Development of a highly sensitive multiplex reverse transcription-polymerase chain reaction (m-RT-PCR) method for detection of three potato viruses in a single reaction and nested PCR

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ABSTRACT

Potato virus Y (PVY), potato virus X (PVX) and potato leaf roll virus (PLRV) are the most economically important viruses in commercial potato production in Egypt. The m-RT-PCR assays were used for the detection of these three viruses in one reaction using three specific primer pairs designed to amplify the full-length of the coat protein gene of each virus (810 bp for PVY, 700 bp for PVX, 548 bp for PLRV). Specificity of the three primer pairs was confirmed by the nested PCR (nPCR) assay using three internal specific primer pairs designed to amplify 480, 360, and 420 bp from the internal sequence of the cp gene of PVY, PVX and PLRV, respectively. The amplified products were sequenced and analyzed. This m-RT-PCR method detected all three viruses in a single reaction mixture in naturally infected field grown potatoes and nPCR assay can confirm the presence of virus infection without needing for sequence analysis. The m-RT-PCR assays developed is a reliable, rapid and sensitive method for the detection of these three viruses in one plant sample. The use of the m-RT-PCR and nPCR assays are recommended for applications Since they improved sensitivity over standard RT-PCR and necessary for the early detection of infection and for quarantine and breeding purposes.

***Key words**: Potato, PVY, PVX, PLRV, m-RT-PCR, nPCR, sequence analysis.*

INTRODUCTION

Potato *(Solanum tuberosum* L.) often becomes infected with two to three different viruses (McDonald, 1984), resulting in a decreased quality of planting potato tubers. The most common viruses affecting potato crops throughout the world are potato virus Y (PVY-potyviruses), potato virus X (PVX-potexvirus) and potato leaf roll virus (PLRV-luteovirus) (Beemster & Rozendaal, 1972; Singh, 1999). In Egypt, potato is considered as one of the most important and economic vegetables that is attacked with different viral diseases causing a serious damage on potato production (Shalaby, 1993; Shalaby *et al.*, 1997a,b; Soliman *et al.*, 2000). Polymerase chain reaction (PCR) and reverse

transcription PCR (RT-PCR) have been used for detection of DNA (Lopez-Moya *et al.*, 1992; Shalaby *et al.*, 1997c) and RNA viruses (Hadidi *et al.*, 1995; Singh, 1998; Shalaby *et al.*, 1999). Multiplex PCR is used if more than one virus is amplified simultaneously in a single reaction, resulting in considerable savings of both time and effort (Chamberlain *et al.*, 1988). Multiplex polymerase chain reaction (m-PCR) accommodates several pairs of primers in one reaction, resulting in reduced material costs and time when compared to several individual PCR reactions. Amplification of RNA templates, requiring reverse transcription (RT) prior to amplification by PCR. m-RT-PCR, has been limited to five viruses using purified RNA (Bariana *et al.*, 1994) and two or three viruses using total nucleic acid extract (Singh *et al.*, 1996; Jacobi *et al.*, 1998). Furthermore, during RT ascertaining the desired concentrations of various reagents and primer combinations, significantly affecting the subsequent amplification process, it is a lengthy process (Singh *et al.*, 2000). Multiplex - polymerase chain reaction (m-PCR) has been developed for some plant viruses, and allows the detection of more than one virus in a single PCR (Bariana *et al.*, 1994; Minafra & Hadidi, 1994; Grieco & Gallitelli, 1999). In efforts to reduce extensive yield losses due to viral diseases in subsequent potato crops, seed tubers are tested for the presence of viruses and the resultant virus-free seed tubers are used for planting (Nie & Singh, 2000). The RT-PCR procedure has been developed to detect individual viruses from dormant tubers (Singh and Singh, 1996; Watts *et al.*, 1997 and Singh, 1998). Nested PCR means that two pairs of PCR primers were used for a single locus. The first pair amplified the locus as seen in any PCR experiment. The second pair of primers (nested primers) bind within the first PCR product and produce a second PCR product that will be shorter than the first. The logic behind this strategy is that if a wrong locus was amplified by mistake, the probability is very low that it would also be amplified a second time by a second pair of primers (Morris *et al.*, 2001). This paper describes the developing and application of multiplex RT-PCR for the simultaneous detection of three viruses of potato (PVY, PVX and PLRV) in infected potato samples in one step reaction and confirmed with nested PCR assay and sequence analysis.

MATERIALS AND METHODS

Virus isolates

Samples of PVY, PVX and PLRV infected plant tissues and tubers were collected locally and stored at -80°C or as a freeze-dried material at -20°C for subsequent studies.

Extraction of total nucleic acids

Viral RNAs were extracted from potato samples infected with potato viruses (PLRV, PVX and PVY) using RNeasy Plant Mini kit (QIAGEN® cat# 74903) according to manufacturer instructions.

Virus specific primers

Primers were designed for the full length of the coat protein gene for PVY, PVX and PLRV according to available sequences from the GenBank of the National Center for Biotechnology Information and synthesized in Life Tech. Inc., Rockville, MD, USA (Table 1a). On the other hand, The new antisense and sense primers used in nested PCR for PVX and PLRV were designed to amplify 360 and 420 bp respectively from the full length of the coat protein gene for PVX and PLRV. Primers designed by Singh *et al.* (1995) were used to amplify a fragment of 480 bp from the coat protein gene of PVY. Table (1) shows the primer sequences, primer positions and the

expected amplified fragment in m-RT-PCR and n-PCR.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA extracted from infected potato material was used as templates for one-tube RT-PCR amplification reactions (one-Step RT-PCR system, QIAGEN®) for each virus. The sequences of the different primers used are listed in Table (1a). The following cycling scheme was used: 30 min at 50°C and 15 min at 95°C (for RT Reaction) followed by 30 cycles, each cycle consisted of denaturation 94°C (1 min), primer annealing at 55°C (1 min) and primer extension at 72°C (1 min), with final step for 10 min at 72°C.

Multiplex reverse transcription polymerase chain reaction (m-RT-PCR)

First, three primer mixes (Table 1a) were made up with each primer at a final concentration of 0.45 µM. The primer mixtures could be stored frozen at -20 °C or for long term storage, freeze dried for resuspension to the same concentration at a later date. The one step RT-PCR kit and protocol (QIAGEN) was used according to Manufacturer instructions as follows: Fifty nanograms of viral RNA in 5 µl was mixed with 10 µl of 5x buffer, 2 µl of dNTPs mix, 10 µl of 5x Q-solution, 3 µl of 10 µM of each viral sense primer, 3 µl of 10 µM of each complementary primer, 2 µl of QIAGEN enzyme mix, 0.2 µl of RNase inhibitor and water up to 50 µl. The one-step RT-PCR mixture varied depending on the number of intended viruses to be detected and the number of primers involved. The PCR parameter starting with 30 min at 50°C and 15 min at 95°C (for RT Reaction) followed by 30 cycles, each cycle consisted of denaturation at 94°C (1 min), primer annealing at 55°C (1 min) and extension at 72°C (1 min), with final step for 10 min at 72°C.

Nested polymerase chain reaction (nPCR)

Nested PCR was performed for simultaneous detection and confirming potato viruses from plant materials. For the first amplification, each external primer (Table 1a) and the cycling scheme used before in RT-PCR amplification were applied. For the second amplification, 0.5 µl of the amplified product from the first round of amplification was used as a template were added to the reaction mixture containing each of internal primers (Table 1b). The reaction mixture contained 250 mM dNTPs and 0.5 U of Taq DNA polymerase in a final volume of 50 µl. Thermocycling was performed as follows; 30 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 2 min, followed by 72°C for 7 min. In all reactions, healthy plant material and sterile distilled water controls from the first round of PCR were used. All PCR experiments were done in a PCR system UNO II - Thermoblock Cycler (Biometra Germany).

Electrophoresis analysis

Amplified products were electrophoresed on 1.0 % agarose gels, stained with ethidium bromide and photographed under UV light using Gel Documentation System (GELDOC 2000, Bio-Rad, USA) and determined using the 1 kb DNA Ladder Molecular Weight Marker (Promega Corp., Madison, WI).

Table (1): The oligonucleotide sense and antisense primer pairs used for m-RT-PCR and n-PCR.

a- The oligonucleotide sense and antisense primer pairs used for m RT-PCR			
Primer name	Nucleotide sequence (5` to 3`)	Polarity	Expected amplicon size (bp)
Potato virus Y			
PVYCPv*BamH1*	5' TCAAGGATCCGCAAATGACACAATTGATGCAGG 3'	sense	801
PVYCPc*EcoR1*	5' AGAGAGAATTCATCACATGTTCTTGACTCC 3']	antisense	
Potato virus X			
PVXCPv*EcoRI*	5`-GATAGAATTCAGATGACTACACCAGCCAACACC-`3	sense	700
PVXCPc*NcoI*	5`-TACGCGTCGGTTCCATGGACGTAGTTATGG TGG-`3	antisense	
Potato leaf roll virus			
PLRVCPv *EcoR1*	5'AATAGAATTCTAATGAGTACGGTCGTGGTTARAGG 3	sense	548
PLRVCPc *NCo1*	5'AAAACCATGGCTATYTGGGGTTYTGCARAGCYAC-3	antisense	
b- The oligonucleotide sense and antisense primer pairs used for n-PCR			
Potato virus Y (*Singh et al., 1995*)			
PVY 3S	5`-ACGTCCAAAATGAGAATGCC- 3	sense	480
PVY-A4	5`-TGGTGTTCGTGATGTGACCT-3`	antisense	
Potato virus X			
PVXv1	5`- GAYACNATGGCNCARGCNGCNTGG-3`	sense	360
PVXc2	5`-YTGNGCNGCRTTCATYTCNGCYTC-3`	antisense	
Potato leaf roll virus			
PLRVv1	5` GTNCARCCNGTNGTNATGGTNAC 3	sense	420
PLRVc2	5` RTGCCAYTCNACNCCRTTDATCAT 3	antisense	

CP = coat protein gene; v = Viral sense primer, c = Complementary sense primer, Restriction enzyme sites are underlined.

Cloning of the amplified fragments

DNA amplified fragments were ligated directly into pGEM®-T Easy plasmid (Promega Corp., Madison, WI). *Escherichia coli* strain XL1-blue (Stratagene) was transformed with the recombinant plasmid. White bacterial colonies were selected based on the loss of β-galactosidase activity. DNA was prepared from selected white colonies, digested with *Eco*RI and fractionated on agarose gels. Restriction enzyme digestion, ligation reactions, transformation of *E. coli*, and other molecular biology techniques were done according to Sambrook *et al.* (1989).

DNA sequencing and computer analysis

The nucleotide sequences of viral DNA inserts were done at the University of Wisconsin, Biotechnology Center using ABI PRISM™ Dye Terminal Cycle Sequencing Ready Reaction Kit with AmpliTag® DNA Polymerase, FS. Sequencing was performed on 377 DNA Sequencer (Applied Biosystem Co.). Sequence information as stored, and multiple sequence alignments were done using DNAMAN sequence analysis software program (Lynnon Biosoft, Canada), with those of PVY, PVX and PLRV available in GenBank.

RESULTS AND DISCUSSION

Viral fragments from the three potato viruses (PVY, PVX and PLR) were successfully amplified using cDNAs primed by specific sense and antisense primers. Bands of expected approximate sizes for PVY 801 bp (lane 1), PVX 700 bp (lane 3), and PLRV 548 bp (lane 5) were observed in RT-PCR detection. Figure (1) shows the detection of potato virus Y (PVY), potato virus X (PVX) and potato leaf roll virus (PLRV) individually by RT-PCR and agarose gel electrophoresis analysis. Lane M, 1 kb DNA ladder; lanes 1, 3 and 5 healthy potato; lane 2, 4 and 6 potato infected with PVY, PVX and PLRV, respectively. Fragments of PVY 801 bp (lane 2), PVX 700 bp (lane 4), and PLRV 548 bp (lane 6) were amplified by their specific primer pairs as shown in Table 1a. Theoretically, any part of a viral genome can be amplified by RT-PCR if full length cDNAs are used, because the poly (A) is located at the 3` end of the viral genome. However, the genomes of the viruses of interest are usually several thousands bases long, and reverse transcription might be terminated prematurely, resulting in cDNAs of various lengths, starting from the 3` end of the genome. RT-PCR determines the size of the cDNA, as well as defining regions that would be most suitable for diagnosis of viruses under tesing (Puurand *et al.,* 1994). Using the optimized m-RT-PCR protocol, all three viruses were detected from the viral samples and the three amplified fragments, shown in Fig. 2, were located at different part of each of the PVY, PVX and PLRV genome, e.g. PVY (801 bp), PVX (700 bp) and PLRV (548 bp). Successful amplifications of all three fragments in one reaction and one lane were observed when cDNAs primed by the specific sense and antisense primers. Fig. (2) shows that one to three viral fragments, representing PVY, PVX and PLRV, can be amplified from infected tissues and tuber samples, indicating the presence of these viral pathogens. Amplification of cDNAs primed by RT-PCR and m-RT-PCR methods were equal in band intensity. Thus, the protocol appears suitable

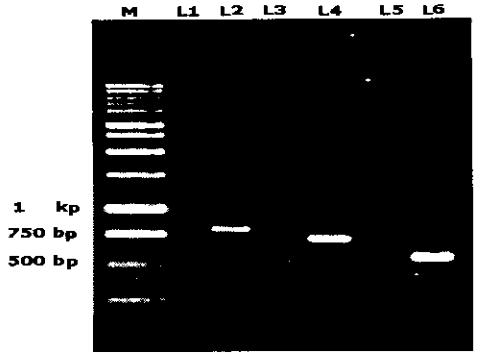

Fig. (1): Detection of potato virus Y (PVY), potato virus X (PVX) and potato leaf roll virus (PLRV) individually by RT-PCR and agarose gel electrophoresis analysis. Lane M, 1 kb DNA ladder; lanes 1, 3 and 5 healthy potato; lane 2, 4 and 6 potato infected with PVY, PVX and PLRV respectively. Fragments. of PVY 801 bp (lane 2), PVX 700 bp (lane 4), and PLRV 548 bp (lane 6) were amplified by their specific primer pairs as shown in Table 1a.

for application to leaves and dormant tubers containing a mixture of viruses. The multiplex RT-PCR saves time and reagent costs compared with monospecific RT-PCR technique, which requires several reactions for the same number of tests (Nie and Singh, 2000). Occasionally, this method has been used for some plant viruses (Pappu *et al.,* 1993; Gibbs and MacKenzie, 1997). It is concluded that the use of multiplex assays can facilitate multivirus detection in potato leaves and tubers, reducing procedural costs and time, and also increasing the number of viruses that

can be tested simultaneously. This method could be further extrapolated to detect viruses from other vegetatively propagated crops and horticultural trees, which are succumbed to multiple virus infection through many years of propagation. In addition, this method should

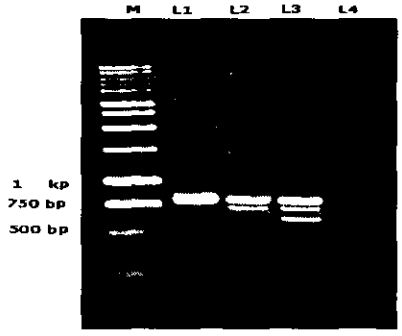

Fig. (2.): Detection of PVY, PVX and PLRV by RT-PCR and multiplex-RT-PCR from cDNAs primed by specific sense and antisense primers for each virus as shown in table 1. Lane M, DNA Ladder molecular size markers; the sizes in bp are indicated on the left hand margin. Lane 1, amplified PVY by RT-PCR, lane 2, multiplex RT-PCR in which two pairs of viral specific primers for PVY and PVX were present in the reaction and two viral RNAs and lane 3, multiplex RT-PCR in which three pairs of viral specific primers for PVY, PVX and PLRV were present in the reaction. three viral RNAs. The amplified products were of: PVY (801 bp), PVX (700 bp) and PLRV (548 bp).

prove useful for animal and fungal viruses, where a vast majority of them have adenylated sequences at their 3` end (Shatkin, 1974). Multiplex PCR is increasingly used because it improves the efficiency of diagnostic PCR (Johnson, 2000). In the near future, multiplex PCR will probably be adapted for the simultaneous detection of viruses of one particular crop and for the simultaneous detection of other major plant pathogens such as viruses, viroids, bacteria, fungi in the same reaction as already demonstrated for viruses and viroids (Nie and Singh, 2001). An increase in sensitivity would quite probably be achieved

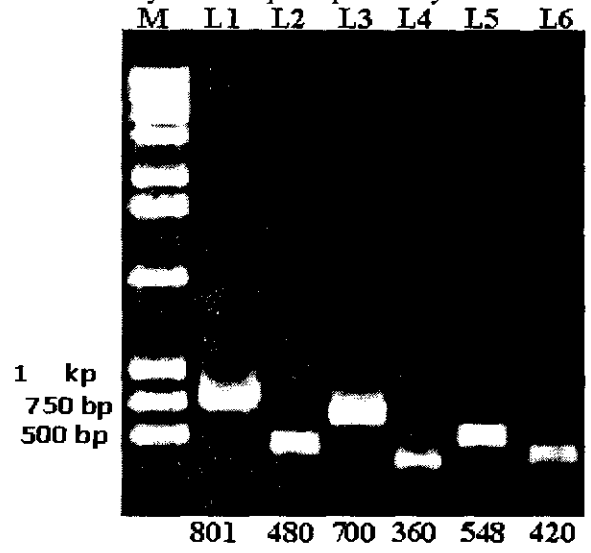

Fig. (3): Detection of PVY, PVX and PLRV by RT-PCR as first round amplification and n-PCR for second round from cDNAs primed by specific external and internal sense and antisense primers for each virus as shown in table 1. Lane M, DNA ladder molecular size markers; the sizes in bp and kb are indicated on the left hand margin. Lane 1,3 and 5 amplified PVY, PVX and PLRV respectively by RT-PCR as first round, While lane 2,4 and 6 nested-PCR for the first amplified product of PVY, PVX and PLRV respectively. The first round amplified products were of: PVY (801 bp), PVX (700 bp) and PLRV (548 bp), while the second round amplified product by n-PCR were of: PVY (480 bp, PVX (360 bp) and PLRV (420 bp), the sizes of each product in bp are indicated on the bottom hand margin.

if multiplex PCR in a single closed tube were developed based on this technology. In nPCR, a second round of amplification was performed using primers, which produce a specific 480 bp product from the coat protein gene of PVY, 360 bp product from the coat protein gene of PVX and a specific 420 bp product from the coat protein gene of PLRV (Fig. 3). When attempts were made to detect potato viruses under study by RT-PCR using the different sets of primers, the results were equally or less sensitive than nPCR. Our results indicated that an extra step to the nested PCR, increases the sensitivity of RT-PCR detection, and another approach to increase the sensitivity level of RT- PCR is to add an extra cycle of PCR with internal primers. This procedure, called nested-PCR, has been used in diverse research fields (Gundersen and Lee, 1996; Lee *et al.*, 1994). These techniques demonstrate the feasibility of multiplex RT-PCR based on an accurate primer design for the identification of several potato viruses, i.e., PVY, PVX and PLRV in a single step reaction. Such a method should increase both the sensitivity and specificity of the diagnosis and thus reduce the possibility of false negatives. In addition, it improves the sensitivity of the existing molecular protocol for potato viruses detection by developing a nested PCR (nPCR) in which primers were designed to anneal internally to the amplicon produced from standard PCR.

Sequence analysis

PVY, PVX and PLRV sequences were aligned with the sequence of PVY, PVX and PLRV available in the GenBank. Sequencing results confirmed the viral origin of the amplified products for each virus isolate and revealed some differences between our PVY, PVX and PLRV isolates (Fig. 4, a, b and c) and their respective reference sequences [GenBank accession numbers g441193 (Robaglia *et al.*, 1989), X88784, and D13954 (Santa Cruz and Baulcombe, 1995) and D00734 (Keese *et al.*, 1990) respectively]. The highest nucleotide sequence identity was 94 % for the Egyptian isolate of PVYn (PVYEgcp1) and PVYn from France (Fig. 4a), 96.7 % for the Egyptian isolate of PVX (PVXegcp1) and PVX from UK (Fig. 4b) and 99 % for the Egyptian isolate of PLRV (PLRVEgcp1) and PLRV from Canada (Fig. 4c).

In conclusion, this report describe the development of multiplex RT-PCR (m-RT-PCR) and nested PCR (nPCR) assays, which improves the sensitivity of PVY, PVX and PLRV detection. The use of these techniques to enable early identification of potato viruses and for quarantine and breeding purposes.

ACKNOWLEDGMENTS

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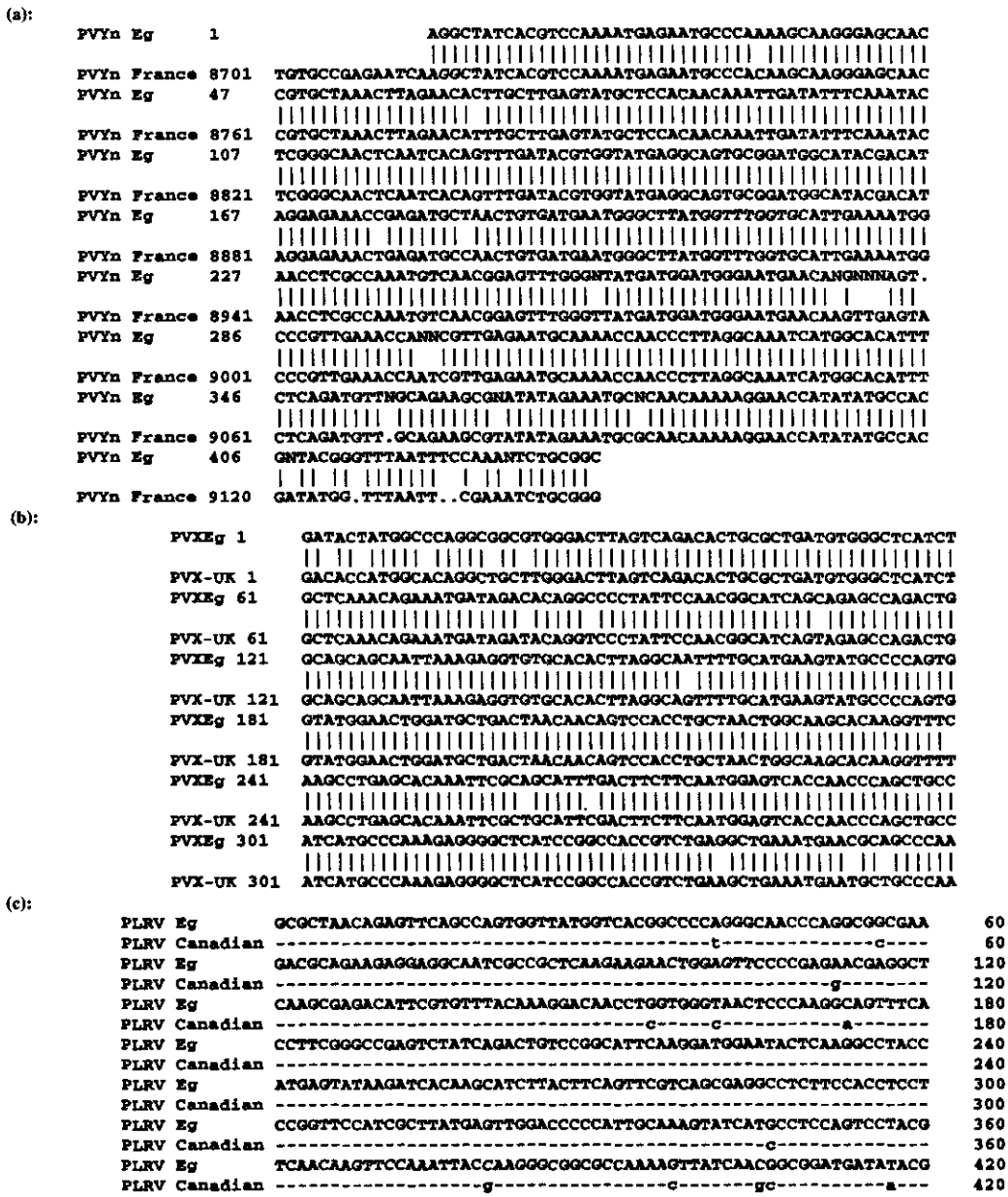

Fig. (4): Nucleotide sequence alignments of the amplified RT-PCR products for PVY, PVX and PLRV of the partial part of coat protein gene (CP-genes for each virus) form the cloned PVY, PVX and PLRV isolated from potato plants in Egypt. a) Multiple sequence alignment between PVYEgcp1 (Uppercase letter) with PVY^{N} strain France isolate (Robaglia et al., 1989), GenBank accession no. g441193, and the highest nucleotide identity was 94 %. b) Multiple sequence alignment between PVXEgcp1 (Uppercase letter) with PVX from United Kingdom (Santa Cruz & Baulcombe, 1995), GenBank accession no. X88784, and the highest nucleotide identity was 96.7%. Multiple sequence alignment between PLRVEgcp1 (Uppercase letter) with PLRV from Canada (Keese et al., 1990), GenBank accession no. D13954 and D00734, and the highest nucleotide identity was 99 %.

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الملخص العربي

تطوير تقنية عالية الحساسية تعتمد على استخدام تفاعل البلمرة المتسلسل المصاحب لتفاعل التناسخ العكسي في تشخيص الإصابة بثلاثة فيروسات تصيب البطاطس في تفاعل واحد متعدد التشابك (m-RT-PCR) والـ nested-PCR

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تعتبر فيروسات X, Y و التفاف اوراق البطاطس PLRV من الفيروسات الهامة التى تؤثر على الانتاجية الاقتصادية لمحصول البطاطس فى مصر. تم استخدام تفاعل متعدد البلمرة المتسلسل PCR المصاحب لتفاعل التناسخ العكسي RT للكشف عن هذه الفيروسات الثلاثة باستخدام بادئات متخصصة صممت لإكثار الجين المسئول عن إنتاج البروتين لكل فيروس (٨٠١ نيوكليوتيدة لفيروس Y البطاطس و ٧٠٠ نيوكليوتيدة لفيروس X البطاطس و٥٤٨ نيوكليوتيدة لفيروس التفاف اوراق البطاطس بالترتيب).

تم التأكد من تخصص البادئات المستخدمة فى التشخيص للفيروسات الثلاثة باستخدام بادئات اخرى داخلية متخصصة تم تصميمها لتفاعل الـ nested-PCR لتأكيد حساسية التشخيص و كذلك تم دراسة التتابع النيوكليوتيدى لنواتج الـ PCR.

تم الكشف عن الفيروسات الثلاثة باستخدام طريقة الـ m-RT-PCR فى خليط تفاعل واحد حيث انة فى حالات الإصابة الطبيعية لنباتات البطاطس فى الحقل فأنها تكون بفيروسين أو ثلاثة على الاقل كعدوى خلية و بالتالى فانه يمكن تشخيص الاصابة بأثنين او ثلاثة فيروسات باستخدام تفاعل واحد متعدد التشابك (m-RT-PCR) . تعتبر طريقة الـ m-RT-PCR سريعة و فعالة و ذات حساسية عالية فى الكشف السريع عن الفيروسات الثلاثة فى عينة نبات واحدة.

أي أن استخدام طريقتى الـ m-RT-PCR والـ nested-PCR يوصى بهما للتطبيقات الكشفية لحساسيتهما العالية اكثر من الـ RT-PCR و خاصة فى الكشف المبكر عن الاصابة و فى الحجر الزراعى وكذلك فى برامج التربية.