

# Repeated conserved sequences as genetic markers for phytoplasma detection

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## Abstract

Phytoplasmas are cell wall less, unculturable plant pathogens that multiply in phloem cells of the plant vascular system and cause diseases affecting plants in agricultural and natural ecosystems. A broad array of phylogenetically diverse phytoplasma strains and species infects plants, and some plant hosts harbor very low titers of phytoplasma, emphasizing the need for highly sensitive methods for their detection. We investigated an approach to simplify and improve the sensitivity of detection of phytoplasmas, through focusing on a class of repeated conserved sequences (RCS) in phytoplasma genomes. Features of the RCS and results from PCR-based assays suggest its use as a genetic marker for phytoplasma detection.

**Key words:** mobile genetic unit, polymerase chain reaction.

## Introduction

Phytoplasmas are cell wall less plant pathogens that multiply in phloem cells of the plant vascular system and cause economic losses in agriculture crops and damage in natural ecosystems. Infected plants often develop characteristic symptoms, such as phyllody and virescence of flowers, flower sterility, yellowing or reduced size of leaves, proliferation of axillary shoots, general stunting, decline, and even plant death.

Recent progress in sequencing and analyses of phytoplasma genomes (Oshima *et al.*, 2004; Bai *et al.*, 2006) have revealed that these plant pathogens experienced substantial evolutionary gene decay (formation of pseudogenes), gene loss, and disruption or loss of important biosynthetic pathways (Davis *et al.*, 2003; 2005). These genomic changes may account for increasing their dependence on plant and insect hosts and likely accounting for the inability to culture phytoplasmas in artificial media *in vitro*. The analyses also revealed another striking feature of phytoplasma genomes: the presence of numerous repeated sequences that are highly conserved among phylogenetically diverse phytoplasmas.

Difficulties continue to confront detection of phytoplasmal infections, especially in woody plants. We investigated an approach to improve the sensitivity and simplify the detection of this group of pathogens through by focusing on a class of repeated conserved sequences (RCS) in phytoplasma genomes.

## Materials and methods

### Phytoplasma strains and nucleic acid preparation

Phytoplasmas in this study were propagated in a white flowered variety of the host plant, *Catharanthus roseus* (Madagascar periwinkle), in an insect proof greenhouse. Seven phytoplasmas, representing diverse 16S rDNA RFLP groups, were studied.

### PCR, cloning of PCR products and sequence analysis

DNA was extracted from plant tissues by using Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania) and was used as template in polymerase chain reactions (PCRs). RCS DNAs were amplified during 35 cycles in an automated thermal cycler with AmpliTaq polymerase (Invitrogen, Carlsbad, CA). PCR mixtures and conditions were as previously described (Gundersen and Lee, 1996; Jomantiene *et al.*, 1998). PCR products (5 µl) were electrophoresed through 1.4% agarose gel. PCR products were cloned in *Escherichia coli*, by using a TOPO TA cloning kit (Invitrogen), according to manufacturers' instructions, and were sequenced using automated DNA sequencing to achieve at least 3-fold coverage per base position in sequencing both strands. Sequences were analyzed using DNASTAR Lasergene software suite. Basic Local Alignment Search Tool (BLAST) searches (Altschul *et al.*, 1990) were carried out at the National Center for Biotechnology Information (NCBI) web site, <http://www.ncbi.nlm.nih.gov>.

## Results

Analysis of the completely sequenced genomes of phytoplasma strains OY-M (GenBank no. NC\_005303) and AY-WB (GenBank no. NC\_007716) revealed the presence of numerous repeated conserved sequences (RCS). Of the several types of repeated sequences observed, one of unresolved origin was selected for further study. The repetitive nature of this RCS suggested its use as a genetic marker for design of new primers to facilitate PCR-based detection of phytoplasmas in plants, especially woody plants.

Based on alignments of RCS sequences from the completely sequenced genomes, RCS primers were designed to yield PCR products that ranged from ca. 530-549 bp.

Using primer pair RCSF1/RCSR1 in PCRs, we amplified genomic DNA sequences from phytoplasmas be-

longing to diverse groups, including groups 16SrI, 16SrIII, 16SrV, 16SrVI, and 16SrX.

Comparisons of the nucleotide sequences amplified from these strains revealed high sequence conservation of RCS segments across these phylogenetically divergent groups. The RCS sequences from different phytoplasmas contained two direct repeats and a palindromic sequence. The OY-M phytoplasma genome contained seven copies of this RCS, and the AY-WB phytoplasma genome contained five copies of the RCS, distributed around the chromosome in both genomes. Features and the distribution of the RCS copies suggest they may represent potential mobile units.

## Discussion

Over the past decade, detection and identification of phytoplasmas has been heavily based on 16S rDNA sequences and other highly conserved gene sequences, some of which have been useful for the design of phytoplasma group- or subgroup-specific PCR primers (Jomantiene *et al.*, 1998; Lee *et al.*, 1998; Marcone *et al.*, 1999; Shao *et al.*, 2006). However, amplification of phytoplasma gene sequences from symptomatic or symptomless plants containing low titers of the pathogen is often fraught with difficulty, particularly in studies of phytoplasmas infecting woody plants. Nested PCR has been applied to overcome problems related to sensitivity of phytoplasma detection, although this approach is more time consuming and subject to template DNA contamination than single PCR. Amplification of phytoplasmal RCS segments offers advantages of increased sensitivity due to multiple copies of target sequence. The RCS appears unique to phytoplasmas, and sequence variability could aid species, group, subgroup, or strain differentiation.

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