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Short Communication

Sequencing of Coat Protein Gene of an isolate of *Cucumber mosaic virus* Infecting Black Pepper (*Piper nigrum* L) in India

A I Bhat*, P S Hareesh and R Madhubala
Division of Crop Protection, Indian Institute of Spices Research, Marikunnu, Calicut 673 012, Kerala, India

The coat protein gene of an isolate of *Cucumber mosaic virus* (CMV) associated with stunted disease affecting black pepper plant was cloned and sequenced. The coat protein gene comprised of 657 nucleotides encoding a protein of 218 amino acids. Sequence analysis showed that the gene was most closely related to CMV infecting Egyptian henbane plant in India, the member of subgroup I of CMV. The amino acid sequence identity with members of subgroup I was 92-99% while that with members of subgroup II was 77-79%. On the basis of sequence homology, it is concluded that CMV infecting black pepper in India is a strain of CMV belonging to subgroup I.

Key words: *Cucumber mosaic virus*, coat protein gene, cloning, sequence analysis.

Cucumber mosaic virus (CMV), the type species of the genus, Cucumovirus in the family of Bromoviridae (1), is one of the most widespread plant viruses in the world. Its genome consists of three plus-sense single stranded RNAs plus a fourth subgenomic RNA. RNAs 1 and 2 are associated with viral genome replication, while the RNA 3 encodes for movement protein and coat protein. CMV has an extensive host range infecting over 1000 species in more than 85 plant families (1). The numerous strains of CMV have been classified into two major subgroups (subgroup I and II) on the basis of serological properties and nucleotide sequence homology (2, 3). The subgroup I has been further divided into two groups (IA and IB) by phylogenetic analyses (4).

Black pepper obtained from dried berries of *Piper nigrum* L, is an important spice of international commerce for India. The crop is mainly grown in the southern states of Kerala and Karnataka. The productivity of the crop is considerably low due to many biotic stresses, including viruses. Recently, association of a CMV isolate with the diseased black pepper in India causing small, crinkled, brittle, leathery leaves with chlorotic patches/streaks along with reduced internodal length and stunting of the vine was established on the basis of biological and serological tests (5). However, the exact identification of the virus and subgroup to which it belongs remained unaddressed. We report here the identification of the virus based on the coat protein sequence identities.

Disease affected black pepper samples collected from Belur (Karnataka state, India) were used in this study. Samples were initially tested for the presence of CMV by double antibody sandwich ELISA (DAS-ELISA) using virus specific immunoglobulin G (Ig G) and enzyme conjugate prepared against black pepper isolate of CMV in our laboratory. Total RNA extracted from ELISA positive black pepper tissues using Nucleospin RNA Plant kit (Macherey-Nagel, Duren, Germany) was used as template for RT-PCR. RT and PCR were performed in the same tube without any buffer changes. The primer pair derived from the coat protein (CP) gene sequence of CMV was used to prime the amplification. 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 last 17 bases of the coding region of the CP gene (Source: GenBank accession number: AF350450). The PCR reaction (100 μl) contained 200 ng each of the primers, 20 U Ribonuclease inhibitor, 10 U AMV reverse transcriptase, 2.5 U Taq Polymerase, 1x PCR buffer, 10 mM Dithiothreitol, and 10 μM each of the dNTPs. PCR mix (27 μl) containing the above components was added to the tubes containing the template RNA (73 μl) resulting in a final reaction volume of 100 μl. Amplification was

*Corresponding author. E-mail: ishwarabhat@iisr.org; aib65@yahoo.co.in
Abbreviations: CMV, *Cucumber mosaic virus*; CP, coat protein.

performed in an automated thermal cycler (Eppendorf Master Cycler Gradient) programmed for one cycle at 42 °C for 45 min for cDNA synthesis and 40 cycles of amplification with the following parameters: 30 sec of denaturation at 94 °C, 1 min of annealing at 50 °C, and 1 min of extension at 72 °C followed by one cycle of final extension for 10 min at 72 °C.

Following PCR, reaction products (20 μl) were analysed by electrophoresis on 1% agarose gel using Tris–acetate EDTA (TAE) buffer containing ethidium bromide. DNA was visualized and photographed using a UV transilluminator and a gel documentation apparatus. The PCR product was purified using Strata Prep PCR purification kit followed by polishing the purified PCR product using P*fu* DNA Polymerase and dNTP mix. The resultant product was then cloned into pPCR Script Amp SK(+) cloning vector using kit, and competent *Escherichia coli* strain DH5α were transformed (6). Recombinant clones were identified by restriction endonuclease digestion, and selected clones were sequenced at the automated DNA sequencing facility at the Avestha GenGraine Technologies Pvt Ltd., Bangalore, India.

Sequence data were compiled using Seqaid Version 3.6 (7). Multiple sequence alignments were made using Clustal X. Sequence phylograms were constructed by Neighborhood Joining Bootstrap method (Bootstrap analysis with 1000 replicates) in Clustal X (1.81), and rooted trees were generated using TREEVIEW software (Win 32) Version 1.6.6 (8). The CP nucleotide and amino acid sequences of other CMV isolates used for comparison (Table 1) were obtained from GenBank. The BLAST programme was used to identify related sequences available from the GenBank database.

RT-PCR was successful in amplifying the CMV coat protein gene from six black pepper samples collected from Belur. A PCR product of expected size (ca. 650 bp) was observed in all the infected samples and not with healthy black pepper samples. The identity of amplicons was confirmed by sequencing. The nucleotide sequence of the CMV isolate was deposited at GenBank and its accession number is listed in Table 1. The sequenced region contained a single open reading frame of 657 bases that could potentially code for a protein of 218 amino acids. The coat protein gene of the black pepper CMV isolate was compared with corresponding gene of CMV isolates belonging to both the subgroups from different hosts and

Table 1. Sources of *Cucumber mosaic virus* coat protein gene sequences used for comparison

CMV Subgroup	Country	Designation	GenBank Accession No.
Subgroup I	China	P1*	AJ006988
	China	ChCu$	X65017
	China	SD$	AB008777
	India	IN-Am	AF198622
	India	IN-Ba	AY125575
	India	IN-Di	AF281864
	India	IN-Hb	AF350450
	India	IN-PhyM$	X89652
	Israel	Ban*	U43888
	Japan	D8*	AB004781
	Japan	Leg*	D16405
	Japan	N*	D28486
	Japan	Pepo*	D43800
	Japan	C7-2$	D42079
	Korea	Kor*	L36251
	Korea	ABI$	L36525
	Taiwan	M48$	D49496
	Taiwan	NT9$	D28780
Subgroup II	Australia	Q	M21464
	Hungary	Irk	L15336
	Japan	M2	AB006813
	Scotland	Kin	Z12818
	South Africa	S	AF063610
	United States	Ls	AF127976
	United States	WI	D00463
	India	IN-Pn	AY545924 (This study)

*belong to subgroup IA according to Roossinck *et al* (ref 4)
$belong to subgroup IB according to Roossinck *et al* (ref 4)

regions including India (Table 1) at the nucleotide and amino acid levels. Sequence phylogram revealed that black pepper isolate was most closely related to members of subgroup I isolates (Fig. 1). The coat protein gene of black pepper isolate shared 89-95% identity with various isolates of CMV belonging to subgroup I while per cent identity ranged from 75 to 76 with various isolates of CMV in the subgroup II. Similarly, comparison of amino acid sequences of the coat proteins revealed that coat protein of black pepper isolate shared 92-99% identity with members of subgroup I and from 77 to 79% identity with members of subgroup II. Identities of 94-95% and 96-99% were observed with the available coat protein gene sequences of CMV subgroup I isolates from India at nucleotide and amino acid level, respectively. The sequence homology of coat protein gene ranged between 96.3% and 99.5% within

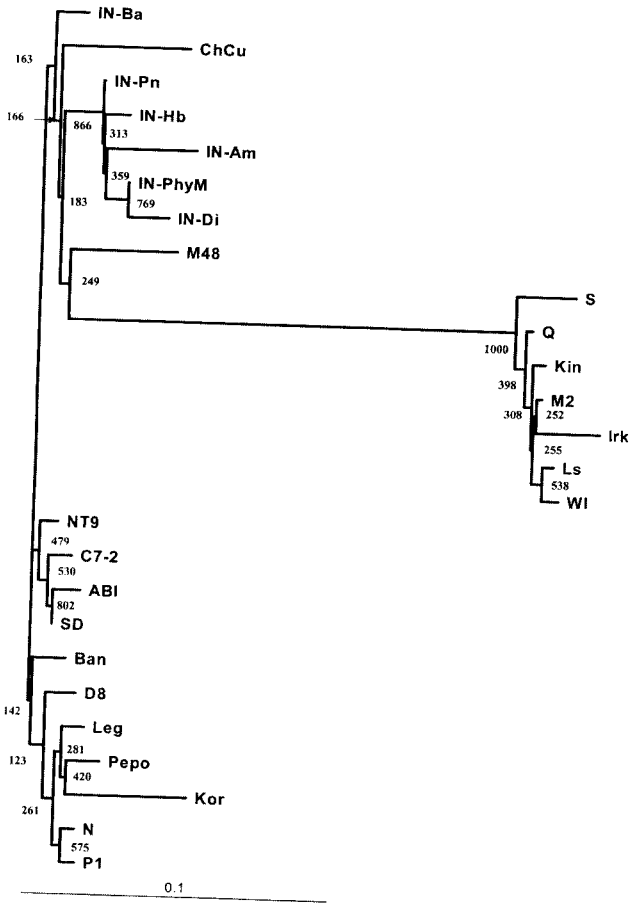

Fig. 1. Phylogram, drawn by Neighborhood Joining Bootstrap method in Clustal X (1.81), illustrating phylogenetic relationships based on the multiple alignments of the coat protein amino acid sequences of 25 distinct isolates of *Cucumber mosaic virus* and black pepper isolate of CMV. Sequences for comparisons were obtained from GenBank and designation given to each of the isolates and their GenBank accession numbers are given in Table 1. The bootstrap values are shown at the individual nodes.

a subgroup and between 76.0 and 77.5% for isolates of different subgroups at the nucleotide level. Homologies in coat protein amino acid sequences were 94.0–99.2% and 79.5%–83.2%, respectively (2). In accordance with this the black pepper isolate tested in this study belongs to subgroup I.

Multiple alignment based on coat protein amino acid sequences revealed large sequence differences between subgroup I and II isolates. Though differences in amino acid residue existed at several positions, N- and C-terminal regions of the coat protein accounted for most of the differences (Fig. 2). As reported by Roossinck *et al* (4), the phylogenetic analysis of CMV isolates belonging to

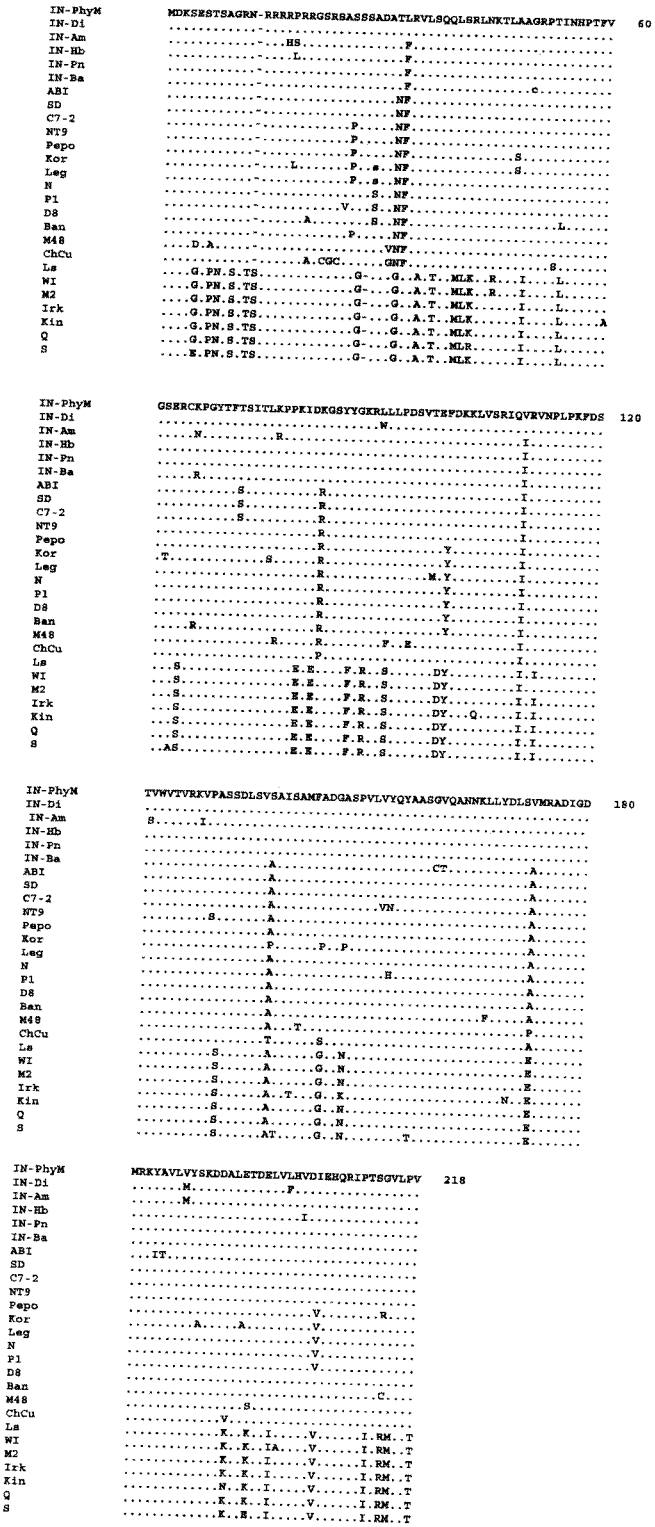

Fig. 2. Multiple alignment of coat protein amino acid sequences (shown as DNA) of *Cucumber mosaic virus* (CMV) isolates used in the study. The alignment was generated by Clustal X. Dot indicates identity and dash indicates deletion at a given position. Designations given to each isolate are given in Table 1.

subgroup I revealed the existence of subclusters within the subgroup I isolates. According to this the black pepper isolate of CMV used in the present study belongs to subgroup IB (Table 1; Fig. 1). Occurrence of CMV on black pepper has also been reported from Brazil (9) and Sri Lanka (10) on the basis of biological and serological tests. As these tests can not differentiate the CMV isolates infecting black pepper from different regions, it is important to clone and sequence these isolates in order to distinguish them.

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