Zagazig J.Agric. Res., Vol. 29 No.(5) 2002 1591-1605

DEVELOPMENT OF A COMPETITIVE REVERSE-TRANSCRIPTION PCR FOR THE DETECTION AND QUANTIFICATION OF BEAN YELLOW MOSAIC VIRUS (BYMV)

El-Said[1] H. M.; M. A. Kheder [1]; M. El-Kady[2] ; Hayam S. Abdelkader[2] M. M. M. Atia[1] and Iman H. Khatab[2]

[1] Agriculture Botany Department, Faculty of Agriculture, Zagazig University.

[2] Molecular Biology Lab., Virus Department, Plant Pathology Research Institute, ARC.

Received 27 / 8 / 2002 *Accepted 7 / 10 / 2002*

ABSTRACT: Competitive Reverse-transcription PCR (RT-cPCR) was developed for the quantification of bean yellow mosaic virus (BYMV) RNA. The construction of an internal DNA standard as a competitor fragment was performed by DNA deletion within the wild-type template followed by cloning into pGEM-T-Easy vector. For the construction of this internal recombinant DNA-fragment, the only primers required are one composite hopping plus primer (P_{31}) composed of nucleotide sequences at positions, (472–489 and 606-623) and two primers used for the amplification of the target sequence of BYMV. During PCR the composite primer induces a net deletion of 116 bp in the BYMV competitor. Competitive RT-PCR depends on the co-amplification of the native target BYMV together with an internal competitor DNA that is structurally related to the target sequence. The advantage of this technique is that the variations in amplification efficiency affect both template and competitive DNA similarly. The concentration of BYMV copy number was calculated based on a standard curve that was generated by the co amplification of internal competitor ($pEHR_{314}$) and target sequences ($pEHR_{34}$) in serial dilutions. The detection limit of the Qc-PCR was $4x10^6$ copies per one gram of infected tissues. The assay is sensitive and reproducible, and will be useful for virological and pathological studies.

Key Words: **BYMV, Bean, RT-PCR, QC-PCR, CRT-PCR**

INTRODUCTION

Bean yellow mosaic virus (BYMV) is a flexuous, rod-shaped virus that belongs to the potyvirus group. The virus is transmitted through sap and by aphids in the nonpersistent manner. More than 20 aphid species are reported to transmit the virus including *Acyrthosiphon pisum, Aphis fabae, Aphis craccivora* and *Myzus persicae* (Bos, 1970).

To measure the BYMV load in infected faba bean plants, direct tests based on PCR (Qc-RT-PCR) should be implemented (Kidd and Ruano, 1995). The polymerase chain reaction (PCR) is a powerful tool to amplify small amounts of DNA or RNA for various molecular analyses (Saiki *et al.* 1985). The PCR primers designed for a region of the coat protein of BYMV were superior in sensitivity and specificity, because of relative conservation of this region (Young *et al.* 1997).

Competitive RT-PCR relies on a competition between a known amount of a competitor DNA and an unknown amount of a target sequence. Relative quantitative RT-PCR uses an internal standard to monitor each reaction and allow comparisons between different reactions to be made (Förster, 1994). A second set of primers is incorporated into the reaction for an invariant, housekeeping message. The difficulty here is matching the level of the internal message to that of the target so that one reaction doesn't dominate. Competitive RT-PCR avoids difficulties created by differences in the efficiency of the PCR reaction itself with different template/primer sets. A competitive template binds the same primers but has been altered in some way (i.e. small deletions or additions) to provide a product that is distinguishable from the target itself (De Francesco, 1998). We developed a competitive RT-PCR to quantitate BYMV/RNA using a competitor template for use with BYMV/CP target. This method provide a general scheme for producing a competitor template (RNA or DNA), identical to the target at its ends (the primers themselves) but shorter in size and, hence, there should be no differences in PCR efficiency. The enzymological principle of the quantitative competitive PCR (QC-PCR) was originally described in 1997 by Schnell and Mendoza proposing a theoretical framework that facilitated quantification in experimental methodologies. QC-

PCR is also very specific, sensitive, easy to perform, reproducible, and rapid compared with conventional methods. This RT-PCR based quantification methods of BYMV viral RNA will be useful for virological and pathological studies.

MATERIALS AND METHODS

Total RNA isolation: Total RNA was extracted from 30 µg of infected and uninfected faba bean leaves using SV Total RNA Isolation System from Promega Corporation, (Madison, WI.) 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, pH 7.5, 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.

cDNA synthesis: Serial dilutions of total RNA (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) were used as templates in reverse transcription reaction (RT) in order to synthesize complementary DNA_s (cDNA).

The moloney murine leukemia virus reverse transc-riptase (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 (M_4) CTAATAC-GAACACCAAGCAT 100 mM DTT and 40U RNase inhibitor as described by Shooman (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.

Detection limit of BYMV by PCR: Five microliters of each cDNAs were used as templates in the PCR process to measure the detection limit of BYMV/CP. The PCR reaction was performed in 100 µl total volume containing 2.5 units of Taq DNA polymerase (Promega) at 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.1% Triton-X-100, and 50 pm/µl of each forward (P_3) AATGC-AAAGCCAACATTC and reverse (M_4) CTAATACGAACACC-AAGCAT primers as described by Barker *et al.*, (1993); Hataya *et al.* (1994) and Shooman, (1997). The PCR cycles were repeated 35 times under the following conditions: denaturation at 94°C for one minute, annealing at 45°C for two minutes and extension at 72°C for two minutes. The PCR products were analyzed by 1% agarose gel electrophoresis and the size of the BYMV/DNA fragment was determined in accordance with the DNA molecular weight markers.

End-point titration and PCR-blot hybridization: The last dilution of RT-PCR product detected by agarose gel electrophoresis was further diluted into (1/2, 1/4, and 1/8) and PCR was performed on each dilution using the primer set P_3 and M_4 and five microliters of each PCR were spotted onto the nitrocellulose

ırane using the protocol bed by Koreschineck *et al.*). The membrane was ıred by using 0.5 M NaOH 5 min followed by lization in 1M Tris-HCl pH then 2X SSC and finally itated by ethanol 95% for 5 nd air dried. The DNA was linked under UV light for 2 before going into the lization process.

ybridization and hybrid-n: The prehybridization, lization, and colorimetric tion procedures were carried using the "Genius II DNA ng and detection kit" ıringer Mannheim IN m). The membranes were bridized for 1-3 hr at 65°C in dization oven (Techne-idizer HB-ID) with at least of prehybridization r/100 cm^2 (6.25 ml of 20X 0.25 ml of 10% N-sacrosine, 0.025ml of 20% 1.25 g blocking reagent). DNA probe was denatured by ıg in water bath for 5 min at C then placed immediately in ıol ice bath. Hybridization performed in about 20ml of ıdization solution containing 5 10ng freshly denaturized dig-dUTP labeled probe and overnight incubation in this solution at 65°C in a hybridization oven (Techne-Hybridizer HB-ID). The membrane was washed twice at room temperature for 5 min with 150 ml 2X SSC 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 1 gm 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 antibodyies were removed and the membrane was 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.

Producing of competitor BYMV /DNA: A competitor DNA molecule distinguished from the wild – type CP genome by a 116 bp deletion was constructed by using the hopping primer approach as described by Schnell and Mendoza, (1997); and Shooman (1997). The composite plus primer (P_{31}) is composed of two parts, nucleotide positions, (472–489 and 606-623) AATGCAAAGCCAA-CATTCGGCCAGGTATGCTTTG A and the M4 primer at nucleotide position (822–802) CTAAATA-CGAACACCAACGAT. During PCR this primers would induce a net deletion of 116 bp. Primers P_{31} and M_4 amplify 236 bp while P_3 and M_4 amplify 352 bp. This difference is suitable for resolving the PCR products (352 bp and 236 bp) on 1% agarose gel.

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 mixture and 50 pm/µl of each forward P_3: AATGCAAAGCCAACATTC and/or P_{31}: AATGCAAAGCC-AACATTCGGCCAGGTATGCT TTGACTAAATACGAACACCA ACGAT and reverse (M_4) CTAATACGAACACCAAGCAT primers as described by Barker *et al.*, (1993); Hataya *et al.* (1994) and Shooman (1997). The PCR cycles were repeated 35 times under the following conditions: Denaturation at 94°C for one minute, annealing at 45°C for two minutes and extension at 72°C for two minutes. The PCR products were analyzed by 2% agarose gel electrophoresis and the size of the BYMV/ DNA fragment was determined in accordance with the DNA molecular weight markers.

Cloning of BYMV/CP gene (wild and competitor): The PCR products of both the wild type BYMV P_3M_4 (352 bp) and the BYMV-DNA competitor $P_{31}M_4$ (236bp) were directly cloned into pGEM-T-Easy cloning vector as described by Sambrook *et al.* (1989), and Shooman, (1997) as shown in Fig. (1). Ligation reactions were incubated overnight at 16°C and contained equi-molar amounts of PCR products (inserts) and vector DNAs. Ligations were

conducted at 300 mM Tris-Cl, pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP and 3 Weiss units/ µl T4 DNA ligase. The ligation reactions were transformed into JM109 strain of *E.coli*. Positive clones were identified by blue/white selection, and *Eco*RI restriction endonuclease digestion. The plasmid DNAs were extracted by using Wizard plasmid mini-preparation system from Promega to be used in co-amplification quantification protocol.

Co-amplification of both target and competitor BYMV plasmids: Wild type of BYMV/DNA pEH_{34} was titrated against varying known amounts of mutant $pEHR_{314}$ (competitor) DNA in competitive PCR assay. Wild type- and competitor DNAs of BYMV coat protein were co-amplified by PCR in a final volume of 50 µl containing 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 0.1 % Triton X – 100, 200 µM each dNTPs, 1.5 µM $MgCl_2$, 1µM of both plus primers (P_3 and/or P_{31}), 1 mM minus primer (M_4), 1.25 units of *Taq* DNA polymerase. The PCR cycles were repeated 35 cycles under the following conditions: Denaturation at 94°C for one minute, annealing at 55°C for two minutes and extension at 72°C for two minutes. The PCR products were analyzed by 2% agarose gel electrophoresis and the size of the wild-type target DNA and the mutated DNA PCR fragments were determined in accordance to the distance between each of the two primers used.

RESULTS

End-point titration of BYMV RNA by RT-PCR: The sensitivity of the RT–PCR assay was evaluated using serial dilutions of RNAs. Using the UV– ethidium-bromide-gel detection scheme, the minimal detectable amount of RNA was at RNA dilution of 10^{-4} which is equivalent to 150 ng of leaf tissues. This is equivalent to the detection of *i.e.* $6X10^5$ copies per one gram of infected tissues (Fig 2).

Titration of BYMV- RNA by PCR-blot hybridization: The minimal dilution (10^{-4}) of RNA detected by end-point RT-PCR and agarose gel electrophoresis was used to estimate the detection limit of BYMV in infected tissues. The detection limit was at 1/8 dilution, which is equivalent to 18 ng fresh tissue which is equivalent to 4.8 $X10^6$ viral particles per one gram-infected tissue. The DNA probe

gave no signal with extracts of healthy faba bean leaves (Fig. 3).

Quantitation of BYMV RNA by Competitive PCR: The BYMV competitor DNA is flanked by the same primers (P3 and M4) that are used to amplify the wild-type BYMV, but constitutes a shorter amplicon (236 bp) than the wild-type target BYMV/CP (352 bp). The two amplicons were cloned into pGEM-T-Easy vector and quantified by co-amplifying the target BYMV/CP ($pEHR_{34}$) and known amounts of internal competitor $pEHR_{314}$ BYMV (Fig. 4). The resulting amplicons were separated on an agarose gel and stained with ethidium bromide. Both bands of target and competitor were quantified and the yields of the two products were compared by plotting the log ratio of the competitor PCR product to target PCR product against the amount of internal competitor used in the Qc-PCR on the x-axis. The ratio plot was extrapolated to r=1, which is the ratio of equal molar amounts of target and competitor BYMV/PCR products.

This equivalence point gives the initial amount of target BYMV in the sample. The equivalence point in the agarose gel electrophoresis was at 10^4 copies of mutant competitor DNA. Therefore, the sensitivity of detection of BYMV was about $4x10^6$ virus particles per gm of infected tissues (Fig. 4).

DISCUSSION AND CONCLUSION

The SV Total RNA Isolation System has been used successfully to isolate RNA from faba bean leaves infected with BYMV as described by Brisco *et al.* (1998). Brisco, *et al.* (1996) used the same method to isolate the total RNA from leaves of tobacco, tomato and *Arabidopsis*. The successful isolation of intact RNA obtained was due to the effective powdering of tissues by liquid nitrogen, and, denaturation of nucleoprotein complexes by the addition of guanidine thiocyanate in association with sodium dodecyl sulphate, precipitation of the RNA by ethanol on the silica surface of glass fibers, inactivation of endogenous ribonucleases, and removal of contaminating DNA and proteins. This procedure yields an essentially pure fraction of total RNA after only a single round of purification without organic extraction or precipitations. The yield of total RNA obtained was determined by

spectrophotometry at 260nm, to be 80μg of RNA/30 μg of tissues.

The polymerase chain reaction (PCR) is an extremely sensitive method owing to the repetitive multiplication of template molecules. This property is a drawback for quantitative measurements because small differences in the multiplication factor lead to large differences in the amount of product (Raeymaekers, 2000). Vunch *et al.* (1990) used RT-PCR to detect low concentration of BYMV in total RNA extracts of infected gladioli leaves. They reported that, combination PCR with molecular hybridization using ^{32}P-labeled probe increased the sensitivity of this method to four to five orders of magnitude as compared to direct molecular hybridization. This enables the detection of up to single picogram quantities of the virus. Theses results were in agreement with our results obtained from combination of end-point titration and PCR-blot hybridization assay using digoxigenin-11-dUTP BYMV/DNA probe. The minimal RNA dilution required to detect BYMV by RT-PCR amplification was (10^{-4}) which is equivalent to 150 ng of leaf tissue, i.e. $6x10^5$ particles/gm of infected tissue. When, the last PCR product detected by agarose gel was serially diluted into 1/2, 1/4, and 1/8 and PCR was performed on each dilution, the detection limit of the PCR-blot hybridization assay was at dilution 1/8 which is equivalent to 18ng of fresh tissues, i.e. 4.8 X 10^6 virus particles per one gram of infected tissues

Two methods have been reported to solve the problem of quantification: Kinetic method based on determination or comparison of the amplification factor; and coamplification method, which compare the amount of competitor to that of simultaneously amplified target template (Orlando *et al.* 1998).

The Qc-PCR is an advanced assay for the quantification of copy number and gene amplification of viral particles present in infected tissues without the requirement of large number of samples. Micheal *et al.* (1993) used the quantitative competitive PCR for the accurate quantification of HIV (Human Immunodeficiency Virus type-I) DNA and RNA species in order to determine viral load and for the overall assessment of clinical status of HIV-type 1 infected patients.

Qc-RT-PCR technique enables laboratory staff to quantify

gene expression more cheaply and more quickly than the classical technique (Möller, *et al.* 1998). In addition, one saves on samples, which is becoming more and more important as, often, only micrograms of infected tissues are required. These advantages can be gained without any lack of specificity and at a very high level of PCR sensitivity (Förster, 1994).

We successfully used the Qc-RT-PCR approach to quantify BYMV in small amounts of infected tissues and our results were as highly sensitive and specific as the smaRT-PCR quantification method described by Marten and Henning, (2001). The method used here to quantitative BYMV by Qc-RT-PCR could be also used in a variety of theoretical and clinical applications as the smaRT-PCR. However, our Qc-RT-PCR has several advantages over the smaRT-PCR. First, it can be performed in every PCR laboratory without the need for any additional equipment. Second, all components are already in use for classical PCR. Third, it is easier to automate procedures during PCR and the calculation of molecules.

The Qc-PCR technique described here is based on the generation of internal competitor BYMV clone (pEHR314) which completed within one day and standard PCR equipment was also used. The results showed that the co-amplification of the Qc-PCR was very efficient, and the ratio between target and competitor PCR products remained constant throughout the amplification. By employing this technique for BYMV quantification, we have shown that the present BYMV/RNA quantification was not necessarily limited to the exponential phase but was also reliable in the plateau phase of PCR. We have been able to accurately quantitate BYMV in a small amount of infected faba bean tissues at which the equivalence point in the agarose gel electrophoresis was at 10^4 copies of mutant competitor DNA. Therefore, the sensitivity of detection of BYMV was calculated $4x10^6$ virus particles per gm of infected tissues Fig (4).

In conclusion, our data on sensitivity, linearity and reproducibility demonstrated that the described competitive RT–PCR assay allows the relatively easy, accurate and absolute quantification of BYMV/RNA with a high sensitivity even for tissues, where the RNA is of low abundance. Because all of the steps of the procedure can be

performed by commercially available kits, the method in this report can be used easily even in laboratories with little experience in molecular biology.

REFERENCES

Barker H. ; Webster K. D. and Reauy B. (1993). Detection of potato virus Y in potato tubers : A comparison of polymerase chain reaction and enzyme linked immunosorbent assay. Potato Res. 36 : 13-20

Bos L. (1970): Bean yellow mosaic virus. Descriptions of Plant Viruses No.40. 4 pp.

Brisco, P., Brian Andersen and Craig Smith, (1996): A Rapid Protocol for the Isolation of Total RNA Suitable for RT-PCR. Promega Notes Magazine Number 55, p.10.

Brisco, P., Sankbeil, J. and Kephart, D. (1998): Promega Notes Number 67, p. 16.

De Francesco, (1998): Taking the measure of the message. The Scientist, 12 (23):20, Nov. 23

Erlinge D, Hou M, Webb TE, Barnard EA, and Moller S. (1998): Phenotype changes of the vascular smooth muscle cell regulate P2 receptor expression as measured by quantitative RT-PCR. Biochem Biophys Res Commun. Jul 30; 248(3):864-70

Förster E. (1994): An improved general method to generate internal standards for competitive PCR. Bio-Techniques,16:18-20.

Hataya T., Kazuko Inous A. and Shikata E. (1994): A PCR-microplate hybridization method for plant virus detection. J. Virol. Methods, 46 : 223-236.

Kidd, K.K. and Ruano, G. (1995): in *PCR2-a practical approach* (McPherson, M.J., Hames, B.D. and Taylor, G.R., eds), pp. 8–15, Oxford. University Press.

Koreschineck, I., Himmler, G., and Sagl, R. (1991): A PCR membrane spot assay for the detection of plum pox virus RNA in bark of infected trees. J. Virological Methods 31, 139-146

Marten, S. and Henning, M. (2001): SmaRT-PCR: a novel application of competitive PCR for mRNA quantitation. Institute of Path physiology, Martin Luther, University. Halle-Wittenberg, Magdeburger Str. 18, D-06097 Halle, Germany, Elsevier Science Ltd.

Mathuresh S.; Singh R. P. and Singh M. (1996): Factors affecting detection of PVY in

lormant tubers by reverse ranscription polymerase chain eaction and nucleic acid spot ıybridization. J. Virol. Method, ı0 : 47-57.

ıeal J. R.; Ka-Cheung; Iuk Bill V. and Jeffrey D. (1993): Quantitative polymerase chain eaction for accurate ıuantitation of HIV DNA and RNA species. Biotechniques, .4, 70-79.

ler S., Adner M. and Edvinsson L. (1998): Increased evels of endothelin ETB eceptor mRNA in human ›mental arteries after organ culture: quantification by competitive reverse ranscription-polymerase chain eaction. Clin Exp Pharmacol Physiol; 25(10):788-94

ındo C, Pinzani P, and Pazzagli M. (1998): Developments in ıuantitative PCR. Clin. Chem.Lab.Med., 36 (5):255-269. Review.

ymaekers L., (2000): Basic principles of quantitative PCR. Mol Biotechnol. Jun; 15 (2):115-22.

i RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, and Arnheim N. (1985): Enzymatic amplification of B-globin genomic sequencesand restriction site analysis for diagnosis of sickle cell anemia. Science, 230:1350-1354.

Sambrook J.; Fritsch E. F. and Maniatis T. (1989): Molecular Cloning: A Laboratory Manual, 2nd ed. New York: Cold Spring Harber Laboratory.

Schnell S. and Mendoza C. (1997): Enzymological considerations for a theoretical description of the Quantitative Competitive Polymerase Chain Reaction (QC-PCR). Journal of theoretical Biology 184, 433-440.

Shooman S. (1997): Studies on the molecular Biology of Broad Bean virus in Egypt. M. Sc. Thesis. Ain Shams University, Faculty of Science, Botany Department, 89 pp.

Vunch R., Rosner A. and Stein A. (1990): Detection of bean yellow mosaic virus in gladioli corms by the polymerase chain reaction. Annals of Applied Biology, 119: 289-297.

Young Y. H. ; Jeon J. H. ; Choi K. H ; Kim H. S. ; YI Y. S. and Young H. (1997): An effective detection of potato virus Y using RT-PCR technique. Korean J. of Plant Pathol., 13 : 219-224.

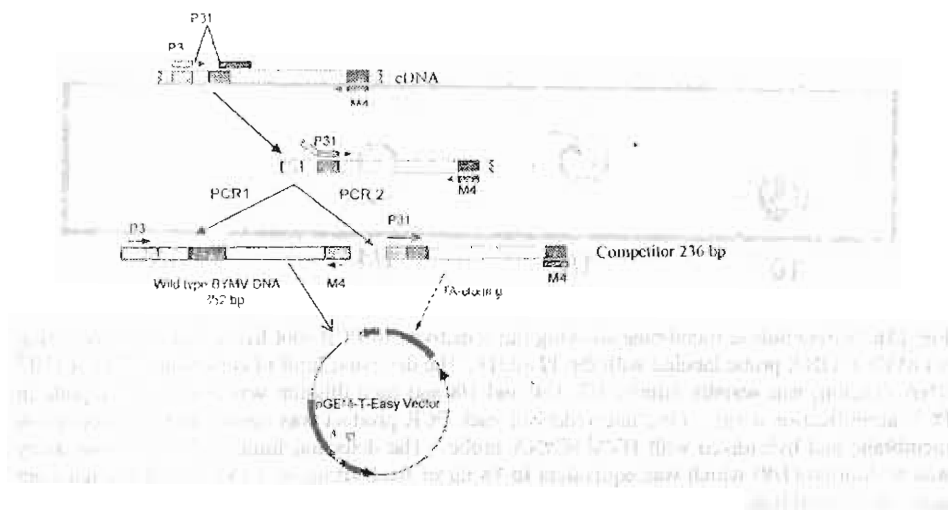

Fig. (1): Flowchart showing the generation of BYMV/DNA-competitor. The primers used are: P_3 as a forward primer, M_4 as a reverse primer, and P31 as a forward composite looping primer. After TA-cloning the competitor fragment and wild type BYMV fragment were co- amplified together in Qc-PCR experiment.

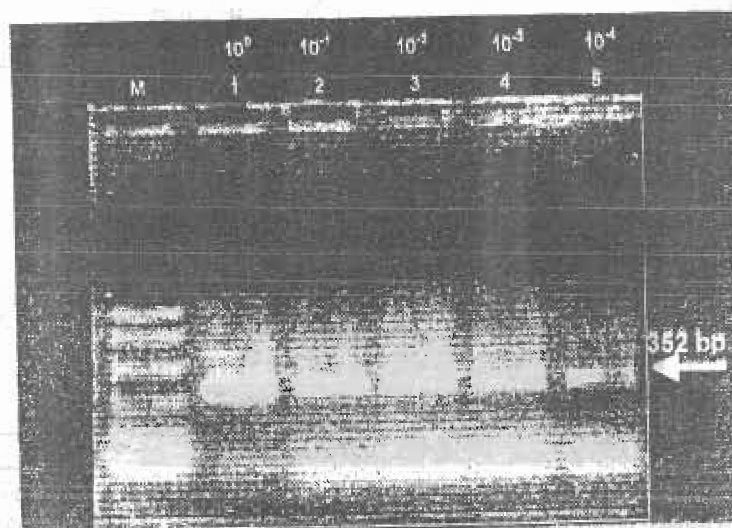

Fig. (2): Agarose gel electrophoresis showing PCR products of amplified BYMV/Cp gene (352 bp of the coat protein gene). Serial dilutions of total RNA were subjected to RT-PCR amplification (Lanes 1 to 5). The BYMV detection limit of RT-PCR is shown in lane 5 at dilution of 10^{-4}. This is equivalent to 150 ng of leaf tissue, $6x10^5$ viral particles/gm of infected tissue. M: Molecular weight DNA Marker. The arrow indicates the 352 bp of the correct size of the expected PCR product.

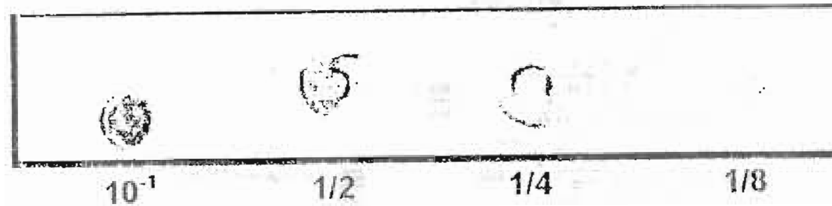

Fig. (3): Nitrocellulose membrane showing the sensitivity of PCR blot hybridization assays using BYMV/CP DNA probe labeled with dig 11-dUTP. The detection limit of end-point RT-PCR (10^{-4} RNA dilution) was serially diluted 1/2, 1/4, and 1/8 and each dilution was used as a template in PCR amplification assay Five microliters of each PCR product was spotted onto nitrocellulose membrane and hybridized with BYMV/DNA probe. The detection limit of the PCR-blot assay was at dilution (1/8) which was equivalent to 18 ng of fresh tissue or 4.8×10^6 viral particles per gram of infected tissue.

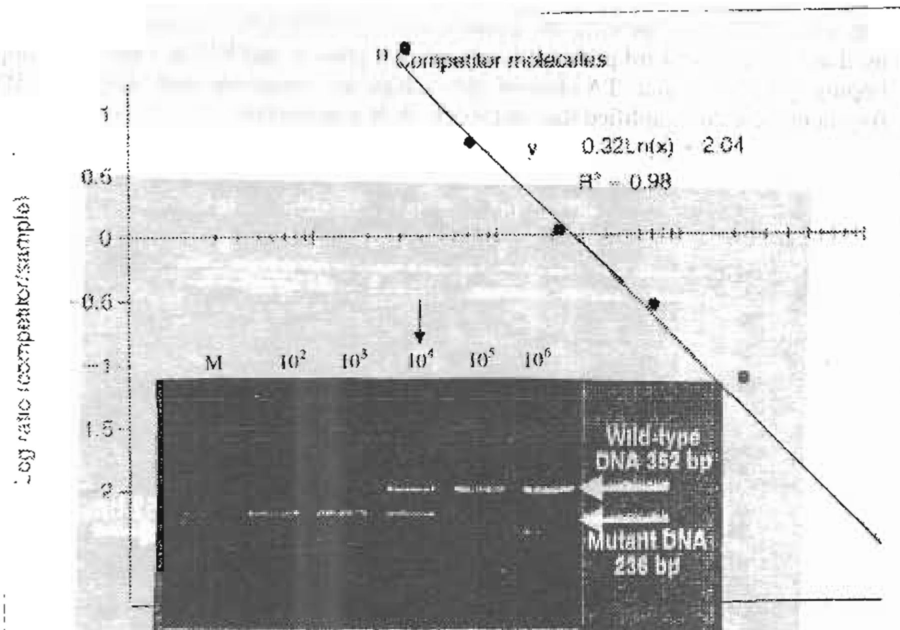

Fig.(4). Competitive RT-PCR was performed as described by Erlinge *et al.* 1998). The PCR products were separated on a 2% agarose gel (Target and competitor). The ratio of competitor BYMV/DNA (236 bp) and target (352 bp) in each reaction was plotted against competitor copy numbers. Using linear regression, the equivalence point (log ratio=0) was determined at 10^4. The copy number of BYMV/DNA molecules in the target) was determined as 4×10^6 copies/1gm of infected tissues. M=PCR size marker.

تطوير طريقة النسخ العكسى التنافسى- تفاعل البلمرة المتسلسل لاستخدامها فى التقدير الكمى والكشف عن فيروس تبرقش الفاصوليا الأصفر

[1]هانى محمد السعيد- [1]محمد عاطف خضر- -[2]مصطفى القاضى- [2]هيام سامى عبد القادر، [1]محمود محمد محمد عطية و [2]ايمان حسن خطاب

[1] قسم النبات الزراعى -كلية الزراعة -جامعة الزقازيق

[2]معمل البيولوجيا الجزيئية - قسم بحوث الفيروس -معهد بحوث أمراض النبات مركز البحوث الزراعية.

تم تطوير طريقة النسخ العكسى التنافسى و تفاعل البلمرة المتسلسل فى التقدير الكمى للحامض النووى الريبونيوكليك اسيد لفيروس تبرقش الفاصوليا الأصفر. تم بناء سلسلة من التتابع النيوكليوتيدى تعرف بـالجزىء المنافس Competitor وذلك بعمل طفرة عن طريق ازالة Deletion جزء من التتابع النيوكليوتيدى للجزىء الطبيعى Wild-Type ثم كلونته داخل الناقل pGEM-T-Easy Vector ويحتاج لبناء هذا الجزىء المنافس الى بادىء واثب مركب composite hoping primer يعرف باسم P31 و هو مركب من تتابعات نيوكليوتيدية فى المواقع (٤٨٩-٤٧٢ و ٦٠٦-٦٢٣) و اثنان من البوادىء لأكثار جزء من التتابع النيوكليوتيدى لجينوم الفيروس الطبيعى Wild-Type وفى أثناء اجراء عملية تفاعل البلمرة المتسلسل نجد أن حجم ناتج الPCR فى جينوم الجزىء المنافس باستخدام البادىء المركب P31 يكون محذوفا منه ١١٦ قاعدة نيتروجينية. وتعتمد طريقة النسخ العكسى التنافسى وتفاعل البلمرة المتسلسل على الاكثار المشترك لجينوم كلا من فيروس التبرقش الأصفر الطبيعى (القالب) مع جينوم الجزىء المنافس و اللذان يكونان مرتبطان تركيبيا فى التتابعات النيوكليوتيدية.

و الجدير بالذكر أن مايميز هذه التقنية هو ان التباين فى كفاءة اكثار Amplification efficiency كلا الجزيئان (الطبيعى و المنافس) تؤثر على كلا منهما بنفس الدرجة. و قد تم حساب تركيز عدد نسخ جزيئات فيروس لفيروس تبرقش الفاصوليا بالاعتماد على المنحنى الناتج بواسطة الاكثار المشترك لكلا الجزيئان المنافس الداخلى (pEHR314) و الجزىء الطبيعى (pEHR34) فى سلسلة من التخفيفات . وقد بلغت حساسية تكنيك النسخ العكسى التنافسى و البلمرة المتسلسل فى التحديد الكمى للفيروس حتى $4x10^6$ فيروس لكل واحد جرام من النسيج المصاب. و يعتبر هذا الاختبار حساس وقابل للتطبيق و سوف يكون مفيدا فى الدراسات الفيروسية و المرضية المستقبلية.