

NEW SENSITIVE ASSAY FOR THE DETECTION OF POTATO LEAFROLL VIRUS BY CONVENTIONAL AND REAL-TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION



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INTRODUCTION

Potato leafroll virus (PLRV) is one of the most important viral pathogens infecting potato. The virus is phloem limited and its titer in infected plants is low. Thus, the pathogen detection is difficult. Here we report on a development of new RT-PCR and real-time RT-PCR assays based on the same primers for very sensitive PLRV detection.

MATERIAL AND METHODS

Total RNA was purified as previously described [3]. RT reactions were carried out with the Reverse Verte KIT (Novazym). cDNA was amplified using Singh primers [2] and designed pairs: L2, L4, L5, L6. PCR was performed using a 25 µl reaction mixture: 2.5 µl of cDNA, 2 mM MgCl₂, 0.2 mM dNTPs containing dATP, dCTP, dGTP and dUTP, 0.4 µM of each forward and reverse primer, 0.5 U Uracil DNA Glycosylase (Bioline), 1xPolymerase Green Buffer and 1.25 U Go-TaqHotStart Polymerase (Promega). The temperature profile followed: initial denaturation at 95°C for 3 min, 35 cycles at 94°C for 10 sec., annealing at 60°C for 20 sec., extension at 72°C for 30 sec. and a final extension at 72°C for 5 minutes.

Real-time PCR was carried out on a CFX96 Touch™ Real-Time PCR Detection System (BioRad Ltd.) using AmpliQ Real-Time PCR OptiProbe kit or AmpIQ Real-Time PCR SybrGreen kit (Novazym). Final reactions contained 2.5 µl of cDNA, 5 µl of respective AmpIQ Real-Time PCR kit, 0,15 µl of 20 µM stock of forward and reverse primers and 0,15 µl of 10 µM probe in probe-based assays, which was supplemented by nuclease-free water in dye-based assays. All reactions were supplemented with nuclease-free water up to 10 µl. Thermal cycling conditions were 10 min at 95°C followed by 60 cycles of 10 s at 95°C and 20 s at 60°C.

Table 1 Primers used for detection of PLRV in this study.

| Primer names | Lenght (bp) | Virus | Position | Tm (°C) | Sequence (5'-3') | Reference |
|--------------|-------------|-------|-------------|---------|-----------------------|---------------------|
| Singh F | 336 | PLRV | 3,669-3,688 | 55,9 | CGCGCTAACAGAGTTTCAGCC | Singh et al. (1995) |
| Singh R | | | 4,004-3,985 | 53,8 | GCAATGGGGTCCAACTCAT | |
| L2 F | 165 | PLRV | 3,869-3,888 | 53,8 | AGACTGTCCGGCATTCGAAG | This paper |
| L2 R | | | 4,033-4,014 | 53,8 | TTGACGTAGGACTGGAGGGA | |
| L4 F | 194 | PLRV | 3,839-3,858 | 53,8 | AGGAAGTTTCACCTTCGGGC | This paper |
| L4 R | | | 4,032-4,013 | 53,8 | TGACGTAGGACTGGAGGGAT | |
| L5 F | 90 | PLRV | 3,785-3,804 | 53,8 | AGGCTCAAGCGAGACATTCG | This paper |
| L5 R | | | 3,874-3,855 | 55,9 | CAGTCTGATAGACTCGGCC | |
| L6 F | 218 | PLRV | 2,150-2,169 | 60,0 | GC CGGAGTCGGCCACTATC | This paper |
| L6 R | | | 2,367-2,348 | 57,9 | TGTGGGGAGGCCATACGCGA | |

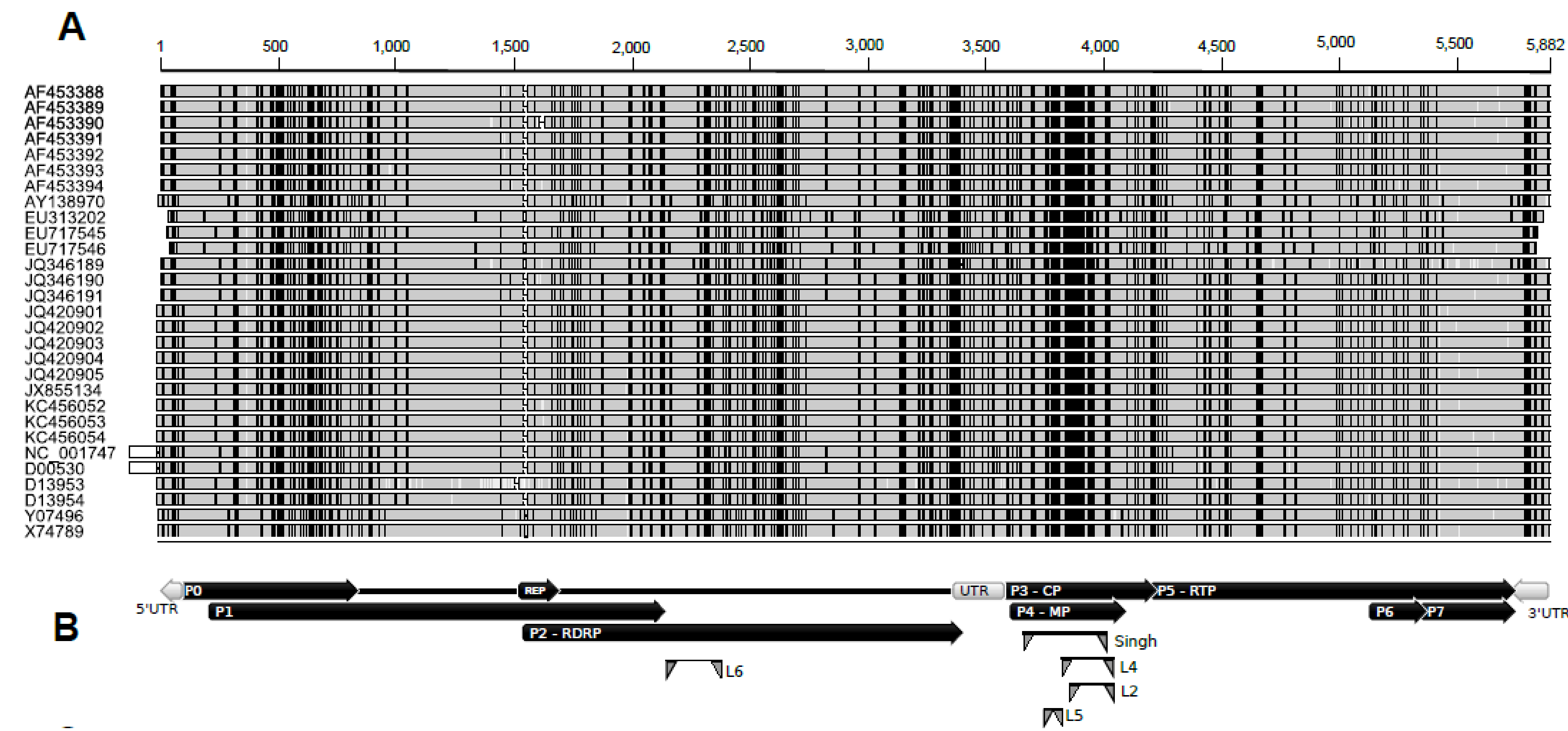


Fig. 1 Multiple sequence alignment of 29 PLRV isolates (A) in scale with scheme of PLRV genome organization (B) having indicated non coding regions (UTRs) and regions coding for specific proteins (P0-P7). REP is replication associated protein (Jaag et al. 2003). RDRP – RNA-dependent RNA polymerase, CP – coat protein, MP – movement protein and RTP – readthrough protein (Taliensky et al. 2003). Position of primers used in this study is also indicated as L2, L4, L5 and L6 for primer set designed in this study and Singh for set designed by Singh et al. (1995) (B). Comparison of the primers sequences with the corresponding genome fragments of PLRV isolates (C). PLRV isolates are indicated by their respective Gene Bank accession number.

RESULTS AND DISCUSSION

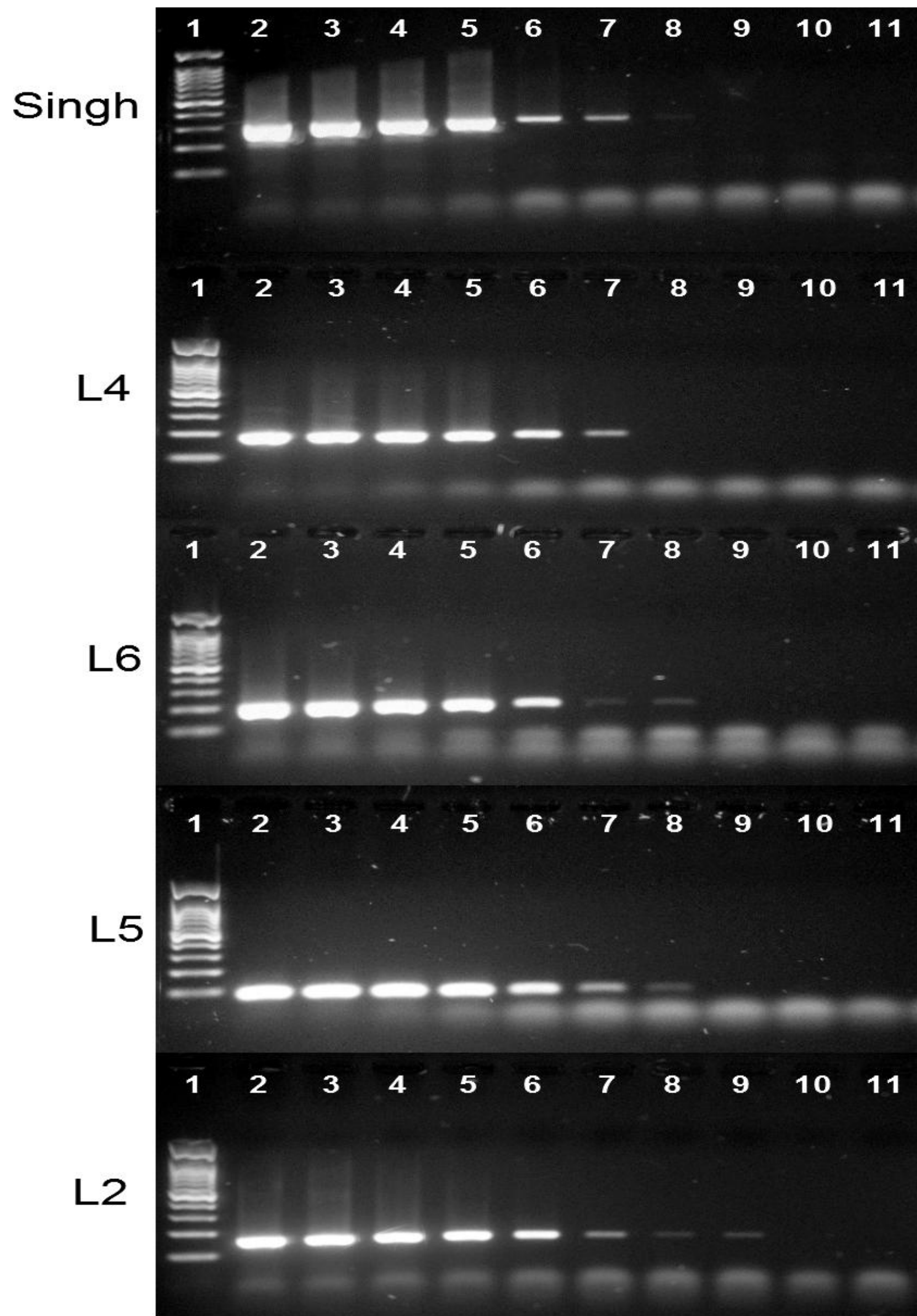


Fig. 2 Influence of primer sets: Singh (A), L4 (B), L5 (C), L6 (D) and L2 (E) on the sensitivity of PLRV detection by RT-PCR. Lane 1-8, 10-fold serial dilutions from 100 ng to 10 fg amount of RNA added to reverse transcription (RT) reaction; lane 9 – RT reaction supplemented with water instead of RNA (RT mock); lane 10 – PCR reaction supplemented with water instead of cDNA (PCR mock), lane 11 – reaction supplemented with RNA isolated from healthy plant, reverse transcribed and amplified by PCR (negative control). M is size marker Nova 100 bp DNA ladder (Novazym).

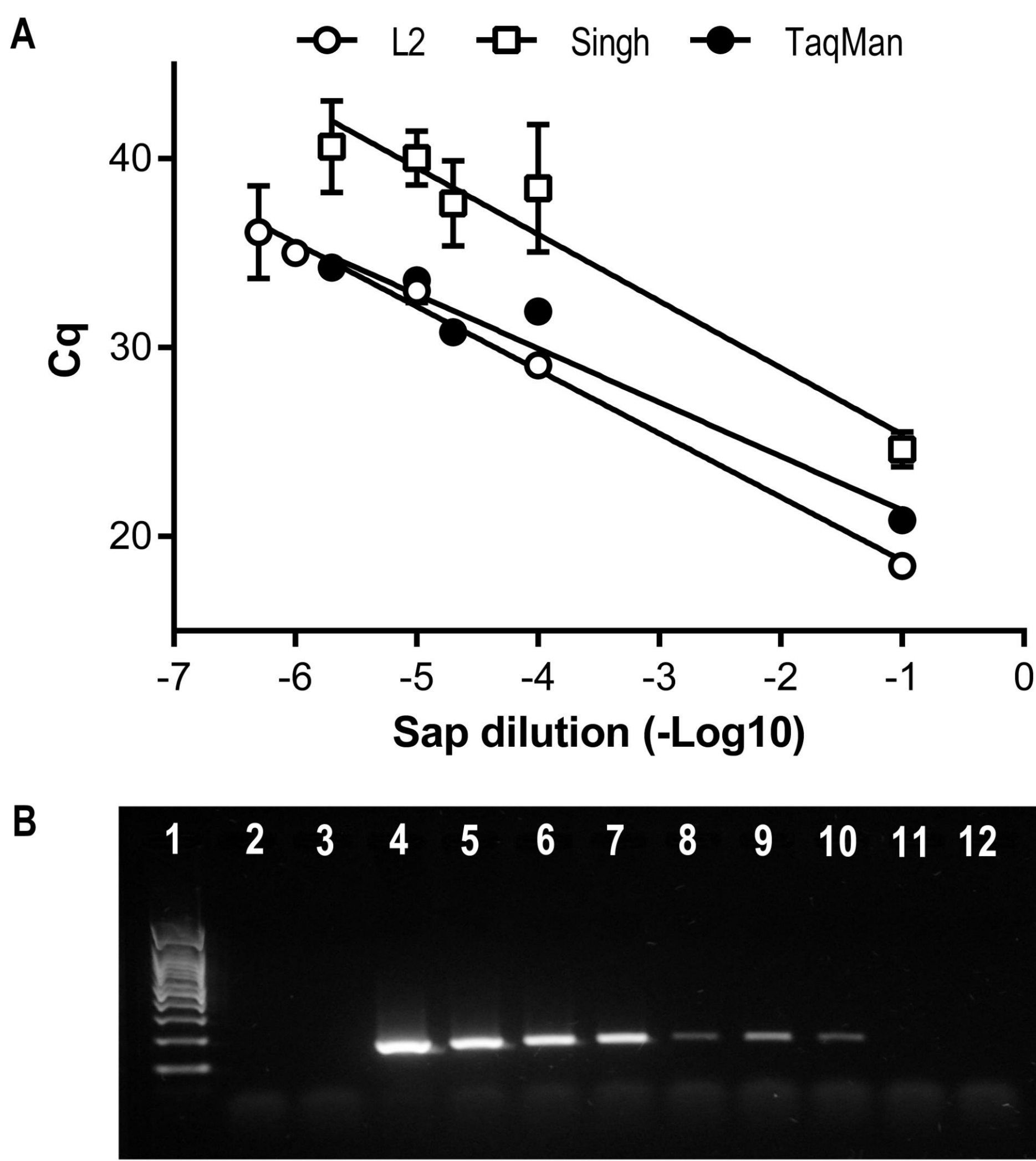


Fig. 3. Effect of primer sets: L2, Singh, TaqMan on PLRV detection sensitivity by real-time RT-PCR (A) and sensitivity of L2 primers in conventional RT-PCR (B).

Primer pair designed for PLRV detection by Singh et al. [2] become one of the most intensively used in PLRV-related works. Thus, we determined if any of designed primers can facilitate more sensitive detection than that set. For L4 primers amplification product was observed up to 1 pg of RNA. Primer set designed by [2], L6, and L5 facilitated PLRV detection up to 100 fg of RNA. L2 set was the most sensitive (10 fg). The sensitivity of PLRV detection using L2 primers was further tested by conventional RT-PCR and real-time RT-PCR. Total RNAs were isolated from sap dilutions and amplified by conventional RT-PCR based on L2 primers and by real-time RT-PCR. The real-time RT-PCR was performed with different sets of primers including (i) the L2 primers in the presence of SybrGreen (SG), (ii) the PLRV-specific primers designed by Singh et al. [2] in the presence of SG and (iii) the PLRV specific primers and TaqMan probe (TM) designed by Boonham et al. [1]. Conventional RT-PCR amplified specific product up to 2x10⁶-fold diluted sap (Fig. 3B). The SG dye and L2 primers based real-time RT-PCR facilitated detection of PLRV in sap diluted up to 2x10⁶-fold. The SG real-time RT-PCR with primers according to Singh et al. [2] and TM real-time RT-PCR with primers and probe according to Boonham et al. [1] detected PLRV in samples diluted up to 5x10⁵ fold.

CONCLUSION

Among tested primers, L2 set facilitated the most sensitive PLRV detection both by RT-PCR and by real-time RT-PCR. The sensitivity of conventional RT-PCR and real-time RT-PCR was the same when both assays utilized L2 primers

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