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'Flavescence dorée' phytoplasma strain differentiation in the translocase (secY) gene

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Abstract

'Flavescence dorée' (FD) associated phytoplasmas are molecularly distinguished in strains belonging to 16SrV-C and -D subgroups that are geographically separated in the majority of the European countries where the disease was reported. While the subgroup differentiation on the 16S ribosomal gene is quite stable, RFLP analyses on the translocase gene allow differentiation of subtypes in both subgroups confirming ability of FD phytoplasmas to rapidly differentiate lineages with possibly diverse aggressiveness.

Key words: 'Flavescence dorée', secY gene, strain differentiation, epidemiology.

Introduction

'Flavescence dorée' (FD) is a quarantine phytoplasma disease in Europe still expanding its geographical distribution despite all the quarantine measures applied in many countries over the last 20 years. The major problem viticulturists are facing is the great ability of phytoplasmas associated with this disease to differentiate new strains in short periods of time. The molecular differentiation of FD strains present in diverse grape growing areas where the disease is present, is therefore of major relevance towards a correct disease management.

Several molecular markers were employed to differentiate FD strains after the first identification of two subgroups in 16S ribosomal gene (Bertaccini *et al*., 1997; Martini *et al*., 1999). One of the most informative and widely employed was shown to be the translocase gene (SecY) (Martini *et al*., 2002; Botti and Bertaccini, 2007; Arnauld *et al*., 2007) therefore a molecular comparison among several FD strains from various locations in Italy and in Serbia, collected in different years was carried out.

Materials and methods

Selected grapevine samples collected in Italy and in Serbia in the period 2002-2010 were employed to verify presence of variability in the secY gene. After total nucleic acid extraction PCR/RFLP analyses on 16S ribosomal gene were carried out to distinguish between 16S ribosomal subgroups 16SrV-C and 16SrV-D. The strains were further examined by PCR/RFLP analyses on SecY (translocase) genes (Martini *et al*., 2002; Angelini *et al*., 2001). To further evaluate variability two strategies (I and II) were employed. In (I) selected FD9f3/r2 amplicons from 16SrV-C strains were purified and cloned by InsT/Aclone PCR Product Cloning Kit (Fermentas, Vilnius, Lituania) specific for PCR fragments. Recombinant clone selection was carried out by PCR amplification using universal M13f/r primers on the colonies and/or nested PCR on M13f/r amplicons diluted 1:30 in sterile water with primers used to generate the PCR fragments cloned. Amplicons obtained from 4 to 20 colonies per cloned fragment were then subjected to RFLP analyses with *Alu*I, *Tru*I, *Taq*I, and *Tsp509*I restriction enzymes (Fermentas, Vilnius, Lithuania) to verify consistence of restriction profiles with those derived from the non-cloned amplicons. In strategy (II) selected FD9f3/r2 amplicons from 16SrV-D strains were produced with nested-PCR amplification carried out at least 3 times from each amplicon obtained from direct PCR and then RFLP analyses were carried out on all amplicons with the above listed enzymes in order to verify profile consistency.

Results

The examined strains belonging to subgroup 16SrV-C showed RFLP polymorphisms with *Tru*I and *Taq*I restriction enzymes on SecY gene that resulted partially related to their geographic origin. In particular strain differentiation was achieved for samples from Serbia and Italy and their tentative grouping showed identity between strains from Aleksandrovac (Serbia) and Tuscany (Italy). RFLP profiles identity was present also among samples from Niš, Irig (Serbia) and Veneto (mainly Treviso province) (Italy). Further RFLP profiles differentiable from each other, and from all the previous ones were also identified in samples from Niš and Irig.

The RFLP analyses on SecY gene from 16SrV-D strains collected in Veneto region show identical profiles with *Tru*I, *Tsp509*I and *Taq*I restriction enzymes with reference strain FD-88 from France (kindly provided by E. Boudon-Padieu). This profile was clearly

differentiable from the one identified in the majority of samples from Emilia (Lambrusco varieties) (Italy).

Some strains from Italy (VR32 and PC4, collected in 2002 in Veneto and Emilia, respecively) showed a 16SrV-C profile in 16S ribosomal gene, but the profiles on SecY gene were either consistent with 16SrV-C or – D, according with the enzyme used. Cloning of strains PC4 and VR32, showed RFLP profiles not always confirming those detected in the non cloned amplicons, although the PCR products were of the expected length, however the most frequent profile was identical to the one of the not cloned amplicons (figure 1). The profile comparison allow to verify that one of the profiles obtained from cloning of sample PC4 is undistinguishable from profiles obtained with the same restriction enzyme from samples of Lambrusco varieties in 2009 and 2010 samples (figure 1). On the other hand the use of strategy II allow the identification of polymorphic profiles in different PCR assays from the same direct amplicons in both samples from Emilia and from Veneto (data not shown).

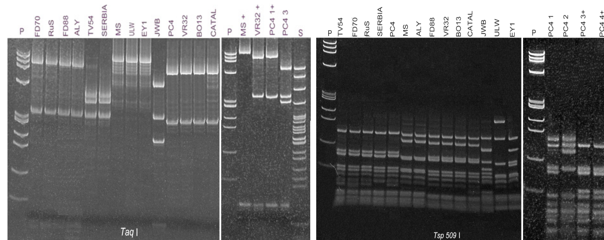

Figure 1. RFLP profiles obtained from FD strains and controls on secY compared withthose on cloned amplicons. +, most common profile in 2002 samples that was retrieved in 2009-2010 samples from Emilia.

Discussion

The RFLP analyses on nested amplicons and on clones or on repeated amplicons allow to verify the presence of variability on SecY gene from 16SrV-C strains from Italy and Serbia and on 16SrV-D strains collected in Veneto and in Emilia. The results not only confirm the strain differentiation in SecY gene of FD-D type phytoplasmas according with geographic distribution and variety (Bertaccini *et al.*, 2009) but also the temporal differentiation of strain population in different geographic areas of Italy. The presence of heterogeneous profiles in the different colonies obtained after cloning and in amplicons from the same template suggests the presence of further variability not observed in 'regular' amplicon RFLP analyses.

These results can be explained with the presence of a population of distinguishable phytoplasmas infecting the same plant (Angelini *et al.*, 2004). The repeated finding of profiles only detected in one clone of a FD-C strain from 2002 in FD-D strains from 2009 and 2010 in the same region indicates the increase in a few years of a phytoplasma population barely detectable about 10 years ago. The results indicate that now this phytoplasma is prevalent in some of the areas where the disease is spread. The presence of phytoplasma population is not very easy to be detected in routine sample analyses, but it is clear that a mixture of heterogeneous phytoplasmas colonizing new environments, or the residues of old phytoplasma populations, that lost transmissibility or virulence characteristics can play important roles in FD epidemiology.

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