Horticulture Innovation Australia

Final Report

RNA silencing based Phytophthora root rot resistant avocado rootstocks – Phase 2

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AV13000

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Contents

Summary

Phytophthora root rot (PRR) is the most serious and widely distributed disease of avocado worldwide. The causal agent, *Phytophthora cinnamomi*, is a primary constraint on avocado productivity in Australia. This project employed a novel strategy that uses RNA silencing technology to obtain avocado rootstocks that are tolerant to PRR.

We now have the proof of concept for *P. cinnamomi* resistant avocado rootstocks of cultivar 'Reed' screened in limited growth cabinet and glasshouse trials. The transformed plants generated in Phase 1 of the project were clonally multiplied using the double grafting method. In addition, we also continued to generate new transformation events and clonal propagation of transformed plants. The plants were screened for resistance in the glass house using the double pot method of inoculation and showed active root growth and larger number of roots than the wild-type (WT) Reed controls. The selected lines continued to show improved resistance over WT non-transformed controls in resistance screening trials in the glasshouse. Molecular analysis of transformed plants is inconclusive at present for the mechanism of resistance.

This project offers a solution to the major problem of lack of tolerance to *P. cinnamomi* in commercial Australian avocado rootstocks. The benefits to industry include long-term cost effective, environmentally friendly solution to the root rot problem by reducing the usage of chemical control measures and reducing the implementation of various cultural practices.

Keywords

Phytophthora root rot (PRR); *P. cinnamomi*; RNA silencing; Clonal propagation; Resistance screening.

Introduction

The Australian avocado industry production has continued to increase with total Gross Value of Production in 2015/16 estimated to be about $460 million, an increase of about 30 per cent on 2014/15. According to the latest industry statistics released by Avocados Australia, domestic production doubled over the past decade and is forecast to double again, with the 100,000 tons mark expected to be reached by 2025. Australian per capita consumption reached 3.27 kg per person in 2015, ranking amongst the highest avocado consumption levels in the English-speaking world.

Factors which limit the production of avocado include diseases such as Phytophthora root rot (PRR), anthracnose and avocado sun-blotch viroid (Marais 2004; Palukaitis *et al*. 1979). Losses caused by these diseases are estimated to be worth millions of dollars and severely affect the growth, production and international trade of avocado. The average yield for avocados across Australian orchards is low at 7 t/ha. To improve to avocado yields, investment in the development of new technology must continue, particularly plant breeding, rootstock selection, orchard management, disease control, integrated pest management (IPM) and post-harvest handling.

Phytophthora root rot

PRR is the most important and significant disease affecting avocado production. The soil borne pathogen which causes this disease, *Phytophthora cinnamomi* (PC), is considered to be one of the world's worst invasive organisms. In the past PRR has destroyed over half of Australia's avocado crop and has crippled important germplasm collections globally. Today, it remains a serious impediment to on-farm productivity gains, causing >$40M per year in losses nationally despite growers spending approximately $10M per year on disease management and chemical prevention practices (www.avocado.org.au; Smith et al., 2011). As such, research towards development of durably resistant rootstock material in Australia is paramount. However, despite is devastating consequences, our understanding of this pathogenic interaction and resistance mechanisms are still indadequate.

PRR Management Strategies

Root rot destroys the tree root system which leads to the inability of the tree to adequately uptake water or nutrients from the soil. Presently root rot is a problem in all Australian orchards and has the potential of making orchards unprofitable if ignored. Successful long-term production requires land with very well-drained soils, which is currently expensive and scarce.

Despite the success of phosphate treatments, an integrated biological and chemical approach is still recommended in high risk subtropical areas of northern New South Wales and Queensland. Integrated management of avocado root rot includes planting clean avocado nursery stock, selecting low hazard

sites, planting on mounds in more hazardous sites, prevention of the introduction of *P. cinnamomi* to clean sites, using resistant rootstocks, appropriate irrigation levels, applying systemic chemicals, treating with gypsum and adding organic mulches. All these practices translate to an increase in production costs to achieve optimum yields.

Resistant rootstocks

The best method for controlling PRR is the use of resistant rootstocks. However, resistance in avocado rootstocks is difficult to find. This is because of the wide host range of *P. cinnamomi* and lack of selection pressure on avocado as the gene pool at centres of origin of avocado in Central America has not developed in the presence of *P. cinnamomi*. Currently the Australian avocado industry relies on a range of rootstocks selected by nurserymen, for which there is little evidence to substantiate their performance. Intensive research and breeding programs (e.g. by the now Department of Agriculture and Fisheries; Whiley et al 2008) has generated valuable rootstock germplasm for PRR resistance. These breeding programs are based on 'escape' trees with increased tolerance under PC pressure. However, the resistance is not absolute and the term tolerance is often used to define the ability of rootstocks to withstand infection.

In Australia, the wide-spread use of these tolerant varieties is limited by import and royalty restrictions, as well as industry-wide bottlenecks in supply due to poor clonal propagation ability. Also it seems that some scion-rootstock combinations are not as physiologically compatible as others and thus the nutrient status and vigour of the roots may be greatly affected depending on grafting success to Hass.

Importantly, despite years of breeding research, the molecular basis for increased PRR tolerance remains unknown. Only recently, advancements in molecular technologies are helping to unravel the underlying molecular responses in tolerant plants. However, to date this has centered on only one tolerant variety (Dusa®) and the results suggest that a number of different genes may be involved at different stages of infection (Mahomed and van den Berg, 2011; Reeksting et al., 2014). The fact that many genes may quantitatively contribute to natural tolerance as opposed to single, major gene contributions makes it more difficult to improve resistance in avocado through targeted breeding for natural resistance.

New technologies based on a natural process in plants called RNA silencing are now paving the way for new innovations in PRR disease control. This project was a pioneer study on RNA silencing for resistance to PRR in avocado.

RNA silencing

RNA silencing, a conserved eukaryotic surveillance mechanism, also known as post-transcriptional gene silencing (PTGS) in plants, has emerged as a potentially powerful strategy to engineer disease resistance against viruses, viroids, nematodes, insects pests and fungal infections in plants (Dietzgen & Mitter 2006,

Dang et al. 2011, Yu et al. 2012, Tamilarasan and Rajam 2013, Zhang et al. 2013).

RNA silencing is thought to play a role in the protection against invading nucleic acids in plants and considerable resources continue to be directed towards its application as a result (Bartel 2004; Baulcombe 2004; Lecellier & Voinnet 2004). Exceptional progress in dissecting the modes of action, pathways and expression of transgene encoding for double stranded (ds) RNA with respect to viral resistance has been made. A key conserved feature is that it is triggered by double-stranded RNA (dsRNA) that is processed into 21-25 nt short interfering (si)RNAs by the activity of an RNase III like enzyme called a Dicer. The siRNA is then incorporated into a RNA-induced silencing complex, so ensuring that it specifically degrades any RNA sharing sequence similarity with the inducing dsRNA. A discussion of RNA silencing mechanisms is beyond the scope of this report and readers are directed to the expert and exhaustive reviews of Baulcombe (2004), Ruiz-Ferrer & Voinnet (2009), Simon-Mateo & Garcia (2011) and Wang et al. (2012).

This technology of host delivered gene silencing (HIGS) can be exploited to control fungal diseases of economic importance. In simple terms, a host plant is typically transformed with a construct whose gene is obtained from the pest of interest which in our case is *P. cinnamomi*. When that gene is transcribed in the plant it produces dsRNA corresponding to the targeted pest gene. The plant recognizes that dsRNA as a foreign molecule and degrades it through a well-characterized process leading to formation of siRNAs. Pests feeding on the host plants ingest those siRNAs that subsequently trigger the pest to degrade the target gene within the pest. Thus, a pest gene critical to the functioning of the pest is "silenced" in the pest, rendering the plant resistant (Fig. 1).

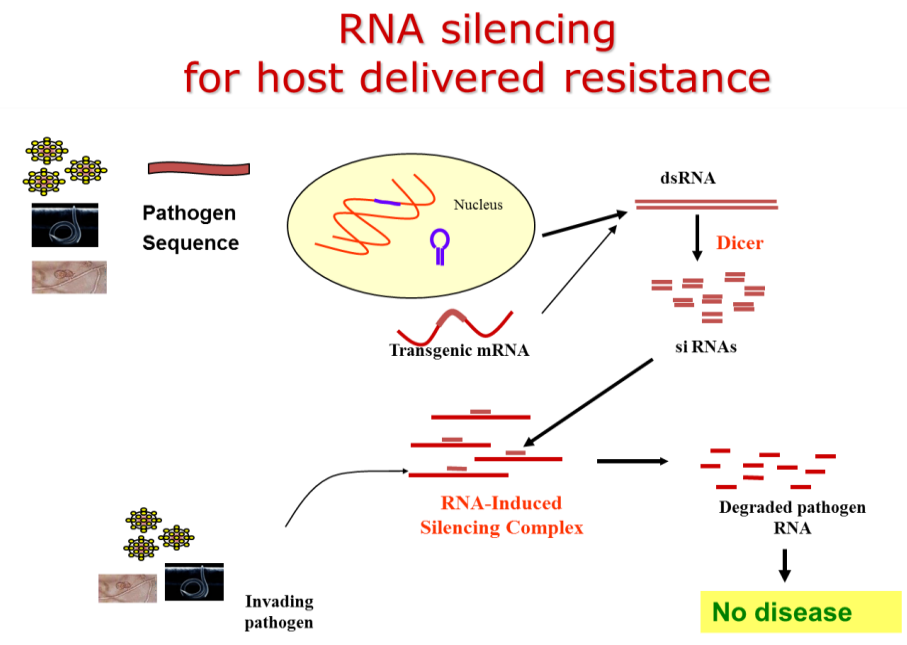

Figure 1: *Host induced gene silencing (HIGS) for resistance to pathogens*

Proof of concept for HIGS based control of fungus gene expression in plants was first provided by Tinoco et al. (2010). They showed tobacco plants carrying the beta-Glucuronidase (GUS) silencing construct were shown to negatively control GUS gene expression in the transformed GUS expressing *Fusarium verticillioides*. This study was followed by the finding that silencing of fungal effector genes in the host plant results in reduced development of the biotrophic fungal pathogen *Blumeria graminis* in wheat and barley (Nowara et al., 2010). In recent years, an increased number of studies using HIGS as a control agent of pathogenic fungus have been reported in several plant species (Table 1). However, the molecule (i.e., dsRNA, small RNA) in which HIGS is mediated in fungal disease suppression, or the pathway in which the silencing molecule is delivered from plant to fungus is yet to be elucidated.

In this project, we utilised HIGS technology as the controlling agent of PRR in avocado plants. The putative transgenic plants were clonally propagated, new independent lines using new transformation events were developed and these putative transgenic plants were screened in glasshouse for *P. cinnamomi* resistance. Furthermore, a DNA extraction method from Avocado tissues was optimised, and molecular analysis of transformed plants was conducted using various approaches, namely Polymerase chain reaction (PCR), southern blot analysis, small RNA Northern blot analysis and small RNA seq analysis.

Table 1. Summary of HIGS application studies for fungal disease control

Target fungal species	Target gene	Host plant	Effect	References
Fusarium verticillioides	*GUS* (reporter gene)	tobacco	*GUS* silencing; Proof of concept	Tinoco et al. (2010)
Blumeria graminis	*Avra10* (effector gene)	barley and wheat	Reduced fungal development (in the absense of the matching resistance gene *Mla10*)	Nowara et al. (2010)
Puccinia striiformis f. sp. tritici	*PsCNA1/PsCNB1* (Calcineurin Homologs)	wheat	Reduced the germination rates of urediospores	Zhang et al. (2012)
P. triticina, P. graminis and P. striiformis	*PtMAPK1* (MAP kinase), *PtCYC1*(cyclophilin) and *PtCNB* (calcineurin B)	wheat	Disease suppression	Panwar et al. (2013)
Fusarium graminearum	*CYP51A*, *CYP51B* and *CYP51C (cytochrome P450 lanosterol C14α-demethylase)*	*Arabidopsis thaliana* and barley	Resistance	Koch et al. (2013)
Fusarium oxysporum f. sp. *cubense*	Velvet and FTF1 (transcription factor)	banana	Resistance	Ghag et al. (2014)
Sclerotinia sclerotiorum	*CHS* (chitin synthase)	tobacco	Resistance	Andrade et al. (2015)
Bremia lactucae	*HAM34* (*Highly Abundant Message #34;* gene of unknown function) and *CES1* (cellulose synthase)	lettus	Resistance	Govindarajulu et al. (2015)
Fusarium graminearum	*CHS3b*	wheat	Resistance	Cheng et al. (2015)
Verticillium dahliae	*VdH1* (hygrophobin)	cotton	Resistance	Zhang et al. (2016)
Fusarium culmorum	Fc*Gls1 (*β-1, 3-glucan synthase*), FcFmk1 (*MAP kinase*) and FcChsV*	wheat	Abbarent hyphae formation	Chen et al. (2016)

Broad Aims for AV13000:

1. Clonally propagate identified PRR tolerant putative transgenic line/s
2. Continue to generate and clonally multiply more independent transformants.
3. Resistance screening of transformed lines in the glass house
4. Molecular analysis of transformed lines.

Methodology

I: Clonal Propagation

The Reed rootstock transformed with RNA silencing constructs targeting critical Gene A and Gene B of PC were clonally propagated via the modified Frolich-Platt grafting technique (Fig. 2) (Ernst, 1999). A nurse seedling was grown until the stem was thick enough to be grafted and the rootstocks were used to graft nurse seedlings. At bud burst (3 to 4 weeks after grafting) the plant was placed in a dark room where shoots could etiolate. When shoots had etiolated to 200 to 300 mm in length (approximately 4 weeks after bud burst) the plant was removed from the darkroom. IBA gel (Clonex® red) was applied to a small incision made at the base of the etiolated shoot, approximately 100 mm above the graft union. A pot was positioned over the etiolated shoot (one per shoot) with a suitable soil. Soil was then packed tightly around the base. The plant, of which only the upper parts of the etiolated shoot and leaves protruding from the medium filled containers, was placed under shade cloth to induce photosynthesis and progressively harden off. Once the shoots reached the desired length after bud burst and the newly developed flushes of approximately 50 mm in length appeared, the avocado plants were severed from the nurse seedling just above the nurse graft and below the pot (approximately 6 weeks after the second grafting). The newly-severed plant was transplanted to a larger pot and kept under 40% shade until it reached the desired stage (Fig. 3). All avocado plants used in this project were grown in a temperature-controlled glasshouse or in a growth chamber at light/dark = 16/8 h; T = 26/22 °C and 70% humidity.

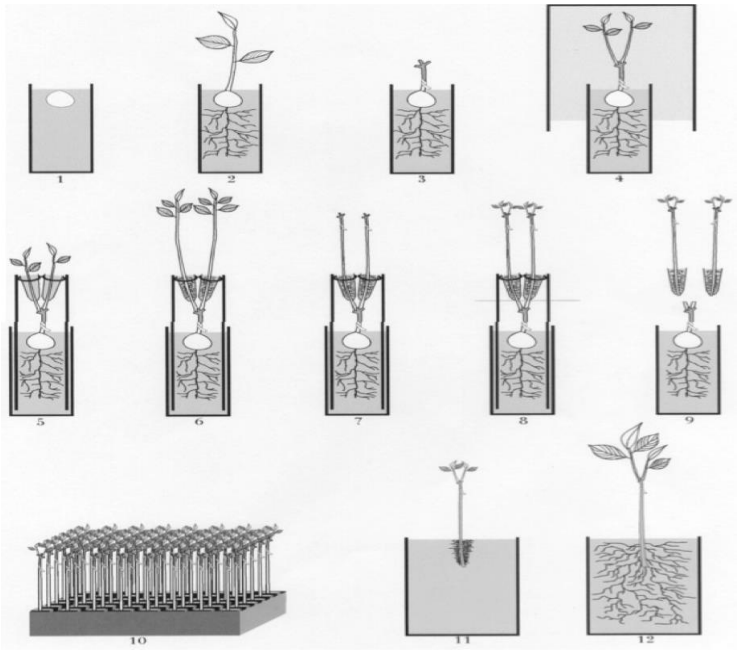

Figure 2: *Modified Frolich-Platt grafting technique, Ernst 1999.*

Figure 3: *Clonal propagation of transformed rootstocks a) Etiolated shoot, (b) Etiolated shoot after application of root hormones, (c) Plant after being severed from the nurse seedling (d) Clonal plant*

II Transformation:

Avocado transformation was done as described in project AVO8002 using *Agrobacterium tumefaciens*.

III. Resistant Screening

Phytophthora inoculum was multiplied on wheat seeds. To a 250mL sterile schott bottle, v/v 100mL wheat seeds plus 100mL water was added and the bottles were then autoclaved. 3-5mm^{2}cube of *P. cinnamomi* was added to the autoclaved wheat seeds. The bottles were then wrapped in aluminium foil and stored at 24°C for 2 weeks. We used plastic seedling trays for creating sick soil. The trays were cleaned and rinsed with 70% ethanol. Soil (UQ MIX) was added to the trays and one bottle of the *P. cinnamomi* culture was added per tray. The trays were wrapped in aluminium foil and stored at 24°C for 2 weeks.

Gene A and Gene B plants multiplied via the Frolich-Platt double grafting technique and non-transformed or wild type (WT) Reed plants from seedlings and tissue culture were screened for resistance using the double pot method (Fig 4). The plants were initially infected with *P. cinnamomi* soil. These plants were then flooded for at least 4 days continuously in trays filled with *P. cinnamomi* infected water. For the remaining 3 days the plants were removed from the trays and maintained on the bench. The plants were re-flooded similarly as mentioned above for the duration of the trial (6-8 weeks). At the end of the trial the plants were maintained in the glasshouse under normal conditions (29^{o}C ± 3^{o}C). Three large-scale experiments were conducted, in the glasshouse, of plants that were clonally derived. Our method of

inoculation allowed root growth to be monitored without disturbing roots and the counting of roots penetrating the primary pot (Fig. 4). Phenotypic observations like number of leaves, leaf characteristics, number of roots protruding through the mesh, dimension of stem at the bottom of plant and dimension of stem a the top of plant were recorded every 2 weeks for up to 8 weeks.

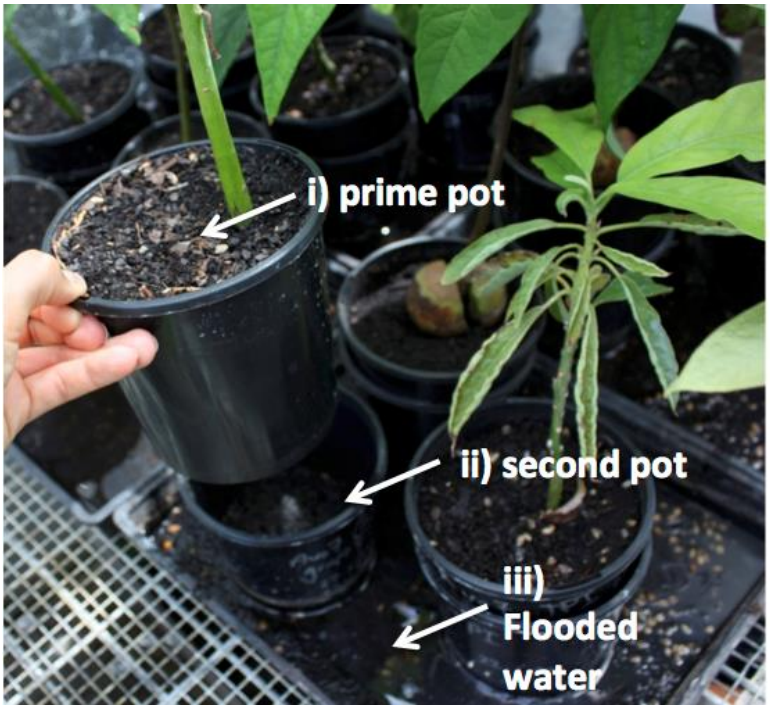

Figure 4: *Double-pot method of PC inoculation – PC resistance screening using the double-pot method for root growth and PC induction. Plants were planted into double-pots and PC inoculation was initiated in three sites i) infected soil at the base of the prime pot ii) infected soil at the base of the second pot in which the growing roots from prime pot were in contact with PC inoculated soil iii) base of the second pot in contact with infected flooded water.*

IV: Molecular analyses

The clonal plants were subjected to molecular analysis using methods such as Polymerase Chain Reaction, Southern blot analysis and Small RNA sequencing analysis.

Outputs

We have shown the proof of concept for *P. cinnamomi* resistant avocado rootstocks of cultivar 'Reed' screened in limited growth cabinet and glasshouse trials. The putatively transgenic plants generated in Phase 1 of the project were clonally multiplied (Fig 3). Clonal propagation of the putative transgenic avocado lines using the Frolich-Platt double grafting technique was successfully achieved by generating more than 10 clonal plants for each independent line. In addition, new transformation events were conducted and independent lines were generated for cultivar 'Reed". These lines were also clonally propagated for resistance screening.

The putatively transgenic plants were screened for resistance using the double pot method of inoculation. The plants were subjected to *P. cinnamomi* inoculum for 6-8 weeks. This was done by flooding the pots in *P. cinnamomi* infected water for 4 out of 7 days per week for a total period of up to 6-8 weeks. These plants were monitored for number of roots protruding through the mesh, number of leaves, dimension of stem at the bottom and top of plant and for overall PC severity.

In two independent *P. cinnamomi* screening trials, the transformed plants with Gene A showed improved resistance to *P. cinnamomi* when compared to non-transformed Reed plants (Fig 5 and Fig 6). The transformed plants maintained healthy root and leaf growth for the duration of the 6-8 week resistance screening. WT plants developed necrotic roots and brown tips on leaves after 2-weeks of *P .cinnamomi* challenge, and leaf colour changed from green to light green by the end of the 6-8 week screening period. Gene B transformed plants did not show improved resistance as compared to untransformed Reed.

A DNA extraction method from Avocado tissues was optimised, and molecular analysis of transformed plants was conducted using various approaches, namely PCR, southern blot analysis, small RNA Northern blot analysis and small RNA seq analysis. However, the results of these analyses were inconclusive indicating either loss of the genetic modification or presence of chimeras leading to lack of repeatability. More in depth investigation is warranted.

Interestingly even though we have not been able to prove the presence of genetic modification in repeat experiments, the transformed lines have performed better as compared to non-transformed lines in resistance screening experiments in the glasshouse. The selected lines continued to show improved resistance over WT non-transformed controls in resistance screening trials in the glasshouse.

Figure 5: *Images of control wild type plants and four Gene A plants after PC inoculation in the glasshouse.*

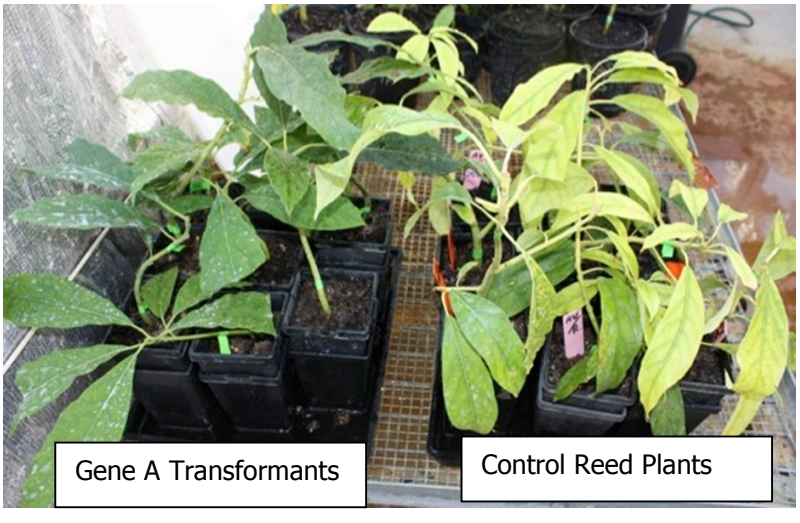

Figure 6: *Images of Gene A transformed rootstocks plants clonally propagated by double grafting method and wild-type control Reed plants that have undergone 6-weeks of PC screening.*

Outcomes

1. Successful clonal propagation of the transformed plants carrying RNA silencing constructs targeting *P. cinnamomi*.
2. New transformation events leading to generation of >10 independent putatively transgenic plants.
3. DNA extraction form Avocado leaf tissue optimised.
4. Optimised method for screening of clonally propagated plants for resistance screening in the glasshouse.
5. Resistance screening trials in the glasshouse with Gene A transformed plants showed resistance/tolerance to *P. cinnamomi* when compared to wild type non- transformed Reed plants.

Evaluation and Discussion

Since the first reports of somatic embryogenesis in avocado using immature zygotic embryos in 1981, long term maintenance and regeneration of plantlets from avocado SE's has been a major barrier in the development of new avocado varieties. During the phase 1 of this project we developed a new and efficient methodology of avocado SE regeneration to improve on existing protocols of maintenance, regeneration and plant recovery. The results from AVO8002 indicated that multiplication of SE's on MMSE significantly improved the proliferation rates over time with the cultivars tested compared to the previously published reports using MSP. A two-step regeneration system involving the transfer of SE's to liquid medium with 1 g/L glutamine for 12-15 days as a step for induction of SE germination between subcultures in solid medium, significantly enhanced shoot/plant development from SE tissue.

In AV08002 we successfully engineered dsRNA constructs to target essential genes in *P. cinnamomi* for the delivery of RNA silencing based resistance to this pathogen in avocado. Screening for silencing efficiency of essential genes was initially carried out by soaking the fungal mycelium in dsRNA and selecting for lethal phenotypes. In the previous project we also developed a system for avocado transformation using cultivar 'Reed" and have two putative avocado transgenic lines transformed with double stranded RNA constructs targeting Gene A and Gene B of *P. cinnamomi.* In the current project we progressed through the stages of multiplication. Multiplications were carried out via the commercially used Frolich-Platt double grafting technique.

P. cinnamomi is known to survive for as long as 6 years in moist soil (Zentmyer and Mircetich 1966) and it is clear that moisture is a key factor in the establishment, spread and longevity of *P. cinnamomi* diseases. A sexual sporulation requires liquid environment, both for the formation of sporangia and for the release and activity of motile zoospores. Disease development is enhanced after heavy rain and in waterlogged soils (Hardham 2005). PC screening experiments were set up by first inoculating the soil with PC inoculum. First primary pot was planted inside the secondary pot. Plants roots were allowed to grow through the cheesecloth and chicken wire into soil into the secondary pot for 5 days. All plants were placed in a container that was flooded with water-inoculated PC for 4 out of 7 days per week for a total period of up to 6-8 weeks. This method of inoculation exposed the plants to PC via three ways and ensured complete inoculation. We optimised the *P. cinnamomi* resistance screening method on Reed control plants and used the same protocol for the transformed plants as they completed the grafting process and developed suitable root systems. The avocado plants generated at various stages of growth, under went PC challenge. Every two weeks, photos and detailed plant descriptions were recorded monitoring disease progression in both putative transgenic and WT Reed plants. The putative transgenic rootstocks that have

non-transgenic scion (like Hass) grafted on to Reed were also evaluated to determine if the grafting process has any effect on the ability of the rootstock to withstand the pressures of *P. cinnamomi*.

Our first two screening experiments clearly showed that the clonally multiplied putative transgenic Gene A plants produced longer roots; roots which appeared to have an overall healthier root structure than control Reed plants. In addition, the putative transgenic plants maintained healthy root and leaf growth for the duration of the PC screening. The control Reed WT plants developed necrotic roots and brown tips on leaves after PC challenge, and leaf colour changed from green to light green by the end the PC screening.

Intriguingly, molecular analysis of the plants to confirm the presence of genetic modification were inconclusive in repeat experiments. It could be that the putative transgenic plants do not harbour or have lost the genetic modification, or the molecular methods use in this study were not sensitive enough to accurately detect the presence of genetic modification in putative transgenic plants. Use of selectable marker, such as antibiotic or herbicide resistance, in genetic transformation system is an essential part for recovering transgenic plants. However, occurrence of escapes (i.e. that is non-transformed regenerants or chimeric plants) within the selected individual are also known to be a common phenomenon (Domínguez et al., 2004; Faize et al., 2010). Chimera is referred to plants made of more than one cell line. In plant transformation, a mixture of transformed and untransformed cells can give rise to a chimeric plant. There are several reasons into which escapes and chimeras can regenerate even when the selection media is used. The most common explanations for generation of chimeras are: transient expression of genetic modification during the early stage of regeneration, and cohabiting *Agrobacterium* in the regenerated plant cells. Selection of the non-chimeric regenerants often requires multiple degree of confirmation. This includes phenotypic (visual or physical) screening with the use of reporter gene (e.g., GUS, Green Fluorescent protein (GFP)), and continuous PCR confirmation for the presence of RNA-seq (Birch 1997; Faize et al., 2010).

If the original putative transformants Gene A and Gene B were chimeric, composed of transformed and untransformed cells, we could have unknowingly manipulated and even diluted the ratio of the transformed to the untransformed cells in the original transformant and its clones overtime by simply growing the plants and making a clonal from the chimeric plant. This can explain the results from the molecular analysis conducted for this report, and the result of the PCR confirmation, which was initially positive for the presence of genetic modification (in project AVO8002).

DNA methylation in eukaryotes plays an important role in maintaining stability and integrity of the genome,

as well as epigenetically regulating developmental processes in an organism. RNA-dependent DNA methylation (RdDM) is a small RNA mediated epigenetic pathway in plants, and is known to primarily target transposons and repetitive sequence (Law and Jacobsen 2010, Eun et al 2012). This can result in direct silencing or repressing the activity of transposable elements, or it can alter the activity of the nearby genes (Eun et al 2012; Matzke and Mosher 2014). To better understand the source of resistant phenotype in the putative transformants that don't seem to harbour or express the genetic modification, sequence data was further investigated for signs of altered methylation status of genome by using the abundance of small RNAs (24nt in particular) as a proxy. Although this idea cannot be ignored, with the current method used and samples tested, we could not identify any clear changes in small RNA abundance methylation status of putative transgenic Gene A plant genome.

In conclusion, although these plants seemed to have maintained resistance towards PPR, we cannot establish the explanation behind this phenotype on the basis of the results from the molecular analysis acquired to current date. Further studies are required to better understand this phenomenon.

The commercial cultivation of GM fruit tree are rare and limited to GM poplar with insect resistance grown in China and GM papaya with virus resistance grown in USA and China. New biotechnological approaches like trans-grafting that involves grafting non – GM scion on a GM rootstock are raising questions about the status of fruit obtained from such a chimeric plant. Whether non-modified upper stem grafted on GM rootstock and their products must be subject to the GMO legislation is principally a legal and political question in addition to the scientific proof for absence of transgene in the scion. In the case of GM rootstock grafting, especially if we are targeting a root pathogen using RNA silencing technology without any effect on endogenous genes or attempt to alter any traits in the scion, the fruit will not contain any novel genetic material and therefore should not be subject to GM labelling. We need to find out if the distinction between a product carrying genetic modification and a product without any modification but resulting from the use of GM techniques is important to the consumer? The dividing line between what is a GMO and what is not is becoming increasingly more intertwined. The development of new techniques demands greater clarity and perhaps also new interpretations of the current legislation and regulations regarding GMOs.

Recommendations

It would be desirable to screen these plants in Phytophthora sick plot to confirm resistant status. However, this will require exemption from OGTR. Though, all our results to date indicate that the genetic modification may not be present, we are not sure if OGTR will give an exemption as the plants have gone through a transformation event and may require more in depth investigation. It is envisaged that acceptance of such a technology will need to go through a rigorous regulatory and acceptance pathway.

It would be fruitful to try innovative topical application of RNA silencing targeting *P.cinnamomi* as opposed to generating transgenic plants. We have developed a patent protected 'BioClay' technology to deliver RNA silencing molecules loaded on clay nanoparticles. This has been shown to be effective against protection form viruses. It will be interesting to investigate if this technology for fungal diseases such as PRR or anthracnose.

Scientific Refereed Publications

The results obtained in this project have not been published due to the work being commercial-in-confidence.

1. **N .Mitter (2016)** Keynote guest speaker at AVOCO | NZ & Australian Avocado Growers' Conference, June 2106, Auckland, New Zealand (fully funded by AVOCO).
2. **N .Mitter (2016)** Keynote guest speaker at Tasmanian Division of the Australasian Plant Pathology Society, Seminar day, April 2016, Hobart, Tasmania (Fully funded by APPS).
3. **N Mitter** (2016) Plenary speaker at 6th International Conference on "Plant, Pathogens and People" with mission "Challenges in Plant Pathology to benefit humankind" to be held from February 23-27, 2016, New Delhi, India
4. **N. Mitter** (2015) Keynote presentation 'Harnessing the power of RNA interference for crop protection' at 27th Annual Meeting of the Thai Society for Biotechnology and International Conference (TSB 2015), 17 -20 November 2015, Bangkok, Thailand.
5. **N Mitter** (2015) Invited speaker at International Avocado Brainstorming Meeting as key representative from Australia, September 2015, Peru.
6. **N Mitter** (2015) Oral presentation 'Cryopreservation of avocado germplasm' World Avocado Congress, 13-18 September 2015, Peru.
7. **N Mitter** (2015) Oral presentation 'Effective host delivered silencing against '*Phytophthora cinnamomi*', World Avocado Congress, 13-18 September 2015, Peru.
8. **N Mitter (2015)** Invited panel member in Agriculture Innovation Showcase organised by Life Sciences Queensland, May 22, 2015, Brisbane, Australia.
9. **N Mitter** (2014) Convenor and speaker, Mango outlook India, Genomics, Physiology, Disease resistance and possibilities, Australia India Horticulture Linkage Workshop, August 17-18, 2014, Brisbane, Australia.
10. **N Mitter** (2014) Invited presentation at OECD session on GM crops, International Horticulture Conference, 17-22 August, Brisbane.
11. **Mitter N (2014)** Key Note invited speaker on 'My affair with avocado- healthy, productive and conserved, AVOCO conference, 5 July 2014, New Zealand.
12. **Mitter N et al** (2014) Phytophthora resistant GM rootstocks for non-GM scion, International Horticulture Conference, 17-22 August, Brisbane.
13. **Mitter N** (2014) Phytophthora resistant avocado rootstocks, Avocado industry meeting, Brisbane, June 19, 2014.
14. **Mitter N** (2013), RNAi for crop protection, *In* : India-Australia Collaborative Meeting to develop collaboration in Biofuels and RNAi, New Delhi, India.

15. **Mitter N (**2012), Innovative platform technologies for disease resistance, *In:* Macadamia Industry Board meeting, Ecosciences precinct, Brisbane, Australia.
16. **Mitter N** (2012), Genetically modified Rootstocks for non-genetically modified fruit, *In:* Gene Technology Workshop, Horticulture Australia Ltd, across Industry Funded Initiative, Canberra, Australia.
17. **Mitter N** (2012)**,** Developing Phytophthora resistant rootstocks using RNA silencing (key note) *In:* Team Avocado Conference, New Zealand.
18. **Mitter N** (2012)**,** New strategies to manage plant diseases, *In:* QAAFI Annual Research Meeting, Gold Coast, Australia.
19. **Mitter N** (2012), Transformation of avocado (key note), *In:* Avocado Genetics and Plant Improvement meeting, California, USA.
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Intellectual Property/Commercialisation

The project outputs are commercial in confidence. Background IP from AVO8002 as per the HIAL agreement.

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