Possible Escape of a Recombinant Isolate of *Potato virus Y* by Serological Indexing and Methods of its Detection

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ABSTRACT

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Surveys of commercial and seed potato fields for virus diseases (1998 to 2002) in Manitoba established that *Potato virus Y* (PVY) is of concern in seed potato production. To determine the prevalence of PVY strains, PVY-infected tubers identified by reverse transcription-polymerase chain reaction (RT-PCR) from surveys (2000 to 2001) were grown for symptom expression and strain characterization by strain-specific RT-PCR, bioassays, and serological assays. Of the samples collected (2000 to 2001) and tested by RT-PCR, 4.0% contained PVY. Further analysis of the PVY-positive samples by a duplex RT-PCR facilitating the simultaneous detection of common (PVY^O) and tobacco vein necrosis strains (PVY^{N/NTN}) indicated that 37.5% contained PVY^O and 63.5% contained PVY^N-type isolates. Analysis of the PVY^N-type samples using three monoclonal antibodies (MAbs) showed that all reacted with only the PVY^O MAbs and not with the PVY^N-specific MAb. Partial nucleotide sequences of both ends of PVY-RNA showed that the PVY^N-type isolates resembled those reported in 1996 from Manitoba. These isolates are designated as PVY^{N:O}. In view of the increased incidence of PVY^{N:O} in one production area, seed tubers imported from other provinces of Canada and the neighboring United States were analyzed for PVY^{N:O}. The PVY^{N:O} was detected in imported seeds from Minnesota, Montana, and North Dakota.

Potato virus Y (PVY) is the type-species of genus *Potyvirus*, a positive strand monopartite RNA virus that infects many important food and vegetable crops worldwide including pepper, potato, tobacco, and tomato (28). Several strain groups of PVY are recognized on the basis of symptoms in naturally infected potatoes (*Solanum tuberosum*) and tobacco (*Nicotiana tabacum*) (7,15). The common strain (PVY^O) induces mild to severe mosaic and leaf drop streaks in potato, and systemic mottle in tobacco. The tobacco vein necrosis group (PVY^N) induces very mild mottling in most potato cultivars with occasional leaf necrosis in some (3,15) and induces severe systemic necrosis of leaf veins and petioles in tobacco (7). The PVY^{NTN} subgroup strains of PVY^N causing potato tuber necrotic ringspot disease induce chlorotic mottle to mosaic symptoms in potato plants and superficial to deeply sunken necrotic rings in tubers, and the

veinal necrosis symptoms in tobacco (17). Some partially characterized strains, including recombinants of PVY^N and PVY^O (2,4,15,20), the PVY^C strains which induce stipple streak symptoms in potato cultivar bearing the *Nc* gene, and the PVY^Z strains which overcome the resistance genes against both PVY^O and PVY^C (12,15), also exist.

PVY^O and the PVY^N strains that infect potato and tobacco have been known for seven decades (39). However, the general group of PVY^N, including PVY^{NTN} (15,17) (referred to here as PVY^{N/NTN}), and other possible recombinants of PVY^O and PVY^N including PVY^Z (2,12), isolate Wilga (4), and the isolates L-56 and L-136 (20,21) (the latter designated as PVY^{N:O} [25]) have been recently recognized. In addition to the increasing number of strains, the PVY^{N/NTN} group has been assigned to European (Eu) PVY^{N/NTN} and North American (NA) PVY^{N/NTN} geographical subgroups (24). The diagnosis of PVY^{N/NTN} strains has become complex due to the incidence of mixed infections (27,41,42) and increasing numbers of strains and substrains (24). Bioassay and serological and molecular assays may be required to provide conclusive identification of strains (36). The complex assay requirement precludes large-scale testing of seed tubers to prevent unwanted strains in planting material.

PVY strains have the potential to significantly reduce potato yield and quality,

and therefore, PVY-free potato seed is required by potato certification programs in many countries (38). Although careful sanitary measures reduce the infection and spread of PVY^O, a minimal number of PVY^O-infected tubers in a seed lot can be tolerated. In contrast, it is a common practice in North America to limit the importation or introduction of seed potatoes from areas where even a low percentage of potatoes may be infected with PVY^N (1,31,33). Consequently, the North American Plant Protection Organization (NAPPO) approved a set of recommendations for PVY^N risk management, which included a panel of three monoclonal antibodies (MAbs) with differing specificity (see the NAPPO website) to screen seed potatoes for PVY^N.

Accurate and speedy diagnosis of PVY and its strains is an important consideration in the effective management of the potato seed crop. Diagnosis based on bioassay is generally reliable but time-consuming (29,36). Enzyme-linked immunosorbent assay (ELISA) is rapid but relies on strain-specific antibodies, with separate tests for different antibodies, and the need for sprouted rather than dormant tubers (11,37). However, false positive results can occur with ELISA, especially for the PVY^{N:O} isolates, which may share PVY^O-like coat protein but cause PVY^N-like symptoms in tobacco (20,21).

Reverse transcription-polymerase chain reaction (RT-PCR) is a powerful tool for potato virus diagnosis (22,23,32,41). The P1 gene of the PVY genome has been proposed as the most variable region among the potyviruses (8), including the various strains of PVY (18). For example, by targeting the P1 gene, a 3-primer system can differentiate between the Eu-type PVY^{N/NTN} and NA-PVY^{N/NTN} (36,43). Similarly, Eu-type, and NA-type PVY^{N/NTN} can be distinguished from all other PVY^N and PVY^{NTN} isolates in a competitive RT-PCR based on sequence variation in the P1 gene (24,25). However, a multiplex RT-PCR protocol, which would facilitate simultaneous detection and differentiation of both PVY^O and PVY^N, including the recombinant (PVY^{N:O}) strains, may be of practical use for large-scale tests to manage PVY^N in seed certification programs. In this study, the distribution of the PVY^{N:O}-like isolates and their possible sources of introduction in Manitoba and

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their detection using a duplex RT-PCR, which simultaneously differentiates between the PVY^O group and the PVY^{N/NTN} group, is described.

MATERIALS AND METHODS

Potato samples and symptom expression. In Manitoba, 6,201 tubers from 34 fields (2000) and 2,383 tubers from 12 fields (2001) were collected randomly and examined for tuber necrosis symptoms. Both seed and processing potato fields were sampled. In 2001, 228 leaf samples biased in favor of mosaic symptoms were also collected from 20 fields. The leaf and tuber samples were first tested for PVY by a duplex RT-PCR devised for the simultaneous detection of *Potato leafroll virus* (PLRV) and PVY (34), and the samples identified as infected by PVY were used for determination of PVY strains.

One seed piece from each PVY-infected tuber was planted in a 15-cm plastic pot containing a soil mix. Plants were grown in a greenhouse with 12 h light ($90 \mu\text{m}^{-2} \text{s}^{-1}$) at 15 to 18°C to optimize virus symptoms (29). Symptoms were recorded every month for 3 months. Progeny tubers were examined for tuber necrosis symptoms.

Search for PVY^{N/O} in potato regions. To determine sources of PVY^{N/O} introduction into Manitoba, where potato seed is imported from a number of Canadian provinces (Alberta, British Columbia, Saskatchewan) and the neighboring United States (Minnesota, Montana, North Dakota), tubers and leaves were tested. Seed tubers were collected prior to planting in late April and early May of 2002, and leaves were collected in the first 2 weeks of July 2002. Except for Montana, two seed lots from each imported and five domestic seed sources were selected. One seed lot was selected from Montana. A random sample of 180 to 210 seed tubers per seed lot were tested. A field survey during the first 2 weeks of July (to avoid current season spread of PVY in fields planted with the imported seed sources) was conducted prior to the onset of aphid flights in Manitoba. Two thousand plants were observed in 10 randomly selected areas in each field, and leaves from plants showing mosaic symptoms were collected. Both seed tubers and leaves were tested, first by duplex RT-PCR described in this study, then by ELISA and bioassay for the strain identification as described below.

Serological assays. Triple antibody sandwich-ELISA was conducted to verify PVY infection and to identify strains, using three PVY MAbs recommended by NAPPO. The MAb 4C3 detects both PVY^O and PVY^N (9), MAb2 is specific for PVY^O (19), and MAb 1F5 (9) is specific for PVY^N. Leaf sap (300 μl) from healthy plants or plants with mosaic or veinal necrosis symptoms was mixed with 300 μl of extraction buffer (20 mM phosphate buffer, pH 7.4, 0.15 M NaCl, 0.05% Tween 20,

2% polyvinyl pyrrolidone [PVP-40], 0.3% skim milk powder) and incubated at room temperature for 3 h. The leaf extract (100 μl) was loaded in duplicate wells coated with a polyclonal PVY antibody (Phytopathogenics Inc., Vancouver, Canada) at 1:5,000 dilution. The loaded plates were incubated at 4°C overnight, and washed three times with double-distilled (dd) H₂O. Specific monoclonal antibodies were added at 1:4,000, 1:2,500, and 1:5,000 dilutions for 4C3, 1F5, and MAb2 (Phytopathogenics Inc., Vancouver, Canada), respectively, and incubated at 37°C for 2.5 h. Plates were washed three times with ddH₂O, and alkaline phosphatase conjugated antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were added at 1:3,000 dilution and incubated at 37°C for 2.5 h. After washing three times with ddH₂O, 0.5% *p*-nitrophenyl phosphate was added to the wells and incubated at room temperature for 1 h. The resultant absorbance at 405 nm was measured. The threshold for a positive reading was 0.100 if the healthy control absorbance was 0.030 or less. If the healthy control average was greater than 0.030, then the mean of the healthy controls was multiplied by four and used as the positive threshold. The PVY^O-139 tobacco culture (30) was used as the positive control for PVY^O, while the PVY^N N-Jg tobacco culture (24) was used as the positive control for PVY^N.

Bioassay. *Nicotiana tabacum* cv. Samsun and *Solanum brachycarpum* were used to determine the pathotype of PVY (29). Seedlings (three-leaf stage) of *N. tabacum* and *S. brachycarpum* grown in 15-cm plastic pots containing a soil mix were mechanically inoculated with PVY extracts. Briefly, leaves of PVY-infected potato plants were ground with a mortar and pestle in the extraction buffer (0.01 M sodium phosphate, pH 7.5, containing 0.4% sodium sulfite) (1:10, wt/vol) and mechanically inoculated to *N. tabacum* and *S. brachycarpum* for symptom expression (29). The inoculated plants were kept in the same conditions as described above. Symptoms were recorded weekly for 5 weeks.

Compositing of tuber, sprout, and leaf samples. To increase the efficiency for a large-scale survey of PVY^{N/O}-like isolates in a potato crop by a duplex RT-PCR (see below), the PVY^{N/O}-infected field grown tubers were used for the production of composite samples that included infected (I) and healthy (H) materials. Infected and healthy tubers, sprouts, and leaves of three cultivars (Chieftain, Norland, and Yukon Gold) from the same seed lot were used to prepare the composites of tissues (data not shown), saps (A), and nucleic acid extracts (B). Buffered saps (see "Nucleic acid extraction") from healthy and infected sprouts or healthy and infected tubers were combined in I:H ratios of 1:10, 1:20, 1:30, 1:40, 1:50, 1:70, and 1:100 and used for

nucleic acid extraction. The ratios were 1:50, 1:100, 1:200, 1:400, 1:600, 1:800, and 1:1,000 for leaf samples. The nucleic acid extracts of individual healthy and infected sample were combined to provide I:H ratios of 1:10, 1:50, 1:100, 1:200, 1:400, 1:800, and 1:1,200 for tubers; 1:10, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1,200, and 1:1,500 for sprouts; and 1:100, 1:500, 1:1,500, 1:2,000, 1:2,500, and 1:3,000 for leaf extracts. Tests with composited samples were repeated by two people and repeated five times by each person. In each test, tubers, sprouts, and leaves from three cultivars were used. Each test had three replicate samples at each I:H ratio.

Nucleic acid extraction. Nucleic acids were extracted from leaves, tubers, and sprouts using sodium sulfite (35). For each sample, 300 μl of extraction buffer (0.1 M Tris-HCl, pH 7.4, 2.5 mM MgCl₂, 0.65% Na₂SO₃, and 6 U of DNase I) was combined with 5 drops (150 to 200 μl) of sap, obtained by a Tuber Slicer (Electrowerk, Behcke and Co., Hannover, Germany). Incubation at 37°C for 10 min was followed by extraction with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, vol/vol/vol) and precipitation of RNA from the aqueous phase with 1 vol of isopropanol in the presence of 0.1 vol of 3 M sodium acetate (−20°C, overnight). The precipitate was collected by centrifugation (12,000 × *g*, 15 min, 4°C), washed with 70% ethanol, dried under a vacuum, and dissolved in 1,000 μl of sterile water for leaf and sprouts and 100 μl for tuber samples.

RT-PCR. RNA extract (2.5 μl , 400 ng) plus 2.5 μl of water was incubated at 65°C for 8 min and chilled on ice for 3 min to denature the RNA. The RT mixture was added to provide a final concentration of 20 ng/ μl of reverse primer (see below) (24,25): 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 2.5 mM MgCl₂, 1.5 mM of each dNTPs, 20 U RNasin (Promega Corp., Madison, WI), and 200 U Moloney murine leukemia virus-reverse transcriptase (Gibco BRL). Samples were incubated 1 h at 42°C for RT and subsequently incubated at 95°C for 3 min to terminate the reaction.

The initial determination of PVY was carried out by a duplex method developed for PLRV and PVY (34), and the second duplex RT-PCR derived from a multiplex competitive RT-PCR (25) was used for PVY strains. It was performed using 2.0 μl aliquots of the cDNA mixture in 25 μl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 100 μM each of dATP, dCTP, dGTP, and dTTP (Promega), 0.1 μg each of the primers (reverse, 5'-CATTGTGTGCCAATTGCC-3' [A]; and forward, 5'-GACAGTTGGACTTTTGCA-ACG-3' [S7] for an amplified product of 281 bp of PVY^O, 5'-GGTGAAGCTAATCATGTCAAC-3' [S6] for an amplified product of 443 bp of PVY^{N/NTN} [25]), and

0.625 U of DNA polymerase (PE Applied Biosystems, Foster City, CA). Samples were amplified for 30 cycles using a Peltier Thermal Cycler (MJ Research, Watertown, MA). Annealing temperature was 62°C for the first 5 cycles, 60°C for the next 5 cycles, 58°C for the following 10 cycles, and 55°C for the last 10 cycles. Each cycle consisted of denaturation (92°C, 30 s), primer annealing (30 s) and primer extension at 72°C (90 s), and a final extension of 10 min at 72°C. Amplified products (8 µl) were separated by electrophoresis in a 1.5% agarose gel containing ethidium bromide at 0.5 µg/ml and photographed under UV illumination with an imaging system (Alpha Innotech IS1000, San Leandro, CA).

Two cDNA clones derived from 5' and 3' ends of four isolates (Mb-20, Mb-23, Mb-112, and Mb-119) from Manitoba causing tobacco vein necrosis or mosaic symptoms were sequenced in each direction as previously described (20,24) to verify the genotype of the isolates (data not shown).

RESULTS

Relative proportions of PVY strains. During 2000 and 2001, leaves and dormant tubers were analyzed for the presence of PVY. Combined samples (8,812) of commercial and seed fields were tested by a duplex RT-PCR for PVY and PLRV (34), and 361 samples (4.0%) were positive for PVY (Table 1, Processing and Seed combined). In seed fields, PVY incidence was 2.3%, and in processing fields, the incidence was 7.7% (Table 1). Of the total 289 (Processing and Seed combined, 2000) RT-PCR positive tubers in 2000, 236 germinated, and the daughter plants were used

for the determination of PVY strains. On the basis of symptoms expressed in the tobacco bioassay, an equal number (50.0%, 75 each) of tobacco plants had mild mosaic or vein necrosis symptoms when the plants were inoculated with the samples from processing fields of year 2000 (Table 2). This indicates an equal proportion of PVY^O and PVY^N-like infection in the samples collected from processing fields in that year. However, when samples collected from seed lots of year 2000 were tested, 36.0% (31/86) contained PVY^O and 64.0% (55/86) had PVY^N-like infection (Table 2). In 2001, about one-third (6/16 and 1/3 for processing and seed samples, respectively) of the PVY-positive leaves collected from processing and seed lots contained PVY^O, whereas two-thirds (10/16 and 2/3 for processing and seed samples, respectively) had PVY^N-like infection. In the tuber samples, the proportion of PVY^N-like infection increased dramatically, reaching over 94.1% (16/17) in the PVY-positive tubers collected from seed potatoes and 100% (36/36) in the processing tubers (Table 2). There was complete agreement in the tobacco bioassay and the RT-PCR results of these samples (Table 2).

A duplex RT-PCR for simultaneous detection of PVY^O and PVY^N group of strains in potato tubers and leaves. The proportion of PVY^N-like infection in tobacco was unexpectedly high in the population of PVY (Table 2) originating from potato samples, and this necessitated a large-scale routine testing of potato seed lots. To facilitate the simultaneous detection of PVY^O and PVY^N-like strains in a routine indexing of potato samples, a modification of the tetraplex-competitive

RT-PCR (25) was carried out. The tetraplex-competitive RT-PCR (25) showed that the PVY^{N/NTN} from Manitoba was amplified by primers specific to Eu-type PVY^{N/NTN} (Fig. 1A, lanes 5 to 7) but not by the NA-type PVY^{NTN} (Fig. 1A, lane 14). Since only two strains were regularly encountered in Manitoba tuber samples, a duplex-competitive RT-PCR derived from the tetraplex PCR, using primer S6 for amplification of PVY^{N/NTN} and S7 for PVY^O (25), was adopted. S6 and S7, when paired with the A, resulted in a fragment of 443 bp for PVY^N-like strains and a fragment of 281 bp for PVY^O (Fig. 1B). Analysis of tobacco plants with mosaic symptoms using duplex RT-PCR confirmed that they contained only PVY^O-specific PCR fragments (Fig. 1B, lanes 8, 12, 15, and 17), while those with the vein necrotic symptoms contained PVY^{N/NTN}-specific fragments (Fig. 1B, lanes 1 and 4). Differentiation of the PVY^O and PVY^{N/NTN} strains on the original potato tuber and leaves was also confirmed by duplex RT-PCR (data not shown). In addition, the duplex-RT-PCR of tobacco leaves showed the existence of several doubly infected (PVY^O + PVY^{N/NTN}) plants (Fig. 1B, lanes 2, 3, 5 to 7, 9 to 11, 13, 14, 16, and 18 to 20), and the virus titer based on band intensity of each strain in the doubly infected plants was variable without favoring a particular strain.

Nature of PVY^N-like isolates. *S. brachycarpum* is an indicator plant in which both PVY^N and PVY^O strains can be differentiated by the lethal necrosis or the mosaic symptom they produce (29). When *S. brachycarpum* was inoculated with tobacco sap from plants used in the RT-PCR studies with mosaic or vein necrosis symptoms (five samples of each), none of them developed lethal necrotic symptoms typical of PVY^N. Although both resulted in mosaic symptoms similar to PVY^O in *S. brachycarpum* plants, there was an increased crinkling of the leaves inoculated with the tobacco plants with necrotic symptoms compared to those with mosaic symptoms.

When ELISA was carried out with various antibodies using tobacco leaves, the MAb 4C3 detected all samples from symp-

Table 1. Incidence of *Potato virus Y* (PVY) in potato in years 2000 and 2001 in Manitoba

Year	Processing ^a		Seed ^a	
	No. tested	PVY	No. tested	PVY
2000 (tubers)	2,277	175 (7.7%)	3,924	114 (2.9%)
2001 (leaves)	67	16 (24.0%)	161	3 (1.9%)
2001 (tubers)	598	36 (6.0%)	1,785	17 (1.0%)
Total	2,942	227 (7.7%)	5,870	134 (2.3%)

^a Samples were tested by a duplex reverse transcription-polymerase chain reaction for PVY and *Potato leafroll virus* (PLRV) (34).

Table 2. Incidence of *Potato virus Y* (PVY) strains in potato in Manitoba as determined by bioassay in tobacco and reverse transcription-polymerase chain reaction (RT-PCR)

Year	Processing			Seed		
	No. tested	PVY ^{Oa}	PVY ^{Na}	No. tested	PVY ^O	PVY ^N
Bioassay						
2000 (tubers)	150	75 (50.0%)	75 (50.0%)	86	31 (36.0%)	55 (64.0%)
2001 (leaves)	16	6 (37.5%)	10 (62.5%)	3	1 (33.0%)	2 (67.0%)
2001 (tubers)	36	0 (0.0%)	36 (100.0%)	17	1 (5.9%)	16 (94.1%)
RT-PCR ^b						
2000 (tubers)	150	75 (50.0%)	75 (50.0%)	86	31 (36.0%)	55 (64.0%)
2001 (leaves)	16	6 (37.5%)	10 (62.5%)	3	1 (33.0%)	2 (67.0%)
2001 (tubers)	36	0 (0.0%)	36 (100.0%)	17	1 (5.9%)	16 (94.1%)

^a PVY^O is the common strain of PVY; and PVY^N is the tobacco vein necrotic strain of PVY.

^b PVY strain specific competitive RT-PCR described in this study.

tomatic leaves in which A_{405} was significantly higher than that of healthy controls as PVY-positive (Table 3). When MAb2, the antibody specific to PVY^O, was used, a high reading of A_{405} was recorded for samples with symptoms, regardless of the symptom type (Table 3). None of the samples reacted with the PVY^N-specific MAb 1F5.

To ascertain if differences at the molecular level existed in PVY^N-like isolates, partial nucleotide sequence of the 3' and 5' ends of mosaic isolates (Mb-20 and Mb-23) and tobacco vein necrotic symptom causing isolates (Mb-112 and Mb-119) was determined (data not shown). The sequences of isolates (Mb-20 and Mb-23) exhibiting mosaic symptoms were identical

to that of PVY^O (30) in both ends of the genome. However, sequences of the isolates (Mb-112 and Mb-119) causing tobacco vein necrotic symptoms were similar to that of PVY^O in the 3' end, but different in the 5' end. In fact, they were similar to Eu-PVY^{N/NTN} sequences such as PVY^N-605 (GenBank accession number: X97895) in the 5' end (data not shown). The isolates appeared to possess similar genomic properties to the ones reported earlier from the same area (21) (data not shown).

Absence of tuber necrosis symptoms.

None of the PVY-infected samples collected from six potato cultivars (Chieftain, Norland, Russet Burbank, Russet Norkotah, Shepody, and Yukon Gold) had rings-

pot necrosis symptoms in the original field-grown tubers, or in the progenies grown in the greenhouse from the infected tubers (data not shown). Thus, these isolates that possess the coat protein region of PVY^O causing vein necrotic symptoms in tobacco similar to PVY^N, and that lack the ability to cause potato tuber ring spot necrosis symptoms, are different from PVY^{NTN}. Because of the unique genomic and pathological characteristics, and to differentiate these isolates from typical PVY^{N/NTN}, they have been referred to as PVY^{N:O} group of isolates.

Sensitivity of PVY^{N:O} detection in composite samples. In the composite tuber sap samples, detection of PVY^{N:O} was unaffected by I:H ratios up to 1:100 in the two cultivars (Norland and Yukon Gold) (data not shown) but it was limited to 1:50 in cv. Chieftain (Fig. 2A, lane 5). In the composites of sprout sap up to 1:100 (the highest tested) (Fig. 2A, lane 14) and in the potato leaf sap up to 1:1,000 (Fig. 2A, lane 21), PVY^{N:O} bands were observed. When extracted RNA from individual samples was used to prepare composites, the sensitivity of detection was extended to 1:1,200 to 1:1,500 in tubers and sprouts (Fig. 2B, lanes 1 to 7, 8 to 15) and over 1:2,000 in leaves (Fig. 2B, lanes 16 to 21). The amplified bands were more intense in the composites of sprouts in contrast to the composites of tuber sap or tuber RNA (Fig. 2A and B). RNA extracts prepared from leaves appeared to be concentrated and sometimes needed further dilution to reduce nonspecific amplifications.

Search for PVY^{N:O} strains in other potato growing areas. Most of the PVY^{N:O}-infected plants developed mosaic symptoms under greenhouse conditions similar to the field symptoms of PVY^O-infected plants. Therefore, it appeared feasible to select potato plants on the basis of leaf symptoms during the growing season and determine the distribution of strains. In 2001, leaf sap from 1,625 leaves with mosaic symptoms from fields in New Brunswick and Prince Edward Island, inoculated to tobacco, failed to develop vein necrotic symptoms. At the same time, 19 leaf samples with mosaic symptoms from a field known to have PVY^{N:O} infection in

Fig. 1. A, Nucleic acid extracts from dormant tubers infected with common strain of *Potato virus Y* (PVY^O), recombinant isolate of PVY (PVY^{N:O}), or doubly infected with PVY^O + PVY^{N:O} were analyzed by a multiplex (tetraplex) reverse transcription-polymerase chain reaction (RT-PCR) for their strain groups. PVY^O, lanes 1 to 3, 13; PVY^{N:O}, lanes 5 to 7; PVY^O + PVY^{N:O}, lanes 8 to 12; North American (NA)-PVY^{N/NTN}, lane 14; European (Eu)-PVY^{N/NTN}, lane 15; negative control, lane 4; and DNA molecular markers, lanes M. **B**, Nucleic acid extracts from potato tubers infected by PVY^O, PVY^{N:O}, or PVY^O + PVY^{N:O} analyzed by a duplex RT-PCR. Top bands indicate PVY^{N/NTN} (all PVY^N-like isolates, including PVY^{N:O}), bottom bands are PVY^O, and presence of two bands indicates double-infection by both strains. Lanes at both ends of gel are DNA size markers.

Table 3. Differentiation of *Potato virus Y* (PVY) strains in potato by a three antibody panel in enzyme-linked immunosorbent assay

Year	Symptoms in tobacco	NAPPO ^a -recommended monoclonal antibodies average $A_{405} \pm SD$			
		No. tested	4C3 ^b (PVY)	MAb2 ^c (PVY ^O)	1F5 ^d (PVY ^N)
2000	Mosaic	105	2.46 $\pm$ 0.50	2.83 $\pm$ 0.50	0.03 $\pm$ 0.02
2000	Necrosis	130	1.72 $\pm$ 0.71	2.74 $\pm$ 0.56	0.02 $\pm$ 0.04
2000	Healthy	50	0.05 $\pm$ 0.02	0.09 $\pm$ 0.05	0.02 $\pm$ 0.02
2001	Mosaic	10	1.48 $\pm$ 0.78	2.86 $\pm$ 0.13	0.03 $\pm$ 0.03
2001	Necrosis	54	1.90 $\pm$ 0.48	2.85 $\pm$ 0.08	0.03 $\pm$ 0.01
2001	Healthy	10	0.02 $\pm$ 0.01	0.01 $\pm$ 0.01	0.01 $\pm$ 0.01

^a North American Plant Protection Organization.

^b Monoclonal antibody 4C3 detects both the common (PVY^O) and the tobacco vein necrotic (PVY^N) strains.

^c Monoclonal antibody MAb2 detects PVY^O strain.

^d Monoclonal antibody 1F5 detects PVY^N strain.

Manitoba caused mosaic symptoms in seven tobacco plants indicative of PVY^O and veinal necrotic symptoms in 12 plants indicative of PVY^{N:O}. This showed that not all symptomatic potato plants are infected

with PVY^{N:O} and symptoms in potato may not provide conclusive identification of this strain.

To further establish the relationship of potato seed sources with the presence of

symptomatic potato plants and the determination of PVY^{N:O} isolates from such sources, 11 imported and five local potato seed sources were tested. Of the 11 imported seed lots, four from Minnesota, Montana, and North Dakota contained PVY^N-like positive tubers in the duplex RT-PCR assay (Table 4, data of lots with 0 positive not shown). Leaves from plants grown from the positive tubers in the greenhouse were tested by ELISA, tobacco bioassay, and RT-PCR, which identified them as PVY^{N:O} (Table 4, right column). Leaf surveys during the growing season of the fields planted with the same seed lots corroborated the presence of infected plants with PVY^{N:O} isolates in most cases (Table 4). However, observations of symptomatic plants alone in cultivars Gold Rush, Sangre, and Viking were difficult and confirmed that symptoms in potato would not be reliable for PVY strain detection. No PVY was detected in eight other seed lots from Alberta, British Columbia, Manitoba, North Dakota, and Saskatchewan (data not shown). Of the remaining domestic seed lots from Manitoba, PVY^{N:O} was detected in four (Table 4). Again, a low infection rate based on visual symptoms in potato plants in the field planted with the same seed sources was confirmed (Table 4).

DISCUSSION

In recent years, extensive surveys for PVY^N strains have been carried out in France (14), Czech Republic (26), Poland (5,40), and Spain (2). Similar work has also been performed in the United States (6). These surveys have reported an increased number of recombinant isolates consisting of PVY^O and PVY^N (5,14,40). The group of recombinant isolates (Wilga, L-56, and L-136) cause systemic mosaic and mottle in potato leaves but no ringspot necrosis in tubers (4,20,21). They are

A. Sap dilution

B. Nucleic acid dilution

Fig. 2. Analysis of recombinant isolate of *Potato virus Y* (PVY^{N:O}) by reverse transcription-polymerase chain reaction (RT-PCR) in composite samples of tubers, sprouts, and leaves, using sap or nucleic acid extracts. **A.** Sap from healthy and PVY^{N:O}-infected tubers were combined to provide infected:healthy (I:H) ratios of 1:10, 1:20, 1:30, 1:40, 1:50, 1:70, and 1:100 for both tubers and sprouts (lanes 1 to 7 and 8 to 14, respectively). For leaves the ratios were 1:50, 1:100, 1:200, 1:400, 1:600, 1:800, and 1:1,000 (lanes 15 to 21). **B.** Individual samples of nucleic acid extracts were mixed to provide I:H ratios of 1:10, 1:50, 1:100, 1:200, 1:400, 1:800, and 1:1,200 for tubers (lanes 1 to 7), and 1:10, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1,200, and 1:1,500 for sprouts (lanes 8 to 15). For leaves the ratios were 1:100, 1:500, 1:1,500, 1:2,000, 1:2,500, and 1:3,000 (lanes 16 to 21). Lanes at both ends of gel contain molecular markers.

Table 4. Determination of the recombinant isolate of *Potato virus Y* (PVY^{N:O}) from samples of imported and domestic potato seed sources that tested positive for PVY^N in the duplex reverse transcription-polymerase chain reaction (RT-PCR) assay

Potato seed source	Cultivar/sample	RT-PCR	ELISA ^a		Tobacco bioassay		Conclusion ^b
		PVY ^N c	PVY ^O c	PVY ^N	PVY ^O	PVY ^N	PVY ^{N:O} c
Minnesota	Gold Rush (tubers)	3	3	0	0	3	3
	Gold Rush (leaves)	3	3	0	0	3	3
Minnesota	Yukon Gold (tubers)	2	2	0	0	2	2
	Yukon Gold (leaves)	1	1	0	0	1	1
Montana	Sangre (tubers)	1	1	0	0	1	1
	Sangre (leaves)	0	0	0	0	0	0
North Dakota	Gold Rush (tubers)	2	2	0	0	2	2
	Gold Rush (leaves)	3	3	0	0	3	3
Manitoba-1	Shepody (tubers)	1	1	0	0	1	1
	Shepody (leaves)	2	2	0	0	2	2
Manitoba-3	Russet Burbank (tubers)	1	1	0	0	1	1
	Russet Burbank (leaves)	0	0	0	0	0	0
Manitoba-7	Nordonna (tubers)	1	1	0	0	1	1
	Nordonna (leaves)	1	1	0	0	1	1
Manitoba-15	Russet Burbank (tubers)	1	1	0	0	1	1
	Russet Burbank (leaves)	7	7	0	0	7	7

^a Enzyme-linked immunosorbent assay. Antibodies used in ELISA were those stated in Table 3.

^b Total number of PVY^{N:O} in conclusion was determined by analysis of combined data of ELISA and RT-PCR.

^c PVY^O is the common strain of PVY; PVY^N is the tobacco veinal necrotic strain of PVY; PVY^{N:O} is the recombinant isolate of PVY.

PVY^O-like both in their 3' end sequence and serology, but differ in the nucleotide sequences of the 5' end of the molecule. In some cases, evolution of such recombinant isolates has been noted under controlled conditions with double-infection of PVY^N and PVY^O during cross-protection studies (13). These new recombinant PVY isolates (PVY^{N:O}-like) often appear to have a high multiplication and spread rate (5,40) and may constitute 90% of the total PVY^N population (5,13). Our findings corroborate those in Europe. PVY^{N:O} isolates seem to predominate the PVY population in some fields (Table 2). There has been an increase in the proportion of recombinant isolates (as determined by serology, RT-PCR, and nucleotide sequences) in years 2000 and 2001 compared with 1996, when only two samples were detected as PVY^{N:O} from 300 PVY samples analyzed (20). In year 2001, tuber samples contained over 90% of PVY^{N:O} strain, in contrast to roughly equal or 64% in the year 2000 (Table 2). This dramatic increase of PVY^{N:O} is of concern because of its PVY^N-like phenotype in tobacco plants, and thus needs reappraisal of the PVY population in every potato growing area, particularly in view of the observation that the PVY^{NTN} strains could have evolved as a result of high infection pressure of the PVY^N isolates in one area (16). Every attempt to reduce the PVY infection pressure irrespective of strain-type needs to be made to limit the formation of recombinants.

In North America and in other countries, the certification of seed tubers is subjected to virus testing using multiple antibodies. This includes a first detection of PVY by ELISA using broad-spectrum antibodies and a second test detecting PVY^N using PVY^N-specific monoclonal antibodies (14; see the NAPPO website). As shown in the present study, the recombinant PVY^{N:O} isolates under this system would be identified as PVY^O by both types of antibodies and thus may not be completely removed from a seed lot. The tolerance level of virus in various classes of seeds varies from country to country or from state to state in one country (38). Therefore, such escaped infected tubers would continue to serve as sources of virus inoculum for the aphids for the next generation. In addition, if such seed lots are exported to other areas, the recombinants would be introduced to a new region.

A limited survey of the recombinant (PVY^{N:O}) strain in this study from imported and domestic seed sources in Manitoba showed that the strain is not limited to only Manitoba. Seed tubers introduced from sources in the United States also contained PVY^{N:O} (Table 4). Therefore, distribution of this strain is much wider than we initially thought, and attempts to ensure that seed sources are free of this strain are urgently needed.

To facilitate routine detection of PVY^{N:O} and at the same time avoid the use of multiple testing with various antibodies to determine the strain types, a multiplex (duplex) RT-PCR protocol was evaluated in this study. To minimize the cost of large-scale testing of seed lots, use of the high sensitivity of RT-PCR has been made through the compositing of samples (Fig. 2). Use of dormant tubers, sprouts, or leaves can be composited, from which an indication of the presence of the PVY^{N:O}-like isolates could be ascertained. Depending on the situation and the class of seed tuber, an actual percentage of individual strains present can be determined by performing tests on the individual extracts.

The recombinant isolates found in Europe have been referred to as PVY^N Wi or Wilga-type (4,13) (named after the cultivar Wilga, in which they were first detected). These isolates are known to cause a lethal necrotic symptom in *S. brachycarpum* identical to PVY^{NTN} (7). However, the PVY^{N:O} isolate found in this study did not cause the lethal necrotic symptoms in *S. brachycarpum*; instead they caused symptoms more like PVY^O (29). Thus, there is a possibility that these isolates are different from those found in Europe, and it is possible that many types of recombinants may exist with varying pathogenicity and recombination sites (10).

In conclusion, a recombinant isolate of PVY (PVY^{N:O}) was identified in Manitoba and several of the neighboring U.S. states by using strain-specific RT-PCR, ELISA, and bioassays. This isolate shares serological properties of PVY^O but causes PVY^{NTN} type of symptoms in tobacco plants, and therefore could escape through the serological indexing in the seed certification program. The competitive-duplex RT-PCR was able to simultaneously differentiate between PVY^O and the PVY^{NTN} strains including PVY^{N:O}, and therefore could be used in large-scale screening and management of PVY^{NTN} in certification programs. With this in mind, further research to monitor the distribution and incidence of PVY^{N:O} and to further evaluate multiplex RT-PCR for large-scale testing of seed lots is warranted.

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