OPTIMIZATION OF DUPLEX RT-PCR FOR SIMULTANEOUS DETECTION OF POTATO VIRUS Y AND S

Baswaraj Raigond¹, Medha Sharma¹, Yamini Chauhan¹, A Jeevalatha¹, BP Singh¹ and Sanjeev Sharma¹

ABSTRACT: A duplex Reverse Transcription Polymerase Chain Reaction (RT-PCR) assay was developed for simultaneous detection of two viruses infecting potato i.e. Potyvirus (Potato virus Y) and Carlavirus (Potato virus S). Specific primers were designed against coat protein gene of both the viruses (PVY and PVS) and uniplex RT-PCR assay was standardized for detection of these viruses (PVY and PVS) individually. Thereafter, an attempt was made to detect both viruses in a single reaction. RT-PCR conditions were optimized by altering PCR mix i.e. dNTPs, primer and Taq DNA polymerase. This optimized PCR mix and PCR conditions showed an expected size of amplicons in duplex RT-PCR with respect to PVY (380 bp) and PVS (567 bp). Robustness of the technique was further validated wherein; duplex RT-PCR was carried out to detect both the viruses in potato tubers and field infected potato plants. It could detect both viruses in field (naturally) infected plants and tubers. It has the detection sensitivity similar to that of uniplex RT-PCR assay for respective viruses. The duplex RT-PCR tested here provides a simple, rapid, sensitive and convenient way for simultaneous detection of PVY and PVS. In addition, it reduces the time and cost of the consumables and can be used for routine detection of both the viruses simultaneously in seed certification programmes.

KEYWORDS: Duplex RT-PCR, PVY, PVS, elongation factor 1-α, potato tubers

INTRODUCTION

Potato is a member of the Solanaceae, family that includes several other economically important species such as tomato, eggplant, tobacco and pepper. Potatoes are infected by more than 36 viruses and a viroid of which eight are reported in India i.e. PVY and PVA (genus Potyvirus), PVS and PVM (genus Carlavirus), PVX (genus Potexvirus), Potato Tomato leaf curl New Delhi virus (genus Begomovirus), PLRV (genus Polerovirus), and Groundnut bud necrosis virus (genus Tospovirus). Potato virus Y (PVY) is the type species of the genus Potyvirus which belongs to Potyviridae family, the largest recognized plant virus family infecting plants and affecting the yield up to 90%. It is transmitted in the non-persistent manner by aphids. Potato virus S (PVS) belongs to the genus Carlavirus and family Betaflexiviridae and is transmitted mechanically, vegetative propagation and by aphids as well in a non-persistent manner to several members of the Solanaceae and Chenopodiaceae.

These viruses cause significant economic losses in potato production in India. Therefore, accurate and timely diagnosis is very much essential for their effective management. Under field conditions, potato plants are often infected with two or more viruses, resulting in degeneration of planting material as well as 30 to 50% yield loss (Sun and Yang, 2004). Use of virus free certified planting material is therefore the best way to reduce the losses due to viruses in successful production of potato. Efficient and robust techniques for detection of viruses in potato plants are, therefore a pre-requisite for taking successful seed production.
Viral diseases can be diagnosed by mosaic patterns on leaves, stunting of the plant, leaf malformations, and tuber malformations. Different techniques such as electron microscopy (EM), Immuno sorbent electron microscopy (ISEM), serology, nucleic acid hybridization and polymerase chain reaction based methods are used to detect the viruses. Among these techniques nucleic acid based (RT-PCR and PCR) and serological based (ELISA) techniques are popular in diagnostic laboratories. PCR is very efficient and specific method for in vitro amplification of DNA templates. The RT-PCR procedure has been developed to detect RNA viruses from dormant tubers (Singh and Singh, 1996) and field infected plants as well.

In case of RT-PCR analysis, false negative results may be produced due to failure in any of the successive steps like RNA extraction, cDNA synthesis and PCR set up. Therefore, use of an internal control can discriminate between a healthy sample and a failed amplification. Usually, housekeeping genes are used as internal control since the expression of the genes is influenced by the biotic and abiotic stress conditions. Du et al. (2006) compared the sensitivity of 18S ribosomal RNA and nad2 mRNA as an internal control during multiplexing of five potato viruses and found that 18S ribosomal RNA is more sensitive than nad2 mRNA. Similarly, Nicot et al. (2005) studied the variability of expression of seven housekeeping genes viz. actin, aprt, 18S rRNA, $ef1\alpha$, tubulin, cyclophilin and the ribosomal protein L2 in RealTime PCR normalization wherein $ef1\alpha$ gene was observed to give stable expression in potato in both biotic and abiotic conditions.

However, in recent times a duplex RT-PCR for the simultaneous detection of Onion yellow dwarf virus (OYDV) and Shallot latent virus (SLV), which allowed the successful identification of both viruses in cloves and leaves has been developed (Majumder et al. 2008). Similarly, Singh et al. (1996) developed duplex RT-PCR for simultaneous detection of PVY and PLRV. A systematic study was initiated to optimize duplex RT-PCR for simultaneous detection of PVY and PVS from infected potato leaf tissues and dormant tubers along with an $ef1\alpha$ gene as an internal control.

**MATERIALS AND METHODS**

The study was carried out in the Division of Plant Protection, Central Potato Research Institute, Shimla (H.P). Pure cultures of the inoculum of *Potato virus Y* and *Potato virus S* were maintained in virus culture facility of Division. The leaves containing infection of respective viruses i.e., PVY and PVS were brought to the laboratory under ice-cold conditions and were ground separately in liquid nitrogen to a fine powder in a sterilized and dried, pre chilled mortar and pestle. Tubers infected with the above viruses stored at room temperature were used for these studies.

**Designing virus specific primers**

Different sets of primers targeting the coat protein region of PVY and PVS were designed by using the sequence information available at NCBI data base. These primer sets were designed by using software Primer3 Input (version 0.4.0). Further the specificity of these designed primer sets were analyzed by BLAST and best combination of the primers were selected for this study. An elongation factor 1-$\alpha$ ($ef1\alpha$) gene from potato (*Solanum tuberosum*) was used as an internal control.

**Sample preparation and reverse transcription**

Total RNA was isolated from the pure cultures of respective viruses from leaves and tubers by using Spectrum™ Plant Total RNA kit (Sigma-Aldrich, USA) by following user protocol. The eluted total RNA was quantified.
by Thermo Scientific Nanodrop 2000. The first strand of c-DNA was synthesized by Revert Aid™ c-DNA synthesis kit (Fermentas Life Sciences) using random hexamer. The Reverse transcription (RT) mixture i.e. 4.0 µl of 5X buffer, 2.0 µl of 10 mM each dNTP mix, 1.0 µl of 20 U/µl RNase inhibitor, 1.0 µl of 0.2 µg/µl Random primer, 6.0 µl of template RNA, 1.0 µl of 200U/µl RT enzyme and 5.0 µl of RNase free water were added to provide a final volume of 20 µl. All the reactions were set up in ice cold condition to avoid premature cDNA synthesis and minimize the risk of RNA degradation. The reaction mixture was mixed, briefly centrifuged and incubated at 25°C for 05 min, 42°C for 59 min, 75°C for 10 min and held at 4°C. Later cDNA was used for further PCR amplification and the remaining quantity was stored at -20°C for further use.

**PCR amplification and sélection of primer**

PCR reactions were carried out in 20 µl reaction volume containing 2.0 µl of cDNA, 2.0 µl of 10X PCR buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 15 mM MgCl₂), 0.5 µl of 2 mM dNTPs, 0.5 µl of 10µM of respective forward and reverse primers, 0.5 µl of Taq DNA polymerase (Bangalore GeNei) and 14.0 µl of sterile double distilled water. Amplification was carried out for PVY and PVS independently from infected leaves as follows; initial denaturation at 94°C for 2min, thirty five cycles of denaturation at 94°C for 30 S, annealing at 58°C for 45 S, extension at 72°C for 1min; and final extension at 72°C for 10 min. About 10 µl of the amplified product from each tube was loaded onto 1.5% agarose gel alongside DNA ladder. The DNA bands were visualized on a UV-transilluminator and the primer pair showing expected size without any multiple bands was selected for further studies.

**Optimization of uniplex RT-PCR**

After selecting a primer pair, gradient PCR was performed with different annealing temperatures using Veriti 96 well thermal cycler (Applied Biosystems). The temperature profile of the PCR cycle was pre-incubation at 94°C for 2 min leading to 35 cycles of melting at 94°C for 30 min, annealing at 56°C, 58°C, 60°C, 62°C and 64°C for 45 min and extension at 72°C for 1 min followed by final extension at 72°C for 10 min. The amplified products were analyzed by electrophoresis wherein a single set of primer with a specific annealing temperature was selected for each target (PVY and PVS), showing single specific amplification. After selection of primer pairs and annealing temperature for respective viruses, it was further confirmed by carrying out PCR reactions with controls i.e. internal control, healthy control and water control. All these reactions were carried out as per the above optimized master mix and PCR conditions. About 10 µl of the amplified product from each tube was loaded onto 1% agarose gel and visualized on a UV-transilluminator.

**Optimization of Duplex RT-PCR**

To optimize duplex RT-PCR, the cDNAs of both the viruses (PVY and PVS) were used for simultaneous detection. The duplex PCR was carried out following the optimized uniplex RT-PCR protocol (as above) with some minor changes i.e., reaction mix containing 2.0 µl of cDNA (both the template), 3.0 µl of 10X PCR buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 15 mM MgCl₂), 0.5 to 1.5 µl of 2 mM dNTPs, 0.5 to 1.0 µl of 10µM of respective forward and reverse primers, 0.5 to 1.0 µl of Taq DNA polymerase (Bangalore GeNei) and sterile double distilled water to make up the volume to 20 µl. Amplification was carried out as above with change in annealing time from 45 s to 1 min. The amplified DNA bands were
Detection of potato virus Y and S using duplex RT-PCR

visualized on a UV-transilluminator and the appropriate reagents, their quantity and PCR conditions were selected.

Detection of PVY and PVS in potato tubers

In order to detect the presence of PVY and PVS in dormant potato tubers, the total RNA from three different tubers was isolated and a complementary strand was synthesized as mentioned earlier. These tubers were screened for the infection of PVY and PVS independently by optimized uniplex PCR conditions. Later, the optimized duplex RT-PCR conditions were used for simultaneous detection of both the viruses (PVY and PVS) from the infected tubers.

Validation of duplex RT-PCR

To validate the sensitivity and robustness of the optimized duplex RT-PCR, it was further used to detect/screen the presence of both the viruses (PVY and PVS) in different field infected (based on visual symptoms) plant samples. All the experiments were repeated twice to confirm the findings.

RESULTS AND DISCUSSION

Potatoes are generally propagated by vegetative means through 'seed' tubers or tissue culture raised planting materials. This leads to degeneration of seed stocks due to viruses infecting potato. Diagnostics of viral disease infected plants and elimination is the key in obtaining virus-free planting material. Most of the virus testing laboratories use serological based techniques for detection of potato viruses. Uniplex RT-PCR protocols have been proved to be more sensitive than serological techniques. However, uniplex RT-PCR are expensive, time and labor consuming when a large number of samples are to be tested for more than one virus. Duplex RT-PCR can overcome this limitation to a greater extent since it can detect two viruses in a single reaction tube. So an attempt was made to develop duplex RT-PCR protocol for simultaneous detection of PVY and PVS along with eflA of potato.

Sample preparation and reverse transcription

During the synthesis of complementary DNA strand, random hexamer primer was used in this study and found successful. Among the various reagents mix used during synthesis of a complementary strand against a RNA template, the quantity and concentration of primer greatly affect the subsequent PCR amplification. Therefore, different strategies like use of common degenerate primer (Saade et al., 2000), Oligo dT primer (Nie and Singh, 2000; Bostan and Peker, 2009) and commercially available random hexamer primer (Nie and Singh, 2001) were tried and found successful in the further PCR amplification.

PCR amplification and selection of primers

PCR reactions were carried out in different combinations of primers so as to select a suitable primer with specific amplicon for detection of respective viruses. Based on specificity and size one set of primer for each target viz. PVY-CP-F/PVY-CP-R, PVS-CP-F/PVS-CP-R were identified (Table 1). This exercise of selecting a suitable primer is very much essential for PCR amplification of the specified target, if not; it may amplify some other targets leading to non-specific bands which are not desired.

Optimization of uniplex RT-PCR

To determine the annealing temperature for selective primers for uniplex PCR detection of both the viruses, a gradient PCR was set up and the results revealed that both the viruses i.e. PVY (380 bp) and PVS (567

bp) were amplified independently, but the amplification was more intense at an annealing temperature of 62°C. It is often necessary to optimize the PCR reagent mix and PCR steps to detect the viral templates in a given sample. Selection of primer and determination of its annealing temperature is an essential and crucial parameter for specific amplification of the target template. Similarly, Singh et al. (2000) and Majumder et al. (2008) reported the significance of annealing temperature during optimization of RT-PCR conditions.

PCR conditions were further validated by PCR amplification with Internal control i.e. elongation factor 1-α (ef1α) gene (from Solanum tuberosum), healthy control, water control and positive control. The results under UV-transilluminator revealed that the internal control and positive control gave specific fragments and there was no band in the healthy and water control. This indicated that, the selective set of primer with optimized PCR conditions can be used further for the detection and diagnosis of PVY and PVS by uniplex RT-PCR. We found the expression of ef1α gene during our experiments in both healthy and infected samples in tubers and leaves. The amplification of ef1α from fresh leaf samples showed sharp and high intensity of band in comparison to the tubers. To trouble shoot the failure in the reaction mix or in PCR cycle and to avoid false negative results we tried to co-amplify ef1α gene of potato which is reported to be a stable housekeeping gene during biotic and abiotic stress conditions (Nicot et al., 2005).

### Optimization of duplex RT-PCR

An attempt was made for simultaneous detection of PVY and PVS by duplex RT-PCR in a single reaction tube with some modifications to the above optimized uniplex RT-PCR. The results revealed the amplification of both the viruses i.e. PVY (380 bp) and PVS (567 bp) indicated that the increased quantity of primers (from 0.5 to 1.0 µl), dNTPs (from 0.5 to 1.5 µl) and Taq Polymerase (from 0.5 to 1.0) gave a sharp amplicon of both the targeted viruses as compared to 0.5 µl each of primer, dNTPs and Taq Polymerase. This indicated that, inputs like primer pair, dNTPs, and Taq DNA polymerase were some of the important factors for successful duplex PCR amplification (Fig. 1). While performing multiplex PCR for simultaneous detection

<table>
<thead>
<tr>
<th>Target Virus</th>
<th>Primer Name</th>
<th>Polarity</th>
<th>Sequence 5'-3'</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVY/CP</td>
<td>PVY-CP-F</td>
<td>Sense</td>
<td>5'-ACGTGGTATGAGCCAGTGCGGA-3'</td>
<td>380</td>
</tr>
<tr>
<td></td>
<td>PVY-CP-R</td>
<td>Antisense</td>
<td>5'-ATGTGCGCTTCCCTAGCCCTCA-3'</td>
<td></td>
</tr>
<tr>
<td>PVS/CP</td>
<td>PVS-CP-F</td>
<td>Sense</td>
<td>5'-TTGGCCCAAATCAAGGGCACGG-3'</td>
<td>567</td>
</tr>
<tr>
<td></td>
<td>PVS-CP-R</td>
<td>Antisense</td>
<td>5'-CGGCCAACCCGCGATTCATTCCATTG-3'</td>
<td></td>
</tr>
</tbody>
</table>

![Fig. 1. Optimization of Uniplex & Duplex RT-PCR. 1-Ladder; 2-Duplex; 3-Negative control; 4-PVY; 5-PVS.](image)
Detection of potato virus Y and S using duplex RT-PCR

of viruses based on the standard PCR, it is often necessary to optimize the reaction parameters (Singh and Nie, 2003). A higher concentration of primer can compensate the lower concentration of the given target or template (Singh et al., 2000). Majumder et al., (2008) reported that a two fold increase in primer concentration increased detection efficiency in duplex PCR for simultaneous detection of OYDV and SLV.

Detection of PVY and PVS in potato tubers

The tubers known to be infected with viruses were screened for the infection of PVY and PVS independently by optimized uniplex PCR for respective viruses wherein the uniplex RT-PCR results indicated the presence of both the viruses. The cDNA of the same tubers were used as a template in duplex PCR. The results indicated a sharp amplicon of both the viruses indicating that duplex RT-PCR can be employed for screening the seed tubers in seed certification programme. Singh et al. (2000) reported the optimized duplex RT-PCR which is capable of detecting PVY and PLRV in composite samples of field grown tubers. Similarly Majumder et al. (2008) optimized the duplex RT-PCR for simultaneous detection of Onion yellow dwarf virus (OYDV) and Shallot latent virus (SLV) in cloves of garlic. Hidayet and Pinar (2009) also reported the use of triplex RT-PCR for accurate and speedy diagnosis of PLRV, PVS and PVX in dormant potato tubers as an integral part of seed certification programs.

Validation of duplex RT-PCR

It is very much essential to validate and assess the usefulness of optimized duplex RT-PCR for routine detection of PVY and PVS. So, a total of 25 suspected potato plant samples collected from field were processed for duplex RT-PCR. Along with the samples, controls of duplex PCR, uniplex PCR (respective viruses) and negative control were tested. The results revealed that the optimized duplex RT-PCR could successfully detect both the viruses in 5th and 12th samples, whereas other samples gave amplification of either PVY of PVS. So, this duplex RT-PCR was most successful and reliable in detecting both PVY and PVS. With respect to controls, specific and desired amplicons were amplified in positive control and no band in negative control (Fig. 2). Singh et al. (2003) used duplex RT-PCR to detect both the common (PVYO) and tobacco veinal necrosis strain (PVY N/NTN) of Potato virus Y. Similarly, Majumder et al. (2008) has optimized the duplex RT-PCR and was used to screen Onion yellow dwarf virus (OYDV) and Shallot latent virus (SLV), wherein it could successfully

![Fig. 2. Validation of duplex RT-PCR of PVY & PVS using field collected samples. 1 Ladder; 2to 14- field collected samples; 15-positive control for duplex; 16- Negative control, 17-18 uniplex for PVS and PVY respectively.](image-url)
detect both the viruses in all the selected lines collected from field.

CONCLUSIONS

Production of virus-free planting material is one of the most important steps in production of potato in generation of healthy planting material. The optimized duplex RT-PCR for simultaneous detection of PVY & PVS in field infected plants and tubers reduce the time and cost of the reagents (chemicals) as compared to uniplex RT-PCR. Therefore, it is suggested that the optimized duplex RT-PCR can be employed to detect single or mixed infections of PVY and PVS in routine screening for virus freedom in seed certification programme.

LITERATURE CITED


MS received: 15 April 2013; Accepted: 22 April 2013