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ORIGINAL ARTICLE

NE-11 represents a new strain variant class of *Potato virus Y*

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Abstract This report describes the characterization by whole-genome sequencing of four PVY isolates with unique combinations of molecular and symptomatic characteristics. Three of these four isolates were of type $PVY^{N:O}$ (ID-1, OR-1, PN10A), including one of "type B", which contains an extra recombination event in the 5′UTR/P1 cistron; the other (NE-11) represents a novel PVY molecular genotype, previously misclassified as a PVY^{NA-NTN} isolate. The full genome sequence of this latter isolate is unique inasmuch as it is nearly identical to that of PVY^{N} isolates for the first 2,000 nucleotides (nts), after which it very strongly resembles PVY^{NA-NTN} isolates for the next 600 nts. For the final 7,000 nts of its genome, NE-11 shares intermediate identity with these other two previously reported classes of PVY^{N} genomes, except for a portion of the capsid protein region in which it resembles neither. Recombination in each of the four isolates was verified by a suite of recombination detection programs. PN10A represents the first complete sequence of a PVY strain variant of the class reported as PVY^{N}-W (or $PVY^{N:O}$) type B. Specific PCR assays for two unique regions of NE-11 are presented that will allow the identification of this strain variant by other researchers.

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Introduction

Among the viruses that affect solanaceous crops, potato virus Y (PVY) and potato leafroll virus (PLRV) are the most widespread and economically destructive (De Bokx and Huttinga 1981). Potato virus Y (PVY) is the type member of the genus *Potyvirus* and infects several important solanaceous crops, including potato, tomato, pepper, and tobacco (De Bokx and Huttinga 1981; Shukla et al. 1994). Although isolates of PLRV have relatively little phenotypic and genotypic diversity (Guyader and Ducray 2002; Taliansky et al. 2003), PVY isolates are considerably more diverse, with several of the reported genetic strains both being symptomatically distinguishable and having distinct economic impacts (De Bokx and Huttinga 1981).

Widely recognized strains of PVY include the common strain (PVY^{O}), the tobacco necrosis strain (PVY^{N}), and the PVY^{C} strain, which causes stipple streak (De Bokx and Huttinga 1981). Although PVY^{O} has been the predominant strain affecting seed potatoes in North America (Draper et al. 2002), other PVY strain variants, such as PVY^{N} and PVY^{NTN}, have also begun appearing in North America (McDonald and Kristjansson 1993; Nie and Singh 2002; Piche et al. 2004). The origins of PVY^{N}, which causes veinal necrosis on the leaves of infected tobacco plants (De Bokx and Huttinga 1981; Robaglia et al. 1989), are difficult to pinpoint, although it may have been detected in South America over 70 years ago (Smith and Dennis 1940; Silberschmidt 1960). PVY^{NTN}, causes potato tuber necrotic

ringspot disease (PTNRD, Bezner et al. 1984; LeRomancer et al. 1994). Another set of strain variants causes tobacco veinal necrosis but has a PVY^{O} serotype (PVY^{N}-Wi; Chrzanowska et al. 1991; $PVY^{N:O}$ Nie and Singh 2002). The PVY^{N}-Wi and $PVY^{N:O}$ variants may, however, represent the same subset of strain variants (Lorenzen et al. 2006a). Another genotype, NA-$PVY^{N/NTN}$, with variants causing both PVY^{N}- and PVY^{NTN}-like symptoms was first discovered in North America but has now also been found elsewhere in the world (Lorenzen et al. 2006a).

Whereas the genome sequence of NA-$PVY^{N/NTN}$ isolates have been reported to contain no obvious evidence of recombination (Nie and Singh 2003a), both PVY^{NTN} and PVY^{N}-Wi/$PVY^{N:O}$ isolates bear indications of recombination between PVY^{O} and PVY^{N} isolates. Although PVY^{NTN} isolates have three clearly identifiable recombination junctions (Glais et al. 2002), both PVY^{N}-Wi and $PVY^{N:O}$ genotypes have a single recombination junction (Glais et al. 2002; Nie and Singh 2003). Some PVY^{N}-Wi and $PVY^{N:O}$ isolates can cause atypical tuber necroses that are somewhat milder than that of classical PVY^{NTN} (Piche et al. 2004; Lorenzen et al. 2006a).

Potato virus Y has been a major recent concern for potato seed producers in North America. We recently confirmed the presence of "European" types of PVY^{N} and PVY^{NTN} strains in North America by whole-genome sequencing (Lorenzen et al. 2006a). One previously reported isolate, NE-11, (Piche et al. 2004) had some anomalies that were not completely consistent with a diagnosis of NA-PVY^{NTN} (Lorenzen et al. 2006b). In this report, we document that NE-11 represents a new, previously undescribed molecular genotype of PVY^{NTN}. The genome of this PVY^{N} variant contains at least three recombination junctions, has a base sequence equidistant between typical PVY^{N} and NA-$PVY^{N/NTN}$ sequences, and has a tract of sequence within the CP cistron that has apparently been obtained by recombination from a divergent, currently uncharacterized PVY genotype. We also report the first full-length sequence for the strain variant termed PVY^{N}-W type B (Glais et al. 2002).

Materials and methods

Virus isolates

The previously described virus isolates, NE-11, OR-1, and ID-1 (Piche et al. 2004) were maintained in lyophilized plant tissue at −20°C. Isolate PN10A was isolated from a tuber originating in a seed test of lots originating from Idaho potato seed growers (Nolte and Lorenzen, unpublished). Control isolate PVY^{O}139 was generously provided by R. Singh and represents a typical 'ordinary' PVY isolate.

RT-PCR and sequencing

Virus extraction, RT-PCR, and sequencing were performed as recently described by Lorenzen et al. (2006a).

Phylogenetic and recombination analysis

ClustalX (Thompson et al. 1997) with default settings was used to align 34 full PVY genome sequences, including the four determined in this study. ClustalX was also used for construction of neighbor-joining phylogenetic trees (1,000 bootstrap iterations, Jukes Cantor distance model). Alignments were checked by eye and recombination detection and analysis were conducted using the RDP (Martin and Rybicki 2000), GENECONV (Padidam et al. 1999), BOOTSCAN (Martin et al. 2005a), MAXCHI (Maynard Smith 1992), CHIMAERA (Posada and Crandall 2001), 3SEQ (Boni et al. 2007) and SISCAN (Gibbs et al. 2000) methods implemented in RDP3 (Martin et al. 2005b). Default RDP3 settings were used throughout except that only recombination events independently identified by three or more methods were taken into consideration. Phylogenetic trees were constructed using the maximum-likelihood method implemented in PHYML (Guindon and Gascuel 2003) using the HKY + gamma model with four rate categories, a gamma shape parameter of two and transition:transversion ratio inferred from the data.

PCR assays

RNA extraction, cDNA synthesis, and multiplex PCR assays were conducted to characterize strain type (PVY^{N}, PVY^{N}-W, PVY^{NTN}, PVY^{O}, NA-$PVY^{N/NTN}$) and test for strain mixtures as has been recently described by Lorenzen et al. (2006b). A specific PCR assay for the O:N recombination junction at genome position ~500 nucleotides (nts) was also conducted according to Lorenzen et al. (2006b). PCR assays were designed to specifically amplify products from the two unique portions of NE-11. Polymerase chain reactions were performed in a 20-µl reaction volume that contained 0.8 µl cDNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.8 mM $MgCl_2$, 0.2 mM (each) dNTP, 0.12 µM each primer (Table 1), and 1.0 unit AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). The "touch-down" PCR program was 94°C for 2 min, 12 cycles of 94°C for 10 s, 66°C for 30 s (−0.5°C per cycle), and 60 s at 72°C, followed by 20 cycles of 94°C for 10 s, 60°C for 30 s, and 72°C for 60 s, ending with a final extension for 7 min at 72°C. The primers for NE-11 were tested both on their own and as part of a four-primer multiplex designed to identify the PVY^{N}/PVY^{NA-N}

Table 1 Specific assays to detect NE-11 at two diagnostic segments of the genome

Assay	Primer name[a]	Primer sequence
NE-11 RJ1	N_1979F	TCGTAATAGGTAATAGTGGCGAC
	NA_2627R	CACAACTTCACTGCAAGC
NE-11 CP	NE_8049	GCGTTCTTTGTTAATGGCGAC
	NE_9026R	GCATTTTCAACAATCGGCTTGA

F indicates forward primer, *R* indicates reverse primer

[a] The number indicates position in genome

recombination breakpoint at genome position 2000 and the unique NE-11 CP region (Table 1). The PCR cycling program was identical to that described above except that the initial and final annealing temperatures were 2°C cooler (64, 58°C, respectively). PCR products were separated on a 1% agarose electrophoretic gel with GelStar (Cambrex, Rockland, ME) prior to fluorescence-based visualization.

Biological assays

Four PVY isolates, PVY^{O}139, NE-11, OR-1 and ID-1, were inoculated into ten potato cultivars. Uninoculated plants served as negative controls. Potato cultivars included Red LaSoda, Red Norland, Ranger Russet, Shepody, Alturas, Atlantic, Yukon Gold, Russet Norkotah, Umatilla Russet and Russet Burbank. Potato plants were approximately 15–20 cm tall at the time of inoculation, with eight to ten full-expanded leaves. Plants were inoculated as previously described (Piche et al. 2004). Each PVY isolate × cultivar treatment combination was replicated three times, and there were three plants per replication. Plants were monitored for foliar and tuber symptom development as previously described in Draper et al. (2002). Symptom development characteristic of PVY (chlorotic mosaic, veinal necrosis and leaf necrosis) was scored from 7 days post-inoculation, and tubers were rated for PTNRD symptoms following 4 weeks of storage at room temperature, between 70 and 90 days post-inoculation. The experiment was performed twice.

Results

Genomic characterization of the four new PVY isolates

We determined the nearly complete genomic sequences of four PVY isolates. The isolates were selected based on a combination of their novel serological and biological characteristics. Whereas it was suspected that isolates ID-1, OR-1and NE-11 might represent novel genotypic variants (Piche et al. 2004), isolate PN10A was interesting because of its unique foliar symptoms. A multiplex PCR assay designed to detect strain mixtures (Lorenzen et al. 2006b) detected no evidence of such in the four isolates. The strain types detected in the multiplex assay were "NA-PVY^{N}-like" (NE-11), with a single band of 328 bp, and $PVY^{N:O}$-like (ID-1, OR-1, PN10A) with two bands of 117 and 689 bp. A subsequent PCR assay yielded a 514-bp amplicon for isolate PN10A that was diagnostic of a previously characterized PVY^{O}/PVY^{N} recombination event in the P1 region (Lorenzen et al. 2006b).

The lengths of the four sequenced genomes (excluding poly-A tails) and their corresponding GenBank accession numbers are: NE-11: 9,701 nts (DQ157180), ID-1: 9,670 nts (DQ157178), OR-1: 9,670 nts (DQ157179), PN10A: 9,666 nts (DQ008213). The last three isolates were sequenced from PCR products that excluded the 5′ 34 nts of the PVY genome All four isolates showed a high degree of similarity with other publicly available sequences and clustered closely in maximum-likelihood (Fig. 1a) and neighbor-joining phylogenetic trees (data not shown) with other previously sequenced PVY isolates. Isolate NE-11 was 97.2% identical to a Swiss PVY^{N} isolate, N605 (gb|X97895, Jakab et al. 1997), 97.0% identical to Mont (gb|AY884983, Lorenzen et al. 2006a), and 96.5% identical to SCRI-N (gb|AJ585197); the next most similar sequences were the $PVY^{NA\text{-}NTN/N}$ isolates N-Jg, SASA-61, Tu660, and RRA-1 (96.0%; AY166867, AJ585197, AY166866, and AY884984). Amino acid identities with these publicly available sequences were higher: N605-99.3%, Mont-99.2%, SCRI-N-98.9%, RRA-1-98.2%. Isolates ID-1 and OR-1 were 99.8% identical to one another and respectively 99.7 and 99.8% identical to the $PVY^{N:O}$ isolate Alt (gb|AY884985), 99.6% identical to isolates L-56 and Mb112 (gb|AY745492, AY745491), 99.5% identical to SASA-207 (gb|AY745492), and 97.3% identical to PN10A (gb|DQ008213, this paper). The amino acid identities among these $PVY^{N:O}$ isolates ranged from 99.8 to 99.9%, except for PN10A (98.6%). Isolate PN10A (gb|DQ008213) was 97.4% identical to the $PVY^{N:O}$ isolates L-56 and SASA-207 (gb|AY745492, AJ584851) and 97.3% identical to the $PVY^{N:O}$ isolates Mb112 and Alt (gb|AY745491, AY884985). The amino acid identities of PN10A to $PVY^{N:O}$ isolates ID-1, OR-1, Alt, L56, Mb112, and SASA-207 ranged from 98.6 to 98.4%. Amino acid identities of PN10A to PVY^{NTN} isolates were 96% or less.

Characterization of recombination events detectable in the four new sequences

Recombination has been previously identified as a feature of PVY evolution, and we therefore examined all publicly

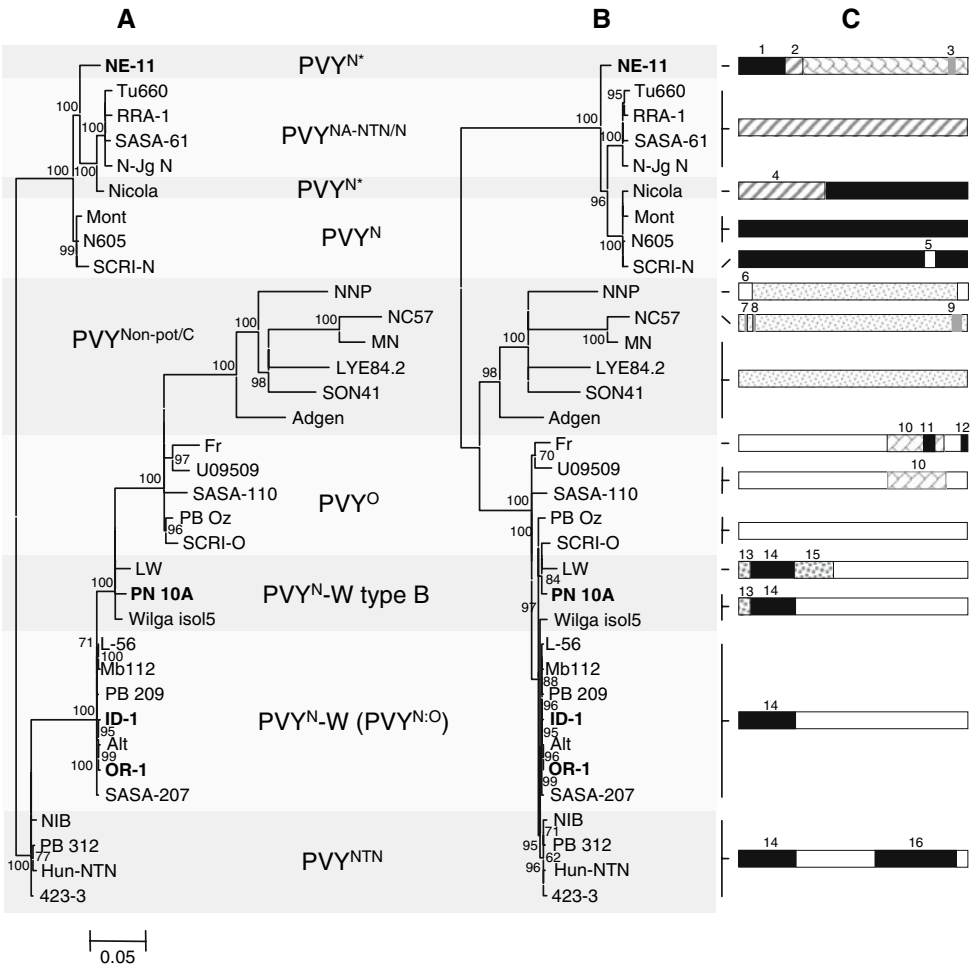

Fig. 1 Phylogenetic relationships among all publicly available full-length PVYgenomes. Sequences determined in this study are listed in bold type. Maximum-likelihood reconstruction of the PVY phylogenies, either (**a**) ignoring recombination or (**b**) taking recombination into account. **c** To-scale genome cartoons depicting recombinant mosaics detect amongst the analysed PVY genome sequences. The color coding is as follows: *yellow* portions of the NE-11 genome that are approximately equidistantly related to $PVY^{NA\text{-}NTN/N}$ and PVY^{N}-like sequences; *dark green* $PVY^{NA\text{-}NTN/N}$-like sequences; *light green* PVY^{N}-like sequences; *orange* PVY^{C}- like sequences; *dark blue* PVY^{O}-like sequences. *Light blue* more divergent PVY^{O}-like sequences; *blue* with stippling denotes detectable recombination events between PVY^{O}-like sequences. *Grey* sequences of unknown origin possibly derived from viruses outside the PVY group. PVY^{N*} indicates recombinants between $PVY^{NA\text{-}NTN/N}$ and PVY^{N}. Construction of the phylogenetic tree taking recombination into account involved removal of the numbered regions in the sequences depicted

available full-length PVY genomes together with our four new sequences for evidence of recombination. We identified 16 obvious recombination signals and, to aid in our identification of parental and recombinant sequences, we constructed a "base PVY phylogeny" that was not as severely affected by recombination as that presented in Fig. 1a. We removed the identified recombination tracts from the multiple sequence alignment (i.e. we treated the recombinant tracts as missing data) and reconstructed the phylogeny (Fig. 1b). As expected, the tree length of the "recombination-free" phylogeny (it is, in fact, only free of the most obvious recombination events) is substantially shorter than that of the phylogeny ignoring recombination (Schierup and Hein 2000)—a good indication that we had indeed removed a substantial proportion of the recombination signals in the alignment.

Our recombination analysis indicated that all four of the PVY sequences determined in this study bear traces of one or more past recombination events (Fig. 1c). The ID-1, OR-1, and PN10A isolates were all of the PVY^{N}-W/$PVY^{N:O}$ type, with a single recombination breakpoint somewhere between nucleotide positions 2396 (2393 for PN10A) and 2412 (event 14 in Fig. 1c; position numbering based on the sequence of Alt [gb|AY884985]). The analysis also revealed that PN10A had an additional recombination breakpoint at approximately nucleotide position 500 (event 13 in Fig. 1c). This event was clearly identified by the RDP ($P = 9.2 \times 10^{-198}$), BOOTSCAN

($P = 1.67 \times 10^{-167}$), GENECONV ($P = 4.4$ E^{-169}), SISCAN ($P = 8.1 \times 10^{-29}$), MAXCHI ($P = 4.2 \times 10^{-45}$), CHIMAERA ($P = 1.6 \times 10^{-45}$), and 3SEQ ($P = 5.98 \times 10^{-297}$) recombination detection methods. Other isolates in GenBank with a nearly identical recombination signal within the P1 cistron included Wilg156var (AJ889868), Wilg156 (AJ889867), Wilga (AJ890350), N-Wi-P (AF248500), Gr99 (AJ890343), 34/01 (AJ890342), AJ889866, LW (AY890349), Pic3 (AY700018), and Vic20 (AY700020).

As mentioned above, isolate NE-11 had been characterized as an NA-PVY$^{N/NTN}$ isolate (Piche et al. 2004). However, recombination analysis indicated three likely recombination events in the NE-11 genome. The two events on the 5′ end of the genome (events 1 and 2 in Fig. 1c) represent introgressions of PVY^{N}- and PVY$^{NA\text{-}N/NTN}$-type sequences. For the first 2,000 nts, NE-11 is nearly identical to the PVY^{N} isolates N-605 and Mont, whereas for the next 680 nucleotides (positions ~2,015 to ~2,696), it closely resembles the NA-PVY^{N} isolates N605 and Tu660 (Fig. 1 c). These recombination events were detected by the RDP ($P = 2.9 \times 10^{-17}$), GENECONV ($P \leq 8.4 \times 10^{-15}$), BOOTSCAN ($P = 8.4 \times 10^{-15}$), MAXCHI ($P \leq 4.3 \times 10^{-11}$), CHIMAERA ($P \leq 3.2 \times 10^{-12}$), and 3SEQ ($P \leq 1.0 \times 10^{-16}$) recombination detection methods. Interestingly, from position 2700 to the CP cistron the three isolates, N605, Tu660, NE-11 are all remarkably similar (>98% identical). Another probable recombination event is detectable in the NE-11 CP/3′UTR with the tract of sequence from genome positions ~8,853 to ~9,620, inferred to have been derived from a divergent, currently uncharacterized PVY genotype (event 3 in Fig. 1c; 5′ and 3′ breakpoint positions could not be accurately identified because a parental donor sequence was absent from the alignment). Despite the absence of a good candidate parental sequence, this event was still clearly detectable by the RDP (5.1×10^{-5}), BOOTSCAN (8.8×10^{-3}), and SISCAN (2.6×10^{-9}), recombination detection methods. While the origin of the recombinant tract is currently unknown, certain Chinese [XCH47 (AY841267), XCH24 (AY841258), Qingzhou (AY742716), Mudanjiang-8-3 (AY742731), Fengyang-8-2 (AY742729), Mudanjiang-8-2 (AY742727), Fengyang-10 (AY742720), Fengyang-6 (AY742718), Shenyang (AY742715), Fengyang-8-1 (AY742719), and Mudanjiang-13-5 (AY742732); Na, unpublished] and Brazilian (N-curl, DQ530509, Sawazaki et al. unpublished) PVY CP sequences share > 98% identity with that determined for NE-11.

The genomic region in which the most PVY recombination events have been reported is that encoding the CP and 3′UTR region. Besides the recombination breakpoints detected in the CP cistron of NE-11, we also detected other previously unreported breakpoints in this region. Analysis of recently submitted GenBank sequences identified recombination breakpoints in isolates XCH30 and XCH44 (Na unpublished) at position 8823, and in isolate Beijing (Na unpublished) at position 8997.

Another noteworthy recombination event was detected in the genomic sequence of isolate Nicola (event 4 in Fig. 1c). Classified by Schubert et al. (2007) as being a PVY$^{NA\text{-}NTN}$ isolate, Nicola actually only resembles PVY$^{NA\text{-}NTN}$ sequences for the 5′ 3,600 nucleotides of its genome. From that point onwards it very closely resembles the sequences of PVY^{N} isolates. This event, with sequences Tu660 (PVY$^{NA\text{-}NTN}$) and N605 (PVY^{N}) identified as possible parents, was detected by the RDP ($P = 9.1 \times 10^{-19}$), GENECONV ($P = 4.7 \times 10^{-19}$), BOOTSCAN ($P = 2.3 \times 10^{-20}$), MAXCHI ($P = 1.8 \times 10^{-16}$), CHIMAERA ($P = 1.9 \times 10^{-17}$), SISCAN ($P = 3.8 \times 10^{-8}$), and 3SEQ ($P = 5.2 \times 10^{-47}$) methods.

Development of a PCR assay for NE-11-like sequences

The previously published PCR assay (Lorenzen et al. 2006b) for the PVY^{N}W-B recombination breakpoint at genome position 500 (Glais et al. 2002) successfully amplified a 514-bp fragment from PN10A but gave no products from the other isolates reported here. Similarly, this assay produced the 514-bp amplicon for 13 isolates in which P1 cistron sequencing had identified the PVY^{N}W-B breakpoint. The amplicon was not, however, produced for any isolates that lacked the PVY^{N}W-B breakpoint. The multiplex assay for NE-11 produced amplicons of 649 and 978 bp for isolate NE-11, but did not produce amplicons for Mont, T2 (PVY^{N}), RRA-1 (PVY$^{NA\text{-}N}$) or the other three isolates characterized here. These assays have also been validated using cDNA from two other isolates provided by a cooperating researcher (Lorenzen and Crosslin, unpublished). Sequencing of the PCR amplicons confirmed their high degree of similarity to NE-11.

Biological characterization of the four new isolates

Symptom development in foliage and tubers caused by isolates NE-11, OR-1 and ID-1 were compared to "an ordinary" isolate, PVY^{O}139. There were statistical differences among PVY isolates in the severity of symptom development among cultivars ($P \leq 0.01$; Table 2). Typical symptoms of mild mosaic in potato foliage, characteristic of PVY^{O}, were evident with all of the PVY isolates studied. The severity of mosaic symptoms caused by OR-1 was significantly less than those caused by the other isolates examined. There were also significant differences among cultivars ($P \leq 0.0001$) in the severity of foliar PVY

Table 2 Severity of "mosaic" symptoms among several *Potato virus Y* isolates and potato cultivars

Cultivar	PVY Symptom development[a]											
	PVY Strain											
	PVY^{o}139[b]		NE-11		OR-1		ID-1		Mean			
Alturas	2.0	a	0.3	d	0.0	b	0.3	c, d	0.4	e		
	A		A		A		A					
Altantic	1.0	a	2.0	b	1.0	a, b	2.3	a	1.6	b		
	B		AB		B		A					
Ranger Russet	1.7	a	0.0	e	0.7	a, b	0.0	d	0.6	d, e		
	A		B		AB		B					
Red LaSoda	1.3	a	1.0	c	0.0	b	1.7	a, b	1.0	c, d		
	A		A		A		A					
Red Norland	1.0	a	0.0	e	2.0	a	1.7	a, b	1.3	b, c		
	AB		B		A		A					
Russet Burbank	0.3	a	1.0	c	0.0	b	0.0	d	0.3	e		
	B		A		B		B					
Russet Norkotah	2.0	a	2.0	b	1.0	a, b	1.0	b, c	1.5	b, c		
	A		A		A		A					
Shepody	0.0	a	1.0	c	1.0	a, b	2.3	a	1.1	b, c, d		
	C		B		B		A					
Umatilla Russet	0.7	a	1.0	c	0.0	b	1.0	b, c	0.7	d, e		
	A		A		B		A					
Yukon Gold	2.0	a	3.0	a	1.7	a	2.3	a	2.3	a		
	A		A		A		A					
Mean	1.1		1.2		0.7		1.3					
	A		A		B		A					

Mean separation based upon Fisher's protected least significant difference (LSD) test ($\alpha = 0.05$). Means within columns followed by the same lower case letter and means within a row with the same upper case letter below the number are not significantly different

A significant cultivar by PVY strain interaction was observed ($\alpha < 0.0001$), as well as among the main effects of cultivar ($\alpha < 0.0001$) and PVY strain ($\alpha = 0.0115$)

[a] Foliar virus symptoms for each isolate compared to non-inoculated control plants of same cultivar based on a rating scale of: 1 = mild mosaic symptoms characteristic of PVY; 2 = moderate mosaic with pronounced interveinal chlorosis; 3 = mosaic symptoms, severe crinkling of leaf tissue and some interveinal necrosis evident

[b] PVY^{o}139 is an "ordinary" strain of PVY causing common mosaic in potato, NE-11 is a new molecular type of PVY^{ntn} (this paper) and OR-1 and ID-1 are isolates of PVY$^{n:o}$

symptoms that developed. Mosaic symptoms caused by all isolates were significantly more severe in cv. Yukon Gold compared to the other cultivars tested (Table 2). Cultivars Russet Burbank and Alturas displayed significantly less severe mosaic symptoms than did nearly all of the other cultivars studied. Interestingly, there were PVY isolate-potato cultivar combinations that resulted in some very severe reactions, as evidenced by a significant PVY strain X–cultivar interaction ($P \leq 0.0001$). For example, PVY isolate ID-1 caused fairly severe mosaic symptoms on cv. Shepody, whereas in this cultivar PVY^{O}139 caused no symptoms (Table 2). In contrast, Ranger Russet is very susceptible to ordinary strains of PVY, as demonstrated in this study (Table 2), but did not develop typical mosaic symptoms when inoculated with any of the other PVY isolates.

Significant differences also existed among PVY isolates in their ability to cause PTNRD ($P \leq 0.001$), although there was also a significant isolate-X–cultivar interaction ($P \leq 0.001$). Necrosis was never observed in tubers obtained from the non-inoculated plants of any tested cultivar. Symptoms were also never observed in any potato plants inoculated with PVY^{O}139 (the ordinary strain; Table 3). PVY$^{N:O}$ isolate OR-1 caused significantly more PTNRD than the other isolates studied. Differences also existed among cultivars with regard to the frequency of PTNRD development. Tuber necrosis was only observed in cvs. Atlantic, Alturas, Red Lasoda and Yukon Gold, with the latter cultivar being the most susceptible to PTNRD development among the PVY isolates tested (Table 3). The other cultivars did not develop any tuber necrosis symptoms following PVY infection.

Discussion

In this paper, we report that a PVY^{N} isolate that produces PVY^{NTN}-like symptoms (Piche et al. 2004) represents a new molecular genotype of PVY that is intermediate between previously reported PVY^{N} and PVY$^{NA\text{-}N}$ isolates. Initial attempts to classify NE-11 using molecular assays yielded ambiguous results. Whereas the multiplex assay of Nie and Singh (2002) identified it as a PVY$^{NA\text{-}N/NTN}$ strain variant (Piche et al. 2004), a second PCR assay using PVY$^{NA\text{-}N/NTN}$-specific primers (Lorenzen et al. 2006b) indicated the isolate was not a PVY$^{NA\text{-}N/NTN}$ strain variant (data not shown). The full genome sequence of NE-11 indicates that it is a novel intermediate to the PVY$^{NA\text{-}N}$ and PVY^{N} strain variants. While clearly recombinant, it is also identical to the PVY^{N} isolates N605 and Mont in the genome region queried by the Nie and Singh (2002) assay, indicating that a PCR artifact may have resulted in its initial misclassification by Piche et al. (2004).

The evidence supporting the occurrence of two recombination events within the 5′ 2,700 nucleotides of the NE-11 genome is overwhelming. It is unclear in which order the events occurred, but it seems likely that the first event has been partially overprinted by the second. Whereas the first 2,000 nucleotides of the NE-11 are PVY^{N}-like, the next 696 nucleotides are PVY$^{NA\text{-}N/NTN}$-like, with the remainder of the genome being intermediate between PVY^{N} and PVY$^{NA\text{-}N/NTN}$. The relatively equidistant

Table 3 Response of potato cultivars to tested isolates of *Potato virus Y* with regard to potato tuber necrotic ringspot disease (PTNRD) symptoms

Cultivar	PTNRD Symptom development[a]											
	PVY Strain											
	PVY^{o}139[b]		NE-11		OR-1		ID-1		Healthy		Mean	
Alturas	–[c]		0.0	b	33.3	b	0.0	b	0.0	a	8.3	b, c
			A		A		A		A			
Altantic	0.0	a	0.0	b	83.3	a	0.0	b	0.0	a	12.8	b
	B		B		A		B		B			
Ranger Russet	–		0.0	b	0.0	b	0.0	b	0.0	a	0.0	c
			A		A		A		A			
Red LaSoda	0.0	a	33.3	a	0.0	b	–		0.0	a	11.1	b
	A		A		A				A			
Red Norland	0.0	a	0.0	b	–		0.0	b	0.0	a	0.0	c
	A		A				A		A			
Russet Burbank	0.0	a	0.0	b	0.0	b	0.0	b	0.0	a	0.0	c
	A		A		A		A		A			
Russet Norkotah	0.0	a	0.0	b	0.0	b	0.0	b	0.0	a	0.0	c
	A		A		A		A		A			
Shepody	0.0	a	0.0	b	0.0	b	0.0	b	0.0	a	0.0	c
	A		A		A		A		A			
Umatilla Russet	0.0	a	0.0	b	0.0	b	0.0	b	0.0	a	0.0	c
	A		A		A		A		A			
Yukon Gold	0.0	a	–		100.0	a	33.3	a	0.0	a	38.9	a
	C				A		B		C			
Mean	0.0		3.8		20.3		1.7		0.0			
	B		B		A		B		B			

Mean separation based upon Fisher's protected least significant difference (LSD) test ($\alpha = 0.05$). Means within columns followed by the same lower case letter and means within a row with the same upper case letter below the number are not significantly different

A significant cultivar by PVY strain interaction was observed ($\alpha < 0.0001$), as well as among the main effects of cultivar ($\alpha < 0.0001$) and PVY strain ($\alpha < 0.0001$)

[a] Percentage of tubers harvested displaying typical symptoms of potato tuber necrosis ringspot disease

[b] PVY^{o}139 is an "ordinary" strain of PVY causing common mosaic in potato, NE-11 is a new molecular type of PVY^{ntn} (this paper) and OR-1 and ID-1 are isolates of PVY$^{n:o}$

[c] No tubers were produced by these cultivars, frequently due to the debilitating nature of the host:virus interaction

genetic relationship of NE-11, PVY^{N} and PVY$^{NA\text{-}N/NTN}$ groups from 2,700 nts to the CP cistron suggests that significant time has passed since the divergence of these or as-yet-unidentified parental lines.

In fact, there is evidence of another recombination event in this region of the NE-11 genome. The event begins just 5′ of and ends near the middle of the CP cistron and also apparently involves a parental sequence that is currently unsampled, although in this case the parent appears to be an even more divergent PVY genotype. A BLAST search of GenBank (http://www.ncbi.nlm.nih.gov/) using an 800-bp NE-11 CP sequence beginning at genome coordinate 8,500 identified several PVY sequences that were nearly identical (99.7%) to NE-11 in the queried region (XCH47, XCH24, Qingzhou; Na unpublished. Eight additional sequences reported by the same authors were >98.5% identical to this portion of NE-11 and showed the same recombination breakpoints, whereas the PVY^{N} and PVY$^{NA\text{-}N/NTN}$ CP sequences in the public sequence database that most resembled the NE-11 query sequence were 94.2 and 93.2% identical to it, respectively. Similarly, a CP sequence from Brazil deposited in 2006 by Sawazaki et al. (unpublished) was 98.0% identical to this portion of NE-11. Therefore, although previously unrecognized, PVY variants with similar CP recombination mosaics to NE-11 already exist in Asia and South America. These CP recombination junctions have not been noted in previous reports of recombination events in the CP region (Revers

et al. 1996; Boonham et al. 2002). The lack of any close relatives of the donor parent in public sequence databases indicates a need for more extensive sampling of members of the potyvirus family.

One of the reasons full-length sequencing was initiated for isolates ID-1 and OR-1 was to identify candidate nucleotide polymorphisms that might be associated with atypical tuber necrosis phenotypes (Piche et al. 2004). Although these two isolates have very similar sequences and are genetically clearly $PVY^{N:O}$ isolates (Fig. 1; Lorenzen et al. 2006a; Nie et al. 2004), they differ significantly in their interactions with host plants. Isolate ID-1 produces significantly more severe foliar symptoms than OR-1 in all ten potato cultivars tested. Isolate NE-11 produced leaf mosaic symptoms intermediate between ID-1 and OR-1 and was indistinguishable from the control PVY^{O} isolate, PVY^{O}139. Conversely, OR-1 caused significantly more severe necrotic ring spot disease in tubers than either ID-1 or NE-11. Although these isolates are phenotypically distinct, nothing in our genetic analysis explains these differences in pathogenicity. Although no pathogenicity-associated nucleotide polymorphisms could be identified, other $PVY^{N:O}$ isolates are being biologically characterized and sequenced to create a broader database that will be more suitable for identifying specific nucleotide sequences responsible for PTNRD.

The current data are also insufficient to speculate as to the role that recombination has had in modifying the infectivity and pathogenicity of the viruses studied. Although PVY^{N} isolates within the N605 genetic clade do not normally cause PTNRD, a portion of PVY^{N} isolates that lacked the PVY^{NTN} recombination event in the CP region also caused PTNRD (Boonham et al. 2002). As has been previously observed (Piche et al. 2004), $PVY^{N:O}$ isolate OR-1 produces atypical PTNRD. However, in contrast to that report, $PVY^{N:O}$ isolate ID-1 also caused similar PTNRD, albeit at a much lower incidence than OR-1. Although not analyzed for symptom severity here, it is interesting to note that isolates of the PN10A variant type have been selected for more detailed analyses in other studies because of the severe or unusual foliar symptoms they cause. These included strain mixtures such as the Unk-4, Unk-7 "isolates" (Piche et al. 2004; Lorenzen et al. 2006b), and other samples we have analyzed because of unusual foliar symptoms (data not presented). PN10A was selected for sequencing from a set of such samples because it did not contain an obvious mixture of genetic variants yet included the recombination junction in the P1 cistron.

Some of the recombination events detected in our analysis have been reported previously. The major recombination junction(s) at or near the HC-Pro-P3 border for both PVY^{NTN} and $PVY^{N:O}$/PVY^{N}-W strain variants was reported by Glais et al. (2002), and a PCR assay was developed for this recombination junction using PVY^{N}- and PVY^{O}- specific primers (Nie and Singh 2003b). The recombination junction in PN10A at 497 nts is apparently identical to that of PVY^{N}-Wi-P and would result in its classification as closely related to the PVY^{N}-W type B variants (Glais et al. 2002). However, this is the first report of a full-length sequence for a PVY^{N}-W strain variant that contains this breakpoint. The full sequence of a similar isolate was recently deposited in GenBank (Wilga isolate 5, gb|AJ890350, Schubert et al. 2007).

As mentioned, the CP region of NE-11 was nearly identical to that of several Chinese isolates that appear to have descended from the same ancestral CP recombinant as NE-11. Examination of a larger set of CP sequences deposited by the same research group provided evidence of other previously undetected recombination events. Isolates XCH30 and XCH44 had a recombination event at position 8823 of an aligned full-length genome, and a set of additional isolates (Beijing, Fenyang-4-1, AFY1, Mudanjiang11-1, others) had a breakpoint at position 8997. These represent new recombination events that can be added to those in the CP region previously reported at positions 8715, 8748, 8891, 9144, 9183, and 9376 (Revers et al. 1997; Boonham et al. 2002; Fanigliulo et al. 2004). Therefore, in addition to the CP/3′UTR recombination events reported by those authors, this paper reports additional recombination events in the CP cistron of PVY.

PN10A is nearly identical to PVY^{N}-W-P (Glais et al. 2002; Schubert et al. 2007). Although PN10A is the first reported full-length sequence for a PVY^{N}-W/$PVY^{N:O}$ type B isolate with a recombination junction between 497 and 500 nts, this is not the first report of such an isolate in North America. Two P1 sequences submitted to GenBank from Mexico, Pic3 and Vic20 (Ramirez-Rodriguez, Ochoa-Sanchez, Frias-Trevino, Flores-Olivas, Silva-Rosales, Gonzalez de la Vara, Rivera-Bustamante, Martinez-Soriano unpublished), show evidence of the same recombination event. These two P1 sequences are almost identical to PN10A and PVY^{N}-W-P for the ten polymorphic bases on either side of the recombination junction, and overall are 98.7 and 96.2% identical to PN10A in the P1 cistron. Therefore, although the origin of this recombination event remains unknown, similar variants must be widely distributed across North America.

In conclusion, the $PVY^{N:O}$ isolates in North America that have been sequenced to date are all very closely related to one another and share very high degrees of sequence identity with European PVY^{N}-W isolates. The NE-11 isolate represents a new category of the PVY^{N} group that is intermediate between previously described PVY^{N} and $PVY^{NA\text{-}N/NTN}$ classes, with multiple recombination events and widespread global distribution. We anticipate that the multiplex PCR assay we have developed

to assess the two unique portions of the NE-11 genome will be useful to other researchers wishing to identify whether this PVY strain variant exists in their collections.

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