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Characterization of *Cucumber mosaic virus* infecting Indian long pepper (*Piper longum* L.) and betel vine (*Piper betle* L.) in India

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Natural infection of *Cucumber mosaic virus* (CMV) in Indian long pepper (*Piper longum* L.) and betel vine (*Piper betle* L.) was detected by reverse transcription polymerase chain reaction (RT-PCR). The coat protein gene sequences of CMV infecting these hosts were amplified. The resulting amplicons were cloned and sequenced. In both the cases coat protein gene consisted of 657 nucleotides, potentially encoding for a protein of 218 amino acids. The sequence comparisons revealed 100% identity of these isolates both at amino acid and nucleotide levels, suggesting a common origin. Sequence analyses with various CMV isolates showed that CMV infecting banana and black pepper in India to be more close to isolates from Indian long pepper and betel vine. Based on the coat protein gene sequence identities, it was concluded that CMV infecting both Indian long pepper and betel vine belong to the subgroup I. This is the first report on molecular characterization of CMV infecting Indian long pepper and betel vine.

Keywords: betel vine, coat protein gene, *Cucumber mosaic virus*, Indian long pepper,
IPC Code: Int. Cl.[7] C12N7/02, 15/09; C12R1:94

Introduction

Cucumber mosaic virus (CMV) is a widely prevalent plant virus because of its extensive host range that spans around 800 species[1]. 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[1,2]. The genome of CMV consists of three genomic RNAs, viz. RNA 1, 2 and 3. The gene 1a in RNA 1 and 2a in RNA 2 are associated with virus replication, while gene 2b in RNA 2 is correlated with long-distance movement of virus[3], host specificity[4] and process of gene silencing suppression[5] in various host systems. The RNA 3 consists of two genes, i.e. 3a and 3b, the latter being translated from the sub genomic RNA 4. The gene 3a codes the product required for cell-to-cell movement and 3b encodes for viral coat protein (CP)[6-8].

Betel vine (*Piper betle* L.) and Indian long pepper (*P. longum* L.) are two important cultivated species of the genus *Piper*, next to black pepper (*P. nigrum* L.). Betel vine is cultivated for its leaves, which are used for mastication due to its stimulatory aromatic taste[9,10]. The plant is also known for its medicinal properties and is useful in catorrhal and pulmonary afflictions[11]. In India, Tamil Nadu, Karnataka, Andhra Pradesh, Kerala and West Bengal have the major areas of cultivation (a total of 43,000 ha) with an annual production of around Rs.7000 millions worth[12]. Indian long pepper is an exceptionally important medicinal plant with anti-HIV constituents[13]. Fruits, stem and roots of this plant are used in traditional Indian medical systems like *Ayurveda*, *Siddha* and *Unani*[14].

Association of two kinds of viruses with isometric (25 nm in diameter) and rigid rod (350 × 15 nm^2) shaped particles have been reported on betel vine from India[15,16]. However, the exact identification of the causal virus remained unaddressed. In addition, occurrence of a Badnavirus, *Piper yellow mottle virus* (PYMV) has been reported on betel vine from Thailand[17]. Recently, association of a CMV isolate with mosaic disease of Indian long pepper was established on the basis of biological and serological tests in India[18,19]. However, the exact identification of the virus and its subgroup remained unaddressed. The authors report here the coat protein (CP) gene sequence based characterization of CMV infecting *P. longum* and *P. betle* in India.

Materials and Methods

Leaves of *P. betle* and that of *P. longum* showing mosaic symptoms, collected respectively from

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Kasaragod and Kozhikode Districts Kerala, India, were used in the study. Both samples showed positive reaction with CMV in DAS-ELISA tests. RNA extraction was done using Nucleospin RNA Plant kit as per supplier's instructions (Macherey-Nagel, Duren, Germany). The isolated RNA was subjected to RT-PCR, which were performed in the same tube as described earlier[20]. The primer pairs used to prime the amplification of CP gene was designed based on multiple sequence alignment of subgroup I of CMV isolates, available in the GenBank[21]. 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. The PCR reaction (100 µL) contained 30 pmole each of the primers, 20 units Ribonuclease inhibitor (Genei, Bangalore, India), 10 units AMV reverse transcriptase (Finnzymes OY, Finland), 2.5 units *Taq* Polymerase (Genei, Bangalore, India), 1X PCR buffer (Genei, Bangalore, India), 10 m*M* Dithiothreitol, (Genei, Bangalore, India) and 10 µ*M* each of the dNTPs (Finnzymes OY, Finland). 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 performed in an automated thermal cycler (Eppendorf Master Cycler Gradient) and the programme consisted of one cycle at 42°C for 45 min for cDNA synthesis, followed by 40 cycle reaction profile involving 30 sec of denaturation at 94°C, 1 min of 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.

The reaction products (20 µL) were run along with standard size 500 bp DNA ladder (Genei, Bangalore, India) on 1% agarose gel containing ethidium bromide in Tris-acetate EDTA (TAE) buffer. The DNA bands were visualized and photographed using a UV transilluminator and a gel documentation apparatus (Alpha Innotech Corporation, CA, USA), The PCR product was purified using Strata Prep PCR purification kit (Stratagene, LaJolla, CA, USA), followed by polishing the purified PCR product using Pfu DNA Polymerase (Stratagene, LaJolla, CA, USA) and dNTPs mix. The resultant product was then cloned into pPCR Script Amp SK(+) cloning vector using pPCR Script Amp SK(+) cloning vector kit (Stratagene, LaJolla, CA, USA) and competent *Escherichia coli* (strain DH5α) were transformed by following standard molecular biology procedures[22]. Recombinant clones were identified by PCR and restriction endonuclease digestion. The 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)[23]. Multiple sequence alignments were made using CLUSTAL W[24]. Sequence phylogram was 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)[25]. The CP nucleotide and amino acid sequences of other CMV isolates used for comparison (Table 1) were obtained from GenBank[21]. The BLAST programme[26] was used to identify related sequences available from the GenBank database.

Results and Discussion

RT-PCR was successful in amplifying the CMV CP gene from both *P. longum* and *P. betle*. A PCR product of expected size (ca. 650 bp) was obtained from infected samples (Fig. 1). The identity of amplicons was confirmed by sequencing. The nucleotide sequences of CMV isolates were deposited at GenBank and their accession numbers are listed in Table 1. The sequenced region in both the cases contained a single open reading frame of 657 bases that could potentially code for a protein of 218 amino acids. Pairwise comparison showed that the CP gene sequence of both the isolates of CMV from betel vine and Indian long pepper were highly conserved with

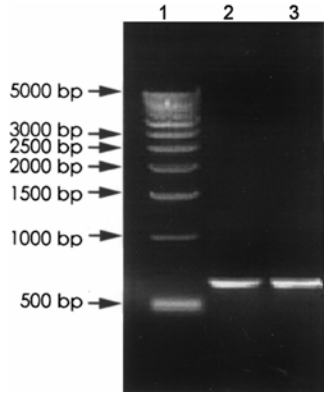

Fig. 1—Agarose gel electrophoresis of RT-PCR products: Lane 1, DNA size markers, values on the left indicate the size of marker bands; Lane 2-3: RNA from CMV infected *P. longum* and *P. betle*, respectively.

100% sequence identities both at nucleotide and amino acid levels (Fig. 2). The sequences of the CP gene of both the isolates were compared with those of other CMV isolates belonging to subgroup I and II from India, and a few representative isolates from other parts of the world (Table 1). CMV isolates of both *P. longum* and *P. betle* showed 93-97% and 95-99% identity at nucleotide and amino acid level, respectively, with CMV isolates belonging to subgroup I from India, while an identity of 71% and 79% was observed with only one available CMV isolate infecting *Lilium*, belonging to subgroup II (Fig. 2). On the other hand, an identity of 89-94% and 93-99% were observed with selected CMV isolates from other parts of the world, at the nucleotide and amino acid levels, respectively, with subgroup I isolates, while it ranged from 75-76% and 77-79% with isolates belonging to subgroup II.

The results of multiple alignments based on amino acid sequences of CP were used to generate a phylogram illustrating their phylogenetic relationship. Phylogram showed two distinct clusters clearly separating isolates belonging to subgroup I and II (Fig. 3). Further, among subgroup I, isolates

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IN-Pb           MDKSESTSAGRN-RRRRPRRGSRSASSSADATFRVLSQQLSRLNKTLAAGRPTINHPTFV
IN-Pl           ************-**************************************************
IN-Ba           ************-**************************************************
IN-PhyM         ************-******************L*****************************
IN-Di           ************-******************L*****************************
IN-Pn           ************-**************************************************
IN-Hb           ************-****L*************************************************
IN-Am           ************-***HS************************************************
In-Li           ****G*PN*S*TS*************G-***GL*A*T**MLK**R***I****LI**S**

IN-Pb           GSERCKPGYTFTSITLKPPKIDRGSYYGKRLLLPDSVTEFDKKLVSRIQIRVNPLPKFDS
IN-Pl           ***************************************************************
IN-Ba           *****R*********************************************************
IN-PhyM         **********************K*************************V**************
IN-Di           **********************K*******W*****************V**************
IN-Pn           **********************K*************************************
IN-Hb           **********************K*************************************
IN-Am           *****N**********R*****K*************************************
In-Li           ***SGN**********N**EFEK***F*R**S******DY**********I********

IN-Pb           TVWVTVRKVPASSDLSVAAISAMFADGASPVLVYQYAASGVQANNKLLYDLSAMRADIGD
IN-Pl           ***************************************************************
IN-Ba           ****************************************T**********************
IN-PhyM         ****************S*********************************V*******
IN-Di           ****************S*********************************V*******
IN-Pn           ****************S*********************************V*******
IN-Hb           ****************S*********************************V*******
IN-Am           S*******I********S*********************************V*******
In-Li           **********S*************G**N************************E*******

IN-Pb           MRKYAVLVYSKDDALETDELVLHVDIEHQRIPTSGVLPV
IN-Pl           ***************************************
IN-Ba           ***************************************
IN-PhyM         ***************************************
IN-Di           *******M*************F*****************
IN-Pn           ***************************************
IN-Hb           **********************I****************
IN-Am           *******M*******************************
In-Li           ************K**K**I*****V******I*RM**T
```

Fig. 2—Multiple alignment of coat protein amino acid sequences (shown as DNA) of *Cucumber mosaic virus* (CMV) isolates from India. Sequences for comparison were obtained from GenBank. The alignment was generated by Clustal W. Asterisk indicates identity and dash indicates deletion at a given position. Designations given to each isolate are given in Table 1.

Table 1—Source of *Cucumber mosaic virus* coat protein gene sequence used for comparisons

CMV Subgroup	Country	Designation	GenBank Acc.No.
Subgroup I	China	PI	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
	India	IN-Pn	AY545924
	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	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	Wl	D00463
	India	IN-Pb	AY690620 (Present study)
		IN-Pl	AY690621 (Present study)

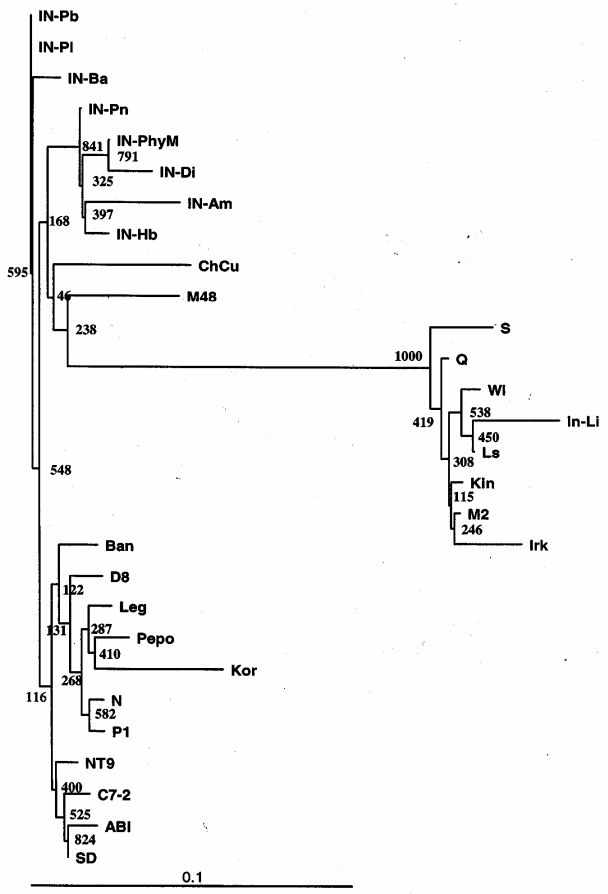

Fig. 3—Phylogram, drawn by Neighborhood Joining Bootstrap Method in Clustal X (1.81), illustrating phylogenetic relationships based on the multiple alignments of the coat protein amino acid sequences of 27 distinct isolates of *Cucumber mosaic virus* (CMV) and Indian long pepper and betel vine isolates of CMV. Sequences for comparisons were obtained from GenBank and designation of each isolate and their GenBank accession numbers are given in Table 1. The boot strap values are shown at the individual nodes.

originating from *P. betle* and *P. longum* were closer to CMV isolate infecting banana, followed by black pepper in India. In general, among subgroup I, CMV isolates originating from the same geographic area were more closely clustered compared to isolates from different geographic area (Fig. 3). The sequence comparisons and phylogram clearly showed that both CMV isolates used in this investigation belong to the subgroup I.

This is the first report on the molecular characterization of *Cucumber mosaic virus* infecting *P. betle* and *P. longum* from India and elsewhere. CMV has a wide host range and is known to infect several crops and weed hosts[1]. Both long pepper and betel vine are grown under mixed cropping systems, wherein banana is one of the most prevalent crops. High sequence identity in the CP gene (99%) observed with CMV isolate from banana indicates that it may act as source of infection for these crops and *vice versa*. However, it remains to be demonstrated whether sequence differences could be applied to resolve pathotypes. Our preliminary experimental evidence clearly suggests that the CMV isolate from *P. longum* could easily infect black pepper (*P. nigrum*) (unpublished findings). However a large number of additional CMV isolates from different hosts and locations will have to be examined to establish correlation between molecular typing and symptom severity.

Acknowledgement

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