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Molecular evidence for the presence of '*Candidatus* Phytoplasma cynodontis', the Bermuda grass white leaf agent, in India

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Abstract

White leaf-affected Bermuda grass plants collected in eastern Uttar Pradesh province of India were examined for phytoplasmal infections with PCR assays using universal phytoplasma primers directed to rDNA sequences. All symptomatic plants examined tested positive. By sequence analysis of the amplified 16S rDNA and 16S/23S rDNA spacer region sequences, the detected phytoplasma proved to be a strain of '*Candidatus* Phytoplasma cynodontis', the causal agent of Bermuda grass white leaf disease.

A yellows disease of Bermuda grass was already known to occur in India. However, molecular detection and characterization of the presumable causal agent had previously not been reported.

Key words: '*Candidatus* Phytoplasma cynodontis', Bermuda grass white leaf, ribosomal DNA, *Cynodon dactylon*.

Introduction

Bermuda grass white leaf (BGWL) is a destructive phytoplasmal disease of Bermuda grass (*Cynodon dactylon*), which is known to occur in several Asian countries, Sudan, Italy, Cuba and Australia (Jung *et al*., 2003; Marcone *et al*., 2004; Arocha *et al*., 2005a). The causal agent, the BGWL phytoplasma, which is a member of the phylogenetic BGWL phytoplasma group or 16SrXIV group, is a discrete taxon at the putative species level, for which the name '*Candidatus* Phytoplasma cynodontis' has been adopted (Marcone *et al*., 2004).

The disease is characterized by extensive chlorosis, proliferation of axillary shoots, bushy growing habit, small leaves, shortened stolons and rhizomes, stunting and death of the plants. In the early stage of the disease, light green to yellow streaks on the leaves are also present. Also, in white leaf-diseased Bermuda grass plants, the '*Candidatus* Phytoplasma graminis' which is a member of the 16SrXVI group was identified in Cuba, whereas a strain belonging to aster yellows group (16SrI) subgroup 16SrI-L, was transmitted from Bermuda grass showing typical BGWL symptoms, in Thailand, to the experimental phytoplasma host *Catharanthus roseus* (periwinkle) by means of dodder (Marcone *et al*., 2000; Arocha *et al*., 2005b).

A disease similar or identical to BGWL, named yellow leaf, was reported to occur in India (Rishi, 1978). This report is based on symptomatology, temporary remission of symptoms after tetracycline treatment of affected plants and electron microscope observations. However, molecular data on the identity of the presumable causal agent and its phylogenetic relatedness to other phytoplasmas have not yet reported. In the work presented here, molecular technologies were employed to detect and identify the phytoplasma infecting Bermuda grass in India.

Materials and methods

Samples from diseased Bermuda grass plants showing typical white leaf symptoms and from non-symptomatic plants of the same species were collected during spring 2007 in several locations near Gorakhpur, eastern Uttar Pradesh. Young shoots including leaves were examined. Total DNA was extracted from approximately 1 g of tissue employing a phytoplasma enrichment procedure as described previously (Ahrens and Seemüller, 1992). For amplification of phytoplasmal ribosomal DNA (rDNA) by PCR assays, the universal phytoplasma primer pairs P1/P7 and P4/P7 (Schneider *et al*., 1995), were used. Primers P1/P7 amplify a fragment of approximately 1,800 bp in length extending from 5' end of the 16S rRNA gene to the 5' region of the 23S rDNA, thus including the 16S/23S rDNA spacer region, whereas the primer P4, in combination with P7, amplifies a fragment of about 530 bp. PCR assays were performed either as one-round PCR (direct PCR) using the primer pair P1/P7 or P4/P7, or as nested PCR by re-amplifying the P1/P7 products with the primer pair P4/P7. For sequencing, PCR products (approximately 5 µg) were separeted by electrophoresis in 1.5% agarose gel. Fragments with sizes corresponding to the expected amplified sequences were excised from the gel and eluted using the QIAquick gel extraction kit (Qiagen). DNA fragments were either sequenced directly or cloned prior to sequencing. For cloning, DNA fragments were ligated into plasmid vector pGEM-T (Promega) and recombinant plasmid used to transform *Escherichia coli* strain DH5α. Selected recombinant clones were screened for phytoplasmal rDNA inserts by PCR using the primer pair P4/P7. Plasmid DNA was purified using the NucleoSpin system kit (Macherey-Nagel). Sequencing of both strands was performed by ABI's AmpliTaq FS dye terminator cycle sequencing chemistry, based on Sanger's Sequencing method, in an automated ABI

3100 Genetic Analyzer. Primers for sequencing PCR products were the same as for PCR amplification whereas the standard primers SP6 and T7 were used for sequencing the cloned fragments. Sequences were then assembled and edited using DNASTAR's LaserGene software (DNASTAR) and consensus sequences were generated. These were then used as query sequences in a BLAST 2.0 search (Altschul *et al*., 1977) and were trimmed to include the 16S and/or 16S/23S rDNA spacer region sequences only. Sequence alignments were performed by using CLUSTAL, version 5, using DNASTAR's LaserGene software (DNASTAR).

Results

All symptomatic Bermuda grass plants examined (15 plants) tested phytoplasma-positive in PCR assays. However, in template DNA isolated from five of them, detectable amplification products were only obtained by nested PCR. Neither by direct ('one-round') nor by nested PCR assays was DNA amplified from template DNA isolated from any of the non-symptomatic plants. Nucleotide sequence analysis of 16S rRNA genes revealed that the phytoplasma detected in diseased Bermuda grass in India, hereafter referred to as 'strain BGWL-In', is nearly identical to strains of the BGWL agent (= '*Candidatus* Phytoplasma cynodontis') whose 16S rDNA sequences are available from GenBank database, sharing with them a sequence similarity which varied from 99.2 (Iranian strain, GenBank accession no. EF444486) to 99.9% (Thai strains, GenBank accession nos. AB052871 and AF248961, respectively). In particular, strain BGWL-In shared a sequence similarity value of 99.7% with the reference strain, BGWL-C1, (GenBank accession no. AJ550984) of the '*Ca.* P. cynodontis'.

At 16S/23S rDNA spacer region sequence level, BGWL-In proved to be identical to BGWL phytoplasma strains from Italy, Sudan, Thailand, Indonesia, Australia and Iran (GenBank accession nos. AJ550984, AJ550985, AJ550986, AF100412, AF248961, Y14645, Y15868 and DQ195215, respectively) as well as to date palm white tip die-back (DP-WTD) and date palm slow decline (DP-SD) phytoplasmas (Marcone *et al*., 2004), whereas it showed a sequence similarity of 99.2% with BGWL strains from China and Iran (GenBank accession nos. AF025423 and DQ195216, respectively) as well as to carpet grass white leaf (CGWL) and brachiaria white leaf (BraWL) phytoplasmas (Marcone *et al*., 2004). The 16S/23S rDNA spacer region of BGWL-In strain is about 250 bp in length and contains the gene encoding $tRNA^{Ile}$. The sequence of this gene was identical in all strains examined.

Discussion

With PCR assays using universal phytoplasma primers directed to rDNA sequences, and sequence analysis of amplified DNA, we identified a strain of '*Ca.* P. cynodontis', the causal agent of BGWL disease, in white leaf-affected Bermuda grass plants in India. The data obtained further show that at 16S rDNA and 16S/23S rDNA spacer region sequence level, Bermuda grass-infecting phytoplasmas of the BGWL phytoplasma group, occurring in various geographical areas, are largely identical and represent a distinct taxonomic entity, *i.e*., the '*Ca.* P. cynodontis'. Also, this is the first report from India on molecular detection and characterization of phytoplasmal infections in Bermuda grass.

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