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Rapid Detection of *Potato Y potyvirus* in Potato Farms of Kermanshah using RT-PCR Amplification of the P1- Protease Gene and Its Cloning

[1]M.H. Mirmomeni, [1]A. Sharifi and [2]S. Sisakhtnezhad
[1]Cell and Molecular Unit, Department of Biology, Razi University, Kermanshah, Iran
[2]Medical Biology Research Center, Kermanshah University of Medical Science, Kermanshah, Iran

Abstract: In this study, the RT-PCR method was used to detect the Y virus in potato tubers and leaves. Samples suspicious of virus infection with symptoms of virus infection were gathered from farms in Kermanshah and placed in plastic bags and kept at -80°C temperature in order to maintain the RNA of the virus until extraction. The extraction and purification of RNA were carried out using Tri-Reagent kit. One of the virus genes is the P1 protease gene which codes a proteinase enzyme. This enzyme plays a role in breaking the initial polyprotein. For amplification of this gene three primer, including primer-1, primer-2 and primer-3, were designed and used. Using primer 1 and reverse transcriptase enzyme, cDNA was synthesized and then PCR was performed using the primers 1, 2 and 3. The PCR products were examined by agarose gel electrophoresis (1%). Consequently, two pieces of DNA (400 and 800 bp) were yielded which were identical to the genome DNA sequencing. Thus, the proposed technique is a convenient method for quick and accurate detection of viruses and, therefore, the application of this method for detecting *Potato Y virus* in potato farms is recommended.

Key words: *Potato Y potyvirus*, PVY, RT-PCR, cloning, P1-protease gene

INTRODUCTION

Potato Y potyvirus (PVY) is one of the most damaging species of genus Potyvirus, family Potyviridea (the largest plant virus family) (Maki-Valkama, 2000; Syller, 2006; Urcuqui-Inchima *et al*., 2001). PVY has been identified as a serious potato pathogen that affects salanuceous crops including potato, tomato and tobacco. It also infected many salanuceous and nonsalanuceous weeds (Varveri, 2000; Moury *et al*., 2002; Maki-Valkama, 2000; Lorenzen *et al*., 2006; Spetz, 2003). PVY is widely distributed through the world (Varveri, 2000; Moran and Rodoni, 2003) and transmitted in a nonpersistant manner by several aphid species, with varied transmission efficiency (Varveri, 2000; Maki-Valkama, 2000; Spetz, 2003).

This virus has a flexuous filaments shape with no envelop. PVY has a single molecule of positive sense RNA (ssRNA) about 9.8 kilobases. PVY genome contain a single Open Reading Frame (ORF) which is flanked by a 3` and 5` NTRs. During the infection process, viral RNA is translated and genomic-derived polyprotein (340-370 kilo Dalton) is cleaved co-and post translationally, by a virus encoded proteinase, in to 10 mature proteins. This proteins include P1, Hc-Pro, P3, 6K1, CI, 6K2, Vpg, Nia-Pro, Nib and CP. All of these proteins and NTRs are essential for virus propagation (Maki-Valkama, 2000; Spetz, 2003; Mestre *et al*., 2003; Carrington *et al*., 1998; Urcuqui-Inchima *et al*., 2001). This virus is one of the most harmful potato viruses which cause the Rogus disease. The symptoms of this disease are brown lines especially in leaves and in severe cases of the disease, falling of leaves occur. PVY symptoms can differ according to the PVY strain, host resistance, time infection during plant growth and environmental condition. Therefore, these traits are used for the classification of PVY strains into different groups. Currently, according to symptomatology and serology, four main strains of PVY include PVY^{O}, PVY^{C}, PVY^{NTN} and PVY^{N} are known (Moury *et al*., 2002; Maki-Valkama, 2000; Moran ana Rodoni, 2003; Boonham *et al*., 2002; Lorenzen *et al*., 2006).

Several detection methods are used for detection PVY. The current method is called ELISA (Shalaby and Mazyad, 2002), but since its sensitivity does not allow it to detect infection in dormant tubers or in aphids and dose not detect these viruses in one step reaction and can not distinguish some strains like PVY^{NTN} (Varveri, 2000), recently, molecular method such as PCR (Akad and Czosnek, 2002; Ghosh *et al*., 2002; Moravec *et al*., 2003), PCR-ELISA (Varveri, 2000; Akad and Czosnekh, 2005), IC-PCR, PC-PCR, PC-PCR-ELISA (Varveri, 2000), foluorogenic 5` nuclease RT-PCR and isothermal NASBA amplification assay (Schoen and Leone, 1998) have been developed and used.

Corresponding Author: M.H. Mirmomeni, Cell and Molecular Unit, Department of Biology, Razi University, Kermanshah, Iran

One of the virus genes is the P1 protease gene which codes a proteinase enzyme. This enzyme plays a role in breaking the initial polyprotein. The P1-proteinase is the least conserved protein among potyviruses (Verchot and Carrington, 1995; Arbatova *et al*., 1998).

The aim of this investigation is to detection of the causal agents for virus-infected potato crops, collected from Kermanshah potato farms, using the RT-PCR assay for P1-protease gene and its cloning.

MATERIALS AND METHODS

Collection potato samples and maintenance: Potato samples, for virus detection were collected from Kermanshah potato farms using visual examination for the apparent symptoms. Samples were saved in -80°C and used during 6-12 month.

Preparation of total RNA: For detection of infected samples, Tri-Reagent Kit was used (Sigma). Total RNA was extracted from potato seeds and leaves by a Tri-Reagent Kit using Akad and Czosnek (2002) method.

Reverse Transcriptase-PCR (RT-PCR): The oligonucleotid primers used for the specific amplification of the P1-protease gene, primer-1 (5'-TTCCAAAGTGTCCTTTGAG-3`), primer-2 (5'-CTTCATCAAACAACTCTTT-3') and primer-3 (5`-TCTGGGCATCAGTCTTG-3`) were designed by Akad and Czosnek (2002). In the first step for cDNA synthesis, small quantity of total RNA (5 μL) was resuspended in ddH_2O (8 μL) and primer-1 (1 μL) and incubated at 70°C for 10 min, then incubated in ice bath for 30 min. In the next step, this solution was added in the mixture contain dNTP mix (1 μL), reverse transcriptase 5X buffer (4 μL), AMV reverse transcriptase (1 μL) and first strand cDNA synthesis was carried out at 42°C for 60 min and at 90°C for 10 min. Using cDNA as template, P1-protease gene was PCR-amplified with Taq DNA polymerase. A volume of 5 μL from the reverse transcriptase reaction was added to reaction mixture containing ddH_2O (25 μL), 10X PCR buffer (2.5 μL), 25 mM dNTPs (0.25 μL), 100 Pmol μL^{-1} each primer and 1 unit Taq DNA polymerase. Amplification was carried out in UK thermocycler (Model FTGRAD2D) with temperature programmed consisting of initial cycle (95°Cfor 3 min, 50°C for 2 min, 72°C for 2 min), 30 amplification cycles (95°C for 1 min, 55°C for 1 min, 72°C for 1 min) and final extension (72°C for 10 min). A volume of 5 μL of the PCR product was analyzed by electrophoresis in 1% agarose gel (Samobrook *et al*., 2001).

Cloning of the small fragment of P1-protease gene in pTZ57R/T vector and its sequencing: The small fragments (400 bp) were obtained by PCR from P1-protease gene, purified from gel and inserted in the corresponding site of cloning vector (pTZ57R/T) (Fig. 1)

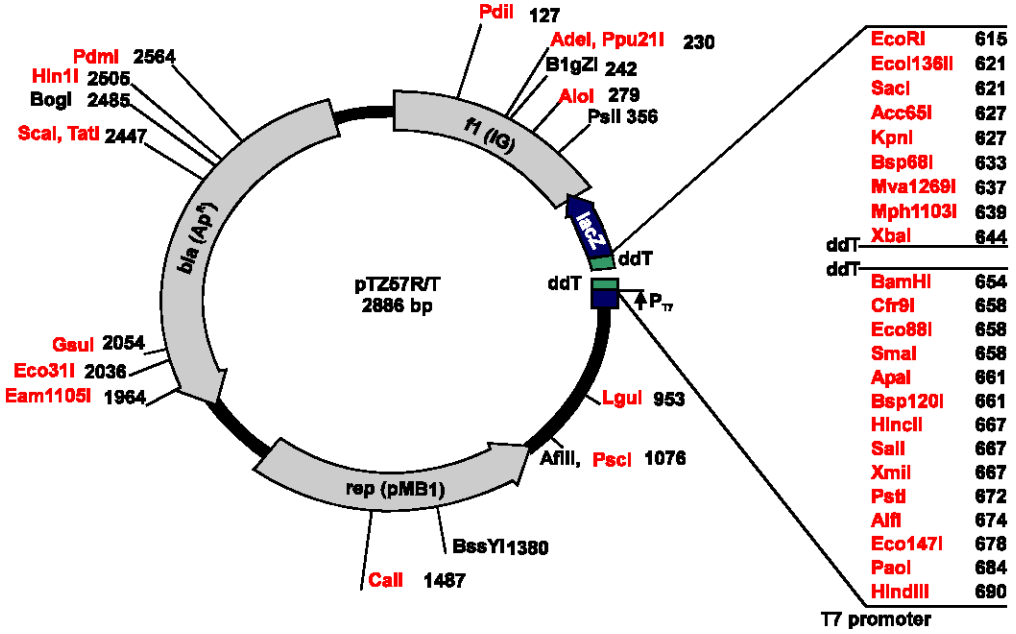

Fig. 1: pTZ57R/T cloning vector and its restriction map. rep (pMB1): A replicon (rep) from the pMBI plasmid is responsible for the replication. bla (AP^R): β-lactamase gene conferring resistance to ampicillin. f1 (IG): Synthesis of a single-stranded DNA. (http://www.fermentas.com/catalog/kits/kitinstaclone.htm)

supplied from Fermentas Inc T/A Clone™ PCR Product Cloning Kit (# K1213). Sequencing was performed by Korean Microgene Co. Ltd with automation dideoxy chain determination method.

RESULTS AND DISCUSSION

Total RNA was extracted from PVY-infected potato samples (Fig. 2) and the cDNA synthesis was performed. Furthermore, PCR reaction was performed using primers 1, 2 and 3 according to the pattern showed in Fig. 2 which resulted in two 400 and 800 bp fragments as expected (Fig. 3). The small fragment (400 bp) was separated from gel and cloned in the corresponding site of cloning vector (pTZ57R/T).

In order to amplify the recombinant plasmids, *E. coli DH5α* bacteria transformed with recombinant vector, via Transform Aid™ Bacterial Transformation System. Recombinant clones were identified by blue/white selection, since the vector is LacZ genetically marked. The correct insertion (400 bp fragment/pTZ57R/T product) was confirmed by comparison of 400 bp fragment/pTZ57R/T product with control plasmid vector pTZ57R without insert and control plasmid pTZ57R/T with 952 bp fragment insert by agarose gel electrophoresis (1%) (Fig. 4). Note that L2 and L3 are control plasmid pTZ57R

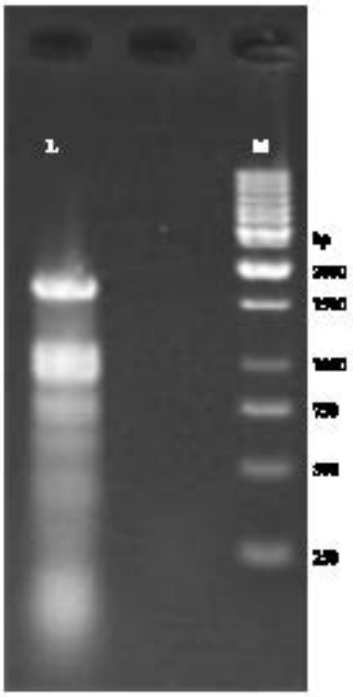

Fig. 2: Total RNA extraction with Tri-Reagent Kit. L and M referred to the total RNA product and DNA marker (GeneRuler™ 1kb DNA Ladder #SM0313), respectively

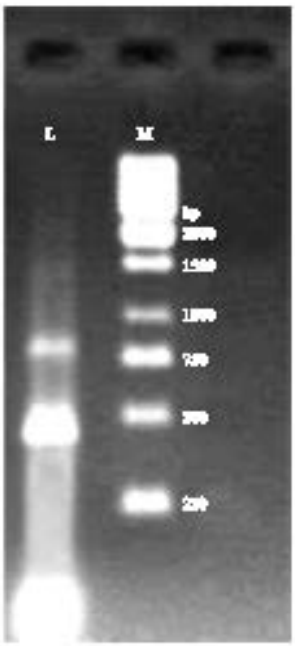

Fig. 3: Specific PCR amplification of PVY P1-protease gene. L and M referred to the PCR product and DNA marker (GeneRuler™ 1kb DNA Ladder #SM0313), respectively

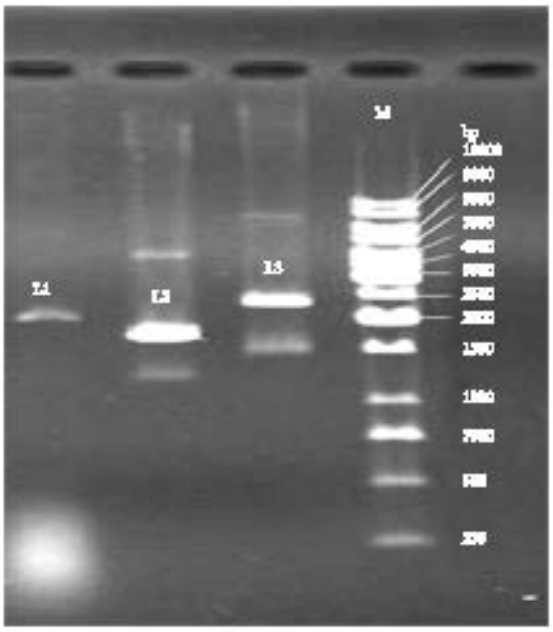

Fig. 4: Comparison of 400bp fragment/pTZ57R/T product with two control plasmid. L1 referred to 400bp fragment/pTZ57R/T product clone. L2, L3 and M referred to the control plasmid pTZ57R without insert, control plasmid pTZ57R/T with 912bp fragment insert and DNA marker (GeneRuler™ 1kb DNA Ladder #SM0313), respectively

TTCCAAAGTGTCCTTTGAGTTTCAATCTTGCGATCGTACTTCCCAGGTGGGATACT
TAATGCTTTCGCTCAGACACACACAGTGTATCGCGTATGTCGAGTATCCATAGTTT
AGGGCGTGGACTACCAGGGTATCTAATCCTGTTTGATCCCCACGCTTTCGTGCCTC
AGTGTCAATATACGCGTAGCCAGCTGCCTTCGCAATTGGTGTTCTATGTCATATCT
AAGCATTTCACCGCTACATGACATATTCCGCTAACCTCCACAATATTCAAGACTG
ATGCCCAGATAATCGGATCCCGGGCCCGTCGACTGCAGAGGCCTGCATGCAAGCT
TTCCCTATANTGAGTCGTATTAGAGCTTGGCGTAATCATGGTCATANCTGTTTCCT

Fig. 5: Nucleotide sequence of P1-protease gene was cloned in pTZ57R/T cloning vector

plasmid, which extracted from transformed *E. coli DH5α*. Moreover, identification of 400 bp fragment/pTZ57R/T product was confirmed by sequencing (Fig. 5).

The damage rate of infection by PVY is high in Iran and worldwide. This virus is one of the most harmful viruses which can reduce the efficiency of producing potato. Molecular techniques are needed to detect the virus promptly and take necessary actions for preventing its spread. The method used is RT-PCR. After the extraction of RNA virus, the process of PCR by 1, 2 and 3 primers was performed. Primers have been designed for virus gene p1 protease.

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