Analysis of 3'-Terminal Region of *Bean common mosaic virus* Strains Infecting Common Bean in India

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Abstract Four strains (NL-1, NL-1n, NL-7 and NL-7n) of *Bean common mosaic virus* (BCMV) prevalent on common bean (*Phaseolus vulgaris*) in Himachal Pradesh, a north-western Himalayan state of India were compared at the 3' terminal region of the viral genome to elucidate variation and relationship among these strains. 3' terminal region comprising of partial nuclear inclusion b (Nlb) (171–233 bp), complete coat protein (CP) (860 bp) and 3' untranslated region (UTR) (208–244 bp) shared 96–98% nucleotide and 96–99% amino acids identity among various strains of BCMV. Multiple alignment as well as cluster dendrograms of the 3' terminal region placed the test isolates in BCMV species of genus *Potyvirus*. Phylogenetic analysis of complete CP as well as 3' UTR also showed Indian strains to be distinct strains. Sequence homology, multiple alignment and evolutionary divergence of 3' terminal region could not differentiate the pathogenic strain groups, thereby establishing least role of this region in strain characterization of the virus. Comparisons of CP and 3' UTR region of BCMV strains and other members of genus *Potyvirus* clearly indicated the little utility of 3' terminal region in distinction of virus strains. Implications

of coat protein region in viral strain distinction are also discussed.

Keywords BCMV · Common bean · Strains · Coat protein · Variability

Introduction

Common bean (*Phaseolus vulgaris* L.) locally called as 'Rajmash', is one of the major food legumes grown throughout the world including India [13, 24]. It is a premier pulse crop of north-western Himalayan states of India and is commercially cultivated in diverse ecological niches falling in altitudinal ranges of 900–3,000 masl. Among various diseases, bean common mosaic caused by *Bean common mosaic virus* (BCMV, Genus *Potyvirus*), a member of family *Potyviridae*, is one of the devastating diseases inflicting both qualitative and quantitative losses in beans [13, 18]. BCMV encodes 8 proteins whose functions have been characterized [3, 8, 29]. The coding ORF is translated into one polyprotein of 340–370 Da which is processed into proteinase (Pro-1), helper component proteinase (Hc-Pro), proteinase (Pro-3), a 6 kDa protein, cylindrical inclusion (CI), a second 6 kDa protein product, nuclear inclusion a (NIa), nuclear inclusion b (Nlb) and CP [8]. BCMV is known to possess high pathogenic variability in Europe, USA and Africa, and virus strains have been identified on bean differentials [6, 7, 12, 19, 22, 28]. There are reports of BCMV occurrence on common bean in India and BCMV recurs regularly in this region with an incidence ranging from 0.5 to 77.0 per cent causing economic losses [23]. The occurrence of four strains NL-1, NL-1n, NL-7, and NL-7n on common bean has been established in Himachal Pradesh based on host differential reaction and

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peptide profiling of the coat protein of the virus isolates [11].

The taxonomy of BCMV has undergone major changes in the recent past as two serotypes, A and B causing temperature insensitive necrosis and mosaic symptoms, respectively on bean differential cultivars [17, 25, 27] have been reclassified as two *Potyvirus* species BCMV and *Bean common mosaic necrosis virus* (BCMNV) based on peptide profiles, molecular wt. of CP, genome size and sequence [14, 26, 30]. Isolates of serotype A (NL-3, NL-5 and NL-8) causing temperature independent necrosis on bean cultivars possessing *I* gene have been placed in BCMNV species, whereas mosaic inducing strains (NL-1, NL-2, NL-4, NL-6, NL-7, US-1, US-2, and US-5) in BCMV (serotype B). Some legume potyviruses like *Blackeye cowpea mosaic virus* (BICMV), *Azuki bean mosaic virus* (AzMV) and *Peanut stripe virus* (PStV) earlier known to be different viruses, are now considered as the strains of BCMV based on their coat protein sequences [14, 15, 21, 31]. Therefore reaction of these legume viruses on differentials may confuse the identity of a particular strain. Also most of the 'new' strains or 'novel pathotypes' identified by different workers do not behave as the traditional BCMV strains which were used for the selection of BCMV differentials [2, 3]. Hence, the reliable criterion for differentiation of viruses/strains is molecular characterization of viral genome sequences. Partial sequence data of the coat protein region can also be used to observe the variability in potyviruses [2, 4]. The objective of this study was to characterize the BCMV strains prevalent in Himachal Pradesh using 3' terminal region of viral genome and to assess the utility of the region in strain differentiation.

Materials and Methods

Virus Strains and Their Maintenance

The cultures of BCMV strains (NL-1, NL-1n, NL-7, and NL-7n) maintained in the Molecular Plant Pathology Laboratory, Department of Plant Pathology, CSK HPKV, Palampur, were obtained from the infected seeds of common bean cv. Jawala. Virus cultures were multiplied and maintained on healthy seedlings of cv. Jawala by sap inoculation under insect proof glass house conditions. Glasshouse was sprayed regularly with hostathion/imidachloropid to avoid vector infestation/transmission. Nine isolates of BCMV strains BCMV-57(NL-1), BCMV-65(NL-1), BCMV-58(NL-1n), BCMV-59(NL-1n), BCMV-60(NL-1n), BCMV-62(NL-7), BCMV-64(NL-7), BCMV-61(NL-7n) and BCMV-63(NL-1n) were used in this study and their identity as BCMV was established through Double antibody sandwich-enzyme linked immunosorbent

assay (DAS-ELISA) using polyclonal antiserum (Loewe Biochemica GmbH, Germany). A standard set of common bean differentials was used to confirm the identity of the virus strains [6]. Virus inoculum was prepared as per standard procedure [11] and five plants from each differential cultivar in host group I–V were inoculated with each isolate using leaf rub method and one un-inoculated plant was kept as control [6]. To identify necrosis inducing isolates, three plants of host group VI to IX, were inoculated and maintained in growth chamber at >30°C with 14–16 h light (Saveer India Ltd). Test isolates were identified as strains and assigned a pathogroup based on their reaction on a particular host group as per the procedure of Drijfhout et al. [6]. Symptomless expression in resistant plants was confirmed by DAS-ELISA and reverse transcription-polymerase chain reaction (RT-PCR) using BCMV specific primers.

RT-PCR Amplification and Cloning

Total RNA of nine BCMV isolates propagated on *P. vulgaris* was extracted from fresh infected leaf tissues using TRI reagent (MRC, USA) following manufacturer's instructions. cDNA was synthesized from 5 to 10 µl of total RNA by reverse transcription using M-MuLV reverse transcriptase (1 µl) (Fermentas, USA) and oligo dT18 primer followed by PCR amplification using BCMV specific primers. These primers (F: 5'TGGCTGCTTGAGA GAGATGA3'); R: (5'ATCACTCTGCATGTCCTCAC3') were designed from the conserved sequences in the N1b and 3' UTR region of BCMV (NCBI) using online Primer3 program [20]. The amplification was carried out in a thermal cycler (Applied Biosystem) programmed as per Xu and Hampton [32] with annealing temperature of 55°C. The PCR products were purified using DNA purification kit (Auprep, Life Technologies, Pvt Ltd.) as per manufacturer's protocol and ligated into the T-tailed cloning vector (pGEM-T Easy, Promega). Ligation reactions were transformed into *E. coli* DH5α using standard transformation techniques and recombinant colonies were identified by colony PCR. Plasmid containing the insert was isolated by Alkali Lysis method and was lyophilized before sequencing on custom basis (Bioserve, India, Pvt. Ltd.).

Sequence Analysis

Sequences were subjected to BLAST analysis using BLASTn program available online (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and deposited in NCBI. Nucleotide sequences were translated into amino acid (a/a) sequences using Translation Expasy Tool (<http://www.expasy.ch/tools/dna.html>). Nucleotide sequences of the deduced a/a sequences were aligned with sequences of other selected

Table 1 Reaction of Indian isolates of *Bean common mosaic virus* used in this study on differential set of bean cultivar

Differential cultivar ^a	BCMV strain groups/Isolates								
	Pathogroup I					Pathogroup II			
	NL1		NL1n			NL7		NL7n	
	BCMV-57	BCMV-65	BCMV-58	BCMV-59	BCMV-60	BCMV-62	BCMV-64	BCMV-61	BCMV-63
Dubbele Witte ^b	S	S	S	S	S	S	S	S	S
Redlands Greenleaf 'C' ^b	R	R	R	R	R	S	S	S	S
Great Northern UI 123 ^b	R	R	R	R	R	R	R	R	R
Sanilac ^b	R	R	R	R	R	R	R	R	R
Monroe ^b	R	R	R	R	R	R	R	R	R
Jubila ^c	R	R	N	N	N	R	R	N	N
Improved Tendergreen40031 ^c	R	R	R	R	R	R	R	R	R
Black turtle soup ^c	R	R	R	R	R	R	R	R	R
Amanda ^c	R	R	R	R	R	R	R	R	R

S host group susceptible to systemic infection, N necrosis above 30°C (susceptible), R resistant, no systemic infection

^a As per Drijfhout et al. [6]

^b Host group: I–IV genotypes with recessive *II* alleles

^c Host group: VI–IX with dominant *II* alleles

strains of BCMV, BCMNV and *Bean yellow mosaic virus* (BYMV), available in the NCBI database. Nucleotide a/a sequences were aligned by ClustalW program (bioedit software). To observe genetic distance, phylogenetic tree were constructed using Maximum Parsimony method of MEGA software package version 4 (<http://www.kumarlab.net/publications>). The repeatability of the branching orders was estimated using the seqboot program of MEGA4 for bootstrapping. Evolutionary divergence analyses between isolates/strains at amino acid level were also calculated using the Poisson correction method in MEGA4 software. The distance was calculated as the number of sites at which two sequences being compared were different. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 341 positions in the final dataset.

Results and Discussion

Virus and Strain Identity

DAS-ELISA test using BCMV specific antiserum established the identity of the test isolates as BCMV and reaction pattern on CIAT bean differential set [6] confirmed the strain identity as NL-1, NL-1n, NL-7 and NL-7n (Table 1). Isolates of NL-1n and NL-7n strain caused local and veinal necrosis on cv. Jubila at high temperature (>30°C) within 24–48 h after inoculation (Table 1), whereas NL-1 isolates

caused mosaic on cv. Dubbele Witte and NL-7 isolates induced mosaic symptoms on cv. Dubbele Witte and Redlands Greenleaf 'C' characteristic of standard NL-1 and NL-7 strains identified by Drijfhout et al. [6].

RT-PCR Amplification and Cloning of the 3' Terminal Region

RT-PCR amplification of nine isolates belonging to four BCMV strains (NL-1, NL-1n, NL-7 and NL-7n) generated an amplicon of ~1,300 bp with BCMV specific primers. The amplified product of each isolate of different strains was cloned in pGEM-T Easy vector and transformed into *E. coli* strain DH5α followed by sequencing using custom services. Nucleotide sequences of the individual strain were submitted in NCBI genbank vide accession no. EU492546 (BCMV-57, NL-1), EU713858 (BCMV-58, NL-1n), FJ157246 (BCMV-59, NL-1n), FJ157245 (BCMV-60, NL-1n), FJ387162 (BCMV-61, NL-7n), GQ456169 (BCMV-63, NL-7n), FJ491262 (BCMV-62, NL-7), GQ356169 (BCMV-64, NL-7), GQ850881 (BCMV-65, NL-1). Sequence analysis of four strains revealed that the 3' terminal region consisted of partial nuclear inclusion b (NIb) (171–233 bp), complete coat protein (CP) (860 bp) and 3' untranslated region (UTR) (208–244 bp). The respective size of NIb, CP and 3'UTR of NL-1 strain isolates BCMV-57 and 65 was 137 and 171 bp, 860 bp each and 244 and 223 bp while it was 220, 210 and 233 bp, 860 bp each and 208, 224 and 208 bp in BCMV-58, 59 and 60, respectively belonging

Fig. 1 Multiple alignment of amino acid sequence of partial Nib and complete coat protein (8813-110054 nucleotides of the type strain, NL1 [AY112735]) of *Bean common mosaic virus* strains from India. Cleavage motif (QS) and aphid transmission motif (DAG) are highlighted

to NL-1n strain. Similarly, BCMV-62 and 64 placed in NL-7 strain possessed 183 and 161 bp Nib, 860 bp each CP and 214 and 224 bp length 3' UTR while it was 198 and 183, 860 bp in each and 239 and 214 bp in isolate BCMV-61 and 63 from strain NL-7n. In all the isolates, the length of CP was 860 bp only while in the length of partial Nib and 3' UTR varied to a considerable extent.

The multiple alignment of deduced a/a sequences of the Indian isolates (Fig. 1) showed 96–100% similarity at coat protein amino acid level and 77–100% at 3' UTR (Data not shown). In all the four strains, DAG motif was present at a/a position 12 of CP which is important for aphid transmission in potyviruses. Q/S polypeptide cleavage site and V residue responsible for cleavage of CP from viral polypeptide were also found in all the 9 isolates (Fig. 1). The DAG motif has also been reported at same position in LM1 and other Peruvian BCMV isolates, further confirming the identity of isolates under study [16]. However, absence of DAG motif was noticed in NY-15 isolate of BCMV [32].

All the strains contained termination codon at variable amino acid positions. Sequence comparisons of test strains

and other BCMV strains from NCBI database at CP amino acid level revealed 98–100% homology of the Indian isolates with strains US-1 (L12740) and US-7 (L19474) from USA (Data not shown). Comparison of 3' UTR also showed similar patterns with Indian isolates exhibiting 75–99% homology with US-1, US-7 and NL-1 strains (Data not shown). Phylogenetic analysis of a/a sequence data exhibited more differences among various test isolates as compared to phylogram constructed from nucleotide sequence phylogram. Consequently, further comparisons were made considering deduced a/a sequence of Nib and CP (3' terminal region) as well as CP alone by MEGA4 software.

The deduced CP amino acid sequence comparisons reflected two major clades accommodating Indian isolates along with BCMV (AY575773) from Taiwan; PSTV (U05771), US-1 (L12740), US-7 (L19474), AzMV (U60100) all from USA, CH-1 (L19539) from Chile, NL-1 (AY11235) from England, NL-4 (DQ666332) from Colombia and NL-7 (U37075) from Peru (Fig. 2a). However, the other type strains and members of Potyviruses

Fig. 2 Phylogenetic tree showing relationship of Indian isolates: BCMV-57(NL-1), BCMV-65(NL-1), BCMV-58(NL-1n), BCMV-59(NL-1n), BCMV-60(NL-1n), BCMV-62(NL-7), BCMV-64(NL-7), BCMV-61(NL-7n) and BCMV-63(NL-1n) with other BCMV strains* at coat protein (CP) only (a) and 3' terminal region (b) (comprising N1b and complete CP) amino acid sequence level (*NCBI genbank

sequence accession numbers: BCMV-NL-4, DQ666332; BCMV-NL-1, AY112735; BCMV-NL-2, L19472; BCMV-NL-7, U37075, BCMNV-NL-3, NC004047; BYMV, NC003492; BCMV-US-1, L12740; BCMV-US-10, U37072; BCMV-US-7, L19474; BCMV-NL-1, L15331; BCMV-NL-1, L15332; BCMV-CH-1, L19539; BICMV, AY575773; PStV, U05771; AzMV, U60100)

were clustered either independently or with other isolates (Fig. 2a, b). Comparison of coat protein sequences of the test strains at nucleotide and a/a levels showed very little differences between the strains which exhibited 91–100 per cent similarity among themselves and were clustered in a single group (Fig. 2a, b). Evolutionary divergence determined through MEGA4 at CP amino acid level ranged from 0.00 to 0.807 and from 0.018 to 3.193 for the 3' terminal comprising partial N1b and CP (Table 2). It clearly indicates that CP region is not able to distinguish strains. This was further strengthened by the evolutionary divergence analysis indicating that CP region alone is unable to distinguish strains barring few exceptions. These results indicate the involvement of some other component of the viral genome in conditioning pathogenicity on the differential hosts which ultimately form the basis for strain differentiation.

Although 3' terminal region is considered as the most variable region of the viral genome yet it failed to distinguish pathogenically discrete strains at molecular level.

This further suggests that 3' terminal region as a whole or only CP has no role in pathogenicity and thereby account for lack of correlation between pathogenicity and 3' terminal region sequence variability. This necessitates full genome sequencing of different strains of BCMV for their region wise sequence comparisons to unravel the real key players involved in pathogenicity. Until all the strains of BCMV are fully characterized it is difficult to establish which part of the viral genome is really responsible for pathogenic variation.

Comparative account of the CP region of the Indian isolates supports Shukla and Ward [26] that strains of the virus shared 90–99 per cent amino acid similarities. Our observations strengthen the view point of Adams et al. [1] regarding the utility of CP gene in virus strain identification. Analysis of CP and CI (Cylindrical inclusion) region of potyvirus genome suggested that CI region provides better insight as it represents the reflection of complete ORF in the absence of full genome and has been regarded as the most accurate region for species identification [1].

Table 2 Estimates of evolutionary divergence between sequences of Indian strains of BCMV based on nuclear inclusion body protein (Nib) and coat protein (CP)

Isolate/ Strain	BCMV-57 (NL-1)	BCMV-59 (NL-1n)	BCMV-58 (NL-1n)	BCMV-60 (NL-1n)	BCMV-61 (NL-7n)	BCMV-62 (NL-7)	BCMV-63 (NL-7n)	BCMV-64 (NL-7)	BCMV-65 (NL-1)
BCMV-57 (NL-1)		0.007	0.014	0.007	0.017	0.007	0.017	0.021	0.784
BCMV-59 (NL-1n)	3.193 ^a		0.014	0.007	0.017	0.007	0.017	0.031	0.784
BCMV-58 (NL-1n)	2.574	2.654		0.014	0.014	0.014	0.014	0.031	0.792
BCMV-60 (NL-1n)	3.193	2.398	2.431		0.017	0.007	0.017	0.031	0.770
BCMV-61 (NL-7n)	2.787	3.059	2.613	2.654		0.017	0.000	0.014	0.014
BCMV-62 (NL-7)	2.398	2.787	2.942	2.574	2.654		0.017	0.031	0.784
BCMV-63 (NL-7n)	2.431	2.787	2.942	2.574	2.654	0.018		0.014	0.807
BCMV-64 (NL-7)	2.741	2.887	2.887	2.787	2.741	2.465	2.465		0.830
BCMV-65 (NL-1)	2.465	2.999	2.654	3.124	2.613	2.654	2.696	2.500	

Values below the diagonal line represent Nib and CP while above the line represents only CP

^a The number of amino acid substitutions per site from analysis between sequences is shown. All results are based on the pairwise analysis of 9 sequences. Analyses were conducted using the Poisson correction method in MEGA4 software. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 341 positions in the final dataset

However, to establish the identity of the newly designated strains, NL-1n and NL-7n needs confirmation by complete genome characterization. NL-1n and NL-7n could be considered as mutants that might have evolved during process of co-evolution of both bean and BCMV in this part of the world [23] or could have evolved due to the recombination of different type strains infecting same cultivar as revealed by Silbernagel et al. [27] and other host-pathosystems [5]. The differences could also be due to mutation of common ancestor virus or by different selection processes into different agro ecological strains. Gibbs et al. [9] corroborate theory of evolution of BCMV lineage in South and East Asia, where cultivated as well landraces are host to these viruses. Moreover, close affinity of coat protein sequence of four strains, differing in pathogenicity indicated that CP region does not account for symptom variation [10]. In conclusion, this study clearly established that coat protein region is unable to delineate the strains of the same virus, however, it clearly distinguishes different viruses of the family *Potyviridae*, thereby indicating the involvement of other regions of the viral genome involved in pathogenicity behaviour of isolates of the same virus.

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