# SENSITIVE DETECTION OF POTATO VIRUS Y AND DIFFERENTIATION OF *O* AND *N* TYPES BY LOOP-MEDIATED ISOTHERMAL AMPLIFICATION

**Krzysztof Treder[1], Joanna Chołuj[1], Bogumiła Zacharzewska[1], Aurelie Rakotondrafara[2], Lavanya Babujee[2]**

[1] – Bonin Research Center, Plant Breeding and Acclimatization Institute - National Research Institute, 76-009 Bonin, Poland.

[2] – Department of Plant Pathology, University of Wisconsin-Madison, 1630 Linden Drive, Madison, WI 53706, USA.

## INTRODUCTION

Potato virus Y (PVY) has been a global challenge for potato production and the main leading cause of seed crop downgrading and rejection for certification. At least nine PVY strains are currently known which differ at the biological and molecular levels but all can be grouped into two serotypes: the O serotype, which includes $PVY^{O}$, $PVY^{N:O}$, $PVY^{N-Wi}$, $PVY^{C}$, and the N serotype, which includes $PVY^{N}$, $PVY^{E}$, $PVY^{Z}$, $PVY^{NA-N}$ and PVY-NE11. Accurate and timely diagnosis is a key to effective disease control. The reverse transcription loop-mediated amplification (RT-LAMP) is gaining recognition as fast and sensitive alternative to RT-PCR in the detection of plant viruses. The RT-LAMP is based on an isothermal auto cyclic strand displacement of DNA synthesis. The high specificity and speed of the method heavily rely on the primer sets designed for the amplification of the targeted regions. Here we designed Y4 primer set and optimized RT-LAMP to detect PVY with high speed and sensitivity, as well as to differentiate the O/N isolates.

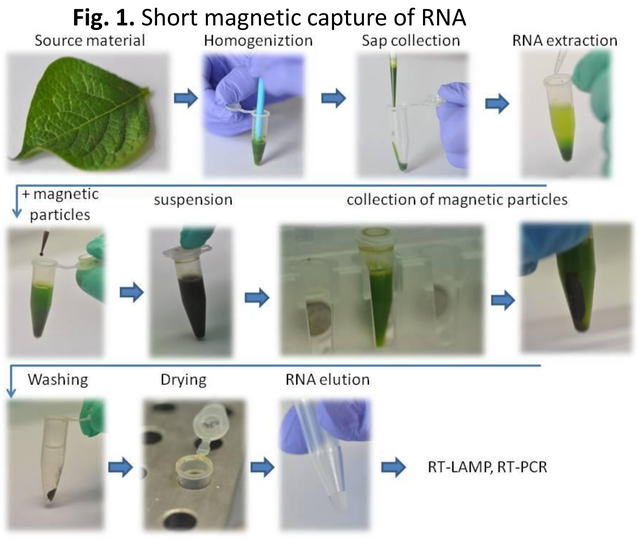
## MATERIALS AND METHODS

**European (EU) PVY isolates.** Potato tubers infected with isolates 12/94, N Nysa, or with isolates Wi and LW were kindly provided by the Młochów Research Center of the Plant Breeding and Acclimatization Institute – National Research Institute. PVY isolates: PV-0403, PV-0410, PV-0348, PV-0345, PV-0343 were purchased from the Plant Virus Collection maintained by Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures. Leave samples infected with PVY isolates Bonin 1-3 were collected in local experimental fields. **North American (USA) PVY isolates** were from the collection held by Dr. A. Rakotondrafara.

**Table 1.** Description of PVY isolates used in the study.

| Isolates | Strain | Serotype | Accession number | Country | Isolate source |
|---|---|---|---|---|---|
| 12/94 | $PVY^{NTN}$ | N | AJ889866 | Poland | Młochów |
| Wi | $PVY^{N-Wi}$ | O | EF558545 | Poland | Młochów |
| N Nysa | $PVY^{N}$ | N | FJ666337 | Poland | Młochów |
| LW | $PVY^{O}$ | O | AJ890349 | Poland | Młochów |
| PV-0403 | $PVY^{NTN}$ | N | – | Hungary | DSMZ |
| PV-0410 | $PVY^{NTN}$ | N | – | Germany | DSMZ |
| PV-0348/ CH-605 | $PVY^{N}$ | N | X97895 | Switzerland | DSMZ |
| PV-0327 | $PVY^{N}$ | N | – | France | DSMZ |
| PV-0345 | $PVY^{O}$ | O | – | Spain | DSMZ |
| PV-0343 | $PVY^{O}$ | O | – | Germany | DSMZ |
| Bonin 1 | $PVY^{O}$ | O | – | Poland | Bonin |
| Bonin 2 | $PVY^{N-Wi}$ | O | – | Poland | Bonin |
| Bonin 3 | $PVY^{NTN}$ | N | – | Poland | Bonin |
| MN-2 | $PVY^{NTN}$ | N | | USA | Madison |
| Tu 660 | $PVY^{NA-NTN}$ | N | AY166866 | USA | Madison |
| NTN40 D8A | $PVY^{NTN}$ | N | - | USA | Madison |
| Pito | - | N | - | USA | Madison |
| PVY:O | - | O | - | USA | Madison |
| CR-1 | $PVY^{N:O}$ | O | | USA | Madison |
| ID-1 | $PVY^{N:O}$ | O | DQ157178 | USA | Madison |
| PB22 | $PVY^{N:O}$ | O | - | USA | Madison |

**Fig. 1.** Short magnetic capture of RNA




**RNA isolation**. *Silica capture* of RNA was according to [1]. *Manufacturer magnetic capture* of RNA was on Novabeads magnetic particles according to the manufacturer (Novazym Polska s. c.). *Short magnetic capture* of RNA was as in [1] but instead of silica, magnetic particles were used and two washes were omitted (Fig. 1). Potato sap was added to tubes containing beads suspended in buffer. Samples were incubated at room temperature, particles were captured on the magnetic stand and the supernatants were removed. The beads were washed with buffer and with ethanol, suspended in RNase-free water. After short incubation the beads were captured, the supernatants were collected and stored.

**Table 2.** Description of Y4 primers set. F1c and B1c regions of FIP and BIP primers are indicated by lower case letters, F2 and B2 regions of FIP and BIP primers are shown as upper case letters. Nucleotide position corresponds to the genome of PVY isolate N Nysa with accession number FJ666337. The Y4 set was designed according to [3].

| Name | Position (nt) | Sequence |
|---|---|---|
| Y4F3 | 8802-8820 | TGC CAA CTG TGA TGA ATG G |
| Y4B3 | 9105-9085 | GTT CGT GAT GTG ACC TCA TAA |
| Y4FIP | F1c 8934-8917<br>F2 8865-8884 | gca ttc tca acg att ggt ACG GAG TTT GGG TTA TGA TG |
| Y4BIP | B1c 8950-8968<br>B2 9027-9008 | gca aat cat ggc aca ttt c CG TGG CAT ATA TGG TTC CTT |
| Y4LF | 8914-8893 | CAA TGG GTA TTC GAC TTG TTC A |
| Y4LB | 8969-8987 | TCA GAT GTT GCA GAA GCG T |

**RT-LAMP assay.** Primer sets used: N [2], Y5 [3], Y4 (Table 2). The regions amplified by Fip and Bip primers of N, Y5 and Y4 sets correspond respectively to base positions nts 8808-8965, nts 8938-9217 and nts 8865-9027 in the genome of isolate N Nysa. The RT-LAMP reaction mixture contained 0.375 µM each of outer primer (F3, B3), 1.5 µM each of inner primer (FIP, BIP), 0.75 µM each of loop primer (LB), 1X Isothermal Master Mix containing proprietary fluorescent dye (Novazym Polska s. c.), 0,25 U AMV reverse transcriptase (Novazym Polska s. c.) with 100 pg of total RNA as a template. The amplification in a Genie II (OptiGene Ltd.) was conducted at 65°C for 30 min. The annealing temperature (Ta) was determined from slow annealing (0.05 °C/s) for 5 min, starting at 95°C and ending at 80°C. The thermal profile in a CFX96 Touch™ (BioRad Ltd) included 60 cycles of 30 sec at 65°C and melting analysis (65°C to 98°C, 0.5°C/sec). Each experiment was conducted in at least two replicates and was repeated independently at least three times. A non-template control (sterile water, ntc), a negative control (total RNA from virus-free plant, nc), and a positive control (total RNA from PVY infected plant) were included for each LAMP run. **Strain identification by multiplex RT-PCR.** The RNA was isolated according to [1] from tobacco cv. Samsun)infected with PVY isolates Bonin 1-3 and with isolates representing $PVY^{O}$ (PV-0345), $PVY^{N-Wi}$ (EF558545), $PVY^{N}$ (PV-0348), and $PVY^{NTN}$ (PV-0403). Reverse transcriptions were carried out with the Reverse Super Verte KIT with random primers (Novazym Poland). The cDNA was amplified by the multiplex PCR according to [4].

## RESULTS

While all primer sets successfully amplified the PVY target sequence as expected, the newly developed Y4 RT-LAMP assay facilitated the fastest PVY detection (Figure 2a). The Y4 assay was one order of magnitude more sensitive than the previously described Y5 RT-LAMP assay, which was already 10-fold more sensitive than the RT-PCR and 1000-fold more sensitive than enzyme immunoassays [3]. The Y4 primers were highly specific to PVY (Fig. 2cd). The strain status of field isolates was confirmed as $PVY^{O}$ for Bonin 1, $PVY^{N-Wi}$ for Bonin 2, and $PVY^{NTN}$ for Bonin 3 (Fig. 3).

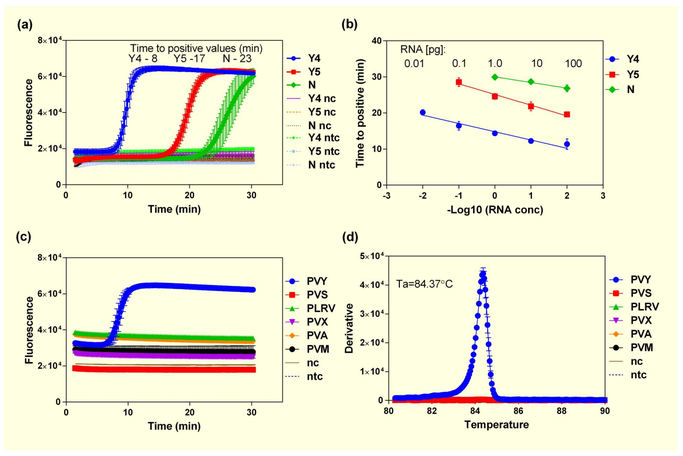



**Fig. 2** Performance of the Y4 set primed RT-LAMP for PVY detection. **(a)** Comparison of the amplification speeds of different primer sets in the RT LAMP assay. Controls: RNA from virus-free plants (nc), water (ntc). The time to positive values are shown above the plot. **(b)** Comparison of the sensitivity of different RT-LAMP assays by total RNA serial dilution. **(c)** Specificity of the Y4 RT-LAMP as the amplification plots **(d)** Second derivatives of annealing curves recorded for reactions from panel (c).

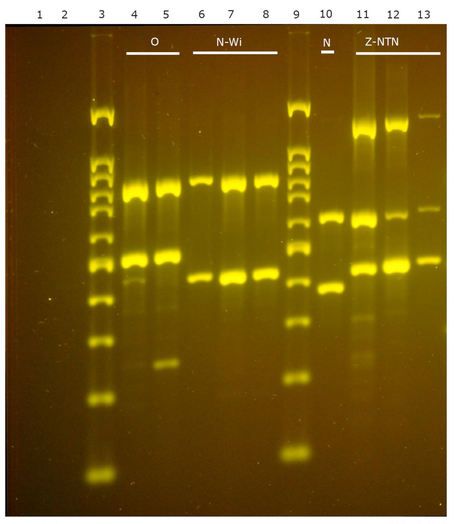



**Fig. 3** Identification of strains by multiplex RT-PCR. 1 – non-template control, 2 – RNA from healthy plant, 4 – PV-0345, 5 – Bonin 1, 6 – Wi, 7 and 8– Bonin 2, 10 - PV-0348, 11 – PV-0403, 12 and 13 – Bonin 3, 3 and 9 - Nova 100 bp, (Novazym Poland).

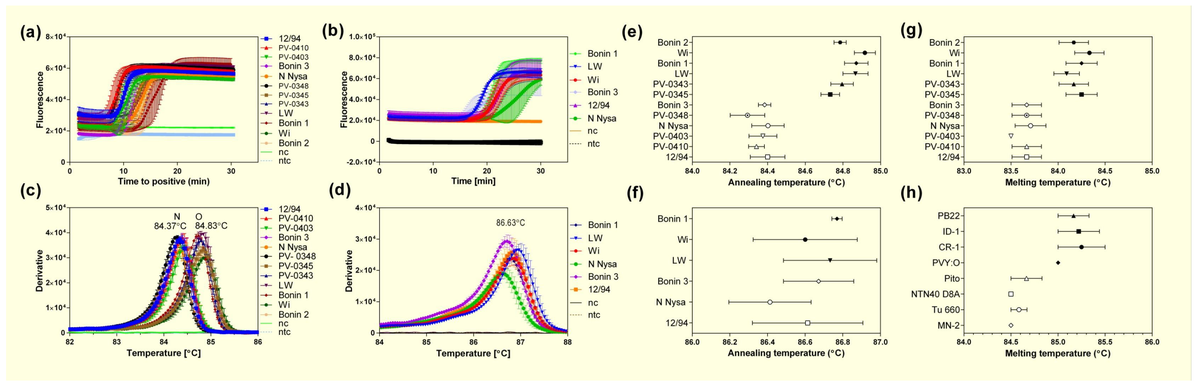



**Fig. 4** Differentiation of PVY types "N" and "O" by Y4 primer set. Amplification plots recorded for Y4 (a) and Y5 (b) primer sets priming RT-LAMP reactions supplemented with total RNA isolated from plants infected with indicated EU PVY isolates, or with total RNA from virus-fee plant (nc), or with water instead of RNA (non-template control – ntc). Second derivatives of annealing curves recorded for amplicons amplified by Y4 (c) and Y5 (d) primer sets in reactions described respectively in panels (a) and (b). Annealing temperature (Ta) ranges of the RT-LAMP products obtained on EU PVY strains by amplification with Y4 (e) and Y5 (f) primer sets. The annealing temperatures were recorded on a Genie II apparatus. Melting temperature (Tm) ranges of the RT-LAMP products obtained on EU (g) or USA (h) PVY strains by amplification with Y4 primer set. The melting temperatures were measured on CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). PVY strains with O or N types of coat protein are indicated respectively by symbols with black and white background. The data are averages from at least two independent experiments performed in triplicate. Error bars indicate standard deviation.

Both Y5 and Y4 RT-LAMP assays detected all tested EU isolates (Figure 4a and 4b). However, analysis of Ta indicated that the products amplified by Y4 primers from isolates with an O serotype had a 0.46°C variation in their Ta values when compared to the N-type isolates ($p<0.0001$, table 3, Fig. 4c and 4e). Such significant variation was not observed with the Y5 primer set (difference 0.14°C, $p=0.2191$, table 3, Figure 4d and 4f). Furthermore, the variation in Tm values was clear between O and N types when using the Y4 primers (difference 0.56°C, $p<0.0001$, Table 3, Fig. 4g). The Y4 RT-LAMP discriminated also USA PVY O and N-types. The O-type had a Tm value of an order of 0.47°C higher than (85.16°C) that of the N-type (84.69°C) ($p=0.0005$, Table 3, Figure 4h).

**Table 3.** Two-tailed unpaired t test analysis of mean Ta and Tm values

| | Y5 RT-LAMP | Y4 RT-LAMP | Y4 RT-LAMP | Y4 RT-LAMP |
|---|---|---|---|---|
| O vs. N | EU Ta | EU Ta | EU Tm | USA Tm |
| P value | 0.2191 | <0.0001 | <0.0001 | 0.0005 |
| P value summary | ns | **** | **** | *** |
| | | | | |
| Mean ± SEM of N isolates | 86.56 ± 0.0786, n=3 | 84.37 ± 0.01746, n=6 | 83.65 ± 0.03038, n=6 | 84.69 ± 0.03961, n=4 |
| Mean ± SEM of O isolates | 86.7 ± 0.05132, n=3 | 84.83 ± 0,02937, n=6 | 84.21 ± 0.03544, n=6 | 85.16 ± 0.05577, n=4 |
| Means difference | 0.1367 ± 0.09387 | 0.4633 ± 0.03417 | 0.56 ± 0.04668 | 0.4712 ± 0.0684 |
| 95% confidence interval | -0.124 to 0.3973 | 0.3872 to 0.5395 | 0.456 to 0.664 | 0.3039 to 0.6386 |
| R squared (eta squared) | 0.3464 | 0.9484 | 0.935 | 0.8878 |

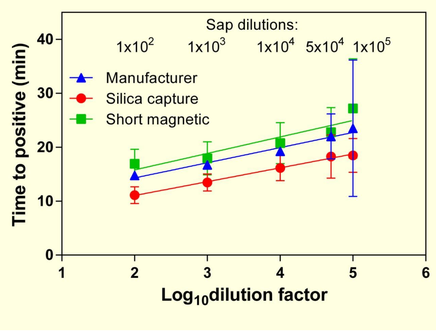



**Fig. 5** An impact of method of RNA isolation on the detection of PVY by Y4 RT-LAMP. Infected sap was serially diluted up to 2x106 and total RNA was purified from each dilution according to (i) manufacturer, (ii) silica capture, and (iii) short magnetic procedures. Time to positive results are shown as a function of the logarithm of the dilution factor. Error bars indicate standard deviation

The impact of three protocols of RNAs isolation on the sensitivity of PVY detection was investigated. The RNAs were isolated from leaf sap dilutions and amplified with the Y4 RT-LAMP assay (Fig. 5). All tested procedures facilitated PVY detection with the same detection limit = 1 x $10^5$-fold diluted sap (Fig. 5).

## CONCLUSIONS

- The Y4 RT-LAMP discriminated the PVY O and PVY N types.
- The Y4 assay facilitated the fastest detection of PVY and highest sensitivity.
- With Y4 RT-LAMP the amplification for very low virus concentrations can be accomplished in 15-20 min.
- The simplification of the procedure of RNA isolation had no negative impact on the sensitivity of PVY detection.
- The entire test can be performed in 40 min.

## REFERENCES

1. Zacharzewska et. al. 2014. Am J Potato Res 91:525–531.
2. Nie X. 2005. Plant Dis 89:605–610.
3. Przewodowska et al. 2015. Am J Potato Res 92:303–311.
4. Chikh-Ali et al. 2010. J Virol Methods 165:15–20.

## ACKNOWLEDGEMENTS

The authors would like to thank Prof. Renata Lebecka and Prof. Ewa Zimnoch-Guzowska for providing tubers infected with PVY isolates and MSc. Danuta Sekrecka for providing virus-free plants and sources of PLRV, PVA, PVM, PVS and PVX. We also want to thank Maria Fedczak for excellent technical help. This study was supported financially by the Ministry of Agriculture and Rural Development, Poland of the project No. 58: "Development of sensitive methods for detection of the most important potato viruses" (4 3 00 7 01) and by National Science Centre, Poland (grant No. 2016/21/B/NZ9/03573) to KT, and by a Hatch Act fund (MSN179998) and the Wisconsin Potato Industry Board grant (MSN191752) to AMR.

EAPR 2017 20th TRIENNIAL CONFERENCE Versailles - 9-14 july 2017

NATIONAL SCIENCE CENTRE POLAND