Detection and genome characterization of *Potato virus Y* isolates infecting potato (*Solanum tuberosum* L.) in La Union (Antioquia, Colombia)

Detección y caracterización del genoma de aislamientos de *Potato virus Y* infectando papa (*Solanum tuberosum* L.) en La Unión (Antioquia, Colombia)

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ABSTRACT

Potato virus Y (PVY) is one of the most severe viruses affecting the production of potato (*Solanum tuberosum*) in the world. This study presents a detailed molecular analysis using next-generation sequencing (NGS), IC-RT-qPCR and RT-PCR on the PVY isolates infecting seed-tubers and foliage of potato plants cv. Diacol-Capiro in La Union (Antioquia, Colombia). Analysis of incidence by IC-RT-qPCR in 15 random leaf samples of three cultivation plots and fifteen sprouting tuber eye-buds reveal infection levels between 13.4 and 80%; a higher incidence of 86.7% was observed in seed-tuber samples with threshold cycle (Ct) values as low as 24.3. Genome assembly from a bulk of foliage samples resulted in a consensus PVY genome (PVY_LaUnionF) of 9,702 nt and 399 polymorphic sites within the polyprotein ORF; while the assembled genome from sprouts of tubers has 9,704 nt (PVY_LaUnionT) and contained only six polymorphic nucleotide sites. Phylogenetic analysis demonstrates that the PVY isolates from leaf samples are in the recombinant PVY[NTN] group (sequence identity >99%); while those from tuber sprouts are in the PVY[N/NTN] group with identities above 95%. Sanger sequencing of viral capsid suggests the presence of a third variant related to PVY[O], a prevalent strain reported in potato fields worldwide.

Key words: ELISA, genomics, plant viruses, potyviruses, variants.

RESUMEN

El *Potato virus Y* (PVY) es uno de los virus más severos que afectan la producción de papa (*Solanum tuberosum*) en el mundo. Este trabajo presenta un análisis molecular detallado utilizando secuenciación de nueva generación (NGS), IC-RT-qPCR y RT-PCR, de aislamientos de PVY procedentes de tubérculo-semilla y tejido foliar de plantas de papa cv. Diacol-Capiro en el municipio de La Unión (Antioquia, Colombia). Las evaluaciones de incidencia de PVY utilizando IC-RT-qPCR en 15 muestras aleatorias de tres lotes, indican niveles variables de infección de este virus (13,4 a 80%), mientras que su ocurrencia fue muy alta (86,7%) en 15 muestras de brotes de tubérculo-semilla, con valores de Ct (ciclo umbral) tan bajos como 24,3. El ensamblaje del genoma viral obtenido a partir de una mezcla de muestras de tejido foliar, resultó en una secuencia consenso (PVY_LaUnionF) de 9.702 nt y 399 sitios polimórficos dentro del marco de lectura abierto (ORF) que codifica para la poliproteína. El genoma ensamblado a partir de la mezcla de brotes de tubérculo-semilla presentó un tamaño de 9.704 nt (PVY_LaUnionT) y tan sólo seis sitios polimórficos. Los análisis filogenéticos indican que los aislamientos procedentes de tejido foliar hacen parte del grupo recombinante de PVY[NTN] (porcentaje de identidad >99%), mientras que los aislamientos de brotes de tubérculos se ubicaron en el clado PVY[N/NTN] con porcentajes de identidad superiores al 95%. La secuenciación de Sanger de la cápside viral detectó una tercera variante relacionada con PVY[O], una raza con altos niveles de prevalencia en cultivos de papa del mundo.

Palabras clave: ELISA, genómica, potyvirus, virus de plantas, variantes.

Introduction

Potato virus Y (PVY), the type species of the genus *Potyvirus* (*Potyviridae*), is one the most important viruses affecting the production of potato (*Solanum tuberosum* L.) worldwide (Karasev and Gray, 2013). Foliar symptoms associated to PVY can result in yield reductions between 40% and 70%, while tuber damage significantly compromises its quality and marketability (Nolte *et al.*, 2004; Fageria *et al.*, 2013). PVY can be transmitted mechanically, in a non-persistent manner by aphids such as *Myzus persicae* or vertically, by infected seed-tubers (Karasev and Gray, 2013). Symptoms induced by PVY are highly dependent on the type of strain and host cultivar and may include leaf mosaics, mottle and crinkling, vein necrosis and necrotic spots; some variants can also induce necrotic ringspots in

the tuber skin (Quenouille *et al.*, 2013). Additionally, mixed infection by PVY and *Potato virus X* (PVX) in several solanaceous crops cause a synergistic interaction that can reduce yields up to 80% (Vance, 1991). PVY can also affect other Solanaceous crops such as tobacco (*Nicotiana tabacum*), pepper (*Capsicum annumm*) and tomato (*Solanum lycopersicum*) (Scholthof *et al.*, 2011).

PVY virions consist of flexuous particles of about 730 nm in length and a diameter of approximately 11 nm and are composed of a positive-sense single stranded RNA of approximately 9,000 nt encapsidated by roughly 2,000 coat proteins subunits (CP) (Ivanov *et al.*, 2014); a covalently linked virus-encoded protein (VPg) is bound to the 5' end (Dougherty and Carrington, 1998; Revers and García, 2015). The PVY genome encodes for a polyprotein of 3060-3063 amino acids, which after processing by three viral encoded proteases, gives rise ten mature protein products: P1, HC-Pro, P3, 6K1, CI, 6K2, NIa-VPg, NIa-Pro, NIb and CP (Dougherty and Carrington,1998; Revers and García, 2015). Recent studies have shown that potyvirus contain two additional ORFs named PIPO (Pretty Interesting Potyviridae ORF) and PISPO (Pretty Interesting Sweet Potato Potyvirus ORF) expressed as a result of RNA polymerase transcriptional slippage (Olspert *et al.*, 2015; Untiveros *et al.*, 2016).

The PVY group consists of at least five strains (PVY[O], PVY[N], PVY[C], PVY[Z] and PVY[E]) differentiated by the symptoms induced on specific hosts, serological typing and/or nucleotide sequence (Singh *et al.*, 2008). PVY[C] and PVY[O] comprise strains that can induce a hypersensitive resistance response (HR) in potato cultivars with the *Nc* and *Ny* genes, respectively (Karasev and Gray, 2013). PVY strains that do not induce HR genes and cause systemic necrosis in tobacco belong to the PVY[N] group, while strains that fail to induce necrosis in tobacco and do not produce the typical symptoms of PVY[O] and PVY[C] in their corresponding cultivars are classified as PVY[Z], as this strain elicits the Nz gene (Singh *et al.*, 2008; Kerlan *et al.*, 2011). During the past decades genome sequencing has revealed that many PVY strains such as PVY[N:O], PVY[N-Wi] and PVY[NTN] are the result of recombination between PVY[O] and PVY[N] (Karasev and Gray, 2013). Recombinant strains have received increased attention as they are associated with tuber necrotic ringspot disease in potato (PVY[NTN]) and tobacco veinal necrosis (PVY[N:O], PVY[N-Wi]) (Ogawa *et al.*, 2008).

Due to the emerging importance of PVY, several studies have been conducted in the potato producing regions of Colombia that demonstrated the global presence of PVY[N] and PVY[NTN] (Gil *et al.*, 2011; Villamil *et al.*, 2014), in addition to two divergent PVY clades named as I-Col y IV-Col (Gallo *et al.*, 2012; Henao *et al.*, 2013). Villamil *et al.* (2014) obtained the first complete PVY genome in Colombia using next-generation sequencing (NGS) of siRNA from *S. tuberosum* plants in Cundinamarca; they also found that *Potato yellow vein virus* (PYVV) and PVY co-infect potato plants with a prevalence of 21% within the Phureja group and 23% within the Andigena group. A recent study using TAS-ELISA and RT-qPCR, revealed widespread PVY infection in *S. tuberosum* seed-tubers used as planting material in the municipalities of La Union and Yarumal in the province of Antioquia (Medina *et al.*, 2015). Further work confirmed the generalized presence of PVY infecting potato crops in Yarumal and next-generation sequencing (NGS) resulted in the assembly of a complete PVY genome with phylogenetic affinity to necrotic PVY[NTN] strains (Muñoz *et al.*, 2016). The aim of this study was to perform a detailed molecular analysis on the PVY isolates infecting commercial seed-tubers and potato plants cv. Diacol-Capiro in three plots from La Union (Antioquia) using ELISA, IC-RT-qPCR, RT-PCR and NGS analysis. The presence of two different PVY strain types with phylogenetic affinities to the PVY[N] and PVY[NTN] is demonstrated and their relationship to previously sequenced genome discussed. Sanger sequencing also suggests the presence of PVY[O] in this Colombian region.

Materials and methods

Plant samples and IC-RT-qPCR

Immunocapture (IC) was conducted following the method of Wetzel *et al.* (1992) using the Triple Antibody Sandwich PVY detection kit from Agdia (Elkhart, IN). Fifteen random leaf samples collected in three (A, B and C) commercial potato cv. Diacol-Capiro fields (for a total of 45 samples), and fifteen sprouting eye-buds (1-2 cm) of seed-tuber samples from storage commercial cellars in La Union (Antioquia) were evaluated; these tubers were not related to plants sampled in the field plots. Positive and negative controls consisted of lyophilized plant tissue purchased from Agdia (Elkhart, IN). For cDNA synthesis, 12.5 µL of sample released after TAS-ELISA using Tris-HCl 10 mM pH 8.0 and 1 % Triton X 100 were used as template. The reaction also included 200 U of Maxima Reverse Transcriptase, 1X RT buffer, 0,5 mM dNTPs, 100 pmol Oligo-dT primers and 20 U of RNAse inhibitor (Thermo Fisher Scientific, Waltham, MA) and was incubated at 65°C for 5 min, followed by 50°C for 30 min and 85°C for 5 min in a T3 thermal cycler (Biometra, Germany). For the qPCR step, the Maxima SYBR Green/ROX qPCR Master

Mix (2X) kit (Thermo Fisher Scientific, Waltham, MA) was used in 25 µL of reaction containing 12.5 µL of mix, 10 µL DEPC water, 20-100 ng cDNA and 0.3 µM of specific primers PVY-1 FP (5′CCA ATC GTT GAG AAT GCA AAA C 3′) and PVY-1 RP (5′ATA TAC GCT TCT GCA ACA TCT GAG A 3′), designed by Singh *et al.* (2013) for specific amplification of PVY strains using RT-qPCR with Taqman probes. Amplification cycles consisted of 10 min at 95°C to activate the polymerase, followed by 35 cycles at 95°C for 15 s and 53°C for 45 s, using a Rotor-Gene Q-5plex Platform (Qiagen, Hilden, Germany); fluorescence was measured after each amplification cycle. The samples were considered positive if they exhibited fluorescence values higher than the threshold before the 35th cycle (Schena *et al.*, 2004). Primer specificity was verified by High Resolution Melting in the 50 and 99°C range and the identity of five amplicons, including the positive control, was confirmed by Sanger sequencing.

Next-generation Sequencing and Sanger sequencing

High-throughput sequencing of the *S. tuberosum* cv. Diacol-Capiro transcriptome was performed on a sample from a bulk of leaves with rugose mosaics, a symptom typically associated with PVY infection, and previously tested using TAS-ELISA with the Triple Antibody Sandwich detection kit specific for PVY strains (SRA 20001/0096) from Agdia (Elkhart, IN). Samples were ground in liquid nitrogen and 100 mg of the remaining powder were used for RNA extraction with the GeneJET Plant RNA Purification Mini Kit (Thermo Fisher Scientific, Waltham, MA). The library was constructed with the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA) and rRNA depleted with the TruSeq Stranded Total RNA with Ribo-Zero Plant kit (Illumina, San Diego, CA). Sequencing was performed with the Illumina HiSeq 2000 system service provided by Macrogen (Seoul, South Korea). A similar procedure was followed for the tuber transcriptome; in this case, a bulk sample consisting of ten asymptomatic sprouting eye-buds (1-2 cm) from different Diacol-Capiro potato seed-tubers were used. Adapter sequences and low quality bases were removed with SeqTK (https://github.com/lh3/seqtk). Sequence assembly was performed with Trinity (Grabherr *et al.*, 2011) and the contigs corresponding to PVY genome were identified by a local BLASTN search. Both PVY genomes assemblies were confirmed by mapping with Bowtie2 (Langmead and Salzberg, 2012) and checked for inconsistencies and assembly artifacts with Tablet (Milne *et al.*, 2010). Consensus sequences were deposited in GenBank under accession codes KR149260 (PVY_LaUnionF) and KX531041 (PVY_LaUnionT). Additionally, the CP (801

nt) region was amplified by RT-PCR in six symptomatic foliage samples obtained from single plants (LU1, LU2, LU3, LU4, LU5 and LU6) from La Union using primers CP PVYCPF (5′ACC ATC AAG SAA ATG ACA CA 3′) and PVYCPR (5′ CGG AGA GAC ACT ACA TCA CA 3′) (Glais *et al.*, 2002) following the protocol reported by Henao *et al.* (2013) and sequenced by the Sanger method (GenBank accession codes KR902698-KR902703) after gel purification using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA).

Bioinformatic analysis

ORFs codifying for viral proteins were identified with BLASTX (Gish and States, 1993). The phylogenetic analysis was performed in MEGA6 (Tamura *et al.*, 2013) by the Neighbor-Joining method with 1,000 bootstrap replicates using the Tamura 3-parameter method (Tamura, 1992). Rate variation among sites was modeled with a gamma distribution (shape parameter = 0.3). Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013). The rates of non-synonymous substitutions per non-synonymous site (Ka) and synonymous substitution per synonymous site (Ks) were estimated by the Nei-Gojobori method using a sliding window of 14 codons (Nei and Gojobori, 1986). The recombination analysis was performed by measuring the local nucleotide percent identity along the genome using in-house perl scripts and plotted with a sliding window of 100 nucleotides; sequences PVY[N] (AY8849983), PVY[O] (EF026074) and PVY[C] (AJ439544) were used as parents. Multidimensional scaling analysis was performed with cmdscale function in R (www.R-project.org) with distances calculated by the Kimura 2-parameter method (Kimura, 1980).

Results

IC-RT-qPCR detected PVY in all plots (Tab. 1). In plot A, five plants out of 15 were found to be infected (33.3%) with Ct values in the 25.9 to 33.2 range and melting temperatures between 76.3 and 76.5°C. Plot B exhibited the highest incidence between foliage samples (12/15, 80%) with Ct values as low as 26.4 and an average Tm of 76.7±0.4°C in agreement with the melting temperatures measured in plot A. Only two out of 15 leaf samples tested positive in plot C (13.3%) with Ct values of 30.1 and 31.4; Tm values were 76.8 and 78.1, respectively. In contrast, the incidence of PVY in tuber samples was 80% (out of 15 samples) with Ct values as low as 23.7 and an average melting temperature of 77.3±0.9°C. An overview of the Tm data reveals a 1.8°C difference between the extreme values (76.3°C and 78.1°C), suggesting the presence of sequence variants.

TABLE 1. IC-RT-qPCR detection of PVY in leaf samples from three potato cv. Diacol-Capiro cultivating plots (A-C) and sprouting eye-buds of tubers (T) from La Union (Antioquia).

	Plot A			Plot B			Plot C			Tubers	
	CT1	Tm2 (ºC)		Ct	Tm (ºC)		Ct	Tm (ºC)		Ct	Tm (ºC)
A1	>35	-	B1	>35	-	C1	>35	-	T1	28.1	77.8
A2	>35	-	B2	29.8	77.2	C2	>35	-	T2	27.1	77.2
A3	>35	-	B3	26.4	76.4	C3	>35	-	T3	30.9	76.6
A4	30.3	76.5	B4	29.1	77.1	C4	>35	-	T4	28.2	78.1
A5	>35	-	B5	29.2	77.2	C5	>35	-	T5	27.4	77.4
A6	28.7	76.5	B6	30.4	77.1	C6	>35	-	T6	27.2	77.6
A7	33.2	76.3	B7	>35	-	C7	>35	-	T7	30.1	77.5
A8	>35	-	B8	28.8	76.4	C8	>35	-	T8	>35	-
A9	>35	-	B9	28.7	76.3	C9	>35	-	T9	32.1	78.1
A10	>35	-	B10	28.3	76.9	C10	30.1	78.1	T10	31.1	77.5
A11	28.2	76.5	B11	29.6	76.3	C11	>35	-	T11	>35	-
A12	>35	-	B12	28.2	76.5	C12	31.4	76.8	T12	>35	-
A13	>35	-	B13	>35	-	C13	>35	-	T13	24.3	77.4
A14	25.9	76.3	B14	29.4	76.56	C14	>35	-	T14	23.7	78.1
A15	>35	-	B15	29.6	76.86	C15	>35	-	T15	25.7	77.6

[1]Ct: threshold cycles below 35 were considered positive.

[2]Tm: melting temperatures.

To get a more detailed characterization of the PVY strains infecting *S. tuberosum* cv. Diacol-Capiro in La Union, a high-throughput transcriptome sequencing was carried out on bulks from both foliage and sprouting eye-buds from tuber samples; PVY infection was confirmed by ELISA prior sequencing. A total of 4,754,725 paired-end reads were obtained from the infected leaf-sample with 7,750 reads per million (RPM) attributed to PVY. Assembly resulted in a consensus PVY genome (PVY_LaUnionF) of 9,702 nt (excluding the poly-A tail) with an average sequence depth of 3,110x (Fig. 1A). The consensus sequence contains 449 polymorphic sites with a transition/transversion ratio of 0.85; the high proportion of polymorphisms suggests the presence of mixed variants within the sample, which is not a surprising result when bulk samples are used for sequencing. The ORF encoding the polyprotein is located at positions 188 and 9,373; frameshift product P3N-PIPO is predicted at nucleotide positions 2,474-2,926. The polyprotein ORF contains 399 polymorphic sites that translate into 67 amino acid changes; as expected, most substitutions map to the third codon position (316). Percentagewise, most changes are amino acid changes are located in the 6K1 (7/52, 13.5%), 6K2 protein (5/52, 9.6%), P1 (25/285, 8.8%) and P3 (25/364, 6.8%). CP (3/267, 1.1%) and NIb (13/519, 2.5%) showed the lowest degrees of variation. Isolate PVY_LaUnionF shares 99.8% sequence identity with a similar isolate (PVY_Yarumal: KT336551) from the municipality

of Yarumal in the north of Antioquia with phylogenetic affinity to PVY[NTN] strains (Muñoz *et al.*, 2016).

The high levels of variation observed in the PVY foliage assembly may be indicative of sequences experiencing diversifying selection. To address this point, the ratio (Ka/Ks) of non-synonymous substitutions per non-synonymous site (Ka) to synonymous substitution per synonymous site (Ks) was measured along the polyprotein coding region using a sliding window of 14 codons (Fig. 2). Ka/Ks values could be determined for 1,831 sites; 1,473 (80.4%) were under strong purifying selection (Ka/Ks=0), 355 were under weak purifying selection (19.4%, 0< Ka/Ks <1) and three sites under either neutral or weak positive selection (Ka/Ks >1). All windows under positive selection were located within the P1 protein and centered at amino acid positions 59 (Ka/Ks=1.05), 60 (Ka/Ks=1.0) and 63 (Ka/Ks=1.09), comprising the amino acid sequence (I/V)RTSK(N/S)G(T/A). A region centered at residue 2,083 within NIa-Pro showed a Ka/Ks very close to the threshold value (0.97) and comprises a region with the sequence (H/D)LFKSLNGS(T/M)E(A/V).

The tuber transcriptome, on the other hand, resulted in a data set comprising 4,754,725 paired-end reads of which 8,256 (1,736 RPM) were attributed to PVY. The assembled genome consists of a 9,704 nt contig (excluding the polyA tail) with average sequence depth of 170x and was named
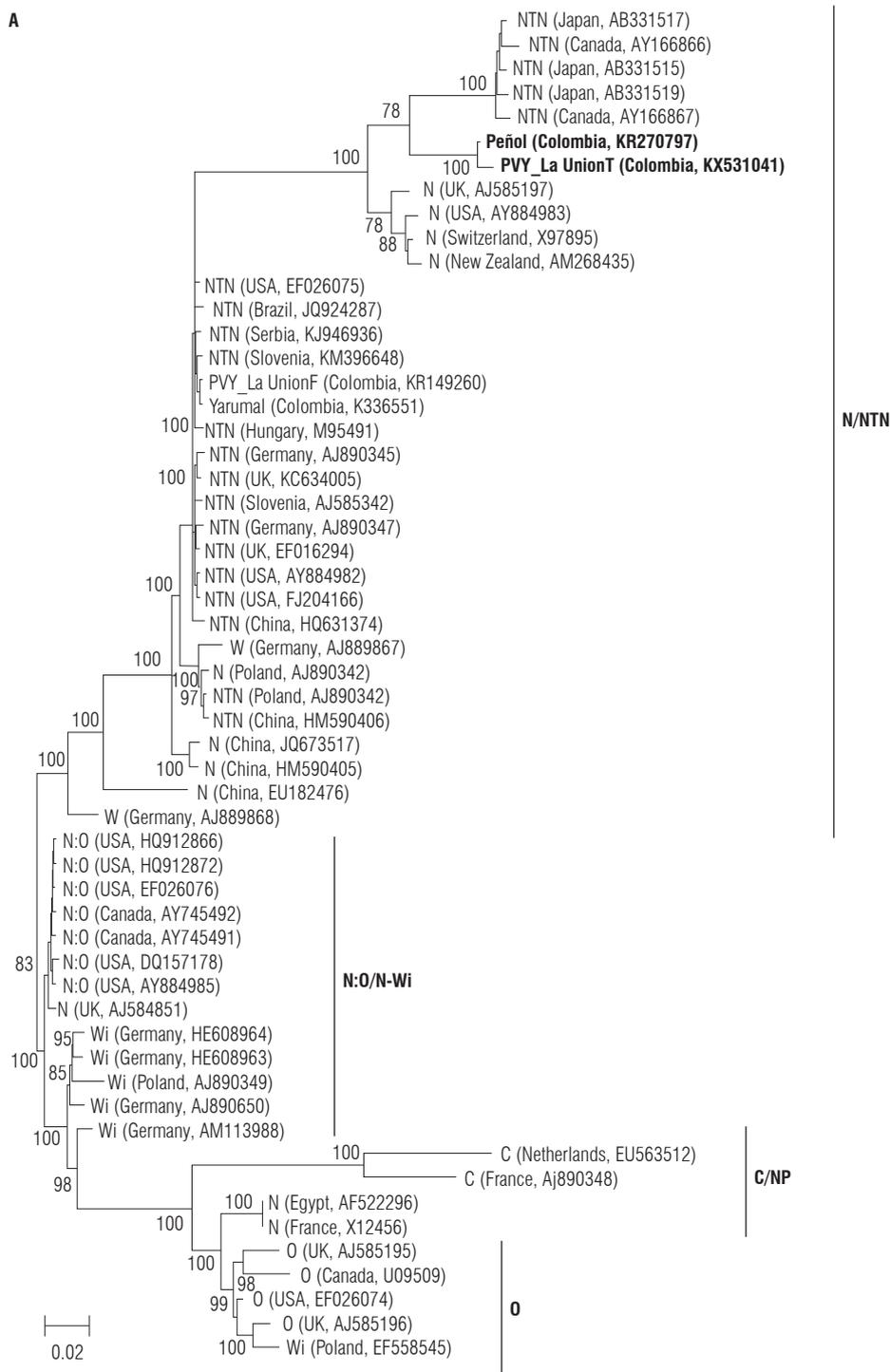
FIGURE 1. Sequence coverage and mapping of the PVY genomes infecting potato foliage and sprouting eye-buds of tubers in La Union (Antioquia). The relative position of each mature protein in PVY is show on top of each panel; viral protease cleavage sites are shown. The PVY isolated from bulked foliage (PVY_LaUnionF, KR149260) exhibits a very high sequence coverage (3110 x) and variability with 449 polymorphic sites suggesting the presence of mixed variants in the bulked sample. In contrast, the PVY strain from a bulk of tuber sprouts (PVY_LaUnionT, KX531041), resulted from an assembly with 170x coverage and only six polymorphisms.
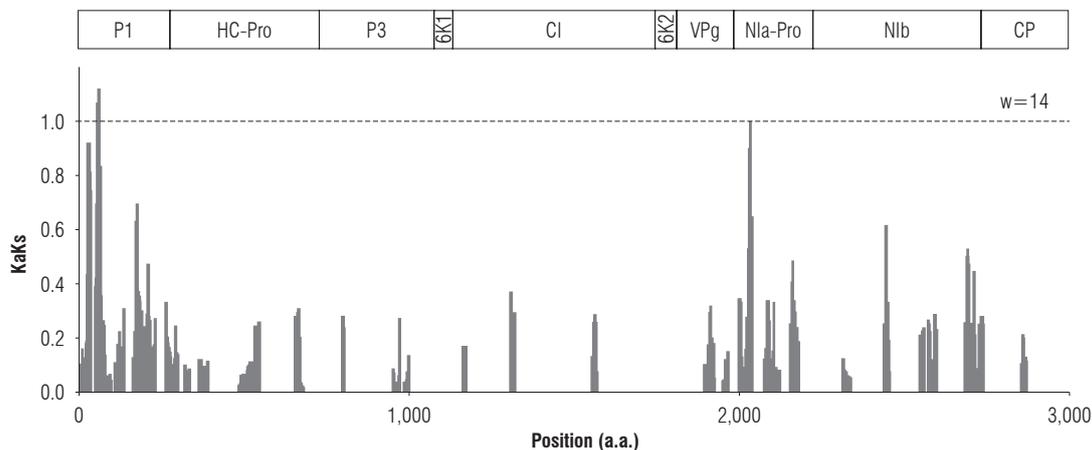
FIGURE 2. Sliding window analysis of genome variants of the PVY consensus isolated from potato foliage in La Union (Antioquia). A window size of 14 was used. Ka/Ks ratios larger than one are indicative of positive selection.

TABLE 2. Pairwise sequence comparison between PVY_LaUnionF (KR149260) and PVY_LaUnionT (KX531041). Values correspond to the percent identity between the consensus sequences at the nucleotide (nt) and amino acid level (a.a.).

		5'UTR	P1	HC-Pro	P3	6K1	CI	6K2	NIa-VPg	NIa-Pro	NIb	CP	3'UTR
% id.	(nt)	87.5	92.2	93.1	85.9	82.7	85.4	84.1	91.9	94.4	95.1	95.6	86.5
	(a.a)	–	92.4	97.6	94.0	86.5	95.4	90.4	96.8	97.5	98.5	99.2	–

A

NTN (Japan, AB331517)
NTN (Canada, AY166866)
NTN (Japan, AB331515)
NTN (Japan, AB331519)
NTN (Canada, AY166867)
Peñol (Colombia, KR270797)
PVY_La UnionT (Colombia, KX531041)
N (UK, AJ585197)
N (USA, AY884983)
N (Switzerland, X97895)
N (New Zealand, AM268435)
NTN (USA, EF026075)
NTN (Brazil, JQ924287)
NTN (Serbia, KJ946936)
NTN (Slovenia, KM396648)
PVY_La UnionF (Colombia, KR149260)
Yarumal (Colombia, K336551)
NTN (Hungary, M95491)
NTN (Germany, AJ890345)
NTN (UK, KC634005)
NTN (Slovenia, AJ585342)
NTN (Germany, AJ890347)
NTN (UK, EF016294)
NTN (USA, AY884982)
NTN (USA, FJ204166)
NTN (China, HQ631374)
W (Germany, AJ889867)
N (Poland, AJ890342)
NTN (Poland, AJ890342)
NTN (China, HM590406)
N (China, JQ673517)
N (China, HM590405)
N (China, EU182476)
W (Germany, AJ889868)
N:O (USA, HQ912866)
N:O (USA, HQ912872)
N:O (USA, EF026076)
N:O (Canada, AY745492)
N:O (Canada, AY745491)
N:O (USA, DQ157178)
N:O (USA, AY884985)
N (UK, AJ584851)
Wi (Germany, HE608964)
Wi (Germany, HE608963)
Wi (Poland, AJ890349)
Wi (Germany, AJ890650)
Wi (Germany, AM113988)
C (Netherlands, EU563512)
C (France, Aj890348)
N (Egypt, AF522296)
N (France, X12456)
O (UK, AJ585195)
O (Canada, U09509)
O (USA, EF026074)
O (UK, AJ585196)
Wi (Poland, EF558545)

N/NTN
N:O/N-Wi
C/NP
O

0.02

FIGURE 3. Phylogenetic and recombination analysis of the PVY isolates from foliage and tubers in La Union (Antioquia). The phylogenetic tree (A) suggest that the foliage isolate has phylogenetic affinity with a clade composed of recombinant PVY[NTN] strains while PVY from tubers is more closely related to strains of the non-recombinant PVY[N/NTN] group. Complete PVY genomes from Colombia are shown in bold. Panels B and C show the analysis of recombination for both strains using PVY[N], PVY[O] and PVY[C] as parents.

PVY_LaUnionT (Fig. 1B). In contrast to the leaf isolate, the tuber PVY consensus contained only six polymorphic nucleotide sites; two of them, U4746G and T6875C,

result in amino acid changes in CI (V1520G) and NIa-Pro (W2230R). The closest PVY isolate available in GenBank corresponds to isolate mar7 (KR270797) infecting *S.*

B Foliage

C Tuber
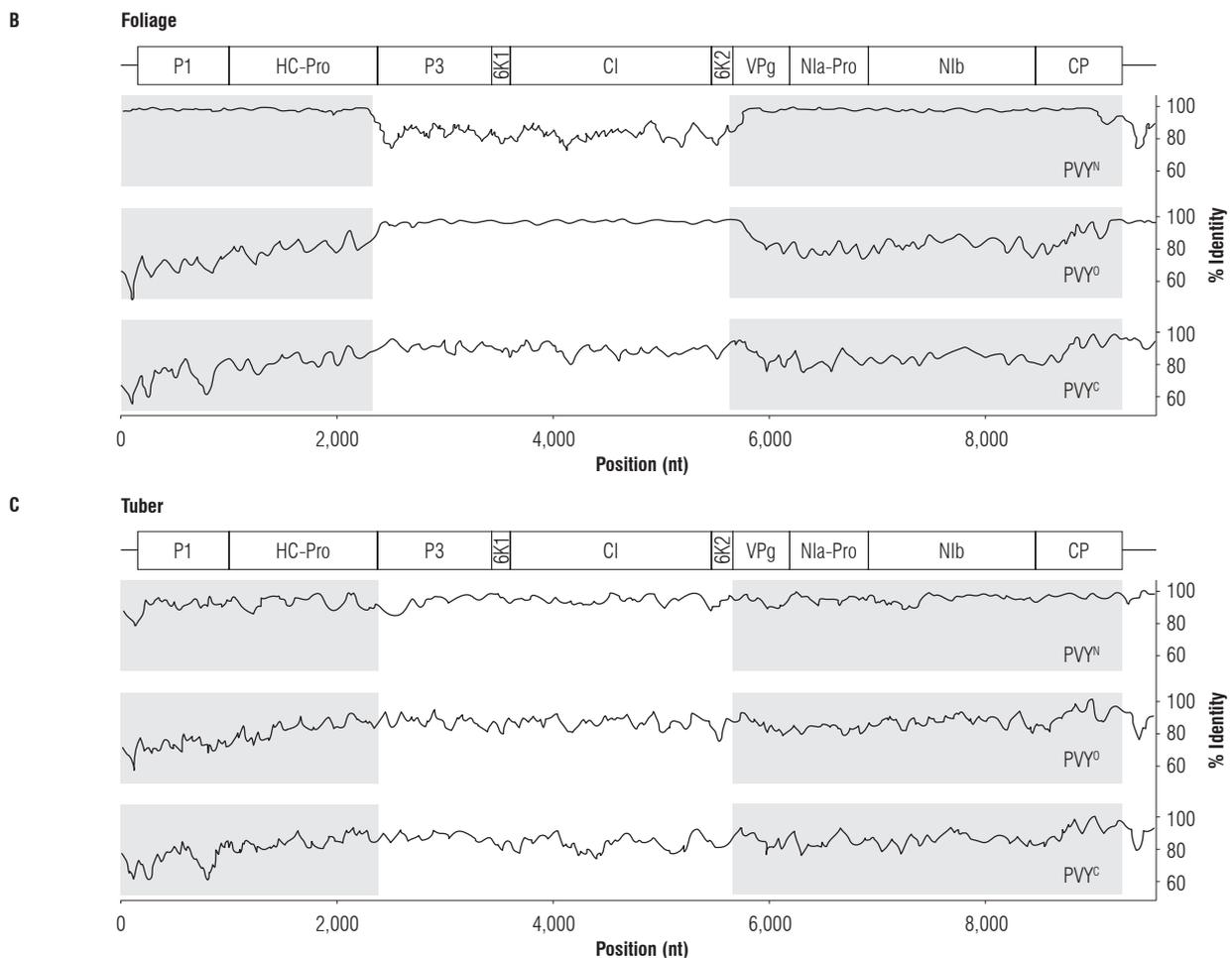
FIGURE 3. (Continuation)

lycopersicum at the municipality of El Peñol (Muñoz-Baena *et al.*, 2016). Isolate mar7 has phylogenetic affinity to the PVY[N] group and shares 99.4% nucleotide sequence identity with PVY_LaUnionT. Pairwise comparison among the genomes from foliage and tubers reveal significant differences in nucleotide and amino acid sequence identity between regions encoding each of the mature proteins (Table 2). The highest differences were found at the 6K2 and P3 proteins with nucleotide (amino acid) percent identities of 85.9 (94.0) and 92.2 (92.4); the best conserved segment correspond to CP (95.6/99.2) and NIb (95.1/98.5). 5' and 3' untranslated regions share 87.5% and 86.6% nucleotide sequence identity, respectively.

Phylogenetic analysis of complete PVY sequences confirmed that PVY_LaUnionF and PVY_LaUnionT cluster in two different clades within the N/NTN group (Fig. 3A). PVY_LaUnionF is part of a group comprising (only) PVY[NTN] recombinant strains and its close evolutionary relationship with the PVY isolate from Yarumal becomes clear as they cluster together within the clade. The tuber strain, on the other hand, belongs to a group comprising PVY[N] strains and other PVY[NTN] strains; as expected, isolate mar7 from tomato also belongs to this group. The recombinant nature of the PVY_LaUnionF was further confirmed by recombination analysis (Fig. 3B). Sequence comparison with PVY[N], PVY[O] and PVY[C] strains reveal three recombination breakpoints at the HC-Pro/P3 and 6K2/NIa-VPg junctions and near the end of the CP region, a pattern typical of PVY[NTN] strains (recombinant junction: NTNa) (Karasev and Gray, 2013). Recombination analysis of the PVY tuber strain failed to reveal recombination breakpoints (Fig. 3C).

To corroborate the diversity of PVY strains infecting *S. tuberosum* (cv. Diacol-Capiro) in La Union, partial Sanger sequencing of the CP region was performed in single leaf samples from different commercial cultivation plots (LU1-6). Multidimensional scaling analysis using sequences from Colombia and the rest of the world reveals seven

distinct PVY clusters (Fig. 4). Cluster A includes sequences belonging to strains PVY[N], PVY[N:O], PVY[W], PVY[C] and to NP isolates (non-potato isolates) from all over the world; Cluster C includes sequences corresponding to strains PVY[N] and PVY[NTN]; the remaining clusters are composed exclusively from Colombian isolates. The CP segment from PVY_LaUnionF clustered in group C, which includes the PVY genome from Yarumal, in agreement with phylogenetic analysis discussed above. This group also includes Sanger sequences LU1, LU2, LU4, LU5 and LU6 and Colombia isolates 13Col, 14Col, 40MY and 454749MY

previosuly deposited in GenBank by Gil *et al.* (2011) and Henao *et al.* (2013). Group C comprises only PVY strains with *S. tuberosum* as isolation host. The CP segment of PVY_LaUnionT clustered in group B composed exclusively by Colombian isolates and includes isolates mar7 (Muñoz-Baena *et al.*, 2016) and 44Col (Jaramillo *et al.*, 2011) infecting tomato and tamarillo (*Solanum betaceum*) respectively; none of the Sanger sequences obtained here was included within this group. Interestingly, sequence LU3 clustered in group A, which corresponds to the ordinary strain of PVY (PVY[O]) and suggests the presence of at least a third PVY
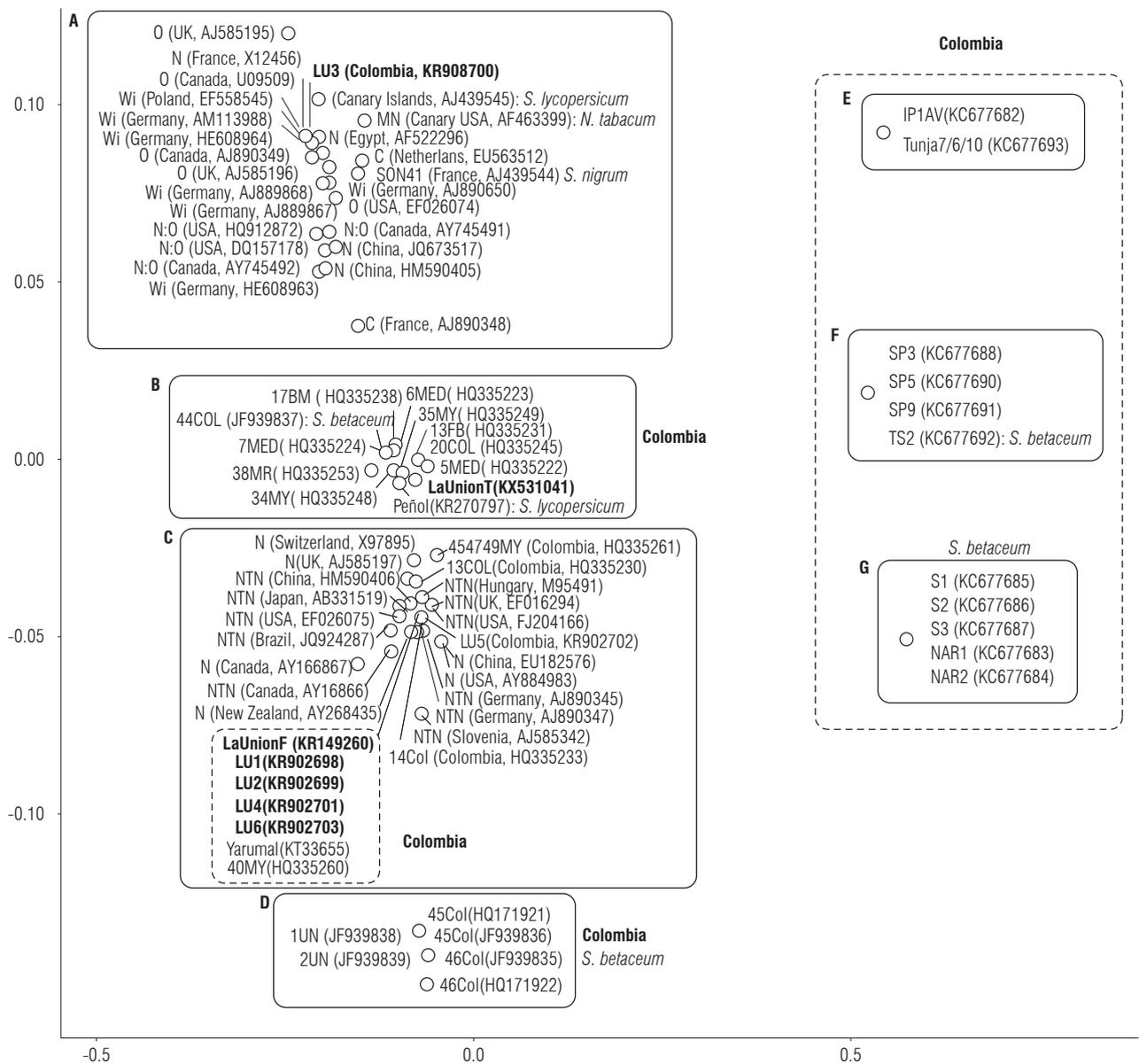
FIGURE 4. Multidimensional scaling visualization of partial PVY capsid sequences. Scale in the diagram directly reflects the genetic distances between sequences. When a PVY isolate infects a host different to *S. tuberosum* the corresponding host is indicated. Sanger sequences obtained in this work are shown in bold.

strain in La Union. No sequences related to groups D-G were found in this study.

Discussion

In this work, we have demonstrated the presence of at least two types of PVY strains infecting *S. tuberosum* (cv. Diacol-Capiro) in the municipality of La Union, the main potato producer of Antioquia. The isolate PVY_LaUnionT, infecting seed-tuber material has phylogenetic affinity with strains of the tobacco veinal necrosis PVY group, PVY^N (Singh *et al.*, 2008). PVY^N isolates were reported for the first time in Peru and Bolivia in the 1940s (Nobrega and Silberschmidt, 1944) followed by reports in the United States and Europe in the 1960s (Kahn and Monroe, 1963). Symptoms caused by PVY^N are not considered to be severe on potato (Singh *et al.*, 2008). Interestingly, Colombian PVY^N isolates form a very distinct cluster with widespread distribution in Antioquia and the rest of the country (Gil *et al.*, 2011; Henao *et al.*, 2013; Villamil *et al.*, 2014). Isolates from this group have also been collected in the provinces of Nariño (17BM, 34MY, and 35MY), Boyacá (17BM) and Cundinamarca (13FB). In Antioquia, members of this group have been collected in the municipalities of Santa Rosa de Osos (6MED), La Ceja (38MR) and Sonsón (5MED), the latter two located in the vicinity of La Union. Group C seems to be able to cross the host barrier as attested by isolates 44Col and mar7 infecting *S. betaceum* and *S. lycopersicum*, respectively (Jaramillo *et al.*, 2011; Muñoz-Baena *et al.*, 2016). PVY_LaUnionT is very closely related to isolate mar7, which together with the geographical proximity of the municipality of El Peñol, suggests that passage of PVY between alternate hosts might be a result of inadequate crop management practices. The infection of PVY^{NTN} and PVY^N in tomato has been previously reported in Spain and Poland (Aramburu *et al.*, 2006; Hasiów-Jaroszewska *et al.*, 2015).

The PVY genome from foliage, PVY_LaUnionF, is clearly a member of the recombinant PVY^{NTN} group of strains and its similarity to a previously sequenced genome from Yarumal in north Antioquia indicate that both isolates probably have a common origin. Analysis of the CP region shows that PVY_LaUnionF is part of a group of PVY strains with worldwide distribution and previously reported in Colombia in Chipaque (Cundinamarca) (Villamil *et al.*, 2014) and the east of Antioquia in the municipalities of Carmen de Viboral (454749MY), Sonsón (13Col and 14Col) and La Ceja (40MY) (Gil *et al.*, 2011; Henao *et al.*, 2013). The majority of Sanger sequences (LU1-2 and LU4-6) clustered within this group thus confirming the high prevalence of this strain in the potato cultivating regions of eastern

Antioquia. Our data indicates that isolate PVY_LaUnionF has high sequence variability and comprises a larger set of sequence variants that PVY_Yarumal, in which only fifteen nucleotide polymorphic sites were observed that translate into three of amino acid changes in P1 (K30E), 6K1 (M1121I) and NIb (K2758R) (Muñoz *et al.*, 2016). The K30E was also found in the PVY_LaUnionF and a M1121V change was observed instead of M1121I; no polymorphism equivalent to K2758R was detected in PVY_LaUnionF. Unfortunately, the genome sequence reported by Villamil *et al.* (2014) is not available in GenBank and could not be compared with the sequences reported in this work.

The results obtained from the sliding window comparison of non-synonymous to synonymous substitution is in agreement with previous reports showing that P1, P3, 6K1, 6K2, and CP genes have more neutral codons than expected (Cuevas *et al.*, 2012). According to our data, P1 is undergoing moderate positive selection in the segment comprising residues 59 to 66. A recent population analysis of PVY from Iran identified that 22 sites in P1 and three in NIa-Pro underwent positive selection (Pourrahim and Farzadfar, 2016). Protein P1 is a serine protease involved in RNA silencing and it has been suggested that modulation of P1 activity has evolved to keep viral amplification below host-detrimental levels therefore helping to maintain a higher long-term replicative capacity (Pasin *et al.*, 2014). It would be interesting if future studies could determine whether slight positive selection observed at the N-terminus of P1 in PVY_LaUnionF is related to fine-tuning of its protease function. The other region undergoing relaxed selection pressure is located at the N-terminus of a second protease, i.e., NIa-Pro, which is required for the proteolytic processing of the most potyviral proteins and has an important effect on infectivity (Ivanov *et al.*, 2014).

Interestingly, sequence analysis of the CP region of sample LU3 reveals that this isolate is most similar to isolates CO1960 (99.7 %), CO1827 (99.7%), CO1801 (99.5%) from the United States (Karasev *et al.*, 2011). These isolates are part of a PVY^O group of strains reported to be spreading across the United States and other countries world-wide. The PVY^O group can cause mild mosaic in tobacco and induces necrosis and severe stunting in potato cultivars carrying the *Ny* gene, such as Desiree and Maris Bard, as a result of eliciting the hypersensitive response (HR) (Karasev *et al.*, 2011). This is the first report in Colombia for this group.

In 2014, Colombia had an estimated yield of 19.9 t ha^{-1} which falls short to the productivity seen in countries such as UK (33.17 t ha^{-1}) and the United States (51 t ha^{-1})

(http://faostaT3.fao.org). It is likely that these low yields are related to the high incidence of viral infections, which might be caused by the lack of adequate seed-certification programs and malpractices in the handling of crops. One of the principal means of virus distribution is through movement of infected propagation material and once a pathogen gets introduced into a new region it may spread rapidly through the action of vectors and cultural malpractices. PVY is a constant threat to potato production as a result of the lack of efficient resistance of local varieties to many of its strains and, for this reason the main control strategy is based on the certification of tuber seed production. The high incidence levels of PVY detected in foliage and seed-tuber samples and the finding of at least three different strains in a single potato-producing municipality in Antioquia reveal a worrying situation regarding the sanitary conditions of this crop. Urgent measures are required to strengthen current seed-certification programs such as the incorporation of detection methods as IC-RT-qPCR that offer higher sensitivity than ELISA and a constant monitoring of the genomic characteristics of the PVY strains present in each potato-producing region. The latter is of the utmost importance as genome information can help to predict the epidemiological impact of PVY infection, since it is possible to associate the strain genotypes with their impact on yield and tuber quality as well as with alternate hosts and transmission efficiency by arthropod vectors.

Conclusions

The genomes of two different PVY infecting *S. tuberosum* in the municipality of La Union were obtained using NGS of leaves and tuber sprouts. These genomes comprised 9,072 and 9,704 nt, respectively and encoded a polyprotein of 3060 residues.

The consensus genome from bulked samples of the infected leaves was highly polymorphic containing 399 variable sites at the nucleotide level which leads to 67 amino acid changes in polyprotein, suggesting the presence of mixed variants within the sample. The genome from infected tubers showed little variability as only six nucleotide substitutions and two amino acid changes were observed. These differences in variability might be a consequence of different transmission methods occurring in foliar tissue (e.g. mechanical transmission, vectors, seed-tuber) in comparison with just the primary inoculum of seed-tuber.

High incidence of PVY were observed by IC-RT-qPCR in fifteen commercial seed-tuber samples from la Union (86.7%) and in two out of three potato cv. Diacol-Capiro

producing plots (33.4 and 80%). These results underscore the necessity of comprehensive reforms to currently available seed-certification programs in Colombia using more sensitive and specific virus detection methods such as real-time RT-PCR.

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