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Diversity among mycoplasma-like organisms inducing grapevine yellows in France

by

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S u m m a r y : As antibodies and molecular probes which were previously obtained for diagnosis of grapevine flavescence dorée (FD; a yellows disease induced by a MLO), showed to be highly specific, a survey of grapevine samples collected in different viticultural areas in France was undertaken, using a PCR method with primers allowing amplification of a part of the 16S rRNA gene of most MLOs, and restriction analyses of the amplified products (AHRENS and SEEMÜLLER 1992). The presence of MLO was established in all the different grapevine samples, and their diversity was demonstrated. The typcial pattern yielded by FD *sensu stricto*-MLO was found in samples from southern vineyards, including a symptomless rootstock. Two different patterns were found in samples affected by bois noir disease of northern French vineyards, one of these patterns being previously undescribed. The present survey was non exhaustive and should be followed in the frame of a large collaboration between viticultural countries. It showed the diversity in causal agents of diseases which converge in symptomatology, and emphasizes on the need of specific diagnosis tools, for identification of each of the vector species, for epidemiological studies, and availability of planting material.

K e y w o r d s : MLO, grapevine yellows, flavescence dorée, bois noir, diagnosis, PCR.

Introduction

Grapevine yellows (GY) diseases occur in several regions of the world (RUMBOS 1988; MAGAREY 1988; GRANATA 1991). In France, two main GY diseases, flavescence dorée (FD) and bois noir (BN), have been reported which differ in their geographic occurrence and epidemiology. The FD disease, an important disease of southern France vineyards, has been fully characterized (CAUDWELL *et al.* 1964, 1970) and is shown to be consistently associated with a mycoplasma-like organism (MLO). Its vector is the leafhopper *Scaphoideus titanus* BALL. The BN disease was found in northern France, in vineyards of Burgundy and Alsace (CAUDWELL *et al.* 1961) and is assumed to be related to Vergilbungskrankheit in Germany. However, although BN is identical in symptomatology to FD, experimental transmission of BN from grapevine to grapevine was unsuccessful using *S. titanus*, and the natural vector of BN has not been identified.

In spite of the inability to culture MLOs *in vitro*, maintenance of FD-MLO, in experimental hosts which contain relatively large amount of MLOs compared to grapevine (CAUDWELL *et al.* 1972), has led to the preparation of FD-specific antibodies and DNA probes (CAUDWELL *et al.* 1982; BOUDON-PADIEU *et al.* 1989; SCHWARTZ *et al.* 1989; DAIRE *et al.* 1992). These diagnostic tools efficiently detect FD-MLO in *S. titanus* collected in GY-affected vineyards from southern France (BOUDON-PADIEU *et al.* 1989) and in symptom-bearing, field-grown cultivars from the same area (CAUDWELL and KUSZALA 1992; DAIRE *et al.* 1992). However, the FD specific antibodies and probes did not detect the presence of MLO in most of grapevine samples with GY symptoms harvested in the northern vineyards of France where the BN disease occurs (CAUDWELL and KUSZALA 1992; DAIRE, unpublished data). These results suggest the need of diagnostic tools with a broader specificity for MLO detection other than that of FD. Recently, studies of the

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16S rRNA gene of MLOs has enabled the design of PCR primers from conserved sequences of this gene. These primers allow amplification of a part of the 16S rRNA gene of most plant MLOs (AHRENS and SEEMÜLLER 1992) and thus can be used as universal primers for PCR detection of MLOs. In the present paper, we report of the use of these primers for the detection of MLOs in GY-diseased grapevines from various vineyards of France which were previously found to be negative when tested by hybridization using FD-specific DNA probes.

Materials and methods

MLO isolates in periwinkle: The following MLO isolates maintained in *C. roseus* were studied: FD was isolated from diseased grapevine using *S. titanus* (CAUDWELL *et al.* 1970, 1972). Phi was isolated from *Euscelis* species in bois noir-affected vineyards (CAUDWELL *et al.* 1971). IPVR (Italian periwinkle virescence) (DAVIS *et al.* 1992) was naturally transmitted to exposed periwinkle in infected vineyards. FDU (flavescence dorée from Udine) (DAVIS *et al.* 1992) was obtained by dodder transmission from grapevine to periwinkle.

Vine samples: Canes from various GY-symptom-bearing Alicante Bouschet and Grenache stocks, and from a symptomless motherplant of rootstock 3309 C, suspected to be infected by FD, were harvested from different locations in southern France where FD has been characterized. GY-symptom-bearing Chardonnay and Pinot noir samples were harvested from different areas of Burgundy and Rhône valley.

DNA extraction: Total DNA from periwinkle was extracted according to ROGERS and BENDICH (1988). DNA was extracted from a MLO-enriched fraction prepared from grapevine main leaf veins as previously described (DAIRE *et al.* 1992).

Dot blot hybridization with FD-specific probe: DNA was blotted onto Biohylon Z^+ nylon membrane (Bioprobe system), according to the instructions of the manufacturer. Hybridization was performed using standard procedures (SAMBROOK *et al.* 1989) under stringent washing conditions (0.2 x SSC for final wash). The ^{32}P-labelled probe was a cloned DNA fragment (FD2, 1.7 kb) specific for FD-MLO (DAIRE *et al.* 1992).

DNA amplification and restriction analysis: The primers were designed by AHRENS and SEEMÜLLER (1992). The same procedure was used, except for a modification of the reaction mixture. Amplification was carried out in a total of 40 µl containing 5-10 ng DNA, 0.25 µM of each primer, 20 mM each dNTPs, 1 unit of Taq DNA polymerase (Appligene), 4 µl of Taq buffer (70 mM $MgCl_2$, 500 mM Tris-Cl, 500 mM KCl, 500 mM $(NH_4)_2SO_4$, 2 mg/ml BSA), and overlaid with 40 µl of mineral oil. 10 µl of the mixture containing amplified DNA was directly digested overnight with 2.5 units of *AluI* restriction enzyme.

Native amplification products were electrophoresed in an agarose (1.5 %) gel, and digested products were analysed using polyacrylamide (10 %) gel electrophoresis, according to standard procedures (SAMBROOK *et al.* 1989). DNA was visualized under UV light following ethidium bromide staining.

Results

Dot blot hybridization: Using a FD-specific probe, positive results were obtained in dot blot hybridization with DNA from FD-infected periwinkle and DNA from all infected samples and symptomless rootstock collected from vineyards of southern France. In contrast, no hybridization was obtained with DNA from other MLO-infected periwinkle and diseased grapevine samples, or with DNA from healthy periwinkle and grapevine. For results see Figure.

Amplification: After 25 cycles, a DNA band of about 560 bp was visible in all samples of infected periwinkle, whereas no amplification occurred with healthy control (Figure A, left).

Following 35 amplification cycles, DNA from all field-collected GY-diseased grapevine or from the symptomless rootstock samples revealed a DNA band of the same size as found with infected periwinkle DNA. A weaker band of the same size appeared in healthy grapevine DNA controls (Figure A, right).

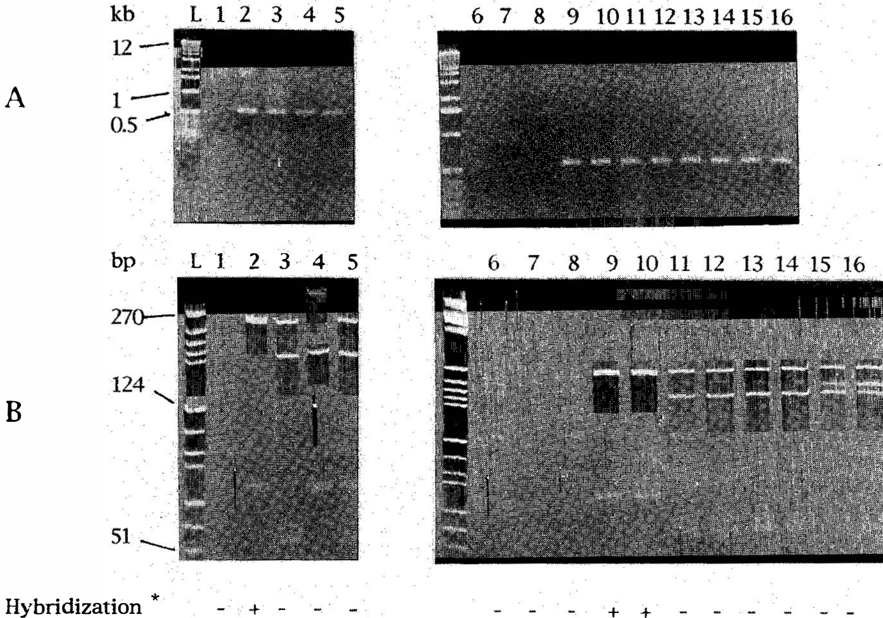

Figure: **A**: agarose gel (1.5 %) electrophoresis of 16S rDNA PCR-amplified fragments (560 bp) from various MLOs in experimentally infected periwinkle or in naturally infected grapevine. **B**: polyacrylamide gel (10 %) electrophoresis of the amplified products shown in A, after *Alu*I restriction. Periwinkles: (1) healthy, (2) infected with FD-MLO, (3) with IPVR-MLO, (4) with FDI-MLO, (5) with Phi-MLO. Healthy grapevines: (6) Chardonnay, (7) Alicante, (8) 3309 C (rootstock). GY-symptom-bearing grapevines: (9) Alicante from southern France locality a**, (10) 3309 C from southern France locality b, (11) Chardonnay from Burgundy locality c, (12) from Rhône valley locality d, (13) from Burgundy locality e, (14) from Rhône valley locality f, (15) from Burgundy locality g, (16) from Rhône valley locality f. L: molecular weight marker. * Total DNA from each sample was hybridized with the specific FD-MLO DNA probe FD2. Positive hybridization (+). Negative hybridization (-). ** a, b, c, d, e, f, g: localities where samples were harvested.

*Alu*I restriction pattern analysis of amplified MLO DNA from periwinkle infected with FD, FDU, IPVR, and Phi, demonstrated 4 distinct patterns (Figure B, left). Each of these patterns was identical to the pattern of one of the 4 MLO groups described by AHRENS and SEEMÜLLER (1992) on the same amplification and *Alu*I restriction basis. The FD pattern resembled that of group IV, FDU of group III, IPVR of group II, and Phi of group I.

*Alu*I restriction analysis of the amplification products obtained from healthy grapevine samples consistently yielded patterns which were different from MLO-DNA like patterns. *Alu*I restriction analysis patterns of DNA amplified from diseased grapevine samples (Figure B, right) could be classified into 3 different types: 2 of the types were similar to the above groups IV and II, respectively, while the 3rd represented a new type, previously undescribed.

The group IV pattern was found in all vine samples obtained from vineyards in southern France (*V. vinifera* and rootstock cvs). Group II pattern was found in all but two samples originating from vineyards in Burgundy and the Rhône valley. The new isolate pattern was found in one of 2 samples from a locality of the Rhône valley (f) and in a sample from one locality in Burgundy (g). Group III and group I patterns were not found in any of the tested samples.

Discussion

FD-specific probes detected MLOs in FD-infected periwinkle and in grapevine samples from southern France, as previously reported (DAIRE *et al.* 1992), but did not detect MLOs in samples of grapevine with GY symptoms from other regions in France. This suggests a diversity in the MLO etiology of GY in France, and was analysed using the methods of MLO typing published by AHRENS and SEEMÜLLER (1992).

AluI restriction analysis of the products amplified in the 4 standard MLOs in periwinkle were consistent with previous data: FD was found to belong to the same group as elm-yellows (group IV), a result consistent with dot blot hybridization data (DAIRE *et al.* 1992) and Southern blot data (DAIRE, unpublished results); FDU belonged to group III, as shown by AHRENS and SEEMÜLLER (1992).

However, it must be reminded that grouping of MLOs on the basis of PCR amplification and restriction analysis does not necessarily imply that these organisms are related in all other respects. Though FD-MLO belongs to the same group as elm yellows (EY), and FD-MLO DNA strongly hybridized with EY-DNA (DAIRE *et al.* 1992, and unpublished results), no serological reactivity in ELISA was found using polyclonal and monoclonal antibodies to FD-MLO with EY antigens (D. L. DAVIES, personal communication). On the other hand, FD and FDU were found to belong to two different groups, which strongly suggests that they are not related, data consistent with reciprocal serological tests using FD-specific or FDU-specific antibodies on FD and FDU antigens (BOUDON-PADIEU, unpublished observations, and T. A. CHEN, personal communication).

Because of low MLO titre, 35 cycles of amplification were required to effectively detect MLO-DNA from grapevine tissues. In healthy grapevine samples, following a high number of amplification cycles, a weak 560 bp DNA band appeared, of uncertain origin. Restriction patterns of such amplified products were different from MLO-DNA like patterns. These results are consistent with previous data.

The *AluI* restriction patterns, obtained in diseased grapevine samples, were always distinct from patterns obtained from healthy grapevine cultivars, and most of them were similar to patterns obtained by AHRENS and SEEMÜLLER (1992) with plant MLOs. For these reasons, we concluded that they were MLO patterns. Two patterns in grapevine were similar to those we obtained with two of the MLOs maintained in periwinkle: one MLO was the well characterized FD-MLO; the other was the IPVR-MLO of a supposed grapevine origin. In addition, a yet undescribed *AluI* profile was found in some grapevine samples from northern vineyards. Neither FDU type nor Phi type were found in the present survey.

The present results confirm that FD disease, associated with *S. titanus*, in southern France, and the GY disease in northern France, are distinct from each other. The results obtained from samples from southern France are in agreement with previous positive hybridization and ELISA data obtained with the same samples. In contrast, grapevine with GY symptoms from vineyards in northern France, tested negative using FD-specific diagnostic tools. These results also show that different grapevine MLOs exist in northern areas and that they occur in close proximity as was found in the Rhône Valley. Hence, the disease bois noir occurring in northern France and previously distinguished from FD because of its inability to be transmitted to plants by *S. titanus*, could be caused by several distinct MLOs.

These data must be considered as preliminary results. A broader survey of MLOs in grapevine in France and other countries, would be most valuable and would provide significant information.

The vectors for grapevine yellows other than FD are still unknown, and their identification would likely provide an explanation of the geographic distribution of the diseases. In this respect, Phi-MLO was included in the present study because of its origin (CAUDWELL *et al.* 1971). The search for occasional or erroneous ampelophagous potential vectors could contribute to understand the transmission of some GY-MLOs. Finally, our results emphasize the need for

obtention of specific genomic and serological tools for further characterization and reliable detection of GY other than FD and to understand the processes of propagation and expression of the diseases.

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