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Final Report

Papaya clean seed program

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Summary

Papaya sticky disease is found across the major papaya growing regions in Australia. Papaya plants infected with the disease have fruit that exudes watery latex which dries on the skin, producing unmarketable fruit. In Australia, the disease is caused by a virus (PMeV2-Aus) that is seed transmitted. The objective of this project was to develop PMeV2-Aus-free parental lines to enable the production of virus-free hybrid seed for growers and develop new knowledge in relation to the virus involved in the disease.

The Australian virus has only recently been discovered, and very little is known about its physical characteristics and epidemiology. A specific, sensitive, and rapid test was developed and validated to diagnose PMeV2-Aus in samples of leaf tissue. A qPCR test was also developed for determining the sex of the plants, which was made into a multiplexed single assay with the virus test. Embryo rescue was used to generate virus-free parent lines, with growth in an insect proof shade house. On average over the 506 plants propagated from the ten different parent lines, 96% of embryos tested negative to PMeV2-Aus. Between six and ten plants of the ten parental lines were entered into tissue culture maintenance to safeguard the industry's investment into developing PMeV2-Aus-free parental lines.

A field trial with virus-free plants saw rapid re-infection with the virus, demonstrating that careful management of the parental lines is required to produce virus-free seed. The benefits of growing clean seed will not be fully realised without proper management plans until localised virus pressures drop.

Characterisation of PMeV2-Aus, has found significant difference to the situation reported overseas. PMeV2-Aus is a double-stranded RNA virus, not a single stranded RNA virus as is reported overseas. The second virus reported overseas as contributing to sticky disease was not found in Australian plants and only PMeV2-Aus was found linked to disease in Australia. There does not seem to be a recognisable coat protein in the virus genome sequence, though virus particles are easily found. This is very unusual for plant viruses and the method by which the virus obtains the proteins to form a capsid is currently still unknown. Many questions remain about the epidemiology of PMeV2-Aus. This can now be explored with a supply of healthy virus-free plant material. Important questions that can impact management decisions include what is spreading virus, how soon after infection can the virus be detected, and how soon after infection can it transmit the virus.

Keywords

Papaya; sticky disease; seed disinfestation; embryo rescue; virus.

Introduction

Papaya plants afflicted with papaya sticky disease have fruit that exudes watery latex which dries on the skin, producing unmarketable fruit (Figure 1a & b). The disease is recognised currently in Brazil and Mexico (C. H. Rodrigues, Ventura, and Maffia 1989; Perez-Brito et al. 2012). The disease is caused by virus infection, which primarily reside in the lactifers (Maciel-Zambolim et al. 2003), both increasing the pressure and fluidity of the latex (Magaña-Álvarez et al. 2016). From research in Brazil, the causal agent was initially thought to be a double-stranded RNA virus nominally related to a group of fungal viruses (Abreu et al. 2015), but it was then found to be a complex of two viruses (Antunes et al. 2016), with the initially discovered virus (papaya meleira virus: PMeV) symptomless, and the second virus (papaya meleira virus 2: PMeV2) required for disease symptoms. PMeV2 is related to Umbraviruses, which do not contain a coat protein sequence and as such requires the help of PMeV to create virus particles, which was verified through protein sequencing of purified particles (Antunes et al. 2016) by researchers in Brazil. Elsewhere in the world, however there has only been viruses similar to PMeV2 described (Zamudio-Moreno et al. 2015; Quito-Avila et al. 2015; Juan F. Cornejo-Franco et al. 2021), with low genomic relatedness between the strains.

Papaya plants in Australia were initially submitted for sticky disease assessment in 2014 and subsequently in 2016 (Figure 1a and b). Two different sized virus particles were observed in the material (Figure 1c), and a viral sequence (PMeV2-Aus) was obtained by high throughput sequencing. Development of a generic test for PMeV2 strains was developed, and initial screening of Australian papaya production areas showed widespread infection with the virus. The virus is reported to be seed transmitted at high levels (Tapia-Tussell et al. 2015), and screening of the Australian papaya seed production nursery found high levels of infection, both the field plants and tissue culture stocks. This project was developed to mitigate the continual introduction of virus infected plants through infected seed, enabling better management options for the disease.

This project was engaged to:

- Develop a clean seed testing protocol targeting causal agent(s) of Papaya Sticky Disease
- Generate clean seed for lines and varieties of importance to industry
- Undertake activities to improve knowledge of Papaya Sticky Disease in Australia

Methodology

Testing protocol

The initial reverse-transcriptase PCR which was designed to amplify all possible known variants of PMeV2 was in a conserved region of the putative RNA-dependent RNA-polymerase gene. Two forward primers and a single degenerate reverse primer (Table 1) were required to amplify all sequences described so far. The primers produce a single band of 570 bp. Initial surveys for the virus were conducted using these primers and a gel-based assay, but a better option was needed for large scale screening of seedlings.

To screen large numbers of plants rapidly and economically for PMeV2-Aus, a multiplex qRT-PCR was chosen. Primers and probes were designed to amplify the virus, an internal control (18S), and a male specific region for sex determination. Primers and probes are listed in Table 1. The virus region was selected after aligning four genomes assembled from a high throughput sequencing run of different samples from 2014 and 2016 infected plants. A coding region of the virus genome was chosen for primer design to limit the possibility of false negative results due to sequence drift in the virus genome, and away from the previous PCR to allow verification of assay results without possible contamination issues. The 18S primers and probe were designed on the papaya 18S genome fragment (Genbank ref: CPU42514), and the sexing primers and probes were designed on a male specific region (Genbank: AY428946). All primers and probes for the qPCRs were designed with Primer3.

Table 1 PCR primers and probes used for diagnostic assays for PMeV2-Aus

Name	Function	Type	Sequence (5' -> 3')
PMeV2DiagF	RT-PCR	For	CATGTGTCGGTTGAGATGTTGAA
PMeV2DiagF2	RT-PCR	For	CACGTAAGTGTTGCGATGTTGAA
PMeV2DiagR	RT-PCR	Rev	GAAAATMGGAACKCCATCACACAA
PMeV2_198F	Multiplex qRT-PCR Virus	For	GCGGAATCGGCAAAGGGTAA
PMeV2_198Fd	Multiplex qRT-PCR Virus	For	GCGGAAYYGGYAMHGGGTAA
PMeV2_322R	Multiplex qRT-PCR Virus	Rev	GGTTGAACGCGTCTCCAAGA
PMeV2_322R2	Multiplex qRT-PCR Virus	Rev	GGTTGAACGCGTCTCCAAGT
PMeV2_227P	Multiplex qRT-PCR IC	Probe	FAM-TGGTTCCTCAAGTTCGTTCCACGCA-BHQ-1
Pcar18S_1260F	Multiplex qRT-PCR IC	For	AGTTGGTGGAGCGATTTGTCT
Pcar18S_1384R	Multiplex qRT-PCR IC	Rev	TATGGCCGCTTAGACCAAGG
Pcar18S_1337P	Multiplex qRT-PCR IC	Probe	TET-CCCTCCACGGCCGGCTTCTT-BHQ1
Nafp265F	Multiplex qRT-PCR Sex	For	TTCACATGCACTTAAATATCCGCTAC
Nafp389R	Multiplex qRT-PCR Sex	Rev	GGGAAAATAGAGTTGGGGTGAC
Nafp322P	Multiplex qRT-PCR Sex	Probe	ROX-TGTGCAGTTTGAGCAGCAGCAATTCTT-BHQ-2

FAM = Carboxyfluorescein; TET = Carboxy-2,4,7,7-tetrachlorofluorescein; ROX = carboxy-X-rhodamine; BHQ-1 = Black Hole Quencher 1™ and BHQ-2™ (Biosearch Technologies, Inc., Novato, CA)

All testing was on a Rotorgene (Qiagen) or QuantStudio5 (ABI) real time thermocycler, using the Promega GoTaq® Probe one-step RT-qPCR kit. For specifics see Appendix 1.

Generation of virus free plants

The paper describing the seed transmission of PMeV in papaya (Tapia-Tussell et al. 2015) reported 83% seed coat infection, and 50% embryo/endosperm infection by RT-PCR. We therefore chose embryo rescue as the initial method to investigate the production of virus free plants, as there was not 100% infection in the embryo there was a very good chance for recovery of some healthy plants. Embryo rescue in papaya is an established technique, as is it routinely required in breeding programs that utilise wild relatives for introgression of disease resistance or other traits (R. A. Drew et al. 2004). Collaboration with Griffith University was established as they have the expertise in papaya tissue culture and embryo rescue techniques. Techniques for virus elimination usually involves some form of tissue culture, usually combined with thermotherapy and or chemotherapy. The first round of embryo rescue trialled the effectiveness of standard

embryo rescue vs the addition of ribavirin to the plating media, which has shown some success as a chemotherapy agent for other RNA viruses (Zapata, J. Creighton Miller, and Smith 1995; Singh 2015). Trial of thermotherapy was not required as other approaches were effective and the application of this technique to a tropical plant has not been demonstrated. Protocol followed is described in Appendix 2.

Ten parent lines were chosen in consultation with industry representatives, and immature fruit suitable for embryo rescue of these lines were provided by Papaya Seeds Australia. The protocol for embryo rescue is modified from protocols published by (Magdalita et al. 1996) and Azad, Rabbani, and Amin (2012), with details contained in Appendix 3. Plantlets regenerated through the system were initially deflasked and grown in insect proof mesh bags (Appendix 4). Following hardening off, these were then transferred to an insect proof screenhouse. Plants that tested positive to the virus were removed and destroyed. After the plants had been screened for virus several times, representatives of the virus free lines were established in tissue culture for security maintenance.

Virus Characterisation

Although papaya sticky disease has been known and researched since 1989 (C. H. Rodrigues, Ventura, and Maffia 1989), it was only in 2016 that the causal agent was definitively defined (Antunes et al. 2016). This means that a lot of the previous work conducted in Brazil may no longer be applicable. Also, the differences in the symptomology and genomes between the different reported strains, means that previous knowledge may not be applicable in the Australian context. Basic virus characterisation was undertaken to close knowledge gaps in the international research and specific for the Australian virus including verification of the genome type and virus purification properties. A relatively clean virus nucleic acid preparation using infected latex from fruit was developed (see Appendix 5), and this material was used to generate high-throughput sequencing libraries to search for the second virus reported as important in sticky disease overseas. As infected latex gave high concentrations of the viral nucleic acid, attempts were made to isolate the virus particles from the latex, as the particles appear to be linked to the latex polymers (S. P. Rodrigues et al. 2009).

Sticky disease knowledge development

There are two viruses reported to be involved in sticky disease in Brazil, with the second virus currently unknown in Australia. Knowledge of the second virus is important, as it appears to be involved in the production of viral particles, the properties of which are fundamental to vector movement. To search for the second virus, targeted high throughput sequencing libraries were produced sequence on highly purified viral nucleic acid preparations. A small-scale insect trapping was conducted in the seed production blocks in both the Tablelands and Innisfail using yellow sticky traps to look for the presence of whitefly, a proposed vector of PMeV. Near the end of the project, virus free plants were tested in a field trial to establish the reinfection rate. Eighty-three plants from six parent lines were established in two locations, one on the tablelands, and one at Innisfail. The plants were sampled before planting, and then three months following planting for the presence of PMeV2-Aus.

Outputs

Development of testing protocol

The initial test developed prior to this project was based on a reverse transcriptase PCR (RT-PCR) assay to detect conserved regions of the RNA-dependent RNA polymerase (RDRP) gene. This test should detect all currently reported variants of PMeV2 that cause sticky disease in papaya. Although this assay was sensitive and robust, a probe-based RT-PCR (qRT-PCR) was developed specific to the Australian PMeV2 isolate to increase specificity and sensitivity and decrease cost and time per assay. The developed primer and probe set seemed to work well, until a loop-mediated amplification (LAMP) assay was developed in a different region of the genome, which gave differing results with some samples. Previous negative samples from a seed grow out experiment tested positive using the LAMP assay. Subsequent sequencing of the virus from these samples showed a number of nucleotide changes at the location of mainly the forward primer that could abolish PCR amplification (Figure 1) and thus generate false negative results. Two other qRT-PCRs were developed for PMeV2-Aus, but both had higher non-specific amplification. Therefore, both the forward and reverse primers were redesigned to account for the newly detected variation seen in the different isolates collected. The previously tested embryo rescue samples were retested with the new primers which confirmed the previous results. This indicated the newly detected sequence variants of the virus were not present in the parent lines. Further analysis showed that the new variants were from isolates collected from a single farm with do not use seed for propagation. Given the parental lines were confirmed negative for the new variants, the screening for seed production was not affected.

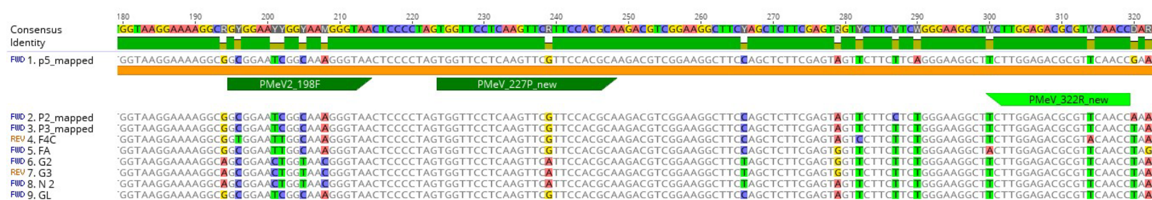

Figure 1 Alignment of PMeV2-Aus isolates under the qRT-PCR primers and probe. Regions of differences are background coloured. The isolates G2, G3 and N2 are the most diverse.

To assist in the screening of plants for PMeV2-Aus, a loop-mediated isothermal amplification (LAMP) assay was developed. The advantages of LAMP for plant viral diagnostics are the lower cost, both for the reagents required for an RNA virus, and the used of crude extracts instead of purified RNA preparations. The disadvantage is the validation process is usually more involved, as due to the large numbers of primers involved, establishing true amplification of a low copy number target versus non-specific amplification can be difficult. The LAMP assay was designed in a virus genome region between the two existing assays (Figure 2), to limit possible contamination, while still in a fairly conserved region of the genome.

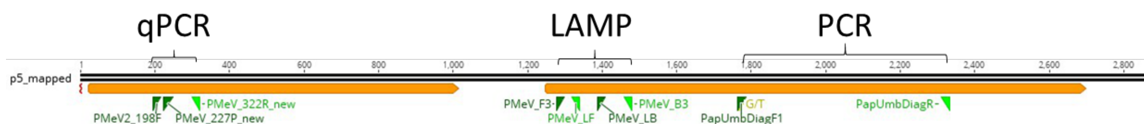

Figure 2 Position of the three diagnostic assays on the 5' portion of the PMeV2-Aus genome.

The LAMP assay was rapid (Figure 3) with the best purified infected RNA extract detected within 5 minutes. All purified RNA extracts were detectable before 20 minutes, which is before an ordinary RT-PCR has even begun cycling. Infected latex was also added directly to the assay without treatment, and it was also successfully detected, though it took nearly double the time of the purified RNA extracts, and the amplification amount was lower. Although the LAMP reaction showed promise, there was limited time and resources to fully validate it for routine diagnostic use. The limits of the assay need to be ascertained, including the detection ability in crude extracts of different plant materials, as well as rate of non-specific amplification. To create this as a field deployable test, additional work would be required including conversion to a visual colour change reaction or testing of combinations of labelled primers to enable the use of lateral flow tests strips for amplification detection.

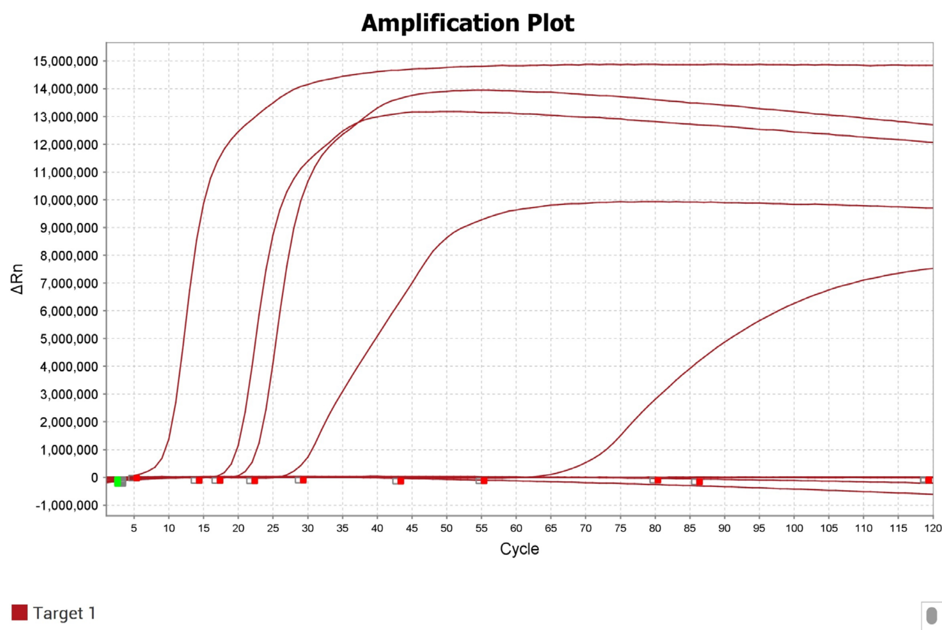

Figure 3 Example amplification plot for PMeV2-Aus LAMP assay. The first four amplification curves from the left were different positive RNA extracts, and the one on the right crude latex added directly to the assay without any treatment. The healthy papaya RNA and not template control failed to amplify. Each cycle is 30 seconds.

Seedling production results

Production of virus free plants was successful using embryo rescue for all ten parent lines. Initial numbers of plantlets recovered was low, until the correct maturity of the fruit was ascertained (approximately 90-120 days after anthesis) to successfully germinate the embryos *in vitro*. A graphic outline of the process of papaya embryo rescue is in Figure 4. The success rates of embryo germination on MS medium supplemented with plant growth regulator were 95 to 100%, and ~70% of these developed roots and shoots on half strength MS medium. Plantlets were observed to carry over the effect of gibberellic acid, with elongated and thin stems apparent until acclimatisation. The survival rate at acclimatisation varied from 20 to 90%. Several factors affected the survival rate, the most significant being the system where the seedlings were kept following acclimatisation. Once the seedlings were transferred from a humidifier cabinet to ambient humidity/temperature, they were kept in a 70% shade-house, and the seedling trays were covered with a net-bag to protect the seedlings from insects. However, the net-bag is not well designed for watering purposes which contributed to loss of seedlings over summer.

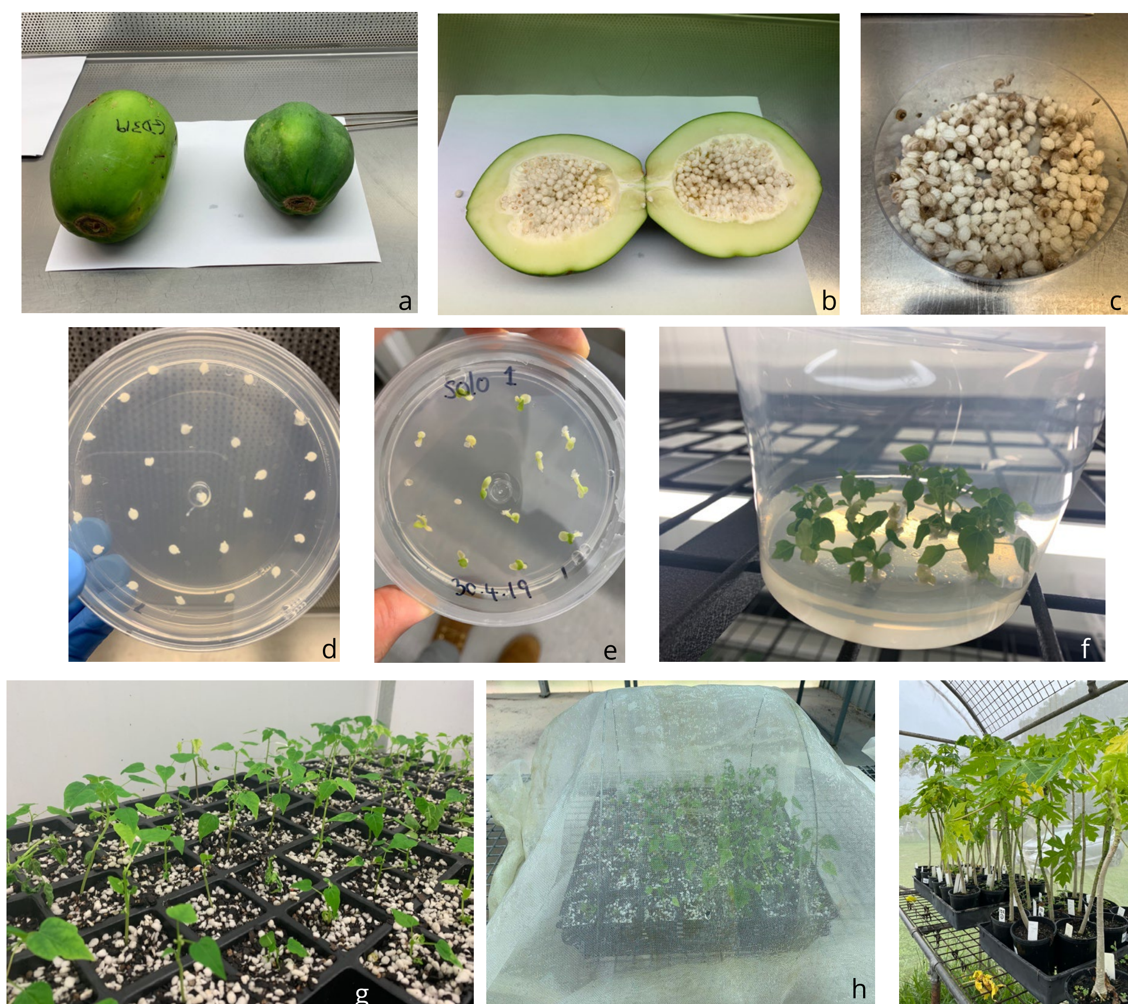

Figure 4 Overview of the process of embryo rescue in papaya. a) immature fruit at 90-100 days after anthesis b) suitable immature seed to embryo rescue c) seed collected from fruit in sterile conditions d) embryos on embryo rescue media e) germinating embryos f) papaya plantlets on growth media g) papaya plants during acclimatisation in a humidity cabinet h) seedlings transferred to 70% shade house and covered with a net bag for insect protection i) mature potted plants.

The use of ribavirin in the germination media was discontinued following the first trial, as the expensive chemical did not substantially increase the numbers of virus free plants over the standard embryo rescue technique. Of the 28 PL1 embryos germinated on ribavirin none were positive for PMeV2-Aus and 1 was

unsure, and of the 26 embryos germinated on media lacking ribavirin, 1 was positive and 6 were unsure.

Table 2 details the numbers and testing results of the plants generated. There were only three plants that tested positive in the qRT-PCR. Plants that tested positive, or unsure (late Ct value) were discarded. The plants were initially tested at about the third leaf stage after hardening off. The relatively early testing was shown to be successful for virus indexing, as there was no increase in positive samples from subsequent testing as the plants matured in the insect proof screenhouse. The most important parent lines for industry were started first through the embryo rescue process, and therefore had the most time in the insect proof screenhouse, and the greatest amount of confirmatory testing.

Table 2 Testing results of the ten parent lines generated through embryo rescue as tested by the qRT-PCR

Parental lines	Total Generated	Male	Female	Negative	Positive	Unsure[1]	% clean	*In vitro* cultures
PL1	106	44	53	97	2	7	91.5%	13
PL2	80	40	27	67	0	2	97.5%	9
PL3	33	3	13	16	0	0	100%	6
PL4	29	11	18	28	0	3	89.7%	6
PL5	44	11	23	33	0	2	95.5%	6
PL6	44	26	18	43	0	1	97.7%	6
PL7	43	23	20	44	0	0	100%	6
PL8	57	45	12	55	0	2	96.5%	6
PL9	37	23	13	35	1	1	94.6%	6
PL11	33	26	7	33	0	1	100%	6
Total	506	252	204	451	3	19	96% Avg	70

[1] Unsure results are plants that came up late in the qRT-PCR, generally after 37 cycles.

Table 2 also outlines the number of each line that were entered into *in-vitro* culture for each of the parent lines. The lines are currently maintained in tissue culture. As papaya cannot be subcultured indefinitely (R. Drew 1988), a protocol has been established that the plants are grown out in an insect proof enclosure to limit the chance of reinfection by PMeV2-Aus.

Virus Characterisation

Prior to this project, the genome of the causal agent of papaya sticky disease in Australia was discovered. It was a 4601 nt genome resembling the viruses reported overseas causing similar symptoms. These viruses are reported to not contain a coat protein and require a second virus, PMeV, to co-opt its coat protein to create virus particles. The goal of this research was to find out more details about the second virus involved in the sticky disease infection.

High throughput sequencing (HTS) was tasked to find the evidence of this second virus. Several methods were trialled to generate a high-quality targeted library for HTS, but most failed to generate an acceptable library, or sequence depth. The final method that yielded quality sequencing results was a high-quality RNA extraction from infected latex, followed by digestion with Shortcut® RNAse III (New England Biolabs) (Figure 5), an enzyme that converts long double-stranded RNA into short (18-25 nt) fragments. The dsRNA fragments were subsequently made into a standard Illumina siRNA library and sequenced. From the 1.2 M reads generated, 966 K mapped to the already sequenced PMeV2-Aus. The remaining 3.1 K reads did not assemble any contigs longer that 100 nt. No reads could be mapped to the PMeV sequence from Brazil.

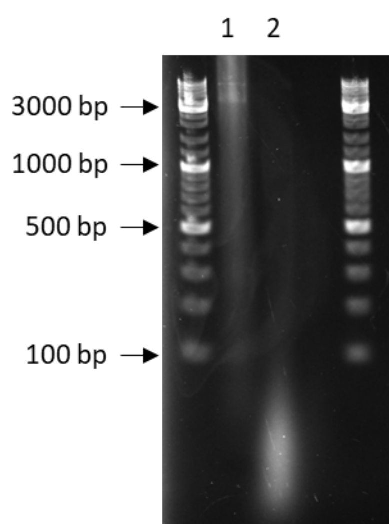

Figure 5 Digestion of latex extraction with ShortCut™ RNAseIII (NEB). RNA sample was digested into ~25 bp fragments. *Lanes 1: undigested latex TNAE 2: Digested w RNAseIII Ladder is 1Kb Plus (NEB) DNA ladder.*

In the literature, it is reported that PMeV2 is a single stranded RNA (ssRNA) virus, but there is no evidence supplied to support this. It seems to be assumed that the virus is ssRNA since there is no recognisable virus coat protein in the genome like the *Umbraviruses* which are ssRNA viruses that are encapsidated by other viruses. To test this, basic investigations into the nature of the virus genome was undertaken. A method for extraction of viral nucleic acid was developed (Appendix 1: Extractions) which included a nuclease digestion step, so only nucleic acid protected by a viral coat was extracted. Running these extractions on a non-denaturing gel usually shows two bands (Figure 6), which was initially thought to be indicative of two different viruses. Following the failure of the HTS to find another virus, this was examined in greater details. Heating the extracts in a denaturant (47.5% formamide), and running on an agarose gel with a ssRNA ladder always showed a single band of the correct size of ~4600 nt. The extracts were subsequently tested with nucleases, DNAse I, RNAse A and P1 Nuclease (Figure 7). DNAse I will only digest double stranded DNA, RNAse A will digest RNA, and P1 nuclease will only digest single stranded RNA or single stranded DNA. Only the RNAse A cut the product indicating that it was neither DNA nor ssRNA, therefore the virus genome is dsRNA. This is also confirmed by the complete digestion of the genome by the Shortcut® dsRNAse. As this is contrary to what is reported in the literature, a further check was made by using two probes designed for the sense strand and the antisense strand. Viral extract was heated and dotted on a membrane, the probes hybridised and developed. Both the sense strand and the antisense strand were visible (Figure 8) indicating that both strands were present.

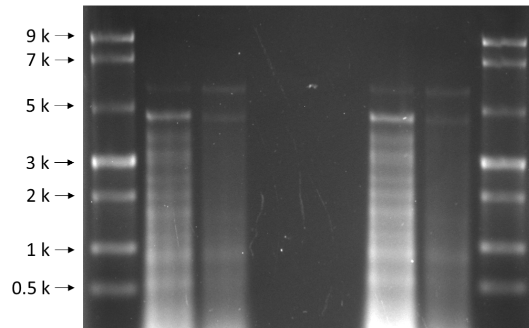

Figure 7 Latex extraction from four infected latex samples. Ladder is high MW ssRNA ladder (NEB). All four samples show the double band pattern typical to virus extractions from latex.

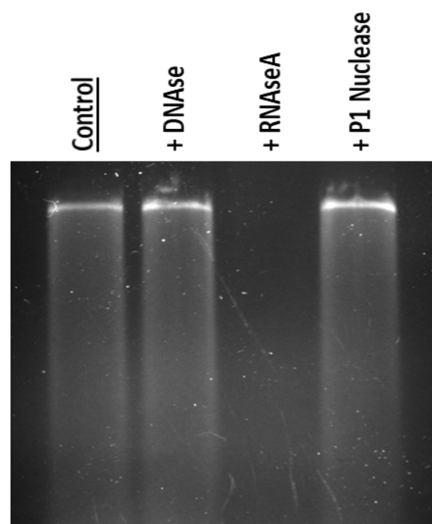

Figure 8 Nuclease assay for assessing PMeV2-Aus genome nucleic acid type. Equal amount of infected latex extraction were digested with DNAse I, RNAse A, and P1 Nuclease, before running on a denaturing gel.

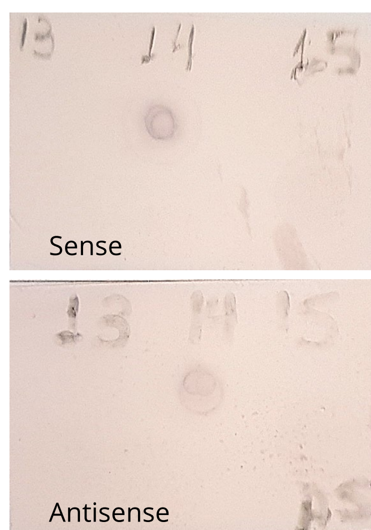

Figure 6 Dot Blot for assay of Infected latex extracts with sense and antisense probes. Samples: 13 – Direct spot of infected latex 14 – 2 µL of TNAE extraction from infected latex. 15 – TNAE from healthy plant. Sense and antisense biotin labelled probes were hybridized, washed, and detected with streptavidin-AP and BCIP/NBT substrate.

When examining the virus particles under TEM, there are two different sized particles ~28 and ~38 nm isometric particles (Figure 9). One possible explanation for the two particle sizes with only a single virus, is that there are full and empty particles, as the proteins in isometric virus particles can self-assemble into virus like particles without a genome. Another likely explanation is that there is encapsidation of both ssRNA and dsRNA genomes that leads to larger and smaller particles. To ascertain this, a large-scale purification of the virus particles is required for downstream analysis. Numerous attempts were made with different conditions to do this from infected latex, but it was ultimately a failure, due to the inability to disassociate the virus particles from the latex solids to which they seem to be bound (S. P. Rodrigues et al. 2009).

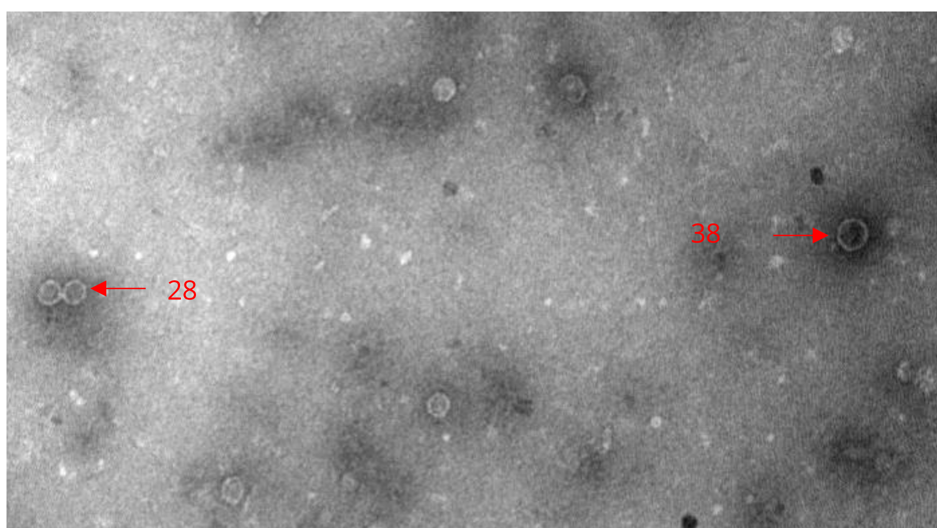

Figure 9 Transmission electron micrograph of virus miniprep from sticky disease infected papaya leaf sample. Two different virus particle sizes are visible, 28 nm and 38 nm. Sample was negatively stained with ammonium molybdate.

The big mystery is that the virus is definitely encapsidated, as virions are visible by electron microscopy and the virus genome is protected from nuclease attack, but where does the coat protein come from? Brazil is the only country to describe another virus, and this virus seems to contribute its coat protein to the other virus (Antunes et al. 2016). Other reports of viruses similar to PMeV2 all report no other virus, even with HTS analysis (Quito-Avila et al. 2015; J. F. Cornejo-Franco, Alvarez-Quinto, and Quito-Avila 2018; Juan F. Cornejo-Franco et al. 2021; Sá Antunes et al. 2020), though most of these analysis are sequences only, and do not show the presence of virions. We have attempted several virus purifications using rate zonal sucrose density gradients and isopycnic caesium sulfate gradients with and without various detergents, ionic dispersants and chaotropic agents, but nothing gave high concentrations of purified virus to allow the positive identification of the viral coat protein vs the proteins present in papaya latex on a denaturing protein gel. The papaya latex could be dispersed with 2% Triton X-100, but the viral particles could not be separated from the latex protein agglomerates.

Prior to the completion of the project, a paper was published from Ecuador that conducted a HTS analysis on babaco, a relative of papaya that is a natural hybrid (Vasconcellea x heilbornii). The plants were showing no symptoms, but the sequence of an umbra-like virus was found. Alignment of the virus with PMeV2-Aus showed a ~95% similarity across the whole genome.

Sticky Disease Knowledge Development

It is reported that silverleaf whitefly (*Bemisia tabaci*) are a vector of PMeV (Vidal, Nascimento, and Habibe 2003), although they are not reported as a pest of papaya. Yellow sticky traps were used to monitor for whitefly in the papaya seed blocks in both the Tablelands and Innisfail. The traps were replaced every two weeks and were inspected for whitefly, however, no whitefly were discovered on the traps. Recently it has been shown in Mexico that a leaf hopper *Empoasca papayae* is a vector of PMeV2 (García-Cámara et al. 2019). Leaf hoppers are a problem in the Tablelands and Innisfail areas, as a species of leafhopper is known to spread the phytoplasma pathogens that cause yellow crinkle, mosaic and dieback in papaya. These pests

were not looked for, as this was published after the trapping and analysis had concluded. Trapping specifically for these pests and virus transmission work with this vector should be considered.

A small study was initiated to test the amount of seed transmission in two parent lines. 120 seeds of PL1 and PL2 were treated with gibberelic acid (GA) as per the industry standard. There was good germination of PL1 (78/120), but very poor germination of PL6 with only 6 plants germinating. These were initially tested at the third leaf stage, and appeared negative for PMeV2-Aus. Some were selected for an infection trial, but then control plants subsequently tested positive for PMeV2-Aus. There are two possibilities for this: the virus is below detectable limits when initially tested or the virus was moved between the infected plants and controls in the glasshouse. A new experiment was designed, including rapid continuous sampling and growth of groups of plants in insect proof enclosures. All seeds failed to germinate this time. New seeds were sought, but the experiment could not be completed due to covid lockdowns. Also, a small host range study was initiated with a selection of viral indicator hosts and mechanical inoculation: tomato, cucumber and *Nicotiana benthamiana* as well as papaya plants from the seed grow out were tested. Leaf material from infected papaya growing in a DAF glasshouse was used to inoculate the plants, with material tested 4 weeks post inoculation for the virus. No plants tested positive for the virus. After testing the innoculum source plants it was initially thought that the reason the infection failed was due to low titre after extended glasshouse cultivation. Later, due to the realisation that there is higher virus diversity than anticipated, it was found that the innoculum source plants were unable to be detected reliably by the assay used. This means that the negative results for the infection trial are not valid. Again, a repeat of the experiment was designed using infected latex as the innoculum source, but covid restrictions meant that it could not be completed.

A small-scale field study was conducted with the excess virus-free plants developed early in the project with the aim of gauging the rate of reinfection with PMeV2-Aus. Eighty-three plants from six parental lines were planted at two locations, on the Tablelands and in Innisfail with the numbers planted, and the results of testing in Table 3. Results of the testing are from the second sampling in February 2021.

Table 3 Number and location and testing results for the field trial of virus free papaya plants

Parental lines	Coast		Tablelands		Total		
	Plants	Positive	Plants	Positive	Plants	Positive	%
PL1	18	3	23	10	**41**	**13**	**31.7**
PL2	8	0	6	5	**14**	**5**	**35.7**
PL3	2	0	3	3	**5**	**3**	**60**
PL4	2	1	2	0	**4**	**1**	**25**
PL5	11	0/2 tested	3	1	**14**	**1**	**7.14**
PL8	2	1	3	0	**5**	**1**	**20**
Total	**43**	**5**	**40**	**19**	**83**	**24**	**28.9 Avg**

All plants established in the field trial tested negative shortly before planting out in the field. On average there was ~29% reinfection within 3 months of planting, which is quite rapid. There looks like more infections on the tablelands, than the coast, but larger numbers would be required for proper analysis. There is also the possibility that the test is more sensitive to the strains of virus on the tablelands, as that is the origin of virus that the characterisation work was done on. The patchy nature, and speed of spread of the infection indicates an aerial insect vector. The rapid spread of the virus into the clean material has large implication of for the management of the clean seed production.

Outcomes

Seed testing protocol development

A robust testing methodology has been developed and validated to routinely diagnose plant infection with PMeV2-Aus, the causal agent of sticky disease in Australia. This test can be used for diagnostics of any strain currently identified in Australia. This test, and the slower more generic test can be accessed by industry through Grow Help, the Department of Agriculture and Fisheries fee-for-service pathology service.

Generation of virus free parental material

Ten parent lines testing clean from PMeV2-Aus have been delivered to industry. These lines were chosen as a priority by industry for production of current hybrids. Representatives of these plants have been entered into tissue culture for long term maintenance. Papaya Seeds Australia, the commercial seed production for Australian Industry, have been informed of methods for production of virus free seed with respect to the current knowledge of virus epidemiology. If recommendations are followed, clean seed will soon be available to growers.

Sticky disease knowledge development

It has been demonstrated that there is only a single, encapsidated virus involved in the papaya sticky disease in Australia, in contrast to the situation in Brazil and elsewhere. The properties of the Australian strain of the virus appear to be different to what is reported elsewhere, or there are assumptions being made about PMeV2. The rapid movement of virus into clean planting material indicates an aerial insect vector. The lack of uninfected plant material has limited the amount of epidemiology that could be performed on the PMeV2-Aus so far. The multiplication of clean parent material by tissue culture can allow this work to begin. Current knowledge of the virus involved in sticky disease and project progress has been disseminated to growers both through the industry communication circular ‘Papaya Press’, and grower meetings throughout the term of the project.

Monitoring and evaluation

How effective was the project?

This project has delivered the key criteria of the project – the ability of the industry to create virus free seed. The ten parental lines that are currently used to create the elite hybrids preferred by industry have been cleaned of PMeV2-Aus and delivered to industry. Knowledge of the speed of virus ingress into the parental lines has been discovered, and they have been informed of management options to develop clean seed.

How relevant was the project to the needs of intended beneficiaries? To what extent has the project met the needs of industry levy payers?

The project achieved its main aims of delivering clean parental lines from which there can be clean seed produced for industry. This is the main objective of industry to allow management options for sticky disease.

Have regular project updates been provided through linkage with the industry communication project?

Report on the project progress has been communicated to stakeholders through the industry communication project. Every issue of the 'Papaya Press' since the commencement of the project has contained an update about the clean seed project.

Did the project engage with industry levy payers through their preferred learning style?

As well as the regular communication with the wider industry through the aforementioned communication project, key growers who are members of the reference committee have had project presentations and informal talks. Numerous in-person meetings (formal and informal) have happened between project personnel and key members of Papaya Seeds Australia about project aims, progress and challenges.

What efforts did the project make to improve efficiency?

We operate under continual improvement of procedural optimisation and efficiency. Development and validation of a multiplex PMeV2-Aus and sexing qPCR increases efficiency and limits errors involved with double handling of the samples. For most testing, the samples collected were processed and dried in the format required for extraction on site in North Queensland, rather than collected and labelled, shipped then reprocessed into the correct format.

Recommendations

As there is rapid movement of virus back into clean planting material, the benefits of growing clean seed will not be fully realised without proper management plans. If possible, new blocks of clean material should be planted as far from existing infected blocks as possible. The application of insecticide before destruction of blocks should be considered to limit large scale migration of possible vectors out of infected blocks into new blocks. The faster the destruction of blocks when picking is completed the better. Also, shorter cropping cycles may be required for a time to limit the build-up of inoculum in the new blocks. Over time, after the innoculum levels drop, the stricter management plans can be revised.

In meetings held with Papaya Seeds Australia, segregation of the papaya seed production from commercial blocks was discussed, either through physical distancing from virus infected blocks, or insect proof structures with high worker hygiene to maintain the virus free plants. The observed rapid infection of these plants highlights this need and planning moving forward. A table of graded recommendations are listed in Appendix 6.

There is a lot of basic epidemiology that still needs to be done to properly inform the management of papaya crops in relation to sticky disease. Simple questions, such as the time between infection and the ability to detect the virus, as well as more complex questions like what the viral vector in Australia is, could not be answered as there was no supply of clean plants. Now there is a supply of clean plants, these fundamental questions can be addressed which would inform basic management decisions in relation to sticky disease. It is recommended that work be funded on the epidemiology of sticky disease in Australia.

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Intellectual property, commercialisation and confidentiality

This report is intended for dissemination.

There are no intellectual property, commercialisation or confidentiality issues to report.

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Appendices

Appendix 1 PCR Methods

All qRT-PCR was on a Rotorgene (Qiagen) or QuantStudio5 (ABI) real time thermocycler, using the Promega GoTaq® Probe one-step RT-qPCR kit. Ordinary One-Step-rt_PCR also used either the Promega kit or MyTaq One-Step RT-PCR kit (Bioline). The PMeV2-Aus probe (PMeV277_P) was labelled with FAM and BHQ-1, And the sexing PCR probe (Nafp322P) was labelled with ROX and BHQ-2.

Template preparation was using the Qiagen Biosprint 15 Plant DNA kit, omitting the RNAse A addition to the RCW buffer. Ten x 5 mm diameter leaf discs were collected by biopsy punch and freeze dried. The dried leaf discs were disrupted with a ball bearing in a Tissuelyser (Qiagen), then the 300 µL of RLT buffer from the kit added. The rest of the protocol was as per the manufacturers protocol, except for the elution was in 100 µL of water.

Duplex qRT-PCR for PMeV2-Aus and Sexing

Reagents	1x
2x Mastermix	7.5 µL
RT-Enzyme	0.3 µL
PMeV322_R	0.3 µL
PMeV322_R2	0.3 µL
PMeV198_Fd	0.6 µL
PMeV322_P	0.6 µL
Nafp265F	0.3 µL
Nafp389R	0.3 µL
Nafp322P	0.3 µL
ddH_2O	3.5 µL
Total	14 µL
Template	1 µL

Reverse transcription	45 °C	15 min
Initial denaturation	94 °C	2 min
Cycling 40x	94 °C	15 s
	62 °C	30 s

One-step RT-PCR for PMeV2

Reagents	1x
One-Step mix (2x)	7.5 µL
PMeVDiagF1	0.6 µL
PMeVDiagF2	0.6 µL
PMeVDiagR	0.6 µL
Reverse Transcriptase (Bioline)	0.15 µL
Ribosafe RNAse Inhibitor (Invitrogen)	0.3 µL
RNAse free water	4.25 µL
Total	14 µL
Template	1 µL

Reverse transcription	45 °C	10 min
Initial denaturation	94 °C	2 min
Cycling 40x	94 °C	15 s
	60 °C	15 s
	72 °C	30 s
Final Extension	72 °C	2 min

Amplification products for the One-step RT-PCR were run on a 1% LB gel for 20 minutes at 300 V. Positive results are a 569 bp band.

Appendix 2 Embryo Rescue Protocol

The protocol for papaya embryo rescue is modified from protocols published by Magdalita et al. (1996) and Azad et al. (2012). An immature fruit (90 – 120 days after anthesis) was harvested from a parental tree. The fruit was then washed with 4% Chlorhexidine solution (Microshield 4 Surgical Hand Wash), surface sterilised with 70% ethanol and dried in a laminar-flow cabinet. The fruit was then bisected in the laminar-flow cabinet, seeds were harvested and kept in a sterile petri dish. Embryos were excised from immature seeds by cutting the seed in half perpendicular to the major ridge with a sterile scalpel, then remove from their endosperm and implanted in a vertical position with their radical embedded in the medium (see table below). Germinated embryos were transferred to half strength MS medium (Sigma Aldrich) without plant growth regulators. Successfully developed plantlets were transplanted and acclimatised following the procedure of Drew (1988). Roots were washed under tap water to remove residual agar. Each plant was placed in a cell of a 42-cell seedling tray (0.06 L/cell) containing steam-pasteurised potting mix (Searles seed raising mix: perlite in the ratio of 1: 1). Plants were gown initially at 90% humidity with a gradual reduction by 5% every day for 7 days or until the humidity in the cabinet reached ambient relative humidity.

Embryo rescue medium

Final concentration	Stock concentration
1X	Murashige and Skoog Basal Medium (M5519, Sigma Aldrich)
0.25 µM	100 µM BAP
0.25 µM	100 µM NAA
10 µM	1000 µM GA3
50 mg/L	50 mg/ml Ribavirin (first experiments only)
2%	Sugar
0.8%	Agar

Appendix 3: Tissue culture Initiation Protocol for papaya

Each macronutrient was prepared in a separate bottle by weighing each chemical as detailed in Table A3.1 and all the micronutrients was prepared by weighting all the chemicals as detailed in Table A3.2 and put in one bottle. The chemical was placed in a 2-Litre beaker, which also contained a magnetic stirrer bar, and dissolved in deionised water. Once the chemical was dissolved completely, the volume was adjusted to one litre in a 1 Litre volumetric flask. The stock was labelled and stored at 4 °C.

Murashige and Skoog (MS) vitamins were used in papaya micropropagation. All the vitamins were prepared in one bottle by weighting all the chemicals as detailed in Table A3.3, placing in a 2-Litre beaker and dissolving in deionised water. Once the chemical was dissolved completely, the volume was adjusted to one litre in a 1 Litre volumetric flask. Sixty ml of the stock was aliquoted into a polypropylene 125 ml container, then labelled and stored at -20 °C. The vitamins were thawed completely in a microwave before used.

Table A3.1: Micronutrients used to prepare stock solution for one litre

Chemical	Stock Concentration	Weight (g)
Potassium Nitrate (KNO_3)	1M (100X)	101.1032
Ammonium Nitrate (NH_4NO_3)	2 M (50X)	160.086
Sodium Dihydrogen Phosphate Dihydrate ($NaH_2PO_4.2H_2O$)	0.8 M (400X)	124.808
Calcium Cholride Dihydrate ($CaCl_2.2H_2O$)	1.2 M (400X)	176.412
Ethylenediaminetetraacetic Acid, Ferric-Sodium Complex (EDTA FeNa)	0.04 (400X)	14.682
Magnesium Sulfate Heptahydrate ($MgSO_4.7H_2O$)	1.2 M (400X)	295.764

Table A3.2: Micronutrients used to prepare stock solution (400X) for one litre

Chemical	Concentration	Weight (g)
Boric Acid (H_3BO_3)	0.06 M	3.7098
Manganese (II) Sulfate Tetrahydrate ($MnSO_4.4H_2O$),	0.04 M	8.922472
Zinc Sulfate Heptahydrate ($ZnSO_4.7H_2O$),	0.014 M	4.6007936
Copper (II) Sulfate Pentahydrate ($CuSO_4.5H_2O$)	0.6 mM	0.149811
Ammonium Molybdate Tetrahydrate [$(NH_4)6Mo_7O_{24}.4H_2O$]	0.4 mM	0.49436
Cobalt Chloride Hexahydrate ($CoCl_2.6H_2O$)	0.4 mM	0.0951356
Potassium Iodide (KI)	2 mM	0.3320056

Table A3.3: Components to prepare MS vitamins stock solution (400X) for one litre

Chemical	Concentration	Weight (g)
Myo-inositol	0.222 M	40
Nicotinic acid	1.625 mM	0.2
Thiamine.HCl	0.1186 mM	0.04
Pyridoxine.HCl	9.7257 mM	2
Glycine	0.01 M	0.8

Day 1: Prepare media

1. **Prepare I-01: Liquid medium for an overnight treatment**

- Add 200 ml of distilled water into a 500 ml volumetric flask, then aliquot each stock as per Table A3.4, **column I-01** in the volumetric flask.

- Adjust volume to 500 ml, close the lid and mix the solution.
- Aliquot 10 ml of the mixed solution into a test tube and close the lid for each test tube.

2. I-02 and I-03: Liquid and solid media

- Add 200 ml of distilled water into a 1000-ml volumetric flask, then aliquot each stock as per Table A3.4, **column I-02 + I03** in the volumetric flask.
- Adjust volume to 1000 ml, close the lid and mix the solution.
- Transfer the **I-02 + I-03 solution** into a beaker and adjust pH to 5.85.

3. Prepare I-02 medium: Liquid medium for 3-day treatment

- Take 250 ml of **the I-02 + I-03 solution** and put into a beaker
- Add 5 gram of sugar and dissolve the sugar
- Aliquot 5 ml of I-02 medium into a test tube and close the lid for each test tube.

4. Prepare I-03 medium: Solid medium for transfer after the 3-day treatment

- Take 750 ml of **the I-02 + I-03 solution** and put into a beaker
- Add 15 gram of sugar and 4.5 gram of agar, then boil the medium to dissolve the sugar and agar.
- Pour about 15 ml of I-03 medium into a small container, then close the lid.

5. Autoclave all the media and 4 L of distilled water

Table A3.4: List of chemicals used to prepare initiation media for 50 samples

Stock		conc.	ml used to prepare			
Bottle #	Chemical		I-01	I-02	I-03	I-02 + I-03
1	KNO_3	50X	10	5	15	20
2	NH_4NO_3	100X	5	2.5	7.5	10
3	$NaH_2PO_4.2H_20$	400X	1.25	0.625	1.875	2.5
4	$CaCl_2$	400X	1.25	0.625	1.875	2.5
5	EDTAFeNa	400X	1.25	0.625	1.875	2.5
6	$MgSO_4.7H_2O$	400X	1.25	0.625	1.875	2.5
7	Micro elements	400X	1.25	0.625	1.875	2.5
8	MS vitamins	400X	1.25	0.625	1.875	2.5
10	BAP	100 µM	2.5	1.25	3.75	5
11	NAA	100 µM	2.5	1.25	3.75	5
	Sugar[1/]			5 g	15 g	
	Agar[1/]				4.5 g	
	PPM solution		20	0.5	1.5	2
Final volume			**500 ml**	**250 ml**	**500 ml**	**250 ml**

[1/] gram used in medium preparation

Day 2: Collect plant samples (Cl_2 solution) and overnight treatment (I-01)

1. Collect plant sample and put in 0.01% chlorine solution
2. In a laminar air-flow cabinet, treat the explants with 0.5% chlorine solution for 10 minutes.
3. In a laminar air-flow cabinet, rinse with sterile distilled water, two times.
4. Trim the explants and put them into I-01 medium.
5. Put test tubes on the roller drum and keep the roller drum on for an overnight treatment.

Day 3: Transfer explants into I-02 medium (3-day treatment)

1. Remove the explants from I-01 medium and trim the base of the explants and transfer to I-02 medium.
2. Incubate the explants on the roller drum for 3 days. Check for contamination and remove them out from

the lab every day.

Day 4: Check for contamination

Day 5: Check for contamination

Day 6: Check contamination and transfer explants into I-03 media

1. Remove the clean explants from I-02 medium and trim the base of the explants and inoculate in to I-03 medium.
2. Incubate the explants in a growth room. Check for contamination and remove them out from the lab every 2-3 days.
3. After 2 weeks, transfer clean culture into multiplication medium and grow the culture on for 2-3 weeks. The culture needs to keep transferring to fresh media every 3-4 weeks by alternating between multiplication and hormone free media. Once the culture produces a long (2 cm) shoot, transfer the shoot to rooting medium and hormone free medium as per routine tissue culture program.

Appendix 4: Acclimatisation protocol for papaya from *in vitro* culture to plantlet

Rooted plants from shoot induction medium were acclimatised following the procedure of Drew (1988).

1. Remove rooted cultures from agar medium very gently.

2. Remove residual agar from the roots by gently wash roots using UV sterile water. Keep the plantlets moist by covering with wet paper towel.

3. Plant the plantlets in a 42-cell seedling tray containing steam-pasteurised potting mix (Searles seed raising mix: perlite in the ratio of 1: 1).

4. Keep the plantlet at 90% humidity with a gradual decrease by 5% every day for 7 days or until the humidity in the cabinet reached ambient relative humidity.

5. Apply ¼ strength of liquid fertiliser at 7 days after transplanting (DAT), ½ strength at 14 DAT and full strength at 21 DAT.

Appendix 5 Papaya Latex Virus Genome Extraction

Take 350 µL of Latex diluted in 1:1 with 0.1 M sodium citrate pH 6.5

Add 20 µL of 2 M $MgCl_2$, 9 µL of 1 M $CaCl_2$

Add 5 µL of DNAseI (NEB) and 2µL of RNAseA (NEB)

Incubate for 20 minutes at 37 °C

Add 350 µL of Extraction buffer (below)

Add 14 µL of Proteinase K (20 mg/ml)

Incubate at 37 °C for 30 minutes

Extract with 1 volume of Tris saturated phenol

Centrifuge for 8K for 4 minutes at room temperature in microfuge

Remove supernatant to a new tube and add 1 volume of chloroform/IAA 24:1

Centrifuge for 8K for 4 minutes at room temperature in microfuge.

Remove ~400 µL of supernatant to a new tube and add 0.1 volumes of 3 M sodium acetate, 2.5 vol of 100% ethanol.

Incubate at room temp for 20 minutes.

Spin down @ 12 k rpm in microfuge for 20 minutes

Remove supernatant and air-dry pellet for 15 minutes

Resuspend in 50 µL of nuclease free 10 mM Tris-HCl, 1 mM NaEDTA pH 8

Appendix 6 Recommendations

Longevity of the parent lines to produce clean seeds can be related to the choices made from the table below i.e. multiple choices from the better or higher category for location, movement, and testing would result in high confidence for long term production of clean hybrid seed. There is an added benefit of even simple insect resistant enclosures should reduce the occurrence of phytoplasma disease in the seed production blocks.

Recommendation Table for Management of Clean Seed Parent Material		
Location	Best	Growth away from production area, in insect proof structures
	Better	Growth away from production areas. Limited contact
	Okay	Growth in production area in insect proof structures
	Not recommended	Growth next to production block with no protection
Movement	Best	No direct contact (equipment/staff) between seed production and production blocks
	Better	Limited contact between seed block and production blocks
	Okay	High worker/equipment hygiene between blocks
	Not Recommended	Free movement of staff and dirty equipment between production blocks and seed blocks
Testing	Best	Three monthly testing of all parent material
	Better	Testing before initial crosses, and every three months after
	Okay	Testing before initial crosses
	Not recommended	Seed batch testing
Triage	Best	Positive trees have knockdown insecticide sprays before removing
	Better	Infected trees are removed
	Not Recommended	Trees are left, but no seed collected from these trees