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Molecular detection and characterization of phytoplasma associated with China aster (*Callistephus chinensis*) phyllody in India

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Abstract: China aster (*Callistephus chinensis* L.) is one of the most popular annual flowering plant grown throughout the world. Phyllody disease of China aster is a phytoplasma associated disease that induces severe economic losses. Phytoplasmal disease in China aster was assessed for phytoplasma by direct polymerase chain reaction primed by using phytoplasma universal primer pairs PI/P7. A 1.8 Kb DNA fragments encoding the portion of phytoplasma 16SrDNA amplified by PCR was cloned and sequenced. Sequencing of the PCR product and BLAST analysis indicated that China aster phyllody phytoplasma strain shared maximum sequence identity (99%) with strains of Peanut Witches' broom (16SrII) phytoplasma group. Phylogenetic relationship of 16SrDNA sequence of China aster phyllody phytoplasma strain in the present study confirmed association of Peanut Witches' broom (16SrII) group of phytoplasmas with China aster phyllody disease in India.

Keywords: *Callistephus chinensis,* Phytoplasma, Phylogenetic analysis, 16SrII

INTRODUCTION

China aster (*Callistephuschinensis* L. Nees.) is one of the important commercial flower crops which belong to the family Asteraceae, grown throughout the world. It stands third next to Chrysanthemum and Marigold. The crop is native to China (Navalinskiene *et al*., 2005). The flowers have long vase life and are used for various decorative purposes. In India, it is grown successfully during kharif, rabi as well as summer seasons for year-round production (Singh, 2006). China aster is also an important commercial flower crop of Siberia, Russia, Japan, North America, Switzerland and Europe. In India, it is grown in an area of 3500 ha with the productivity of 10-12 tonnes/ha. Among various states in India, Karnataka, Tamil Nadu, West Bengal and Maharashtra are the major growing states (Anonymous, 2009). The china aster area, production and productivity has however remained virtually stagnant over recent decades as the crop suffers from many diseases like Fungal wilt, Collar rot, Grey mould, Rust, Leaf spot, Stem rot, Canker and bacterial wilt. Among the major constraints, phyllody is a serious disease in most China aster growing regions (Singh, 2006). Recently, phyllody symptoms in China aster plants have been frequently observed in several fields of the India. Phyllody disease on China aster was first reported during 1986 from Bangalore, India and known to be transmitted by *Orosius albicinctus* (Rangaswamy *et al*., 1988). The disease was characterized by chlorosis, upright growth, small leaf, short internode, stunting, profuse vegetative growth and phylloid flower (transformation of floral organs into leaf-like structures). However little attempts have been made on the characterization of the phytoplasma. Therefore, present work aims were to understand the certain molecular relationship of China aster phyllody phytoplasma with other phytoplasmas.

MATERIALS AND METHODS

The sample of China aster showing typical symptoms of phyllody and healthy plants were collected from the naturally infected field and maintained in green house. The total genomic DNA was extracted from leaf tissues of healthy plants and phyllody infected plants by following the cetyltrimethyl ammonium bromide (CTAB) method described by Namba et al. (1993). The DNAs extracted from a described phytoplasma associated with periwinkle phyllody (Yamini et al. 2009) were used as positive controls. The total genomic DNA from phyllody affected plants was subjected to direct PCR. The phytoplasma universal primer pairs P1 (5°AAGAGTTTGATCCTGGCTCAGGATT 3°) (Deng and Hiruki 1991) and P7 (5° CGTCCTTCATCGGCTCTT 3°) (Kirkpatric et al. 1994) were used to amplify a 1.8 Kb corresponding to 23S rRNA gene of phytoplasma. The PCR reaction (25 µl) contained 14.4 µl sterile distilled water, 2.5 µl 10X PCR buffer, 2.5 mM MgCl2 1.5 µl, 2.5 mM dNTP 2 µl, 1.25 µl of each primer (20mM), 0.1 µl Taqpolymerase and 2 µl DNA template. The PCR was carried out in thermal cycler (Techne Genius/ Eppendorf) by using the following parameters: 94°C for 2 min. 55°C for

2 min. 72°C for 3 min. for one cycle, followed by 34 cycles of 94°C for 2 min. 55°C for 2 min. 72°C for 3 min and in the final cycle 72°C for 10 min. Resulting PCR products were analysed employing electrophoresis through 1%agarose gel stained with ethidium bromide at 80 volts in 10XTBE running buffer and DNA bands were visualized using an UV transilluminator.

DNA from agarose gel in TBE buffer was extracted and purified by using QIA quick gel extraction kit (Cat. No. 28704; Qiagen, Germany) as per the manufacturers recommendations. Eluted product was sent to the National Centre for Biological Sciences, Bengaluru, for sequencing by primer walking method. Obtained sequences were aligned and joined together to get full length sequence using 'nucleotide blast' at basic blast programmes and 'align two (or more) sequences' at specialized blast programmes freely assessing 'Basic Local Alignment Search Tool (BLAST)' at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The phylogenetic neighbor-joining trees and evolutionary analysis were conducted to study the percent homology of China aster phyllody phytoplasma with other phytoplasmas by using MEGA 6.06 software package (Tamura et al. 2013). Robustness of trees was determined by bootstrap sampling of multiple sequence alignment with 1000 replications (see below).

RESULTS AND DISCUSSION

The affected plants showed different types of phyllody disease symptoms. The affected plant showed slight yellowing along the vein followed by proliferation of short upright branches. In advanced stages of infection severe reduction in leaf size and profuse vegetative growth were observed. All the flowers developed into vegetative structures (phyllody). Occasionally partial phyllody was also noticed wherein, a few affected branches showed phylloid flower while the remaining branches produced normal flowers.

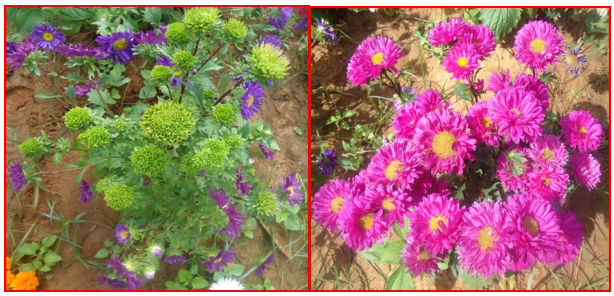

Fig. 1. *Phytoplasma- infected China aster plant showing typical phyllody symptoms: bushy appearance due to Short internode with reduced leaves, Shoot proliferation and transformation of floral organs into leaf like structures (left panel) compared with a healthy plant (right panel).*

Polymerase chain reaction was employed to establish an association of phytoplasma through amplification of phyllody specific PCR product approximately 1800bp fragment of 16SrDNA by using phytoplasma universal primers P1/P7. The PCR amplification products of 1.8 kb from infected China aster and periwinkle a positive control (Fig. 2) corresponding to 16S rDNA gene of phytoplasma was obtained from phyllody infected plants which were absent in healthy plants. After puri-

Table 1. Phytoplasma strains employed in phylogenetic analysis.

S. N.	Phytoplasma strain	Acronym	Accession no.	Group
1.	Pigeon pea witches'-broom	PPWB	AB741637.1	16SrIX
2	Black gram witches'-broom	BGWB	AB690304.1	16SrII
3.	Peanut witches'-broom	PWB	JX871467.1	16SrII
4.	Cauliflower witches'-broom	CWB	KC953000.1	16SrII-A
5.	Aster virescence	AV	AB690303.1	16SrII-A
6.	Tomato big bud	TBB	KC953004.1	16Sr I-B
7.	Periwinkle phytoplasma	PP	KC508644.1	16Sr I
8.	Sweet potato little leaf	SPLL	JQ067649.1	16SrII
9.	Soybean witches'-broom	SWB	KC508646.1	16SrII
10	Crotalaria witches'-broom	CWB	EF656454.1	16SrII
11.	Sweet potato little leaf	SPLL	JQ868446.1	16SrII
12.	Cowpea virescence	CV	KC953001.1	16SrII
13.	Jasmine witches'-broom	JWB	AB257290.1	16SrII
14	Bushehr(Iran) eggplant big bud	BEBB	JX483699.1	16SrII
15.	Eggplant big bud	EBB	JX441321.1	16SrII
16.	Sesame Phyllody	SP	KC920750.1	16SrII
17.	Tomato big bud	TBB	JQ868448.1	16Sr I-B
18.	Eggplant phyllody	EP	FN257482.1	16Sr II
19.	Sesame phyllody	SP	KF429485.1	16SrII
20.	Sesame phyllody	SP	EF193357.1	16SrII
21.	Alfalfa witches'-broom	AWB	AY169322.1	16SrII-D
22.	Cotton phyllody	CP	JQ868439.1	16SrII-C
23.	Candidatus phytoplasma aurantifolia	CPA	JQ868437.1	16SrII-B
24.	Faba bean phyllody	FB	HQ589188.1	16SrII-C

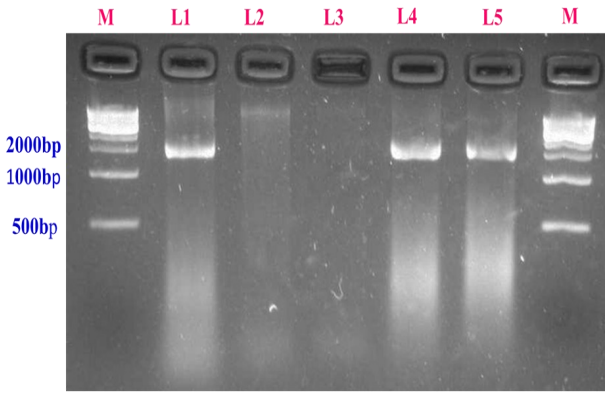

Fig. 2. *Amplification of phytoplasma 23SrRNA by PCR by using phytoplasma universal primers P1/P7 from infected China aster phyllody plants (1,800 bp amplicons). M 1Kb marker (MBI Fermentas Life Sciences, Germany), lanes L1 Undiluted China aster infected plant; L2 Healthy China aster; L3 Water control; L4 1:40dilution of PCR product of aster phyllody; L5 Positive control (Catharanthus roseus).*

fication the PCR amplified product (1.8kb) was sent to the National Centre for Biological Sciences (NCBS), Bengaluru for sequencing and the sequence obtained was subjected to blast in NCBI database to know the percent homology with other phytoplasmas sequences. The BLAST analysis indicates the close relationship with several phytoplasmas belonging to ribosomal group 16SrII and showed more than 99% nucleotide identity. The phylogenetic analysis revealed that China aster phyllody phytoplasma is closely related to the one associated sesamum phyllody (KC920750) and tomato big bud (JQ868448), both members of 16SrII group (Figure 3).

The characteristic symptoms of phyllody disease of China aster observed in present study (yellowing, stunting, proliferation of shoot and phyllody,) were similar to the symptoms of China aster previously described in India (Rangaswamy *et al*., 1988) and Myanmar (Win *et al.,* 2011). Amplification of a phytoplasma characteristic 1.8kb amplicons corresponding to23S rRNA, followed by sequencing and phylogenetic analysis revealed that the phytoplasma associated with China aster phyllody phytoplasma shared more than 99% sequence identity with sesamum phyllody phytoplasma (KC920750) and tomato big bud (JQ868448), indicated that China aster phyllody is closely related to members of Peanut Witches'broom, (16SrII) phytoplasma group. Result similar to the present investigation has already proposed by Win *et al.* (2011) indicated that China aster as a new host for a subgroup 16SrII-A phytoplasma. The 16S rDNA gene has not been sequenced completely for most of the phytoplasmas (Azadvar and Baranwal, 2010). Whether rDNA gene, encoding 16S can be used for finer differentiation of phytoplasmas in a group or not will be known when more sequences of phytoplasma in a group become available.

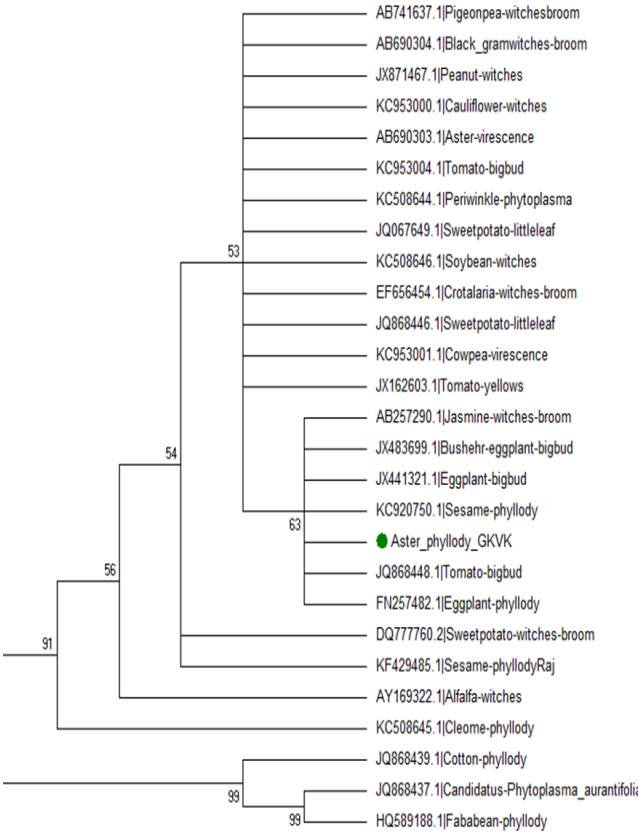

Fig 3. *Phylogenetic tree obtained from comparision of complete nucleotide sequence of China aster phyllody phytoplasma with other phytoplasmas from NCBI database. The dendrograms are calculated using neighbour-joining algorithm of MEGA 6.06 version. Numbers at nodes indicate percentage bootstrap confidence scores (1,000 replications).*

Conclusion

The aster phyllody is an emerging problem with increased incidence in the last few years. The use of infected propagating materials as well as the presence of abundant numbers of *O. albicinctus* may become the main source for spreading this phytoplasma. Based on this result and previous studies, it could be concluded that that China aster plants serve as a potential reservoirs for other phytoplasmas affecting economically important crops.

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