Coat protein gene sequence studies suggest that *Cucumber mosaic virus* infecting paprika (*Capsicum annuum* L.) in India belongs to subgroup IB

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Abstract

Cucumber mosaic virus (CMV) causing mosaic, leaf distortion and stunting on paprika (*Capsicum annuum* L.) in India was detected by direct antigen-coated enyme linked immunosorbent assay (DAC-ELISA) and reverse transcription polymerase chain reaction (RT-PCR). The coat protein (CP) gene of the virus was amplified, cloned and sequenced. Sequenced region contained a single open reading frame of 657 nucleotides potentially coding for 218 amino acids. Amino acid and nucleotide sequence analyses of CMV CP with members of subgroup IA, IB and II revealed that CMV infecting paprika showed the greatest identity with members of subgroup IB. The identity of amino acid and nucleotide sequences ranged from 95% and 91–92% with members of subgroup IA, 94–99% and 93–97% with subgroup IB isolates and 78–82% and 75–76% with members of subgroup II. The results were also confirmed through phylogram based on multiple sequence alignments of coat protein gene. This is the first report on molecular characterization of CMV infecting paprika in India.

Keywords: *Paprika,* Capsicum annuum*,* Cucumber mosaic virus*, coat protein gene, sequence analyses, phylogenetic relationships*

Introduction

Cucumber mosaic virus (CMV) is one of the most widespread plant viruses in the world with extensive host range infecting about 1000 species including cereals, fruits, vegetables and ornamentals (Roossinck et al. 1999). CMV is a multicomponent virus with three genomic RNAs in positive sense. RNAs 1 and 2 are associated with viral genome replication while RNA 3 encodes for movement protein and coat protein. Numerous strains of CMV have been classified into subgroups I and II based on serological properties and nucleotide sequence homology (Palukaitis et al. 1992). The subgroup I has been further divided into two groups (IA and IB) by phylogenetic analyses (Roossinck et al. 1999).

The genus *Capsicum*, commonly known as ''red chile'', ''chilli pepper'', ''red pepper'', ''paprika'', ''cayenne'', etc. belongs to the nightshade family (Solanaceae). The five major species of cultivated capsicum are *C. annuum*, *C. frutescens*, *C. chinense*, *C. pendulum* and *C.*

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pubescens. Despite being a single species, *C. annuum* has many forms such as bell pepper, paprika, jalapeno, pimento and Aleppo pepper. Paprika is a mild or non-pungent variety of Capsicum (Pruthi 1976). It is the term used by the international spice traders for non-pungent (sweet) red capsicum (Shiva et al. 2006). These add colour to food, help preserve food, have medicinal value, are excellent sources of vitamin A and C and have low calorific value (Vellalon 1981). The major chilli growing countries are India, Nigeria, Mexico, China, Indonesia and the Korean Republic (FAO 1998). The major chilli growing states in India are Andhra Pradesh, Maharashtra, Karnataka and Tamil Nadu which together contribute about 75% of the total area. Paprika crop is infected by a number of diseases caused by fungi, bacteria, nematodes and viruses. Nearly 36 viruses were reported to infect paprika or chilli worldwide causing considerable yield loss (Makkouk & Gumof 1974). About six different viruses including CMV were reported to infect chilli in India based on symptomatology, host range, particle morphology and serological affinities (Prasad Rao & Yaraguntaiah 1979). Characterization of coat protein of CMV on chilli in India has been reported (Deyong et al. 2005). Here we present the detection and characterization of CMV infecting paprika and its phylogenetic relationships with other members.

Materials and methods

Plant material

Young leaves from different paprika cultivars/lines exhibiting mosaic and stunting, maintained at the Indian Institute of Spices Research, Calicut were used in this study as a source of virus.

ELISA

Direct antigen coated-enzyme linked immunosorbent assay (DAC-ELISA) was done in a polystyrene (Co-star) immunoplate as described by Clark et al. (1986). Samples were ground in coating buffer (pH 9.6) at a 1:5 ratio containing 2% polyvinyl pyrrolidone (PVP). Polyclonal antiserum raised against CMV in our laboratory (Bhat et al. 2004) was used at dilutions of 1:2000. Goat anti-rabbit IgG conjugated with alkaline phosphatase was used at 1:20 000 (Genei, Bangalore, India) and substrate (*p*-nitrophenyl-phosphate) (0.5 mg/ml) was used in substrate buffer.

Total RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA from healthy and infected paprika leaves was isolated using the modified acid guanidium thiocyanate phenol chloroform method (Siju et al. 2007). The primer pair used to prime the amplification of coat protein (CP) gene was designed based on multiple sequence alignment of subgroup I of CMV isolates deposited in GenBank (Benson et al. 1999). The genome sense primer, 5′ ATGGACAAATCTGAATCAAC 3′ was derived from the beginning of the first 20 bases of the coding region. The genome antisense primer, 5′ TCAAACTGGGAGCACCC 3′ represented the last 17 bases of the coding region of CP gene. A 50 μl RT-PCR reaction contained 1 X PCR reaction buffer (Genei, Bangalore, India), 0.01 M dithiothreitol (DTT), 20 U RNasin (Genei, Bangalore, India), 15 pM each of the primers, 1.5 U Taq DNA polymerase (Genei, Bangalore, India), 10 U M-MuLV RT (Fermentas, USA) and 10 μM each of the dNTPs (Finzymes, Finland). One μl of template total RNA was added to the reaction mix containing the above components and the final volume was made up to 50 μl using ultra-pure water. Amplification was performed in an automated thermal cycler (Eppendorf Mastercycler Gradient, Germany) with a reaction

profile consisting of initial cDNA synthesis at 42°C for 45 min, followed by 40 cycles with each cycle involving 30 s denaturation at 94°C, 1 min annealing at 50°C, and 1 min of extension at 72°C and a single cycle of final extension at 72°C for 10 min. Following RT-PCR, the reaction products and a standard 500 bp DNA ladder (Genei, Bangalore, India) were run on a 0.8% agarose gel containing ethidium bromide in tris-acetate-EDTA (TAE) buffer. The bands were visualized and photographed using a UV transilluminator and gel documentation apparatus (Alpha Innotech Corporation, CA, USA).

Cloning and sequencing

The amplified PCR product was excised from the gel and eluted using perfect prep gel extraction kit (Eppendorf, Germany) as per the instructions. The PCR product was ligated to pTZ57R/T vector (InsTA clone, Fermentas, USA) along with a control reaction as described in the User's manual. Competent *Escherichia coli* (strain DH5α) were transformed by standard molecular biology procedures (Sambrook & Russell 2001). Recombinant clones were confirmed by PCR and restriction digestion. A selected clone was sequenced at the automated DNA sequencing facility available at Genei, Bangalore, India. Sequence data was compiled using Seqaid Version 3.6 (Rhoads & Roufa 1985) Multiple sequence alignments were made using Clustal X (1.81). Percent identities were determined using Bio-Edit Sequence Alignment Editor. Sequence phylogram was constructed by Neighbourhood joining Bootstrap method (Bootstrap analysis with 1000 replicates) in Clustal X (1.81) and rooted trees were generated using TREEVIEW software (Win 32) (Page 1996). The CP nucleotide and amino acid sequences of other CMV isolates used for comparisons (Table I)

Table I. Source of coat protein nucleotide and amino acid sequences for comparison obtained from GenBank. *Peanut stunt virus* was used as outgroup.

CMV Subgroup	Country	Designation	GenBank Accession No.
Subgroup I A	China	P1	AJ006988
	Japan	Ban	U43888
	Japan	Leg	D16405
Subgroup I B	India	IN-Pn	AY545924
	India	IN-Am	AF198622
	Korea	C7-2	D42079
	Taiwan	M48	D49496
	Taiwan	NT9	D28780
	Taiwan	TA-Ca	AJ810266
	China	CH-Ca	AJ810261
	India	IN-Ca	AJ810260
	Thailand	TH-Ca	AJ810259
	India	IN-Jc	EF153739
	India	IN-Rs	DG914877
Subgroup II	India	IN-Li	AJ585086
	Australia	Q	M21464
	Hungary	Irk	L15336
	Japan	M2	AB006813
	Scotland	Kin	Z12818
	South Africa	S	AF063610
	United States	Ls	AF127976
	United States	W1	D00463
	Korea	KO-Pa	AB109908
	India	**IN-Pa**	**EF583882 (under study)**
Peanut stunt virus	China	PSV	AJ222804

were obtained from GenBank. The BLAST program (Altshul et al. 1997) was used to identify related sequences available from the GenBank database.

Results and discussion

Of the 10 infected paprika cultivars/lines tested through DAC-ELISA, four reacted positively to CMV antiserum. RT-PCR was successful in amplifying the CP gene of CMV from DAC-ELISA positive paprika samples. A PCR product of an expected size of 650 bp was obtained with infected paprika while no such product was seen from healthy sample (Figure 1). The amplified product was cloned and sequenced. The sequence contained a single open reading frame of 657 bases that could code for a protein of 218 amino acids. Pairwise comparisons of paprika CMV CP with members of subgroups I and II from different parts of the world showed that CMV infecting paprika shared highest amino acid and nucleotide sequence identities with members of subgroup I (Table II). Further, sequence identities with members

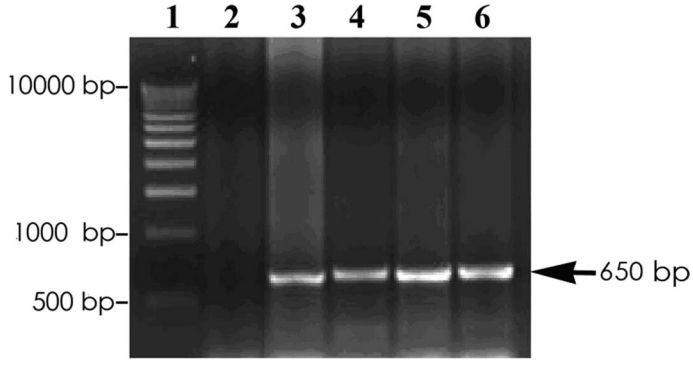

Figure 1. RT-PCR products of healthy and infected paprika samples run along with DNA size markers on a 0.8% agarose gel. Lane 1: 1 kb ladder marker; lane 2: healthy paprika sample; lanes 3–6: infected paprika samples.

Table II. Percent coat protein nucleotide sequence (above the diagonal) and amino acid sequence (below the diagonal) identity of CMV isolate on paprika (IN-Pa) with other CMV isolates belonging to subgroup I and II. Designation given to each of the isolates and their GenBank accession numbers are given in Table I.

	Subgroup II					Subgroup IB							Subgroup IA		
Strain	S	KO-Pa	Irk	Kin	IN-Li	IN-Rs	TH-Ca	IN-Pa	IN-Ca	IN-Pn	TA-Ca	CH-Ca	Leg	P1	Ban
S		99	98	98	98	74	75	75	75	75	75	76	76	76	76
KO-Pa	97		99	99	98	75	76	76	76	76	76	77	77	77	77
Irk	95	97		98	97	75	75	75	75	75	75	76	76	76	76
Kin	97	99	97		98	76	76	76	76	76	76	76	76	76	76
IN-Li	94	96	94	96		75	75	75	75	75	75	75	75	75	75
IN-Rs	81	83	80	82	79		98	97	96	95	93	93	91	91	92
TH-Ca	81	83	81	83	79	99		96	96	95	93	93	91	90	92
IN-Pa	80	82	79	81	78	99	99		95	95	93	93	92	91	92
IN-Ca	81	83	80	82	79	98	98	97		96	93	94	93	92	93
IN-Pn	81	83	80	82	79	100	99	99	98		93	94	92	92	93
TA-Ca	79	81	79	81	78	95	96	94	97	95		96	92	92	92
CH-Ca	81	83	80	82	79	98	98	97	100	98	97		93	93	93
Leg	81	82	80	79	79	96	95	95	96	96	93	96		99	98
P1	81	83	80	82	79	96	96	95	97	96	94	97	99		98
Ban	80	82	79	81	79	96	96	95	96	96	94	96	97	97	

of subgroups IA and IB showed that paprika CMV CP gene (IN-Pa) shared greatest identity with members of subgroup IB. Sequence identity of amino acid and nucleotide were in the range of 95% and 91–92% with subgroup IA isolates while it was 94–99% and 93–97% with subgroup IB isolates, respectively. An identity of 78–82% and 75–76% was observed with subgroup II isolates at amino acid and nucleotide levels, respectively (Table II). Comparison of CP gene sequence with five different isolates of CMV infecting chilli and paprika from other parts of the world revealed that Indian paprika CMV shared identities ranging from 94 to 99% and 93–96% at amino acid and nucleotide levels with four of the five isolates belonging to subgroup I, while the least identity (82% and 76%) was observed with a Korean

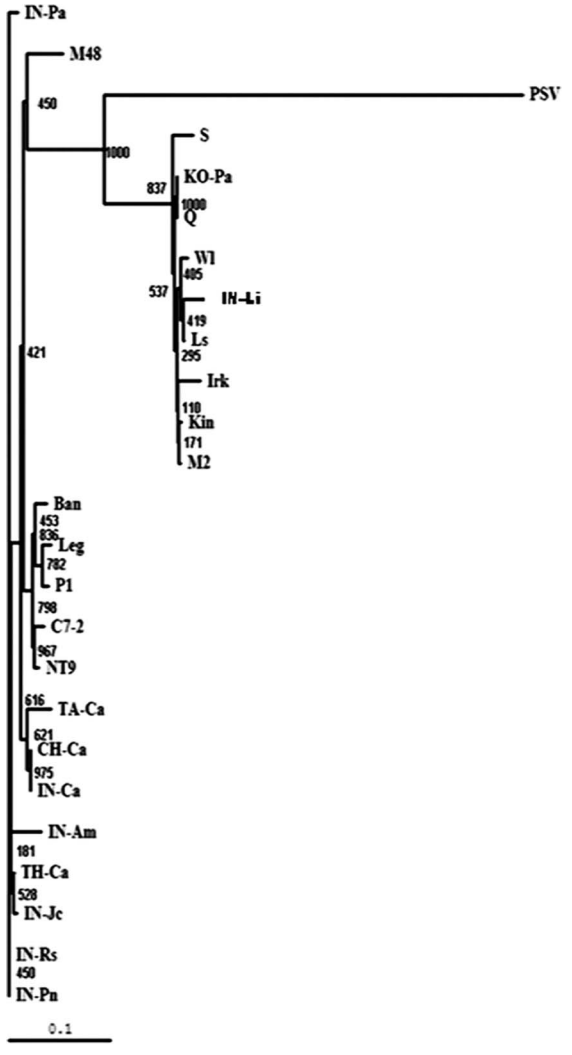

Figure 2. Phylogram drawn by neighbourhood joining bootstrap method in clustal X (1.81) illustrating phylogenetic relationships based on the multiple sequence alignment of the coat protein amino acid sequence of isolates of CMV (subgroups I and II) and paprika isolate of CMV (IN-Pa). Sequences for comparisons were obtained from GenBank and designations given to each of the isolates and their GenBank accession numbers are given in Table I. Boot strap values are given at the nodes. PSV was used as outgroup.

isolate belonging to subgroup II. Among the five chilli isolates, the Indian paprika isolate shared greatest identity with the Thailand isolate at 99% and 96% while the chilli isolate from India shared an identity of 97% amino acid and 95% nucleotide identities, respectively. Further comparison with members of CMV infecting other hosts revealed that the Indian isolate showed a greatest identity of 99% and 97% with *Rauwolfia serpentina* (Sarpagandha) and 99 and 95% with *Piper nigrum* (Black pepper) both at amino acid and nucleotide levels, respectively. The above results suggest that CMV isolates infecting different hosts in a particular region are highly conserved.

Multiple sequence alignment based on predicted CP amino acid sequences among chilli isolates showed that CMV infecting paprika in India and Thailand differed only at one position when compared to other chilli isolates that differed at two positions. The paprika isolate from Korea (belonging to subgroup II) showed more difference with Indian isolate, with many amino acid substitutions. From the CMV isolates infecting other hosts, the Indian paprika isolate showed high sequence similarity with CMV infecting *R. serpentina* and *P. nigrum*, both from India. A phylogram illustrating the phylogenetic relationship among CMV isolates generated based on CP amino acid sequences showed that CMV infecting paprika in India was most closely related to the members of subgroup I (Figure 2). In contrast, CMV isolates belonging to subgroup II formed a different cluster, well separated from members of subgroup I. Within subgroup I, CMV on paprika in India showed close phylogenetic relationship with members of subgroup IB than IA, which formed a separate cluster. Among the CMV isolates infecting other hosts, IN-Pa showed a maximum phylogenetic relationship with IN-Rs and IN-Pn. Among the isolates from chilli, IN-Pa showed a close evolutionary relationship with CMV isolate from Thailand while it was distant to the isolate from Korea, which formed a different cluster.

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