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Genetic diversity of Italian and French "bois noir" phytoplasma isolates

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Abstract

Genetic diversity of "bois noir" phytoplasma (BNp) isolates was determined by PCR-RFLP of the stolbur specific *stol-1H10* gene encoding a putative membrane protein. BN-infected grapevines were collected from the most representative Italian and French wine producing regions. Weeds and vectors collected in infected vineyards from different Italian regions were also analysed for BN characterization. Size polymorphism of *stol-1H10* gene was obtained from PCR assays. Twelve different RFLP patterns were detected in BNp grapevine isolates, one in *Urtica dioca* and four in *Convolvolus arvensis* isolates. *Hyalesthes obsoletus* BNp isolates with two prevalent RFLP profiles were detected in grapevines and in herbaceous hosts from the same vineyards. *Stol-1H10* represents a useful non-ribosomal marker to type BNp in plant and insect hosts.

Key words: Grapevine, "bois noir", *stol-1H10,* PCR-RFLP.

Introduction

Phytoplasmas belonging to the 16SrXII-A ribosomal subgroup are associated with several diseases of wild and cultivated plants such as grapevine "bois noir" (BN) or grapevine Vergilbungskrankheit (VK). The cixiid planthopper *Hyalesthes obsoletus* Signoret is the vector of BN phytoplasma (BNp) to grapevine in the Euro-Mediterranean region.

RFLP analyses of 16SrDNA evidenced low variability among stolbur isolates from different areas and host plants. However biological and genomic diversity have been evidenced. Periwinkle plants infected by different stolbur isolates can show different symptoms and variation in the chromosome size of stolbur isolates has also been reported (Marcone *et al*., 1999).

Based on PCR-RFLP analysis of non ribosomal *loci*, three VK isolates (tuf-type) have been recently characterised in Germany in the vector *H. obsoletus* as well as in infected grapevines and wild hosts (Langer and Maixner, 2004). Each VK isolate showed a specific association with an herbaceous host (*Urtica dioica*, *Convolvolus arvensis* and *Calystegia sepium*) and a vector population, so that three different natural cycles of BNp have been proposed for the disease in German vineyards.

In a previous work, genetic diversity of different periwinkle-maintained stolbur isolates was determined by PCR-RFLP of the *stol-1H10* gene encoding a putative membrane protein with no homologue in the '*Candidatus* Phytoplasma asteris" genome (Pacifico e*t al.,* 2006). In this work, PCR-RFLP of *stol-1H10* genes was used to investigate genetic diversity of French and Italian BNp isolates.

Materials and methods

Leaf samples were collected from 131 yellows symptomatic grapevines from seven Italian grapevine-growing regions. One hundred and nineteen French BN isolates, collected in 10 wine producing areas, were also included. *C. arvensis* and *U. dioica* samples were collected in BN-infected vineyards of North-western Italian regions. Adult *H. obsoletus* were sweep-collected from nettle in Lazio, Piemonte, and Valle d'Aosta and from bindweed in Piemonte.

Total DNA was extracted from 1.5 g of grapevine and *U. dioca* midribs and from leaves, stems and petioles of symptomatic *C. arvensis* plants (Marzachì *et al.,* 1999), and from single *H. obsoletus* individuals, fresh or stored in 70% ethanol at 4 °C (Marzachì *et al.*, 1998).

For preliminary BN diagnosis, DNA extracts were amplified with the universal ribosomal primers P1/P7 (Schneider *et al.*, 1995) followed by nested-PCRs with group-specific ribosomal primers R16S(I)F1/R1 (Lee *et al.*, 1994). To confirm each isolate as a member of the 16SrXII-A subgroup, nested PCR amplicons were digested with *Mse*I. Grapevines without symptoms and healthy *Catharanthus roseus* seedlings were used as negative control in PCR assays.

For BN characterization on the *tuf* gene, direct and nested PCR assays (Schneider *et al.*, 1997) were run as detailed in the original papers and VK type was then determined by RFLP analysis with *Hpa*II. Stolbur specific primers H10F1/H10R1 and H10F2/H10R2 (Pacifico *et al.*, 2006) were used to amplify the *stol-1H10* gene from Italian and French BN isolates. "V" type was determined by RFLP analysis of *stol-1H10* amplicons after digestion with *Rsa*I or *Alu*I.

Results

BN-specific RFLP profiles were identified from all symptomatic field-collected French and Italian grapevines, 90% *C. arvensis* samples, 25% *U. dioica,* and 39% *H. obsoletus*. No amplification was obtained from negative controls and weeds without symptoms.

Tuf gene was amplified from all Italian grapevines, 90% *C. arvensis*, 25% *U. dioica*, and 86% *H. obsoletus* individuals. RFLP analysis showed the presence of VK types I and II in grapevines from Piemonte and Valle d'Aosta. VK type II was detected in grapevines from Sicilia, Sardegna and Toscana, while VK type I was found in Lazio and Liguria. VK type I was present in *U. dioica* in Valle d'Aosta while in *C. arvensis* from Piemonte only VK type II was detected. Only VK type I was detected in *H. obsoletus* BNp isolates collected on nettle in Lazio, Piemonte and Valle d'Aosta. VK type II was detected in vectors sampled on *C. arvensis* in Piemonte.

Stol-1H10 amplicons of two different sizes (1,570 bp and 1,820 bp) were amplified from French grapevines. Amplicons of three different sizes were obtained from Italian grapevines (1,570 bp, 1,820 bp and 2,070 bp). RFLP analysis with *Rsa*I and *Alu*I of grapevine *stol-1H10* amplicons evidenced 12 distinct patterns (V1-V12 types). V3 and V5 profiles were unique to VK type I isolates, V1 was present both in VK type I and II isolates, while the remaining V profiles were associated with VK type II isolates.

Middle sized PCR amplicons were obtained from weeds and vectors captured on *U. dioica*. Large amplicons were only obtained from *C. arvensis* from Piemonte and three vector individuals collected on bindweed from the same Region. Isolates from BN-infected *U. dioica* sampled in Valle d'Aosta and from 98% of insects collected on this species were characterized as V3. *C. arvensis* BNp isolates were characterized as V9, V10, V11 or V12 types. BNp isolates from vectors collected on bindweed were all characterised as V12 type.

Discussion

Non-ribosomal markers are needed for a finer molecular differentiation of phytoplasmas within the subgroup 16SrXII-A. As all *Mollicutes,* phytoplasmas lack a cell wall so that membrane proteins play a central role in regulating phytoplasma-host interactions. Variation in size, sequence and structural organization of membrane proteins may therefore reflect isolate-related biological and ecological properties such as association to different plant species or vector populations. *Stol-1H10* gene represents a genetic marker more variable than other non ribosomal markers, due to its size polymorphism and variability in restriction sites.

At least three different *stol-1H10* V types are associated with a single VK type. The highest level of *stol-1H10* genetic variability is associated with BNp isolates characterized as VK type II. BNp isolates from grapevine showed the highest variability, followed by isolates from *C. arvensis*; the lack of variation in isolates from *U. dioica* may reflect a strict association of this plant with the isolate. Inaccurate evaluation of the diversity of BNp isolates from this species, due to the low number of affected plants found in our surveys, cannot be ruled out. Nevertheless, only two prevalent V types were present in different vector populations collected where *U. dioica* or *C. arvensis* were the predominant host species and from plant samples from the same vineyards. Our result shows that PCR-RFLP analysis of the *stol-1H10* gene represents a suitable tool for the molecular characterization of stolbur phytoplasmas associated with grapevine BN disease.

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