Microrna regulation of avocado adventitious rooting for clonal rootstock propagation

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Persea americana, common name avocado, contributes around $300 million at the farm gate and $540 million at retail to the Australian economy. Avocado is a woody tree species that is commercially grafted for obtainment of both desirable fruiting and rootstock resilience qualities. Clonal propagation of rootstocks has been reported by industry to yield three times higher than sexually reproduced however, the difficulty of adventitious root generation from avocado stem cuttings makes propagation a significant challenge. The Frolich and Platt double grafting protocol (Frolich and Platt, 1965 and derivatives thereof e.g Brokaw 1977 patent #4012866, Ernst 1999) is the standard practice for rootstock propagation and whilst industry reports an un-interrupted etiolation step is crucial for rooting phenotypes, it is not yet understood why this is so. To address this issue, we looked at miR160, a proven adventitious rooting promoter in *Arabidopsis thaliana*, expression in avocado scions grafted for root clonal propagation. Previous work has determined that etiolation supports a spatiotemporal accumulation of miR160 in basal tissue compared to apical, a trend that was not identified in non-etiolated equivalent scions. As this initial experiment only sampled tissue at the completion of the etiolation step, a time course assay sampling throughout the etiolation and de-etiolation steps has been performed to determine when key molecular changes are occurring, the results for which will be discussed. Understanding the molecular regulation of adventitious rooting in avocado will allow informed and calculated decisions for improving clonal rootstock propagation and may lead to the enhanced rooting efficiency for farmers in the future.

Key Words: Clonal rootstocks, Adventitious roots, MiR160, *ARF17*, Etiolation, De-etiolation, Auxin.

INTRODUCTION

Avocado is an open pollinating crop and thus offspring are often highly heterozygous for commercially valuable characteristics (Alcaraz and Hormaza 2009). Clonal propagation is therefore the preferred method of plant multiplication, as it allows complete preservation of desirable parental characteristics (Noguera et al. 2011); however, clonal rootstock propagation is a significant challenge. Avocado is highly recalcitrant to the production of adventitious roots, which are roots generated from non-root tissues such as stems. As such, industry relies on the Frolich and Platt double grafting protocol ((Ernst 1999; Frolich and Platt 1972), a protocol that has remained largely unchanged since the 1970's. Briefly, mature budwood from the rootstock to be clonally propagated is grafted to a young seedling which serves as a nurse plant. After allowing the graft to heal, the grafted plant is etiolated in complete and un-interrupted darkness for two weeks. Upon de-etiolation, the basal section of the etiolated budwood is wounded and exogenously treated with auxin prior to pot and soil placement. Once the budwood has produced roots, the fruiting scion can be grafted and the plant, supported by its newly formed roots, can be removed from the nurse plant. Due to its high time and labour inputs, trees supported by clonal rootstocks are nearly twice as expensive as seedling rootstock equivalents. Consequently the majority of the Australian avocado industry is based on seedling rootstocks, despite clonal rootstocks reporting up to three times higher yields (Anderson 2012; Smith et al. 2011).

Perhaps the biggest limitation towards improving the efficiency of the current clonal rootstock propagation protocol is a lack of understanding of adventitious root initiation and growth at the molecular level. In the pursuit of understanding this process, microRNAs make excellent candidates of study. MicroRNAs (miRNAs) are 20-25 nt single stranded RNA molecules that negatively regulate endogenous transcript abundance in a highly specific manner (Bartel 2004; Baulcombe 2004; Gleeson *et al.* 2014; Mallory and Vaucheret 2006). Generated from self-complementary RNA transcripts that fold into double-stranded RNA hairpins, miRNAs are processed into 21 nucleotide duplexes by Dicer-Like proteins (Kurihara and Watanabe 2004; Voinnet 2009). A single strand from the duplex is incorporated into the RNA-Induced Silencing Complex (RISC), and guides this protein complex to complimentary messenger RNA molecules in the cell (Bartel 2004). If complementarity is achieved between the mRNA transcript and miRNA, the RISC either transcriptionally or translationally inhibits the complement preventing the synthesis of the encoded protein and ultimately its cellular function (Rajagopalan *et al.* 2006; Voinnet 2009). MiRNAs commonly target transcription factors, making them central to many developmental regulatory pathways, including rooting. Furthermore, they are highly conserved across the plant kingdom, with the mechanisms elucidated in model systems for adventitious rooting likely to be conserved in avocado (Gleeson *et al.* 2014; Gutierrez *et al.* 2009; Rhoades *et al.* 2002).

In *Arabidopsis*, miR160 is a positive regulator of adventitious rooting whereas miR167 is a negative regulator (Gutierrez *et al.* 2009). MiR160 and miR167 regulate the transcript abundance of *Auxin Response Factors* (ARFs) (Bellini *et al.* 2014; Gutierrez *et al.* 2009; Mallory *et al.* 2005). ARFs are a family of transcription factors that regulate the expression of auxin responsive genes (Guilfoyle and Hagen 2007; Tiwari *et al.* 2003). Auxin, a plant hormone shown to promote the initiation of adventitious roots in a number of woody tree species including avocado, controls activity of ARFs though Aux/IAA protein interactions (García-Gómez *et al.* 1994; Guilfoyle and Hagen 2007). Under low auxin conditions, Aux/IAA proteins bind to ARFs to inhibit their transcription factor function. However, when the auxin content of the cell is raised, there is an increased affinity between the Aux/IAA proteins and the TIR1 receptor of the SCF complex, leading to ubiquitination and thus degradation of the Aux/IAA proteins. This allows the ARFs to homo/heterodimerise and thus exert their promotive or suppressive function over auxin responsive genes (Vernoux *et al.* 2011). *Arabidopsis* over-expressing and knockout lines have shown that ARF6 and ARF8, which are regulated by miR167, promote adventitious rooting and *ARF17*, which is regulated by miR160, suppresses adventitious rooting (Gutierrez *et al.* 2009; Rhoades *et al.* 2002).

This study aimed to profile the expression of miR160 and *ARF17* in avocado rootstock cuttings grafted to nurse plants for the clonal propagation. Plants were harvested throughout the Frolich and Platt protocol with the goal of identifying when key molecular changes were occurring. Furthermore, upon de-etiolation plants were divided into either auxin or non-auxin treatment groups, as avocado will not produce adventitious roots without the addition of exogenous auxin (Haissig and Davis 1994). By using two treatment groups that produce opposite adventitious rooting phenotypes, we aimed to correlate molecular profiles with rooting and identify when key changes occur.

METHODS

'Reed' budwood cuttings were slot and peg grafted to 3 month old 'Reed' seedlings. The graft union was allowed to heal under ambient temperature, humidity and shaded light for three weeks during January at Andersons HorticulturePTY LTD, Duranbah in New South Wales, Australia. Grafted plants were then etiolated in complete darkness at 25 ˚C for 14 days with a subset harvested at day 13. Upon de-etiolation, remaining plants were lightly scrapped on the basal section of etiolated tissue and treated +/- auxin (CLONEX® by Growth TechnologyLTD, Somerset, England) prior to secondary pot and soil placement. A subset of plants were sampled from each treatment group at 3 days post de-etiolation and remaining plants were assessed for adventitious rooting phenotype 6 weeks post de-etiolation.

For each harvest, the apical and basal 2 cm of each etiolated scion was collected in liquid N2; basal tissue corresponded to the site of wounding and +/-auxin treatment for root induction. Tissues were pooled in three groups of three individuals (3 biological replicates with 9 individuals per treatment group). Harvested tissues were stored at -80 ˚C.

Total RNA was extracted from samples, ground under liquid nitrogen to a fine powder, using the Plant RNA Purification Kit® by MasterPureTM with DNase treatment (Epicentre by Illumina, Maddison, WI, USA). RNA was transcribed into miRNA-cDNA using the Plant miScript Kit (Qiagen, Limberg, Netherlands), as per manufacturer's instructions. Expression of miR160 was determined using qRT-PCR in 20 μL reactions (1x QuantiTect SYBR green mastermix, 0.8 μM forward primer, 1x miScript universal reverse primer, 2 μL template) in technical duplicate. Thermal cycling was 95 ˚C for 15 minutes followed by 40 cycles of 94 ˚C for 30 seconds, 55 ˚C for 30 seconds, and 70 ˚C for 30 seconds. Forward primer sequences were as per Table 1 and snoR41Y and snoR66 were used as housekeeping genes. For *ARF17* expression, high molecular weight RNA was transcribed into cDNA using Invitrogen's SuperScript® III First-Strand Synthesis for RT-PCR kit (Life Technologies, Carlsbad, MA, USA), as per manufacturer's instructions. *ARF17* relative expression was determined using qRT-PCR in 20 μL reactions (1x QuantiTect SYBR green mastermix, 0.5 μM forward primer, 0.5μM reverse primer, XX ng template) in technical duplicate. Thermal settings were 95 ˚C for 15 minutes and 40 cycles of 95 ˚C for 10 seconds and 55 ˚C for 20 seconds. ACTIN and GAPDH were assayed as housekeeping genes (all primer sequences Table 1). Melt curve analysis was included for all products in all runs.

Table 1: Sequences of primers used for determining relative expression of miR160 and RF17

Gene	Forward (5'-3')	Reverse (5'-3')
snoR41Y	ATACCTCCTACTCATTCTGAGTG	miScript Universal Reverse
snoR66	GCTATAAGCATTTTTACTCTGAGGAT	miScript Universal Reverse
miR160	TGCCTGGCTCCCTGTATGCCA	miScript Universal Reverse
Actin	GCTGCTTCTGCTCTCTCTCTC	CAGCCTTAACCATTCCAGTT
GAPDH	TGGGAAACTTACAGGAATGG	GTCACCCACAAAGTCAGTAGAA
ARF17	TGATTGGGAACATGGATCCCTCG	AGAGTACAGCAACTGATGGTCG

qRT-PCR Clipped data was first passed through LinReg software (University of Amsterdam, The Netherlands) to determine reaction efficiency; reactions with R^2 values less than 0.995 were excluded from analysis. LinReg PCR efficiency and CT values were averaged for technical replicates and relative expression (RE) calculated for each biological replicate set (n=3) with $RE=PE_{GOI}\hat{}(-Ct_{GOI})/PE_{HK}\hat{}(-Ct_{HK})$ where PE is primer efficiency, Ct is cycle threshold, GOI is gene of interest and HK is mean value for the multiple housekeeping genes. Statistical significance was determined using one-way ANOVA with Bonferroni-Holm post-hoc test in Microsoft Excel 2010 (Microsoft Corporation, USA) with Daniel's XL Toolbox addin for Excel, version 6.60 (Daniel Kraus, Würzburg, Germany). Mean relative expression values were plotted in Excel ± standard error.

RESULTS

The molecular signalling behind adventitious root production in avocado scions grafted to nurse plants for clonal propagation of rootstocks remains poorly understood. MicroRNAs, specifically miR160 and miR167, have shown to be influential factors in Arabidopsis adventitious rooting through their regulation of ARF 17 and ARF6/8 respectively. To determine if molecular factors known to control adventitious root production in model species play a similar role in avocado, miR160 and ARF17 expression were profiled in avocado scions throughout the clonal rootstock propagation protocol.

It was found that grafted plants etiolated for 13 days had relatively low miR160 expression with no spatial difference between basal and apical tissues of the grafted scion (Figure 1a). However, upon de-etiolation, a significant increase (p=0.0007) in miR160 expression was noted in basal tissues treated with auxin. This increase was not seen in non-auxin treated basal tissues. Furthermore, miR160 was found to be twice as abundant in basal tissue than apical in these auxin-treated scions (statistically significant difference, p=0.0033). No statistically significant differences could be detected between auxin-treated basal tissue and non-auxin-treated apical tissue, or, between auxin treated apical tissue and non-auxin-treated basal tissue. Thus, the maximum level of miR160 expression was similar between treatment groups, however, the spatial accumulation was redefined.

Analysis of *ARF17* expression showed inverse expression patterns when compared to miR160, as expected for a miR-target interaction (Figure 1b). Scions of grafts etiolated for 13 days had relatively high *ARF17* accumulation in both apical and basal tissues, correlating to when miR160 was lowest. As seen with miR160, a significant spatial difference in the accumulation of *ARF17* in auxin-treated, de-etiolated scions was seen, with 2.7 fold lower expression in basal tissue than apical (p=0.0117). This spatial downregulation of *ARF17* in basal tissues was found to be an auxin-treated specific trend, with no significant difference between basal and apical tissues in the non-auxin treated scions (p=0.305, Figure 1b). Furthermore, this same level of *ARF17* in both apical and basal tissues of non-auxin treated scions was as high as in the apical tissue of auxin treated scions.

Phenotyping revealed root production in the basal portion of 89% of auxin-treated, de-etiolated scions, as opposed to 0% rooting by non-auxin-treated de-etiolated scions (Figure 2b). This correlates to the spatially-specific accumulation of miR160 in auxin-treated basal tissue, and the corresponding decrease in *ARF17* accumulation, as exemplified by Figure 2a.
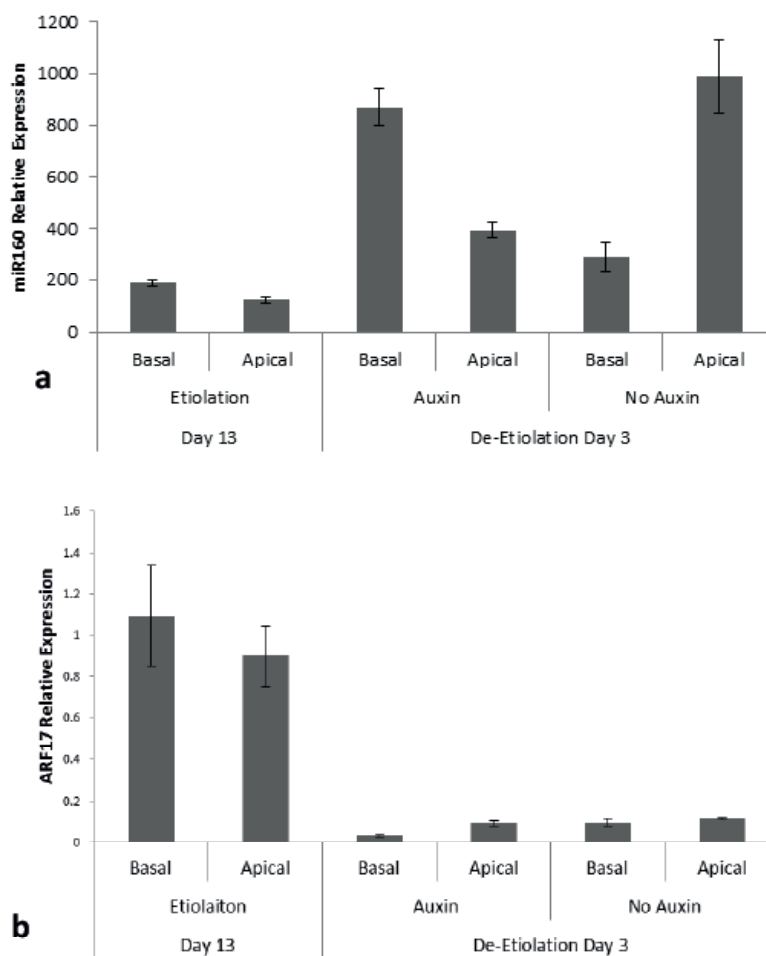

Figure 1: Relative expression of a) miR160 and b) ARF17 in basal and apical tissues of avocado 'Reed' scions grafted for clonal rootstock propagation. Samples were harvested at 13 days into etiolation and 3 days into de-etiolation, where de-etiolated plants were divided into auxin or minus auxin treatment groups. Bars represent mean expression values (n=3, samples harvested in three pools of three) ± standard error.

Figure 2: Phenotype of a) auxin and b) minus auxin treated scions grafted for clonal rootstock propagation. Phenotyping was performed 6 weeks post auxin treatments and de-etiolation.

DISCUSSION AND CONCLUSION

The protocol used by industry for the clonal propagation of avocado rootstocks has remained largely unchanged since the 1970's. Furthermore, many improvements to date have been through trial and error. By improving our understanding of the molecular events underpinning adventitious root production, more informed decisions can be made to improve the efficiency of the existing protocol. MicroRNAs make excellent candidates of study for this purpose as they are often core regulators, are highly conserved across the plant kingdom and have previously been identified as important regulators of adventitious rooting in model species (Gleeson *et al.* 2014; Gutierrez *et al.* 2009; Rhoades *et al.* 2002). In this study, avocado scions grafted to nurse plants for the clonal propagation of rootstocks were analysed throughout the existing propagation protocol to identify key molecular changes associated with adventitious rooting.

It was found that miR160 is more abundant in the basal than apical tissues of auxin treated scions three days post auxin treatment and de-etiolation. As miR160 is a positive regulator of adventitious rooting in *Arabidopsis* studies (Bellini *et al.* 2014; Gutierrez *et al.* 2009; Gutierrez *et al.* 2012), accumulation at the site of auxin-treatment and adventitious root production in avocado is promising evidence for conservation of this process in avocado. Furthermore, this may be mediated in part by regulation of *ARF17*, with an inverse correlative relationship noted between miR160 and *ARF17*. A role for *ARF17* in adventitious rooting has been established in *Arabidopsis*, with *ARF17*-overexpression lines showing reduced AR production. Taken together, the site-specific increase in miR160 and decrease in *ARF17* expression in the basal stem of avocado cuttings induced to root supports a mechanistic relationship between these genes in avocado adventitious rooting.

Interestingly, the correlative trends identified between miR160 and *ARF17* expression and rooting were found to be auxin-treatment specific. In the absence of auxin, miR160 accumulated in apical tissue but not basal tissues of scions 3-days post-etiolation; opposite to the basal accumulation observed in auxin treated scions. Furthermore, this apical accumulation of miR160 did not coincide with a decrease in *ARF17* in the apical tissue, or an adventitious rooting phenotype. In the absence of auxin therefore, it is suggested that other *ARF17* regulatory factors are more influential than miR160, preventing detection of this regulator-target correlative relationship. In all, it appears that a functional interaction between miR160 and *ARF17*, and any other molecular signals, in avocado adventitious root initiation and emergence, is dependent upon exogenous applications of auxin.

Evidence from this work suggests that auxin application to avocado scions prior to de-etiolation, as per the industry practice, is required for adventitious rooting and an associated accumulation of miR160 and reduction in *ARF17* at the site of root production. Whilst there are undoubtedly numerous other molecular factors influencing adventitious root production in avocado, miR160 and *ARF17* may be crucial first steps in unravelling these complex signalling pathways. A better understanding of the molecular pathways behind adventitious rooting will result in more efficient and strategic improvements to the existing protocol used by industry to clonally propagate avocado rootstocks.

ACKNOWLEDGEMENTS

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