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PARTIAL NUCLEOTIDE SEQUENCING OF THE COAT PROTEIN GENE OF AN EGYPTIAN ISOLATE OF BEAN YELLOW MOSAIC VIRUS

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ABSTRACT: Partial nucleotide sequence of the coat protein gene of an Egyptian isolate of bean yellow mosaic virus (BYMV/CP-E) was determined. The full length of the coat protein gene was 822bp corresponding to a 274 amino acids. A reverse transcription polymerase chain reaction (RT-PCR) method has been developed to amplify 352 bp fragment of BYMV/CP. BYMV/CP-E fragment was cloned and sequenced using an ABI-PRISM 310 genetic analyzer. Comparison of the BYMV/CP-E sequence with those of the American (A), Netherland (N), and Japanese (J) BYMV isolates revealed that coat protein is highly conserved, with nucleotide sequence identities of 90-100%. The Netherland and American isolates were identical to the Egyptian isolate, indicating that these isolates might have evolved from a common ancestor. Molecular hybridization assay using digoxigenin-labeled probe has been developed and applied for the detection of BYMV in a small amounts of infected faba bean tissues (30µg). The assays used in this study are accurate, easy to perform, and applicable for screening of BYMV in imported faba bean cultivars. This virus now can be positively identified from infected faba bean tissues in a few hours instead of a few months by symptoms on indicator plants.

Key words: BYMV, RT-PCR, faba bean, gene sequence, hybridization

INTRODUCTION

Bean yellow mosaic virus (BYMV) is a member of the genus *Potyvirus* (Bos, 1970, and Bos *et al.*, 1974). The potyviral genome consists of a positive-sense RNA of approximately 10Kb with a genome-linked protein (VPg) at the 5' terminus and a poly (A) tract at the 3' terminus (Dougnerty and Carrington, 1988). The complete nucleotide sequences of several Potyvirus genomes have also been determined; e.g. tobacco etch virus (TEV), tobacco vein mottling virus (TVMV), plum pox virus (PPV), potato virus Y (PVY),pea seed-born mosaic virus (PSbMV), pepper mottle virus (PepMoV), papaya ringspot virus (PRSV), turnip mosaic virus (TuMV), soybean mosaic virus (SMV), Johnson grass mosaic virus (JGMV), potato virus A (PVA) and peanut stripe virus (PStV) (Bryan *et al.* 1992). The genome organization of all potyviruses is similar and the polypeptide is proteolytically processed into at least eight mature proteins; the N-terminal protein (P1), the helper component-protease (HC-Pro), the P3 protein, the cytoplasmic inclusion protein (C1), the nuclear inclusion protein a (NIa), which consists of VPg domain (NIa-VPg) and a protease domain (NIa-Pro), the nuclear inclusion protein b (Nib) and the coat protein (CP) (Nakamura *et al.* 1996). The polyprotein also contain conserved motifs, such as "GDD" involved in polymerase activity, described for plant RNA viruses (Hammond and Hammond, 1989). BYMV is a member of a subgroup consisting of BYMV and clover yellow mosaic vein viruses (CYVV), which are serologically related and have similar host ranges (Uyeda, 1992). Currently the viral disease status of seed borne viruses such as bean yellow mosaic potyvirus (BYMV), alfalfa mosaic alfamovirus (AMV), cucumber mosaic cucumovirus (CMV) and subterranean clover mottle sobemovirus (SCMoV) are evaluated by visual observation of plants grown for seed propagation. Suspect plants are sometimes tested by ELISA. Screening for all seed borne viruses by virus specific ELISA tests for each sample is both prohibitively expensive and labour intensive (Rosner *et al.* 1994). Furthermore, antisera to some of these viruses are not readily available. Polymerase chain reaction is a powerful technique used for the amplification of a specific DNA sequences and is capable of enrichment by a factor of 10^6 to

10^9, enabling the detection of a few target nucleotides (Saiki *et al.* 1985 and 1988). Reverse transcription followed by polymerase chain reaction (RT-PCR) has been used to detect BYMV with sensitivity in the range of 1 pg quantities of virus (Vunch *et al.*, 1990). Here, we determined for the first time the partial nucleotide sequence of BYMV capsid protein gene of an Egyptian isolate and elucidated the genetic relationship between BYMV-CP-E and other BYMV isolates.

MATERIALS AND METHODS

Source of Egyptian isolate of BYMV: Faba bean leaves showing typical symptoms of BYMV were obtained from El-Sharkia (Zagazig) and Beni-suef (Sads) governorates as a source of BYMV infection during November, 1997.

ELISA: About 0.5 gm of leaf samples showing typical symptoms of BYMV (mild mosaic followed by narrowing) were assayed by double antibody sandwich ELISA with a BYMV detection kit (Boehringer GmbH). The test was performed according to Clark and Adams, (1977). Absorbance at 405 nm was determined with the aid of an automated microplate ELISA reader (DYNATECH, USA). A reaction was considered positive if the absorbance was 2.5 times that of a healthy sample.

Total nucleic acid extraction from plant tissue: Total RNA was extracted from 30 µg of infected and uninfected faba bean leaves using SV Total RNA Isolation System from (Promega Corporation, Madison, WIS.) as described by Brisco *et al.* (1998). Tissues were frozen in liquid nitrogen and ground to a fine powder. 175 µl of RNA lysis buffer (4M gaunidine thiocyanate, 0.01M Tris-HCl, pH 7.5, 0.97 % β–mercaptoethanol) was added to the ground tissue, mixed thoroughly, and 350 µl of RNA dilution buffer was added. The mixture was mixed by inversion and centrifuged at 14000xg for 15 minutes. The cleared lysate was mixed with 200 µl of 95% ethanol and centrifuged for 10 minutes at 12.000-14.000 xg at 4°C. The cleared lysate solution was transferred to the spin basket Assembly and centrifuged at 12000-14000 xg for one minute. The eluate was decanted and 600 µl SV RNA wash solution (600mM potassium acetate, 10mM Tris-HCl, pH7.5, 60% ethanol) was added twice. The DNase 1

mix was prepared by combining 40-µl yellow core buffer, 5 µl 0.09 M $MnCl_2$ and 5 µl of Dnase 1 enzyme and 5 µl of this freshly prepared Dnase 1 mix was added directly to the membrane inside the spin basket and the mixture was incubated at 25°C for 15 minutes. 200 µl of SV Dnase stop–solution was added to the spin basket and centrifuged at 12.000 xg for one minute. 100-µl nuclease free water was added to the membrane in the spin basket and centrifuged at 14.000xg for one minute. The spin basket was removed and the elution tube containing the purified RNA was capped and stored at -70°C.The yield of total RNA obtained was determined by spectrophotometer at 260nm (A_{260}), where 1 absorbance unit equals 40µg of single-stranded RNA/ml.

Reverse-transcriptase polymerase chain reaction (RT-PCR): The total RNA extracted from infected and healthy faba bean plants by using SV total RNA isolation system was used as a template in reverse transcription reaction (RT) in order to synthesize complementary DNA_s (cDNA). The moloney murine leukemia virus reverse transcriptase M-MLV from Promega was used in the cDNA synthesis system as described by Mathuresh *et al.* (1996) and Young *et al.*, (1997). The secondary structure of the RNA was eliminated by heat treatment to allow the M-MLV, reverse transcriptase to produce longer cDNA molecules. Buffers and enzymes needed to generate high quality cDNA were from Promega. Reverse transcription reactions (20 µl) were performed in 50 mM Tris-cl pH 8.3, 75 mM KCl, 15 mM $MgCl_2$, 500 µM dNTPs, 10 units of MMLV reverse transcriptase, 80 µg of total RNA dissolved in deionized autoclaved DEPC treated water, 1.0µM of minus primer (M2), CTAGCA-CGCACAGGAGTTCT, 100 mM DTT and 40U Rnase inhibitor as described by Shooman (1997); and Abdelkader (1997). Reagents were mixed by tapping, spun briefly and placed in a 42°C water bath for 60 minutes followed by incubation at 95°C for three minutes to inactivate the Rtase and to denature the RNA-cDNA hybrids. The reaction was spun briefly and placed quickly on ice. PCR was performed in 100 µl total volume containing 2.5 units of Taq DNA polymerase (Promega) at 10mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5mM $MgCl_2$, 0.2 mM of each dNTPs, 0.1% Triton-X-100, 5 µl of reverse transcription reaction

re and 50 pM/µl of each rd (P_1) ATGTC (AT) GA CAAGA (AG) (CA) TCT and reverse (M_4) ATACGAACACCAAGCAT rs as described by Barker et 1993); Hataya et al. (1994) Shooman (1997). The PCR s were repeated 35 times the following conditions: uration at 94°C for one e, annealing at 45°C for two es and extension at 72°C for minutes. The PCR products analyzed by 1% agarose gel ophoresis and the size of the ength BYMV DNA fragment letermined in accordance with DNA molecular weight ers. In hemi–nested PCR PCR), the templates produced ie first PCR by the outer ers (e.g. P_1M_4) were re-ified by reusing one of the primers (M_4) with a new d (inner) plus primer (e.g. P_3: GCAAAGCCAACATTC). primer CTAGCACGCACA-AGTTCT is situated internal to M_4 primer. The aim of the d second round of PCR is to ease the specificity and itivity of the RT-PCR and to late the products amplified in first round of PCR. The ocol of hn-PCR was described Nontasser et al. (1992), Hadidi al. (1993), and Abdelkader (1997). Nucleotide sequences of the aforementioned primers were used for the amplification of BYMV/CP gene fragments. P and M stand for plus and minus primers, respectively. Primer P_1 has an (ATG) add-on triplet preceding the sequence. Primer M_4 has (CTA) triplet at its 5`-end, complementary (TAG) stop codon at the 3`-end of the viral genome.

Molecular cloning of BYMV/ CP gene: The PCR product P_3M_4 (352 bp) of BYMV-E was directly cloned into pGEM-T-Easy cloning vector (Promega Corporation, Madison, WIS) according to Sambrook et al., (1989). Ligation reaction was incubated overnight at 16°C and contained equi-molar amounts of PCR product (insert) and vector DNAs. Ligations were conducted at 300 mM Tris-Cl, pH 7.8, 100mM $MgCl_2$, 100mM DTT, 10mM ATP and 3 Weiss units/ □l T4 DNA Ligase. The ligation reaction was transformed into strain JM109 of *E.coli*. Positive clones were identified by blue/white selection, *Eco*RI restriction endonuclease, and DNA sequence analysis. The plasmid DNAs were extracted by using Wizard plasmid mini-preparation system from Promega as recommended and used for probe preparation.

Probe preparation: High specificactivity DNA probe was synthesized as reported by Thomson et al. (1995) using digoxigenin labeled nucleotides (Boehringer Mannheim, Indianapolis). The PCR reaction was performed in 50ul total volume containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 1ng of BYMV-CP clone (pEHR34), 1X dNTPs labeling mixture (10mM dATP, 10mM dCTP, 10mM dGTP, and 6.5 mM dTTP, 3.5 mM Dig-11dUTP, in Tris-HCl, pH 7.5), 1μ M of minus sense primer M_4 (5'-CTAATACGAACACCAAG-CAT-3'), 1uM of plus sense primer P_3 (5'-AATGCAAAGCCAAC-ATTC-3'), and 1.25 U Taq DNA polymerase and then the volume was completed to 50ul with nuclease free water. The PCR reagents were obtained from (Promega Corporation, Madison, WI). The DNA thermal cycler UNOII from Biometra, Inc., with heated lid was used with heated lid for 35 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 2 min and extension at 72°C for 2 min.

Southern blotting: Southern blot hybridization technique (Southern, 1975) was used to confirm that amplified fragments by PCR were BYMV-CP gene fragments using the non-radioactive digoxigenin-labeled DNA probe. The gels were placed in a glass dish containing 200 ml denaturation solution (1M NaCl and 0.5 M NaOH) for 15 minutes with gentle shaking at room temperature. The gel was neutralized by soaking twice for 15 minutes in neutralization solution (1.5 M NaCl, 0.5 M Tris-Cl, pH 7.0). In a blotting apparatus the reservoir was filed with 500 ml 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) and set on three sheets of 3 mm paper which were rinsed with the 20X SSC. A piece of nitrocellulose membrane (milli-pore) of the exact gel size was cut and wetted in 20X SSC. The gel was placed on the saturated 3-mm paper to the level of platform. The wetted membrane was placed on the top of the gel and then stack of paper towels and 500 gm weight were placed on the top. The apparatus was left overnight to allow transfer of DNA from the gel to the membrane. The DNA was fixed on the membrane using UV cross-linker used with a non-radioactive hybridization technique.

Dot-blotting: The preparation of tissue extracts for dot-blot was carried out according to the method of Loebenstein and Akad

(1997). Eight millimeter diameter leaf tissue of broad bean was ground in 100µl of extraction buffer (0.2M potassium phosphate containing 5 mM dithiothreitol, 0.1% triton X-100 and 10 mM 2-mercaptoethanol, pH 8.3 (Crosslin *et al.* 1992). An equal volume of Denaturation solution, 8X SSC (1XSSC is 150mM NaCl plus 15mM Sodium Citrate, pH 7.0) 10% formaldehyde was added to the homogenate, then heated to 60°C for 15 minutes and then kept on ice. Dilutions of the extract from 1:1000, 1:10,000, and 1:100,000 were prepared with 4XSSC containing 5% formaldehyde (v/v). 5µl of each dilution of the tissue extracted was spotted onto nitrocellulose membrane.

Pre-hybridization and hybridization: The rehybridization, hybrdization, and immunological detection were carried out using the "Genius II DNA labeling and detection kit" (Boehringer Mannheim IN system). The membranes were prehybridized for 1-3 hr at 65C in a hybridization oven (Techne-Hybridizer HB-ID) with at least 25ml of prehybridization buffer/100 cm^2 (6.25 ml of 20X SSC, 0.25 ml of 10% N-laurysacrosine, 0.025ml of 20% SDS, 1.25 g blocking reagent). The DNA probe was denatured by boiling in water bath for 5 min at 100°C then placed immediately in ethanol ice bath. Replacing the membrane with about 20ml of hybridization solution containing 5 µl of 10ng freshly denaturized dig-11 dUTP labeled probe and the membranes were incubated with this hybridization solution overnight at 65°C in hybridization oven (Techne-Hybridizer HB-ID). The membrane was washed twice at room temperature for 5 min with 150 ml 2XSSC containing 1.0% SDS (w/v) at 25°C and another two times 2 x 15 min with 100ml 0.1XSSC containing 0.1% SDS at 25°C. Nitrocellulose membranes were washed briefly for 1 min in buffer I (0.1 M maleic acid 0.15 M NaCl, pH 7.5) and incubated for 30 min with 100 ml buffer 2 (100ml buffer I and 1gm blocking reagent). The membranes were incubated for 30 min at 25°C with anti-dig-alkaline phosphatase conjugate diluted 1-5000 in buffer 2. The unbound antibody–conjugate were removed and equilibrated by washing 2 times for 15 min with 100 ml of buffer (1), then washed for 2min with 20 ml of buffer (3) containing (100 mM Tris-HCl, 100 mM NaCl, 50mM $MgCl_2$, pH 9.5) at 25°C. The membranes were incubated in 10 ml of color solution containing

45 µl NBT (nitro blue tetrazolium) and 35 µl X-phosphate (5-Bromo-4-chloro-3-indolyl phosphate), dissolved in 10 ml buffer (3) in a plastic suitable box in the dark. The membranes were incubated for 30 min to 1 hour for the color reaction development. The reaction was stopped when desired signals were obtained using Genius buffer IV (10mM Tris-HCl, 1mM EDTA, pH 8.0) for 5 minutes. The membranes were air dried and stored at room temperature.

Nucleotide sequencing of BYMV/CP gene: BYMV- DNA sequencing was done on ABI Prism 310 Genetic Analyzer (PE Applied Biosystems) using ABI Prism® BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). The dye terminator is high sensitive dye containing fluorescein donor dye (6-FAM), linked to rhodamine acceptor dye. The maximum excitation is that of the fluorescein donor, and the emission spectrum is that of the dRhodamine acceptor. The BigDye terminators are 2-3 times brighter than the rhodamine dye terminators when incorporated into cycle sequencing products. After the plasmid DNA of BYMV was purified using spin column procedure described before, the sequencing reaction was performed in a total volume of 40µl containing 16µl of Terminator Ready Reaction Mix (dye terminators, deoxynucleotide triphosphates, AmpliTaq DNA Polymerase, FS, *Tth* pyrophosphatase, magnesium chloride, and buffer), 2-3 µg of BYMV-DNA, 10 pmol of the minus specific primer (M4) and placed on GeneAmp PCR System 9700 PE to perform cycle sequencing for 45 cycles at 95°C for 30 sec, 55°C for 20 sec, 60°C for 4 min. Purification of the extended PCR product was done by Centri-Sep spin columns as recommended by the ABI Prism 310 Genetic analyzer User's Manual (P/N 903565). The samples were resuspended in 20ul of TSR (supplied with the polymer), vortexed and heated to 95°C for 2 min and placed on ice before loading. The samples were electrophoresed on the ABI Prism 310 Genetic Analyzer according to the user's manual (P/N 903565).

RESULTS

Detection of BYMV by PCR: Reverse transcription–polymerase chain reaction (RT-PCR) was performed on total RNA extracted from 30µg leaf tissues by the SV total-RNA extraction method (Brisco, 1998). The RNA was reverse transcribed by the MMLV

reverse transcriptase. The reverse transcription reaction was primed with either the M_2 or M_4 minus-sense primers. The resulting complementary DNA (cDNA) was amplified by PCR after adding either P_1 or P_3 plus-sense primers. Figure (1) shows the amplification products obtained from infected faba bean by using primers P_1 or P_3 with M_2 or M_4, primers. The expected product size of P_1M_4 is 825 bp, P_1M_2 is 665 bp, P_3M_4 is 352 bp, and P_3M_2 is 191 bp.

The hemi–nested–PCR was performed by using P_3 and M_2 primers in order to validate the authenticity of the DNA amplified in the first round of PCR. The PCR products obtained by using P_3 and M_2 and P_3 and M_4 as plus–sense and minus – sense primers in a second round of PCR is shown in Figure (1). It appears from Figure (1) that the size of the PCR fragments were in agreement with those expected from the sequence (191 bp and 352 bp).

Southern blot analysis of PCR products: Southern blot hybridization technique was used to confirm the authenticity of the PCR products of BYMV/CP-E obtained in Figure (2). The BYMV/CP PCR products were resolved on 1% agarose gel and transferred onto a nylon membrane as described under the Materials and Methods. Hybridization was performed by using the BYMV specific digoxigenin labeled DNA probe. Figure (2) shows that the DNA probe was successfully hybridized with all PCR products of the coat protein gene of BYMV amplified with the primers P_1 and M_4 (825 bp), P_1 and M_2 (665 bp), P_3 and M_4 (352bp), and P_3 and M_2 (191 bp).

Cloning of BYMV/CP-E gene into *E. coli* and preparation of dig-11 dUTP probe: The strategy used for cloning of BYMV/CP gene in *E. coli* was based on direct cloning of PCR product in PGEM–T–Easy vector (3018 bp). P_3M_4 PCR product of BYMV/CP gene was cloned and ligated directly into a pGEM-T-Easy cloning vector. The ligation products were transformed into *E. coli* JM109 component cells. White colonies resistant to ampicillin containing recombinant plasmids were selected for isolation of recombinant DNA plasmids containing the CP gene of BYMV by plasmid mini-prep method described under the Materials and Methods, while the blue colonies were also screened for the empty pGEM–T plasmid (without CP inserts) as a plasmid DNA control. The restriction digestion analysis

of the selected white colonies with *Eco*R1 restriction enzyme (Fig. 3) indicated that the plasmid plus insert equal 3370

(lane 1) and the insert size was 352 bp as shown in lanes 3 and 4. The recombinant plasmids which containing the cp insert shown in Fig (3) were called pEHR34 and pEHR44 (lanes: 3 and 4) and selected for further validation by PCR using the primers P_3 and M_4.

Validation of cloning by PCR. The recombinant plasmids prepared by plasmid min-preparations were validated by PCR using primers P3 and M4 for amplifying BYMV-CP conducted on presumptive recombinant plasmids tested previously by *Eco*R1 enzyme restriction digestions (Fig. 3). In Fig. (4), the presumptive clones pEHR34, pEHR44 and some other randomly selected clones were tested by PCR amplification using the two primers P_3 and M_4 to detect which of these clones contained the right CP gene insert. Figure (4), shows that three clones (lanes: 2, 4 and 5) were given the correct size (352 bp) of the coat protein gene after PCR amplification and agarose gel electrophoresis analysis.

Sensitivity of dotblot hybridization assay versus ELISA: BYMV-infected and un-infected leaf extracts were used for dot blot hybridization experiment and also assayed by DAS-ELISA at the same time. 10ul of leaf extract were spotted onto nitrocellulose membrane at dilutions 1:100, 1:1000, 1:10,000, and 1:100,000 (Table 1). The DNA probe enabled detection up to 1:10,000 dilutions while no reaction was observed at this dilution with ELISA. This is equivalent to $2X10^2$ particles/spot (30 µg of fresh tissue.

Table (1): Comparison between the sensitivity of dot blot and ELISA assays in detecting BYMV at serial dilutions of infected faba bean leaf extracts

Leaf extracts dilution	ELISA	Dot-blot
1/100	++	+++
1/1000	+	++
1/10.000	-	+
1/100.000	-	-

- = negative reaction

Sequence Analysis of BYMV/CP: Multiple sequences were aligned by the PILEUP program of the GCG package (Version 10.0, 1999, Genetics Computer Group, Madison, WI., USA). Distance matrices for complete coat protein sequences

were calculated from alignments based on the Dayhoff PAM matrix (Dayhoff *et al.*, 1983), using the program PROTDIST in PHYLIP software package Version 3.5c (Kimura, 1980).

The GAP program in the GCG package was used to determine the degrees of amino acid and nucleotide homology of BYMV coat protein with those of other BYMV isolates. Sequence for alignments were obtained from the GenBank database under the following accession numbers including (N) (AJ289199) (Van der Vlugt, 2000) and (A) (S77515) (Tracy *et al.*, 1992), and (J) (AB041972) (Wada *et al.*, 2000)

Partial Nucleotide Sequence of BYMV/ CP-E: One of the two cDNA clones, the pEHR34 was used to determine the partial nucleotide sequence of *BYMV/CP-E*. The partial nucleotide sequence was determined to be 349 nucleotides in length, (Figure 5). The Egyptian isolate of BYMV/CP nucleotide sequence was determined by using ABI PRISM 310 Genetic Analyzer. The nucleotide sequence of the CP of BYMV-E reported in this study was compared with other BYMV/CP isolates published from GenBank (NCIB) using a composite alignment computer program. The degree of homology between sequences was found to be directly conserved in all four sequences with more than one sequence gap in the Japanese isolate. The sequence similarities were 100 % identity with the Netherland isolate and with the American isolate, whereas it showed 90% identity with the Japanese isolate Fig (5).

DISCUSSION AND CONCLUSION

The major problem in the detection of BYMV in faba bean plants is the low concentraion of the virus present in infected tissue. The commonly used diagnostic method, ELISA, failed to detect the low virus concentration in infected tissue. Our RT-PCR and nucleic acid hybridization assays were more sensitive than ELISA and can detect and identify any seed born viruses. Because our primers were designed in the most conserved region in viral genome (coat protein region), our DNA probe was able to detect and hybridize to a wide range of different strains of the virus.

The present study demonstrates successful use of RT-PCR and nucleic acid hybridization assays to detect BYMV in total RNA isolated from infected faba bean tissues and

indicates feasibility as a rapid and accurate laboratory assays for early detecting BYMV from field samples. Only a few hours are required for positive identification of the virus in infected plants. The sizes of the PCR products from BYMV/CP-E (825 bp, 665 bp, 352 bp, and 191 bp) were identical to that obtained by Shooman (1997) using exactly the same primers. These results indicated the successful isolation of undegraded RNA from BYMV infected tissues using the SV-total RNA isolation method as well as the specific priming of efficient RT-reactions. In Southern hybridizations, the amplified PCR products of the coat protein gene specifically hybridized to the BYMV/CP DNA probe indicating that authentic BYMV sequences were amplified. Although, the RT-PCR was more sensitive than dot-blot hybridization assay, the later procedure was adequate for the detection of BYMV infection in faba bean plants.

Spotting of 10ul of diluted leaf extracts of bean plants infected with BYMV in dot blot hybridization enabeled detection of 1:10,000 dilution while, there was no reaction observed at this dilution in DAS-ELISA (Table 1). This is equivalent to $2X10^2$ particles/spot (30 µg of fresh tissue). Therefore, the detection limit of the Dig-labeled DNA probe was approximately (3×10^4 particles) per one gram of infected tissue. These indicates that dot-blot hybridization is more sensitive and easier to perform than ELISA.

Of the many parameters available to establish the taxonomic status of potyvirus isolates, coat protein and gene sequence data have been shown to be most useful and have been sucessfully applied to discriminate between potyviruses and strains. For the coat protein, the nucleotide sequences of the Egyptian isolate of BYMV/CP was determined by using ABI PRISM 310 Genetic Analyzer. The nucleotide sequence of the CP of BYMV-E reported in this study was compared with other BYMV/CP isolates published in the GenBank using a composite alignment computer program. The degree of homology between sequences indicated that the sequences were conserved in all four isolates with more than one sequence gap in the Japanese isolate. American and Netherland strains of bean Yellow mosaic virus (BYMV) showed a 100% identity, however, while only a 90% identity was found between the coat protein sequence of Japanese isolate (J) and Egyptian isolate (Fig 5). These results were

in agreement with that obtained by other researchers (Dougherty &Hiebert, 1980; Yeh & Gonsalves, 1985; Alison *et al.*, 1985; Hellman *et al.*, 1986; Domier *et al.*, 1986; and Hammond & Hammond., 1989). Shukla and Ward (1988 and 1989) concluded that amino acid identities of CPs between different potyviruses ranged from 38% to 71%, whereas they ranged from 90% to 99% between strains of each species. This observation suggests that the Egyptian isolate of BYMV/CP is closely related to both isolates of Netherlands and American more than that of the Japanese isolate and might have evolved from a common ancestor based on CP sequence alignments. On the other hand, the variation in coat proteins may reflect the variation in symptom development, host range, and geographical distribution (Tordo *et al.*, 1995; Lee and Wong, 1998). It is apparent that these parameters for the BYMV isolate from Japan is quite different from those for Netherland, US, and Egyptian isolates.

A new standard should be established to distinguish between different strains of viruses in the genus *Potyvirus* when more sequence information for different coat proteins becomes available. Nevertheless, the diversity of the CP protein should be taken into consideration regarding taxonomy of potyviruses. Also the CP gene can be used as a target for development of specific probes to distinguish strains of potyviruses. Furthermore, the complete genome sequence of BYMV/CP-E provides an important base for construction of the *in vitro* and *in vivo* infectious clones at the cDNA level. The infectious clones of BYMV/CP-E can be used as tools, through recombination at the cDNA level, to possible study the differences of nucleotides and amino acids among different strains for their biological functions.

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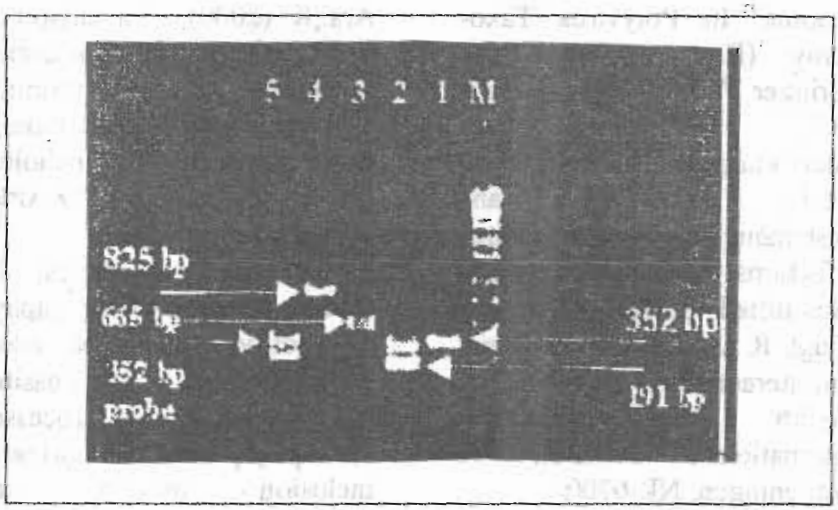

Fig. (1): 1% agarose gel electroporesis showing the PCR amplification products of BYMV coat protein gene. Lane 1:PCR product using primers P3 and M4 (352 bp), Lane 2:PCR product using primer set P3 and M2 (191 bp), Lane 3:PCR product using primer set P1 and M2 (665 bp), Lane 4:PCR product using primer set P1 and M4 (825 bp), and lane 5: PCR probe using P3 and M4 primers (352 bp) M:1 kbp ladder DNA marker.

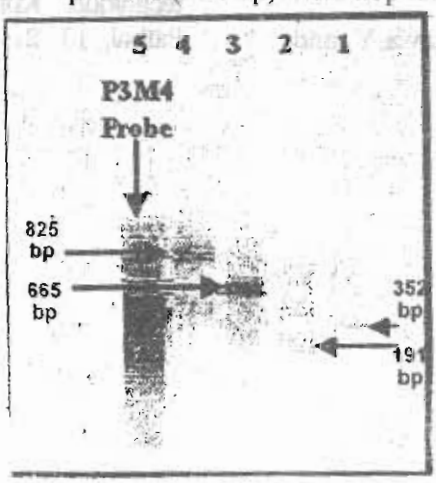

Fig. (2): Southern-blot hybridization of PCR products hybridized with digoxigenin-labeled DNA probe specific for BYMV coat protein. Lane 1:PCR product using primers P3 and M4 (352 bp), Lane 2:PCR product using primer set P3 and M2 (191 bp), Lane 3:PCR product using primer set P1 and M2 (665 bp), Lane 4:PCR product using primer set P1 and M4 (825 bp), Lane 5: self hybridization of P3M4 probe.

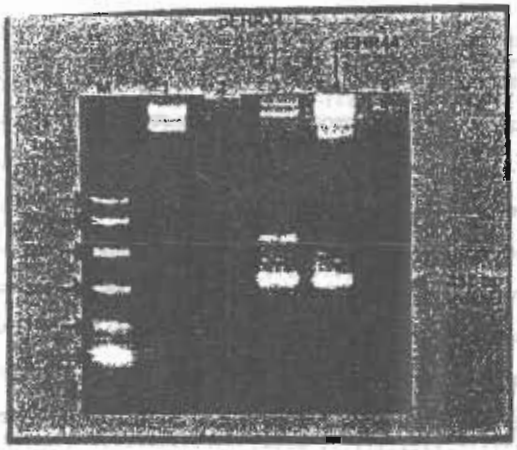

Fig. (3): Screening of pGEM-T-Easy transformed E.coli colonies by plasmid mini-preparation and restriction digestion with EcoRI. Analysis was performed on 1% agarose gel electrophoresis. Lane 1: undigested plasmid, Lane 2 and 5: digested plasmids without inserts, Lanes: 3 and 4: digested plasmids containing BYMV-E-Cp gene fragment of the correct size (352 bp). M: PCR Molecular weight DNA Marker (Promega).

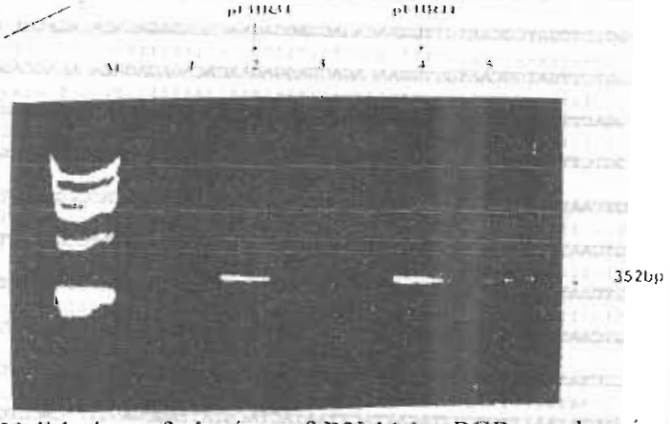

Fig. (4): Validation of cloning of P3M4 by PCR product into pGEM-T-Easy by PCR. Transformed colonies subjected to restriction digestion were also subjected to PCR amplification by using P3 and M4 primers. The expected product was 352 bp. The PCR products were analyzed by 1% agarose gel electrophoresis. Lanes2, 4 and 5 showing the correct size of CP gene fragment of BYMV (352 bp). The other PCR products are not correct in size (non-recombinant plasmids). M : 1 Kb DNA ladder molecular weight marker.

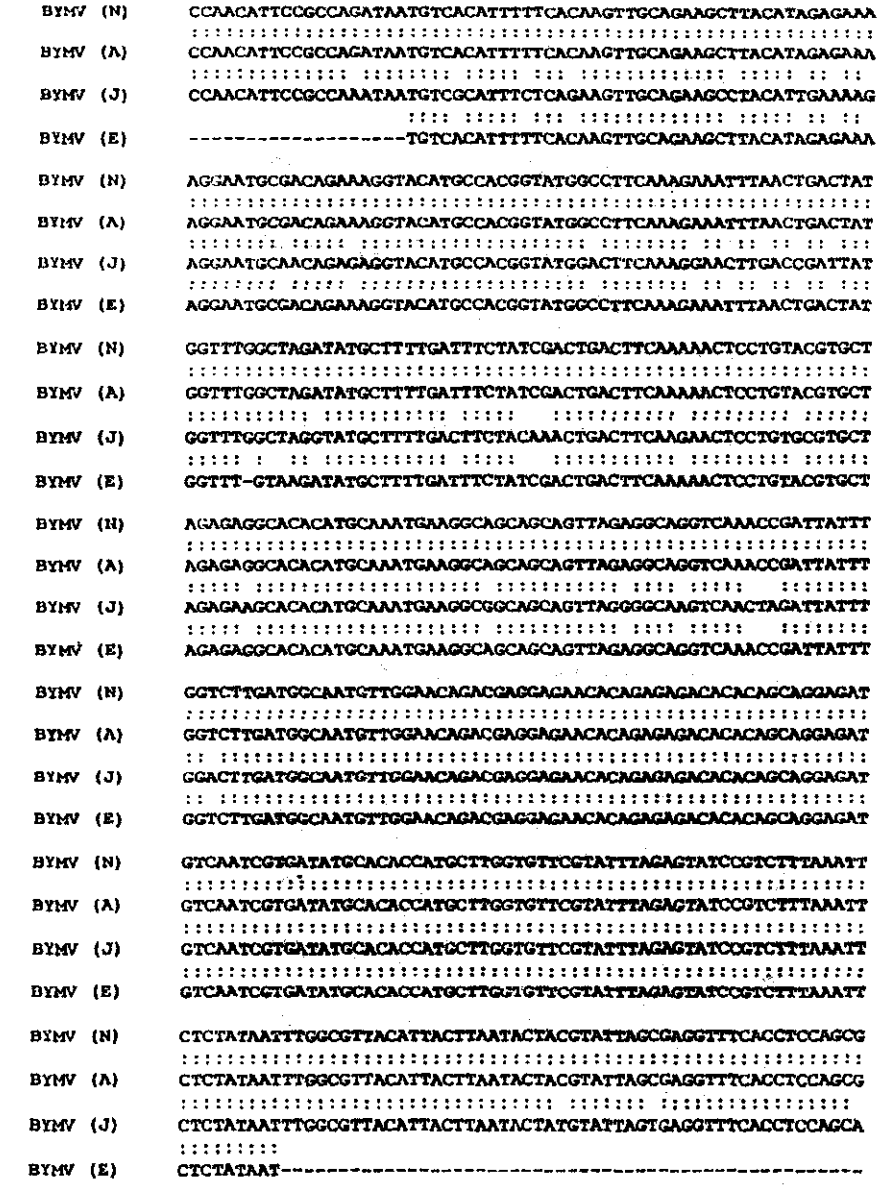

Fig.5: Alignment of partial nucleic acid sequences encoding the coat proteins of the published BYMV strains with the Egyptian strain: Netherlands (N), American (A), Japan (J), and Egypt (E) (Current study).

التتابع النيوكليوتيدى الجزئى لجين الغلاف البروتينى لعزلة مصرية لفيروس تبرقش الفاصوليا الأصفر

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مركز البحوث الزراعية.

تم تحديد التتابع النيوكليوتيدى لجين الغلاف البروتينى للعزلة المصرية لفيروس تبرقش الفاصوليا الأصفر جزئيا , حيث أن الطول الكامل لجين الغلاف البروتينى ٨٢٢ نيوكليوتيدة و التى تقابل ٢٧٤ حامض أمينى.و قد تمكننا باستخدام طريقة مطورة من النسخ العكسى و تفاعل البلمرة المتسلسل من اكثار جزء طوله ٣٥٢ قاعدة نيتروجينية من جين الغلاف البروتينى لفيروس تبرقش الفاصوليا الأصفر. و قد تم كلونة هذا الجزء من جين الغلاف البروتينى لفيروس تبرقش الفاصوليا الأصفر و تحديد التتابع النيوكليوتيدى له. وقد اتضح بمقارنة التتابع النيوكليوتيدى لجين الغلاف البروتينى للعزلة المصرية مع التتابع النيوكليوتيدى لنفس الجزء من جين الغلاف البروتينى لعزلات فيروس تبرقش الفاصوليا الأصفر الأمريكية و الهولندية و اليابانية أن جين الغلاف البروتينى ثابت بدرجة عالية مع تطابق ملحوظ فى التتابع النيوكيوتيدى بنسبة تتراوح من ٩٠-١٠٠ %.

و قد أظهرت النتائج أن التتابع النيوكليوتيدى للعزلات الأمريكية و الهولندية يتطابق تماما مع التتابع النيوكليوتيدى لجين الغلف البروتينى للعزلة المصرية لفيروس تبرقش الفاصوليا الأصفر مما يوضح أن هذه العزلات قد تكون نتجت من أصل مشترك. و قد تم تطبيق استخدام طريقة تهجين الحامض النووى باستخدام مجسات معلمة ب **Digoxygeninn-labeled** للكشف عن وجود الفيروس فى كمية صغيرة جدا من انسجة نبات الفول البلدى المصاب (٣٠ ميكروجرام).

وقد تبين أن التقنيات المستخدمة فى هذه الدراسة دقيقة و سهلة الأجراء والتطبيق لتشخيص فيروس تبرقش الفاصوليا الأصفر فى أصناف الفول البلدى المستوردة. و يمكن تشخيص هذا الفيروس الآن بطريقة ايجابية فى أنسجة الفول البلدى المصابة خلال ساعات قليلة بدلا من الأنتظار شهور حتى تظهر الأعراض المرضية على العوائل المشخصة.