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SHORT COMMUNICATION

DETECTION OF PHYTOPLASMAS ASSOCIATED WITH GRAPEVINE FLAVESCENCE DORÉE DISEASE USING REAL-TIME PCR

P.A. Bianco[1], P. Casati[1] and N. Marziliano[2]

[1] *Istituto di Patologia Vegetale, Università degli Studi di Milano, Italy*
[2] *SDS/GA/SPS Core Lab, Applera Italia, Monza, Italy*

SUMMARY

Flavescence dorée (FD) of grapevine is a serious disease caused by phytoplasmas. Currently available protocols for phytoplasma detection in grapevine are complex and time consuming. This work reports the results of a real-time PCR assay (TaqMan®) for detecting phytoplasmas in grapevines affected by FD. We obtained specific detection in samples from seven FD-affected grapevines. This method gave no signal with phytoplasmas associated with another grapevine disease (Bois noir) or with eleven reference phytoplasma strains grown in periwinkle. We propose Real-time PCR as an alternative and rapid method for the detection of phytoplasmas in grapevine.

Key words: grapevine, flavescence dorée, phytoplasma detection, real-time PCR

Flavescence dorée (FD) is considered one of the most severe threats to viticulture in all areas where it has been reported (Boudon-Padieu, 2003). It is caused by phytoplasmas transmitted by the leafhopper *Scaphoideus titanus* Ball. Crop losses and environmental impact are considerable since control is based on elimination of the vector with insecticides during the growing season. In addition, to limit the spread of the disease, the use of healthy planting material (rootstocks, cuttings and grafted vines) is necessary. One of the main constraints in FD prevention is the lack of rapid and sensitive tools for phytoplasma detection and identification. Until now, the protocols in use for phytoplasma detection in grapevine have been based on nested PCR using primers specific for phytoplasmal 16SrDNA ribosomal sequences (Ahrens and Seemüller, 1992; Bianco *et al.*, 1993; Daire *et al.*, 1993; Prince *et al.*, 1993) or non-ribosomal genes (FD9) (Daire *et al.*, 1997; Angelini *et al.*, 2001; Clair *et al.*, 2003). RFLP analysis of PCR products using different restriction enzymes, is then required in order to distinguish phytoplasmas belonging to subgroup 16SrV-A, which rarely occurs in grapevines (Bianco *et al.*, 1996), from those associated with FD (Daire *et al.*, 1997) which are assigned to subgroup 16SrV-C and 16SrV-D (Martini *et al.*, 1999; Angelini *et al.*, 2001). Other phytoplasmas that infect grapevine and cause symptoms identical to FD (Boudon Padieu, 2003), are those causing Bois noir (BN), which belong to subgroup 16SrXII-A (Daire *et al.*, 1993; Maixner *et al.*, 1995a; Daire *et al.*, 1997). Thus the situation is complex and required until now methods that are time consuming and include use of toxic reagents.

The present work aimed to develop a specific and rapid method for detecting phytoplasmas involved in FD aetiology. A specific 5' Nuclease Assay® (TaqMan® assay) was developed on the basis of the 16Sr DNA sequence of the phytoplasmas so far detected in grapevine.

DNA samples were extracted from the leaves of eight different grapevine plants showing typical symptoms of FD, collected in two different areas of Northern Italy (Oltrepó pavese for cvs Chardonnay and Pinot bianco, and Garda bresciano for cv Sangiovese). The phytoplasma references used were severe western aster yellows (SAY, 16SrI-B), Clover phyllody (KVG, 16SrI-C), tomato big bud (TBB, 16SrII), green valley X disease (GVX, 16SrIII), elm yellows (EY1, 16SrV-A), elm witches' broom (ULW, 16SrV-A), beet leafhopper transmitted virescence (BLTVA, 16SrVI), ash yellows (ASHY, 16SrVII), peach yellow leaf roll (PYRL, 16SrX), apple proliferation (AT, 16SrX-A), and grapevine yellows (GY, 16SrXII-A). All were kindly provided by Dr. E. Seemüller. DNA extracted from healthy grapevine seedlings was used as control.

Total nucleic acids (TNA) were extracted from 100 mg of petioles and veins of grapevine or periwinkle leaves, using Plant Dneasy Mini Kit (Qiagen®) according to the manufacturer's instructions. One microlitre of ten-times-diluted total DNA from each sample was added to a standard PCR reaction mixture as reported by Schaff *et al.* (1992). Two different primer sets, 16SrF2n/16SrR2 (abbreviated F2/R2) (Lee *et al.*, 1993) or P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*,

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Corresponding author: P.A. Bianco
Fax: +39.02.50316781
E-mail: piero.bianco@unimi.it

1995), designed for amplification of the 16Sr DNA from all known phytoplasmas, were used for the first round of amplification. The amplicons were subjected to nested PCR using primers R16(I)F1/R1 designed for the specific amplification of rDNA from aster yellows and stolbur group (Davis *et al.*, 1997), or primers R16(V)F1/R1 designed for the specific amplification of the elm yellows group (Lee *et al.*, 1994). The primers $16R_{758f}/V_{1731r}$ were used to determine subgroup affiliation in the 16SrV group (Martini *et al.*, 1999). Additional PCR assays were conducted using non-ribosomal primers FD9f2/FD9r followed by nested PCR with primers FD9f3/FD9r2 (Angelini *et al.*, 2001).

Three to 5 µl of each DNA product from nested PCR of the grapevine samples were singly digested with restriction endonucleases *Alu*I, *Mse*I, *Bfa*I and *Taq*I. The RFLP profiles were resolved by electrophoresis through 5% polyacrylamide gel and staining with ethidium bromide.

In order to develop a real-time PCR assay for simultaneous detection of phytoplasmas belonging to the 16SrV-C and 16SrV-D subgroups, specific primers were designed through computer analysis using the software Primer Express (Applied Biosystems, Foster City, CA, USA). The primers are shown in Fig. 1. The expected amplified fragment of 89 bp is located between positions 1024 (primer forward, F1024) and 1112 (primer reverse, F1112) on the phytoplasma sequences deposited in GenBank (accession No. X76560 and AF176319). The labeled internal 23-bp probe (iProbe) used in this study was complementary to positions 1057-1079. This fragment contains the *Bfa*I cleavage site that is present in both the 16SrV-C and 16SrV-D subgroups. In the same fragment a single base mutation (T instead of C) occurs at position 1068 of EY1 and ULW reference strains thus disrupting the *Bfa*I restriction site (Fig. 1). The presence of this restriction site has been suggested as a distinctive marker for the identification of phytoplasmas related to the FD agents (Bianco *et al.,* 1996; Lee *et al.*, 1998) and was recently adopted for identification of phytoplasmas associated with FD in Italy (Pasquini *et al.*, 2001).

Real-time PCR was performed on a MicroAmp optical 96-well plate (capped with MicroAmp optical caps) using the automated ABI Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA, USA).

Table 1. Results of PCR and RFLP tests. The second column shows the results RFLP analysis. The remaining columns report the results of the real-time PCR tests done with 16SrF2/R2 amplicons. The values are those of the threshold cycle (that is the amplification cycle giving a signal 10 standard deviations above the background noise).

Sample or strain	Phytoplasma detected	Ct values at annealing temperatures		
		60°C	62°C	64°C
NTC[1]	-	-	-	-
NAC[2]	-	-	-	-
HG[3]	-	-	-	-
SAY	16SrI-B	-	-	-
KVG	16SrI-C	-	-	-
TBB	16SrII	-	-	-
GVX	16SrIII	-	-	-
EY1	16SrV-A	28.76	42.67	-
ULW	16SrV-A	29.45	45.67	-
BLTVA	16SrVI	-	-	-
ASHY	16SrVII	28.87	47.75	-
PYRL	16SrX	-	-	-
AT	16SrX-A	-	-	-
GY	16SrXII-A	-	-	-
n. 63 (Chardonnay)	16SrV-C	12.00	nt[4]	nt
n. 69 (Chardonnay)	16SrXII-A	-	nt	nt
n. 82 (Chardonnay)	16SrV-C	17.97	28.45	34.15
n. 83 (Pinot blanc)	16SrV-C	14.97	26.41	33.87
n. 84 (Pinot blanc)	16SrV-C	15.08	25.15	32.65
n. 262 (Sangiovese)	16SrV-D	13.89	25.00	32.43
n. 264 (Sangiovese)	16SrV-D	10.82	25.82	31.32
n. 265 (Sangiovese)	16SrV-D	10.06	32.31	34.21

[1] No-template control
[2] No-amplification control
[3] Healthy grapevine
[4] not tested

Fifty microlitres of reaction volume were loaded in each well containing 5 µl of DNA template, 1xTaqMan® Universal Master Mix (Applied Biosystems, Foster City, CA, USA), 200 nM of FAM/TAMRA labeled probe (iProbe), and 900 nM of each primer (F1024 and R1112). F2/R2 amplicons were used as template for both grapevine and periwinkle strains.

The PCR consisted of a preliminary cycle at 50°C for 2

1024 1068 1112
X76560 (16SrV-D) gtgagatgttaggttaagtcctaaaacgaacgc*aacccctgtcg***c***tagttgccagc*acgtaatggtggggactttagcgagactgccaa
AF176319 (16SrV-C) gtgagatgttaggttaagtcctaaaacgaacgc*aacccctgtcg***c***tagttgcca*gcacgtaatggtggggactttagcgagactgccaa
AF189214 (16SrV-A) gtgagatgttaggttaagtcctaaaacgaacgc*aacccctgtcg***t***tagttgcca*gcacgtaatggtggggactttagcgagactgccaa

Fig. 1. Alignment of the DNA sequence fragments obtained from three different subgroups by amplification with primers F1024/R1112 (underlined sequences). The sequence of the iProbe used for the real-time visualization is in italics. The nucleotide marked in bold is the mutation that forms the *Bfa*I restriction site.

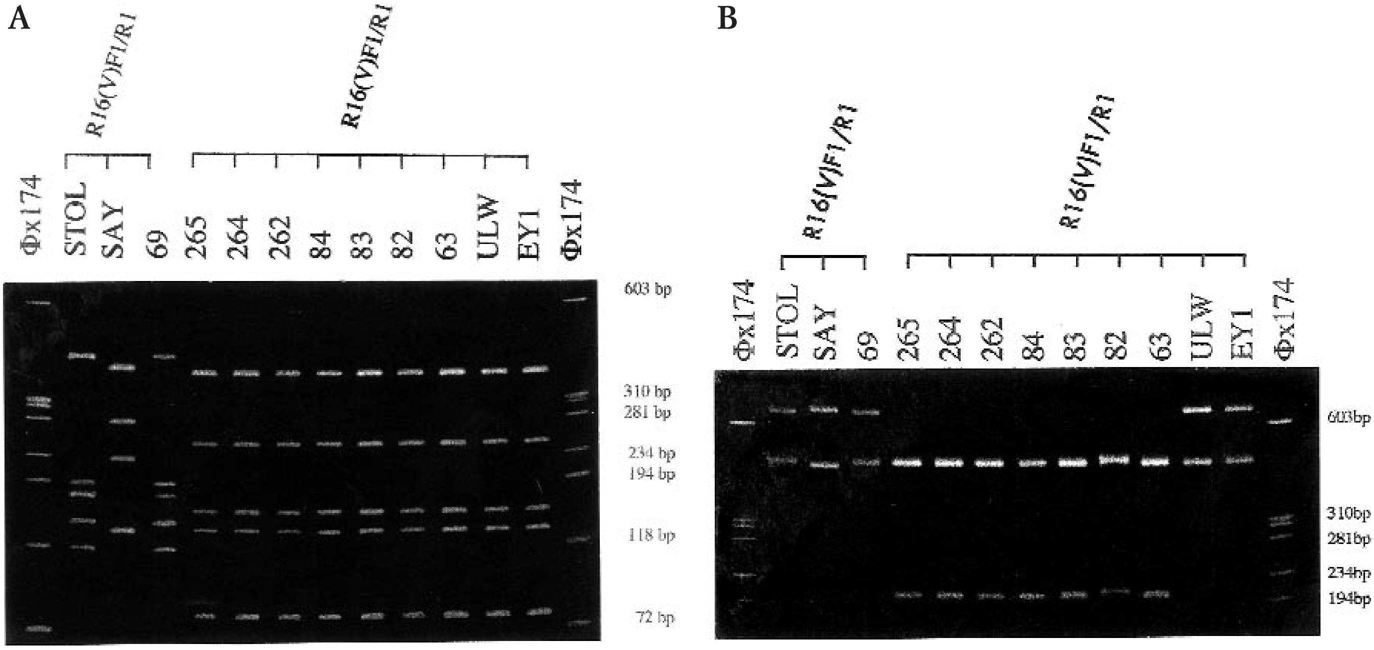

Fig. 2. Results of RFLP analysis of the amplicons obtained by PCR from grapevine samples and phytoplasma strains EY1 and ULW (16SrV-A), SAY (16SrI-B) and STOL (16SrXII-A). Restriction enzymes were *Mse*I (**A**) and *Bfa*I (**B**). The molecular weight marker is Fx174 RFI *Hae*III digested.

min and 95°C for 10 min followed by fifty cycles of 95°C for 15 seconds and 60°C or 62°C or 64°C, for 1 min. The fluorescent light emission of each well was recorded in real-time by generating overlapping spectra in the wavelength range between 500 nm and 660 nm and the data were automatically analyzed by the ABI Prism® 7700 Sequence Detection System software (version 1.9). Two PCR controls were included: (a) no template controls (NTC) with no DNA added to the reaction mix and (b) no amplification control (NAC) with the addition of 1 µl SDS (0.5% w/v) to the reaction mix in order to denature the AmpliTaq Gold® DNA polymerase.

The results of RFLP analysis of grapevine samples are shown in Figs. 2 and 3, and summarized in Table 1. Grapevine sample 69 showed a profile consistent with the presence of phytoplasmas of the 16SrXII-A subgroup (Fig. 2A), to which the agent of BN is assigned. The remaining grapevine samples (63, 82, 83, 84 262, 264 and 265) all proved to be infected by phytoplasmas belonging to the 16SrV group (Fig. 2A). Fig. 3 shows that phytoplasmas present in samples 262, 264 and 265 belong to subgroup 16SrV-D, while samples 63, 82, 83 and 84 belong to subgroup 16SrV-C. Moreover RFLP profiles obtained with the restriction enzyme *Bfa*I showed the phytoplasma reference strains EY1 and ULW (16SrV-A) can be distinguished from the phytoplasmas detected in grapevine samples (Fig. 2B).

These data were used as starting point to verify the applicability of real-time PCR for specific and simultaneous identification of phytoplasmas involved in FD (16SrV-C and 16SrV-D). The results of the real-time PCR experiments using F2/R2 amplicons as templates are shown in Table 1. None of the samples used as negative controls yielded any detectable signal in any of the three experiments carried out at different annealing temperatures. No increase in fluorescence was obtained with DNA samples from phytoplasma reference strains belonging to groups or subgroups 16SrI-B (SAY), 16SrI-C (KVG), 16SrII (TBB), 16SrIII (GVX), 16SrVI (BLTVA), 16SrX (PYRL), 16SrX-A (AT), and 16SrXII-A (GY). Fluorescence was detected between cycle thresholds (Ct) 28.76 and 29.45 with templates from subgroups 16SrV-A (EY1 and ULW) and 16SrVII-A (ASHY) when primer annealing was done at 60°C. At 62°C the fluorescence increase was recorded very close to the last amplification cycle (between Ct 42.67 and 47.75) and was probably not significant. No fluorescence increase was observed with these samples when annealing was done at 64°C.

With DNA from infected grapevines no detectable signals were obtained from sample 69, which was infected by the BN agent (16SrXII-A subgroup). By contrast, the remaining grapevine samples 63, 82, 83, 84, 262, 264 and 265, all belonging to the 16SrV group, responded positively. These samples were infected by FD-related phytoplasmas and showed Ct values between 25 and 34.21 at annealing temperature of 62°C or 64°C. We also correlated the Ct values with the end-point fluorescence values (Rn) (Fig. 4A). The Rn values are an indicator of the overall robustness of amplification. Fig. 4A shows the scatter plots for EY1, ULW and ASHY

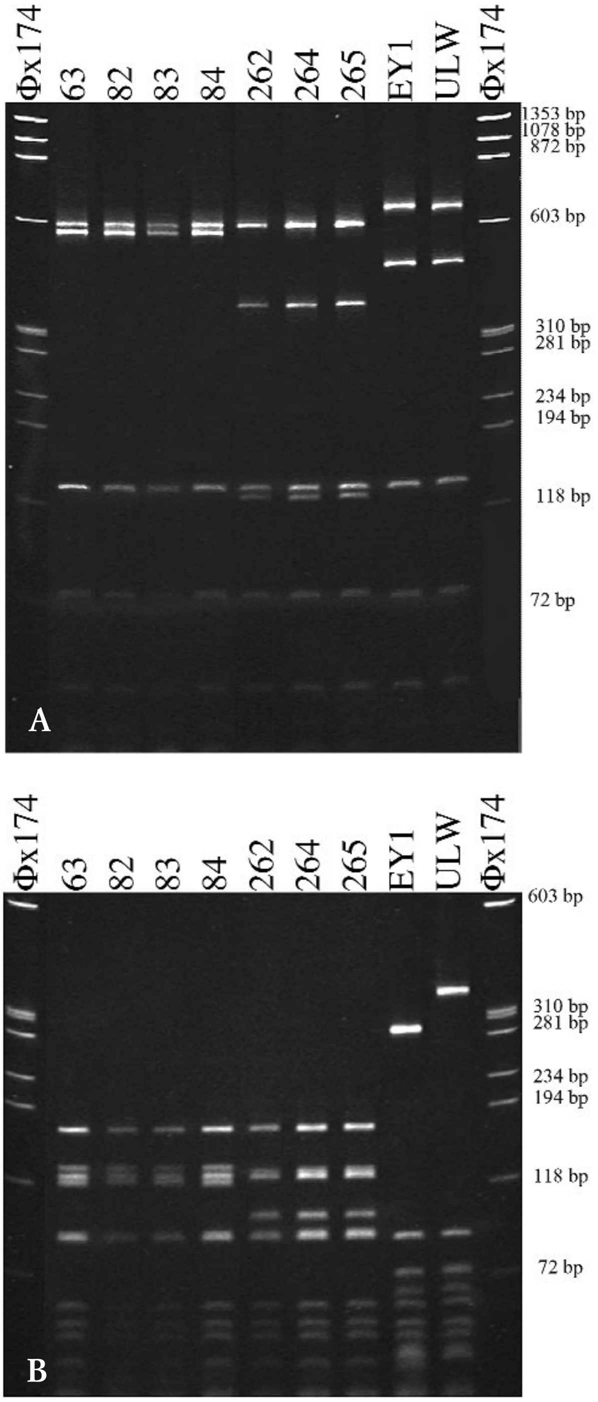

Fig. 3. Results of RFLP analysis of the FD9f3/fd9r2 amplicons obtained by PCR from grapevine isolates and phytoplasma strains EY1 and ULW (16SrV-A). Restriction enzymes were *Alu*I (**A**) and *Mse*I (**B**).

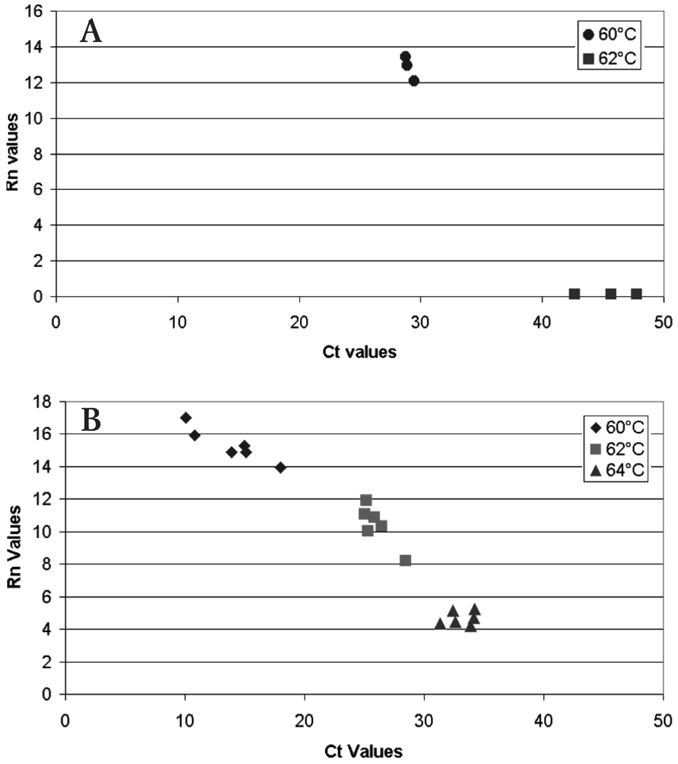

Fig. 4. Correlation between the Ct (cycle threshold) values and Rn (end-point of fluorescence) values of phytoplasma isolates EY1, ULW, and ASHY (A) and FD-associated phytoplasma isolates (B). These data refer to the Ct values of the samples listed in Tab. 1 to which the related Rn values were added in the scatter plot.

indicating decreasing Ct values due to the increased annealing temperature that correlates with the loss of amplification signals. Decreasing Ct values at higher annealing temperatures were also observed with FD-associated phytoplasmas (Fig. 4B). As expected, the higher the annealing temperature, the greater the loss of fluorescence: the Rn vs Ct correlation values were, at 60°C, $y=-0.3321x + 19.898$ $R^2 = 0.8877$, at 62°C $y = -0.8539x + 32.638$ $R^2 = 0.7792$ and at 64°C $y =-0.00985x + 14.1$ $R^2 = 0.752$.

Use of this real-time PCR protocol, with the F1024-R1112 primers and the TaqMan® probe described, allowed selective detection of the 16SrV-C and 16SrV-D phytoplasmas associated with FD, thus providing a reliable alternative to PCR-RFLP tests. Further experiments will be necessary to develop a suitable method for subgroup determination in order to distinguish between subgroups 16SrV-C and 16SrV-D.

There are reports of a further phytoplasma in grapevine, i.e. ALY associated with alder yellows (Maixner *et al.*, 1995a; Maixner *et al.*, 1995b; Maixner *et al.*, 2000, Angelini *et al.*, 2001). In real-time PCR this phytoplasma reacts identically to the group 16SrV phytoplasmas infecting grapevines. However, ALY seems to be rare in grapevine and further studies are needed to understand its role in grapevine yellows etiology.

In conclusion, the real-time PCR protocol reported here should be considered as a useful tool for fast and reliable diagnosis of FD. The protocol is rapid because it does not require DNA visualisation by gel electrophoresis. Also it does not use toxic chemicals like ethidium bromide and polyacrylamide. This protocol could thus facilitate the introduction of molecular methods in laboratories devoted to plant health certification and plant quarantine services.

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