Development of multiplex RT-PCR and real-time RT-PCR assays to detect three potato viruses

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INTRODUCTION

Due to the vegetative way of propagation, the potato plant is a host to more than forty viruses. Among them, the most important is potato virus Y (PVY), which can reduce the tuber yield up to 80%. Similarly, a devastating impact can be also imposed by potato leafroll virus (PLRV). The dependence of this virus on its aphid vector facilitated effective control through the use of aphicides. However, it is still circulating in the environment and can propagate in seed tubers. Potato virus M (PVM) is less dangerous in terms of direct yield losses but infected plants attract fungal and bacterial pathogens contributing to the economic importance of this virus. Production of healthy seed tubers requires testing for the most important viruses, usually by DAS-ELISA. Here, we present our data on the development and optimization of sensitive and simultaneous detection of PVY, PLRV and PVM by conventional multiplex RT-PCR and started the optimization of the assay to the real-time RT-PCR format.

MATERIALS AND METHODS

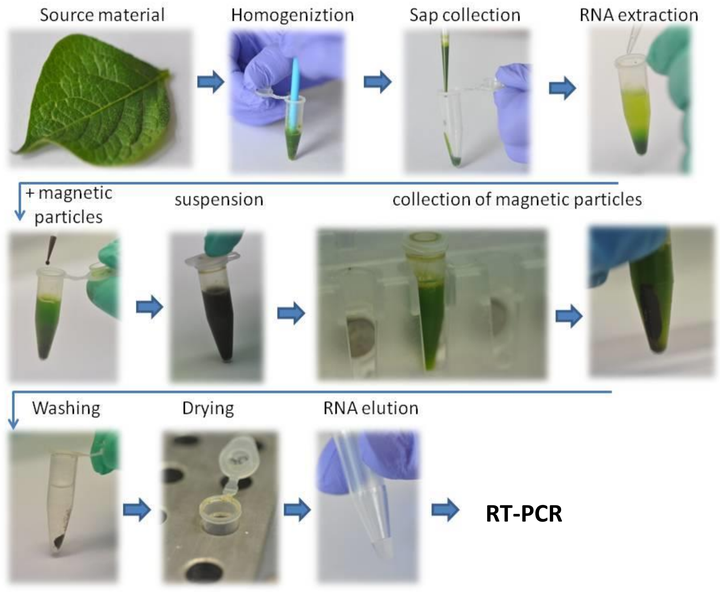

Fig. 1. Magnetic capture of RNA [1]

Table 1. Primers used in this study

Name	Sequence (5'-3')	Product (bp)	Reference
L2F	AGACTGTCCGGCATTCAAGG	165	This work
L2R	TTGACGTAGGACTGGAGGGA		This work
RS Y1F	CCAATCGTTGAGAATGCAAAAC	74	[2]
RS Y1R	ATATACGCTTCTGCAACATCTGAGA		[2]
M7F	GAyGGyGTGCAGwGCGAGGG	404	This work
M7R	TGrATCACCAGCCGCGCGAA		This work
Plant Uni	TGGGAAGTCCTCGTGTTGCA	260	[3]
Plant Uni	TTTAGTGCTGGTATGATCGC		[3]

The total RNA was isolated according to [1] from virus-infected and healthy control potato plants (Fig. 1). The RNA was adjusted to 100 ng/µl. For sensitivity assays it was serially diluted to cover range from 10 ng/µl to 10 fg/µl. Dilutions were tested both by uniplex and by multiplex RT-PCR. The experiments were performed in three independent biological replicates. The cDNA synthesis was performed using the Reverse Verte Kit with random hexamers (Novazym). The PCR mixture in the final volume of 10 µl contained: cDNA (1 µl), 2 mM $MgCl_2$; nucleotide triphosphates (dATP, dCTP, dGTP and dUTP) 0.2 mM each; 0.5U Uridine N-glycosidase (Bioline); primers 0.4 µM each, 1x polymerase buffer and 1.25 U GoTaq® Hot Start polymerase (Promega). The reaction was carried out according to the following thermal profile: initial denaturation - 3 min at 95 ° C, 35 cycles of denaturation for 1 min at 94 ° C; attaching the primers at 55 ° C for 1 min, primer extension at 72 ° C for 1 min. The final extension was carried out for 5 min at 72 ° C. The reaction products were separated by electrophoresis in 1.5% agarose (140V for 40 min). The gels were stained with a GelGreen dye (Biotium), and the reaction product was visualized by excitation of fluorescence of the dye complex with DNA at 470 nm using a Blue Box transilluminator (Invitrogen). Gels were documented using a Nikon D90 camera using an orange filter. As part of the research, reaction conditions were optimized to obtain optimal amplification of all viruses. For real-time RT-PCR conditions were as described in [1]. Primers L2 (table 1) and Y4 [1] were compared with primers described for PLRV [4] and PVY [2] detection by real-time RT-PCR.

RESULTS

Plant Uni primers effectively amplified 5S RNA both in the virus-free (Fig. 2A, samples 1-5) and in virus-infected plants. This result indicates that the quality of RNA isolated by magnetic capture (Fig. 1) was good. In plants infected by PVY, PLRV and PVM (Fig. 2A, PC1) or by PVY and PLRV (Fig. 2A, PC2) both PVY and PLRV were correctly detected in the presence of Plant Uni primers. Also, singular infections by PVY (Fig. 2A, PC4) or PLRV (Fig. 2A, PC3) were correctly detected. However, Plant Uni primers had a negative impact on the detection of PVM (Fig. 2A, PC1). Excluding the Plant Uni primers from the primer mix had a positive effect on detection of PVM in the mixed infection (Fig. 2B, PC1). In multiplex reaction, all viruses were detected in the total RNA diluted up to 100 pg/µl (Fig. 3D) and sensitivity of the detection in uniplex and multiplex RT-PCR reactions was comparable (Fig. 3). The multiplex RT-PCR was applied to test 200 potato genotypes from 1600 total stored in the Potato Gene Bank (Bonin Research Center, Laboratory of In Vitro Cultures). The example of this testing is shown in Fig. 4. All tested genotypes were confirmed to be virus-free. Positive results were only obtained for plants from the virus collection (Fig. 4, samples 30-35). Preliminary results show that selected primers are appropriate for real-time RT-PCR (Fig. 5). However, further work is needed to develop the real-time multiplex assay.

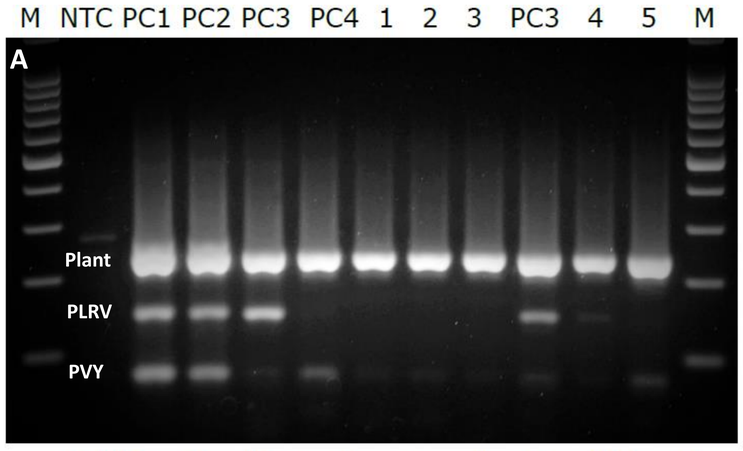

Fig. 2. The detection of PVY, PLRV and PVM in the presence (A) or absence (B) of Plant Uni primers. Non-template control – NTC, plant infected simultaneously by PVY, PLRV and PVM – PC1, double infection by PVY and PLRV – PC2, singular infection by PLRV – PC3 and by PVY – PC4. Markers 100-1500 bp – M.

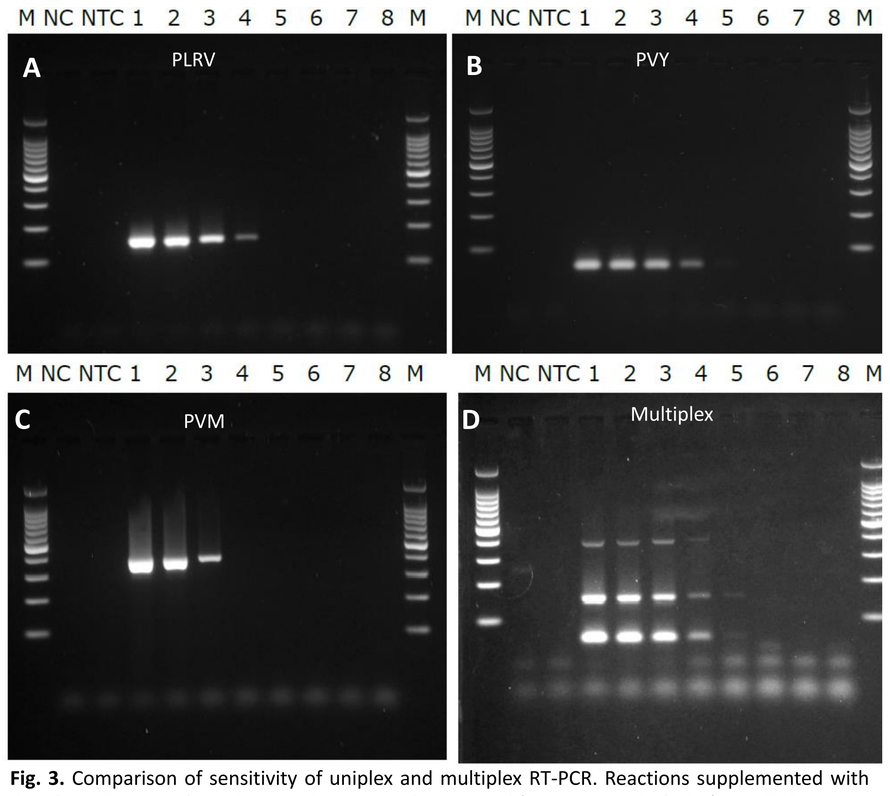

Fig. 3. Comparison of sensitivity of uniplex and multiplex RT-PCR. Reactions supplemented with RNA from the virus-free plant – NC, non-template control (water instead of RNA) – NTC. Reactions supplemented 1 ul of RNA dilutions from virus-infected plants containing 1 – 100ng, 2 – 10 ng, 3 – 1 ng, 4 – 100pg, 5 – 10 pg, 6 – 1 pg, 7 – 100 fg, 8 – 10 fg of total RNA.

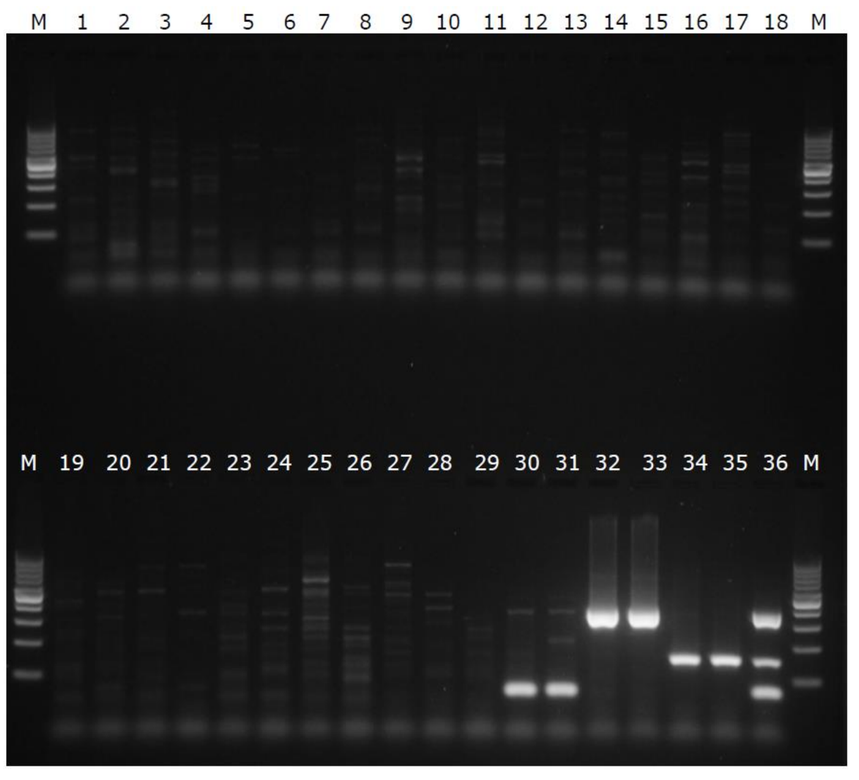

Fig. 4. Example of testing the collection of potato genotypes stored in the Potato Gene Bank by multiplex RT-PCR. Samples from the virus collection - 30-35.

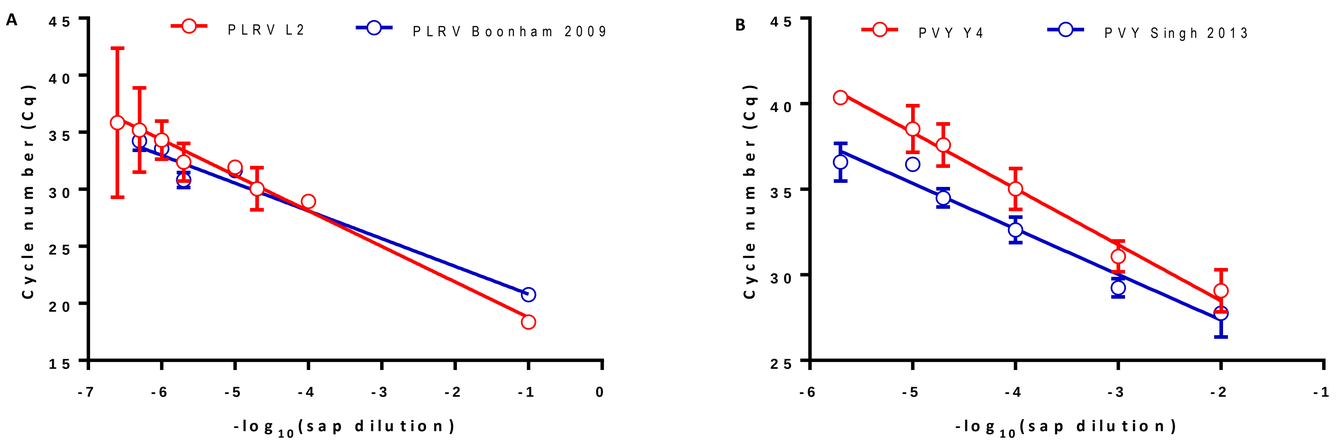

Fig. 5. Comparison of sensitivity of PLRV (A) and PVY (B) detection by primers L2 and described in [4] for PLRV and primers Y4 [1] and published in [2] for PVY. The total RNA was isolated as in [1] from sap obtained from PLRV and PVY-infected plants and serially diluted up to 8 mln fold. The PLRV was detected by L2 up to 4 mln-fold diluted sap and by primers described in [4] up to 1 mln-fold diluted sap. Both tested primers detected PVY up to 0.5 mln-fold diluted sap.

CONCLUSIONS

- The Plant Uni primers had a negative impact on PVM detection.
- The multiplex RT-PCR assay has comparable sensitivity to uniplex RT-PCR.
- The developed method is appropriate for certification of genotypes introduced to the gene bank.
- Further simplification of the assay can be achieved by switching to the real-time detection format but requires further work.

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