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Molecular diversity in '*Candidatus* Phytoplasma mali' in Lombardia

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Abstract

Apple proliferation (AP) was first described in Italy in 1950, and occurs in many European pome fruit growing areas. In the last years new epidemics of the disease were reported in northern Italian regions. At least three phytoplasma subtypes have been reported in Europe, classified on the basis of the polymorphisms detected in the nitroreductase gene of '*Candidatus* Phytoplasma Mali'. This work reports the results of the presence of AP in Lombardia and the characterization of the phytoplasma ribosomal protein subtypes associated with the disease in this region.

Key words: Apple proliferation phytoplasma, rpl22-rps3 genes, PCR/RFLP analyses, Lombardia, Italy.

Introduction

Apple proliferation (AP), first described in Italy in 1950, occurs in many European apple fruit growing areas. In the last years, new epidemics of the disease were reported in Italian northern regions, such as Friuli-Venezia Giulia, Veneto, Trentino Alto Adige and Valle d'Aosta.

AP is a quarantine phytoplasma disease of economically important relevance, which causes vigor depletion of the trees whose fruit cannot be commercialized because of their poor organoleptic qualities. Phytoplasmas are spread in a persistent-propagative manner by phloem-feeding insects; psyllids have a crucial role in the transmission of phytoplasmas belonging to the apple proliferation ribosomal group, in particular *Cacopsylla picta* (Förster) in northeastern Italy and *Cacopsylla melanoneura* (Förster) in northwestern Italy.

Classification of phytoplasmas is based on molecular analysis of 16S ribosomal gene (16SrDNA). The causal agent of AP was proposed to represent the '*Candidatus* Phytoplasma mali' ribosomal group (16SrX). Recent studies on a non-ribosomal fragment, which is composed of three putative genes (PR-1, PR-2 and PR-3), have suggested that there is high genetic variability among 16SrX phylogenetic group, and less variability between AP phytoplasma isolates. PCR amplification of PR-2/PR-3 spacer and PR-3 gene, followed by restriction analysis of the PCR products, has allowed to evidence differences between '*Ca*. P. mali' strains. Indeed, three AP subtypes, named AT-1, AT-2 and AP, were described (Jarausch *et al*., 2000).

The aims of this study were therefore to evaluate the presence of AP in apple growing areas of Lombardia.

Materials and methods

During 2006, collections of symptomatic and asymptomatic plants were carried out in different apple orchards in Sondrio, Como, Brescia and Mantova provinces.

Total DNA was extracted from each sample using 500 mg of ribbings or wood.

Phytoplasma detection and molecular characterization was obtained by nested PCR assay and RFLP analysis on three different genome fragments: 16SrDNA, ribosomal protein and putative nitroreductase gene. Identification on the basis of 16SrDNA gene was conducted with the universal primer (r16)-P1/P7 (Lee *et al*., 1998), for the first round of PCR, followed by a second cycle with specific primers R16fAT/rAS and R16(X)F1/R1 (Smart *et al*., 1996; Lee *et al.,* 1995).

For amplification of the putative nitroreductase gene primers AP10/AP13 and AP14/AP15 (Jarausch *et al*., 1994; Jarausch *et al*., 2000) were used, respectively for direct and nested PCR assay. Rpl22 and rps3 genes were amplified according to Martini (2004) and Martini *et al.* (2005), with rpL2F3/rp(I)R1A and rpAP15f/rpAP15r primers. In all PCR tests positive controls (AT-1 and AP-15 phytoplasma isolates from infected periwinkle) and negative controls (DNA extracted from apple seed and the sample without DNA) were used. Results were visualized by electrophoresis in agarose gel.

RFLP analysis was conducted on the amplicons obtained from each sample. In particular DNA fragments of 16SrDNA gene were digested with *Ssp*I endonuclease, putative nitroreductase gene with *Hinc*II and *Pag*I endonucleases and rpl22-rps3 fragments with *Alu*I.

Polymorphisms were visualized after polyacrylamide gel electrophoresis.

Results

Table 1 reports the results of the examined samples: '*Ca*. P. mali' was the only phytoplasma identified in our field samples on the basis of 16SrDNA analysis (PCR and RFLP analysis). AT-1 subtype was the only subtype which was identified in apple tree samples, harvested in four different areas of Lombardia region. RFLP analysis

Table 1. Results of the PCR/RFLP assays conducted on field samples collected from AP diseased plants in different provinces of Lombardia region.

Province	no. of samples	16srDNA gene	Putative nitroreductase gene	Ribosomal protein genes
Brescia	4	16SX-A	AT-1	rpX-A
Como	10	16SX-A	AT-1	rpX-B / rpX-C
Mantova	2	16SX-A	AT-1	rpX-C
Sondrio	12	16SX-A	AT-1	rpX-A / rpX-C /rpX-D

on ribosomal protein gene with *Alu*I endonuclease enzyme showed the presence of four different profiles, rpX-A, rpX-B, rpX-C and rpX-D, very similar to those reported by Martini *et al.*, 2007.

Discussion

The present work shows the preliminary results obtained about the aetiology of apple proliferation in different areas of Lombardia region. Only '*Ca.* P. mali' was identified in symptomatic plants on the basis of 16S ribosomal gene analysis. Moreover, only AT-1 subtype was found in all samples on the basis of the *Hinc*II and *Pag*I restriction analysis on the putative nitroreductase gene. Higher variability was observed with rpl22-rps3 gene analysis. In fact, RFLP patterns with *Alu*I enzyme identified four different phytoplasma subtypes according to those reported by Martini *et al.* (2007): rpX-A, rpX-B, rpX-C and rpX-D.

Further investigation will be carried out in order to evaluate the frequency and the distribution of the phytoplasma subtypes identified in AP affected apple trees. Also, field observations will be conducted about the presence and distribution of possible phytoplasma vectors of AP in Valtellina (Sondrio) and in other areas of Lombardia.

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