Whole-tree and tension wood-associated expression profiles of microRNAs in *Eucalyptus* trees

by

Grant R. McNair

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In the Faculty of Natural and Agricultural Sciences

Department of Genetics

University of Pretoria

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Under the supervision of Prof Alexander A. Myburg

Co-supervisor Prof Jacques Theron

DECLARATION

I, the undersigned, hereby declare that this dissertation submitted herewith for the degree M.Sc to the University of Pretoria, contains my own independent work and has not be submitted for any degree at any other university.

Grant McNair

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DISSERTATION SUMMARY

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Grant McNair

Supervised by **Prof Alexander A. Myburg** and **Prof Jacques Theron**

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Summary

Trees are large, biologically complex multi-cellular organisms that have adapted to terrestrial growth. This places specific demands on their physiology such as the ability to transport water over long distances and the ability to withstand extreme mechanical forces. Wood formation (xylogenesis) is the development of the secondary vascular system within trees, which mainly addresses these two physiological needs. Xylogenesis is a highly ordered developmental process, consisting of a number of overlapping yet distinct developmental phases. These phases are strictly regulated through a combination of biochemical signalling networks and gene expression regulation.

microRNAs (miRNAs) are endogenous non-coding, small (~22 nt) RNAs that function predominantly as negative regulators of gene expression at the post-transcriptional level. They

have been implicated in the regulation of plant developmental processes, including determining cell fate in the apical meristem and timing of developmental events such as flowering and leaf morphogenesis. miRNAs have recently been found to have regulatory roles in plants placed under various conditions of abiotic stress such as drought, mechanical stress, cold and high levels of salinity.

Trees placed under mechanical stress produce a specialised form of wood called reaction wood. Reaction wood is referred to as tension wood in angiosperms, as it forms on the outside of a bent trunk or branch in order to correct for the non-vertical growth. The formation of tension wood requires extensive reprogramming of wood development processes. This makes tension wood induction an ideal tool to study and refine our understanding of wood development. Recently, miRNA regulation has been implicated in the control of normal and tension wood formation in trees, but the full extent to which miRNAs are involved in tension wood induction is not known.

To identify miRNAs potentially involved in the regulation of normal and tension wood development in fast-growing *Eucalyptus* plantation trees, real-time quantitative polymerase chain reaction (RT-qPCR) and Northern blot analyses were performed for a number of conserved and putatively novel *Eucalyptus* miRNAs. A total of 12 miRNAs representing 12 distinct miRNA families were profiled, including three novel miRNAs that are putatively specific to *Eucalyptus* trees. To more fully understand miRNA function in terms of tree development, the abundance profiles of the selected miRNAs were first determined at the whole-tree level. Of the conserved miRNAs profiled, five (miR160, miR166, miR167, miR172, miR408) were found to have abundance profiles consistent with their predicted roles in plant development. At the whole-tree level, miR90, putatively novel to *Eucalyptus,* was predominately expressed in the mature leaves and flowers. miR90 may target a MADS-box transcription factor which is not required for mature leaf and flower growth. miR408, a regulator of the expression of a plastocyanin gene involved in

lignin polymerisation, was expressed at low levels in the immature and mature xylem, where cell lignification is most prominent. In the tension wood, miR166, a known regulator of wood development and miR408 displayed similar increasing abundance over time in tension wood xylem. These profiles support their potential role in wood development. miR160 and miR167, which target auxin response factors responded early to bending stress, with their abundance reaching maximum levels six hrs post-induction before decreasing again. This is consistent with the observed role of auxin response factors as a mechanism to rapidly respond to stimuli, such as bending.

The miRNAs abundance profiles generated in this study suggest that some miRNAs do indeed play a role in normal and tension wood development, though not necessarily directly. These results provide further insights into the complex nature of miRNA regulation and their hypothesised roles in wood development. The miRNAs highlighted herein are strong candidates for further functional studies as their abundance profiles and predicted targets are consistent with roles in wood development.

PREFACE

Plants are found in aquatic and terrestrial environments, each environment placing specific demands on plant physiology for plant survival. In terrestrial environments, such physiological developments essential for plant survival are maintained e.g. vertical growth and transport of water and nutrients throughout the plant. This is most clearly visible in trees, which can attain heights in excess of 100 m. The necessity for vertical growth led to the evolution of unique developmental biology i.e. wood formation (xylogenesis). The primary vasculature of a tree is derived from the procambium that, in turn, gives rise to the vascular cambium. Bi-directional differentiation of the vascular cambium results in the development of the phloem and xylem, with the xylem forming the wood of the tree. This developmental process has sequential steps that are strictly co-regulated, as well as independently regulated. The coordination of the strict regulation of xylogenesis is achieved through differential gene expression and numerous signalling molecules of which some have been identified. The exact mechanisms that control xylogenesis, particularly the genetic component, are yet to be fully resolved or defined.

microRNAs (miRNAs) are a class on endogenous non-coding, small RNAs ~ 22 nt in length. They function as negative regulators of gene expression at a post-transcriptional level in a timing-and tissue-specific manner. miRNAs function in the majority of plant developmental processes, including wood formation, where they regulate meristem cell differentiation and subsequent tissue patterning. The increasing number of miRNAs being identified in plants has resulted in a backlog in the more tedious process of their characterisation. Of those characterised, it has become evident that miRNAs respond to various biotic and abiotic stresses. Mechanical stress (bending) of trees results in the development of a unique form of wood, termed reaction wood. The reaction wood formed in angiosperms is referred to as tension wood, as it develops on

the upper side of a bent trunk and attempts to "pull" the tree upright. The development of tension wood requires the reprogramming of wood development and formation. Therefore, tension wood is an excellent means of studying wood formation, particularly the genetic regulatory components, in this case miRNAs. In order to develop a more complete understanding of miRNA function in wood formation, one should also take into account the complexities of plant development as a whole.

The **aim of this M.Sc** study is the determination of miRNA abundance patterns at the whole tree levels of a *Eucalyptus grandis* x *Eucalyptus urophylla* (GU) hybrid paying particular attention to the xylogenic tissues in order to identify miRNAs potentially involved in wood development. To further refine the roles of these miRNAs in wood formation their abundance patterns were investigated in response to mechanical stress.

Chapter 1 of this dissertation is a review of the literature published to date regarding plant miRNAs with a focus on miRNAs with the potential for involvement in wood development and formation. To establish an understanding of miRNA function, their biogenesis and evolution are discussed. This is followed by reviewing of miRNAs with potential for involvement in the regulation of wood development. These miRNAs are divided into two groups i.e. regulators of transcription factors and miRNAs responding to stress or external stimuli. These two groups are discussed in some detail for the benefit of the discussion in Chapter 2. This review further describes in brief wood development, with special attention to xylogenesis and tension wood formation.

The complexities of wood formation place a premium on the correct regulation of this developmental process. Correct regulation of wood formation requires cross-talk between and within signalling networks and pathways, and the integration of both genetic and external signalling components of gene regulation. In *Populus,* two recent studies identified a number

miRNAs with the potential to be regulators of secondary growth, specifically xylogenesis. **Chapter 2** of this dissertation describes the comprehensive characterisation of miRNA abundance patterns at the whole-tree level of a field-grown clonal GU hybrid. To further characterise miRNAs with regard to their potential as regulators of wood development, miRNA abundance profiles in the xylogenic tissues of clonally propagated GU trees placed under mechanical stress in a time-course experiment for the period of one month were determined.

The findings presented in this dissertation represent the outcomes of a study undertaken from March 2006 to July 2009 in the Department of Genetics, University of Pretoria, under the supervision of Prof A.A. Myburg. Chapter 2 has been prepared in manuscript format for submission to a peer-reviewed research journal, as is the standard procedure in our research group. Therefore, a certain degree of redundancy may exist between the introductory section of Chapter 1 and Chapter 2. The following posters were generated based on preliminary results obtained in this M.Sc study:

McNair G.R., Victor M., Myburg A.A. 2007. Expression profiling of miRNAs in *Eucalyptus* trees under normal and tension wood formation. IUFRO Tree Biotechnology, Azores, Portugal, 3 - 8 June 2007 (Poster).

McNair G.R., Victor M., Myburg A.A. 2007. Expression profiling of miRNAs in *Eucalyptus* trees under normal and tension wood formation. IUFRO Improvement and Culture of Eucalypts 2007, Durban, 22 – 26 October 2007 (Poster).

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LIST OF ABBREVIATIONS

ARF	Auxin Response Factor
BDS	Bi-directional Developmental Series
GA	Gibberrelin
miRNA	microRNA
miRNA*	Small RNA derived from the opposite stem-loop arm
OP	Opposite Wood
OPX	Opposite Wood Xylem
OPP	Opposite Wood Phloem
PAPS	3'-phosphoadenosine 5'-phosphosulphate sulfotransferase
RISC	RNA Induced Silencing Complex
rRNA	Ribosomal RNA
RT-qPCR	Reverse Transcriptase quantitative PCR
RT-PCR	Real Time PCR
siRNA	Small interfering RNA
TW	Tension Wood
TWX	Tension Wood Xylem
TWP	Tension Wood Phloem

Chapter 1

LITERATURE REVIEW

microRNA biogenesis, evolution and function

1.1 Introduction

The discovery that small, non-coding RNAs of 21-24 nt in length, termed small interfering RNAs (siRNAs), play important roles in gene regulation has led to the re-evaluation of the long-accepted central dogma that dictated the flow of genetic information from DNA to RNA to protein. siRNAs were discovered by chance during attempts to engineer petunia flowers that had greater purple colouring (Napoli *et al*. 1990). In this study the coding region of *ChsA*, the gene responsible for purple flower colour, was placed under the control of the 35S promoter. However, over-expression of the *ChsA* gene resulted in flowers that were varigenated or had white sectors (Agrawal *et al*. 2003). This silencing effect was termed post-transcriptional gene silencing (PTGS) and this phenomenon was later also observed in the kingdom Fungi and Animalia (Agrawal *et al*. 2003; Caplen *et al*. 2001; Cogoni *et al*. 1994). Subsequently, a further group of 21-24 nt small, non-coding RNAs were identified. These were termed microRNAs (miRNA) and were found to have several similarities and differences when compared with siRNAs (Agrawal *et al*. 2003). The most notable difference was that siRNAs regulate the same genes as from which they are derived, while miRNAs regulate genes other than those from which they were encoded. The first miRNAs were discovered in the nematode *Caenorhabditis elegans* (Lee *et al*. 1993). It was found that *lin-4* mutants showed disruption of temporal regulation of *C.elegans* larval development. The *Lin-4* gene was found to encode a 22 nt non-coding RNA. This RNA showed complementarity to several conserved sites in the 3` untranslated region (UTR) of the *Lin-14* gene (He and Zhang 2004). Further studies showed that Lin-4 was responsible for the down-regulation of *Lin-14* and early entrance into the second larval stage. Thus, the miRNA *Lin-4* negatively regulates the expression of the *Lin-14* gene (He and Zhang 2004).

miRNA have since been defined as endogenous ~ 22 nt RNAs that play important regulatory roles in plants, as well as other organisms by targeting mRNAs for cleavage or translational repression (Bartel 2004). The endogenous miRNA precursors form hairpin structures that are recognised in the nucleus by a member of the Dicer RNaseIII enzyme family (Schauer *et al*. 2002). This ~22 nt duplex is then recognised by a RISC-like complex, following which one of the strands of

the duplex is incorporated into the RISC complex. The miRNA guides the RISC complex to the target mRNA, where it binds to the target mRNA and either directs its cleavage or prevents the translation of the mRNA transcript (Park *et al*. 2002). miRNA-directed degradation of target transcripts is the primary means of regulation, with only a handful of miRNAs being implicated in translation inhibition in plants (Chen 2004; Hake 2003; Jiao *et al*. 2008; Park *et al*. 2002). This has been ascribed to a very high degree of complementarity of miRNAs with their target mRNA transcripts. This has facilitated the development of bio-informatic tools to identify miRNAs, in addition to those identified by traditional approaches such as cloning and genetic approaches (Bartel and Bartel 2003; Bartel 2004). The development of high-throughput DNA sequencing technologies has resulted in a dramatic increase in the number of miRNAs identified (Fahlgren *et al*. 2007; Lu *et al*. 2005a).

The majority of plant miRNAs initially discovered was highly conserved and approximately half have transcription factors as targets. These transcription factors are usually involved in cell fate determination, developmental patterning and cell differentiation (Bartel 2004). Interestingly, miRNAs have been implicated in the regulation of their own biogenesis. As the number of miRNAs identified increased, so did the number of predicted pathways and responses that they are involved in regulating. In many cases, multiple loci encoding the same or near-identical mature miRNA sequence were identified. These loci were placed into the same miRNA gene family. Examples include cell metabolism, and abiotic and biotic stress responses (Chiou 2007; Chiou *et al*. 2006; Jones-Rhoades and Bartel 2004; Jones-Rhoades *et al*. 2006; Liu *et al*. 2008; Lu *et al*. 2005a; Lu *et al*. 2008; Lu *et al*. 2005b; Lu and Huang 2008; Sunkar and Zhu 2004; Zhou *et al*. 2008; Zhou *et al*. 2007). This makes miRNAs viable candidates for genetic engineering to produce plants with favourable phenotypes or characteristics, which is particularly applicable to the forestry and agricultural industry.

Plants have adapted to survive in most environments on earth, many of them extreme. This includes both the aquatic and terrestrial environment, each with their associated difficulties that need to be overcome. The terrestrial environment places several requirements on plants, including the uptake of water and nutrients from the soil, transport of the above to the remainder of the plant, maintenance of vertical growth, and the ability to survive extremely variable conditions. The

necessities for a transport system within plants, as well as a means of structural support during plant growth led to the development and specialisation of the vascular tissues. The vascular tissues of modern plants such as trees consist of xylem and phloem that are derived from the vascular cambium, with the xylem forming to the inside of the vascular cambium and the phloem developing to the outside. These tissues are commonly referred to as wood. The function of the vascular tissues, both structurally and as a transport system, calls for rigid and specialised structures. The necessity to adapt to changing conditions, pathogens and abiotic stress requires a degree of plasticity in the vascular tissues for the appropriate response to be achieved (Fagard *et al*. 2000; Mellerowicz *et al*. 2001; Mellerowicz and Sundberg 2008). Gymnosperm and angiosperm trees can attain vertical growth in excess of 50 m, which places great corrective stress on the trees to maintain the optimal vertical growth. Angiosperms such as *Eucalyptus* correct for non-vertical growth through the reprogramming of vascular tissues to give rise to reaction wood (Timell 1969; Timell 1986). This reaction wood in angiosperms is referred to as tension wood, as it serves to create tension within the tree to pull the tree upright (Baba K. 1995; Timell 1969). The study of the reprogramming of the vascular cambium provides an opportunity to unravel some of the intricacies of the mechanisms and processes of wood formation.

In this review, only a brief overview of the process and criteria required in the identification of plant miRNAs is given, as this is not the focus of this M.Sc dissertation. Reviews covering this aspect of miRNA research include Meyers *et al* (2008). Following this, miRNA biogenesis is explained, as well as the potential evolution history of miRNAs. The review then changes direction, focusing on trees and wood formation with specific reference to tension wood, (see Plomoin *et al* 2001; 2003 and Du 2007). Subsequently, miRNAs of potential interest to this research project are discussed in some detail with a focus on miRNA function and diversity, target genes and these targets' predicted functions. Reviews of interest include Jones-Rhoades *et al* (2006) and Lu and Huang (2008).

1.2 miRNA Biogenesis

1.2.1 miRNA transcription and processing

Although miRNA and siRNA have different origins, they undergo similar processing steps (Table 1.1) and are in many cases functionally interchangeable (Carrington and Ambros 2003). The majority of miRNA genes are located in regions of the genome previously described as intergenic regions. Animal miRNA genes are also often located within introns, sometimes within an intron of the target gene itself (Jones-Rhoades *et al*. 2006). Plant miRNA genes are typically larger than their animal counterparts and are often larger than 1 kb. This facilitates the folding and formation of secondary structures required for miRNA transcript recognition and processing. Included in this are mismatches between the mature miRNA (miRNA) and its complementary sequence (miRNA*) that result in bulges. As the miRNA transcripts (pri-miRNA) have 5' caps and polyadenylated 3' ends, it is thought that most miRNA genes are transcribed by RNA polymerase II (Xie *et al*. 2005). In plants, there are three processing steps, all taking place within the nucleus (Figure 1.1). The first two steps are performed by a single enzyme known as Dicer-like 1 (DCL1), which has both Drosha and DICER enzymatic functions (Jones-Rhoades *et al*. 2006; Papp *et al*. 2003), and is located within the nucleus. Mutant studies have shown DCL1 to be the only one of the four DCL proteins identified in *Arabidopsis* that is required for miRNA processing (He and Zhang 2004). DCL1 first produces the pre-miRNA, which is then processed to yield a 21-24 nt mature miRNA (Kidner and Martienssen 2003; Kidner and Martienssen 2005a; Kidner and Martienssen 2005b). Pre-miRNAs are often not detected, or when detected are in extremely low quantities (Bartel 2004). This suggests that pre-miRNAs are rapidly processed to a mature miRNA.

Table 1.1 Differences between siRNAs and miRNAs in plants

siRNA	**miRNA**
Derived from target gene transcript	Encoded by is own gene
Targets the same gene as from which it was derived	Targets genes other than its own MIR sequence
Pairs precisely with target sequence	Pairs imprecisely with the target sequence
Two processing steps to yield a mature siRNA	Three processing steps to yield a mature miRNA
First step occurs in the nucleus, second in the cytoplasm	All processing takes place in the nucleus

In order to produce mature miRNAs, a third step is required. HUA Enhancer 1 (HEN1) has a methyl transferase activity and performs the final processing step (Figure 1.1) to yield the mature miRNA duplex (Kidner and Martienssen 2005a; Park *et al*. 2005). The DCL1 final product has a 2-nt overhang at each 3' end. HEN1 targets this 3' overhang and methylates the terminal 2'-hydroxyl group (Kidner and Martienssen 2005a; Park *et al*. 2005; Park *et al*. 2002). Park *et al* (2005) demonstrated that the 2-nt overhang was an absolute requirement for methylation, but miRNA length and the mismatch bulges also play a role in determining the methylation status of the miRNA duplex (Park *et al*. 2005). The function of this methylation is still unclear, but it may be required for miRNA duplex stability by protecting the 3' end from uridylation (Jones-Rhoades *et al*. 2006; Li *et al*. 2005) and recognition by RISC.

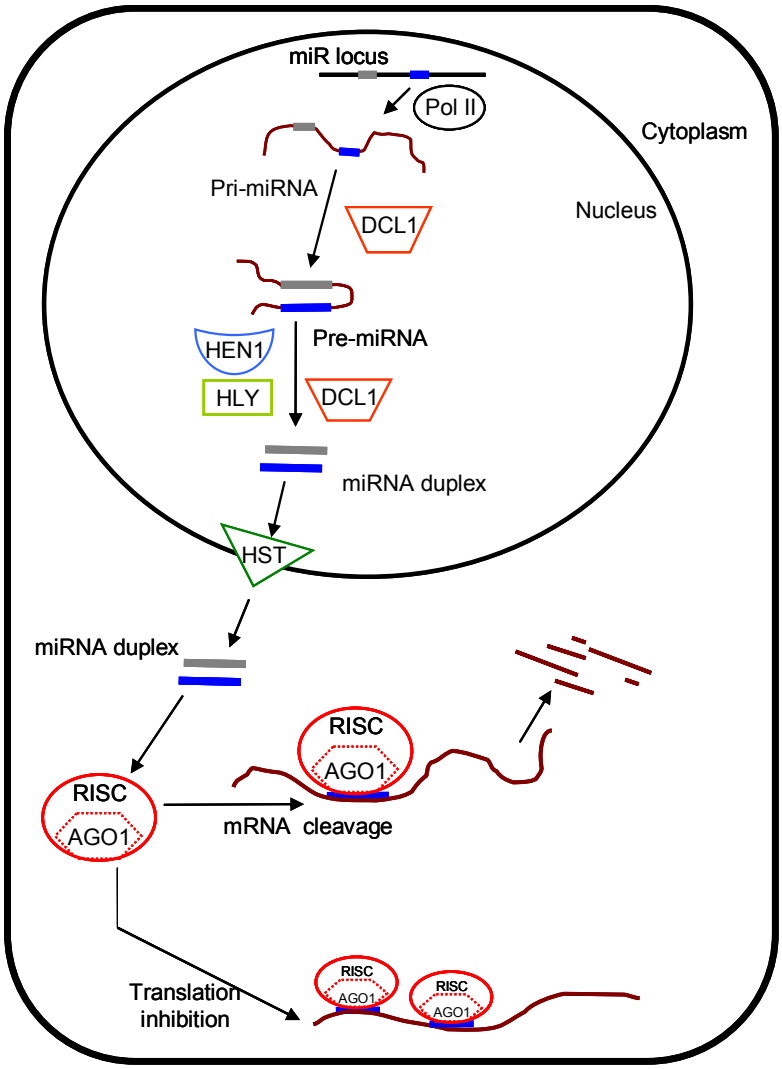

Figure 1.1. The biogenesis and action of miRNAs in plants. Transcription of miR loci by Pol II yields a pri-miRNA. This pri-miRNA is processed by DCL1 and potentially associated proteins to produce the fold-back precursor pre-miRNA. The pre-miRNA remains in the nucleus and is further processed by DCL1, HLY and HEN1. These proteins then further process the pre-miRNA yield the miRNA–miRNA imperfect duplex, which is transported to the cytoplasm by HST where the mature miRNA is incorporated into RISC. The RISC includes the AGO1 protein and the RISC complex is guided by the miRNA to the target mRNA. The miRNA/RISC complex can then either direct cleavage of or translation inhibition of the target transcript (simplified from Kidner and Martiemssen 2005).

The mature miRNA is then transported to the cytoplasm by HASTY (HST), a nucleocytoplasmic transporter belonging to the importin β family and a homologue of the animal R1D1, HYPONASTIC1 (HYL1) protein (Kidner and Martienssen 2005a). Mutant studies with non-functional HST resulted in reduced levels of miRNA accumulation. The incorrect regulation or functioning of any of the components of miRNA biogenesis results in reduced quantities of mature miRNAs and loss of regulatory control over target gene expression. This results in a pleiotropic phenotype, with plants displaying a wide range of phenotypic abnormalities and is symptomatic of the loss of regulatory control over multiple genes (Jones-Rhoades *et al*. 2006). Since any failure of miRNA biogenesis would result in the detection of altered miRNA abundance patterns for all miRNAs, it is therefore important to ensure that plants used in miRNA abundance profiling are phenotypcally normal. Once the mature miRNA duplex has been exported from the nucleus to the cytoplasm, the miRNA molecule is incorporated into a RNA-induced silencing complex (RISC). The miRNA serves as a guide for RISC by directing it to the target mRNA transcript. The biogenesis of miRNAs is in the process of being more finely resolved so that each component will be identified and its function determined, as well as the interactions with other components of the small RNA biogenesis machinery (Mallory *et al*. 2008).

1.2.2 miRNA incorporation into RISC

The miRNA/miRNA* duplex is only detected at low levels and appears to be a transient intermediate (Bartel 2004). This suggests that the duplex is rapidly recognised by RISC. Upon recognition, one of the strands of the duplex is incorporated into the RISC complex (Figure 1). A strand bias has been identified that ensures incorporation of the correct sequence (Khvorova *et al*. 2003). This bias favours the incorporation of the miRNA strand over the miRNA* strand. This appears to be the result of an energetic asymmetry within the duplex (Khvorova *et al*. 2003; Schwarz *et al*. 2003), as the 5' end of the miRNA strand has more mismatches than that of miRNA*. This results in preferential separation at the miRNA 5' end. Once the miRNA strand is incorporated into RISC, the miRNA* strand is rapidly degraded. This is supported by a miRNA* recovery rate that is up to 100-fold lower than that of miRNA (He and Zhang 2004).

The miRNA-RISC complex is now able to recognise and regulate the expression of the appropriate target gene. To date, the exact make-up of the RISC complex and its associated proteins is yet to be fully resolved. It is thought that Argonaute 1 (AGO1) is a major component of RISC. AGO1 is a member of the Argonaute protein family. In *Arabidopsis*, 10 AGO-like genes have been identified (Kidner and Martienssen 2005a; Kidner and Martienssen 2005b). It is thought that AGO1 may also function upstream of the RISC and be involved in the accumulation of miRNAs (Kidner and Martienssen 2005a; Kidner and Martienssen 2005b). Interestingly, AGO1 itself is regulated by a miRNA in plants, miR168 (Vaucheret 2006). The AGO1 protein has PAZ and PIWI domains (Bartel 2004). These domains are required for miRNA incorporation into the RISC complex. The PAZ domain is a putative RNA-binding domain and is probably needed to hold the miRNA within the complex, while the PIWI domain is thought to have a RNase H activity and may be responsible for cleavage of the target mRNA sequence (Jones-Rhoades *et al*. 2006; Song *et al*. 2004).

1.3 miRNA gene expression

Animal miRNAs have been found to be arranged in clusters of related genes that may or may not be transcribed together to yield polycistronic RNA sequences that are later cleaved (Hayashita *et al*. 2005; Lagos-Quintana *et al*. 2001; Suh *et al*. 2004). Alternately, the mRNAs within the cluster may be individually transcribed in such a way that they are co-expressed (Agrawal *et al*. 2003). In contrast, plant miRNA genes are usually not clustered and therefore must rely on various signals and the appropriate combination of *cis*-elements to ensure correct spatial and temporal miRNA expression. These elements also ensure the appropriate miRNA response to various abiotic and biotic conditions (Jones-Rhoades and Bartel 2004; Jones-Rhoades *et al*. 2006; Liu *et al*. 2008; Zhou *et al*. 2008; Zhou *et al*. 2007). In animals, certain miRNA are found at copy numbers as high as 50000 per cell (Jones-Rhoades *et al*. 2006). Accordingly, a number of plant miRNA will surely represent some of the most highly expressed plant RNAs. The expression of a number of miRNA genes is regulated by phytohormones or growth conditions (Jones-Rhoades *et al*. 2006). Examples include miR160 and

miR167 which regulate auxin response factors (Bartel and Bartel 2003; Mallory *et al*. 2005; Reinhart *et al*. 2002), and miR159, which is dependent on gibberellin (Achard *et al*. 2004). An exceptional example of miRNA expression being determined by environmental conditions is miR395, which is not expressed under normal plant growth conditions. However, when the plant is under sulphur starvation, it is up-regulated 100-fold in order to down-regulate a SO_2 transporter, *AST68* (Adai *et al*. 2005; Chiou 2007; Jones-Rhoades *et al*. 2006). The coordinated regulation of miRNAs and their targets are becoming more apparent. This resulted in an increasing number of investigations aimed at further defining the role of miRNAs during developmental processes, plant growth and in response to external stimuli such as stress (Garcia 2008).

1.4 miRNA regulation of target gene expression

The knowledge of siRNA regulatory mechanisms and the observed similarity between siRNAs and miRNAs in animals was used in initial animal miRNA studies to identify translation repression as the mechanisms by which miRNAs regulated target genes (Doench *et al*. 2003; Pillai *et al*. 2005). It was found that in *C. elegans*, the RISC-miRNA complex recognised the target miRNA in a sequence-specific manner through complimentary base pairing (Cullen 2004). In *C. elegans*, miRNAs could regulate the expression of the target gene by translational inhibition through imperfect binding to the mRNA, resulting in mismatches and bulges between the miRNA and the target mRNA. This is the dominant form of miRNA regulation in animals (Bartel 2004; Doench *et al*. 2003). In translational inhibition, a number of RISC-miRNA complexes bind to a series of complimentary binding sites (Cullen 2004) that are usually located in the 3' UTR of the target gene (He and Zhang 2004). The second means of regulation is achieved by directing the cleavage and subsequent degradation of the target mRNA (Bartel 2004). Cleavage occurs when there is a high degree of complementarity between the miRNA and the target mRNA. Not surprisingly, these binding sites are highly conserved (Jones-Rhoades *et al*. 2006).

In plants, most miRNAs have been found to direct cleavage of the target mRNA and plant miRNAs have a very high degree of complimentarily to their target mRNA. miR172 is an exception as it primarily acts as a translational repressor (Aukerman and Sakai 2003; Bartel 2004; Chen 2004). The RISC-miRNA complex cleaves the target mRNA between the 10th and 11th nt of the miRNA (Hutvagner *et al*. 2004; Kasschau *et al*. 2003; Llave *et al*. 2002). This cleavage is performed by the "Slicer" activity of the AGO component of RISC, which cleaves a single phosphodiester bond (Jones-Rhoades *et al*. 2006). This results in the rapid degradation of the cleaved mRNA by the RNA-degrading machinery of the cell. A study investigating miRNAs and DNA methylation indicated that miRNAs may interact with the spliced mRNA to direct DNA methylation of the target genes (Bao *et al*. 2004). The varied mechanisms through which miRNAs act make it possible for miRNAs to regulate developmental processes at several points to achieve rapid and fine regulation of these processes.

1.5 miRNA evolution

miRNAs are thought to have first evolved more than 400 million years ago (Bowman 2004; Pasquinelli 2002; Pasquinelli and Ruvkun 2002). The evolution of modern plants from green algae would have required the rapid diversification of algae genes, along with the appearance of numerous new genes in order to produce new cellular structures, cell types and tissues (Fattash *et al*. 2007). This would have necessitated the development of novel regulatory mechanisms and the adaptation of old mechanisms to ensure that the newly found complexity is maintained and expanded (Mattick 2004). One such novel mechanism appears to be miRNA regulation of gene expression, as to date no miRNAs have been identified in green algae (Fattash *et al*. 2007). Plant miRNAs are hypothesised to have evolved from inverted duplication events, consisting of the target gene or a fragment thereof that are transcribed to form hairpin structures that are recognised by the miRNA biogenesis machinery (Allen *et al*. 2004; Fattash *et al*. 2007; Rajagopalan *et al*. 2006). Axtell and Bartel (2005) examined 23 miRNA families identified in *Arabidopsis*. Of these, 11 had homologues in a gymnosperm and eight

in a fern, indicating miRNA conservation since the emergence of flowering plants and making it apparent that miRNAs are an evolutionary conserved mechanism of gene regulation in plants (Axtell and Bartel 2005). Subsequent work by Fattash *et al.* (2007) identified 17 miRNA families in moss that had previously been identified in higher plants.

High-throughput sequencing has allowed for deep sampling of expressed small RNAs. This has made it possible to detect rare miRNAs that escaped the cloning and bioinformatic approaches. Using this approach, Fahlgren *et al.* (2007) identified 48 non-conserved miRNAs in *Arabidopsis*, the majority of which were represented only once in the genome. Some miRNAs target the genes from which they were originally evolved, while others have lost specificity and no longer interact with their parental genes (Fahlgren *et al*. 2007). They proposed that miRNAs are continually evolving, but that only a limited number are maintained and serve as functional regulators of gene expression (Axtell 2008; Axtell and Bowman 2008). Maher *et al.* (2006) found that the evolution of a number of rapidly evolving miRNA families in *Arabidopsis* was driven by processes similar to those associated with protein gene families. The expansion of the miRNA families was the result of various duplication events that include genome-wide, segmental and tandem duplication events that then underwent diversification and dispersion. These duplications and resulting expansions of miRNA gene families have resulted in subtle differences in the spatial and temporal expression of individual family members, although varying levels of redundancy is still observed (Axtell 2008; Axtell and Bowman 2008; Maher *et al*. 2006).

A large number of miRNAs are conserved across plant species and target homologous genes in the different species (Jones-Rhoades *et al*. 2006). With the deep sequencing capabilities now easily accessible, an increasing number of novel, species-specific miRNAs have been identified. The conserved miRNAs often do not share the same expression patterns or levels across species. Similarly, conserved miRNAs or family members may function at different developmental stages or tissues in different species (Ha *et al*. 2008). They go on to suggest that divergence of miRNA sequence and expression observed at the interspecies levels may affect the regulation of miRNA-targeted genes in interspecific hybrids and allopolyploids with divergent genomes. This may result in

phenotypic and developmental changes being observed in the hybrids (Ha *et al*. 2008). It is therefore necessary to be cautious when ascribing functional importance to novel miRNAs without a complete evaluation. It stands to reason that miRNAs should be evaluated at the species level, where ideally individual miRNA family members are characterised. These observations further present difficulties when assigning miRNA function in plants in general, particularly in highly specialised conditions and responses.

1.6 Trees and wood development

Trees are complex multi-cellular organisms that have been evolving and adapting to ever-changing terrestrial environments. One such adaptation, essential for terrestrial growth, is the ability to transport water and nutrients throughout the plant, from the roots to the apical meristem and from the source organs such as leaves to the remainder of the plant. This led to the development of the vascular tissues present within plants such as ferns, gymnosperms and angiosperms. The vascular tissue of trees consists of xylem and phloem that are derived from the procambium, which gives rise to the primary vascular tissues in vascular bundles, but eventually also to secondary vascular tissues, which give rise to wood. The vascular cambium forms once the interfascicular cells between the vascular bundles differentiate to form a continuous ring of cambial cells. The secondary vascular tissues are derived from the vascular cambium, which separates the xylem from the phloem. Gymnosperms and angiosperms are of particular importance to man as a result of their ability to form wood, essentially secondary xylem.

1.6.1 Wood development

Wood consists of secondary xylem that is derived from a secondary meristem, the vascular cambium (Plomion *et al*. 2001). Several steps are required to produce wood and these are extensively regulated by various signalling pathways, either independently or through cross-talk at various levels (Fukuda 2004). From the procambium the xylem and phloem cells differentiate asymmetrically within the

vascular bundles (Carlsbecker and Helariutta 2005), with the xylem mother cells having a greater rate of division than the phloem mother cells (Plomion *et al*. 2001). The xylem and phloem form on opposite sides of the procambium, with the phloem forming towards the outside and the xylem towards the inside of the procambium in the vascular bundles (Figure 1.2A).

The vascular cambium (Figure 1.2B) develops from the procambium (Plomion *et al*. 2001). The determination of adaxial/abaxial patterning within the procambium and vascular cambium requires the antagonistic action of several classes of transcription factors. The first being the class III Homeodomain-Leucine Zipper transcription factor family (HD-ZIP III), which include *REVOLUTA* (*REV*), *PLABULOSA* (*PHB*) and *PHAVOLUTA* (Carlsbecker and Helariutta 2005). HD-ZIP III transcription factors promote adaxial development in the pro-and vascular cambium, while the second class, the *KANADI* transcription factors are abaxial-promoting (Emery *et al*. 2003). Several other factors have been associated with the adaxial/abaxial patterning of the plant vasculature, including *ASYMMETRIC LEAVES1* (*AS1*) and *AS2* factors, and Altered Phloem Development (APL) and cytokinin signals (Bonke *et al*. 2003; Carlsbecker and Helariutta 2005; Waites and Hudson 1995).

The secondary vascular tissues are derived from the vascular cambium, which separates the xylem from the phloem. This is achieved through the interfascicular cells between the vascular bundles differentiating to form a continuous ring (Figure 1.2B) of cambial cells (Plomion *et al*. 2001). Auxin is required for the continuous vascular pattern formation. This is achieved by polar auxin flow, as set forth by the auxin-flow canalisation hypothesis (Fukuda 2004). The vascular cambium is responsible for secondary growth, in other words, the increase in diameter of the tree through the formation of secondary xylem and phloem (Figure 1.2B).

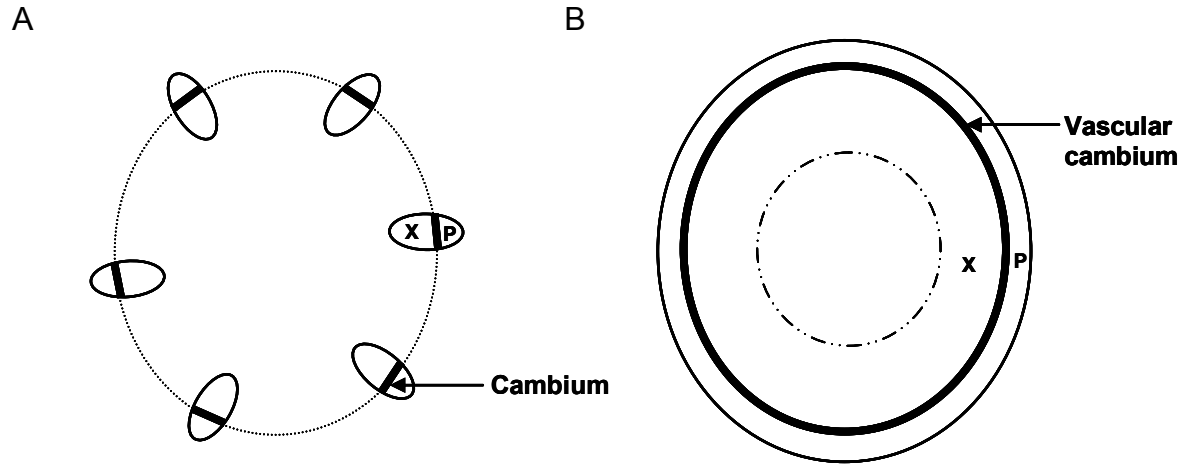

Figure 1.2 (A) Development of the vascular tissues (which is derived from the procambium) within the vascular bundle. (B) The continuous development of the xylem (X) and phloem (P) from the vascular cambium.

Once differentiation of xylem cells from the vascular cambium has occurred, secondary xylem formation takes place. Secondary xylem forms during the secondary phase of tree development where it has a supportive role and serves as the water transport system within the tree (Nieminen *et al*. 2004). Xylem is the collective name for xylem fibres, treachery elements and non-conducting xylem parenchyma cells that are derived from xylem precursor cells (Fukuda 2004). There are five major steps that lead to secondary xylem (wood) formation, namely: division of cambial initials, cell expansion, secondary cell wall deposition, lignification and programmed cell death (Plomion *et al*. 2001; Scarpella *et al*. 2004; Ye 2002; Ye *et al*. 2002). The first step is cambial cell division. This is followed by cell expansion, during which the xylem cells expand both radially and longitudinally. This expansion occurs during the deposition of the primary cell walls, and requires enzymes such as endoglucases and expansins (Sampedro and Cosgrove 2005), along with structural proteins such as extensins and arabinogalactan (Darley *et al*. 2001). The major components of the primary cell wall are cellulose fibres, hemicelluloses that serve to link the cellulose fibres, and pectin polysaccharides (Darley *et al*. 2001). These components also make up the secondary cell wall, along with several others, including lignin.

Following the expansion step, deposition of the secondary cell wall takes place. It consists of three layers S1, S2 and S3 (Figure 1.3A), and develops between the plasma membrane and primary cell wall (Andersson-Gunneras 2005). This requires coordinated expression of genes involved in the production of polysaccharides, lignins and cell wall proteins (Plomion *et al*. 2001). Secondary cell wall cellulose synthase (CESA) proteins are required for the production of the cellulose microfibrils, which are the major component of secondary cell walls (Fukuda 2004). Cellulose organisation differs between primary and secondary cell walls with regard to orientation of the microfibrils relative to that of the cell wall. This is known as the microfibril angle and contributes to the rigidity of the cell wall (Chaffey *et al*. 2000). The microfibrils are deposited transversely in the S1 layer, longitudinally in the S2 layer and again transversely in the S3 layer (Andersson-Gunneras 2005).

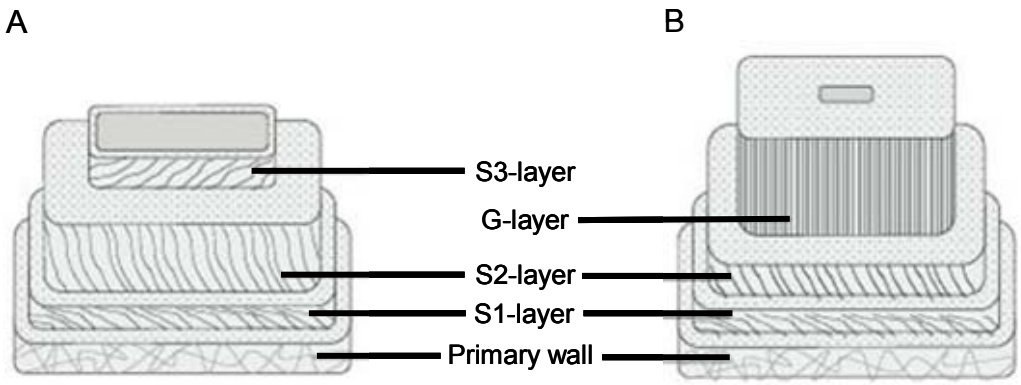

Figure 1.3 A diagrammatic representation of the differences between normal wood (A) and tension wood (B). The cell wall consists of three layers, each with a different microfibril angle. In tension wood the G-layer replaces most of the S2 and S3 layers, and is characterised by a small microfibril angle (Andersson-Gunnerås, 2005).

Following cellulose deposition, the cells undergo lignification. Lignification provides compressive strength, hydrophobicity and rigidity to the wood (Plomion *et al*. 2001). The lignification process itself has three steps: synthesis of the monolignols, transport and secretion of the monolignols and finally polymerisation of the monolignols within the secondary cell wall (Boerjan *et al*. 2003; Minorsky 2002). The lignin biosynthetic pathway generates three different subunits: *p*-hydroxyphenyl

(H), guaiacyl (G) and syringyl (S) phenylpropanoid subunits. Polymerisation of the monolignols may be achieved via the enzymatic activity of peroxidases and laccases (Boudet *et al*. 2003; LaFayette *et al*. 1999). Both MYB and LIM transcription factors are required for the up-regulation of genes involved in lignin precursor biosynthesis (Fukuda 2004). They serve to activate transcription of genes encoding the enzymatic components of lignin biosynthesis, including cinnamyl alcohol dehydrogenase (*CAD*) and hydroxycinnamoyl-CoA reductase (Boudet *et al*. 2003). The final step in xylem formation is programmed cell death that gives rise to mature xylem (Plomion *et al*. 2001). The availability of the *Arabidopsis* genome sequence and its ability to undergo secondary growth, including secondary xylem formation (Chaffey *et al*. 2002), allows for comparative studies of wood formation within the genera *Populus* and *Eucalyptus*. Thereby making it possible to identify candidate genes for wood formation, as well as their regulators, which include miRNAs.

1.6.2 Tension wood formation

As trees are living in an ever-changing environment, numerous factors alone or in combination subject them to stress. One such stress is the disruption of normal vertical plant growth. This may be due to prevailing winds, trauma or due to sloping growth sites (Lafarguette *et al*. 2004). In order to correct for non-vertical growth, woody plants such as trees respond by developing a specialised type of wood, known as reaction wood (Scurfield 1973; Timell 1969), which serves to correct the orientation of the stem or branch, or at least resists the mechanical stress. Reaction wood takes different forms in gymnosperms and angiosperms (Timell 1969).

Gymnosperm reaction wood is referred to as compression wood (CW) while in angiosperms it is known as tension wood (TW). Compression wood forms on the underside of the bent stem or branch and differs both physically and chemically from normal wood. CW typically consists of short, round trachieds that have higher cell wall thickness to cell diameter ratio, increased levels of lignin and an increased microfibril angle in the S2 layer with regard to the fibre axis (Hellgren *et al*. 2004; Timell 1969). In angiosperms, TW forms on the upper side of a bent stem or branch. TW is characterised by having longer and increased numbers of fibres, with a decrease in the number of

vessel elements. There is a decrease in the lignin content and an increase in the cellulose content. This is primarily due the presence of an additional "gelatinous layer" (G-layer, Figure 1.3B) that replaces the S3 layer located inside of the S2 layer of fibres (Timell 1969; Timell 1979; Timell 1986). This G-layer consists primarily of pure cellulose that is highly crystalline. Syringyl lignin makes up the majority of the lignin present in the G-layer (Joseleau *et al*. 2004; Young *et al*. 1971). The S1 and S2 layers are thinner in tension wood in which the G-layer is present, but have normal amounts of lignin with a syringyl (S) to guaiacyl (G) lignin ratio expected in normal wood. This would explain the increased S:G ratio in TW. The presence of the G-layer is not necessary for reaction wood to be defined as tension wood. In cases when trees do not form TW. Yoshizawa *et al.* (2000) found that the S2 layer developed characteristics similar to those of the G-layer, namely increased cellulose levels and altered lignin ratios (Yoshizawa *et al*. 2000).

To date, three hypotheses have been put forward for the development of tension wood. Initially, Boyd and Schuster (1972) proposed the "lignin swelling hypothesis" where growth stress is attributed to the presence of large amounts of lignin in the S1 and S2 layers compared to that in the G-layer, therefore the S1-S2 layers should swell once lignified. This directs the force transversely as the G-layer resists longitudinal expansion (Boyd and Schuster 1972). Bamber *et al.* (2001) followed with the "cellulose tension hypothesis". Here it was pointed out, as was suggested by Wardrop and Harada (1965), that as microfibrils are formed and polymerised (crystallising) they would contract longitudinally creating tension, while lignin in the S1-S2 layer would inhibit crystallisation of the cellulose, thus reducing the longitudinal contraction forces (Bamber 2001; Pilate *et al*. 2004a; Wardrop and Harada 1965). In 1994, Okuyama *et al.* (1994) presented the "unified hypothesis". Here compressive stresses are created through the deposition of lignin between the microfibrils, while tensile stresses are produced between the microfibrils such that the stresses are positive with regard to microfibril axial direction. Together, these stresses generate the plant's growth stresses (Okuyama *et al*. 1994; Pilate *et al*. 2004a).

It is thought that auxin plays a role in the development of tension wood (Wilson and Archer 1977), as auxin is required for normal xylem development in wood (Berleth *et al*. 2004; Tuominen *et*

al. 1997). This thinking was not supported by gravitropic responses in roots and shoots being associated with the redistribution of auxin, and auxin distribution across the vascular tissues of poplar (Hellgren *et al*. 2004). Teichmann *et al.* (2008) investigated the role of auxin in tension wood. A GUS gene construct was placed under control of the GH3 auxin response factor promoter. This revealed that despite high concentrations of auxin in the cambium, there was no induction of GH3::GUS until bending stress was applied to the transgenic poplars. This supports the hypothesis that it is the change in auxin concentration, rather than the presence or absence of auxin that determines the response to mechanical stress. Another phytohormone, ethylene, has been implicated in tension wood development, as there is a great increase in the production of ethylene in *Eucalyptus* stems that form tension wood (Nelson and Hillis 1978). This is further supported by the increased expression levels of 1-aminocyclopropane-1-carboxlate (ACC) oxidase, which is responsible for ethylene production in poplar, when poplar is induced to produce tension wood (Andersson-Gunneras *et al*. 2003; Du and Yamamoto 2007). There is tentative evidence that auxin plays a role in signalling during tension wood formation, while ethylene appears to be definitively involved in tension wood formation.

To date there have been a number of studies investigating gene expression in reaction wood. The sequencing of the poplar genome has resulted in increased research in angiosperms and consequently, tension wood. The ability to contrast expression profiles between gymnosperms and angiosperms has allowed for the identification of distinct differences in gene expression in trees under bending stress. Examples include genes in the monolignol biosynthesis pathway. In gymnosperms, representatives of the *4-coumarate CoA ligase* (*4CL*), *Caffeoyl CoA O-methyltransferase* (*CCoAOMT*) and *S-adenosyl methionine synthetase* were up-regulated in compression wood (Whetten *et al*. 2001). In angiosperms, two of three *CCoAOMT*s were down-regulated as was a *4CL* (Andersson-Gunneras *et al*. 2006 Pilate 2004). Andersson-Gunneras *et al* (2006) found that cinnamoyl-CoA reductase (CCR), caffeic acid O-methyltransferase (COMT) and ferulate 5-hydroxylase (F5H) were also down-regulated in tension wood. Cellulose synthase (CesA) genes, responsible for producing cellulose microfibrils were found to be up-regulated in tension wood (Andersson-Gunneras, 2006; Paux *et al*. 2005; Paux *et al*. 2004; Pilate *et al*. 2004b) as were the

Sucrose Synthase (*SuSy*) genes that produce the CESA substrate, UDP-glucose. Lafarguette *et al.* (2004) identified several *Fascilin-like arabinogalactan* (*FLA*) genes to be highly expressed in poplar tension wood. The exact function of these *FLAs* is yet to be resolved, but they are thought to be involved in tension wood xylem formation as this is where they appear to accumulate.

Investigations into the proteomics of tension wood induction are limited to two studies. The first identified five tension wood-specific proteins (Baba *et al*. 2000). The second study by Plomion *et al.* (2003) identified 12 proteins from *Eucalyptus* that are putatively associated with growth stress (Pilate *et al*. 2004a; Plomion *et al*. 2003). To date, two studies have investigated the role of miRNAs in tension wood formation and the effect of tension wood induction on miRNA expression. Lu *et al.* (2005) identified 22 poplar miRNAs of which 10 appeared to be novel. The majority of the miRNAs appeared to be up- or down-regulated in woody tissues in response to tension wood induction. Of the conserved miRNAs, miR172 appeared to be down-regulated in opposite wood xylem relative to tension wood xylem and upright control xylem. miR159 appeared to only be highly expressed in opposite wood xylem, while miR408 was predominantly detected in tension and opposite wood xylem (Lu *et al*. 2005b; Lu and Huang 2008). Interspecies variation of miRNA expression patterns may be subtle. These differences in expression may result in changes in plant development and their response to external stimuli. This makes it necessary to investigate each species for which finer miRNA functioning is being assigned. The subsequent comparison of such studies will serve to increase our understanding of miRNA function.

1.7 Biological roles of miRNAs

More than 70% of the initial plant miRNAs detected had transcription factors as predicted targets (Jones-Rhoades *et al*. 2006; Jover-Gil *et al*. 2005). It is therefore not surprising that many miRNAs are involved in plant development. Similarly, 62 of the 88 *C. elegans* miRNAs were expressed during larval development. Two thirds of these miRNAs were continually detected once expression was initiated (Lim *et al*. 2003). Transcription factors targeted are generally members of large multi-gene

families where one or several family members are regulated by a particular miRNA. These miRNA-targeted gene family members often act in the same functional pathways with some degree of redundancy (Jover-Gil *et al*. 2005). miRNAs also play a crucial role in the regulation of both the timing and patterning of plant development (Jones-Rhoades *et al*. 2006).

miRNA function can be elucidated in a number of ways. Forward genetics mutation screening is not considered a feasible means of assigning miRNA function. This is due to the high level of redundancy in miRNA family members (Sieber *et al*. 2007). To date, a limited number of miRNAs have been identified using forward genetics. miR164c has been associated with a loss-of-function phenotype, described as *early extra petals* (*eep1*) in *Arabidopsis* (Baker *et al*. 2005). Over-expression, usually in a constitutive manner, with a 35S promoter is the most common method of validating miRNA function (Aukerman and Sakai 2003; Llave *et al*. 2002; Palatnik *et al*. 2003; Schwab *et al*. 2005; Sunkar *et al*. 2006). miRNA specificity can be investigated using tissue-specific promoters (Schwab *et al*. 2006). Another approach is to design cleavage-resistant forms of the target gene/s that are under the control of the 35S promoter or a tissue-specific promoter, if required (Jones-Rhoades *et al*. 2006). Over-expressing miRNA plant lines can be used to restore to the normal phenotype through simultaneous over-expression of the target mRNA. This has been undertaken for a number of miRNAs, including miR159, miR160, miR166 and miR172 (Aukerman and Sakai 2003; Chen 2004; Mallory *et al*. 2005; Mallory *et al*. 2004; Palatnik *et al*. 2003).

The following is a review of conserved miRNAs, with a primary focus on those investigated in this study. These miRNAs are divided into two categories. The first being miRNAs that target transcription factors, including miR159, miR164, miR166 and miR172. The second category consists of miRNAs that target genes that respond to stress or external stimuli. These miRNAs are miR160, miR167, miR168, miR408 and miR472.

1.7.1 miRNA regulation of transcription factors

miR159

miR159 regulates a number of non-overlapping targets in *Arabidopsis* (Table 1.2), including *MYB33* and *MYB65* (Achard *et al*. 2004; Jover-Gil *et al*. 2005; Palatnik *et al*. 2003), which are members of the small gene family of *GAMYB5* found in *Arabidopsis* (Millar and Gubler 2005). GAMYBs encode transcription factors that function as gibberillin (GA)-responsive transcriptional regulators and are responsible for the activation of a number of the genes that are also regulated by gibberillin (Achard *et al*. 2004; Jover-Gil *et al*. 2005). In plants, GA is required for the normal progression and maintenance of a large number of plant developmental and growth processes, including stem elongation, circadian regulation, seed germination and the regulation of flower development (Olszewski *et al*. 2002). *MYB33* and *MYB65* are required for anther development and show redundancy with regard to this. *MYB33* expression occurs at the onset of flowering in the shoot apex in response to an increased GA level. Only when in the reproductive phase did rice lines with disrupted GAMYB expression have reduced internode length and floral organ defects, particularly in anther development (Millar and Gubler 2005).

Achard *et al.* (2004) over-expressed miR159 in *Arabidopsis* and the resultant phenotype resembled that of the *myb33 myb65* double mutant. This work and that of Millar and Gubler (2005) show that miR159 is essential for the spatial regulation of *MYB33* and *MYB65* expression. Millar and Gubler (2005), with the use of miR159 cleavage-resistant *MYB33* genes linked to GUS, demonstrated that the unregulated expression of *MYB33* resulted in seedling arrest and failure of cotyledon expansion. All of the mutant plants that survived were substantially smaller than wild-type plants and this appears to contradict the predicted role of *MYB33* in plant development. An interesting observation was the absence of full-length *MYB33* transcripts in certain tissues, particularly the root tips and SAM, even though *MYB33* was transcribed in these tissues. These transcripts were cleaved by RISC-miR159, possibly to rapidly remove *MYB33* transcripts in cells with high division rates (Millar and Gubler 2005; Reyes and Chua 2007). The miR159-directed regulation of genes involved in GA

signalling is of primary interest in this study, as GA signalling plays a role in normal growth, particularly stem elongation and potentially wood development.

miR164

Computational analysis of the *Arabidopsis* genome identified 110 predicted NAC genes (Mitsuda *et al*. 2005). NAC genes are a family of transcription factors that are functionally diverse and structurally distinct, and are unique to plants. The NAC family is divided into a number of groups and subgroups, including the non-apical meristem (NAM) subgroup (Kikuchi *et al*. 2000; Laufs *et al*. 2004; Olsen *et al*. 2005). Within the NAM subgroup are the *CUP-SHAPED COTYLEDONA* (*CUC*) genes, namely *CUC1*, *CUC2* and *CUC3* (Table 1.2). *CUC1* and *CUC2* show a degree of redundancy in their requirement for shoot apical meristem (SAM) formation (Jover-Gil *et al*. 2005; Mallory *et al*. 2004). *cuc1* and *cuc2* mutants have phenotypes that include cotyledon and floral organ fusions (Jover-Gil *et al*. 2005; Mallory *et al*. 2004). This suggests that CUCs specify the boundaries of gene expression of the genes under their control (Aida *et al*. 1997; Aida *et al*. 1999; Laufs *et al*. 2004; Olsen *et al*. 2005).

CUC1 and *CUC2* are targets of miR164 regulation. Cleavage-resistant *CUC1* resulted in abnormal floral phenotypes, which included increased petal number, the loss of sepals and larger mature flowers (Mallory *et al*. 2004). Over-expression of miR164a and miR164b resulted in phenotypes similar to *cuc*1 *cuc2* double mutant phenotypes (Mallory *et al*. 2004). Therefore, miR164 serves to constrain the expression boundaries of *CUC1* and *CUC2* (Aida *et al*. 1997; Aida *et al*. 1999; Laufs *et al*. 2004; Olsen *et al*. 2005). Additional phenotypes not consistent with the *cuc*1 *cuc2* mutant were present in the miR164b over-expression lines. The fused rosette leaves and fused stem-pedicle phenotypes (Mallory *et al*. 2004) were attributed to miR164b-directed cleavage of an additional NAC gene, *NAC1*. This demonstrates the specificity of miRNA regulation, as different miRNA family members can regulate specific target genes within the same gene family (Guo *et al*. 2005; Laufs *et al*. 2004). *NAC1* is required for the induction and development of lateral roots in *Arabidopsis* in an auxin-responsive manner (Eckardt 2005; Guo *et al*. 2005; Xie *et al*. 2000; Xie *et al*. 2005). As mechanical

stress results in changes to the distribution of auxin, there is the potential for changes in *NAC1* expression. This may require changes in miR164 expression to ensure the correct regulation of *NAC1*. *NAC1* or a *Eucalyptus* homologue may have additional roles in the development of trees.

miR166

The expression of several plant Homeobox leucine-zipper (HD-Zip) class III genes are regulated by miR166 (Table 1.2). In *Arabidopsis,* these are *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REVOLUTA* (*REV*), *ATHB-8* and *ATHB*-15 (Aukerman and Sakai 2003; Emery *et al*. 2003; Rhoades *et al*. 2002). Initial studies identified miR166 as a regulator of the timing of flowering and the determination of leaf polarity in a number of plants (Aukerman and Sakai 2003; Emery *et al*. 2003; Juarez *et al*. 2004; Kidner and Martienssen 2004; Mallory *et al*. 2004; McConnell and Barton 1998; Nogueira *et al*. 2007; Nogueira *et al*. 2006). Follow-up studies identified miR166 as a regulator of vascular development in *Arabidopsis* and tobacco (Kim *et al*. 2005; McHale and Koning 2004).

Over-expression of *PHB* in *Zinnia* resulted in xylem forming around phloem in the leaf veins. *rev* mutants showed a decrease in the number tracheary elements (TE) within the xylem (Ohashi-Ito and Fukuda 2003). *PHB*, *PHV* and *REV* have overlapping expression patterns in vascular bundles and apical meristem. Loss-of-function mutant studies involving single, double and triple mutants (*rev*, *phb* and *phv*) demonstrated that *REV*, *PHV* and *PHB* were redundant with regards to the regulation of tissue pattern formation within the vascular bundle (Ohashi-Ito *et al*. 2005). The expression of *ZeHB-10*, the *Zinnia* homologue of *ATHB-8,* is detected specifically in xylem precursor cells. Over-expression of *ZEHB-10* results in increased numbers of TEs within the vascular bundles. This suggests that *ZeHB-10* promotes the differentiation of xylem precursor cells to TEs. In contrast, *ZEHB-12*, the *Zinnia* homologue of *REV,* when over-expressed, resulted in the formation of xylem vessels and not TEs. The expression of *ZEHB-12* occurs in the early stages of xylem development once TE differentiation is almost complete. Therefore, ZEHB-12 may play a role in differentiation of xylem parenchyma and xylem precursor cells (Ohashi-Ito *et al*. 2005).

A homologue of *REVOLUTA*, *PtaHB1,* was identified in a *Populus tremula* x *P. alba* cross by Ko *et al.* (2006). As was the case in *Zinnia*, *PtaHB1* was primarily expressed in secondary xylem tissue, although *PtaHB1* transcripts were also detected in the transition between primary and secondary growth. *In situ* hybridization confirmed the preferential expression of *PtaHB1* on the xylem side of the vascular cambium. Ko *et al.* (2006) went on to demonstrate that *PtaHB1* expression is regulated through miR166-directed cleavage. The highest levels of miR166 were detected in the bark, with miR166 being absent in the xylem. Corresponding to this, *PtaHB1* was detected at low levels in the bark and at the highest in the xylem (Ko *et al* 2006). In *Medicago truncatula*, miR166 regulates root development and the associated symbiotic nitrogen-fixing nodules through co-expression of miR166 with its targets in the vascular bundles and apices of the root and associated nodules (Boualem *et al*. 2008). With the changes in vessel to fibre ratio associated with tension wood development, changes in miR166 target gene expression are likely to be required.

miR172

In plants, there are a number of core regulatory genes involved in the major developmental processes and stages of plants. Included in this group is *APETALA2* (*AP2*). *AP2* encodes a homeotic transcription factor and is a member of the floral organ identity (ABC) genes (Drews *et al*. 1991; Weigel and Meyerowitz 1994), specifically class A (Table 1.2). These three classes of genes work in various combinations to specify the four types of floral organs (Chen 2004). Unlike the other ABC genes, *AP2* is expressed in almost all plant organs in every stage of development. Accordingly, loss-of-function *ap2* mutants not only affect floral organ development, but also various other aspects of plant (Lohmann and Weigel 2002)

Chen (2004) demonstrated that miR172 is involved in the regulation of *AP2* expression. Regulation is by translation inhibition, rather than cleavage of the *AP2* mRNA (Aukerman and Sakai 2003; Chen 2004; Jiao *et al*. 2008; Jover-Gil *et al*. 2005). Constitutive over-expression of miR172 in *Arabidopsis* and tobacco resulted in accelerated floral transition, as well as phenotypes associated with *ap2* loss-of-function mutants (Chen 2004; Mlotshwa *et al*. 2006). In contrast, cleavage-resistant

AP2 sequences resulted in enlarged floral meristems and the loss of floral determinacy. Therefore, miR172 is required for the regulation of floral organ identity and flowering time (Jover-Gil *et al.* 2005). Jung *et al.* (2007) found that *GIGANTEA* (*GI*) regulated the abundance of miR172 in a photoperiodic manner in *Arabidopsis*. This may regulate photoperiodic flowering by inducing *FLOWERING LOCUS* (*FT*) independent of *CONSTANS* (*CO*). GI-mediated photoperiodic flowering is though to be regulated through the coordination of two genetic pathways: one mediated via *CO* and the other through miR172 and its targets (Jung *et al*. 2007). Lauter *et al.* (2005) identified the maize *AP2*-like homologue, *Glossy15*, which was involved in leaf maturation and was regulated by miR172. When *Glossy15* is not cleaved by miR172 the leaf fails to progress from the juvenile stage to the adult mature leaf stage (Lauter *et al*. 2005).

1.7.2 miRNAs responding to stress or external stimuli

As the number of miRNAs identified in plants increase, there is an increasing number of predicted miRNA targets not involved in developmental regulation. A large proportion of these are involved in responses to stresses, such as drought, cold, phosphate starvation, oxidative stress and mechanical stress (Chiou *et al*. 2006; Fujii *et al*. 2005; Lu *et al*. 2005b; Lu and Huang 2008; Sunkar *et al*. 2006; Sunkar and Zhu 2004). Other processes under predicted miRNA regulation are cellular and metabolic processes, and disease resistance (Allen *et al*. 2004; Jones-Rhoades and Bartel 2004; Lu *et al*. 2005a; Sunkar *et al*. 2005; Sunkar and Zhu 2004).

miR160

Auxin, a phytohormone, is involved in the regulation of key developmental and non-developmental processes through the interaction with numerous auxin response factors (ARFs). Early response genes are one of the main targets of auxin signalling and, as the name suggests, these genes are required for rapid response to external and internal stimuli (Ulmasov *et al*. 1999). These genes, in turn, activate various signalling cascades (Swarup *et al*. 2002), including GH3-mediated homeostasis required for response to various stresses via complex auxin signalling networks (Park *et al*. 2007). ARFs function by binding to auxin response elements (AuxREs). These are conserved sequences within the

promoters of auxin-responsive genes (Hagen and Guilfoyle 2002; Liscum and Reed 2002). Auxin plays a role in the regulation of gravitropic and phototropic responses, stem elongation, adventitious root and wood formation (Uggla *et al*. 1996). ARFs do not directly respond to auxin, but rather form dimers with auxin/indole acetic acid (IAA) repressors (Guilfoyle *et al*. 1998; Guilfoyle 1998), with the change in auxin levels rather than the degree of change in auxin levels initiating an auxin response (Nilsson *et al*. 2008).

miR160 (Table 1.2) targets a few ARFs, namely *ARF10*, *ARF16* and *ARF17* (Mallory *et al*. 2005; Rhoades *et al*. 2002). Failure of miR160 to cleave *ARF17* mRNA resulted in altered levels of a number of auxin-inducible GH3-like transcripts. This led to a number of altered phenotypes that included premature inflorescence development, altered phylotaxis, reduced numbers of lateral roots and embryo abnormalities (Mallory *et al*. 2005; Sorin *et al*. 2005). Liu *et al.* (2007) demonstrated that miR160 repression of *ARF10* is essential for seed germination. In addition, a cleavage-resistant form of *ARF10* resulted in plants with contorted flowers, twisted siliques and curled stems. Interestingly, the over-expression of miR160 resulted in reduced sensitivity to ABA during germination, while ABA-responsive transcript levels increased (Liu *et al*. 2007). *ARF10* and *ARF16* function to restrict stem cell niche differentiation, while promoting that of columella cells. Although functionally redundant, the two ARFs are both required for root cap development by preventing the auxin signal from bypassing them to initiate columella cell production. In roots, *ARF10* and *ARF16* are regulated independently by auxin and miR160 (Wang *et al*. 2005). The described role of auxin in wood formation, coupled with the miR160 regulation of GH3-like transcripts, raises the question of miR160-mediated regulation in normal and tension wood formation.

miR167

miR167 regulates two additional ARFs, namely *ARF6* and *ARF8* (Table 1.2), which regulate development in immature flowers (Nagpal *et al*. 2005; Wu *et al*. 2006). These two ARFs, in turn, activate their respective targets by binding to *cis*-elements as dimers (Hagen and Guilfoyle 2002). *ARF8* was the first ARF identified in the regulation of GH3s in *Arabidopsis,* where it positively

regulates three GH3 genes in response to auxin signalling (Tian *et al*. 2004), with the GH3s, in turn, inactivating auxin. In this way, *ARF8* regulates auxin levels through a negative feedback mechanism via the GH3 gene family (Staswick *et al*. 2005). Yang *et al.* (2006) showed that an increase in auxin levels was followed by an increase in miR167 transcripts. This was followed by a decrease in *ARF8* transcript levels and a corresponding decrease in *OsGH3-2* transcript levels, *OsGH3-2* encodes an IAA-conjugating enzyme. This may be a mechanism to regulate cellular auxin levels that in turn, determine the specific auxin responses (Tian *et al*. 2004; Yang *et al*. 2006). These studies demonstrate the close link between auxin responses and changes in auxin concentration (Woodward and Bartel 2005a; Woodward and Bartel 2005b).

The exogenous application of auxin and GA to poplar has a synergistic effect on the initiation/activation of cell division from the cambial meristem and subsequent xylogenesis, with auxin being required for xylem differentiation. This is partially explained by GA being responsible for the stimulation of polar auxin transport in poplar (Bjorklund *et al*. 2007; Digby and Wareing 1966). Nilsson *et al.* (2008) noted that there was little correlation between auxin concentration in the vascular cambium and its derived xylogenic tissues with the level of gene expression in those tissues. In contrast to this, the maturing secondary xylem cells expressed auxin-responsive genes to higher levels than in the vascular cambium (Nilsson *et al*. 2008), pointing to the role for auxin in xylogenesis.

Ru *et al.* (2006) demonstrated that miR167 directs the degradation of *ARF8* and the translational repression of *ARF6,* with the constitutive over-expression of miR167 resembling that of the *arf8/arf6* double mutant. The mutant lines showed abnormal development in all four whorls, resulting in defects affecting plant fertility (Ru *et al*. 2006). Wu *et al.* (2006) subsequently expanded on this, showing that miR167 is essential for correct patterning of gene expression and accordingly, the fertility. In contrast to this, Oh *et al.* (2008) analysed *ARF8L* mRNA (a potential Pta-miR167 target) and found it to be cleaved in zygotic embryos but not in female gametophytes of *Pinus taeda* (Oh *et al*. 2008). miR167 regulation of tissue patterning through ARFs and their downstream targets such as GH3s creates the potential for regulation of tissue patterning in more than the floral organs, this may include the vascular tissue.

miR168

Argonaute (AGO) proteins have been identified in *Arabidopsis* to play a role in small RNA biogenesis and regulation (Hunter and Poethig 2003; Hutvagner and Simard 2008; McConnell and Barton 1998; Vaucheret *et al*. 2004; Vazquez *et al*. 2004; Zheng *et al*. 2007; Zilberman *et al*. 2003). Agronaute 1 (AGO1) is not only a key component of the RISC complex, but is also the target of a regulatory feedback mechanism of miRNA gene regulation. This is achieved through miR168 (Table 1.2). Incorrect regulation of AGO1 results in aberrant functioning of the miRNA biogenesis machinery, in particular, the assembly of the RISC complex and processing of miRNAs (Rhoades *et al*. 2002). Varied and severe phenotypes are associated with the loss of this feedback mechanism, resulting from the over-expression of the various targets (Vaucheret 2006; Vaucheret *et al*. 2004).

Two additional regulatory requirements for correct AGO1 function have since been set forward by Vaucheret *et al.* (2006). These are the co-regulation of miR168 and AGO1 transcription, followed by the post-transcriptional stabilisation of miR168. Post-transcriptional stabilisation is most likely to take place at the level of RISC assembly (Vaucheret 2006; Vaucheret *et al*. 2004). Correct miRNA stability ensures that the mature miRNA is present within the cell only for the specific period of time that it is required to regulate a particular developmental stage or plant response. Continued characterisation of AGO proteins and their interaction with other components of the small RNA mechanism will be required to understand the complex nature of AGO proteins (Mallory *et al*. 2008) and the function of miR168 in miRNA, regulated responses.

miR408

One of the miRNAs that do not regulate transcription factors is miR408 (Table 1.2). It is one of the few plant miRNAs that are not located in intergenic DNA, but within the 3' UTR of a gene (At2y47020) in an antisense polarity (Sunkar and Zhu 2004). miR408 is highly expressed in seedlings, while being poorly expressed in all adult tissues of *Arabidopsis* (Sunkar and Zhu 2004). A putative target of miR408, a plastocyanin-like protein, was identified in poplar by Lu *et al.* (2005). These proteins have been implicated in the lignin polymerisation process. This is supported by the

failure to detect miR408 in xylem tissue, which is the major target for lignification. However, miR408 was detected in phloem and leaf tissues. Of particular interest to researchers in wood formation was the observation that miR408 was substantially up-regulated in tension and opposite wood, thereby suppressing lignin deposition in these reaction wood tissues (Lu *et al*. 2005b). A laccase is also a confirmed target of miR408 (Schwab *et al*. 2005).

miR472

miR472 (Table 1.2) is predicted to target several related disease resistance proteins in poplar, including two homologues of predicted targets in *Arabidopsis*, CC/NBS/LRR and TIR/NBS/LRR disease resistance proteins (Deyoung and Innes 2006; Lu *et al*. 2005b; McHale *et al*. 2006). Plant defence responses are costly, as resources are redirected away from conventional sinks such as growth and reproduction. The comparatively inexpensive nature of the miRNA regulatory system is a means of regulating specific plant defences required for rapid response. The evolutionary conservation of miR472 among plants is in concordance with continual conflict between plant and pathogen and adaptation of defence responses to pathogen attack (Lu *et al*. 2007; Lu *et al*. 2005b) many of these responses involve modification to the plant cell wall. To date no functional testing of miR472 has taken place.

Other examples of miRNAs that are not directly involved in plant development are miR406, which has two spliceosome-related proteins as potential targets, and miR400. miR400 is predicted to have target sequences in at least 10 pentatricopeptid repeat protein (PPR) family members (Sunkar and Zhu 2004). PPRs have been implicated in the mediation of macro molecular interactions (Sunkar and Zhu 2004). Lu *et al.* (2005) identified a further three miRNAs, namely miR474, miR475 and miR476, that have seven predicted PPR targets. PPR genes have a number of predicted miRNA target sites. This raises the possibility that miRNA may function in redundant or co-ordinated pathways.

Table 1.2 miRNAs, targets and predicted target function

microRNA	**TARGETED GENE FAMILY**	**PREDICTED TARGET FAMILY FUNCTION**	**TARGETED GENE/S**	**PREDICTED TARGET FUNCTION**	**NOVEL *Eucalyptus* miRNA**
miR90	MADS-box family protein, ubiquitin protein ligase 1	Transcription Factor Protein degradation	unknown	unknown	Yes
miR159	MYB gene family	Transcription Factor-plant growth, flower timing	*MYB33* *MYB65*	Gibberillin-responsive TF Flower development	No
miR160	Auxin response factor	Transcription Factor-response to phytohormones	*ARF10, ARF16* *ARF17*	Root, stem, embryonic, inflorescence development	No
miR164b	NAC	Transcription Factor-control of boundary size during floral development	*CUC1, CUC2* *NAC1*	Shoot apical meristem formation Lateral root formation	No
miR166	HD-ZipIII	Transcription Factor-vascular development,	*PHABULOSA*, *PHAVOLUTA*,	Leaf polarity, Patterning within vascular	No

		leaf polarity	*REVOLUTA*, A*THB*-8, *ATHB*-15	bundles	
miR167	Auxin response factor	Transcription Factor-auxin signalling	*ARF6* *ARF8*	Floral development, Fertility	No
miR168	AGO1	miRNA biogenesis	*AGO1*	Component of RISC	No
miR172	Homeotic protein	Transcription Factor-	*APETALA2*	Floral organ identity Flower timing	No
miR293a	Disease resistance protein PAPS reductase Protein kinase	NBS-LRR proteins Biosynthesis of molybdopterin	unknown	unknown	Yes
miR362	Calcium-binding calmodulin protein Ras GTPase family protein	Signal transduction Signal transduction	unknown	unknown	Yes
miR408	Plastocyanin-like protein	Lignin polymerisation	At2g47020 At2g02850	Lignin polymerisation	No

miR472	Serine/threonine protein kinase	Disease resistance, protein phosphorylation	At5g17680	TIR/NBS/LRR disease resistance protein	No

1.8 Cloning of miRNAs in *Eucalyptus* trees

In a previous study in our laboratory, Victor (2006) identified 48 predicted *Eucalyptus* miRNAs from the xylogenic tissues of *E. grandis*. These 48 putative miRNAs were placed into 13 gene families. Of these, eight represented potentially novel *Eucalyptus* miRNA gene families, here named miR31, miR90, miR140, miR200, miR293, miR320, miR359 and miR362. The remaining five families were conserved among plants, namely miR159, miR160, miR168 and miR172 and miR472. The eight potentially novel gene families consist of 28 miRNAs with the remaining 20 miRNAs belonging to the five conserved miRNA gene families.

The isolation and identification of novel and conserved miRNAs from the woody tissues of *Eucalyptus* trees raises questions regarding their roles in tree development in *Eucalyptus* and, in particular, their potential function in the processes governing xylogenesis. To further refine the role of miRNAs in plant development and then specifically in tension wood formation, we undertook to determine the abundance profiles of a number of novel and conserved miRNAs. This was at the whole-tree level and within the xylogenic tissues of tension wood-induced *Eucalyptus* trees.

1.9 Conclusion

miRNA research has identified many of the fundamental components of miRNA biogenesis, function and regulation. This has led to the understanding of how complex the miRNA regulatory mechanism is with subtle alterations to this mechanism resulting in changes in plant development, growth and response to external stimuli. The recent advances in the development of tools required for small RNA research has made it possible to study miRNAs on a larger scale and in finer detail. High-throughput sequencing has made the isolation and identification of miRNAs far more efficient, with large numbers of novel miRNAs being identified in various plant genera, and miRNAs previously thought to be novel have been found to be conserved among plant species. The rapid increase in miRNA numbers has resulted in functional analysis of miRNAs lagging behind, with relatively few miRNAs having been functionally characterised. This has led to the understanding that conserved miRNAs may have differing expression patterns across

plant species. Therefore, one cannot apply the expression pattern observed in one plant species to all plant species. Conserved miRNAs do, in general, perform the same function across species, but subtle differences are observed, particularly with regard to tissue specificity.

A number of studies have recently been undertaken to evaluate these subtle differences, including miRNA response to various abiotic and biotic stresses. These studies have found that several miRNAs respond to one or more stresses. One such stress is mechanical stress (bending). Mechanical stress causes a reprogramming of wood formation processes. This, in turn, alters the cellular structure and chemistry of the subsequently developing tension wood. From the onset of mechanical stress, it takes approximately three weeks for tension wood fibres to form. Tension wood formation requires adjustments and changes throughout xylogenesis. This begins with altered differentiation of the vascular cambium, the xylem mother cells giving the distinctive vascular patterning and structure of tension wood. At the cellular level, changes in cell wall biosynthesis-associated pathways alter the structure and chemistry of the developing wood cells.

The ability to induce tension wood formation under experimental conditions makes it an attractive system to study wood formation. This makes it possible to investigate the role of regulatory mechanisms such as miRNAs in governing wood and specifically tension wood formation. The study of miRNAs in normal and tension wood development can help answer these questions, such as what mechanisms regulate normal and tension wood formation, what components of the wood formation they regulate, are these regulators required throughout normal and tension wood development or only at specific points, and what are the regulatory differences between normal wood development and tension wood development.

To date, studies by Lu *et al.* (2005, 2008) found that a number of novel and conserved miRNAs were differentially expressed between normal and tension wood. miRNA expression also differed between tension wood and opposite wood xylem. However, these studies only examined the endpoint in tension wood formation, three weeks post-induction and only in the xylem, thus potentially missing miRNA regulatory involvement of the numerous intervening steps leading up to this. A study of miRNA abundance profiles in *Eucalyptus* will advance our understanding of miRNA function in trees and plants.

As this research group's primary research interests lie in understanding wood development, determining miRNA abundance profiles in the vascular tissue of *Eucalyptus* undergoing tension wood formation will advance our understanding of tension wood development. When these results are compared to those of normal wood development, it may reveal mechanisms underlying tissue patterning and development of the vascular tissues. This, in combination with previous research, may then be used to extrapolate in which tissues each miRNA may act, what their targets are and where these targets may function.

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Chapter 2

Expression profiling of conserved and novel *Eucalyptus* miRNAs in a *E. grandis* x *E. urophylla* hybrid clone during normal growth and development and in response to tension wood induction

Grant McNair, Michelle Victor, Alexander Myburg

Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa

Insights and contributions

This chapter is prepared in the format of a manuscript for a peer-reviewed research journal. I designed the experiment, performed all experimental procedures and analyzed the data generated. Ms. Michelle Victor provided guidance and assistance in RNA isolation and RT-qPCR analysis of small RNAs. Ms. Victor also provided background information about the miRNAs that she isolated and sequenced from the xylogenic tissues of *E. grandis*. Ms. Sheena Saayman, Prof. Patrick Arbuthnot and Dr Marco Weinberg at the University of the Witwatersrand provided guidance in the development of a miRNA Northern blot analysis protocol. As main supervisor Prof. Alexander Myburg provided guidance throughout the course of the study and was instrumental in the development of the experimental design and strategy, editing of the chapter and funding of the work.

2.1 Abstract

microRNAs (miRNA) are highly specific, small, non-coding RNAs of approximately 22 nt in length, which are processed from endogenous miRNA genes. They mostly function as negative regulators at the post-transcriptional and translational levels. miRNAs regulate numerous developmental processes in plants including the determination of meristem and floral organ identity, timing of developmental processes and regulation of leaf morphogenesis. They have been linked to another key developmental process in plants, namely xylogenesis or vascular development. In trees, this process underlies the formation of wood. Genes expressed during the different phases of xylogenesis ultimately determine the physical and chemical properties of wood. Tension wood is a form of reaction wood produced by angiosperms on the outside or topside of a bent trunk or branch to correct for the non-vertical growth, thus serving to "pull" the tree upright. To investigate the potential role of miRNAs in normal and tension wood development, RT-qPCR and Northern blot analysis was performed for a number of conserved and putatively novel *Eucalyptus* miRNAs. A total of 12 miRNAs representing 12 distinct miRNA families were profiled, including three novel that are species specific *Eucalyptus*. A number of these profiles were consistent with the predicted roles of the miRNAs. At the whole-tree level, miR90, novel to *Eucalyptus* was predominately expressed in the mature leaves and flowers. miR90 may target a MADS-box transcription factor. miR408, regulator of the expression of a plastocyanin involved in lignin polymerisation was expressed at low levels in the immature and mature xylem, where cell lignification is most prominent. In the tension wood profiling miR166, a known regulator of wood development and miR408, predicted to function in wood formation displayed similar patterns in tension wood xylem, that of increasing abundance over time. miR160 and miR167, which target auxin response factors responded early to bending stress, increasing 6 hrs post-induction before decreasing again. The abundance profiles of these and other miRNAs suggest that miRNAs do indeed play a role in wood development, though not necessarily directly. These roles are yet to be studied in functional genetic experiments.

2.2 Introduction

The genus *Eucalyptus* is indigenous to Australasia with more than 700 species being described (EUCLID, 2009) and only a small number not indigenous to Australia itself. One such example is *E. urophylla,* which is indigenous to the Indonesian islands and Timor (Campinhos 1999). *Eucalyptus* is a fast-growing genus of economically important forest trees and one of the dominant genera in commercial forestry. *Eucalyptus* species are cultivated world-wide in tropical and subtropical regions and some temperate regions. A number of factors contribute to this, such as the diversity within the genus allowing for cultivation in a wide range of climates, the hybrid vigour associated with a number of hybrid crosses, including the *E. grandis* x *E. urophylla* hybrid and favourable wood properties (Plomion *et al*. 2001). *Eucalyptus* is of commercial value as a source of timber and pulping material (Plomion *et al*. 2001). *Eucalyptus* has also been identified as a potential sustainable source of lignocellulosic biomass to supply the bio-fuel industry.

Xylogenesis is a complex developmental process describing the secondary plant growth originating from the lateral meristem of woody plants. The lateral meristem (vascular cambium) gives rise to xylem and phloem, with the xylem differentiating to the inside and the phloem differentiating to the outside (Mellerowicz and Sundberg 2008; Minorsky 2002; Nieminen *et al*. 2004; Plomion *et al*. 2001; Wardrop and Harada 1965; Whetten *et al*. 2001; Ye 2002). The differentiation to mature xylem requires complex signalling and cross talk between signalling pathways and gene expression networks (Schrader *et al*. 2004). A number of experiments investigating gene expression across the cambial zone have identified numerous genes potentially involved in the regulation of wood formation (McCann and Carpita 2008). It has been estimated that 10% of the plant genome encodes genes required for cell wall development and subsequent re-arrangements (Henrissat *et al*. 2001). Not surprisingly, a large proportion of these are carbohydrate active enzymes (CAZymes, Geisler-Lee *et al*. 2006; Karpinska *et al*. 2004; Ko *et al*. 2006; Mitsuda and Ohme-Takagi 2008), as well as transcription factors representing various families, including MYBs, NACs and HD-ZIPs (Scurfield 1973; Timell 1969). The exact mechanisms that govern and regulate the initiation and development of

wood are yet to be to be fully resolved, although miRNAs have been implicated in this process. Further investigation miRNAs in xylogenic tissues may identify further roles for these negative regulators.

Reaction wood formation is a corrective response to bending stress or non-vertical growth experienced by woody plants. In angiosperms, this reaction wood is termed tension wood (Bamber 2001; Hellgren *et al*. 2004; Joseleau *et al*. 2004; Timell 1969; Whetten *et al*. 2001). The development of tension wood requires the reprogramming of wood formation, beginning with altered proliferation rates on either side of the vascular cambium and culminates with altered secondary cell wall structure and chemistry. Some changes include increased fibre to vessel ratios, replacement of the S3 layer of the cell wall by a gelatinous G-layer and reduced lignin levels (Allona *et al*. 1998; Andersson-Gunneras *et al*. 2003; Andersson-Gunneras *et al*. 2006; Baba *et al*. 2000; Du and Yamamoto 2007; Paux *et al*. 2005; Paux *et al*. 2004; Pilate *et al*. 2004). Several studies have investigated tension wood formation through transcript profiling, biochemical and chemical analysis to begin to unravel the complexities of wood formation (Plomion *et al*. 2001; Scarpella *et al*. 2004; Ye 2002; Ye *et al*. 2002). These studies revealed the re-direction of carbon flux from hemicellulose and lignin production to that of cellulose, differential regulation of lignin biosynthetic genes, the role of GA, auxin and ethylene-related genes in the mediation and regulation TW formation. Many of these transcription factor families and signaling pathways have family members/components that fall under miRNA regulation. This makes the combination of miRNA directed regulation and tension wood development attractive targets of for further study. Such a study would serve to advance our understanding or miRNAs and wood development. Tension wood formation results in the reprogramming of the five steps of xylogenesis. There are five stages in wood development, namely: division of cambial initials, cell expansion, secondary cell wall deposition, lignification and programmed cell death (Bartel and Bartel 2003; Carrington and Ambros 2003; Chiou 2007; Jones-Rhoades *et al*. 2006; Kidner and Martienssen 2005a; Ko *et al*. 2006; Lu *et al*. 2008; Lu and Huang 2008; Mallory *et al*. 2005; Sunkar and Zhu 2004). Accordingly one would expect early, intermediate and late responses to occurring during tension wood development.

Unravelling the genetic basis of these processes is far from complete and most likely involves several recently identified regulatory mechanisms. One of these mechanisms, miRNA-based gene regulation, involves small (~21 nt) non-coding RNA molecules acting as negative regulators mostly through directed cleavage of target transcripts. miRNA regulation has been implicated in many facets of plant development and survival. Examples include the regulation of plant developmental processes and maintenance of homeostasis and response to stress (Axtell 2008; Axtell and Bartel 2005; Axtell and Bowman 2008; Fahlgren *et al*. 2007; Fattash *et al*. 2007; Maher *et al*. 2006). It has become clear that miRNAs function predominately as regulators of early developmental processes such as meristem cell fate. miRNAs are placed into gene families based on the sequence of the mature miRNA with identical or near identical miRNAs being place in the same family. miRNA family members in most cases regulate the same or closely related target genes, often in a tissue-specific manner (Ko *et al*. 2006).

Only one miRNA, miR166, has been functionally confirmed as playing a role in wood development through the spatial restriction of a HD-ZIP III transcription factor (Ko *et al* 2006, Lu *et al*. 2008; Lu *et al*. 2005b). Several other miRNAs have been potentially implicated in the regulation of wood development. Some of these miRNAs including miR160, miR167 and miR408 may play a role in tension wood formation (Chang *et al*. 1993, Lu *et al*. 2008; Lu *et al*. 2005b). Signalling pathways (auxin) and transcriptional regulators (HD-Zips and MYBs) known to play a role in wood development have also been identified as targets of miRNA regulation. Further research of these topics may reveal potential roles for miRNA regulation in the processes of wood formation.

A recent study by Victor *et al* (2006) identified 48 predicted *Eucalyptus* miRNAs from *E. grandis* that could be grouped into 13 miRNA gene families. Of these, eight families represented potentially novel *Eucalyptus* miRNA gene families, with the remaining five families being conserved among plants. The eight potentially novel families consisted of 28 miRNAs with the remaining 20 miRNAs belonging to the five conserved miRNA gene families. The 13 families target a number of distinct classes of genes including disease resistance, signal transduction and several transcription factor families.

A necessary requirement for miRNA classification is the confirmation that the putative miRNAs are expressed and processed to a ~22 nt mature miRNA sequence. This is generally assessed by means of Northern blot analysis and RT-qPCR. The abundance profiles generated can be used to infer the potential biological roles of the miRNAs. miRNAs identified with potential roles in wood development could be studied further to fully characterise their function. The aim of this study is to determine the abundance of a number of novel *Eucalyptus* and conserved miRNAs in *E. grandis* development, specifically that of wood formation. The miRNA abundance profiles were first determined at the whole-tree level. This was done to gain an understanding of miRNA abundance patterns throughout the tree. Once completed, the vascular tissues of tension wood induced trees were profiled at four time-points over a period of one month. The tension wood results were compared to those of trees sampled at the same time, but growing under normal conditions. The whole-tree and tension wood data were interpreted, while taking previous research into account.

2.3 Materials and methods

2.3.1 Plant materials

All of the tissues used in this study were collected from ramets of a single clonal *E. grandis* x *E. urophylla* (GU) hybrid genotype (GUSAP1, Sappi Forest Research). For whole-tree miRNA abundance profiling, actively growing five-year-old ramets were sampled. A total of nine tissues were collected, namely shoot tips (ST), flower buds (FL), mature leaves (ML), maturing xylem (X), immature xylem (IX), phloem (P), bark (B), roots (R) and green twigs (TG) respectively (Figure 2.1). The samples were immediately frozen in liquid nitrogen, transported on dry ice and finally stored in a -80°C freezer for later RNA extraction. The bark and phloem were peeled from the stem (Figure 2.1 & 2.2), after which the differentiating phloem was lightly scraped from the inside of the bark. The bark was then cut into strips and frozen (Figure 2.1E). The immature xylem was collected by light scraping of the early developing xylem (1-2 mm) from the surface of the exposed tree trunk (Figure 2.1C), after which the maturing xylem (Figure 2.1D) was sampled by deeper scraping of the later

developing xylem (~2-5 mm). Flower buds (Figure 2.1H) and shoot tips were individually picked and frozen. The green twigs were collected from newly developing branches with the section between the last node and the shoot tip, but excluding the shoot tip itself. The flowers and green twigs contain vascular tissue, including lignified cell walls. The roots (Figure 2.1.I) were collected from one-year-old potted GU ramets, washed under running water and then immediately frozen in liquid nitrogen. The ramet sampled for use in the whole-tree profiling was from a clonal field trial in a sub-tropical site, near Kwambonambi, KwaZulu-Natal on the eastern coastal plain of South Africa.

Figure 2.1 Plant materials for whole-tree expression profiling (A) the GUSAP1 clonal trial from which the whole-tree was sampled, (B) separation of the phloem and bark from the immature and mature xylem, collecting of the: (C) immature xylem, (D) mature xylem, (E) bark, (F) immediate freezing of samples in liquid N_2, (G) mature leaves, (H) developing flowers and (I) root

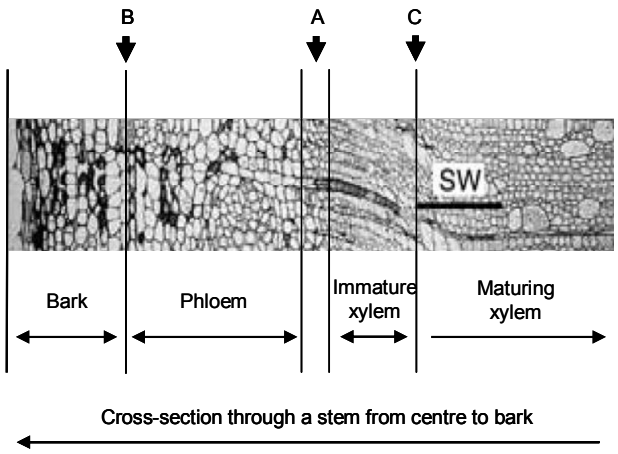


Figure 2.2 A cross-sectional view of the bidirectional developmental series of wood formation showing sites of tissue separation during sampling. (A) Separation of the bark and phloem from the xylem occurs at the vascular cambium, from here (B) the phloem consisting of phloem mother cells and phloem are separated from the bark. (C) The immature xylem consisting of xylem mother cells and immature xylem are then collected from the exposed stem after which the maturing xylem is collected (image modified from Anderson-Gunneras 2003)

For the tension wood experiment, two-year-old GUSAP1 clonal ramets were sampled. The potted ramets were grown and bent under shade netting. Four time points were sampled: 6 hrs, 48 hrs, one week and one month after bending. Four potted trees were used as biological replicates at each time point. The two trees in each biological replicate were positioned opposite one another and anchored at their bases. Bending of the stems was achieved by attaching a rope to the two tree tops and pulling the crowns together. The rope was then tensioned to the point where each main stem was bent at approximately a 45° angle (Figure 2.3A & B). Xylem (outer 4 mm) comprising the early and late maturing xylem and phloem samples were collected in the same manner as above from the upper tension wood (TW) side where each main stem was bent approximately at 45° from vertical (Figure 2.3B) and from the underside (opposite wood, OP).

Figure 2.3 Tension wood plant materials: (A) a pair of bent trees as well as two vertical control plants, (B) the bent region of the stem that was sampled, (C) the development of tension wood on the upper side of the stem in a two year-old bent stem and (D) sampling of the xylem from a bent stem.

2.3.2 RNA isolation

For total RNA extraction from the respective tissues, several protocols were tested, with the modified CTAB RNA extraction method described by Chang *et al* (1993) being the most successful in recovering RNA from non-lignified and lignified tissues (Shi and Chiang 2005). The quality and quantity of the total RNA isolated was determined using the ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies) followed by resolving the total RNA on a 1.5% RNase-free agarose gel.

2.3.3 Northern blot analysis of miRNAs

Northern blot analysis of the whole-tree experiment required 25 µg of total RNA from each tissue. For the whole-tree experiment, one tree was comprehensively sampled. The RNA was resolved for four hrs at 300V on 15% urea denaturing polyacrylamide gels using the Biorad ProteanII xi gel system. To confirm equal loading, the gel was stained with EtBr, visualised and photographed. Thereafter the gel was trimmed and measured. Chromatography filter paper (3 mm) cut approximately 7-9 mm wider and longer than the trimmed gel was soaked in 0.5X TBE along with a nitrocellulose membrane of the same dimensions and used to make a gel sandwich. The gel sandwich was placed on a semi-dry blotter (Sigma). The total RNA was transferred to the nitrocellulose membrane (GE Healthcare, Amersham Hybond^{Tm}-N^{+}) for one hour at 500 mA. The RNA was then UV cross-linked to the membrane using 0.2 J (UVItec, UV link CL 508 ultraviolet crosslinker) after which the membrane was baked for 1 hour at 80°C. A radioactively labelled RNA molecular weight standard

(Decade marker system, Ambion) was used as a size standard. The molecular weight standard was prepared as directed in the user manual, with 6 µl being loaded per gel.

The probes synthesized were complimentary to the mature miRNA sequence (Table S2.1) and radioactively labelled at the 5' end using [γ-^{32}P] ATP as follows: 20 µM nonphosphorylated probe, 2 µl 10X Kinase buffer (Promega), 10 units T4 polynucleotide kinase (Promega), 2-5 µl [γ-^{32}P] ATP (5 µCi.ml^{-1}) depending on the age of the radioactive isotope. RNase free H_2O was used to make up a final volume of 20 µl. Labelling was done at 37°C for 1 hour, after which the probe was purified using the Nanosep 3k Omega Column (PALL) as per the manufacturers' instructions. All probes and primers were synthesised and cartridge-purified by Inqaba Biotechnical Industries.

The membranes were pre-hybridised in Amersham™ Rapid-Hyb buffer (GE Healthcare) for 20 min. Following that, the labelled probes were added to the Rapid-Hyb buffer and the hybridisation occurred overnight. Once hybridisation was completed the membrane was washed three times. The first wash was in 50 ml of 5X SSC and 0.1% SDS solution for 20 min at room temperature. The subsequent two washes were in 50 ml 1X SSC and 0.1% SDS at 42°C for 15 min each. Each membrane was exposed to a phosphor plate (Kodak) for 1-3 days after which the image was visualised on the Phosphor-Imager (BioRad) using Quantity One (Version 4.4.0) software (BioRad). The membranes were stripped in 50 ml 1% SDS at 80°C for 30 min and re-probed with another miRNA probe. This process was repeated up to four times per membrane.

2.3.4 cDNA synthesis for RT-qPCR analysis

Reverse transcribed quantitative PCR analysis was used as an independent alternative for the Northern blot analysis and to profile those miRNAs expressed at levels too low for Northern blot analysis. The protocol developed by Shi *et al* (2005) was used for its sensitivity in detecting mature miRNAs. The total RNA of the four potted plants for each time point was pooled and then processed for RT-qPCR analysis. In overview, 2 µg total RNA was treated with DNaseI to remove any DNA contaminating the samples as follows: 80 U RNasin® (Promega) and 30 U RNase free DNaseI (Roche) was

combined with 2 µg total RNA in a 20 µl reaction and incubated at 37°C for 30 min. The Poly (A) Tailing Kit (Ambion) was used to polyadenylate 1 µg DNaseI digested total RNA in a 20 µl reaction for 1 hour at 37°C as directed in the manufacturers' protocol. The polyadenylated total RNA was then purified by phenol-chloroform extraction and subsequently recovered through ethanol precipitation. The recovered RNA was then used in a reverse transcription reaction using 200 U SuperScript™ III (Invitrogen) reverse transcriptase and 0.5 µg of a polyT-VN anchored adaptor oligonucleotide (Table S2.2) as per the manufacturers' instructions, to generate first strand cDNA. First strand cDNA samples from all of the samples were then used for expression analysis of the miRNAs using quantitative real-time PCR (Lu *et al*. 2005b).

2.3.5 RT-qPCR analysis of miRNA abundance levels

The mature miRNA sequences were used as the forward primer (Table S2.2). If the miRNA sequence had a run of three or more G/C bases within the last five bases an additional A was added to ensure specificity of priming and amplification. A primer complimentary to that of the poly-T adaptor used in the first strand cDNA synthesis served as the reverse primer in the RT-qPCR reaction (Figure 2.4). The 5.8S rRNA was selected as the endogenous reference gene against which the miRNA results were normalized. To avoid skewing of miRNA abundance patterns resulting from differences in amplicon size, the 5.8S forward primer was designed to yield a product of the same approximate size as that of the miRNA amplicon.

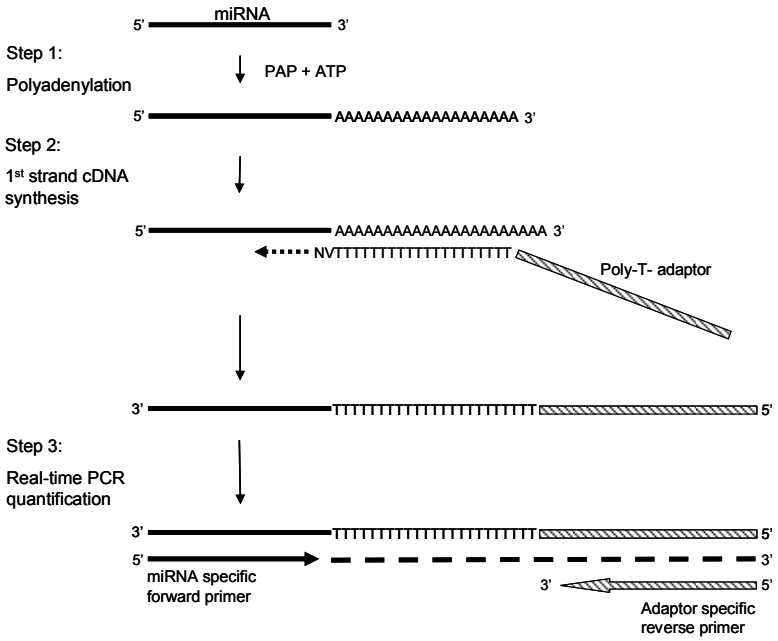


Figure 2.4 A diagrammatic representation of the polyadenylation and real-time PCR process used in the RT-qPCR analysis of the miRNAs in the GU hybrid (simplified from Shi and Chiang 2005). PAP = 3'-phosphoadenosine 5'-phosphosulphate sulfotransferase

Real-time quantification was performed using the LightCycler® 480 instrument (Roche) and the LightCycler® 480 SYBR Green I master kit (Roche). Half reaction volumes (10 µl) of the kit were used for miRNA quantification. The RT-qPCR reactions were optimized for the amount of template required per reaction by means of a serial dilution for each template: a 1:10 dilution was found to be optimal. A reaction consisted of 5 µl 2X LightCycler® 480 SYBR Green I master mix (Roche), 1 µl 1:10 diluted cDNA, 0.5 µM of both the forward and reverse primer, and water was used to make the reaction up to a final volume of 10 µl. The PCR conditions were as follows: sample denaturation and enzyme activation for 10 min at 95°C; 40 cycles of amplification for 5 s at 95°C, primer annealing for 10 s at 60°C, elongation for 5 s at 72°C; followed by melting curve analysis for 20 s at 95°C and for 20 s at 60°C and thereafter continued increase to 95°C with 10 acquisitions per 1°C. The RT-qPCR products were then resolved on a 2% agarose gel to confirm the presence of a single PCR product for

each of the primer combinations. Internal standards were generated by using the gel purified PCR products recovered using the Qiaquick® gel extraction kit (Qiagen) in a serial dilution. This was performed for each of the miRNAs being investigated. The initial concentration for each of the PCR products post gel purification was calculated using the ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies). Standard curves were produced using the second derivative maximum method included in version 1 of the LightCycler 480 software package (Roche). For normalization of the expression data, the 5.8S rRNA was selected as the reference gene. All reactions were done in triplicate and negative controls were included for all primer combinations. These consisted of water replacing the first strand cDNA template.

Standard curves were generated for each of the miRNAs and the 5.8S rRNA reference gene from a serial dilution. Relative quantities of each miRNA and the 5.8S rRNA were generated using version 1 of the LightCycler 480 software package (Roche). The relative quantity data were exported to Microsoft Excel where graphical representations of the relative quantities were generated.

2.4 Results

2.4.1 Whole-tree profiling of conserved and putatively novel miRNAs in *Eucalyptus*

We investigated whether any of the miRNAs were differentially expressed in a total of nine tissues (shoot tip, flowers, mature leaves, twigs, xylem, immature xylem, phloem, bark and roots). These where analysed to ensure that a representative whole-tree expression profile was established for each of the miRNAs. The tissues were compared in three broad groups, the bi-directional cambial developmental series (BDS, xylem, cambium and phloem, Figure 2.2), the green crown tissues (shoot tip, flower, mature leaf and twigs), or in the bark and roots. An increase or decrease of a particular miRNA in the xylogenic tissues (BDS) relative to the non-xylogenic tissues, or across the BDS, was used as an indication of potential miRNA involvement in the regulation of wood formation. The

presence of significant levels of vascular tissue in the green twigs and flowers may result in the abundance profiles of some of the miRNA analysed to be more similar to those of the BDS than the other crown tissues. To ensure the reliability of the findings, Northern blot and RT-qPCR analyses were performed. The high sensitivity of RT-qPCR analysis allowed the detection of miRNAs that were too lowly expressed to be detected by Northern blot analysis. Beside the differences in sensitivity, the Northern blot and RT-qPCR results were highly comparable, with only a small number of discrepancies being observed between the two techniques.

RNA isolation from whole-tree samples

Total RNA was isolated from the nine whole-tree tissues listed above. Yields ranged from 2-4 $\mu g.ul^{-1}$ and 200-400 µg per sample. To determine and ensure the quality of the total RNA, the samples were resolved on a 1.5% RNase-free agarose gel (Figure 2.5) and NanoDrop optical density (OD) ratio 260/280 readings of between 1.9-2.1 were recorded.

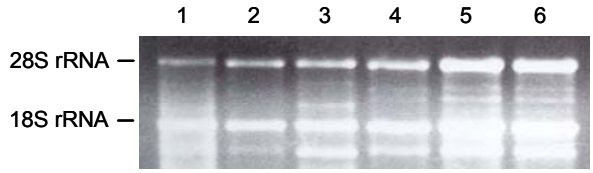


Figure 2.5 Examples of total RNA samples for the whole-tree experiment, lane 1- shoot tip, lane 2- twigs, lane 3- mature leaves, lane 4- xylem, lane 5- immature xylem, lane 6- phloem

Northern blot and RT-qPCR analysis

All miRNAs were detected by RT-qPCR. Northern blot analysis detected all but two miRNAs, miR90 (Figure 2.8J) and miR293 (Figure 2.8K), with a third, miR362 (Figure 2.8L), being too poorly detected to be used for analysis (results not shown). This is reflected in the low RT-qPCR relative abundance levels of miR90 and miR293, but not miR362. miR168 (Figure 2.8H) and miR408 (Figure 2.8G) were also poorly detected by Northern blot, but the results are shown. In general, there was no clear clustering of the miRNA abundance profiles across tissues, but within the three broad categories

(crown, developmental series and other) several patterns discussed below became evident. The miRNAs exhibited a wide range of relative expression values across the tissues sampled. There were some contrasting patterns of miRNA abundance such as miR166 (Figure 2.8E), which was barely detectable across the xylem-phloem developmental series, while miR172 (Figure 2.8F) was detected at relatively high levels in these tissues.

Normalisation of miRNA abundance profiles at the whole-tree level

To ensure equal loading of the total RNA on the PAGE gels, the gels were stained in EtBr and visualised under UV light. Equal levels of fluorescence of the 28S, 18S and 5.8S rRNA bands across the whole-tree tissue series were used as an indication of equal loading (Figure 2.6). To confirm equal transfer of the total RNA from the gel to the membrane, a 5.8S rRNA-specific probe was used to detect the rRNAs levels in each of the samples. For RT-qPCR analysis, equal amounts of total RNA were used as input for the first strand cDNA synthesis. The 5.8S rRNA was once again used as the reference gene (Figure 2.7). This was done to control for efficiency of cDNA synthesis (reverse transcription) and loading differences across the wells of the real-time PCR plate.

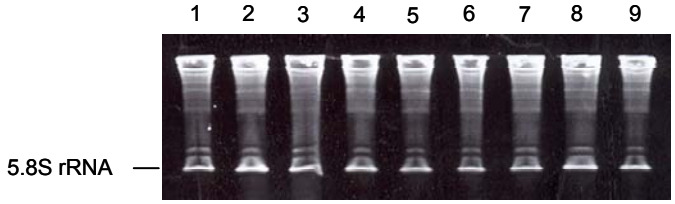


Figure 2.6 EtBr staining and visualisation of the nine whole-tree tissues total RNA on a 12% denaturing PAGE gel prior to transfer to a nitrocellulose gel. lane 1- shoot tip, lane 2- flowers, lane 3- mature leaves, lane 4-twigs, lane 5- xylem, lane 6- immature xylem, lane 7- phloem, lane 8- bark, lane 9- roots

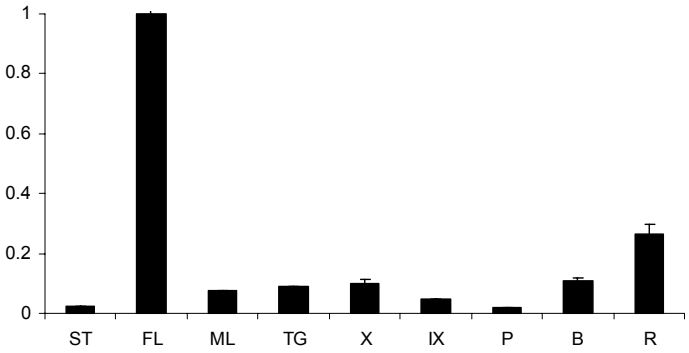


Figure 2.7 Raw 5.8S rRNA values used to normalise the relative abundance of the miRNAs in the real-time PCR analysis. Comparatively high levels of 5.8S rRNA were detected in the flower tissue. Independent replication confirmed this to be the correct value for the flower tissue. The x-axis represents the nine tissues sampled and the y-axis represents the raw values generated (relative to that of the leaf tissue) for the 5.8S rRNA standard curve. The error bars represent ± SE of three technical repeats.

miRNA abundance profiles at the whole-tree level

The first group of miRNAs profiled were those predicted to play a role in phytohormone signalling. RT-qPCR analysis of miR159 (Figure 2.8A), known to target gibberellin responsive MYBs, suggested that miR159 was slightly more abundant in the crown tissues, with highest abundance in the mature leaves. The Northern blot results confirmed this. miR167 (Figure 2.8B), which was previously shown to target auxin response factors (ARFs), was much more abundant in the green crown tissues, except in the twigs. Northern blot analysis also suggested increased expression in the roots, but RT-qPCR did not detect a similar increase. Across the BDS, miR167 is comparatively lowly expressed. In contrast to this, the levels of miR160 (Figure 2.8C), which targets different ARFs, where highest in the phloem and then decreased across the BDS with the lowest levels detected in the maturing xylem. This is possibly an example of regulation through an expression gradient. The lowest levels of miR160 were detected in the bark and roots. This was confirmed by the Northern blot analysis.

Of the miRNAs targeting transcription factors (TFs) miR164 (Figure 2.8D) regulates three NAC TFs. miR164 has a similar abundance pattern to miR160 at the whole-tree level with it being

differentially expressed across the BDS with the highest levels detected in the phloem. Comparatively low levels of miR164 were observed in the developing flowers and roots. The flower and root levels were confirmed by Northern blot analysis. However, Northern blot analysis suggested much higher expression of miR164 in the bark than detected by RT-qPCR analysis. The next two miRNAs, namely miR166 (Figure 2.8E) which regulates a number HD-ZIP III TFs and miR172 (Figure 2.8F) regulator of the floral ABC gene *AP2*. These miRNAs had opposing patterns of mature miRNAs levels. miR166 was most abundant in the crown tissues (except flowers), while miR172 was highest in the BDS with that of the phloem being the highest. miR166 was barely detectable in the BDS of the GU hybrid and was absent from the xylem and immature xylem tissues. Northern blot analysis suggested higher expression of miR166 in the bark than in the shoot tip and leaf tissues, but this was not supported by RT-qPCR analysis, which failed to detect the expression of miR166 in the bark.

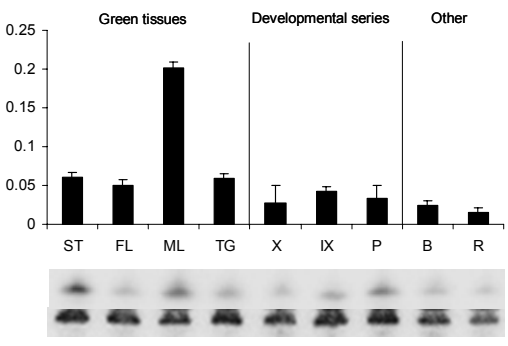
miR408 (Figure 2.8G) regulates a lignin polymerising plastocyanin, which makes it a strong candidate for involvement in wood formation (Kim *et al*. 2005; Ko *et al*. 2006) miR408 had a similar pattern of miRNA abundance to that of miR166 (Figure 2.8E), which has also been implicated in the control of xylem development (Rhoades *et al*. 2002). The highest levels of miR408 were observed in the crown tissues, excluding the developing flowers, while low levels of miR408 (up to four-fold lower) were observed in the immature xylem and xylem with slightly higher levels in phloem. In contrast to miR166, miR408 abundance levels in roots were similar to that in the green tissues. Due to the repeated poor quality of the blots for miR408 (Figure 2.8E) more emphasis was placed on the RT-qPCR results.

Difficulty was also experienced in the detection of miR168 (Figure 2.8H) using Northern blot, so the RT-qPCR results was used as primary data. miR168 regulates the expression of AGO1, serving as a negative feed back mechanism of miRNA biogenesis (Jones-Rhoades and Bartel 2004). The highest levels of miR168 were detected in the green tissues (shoot tips and mature leaves) of the tree. The lowest levels were observed in the maturing and immature xylem within the BDS with similar levels observed in the bark and roots.

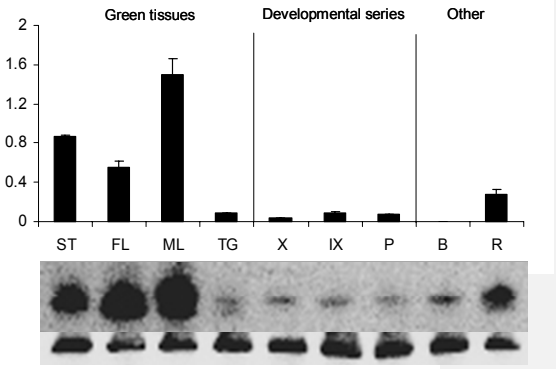
A number of related disease resistance genes are targets of miR472 regulation (Ko *et al*. 2006). Relatively consistent levels of miR472 (Figure 2.8I) were observed in all tissues during Northern blot analysis with slightly lower levels being detected in the shoot tips and roots. RT-qPCR did however not detect the same levels of miR472 in the flowers, bark and roots. miR293 (Figure 2.8J) is also predicted to target a disease resistance gene, but (except for equally low expression in bark and roots) its whole-tree abundance profile was quite different to that of miR472 (Figure 2.8I). The RT-qPCR results indicated that the highest level of the miR293 was present in the shoot tip and flowers and it was present in comparatively low levels in the maturing xylem. The different abundance profiles of miR472 and miR293 may reflect differences in the expression of their target genes.

miR362 (Figure 2.8K), a putatively novel *Eucalyptus*-specific miRNA had comparatively high levels of mature miRNA in the bark and roots relative to the remaining whole-tree tissues. The extremely low levels to which miR90 (Figure 2.8L), another putative novel *Eucalyptus*-specific mRNA was expressed (the lowest detected during RT-qPCR analysis), it was not possible to detect it using Northern blot analysis. miR90 appeared to have a green tissue-specific (flowers and mature leaves) expression pattern, with it being barely detectable or undetectable in the remaining tissues investigated.

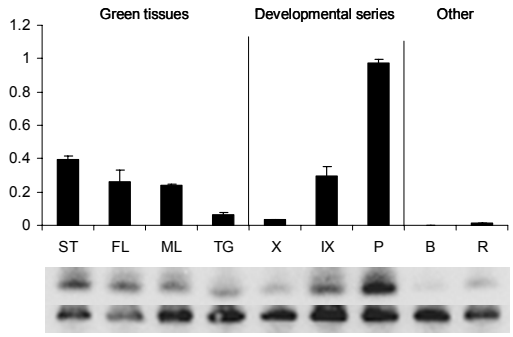
A- miR159



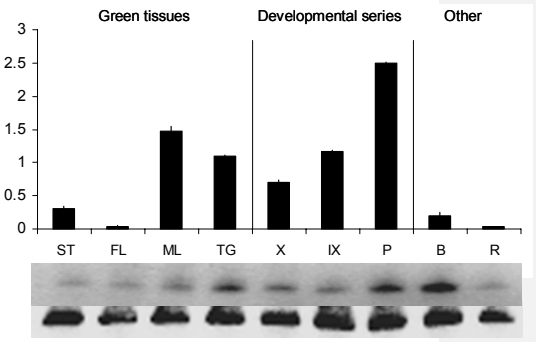
B- miR167



C- miR160



D- miR164



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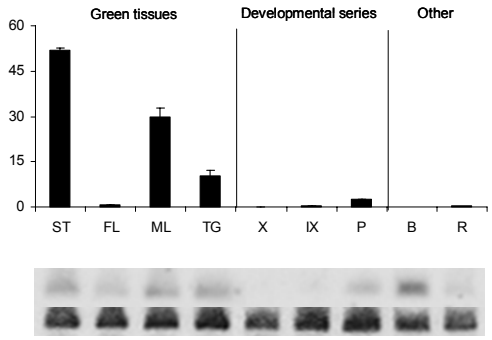
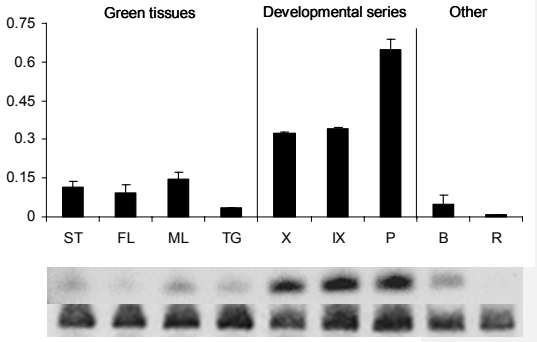


F- miR172



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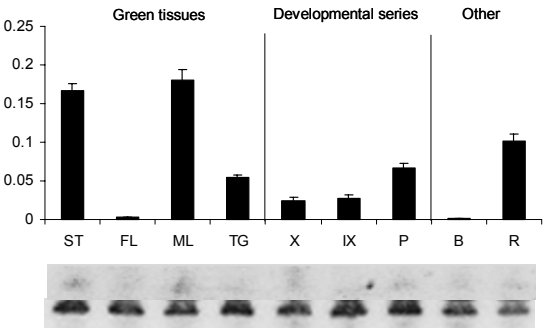

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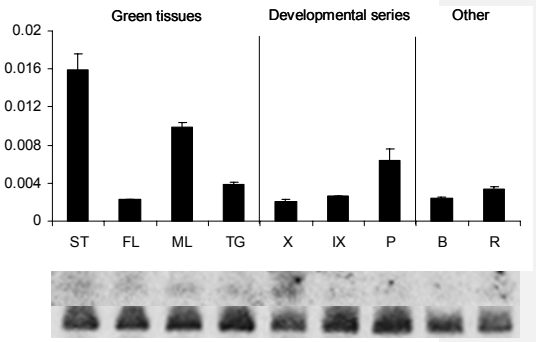
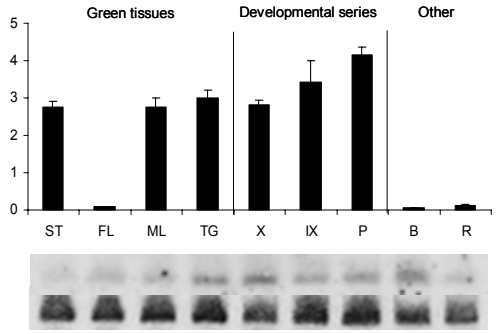


I- miR472



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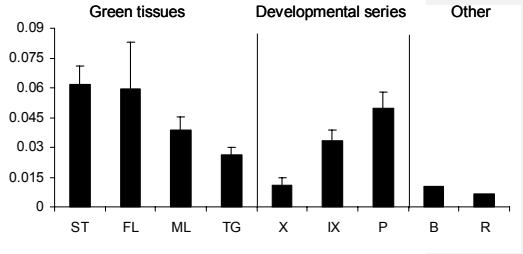
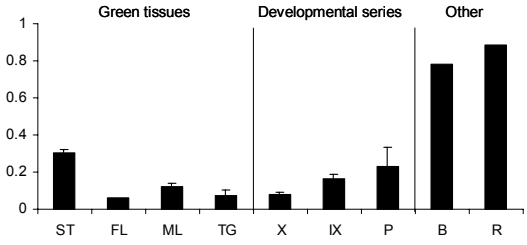


K- miR362



L- miR90

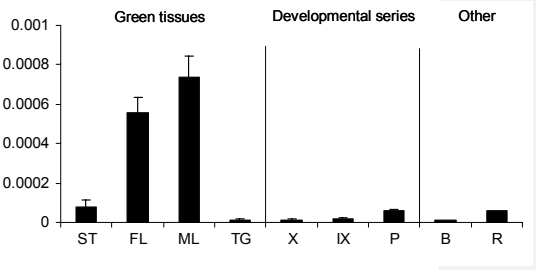


Figure 2.8 Whole-tree abundance profiling of *Eucalyptus* miRNAs. The expression levels were determined by RT-qPCR (graphs) and Northern blot analysis (gel images below graphs). (A) miR159, (B) miR167, (C) miR160, (D) miR164, (E) miR166, (F) miR172, (G) miR408, (H) miR168, (I) miR472, (J) miR293, (K) miR362, (L) miR90. The RT-qPCR results of the miRNAs were standardised to the 5.8S rRNA reference gene levels in the same tissue (Figure 2.7). The x-axis represents the tissues analysed and the y-axis the relative abundance values. The error bars represent ± SE of three technical replicates. The Northern blots results are set out with the miRNA blot above that of the control 5.8S blot. A single 5.8S blot was performed for each membrane. This was used for all miRNAs analysed on the same membrane.

2.4.2 Expression profiling of miRNAs in response to tension wood induction

Tension wood (TW) is characterised by many chemical and morphological changes not associated with normal wood development. Opposite wood (OP) does not undergo the same marked changes, but may still differ in gene expression and development from normal wood. This suggests that changes in signalling and the subsequent gene expression in the OP and TW side of the stem or branch may play a role in the maintenance of apparently normal growth in OP, but result in radical changes to the TW side of the branch or stem. It was of interest to determine if any of the miRNAs that were differentially expressed over the bi-directional developmental series (BDS, xylem-cambium-phloem) in normal tissues were also differentially expressed on both sides of the bent stems.

Experimental design

A significant increase or decrease of a particular miRNA across the BDS in bent stems was used as an indication of potential miRNA involvement in the regulation of tension wood formation. The TW experiments results were further compared to miRNA levels in gravity (horizontal) and vertical (unbent) controls. This was done to determine if the observed changes in miRNA levels were results of gravitational or mechanical stress responses in *Eucalyptus*.

The experimental design used to evaluate the response of miRNAs to mechanical stress was to bend four potted ramets for each of the predetermined time periods (6 hrs, 48 hrs, one week and one month) such that all of the trees could be sampled on the same day in the shortest period of time (i.e. bending was started at different times starting from one month to 6 hrs before sampling). This was done to remove or minimise the possible diurnal and temporal effects during sampling and allow comparison of all of the time points to the same upright control. The upright control was represented by two ramets grown alongside the bent trees in order to compare normal wood development to that of TW (and the horizontal gravity controls). This would enable us to differentiate between miRNA abundance patterns in normal wood and TW development. Gravity controls were included at 48 hrs and one week. Each gravity control consisted of two ramets placed on the ground, with their stems fully supported along the length to ensure no mechanical stress and that the stems were parallel to the

ground. Gravity controls were not included at the one-month time point as gravity responses are predominately short term in nature. Here upper and lower samples were collected from the xylem for the same number of trees as the bending experiment. The data generated from this assisted us in differentiating between gravity and mechanical stress-induced changes in miRNA abundance. This study provides a series of snap shots into miRNA responses to bending stress and gravity responses.

RNA isolation from TW and OP xylem and phloem samples

Total RNA was isolated for all bent ramets from each of the sampling time points. In each case, four tissues were collected (tension wood xylem, TWX, opposite wood xylem, OPX, tension wood phloem, TWP and opposite wood phloem, OPP). The total RNA from the vertical (unbent) and horizontal (gravity) controls was isolated in the same manner, except for the vertical control, where xylem and phloem samples were bulked from all sides of the stem. Yields ranged from 2-4 $\mu g.\mu l^{-1}$ and 200-250 µg per sample. To determine and ensure the quality of the total RNA, the RNA was resolved on 1.5% RNase-free agarose gels (Figure 2.9A & B) and NanoDrop optical density (OD) at ratio 260/280 readings of between 1.9-2.2 were observed.

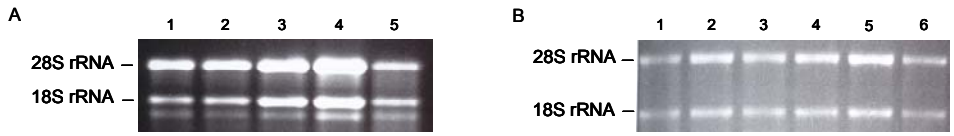


Figure 2.9 Examples of (A) total RNA isolated for the tension wood experiment in xylem (lane 1- TWX 0 hr, lane 2- OPX 0 hr, lane 3- TWX 6 hrs, lane 4- OPX 6 hrs, lane 5- TWX 48 hrs) and (B) Tension wood experiment in phloem (lane 1- TWP 0 hr, lane 2- OPP 0 hr, lane 3- TWP 6 hrs, lane 4- OPP 6 hrs, lane 5- TWP 48 hrs, lane 6- OPP 48 hrs).

RT-qPCR analysis of miRNA abundance in the tension wood induction series

The total RNA from the same tissues for each of the four ramets sampled at the same time point were pooled and used in the RT-qPCR analyses of all 11 miRNAs profiled. The raw 5.8S values (Figure

2.10) indicated that for the xylem samples 5.8S was more abundant in the TWX compared to the OPX (Figure 2.10A). There was no clear pattern in the OP samples (Figure 2.10B) for raw 5.8S values. The variation observed between the OP and TW 5.8S levels was not expected as it is considered a housekeeping gene. It was therefore assumed that the differences observed were truly results of differences in the efficiency of the various steps involved in cDNA template generation for miRNA profiling (e.g. polyadenylation and reverse transcription) and that these differences would affect the miRNAs in the same tissue equally. The remaining five miRNAs were analysed using RT-qPCR only. The same methods of normalisation as those used in the whole-tree profiling were applied to the tension wood experiment. When comparing transcript abundance of a particular miRNA across the xylem and phloem samples, some miRNAs had similar patterns across the time series, while others had opposite abundance profiles (e.g. miR159, Figure 2.11A). Within the xylem and phloem tissues, miRNAs had a number of distinctive patterns including differential expression between TW and OP samples (e.g. miR167, Figure 2.11C), early or late responses to bending (e.g. miR408, Figure 2.11E) and, in a few cases, miRNAs did not respond to bending (e.g.miR167, phloem, Figure 11C).

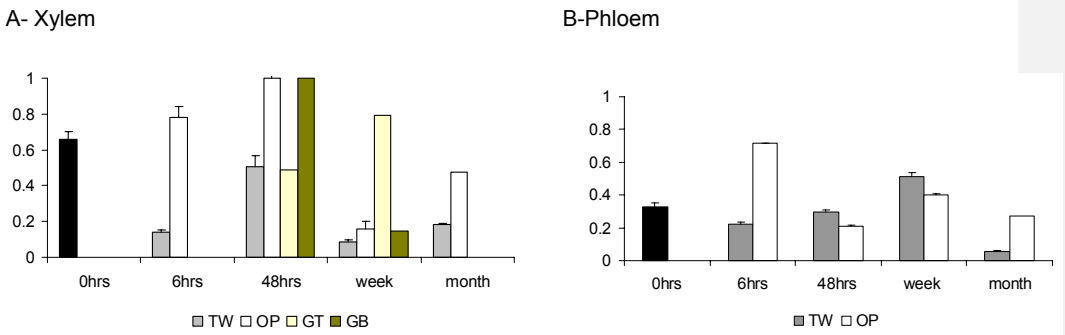


Figure 2.10 Raw 5.8S rRNA values used to normalise the relative abundance of the miRNAs in the real-time PCR analysis. (A) TWX and OPX values, (B) TWP and OPP values. The x-axis represents the tissues and time points analyzed and the y-axis the raw 5.8S abundance values. The error bars represent ± SE of three technical repeats.

miRNA abundance profiles in response to bending

Three of the miRNAs profiled in the TW series were predicted to respond to phytohormone signalling. miR159 (Figure 2.11A) regulates two MYB TFs that function in GA signalling pathway. The TWX and OPX samples showed an early (6 hr) decrease of approximately 0.5 fold in miRNA levels when compared to the upright control. After 48 hrs, miR159 levels in the OPX returned to normal, while TWX levels continued to decrease to a minimum at one week. In the phloem samples, miR159 levels in OP remained at that of the upright control, while there was a two-fold increase in miR159 abundance 48 hrs post bending in the TWP. The 48 hr xylem gravity controls revealed a reduction in miR159 abundance on the upper and lower sides of the stem which was similar to the reduction observed in TWX. This may point to miR159 in some way being associated with a response to gravitational stimuli at early time points. Additional reduction of TWX abundance of miR159 after one week may reflect an additional effect of mechanical stress and TW induction (over the effect of gravity).

miR160 (Figure 2.11B) and miR167 (Figure 2.11C) target several ARFs in auxin signalling pathways and so may be involved in stem elongation and secondary growth. As auxin is known to play a central role in a trees response to gravity these two miRNAs may also serve as indicators of the ability to distinguish between miRNA gravity and tension wood responses. The levels of both miRNAs increased four to six-fold in the TWX relative to the control 6 hrs post induction, but by 48 hrs both were approaching normal levels again. miR160 levels again increased up to six-fold by the one-week time point, whereas miR167 only increased about 0.5 fold at the same time. For both miRNAs, the lower xylem gravity controls showed significantly higher abundance than the upper gravity controls at 48 hrs suggesting a strong response to gravity. The lower gravity control sample for miR160 in particular showed a strong (five fold) increase in miRNA abundance over the upright control (Figure 2.11B). The increased levels of both miRNAs in the lower gravity samples suggest that they both restrict the expression of their targets in response to gravitational stimulus in the underside of horizontal stems. However, there seemed to be an opposite effect in the bent samples which experienced a mixture of gravity and tension stimuli.

miR160 abundance in OPX samples did not differ significantly from that of the vertical control at 6 hrs and 48 hrs, but after this the levels increased three fold after one week of bending. In contrast to the TWX sample, the TWP sample showed 50% reduction in miR160 abundance 6 hrs post bending where after it increased to levels only marginally above that of the vertical sample at 48 hrs. From this point the TWP and OPP samples showed decreasing levels of miR160. miR167 levels in the phloem fluctuated around that of the vertical control for the duration of the trial.

miR166 (Figure 2.11D) and miR408 (Figure 2.11E) are predicted to play roles in vascular patterning of wood and secondary cell wall structure respectively. This is achieved by negative regulation of several HD ZIPs III TFs (Bowman 2004; Pasquinelli 2002; Pasquinelli and Ruvkun 2002) and predicted lignin polymerising plastocyanins. miR166 levels remained unchanged over the 6 hrs of bending relative to the vertical control. After 48 hrs of mechanical stress miR166 levels doubled in TWX and OPX samples, while the levels of the corresponding gravity samples remained similar to that of the vertical control. By one week miR166 levels in the gravity upper and lower xylem had increased eight-and four-fold respectively with the upper gravity levels being three-fold higher than the TWX. At the same time point TWX levels had not changed from that observed at 48 hrs, while OPX levels increased to the same level as the gravity lower sample. By the end of the trial miR166 TWX levels were six-fold of the vertical control and the OPX eight-fold. In the phloem samples miR166 increased eight-fold in the TWP after 48 hrs of mechanical stress relative to the vertical control. At the remainder of the time points investigated, the phloem sample levels of miR166 were similar to the vertical control. This data suggests that miR166 may initially respond to TW induction in the phloem, but later response in the xylem appeared to be driven by gravity induced signalling

miR408 TWX levels initially decreased (four-fold) at 6 hrs relative to the vertical control, then increased to maximum levels, three-fold that of the vertical control one week after induction. OPX levels returned to that of the vertical control by this time. miR408 levels were reduced

approximately three-fold relative to the vertical control in all gravity samples. In the TWP miR408 was initially reduced (6 hrs), then stabilised around that of the vertical control, miR408 responded in a similar manner in the first 48 hrs, thereafter it increased reaching levels twice that of the vertical control one week after TW induction. These results point to a potential role for miR408 regulation in TW development particularly a week into tension wood induction.

The conserved miRNAs miR172 (Figure 2.11F) and miR472 (Figure 2.11G) target unrelated genes, miR172 the floral regulator *AP2* and miR472 a number of related disease resistance genes. Yet they had similar abundance patterns in the xylem for the first 48 hrs during which the miRNAs levels decreased to approximately 50% of the vertical control. miR172 subsequently peaked (two-fold increase relative to the vertical control) one week after bending, while miR472 levels returned to normal. After one month, the levels of both miRNAs were approaching those observed 48 hrs into the bending experiment. The miR172 TWP and OPP samples had opposite patterns of miRNA abundance. TWP levels of miR172 peaked 48 hrs and one month post–induction. Whereas OPP levels peak one week after tension wood induction. miR172 gravity control levels fluctuated between 90% and 60% relative to the vertical control. The reduced levels of miR172 a week after gravity induction and the increased (two-fold) levels in the TWX and OPX at the same time point suggest opposite response to bending and gravity by miR172 in the later stages of TW development. The opposite expression of TWP and OPP may indicate that miR172 was required in different samples at different times to modulate the trees response to mechanical stress. The miR472 levels in the gravity control samples remained consistently below 50% that of the vertical control possibly indicating a miR472 response to gravity stimulus in *Eucalyptus*.

In the case of miR168 (Figure 2.11H), which regulates miRNA biogenesis via a negative feedback mechanism by regulating *AGO1* expression. The gravity controls samples did not show any response to being placed horizontal relative to the ground. The TWX abundance profile of miR168 appeared to peak one week post-induction (2.5-fold), while the OPX levels remain relatively constant around that of the vertical control. TWP levels peaked 48 hrs after bending commenced after which miR168 levels decreased to well the below those detected for the up-right control. TWP levels

decreased to a third of that observed in the vertical control after a month of mechanical stress. The increased levels of miR168 in the TWX at one week, the minimal changes in levels of the gravity controls and the reduced levels in late TWP may indicate miR168 involvement in TW responses during bending.

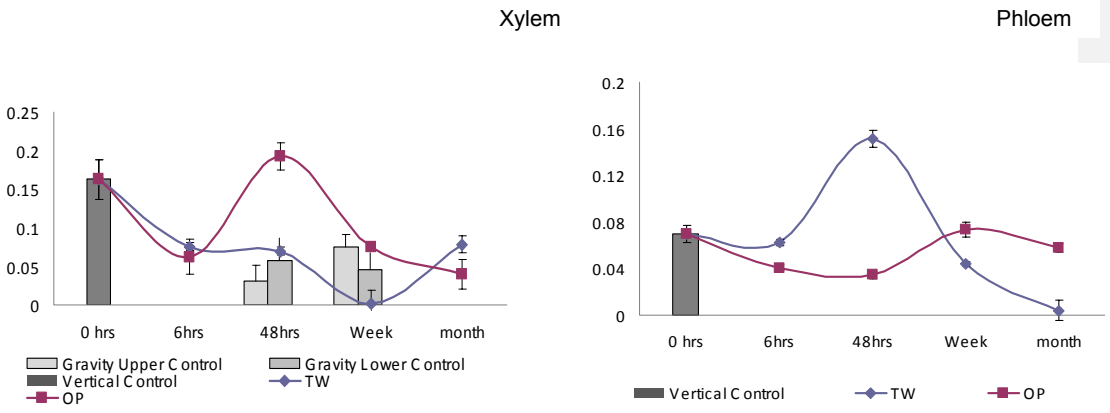
The regulation of spatial expression of three NAC TFs is achieved by miR164 (Figure 2.11I). miR164 levels increased nine-fold 48 hrs post-induction in the TWX and 12-fold in OPX relative to the vertical control. Both tissues then showed a reduction from 48 hrs miRNA abundance by a little over 50% a week into the bending experiment. After one month miR164 abundance increased again. The analysis of miR164 in the phloem tissues found that levels increased in TWP by three-fold within 48 hrs of initiating bending stress. Thereafter the transcript levels stabilized at that just below that of the upright tree. In the controls for miRNA response to gravity, there was a four-to five-fold increase in miR164 abundance. Forty eight hours after induction of the bending experiment TWX and OPX levels were twice that of their corresponding gravity controls. After a week OPX levels in the bending experiment were reduced to those of the gravity control, with the TWX levels below those of the upper gravity sample. It appears that miR164 responds to both gravitational and bending, with gravity probably the primary induction agent. However, the up-regulation of miR164 in OPX and TWP after 48 hrs of bending may also point to tension wood response in early tension wood development in the phloem.

The three putatively novel *Eucalyptus* miRNAs were profiled. Bioinformatics analysis predicted miR90 (Figure 2.11J) to regulate a MADS-box family protein or an ubiquitin protein ligase 1. miR90 OPX levels remained near to that of the vertical control. The TWX miR90 levels increased at 48hrs after bending, but returned to approximately normal a week of bending. At one week miR90 levels in the upper gravity control were elevated to twice that of the vertical control. In the phloem tissues, miR90 increased in abundance two-fold relative to the vertical control 6 hrs post-bending in the TWP. Thereafter TWP miR90 levels continue to decrease throughout the period of the trial, decreasing to a quarter of vertical controls level. The miR90 OPP level increased after 48 hrs of

bending and then decreased. The up-regulation of miR90 at 48 hrs in TWX and 6 hrs in TWP suggest miR90 responds to mechanical stress, primarily in early TW development.

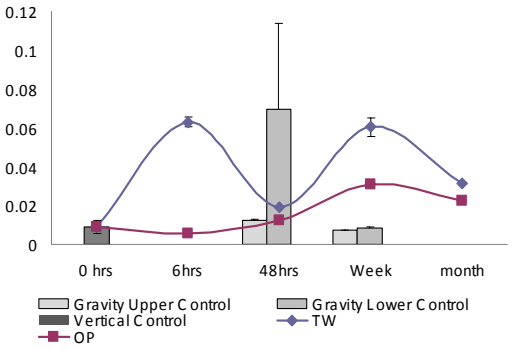
In TWX, the expression of miR293 (Figure 2.11K) and miR362 (Figure 2.11L) were similar and peaked twice during the trial period at 6 hrs and one week respectively. OPX levels remained constant, at the level observed in the vertical control for miR293. miR362 OPX abundance decreased slightly for the first 48 hrs, thereafter miR362 reach a maximum level, twice that of the vertical control after a week of bending. By one month, OPX levels were back to normal. Within 48 hrs of bending, miR293 decreased in abundance in the phloem tissues to 30% of that of the vertical control. After a week of bending TWP miR293 levels increased to 50% more than that of the vertical control, while OPP levels remained below 50% of the vertical control. At the last sampling, miR293 levels were back to those observed 48 hrs post-induction. miR362 levels in the TWP decreased 50% and OPP 70% relative to the vertical control after 6 hrs of bending. OPP levels varied slightly around this level for the remainder of the trial period. After a week of bending TWP levels increased to 60% of the vertical control and remain in this region for the remainder of the trial. These finding may indicate that miR293 and miR362 play a role in TW xylem development by clearing target transcripts from the TWX but not OPX.

A - miR159

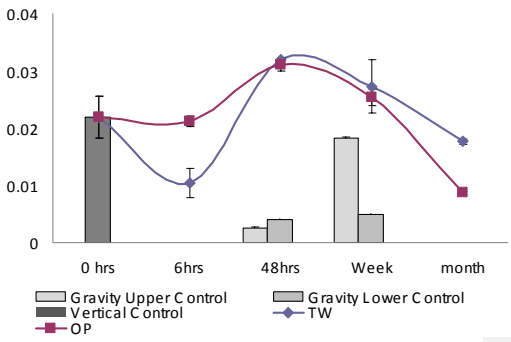


B - miR160

Xylem

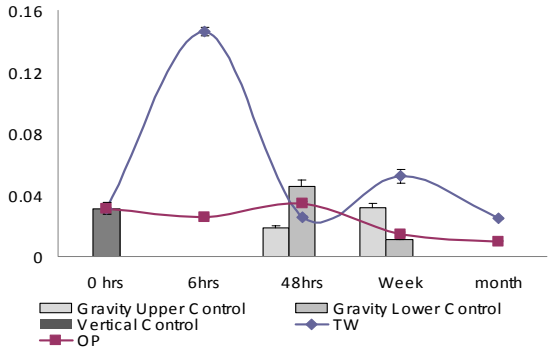


Phloem

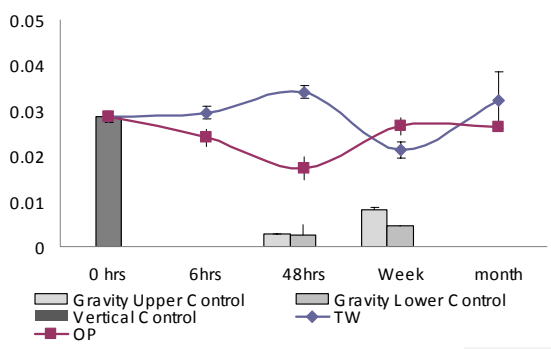


C - miR167

Xylem

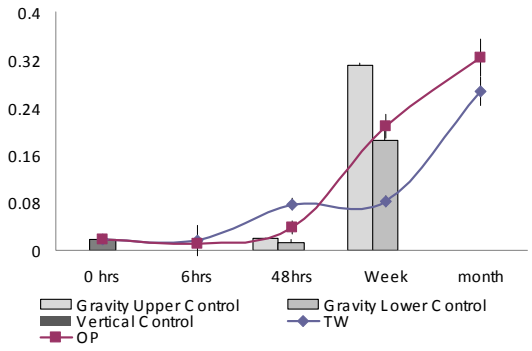


Phloem

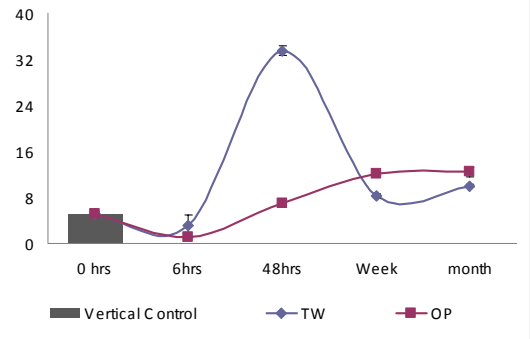


D - miR166

Xylem



Phloem



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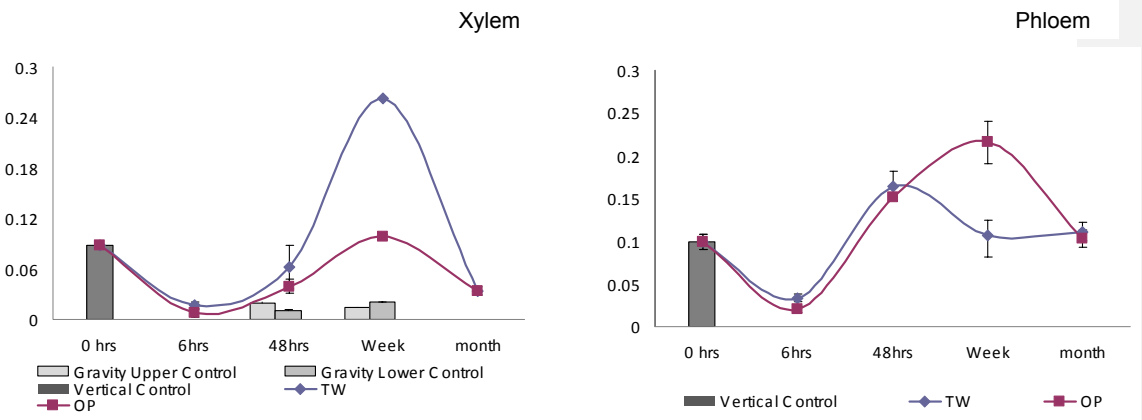
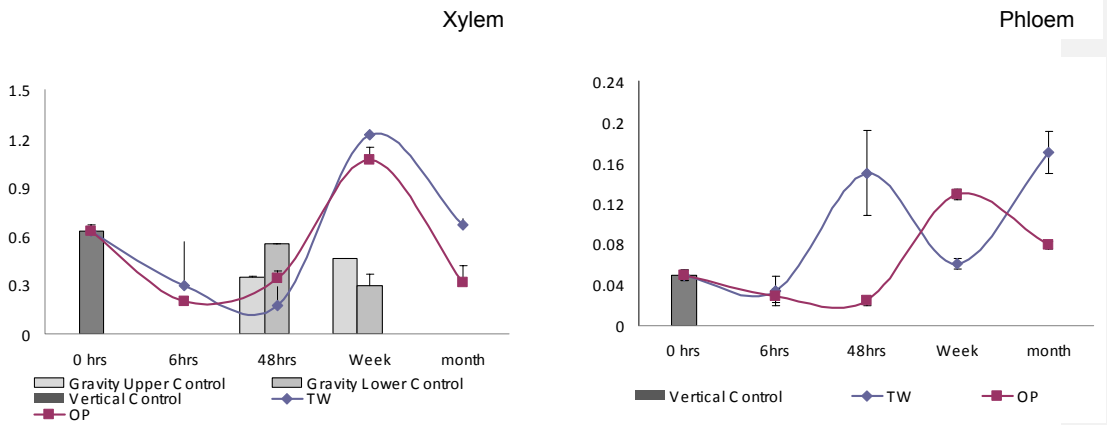
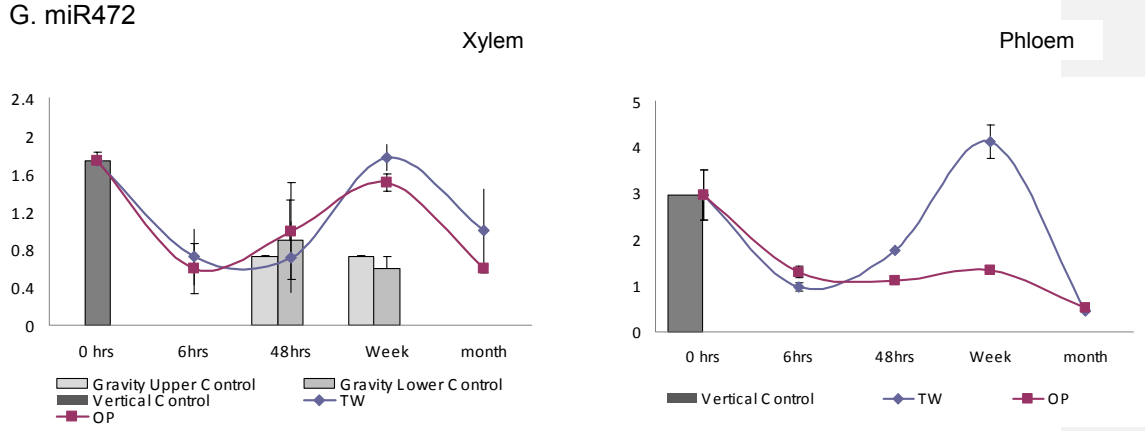


F - miR172

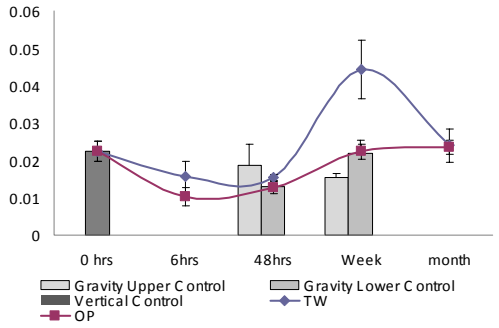


G. miR472

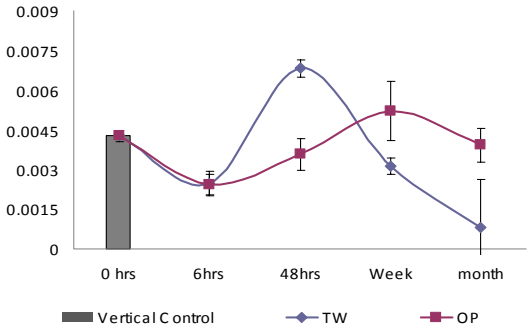


H - miR168

Xylem

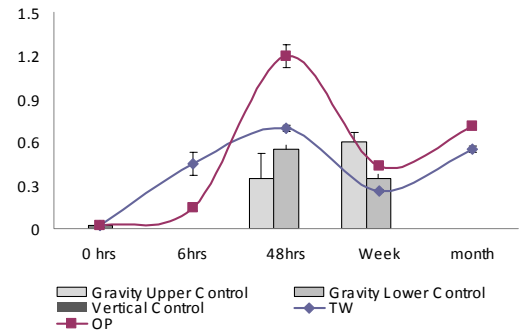


Phloem

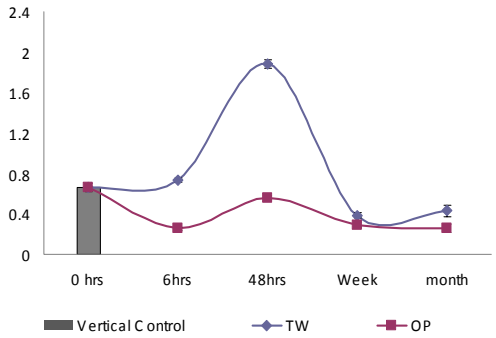


I - miR164

Xylem

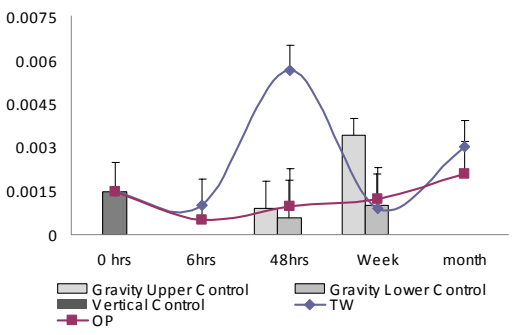


Phloem

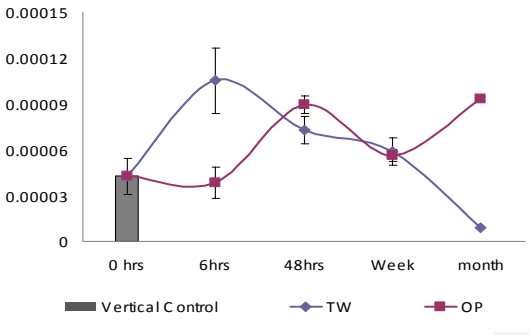


J - miR90

Xylem



Phloem



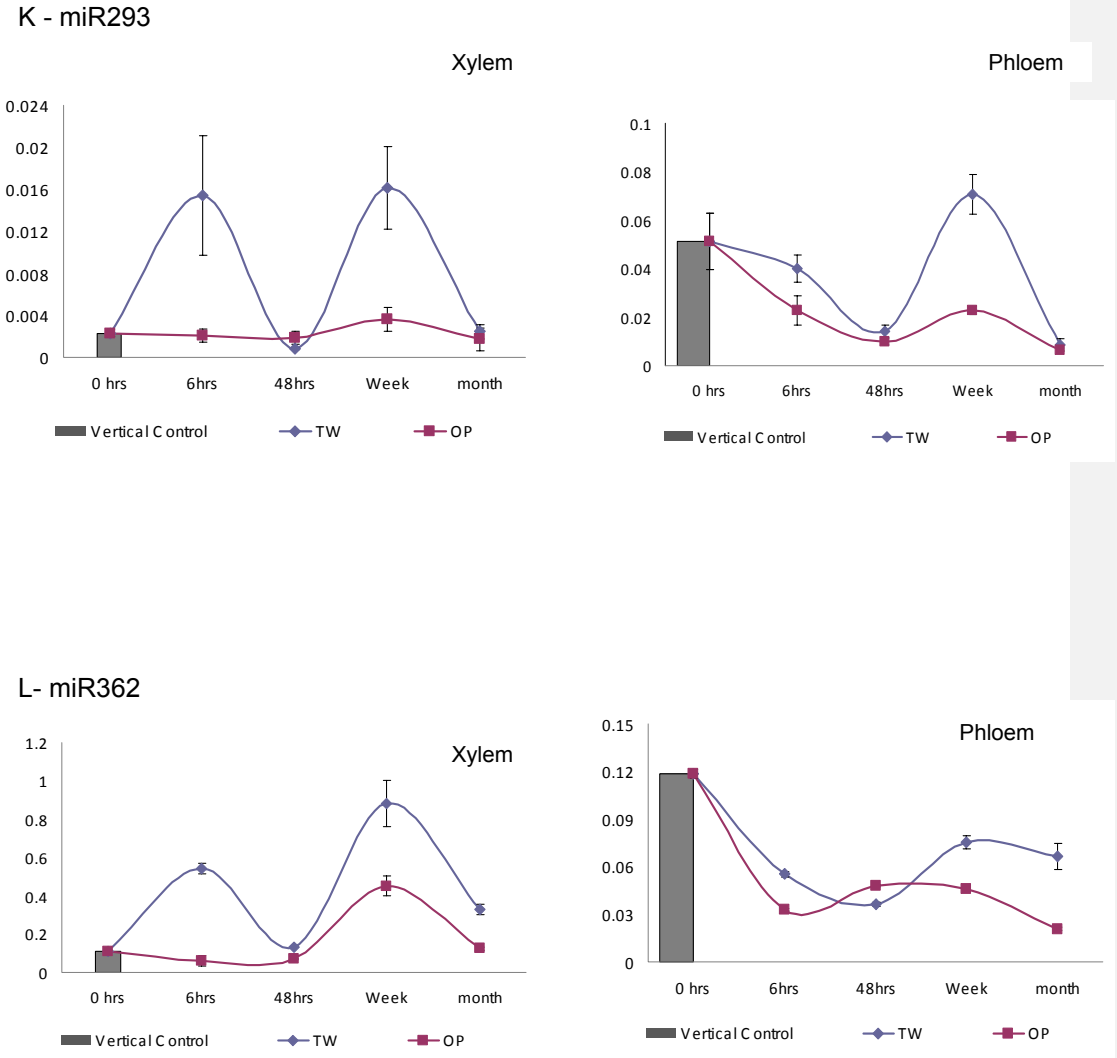


Figure 2.11 Expression profiles of *Eucalyptus* miRNAs in response to tension wood induction and formation and to gravity. The expression levels were determined by RT-qPCR. (A) miR159, (B) miR160, (C) miR167, (D) miR166, (E) miR408, (F) miR172, (G) miR472, (H) miR168, (I) miR164, (J) miR90, (K) miR293, (L) miR362. The RT-qPCR results of the miRNAs were standardised to the levels of the 5.8S rRNA reference gene levels in the same tissue (Figure 2.10). The x-axis represents the tissues analysed and the y-axis the relative expression values. The error bars represent ± SE of three technical replicates.

2.5 Discussion

microRNAs are a functionally diverse group of small non-coding RNAs that are of ancient origins (Jones-Rhoades *et al*. 2006). A number of miRNAs have been functionally characterised in plants. These only represent a small fraction of miRNAs identified in plants and are mostly comprised of conserved miRNAs (Chiou 2007; Chiou *et al*. 2006; Jones-Rhoades and Bartel 2004; Jones-Rhoades *et al*. 2006; Liu *et al*. 2008; Lu *et al*. 2005a; Lu *et al*. 2008; Lu *et al*. 2005b; Lu and Huang 2008; Sunkar and Zhu 2004; Zhou *et al*. 2008; Zhou *et al*. 2007). This leaves large numbers of miRNAs, particularly those thought to be novel, in need of further study and characterisation. To begin to understand the biological roles of miRNAs, it is necessary to determine where, when and to what level miRNAs are expressed within the various tissues and organs of the plant (Bartel and Bartel 2003; Carrington and Ambros 2003; Chiou 2007; Jones-Rhoades *et al*. 2006; Kidner and Martienssen 2005a; Ko *et al*. 2006; Lu *et al*. 2008; Lu and Huang 2008; Mallory *et al*. 2005; Sunkar and Zhu 2004), and how miRNAs respond to various stresses including mechanical stress (Ha *et al*. 2008). The task of assigning function is further complicated by interspecies differences in miRNA expression that in turn results in varying degrees of functional differentiation between species (Aukerman and Sakai 2003; Emery *et al*. 2003; Juarez *et al*. 2004; Kidner and Martienssen 2004; Ko *et al*. 2006; Mallory *et al*. 2004; McConnell and Barton 1998). This makes it difficult to assign functions to miRNAs in an automated fashion and further necessitates the evaluation miRNA function at the genus level at the very least. miRNAs function in most plant developmental processes, including the development of wood (Ko *et al*. 2006). Apart from miR166, no other miRNAs are confirmed to function in wood development although several have been suggested to play a role in wood developmental processes and stress responses associated with wood formation (e.g. miR408, miR160, miR167, reviewed by Lu and Huang 2008). The complexity of, and sequential overlapping nature of wood development processes makes this a tantalising target for miRNA mediated regulation. The precision of miRNA regulation, achieved by spatial and temporal regulation of miRNA expression, the stability of the mature miRNAs make then ideal candidates for the regulation of wood development.

The aim of this study was the determination of miRNA levels and subsequent abundance profiles in *Eucalyptus*. This was done at the whole-tree level and in the xylogenic tissues of trees under mechanical stress. The purpose was the further refining of the role of the miRNAs in *Eucalyptus* development and particularly wood formation. Whole-tree miRNA abundance profiles were generated to build an understanding of miRNA abundance patterns throughout the tree. Once completed, the vascular tissues of tension wood induced trees were analysed at four time-points over a period of one month, potentially identifying miRNA regulatory role in wood development. The results were compared to those of trees sampled at the same time growing under normal conditions. To distinguish between tension wood and gravity induced responses in the trees, gravity controls were included at 48 hrs and one week. These consisted of potted ramets being placed on their sides, parallel to the ground. The whole-tree, tension wood and gravity data were interpreted, while taking previous research into account to infer possible role for the profiled miRNAs in wood development under normal and mechanical stress conditions.

For the quality control of the total RNA use in this study, the total RNA was resolved on agarose gels and quantified using NanoDrop technology. Both confirmed the high quality of the total RNA isolated for this study. The used of a single tree in the whole-tree experiment is a limiting factor, preventing biological replication of these results. The decision to pool the total RNA from each of the tissues in biological replicates of the tension wood experiment was because of cost and capacity restrictions. The discrepancies observed between the RT-qPCR and Northern blot results may be explained by hybridization of the probe or RT-qPCR primer to more than one member of a miRNA family. Generally RT-qPCR primers are specific to a particular family member. Probe and primer stability may also partially explain these differences as one can only use the mature miRNA sequence or its compliment when designing the primers and probes respectively. Furthermore, the RT-qPCR results were normalised to the abundance of the 5.8S rRNA in that sample, whereas the Northern blot results were visually normalised to the 5.8S levels of that particular Northern blot.

2.5.1 A role for miRNAs in the development of wood in *Eucalyptus* as observed at the whole-tree level?

The whole-tree expression profiling experiment sampled clonally propagated *Eucalyptus* hybrid genotypes to remove genotypic variation from the study. The use of the same clonal genotype in the whole-tree and TW experiments made it possible to compare the results directly. During the sampling process, all possible measures were taken to prevent degradation of the plant material. Plant materials were collected at a single time point to limit the effect of circadian and diurnal rhythms. This sampling approach therefore only allows for a glimpse into miRNA functioning at the point of sampling. It is highly likely that at least some miRNAs will be regulated in a circadian or diurnal fashion and this will not be detected in this study. qRT-PCR results may be skewed by differences in amplicon size, to avoid this the 5.8S forward primer was designed to yield a product of the same approximate size as that of the miRNA amplicons. For normalization of the expression data, the 5.8S rRNA gene was selected as the reference gene.

Discussion and interpretation of whole-tree abundances profiles in *Eucalyptus*

To more fully understand miRNA function in terms of wood formation and whole-plant development the abundance profiles of the selected miRNAs were first determined at the whole-tree level in order to better interpret the results observed in the vascular tissues of *Eucalyptus* during the mechanical stress experiment. The conserved miRNAs, most have been functionally characterised in non-woody plant models. An exception is miR166 which has been functionally characterised in *Populus* (Aloni 1979; Millar and Gubler 2005; Rhoades *et al*. 2002; Ridoutt *et al*. 1996; Uggla *et al*. 1996). Therefore, it was necessary to determine the abundance patterns of the miRNAs (miR90, miR159, miR160, miR164, miR166, miR167, miR168, miR172, miR293, miR362, miR408 and miR472) in *Eucalyptus*.

The TW results were interpreted as described in the results section of this chapter. For the discussion of the results to follow it is important to note that transcriptional regulation of the target genes is primarily through its promoter and the regulatory mechanisms associated with the promoter. miRNA regulation serves to fine-tune this expression, allowing for rapid clearance of target

transcripts from the cell or tissue type facilitating the correct spatial and temporal expression of the target gene. An increase or reduction in mature miRNA levels does not necessarily indicate miRNA regulation of the corresponding target gene, although this is generally the case.

miR159, miR160 and miR167 are known to function in the auxin and gibberellin signalling pathways (Harrison and Klein 1979; Jacobs and Case 1965; Little *et al*. 2002). These pathways together and singly have been found to play important roles in plant development and specifically wood formation (Millar and Gubler 2005). miR159 is a confirmed negative regulator of *MYB33* and *MYB65* which are gibberellin (GA) responsive transcription factors (Olszewski *et al*. 2002). In plants, GA is essential for the normal progression and maintenance of the majority of plant developmental and growth processes, including stem elongation, circadian regulation, seed germination and the regulation of flower development (Aloni 1979; Digby and Wareing 1966; Eriksson *et al*. 2000; Harrison and Klein 1979; Jacobs and Case 1965; Little *et al*. 2002; Ridoutt *et al*. 1996). In wood development, GA signalling networks regulate the development of the xylem and phloem from the vascular cambium and play a role in xylem fibre elongation (Jover-Gil *et al*. 2005). This was further supported by Eriksson *et al* (2000), who generated transgenic hybrid poplar lines over-expressing GA. These lines had an increase in biomass and growth rate, but negatively affected root initiation.

MYB33 and *MYB65* have been implicated in the regulation of floral organ development, in particular that of the anthers (Millar and Gubler 2005). Millar and Gubler (2005) found that *MYB33* was completely absent in the shoot apical meristem (SAM). It is likely that a similar situation exists in *Eucalyptus* as the highest levels of miR159 detected by RT-qPCR analysis were in the mature leaves (Figure 2.8A, Table 2.1). Interestingly, the low levels of miR159 were detected in the BDS, particularly the mature xylem when compared to the crown tissues. This may point to the role miR159 plays in the regulation of the GA responsive transcription factors in the cell elongation process. The observation that cleavage resistant *MYB33* mutant *Arabidopsis* lines were smaller than the wild type controls (Hagen and Guilfoyle 2002; Liscum and Reed 2002) indicates the importance of correct miR159 regulation in plant growth, particularly stem elongation and potentially a role for miR159 in stem elongation.

The phytohormone auxin is involved in the regulation of numerous developmental and growth process through the interaction with various ARFs. ARFs function by binding to AuxREs, these are conserved sequences located within the promoters of genes required for the appropriate response to a specific stimuli (Ulmasov *et al*. 1999). One of the main targets of auxin are early response genes i.e. genes that are required for the rapid response to external and internal stimuli (Swarup *et al*. 2002). These genes in turn activate various signalling cascades (Wang *et al*. 2008), including GH3-mediated homeostasis required for response to various stresses via complex auxin signalling networks. GH3 belong to the third class of ARFs, which responsible for the catalizating the adenylation of specific substrates so modulating auxin homeostasis (Hardtke and Berleth 1998; Little *et al*. 2002). These hormone-gene interaction cascades are centred around interactions of ARFs and AUX/IAA transcriptional regulator proteins (Uggla *et al*. 1996). As with GA, auxin plays a role in the regulation of stem elongation and potentially wood formation (Hartweck 2008). GA has been found to positively affect the production of auxin and vice versa (Friml 2003) and as such they are integrally linked in wood development.

Auxin is produced in the shoot tips and then transported to its point of action via the phloem (Nilsson *et al*. 2008). This may explain the high levels of miR160 (Figure 2.8B) present in the phloem as reduced sensitivity may be required to prevent erroneous activation of auxin induced responses in the transport tissue. It has been suggested that it is not the degree of change in auxin levels, but rather the change itself that initiates an auxin response (Teichmann *et al*. 2008). Further support for the role of miR160 as a regulator of wood development is the decreasing levels of miR160 from the phloem to the mature xylem (Table 2.1). The levels of miR160 in the phloem were the highest detected in the GU hybrid for miR160 (Figure 2.8B) and is supported by the Teichmann (2008) report that a GH3::GUS construct in poplar was highly expressed in the maturing vessels and in cells surrounding maturing phloem, but not in the immature phloem itself, despite high auxin levels (Mallory *et al*. 2005). These findings further support a role for miR160 in xylogenesis as comparatively low levels of mir160 were observed in the xylem, similar results were observed in poplar by Lu *et al* (2005). *ARF17* is a positive regulator of two further GH3s involved in stem elongation and potentially

secondary cell wall development, *GH3*-2 and *YDK1* (Park *et al*. 2007; Wang *et al*. 2008). Mutation studies of these genes result in plants with shortened hypocotyls when grown both in the light and dark (Tian *et al*. 2004). Consequently, alterations to *ARF17* expression would in turn affect the expression down stream targets. The above results along with previous research supports the role of miR160 regulation of auxin responses and GH3s, so regulating plant growth, particularly stem elongation.

ARF8 was the first ARF identified in the regulation of GH3s in *Arabidopsis* where it positively regulates three GH3s in response to auxin signalling (Staswick *et al*. 2005) with the GH3s, in turn inactivating auxin (Yang *et al*. 2006). miR167 (Figure 2.8C) acts as a negative regulator of *ARF6* and *ARF8* (Woodward and Bartel 2005a; Woodward and Bartel 2005b). This demonstrates the close link between auxin responses and changes in auxin concentration (Nagpal *et al*. 2005) and the fine tuning required. The justification for the strict regulation of ARF6 and ARF8 is made apparent by Nagpal *et al* (2005) who demonstrated that the two genes regulate the timing of flower maturation in a dosage dependent manner (Sundberg and Uggla 1998). This explains the high levels of miR167 observed in the developing flowers (Figure 2.8C).

The high concentration of miR167 in the mature leaves, flowers and shoot tip (Table 2.1) may be explained by the fact that auxin is produced in these meristematic tissues of theses organs and is subsequently distributed to the rest of the plant. The major source of polar transported Indole acetic acid (IAA) for secondary stems is the apical shoot (Bjorklund *et al*. 2007). Little *et al* (2002) showed that auxin flow from the apex promotes the growth of interfasicular cambium in *Arabidopsis* stems and is required for correct secondary xylem development. The observed low levels of miR167 in the green twigs (representing internode one and two) are consistent with these findings as interfasicular cambium is initiated very early on in (likely in the first internode) in *Eucalyptus*. The observed high levels of miR167 in the mature leaves and shoot tip are likely to prevent erroneous induction of auxin responses in the tissues that produce auxin.

A major sink for auxin in polar transport is the cambial meristem and the xylogenic tissues derived from it and it has been postulated that auxin is an endogenous regulator of wood formation processes (Nilsson *et al*. 2008). Nilsson *et al* (2008) observed that there was little correlation between auxin concentration in the vascular cambium and its derived xylogenic tissues with the level of gene expression in those tissues. The maturing secondary xylem cells expressed auxin responsive genes to higher levels than those observed in the cambium (Baima *et al*. 2001; Emery *et al*. 2003). This may explain the low levels of miR167 in the BDS as well as the twigs and roots of the *Eucalyptus* hybrid (Figure 2.8C), which both have significant amounts of secondary vasculature. This may in turn indicate a requirement for miR167 regulation of auxin signalling in the development of secondary vasculature in *Eucalyptus*.

miR166 and miR408, target genes directly involved in wood development and cell wall formation processes. Several HD-ZIP III proteins are required for establishing the central identity of the stem through correct vascular bundle patterning (Carlsbecker and Helariutta 2005; Emery *et al*. 2003; Engstrom *et al*. 2004; Fukuda 2004). These same HD-ZIPs III subsequently determine of the adaxial fate of the lateral organs (Bartel and Bartel 2003; Ko *et al*. 2006; Ohashi-Ito *et al*. 2005). Three HD-ZIPs III, *REV, PHB* and *PHV* are all targets of miR166 regulation (Aukerman and Sakai 2003; Emery *et al*. 2003; Juarez *et al*. 2004; Kidner and Martienssen 2004; Mallory *et al*. 2004; McConnell and Barton 1998; Nogueira *et al*. 2007; Nogueira *et al*. 2006). Ko *et al* (2006) showed the regulation of *PtaHB1* to be similar to that of adaxial–abaxial control of vascular development, thus regulating bark to xylem differentiation from the cambial meristem. Our results supports this, the highest levels of miR166 (Figure 2.8E, Table 2.1) were detected in the bark, with lower levels being present in the phloem, while miR166 was undetectable in the mature and immature xylem. The detection of miR166 in the mature leaves is also consistent with its regulation of adaxial–abaxial development of leaves. Failure of miR166 regulation in leaves results in strongly adaxialised leaves (Lu *et al*. 2005b; Sunkar and Zhu 2004).

Plastocyanins are predicted to play a role in lignin polymerisation during xylogenesis (Lu *et al*. 2005b). Two plastocyanins (At2g47020, At2g02850) were validated as targets of miR408

regulation (LaFayette *et al*. 1999; Mayer and Staples 2002). One would therefore expect to detect lower levels of miR408 (Figure 2.8G) in the xylogenic tissues of *Eucalyptus*. This was indeed the case with the lowest levels being detected in the maturing xylem, followed closely by the immature xylem (Table 2.1). The relatively high levels observed in the shoot tip further supports the potential for the targeted plastocyanins to be involved in xylogenesis and that miR408 plays an active role in the regulation of these plastocyanins. Schwab *et al* (2005) confirmed through modified 5' RACE that a laccase is a predicted target in *Arabidopsis*. Laccases along with peroxidases are secreted into the secondary cell walls of xylogenic tissues where they polymerize monolignols in the presence of oxygen (Kidner and Martienssen 2004; Kidner and Martienssen 2005b; Mallory *et al*. 2008). Therefore, it is likely that miR408 plays an integral role in the later stages of xylogenesis. The low levels of miR408 in the bark are in line with the highly lignified nature of bark, while the low levels of miR408 in the flowers is likely a result of their morphology in *Eucalyptus*. *Eucalyptus* flowers are very woody and have large amounts of secondary cell wall material.

miR168 is essential for maintaining a functioning miRNA regulatory pathway. miR168 over-expression mutants resemble those of *AGO1* loss-of-function mutants (Liu *et al*. 2008; Lu and Huang 2008; Zhou *et al*. 2007) resulting in a large number of plant abnormalities indicative of the diverse and vital roles of miRNAs in plant growth and development. Studies investigating the role of miRNAs in response to stress found that miR168 potentially responds to certain stresses, including UV-B radiation, drought, low temperature and high salinity (Jones-Rhoades *et al*. 2006; Vaucheret 2006; Vaucheret *et al*. 2004). From these studies it is clear that miR168 (Table 2.1) is required for more than just the maintenance of miRNA regulation, but rather plays an essential role in the fine tuning of miRNA regulation within the plant, in response to stress, in the various organs and tissues and at the cellular level (Olsen *et al*. 2005). The variable expression pattern observed at the whole-tree level for miR168 (Figure 2.8H) would be consistent with this hypothesis.

miR164 and miR172 target transcription factors that belong to families known to play roles in wood development, but they target family members not known to directly play a role in wood development. miR164 is confirmed as the negative regulator of *CUC1* and *CUC2* TFs. It is required

in combination with its targets to specify boundaries of gene expression with regard to cell differentiation in the shoot apical meristem (Laufs *et al*. 2004). miR164 was also found to target *NAC1* for translational regulation, but only by miR164b. Over-expression of cleavage resistant *CUC2* resulted in overall smaller plants, with numerous leaf abnormalities (Guo *et al*. 2005; Laufs *et al*. 2004; Mallory *et al*. 2004). The low levels of miR164 (Figure 2.8D, Table 2.1) in the shoot tip, flowers and roots are consistent with miR164 role in the normal development and maintenance of these tissues (Eckardt 2005; Guo *et al*. 2005; Mallory *et al*. 2004; Rhoades *et al*. 2002; Xie *et al*. 2000). It is also tempting to speculate that miR164 may play a role in xylem development, as the levels of miR164 were comparatively low in the xylem, while that in the phloem and bark were the highest levels in the tree according to the Northern blot results. *NAC1* cleavage by miR164 down-regulates auxin signals in the roots to induce lateral root development, therefore NAC1 functions through auxin induction pathways (Jofuku *et al*. 2005).

The primary role of *AP2* in plant development is the regulation of floral organ development, although it is expressed in all *Arabidopsis* tissues (Aukerman and Sakai 2003; Chen 2004; Jung *et al*. 2007; Lauter *et al*. 2005). This is confirmed by *ap2* mutant lines that have morphological defects other than those associated with flowering (Jiao *et al*. 2008). This taken with the fact that miR172 preferentially binds to target transcripts for translational inhibition rather than target degradation, would suggest that miR172 concentrations are of paramount importance for appropriate *AP2* regulation (Fattash *et al*. 2007; Itaya *et al*. 2008; Lu *et al*. 2007; Lu *et al*. 2005b). The whole-tree profile of miR172 in *Eucalyptus* is interesting. The low level of miR172 (Figure 2.8G) expression in the flowers is in line with the role of *AP2* in flower development. The very high levels of miR172 (Table 2.1) in the xylogenic tissues correspond to the inhibition *AP2* in woody tissues. The failure to detect miR172 in the roots is unexpected, as no function has been attributed to *AP2* in roots. This does not exclude the possibility of alternate forms of gene regulation suppressing *AP2* expression in the roots.

miR472 is predicted to regulate a number of CC/NBS/LRR and TIR/NBS/LRR disease resistance genes and is conserved among plants (Deyoung and Innes 2006; McHale *et al*. 2006). To

date, no cleavage analysis or functional investigations of miR472 have been reported. Two of the predicted targets of miR472 have been investigated in *Arabidopsis*; a CC/NBS/LRR and a TIR/NBS/LRR disease resistance protein respectively (Mendel 1997; Mendel and Schwarz 1999). miR472 (Figure 2.8I, Table 2.1) expression groups into two groups in the Northern blot analysis, the first expressed at a slightly lower level (shoot tip, mature leaves, flowers and roots) than the second group (twigs, xylem, immature xylem, phloem and bark). The highest levels were detected in the xylem tissue and bark. Interestingly, Lu *et al* (2007) failed to clone and identify miR472 from the xylem of pine trees infected with fusiform rust disease making it possible for the targeted resistance genes to be expressed.

The remaining miRNAs discussed are three putatively novel miRNAs, miR90, miR293 and mir362. miR90 was the lowest expressing miRNA profiled. miR90 (Figure 2.8J) was relatively highly expressed in the mature leaves and flowers (Table 2.1) when compared to the remainder of the tissues where miR90 was barely detected using RT-qPCR. miR90 has several predicted targets based on *Arabidopsis* and poplar genome sequence data (Victor *et al*, 2007). These include a MADs-Box transcription factor protein and an ubiquitin protein ligase in *Arabidopsis* as well as unknown proteins in poplar. miR90 may functions to repress its target gene/s in mature leaves and flowers, with the target gene/s being required in the remaining plant tissues.

miR293 has three predicted targets in the poplar genome with none being identified in *Arabidopsis*. They are a disease resistance protein, PAPS reductase involved in the biosynthesis of molybdopterin cofactor (Hancock 2003) and a protein kinase. The high levels of miR293 (Figure 2.8K) detected in the shoot tip and the comparatively low levels profiled in the mature xylem may point to the necessity for the target gene not to be expressed in the shoot tip and mature leaves (Table 2.1). As with miR293, miR362 (Figure 2.8L) only has predicted targets in poplar. These include a calcium-binding calmodulin protein and a member of the Ras GTPase protein family, both involved in signal transduction. Ras GTPases play a role in intracellular signal transduction by associating with the cell membrane (Ehrhardt *et al*. 2002; Reuther and Der 2000). Members of this family are thought to be involved in the regulation of haematopoietic stem cells, and may function in their growth,

survival, differentiation and cytokine production (Liu *et al*. 2008; Zhang *et al*. 2008b; Zhou *et al*. 2008; Zhou *et al*. 2007). The high levels of miR362 in the roots and bark (Table 2.1) suggest that the Ras GTPase or calcium-binding calmodulin genes are not to be expressed in these tissues.

2.5.2 miRNA response and putative involvement in tension wood development in *Eucalyptus*

Recently, there have been a number of investigations directed at discerning the roles of miRNAs in the regulation of responses of plants to various forms of abiotic and biotic stress. These include disease resistance, response to changes in salinity, drought, submergence, UV-B exposure, cold and mechanical stress (Liu *et al*. 2008; Lu and Huang 2008; Reyes and Chua 2007). *Eucalyptus* is a major tree genus used in plantation forestry as its species and hybrids are fast growing and have favourable wood properties. Like other angiosperms, it produces a modified wood tissue in response to mechanical stress. Tension wood differs on a structural and chemical level from normal wood. The reprogramming of wood formation during tension wood production makes it possible to study wood development from a different view. This provides an opportunity to refine our understanding of miRNA function in wood formation. For these reasons, miRNA abundance profiles were determined for the vascular tissues of *Eucalyptus* during a mechanical stress experiment.

The bending schedule was set up in a manner that allowed for the sampling of all trees on the same day over a short period. This made it possible to minimise the potential effects of temporal (diurnal and circadian) variation and allowed us to compare all time points to the same vertical control. The upright control was represented by two ramets grown alongside the bent trees in order to compare normal wood development to that of TW. This was done to identify differences in miRNA abundance patterns in the vascular tissues of trees undergoing normal and TW development. Additional controls for the effects of gravity on miRNA abundance profiles were included in this study as the bending of trees initiates both mechanical stress and gravity in the plant. The time points sampled were 48 hrs and one week, here upper and lower samples were collected from the xylem and phloem for the same number of trees as the bending experiment. The data generated from this would assist in the

differentiation between gravity and mechanical stress-induced changes in miRNA abundance in the bending experiment. This study provides a series of snap shots of miRNA responses to bending stress.

This study aimed to identify differential expression of miRNAs on both sides of the bent stems, in the phloem and xylem. An increase or decrease of a particular miRNA in bent stems was compared to the vertical and horizontal controls used as an indication of potential miRNA involvement in the regulation of tension wood formation. Within the xylem and phloem, differential expression between the tension wood and opposite wood samples was used as an indicator of possible miRNA function in response to mechanical stress.

The potted ramets were grown under shade cloth. Four time points were sampled: 6 hrs, 48 hrs, one week and one month. These time points allow one to detect early and late responses to mechanical stress i.e. regulation during tension wood induction and the subsequent regulation of tension wood production. Previous studies of miRNA response to bending only examined miRNA abundance at a single time point, once tension wood was visible (after three weeks), so skipping over the necessary reprogramming of wood development leading to the production of mature tension wood. Paux *et al* (2005) analysed gene expression in *Eucalyptus* undergoing TW formation at three points during the first week of bending. They found that individual gene expression did indeed vary across this time. Four potted trees were used as biological replicates at each time point although the total RNA isolation were pooled for this study as previously explained. The pooling of the total RNA prevented the biological replication of the analysis. This was done as the number of samples exceeded the capacity and funds to analyse them independently. This study represents the most detail study of miRNAs in TW responses in trees to date, the inclusion of gravity and vertical controls make it possible to distinguish between gravitational and mechanical stress responses observed in the plants.

Discussion and interpretation of miRNA mechanical stress responsive abundances profiles in *Eucalyptus*

miR159 is a negative regulator of GA responsive *MYB33* and *MYB65* TFs. A number of recent studies attempting to identify miRNAs involved in various stress responses including; high soil salinity, cold, submergence and exposure to UV-B (Funada *et al*. 2008) found miR159 to respond in these studies. The wide range of stresses with which miR159 has been implicated is potentially explained by the targets, transcription factors required for gene regulation and signal transduction (Lu and Huang 2008; Reyes and Chua 2007; Zhang *et al*. 2008a).

Tension wood induction is a response to mechanical stress and miR159 (Figure 2.10A) may be involved in the regulation of responses to bending. It was demonstrated that the application of GA to one side of an upright poplar stem was sufficient to induce tension wood formation (Hartweck 2008; Oh *et al*. 2008). Over-expression of miR159 results in transgenic plants being hyposensitive to ABA (Zhang *et al*. 2008a). It is also known that the ABA signalling pathway acts in an antagonistic manner with the GA signalling pathway (Nilsson *et al*. 2008; Swarup *et al*. 2002; Wang *et al*. 2008). Zhang *et al* (2008) further investigated the role of the small RNA-ABA interaction in *hen* and *dcl1* mutant *Arabidopsis* lines respectively. These mutants were hypersensitive to ABA with plants having increased expression of ABA and stress responsive genes and, as expected, the plants where demonstrated to be more sensitive to osmotic and salt stress (Bjorklund *et al*. 2007). These finding make miR159 a potential candidate as a regulator of mechanical responses. The general two-fold reduction in miR159 (Table 2.1) transcripts at 6 hrs and 48 hrs in the TWX was similar to that in the gravity control at this point (Figure 2.10A). The reduction in miR159 levels during mechanical stress and in response to gravity suggests the observed results are most likely associated with the activation of the gravity responses. The lack of miR159 abundance across the BDS in the whole-tree experiment does not suggest a role for miR159 in normal wood development, while the increased abundance (two-fold) of miR159 in the TWP 48 hrs into the bending trial when taking the corresponding decrease in the TWX may indicate a role for miR159 in the differential regulation of TW responses

Auxin functions through changes in its concentration within and across tissues initiating a rapid response and specific actions to counteract and overcome the causative stress (Du *et al*. 2004). One would to expect that a miRNA which regulates the expression of ARFs to respond to mechanical stress, such as miR160. Auxin is also believed to play a central role in the regulation of cambial cell division (Bartel 2004; Jones-Rhoades *et al*. 2006). Du *et al* (2004) suggested that auxin and GA regulate the quantitative production of tension wood fibers and therefore are essential for the formation of tension wood (Mallory *et al*. 2005). Teichmann *et al* (2008) found that despite high concentrations of auxin in the cambium, there was no induction of GH3::GUS until bending stress was applied to the transgenic poplars. We observed a five-fold increase in miR160 (Figure 2.10B, Table 2.1) transcript levels after 6 hrs in the TWX, while at the corresponding time there was a 50% decrease in miR160 levels in the OPX relative to the vertical control. This is in line with the role of miRNAs as a rapid response regulatory mechanism (Park *et al*. 2007; Wang *et al*. 2008). miR160 regulates *ARF17* expression which in turn positively regulates, *GH3*-2 and *YDK1* (Hellgren *et al*. 2004; Park *et al*. 2007; Wang *et al*. 2008) which are required for hypocotyl elongation. *ARF17* also negatively regulates *GH3*-5 and *DFL1* which have been linked to stress responses (Liu *et al*. 2008; Lu *et al*. 2005b; Lu and Huang 2008; Zhou *et al*. 2008; Zhou *et al*. 2007). These findings support a role for miR160 in the regulation of plant elongation and stress response and potentially in regulating TW formation. Further functional characterisation of this miRNA may confirm it as a regulator of TW development

The second increase of miR160 of TW a week post induction may be required for the regulation of genes involved in cellular expansion and growth in the newly forming tension wood as genes involved in cell wall synthesis often only start to show changes in expression a week to three weeks after induction. These include the CesA genes (Paux *et al*. 2005). Hellgren *et al* (2004) observed lower levels of auxin on the OPX side of bent stems than that

of the cambium and the TWX side 15 days after tension wood induction in poplar. This may explain the lower levels of miR160 in the OP samples of both the phloem and xylem as ARFs modulate auxin accumulation and homeostasis. The binding of ARFs to free cellular auxin may be a mechanism for the regulation of free cellular auxin levels (Liu *et al*. 2008) and there a candidate for follow-up investigation.

miR167 is suggested to be a regulator of several stress responses across a number of plant species through the negative regulation of two ARFs, *ARF6* and *ARF8*. These include cold, salinity, mechanical, UV-B and drought stress in *Arabidopsis* and poplar (Zhang *et al*. 2008b). Furthermore, some of the above authors investigated entire miRNA families, finding that different family members showed different responses to a particular stress. The miR167 family is one such miRNA family with miR167a and miR167c responding to cold, drought and salinity stress, while miR167d did not respond to cold stress in *Arabidopsis* (Kim *et al*. 2005; Ko *et al*. 2006). Zhou *et al* (2007) identified a single family member, miR167d to potentially respond to UV-B stress. They went on to identify stress-related motifs present in promoters of miR167d and its target genes. When maize was placed under submersion stress, miR167 was found to be among the early responsive miRNAs (Baima *et al*. 2001; Kang and Dengler 2002; Prigge *et al*. 2005; Zhou *et al*. 2008). miR167 (Figure 2.10C) TWX abundance peaks four-fold higher than that of the vertical control 6 hrs post induction. Subsequently miR167 levels reduced to near that of the vertical control (Table 2.1). The minimal change in OPX miR167 levels and the fluctuation of gravity controls around that of the vertical control may point to a role in early TW formation. miR167 has been implicated in responses to several forms of stress and it is likely that miR167 plays a role in mechanical stress responses.

RT-qPCR analyses of the TWX and OPX, miR166 (Figure 2.10D) increased gradually for the first week of the trial. Thereafter miR166 expression levels increased to 15 times that of the vertical control in the OPX while the TWX increased to a lesser extent. As miR166 regulates xylem formation from the vascular cambium and cell division occurs at a greater rate on TW side of trees under mechanical stress. Changes in *REV* may alter the cellular proliferation rates within xylogenic tissues

as it plays a role in xylem fiber and vessel differentiation (Xie *et al*. 2000; Xie *et al*. 2002). One may expect lower levels of miR166 in TW xylem tissue where increased amount of xylem is required to generate the tensile forces required to right the tree. Zhou (2008) identified three miR166 family members to be putatively involved in response to cold stress and went on to suggest that this regulation may affect auxin signalling pathways. miR166 is the negative regulator of ATHB-8, with ATHB-8 in turn being positively regulated by auxin which results in vascular differentiation and an increase in xylem (Liu *et al*. 2008; Zhou *et al*. 2007). These findings illustrate the interaction of hormone signalling and transcription regulation in wood development. As both these are also targets for miRNA regulation this may represent miRNA regulation at multiple levels. No gravitational response was observed in the first 48 hrs of the trial, by one week miR166 levels in the upper side of the stem were 15 times higher than that of the vertical control and three-fold higher than the TWX at the same time point (Table 2.1). This suggests that the miR166 response observed in the TW trial are likely to be the result of gravity stimuli. The observation that when miR166 is up-regulated in TWX in is down-regulated in the TWP along with further investigation into the role of miR166 in TW development may reveal a TW component to the miR166 response.

AP2, an ABC gene involved in floral development is the target of miR172 regulation. There was however a 50% reduction in miR172 (Figure 2.10G) levels for the first 48hrs post induction in the xylem, followed by increase at one week after which the levels begin to approach that of normal growth again. As miR172 acts predominantly through translational repression, a decrease in miR172 abundance would be expected to result in increased translation of the *AP2* transcripts, with increased levels of miR172 suggesting increasing translation repression. In the phloem tissues, OPP had similar abundance to that of OPX, with the distinction that OPP levels did not drop below that of the vertical. The TWP and OPP had alternation peaks of expression, OPP peaked at one week, while TWP peaked at 48 hrs and one month respectively (Table 2.1). The gravity control levels did not exceed that of the vertical control or drop below 50% of the vertical control level. From this data it is not likely that miR172 is involved in TW development and that changes in miRNA levels may be due to gravitational stimuli.

miR164 targets three NAC transcription factors family. Zhou *et al* (2008) found that the expression levels of miR164 in control and cold stressed plants did not differ significantly. They suggested that miR164a may interact with auxin pathways through *NAC1* as *NAC1* can be induced by an auxin repressor (Lu *et al*. 2005b; Sunkar and Zhu 2004). The observation that miR164 (Figure 2.10I) levels were elevated in the gravity and bending experiment xylem samples relative to that of the vertical control (Table 2.1) suggests miR164 responded predominantly to gravity. The up-regulation of miR164 in the TWP at 48 hrs may point to a role of miR164 regulation in phloem responses to bending. This is interesting as in the whole tree profile miR164 is relatively highly expressed in the phloem when compared to the remainder of the tissues. This might point to a requirement of *NAC1*, *CUC1* or *CUC2* in the re-organization of secondary growth in the stem, possibly in response to auxin signalling in the case of *NAC1*.

As miR168 is involved in the feedback regulatory mechanism of miRNAs and consequently respond to a number of forms of stress including abiotic stress conditions (LaFayette *et al*. 1999; Schwab *et al*. 2005). The approximately stable levels of miR168 in the OPX (Figure 2.10F, Table 2.1) and elevated levels in the TWX a week after bending began along with the reduced levels observed in the TWP point to a potential role for miR168 in the regulation of later TW development.

miR408 is predicted to regulate a plastocyanin that functions in lignin polymerization (Plomion *et al*. 2001) and several laccases (Pilate *et al*. 2004; Timell 1969). Lignin deposition occurs in the last stages of secondary cell wall formation, just prior to programmed cell death (Lu *et al*. 2005b; Paux *et al*. 2005) and one would expect plastocyanins to primarily be required in these latter stages. TW is characterised by massive increases in cellulose, while lignin levels remain unchanged or in many cases is reduced (Lu *et al*. 2005b; Lu and Huang 2008). Tension wood can already be observed by three weeks after bending was commenced (Ko *et al*. 2006). By this point, the xylem fibres and vessels cells have undergone PCD and are functioning to right the tree. Accordingly, one would expect miR408 (Figure 2.10E) levels similar to that of the vertical initially and that once the trees have been placed under mechanical stress, miR408 levels should increase. This was the findings of this study, miR408 levels were elevated in the TWX one week after bending commenced (Table

2.1), Lu *et al* (2005) found miR408 to be up regulated in TWX and OPX. Paux *et al* (2005) found that some lignin biosynthetic genes in *Eucalyptus* undergoing mechanical stress were up regulated in the first 24hrs after which their levels stabilised or decreased,. Our data revealed that miR408 levels were reduced for the first 48 hrs after bending and subsequently increased to maximum levels a week after induction. The reduction of mir408 levels in the gravity xylem samples while miR408 levels were increasing suggested that miR408 serves to down-regulate its targeted plastocyanins in tension wood formation but not in response to gravitational signalling.

The three novel miRNAs were the last to be analyzed. miR90 was the first of the novel miRNAs identified in *Eucalyptus* in this study. Initially miR90 (Figure 2.10J) increases two-fold in the TWP, whereafter it approaches normal levels a week after bending, and then decreasing to 20% of the normal level. In the xylem, TWX increased in abundance to three times that of the vertical control (Table 2.1), whereafter the levels again decreased. Increases in miR90 transcripts may point to the need for the target gene to be down regulated for TW development to proceed. Expression of miR293 (Figure 2.10K) peaked twice in the TWX (Table 2.1), at 6 hrs and a week respectively, a similar pattern is observed for miR362 (Figure 2.10L, Table 2.1) in the TWX. miR293 OPX levels remain at those observed in the vertical control. This suggests miR293 may regulate gene expression at two points in TW formation. One of the predicted targets may not be required for early response to bending, but rather in the later stages of xylem development. miR362 predicted targets are involved in signal transduction. miR362 (Figure 2.10L) levels in the phloem were reduced to less than 50% that of the vertical control within 6 hrs of bending. By one month OPP levels have decreased further to 25% of that seen in the vertical control. This reduction in miR362 levels in the phloem may indicate the requirement of its target in TW responses in the phloem.

Table 2.1 Summary of whole-tree and tension wood miRNA abundance profiling results

miRNA	Whole-tree experiment	Tension wood experiment
miR90	Up-regulated in FL and ML	Up-regulated in TWX (48 hrs) and TWP (6 hrs). Indicating potential involvement in early TW development
miR159	Up-regulated in ML	Appears to primarily respond to gravity stimulus. Potential additional TWX regulation in later development (one week)
miR160	Expression gradient across IX to P	Mixed bending and gravity response in TWX. Reduced levels in gravity phloem samples
miR164	Expression gradient across IX to P	Up-regulated in the mechanical stress and gravity xylem tissue, with largest increase in the OPX. Up-regulated in TWP (48 hrs)
miR166	Absent from IX and X Up-regulated in crown tissues	Up-regulation in early (48 hrs) TWP, late responses in TW appear to be the result of gravity-induced signalling
miR167	Up-regulated in ST, ML and FL	Early (6 hrs) up-regulation response in TWX. Reduce levels in gravity phloem samples.
miR168	Up-regulated in ST	Up-regulate in TWX at one week, down regulated from one week to a month in TWP

miR172	Up-regulated IX, X and P	Up-regulation in bending xylem (one week). Slightly reduced levels in gravity controls
miR293	Low levels in B and R	Up-regulated in TWX (6 hrs and week), may play a role in TWX development
miR362	Up-regulated in B and R	Up-regulated in TWX (6 hrs and week), reduced levels in OPP and TWP
miR408	Low levels in FL IX, X and B Up-regulated in ST and ML	Up-regulated in TWX (one week), reduced levels in xylem gravity controls. Potential regulator of TW cell wall deposition
miR472	Two levels of expression, higher in TG, X, IX, P and B	Down-regulation (50%) in xylem gravity controls. Reduced levels in OPP (throughout)

shoot tips (ST), flowers (FL), mature leaves (ML), maturing xylem (X), immature xylem (IX), phloem (P), bark (B), roots (R) and green twigs (TG)

tension wood (TW), opposite wood (OP), vertical control (vert), tension wood xylem (TWX), opposite wood xylem (OPX), tension wood phloem (TWP), opposite wood phloem (OPP), gravity upper surface (GUPS) and gravity lower surface (GLS)

2.6 Conclusions

This M.Sc study has contributed to the characterization of miRNAs in trees and specifically, fast-growing *Eucalyptus* plantation trees that are of commercial importance for pulp and paper production and also holds potential for future bioenergy production. This study followed on a pioneering M.Sc. study (Victor 2006) in which conserved and putatively novel miRNAs were isolated from the vascular tissues of *E. grandis*. The miRNAs selected for this study included some of the novel and conserved miRNAs identified by Victor (2006) as well as some other miRNAs known to be conserved within plants. The miRNAs selected are negative regulators of genes encoding transcription factors known to function in plant development, defences related genes, genes required in cell wall biosynthesis and miRNA biogenesis genes. The two main aims of this study were firstly the characterisation of the expression profiles of the selected miRNAs at the whole-tree level, and secondly, characterisation of their expression profiles in developing wood during response to mechanical stress over a period of one month. The results of this research project provides further insights into miRNA function in *Eucalyptus* and, where applicable, trees in general with specific reference to wood development.

To more fully understand the functions of the selected miRNAs in terms of general tree development, the miRNA abundance profiles were first determined in a series of primary and secondary tissues collected at the whole-tree level. This allowed for more accurate interpretation of the expression profiles observed in response to mechanical stress. The miRNAs were found to have diverse expression profiles in the whole-tree study. Some expression profiles clustered together. Examples of this are miR160 and miR164 that were differentially expressed across the BDS with the highest levels detected in the phloem. miR160 regulates the expression of ARFs potentially involved wood development and miR164 regulates three NACs involved in floral and root development. Other miRNAs were unique in their abundance profiles when examined at the whole-tree level. This was clearly observed for miR166 and miR167. The former was detected at the highest levels in the green tissues, with comparatively low levels detected across the BDS, while miR167 was most abundant in the BDS. These observed abundance profiles are consistent with the predicted role of miR166 in vascular patterning and supports a role for miR167 in wood formation as it regulates ARFs, and auxin signalling is essential for correct wood development. These two miRNAs would make good targets for further study when taking the data

generated in this study into account. The identification of the *Eucalyptus* orthologues of these miRNA targets would facilitate further study of these miRNAs in *Eucalyptus*. The use of cleavage resistant target genes or miRNA over-expression constructs under the control promoters that direct xylem and or phloem specific expression would further resolve their function in xylogenic tissues.

miRNAs also displayed differential expression within the tissue groupings. These include miR159, which was up-regulated only in mature leaves. miR90 was predominantly expressed in the mature leaves and young flowers while miR362 was detected at highest levels in the bark and roots of *Eucalyptus*. The miR168 whole-tree profile was unique and did not fit into any of the patterns of miRNA expression observed in this study. miR168 function as a regulator of miRNA biogenesis to ensure the correct processing of miRNAs in a particular tissue or point in time and therefore expression would be expected to vary across tissues to ensure the spatial and temporal regulation of miRNA expression. The abundance patterns detected for miR166 and miR408 were consistent with their predicted function as well as that of their targets in wood development (Ko *et al* 2006, Lu *et al* 2005). This serves the dual function of confirming the observations noted in previous studies and confirming that validity of this study.

The whole-tree profiling of the selected miRNAs led to the identification of several miRNAs potentially involved in xylogenesis, namely miR160, miR166, miR167 and miR408. Despite the primary focus of this studying being the identification of miRNAs as potential regulators of wood development, the whole-tree profiling will also serve as a guideline for researchers interested in investigating alternative roles for miRNAs in tree development such as leaf and flower development (miR90), primary growth (miR167) and miR362 in bark and root development.

In the second part of this study, the abundance profiles for the same set of miRNAs were determined for a range of periods up to one month during tension wood formation. miRNAs are known to respond to various abiotic stresses including mechanical stress. The reprogramming of wood formation during stem bending makes it possible to study wood development from a perspective that is not possible in normally developing tissues. This provides an opportunity to refine our understanding of the regulation of wood formation. The profiling of miRNAs at four time points over the period of one month was a first

as previous studies only profiled miRNAs at a single point in time, which was three to four weeks after bending when the first tension wood is usually visible. This allowed a greater resolution in our understanding of miRNA expression dynamics during tension wood induction. We found that many miRNAs were differentially expressed during tension wood formation and that the expression profiles were much more complex than previously inferred from single time point studies. Profiles of interest were identified for miR159, miR160, miR166, miR167 and miR408. microRNAs miR159, miR160 and miR167 are known regulators of transcription factors involved in gibberellin and auxin phytohormone signalling pathways. Both of these hormones have been found to play roles in tension wood formation. In this study, miR160 and mR167 showed an early response to tension wood induction, increasing in abundance potentially functioning in the early regulation of tension wood responses in *Eucalyptus* mediated by the auxin signalling pathway. The potential to regulate this pathway, particularly the GH3 pathway which has been shown too responded to bending stimuli further supports the role for miRNA mediated regulation. Levels of miR159, a negative regulator of two MYBs, decreased in TWX but increased in TWP during bending, this when consider together with the whole-tree profile where little variation was observed across the BDS may implicate miR159 in TW development regulation. These findings suggest that these miRNAs function in the regulation of tension wood development. miR166 and miR408 levels increased during the period of mechanical stress. miR166 determines vascular patterning in the xylem with altered target HD-ZIP expression resulting in changes in fibre to vessel ratios. The homologues of *AtHB*-8 directs fibre development while *REV* directs vessel development in *Zinnia*, both of these genes are under miR166 regulation. The results presented here support the further fine scale mapping of this miRNAs expression to fully understand the spatial dynamics of its expression and subsequent effect on cell fate. The increased abundance of miR408 was expected as miR408 represses the expression of plastocyanin genes involved in lignin polymerisation and this correlates with chemical studies of tension wood that found lignin levels to be reduced. Together these miRNAs represent subjects for further study using the same approaches as those suggested for miRNAs identified as potential targets for further research.

Comment [Z1]: This conclusion seems fairly generic and I am not sure that you gave me enough specific information in the preceeding sentences to make me believe you (although you may be correct).

Comment [Z2]: Too general. Did we not decide to distinguish between early and late responses. At least give some indication of the specific time points otherwise the benefit of the resolution of your experiment is lost.

Comment [Z3]: Is the observed direction of miRNA increase consistent with the previously observed increase in fibre to vessel ratio in tension wood?

The *Eucalyptus* genome sequencing initiative undertaken by the Department of Energy in the USA will soon allow genome-wide identification and characterisation of *Eucalyptus* miRNA genes and

their promoters. Using the *cis*-elements identified in these promoters, links between transcriptional regulation and the abundance profiles generated in this study could be established to provide a more complete picture of the positions and roles of miRNAs in transcriptional networks. Deep, whole-transcriptome sequencing and profiling currently being undertaken in our research group will provide the transcript sequences and detailed expression profiles of the predicted miRNA targets. This data can be used to validate both conserved and putatively novel miRNA target genes by performing 5'RACE cleavage assays and RT-qPCR. The latest in sequencing technologies will facilitate the identification of miRNAs that were not detected in this study or identified by Victor *et al* (2006).

In conclusion, this study provides insights into the complex nature of microRNA directed regulation in *Eucalyptus* and trees in general. The whole-tree profiling of the miRNAs selected for this study has led to a better understanding of their expression across various tree tissues. The abundance profiles generated here have provided insights into these miRNAs potential functions, support for previously published findings in poplar and *Arabidopsis* (see reviews Jones-Rhoades *et a*l 2006 and Lu *et al* 2008) and allow for hypotheses regarding their function in *Eucalyptus* and potentially wood formation to be generate. The tension wood study further evaluates the potential for miRNA involvement in TW development. The time trial design of this study allowed for the spatial and temporal nature of miRNA expression during TW development to be determined at four points during xylogenesis so building on studies that only evaluated miRNA expression at a single point during TW development, in most cases once the G-Layer had formed (Lu *et al* 2005, 2008). Therefore this study provides information pertinent to future miRNA research and studies of wood formation.

2.7 Acknowledgements

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2.9 Supplementary material

Table S2.1 Probes used for *Eucalyptus* miRNA Northern blot analysis

Probe name	Sequence (5'-3') [c]
Egr-miR90 [a]	CCACCGCCAAGCACCAACCT
Egr-miR159 [a]	GGAGCTCCCTTCAGTCCAAG
Egr-miR160 [a]	TGGCATACAGGGAGCCAGGCA
Ptc-miR164 [b]	TGCACGTGCCCTGTTTCTCCA
Ptc-miR166 [b]	GGGGAATGAAGCCTGGTCCGA
Ptc-miR167 [b]	TAGATCATGCTGGCAGCTTCA
Egr-miR168 [a]	TCCCGACCTGCACCAAGCGA
Egr-miR172 [a]	ATGCAGCATCATCAAGATTCT
Egr-miR293 [a]	CCAGCCATGGCGAGGCCGACC
Egr-miR362 [a]	CAGGATGTTGAGTTCCAAGA
Ptc-miR408 [b]	TGCCAGGGAAGAGGCAGTGCAT
Egr-miR472 [a]	GGAATGGGCGGCCTTGGGAAA
Egr-5.8S rRNA	TATCCGTTGCCGAGAGTCGTTA

[a] Egr = *Eucalyptus grandis*
[b.] Ptc= *Populus tricocarpa*
[c.] Sequence complimentry to mature miRNA

Table S2.2 Primers used for RT-qPCR analysis of *Eucalyptus* miRNA

Primer name	Primer sequence (5'-3') [c]
Egr-miR90 [a]	GACCACGCGTATCGATGGCTCA
Egr-miR159 [a]	CTTGGACTGAAGGGAGCTCCA
Egr-miR160 [a]	TGCCTGGCTCCCTGTATGCCAA
Ptc-miR164 [b]	TGGAGAAACAGGGCACGTGCA
Ptc-miR166 [b]	TCGGACCAGGCTTCATTCCCCAA
Ptc-miR167 [b]	ATGAAGCTGCCAGCATGATCTGGA
Egr-miR168 [a]	TCGCTTGGTGCAGGTCGGGAA
Egr-miR172 [a]	AGAATCTTGATGATGCTGCATA
Egr-miR293 [a]	GGTCGGCCTCGCCATGGCTGGA
Egr-miR362 [a]	TCTTGGAACTCAACATCCTGA
Ptc-miR408 [b]	ATGCACTGCCTCTTCCCTGGCA
Egr-miR472 [a]	TTTCCCAAGGCCGCCCATTCCA
Egr-5.8S rRNA_Forward	ACGTCTGCCTGGGTGTCACAA
Poly-T adaptor	GACCACGCGTATCGATGGCTCAT16V*
Poly-T adaptor_Reverse	GACCACGCGTATCGATGGCTCA

*V=A,G,C
[a] Egr = *Eucalyptus grandis*
[b] Ptc= *Populus tricocarpa*
[c] mature miRNA sequence