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Nucleotide and Amino Acid Sequences Comparison between Two Strains of *Barley Stripe Mosaic Hordeivirus* (BSMV)

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Nucleic acid sequence comparison between the Egyptian strain (G119) and the American strain (ND18) of *Barley Stripe Mosaic Virus* (BSMV) revealed a high degree of sequence similarity of γ RNAs, comparing to βRNAs of both strains. On the other hand nucleotide and amino acid sequences of the two strains revealed differences in the 5' untranslated region (UTR) between the coat protein and βb gene. It was clear that, five bases, and 18 out of 116 amino acids were found different, three amino acids insertion, and one amino acid deletion. Little differences were also observed regarding the 5' UTR of RNA γa, as six nucleotides were changed. 12 out 162 amino acids were differed and one amino acid deletion. G119 strain was cross reacted specifically with the monoclonal antiserum raised against the coat protein of ND18 stain and the two coat proteins were indistinguishable in their sizes

INTRODUCTION

Barley Stripe Mosaic Hordeivirus (BSMV) is one of the most dangerous viruses affecting barley and wheat all over the world. The importance of this virus comes from its seed transmissibility in extremely high percentage (Brunt *et al.*, 1996, Sutic *et al.*, 1999, and Zein, Salwa, 2002). BSMV is a positive sense-stranded RNA virus composed of three genomeα,β, and γ that encode a total of eight proteins (Donald *et al.*, 1997). BSMV has an attractive system for dissecting components, which contribute to common mechanisms of viral replication and movement. In addition to the ease with which biologically active recombinant DNA clones can be isolated from different strains of BSMV (Donald *et al.*, 1993). Recently, distinction among related viruses and related strains based on nucleotide sequence analysis of viral coat protein genes provide more critical examination of intravirus, interogroup, and intergroup variations than previously had been possible (Solovyev *et al.*, 1999). In the present study PCR technique was applied to differentiate between the Egyptian strain G119 and the American strain ND18 of BSMV. The sequence analysis of portions of the genome of strain G119 was compared with the corresponding of ND18 strain. Moreover the SDS-PAGE eluted protein of G119 was reacted specifically with the monoclonal antibodies raised against ND18 using western blotting technique. The molecular weight of the coat protein of the Egyptian strain (G119) was identical to that of the American strain (ND18), it was 22KDa.

The *barley stripe mosaic virus* (BSMV) genome

MATERIALS AND METHODS

Virus isolates

The Egyptian strain (G119) of BSMV previously isolated and identified by Zein Salwa (2002) was propagated on barley plants cv. G119. The American strain (ND18) provided by Jackson's laboratory at the Univ. Of California, Berkeley, USA was propagated on barley plants cv. Black hulless. The infected plants were kept under greenhouse conditions (27-30 °C). Barley leaf seedlings of cv. G119 emerging from infected seeds were used for western blotting assay.

Oligonucleotide primers

In the present work four primers kindly provided by Jackson were used as shown in Table (1).

Nucleic acid extraction and reverse transcription/polymerase chain reaction (RT/PCR)

The total nucleic acids were extracted from barley leaves infected by the Egyptian strain (G119) and other infected by the American strain (ND18) following the method described by Halpern and Hillman (1996). The reverse transcription reaction (RT) was performed in 20 µl total volume using the Thermoscript RT-PCR reaction Kit (Gibco-BRL). 50 ng of random primer was incubated with 10 pg-5 µg of total nucleic acid and /or ND18 viral RNA (provided by Jackson's laboratory) at 65°C for 5 min and the mixture was placed on ice. Ten µl of the reverse transcription reaction mixture contained 0.01 M DTT, 400 U/µl RNase OUT, 2 mM dNTPs mix, and 15 U/µl Thermoscript RT enzyme was added. The reaction mixture was transferred to a thermal cycler preheated to the following conditions: 25°C for 10 min, followed by 20-50 min at 50°C, then the RT-reaction was terminated by incubation at 85°C for 5 min (Donald *et al.*, 1997). Four primers were used in the present study 10 ng/µl of each of oligoncleotide 492 base pair fragment of the β genomic the upstream primer –660 downstream primer bb-4 as shown in (Table 1) and base pair of oligonucleotide 559 bp fragment of the γ genomic upstream primer 5' gamma and downstream +557 as shown in (Table 1). The reaction mixture was incubated at 95°C for 1 min, annealing at 48°C for 1 min and extension 73°C for 3 min for 25 cycles. The PCR products were electrophoresed in agarose gel. Ten µl of the reaction mixture containing 8 µl of the PCR product for βb+UTR for genomicβ and γa+UTR for genomic γ and 2 µl of loading dye were analyzed on 1% agarose gel dissolved in 1X TAE buffer (Sambrook *et al.*, 1989 and El-Banna, Om-Hashem *et al.*, 2000) in the presence of 0.5 mg/ml ethidium bromide and photographed with MP4 Polaroid camera under UV transilluminator (wavelength 245 nm). The size of the PCR products was determined according to the molecular weight of DNA markers 1 Kilo base.

Table (1): The nucleotide sequences of the four primers are illustrated.

Primer	Nucleotide sequence
(1) –660	5' TCATTTCCAGATGCCC 3'
(2) bb-4	5' CGCCTTTAATCATTGG 3'
(3) 5'gamma	5' GTATTAGCTTGAGCATT 3'
(4) +557	5' GAACCTTTACTTTGAG 3'

Cloning

1-Ligation:

The amplified PCR products of β and γ genomes were cloned directly

into TOPO-TA cloning kit (Invitrogen company pamphlet). The PCR products TOPO is linearized with 3' overhang of (T) residues, this allows the PCR inserts to ligate efficiently with the vector. The ligation reaction was performed in 5 µl total volume reaction, 10 ng of PCR products and 10 ng/µl of TOPO vector. The ligation reaction was incubated for 5 min at room temperature and then placed on ice until one shot transformation reaction starts. TOPO™ cloning provides a highly efficient, 5 min, one step cloning strategy for the direct insertion of Taq polymerase-amplified PCR product into a plasmid vector.

TOPO™ cloning exploits the ligation activity of topoisomerase by providing an activated, linearized TA vector using proprietary technology (Shuman, 1994).

2- Bacterial transformation

Two µl of the ligation reaction was carefully transferred into .50µl competent *E. coli* bacteria, strain DH5α into tube. The tube was shaken gently and placed on ice for 30 min. The mixture was heat-shocked for 2 minutes in a water bath at 37°C. After adding 500 µl LB medium at room temperature, the mixture was incubated for 1h at 37 °C with shaking. 50µl aliquots of the transformation culture and 40 µl X-gal were plated together onto duplicate LB-medium plates. The plates were incubated overnight at 37 °C. White colonies containing recombinant plasmid were tested by restriction analysis of mini-preps.

3-Isolation of recombinant plasmid DNA preparation

Single white colony was inoculated separately into LB-medium with ampicillin (80mg/ml) overnight, at 37°C in shaking water bath. Recombination plasmids were extracted using alkaline lysis mini preparation procedure (Maniatis *et al.*, 1982).

4-Restriction analysis of minipreparation of plasmid DNA (digestion)

Mini preparations were analyzed by digestion with restriction enzyme and gel electrophoresis (Maniatis *et al.*, 1982). Restriction enzymes used for recombinant plasmid digestion for each clone is listed in table (2). The digestion reactions were heated at 37 °C for 1h, then terminated by addition 3 µl loading buffer and electrophoreses in agarose gel in TAE buffer. The minipreparation was used for kit sequencing.

Table (2): Restriction enzyme used for recombinant plasmid digestion in minipreparation

Clone	Vector	Restriction enzyme
(βb+UTR) for genomicβ	TOPO-TA	EcoR1, NocI
(γa+UTR) for genomic γ	TOPO-TA	EcoR1, EcoR1

DNA Sequencing

Automated DNA sequencing reactions were performed according to Rubio *et al.* (1999) using the M13 reverse primer. Sequencing reactions contained 3µl sequencing mix (Big Dye Terminator Cycle Sequencing Ready reaction Kit in conjunction with the ABI PRISM TM 337 Genetic Analyzer from PEPKIN- ELMER (Applied Biosystem Division Foster City 6, CA, USA), 3 µl sequencing buffer, 0.7 µl 5 pmol/µl primer, and 1 µl DNA. Sequencing reactions were

placed in a thermal cycler for 25 cycles as follows: 95°C for 30 sec, 50°C for 15 sec, and 60°C for 4 min. Reactions were precipitated with 5 µl 3 M sodium acetate pH 5.2, 50 µl 100% ethanol, and incubated at room temperature for 15 min. Samples were centrifuged at 14,000 rpm for 20 min, rinsed with 7% ethanol and dried. The sequencing reactions were analyzed on a 5% sequencing gel and compared the sequence between the RNA of the Egyptian strain (G119) and the American strain (ND18).

Western blotting for protein analysis

Ten barley grain cv G119 were separately planted and the leaf tissues were homogenized during 10-14 days after planting according to the method of Donald *et al.* (1993). Samples were centrifuged at 14,000 rpm for 3 min and the supernatants were separated using a polyacrylamide gel electrophoresis (SDS-PAGE) composed of 10% resolving gel and 5% stacking gel. The running parameters were 70V/U using Biometera vertical cell. Proteins were transferred onto nitrocellulose membrane using transfer buffer at 100V/1h: 30min, the membranes were blocked overnight in 3% (w/v) milk powder in TBS buffer at 4°C. Followed by washing in TBS 3 times 5 min each. The membranes were probed with an monoclonal antibody (provided by Jackson's lab) diluted (1:3000) raised in mouse against the BSMV coat protein then washed 3 times 5 min each. The membranes were probed using a secondary antibody, goat antimouse conjugate with horseradish peroxidase (1:7000) and developed using West Dura extended (Pierce) and photographed by X- Ray film Radiogaphique Fujimedical. The size of the protein was determined according to the molecular weight of protein ladder Prestained Protein Ladder (molecular weight ranging from 10-200 KD) using SDS-PAGE.

RESULTS AND DISCUSSION

Nucleic acid extraction and reverse transcription polymerase chain reaction (RT-PCR)

RNA extracted from barley plants infected with the Egyptian strain of BSMV and others infected with the American strain ND18 were reverse transcribed using random primers followed by polymerase chain reaction (PCR) which amplified BSMV cDNAs. No PCR products were obtained when cDNA generated from uninfected barley plants using the same procedures (Fig.1). The results show that the two strains have approximately 492, 559 each when compared with 1kb DNA ladder that used as a control. Similar results had reported by Donald *et al.* (1997). On the other hand UTR, βb gene didn't gave good band in order to appear obviously because the primer designed to ND18 strain which, confirmed when we performed sequence to this gene which indicated that there were differences in nucleotides between them.

Cloning and sequence comparison between the two strains

The PCR products amplified fromβ, and γ cDNAs of the G119 and ND18 strains were cloned and sequenced. The sequence of the Egyptian strain (G119) was compared with the American strain (ND18). The sequences of two strains were highlighted (Fig 2 and 3). In particular, when the the untranslated region between the coat protein and βb gene of the two isolates were aligmed. Data presented in (Fig 4) show five bases that differed. Moreover, amino acid sequence analysis for the two isolates

in the same region revealed 18 out of 116 amino acid were different, three amino acids insertion and one amino acid deletion (Fig. 5). In the case of the 5' untranslated region the nucleotide sequence analysis of RNAγa of the two isolates revealed that six nucleotides were changed (Fig 6). Whereas amino acids sequence analysis showed 12 out of 162 amino acid were differed and one amino acid deletion (Fig 7).

TOPO™ cloning provides a highly efficient, 5 min. one step cloning strategy for the direct insertion of Taq polymerase-amplified PCR product into a plasmid vector. TOPO™ cloning exploits the ligation activity of topoisomerase by providing an activated, linearized TA vector using proprietary technology (Shuman, 1994).

In the present work, an attempt was made to compare the Egyptian strain (G119) strain of BSMV, which is prevalent in barley in Egypt with the North Dakota (ND18) strain. Data revealed a high degree of sequence similarity of γ RNAs, comparing to β RNAs of both strains. This result is in agreement with Jackson *et al.* (1991) and Solovyev *et al.* (1996). Nucleotide and amino acid sequences of the two strains (G-119 and ND18) revealed differences in the untranslated region (UTR) between the coat protein and βb gene, five bases were differed, 18 out of 116 amino acids were differed, three amino acids insertion, and one amino acid deletion. Little differences were also observed regarding the 5' UTR of RNA RNAγa, six nucleotides were changed, 12 out of 162 amino acids are differed and one amino acid deletion. The N-terminal domain found in BSMV shown to have a role in the BSMV βb protein RNA binding activity in vitro, and these domains have little sequence similarity among the BSMV βb proteins. Therefore exchanges of the βb N-terminal domains between G119 and ND18 could provide clues to their specificity in interaction with virus RNA in vivo (Solovyev *et al.*, 1996 and 1999). This hypothesis is in agreement with data showing that BSMV βb bound RNA without any specificity in vitro (Donald *et al.*, 1997). These differences in strain character provide an excellent opportunity for structure studies of the genome and genetic analyses of biological phenomena (Jackson *et al.*, 1991 and Sutic *et al.*, 1999). Nevertheless, comparison between virus strains will undoubtedly lead to the better understanding, how virus structure is related to biological activity.

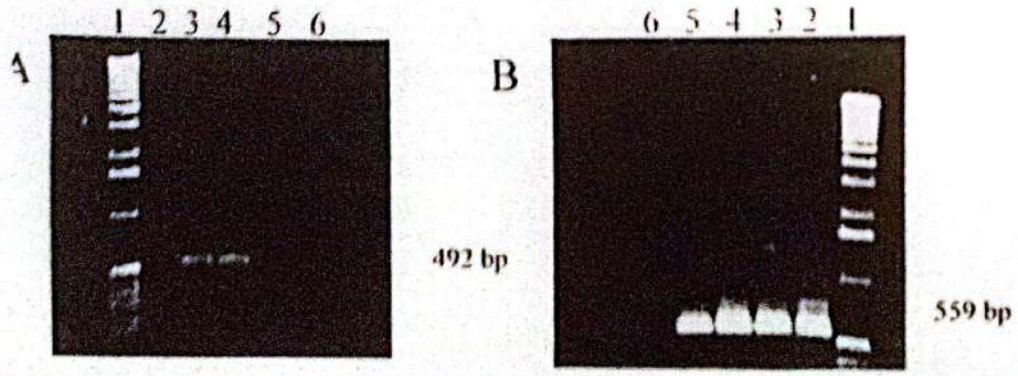

Fig.(1): PCR amplication of portions of the β and γ cDNAs from the BSMV ND18 and G119 isolate. RNA extracted from BSMV-infected plants or viral RNA was reverse transcribed using random primer followed by PCR amplification using the primers -660 and bb-4 (A), or 5'gamma and +557 (B) PCR products were analyzed on a 1% agarose gel. Lanes1, size markers; lanes 2, ND18 cDNA clones; lanes 3, ND18 vRNA; lanes 4, ND18 infected barley; lanes 5, G119 infected barley; lanes 6, uninfected barley.

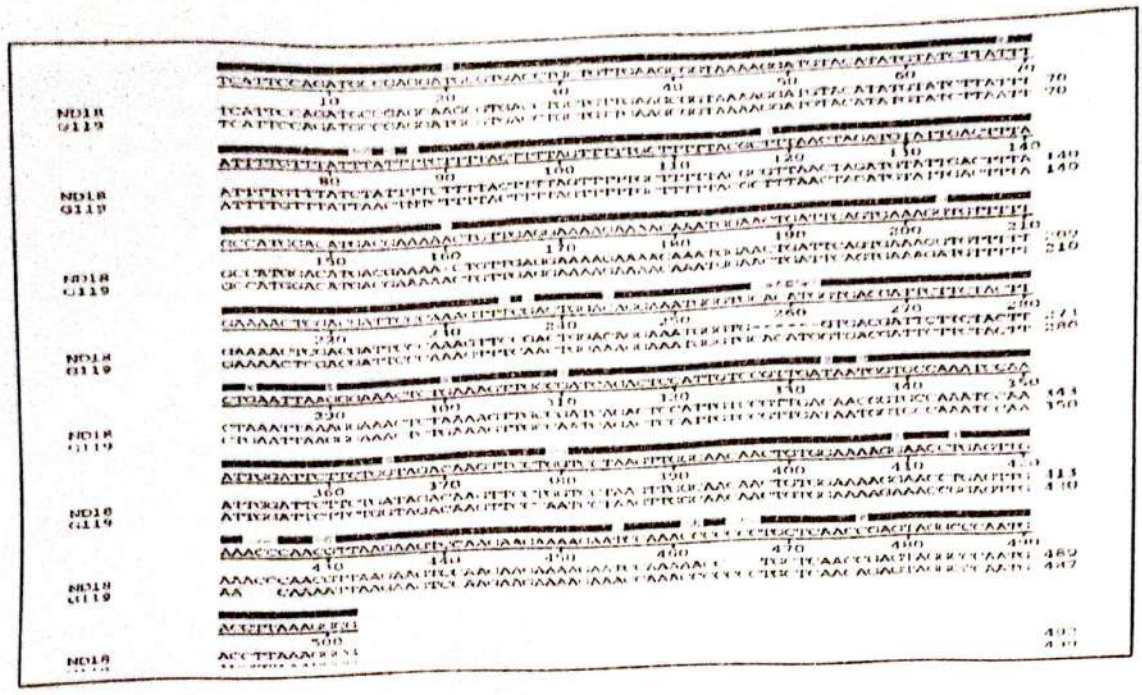

Fig. (2): An alignment of the nucleotide sequence of ND18 and G119 strains in a region of RNAβ, that includes the untranslated region between the coat protein and βb gene and the 5' terminus of the βb gene.

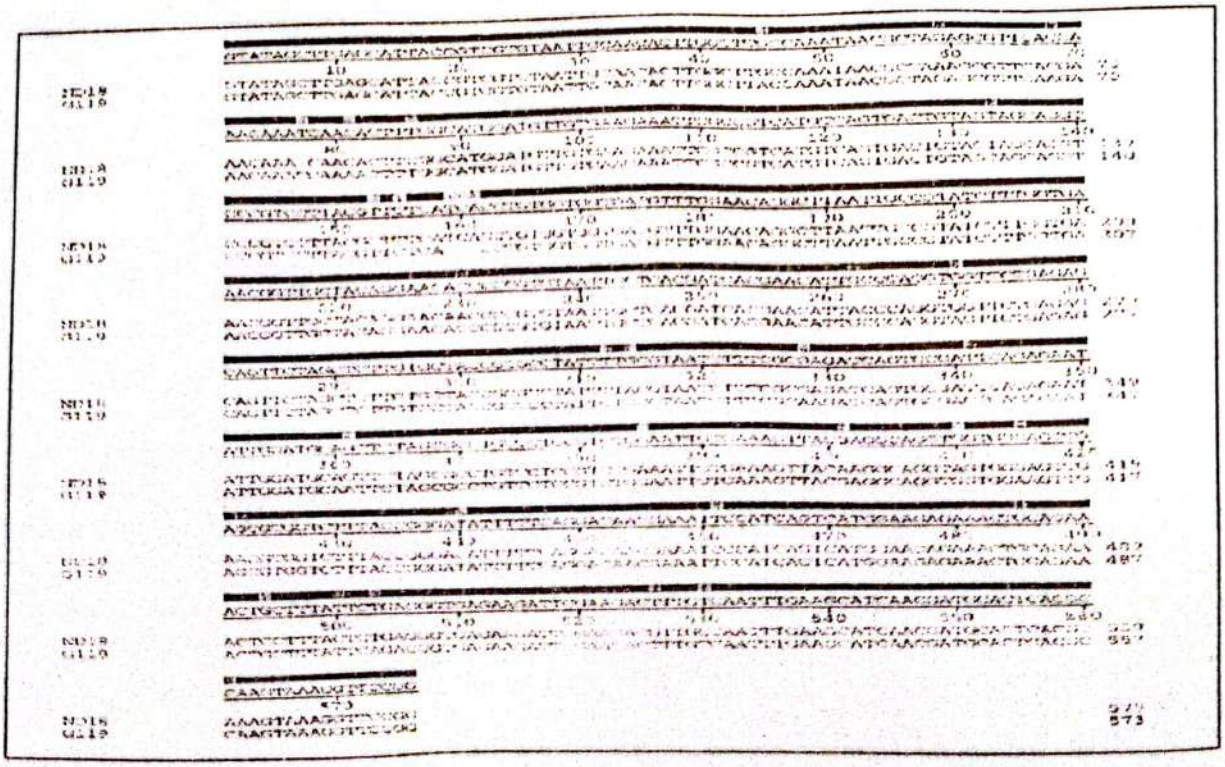

Fig. (3): An alignment of the nucleotide sequence of ND18 and G119 strains in a region of RNAγ, that includes the 5' untranslated region, 5' terminus of the γa gene.

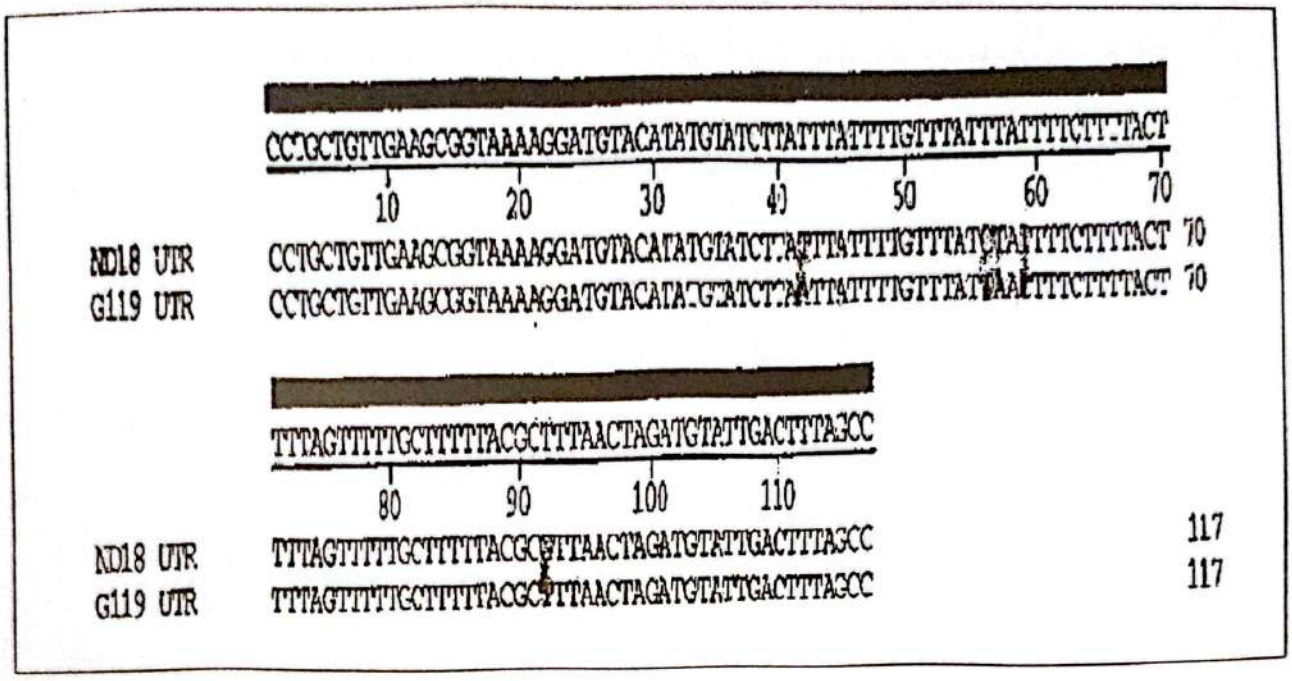

Fig. (4): An alignment of the nucleotide sequence of the untranslated region between the coat protein and βb gene in RNAβ of the ND18 and G119 strains.

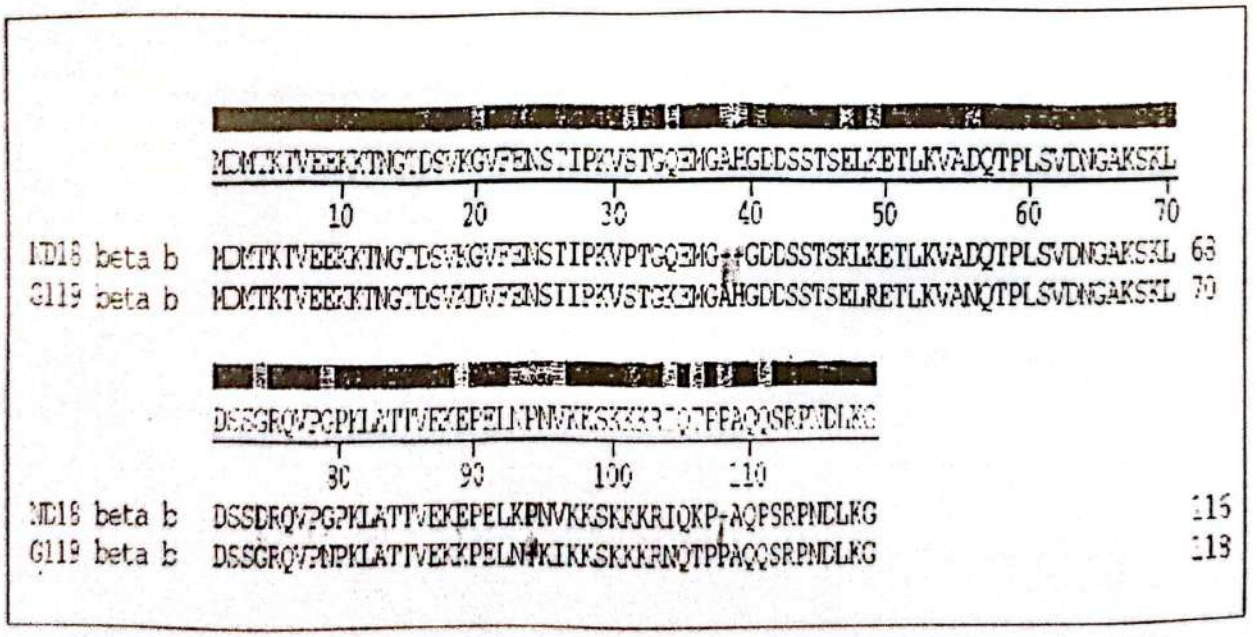

Fig (5): An alignment of the amino acid sequence of the amino-terminus of βb proteins of the ND18 and G119 strains.

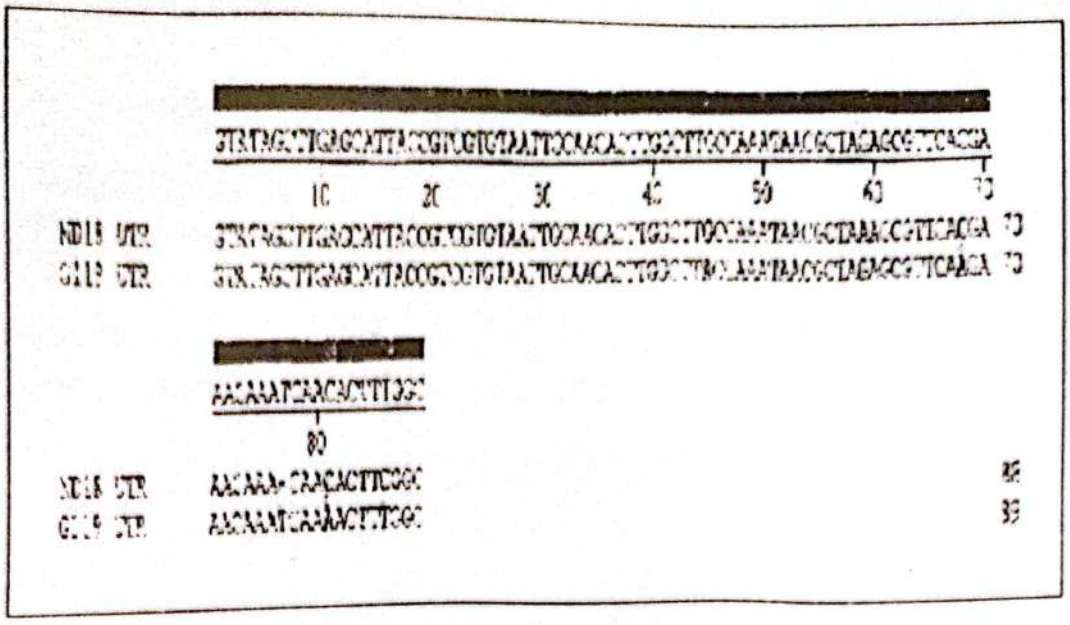

Fig. (6): An alignment of nucleotide sequence of the 5'untranslated in ya of theND18 and G119 strains

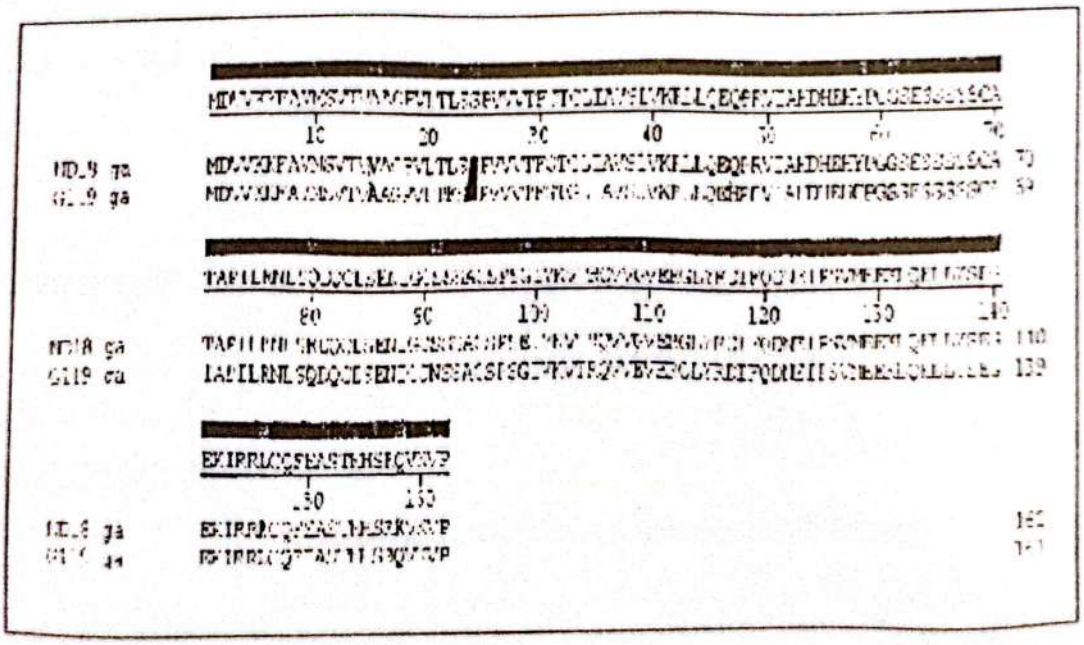

Fig. (7): An alignment of amino acid sequence of amino-terminus of the ya proteins of the ND18 and G119 strains.

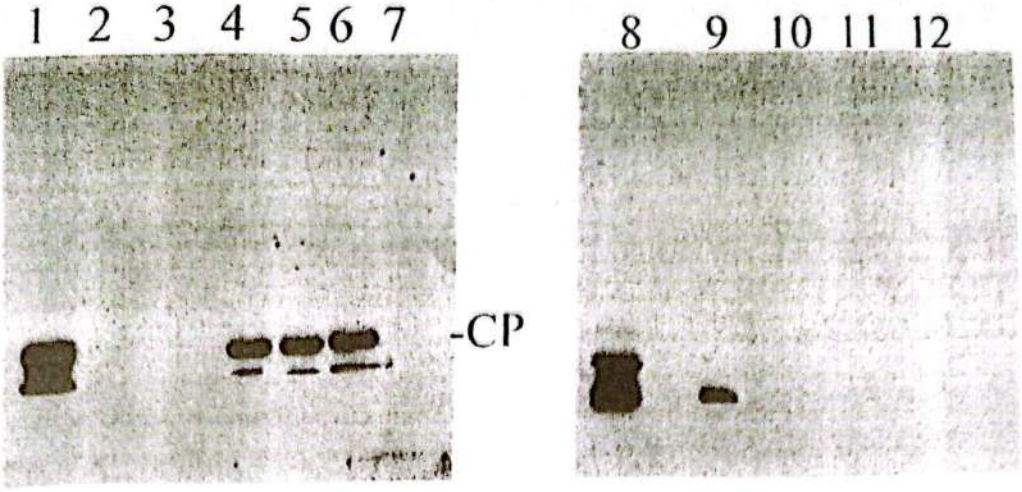

Fig. (8): Analyses of G 119 infected seed. Samples were analyzed by SDS-PAGE followed by western blotting using antiserum Raised against the coat protein of the ND18 strain of BSMV. Lanes1, 8. ND18 infected black hulless barley; lanes 2-12, leaf samples grown from G119 infected seed: lanes 7, 14 uninfected black hulless barley.CP = coat protein

Western Blotting for protein analysis

The coat protein of the Egyptian strain of BSMV purified by SDS-PAGE was cross reacted specifically to the antiserum raised against the coat protein of American strain North Dakato 18 (ND18) isolate of BSMV, using western blotting analysis. Four out of ten plants grown from cv Giza 119 cultivar infected seeds reacted positively in the presence of the coat protein of BSMV strain.

In these experiments Black Hulles barley cv infected with the ND18 isolate of BSMV was used as a positive control (Fig 8). The molecular weight of the coat protein of the Egyptian strain G119 was identical to that of the American strain ND18 being 22 KD. Solovyev *et al.* (1999) used western blotting analysis for studying the BSMV triple gene block (TGB) and compared it with *poa semi latent Hordeivirus* (PSLV) and *Lychnis ring spot Hordeivirus* (LRSV).

REFERENCES

Brunt, A.A.; Crabtree, A.; Dallwitz, M.J.; Gibbs, A.J. and Watson, L. (1996). Viruses of plants. Description and lists from the VIDE Database. CAB International Walling for U.K., 1484 pp.

Donald, R.G.K.; Lawrence, D. M. and Jackson, A.O. (1997). The *Barley stripe mosaic virus* 58-kilodalton beta b protein is a multifunctional RNA binding protein. J. virology 71: 1538-1546.

Donald, R.G.K.; Zhou, H., and Jackson, A.O. (1993). Serlogical analysis of *Barley stripe mosaic virus*-encoded proteins in infected barley. Virology 195: 659-668.

EL-Banna,Om-Hashem M.; Sabek, A.M.; Ibrahem, I.A. and Farag, A.G. (2000). Detection of *Zucchini yellow mosaic vrus* (ZYMV) by RT-PCR and nucleic acid hybridization. Egypt.J. Appl.Sci. 15: 20-35.

Halpern, B.T. and Hillman, B.I. (1996). Detection of *Blueberry scorch virus strain* NJ12 by reverse transcriptase-polymerase chain reaction amplification. Plant Dis., 80:219-222.

Jackson, A.O.; Petty, I.T.D.; Jones, R.W.; Edwards, M.C., and French, R. (1991). Analysis of *Barley*

stripe mosaic virus pathogenicity. Semin. Virol., 2: 107 –119.

Maniatis, T.; Fritsch, E.F. and Sambrook, J. (1982). Molecular cloning: A laboratory Manual. New York: Cold Spring Harbor Laboratory.

Rubio, T.; Borja, M.; Scholthof, H.B.; Feldstein, P.A.; Morris, T.J. and Jackson, O.A. (1999). Broad–spectrum protection against *tombusviruses* elicited by defective interfering RNAs in transgenic plants. J.Viroloy 73: 5070-5078.

Sambrook, J.; Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning: A laboratory Mannual, 2nd ed. New York: Cold Spring Horber laboratory.

Shuman, S. (1994). Novel approch to molecular cloning and polynucleotide synthesis using vaccinia DNA tosiscmerase. J. Biol. Chem., 269. 32678-32684.

Solovyev, G.A.; Savenkov, I.E.; Agranovsky, A.A. and Morozov, Yu.S. (1996). Comparisons of the genomic Cis-elements and coding regions in RNAβ components of the *hordeivirus*, lychnisspot, and *Poa semilatent virus*. Virology 219:9-18.

Solovyev, G.A.; Savenkov, I.E.; Grdelishvili, Z.V.; Kalinina, O.N. and Morozov, Yu. S. (1999). Movement of hordeivirus hybrids with exchanges in triple gene block. Virology 253:278-287.

Sutic, D.D.; Ford, R.E. and Tosic, M.T. (1999). Plant virus diseases Press New york Washington, D.C 553pp.

Zein, Salwa N. (2002). Advanced studies on *Barley stripe mosaic virus* (BSMV). Ph.D thesis, Fac Agri., Cario Univ., Egypt.137pp.