Anales del Jardín Botánico de Madrid 62(2): 127-133
www.rjb.csic.es

Grapevine yellows diseases in Spain: eight year survey of disease spread and molecular characterization of phytoplasmas involved

by

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Abstract

Among grapevine yellows phytoplasma diseases in Europe, flavescence dorée (FD) is the most devastating and in the last decade has reached Spanish vineyards, mainly in Catalonia. An eight-year survey was carried out in the areas where the disease has spread (Alt Empordà, Catalonia, Northern Spain) and in the remaining vine-growing areas of Catalonia. Sequence analyses of a portion of the 16S-23S ribosomal DNA cistron, from selected grapevine samples from Catalonia, showed that the phytoplasmas involved in grapevine yellows belong to 16S ribosomal subgroups V-D (flavescence dorée, FD) and XII-A (bois noir, BN). A set of Spanish FD isolates collected during these years were further studied by RFLP analyses of the 16S-23S ribosomal DNA fragment, as well as the rpS3 and SecY genes. All the FD phytoplasma strains studied were related to phytoplasmas belonging to ribosomal protein subgroup rp-E.

Keywords: Flavescence dorée, bois noir, molecular detection, *Vitis*.

Resumen

La flavescencia dorada (FD) es la enfermedad más agresiva de entre todas las enfermedades de fitoplasmas que causan amarilleos de vid en Europa, y que en la última década ha alcanzado también a los viñedos de España, principalmente en Cataluña. Se ha realizado un seguimiento durante ocho años en las zonas donde la enfermedad se había difundido (Alt Empordà, Cataluña) y en el resto de zonas con cultivo de vid de Cataluña. El análisis del fragmento del gen DNA ribosomal 16S-23S, de una selección de muestras de vides de Cataluña, indica que los fitoplasmas que están implicados en los amarilleos de vid pertenecen a los subgrupos ribosomales 16S V-D (flavescencia dorada, FD) y XII-A (bois noir, BN).

Una selección de aislados españoles de FD obtenidos durante estos años se ha examinado mediante análisis RFLP del fragmento del gen ribosomal 16S-23S, y de los genes rpS3 y SecY. Todos los aislamientos de fitoplasmas FD estudiados están relacionados con fitoplasmas pertenecientes al subgrupo de proteína ribosomal rp-E.

Palabras clave: Flavescencia dorada, bois noir, detección molecular, vid.

Introduction

Two phytoplasmas associated with diseases in cultivated grapevines, flavescence dorée (FD) and bois noir (BN), were reported in recent years in Spain (Abad & al., 1998; Batlle & al., 1997, 2000; Laviña & al., 1996). The former is classified in the 16SrV group and the latter in the 16SrXII-A/stolbur group. Symptoms of both diseases are similar and mainly involve plant decline, leaf rolling, shrivelled grapes, unripened shoots and reddening on red wine cultivars (Fig. 1) or yellowing on white wine cultivars (Fig. 2). Molecular methods have been developed to differentiate and identify the diverse phytoplasmas involved in grapevine yellows diseases (Padovan & al., 1995; Daire & al., 1997; Lee & al., 1998; IRPCM, 2004).

Fig. 1. Symptoms of decline and reddening on a red wine grapevine cultivar.

Fig. 2. Symptoms of leaf rolling and yellowing on a white wine grapevine cultivar.

FD is the most aggressive phytoplasma among those associated with grapevine diseases, and is thus subject to quarantine restrictions. Genetic relatedness of this pathogen, revealed by 16Sr DNA analyses, showed that two different ribosomal subgroups (16SrV-C, also known as FD-C, and 16SrV-D, also known as FD-D) are associated with outbreaks in Italy and both are transmitted by the vine leafhopper insect *Scaphoideus titanus* Ball (Bertaccini & al., 1997; Martini & al., 1999; Mori & al., 2002). In recent years outbreaks associated with FD-D type phytoplasmas were observed in several regions of Northern Italy and in France (Angelini & al., 2001; Bertaccini, 2002; Boudon-Padieu, 2003).

In Spain *S. titanus* was identified in the four provinces of Catalonia (NE Spain): Barcelona, Gerona, Lérida and Tarragona (Barrios & al., 1998). The first FD outbreak occurred in Gerona (Laviña & al., 1996), which forced the Catalonian administration to legislate a compulsory program to eradicate FD at Alt Empordà (Rahola & al., 1997). In application of this program, vineyards were sprayed (via helicopter) with a pyrethroid insecticide to control *S. titanus* in areas ranging from 1900 Ha in 1997 to 2030 Ha in 1999.

In this work, we surveyed ca. 63 027 Ha of vineyards in Catalonia (Spain) for phytoplasmas involved in grapevine yellows diseases. Molecular identification of phytoplasmas was performed from selected positive samples collected during different years of the epidemic.

Materials and methods

Monitoring the presence of phytoplasma and plant material used for phytoplasma detection

In the Alt Empordà (Gerona), foci of FD were discovered by visual inspection of plots having more than 20% of their grapevine plants with symptoms of yellowing or reddening. This was done whenever possible by observing the plots from a high vantage point. Otherwise, the perimeters of the plots were surveyed to find symptomatic plants. Once one focus was found, plots at a distance of 500 m from the outer plants in the infected focus were inspected plant by plant. The rest of the plots in the area at risk (Alt Empordà) were examined at random. In the remaining vine-growing areas of Catalonia, samples expressing symptoms were selected.

Samples from 112 symptomatic grapevines were collected in the affected provinces during July and September for the seven year period of 1996-2002.

DNA extraction, PCR amplification and RFLP and sequence analyses

Leaf mid-vein tissues were subjected to DNA extraction using a combined method, a concentration of phytoplasmas with PGB (Ahrens and Seemüller, 1992) and a DNA extraction performed with the E.Z.N.A.® Plant MiniPrep Kit (Omega Biotek), as described by Martín & Torres (2001).

PCR was performed with Ready-to-Go PCR Beads (Amersham Biosciences) as previously described (Martín & Torres, 2001). Samples lacking DNA were used in each experiment as negative controls; positive and reference controls were as described in Fig. 3. Amplification of a partial rDNA cistron (16S rDNA, 16S/23S rDNA spacer region, trnI and the 5' part of 23S rDNA representing ca. 1800 bp) with phytoplasma-universal primer pairs P1 (Deng & Hiruki, 1991) and P7 (Schneider & al., 1995). This was followed by nested PCR reactions using the R16(V)F1 and R16(V)R1 primer pairs specific for group 16SrV and the R16(I)F1 and R16(I)R1 primer pairs specific for groups 16SrI and 16SrXII (Lee & al., 1994). Then selected grapevine samples that gave positive results with the R16(V) primers (Tab. 1) were further analysed using nested-PCR with primers $16R_{758f}$ and $M23SR_{1804r}$ (Martini & al., 1999) on the P1/P7 amplicon. Moreover, these samples were analysed by nested PCR with primers FD9f3 and FD9r2 on FD9f2/FD9r amplicons , which represent the entire SecY gene and a portion of the rpL15 gene (Angelini & al., 2001). In addition, PCR reactions using the rp(V)F1 and rpR1 primers gave a product representing part of the ribosomal protein rpS3 gene (Lee & al., 1998). About 200 ng of each positive product was each separately digested with *Tru*I, *Taq*I, *Alu*I and *Tsp509*I restriction enzymes (Fermentas, Vilnius, Lithuania) for at least 16 hours, following manufacturer instructions.

All PCR products that were sequenced were first cleaned using the E.Z.N.A. Clean kit (Omega Biotech). Fragments were sequenced using the Big Dye Terminator reaction kit (Applied Biosystems) in an ABI Prism 377 (Applied Biosystems). Sequencing

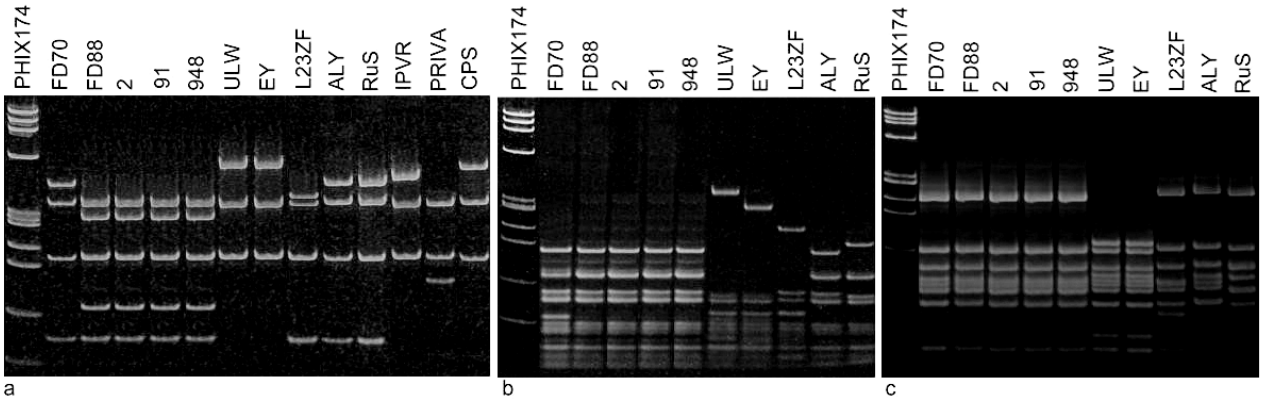

Fig. 3. Polyacrylamide gels showing the RFLP patterns of: **a,** 16Sr DNA plus spacer of phytoplasmas from reference strains and some of selected grapevine samples listed in Table 1 amplified with primers M1/B6 ($16R_{758f}$/$M23SR_{1804r}$) and digested with *Taq*I; **b,** secY phytoplasma gene amplified with the FD9f3 and FD9r2 primers digested with *Tru*I; **c,** rpS3 gene amplicons digested with *Tsp509*I. Reference samples in periwinkle (*Catharanthus*): EY, elm yellows from US (16SrV-A); ULW, elm yellows from EU (16SrV-A); IPVR, Italian periwinkle virescence (16SrXII-A); ALY, alder yellows (16SrV-C), PRIVA, primula yellows (16SrI-B), CPS, *Catharanthus* phyllody from Sudan (16SrVI-C), RuS, rubus stunt (16SrV-E). Reference samples in other species: FD-70 and FD-88, grapevine infected with flavescence dorée from France (respectively 16SrV-C and 16SrV-D); L23ZF, jujube infected with witches' broom (16SrV-B). ØX174, marker *Hae*III digested; fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72.

Table 1. Description of selected flavescence dorée phytoplasma isolates analysed by RFLP. Genbank accession numbers from sequences of some isolates are shown.

Reference	Cultivar	Municipality	Year	Sequence
1	Garnatxa grisa	Agullana	1996	AJ548788*
2	Carinyena	Agullana	1996	AJ548789*
62	Macabeo	Agullana	1999	AJ548790*
91	Garnatxa blanca	St. Climent	1999	AJ548791*
947	Garnatxa blanca	Masarac	2001	AJ548792*
948	Garnatxa blanca	Masarac	2001	–
949	Garnatxa blanca	Masarac	2001	–
951	Carinyena	Cabanes	2001	AJ548793*
952	Garnatxa blanca	Cabanes	2001	AJ548794*
1487	Macabeo	Agullana	2002	AJ548787**

*sequence of R16F2/R2 amplicons.
**sequence of P1/P7 amplicon.

of both strands was performed using the R16F2 and R16R2 (Lee & al., 1995) or P1 and P7 primers. Bioedit™ software was used to identify the consensus sequence from the two strands of each amplification product.

Results

Monitoring of phytoplasma presence

In Alt Empordà (Gerona) the number of plots having more than 20% of their grapevine plants with phytoplasma symptoms was zero in 1998, starting from values of 29 in 1996, and 20 in 1997. From 1998 onwards, there has only been one outbreak, this one occurring in 2001 (Tab. 2). There are several municipalities in which scattered plants affected by yellows were detected: Agullana, Biure, Cabanes, Cantallops, Espolla, Masarac, Pau, Peralada and St. Climent. Affected municipalities form a triangle whose base is at the Pyrenees (natural border with the South of France) and its opposite vertex towards the South of Spain (Fig. 4).

In the remaining Catalonian vineyards only isolated grapevines showed symptoms of phytoplasma disease.

Phytoplasma detection

Nested-PCR with the R16(V) and R16(I) primers showed that 45 samples over 112 analysed were positive for the presence of phytoplasma. The 20 samples that gave the PCR products of expected size using the R16(V) specific primers came from Gerona, while the 25 samples that gave PCR products of expected size with R16(I) specific primers were distributed between the four provinces.

Table 2. Occurrence of flavescence dorée, in productive vineyards from Alt Empordà (Gerona), from 1996 to 2003.

Year	> 20% symptomatic plants		< 20% symptomatic plants	
	Area (Ha)	Nº plots	Area (Ha)	Nº plots
1996	23.85	29	77.4	87
1997	15.38	20	135.41	154
1998	0	0	82.81	92
1999	0	0	41.98	45
2000	0	0	31.48	33
2001	0.45	1	28.99	29
2002	0	0	23	23
2003	0	0	12.2	6

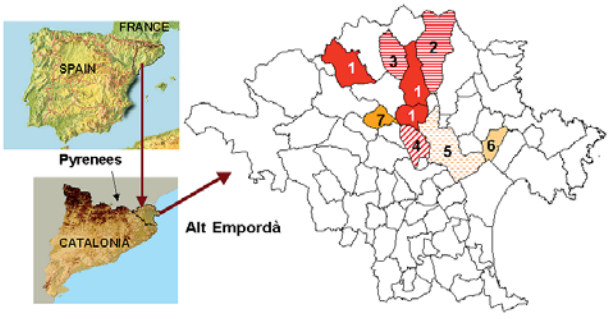

Fig. 4. Distribution of flavescence dorée phytoplasma at Alt Empordá since 1996; the most affected cultivars were Carinyena, Chardonnay, Garnatxa blanca, Garnatxa grisa, and Macabeo. **(1)** Focus in 1996 and 1997, <20% since 1998 to 2003; **(2)** Focus in 1996, <20% since 1997 to 2001; **(3)** Focus in 1997; **(4)** Focus in 2001, <20% in 2000; **(5)** <20% since 1996 to 2002; **(6)** <20% since 1999 to 2000; **(7)** <20% since 2000 to 2002.

FD identification

RFLP analyses of SecY and rpS3 amplicons from ten selected samples that were positive with the

R16(V) primers indicated that these phytoplasmas were FD and were molecularly undistinguishable from each other and from reference strain FD88. In all the genomic fragments analysed, they were referable to phytoplasmas belonging to the ribosomal subgroup 16SrV-D and to rp subgroup E (Fig. 3) (Martini & al., 2002).

No sequence differences were seen among the eight P1/P7 or R16F2/R2 amplicons (Tab. 1). The BLAST search of sequence AJ548787 (1782 bp) from the Spanish isolate FD1487 showed highest similarity (99.9%) with the strain FD70 (AF176319) (Davis & Dally, 2001) from FD infected grapevines. The alignment of both sequences showed only two nucleotide differences, in positions 1425 and 1594 in the Spanish isolate FD1487. In agreement with the RFLP analysis of the ribosomal DNA fragment, the difference in nucleotide position 1425 corresponds with a putative restriction site for *Taq*I in FD1487 that is not present in FD70. The difference in position 1594 was due to a poliA (seven A in FD1487 and eight A in FD70). A complete 16S rDNA sequence of FD88 was not available, but AF458380, a partial 16S, 16S-23S intergenic spacer and 23S rDNA sequence of strain FD92 (=FD88) (Angelini & al., 2003), made it possible to compare 1020 nucleotide positions of AJ548787 (FD1487) with AF458380 (FD92=FD88). These sequences were 97.9% similar, however, both strains show the presence of a putative restriction site for *Taq*I at position 1425 that allows detection of identical profiles after RFLP analyses (Fig. 3).

BN identification

No significant sequence differences were seen among the P1/P7 amplicons of isolate BN2642 and the R16F2/R2 amplicons from isolates BN974, BN1536, BN2001 and BN 2002. The BLAST search of sequence AJ964960 (1684 bp) from Spanish isolate BN2642 showed highest similarity (99.5%) with sequence AF248959 from strain STOL, a member of the 16SrRNA RFLP subgroup XII-A (Davis & Dally, 2001). The alignment of both sequences showed only five nucleotide differences, in position 623, 714, 1007, 1013 and 1203 in isolate BN2642.

Discussion

During the monitoring of phytoplasma presence in the Catalonian provinces at risk (Barcelona, Gerona, Lérida and Tarragona), only grapevines at Alt Empordà (Gerona) were positive for disease caused by phytoplasmas from the 16SrV-D/elm yellows subgroup (flavescence dorée, FD). No phytoplasmas belonging to this group were detected in the grapevines of the remaining plots of Gerona and of the other three Catalonian provinces where only phytoplasmas belonging to the 16SrXII-A/stolbur subgroup (Bois noir disease) were identified.

Bois noir disease is distributed in more viticultural areas, but with a low incidence level, and it is responsible for minor economic losses (observations of Servei Sanitat Vegetal, DARP). Moreover, flavescence dorée, a destructive phytoplasma that is subjected to quarantine regulations, is confined to a small part of the Alt Empordà near the French border.

The FD disease incidence in Spain has drastically decreased since 1996 when the first focus appeared. The restricted spread of FD could be a result of the effectiveness of the measures adopted (Rahola & al., 1997) such as the elimination of affected grapevines and the containment of *S. titanus*, even though the confluence of other natural factors that could have accounted for the decreased incidence can not be excluded.

The IRPCM Phytoplasma/Spiroplama Working Team-Phytoplasma Taxonomy Group (2004) established the basis to identify '*Candidatus* Phytoplasma' species by the level of sequence identity of their 16S rRNA genes. The phytoplasma 16S rRNA sequences obtained from Spanish grapevines shows highest similarity with sequences that are related to two '*Ca*. Phytoplasma' species that are not yet formally described but for which names were proposed at the Tenth International Congress of the International Organization of Mycoplasmology held in 1994 in Bordeaux, France. These names are '*Ca*. Phytoplasma vitis' and '*Ca*. Phytoplasma solani'. All sequences from samples with positive R16(V)F1/R1 amplicons are related to '*Ca*. Phytoplasma vitis' of 16SrV/elm yellows group; sequences from samples with positive R16(I)F1/R1 amplicons are related to '*Ca*. Phytoplasma solani' of 16SrXII/stolbur group. Both '*Ca.* Phytoplasmas' reported here are incidental citations which do not constitute prior citations, according to rule 28b of the bacteriological code (Lapage & al., 1992).

Previous work has been conducted to define the subgroups of FD phytoplasmas. Davis & Dally (2001) classified two FD strains, based on 16S rRNA gene RFLP patterns, as members of two distinct subgroups: FD70 as 16SrV-C and X76560 as 16SrV-D. Genetic variability among FD phytoplasmas, based on RFLP analyses from three DNA fragments (partial rDNA operon, partial rps3 operon and partial SecY gene) was also reported by Martini & al. (1999, 2002) and Botti & Bertaccini (2003). Based on *Taq*I restriction site data, the latter study allowed the differentia-

tion of two rRNA subgroups from grapevine samples, 16SrV-C and 16SrV-D, and allowed differentiation of seven FD phytoplasma variants belonging to the 16SrV-C (including strain FD70) and two FD variants belonging to the 16SrV-D (including strain FD88) subgroups. The subgroups defined by Davis and Dally (2001) are not equivalent to those described by Martini & al. (1999, 2002), since they were based on RFLP (*TaqI*) patterns of the 16S rDNA cistron in which only two strains belonging to subgroup 16SrV-C were compared.

Direct comparison or phylogenetic analysis of 16S rDNA provides a good tool to define phytoplasma groups and to identify '*Ca*. Phytoplasma' taxa (IRPCM, 2004), but the high levels of similarity among different 16SrV phytoplasmas using this DNA fragment preclude its use in subgroup classification (Davis & Dally, 2001). It seems that the rps3 gene shows more variability and can be employed to differentiate closely related FD strains (Martini & al., 2002).

Based on RFLP analyses of the 16S rDNA cistron and of the rps3 and SecY genes, FD phytoplasmas identified in Spain over an eight-year period (1996-2003) belong to subgroup 16SrV-D (Martini & al., 2002) and to ribosomal protein subgroup E (Lee & al., 2004). Moreover, they are genetically indistinguishable from the FD88 strain from France (kindly provided by E. Boudon-Padieu, INRA, Dijon, France and described in Martini & al., 2002) and from other reference strains that show epidemic activity in France and Italy (data not shown).

Acknowledgments

The authors thank technicians from the Plant Protection Service (Generalitat de Catalunya), especially Gonçal Barrios and Joan Reyes who brought the symptomatic grapevines to our attention. Thanks to Dr. Dan Nickrent (Southern Illinois University, Carbondale, USA) and the referees for their helpful comments on the manuscript. This work was partially supported by a FEOGA grant (number FEDER: 94 00 10 038).

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Editor asociado: D.L. Nickrent
Recibido: 29-VII-2005
Aceptado: 15-X-2005