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Uniplex and duplex PCR detection of geminivirus associated with potato apical leaf curl disease in India

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ABSTRACT

Apical leaf curl disease has emerged as a new disease in potato during the last decade in India due to a change in planting date and an increased whitefly population. Its incidence is on the rise threatening the cultivation of potato across the country. Hence, a PCR assay was developed for the detection of *Tomato leaf curl New Delhi virus*-potato (ToLCNDV-Potato) which is the causal agent of apical leaf curl disease in potato. Primers specific to the coat protein (AV1) and replicase (AC1) gene regions were designed and used for standardization of the PCR. Some of the primers (LCVCPF1/LCVCPR1, LCVREPF2/LCVREPR2, LCrep1F/LCrep2R) could detect the virus in 2.4–0.24 pg of total DNA of infected plant. A duplex PCR assay was optimized with the selected coat protein gene specific primers and primers specific to potato urease gene, a housekeeping gene served as an internal check. The suitability of these primers was examined for detection of the virus in 80 potato apical leaf curl disease samples from 11 different potato growing states of India and also from micro-plants grown in tissue culture. The selected coat protein primer pair (LCVCPF1/LCVCPR1) was found to be conserved in all 80 isolates except for a few isolates, which had a single nucleotide substitution in the forward primer sequence. These substitutions did not interfere with amplification of the coat protein gene. The primers could detect the virus using a print-capture PCR assay both in the presence and absence of an internal control. These results indicate the robustness of the PCR assay for virus indexing of mother stocks in the seed production system.

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1. Introduction

Potato apical leaf curl disease was first reported in northern India by Garg et al. (2001). The association of a geminivirus with this disease has been found by immunoelectron microscopy using polyclonal antibodies of the *Indian cassava mosaic virus* and based on symptoms, the virus was named tentatively as *Potato apical leaf curl virus* (Garg et al., 2001). Later, Usharani et al. (2003) confirmed that this virus is a strain of *Tomato leaf curl New Delhi virus* (ToLCNDV) belonging to the genus *Begomovirus* within the family *Geminiviridae*. The virus has 93–95% sequence identity with ToLCNDV isolates and <75% sequence identity with other *Tomato leaf curl virus* isolates and *Potato yellow mosaic virus* (Usharani et al., 2003). The affected plants show curling/crinkling of apical leaves with a conspicuous mosaic symptom. When the infected tubers are used for planting, the entire plant show symptoms with severe stunting due to high virus titre (Fig. 1). The plants recover from symptoms as the maximum temperature falls below 25 °C. Recently, high prevalence of this disease was recorded in some of the popular high yielding commercial potato varieties from different potato growing regions of the country. The virus is transmitted by whiteflies and the infection is more common in crops planted during October than in November because of the large whitefly population (Chandel et al., 2010). The incidence correlates positively with the whitefly population and the whitefly infestation period of potato crops (Lakra, 2003a). Around 40–75% of infections were recorded in the cultivars grown in Indo-Gangetic plains of India (Venkatasalam et al., 2005). Lakra (2002) reported even up to 100% of infection from the Hisar (Haryana) area of India causing heavy yield losses in susceptible varieties. The primary infection in the field appears within 40–45 days after planting. Infection results in significant decrease in size and number of tubers (Lakra, 2003b). Losses in marketable yield were reported to be as high as 50% in early planted susceptible cultivars (Lakra, 2002).

In potato seed production programme, enzyme linked immunosorbent assay (ELISA) is being used for indexing of tubers for viruses. To augment the seed production, the institute is shifting from conventional system to micropropagation in which the virus titre goes very low in microplants. In this circumstance, the ELISA may fail to detect the virus and give false negative results. Therefore, a polymerase chain reaction (PCR) technique

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Fig. 1. Infected potato plants showing severe symptoms of apical leaf curl disease.

was standardized for its detection. Standard RT-PCR protocols are available for the detection of potato viruses (Nie and Singh, 2001; He et al., 2006; Schubert et al., 2007; Nie et al., 2008; Ali et al., 2010). Print-capture PCR and conventional PCR detection of ToLCNDV-Potato was reported earlier (Gawande et al., 2007 and Venkatasalam et al., 2011) but using a single primer pair and few samples. The present study was carried out to test different primer pairs, to develop a reliable PCR assay and to validate the assay for its routine use. A duplex PCR assay along with urease gene of potato as an internal control to avoid false negative results is also described.

2. Materials and methods

2.1. Designing of primers

The complete nucleotide sequence of DNA A of ToLCNDV-Potato (AY286316) was used to design the primers. Eight pairs of primers, two each from the coat protein (LCCP1F/LCCP1R and LCCP3F/LCCP3R) and replicase gene (LCrep1F/LCrep2R and LCrep2F/LCrep2R) regions were designed using Primer 3 Input (version 0.4.0) software and two each from the coat protein (LCVCPF1/LCVCPR1 and LCVCPF2/LCVCPR2) and replicase gene (LCVREPF1/LCVREPR1 and LCVREPF2/LCVREPR2) regions were designed manually and properties were analyzed with online software, Oligo Calc: Oligonucleotide properties calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html). For urease gene, a forward primer Ure-f1 reported by Bradeen et al. (2009) was used along with a reverse primer designed in this study by using sequence information of λ2 region of urease gene (AJ276864). Primer sequences, locations and expected amplicons according to the target sequences are shown in Table 1.

2.2. Source of plant materials

Tubers were collected from the potato plants showing typical symptoms of apical leaf curl disease, planted in insect proof glass house and leaves from these plants were then used to standardize the PCR. Eighty leaf samples collected from fields and experimental farms in 11 different potato growing states of India during 2010–2011 were used for validation of the selected primers. In all experiments, virus free micro plants grown in tissue culture were used as negative control.

2.3. Standardization of PCR conditions and selection of primers

Total DNA was extracted from healthy and infected leaves using GenElute™ Plant Genomic DNA Miniprep kit (Sigma-Aldrich, Missouri, USA) following the manufacturer's instructions and the concentration and quality of DNA was checked using a Nanodrop 2000 spectrophotometer (Thermoscientific, Leon-Rot, Germany). PCR was carried out in 20 μl reaction volume containing 2.0 μl of DNA (≤100 ng of total DNA), 2.0 μl of 10× PCR buffer (100 mM Tris–HCl pH 9.0, 500 mM KCl, 15 mM $MgCl_2$), 1.0 μl of 2 mM dNTPs, 1.0 μl of 10 μM of respective forward and reverse primers, 0.5 μl of 1.5 U *Taq* DNA polymerase (GeNei, Bangalore, India) and 12.5 μl of sterile double distilled water. As the range of calculated annealing temperature of the designed primers was from 55 °C to 65 °C, gradient PCR was performed with all eight primer pairs at different annealing temperatures, *viz*., 53 °C, 55 °C, 57 °C, 59 °C, 61 °C and 63 °C in a Veriti 96 well thermal cycler (Applied Biosystems) to select the optimum annealing temperature. The temperature profile of the PCR cycle was pre-incubation at 94 °C for 5 min leading to 35 cycles of melting at 94 °C for 1 min, annealing at the above mentioned temperatures for 1 min and synthesis at 72 °C for 1 min followed by an extension of 72 °C for 10 min. All other PCR were carried out using GeneAmp PCR system 9700 (Applied Biosystems). The amplified products were analyzed by electrophoresis in a 1% agarose gel in 1× TAE (0.04 M Tris–acetate, 1 mM EDTA, pH 8) buffer and visualized with UV light after ethidium bromide staining.

2.4. Sensitivity of the selected primers

The sensitivity of the selected primer pairs was evaluated by making tenfold dilutions ($1/10^2$–$1/10^7$) of the total DNA isolated from the infected leaf sample in sterile distilled water which is approx. 24 ng, 2.4 ng, 0.24 ng, 24 pg, 2.4 pg and 0.24 pg of total DNA in 2 μl, respectively and PCR were carried at annealing temperatures, *viz*., 57 °C and 61 °C, with the same PCR mix and cycle conditions. The selected primer pairs were checked for the absence of any non-specific amplification in 24 commercial varieties under micro-propagation during which the annealing temperature was

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