Plant Pathology (2006) **55**, 25–28 Doi: 10.1111/j.1365-3059.2005.01294.x

Nested PCR assay for detection of sugarcane grassy shoot phytoplasma in the leafhopper vector *Deltocephalus vulgaris*: a first report

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Nymphs of *Deltocephalus vulgaris*, the leafhopper vector of sugarcane grassy shoot (SCGS) disease, fed on SCGS-infected and healthy sugarcane leaves, and SCGS-infected and healthy plant tissue of sugarcane cv. CoLk 8102, were examined by nested PCR using phytoplasma-specific rRNA operon primers for detection of the SCGS phytoplasma. Samples of SCGS-infected plants with symptoms and SCGS-exposed *D. vulgaris* nymphs yielded SCGS-exclusive DNA bands when nested PCR was performed. Negative results were obtained when symptomless plant host and unexposed insect vector samples devoid of phytoplasma DNA templates were used. Such a reliable molecular tool for the precise detection of SCGS phytoplasma in the *D. vulgaris* population would help forecast the potential of secondary spread of SCGS in a susceptible sugarcane variety, and may facilitate control of the disease.

Keywords: grassy shoot disease, insect vector, molecular detection, rRNA, *Saccharum*, SCGS

Introduction

Phytoplasmas are a group of plant-pathogenic, phloem-restricted prokaryotes belonging to the class Mollicutes, and are characterized by a lack of cell walls and remarkably small (500–1350-bp), AT-rich genomes (Razin *et al*., 1998). Phytoplasmas are the causal agents of diseases in hundreds of plants (Corbett *et al*., 1971; McCoy *et al*., 1989; Ahrens & Seemüller, 1992). They are transmitted from plant to plant by grafting and other vegetative propagation techniques, and by specific phloem-feeding insects, especially leafhoppers, planthoppers and psyllids (McCoy *et al*., 1989). Many diseases caused by phytoplasmas are of great economic importance, in particular those of fruit and ornamental trees, perennials, high-value vegetable crops (Ahrens & Seemüller, 1992), and sugarcane (Rishi & Chen, 1989; Singh *et al*., 2002; Srivastava *et al*., 2003).

Sugarcane grassy shoot (SCGS) disease is an economically important and widely occurring phytoplasma disease of sugarcane in several countries, including India (Rishi & Chen, 1989; Singh *et al*., 2002; Srivastava *et al*., 2003). SCGS-affected sugarcane is typically recognized by the presence of profuse tillering and narrow green or pale green leaves. Unchecked spread of the disease may result in heavy losses in cane yield and sucrose content in affected plants. In sugarcane crops, SCGS-infected plants are typically limited in number, but incidence increases by up to 60–80% in ratooned crops through secondary spread by insect vectors. Insect vectors of the phytoplasma are restricted to phloem-feeding leafhopper and planthopper members of the Cicadellidae, Fulgoroidea and Psylloidea, which transmit the phytoplasma in a persistent propagative manner. Successful transmission of the disease is dependent on acquisition of the phytoplasma by the vector during feeding (acquisition access period); passage of the phytoplasma from the insect gut through the haemocoel and passage across the salivary gland cell membranes (latent period); and inoculation of a healthy plant during subsequent feeding (inoculation access period). One leafhopper species, *Deltocephalus vulgaris*, has been found to transmit the SCGS phytoplasma successfully to sugarcane cv. CoLk 8102 (Singh *et al*., 2002). Observations on the development of SCGS symptoms on insect-fed plants were recorded in planted and first ratooned crops. Nymphs of *D. vulgaris* were found to be more efficient than adults in transmitting the SCGS phytoplasma.

The detection and identification of SCGS phytoplasma are necessary for accurate disease diagnosis and management. Because it is imperative to assess the proportion of the population of vectors carrying SCGS phytoplasma, in order to forecast the potential of secondary spread of the disease and to initiate a disease management strategy, the aim of the present study was to develop a PCR assay for

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Accepted 1 July 2005

the SCGS phytoplasma. Currently, phytoplasma detection and characterization are based predominantly on PCR amplification of rRNA genes (rDNAs), especially the 16S rDNA (Seed *et al*., 1994; Bertaccini *et al*., 1995, 1997; Lloyd-Macglip *et al*., 1996; Jomantiene *et al*., 1998; Razin *et al*., 1998; Griffiths *et al*., 1999). The 16S rRNA genes, 16S-23S rRNA intergenic spacer regions and 23S rRNA genes have been widely used as targets to detect and identify many different types of phytoplasma (Gundersen *et al*., 1996). In the present study, universal phytoplasma-specific primer pairs P1 and P7 (Deng & Hiruki, 1991; Smart *et al*., 1996), derived from highly conserved ribosomal sequence primers within the 16S and 23S RNA gene sequences and intergenic spacer regions, were used in direct PCR. The P4 and P7 primers (Smart *et al*., 1996) were used for nested PCR assays that successfully detected the SCGS phytoplasma in *D. vulgaris* for the first time.

Materials and methods

Rearing test plants and insects

The SCGS-susceptible sugarcane variety CoLk 8102 (*Saccharum* spp. hybrid) was used throughout the study. Three sets each of healthy as well as SCGS-infected plants were maintained separately in the glasshouse. Healthy plants were raised from seed that was subjected to moist hot-air treatment at 54°C for 2·5 h to eliminate any SCGS phytoplasma, and were never exposed to disease-transmitting insects. Healthy plants and those showing SCGS symptoms were maintained in separate sections of an insect-free glasshouse at 25–28°C and 65–70% RH. One-month-old diseased plants were utilized for acquisition feeding by *D. vulgaris*. The population of *D. vulgaris* was maintained in cages (60 cm high and 25 cm in radius) made up of transparent sheets of glass fibre. Top and side openings of the cage were covered with fine nylon mesh for aeration. Caged *D. vulgaris* colonies were fed leaves of healthy sugarcane plants. To obtain inoculative nymphs, 4th instar nymphs were given a 15-day acquisition access period on leaves of three sets of SCGS-infected (showing symptoms) CoLk 8102 plants.

Extraction of DNA

DNA was extracted from SCGS-exposed and non-exposed *D. vulgaris* nymphs, leaves of healthy sugarcane and sugarcane experimentally infected with the SCGS phytoplasma, using a phytoplasma-enrichment procedure (Dellaporta *et al*., 1983).

Amplification reactions

Oligonucleotide primer pair P1 (5′-AAGAGTTTGATCCTGGCTCAGGATT-3′)/P7 5′-CGTCCTTCATCGGCTCTT-3′) (Deng & Hiruki, 1991; Smart *et al*., 1996) was used in direct PCR to amplify SCGS phytoplasma 16S and 23S rDNA intergenic regions from infected leaves and insect vectors. Assays were performed in 10-μL volumes containing 15 ng template DNA, 100 ng of each primer; 150 μM of each dNTP (adenosine, thymine, guanine and cytosine), and 4 mM $MgCl_2$, 0·6 units *Taq* Polymerase (Bangalore Genei, India) with 1 × PCR buffer provided with the enzyme. Reaction mixtures containing only healthy plant or nonexposed insect DNA served as negative controls. Cycling was performed in a PTC 200 thermocycler (MJ Research) as follows: 5 min denaturation at 95°C followed by 35 cycles of 95°C for 30 s, 58°C for 1 min, 72°C for 1 min and elongation for 10 min at 72°C followed by a 15-min cooling-down period at 4°C. Following a first-round PCR with primer pair P1/P7, a 1-μL aliquot of PCR-amplified product was used in the nested PCR reaction using primer pair P4 (5′-GAAGTCTGCAACTCGACTTC-3′)/P7 (5′-CGTCCTTCATCGGCTCTT-3′) (Smart *et al*., 1996). The thermocycler was programmed as above, except the annealing temperature was 55°C for 1 min. The experiment was repeated three times using fresh samples (five leaf samples and three insect samples per experiment) each time to confirm the presence/absence of phytoplasma.

Analysis of PCR amplification products

Volumes of 5 μL nested amplicon were resolved on a 2% (w/v) agarose gel containing 1 μg mL^{-1} ethidium bromide. Electrophoresed samples were photographed using an AlphaImager TM Gel (Alpha Innotech Corporation) documentation system.

Results and discussion

There was no amplification of phytoplasma DNA from healthy sugarcane or insects in direct PCR assays using the primer pair P1/P7. A product of *c*. 1600 bp was amplified from all the samples of SCGS-infected sugarcane leaves. The inability of direct PCR to detect phytoplasma necessitated nesting of amplified products with another universal phytoplasma-specific primer pair. Nesting of P1/P7-amplified product with P4/P7 primers resulted in amplification of *c*. 500-bp products in both SCGS-infected leaves and exposed insect vectors. All the samples of SCGS-infected leaves and exposed insect vectors tested positive for the presence of the *c*. 500-bp product. No PCR products were amplified in any of the samples when DNA extracts from healthy plants and unexposed vectors were assayed by the nested PCR primer pairs.

Prior to this work molecular detection of SCGS phytoplasma in insect vectors had not been accomplished, although the disease was diagnosed almost 50 years ago and is prevalent in almost all sugarcane-growing countries, causing economic losses. Serology and DNA tests have been developed for diagnosis of phytoplasma diseases in sugarcane (Ratana, 2001; Srivastava *et al*., 2003). Of these techniques, PCR testing was found to be the most sensitive and reliable. Seasonal identification of SCGS-transmitting vectors is of prime importance for timely control of this disease (Blanche *et al*., 2003). Detection of phytoplasma may enable the identification of insect vectors in plant disease systems where vectors have not been determined, such as in Australia, where the grassy shoot disease phytoplasma has been found in grasses. Because a single round of PCR with the primer pair P1/P7 was not able to detect low-titre phytoplasma infection in *D. vulgaris*, a second round of PCR was necessary. A nested PCR approach is often needed for detection of phytoplasmas (Schneider & Gibb, 1997) when they occur at low levels or are distributed unevenly in their plant or insect hosts (Goodwin *et al*., 1994; Andersen *et al*., 1998). Poor amplification of target DNA by direct PCR is sometimes attributed to inhibitors present in host plant/vector tissues (Cheung *et al*., 1993; Schneider & Gibb, 1997).

Nested PCR using P1/P7 and P4/P7 has proven to be a reliable molecular tool for detection of the SCGS phytoplasma in *D. vulgaris*. Sequence analysis of the phytoplasmal DNA would help in verifying the identity, phylogenetic association and genetic relatedness of SCGS phytoplasmas. It is likely that rapid and sensitive PCR testing of important breeding lines of sugarcane and insect vectors will augment, or perhaps even replace, the traditional indexing procedures that have been used historically to detect SCGS phytoplasmas.

Acknowledgements

The authors are highly grateful to Dr R.L. Yadav, Director, IISR, Lucknow for providing the necessary facilities to carry out the experiments and encouragement during the course of this investigation.

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