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Journal of Virological Methods 116 (2004) 139–146
Journal of Virological Methods
www.elsevier.com/locate/jviromet

Development of a real-time RT-PCR assay for the detection of Potato spindle tuber viroid

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Received 18 September 2003; received in revised form 10 November 2003; accepted 11 November 2003

Abstract

Potato spindle tuber viroid (PSTVd) is a quarantine pathogen in the European Union and causes damaging diseases of solanaceous crops. Under the EU Plant Health directive 2000/29/EC, countries must have the ability to detect and identify accurately and rapidly the introduction of harmful organisms in plants or plant products; furthermore, if the quarantine pathogen is found, be able to survey extensively for it. In this respect, PSTVd poses an interesting technical problem, since its RNA does not code for any proteins and thus any diagnostic method must be based on the detection of the RNA and be suitable for scaling up to testing large sample numbers. With this in mind a one-tube real-time RT-PCR assay based on TaqMan™ chemistry was developed. Investigations were carried out into various aspects of the assay relevant to the efficient amplification of targets that have a significant amount of secondary structure such as viroids. Thus comparisons were made of reverse transcription temperature, concentration and type of reverse transcriptase, RNA denaturation, sample purity and single versus two-tube reaction format. The assay developed was shown to be able to detect a wide range of isolates of PSTVd and in comparison with a chemi-luminescent hybridisation system was shown to be 1000-fold more sensitive. A further significant advantage of this assay format compared with hybridisation is that it is suitable for scaling up to large sample numbers using robotic liquid handling systems.

Keywords: Potato spindle tuber viroid, PSTVd; Real-time PCR, RT-PCR; TaqMan

1. Introduction

Potato spindle tuber viroid (PSTVd) is the type member of the genus *Pospiviroid* (family Pospiviroidae) and consists of a single-stranded, circular RNA molecule, measuring between 356 and 361 nucleotides in length. In common with other viroids, this RNA contains no open reading frames (ORFs) and is not known to code for any protein products. Due to a high degree of internal sequence complementarities, PSTVd RNA has a complex secondary structure and as a result is extremely stable ex planta (Gast et al., 1996). PSTVd is highly infectious, being spread rapidly by contact, through pollen, true seed and by aphids, through transencapsidation with Potato leaf roll virus (PLRV) (Grasmick and Slack, 1986; Salazar et al., 1995). Losses in potato caused by PSTVd can vary with isolate and cultivar, but can decrease yield by up to 64% (Pfannenstiel and Slack, 1980). PSTVd has a narrow natural host range being found in potato (and other tuber forming *Solanum* spp.) avocado, pepino and tomato (Puchta et al., 1990; Querci et al., 1995; Verhoven and Roenhorst, 1995; Mackie et al., 2002). At present, naturally resistant varieties are unavailable and as a result control centres around either national (e.g. seed certification schemes) or international (e.g. quarantine restrictions) control measures. PSTVd is a listed quarantine organism for many regions, including the European Union.

Whatever scheme is employed, the starting point for the control of PSTVd is the use of a rapid and reliable detection method. Since viroid RNA does not code for any proteins, diagnostic approaches based upon serology are not applicable. As a result a variety of alternative diagnostic approaches have been used for detecting PSTVd, including bio-assays (Raymer and O'Brien, 1962), return-polyacrylamide gel electrophoresis (R-PAGE) (Schumacher et al., 1986;

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doi:10.1016/j.jviromet.2003.11.005

Schroeder and Weidemann, 1989) and nucleic acid hybridisation (Owens and Diener, 1981; Welnicki and Hiruki, 1992; Nakahara et al., 1998). In recent years, the polymerase chain reaction (PCR), combined with reverse transcription (RT-PCR) has been used for the detection of PSTVd (Joung et al., 1997; Weideman and Buchta, 1998; Shamloul et al., 2002). However, while PCR-based methods have a number of advantages over other techniques, they also carry some practical limitations for routine diagnostic use, in particular they tend to be labour-intensive and since the methods are 'open tube' they run the risk of carry-over contamination and the possibility of false positive results. The use of homogeneous assay systems such as TaqMan has been successfully applied to the detection of plant viruses (Mumford et al., 2000; Korimbocus et al., 2002; Boonham et al., 2002). This technique combines a probe (labelled at each end with a reporter and a quencher dye and which is designed to anneal to a sequence internal to the PCR primers) with the 5′ exonuclease activity of Taq polymerase (Holland et al., 1991). While the probe is intact, fluorescence emitted by the reporter is absorbed by the quencher (fluorescent resonance energy transfer). During amplification the probe is cleaved separating the dyes, resulting in the release of fluorescence related to the amount of product amplified. The increase in reporter fluorescence is monitored in real time during amplification, using a combined thermal cycler and fluorescence reader e.g. the ABI PRISM™ 7700 or 7900HT sequence detection system. As a result, TaqMan® assays are closed-tube and no post PCR manipulations (e.g. gel running) are required, hence removing many of the problems associated with conventional PCR.

This paper describes the development of an assay for the routine detection of PSTVd, based upon real time RT-PCR and TaqMan chemistry.

2. Material and methods

2.1. Plant material

Infected material (or plasmid clones) was gratefully received from various sources (see Table 1). Infected leaf material was inoculated onto tomato plants and kept at a controlled temperature of 18 °C with a 12 h photoperiod.

2.2. Nucleic acid extractions

All centrifugation steps were carried out in a microcentrifuge at 11,000 × *g* and at 4 °C. For the analysis of potato in vitro plants and mature potato leaves 0.5 g of tissue was taken.

In order to investigate the effect of RNA purification on the detection of PSTVd by real time PCR, the method of Salazar et al. (1988) was used. Further purification was carried out by vortexing the extract with 1 volume of phenol:chloroform (1:1). The aqueous phase was recovered by

Table 1
List of isolates tested using the PSTVd TaqMan assay, including sequence accession number and type of tissue tested where available

Isolate	Result	Tissue tested	Origin
cv. Morning gold	+	Potato tuber	R.P. Singh, Agriculture and Agri food, Fredericton, Canada
PV86	+	Tomato leaf	American Type Culture Collection (ATCC), Manassas, USA
Peru	+	Tomato leaf	J. Martin, La Station de Quarantaine Pomme de Terre, Le Rhue, France
Mild strain	+	Tomato leaf	P. Khurana, Central Potato Research Institute, Shimla, India
CSL-PSTVd	+	Potato leaf	Central Science Laboratory (CSL), York, UK
Russian	+	Tomato leaf	T. James and C. Jefferies, Scottish Agricultural Science Agency (SASA), Edinburgh, UK
PV-0064	+	Tomato leaf	Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany
Howell	+	Tomato leaf	J.W. Roenhorst and J.Th.J. Verhoeven, PPS, Postbus 9102 6700, H.C. Wageningen, The Netherlands
NS3 (X17268)	+	Tomato leaf	J.W. Roenhorst and J.Th.J. Verhoeven, PPS, Postbus 9102 6700, H.C. Wageningen, The Netherlands
PSTVd NZ (AF369530)	+	Tomato leaf	David Elliott, MAF Operations, National Plant Pest Reference Lab, 131 Boundary Road, Blockhouse Bay, P.O. Box 2095, Auckland 1015, New Zealand
PSTVd (true seed)	+	Potato true seed	Centro Nacional de Sanidad Agropecuaria (CENSA), Carretera de Tapaste, Apartado 10, San José de las Lajas, CP 32700, La Habana, Cuba
PSTVd (in vitro)	+	In vitro potato leaf	Centro Nacional de Sanidad Agropecuaria (CENSA), Carretera de Tapaste, Apartado 10, San José de las Lajas, CP 32700, La Habana, Cuba
RG1 (U23058)	+	Plasmid DNA	R. Owens, Molecular Plant Pathology Laboratory, Beltsville, USA
M5 (M93685)	+	Plasmid DNA	R. Owens, Molecular Plant Pathology Laboratory, Beltsville, USA
Tomato chlorotic dwarf viroid	+	Tomato leaf	J.W. Roenhorst and J.Th.J. Verhoeven, PPS, Postbus 9102 6700, H.C. Wageningen, The Netherlands
Citrus exocortis viroid	−	Tomato leaf	J.W. Roenhorst and J.Th.J. Verhoeven, PPS, Postbus 9102 6700, H.C. Wageningen, The Netherlands
Chrysanthemum stunt viroid	−	Chrysanthemum leaf	Central Science Laboratory (CSL), York, UK

centrifugation for 5 min and sodium acetate (0.1 volume of 3 M) and ice-cold ethanol (2.5 volume) were added and incubated at −20 °C for 1 h. Following centrifugation for 10 min the nucleic acid was resuspended in 100 μl of sterile distilled water (SDW) and a second precipitation was carried out by adding *iso*-propanol (2 volume) and sodium chloride (0.5 volume of 5 M). Following overnight incubation at −20 °C the nucleic acid was recovered by centrifugation for 10 min, the pellet was washed with ethanol (70%), dried and re-suspended in 50 μl of water.

For general diagnostic testing a method adapted from Chang et al. (1993) was used. Prior to extraction it was necessary to pre-process potato true seed as follows: individual seeds were soaked in SDW for 24 h; the water was then removed before processing. Tissue (100–200 mg) was ground to a fine powder in liquid nitrogen in a pestle and mortar and placed in a sterile microcentrifuge tube. The ground tissue was mixed with 1ml of homogenising buffer (2% hexadecyltrimethyl ammonium bromide, 100 mM Tris–HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1% Na_2SO_3, 2% PVP-40). After incubation at 65 °C for 10–15 min, two chloroform:isoamyl alcohol (24:1) extractions were performed. RNA was precipitated from the aqueous layer overnight at 4 °C with an equal volume of 4 M lithium chloride. The pellet obtained by centrifugation for 30 minutes was resuspended in 200 μl of TE buffer containing 1% sodium dodecyl sulphate and incubated at −20 °C for 30 min with 100 μl of 5 M NaCl and 300 μl of ice cold *iso*-propanol. Following centrifugation for 10 min the pellet was washed with 70% ethanol, repelleted and dried. Finally the pellet was resuspended in 50 μl DEPC-treated water and stored at −80 °C.

2.3. TaqMan primer and probe design

A multiple sequence alignment of 74 PSTVd sequences available from the EMBL database was made using the Clustal V method from the MegAlign multiple alignment package (DNA Star). Conserved regions were identified from the multiple sequence alignments and probes and primers were designed using Primer Express software (Applied Biosystems). The sequences of the primers and probes used for viroid detection are given in Table 2. The software most importantly allows accurate T_m calculations to be made for sets of primers and probes such that the primers are within the range 58–60 °C and probes are 10 °C higher at 68–70 °C, to improve specificity. In addition the total length of each amplicon should be below 150 bp to allow efficient amplification. The 5′ terminal reporter dye used was FAM (6-carboxyfluorescein) and the 3′ quencher dye TAMRA (tetra-methylcarboxyrhodamine) was used. All probes and primers were supplied by Applied Biosystems.

Table 2
Sequence of the primers and probe for the TaqMan real-time RT-PCR, including the position (in the primer/probe name) on sequence accession AF483471

Primer name	Orientation	Sequence (5′–3′)
PSTV-231F	Forward	GCC CCC TTT GCG CTG T
PSTV-296R	Reverse	AAG CGG TTC TCG GGA GCT T
PSTV-251T	Probe	CAG TTG TTT CCA CCG GGT AGT AGC CGA

2.4. TaqMan assay setup

Due to the secondary structure of the viroid genome a number of different reverse transcription parameters and RNA denaturation steps were optimised. Comparisons were made of different cDNA synthesis temperatures, denaturation of RNA prior to cDNA synthesis, the type and concentration of reverse transcriptase used, and single compared with two-tube RT-PCR. The TaqMan PCR reactions (total volume: 25 μl) were set up in 96-well reaction plates using PCR core reagent kits (Applied Biosystems). The optimal one tube conditions were as follows: 1 μl of RNA, 1× Buffer A (50 mM KCl, 10 mM Tris–HCl pH 8.3, ROX passive reference dye (concentration undisclosed by manufacturer), 5.5 mM $MgCl_2$, 0.2 mM each dNTP, 300 nM each primer, 2.5 pmol probe, 0.625 U AmpliTaq Gold and 10 U MMLV (Promega). The assays were carried out on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) at generic cycling conditions (Mumford et al., 2000). The threshold cycle (C_t) is the cycle at which a significant increase in fluorescence occurs; hence a C_t value below 40 indicates a positive result. The change in normalised fluorescence (ΔR_n) records the amount of product amplified.

2.5. Chemi-luminescent hybridisation

A chemi-luminescent hybridisation protocol similar to that of Podleckis et al., 1993 was used as follows. Samples (3 μl of RNA) diluted in water were applied to nylon membrane (Agdia, Elkhart, Indiana, USA) and air-dried at room temperature. To cross-link the RNA, the membrane was placed on filter paper soaked in 20× SSC buffer (0.15 M NaCl, 0.015 M sodium citrate ($C_6H_5Na_3O_7$) pH 7) in a UV crosslinker set at 6×10^4 μJ/cm^2. The membrane was then placed into a glass hybridisation tube (Hybaid) with 4 ml of DIG Easy-Hyb buffer (Roche Diagnostics). The lyophilised dig-labelled PSTVd probe (Agdia) was resuspended in 100 μl of DIG Easy-Hyb buffer (Roche Diagnostics) and added to the hybridisation tube. The tube was incubated in a rotating hybridisation oven (Hybaid) at 55 °C for 18 h. All wash steps were performed in a sealed plastic box on an orbital shaker at 100–150 rpm. The membrane was removed from the tube and washed for 5 min at room temperature in wash buffer 1 (2× SSC, 0.1% SDS), the wash buffer was replaced with 200 ml of wash buffer 1 containing 1 μg/ml RNase A and washing continued for 15 min at room temperature. The membrane was then washed twice for 15 min per wash in wash buffer 2 (0.1× SSC, 0.1% SDS) preheated to 65 °C. The membrane was rinsed in 1× maleic

acid buffer (100 mM maleic acid, pH 7.5, 150 mM NaCl) for 1 min at room temperature, before being placed in 1× blocking buffer (Roche Diagnostics) for 2 h at room temperature. Anti-DIG alkaline-phosphatase (Roche Diagnostics) was spun at 11000 × *g* in a microcentrifuge for 1 min, prior to being added to the blocking solution used in the previous step at a dilution of 1:10,000. The membrane was incubated for 30 min at room temperature, before being washed twice in 1× maleic acid buffer for 15 min at room temperature and then once in detection buffer (100 mM Tris–HCl, pH 9.5, 100 mM NaCl) for 5 min at room temperature. The substrate (CSPD®; Roche Diagnostics) was diluted 1:100 in detection buffer and pipetted directly onto the membrane, which was then sealed in cling film making sure the substrate was evenly distributed over the membrane. The membrane was then exposed to photographic film for 2–3 h at room temperature, and the film was developed.

3. Results

3.1. Sample preparation

TaqMan results (C_t and ΔR_n) were obtained for PSTVd infected potato leaf and potato in vitro plant material, extracted using a simple method (referred to as semi-purified) and with further purification (referred to as purified); in each comparison equivalent amounts of plant tissue was processed. The results (see Fig. 1) show that efficient DNA amplification is dependant on the quality of the nucleic acid preparations used, generally further purification results in a reduction in C_t values and an increase in ΔR_n values. The extra purification step has a greater effect on both ΔR_n and C_t values when mature potato leaf is extracted than when in vitro plant material is extracted.

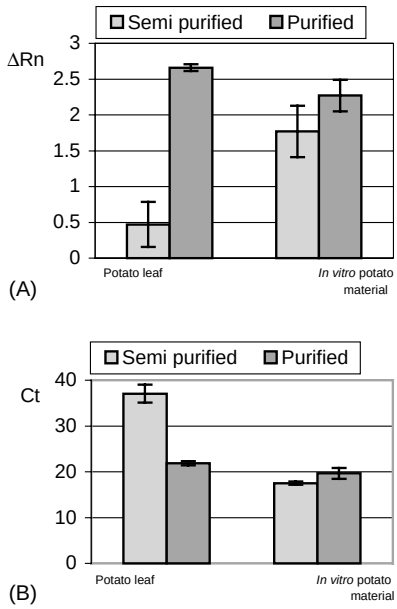

Fig. 1. Effect of sample preparation on TaqMan real-time RT-PCR for: (A) ΔR_n values and (B) C_t values.

3.2. Optimisation of cDNA synthesis

A number of different parameters involved in cDNA synthesis were investigated, in order to overcome potential problems associated with the complex secondary structure associated with PSTV RNA. The C_t values following real time PCR were used to indicate if the changes were improving the assay and giving greater sensitivity (decreasing C_t) or if they were detrimental to the assay and giving lower sensitivity (increasing C_t). In each case, the same three RNA preparations (A, B and C) were tested, extracted from tomato leaf material infected with isolate 'Russian' (T. James and C. Jefferies, Scottish Agricultural Science Agency, Edinburgh, UK).

3.2.1. Type of reverse transcriptase

A range of seven different reverse transcriptase enzymes were compared using the single tube real time RT-PCR. The enzymes compared were of different types or formulations that should give different properties. The enzymes were: standard recombinant Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega); MultiScribe (Applied Biosystems) recombinant MMLV reverse transcriptase; Superscript II (Invitrogen) MMLV enzyme with RNase H deletion; Q-Thermo RT (Qbiogene) thermostable MMLV enzyme with RNase H deletion; Expand RT (Roche) MMLV enzyme with RNase H deletion; Avian Myeloblastosis Virus (AMV) reverse transcriptase (Promega); Thermoscript (Invitrogen) AMV enzyme with RNase H deletion. Little difference was observed between the reverse transcriptase enzymes compared, when used in a one tube RT-PCR (see Fig. 2). With each of the RNA samples MultiScribe gave the lowest C_t values, whilst Thermoscript gave the highest C_t values.

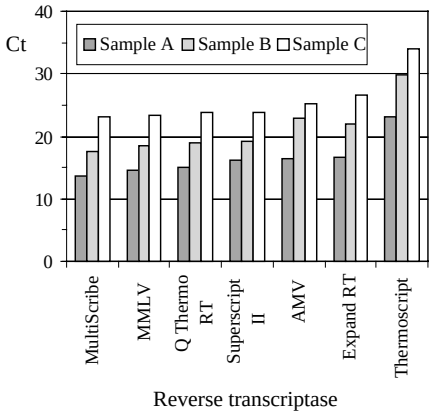

Fig. 2. Effect of type of reverse transcriptase on the C_t values for three RNA samples when tested using a one tube real-time RT-PCR.

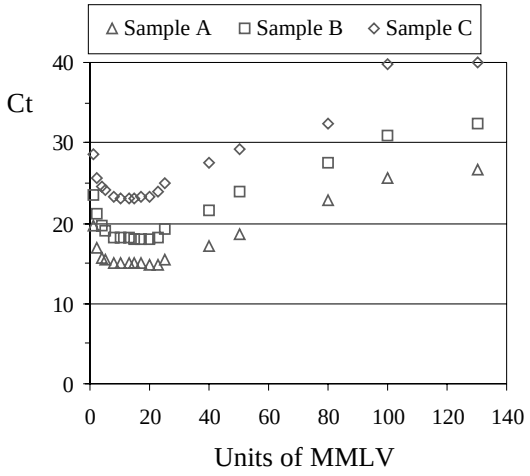

Fig. 3. Effect of amount of MMLV (Promega) on the C_t values for three RNA samples when tested using a one tube real-time RT-PCR.

3.2.2. Concentration of reverse transcriptase

The amount of reverse transcriptase added to a one tube RT-PCR was compared for a range from 1.2–130 U. The results (see Fig. 3) show that at low and high concentrations of MMLV the enzyme increases the C_t values for each of the RNA samples tested, and that the lowest C_t values were achieved at a range of MMLV of 8–23 U.

3.2.3. cDNA synthesis temperatures

The temperature of the reverse transcription step of a one tube RT-PCR was varied from the standard temperature (Mumford et al., 2000), to higher temperatures, which would relax the secondary structure of the RNA and potentially allow more cDNA to be copied from the RNA. The results (see Table 3) show that reverse transcription temperatures higher than 48 °C were found to give higher C_t values for each of the RNA samples tested, suggesting that the copying of RNA into cDNA was less efficient at the higher temperatures. In each case a shift of between three and four cycles was observed when the temperature was changed from 48 to either 55 or 60 °C, which equates to approximately a 10-fold decrease in the amount of cDNA synthesised (3.3 cycles equates to approximately a 10-fold difference in amount).

3.2.4. Denaturation of RNA prior to cDNA synthesis

The one tube RT-PCR protocol was compared with extracted RNA added directly to the PCR master mix, or with the RNA heat denatured (heated to 95 °C for 5 min) prior to adding to the PCR master mix. The results (Table 3) show that heat denaturation reduced the C_t values by between 2.5 and 4 cycles, with the RNA samples tested.

3.2.5. Single compared with two-tube RT-PCR

Following evaluations of the single tube RT parameters, the best conditions for large scale testing (most sensitive, practical and less expensive) were selected, and compared to the standard (following manufacturers protocols) two-tube RT-PCR, to compare how it performed against this benchmark. The results (see Table 3) show that the one tube reaction reduced the C_t values by between 0.9 and 2.7 cycles with the RNA samples tested compared with the standard two-tube system.

3.3. Sample testing

A total of 13 isolates of PSTVd from a broad geographic range were tested using the TaqMan assay (Table 1); in each case a positive result was achieved. In addition three other pospiviroids were tested; Tomato chlorotic dwarf viroid gave a positive result, whilst Citrus exocortis viroid and Chrysanthemum stunt viroid both gave negative results. In addition to these isolates tested it is possible to predict by analysing nucleotide variation within the primers and probe how many of the previously sequenced isolates would be detected by the assay (see Table 4). Of the 74 isolates present on the sequence database, 62 have 100% sequence identity with the primers and probe designed, it is expected that all of these isolates would be detected using this assay. The remaining 11 isolates have nucleotide mismatches, two of these isolates and also the related Tomato chlorotic dwarf viroid have been tested and give positive results, allowing predictions to be made with the remaining nine isolates. Isolates NS3 and PSTVd NZ both have three nucleotide mismatches and give positive results, thus it is expected that isolates corresponding to accession numbers X76846, X52038 and X52040, which have similar sequences would all be detected. In addition since Tomato chlorotic dwarf viroid

Table 3
Effect of reverse transcription temperature, heat denaturation of RNA prior to set up (using a one tube real-time RT-PCR) and format of the assay (optimised single tube RT-PCR vs. a standard two tube RT-PCR) on the C_t values generated for the PSTVd assay on three infected RNA samples

Sample	C_t values						
	Reverse transcription (RT) temperature			Heat denaturation (95 °C, 5 min)		Assay format	
	48 °C	55 °C	60 °C	+	−	One tube	Two tube
A	14.7	17.0	17.0	12.5	15.8	15.5	16.4
B	18.0	21.0	20.5	16.0	19.0	18.5	21.3
C	22.0	26.0	25.0	22.0	24.5	23.5	25.1
Water	40.0	40.0	40.0	40.0	40.0	40.0	40.0

Table 4
Multiple sequence alignments and TaqMan results (were tested) for isolates of PSTVd, where published sequence shows mismatches with the TaqMan assay designed

Accession number	*Forward primer*	**Probe**	*Reverse primer*	*TaqMan Result*
U23058	GCCCCCTTTGCGCTGT	TCGGCTACTACCCGGTGGAAACAACTG	AAGCTCCCGAGAACCGCTT	+
Y09890	---A----------	--------------------------	------------------	nt
M88677	----------	--A----------	----------	nt
X97387	----------	-------T----------	----------	nt
AJ007489	----------	--------------------------	---T----------	nt
M88681	----------	----A–T----------	----------	nt
X76846	CG----------	--------------------------	----------	nt
X52038	CG----------	--------------------------	----------	nt
AF369530	CG----C---------	--------------------------	----------	+
X17268	CG----C---------	--------------------------	----------	+
X52040	CG----C---------	--------------------------	----------	nt
AF536193	CG----------	-----A----------	----------	nt
U51895	CG----C---------	---A----------	----------	nt
AF162131	CG----C---------	-----A----------	----------	+
L78460	CG--T--C--------	--------------------------	----------	nt
K00817	--TT-TC---------	----AG----------	--------A–CG-------	nt
J02053	C–-T-G---G-AGCT-	CT–A----------	-----T-AACCCCAAA-CG	-
AF394453	C–-GGTC–-CGAAGC-	-T----------	-----T-AAC-CCTTTT--	-

Only polymorphic nucleotides are shown; (–) indicates 100% nucleotide identity with isolate RG1 (corresponding to U23058) and 61 other isolates where sequence is available. The accessions AF162131, L78460, K00817, J02053, AF394453 correspond to Tomato chlorotic dwarf viroid, Mexican papita viroid, Tomato planta macho viroid, Citrus exocortis viroid and Chrysanthemum stunt viroid, respectively. *Note*: nt indicates isolates not tested.

gave a positive result and has four nucleotide mismatches, it is expected that isolates corresponding to accession numbers AF536193 and U51895, which have similar sequences would also be detected. Of the remaining five isolates, three only have a single nucleotide mismatch in the 5′ end of the forward primer or the 3′ end of the probe and one has a double nucleotide mismatch at the 3′ end of the probe; it is expected that all of these four isolates would be detected, since other isolates, with more mismatches but in similar positions are also detected. Only one isolate has a nucleotide mismatch, which may affect amplification (isolate KF7, accession AJ007489 has a mismatch near the 3′ end of the reverse primer).

3.4. Sensitivity comparisons

A comparison was made between the sensitivity of the real time RT-PCR assay and a nucleic acid hybridisation assay (see Fig. 4). The real time RT-PCR was shown to be 1000 times more sensitive than the hybridisation method used.

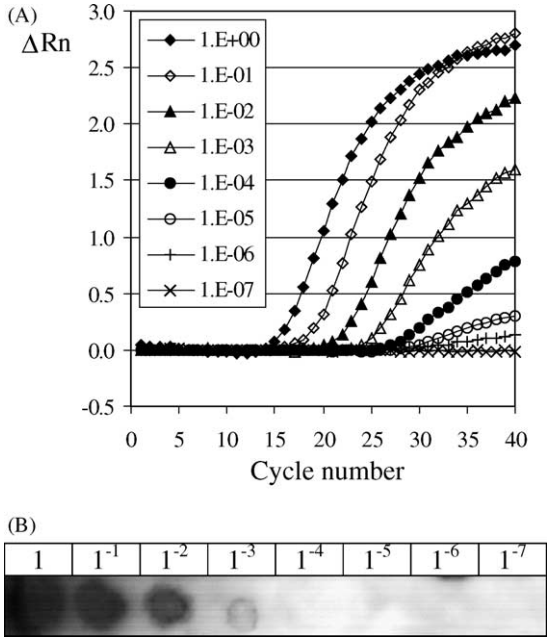

Fig. 4. Sensitivity comparison using a dilution series of infected RNA between the real time PCR assay and the chemi-luminescent hybridisation method for the detection of PSTVd. (A) Amplification plots from the real time RT-PCR, showing cycle number vs. normalised fluorescent values (ΔR_n), illustrating the detection of PSTVd in a 1×10^6 dilution of RNA. (B) Autoradiograph following hybridisation to the same samples, illustrating the detection of PSTVd in a 1×10^3 dilution of RNA.

4. Discussion

The results presented show that the TaqMan® assay allows the specific and sensitive detection of PSTVd. The new assay has been tested against a large number of isolates from a range of different geographical locations around the world and was shown to detect all those obtained. In addition, by database searching with the sequences of the

primers and probes it is possible to predict that the assay will quite likely be able to detect a further 68 isolates that have been sequenced. This demonstrates that the assay has a broad-specificity. In addition to being able to detect a broad range of isolates, the assay has been shown to do so from infected tomato leaves; potato tubers, in vitro plants and also potato true seed.

In the development of the method, a number of different aspects were examined in more detail. Firstly, the effect of sample purification on a real time RT-PCR assay was examined. Whilst viroid could be detected directly from in vitro plant material without further purification, when mature potato leaves were tested much greater discrimination between healthy and infected material was achieved if the RNA was purified further. It is assumed that the additional purification steps used are removing more enzyme inhibitors from the RNA sample, potentially more evident in the mature leaves than in in vitro material. It is difficult to speculate what the inhibitors might be, but the results show that the quality of nucleic acid preparations is clearly a determining factor when these are going to be used in assays based on PCR. It has been shown previously that amplification can be inhibited by diverse factors such as divalent cations (Wiedbrauk et al., 1995), polysaccharides, secondary metabolites and polyphenols (Demeke and Adams, 1992; Koonjul et al., 1999).

One advantage of real time PCR assays using TaqMan chemistry noted previously has been that of generic cycling conditions (Mumford et al., 2000). This involves designing the primers and probes around a set of standard conditions, leaving very little optimisation to be done. Viroids however pose a slightly different challenge during assay development, that of extremely complex RNA secondary structure. The secondary structure could prevent enzymes that work at low incubation temperatures (e.g. reverse transcriptase enzymes) from interacting with the RNA. Thus during the development of the real time assay, a number of different approaches were examined in order to reach an optimum protocol which had both good sensitivity and also practicality when faced with large sample numbers. The results show that simply increasing the temperature at which the reverse transcription reaction is done decreased the sensitivity of the assay, presumably due to the deviation from the optimum temperature under which the enzyme synthesises cDNA. Denaturing the sample prior to cDNA synthesis on the other hand gave a maximum increase in sensitivity of approximately 10-fold. The later modification may be useful when testing samples that require extreme sensitivity, but may prove difficult to implement when testing very large sample numbers. When the amount and type of reverse transcriptase enzyme was compared again differences were noted. When comparing different types of reverse transcriptase with different properties only small difference was observed, the MMLV enzymes performed better than the AMV enzymes in the one tube system, whilst standard MMLV enzymes performed better than those with deletions in the RNase H region. The latter may be due to the fact RNase H minus enzymes are designed to give much longer transcripts, this is superfluous in an assay of this kind where the target to be amplified is only 65 bp in length. Addition of either too much or too little reverse transcriptase to the one tube assay resulted in either decreased sensitivity or in extremis complete failure to detect the target, this is thought to be due to reverse transcriptase inhibiting Taq polymerase at high concentrations (Sellner et al., 1992) and at low concentrations being unable to transcribe enough cDNA. When an optimum one tube RT-PCR assay protocol had been reached, a comparison was then made between the one-tube assay and a standard two-tube protocol. The results show that the one tube assay is more sensitive. This can be partly accounted for since only part (1/4) of the cDNA synthesised is added to a PCR reaction in the two-tube system, so that the PCR reaction conditions are not adversely altered.

Finally, when an optimised one-tube RT-PCR protocol had been reached it was compared to a widely used nucleic acid hybridisation method, based around the use of a DIG-labelled RNA probe and chemi-luminescent detection. The real time RT-PCR assay was shown to be 1000-fold more sensitive than the hybridisation protocol. In addition the real time PCR approach has several other practical advantages when compared to a hybridisation method. Firstly, the real time assay uses only reagents purchased from a supplier and commonly synthesised and purified to a known concentration (i.e. primers probes and other PCR reagents). In contrast hybridisation is based around the synthesis of a DIG labelled probe, commonly by the user, prior to the assay being carried out. The synthesis of which can be a difficult process to control accurately and reliably. Secondly, the real time RT-PCR assay is based on a standard 96 or 384 well format and requires no post PCR manipulation and thus can be set up reliably using robotic laboratory liquid handling systems. On the other hand, hybridisation assays would be difficult or impossible to automate using robotics, requiring many complex ‘hands on’ manipulations. Finally, and perhaps most importantly, interpretation of real time RT-PCR assays using C_t values is very simple (i.e. C_t values <40 are positive whilst values of 40 are negative). In comparison, interpretation of hybridisation results requires interrogation of each spot on the autoradiograph by eye; a process that is both subjective (potentially different between different users) and open to errors from external factors such as background luminescence on hybridisation filters.

The real time RT-PCR assay developed should be well suited to large-scale detection of PSTVd providing the perennial problem of large-scale nucleic acid extraction can be solved. It offers a number of advantages over other systems currently available to diagnosticians, including improvements in sensitivity, a format suitable for automation using laboratory liquid handling robotics and results that are easily interpreted.

Acknowledgements

Funded by Defra Plant Health Division. The authors would also like to thank Linda Kox (PPS, Wageningen, The Netherlands) for supplying viroid infected material.

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