Development of TaqMan® Assays Towards the Detection of *Parsnip yellow fleck virus* and *Anthriscus yellows virus*

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Abstract: *Parsnip yellow fleck virus* (PYFV), type member of the genus *Sequiviridae*, may be transmitted mechanically or vectored semi-persistently by the carrot-willow aphid (*Cavariella aegopodii* Scopoli) (Murant, Gould, 1968). Vector transmission can only occur after the acquisition of the waikavirus helper, *Anthriscus yellows virus* (AYV) (Murant, Gould, 1968). In the late 1990's, both the UK and the Netherlands experienced PYFV epidemics in carrot crops and although sporadic, infections caused significant crop losses resulting in up to 30% plant death. Use of conventional pesticides has failed to depress vector activity sufficiently to control the spread of the virus. While investigating the epidemiology of the anthriscus strain of PYFV and the phenology of *C. aegopodii*, it became clear that cited symptoms such as leaf reddening and apical necrosis were not consistent in host plants. To enable further progress with laboratory trials, it became necessary to develop reliable diagnostic tools. In preference to traditional PCR methods, which were unsuitable for testing large sample numbers, TaqMan® assays were developed to detect both the AYV and PYFV isolates used in transmission studies. Both assays have been invaluable for ongoing investigations in the laboratory, but due to the high variability between different PYFV isolates, will need further development before they can be used for the robust diagnosis of virus in field samples.

Key words: *Parsnip yellow fleck virus*, TaqMan®, virus detection, *Cavariella aegopodii*.

Introduction

Parsnip yellow fleck virus is sap transmissible with isometric particles approximately 30nm in diameter (Murant, Gould, 1968) and is type member of the genus Sequiviridae. The carrot-willow aphid (*Cavariella aegopodii*) is the primary vector, transmitting PYFV semi-persistently; but only in the presence of the waikavirus helper, *Anthriscus yellows virus* (AYV). PYFV was first reported on parsnip in the UK by Murant and Gould (1968). Subsequent investigations revealed that there are two distinct serotypes; the parsnip strain (PYFV-P), transmissible to parsnips and the *Anthriscus* strain (PYFV-A) from cow parsley (*Anthriscus sylvestris*), transmissible to carrots. These two strains differ from each other in host range, with all natural hosts occurring in the Umbelliferae family (Hemida, Murant, 1989). PYFV-A was the cause of the virus epidemic of 1998 in carrots, with crop losses estimated in the region of 4%, which equates to nearly £5M in that year. Symptoms observed in carrots ranged from leaf yellowing and flecking to severe stunting and eventual death of individual plants, leading to lack of size control in premium crops. PYFV is also widely thought to be the causal agent of a carrot root disorder, similar in appearance to crown rot, in which infected carrot roots assume a cigar shape and on dissection, reveal brown, occasionally circular patches (Tyler, 1998).

Recent investigations in the epidemiology of PYFV-A and phenology of its vector highlighted the need for robust diagnostic tools for use in controlled laboratory conditions. Historically, studies of PYFV and AYV relied heavily upon traditional virological methods, in particular, the use of sap inoculations to indicator plants for virus detection (Murant, Gould, 1968; El Nagar, Murant, 1974; Murant, 1974; El Nagar, Murant, 1976a; El Nagar, Murant, 1976b). This method has since proved

unreliable, as symptoms are inconsistent in host plants. In addition, it is highly labour intensive and runs the risk of false negative results, as some sap inoculations may be unsuccessful. Diagnostic methods such as enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) are also unsuitable. PYFV isolates have proved highly variable serologically and no suitable panel of antibodies capable of detecting all isolates has been developed. Furthermore, PCR is not suited to testing large sample numbers due to the necessity for post-PCR manipulations, which also increase the risk of contamination and false-positive results (Mumford et all., 2000).

This paper describes the development of a new diagnostic tool for the detection of both PYFV and AYV using TaqMan® technology. These real-time fluorogenic reverse-transcription-PCR (RT-PCR) -based assays, modifications of traditional PCR, are highly sensitive, less labour intensive and give quick, easily analysed results for qualitative and/or quantitative interpretation. TaqMan® chemistry links together PCR with fluorescent detection using a dual-labelled fluorogenic probe that anneals to the target DNA within the region bordered by two oligonucleotide primers (Fig.1). The probe is linked to two dyes, a reporter dye at the 5' nucleotide and a quencher dye at the 3' nucleotide. While the probe is intact, the close proximity of the two dyes allows absorption of fluorescent emissions from the reporter dye by the quencher dye (fluorescent resonance energy transfer). During amplification, the 5' nuclease activity of the Taq DNA polymerase cleaves the probe, separating the two dyes, which results in an increase in reporter fluorescence. The PCR cycling results in an exponential amplification of the product that is analogous to the increase in fluorescence intensity (Weller et all., 2000). Fluorescence is monitored in real time during amplification, which obviates the need for post-PCR manipulations (Mumford et all., 2000).

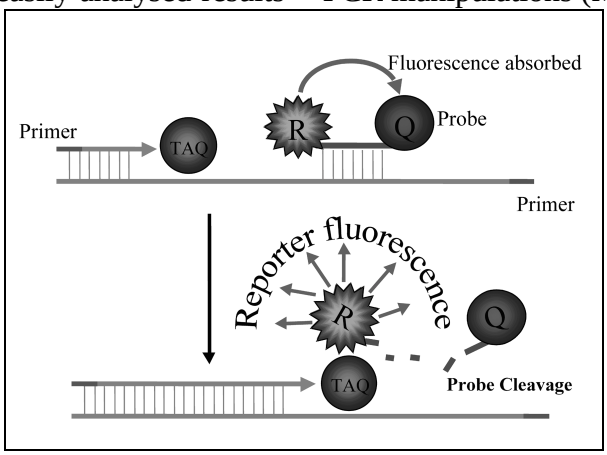

Figure 1. Schematic of TaqMan® chemistry.

Methods

The virus isolates AYV C39-12 and PYFV 489-H were used in all virus detection studies. Both viruses were supplied by Horticulture Research International (HRI), Wellesbourne, Warwickshire. AYV C39-12 was isolated from a wild *A. sylvestris* sample with a mixed infection of AYV and PYFV, collected at Wellesbourne. PYFV 489-H was also isolated from a wild *A. sylvestris* sample collected at Charlecote, near Wellesbourne. Infected *A. cerefolium* and *A. sylvestris* were used as virus source plants for vector acquisitions and transmissions of AYV and PYFV. *N. occidentalis* were used as PYFV source plants for mechanical transmissions. PYFV source plants were maintained in a Sanyo MLR 350 CE cabinet and AYV source plants were maintained in a glasshouse, both at 18ºC ± 2ºC with a 16-hour photoperiod.

RNA was extracted from infected plant material using a method modified from that of Chang et all., (1993).

Primers and probes were designed to sequence within the polymerase gene for both viruses using Primer Express™ Version 1.0 software (PE Biosystems, Foster City, CA, USA). The

PYFV primers and probe were designed to the sequence of the isolate 489H within the most conserved region of the genome, identified from a multiple sequence alignment of twenty PYFV isolates (Tab. 1). The AYV primers and probe were designed to the sequence of the isolate C39-12. For both assays, the probes were covalently labelled at the 5´ terminal nucleotide with the 6-carboxyfluorescein (FAM) reporter dye and at the 3´ terminal nucleotide with the tetra-methylcarboxyrhodamine (TAMRA) quencher dye.

For robustness, a previously designed RNA-specific internal control for the TaqMan® assays was used. This constituted a primer-probe combination designed to the cytochrome oxidase (COx) gene, labelled with the VIC (PE Biosystems) reporter dye at the 5′ terminal nucleotide and the TAMRA quencher dye at the 3′ terminal nucleotide (Weller et all., 2000).

The TaqMan reactions were performed in 25 µl volumes for each well using MicroAmp Optical 96-well plates and MicroAmp Optical Caps (Applied Biosystems, Foster City, CA, USA). Reagents were obtained from the TaqMan Core PCR Reagent Kit (Applied Biosystems, Foster City, CA, USA), with the addition of 200 U/µl of *Moloney murine leukemia virus* (M-MLV) reverse transcriptase (Promega Corp.). For each reaction, 1 µl of RNA extract was added, giving a final volume of 25 µl. Plates were then cycled at the generic RT-PCR system conditions (48ºC for 30 min., 95ºC for 10 min., and 40 cycles of 60ºC for 1 min. followed by 95ºC for 15 sec.) within the 7700 Sequence Detection System (PE-Biosystems), using real-time data collection.

To optimise the performance of the PYFV and the AYV assays, primer concentration matrices were carried out, using a 3 x 3 primer concentration matrix of 50-, 300- and 900 nM. For each assay, the optimum primer concentrations were selected as the lowest concentrations that gave the highest normalised reporter fluorescence (ΔR_n) and the lowest threshold cycle (C_T). Following primer limitation the primer concentration was selected that gave a reduced normalised reporter fluorescence (ΔR_n) but which did not cause an increase in C_T. Any further decrease in primer concentration in each case saw an increase in C_T and therefore a reduction in assay sensitivity.

Table 1. Panel of *Parsnip yellow fleck virus* isolates used in the design of Primers and probe for the PYFV TaqMan assay.

Isolate	**Original host**
489C1	*Anthriscus*
486 B1	*Anthriscus*
489 e2	*Anthriscus*
519 E	Carrot
519 G	Carrot
486 B4	*Anthriscus*
489 H-c	*Anthriscus*
V2005207	Carrot
527 F	Carrot
wis-C	Carrot
V2003614	Carrot
CV065	Celery
508	Celery
P121	Parsnip
cv-506	Celery
513c	Celery
513B	Carrot
A421	*Anthriscus*
516b	Carrot
515a	Carrot

To increase efficiency, the AYV and COx internal control assay were multiplexed. Using a ten-fold dilution series of a positive template, the performance of the multiplexed assay was compared to those of the corresponding simplex assays, to ensure that the internal control assay would not out-compete the AYV assay for qualitative results analyses. The sample concentration was transformed using Log^{10} and plotted against the C_T values. The resulting gradient (y) and correlation (R^2) difference between the multiplexed assay and the corresponding simplex assay, was compared (Figs. 2, 3). Observation of relatively unchanged C_T values between both multiplexed and corresponding simplex assays is considered sufficient for effective qualitative analyses.

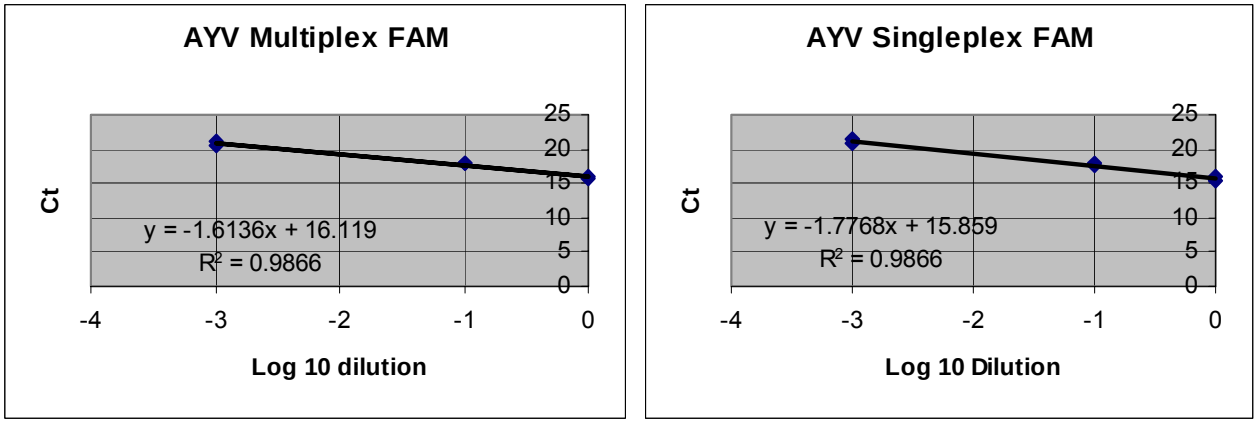

Figure 2. Comparison between AYV multiplex assay and corresponding singleplex assay.

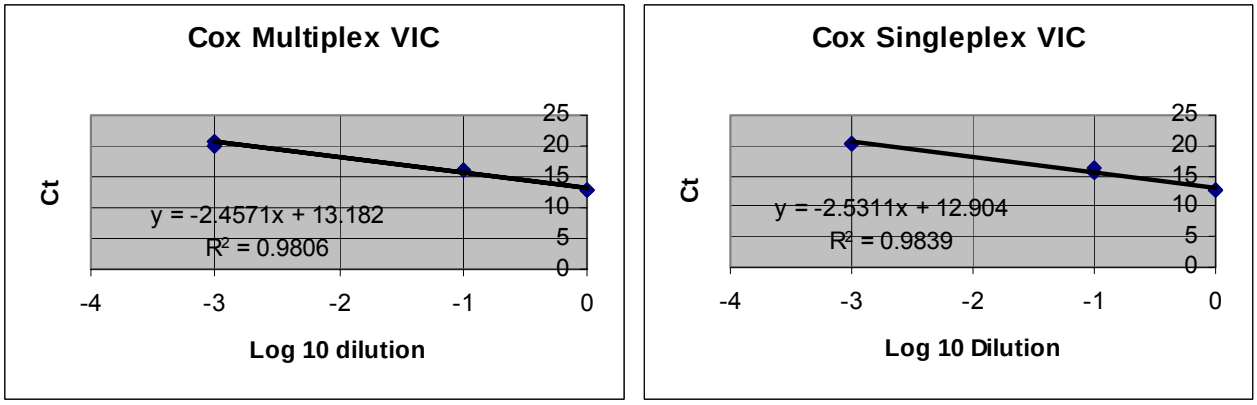

Figure 3. Comparison between COx multiplex assay and corresponding singleplex assay.

Results

Initially, primer concentration matrices were carried out separately for each assay. The optimum primer concentration varied in each case; 900/900nM for AYV; 300/300nM for PYFV and 60/100nM for the COx assay. After multiplexing the AYV and internal control assays, primer limitation experiments were conducted to ensure that one assay would not out-compete the other reaction (Fig. 2, 3).

After primer optimisation, the PYFV assay was tested for specificity against a collection of twenty PYFV isolates. Six of these isolates were found to be positive, two of which were strongly positive, including as expected, the 489-H isolate, to which the primers were originally designed, and four were weakly positive. All other isolates were undetected. The results of this specificity trial indicate that the region of sequence used in the design of probes and primers is too variable to detect all isolates within the collection.

Discussion

The development of new TaqMan® assays for the detection of both AYV and PYFV in plant material has proved invaluable in the laboratory situation. Confirmation of infection with either virus in plant material can be obtained conclusively within a short timescale prior to using source plants for vector transmission experiments, even when virus titre is very low. In addition, it has been possible to extend analyses of results to determine the 'best' plants for use in these experiments, where source plants with a high virus titre are preferred. The sensitivity of all three assays has been especially highlighted by subsequent experiments in which virus was detected in individual viruliferous aphids (data not shown).

As the AYV and COx assays have been multiplexed, reagent and labour costs have been reduced to a minimum; just two rather than three reactions are required to detect both viruses and the internal control. Thus far, TaqMan® technology does not allow multiplexing of more than two assays due to the limited availability of reporter dyes. However, with further optimisation alternative multiplex assays may be developed to detect either AYV/PYFV mixed infections or PYFV with the COx internal control.

The PYFV region sequenced hitherto has proved to be highly variable, illustrated by the results of the specificity trials in which very few PYFV isolates from the collection were detectable. This limitation has not restricted the use of the assay in the laboratory as the primers and probes were specifically designed to the sequences of the key isolates in use. Furthermore, this assay could be the foundation for further developments in PYFV detection, whereby additional sequencing of the PYFV genome may reveal a more conserved region to which new primers and probes may be designed. Alternatively, using a combined approach, multiple assays may be developed for the detection of all the isolates sequenced for use in a single 'cocktail' assay.

In conclusion, the AYV multiplex and PYFV singleplex assays described in this paper provide reliable, robust alternative tests for the detection of both viruses. While the PYFV singleplex assay is currently unable to detect all known isolates, it is sufficiently sensitive for use in the routine testing of virus source plants for controlled laboratory experiments, reducing the risk of false negative results and allowing for a rapid turnaround of results.

Acknowledgements

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References

Chang S., Puryear J., Cairney J. 1993. A simple and efficient method for isolating RNA from pine trees. - Plant Molecular Biology Reporter, 11, 2: 113-116.

El Nagar S., Murant A.F. 1974. Viruses from umbelliferous plants. - Report of the Scottish Horticultural Research Institute for 1973: 1-66.

El Nagar S., Murant A.F. 1976a. Relations of the semi-persistent viruses, parsnip yellow fleck and Anthriscus yellows, with their vector, *Cavariella aegopodii*. - Annals of Applied Biology, 84: 153-167.

El Nagar S., Murant A.F. 1976b. The role of the helper virus, anthriscus yellows in the transmission of *Parsnip yellow fleck virus* by the aphid *Cavariella aegopodii*. - Annals of Applied Biology, 84: 169-181.

Hemida S.K., Murant A.F. 1989. Host ranges and serological properties of eight isolates of *Parsnip yellow fleck virus* belonging to the two major serotypes. - Annals of Applied Biology, 114:101-109.

Mumford R.A., Walsh K., Barker I., Boonham N. 2000. Detection of *Potato mop top virus* and *Tobacco rattle viru*s using a multiplex real-time fluorescent reverse-transcriptase polymerase chain reaction assay. - Phytopathology, 90, 5: 448-453.

Murant A.F. 1974. Parsnip yellow fleck virus. - CMI/AAB Descriptions of Plant Viruses, 129: 1-4.

Murant A.F., Goold R.A. 1968. Purification, properties and transmission of parsnip yellow fleck, a semi-persistent, aphid-

borne virus. - Annals of Applied Biology, 62: 123-137.

Tyler F. 1998. Yellow fleck: Reading the signs. - Grower, October 29/Week 44: 14.

Weller S.A., Elphinstone J.G., Smith N.C., Boonham N., Stead D.E. 2000. Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) Assay. - Applied and Environmental Microbiology, 66, 7: 2853-2858.

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