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NUCLEOTIDE SEQUENCE OF THE CAPSID PROTEIN GENE OF BEAN YELLOW MOSAIC VIRUS CHLOROTIC SPOT STRAIN

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Introduction

The potyviruses including bean yellow mosaic virus (BYMV) have a flexuous thread particle of about 750 nm in lengths.²¹⁾ The virion contains a single component of (+)sense single-stranded RNA genome of approximately 10K nucleotides.^{3,4,6,27)} These RNAs are thought to be covalently linked to a small protein (VPg)^{16,33)} at the 5' terminal nucleotide and have a polyadenylated track at the 3' terminus.^{16,18)}

So far, seven virus-encoded polypeptides were found to be associated with potyviral infections: the coat protein (CP), helper component (HC), VPg, cylindrical inclusion (CI), cell to cell transfer function protein (28-34K) and two nuclear inclusion proteins (NIa and NIb). The cistrons for each of these genes have been mapped to the potyviral genomes.^{3,5,6,9,18,27)} Genomes of potyviruses are expressed through the production of a large polyprotein which is proteolytically cleaved in a similar manner to that of picorna-, toga- and comoviruses.^{11,17,40,45)}

These studies together with the sequence analyses of the 3' terminal region of some potyviruses revealed that the predicted coat protein cistron was located upstream from the polyadenylated 3' terminus of the genome.^{1,2,3,6,8,12,14,25,27,41)}

Amino acid sequence of the coat protein is an increasingly important taxonomic criterium of the potyvirus as the sequence informations are accumulating and comparison of major potyviruses and their strains become available. It has been pointed out that N-terminus of the amino acid sequence is specific for each potyvirus.^{20,36,37,38)}

In this paper, we report the nucleotide sequence of the 3' terminal region of the BYMV-CS. Coat protein gene is mapped in the deduced sequence and its amino acid sequence was determined.

Materials and Methods

Virus and Viral RNA extraction. BYMV-CS was originally isolated from

red clover²³⁾ and maintained in broad bean (*Vicia fava* cv. Wase-Soramame). The virion was purified from infected broad bean leaves by a procedure described previously.³⁹⁾ Genomic RNA from purified BYMV-CS was prepared by a method similar to that described previously.^{4,7,17)} To a 2 mg/400 μ l suspension of the virus was added an equal volume of 200 mM ammonium carbonate (pH 9.0) containing 2 mM EDTA, 2% SDS, 200 μ g/ml bentonite and 10 μ g/ml Proteinase K. After incubation for 20 min at room temperature, the mixture was layered (1 ml/tube) onto a linear-log 7.5%-33.4% (w/v) sucrose gradient containing 150 mM NaCl, 15 mM sodium citrate (pH 7.0). Gradients were centrifuged for 9 hr at 24,000 rpm in a Hitachi RPS-27 rotor. Viral RNA, which sedimented as a single sharp zone, was recovered by precipitation with 2.5 volumes of ethanol, and used for cDNA synthesis.

Complementary DNA synthesis. The first strand of complementary DNA was synthesized¹³⁾ in a reaction mixture containing 50 mM tris-HCl (pH 7.9 at 42°C), 10 mM MgCl₂, 30 mM KCl, 1 mM DTT, 1 mM each of dATP, dCTP, dGTP, dTTP, 80 μ g/ml of oligo (dT₁₂₋₁₈), 50 μ g/ml actinomycin D, 100 μ Ci/ml of [α -³²P] dCTP, 1600 U/ml reverse transcriptase (BRL) and 200 μ g/ml of viral RNA and incubated at 42°C for 40 min. After phenol-chloroform extraction and ethanol precipitation, the first strand cDNA was converted to the double stranded cDNA in a reaction mixture containing 100 μ Ci/ml [³H] dCTP, 20 mM tris-HCl (pH 7.4), 5 mM MgCl₂, 10 mM ammonium sulfate, 100 mM KCl, 15 μ M β -NAD, 50 μ g/ml BSA, 10 μ M dNTPs, 4.3 U/ml RNase H (Takara shuzo Co.), 115 U/ml *E. coli* DNA polymerase I (Takara shuzo Co.), 6 U/ml *E. coli* DNA ligase (BRL). The mixture was incubated sequentially for 1 hr at 12°C and for 1 hr at 22°C. In a second experiment, cDNA was synthesized using a cDNA Synthesis System Plus purchased from Amersham Corp. After chromatography on Sephadex G-50, the double stranded cDNA was tailed with dCTP using terminal deoxynucleotidyl transferase (Takara shuzo Co.) and annealed with *Pst* I cut dG-tailed pBR 322 (BRL) and recombinant cDNAs were used to transform *Escherichia coli* HB 101 cells made competent by CaCl₂.²⁸⁾

Southern blot hybridization. Recombinant DNAs were screened for cDNA inserts by a southern blot hybridization.³⁸⁾ Viral RNA in 5 mM MgCl₂ and 25 mM glycine-NaOH (pH 9.0), was incubated at 37°C for 3 hr³²⁾, and precipitated by ethanol. Magnesium cleaved viral RNA was labeled using [γ -³²P] ATP and T4 polynucleotide kinase (Takara shuzo Co.) and used as a probe.

cDNA sequence determination. Insert cDNAs digested with *Pst* I were ligated into the polylinker region of the replicative form of the bacteriophage M13mp19 and it was used to transform *E. coli* JM109 made competent by RuCl₂.^{15,19)} An ordered set of deleted clones was made by exonuclease-III attacking method¹⁹⁾ and sequenced by dideoxy chain termination method using a sequencing kit (Takara shuzo Co.).

Sequences were compiled and analysed using a NEC 9801 XL computer and a software programmed by GENETYX (SDC Software Development Co.).

Characterization and amino acid sequencing of the capsid protein. Immediately after the purification, 1 mg of purified virus was resuspended in 2 ml of 50 mM sodium borate buffer at pH 8.0, and incubated with 2 μ g of trypsin (Sequence grade, Boehringer Mannheim) for 30 min at room temperature.^{2,36} The trypsin-resistant particles which contain core subunits was collected by centrifugation at 40,000 rpm for 2 hr at 4°C in Hitachi RP-65 rotor. The final pellet was resuspended in double distilled water. SDS-PAGE of BYMV-CS intact or core capsid proteins was carried out in 12.5% polyacrylamide gel electrophoresis system of LAEMMLI.²⁴ Amino acid composition of BYMV-CS intact coat protein was determined by using hydrolysate treated in 6N HCl at 110°C for 24 hr, and the amino acid analysis was performed by a Hitachi-835 analyzer.²⁶ The N-terminal sequence of intact or trypsin treated virion were performed by a sequential Edman degradation method in 477A Protein Sequencer and 120A PTH Analyzer (Applied Biosystems).

Results

Recombinant DNA Molecules Containing BYMV-CS

About four hundreds of recombinant DNA plasmids containing cDNA inserts derived from BYMV-CS RNA were identified. The cDNA inserts of those plasmids ranged in size from 500 to 2,200 base pairs (bp), and were analyzed by

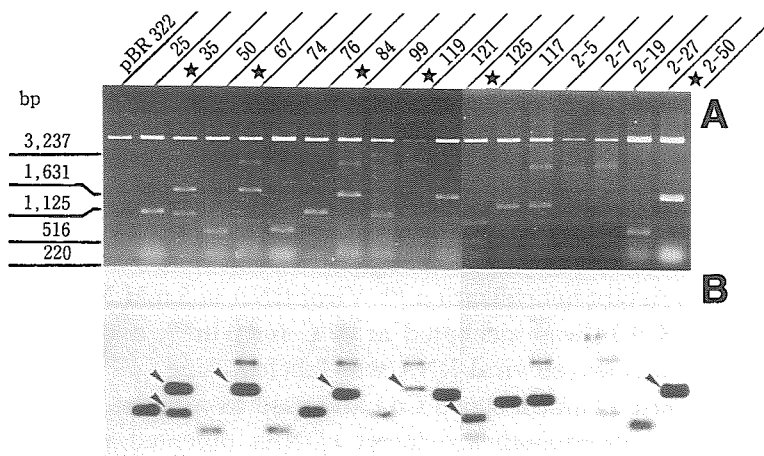


Fig. 1. Restriction analysis and southern blots of DNA from the clones. Plasmid DNAs containing cDNA inserts were restricted with *Pst*I and electrophoresed in 1.5% agarose gel (A). DNAs were transferred onto nitrocellulose, hybridized with ³²P labeled genomic RNA and autoradiographed (B).

Southern blot hybridization with 5' ^{32}P -labelled genomic RNA probe as shown in Fig. 1. In this study, we selected 6 recombinant cDNA clones which covered 3' terminal portion (pCS35, pCS67, pCS84, pCS119, pCS125, pCS2-50,) of the genome RNA. The relationships between the cDNA inserts and the BYMV-CS genomic RNA are presented in Fig. 2, along with a strategy used to determine the nucleotide sequence (Fig. 3). Since these clones did not contain the 3' terminus as they lacked the poly A sequence up stream of the oligo dC tail, the second cDNA synthesis was performed using a kit purchased from Amersham Corp. The plasmid pCS201 was found to contain the poly A sequence.

DNA Sequencing

The 1,392 nucleotides sequence determined from pCS119 and pCS201 was presented in Fig. 4. The DNA sequence contains one large open reading frame of 1,218 nucleotides which is terminated by a single TAG codon (Fig. 4). The

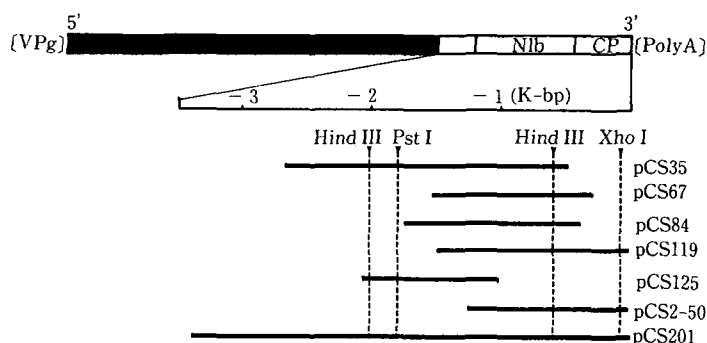


Fig. 2. The physical maps and assignment of complementary DNA recombinant molecules to BYMV-CS genomic RNA.

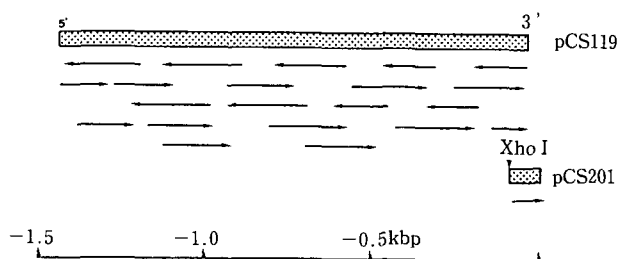


Fig. 3. The nucleotide sequencing strategy of 3' terminal region of BYMV-CS. The horizontal arrows indicated the direction and extent of sequence determination. Since pCS119 did not contained poly A sequence, poly A and upstream sequences were determined using a fragment of pCS201 digested with *Pst*I and *Xho*I.

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-406 Lys Glu Lys Gly Asp Leu Trp Phe Met Ser His Arg Gly Ile Gln Val Asn Gly Met Trp
-1392 AAA GAG AAG GGT GAC TTG TGG TTT ATG TCA CAT AGA GGG ATA CAA GTG AAT GGT ATG TGG

-386 Ile Pro Lys Leu Glu Glu Glu Arg Ile Val Ser Ile Leu Glu Trp Asp Arg Ala Leu Gln
-1332 ATT CCA AAG CTG GAG GAA GAG CGA ATT GTA TCA ATT CTT GAA TGG GAT AGA GCC CTC CAA

-366 Pro Glu His Arg Leu Glu Ala Ile Cys Ala Ser Met Ile Glu Ala Trp Gly Tyr Pro Glu
-1272 CCA GAA CAC AGG CTT GAA GCA ATT TGT GCA TCC ATG ATA GAA GCA TGG GGT TAC CCT GAA

-346 Leu Leu Asn His Ile Arg Lys Phe Tyr Leu Trp Val Leu Gly Gln Ala Pro Tyr Ser Gln
-1212 TTA CTG AAC CAT ATC CGT AAA TTC TAC CTC TGG GTC TTG GGT CAG GCA CCA TAC AGT CAA

-326 Leu Ser Ala Glu Gly Lys Ala Pro Tyr Ile Ser Glu Val Ala Leu Lys His Leu Tyr Thr
-1152 CTC AGT GCT GAA GGA AAA GCA CCA TAC ATA TCA GAG GTT GCC CTC AAG CAT TTG TAC ACA

-306 Glu Glu Lys Ile Thr Pro Ala Glu Leu Glu Arg Tyr Asn Val Ala Leu Val Asp Cys Ala
-1092 GAA GAA AAG ATC ACA CCG GCA GAG CTT GAA AGG TAC AAC GTA GCC CTA GTT GAT TGT GCG

-286 Glu Pro Glu Glu Asp Glu Val Ile Leu Cys Cys Phe Gln Ser Asp Gln Glu Lys Leu Asn
-1032 GAG CCA GAA GAA GAT GAA GTA ATT CTG TGC TGT TTC CAA TCA GAC CAA GAG AAA CTC AAT

-266 Ala Ser Glu Lys Lys Lys Asp Lys Asp Lys Lys Val Glu Asp Gln Ser Thr Lys Glu Ser
-972 GCT AGT GAA AAG AAG AAG GAT AAG GAC AAG AAG GTT GAA GAT CAA TCA ACC AAA GAA AGT

-246 Glu Gly Gln Ser Ser Lys Gln Ile Ile Pro Asp Arg Asp Val Asn Ala Gly Thr Thr Gly
-912 GAG GGG CAG AGT AGC AAA CAA ATC ATA CCT GAT CGG GAC GTG AAT GCA GGA ACA ACT GGA

-226 Thr Phe Ser Val Pro Arg Leu Lys Lys Ile Ala Gly Lys Leu His Ile Pro Lys Val Asn
-852 ACT TTC TCA GTC CCA AGA CTC AAG AAG ATA GCA GGA GGG CTC CAC ATT CCG AAG GTG AAT

-206 Gly Lys Ile Val Leu Asn Leu Asp His Leu Leu Glu Tyr Asn Pro Ser Gln Asp Asp Ile
-792 GGA AAG ATT GTA CTA AAC CTG GAC CAC TTG TTG GAG TAC AAT CCG TCA CAG GAT GAT ATC

-186 Ser Asn Thr Ile Ala Thr Asp Glu Gln Phe Lys Ala Trp Tyr Asn Gly Val Lys Gln Ala
-732 TCA AAT ACC ATT GCA ACT GAT GAA CAA TTT AAA GCA TGG TAC AAT GGA GTG AAG CAG GCT

-166 Tyr Glu Val Glu Asp Ser Gln Met Ser Ile Ile Leu Asn Gly Leu Met Val Tyr Cys Ile
-672 TAT GAA GTT GAA AGT TCA CAG ATG AGC ATT ATT TTG AAT GGG TTG ATG TGA TGG TGT ATC

-146 Glu Asn Gly Thr Ser Gly Asp Leu Gln Gly Glu Trp Thr Met Met Asp Gly Asp Glu Gln
-612 GAA AAT GGA ACA TCA GGT GAC TTA CAA GGT GAG TGG ACC ATG ATG GAT GGT GAT GAA CAA

-126 Val Thr Tyr Pro Leu Lys Pro Ile Leu Asp Asn Ala Lys Pro Thr Phe Arg Gln Ile Met
-552 GTG ACA TAC CCC CTG AAA CCC ATC TTA GAT AAT GCA AAG CCA ACA TTT CGC CAG ATA ATG

-106 Ser His Phe Ser Gln Val Ala Glu Ala Tyr Ile Glu Lys Arg Asn Ala Thr Glu Arg Tyr
-492 TCG CAC TTT TCA CAG GTT GCT GAA GCT TCT ATA GAG AAG AGG AAT GCA ACT GAG AGG TAT

-86 Met Pro Arg Tyr Gly Leu Gln Arg Asn Leu Thr Asp Thr Gly Leu Ala Arg Tyr Ala Phe
-432 ATG CCG CGT TAT GGC CTC CAG AGG AAC TTA ACT GAC TAT GGT TTG GCT AGA TGT GCT TTT

-66 Asp Phe Tyr Arg Leu Thr Ser Arg Thr Pro Val Arg Ala Arg Glu Ala His Met Gln Met
-372 GAT TTC TAC AGG CTA ACT TCG AGA ACT CCT GTG CGT GCT AGG GAA GCA CAT ATG CAG ATG

-46 Lys Ala Ala Ala Ile Arg Gly Lys Ser Asn Arg Leu Phe Gly Leu Asp Gly Asn Val Gly
-312 AAG GCA GCA GCA ATT AGA GGC AAG TCA AAC CGA TTA TTT GGT CTT GAT GGA AAT GTT GGA

-26 Thr Asp Glu Glu Asn Thr Glu Arg His Thr Ala Gly Asp Val Asn Arg Asp Met His Thr
-252 ACA GAC GAG GAG AAC ACA GAA AGA CAC ACA GCA GGA GAT GTC AAT CGT GAT ATG CAC ACC

-6 Met Leu Gly Val Arg Ile ***
-192 ATG CTT GGT GTT CGT ATT TAG A GTATCCGTCT ATAAATTCTC TGAAATTGG CGTTACATTA CTAA

-125 TACTA TGATTAGCG AGGTTTACC TCCAGCATTT TAAATTCAGT AAGTGTTTCA TTCTCTCTAC TCTGACA

-53 GGG TAAGCTGTTA GTGAGGTTTC CTCGAGTGGG CCTGATCTTT GTAGAGCGAC PolyA

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Fig. 4. The cDNA sequence of BYMV-CS as the genomic RNA sense. The predicted amino acid sequence of the large open reading frame is presented in the standard three letter code above the nucleotide sequence. The cleavage site is indicated by black arrow.

polypeptide encoded for is 406 residues long (Fig. 4.). The other reading frames contain numerous stop codons.

Coat protein analysis

BYMV-CS coat protein has a molecular weight of about 30,808 which was estimated from amino acid composition analysis (Table 1). This value is in good agreement with that estimated from SDS-PAGE analysis (Fig. 5). An attempt to

TABLE 1. Amino Acid Composition of the BYMV-CS Coat Protein.

| Amino acid residue | BYMV-CS | |
|--------------------|-------------------|-----------------------|
| | Predicted NA Seq. | Determined chemically |
| Met | 10 | 9 |
| Asx | 37 | 36 |
| Thr | 18 | 17 |
| Ser | 16 | 15 |
| Glx | 33 | 34 |
| Gly | 19 | 21 |
| Ala | 18 | 19 |
| Cys | 1 | 1 |
| Val | 14 | 14 |
| Ile | 15 | 13 |
| Leu | 19 | 20 |
| Tyr | 10 | 10 |
| Phe | 7 | 7 |
| Lys | 21 | 21 |
| His | 6 | 6 |
| Arg | 17 | 17 |
| Pro | 9 | 10 |
| Trp | 3 | N |
| mol. wt. | 30,998 | 30,808 |

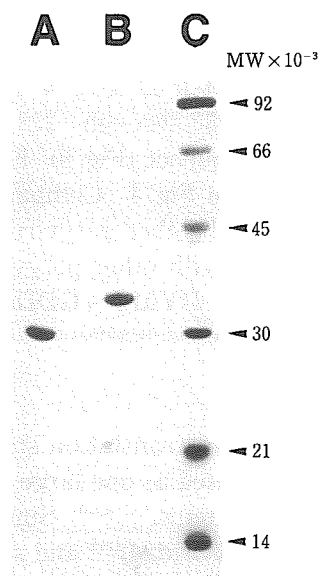


Fig. 5. Polyacrylamide gel electrophoresis of intact(B) and trypsin treated(A) coat protein of BYMV-CS. Numbers on the right are Mw of marker proteins(C)

determine the amino acid sequence of the N-terminal portion of purified BYMV-CS coat protein was unsuccessful, presumably due to a blocked N-terminus. Similar situations are also encountered with those of TEV-HAT, TVMV and PVY.^{2,6,35).}

But amino acid sequence of these viruses were obtained after removing the N-terminal about 20 to 30 amino acid residues by partial digestion with trypsin. When purified BYMV-CS was likewise treated with trypsin, the intact 32K coat protein resulted in the generation of a "trypsin resistant core" with an estimated molecular weight of 29K as determined by SDS-PAGE (Fig. 5). The sequence of

Fig. 6. Comparison between the N-terminal portion of the amino acid sequence predicted from the nucleotide sequence and that determined by a direct chemical method. The amino acid sequence of the core casid protein determined by the direct chemical method was boxed.

Cell free translation studies of some potyviruses indicated that the genome was generated as one large protein which was subsequently processed to give the mature proteins.^{5,7,18,40). Simmilar gene expression mechanism was demonstrated for picornaviridae, ^{22). and subsequently been found for cowpea mosaic virus^{42). or other plant viruses.^{43). It was shown that the enzymes involved were cystein like proteinase and catalysis occured on the carbonyl side of a glutaminy residue. In potyviruses, the protein cleavages occured on Gln-Gly, Gln-Ser or Gln-Ala^{1,2,3,6,8,12,27,31). and amino acids with "bulky" side chains are found adjacent to the glutamine residue and these amino acids are tyrosine, phenylalanine or histidine of aromatic amino acids.^{1). In our amino acid sequence predicted from the nucleotide sequence, these glutamine-X dipeptide were found at five positions. The molecular weight of the BYMV-CS protein was estimated to be 32K by polyacrylamide gel electrophoresis. The value suggests that the probable cleavage site is between the glutamine and serine residues found at amino acid position -273 to -272. This would result in a 30,998 molecular weight capsid protein molecule, which is in good agreement with the molecular size of 30,808 estimated by amino acid composition analysis and also 32K estimated by polyacrylamide gel electrophoresis. In addition, chemical sequencing of the protein digested with trypsin yielded amino terminal protein of ten amino acids which was identical to amino acid residues -237 to -228 of that deduced from the nucleotide sequence. This ten amino acid sequence was not found at any other locations in the putative}}}}}}

polyprotein. Tryptic cleavage after the lysine residue at -238 of the predicted coat protein would yield a trypsin resistant core protein with molecular weight of 27,479 similar to that estimated from polyacrylamide gel electrophoresis. Thus, we concluded that coat protein is produced by proteolytic cleavage of BYMV-CS polyprotein between the glutamyl residue at amino acid position -274 and the following seryl residue. The same dipeptide was apparently cleaved in TEV-HAT and TVMV. Alignment of amino acid sequences showed that BYMV-CS coat protein has significant homologies with other potyvirus coat proteins. The inner portion of amino acid sequences have higher homologies than that of N- and C-terminal sequences between different viruses (Fig. 7). Comparison of the amino acid sequence between BYMV-CS and BYMV-GDD showed that extensive homology existed even in N- and C-terminal regions. These results were also consistent with the notion that immunological specificities of potyviruses were located in N- and C-terminal arms arranged on the surface of virus particle as proposed previously.^{20,36,37,38).}

Nucleotide sequence of the 3'noncoding sequence of BYMV-CS has no homology to that of other potyviruses sequenced to date and no polyadenylation signals were found. However significant homology in the 3'noncoding region between BYMV-CS and BYMV-GDD^{14).} was found.

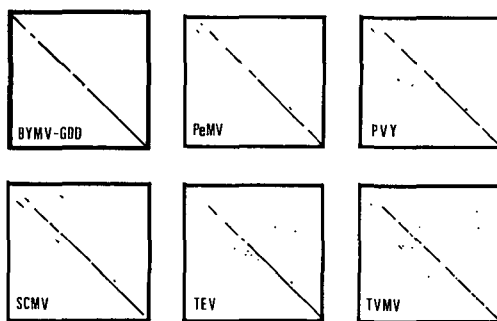


Fig. 7. Harr Plot analysis of six potyvirus(vertical) coat proteins with BYMV-CS(horizontal). The dot-plots were made using a window of 10 and stringency of 0.7.

Summary

The sequence of 1,392 nucleotides of the 3' region of the bean yellow mosaic virus chlorotic spot strain (BYMV-CS) RNA has been determined. There is a single open reading frame (ORF), from the 5' end, of 1,218 nucleotides upstream from a 173 nucleotides non-coding region that is polyadenylated. The ORF encodes a polypeptide of 406 amino acids. The cistron of the genome encoding a

potyviral capsid protein is adjacent to the 3'non-coading region. The chemically determined amino acid composition of the coat protein was similar to that predicted from the nucleotide sequence. The sequence of the 10 amino terminal amino acids of the BYMV-CS coat protein was determined chemically after removing blocked amino terminal oligo-peptides. Our data suggests that the BYMV-CS coat protein, like that of other potyviruses, is a product of the maturation of a large polyprotein. The putative cleavage site is at a glutamine-serine dipeptide. The capsid protein gene consists of 819 nucleotides and corresponds to a region coding 273 amino acids which have a calculated molecular weight (Mw.) of 30,998. The amino acid sequence of BYMV-CS coat protein is 53 to 64% homologous to other potyvirus coat proteins and most of the variations are found in the amino terminal region. And it was 92% identical to coat protein gene of BYMV-GDD.

Key words: BYMV, Capsid protein, Nucleotide Sequence.

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