Molecular and Symptom Analysis Reveal the Presence of New Phytoplasmas Associated with Sugarcane Grassy Shoot Disease in India

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ABSTRACT

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A total of 240 sugarcane (*Saccharum officinarum*) plants showing phenotypic symptoms of sugarcane grassy shoot (SCGS) disease were collected from three states of India, Maharashtra, Karnataka, and Uttar Pradesh. Phytoplasmas were detected in all symptomatic samples by the polymerase chain reaction (PCR) amplification of phytoplasma-specific 16S rRNA gene and 16S-23S rRNA spacer region (SR) sequences. No amplification was observed when DNA from asymptomatic plant samples was used as a template. Sixteen samples were selected on the basis of phenotypic symptoms and geographic location, and cloning and sequencing of the 16S rRNA and spacer regions were performed. Multiple sequence alignments of the 16S rRNA sequences revealed that they share very high sequence similarity with phytoplasmas of rice yellow dwarf, 16SrXI. However, the 16S-23S rRNA SR sequence analysis revealed that while the majority of phytoplasmas shared very high (>99%) sequence similarity with previously reported sugarcane phytoplasmas, two of them, namely BV2 (DQ380342) and VD7 (DQ380343), shared relatively low sequence similarity (79 and 84%, respectively). Therefore, these two phytoplasmas may be previously unreported ones that cause significant yield losses in sugarcane in India.

Phytoplasmas of sugarcane (*Saccharum officinarum* L.) are reported to be associated with three major diseases: sugarcane white leaf (SCWL), sugarcane grassy shoot (SCGS), and sugarcane yellow leaf (SCYL). In many parts of Asia, SCGS and SCWL diseases are becoming a major threat to sugarcane cultivation. SCGS and SCWL are characterized by white leaves with complete loss of chlorophyll and profuse tillering, giving the plant a grassy appearance (3). In India, SCGS phytoplasmas are spreading at an alarming rate, adversely affecting yield of the sugarcane crop. Sugarcane is a vegetatively propagated crop, and therefore phytoplasmas can be transmitted via propagation material, although phytoplasmas are also

transmitted by phloem-feeding leafhopper vectors (22,24,28,29). The SCGS and SCWL phytoplasmas are closely related to phytoplasmas associated with rice yellow dwarf (RYD) disease, annual bluegrass white leaf (ABGWL), bermuda grass white leaf (BGWL), and brachiaria grass white leaf (BraWL) (8,11). SCGS and SCWL phytoplasmas have been classified under the 16SrXI-B subgroup of the rice yellow dwarf (RYD) group (16SrXI) (8,13).

Extensive field surveys in three major sugarcane growing states of India, namely Maharashtra, Karnataka, and Uttar Pradesh, revealed that phytoplasma infection in sugarcane exhibits different phenotypic symptoms. These observations led to speculation that there might be more than one phytoplasma infecting sugarcane crops in India and prompted us to determine if there was genetic variation in the phytoplasma. The goal of this study was to isolate, clone, and sequence the 16S rRNA gene and the 16S-23S spacer region (SR) from sugarcane plants from different regions of India showing phytoplasma-like symptoms.

MATERIALS AND METHODS

Collection of plant material. Sugarcane (*S. officinarum*) plant samples exhibiting at least one of the following symp-

toms—creamy leaves, grassy shoot with white leaves, stunted plants with yellow leaves, and white leaf in the whorl of green leaves,—were collected from Maharashtra, Karnataka, and Uttar Pradesh states during July 2003 to December 2005. From each location, three to four samples were collected. Corresponding asymptomatic samples were also collected.

DNA extraction and polymerase chain reaction (PCR) amplification. Total DNA from leaves of healthy and infected sugarcane plants was extracted by the method of Ahrens and Seemüller (1). Concentration of DNA was estimated spectrophotometrically. DNA extracted from symptomatic sugarcane plants was used as template for PCR, while DNA extracted from corresponding asymptomatic plants was used as negative control. The 16S rRNA gene was PCR amplified using the SN910601 (GTTTGATCCTGGCTCAGGATT) and SN910502 (AACCCCGAGAACGTATT CACC) primers (16). The 16S-23S rRNA SR was PCR amplified using P3 (GGA TGGATCACCTCCTT) and P7 (CGTCTT TCATCGGCTCTT) primers (23). The PCR reaction was performed with 1× PCR buffer (Fermentas), 1.25 mM MgCl₂, 50 ng template DNA, 0.2 mM of each dATP, dGTP, dTTP, and dCTP, 0.1 μM of each primer, and 1 U of *Taq* DNA polymerase (Fermentas) in a final reaction volume of 50 μl. The PCR reaction was carried out in a PTC-100 thermocycler (MJ Research) for 35 cycles comprising denaturation at 94°C for 30 s; annealing at 55°C (16S rRNA gene) or 57°C (16S-23S rRNA SR) for 45 s; extension at 72°C for 90 s (16S rRNA gene) or 60 s (16S-23S rRNA SR); and final extension at 72°C for 5 min. The reaction mixture was electrophoresed in 1% agarose gel, and the PCR products were visualized by UV transillumination after staining with ethidium bromide. Restriction fragment length polymorphism (RFLP) analysis was performed on the PCR amplified 16S rDNA fragment using *Hpa*II, *Sau*3AI, *Taq*I, *Tas*I, and *Tau*I restriction enzymes according to the manufacturer's (New England BioLabs) instructions.

Cloning and sequencing. DNA fragments of approximately 1.3 kb (16S rRNA

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*The e-Xtra logo stands for "electronic extra" and indicates that Figure 1 appears in color in the online edition.

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gene) and 0.32 kb (16S-23S rRNA SR) were purified using QIAquick gel extraction kit (QIAGEN) and ligated to pGEM-T vector according to the manufacturer's instructions (Promega Corp.). The recombinant plasmid was used to transform *Escherichia coli* strain DH5 α . Plasmid DNA from individual clones was extracted with a Miniprep kit (QIAGEN) and checked for the presence of the insert by digesting the plasmid DNA with *NotI* and *EcoRI* (New England BioLabs) (data not shown). The inserts were sequenced from both directions using universal SP6 or T7 primers by the standard procedure (19).

Phylogenetic analysis. The sequences of 16S rRNA gene and 16S-23S rRNA SR from the present study were deposited in NCBI GenBank, and the accession numbers are given in Table 1. BLAST (Basic Local Alignment Search Tool) search (2) for 16S rRNA gene and 16S-23S rRNA SR sequences was performed to identify closely related nucleotide sequences available in the database (<http://www.ncbi.nlm.nih.gov>).

The nucleotide sequences were aligned using ClustalW program with MacVector 9.0 software. The genetic distances and similarities were calculated using the Kimura 2-parameter. The phylogenetic trees were constructed using neighbor-joining by MEGA-3.1 software (12) with 1,000 bootstrap replications.

RESULTS

Disease symptoms and PCR amplification. The survey of various fields showed that grassy shoot with chlorotic or creamy white leaves was the most prevalent symptom of phytoplasma infection. All the sugarcane plant samples that exhibited one or more of the four major symptom types—cream-colored leaves, grassy shoots with white leaves, stunted plants with yellow leaves, and white leaves in a whorl of green leaves (Fig. 1)—were positive for PCR amplification of phytoplasma-specific DNA products of 1.3 and 0.32 kb in length representing 16S rRNA gene and 16S-23S rRNA SR, respectively (data not shown). A total of 240 samples

(including 42 that were asymptomatic), which were collected from three different states of India, were analyzed. No PCR amplification was observed with either set of primers when DNA from the asymptomatic samples collected from the same fields was used as template.

In an attempt to detect the polymorphism in the 16S rDNA, the PCR products were subjected to RFLP analysis. However, no significant polymorphism was detected using five restriction enzymes (*HpaII*, *Sau3AI*, *TaqI*, *TasI*, and *TauI*, data not shown).

Analysis of 16S rRNA gene sequences. Partial sequence (1,183 bp) of 16S rRNA gene was determined for the selected phytoplasmas listed in Table 1. The multiple sequence alignment revealed that the phytoplasmas collected from different districts as well as from a single district, although producing different symptoms, shared a very high (>99%) sequence identity. BLAST searches for the 16S rRNA gene sequences reported in this paper (DQ459438, DQ459439, AM261829,

Table 1. Predominant symptoms of the phytoplasma-infected plants collected, their location of collection, variety of host plant, and accession numbers of the 16S-23S rRNA spacer region (SR) and 16S rRNA gene sequences reported in the present study

S. no.	Isolate	Symptoms	State	Variety of host plant	Accession no.	
					16S-23S rRNA SR	16S rRNA
1	NS1	Cream colored leaves	Karnataka	Co 86032	DQ380336	DQ459438
2	NS5	Grassy shoot with white leaves	Karnataka	CoC 671	DQ380337	
3	ND1	Stunted plants with yellow leaves	Karnataka	CoC 671	DQ380338	AM269744
4	ND7	White leaf in the whorl of green leaves	Karnataka	Co 94012	DQ380339	AM269745
5	VG4	Grassy shoot with white leaves	Maharashtra	Co 86032	DQ380340	AM269746
6	GD3	Grassy shoot with white leaves	Maharashtra	Co 94012	DQ380341	AM269747
7	BV2	Grassy shoot with white leaves	Maharashtra	Co 86032	DQ380342	DQ459439
8	VD7	Grassy shoot with white leaves	Maharashtra	Co 92004	DQ380343	AM261831
9	DR2	Grassy shoot with white leaves	Maharashtra	Co 86032	DQ380344	AM269748
10	SV3	Grassy shoot with white leaves	Maharashtra	Co 86032	DQ380345	
11	BO1	Grassy shoot with white leaves	Maharashtra	CoSi 98071	AM269739	AM266588
12	MA4	Stunted plants with yellow leaves	Maharashtra	CoC 671		AM269749
13	SV1	White leaf in the whorl of green leaves	Maharashtra	Co 94012	AM261742	AM261830
14	PW12	Grassy shoot with white leaves	Uttar Pradesh	CoC 671	AM261833	AM269751
15	CM1	White leaf in the whorl of green leaves	Uttar Pradesh	CoS 675	AM269740	AM261829
16	CM6	White leaf in the whorl of green leaves	Uttar Pradesh	CoS 8436	AM269741	AM269750

Fig. 1. Different symptoms of sugarcane grassy shoot disease: **A**, cream colored leaves; **B**, grassy shoot with white leaves; **C**, grassy shoot with yellow leaves; **D**, white leaf in the whorl of green leaves.

AM261830, and AM261831) indicated 99% sequence identity with SCWL (AB052874) and Mollicutes from *S. officinarum* (X76432), 98% with sorghum grassy shoot (SGS) phytoplasma (AF509325) and BGWL phytoplasma (AF248961), and 97% with RYD phytoplasma (D12581). This confirmed that the

phytoplasmas tested in this study belong to RYD 16S-group of '*Candidatus phytoplasma*'. The sequence identity among the 16S rRNA gene sequences reported in this paper was more than 99%.

Besides this, the SCGS and SCWL shared one signature sequence, 5'-TTA-3' (positions 520–522), and three single-

nucleotide alterations unique for the group: 'C' at position 119, 'C' at 1018, and 'T' at 1172. These sequence alterations clearly distinguish the SCGS and SCWL phytoplasmas from other phytoplasmas. Importantly, the signature sequences that are unique to members of the RYD group, such as 5'-AACACTG-3' and 5'-GCAA-3'

Fig. 2. Phylogenetic tree constructed by neighbor-joining method, comparing the 16S rRNA gene sequences of sugarcane phytoplasma with those of other related phytoplasmas from GenBank. 16S rRNA gene sequences of phytoplasmas determined in this study are in bold. Bar represents phylogenetic distance of 1%. Numbers on branches are confidence percentage obtained from 1,000 bootstrap replicates.

(13), are also present in the AM261830, SV1; AM261829, CM1; AM261831, VD7; DQ459438, NS1; and DQ459439, BV2 phytoplasmas, reported in our samples.

To study the phylogenetic relationship of our phytoplasmas to SCGS, SCWL, and other RYD 16S-group phytoplasmas, partial 16S rRNA gene sequences (1,183 bp) of 38 phytoplasmas were aligned using ClustalW. A phylogenetic tree, constructed by neighbor-joining method with 1,000 bootstrap replications, is presented in Figure 2. All the SCGS, SCWL, and SGS formed a stable phylogenetic subcluster judged by branch length and bootstrap values of 100% in RYD 16S-group.

To rule out the possibility of amplification of the host plastid 16S rRNA gene in PCR, we performed a direct comparison of 16S rRNA gene sequences of sugarcane plastome and SCGS phytoplasma. The results showed a significant difference between the two 16S rRNA gene sequences, indicating that the cloned 16S rRNA gene represents the SCGS phytoplasma, not the host plant chloroplast genome.

Analysis of 16S-23S rRNA SR. Multiple sequence alignment of 16S-23S rRNA SR sequences, listed in Table 1, indicated that 16S-23S rRNA SR of all phytoplasmas were almost identical (315 bp) except for those of BV2 and VD7 (312 bp). For further analysis, 16S-23S rRNA SR sequence of NS1 isolate was taken as a representative for all the similar sequences. The NS1 (DQ380336) shared 79 and 84% sequence identity with those of BV2 (DQ380342) and VD7 (DQ380343), respectively (Fig. 3). The BV2 and VD7

shared 83% sequence identity with each other. There was no correlation between phenotypic symptoms and SR sequence variations. For example, NS1, NS5, ND1, ND7, and SV3 exhibited different phenotypic symptoms, but had identical 16S-23S rRNA SR sequences. On the contrary, BV2 and VD7 showed similar phenotypic symptoms with those of NS5, VG4, GD3, DR2, SV3, and PW12, but represented sequence variation in 16S-23S rRNA SR.

The 16S-23S rRNA SR sequences were aligned with previously reported sequences in the database. The results showed that NS1 (DQ380336) shared 99% sequence identity with SCGS (AB243298 and AM085763) from India and with SCWL (AF037595) from the United States. It also showed 98% sequence identity with SCGS (AM085764) from India and SCWL (AY139874) reported from Taiwan. In the phylogenetic analysis (Fig. 4), NS1 (DQ380336), the SCGS phytoplasmas (AB243298, AM085763, and AM085764) from India, and SCWL reported from Taiwan (AY139874) and the United States (AF037595) grouped together with a bootstrap value of 92. In contrast, BV2 (DQ380342) and VD7 (DQ380343) diverged from the other phytoplasmas and grouped together with a bootstrap value of 89%. The long branching supports recent divergence of BV2 and VD7, suggesting that these phytoplasmas might be new diverging strains from the more prevalent strains represented by NS1 isolate.

DISCUSSION

Phytoplasma diseases of sugarcane (SCGS and SCWL) have been reported to

cause substantial losses in the sugarcane crop all over Asia (4–6,18,20,27). The chance of their transmission to other geographical regions seems to be high, given the large phytoplasma reservoir already revealed, the propensity of new phytoplasma strains to evolve (14), and the ability of leafhoppers, the most common insect vectors of phytoplasma, to migrate long distances (25,26) and switch to new host plants (17). In the present study, 198 sugarcane plant samples exhibiting different symptoms tested positive for phytoplasma through PCR amplification of 16S rRNA gene and 16S-23S rRNA SR using primers specific for phytoplasmas. The presence of various symptoms in phytoplasma-infected plants led to the hypothesis that multiple strains of sugarcane phytoplasma were infecting the sugarcane crop in India. If multiple strains were present, identification and characterization of the strains in particular host plant species would be needed so that effective and early diagnostic methods, based on genetic heterogeneity, could be developed. In view of reports that some ash yellows phytoplasmas are more aggressive and can cause serious damage to less tolerant *Fraxinus* species (21), it is important determine whether any of the newly identified sugarcane phytoplasmas show differential aggressiveness in causing grassy shoot disease and yield losses.

A BLAST search for the 16S rRNA gene sequences reported in this paper showed that they shared more than 99% sequence identity among themselves and 97 to 99% identity with those of other phytoplasmas in the RYD 16S-group. This confirmed

Fig. 3. Multiple sequence alignment of 16S-23S rRNA spacer region sequences of sugarcane grassy shoot (SCGS) phytoplasmas NS1, BV2, and VD7 by ClustalW.

that the present SCGS phytoplasmas belong to RYD 16S-group of '*Candidatus phytoplasma*'. Jung et al. (10) reported that two phytoplasmas should be grouped under the same species-level taxa if they have 16S rRNA gene sequence identity more than 97.5%. The sequence homology in the present SCGS-causing phytoplasma is more than 99%, and their homology with SCWL and BGWL is from 98 to 99%. Therefore, it can be concluded that SCGS, SCWL, SGS, and BGWL belong to the same species-level taxa. The phylogenetic tree computed by the neighbor-joining method also revealed that all the phytoplasmas causing SCGS, SCWL, SGS, and BGWL form a stable phylogenetic sub-cluster, as judged by branch length and bootstrap values of 100% in the RYD 16S-group clusters. This indicated that these

phytoplasmas are closely related and share a common ancestor. However, Fox et al. (9) reported that 16S rRNA gene sequence homology may not be able to distinguish between recently diverged species. Smart et al. (23) also reported that genetic variation of 16S rRNA gene sequences within a given group of phytoplasmas is difficult because of the high level of sequence conservation. Instead, sequence diversity of 16S-23S rRNA SR could be used to identify different strains of phytoplasmas classified within the same group. In the present paper, we report that 16S-23S rRNA SR sequence diversity could be used to distinguish recently diverged strains within a given group of phytoplasmas in a sugarcane crop. In most of the phytoplasmas, the 16S-23S rRNA SR sequences were identical, regardless of the phenotypic

symptoms of the infected plants. Similarly, closely related phytoplasmas have been reported to induce different phenotypic symptoms, and different types of phytoplasmas to induce similar symptoms (7,8,14,15).

In the present study, we found that two phytoplasmas, BV2 (DQ380342) and VD7 (DQ380343), showed similar phenotypic symptoms, but the sequence similarity with that of other phytoplasmas from the present study was only 79 and 84%, respectively. Figure 3 also shows that BV2 (DQ380342) and VD7 (DQ380343) diverged to the separate subcluster in the cluster shared by NS1 (DQ380336) and other SCGS and SCWL isolates. The long branching of BV2 (DQ380342) and VD7 (DQ380343) supports recent divergence from the SCGS phytoplasma.

Fig. 4. Phylogenetic tree constructed by neighbor-joining method, comparing the 16S-23S rRNA spacer region (SR) sequences of sugarcane phytoplasma with those of other related phytoplasmas from GenBank. 16S-23S rRNA SR gene sequences of phytoplasmas determined in this study are in bold. Bar represents phylogenetic distance of 2%. Numbers on branches are confidence percentage obtained from 1,000 bootstrap replicates.

From the present investigation, it is clear that there is one phytoplasma (NS1) that is more prevalently associated with SCGS disease in India, but there are two other phytoplasmas, which have diverged from the existing NS1 in their 16S-23S rRNA SR sequence. This study adds to our knowledge of the diversity of phytoplasma infecting sugarcane crop in India. We also report two new SCGS phytoplasmas never reported earlier.

It may also be concluded that although 16S rRNA sequence can be used routinely to distinguish and establish relationships between genera and well-resolved phytoplasma species, very recently diverged species may not be recognized by this criterion. On the other hand, the 16S-23S rRNA SR provides greater genetic variation than 16S rRNA sequence; hence, it is more suitable for distinguishing newly evolved strains within a given species.

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