Final Report

Industry Development and Research in the Papaya Industry

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Papaya Industry Australia

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Summary

This project encompassed two sub-projects: an Industry Development Officer (IDO), and a one- year project for a papaya researcher.

The aims for the IDO component were to develop a good working knowledge in all aspects of commercial papaya production in Australia, to tailor make varieties suitable for different growing regions. Also to work on solutions for papaya industry problems and liaise with growers and papaya industry groups. A close working relationship was developed with researchers, grower organisations (Innisfail pawpaw growers, Mareeba fruit and vegetable association), Papaya Australia and Papaya IAC. The role included visiting farms on the Tablelands and the coastal areas, and collaboration with those involved in the supply chain for the industry. Field days were organised in each area to develop a better understanding of papaya production and to provide a forum for discussion and knowledge exchange. Reports were also be submitted to Papaya Post.

The objectives for Papaya Researcher component were to continue relevant research in papaya breeding using micropropagation techniques and molecular marker assisted breeding selection (MAS). It was proposed to refine the current papaya tissue culture protocols to the production on a commercial scale by aiming for higher efficiency and commercially viability. This covers initiation of parent trees in the field, establishment cultures in a laboratory, deflasking, acclimatisation and hardening for field planting.

Tissue culture protocols from field initiation through acclimatisation were developed for commercial scale production. The protocols were adopted from Drew 1988 with minor modification to be suitable to a commercial system. More than 50% of cultures were successfully initiated from field to laboratory and 100% of selected trees were established in tissue culture.

Preliminary studies showed 100% accuracy of markers to papaya using trees of known sex. Sex linked DNA markers were screened on approximately 2000 seedlings of breeding lines. The accuracy of the markers to expression of sex of papaya were confirmed of 100% accuracy.

Keywords

Papaya; DNA marker; Tissue culture; Industry development; Farm visit

Introduction

Unavailability of a long term multifaceted researcher has been identified as one of the problems for papaya industry in Australia. The industry requires a researcher who is knowledgeable about problems of industry production, who understands how research can be applied to make improvements in papaya, and who can improve and extend communication between relevant organisations. There is an opportunity to develop a potential long-term researcher for Papaya Industry Australia. The employment of an IDO has encouraged a better understanding of papaya production systems for each growing region and problems in each growing area have been identified and research to tackle those has been conducted suitably.

Breeding is one of the key research strategies for papaya crop improvement and a genetic inheritance study is necessary in a breeding program. To be able to study genetic inheritance of trait of interest, evaluation of parental lines and all the progenies must be done at the same time to eliminate most environmental factors. The genetics of parental lines must be duplicated and tissue culture is the ideal method. Initiation process of material from a field into a laboratory was slow and, from literature reviews, the most difficult process to establish the culture. The initiation protocol was refined in this research.

A breeding program to improve eating quality of papaya has been funded by Horticulture Innovation Australia Limited (previously Horticulture Australia Limited) since 2005 (Hansen 2005; Drew 2005). The selection process is the most crucial procedure for a breeding program. Conventional breeding relies on phenotypic expression which takes time to express and is likely greatly influenced by the environment. DNA marker assisted selection can be done at any stage of plant development, without having to wait for the desired traits to be expressed.

Fruit shape of papaya is related to the sex of papaya trees. The preferences in Australian domestic market are female fruits for yellow papaw, and hermaphrodite fruits for red papaya. As a result, growers have to grow multiple seedlings in each planting site to achieve the maximum number of the desirable sex of trees per plantation. This means more seedlings have to be planted than are kept, more labour invested just to cull the unwanted trees and worst of all, roots of old stumps can be a source of infection for root diseases. One simple conclusion is this that this costs more money for growers. Advances in the study of papaya genetics is providing us a map of its genome and the research about sex of papaya is very well documented. This research is the demonstration of the application of the DNA markers to assist farm management. The accuracy of the marker to detect sex of papaya will be evaluated in trees of unknown sex when those trees reveal their sexes.

Both the IDO and papaya researcher roles were delivered by Dr Chat Kanchana-udomkan.

Methodology

Industry Development Officer: IDO Project

Activities related to the IDO were as listed below

- visit farms in Mareeba, Dimbulah, Innisfail and Tully
- write articles for Papaya Post
- attend meetings
- organise field days.

Papaya research project:

1. Refinement of Protocol for Papaya Tissue Culture

1.1 Initiation process

Field collection

- In a field, shoots were collected and soaked in 0.01% chlorine solution plus few drops of detergent.
- In a laboratory, the treated samples were rinsed using sterile water.
- In a laminar air-flow cabinet, the samples were treated with 0.5% chlorine solution plus few drops of detergent for 5 minutes then rinsed three times using sterile water.

Overnight treatment

- In a laminar air-flow cabinet, the explants were cut into 2 cm length. Then they were out into sterile 1X DS minerals (Stock #3) supplement with MS vitamins and 4% PPM solution.
- The samples were incubated overnight on a roller drum at 26ºC ± 1ºC.

Transfer of samples to liquid medium

- The samples from overnight treatment were rinsed in sterile water, the base of the explants were trimmed and cut into the length of 1 cm.
- The samples were grown in sterile liquid medium (multiplication medium supplement with 0.2% PPM solution and 2% sucrose, pH 5.65) and incubated on a roller drum in a growth room (27ºC, 16/8 hours light/dark) for 3 days. The explants were transferred to solidify media (multiplication medium supplement with 0.2% PPM solution, 2% sucrose and 0.8% agar, pH 5.65).

1.2 Micropropagation process

Apically dominant plants were dissected into nodal sections and sub-cultured in a multiplication medium for 4 weeks (Appendix 1). Shoots were removed from the nodes and transferred to a root induction medium for 3 days (Appendix 1). They were then transferred to a plant growth regulator-free medium (Appendix), also known as “single shoot medium” for 3-4 weeks. Then, the cycle of micropropagation was repeated by multiplication, root induction and shoot induction (Figure 1) to multiply the number of plants that were required. All plant sections were incubated under conditions of 16 hours light from fluorescent lamps and 8 hours dark at 25ºC ± 1ºC.

1.3 Acclimatisation

Rooted plants from shoot induction medium were acclimatised following the procedure of Drew (1988). Roots were washed using UV sterile water to remove residual agar. A plant was planted in 42-cell seedling tray containing steam-pasteurized potting mix (Searles seed raising mix: perlite in the ratio of 1: 1). Plants were gown initially 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.

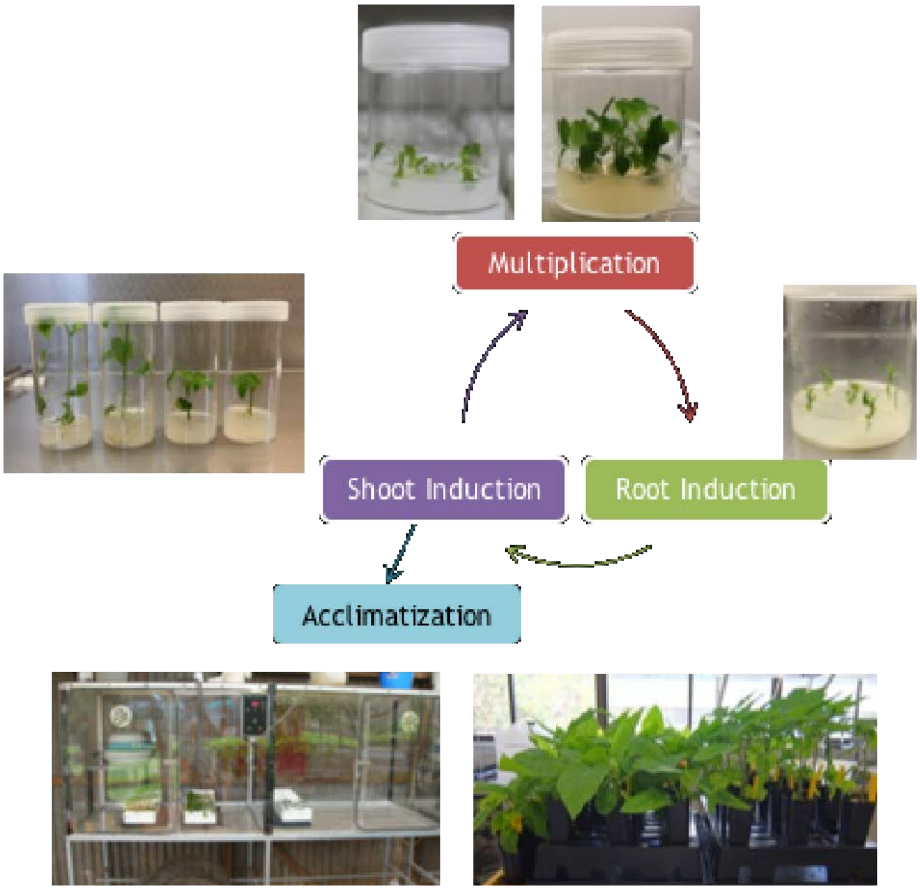

Figure 1: Procedure of papaya *in vitro* propagation

2. Papaya sex determination using DNA markers

Seeds of 11 crosses resulted from Marker Assisted Breeding Project: PP10005 were sown at Lecker Farming, Mareeba, QLD in February 2015. These seedlings were used as plant materials to screen for their sexes. Genomic DNAs of all samples were extracted using the protocol by Dellaporta et al (1983) with a minor modification as detailed in Appendix 2. To speed up the assay, the measurement of DNA quantification and qualification was applied to few samples to estimate the concentration of DNA as a whole and applied this concentration to all the samples.

2.1 PCR assay

Each reaction contained 1 µl of genomic DNA, 0.3 unit MyTaq Red DNA Polymerase (Bioline), 1X MyTaq Red Reaction Buffer (1 mM dNTPs, 3mM MgCl2, stabilizers and enhancers; Bioline) and 0.3 µM each of forward and reverse primers (Table 1). PCR cycles were carried out in a thermal cycler (MyCycler, BioRad) using a traditional PCR cycle, which was programmed as detailed in Figure 2.

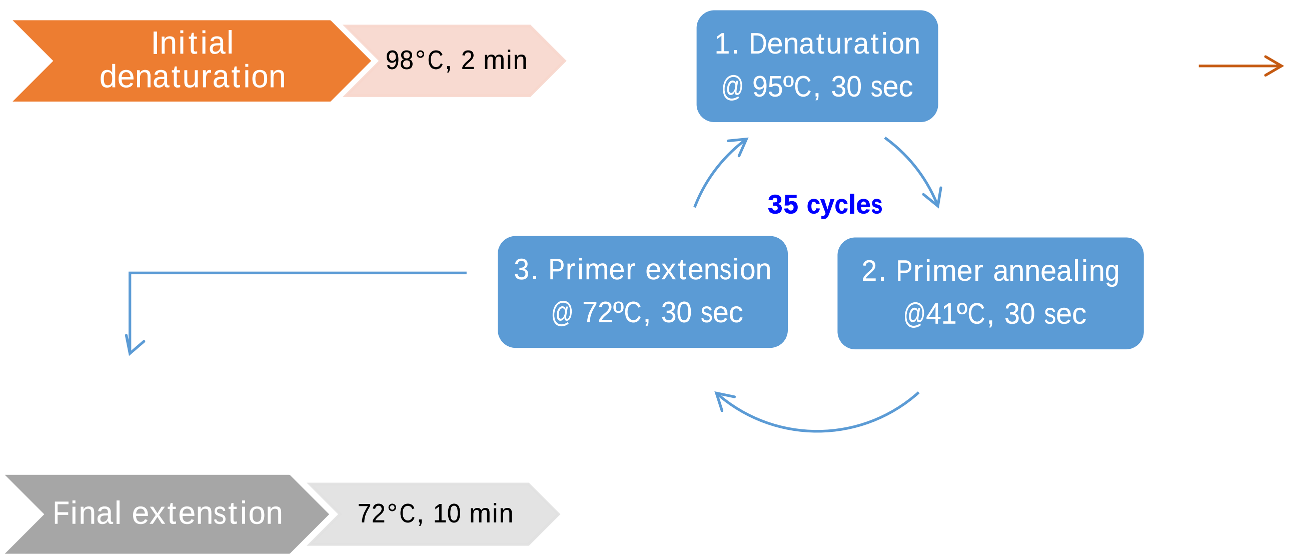

Figure 2: The PCR cycle used for screening of papaya seedlings for sex identification.

2.2 Agarose gel electrophoresis

PCR amplification products were separated by electrophoresis in a 1.5% agarose gel in TAE buffer (40 mM Tris-acetate [pH 8.0], 1 mM EDTA) at 100 V for 1 hour, and stained with with RedSafe™ Nucleic Acid Staining Solution 20,000X (iNtRON Biotechnology, Korea). The gel was then visualised under ultraviolet light.

Table 1: Primers used for papaya sex determination

Primer Name	Primer sequences:	PCR size	Type of marker	Sex detection	References
T1	T1F: TGCTCTTGATATGCTCTCTG T1R: TACCTTCGCTCACCTCTGCA	1.3 kb	SCAR	All sex	
T12	T12F: GGGTGTGTAGGCACTCTCCTT T12R: GGGTGTGTAGCATGCATGATA	800 bp	SCAR	Hermaphrodite and Male	Deputy et al. (2002)
W11	W11F: CTGATGCGTGTGTGGCTCTA W11R: CTGATGCGTGATCATCTACT	800 bp	SCAR	Hermaphrodite and Male	
SCARps	SDP-F: GCACGATTTAGATTAGATGT SDP-R: GGATAGCTTGCCCAGGTCAC	225 bp	SCAR	Hermaphrodite and Male	(Urasaki et al. 2002b)
SCARpm	SDP1-F: GGATAGCTTGCCCAGGTCAC SDP1-R: GGTAAGAGTTTTTCCCAAGC	347 bp	SCAR	Hermaphrodite and Male	(Urasaki et al. 2002a)
SCAR SDSP	C-F: AAACTACCGTGCCATTATCA C-R: AGAGATGGGTTGTGT CACTG	369 bp	SCAR	Hermaphrodite and Male	(Chaves-Bedoya and Nuñez 2007)
OPE03-0.4	OPE03: CCAGATGCAC	0.4 kb	RAPD		
OPC09-1.7	OPC09: CTCACCGTCC	1.7 kb	RAPD		(Niroshini et al. 2008)
C09/20	C09/20-F: CTCACCGTCCATTTTAATTA C09/20-R: CTCACCGTCCGCGGCATCAATGTA	978 bp	SCAR	Hermaphrodite and Male	
OPF2-0.8	F-Napf-70: GGATCCCT ATTAG R-Napf-71: GAGGATCCCTTTTGC	0.83 kb	SCAR	Male	(Parasnis et al. 2000)

Note: Highlighted primers can detect polymorphisms between female and hermaphrodite/male bulk DNA

Outputs

IDO project:

- 3 articles for Papaya Post:
    - Report from the IDO: The first report from Industry Development Officer, Papaya Post, Edition 3, December 2014
    - Report from the IDO: Papaya in Thailand, Papaya Post, Edition 1, April 2015
    - Report from the IDO: Papaya Seedlings Sex Determination using DNA Markers, Papaya Post, Edition 2, June 2015
- A trip to papaya farms in Thailand was made in March 2015. The IDO covered all of their own expenses related to this trip. Two farms were visited and a power point presentation of "Australian Papaya and Its Research" was presented to growers and researchers in Thailand. The detail of the visit was presented in Papaya Post, Edition 1, April 2015. An article about the presentation and the visit was also published in Thai in Kehakaset Agricultural Magazine, Volume 39, issue 5, May 2015 (ISSN 01258877).
- 3 presentations:
    - "Industry breeding program and tissue culture" at South Johnstone research station, Innisfail.
    - "Australian Papaya and Its Research" at Kehakaset Farm, PathumThani, Thailand.
    - "Marker Assisted Breeding of Papaya to Develop New Commercial Lines" Papaya Interim Industry Advisory Meeting, Cairns.
- 2 field days in both regions, Tableland and Innisfail: August 2014 and November 2015
- Market visits: Aiming to keep in contact with agents, and to get a better understanding of the market chain for papaya in Australia. Three visits were made in this project.

Papaya research project:

- A refined micropropagation protocols for papaya production for a commercial scale operation.
- DNA markers for sex determination to be used to assist selection in future breeding programs.
- An academic paper relating to research will be submitted to present in an International Symposia on Tropical and Temperate Horticulture in Cairns, Australia in November 2016.

Outcomes

IDO project:

A researcher who: is familiar with, and has a better understanding in all aspects of papaya production in Australia, understands the industry dynamics and can identify problems for each region, improve communication with relevant R&D to growers and supply chain partners and, assist and extend R&D to combat grower production problems. This will facilitate the breeding programs to tailor-made varieties to meet specific requirements in each growing region.

Papaya research project:

Knowledge related to papaya breeding, molecular breeding and tissue culture of papaya crops has been transferred to papaya breeders, growers and researchers through scientific publications, industry communications and direct networking.

The availability of molecular markers to select for sex of papaya can save costs associated with farm management and space to plant number of tree in breeding trials.

Evaluation and Discussion

IDO:

Farm visits: Several farms have been visited regularly aiming to keep in contact with growers, observe their production systems and introduce DNA technology to aid farm management. Good relationships between the IDO and most growers have been developed (please refer to the Acknowledgements).

Market visits: Aiming to keep in contact with agents, and to get a better understanding of the market chain for papaya in Australia. Three visits were made in this project.

Field day organisation: Two field days were organised for researchers to visit farms in Innisfail and Mareeba.

Papaya Research Project:

Tissue culture

Approximately 3000 plants of commercial red papaya (RB1 and RB4) were planted at Lecker Farming, Mareeba as part of the result from VC funded component of the project. One hundred tissue cultured plants of RB1 and RB4 in total (10 plants of each variety at each farm) were also planted at four different farms in Innisfail, Joe Zappala, Carolyn Broom, Michael Oldano and Eddie Mizzi, and one farm in Dimbulah, Michael Canzian. The tissue culture trees produced higher yields compared to seed propagation trees.

The refinement of the initiation process from this research enabled researchers to bring explants from a field directly to a laboratory. The percentage of clean culture depended on time and season to collect the samples from fields. Higher percentage of clean culture was presented when the samples were collected during dry weathers.

The multiplication medium was optimised by reducing concentration of plant growth regulators. Plants can produce strong shoots when using optimum concentration of 0.25 µM NAA and 0.25µM BAP. Rooting process is under a refinement process. Incubation of cultures in rooting media at low concentration of IBA (10 µM) for a longer period of time can increase root production in the cultures.

Sex determination using DNA markers

Five out of 10 primers can differentiate between female to hermaphrodite/male tree in bulked DNA (Figure 3). From the preliminary experiment, the marker can correctly identify female and male/hermaphrodite trees with an accuracy of 100% using 100 trees of known sexes (Figure 4). The reactions that distinguish between female and male/hermaphrodite were applied to seedlings from the breeding project. Their sex was unknown. Over 2,000 seedlings were tested and the tested plants were distributed to four farms in Innisfail, one farm in Tully and two farms on the Tableland. The accuracy of the marker to detect sex of papaya, evaluated from the sex of these unknown seedlings showed 100% accurate to phenotype. This marker can be useful to screen sex of seedlings before field planting especially in breeding program that only hermaphrodite red and female yellow will be planted for evaluation.

Hofmeyr (1938) and Storey (1938) proposed the genotype of male, hermaphrodite and female plants are Mm (M^1m), M^hm (M^2m) and mm respectively, and explained that homozygous dominant alleles are lethal. Therefore, segregation of sex type from selfed hermaphrodite trees is 2 hermaphrodites: 1 female. Whereas seeds from female trees segregate at the ratio of 1:1 female: hermaphrodite if the plant is crossed with a hermaphrodite tree, but that of 1:1 female: male when it is crossed with a male tree. The segregation of markers linked to sex of each cross agreed to the proposed ratio.

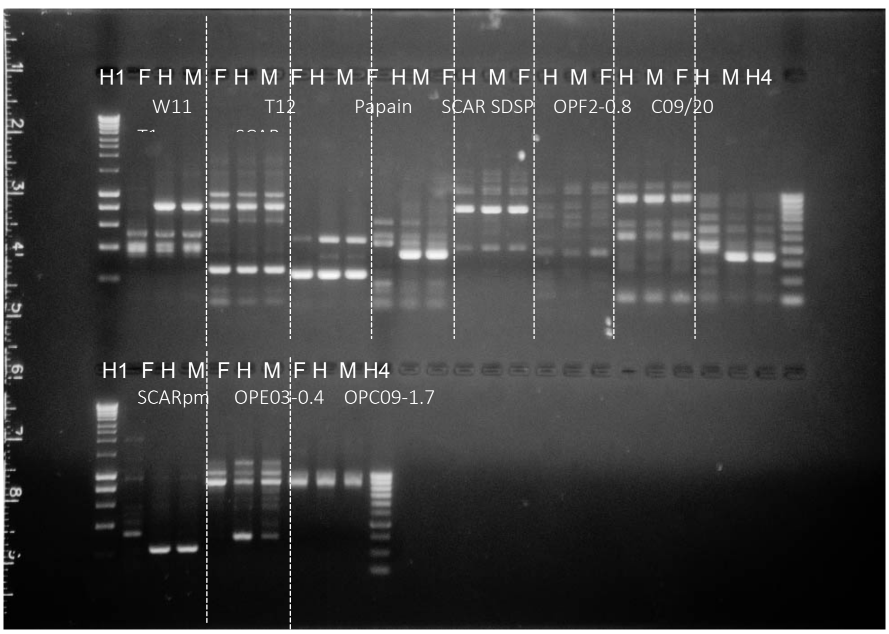

Figure 3: Sex determination using bulk DNA of female (F), hermaphrodite (H) and male (M) plants using 11 primers.

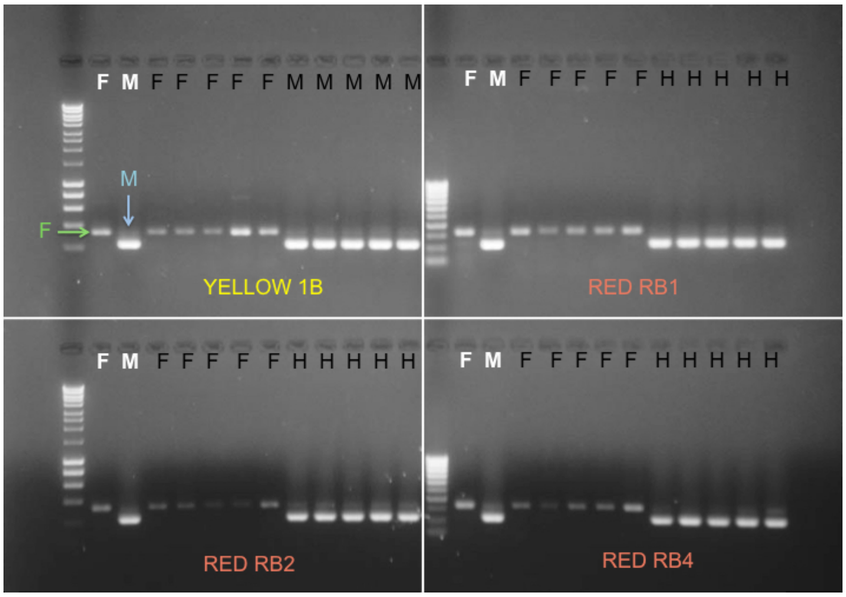

Figure 4: Sex determination using SDP-1 primer in known sex of commercial papaya, 1B, RB1, RB2 and RB4. Lane ‘F’ indicated pooled DNAs of known female trees and Lane ‘M’ indicated pooled DNAs of known male trees. The markers at blue arrow (→) indicates male trees and green arrow (→) indicates female trees.

Recommendations

- Field Days should be organised at least once a year to keep growers up to date with research outcomes and this can be a showcase for growers who demonstrate success in their farming systems.
- Sex determination markers should be applied at the farm level as this can reduce costs related to management. However, cost analysis should be done to compare the benefit of using DNA markers and traditional multiple seedling system.
- Other DNA markers should be developed and applied to breeding program and farm management.

Scientific Refereed Publications

Kanchana-udomkan C., Drew R. and Ford R., (Submitted) Papaya sex determination using DNA markers. In preparation for International Symposia on Tropical and Temperate Horticulture in Cairns, Australia.

Note: This article will be submitted as an oral presentation and if accepted, it will be published in Acta Horticulturae.

Intellectual Property/Commercialisation

No commercial IP generated.

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1. Carolyn and Bob Broom
2. Michael Canzian
3. Gerard Kath
4. Eddie Mizzi
5. Miclhael Oldano
6. Joe Zappala

We thank the members of the plant breeding advisory group who met regularly with the project team and contributed valuable inputs towards breeding objectives and evaluation of trial plantings. We thank the following members of this group:

1. Carolyn and Bob Broom
2. Chris Cini
3. Hayden Darvenisa
4. Gerard Kath
5. Miclhael Oldano
6. Joe Zappala

Appendices

Appendix 1: In vitro propagation

Apically dominant plants were dissected into nodal sections and sub-cultured in a multiplication medium for 4 weeks. Shoots were removed from the nodes and transferred to a root induction medium for 7 days. They were then transferred to a plant growth regulator-free medium, also known as “single shoot medium” for 3-4 weeks. Then, the cycle of micropropagation was repeated by multiplication, root induction and shoot induction (Figure 1) to multiply the number of plants that were required. All plant sections were incubated under conditions of 16 hours light from fluorescent lamps and 8 hours dark at 26ºC ± 1ºC.

1.1 Multiplication process

To prepare the multiplication medium; 10 ml each of macronutrients stock solutions #3, micronutrients stock solutions #3 and MS vitamins (except potassium nitrate used 20 ml/litre) was aliquoted and dispensed into a 1-litre volumetric flask. The final concentration each of nutrient was detailed in Table 1. The medium was supplemented with 0.25 µM 1-naphthaleneacetic acid (NAA) and 0.25 µM 6-benzylaminopurine (BAP) and then it was adjusted the volume to one litre. In a 2-litre glass beaker, which had contained a magnetic bar, the medium was transferred into the beaker and pH was adjusted to 5.65. After that, 20 g (2% w/v) of sugar and 8 g (0.8% w/v) of agar were added into the solution and the solution was boiled either in a microwave or on a hotplate stirrer until sugar and agar were totally dissolved. One hundred ml of the medium were dispensed into a 600-ml round takeaway container; the lid was closed and the container was sterile in an autoclave at 121ºC for 15 minutes. The preparation of stock solutions #3 and MS vitamins detailed in 3.1.1.1 and 3.1.1.2, respectively.

1.1.1 Preparation of stock solution #3

Each of macronutrient was prepared in a separate bottle by weighing each chemical as detailed in Table 2. The chemical was placed in a 2-Litre beaker, which had contained a magnetic stirrer bar, and dissolved in deionised water. All of the microelements were prepared in one bottle by weighting all the chemicals as detailed in Table 3 and placing in the same beaker. 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 4ºC.

Table 1: Final concentration each of macronutrient, micronutrient and vitamin in multiplication medium

Chemical	Formula	Concentration
Ammonium Nitrate	NH_4NO_3	0.02 M
Potassium Nitrate	KNO_3	0.02M
Sodium Dihydrogen Phosphate Dihydrate	$NaH_2PO_4.2H_2O$	2 mM
Calcium Cholride Dihydrate	$CaCl_2.2H_2O$	3 mM
Ethylenediaminetetraacetic Acid, Ferric-Sodium Complex	EDTA FeNa	0.1 mM
Magnesium Sulfate Heptahydrate	$MgSO_4.7H_2O$	3 mM
Boric Acid	H_3BO_3	0.15 mM
Manganese (II) Sulfate Tetrahydrate	$MnSO_4.4H_2O$	0.1 mM
Zinc Sulfate Heptahydrate	$ZnSO_4.7H_2O$	0.04 mM
Copper (II) Sulfate Pentahydrate	$CuSO_4.5H_2O$	1.5 µM
Ammonium Molybdate Tetrahydrate	$(NH_4)6Mo_7O_{24}.4H_2O$	1 µM
Cobalt Chloride Hexahydrate	$CoCl_2.6H_2O$	1 µM
Potassium Iodide	KI	5 µM
Myo-inositol		0.28 mM
Nicotinic acid		2 µM
Thiamine HCl		0.15 µM
Pyridoxine HCl		1.5 µM
Glycine		0.013 mM
1-naphthaleneacetic acid	NAA	0.25 µM
6-benzylaminopurine	BAP	0.25µM

Table 2: Macronutrients used to prepare stock solution #3 (100X)[1/] for one litre

Chemical	Concentration	Weight (g)
Ammonium Nitrate (NH_4NO_3)	2 M	160.1000
[1/]**Potassium Nitrate (KNO_3)**	1M	101.1100
Sodium Dihydrogen Phosphate Dihydrate ($NaH_2PO_4.2H_2O$)	0.2 M	31.2020
Calcium Cholride Dihydrate ($CaCl_2.2H_2O$)	0.30 M	44.1000
Ethylenediaminetetraacetic Acid, Ferric-Sodium Complex (EDTA FeNa)	0.01 M	3.6706
Magnesium Sulfate Heptahydrate ($MgSO_4.7H_2O$)	0.3 M	73.9500

Note: [1/] KNO_3 stock solution #3 was prepared for 50X

Table 3: Micronutrients used to prepare stock solution #3 (100X) for one litre

Chemical	Concentration	Weight (g)
Boric Acid (H_3BO_3)	0.015 M	0.9276
Manganese (II) Sulfate Tetrahydrate ($MnSO_4.4H_2O$),	0.01 M	2.2306
Zinc Sulfate Heptahydrate ($ZnSO_4.7H_2O$),	0.004 M	1.1502
Copper (II) Sulfate Pentahydrate ($CuSO_4.5H_2O$)	0.15 mM	0.0374
Ammonium Molybdate Tetrahydrate [$(NH_4)6Mo_7O_{24}.4H_2O$]	0.1 mM	0.1236
Cobalt Chloride Hexahydrate ($CoCl_2.6H_2O$)	0.1 mM	0.0238
Potassium Iodide (KI)	0.5 mM	0.0830

1.1.2 Preparation of vitamins

Murashige and Skoog (MS) vitamins were used in the media preparation of papaya micropropagation. All the vitamins were prepared in one bottle by weighting all the chemicals as detailed in Table 4, 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. The stock vitamin was labelled and stored at 4ºC.

Table 4: Components to prepare MS vitamins stock solution (100X) for one litre

Chemical	Concentration	Weight (g)
Myo-inositol	0.028 M	10.00
Nicotinic acid	0.2 mM	0.05
Thiamine.HCl	0.015 mM	0.01
Pyridoxine.HCl	0.15 mM	0.05
Glycin	1.3 mM	0.20

1.2 Root induction process

To prepare the root induction media; 10 ml each of macronutrients stock solutions #2, micronutrients stock solutions #2 and MS vitamin was aliquoted and dispensed into a 1-litre volumetric flask. The final concentration each of nutrient was detailed in Table 5**.** The medium was supplemented with 10 µM Indole-3-butyric acid (IBA) then it was adjusted the volume to one litre. In a 2-litre glass beaker, which had contained a magnetic bar, the medium was transferred into the beaker and pH was adjusted to 5.65. Then 20 g (2% w/v) of sugar and 8 g (0.8% w/v) of agar were added into the solution and the solution was boiled either in a microwave or on a hotplate stirrer until sugar and agar were totally dissolved. Twenty-five ml of the medium was dispensed into a 100-ml round takeaway container, the lid was closed and the container was sterile in an autoclave at 121ºC for 15 minutes. The preparation of stock solutions #2 and MS vitamins detailed in 3.1.2.1 and 3.1.1.2, respectively

1.2.1 Preparation of stock solution #2

Each of macronutrient was prepared in a separate bottle by weighing each chemical as detailed in Table 6. The chemical was placed in a 2-Litre beaker, which had contained a magnetic stirrer bar, and dissolved in deionised water. All of the microelements were prepared in one bottle by weighting all the chemicals as detailed in Table 7 and placing in the same beaker. 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 4ºC.

Table 5: Final concentration each of macronutrient, micronutrient and vitamin in root induction medium

Chemical	Formula	Concentration
Ammonium Nitrate	NH_4NO_3	0.01 M
Potassium Nitrate	KNO_3	0.01M
Sodium Dihydrogen Phosphate Dihydrate	$NaH_2PO_4.2H_2O$	1 mM
Calcium Cholride Dihydrate	$CaCl_2.2H_2O$	2 mM
Ethylenediaminetetraacetic Acid, Ferric-Sodium Complex	EDTA FeNa	0.05 mM
Magnesium Sulfate Heptahydrate	$MgSO_4.7H_2O$	1.5 mM
Boric Acid	H_3BO_3	0.05 mM
Manganese (II) Sulfate Tetrahydrate	$MnSO_4.4H_2O$	0.05 mM
Zinc Sulfate Heptahydrate	$ZnSO_4.7H_2O$	0.02 mM
Copper (II) Sulfate Pentahydrate	$CuSO_4.5H_2O$	1.5 µM
Ammonium Molybdate Tetrahydrate	$(NH_4)6Mo_7O_{24}.4H_2O$	0.01 µM
Cobalt Chloride Hexahydrate	$CoCl_2.6H_2O$	5 µM
Potassium Iodide	KI	2.5 µM
Myo-inositol		0.28 mM
Nicotinic acid		2 µM
Thiamine HCl		0.15 µM
Pyridoxine HCl		1.5 µM
Glycine		0.013 mM
Indole-3-butyric acid	IBA	10 µM

Table 6: Macronutrients used to prepare stock solution #2 (100X) for one litre

Chemical	Concentration	Weight (g)
Ammonium Nitrate (NH_4NO_3)	1 M	80.0500
Potassium Nitrate (KNO_3)	1M	101.1100
Sodium Dihydrogen Phosphate Dihydrate ($NaH_2PO_4.2H_2O$)	0.1 M	15.6010
Calcium Cholride Dihydrate ($CaCl_2.2H_2O$)	0.20 M	29.4000
Ethylenediaminetetraacetic Acid, Ferric-Sodium Complex (EDTA FeNa)	5 mM	1.8353
Magnesium Sulfate Heptahydrate ($MgSO_4.7H_2O$)	0.15 M	36.9750

Table 7: Micronutrients used to prepare stock solution #2 (100X) for one litre

Chemical	Concentration	Weight (g)
Boric Acid (H_3BO_3)	5 mM	0.3092
Manganese (II) Sulfate Tetrahydrate ($MnSO_4.4H_2O$),	5 mM	1.1153
Zinc Sulfate Heptahydrate ($ZnSO_4.7H_2O$),	0.002 M	0.5751
Copper (II) Sulfate Pentahydrate ($CuSO_4.5H_2O$)	0.15 mM	0.0025
Ammonium Molybdate Tetrahydrate [$(NH_4)6Mo_7O_{24}.4H_2O$]	10 µM	0.0124
Cobalt Chloride Hexahydrate ($CoCl_2.6H_2O$)	0.05 mM	0.0119
Potassium Iodide (KI)	0.25 mM	0.0415

1.3 Shoot induction process

To prepare single shoot medium, stock solution #3 was used and followed the instruction of the preparation of multiplication medium except no plant growth regulator was added into this medium. After sugar and agar were dissolved, 100 ml of the medium were dispensed into a 100-ml round takeaway container, the lid was closed and the container was sterile in an autoclave at 121°C for 15 minute

Appendix 2: Genomic DNA extraction

Total genomic DNA of papaya was extracted from leaves of 2-month old seedlings using the protocol by Dellaporta et al (1983) with a minor modification. Before the start of the extraction process, the extraction buffer was preheated to 65ºC in a water bath and isopropanol was chilled to -20ºC.

For each sample, 600 mg of fresh leaf sample was collected and placed in a sterile 2 ml microcentrifuge tube. The sample and the tube were snap frozen in liquid nitrogen and the sample was then ground to fine a powder using a sterile micropestle. Eight hundred µl of preheated 2% CTAB extraction buffer (2 g of CTAB hexadecyl-trimethyl-ammonium bromide, 10 mM Tris, 50 mM EDTA [Ethylene diaminetetraacetic acid], 0.7 M NaCl) was added and the sample was vortexed. Then, 60 µl of freshly prepared 10% (w/v) N-laurylsarcosine, 60 µl of freshly prepared 10% (w/v) poly-vinyl-pyrrolidone (PVP) and 40 µL of 10% (w/v) sodium dodecyl sulfate (SDS) were added to the CTAB/leaf mixture, after which the tube was vortexed briefly. The mixture was incubated at 65ºC for 1 hour, and vortexed every 15-20 minutes. The gDNA was purified by adding an equal volume (approximately 900 µl) of phenol: chloroform: isoamyl alcohol mixture (25:24:1) to the leaf/extraction buffer mixture. The mixture was combined on an orbital shaker at room temperature for 30 minutes, and then centrifuged at 13,000 rpm for 15 minutes at room temperature.

The upper aqueous phase (that contained DNA) was transferred by pipetting (approximately 700 µl) into a sterile microcentrifuge tube. The purification process was repeated. Approximately 600 µl of the upper aqueous phase was transferred to a sterile microcentrifuge tube. DNA was precipitated by adding 800 µl of cold isopropanol (approximately 1.25-1.5 time volume of the upper aqueous solution) and 80 µl of 3M sodium acetate. The mixture was gently mixed by inverting the tube until DNA precipitated, and then the mixture was placed on ice for 1 hour to complete the DNA precipitation reaction. The DNA pellet was collected by centrifuging the samples at 10,000 rpm for 5 minutes and carefully discarding the supernatant. The pellet was then washed with 300 µL of 70% ethanol and the tube was inverted once. The samples were centrifuged at 13,000 rpm for 15 minutes and the supernatant was discarded. The DNA pellet was air dried at room temperature to remove any trace of ethanol, then was resuspended in 50-100 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and incubated at 45ºC for 1 hour. The final step was to eliminate RNA by adding 5 µL of 10 mg/ml Rnase A and the sample was incubated at 37ºC for 15 minutes. The DNA solution was kept at -20ºC for long term storage.