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Development of an immunomagnetic capture-reverse transcriptase-PCR assay for three pineapple ampeloviruses

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A B S T R A C T

A semi-automated, immunomagnetic capture-reverse transcription PCR (IMC-RT-PCR) assay for the detection of three pineapple-infecting ampeloviruses, *Pineapple mealybug wilt-associated virus-1*, *-2* and *-3*, is described. The assay was equivalent in sensitivity but more rapid than conventional immunocapture RT-PCR. The assay can be used either as a one- or two-step RT-PCR and allows detection of the viruses separately or together in a triplex assay from fresh, frozen or freeze-dried pineapple leaf tissue. This IMC-RT-PCR assay could be used for high throughput screening of pineapple planting propagules and could easily be modified for the detection of other RNA viruses in a range of plant species, provided suitable antibodies are available.

1. Introduction

Immunocapture-PCR (IC-PCR) has gained popularity as a detection method for plant viruses as it often improves the sensitivity and specificity of the assay, reduces problems from PCR inhibitors in the sample and provides a faster and cheaper method of preparing template for amplification (Geering et al., 2000; Sharman et al., 2000; Wetzel et al., 1992). In its most typical format, antibodies immobilised on the inside surface of a microfuge tube are used to capture virus particles from a plant extract, the remaining plant components are removed and the viral nucleic acid released into the PCR mixture through application of heat.

A variation on IC-PCR is immunomagnetic capture-PCR (IMC-PCR), which differs through the use of paramagnetic beads as the solid phase for capture instead of a microfuge tube. Pathogen-specific capture antibodies are either covalently linked to tosylactivated beads (Milne et al., 2006), indirectly bound via sheep or goat anti-rabbit immunoglobulin G that is linked covalently to the beads (Dittapongpitch and Surat, 2003) or linked non-covalently to protein A-coated or streptavidin-coated beads following biotinylation of the antibodies (Fu et al., 2005; Jothikumar et al., 1998; Suñén et al., 2004). To allow washes and exchange of buffers, the beads are simply immobilised using a magnet. IMC-PCR is advantageous over IC-PCR for detecting pathogens where sample volumes are very large and there is a need to concentrate the pathogen whilst simultaneously removing inhibitors (Casas and Suñén, 2002; Fu et al., 2005; Jothikumar et al., 1998; Sweeney et al., 2007). For routine pathogen detection, IMC-PCR also provides the potential for high throughput assays using robots that transfer the beads through the various steps in the assay. Finally, as the beads can move within three dimensions, antibody–antigen interactions are likely to be much faster and therefore the incubation steps shorter.

Pineapple is host to at least seven plant viruses in the genera *Ampelovirus* (family *Closteroviridae*) and *Badnavirus* (family *Caulimoviridae*) (Gambley et al., 2008a,b; Sether et al., 2001, 2005a,b; Thomson et al., 1996; Wakman et al., 1995). Mealybug wilt disease (MWD), one of the most important field diseases of pineapple in the world, is believed to have a viral aetiology. Although the exact cause of the disease is uncertain, strong correlations between disease symptoms and *Pineapple mealybug wilt-associated virus-2* (PMWaV-2) infection have been observed in Hawaii (Sether and Hu, 2002) and, additionally, with *Pineapple mealybug wilt-associated virus-1* (PMWaV-1) and *-3* (PMWaV-3) infection in Australia (Gambley et al., 2008a). Another two ampeloviruses, Pineapple mealybug wilt-associated viruses -4 and -5 (PMWaV-4 and -5), have been described but as yet neither virus has been implicated as a causal agent of MWD (Gambley et al., 2008a; Sether et al., 2005b).

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Pineapple is vegetatively propagated and therefore virus infections are perpetuated in planting material from one generation to the next. Plants affected by MWD sometimes recover and show no signs of the disease (Carter, 1963; Ito, 1959). This complicates the control of MWD as symptomless propagules collected from recovered plants may succumb to the disease in subsequent plantings. A rapid, high throughput assay for screening in vitro plantlets for PMWaV-1, -2 and -3 would expedite the selection of virus-free planting material, thus aiding in the control of the disease. Full or partial automation of the assay would also reduce risks of cross-contamination between samples as compared with conventional IC-RT-PCR.

In this paper, a semi-automated IMC-RT-PCR assay for the detection of three ampeloviruses from pineapple is described. The assay can be used in either one- or two-step RT-PCR formats.

2. Materials and methods

2.1. Source of plant samples

To optimise the assay, fresh, frozen (−80 °C) or lyophilised leaf samples were used, all obtained from south-east Queensland (QLD). Sample D10, which was infected with PMWaV-1, -2 and -3 and sample C8G2, which was infected with PMWaV-1, were from commercial, clonal lines of *Ananas comosus* var. *comosus* cv. Smooth Cayenne. Sample FrD1225, which was infected with PMWaV-1, -2 and -3, was from a germplasm accession of *Ananas comosus* var. Queen cv. Alexander maintained at the Maroochy Research Station (MRS), Department of Primary Industries and Fisheries (DPI&F).

To validate the assay, a range of plant samples collected previously from crops in south-east QLD and stored at −80 °C, and from the germplasm collections at the MRS and the Centre de coopération internationale en recherche agronomique pour le développement (CIRAD), Martinique, and stored as freeze-dried cultures (Gambley et al., 2008a), were used (Table 1). In addition, 13 fresh crowns of a clonal line of cv. Smooth Cayenne, which originated from a commercial property located in south-east QLD, were obtained from a fresh-fruit market in Brisbane. The virus and MWD status of these plants was unknown.

2.2. Sample extraction

Sap extracts were prepared by grinding 0.1 g of white basal leaf tissue in 1 ml of extraction buffer (0.5 M potassium phosphate

Table 1
Ampeloviruses detected in accessions from germplasm collections maintained at the Maroochy Research Station (MRS), Australia and at the Centre de coopération internationale en recherche agronomique pour le développement (CIRAD), Martinique and in samples collected from commercial properties located in south-east Queensland. For field samples, the location listed refers to the nearest township to the commercial property from where it was collected.

Species	Cultivar—clonal line	Location	PMWaV-status RT-PCR-MTPD	PMWaV-status IMC-RT-PCR
A. comosus var. *comosus*	Perolera	MRS	1, 2 and 3	1, 2 and 3
A. comosus var. *comosus*	Eleuthera	MRS	1, 2 and 3	1, 2 and 3
A. comosus var. *comosus*	Alexander Queen	MRS	1, 2 and 3	1, 2 and 3
A. comosus var. *comosus*	Mauritius Queen	MRS	1, 2 and 3	1, 2 and 3
A. comosus var. *comosus*	Masmerah	MRS	1, 2 and 3	1, 2 and 3
A. comosus var. *erectifolius*	Selvagem 6	MRS	1, 2 and 3	1 and 2
A. comosus var. *comosus*	Cayenne	CIRAD	1, 2 and 3	1, 2 and 3
A. comosus var. *comosus*	Red Spanish	CIRAD	1, 2 and 3	1, 2 and 3
A. comosus var. *comosus*	Queen	CIRAD	1, 2 and 3	1, 2 and 3
A. comosus var. *comosus*	Red Spanish	CIRAD	1, 2 and 3	1, 2 and 3
A. comosus var. *comosus*[1]	Cayenne-hybrid	Beerwah	3	3
A. comosus var. *comosus*[1]	Cayenne-hybrid	Beerwah	1	1
A. comosus var. *comosus*[1]	Cayenne-hybrid	Beerwah	None	None
A. comosus var. *comosus*	Cayenne-hybrid	Beerwah	None	None
A. comosus var. *comosus*	Cayenne-hybrid	Beerwah	None	None
A. comosus var. *comosus*	Cayenne-hybrid	Beerwah	1, 2, and 3	1, 2 and 3
A. comosus var. *comosus*	Cayenne-hybrid	Beerwah	1, 3 and 5	1 and 3
A. comosus var. *comosus*[1]	Cayenne-C30	Glasshouse Mountains	1, 2, 3 and 5	1, 2 and 3
A. comosus var. *comosus*	Cayenne-C30	Glasshouse Mountains	1, 2, 3 and 5	1, 2 and 3
A. comosus var. *comosus*	Cayenne-C30	Glasshouse Mountains	1, 2, 3 and 5	1, 2 and 3
A. comosus var. *comosus*[1]	Cayenne-C30	Glasshouse Mountains	1, 2 and 3	1, 2 and 3
A. comosus var. *comosus*	Cayenne-C30	Glasshouse Mountains	1, 2, 3 and 5	1, 2 and 3
A. comosus var. *comosus*	Cayenne-C30	Glasshouse Mountains	1, 2 and 3	1, 2 and 3
A. comosus var. *comosus*	Cayenne-C30	Glasshouse Mountains	1, 2 and 3	1, 2 and 3
A. comosus var. *comosus*	Cayenne-C30	Glasshouse Mountains	1, 2 and 3	1, 2 and 3
A. comosus var. *comosus*	Cayenne-C30	Glasshouse Mountains	1, 2 and 3	1, 2 and 3
A. comosus var. *comosus*	Cayenne-C30	Glasshouse Mountains	1, 2, 3 and 5	1, 2 and 3
A. comosus var. *comosus*	Cayenne-F180	Glasshouse Mountains	1	1
A. comosus var. *comosus*[1]	Cayenne-F180	Glasshouse Mountains	1 and 3	1 and 3
A. comosus var. *comosus*[1]	Cayenne-F180	Glasshouse Mountains	1 and 2	1 and 2
A. comosus var. *comosus*[1]	Cayenne-F180	Glasshouse Mountains	2	2
A. comosus var. *comosus*	Cayenne-F180	Glasshouse Mountains	1, 3 and 5	1 and 3
A. comosus var. *comosus*	Cayenne-F180	Glasshouse Mountains	1, 2, 3 and 5	1, 2 and 3
A. comosus var. *comosus*	Cayenne-F180	Glasshouse Mountains	1, 2 and 3	1, 2 and 3
A. comosus var. *comosus*	Cayenne-F180	Glasshouse Mountains	3	3
A. comosus var. *comosus*	Cayenne-F180	Gympie	1, 2 and 3	1, 2 and 3
A. comosus var. *comosus*	Cayenne-F180	Gympie	1 and 3	1 and 3
A. comosus var. *comosus*	Cayenne-F180	Gympie	None	None
A. comosus var. *comosus*	Cayenne-F180	Gympie	2	2
A. comosus var. *comosus*	Cayenne-F180	Gympie	None	None
A. comosus var. *comosus*	Cayenne-F180	Gympie	1	1
A. comosus var. *comosus*	Cayenne-F180	Gympie	None	None

[1] IMC-RT-PCR indexing result is shown in Fig. 3.

buffer, pH 8.0, containing 0.5% (w/v) Na_2SO_4 and 4% (v/v) Triton X-100), using a mortar and pestle. Alternatively, a TissueLyser (QIAGEN, Hamburg, Germany) was used, commencing with 0.03 g of tissue, which was macerated in 300 μl of extraction buffer by shaking a 2-ml tube containing a 5-mm diameter steel ball at a frequency of 30 cycles s^{-1} for two periods of 1 min each. After brief centrifugation at 13,000 rpm, a 100-μl aliquot of each extract was used for subsequent immunocapture steps.

2.3. Primers used in PCR

The primers PMW1dF (5′-AGTGAAGTGTTGGTGGATCCT-3′) and PMW1dR (5′-TTTGTGTACGACTGTGCGCAAT-3′) were designed based on published sequences of PMWaV-1 (GenBank accessions EF463006, EF467923, EF467924 and EF467925) for amplification of a fragment of the RNA-dependent RNA polymerase (RdRp) region of the viral genome. Similarly, the primers Wilt3dF (5′-GAATGGTTTCAAAGTAGGGTTCCA-3′) and Wilt3dR (5′-GACGGTATATTTCGAGTTCCAC-3′) were designed based on the published sequences of PMWaV-3 (GenBank accessions EF467918 and EF467919) for amplification of region within the RdRp region. Primers PMW2223 and PMW2224 reported by Sether et al. (2001) were used to amplify a region of the heat shock protein 70 homologue gene of PMWaV-2. The published sequence of primer PMW2223 contains an error (DM Sether pers. comm.) and the corrected sequence used was 5′-CCATCCACCAATTTTACTAC-3′.

To confirm specificity of the three primer sets, representative PCR products were directly sequenced using each amplification primer at the Australian Genome Research Facility, The University of Queensland. The identity of sequences was confirmed by pairwise comparison with published sequences using the Vector NTI Advance 10 software package (Invitrogen, Carlsbad, USA).

2.4. Immunomagnetic capture-reverse transcriptase-PCR (IMC-RT-PCR)

Dynabeads protein G (Invitrogen) suspended in phosphate-buffered saline (PBS) were coated with polyclonal antibodies (Abs) to viruses from QLD pineapples (bleed 13/01/1993, Wakman et al., 1995). A 1.5-μl aliquot of undiluted antiserum was added directly to 25 μl of the bead suspension and incubated at room temperature for 30–40 min with continuous shaking. The beads were then washed three times with 400 μl of PBS containing 0.5% Tween-20 (PBST), each time using a magnetic rack (QIAGEN) to immobilise the beads to allow buffer exchanges. The beads were then resuspended in 25 μl of PBST.

A 5-μl aliquot of the Ab-coated beads was incubated with 100 μl of sap extract for 30 min at room temperature with continuous shaking. The beads were then washed twice with 400 μl of PBST, once with 400 μl of sterile water and then resuspended in 50 μl of sterile water. Each wash was for 2 min at room temperature and all washes were done using a Biosprint 15 robot (QIAGEN), operated using a custom designed program to stir the beads within each wash solution and to transfer the beads between wash solutions.

To produce cDNA, 24.5 μl of the final bead resuspension was incubated with 250 ng of random hexamers at 80 °C for 5–10 min, snap chilled on ice and 8 μl of 1× RT buffer (Invitrogen), 100 U of SuperScript™ III (Invitrogen), 4 mM DTT, 400 μM dNTPs and water added to a total volume of 40 μl. Reactions were then incubated at 50 °C for 50 min then at 72 °C for 15 min.

Each PCR mixture contained 2 μl of cDNA, 1× PCR buffer (Invitrogen), 1.75 mM $MgCl_2$, 200 μM dNTPS, 1.5 U of Taq DNA polymerase (Invitrogen), 2.5 pmoles each of the primers PMW1dF and PMW1dR and 5 pmoles each of the primers PMW2223, PMW2224, Wilt3dF and Wilt3dR, and water to a total volume of 25 μl. Thermocycling conditions were 94 °C for 1 min, then 40 cycles of 94 °C for 20 s, 54 or 56 °C 20 s and 72 °C for 30 s followed by a final incubation at 72 °C for 5 min.

Alternatively, a SuperScript™ III one-step RT-PCR system with Platinum Taq DNA polymerase (Invitrogen) was used. Each 50 μl RT-PCR mixture contained 18 μl of the final bead resuspension, 5 pmoles of each primer, 1× RT-PCR buffer (containing 0.4 mM of each dNTP and 3.2 mM $MgSO_4$; Invitrogen), 2.5 μl of SuperScript III reverse transcriptase/Platinum Taq DNA polymerase mix and water. Thermocycling parameters were 30 min at 50 °C for cDNA synthesis, then 2 min at 94 °C to stop the reaction, followed by 40 cycles at 94 °C for 15 s, 52 °C 30 s and 68 °C for 1 min for PCR and a final incubation at 68 °C for 5 min.

To determine the sensitivity of the assays using the optimised IMC-RT-PCR protocol, sap extracts prepared from two samples infected with all three viruses were diluted serially (10-fold dilutions) in sap extracts prepared from an uninfected seedling pineapple plant. One sample was lyophilised and the other frozen leaf tissue.

2.5. Optimisation of IMC-RT-PCR

For optimisation experiments, random hexamers (Sigma Genosys, Australia) were used to initiate cDNA synthesis and only the primers specific for PMWaV-1 were used in PCR unless otherwise stated. Sap extracts were prepared from a plant infected with PMWaV-1 at a dilution of 1:10 and further dilutions of this extract were prepared in healthy sap extracts.

To optimise the incubation time for immunocapture, aliquots of the coated beads were resuspended in either a 1:10 or 1:10,000 dilution of sap extract, then either immediately washed (0 min) or incubated at room temperature for 5, 15 or 30 min. The beads were then washed and RT-PCR done as described above, with the exception that 20 pmoles of PMW1dR was used instead of random hexamers to prime first strand cDNA.

To optimise the amount of Ab-coated beads required to capture the virions, 5 or 10 μl of beads were incubated for 30 min with a 1:100 dilution of sap extract. Additionally, a fresh extract from the same sample was diluted serially (10-fold dilutions) and used to compare the amount of PCR amplification obtained when either 100 or 200 U of reverse transcriptase was used for cDNA synthesis. In the same experiment and using 200 U of reverse transcriptase, the 1:10,000 dilution of sap extract was used to evaluate the amount of PCR amplification obtained when either 5, 15 or 24.5 μl of the final bead resuspension was used in cDNA synthesis.

To optimise primer concentrations for the triplex PCR assay, virions were trapped from a 1:100 dilution of sap extract from a sample containing all three viruses, cDNA prepared using random hexamers and the concentrations of PMWaV-2 and -3 primers varied.

To test the specificity of the antibodies for PMWaV-1, -2 and -3, fresh 1:10 and 1:100 dilutions of the same sample were tested in duplicate reactions using either uncoated beads or Ab-coated beads and assayed by PCR using all three primer pairs.

2.6. Immunocapture PCR (IC-PCR)

For IC-PCR, 0.2 ml microfuge tubes were incubated with 50 μl of the same antiserum as described above, diluted to 1:300 in 0.05 M sodium carbonate buffer, pH 9.6, at room temperature for 2 h. The tubes were then washed three times with 200 μl of PBST and then incubated for 3 h at room temperature with 50 μl of sap extract. The tubes were washed as before, rinsed with sterile water, and 50 μl of SuperScript™ III one-step RT-PCR reagent added. The RT-PCR reagents and thermocycling parameters were as described above for the one-step RT-PCR triplex assay.

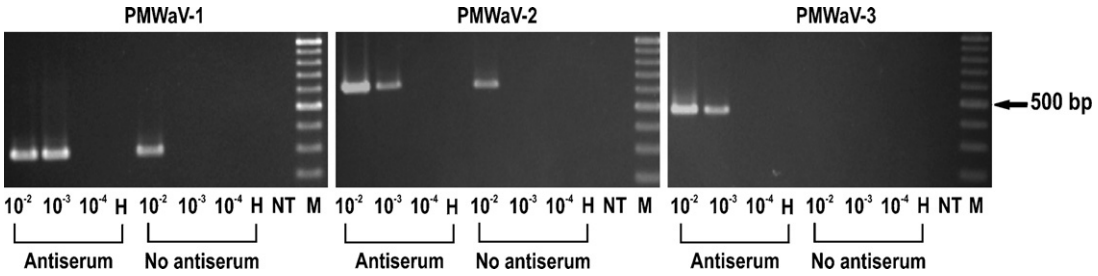

Fig. 1. Analysis of the specificity of the antibodies for capture of *Pineapple mealybug wilt-associated virus 1, 2* and *3* (PMWaV-1, -2 and -3). Immunomagnetic capture-reverse transcription-PCR was done on pineapple sample FrD1225 using Dynabeads protein G that were either coated with antibodies or uncoated. The sample sap extract was diluted serially in ten-fold steps (10^{-1} to 10^{-3}) in an extract from an uninfected pineapple sample (H) (dilutions shown below lanes). The no template control (NT) and 100 bp DNA Plus (Fermentas) marker (M) are also shown below their respective lanes.

The sensitivity of IC-PCR was evaluated using sap extracted from the D10 sample diluted serially to 1:1000 in healthy extract.

3. Results

3.1. Optimisation of IMC-RT-PCR

Parameters for IMC-RT-PCR such as the incubation period, amount of Ab-coated beads, units of reverse transcriptase enzyme, and amount of the final bead resuspension were optimised for the detection of PMWaV-1 by IMC-RT-PCR. These parameters were then used to develop a triplex assay for the detection of PMWaV-1, -2 and -3.

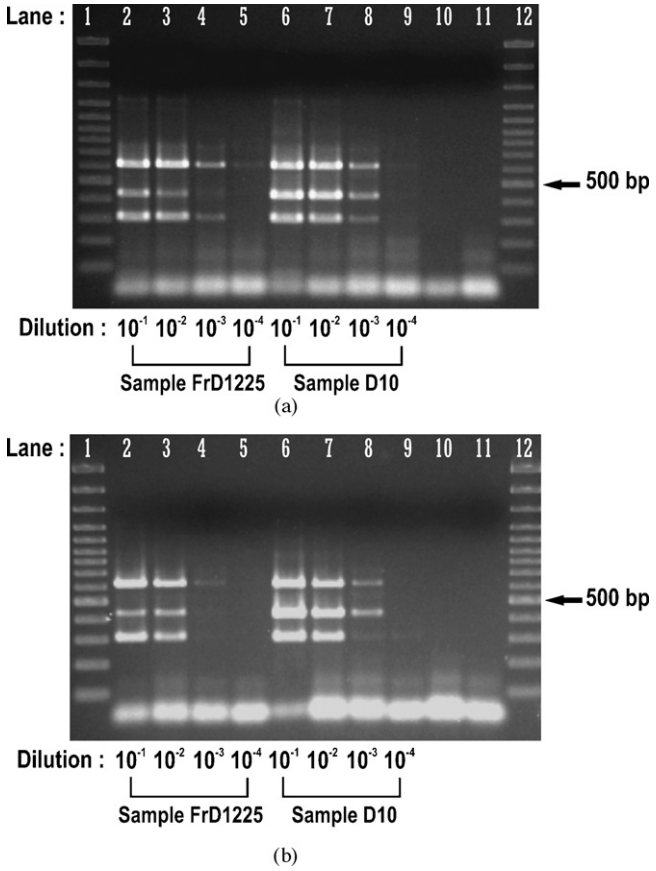

Fig. 2. Comparison of two-step (panel a, cDNA primed with random hexamers) and one-step (panel b, cDNA primed with reverse amplification primer) reverse transcriptase-PCR formats. Pineapple samples FrD1225 and D10 were used for the tests, which were infected with *Pineapple mealybug wilt-associated virus-1*, *-2* and *-3* (PMWaV-1, -2 and -3). The infected sap extracts were diluted serially in 10-fold steps in an uninfected pineapple extract to a final dilution of 1:10,000 (10^{-1} to 10^{-4}, shown below lanes). Lane 10 is the diluent, lane 11 is the no template PCR control and lanes 1 and 12 are the 100 bp DNA Plus marker (Fermentas).

PMWaV-1 was detected from sample C8G2 when an undiluted sap extract was incubated with Ab-coated beads for all time periods (0, 5, 15 and 30 min) tested but only after a 30-min incubation period when the sap extract was diluted 1:1000 (data not shown). This 30 min incubation period was thereby adopted for the standard assay.

From a comparable experiment, the use of less than 5 μl of Ab-coated beads in IMC-PCR adversely affected the detection of a badnavirus, *Banana streak GF virus*, from infected banana (authors, unpublished). Therefore, in this study, only volumes of 5 and 10 μl of Ab-coated beads were compared. PMWaV-1 was detected from a 1:100 dilution of sample C13Gy1 when either volume was used (results not shown), so the lesser volume was adopted for the standard assay.

PMWaV-1 was detected from all serial dilutions of sap extract from sample C13Gy1 using either 100 or 200 U of reverse transcriptase (results not shown). PMWaV-1 was also detected by PCR when 5, 15 or 24.5 μl of the final bead resuspension were used to produce cDNA, but PCR amplification was greatest when the highest volume was used (results not shown). Therefore, 100 U of reverse transcriptase and 24.5 μl of final bead resuspension were adopted for the standard two-step IMC-RT-PCR assay. For one-step IMC-RT-PCR, 18 μl of the final bead resuspension was used as this was the maximum volume which could be added to the reaction.

PMWaV-1, -2 and -3 were detected simultaneously from sample FrD1225 when 2.5 pmoles of each PMWaV-1 primer and 5 pmoles of each PMWaV-2 and -3 primer, were used (results not shown). These primer amounts were adopted for the standard assay as any more had either a negligible effect or was deleterious to PCR amplification.

Some direct binding of virions and/or viral RNA to the beads was detected. Although PMWaV-3 was not detected when the beads were not coated with antibodies, PMWaV-1 and -2 were still detected at a 1:100 sap dilution (Fig. 1).

3.2. Validation of IMC-RT-PCR

The optimised protocol for IMC-RT-PCR was to use 5 μl of Ab-coated beads, 30 min of sap incubation, 100 U of reverse transcriptase and either 18.0 or 24.5 μl of the final bead resuspension in a one- or two-step RT-PCR, respectively. The optimal virus-specific primer amounts for the triplex PCR assay were 2.5 and 5 pmoles of primer for detection of PMWaV-1 and the remaining two viruses, respectively. Using this protocol, but with individual virus-specific PCR assays, DNA fragments of 281, 610 and 402 bp were amplified from sample FrD1225, sequenced and confirmed to be PMWaV-1, -2 and -3, respectively. Using the same assays, all

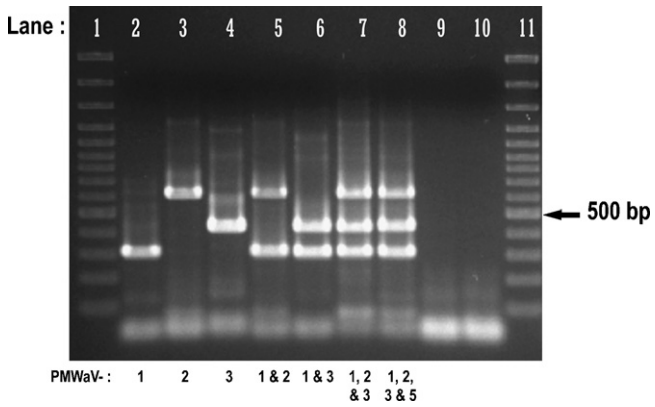

Fig. 3. Results from selected samples used to validate the immunomagnetic capture-reverse transcriptase-PCR triplex one-step assay. Samples were either infected with one or combinations of viruses as indicated below each lane. Lane 9 was from an uninfected pineapple and lane 10 was the no template control. Lanes 1 and 11 are the 100 bp DNA Plus marker (Fermentas). The details of the samples shown in this figure are listed in Table 1.

three viruses were detected from sample FrD1225 diluted 1:10 and 1:100 (Fig. 1).

To increase the speed of the assay, a one-step IMC-RT-PCR was developed. Using this assay, all three viruses were detected from a 1:1000 dilution of sap extracted from frozen (sample D10) or lyophilised leaf tissue (sample FrD1225). This one-step assay was slightly more sensitive than when IMC-RT-PCR was done as a two-step assay (Fig. 2). The detection of all three viruses using both IMC-RT-PCR and IC-RT-PCR was similar (results not shown).

A selection of 42 virus samples tested previously from commercial crops in south-east QLD and germplasm collections (Gambley et al., 2008a), were re-indexed using the IMC-RT-PCR triplex primer assay (Table 1). For PMWaV-1 and -2, there was a 100% correlation of results, but a single PMWaV-3-infected sample was not detected using this assay. The related ampelovirus, PMWaV-5, was present in eight of the samples indexed but did not interfere with the detection of PMWaV-1, -2 or -3 by IMC-RT-PCR (Fig. 3).

Freshly collected pineapple crowns from a clonal line of the cultivar Smooth Cayenne were also indexed for PMWaV-1, -2 and -3 using the triplex ICM-RT-PCR assay. PMWaV-2 was not detected in any of the 13 samples tested. PMWaV-1 was detected in 12 of the 13 samples, and PMWaV-3 in 10 of the 12 PMWaV-1 infected samples.

4. Discussion

This study describes a new method for detection of PMWaV-1, -2 and -3 and has advantages over pre-existing methods as it is semi-automated, allowing large numbers of plants to be screened as part of a clean planting material scheme. This method could be used to screen traditional planting propagules such as suckers and fruit crowns or those produced using the meristem tip *in vitro* culture method developed by Sether and Hu (2001). Identification of virus-free plants would benefit future research into the etiology of MWD and also have a direct benefit to pineapple industries worldwide, as even in the absence of MWD symptoms, PMWaV-1 can affect yield (Sether and Hu, 2001).

IMC-RT-PCR was used to detect PMWaV-1, -2 and -3 from fresh, frozen and lyophilised pineapple leaf samples and the assay was equivalent in sensitivity to conventional IC-RT-PCR. The complete range of specificities of the antiserum used is not known, as it was produced from a number of different field-infected pineapple plants, possibly containing different combinations of viruses. However, use of this antiserum in IMC-RT-PCR demonstrated the presence of antibodies to PMWaV-1, -2 and -3. Although not shown in this study, the antiserum also contains antibodies specific to Pineapple bacilliform comosus virus (authors, unpublished). The antiserum was not evaluated for the presence of antibodies specific to the two remaining pineapple-infecting ampeloviruses, PMWaV-4 and -5 or to the other known pineapple-infecting badnavirus, Pineapple bacilliform erectifolius virus.

With the exception of a single result, there was complete correlation between results obtained using IMC-RT-PCR and those from previous indexing experiments. The published amino acid sequences of PMWaV-3 within the RdRp region differ by up to 7.8% (Gambley et al., 2008a) and the failure to detect this virus from one sample was due most likely to a mismatch between the PMWaV-3 nucleotide sequence present in the sample and the specific primers used in the IMC-RT-PCR assay. Potential mismatches between the PMWaV-1 and the specific primers used in this study are also possible as the published amino acid sequences of PMWaV-1 within the RdRp differ by up to 4.2% (Gambley et al., 2008a). By contrast to PMWaV-1 and -3, the few published nucleotide sequences available for PMWaV-2 are nearly identical.

Magnetic capture PCR assays have been described for the detection of other plant viruses, including two ampeloviruses that infect grapevine and several potexviruses of ornamental bulb crops (Fazeli et al., 1998; Little and Rezaian, 2006; Miglino et al., 2007). These assays used either paramagnetic beads coated with anti-mouse IgG to attach specific monoclonal antibodies for the isolation of virions or streptavidin-coated beads to isolate viral RNA molecules bound to virus-specific biotin-labelled oligonucleotides. In this study, an alternative method is provided for magnetic capture, which could easily be modified for the detection of other viruses in a range of plant host species as it uses protein G coated beads to attach virus-specific antibodies. Protein G binds IgG from a broad range of animal species and thus is a more versatile system than using anti-mouse IgG or antibody binding agents such as protein A. Protein A does not bind IgGs from rat, chicken and goat and only binds to some but not all subclasses of IgG from other species (Page and Thorpe, 2002). In comparison to the immunocapture methods, the assays that use streptavidin beads to trap biotin-labelled probes are more complex but are not dependent on the availability of suitable antisera, which is a major limiting factor in the use of immunocapture methods.

IMC-RT-PCR is well-suited to automation and high throughput screening procedures, which reduce processing time, operator error and contamination. This study has provided a fast method to index for the three viruses associated with MWD in Australia. Individual virus-specific RT-PCR assays have been published previously for the detection of four of the five ampeloviruses known to infect pineapple (Gambley et al., 2008a; Sether et al., 2001, 2005a), but using these assays for high throughput screening would be more costly than the triplex IMC-RT-PCR assay. Once suitable antiserum became available, specific primers for detection of PMWaV-4 and -5 could also be incorporated into the IMC-RT-PCR.

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