Variability of *Potato virus Y* in Tomato Crops in Poland and Development of a Reverse-Transcription Loop-Mediated Isothermal Amplification Method for Virus Detection

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ABSTRACT

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A collection of 147 *Potato virus Y* (PVY) isolates from tomato, originating from several commercial fields and greenhouses in different regions of Poland, was tested for the presence of PVY by reverse-transcription polymerase chain reaction. However, in some cases, the results obtained were

ambiguous. Therefore, a sensitive reverse-transcription loop-mediated isothermal amplification method was developed for rapid detection of PVY isolates. Phylogenetic and recombination analyses were performed based on sequences of the coat protein gene. In comparison with results obtained in 2008, the presence of other strains besides PVY^NWi-P was confirmed. A novel recombinant between PVY^{NTN} and PVY^NWi-P strains was detected. Our results indicate an increasing distribution and variability of the PVY population on tomato in Poland.

Additional keywords: PVY strains, RT-LAMP.

Potato virus Y (PVY) is the type species of the genus *Potyvirus* in the family *Potyviridae* (Hall et al. 1998). PVY is probably the most widespread virus infecting potato as well as other solanaceous crops, such as tomato and pepper (Cuevas et al. 2012). The virus has filamentous particles of approximately 700 to 750 nm in length. The genome consists of one positive-sense single-stranded RNA of approximately 9.7 kb in length, with the VPg protein covalently attached to its 5' end and a poly-A tail at its 3' end. The viral RNA encodes a single large polyprotein, which is cleaved by three virus-encoded proteases (P1, HC-Pro, and NIa-Pro) into 10 products: P1, HC-Pro, P3, 6K1, CI, 6K2, NIa-Pro, VPg, Nib, and CP (Chung et al. 2008). PVY is known for its high level of genetic diversity. The virus was originally classified into three strains (PVY^C, PVY^O, and PVY^N) according to biological properties or genome sequences (Singh et al. 2008). Later, two subgroups belonging to the PVY^N strain were identified in potato: PVY^{NTN} and PVY^NWi (Chrzanowska 1991). Within PVY^NWi, two subgroups can also be distinguished: PVY^NWi-P and PVY^NN242 (Rigotti and Gugerli 2007). PVY is a serious threat for tomato crops and causes large yield losses in different countries (Abad and Jordá 2000; Rosner et al. 2000). The virus is transmitted mechanically and by different aphid species (Boquel et al. 2011). In recent years, climate conditions (early, warm spring) have led to the occurrence of a large number of aphids in Poland. Indeed, high incidences of PVY were observed in greenhouse and field tomato plants in 2012 and 2013 in Poland. PVY was first reported in Poland on tomato in 2002 (Pospieszny 2003). The presence of PVY has only occasionally been reported since then. In 2008, increased distribution of PVY was reported; however, only PVY^N-Wi was identified in tomato plants under greenhouse conditions (Pospieszny et al. 2009). PVY was not found in plants from

cultivated fields at that time. In order to analyze and establish the diversity among PVY isolates, a survey was conducted in tomato production fields and greenhouses in different regions of Poland. Moreover, we screened the population in terms of the occurrence of potential novel recombinants. Recombination is very common in PVY and is likely to impact the evolution of the virus, because most of the currently present isolates are recombinant forms. The presence of PVY in collected samples was verified by reverse-transcription polymerase chain reaction (RT-PCR); however, in a few cases, the results were ambiguous. Hence, we aimed to develop a very sensitive and rapid reverse-transcription loop-mediated isothermal amplification (RT-LAMP) method for the diagnostic of PVY isolates from tomato. The LAMP method has been previously developed for PVY isolates originating from potato; however, the viral population may differ among the hosts (Almasi and Dehabadi 2013; Nie 2005). The most significant advantage of the LAMP is the ability to amplify target DNA under isothermal conditions (63 to 65°C) in less than 1 h. The products of LAMP are steam-loop DNA structures with several inverted repeats of the target, and cauliflower-like structure with multiple loops. Thus far, many viral species have been successfully detected using this method (Hasiów-Jaroszewska and Borodynko 2013; Lee et al. 2011; Peng et al. 2012). This article presents the results of molecular analyses of a collection of tomato PVY isolates and development of the RT-LAMP method for rapid detection of the virus present in samples at low concentrations.

MATERIALS AND METHODS

Survey and PVY detection using conventional RT-PCR.

In 2012 to 2013, a survey was conducted in 25 commercial production fields and 10 greenhouses located in the Wielkopolska, Dolnośląskie, Lubuskie, and Mazowieckie regions. Altogether, 176 samples from tomato with symptoms of leaf malformation and crinkling were collected. Among them, 44 samples came from

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greenhouses (Table 1). Field and greenhouse samples showing symptoms of leaf distortion, stunting, and mosaic were collected from individual tomato plants (Fig. 1A, B, and C). For the detection of PVY, total RNA was isolated from 100 mg of tissue using the RNeasy Plant Mini Kit (Qiagen, Hilde, Germany) according to the manufacturer's instructions. The RNA was used in one-step RT-PCR reactions using three sets of primers: PVY3+/PVY3-, PVYc3/f, and PVY CP2+/1- designed by Rigotti and Gugerli (2007) to detect different PVY strains (Table 2). One-step RT-PCR was performed in a 50- μ l reaction mixture containing 2 μ l of RNA, 25 μ l of DreamTaq PCR Master Mix (2 $\times$) (Thermo Scientific, Wilmington, DE), 1 μ l of each primer (10 μ M), and 1 μ l of M-MuLV reverse transcriptase (200 U) (Thermo Scientific). Amplifications were carried out as described previously (Rigotti and Gugerli 2007). The RT-PCR products were separated on 1.5% agarose gel stained with Midori Green (NIPPON Genetics, Dürren, Germany).

Phylogenetic and recombination analyses. To assess genetic composition of the PVY population in samples collected, the coat protein (CP) gene region (1.2 kbp) was amplified. Total RNA was first transcribed using 2 μ l of 10 μ M oligo(dT) primer and M-MuLV reverse transcriptase (Thermo Scientific) according to the manufacturer's recommendations. The CP gene was amplified from the resulting cDNA using primers designed by Boonham et al. (2002) (Table 2). The PCR reaction mixture contained 1 μ l of cDNA, 25 μ l of DreamTaq PCR Master Mix (2 $\times$) (Thermo Scientific), and 1 μ l of each primer (10 μ M). PCR products were purified using the Qiaex II gel extraction kit (Qiagen). The resulting DNA was ligated into the pGEM-T Easy vector (Promega Corp., Madison, WI) and transformed to *Escherichia coli* Dh5 α (Life Technologies, Carlsbad, CA) according to the manufacturers' instructions. The presence of the insert was verified by digestion using *Eco*RI (Thermo Scientific). The selected plasmids were sequenced using a Beckman Coulter CEQ8000 sequencer

TABLE 1. Origin and number of the samples used in this study

Region of Poland	Year of collection	Number of samples from fields	Number of samples from greenhouses	Tomato cultivars in greenhouses ^a	Tomato cultivars in fields ^b
Wielkopolska	2012	30	10	Growdena	Polfast, Etna, Hektor, Dyno, Rumba, Polbig
Wielkopolska	2013	30	7	...	...
Dolnośląskie	2012	7	3	—	Polfast
Dolnośląskie	2013	7	3	—	...
Lubuskie	2012	10	4	—	Babinicz, Polbig, Hektor, Olga
Lubuskie	2013	10	3	—	...
Mazowieckie	2012	15	7	Tomimaru Muchoo, Admiro, Foronti	Polbig, Koneser
Mazowieckie	2013	23	7	...	...

^a Symbol: — indicates lack of information.

^b Partial information.

Fig. 1. A, Example of 'Polbig' tomato in the field in the Wielkopolska region infected with *Potato virus Y* (PVY). B, Polbig tomato plant infected with PVY. C, Tomato plant (unknown cultivar) infected with PVY in greenhouse.

TABLE 2. Primers used in this study

Primer name	Sequence 5'–3'	Temperature of annealing (°C)	Reference
PVY3+	TGTAACGAAAGGGACTAGTGCAAAG	57	Rigotti and Gugerli 2007
PVY3-	CCGCTATGAGTAAGTCCTGCACA	...	...
PVYc3	CAACGCAAAAACACTCA(CT)AA(AC)GC	...	...
PVYf	TAAGTG(AG)ACAGACCCTCT(CT)TTCTC	...	...
PVYCP2+	CCAGTCAAACCCGAACAAAGG	...	...
PVYCP1-	GGCATAGCGTGCTAAACCCA	...	...
CPF	AACTGGAAGAATTGGCGGCCGAGGAAT TTTTTTTTTTTTTTTT	53	Boonham et al. 2002
CPR	RGCTTCACTGAAATGATGG	...	...
FIP	GCGCATTTCTATATACGCTTCACCAACC CTTAGGCAAATCAT	63	This study
BIP	CGCAGCATTGAAATCAGCTGTGTCTCC TCTTGTGTACTG	...	...
LoopF	TGCAACATCTGAGAAATGTGC	...	...
LoopB	CTCGACTTTTCGGGTGGA	...	...
F3	CGTTGAAACCAATCGTTGAGAA	...	...
B3	GACATCCTCGGTGGTGTG	...	...

(Beckman Coulter, London, UK). The compilation and editing of sequences was performed in BioEdit, version 7.2.5 (Hall 1999). In order to carry out a detailed comparison of the CP gene of PVY, sequences of 198 different isolates were compared. These included 180 isolates obtained from the GenBank along with 18 new sequences described in this article (accession numbers KJ746439 to KJ746456). Multiple alignment was performed using ClustalW. Evidence of recombination events in the CP sequences was identified using Recombination Detection Program (RDP), version 4.22 Beta (Martin et al. 2010). RDP4 incorporates several recombination detection methods into a single suite of tools: RDP, GENECONV, BOOTSCAN, MAXCHI, CHIMAERA, SISCAN, and 3SEQ. Maximum likelihood trees were inferred for the nucleotide alignment using MEGA, version 6.0.5 (Tamura et al. 2013). The Tamura-Nei model of nucleotide substitution was estimated as the most appropriate using MEGA 6 and chosen for construction of phylogenetic tree. Confidence in branch points in the phylogenetic tree was assessed by the bootstrap method, with 1,000 pseudorandom replicates. The ratio between the nonsynonymous (d_N) and synonymous (d_S) substitutions rates ($\omega = d_N/d_S$) was used as a proxy to evaluate the intensity and sign of selection operating on CP. Values of $\omega = 1$, $\omega < 1$, and $\omega > 1$ indicate neutral, purifying or negative selection, and diversifying or positive selection, respectively. Selective pressure at the codon level was estimated using the SLAC, FEL, IFEL, and MEME methods from the Datamonkey server (Kosakovsky Pond and Frost 2005) and MEGA 6. In order to identify correlated variation among amino acid sites, in particular those with evidence of selection pressure, we performed analyses of coevolution within the CP using CAPS (coevolution analysis using protein sequences) software, version 1 (Fares and McNally 2006).

RT-LAMP method. Isolates representing the four different PVY strains identified and two samples that were problematic in RT-PCR were subjected to RT-LAMP. To verify specificity of the

reaction, RT-LAMP was also carried out using another virus species infecting tomato, *Tobacco etch virus* (TEV). RNA of TEV was produced from a full-length plasmid clone kindly supplied by Prof. Santiago Elena, IBMCP, Valencia, Spain. RT-LAMP primers were designed using Primer Explorer software, version 4, based on CP genes of PVY (Table 2) and the aligned nucleotide sequences of PVY CP obtained in this article and others retrieved from GenBank. Each 25- μ l LAMP reaction consisted of 2 μ l each of FIP and BIP primers (10 μ M), 0.5 μ l each of F3 and B3 primers (10 μ M), 1 μ l each of LoopF and LoopB primers (10 μ M), 15 μ l of Isothermal Mastermix (ISO-001t) (OptiGene, Horsham, UK) 1 μ l of the target RNA, and 1 μ l (20 U) of Transcriptor Reverse Transcriptase (Roche, Mannheim, Germany). The mixture was incubated at 63°C for 40 min in a thermoblock (Biometra, Göttingen, Germany). Products were verified by electrophoresis on 1.5% agarose gels with Midori Green (NIPPON Genetics) or directly visualized in UV light by addition of 3 μ l of SYBR Green I (diluted 1:1000) (Novazym, Poznań, Poland). To determine the sensitivity of the PVY LAMP assay, the plasmid bearing full-length CP was used to make serial dilutions. The recombinant plasmid DNA was measured using a NanoDrop spectrophotometer (Thermo Scientific), adjusted to 1 μ g/ μ l, and serially diluted to serve as standards determining assay sensitivity. The plasmid DNA was used for both LAMP and conventional PCR. The conventional PCR was performed using 1 μ l of CP primers (Boonham et al. 2002), 25 μ l of DreamTaq Green PCR Master Mix (Thermo Scientific), and 1 μ l of template DNA (serial 10-fold dilutions of plasmids) in a total volume of 50 μ l. The LAMP method was performed using the above-mentioned primers, 15 μ l of Isothermal Mastermix (ISO-001t) (OptiGene), and 1 μ l of template DNA (serial 10-fold dilutions of plasmids) in a total volume of 25 μ l. Products of both reactions were compared by

TABLE 3. Identification of *Potato virus Y* (PVY) isolates collected during 2012 to 2013 by one-step reverse-transcription polymerase chain reaction (RT-PCR)^a

PVY strain	Amplification bands (bp)	Number of isolates
PVY ^N Wi-P	530	74 (+2)
PVY ^{NN} 242	530, 440	30
PVY ^O	530, 660	14
Recombinant PVY ^{NTN}	440	27
PVY ^C	660	0
PVY ^N	440, 1100	0
Sum	...	147

^a One-step RT-PCR was conducted according to Rigotti and Gugerli (2007); (+2) = two isolates detected after passaging on tobacco plants.

Fig. 3. Schematic representation of recombinant isolate Br 2.1 through recombination between the Polish *Potato virus Y* (PVY) PC6 and W4 isolates, representing PVY^{NTN} and PVY^NWi-P, respectively. The recombination breakpoints were at 12 and 351 nucleotides.

Fig. 2. Identification of *Potato virus Y* (PVY) strains by one-step reverse-transcription polymerase chain reaction (RT-PCR) conducted according to Rigotti and Gugerli (2007). **A**, RT-PCR amplification using the primers CP2+/1- (lanes 2 to 8) and PVYc3/f (lanes 9 to 15) on total RNA of *Solanum lycopersicum* infected with PVY^{NN}242 (lanes 3 and 10), PVY^NWi-P (lanes 4 and 15), PVY^{NTN} (lanes 6 and 13), PVY^O (lanes 7 and 14). Lane 1, HyperLadder IV (Bioline); lanes 2 and 9, negative control, total RNA from healthy tomato; lanes 5 and 12 plus 8 and 11, problematic samples, unclear identification of PVY strains. **B**, RT-PCR amplification using the primers CP2+/1- on total RNA from *Nicotiana tabacum* ‘Xanthi’ plants after the reinoculation of samples which gave ambiguous result in previous RT-PCR. Lane 1, HyperLadder IV (Bioline); lane 2, negative control, total RNA from healthy tobacco; lanes 3 and 4, PVY^NWi-P.

electrophoresis on 1.5% agarose gel with Midori Green (NIPPON Genetics). Because the quality and quantity of template may have great influence on the method, we also compared conventional RT-PCR with RT-LAMP. The serial 10-fold dilutions of total RNA

(adjusted to 100 ng/μl) of the PVY^NWi-P isolate were prepared and used in RT-PCR with CP primers (Boonham et al. 2002) and in RT-LAMP. Similarly, the detection limit was approved by electrophoresis on 1.5% agarose gel.

Fig. 4. Maximum likelihood phylogenetic tree for full-length coat protein gene sequences of 13 new Polish *Potyvirus Y* (PVY) isolates and 101 other PVY isolates retrieved from GenBank. The tree was generated using Tamura-Nei substitutions model and MEGA6 software. Branches with <80% bootstrap support were collapsed. The scale bar represents a genetic distance of 0.05. New sequences of the Polish isolates deposited in the GenBank under accession numbers KJ746439 to KJ746456 were marked with different symbols (circle = PVY^O, square = PVY^NWi-P, and triangle = PVY^NN242). The other sequences show their GenBank codes, countries of origin, and host collection data.

RESULTS

Survey and PVY detection using conventional RT-PCR.

The samples were collected from plants showing the symptoms in the form of growth reduction and severe malformation in the fields and mosaic and malformation and crinkles in greenhouses. Among the 176 samples collected (Table 1) in the fields, 145 gave positive results in RT-PCR whereas, in 2 samples, the results were ambiguous (Table 3). Twenty-nine samples were clearly negative. The primer sets developed by Rigotti and Gugerli (2007) distinguished among the PVY strains according to banding patterns obtained after RT-PCR (Fig. 2A; Table 3). Four banding patterns were observed using RT-PCR analysis of RNA isolated from symptomatic plant tissues: a 0.44-kb fragment corresponding to PVY^{NTN} in 27 samples (23 from fields and 4 from greenhouses), two bands of 0.66 and 0.53 kb corresponding to PVY^O in 14 samples (12 from fields and 2 from greenhouses), 0.53 kb corresponding to PVY^{NWi-P} in 74 samples (59 from fields and 15 from greenhouses), and two bands of 0.53 and 0.44 kb corresponding to PVY^{N242} in 30 samples (22 from fields and 8 from greenhouses). The presence of PVY^C and PVY^N was not confirmed in any of the tested samples.

Recombination and phylogenetic analyses. To establish diversity among PVY isolates and the relationships between them, we performed phylogenetic analyses based on the CP gene. We amplified and sequenced the CP gene from all samples that tested positive in RT-PCR or RT-LAMP (147 samples in total). The majority of PVY isolates representing a particular strain showed a very high level of identity ranging from 99.8 to 100%. For that reason, the sequences of PVY representing particular variants (18 in total) were selected from among the 147 sequences for further analysis. The sequences have been deposited in the GenBank database under accession numbers KJ746439 to KJ746456. The sequences of the CP genes of 180 PVY isolates found worldwide were retrieved from the GenBank and analyzed together with sequences obtained in this study. As expected, several recombinants were detected among the PVY sequences obtained in this study by at least three of the eight statistical methods implemented in RDP4. The analysis revealed that one Polish sample designated as Br2.1, that was originally classified as PVY^{NTN}, is a recombinant between

PVY^{NTN} and PVY^{NWi-P} isolates, with the recombination breakpoints in the 12- and 351-nucleotide positions (Fig. 3). Moreover, the presence of isolates belonging to recombinant PVY^{NTN} strain was confirmed. All recombinant and identical sequences were excluded, reducing the dataset to 114 isolates (13 from this study and 101 retrieved from GenBank). In the phylogenetic tree, the Polish isolates clustered with PVY^{NWi-P}, PVY^{N242}, or PVY^O strains (Fig. 4). The Polish PVY^{NWi-P} and PVY^{N242} strains were closely related to the isolates from Germany and Brazil collected from potato and isolates collected from tomato in Poland. Strain PVY^O was very closely related to another Polish isolate collected from tomato in 2008. Most of the sequences retrieved from GenBank referred to isolates collected from potato. The normalized $d_N - d_S$ value was calculated for each codon of CP. For most of the codons, this value was less than one, indicating purifying (stabilizing) selection. Negatively selected positions were scattered along the open reading frame. The codon at nucleotide 193 was under positive selection by two (IFEL and MEME) of the four methods used and by MEGA 6 ($d_N - d_S = 2.65$; $P = 0.194$). Finally, six groups of covariation were detected with a total of 14 amino acids involved, and codon 193 was a member of two of these covariable groups (Fig. 5).

RT-LAMP assay. RT-LAMP primers for amplification of all PVY strains were designed based on CP gene sequences. The RT-LAMP assay developed allowed us to detect the four PVY strains that occur in Poland (Fig. 6A). After amplification, RT-LAMP products were observed for PVY RNA, whereas no amplicons were detected for TEV or RNA from uninfected plants. Similarly, the color of RT-LAMP products changed from orange to green when PVY was detected with SYBR Green I, whereas the original orange color remained when the healthy control and TEV were tested (Fig. 6B). Furthermore, the RT-LAMP was compared with conventional RT-PCR. RT-LAMP and RT-PCR amplification of serial dilutions of total RNA isolated from a plant infected with PVY^{NWi-P} indicated that RT-LAMP detection sensitivity was 10-fold higher than that of the RT-PCR (Fig. 7A and B). Amplification of plasmid DNA using the LAMP method detected DNA at as little as 1 fg/μl, while the detection limit of PCR was approximately 100 fg/μl (Fig. 8A and B). For the two tomato leaf samples that gave ambiguous results in RT-PCR, RT-LAMP indicated the presence of PVY (Fig. 6A and B). When sap obtained from these plants was used to inoculate *Nicotiana tabacum* 'Xanthi' plants, the presence of PVY^{NWi-P} was confirmed by RT-PCR (Fig. 2B).

DISCUSSION

In 2012 and 2013, a collection of 147 PVY isolates was obtained from tomato plants growing in commercial fields and greenhouses in different regions of Poland. The phylogenetic analysis based on CP sequences gave a complete overview of the PVY populations these isolates. We were able to group tomato isolates into four different strains: PVY^{NTN}, PVY^{N242}, PVY^O, and PVY^{NWi-P}. Moreover, we found a novel recombinant isolate between the PVY^{NTN} and PVY^{NWi-P} strains. The analysis revealed the presence of different virus strains in individual fields. Nevertheless, the majority of PVY isolates representing a particular strain and collected from the same field had a high level of sequence identity. Plants infected with any of the four PVY strains exhibited similar symptoms of growth reduction, leaf crinkling, and mosaics and, for that reason, the symptoms were not useful for diagnosis. The structure of the PVY populations in isolates collected in greenhouses and fields was also very similar, with a predominance of the PVY^{NWi-P} strain. Interestingly, PVY^{NWi-P} was also detected on most infected potato samples collected in Poland (Yin et al. 2012). These results might suggest that the virus can be transferred by aphids from potato to the neighboring tomato plants, because PVY incidence can reach 40% in potato cultivars (Kostiw 2011). Aphids could be the main source of PVY spread in Poland, because none of

Fig. 5. Network of coevolving residues in the *Potato virus Y* coat protein. Coevolutionary relationships are represented as networks, with amino acid sites represented as circles. The position of the site in the multiple sequence alignment is indicated within the circle, and coevolutionary links between pairs of sites are represented as edges.

the nonaphid-transmitted PVY^C isolates were found on tomato. In both 2012 and 2013, the climate conditions (a warm, wet spring and a warm summer) were conducive to the appearance of aphids.

The analysis of PVY population structure on tomato crops in northeast Spain revealed the presence of genetically different PVY isolates, mixed infections, and recombinants from other hosts such as potato or pepper and natural weeds (Aramburu et al. 2006). Because of this, the previous classification of PVY isolates, which is based on isolates from potato and pepper, might not be useful for PVY from tomato. Our results confirmed that distribution of potato and nonpotato isolates was intermingled in the tree, with the exception of isolates from *Solanum betaceum*, which formed separate cluster. Interestingly, the Polish PS4 isolate which belongs to PVY^O strain was very closely related to another Polish isolate collected from tomato in 2008. This suggests that particular variants persist over time. The role of the CP in potyvirus pathogenicity has been previously shown. The N terminus of the CP plays multifunctional roles. It is exposed on the virion surface and is involved in vector transmission and virus movement in the plant (Andersen and Johansen 1998), thus becoming a potential target of selection at both vector and plant levels. Our analysis revealed that the main

force driving CP evolution is purifying selection; however, in our and other authors' (Cuevas et al. 2012) analyses, some codons under positive selection were also detected. In both potato and nonpotato isolates, codon 193 was under diversifying selection.

A diversity of PVY strains infect tomato and other plant species that might act as viral reservoirs, presenting a major problem for the development of resistance strategies for disease control as well as development of diagnostic tools to detect PVY. Aramburu et al. (2006) showed that RNA amplification by RT-PCR could be biased toward specific sequences and that the results may vary depending on the primers used. Similarly, in our study, RT-PCR amplification assays using primers designed by Rigotti and Gugerli (2007) provided ambiguous results from some of the collected samples. The RT-LAMP assay developed here enabled detection of four PVY strains using a single primer set in half an hour, requiring only a conventional water bath or heating block. The sensitivity of RT-LAMP was also demonstrated using field samples, from which we were able to detect PVY^{NWi-P} isolates in two samples that gave ambiguous results in RT-PCR. Thus, RT-LAMP has a great potential for use in routine detection of PVY in the fields and greenhouses.

Fig. 6. Detection of *Potato virus Y* (PVY) strains in selected tomato leaf samples by reverse-transcription loop-mediated isothermal amplification (RT-LAMP). **A**, Analysis of RT-LAMP products on agarose gels: lane M, Hyper-Ladder IV (Bioline); lane 1, negative control, healthy tomato sample; lane 2, *Tobacco etch virus*; lane 3, PVY^{NTN}; lane 4, PVY^{NWi-P}; lane 5, PVY^O; lane 6, PVY^{NN242}; lanes 7 and 8, problematic samples in reverse-transcription polymerase chain reaction. **B**, Visual detection of RT-LAMP products using SYBR Green I. Numbers correspond to the gel lanes in A.

Fig. 7. Comparison of sensitivity of reverse-transcription loop-mediated isothermal amplification (RT-LAMP) and reverse-transcription polymerase chain reaction (RT-PCR) methods. **A**, Detection limit of the RT-LAMP assay using total RNA isolated from leaves of tomato infected with PVY^{NWi-P}. Lane 1, HyperLadder IV (Bioline); lane 2, negative control, water; lanes 3 to 9 correspond to serial 10-fold dilutions of RNA (ranging from 100 ng/μl to 100 fg/μl). **B**, Detection limit of the RT-PCR assay using total RNA isolated from tomato infected with PVY^{NWi-P}. Lane 1, HyperLadder III (Bioline); lane 2, negative control, water; lanes 3 to 8 correspond to serial 10-fold dilutions of RNA (ranging from 100 ng/μl to 1 pg/μl).

Fig. 8. Comparison of sensitivity of loop-mediated isothermal amplification (LAMP) and polymerase chain reaction (PCR) methods. **A**, Detection limit of the LAMP assay using serial dilution of coat protein (CP) plasmid. Lane 1, HyperLadder IV (Bioline); lane 2, negative control, water; lanes 3 to 13 correspond to serial 10-fold dilutions of CP plasmid (ranging from 1 μg/μl to 0.1 fg/μl). **B**, Detection limit of the PCR assay using serial dilution of CP plasmid. Lane 1, HyperLadder III (Bioline); lane 2, negative control, water; lanes 3 to 11 correspond to serial 10-fold dilutions of CP plasmid (ranging from 1 μg/μl to 10 fg/μl).

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