An Improved Multiplex IC-RT-PCR Assay Distinguishes Nine Strains of *Potato virus Y*

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Abstract

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A multiplex reverse-transcription polymerase chain reaction (RT-PCR) assay was previously developed to identify a group of *Potato virus Y* (PVY) isolates with unusual recombinant structures (e.g., PVY^{NTN-NW} and SYR-III) and to differentiate them from other PVY strains. In the present study, the efficiency of this multiplex RT-PCR assay was validated and extended considerably to include five additional strains and strain groups not tested before. To make the multiplex RT-PCR assay more applicable and suitable for routine virus testing and typing, it was modified by replacing the conventional RNA extraction step with the immunocapture (IC) procedure. The results obtained using well-char-

acterized reference isolates revealed, for the first time, that this multiplex RT-PCR assay is an accurate and robust method to identify and differentiate the nine PVY strains reported to date, including PVY^O (both PVY^O and PVY^{O-O5}), PVY^N, PVY^{NA-N}, PVY^{NTN}, PVY^Z, PVY^E, PVY-NE11, PVY^{N-Wi}, and PVY^{N:O}, which is not possible by any of the previously reported RT-PCR procedures. This would make the IC-RT-PCR procedure presented here a method of choice to identify PVY strains and assess the strain composition of PVY in a given area. The IC-RT-PCR protocol was successfully applied to typing PVY isolates in potato leaf tissue collected in the field.

Potato virus Y (PVY) is one of the most common and destructive viruses infecting potato worldwide (11,37). It is the type species of the genus *Potyvirus*, family *Potyviridae*. PVY has a single-stranded positive-sense genomic RNA of approximately 9.7 kb, with a VPg protein covalently linked to the 5' terminus and a poly(A) tail at the 3' terminus (3). Potato isolates of PVY have been classified into five strain groups (PVY^O, PVY^C, PVY^N, PVY^Z, and PVY^E) based on reactions in potato indicators carrying the resistance genes *Ny*, *Nc*, and *Nz* and tobacco (*Nicotiana tabacum*) symptoms (9,16,20, 21,37). In addition, several types of PVY recombinants have been identified based on genome sequences, including PVY^{NTN}, which has two recombination types: type A, with three recombination junctions (RJs) at the HC-Pro/P3, VPg-N1a, and C terminus of the coat protein region (CP) and type B, with four RJs at the P1, HC-Pro/P3, VPg/N1a, and C terminus of the CP gene; PVY^{N-Wi}, with two RJs at P1 and HC-Pro/P3, and also referred to as PVY^{N-Wi}(B) or PVY^{NWi-P}; PVY^{N:O}, with one RJ at HC-Pro/P3, and also referred to as PVY^{N-Wi}(A) or PVY^{N242}; and PVY^{NTN-NW} (2,5,7, 8,12,14,26,27,34,37). All these recombinants were found to have mosaic genomes built of fragments of parental PVY^O and PVY^N sequences. The recombinant types in potato isolates of PVY were grouped in nine main recombination patterns (13), and positions of the main recombinant RJs were found to be relatively conserved. Additional PVY genotypes were described, sufficiently different from nonrecombinant PVY^O and PVY^N and from other simple O/N recombinants, such as PVY^{NA-N} (29,33) and PVY-NE11 (22).

The efficient control of PVY requires the availability of reliable detection and identification methods of PVY strains but the genetic

diversity of PVY complicates the identification methods. Several multiplex reverse-transcription polymerase chain reaction (RT-PCR) assays have been developed to distinguish PVY strains based on nucleotide polymorphisms around the RJs in PVY recombinants (6,24,28,30,35). However, most of these methods probed a single RJ and, thus, were only able to distinguish certain recombinant PVY strains, particularly PVY^{N-Wi} or PVY^{NTN} (28,30). The single-step triplex PCR assay reported by Rigotti and Gugerli (35) distinguishes several PVY strains but targeted only the RJ in the P1 found in PVY^{N-Wi} and PVY^{NTN}(B). In addition, these methods are not able to effectively detect mixed strain infections.

The multiplex PCR assays reported by Lorenzen et al. (24) and Chikh Ali et al. (6) are widely used to distinguish among a broader range of PVY strains. The multiplex PCR assay reported by Lorenzen et al. (24) targets two RJs in PVY genome, between HC-Pro and P3, and within the VPg, which allows several strains to be separated but is limited in its ability to distinguish among some of the PVY strains that are common in the United States. For example, PVY^{N-Wi} and PVY^{N:O} cannot be differentiated. In addition, PVY^{NTN-NW} would be misidentified as PVY^{NTN}, and PVY-NE11 and PVY^E would be misidentified as PVY^{NA-N} and PVY^{NTN}, respectively. The multiplex RT-PCR assay reported by Chikh Ali et al. (6) was designed to target five RJs, allowing it to distinguish additional PVY strains and variants occurring alone and in combination with one another in infected potato. The RT-PCR based assay was designed based on the most current PVY sequence and strain information available in 2010, and tested on PVY isolates available in the Laboratory of Plant Pathology at Utsunomiya University, Japan (6). Since this RT-PCR assay was reported, sequences of other PVY strains became available, including PVY^Z and PVY^E (9,20). Moreover, this method was tested on a limited set of PVY strains available in Japan at the time. Although the banding patterns of other significant strains and variants (e.g., PVY^N and PVY^{N:O}) were inferred from sequence data they were not tested using this multiplex method. In the present study, the applicability of the multiplex RT-PCR assay (6) was validated with

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additional PVY strains and genotypes such as PVY^N, PVY^Z, PVY^E, PVY^{O-O5}, PVY-NE11, and PVY^{N:O}. In addition, this methodology was demonstrated to successfully work in the immunocapture (IC)-RT-PCR format.

Materials and Methods

Virus isolates. Twenty-two reference isolates of PVY from the University of Idaho, Plant Virology Laboratory virus collection representing PVY^O (including PVY^{O-O5}), PVY^N, PVY^{NA-N}, PVY^{N-Wi}, PVY^{N:O}, PVY-NE11, PVY^Z, and PVY^E (Table 1) were tested. These isolates were maintained in tobacco (*N. tabacum* 'White Burley') in a climate-controlled, insect-free growth chamber as described previously (19). In addition to the reference isolates, 22 PVY-infected potato leaf samples were collected from potato seed lots grown at the Othello seed lot trial field in Othello, WA, in June 2012. The infection status of tobacco and potato samples was confirmed by triple antibody sandwich enzyme-linked immunosorbent assay using a series of PVY-specific polyclonal and monoclonal antibodies as described (32).

IC-RT-PCR. The IC step was done by coating wells of twin.tec PCR 96-well plates (Eppendorf) with 100 µl of the R8 rabbit polyclonal antibodies (19) at 1:1,000 dilution in 20 mM sodium carbonate buffer (pH 9.6) and incubated at 37°C for 2 to 4 h or 4°C overnight. Plates were washed three times using phosphate-buffered saline (1× PBS) with 0.1% Tween 20 (PBST). Leaf samples were ground in extraction buffer (1× PBS, 0.3% dry milk, and 2% polyvinylpyrrolidone) at a 1:10 (wt/vol) ratio. Sample extracts (100 µl each) were added to the coated plates and incubated 37°C for 2 to 4 h or 4°C overnight. Plates were washed five times using 1× PBST and then rinsed with deionized water. The RT mixture was prepared using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega Corp.) according to the manufacturer's instructions and a mixture of oligo-dT and random hexamer primers, each at 3 µM final concentration. The RT program for first-strand cDNA synthesis started at 25°C, increased to 42°C at 1°C per 30 s, held at 42°C for 45 min, and then increased to 60°C at 1°C per 2 min. The transcriptase was inactivated by incubation at 70°C for 10 min. The

PCR step was carried out using GenScript Taq DNA Polymerase kit (GenScript). Twelve primers (6) designed to detect five RJs and differentiate them from nonrecombinant sequences in the P1 (two RJs targeted by three primers), HC-Pro/P3 (four primers), VPg/NIa (two primers), and NIb/CP (three primers) regions of PVY genome (6) were used at a final concentration of 0.2 µM each. cDNA solution (3 µl) was added to a PCR tube which contained 2 µl of 10× GenScript buffer, 1.6 µl of dNTPs (2.5 mM each), primers, and 0.3 µl (5 U/µl) of GenScript Taq polymerase. The volume of the mixture was adjusted to 20 µl using MilliQ H₂O. PCR amplification was performed in a DNA Engine Dyad Thermal Cycler (Bio-Rad Laboratories, Inc.) using the PCR program described previously (6). PCR products were separated in 2% agarose gels containing ethidium bromide at 10 µg/ml and visualized under UV illumination.

Results

Twenty-one isolates from the laboratory collection represented nine strains of PVY (17; Table 1). Four of these genetic types (PVY^N, PVY^E, PVY-NE11, and PVY^{N:O}) had not been previously analyzed by our multiplex RT-PCR assay. Genomic organization of the PVY strains and variants analyzed is presented in Figure 1, with positions and sizes of the expected PCR products indicated. The observed band patterns of all strains and genotypes tested were in agreement with the expected patterns derived from the genomic organization (Figs. 1 and 2; Table 1). PVY^O and PVY^{O-O5} isolates produced expected bands of 853 and 532 bp. PVY^N isolates produced bands of 1,307, 633, and 398 bp (Fig. 2; Table 1). Two PVY-NE11 isolates produced one band of 633 bp. This could be proposed as a unique banding pattern that may be now considered diagnostic for this type of PVY genome. Having the same genome structure, PVY^{NTN(A)} and PVY^Z produced three bands of 1,307, 633, and 441 bp (Fig. 2; Table 1). PVY^{N:O} produced three bands of 853, 633, and 441 bp while PVY^{N-Wi} produced two bands of 853 and 441 bp (Fig. 2; Table 1). This RT-PCR typing methodology was able to distinguish PVY^{N:O} isolates from PVY^{N-Wi} based on the presence or absence of an additional recombinant junction in the

Table 1. Summary of evaluated *Potato virus Y* (PVY) isolates testing^a

Isolate name	Genotype	Reference	Geographic origin	Band size (bp)						RT-PCR typing ^b
				398	441	532	633	853	1,307	
Mont	→ PVY ^N	23	United States	P	N	N	P	N	P	→ PVY ^N
N605	→ PVY ^N	15	Switzerland	P	N	N	P	N	P	→ PVY ^N
NzF	→ PVY ^N	36	New Zealand	P	N	N	P	N	P	→ PVY ^N
NE11	→ PVY-NE11	22	United States	N	N	N	P	N	N	→ PVY-NE11
ID20	→ PVY-NE11	18	United States	N	N	N	P	N	N	→ PVY-NE11
RRA-1	PVY ^{NA-N}	23	United States	N	N	N	N	N	P	PVY ^{NA-N}
HR1	→ PVY ^Z -NTN	12–14, 20	United States	N	P	N	P	N	P	→ PVY ^{NTN}
L26	→ PVY ^Z -NTN	12–14, 20	United States	N	P	N	P	N	P	→ PVY ^{NTN}
N4	PVY ^{NTN}	12–14	United States	N	P	N	P	N	P	PVY ^{NTN}
F2M3	PVY ^{NTN}	Unpublished	Mexico	N	P	N	P	N	P	PVY ^{NTN}
423.3	PVY ^{NTN}	23	United States	N	P	N	P	N	P	PVY ^{NTN}
AST	PVY ^{NTN}	9	Brazil	N	P	N	P	N	P	PVY ^{NTN}
MON	→ PVY ^E	9	Brazil	N	P	N	P	N	N	→ PVY ^E
AGA	→ PVY ^E	9	Brazil	N	P	N	P	N	N	→ PVY ^E
Alt	→ PVY ^{N:O}	23	United States	N	P	N	P	P	N	→ PVY ^{N:O}
N1	PVY ^{N-Wi}	18	United States	N	P	N	N	P	N	PVY ^{N-Wi}
MF-VOY	PVY ^{N-Wi}	10	Brazil	N	P	N	N	P	N	PVY ^{N-Wi}
ID269	→ PVY ^{O-O5}	18, 19	United States	N	N	P	N	P	N	→ PVY ^O
ME173	→ PVY ^{O-O5}	18, 19	United States	N	N	P	N	P	N	→ PVY ^O
Oz	PVY ^O	1	United States	N	N	P	N	P	N	PVY ^O
CW	PVY ^O	18	United States	N	N	P	N	P	N	PVY ^O
Tb60	PVY ^O	Unpublished	United States	N	N	P	N	P	N	PVY ^O

^a Isolates are listed according to their strain and genotype assignment in corresponding references. Geographic origin and expected number and size of polymerase chain reaction (PCR) fragments are indicated. P = positive, N = negative, and arrows mark genotypes and strains not previously tested with this assay. "Unpublished" refers to PVY isolates identified by whole genome sequencing but not published yet.

^b Reverse-transcription polymerase chain reaction.

Fig. 1. Genomic structure of *Potato virus Y* (PVY) strains tested using immunocapture reverse-transcription polymerase chain reaction (IC-RT-PCR) assay with primer locations (arrows) and expected products (black bar). Numbers near the black bars indicate the expected fragment size. Strains and genotypes tested in this report are labeled with dotted arrows. Strains and genotypes tested previously (6) but not tested in the current study are labeled with asterisks. The numbering system of recombination junction (RJ)1 to 4 was according to Karasev and Gray (17). RJ1, RJ1a, RJ2, RJ3, and RJ3a refer to recombination junctions targeted by this IC-RT-PCR. RJ1a and RJ3a were not tested in the current study but were tested previously (6).

P1 region (Figs. 1 and 2). Although the differentiation of PVY^{N:O} isolates from PVY^{N-Wi} was inferred from sequence data (6), they were not tested using this multiplex method in our prior studies. Two isolates of PVY^E strain produced two bands of 633 and 441 bp (Fig. 2; Table 1), providing a thus-far-unique pattern which may be considered diagnostic for this PVY strain. PVY^{NA-N} produced a single band of 1,307 (Figs. 1 and 2; Table 1), as expected and as was demonstrated previously (6), distinct from PVY-NE11 and from PVY^E (Fig. 2; Table 1). In field potato samples, 14 samples produced two bands of 853 and 441 bp typical of PVY^{N-Wi} (Fig. 3); 6 samples produced three bands of 1,307, 633, and 441 bp typical of PVY^{NTN} of A type (Fig. 3); and two samples produced three bands of 853, 633, and 441 bp typical of PVY^{N:O} (Fig. 3).

Discussion

Biological identification using differential potato cultivars along with tobacco can be used to differentiate some PVY strain groups but this method is not practical for many recombinant PVY isolates, because the biology of PVY recombinants in potato has not been well studied. Serological methods using monoclonal antibodies have limited value for typing the recombinant PVY strains due to a high degree of amino acid sequence identity in the PVY capsid protein. PVY^N and PVY^O serotypes can be distinguished but these serotypes may be associated with different recombinant and nonre-

combinant genotypes (4,19,31). Moreover, serological properties may change dramatically due to a single point mutation (4,10,19), which often leads to discrepancy between the serological profile and the genomic structure of PVY strains.

Molecular identification methods using RT-PCR are fast and robust, and can reliably differentiate PVY strains and variants. However, the vast pool of nonrecombinant and recombinant PVY strains requires comprehensive and reliable RT-PCR assays that can detect and differentiate the known PVY strains and common variants. All multiplex RT-PCR assays reported thus far can identify a limited number of PVY strains due to the limited number of the RJs targeted in the PVY genome (24,30,35). The multiplex RT-PCR reported by Chikh Ali et al. (6) was originally developed to distinguish a group of PVY isolates with unusual recombinant structures (5) (e.g., PVY^{NTN-NW} and SYR-III) in order to differentiate them from the other, more ordinary PVY strains. The assay targeted five RJs along the PVY genome (13) and, thus, was unique among the RT-PCR methods compared with other methodologies available (24,30,35). Targeting these five RJs represents a very powerful general tool for differentiating most of the known PVY strains and strain variants beyond the group of Syrian isolates (6,25). However, the efficacy of this RT-PCR was originally assessed and validated using only a limited set of PVY strains which excluded significant and widely distributed strains and strain groups, such as PVY^{O-O5}, PVY^N, PVY^{N:O}, and PVY-NE11 (6). It

Fig. 2. Results of the multiplex polymerase chain reaction assay using tobacco samples infected with reference *Potato virus Y* (PVY) isolates. Lane 1, 100-bp DNA ladder; lane 2, PVY^N (Mont); lane 3, PVY^N (N605); lane 4, PVY^N (NzF); lane 5, PVY-NE11 (NE-11); lane 6, PVY-NE11 (ID20); lane 7, PVY^{NA-N} (RRA-1); lane 8, PVY^Z-NTN (HR1); lane 9, PVY^Z-NTN (L26); lane 10, PVY^{NTN} (A, N4); lane 11, PVY^{NTN} (A, F2M3); lane 12, PVY^{NTN} (A, 423.3); lane 13, PVY^{NTN} (A, AST); lane 14, PVY^E (MON); lane 15, PVY^E (AGA); lane 16, PVY^{N:O} (Alt); lane 17, PVY^{N-Wi} (N1); lane 18, PVY^{N-Wi} (MF-VOY); lane 19, PVY^O-O5 (ID269); lane 20, PVY^O-O5 (ME-173); lane 21, PVY^O (Oz); lane 22, PVY^O (CW); lane 23, PVY^O (Tb60); lane 24, a 1-kb DNA ladder; lane 25, healthy potato control. Vertical arrows above lanes indicate novel patterns reported here.

Fig. 3. Results of the multiplex polymerase chain reaction assay using field potato samples. Lanes 1 and 25, 100-bp DNA ladder; lanes 2, 5, 6, 8, 11–15, 17, 18, 20, 22, and 23, *Potato virus Y* (PVY) ^{N-Wi}; lanes 3, 4, 9, 10, 16, and 19, PVY^{NTN}(A); lanes 7 and 24, PVY^{N:O}; lane 21, healthy potato control.

was not tested on isolates from recently sequenced strains PVY^Z (20) and PVY^E (9).

In the present study, the efficiency of this multiplex RT-PCR method was extended to include five additional strains and strain variants not previously tested. The RT-PCR test developed initially to distinguish rare Syrian isolates (6) can differentiate at least nine PVY strains and strain groups. The results obtained using well-characterized reference isolates and potato field samples confirmed the accuracy and robustness of this assay to identify PVY strains reported thus far, including PVY^O (both PVY^O and PVY^O-O5), PVY^N, PVY^{NA-N}, PVY^{NTN}, PVY^Z, PVY^E, PVY-NE11, PVY^{N-Wi}, and PVY^{N:O}, which is not possible by any of the previously reported RT-PCR methods. This would make this RT-PCR procedure a method of choice to identify PVY strains and assess the strain composition of PVY in a given area.

Hence, this is the first multiplex RT-PCR assay that identifies and differentiates a potato tuber necrotic ringspot disease-inducing PVY^E strain from other PVY strains. This is an important practical advancement, because PVY^E can overcome potato resistance genes *Ny*, *Nc*, and *Nz* causing symptomless infection or mild foliage symptoms in potato (9) and, thus, escape the field inspection. The multiplex RT-PCR described here can also differentiate PVY^{N-Wi} and PVY^{N:O}, which is important due to the differences in the biological properties and evolutionary origin between PVY^{N-Wi} and PVY^{N:O} (11,19,31,38). An additional unique and quite significant feature of this assay is its ability to identify and differentiate PVY-NE11 from PVY^{NA-N}, which was not accomplished by previously reported multiplex RT-PCR assays and required additional RT-PCR tests with a different primer set (22). This feature of our assay would help to identify the PVY-NE11 strain in the field samples and estimate its significance in potato.

To improve the efficiency and ease of this multiplex RT-PCR assay, the conventional RNA extraction requirement was replaced with the IC format. The present study has demonstrated that the previously reported multiplex RT-PCR assay (6) is a robust and universal method for the detection and identification of the nine PVY strains reported to date, including PVY^O (both PVY^O and PVY^O-O5), PVY^N, PVY^{NA-N}, PVY^{NTN}, PVY^Z, PVY^E, PVY-NE11, PVY^{N-Wi}, and PVY^{N:O}.

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