Detection and Quantification of *Potato virus Y* Genomes in Single Aphid Stylets

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Abstract

Typically, the detection of a plant virus within its vector is carried out on the entire insect body. This process can be a possible source of confusion in the quantification of transmissible virus particles for styletborne viruses such as *Potato virus Y* (PVY), since the transmissible virus fraction is the one only retained in the aphid vector's mouthparts. The objective of this study was to develop and validate the quantitative PCR method for the detection and quantification of PVY in the vector's stylet. Using a specific method based on TaqMan chemistry with higher sensitivity than conventional reverse transcription PCR, this study reveals that

a significant amount of the virus is enclosed within the dissected stylets of *Myzus persicae*. Because this quantification only concerns the portion of the virus attached to the stylets, uniformity was observed in the recorded numbers of virus targets. This novel assay is applicable to several PVY strains as a rapid and sensitive detection method for use in PVY research and offers a convenient tool for deciphering the mechanism of *Potyvirus* acquisition.

Keywords: absolute quantification, transmission, virus RNA copies

Potato virus Y (PVY), a type member of the genus *Potyvirus*, is one of the most important viral pathogens affecting the yield and quality of potato production worldwide (Karasev and Gray 2013; Quenouille et al. 2013; Singh et al. 2008). PVY has a single-stranded positive-sense RNA genome approximately 10 kb in size, which is translated into a single polypeptide cleaved into 10 multifunctional proteins (Dougherty and Carrington 1988). PVY isolates from potato strains are classified into seven strain groups (PVY^O, PVY^C, PVY^N, PVY^{NTN}, PVY^{N-Wi}, PVY^E, and PVY^Z) according to symptoms induced on tobacco and potato host plants and according to the presence or absence of recombination events in their genome (Glais et al. 2017; Green et al. 2018; Singh et al. 2008). Genomes of the PVY^O, PVY^C, and PVY^N groups are nonrecombinant, whereas those of PVY^{NTN}, PVY^{N-Wi}, PVY^E, and PVY^Z are recombinants from the PVY^N and PVY^O parental strain groups (Boonham et al. 2002; Glais et al. 2002; Green et al. 2018; Lorenzen et al. 2008; Nie et al. 2004). Compared with the other recombinant PVY isolates, PVY^{NTN} and PVY^{N-Wi} isolates are considered as common recombinants found in many geographical locations (Green et al. 2017). Data collected in U.S. potato-growing regions showed a PVY strain composition shift from the previously dominant strain PVY^O to the two recombinant PVY^{NTN} and PVY^{N-Wi} strains, and the latter has become the most prevalent strain (Funke et al. 2017). The reasons for these changes in the relative abundance of PVY can be interpreted as a strain-specific resistance against PVY^O exhibited by the varieties most used in potato field production areas (Funke et al. 2017; Rowley et al. 2015).

This complex diversity within PVY species was highlighted using numerous methods, specifically reverse transcription (RT)-PCR and RT quantitative PCR (qPCR) developed to detect PVY in plants or tubers (Balme-Sinibaldi et al. 2006; Boonham et al. 2009; Chikh Ali et al. 2010, 2013; Glais et al. 1998, 2002; Jacquot et al. 2005; Kogovšek et al. 2008; MacKenzie et al. 2015; Nie et al. 2013; Rolland et al. 2008) and more recently using droplet digital PCR (Mehle et al. 2018). PVY is transmitted by more than 65 aphid species, including potato-colonizing and noncolonizing species (Nault 1997; Pelletier et al. 2012; Radcliffe and Ragsdale 2002; Ragsdale et al. 2001; Singh and Boiteau 1986; Verbeek et al. 2010), and its transmission efficiency depends on many factors among others aphid species, PVY isolates, and their host plants. Transmission tests assessed with several aphid species and PVY strains highlighted that *Myzus persicae* and *Rhopalosiphum padi* transmit PVY^{NTN} isolates with the greatest efficiency followed by PVY^O and PVY^{N:O} isolates (Mondal et al. 2016). PVY is transmitted in a nonpersistent manner (reviewed in Dietzgen et al. 2016) according to the “helper strategy,” meaning that the viral helper protein HC-Pro (Pirone and Blanc 1996) mediates binding of virus particles to the vector stylets by forming an intermediary bridge. HC-Pro is a 50-kDa protein containing the conserved motif KITE at its N-terminal end (Robaglia et al. 1989) and PTK at its C-terminal end (Peng et al. 1998), which are involved in the binding to the aphid and to the consensus DAG motif of the coat protein, respectively (Blanc et al. 1997).

Improving our understanding of PVY transmission requires methods to detect the virus in the vector. To this end, various tools have been reported. Conventional RT-PCR techniques associated with different procedures allowing rapid viral RNA extraction were developed to detect PVY from a single aphid (Kim et al. 2016) and to define aphid species potentially able to acquire the virus (Singh et al. 1996).

A similar approach based on real-time PCR was developed by Olmos et al. (2005) for the quantitative detection of another *Potyvirus* species, the *Plum pox virus* (PPV), in single aphids. In this study, viral RNA targets were detected in individuals of the PPV vector *M. persicae* and the nonvector *Aphis nerii*, with a sensitivity 100 times greater than with conventional RT-PCR and 1,000 times higher than with the triple antibody sandwich enzyme-linked immunosorbent assay. One-step real-time RT-PCR was also developed to detect the persistently transmitted *Barley yellow dwarf virus* (BYDV) in single aphids, with detection and quantification ranging from 10² to 10⁸ BYDV RNA copies. This confirms the high sensitivity of the technique compared with conventional RT-PCR (Fabre et al. 2003).

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Funding: This work was conducted as part of the Vegephy project funded by the Conseil Régional de Picardie and the Comptoir Commercial des Lubrifiants and SIPRE companies.

*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary figures and one supplementary table are published online.

The author(s) declare no conflict of interest.

Accepted for publication 3 April 2019.

Yet the viral detection assays described above were conducted using whole aphids and could therefore detect virus particles retained in the aphid stylets, as well as ingested virus particles remaining in the gut. However, for nonpersistent viruses, only viruses attached to the aphid stylets, and not those in the digestive tract, are transmitted (Powell 2005). This may be a source of confusion regarding the vector's ability to carry the virus and its capability for transmission. Zhang et al. (2013) highlighted this crucial difference and consequently developed a procedure to detect PVY in the stylets of a single aphid. Although this technique has enabled detection of the qualitative virus presence or absence within stylets using one-step conventional RT-PCR, it is not capable of obtaining quantitative parameters of the viral presence within aphid stylets. To address this issue, this study aims to improve the existing method of PVY detection in stylets by offering a more sensitive technique with a quantitative output to provide an opening for better understanding of the mechanism of virus acquisition.

Materials and Methods

Virus origin and aphids. Five previously characterized potato PVY isolates were used in this study (Table 1): one PVY^N isolate (N605), one PVY^{NTN} isolate (FrOrl), one PVY^{O-05} isolate (O-139), and two PVY^{N-Wi} isolates (Lw and Sp17) (Glais et al. 1996; Green et al. 2017, 2018; Jakab et al. 1997). These isolates were obtained from L. Glais of the French Federation of Seed Potato Growers (FN3PT). These isolates were maintained by mechanical inoculation on potato plants of cultivar Bintje or on *Nicotiana tabacum* 'Xanthi' under controlled conditions at 20 ± 1 °C, with 60 ± 5% relative humidity under a 16-h/8-h light/dark photoperiod.

The *M. persicae* aphid colony was initiated from a single apterous parthenogenetic female from a clone collected in 1999 in a potato field in Loos-en-Gohelle, France. Aphids were reared on 2- to 3-week-old turnip plants (*Brassica rapa* L. var. *rapa*) in soil placed in 9-cm square plastic pots enclosed in Plexiglas cages and maintained in a growth room at 20 ± 1 °C under a 16-h/8-h light/dark photoperiod to induce parthenogenesis. Adults obtained from larvae raised for 8 days on turnip leaves and kept on agar medium in Petri dishes were used for experiments.

Virus acquisition and stylet preparation. A group of starved *M. persicae* were allowed to probe the PVY^{NTN}FrOrl-infected potato leaflet or a healthy control for 10 minutes as soon as they stopped moving. The virus amount acquired during aphid feeding was assumed to be highly dependent of the viral titer in the plant; thus, only one leaflet was used during acquisition to reduce variability in the virus amount acquired by the aphids (Rotenberg et al. 2009). The aphids were then collected and stored at -20 °C in 95% (vol/vol) ethanol (Zhang et al. 2013). Each aphid was dissected using miniforceps under a stereomicroscope (SteREO Discovery; Carl Zeiss) in order to separate the head from the stylets. The stylet bundle was removed from the proboscis and the stylets of each aphid, as well as the remaining body parts, were individually placed in a microfuge tube. Both were kept at -20 °C for total RNA extraction.

Total RNA extraction from plants and aphids. The five PVY isolates were extracted from infected tobacco or potato plants 2 weeks after their mechanical inoculation. Two pooled symptomatic leaves were ground in liquid nitrogen, and total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) and subsequently eluted in 30 µl of nuclease-free water, according to the manufacturer's instructions.

RNA was extracted from the stylets and the aphid body using the NucleoSpin RNA XS kit (Macherey-Nagel). It was then eluted in

15 µl of RNase-free water, according to the manufacturer's recommendations. The total RNA recovered from stylets was between 10 and 30 ng/µl.

RT-PCR assay. Conventional RT-PCR was applied to detect PVY in plants (potato, tobacco) and in aphid stylets. The primers (forward Yo3S, reverse Yo4A), reaction mix, RT-PCR kit, and PCR program were as described by Zhang et al. (2013), except that 2 and 4 µl of total plant RNA extract and total stylet RNA extract were used, respectively. With this procedure, a PCR product of 480 bp extending from nucleotides 8,717 to 9,196 of the coat protein (CP) gene (Singh and Singh 1996) was generated. A positive control (RNA extract from the PVY^{NTN}FrOrl-infected plant and positive stylets) was included, and RNA extracted from the stylets of aphids maintained on uninfected plants was used as a negative control.

TaqMan primers and probe. Nucleotide sequences 8,717 to 9,196 of 18 coat protein genes of PVY isolates selected from the main PVY strain groups in the NCBI database (Green et al. 2017) were aligned using the BioEdit program and were compared with the homologous nucleotide sequence of the five PVY isolates sequenced in this study (N605, O-139, Lw, Sp17, and FrOrl) (Fig. 1). Within this sequence of 480 nucleotides, a narrow region corresponding to the central part of the CP gene (at positions 8,863 to 8,984) was precisely analyzed for selection of the primers and probe because it is known to be highly conserved between most PVY stain groups (MacKenzie et al. 2015). Thus, the optimal primers and probe for hybridization to all expected PVY isolates were designed from these sequence data using Primer Express software (Applied Biosystems). The selected CPF8869 (5'-GCATACGACATAGGAGAACTGA-3') and CPR8977 (5'-CCATCATAACCCAACTCCGT-3') primers amplified a 109-bp fragment (nucleotide positions according to the PVY^N-605 isolate; Jakab et al. 1997). The hydrolysis probe CPprobe8924 (5'-TGCATTGAAAATGGAACCTCGCCA-3') was labeled with the fluorescent dyes 6-carboxyfluorescein (FAM) on the 5' end and Black Hole Quencher BHQ-1 on the 3' end (MWG Biotech).

Preparation of PVY RNA standard. For absolute quantification, it is essential to use a standard curve with a known concentration of RNA target in order to transform the qPCR output of amplification cycles (*C_p*) into target copies. The RNA standard was obtained by in vitro transcription from the T7 RNA polymerase-dependent plasmid system as follows. One-step RT-PCR was performed on 2 µg of total nucleic acid extracted from PVY^{NTN}FrOrl-positive potato plants as described by Singh et al. (1996), using the One-Step RT-PCR kit (Qiagen). A CP nucleotide sequence (480 bp) was reverse transcribed and amplified with forward primer Y³SS5'-ACGTCCAA AATGAGAATGCC-3' and reverse primer Y⁴A5'-TGTTGTTC GTGATGTGACCT-3' (Singh et al. 1996), allowing amplification of a fragment located in the central position of the coat protein (CP), which is highly conserved between PVY stains (MacKenzie et al. 2015). The RT-PCR product was then purified from agarose gel using an Ultra Clean Gel Spin Purification kit (Mo Bio). Subsequently, 100 ng of cDNA was inserted into TOPO TA cloning vector 2.1 (Invitrogen) according to the manufacturer's recommendations and cloned into *Escherichia coli* JM109 competent cells (Promega Inc.). Transformants were selected by ampicillin resistance in order to obtain the pTopoCP plasmid.

To generate a linear DNA template for run-off transcription, 0.5 µg of pTopoCP plasmid template was digested with 1 U of *Spe*I restriction enzyme (New England BioLabs Inc.). After complete digestion, the restriction enzyme was heat deactivated according to the manufacturer's recommendations. PVY RNA transcripts were synthesized using 10 U of T7 RNA polymerase (SP6/T7 Transcription Kit; Roche Applied Science) in a 25-µl reaction at 37 °C for 1 h, followed by DNase treatment and heat inactivation. The RNA was then phenol-chloroform extracted, ethanol precipitated, and resuspended in 20 µl of RNase-free water. An additional treatment with Turbo DNA-free (Ambion) was performed to eliminate all remaining plasmid DNA. The PVY RNA concentration was then determined by spectrophotometry. A dilution from 45 × 10⁷ to 45 copies of the RNA transcript was performed for the standard.

Table 1. Strain group, related classification publication, and GenBank accession numbers of *Potato virus Y* isolates used in this study

Isolate name	Strain group	GenBank accession no.	Reference
FrOrl	NTN	AF228634	Glais et al. 1996
N605	N	X97895	Jakab et al. 1997
O-139	O5	U09509	Green et al. 2017
Lw	N-Wi	AJ890349	Green et al. 2018
Sp17	N-Wi	No data	Glais et al. 1996

Definition of PVY RNA copy number. The amount of standard RNA transcript quantified by a NanoDrop spectrophotometer (Thermo Scientific) was converted to a copy number by taking into account the molecular weight of an RNA nucleotide (340 Da), the fragment length (605 bases) of the in vitro transcript (extending from the T7 transcription factor to the *SpeI* restriction site), and the Avogadro constant (6.023×10^{23} molecules/mol) (Avogadro 1811). The copy number was calculated per 2.5 μ l, the same volume used for the template in each real-time qRT-PCR. Ten-fold serial dilutions of in vitro RNA transcript were prepared (from 45×10^7 to 45 copies), aliquoted, and stored at -80°C until use.

One-step real-time RT-PCR assay. Quantitative real-time RT-PCR was performed with a LightCycler480 II (Roche Applied Science) on 384-well plates in a final volume of 10 μ l with 4 μ l of RNA extraction from stylets or 2.5 μ l each of plant RNA and standard RNA transcript, added in LightCycler Multiplex RNA Virus Master mix (Roche Applied Science) and 500 nM each of probe and specific sense and antisense primers. Two technical replicates were performed for each sample except for the viral transcripts, which were processed in triplicate. The thermocycler was programmed for 10 min of RT at 50°C , followed by 30 s of initial

denaturation at 95°C , and finally 45 cycles at 95°C for 5 s, 60°C for 30 s, and cooling at 40°C for 30 s. Fluorescence intensity of the FAM dye was recorded during the amplification process in the "Single" mode. *C_p* values for each sample were calculated with LightCycler 480 software (version 1.5.1) using the absolute quantification/fit points method (Roche Applied Science). The slopes obtained from amplification of the successive RNA dilution series were used to calculate the efficiencies according to the following mathematical formula: slope = $-1/\log$ (efficiency).

Results

Specificity of the real-time RT-PCR assay. According to in silico alignments achieved on the 18 PVY sequences (Fig. 1), primer CPF8869 and probe CPprobe8924 had a 1 to 2 mismatch with Adgen, PRI-509, PVY-MON, Ditta, 12-94, and MB112 sequences, which are positioned mostly in the central part of the primers and should not, in theory, prevent hybridization. For primer CPR8977, the mismatch observed on N605 and 12-94 should not prevent hybridization; in contrast, there could be some lack of attachment on TU-660 and N-Jg isolates, owing to a mutation in the 3' primer region at position 20 of primer hybridization (T instead of C) and at

Fig. 1. Multiple alignment of *Potato virus Y* (PVY) capsid protein (CP) fragments between nucleotides 8,863 and 8,984. The comparison was achieved using sequences retrieved from GenBank except for isolates with an asterisk, which are isolates sequenced as part of this study. Because a mutation was observed in the sequenced N605 isolate at nucleotide position 8,970 compared with the GenBank sequence, the two sequences are represented. Dots symbolize nucleotides that are present in all PVY sequences and are identical to those of PVY^{NTN}FrOrl isolate. Nucleotides that differ from the FrOrl sequence used as standard sequence are represented. Accession numbers are provided in parentheses for the following isolates: FrOrl (AF228634), 139 (U09509), Lw (AJ890349), Oz (EF026074), SCRI-O (AJ585196), N605 (X97895), Tu660 (AY166866), MONT (AY884983), N-Jg (AY166867), Adgen (AJ890348), PRI-509 (EU563512), PVY-MON (JF928458), Ditta (AJ890344), HN1 (HQ631374), GR99 (AJ890343), 12-94 (AJ889866), HN2 (GQ200836), Wilga (AJ890350), N1 (HQ912863), L56 (AY745492), and MB112 (AY745491). The nucleotide positions are based on the PVY^N605 sequence (Jakab et al. 1997). Forward primer CPF8869, reverse primer CPR8977, and hybridization probe CPprobe8924 are indicated above in gray boxes and strain groups are shown on the left. Sequence alignment was processed using the BioEdit sequence alignment editor.

Table 2. Raw data for standard curves performed in triplicate on viral transcripts and plant RNA extracted from leaves infected with the PVY^{NTN}FrOrl isolate giving amplification efficiency of $E = 2.02$ and $E = 1.98$, respectively

Template	Sample	Theoretical concentration	Mean crossing point	Mean concentration	S.D. concentration
RNA transcript ^a	Dilution 1	45×10^7	15.12	45.74×10^7	27.81×10^6
	Dilution 2	45×10^6	18.21	52.95×10^6	13.27×10^6
	Dilution 3	45×10^5	21.65	48.21×10^5	17.33×10^5
	Dilution 4	45×10^4	24.83	50.47×10^4	12.28×10^4
	Dilution 5	45×10^3	28.26	44.51×10^3	36.05×10^2
	Dilution 6	45×10^2	31.57	45.21×10^2	14.47×10^2
	Dilution 7	22.5×10^2	33.49	13.10×10^2	94.88×10
	Dilution 8	45×10	34.36	79.60×10	62.97×10
	Dilution 9	4.5×10	38.30	3.84×10	0.57×10
Plant RNA ^b	Dilution 1	15	8.87	13.8830	2.9485
	Dilution 2	2.5050	11.22	2.7141	0.5496
	Dilution 3	0.4170	13.01	1.5258	2.0349
	Dilution 4	0.0694	16.32	0.0830	0.0349
	Dilution 5	0.0011	19.17	0.0113	0.0032
	Dilution 6	0.0019	21.68	0.0019	0.0003

^a Theoretical concentration and mean concentration values for RNA transcripts are presented as copy numbers per reaction.

^b Theoretical concentration and mean concentration values for plant RNA are presented in nanograms per reaction.

positions 14 and 17 for the PVY-MON isolate. The selected primers and probe appear to be suitable for detection of the other isolates. Amplified RNA extracted from infected leaves of the five PVY isolates (FrOrl, O-139, N605, Lw, and Sp17) confirms good amplification of each isolate with almost the same efficiencies (1.98, 1.98, 1.97, 1.97, and 1.96, respectively; Supplementary Fig. S1), making this tool effective for detection of PVY strain groups N, NTN, O5, and N-Wi.

Sensitivity of the real-time RT-PCR. To determine the real-time RT-PCR sensitivity, 10-fold serial dilutions of the previously prepared transcript standards were made from 45×10^7 to 45 copies. The reliability of real-time RT-PCR using the viral transcript was

then tested in an experiment conducted with three repetitions of each sample.

The standard curve revealed a high amplification efficiency ($E = 2.02$), which is in the range of 45×10^7 to 45 viral RNA copies per reaction (Table 2). In our conditions, the detection threshold was fixed to 45 copies; since the amplification efficiency was around 100%, it was possible to estimate the copy number with high reliability.

The amplification efficiencies of the standards and PVY-infected aphid stylets were compared according to Rieu and Powers (2009). Successive RNA dilutions could not be made because of the low amount of RNA extracted from the stylets; therefore, RNA extracted from leaves infected with the PVY isolates was used as an alternative, giving an efficiency of around 1.98 (Table 2), which is close to the standard efficiency value.

Sensitivity of the conventional RT-PCR. The previously prepared transcript diluted from 45×10^7 to 45 copies (Table 2) was subjected to conventional RT-PCR. Electrophoresis analysis revealed no detection using the more concentrated sample (Fig. 2; lane 1). By contrast, we observed a 480-bp product with a decreasing intensity corresponding to viral transcripts from 45×10^6 to 22.5×10^2 template molecules per reaction and nothing below the latest value corresponding to 45×10 and 45 template molecules per reaction (Fig. 2; lanes 8 and 9).

Detection in stylets using the real-time RT-PCR assay. RNA samples extracted from stylets of healthy aphids or aphids fed on PVY^{NTN}FrOrl-infected plants were used as templates and samples from healthy or PVY-infected plants were included as controls. The amplification plot shows that only RNA extracted from PVY-infected plants and from aphids that had fed on the same infected plant (Supplementary Fig. S2) could be detected. Amplification in the stylet sample curve was slightly delayed owing to the small amount of virus enclosed in the sample compared with the infected plant. As expected, RNA from healthy plants and aphids fed on the same plants was not amplified.

Quantification of viral targets was performed on stylets from 21 aphids after 10 min of acquisition on the PVY-infected leaflet (Supplementary Table S1). Of the tested aphids, 12 (57%) were positively amplified. Among the 12 stylets detected positively in PVY, all presented a number of targets between 1,141 to 1,904 copies, except for one stylet that reached 3,327 copies (Fig. 3). Otherwise, the total volume of RNA extracted from the stylet could be used to estimate the total amount of virus enclosed in each stylet, and the value oscillated between 4,000 and 12,300 RNA targets (Fig. 3).

The same RNAs used for quantification were then subjected to conventional RT-PCR detection. The signal generated from the amplification product staining at 480 pb (Fig. 4) provided low intensity because of the small virus amount enclosed in the stylets, unlike the PVY-infected leaf that served as a positive control (Fig. 4; lane Pp). PVY was detected on eight stylets (Fig. 4; lanes 4, 6, 7, 8, 11, 18, 19, and 21), representing 38% of the total sample. Surprisingly, stylet

Fig. 2. Amplification efficiency using conventional reverse transcription PCR (RT-PCR). Successive dilutions containing viral transcripts from 45×10^7 to 45 template molecules per reaction were amplified by one-step RT-PCR targeting the *Potato virus Y* (PVY) coat protein gene. The amplification product was subjected to 1.2% agarose gel electrophoresis analysis. The arrow indicates the 480-bp RT-PCR product. B = RT-PCR mix without matrix and L = 100-bp DNA ladder ready to load (Solis BioDyne).

Fig. 3. Absolute quantification of RNA targets in aphid stylets after 10-min acquisition on a *Potato virus Y* (PVY)-infected leaf. The mean number of RNA targets recorded in 4 µl of extracted RNA is shown for each positive stylet (light gray bars), with the standard error between technical duplicates. The dark bars represent an estimate of the number of targets in whole stylets (15 µl of RNA extraction).

Fig. 4. The 1.2% agarose gel electrophoresis analysis showing detection of *Potato virus Y* (PVY) from the stylets of single *Myzus persicae* using a one-step reverse transcription PCR (RT-PCR) assay targeting the PVY coat protein gene. Lanes 1 to 21 are different individual stylets. The arrow indicates the 480-bp RT-PCR product. N = negative control, Pp = positive control (PVY-infected plant), B = RT-PCR mix without matrix, P = positive control (PVY-infected aphids), and L = 100-bp DNA ladder ready to load (Solis BioDyne).

sample 7 was only positive when conventional RT-PCR was used (Fig. 4).

Virus quantification in aphids. To know what represent the stylets virus contain compared with the rest of the aphid body, quantification of viral targets separately from the stylets and bodies of 29 aphids after 10 min of continuous PVY^{NTN}FrOrl probing was carried out. Of 29 aphids, 27.58% displayed the virus in both the stylets and body, compared with 44.82 and 17.24% in the body and stylets alone, respectively. The total proportion of aphids with PVY in their stylets increased to 44.82% when aphids carrying the virus in both their stylets and body were taken into account (Table 3). In that experiment, 10.34% of samples (3 of 29) were not detected as PVY positive whether in the bodies or stylets. Moreover, concerning the number of viral targets detected in the two different parts of the aphids, the results showed that this average number was statistically greater in the aphid body (16,200 copies) than in the stylets (524 copies) (Table 3) (*t* test, *P* = 0.0059). Indeed, the copy number ranged from 1,090 to 74,500 in aphid bodies detected as positive, whereas it ranged from 105 to 2,310 in stylets (Table 4).

Discussion

Acquisition of a virus by its aphid vector occurs during ingestion of plant sap from virus-infected plants. For nonpersistent viruses such as PVY, the fraction of acquired virus that actually contributes to its transmission is represented by what is retained at the stylet level (reviewed in Blanc et al. 2014). This retention requires the viral helper protein HC-Pro, which forms a molecular bridge between the receptors lining the stylets and the viral particles (Ammar et al. 1994). Although the distribution of PVY viral particles along the stylets remains unknown, they can be found along the length of the maxillary stylets (Ammar et al. 1994; Berger and Pirone 1986), retained at the distal extremity of the food canal (Bradley 1966; Wang et al. 1996), or in the common duct (Martín et al. 1997; Powell, 2005). The exclusive attachment of the *Cauliflower mosaic virus* helper protein is limited to the acrostyle, a specific zone in the common duct of the maxillary stylets (Uzest et al. 2010; Webster et al. 2017). This spatial specificity of fixation has not yet been demonstrated for potyviruses. However, based on similarities in the transmission mode of nonpersistent viruses that rely on the helper protein, it cannot be excluded that PVY binds to the acrostyle. If so, it is expected that the amount of virus inside the stylets is very low, so it is essential to have a tool that is both sensitive and specific to detect it.

Jacquot et al. (2005) developed a sensitive qRT-PCR tool targeting the nucleotide region coding for the HC-Pro PVY protein, which is known to be variable between the different PVY strains. Likewise, Kogovšek et al. (2008) developed a qRT-PCR tool targeting the conserved CP region but without an absolute quantification output. Thus, it seemed necessary to develop a specific approach to record viral quantitative data in aphid stylets.

The target region used for quantification in this study is in the core coat protein coding region, which is highly conserved across most PVY strain groups (Kim et al. 2016; MacKenzie et al. 2015). Indeed, nucleotide sequence alignment indicates a slight variability with a degree of homology of at least 97% in this region, making the primers and probe able to detect in silico various isolates among PVY strain groups. This ability was confirmed by checking this tool against five

PVY isolates of different strain groups. All of them were amplified with the same efficiencies.

The technique developed here shows an improvement in sensitivity compared with conventional RT-PCR detection of the virus in the stylets previously proposed by Zhang et al. (2013). In the present study, even if a quantification with high reliability in the range of 45×10^7 to 45 RNA copies per reaction was recorded, we did not reach the 0.5 RNA copy number reported by Mehle et al. (2018) for the absolute quantification of RNA extracted from PVY^{NTN}-infected leaves; this high sensitivity is achieved by combining qRT-PCR with RT droplet digital PCR.

Under the conditions used in this study, the quantification assay was capable of detecting viral RNA in total stylet content. Because this comprises virus bound to the stylets as well as virus contained in the stylet lumen, it remains unclear which fraction of the detected virus was actually bound to the stylets and available for transmission. Purging the aphids from plant sap by membrane feeding on virus-free solutions may help solve this issue in future experiments, although this might be a delicate approach because potyviruses are flushed out during saliva secretion (Martín et al. 1997).

RNA targets recorded in the stylets of the analyzed aphids oscillated between 1,100 and 3,300 viral targets per stylet, although these values depend on the plant viral titer during the aphids' ingestion. The slight interstylet variability observed may be attributable to the fact that neither the real feeding period nor the volume ingested by aphids was controlled. Indeed, an electrical penetration graph used to monitor insect probing and feeding behavior (Tjallingii 1988) may provide an accurate comparison between aphids. In the present

Table 4. Mean values of RNA target quantification recorded in two technical replicates^a

Aphid no.	Mean of RNA targets recorded	
	Stylets	Body
1	0	2,595
2	237	0
3	0	9.79 ^b
4	296.5	829
5	0	0
6	394	0
7	0	2,675
8	0	4,630
9	1,240	865
10	2,310	43,500
11	0	1,090
12	0	0
13	0	6,930
14	977	13,925
15	0	2,320
16	155	12,850
17	0	59,300
18	0	3,570
19	211	0
20	0	0
21	0	61.4
22	258	0
23	105	52,350
24	0	24,000
25	155	74,500
26	0	223
27	367	1,755
28	106	0
29	0	16,200

^a Recordings were made from 29 aphids after 10 min of sustained feeding on a *Potato virus Y* (PVY)-FrOrl-infected leaf. RNA was extracted separately from the stylets and body of each aphid. Quantification was then performed on a 4-μl extraction for each sample. The standard curve of the RNA transcript was used to calculate the PVY targets for each sample.

^b Value below the amplification sensitivity threshold and considered as negative.

Table 3. Qualitative and quantitative detection of *Potato virus Y* (PVY) in 29 aphids after 10-min acquisition on an infected leaf

Detection on	PVY-positive samples (%)	PVY-negative samples (%) ^a	Average no. of copies
Stylet	17.24 (5/29) ^b	–	524 ± 176 ^c
Body	44.82 (13/29)	–	16,200 ± 5,056
Stylet + body	27.5 (8/29)	10.34 (3/29)	–

^a Dashes indicate no value.

^b Number of PVY-positive or PVY-negative samples/total number of tested samples.

^c Standard error between the positive samples.

study, quantification of PVY RNA copies was only achieved on a small part of RNA extracted from the stylets; extrapolating the estimate to the whole fraction suggests that the RNA target number could range from 4,000 to 12,300 copies. A plausible explanation for the much greater variability observed in previous studies is that the authors did not discern between styletborne and ingested viruses. Pirone and Thornbury (1988) detected between 15 and more than 20,000 virions in individual aphids fed on ¹²⁵I-labeled *Tobacco vein mottling virus* particles. Similarly, the number of PPV targets carried by individual *M. persicae* or *A. nerii* insects was reported to range from 40 to 2,381 (Olmos et al. 2005). In this study, comparing target numbers recorded in stylets to aphid bodies revealed a greater number in the bodies (Tables 3 and 4). Moreover, it was observed in a nonnegligible number (44.82%) of tested aphids that the body carried the virus, whereas no virus was detected in the corresponding stylets. This result may be attributable to a lack of transmission complex formation in the stylets during feeding. Interestingly, the same observation was made by Zhang et al. (2013), who reported that the virus was detected in all aphid bodies and only in 70% of the tested stylets after a sustained feeding period of *R. padi* on potato PVY-infected potato leaves, which means that the virus was not always retained in the stylets or was at a concentration below the detectable limit. This suggests that the number of viruliferous aphids could be overestimated by quantifying the virus in the whole insect. Considering the large number of viral copies found in the stylets after acquisition, it is important to determine the epidemiological significance of these values, since only a few PVY viral particles are sufficient to initiate infection (Moury et al. 2007). Indeed, using a mathematical model, Moury et al. (2007) focused on population bottlenecks during horizontal transmission and determined that an average release of 0.5 to 3.2 viral particles by aphids was sufficient to initiate plant infection, which is extremely low compared with the value recorded from the stylets in the present study.

This method enables substantial time-savings through the complete elimination of the period occurring between virus inoculation by the vector and the appearance of foliar symptoms and could help to determine the efficiency of virus acquisition between several vectors. It could also be an effective tool to screen candidate molecules that block the interaction between the virus and its receptor within the vector (e.g., mineral oil as well as molecules that act as potential antivirals). In addition to improving the sensitivity of existing techniques, the method described here will provide a useful tool for understanding the mechanisms of PVY acquisition.

Acknowledgments

The author thanks Laurent Glais and Martin Drucker for critical reading of the manuscript and once again Laurent Glais from FN3PT/RD3PT for providing PVY isolates. The author is indebted to the Bioécologie des Insectes Phytophages et Entomophages laboratory for providing the aphid clone and Brandon Loveall of Improvement for English proofreading services.

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