

Research Note

The Nucleotide Sequence of the Coat Protein Gene and 3' Untranslated Region of Azuki Bean Mosaic Potyvirus, a Member of the Bean Common Mosaic Virus Subgroup

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The relationship of azuki bean mosaic potyvirus (AzMV) to members of the bean common mosaic virus (BCMV) subgroup has been unclear. Degenerate oligonucleotide primers and the polymerase chain reaction were used to amplify and clone the coat protein (CP) gene and 3' untranslated region (UTR) of AzMV. The deduced amino acid sequence of the CP is 94% identical to that of dendrobium mosaic virus, establishing the two as strains of the same virus. While the CP amino acid identities between AzMV and potyviruses of the BCMV species are at or below 90%, the 91 to 94% identity between their UTRs suggests that AzMV could be considered a strain of BCMV. Interestingly, the grouping of potyviruses within the greater BCMV subgroup on a coat protein amino acid tree correlates with a grouping based on the response elicited on bean containing the *I* gene for resistance to BCMV.

Additional keywords: coat protein phylogeny, *I* gene of *Phaseolus vulgaris*, phylogenetic tree, plant virus, taxonomy, temperature-sensitive resistance.

It has been well recognized that the taxonomy of the potyviruses, the largest group of plant viruses, is in a "very unsatisfactory state"; the size of the group and the extensive variation therein have made it difficult to reliably identify individual members and particularly to find characters that distinguish distinct potyviruses from strains (Ward et al. 1992 and references therein). While some have predicted that a "continuum" of strains may make such a distinction impossible (Bos 1970; Matthews 1979; Hollings and Brunt 1981; Harrison 1985), others have presented analyses of sequence homologies that argue for a demarcation between distinct viruses. Shukla and Ward (1988) found that pairwise coat protein (CP) amino acid sequence comparisons among 17 strains of eight distinct poty-

viruses showed a bimodal distribution—sequence identities ranged from 38 to 71% for distinct viruses (species) as opposed to 90 to 99% for strains of the same virus. Further work has revealed an additional cluster in the 75 to 89% range, which Ward and Shukla have grouped with the former (distinct species) rather than the latter (strains of the same species) (see Fig. 3 in Mink et al. 1994). Frenkel et al. (1989) had earlier suggested that the 3' untranslated region (UTR) may be useful for determining relationships among viruses whose CP amino acid sequence homology falls midway between the original bimodal distribution. In that work they found that nucleotide sequence homology ranged from 39 to 53% for distinct viruses, in contrast to 83 to 99% for strains.

One group of potyviruses for which sequence comparisons could prove particularly useful includes the many biologically distinct isolates of several potyviruses that naturally infect legumes. While it is clear that a number of these, including bean common mosaic virus (BCMV), blackeye cowpea mosaic virus (BICMV), azuki bean mosaic virus (AzMV), cowpea aphid-borne mosaic virus (CABMV), peanut stripe virus (PStV), and soybean mosaic virus (SMV) are closely enough related to be included in a BCMV subgroup of potyviruses (Dijkstra and Khan 1992), there has been disagreement about which sets of isolates constitute distinct viruses. Recently, BCMV serotypes A and B were formally separated into two distinct species, BCMNV (bean common mosaic necrosis virus) and BCMV, respectively (Mink et al. 1994). Numerous studies of the biology, serology, and CP peptides of the other potyviruses named above have failed to find "characteristics that can be used to distinguish unequivocally" among them (Mink and Silbernagel 1992, and references therein), and recent data have led to the proposal that AzMV, BICMV, and PStV be considered isolates of the newly defined BCMV species (McKern et al. 1992; Huguenot et al. 1994; Mink et al. 1994).

Consistent with this proposal, sequence data provided the basis for a claim to finally "settle the taxonomic position" of certain BCMV and BICMV isolates as strains of the same virus (Khan et al. 1993) and for inclusion of a new member, dendrobium mosaic virus (DeMV; Hu et al. 1995), in the

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BCMV subgroup. The present study was undertaken to provide similar sequence data for AzMV, a potyvirus related in our studies to certain BCMV serotype B isolates, CABMV, and BICMV in terms of the response elicited on bean containing the *I* gene for resistance to BCMV (Fisher and Kyle 1994).

AzMV was obtained from M. J. Silbernagel (USDA WSU-IAREC, Prosser, WA) and maintained on *Phaseolus vulgaris* 'BT-2'. A crude nucleic acid extract from systemically infected leaf tissue served as the starting material for a two-step procedure (modified from Pappu et al. 1993) that included reverse transcription and PCR-based amplification of approximately 1,370 nucleotides of the 3' end of AzMV RNA (containing the complete CP gene and 3' UTR). Ten microliters of the Sephadex G-50 column flowthrough was mixed with 2 µl of a 50 µM, 25-base poly-T oligonucleotide primer (CWC-111) containing a 3' G (previously determined to prime reverse transcription on AzMV RNA), incubated at 70°C for 5

min, cooled, and then incubated at 42°C for 50 min following addition of the following components to a total reaction volume of 30 µl: 6 µl 5× reverse transcription buffer (Promega, Madison, WI), 3 µl dNTPs (10 mM each), 3 µl 100 mM DTT, 3 µl 40 mM sodium pyrophosphate (prewarmed to 42°C), 30 units RNasin (Promega), and 18 units AMV reverse transcriptase (Promega).

Following reverse transcription, 3 µl of the reaction mix was used directly in a polymerase chain reaction containing the following additional components in a total volume of 50 µl: 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 2.0 mM MgCl₂, 120 µM each dNTP, 75 pmole primer CWC-111, 75 pmole of a degenerate oligonucleotide primer (CWC-107) for the Nib gene (5'-CAYKGN YTN GARGC NATYTGYGC-3'; Cassidy et al. 1993), and 2.5 units of Taq polymerase (Boehringer-Mannheim). (Degenerate positions in oligonucleotide primers are represented by the following single letter codes: Y = C and T; R = A and G; K = A and C; H = A, C, and T; N = A, C, G,

TTTGATTAT	GAGATTGGG	TGCGGAGAA	TCTGTGCAC	CTACA <u>TGA</u>	GGAATCAAC	CAGCCACCA	63
				S	G	I	7
GAAGTGGAT	GCTGTATA	GACACAGGC	AAGGATGAG	AAGAAGAAA	AGCAGCAAA	GGAAAAGGC	126
E	V	D	A	G	I	D	28
CCTGAGAGC	AAAGAAGGG	TCAGGCAAC	AACAGCCGA	GGAGCGGAA	AACTCAACA	ATGAGAGAC	189
P	E	S	K	E	G	S	49
AAGGATGTA	AATGCTGGT	TCCAAAGGG	AAGGTTGTT	CCTCGGCTT	CAAAGGATC	ACAAGAAAG	252
K	D	V	N	A	G	S	70
ATGAATATG	CCCACGGTA	AGGGGGAAT	ATGATCCTA	AATTTAGAT	CATCTATTG	GATTACAAG	315
M	N	M	P	T	V	R	91
CCAGAACAA	ACTGATCTT	TTTAACACA	AGAGCAACA	AAGATGCAG	TTTGAAATG	TGGTACAAT	378
P	E	Q	T	D	L	F	112
GCTGTGAAG	GGCGAGTAT	GAGATAGAT	GATGAACAG	ATGTCAATT	GTGATGAAT	GGCTTTATG	441
A	V	K	G	E	Y	E	133
GTGTGGTGC	ATTGACAAT	GGTACTTCA	CCAGATGTG	AATGGTACC	TGGGTCATG	ATGGATGGA	504
V	W	C	I	D	N	G	154
GATGAGCAG	GTGGAATAC	CCACTCAAA	CGAATGGTT	GAAAAATGCA	AAGCCAACA	CTCCGTCAA	567
D	E	Q	V	E	Y	P	175
ATCATGCAC	CATTCTCTA	GATGCAGCT	GAAGCATAC	ATTGAGATG	AGAAATTCC	GAGAGGCCG	630
I	M	H	H	F	S	D	196
TATATGCCT	AGGTACGGA	CTACTCCGG	AATTGAGG	GACAAAAAC	CTAGCTCGC	TACGCTTTT	693
Y	M	P	R	Y	G	L	217
GATTCTTAC	GAAGTGACA	TCCAAAACA	TCGGATCGA	GCAAGAGAA	GCAGTAGCA	CAGATGAAG	756
D	F	Y	E	V	T	S	238
GCAGCAGCC	CTCAGCAAC	GTTACCAGC	AAGTTATTT	GGAATTGAT	GGTAATGTG	GCAACAACC	819
A	A	A	L	S	N	V	259
AGCGAGAAT	ACTGAAAGC	ACACTGGCA	AGGGACGTC	AATCAGAAC	ATGCACTCA	CTTCTTGGC	882
S	E	N	T	E	S	T	280
ATGGGTTCC	CGGCAGTAA	AGATTGGGT	CAACCGATC	ACAGTTAGC	ATCTCGCGT	CGGTGAATA	945
M	G	S	P	Q	#		285
ATTCATAT	AGTAATCTT	TTATGTTCT	CTTTAGTTT	CAGTGTGGT	TTTACCACC	TTTGTGTTA	1008
CTATTGTGA	TAGTGTGGT	TGGCCACCC	AACATATTG	TGACTACTT	TATGTTTAT	GAGTAAGCC	1071
GGAAGAACC	ATTGCAATG	CGGAGGGCA	TGCAGAGTG	GTTGCACCA	CGCGTTATG	GAGTAGCTA	1134
CGGCAATGT	TTGTTGTTCC						1153

Fig. 1. DNA sequence corresponding to the 3' terminus of azuki bean mosaic virus (AzMV) RNA, beginning 42 nucleotides 5' to the assumed start of the CP gene (codon underlined) and continuing to the 3' poly-A tail. Underlined individual nucleotides are sites where variation was observed in partial clones (see text). The # denotes the stop codon at the end of the CP gene. The deduced CP sequence is presented; the DAG sequence is underlined. GenBank (EMBL) Data Library accession no. U60100.

Table 1. Amino acid and nucleotide sequence identities (%) between the coat proteins and 3'-UTRs of AzMV and other selected potyviruses^a

Viruses	AzMV	DeMV	BCMV US-1	PStV	BCMV NL-4	BICMV	BCMV NY-15	BCMV NL-1	SMV	BCMV NL-3	PWV-K	WMV	ZYMV	SAPV	BYMV
AzMV		93.9	90.3	89.7	89.4	88.7	88.0	86.9	78.2	77.2	76.4	74.2	70.9	68.8	59.9
DeMV	94.6		90.6	89.9	89.6	89.2	88.5	87.8	79.1	78.1	77.3	75.1	71.9	71.1	60.1
BCMV-US-1	93.6	92.0		91.3	91.7	94.1	96.5	96.2	78.5	77.3	76.3	74.5	73.7	70.2	62.0
PStV	90.8	91.6	90.7		90.6	89.6	88.9	87.8	76.7	77.4	75.3	73.8	72.7	70.2	60.5
BCMV-NL-4	94.0	92.0	98.8	91.0		91.0	89.6	88.9	77.8	76.7	74.9	73.8	72.7	69.8	61.3
BICMV	93.3	93.0	95.5	90.4	96.7		92.4	91.3	78.1	77.8	75.3	73.1	73.0	70.2	61.0
BCMV-NY-15	93.4	90.7	97.9	89.3	97.5	94.5		95.8	77.4	77.4	75.3	74.2	72.7	69.8	62.0
BCMV-NL-1	92.3	90.8	96.9	89.8	98.0	95.1	95.9		76.7	76.4	75.3	72.8	73.0	68.8	60.6
SMV	59.8	58.4	61.6	58.9	60.8	60.0	60.6	60.9		78.9	79.6	87.6	75.4	75.4	63.6
BCMV-NL-3	57.1	57.3	54.2	57.0	54.2	55.7	55.1	52.6	50.7		80.0	77.4	75.0	90.6	61.3
PWV-K	51.7	50.3	51.6	48.4	51.2	50.7	52.5	50.8	49.7	49.2		74.4	72.9	71.4	59.2
WMV	63.3	60.7	63.9	60.4	64.3	63.5	63.3	64.0	76.6	55.9	50.9		73.9	74.3	63.2
ZYMV	38.4	37.1	38.3	37.2	37.6	38.2	38.0	37.8	33.3	36.0	32.3	38.8		70.2	60.5
SAPV	46.5	47.1	44.3	46.8	43.9	45.0	44.9	43.5	40.3	62.3	39.0	46.9	35.1		61.2
BYMV	34.7	33.6	35.1	34.2	35.1	35.4	34.7	35.4	32.4	28.5	28.8	35.2	40.9	25.6	

^a CP amino acid sequence alignments were constructed in the GCG program PILEUP (Devereux et al. 1984) with a gap weight of 3 and a gap length weight of 0.1. Nucleotide sequence alignments of the 3' UTR were constructed initially in PILEUP with a gap weight of 4 and a gap length weight of 0.1 and then were edited manually for better alignment. Pairwise identity between each of the viruses was determined using the GCG DISTANCES program with the average sequence length as the denominator. CP amino acid comparisons are above the diagonal; 3'-UTR nucleotide comparisons are below the diagonal. The fourteen potyviral coat protein and 3'-UTR sequences are from the following GenBank accession numbers or publications: dendrobium mosaic virus (DeMV; U23564); bean common mosaic virus (BCMV) strain US-1 (L12740); BCMV-NL-4 (L21766); BCMV-NY-15 (S66252); BCMV-NL-1 (S66251); bean common mosaic necrosis virus (BCMV) strain NL-3, formerly designated BCMV NL-3 (U19287); peanut stripe virus blotch isolate (PStV; U05771); blackeye cowpea mosaic virus (BICMV; S66253); soybean mosaic virus strain G2 (SMV; S42280); passion-fruit woodiness virus strain K (PWV-K; Gough and Shukla, 1992); watermelon mosaic virus (WMV; D00592); zucchini yellow mosaic virus (ZYMV; L29569); South African passiflora virus (SAPV; D10053); bean yellow mosaic virus (BYMV; X63358).

and T.) The reaction was overlaid with mineral oil and cycled 30 times (2 min 94°C, 2 min 42°C, 2 min 72°C) followed by elongation at 72°C for 10 min. Alternatively, partial, overlapping clones containing 5' nucleotides 1-764 or 3' nucleotides 445-1153 (numbering as in Fig. 1) were made using degenerate oligonucleotide primers CWC-107 (5'; above) and CWC-106 (3'; GCNGCNGYYTTCATYTG); or CWC-104 (5'; TGGTGYATHGANAAYGG) and the poly-T primer (3'; above), respectively. Primers CWC-104 and CWC-106 were modified slightly from Langeveldt et al. (1991) and Pappu et al. (1993). For the 5' partial clone, the annealing temperature in the PCR reaction was changed from 42°C to 46°C.

Each reaction product was purified using GeneClean II (BIO 101 Inc., Vista, CA), ligated into a pCR II vector and transformed into competent *E. coli* using a TA Cloning Kit (Invitrogen, San Diego, CA). Clones were screened for inserts of the correct size on agarose gels following *EcoRI* digestion of plasmid DNA purified by an alkaline lysis procedure. The cloned AzMV cDNA was sequenced using a Sequenase 2.0 DNA Sequencing Kit (U.S. Biochemical, Cleveland, OH) and 5% Long Ranger sequencing gels (AT Biochem) according to the manufacturer's directions.

The sequence of the coding region of the CP (855 nucleotides) and the 3' UTR (256 nucleotides excluding the poly-A tail), as determined from a full-length clone of the region, is presented in Figure 1, along with the deduced 285 amino acids of the AzMV CP. Two noteworthy features of the N-terminal region of the CP are the presence of the DAG sequence associated with aphid transmissibility and the fact that the AzMV CP is two amino acids shorter than BCMV CPs (e.g., BCMV-NY-15) in this region. Partial clones made a year earlier of the 5' or 3' region of AzMV as described above contained the following nucleotide substitutions, respectively: C to T at position #675; A to G at #575, C to T at #675, G to A at #708, C to T at #731, C to T at #1030.

The AzMV CP sequence and the 3' UTR nucleotide sequence were compared with sequences of 14 other potyviruses, selected from available databases as representative of either closely related potyviruses, legume-infecting potyviruses, or other potyviruses that had been tested for the response(s) elicited on bean cultivars possessing the *I* gene for resistance to BCMV (Fisher and Kyle 1994). In an attempt to apply Shukla and Ward's (1988) criteria for distinguishing strains of the same virus from distinct viruses, the pairwise percent identity of CP amino acids and of the 3' UTR nucleotides between each of the viruses was determined (Table 1) using the GCG Distances program from the University of Wisconsin Genetics Computer Group (Devereux et al. 1984). Phylogenetic (evolutionary) relationships among the 15 potyviruses were examined by parsimony analysis of aligned CP amino acid sequences using the Phylogenetic Analysis Using Parsimony (PAUP) software package (Swofford 1991; see Fig. 2).

The pairwise comparisons of the CP sequence data of the related potyviruses (Table 1) show that AzMV is most closely related to DeMV, with 94% coat protein identity consistent with the view that these isolates are strains of the same virus. Further, the phylogenetic tree generated from the CP amino acid sequence data (Fig. 2) clearly shows a close relationship between AzMV and DeMV and their more distant relationship to the other members of the newly defined BCMV species (including BCMV NY-15, BCMV NL-1, BCMV US-1, BICMV, BCMV NL-4, and PStV). When the first four of these latter isolates are considered in pairwise comparisons of sequence identity, the values are greater than 90%. When BCMV NL-4 and PStV are added to the group, values drop to 89% or 88%, respectively. When AzMV is included, percent identities range from as low as 87% (with BCMV NL-1) to 90% (with BCMV US-1), somewhat below Ward and Shukla's cutoff point for strains of the same virus (Fig. 3 of

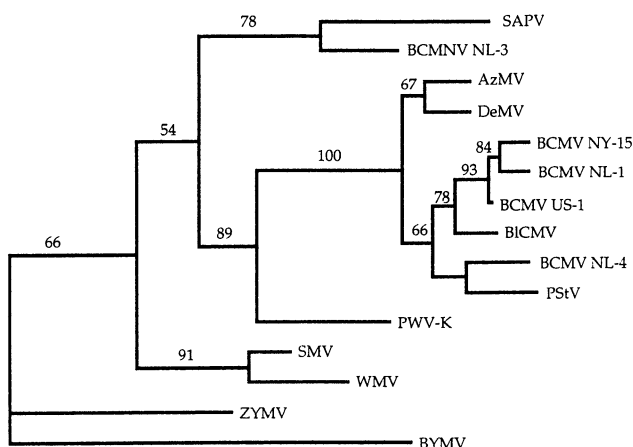


Fig. 2. Single most parsimonious tree of selected (see Table 1) potyviral coat protein amino acid sequences constructed using the PAUP software package version 3.0r (497 steps, CI = 0.829, HI = 0.171). One hundred replications of the heuristic search procedure were performed with random stepwise addition. The tree was rooted with BYMV. Numbers above the nodes represent results of 100 bootstrap replications; branches without numbers were inconsistent with the bootstrap majority rule tree. Branch lengths are proportional to the number of substitutions that occur along the branches.

Mink et al. 1994). When only the amino acids of the variable N-terminus of the CPs are compared (e.g., the first 50 amino acids of AzMV CP), the percent identity drops substantially (e.g., 65% identity for AzMV and BCMV US-1; data not shown). The 91 to 94% identities between the 3' UTRs of these seven isolates (Table 1), however, support the idea that they all could be considered strains of BCMV.

We have noted previously a correlation between relationships among legume potyviruses based on CP sequence and similarity of host responses on various genotypes of *P. vulgaris* (Fisher and Kyle 1994, 1996). All viruses that group by CP sequence with the newly defined BCMV species (including AzMV) and that have been tested carefully on bean cultivars with and without the I gene (e.g., 'BT-1' and 'BT-2') show a temperature-sensitive resistance/systemic necrosis response (i.e., AzMV, BICMV, and BCMV strains NY-15, NL-1, US-1, NL-4). In contrast, potyviruses that group with the newly defined BCMNV species (e.g., BCMNV NL-3, formerly designated BCMV NL-3) cause temperature-insensitive necrosis on BT-1 bean. Even when only the variable N-terminus of the CP of the nine BCMV subgroup viruses included on Figure 2 is used, the resulting tree is almost identical (data not shown). The availability of appropriate infectious clones will allow a direct examination of the basis for this correlation between CP sequence and host response.

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