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DEGENERATE-PRIMED POLYMERASE CHAIN REACTION FOR EARLY DETECTION OF FIG MOSAIC VIRUS

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ABSTRACT

Samples of naturally infected fig plants were collected from different gardens and greenhouses in Egypt, mostly in north coast of the western desert which extends from the west of Alexandria to the Marsa Matrouh. dsRNA was isolated from Viral, healthy and infected fig tissue. Reverse transcription reactions were performed. Universal primers of potyvirus were used in RT-PCR to amplify 969 bp. within the open reading frame of NIb and CP genes. Two degenerate primers were designed and used to amplify 374 bp. within the open reading frame of the coat protein gene of FMV (CP-like gene). The two amplified PCR products of conserved and universal primers were sequenced using forward primers and sequence analyses. The present study introduced a candidate for effective, sensitive and reproducible diagnostic tools for screening plant tissues for potyvirus infections and may be important in controlling (preventing/ enhancing) potyvirus infection. The availability of CP-like products would be helpful in studies concerning ELISA, PCR and other related molecular techniques.

INTRODUCTION

According to FAO, the Mediterranean basin area is known to produce 80% of global production of fig. Turkey provides 27%, Europe 15% and Egypt 11% , in addition to the other countries. Fig enjoys high quality in almost all environments and does not require large production requirements. It has the ability to adapt and live in harsh environmental conditions. It also does not require large amounts of water, fertilizers and resistance to pests and diseases. These entire qualities make this tree occupies a prominent place and plant material worth study and attention.

The first known reports of fig mosaic disease (FMD) were made by Condit in 1922 and Swingle in 1928 (Alfieri, 1967), but the first critical study was conducted by Condit and Horne (1933). FMD has been widespread in several fig growing countries, including Egypt. Although it was not sap- or seed-transmissible (Martelli et al., 1993; Elbeaino et al., 2006), successful transmission of the disease by the eriophyid mite, Aceria ficus Cotte, has been reported by Flock and Wallace (1955).

Putative potyviruses were reported from Croatia (Grbelja, 1983) and then the pathogen was assumed as a member of the Potyviridae family by Brunt et al. (1996). After years earli-

er, some double membrane-bound bodies (DMBs) and rod-shaped virus particles (720 nm in size) showing a tail of 230 were described as possible agents of the disease by a Spanish scientist (Serrano et al., 2004). Double stranded RNAs (dsRNA) with a size ranging from 0.6 to 6.6 kb were obtained from infected trees in Turkey (Açıkgoz and Döken, 2003). The main objective of the present work is the early detection of the fig mosaic virus in infected tissues.

MATERIALS AND METHODS

Sample collection :

During summer 2008, samples from naturally symptomatized fig (Ficus carica) plants, exhibiting the characteristic fig mosaic symptom (chlorotic spotting of leaves), were collected from different gardens and greenhouses in north coast of the Egyptian western desert (from the west Alexandria to Marsa Matrouh). Healthy material was obtained from seedling, or by shoot-tip tissue culture.

Fig. (1): Infected and healthy fig plant leaves. The infected leaf (left) exhibited typical symptoms of FMD (chlorotic spotting of leaves). The healthy non-symptomized leaf exhibited homogenous distribution of chlorophyll.

Extraction of total RNA :

dsRNA isolation from fig tissue (healthy & infected) were done using RNeasy Mini Kit according to manufacturer's instructions (QIAGEN, Germany). The RNA was dissolved in DEPC-treated water, quantities spectrophotometrically and analyzed on 1.2 % agarose gel.

Extraction of vial RNA :

Viral RNA was isolated from partially purified virus preparations using QIAamp viral RNA isolation kit according to the manufacturer's instructions (QIAGEN, Germany).

Reverse transcription-polymerase chain reaction (RT-PCR) :

First-strand cDNA was synthesized using Moloney Murine Leukemia Virus reverse transcriptase (Fermentas, USA). Reverse transcription reactions were performed using primer oligo dT primer (5`-TTTTTTTTTTTTTT-3`). Each 25 µl reaction mixture containing 2.5 µl of 5x buffer with $MgCl_2$, 2.5 µl of 2.5 mM dNTPs, 1 µg of primer, 2 µg RNA, 200 U reverse transcriptase enzyme. RT-PCR amplification was performed in a Mastercycler Personal (Eppendorf, Germany) programmed at 42ºC for 1 hr, 72ºC for 10 min. cDNA was then stored at -20ºC until used.

Detection of FMV using universal primer of *Potyviruses* :

Universal primers of potyviridae designed by (Chen et al., 2001) were used to detect FMV in the infected tissue and partially purified virus. 2 µl randomly primed cDNA (from total RNA of healthy, infected tissues and viral

RNA) were added to 2.5 µl Taq polymerase buffer 10x (Promega, Madison, USA) containing a final concentration of 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM each specific primer and 0.2 µl Taq polymerase (5 U/ µl) in a final reaction volume of 25 ml. PCR conditions were initial denaturation at 95°C for 5 min, followed by 34 cycles at 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min. Final extension was at 72°C for 10 min. Amplification products were visualized in 1.5 % agarose gel run in 0.5x TBE buffer.

Detection of FMV using degenerate primers based on a conserved region within the coat protein

Based on a conserved region of the mosaic virus coat protein sequences available in GenBank, two degenerate primers were designed and used to amplify DNA fragment within the open reading frame of the coat protein gene for the infected tissues. The forward and reverse primers were designated as CP_1 and CP_2, respectively. CP_1 was (5'-ZAY GGX GAX GAZ CAZ GTG-3') and CP_2 was (5'AAZ GCX GCZ GCX ATY AAY-3'). 2 µl randomly primed cDNA (from total RNA of healthy, infected tissues and viral RNA) were added to 2.5µl Taq polymerase buffer 10x (Promega, Madison, USA) containing a final concentration of 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM each specific primer and 0.2 µl Taq polymerase (5 U/µl) in a final reaction volume of 25 ml. PCR conditions were initial denaturation at 95°C for 5 min, followed by 34 cycles at 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min. Final extension was at 72°C for 10 min. Amplification products were visualized in 1.5 % agarose gel run in 0.5x TBE buffer.

Nucleotide sequence and sequence analyses :

The two amplified PCR products of conserved and universal primers were sequenced using forward primer. Sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and model 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Blast search for alignment of the obtained sequence with the published ones was done using database of National Centre for Biotechnology Information (NCBI). These DNA fragments *(coat protein-like gene and NIb)* were deposited in GenBank under the GQ288368 and GQ871933 accession numbers, respectively.

RESULTS

Disease Incidence :

Fig mosaic symptoms were observed in all the fields surveyed. The average incidence was 99% and the resulting average severity was 50% with a standard deviation of 6.8%. A total of 30 fig cultivars were assessed, 99% of which exhibited symptoms of FMV.

Detection of FMV using universal primer of Potyvirus using RT-PCR :

Universal primers of Potyviridae, designed by (Chen et al., 2001), were used and successfully amplified 969 bp of nucleocapsid protein gene of FMV in the infected tissue. PCR analysis of the result results revealed that this DNA fragment was amplified within the orf of the nuclear inclusion body (NIb) and CP genes (Fig. 2).

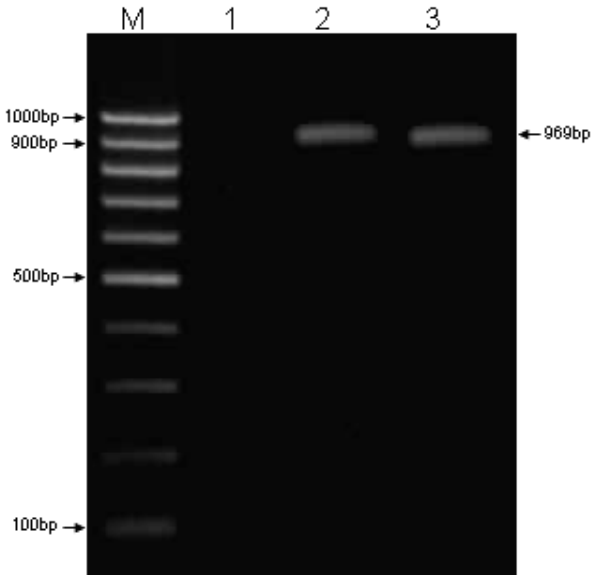

Fig. (2): RT-PCR for the NIb gene of the FMV amplified by universal primer of Potyviruses. Lanes; M. DNA marker 100 bp ladder, lane 1: healthy fig plant, lane 2: infected fig leaves and lane 3: partial purified virus.

Viral nucleic acid and RT-PCR amplification of a conserved region within the coat protein of FMV :

Neither dsRNA nor ssRNA extraction allowed identification of the genomic characteristics of the putative FMV. Only the two degenerate oligonucleotide primers designed to amplify a conserved region within the open reading frame (orf) of the coat protein gene of potyviridae were successfully used in RT-PCR. PCR analysis of our results revealed that a DNA fragment of only 374 bp was amplified within the orf (beginning about 1 - 146 codons after the starting codon, AUG) of the FMV coat protein (CP) gene (Fig. 3).

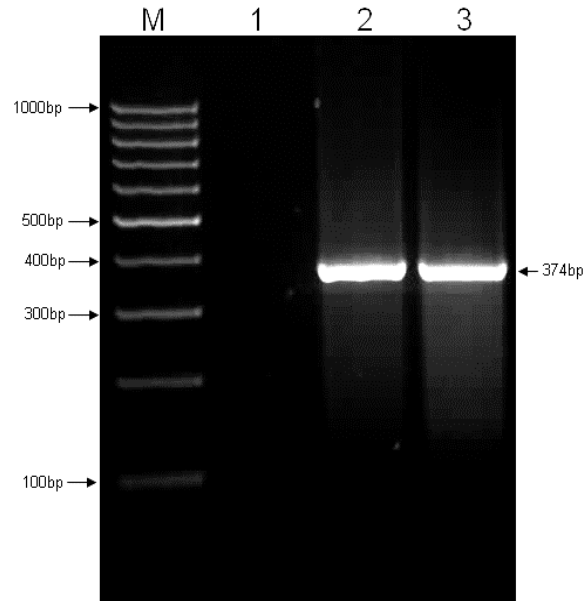

Fig. (3) : RT-PCR for the coat protein gene of the FMV amplified by degenerate primers. Lanes M: DNA marker 100 bp ladder, lane 1: healthy fig plant, lane 2: infected fig leaves and lane 3: partial purified virus.

Nucleotide sequence and sequence analyses of NIb gene

The nucleotide sequence of NIb was shown in Fig (4). A single open reading frame (orf) that could encode a polypeptide of 333 amino acids was detected. One stop codon was found all over the sequence (end of NIb and beginning of CP).

AGA TGG CGG GCT CTG AAC CTT GTT CCC TCC ACA CGA
GCC GCC GAC GCG TCG TGG TGG CTG TGC GCG AGT TTA
CCT CGC GCG CGG AGA AAA CGC GGG CGG GGC GCG GGC
CGC GCC GTG TTG TGG TTG GAG AGA AAT AGA GAG CAC
GCG CGC CGA GTG AAT GTA ACA GCA TTT ATT ATA CAT
TGT GCG GGG CGC CCC CTC CAT TCC TAC CCT GTG CAC
GTA GCT GGC GGC GGG GTG TCC GTC CGT CCT CAC GTG
GTA CCC GAA GCT CTT CGC TGT GAT AAT CTG CGG AAC
ACC ACG GGA GAC CGT GTC CCG CCC GAC GAG CAA CAA
GTT CGT GGT GAG GTC TGG GTT GTG GAG AGT AGG GAC
ACG ATT AAC ATG CTC ACC ATC CTC ATG AAT GAG TTT
CCG CTC TTA TGG CTC TGT TCT GCG GCT ACG CTG CTA
CCC GAT TGT GTC AAT ACG GGT CTG GCA GAA GCA TTA
AGG GCC TGG CAC ATA TCC TCT CAT GTA CTG GAT GTG
TCG CTA TGG AGT TCT GCA TCC GTC CAG TTA AAT TAG
ATA CTA ACT TAT CAC CAC CCT ACT CCT GCG CTC TCT
CGG TCC GGC TTA TGT ATG TGG CTT AAT ATA CCC CTG
TAC CAC ACC CTT GAA CAG GTG ACG TTA GTG ATA TCG
AGG CTT ACC TCT GCT TCA GGG GAG CTC TGT GGG AGT
GAC TCA TAC TTC AGT ATA TAT ATT CAG CTG CTG CGT
CTA ACT CCC GTC CTT CGA ATA ATG GGA GCG TCT CTT
ATT GCG CTT GGA GAG GTC CCT CTA GTC TGC TTA GAG
GTG TGT ATT GTT CTC TTG ACG ATT TGC GCT ATC TTC
TCT TCG TGT TAT AGT AGA GTC ATC TAT AAA TCT GCG
CTC TAT GAG GAG TTA TTG AAG GAG GTG GGC AGA GAA
ACT CTC TAT TAC TTG GCT ACA AAA GCT GAA GAT GAA
TGC TTC TCT ACA GTA GGC ACA CTC TTG TCT CCT

Fig. (4) : The nucleotide sequence of NIb gene.

Nucleotide sequence and sequence analyses CP gene :

The nucleotide sequence of CP was shown in Fig (5). A single open reading frame (orf) that could encode a polypeptide of 124 amino acids was detected. No stop codon was found all over the sequence. The nucleotide sequence of CP-like gene was blasted in GenBank database and compared to all available sequences.

TCT GGA TCT GAC GTA CGG ATC CAG TAG CTG ACT GCA
GCG TAG CAG TCG TAC GCA GAT GCG AGT AGC CAG TCC
GCA ATG CCA TGC TGC TGA TCA GCG TCC AGG ATG CAG
TAC AGG TAC TGT ATC AGT CAG GTC AGG CAT TGC ACG
CTA CGC CGT ACT ACA GTA AGT CAC GGA CTG CTA GTC
CAG GCA CGT AGC GTC GAT AGC ATC GTA GAT GAG ACT
GAT AGT AGA TTC GGT ACG ACT TGC ATG ACT ACG TGA
GGC ATG ATC AGA TAC TGG CTT AGT GAC TGA TGC ACG
TCA CGC TAG GAT CTG CGT AGC TCG ATC AGC CCG TAT
CGC GAT CCG TCG ATA GTC AGT CTA CGC ACC ATA GAC
CTC GTG TAT GAC GA

Fig. (5) : The nucleotide sequence of CP-like gene.

DISCUSSION

According to FAO, the Mediterranean basin area is known to produce 80% of global production of fig. Turkey provides 27%, Europe 15% and Egypt 11% in addition to the other countries. In the present work, a novel primer set was developed to amplify 374 bp within the CP-like gene from both infected tissue and partial purified virus. This amplified product represents more than 8% of the whole genome. It is sufficient sequence to determine the species of the virus and thus potentially to identify unrecognized potyviruses. One major problem with degenerate primers is that the concentration of some permutations in the mixture is so small, due to their great multiplicity, that amplification is effectively inhibited. For any given viral RNA target only a proportion of the primer may participate in the initiation of high efficiency extension in the early rounds of PCR. It was believed that the redundancy of the CP1 and CP2 was insufficient to cause this problem (Knoth et al., 1988). Traditional serological methods based on neutralization and fixed cell ELISA have proven effective for identifying potyviruses and indeed classifying them. However, some were not classified using this technology due to difficulties in interpreting antigenic cross reactivity or failure to identify

relatively close antigenic relationships that depend on epitopes encoded by regions of the genome that do not reflect the serological tests. Moreover, serology is time consuming, requires highly experienced personnel and is less precise than nucleotide sequence determination.

Using molecular methods, it is now possible to analyze archival material and confirm the identification of tentatively identified viruses. The capacity of the CP1 and CP2 primers to potentially amplify large numbers of potyviruses makes them valuable diagnostic and taxonomic tool for virology.

CONCLUSION

In this paper, a novel primer set was developed to amplify 374 bp within the CP-like gene from both infected tissue and partial purified virus of an Egyptian FMV isolate. To our knowledge, this is the first report that designs primers to amplify the sequence of this conserved region in an Egyptian isolate. Further studies to develop kits for ELISA, Western and dot blotting, hybridization as well as potential biocontrol agent are switched on. The availability of CP-like products would be helpful in studies concerning ELISA, PCR and other related molecular techniques. In addition, it provides a candidate for effective, sensitive and reproducible diagnostic tools for screening plant tissues for potyvirus infections and may be important in controlling potyvirus infection. Also, it may be useful in monitoring the distribution of FMV, the fate of genes and release of wild type as well as genetically engineered FMV. Furthermore, it will facilitate risk assessment, ecological and viral epidemiological studies.

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الملخص العربى

تخليق بادىء جديد للكشف عن ڤيروس تبرقش نبات التين باستخدام تفاعل البلمرة المتسلسل

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تم جمع عينات التين المصابة طبيعياً من مختلف الحدائق والمزارع الزراعية فى مصر، ومعظمها فى الساحل الشمالى للصحراء الغربية التى تمتد من غرب الإسكندرية إلى مرسى مطروح، كما تم عزل الحمض النووى أر إن إيه (RNA) من الڤيروس وأنسجة التين السليمة والمصابة، وبينت الدراسة الجزيئية التى تمت على الجينوم الڤيروسى باستخدام اختبار النسخ العكسى (RT-PCR) وتفاعل البلمرة المتسلسل أن البادىء المتخصص لمجموعة بوتى ڤيروس (*Potyvirus*) له القدرة على التعرف على جزء الحمض النووى دى إن إيه (cDNA) وتكبير منطقة ٩٦٩ زوجاً من القواعد النيتروچينية لكل من الڤيروس وللنبات المصاب فى حين لم يظهر النبات السليم أى تفاعل.

وباستخدام البادىء المخلق والمصمم فى هذه الدراسة تم تكبير ٣٧٤ زوجاً من القواعد النيتروچينية داخل چين الغلاف البروتينى للڤيروس (*Coat Proteingene*) وأعطى نتيجة إيجابية مع كل من الڤيروس والنبات المصاب فى حين لم يظهر النبات السليم أى تفاعل.

هذه الدراسة قدمت طريقة فعالة وحساسة وقابلة للتكرار كأداة من الأدوات التشخيصية لفحص الأنسجة النباتية المصابة بڤيروسات مجموعة potyvirus، كما أنه من الممكن استخدامه فى مكافحة العدوى الڤيروسية لنبات التين.

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