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Optimisation of a one-step PCR assay for the diagnosis of Flavescence dorée-related phytoplasmas in field-grown grapevines and vector populations

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Summary

Field-infected grapevines and natural populations of *Scaphoideus titanus* have been analysed to detect group V phytoplasmas associated with flavescence dorée in north-western Italy using nested PCR. A first amplification driven by universal ribosomal primers R16SF2/R2 was followed by a second round assisted by R16(V)F1/R1 primers and subsequent RFLP analysis. To optimize the test, nested PCRs were compared with direct amplification assisted by the group V-specific fAY/rEY primer pair, directed towards other ribosomal sequences. In nested and direct PCRs, respectively, DNAs from 71 and 57 out of 96 grapevines (*i.e.* 73.9 and 59.3 %) and 51 and 50 out of 108 insects (*i.e.* 47.2 and 46.3 %) reacted positively. Although it was not possible to determine the subgroup of the phytoplasmas after fAY/rEY amplification, these primers could be used successfully in mass screening of plant material and insect populations. They could detect, in single-step amplification, the phytoplasmas in 80 and 98 % of the plant and insect samples, respectively, that were already indexed as positive using nested PCR. This strongly reduced the number of samples requiring the nested approach, with beneficial effects on costs, labour and risks of the analysis.

K e y w o r d s : grapevine, flavescence dorée, *Scaphoideus titanus*, fAY/rEY primer pair, direct PCR, mass diagnosis.

Introduction

Phytoplasma-induced grapevine yellows diseases (GYs) can be etiologically different but symptomatically similar. Plants may exhibit, singly or in various combinations, interveinal yellowing or reddening, pronounced or mild leaf rolling, thickening and hardening of leaf margins, vein necrosis, black pustules along the shoots, incomplete or retarded shoot lignification, necrosis of the inflorescence apex, and bunch withering. Phytoplasmas of diverse 16SrDNA RFLP subgroup affiliations (*sensu* Lee *et al.* 1998), have been associated, in single or mixed infection, with GY-affected grapevines in Australia, North America and Europe, where bois noir and flavescence dorée are the reported diseases (Daire *et al.* 1993, 1997 a, b*;* Davis *et al.* 1993 a, b, 1997, 1998; Prince *et al.* 1993; Bertaccini *et al.* 1995; Maixner *et al.* 1995 a, b; Padovan *et al.* 1995, 1996; Albanese *et al.* 1996; Alma *et al.* 1996; Bianco *et al.* 1996 a, b; Marcone *et al.* 1996 a; Batlle *et al.* 1997, 2000; Kölber *et al.* 1997; Gibb *et al.* 1999; Martini *et al.* 1999; Marzachì *et al.* 2000, 2001; Seruga *et al.* 2000).

Bois noir (BN) in France (Daire *et al.* 1993, 1997 a, b) and the equivalent disorders in Germany (Vergilbungskrankheit (VK); Maixner *et al.* 1995 a), Hungary (Kölber *et al.* 1997), Italy (legno nero) and the whole Mediterranean Basin (Davis *et al.* 1993 a, b, 1997; Albanese *et al.* 1996; Marcone *et al.* 1996 a; Daire *et al.* 1997 a, b; Batlle *et al.* 2000; Marzachì *et al.* 2000; Seruga *et al.* 2000) are consistently associated with XII-A subgroup members (stolbur). VK and BN phytoplasmas are transmitted to grapevine in nature by the cixiid planthopper *Hyalesthes obsoletus* Signoret (Maixner 1994; Sforza *et al.* 1998). However, the disease appears to be present and spreading in areas where this vector has not been found. The phytoplasma has been detected in hoppers different from the above, suggesting that other vectors may well exist (Batlle *et al.* 2000).

Flavescence dorée (FD) was originally described in southern France, and is now also reported from northern Spain and Italy (Bertaccini *et al.* 1995; Bianco *et al.* 1996 a, b; Batlle *et al.* 1997, 2000; Marzachì *et al.* 2001). It is caused by phytoplasmas of subgroups V-C and –D (Martini *et al.* 1999). The natural vector is the monophagous grapevine-limited leafhopper *Scaphoideus titanus* (=*littoralis*) Ball (Caudwell *et al.* 1970). A GY present in the Pfalz area, Palatinate GY (PGY; Maixner *et al.* 1995 b), caused by a subgroup V-C phytoplasma, appears to differ from FD *sensu stricto* in both RFLP pattern of a non-ribosomal DNA fragment (Daire *et al.* 1997 b) and specific vector, being transmitted by *Oncopsis alni* Schrank, like alder yellows phytoplasma (Maixner *et al.* 2000).

FD represents perhaps the biggest threat to viticulture and is one of the most treated diseases for quarantine in Europe, therefore quick and reliable diagnostic methods are needed. Although primers for amplifying non-ribosomal FD DNA in direct PCRs have been published (Daire *et al.* 1997 b), they have not been widely used, possibly because of lack of sensitivity and specificity (Clair *et al.* 2000). Consequently, FD diagnosis still largely relies on nested or serially nested amplifications of ribosomal or chromosomal DNA driven by various combinations of primer pairs (Bertaccini *et al.* 1995; Bianco *et al.* 1996 b; Batlle *et al.* 1997, 2000; Daire *et al.* 1997 a; Martini *et al.* 1999; Clair *et al.* 2000; Marzachì *et al.* 2001). Recently, a group V-specific primer pair, directed to 16S rDNA sequences (fAY/rEY; Marcone *et al.* 1996 b, c), has been used to detect PGY in direct PCRs in plants and insect vectors (Maixner and Reinert 1999;

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MAIXNER *et al.* 2000). In this paper we report the optimisation of PCR conditions for the fAY/rEY primer pair in order to render them amenable to one-step PCR using as templates DNA from field-grown grapevines and natural vector populations. A more rapid and simpler method, equal in sensitivity but reducing manipulations and costs associated with nested PCR, would be of obvious advantage in FD mass screening.

Material and Methods

Plant and insect samples, reference phytoplasmas and DNA isolation: Leaf samples were collected from healthy-looking and 96 GY-symptomatic grapevines, cvs Arneis, Barbera, Bonarda, Brachetto, Chardonnay, Chenin, Cortese, Croatina, Dolcetto, Erbaluce, Favorita, Freisa, Grignolino, Malvasia, Moscato Bianco, Nebbiolo, Pinot nero and Ruchè from several commercial vineyards in southeastern Piemonte, northwestern Italy, during May-October 2000 (MARZACHÌ *et al.* 2001). Adults of *Anoplotettix fuscovenosus* (Ferrari), *Euscelis incisus* Kirschbaum, *Euscelidius variegatus* Kirschbaum, *H. obsoletus*, *Philaenus spumarius* (L.), *Psammotettix alienus* (Dahlbom) and *S. titanus* were sweep-captured alive in some of the most severely affected vineyards, frozen immediately and stored at -20 °C, either as such or in acetone, till use.

Isolates (taxonomic assignments according to LEE *et al.* 1998) of European aster yellows, American elm yellows, and Serbian stolbur from pepper (EAY, subgroup I-B; EY-1, V-A and Stol; XII-A, respectively) were propagated and maintained in periwinkle (*Catharanthus roseus* (L.) G. Don). They had been originally supplied by Drs. M.F. CLARK (Horticulture Research International, East Malling, UK – group XII) and E. SEEMÜLLER (Biologische Bundesanstalt, Institut für Pflanzenschutz im Obstbau, Dossenheim, Germany, group V). DNAs from jujube (*Zizyphus jujuba* Miller) and wild blackberry (*Rubus fruticosus* L.), naturally infected by jujube witches'-broom (JWB; V-B) and rubus stunt (RS; V-C) phytoplasmas (PASQUINI *et al.* 2000) were also used for comparison. Samples from seedlings grown in the glasshouse were used as negative controls.

DNAs were extracted either from individual insects by the method of DOYLE and DOYLE (1990) as adapted by MARZACHÌ *et al.* (1998), or as detailed by MARZACHÌ *et al.* (1999) from 1.5 g midribs if possible or, with severely affected grapevines, blackberry and jujube samples, from whole reduced leaves. Final products were suspended in 100 µl sterile double distilled water and aliquots (typically 2 µl) used in PCR experiments.

Primers, PCR amplification and RFLP analysis: The universal primer pair R16F2/R2 (LEE *et al.* 1993; yielding a product of 1225 bp) was used in direct PCR assays. Irrespective of results, reaction products were diluted 1:40 and used as templates in nested reactions driven by primer pair R16(V)F1/R1 (LEE *et al.* 1994; 1100 bp). Samples not responding positively to this primer pair were subjected to nested amplification with primer pair R16(I)F1/R1 (LEE *et al.* 1994) and corresponding undiluted DNAs were also directly amplified with Stol-specific primers M1/P8 (MARZACHÌ *et al.* 2000; 2001). Reactions and cycling conditions are detailed in the original papers.

With primer pair fAY/rEY (~300 bp; MARCONE *et al.* 1996 b), initial denaturation for 4 min at 95 °C was followed by 4 cycles at 95 °C for 30 s, 65 °C (with a decrease of 0.5 °C at each cycle) for 60 s and 72 °C for 60 s, then by 31 cycles at 95 °C for 30 s, 63 for 1 min and 72 °C for 1 min (5 min in the last cycle). In addition to DNA (1:50 of the total extract), 50 µl reaction mixture contained 0.125 mM of the 4 deoxynucleotides, 2 mM $MgCl_2$, 0.50 µM of each primer and 1 unit of *Taq* DNA polymerase (Polymed, Sambuca, Firenze, Italy).

Reaction products were analysed in 1 or 1.5 % agarose gels buffered in 0.5 x TBE (SAMBROOK and RUSSEL 2001) along with 1 kb DNA size marker (BRL-Life Technologies, Gaithersburg, MD, USA) and visualised by UV-light after staining with EtBr.

Nested-amplified fragments were digested according to the producer's recommendations, for 16 h with 10 units of the restriction enzyme *Bfa*I (New England Biolabs, Beverly, MA, USA). Products were resolved in 5 % polyacrylamide gels buffered in 1 x TBE along with PCR 100 bp Low Ladder size marker (Sigma Chemical Co., St Louis, MO, USA) and visualised as above.

Cloning and sequencing of EY–1 DNA fAY/rEY amplicon: DNA from EY-1 was amplified as above and amplicons purified using the Geneclean kit (Bio 101, Carlsbad, CA, USA) following the producer's instructions. After repair and phosphorylation reactions, the purified band was inserted at the *Sma*I site in the polylinker region of pBluescript SK (+) phagemid vector (Stratagene, La Jolla, CA, USA). The complete nucleotide sequence of the cloned pFAYREY-1 was determined directly on the intact plasmid in both directions in a Beckman-Cheq 2000 DNA analysis system using fAY and rEY primers and dichlorhodamine dye terminators (Bioindustry Park, Colleretto Giacosa, Torino, Italy), assembled and computer analyzed (PC Gene, Intelligenetics Inc., Mountain View, CA, USA).

Results

DNA amplification and characterisation: Irrespective of the primer pair used, target DNAs were never amplified from (i) asymptomatic plants collected in the field, (ii) healthy greenhouse-grown seedlings, (iii) healthy leafhoppers reared under controlled conditions or (iv) field-collected leafhoppers different from *S. titanus*, not known as FD vectors (not shown).

PCR results obtained with DNAs from symptomatic grapevine samples and field-collected *S. titanus* are summarised in the Table. In nested PCR assays, DNAs from 71/96 grapevines and 51/108 *S. titanus* (74.0 and 47.2 %, respectively) yielded amplified fragments of the correct size. RFLP analysis showed that *Bfa*I-digested amplicons appeared identical to that of RS, indicating that phytoplasmas of the same subgroup as FD were carried in both plants and insects (Fig. 1).

Target DNAs from plants (57/96; 59.4 %) and insects (50/108; 46.3 %) were amplified in direct PCRs driven by

T a b l e

Amplified/tested phytoplasma DNAs from symptomatic field-grown grapevines and *S. titanus* in group V-specific nested and fAY/rEY single step PCRs

Primers	Grapevines	*S. titanus*
Nested R16(V)F1/R1	71/96	51/108
FAY/rEY	57/96	50/108

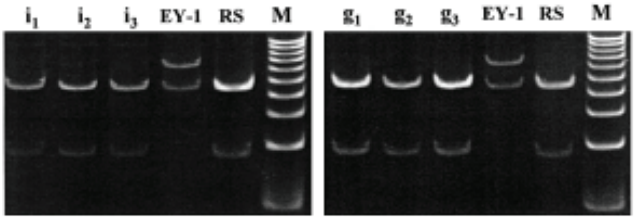

Fig. 1: *Bfa*I-restricted R16(V)F1/R1 amplicons obtained from representative *S. titanus* (i) and symptomatic grapevines (g). EY-1 and RS are phytoplasma reference strains. M: PCR 100 bp Low Ladder (Sigma Chemical Co.).

primer pair fAY/rEY, although using the cycling conditions of the original papers (Marcone *et al.* 1996 b, c) DNAs from symptomless plants, non-vector insects or other than group V phytoplasma-infected control plants yielded products of electrophoretic mobility within the range of that of the expected amplicons (Fig. 2 A). These products disappeared when the annealing temperature was increased to 63 °C (Fig. 2 B).

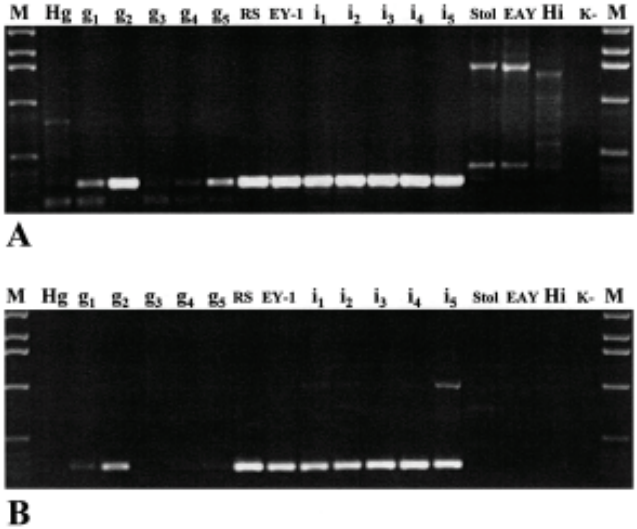

Fig. 2: Agarose gel electrophoresis of fAY/rEY amplificons from representative symptomatic grapevines (g) and *S. titanus* (i), healthy grapevines (Hg) and healthy *S. titanus* (Hi). Phytoplasma reference strain acronyms are given in the text. K^-: negative control; M: 1kb DNA size marker (Life Technologies). Annealing temperatures were 59 °C (**A**) and 63 °C (**B**).

S e q u e n c i n g : The nucleotide sequence (301 bp, 47 % G+C content) of the fAY/rEY amplified DNA fragment from EY-1 was determined and a restriction map produced. A number of restriction enzyme recognition sequences was present, but enzymatic digestion indicated no differences among amplified fragments from group V phytoplasma reference isolates, grapevines and *S. titanus* (not shown). As expected from previous work (Marcone *et al.* 1996 b), high homologies were found with 16S rDNA nucleotide sequences of other group-V phytoplasmas, indicating that fAY primes at nucleotide position 1116 and rEY at nucleotide position 1437 of the 16S rDNA region of EY-1 (Fig. 3).

Discussion

When DNAs from plant and insect samples that were group V-positive by nested PCR were directly amplified in one step with the fAY/rEY primer pair, 80 and 98 %, respectively, yielded a specific DNA fragment of the expected size. The differences in response of the two hosts could be explained by the different concentrations of pathogen DNA carried in the processed tissues or to DNA losses due to the isolation protocols. However, in practical terms, direct amplification with the fAY/rEY primer pair, reduces the need to use nested PCR analysis to only about 20 % of the plant samples.

Enzymatic restriction of the amplified fAY/rEY fragments did not enable us to tell whether the amplified phytoplasmas belonged to distinct subgroups within group V (Bianco *et al.* 1996 a; Martini *et al.* 1999; Marzachì *et al.* 2001). However, subgroup VA phytoplasmas appear to occur very rarely in grapevines (Bianco *et al.* 1996 a; Marzachì *et al.* 2001) and their involvement in disease development has yet to be proven, whereas those belonging to the recently proposed VD subgroup have been detected in northeastern Italy only (Martini *et al.* 1999). Digestion with *Bfa*I of R16(V)F1/R1 nested-amplified fragments (Fig. 1) would not be the strategy of choice to ascertain the presence of putative subgroup VD phytoplasmas, whose possible presence in the infected grapes and insects can therefore not be ruled out.

However, in the face of FD epidemics, subgroup affiliation is a secondary consideration and a simple, quick and reliable method for mass diagnosis becomes urgently necessary. Primers fAY/rEY have been used for the detection of group V phytoplasmas in grapevines and vectors (Maixner and Reinert 1999; Maixner *et al.* 2000), and we have optimised the method. This optimised protocol never gave false positive responses either with putative non-vector insects or symptomless field grapevines or glasshouse-cultivated periwinkles experimentally infected with phytoplasmas belonging to groups other than 16SrV, or healthy grape seedlings. Furthermore, results obtained with the fAY/rEY primer pair have been consistently confirmed in traditional nested amplifications driven by primers R16(V)R1/F1.

Negative results obtained with field-collected symptomatic grapevines, both in traditional group V-specific nested and direct fAY/rEY amplifications, can be ascribed to either Stol infection or other conditions (viruses, chemical treatments) causing symptoms in the plants (Marzachì *et al.* 2001). About 50 % of the DNAs extracted from *S. titanus* captured in heavily FD-infected vineyards, irrespective of the method used for amplification, did not yield group V-specific amplicons. It should be noted that 38 and 11 %,

EY(L33763)	AGTCCTAAAACGAACGCAACCCCTGTCGTTAGTTGCCA**GC**	1069
pFAYREY		
FD(X76560)	AGTCCTAAAACGAACGCAACCCCTGTCGcTAGTTGCCA**GC**	1079
EY(L33763)	**ACGTAATGGTGGGGACTT**TAGCGAGACTGCCAATTAAACA	1109
pFAYREY	**TT**TTAGCGAGACTGCCA.TAAACA	21
FD(X76560)	**ACGTAATGGTGGGGACTT**TAGCGAGACTGCCAATTAAACA	1119
EY(L33763)	TTGGAGGAAGGTGGGGATAACGTCAAATCATCATGCCCCT	1149
pFAYREY	TTGGAGGAAGGTGGGGATAACGTCAAATCATCATGCCCCT	61
FD(X76560)	TTGGAGGAAGGTGGGGATAACGTCAAATCATCATGCCCCT	1159
EY(L33763)	TATGATCTGGGCTACAAACGTGATACAATGGCTATTACAA	1189
pFAYREY	TATGATCTGGGCTACAAACGTGATACAATGGCTATTACAA	101
FD(X76560)	TATGATCTGGGCTACAAACGTGATACAATGGCTATTACAA	1199
EY(L33763)	AGAGTAGCTGAAACGCGAGTTTTTAGCCAATCTCAAAAAG	1229
pFAYREY	AGAGTAGCTGAAACGCGAGTTTTTAGCCAATCTCAAAAAG	141
FD(X76560)	AGAGTAGCTGAAACGaGAGTTTTTAGCCAt..TCAAAAAG	1237
EY(L33763)	GTAGTCTCAGTACGGATTGAAGTCTGCAACTCGACTTCAT	1269
pFAYREY	GTAGTCTCAGTAgGGATTGAAGTCTGCAACTCGACTTCAT	181
FD(X76560)	GTAGTCTCAGTACGGATTGAAGTCTGCAACTCGACTTCAT	1277
EY(L33763)	GAAGCTGGAATCGCTAGTAATCGCGAATCAGCATGTCGCG	1309
pFAYREY	GAAGCTGGAATCGCTgGTAATCGCGAATCAGCATGTCGCG	221
FD(X76560)	GAAGCTGGAATCGCTAGTAATCGCGAATCAGCATGTCGCG	1317
EY(L33763)	GTGAATACGTTCTCGGGGTTTGTACACACCGCCCGTCAAA	1349
pFAYREY	GTGAATACGTTCTCGGGGTTTGTACACACCGCCCGTCAAA	261
FD(X76560)	GTGAATACGTTCTCGGGGTTTGTACACACCGCCCGTCAAA	1357
EY(L33763)	CCACGAAAGTTAGCAATACCC**GAAAGCAGTGGCTTAACTT**	1389
pFAYREY	CCACGAAAGTTAGCAATACCC**GAAAGCAGTGGCTTAACTT**	301
FD(X76560)	CCACGAAAGTTAGCAATACCC**GAAAGCAGTGGCTTAACTT**	1397
EY(L33763)	**CG**CAAGAAGAGGGAGCTGTCTAAGGTAGGGTTGATGATTG	1429
pFAYREY		
FD(X76560)	**CG**CAAGAAGAGGGAGCTGTCTAAGGTAGGGTTGATGATTG	1437

Fig. 3: Alignment of 16S ribosomal nucleotide sequences from EY-1, FD and pFAYREY (insert). Primer sequences in bold.

respectively, of field-collected *H. obsoletus* and *O. alni* carried Stol and PGY (MAIXNER *et al.* 1995, 2000; SFORZA *et al.* 1998). Although carrier status does not necessarily mean that the insect transmits the phytoplasma, these findings would reinforce the suggestion that *S. titanus* is heavily involved in FD epidemics in southeastern Piemonte (MARZACHÌ *et al.* 2001).

In conclusion, primers fAY/rEY can be used in mass screening for FD-related phytoplasmas in plant material and vector populations in order to reduce costs, contamination risks, effort and time needed to evaluate a large number of samples. On the down side, subgroup affiliation of the phytoplasmas cannot be determined, and a residual 20 % of the plant samples may have to be re-tested by nested PCR.

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