Journal of Virological Methods 211 (2015) 51–54

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Short communication

Quantitation and localization of pospiviroids in aphids

N. Van Bogaert[a,b], K. De Jonghe[b], E.J.M. Van Damme[c], M. Maes[b], G. Smagghe[a,*]

[a] Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium
[b] Plant Sciences Unit – Crop Protection, Institute for Agricultural and Fisheries Research (ILVO), Burgemeester Van Gansberghelaan 96, 9820 Merelbeke, Belgium
[c] Department of Molecular Biotechnology, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium

Article history:
Received 10 June 2014
Received in revised form 6 October 2014
Accepted 10 October 2014
Available online 18 October 2014

Keywords:
Tomato apical stunt viroid
Potato spindle tuber viroid
Myzus persicae
Fluorescent in situ hybridization
Transmission

A B S T R A C T

In this paper, the potential role of aphids in viroid transmission was explored. Apterous aphids were fed on pospiviroid-infected plants and viroid targets in the aphids were consequently quantified through RT-qPCR and localized within the aphid body using fluorescence in situ hybridization (FISH). Based on the analytical sensitivity test, the limit of detection (LOD) was estimated at 1.69×10^6 viroid copies per individual aphid body. To localize the viroids in the aphids, a pospiviroid-generic Cy5-labelled probe was used and the fluorescent signal was determined by confocal microscopy. Viroids were clearly observed in the aphid's stylet and stomach, but not in the embryos. Viroids were detected in 29% of the aphids after a 24 h feeding period, which suggests only a partial and low concentration viroid uptake by the aphid population including viroid concentrations under the LOD. However, these results show that viroids can be ingested by aphids while feeding on infected plants, thus potentially increasing the transmission risk. The combination of FISH and RT-qPCR provides reliable and fast localization and quantitation of viroid targets in individual aphids and thus constitutes a valuable tool in future epidemiological research.

Viroids are non-protein encoding, single-stranded RNA molecules, currently considered as the smallest plant pathogens (Diener, 1971, 2003; Flores et al., 2005; Navarro et al., 2012). They cause disease in economically important crops, most notably in potato and tomato (Flores et al., 2005). Additionally, they are widespread in many ornamentals where they occur latently (Verhoeven et al., 2008, 2013). Between plants, viroid movement occurs predominantly by mechanical transmission (i.e. physical contact with contaminated sources, such as infested pruning gear) (Singh, 2006; Verhoeven et al., 2010). Transmission routes involving different insect species, as well as more complex interactions with viruses, have been proposed over the years (Singh et al., 1992; Querci et al., 1997; Van Bogaert et al., 2014). So far, no insect vectors have been identified for viroids (Nielsen et al., 2012). However, several reports do indicate that arthropods might play a role in viroid transmission (Schumann et al., 1980; Antignus et al., 2007; Matsuura et al., 2010). It is conceivable that viroids are spread purely mechanically by contaminated insect parts, surviving and causing infection when new plants are visited. This type of transmission may be similar to that of certain non-persistent viruses, where the vector acts as a contaminated needle.

For detection and quantitation of viroids in plant tissues and seeds, several PCR-based assays have been developed over the recent years (Boonham et al., 2004; Monger et al., 2010; Verhoeven et al., 2010; Botermans et al., 2013). However, these assays have not yet been validated for insect matrices. Additionally, it is currently unknown whether insects can take up viroids while feeding on infected plants, and if so, in which concentrations. Detailed information on the (ultrastructural) location of viroids in plants has been obtained in the past by fluorescent in situ hybridization (FISH) in conjunction with microscopy (McFadden, 1991). *Avocado sunblotch viroid* (ASBVd) detection in avocado leaves was realized using dioxygenin (DIG)-labelled RNA probes (Lima et al., 1994). *Coconut cadang cadang viroid* (CCCVd) and *Citrus exocortis viroid* (CEVd) have been located in plant tissues both ultrastructurally and histologically, using transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM), respectively (Bonfiglioli et al., 1996).

In this paper we describe experiments wherein viroids were quantified and localized in an insect using RT-qPCR and FISH. Two pospiviroids were used, namely the *Potato spindle tuber viroid* (PSTVd) and *Tomato apical stunt viroid* (TASVd) and the green peach aphid *Myzus persicae* (Sulzer) was chosen as a typical pest model. In the experiments the aphids were allowed to feed on viroid-infected plants. After feeding, RNA was extracted from the aphids and used in different RT-qPCR assays to quantify viroid copies. In parallel,

* Corresponding author. Tel.: +32 0 9 264 61 50; fax: +32 0 9 264 62 39.
E-mail address: guy.smagghe@ugent.be (G. Smagghe).

http://dx.doi.org/10.1016/j.jviromet.2014.10.003
0166-0934/

FISH analyses were performed with viroid-specific probes to localize the viroids in the aphid's body by means of confocal microscopy. To the best of our knowledge, this is the first study that endeavours visualizing viroids in insects.

A colony of *M. persicae* was maintained on zucchini (*Cucurbita pepo*, L.) plants during the entire course of the experiments. Pathogen-free tomato plants (*Solanum lycopersicum*, L., Cv. Marmande) and Jasmine nightshade (*Solanum jasminoides*, Paxton) were mechanically infected with PSTVd (Genbank Accession: KF49372.1) and TASVd (Genbank accession: KF484878) using carborundum, and they were tested viroid-positive before starting the experiments. Both the PSTVd- and TASVd-infected plants were used for the RT-qPCR detection experiments. For the localization experiments by FISH and CLSM, we made use of the TASVd-infected plants. Fifty adult apterous *M. persicae* were placed onto pospiviroid-infected and non-infected plants, which were separated over two gauze cages to avoid cross-contamination. Aphids were placed into small feeding tubes that were installed upon specific plant leaves. After a feeding period of 24 h, aphids observed feeding on the leaf were selected for RT-qPCR and FISH.

For quantitation purposes and to estimate the LOD, we made use of a standard serial dilution of pospiviroid RNA-transcripts, which were synthesized through transcription of a cloned PSTVd genome (357 nt). This sequence was inserted into the pGEM 1.2/blunt vector (Promega, Madison, WI, USA) and subsequently transformed into *Escherichia coli* TOP10 by electroporation. Transformants were selected by ampicillin resistance. Plasmids were linearized by XbaI digestion and used as a target in an in vitro transcription reaction using the Megascript T7 kit (Thermo Fisher Scientific, Lafayette, CO, USA) followed by TURBO DNase digestion at 37 °C for 15 min. The synthesis of the 418 nucleotide RNA (357 nt PSTVd RNA + 61 nt vector RNA) was confirmed using capillary electrophoresis (i.e. QIAxcel Advanced System, Qiagen, Hilden, Germany). RNA recovery was performed using a phenol–chloroform extraction and RNA concentration (ng/μl) was measured using a ND-1000 NanoDrop® spectrophotometer (Isogen Life Science, De Meern, the Netherlands). Finally, from this transcript, a ten-fold dilution series (10^{-1} until 10^{-10}) was prepared. To make an analogue dilution series in an aphid matrix, 2 μl of each RNA dilution was spiked onto a non-infected aphid individual. The 10 resulting aphid samples were then extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The theoretical and analytical sensitivity and specificity of the RT-qPCR were established (data not shown).

The sets of primers and probes used for the specific detection of PSTVd and TASVd are based on Boonham et al. (2004) and Monger et al. (2010), respectively. Next to these specific tests, generic primers and pUCCR-probe from Botermans et al. (2013) were used as an alternative confirmation test. For each of these assays, at least three different assays with aphids fed on infected and non-infected plants, were carried out. We calculated the amount of viroid particles present in a sample based on the average ribonucleotide molecular weight (Olmos et al., 2005), presenting results for one of the RT-qPCR assays (Table 1). Statistical analysis of the results was done with the SPSS software (IBM Corporation, Armonk, NY, USA).

For viroid localization by FISH, the aphid's digestive system, stylet and embryos were first dissected in phosphate buffered saline (PBS, pH 7.4) by means of two BD Microlance needles (25Gx5/8, Beckton Dickinson & Co, Franklin Lakes, NJ, USA) and fixed in Carnoy's fixative (chloroform:ethanol:glacial acetic acid, 6:3:1, v/v) for 5 min at room temperature. Samples were then washed 3 times for 1 min in hybridization buffer (HB) [20 mM Tris–HCl pH 8.0, 0.9 M NaCl, 0.01% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) formamide], and hybridized overnight with 10 pmol fluorescent probe/ml in HB (Ghanim et al., 2009).

Table 1
RT-qPCR analysis of PSTVd transcripts in green peach aphid. Mean Cq values, calculated concentrations of viroid RNA and number of viroid copies are shown. Analyses were performed with single and 10 pooled *M. persicae* aphids that fed on PSTVd infected plants and with a PSTVd RNA dilution series (10^{-2} until 10^{-7}) in an aphid matrix after a one-step qPCR-assay (Agpath ID™ one-step RT-PCR kit, Applied Biosystems) using the primers used by Botermans et al. (2013). The following thermal cycling conditions were used: reverse transcription (RT) at 45 °C for 10 min, reverse transcription inactivation at 95 °C for 10 min, amplification for 40 cycles at 95 °C during 15 s and 60 °C for 45 s. The PSTVd-transcript standard curve has a R^2 of 0.998 and a slope of −3.56.

Sample contents	Cq-value	Concentration (ng/μl)	Number of copies (per μl)
1× *M. persicae*	30.57	0.00398	1.69×10^7
10× *M. persicae*	23.64	0.352	1.49×10^9
PSTVd-transcript 10^{-2}	19.29	3.96	1.68×10^{10}
PSTVd-transcript 10^{-3}	24.46	0.396	1.68×10^9
PSTVd-transcript 10^{-4}	26.77	0.0396	1.68×10^8
PSTVd-transcript 10^{-5}	30.54	0.00396	1.68×10^7
PSTVd-transcript 10^{-6}	34.03	0.000396	1.68×10^6

Far-red Cyanine-5 (Cy5) was selected as the fluorochrome conjugated to a short oligonucleotide probe, based on an existing pospiviroid qPCR-probe (Cy5-5′-CCGGGGAAACCQGGA-3′, Botermans et al., 2013). After hybridization, the samples were washed 3 times in HB for 1 min, and then whole-mounted and viewed under a confocal microscope. We used the NIS Advanced Research (AR) 4.13 software connected to a Nikon A1R confocal microscope (Nikon Instruments, Paris, France) and made use of two excitation lasers, exciting at 488 nm and 639 nm for detecting autofluorescence and the Cy5-signal, respectively. The acquisition settings and scanning settings were kept fixed throughout all experiments (i.e. scan size 512, scan speed ¼ and count 4). ROI statistics were analyzed for the Cy5-channel of each picture. Dissected stylets, guts and embryos of in total 20 aphid individuals that were fed on PSTVd-infested tomato plants were pooled per 10 in Eppendorf tubes (i.e. two tubes of 10 guts, two tubes of 10 stylets and two tubes of 10 embryos). These samples were tested in duplo in a one-step RT-qPCR assay (Boonham et al., 2004, Agpath ID™ one-step RT-PCR kit, Applied Biosystems) in order to confirm viroid presence or absence in these body parts. PCR cycling conditions and quantitation using the PSTVd-dilution series in aphid matrix were identical to the experiments described for whole aphid bodies (see above and Table 1).

The two RT-qPCR assays suitable for TASVd-detection (Monger et al., 2010; Botermans et al., 2013) resulted in comparable mean Cq-values when applied on aphids fed on TASVd-infected plants ($p = 0.055$, Kruskal–Wallis). The average Cq-value for all aphid samples (individual and pooled) over the three assays was 31.0 ± 3.9. This Cq-value did not seem to be influenced by the number of aphid individuals per tube, since 1 single aphid could yield a similar, or even higher, Cq-value compared to a pool of 10. The calculated numbers of transcripts for 1 single individual and 1 pooled sample of aphids (i.e. 10 individuals) after conducting an analytical sensitivity assay using the Botermans-primers (one-step RT-qPCR), are presented in Table 1. Based on these data, the LOD was estimated at 1.69×10^6 copies for one whole aphid body. The RT-qPCR efficiency of the standard dilution series was 91% (calculated by equation: $efficiency = -1 + 10^{(-1/slope)}$, Table 1), making this series suitable for relative viroid quantitation in aphid individuals. Detection of PSTVd using the RT-qPCR technique as described by Boonham et al. (2004) in aphids that fed on PSTVd-infected tomato plants also resulted in comparable Cq values.

For the localization experiments using FISH and CLSM, the specificity of detection was confirmed using the appropriate controls (Table 2). A clear Cy5 fluorescent signal was observed in the foregut of a probe-treated aphids that had fed on a TASVd-infected plant (Fig. 1A and B). Limited background autofluorescence was