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Molecular characterization of a phytoplasma causing Phyllody in clover and other herbaceous hosts in Northern Italy

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Abstract

Red clover (*Trifolium pratense*) and Ladino clover (*Trifolium repens*) plants showing phytoplasma-associated symptoms (yellowing/reddening, virescence and phyllody) have been recovered in Friuli-Venezia Giulia, Italy. Using *Alu*I RFLP analysis of PCR amplified 16S rDNA we showed that the disease can be caused independently by two phylogenetically distinct phytoplasmas. One of them showed the very typical 16S rDNA RFLP pattern of the agent of Clover Phyllody in Canada (CCPh). The 16S rDNA of the other phytoplasma (Italian Clover Phyllody phytoplasma, ICPhp) has been PCR amplified, cloned and sequenced. The sequence revealed high similarity (>98%) with phytoplasmas belonging to the X disease cluster, which includes organisms not reported to cause phyllody on their hosts. The analysis by *Alu*I RFLP of the PCR amplified pathogen 16S rDNA from other herbaceous plants (*Crepis biennis, Taraxacum officinale, Leucanthemum vulgare*) collected nearby with phytoplasma-associated symptoms showed similar patterns. Southern blot hybridization of their *Eco*RI digested total DNA revealed identical RFLP patterns, suggesting that the causative agent may be the same organism.

Abbreviations: PCR – Polymerase Chain Reaction; rDNA – gene for the small subunit ribosomal RNA; RFLP – Restriction Fragment Length Polymorphism.

Introduction

The recently introduced trivial name 'phytoplasmas' refers to a group of mycoplasmalike organisms which cause diseases in several hundred plant species (McCoy et al., 1989). Clovers (*Trifolium* spp.) may be affected by several phytoplasma diseases, which have been named according to the symptoms they induce. The Clover Yellow Edge (CYE) floral symptoms consist of a reduction in colour and size, but never phyllody or virescence (Chiykowski, 1981). Conversely virescence and phyllody are typical symptoms of Clover Phyllody (CPh) (Chiykowski, 1962; Krczal, 1960). The phytoplasmas associated with the named diseases have been transmitted to a variety of experimental hosts, including periwinkle (*Catharantus roseus* L.), and the symptoms shown on those hosts were consistant with those shown on the natural host (Chiykowski, 1962, 1981). In addition to the former ones, clover has been reported to host other phytoplasma diseases (i.e. Clover Proliferation, Chen and Hiruki, 1975), including diseases typical of woody plant such as the X disease (Chiykowski and Sinha, 1982).

By the introduction of molecular techniques it has been shown that CYE and CPh are caused by taxonomically distantly related phytoplasmas. In the 16S rDNA *Alu*I restriction analysis carried out by Schneider et al. (1993) a European isolate of CPh (KV) showed a pattern identical to typical Aster Yellows strains. In a similar work, Lee et al. (1993) found that a Canadian isolate of CPh (CCPh) differed from the typical Aster Yellows strains by having an unique pattern due to the absence of an *Alu*I site in only one of its two rRNA genes, while CYE was related to the X disease phytoplasmas. Finally, Gundersen et al. (1994) examined CYE and CCPh by 16S rDNA sequencing and assigned

them to their cluster (iv) (X disease and related strains) and (i) (Aster Yellows and related strains) respectively. These results were in agreement with those obtained by Southern blot hybridization studies (Lee et al., 1992a; Lee et al., 1992b).

While CYE was only recovered in North America, CPh was reported in Europe (Carraro et al., 1991; Krczal, 1960). It is widely spread in Northern Italy, where *T. repens* and *T. pratense* are often infected. We found that only part of the clover showing phyllody and virescence symptoms in Northern Italy were actually affected by a phytoplasma related to a previously reported agent of CPh (CCPhp). In this paper we reported the characterization by 16S rDNA sequence analysis of the additional phytoplasma which causes Clover Phyllody in Northern Italy and which is called Italian Clover Phyllody phytoplasma (ICPhp).

Materials and methods

Origin of samples

About 50 samples of clover (*Trifolium pratense* L. and *T. repens* L.), *Leucanthemum vulgare* L., *Taraxacum officinale* Weber and *Crepis biennis* L. showing yellowing, virescence and phyllody symptoms were collected during 1992, 1993 and 1994 from different sites in the region Friuli-Venezia Giulia (Italy), mostly from two sites named Cussignacco and Cavazzo Carnico. The phytoplasmas were transmitted from the natural herbaceous host to periwinkles by a dodder bridge (Osler et al., 1994). The following phytoplasma infected periwinkles were also included as reference in the 16S rDNA-RFLP study: CCPh phytoplasma (CCPh) (donated by Dr. T.A. Chen, Dept. of Plant Pathology, Rutgers University, New Brunswick), Western X disease phytoplasma (WX), and Aster Yellows phytoplasma (donated by Dr. E. Seemüller, Institut für Pflanzenschutz im Obstbau, Dossenheim, Germany).

DNA extraction and amplification

Nucleic acids were extracted from 300 mg of plant tissue using previously published methods (Firrao et al., 1993; Firrao and Locci, 1993). Due to the presence of *Taq* polymerase inhibitors, plants other than clover and periwinkle were extracted with phenol followed by chlorophorm:isoamyl alchol (24:1). The DNA was then recovered using the Gene Clean kit (BIO101, La Jolla, CA). The 16S rDNA amplification by polymerase chain reaction was carried out using the protocol and the primer pair P1/P4 according to Firrao et al. (1993). Three μl of the PCR products were run on an 1% agarose gel in the presence of 0.5 $\mu g\ ml^{-1}$ ethidium bromide to quantify the amount of DNA amplified.

The product of the amplification with P1/P4 spawns a region of the phytoplasma 16S rDNA which includes the restriction endonuclease *Alu*I recognition sites named c, d, e, f, g, and h by Schneider et al. (1993).

Amplified 16S rDNA-RFLP

Four to 15 μl of the PCR product were digested with 5U *Alu*I endonuclease for three hours and then loaded onto a 1% GTG + 3% NuSieve agarose (FMC, Rockland, ME) gel in TBE (89 mM Tris-borate, 2 mM EDTA) buffer. The gel was run for 5 h at 7 Vl cm^{-1}, then stained with ethidium bromide and visualized under UV light.

Cloning and sequencing

The product of a PCR amplification from ICPh infected periwinkle DNA carried out using primers P1 and P4 (Firrao et al., 1993) was incubated with 2U of *E. coli* polymeraseI Klenow fragment (Boehringer Mannheim, Germany) for 15 min in the presence of 0.1 mM dNTPs. The reaction was electrophorized on an agarose gel and the 860bp DNA fragment was purified using the Gene Clean kit (BIO101, La Jolla, CA). It was then ligated to a *Sma*I-cut pBluescript plasmid vector and transformed in *E. coli* DH5α competent cells. A recombinant plasmid was chosen for subcloning and sequencing, which were carried out according to standard techniques (Ausubel et al., 1992). From the resulting sequence a specific oligonucleotide was synthesised and used in conjunction with the primer P1 of Deng and Hiruki (1991), to amplify a 550 bp fragment corresponding to the 5′-end of ICPh 16S rDNA. This DNA fragment was cloned and sequenced using the same methods used for the 860 bp fragment. The sequence of the 3′-end was obtained together with that of the 16S/23S spacer region (Kirkpatrick et al., 1994) and provided by courtesy of C. Smart (Dept. of Plant Pathology, University of California, Davis, CA).

Sequence analysis was carried out using the package developed by the Genetic Computer Group at the University of Wisconsin (Devereux et al., 1984). The phylogenetic analysis was performed using the bootstrap option of the program PAUP (phylogenetic analysis using parsimony) for Macintosh (Swofford, 1991).

Chromosomal RFLP
Total nucleic acids were extracted from the periwinkles infected by the phytoplasma transmitted from infected herbaceous plants (*Trifolium repens, Crepis biennis, Taraxacum officinale, Leucanthemum vulgare*) using the Doyle and Doyle (1990) method. After RNase treatment and ethanol precipitation, about 5 μg of total DNA were digested with 10U *Eco*RI for 4 h, then loaded in an 1% agarose gel in TBE buffer and run for 12 h at 2.5 V cm^{-1}. The Southern blot was carried out according to the standard protocol (Ausubel et al., 1992) and the hybridization with digoxigenin-labelled probes following the manufacturer's instruction (Boehringer Mannheim, Germany). A mixture of 4 Western X disease phytoplasma cloned chromosomal fragments (Kirkpatrick et al., 1987), kindly supplied by B.C.Kirkpatrick (University of California, Davis, CA), was used as the probe.

Results

16S rDNA-RFLP
The PCR amplification of DNA extracted from symptomatic clover plants using the primer pair P1/P4 resulted in the amplification of DNA fragments of about 860 bp (not shown). The amplification products were then digested with *Alu*I restriction endonuclease and the samples subdivided into two different groups according to the RFLP pattern obtained. The first group (lane 3 of Figure 1) included the samples which gave a RFLP pattern similar to that obtained by digestion of the PCR amplified 16S rDNA of the CCPhp (lane 5 of Figure 1), and the second group (lane 2 of Figure 1) the samples with RFLP patterns similar to that of the WXp (lane 6 of Figure 1). All the samples collected from the site of Cussignacco showed a RFLP pattern of the second group. The samples collected in the site of Cavazzo Carnico showed RFLP patterns of both groups in 1992, but only of the first group in 1994 (no symptomatic clover samples could be collected in 1993 from this site). In one of the samples from Cavazzo Carnico, a RFLP pattern consistent with a double infection was detected (lane 4 of Figure 1).

Phylogenetic position of ICPh
The entire 16S rDNA of ICPh was cloned and sequenced. The sequence was submitted to the GenBank – EMBL database with the accession number X77482. The comparison with recently obtained sequences of the 16S rDNA of various phytoplasmas

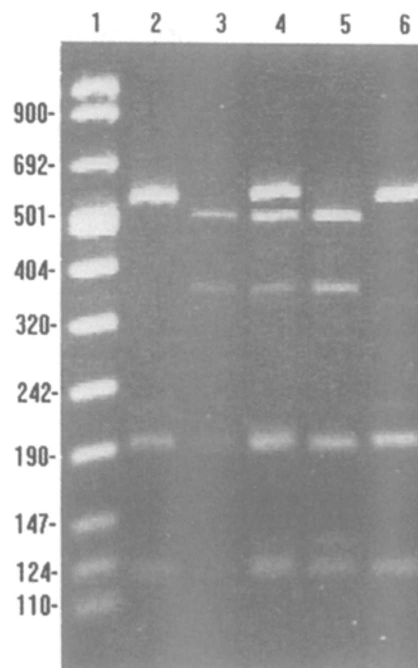

Figure 1. Agarose gel electrophoresis of the *Alu*I digestion of PCR amplified DNA extracted from different clover (lanes 2, 3 and 4) and periwinkle (lane 5: CCPh infected; lane 6: WX infected) plants. Lane 1: molecular weight marker.

(Gundersen et al., 1994; Kuske and Kirkpatrick, 1992; Lim and Sears, 1989; Namba et al., 1993; Seemüller et al., 1994) indicated high similarity with WXp, blueberry witches' broom phytoplasma (VACp), tsuwabuki witches' broom phytoplasma (TWp) and CYEp, ranging from 98.30% (with WXp) to 98.85% (with VACp). Although the significant number of differences suggests that ICPh should be taken as a different organism from CYEp (98.33% similarity), the phylogenetic analysis using PAUP showed that these two phytoplasmas isolated from clover are phylogenetically closely related (Figure 2).

Symptoms on clover and periwinkle
The two phytoplasmas causing Clover Phyllody in Northern Italy could not be distinguished by symptom evaluation on the natural host. The early symptoms were the yellowing/reddening of leaves, followed by virescence and phyllody. However differential symptoms were observed when both phytoplasmas were transmitted to periwinkle: ICPh infected plants had elongated leaves, while those infected by the CCPh related phytoplasma had round leaves. A detailed description of the symptoms caused on natural and

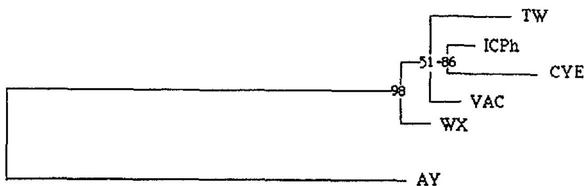

Figure 2. Phylogram resulting from bootstrap parsimony anylisis of 16S rDNA sequences of selected phytoplasmas. Numbers are bootstrap values. TW = Tsuwabuki witches' broom (accession no. D12580); ICPh = Italian Clover Phyllody (accession no. X77482); CYE = Clover Yellow Edge (accession no. L33766); VAC = Blueberry witches' broom (accession no. X76430); WX = Western X disease (accession no. L04682).

experimental hosts by the phytoplasmas isolated from herbaceous hosts in Friuli-Venezia Giulia is given elsewhere (Osler et al., 1994). The phytoplasmas from the donor plant and the periwinkle were identical as confirmed by 16S rDNA-RFLP (not shown).

Host range of ICPh

The same *Alu*I PCR-RFLP pattern obtained from ICPh infected clover was also generated by the digestion of the amplification products obtained from DNA extracted from *Leucanthemum vulgare*, *Taraxacum officinale* and *Crepis biennis* which occurred in the same area (not shown). The associated phytoplasmas were transmitted to periwinkles where symptoms similar to those caused by ICPh were produced (Osler et al., 1994). Identical RFLP patterns were also obtained by hybridization of the *Eco*RI digested DNA with a cocktail of cloned chromosomal fragments of WXp (Figure 3). Conversely the RFLP pattern of the WXp was completly different, suggesting that the genetic differences among ICPh and WXp were significant.

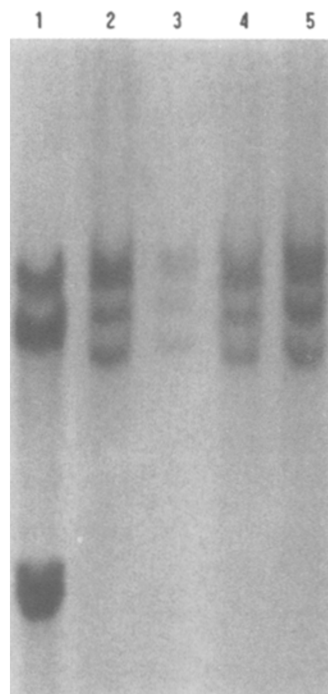

Figure 3. Southern blot hybridization of *Eco*RI digested DNA from periwinkle infected by WXp (lane1), and phytoplasmas transmitted from *Leucanthenum vulgare* (lane 2), *Taraxacum officinale* (lane 3), *Trifolium repens* (lane 4) and *Crepis biennis* (lane 5).

Discussion and conclusion

In the past years certain biological properties such as host induced symptom expression and vector specificity have been regarded as useful tools to distinguish phytoplasmas (Chiykowski and Shina, 1990; Kirkpatrick, 1991; Marwitz, 1990). More recently the taxonomy of this group of fastidious prokaryotes has been revised on the basis of the results obtained by nucleic acid hybridization and 16S rRNA sequencing (Davis et al., 1988; Gundersen et al., 1994; Seemüller et al., 1994). As a rapid method for distinguish phytoplasmas, the restriction endonuclease digestion of PCR amplified 16S rDNA has been suggested and widely applied (Lee et al., 1993; Schneider et al., 1993).

By the use of *Alu*I PCR-RFLP we showed here that the clover plants with symptoms of phyllody found in Northern Italy can actually be infected by two phylogenetically different phytoplasmas. One of the two phytoplasmas could be easily identified because it showed the very typical pattern of CCPh. This pattern is unique among the strains examined to date, its peculiarity being that the sum of the fragments derived from the *Alu*I digestion exceeded the size of the amplified fragment. In the case of the amplification with the primer pair P1/P4 the sum of the digested fragments is 1370 bp instead of the expected 860bp. It has been suggested that this is due to the presence in the CCPh chromosome of two 16S rRNA genes whose sequence differs in correspondence of an *Alu*I site (Lee et al., 1993). The second phytoplasma causing phyllody on clover appeared to be a newly discovered phytoplasma and it was classified, as suggested by the Subcommittee on the Taxonomy of Mollicutes of the International Committee on Systematic Bacteriology (Tully, 1993), by 16S rDNA sequence analysis.

It resulted to belong to the WXp cluster as defined by Seemuller et al. (1994), but clearly distinct from the other representatives of thise group. The phytoplasmas belonging to X disease cluster have been found predominantly in North America; their presence in Europe seemed to be restricted to the blueberry witches brooms phytoplasma. Recently several additional phytoplasmas have been identified which appear to be related to the WXp cluster on the basis of 16S rDNA- RFLP studies (Griffiths et al., 1994; Gundersen et al., 1996), but none of them is of European origin. Conversely, the ICPhp appears to be widely spread in Northern Italy. In a survey of plant pathogenic phytoplasmas infecting weeds in Friuli Venezia Giulia carried out using *Alu*I PCR-RFLP, several species were found infected by phytoplasma of the WXp cluster (Osler et al., 1994). Three of them, examined in this study, showed identical RFLP patterns when hybridized with a cocktail of four WXp chromosomal fragments. It is therefore likely that ICPh is very actively spread by insect vectors to many different plant species.

The WXp cluster includes phytoplasmas not known to cause phyllody on their host plant. Although the presence of phyllody seemed to be restricted to some well characterized groups of phytoplasmas (Aster Yellows, Sunhemp Phyllody), more recent molecular information had given limited support to the hypothesis that differences in symptom expression can actually reflect substantial taxonomic differences (Lee et al., 1992a; Schneider and Seemüller, 1994). The results reported herein showed that two only distantly related phytoplasma may naturally infect and cause phyllody in the same host in the same geographic area. It is also interesting to note that the two phytoplasma may be found together in the same plant and that the organism phylogenetically closest to ICPhp is the CYEp, another clover pathogen. These findings suggest that virescence and phyllody may be the result of the expression of a limited genetic pool, which could be transmitted horizontally to phylogenetically distant phytoplasmas sharing the same hosts. The finding that both the phytoplasmas examined in this study can be transmitted experimentally by the leafhopper *Euscelidius variegatus* (L. Carraro, unpublished results) further supports this hypothesis.

Aknowledgements

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