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SHORT COMMUNICATION

Evidence of Recombinant Isolates of Potato Virus Y (PVY) in Argentina

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Abstract In order to make a first approach in the identification of the genetic diversity of Potato virus Y (PVY) in Argentina, 46 PVY isolates from different potato growing regions of Argentina were characterized both, biological and serologically. Five of them (ST11, RCA5a, RCA6, RCA14b and SSF6) were selected for further genomic analyses. Four genomic fragments containing hot spot regions of recombination (HSR) reported previously were sequenced in each isolate and compared to PVY^N (CS434575.1) and PVY^O (U09509.1) reference genomes looking for genomic recombinations. Isolates with one, two or three recombination points were identified among these, including the two strains considered typical PVY^N (RCA5b) and PVY^O (SSF18) used as controls. This is the first report of the presence of recombined PVY in Argentina using a combination of biological, serological and molecular tools that shed light on the genetic diversity of PVY viruses in this country.

Resumen Con el fin de hacer un primer acercamiento en la identificación de la diversidad genética del virus Y de la Papa (PVY) en Argentina, se caracterizaron 46 aislamientos de PVY de diferentes regiones productoras de papa de Argentina, tanto biológica como serológicamente. Se seleccionaron cinco de ellos (ST11, RCA5a, RCA6, RCA14b y SSF6) para realizar análisis genómicos posteriores. De cada aislamiento se secuenciaron cuatro fragmentos genómicos que contenían cuatro regiones claves de recombinación (HSR) reportadas previamente y se compararon con los genomas de referencia PVY^N (CS434575.1) y PVY^O (U09509.1) en busca de recombinaciones genómicas. Entre estas, se identificaron aislamientos con uno, dos o tres puntos de recombinación, incluyendo en las dos razas consideradas típicas, PVY^N (RCA5b) y PVY^O (SSF18), usadas como controles. Este es el primer reporte de la presencia de PVY recombinante en Argentina usando una combinación de herramientas biológicas, serológicas y moleculares, que arrojaron luz en la diversidad genética de los virus PVY en este país.

Keywords Hot spot recombination · Sequencing data · PTNRD

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Introduction

Potato virus Y (PVY) is one of the most common disease agents of potato production worldwide. PVY is ubiquitous in potato growing areas and this expansion is probably due to the spread of *inoculi* through asymptomatic potato cultivars (Piche et al. 2004). This plant virus -transmitted by aphids- is the type member of the genus *Potyvirus* (family *Potyviridae*) (De Bokx and Huttinga 1981).

Originally, Singh et al. (2008) described five different strains PVY^C, PVY^O, PVY^N, PVY^Z and PVY^E based on hypersensitive resistance (HR) in potato cultivars harboring

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specific N genes and by their different symptoms in *Nicotiana tabacum* cv. Samsun: PVY^{N} was characterized by producing vein necrosis (VN) on *N. tabacum* leaves, where PVY^{C}, PVY^{O}, PVY^{Z} and PVY^{E} induce mosaic symptoms. Genomic characterization of PVY isolates revealed that many of them have recombinant genomes composed of fragments of PVY^{O} and PVY^{N} sequences (Lorenzen et al. 2006, 2008; Karasev and Gray 2013). Lorenzen et al. (2006) described genome regions where recombination takes place frequently, and named these regions as Hot Spot Regions (HSR).

The three best-studied recombinants are PVY^{NTN} (which contain three to four recombination junctions), $PVY^{N\text{-}Wi}$ and $PVY^{N:O}$, which have two and one recombinant junctions, respectively (Glais et al. 2002; Nie et al. 2004; Lorenzen et al. 2006; Singh et al. 2008). PVY^{NTN} is capable of inducing tuber necrosis, and this symptom is often referred to as potato tuber necrotic ringspot disease (PTNRD) (Le Romancer et al. 1994). $PVY^{N\text{-}Wi}$ was assumed to be a more virulent and aggressive isolate of PVY^{N}, but serologically related to PVY^{O} (Chrzanowska 1994). Since then, isolates with the same characteristics were reported in Canada (McDonald and Singh 1996), Spain (Blanco-Urgoiti et al. 1998) and France (Kerlan et al. 1999). In Canada and USA isolates presenting similar characteristics as $PVY^{N\text{-}Wi}$ were called $PVY^{N:O}$ (Singh et al. 2003). Moreover, isolates of PVY^{Z} and PVY^{E} strain groups presented recombinant genomes, and some of them were found to be non-necrotic in tobacco (Hu et al. 2009; Kerlan et al. 2011; Galvino-Costa et al. 2012a). In South America, PVY^{NTN} has been reported in Peru, where it does not cause necrotic rings in native Andean cultivars, but induces symptoms that may be confused with those caused by other pathogens (Salazar et al. 2000). The presence of the PVY^{NTN} variants has been also confirmed in commercial potato fields in Mexico (Hernandez de la Cruz et al. 2007; Quintero-Ferrer and Karasev 2013), in Brazil (De Ávila et al. 2009; Galvino-Costa et al. 2012b) and Colombia (Gil et al. 2011).

In potato production fields of Argentina, PVY^{N} and PVY^{O} had coexisted with a historic prevalence of PVY^{N} strain (Butzonitch et al. 1996). Since 1996, visual assessments on tubers coming from different areas of the country showed symptoms of PTNRD, and they were related to PVY by serological tests (Colavita et al. 2007). The presence of PVY^{NTN} isolates was later confirmed by amplification with specific primers designed by Schubert et al. (2007), (Colavita 2010). Although some preliminary characterization of a few isolates from Argentina was conducted in the past, no systematic screening of PVY isolates from potato in Argentina was carried out. Moreover, there was no complete information available about PVY isolates present in Argentina, or a systematic characterization using biological indicators such as tobacco or susceptible potato cultivars, combined with modern molecular tools.

Thus, the aim of this work is to confirm for the first time in Argentina the presence of recombinant PVY isolates within 46 PVY isolates from an Argentinian potato collection using biological, serological and molecular data.

Materials and Methods

Forty-six isolates of PVY were obtained in a nine-years period (1996–2005) from *Solanum tuberosum* samples presenting PVY symptoms in leaves and/or tubers. These samples were collected from potato fields at different regions of Argentina and different potato cultivars (Table 1). All the PVY isolates were tested for viral diseases at the Seed Analysis Laboratory of the National Institute of Agricultural Technology of Balcarce by DAS-ELISA.

PVY isolates were biologically characterized by visual description of symptoms in leaves of *Nicotiana tabacum* cv. Xanthi (tobacco), (three replicates per sample) infected mechanically with a mix of 1 g of PVY infected potato leaves and 5 ml of sodium phosphate buffer (Le Romancer and Kerlan 1991).

At the same time, in two separate tests conducted during 2010 and 2011, potato cv. Calén INTA was inoculated to observe the presence of PTNRD symptoms. This cultivar was previously described to be susceptible and to develop PTNRD (Colavita et al. 2007; Colavita 2010). Previous to inoculation, all plants were tested for PVY presence by DAS-ELISA. Mock-inoculated tobacco and potato plants as negative controls were included. Plants were maintained in a greenhouse free of aphids at 22 °C with a 16:8-h photoperiod. All plants were evaluated from the 7th day after inoculation up to the end of life cycle. Mature potato tubers were harvested and stored at 20 °C in darkness for 2 months to allow intensification of necrotic ringspots.

Three types of commercial antibodies were used to determine the behavior of PVY isolates: an antibody polyclonal (pAbs PVY), an antibody cocktail of monoclonal (mAbs PVY-cock), both from BIOREBA AG (Switzerland), and monoclonal antibodies specific MAb 1 F5 (reacts with isolates belonging to the PVY^{N}, PVY^{NTN}, $PVY^{NA\text{-}N}$, and PVY^{O}-O5 strains), MAb2 (reacts with PVY^{O}, PVY^{O}-O5, $PVY^{N\text{-}Wi/N:O,}$ and PVY^{C} strains) and mAbs PVY^{C}, from AGDIA, Elkhart, IN (USA) according to manufacturer's descriptions. Serologic testing was performed by the method ELISA double sandwich of antibodies (DAS-ELISA) according to Clark and Adams (1977), and triple sandwich (TAS-ELISA).

Seven isolates were selected for further molecular analyses (ST11, RCA5a, RCA6, RCA14b, SSF6, SSF18 and RCA5b). Total RNA of each isolate was extracted from 100 mg of infected leaves tissue of tobacco plants using the Trizol® Reagent method (Invitrogen, USA). Four

Table 1 Serological and biological characterization of 46 PVY isolates

Isolate	Cultivar	Origin	ELISA	Biological characterization	
				Symptoms on *N. tabacum* cv. Xanthi	Symptoms on potato tubers cv. Calén INTA
ST 1	Kennebec	SE Bs. As.	N	VN	0
ST 3	Spunta	Mendoza	N	VN	PTNRD
ST 4	Spunta	Mendoza	N	VN	PTNRD
ST 5	Araucana INTA	SE Bs. As.	N	VN	PTNRD
ST 7	Calén IINTA	SE Bs. As.	O	MO	PTNRD
ST 8	Calén IINTA	SE Bs. As.	N	VN	PTNRD
ST 11	Kennebec	SE Bs. As.	N	VN	PTNRD
ST 12	unknown	unknown	N	VN	PTNRD
MP 5	Spunta	SE Bs. As.	N	VN	0
MP 6	Kennebec	SE Bs. As.	N	VN	PTNRD
MP 7	Kennebec	SE Bs. As.	N	VN	PTNRD
MP 14	Huinkul	SE Bs. As.	O	VN	PTNRD
MP 16	Huinkul	SE Bs. As.	O	MO	PTNRD
MP 17	Huinkul	SE Bs. As.	O	VN	PTNRD
MP 19	Spunta	W Bs. As.	N	VN	PTNRD
MP 20	Spunta	W Bs. As.	N	VN	PTNRD
MP 21	Spunta	W Bs. As.	N	MO	PTNRD
MP 23	Spunta	SE Bs. As.	N	VN	0
RCA 1	Nicola	SE Bs. As.	N	VN	0
RCA 2	Nicola	SE Bs. As.	O	VN	0
RCA 5a	Americana INTA	Mendoza	O	VN	0
RCA 5b	Americana INTA	Mendoza	N	VN	0
RCA 6	Spunta	SE Bs. As	N-O/O5	VN	PTNRD

regions previously described as HSR (Glais et al. 2002; Lorenzen et al. 2006) were amplified by PCR with primers designed using FastPCR software (Kalendar et al. 2009; Table 2), using an alignment of complete genomes of PVY deposited in NCBI (www.ncbi.nlm.nih.gov) at the time of performing this study.

Each PCR product was sequenced in a megaBACE automatic sequencer (GE, Switzerland) in both directions, using amplification primers as sequencing primers (Table 2). Sequence similarity was performed using Basic Local Alignment Search Tool (BLAST) from NCBI site (http://www.ncbi.nlm.nih.gov/), and recombinant searches

Table 1 (Continued)

Isolate	Cultivar	Origin	ELISA	Biological characterization	
				Symptoms on *N. tabacum* cv. Xanthi	Symptoms on potato tubers cv. Calén INTA
RCA 7	Spunta	SE Bs. As	N	VN	PTNRD
RCA 8	Kennebec	SE Bs. As	O	VN	0
RCA 14b	Kennebec	SE Bs. As	C	VN	0
RCA 15	Kennebec	SE Bs. As	N	VN	PTNRD
RCA 16	Kennebec	SE Bs. As	O	VN	0
RCA 19a	Kennebec	SE Bs. As	O	VN	0
RCA 20	Kennebec	SE Bs. As	N	VN	0
SSF 1	Spunta	SE Bs. As	N	VN	PTNRD
SSF 2	Spunta	SE Bs. As	N	VN	PTNRD
SSF 3	Spunta	SE Bs. As	N	VN	PTNRD
SSF 4	Spunta	SE Bs. As	N	VN	PTNRD
SSF 5	Spunta	SE Bs. As	N	VN	PTNRD
SSF 5	Spunta	SE Bs. As	N	VN	PTNRD
SSF 6	Spunta	SE Bs. As	N-O/O5	MO	PTNRD
SSF 7	Spunta	SE Bs. As	N	VN	PTNRD
SSF 8	Spunta	SE Bs. As	N	VN	PTNRD
SSF 9	Spunta	SE Bs. As	N	VN	PTNRD
SSF 11	Spunta	SE Bs. As	N	VN	PTNRD
SSF 13	Spunta	SE Bs. As	N	VN	PTNRD
SSF 14	Spunta	SE Bs. As	N	VN	PTNRD
SSF 16	Spunta	SE Bs. As	N	VN	PTNRD
SSF 17	Spunta	SE Bs. As	N	VN	PTNRD
SSF 18	Russet Burbank	SE Bs. As	O	MO	0

The symptoms on tobacco were recorded after a week post inoculation. The PTNRD symptoms on potato tubers cv. Calén INTA were recorded after 6 weeks post harvest and stored in darkness. The fourth column shows the performance with monoclonal antibodies from AGDIA Inc. Grey color shows the isolates used for molecular analysis

SE Bs. As South East Buenos Aires, *W Bs. As* West Buenos Aires, *VN* vein necrosis, *MO* mosaic, *PTNRD* potato tuber necrotic ring spot disease

Table 2 Four primer pairs designed to amplify four different fragments of PVY genome. Each primer pair contains only one Hot Spot Regions (HSR)

Name of primer	Sequence (5′ → 3′)	PCR fragment size (bp)	Genomic location[a]	Estimated position of the HS (bp)[b]
12- HS1 F	G_{233}CAARCTACCATACTCACCC	664	233–252	499–502
12- HS1 R	C_{896}CACTATCGCCCTTTCGTA		878–896	
12- HS2 F	G_{2109}GAGGATGCCAAGGATTTCAC	612	2109–2129	2320–2328 or
12- HS2 R	T_{2720}CTGCTGCTGACACTCGTA		2702–2720	2392–2419
12- HS3 F	C_{5403}ACTCTYAGGGCTAGATATGC	591	5403–5423	5804–5837
12- HS3 R	T_{5993}CAGGATAGACGTTCTCTTCA		5973–5993	
12- HS4 F	A_{8899}CTGTGATGAATGGGCTTATGG	445	8899–8920	9169–9193
12- HS4 R	T_{9343}ACTTGGAGAGACATCCTCGG		9323–9343	

[a] Numbered according to alignment of all sequences used to design four primer pairs

[b] According to HSR described by Glais et al. (2002) and Hu et al. (2009)

were performed with RDP3 software (*Recombination Detection Program, version 3.44*, Martin et al. 2010).

Results and Discussion

Biological Description of Viral Isolates Inoculated on Tobacco and Potato

Considering that in this work we did not intend to reach to a final strain classification of isolates, but to detect potential PVY recombinants, we used tobacco and potato cv. Calén INTA, along with serological behavior.

Out of the 46 PVY isolates studied, 41 (89.6%) showed VN and death of lower leaves, which remained hanging along the stem in tobacco (Table 1; Fig. 1a), while the other 5 induced systemic mosaic and mottle symptoms (Table 1).

The results of the inoculation of potato cv. Calén INTA showed a variety of symptoms including mosaic, necrosis or premature death of plants 30 days post-inoculation (Table 1; Fig. 1b, c). We found that 33 out of 46 isolates produced PTNRD symptoms (Table 1; Fig. 1d). Within these, ST7, MP16, MP21 and SSF6 isolates showed a mosaic symptom with no VN in tobacco. Only the isolate SSF18 showed mosaic symptoms in tobacco with no PTNRD in Calén INTA.

Serological Identification of Viral Isolates

Analyses with monoclonal antibodies identified epitopes of three strains PVY^N, PVY^O and PVY^C. We found 32 isolates

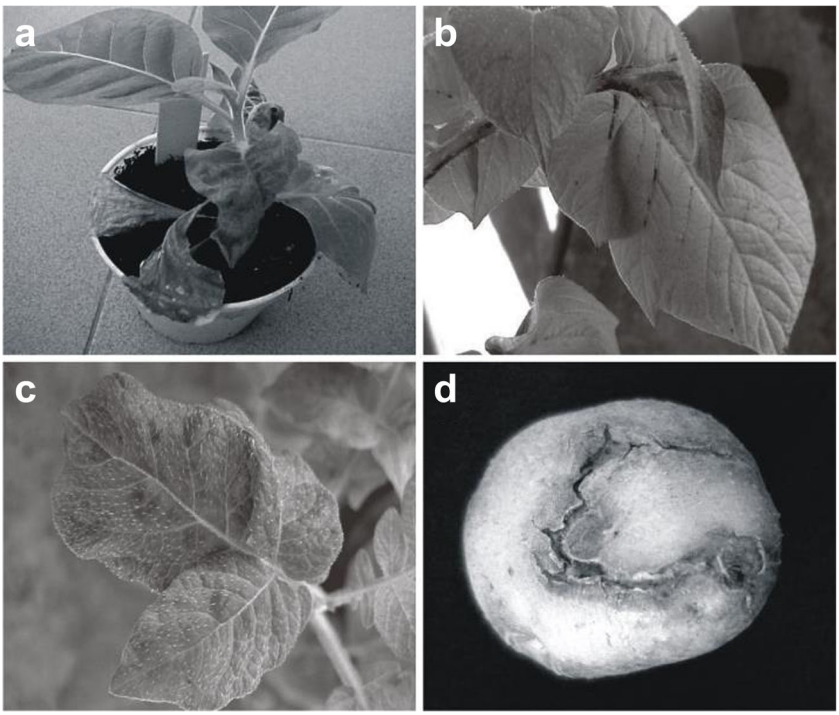

Fig. 1 Symptoms in tobacco and potato cv. Calén INTA of PVY isolates. **a** vein necrosis and death of inferior leaves in plants of *N. tabacum* cv. Xanthi, **b** necrosis in leaves of potato cv. Calén INTA, **c** mosaic in leaves of potato cv. Calén INTA and **d** PTNRD on tuber of potato cv. Calén INTA

Table 3 Summary of Recombination breakpoints (RBs) described in 7 PVY isolates over 4 Hot Spot Regions (HSR). RBs were identified by the RDP3 software

Isolate	HSR	Sequence length[a] (bp)	Position in reference genome[b] (bp)	Statistical Values[c] (P)	Switch[d]
SSF18	2	571 (214)	2347	1.3×10^{-10}– 1.54×10^{-14}	N-O
RCA14b	2	351 (114)	2465	$2.6 \times 10^{-3} - 2.4 \times 10^{-7}$	N-O
RCA5a	2	564 (282)	2418	$1 \times 10^{-9} - 2.1 \times 10^{-13}$	N-O
	3	529 (390)	5825	$2 \times 10^{-3} - 1.1 \times 10^{-6}$	O-N
RCA5b	2	564 (282)	2418	$1 \times 10^{-9} - 2.8 \times 10^{-14}$	N-O
	4	378 (240)	9175	$1.5 \times 10^{-3} - 5.9 \times 10^{-6}$	N-O
RCA6	2	555 (190)	2328	$3 \times 10^{-8} - 3.6 \times 10^{-11}$	N-O
ST11	2	574 (285)	2420	$1.8 \times 10^{-6} - 5.8 \times 10^{-12}$	N-O
	3	526 (368)	5804	$4.3 \times 10^{-5} - 3.8 \times 10^{-7}$	O-N
SSF6	2	565 (191)	2327	$1.1 \times 10^{-7} - 1.2 \times 10^{-9}$	N-O
	3	547 (388)	5804	$2.1 \times 10^{-3} - 2.6 \times 10^{-7}$	O-N

[a] Sequence length of each fragment. In brackets, position in each fragment where RB was detected

[b] Position of RB in genome alignment between PVY^{O} (U09509.1), the isolate and PVY^{N} (CS434575.1)

[c] Range of P values of 7 different statistical analysis used in RDP3. All of them detected the RB

[d] Taking into account percentage of identity of fragments to PVY^{O} or PVY^{N} reference genomes at both sides of the RB (data not shown)

with concordance in symptoms and serology data: 26 isolates reacted to mAb-PVY^{N} and showed VN in tobacco and PTNRD, and 6 isolates reacted to mAb-PVY^{N} and presented VN in tobacco and no PTNRD. The isolates MP14, MP17, RCA2, RCA5a, RCA8, RCA16 and RCA19a reacted to mAb-PVY^{O}, but they induced VN in tobacco (Table 1). Similar behavior were reported in recombinant $PVY^{N:O}$ variants (Singh et al. 2003) and $PVY^{N\text{-}Wi}$ (Chrzanowska 1994). In addition, isolates MP14 and MP17 showed PTNRD. Also, ST7 and MP16 despite being positive to mAb PVY^{O} and present mosaic symptoms in tobacco, they induced PTNRD, attributable to a PVY^{NTN} variant. The isolate RCA14b reacted to mAb PVY^{O+C} and it showed VN in tobacco with no PTNRD.

These results suggest either the occurrence of recombinant strains or specific point mutations that affect symptomatology and serological reactivity. Previous studies have described that specific changes in lysine (K400) and glutamic acid (E419) in the C-terminus of HC-Pro protein can result in the loss of ability to induce necrosis in tobacco (Tribodet et al. 2005). Additionally, Hu et al. (2009) reported that the substitution of aspartic acid (D205) to glycine (G205) in the HC-Pro protein is also associated with loss of induction of necrosis in tobacco. Any of these changes would generate a PVY^{O} -like strain with novel capacity to induce VN in tobacco; or a PVY^{NTN} -like strain without capacity to induce VN in tobacco.

Also, we found two isolates (RCA6 and SSF6) that showed simultaneous positive reactions with mAb PVY^{N} and mAb PVY^{O+C} evidencing the presence of epitopes for both antibodies. Both isolates presented symptoms in tubers, but RCA6 caused VN while SSF6 showed mosaic in tobacco leaves. These results suggest a possible mixture of strains. However, the chromatograms derived from PCR sequencing of this isolates showed no overlapping peaks along the sequences at the HSR studied (work not shown). Alternatively, these results can be explained by a recombinant nature of the isolates or specific mutations that affect serological reactions and/or the capability to produce symptoms in tobacco (Chikh-Ali et al. 2007; Karasev et al. 2010). Moreover, Tribodet et al. (2005) reported a PVY^{NTN} -like strain without capacity to induce VN in tobacco. On the other hand, we cannot dismiss the possibility that these isolates could be a variant of $PVY^{N\text{-}Wi}$ with PVY^{O} -05 serological reactivity described in Brazil (Galvino-Costa et al. 2012b), due to both, Mab2 and mAb 1 F5 used to detect PVY^{N} can react with the PVY^{O} -05 serotype (Karasev et al. 2010, 2011; Nikolaeva et al. 2012).

Recombination-Detection Analyses

Based on symptoms and serology, we selected five possible recombinant isolates (ST11, RCA5a, RCA6, RCA14b and SSF6) to investigate the presence of recombinant sequences around reported HSR in the PVY genome, included two putative "non recombinant" isolates: SSF18 (as typical PVY^{O} strain) and RCA5b (as typical PVY^{N} strain). The percentage of identity with PVY^{N} and PVY^{O}, the position of recombination breakpoint (RB), the length of the analyzed fragment and the tentative orientation of the recombination are shown in Table 3. The sequences of the amplicons obtained from all isolates were deposited in NCBI under accession numbers KF978106 through to KF978123 and KF986217 through to KF986224.

We found recombination events in at least one previously defined HSR (Glais et al. 2002; Lorenzen et al. 2006) in all isolates studied, including the two isolates SSF18 and RCA5b

chosen as reference (Table 3). We detected three patterns of recombination: i) isolates with one recombination event (SSF18, RCA14b and RCA6), ii) isolates with two recombination events (RCA5, ST11 and SSF6) and iii) one isolate with two recombination events, different from ii) (RCA5b). In particular, HSR1 did not show recombination events in any of the isolates, and all of them showed PVY^N sequences. HSR2 presented recombination in all seven isolates, with a sequence that shifts from PVY^N to PVY^O. We found three isolates with recombination in HSR3 (RCA5a, ST11 and SSF6) that reverse sequences from PVY^O back to PVY^N. Finally, only one isolate (RCA5b) showed recombination in HSR4 with a sequence that suggests a shift from PVY^N to PVY^O, this segment encodes part of the capsid protein, involved in the ability to generate PTNRD in PVY^{NTN} strains (Boonham et al. 2002).

RCA5b presented the same direction change (PVY^N to PVY^O) in HSR2 and HSR4, suggesting the occurrence of an undetected recombination event between these two regions, that would shift from PVY^O to PVY^N. We re-analyzed the sequence of HSR3 for this isolate, reducing the stringency of the parameters employed by RDP3 software. We have detected one possible recombination event in the last 40 nucleotides of the amplicon corresponding to a change from PVY^O to PVY^N in HSR3, with three of the statistical methods used by the program, with p-values between 10^{-5} and 10^{-3}. This could be indicating that RCA5b presents three recombination events.

There are growing evidences -since Koonin (1991) to Karasev and Gray (2013)- that recombination is a major factor in development of plant RNA viruses. These sequence variations can lead to changes in virulence, symptomatology and the emergence of new viral strains (Chare and Holmes 2006).

This study reports for the first time the presence of recombined PVY in Argentina. Moreover, recombination was also detected in the isolates selected as "controls" due to their biological and serological behavior as typical PVY^N or PVY^O strains, suggesting that recombination in virus strains is a frequent event that can go undetected, based solely on biological and ELISA results. For a proper strain classification, multiplex RT-PCR (Chikh-Ali et al. 2013) and complete sequence data for each isolate would be appropriate (Massa et al. 2008).

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