IIMMUNOCAPTURE RT-PCR PROBING OF POTATO VIRUS Y ISOLATES

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Summary. – Twenty potato virus Y (PVY) isolates were characterized. They represented two strains only, PVY^O (three isolates) and PVY^N (17 isolates). However, application of serological and molecular genetic methods led to a more complicated characterization. For example, five isolates induced necrotic symptoms on tobacco plants typical of PVY^N, despite reacting as PVY^O serologically. Moreover, the PVY isolates were not identical according to molecular genetic properties. Typical PVY^{NTN} PCR products were observed for 14 isolates, but five of them (Hr 220-5, Hr 387-7, Nord 242, Syn1Scot, and 41-97) did not produce potato tuber necrotic symptoms in infected cultivars. An immunocapture reverse transcription–polymerase chain reaction (RT-PCR) probing was developed using a set of 24 primer pairs derived from eight regions of the PVY genome. Using this method, five out of seven PVY^{NTN} isolates including the Czech standard PVY^{NTN} from the potato cv. Nicola were found to be identical. However, two PVY^{NTN} isolates and all the other probed PVY samples showed unique patterns, suggesting specific differences at the nucleotide level. This method enabled specific identification of individual isolates variability even within different PVY strains.

Key words: potato virus Y; Solanum tuberosum L; immunocapture RT-PCR; virus genome

Introduction

PVY (species *Potato virus Y*, genus *Potyvirus*, family *Potyviridae*) (van Regenmortel *et al.*, 2000) isolates have been subdivided into three strains, PVY^O, PVY^N and PVY^C on the basis of symptoms on *Nicotiana tabacum, Physalis floridana* and potato cultivars (de Bokx and Huttinga, 1981). In addition, PVY strains have been differentiated by the reaction of other indicator plants and potato cultivars carrying hypersensitive genes directed against different PVY strains (Jones, 1990; Valkonen *et al.*, 1994). Strain-specific monoclonal antibodies (MAbs) have also been utilized (Gugerli and Fries, 1983; Rose and Hubbard, 1986; Singh *et al.*, 1993).

At the beginning of the 1980s, potato tuber necrotic ringspot disease (PTNRD), caused by PVY^{NTN}, a new variant related to PVY^N (Le Romancer *et al.*, 1994; van den Heuvel *et al.*, 1994), has been reported, affecting potato crops in Hungary (Beczner *et al.*, 1984). The disease subsequently spread widely across Europe, being reported in many countries, namely Germany (1984), Czechoslovakia (1988), Austria (1990), Yugoslavia (1990), France (1991), Belgium (1992), Great Britain (1992) and Denmark (1992) (Weidemann and Maiss, 1996). An additional distinct variant related to PVY^N has been reported in Poland (Chrzanowska, 1991) and then in France (Glais *et al.*, 1998) and Czech Republic (Dědič and Ptáček, 2000).

Identification of PVY^{NTN} is based primarily on the development of necrotic symptoms on tubers of inoculated sensitive potato cultivars, but this is time-consuming. It has not been possible to select indicator plants or to produce

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Abbreviations: ELISA = enzyme-linked immunosorbent assay; MAbS = monoclonal antibodies; PVY = potato virus Y; PTNRD = potato tuber necrotic ringspot disease; RT-PCR = reverse transcription–polymerase chain reaction

MAbs that reliably distinguish PVY^{NTN} isolates from other members of PVY^{N} strain group. The methods based on the variability of genomic sequence have been more successful. Blanco-Urgoiti *et al.* (1996) have developed a molecular typing method for classifying PVY isolates using the coat protein region. Weidemann and Maiss (1996), Glais *et al.* (1996) and Weilguny and Singh (1998) utilized RT-PCR directed to 5′-non-translated region 5′-NTR and P1 protein for strain-specific differentiation of PVY^{NTN}. Differentiation of PVY^{NTN} isolates was achieved by Boonham *et al.* (1999) too, but using primers derived from the CP region. However, the RFLP analysis of the whole genome of a group of sequenced PVY isolates (Glais *et al.*, 1998) has indicated a more complex nature of PVY genome possibly because of recombination events. This suggested that PCR analyses based on single primer pairs are not sufficient for characterization of a particular isolate. Moreover, previous work using DNA heteroduplexes has shown that PVY^{N} including $PVY^{N}W$ forms populations of mixed variants or quasi-species (Matoušek *et al.*, 2000).

In this study a number of PVY isolates was investigated by biological, serological and molecular genetic methods. In order to characterize individual isolates, a system of immunocapture RT-PCR probing of the PVY genome has been developed. This system utilizes a set of primers, designed from the PVY genome, which proved useful in characterization of PVY isolates.

Materials and Methods

PVY isolates. Twenty virus isolates (17 from PVY^{N} strain and 3 from PVY^{O} strain) originating from Canada, Czech Republic, France, Poland and Slovenia were collected and maintained *in vitro* usually as infected potato microplants. Plants of *N. tabacum* cv. Samsun were mechanically inoculated with the PVY isolates at the 3–4 leaf stage and maintained in a greenhouse under natural photo-period and temperatures. The presence of PVY was determined 10–21 days later by the development of characteristic symptoms and by a positive reaction in an enzyme-linked immunosorbent assay (ELISA). The isolates were then transmitted from tobacco to the potato cvs Eersteling (hypersensitive to PVY-C), Desirée (hypersensitive to PVY-O), Maris Bard (hypersensitive to PVY-O, PVY-C and PVY-Z (Jones, 1990)). The cv. Lukava was included as a sensitive host plant for PTNRD. The plants, daughter tubers and plants grown from the daughter tubers were scored repeatedly for visual symptoms.

Double antibody sandwich ELISA (DAS-ELISA). A broad spectrum of PVY antibodies (Bioreba or IPO-DLO, Germany) and PVY-N, PVY-O/C MAbs (Bioreba, Germany) were used (Clark and Adams, 1977) for serotyping of PVY isolates.

Immunocapture RT-PCR. Primer sets suitable for differentiation of PVY isolates, selected from the 5′-NTR or P1 protein region, were derived from the PVY^{NTN} genome sequence (Weidemann and Maiss, 1996; Glais *et al.*, 1996; Weilguny and Singh, 1998). For the RT-PCR probing system, the PCR and RT-PCR primers were derived from the sequence of a Hungarian isolate of PVY^{NTN} (EMBL database, Acc. No. M95491). Eight regions, numbered 1–8, located throughout the PVY genome were selected from alignment of PVY^{NTN} with PVY^{O} (Acc. No. D00441) at positions where the maximum difference in restriction sites was observed. Each of these regions was subdivided into parts I-III to obtain three fragments ranging in length from 440 to 220 bp. Twenty four primer pairs in total were derived from the PVY genome. The thermodynamic properties and structural features of individual primer pairs were calculated using the Gene Runner version 3.02 (Hastings Software, Inc.) in order to minimize differences in the annealing temperatures and the effect of complicated secondary structures. The primer pairs are listed in Table 1. We used either the RNeasy Plant Total RNA Kit (Qiagen) for total RNA isolation or an immunocapture on ELISA plates for template preparation. First-strand cDNAs were synthesized by the Enhanced Avian RT-PCR Kit (Sigma) using reverse primers or random hexanucleotides in 0.2 ml PCR tubes or in ELISA microplates. For the PCR, 5 µl of the reverse transcriptase reaction mixture (cDNA) was amplified in a total volume of 50 µl using the Enhanced Avian RT-PCR Kit (Sigma) and the primers selected by Weidemann and Maiss (1996), Glais *et al.* (1996) and Weilguny and Singh (1998): The amplification products were analyzed in a 1% agarose gel stained with ethidium bromide. The RT-PCR was performed in a PTC-100 thermocycler (MJ Research), and gels were visualized by documentation system KS-3000 (Ultralum). Aliquots (10 µl) of PCR products, amplified using the procedure of Glais *et al.* (1996), were digested with 4 units of *Taq*I endonuclease at 65°C for 2 hrs.

Results and Discussion

Because of the economic importance of PVY^{N} to potato production, isolates mostly identified biologically as PVY^{N} were selected for this study. For comparison, two classical PVY^{O} isolates, Irlande O and Hr 469-13 and the unusual isolate 41-97 from Poland, having biological and serological properties of PVY^{O}, were also included. From serological analyses and tests on potato cultivars the 17 PVY^{N} isolates could be split into several subgroups (Table 2). The first subgroup included nine isolates typical of PVY^{NTN} from which characteristic PCR products were obtained, using three published techniques for differentiating PVY^{NTN} (Weidemann and Maiss, 1996; Glais *et al.*, 1996; Weilguny and Singh, 1998). From this subgroup the isolate from the cv. Nicola was selected as the standard PVY^{NTN}. The second subgroup of four isolates designated $PVY^{N}W$ (Chrzanowska, 1991) showed a positive reaction with PVY^{O}-specific antibody, and did not produced PTNRD symptoms. According to RT-PCR analysis, $PVY^{N}W$ isolates could be subdivided into two sets. The first produced PVY^{NTN}-specific RT-PCR products, while the second did not (Table 2). The properties of four remaining isolates, Tu 660 Cal, Syn 1 Scot, 41-97 and 12-94, were unusual. Tu 660 Cal, in contrast to

Table 1. Primers and their characteristics

Primer groups, primer designation, primer sequence	Position[a]	Gene region[b]	Annealing temperature[c] (°C)
Group 1		P1, HC-pro	57
1-Ipl: 5′CTTCCATACTCACCCGCTCC3′	238-257		
1-Imi·5′CACTTGACGCAGCCATTTG3′	490-472		
1-IIpl:5′GCGCGAGGAGAGAGAAAGAG3′	438-457		
1-IImi:5′GCGATGTGATCCCAAAACTT3′	744-724		
1-IIIpl:5′GGTCTGTTCAACTGATTAGCAAG3′	677-699		
1-IIImı:5′AGCGCTTGAGAACTGAACCA5′	1050-1031		
Group 2		HC-pro	53
2-Ipl:5′CAGGCATACCAGTTGAAGAC3′	1121-1131		
2-Imi:5′CTCAGATCAACCGGTTCAGT3′	1370-1351		
2-IIpl.5′GGCAGACAAAGATCGCTT3′	1293-1310		
2-IImi:5′CAATTTGCTTTGGCAGATAG3′	1607-1588		
2-IIIpl:5′CAAGAAAGGAGACATCTCGTT3′	1554-1574		
2-IIImı:5′GGTGCTTCTTAGTTGGCG3′	1978-1961		
Group 3	HC-pro, P3	55	
3-Ipln:5′CTCGAAGGATGGAAACTACG3′	1881-1900		
3-Imin:5′TGCGTTTCGTGATCGACTAG 3′	2276-2257		
3-IIpl:5′ATTAGTGAGGAAGATGCAAAGG3′	2101-2122		
3-IImi:5′GTGCTTAATGTCAGACTCCAAC3′	2398-2376		
3-IIIpln:5′TTGAAAGCATCTAGCGTGTCC3′	2329-2349		
3-IIImin:5′CAAGGAGATCTGTAGCTGCAGT3′	2773-2752		
Group 4		CI	54
4-Ipl:5′TCCACATTACAGAACCGAGG3′	3807-3826		
4-Imi:5′TCCACGCATACGCAGTGTT3′	4071-4053		
4-IIpl:5′GCGACCACTAGCGGAGAA3′	3993-4010		
4-IImi:5′ACTTGACTGGTTGCTGTGTTG3′	4321-4301		
4-IIIpl:5′TCAGCTACTCCAGTGGGAAG3′	4267-4286		
4-IIImi:5′GAACGGTGAGACTTTCAACC3′	4647-4628		
Group 5		6K2, Nia	52
5-Ipl:5′TGGAAGAAGTCATTAGTGGC3′	5605-5624		
5-Imi:5′CAACAGTGGTGCCTTTACC3′	5872-5854		
5-IIipl:5′TGGATCTGCATACAGGAAGA3′	5826-5845		
5-Iimı:5′TCACAGATTTTGAGTGGGTT3′	6170-6151		
5-IIIpl:5′TTGGTCTGACAAGGCTCTAA3′	6111-6120		
5-IIImi:5′ATGATGATAATGTCTCTGCCT3′	6536-6516		
Group 6		NIa, NIb	52
6-Ipl:5′GTCTGCTGAATATGGAACGT3′	6350-6360		
6-Imi:5′GAGCTCGGAAGTGCAGTT3′	6589-6572		
6-IIpl:5′CGATCAAAGGCAGAGACAT3′	6509-6527		
6-IImi:5′ATCCTCATCAAAGGCTGAA3′	6837-6819		
6-IIIpl.5′AGTTGGAATACACAGCTTGG3′	6771-6780		
6-IIImi.5′CTCTTTGAAGTGCCGACAC3′		7134-7126	
Group 7		NIb, CP	54
7-Ipl:5′AGAAATCGACAGCACGTGTG3′	8031-8050		
7-Imi:5′GATCAGCTCTATCCCATTGC3′	8290-8271		
7-IIpl:5′AATCGAGGGTATGTACGTGC3′	8217-8236		
7-IImi:5′AGTTCATCATCCAAGGCAAC3′	8540-8521		
7-IIIpl:5′ACAGGACAGTAGATGAGGAGGA3′	8474-8495		
7-IIImi:5′CCACGTATCAAACTGTGATTGA3′	8850-8829		
Group 8		CP, 3′UTR	53
8-Ipl:5′TCGAGTATGCTCCACAGCAA3′	8783-8802		
8-Imi:5′GGTTTCAGTGGGTATTCGACT3′	9011-8991		
8-IIpl:5′GGGTTATGATGGATGGAGATG3′	8966-8986		
8-IImi:5′GTGTACTAATGCCACCGTCC3′	9295-9276		
8-IIIpl:5′TTCAAATGAAGGCCGCAG3′	9224-9241		
8-IIImi:5′ACAACACCACCCAAGCAGA3′	9662-9644		

[a]Position (nucleotide numbering) according to the Hungarian isolate of PVY^{NTN} (EMBL Acc. No. M95491).
[b]Gene regions covered by the primer groups.
[c]Annealing temperature which was used for RT-PCR is given.

Table 2. Biological, serological and molecular genetic characterization of PVY isolates

Isolates	Origin	Serological characteristics			Biological characteristics					RT-PCR with combination of primers						
		Monoclonal antibodies			Tobacco	Indicator plants			Potato tubers	Glais *et al.* (1996)			*TaqI*	W&M	W&S	
		PVY-N	PVY-N	PVY-O/C		Potato plants				c-d	a-d	b-d		1-2	3-4	
		Bioreba	Adgen	Adgen	Samsun	Erstling	Desireé	Maris Bard	Lukawa	PVY	PVY-N	PVY-O	Y-NTN	Y-NTN	Y-NTN	
Nicola	Czech	+	+	–	VN	M	M	M	PTNRD	+	+	–	+	+	+	
Igor	Slovenia	+	+	–	VN	M	M	M	PTNRD	+	+	–	+	+	+	
Granola	Czech	+	+	–	VN	M	M	M	PTNRD	+	+	–	+	+	+	
Hr 193/2	Czech	+	+	–	VN	M	M	M	PTNRD	+	+	–	+	+	+	
Orleans	France	+	–	–	VN	M	M	M	PTNRD	+	+	–	+	+	+	
Karmela	Czech	+	+	–	VN	M	M	M,LL	PTNRD	+	+	–	+	+	+	
Ny	Poland	+	+	–	VN	M,NSt	M	M,LD	PTNRD	+	+	–	+	+	+	
Y-N Weq	Poland	+	–	–	VN	M,NSt	M	M,LL	PTNRD	+	+	–	+	+	+	
TU 64-8	Canada	+	+	–	VN	M,NSt	M	M	PTNRD	+	+	–	+	+	+	
Syn 1 Scot	Canada	+	+	–	VN	M	M	M	–	+	+	–	+	+	+	
PI3 (41/97)	Poland	–	–	+	M	M	M	M	–	+	+	–	+	+	+	
PI5 (12/94)	Poland	+	+	–	VN	M	M,LD	M	PTNRD	+	–	+	–	–		
TU 660 Cal	Canada	+	+	–	VN	M,LD	M	VN,LD	–	+	+	–	–	–		
Hr 220/5	Czech	–	–	+	VN	M	M	M	–	+	+	–	+	+	+	
Hr 387/7	Czech	–	–	+	VN	M	M	M	–	+	+	–	+	+	+	
Nord 242	France	–	–	+	VN	M	M	M	–	+	+	–	+	+	+	
Hr 21/2	Czech	–	–	+	VN	M	M	M	–	+	–	+	–	–		
Y-N Wi	Poland	–	–	+	(VN)	M	M	M	–	+	–	+	–	–		
Hr 469/13	Czech	–	–	+	M	M	M	M,LD	–	+	–	+	–	–		
Irlande O	France	–	–	+	M	M	LL	M	–	+	–	+	–			

Reaction of tobacco and potato plants: M = mosaic; LL = local lesions; VN = vein necrosis; NSt = stem necrosis; LD = leaf dropping; PTNRD = tuber necrotic ringspot symptoms; W&M = Weidemann and Maiss, 1996; W&S = Weilguny and Singhungh, 1998; (+) = positive; (–) = negative.

the published data (McDonald and Singh, 1996), did not produce PTNRD on the sensitive potato cvs used in this study. In addition, no PVY^{NTN}-specific RT-PCR products were observed, thus supporting the results of Weidemann and Maiss (1996). Isolate 12-94 showed biological and serological reactions typical of PVY^{NTN} (including PTNRD), but specific PVY^{NTN} RT-PCR products were not observed. For Syn 1 Scot, PTNRD was not observed, although it was positive for PVY^{NTN} in RT-PCR. The same results were observed for isolate 41-97, although its biological and serological properties corresponded to PVY^{O}. This isolate is quite unusual since it has properties typical of PVY^{O} and yet gives PVY^{NTN}-specific RT-PCR products. It can be concluded that the RT-PCR methods evaluated do not reliably differentiate PVY isolates and that the PVY^{N} subgroup is very variable, thus confirming the work of others (Glais *et al.*, 1998; Dědič and Ptáček, 1998; Singh *et al.*, 1998). The high diversity of PVY may mean that the virus undergoes fast evolutionary changes.

In order to identify PVY isolates quickly, without the need for sequencing, the method of immunocapture RT-PCR probing, using a set of 24 pairs of primers covering eight regions throughout the whole PVY genome was developed. Each region was subdivided into three parts in order to form three distinct cDNA products by RT-PCR, ranging from 220 to 440 bp. These products can be

analyzed easily on both agarose and polyacrylamide gels. Polyacrylamide gel must be used, if thermodynamic analysis of cDNA by TGGE or DNA heteroduplexes (Matoušek *et al.*, 2000) is done. With a broad set of primers, it is possible to easily score individual products in a large range of isolates.

Initial experiments, using the standard PVY^{NTN} isolate of Czech origin from the cv. Nicola, showed that all selected primers led to amplification of specific products (Fig. 1). Five out of nine PVY^{NTN} isolates were identical (Table 3) and were different from the four other PVY^{NTN} isolates, Tu 64-8, Karmela, Ny and Y-N Weq. In Tu 64-8 differences are probably due to nucleotide changes in the genomic regions covering CI, 6K2 ORFs and the 3'UTR. Some differences for the Karmela and Y-N Weq isolates can be predicted from differences in the 6K2, NIa, NIb and CP regions and in the boundary between HC-Pro/P3. In contrast, for the Ny isolate some nucleotide differences were predicted only in the HC-Pro/P3 boundary. As expected, the lowest scores of 12.5 and 29% were observed for the PVY^{O} isolates, Irlande O and Hr 469-13 because the probing system was specifically designed from PVY^{NTN}. These PVY^{O} isolates differed from each other in several regions (Table 3),

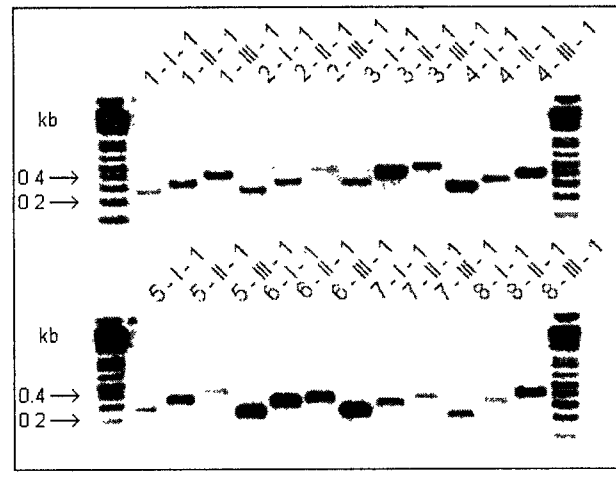

Fig. 1

Analysis of cDNA fragments of PVY^{NTN} Nicola using selected primers covering eight regions of PVY genome (see Material and Methods)

Reaction products (10 µl) were analyzed in 2% agarose gel. The gel was stained for nucleic acids with ethidium bromide and is presented as negative image.

Table 3. Immunocapture RT-PCR probing of PVY isolates

	Primers																							
Isolates	1-I	1-II	1-III	2-I	2-II	2-III	3-I	3-II	3-III	4-I	4-II	4-III	5-I	5-II	5-III	6-I	6-II	6-III	7-I	7-II	7-III	8-I	8-II	8-III
Nicola	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Igor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Granola	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hr 193/2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Orleans	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Karmela	+	+	+	+	+	+	+	–	+	+	+	+	–	–	–		+	–	–	+	+	+	–	+
Ny	+	+	+	+	+	+	+	–	+	+	+	+	+	(+)	+	+	+	+	+	+	+	(+)	(+)	+
Y-N Weq	(+)	+	+	+	+	–	+	–	+	+	+	+	–	–	–	–	–	–	–	(+)	–	(+)	–	(+)
TU 64-8	+	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–
Syn 1 Scot	(+)	+	+	+	+	+	–	+	–	–	+	–	–	+	–	+	–	–	–	+	–	+	+	–
PI3 (41/97)	+	+	+	+	+	+	+	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–
PI5 (12/94)	(+)	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TU 660 Cal	(+)	+	+	(+)	+	+	–	+	–	–	–	(+)	–	+	+	+	+	+	+	+	+	+	+	–
Hr 220/5	+	+	+	+	–	–	+	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
Hr 387/7	+	+	+	+	+	+	+	–	+	+	(+)	+	+	–	–	–	–	(+)	–	–	–	(+)	+	(+)
Nord 242	+	+	+	+	+	+	+	–	+	+	+	+	+	–	–	–	–	–	–	–	–	+	+	+
Hr 21/2	–	–	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	–	–	+	+	+
Y-N Wi	–	–	+	+	+	–	+	–	+	+	(+)	–	–	–	–	+	–	–	–	–	–	–	–	(+)
Hr 469/13	–	–	(+)	(+)	–	–	–	–	–	–	–	(+)	–	–	–	–	–	–	–	–	–	–	–	–
Irlande O	–	–	–	–	–	–	–	–	–	+	+	+	+	(+)	–	–	–	–	–	–	–	–	+	+

+ = positive reaction; (+) = weak positive reaction; – = negative reaction.

suggesting more nucleotide differences. The remaining nine probed PVY samples showed unique patterns, suggesting specific differences at the nucleotide level. Significant parts of these isolates belongs to PVY^{N}W (Chrzanowska, 1991). The greatest differences between PVY^{N}W and the standard PVY^{NTN}, were found in the regions covering NIa, NIb and CP, while the most of positive reactions were observed in the 5′ portion of PVY genome. According to our analyses (Table 2), the remaining isolates have unique combinations of biological, serological and RT-PCR properties. The probing spectra of these isolates are also unique. Isolate 12-94 is very similar to standard PVY^{NTN}, except in the P1 region (Table 3). Because this isolate also elicits PTNRD it is most probably a true PVY^{NTN} isolate. Differences in Tu 660 Cal, Syn 1 Scot and 41-97 occur in various parts of the genome. Our analyses support the theory that some isolates might be recombinants as proposed by Glais *et al.* (1998).

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