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Reverse transcription–detection of immobilized, amplified product in a one-phase system (RT–DIAPOPS) for the detection of potato virus Y

M. Nicolaisen[a]*†, H. N. Rasmussen[b], K. Husted[a] and S. L. Nielsen[a]

[a]*Danish Institute of Agricultural Sciences (DIAS), Department of Plant Protection, Research Centre Flakkebjerg, Slagelse 4200; and* [b]*NUNC A/S, Kamstrupvej 90, Postbox 280, Roskilde 4000, Denmark*

An RT–DIAPOPS (reverse transcription–detection of immobilized, amplified product in a one-phase system) assay was developed for the detection of potato virus Y (PVY). The sensitivity of the assay was 0·5 pg when virions were added directly to the RT reaction. The assay was capable of detecting all isolates investigated, representing several taxonomic groups of PVY. The RT–DIAPOPS offers an attractive alternative to RT–polymerase chain reaction (PCR) as it does not depend on gel electrophoresis to detect virus-specific PCR products. Finally, as amplicons are covalently attached to a solid phase, it minimizes the risk of contamination.

Keywords: DIAPOPS, PCR, potato virus Y

Introduction

The reverse transcriptase polymerase chain reaction (RT–PCR) provides a sensitive method for detecting specific RNA sequences such as the genomes of many plant viruses. This method has, among numerous other applications, recently been applied to detecting potato virus Y (PVY) (Barker *et al*., 1993; Singh & Singh, 1996). However, the RT–PCR procedure is not suited to routine testing of a larger number of samples, primarily due to the time-consuming gel electrophoresis prior to visualization. This has led to the development of several second-generation PCR procedures such as the fluorogenic 5′ nuclease RT–PCR assay (TaqMan) (Schoen *et al*., 1996) and PCR–enzyme linked immunosorbent assay (ELISA) (Alard *et al*., 1993). The TaqMan assay unfortunately depends on expensive equipment, whereas PCR–ELISA requires transfer of PCR products before detection, thus increasing the risk of contamination.

An alternative assay, detecting immobilized, amplified products in a one-phase system (DIAPOPS), has been developed for the detection of DNA templates (Rasmussen *et al*., 1994). This assay utilizes equipment already present in most virus-testing laboratories. Like TaqMan and PCR–ELISA, it does not depend on gel electrophoresis, but unlike PCR–ELISA this assay does not require the transfer of PCR products prior to detection. NucleoLink™ Strips (NUNC A/S, Roskilde, Denmark) are covalently coated with an oligonucleotide which serves as one of two primers in the following PCR. During PCR amplicons are thus covalently attached to the microwell surface. These amplicons serve as targets for a biotin-labelled probe which can subsequently be detected in an ELISA reader after the addition of a suitable streptavidin–enzyme/substrate combination. The use of a solid phase eliminates transfer steps and thereby reduces the risk of contamination.

The possibility of exploiting this method in plant virus was tested by including an RT step prior to DIAPOPS and optimizing and applying the assay in the detection of PVY. This type virus of the Potyvirus group is one of the most important pathogens of potato, causing substantial losses. Based on symptom development in *Nicotiana tabacum* and *Physalis floridana*, PVY has been divided into PVY^{O} (common strain), PVY^{N} (tobacco veinal necrosis strain), PVY^{C} (stipple streak strain) (Beemster & de Bokx, 1987), and recently PVY^{NTN} (tuber necrotic strain) (Le Romancer *et al*., 1994).

Materials and methods

Viruses

Virus isolates were obtained from various sources as described in the results. PVY^{O} PV0077 was used to determine the sensitivity of the test. PVY virions were purified as described (Okamoto *et al*., 1996), and

*To whom correspondence should be addressed.

E-mail: mogens.nicolaisen@agrsci.dk

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Table 1 Characteristics of primers and RT–DIAPOPS probe used for amplification and detection of PVY

Primer	Sequence[a]	Position[b]
Downstream R5	5′-ACATCCTCGGTGGTGTGCCTCTCTG	9309–9333
Upstream D1	5′-**TTTTTTTTTT**CTGTGATGAATGGGCTTATGGTTTGGTG	8901–8928
Internal R7	5′-GCCACCGTCCAACCCGAAAAGTC	9264–9286

[a]Linker thymidines are shown in bold.

[b]Nucleotide position according to EMBL accession number X12456 (Robaglia *et al*., 1989).

resuspended in 10 mM sodium citrate pH 7·4, 1 mM EDTA, and 0·1% mercaptoethanol. The concentration of virions was estimated by measuring the absorbance at 260 nm (A_{260}) of the purified extract using the assumption that A_{260} of a 0·1% solution through a 1 cm path is 2·9 for PVY (de Bokx & Huttinga, 1981). Glycerol was added (50% v/v) and the virus suspension stored at −20°C.

Primer design

A search was made for published sequence data of the PVY coat protein in the GenBank database. On the basis of aligned sequences from 22 isolates, a universal primer set (upstream D1 and downstream R5) was designed (Table 1) and used for PCR and RT–DIAPOPS. An internal probe R7 was designed for RT–DIAPOPS.

RNA extraction from plant material

A modified version of the RNA extraction procedure described by Spiegel & Martin (1993) was used. Leaf or tuber tissue (0·3 g) was homogenized in 0·9 mL extraction buffer [0·2 M Tris–HCl pH 8·5, 15 g L^{-1} lithium dodecylsulphate, 0·375 M LiCl, 10 g L^{-1} sodium deoxycholate, 1% Igepal CA-630 (Sigma, St Louis, MO, USA), 10 mM EDTA] in a plastic bag using an electric roller (Bioreba AG, Basel, Switzerland). From this extract, 500 μL was precipitated with 750 μL 5 M potassium acetate pH 6·5 and centrifuged at 14 000 r.p.m. (20 800 ***g***) for 10 min at 4°C. From the supernatant, 600 μL was then precipitated with 500 μL cold isopropanol and centrifuged at 14 000 r.p.m. for 20 min. The pellet was washed with 70% ethanol, dried, and finally resuspended in 20 μL sterile double-distilled H_2O.

Reverse transcription of RNA

Synthesis of cDNA was carried out for 1 h using 1·4 μL RNA (0·5 μg μL^{-1}) at 37°C in a final volume of 10 μL containing 50 mM Tris pH 8·3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0·5 mM of each dNTP, 20 pmol oligo dT_{20}, 4 units of RNasin (Promega, Madison, WI, USA), and 26 units of M-MLV reverse transcriptase (Life Technologies, Rockville, MD, USA). RNA from virus-free tissue and RNA-free reactions were included in each experiment as negative controls.

PCR

For comparison with RT–DIAPOPS, a standard PCR procedure was run in parallel. Amplification was performed with 2·5 μL cDNA in a final volume of 25 μL containing 10 mM Tris pH 8·3, 50 mM KCl, 1·5 mM $MgCl_2$, 0·15 mM of each dNTP, 1 μM downstream primer (R5), 1 μM upstream primer (D1) and 0·5 units of Taq DNA polymerase (Life Technologies). Following an initial denaturation at 94°C for 2 min, PCR conditions were as follows: 35 cycles at 94°C, 30 s; 70°C, 15 s; and 72°C, 15 s, and a final extension for 5 min at 72°C. PCR products were analysed by electrophoresis through a 1·5% agarose gel followed by staining with ethidium bromide and visualization of DNA bands using a UV transilluminator.

RT–DIAPOPS

A DIAPOPS assay developed by NUNC A/S (Rasmussen *et al*., 1994) was optimized for detection of PVY.

Coating

NucleoLink™ Strips (NUNC A/S) were coated by adding 100 ng phosphorylated D1 in 100 μL 10 mM 1-methylimidazole, 10 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide before incubation at 50°C for 5 h and washing seven times with 0·4 N NaOH, and three times in H_2O according to the manufacturer's protocol.

Amplification

Just before use, strips were blocked with 10 g L^{-1} bovine serum albumin (BSA) in Tris–saline buffer (0·1 M Tris–HCl pH 7·5, 0·15 M NaCl, 0·1% Tween 20) for 30 min at room temperature (20°C). Amplification was carried out in 27·5 μL using 2·5 μL cDNA in 10 mM Tris–HCl pH 8·3, 0·1% Tween 20, 50 mM KCl, 1·5 mM $MgCl_2$, 0·15 mM of each dNTP, 0·5 μM R5, 0·06 μM D1 and 0·5 units of *Taq* DNA polymerase (Life Technologies). Cycle conditions were as follows: an initial denaturation at 94°C for 2 min, followed by 20 cycles at 94°C, 1 min; 72°C, 1·5 min; 20 cycles at 94°C, 1 min; 65°C, 1 min; 72°C, 1 min; and a final extension for 10 min at 72°C. After amplification the plates were washed in 0·2 N NaOH, 0·1% Tween 20 followed by washing in Tris–saline buffer.

Table 2 Detection of PVY isolates using RT–DIAPOPS

Strain or group	Isolate	Origin	RT–DIAPOPS	Gel
PVY^{O}	53-15	Denmark (DIAS)	1511[b]	++[c]
PVY^{O}	53-16	Denmark (DIAS)	2784	+++
PVY^{O}	53-19	Denmark (DIAS)	1822	+++
PVY^{O}	53-33	Denmark (DIAS)	2672	+++
PVY^{O}	PV0077	DSM	1899	+++
PVY^{N}	53-18	Denmark (DIAS)	1581	+++
PVY^{N}	53-24	Denmark (DIAS)	2050	+++
PVY^{N}	53-26	Denmark (DIAS)	2368	+++
PVY^{N}	53-48	Denmark (DIAS)	1733	+++
PVY^{NTN}	PVY^{NTN} LB	Lebanon (Le Romancer)	837	+
PVY^{NTN}	PVY^{NTN} R	Romania (Weidemann)	1566	++
PVY^{NTN}	PVY^{NTN} N	Germany (Weidemann)	789	+
PVY^{NTN}	PVY^{NTN} H	Hungary (Horvath)	1252	++
PVY^{NTN}	PVY^{NTN} Attica	Hungary (Zoltan Bözce)	1260[d]	++
PVA	48-1	Sweden (Akius)	323	–
PVV	PV0319	DSM[e]	270	–
PVV	PV0318	DSM	297	–
N. t. 'Xanthi'[a]			460	–
N. benthamiana			343	–

[a]Healthy controls.

[b]Absorbance values at A450 nm ×1000, mean of two wells.

[c]Visualized cDNA after PCR amplified and gel electrophoresis: –, no visible band; +, faint band, ++, moderately intense band, +++, intense band.

[d]One well only.

[e]Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

Detection

Biotin was linked to the probe R7 at the carbon-5 of the 5′ ribose via a C6-phosphate linker at DNA Technology (Århus, Denmark.).

Hybridization with 100 nM biotinylated detection probe R7 was carried out in 100 μL 6 × SSC (900 mM NaCl, 90 mM sodium citrate), 5 × Denhardt's solution (1 g L^{-1} Ficoll, 1 g L^{-1} polyvinylpyrrolidone (PVP-360, Sigma, Uppsala, Sweden), 1 g L^{-1} (BSA) at 50°C for 2 h. Strips were washed three times in 0·1 × SSC, 0·1% Tween 20 followed by incubation at 37°C for 15 min and three more washes using the same buffer. Strips were incubated with horseradish peroxidase-labelled streptavidin (DAKO A/S, Glostrup, Denmark) diluted 1 : 5000 in Tris–saline buffer at room temperature for 1 h. After washing in Tris–saline buffer, 100 μL TMB-substrate (3,3′, 5,5′-tetramethylbenzidine, hydrogen peroxide) was added. After incubation at room temperature for 30 min the reaction was stopped by the addition of 100 μL 0·1 M H_2SO_4, and the absorbance at 450 nm was read in an ELISA reader (Multiskan MCC/340, Labsystems, Helsinki, Finland). All samples were duplicated.

Results

Design of primers and test of specificity

Based on the alignment of 22 nucleotide (nt) sequences published in the GenBank covering the coat protein region of different PVY isolates of the strains PVY^{N} and PVY^{O}, consensus regions were identified. From these regions, primers with an expected product of 432 nt were designed for optimal amplification (Table 1). The internal probe R7 was designed for detection in RT–DIAPOPS. Among the 22 sequences there were differences at two nucleotide positions in both the downstream primer R5 sequence and the probe R7. The differences were all found in PVY^{N} isolates. No differences were found in the upstream primer D1. Since the initiation of this work, several PVY sequences, including PVY^{C} and PVY^{NTN}, have been added to the GenBank, but these sequences have not added further divergence in the primer/probe regions.

A panel of isolates belonging to all PVY groups and to closely related potyviruses infecting potato, namely potato virus A (PVA) and potato virus V (PVV), was used to test the specificity of the assay. The tested primers/probe detected all PVY isolates from the PVY^{N} and PVY^{O} strains and the PVY^{NTN} group, although two PVY^{NTN} isolates (LB and N) showed absorbance values close to the detection limit (Table 2). PVA and PVV were not detected. Plants inoculated with PVY^{C} were not included in the experiments presented in Table 2. Later experiments showed that PVY^{C} (isolates DSM PV0346 and DSM PV0347) could be detected with absorbance values about three times higher than healthy controls. For comparison, a standard PCR combined with gel electrophoresis was performed in parallel. The band intensities from this experiment are shown in Table 2. High band intensities typically corresponded to high absorbance values in RT–DIAPOPS, and *vice*

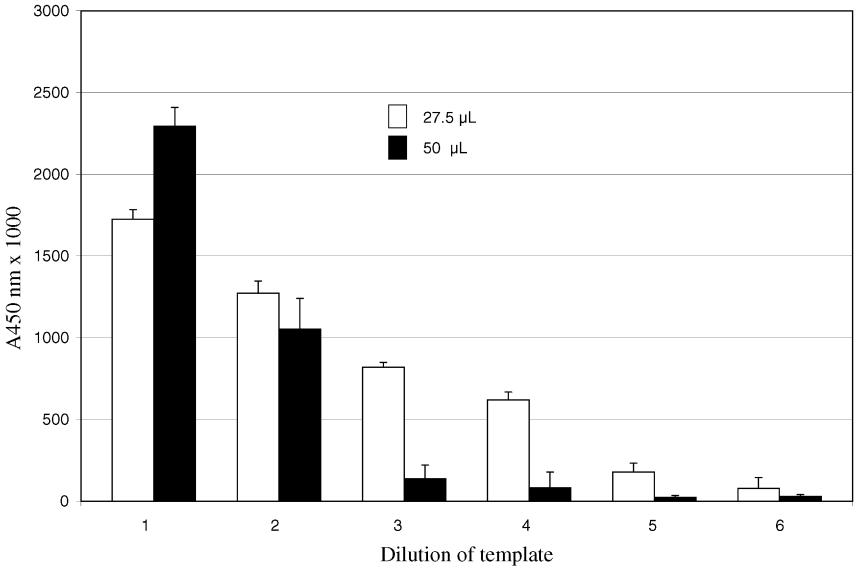

Figure 1 Effect of reducing volume from 50 to 27·5 μL using different amounts of template. (1) 10^{-4} dilution of template; (2) 10^{-6}; (3) 10^{-8}; (4) 10^{-9}; (5) 10^{-10}; (6) water control. Each bar represents the average of three individual experiments. Standard errors are indicated on top of the bars.

versa. All PVY isolates were detected using this standard PCR.

Optimization of RT–DIAPOPS

The original DIAPOPS procedure (Rasmussen *et al.*, 1994) was optimized for the detection of PVY.

In order to reduce costs, the amplification volume was reduced from 50 to 27·5 μL per well. An experiment was performed in which decreasing amounts of template (in this case a specific PCR product instead of cDNA was used) were tested in 27·5 or 50 μL total volume. The template concentration was the same at the two amplification volumes. The signal intensity when using low amounts of template was higher when using 27·5 μL total volume compared with 50 μL, whereas the opposite effect was observed at higher amounts of template (Fig. 1).

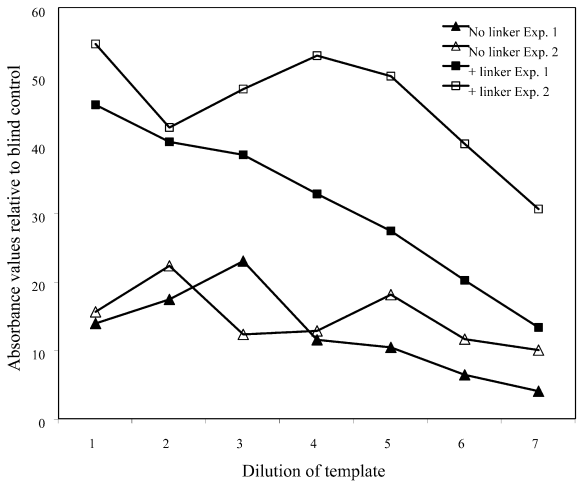

Figure 2 The effect of a introducing a 10 dT linker to the 5′ end of primer D1 on a template dilution series shown as absorbance values relative to blind controls (no template). Two individual experiments (Exp. 1 and 2) were performed. NucleoLink™ strips 1 (Exp. 1) and 2 (Exp. 2) were coated with D1 plus linker; strips 3 (Exp. 1) and 4 (Exp. 2) with D1 without linker. (1) 10^{-2} dilution of PCR template; (2) 10^{-3}; (3) 10^{-4}; (4) 10^{-5}; (5) 10^{-6}; (6) 10^{-7}; (7) 10^{-8}.

It has been shown that the addition of a 10-thymidine residue linker to the coating primers results in improved detection (Oroskar *et al.*, 1996). An elongation of D1 by the addition of 10 dTTP to the 5′ end (the end that is covalently attached to the microwell) resulted in three to five times higher absorbance values in this system in two individual experiments (Fig. 2).

Two different combinations of enzyme and substrate were tried, namely alkaline phosphatase together with the substrate 4-methylumbelliferyl phosphate, and horseradish peroxidase together with the substrate TMB. No significant differences were observed in the performance of the two enzyme/substrate combinations with regard to the signal ratio between healthy and diseased samples (data not shown).

Sensitivity

The sensitivity of RT–DIAPOPS was estimated either by the addition of different amounts of PVY^{O} PV0077 virions to healthy tuber tissue (cv. Bintje) before the extraction of RNA or by the addition of virions directly to the RT reactions. When virions were added to the RT reaction, the detection limit was in the range 0·1–0·5 pg of PVY virions (Fig. 3). When virions were added to

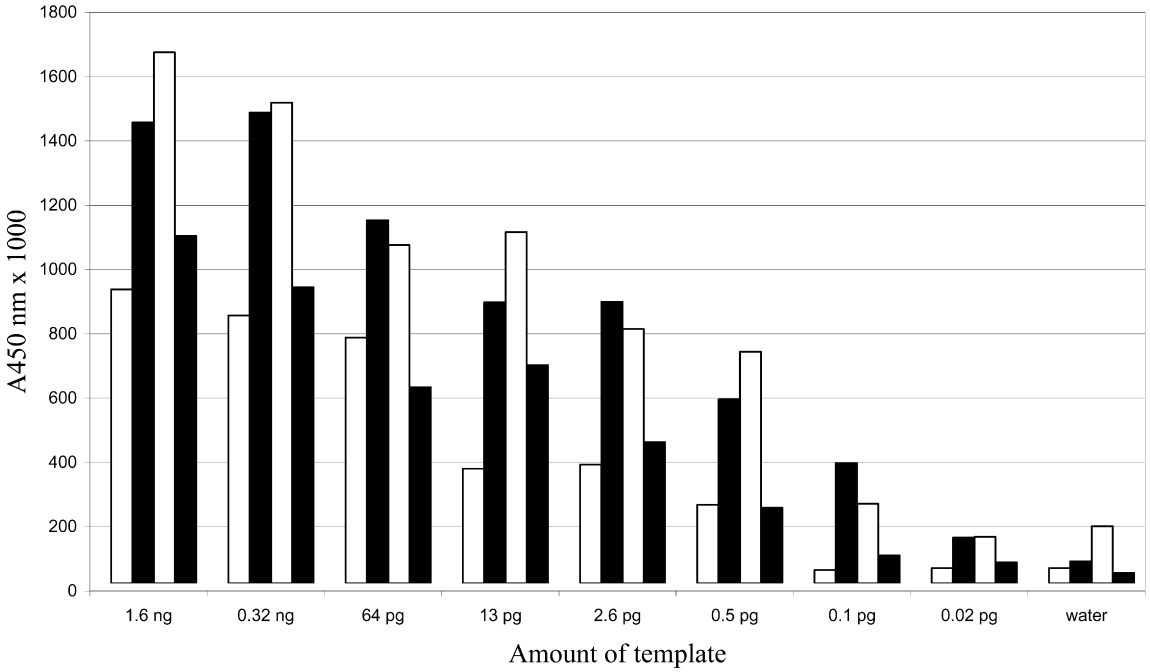

Figure 3 Detection by RT–DIAPOPS of purified PVY virions added directly to the RT reaction measured at A_{450}. The four bars in each dilution represent four independent experiments.

tuber extracts before RNA extraction, the detection limit was about 10 pg in three experiments.

Discussion

The development of RT–PCR for the detection of plant RNA viruses has, in numerous cases, led to improved assay sensitivity. Unfortunately, RT–PCR is not easily automated and problems with contamination are often encountered. The RT–DIAPOPS technique presented here offers a colorimetric alternative to electrophoretic analysis which can be performed using equipment already present in most virus-testing laboratories. Furthermore, the procedure minimizes the risk of contamination as PCR products are covalently attached to a solid surface, and absorbance readings are performed directly in the wells thus eliminating transfer of amplified products.

To assess the possibility of using the method for detecting plant RNA viruses, the assay was optimized for detection of PVY. The procedure should be sensitive, specific, reliable and suitable for high-throughput analysis. Sensitive and specific RT–PCR procedures for PVY have been described (Barker *et al*., 1993; Singh & Singh, 1996). However, these procedures are not suitable for large-scale testing, mainly because of many liquid-transfer steps and dependence on post-PCR electrophoretic analysis.

A set of primers and a probe were designed for optimal specificity in the detection of PVY using RT–DIAPOPS. These oligonucleotides were tested against an array of potyvirus isolates, and it was demonstrated that isolates of PVY^{O}, PVY^{C}, PVY^{N} and PVY^{NTN} were all detected, whereas isolates of the potato-infecting potyviruses PVA and PVV were not. A standard PCR which was run in parallel also detected all PVY isolates. The presence of two nucleotide differences in some of the published sequences from different isolates compared with the oligonucleotide primer R5 and probe R7 apparently did not affect detection, although none of the isolates in this study was actually sequenced. As the coat-protein coding sequence of a large number of PVY isolates has been sequenced and the region has been found to be highly conserved, it is not likely that new isolates will emerge that will escape detection.

The RT–DIAPOPS procedure was optimized with respect to several parameters. In order to reduce costs, the volume of the RT–DIAPOPS reaction was reduced. Surprisingly, this reduction in reaction volume resulted in an increased signal at low concentrations of template, whereas higher template concentrations resulted in weaker signals. No explanation is put forward for this phenomenon. The addition of a 10-thymidine residue linker to the 5′ end of D1 resulted in three- to fivefold increased signals. This effect has been described previously (Oroskar *et al*., 1996), and is probably caused by the specific sequence becoming more accessible for cDNA templates to hybridize.

The sensitivity of the method was estimated in two ways. Purified and diluted virions were added to healthy tuber material before RNA extraction or directly to the RT reaction mixture. As expected, sensitivity was lower when the virions were added before the RNA extraction,

as there will inevitably be a loss of viral RNA during RNA extraction. When the virions were added before RNA extraction sensitivity was 10 pg, whereas it was at least 0·5 pg when the virions were added directly to the RT reaction mixture. This detection limit is better than the results obtained by Singh & Singh (1996) who detected 1 pg purified PVY^O RNA using RT–PCR. For comparison, the antiserum described here typically detects 10 ng virions in ELISA.

RT–DIAPOPS offers an attractive alternative to conventional RT–PCR methods for the detection of plant viruses, as it is comparable in sensitivity to RT–PCR; it detects all investigated isolates of PVY; it is reliable; and it is suitable for high-throughput analysis. If precoated microwells are available (coated microwells can be stored for up to 1 years; H.N. Rasmussen, unpublished results), RT–DIAPOPS can be performed in 1–2 days.

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References

Alard P, Lantz O, Sebagh M, Calvo CF, Weill D, Chavanel G, Senik A, Charpentier B, 1993. A versatile ELISA–PCR assay for mRNA quantification from a few cells. *Biotechniques* **15**, 730–7.

Barker H, Webster KD, Reavy B, 1993. Detection of potato virus Y in potato tubers: a comparison of polymerase chain reaction and enzyme-linked immunosorbent assay. *Potato Research* **36**, 13–20.

Beemster ABR, de Bokx JA, 1987. Survey of properties and symptoms. In: de Bokx JA, van der Want JPH, eds. *Viruses of Potatoes and Seed-Potato Production*. Wageningen, The Netherlands: PUDOC, 84–113.

de Bokx JA, Huttinga H, 1981. *Potato Virus Y*. Descriptions of Plant Viruses no. 242. Kew, UK: CMI/AAB.

Le Romancer M, Kerlan C, Nedellec M, 1994. Biological characterization of various geographical isolates of potato virus Y inducing superficial necrosis on potato tubers. *Plant Pathology* **43**, 138–44.

Okamoto D, Nielsen SVS, Albrechtsen M, Borkhardt B, 1996. General resistance against potato virus Y introduced into a commercial potato cultivar by genetic transformation with PVY^N coat protein gene. *Potato Research* **39**, 271–82.

Oroskar AA, Rasmussen S-E, Rasmussen HN, Rasmussen SR, Sullivan BM, Johansson A, 1996. Detection of immobilized amplicons by ELISA-like techniques. *Clinical Chemistry* **42**, 1547–55.

Rasmussen SR, Rasmussen HB, Larsen MR, Hoff-Jørgensen R, Cano RJ, 1994. Combined polymerase chain reaction-hybridization microplate assay used to detect bovine leukemia virus and *Salmonella*. *Clinical Chemistry* **40**, 200–5.

Robaglia C, Durand-Tardif M, Tronchet M, Boudazin G, Astier-Manifacier S, Casse-Delbart F, 1989. Nucleotide sequence of potato virus Y (N strain) genomic RNA. *Journal of General Virology* **70**, 935–47.

Schoen CD, Knorr D, Leone G, 1996. Detection of potato leafroll virus in dormant potato tubers by immunocapture and a fluorogenic 5′ nuclease RT–PCR assay. *Phytopathology* **86**, 993–9.

Singh M, Singh RP, 1996. Factors affecting detection of PVY in dormant tubers by reverse transcription polymerase chain reaction and nucleic acid spot hybridization. *Journal of Virological Methods* **60**, 47–57.

Spiegel S, Martin RR, 1993. Improved detection of potato leaf roll virus in dormant potato tubers and microtubers by the polymerase chain reaction. *Annals of Applied Biology* **122**, 493–500.