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Full Length Research Paper

The feasibility of tetraplex RT-PCR in the determination of PVS, PLRV, PVX and PVY from dormant potato tubers

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Dormant potato tubers belonging to cvs., namely, Agria, Granola and Marfona known to be infected with potato viruses (*Potato leafroll virus*, PLRV; *Potato virus S,* PVS; *Potato virus* X, PVX and *Potato virus Y*, PVY) were tested with uniplex RT-PCR and strong bands specific to each virus were obtained from cultivars. When cDNA synthesized for uniplex RT-PCR was used for tetraplex RT-PCR, the bands obtained from PVS, PLRV and PVX were too faint to be photographed and there is no any observed band for PVY. To improve the band density, the concentration of oligo dT primer in RT was increased from 20 to 40 ng in the subsequent experiments. The increasing of oligo dT primer concentration in RT increased the band density for PVS and PVX, but not PVY. Upon this, different amount of total RNA were tested in RT stage. The best result was obtained from 5 µl of total RNA and followed by 3.5 and 2.5 µl applications. In order to determine the effect of cDNA amount in PCR, 2 µl cDNA + 23 µl PCR, 5 µl cDNA + 20 µl PCR and 5 µl cDNA + 25 µl PCR mixture were compared. However, no distinct differences were observed among various cDNA amounts. As a result, instead of tetraplex RT-PCR, it is suggested the use of triplex RT-PCR for reliable detection of PLRV, PVS and PVX. However, uniplex PCR could be suggested for reliable detection of PVY from this study by using the same cDNA.

Key words: Potato viruses, detection, dormant tubers, tetraplex RT-PCR.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the most widely grown field crops in Turkey, and annual production is approximately 5 million tons from 200,000 ha (Anonymous, 2003). Turkey needs approximately 125,000 - 150,000 tons potato seed per year and there is no state company that produced diseases-free seed potato in Turkey (Bostan and Haliloğlu, 2004). To meet the demand of farmers, seed potatoes have been imported from other countries (mainly European), multiplied and then have been distributed to the producers by some private companies. But, a vegetative propagated potato crop presents ample opportunity for the accumulation of viral diseases with each multiplication in the field (McDonald, 1984; Singh, 1999). Although there are more than 30 viral pathogens that can impact potato worldwide (Salazar, 1996); *Potato leafroll virus* (PLRV, a *Polerovirus*), *Potato virus S* (PVS, a *Carlavirus*), *Potato virus X* (PVX, a *Potexvirus*) and *Potato virus Y* (PVY, a *Potyvirus*) are the most common viruses economically affectting potato crops. These viruses can occur in single or as mixed infections within the potato crop (Singh, 1999). In an effort to reduce extensive yield losses due to viral diseases in subsequent potato crops, seed tubers are tested prior to planting for the presence of viruses and the resultant virus-free tubers are planted (Jones, 1988; Spiegel and Martin, 1993). However, routine diagnosis of potato viruses from dormant seed tubers in post-harvest potato indexing requires reliable, sensitive, inexpensive and rapid procedures. The RT-PCR assays offer the possibility of improved sensitivity and a more rapid diagnosis of individual viruses from various tissues of potato (Barker et al., 1993; Spiegel and Martin, 1993; Singh et al., 1997; Singh and Singh, 1998; Walsh et al., 2001) and aphid vectors (Singh et al., 1995; Singh and Singh, 1996; Singh et al., 1997). However, detection of several individual viruses separately by RT-PCR reac-

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Table 1. The primer pairs used for PVS, PLRV, PVX and PVY in RT-PCR.

Virus	Sequens	Polarity	Fragment
PVS	5'-TGGCGAACACCGAGCAAATG-3' 5'-ATGATCGAGTCCAAGGGCACTG-3'	Sense Antisense	187 bp
PLRV	5'- CGCGCTAACAGAGTTCAGCC-3' 5'-GCAATGGGGGTCCAACTCAT-3'	Sense Antisense	336 bp
PVX	5'-TAGCACAACACAGGCCACAG-3' 5'-GGCAGCATTCATTTCAGCTTC-3'	Sense Antisense	562 bp
PVY	5'-AAGCTTCCATACTCACCCGC-3' 5'-CATTTGTGCCCAATTGCC-3'	Sense Antisense	856 bp

tions is expensive and time consuming.

In order to reduce the labor and equipment costs for test, a duplex RT-PCR (d-RT-PCR) detection of PLRV and PVY has been reported (Singh et al., 1996; Russo et al., 1999; Singh et al., 2000), a multiplex RT-PCR (m-PCR) protocols were developed for the detection of 5 viruses (PVA, PVS, PVX, PVY and PLRV) in potato tubers and simplified by using an oligo (dT) in the synthesis of cDNA for potato viruses (PVX, PVS, PVY and PVA) RNAs (Nie and Singh, 2000; Singh and Nie, 2003). In addition, multiplex RT-PCR was used to differentiate strains of PVY (Nie and Singh, 2002; Singh and Nie, 2003; Lorenzen et al., 2006; Rigotti and Gugerli, 2007).

The main objective of this study was to evaluate the feasibility of tetraplex RT-PCR in the determination of potato viruses (PVS, PLRV, PVX and PVY) in dormant seed potato tubers belonging to some potato cultivars grown commercially in Turkey as a routine for certification.

MATERIALS AND METHODS

Virus sources

Dormant tubers belonging to cvs., Agria, Granola and Marfona known to be infected with PVS, PLRV, PVX and PVY were obtained from our previous studies and multiplied in greenhouse and field. All tubers were tested previously by ELISA (Bostan and Haliloğlu, 2004) and then tested by RT-PCR in our laboratory to identify of particular viruses (Bostan et al., 2006).

RNA isolation from dormant tubers

RNA was extracted from tubers as described previously (Singh et al., 2002). Briefly, 6 drop of plant sap (150 - 200 µl) was obtained from tuber tissues by passing the tissue through a tissue slicer/ grinder (Electrowerk, Behncke and Co., Hannover, Germany) and combined with 300 µl of extracting buffer (0.1 M Tris HCl, pH 7.4, 2.5 mM $MgCl_2$, 0.65 % Na_2SO_3 containing 6 U of RNase-free DNase I) (Roshe Molecular Bochemicals). The mixture was vortexed for 10-15 s and then incubated at 37°C for 10 min. Nucleic acids were extracted with an equal volume phenol:chloroform: isoamyl alcohol (25:24:1) and then precipitated with 1 vol. of isopropenol in the presence of 0.1 volume of 3 M sodium acetate (-20°C overnight). The precipitate was collected by centrifugation (12,000 g, 15 min, 4°C), washed with 70% ethanol, dried under vacuum and dissolved in 100 µl of distilled water.

Reverse transcription polymerase chain reaction (RT-PCR)

For the reverse transcription (RT), 2.5 µl of RNA extract was incubated at 65°C for 5 min, and then chilled on ice for 10 min to denature the RNA. The RT mixture of 7.5 µl was added to provide a final concentration of 20 ng/µl of reverse primer specific to PLRV, 20 ng of oligo (dT) primer (Roshe), 50 mM Tris-HCl pH 8.3, 75 mM KCl, 10 mM dithiothreitol (DDT), 2.5 mM $MgCl_2$, 1.5 mM of each dNTPs (Promega), 200 U RNasin (Promega, Madison, WI) and 20 U Moleney Murine Leukemia virus-reverse transcriptase (MMLV-RT) (Invitrogen). Samples were incubated for 1 h at 42°C for RT and incubated subsequently at 95°C for 3 min to terminate the RT reaction.

PCR was carried out using 2 µl aliquots of the cDNA mixture in 23 µl containing 10 mM Tris-HCl pH 8.3, 50 mM, 3.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.2 mM of each of antisense and sense primers and 0.625 U of Taq polymerase (Sigma). Thermo-cycling was performed as described previously (Nie and Singh, 2001, 2002). Samples were amplified in 35 cycles using a Peltier thermal cycler (MJ Research, Watertown, MA). Annealing temperature was 62°C for the first 10 cycles, 60°C for the next 10 cycles and 58°C for the last 15 cycles. Each cycles consisted of denaturation (92°C, 30 s), and primer annealing (30 s) and primer extension at 72°C (90 s) as well as a final extension for 10 min at 72°C. 20 µl of amplified product containing bromophenol blue tracking dye was separated by electrophoresis in a 1.5% agarose gels containing 0.5 µg/ml ethidium bromide and photographed under UV illumination with an imaging system. Positive control for the viruses under study was included in each experiment. In order to determine of the size of amplified products in the gel, as a marker, a low mass ladder (Invitrogen) was used. The antisense and sense primers used for PLRV (Singh et al., 1995), PVS (Matoušek et al., 2000), PVX (Skryabin et al., 1988) and PVY (Nie and Singh, 2001) were given in Table 1.

RESULTS AND DISCUSSION

When dormant tubers known to be infected with PLRV, PVS, PVX and PVY viruses were tested in uniplex RT-PCR, strong bands specific to each virus were obtained from cultivars and it was not observed a significant difference between the cultivars and viruses (Figure 1). Obtaining strong bands from uniplex RT-PCR application for each of virus shows that the cultivars used as material did not contain any substances to inhibit RT-PCR.

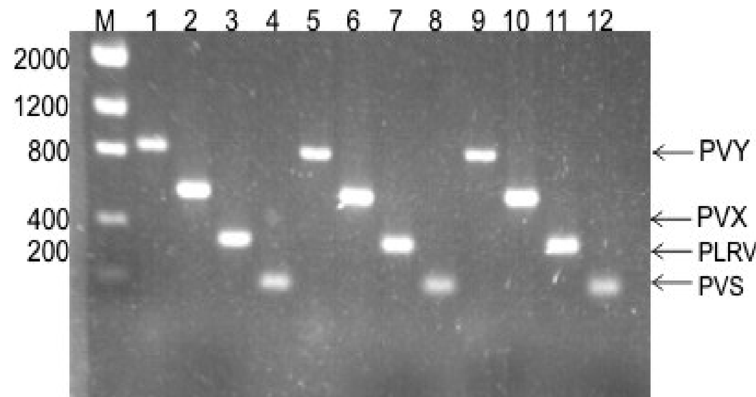

Figure 1. Uniplex RT-PCR products from Agria, (PVY: Lane 1, PVX: Lane 2, PLRV: Lane 3, PVS: Lane 4), Granola, (PVY: Lane 5, PVX: Lane 6, PLRV: Lane 7, PVS: Lane 8), Morfona, (PVY: Lane 9, PVX: Lane 10, PLRV: Lane 11, PVS: 12), M: Marker.

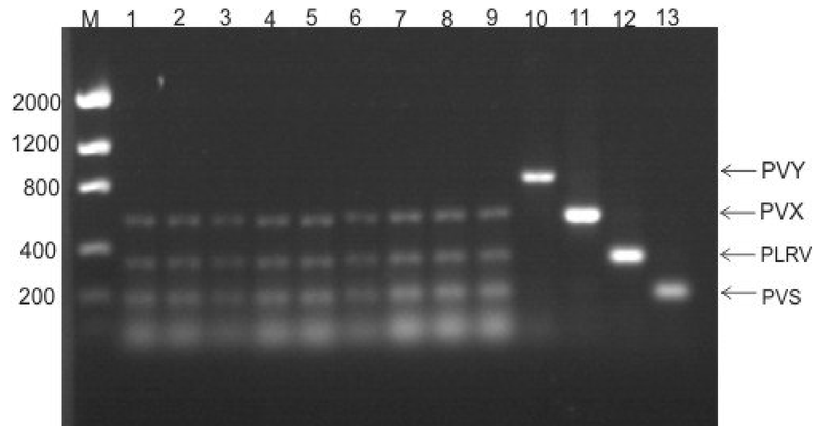

Figure 2. Detection of PVY, PVX, PLRV and PVS from different cultivars by using different amount of total RNA in RT: when 2.5 µl total RNA was used (Agria: Lane 1, Granola: Lane 2, Morfona : Lane 3), 3.5 µl of total RNA (Agria: Lane 4, Granola: Lane 5, Morfona: Lane 6), 5.0 µl total RNA (Agria: Lane 7, Granola: Lane 8, Morfona: Lane 9), positive control for PVY: Lane 10, for PVX: Lane 11, for PLRV: Lane 12, for PVS: Lane 13, Marker: M.

When cDNA synthesized for uniplex RT-PCR was used for tetraplex RT-PCR (2 µl cDNA + 23 µl PCR mixture), the bands obtained from PLRV, PVS and PVX were too faint to be photographed and no band was observed for detection of PVY (data is not shown). In order to improve the band density, the concentration of oligo dT primer in RT was increased from 20 to 40 ng in the subsequent experiments. The increasing of oligo dT primer concentration in RT has increased the band intensity of PVS and PVX, but not PVY (data is not shown). When 2.5, 3.5 and 5 µl of total RNA, 2 µl of cDNA + 23 µl PCR mixture was used in RT, the best result were obtained from 5 µl of total RNA and followed by 3.5 and 2.5 µl applications (Figure 2). Upon this, the following applications, 5 µl of the total RNA was used for cDNA synthesis.

In order to determine the effect of cDNA amount in PCR, 2 µl cDNA + 23 µl PCR, 5 µl cDNA + 20 µl PCR and 5 µl cDNA + 25 µl PCR mixture were compared, any distinct differences was not onserved among these applications as seen on the gel below. Any band was not seen for PVY (Figure 3).

To assess the usefulness and sensitivity of tetraplex RT-PCR for routine detection of PLRV, PVS, PVX and PVY, uniplex and tetraplex RT-PCR experiments were carried out in parallel using the same nucleic acid. For this purpose, a total of 100 field-grown tubers known to be infected with one or more viruses tested after 45 days of harvesting by tetraplex RT-PCR. When tubers were tested by uniplex RT-PCR, a total of 61 tubers were positive for PLRV, 47 for PVS, 19 for PVX and 78 for

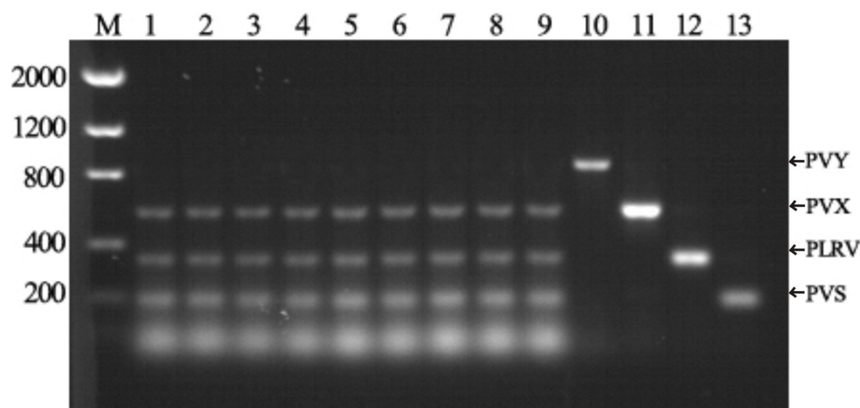

Figure 3. The effect of cDNA amount and PCR mixtures on the band density for viruses: 2 µl cDNA + 23 µl mix (Agria: Lane 1, Granola: Lane 2, Morfona: Lane 3), 5 µl cDNA + 25 µl PCR mix (Agria: Lane 4, Granola: Lane 5, Morfona: Lane 6), 5 µl cDNA + 20 µl PCR mix (Agria: Lane 7, Granola: Lane 8, Morfona: Lane 9), positive controls (PVY: Lane 10, for PVX: Lane 11, for PLRV: Lane 12, for PVS: Lane 13), Marker: M.

PVY. However, when the same tubers were tested by tetraplex RT-PCR, 54 samples were positive for PLRV, 43 for PVS, 15 for PVX but not PVY.

While obtaining the bands from PVS, PLRV and PVX, one of the reason why no bands were obtained from PVY is that, the concentrations of these viruses belonging to different groups were different in tissue, that is, the concentration of PVY might be lower than the other viruses in tissue (Brunt, 1988). In addition to this, it was reported that potato cultivars vary in PVY concentrations depend on storage conditions in the tubers (Barker et al., 1993; Singh and Singh, 1996, 1997). Another possible reason might be the difference between the fragment sizes amplified by primer pairs. While the fragment lengths that the primer pairs used for PVS, PLRV and PVX amplified were 187 bp, 336 bp and 562 bp respectively, this size was 856 bp for PVY. As reported previously (Singh and Singh, 1997), the difference in the sensitivity between the largest (1040 bp) and smallest (217 bp) PCR product size was over 1000 times, and therefore, it was suggested that primers producing fragments of about 400 bp will detect PVY most sensitively. In addition to the size differences of amplified products, it is possible that the location of the target sequence on the RNA molecule may influence the sensitivity of virus detection. Although the bands specific to PVY were obtained in the multiplex RT-PCR study carried out by (Nie and Sing, 2001), any band specific to PVY was not seen in our study. This could be due to several reasons such as the use of less sap during total RNA extraction procedure, not including additional purification step, primer pairs (amplifies 856 pb fragment) used specific to all strain of PVY and differences in PCR conditions. As reported previously by Singh et al. (2000), the concentration of various reagents and primers affect significantly subsequent amplification process.

As a result, accurate and speedy diagnosis of PVY or other viruses (PVS, PLRV, and PVX) in dormant potato tubers is an integral part of certified seed programs. Therefore, it is suggested that, the use of triplex RT-PCR for reliable detection of PVS, PLRV and PVS for large-scalar survey. However, PVY should be tested simplex RT-PCR by using the same cDNA. In order to reduce the labor and equipment costs for test, multiplex real-time RT-PCR should be suggested for detection in single and mixed infections of these viruses (PVY, PVX, PVA and PVS), since it was reported that the real-time RT-PCR is 100 fold more sensitive than conventional RT-PCR (Boonham et al., 2004; Klerk et al., 2001; Bright et al., 2007).

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REFERENCES

Anonymous (2003). Türkiye İstatistik Yıllığı. T.C. Başbakanlık Türkiye İstatistik Kurumu. Yayın No: 2776, Ankara.

Barker H, Webster KD, Reavy B (1993). Detection of potato virus Y in potato tubers: a comparison of polymerase chain reaction and enzyme-linked imminosorbent assay. Potato Res. 36: 13-20.

Boonham N, Ganzález PL, Mendez MS., Lila PE, Blockley A, Walsh K, Barker I,Mumford RA (2004). Development of a real-time RT-PCR assay for the detection of potato spindle tuber viroid, J. Virol. Methods, 116: 139-146.

Bostan H, Haliloğlu K (2004). Distribution of PLRV, PVS, PVX, and PVY (PVY^N, PVY^o and PVY^c) in the seed potato tubers in Turkey. Pak. J. Biol. Sci. 7(7): 1140-1143.

Bostan H, Guclu C, Ozturk E, Ozdemir I, Ilbagi H (2006). Influence of Aphids on the Epidemiology of Potato Virus Diseases (PVY, PVS and PLRV) in the High Altitude Areas of Turkey. Pak. J. Biol. Sci. 9(4):

759-765.
Bright OA, Patrick JS, Philip HB (2007). Simultaneous detection of potato viruses, PLRV, PVA, PVX and PVY from dormant potato tubers by TaqMan real-time RT-PCR. J. Virol. Methods, 142: 1-9.
Brunt AA (1988). Purification of filamentous Viruses and Virus-Induced Noncapsid Proteins. In. The Plant Viruses 4 (ed. R. G. Milne). Plenum Press. Newyork and London, pp. 85-110.
Jones ED (1988). A current assessment of *in vitro* culture and other rapid multiplication methods in North America and Europe. Am. Potato J., 65: 209-220.
Klerk MM, Leone GOM, Verbeek M., van den Heuvel JFJM, Schoen CD (2001). Development of a multiplex AmpliDet RNA for the simultaneous detection of potato leafroll virus and potato virus Y in potato tubers, J. Virol. Methods, 93: 115-125.
Lorenzen JH, Piche LM, Gudmestad NC, Meacham T, Shiel P (2006). A multiplex PCR assay to characterize *Potato virus Y* isolates and identify strain mixtures, Plant Dis. 90: 935-940.
Matoušek J, Schubert J, Didic P, Ptáèek J (2000). A broad variability of potato S carlavirus (PVS) revealed by analysis of RT-PCR-amplified virus sequences. Can. J. Plant Pathol. p. 22.
McDonald JG (1984). Viruses associated with mosaic symptoms in Russet Burbank potato. Can. J. Plant Pathol. 6: 224-226.
Nie X, Singh RP (2000). Detection of multiple potato viruses using an oligo (dT) as a common cDNA primer in multiplex-RT-PCR. J. Virol. Methods, 86: 179-185.
Nie X, Singh RP (2001). A novel usage of random primers for multiplex RT-PCR detection of virus and viroid in aphids, leaves and tubers. J. Virol. Methods, 91: 37-49.
Nie X, Singh RP (2002). A new approach for the simultaneous differentiation of biological and geographical strains of *potato virus Y* by uniplex and multiplex RT-PCR. J. Virol. Methods, 104: 41-54.
Rigotti S, Gugerli P (2007). Rapid identification of potato virus Y strains by one-step triplex RT-PCR. J. Virol. Methods, 140: 90-94.
Russo P, Miller L, Singh RP, Slack SA (1999). Comparison of PLRV and PVY detection in potato seed samples tested by Florida winter field inspection and RT-PCR. Amer. J. Potato Res., 76:313-316.
Salazar LF (1996). Potato Viruses and Their Control. CIP, Lima. p. 214.
Singh RP, Kurz J, Boiteau G, Bernard G (1995). Detection of potato leafroll virus in single aphids by reverse transcription polymerase chain reaction and its potential epidemiological application. J. Virol. Methods, 55:133-143.
Singh RP, Kurz J, Boiteau G (1996). Detection of stylet–borne and circulative potato viruses in aphids by duplex reverse transcription polymerase chain reaction. J. Virol. Methods, 59:189-196.
Singh M, Singh RP (1996). Factors affecting detection of PVY in dormant tubers by reverse transcription polymerase chain reaction and nucleic acid spot hybridization. J. Virol. Methods, 60:47-57.
Singh M, Singh RP (1997). Potato virus Y detection: sensitivity of RT-PCR depends on the size of fragment amplified. Can J. Plant Pathol. 19: 149-155.
Singh RP, Kurz J, Boiteau G, Moore LM (1997). Potato leafroll virus detection by RT-PCR in field-collected aphids. Am. Potato J. 74: 305-313.
Singh RP, Singh M (1998). Specific detection of potato virus A in dormant tubers by reverse transcription polymerase chain reaction. Plant Dis. 82: 230-234.
Singh RP (1999). Development of the molecular methods for potato virus and viroid detection and prevention. Genome, 42:592-604.
Singh RP, Nie X, Singh M (2000). Duplex RT-PCR: reagent concentrations at reverse transcription stage affect the PCR performance. J. Virol. Methods, 86: 121-129.
Singh RP, Nie X, Singh M, Coffin R, Duplessis P (2002). Sodium Sulphite inhibition of potato and cherry polyphenolics in nucleic acid extraction for virus detection by RT-PCR. J. Virol. Methods, 99: 123-131.
Singh RP, Nie X (2003). Multiple virus and viroid detection and strain separation via multiplex reverse transcription-polymerase chain reaction, Can. J. Plant Pathol. 25: 127-134.
Skryabin KG, Kraev AS, Morozov S, Yu Rozanov MN, Chernov BK, Lukasheva LI, Atabekov JG (1988). The nucleotide sequences of potato virus X RNA. Nucl. Acid Res. 16: 10929-10930.
Spiegel S, Martin RP (1993). Improved detection of potato leafroll virus in dormant potato tubers and micro tubers by the polymerase chain reaction and ELISA. Ann. Appl. Biol. 121: 493-500.
Walsh K, North J, Barker I, Boonham N (2001). Detection of different strains of *Potato virus Y* and their mixed infections using competitive fluorescent RT-PCR. J. Virol. Methods, 91: 167-173.