Molecular and Serological Typing of *Potato virus Y* Isolates from Brazil Reveals a Diverse Set of Recombinant Strains

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Abstract

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In Brazil, *Potato virus Y* (PVY) currently presents a significant problem for potato production, reducing tuber yield and quality. Recombinant tuber necrotic isolates of PVY had been reported to occur in the country but no systematic study of the PVY isolate diversity was conducted thus far. Here, a panel of 36 PVY isolates, randomly collected in Brazil from potato between 1985 and 2009, was subjected to a systematic molecular and serological typing using reverse-transcription polymerase chain reaction and a series of PVY^O- and PVY^N-specific monoclonal antibodies. The data collected were combined with biological characterization of the same isolates in tobacco. Of the 36 isolates tested, 3 were typed as PVY^O, 10 as PVY^{N:O/N-Wi}, 21 as PVY^{NTN}, and 2 as “unusual” or inconclusive. Of the 10 isolates from the recombinant PVY^{N:O/N-Wi} strain group, 1 isolate, MAF-VOY, was found to have an unusual serological profile identical to the nonrecombinant PVY^O-O5 strain group. The 21 tested PVY^{NTN} isolates included 1

isolate that did not induce vein necrosis in tobacco and 2 isolates with an unusual serological profile (i.e., displaying negative reactivity to one commercial PVY^N-specific monoclonal antibody). Whole genome sequences were determined for four PVY isolates from Brazil, representing PVY^O, PVY^{NTN}, and PVY^{N-Wi} strains. The genome of the MAF-VOY isolate was found to be recombinant, having characteristic N-Wi structure with two recombinant junctions and carrying a single mutation in the capsid protein at position 98, which led to an unusual O5 serological reactivity. Taken together, the data obtained suggest that the two recombinant strains, PVY^{NTN} and PVY^{N:O/N-Wi}, now are apparently dominant in the Brazilian potato crop. The data also suggest that recombinant isolates in Brazil often have unusual serological reactivity which may hamper their correct identification by conventional typing based on enzyme-linked immunosorbent assay.

Potato virus Y (PVY) is one of the most important pathogens in potato worldwide (22,29). It affects potato tuber yield (44) and quality, inducing potato tuber necrotic ringspot disease (PTNRD), which makes tubers of susceptible cultivars unmarketable as fresh potato (4). PVY belongs to the genus *Potyvirus*, family *Potyviridae*, and, under natural conditions, is transmitted by more than 50 species of aphids in a nonpersistent manner (29).

Difficulties in managing PVY in potato and other crops susceptible to PVY (such as pepper, tomato, and tobacco) stem, to a large extent, from a huge diversity in PVY strains and multiple recombinant virus genomes. Five genetic strain groups of PVY were defined based on hypersensitive response reactions in a set of potato indicators carrying different *N* resistance genes, and reactions in tobacco: these were PVY^O, PVY^C, PVY^N, PVY^Z, and PVY^E (2,10,26,48). In addition to these genetic strains of PVY, multiple recombinants were found carrying segments of PVY^O and PVY^N genomes in various combinations. The most common recombinants found in Europe and North America were PVY^{N:O} (one recombinant junction [RJ]), PVY^{N-Wi} (two RJs), and PVY^{NTN} (three or four RJs) (21,23). Additional recombinants and variants were reported, such as PVY^{NA-N} and PVY-NE-11 (32,33). Since about early 1980s, a transition was observed in Europe and elsewhere from predominantly nonrecombinant strains such as PVY^O, PVY^C, and PVY^N to recombinant variants such as PVY^{NTN} and PVY^{N-Wi}/PVY^{N:O}, often associated with PTNRD syndrome (4,22,30). Changes in PVY strain composition had important con-

sequences for the potato industry in all countries affected by PVY, leading to changes in potato certification systems and complete removal from production of potato cultivars susceptible to PTNRD.

To distinguish PVY strain groups and variants, several approaches can be utilized. In addition to the potato indicator test, reactions in tobacco are often used to broadly divide PVY isolates into those inducing vein necrosis and those inducing only mosaic and vein clearing. Isolates inducing vein necrosis in tobacco generally belong to the PVY^N strain group, or have recombinant genomes, such as PVY^{NTN}, PVY^{N-Wi}, or PVY^{N:O}. Nevertheless, recently, PVY^Z and PVY^E strain groups were demonstrated to have recombinant genomes, and some isolates from these strain groups were found to be non-necrotic in tobacco (19,24,30). Two basic laboratory approaches are also used to distinguish PVY strain groups and variants: serology with PVY^N- and PVY^O-specific monoclonal antibodies (MAbs) (12,28), and reverse-transcription polymerase chain reaction (RT-PCR) methodology utilizing nucleotide polymorphism characteristic of specific PVY strains and recombinant variants (6,8,34). Tremendous diversity in PVY strains and rapid evolution of the virus through recombination and mutations created an interesting situation where no single method of PVY strain differentiation is sufficient to type a field isolate of PVY, and multiple methods are necessary to confidently assign such an isolate to a particular strain group (22).

In South America, PVY was first described in the early 1940s; however, it was not considered a problem for potato production in Brazil at that time (43). Until the early 1990s, PVY was rarely detected in the Brazilian potato fields and the *Potato leafroll virus* was considered, for a long time, to be the only potato virus related to yield losses. Nevertheless, between 1985 and 1987, sporadic PVY outbreaks were recorded in some experimental fields with the extremely susceptible cultivar ‘Chiquita’, located at Minas Gerais State. At that time, the majority of PVY isolates were assumed to represent PVY^O, because they did not induce vein necrosis in

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tobacco, and only a few of them were characterized as necrotic isolates, based on the vein necrosis in tobacco (17,18). However, about a decade later, the Brazilian potato growers started to report disease symptoms associated with tobacco necrotic PVY isolates in the commercial potato fields, especially in the fields planted with imported seed (15,50). These necrotic PVY isolates apparently spread quickly and, in 1996, several Brazilian states reported problems in potato related to PVY. It is not clear whether any of the isolates causing vein necrosis in tobacco, described during this early PVY history in Brazil, belonged to the recombinant PVY strains or the nonrecombinant PVY^N strain. Hence, the first official evidence of the presence of PVY^{N-Wi} and PVY^{NTN} recombinant strains in Brazil was reported by two different laboratories, independently, in 1997, both in potato seed increases derived from seed potato imported from Europe or North America (37–39,49). Currently, PVY^{NTN} and PVY^{N-Wi} have been reported to be the predominant PVY recombinant strains in potato in almost all Brazilian states (1,7,13,45,46). Nevertheless, only a limited set of tools was applied to PVY isolate characterization in Brazil thus far, and the molecular, serological, and biological diversity among the PVY isolates circulating in Brazil in potato crops was not studied on a systematic basis up until very recently (19).

A recent initial systematic study of just three recombinant PVY isolates collected from potato in 2008 demonstrated the occurrence of a rare PVY^E genetic strain in Brazil, and an unusual serological profile or recombinant structure characteristic of PVY^E and PVY^{NTN} isolates circulating in Brazilian potato (19). These initial findings suggested a substantial diversity among potato PVY isolates in Brazil and circulation of rare and unusual types of PVY recombinants, and prompted our study of a larger set of 36 PVY isolates collected from field potato and maintained in the Plant Pathology Laboratory in the Federal University of Lavras, Minas

Gerais, Brazil. Here, we describe the diversity of potato PVY isolates having circulated in Brazil over a span of 24 years, from 1985 to 2009, using a combination of biological, serological, and molecular techniques. The data obtained suggested a large diversity of recombinant PVY isolates circulating in Brazil, and allowed us to speculate on an approximate history of changes in PVY strain composition in potato in Brazil between 1985 and 2009. This is the first comprehensive study of potato isolates of PVY conducted on 36 random field samples.

Materials and Methods

Virus isolates: origin and maintenance. The 36 Brazilian PVY isolates studied were collected between 1985 and 2009. All PVY isolates were initially collected by seed potato growers who submitted tubers for post-harvest testing to the enzyme-linked immunosorbent assay (ELISA) Laboratory (Centro de Indexação de Vírus—CIV/MG) at the Federal University of Lavras, and, thus, were considered random samples. The majority of the samples, 33, came from the state of Minas Gerais, where the main production of Brazilian potato occurs; however 2 samples were received from São Paulo, and 1 sample from Paraná. Locations of the initial field collections and potato cultivars where these isolates originated are listed in Table 1. The infected tubers were transplanted to pots in an insect-proof greenhouse, and emerging plants were tested serologically for four potato viruses (PVY, *Potato leafroll virus*, *Potato virus S*, and *Potato virus X*) and confirmed to be infected only with PVY. Tubers obtained from these original infected potato plants were stocked and had been periodically multiplied to maintain the original inoculum. Potential PVY strain mixtures were detected using either strain-specific triple-antibody sandwich (TAS)-ELISA (28) or the standard RT-PCR assay according to Lorenzen et al. (34). The standard PVY isolates Tb60 and Oz (PVY^O), ID269

Table 1. List of *Potato virus Y* isolates studied, grouped according to the year of collection^a

Isolate ID	Year collected	Cultivar	Location (city/state)
YO-ANT25	1985	Achat	Maria da Fe/Minas Gerais
YN-UFLA#1	1987	Unknown	Southern Minas Gerais
YN-UFLA#2	1987	Unknown	Southern Minas Gerais
PVY-FORTE	1996	Baraka	Maria da Fe/Minas Gerais
Y-BR	1996	Achat	Itajubá/Minas Gerais
YN-IBIA	1997	Monalisa	Ibiá/Minas Gerais
YN-SP22	1999	Jaette Bintje	Jose Furtado/Sao Paulo
YN-VELOX	1999	Velox	Três Corações/Minas Gerais
YO-SP08	1999	Bintje	São Paulo
PVY ^N -MG	1999	Bintje	Tiros/Minas Gerais
Y-BR2	2000	Achat	Itajubá/Minas Gerais
SGS-AG	2007	Agata	São Goncalo Sapucaí/Minas Gerais
ALF-VI	2007	Vivaldi	Alfenas/Minas Gerais
ARA-MO	2007	Monalisa	Araxá/Minas Gerais
TAP-AG	2007	Agata	Tapira/Minas Gerais
COR-AG	2007	Agata	Coromandel/Minas Gerais
IPU-MA	2007	Markies	Ipuína/Minas Gerais
LAV-CL	2007	Unknown	Lavras/Minas Gerais
MFE-AG	2007	Agata	Maria da Fe/Minas Gerais
SGS-MO	2008	Monalisa	São Goncalo Sapucaí/Minas Gerais
MF-AST	2008	Asterix	Maria da Fé/Minas Gerais
PA2-AGA	2008	Agata	Pouso Alegre/Minas Gerais
LA-MON	2008	Monalisa	Lavras/Minas Gerais
ES-AGA	2008	Agata	Estiva/Minas Gerais
SA-AST	2008	Asterix	Senador Amaral/Minas Gerais
MU-AGA	2008	Agata	Muzambinho/Minas Gerais
LUI-AGA	2009	Agata	Luiziana/Parana
PEN-AG2	2009	Agata	Pedrinópolis/Minas Gerais
PED-AX	2009	Asterix	Pedralva/Minas Gerais
MAF-CA	2009	Caesar	Maria da Fe/Minas Gerais
PED-CA	2009	Caesar	Pedralva/Minas Gerais
CRI-AX	2009	Asterix	Cristina/Minas Gerais
MAF-VOY	2009	Voyager	Maria da Fé/Minas Gerais
DOV-AG	2009	Agata	Dom Viçoso/Minas Gerais
PED-AG	2009	Agata	Pedralva/Minas Gerais
MAF-VIV	2009	Vivaldi	Maria da Fé/Minas Gerais

^a Potato cultivars from which the isolate was collected and location of the initial collection are also listed.

(PVY^O-O5), Mont (PVY^N), 423-3, N4, and L26 (all PVY^{NTN}), and PVY-NE-11 used for comparisons were from the University of Idaho Plant Virology Laboratory collection (24,28,33).

Biological characterization in tobacco. To investigate the pathotype of Brazilian isolates, ‘Burley’ and ‘Samsun’ tobacco were used. In each experiment, four plants per isolate were inoculated, and testing was repeated three times for each isolate. All plants were mechanically inoculated at a four- to six-leaf stage as described previously (24). Control PVY isolates from the laboratory collection were always included in the experiment. Visual scoring of PVY symptoms in tobacco commenced 5 days post inoculation (dpi) and continued for 4 to 5 weeks. Each plant was scored daily for visual symptoms starting at 5 dpi, and later tested by TAS-ELISA and RT-PCR in order to confirm the PVY infection status and to type the PVY isolate infecting the specific plant. All plants were grown in an insect-proof greenhouse before and after inoculations under natural light supplemented by fluorescent and incandescent lamps with a day-and-night cycle of 18 and 6 h, respectively, and maintained at 24 to 26°C (day) and 16 to 18°C (night) throughout the experiment.

Serological analysis. The serological reactivity of PVY isolates was tested in a TAS-ELISA format, as described by Karasev et al. (28). All tests included control PVY isolates from the laboratory collection, displaying distinct serological patterns characteristic of PVY^O, PVY^N, and PVY^O-O5 strain groups. In addition to a polyclonal antiserum (28), three strain-specific MAbs were used: MAb2 recognizing PVY^O, PVY^O-O5, PVY^{N-Wi/N:O}, and PVY^C (35)

(Agdia); 1F5 recognizing PVY^N, PVY^O-O5, and PVY^{NTN} (12,28,42) (Agdia); and SASA-N recognizing PVY^N and PVY^{NTN} (42) (Scottish Agriculture Science Agency).

RT-PCR and differentiating primers. To classify all 36 isolates, and check for possible mixed infections, a multiplex assay described by Lorenzen et al. (34) was used. In this assay, eight primers are combined in a single multiplex RT-PCR test to differentiate PVY^O, PVY^N, PVY^{N:O/N-Wi}, PVY^{NTN}, and PVY^{NA-N}, or mixtures of those. An additional primer pair was used to differentiate PVY^E genome from PVY-NE-11 and ordinary PVY^{NTN} isolates, as described elsewhere (19). Virus RNA extraction and the multiplex RT-PCR assay were performed as described (34). About 100 mg of tobacco leaf tissue was homogenized using a mini-pestle in an Eppendorf tube containing 400 µl of fresh extraction buffer (Dellaporta I: 100 mM Tris [pH 8.0], 50 mM EDTA, 500 mM NaCl, and 10 mM 2-mercapto-ethanol); then, 52.8 µl of 10% sodium dodecyl sulfate (SDS) was added, followed by vortexing and incubating at 65°C for 10 min. After the addition of 128 µl of 5 M potassium acetate, the mixture was vortexed and subjected to low-speed centrifugation for 10 min. The resulting supernatant was carefully removed and nucleic acids precipitated with isopropanol. The pellet was washed twice in 70% ethanol, air dried, and resuspended in 80 µl of diethylpyrocarbonate-treated water. Reverse transcription was performed using 1 µl of the total nucleic acid extract in a 15-µl reaction volume that contained 5× first-strand buffer (Invitrogen), 1 mM (each) dNTP, 0.12 µM oligo-dT primer mix, 6 units of RNase Out Ribonuclease Inhib-

Table 2. Summary of biological, serological, and molecular typing of the 36 Brazilian isolates studied

Isolate ID	Symptoms ^b	Serology ^a			RT-PCR ^c	Final call
		MAb2	1F5	SASA-N		
YO-ANT25	M	+	–	–	O	O
YN-UFLA#1	SM	+	–	–	NA-N	?
YN-UFLA#2	FM	+	–	–	O	O
PVY-FORTE	VN	–	+	+	NTN	NTN
Y-BR	SM	+	–	–	NA-N/O	?
YN-IBIA	VN	–	+	+	NTN	NTN
YN-SP22	VN	–	+	+	NTN	NTN
YN-VELOX	VN	–	+	+	NTN	NTN
YO-SP08	M	+	–	–	O	O
PVY ^N -MG	VN	–	+	+	NTN	NTN
Y-BR2	VN	+	–	–	N:O	N:O/N-Wi
SGS-AG	VN	+	–	–	N:O	N-Wi
ALF-VI	VN	–	+	+	NTN	NTN
ARA-MO	VN	–	+	+	NTN	NTN
TAP-AG	VN	–	+	+	NTN	NTN
COR-AG	VN	–	+	+	NTN	NTN
IPU-MA	VN	–	+	+	NTN	NTN
LAV-CL	FM	+	–	–	N:O	N:O/N-Wi
MFE-AG	VN	+	–	–	N:O	N:O/N-Wi
SGS-MO	VN	–	+	–	NTN	NTN/AST
MF-AST	M	–	+	+	NTN	NTN/Z
PA2-AGA	VN	–	+	+	NTN	NTN
LA-MON	VN	–	+	+	NTN	NTN
ES-AGA	VN	–	+	+	NTN	NTN
SA-AST	VN	–	+	+	NTN	NTN
MU-AGA	VN	–	+	–	NTN	NTN/AST
LUI-AGA	VN	–	+	+	NTN	NTN
PEN-AG2	VN	–	+	+	NTN	NTN
PED-AX	VN	+	–	–	N:O	N:O/N-Wi
MAF-CA	VN	+	–	–	N:O	N:O/N-Wi
PED-CA	VN	+	–	–	N:O	N:O/N-Wi
CRI-AX	VN	–	+	+	NTN	NTN
MAF-VOY	VN	+	+	–	N:O	N-Wi/O5
DOV-AG	VN	+	–	–	N:O	N:O/N-Wi
PED-AG	VN	–	+	+	NTN	NTN
MAF-VIV	VN	+	–	–	N:O	N:O/N-Wi

^a Serological reactivity in triple-antibody sandwich enzyme-linked immunosorbent assay when the listed monoclonal antibodies (MAbs) were used as detecting antibodies.

^b Tobacco reaction in ‘Burley’ and ‘Samsun’: M = mosaic/vein clearing and VN = vein necrosis; intensity of mosaic reaction: F = faint, M = regular, and S = severe.

^c Reverse-transcription polymerase chain reaction (RT-PCR) typing was conducted according to Lorenzen et al. (34).

itor (Invitrogen), and 60 units of SuperScript II reverse transcriptase (Invitrogen).

Sequencing and sequence analysis. The whole-genome sequences were obtained by sequencing RT-PCR products for Y^O-ANT, ALF-VI, SGS-AG, and MAF-VOY isolates representing PVY^O, PVY^{NTN}, and PVY^{N-Wi} types, respectively, found in Brazil. The sequencing was conducted essentially as described by Hu et al. (24). Some additional primers were designed to complete the genome sequences of MAF-VOY and Y^O-ANT. All PCR products were purified using the ExoSAP-IT for PCR Product Clean-Up (USB Products; Affymetrix Inc.), mixed with appropriate primers and submitted for sequencing to Genewiz. The contigs obtained were assembled using the Lasergene Core Suite Software (DNASTAR), and the full-length sequences assembled were aligned using the CLUSTALW2 program with default settings. The

sequence analysis and generation of phylogenetic trees were performed as described previously by Karasev et al. (27), and the recombination analysis as described by Galvino-Costa et al. (19) using the RDP4 package.

Results

Biological characterization in tobacco. All 36 isolates were tested in two tobacco cultivars, Burley and Samsun, following mechanical inoculation at the four- to six-leaf stage. Symptoms were consistent between Burley and Samsun, and both the type of symptoms and timing of their appearance were very similar for each isolate. Seven of the isolates tested induced vein clearing, various degree of mosaic, and leaf mottling but no vein necrosis (Table 2). On the other hand, 29 of the isolates induced vein necrotic reaction in both tobacco cultivars, sometimes visible in just 5 to 7 dpi, in addition to the vein clearing and mosaic. This vein necrosis generally coincided with stunting, sometimes very severe, and often with stem necrosis. If a younger plant was inoculated, at a four-leaf stage, it was completely necrotized and dead at about 10 dpi. Each plant was scored for visual symptoms and later tested by TAS-ELISA and RT-PCR in order to confirm the PVY infection status, and to type the PVY isolate infecting the specific plant. No inconsistencies or cross-contaminations were identified for more than 430 inoculated tobacco plants. In just a few cases, inoculations failed, and these plants were excluded from further experiments.

Serology of the Brazilian isolates. Serological typing of the Brazilian isolates under study is summarized in Table 2. Of the 36 isolates analyzed, 15 reacted with the PVY^O-specific antibody MAb2 and, thus, were typed as having the PVY^O serotype. In all, 22 isolates reacted positively to the PVY^N-specific MAb 1F5, and 19 of these were also positive against another PVY^N-specific MAb, SASA-N. One of the 1F5-positive isolates, MAF-VOY, was also positive against PVY^O-specific MAb2 and negative against MAb SASA-N; thus, it was typed as having a characteristic PVY^O-O5 serotype (28). Two other 1F5-positive isolates, SGS-MO and MU-AGA, did not bind the second PVY^N-specific antibody, SASA-N, while being MAb2 negative; this identified both isolates as having a characteristic PVY-AST serotype, as described elsewhere (19). In summary, 14 isolates were typed as having PVY^O serotype, 1 was typed as PVY^O-O5, and 21 isolates as having broadly defined PVY^N serotype (i.e., MAb2-negative, 1F5-positive, SASA-N-positive or negative; Table 2).

Molecular typing by RT-PCR. The 36 isolates were typed using the multiplex assay developed by Lorenzen et al. (34) which can distinguish between main recombinant and nonrecombinant potato PVY strain groups and their mixtures. The summary of this RT-PCR typing is presented in Table 2. In total, 3 isolates were typed as nonrecombinant PVY^O type, 1 as PVY^{NA-N} type, 10 as recombinant PVY^{N:O} type, 21 as recombinant PVY^{NTN} type, and 1 (Y-BR) as an unusual type. This unusual RT-PCR profile included only one of the two bands characteristic of PVY^O, and the PVY^{NA-N} band; in this case, the possibility of mixed infection was excluded, or at least greatly reduced, based on the clear PVY^O serological typing for this isolate. To help further type this isolate, Y-BR, we used individual primer pairs designed to type PVY^O and PVY^{NA-N} in four different PCR assays (34). In the first assay, the isolate was tested using a single primer pair specific to PVY^{NA-N} (n_5707+A6032m), which consistently amplified a characteristic NA-N band (328 bp) and, in the second assay, two specific PVY^O primer pairs (S5585m+o6266c and o2172+o2439) were used separately, with only o2172+o2439 primers generating one band (267 bp) characteristic of PVY^O. Thus, the primer pair responsible for amplification of the larger band, 689 bp (S5585m+o6266c) characteristic of the PVY^O sequence, consistently failed to produce the respective amplicon for Y-BR in RT-PCR.

Isolate MAF-VOY, which was found to induce vein necrosis in tobacco (Fig. 1) and to have a characteristic PVY^O-O5 serotype (Table 2), was identified as a typical N:O recombinant producing 181- and 689-bp bands (Fig. 2). No evidence of mixed infection

Fig. 1. Symptoms induced by five Brazilian isolates in tobacco ('Samsun'). A, MAF-VOY (Potato virus Y [PVY]^{N-Wi}-O5); B, TAP-AG (PVY^{NTN}); C, YO-SP08 (PVY^O); D, LAV-CL (PVY^{N:O}-minus); and E, MF-AST (PVY²-NTN).

was found during either RT-PCR typing in multiplex assay of Lorenzen et al. (34) (Fig. 2) or serological typing with PVY^N- and PVY^O-specific MAbs. This RT-PCR typing was consistent with MAF-VOY's ability to induce vein necrosis in tobacco; however, this N:O type of recombinant genome was not associated with the PVY^O-O5 serological marker before.

Whole-genome sequences. The entire genome was sequenced for four Brazilian isolates representing main PVY strains found in Brazil. The sequences of the Y^O-ANT (9,649 nucleotides [nt]), ALF-VI (9,698 nt), SGS-AG (9,674 nt), and MAF-VOY (9,691 nt) were deposited in the GenBank database and would appear under accession numbers JQ924285, JQ924287, JQ924288, and JQ924286, respectively. The four Brazilian whole-genome sequences were subjected to a phylogenetic analysis against 45 full-length PVY sequences from the GenBank, including all major strains of PVY (Fig. 3). As expected, the four Brazilian isolates studied here clustered into the respective strain groups, with isolate ALF-VI grouped in the same branch as PB312, PVY-AST, HunNTN, 423-3, and other typical PVY^{NTN} isolates from GenBank (approximately 99% identity). Similarly, MAF-VOY and SGS-AG isolates clustered into the PVY^{N-Wi} group and Y^O-ANT clustered into the PVY^O group. The Y^O-ANT isolate displayed 98% identity with PB-Oz and SCRI-O, both typical PVY^O isolates.

The RDP analysis showed that isolate ALF-VI had a regular PVY^{NTN} genome with the typical three RJs located at HC-Pro/P3, CI/N1a, and CP/3'UTR, at positions 2,413, 5,827, and 9,173, respectively, in the parental genome of isolate Mont. Positions of these RJs were very close to the position of RJs in the reference PVY^{NTN} isolate 423-3 (32). SGS-AG and MAF-VOY had the PVY^{N-Wi} genome with two RJs (at P1 and HC-Pro/P3). Positions of these RJs in the SGS-AG genome, at nucleotides 462 and 2,356 in the parental genome of isolate Mont, were almost identical to the reference PVY^{N-Wi} isolate PN10A (32) whereas, in the genome of MAF-VOY, positions of RJs at nucleotides 479 and 2,303 were shifted relative to the reference PVY^{N-Wi} isolate PN10A (32). In all three cases, PVY^N (Mont) and PVY^O (PB-Oz) were easily identified as parents of ALF-VI, SGS-AG, and MAF-VOY. The analysis also showed that the genome of Y^O-ANT was nonrecombinant and belonged to the PVY^O strain group. Although both SGS-AG and MAF-VOY shared the same recombinant genomic structure, MAF-VOY had a single amino acid substitution located at the 98th position of the coat protein (Q instead of R) (28), which explained the unusual O5 serotype identified in this N-Wi isolate by TAS-ELISA (Table 2).

Discussion

Brazil is one of the largest producers of ware potato in South America; however, it is currently dependent on seed potato importation necessary for subsequent multiplication and propagation in certified seed potato fields. Large volumes of imported potato seed may certainly carry a risk of introduction of potato pathogens, including the new recombinant PVY strains. The main sources of seed potato imported by Brazilian producers are located in Europe, Canada, and the United States (14,16), where a wide range of PVY types have been reported since the 1980s (2-4,9,20,21,23,31,32,36,40,41,47). Recently, it was shown that the PVY isolates in Brazil were dominated by recombinant strains PVY^{NTN} and PVY^{N-Wi} (1), similarly to what was reported in Europe and North Africa (5,11). In particular, PVY^{NTN} was found to be the most frequent among these recombinant strains, in at least seven different states of Brazil (1). Galvino-Costa et al. (19) reported on a rare PVY^E strain found in two States in Brazil, and on an unusual serological profile characteristic of two isolates from PVY^E and PVY^{NTN} strains. However, the presence of other PVY strains and variants, such as PVY^{N:O}, PVY^Z, PVY^{O-O5}, and PVY^{NA-N}, as well as the general PVY diversity among isolates circulating in Brazilian potato, have not been studied so far.

In Brazil, more than 80% of seed potato is produced in only four states, with Minas Gerais being the largest potato-producing state, with a share of about 30% of national production (25). In order to

address the diversity of PVY isolates circulating in potato in Brazil, we systematically characterized the set of 36 potato PVY isolates, mostly from seed potato fields in the Minas Gerais State of Brazil, with 3 isolates from the states of São Paulo and Paraná collected during the span of 24 years between 1985 and 2009. Because all these isolates came to the Plant Pathology Laboratory of the Federal University of Lavras in infected potato tubers submitted directly by growers for post-harvest testing, this set may be considered random and, thus, roughly representing isolates of PVY in circulation in Brazilian potato over the years. All 36 isolates were tested for their symptoms in tobacco, subjected to serological profiling using three MAbs specific to PVY^O or PVY^N serotypes, and molecularly typed using the standard, multiplex RT-PCR protocol of Lorenzen et al. (34). No nonrecombinant PVY^N strain isolates were found among the 36 isolates in the collection, and only three nonrecombinant isolates belonging to the ordinary strain PVY^O were identified (Tables 1 and 2). Interestingly, all three PVY^O isolates were collected between 1985 and 1999 and no PVY^O isolate was collected after 1999. The first recombinant isolate, PVY-FORTE, collected originally in 1996 from potato 'Baraka', had typical PVY^{NTN} features (i.e., PVY^N serotype, two characteristic RJs in its genome identified by RT-PCR, and induced vein necrosis in tobacco; Table 2). Four additional PVY^{NTN} isolates (YN-IBIA, YN-SP22, YN-VELOX, and PVY^N-MG) were collected between 1997 and 1999 from four different potato cultivars, suggesting the presence of PVY^{NTN} isolates in the seed potato system in Brazil since at least 1996. Another recombinant PVY type, PVY^{N:O/N-Wi}, was first collected later, in 2000 (isolate Y-BR2).

Two of the isolates from these early collections conducted between 1987 and 1996 (YN-UFLA#1 and Y-BR) seem to be of an unusual PVY type, simultaneously displaying features typical for PVY^{NA-N} and PVY^O (Table 2). Both isolates induced severe mosaic and crinkling symptoms in tobacco but no vein necrosis was observed, and both reacted only with MAb2 showing serotype PVY^O; however, the RT-PCR pattern in the multiplex assay according to Lorenzen et al. (34) revealed a single, 328-bp amplicon char-

Fig. 2. Molecular typing of seven representative Brazilian *Potato virus Y* (PVY) isolates using multiplex reverse-transcription polymerase chain reaction (32,34). Lane M, 100-bp ladder marker; lane 1, Y-BR ("unusual" PVY^{NA-N:O} pattern); lane 2, MAF-VOY (PVY^{N:O-O5}); lane 3, MF-AST (PVY^{Z-NTN}); lane 4, YO-SP08 (PVY^O); lane 5, CRI-AX (PVY^{NTN}); lane 6, MAF-VIV (PVY^{N:O}); lane 7, YN-UFLA#1 (PVY^{NA-N}). Positions of characteristic amplicons are listed on the right.

acteristic of PVY^{NA-N}. In the case of isolate Y-BR, this 328-bp amplicon was present in addition to the 267-bp amplicon characteristic of the PVY^O type (Table 2). All possibilities of mixtures were tested: (i) of the seven different MAb (including PVY^N-, PVY^O-, and PVY^{O/C}-specific MAbs) tested, only the O-specific antibodies reacted with both YN-UFLA#1 and Y-BR; and (ii) a series of RT-PCR assays using the primer pairs specific to PVY^{NA-N} and PVY^O types were conducted separately (not in a multiplex format), always producing the same bands amplified in a multiplex assay. Isolates YN-UFLA#1 and Y-BR have been currently assigned an “unusual” status, and the final classification of

these two isolates will await whole-genome sequencing and subsequent recombination analysis.

A large group of 25 PVY isolates collected between 2007 and 2009 contained only the PVY^{NTN} and PVY^{N:O/N-Wi} recombinant strains, with PVY^{NTN} being almost twice as frequent as the PVY^{N:O/N-Wi} strain (Table 2). Among the PVY^{N:O/N-Wi} isolates recognized only by the O-specific MAb, one isolate, LAV-CL, induced only mosaic and vein clearing in tobacco. Although this isolate did not induce vein necrosis in tobacco, it had a typical N:O/N-Wi profile amplified in the RT-PCR multiplex assay (34), which indicated that it belonged to a relatively rare PVY strain

Fig. 3. Cladogram of the whole-genome nucleotide sequences of *Potato virus Y* (PVY) isolates. Phylogenetic trees were constructed using the neighbor-joining algorithm in the MEGA5 program. Bootstrap values are given as percentage of the 2,000 tree replicates. PVY isolate names and respective accession numbers are indicated on labels. Positions of the four Brazilian isolates sequenced in this work (ALF-VI, SGS-AG, MAF-VOY, and Y^O-ANT) are highlighted with squares.

group called “N:O-minus” (22). Such an isolate could be easily misidentified as an ordinary PVY^O if only biological and serological diagnostic tests had been taken into account. One recombinant isolate, MAF-VOY, displayed a typical N:O/N-Wi band pattern in the multiplex RT-PCR assay (Table 2) but had a recently described serotype PVY^O-O5 (e.g., reacting to MAb2 and 1F5 but not to SASA-N; Table 2) (28). Up until now, this specific serotype, PVY^O-O5, had never been reported outside North America (22,27,28). Isolate MAF-VOY is especially unusual because this is the first documented case where PVY^O-O5 serotype is associated with a recombinant PVY genome, PVY^{N:O/N-Wi} in this case, whereas all previous reports found this serological marker exclusively in nonrecombinant, PVY^O-type genomes (27,28).

Of the 21 recombinant isolates typed as PVY^{NTN}, 20 induced severe vein necrosis in tobacco. One isolate, MF-AST, did not induce vein necrosis in tobacco and, thus, was provisionally classified as PVY^Z-NTN due to a typical PVY^{NTN} band pattern in RT-PCR and a typical PVY^N serotype (Table 2). This assignment is based on a recent characterization of the molecular make-up of the PVY^Z strain group, which apparently comprises recombinant PVY^{NTN} isolates which do not induce vein necrosis in tobacco (24,30). Two other PVY^{NTN} isolates, SGS-MO and MU-AGA, shared an unusual serotype recently described for a recombinant PVY^{NTN} isolate, PVY-AST, from Brazil (MAb2-negative/1F5-positive/SASA-N-negative) (19), indicating that this serotype may be relatively common in the PVY isolates circulating in Brazil.

Whole-genome sequence analysis conducted on four Brazilian isolates (Y^O-ANT, ALF-VI, SGS-MO, and MAF-VOY) revealed two types of recombinant PVY genomes, PVY^{NTN} (ALF-VI) and PVY^{N-Wi} (SGS-MO and MAF-VOY), and a nonrecombinant PVY^O genome (Y^O-ANT). All four sequences clustered into corresponding phylogenetic clades on the whole-genome tree (Fig. 3), representing three PVY strain groups present in Brazil. This sequence analysis was fully consistent with RT-PCR typing performed on these isolates (Table 2). Although the whole genome of a Brazilian PVY^{NTN} recombinant, PVY-AST, had been reported before (19), this is the first report of the whole-genome sequences for two PVY strains collected in Brazil, PVY^O (Y^O-ANT) and PVY^{N-Wi} (SGS-MO and MAF-VOY). One isolate, MAF-VOY, while being typed as belonging to the PVY^{N-Wi} strain, displayed an unusual O5 serology apparently associated with a single amino acid substitution in its capsid protein, R⁹⁸ to Q⁹⁸ (28). These data indicate that PVY isolates circulating in Brazil often have unusual serological properties which may complicate or hamper their differentiation.

If the entire set of the PVY isolates collected over the years in Brazil and maintained at the Federal University of Lavras col-

lection (Tables 1 and 2) is analyzed from a historical perspective, we can speculate that an influx of recombinant PVY strains in the mid- to late 1990s (50) changed composition of PVY strains circulating in potato in Brazil. Currently, the PVY^{NTN} is considered prevalent in several local Brazilian PVY populations (1); however, among the 10 isolates collected by us in 2009, only 4 belonged to the PVY^{NTN} recombinant strain group while the other 6 belonged to PVY^{N:O/N-Wi} (Table 2). That is the first time that PVY^{N-Wi} was found more frequently than PVY^{NTN} in potato in Brazil, although the number of samples is certainly not sufficient to formulate a definite conclusion about the current epidemiologic distribution of PVY strains in the country. This study should be considered only a preliminary indication that the PVY strains and recombinant populations in Brazil are not static and continue to evolve over time. It may seem that, for some reason, the population of PVY^{N-Wi}, which has long been known to have entered the country in the mid-1990s (37–39), started to outperform PVY^{NTN} isolates. Careful analysis of the diversity of the PVY strains and variants uncovered in this work (Table 3) suggests that the overall PVY diversity in Brazil is close to the one observed in both Europe and North America, although some of the PVY strains and types (e.g., nonrecombinant PVY^N, PVY^C, and PVY^O-O5 and recombinant PVY^{N:O}) have yet to be found in Brazil. However, it must be pointed out that at least one unique variant, PVY^{N-Wi}-O5, was found among Brazilian isolates analyzed (Tables 2 and 3) and, on the other hand, only a small number of Brazilian PVY isolates were subjected to a rigorous analysis in this work.

The introduction of recombinant strains in Brazil led to many changes in the potato production system, both seed and ware potato; it especially affected the choice of cultivars, shifting it to those less susceptible to PTNRD. One of the unintended consequences of this was the introduction and spread of ‘Agata’ due to its failure to show foliar and tuber symptoms when infected by the majority of PVY strains and recombinants. Although the official report of necrotic recombinant strains had been published in the mid- to late 1990s (37,38,49), these recombinant isolates could have been present in Brazil a few years before that, as exemplified by the isolate PVY-FORTE collected in 1996. The principal route of entry for all these PVY strains and recombinants into Brazil has likely been via seed potato importation, and it still is an open door for new recombinants of PVY to enter and evolve under the favorable conditions of the tropical potato production system in Brazil.

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Table 3. Comparison of the *Potato virus Y* (PVY) strain groups and variants found in potato in Brazil (as of early 2012) with those found in potato in North America and Europe^a

PVY strains or types	Brazil ^b	North America ^c	Europe ^d
PVY ^O	Y	Y	Y
PVY ^O -O5	N	Y	N
PVY ^C	N	N	Y
PVY ^Z	Y	Y	Y
PVY ^N	N	Y	Y
PVY ^E	Y	N	Y
PVY ^{N-Wi/N:O}	Y	Y	Y
PVY ^{N-Wi/N:O5}	Y	N	N
PVY ^{N-Wi/N:O} (minus) ^e	Y	Y	Y
PVY ^{NTN}	Y	Y	Y
PVY ^{NA-N}	Y	Y	Y
PVY ^{NE-11}	N	Y	N

^a Y = this PVY strain or type has been reported and N = this PVY strain or type has not been reported.

^b References 1, 18, 19, 45, and this work.

^c References 22, 24, 27, 28, 30, 32, 33, 36, 40, and 41.

^d References 3–6, 10, 11, 20, 21, 26, 29, 31, 46, and 47.

^e Unique to Brazil.

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