THE COMPLETE NUCLEOTIDE SEQUENCE OF THE GENOMIC RNA OF *BEAN COMMON MOSAIC VIRUS* STRAIN NL4

by

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Abstract

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The complete nucleotide sequence of the genomic RNA of the NL4 strain of *Bean common mosaic virus* (BCMV-NL4) was determined. The viral genome is 10037 nucleotides in length, excluding the 3' terminal poly (A) tail, and contains a single open reading frame (ORF) of 9666 nucleotides encoding a polyprotein of 3222 amino acids. . The ORF is flanked by 5' and 3' untranslated regions (UTRs) of 133 and 235 nucleotides, respectively. Comparative analyses of the predicted BCMV-NL4 polyprotein with other species of the genus *Potyvirus* revealed nine cleavage sites resulting in ten functional proteins. Nucleotide and amino acid sequence identities indicated a close relationship between BCMV-NL4 and BCMV-NL1. Blast comparisons using coat protein (CP) and 3'UTR sequences showed 100% identity between BCMV-NL4 and the Mexican variant (US6) of this strain.

Key words: BCMV-NL4, potyvirus, genomic RNA, complete sequence, mexican strain.

Resumen

Se determinó la secuencia completa del ARN genómico del Virus del mosaico común del fríjol (*Bean common mosaic virus*) cepa NL4 (BCMV-NL4). El genoma viral consta de 10037 nucleótidos excluyendo la cola de poli (A) y contiene una trama de lectura abierta (ORF) de 9666 nucleótidos que codifica una poliproteína de 3222 aminoácidos. La ORF está flanqueada por dos regiones no traducidas (UTR): la 5'UTR de 133 nucleótidos y la 3'UTR de 235 nucleótidos. Análisis comparativos de la poliproteína del BCMV-NL4 con otras especies del género *Potyvirus*, reveló nueve sitios de clivaje que resultaron en las diez proteínas funcionales características de estos virus. Alineamientos múltiples de las secuencias de nucleótidos del ARN genómico y de aminoácidos de la poliproteína del BCMV-NL4 con las de otros miembros de la familia *Potyviridae* mostraron una relación estrecha entre el BCMV-NL4 y el BCMV-NL1. Las comparaciones mediante el uso del programa Blast de las secuencias de la proteína de la cápsida (CP) y de la 3'UTR, mostraron una identidad del 100% entre el BCMV-NL4 y la variante mexicana (US6) de esta cepa.

Palabras clave: BCMV-NL4, potyvirus, ARN genómico, secuencia completa, cepa mexicana.

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Introduction

Bean common mosaic virus (BCMV) is a species of the genus *Potyvirus* within the family *Potyviridae.* BCMV consists of flexuous filaments approximately 15 x 750 nm containing a molecule of single-stranded, positive-sense RNA of approximately 10 kb. The viral genome is expressed as a polyprotein of about 370 kDa that is cleaved by viral-encoded proteinases into ten functional proteins. A protein (VPg) is attached to the 5'-end of the genomic RNA and it has a poly (A) tail at its 3'-end[7, 20].

Plant viruses are characterized according to different criteria, including their physicochemical properties, nucleic acid, proteins, genome organization, antigenic and biological properties. The characterization and taxonomic classification of viruses, however, requires the molecular analysis of the viral genome. In the case of potyviruses, the 3'UTR and coat protein gene have been often used to produce partial sequences for taxonomic purposes[11, 24]. To date, the complete genomes of over 50 different potyviruses have been sequenced, including the Type strain (NL1) of BCMV and some legume potyviruses recently re-classified as strains of BCMV (e.g. Blackeye cowpea mosaic virus), and other BCMV isolates, including BCMV-RU1, BCMV- cowpea isolate R, and BCMV-cowpea isolate Y.

The NL4 strain of BCMV was first isolated in 1963 by Hubbeling in the Netherlands, from the common bean cultivar Great Northern 123[10]. A similar BCMV strain was identified the following year by Silbernagel in a common bean line from Mexico (the 'Mexican strain')[23]. These viruses were considered as variants of a single BCMV strain belonging to pathogenicity group VII[10]. Both viruses were later shown to be pathogenic to group 6 of common bean genotypes which possess the resistance gene *bc 2*[2], that confers resistance to necrosis-inducing strains of BCMV and *Bean common mosaic necrosis virus* (BCMNV). The Great Northern and Mexican variants also induce mosaic in common bean genotypes possessing resistance genes *bc 1* and *bc 1*[2 10]. In this study, the nucleotide sequence of the complete genome of BCMV-NL4 is reported and its genomic structure is compared to other BCMV strains and BCMNV.

Materials and methods

The BCMV-NL4 isolate used in this investigation has been maintained at the Virology Research Unit of CIAT in infected seeds of the common bean cultivar *Dubbele Witte* since the late 1970s. The virus was replicated by mechanical inoculation onto primary leaves of seedlings of bean cultivar *Redlands Geenleaf C* under glasshouse conditions[18]. Virus particles were partially purified following an existing protocol[17] and the RNA was extracted using the RNAeasy kit (QIAGEN). Twenty two pairs of primers were designed, based either on the conserved genomic regions identified from multiple alignments of reported sequences of potyviruses, or based on the partial sequences obtained in this investigation for BCMV NL4. The primers that were used for the complete genome amplification and their location are shown in Table 1 and Figure 1, respectively.

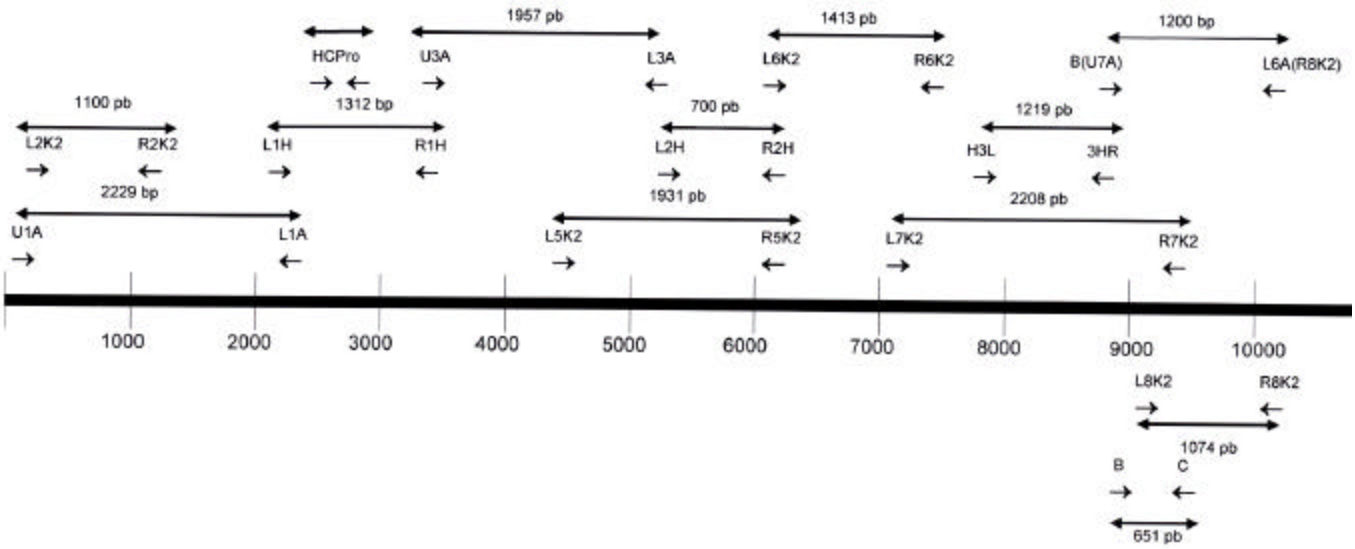


Figure 1. Positions of the primers used for the amplification of genome of BCMV-NL4

Table 1. Primers used for the amplification of the BCM -NL4 genome.

Primer	Sequence 5'? 3'	Position	Direction
U1A	AAAAATTAAAACAACTCAT	1-19	sense
L1A	AAGGGCACTATCAAAGATC	2229-2211	antisense
L2K2	CTGTAGGATGCTCAGCACGA	279-297	sense
R2K2	TGACATAAAGGGCTGTTGAGTG	1383-1367	antisense
HCProL3	GGATGTACATTGCAAAAGAAGG	2463-2484	sense
HCProR2	TCAGGAGCGAAGGTGAAACT	2966-2948	antisense
L1H	CCTAATGGGCAAAGGGAGTT	2183-2201	sense
R1H	CGCAGTTGTGAAACAATCGT	3469-3451	antisense
U3A	TAAGCTTGTTGGAAAAATC	3329-3348	sense
L3A	AGTGGTGTAAGCTCAAAAT	5304-5285	antisense
L5K2	AATCAACAAGCCTCCCACAC	4302-4320	sense
R5K2	TGCTGCCTTTTTGCTTACCT	6233-6215	antisense
L2H	TGCGATGCATCCTGAGATTC	5335-5353	sense
R2H	ATCCAGCCACCACCAAGCAG	6042-6024	antisense
L6K2	TGTTCACACTGCTTGGTGGT	6015-6033	sense
R6K2	TTTCCTTCGGGCTGATTCTA	7428-7409	antisense
L7K2	TGGGCTCATAGTTGGGTTTC	7141-7159	sense
R7K2	CCACACCATAAAGCCATTCA	9349-9331	antisense
H3L	GCAGTCGGAGCACAATACAA	7792-7810	sense
3HR	TCCACACCAGCATCCACTAC	8985-8967	antisense

The viral RNA was amplified using RT-PCR and the products were cloned using the PCR TA Cloning Kit (Invitrogen). About fifty clones with overlapping sequences comprising the whole viral genome were sequenced. Additional sequence infomation was generated by direct sequencing of selected PCR products. The sequence was determined by using the program Sequencher 4.1 (Gene Code Corporation). The program DNAMAN 4.13 (Lynnon BioSoft) was used to convert the nucleotide sequences to amino acids.

Results and discussion

The full-length nucleotide sequence of BCMV-NL4 was deposited in the GenBank with the accession number **DQ666332**. The full-length genomic sequence (excluding the poly A tail) is 10037 nucleotides long and has a 5' untranslated (UTR) region of 133 nt, a single open reading frame (ORF) and a 3'UTR of 235 nt. The ORF has two possible translation start codons. The first AUG codon is at position 134 to 136, and the second codon is at position 146 to 148. Only the first codon AUG is located in the optimum context (A/G)AAAUGGC) for initiation of translation in dicotyledon plants and many potyviruses[9, 13]. Using this codon, the ORF (nucleotides 134 to 9799) encoded a polyprotein of 3222 amino acids. The 5'UTR has a high content of AU (68.4%) and a low content of GC (31.6%) similar to values reported for other potyviruses; and included the sequences AAGACAACA (9 nt, from 21 to 29) and UCAAGCAA (8 nt, from 68 to 75), which correspond to "potybox a" and "potybox b", respectively[26]. The 5'UTR is also rich in CAA, similar to the translation regulatory elements found in the leader sequence of *Tobacco mosaic virus* (*Tobamovirus*), which functions to stimulate translation[13]. Analysis of the BCMV-NL4 5'UTR with the RegRNA program (http://regrna.mbc.nctu.edu.tw/) showed two pyrimidine base rich tracts: CUUCUUUCUCUCG (13 nt, from 52 to 64) and CUUUCUUUG (9 nt, from 111 to 119). These tracts are similar to the "terminal oligopyrimidine tract (TOP)" found at the 5' terminal region of mRNAs of many vertebrates, which has been associated with translation control[15]. The 3'UTR contains a yeast-like signal consense sequence for the poly (A) tail, UAUGA at position 9962 to 9966[13]. A comparison of the BCMV-NL4 polyprotein with those of other potyviruses showed nine cleavage sites for viral proteinases with the capacity to produce ten proteins characteristic of potyviruses (Table 2 and figure 2).

Several potyvirus-conserved motifs were identified in the BCMV-NL4 polyprotein sequence. The motifs H-X_{10}-D-X_{27}-G-D-S-G (amino acids 356 to 398) and FMIIRG (amino acids 416 to 421) were identified as the conserved proteolytic domains of serine proteases, which are located in the carboxylic end of P1 protein[21]. In the amine end region of the HC-Pro protein, the motif C-X_8-C-X_{18}-C-X_2-C (amino acids 471 to 502) was identified and it corresponded to the "zinc finger"[21], and the motif KLSC (496 to 499) associated

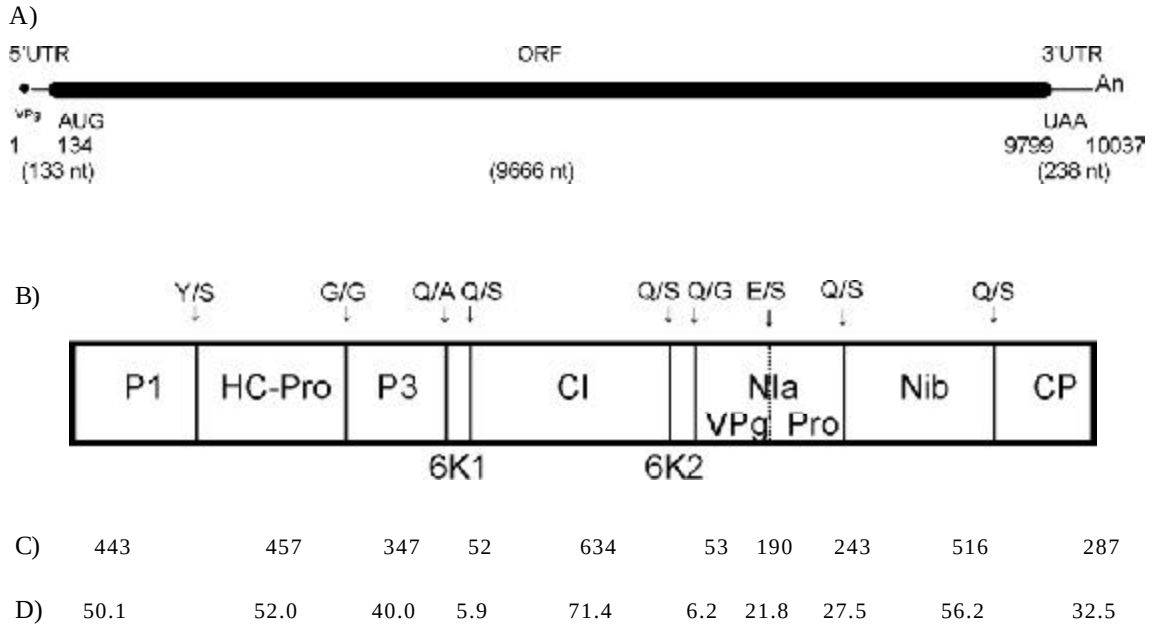


Figure 2. Organization of Bean Common Mosaic Virus NL4 strain genome. A) Structure of genomic RNA. B) Structure of the polyprotein indicating the amino acids at the cleavaje sites for viral proteinases. Number of amino acids (C) and calculed molecular mass in kDa (D) of the mature proteins based on the translation of the genomic sequence.

Table 2. Cleavage sites in BCMV-NL4 polyprotein. Residues strongly conserved at the positions -4 and -2 in the context sequences are underlined.

Position in the polyprotein	Cleavaje sites (amino acids)	Mature protein	Size (amino acids)
443-444	IHHY/S	P1	443
900-901	YRVG/G	HC-Pro	457
1247-1248	VSVQ/A	P3	347
1299-1300	VQVQ/S	6K1	52
1933-1934	VRLQ/S	CI	634
1986-1987	VTTQ/G	6K2	53
2176-2177	VTTE/S	NIa-Vpg	190
2419-2420	VATQ/S	NIa-Pro	243
2935-2936	VHLQ/S	NIb	516
		CP	287

with aphid transmission[19]. In the middle region of HC-Pro, the motif FRNK (623 to 626) was identified, which has been associated with aphid transmission and symptom expression[12]. Towards the carboxylic end of HC-Pro (734 to 736), the motif CCC, which is implicated in systemic movement and interaction with the coat protein[4, 27], and the motif GYCY (784 to 787), which is conserved at the active site of cysteine proteinases[4], were identified. In the amine end region of the P3 protein, the sequence DPY-X_7-SP-X_2-L-X-H-X_2-R-X-R-X_2-E-X_5-W (amino acids 930 to 960) was found, which is similar to the conserved motif EPYX$_7$SPX$_2$LXAX$_2$NXGX$_2$EX$_5$N[20]. Two conserved motifs for binding of nucleotides, characteristic of RNA helicases, were found at the amine end region of the CI protein: GAVGSGKST (amino acids 1384 to 1392) and PTR (amino acids 1410 to 1412)[14]. The sequences KVSAT (1500 to 1504), LVYV (1551 to 1554), VATNIIENGVTL (1602 to 1613) and GERIQRLGRVGR (1646 to 1657), conserved in protein helicases[16] were identified in the CI protein. The residues H_{2222}, D_{2257} and C_{2326} found in the NIa protease of BCMV-NL4 constitute a triad in the catalytic domain of NIa proteinases[8]. The motifs HCHADGS (2662 to 2668), GNNSGQPSTVVDNTLMV (2725 to 2741) and GDD (2769 to 2771), conserved at the catalytic domains of RNA dependent RNA polymerases (RdRpol)[6, 27], were identified in the NIb protein. The motif DAG (2947 to 2949), which has been implicated in aphid transmission, was located in the amine end region of the CP. The conserved residues R_{3128} and D_{3165} were also found and are considered essential for virion assembly[27].

The complete nucleotide sequences and the predicted amino acid sequences of 47 viruses of the family *Potyviridae* and 7 strains or isolates of BCMV were aligned and were compared with the sequence of BCMV-NL4 using the CLUSTALW multiple sequence alignments pro-

gram. The viruses were: *Agropyron mosaic virus* (AgMV, AY623626), *Bean common mosaic necrosis virus* (BCMNV, U19287), *Bean yellow mosaic virus* (BYMV, D83749), *Beet mosaic virus* (BtMV, NC_005304), *Brome streak mosaic virus* (BrSMV, NC_003501), *Chilli veinal mottle virus* (ChiVMV, AJ237843), *Clover yellow vein virus* (ClYVV, AB011819), *Cocksfoot streak virus* (CSV, AF499738), *Cowpea aphid-borne mosaic virus* (CABMV, AF348210), Daphne virus Y (DVY, DQ299908), *Dasheen mosaic virus* (DsMV, AJ298033), *East Asian passiflora virus* (EAPV, AB246773), *Hordeum mosaic virus* (HoMV, AY623627), *Japanese yam mosaic virus* (JYMV, AB027007), *Johnsongrass mosaic virus* (JGMV, Z26920), *Konjak mosaic virus* (KoMV, AB219545), *Leek yellow stripe virus* (LYSV, AJ307057), *Lily mottle virus* (LMoV, AJ564636), *Maize dwarf mosaic virus* (MDMV, AJ001691), *Onion yellow dwarf virus* (OYDV, AJ510223), *Papaya leaf distortion mosaic virus* (PLDMV, BD171712), *Papaya ringspot virus* (PRSV, X67673), *Pea seed-borne mosaic virus* (PSbMV, D10930), *Peanut mottle virus* (PeMoV, AF023848), *Pennisetum mosaic virus* (PenMV, AY642590), *Pepper mottle virus* (PepMoV, M96425), *Peru tomato mosaic virus* (PTMV, AJ437280), *Plum pox virus* (PPV, D13751), *Potato virus A* (PVA, AJ296311), *Potato virus V* (PVV, AJ243766), *Potato virus Y* (PVY, X12456), *Scallion mosaic virus* (ScaMV, AJ316084), *Shallot yellow stripe virus* (SYSV, AJ865076), *Sorghum mosaic virus* (SrMV, AJ310197), *Soybean mosaic virus* (SMV, D00507), *Sugarcane mosaic virus* (SCMV, AJ97628), *Sweet potato feathery mottle virus* (SPFMV, D86371), *Thunberg fritillary mosaic virus* (TFMV, AJ851866), *Tobacco etch virus* (TEV, M11458), *Tobacco vein mottling virus* (TVMV, X04083), *Turnip mosaic virus* (TuMV, AF169561), *Watermelon mosaic virus* (WMV, AY437609), *Wild potato mosaic virus* (WPMV, AJ437279), *Wisteria vein mosaic virus* (WVMV, AY656816), *Yam mosaic virus*, (YMV, U42596) and *Zucchini yellow mosaic virus* (ZYMV, AF127929). The BCMV strains and isolates in this analysis were: *Bean common mosaic virus* (BCMV, AJ312437), BCMV-NL1 (AY 112735), BCMV-RU1 (AY 863025), BCMV-cowpea isolate R (AJ 312437), BCMV-cowpea isolate Y (AJ 312438), BCMV-Blackeye cowpea mosaic virus (BlCMV, AY 575773), and BCMV-Peanut stripe virus (PStV, U 34972).

The viruses which showed the highest percentage of nucleotide identities and amino acid similarity with BCMV-NL4 were: BCMV-NL1 (86nt, 92aa), BCMV cowpea isolate Y (85, 91), BCMV cowpea isolate R (84, 88), BCMV-BlCMV (84, 88), BCMV-RU1 (82, 88) and BCMV-PStV (82, 87). The percentages of identity with the genomic sequences of the other selected viruses ranged from 6 to 69% (table 3). The percentages of similarity with the polyproteins of the other selected viruses ranged from 19 to 71% (table 3). The lowest scores were obtained for the polyproteins of viruses belonging to different genera of viruses in the *Potyviridae*. Among species of the genus *Potyvirus*, amino acid sequence similarities with the polyprotein of BCMV-NL4 ranged from 39 to 71% (Table 3). These results fit well the criteria for differentiation of species in the different genera of the *Potyviridae*: <76% nucleotide identity and <82% amino acid sequence identity of the ORF[1]. The lowest scores of amino acid sequence similarity were with HoMV (38%) and AgMV (37%) of the genus *Rymovirus* and with BrSMV (19%) of the genus *Tritimovirus*.

A cladogram constructed based on multiple sequence alignments of the polyproteins of BCMV-NL4 and 54 other viruses using the Neighbour-Joining method[25] with BrSMV as the outgroup, showed that BCMV-NL4 is most closely related to BCMV-NL1, and was clustered with the other strains and isolates of BCMV into a broader group of potyviruses infecting legumes and other dicotyledons plants (Figure 3). Other clusters included viruses infecting monocotyledons plants, i.e. the *Sugarcane mosaic virus*[22], the cluster of AgMV and HoMV of genus *Rymovirus*, the cluster of viruses OYDV and SYSV, and the cluster of potyviruses infecting solanaceous plants: PTV, PVV, WPMV, PVY, PepMoV and PVA.

Sequences of the coat protein and the 3'UTR have been considered a valuable taxonomic indicator for determination of genetic relatedness and to discriminate among species and strains of potyviruses[2, 11]. Several viruses, for which there is not complete sequence information, were included in the analysis of the CP and UTR regions. The comparison of BCMV-NL4 coat protein sequence with corresponding sequences of other potyviruses from GenBank using blastp, showed 100% identity with the Mexican variant (L11890) and 99% with the NL4 isolate (L21766), reported by Wyatt and Berger in 1993. Analyses of the 3'UTR with blastn showed 100% identity with the Mexican variant, 98% with the Florida strain (US5) (L19473) and 95% with the NL4 isolate (L21766),

When a multiple sequence alignment was performed using CP sequences of BCMV-NL4 and other strains and isolates of BCMV, higher CP amino acid identities were found between the BCMV-NL4 strain selected for this investigation, and: the Mexican (US6) variant (100%), BCMV-NL4 [L21766] (99%), BCMV-US5 (98%) and BCMV-

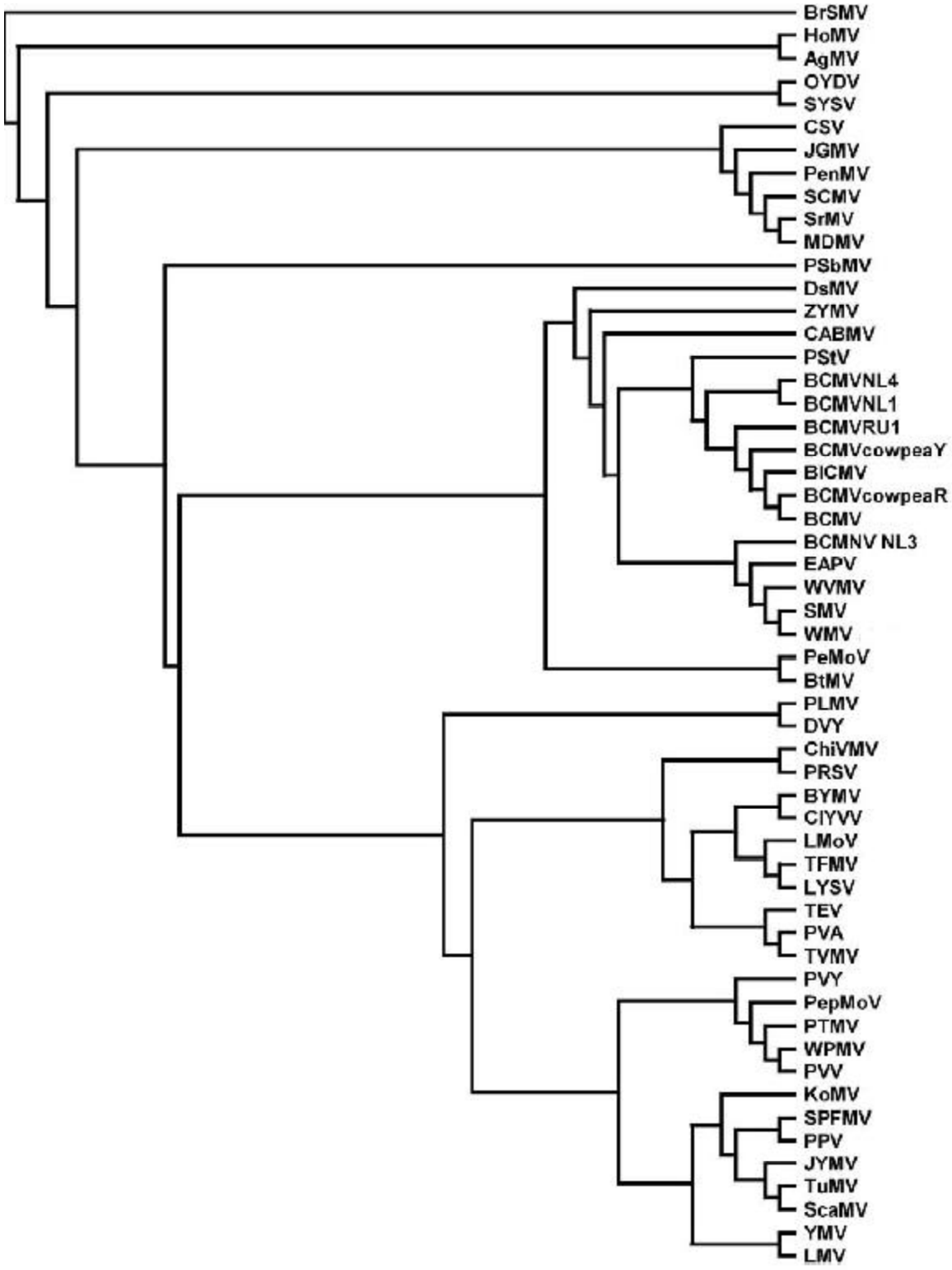


Figure 3. Cladogram resulting from multiple sequence alignments of polyproteins of BCMV-NL4 and 54 other viruses of the family *Potyviridae* reported to GenBank. The tree was constructed using the Neighbour-Joining method and viewed with the TreeView program. Sequences used are detailed in the text.

Table 3. Scores of nucleotide identity and amino acid similarity sequences of BCMV-NL4 with other viruses of the family *Potyviridae* calculated using the CLUSTALW multiple sequence alignment program.

Virus	Scores of nucleotide sequences	Scores of amino acid sequences	Virus	Scores of nucleotide sequences	Scores of amino acid sequences
BCMV-NL1	86	92	TVMV	49	44
BCMVcowpeaY	85	91	TuMV	48	44
BCMVcowpeaR	84	88	JYMV	47	44
BCMV	84	88	LMoV	47	44
BCMV-RU1	82	88	PenMV	49	43
BlCMV	84	88	ChiVMV	48	43
PStV	82	87	PepMoV	47	43
WMV	69	71	SCMV	48	43
SMV	66	69	MDMV	48	43
BCMNV	66	68	PVY	47	43
WVMV	64	68	PTV	46	43
EAPV	67	67	WPMV	46	43
CABMV	63	66	PVV	46	43
ZYMV	59	61	TFMV	47	43
DsMV	56	59	DVY	47	43
PeMoV	53	50	SrMV	49	42
BtMV	53	50	LMV	46	42
YMV	49	46	LYSV	46	42
SPFMV	48	45	JGMV	47	41
PPV	48	45	PRSV	46	41
TEV	50	45	PSbMV	46	41
ScaMV	50	45	CSV	46	41
ClYVV	48	45	SYSV	34	40
PLMV	46	45	OYDV	34	39
KMV	48	45	HoMV	36	38
BYMV	49	44	AgMV	36	37
PVA	49	44	BrSMV	6	19

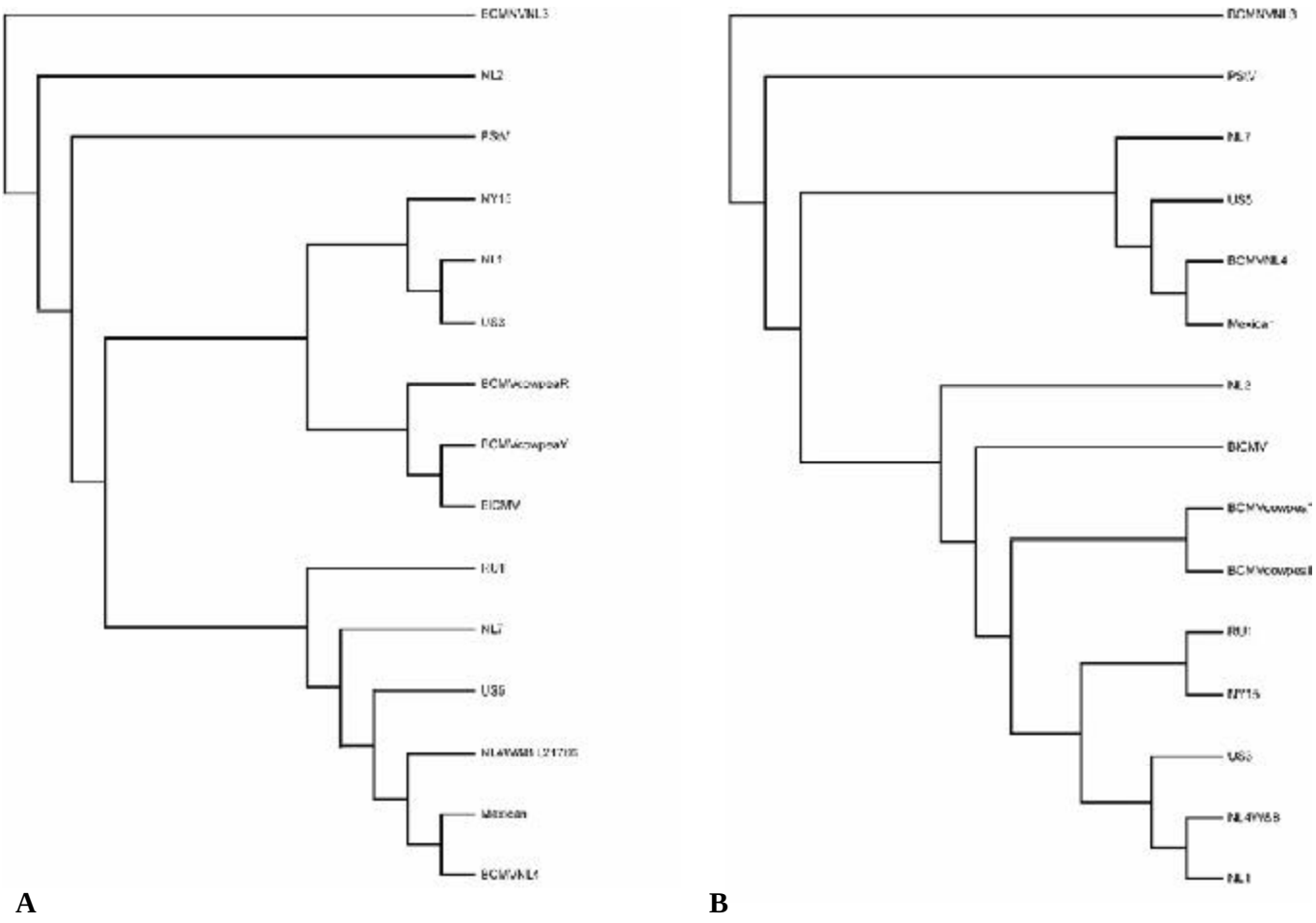
NL7 (96%). Similar results were obtained when their 3'UTRs were compared: BCMV-NL4 showed higher identity (95%) with the Mexican variant, BCMV-US5 (93%), BCMV-NL7 (89%) and BCMV-NL4 [L211766] (88%) (Table 4 and Figure 4).

The results obtained in this investigation closely agree with the biological properties of the known BCMV strains originally described by Drijfhout[10], particularly the high degree of nucleotide and amino acid sequence identity found between the Great Northern (NL4) and Mexican (US6) isolates representing pathogroup VII of BCMV. These two isolates can be considered as variants of a recognized strain of BCMV known as US6 in the United States, and NL4 in Europe. This BCMV strain is particularly important due to its pathogenicity gene $P2^2$, which attacks the recessive gene *bc* 2^2, widely used in common bean improvement projects to protect the dominant necrosis gene (*I*) from necrosis-inducing strains of BCMV and BCMNV. The *bc* 2^2 gene is also present in many common bean cultivars (devoid of the *I* gene), derived from the original Great Northern and Red Mexican common bean genotypes[5].

The close relationship observed in this study between BCMV-NL4 and BCMV-NL1 was expected, considering that BCMV-NL1 is the Type strain of BCMV, and the only strain of the original seven BCMV strains from common bean described by Drijfhout[10] that had

Table 4. Similarity scores of 3'UTR nucleotide sequences and coat protein amino acidic sequences of BCMV-NL4 with sequences of the genomic 3' end from strains of BCMV and BCMNV, using the CLUSTALW multiple sequence alignment program.

Strain / accession number	Pathogenicity group	3'UTR	Coat protein
Mexican / L11890	VII	95	100
NL4 / L21766	VII	88	99
US5 / L19473	IVa	93	98
NL7 / U37075	II	89	96
RU1 / U37077		86	95
NY15 / AF083559	Va	87	91
NL1 / AY112735	I	87	90
NL2 / L19472	Vb	88	90
BCMVcowpeaR / AJ312437		86	90
BCMVcowpeaY / AJ3122438		86	90
PStV / AY968604		84	90
BlCMV / AY575773		87	90
US3 / U37073	IVb	85	89
BCMNV-NL3 / NC_004047	VIa	64	77



A **B**

Figure 4. Cladograms resulting from multiple sequence alignments of coat proteins (A) and 3'UTR (B) sequences of BCMV-NL4 and other strains of the BCMV reported to GenBank, using BCMNV-NL3 as an outgroup. The trees were constructed using the Neighbour-Joining method and viewed with the TreeView program. Sequences used are indicated in the table 4.

been completely sequenced. The remaining fully-sequenced BCMV strains compared here with BCMV-NL4, are legume BCMV strains isolated from *Vigna* spp. (with the exception of BCMV-RU1), which formed a close but separate group of BCMV strains, referred to as the "blackeye cowpea mosaic virus strain subgroup"[28]. The high (>95%) CP and 3' UTR sequence identities observed between BCMV-NL4 and the Florida strain of BCMV, demonstrate that BCMV-NL4 belongs to the common bean subgroup of BCMV strains[3].

Finally, the close relationship observed between the BCMV-NL4 strain selected for this investigation and the Mexican (US6) variant, suggests that this is the origin of the BCMV-NL4 strain maintained at CIAT and characterized here. Nevertheless, considering that the BCMV-NL4 strain was described before the US6 variant, we propose that BCMV-NL4 should be the representative strain of the pathogenic group VII, indicating the variant [Great Northern or Mexican] whenever required.

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