

## CHARACTERIZATION OF AN ASTER YELLOWS PHYTOPLASMA ASSOCIATED WITH CATHARANTHUS LITTLE LEAF IN ARGENTINA

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### SUMMARY

Periwinkle plants showing severe chlorosis and little-leaf symptoms were observed in Córdoba (Argentina). These symptoms have previously been correlated with a phytoplasma infection based on electron microscopy, serology and PCR techniques. The aim of the present work was to characterize the Argentinian catharanthus little leaf phytoplasma (ACLL) at molecular level using PCR-RFLP. Using the universal phytoplasma primers P1/P7 and R16F2/R16R2, we amplified the 1.8 kb fragment extending from the 5'-end of the 16S rRNA gene to the 5'-region of the 23S rDNA, and a 1.2 kb fragment from the 16S rRNA gene. The specific aster yellows primers P1/AYint were also used and the expected amplified fragment of 1.5 kb was obtained. The P1/P7 and R16F2/R16R2 fragments were digested with 9 and 10 restriction endonucleases respectively. The RFLP analysis revealed, for most of the enzymes assayed, identical patterns to those of members of the aster yellows group (16SrI), subgroup B, except for *Rsa* I and *Hae* III, which generated profiles similar to those presented by phytoplasmas from subgroup C. These differences could be explained by sequence heterogeneity of the two rRNA operons that are present in all phytoplasmas. Confirmation of the ACLL phytoplasma in Argentina expands southwards the already wide geographical distribution and diversity of the 16SrI-B (Aster Yellows) group.

**Key words:** PCR-RFLP, periwinkle little leaf, rRNA operon heterogeneity.

### INTRODUCTION

Phytoplasmas are prokaryotes lacking a cell wall and responsible for numerous plant diseases all over the world (McCoy *et al.*, 1989). Detection and identification of phytoplasmas were for a long time based on their biological characteristics and related diseases because it

has not been possible to isolate and study the phytoplasmas in pure culture (Lee *et al.*, 2000). PCR-RFLP and sequence analysis of the PCR-amplified 16S rRNA gene, the ribosomal protein gene and the elongation factor TU (*tuf* gene) have become essential tools for the molecular identification, characterization and classification of phytoplasmas. This kind of analysis provides at least a provisional classification scheme for phytoplasmas (Lee *et al.*, 1998; Jomantiene *et al.*, 2002).

Based on 16S rDNA RFLP patterns, 15 main phytoplasma groups (putative species) and at least 40 subgroups have been described (Lee *et al.*, 1998; Montano *et al.*, 2001) that largely match phytoplasma subclades delineated by phylogenetic analysis of full-length or nearly full-length sequences of 16SrDNA (Lee *et al.*, 1998; Marcone *et al.*, 2000). The occurrence of two ribosomal gene sets in the phytoplasma genome has been demonstrated (Schneider and Seemüller, 1994; Oshima *et al.*, 2004), and small differences have been found between the sequences and RFLP patterns, which have been taken into account in some cases for classification at the subgroup level (Lee *et al.*, 1993; Liefing *et al.*, 1996; Marcone *et al.*, 2000).

The 16SrI aster yellows phytoplasma group, widely distributed and with more than 100 isolates studied, has been separated into at least six well-defined subgroups according to RFLP patterns and nucleotide sequence of the 16S rDNA (Jomantiene *et al.*, 1998; Lee *et al.*, 1998). The classification was consistent with that proposed by Marcone *et al.* (2000) based on the RFLP patterns of the *tuf* gene, and a longer rDNA fragment that includes the 16S-23S rRNA spacer region and the 5' end of the 23S rRNA gene.

Periwinkle (*Catharanthus roseus* L.G. Don) is an ornamental plant reported in different world regions as a natural phytoplasma host showing symptoms such as little leaf, witches'-broom, yellowing, virescence and phyllody. The phytoplasmas associated with these different diseases have been identified as members of the 16SrI-Aster yellows group (Lee *et al.*, 1993; Marcone *et al.*, 2000), 16SrVI-Clover proliferation (Lee *et al.*, 1998) and 16SrXIII-Mexican periwinkle virescence (Gundersen *et al.*, 1994).

In Argentina, the presence but not the identity of phytoplasmas in periwinkle has been determined in

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plants with severe little leaf and chlorosis symptoms, by electron microscopy (EM), serology and PCR (Gomez *et al.*, 1996). In the present work, we established the group and subgroup affiliation of the Argentinian catharanthus little leaf phytoplasma by PCR-RFLP analysis of the rDNA region.

## MATERIALS AND METHODS

**Plant and phytoplasma sources.** Five periwinkle plants naturally infected with phytoplasma were collected from private gardens in the suburbs of Córdoba (Argentina). The pathogens were maintained by successive grafting to healthy periwinkle plants produced by cuttings and grown in a greenhouse under controlled conditions. Healthy periwinkle plants were also kept in the greenhouse to be used as negative controls. Symptoms and disease progress were periodically checked in the grafted plants. DNA of the American aster yellows phytoplasma (AAY), kindly provided by Dr. E. Seemüller (Biologische Bundesanstalt für Land und Forstwirtschaft, Institut für Pflanzenschutz im Obstbau, Dossenheim, Germany) was used as reference strain.

**Primers and PCR conditions.** Fresh shoots and petiole tissue (0.5 g) from the middle and upper parts of the infected plants were ground in sterile mortars with liquid nitrogen. DNA was extracted according to Doyle and Doyle (1990), based on the use of hexadecyltrimethyl-ammonium bromide (CTAB). DNA quality and concentration were evaluated by spectrophotometer and 1% agarose gel electrophoresis, stained with ethidium bromide (EtBr) and visualized under UV light (Sambrook *et al.*, 1989). For ACLL and AAY rDNA amplification, universal primers R16F2/R16R2 (Lee *et al.*, 1993) and P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) were used to amplify respectively a 1.2 kb fragment from the 16S rRNA gene, and a 1.8 kb fragment from the 5'-end of the 16S rRNA gene to the 5'-region of the 23S rDNA. Primers P1/AYint (Deng and Hiruki, 1991; Kuske and Kirkpatrick, 1992), specific for the 16SrI aster yellows group, were also used to amplify a 1.5 kb fragment from the 16S rRNA gene and part of the 16S-23S spacer region.

The DNA extracted from healthy and diseased plants was resuspended in double distilled water to a final concentration of 40 ng  $\mu\text{l}^{-1}$  for direct PCR and each reaction was performed according to Lee *et al.* (1993). The cycling conditions for the R16F2/R16R2, P1/P7 and P1/AYint primers were those described by Lee *et al.* (1993), Schneider *et al.* (1995) and Deng and Hiruki (1991), respectively, with minor modifications in order to obtain sharper bands after the gel electrophoresis (R16F2/R16R2: annealing 54°C, 2 min; P1/P7: annealing 54°C, 2 min).

**RFLP analysis of PCR products.** The RFLP analyses of the ACLL and AAY phytoplasmas were performed with 150-200 ng of the amplified DNA (with primers R16F2/R16R2 and P1/P7). The 1.2 kb fragment was digested with *Alu* I, *Hba* I, *Hpa* II, *Kpn* I, *Mse* I, *Rsa* I, *Hae* III, *Taq* I, *Hinf* I and *Sau* 3AI (New England Biolabs Inc., Beverly, MA, USA) while the 1.8 kb amplified fragment was digested with *Alu* I, *Mse* I, *Hae* III, *Rsa* I (New England Biolabs Inc., Beverly, MA, USA), *Hba* I, *Hpa* II, *Hinf* I, *Sau* 3AI, and *Taq* I (Promega, Madison, WI, USA). The enzyme reactions were separately incubated according to the manufacturers' recommendations. The RFLP patterns of *Alu* I, *Hba* I, *Hpa* II, *Kpn* I, *Mse* I (1.2 kb fragment) and *Alu* I, *Taq* I, *Mse* I (1.8 kb fragment) were analysed by 8% polyacrylamide gel electrophoresis in 1xTBE buffer. DNA digested with *Hae* III, *Rsa* I, *Taq* I, *Hinf* I, *Sau* 3AI (1.2 kb fragment) and *Hinf* I, *Hpa* II, *Sau* 3AI, *Hae* III, *Rsa* I, *Hba* I (1.8 kb fragment) was analysed in 1.5% agarose + 0.5% Metaphor agarose (BioWittaker Molecular Applications, Rockland, ME, USA) in 1xTBE buffer. The ACLL profiles were compared with those of the AAY reference strain and with those proposed by Lee *et al.* (1998) and Marccone *et al.* (2000).

## RESULTS

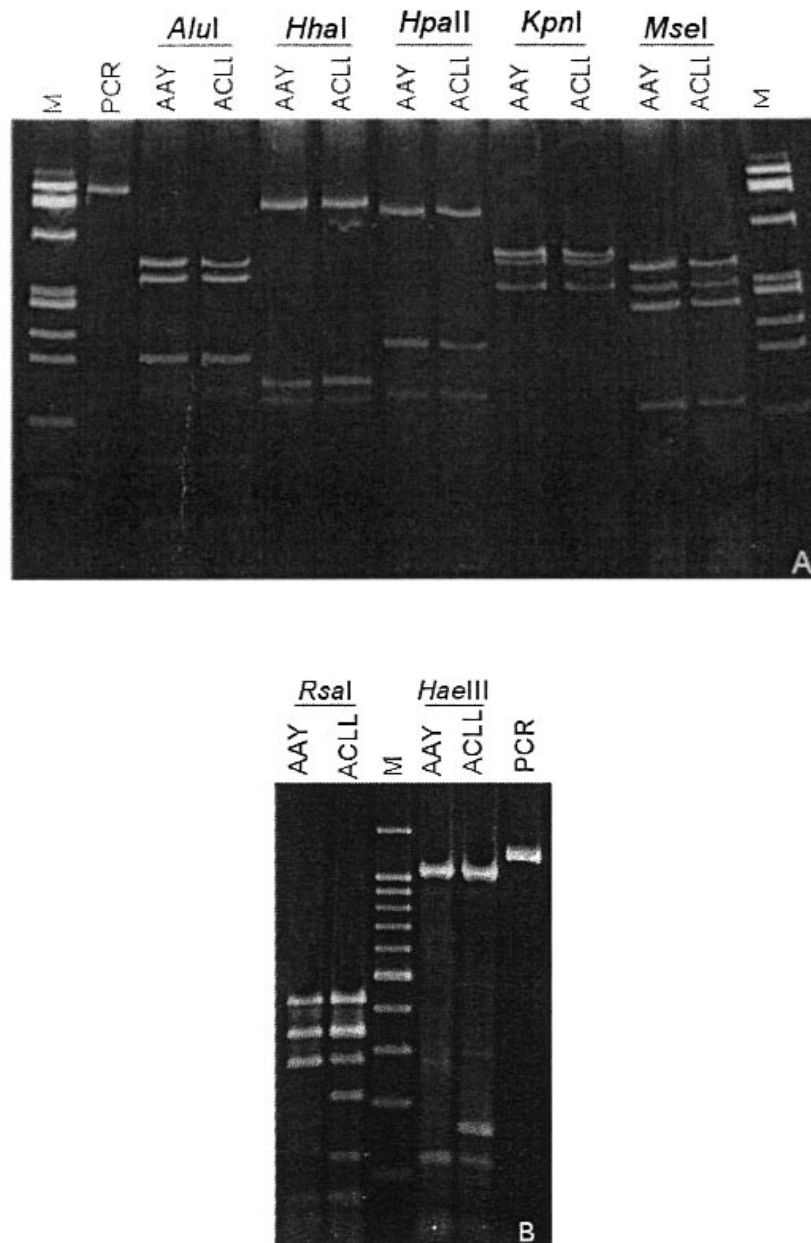
Little-leaf symptoms were observed forty days after grafting the periwinkle plants, and the disease progressed towards the young vegetative organs. Virescence and phyllody became evident in the flowers, and finally abortion of the reproductive structures was observed.

**RFLP analysis.** PCR amplifications were obtained when ACLL and AAY DNA were used as template; no amplification was observed in the negative controls with healthy periwinkle DNA or without DNA template. Universal phytoplasma primers R16F2/R16R2 and P1/P7 gave amplification of the expected fragments of 1.2 and 1.8 kb, respectively, corresponding to the partial 16S rRNA gene, and the entire 16S rRNA gene plus the 16S-23S spacer region and the 5'-end of the 23S rDNA. A 1.5 kb fragment from the 16S rRNA gene and part of the 16S-23S spacer region was also amplified with the specific AY-group primers P1/AYint. Digestion of the ACLL 1.2 kb fragment with *Alu* I, *Hba* I, *Hpa* II, *Kpn* I, *Mse* I (Fig. 1A), *Taq* I, *Hinf* I and *Sau* 3AI (data not shown) generated profiles identical to those of AAY and other members of the 16SrI-AY-group (Lee *et al.*, 1998). However, the patterns originated by *Rsa* I and *Hae* III differentiated between ACLL and AAY (Fig. 1B). Digestion of ACLL rDNA with *Rsa* I produced a 5-band pattern (approximately 110, 210, 270, 340 and 420 bp) in which the sum of the band sizes was greater than the amplified fragment size. The ACLL pattern had two ex-

tra bands besides those coinciding with the AAY pattern (Fig. 1B). The *Hae* III RFLP pattern showed an extra fragment of about 170 bp with respect to AAY (Fig. 1B), resulting in a similar pattern to that proposed for the Clover phyllody phytoplasma (CPh) of subgroup 16SrI-C (Lee *et al.*, 1998).

The ACLL 1.8 kb PCR product digestion with *Hinf* I, *Hpa* II, *Sau* 3AI, *Hba* I, *Alu* I, *Taq* I and *Mse* I, coinci-

dently with those observed in the 1.2 kb RFLP fragment, showed patterns indistinguishable from the AAY reference strain. On the other hand, the *Hae* III and *Rsa* I profiles were slightly different from AAY because of the presence of extra bands (data not shown). The entire RFLP patterns for these enzymes were similar to those proposed by Marcone *et al.* (2000) for CPh, a member of the 16SrI-C subgroup.



**Fig. 1.** RFLP analyses of 1.2 kb PCR-amplified phytoplasmal 16S rDNA with R16F2/R16R2 primer pair. **A)** PCR: amplified product of 1.2 kb from ACLL. M: molecular weight marker  $\Phi$ X174 DNA *Hae* III digest (Promega), fragment size (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72. AAY (American Aster Yellows); ACLL (Argentinian Catharanthus Little Leaf) digested with *Alu* I, *Hba* I, *Hpa* II, *Kpn* I, *Mse* I restriction enzymes. **B)** AAY and ACLL digested with *Rsa* I and *Hae* III. M: 100 pb DNA Ladder (Promega), fragment size (bp) from top to bottom: 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100. PCR amplified product of 1.2 kb from ACLL.

## DISCUSSION

The ACLL phytoplasma is the causal agent of little-leaf symptoms in Argentinian periwinkles. These symptoms were reproduced in grafted periwinkle plants. There have been many reports of phytoplasma diseases that induced similar symptoms in periwinkle; the phytoplasmas responsible have been classified in different 16Sr-groups. In North America, Periwinkle little leaf, American aster yellows (Lee *et al.*, 1998) and Periwinkle virescence (Lee *et al.*, 1991) phytoplasmas have been identified as members of the 16SrI-group, and the Mexican periwinkle virescence (Gundersen *et al.*, 1994) as belonging to 16SrXIII-group. In South America, the *Catharanthus virescence* agent has been cited by Schneider *et al.* (1993) as an aster yellows phytoplasma, later included as a member of the 16SrI-AY group by Marcone *et al.* (2000); also, Barros *et al.* (1998) associated the Superbrotamiento of *Catharanthus* with the presence of phytoplasmas from groups 16SrII and 16SrIX. The causal agents of virescence and phyllody of periwinkle in Europe, the Italian periwinkle witches'-broom and the *Catharanthus virescence* from Asia, have been cited as members of the 16SrI-AY group by Marcone *et al.* (2000). The similar symptomatology shown by plants infected with phytoplasmas from different 16Sr-groups further complicates differentiation and classification of the pathogen.

In the present work we report a disease caused by the ACLL phytoplasma, which developed little-leaf symptoms in periwinkle. The pathogen's identity was confirmed by PCR with primers P1/AYint specific for the AY-group and by RFLP of rDNA fragments amplified with universal phytoplasma primers R16F2/R16R2 and P1/P7.

The collective RFLP patterns of ACLL phytoplasma rDNA were highly similar to the AAY reference phytoplasma, which belongs to subgroup 16SrI-B. However, *Hae* III and *Rsa* I digestions resulted in differences between the ACLL and AAY patterns. The ACLL profiles for these enzymes were similar to those reported by Marcone *et al.* (2000) for Clover phyllody (KVM) from France, Clover phyllody (KVG), and Leafhopper-borne (CVA) phytoplasmas from Germany, belonging to 16SrI-C subgroup. The authors distinguished nine 16SrI subgroups by digestion of the rDNA fragment amplified with primers P1/P7 with only four restriction enzymes (*Sau* 3AI, *Hinf* I, *Hpa* II and *Hae* III) since each subgroup possesses a unique combination of electrophoretic patterns (Marcone *et al.*, 2000). The ACLL RFLP patterns did not exactly correspond with any of the profiles described for the members of 16SrI group. The resulting patterns with unexpected extra bands for *Hae* III and *Rsa* I could be attributed to sequence heterogeneity between the rRNA gene operons, more than to mixed infection. If that was the case, the distinct

RFLP patterns of each presumptively infecting phytoplasma would have been observed when different parts of the plant were analysed, because of an uneven distribution of the phytoplasmas (Jomantiene *et al.*, 2002). Otherwise, overlapping but well-defined patterns for a group or subgroup could have been observed, as suggested by Bianco *et al.* (1993), Lee *et al.* (1995) and Stanulis *et al.* (2000). None of these results were obtained when different plants and plant parts were analysed, and the same RFLP patterns were observed in all cases, supporting the occurrence of nucleotide sequence heterogeneity between the two ACLL rRNA operons.

In this work, the RFLP patterns of subgroup 16SrI-B were observed for the digested 1.2 kb and 1.8 kb fragments of the ACLL rRNA gene except for *Rsa* I and *Hae* III restriction types. Since only two restriction enzymes produced different patterns, which could be explained by the occurrence of sequence heterogeneity between operons, this would not be enough evidence to define a new subgroup for the ACLL phytoplasma within the 16SrI-Aster yellows group. A similar situation has been considered for phytoplasmas AV2192 and AVUT from group 16SrI subgroups L and M, respectively, by Marcone *et al.* (2000). The authors did not find differences in phytoplasma pathogenicity and proposed that operon heterogeneity and the small differences observed at nucleotide level between them with members of subgroup B, should not be overemphasized when defining subgroups.

Our results confirm that *C. roseus* is a natural host of the ACLL phytoplasma which belongs to 16SrI Aster yellows group. The RFLP patterns mostly coincident with subgroup B, suggest the inclusion of ACLL in this subgroup. Since this new AY genotype could have taxonomic relevance we should not exclude the possibility that the ACLL phytoplasma may represent a new subgroup, so further work is required to show if formal differentiation of the ACLL agent from subgroup B is justified. Members of subgroup B are distributed in America, Europe and Asia (Schneider *et al.*, 1997; Marcone *et al.*, 2000). In South America, a phytoplasma from group 16SrI-B has been detected in periwinkle in Peru (Marcone *et al.*, 2000), and Maize bushy stunt has been reported in maize in Brazil (Bedendo *et al.*, 2000). The finding of the ACLL phytoplasma in Argentina expands southwards the already wide geographical distribution and diversity of the 16SrI-B (Aster Yellows) phytoplasmas.

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