stolbur-specific DNA sequence (AJ272132; Marzachì *et al.*, 2000) with Primer3 software (http://www.broad.mit.edu/cgibin/primer/primer3.cgi) and used for BN phytoplasma diagnosis. Three different concentrations (40 fg μL^{-1}, 4 fg μL^{-1} and 400 ag μL^{-1}) of the plasmid pStol224, containing the stolbur-specific DNA sequence recognised by StolFw/Rev (Marzachì *et al.*, 2000), were used in each reaction as positive controls.

Primers fAP_2/rAP_2 (Table 1) were designed on the nitroreductase AP-phytoplasma-specific DNA sequence (L22217, Jarausch *et al.*, 2000) with Primer3 software and used for AP phytoplasma diagnosis. Primers APFw/rAP_1 (5′-CAATCAACAATTAAGTTAAAG-3′ and 5′-CCACCAATAGGACATGTTTC-3′), designed on the same

Table 1 Primers designed for specific amplification of stolbur and apple proliferation phytoplasmas

Primer	Specificity of 16S rDNA	Sequence 5′–3′	Localisation	Amplicon Length
StolFw	XII	AACCGCTCGCAAACAGC	Genomic DNA (AJ272132)	270 bp
StolRev		ATTAGCGCCTTAGCTGTG		
fAP_2	X	AAGAGCAATTCGTACTTTCG	Nitroreductase gene (L22217)	200 bp
rAP_2		GCCGAACTAGTTTCTAATTGAC		

DNA sequence and including fAP_2/rAP_2, were used to amplify a 500-bp fragment from total DNA of an AP-phytoplasma-infected apple tree. The fragment was isolated from agarose gel with Gel Extraction Kit (Qiagen), cloned into pGemT plasmid (EasyVector System, Promega) and named pAP. Three different concentrations of pAP (400 fg μL^{-1}, 400 fg μL^{-1} and 4 ag μL^{-1}) were used in each AP reaction as positive controls.

Real-time PCR assay and melting curve analysis

Real-time PCR assays were performed in 96-well plates in a Bio-Rad iCycler (Bio-Rad, Hercules, CA, USA) Reaction and cycling conditions for universal diagnosis were as detailed in Marzachì & Bosco (2005). Three samples of each phytoplasma–host plant–insect vector combination, selected according to the varying threshold cycles (CTs) (low, medium and high) obtained in the group-specific real-time PCR assays, were used as templates for the universal diagnosis.

The same mixture was prepared for each group-specific diagnostic assay. Primers were added to iQ™ SYBR Green I Supermix (Bio-Rad) at a final concentration of 300 nM together with 5 μL of the corresponding template, in a final volume of 25 μL. Cycling conditions for FD phytoplasma diagnosis with primers fAY/rEY (Marcone *et al.*, 1996) were adapted from Marzachì *et al.* (2001) and were as follows: one cycle at 95°C for 5 min, then four cycles at 95°C for 15 s and 65°C for 1 min (with a decrease of 0.5°C at each cycle) and finally 35 cycles at 95°C for 15 s and 63°C for 1 min.

Reaction conditions for primers StolFw/Rev were optimised as in conventional PCR and were as follows: one cycle at 95°C for 5 min, then 10 cycles at 95°C for 15 s and 70°C for 1 min (with a decrease of 0.5°C at each cycle) and finally 30 cycles at 95°C for 15 s and 65°C for 1 min.

Reaction conditions for fAP_2/rAP_2 were optimised as in conventional PCR and were as follows: one cycle at 95°C for 5 min, then 45 cycles at 95°C for 15 s and 57°C for 1 min.

All samples were run in triplicate in the same plate. The PCR mix with SDW instead of DNA was used as negative control in each assay. Threshold levels, CTs and standard curves were automatically calculated by the Bio-Rad iCycler software, version 3.06070.

Melting curves were analysed at the end of each group-specific real-time amplification. The PCR products were heated to 95°C for 1 min, cooled at 65°C for 1 min and then slowly heated back to 95°C at a rate of 0.5°C/cycle. Melting temperature (MT) and melting peak (MP) of each PCR product, including standards, were calculated by plotting the negative derivative of the fluorescence over temperature versus the temperature by the Bio-Rad iCycler software (version 3.06070). MTs and MPs of each sample were compared with those produced by amplification of the standards in each specific assay.

Results

Conventional PCR diagnosis

Results of specific detection of FD, BN and AP phytoplasmas from field-collected plants and insect vectors are presented in Table 2. Restriction polymorphism analysis with *Taq*I of 16Sr(V)F1/R1 (Lee *et al.*, 1994) amplicons from FD-phytoplasma-infected grapevines showed that all samples shared the same profile as FD-C (Strain from Piemonte, Italy; data not shown). Restriction polymorphism analysis with *Ssp*I of fO1/rO1 (Lorenz *et al.*, 1995) amplicons from AP-phytoplasma-infected apple trees and *C. melanoneura* showed that all samples shared the same profile as the AP reference isolate (data not shown).

Universal real-time PCR diagnosis

CYS2Fw/Rv primers and TaqMan probe CYS2 (Marzachì & Bosco, 2005) were originally designed to amplify the 16S rRNA gene of CY phytoplasma. All the reagents differed by one mismatch from the sequences of Stol, FD and EY (CYS2Fw), ESFY-G1 and ESFY-G2 isolates, AP and PD (CYS2 probe) and FD and EY (CYS2Rv) (Fig. 1), promising good universal diagnostic properties. Phytoplasma-specific amplification was obtained in real-time PCR with these reagents from EAY, EY, Stol and AP periwinkle-maintained reference isolate total DNAs.

Table 2 Specific detection of FD, BN and AP phytoplasmas by conventional methods in field-collected grapevines, apple trees, and insect vectors *Scaphoideus titanus*, *Hyalesthes obsoletus* and *Cacopsylla melanoneura* (positive samples/tested samples)

Year	FD		BN		AP	
	Vitis vinifera	*S. titanus*	*V. vinifera*	*H. obsoletus*	*Malus x domestica*	*C. melanoneura*
2001	—	—	5/23	—	—	—
2002	—	—	16/17	—	—	—
2003	26/33	17/18	11/31	7/31	5/5	3/6
2004	20/43	—	47/62	—	15/15	13/16
Total	46/73	17/18	79/136	7/31	20/20	16/22

FD, flavescence dorée; BN, bois noir; AP, apple proliferation.

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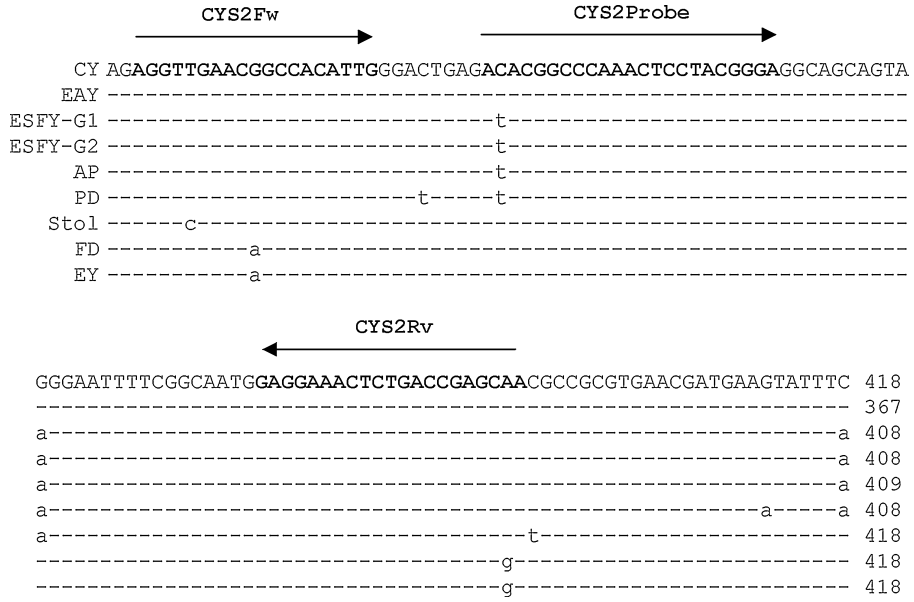

Figure 1 Binding sites of primers CYS2Fw/Rv and CYS2 probe on the nucleotide alignment of partial 16S rRNA gene sequences of CY (AY265214), EAY (AF503568), ESFY-G1 (AJ542544), ESFY-G2 (AJ542545), AP (AF248958), PD (AJ542543), Stol (AF248959), FD (AF176319) and EY (AY197647). Nucleotide changes between sequences are presented. CY, chrysanthemum yellows; EAY, European aster yellows; ESFY, European stone fruit yellows; AP, apple proliferation; PD, pear decline; FD, flavescence dorée; EY, American elm yellows.

Mean CTs were 11.93 ± 0.37 (EAY), 10.43 ± 0.10 (EY), 11.95 ± 0.12 (Stol) and 19.18 ± 0.39 (AP). No amplification was obtained from healthy periwinkles.

The same reagents detected phytoplasma DNA in all test plants (3/3) and test insect vectors (3/3) infected with FD, BN and AP phytoplasmas. Mean CTs of FD-phytoplasma-infected grapevines and *S. titanus* varied from 30.18 to 36.21 and from 20.01 to 31.29, respectively. Mean CTs of BN-phytoplasma-infected grapevines and *H. obsoletus* varied from 30.36 to 33.59 and from 38.99 to 40.50, respectively, while mean CTs of AP-phytoplasma-infected apples and *C. melanoneura* varied from 28.85 to 30.57 and from 22.42 to 37.50, respectively.

Group-specific real-time PCR primers

For stolbur phytoplasma DNA detection, two primers, StolFw and StolRev, flanking a 270-bp region of non-ribosomal DNA (Marzachì *et al.*, 2000) were designed. These primers amplified a fragment of the expected size from total DNA of Stol-phytoplasma-infected periwinkle and pStol224 (Marzachì *et al.*, 2000), and gel electrophoresis confirmed the absence of non-specific products (Fig. 2, panel A). No amplification was obtained from total DNA of phytoplasmas belonging to 16Sr-V and 16Sr-I that have been reported in grapevine in Italy (Boudon-Padieu, 2003) and from total DNA of healthy periwinkle (Fig. 2, panel A).

Mean CTs for different dilutions of pStol224 were 22.18 ± 0.58 (40 fg μL^{-1}), 24.72 ± 0.08 (4 fg μL^{-1}) and 30.82 ± 1.66 (400 ag μL^{-1}) (data not shown). Melting curve analysis of the amplicons obtained from pStol224 confirmed the presence of a single PCR product with a specific MP at 81°C (Fig. 2, panel B).

For AP phytoplasma diagnosis, two primers, fAP_2 and rAP_2, flanking a 200-bp region of the AP phytoplasma nitroreductase gene (Jarausch *et al.*, 2000) were designed. These primers amplified a fragment of the expected size from pAP and total DNA of periwinkles infected with both strains of AP phytoplasma (AP and AT); gel electrophoresis confirmed the absence of non-specific products (Fig. 3, panel A). No amplification was obtained from total DNAs of other phytoplasmas of the same taxonomic group, 16Sr-X (PD and ESFY), or other taxonomic groups (16Sr-V and 16Sr-XII) and from total DNA of healthy periwinkle (Fig. 3, panel A).

Mean CTs for different dilutions of pAP were 11.00 ± 0.39 (400 pg μL^{-1}), 20.22 ± 0.34 (400 fg μL^{-1}) and 28.55 ± 0.37 (4 fg μL^{-1}) (data not shown). Melting curve analysis of the amplicons obtained from pAP confirmed the presence of a single PCR product with a specific MP at 78°C (Fig. 3, panel B).

For FD phytoplasma diagnosis, the group-V-specific primers fAY/rEY (Marcone *et al.*, 1996; Marzachì *et al.*, 2001) were used coupled with SYBR Green I amplicon detection. Mean CTs for different dilutions of pFD81-03 were

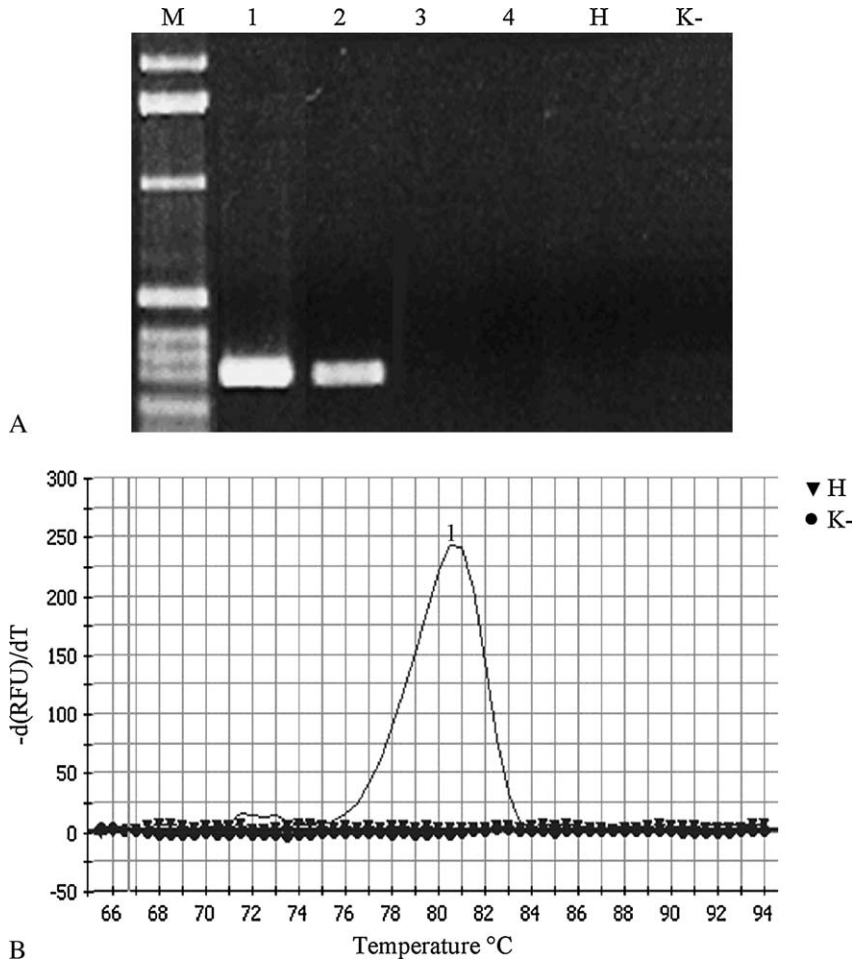

Figure 2 Panel A: gel electrophoresis analysis of amplicons obtained with primers StolFw/Rev from plasmid pStol224 (lane 1) and total DNAs of periwinkle-maintained Stol, EAY and EY reference isolates (lanes 2–4). Panel B: melting peak analysis of amplicon obtained in real-time PCR with primers StolFw/Rev from 2 fg of plasmid pStol224 (curve 1). EAY, European aster yellows; EY, American elm yellows; H, healthy periwinkle; K-, water control; M, 1-kb DNA size marker (Gibco BRL).

8.83 ± 0.12 (400 pg μL^{-1}), 19.77 ± 0.01 (400 fg μL^{-1}) and 27.86 ± 0.24 (4 fg μL^{-1}) (data not shown). Melting curve analysis of the amplicon obtained from pFD81-03 confirmed the presence of a single PCR product with a specific MP at 86°C (Fig. 4).

Group-specific real-time PCR diagnosis

Real-time PCR amplifications of FD, BN and AP phytoplasmas from field-collected plants and insect vectors are presented in Table 3.

Primers fAY/rEY (Marcone *et al.*, 1996; Marzachì *et al.*, 2001) detected the presence of FD phytoplasma in 93.5% of infected grapevines and in 82.3% of infected *S. titanus* adults. Amplification of 14 field-collected grapevines and three *S. titanus* resulted in mean CTs higher than the least concentrated standard (4 fg μL^{-1}). Melting curve analysis of these amplicons showed a single amplicon with the same MP as pFD81-03 for five grapevines and two *S. titanus*, which were then considered as FD phytoplasma infected. The remaining samples produced non-specific amplicons with MPs below 84°C and therefore were not considered infected (Fig. 5, panel A). Gel electrophoresis analysis of the aforementioned PCR products also confirmed these results (Fig. 5, panel B).

Primers StolFw/Rev detected the presence of BN phytoplasma in 92.4% of infected grapevines and in all infected *H. obsoletus* adults (Table 3). Amplification of 13 field-collected grapevines and four *H. obsoletus* resulted in mean CTs higher than the least concentrated standard (400 ag μL^{-1}). Melting curve analysis of these amplicons showed a single PCR product with the same MP as pStol224 for nine grapevines and one *H. obsoletus*, which were then considered as BN phytoplasma infected. The

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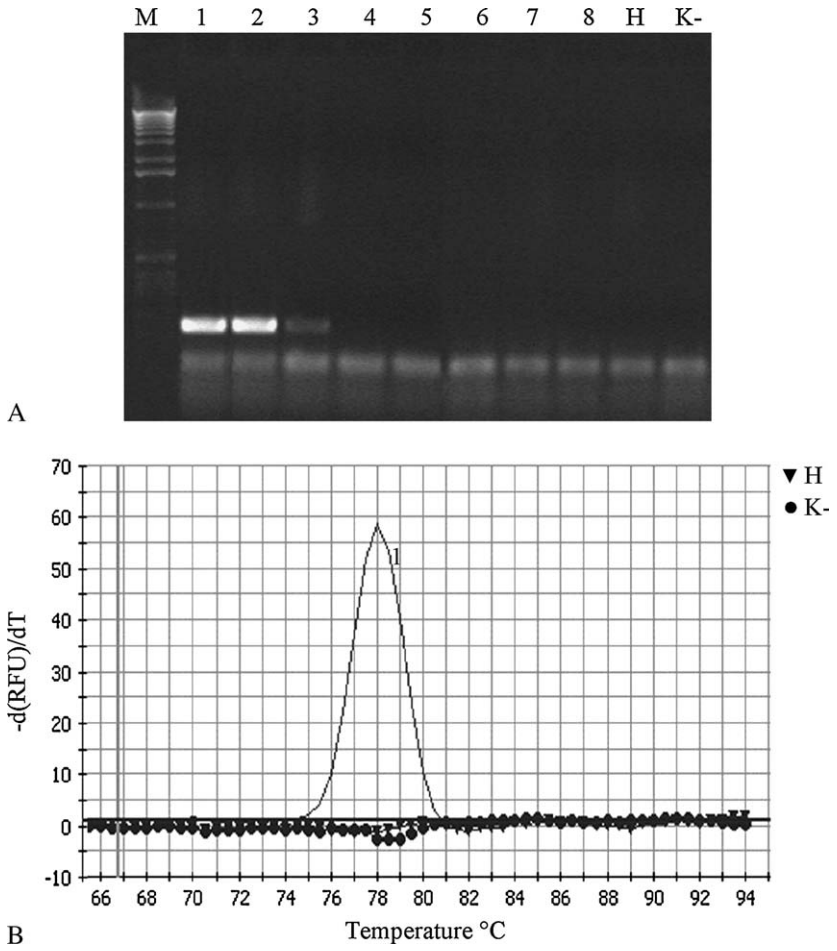

Figure 3 Panel A: gel electrophoresis analysis of amplicons obtained with primers fAP_2/rAP_2 from plasmid pAP (lane 1) and total DNAs of periwinkle-maintained AP, AT, EAY, EY-1, Stol, ESFY, and PD reference isolates (lanes 2–8). Panel B: melting peak analysis of amplicon obtained in real-time PCR with primers fAP_2/rAP_2 from 20 fg of plasmid pAP (curve 1). AP, apple proliferation; EAY, European aster yellows; EY, American elm yellows; ESFY, European stone fruit yellows; AP, apple proliferation; PD, pear decline; FD, flavescence dorée; EY, American; PD, pear decline; H, healthy periwinkle; K-, water control; M, 1-kb DNA size marker (Gibco BRL).

remaining samples produced non-specific amplicons with MPs below 80°C and therefore were not considered infected. Gel electrophoresis analysis of the aforementioned PCR products also confirmed these results (data not shown).

Primers fAP_2/rAP_2 detected the presence of AP phytoplasma in all infected apple trees and *C. melanoneura* samples (Table 3). Amplification of seven field-collected *C. melanoneura* adults resulted in mean CTs higher than the least concentrated standard (4 fg μL^{-1}). Melting curve analysis of these amplicons showed a single PCR product with the same MP as pAP, and these insects were then considered as AP phytoplasma infected. Gel electrophoresis analysis of the aforementioned PCR products also confirmed these results (data not shown).

Discussion

Phytoplasmas are often present in low concentration in infected plants (Lee *et al.*, 2000); therefore, nested PCR is required to obtain reliable diagnostic results. This is time consuming, requires postamplification steps and increases the risk of carry-over contamination. To replace this, we have developed three single-step real-time PCR assays to specifically detect FD, BN and AP phytoplasmas in field-collected host plants and insect vectors and also a single-step real-time PCR assay for universal diagnosis of phytoplasmas, irrespective of taxonomic affiliation.

Different primers have been proposed for the specific detection of FD phytoplasma in field-collected grapevines (Lee *et al.*, 1994; Marcone *et al.*, 1996; Daire *et al.*, 1997;

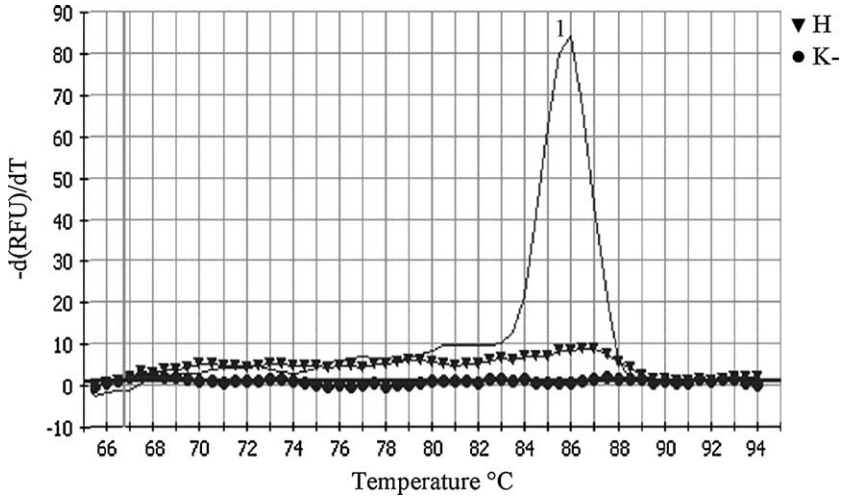

Figure 4 Melting peak analysis of amplicon obtained in real-time PCR with primers fAY/rEY from 20 fg of plasmid pFD81-03 (curve 1). H, healthy periwinkle; K-, water control.

Angelini *et al.*, 2001). The efficiency of the ribosomal-based primers fAY/rEY (Marcone *et al.*, 1996; Marzachì *et al.*, 2001) in detecting group 16Sr-V phytoplasmas in direct conventional PCR (Marzachì *et al.*, 2001) prompted us to test them in real-time assay. We detected FD phytoplasma in 94% of the field-collected infected grapevines, showing the test to be comparable in sensitivity to conventional nested assays. The efficiency of FD phytoplasma detection by real-time PCR was lower on insect vectors (82%). The same primers were more efficient when used in conventional detection of FD phytoplasma in infected *S. titanus* (Marzachì *et al.*, 2001; Bertin *et al.*, 2003). This may be because of the presence of inhibitors in insect DNA extracts on the master mix used for the real-time PCR experiments. Amplification with fAY/rEY does not indicate whether the organism belongs to a distinct subgroup within 16Sr-V (C or D) (Martini *et al.*, 2002), and until now, RFLP analysis of ribosomal PCR products has been the best method to distinguish between subgroups. However, subgroup 16Sr-VA phytoplasmas rarely occur in grapevines (Marzachì *et al.*, 2001), while those associated with FD in Italy belong to subgroups C and D (Angelini *et al.*, 2001). Presence of the restriction site *Bfa*I characterises the ribosomal sequences of these two subgroups (Lee *et al.*, 1998), and it has been recently included in a probe designed to detect FD phytoplasma in a real-time PCR nested assay (Bianco *et al.*, 2004). However, in the face of FD epidemics, subgroup affiliation is a secondary consideration and a simple, direct and reliable method for mass diagnosis is the most useful.

When DNAs from field-collected, BN-phytoplasma-infected plants and *H. obsoletus* hoppers were directly amplified by real-time PCR with primers StolFw/Rev, all the samples yielded an amplicon with the expected melting profile. This is the first report of real-time-based detection of the stolbur phytoplasma.

The efficiency of AP phytoplasma detection was identical to that of conventional nested PCR with group-specific primers in field-collected apple tree samples and *C. melanoneura* adults. Recently, it has been shown that AP phytoplasma can be detected by a quantitative real-time PCR using primers designed on a single-copy gene in all AP-phytoplasma-infected, field-collected *C. costalis* (Jarausch *et al.*, 2004). The same authors showed that phytoplasma titre was variable in different insects, as also confirmed by

Table 3 Group-specific real-time PCR amplification of FD, BN and AP phytoplasmas in field-collected infected grapevines, apple trees, *Scaphoideus titanus*, *Hyalesthes obsoletus* and *Cacopsylla melanoneura* (positive samples/number of samples found positive in conventional diagnostic assays)

Year	FD		BN		AP	
	Vitis vinifera	*S. titanus*	*V. vinifera*	*H. obsoletus*	*Malus x domestica*	*C. melanoneura*
2001	—	—	4/5	—	—	—
2002	—	—	16/16	—	—	—
2003	24/26	14/17	10/11	7/7	5/5	3/3
2004	19/20	—	43/47	—	15/15	13/13
Total	43/46	14/17	73/79	7/7	20/20	16/16

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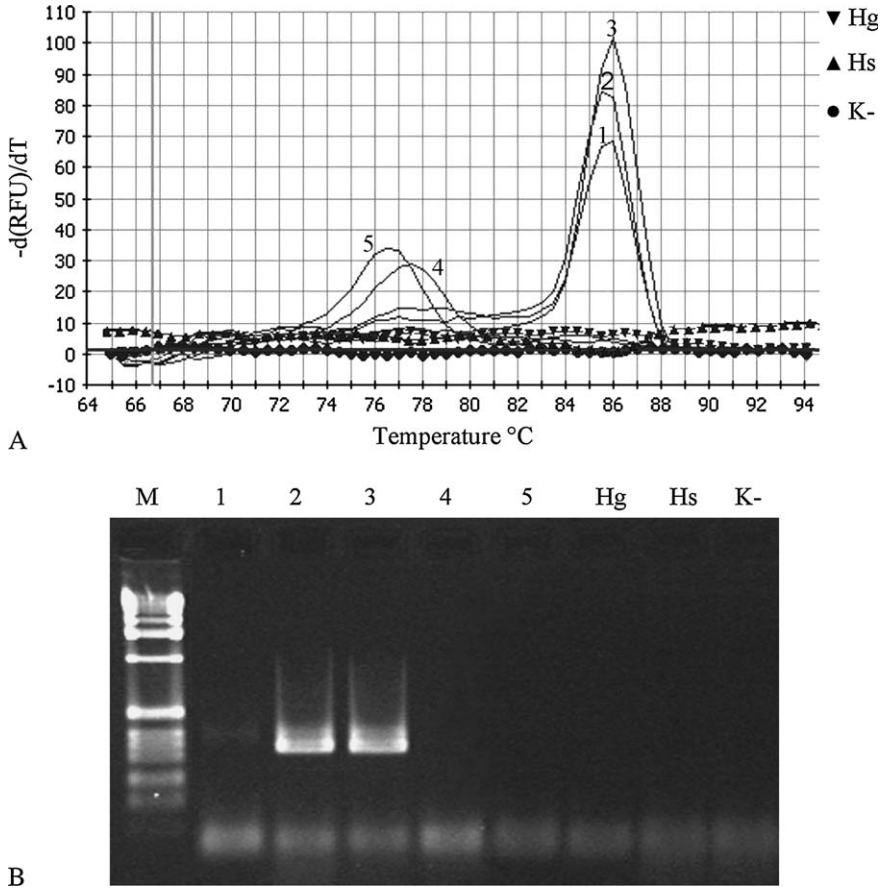

Figure 5 Panel A: melting peak analysis of amplicons obtained in real-time PCR with primers fAY/rEY from 20 fg of plasmid pFD81-03 (curve 1), total DNA of one infected (curve 2) and one negative (curve 4) field-collected grapevines, one infected (curve 3) and one negative (curve 5) field-collected *Scaphoideus titanus*. Panel B: gel electrophoresis analysis of the same amplicons obtained in real-time PCR with primers fAY/rEY. Hg, healthy grapevine; Hs, healthy *S. titanus*; K-, water control; M, 1-kb DNA size marker (Gibco BRL).

us. To distinguish between uninfected apple trees and false-negative results in real-time PCR, the use of an internal positive control based on plant DNA sequences has been suggested (Baric & Dalla-Via, 2004). Our real-time PCR assay detected AP phytoplasma in all apple trees tested without false negatives. Dilution of psyllid samples was required to prevent PCR inhibition due to the high lipid content of their DNA extracts.

A few plant and insect samples infected with FD, BN, and AP phytoplasmas produced CTs higher than the least concentrated corresponding standards. Analysis of the melting curves allowed us to identify the specific amplicons. A low level of infection in these samples can explain our results because they also provided conventional amplification signals only in nested assays. Analysis of the melting curves showed that specific MPs were obtained for two FD-phytoplasma-infected *S. titanus*, although they did not produce any CT in the real-time PCR. We concluded that these insects were also carrying FD phytoplasma but at a level below our detection threshold. A low phytoplasma titre in vector insects is common among field-collected hoppers because they may have been collected only a few days after feeding on infected plants, when the phytoplasmas have had little time to multiply (Palermo *et al.*, 2001). Thus, melting curve analysis is a prerequisite for correct identification of amplicons generated in different real-time PCR detection systems because phytoplasmas are usually present in low concentrations in woody hosts, while their titre is more variable in the insect vectors (Jarausch *et al.*, 2004). Low levels of target template result in high CTs corresponding to late phases of amplification; under these conditions, amplification of non-specific DNA can also occur, resulting in high CTs. This specially occurs in field-collected woody plant or vector samples, where bacterial contamination is common (Skrzeczkowski *et al.*, 2001). For these reasons, melting curve analysis or gel electrophoresis is necessary to avoid false positives.

Melting curve analysis is a rapid, easily programmable final step of the real-time PCR and does not require any post-PCR manipulation.

When DNAs from field-collected plants and vectors infected with FD, BN or AP phytoplasma were directly amplified by real-time PCR using primers and a TaqMan probe designed on a highly conserved ribosomal sequence, all yielded a phytoplasma-specific amplicon. Comparison of CTs obtained with plant and insect samples in universal and in the corresponding group-specific assays showed that the universal assay was less sensitive. The presence of one mismatch in the primer or probe sequences from phytoplasmas included in this study may explain this reduction in efficiency, although the reagents used for the universal assay were designed on the phytoplasma 16S rDNA sequence (Marzachì & Bosco, 2005), which is present in double copy in the phytoplasma genome (Schneider & Seemüller, 1994). Bianco *et al.* (2004) detected phytoplasmas belonging to different subgroups in the 16Sr-V group, in the presence of one mismatch in the TaqMan probe sequence, by decreasing the annealing temperature. Nevertheless, our universal real-time PCR assay detected phytoplasmas belonging to three different taxonomic groups even when the pathogen was present in very low concentrations.

High sensitivity, easy handling, absence of postamplification manipulations and a potential for high throughput make real-time PCR a promising tool for the study of relationships among pathogens and their hosts. Moreover, phytoplasma diagnosis in a one-step assay coupled with real-time detection avoids the risk of contamination and is less time consuming than conventional methods. These new protocols can be applied for mass diagnostic screenings, e.g. on propagation material, and for identification of new host plants and putative new insect vectors.

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