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RESEARCH ARTICLE

Use of *secA* Gene for Characterization of Phytoplasmas Associated with Sugarcane Grassy Shoot Disease in India

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Abstract Surveys of commercial sugarcane varieties were conducted to the phytoplasma disease incidence in eight major sugarcane growing states of India (Uttarakhand, Uttar Pradesh, Maharashtra, Bihar, Assam, Chhattisgarh, Haryana and Tamil Nadu) during 2014–2015. Leaves from 24 symptomatic sugarcane plants of eight varieties showing grassy shoot and chlorosis symptoms, and of 8 non-symptomatic plants were collected and analyzed for phytoplasma presence using *16S rRNA* and *secA* gene-specific primers. Amplification of 1.8- and 1.2-kb products using nested primers (P1/P7 and R16F2n/R16R2) of *16S rRNA* gene and 880- and 480-bp products using *secA* gene-specific primer pairs (SecAfor1/SecArev3 and SecAfor2/SecArev3) was obtained for all the 24 symptomatic sugarcane samples. Pairwise sequence comparison, phylogenetic and in silico RFLP analysis of partial *16S rRNA* and *secA* gene sequences of eight strains of sugarcane grassy shoot phytoplasma representative of the eight states confirmed the association of '*Candidatus* phytoplasma oryzae'-related strains (16SrXI-B) with symptomatic sugarcane varieties. The study confirmed that *secA* gene-specific primers could be employed for molecular characterization of phytoplasmas associated with sugarcane grassy shoot phytoplasmas belonging to 16SrXI group.

Keywords Sugarcane phytoplasma · *16S rRNA* gene · Phylogeny · RFLP · *secA* gene

Introduction

Sugarcane grassy shoot (SCGS) disease associated with phytoplasma presence is the most destructive disease of sugarcane in recent years and a serious threat to sugarcane cultivars in Asian countries (Marcone 2002; Rao et al. 2012, 2014; Zhang et al. 2016). SCGS disease is mainly characterized by production of large number of thin, slender, adventitious tillers from the base of the affected stools. This profuse growth gives rise to a dense or crowded bunch of tillers bearing chlorotic leaves. The affected plants do not produce millable canes and results in decrease of productivity of cane yield and sugar (Rao et al. 2012). In sugarcane, phytoplasmas are mainly transmitted through propagation material and phloem-feeding leafhoppers (Srivastava et al. 2006; Wongkaew et al. 1997; Rao et al. 2014; Tiwari et al. 2016, 2017). The main phytoplasma associated with SCGS disease is '*Candidatus* phytoplasma oryzae'-related strain (Marcone et al. 2001; Nasare et al. 2007; Viswanathan et al. 2011; Rao et al. 2014; Zhang et al. 2016). Classification of phytoplasmas mainly relies on comparison of *16S rRNA* gene sequence, phylogenetic analysis and RFLP profile pattern (Lee et al. 1998; Wei et al. 2007). However, recently other housekeeping and protein coding genes which are less conserved were also used for better resolution of phytoplasma groups and subgroups (Botti and Bertaccini 2003, Lee et al. 2006; Streten and Gibb 2005; Shao et al. 2006; Hodgetts et al. 2008;

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Ramaswamy et al. 2013). Non-ribosomal single-copy genes such as *secA*, which encodes the ATP-dependent energy generator in the bacterial precursor protein translocation cascade system (secA) has been used to cross confirm the phytoplasma classification based on the *16S rRNA*gene (Hodgetts et al. 2008; Bila et al. 2015). Bekele et al. (2011) utilized *secA* gene for characterization of phytoplasmas associated with sugarcane grassy shoot disease from Sri Lanka, India and Vietnam on the basis of a limited number of SCGS strains to confirm its validity for strain characterization. The present study was carried out to confirm the usefulness of *secA* gene-specific primers for detection and characterization of phytoplasma strains belonging to 16SrXI group.

Materials and Methods

A total of 24 commercially grown sugarcane leaf samples of eight varieties (CoC671-Tamil Nadu, Co997-Assam, CoLK94184-Uttarakhand, CoSe92423-Chhattisgarh, CoS8436-Uttar Pradesh, CoLK09202-Haryana, CoS07250-Bihar, Co86032-Maharashtra) showing characteristic symptoms of sugarcane grassy shoot disease were collected from eight major sugarcane growing states of India (Table 1). Three leaf samples from each symptomatic and one non-symptomatic sugarcane of same variety were collected during a survey from eight sugarcane growing states of India and were analyzed for phytoplasma identification. Disease incidence was recorded using percentage plants from each sugarcane field. Total genomic DNA was extracted from all the samples following a described procedure (Ahrens and Seemüller 1992). The phytoplasma *16S rRNA* gene-specific universal primer pairs P1/P7 (Deng and Hiruki 1991; Schneider et al. 1995) followed by R16F2n/R16R2 (Gundersen and Lee 1996) and *secA* gene-specific primers SecAfor1/SecArev3 (5′GARATGAAAACT GGRGAAGG3′/5′GTTTTRGCAGTTCCTGTCATCC3′) followed by SecAfor2 (5′GATGAGGCTAGAACGC CT3′)/SecArev3 were used (Hodgetts et al. 2008) in nested PCR assay. The DNA extracted from sugarcane (variety CoS 07250 infected with a '*Ca*. P. oryzae'-related strain, GenBank Acc. No. JX862179) (Rao et al. 2014) and maintained in greenhouse, was used as positive control. The DNA extracted from non-symptomatic sugarcane leaves and non-template reactions containing sterile distilled water in place of template DNA were used as negative control.

PCRs were carried out in a Mastercycler (Eppendorf, Germany) in 25 µl volumes containing 100 ng DNA template, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each primer, 150 mM each dNTP, 0.5 U Taq DNA polymerase (G-Biosciences). PCR assay was performed with: initial denaturation at 94 °C for 5 min, followed by 30 cycles consisting of denaturation at 94 °C for 45 s, annealing at 56 °C (53 °C for secAfor1/secArev3) for 30 s, and extension at 72 °C for 2 min (1 min for secAfor1/secArev3), with extension in the final cycle for 10 min. First round P1/P7 and secAfor1/secArev3, PCR products were diluted in distilled water in 1:10 ratio and 1 µl of the diluted template was used in nested PCR using R16F2n/R16R2 and secAfor2/secArev3 primer pairs, respectively. Reaction mixture and condition of nested PCR assays were similar as above except for the annealing at 55 °C for 30 s using R16F2n/R16R2. A 5 µl of each PCR product was subjected to electrophoresis in a 1.0% (w/v) agarose gel, stained with ethidium bromide and visualized in a UV transilluminator.

The PCR amplified *16S rRNA* and *secA* gene products of all 24 phytoplasma strains was purified using the SV wizard purification system (Promega, Madison, USA) and directly sequenced in both directions using nested primer pairs specific for *16S rRNA* and *secA* gene, respectively. The obtained *16S rRNA* and *secA* gene sequences were

Table 1 Distribution and incidence of sugarcane grassy shoot disease in different states of India along with GenBank accession number and phytoplasma identification in different sugarcane varieties

S. no.	Place and state	Sugarcane variety	Symptoms observed	Disease incidence (%)[a]	GenBank accession no.	
					16S rRNA	*secA*
1	Kashipur, Uttarakhand	CoLK94184	Chlorotic and grassy shoot	16–19	KU131037	KY491737
2	Lucknow, Uttar Pradesh	CoS8436	Grassy shoot	33–39	KJ435299	KY491732
3	Uchhani, Haryana	CoLK9202	Chlorotic leaves and grassy shoot	13	KU131036	KY491739
4	Seetamarhi, Bihar	CoS07250	Chlorotic leaves	22	KY468266	KY491734
5	Raipur, Chhattisgarh	CoSe92423	Chlorotic leaves	17	KY921760	KY491733
6	Tinsukia, Assam	Co997	Grassy shoot	8	KY468265	KY491736
7	Ahmednagar, Maharashtra	Co86032	Chlorotic leaves	23	KJ698651	KY491735
8	Coimbatore, Tamil Nadu	CoC671	Chlorotic leaves	26	KY468267	KY491738

[a] Incidence was calculated on the basis of symptoms recorded per hundred plants

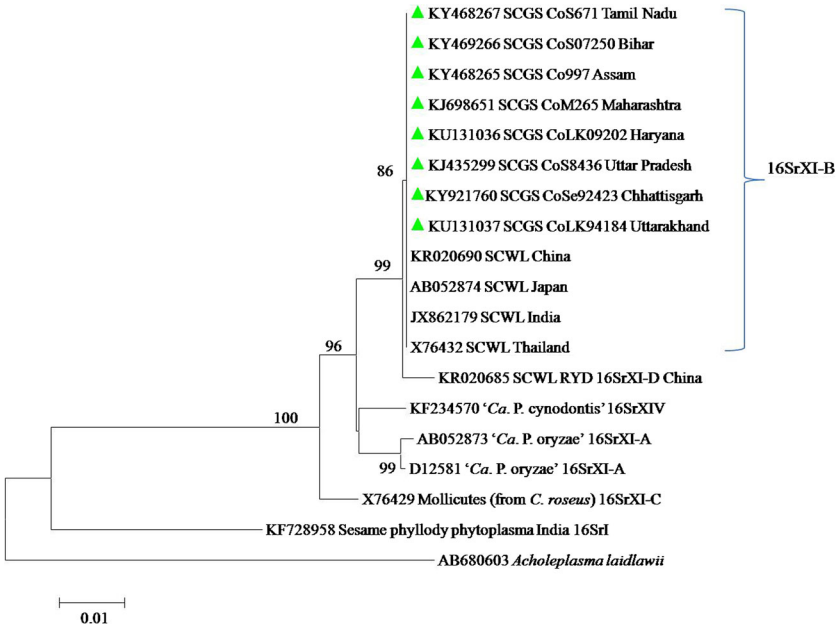

Fig. 1 Phylogenetic tree constructed by the neighbor joining method using 16S rDNA sequence of the sugarcane grassy shoot phytoplasma from eight Indian states and other related phytoplasmas. Bootstrap values (1000 replications) are shown as percentages at the branch points of the phylogenetic tree. *Acholeplasma laidlawii* is used to root the tree

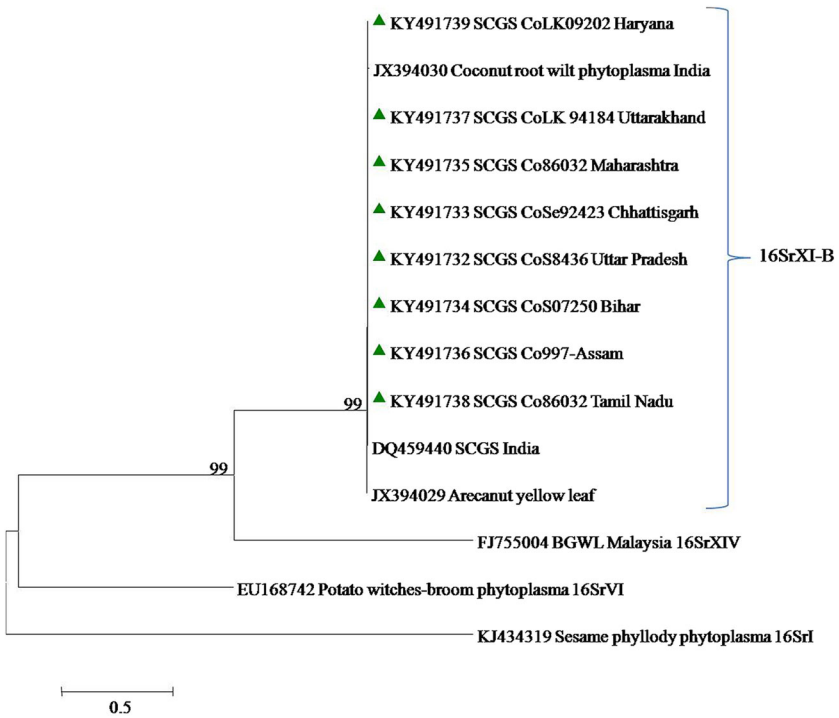

Fig. 2 Phylogenetic tree constructed by the neighbor joining method from nested PCR fragments of *secA* gene sequences of the sugarcane grassy shoot phytoplasma strains from eight Indian states and other related phytoplasmas. Bootstrap values (1000 replications) are shown as percentages at the branch points of the phylogenetic tree. A sesame phyllody phytoplasma strain (16SrI group) is used to root the tree

assembled by DNA Baser 4.7 version, and BLAST analysis was performed with homologous sequences retrieved from GenBank (https://blast.ncbi.nlm.nih.gov). Sequences were aligned using ClustalW software programme (Thompson et al. 1994) and used for the construction of phylogenetic trees using neighbor joining method with MEGA 6.0 (Tamura et al.2013) software with 1000 bootstrap replications. *Acholeplasma laidlawii* (GenBank Acc. No. AB680603) and sesame phyllody phytoplasma (GenBank Acc. No. KJ434319) were used to root the trees in phylogeny for *16S rRNA* and *secA* gene, respectively. The partial sequences of the *16S rRNA* gene were subjected to in silico RFLP analysis using pDRAW32 software (http://www.acaclone.com) and compared with selected strains of phytoplasmas related to '*Ca.* P. oryzae' and belonging to 16SrXI-B subgroup (GenBank Acc. No. JX862179).

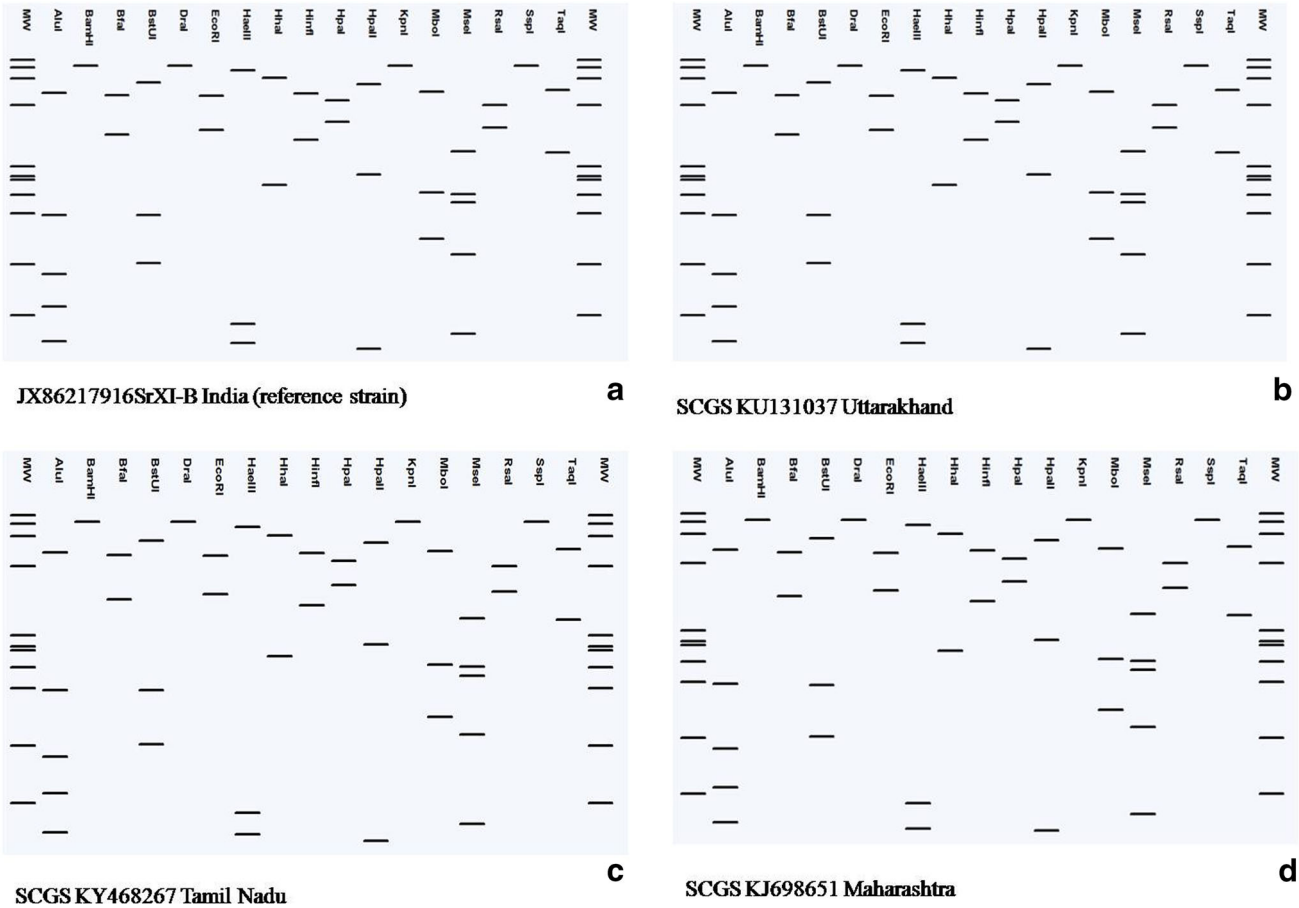

Fig. 3 Virtual RFLP pattern generated by in silico digestion of *16S rRNA* gene fragments of SCGS phytoplasma strains from 4 representative states viz, **a** reference strain SCGS 16SrXI-B subgroup (Acc. No. JX862179), **b** Uttarakhand SCGS strain, **c** Maharashtra SCGS strain, and **d** Tamil Nadu SCGS strain infecting sugarcane crop with 17 restriction enzymes (*Alu*I, *BamH*I, *Bfa*I, *BstU*I, *Dra*I, *EcoR*I, *Hae*III, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Mbo*I(*Sau3A*I), *Mse*I, *Rsa*I, *Ssp*I, *and Taq*I) using pDRAW software. MW: molecular size marker, standard PhiX174 *HaeIII* digested

Results and Discussion

In the survey made in sugarcane fields of eight major sugarcane growing states of India, phytoplasma symptoms of grassy shoot with chlorotic, white leaves and stunted growth were observed with incidence of 8–39% in different surveyed field (Table 1).

PCR analysis using phytoplasma-specific primer pairs of all 24 symptomatic samples yielded 1.8-kb amplicons in direct (with P1/P7 primers) and 1.2 kb in nested PCR assay (data not shown). PCR results using *secA* gene-specific primer pair also produced amplifications of 880 and 480 bp, respectively, with all the 24 symptomatic samples (data not shown). No products were amplified in direct and nested PCR assay when the same sets of primer pairs were used with DNA from non-symptomatic sugarcane plants and with non-template reactions.

Nested PCR amplified products of partial *16S rRNA* and *secA* gene of 1.2 kb and 480 bp, respectively, were submitted to GenBank (Table 1). Since the SCGS strains from different varieties in eight different states showed 100% sequence identity of *16SrRNA* and *secA* genes among themselves, only one sequence of SCGS strain from each variety of eight different locations was submitted to GenBank (Table 1). Pairwise sequence comparison through BLASTn search of partial *16S rRNA* gene sequence of the eight SCGS strains showed 99–100% sequence identity with strains of '*Ca*. P. oryzae' viz., SCGS (GenBank Acc. No. KJ435297), areca nut yellow leaf (GenBank Acc. No. KM593238) and coconut root wilt (GenBank Acc. No. JS273772). The phylogenetic analysis of *16S rRNA* gene sequences confirmed that these strains clustered together with phytoplasma strains enclosed in subgroup 16SrXI-B (Fig. 1).

The pairwise sequence comparison of partial *secA* gene sequences showed 100% identity with reported *secA* gene sequences of strains of SCGS phytoplasma (GenBank Acc. No. KX215775), 99% identity with sugarcane leaf yellows

(GenBank Acc. No. KT335270), areca nut yellows (GenBank Acc. No. JN967911), coconut root wilt (GenBank Acc. No. JS273772) and SCWL Thailand strain (GenBank Acc. No. FM208259), all members of 16SrXI group. In the *secA* gene-derived phylogenetic analysis, the eight SCGS phytoplasma strains identified in the study also clustered with phytoplasma strains in 16SrXI-B subgroup and are in agreement with the phylogenetic results based on *16S rRNA* sequences (Fig. 2).

Virtual RFLP analysis of partial *16S rRNA* gene sequences of all the SCGS strains using 17 restriction endonuclease enzymes showed that all the SCGS strains produced identical RFLP profiles to the strain of SCGS (GenBank Acc. No. JX862179) '*Ca.* P. oryzae'-related (16SrXI-B subgroup) (Fig. 3).

SCGS disease is a major constraint to sugarcane cultivation and production faced by farmers in South East Asia and India. Earlier reports based on *16S rRNA* gene sequence suggested that 16SrXI-B, 16SrXI-D and 16SrXI-F subgroups of phytoplasmas are associated with SCGS and white leaf disease in South Asian countries including India (Nasare et al. 2007; Rao et al. 2008, 2014; Zhang et al. 2016; Yadav et al. 2016). In the present study, the utilization of *secA* gene-specific primers was validated for these phytoplasma strains identification and proven as suitable alternative to the *16S rRNA* gene-based identification (Hodgetts et al. 2008; Bekele et al. 2011; Valiunas et al. 2015; Madhupriya et al. 2015; Al-abadi et al. 2016). Other phytoplasmas have also been successfully detected and characterized on the basis of the *sec*A gene sequences in India such as those associated with 16SrI-B and 16SrII-C subgroup detected in oil palm stunting (Mehdi et al. 2011), sesame phyllody and witches' broom (Nabi et al. 2015) and brinjal little leaf (Rao and Kumar 2017) diseases.

In India, SCGS disease have been reported to cause substantial losses in infected sugarcane crop in terms of cane weight and sucrose content (Tiwari et al. 2012). So far, the identification and characterization of SCGS phytoplasma strains in India was carried out using 16S ribosomal specific primers only. Therefore, the results of this study further confirm that use of *secA* gene-specific primers is useful for detection of SCGS phytoplasma under field condition and can replace the ribosomal primers.

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