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RESEARCH ARTICLE

Biological and Molecular characterization of *Cowpea aphid-borne mosaic virus* causing severe mosaic disease in cowpea, India

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Abstract

A virus showing mosaic and leaf curling on cowpea (*Vigna unguiculata)* was isolated from Rajasthan province of India. Subculture of the virus was easily maintained on *Nicotiana benthamiana*. The isolated virus showed flexuous filamentous particles of 790 × 15 nm, similar to the members of family *potyviridae*. Further, serological test by PAT-ELISA with group specific antiserum confirmed the presence of *Potyvirus* infecting cowpea. A 723 bp DNA fragment was amplified from these plants by reverse-transcription polymerase chain reaction (RT-PCR) using a pair of degenerate primers specific for coat protein (CP) and 3' UTR. The PCR product was cloned and sequenced for analysis. The sequence revealed that this *Cowpea aphid-borne mosaic virus* CABMV-CmRp (KC753448) is closely related to an isolate of Zimbabwe, *Cowpea aphid-borne mosaic virus* CABMV-Z[ZB] (AF348210) with 99% similarity and 81% identity with *Bean common mosaic virus* BCMV-NL-4[IN] (JN692258) (J&K) India.

1. Introduction:

The cowpea (*Vigna unguiculata*) is one of the several species of the widely cultivated genus *Vigna*. It is the most important proteinaceous legume (pulse) crop in the semiarid tropics covering Asia, Africa, Southern Europe and Central and South America. Besides providing a variety of foods, is a source of income and livestock feed (Popelka, 2004). In India, farmers not only use the beans for human consumption and animal feed, but also use the leaves and fruits for cooking. Viral diseases are significantly causative to the reduced yield of cowpea in Asia, Africa and Latin America. Worldwide, more than 20 viruses have been identified as naturally infecting cowpea (Mali and Thottappilly, 1986) and many are transmitted through seeds (Hampton, 1983). *Cowpea aphid-borne mosaic virus* of the genus *Potyvirus* causes severe diffused chlorotic patches, deformation on leaves and growth stunting. The main characteristics considered to define a *Potyvirus* are particle morphology, cytopathology, mode of transmission, serology and molecular characteristics such as genome structure and organization (Ward et al., 1992). Detecting the causal agent through visual assumption is very difficult. Now several diagnostic methodologies are available for the identification of *Potyvirus* including degenerate primers against conserve motif of CP gene and 3' UTR.

During our extensive survey in Rajasthan (India), we found that cowpea plants were severely affected by mosaic and retardation of the plant growth. Here we described potyviruses from cowpea which induce mosaic and leaf curling. A serological identification, mechanical transmission and comparative description of a molecular analysis of a conserved part of the coat protein were used for taxonomic classification of this virus.

2. Materials and Methods:

2.1 Survey and Collection of Samples:

In the year of 2012, a survey conducted in cowpea-growing areas of Rajasthan province of India. Total 35 fields covering 7 villages were observed for the virus incidence. The incidence of virus-like symptoms in the fields were visualized and recorded. Total 80 leaf samples showing leaf curling and mosaic diseases (Fig. 1a) were collected for the present study.

2.2 Serological tests:

The plate trapped antigen enzyme-linked immunosorbant assay (PTA-ELISA) was used for the serological study of this virus by using *Potyvirus* group specific antiserum (Mowat and Dowsan, 1987). The antibody was purchased from LIFE TECHNOLOGIES (INDIA) PVT. LTD, New Delhi, India and used at a dilution of 1:1500 as per the manufacture's instruction. The absorbance was measured at 405 and 450 nm in a Labtech LT-4000 ELISA Reader respectively.

2.3 Mechanical inoculation:

Virus inoculum was prepared by grinding 1 g of young diseased leaves of cowpea in 5 ml of 0.04 M $NaHPO_4$ containing 0.2% sodium diethyldithio carbamate. Prior to inoculation, 75 mg/ml of carborundum and of activated charcoal were added to the sap extract (Morel et al., 2000). Near about 50 *N. benthamiana* plant of four leaves stage grown on insect-free green house were taken for mechanical transmission study. Evaluations were based on the development of local and/or systemic symptoms as well as by serological assays.

2.4 Electron microscopy:

Leaves harvested from naturally infected cowpea (*V. unguiculata*) and mechanically inoculated *N. benthamiana* at four week post-inoculation were used for virus particle purification as described by (Chen et al., 2003). Carbon shadowed formavar coated grids applied with purified CABMV were negatively stained 1% uranyl acetate (Christie et al., 1987) in water and examined under a JEOL 100S electron microscope operating at 80 to 100 KV.

2.5 RNA extraction and cDNA amplification:

Total RNA was extracted from 100 mg fresh leaf samples with the RNEasy Plant Mini Kit (Qiagen, USA) according to the manufacturer's protocol and resuspended in 40 µl of nuclease free water. The 3 µl of RNA was added to 22 µl of RT mixture component (5 µl of RNA, 1 µl of oligo $d(T)_{16,}$ 5 µl of 5X reaction buffer, 2 µl of 2.5 mM dNTP, 0.5 µl of AMV Reverse Transcriptase and 1 µl of 25 mM $MgCl_2$), and subjected to reverse transcription process following the programme: 42 °C for 60 min and 72 °C for 10 min to yield cDNA. PCR reaction was performed in a 25 µl reaction mixture containing 3 µl of cDNA, 1µl of 20 pmol each of potyvirus group specific primers WCIEN-1: 5'-TCGTGIATHGANAATGG-3' and WCIEN-2: 5' $(T)_{21}$V'-3' (Chen and Adams, 2001), 2.5 µl 10× reaction buffer, 2.5 µl of 2.5 mM dNTPs and 1 µl 25 mM $MgCl_2$. PCR was performed in thermal cycler with the programme 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 55 °C for 1 min, 72 °C for 1 min and a final extension of 72 °C for 10 min. The resulting amplicon of ~ 723 bp fragments, corresponding to partial CP gene and 3' UTR was visualized on 1 % agarose gel, stained with ethidium bromide.

2.6 Cloning and Sequencing:

The PCR products cloning were carried out using pGEMT-Easy vector system (Promega). The ligated mix were used to transformed *Escherichia coli* DH5α. Resulting recombinants clones were selected on Luria agar medium containing Ampicillin (100 µg/ml) and X-gal/IPTG (50 µg/ml:40 mM) (Sambrook and Rusell, 2001). Transformed colonies were selected and high purity plasmid DNA minipreps were used for DNA sequencing.

2.7 Phylogenetic Analysis:

The nucleotide sequence was compared with other sequences from NCBI databases using the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/blast). Highest and lowest match scores and closest matching sequences from different isolates of potyviruses were considered. From the blast sequences a phylogenetic tree was constructed with the neighbor-joining method using MEGA 4.0 software (Tamura et al., 2007).

3. Results & Discussion:

3.1 Particle morphology & biological properties:

Our survey for the incidence of mosaic diseases associated with cowpea plants in Rajasthan, India showed that CABMV were the predominant mosaic virus. The results of the electron microscopy (Fig. 1b) showed that particles were flexuous rods with an average dimension of 790 × 15 nm, similar to the members of family *potyviridae*. Out of 50 *N. benthimiana* plants 39 were shown typical curling and mosaic symptoms caused by virus inoculums. This resulted in the development of same kind of symptoms as observed in naturally infected cowpea plants in the fields.

3.2 Detection and identification of plant viruses by PTA-ELISA

Cowpea aphid-borne mosaic virus a species of *Potyvirus* groups were detected by PTA-ELISA using the procedure described above (Table 1). Absorbance values were accepted as positive when the reading was greater than twice the mean absorbance of the virus-free control sample. The absorbance values of the wells receiving sample extract and substrate were only similar to those of wells containing extracts from virus-free tissue and extraction buffer controls. Serological results shown absorbance values at least twice the mean for virus-free samples were obtained with both dilutions of each sample therefore method was particularly valuable in identifying isolates of *Cowpea aphid-borne mosaic virus*.

Table1. Absorbance values obtained by PTA-ELISA with two proteins A enzyme conjugates and the reaction of their substrates with adsorbed components from sample extracts.

Virus & antiserum (host plant)	Sample & dilution[a]	A_{405} values[b]		A_{450} values[b]	
		PA-ALP[c]	NPP[d] only	PA-HRP[c]	TMD[d] only
Potyvirus group (*V. unguiculata)*	I	1.85	0.13	0.85	0.18
	I/10	1.78	0.15	0.88	0.21
	H	0.25	0.13	0.16	0.11
	B	0.15	0.13	0.10	0.10
Potyvirus group (*N.benthamiana)*	I	1.38	0.22	0.85	0.18
	I/10	1.08	0.15	0.76	0.19
	H	0.22	0.16	0.23	0.18
	B	0.16	0.13	0.12	0.10

Note: [a] I = sap from infected leaf extracted in 5 mL of carbonate buffer (pH 9.8) per g of tissue; I/10 = I diluted 1.10 with extraction buffer; H = sap from uninfected leaf extracted as I; B = extraction buffer control.
[b] A_{405} values were recorded after overnight incubation of substrate at 5°C. A_{405} values were recorded after 20 min incubation of substrate at ambient temperature. Absorbance values are the means of 3 wells.
[c] PA-ALP = Protein A-alkaline phosphatase conjugate; PA-HRP = Protein A-horseradish peroxidase conjugate. Wells received sample followed by detecting antiserum, protein A enzyme conjugate and substrate.
[d] NPP = p-nitrophenyl phosphate; TMB = 3,3̀ , 5, 5̀ -tetramethyl benzidine; wells received successively sample and substrate.

3.3 Characterization of viral genome:

The positive amplified product of a 723 bp fragment spanning the conserved motif WCIEN to 3' UTR of potyviruses (Fig. 1c).The recombinant plasmids harboring cDNA inserts (723 bp) was sequenced as described in the methods section. The authenticity of these clones was confirmed by sequenced using T7 and SP6 universal primers and the sequence was deposited in the GenBank (KC753448). After sequencing, the consensus sequence was compared to the sequences

registered in the NCBI database using the Basic Local Alignment Search Tool (blastn; http://www.ncbi.nlm.nih.gov/blast). Sequencing and BLAST analysis confirmed the presence of *Cowpea aphid-borne mosaic virus*. Table 2 summarizes the relative nucleotide sequences similarities between CP genes of our isolate under study with other isolates available in GenBank. *Cowpea aphid-borne mosaic virus* CABMV-CmRp[IN] (KC753448) shared the highest nucleotide sequence identity 99 % with *Cowpea aphid-borne mosaic virus* CABMV-Z[ZB] (AF348210) from Zimbabwe . Number of Brazilian isolates of *Cowpea aphid-borne mosaic virus* infecting different host were showed 91-96 % identity with our isolate (Table 2). *Cowpea aphid-borne mosaic virus* CABMV-Ibadan [UK] shared the 90% identical to CABMV-CmRp[IN] respectively. Interestingly, an Asian isolates of *Potyvirus* like *Bean common mosaic virus* BCMV-NL-4[IN] (JN692258) India (J&K) and *Bean common mosaic virus* BCMV-A[NP] (AB735585) shared 81 % sequence homology with CABMV-CmRp[IN] (Table 2). CABMV-CmRp[IN] clustered with European, American and Asian subcontinents *Potyvirus* isolates strongly supported its worldwide spread and long-distance migration, either through aphid transmission, or most probably, through human activities. Remarkable diversity at *CP* gene level among their geographical isolates may be a major cause of the emergence of new *Potyvirus* diseases of crops introduced in India. It would be interesting to confirm that CABMV has a world-wide geographic distribution and to try to understand what increases its spread.

Figure 1

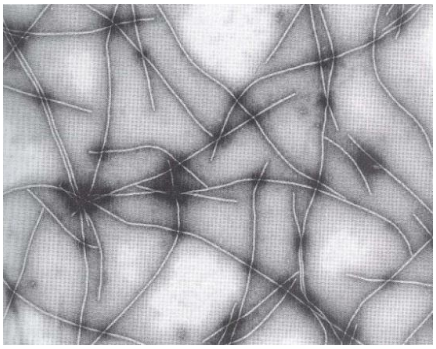

Figure 2

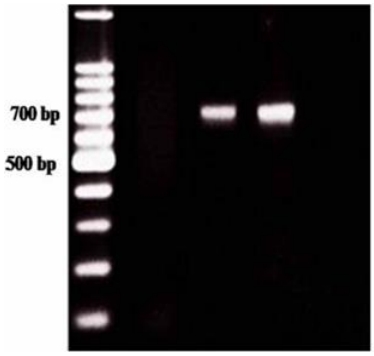

Figure 3

Figure 1. a. Cowpea plant showing characteristics mosaic pattern. **b**. Electron micrograph of purified virus particles causing mosaic disease on cowpea. **c**. RT-PCR analysis of infected cowpea plants with *Potyvirus* group specific primers WCIEN1 & WCIEN2 lane M: 100 bp marker Lane +ve Healthy plant sample Lane 1-2 infected samples.

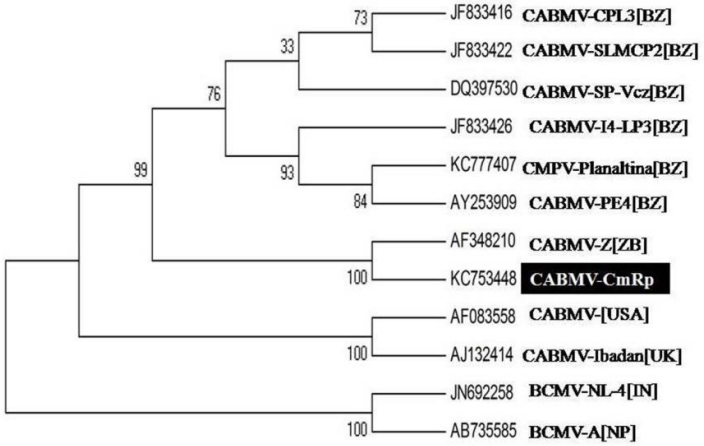

Figure 2 Neighbor-joining phylogenetic dendrogram based on pair-wise and multiple alignment of nucleotide sequences of potyviruses predicted *CP* gene sequences with other reference *Potyvirus* CP sequence from gene bank database. Bootstrap values are placed on major node in accordance with 50% confidence statistics. (Accession no of virus strain and Description are given in Table 2).

Table 2. Origins of disease isolates and nucleotide sequence identity of coat protein (CP) genes of present *Potyvirus* with other potyviruses.

S.No	Viral isolate	Host	Accession No.	% nsi	Country	Abbreviation
1	*Cowpea Aphid-Born mosaic virus*	Cowpea	AF348210	99	Zimbabwe	CABMV-Z[ZB]
2	*Cowpea Aphid-Born mosaic virus*	Passiflora	JF833426	96	Brazil	CABMV-I4-LP3[BZ]
3	*Cowpea Aphid-Born mosaic virus*	Cowpea	DQ397530	95	Brazil	CABMV-SP-Vcz[BZ]
4	*Cowpea Aphid-Born mosaic virus*	Passian	KC777407	94	Brazil	CMPV-Planaltina[BZ]
5	*Cowpea Aphid-Born mosaic virus*	Cowpea	JF833422	92	Brazil	CABMV-SLMCP2[BZ]
6	*Cowpea Aphid-Born mosaic virus*	Cassia	JF833416	92	Brazil	CABMV-CPL3[BZ]
7	*Cowpea Aphid-Born mosaic virus*	Passian	AY253909	91	Brazil	CABMV-PE4[BZ]
8	*Cowpea Aphid-Born mosaic virus*	Cowpea	AJ132414	90	UK	CABMV-Ibadan[UK]
9	*Cowpea Aphid-Born mosaic virus*	Cowpea	AF083558	88	USA	CABMV-[USA]
10	*Bean common mosaic virus*	Bean	JN692258	81	India (J&K)	BCMV-NL-4[IN]
11	*Bean common mosaic virus*	Lablab	AB735585	81	Nepal	BCMV-A[NP]

Note: nsi= nucleotide sequence identity

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