



Detection of four major potato viruses in Japan using a simple RNA preparation and one-step multiplex RT-PCR

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Abstract

A high-throughput alternative to ELISA to detect potato leafroll virus (PLRV), potato virus S (PVS), potato virus X (PVX), and potato virus Y (PVY), economically important viruses in Japan, has been needed for seed potato production. To develop an alternative using a multiplex reverse transcription polymerase chain reaction (mRT-PCR), we verified reported primers by two-step mRT-PCR and designed new primers for PVS and PVY based on the conserved region of genome sequences among three lineages of PVS or six strains of PVY. In addition, primers specific for potato and tobacco elongation factor 1 alpha were designed as an internal control for mRT-PCR. With these primers, one-step mRT-PCR detected all reference isolates of the four viruses. A published paper-based RNA preparation was modified for use in a single tube with an elution procedure to preserve RNA as evidence. As a rinse solution to wash away contaminants, 50% (v/v) isopropanol and 75% (v/v) ethanol resulted in a sensitivity for all viruses was tenfold higher than with the buffered detergent. In the mRT-PCR, the optimized paper-based RNA preparation gave tenfold higher sensitivity than the other two RNA preparation methods. Compared with ELISA, the new RNA preparation with the mRT-PCR was tenfold more sensitive for PVY and PLRV, 1,000-fold more for PVX, but the same for PVS. The new detection method simultaneously detected the four potato viruses from potato leaf by group test and is efficient and sensitive enough to detect the four potato viruses for seed potato certification, quarantine, breeding, and field surveys.

Keywords Conventional RT-PCR · Simple RNA extraction · Filter paper · Internal control · Seed potato production

Introduction

Potato (*Solanum tuberosum* L.) is susceptible to more than 30 virus species (Kumar et al. 2017; Stevenson et al. 2001). In Japan, 12 of these viruses have been isolated from potato plants: alfalfa mosaic virus (AMV), cucumber mosaic virus (CMV), potato aucuba mosaic virus (PAMV), potato leafroll

virus (PLRV), potato mop-top virus (PMTV), potato virus A (PVA), potato virus M (PVM), potato virus S (PVS), potato virus X (PVX), potato virus Y (PVY), tomato ringspot virus (ToRSV), and tomato spotted wilt virus (TSWV) (Maoka et al. 2010). Of the 12, PLRV, PVS, PVX, and PVY predominate in potato fields (Sato et al. 2000). They are vertically transmitted in seed tubers and reduce the quality and yield of potato (Stevenson et al. 2001). Therefore, the use of healthy seed potato is important for their control.

In Japan, the Center for Seed and Seedlings (NCSS) of the National Agriculture and Food Research Organization (NARO) contributes to stable potato production and supply through a seed potato production system (Kawakami et al. 2015). In this system, virus-free seedlings are tissue-cultured from the shoot apical meristem, then virus-free minitubers are produced in the greenhouse. Seed potatoes are propagated from minitubers over two cultivation seasons in the field and finally distributed to prefectural governments. At each stage of propagation, the seed potatoes are tested for virus and certified as virus-free. Although ELISA is a

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simple method for virus detection, antibodies for each virus are needed to detect the four viruses, and it cannot detect multiple viruses in a single assay, so it is expensive and time-consuming for certifying a large number of samples. Thus, an efficient alternative is required to reduce the cost and labor.

A potential alternative to detect multiple viruses is the multiplex reverse transcription polymerase chain reaction (mRT-PCR). Several mRT-PCR assays for potato viruses, which simultaneously detect more than four viruses, have been developed (Bostan and Peker 2009; Du et al. 2006; Nie and Singh 2000; Zhang et al. 2017). The target viruses for mRT-PCR assays are different from each other, and two assays have been developed to detect PLRV, PVS, PVX, and PVY. The assay reported by Bostan and Peker (2009) is easier for separating amplicons by electrophoresis than the other, so their assay would be useful for routine virus detection. Because PVS has been divided into three lineages (Vallejo et al. 2016) and PVY is actually a complex of strains (Green et al. 2017), the specificity of primers for at least two viruses should be verified.

Some simple RNA preparation methods are reported (Agindotan et al. 2007; Nakaune and Nakano 2006; Shi and Panthee 2017; Singh et al. 2004; Zou et al. 2017). A paper-based RNA preparation, in which nucleic acids from a plant homogenate are immediately captured on cellulose filter paper, then slowly released into an external solution, is simple, fast, and inexpensive (Zou et al. 2017). The same principle is used in a commercial product, FTA card technology (GE Healthcare UK Ltd, Amersham, UK), so the principle is suitable for RNA preparation. On the other hand, the method is not suitable for preserving the RNA solution as evidence of certification; because the filter paper is used as the template for RT-PCR, the RNA must first be eluted from the filter paper to be preserved.

Here we report a high-throughput detection method for major Japanese potato viruses from leaf samples using a paper-based RNA preparation for a one-step mRT-PCR. The specificity of primers was verified using genomic sequences of all lineages or strains of each virus. This one-step mRT-PCR can simultaneously detect the four viruses and one

plant gene as an internal control. We altered a previously reported paper-based RNA preparation so that the RNA solution can be preserved and optimized for one-step mRT-PCR. We also compared the sensitivity of the new detection method to that of the ELISAs for these viruses.

Materials and methods

Virus sources

The potato viruses used in this report are listed in Table 1. All viruses were isolated from *S. tuberosum* in Japan. PVY-NTND6 was isolated in Kyushu island, and was kindly provided by Dr. Ogawa, Nagasaki Agriculture and Forestry Experiment Station. The others were isolated in Hokkaido and kindly provided by Mr. Fuwa and Mr. Aono, NCSS. The PLRV isolate was maintained in its source potato cultivar (*S. tuberosum* cv. Toyoshiro). PVS isolates were propagated in *Nicotiana occidentalis* and PVX and PVY isolates in *N. tabacum* cv. Xanthi nc. Virus-infected leaves were stored at -80°C until use.

Primer design

Genomic sequences of potato viruses and plants were retrieved from the website of National Center for Biotechnology Information (NCBI, <https://www.ncbi.nlm.nih.gov/>), and the conserved region of each virus and plant gene was determined using MEGA-X program (<https://www.megasoftware.net/>). Primer melting temperature was calculated using the online program Oligo Calculator (Nihon Gene Research Laboratories, Inc., <https://www.ngrl.co.jp/tools/0217oligocalc.htm>).

For designing PLRV-specific primers, eight genomic sequences were used: 14.2 (GenBank accession AF453394), D00530 (X14600), GAF318-4.2 (KU586454), JPI-1 (JQ420901), PLRV165 (MG356502), PLRV-IM (KC456052), Polish isolate (X74789), and VIRUBRA_1/046 (EU717546) were used; for PVS, nine genomic sequences: Antioquia (KR152654), BB-AND (JX683388), HU1

Table 1 Information on isolates of potato viruses used in this study

Virus	Isolate	Strain / Lineage	Accession ^a	References
PLRV	ChLR_2	unclassified	LC501445	This study
PVS	M	Ordinary	AB451180	Maoka et al. (2010)
	ChS_3	Andean	LC492754	This study
PVX	O-IC249	unclassified	AB451181	Maoka et al. (2010)
PVY	Y28	O	AB451182	Maoka et al. (2010)
	PVY-NTND6	NA-N	AB331515	Ogawa et al. (2008)
	Eu-12Jp	NTN	AB702945	Chikh-Ali et al. (2013)

^aDDBJ/EMBL-Bank/GenBank accessions

(HF571059), Id4106-US (FJ813513), Leona (AJ863509), RL5 (JX683388), RVC (JX419379), Vltava (AJ863510), and Yunnan YN (KC430335): for PVX, 10 sequences: BH (AB195999), CH (MF405302), FX21 (EF423572), GAF318-4.1 (KU586452), Iran isolate (FJ461343), JAL-2 (KR605396), Korean isolate (AF373782), Taiwan isolate (AF272736), Tn148 (MF682528), and Tomato Antioquia (MH282866): and for PVY, 12 sequences: NC57 (DQ309028), Adgen (AJ890348), CA14 (KY847936), ME_236 (KY847962), CO 11 (KY847987), MT100010 (KY847937), MON (JF928458), AGA (JF928459), HO090 (AB331517), PVY-NTND6 (AB321515), MSU_45_384a (KY847984), and MT100017 (KY847988). For the internal control, six sequences of mRNA of potato elongation factor 1 alpha (*EF1 α*) (DQ228326, DQ228328, DQ222490, AJ536671, AB061263 and KF573426), and two of tobacco *EF1 α* (NM_001326165 and U04632) were used.

Establishment of one-step mRT-PCR

Total RNA was extracted from about 25 mg of frozen leaf tissue using PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA), a spin-column-based RNA extraction kit, according to the instructions. The concentration of total RNA was measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). RNA solutions were diluted to 100 ng/ μ l with RNase-free water and used in subsequent experiments.

Primer specificity was confirmed using a two-step RT-PCR. For cDNA synthesis, 100 ng of total RNA was used. Templates and primers were mixed and denatured at 65 °C for 5 min, then chilled on ice at least 1 min. Because PVS, PVX, and PVY have a poly-A tail in their genomic sequence and PLRV does not, an oligo(dT)₁₅ primer and PLRV-specific reverse primer were used in the reverse transcription (RT) reaction. First strand cDNA was synthesized in 10 μ l of the reaction mixture, containing 2.5 μ M oligo(dT)₁₅ primer, 0.2 μ M PLRV reverse primer, 0.5 mM dNTPs, 5 mM dithiothreitol (DTT), and 10 U of SuperScript III reverse transcriptase (Thermo Fisher Scientific). The reaction mixture was incubated at 50 °C for 60 min, followed by 70 °C for 15 min to deactivate the transcriptase. One microliter of cDNA solution was used in the following reaction. PCR was performed in 10 μ l of total volume reaction mixture, which contained 0.2 μ M each virus-specific primer, 0.4 μ M *EF1 α* -specific primers and 1 \times PrimeSTAR max DNA polymerase (Takara Bio Inc., Kusatsu, Japan). The thermal cyclers were programmed for 30 cycles of 98 °C for 10 s and 64 °C for 30 s. For the detection of four viruses by one-step RT-PCR, PrimeScript one-step RT-PCR kit ver. 2 (Dye Plus) (Takara Bio) was used, with 100 ng of total RNA as the template. The 10 μ l of reaction mixture contained 1 \times reaction buffer, 0.4 μ l enzyme mixture, 0.2 μ M each virus-specific primer,

and 0.4 μ M *EF1 α* -specific primers. The reaction condition was programmed for 50 °C for 60 min and 94 °C for 2 min for reverse transcription; 35 cycles of 98 °C for 10 s and 64 °C for 1 min. The RT reaction, PCR, and RT-PCR were performed in a Veriti thermal cycler (Thermo Fisher Scientific) unless otherwise stated.

Five microliters of PCR product was separated electrophoretically in 1.5–2.0% agarose, then stained with Midori Green Xtra (Nippon Genetics Co., Ltd., Tokyo, Japan) according to the instructions and visualized under 510 nm illumination with WSE-5200 Printgraph 2 M (ATTO Corp., Tokyo, Japan).

Optimization of simple RNA extraction using filter paper

The paper-based RNA preparation reported by Zou et al. (2017) was modified (Fig. 1) so that RNA was extracted in one tube, the efficacy of the rinse solution was verified, and an elution procedure was added after the rinse procedure. Leaf tissue (25 mg) was ground in 1 ml of Extraction Buffer #2 [800 mM guanidine isothiocyanate, 50 mM Tris-HCl pH 8.0, 0.5% (v/v) Triton X-100, 1% (v/v) Tween 20] as in the original method (Zou et al. 2017). A 3-mm-diameter disc was punched from Whatman Grade 1 filter paper (GE Healthcare UK) using a 3.0 mm Harris Uni-core Punch

1. Sample preparation
 - 25 mg Leaf tissue
 - | Add Extraction Buffer #2*
 - Homogenize
2. RNA extraction from homogenate
 - 3-mm-diameter paper disc in 0.2 ml tube
 - |
 - Add 100 μ l of homogenate and mix by pipetting
 - |
 - Remove the homogenate with pipet
 - |
 - Rinse with 200 μ l of 50% (v/v) isopropanol (or wash buffer*) by pipetting
 - |
 - Rinse with 200 μ l of 75% (v/v) ethanol (or wash buffer) by pipetting
 - |
 - Add 50 μ l of RNase-free water
 - |
 - Elution of RNA (95°C, 5 min) with thermal cycler
 - |
 - Supernatant (1 μ l) is used as template for RT-PCR
 - |
 - Remainder is stored at -80°C until use

Fig. 1 Flow chart of optimized paper-based RNA preparation. A filter paper disc was placed in a tube, and solutions were added and removed one after another, then RNA was eluted in RNase-free water from the disc, and the supernatant was used as the template for RT-PCR or stored at -80 °C until use. The underline indicates the steps that were added to the original method of Zou et al. (2017). * Contents are shown in “Materials and Methods”

(GE Healthcare) and placed in a 0.2 ml tube. One hundred microliters of the homogenate was added to the tube, then immediately removed from the tube and discarded. The disc was rinsed with 200 μ l of wash buffer (10 mM Tris–HCl buffer, pH 8.0, with 0.1% [v/v] Tween-20) or alcohols (50% [v/v] isopropanol and 75% [v/v] ethanol). Fifty microliters of RNase-free water was added, then total RNA was eluted at 94 °C for 5 min. One microliter of the supernatant was used as template for RT-PCR. To compare the wash buffer and the alcohols as a rinse solution, a tenfold dilution series was prepared with a homogenate of a virus-infected leaf and a healthy potato leaf, and RNA was extracted from each dilution and the viruses detected by one-step mRT-PCR. For the nitrocellulose-based RNA preparation, the protocol for the paper-based RNA preparation was followed, but using a nitrocellulose membrane (Hybond-C Super 0.45-micron, GE Healthcare) instead of filter paper and rinsing with the alcohols. For the heated RNA preparation, the protocol of Nakaune and Nakano (2006) was used with 25 mg of leaf tissue ground in 1 ml of extraction buffer [TE buffer pH 8.0 containing 4% (w/v) polyvinylpyrrolidone K40, 10 mM DTT and 0.1% (v/v) Triton X-100; modified from the solution of Nakaune and Nakano (2006)]. The crude extract was centrifuged (2,500 \times g, 5 min, 4 °C), then heated at 75 °C for 5 min. One microliter of the supernatant was used as the template.

To compare sensitivity among the three RNA preparation methods, we ground 50 mg of PVY-infected leaves or healthy potato leaves in 1 ml of ice-cold distilled water. Plant debris was removed by centrifugation (2500 \times g, 5 min, 4 °C). A tenfold dilution series was prepared with the supernatant of the virus-infected and the healthy leaf homogenate, then each dilution was divided into four tubes. For each method, an equal volume of 2 \times extraction buffer was added to diluted samples, then total RNA was extracted according to the procedures described above. For the PureLink RNA Mini Kit, 1 \times lysis buffer was added to each dilution, then total RNA was extracted according to the product instructions. PVY and *EF1 α* were detected by one-step mRT-PCR. The tests were done three times.

Comparison of sensitivity between RT-PCR and ELISA

Sensitivity of the one-step mRT-PCR and the ELISA for the four potato viruses were compared using 50 mg leaf tissue ground in 1 ml of phosphate-buffered saline pH 7.4 containing 0.05% (v/v) Tween 20 (PBST). A tenfold dilution series was prepared with a homogenate of the virus-infected leaves and the healthy potato leaves, then the diluted samples were divided into two tubes, one for RT-PCR and the other for ELISA. Equal volumes of 2 \times extraction buffer #2 or general extraction buffer (GEB) [0.13% (w/v) sodium sulfate,

2% (w/v) polyvinylpyrrolidone (MW 40,000), 0.02% (w/v) sodium azide, 0.2% (w/v) egg albumin, and 2% (v/v) Tween 20 dissolved in PBST], respectively, was added to samples for RNA extraction or ELISA. For RT-PCR, RNA was prepared using the optimized paper-based method and used in the one-step mRT-PCR. Commercial ELISA kits (Agdia Inc., Elkhart, IN, USA) for each virus were used according to the instructions for the comparison. Absorbance at 405 nm (A_{405}) was measured by an Infinite M200 Pro Multimode Microplate Reader (TECAN Group Ltd., Männedorf, Switzerland) at 30 and 60 min after addition of the substrate solution, which was PNP buffer (pH 9.8) [0.01% (w/v) magnesium chloride hexahydrate, 0.02% (w/v) sodium azide and 9.7% (v/v) diethanolamine] containing 1 mg/ml *p*-nitrophenyl phosphate. This comparison was done three times.

Simultaneous detection from virus-infected potato leaves

The model detection system was verified using a mock potato seed certification and a test group of ten potato plants that included 1 infected plant. Virus-contaminated samples were prepared by mixing healthy and virus-infected potato leaf homogenates. Virus-infected leaves were collected from potato fields; because PVX was not found in the potato fields, an artificially infected potato leaf was used. Each potato leaf was ground in the Extraction Buffer #2 as described above. To prepare the various combinations of virus-infected-tissue homogenates, homogenate from a virus-infected leaf was added at one tenth of the final volume to the virus-free-tissue (“healthy”) homogenate. For example, PVS + PVY sample was prepared by adding 20 μ l of PVS-infected and 20 μ l of PVY-infected homogenate to 160 μ l of healthy homogenate. Total RNA was extracted from each 100 μ l of homogenate using the paper-based RNA preparation, and 1 μ l of total RNA was used for the one-step mRT-PCR.

Results

Establishment of one-step mRT-PCR

When the specificity of the primers published by Bostan and Peker (2009) was checked using Japanese isolates of the four viruses in a two-step tetraplex RT-PCR, the Andean lineage of PVS (PVS^A) and the ordinary strain of PVY (PVY^O) were not detected (data not shown). When the primers and genomic sequences were compared by the MEGA-X program, the nucleotide sequences of the PVS- and PVY-specific primers did not match the respective PVS^A and PVY^O genomes. PLRV- and PVX-specific primers matched the respective genomes, and both were selected for mRT-PCR.

PVS- and PVY-specific primers were newly designed for melting temperatures from 65 to 68 °C by Oligo Calculator, because the temperatures for the other viruses are in this range. To identify a conserved region among three lineages of PVS (ordinary lineage PVS^O, Andean lineage PVS^A, and phureja lineage PVS^P; Vallejo et al. 2016), we compared the genomic sequences of nine isolates chosen from the three lineages and recombinant strains. The forward primer was designed by the substitution of two nucleotides from original primer PVS_gen_F, which was reported by Vallejo et al. (2016) to match the PVS^O sequence, and the reverse primer was newly designed from a conserved region of the gene for the coat protein (CP). Based on the nucleotide sequences of the CP gene, PVY is classified into six types; PVY^C, PVY^O, PVY^{O5}, PVY^E, PVY^{NA-N}, and PVY^{Eu-N} (Green et al. 2017).

PVY primers, designed based on a region in the CP that is conserved among the 12 isolates, were randomly derived from two isolates per each of the above six strains. For the internal control, primers were designed from the conserved region of six potato and two tobacco mRNA sequences for *EF1α*. Primer sequences and expected product sizes are shown in Table 2.

PCR conditions were optimized using cDNA of each virus. The amount of amplicon was increased by adoption of a shuttle PCR program, which comprised a denaturation phase and annealing–elongation phase. The temperature of the annealing–elongation phase was tested every 2 °C from 62 to 68 °C, and 64 °C was the best. In the optimized RT-PCR, all reference isolates were detected by the two-step RT-PCR (Fig. 2a). Fragments of PVS^A and PVY^O

Table 2 Primers for multiplex RT-PCR

Target	Primer	Polarity	Sequence ^a	Size (bp)	References
PLRV	PLRV-F	Sense	CGCGCTAACAGAGTTCAGCC	336	Singh et al. 1995
	PLRV-R	Antisense	GCAATGGGGGTCCAACATCAT		
PVS	PVS-F ^b	Sense	ATGSCGCCYAAACCRGATCC	668	Vallejo et al. (2016) This study
	PVS-R	Antisense	GTGTCAAAGWCGGCGAARCG		
PVX	PVX-F	Sense	TAGCACAACACAGGCCACAG	562	Nie and Singh (2000)
	PVX-R	Antisense	GGCAGCATTTCAGCTTC		
PVY	PVY-F	Sense	GCCAACTGTGATGAATGGGC	438	This study
	PVY-R	Antisense	ACATCCTCGGTGGTGTGCC		
Potato	EF1a-F	Sense	TCAAGCCTGGTATGGTTGTGAC	187	This study
	EF1a-R	Antisense	CTGGGTCATCCTTGGAGTTTG		

^aNucleotides are indicated according to The International Union of Pure and Applied Chemistry (IUPAC) degeneracies code

^bTwo nucleotides from the original primer designed by Vallejo et al. (2016) were replaced

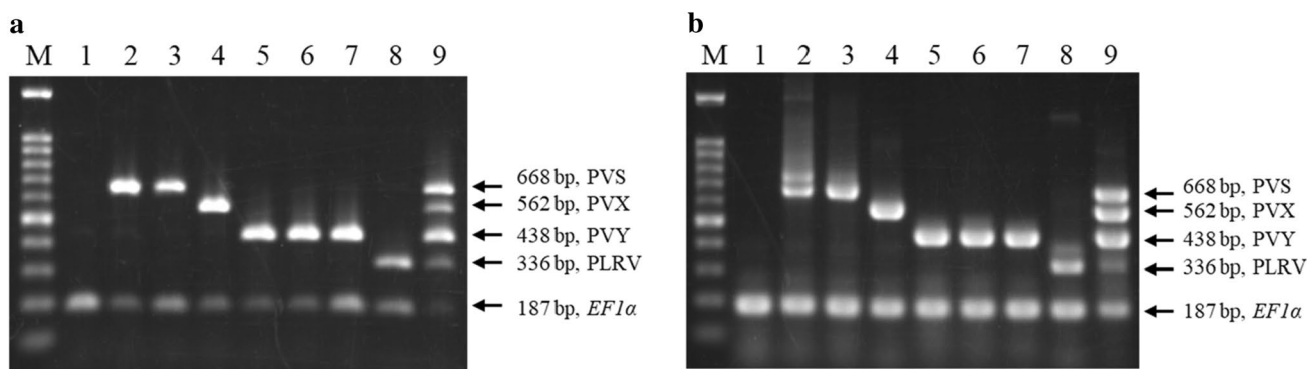


Fig. 2 Detection of reference isolates by multiplex RT-PCR. Total RNA was extracted using PureLink RNA Mini Kit (Thermo Fisher Scientific), then RNA solution was diluted to 100 ng/μl with RNase-free water. One microliter of diluted RNA solution was applied to either **a** two-step mRT-PCR using SuperScript III (Thermo Fisher Scientific) and PrimeSTAR Max DNA Polymerase (Takara Bio) or **b** one-step mRT-PCR using PrimeScript one-step RT-PCR kit ver. 2 (Dye plus) (Takara Bio). Lanes: M, 100 bp DNA ladder RTU

(Bio-helix Co., LTD., Keelung, Taiwan); 1, healthy potato (cv. Irish Cobber); 2, PVS (isolate M); 3, PVS (ChS_3); 4, PVX (O-IC249); 5, PVY (Y28); 6, PVY (PVY-NTND6); 7, PVY (Eu-12jp); 8, PLRV (ChLR_2); 9, mixture of the four total RNA solutions extracted from a leaf singly infected with PVS (M), PVX, PVY (Eu-12jp), and PLRV. *EF1α* was used as the internal control. Black arrows: expected product size of each target

were amplified by the newly designed primers, and that of the plant gene was also amplified in all samples. Next, we confirmed whether a one-step mRT-PCR could detect the reference isolates or not. The annealing–elongation phase was extended from 30 to 60 s for adaptation to the DNA polymerase in PrimeScript one-step RT-PCR kit ver. 2 (Dye plus) (Takara Bio). One-step RT-PCR detected all isolates of four viruses (Fig. 2b). Thirty cycles of amplification seemed to be sufficient to detect viruses from total RNA, which was extracted using the commercial spin column kit; however, the amount of the amplicon of PLRV was slightly lower than for the others. Thirty-five cycles resulted in some nonspecific bands, but nonspecific bands were distinguishable from the specific by amplicon size, so we selected 35 cycles. The specificity and sensitivity of the one-step RT-PCR was also confirmed using a GeneAmp 9700 thermal cycler (Thermo Fisher Scientific), which has a different ramp rate (1.5 °C/s) than the Veriti thermal cycler (3.35 °C/s). The same result was obtained using both thermal cyclers, so the difference in ramp rate did not seem to affect specificity and sensitivity. The difference in lineages of PVS and strains of PVY did not affect the amplification of each virus, so PVS^O (isolate M) and PVY^{NTN} (Eu-12Jp) were used in the following tests.

Optimization of simple RNA extraction for the one-step mRT-PCR

Although the paper-based RNA preparation reported by Zou et al. (2017) is simple, fast, and inexpensive, it is not suitable for a large number of samples and preserving the RNA solution, because the filter paper is transferred from tube to tube and directly used as the template for RT-PCR. To solve the problem, we changed their procedure to use

only one tube, solutions were added or removed by pipetting, and an RNA elution step was added (Fig. 1). When the paper disc was rinsed with the original wash buffer, the sensitivity of the one-step mRT-PCR was unstable among replications for each virus. Comparing the dilution series prepared using healthy potato homogenate and using Extraction Buffer #2, the sensitivity for PVY was 10⁰ and 10⁻⁴ dilution, respectively (data not shown), indicating that unstable amplification was caused by inhibitors contaminating the RNA solution. In a previous study, a rinse with 70% (v/v) ethanol during DNA extraction using filter paper washed away proteins and contaminants (Shi and Panthee 2017). Ethanol can also remove guanidine isocyanate, which is present in Extraction Buffer #2 to denature proteins. Therefore, we used 50% (v/v) isopropanol followed by 75% (v/v) ethanol for the alcohol precipitation in the basic RNA extraction method. As a result, the sensitivity was more stable (Table 3). In addition, the sensitivity for each virus improved 10 times. The difference in virus species did not seem to affect the efficiency of RNA extraction, so only PVY was used to compare the simple RNA extraction methods.

The sensitivity of the optimized paper-based RNA preparation for PVY was compared with that of nitrocellulose-based RNA preparation and heated RNA preparation using the one-step mRT-PCR (Fig. 3). The internal control (*EFlα*) was detected from all samples of each method. The sensitivity of the optimized paper-based RNA preparation was 10 times higher than that of the nitrocellulose-based RNA preparation and the heated RNA preparation and the same as that of the spin column kit. Of the three methods, the optimized paper-based RNA preparation was the most suitable for the one-step mRT-PCR.

Table 3 Detection sensitivity for each dilution of target virus after rinsing with wash buffer or alcohols

Target	Rinse ^a	No. positives/No. replications					Sensitivity ^b
		10 ⁰	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	
PLRV	Wash buffer	4/4	2/4	0/4	nt ^c	nt	10 ⁰
	Alcohols	4/4	4/4	1/4	nt	nt	10 ⁻¹
PVS	Wash buffer	4/4	4/4	2/4	0/4	nt	10 ⁻¹
	Alcohols	4/4	4/4	4/4	0/4	nt	10 ⁻²
PVX	Wash buffer	4/4	4/4	4/4	3/4	0/4	10 ⁻²
	Alcohols	4/4	4/4	4/4	4/4	0/4	10 ⁻³
PVY	Wash buffer	6/6	6/6	1/6	0/6	nt	10 ⁻¹
	Alcohols	6/6	6/6	6/6	0/6	nt	10 ⁻²

^aWash buffer: paper disc was rinsed twice with wash buffer (10 mM Tris–HCl buffer pH 8.0 with 0.1% [v/v] Tween 20); alcohols: the disc was rinsed once with 50% (v/v) isopropanol, then once with 75% (v/v) ethanol. Except for the rinse procedure, the RNA was prepared as shown in Fig. 1

^bSensitivity (bolded) is the lowest dilution that yielded positives in all replications

^cNot tested

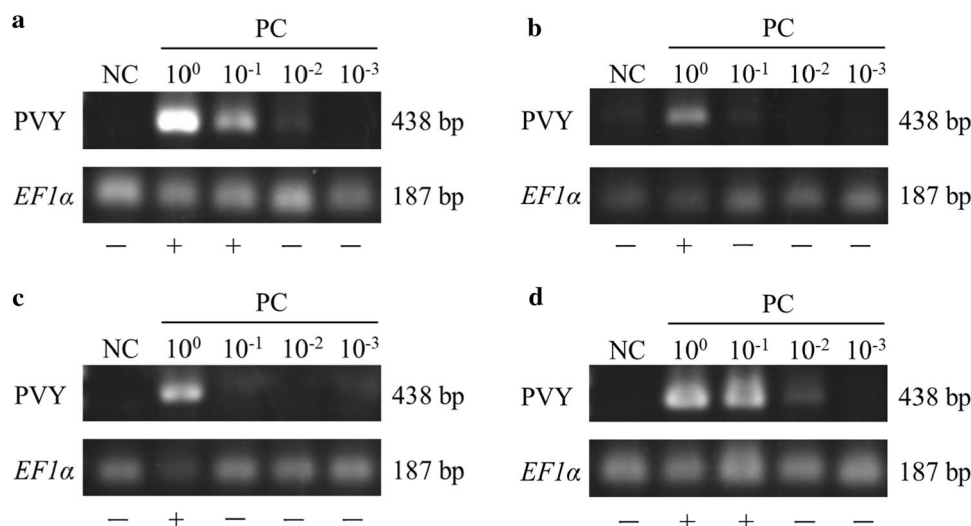


Fig. 3 Results of RT-PCR using the RNA from the four RNA extraction methods. A tenfold serial dilution of PVY was prepared using a homogenate of healthy potato leaf and a PVY-infected tobacco leaf. Total RNA was extracted from **a** RNA preparation using filter paper (Whatman Grade 1, GE Healthcare) or **b** RNA preparation using nitrocellulose membrane (Hybond-C Super 0.45-micron, GE

Healthcare), **c** heated RNA preparation or **d** PureLink RNA Mini Kit (Thermo Fisher Scientific). One microliter of the RNA solution was added to 9 μ l of reaction mix, then one-step mRT-PCR was conducted. Lanes; NC, healthy potato (cv. Irish Cobber); PC, dilution series of PVY. Distinct bands were considered positive (+), indistinct as negative (-). *EFlα* was used as the internal control

Comparison of sensitivity between mRT-PCR and ELISA

The new detection method, composed of the optimized paper-based RNA preparation and one-step mRT-PCR, was compared with ELISA for sensitivity for each virus (Fig. 4). The threshold of absorbance value at 405 nm (A_{405}) was determined as ≥ 1.5 -fold than that for the healthy sample in ELISA. A result with distinct bands (rather than a faint band) was considered positive in RT-PCR. Our new protocol detected PVS, PVX, PVY, and PLRV in dilutions as low as 10^{-3} , 10^{-5} , 10^{-3} , and 10^{-1} , respectively. In contrast, ELISA detected the respective viruses to 10^{-3} , 10^{-2} , 10^{-2} , and 10^0 dilutions. Thus, the sensitivity of the new detection method for PVX was 1,000-fold higher than that of ELISA and tenfold higher for PVY and PLRV. Both methods had the same sensitivity for PVS. The same results were obtained in three replications. Overall, the new detection method was sensitive enough to detect the four viruses.

Confirmation of simultaneous detection from virus-infected potato leaf

In Japan, mixed virus infections are rarely found in potato plants, except for local varieties, and a group of plants are tested for seed potato certification. To consider these factors in our testing method, we used a mock group test of ten plants, which included one virus-infected plant to confirm that the new detection method was suitable for a practical

situation. In this test, all possible combinations of viruses were accurately detected by the new detection method, even when four viruses were present in one homogenate (Fig. 5). The amount of RT-PCR products for each virus seemed unaffected by the amplification of other products for viruses, other than yielding a slightly lower yield of product for PVS in the mixtures PVS + PVX + PLRV (lane 13) and PVS + PVX + PVY + PLRV (lane 16) than in the PVS alone (lane 2). These results indicated that the new detection method can detect the four viruses simultaneously from a potato leaf and is suitable for practical situation.

Discussion

In this study, we established a protocol that met four requirements for detecting four potato viruses for seed potato production. First, all lineages and strains of four potato viruses should be detected, because the detection method is used in the seed potato production system in Japan. Second, the detection method should have an internal control for the RT-PCR to assure that the assay is undoubtedly successful. Third, it must be more sensitive than the current ELISA. Fourth, the method should be as inexpensive and simple as possible to reduce cost and labor.

For the first requirement, the specificity was verified by comparison of primer and genomic sequences, then the reactivity was verified using the two-step RT-PCR with reference isolates (Fig. 2). Because the three lineages of PVS have

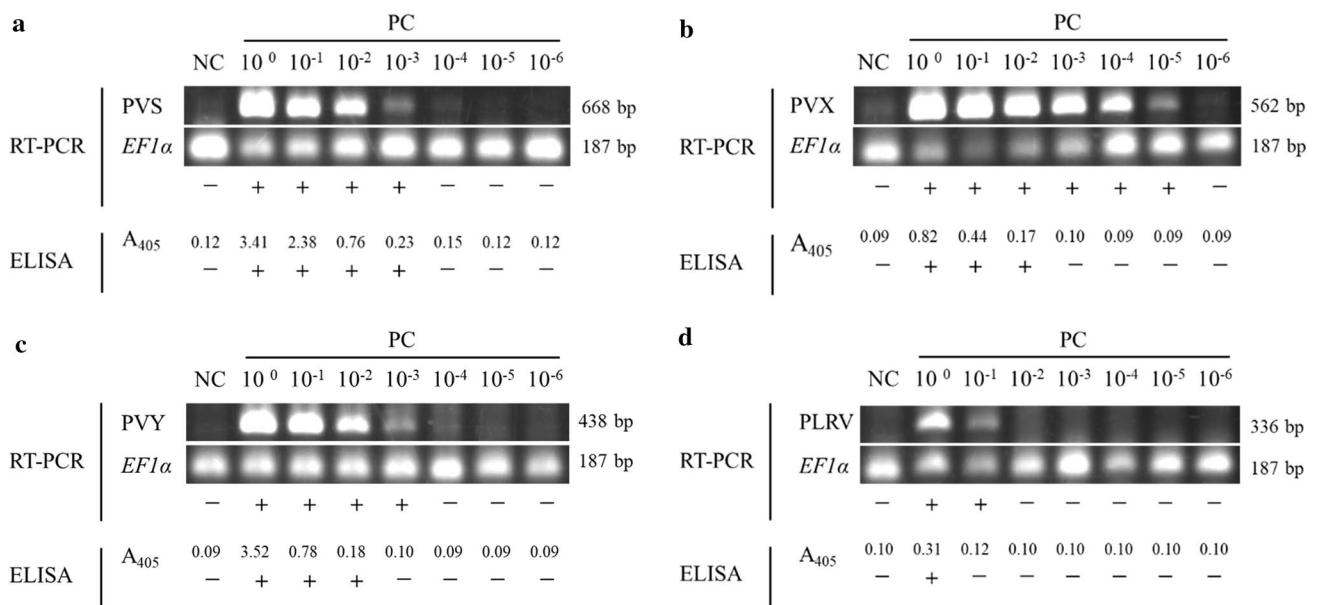


Fig. 4 Comparison between RT-PCR and ELISA. A tenfold serial dilution was prepared using a homogenate of healthy potato leaf and a virus-infected tobacco leaf, except for PLRV-infected potato leaf. Equal volume of 2×Extraction Buffer #2 or GEB was added to the dilution series for RT-PCR or ELISA, respectively. Total RNA was extracted using the optimized paper-based RNA preparation, and one-step mRT-PCR. ELISA was done according to kit instructions. Lanes;

NC, healthy potato (cv. Irish Cobber); PC, dilution series of each virus: **a** PVS, **b** PVX, **c** PVY, and **d** PLRV. In RT-PCR, distinct bands were considered positive (+); indistinct were negative (-). *EF1α* was used as the internal control. In ELISA, A₄₀₅ is the absorbance value at 405 nm at 1 h after addition of substrate solution, and the threshold was 1.5-fold higher than the A₄₀₅ of the healthy sample. Result for each sample is indicated as positive (+) or negative (-)

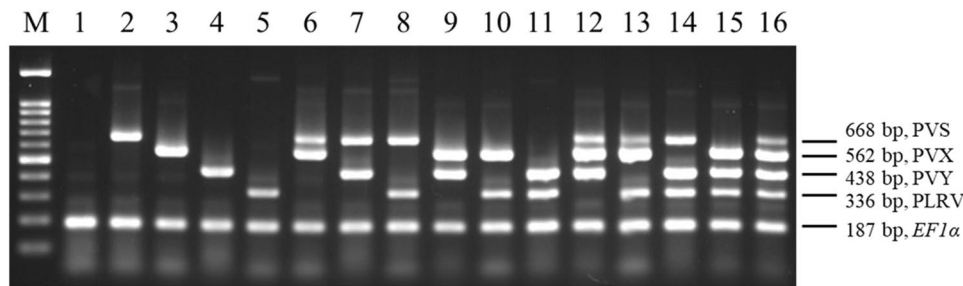


Fig. 5 Detection of four viruses from virus-infected potato leaf using the new protocol. Total RNA was extracted from mixed homogenates of healthy and virus-infected potato leaves using the paper-based RNA preparation, then subjected to the one-step mRT-PCR. Lanes; M, 100 bp ladder maker (Bio-helix); 1, healthy

potato leaf; 2, PVS; 3, PVX; 4, PVY; 5, PLRV; 6, PVS+PVX; 7, PVS+PVY; 8, PVS+PLRV; 9, PVX+PVY; 10, PVX+PLRV; 11, PVY+PLRV; 12, PVS+PVX+PVY; 13, PVS+PVX+PLRV; 14, PVS+PVY+PLRV; 15, PVX+PVY+PLRV; 16, PVS+PVX+PVY+PLRV

relatively different nucleotide sequences compared with the other three viruses, degenerate primers must be based on the most conserved region (Table 2). We proved that two lineages of PVS were detected with our primer set. The reactivity of our primers with PVS^P was confirmed in silico, because this lineage is not present in Japan. To our knowledge, only our primer set can detect the three lineages. In addition, the PVY complex consists of more than 10 major strains (Green et al. 2017), so the primers were designed for a conserved region of the CP to detect all strains. This

primer set detected the three major strains of PVY in Japan, and the reactivity to other strains was also confirmed in silico. Sequence analysis showed that PVX and PLRV have less genetic diversity than do the other two viruses, so primers for these viruses match most isolates, so we used previously reported primers (Nie and Singh 2000; Singh et al. 1995; Table 2).

For the second requirement, the first step was selection of a suitable plant gene for the internal control. A few mRT-PCR assays have used primers that targeted a plant gene as

internal control (Du et al. 2006; Kumar et al. 2017), and *EF1 α* was the most stably expressed gene in potato plants under biotic and abiotic stresses among seven housekeeping genes in a quantitative RT-PCR (Nicot et al. 2005). This gene was consistently expressed, except for slightly lower expression in tubers (Kumar et al. 2017). In addition, in our preliminary experiment, this gene was also detected by the one-step mRT-PCR from a potato leaf singly infected with PLRV, PVS, PVX, and PVY and from a leaf doubly infected with PVS + PVX, PVS + PVY, or PVX + PVY, although the expression level of *EF1 α* was not determined. Therefore, this gene is suitable as an internal control for RT-PCR.

For the third requirement, we compared the sensitivity of the new detection method, i.e., optimized paper-based RNA preparation and one-step mRT-PCR, with that of ELISA for the four viruses (Fig. 4). In previous reports on the development of RT-PCR, the assay's sensitivity for a target virus was confirmed with a known concentration of viral cDNA (Cheng et al. 2013; Du et al. 2006; Zhang et al. 2017), but our detection method was an alternative to the ELISA, so the sensitivity of the assay needs to be compared with that of the ELISA. The sensitivity for PLRV seemed to be lower than that of the other viruses in both RT-PCR and ELISA, although the RT-PCR had higher sensitivity than the ELISA (Fig. 4), perhaps due to a lower viral concentration in the potato plant, because the same result was obtained with samples from other individual plants or another plant infected with a PLRV isolate. For PVS, the new detection method had the same sensitivity as ELISA. We designed degenerate primers for PVS to match the three lineages. The primer that was specific for each isolate made up one fourth or one eighth of the total primers, thus limiting the sensitivity for PVS. Both methods detected PVS at 10^{-3} dilution, which seemed to be sufficient for detection, so we did not change the concentration of the PVS primer set. The new detection method is more sensitive for PVX and PVY than ELISA is, and no improvement seemed to be required. Overall, the new detection method was sensitive enough for the four viruses.

For the fourth requirement, a one-step mRT-PCR and simple protocol to preserve the RNA to reduce cost and labor, we used a commercial one-step RT-PCR kit containing loading dye, and the assay successfully detected all isolates of four viruses (Fig. 2b), a great advantage to two-step RT-PCR or a one-step RT-PCR that does not contain loading dye. Fortunately, the conditions for the one-step RT-PCR were almost the same as those for the two-step RT-PCR, although the one-step RT-PCR produced a nonspecific band that did not affect the detection result. Because a nonspecific band did not appear in the two-step RT-PCR, this phenomenon is likely to be caused by multiple primers in the reverse transcription reaction.

The use of two kinds of alcohols instead of the wash buffer as the rinse solution also improved the assay's

sensitivity (Table 3). In a preliminary comparison of five rinse solutions [the original wash buffer by Zou et al. (2017), 50% (v/v) or 100% (v/v) isopropanol and 75% (v/v) or 100% (v/v) ethanol] using absorbance at 230, 260, and 280 nm, the alcohols were selected for their ability to precipitate RNA to avoid loss of RNA during the rinse. Compared with the original wash buffer, four kinds of alcohols yielded more RNA and less contamination. For the alcohols, the difference in alcohol concentration had little effect on RNA yield, and the lowest tested concentration washed away contaminants. Isopropanol tended to be superior for RNA yield, ethanol for removal of contaminants. Two rinses were required to remove chlorophylls, which inhibit DNA polymerase activity, from the paper disc. Because enzymatic reactions are also inhibited by alcohols, especially by isopropanol, the disc was rinsed with 50% isopropanol followed by 75% ethanol in the optimized method to minimize any negative effect of alcohols to subsequent reactions.

Dormant tubers have also been tested as source material for high-throughput potato virus detection to reduce cost and labor of growing plants (Agindotan et al. 2007; Stammer et al. 2018). The efficiency of PVY detection from dormant tubers decreases during storage (Fox et al. 2005). According to Fox et al. (2005), the detection of PVY from the leaf of a growing plant ("growing-test") using ELISA is more reliable than direct detection from a dormant tuber using real-time RT-PCR or ELISA at 6 weeks after harvest. Because our new detection method was designed for seed potato production, the growing-test was suitable for our purpose, so we did not test dormant tubers. Because dormant tubers are useful for surveys, screening, or other purposes, our new detection method should be tested using dormant tubers.

To our knowledge, we have developed the first one-step mRT-PCR that can detect the four potato viruses, including all lineages of PVS and strains of PVY, and uses a plant gene as an internal control. The RNA preparation method does not require expensive supplies or equipment, so it can be used in most laboratories. Our new protocol for simple RNA preparation and one-step mRT-PCR is more sensitive than ELISA and can detect the four potato viruses simultaneously from potato samples. Moreover, according to our estimates, the cost of the method is equivalent to that of ELISA to detect the four potato viruses. Thus, this new detection method can be used for seed potato certification, quarantine, breeding, and field surveys.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

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