European Journal of Plant Pathology **103:** 251–254, 1997.

Detection and characterization of a phytoplasma associated with annual blue grass (*Poa annua*) white leaf disease in southern Italy

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Accepted 6 November 1996

Key words: phytoplasma 16S rRNA group, rice yellow dwarf phytoplasma, sugarcane white leaf phytoplasma

Abstract

A phytoplasma was detected in annual blue grass (*Poa annua* L. Fienardo), exhibiting white leaf symptoms, that was grown in the fields near Caserta in southern Italy. Based on restriction fragment length polymorphism analysis of PCR-amplified 16S rDNA sequences, the phytoplasma associated with annual blue grass white leaf disease was identified as a new member of phytoplasma 16S rRNA group XI (16SrXI) (type strain, rice yellow dwarf phytoplasma). The annual blue grass white leaf phytoplasma is most closely related to Bermuda grass white leaf phytoplasma found in Asia. Annul blue grass white leaf and Bermuda grass white leaf phytoplasmas were designated as the third subgroup (16SrXI-C) of group XI. This is the first report that a plant pathogenic phytoplasma belonging to group 16SrXI is present on the European continent.

Introduction

Bermuda grass white leaf disease (BGWL), commonly present in Southeastern Asia and Africa, is believed to be caused by a phytoplasma. This phytoplasma is phylogenetically related to sugarcane white leaf (SCWL), sugarcane grassy shoot (SCGS), and rice yellows dwarf (RYD) phytoplasmas (Schneider et al., 1995; Nakashima et al., 1996; Dafalla and Cousin, 1988; R.E. Davis, unpublished). All four phytoplasmas are found in tropical regions and they represent a distinct phylogenetic cluster (Kirkpatrick et al., 1994; Namba et al., 1993; Seemüller et al., 1994). Based on phylogenetic and restriction site analyses of 16S rRNA gene sequences, a new 16S rRNA group XI was proposed to distinguish RYD phytoplasma from other major phytoplasma groups (Gundersen et al., 1994; Schneider et al., 1993). Thus far, diseases caused by similar phytoplasmas have not been found in temperate regions.

In the fall of 1995, we found patches of annual blue grass (*Poa annua* L. Fienardo) growing in the fields near Caserta in southern Italy that exhibited white leaf disease symptoms. On the basis of these symptoms, we suspected a phytoplasmal etiology.

The objectives of this study were to detect and identify the presumed causal agent and, if a phytoplasma, to compare this temperate strain with other tropical strains. Based on extensive restriction fragment length polymorphism (RFLP) analyses of polymerase chain reaction (PCR)-amplified 16S rNRA gene sequences, we conclude that a phytoplasma is associated with annual grass white leaf (ABGWL) disease and that this phytoplasma is related to but distinct from RYD phytoplasma, the only previously established member of 16S rRNA group XI, and from SCGS (R.E. Davis, unpulbished). The ABGWL phytoplasma, therefore, represents a new phytoplasma subgroup within 16S rRNA XI. This is the first report that a disease associated with a phytoplasma belonging to 16S rRNA group XI is present in Europe.

Materials and methods

Sources of infected annual blue grass and reference phytoplasmas. Five samples of annual blue grass showing white leaf symptoms and eleven showing no apparent symptoms were collected in the fall of 1995 from fields near Caserta in southern Italy. Nucleic acid samples of reference phytoplasmas rice yellows dwarf (RYD-In1) and sugarcane grassy shoot (SCGS-In1) from India were prepared in a separate study by R.E. Davis, and sugarcane white leaf (SCWL) and Bermuda grass (BGWL) phytoplasma DNA samples were provided by S. Attathom.

Direct and nested-PCR. The universal primer pairs R16mF2/R1 and R16F2n/R2 previously designed on the basis of phytoplasma 16S rRNA sequences (Gundersen and Lee, 1996; Lee et al., 1993a) were used in PCR assays to detect the presence of putative phytoplasma(s) in annual blue grass tissues. In direct PCR (single amplification) primer pair R16mF2/R1 was used, while in nested-PCR the primer pair R16mF2/R1 was used for the initial amplification and a secnod primer pair R16F2n/R2 was employed for the second amplification (Gundersen et al., 1996; Lee et al., 1995). For PCR, total nucleic acid was extracted from symptomatic and asymptomatic grass tissues according to procedures described elsewhere (Lee et al., 1993b). Nucleic acid samples were diluted 1 in 30 in sterile deionized water or diluted to give a final concentration of 20 ng/ul. PCR assays were performed as previously described (Lee et al., 1995). Thirty-five PCR cycles were conducted in an automated thermocycler (Perkin-Elmer Cetus, Norwalk, CT). The following parameters were used: 1 min (2 min for the first cycle) denaturation at 94 °C, annealing for 2 min at 50 °C (60 °C for second amplification with primer pair R16F2n/R2), and primer extension for 3 min (10 min in final cycle) at 72 °C. Tubes with the reaction mixture devoid of DNA templates were included in each experiment as negative controls. PCR products were analyzed by electrophoresis and visualization of DNA bands using a UV transilluminator.

RFLP analyses of PCR products. Phytoplasma 16S rDNA sequences amplified by nested-PCR using the primer pair R16F2n/R2 were analyzed by restriction enzyme digestion. Three ul of each PCR product was digested separately with two selected restriction enzymes, *Rsa*I (GIBCO/BRL, Gaithersburg, Md) and *Mse*I (New England Biolabs, Beverly, MA) (Lee et al., 1993a). PCR products from two selected samples (G1 and G2) were digested separately with 14 restriction enzymes (*Rsa*I, *Alu*I, *Eco*R1, *Mse*I, *Hpa*I, *Taq*I, *Tha*I, *Hpa*II, *Hae*III, *Dra*I, *Sau*3A, *Hha*I, *Kpn*I and *Hinf*I) to give representative RFLP profiles for the putative phytoplasma associated with the infected grass. Selected enzymes (*Mse*I, *Hae*III, *Alu*I, and *Taq*I) were used to differentiate the ABGWL phytoplasma from RYD, SCWL, BGWL, and SCGS phytoplasmas. The restriction products were then separated by electrophoresis through a 5% polyacrylamide gel followed by staining in ethidium bromide and visualization of DNA bands using a UV transilluminator.

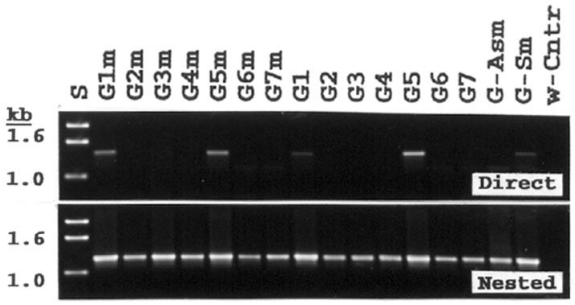

Figure 1. Direct and nested polymerase chain reaction (PCR) amplification of phytoplasma 16S rDNA sequences from annual blue grass, *Poa annua*, total nucleic acid samples prepared from symptomatic (G1m, G5m, G1, G5, and G-sm) and asmptomatic (G2m, G3m, G4m, G6m, G7m, G2, G3, G4, G6, G7, and G-Asm) plants. Direct PCR was performed by using the primer pair R16mF2/R1 and nested PCR by using R16mF2/R1 followed by the primer pair R16F2n/R2. S = DNA fragment size standard; w-Cntr = water control.

Results and discussion

Direct PCR (single amplification) using universal primer pair R16mF2/R1 primed amplification of 16S rDNA sequences from five symptomatic and eleven asymptomatic grass samples. The five symptomatic ones (G1m, G5m, G1, G5, and G-Sm) yielded visible DNA bands and one asymptomatic sample (G3m) also yielded a very faint band on a agarose gel (Figure 1). However, nested-PCR detected phytoplasmas from symptomatic as well as asymptomatic samples. Expression of white leaf symptom on grass seemed to correlate with higher titers of ABGWL phytoplasma in infected grass as indicated in the positive results by direct PCR assays, suggesting a pathogenic role of this phytoplasma. It was surprising to note that 100% of the grass samples, including symptomless

ones, were infected. It is possible that asymptomatic plants are in the state of the incubation period or that they may be more tolerant. Annual blue grass is widely grown as weeds in temperate regions. This is the first report to note the occurrence of white leaf disease in annual blue grass in Italy and on the European continent. The white leaf disease is not known to occur in the United States.

RFLP analyses of nested-PCR products with restriction enzymes *Mse*I and *Rsa*I indicated that all the annaul blue grass plants contained a very similar or identical phytoplasma (data not shown). An unidentified secondary phytoplasma was present in two of the asymptomatic samples. Based on RFLP profiles derived from analyses with 14 restriction enzymes, the principal phytoplasma associated with diseased annual blue grass was identified as a new member of phytoplasma 16S rRNA group XI (16SrXI) (type strain, RYD phytoplasma) (Figure 2). Based on RFLP analyses with *Alu*I, *Hae*III, *Mse*I and *Taq*I (Figure 3) and other enzymes (data not shown), the Italian ABGWL phytoplasma is most closely related to BGWL phytoplasma, which is found in tropical regions in Asia. ABGWL and BGWL represent a new subgroup, 16SrXI-C, distinct from other subgroups, 16SrXI-A (RYD phytoplasma) and 16SrXI-B (SCGS phytoplasma), identified previously (R.E. Davis, unpulblished). In this study, based on RFLP profiles we also designated sugarcane white leaf (SCWL) phytoplasma in Thailand as a new member of subgroup 16SrXI-B. The Italian ABGWL and BGWL phytoplasmas can be differentiated from other members of group 16SrXI by RFLP analysis with restriction enzyme *Mse*I or *Hae*III. Now the group 16SrXI has expanded to include three subgroups and eight phytoplasma strains including strain BVK previously detected in leafhopper from Germany and two phytoplasma strains, BraWL and DacWL, associated with white leaf diseases in two gramineous weeds (*Brachiaria* sp. and *Dactyloctenium aegypcium* in Family Poaceae) (Nakashima et al., 1996). On the basis of putative restriction sites determined by analyzing 16S rDNA sequences from gene bank, phytoplasma strain BVK may represent another subgroup in group 16SrXI. More new subgroups within each of 11 major phytoplasma 16S rRNA groups have been identified (Gundersen et al., 1996). This information permits us to differentiate phytoplasmas at a finer level and has contributed toward the establishment of a comprehensive classification scheme for the uncultured phytoplasmas.

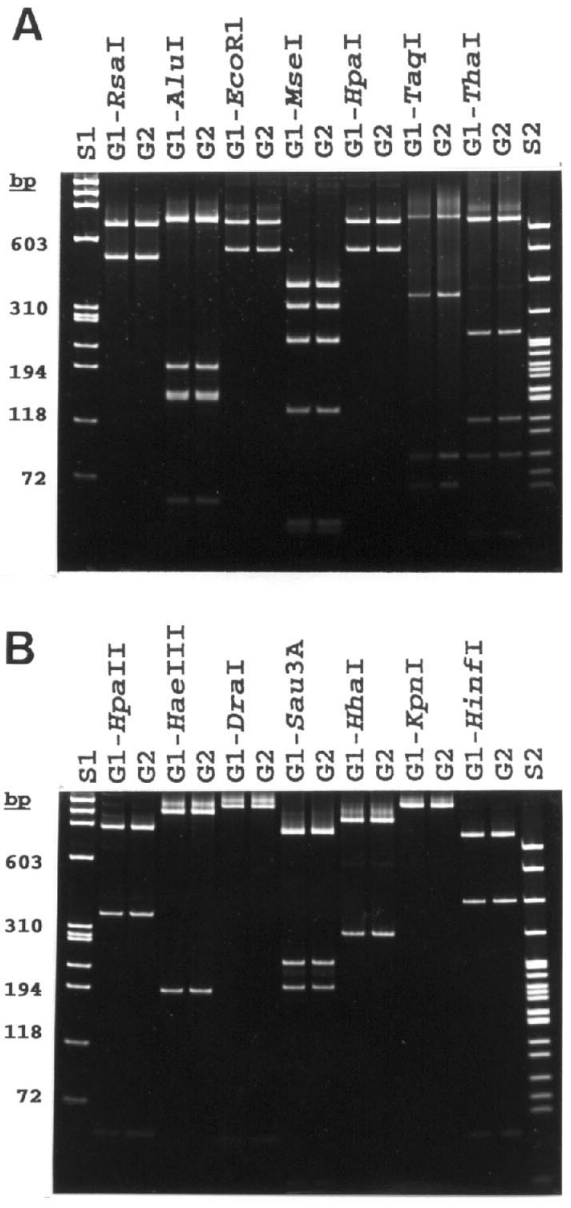

Figure 2. Restriction fragment length polymorphism analyses of 16S rDNA amplified by nested PCR from two annual blue grass samples, G1 (with white leaf symptoms) and G2 (asymptomatic). DNA products were digested with restriction enzymes as listed in (A) and (B) and electrophoresed through a 5% polyacrylamide gel. Lane S1 and lane S2 are molecular weight markers: lane S1, ϕX174 RFI *Hae*III digest, fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 301, 281, 271, 234, 194, 118, and 72; lane S2, pBR322 DNA *Msp*I digest, fragment sizes in base pairs from top to bottom: 622, 527, 404, 307, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34, 26, 15, and 9.

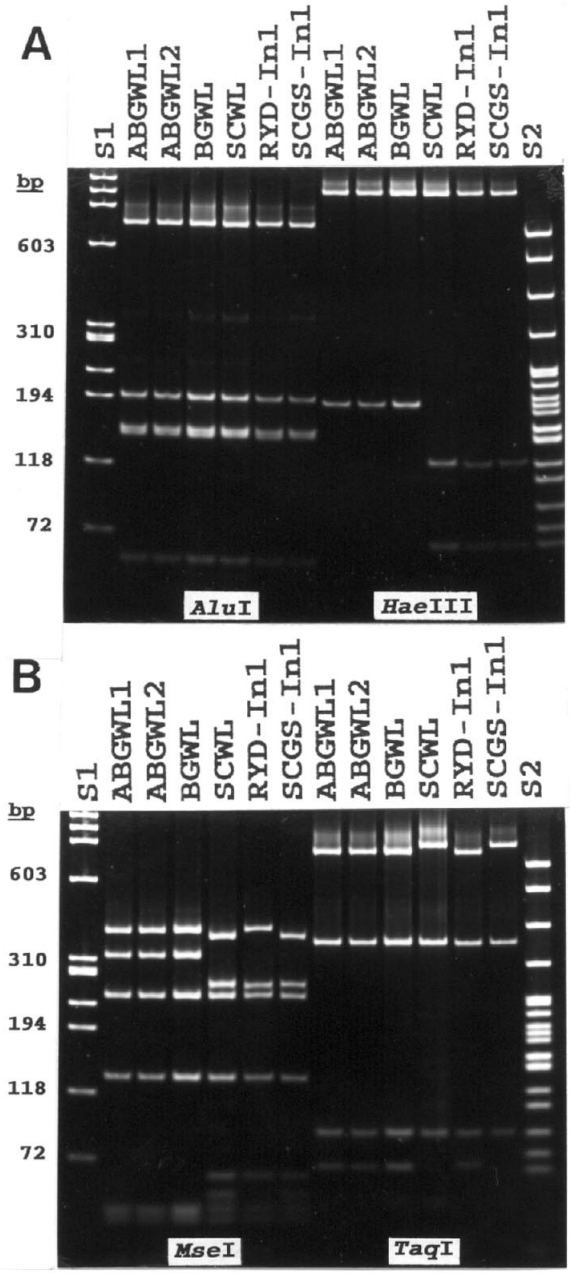

Figure 3. Restriction fragment length polymorphism analyses of 16S rDNA amplified by nested PCR from two annual blue grass white leaf (ABGWL) phytoplasmas and reference phytoplasmas, Bermuda grass white leaf (BGWL), rice yellow dwarf (RYD-In1), sugar cane white leaf (SCWL), and sugar cane grassy shoot (SCGS-In1). DNA products were digested with restriction enzymes as listed in (A) and (B) and electrophoresed through a 5% polyacrylamide gel. Lanes S1 and S2, molecular weight markers are as described in Figure 2.

Acknowledgements

We thank those who kindly provided the strains used in this study and Irena M. Bartoszyk for excellent technical assistance.

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