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IDENTIFICATION OF TWO *SUGARCANE MOSAIC VIRUS* (SCMV) VARIANTS FROM NATURALLY INFECTED SUGARCANE CROP IN PAKISTAN

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Abstract

Leaf samples of sugarcane were collected from symptomatic and non-symptomatic plants. Total RNA was extracted and purified from sugarcane leaves samples. Presence of mosaic virus was confirmed by RT-PCR amplification using primers designed to the conserved regions of the coat protein genes of *Sugarcane Mosaic Virus* (SCMV). An amplification product of expected size (approx. 900 bp) was achieved from symptomatic samples but no amplification was detected from non-symptomatic plant samples. RT-PCR amplified DNA fragments were cloned and sequenced in both directions. DNA sequence from two virus isolates from sugarcane cultivars CSSG676 and CSSG668 showed highest level of sequence identity (97% and 96%, respectively) to SCMV (Bundaberg isolate), indicating that the virus isolates infecting sugarcane varieties are variants of SCMV in Pakistan.

Introduction

Sugarcane (*Saccharum officinarum* L.) is a major sugar crop in Pakistan, cultivated on about 1.3 million hectare (Anon., 2007). Mosaic is amongst the major diseases of sugarcane in the country, a decline due to mosaic was recorded in cane yield from 10-32 % and in sugar yield from 6-10% (Anwar, 2005). Most of the sugarcane genotypes grown in Pakistan are susceptible to mosaic. Affected sugarcane plants show symptoms like leaf lamina of contrasting shades of green or yellowish chlorotic areas and may be accompanied by varying degree of reddening or necrosis (Fig. 1). The chlorotic areas are most evident in young rapidly growing leaves and are particularly distinct in the basal portion of the leaves.

Yields are significantly reduced when infection level reaches 50% (Husnnain & Afghan, 2004). The causal agent of sugarcane mosaic was attributed to a potyvirus called S*ugarcane mosaic virus* (SCMV) family *Potyviridae*, genus *potyvirus*. The virions are flexuous rods, roughly 750 nm in length and have a genome of positive-sense RNA, approximately 10 kb long with a viral encoded protein (VPg) covalently attached to the 5' terminus. There is single long open reading frame (ORF) encoding a polyprotein that is post-translationally processed into the individual gene products by viral proteases. These viruses also characteristically induce the formation of nuclear and cytoplasmic inclusion bodies in the host's cells (Shukla *et al*., 1994).

This is the largest and economically most important family of plant viruses. It accounts for nearly 25% of the known plant viruses and causes disease in almost all commercial crops. SCMV has been reported in more than 70 countries (Jeffrey *et al*., 1998). The present study of SCMV was carried out against different sugarcane cultivars grown in Pakistan.

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Fig. 1. Infected sugarcane leaf exhibiting mosaic symptoms.

Material and Methods

Sample collection: Sugarcane cultivars were planted at the experimental area of School of Biological Sciences, University of the Punjab, Lahore in the year 2004 (autumn season). Leaf samples of sugarcane were collected during spring 2005 from symptomatic (mosaic) (Fig. 1) and non-symptomatic plants. Samples were either directly processed for RNA isolation or stored at -80 °C to avoid degradation of RNA by RNases.

Isolation and purification of viral RNA: Total RNA was isolated from freshly collected SCMV infected sugarcane leaf samples using Tri Reagent Kit (Molecular Research Center, Inc. U.S.A), according to the instruction of the manufacturer. The quality of total RNA was checked in denaturing formaldehyde agarose gel electrophoresis and quantity was determined spectrophotometrically.

RT-PCR of SCMV coat protein gene: Using the reported coat protein gene sequences of SCMV (Algeria *et al*., 2003) the forward primer (5'-GTTTYCACCAAGCT GGAACAGTC-3') and the reverse primer (5'-AGCTGTGTGTCTCTCTGTATTCTCT-3') got synthesized commercially. About 2µg of purified RNA, 200U of M-MLV reverse transcriptase, 50 pmol of antisense primer and 1mM dNTPs were used for RT-PCR. First strand cDNA was synthesized by incubating the above mixture at 42°C for 1 h. The mixture was heated at 70°C for 10 min to stop the reaction. cDNA thus obtained was PCR amplified using *Taq* DNA polymerase. PCR reaction contained 5µl of 10X PCR buffer with ammonium sulphate, 50 pmol of each of the primer pair, 0.2 mM dNTPs, 2.5 U of *Taq* polymerase and 6 µl (approx. 100 ng) of template cDNA with 2.5 mM $MgCl_2$.

After initial denaturation at 94°C for 5 min, PCR reaction was run for 30 cycles with each cycle of denaturation at 94°C for 45 sec, annealing at 58°C for 45 sec and extension at 72°C for 1 min. The final extension was done for 15 min.

Cloning and sequencing of SCMV coat protein gene: The PCR product was eluted from the gel using DNA Extraction Kit and cloned into pTZ57R/T using InsT/A PCR Product Cloning Kit as described by the manufacturers. White transformants were examined for the presence of inserts by colony-pick PCR. Plasmid was isolated from the trnsferments appeared positive in colony-PCR, using Qiagen plasmid isolation kit.

Purified plasmid were sequenced using M13 forward and reverse primers in a Beckman Coulter CEQ™ 8000 Genetic analysis system. Sequencing reaction was prepared using DTCS Quick Start Kit.

Sequence analysis: Sequences were assembled and analysed using DNASTAR (Lasergene). Multiple sequence alignments and phylogenetic trees were produced using ClustalX program. Phylogenetic trees were constructed, using Treeview program (Page, 1996).

Results and Discussion

Gene amplification, cloning, restriction digestion and sequencing: Total RNA was extracted and purified from leaf samples of two sugarcane cultivars (CSSG 676 and CSSG 668), with and without symptoms of sugarcane mosaic virus infection. The presence of SCMV was confirmed by RT-PCR. An amplification product of 900 bp was produced from the samples showing SCMV infection while no amplification was recorded from symptom-less plant samples (data not shown). RT-PCR product was cloned and confirmed by colony-PCR and double digestion (data not shown). After confirmation, DNA sequence of the cloned fragment was determined. The DNA sequences of coat protein gene from SCMV infecting CSSG676 and CSSG668 have been submitted to GenBank and are available under the accession numbers AM040436 and DQ648195, respectively.

Sequence analysis: The nucleotide sequences of the coat protein gene infecting CSSG 676, and CSSG 668 were analyzed for homology search using NCBI-BLAST. Comparison of the nucleotide sequences of SCMV infecting CSSG 676 (AM040436) and CSSG 668 (DQ648195) showed that they were 98% identical. However, these were 95-98% homologous with coat protein genes of various SCMV isolates present in different parts of the world (Table 1). The deduced amino acid sequences (296 aa) of SCMV infecting sugarcane CSSG 676 and CSSG 668 cultivars ranged from 98 -100% identity with the other 23 deduced amino acid sequences to which it was compared.

The nucleotide sequence of SCMV CSSG 676 and CSSG 668 showed the highest level of sequence identity 97% and 96%, respectively to SCMV from Australia (Bundaberg isolate) , indicating that both of these isolates are variant of SCMV infecting sugarcane in Pakistan.

Phylogenetic analysis of the nucleotide sequences is shown in Fig. 2. The phylogenetic tree clearly separated these two strains from the others included in this analysis.

Bootstrapping analysis by MEGA program of the 23 selected SCMV subgroup members together with two Pakistani isolate sequences gave a phylogenetic tree with three main clusters, top to bottom, in addition to some minor branches (Fig. 2). The cluster I comprises of SCMV members mainly from India together with two Pakistani isolates along with two members from Congo and one from China. Cluster II contains members mainly from Australia along with two each from Iran and China. However, cluster III encompass members only from New World (Americas) including two from Brazil and one from USA. Overall geographic impact is being reflected from phylogenetic behaviour of these isolates although some exceptions are there. The possible reason for the scattered distribution of the SCMV isolates evidents the fuzz (seed) transportation within the continents and to other countries, since SCMV is believed to be seed transmitted as well.

Table 1. Different SCMV isolates (nucleotide sequence) used in comparative study together with Pakistani SCMV isolates. Accession nos. along with their geographic distribution and sequence homology within the isolates are described.

N0.	Accession No.	% Homology with AM040436 (PAK)	Country
1.	DQ648195	98	Pakistan
2.	AF006738	96	Australia
3.	AF006737	96	Australia
4.	D00948	96	Australia
5.	AF006735	97	Australia
6.	AF006728	97	Australia
7.	AF006731	97	Australia
8.	AJ278405	97	Australia
9.	AF006734	97	Australia
10.	EF443055	96	India
11.	DQ866747	96	India
12.	DQ866745	96	India
13.	DQ842502	96	India
14.	DQ343236	95	India
15.	AY590778	96	China
16.	AY953351	96	China
17.	DQ316248	96	China
18.	DQ438949	95	Iran
19.	DQ369960	95	Iran
20.	AY819716	95	Brazil
21.	AY819718	96	Brazil
22.	AJ491954	96	Congo
23.	AJ491950	96	Congo
24.	AY836523	96	USA

Although sequence homology among SCMV strains/variants is usually very high (~90-98%), but they show different biological properties such as variation in symptom severity and patterns as well as host range. The molecular basis of these variations is still unclear although the difference of sequences between these strains can be established. If more sequences of different strains of SCMV can be obtained from a region, it may be possible to identify the substitution which modify gene functions and give rise to the variations of biological properties. This information will particularly be helpful for understanding the functions of virus genes and hopefully will lead to the development of new methods for the control of SCMV. Studies are now underway to clone and sequence rest of the genome of these two isolates and to extend this survey for other cultivars of sugarcane grown in the country.

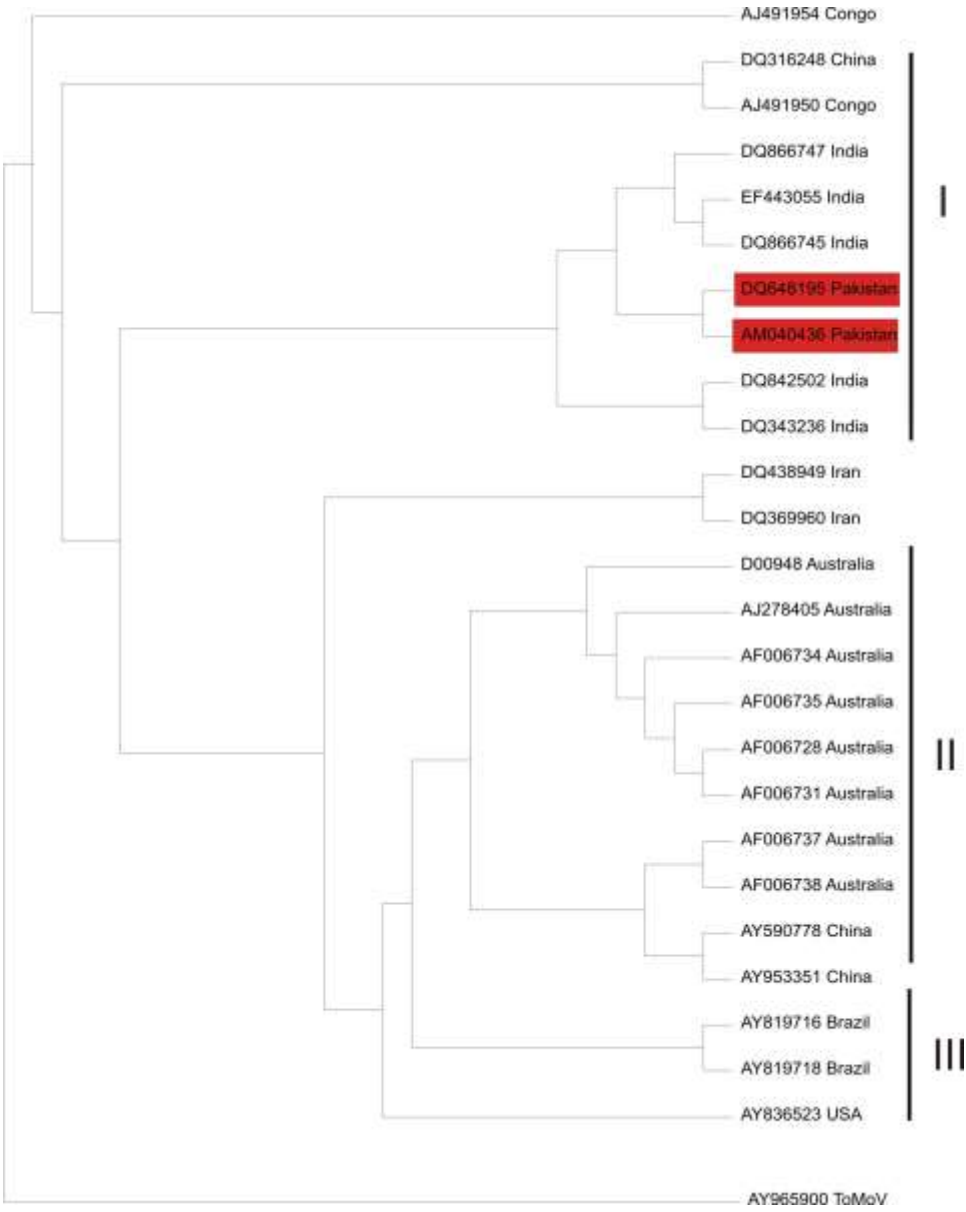

Fig. 2. Phylogenetic dendrogram based upon alignments of the coat protein sequences of SCMV. For brevity and comparison the top most similar sequences in a Blast similarity search with the SCMV coat protein sequence were chosen. The sequences from Pakistan are highlighted in shaded boxes. The tree was arbitrarily rooted on the sequence of the Tomato mottle virus (ToMoV).

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