

Molecular characterization of phytoplasmas in Chilean grapevines

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Abstract

Extensive survey to detect and identify phytoplasmas associated with Chilean grapevines was performed from 2003 to 2005 sampling symptomatic or asymptomatic grapevine plants. Nested PCR amplification allow the detection of phytoplasmas in 28 out of 90 samples. RFLP analyses identified phytoplasmas as belonging to ribosomal subgroups 16SrI-B, 16SrI-C, 16SrVII-A and 16SrXII-A. Cloning and sequencing of selected strains allow to detect genetic variability in 16SrXII-A strains.

Keywords: Phytoplasmas, grapevine yellows, nested-PCR, RFLP, sequencing.

Introduction

Grapevine (*Vitis vinifera* L.) is widely cultivated in Chile and visual detection of yellows symptoms was reported (Caudwell, 1988). More recently, plants with symptoms of grapevine yellows (GY) in both table and wine grape varieties were observed and preliminary phytoplasma identification was reported (Gajardo *et al.*, 2003). Further studies carried out on selected phytoplasmas allow to verify the presence of genetic variability in 16S ribosomal gene of Chilean grapevine strains (Bertaccini *et al.*, 2004).

To identify phytoplasmas present in symptomatic and/or asymptomatic grapevines, surveys were intensified in five regions of Chile (Coquimbo, Valparaíso, Metropolitana de Santiago, Libertador General Bernardo O'Higgins, Maule), where the main grapevine production is concentrated.

Materials and methods

The optimal time-window to visualize yellows in Chile is from the end of January to middle of May. Samples from 90 symptomatic or asymptomatic grapevine plants belonging to table grape: Autumn Royal, Thompson Seedless, Crimson Seedless, and wine grape: Chardonnay, Petit Syrah, Syrah, Cabernet Sauvignon, Merlot, Carménère were collected from summer 2003 to summer/fall 2005. Samples consist of branches with mature leaves, from which both leaf mid-ribs and phloem were quickly separated, tissues that are then immediately frozen in liquid nitrogen and stored at -80°C .

Total nucleic acids were extracted from 1 g of mixture of main leaf midribs and phloem tissues (Prince *et al.* 1993), dissolved in Tris-EDTA pH 8 buffer, and maintained at 4°C ; 20 ng/ μl of nucleic acid were used for amplification.

After direct PCR with primer pair P1/P7, nested PCR with R16F2n/R2 primers (Gundersen and Lee, 1996) or

F1/B6 (Duduk *et al.*, 2004) was performed. Further nested PCR were performed with R16(I)F1/R1 (Lee *et al.*, 1995) and 16R_{758f}/16S_{1232r} (Gibb *et al.*, 1995) primer pairs. PCR and nested PCR reactions were carried out following published protocol (Schaff *et al.*, 1992). Identification of detected phytoplasmas was done using RFLP analyses on amplified ribosomal DNA fragments with *TruI*, *RsaI*, *HhaI*, *Tsp509I*, *TaqI*, *AluI* (Fermentas, MBI, Vilnius, Lithuania) restriction enzymes.

Selected F1/B6, R16F2/R2 and R16(I)F1/R1 amplicons from the diverse phytoplasmas identified after RFLP analyses were purified using Concert Rapid PCR Purification System (Life Technologies, Gaithersburg, MD, USA). DNA fragments were ligated into T/A cloning vector pTZ57R/T following the instructions of the InsT/Aclone PCR Product Cloning Kit (Fermentas, MBI, Vilnius, Lithuania). Putative recombinant clones were analysed by colony-PCR, sometimes in combination with the insert-specific primers. Amplicons obtained from 5 colonies per cloned fragment were subjected to RFLP analyses, as described above. Selected fragments from cloned DNAs were sequenced in both directions using the BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK). The sequences were then aligned with BLAST engine for local alignment (version Blast N 2.2.12).

Results and discussion

Positive results were obtained only after nested amplification on P1/P7. Among the 90 samples tested 28 resulted infected by phytoplasmas. The R16F2n/R2, R16(I)F1/R1 and/or 16R_{758f}/16S_{1232r} nested PCR products were subjected to RFLP analysis that allow the assignment of phytoplasmas to different ribosomal subgroups: 16SrI-B (related to Aster yellows), 16SrI-C (related to Clover Phyllody), 16SrVII-A (related to Ash yellows) and 16SrXII-A (related to Stolbur). In particular 16SrXII-A phytoplasmas were identified in 10 sam-

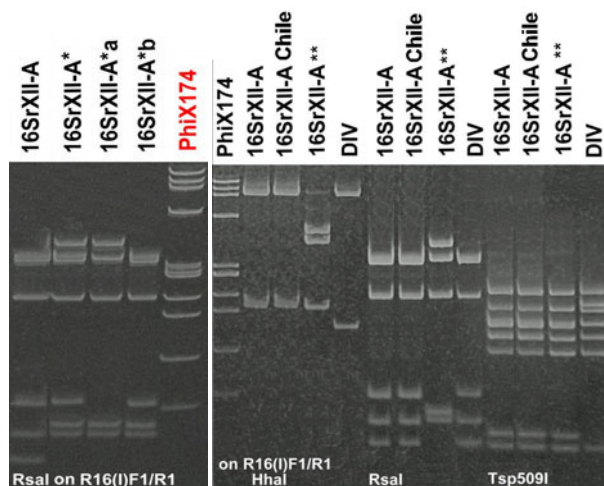


Figure 1. RFLP profiles of phytoplasmas 16SrXII-A from Chilean grapevines. Controls: 16SrXII-A; DIV, *Diplotaxis virescence* (16SrI-B).

ples, 16SrXII-A and 16SrI-B were present in mixed infection in 1 sample, 16SrVII-A was detected in 8 samples, and mixed infection of 16SrI and VII-A (2 samples) and 16SrI-B and 16SrVII-A (1 sample), group 16SrI was also identified in 4 samples. A Chilean isolate referred after RFLP to 16SrXII-A was sequenced and deposited in GenBank (DQ177148).

In one further sample a *RsaI* variability in the RFLP profile of R16(I)F1/R1 was observed; molecular variability was also present in different cloned fragments from the same PCR product. After RFLP with *RsaI*, *HhaI*, *TruI*, and *Tsp509I*, two molecular 16SrDNA variants were identified, revealing that the new profile was a mixed population of two phytoplasmas named 16SrXII-A*a and 16SrXII-A*b (figure 1). After sequencing both phytoplasmas showed 98% of homology with phytoplasma AF248959 clone STOL11 (16SrXII-A). They were submitted to GenBank with the accession code AY739654 and AY739653. A last positive sample, named 16SrXII-A**, collected in the same vineyard as the previous one, showed *HhaI* and *RsaI* variability (figure 1). Sequencing of relevant strains from ribosomal subgroup 16SrI-B (AY741532 and DQ177149) and 16SrVII-A (AY741531, DQ194355 and DQ232752) showed respectively 99-100% homology with 16SrI-B phytoplasma sequences world wide from different hosts, and 97-99% similarity with Ash yellows strains from USA, Colombia, Argentina and Brazil in different hosts.

During the extensive survey performed no evidence of “flavescence dorée” was verified; 16SrXII-A phytoplasmas are worldwide spread in viticultural areas therefore their detection in Chilean grapevine was not unexpected, however the presence of variability in the 16Sr gene was never reported before for this phytoplasma (Bertaccini *et al.*, 2006). The finding of 16SrVII-A phytoplasmas is new for grapevine while the phytoplasma appear to be mainly detected in America

areas in different host species. The 16SrI-B and 16SrI-C phytoplasmas were already reported in some vineyards in Italy where no clear relationship with symptoms was determined. There is no evidence of epidemic spreading of yellows symptoms in the surveyed vineyards, however survey to verify presence and identity of insect vectors or potential vectors are in progress.

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