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Occurrence of a new stolbur strain in tobacco in Serbia

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Abstract

Stolbur phytoplasmas are associated with several important diseases on different crops worldwide. Although stolbur phytoplasmas are known to have low variability in 16S rDNA, some RFLP and single nucleotide polymorphisms among them were reported. To verify the presence and determine the identity of phytoplasmas present in tobacco in Serbia, PCR-RFLP and sequence analyses were performed on DNA extracts from 17 symptomatic plants. RFLP profiles of all positive samples except one were identical to those of 16SrXII-A subgroup, while strain 284/09 showed slightly different profile than others. The obtained results confirm presence and also show that there is variability in 16S rDNA among stolbur phytoplasmas in tobacco in Serbia. The revealed SNP was never reported in stolbur phytoplasmas before and is not present in any sequence deposited in the Genbank, which results in a unique RFLP profile. It is confirmed that the SNP is inside the 16S rDNA and is confirmed with PCR-RFLP analyses on three separate extractions, of which one was of the seedling after grafting with infected plant tissue. Relation of the SNP in the 16S rDNA with possible variations in other marker genes or some ecological properties of the strain are still to be defined.

Key words: stolbur, *Nicotiana tabacum*, PCR/RFLP.

Introduction

Stolbur phytoplasmas (16SrXII-A) are associated with several important diseases of both annual and perennial crops worldwide. In Serbia stolbur phytoplasmas were also reported in various plants and are associated with several economically important diseases, such as bois noir, corn reddening, stolbur on pepper (Martinović and Bjegović, 1950; Duduk *et al*., 2004; Duduk and Bertaccini, 2006). Although stolbur phytoplasmas are known to have low variability in 16S rDNA, some RFLP and single nucleotide polymorphisms (SNPs) among them were reported (Quaglino *et al*., 2009). However, non ribosomal DNA was also often tested for variability among stolbur phytoplasmas (Langer and Maixner, 2004; Pacifiko *et al*., 2006).

Survey for phytoplasma presence, identification and possible variability in tobacco in Serbia was performed.

Materials and methods

To verify the presence and determine the identity of phytoplasmas present in tobacco, molecular assays were performed on DNA extracts from 17 symptomatic plants collected during 2009 in Ečka, Serbia. Total DNA extraction was performed using CTAB protocol described by Angelini *et al*. (2001). Polymerase chain reaction (PCR) was performed, for amplification of phytoplasma 16S rRNA gene, spacer region and part of 23S rRNA gene, using phytoplasma-universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995). The reaction conditions were as reported in literature (Lee *et al*., 1995). Products amplified by PCR assays were visualised and the positive ones were subjected to the restriction fragment length polymorphism (RFLP) analysis. *Tru1*I (Fermentas, Vilnius, Lithuania) restriction endonuclease was used, according to the manufacturer's instructions. For a selected strain (284/09) nucleic acids extraction and PCR-RFLP analyses was repeated on the same plant and on a seedling plant after stem tissue grafting and symptoms appearance. The P1/P7-amplified products of two selected samples, were purified using Metabion mi-PCR purification kit (Metabion International AG, Martinsried, Germany) and sequenced in both directions with two forward primers P1 and R16F2 (Lee *et al*., 1995) and one reverse primer P7, using the BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK). The sequences were assembled using Pregap4 from the Staden program package (Staden *et al*., 2000), aligned using Clustal X (Thompson *et al*., 1997) and searched for SNPs in Bioedit program (Hall, 1999). The obtained sequences were compared with 16Sr sequences of phytoplasmas in the GenBank database using blast (v. Blast N 2.2.18) at the National Center for Biotechnology Information.

Results

RFLP profiles of all positive samples except one, were identical to those of 16SrXII-A subgroup (Lee *et al*., 1998), while strain 284/09 showed slightly different profile than others (figure 1a), suggesting the presence of a new restriction site. To verify this finding new extraction was carried out and the results were confirmed. In order to maintain the strain and to verify persistence of this polymorphism, grafting of the plant material was performed on a tobacco seedling. One month after grafting and symptoms expression, nucleic acids were extracted and the presence of the same strain in grafted plant was confirmed (figure 1b).

Alignment of the sequences obtained from two samples 142/09 and 284/09 (1,704 and 1,703 bp respectively) showed SNP only on one position [184 (A/G)] which is a recognition site for the *Tru*I restriction enzyme and is inside of the 16SrRNA gene.

Blast search of 142/09 and 284/09 phytoplasma 16S

rDNA sequences showed 100% and 99% respectively, homology with a stolbur phytoplasma strain from potato from Russia (EU344887). It was also observed that on the position 184, nt A was present only in 284/09 strain, while 142/09 and all other sequences of stolbur deposited in the GenBank had nt G in that position.

Tobacco seedling grafted with 284/09, together with the sequenced field infected sample (142/09) was transferred to *in vitro* in MS medium and deposited to Phytoplasma collection at the Plant Pathology, DiSTA - *Alma Mater Studiorum* - University of Bologna, Italy (Bertaccini, 2010).

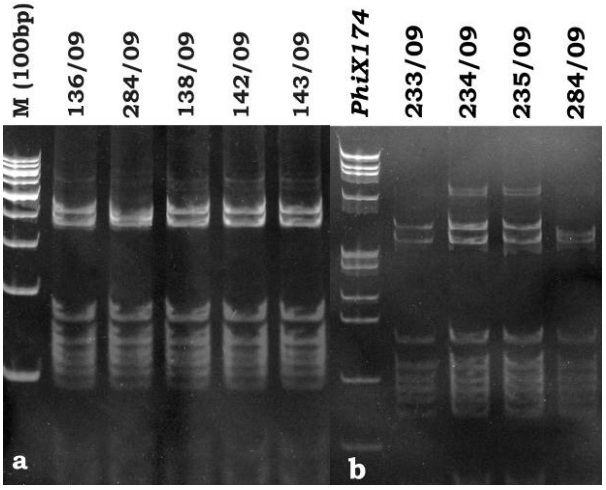

Figure 1. Differential *Tru*I RFLP profiles obtained from P1/P7 amplicons of phytoplasmas from tobacco.

Discussion

The obtained results confirm presence of stolbur phytoplasma in tobacco plants in Serbia. It is also shown that there is variability in 16S rDNA among stolbur phytoplasmas in tobacco in Serbia. While strain 142/09 has regular stolbur RFLP profile with 100% homology of 16S rDNA sequence with a stolbur strain from the Genbank, the strain 284/09 represents a variant of stolbur phytoplasma with a SNP on *Tru*I restriction site. The SNP on the position 184 was never reported in stolbur phytoplasmas before and is not present in any sequence deposited in the Genbank, which results in a unique RFLP profile. It is confirmed that the SNP is inside the 16S rDNA (also inside the 16RF2/R2 amplified region) and is confirmed with PCR-RFLP analyses on three separate extractions, of which one was of the seedling after grafting with infected plant tissue.

Relation of the SNP in the 16S rDNA with possible variations in other marker genes or some ecological properties of the strain are still to be defined.

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