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Real-time assay for quantitative detection of non-persistently transmitted *Plum pox virus* RNA targets in single aphids

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Abstract

A TaqMan real-time RT-PCR was developed to detect and quantify RNA-targets from the non-circulative, non-persistently transmitted *Plum pox virus* (PPV) in individual fresh or aphids captured previously and squashed on paper. Reliable quantitation ranged from 40 up to 4×10^8 copies of control transcripts. This technique was applied successfully to plant material and to individual PPV vector (*Myzus persicae*) and non-vector of PPV (*Aphis nerii*) aphid species demonstrating acquisition of viral targets by both vector and non-vector aphids. The number of viruliferous aphids detected by real-time RT-PCR and nested RT-PCR in a single closed tube was similar in parallel assays, nevertheless the sensitivity provided by real-time RT-PCR was 100 times higher than nested RT-PCR and 1000 times higher than DASI-ELISA and conventional RT-PCR. The quantities of PPV-RNA targets detected in a single aphid ranged from 40 to more than 2×10^3 units. The combined system (immobilization of targets on paper by squash capture and real-time RT-PCR) allows, for the first time, reliable quantitation of PPV targets acquired by individual aphid species and constitute an excellent tool for understanding better PPV epidemiology.

Sharka disease caused by *Plum pox virus* (PPV) is one of the most detrimental and important viral infections of stone fruit trees. It produces high economic and agronomic losses (Németh, 1994). In addition to conventional indexing, several methods based on serology, molecular hybridization or amplification by PCR and real-time PCR have been described previously in an attempt to develop sensitive techniques for detection and characterization of PPV isolates (Varveri et al., 1988; Wetzel et al., 1992; Cambra et al., 1994; Olmos et al., 1997, 1999, 2002, 2003; EPPO, 2004; Schneider et al., 2004; Varga and James, 2005).

PPV is transmitted by a number of aphid species with worldwide distribution in a non-circulative, non-persistent manner (Kunze and Krczal, 1971; Ng and Perry, 2004). PPV-viruliferous aphids may infect a tree during a simple feeding testing (Shukla et al., 1994). However, there are no estimations of the number of PPV virions or RNA targets that a single aphid might carry and transmit.

There are few reports demonstrating the feasibility of detecting non-persistently transmitted viruses in the aphid vectors using PCR-based assays (Singh et al., 1996, 2004; Singh, 1998; Olmos et al., 1997; Nie and Singh, 2001; Cambra et al., 2004), but the information provided to date is only qualitative. Real-time quantitative RT-PCR is a sensitive method for nucleic acids detection because it allows a large dynamic range of target quantification. The method has been applied to quantify RNA copies of a circulative, non-propagative virus in aphids (Fabre et al., 2003). In this study, a real-time quantitative RT-PCR assay, based on the sensitive TaqMan chemistry, was developed for the detection and quantification of the main types of PPV, D and M (Candresse et al., 1998). To design appropriate primers and probe, the nucleotide sequence flanked by the universal primers P1 and P2 (Wetzel et al., 1991) was selected. Alignment of 59 nucleotide PPV sequences recovered from databases GenBank, EMBL and DDBJ were carried out. Primer Express software (Applied Biosystems) was used to obtain the optimal oligo sequences. The primers P241 (5′-CGT TTA TTT GGC TTG GAT GGA A-3′), P316D (5′-GAT TAA CAT CAC

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CAG CGG TGT G-3′) and P316M (5′-GAT TCA CGT CAC CAG CGG TGT G-3′) generate a 76 bp DNA fragment after amplification. The TaqMan probe, PPV-DM (5′-CGT CGG AAC ACA AGA AGA GGA CAC AGA-3′) was labeled with fluorescent dyes, 6-carboxyfluoroscein (FAM) on the 5′-end and *N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3′-end.

A specific PPV nucleotide sequence (243 bp) amplified by RT-PCR (Wetzel et al., 1992), consisting of the target region for the real-time primers, was inserted into the vector pGem-T (Promega Inc.) and cloned into *E. coli* JM-109. Transformants were selected by ampicillin resistance and the orientation of the fragments verified by PCR using forward T7 and P1 (Wetzel et al., 1991) primers. The plasmid was linearized at the *Not I* site and used as target in an in vitro transcription reaction performed with Megascript T7 kit (Ambion Inc., TX) followed by *Dnase I* digestion at 37 °C for 30 min. The synthesis of the 304 nucleotide RNA (243 nucleotide bases of the PPV target + 63 nucleotide bases of the pGem-T vector) was confirmed by electrophoretical analysis in a 2% agarose gel. RNA was purified by phenol–chloroform extraction and ethanol precipitation. The amount of RNA (μg) was quantified by UV densitometry. Conversion of microgram of single stranded RNA to picomole was performed considering the average molecular weight of a ribonucleotide (340 Da) and the number of bases of the transcript (N_b). The following mathematical formula was applied: pmol of ssRNA = μg (of ssRNA) × (10^6 pg/1 μg) × (1 pmol/340 pg) × ($1/N_b$). Avogadro constant (Avogadro, 1811) was used to estimate the number of transcripts (6.023×10^{23} molecules/mol). Number of transcripts was calculated per 5 μl, which was the volume used as template in each quantitative real-time RT-PCR. Ten-fold serial dilutions of the transcripts were prepared from 4×10^{12} to 4×10^{0}, aliquoted and stored at −80 °C until use. Dilutions from 4×10^{8} to 4×10^{0} were employed to generate the standard curve. TaqMan assay for quantitative real-time RT-PCR was carried out in an ABI Prism 7000 (Applied Biosystems). The reaction cocktail contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 1× MultiScribe and RNase Inhibitor Mix (Applied Biosystems), 1 μM primer P241, 0.5 μM primer P316D, 0.5 μM primer P316M and 200 nM TaqMan probe and 5 μl of sample. RT-PCR protocol consisted of one step of 48 °C for 30 min and 95 °C for 10 min followed by 40 cycles of amplification (95 °C for 15 s and 60 °C for 1 min). Data acquisition and analysis were performed with the ABI Prism 7000 software. The default threshold set by the machine was slightly adjusted above the noise to the linear part of the growth curve, at its narrowest point according to the ABI Prism 7000 manufacturers. To determine the theoretical sensitivity and the reliability of the real-time RT-PCR, four repetitions of assay were undertaken using the 10-fold serial dilutions of the transcripts previously prepared. Although it was possible to detect as low as four transcript copies in two out of the four performed assays, the quantitation range was established from 40 copies up to 4×10^{8}, due to of the reliability of the four repetitions (Fig. 1).

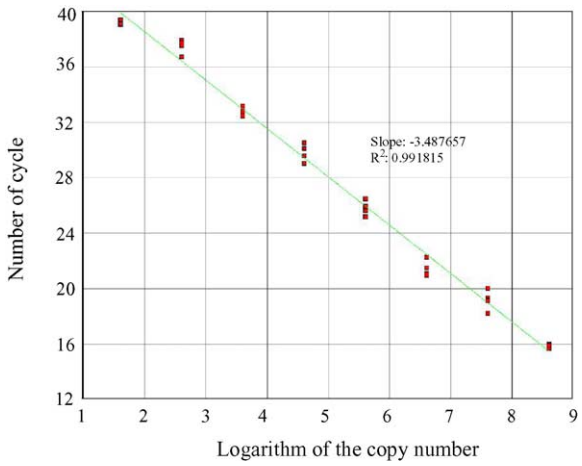

Fig. 1. Quantification range of the real-time assay based on the standard curve obtained with four repetitions of 10-fold serial dilutions of *Plum pox virus* control transcripts.

Subsequently, the method was applied to plant extracts from infected *N. benthamiana* with isolate D 3.3 RB/Nb (NAT) diluted in *N. benthamiana* extracts prepared from healthy plants (1:10 and 1/2 serial dilutions from 1:250 to 1:65,536,000, w/v). Plant extracts were prepared in individual plastic bags by grinding 1:10 (w/v) in PBS, pH 7.2, supplemented with 2% (w/v) PVP-10 and 0.2% (w/v) DIECA (Cambra et al., 1994). Viral RNA was purified with the RNeasy plant mini kit (Qiagen) from each sample, with minor modifications (Bertolini et al., 2001). Briefly, plant extract (200 μl) was mixed with RLT buffer (350 μl) and lysate applied to QIAshredder spin column, recovering the flow-through. Subsequently, absolute ethanol (100%) (0.5 vol.) was added to the lysate and the mixture applied to RNeasy column. After washing with RW1 and RPE buffer, RNA was finally eluted with 50 μl of RNase-free water, and stored at −20 °C until use.

The reliability of the real-time RT-PCR using plant material was tested in an experiment conducted with three repetitions of each sample. To evaluate the suitability of the standard curve generated with the transcripts for an accurate quantitation of PPV targets, amplification efficiencies of standards and field samples reactions were compared according to Rasmussen (2001). The slopes of the calibration curve of the standards (−3.48) and field samples (−3.52) were used to calculate efficiencies using the mathematical formula: slope = −1/log (efficiency). The efficiencies obtained were 1.93 and 1.92 for transcripts and field samples, respectively. The sensitivity of the real-time RT-PCR was compared with that obtained by double-antibody sandwich indirect (DASI)-ELISA using the monoclonal antibody 5B-IVIA (Cambra et al., 1994) (Durviz kit), conventional RT-PCR (Candresse et al., 1995) and nested RT-PCR in a single closed tube (Olmos et al., 2003). The comparison of the sensitivities provided by different techniques is shown in Table 1. It was possible to

Table 1
Comparison of DASI-ELISA, conventional RT-PCR, nested RT-PCR and real-time RT-PCR sensitivities for the detection of *Plum pox virus* isolate 3.3 RB/Nb in serial dilutions of an extract of infected *Nicotiana benthamiana*

Dilution (w/v)	DASI-ELISA values (5B-IVIA antibodies) 405 nm $X \pm$ S.E.[a]	RT-PCR[b]	Nested RT-PCR[b]	Real-time RT-PCR by D + M TaqMan probe (number of copies) $X \pm$ S.E.[c]
1:10	5048.6 ± 98.9	++[d]	++	$351.3 \times 10^6 \pm 24.1 \times 10^6$
1:250	4963.6 ± 40.8	++	++	$117.3 \times 10^5 \pm 0.8 \times 10^5$
1:500	5346.0 ± 23.8	++	++	$51.9 \times 10^5 \pm 9.0 \times 10^5$
1:1000	4969.6 ± 93.1	++	++	$41.9 \times 10^5 \pm 3.1 \times 10^5$
1:2000	3413.3 ± 26.1	++	++	$11.1 \times 10^5 \pm 1.4 \times 10^5$
1:4000	2001.0 ± 18.5	++	++	$5.3 \times 10^5 \pm 0.6 \times 10^5$
1:8000	1178.0 ± 8.1	+	++	$24.3 \times 10^4 \pm 1.0 \times 10^4$
1:16000	787.3 ± 27.3	+	++	$11.3 \times 10^4 \pm 0.8 \times 10^4$
1:32000	673.3 ± 20.0	–[e]	++	$56.8 \times 10^3 \pm 6.9 \times 10^3$
1:64000	515.3 ± 11.3	–	++	$25.7 \times 10^3 \pm 0.4 \times 10^3$
1:128000	542.6 ± 23.6	–	+	$20.7 \times 10^3 \pm 2.7 \times 10^3$
1:256000	490.0 ± 15.1	–	+	5244.7 ± 278.9
1:512000	520.6 ± 33.6	–	–	9418.2 ± 487.6
1:1024000	490.0 ± 15.2	–	–	5973.1 ± 2188.5
1:2048000	510.3 ± 26.8	–	–	1081.4 ± 79.6
1:4096000	481.6 ± 12.4	–	–	721.1 ± 327.0
1:8192000	N.T.[f]	N.T.	N.T.	107.3 ± 3.8
1:16384000	N.T.	N.T.	N.T.	38.4 ± 4.7
1:32768000	N.T.	N.T.	N.T.	111 ± 27.4
1:65536000	N.T.	N.T.	N.T.	(40.5, 8.3, undet[g,h])
Healthy control	478.8 ± 11.7	–	–	Undet

[a] *X*, average of three repetitions using two ELISA wells per assay; S.E., standard error. Measured at 2:30 h.
[b] Three repetitions with coincident result.
[c] *X*, average of three repetitions; S.E., standard error.
[d] Number of crosses from ++ to + indicate the relative intensity of electrophoretical band of the amplified products obtained in an agarose gel.
[e] No amplification.
[f] N.T., no tested.
[g] Undet, no fluorescent signal from the lysis of the TaqMan probe was detected by ABI PRISM 7000.
[h] Neither average of the three repetitions nor standard error was given because one of the repetitions was undetermined by ABI PRISM 7000.

consistently detect PPV by DASI-ELISA and RT-PCR at the same level of dilution 1:16,000 (w/v). Nested RT-PCR in a single closed tube allowed to detect PPV targets at the dilution 1:256,000 (w/v). Real-time RT-PCR with the TaqMan probe was the most sensitive technique allowing reliable detection of PPV targets at the dilution of 1:32,768,000. It was possible to detect PPV targets at the dilution 1:65,536,000 by real-time RT-PCR in two out of the three repeat tests undertaken, suggesting that the detection limit of the technique was reached. Consequently, the sensitivity provided by real-time was 100 times higher than nested RT-PCR and 1000 times higher than DASI-ELISA or conventional RT-PCR. Fabre et al. (2003) evaluated the sensitivity of real-time PCR and found that it was ten and 1000 times higher than RT-PCR and ELISA techniques, respectively, for the detection of *Barley yellow dwarf virus*.

To test the method in woody plants, GF305 peach seedlings infected with viral isolates 3.3 RB/GF, 3.4 RB/GF, Mp19 (PPV-D types) and Ms89, A1, 1M (PPV-M types) kept in collection under quarantine screenhouse facilities at IVIA were used. Successful detection and quantification was obtained from infected plant material with all assayed PPV types. The method was also tested with aphids. Adult wingless *Myzus persicae* and *Aphis nerii* were fed at different acquisition periods (5, 15, 30, 60 and 120 min) on detached leaves of GF305 peach seedlings infected with PPV D isolate 3.3 RB/GF. Ten individual aphids of each species were selected after each acquisition time. Control aphids were fed for the same periods on healthy plants. Aphids were individually squashed on Whatman 3MM paper with the round bottom of different plastic Eppendorf tubes to facilitate the complete disruption of each aphid and to avoid contaminations (Cambra et al., 2000). The filter papers with squashed aphids were stored in a dry place at room temperature until use. The piece of paper harboring each individual squashed aphid was introduced in an Eppendorf tube and 50 μl 0.5% Triton X-100 was added, vortexed and incubated for 2 min at room temperature (Olmos et al., 1996, 1997). Triton extract (5 μl) was used directly as template for nested RT-PCR and real-time RT-PCR assays. Table 2 shows the results obtained after different acquisition periods with PPV vectors and non-vectors species. In the case of the vector aphid, the level of detection had a significant increase at 2 h. However, the number of targets appeared to be very variable (from 54 to 2381) and not related with acquisition time. In the case of *A. nerii*, the detection of the targets was similar after any acquisition period and higher than in the case of *M. persicae*, although the number of targets was very variable too (ranking from 40 to 1032). The percentage of positive amplifications of PPV targets from the total number of individual aphids tested after

Table 2
Analysis of the RNA targets of *Plum pox virus* acquired by *Myzus persicae* (PPV vector) and by *Aphis nerii* (non-vector) on peach seedlings GF305 infected with PPV after different acquisition periods

Aphid		Acquisition period				
		5 min	15 min	30 min	1 h	2 h
M. persicae	Detection[a]	1/10	2/10	2/10	1/10	5/10
	Quantitation[b]	64	67, 159	108, 1780	73	54, 65, 82, 111, 2381
A. nerii	Detection[a]	3/10	2/10	3/10	3/10	4/10
	Quantitation[b]	43, 120, 1032	90, 361	41, 51, 57	40, 83, 174	54, 67, 73, 104

[a] The results were coincident between nested RT-PCR in a single closed tube and real-time RT-PCR.
[b] Number of PPV-RNA copies estimated.

different acquisition periods were 22% (11 out of 50) for *M. persicae* and 30% (15 out of 50) for *A. nerii*. Consequently, only part of the total number of aphids submitted experimentally to different feeding periods (from 5 min to 2 h) acquired detectable levels of RNA-PPV. The highest percentage of amplification obtained under our conditions with PPV was 56% (Olmos et al., 1997). Considering the high sensitivity and reliability of the assay, these results suggest that not all the aphids fed on infected leaves sting into cells containing virions. In fact, it is known the uneven distribution of PPV in the infected leaves.

No amplification was obtained from the control aphids, fed on healthy plants. The variability in the number of targets detected after each acquisition time might be due to the fact that the real feeding period and/or the volume ingested by the aphid was not controlled individually in each aphid. The detection of PPV targets in non-vector aphid species is in agreement with Olmos et al. (1999).

The method was applied to *M. persicae* individuals captured on sticky shoots (Marroquín et al., 2004) from a Japanese plum orchard with high incidence of sharka infection and results were compared with nested RT-PCR in a single closed tube. Nested RT-PCR detected amplifiable RNA targets of PPV in 7 out of 50 individuals tested. The same Triton extract was used for real-time RT-PCR, which was able to detect and quantify targets in the same aphids. The estimation of the number of targets in each reaction was <40 (6, 33), 49, 63, 65, 79 and 109. The number of targets per aphid in this assay varied from 60 to 1090 taking into account that only 5 μl out of the 50 μl of the Triton extract were used as sample.

The TaqMan assay developed was applied successfully for the detection and estimation of the RNA copies that a non-circulative, non-persistently transmitted virus (PPV) is carried by a single aphid species. The method confirmed the results obtained by nested RT-PCR in a single closed tube (Olmos et al., 2003) using immobilized targets from squashed aphids. But on the other hand, the sensitivity provided by real-time RT-PCR was 100 times higher than nested RT-PCR and permitted for the first time, an estimation of the number of PPV targets carried by individual aphids, that ranged from 40 to 2×10^3. In other assays with the circulative, non-propagative *Barley yellow dwarf virus*, the estimated mean number of viral targets in a single viruliferous *Rhopalosiphum padi* was 6.4×10^6 (Fabre et al., 2003), 3000 times higher than our highest estimations with the non-circulative non-propagative PPV. For an accurate estimation of the real number of viral targets acquired at different feeding periods, the use of Electrical Penetration Graph for monitoring insect probing and feeding behaviour (Tjallingii, 1988; Fereres and Collar, 2001) would be very convenient. Also, the use of artificial feeding with virions from purified solutions through membranes would be convenient to ensure virus acquisition in all the aphids. Nevertheless, it has been demonstrated the possibility of quantification of viral targets from fresh individual aphids as well as from aphids previously captured on sticky traps, without the need of previous RNA extraction. These combined technologies (squash capture and real-time amplification) open possibilities for a better understanding of the role of vectors in spreading non-persistent/stylet-borne viruses. The benefits of quantitative determination of the number of virions carried by a single aphid concern multiple topics ranging from studies on virus replication to epidemiological studies (Fabre et al., 2003). In addition, accurate evaluation of the number of virions used to challenge (by graft, mechanical or vector inoculation) the resistance of plants obtained from conventional breeding programmes or after genetic manipulation, would be essential in future.

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