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Detection of different strains of *Potato virus Y* and their mixed infections using competitive fluorescent RT-PCR

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Abstract

A competitive fluorescent RT-PCR assay (CF RT-PCR) was developed for the rapid and reliable detection and discrimination of the two most common strains of *Potato virus Y* (PVY) found in potato (necrotic and ordinary). The assay incorporates two strain specific primers labelled with fluorescent labels, used in conjunction with a universal PVY primer. The strain specific primers compete for the same annealing site which further increases specificity. Discrimination is conferred by the fluorescent labels; green PCR products for PVY^O and red for PVY^N, whilst mixed infections are detected as orange PCR products without the need for staining agarose gels. The assay can be scaled up for the processing of 96 samples simultaneously, with the detection of PCR products directly using a fluorescent microtitre plate reader. The assay successfully discriminated between 20 isolates of PVY tested, and could be used for the direct detection of PVY in potato tubers.

Keywords: Competitive PCR; Fluorescent PCR; Potyvirus; PVY; Viral diagnostics

1. Introduction

Potato virus Y (PVY) is the type member species of the genus *Potyvirus*, family *Potyviridae*. PVY has a long filamentous particle containing a single stranded, positive sense RNA genome of approximately 9.7kb. PVY infects solanaceous plants such as tobacco, tomato, pepper and potato and has a significant impact on potato and other solanaceous plants worldwide and in the UK, causing both qualitative and quantitative damage. Yield losses of 10–80% in potato have been reported (De Bokx and Huntinga, 1981) and together with *Potato leaf roll virus* and *Potato virus X*, PVY accounts for crop losses of £30–50 million per year (Hull, 1984). PVY is widespread, being transmitted by 40 species of aphid in a non persistent manner (Sigvald, 1984) and to subsequent generations via the tuber. Attempts to reduce the spread of the virus by insecticide based vector control are ineffective, therefore PVY is largely controlled through seed health schemes, which often rely upon accurate detection of the virus in the seed tubers post harvest. The two

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common PVY strains in the UK, are described on the basis of hypersensitive reactions in standard cultivars of *Solanum tuberosum* bearing different resistance genes, and on symptoms induced on *Nicotiana tabacum*. The ordinary or common strain of PVY (PVY^O) elicits N_{tbr} (De Bokx and Huntinga, 1981; Jones, 1990), whilst the tobacco veinal necrosis strain of PVY (PVY^N) does not elicit a hypersensitive resistance in *S. tuberosum*, but causes a distinctive veinal necrosis in *N. tabacum* (De Bokx and Huntinga, 1981). Symptoms of PVY^O on potato include severe streak, rugosity or leafdrop streak whilst PVY^N produces mostly mild mottling (De Bokx and Huntinga, 1981; Glais et al., 1996). Symptoms vary greatly between cultivars; hence strain differentiation is an important consideration during testing. Present assays for PVY involve growing up eye core samples (Vetten et al., 1983), followed by ELISA of the grown-on shoots which can take 4–6 weeks.

The ability to detect PVY by Polymerase Chain Reaction (PCR) is well documented (Singh, 1998; Singh et al., 1999). However, the ability to use these methods for routine testing is limited due to problems in handling many samples, for example through laborious RNA extraction procedures and the need to run gels using hazardous chemicals. The aim was to develop a rapid PCR based method for the detection and differentiation of PVY strains, with the potential for higher through-put testing on a microtitre plate format that may be of use for dormant tubers. The paper describes a method based on competitive fluorescent PCR, using two strain specific upstream primers designed to the same region, with a single conserved base change at the 3′ end of the primer, and a universal downstream primer. The strain specific primers were each labelled with a different fluorescent dye, such that the PCR products for each strain could be identified by colour on an unstained agarose gel. Preliminary results are also presented which show that the PCR products can also be detected and the strains separated using a fluorescent microtitre plate reader; further developments in this area would help to streamline testing and be of use in high throughput applications such as tuber indexing.

2. Materials and methods

2.1. Isolates

Field isolates were obtained at the Central Science Laboratory from the Plant Health and Seeds Inspectorate. Other isolates were obtained from R. Bolton (PBI, Cambridge UK), A. Philips (ADAS, UK), D. Govier (Rothamsted Experimental Station, UK), S.L. Nielson (Danish Institute of Agricutural Sciences, Denmark), M. Kuc (Slovenia) and J. Horvath (Institute for Plant Protection, Hungary). Virus isolates were maintained in mechanically infected *N. tabacum* at a controlled temperature of 18°C with a 12 h photoperiod.

2.2. Serological testing

The presence of virus in plant samples was determined by a standard direct double antibody sandwich ELISA, as described by Clark and Adams (1977). Microtitre plates (Nunc, Immunoplate II) were coated with polyclonal antiserum to PVY at a concentration of 1 μg/ml in carbonate buffer for 3 h at 33°C. Plates were then washed three times with PBS–Tween (PBSt). Samples were prepared for ELISA testing by grinding in PBSt + 2% (w/v) polyvinylpyrrolidone (PVP). Plant extracts were incubated overnight at 4°C. Plates were washed three times with PBSt, and PVY monoclonal antibodies conjugated to alkaline phosphatase were added to the plates at the recommended concentration in PBSt containing 5% milk powder and incubated for 2 h at 33°C. The plates were washed three times with PBSt and the substrate (*p*-nitrophenyl phosphate, 0.6 mg/ml) was added. A positive result was taken as an absorbance (405 nm) of two times the corresponding negative control after incubation for 1 h at room temperature. Strain specific monoclonal antibody conjugates were obtained from Adgen Diagnostics, UK, [PVY^N specific (cat:1051-03) and $PVY^{O/C}$ specific (cat:1052-03)].

2.3. Separation of PVY strains in a mixed infection

Isolates designated as a mixed infection by ELISA were inoculated onto a local lesion host *Chenopodium quinoa*, individual lesions were punched out and used to mechanically infect *N. tabacum*. The resulting infected plants were differentiated into PVY^O and PVY^N strains by symptomology and sequence analysis of the coat protein region.

2.4. RNA extraction

Samples (2.5 mg) of tissue from potato tubers or leaves were frozen in liquid nitrogen and ground to a fine powder with a hand roller. RNA was extracted using PolyATract Series 9600 mRNA Isolation System (Promega), which allows the simultaneous extraction of 96 samples.

2.5. Sequence analysis

Multiple sequence analysis of the coat protein gene of 36 isolates of PVY available from the EMBL database was carried out using the Clustal V method (Higgins and Sharp, 1989) from the MegAlign package (DNA Star). Areas of conserved and divergent sequence were identified between strains.

2.6. Primer design

Within the coat protein region a single completely conserved base pair difference that could be used to confer strain specificity was identified. This base pair was incorporated at the 3′ end of the strain specific primers such that a mismatch would destabilise the polymerisation complex enough to prevent primer extension. When used in combination the two primers compete for the binding site and the mismatched primer will not anneal (Fig. 1) (Chehab and Kan, 1990; Harada et al., 1992; Zhu and Clark, 1996). The strain specific primers were linked at the 5′ end to fluorescent dyes [Genosys Biotechnologies (Europe) Ltd]; fluorescein labelled 5′-ACA TCT GGR ACA CAT ACW GTR CCR A-3′ was specific to PVY^O, and rhodamine labelled 5′-ACA TCT GGA ACT CAY ACT GTG CCA C-3′ was specific to PVY^N. The strain specific primers were included in a single, one-tube reverse transcription–PCR (RT-PCR) with a universal first strand primer (5′-CGA ACT AAA CCA TAT CGT GGC-3′).

2.7. RT-PCR

To a Ready-To-Go PCR Bead tube (Amersham Pharmacia Biotech) was added: 1 μl of RNA extract; 2.5 units of AMV reverse transcriptase (Promega Express); 10 pmol of the first strand primer; 10 pmol of each of the two competitive upstream primers and the volume was made up to 25 μl with nuclease free water. The tubes were left to stand for 1 min at room temperature, vortexed and briefly centrifuged. RT-PCR conditions were 37°C for 1 h; 94°C for 5 min; then 30 cycles of 94°C for 30 sec, 58°C for 1 min, 72°C for 30 sec; followed by 5 min at 72°C.

2.8. RT-PCR product detection

PCR products were visualised on a UV transilluminator following electrophoresis through a 1.2% agarose gel in 1 × TBE without the need for ethidium bromide to stain the gel. For use in a microtitre plate format PCR products were separated from excess primers using the Prep-A-Gene kit (BioRad Laboratories Ltd.) and pipetted onto opaque white microtitre plates. The relative

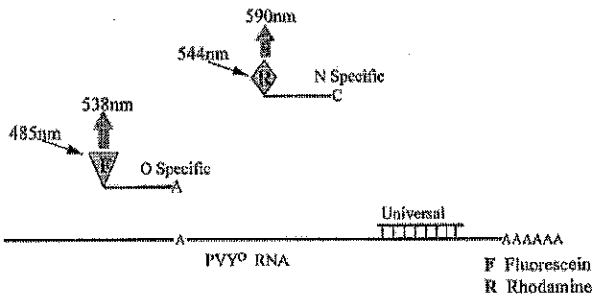

Fig. 1. The conserved single base pair difference between the two strains PVY^O and PVY^N is exploited in competitive fluorescent RT-PCR. Two competitive upstream primers each labelled with a fluorophore are included in a single RT-PCR reaction.