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To cite this version:

Frederic Fabre, Christine Kervarrec, Lucie Mieuzet, Gerard Riault, Aude Vialatte, et al.. Improvement of Barley yellow dwarf virus-PAV detection in single aphids using a fluorescent real time RT-PCR. Journal of Virological Methods, 2003, 110 (1), pp.51-60. 10.1016/S0166-0934(03)00097-1 . hal-02681094

HAL Id: hal-02681094

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Postprint
Version définitive du manuscrit publié dans / Final version of the manuscript published in : Journal of Virological Methods, 2003, vol.110, no. 1, 51-60

Improvement of *Barley yellow dwarf virus*-PAV detection in single aphids using a fluorescent real time RT-PCR

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Summary

One of the major factors determining the incidence of *Barley yellow dwarf virus* (BYDV) on autumn-sown cereals is the viruliferous state of immigrant winged aphids. This variable is routinely assessed using the enzyme-linked immunosorbant assay (ELISA). However, the threshold for virus detection by ELISA can lead to false negative results for aphids carrying less than 10^6 particles. Although molecular detection techniques enabling the detection of lower virus quantities in samples are available, the relatively laborious sample preparation and data analysis have restricted their use in routine applications. A gel-free real-time one-step reverse transcription polymerase chain reaction (RT-PCR) protocol is described for specific detection and quantitation of BYDV-PAV, the most widespread BYDV species in Western Europe. This new assay, based on TaqMan® technology, detects and quantifies from 10^2 to 10^8 BYDV-PAV RNA copies. This test is 10 and 10^3 times more sensitive than the standard RT-PCR and ELISA assays published previously for BYDV-PAV detection and significantly improves virus detection in single aphids. Extraction of nucleic acids from aphids using either phenol/chloroform or chelatin resin-based protocols allow the use of pooled samples or of a small part (up to 1/1600th) of a single aphid extract for efficient BYDV-PAV detection.

Key words : TaqMan probe, viruliferous aphids, virus quantitation, real-time PCR, BYDV-PAV

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1. Introduction

Any virus-based research programme, ranging from virus replication process to epidemiological studies, would not be possible without qualitative or quantitative assays for detecting and/or characterizing the pathogen. The improvement of diagnostic assays is crucial for the development of more effective measures to control any plant virus disease.

Barley yellow dwarf disease (BYDD) was observed for the first time in the early 50s based on physiological disturbances of barley plants (Oswald and Houston, 1951). Characterization of its viral agent, *Barley yellow dwarf virus* (BYDV), was performed using biological, biochemical and biophysical techniques (Rochow and Brakke, 1964; see Smith and Barker, 1999), serological assays (for a review, see French, 1995) and molecular analysis of the BYDV genome (Miller *et al*., 1988; Rizzo and Gray, 1990; Vincent *et al.,* 1991; Chaloub *et al*., 1994; Domier *et al.,* 1994; Bencharki *et al.*, 1999; Chang *et al.,* 1999). Taken together, the data allowed the identification of six distinct species of BYDV including BYDV-PAV, the most widespread in Western Europe (Lister *et al*., 1990; Leclercq-Le Quillec *et al.,* 1995). Although the disease is often sporadic, varying from field to field and from year to year, BYDD is one of the most important in small grain cereals (Lister and Ranieri, 1995).

BYDV is transmitted persistently by various aphid species feeding on Poaceae (Rochow, 1970). As no resistant cultivar is available, BYDD control mainly relies on the use of aphicide sprays. Due to the sporadic character of BYDV epidemics, insecticide treatments are not routinely required. Thus a considerable reduction of the number of sprayings could be obtained by applying insecticide only when necessary. Such a reduction can be achieved by the development of reliable risk assessment tools. However, the latter requires the understanding of factors involved in the epidemiological process. Three major components are suspected to determine the incidence of BYDV in field: (i) the number of immigrant winged aphids and the timing of the colonisation of autumn-sown cereals, (ii) their viruliferous state and (iii) the dynamics of the secondary epidemics spread (Plumb, 1990). Thus, the assessment of infectivity of aphids appears to be one of the major factors for the development of any decision making-system to improve BYDV control strategies, and more generally of any integrated pest management (IPM) strategies for BYDV control. At first, infectivity of aphids was assessed using assays essentially based on the observation of symptoms on plants (French, 1995). From the end of the 70s, enzyme-linked immunosorbant assays (ELISA), a less costly and time-consuming technique, has been used routinely for BYDV detection in single aphids (Lister and Rochow, 1979). However, the lower limit for virus detection corresponds to one million BYDV particles per sample (Canning *et al.,* 1996). This threshold induces problems of false negatives for viruliferous aphids containing a lower quantity of virus.

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Aphid virus load is variable and depends on (i) aphid species and clones (Sadeghi *et al.,* 1997a) and (ii) aphid feeding history (Bencharki *et al.,* 2000). Thus, ELISA cannot be used as the only tool to evaluate accurately the viruliferous state of aphids. In the last decade, the application of molecular techniques for virus detection such as dot-blot hybridization and reverse transcription polymerase chain reaction (RT-PCR) with or without an immunocapture (IC) step, made it possible to detect approximately 1000 genome copies in samples (Canning *et al.,* 1996). However, the requirement of running gels combined with potential problems of contamination during post-PCR manipulations made standard RT-PCR unusable for numerous samples. Thus, due to its simplicity, ELISA remains used widely for large scale testing. The more recently developed quantitative real-time PCR technology can detect as few as 10 copies of a target sequence (Puig *et al.,* 2002). As real-time PCR assay monitors PCR product accumulation during amplification by fluorescence measurement in a closed tube, no post-PCR manipulations are required to validate both detection and quantitation of the target. Real-time PCR technology has already proved to be efficient for the detection of plant RNA and DNA viruses: *Tomato spotted wilt virus* (Tospovirus) (Roberts *et al.,* 2000; Boonham *et al.,* 2002), *Tobacco rattle virus* (Tobravirus) and *Potato mop top virus* (Pomovirus) (Mumford *et al.,* 2000), *Maize streak virus* (Mastrevirus) (Lett *et al.,* 2002), *Cymbidium mosaic virus* (Potexvirus) and *Odontoglossum ringspot virus* (Tobamovirus) (Eun *et al.,* 2000), and *Sugarcane yellow leaf virus* (family *Luteoviridae*) (Korimbocus *et al.,* 2002). This described previously technology may improve upon BYDV detection techniques. Thus, a one-step real-time RT-PCR assay using TaqMan® chemistry for the detection and quantitation of the BYDV-PAV isolates was developed and optimized for detection of the virus in single aphids. The sensitivity, reproducibility and specificity of this new detection method for BYDV-PAV were assessed and compared to published previously ELISA and RT-PCR techniques.

2. Materials & Methods

2.1 Viruses, aphids and plants

The BYDV-PAVb isolate, collected from barley plants in Bourgogne (Central France) in 1996 (Papura *et al.,* 2002), causes severe dwarfing and yellowing of susceptible barley cv. *Express* (Leclercq-Le Quillec, 1992). Isolates PAVvd29, PAVc9, PAV21 and PAVB12 were collected in 1997 from maize in Vendée (western France), Alsace (eastern France), Drôme (southeastern France) and Haute Garonne (southwestern France), respectively. BYDV-PAVPf was collected from wheat in Ille et Vilaine (western France) in 2000. The *Cereal yellow dwarf virus*-RPV (CYDV-RPV) RG isolate was collected from rye-grass in 1988 in Yvelines (central

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France). Finally, BYDV-RMV306 is an isolate collected in Algeria in 1998. Since they were collected, these isolates have been separately maintained in a program-controlled chamber at 20° C, L :16/D :8 on the barley cv. *Express* infested by the Rp1 clone of *Rhopalosiphum padi* (Simon *et al.,* 1991), an efficient BYDV-PAV vector (Sadeghi *et al.,* 1997a). Aphids (Fourth instar or adult) born on these infected plants were used as viruliferous samples for BYDV-PAV detection. Healthy barley seedlings and virus-free *R. padi* were used as control in BYDV detection and quantitation experiments. BYDV-PAV Mex and CYDV-RPV Mex infected leaves were supplied by M. Henry (Mexico strains, Centro Internacional de Mejoramiento de Maïz y Trigo, Mexico).

2.2 Sample preparation

Plant sap was extracted from 100 mg of healthy or infected plants by squeezing leaves in a cylinder press in the presence of 1 ml of grinding buffer (PBS, 0.05 % (v/v) Tween 20 and 2 % (w/v) polyvinylpyrrolidone [PVP K40, Sigma]). Individual aphids were placed in a microcentrifuge tube and ground with a pestle in 100µl of grinding buffer. The resulting samples were immediately used for ELISA tests or nucleic acid extraction.

2.3 Virus purification

The BYDV-PAVb isolate was semi-purified from infected barley cv. *Express* using a variation of a previously published purification protocol (Filichkin *et al.,* 1994). Briefly, 4 g. of infected plant material (leaves and roots) was squeezed in the presence of 40 ml of grinding buffer and homogenised immediately with 0.5 % (w/v) cellulase (8.9 u/mg), 0.75 % (w/v) hemicellulase (9 u/mg) and 0.3 % (w/v) driselase for 3 h at 37° C and left overnight at 4° C. Cell debris was pelleted by centrifugation at 5 000 g. The supernatant was vortexed with 0.2 volume of chloroform/isoamyl alcohol (2:1) and left on ice for 45 min. Centrifugation at 8 000 g for 10 min at 4° C removed insoluble debris. Virus present in the supernatant was precipitated by addition of 10 % (w/v) polyethylene glycol (PEG) 8 000 g and 0.25 M NaCl. After centrifugation at 8 000 g for 15 min at 4° C, the pellets were resuspended in cold sodium phosphate buffer (0.1 M, pH 6.0) and viral particles were purified through a 10-50 % sucrose density gradient as described by Hammond *et al.* (1983). Gradients were collected in 1 ml fractions. The latter were concentrated using a Microcon 100 filter [Millipore] to a volume of 40 µl.

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2.4 Nucleic acid extraction

Two different techniques using either phenol/chloroform or chelex® were used to extract nucleic acids from samples. Firstly, total nucleic acids were extracted from plant sap, ground aphids or semi-purified BYDV-PAVb. Sap (200 µl) of from healthy or infected plants, 50 µl of macerated viruliferous or virus-free aphids and 10 µl of purified viral particles were separately mixed with one volume of phenol/chloroform/isoamyl alcohol (25:24:1), vortexed for 10 sec and centrifuged at 12 000 g for 10 min at 4° C. One volume of chloroform/isoamyl alcohol (24:1) was added to the resulting aqueous layer. After a quick vortexing step, the mixture was centrifuged at 12 000 g for 10 min at 4° C. Nucleic acids present in the aqueous phase were precipitated in the presence of 10 µg of glycogen by the addition of 2 volumes of cold absolute ethanol and 0.1 volume of 3 M sodium acetate, pH 5.5. The precipitation step was performed at –20° C for 2 h. After pelleting at 12 000 g, the supernatant was discarded and nucleic acids were washed with 70 % ethanol, dried, resuspended in 50 µl of RNase-free water and stored at –20° C until used. Secondly, a method using chelatin resin (Chelex® 100, [BIORAD]) was used as described by Boonham *et al.* (2002). Macerated viruliferous or virus-free aphids (50 µl) were mixed with 100 µl of 25 % (w/v) Chelex® resin, heated at 94° C for 5 min and centrifuged for 5 min at 8 000 g. The pellet was discarded and the supernatant was stored at –20° C until used.

2.5 Enzyme Linked Immunosorbant Assay (ELISA)

BYDV-PAV detection in plants was performed using a double antibody sandwich (DAS) ELISA protocol whereas detection of low virus load in aphids was optimized by the use of a triple antibody sandwich (TAS) assay. Microtitre plates were coated with 1 µg/ml of BYDV polyclonal PAV52 antibody (supplied by H. Lapierre, INRA Versailles, France) in carbonate buffer (pH 9.6) for 2 hours at 37° C. Between each step of the ELISA protocol, plates were washed three times with PBST buffer (PBS, 0.05 % (v/v) Tween 20). Samples (100 µl and 50 µl for plant and aphid, respectively) were added in coated wells and left overnight at 4° C. For BYDV detection in aphids, a mouse-derived monoclonal antibody PM63 (supplied by H. Lapierre, INRA Versailles, France) was diluted 1/10 in grinding buffer and 50 µl were added to the plate wells for 2 hours at 37° C. Alkaline phosphatase conjugated to IgG-PAV52 and to anti-mouse antibody [Sigma], diluted 1/1000 and 1/10000, respectively, with grinding buffer supplemented with 0.2 % (w/v) ovalbumin, were respectively used in DAS- and TAS-ELISA tests. After 2 hours at 37° C, plate wells were filled with 100 µl of *p*-nitrophenyl phosphate (1 mg/ml) in substrate buffer (1 N diethanolamine, pH 9.6). The colorimetric reaction spent for 2 hours at room temperature. The absorbance of samples was read at 405 nm using a microplate reader (Titertek Multiscan[MCC]).

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2.6 Standard reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription of the viral RNA was carried out with 3 U of AMV reverse transcriptase [Promega], 10 pmol of the oligonucleotide 5'-^{5331}CAATCCTGACGATCGGGTGTACAT^{5308}-3' (nucleotide positions according to BYDV-PAV vic1 isolate (Miller *et al.,* 1988; Genbank accession number X07653)), 20 nmol of each dNTP, 20 U of RNasin [Promega] and 10 µl of the total nucleic acid extract. The reaction was performed according to the enzyme manufacturer's instructions in a final volume of 20 µl. The cDNA region corresponding to the coat protein (nt 2857–3457) was then amplified by PCR using 2.5 U of the AmpliTaq polymerase [Applied Biosystems], 40 pmol of forward primer (5'-^{2857}ATGAATTCAGTAGGTCGTAGAGG^{2879}-3') and reverse primer (5'-^{3459}CTATTTGGCCGTCATCAAACTG^{3438}-3'), 20 nmol of dNTP, 75 nmol $MgCl_2$, 10 µl of cDNA and adjusted with sterile water to a final volume of 50 µl. The reactions were cycled in a Hybaid Express thermal cycler for 40 cycles of 94° C for 1 min, 52° C for 1 min and 72° C for 1 min. The PCR products (10 µl) were analyzed by electrophoresis in a 1 % agarose gel, stained with ethidium bromide and observed under UV illumination.

2.7 Preparation of viral RNA standards for real-time RT-PCR assay

The plasmid pPAV6, containing full-length BYDV-PAV cDNA (Mohan *et al*., 1995), was used to produce viral RNA transcripts. One µg of pPAV6 was linearized by *Sma*I and purified using the phenol/chloroform extraction protocol described above. BYDV-PAV RNA transcripts were generated in the presence of the m^7GpppG cap analogue [Biolabs] at 37° C for 3 hours with the Ribomax T7 Kit [Promega] according the manufacturer's instructions. The *in-vitro* transcription process was ended by digestion of the pPAV6 plasmid using RNase-free DNase I for 15 min at 37° C. Viral transcripts were extracted using the phenol/chloroform protocol, ethanol precipitated and resuspended in 100 µl of RNase-free water. RNA concentration was determined by spectrophotometry.

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2.8 Designing primer and probe for real-time RT-PCR assay

A highly conserved region in the BYDV-PAV ORF 3 (Fig. 1A), identified by alignment of 9 published coat protein sequences, was used to define forward (Fp) and reverse (Rp) primers and a TaqMan® FAM-labelled (TMp) probe (Fig. 1B). Two primers and one probe were selected from the 200 combinations proposed by the Primer Express software [Applied Biosystems].

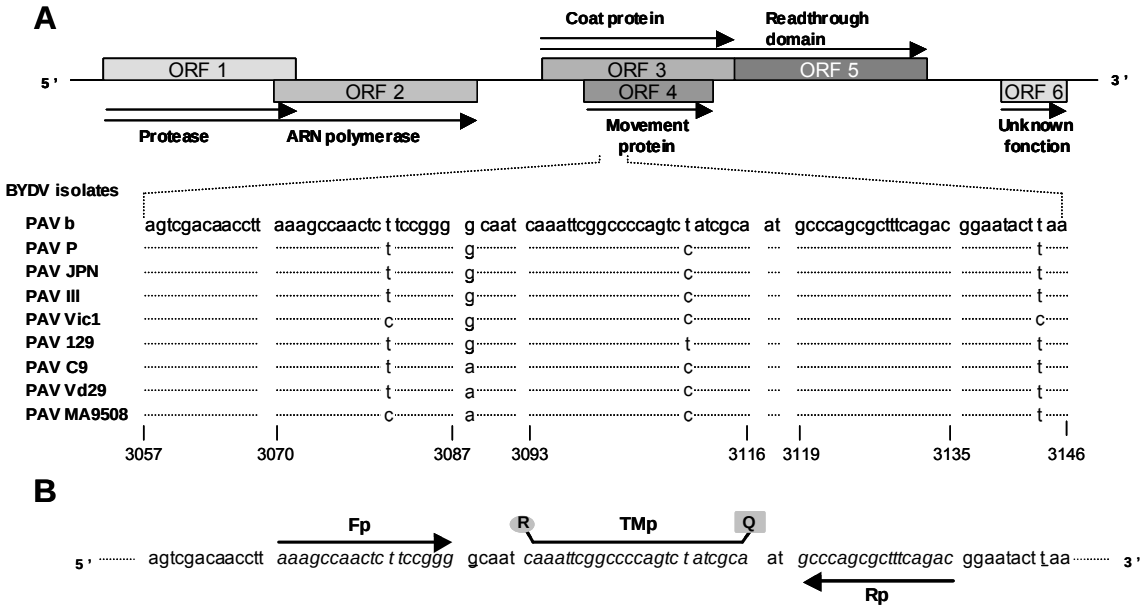

Figure 1: Identification of a BYDV-PAV sequence used as target in a real-time RT-PCR assay. A. BYDV-PAV genomic organisation. Grey boxes illustrate open reading frames (ORF1 to ORF6). The function of the corresponding proteins are denoted. Partial nucleotide sequence alignment of BYDV-PAV isolates (isolate, Genbank accessions: BYDV-PAV b, AY040344; P, D11032; JPN, D85783; III, AF235167; Vic1, X07653; 129, NC 002160; C9, AY167108; Vd29, AY167109; MA9508, AJ007921) is illustrated. Nucleotide changes between isolates are presented. The BYDV-PAVb, P, JPN, III and vic1 isolates belong to the PAV Cp A group whereas isolates 129, C9, Vd29 and MA9508 are BYDV-PAV Cp B isolates according to Miller *et al.,* (2002) and Lapierre H. (INRA, Versailles France, personal communication). B. Binding sites for forward primer (Fp), reverse primer (Rp) and TaqMan® probe (TMp). The fluorescent reporter (R) and quencher (T) linked respectively to the 5' and 3' extremity of the TMp are denoted.

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2.9 TaqMan® one step RT-PCR assay

TaqMan® RT-PCR reactions were performed in a final volume of 25 µl using the One-Step RT-PCR Master Mix Reagents Kit [Applied Biosystems] according to the manufacturer's instructions. The reactions, carried out with 2.5 µl of total nucleic acids from plants, aphids or virus particles, were performed on the ABI Prism 7700 Sequence Detection System [Applied Biosystem]. The reverse transcription of the viral RNA was achieved at 48° C for 30 minutes. PCR performed with the hot-start Ampli Taq polymerase [Applied Biosystem] included an enzyme activation step (10 min at 95° C) followed by 40 cycles of denaturation/annealing/extension (15 sec at 95° C; 1 min at 60° C). During the amplification process, the fluorescent intensities of the reporter dye (FAM), the quencher dye (TAMRA) and the passive dye reference (ROX) were recorded. These data allowed calculation of the normalised reporter signal (ΔR_n) which is linked to the amount of product amplified. The threshold cycle (C_T) refers to the number of amplification cycles required for a significant increase in the reporter's fluorescence (Mumford *et al.,* 2000).

3. Results

3.1. Optimisation and specificity of TaqMan® assay

According to the manufacturer's recommendations, the TMp TaqMan® probe was introduced initially in real-time RT-PCR reactions at 250 nM. The Fp and Rp primers (Fig. 1B) were subjected to an optimisation of concentration using a 3 X 3 matrix of 50, 300 and 900 nM for each primer concentration. This procedure was carried out using BYDV RNA resulting from *in vitro* transcription of the pPAV6 plasmid. The optimum concentration was found to be 300 nM for both forward and reverse primers (not illustrated). TaqMan® probe concentration was then optimised in order to reduce the quantity used in reactions. Detection of the BYDV target by real time RT-PCR was efficient and reproducible with 100 nM of TaqMan® probe.

Quantitation of molecular targets using real-time RT-PCR requires a linear relationship between the threshold PCR cycle (Ct) defined during the exponential increase of the reporter's fluorescence and the logarithm of the initial target quantity in the corresponding sample. Serial dilutions of BYDV-PAV RNA was performed in order to obtain solutions containing 10^8, 10^7, 10^6, 10^5, 10^4 or 10^3 copies of the viral RNA in 2.5 µl. When such samples were tested in real-time RT-PCR assays (Fig. 2), the expected relationship was observed (not illustrated). The slope (-3.325) and the correlation coefficient (R^2=.997) of the latter show that this molecular assay can be used to quantify BYDV-PAV RNA in samples containing from 10^3 to 10^8 copies.

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Moreover, diluted samples containing as low as 10^2 BYDV-PAV RNA copies/2.5 µl were efficiently detected and quantified (not illustrated). The specificity of the designed primers and probe for BYDV-PAV was tested using BYDV-MAV, BYDV-RMV and CYDV-RPV isolates, and a wide range of BYDV-PAV isolates including 8 from Europe and 3 from America and belonging, according to Mastari *et al.* (1998) and H. Lapierre (personnal communication), to BYDV PAV-A or -B groups (Table 1). The assay is restricted to detection of BYDV-PAV isolates (Table 1).

Table 1. BYDV-PAV species and strains used to test the specificity and sensitivity of the developed real-time RT-PCR assay.

BYDV species	Isolate	Cp	Origins	Taq Man detection	Reference
PAV	6	A	USA[a]	+	Mohan *et al*., 1995
	Cloutier	A	Canada	+	Rochow and Carmichael, 1979
	Mex	A	Mexico	+	XXX
	B	A	France	+	Papura *et al*., 2002
	13	A	France	+	Papura *et al*., 2002
	4	A	France	+	Papura *et al*., 2002
	21	A	France	+	Unpublished
	Pf	A	France	+	Unpublished
	C9	B	France	+	Described in this report
	Vd29	B	France	+	Described in this report
	B12	B	France	+	Unpublished
MAV	2		France	-	Sadeghi *et al*., 1997 (b)
	11		France	-	Sadeghi *et al*., 1997 (b)
RPV	RG		France	-	Unpublished
	Mex		France	-	XXX
	306		France	-	Unpublished

[a] Infectious clone

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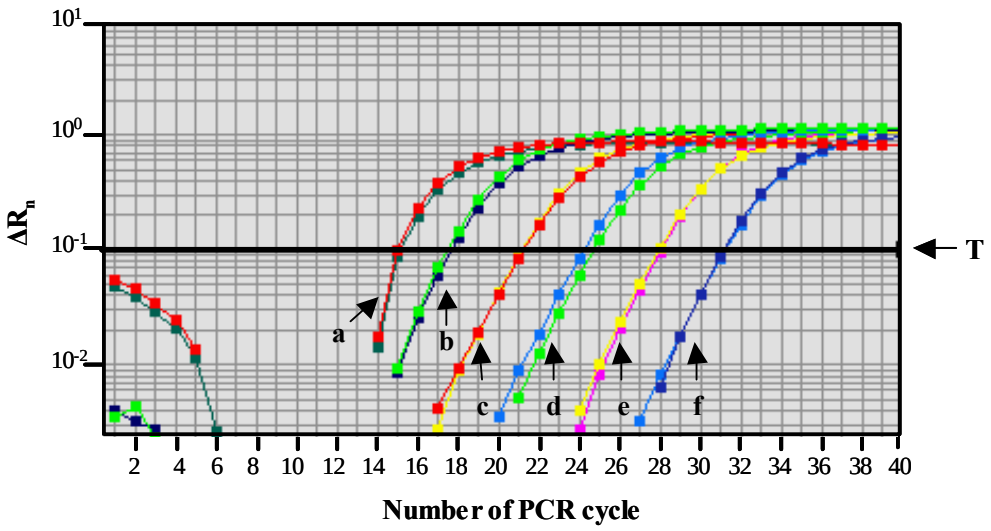

Figure 2: Real-time RT-PCR quantitation of BYDV-PAV RNA. Amplification plots showing the testing in duplicate of a 10-fold dilution serie containing viral transcripts at : 10^8 (a), 10^7 (b), 10^6 (c), 10^5 (d), 10^4 (e) and 10^3 (f) template molecules/reaction. The threshold (T) of normalized reporter fluorescence (ΔRn) used for Ct calculation is represented with a black horizontal line.

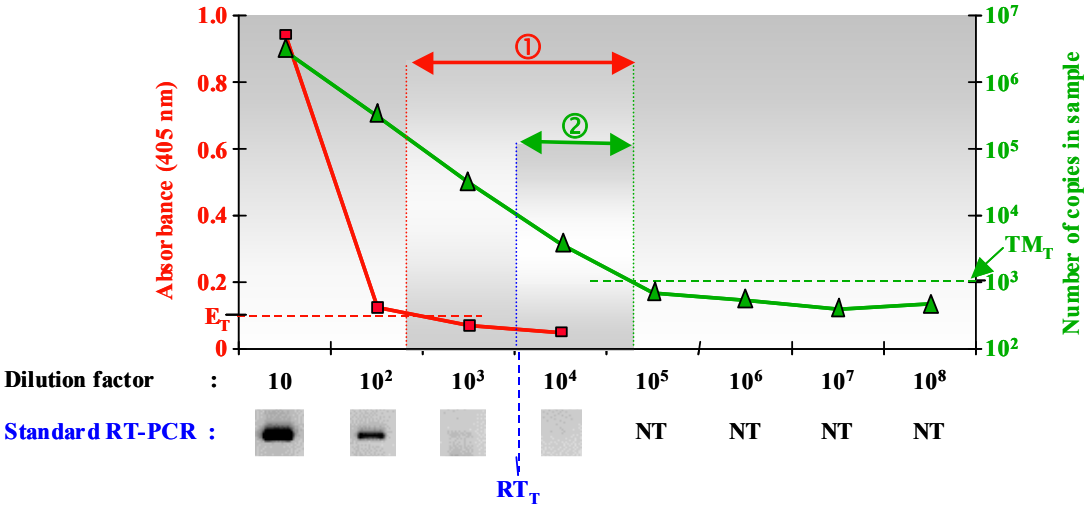

Figure 3: Comparison between end-point dilutions of serological (ELISA) and molecular (standard and real-time RT-PCR) detection of BYDV in plant extracts. ELISA was performed on crude sap whereas molecular detection of BYDV RNA were performed on total nucleic acids extracts. Dilution factors of sample was reported according to various volumes used in phenol/chloroform extraction process and quantities required to perform assays. Quantitation (number of copies in sample) data are resulting from real-time RT-PCR assay; Absorbance (405nm) are obtained using ELISA tests. Standart RT-PCR product were visualised under UV illumination after agarose gel electrophoresis. E_T, TM_T and RT_T denote the ELISA, real-time RT-PCR and standard RT-PCR thresholds for BYDV detection, respectively. ① and ② correspond to the difference of sensitivity between real-time RT-PCR and ELISA or standard RT-PCR, respectively. NT: not tested.

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3.2. Testing the BYDV-PAV TaqMan® assay sensitivity on plants

In order to assess if the TaqMan® assay could detect lower virus concentrations than ELISA and RT-PCR, the end-point dilution of the three techniques was determined on plant extracts. Ten-fold serial diluted fractions of crude sap (using the grinding buffer) or of total nucleic acids extracts from BYDV-PAVb infected plants (using RNase-free water) were produced and tested using the three detection techniques. In these experimental conditions, the threshold values for ELISA and TaqMan® assays were respectively defined as A_{405}=0.1 and 10^3 copies of BYDV template. The standard RT-PCR threshold was determined by the sensitivity of the camera used to visualize double-stranded DNA fragments in the presence of ethidium bromide after agarose gel electrophoresis. End-point dilution factors for DAS-ELISA, standard RT-PCR and real-time RT-PCR were in the range [10^2 – 10^3], [10^3-10^4] and [10^4-10^5] respectively (Fig. 3). Thus, real-time RT-PCR was close to 10^3- and 10-times more sensitive than ELISA and standard RT-PCR, respectively (Fig. 3, ① and ②, respectively).

3.3. Improvement of BYDV-PAV detection in a single aphid

TAS-ELISA, standard RT-PCR and real-time RT-PCR techniques were compared, using serial dilutions of either crude aphid homogenates (using the grinding buffer) or total nucleic acid extracts from viruliferous insects (using RNase-free water), to investigate their capacity to detect BYDV-PAV in a single aphid.

3.3.1 Serological detection of BYDV-PAV

When whole viruliferous aphids were individually used as sample, TAS-ELISA allowed the detection of viral particles in most (43/45; 96 %) virus-containing aphids with a mean A_{405} value of 0.476 (Table 2). Fifty-three viruliferous aphids were individually ground and the resulting homogenates were serially diluted to obtain fractions corresponding from half to 1/16th of an entire aphid. These fractions were used to test the efficiency of ELISA to detect BYDV-PAV in a single aphid. Data resulting from samples corresponding to half an aphid extract range from 0.866 to 0.020 (not illustrated). As the positive threshold of ELISA in our experimental conditions corresponds to A_{405}>0.1, only 33 out of the 53 (62 %) twofold-diluted samples were considered as virus-containing samples. Reduction of the aphid extract fraction used for the serological test produces a decrease of the number of viruliferous aphids from 33 for half aphids to 0 when the samples corresponded to only 1/16th of aphid homogenate. The end-point dilution to achieve detection of BYDV in single insects depends on the initial virus load of the tested aphid. At a high virus load, at least 1/8th of the crude homogenate must be used to obtain effective detection of BYDV-PAV particles with ELISA.

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Table 2. Detection of BYDV-PAV particles in whole or part of single Rhopalosiphum padi

State of tested aphids	Viruliferous					Virus-free
Fraction of aphid	whole	half	1/4th	1/8th	1/16th	half
Detection of BYDV using TAS-	13/16	33/53	12/53	5/53	0/53	0/10
Mean of A_{405}	0.207	0.286	0.243	0.146	0.005	0.001

[a] : Number of positive samples/Number of tested aphids

3.3.2 Molecular detection

Nucleic acids from virus-free or viruliferous aphids were extracted using a phenol/chloroform procedure, serially diluted and used to amplify BYDV RNA using standard or real-time RT-PCR assays. Taking into account the various volumes used in extraction of nucleic acids from single aphids and quantities require to perform assays, standard RT-PCR allows efficient detection of BYDV-PAV in 1/16th of a whole viruliferous aphid extract (Table 3). Moreover, detection of viral RNA can be achieved using only 1/160th of the aphid homogenate (2/12, Table 3). The newly developed TaqMan® assay was performed on single aphid extracts using the same diluted fractions. Detection and quantitation of BYDV genome was successfully achieved with the 1/16th, 1/160th and 1/1600th fractions. Some viruliferous aphids gave positive results when detection of BYDV-PAV was performed on samples diluted ten more times (2/12, 1/16000th). The quantitation of viral particles in all the tested samples were in accordance with the 10-fold dilution series, making it possible to determine the mean number of BYDV particles in the sampled viruliferous *R. padi* as 6.4×10^6 particles per aphid. As expected, no BYDV was detected in virus-free aphids using standard RT-PCR. However, TaqMan® assays performed with fractions from control aphids diluted 16 times produce fluorescence during amplification that was translated by the Sequence detection system software as 8×10^2 BYDV-PAV copies (Tab. 3). This background fluorescence, probably due to phenol traces in samples was effectively eliminated (1.4 copies) in the fractions diluted 160 times.

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Table 3. Detection and quantification of BYDV-PAV RNA in part of single *Rhopalosiphum padi* using standard or real-time RT-PCR assays

State of tested aphids	Viruliferous				Virus-free	
Fraction of aphid	1/16th	1/160th	1/1600th	1/16000th	1/16th	1/160th
Detection of BYDV using Standard	12/12	2/12	0/12	0/12	0/4	0/4
Detection of BYDV using real-time	12/12	12/12	12/12	2/12	0/4	0/4
Mean of BYDV copies in tested	$3.5\ 10^5$	$4.0\ 10^4$	$4.5\ 10^3$	$4.26\ 10^2$	$8.0\ 10^2$	$1.4\ 10^0$

[a] : Number of positive samples/Number of tested aphids

Similar results were obtained with nucleic acids from viruliferous aphids extracted using the fast Chelex®-based extraction protocol (not illustrated). When the two extraction procedures were performed simultaneously on the two halves of individual aphid extracts, calculated extraction yields based on quantitation of viral particles indicated that quantitation of BYDV-PAV particles using Chelex®-derived samples was similar to that obtained using phenol/chloroform. Moreover, the undiluted control samples obtained after Chelex® extractions were not associated with any background fluorescence.

4. Discussion

In the present study, we describe a one-step real-time RT-PCR assay for the specific detection of BYDV-PAV in infected plants and in viruliferous aphids. The developed protocol is able to detect and quantify the targeted virus in samples containing from 10^2 to 10^8 genome copies. Real-time RT-PCR detection of BYDV-PAV using the described primers and probe was found to be from 10- to 100-times more sensitive than standard RT-PCR assays. Such improvement of sensitivity for plant virus detection using real-time RT-PCR (TaqMan-based technology) has been reported (Mumford *et al.,* 2000; Korimbocus *et al*., 2002; Eun *et al.,* 2000; Roberts *et al*., 2000). However, in these reports, the increase in sensitivity was especially relevant for detection of virus in the host plants or vegetative organs. For our detection assay for BYDV-PAV, real-time RT-PCR technology is, above all, relevant for the efficient and sensitive detection of virus in single aphids.

The decrease of threshold for virus detection leads to an improvement of control schemes for plant virus diseases, especially for perennial cultures where control is often based on the early eradication of infected plants (Thresh, 1988). However, the success of such methods mostly depends on the possibility of early diagnosis of the infection. Highly sensitive

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molecular detection methods (PCR, RT-PCR or hybridization assays) have allowed the detection of pathogens in infected hosts earlier than did serological or biological detection protocols (Korschineck *et al*., 1991; Muller *et al.,* 2001). When applied to viruses with slow infection and/or symptom expression processes, this reduction in the time between inoculation and detection will help to reduce secondary spread of the pathogen and increase the efficiency of the eradication process. This motivates the use of new methods to develop more powerful and cost-effective diagnostic tools. In the case of insect-transmitted viruses, numerous strategies have been developed to avoid plant damage (Maelzer, 1986). Some of them require to assess the viruliferous state of insects (Miyai *et al*., 1986; Burgess *et al.,* 1999; Dewar and Smith, 1999). However, virus loads of insects depend on the kinetics of virus transmission, the ability of virus to replicate in insects and on the feeding history of the latter. Members of the *Luteoviridae* are transmitted by aphids in a persistent non propagative manner. Aphids acquire a defined number of viral particles during feeding on infected plants that will progressively decrease due to enzymatic degradation, elimination in the aphid honeydew (Power and Gray, 1995) or by inoculation into new plants. Quantitation of BYDV-PAV particles in viruliferous aphids using the described TaqMan® protocol indicates that the mean number of viruses per aphid was 6.4×10^6. Thus, the identification of viruliferous vectors requires the use of a sensitive technique allowing the detection of less than 10^6 particles. Up to now, studies performed on field-collected aphids using serological assays (DAS- or TAS-ELISA) have indicated that between 0 to 20% of the insects were viruliferous (Kendall and Chinn, 1990 ; Foster *et al.,* 1993 ; Leclercq-Le Quillec *et al.,* 1995). However, taking into account the detection threshold of ELISA and data on BYDV load capacities of aphids assessed in this report, ELISA is probably unable to identify all viruliferous aphids and most of the published data concerning frequencies of natural viruliferous aphids are likely to have been underestimated. From a technical point of view, real-time RT-PCR combines the high sensitivity and specificity of molecular detection techniques (as low as 100 copies detected) with rapidity (up to 96 assays performed in less than 3 hours), simplicity (one-step and gel-free) and compatibility with robotic workstations of serological assays. The protocol presented in this paper has the potentiality to permanently replace ELISA for routine BYDV-PAV detection. The RNA extraction step which could be time- and resource-consuming might be replaced by the use of a quick Chelex®-based protocol. Moreover, the latter minimizes the use of toxic chemicals. Finally, the one-step concept of this test and the elimination of post-PCR processing reduce cross-contamination risks and facilitate processing of high number of samples.

Beside the threshold sensitivity of the diagnostic test used to determined the viruliferous state of collected aphids, the accuracy of the risk index also depends on the number of samples taken from the field. For economical reasons, this number is often limited. When

hundreds of samples have to be tested, assays are often performed on pooled batches rather than individual insects. However, dilution effects resulting from the pooling procedure require the use of a detection method sensitive enough to detect a positive insect with low virus load among a pool of virus-free insects. As real-time RT-PCR was carried out successfully using 1/1600th of the total nucleic acids extracted from a single aphid, this technique will allow the detection of a single viruliferous aphid mixed with many virus-free insects. Finally, the improvement of virus detection sensitivity offered by the TaqMan assay goes beyond virus detection in single aphids. Indeed, the use of only part of individual aphid extract for virus detection will allow the genotyping of the insect to provide a better understand of the relationship between genotype and virus transmission (Vialatte *et al.,* 2002). Studying these relationships is important for evaluating the contribution of the aphid genotypes within a species to virus spread (Gray *et al.,* 2002). The detection of as few as a hundred BYDV-PAV particles will facilitate studies on BYDV biology, and in this respect real-time RT-PCR will provide a quantitative approach for examining the initial steps in BYDV replication in susceptible or resistant plants, as noted by Balaji *et al.* (2002).

In conclusion, the RT-PCR assay described has the required sensitivity for detecting low quantities of virus and is at present the best tool to evaluate the presence of viruliferous vectors in the field. Moreover, the numerous technical advantages of real-time RT-PCR makes it compatible with the requirements of routine use. A further step toward the improvement of BYDV detection in single aphids for risk assessment purpose will be the development of multiplex real-time RT-PCR to detect simultaneously and specifically more than one virus species in a single assay. Although BYDV-PAV is prevalent in Europe, *R. padi* is able to transmit BYDV-MAV (Sadeghi *et al.*, 1997b) and CYDV-RPV.

Acknowledgements

We are grateful to André Comeau, Charles-Antoine Dedryver, Cécile Desbiez and Danièle Giblot-Ducray for critical reading of the manuscript. This work was supported by the Institut National de la Recherche Agronomique (France), the Ecole Nationale Supérieure d'Agronomie (Rennes, France) and Bayer CropScience.

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