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Optimization of an isothermal recombinase polymerase amplification method for real-time detection of Potato virus Y O and N types in potato

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ABSTRACT

Potato virus Y (PVY) is a global challenge for potato production and the leading cause of seed crop downgrading and rejection for certification. Accurate and timely diagnosis is key to effective control of PVY. Here we optimized the isothermal recombinase polymerase amplification (RPA) for accurate detection of different PVY O and N types that were tested, present in different tissues of potato plants including tubers with a primer set that specifically targets the highly conserved pipo region within the viral genome. Combined with a simplified preparation of the template by tissue homogenization, we established a rapid RPA procedure, which can allow real time detection in less than 10 min with a fluorescent probe. Specificity of the reaction was determined by the lack of cross-reactivity with other common potato viruses. Although RPA reagents remain more expensive than PCR reagents, RPA technology is equivalent in that results can be visualized by gel electrophoresis or with a fluorescent probe with greater sensitivity; and it is superior to the common PCR-based assay in its versatility, speed, and lack of need for a highly purified RNA template.

1. Introduction

Potato is the most important dicot food crop worldwide and about \$3.4 billion in potato is produced in the United States annually. The potyvirus Potato virus Y (PVY) is the most important potato virus in North America and the most common reason for rejection of seed potato lots from certification (Frost et al., 2013), which is one of the reasons that PVY is considered to be among the 10 most important viruses in the world (Scholthof et al., 2011). PVY has become increasingly important in the United States since recent regulatory changes require that seed must be certified for low levels of PVY prior to crossing state borders or being planted in fields greater than 1–10 acres, depending on the state. As a result, the need for rapid, inexpensive, and accurate PVY diagnosis has escalated.

PVY causes tuber yield reduction of up to 80% depending on the varieties and time of infection (Nolte et al., 2003; Whitworth et al., 2006; Bantarri et al., 1993; Hane, 1999). Several major changes have contributed to the re-emergence of PVY as a serious disease threat in the United States. An important component is the popularity of several russet varieties (Hane and Hamm, 1999; Rykbost et al., 1999) that show mild or no PVY foliar symptoms but serve as large reservoirs for PVY.

One of the challenges in the control of PVY is the emergence of new recombinants of the virus, which are derived from the ordinary PVY^O strain and the necrotic PVY^N strain (Green et al., 2017, 2018; Karasev and Gray, 2013). These recombinant strains cause mild foliar symptoms on potato, may cause necrosis on tubers, and unlike PVY^O, are not self-limiting in varieties that encode N-gene mediated resistance (Quenouille et al., 2013).

Potato virus Y is the type member of the genus *Potyvirus*, family *Potyviridae*. It is a positive sense RNA virus of about 9700 nts, which encodes a single large polypeptide, which is then processed into nine multifunctional proteins. A second small open reading frame (ORF) termed pipo, predicted to encode a 7 kDa protein, was discovered to overlap with the P3 coding region as fusion to the N-terminal part of P3 protein in all members of the *Potyviridae* family (Chung et al., 2008). This highly conserved ORF is an ideal target for development of detection assays.

Rapid and accurate diagnosis of PVY is key for effective disease control. While the double sandwich enzyme-linked immunosorbent assay (DAS-ELISA) is routinely used to test seed potato crops as part of seed certification, many nucleotide-based procedures with high specificity and automation are also available including RT-PCR and

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isothermal-detection based assays (Nie, 2005; Przewodowska et al., 2015; Singh et al., 2013; Singh, 1999; Treder et al., 2018; Zacharzewska et al., 2014; Glais and Jacquot, 2015). Such methods have not yet been adapted for routine diagnostic schemes. They are hindered largely by the existing challenges in isolating high quality RNA from potato tubers required for PCR-based amplification, the presence of inhibitory compounds and/or the low virus titer in dormant tubers and the time-consuming sample preparation for the assays (Boonham et al., 2008).

Here we describe the optimization of the isothermal-based reverse transcription recombinase polymerase amplification assay (RPA) for robust and rapid detection of PVY from crude extracts of potato leaves and tubers. Isothermal DNA amplification methods such as the loop-mediated isothermal amplification (LAMP) and RPA represent alternatives to PCR-based assays (Magrina Lobato and O'Sullivan, 2018). Their reactions are set in one single temperature without the need of sophisticated equipment. While the LAMP approach is based on six different primers that recognizes distinct regions of the target sequence and relies on a DNA polymerase with strand displacement activity at 60–65 °C, the RPA reaction involves a specific combination of two core enzymes and a protein for simple amplification of molecular targets at a single temperature (Magrina Lobato and O'Sullivan, 2018). This includes the recombinase, which pairs a single set of primers to the homologous sequences; the single stranded DNA binding protein, which displaces the strands of the DNA; and the strand-displacing polymerase, which initiates DNA synthesis from the bound primers. For RNA samples, the reverse transcription can be synchronistically performed in the same reaction just by adding the reverse transcriptase enzyme. Here we designed specific PVY primers that target a consensus region among different PVY strains within the highly conserved pipo coding sequence present in all members of the *Potyviridae* family (Chung et al., 2008). The specificity of the assay for PVY was validated by detection of different strains of PVY and the lack of cross-reactivity with other potato-infecting viruses, including other potyviruses. The power of the assay is based on the fluorescent probe system that allowed real time detection of the PVY amplicon up to 10000-fold dilution of the total RNA sample, in less than 10 min. In addition, the assay was sensitive enough to allow the detection of PVY when using crude extract from peridermal tuber tissues. The present study offers an alternative procedure to conventional nucleic acid-based assays for successful and rapid detection in low viral titer samples, and an optimization of a previously reported RPA approach (Glais and Jacquot, 2015), which now eliminates the need of additional purification step of the products prior visualization and works with low quality RNA materials.

2. Materials and methods

Frozen PVY^O (isolate NY090031), PVY^{N:O} (isolate NY090029) and PVY^{NTN} (isolate NY090004) were maintained in tobacco *Nicotiana benthamiana* leaves at – 80 °C. Potato tubers infected with the known strains of PVY (PVY^O, PVY^{N:O}, PVY^{WI}, PVY^{NTN}) were kindly provided by Stewart Gray (Cornell University) to Amy Charkowski.

Frozen PVY-infected *Nicotiana benthamiana* leaves were used for extracting total RNA for the optimization of the designed primers, and as source of inoculum for rub-inoculation of potato plants cv Atlantic. Tissue cultured potato plantlets infected with PVY^C and PVY^N isolates were obtained from the Wisconsin Seed Potato Certification Program. Other viruses, including the potyvirus *Potato virus A* (PVA C1872), the carlaviruses *Potato virus M* (PVM C1459) and *Potato virus S* (PVS C2283), the potexvirus *Potato virus X* (PVX C634), and the polerovirus *Potato leafroll virus* (PLRV C1899) were obtained from Agdia.

2.1. Total RNA extract preparation

Total RNA was prepared either using the RNA Qiagen kit as directed by the manufacturer or according to the method of Dellaporta et al. (Dellaporta et al., 1983). Briefly, we ground about 100 mg plant

material (leaves or periderms peeled from potatoes) in 500 µL Dellaporte solution (10 mM Tris, 5 mM EDTA, 62.6 mM NaCl and with 10 µL 2-mercaptoethanol in a total 10 ml volume) and added 70 µL of 10% SDS, followed by an incubation at 65 °C for 10 min. We next added 200 µL of 5 M potassium acetate solution and incubated the mixture on ice for 5–10 min followed by centrifugation 10 min at 16,000 g at 4 °C. We then collected the supernatant and mixed it with 300 µL of cold isopropanol on ice for about 5 min. The total RNA was pelleted following centrifugation at 16,000 g, ethanol-washed with a 70% ethanol, dried and resuspended in 100 µL of nuclease free water. For the serial dilution assays, the total RNAs were diluted in water with an initial concentration of 100 ng. As a negative control, 100 ng of total RNA was used as a final concentration for the healthy potato plant samples. For the RNA isolation from potato tubers, RNA was extracted either from a thin or a thicker peel of about 4 cm in length. The thin peel was obtained from the periderm tissue only. The thicker peel included the periderm and a thin layer, of about 1–2 mm in thickness, of the tuber flesh.

2.2. Crude nucleic acid extract preparation from potato leaves and tubers

Crude extracts from freshly harvested or frozen leaves were prepared as follows: one leaflet (~300 mg fresh weight) was ground in a mortar with a pestle at room temperature in 1 ml of the general extraction buffer (GEB3, Agdia, USA) with the addition of 2% v/v Tween 20 and 0.5% (w/v) sodium sulfite. The extract was clarified by centrifugation at maximum speed in a benchtop centrifuge for 2 min. The supernatant was used either immediately or stored at -20 °C until further use. For the serial dilution assays, the samples were further diluted in the GEB3 buffer.

For the preparation of crude extract from potato tuber periderm, we removed about 4 cm of the potato periderm by carefully avoiding any starch tissue. The protocol described above was followed, except that the extracts were kept on ice for 30 min before centrifugation. The ratio of potato periderm to buffer was 100 mg/ 1 ml GEB3 buffer.

2.3. Primers design and sequence

Three primer sets were manually designed to target the conserved pipo open reading frame of PVY based on consensus sequences of strains of PVY available in the NCBI database (Fig. 1). They were next validated with the multi-primer analyzer software from ThermoFisher for standard RPA primer designs. The primer sequences were:

PIPO F1 (nts 2928-2957) 5'-TTATCTAAATCTCTTGAACGATGC TTG GAA 3'

PIPO F2 (nts 2940-2969) 5'- CTTGAACGATGCTTGAAAGATTTAA CTTG-3'

PIPO F3 (nts 2935-2964) 5'-AATCTCTTGAACGATGCTTGAAAGA TTTA-3'

PIPO R1 (nts 3052-3080) 5'-TGGTGATATGTTGTATAACCCTTCA AATC-3'

PIPO R2 (nts 3062-3093) 5'-CAAGAATGCTTGTGGTGATATGTTGT ATAACC-3'

PIPO R3 (nts 3049-3078) 5'-TGATATGTTGTATAACCCTTCAAAT CTGC-3'

The nucleotide positions are based on the PVY genome sequence accession [MF405303](#).

2.4. RPA reaction

RPA was performed using the AmplifyRP Acceler8 discovery kit (Agdia) according to the manufacturer's protocol. Each 10 µL reaction contained 0.42 µL of 10 µM forward and reverse primer, 0.2 µL reverse transcriptase (Superscript III, Thermo Fisher), 5.9 µL rehydration buffer, 1 µL template, and nuclease-free water to 9.5 µL. The reaction mixture was added to rehydrate AmplifyRP Acceler 8 reaction pellets,

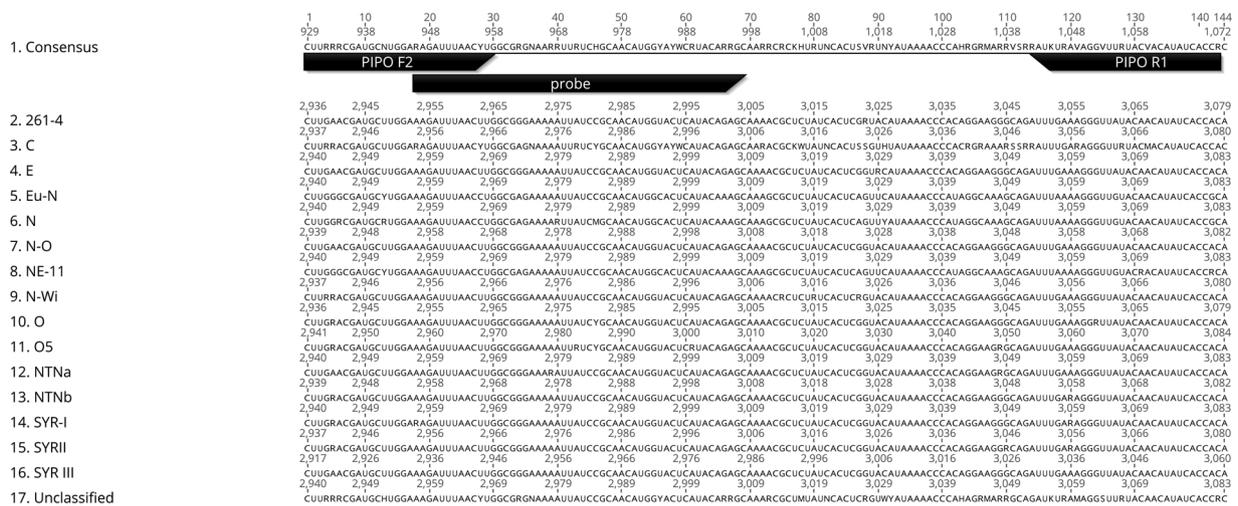


Fig. 1. Nucleotide positions of the F2/R1 primer set and the amplicon probe within a generally conserved region of the pipo-coding frame in different Potato virus Y (PVY) strains. The consensus sequence alignment was generated using the Geneious software. The PVY strains and their accession numbers used for the sequence alignment are: 261-4 (JF92 7755, AM113988), C (DQ309028, AJ890348, AJ439544, EU563512), E (JF928459, JF928458), Eu-N (JQ969036, AY884983, X97895, AM268435), N strain (AB331515, AB331516, AB331517, AB331518, AB331519, AB711144, AJ585198, AY166866, AY166867, AY884984, JN936422), N-O (AY884985, HQ912871, AY745491, AY745492, HQ912872, HQ912870, HQ912866, EF026077), Ni-W (JN083841, JN034046, JQ969039, JQ969041, JF927751, JF927753, HQ912896, HQ912863, HQ912868, JQ924286, JQ924288, JF795485, AJ890349, AJ890350, HE608963, HE608964), NE-11 (HQ912867, JQ917195, DQ157180), NTNn (KF850513, KC634007, KC634005, KC634004, KC296441, KC296437, JQ969035, JQ969034, JQ924287, JF928460, JF927763, JF927761, JF927759, JF927757, JF927752, HQ912869, HQ631374, FJ204166, FJ204165, FJ204164, EF026075, EF016294, AY884982, AJ890345, AJ890344, AB702945), NTNb (JQ969037, AJ890343, AJ890342, AJ889866, HM590406), O strain (AJ585196, EF026074, FJ643479, HM367075, HM367076, HQ912864, HQ912865, HQ912888, HQ912889, HQ912890, HQ912891, HQ912892, HQ912893, HQ912894, HQ912895, HQ912897, HQ912913, HQ912914, JQ924285, JX424837), O5 (PVU09509, HQ912905, HQ912911, HQ912904, HQ912909, HQ912910, HQ912898, HQ912898, HQ912912, HQ912906, HQ912915, HQ912915, HQ912907, HQ912899, HQ912900, HQ912903, HQ912902, HQ912908, HQ912885, HQ912880, FJ643477, HQ912881, HQ912879, HQ912862, HQ912886, HQ912883, HQ912887, HQ912882, HQ912878, FJ643478, HQ912876, HQ912874, HQ912875, HQ912877, HQ912873, HQ912884, HM367076), SYR-I (KC296435, GQ200836, KC296433, AB270705, KC296434), SYR-II (AB461453, AJ889867, AB461451, AB461452, KC296438, KC296440), SYR-III 1 (AB461454), and unclassified (AB714135, PVYAAA, HM991453, HM991454, JQ969040, FJ666337, AJ585197, KC296436, KC296439, KC296432, AF522296, EU182576, AF237963, AJ890346, AJ889868), as characterized in Green et al. (2017) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

and 0.5 µL of 280 mM magnesium acetate was added to the top of reaction tube lids. The magnesium acetate droplets were then spun down using a minifuge and a timer immediately started. The reactions were transferred to a heat block set to 39 C for 20 min or performed in a CFX96 C100 thermal cycler (Eppendorf Corp) at 39 C with 20 s cycle repeated 54 times. The amplified products were directly run on 2–4 % MetaPhor Agarose (Lonza) gel without any additional purification step, unlike previously RPA assay (Glais and Jacquot, 2015) and the amplicons were visualized under UV light.

For real time detection, the 49-nt fluorescently labelled probe that targeted the pipo open reading frame region at nucleotide position 2957–2991 was synthesized at IDT-DNA. The fluorophore/quencher probe was designed according to the TwistAmp instruction (www.twistdx.co.uk). The oligonucleotide backbone is flanked by a dT-fluorophore and its corresponding dT-quencher. In addition, to prevent extension of the probe by the polymerase, an amino acid modifier was added. A THF residue was included in the sequence. When the probe pairs to its target sequence within the amplification product, the probe is cleaved at the THF position, which thus separates the fluorophore and the quencher and generates a fluorescent signal. The probe recognizes nt 2957–2991 of the PVY genome (accession MF405303) within the amplicon product. The probe sequence was:

5'-AAGATTTAACTTGCGGGGAAAATTATCGCAACA/iFluorT/G/idSp//iBHQ-1dT/ACTCATAACAGAGC/3 AmMO/-3'

The fluorophore 6 carboxyfluorescein [FAM-dT] = /iFluorT/, the tetrahydrofuran spacer [THF] = /idSp/, the black hole quencher [BHQ-dT] = /iBHQ-1dT/

3' amino modifier extension blocker = /3 AmMO/

0.6 µl of the 49-nt fluorescently labeled probe was added to the isothermal amplification reaction. The fluorescence intensity of FAM was determined every 20 s for a total of 54 cycles or longer depending

on the samples.

3. Results

3.1. Detection of the different PVY strains with PIPO-targeting specific primers

The RPA assay is based on an isothermal single stranded displacement of DNA. The high performance of the method heavily relies on the efficacy of the recombinase enzyme to successfully pair the designed primers to the target sequence. We thus designed three sets of primers, which targeted a consensus sequence among the PVY strains available in the NCBI database within the pipo open reading frame (Green et al., 2017) (Fig. 1). We tested different forward and reverse primer combinations in the RPA assay to identify the best primer pair for its ability to amplify PVY sequence from viral-infected *Nicotiana benthamiana* leaves. No striking difference was observed among the amplified DNA fragments with the sets of primers used (data not shown). We thus arbitrarily selected primers F2/R1 set for the next experiments, which amplified a 158-nt product (Fig. 1).

To further analyze the selected F2/R1 primer set, we performed the RPA reaction on different PVY strains (PVY^O, PVY^{N:O} and PVY^{NTN}) (Fig. 2A). The result showed the amplification of the PVY product in all reactions. Only primer dimers were observed in the negative control reaction, which lacked RNA (Fig. 2A).

When we tested unrelated viruses commonly found in potatoes, including *Potato virus A* (PVA, potyvirus), *Potato virus M* (PVM, Carlavirus), *Potato virus S* (PVS, *Betaflexiviridae* family), *Potato virus X* (PVX, Potexvirus), and *Potato leafroll virus* (PLRV, Polerovirus), no cross reactivity was observed (Fig. 2B). The RPA amplicon was detected only in infected samples with PVY^C, PVY^N and PVY^O strains (Fig. 2B).

We repeated the above assay in the presence of the 49-nt fluorescent probe for real-time detection (Fig. 2C). PVY was detected after 10 cycles (~3.5 min) in the PVY^O-infected *N. benthamiana* leaves, and after 20 cycles in the infected potato leaf samples. No signal was picked up with the unrelated viral samples above the control RNA from healthy potato plants. A weak signal was detected for PLRV sample after 40 cycles.

3.2. Sensitivity PVY detection by RPA

We next assessed the sensitivity of the RPA assay by performing a serial dilution of the purified total RNA from potato leaves inoculated with PVY^O (Fig. 3A). Our result showed that we amplified the specific PVY band up to 100-fold dilution of the total RNA. Only the primer dimers were observed in the healthy plant control RNA.

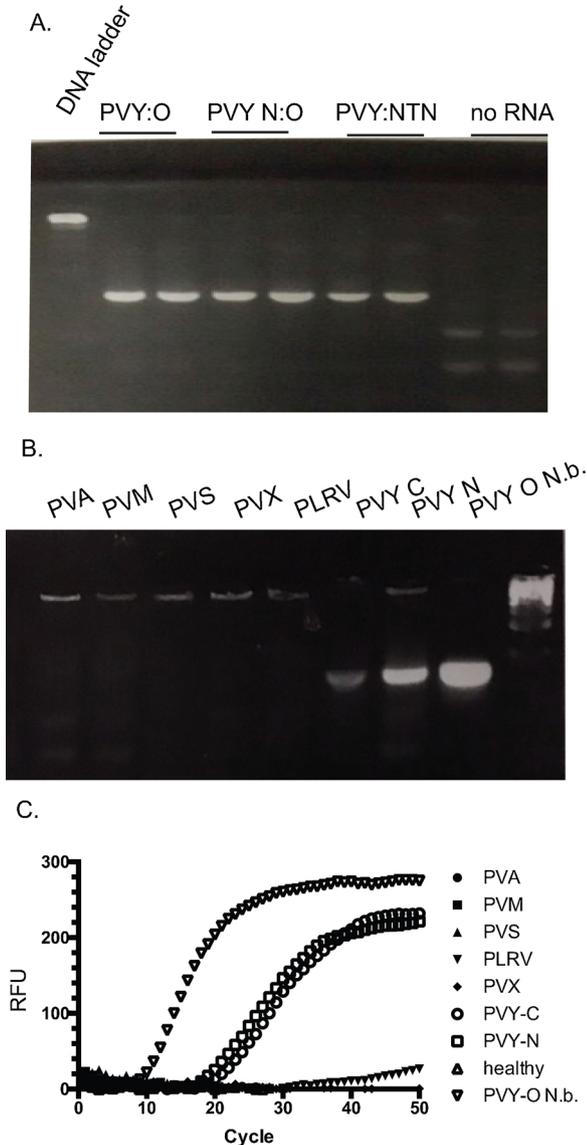
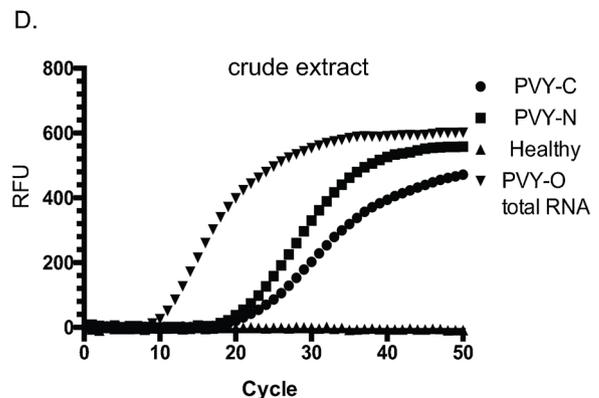
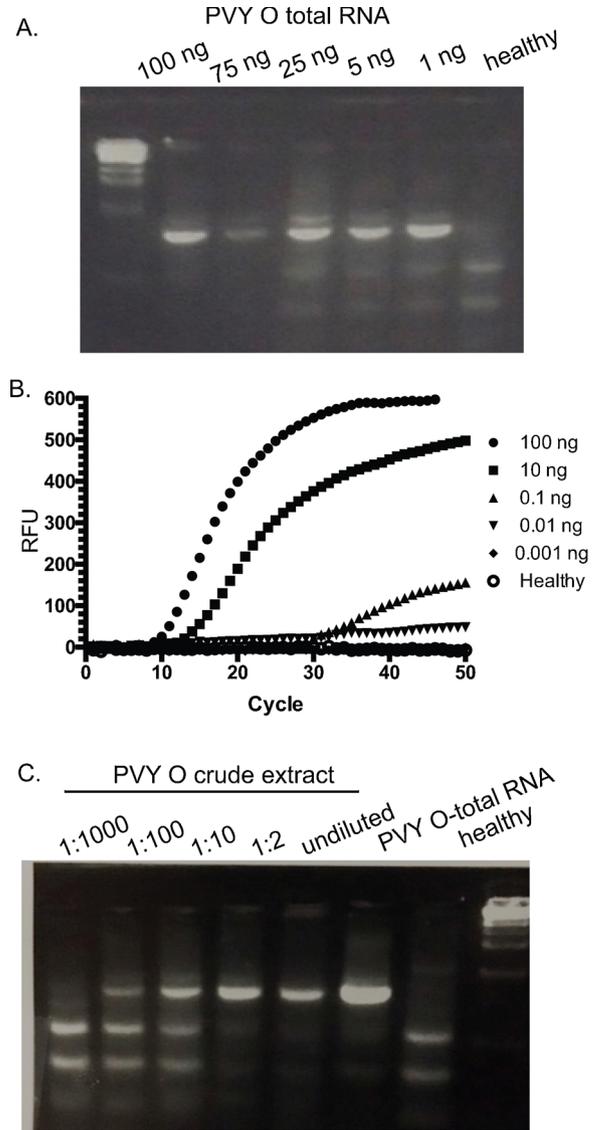


Fig. 2. Detection of the different strains of PVY by RPA targeting region of the pipe open reading frame. A. The RPA assay for the detection of PVY was performed on total RNA extracted from PVY^O; PVY^{N:O}; PVY^{NTN} infected *Nicotiana benthamiana* leaves using the F2/R1 primer set. The reactions were then loaded on a 4% metaphor gel. The expected amplification band was of 158 nucleotides. B. The RPA was performed on total RNA extracts from potato plants infected with different viruses of potato including *Potato virus A* PVA, *Potato virus M* PVM, *Potato virus S* PVS, *Potato virus X* PVX, and *Potato leafroll virus* PLRV. As a control, we included total RNA extracts from PVY-infected *N.benthamiana* leaves as well as PVY^C and PVY^N infected potato plantlets. The reactions were next loaded on a 4% metaphor gel. The expected amplification band was of 158 nucleotides. C. The real time detection of PVY was performed on total RNA extracts from potato plants infected with different virus of potato including *Potato virus A* PVA, *Potato virus M* PVM, *Potato virus S* PVS, *Potato virus X* PVX, *Potato leafroll virus* PLRV in the presence of a 49-nt fluorescent probe for the PVY amplicon. As a control, we included total RNA extracts from PVY-infected *N.benthamiana* leaves as well as from PVY^C and PVY^N infected potato plantlets. The fluorescence intensity of FAM was measured every 20 s for a total of 54 cycles. RFU stands for relative fluorescence unit.



(caption on next page)

Fig. 3. Sensitivity of PVY detection in serially diluted RNA samples. A. The RPA assay was performed with different final concentrations of the total RNA extracted from PVY^O-inoculated potato leaves (100 ng, 75 ng, 25 ng, 5 ng, 1 ng). As a control was included total RNA from healthy potato leaves. The reactions were next loaded on a 4% metaphor gel. B. Real time detection of PVY was performed in the presence of fluorescently labelled probe for the PVY amplicon with different concentrations of total RNA extracts from PVY-inoculated potato leaves (100 ng, 10 ng, 0.1 ng, 0.01 ng, 0.001 ng). C. The RPA assay was performed on serial dilution (0; 1:2, 1:10, 1:100; 1:1000) of crude sap extract from PVY-inoculated potato leaves. The reactions were next loaded on a 4% metaphor gel. D. Real time detection of PVY was performed on crude extract from the PVY^C and PVY^N infected potato plantlets in the presence of the fluorescent probe. As a control, we included total RNA extract from PVY-inoculated leaves and from healthy plants.

We repeated the RPA assay with the fluorescent probe for real time detection of the PVY amplicon (Fig. 3B). PVY was detected after 10 cycles at a 10-fold dilution. After 30 cycles, amplification of PVY was observed up to 1000-fold dilution samples. No detection was observed at the lower dilution point. Our results show that the use of fluorescent probe definitely increases the sensitivity of the RPA for detection of the PVY amplicon.

To streamline the procedure, we repeated the RPA assay on crude sap extract, thus skipping the total RNA isolation step. We serially diluted the extract up to 1000-fold (Fig. 3C). As a control, we included total RNA extract from the PVY-infected leaves and from healthy potato plants. We successfully amplified a PVY amplicon of the expected size in up to a 100-fold dilution of the crude extract. However, specific detection was lost at a 1000-fold dilution. At this dilution point, the band patterns were similar to that observed from the total RNA extract from healthy plants. We also tested the crude extract using the fluorescent probe (Fig. 3D). PVY^C and PVY^N were detected at different cycle points, which reflected the difference in intensity we observed when examining the DNA amplicons from total RNA extract with gel electrophoresis (Fig. 2C).

3.3. Real time detection of PVY in potato peels as an alternative to tuber extract

To estimate the reliability of the RPA assay for PVY detection in dormant tubers, we first tested total RNA extracted from periderms (peels) of PVY^O-infected potato tubers (Fig. 4A). Two different RNA extracts (RNA 1, RNA 2) were used as template in the real-time RPA assay. They differed in the thickness of the peel used for the extraction. As a control, we included total RNA extract from PVY^O-inoculated potato leaves. We detected PVY in both periderm total RNA extracts (Fig. 4A). The thicker sample (RNA 2), which included the periderm and a thin layer of tuber flesh, resulted in a fast detection of the amplicon.

We next prepared crude extract from different periderms peeled from potatoes infected with various strains of PVY (PVY^O, PVY^{N:O}, PVY^{Wi}; PVY^{NTN}) as a template for the real-time RPA assay (Fig. 4B). PVY detection using potato crude extract had weaker signal intensity based on the relative fluorescence unit (RFU) of the assay and required more cycles, when compared to the levels observed with the purified RNA samples (Fig. 4A and B). However, the different strains of PVY were still detected in the tested infected potato tubers samples, with a signal well above that from the healthy potato control.

4. Discussion and conclusion

We optimized the RPA assay for PVY detection in potato leaves and tubers. We validated a set of primers that specifically amplified all PVY strains tested with no cross-reactivity with other potato viruses including potyviruses. Here we revealed that the PVY amplicon can be directly and easily visualized on a gel electrophoresis. This is in contrast

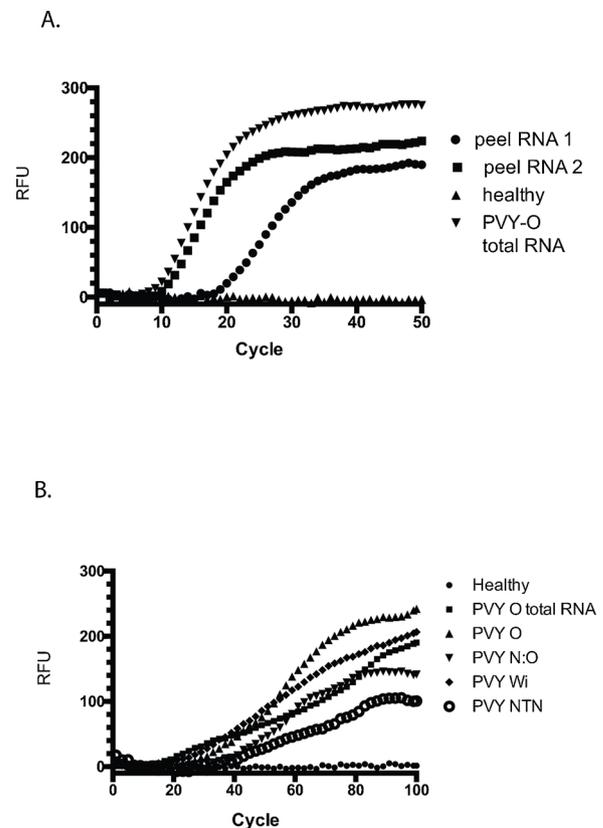


Fig. 4. Sensitivity of PVY detection in potato tubers. A. The real time detection of PVY was performed in the presence of the fluorescent probe on total RNA extract from periderm tissues of PVY^O infected tubers. We tested two different RNA extracts from a thin (RNA1) and a thicker (RNA 2) peel of about 2 in. The thin peel was obtained from the periderm tissue only. The thicker peel included the periderm and a thin layer of the tuber flesh. As a control we included total RNA extract from PVY-inoculated potato leaves. B. The RPA assay was performed on crude extract of periderm peels from potato infected different strains of PVY (PVY^O, PVY^{N:O}, PVY^{Wi}, PVY^{NTN}). We included total RNA extract from potato peels of PVY^O infected and healthy tubers.

with a previous report of PVY detection by RPA, which required an additional purification step (Glais and Jacquot, 2015). The real-time detection of the target sequence with the fluorescent probe enhanced the sensitivity and the performance of the assay. The latter allowed faster detection of PVY in less than 10 min in low viral titer samples. Another advantage of RPA is its lack of requirement for high-quality RNA preparation, which is often necessary for other PCR-based amplification, and the reverse transcription reaction takes place in the same reaction tube. Because the preparation of crude extract is relatively inexpensive and simple, and because the RPA assay can be completed in just few minutes, it brings the cost of the RPA assay close to that of currently used methods when labor costs are considered. The ability of the RPA assay to detect PVY directly from tubers is powerful. Due to the high starch content and phenolic compounds, the quality of the RNA is often compromised when extracted from potato tubers and thus a limiting factor for most nucleic acid-based detection assays. Here we showed that PVY was successfully amplified from different tissues from the periderms of the potato tubers. It is clear that total RNA extraction from the periderm results in a faster and a more sensitive level of detection when compared to the crude extract. However we were still able to detect PVY in the crude extract above background level by increasing the number of cycles up to 100 cycles, which still corresponded to a 30-minute reaction. While the technique shows great potential for routine diagnosis method instead of the grow-out test assay, further optimization may be recommended for high-throughput application.

The flexibility of the RPA assay is also an advantage since amplicons can be detected with gel electrophoresis, with real-time PCR thermocyclers, or with a relatively inexpensive fluorescence detection machine.

Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors.

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