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Functional Analysis of *Eucalyptus grandis* Tubulin Genes Involved in Wood Formation

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Department of Forest and Ecosystem Science

Melbourne School of Land and Environment

The University of Melbourne

Abstract

Knowledge of the process of wood formation and the molecular control of wood quality determination are fundamental prerequisites for our understanding of the evolutionary success of trees in terrestrial ecosystems and for meeting the performance demands for commercial wood and fibre products in the future. In this context, the most important wood quality trait after density is cellulose microfibril angle (MFA). MFA influences wood fibre strength and flexibility which are important in supporting tree architecture and form, as well as for solid wood and pulp and paper products. Research in a number of plant species has provided evidence that MFA is linked to cortical microtubules and might be influenced by tubulin gene activity, a concept that is further explored in this thesis.

This thesis therefore reviews the current literature on plant microtubules, tubulin genes and cellulose microfibril deposition, aims to experimentally characterise alpha and beta-tubulin gene function and tries to determine if a link exists between tubulin gene phylogeny and their specific role during secondary cell wall formation.

In vivo tree transgenesis was used to create transgenic wood sectors and to examine the roles of various *Eucalyptus grandis* tubulin genes in wood fibre MFA determination. The effect of *E. grandis BETA-TUBULIN 1* (*EgrTUB1*), which had been shown by Spokevicius *et al.* (2007) to influence MFA, was re-examined and confirmed. This work was expanded by using both over-expression and down-regulation methods to produce wood fibres with altered MFA. In all cases MFA was found to be increased indicating that beta-tubulin was down-regulated in transgenic sectors and that the use of over-expression vectors led to homology dependent gene silencing (HDGS). Down-regulation was achieved by using artificial micro RNA constructs, representing one of the first examples of successful application of this technology in a perennial forest tree species. Similar results were obtained by using the *E. grandis ALPHA TUBULIN* genes *EgrTUA1* and *EgrTUA2*. Again, MFA was found to be increased in transgenic sectors indicating that alpha and beta tubulin play similar roles in MFA determination and that the balance between them is of primary importance.

This is further highlighted by experiments altering the expression of both alpha and beta tubulin genes simultaneously which produced wood fibres with increased MFA when both

genes were down regulated and decreased MFA when both genes were up regulated. The biological and physical relevance of these changes to MFA were examined in collaboration with the Max Plank Institute of Colloids and Interfaces in Germany and preliminary results for transgenic fibre tensile strength and flexibility are included as an appendix to this thesis.

Finally, in an attempt to identify potential links between specific beta tubulin gene function and the phylogenetic relationship between their products, micro RNA-based down-regulation of six *E. grandis* beta tubulin genes was assessed with regard to its effects on MFA in secondary fibre cell walls. Down-regulation of three beta tubulin genes, *EgrTUB1*, *EgrTUB5* and *EgrTUB6*, resulted in increased MFA while the remaining three, *EgrTUB2*, *3* and *4*, showed no effect, indicating the existence of possible secondary cell wall specific isoforms and a distinct functional divergence within the eucalypt beta tubulin gene family.

Declaration

This is to certify that:

i. This thesis comprises only my original work towards the PhD.

ii. Due acknowledgment has been made in the text to all other material used.

iii. This thesis is less than 100,000 words in length, exclusive of tables, figures, bibliographies and appendices.

Lynette Taylor

July, 2010

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Chapter 1 Introduction

Tree species are grown around the world to produce timber for solid wood products, fire wood, bio-energy and fibre pulp and paper manufacture. The demand for wood and wood products from plantations and managed native forests is increasing. Selective breeding of hardwood and softwood tree species in order to meet the growing demand for wood products is a relatively recent venture (Raymond 2002; Dungey *et al.* 2006), compared to other agricultural crop species which have been selectively bred for thousands of years. Breeding programs are focussing on the improvement of wood quality traits, and molecular research is directed at improving our understanding of the genes that control wood formation.

Desirable wood quality traits vary depending on the end use of the timber. For example, basic density, pulp yield, cellulose content and fibre length are important for pulp and paper, while basic density, microfibril angle, fibre strength and stiffness are some of the properties important for sawn timber (Raymond 2002). In most tree species little is known about the degree of genetic variation or the heritability of these wood quality traits (Raymond 2002; Dungey *et al.* 2006; Gonzalez-martinez *et al.* 2007). Attempts to close this knowledge gap in recent years have been the drivers behind the study of molecular markers, the identification of quantitative trait loci (QTL), genetic association studies and the identification of candidate genes, for example through microarray-based gene expression studies (Hertzberg *et al.* 2001; Raymond 2002; Qiu *et al.* 2008; Stackpole *et al.* 2010a; Stackpole *et al.* 2010b).

Cellulose is the main structural component of plant cell walls in the form of cellulose microfibrils. The wood quality trait microfibril angle (MFA) corresponds to the angular deposition of cellulose within the secondary cell wall of wood fibres. Cellulose microfibril angle influences the tensile strength and flexibility (modulus of elasticity) of wood fibres, and contributes to the overall strength of sawn logs and paper products (Page and El-Hosseiny 1983; Batchelor *et al.* 2000; French *et al.* 2000; Burgert *et al.* 2002).

Tubulin proteins, which are encoded for by tubulin genes, form microtubules. The involvement of cortical microtubules, a cytoskeleton macromolecule, in the orientation of

cellulose microfibrils within plant cell walls has been established since the 1960's (Green 1962; Ledbetter and Porter 1963). Microtubules and more specifically cortical microtubules during both primary and secondary cell wall formation have been shown to influence cellulose microfibril deposition in *Arabidopsis thaliana* (Oda *et al.* 2005; Oda and Hasezawa 2006; Paradez *et al.* 2006a; Paradez *et al.* 2006b). Furthermore, tubulin gene expression has been linked to the cellulose biosynthesis machinery during secondary cell wall formation in expression studies in *Gossypium hirsutum* and *Populus trichocarpa* (Dixon *et al.* 1994; Dixon *et al.* 2000; Oakley *et al.* 2007).

Microarray analyses of hardwood trees such as Poplars and Eucalypts during wood formation (Hertzberg *et al.* 2001; Qiu *et al.* 2008), have identified a large number of candidate genes involved in specific stages of phloem and xylem development. For example during differentiation, expansion, secondary cell wall formation to maturation, and during tension wood formation; the upper side of branches and leaning stems contain tension wood which is characterised by wood fibres with greater tensile strength, increased cellulose content, smaller MFA, reduced vessel numbers, asymmetric growth and often G-layer formation (Wardrop and Dadswell 1957; Clair *et al.* 2006; Qiu *et al.* 2008). On the lower side, opposite wood is formed, conversely, wood fibres are under greater compressive forces, and this wood is characterised by larger MFA, shorter tracheids or fibres and increased lignin content (Qiu *et al.* 2008).

Qiu *et al.* (2008) compared the changes in expression of genes active in the upper side and lower side of branches compared to the trees stems which possess different wood properties. The candidate genes identified include *Eucalyptus grandis FASCICLIN –LIKE ARABINOGALACTAN* (*EgrFLA1* and *EgrFLA2*), *CELLULOSE SYNTHASE* (*EgrCesA2*) and two tubulin genes *ALPHA TUBULIN 1* (*EgrTUA1*) and *BETA TUBULIN 1* (*EgrTUB1*) genes, which had increased expression on the upper side (low MFA) and decreased expression on the lower side (high MFA) of *E. nitens* branches (Qiu *et al.* 2008). Additionally, many other genes and proteins, such as PECTINESTERASE, XYLOGLUCAN ENDOTRANSGLYCOSYLASE and EXTENSIN proteins were also identified (Qiu *et al.* 2008). Similarly, one of the candidate gene families identified to be involved in cell expansion and secondary cell wall formation in poplar by Hertzberg *et al.* (2001) was tubulin.

Furthermore, tubulin gene activity has been shown to influence the orientation of cellulose microfibrils in secondary cell walls of eucalypt wood fibres (Spokevicius *et al.* 2007a). These authors functionally characterised the *Eucalyptus grandis BETA TUBULIN 1* gene (*EgrTUB1*) in stem tissue of eucalypt trees by using Induced Somatic Sector Analysis (Spokevicius *et al.* 2007a). The resulting transgenic wood fibres in this study were characterised by increased cellulose microfibril angle, with an average change of 5^{o}. This result confirmed a role of *EgrTUB1* in determining MFA in secondary wood fibres. However, the role of *E. grandis ALPHA TUBULIN* genes during wood formation is yet to be investigated and constituting the subject matter of this thesis.

In plants, large tubulin gene families encode alpha and beta tubulin proteins that contribute to microtubule formation. In *Populus tremuloides* for example the differential expression of alpha and beta tubulin genes in specific plant tissue types and during tension wood formation has been described by Oakley *et al.*(2007). These authors showed that tubulin genes are both spatially and temporally regulated, with specific genes highly up-regulated in xylem tissue and during tension wood formation (*PoptrTUB9*, *PoptrTUB15*, *PoptrTUA1* and *PoptrTUA5*). Another example are *Gossypium hirsutum* (cotton) fibre cells, where *GoshiTUA8* and *GoshiTUA9* were predominantly expressed during stages of rapid cell elongation, while *GoshiTUB6* and *GoshiTUB7* were expressed during secondary cell wall formation (Dixon *et al.* 1994; Dixon *et al.* 2000). E. grandis also contains a multi-gene tubulin family, which have been shown to be differentially expressed during wood formation in association with MFA (Qiu *et al.* 2008). However, the role of other members of the *E. grandis TUBULIN* multi-gene family, apart from *EgrTUB1*, during wood formation is yet to be investigated, and constituting the subject matter of this thesis.

This thesis aims therefore to improve our understanding of wood formation by investigating the genetic control of the commercially important wood quality trait microfibril angle (MFA), using eucalypts as a model species. One research questions explored in this thesis was if alpha tubulin, like beta tubulin (as shown by Spokevicius *et al.* (2007a)) influence MFA? In addressing this question the activity of alpha and beta tubulin genes both as individuals and in combination were experimentally altered through the use of both established and new molecular biology techniques (synthetic-microRNAs) (Chapter 6). Following this it was hypothesised that individual members of the *E. grandis BETA TUBULIN*

gene family may portray specificity towards influencing microfibril angle (Chapter 7). Findings are discussed with regard to phylogeny and the overall number of members of the *E. grandis TUBULIN* gene family. Gaining a greater understanding of the genetic control of wood formation, the wood quality trait MFA and its influence on wood fibre strength is an important step towards improving our understanding of how trees grow and react to the environment around them, as well as for future selection of molecular markers to be used in breeding programs, and the future development of designer wood for fibre products, biofuel or carbon sequestration.

1.1 Thesis Outline

This thesis is structured in two parts, (i) the theoretical, and (ii) the experimental chapters. Chapters 2, 3 and 4, are theoretical in nature and present a summary of the literature. In Chapter 2 the molecular mechanisms involved in the formation and function of plant cortical microtubules are outlined. Chapter 3 provides a detailed account of tubulin gene family size, structure and phylogeny for a range of plant species, and the spatial and temporal expression patterns of these genes are described. In Chapter 4 cortical microtubule and cellulose microfibrils interactions, during plant primary and secondary cell walls formation are explained. In Chapter 5 the background to the *Agrobacterium* transformation methodology used in this project and the selection of vector based delivery systems is outlined.

The experimental work of this thesis is described in Chapters 6 and 7. Chapter 6 investigates the effects of altered gene activity of three *E. grandis* tubulin genes during wood formation. The use of Induced Somatic Sector Analysis (ISSA) for the creation of transgenic wood fibres, provides insight into the molecular control of microfibril angle formation by tubulin gene activity. In Chapter 7 the specificity of an additional five *E. grandis* beta tubulin genes is investigated using the same methodology. The temporal activity of *E. grandis* tubulin genes is compared and phylogenetic analyses give insight into the evolution of the plant tubulin gene family. Additional information on experimental and statistical design, gene sequences and accession numbers, miRNA development and a preliminary study into wood fibre tensile strength is provided in the form of appendices (A-H).

Chapter 2 Review of microtubule formation, dynamics and function

2.1 Introduction

Microtubules are found in all plant cells and play a key role in cellular processes such as cell division, cell wall formation, cytoplasmic streaming and organelle transport (Wasteneys and Yang 2004). During cell division different microtubule arrays are involved in aspects of mitosis, spindle formation and the development of the phragmoplast, whereas during cell wall formation cortical microtubules, found in the plant cells cortex just beneath the plasma membrane, form cortical microtubule arrays that are involved in the deposition of plant primary and secondary cell walls. All microtubules are comprised of heterodimers of alpha and beta tubulin proteins and these tubulin dimers are arranged in a head to tail manner forming protofilaments (Lloyd *et al.* 2000). Multiple protofilaments bind together, forming a polar cylindrical polymer known as a microtubule. These microtubules feature a positively charged growing end and a negative shrinking end, which have different structural and functional roles (Nogales 1999; Nogales and Wang 2006a). Plant microtubules are formed or nucleated at various locations throughout the plant cell, including the nuclear envelope and cell cortex (Liu *et al.* 1994).

This chapter explores microtubule formation and tubulin proteins, with specific emphasis on cortical microtubules and cortical microtubule arrays. These arrays are formed by bundling together cortical microtubules, which then display co-ordinated dynamic behaviour (Chan *et al.* 2007). Cortical microtubule arrays are one part of a plant's cytoskeleton, which also includes other polymers such as actin filaments.

Cortical microtubules are nucleated (formation initiated) by binding of an additional tubulin protein, gamma tubulin. The nucleation process occurs in two different ways. *De novo* formation where new cortical microtubules form within the cell's cortex (Pastuglia *et al.* 2006) and microtubule-dependent nucleation (branching) where new cortical microtubules form along the length of an already existing cortical microtubule (Murata *et al.* 2005). The process of microtubule nucleation is also involved in array formation and their self-alignment (Nick 2008). Cortical microtubule formation is also influenced by post-translational modification (Nissan et al. 2008) and interactions with microtubule associated

proteins (MAPs) (Hussey *et al.* 2002). Examples of MAPs governing the assembly and disassembly of microtubules as well as anchorage and cross-linking (stability) of microtubules within plant cells, including cortical microtubules are presented in this chapter.

2.2 Tubulin gene and protein structure and the formation of dynamic microtubules

Plant microtubules are composed of highly conserved alpha and beta tubulin proteins which are coded for by tubulin genes. Plant alpha and beta tubulin gene families vary in size, and family members commonly contain between one and eight introns (Liaud *et al.* 1992). The size and position of these introns is often found to be conserved within species and divergent between them (Wolfgang *et al.* 2004). Tubulin genes are highly conserved however contain a hypervariable region at the C-terminus which distinguishes the various family members. Differences in intron position and variability at the C-terminal end influence tubulin gene structure (Nick 2008).

After transcription and translation tubulin proteins undergo additional post-translational modification such as phosphorylation, tyrosination, detyrosination, polyglycylation and polyglutamylation (Liaud *et al.* 1992; Hammond *et al.* 2008). Tubulin amino acids at the 5' N-terminus of the protein are more highly conserved than those at the 3' C-terminus hyper-variable region. The amino acid sequences at the C-terminus of tubulin proteins are important as they are involved in microtubule interactions with MAPs and are targeted for posttranslational modification in plants (MacRea 1997; Dixon *et al.* 2000; Wasteneys *et al.* 2004). Although in general tubulin proteins are highly conserved, differences in posttranslational modification and their specific interactions with other proteins affect microtubule function (Nick 2008).

The three dimensional structure of eukaryotic TUBULIN subunits governs their binding ability during heterodimer formation and microtubule assembly (Nogales *et al.* 1998; Nogales 1999). Electron crystallography of alpha and beta tubulin monomers identified that they contain a core of beta-sheets surrounded by alpha-helices (Nogales *et al.* 1998) (Figure 2.1). The monomers contain three functional domains; the amino-terminal domain where

GTP is bound; the intermediate binding domain were Taxol (a microtubule stabilising agent) has been identified to bind; and the carboxy-terminal domain, involved in MAP and motor protein binding (Nogales *et al.* 1998). Microtubule heterodimers of alpha and beta tubulin monomers join together through the binding of the alpha tubulins GTP at the monomer-monomer interface, which becomes buried and non-exchangeable (Nogales and Wang 2006a). New heterodimers are added to the positively charged end of an emerging microtubule during polymerisation (Dixit and Cyr 2004). Microtubules undergo cyclic polymerisation and depolymerisation; the disassembly of heterodimers (Dixit and Cyr 2004). This cyclic process, also known as dynamic instability, involves three distinct phases; a growth phase (net addition), a shrinkage phase (net reduction), and a pause phase (no net change in length) (Chan *et al.* 2003; Shaw *et al.* 2003; Dixit and Cyr 2004). Dynamic instability allows microtubules to respond to changing internal and external signals during the formation and remodelling of a plant's cytoskeleton (Lloyd *et al.* 2000; Nick 2008).

The assembly properties of microtubules are governed by the binding and hydrolysis of guanosine 5′-triphosphate (GTP) (Nogales 1999). GTP bound by alpha tubulin at the N-site of the polymer is non-exchangeable, whereas the GTP bound by beta tubulin at the E-site is exchangeable (Nogales 1999). GTP is required at the E-site for polymerisation to occur, subsequently the E-site GTP undergoes hydrolysis to form GDP providing the energy for polymerisation (Nogales 1999). In addition, the outer layers of microtubules are believed to be covered with heterodimers with bound GTP (GTP cap), which is suggested to act as a microtubule stabiliser (Nogales and Wang 2006a). Loss of this stabilising cap results in rapid depolymerisation, as the underlying GDP bound heterodimers energetically favour depolymerisation (Nogales and Wang 2006a).

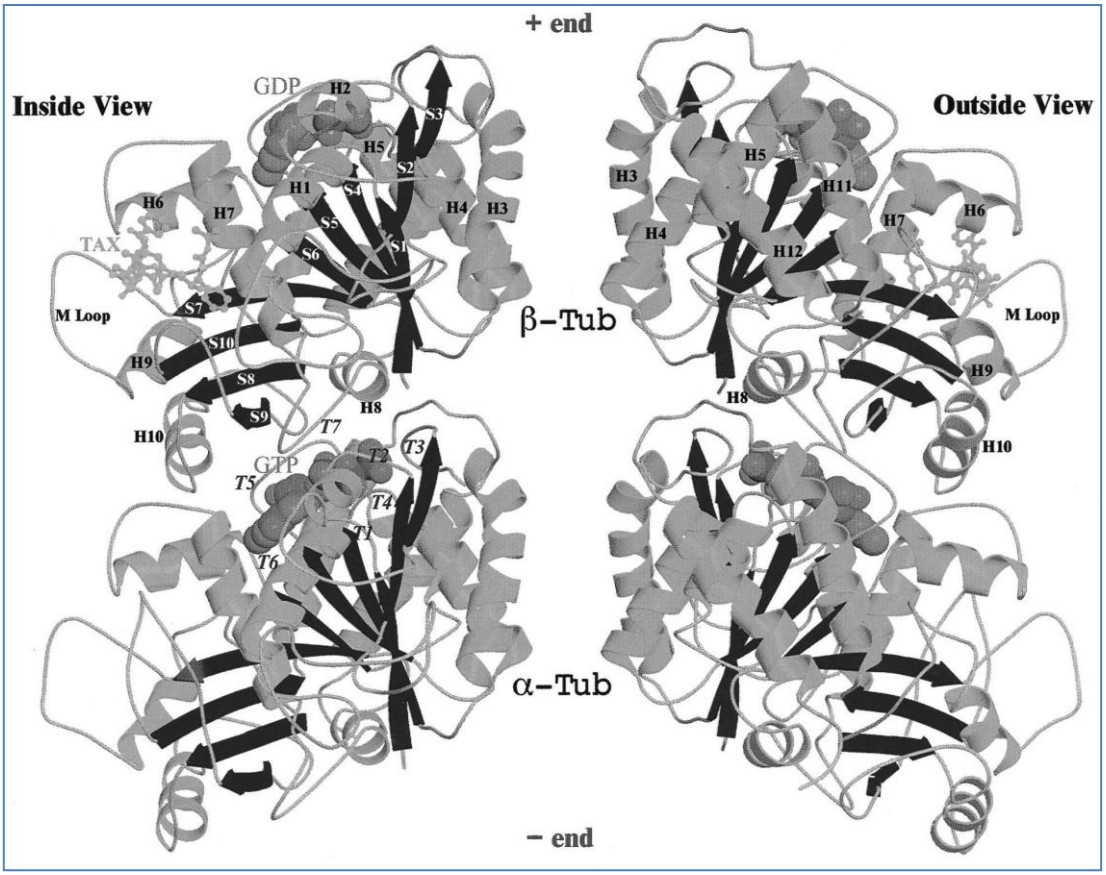


Figure 2.1 Electron crystallography structure of eukaryotc tubulin dimer (Nogales 1999). Two complementary views corresponding to the inside and outside of a microtubule heterodimer are shown. The different secondary structural elements have been labelled including polarity, the beta sheets (S1-S10) represented by dark arrows, the helices (H1-H12), M loops involved in nucleotide binding, and alpha tubulin N-Site (GTP) and beta tubulin E-site (GDP) binding sites.

2.3 Gamma tubulin is central to cortical microtubule nucleation and alignment

Within the cell cortex, cortical microtubules are nucleated by a gamma tubulin ring complex, which contains between 10 and 13 gamma tubulin proteins per complex (Seltzer *et al.* 2003; Murata *et al.* 2005; Pastuglia *et al.* 2006) and this ring complex interacts with the intermediate binding domain of alpha and beta tubulin heterodimers (Nogales and Wang 2006b). Gamma tubulin shows 30% sequence homology with alpha and beta tubulin proteins (Oakley 1992), has been linked to plant microtubule formation in a variety of species including *Arabidopsis*, *Allium* (onion), *Glycine* (soybean) and *Nicotiana* (tobacco) (Liu *et al.* 1993) and appears to have been conserved through eukaryotic evolution (Liu *et al.* 1994).

As previously stated microtubules are formed by two different processes, *de novo* (new) (Pastuglia *et al.* 2006) and through microtubule-dependent nucleation (branching) (Murata *et al.* 2005), which are both governed by gamma tubulin. Branching microtubules form from an already existing microtubule at a divergent angle of approximately 40 degrees. This angle is important as it is the maximum angle at which microtubule-microtubule collision results in self alignment and not depolymerisation (Murata *et al.* 2005). The role of gamma tubulin in the self alignment of cortical microtubules is important as the alignment of cortical microtubules during plant cell wall formation is vital in determining plant cell shape and cell wall structural integrity (Nick 2008). This involvement of cortical microtubules in plant cell wall formation is discussed further in Chapter 4.

Knowledge of the structural characteristics of TUBULIN proteins and on how gamma tubulin interacts with their intermediate binding domain is important in understanding microtubule formation and function (Paluh *et al.* 2000; Jung *et al.* 2001; Pastuglia *et al.* 2006) as specific isoforms of gamma tubulin are involved in the spatial and temporal regulation of microtubule assembly (Liu *et al.* 1994). For example it has been shown that altered gamma tubulin activity influences microtubule function and organisation in *Arabidopsis* causing aberrant nuclei division which influences seedling, pollen and root development, with uneven chromosomal segregation and radial swelling of plant roots (Pastuglia *et al.* 2006). Hence, gamma tubulin is important in regulating aspects of microtubule nucleation, self-alignment and function.

2.4 Organisation and formation of cortical microtubule arrays

Cortical microtubule arrays are made up of bundles of polarised microtubules that display dynamic behaviour, similar to but slower than that of individual microtubules (Chan *et al.* 2007). Microtubule arrays themselves have an overall directional charge, although within an array, individual bundles of microtubules can have differing polarity (Chan *et al.* 2007). It has been hypothesised that nucleation of microtubules in line with pre-existing cortical microtubule bundles is required to maintain array organisation, while nucleation at a divergent angle contributes to array disorganisation (Dixit and Cyr 2004). This divergent nucleation has been suggested to result in array reorientation (Dixit and Cyr 2004; Ehrhardt 2008).

Bundling of microtubules occurs when growing microtubule plus ends encounter other microtubules and subsequently interact and change their trajectory to co-align with that of encountered microtubules (Dixit and Cyr 2004; Ehrhardt 2008). The angle at which the microtubules encounter each other is critical in determining the outcome of microtubule-microtubule interactions, as bundling only occurs if the encounter is at an angle of less than 40 degrees. Otherwise microtubule depolymerisation or crossing over of the encountered microtubule can occur depending on cell type (Wasteneys and Ambrose 2009).

The role of microtubule nucleation in bundling of microtubules has been investigated by Chan *et al.* (2009), who used real-time imaging and showed that new microtubules not only nucleate or branch from pre-existing microtubules, as observed previously (Shaw *et al.* 2003; Murata *et al.* 2005; Ehrhardt 2008) but that they do this in an angle dependent manner. When new microtubules form within a pre-existing region of bundled microtubules, they do this in a predominantly forward facing manner, e.g. towards the plus end of the existing bundled microtubules (Chan *et al.* 2009). These findings were confirmed by recording the average angle of emerging microtubules, which were relatively consistent with what is known about the angle depended bundling of microtubules at 40 degrees or less (Murata *et al.* 2005; Ehrhardt 2008; Chan *et al.* 2009).

It is likely that specific gamma tubulin proteins play a role in perpetuating this angle-dependent nucleation within microtubule arrays (Shaw *et al.* 2003; Van Bruaene *et al.* 2004; Murata *et al.* 2005), while MAPs such as MOR1 increase the frequency of microtubule-microtubule encounters which is believed to promote self-organisation (Whittington *et al.* 2001; Wasteneys *et al.* 2004; Kawamura and Wasteneys 2008). However, the exact molecular mechanism by which gamma tubulin and MOR1 might regulate microtubule dynamics remains unclear.

Cortical microtubule arrays also undergo rotary movements (Chan *et al.* 2007). This occurs as groups of arrays, known as polarised domains, predominantly move in the same direction throughout the cell cortex (Chan *et al.* 2007). Although individual microtubules form in straight lines, both microtubule bundles and arrays follow curved trajectories resulting in multiple polar domains with differing curved trajectories moving around the plant cell

cortex in what appears as fluid-like clockwise or counter clockwise rotational movements (Chan *et al.* 2007) (Figure 2.2).

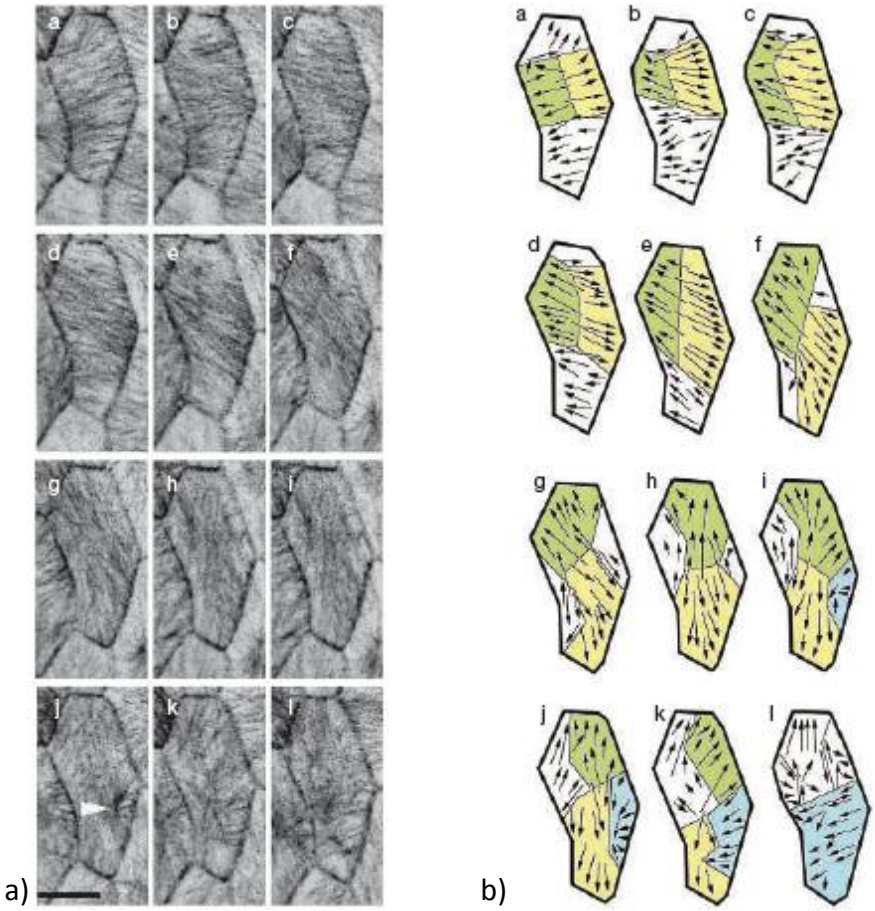


Figure 2.2 Microtubules moving in a mosaic of polarised domains (Chan *et al.* 2007). a) Live cell imagery of GFP-labelled *Arabidopsis* microtubule end-binding protein 1a (AtEB1a-GFP) in hypocotyl epidermal cells. b) Schematic based on the life cell imagery of continues movement of the polar domains around the cell.

Polar domains can display opposing directional rotation in neighbouring elongating cells, and are able to change direction within individual cells (Chan *et al.* 2007). Chan *et al.* (2007) observed that once elongation ceases, the polar domains rest in a steeply pitched, almost longitudinal orientation. The fact that during specific developmental stages microtubule arrays undergo both rotary and fixed trajectory movements is also likely to play a fundamental role in the organisation of cellulose microfibrils during plant cell wall development (Nick 2008). For example, in plant primary cell walls cellulose microfibrils are deposited with various alignments 'random', whereas in secondary cell walls the guided deposition of cellulose produces progressive cell wall layers with alternate helical cellulose microfibril orientation (Abe *et al.* 1995; Nick 2008). The link between cortical microtubules

and cellulose microfibril orientation during primary and secondary cell wall formation is detailed in Chapter 4.

2.5 Post-translational modification of tubulins influences their spatial and temporal activity and abundance

Many molecular mechanisms influence the amount and composition of intracellular tubulin and microtubules including transcription, translation, post-translational modification and their interaction with microtubule associated proteins (MAPs) (Hussey *et al.* 2002; Wang *et al.* 2004; Wasteneys *et al.* 2004; Giani *et al.* 2009). Here the link between post-translational modification, tubulin gene expression and protein abundance will be discussed while the role of MAPs will be specifically explored in the next section.

TUBULIN proteins undergo a range of post-translational modifications of their hypervariable C-terminal regions (Wang *et al.* 2004; Hammond *et al.* 2008). For example, alpha tubulin proteins undergo tyrosination and detyrosination which is the cyclic addition and removal of tyrosine, as well as acetylation, which is the covalent attachment of an acetyl group of a lysine residue (Hammond *et al.* 2008). Other modifications include phosphorylation, which is the addition of phosphate groups, and polyglutamylation and polyglycylation, the addition of chains of glutamate and gylcine residues near the C-terminus of both alpha and beta tubulin (Hammond *et al.* 2008). These types of posttranslational modifications are found both for plant and animal microtubules (MacRea 1997; Hammond *et al.* 2008) and are believed to play important roles in the arrangement and spatial and temporal abundance of microtubules, coinciding with tubulin gene expression (Wang *et al.* 2004). In *Zea mays* (maize), for example Wang *et al.* (2004) showed that post-translational C-terminal modification of alpha-tubulin was highly tissue specific coinciding with differential expression of different alpha tubulin genes. The *Zeama*TUA6 alpha tubulin protein was found to be detyrosinated and highly abundant in anthers, while it was less abundant in pollen tissue were it was tyrosinated (Wang *et al.* 2004).

The specific type of posttranslational modification does not seem to determine abundance nor is one type of modification specific to any given tissue type. For example, in *Zea mays* leaves two leaf specific alpha tubulin isoforms, *Zeama*TUA7 and *Zeama*TUA8, were

observed to be acetylated, whereas the non- leaf specific *Zeama*TUA9 was found to be simultaneously acetylated and detyrosinated and had undergone polyglutamylation (Wang *et al.* 2004). The lack of specificity of a given type of post-translation modification to a particular tissue type has also been observed in plant root. In *Pinus radiata* and *Pinus contorta* acetylation was observed in all microtubules, including root tip cortical microtubules (Gilmer *et al.* 1999). In contrast, no acetylated or detyrosinated tubulin proteins were detected in maize roots (Wang *et al.* 2004). These studies highlight the spatial complexity of post-translational modification of tubulin proteins and microtubules within and between plant species.

Temporal specific post-translational modification has been observed in *Gossypium hirsutum* (cotton) fibres by Dixon *et al.*(2000). Two different tubulin genes, *ALPHA TUBULIN GoshiTUA6* and BETA TUBULIN *GoshiTUB8*, were found to be expressed specifically during two different developmental stages corresponding to cellular elongation and secondary cell wall formation, respectively (Dixon *et al.* 1994). In these cotton fibres microtubules containing *Goshi*TUA6 proteins were found to be detyrosinated only during fibre cell elongation, while microtubules containing *Goshi*TUB8 proteins were detyrosinated only during secondary cell wall formation (Dixon *et al.* 2000).

Another type of post-translational modification is phosphorylation. Phosphorylation has been observed for both alpha and beta tubulin proteins in a number of species including *Arabidopsis thaliana*, *Nicotiana tobaccum* (tobacco), and *Daucus carota* (carrot) (Nuhse *et al.* 2007; Blume *et al.* 2008). For example in *Arabidopsis thaliana* phosphorylation has been linked to microtubule organisation (Nissan *et al.* 2008). In this example the CKL6 protein kinase enzyme was shown to regulate cortical microtubule alignment by phosphorylating two serine residues near the proteins C-terminal hypervariable region (Nissan *et al.* 2008).

This link between posttranslational modification, tubulin proteins and microtubules during specific developmental stages was described by the following authors (Dixon *et al.* 1994; Dixon *et al.* 2000; Wang *et al.* 2004; Blume *et al.* 2008). These different modifications are believed to play an important role in the interaction between microtubules and MAPs, which in turn influence microtubule stability (Wicker-Planquart *et al.* 2004). The spatial and temporal expression of individual tubulin genes within plant tubulin gene families is detailed

in depth for *Zea mays*, *Oryza sativa*, *Arabidopsis thaliana*, *Gossypium hirsutum*, *Populus tremuloides* and *Eucalyptus grandis* in Chapter 3.

2.6 Interactions between microtubules and microtubule associated proteins influence structure and function

Microtubule associated proteins (MAPs) are involved in governing a wide range of microtubule functions and their cellular interactions. This section will describe examples of MAPs involved in microtubule assembly (MOR1) and microtubule anchorage (CLASP1), which is believed to be important during cell division and governing the self-alignment of microtubules, as well as the cross-linking (stability) of microtubules (MAP65) and finally a specific MAP, MAP20 which has been suggested to link cortical microtubules to cellulose microfibrils during secondary cell wall formation.

Microtubule assembly is influenced by *MICROTUBULE ORGANISATION 1* gene (MOR1) expression (Whittington *et al.* 2001). Live cell imagery of MOR1 proteins identified them to be involved in rapid cortical microtubule growth and shrinkage with reduced occurrences of pause phases (Whittington *et al.* 2001; Wasteneys *et al.* 2004; Kawamura and Wasteneys 2008). This was validated in an *Arabidopsis* mutant study where a causative amino acid substitution within the MOR1 protein resulted in a temperature-sensitive cortical microtubule phenotype (Wasteneys *et al.* 2004; Kawamura and Wasteneys 2008). These aberrant MOR1 proteins produced an opposite effect in the resulting microtubules which showed reduced growth and increased pause phases (Wasteneys *et al.* 2004; Kawamura and Wasteneys 2008).

Other examples of MAPs which act on microtubule assembly dynamics include *END* BINDING 1 (EB1), MAP18, MAP60, MAP70-5, SPIRAL1 and SPIRAL2 (Hussey *et al.* 2002; Wasteneys and Ambrose 2009). Studies identifying these additional MAPs have shown that some of them affect microtubules in opposite ways to microtubules bound by MOR1 (Hussey *et al.* 2002).

Plant microtubules at certain times are more highly organised than at others and it seems likely that during some stages of plant cell development cortical microtubules are anchored or attached to the internal surface of a plant cell in order to achieve this organisation

(Ambrose and Wasteneys 2008). One protein involved in microtubule anchorage is *Arabidopsis* CLASP1 which has been shown to mediate microtubule-cortex binding (Ambrose *et al.* 2007). CLASP1 does this in two ways; (i) by binding the plus ends only or (ii) by binding the full length of cortical microtubules. These distinct mechanisms are likely to be differentially advantageous in different cell types or during different developmental stages (Ambrose and Wasteneys 2008). For example, CLASP1 end-on attachment maybe involved in the search and capture behaviour of microtubules (Ambrose and Wasteneys 2008). This behaviour is observed during mitotic spindle formation, where microtubules are observed to be tethered to centrosomes and probe the cytoplasm with their growing ends, which attach to chromosomal kinetochores and actively pull apart the chromosomes (Ambrose and Wasteneys 2008).

Alternatively, CLASP1 lengthwise microtubule anchorages may play an important role in cellulose microfibril alignment within plant cell walls (Ambrose *et al.* 2007). The regular spacing between cortical microtubules has been shown to guide the movement of cellulose synthase rosettes which synthesise ordered cellulose microfibrils (Hussey *et al.* 2002; Paradez *et al.* 2006a). Ambrose and Wasteneys (2008) showed that modulation of CLASP1 protein activity governs the behaviour of the microtubules. Strong CLASP1 attachment for example resulted in microtubules forming a net-like structure, whereas partial detachment resulted in parallel microtubule alignment with complete detachment resulting in randomised cortical microtubules (Ambrose and Wasteneys 2008). The partial detachment of cortical microtubules, which results in parallel microtubule alignment, is believed to be caused by the increased capacity of the microtubule's free end to move about resulting in self alignment (Ambrose and Wasteneys 2008). This demonstrates a mechanism for self-organisation of microtubules in plants, as plant microtubules lack a 'central' microtubule organisation centre (MTOC) as observed in animal cells (Pastuglia *et al.* 2006; Ambrose and Wasteneys 2008). Therefore, CLASP1 and similar MAPs yet to be identified might be involved in a wide range of cellular processes such as mitosis and primary and secondary cell wall formation.

The MAP65 gene family has been extensively studied in *Nicotiana* (tobacco), *Daucus* (carrot) and *Arabidopsis* (Hussey *et al.* 2002; Van Damme *et al.* 2004; Wicker-Planquart *et al.* 2004; Li *et al.* 2009). In these studies MAP65 proteins were identified to cross-link (bind or

stabilise) microtubules to other microtubules or cellular structures. *Nicotiana* MAP65 family members exert varying effects on microtubules and were found to associate with different microtubule arrays (Smertenko *et al.* 2000; Van Damme *et al.* 2004; Wicker-Planquart *et al.* 2004). For example, *in vivo* studies in *Nicotiana* showed that MAP65-1 binds and possibly bundles plant microtubules (Van Damme *et al.* 2004). Similarly, MAP65 was shown to decorate the outer surface of bundled microtubules in *Daucus* (Van Damme *et al.* 2004). In contrast, two members of the *Nicotiana* MAP65 gene family displayed differing effects, MAP65-1a enhances microtubule polymerisation (Smertenko *et al.* 2000) whereas MAP65-1b is involved in bundling and stabilising microtubules (Wicker-Planquart *et al.* 2004).

In *Arabidopsis* MAP65 proteins were found to be associated with different microtubule arrays or organelles including MAP65-1 (interphase MTs, anaphase spindle and phragmoplast), MAP65-3 (endocytotic MTs), MAP65-4 (spindle poles), MAP65-5 (interphase MTs), and MAP65-6 (mitochondria) (Van Damme *et al.* 2004). In a separate study MAP65-2 was found to be associated with cortical microtubules (Li *et al.* 2009). This role of MAP65-2 in stabilising plant microtubules has been studied both *in vivo* and *in vitro* in *Arabidopsis* cells where MAP65-2 was observed to act as a strong stabiliser of cortical microtubules during cold and drug treatments (Li *et al.* 2009). From these studies it is clear that the large family of MAP65 proteins allows them to act on plant microtubules in different ways, highlighting both the spatial distribution of MAP65 proteins and their functions.

In *Populus* the *PttMAP20* gene, in combination with secondary cell wall *CESA* genes, was found to be highly up-regulated during secondary cell wall formation and the MAP20 protein was identified to bind to cortical microtubules (Rajangam *et al.* 2008). Rajangam *et al.* (2008) further demonstrated that PttMAP20 was also able to bind to cortical microtubules in *Arabidopsis* and *Nicotiana* suggesting a conserved function between plant species.

PttMAP20 was identified as a target of DCB (2,6-dichlorophenyazide), a herbicide known to inhibit cellulose synthesis in plants (Delmer *et al.* 1987). When plant cells are treated with DCB, DCB acts on MAP20 and disrupts cellulose biosynthesis while not affecting the binding of MAP20 to cortical microtubules (Rajangam *et al.* 2008). In light of the role of cortical microtubules in guiding the deposition of cellulose microfibrils (detailed in Chapter 4) these

observations provide strong evidence for a link between PttMAP20 and the cellulose synthase complex, possibly identifying a direct functional link between microtubules and the formation of cellulose microfibrils (Rajangam *et al.* 2008).

In summary MAPs in a range of plant species have been shown to govern microtubule assembly and regulate microtubule behaviour (MOR1) as well as cross-link and bind to microtubules (MAP65 and PttMAP20), while others are involved in microtubule anchorage and parallel organisation (CLASP1). These examples show that the spatial organisation of plant microtubules including cortical microtubules is governed by a number of interactions with a wide range of MAPs.

2.7 Summary

Microtubule formation in plants is both a dynamic and a complex process. Although cortical microtubules are formed from two types of tubulin proteins, their dynamic behaviour, post-translational modifications and interactions with gamma tubulin and MAPs highlight the complexity of the molecular mechanisms that influence their organisation and function.

Chapter 3 The tubulin gene family in plants

3.1 Introduction

In plants multiple genes encode either alpha or beta tubulin proteins contributing to microtubule formation. Over the past three decades gene expression studies have provided insights into the temporal and spatial expression of tubulin genes. This chapter explores inter-species variation of tubulin gene family size and structure and determinants of their evolutionary divergence among plants. On this basis current knowledge regarding gene expression patterns during both primary and secondary plant growth is explored. The expression of tubulin genes in plant tissues is also governed by a range of external stimuli which will also be discussed.

3.2 Tubulin gene family size differs between plants undergoing primary and secondary growth

The most significant differences in tubulin gene family sizes are seen between plants that predominantly undergo primary growth and those that also undergo secondary growth. Primary growth is driven by the primary meristem and involves the production of primary xylem and phloem, whereas secondary growth progress from a secondary meristem which produces secondary xylem and phloem and increases the girth of plants. Monocotyledonous plant species only undergo primary growth while a range of dicotyledonous plants also undergo secondary growth. In monocotyledonous species the tubulin gene families of *Zea mays* (maize) and *Oryza sativa* (rice) have been studied. Maize has been identified to contain six alpha tubulin genes (*ZeamaTUA1-6*) and eight beta tubulin genes (*ZeamaTUB1-8*) (Hussey *et al.* 1990; Rogers *et al.* 1993). In rice, only three TUA genes (*OsTUA1-3*) have been identified to date, together with, and equal to the number observed for maize, eight TUB genes, *OsTUB1-8* (Yoshikawa *et al.* 2003).

Tubulin gene families in dicotyledonous plants including herbaceous and woody plants have been studied in *Arabidopsis thaliana*, *Gossypium hirsutum* (cotton) and *Populus trichocarpa* (poplar). Six TUA genes, *ArathTUA1-6*, and nine TUB genes, *ArathTUB1-9* have been described in *A. thaliana* (Kopczak *et al.* 1992; Snustad *et al.* 1992). The *A. thaliana* tubulin

gene family is more similar in size to that observed for monocotyledonous plants, whereas in *G. hirsutum* 12 TUA genes, *GoshiTUA1-12*, and 19 TUB genes, *GoshiTUB1-19*, have been described (He *et al.* 2008). This increase in gene family size continues with *Populus trichocarpa* (poplar), as *P. trichocarpa* has one of the largest known tubulin gene families today containing 20 TUB genes, *PoptrTUB1-20*, and eight TUAs, *PoptrTUA1-8*, and this has been linked to a recent genome duplication and expansion event (Tuskan *et al.* 2006; Oakley *et al.* 2007). From these studies it appears that greater complexity and plant size coincides with larger tubulin gene families.

Also in other woody plant species such as *Eucalyptus grandis* (eucalypt) tubulin genes have been identified, including four TUA genes, *EgrTUA1-4*, and six TUB genes, *EgrTUB1-6* (Spokevicius *et al.* 2007a; Qiu *et al.* 2008). The eucalypt gene family size could expand with the expected publication of the completed Eucalyptus genome sequences in 2011 (http://web.up.ac.za/eucagen/). It is also possible that the tubulin gene families in this species would be of similar size to poplar given their complexity, longevity and extensive secondary growth. Understanding of the tubulin gene families in other tree species is limited, and to date only one TUA gene has been identified in *Picea wilsonii* (*PwTUA1*) (Yu *et al.* 2009).

3.3 Tubulin protein family structure and phylogeny within diverse genera

The classification and structure (ancestral lineage) of diverse tubulin gene families in *Arabidopsis thaliana*, *Pediastrum* (green algae), *Physcomitrella patens* (moss) and *Populus trichocarpa* (poplar) are explored in this section. In addition, current phylogenetic analysis of plants tubulin genes families will be discussed in relation to evolutionary divergence and tubulin gene classification.

Within dicotyledonous plants, the *A. thaliana* and *P. trichocarpa* species are believed to have diverged approximately 100 to 120 million years ago (Tuskan *et al.* 2006). Prior to this the *A. thaliana* and *P. trichocarpa* genomes appear to have undergone a similar and possibly shared genome wide duplication event around the time of their divergence, known as the 'beta' or 'eurosid' duplication event (Tuskan *et al.* 2006). Since then, *A. thaliana* and

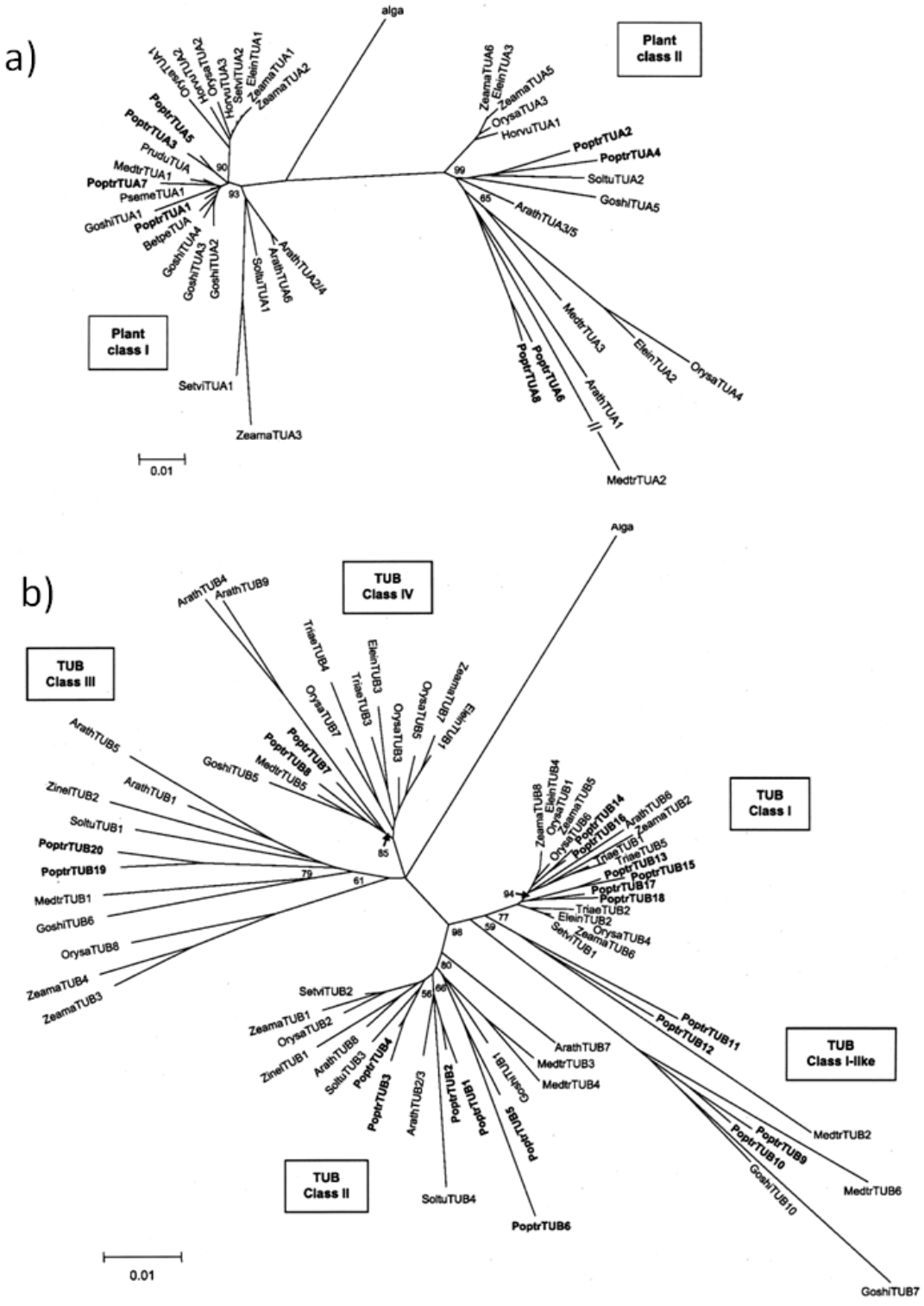
P. trichocarpa have undergone independent genome duplication events, known as 'alpha' and 'salicoid' duplication events, respectively (Tuskan *et al.* 2006). Both duplication events were superseded by an additional ancient genome duplication event (Tuskan *et al.* 2006). These genome wide events add to the complexity of their chromosomal structure and gene families.

The *A. thaliana* tubulin gene families (*ArathTUA* and *ArathTUB*), have high sequence homology at the nucleotide level with, 74 to 98% and 76 to 98%, respectively (Kopczak *et al.* 1992; Snustad *et al.* 1992). Similar homology has been observed for *P. trichocarpa* poptrTUAs 89%-98% and poptrTUBs 76%-96% (Oakley *et al.* 2007). These tubulin genes are classified (grouped) according to gene structure. They are divided into two subclasses with Class I containing *ArathTUA2*, *ArathTUA4* and *ArathTUA6*, and Class II *ArathTUA1*, *ArathTUA3* and *ArathTUA5* (Figure 3.1, a). The tubulin genes in these classes have been suggested to have evolved from two putative ancestral genes (Kopczak *et al.* 1992). Similarly the *P. trichocarpa* TUA genes group into two classes; *PoptrTUA1*, *PoptrTUA3*, *PoptrTUA5* and *PoptrTUA7* belonging to Class I and *PoptrTUA2*, *PoptrTUA4*, *PoptrTUA6* and *PoptrTUA8* to Class II, providing support for TUA genes in more complex plants having evolved from a few shared ancestral genes (Oakley *et al.* 2007) (Figure 3.1,a).

The *A. thaliana ArathTUB* gene family is larger and more diverse than the *ArathTUA* gene family comprising nine members. The nine *ArathTUB* members are divided into three sub-classes with *ArathTUB1* and *ArathTUB5* belonging to Class I, *ArathTUB4* and *ArathTUB9* to Class II and *ArathTUB2*, *AarthTUB3*, *ArathTUB6*, *AarthTUB7* and *ArathTUB8* to class III, suggesting they might have evolved from at least three putative ancestral genes (Snustad *et al.* 1992). In *P. trichocarpa* the beta-tubulin gene family which contains 20 *PoptrTUB* is divided into five classes *PoptrTUB13-18*, Class I; *PoptrTUB9-12*, Class I-like; *PoptrTUB1-6*, Class II; *PoptrTUB19-20*, Class III and *PoptrTUB7-8*, Class IV (Oakley *et al.* 2007). In Figure 3.1, b) *A. thaliana* TUB Class I is encompassed within *P. trichocarpa* TUB Class III, and *A. thaliana* TUB class II within *P. trichocarpa* Class IV, while *A. thaliana* TUB Class III proteins are encompassed within both *P. trichocarpa* Classes I and II, with no *A. thaliana* TUB genes present within *P. trichocarpa* Class I-like TUBs clade. Unlike Snustad *et al.* (1992), Oakley *et al.* (2007) suggested that the distribution of *A. thaliana* and other dicotyledonous and monocotyledonous tubulin genes represented within *P. trichocarpa* classes (phylogenetic

clades) is representative of angiosperm TUB genes having evolved from a single ancestral TUB gene.

The tubulin gene family structure of *A. thaliana* and *P. trichocarpa* are in contrast to *Pediastrum* (green algae), which like most other protists, contains only one or two identical or nearly identical tubulin genes (Luduena 1993; Luduena 1998). The tubulin family of the moss *Physcomitrella patens* for example contains both alpha and beta tubulin proteins, however only the beta tubulin proteins (*Pp*TUB1-6) have been investigated in detail and all members show high sequence homology 90.3% to 96.4% (Wolfgang *et al.* 2004). With six members in its beta tubulin family, *Physcomitrella patens* has less family members compared to *A. thaliana*, yet more than green algae, which is consistent with the observation that lower order plants in general feature smaller tubulin families. The six PpTUB proteins form an individual phylogenetic clade separate from alga and vascular plant clades (Figure 3.1, c), which is consistent with *Physcomitrella patens* being representative of an intermediate evolutionary stage between algae and higher order plants (Rensing *et al.* 2002).


a)
alga
Plant class II
ZeamaTUA6
EleinTUA3
ZeamaTUA5
ZeamaTUA3
OrysaTUA3
HorvuTUA1
PoptrTUA2
PoptrTUA4
SoltuTUA2
GoshiTUA5
ArathTUA3/5
MedtrTUA3
EleinTUA2
OrysaTUA4
PoptrTUA8
PoptrTUA6
ArathTUA1
MedtrTUA2
OrysaTUA2
HorvuTUA3
SetviTUA2
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ZeamaTUA2
PoptrTUA5
PoptrTUA3
PruduTUA
MedtrTUA1
PoptrTUA7
PsemeTUA1
GoshiTUA1
PoptrTUA1
BetpeTUA
GoshiTUA4
GoshiTUA3
GoshiTUA2
ArathTUA2/4
ArathTUA6
SoltuTUA1
Plant class I
SetviTUA1
ZeamaTUA3
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93
99
65
0.01
b)
Alga
TUB Class IV
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ArathTUB9
TriaeTUB4
EleinTUB3
TriaeTUB3
OrysaTUB7
OrysaTUB5
ZeamaTUB7
EleinTUB1
OrysaTUB3
TUB Class III
ArathTUB5
PoptrTUB7
PoptrTUB8
GoshiTUB5
MedtrTUB5
ZinelTUB2
ArathTUB1
SoltuTUB1
PoptrTUB20
PoptrTUB19
MedtrTUB1
GoshiTUB6
OrysaTUB8
ZeamaTUB4
ZeamaTUB3
85
79
61
TUB Class I
ZeamaTUB8
EleinTUB4
OrysaTUB1
ZeamaTUB5
OrysaTUB6
PoptrTUB14
PoptrTUB16
ArathTUB6
ZeamaTUB2
TriaeTUB1
TriaeTUB5
PoptrTUB13
PoptrTUB15
PoptrTUB17
PoptrTUB18
TriaeTUB2
EleinTUB2
OrysaTUB4
ZeamaTUB6
SetviTUB1
94
77
59
98
80
56
60
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ZinelTUB1
ArathTUB8
SoltuTUB3
PoptrTUB4
PoptrTUB3
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PoptrTUB5
PoptrTUB6
SoltuTUB4
TUB Class II
ArathTUB7
MedtrTUB3
MedtrTUB4
PoptrTUB11
PoptrTUB12
TUB Class I-like
MedtrTUB2
PoptrTUB9
PoptrTUB10
GoshiTUB10
MedtrTUB6
GoshiTUB7
0.01

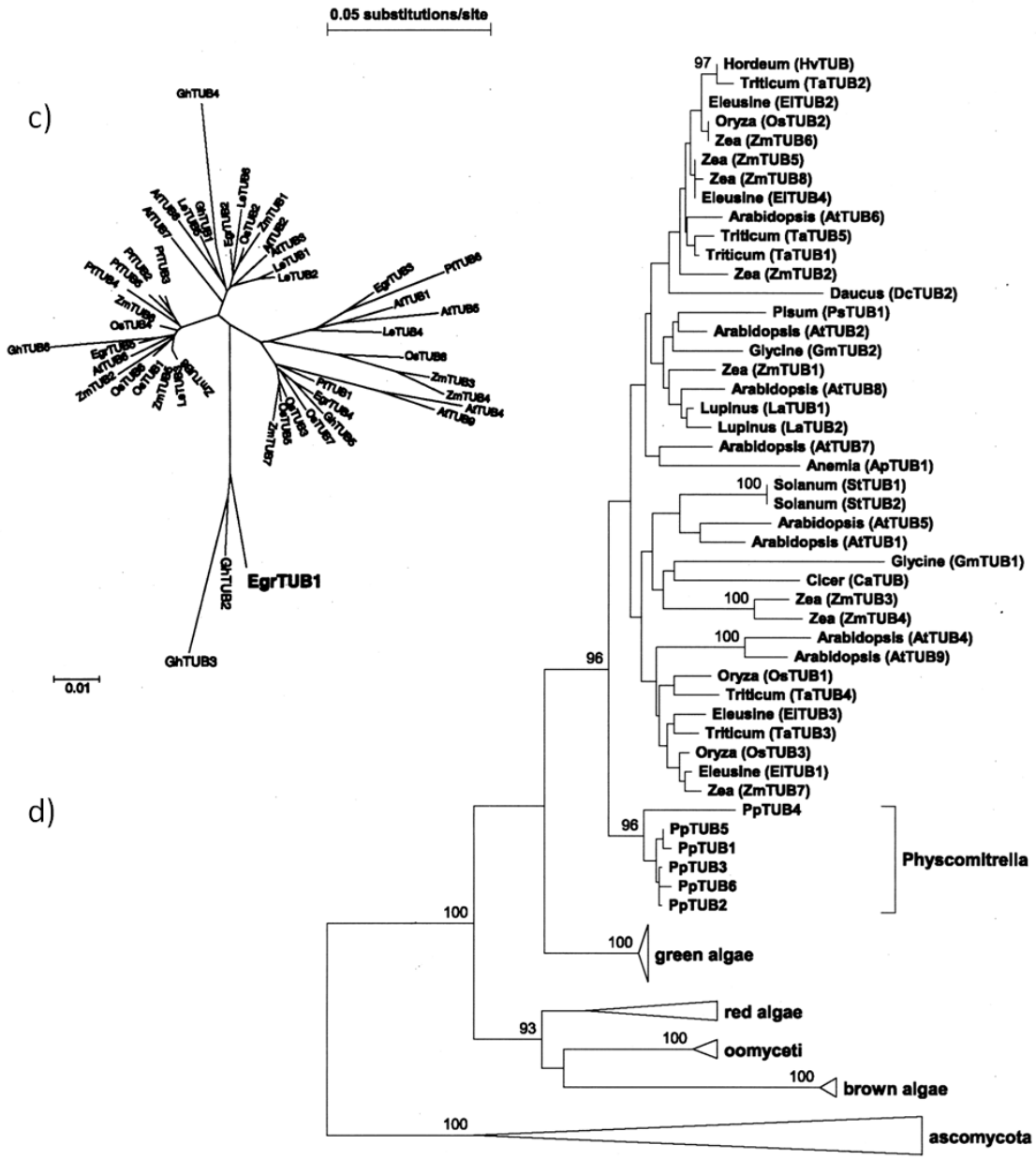


Figure 3.1 Four different phylogenetic trees representing a wide range of plant tubulin proteins. a) Minimum-evolution tree of representative full-length plant TUA proteins. Green algal (C. reihardtii) was used as an Outgroup. The bolded *Poptr*TUA proteins represent the different *Populus* Class I and Class II proteins which are expressed in xylem only and other tissue types respectively. The numbers on major branches indicate percentage support of interior-branch test using 1,000 replicates (Oakley *et al.* 2007). b) Minimum-evolution tree of representative full-length plant TUB proteins. Green algal (*C. reinhardtii*) TUB was used as an Outgroup. The bolded *Poptr*TUB proteins represent different *Populus* Classes I, I-like, II, III, IV. Numbers on major branches indicate percentage support of interior-branch test using 1,000 replicates (Oakley *et al.* 2007). c) Unrooted phylogenetic tree which includes *Egr*TUB1 (Spokevicius *et al.* 2007a) d) Neighbour-joining tree created with full length sequences excluding C-termini. Giving comparisons between beta tubulin proteins from algae, moss and vascular plants, *Ascomycota* was used as an Outgroup. Bootstrapped values are given for branches above 90% (Wolfgang *et al.* 2004).

Here four published phylogenetic trees are presented; each tree gives a slightly different representation of the plant alpha or beta tubulin families depending on the tubulin proteins included and the phylogenetic analysis undertaken. In Figure 3.1 a) and b) displays both monocot and dicot alpha and beta tubulin proteins and their grouping as per the *populus* classification, respectively. c) displays a different selection of monocot and dicot beta tubulin proteins, which include *Eucalyptus Egr*TUB1 however excludes all *Populus* proteins (Spokevicius *et al.* 2007a). d) displays yet another different selection of monocot and dicot beta tubulin proteins, however includes the ancestral moss and algae proteins (Wolfgang *et al.* 2004).

Phylogenetic trees give insight into the evolutionary lineage of plants and their proteins, for example within vascular plants, monocotyledonous and dicotyledonous plants are predicted to have evolved from a common ancestor some 130 to 240 million years ago (Paterson *et al.* 2000; Tuskan *et al.* 2006), and phylogenetic analysis suggests that alpha and beta tubulin families in vascular plants originated prior to the divergence of monocotyledonous and dicotyledonous species (Luduena 1998). This is reflected in the distribution of alpha and beta tubulin familiy members of multiple species found throughout each clade within the separate trees presented (Figure 3.1). Furthermore the separate lineages of the *Physcomitrella* TUB clade from both green algae and vascular plants is clearly depicted in Figure 3.1d (Wolfgang *et al.* 2004). This fits with the fact that speciation of *Physcomitrella* (mosses) happened around 450 million year ago (Wolfgang *et al.* 2004).

3.3.1 Introns and C-terminus are important determinants of tubulin gene evolution

In plants, tubulin genes and proteins are classified according sequence homology as described above, however intron number and position as well as the proteins C-terminus are also important in determining tubulin evolution and are therefore considered during classification, and will be described in detail in this section (Kopczak *et al.* 1992; Snustad *et al.* 1992; Wolfgang *et al.* 2004; Oakley *et al.* 2007). Overall intron position has been widely conserved during tubulin evolution (Wolfgang *et al.* 2004; Oakley *et al.* 2007), and a change in the number and/or position of an intron within gene family members is used to distinguish the various isoforms.

In *Arabidopsis,* for example, TUA genes contain between one and four introns at varying positions; one to three introns in Class I and four in Class II (Kopczak *et al.* 1992). Whereas TUB gene's in this species contain only two introns at conserved sites, with the exception of *ArathTUB1* and *ArathTUB5* (Snustad *et al.* 1992). In *Populus*, Class I TUAs contain three introns while Class II TUAs contain four, with the second intronic position conserved in all of the *Populus* TUAs (Oakley *et al.* 2007). All Classes of *Populus* TUBs (I, I-like, II, III, IV) contain two introns at conserved positions, the exception being Class I TUBs which contain an additional intron in the 5'UTR (untranslated region) (Oakley *et al.* 2007).

The two conserved intronic position in TUB genes are found not only in *Arabidopsis*, *Populus* and many other vascular plants but also in the moss *Physcomitrella patens* (Wolfgang *et al.* 2004; Oakley *et al.* 2007). This conserved number and position of introns further supports Luduena's (1998) conclusion that the TUB gene family observed in vascular plants originated before the divergence of monocot and dicot plants and even before their separation from mosses.

The length and amino acid sequence at the C-terminus also acts as a determinant of evolutionary divergence and is considered during classification (Wolfgang *et al.* 2004). The C-terminus of TUA and TUB proteins extrudes from the surface of assembled microtubules (Nogales *et al.* 1998; Nogales 1999), is involved in microtubule interactions with MAPs (Wasteneys *et al.* 2004) and is the target for posttranslational modification (MacRea 1997; Dixon *et al.* 2000) (see Chapter 2). Their terminal amino acid of plant alpha and beta tubulins is an important part of the hypervariable region (Oakley *et al.* 2007). All *Arabidopsis* and *Zea mays* TUA proteins for example have tyrosine (Tyr) as their terminal amino acid (Kopczak *et al.* 1992; Villemur *et al.* 1992). In *Populus* however, alpha tubulin proteins contain Tyr (known as Y-type) as well as Met (M-type) and Glu/Gln (Q-type) terminal amino acids (Oakley *et al.* 2007). In *Physcomitrella patens* (moss) and green algae, however, the extreme similarity between TUBs, including intron position, size and sequence of the C-termini, are a sign of functional redundancy (Wolfgang *et al.* 2004). Wolfgang *et al.*(2004) suggests that high instances of C-terminal variability in higher plants are linked to more complex regulatory demands of tubulins in these plants.

3.4 Spatial and temporal patterns of tubulin gene expression

Differential expression of tubulin genes has been reported for many plant species, including *Arabidopsis thaliana*, *Zea mays*, *Oryza sativa*, *Gossypium hirsutum*, *Eucalyptus grandis* and *Populus tremuloides*. Tubulin gene expression patterns are described in this section as tubulins contribute to a range of cellular and adaptive processes (Nick 2008). It is important to remember that the naming of tubulin genes in different species has usually followed their order of discovery and does not indicate sequence or functional similarity to similarly named genes (e.g. *EgrTUB1* is not the most similar to *ArathTUB1* or *PoptrTUB1*).

3.4.1 Differential expression of tubulin genes in mocot and dicot plant species

Zea mays and *Oryza sativa* TUA and TUB genes are differentially expressed in distinct tissues (Hussey *et al.* 1990; Rogers *et al.* 1993; Uribe *et al.* 1998; Yoshikawa *et al.* 2003; Giani *et al.* 2009). For example, *ZeamaTUB1* is specifically expressed in pollen while *ZeamaTUB2-5* are all expressed in roots (Hussey *et al.* 1990). In contrast to both those examples, *OrysaTUA2* and *OrysaTUA3* are expressed semi-constitutively (Rogers *et al.* 1993). Although these observed spatial tissue expression profiles differ, they are consistent with extensive microtubule activity within these respective tissues, as they contain rapidly dividing and elongating cells.

The spatial and temporal expression of TUA and TUB genes in *Oryza sativa* has been further shown to be influenced by intron position and number (Jeon *et al.* 2000; Fiume *et al.* 2004; Giani *et al.* 2009). The *OrysaTUBA1* gene for example contains three intronic sequences (Jeon *et al.* 2000). Intron mediated enhancement (IME) of expression conferred by intron 1 in this gene increased GUS expression by 34 fold compared to the intron-less construct, whereas introns 2 and 3 conferred expression levels at 20% of that achieved by intron 1 (Jeon *et al.* 2000). IME from *OrysaTUBA1* gene intron 1 has also been shown to enhance expression of two different rice alpha tubulin genes (*OrysaTUBA2* and *OrysaTUBA3*) (Fiume *et al.* 2004). IME is position dependent. That is, when the conserved intron position was altered, no enhancement was observed (Jeon *et al.* 2000). While a link between intron characteristics and the enhancement of gene expression has been associated with an

increase in steady-state mRNA (Tanaka *et al.* 1990) the exact molecular mechanism governing intron mediated enhancement (IME) remains largely unidentified.

The presence or absence of an intron upstream of the promoter region (leader intron) within an *Oryza sativa* TUB gene affected the gene's expression profile in root tips, leaf sheath and node (Giani *et al.* 2009). Removal of the intron resulted in aberrant expression in the leaf sheath, leaf blade, node, internodes and hulls. Giani *et al.*(2009) termed this phenomenon Intron Dependent Spatial Expression (IDSE). In this case the *OrysaTUB4* leader intron contained an additional promoter sequence, which drove the transcription of two alternative *OrysaTUB4* mRNAs. It was therefore hypothesised that this leader intron was involved in a negative regulatory mechanism, which when removed resulted in the aberrant expression pattern (Giani *et al.* 2009). Overall introns in *O. sativa* tubulins have been shown to influence both the level and pattern of tubulin gene expression, demonstrating their roles in both positive and possible negative regulation of gene expression.

Studies in *Arabidopsis* show similar spatial tubulin gene expression patterns to that of some monocotyledonous plants (Carpenter *et al.* 1992; Carpenter *et al.* 1993; Chu *et al.* 1998; Bao *et al.* 2001; Cheng *et al.* 2001; Yoshikawa *et al.* 2003; Giani *et al.* 2009). For example, *O. sativa* beta tubulin *OrysaTUB8* was expressed in pollen tissue (Yoshikawa *et al.* 2003) along with two alpha tubulin genes *OrysaTUA2* and *OrysaTUA3* in spikelets that contained pollen producing anthers (Giani *et al.* 2009). Similarly, *ArathTUB9* and *ArathTUA1* have been identified as pollen specific tubulin Isoforms (Carpenter *et al.* 1992; Cheng *et al.* 2001). As *OrysaTUB8* and *ArathTUB9* also seem to have derived from a common ancestral gene (Figure 3.1b and c), this might suggest that these genes contribute to the formation of microtubules with a unique function during microspore and/or pollen tube growth.

Spatial and temporal expression of tubulin genes is also diverse during *Gossypium hirsutum* development (Dixon *et al.* 1994; Dixon *et al.* 2000; Li *et al.* 2007; He *et al.* 2008). For example *GoshiTUA5-11* has specific expression within a certain cell type (fibres) which is in contrast to semi-constitutive expression of *GoshiTUA13* which is expressed in most tissue types. Similar fibre specific expression is also observed for some *GoshiTUB* genes. Furthermore, in an immunological study, the *Goshi*TUB4 protein was found to be accumulated in leaves and roots (Dixon *et al.* 1994). Whereas *Goshi*TUA8 and *Goshi*TUA9

were predominantly present during stages of rapid cell elongation of cotton fibre cells, with *Goshi*TUB6 and *Goshi*TUB7 present during secondary cell wall formation (Dixon *et al.* 1994; Dixon *et al.* 2000).

RT-PCR analysis of *GoshiTUA9* further demonstrated its specific developmental regulation, with significant down-regulation in fibre cells at the end of the cellular elongation phase, just before the onset of secondary cell wall formation (Li *et al.* 2007). This stands in contrast to other *GoshiTUA* genes that remained highly active throughout fibre development (*GoshiTUA2/3* and *GoshiTUA4*) (Li *et al.* 2007), suggesting a specific functional role for GoshiTUA9 during cellular elongation and primary cell wall development (Whittaker and Triplett 1999). Similar to the *GoshiTUA* gene family, also the nineteen-member *GoshiTUB* gene family has been characterised by RT-PCR, whereby nine TUB genes were found to be preferentially expressed in cotton fibres (He *et al.* 2008). Within these fibres *GoshiTUB1,7-9* for example were observed to be preferentially expressed at days post-anthesis, which corresponds with secondary cell wall formation (He *et al.* 2008). Therefore, this RT-PCR experiment further supports the suggestion that temporal expression of tubulin genes is indicative of tubulin gene function.

3.4.2 Differential expression of tubulin genes in woody dicotyledonous species

The eucalypt genes *EgrTUA1* and *EgrTUB1* are highly expressed in the xylem of eucalypt branches (Oakley *et al.* 2007; Qiu *et al.* 2008). On the upper side of the branch compared to the lower *EgrTUB1* and *EgrTUA1* were found to be up-regulated 1.5 and 1.4 times, respectively (Qiu *et al.* 2008). The specific role of a *Eucalyptus grandis EgrTUB1* homolog from *Eucalyptus nitens* was investigated in a promoter study using tobacco plants, which showed that *Eucalyptus nitens BETA TUBULIN 1* (*EniTUB1*) was expressed during the later stages of xylogenesis (Spokevicius *et al.* 2007a). Similar to *Eucalyptus grandis*, the *Populus tremuloides* Class I TUA and TUB and Class I-like TUB gene family members were found to be highly expressed in xylem tissue (Figure 3.1, b). Class I *PoptrTUA1* and *PoptrTUA5* genes were up-regulated 2- to 4 fold in xylem during tension wood formation and similarly, *PoptrTUB9* (Class I), *PoptrTUB15* (Class I) and *PoptrTUB16* (Class I-like) were also up-regulation during tension wood formation (Oakley *et al.* 2007). This suggests a role for these

genes during tension wood formation. In contrast, *Populus* Class II, Class III and Class IV TUB genes were expressed at lower levels in xylem, but highly expressed in other tissue types (Oakley *et al.* 2007). *PoptrTUB7* and *PoptrTUB8* (Class IV), and *PoptrTUB19* and *PoptrTUB20* (Class III) for example were highly expressed in pollen, consistent with their clustering with several other pollen specific genes (Figure 3.1b).

Finally immunolocalisation of *Populus* TUA and TUB proteins confirmed their abundance in phloem and xylem fibres undergoing secondary cell wall formation (Oakley *et al.* 2007). This result, along with individual RT-PCR expression profiles in *Populus* and microarray analysis in *Eucalyptus* provide evidence that xylem specific TUA and TUB genes play an essential role during secondary cell wall formation (Oakley *et al.* 2007).

3.5 Tubulin genes and microtubule responses to environmental stimuli

The ability of plants to detect and respond to environmental conditions, enables them to withstand substantial changes in water availability, light, temperature, nutrient levels, prevailing winds and gravity. Cortical microtubules play an important role in this resilience (Nick 1998; Abdrakhamanova *et al.* 2003; Shoji *et al.* 2006; Matsumoto *et al.* 2007; Wang *et al.* 2007). The role of tubulin gene activity and microtubule formation in response to saline conditions and cold acclimation is described below, and the usefulness of inducible gravitropism for investigations into the role of tubulins is discussed.

Recent studies in *Arabidopsis* plants demonstrated that cortical microtubules are affected indirectly by salt stress (Shoji *et al.* 2006). Microtubules depolymerised under high salinity conditions, possibly triggered by the activation of ethylene and ABA signalling pathways (Achard *et al.* 2006). Cortical microtubules in *Arabidopsis* seedlings that withstood and recovered from salt stress were observed to undergo depolymerisation followed by re-organisation, and it was suggested that this response enables plants to limit cell growth during times of salt stress (Wang *et al.* 2007).

Similarly, during cold acclimation (tolerance acquired during chilling prior to freezing) in winter wheat (*Triticum aestivum* L.) microtubules underwent partial depolymerisation followed by re-organisation (Abdrakhamanova *et al.* 2003). This process produced cold-

stable microtubules (Abdrakhamanova *et al.* 2003; Christov *et al.* 2008). A change in tubulin gene expression in three *Triticum aestivum* L. cultivars during cold acclimation was identified. In the frost tolerant cultivars tubulin isotypes from the *TUBA2* group were down-regulated (Abdrakhamanova *et al.* 2003). In contrast, expression of *TUBA3* isotypes remained constant or decreased in frost tolerant cultivars, while it substantially increased in the sensitive cultivar. Abdrakhamanova *et al.*(2003) suggested that although changes in tubulin expression and microtubule properties were observed during cold-acclimation that this was a downstream effect and not a direct cause. However, in their study, the cold-stable microtubules were vital for the plant's ability to adapt to cold temperatures. Abdrakhamanova *et al.*(2003) further concluded that MAPs and the tyrosinylation of tubulins are as important as specific isotype expression in the formation of cold-stable microtubules. Changes in tubulin gene expression in response to cold temperatures has also been observed for spinach leaves (Bartolo and Carter 1991), rye root tips (Kerr and Carter 1990) and *Arabidopsis* leaves (Chu *et al.* 1993). In the latter study, Chu et al (1993) exposed *Arabidopsis* leaves to 4^{o}C for 14 days over which time the expression of all alpha tubulin genes showed little change. In contrast, beta tubulin genes showed differential accumulation of transcripts (Chu *et al.* 1993). Transcript levels of *ArathTUB2*, *ArathTUB3*, *ArathTUB6* and *ArathTUB8* decreased, whereas *ArathTUB9* increased. The change in *ArathTUB8* gene expression was explained by transcriptional down-regulation (Chu *et al.* 1993), while *ArathTUB9* gene transcripts accumulated, and formed more cold-stable microtubules with increased stability at 4^{o}C compared to 25^{o}C (Chu *et al.* 1993).

Gravitropic stress influences early root and shoot development as well as plant stem and branch architecture (Baskin 2001). Gravitropic stress can be induced for example as hypergravity through centrifugation. To mimic gravitropic effects, Matsumoto *et al.*(2007) centrifuged *Arabidopsis* seedlings in the dark at 25^{o}C for a day, at speeds from 1 to 300 g. In this study, altered cortical microtubule arrangement in the hypocotyl region of *Arabidopsis* seedlings resulted in left-handed helical growth which was linked to increased expression of alpha and beta tubulin genes (Matsumoto *et al.* 2007). In another study, under the same centrifugal conditions, an increase in two gamma-tubulin proteins in azuki bean epicotyls was detected (Soga *et al.* 2008). The specific role of gamma tubulin was discussed in detail in Chapter 2.

Another way to induce gravitropic stress experimentally is *via* bending of the plant stem which induces tension wood formation in plants which undergo secondary growth (Pilate *et al.* 2004). Tension wood formation also naturally occurs in branches and stems in response to gravity, and is characterised by increased xylem formation on the upper side of stems and branches when compared to the lower side (opposite wood). Other characteristics include thickened cell walls, a gelatinous layer (G-Layer), increased fibre length, reduce vessel area and smaller cellulose microfibril angle (MFA) (Qiu *et al.* 2008).

A recent microarray study compared *Eucalyptus grandis* tubulin gene expression between the stem, and the upper and lower side of branches (Qiu et al. 2008). Branch wood in this study was characterised by altered physical and chemical properties on their upper side (tension wood) compared to their lower side (opposite wood) and the main stem. The upper compared to the lower side of the branch also showed reduced vessel area and a slight increase in density, with 17-24% more cellulose and 8-12% less lignin. The most significant difference however was found in cellulose microfibril angle (MFA), ranging in the upper side of branch wood from 12-25^{o} and in lower side branch wood from 38-52^{o} with an MFA in the main stem of approximately 25^{o} (Qiu et al. 2008). Of the four alpha tubulin and six beta-tubulin genes described by Qiu et al. (2008), *EgrTUB1* was up-regulated on the upper side of branches and down-regulated on the lower, whereas the *EgrTUA1* was down-regulated on the lower side of branches (Qiu et al. 2008). Similar changes in *Populus* tubulin gene expression during tension wood formation has been observed and was outlined in the previous section (Oakley *et al.* 2007).

3.6 Summary

The tubulin gene families of different plants vary in size and structure, and genomic and phylogenetic analysis has provided insight into their complexity and evolution. Furthermore, the differential expression of alpha and beta tubulin genes has been shown to be common in land plants. The diversity in tissue and developmental stage specific expression patterns highlights potential roles for these genes in specific cellular and adaptive processes, such as pollen formation and primary and secondary cell wall formation. Finally, the influence of environmental stimuli on tubulin gene expression suggests that plant microtubules perform specific cellular functions during environmental stress.

Chapter 4 Plant cell walls and the deposition of cellulose microfibrils

4.1 Introduction

Interactions between cortical microtubules and cellulose microfibrils significantly influence primary and secondary cell wall formation. This chapter explores the composition and function of plant cell walls and outlines functional studies which demonstrate the link between cortical microtubule characteristics and cellulose microfibril orientation during both primary and secondary cell wall formation. As cellulose microfibrils are central to understanding the economically important wood quality trait MFA under investigation in this thesis, the differential expression of cellulose synthase genes, like tubulin genes elaborated on in the previous chapter, their involvement in cellulose microfibril formation and their phylogeny are also described.

4.2 Structure and function of plant cell walls

Plants at a physiological level need to be highly adaptive to their environment. Examples of short term adaptation by plants include spatial and temporal control of above and below ground biomass production, nutrient up-take and photosynthesis (Schurr *et al.* 2006). Throughout their life plants also adapt to changes in environmental conditions and pathogen attack through remodelling and reconstruction of cell walls (Sarkar *et al.* 2009). This includes changing structure and composition by altering the relative amount of celluloses, lignins, phenolics and pectins. Altering cell wall number, thickness and tensile strength can provide both a physical barrier against herbivore attack and the mechanical strength to withstand gravity, strong winds and allows plants to outgrow competitors (Sarkar *et al.* 2009).

Plant primary cell walls are typically thin and flexible (0.1-1 µm) and consist of complex polysaccharides and structural proteins (Cosgrove 2005). The major macromolecules that make up a plant cell wall are crystalline cellulose microfibrils, which act as the structural rods within the cell wall, pectins, which make up part of the matrix which surrounds microfibrils and aid in gluing them in place as well as involvement in cell to cell attachment

in the middle lamella, and hemicelluloses, another microfibril crosslinking polysaccharide that aids in the strength and flexibility of primary cell walls (Cosgrove 2005). There are many other polysaccharides that contribute in lesser amounts to plant cell wall composition such as xyloglucans, arabinoxylans, arabinogalactans and mannans (Sarkar *et al.* 2009).

Cellulose microfibrils and the other polysaccharides are formed by different pathways. Cellulose microfibrils are synthesised by large membrane complexes which extrude the microfibril from the plasma membrane into the cell wall. In contrast, polysaccharides are synthesised in the Golgi apparatus and are then transported by vesicles that fuse with the plasma membrane, thereby depositing them within the cell wall (Cosgrove 2005).

Cellulose is the major structural component found in plant cell walls, and in being so is the most abundant biopolymer on earth (Perrin 2001). Cellulose microfibrils wrap around the plant cell in a helical manner providing stiffness and strength (Plomion *et al.* 2001a). Cellulose microfibrils are formed from crystallised chains of β, 1-4 glucose monomers, and are synthesised from the outer surface of the plasma membrane by transmembrane proteins, called cellulose synthase A (CesA) (Delmer 1999; Somerville *et al.* 2004). Six CesA proteins form a hexagonal rosette subunit and six rosette subunits form one cellulose synthase complex (CSC). Each CesA rosette produces a single glucan chain. Therefore, a cellulose microfibril is believed to consist of 6x6=36 glucan strands (Delmer 1999; Cosgrove 2005) (Figure 4.1).

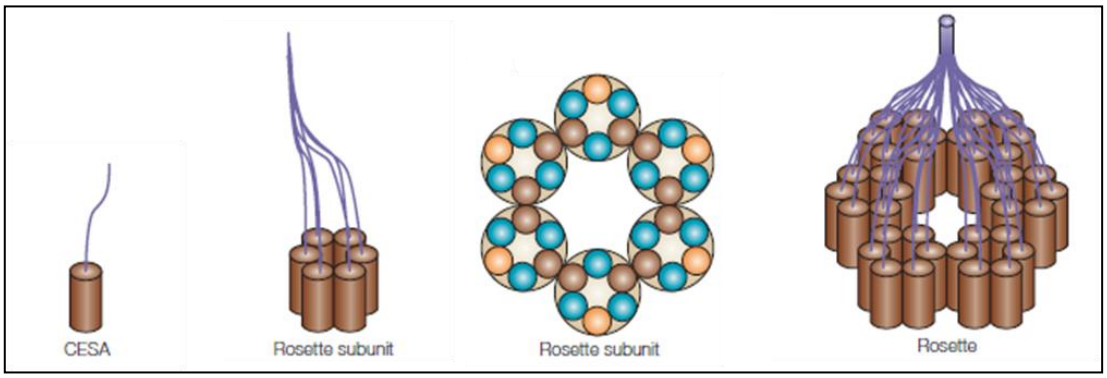


Figure 4.1 Cellulose microfibril formation and rosette structure. A single CesA protein forms a single β, 1-4-glucan chain (purple stands), a single rosette subunit forms one strand and six subunits form a cellulose synthase complex (rosette) which produced a single cellulose microfibril. The view from above a rosette subunit shows a possible composition of different CesA proteins; orange, brown and blue (Cosgrove 2005).

In plant primary cell walls cellulose microfibrils not only provide structural support but also aid in directing cell expansion by providing a plain of resistance to cell expansion, thereby directing the elongation of the cell perpendicular to the orientation of the microfibrils (Baskin 2005; Burgert and Fratzl 2010). Other cell wall constitutents are also involved in cell expansion. For example, the aptly named expansions, which are involved in the loosening and relaxation of cell walls (Sarkar *et al.* 2009), and pectins, which are also involved in pushing apart microfibrils and aiding in their sideways slippage during cell growth (Cosgrove 2005).

Plants also produce secondary cell walls in particular in cells that require greater mechanical strength and therefore greater structural reinforcement (Barnett and Bonham 2004). Secondary cell walls are deposited after cell elongation has ceased (Cosgrove 2005) and are much like primary cell wall with regard to their cell wall constitutents. Plant secondary cell walls however differ from primary cell walls in thickness; as they contain more cellulose in the form of cellulose microfibrils, and through the addition of the macromolecule lignin. Lignin is formed by up to three monolignol monomers, which vary depending on the plant species, and is involved in cross linking cellulose microfibrils and other cell wall components (Boerjan *et al.* 2003). Lignin is involved in cell wall extension mechanics, particularly in opposite and compression wood in tree species, as well as hindering the degradation of plant cell walls by pathogens (Boerjan *et al.* 2003; Sarkar *et al.* 2009).

In the primary cell wall of plants, as outlined, the microfibrils are arranged in various orientations. In contrast, in secondary cell walls microfibrils have distinct and ordered orientations between the multiple cell wall layers (Abe *et al.* 1995; Nick 2008). Wood fibres for example contain a primary wall and up to three secondary cell wall layers (Prodham *et al.* 1995). The inner and outer layers, S1 and S3, feature a larger microfibril angle, almost perpendicular to the cell axis (Burgert and Fratzl 2010). The middle or S2-layer accounts for approximately 80% of the secondary cell wall making the S2 layer the main contributor to a cell's overall strength (Plomion *et al.* 2001a) (Figure 4.2, a). In wood fibres, MFA in the S2 layer can vary between ~0^{o} and ~60^{o} ultimately influencing the mechanical properties of stem and branch wood (Plomion *et al.* 2001a; Burgert and Fratzl 2010) (Figure 4.2, b).

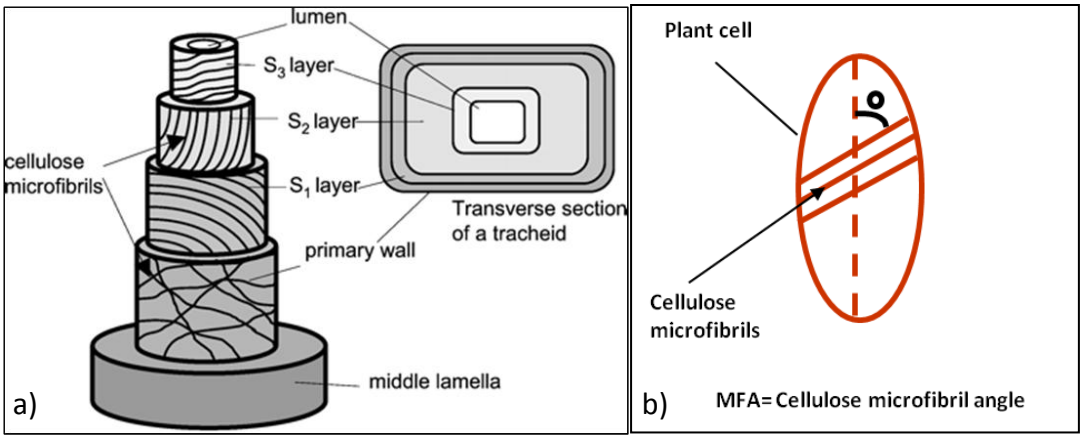


Figure 4.2 Plant cell wall MFA. a) Three-dimensional structure of the secondary cell wall of a tracheid (xylem cell) (Plomion *et al.* 2001b). This figure shows the different orientations of cellulose microfibrils in the primary wall (various alignments or random) and secondary cell wall (parallel angular alignment); S1, S2 and S3 layers. b) Cellulose microfibril angle is the difference in orientation of the cellulose microfibrils from the longitudinal axis of the plant cell.

4.3 Interactions between cortical microtubules and cellulose microfibrils

Almost 50 years ago, the existence of a cytoskeletal polymer now known as 'cortical microtubules' was hypothesised by Green (1962), as algal cells treated with colchicine (a microtubule destabilising drug) displayed altered orientation of cellulose microfibrils in their cell walls. In 1963, Ledbetter and Porter, using electron microscopy, provided the first evidence of a link between cortical microtubules and the orientation of cellulose microfibrils within plant cell walls.

It has been shown in many studies since that cortical microtubules appear to guide cellulose microfibrils (Seagull and Heath 1980; Seagull 1986; Seagull 1992; Prodham *et al.* 1995; Funada 2001; Chaffey and Barlow 2002; Paradez *et al.* 2006a; Paradez *et al.* 2006b) and while some exceptions have been reported were no parallel alignment of cortical microtubules and cellulose microfibrils was found (Preston 1988; Emons *et al.* 1992; Wasteneys 2000), the resounding and more current literature clearly supports the view that

cortical microtubules do in fact guide the alignment of cellulose microfibrils (Baskin 2001; Oda and Hasezawa 2006; Paradez *et al.* 2006a).

Cortical microtubules are not always required for maintaining cellulose microfibril alignment and deposition. For example, drug-dependent disassembly and/or stabilisation of microtubules did not abolish the oriented deposition of cellulose microfibrils during primary cell wall formation in pine seedlings (Itoh 1976), poplar parenchyma cells (Itoh and Shimeji 1976), lettuce hypocotyls (Srivastava *et al.* 1976), adzuki bean epicotyls (Takeda and Shibaoka 1981), cotton hairs (Yatsu and Jacks 1981), maize seedlings (Mueller and Brown 1982) or arabidopsis root cells (Baskin *et al.* 1994; Sugimoto *et al.* 2003). Instead these studies proposed that cellulose microfibrils can largely self-align into parallel order in the absence of microtubules (Paradez *et al.* 2006a). Earlier, a number of *Arabidopsis* cellulose mutant studies already compared the rate of cellulose synthesis, the directional movement of the CSCs and the density of the cellulose microfibril deposition within the primary cell walls and suggested that this may be sufficient to control the orientation of the microfibrils alone (Emons and Mulder 1998; Emons and Mulder 2000; Mulder and Emons 2001; Sugimoto *et al.* 2001; Sugimoto *et al.* 2003).

Furthermore, many studies identified proteins associated with cortical microtubules that may directly link them to cellulose microfibril deposition and orientation like, for example a kinesin-like protein in the *fragile fibre*1 (fra1) *Arabidopsis* mutant, which contains an N-terminal microtubule binding motor domain (Zhong *et al.* 2002). A mutation in this protein was shown to cause altered orientation of cellulose microfibrils in the secondary cell wall of fibres and significantly reduced the mechanical strength of plant stems (Zhong *et al.* 2002). Another example is the katanin-like microtubule severing protein AtKTN1 found in the *fragile fibre*2 (fra2) *Arabidopsis* mutant which has been shown to result in the aberrant deposition of cellulose microfibrils in the primary wall of elongating cells and the secondary cell walls of fibres (Burk *et al.* 2001; Burk and Ye 2002). In addition, microtubule associated proteins (MAPs) such as MAP20 are also likely candidates to be involved in directly linking cortical microtubules and cellulose microfibrils (Rajangam *et al.* 2008) (for descriptions of MAP studies refer to Chapter 2).

In the past, two theories have been widely postulated which include the so called monorail model by Heath (1974), whereby cellulose synthase complexes are proposed to be linked to cortical microtubules by microtubule associated proteins (MAPs) or motor proteins, which act to pull or guide cellulose synthase complexes along cortical microtubules, and the guardrail model (Herth 1980; Giddings and Staehelin 1991), which states that cellulose synthase complexes do not directly interact with cortical microtubules. Instead the microtubules act to delimit path and movement of these cellulose synthase complexes between cortical microtubules. For both these models the driving force of the movement of the cellulose synthase complexes is believed to be the polymerisation and crystallisation process of cellulose microfibrils with or without the aid of cortical microtubules.

Recent studies have shown support for aspects of both models. For example, work by Paradez *et al.* (2006a) showed that CSCs move along the plasma membrane laying down the cellulose microfibrils behind them during primary cell wall formation. The functional link between these cellulose synthase complexes and cortical microtubules was visualised in *Arabidopsis thaliana* hypocotyl cells by using fluorescently labelled CELLULOSE SYNTHASE-6 (CesA6-YFP) and ALPHA TUBULIN-1 (TUA1-CFP) proteins (Paradez *et al*. 2006a; Paradez *et al*. 2006b). In these studies live cell imagery showed the alignment of cortical MTs with the movement of cellulose synthase rosettes, leading to a number of observations. Firstly, that CesA complexes move through the plasma membrane at a constant rate in linear trajectories that coincide with cortical microtubule orientation and secondly, that cellulose synthase rosettes change direction concurrently with cortical microtubules (Paradez *et al*. 2006a; Paradez *et al*. 2006b). Thirdly, the assembly of new cortical microtubule arrays precede the assembly of the cellulose synthase rosettes (Paradez *et al.* 2006a). These observations serve as evidence that cortical microtubules and cellulose microfibrils are both spatially and mechanistically linked.

While the importance of cortical microtubules in directing cellulose microfibril deposition in the primary cell wall has been demonstrated, Paradez *et al.*(2006a) also showed that cellulose synthase rosettes remained ordered for a period of time after cortical microtubules had depolymerised, suggesting that microtubules were not essential to maintain microfibril organisation and that some other mechanism can take over in their absence (as suggested in previously discussed microtubule drug studies). This demonstrated

that the amount of depolymerisation of microtubules (drug treatment) influences the alignment mechanism of microfibrils, because when microtubules are present they are observed to align microfibrils, however, when microtubules are partially destabilised the microfibrils become randomly aligned. Once microtubules are completely depolymerised the ordered alignment of microfibrils returns. This model of independent cellulose microfibril organisation is further supported by evidence of bidirectional communication between microfibrils and microtubules, as reported by Fisher and Cyr (1998) and later observed by Chu *et al.*(2007). In these studies a CESA inhibitor (isoxaben) in tobacco protoplasts and a null mutation of CesA2 in arabidopsis, respectively showed that altering CesA function triggered cortical microtubule disorganisation.

The mechanism by which cellulose microfibrils are aligned in the absence of microtubules is unknown, however, one possible mechanism was hypothesis by Diotallevi and Mulder (2007) who suggested that cellulose microfibrils are able to maintain their own trajectory after the initial deposition of glucose chains through the process of crystallisation and self propulsion. Using a biophysical model, Diotallevi and Mulder (2007) simulated cellulose microfibril polymerisation from cellulose synthase complexes and showed that the CSCs would indeed be able to self-propel across the plasma membrane, as suggested by Herth (1980) and Mogilner and Oster (2003). In their model, ATP energy is gained during cellulose microfibril polymerisation and cellulose crystallisation. Both processes result in an increased 'state of stress' within the glucan polymers (Diotallevi and Mulder 2007), making the hypothesised motion reaction energetically favourable. This energy gain, along with other observed thermal energy fluctuations in the plasma membrane, could then be converted from chemical energy into motion, resulting in the forward movement of the CSC (Diotallevi and Mulder 2007).

This proposed self-propulsion ability of the CSC provides a possible explanation as to how in the absence of cortical microtubules (for example following a microtubule destabilising treatment; colchicines) CSC's continued to move (Baskin *et al.* 1994; Sugimoto *et al.* 2003; Paradez *et al.* 2006a). Cellulose microfibrils crystallisation (rigidity) is also predicted to be sufficient to maintain the linear trajectories of CSCs observed in the absence of microtubules (Paradez *et al.* 2006a; Diotallevi and Mulder 2007). Finally, the modelled speed of the self propelled CSCs was estimated to be ~4.5×10^{-9}m/s, which is comparable to the

measured speed of CSC movement *via* real-time visualisation in arabidopsis of ~5.8x10^{-9}m/s (Paradez *et al.* 2006a; Diotallevi and Mulder 2007).

During the transition from primary to secondary cell wall formation, the cell's biosynthetic machinery (including for example CESA gene expression; see next section) undergoes dramatic changes shifting from supporting active cell expansion to supporting the structural assembly of the secondary cell wall (Samuels *et al.* 2006). Like in the primary cell wall, the exact mechanistic link between cortical microtubules and cellulose microfibrils during secondary cell wall formation remains elusive. In the following paragraphs some studies are described that have investigated the role of cortical microtubules during secondary cell wall formation.

The organisation of cortical microtubules during secondary xylem and phloem differentiation in *Aesculus hippocastanum* L. (horse-chestnut) using immune-fluorescence microscopy of alpha-tubulin showed that cortical microtubules rearrange and become more helically orientated as secondary cell wall formation began (Chaffey *et al.* 2000). Specifically in fibre cells a significant increase in the density of cortical microtubules in helical arrays was also observed (Chaffey *et al.* 2000), while in phloem parenchyma cells rings of cortical microtubules, distinct from the helically orientated microtubule arrays, were observed to form around developing pits, which lack secondary cell walls. Similar observations have been made in *Populus tremula* L. x *P. tremuloides* Michx and *Pinus pinea* L. developing secondary cell walls of xylem and phloem cells (Chaffey and Barlow 2002). Pits are used as channels to transport nutrients between cells and consists of small round or oval shaped regions of primary cell wall surrounded by thickened secondary cell wall (Abe *et al.* 1995; Carlquist 2007).

Similarly, a quantitative electron microscopic study has also given insight into changes in microtubule array and cellulose microfibril orientation during *Gossypium hirsutum* L. (cotton) fibre development (Seagull 1992). During fibre initiation and early elongation the microtubule arrays were observed to have various orientations while in the latter stages of elongation the microtubule arrays were orientated almost transversely. The transition from primary to secondary cell wall formation saw microtubule arrays become longitudinally

aligned to the cells elongation axis (Seagull 1992). During this same developmental stage microtubules were also observed to exhibit similar changes (Seagull 1992).

At the start of secondary cell wall synthesis a fourfold increase in the number of cortical microtubules was also observed (Seagull 1992). Similar changes in the density of cortical microtubules during secondary cell wall formation in *A. hippocastanum* (Chaffey *et al.* 2000) and *G. hirsutum* (Seagull 1992) have also been observed in *Fraxinus mandshurica* var. *Japonica* (Japanese ash) (Prodham *et al.* 1995). In this study the increase in density of cortical microtubules was suggested to play a significant role in regulating the orientation of cellulose microfibrils during secondary cell wall formation of wood fibres. The density of microtubules during secondary cell wall formation of conifer tracheids has been found to be 5-9 microtubules per µm (Abe *et al.* 1994). While during tension wood formation in *Populus euramericana* which is characterised by increased number of highly ordered cellulose microfibrils, wood fibres contained approximately 20 microtubules per µm of cell wall (Fujita *et al.* 1974). Likewise, Prodham *et al.* (1995) observed a high density of microtubule arrays, with 17-18 microtubules per µm of cell wall in tension wood fibres. In this case, this increase in microtubule density was also associated with increased longitudinal orientation of cellulose microfibrils (MFA).

The role of cortical microtubules during secondary cell wall formation has also been investigated in detail in *A. thaliana* cell suspension cultures (Oda *et al.* 2005). In these cultures tracheary element differentiation is induced together with secondary cell wall thickening. Using this system, cortical microtubules where shown to be important in determining not only the region (surface area) of secondary cell wall deposition but also its integrity and width of deposition in tracheary elements (Oda *et al.* 2005; Oda and Hasezawa 2006). Cortical microtubules and microtubule bundles were visualised by confocal microscopy and were shown to appear prior to secondary cell walls and at specific sites where secondary cell walls and bridges (regions joining bands of secondary cell wall) were subsequently deposited (Oda *et al.* 2005). Cortical microtubule bundles were also observed to split and align themselves on either side of the forming secondary cell walls, acting as a boundary to designate regions for secondary cell wall formation (Oda *et al.* 2005; Oda and Hasezawa 2006), which is likely to explain the previously observed helical arrangement of

microtubule arrays surrounding cell wall pits (Abe *et al.* 1995; Chaffey *et al.* 2000; Chaffey *et al.* 2002; Carlquist 2007).

4.4 Cellulose synthase gene families and differential expression

CELLULOSE SYNTHASE A (*CesA*) genes and proteins have been characterised in a wide range of plant species, 6 CesA genes have been identified in *Physcomitrella patens*, 10 in the *Oryza sativa* and *Arabidopsis thaliana* genomes, 17 in the *Populus trichocarpa* genome, while 11 and 6 were identified in *Pinus radiata* and *Eucalyptus grandis*, respectively (Ranik and Myburg 2006; Somerville 2006; Tuskan *et al.* 2006; Kumar *et al.* 2009). Like the tubulin genes discussed in Chapter 3, CESAs are differentially expressed during primary and secondary cell wall formation, where by some CesA genes are expressed during primary cell wall synthesis and make up these rosettes, while others are expressed during secondary cell wall formation and contribute then (Taylor *et al.* 1999; Scheible and Pauly 2004; Taylor *et al.* 2004; Chu *et al.* 2007; Daras *et al.* 2009). Expression patterns of both gene families and related protein distribution overlap (Gardiner *et al.* 2003; Paradez *et al.* 2006b). Examples of differential expression of CesA genes and protein distribution in a range of species are briefly described below.

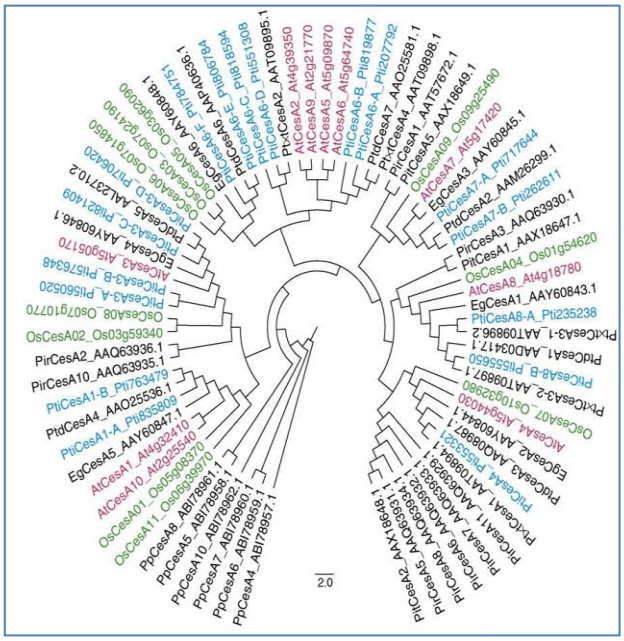
A. thaliana mutant studies have identified CesA genes with specific expression during primary growth including *AtCesA1* (*rsw1* mutant), *AtCesA2*, *AtCesA3* (*ixr1*, *cel1*, *eli1* mutants), *AtCesA5*, *AtCesA6* (*prc1*, *ixr2* mutants), *AtCesA9* and *AtCesA10*, while others including *AtCesA4* (*irx5* mutant), *AtCesA7* (*irx3* mutant) and *AtCesA8* (*irx1* mutant) are expressed during secondary growth (Taylor *et al.* 1999; Scheible and Pauly 2004; Taylor *et al.* 2004; Chu *et al.* 2007; Daras *et al.* 2009). Similarly, in *Hordeum vulgare* L. (barley) three CesAs have been identified, which are preferentially expressed during primary or secondary cell wall formation (Burton *et al.* 2004).

In *A. thaliana* specific CesA genes have been identified to be required for correct cellulose synthase function during primary growth (*AtCesA1* and *AtCesA3*) while partial redundancy was observed for others (*AtCesA2*, *AtCesA5* and *AtCesA6*) (Desprez *et al.* 2007). Temporal expression patterns of *AtCesA2*, *5* and *6* during primary cell wall synthesis in the hypocotyl and root, suggest that this partial redundancy might be caused by developmental stage

specific regulation (Desprez *et al.* 2007). In contrast, all three CesAs (*AtCesA4*, *AtCesA7* and *AtCesA8*) are required during secondary growth (Atanassov *et al.* 2009).

In other species differential expression of individual CesA genes has also been observed. In *E. grandis* for example three primary growth specific CesAs have been identified, *EgCesA4*, *EgCesA5* and the less active *EgCesA6* (Ranik and Myburg 2006). All other *E. grandis* CesA genes are secondary cell wall specific (*EgCesA1* to *3*) (Ranik and Myburg 2006). In *P. trichrocarpa*, microarray profiling demonstrated strong differential expression of 5 out of the 17 CesA genes during xylem secondary cell wall formation. They were *PtiCesA4*, *PtiCesA7-A* and *B*, *PtiCesA8-A* and *B* (A and B homologs are present due to a recent poplar genome wide duplication event) (Kumar *et al.* 2009).

Phylogenetic analysis conducted by Ranik and Myburg (2006) identified clades containing both monocot and dicot CesA proteins with higher similarity between species than within species. For example, *Eucalyptus grandis Eg*CesA proteins displayed low amino acid similarity to each other (61-70%), but relatively higher similarity to *A. thaliana* (71-86%) and *P. trichrocarpa* (81-90%) CesAs, suggesting the presence of a common ancestral gene specific to primary and secondary cell wall formation that predates the monocot-dicot divergence. Figure 4.3 shows the phylogenetic distribution of plant CesA genes (Kumar *et al.* 2009).


PtrCesA2_AAT09895.1
AtCesA2_At4g39350
AtCesA9_At2g21770
AtCesA5_At5g09870
AtCesA6_At5g64740
PtiCesA6-B_Pti819877
PtiCesA6-A_Pti207792
PtdCesA7_AAO25581.1
PtdCesA4_AAT09898.1
PtiCesA5_AAX18649.1
OsCesA09_Os09g25490
AtCesA7_At5g17420
EgCesA3_AAY60845.1
PtiCesA7-A_Pti717644
PtdCesA2_AAM26299.1
PtiCesA7-B_Pti262611
PtdCesA3_AAQ63930.1
PirCesA1_AAX18647.1
PtiCesA04_Os01g54620
OsCesA8_At4g18780
AtCesA8_At4g18780
EgCesA1_AAY60843.1
PtiCesA8-A_Pti235238
PtdCesA3-1_AAT09896.2
PtdCesA1_AAD03417.1
PtiCesA8-B_Pti555650
PtdCesA3-2_AAT09897.1
OsCesA07_Os10g32980
AtCesA4_At5g44030
EgCesA4_AAY60844.1
AtCesA7_At5g05170
EgCesA2_AAO63923.1
PtdCesA1_AAT09894.1
PtiCesA4_Pti552321
PtdCesA2_AAO63922.1
PtiCesA1_AAO63933.1
PtiCesA11_AAO63934.1
PtiCesA7_AAO63931.1
PtiCesA8_AAO63932.1
PtiCesA5_AAX18648.1
PtiCesA2_AAO63929.1
PtiCesA3_AAO63930.1
PtiCesA6_AAO63928.1
PtiCesA9_AAO63927.1
PtiCesA10_AAO63926.1
PtiCesA12_AAO63925.1
PtiCesA13_AAO63924.1
PtiCesA14_AAO63921.1
PtiCesA15_AAO63920.1
PtiCesA16_AAO63919.1
PtiCesA17_AAO63918.1
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PtiCesA23_AAO63912.1
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PtiCesA30_AAO63905.1
PtiCesA31_AAO63904.1
PtiCesA32_AAO63903.1
PtiCesA33_AAO63902.1
PtiCesA34_AAO63901.1
PtiCesA35_AAO63900.1
PtiCesA36_AAO63899.1
PtiCesA37_AAO63898.1
PtiCesA38_AAO63897.1
PtiCesA39_AAO63896.1
PtiCesA40_AAO63895.1
PtiCesA41_AAO63894.1
PtiCesA42_AAO63893.1
PtiCesA43_AAO63892.1
PtiCesA44_AAO63891.1
PtiCesA45_AAO63890.1
PtiCesA46_AAO63889.1
PtiCesA47_AAO63888.1
PtiCesA48_AAO63887.1
PtiCesA49_AAO63886.1
PtiCesA50_AAO63885.1
PtiCesA51_AAO63884.1
PtiCesA52_AAO63883.1
PtiCesA53_AAO63882.1

Chapter 5 Selection of genetic transformation methodology

5.1 Introduction

The manipulation of gene expression through *Agrobacterium* based approaches has been used as a means to determine plant gene function since *Agrobacterium* was first discovered to contain circular DNA (Zaenen *et al.* 1974). A range of transgenic methods are available for investigating the role of genes in wood formation including whole plant transformation, partial transformation or tissue culture procedures, and these need to be assessed in light of the candidate gene and/or developmental process under investigation (for a recent review see Spokevicius *et al.* (2007b)). Whole plant transgenics have been applied to a range of tree species including poplar, eucalypts and conifers (Parsons *et al.* 1986; Fillatti *et al.* 1987; Ho *et al.* 1998; Moralejo *et al.* 1998; Tang and Newton 2003) as well as many others (see review by Herschbach and Kopriva (2002)). *In vitro* tissue culture methods have also been used for studying wood formation in conifers and eucalypts (Moller *et al.* 2003; Spokevicius *et al.* 2005). All these methods have limitations such as transplant recovery efficiency and the need to use aseptic techniques which are time consuming. The use of *Agrobacterium* to create transgenic tissue sectors (regions of transgenic wood within an already developed tree) has been applied to poplar, eucalypts and conifers (Spokevicius *et al.* 2006; Van Beveren *et al.* 2006; Spokevicius *et al.* 2007a). This approach, known as Induced Somatic Sector Analysis, has also been used in this thesis.

The choice of vectors for manipulating expression depends on the candidate gene and/or the molecular process under investigation, and in this thesis two different vector based delivery systems have been used leading to over-expression and down-regulation, respectively. The assumption that the use of over-expression vectors will lead to an increase in gene activity is not always correct, as the phenomenon of gene silencing can result in reduced transcript abundance (Napoli *et al.* 1990). Down-regulation vectors can take several forms and therefore the molecular background to this technology and the vector based systems selected to be used in this project are described together with a brief review of Agrobacterium mediated gene transfer.

5.2 *Agrobacterium*-mediated DNA transfer

Agrobacterium tumefaciens is a common soil bacterium that causes crown gall disease in a wide range of plant species (Hooykaas and Schilperoort 1992; Zupan and Zambryski 1995). This versatility makes it an ideal DNA delivery system for this study as it has been shown to efficiently transform eucalypt trees (Spokevicius *et al.* 2006; Van Beveren *et al.* 2006; Spokevicius *et al.* 2007a). *Agrobacterium* contains specialised Ti (tumour-inducing) plasmids (circular-DNA), which transfer a specific segment of T-DNA (transfer-DNA) into the plant cells (Zupan *et al.* 2000). This process of precise DNA transfer has been harnessed to experimentally transfer foreign DNA into plant cells, enabling the study of gene expression and protein function in transgenic plants.

Two regions within the Ti-plasmid are of particular relevance. Firstly, the T-DNA region which contains single stranded DNA (ssDNA) fragments for transfer, flanked by 25bp left and right borders which facilitate the transfer of ssDNA within these borders directly into the host cell (Zupan *et al.* 2000). A second region, the virulence region, contains an operon of genes known as virulence genes (*vir* genes) which are involved in the cleavage, packaging and delivery of T-DNA. Expression of *VirA* and *VirG* genes is induced by the presence of phenolic compounds released by the plant as part of its response to wounding, as well as the phyto-hormones auxin and cytokinin. These compounds further activate other virulence genes *via* auto-phosphorylation (Zupan *et al.* 2000). These other virulence genes *VirD1/D2* nick the T-DNA at the border regions to release the single stranded DNA. After cleavage, VirD2 remains tightly associated with the 5' end of the T-DNA complex giving it a polar characteristic (Zupan *et al.* 2000). The VirE2 DNA binding protein then coats the T-DNA preventing any degradation of this complex by nucleases, as it travels from the *Agrobacterium* into the host cell where it is integrated into the plant genome (Zupan and Zambryski 1995; Zupan *et al.* 2000).

Strains of *Agrobacterium* used for genetic modification have been altered so that they can be easily harnessed for DNA transfer applications. They no longer cause crown gall disease, a modification achieved by removing unfavourable genes, such as tumour inducing and opine genes. Subsequently, the Vir genes were separated from the T-DNA region creating two separate plasmids, known as the binary vector system - in that the Vir-plasmid now

acts on the T-DNA plasmid to induce T-DNA transfer (Hoekema *et al.* 1983). This system revolutionised microbiology by enabling easy control of *Agrobacterium* mediated DNA transfer into plants. The altered small T-DNA plasmids contain multiple restriction sites allowing genes of interest to be easily cloned into the binary vector (Gelvin 2003). Today a wide range of binary vectors are commercially available to suit a diverse range of applications.

5.3 ISSA transformation methodology

A specialised addaptation of *Agrobacterium*-mediated transformation to tree species by Van Beveren *et al.* (2006) resulted in a simple, yet powerful methodology called Induced Somatic Sector Analysis (ISSA). ISSA involves the direct transformation of cambial cells in tree stems, *via* inoculation with *Agrobacterium*, to create small transgenic sectors (including woody tissue) surrounded by untransformed control tissue.

Advantages of this methodology include its simplicity and its suitability to a wide range of tree species for the specific investigation of genes controlling wood formation. Also results are obtained within a few months, comparing favourably to whole plant transformation methods (Spokevicius 2006). An often underestimated advantage of the ISSA approachis the fact that lends itself to paired statistical analysis, as transgenic sectors created are surrounded by untransformed tissue, with the presence of the transgene being the only difference between a sector and the control tissue. This paired experimental design (treatment Vs control) enables much of the background variation to be removed during data analysis which has, for example enabled the detection subtle changes in MFA within a highly variable system (Spokevicius *et al.* 2007a). One significant disadvantage of ISSA is the small size of the transgenic sectors produced, making isolation of RNA and downstream phenotypic analysis difficult.

5.4 Binary vector selection - Over-expression and down-regulation vectors

Over-expression vectors contain candidate gene(s) (full cDNA sequence), usually under the control of tissue specific, or constitutively active promoters such as the cauliflower mosaic virus 35S promoter (CaMV 35S promoter) (Odell *et al.* 1985). Transgenes under the control of the CaMV 35S promoter characteristically have increased gene expression in all plant

tissues, including increased transcript number and protein production (Guilley *et al.* 1982; Odell *et al.* 1985; Kay *et al.* 1987). In some cases, however, the transgene shares high sequence similarity with, or represents an additional copy of an endogenous gene, which can give rise to gene silencing (Shirsat *et al.* 1989; Hobbs *et al.* 1990).

Gene silencing as a result of transgene over-expression was first reported by Napoli *et al.* (1990) and Van Der Krol *et al.* (1990). They showed that the over-expression of a chalcone synthase gene which was predicted to increase the occurrence of purple pigment in petunia petals, gave rise to petals containing white segments. Analysis of gene expression within the white petal segments revealed that silencing of both the transgene and the endogenous gene had occurred (Napoli *et al.* 1990; Van Der Krol *et al.* 1990). Homology dependent gene silencing (HDGS) has since been observed in a wide range of plant species (Matzke and Matzke 1995; Cogoni and Macino 1999; Kohli *et al.* 1999). In tobacco plants for example, gene silencing of gamma-tubulin resulted in aberrant plant growth (Zhao *et al.* 2009). In tree species HDGS was suspected in eucalypts as Spokevicius *et al.*(2007a) observed the opposite phenotype to that expected for the over-expression of *Eucalyptus grandis BETA TUBULIN 1*.

Two different mechanisms have been described for HDGS, transcriptional gene silencing (TGS) and post transcriptional gene silencing (PTGS), which act by blocking the transcription of mRNA in the nucleus and by targeting transcripts for degradation in the cytoplasm, respectively (Cogoni and Macino 1999; Bernstein *et al.* 2001a). Genes can be targeted for HDGS based on transgene copy number or insertion position within the genome (Yang *et al.* 2005).

TGS involves RNA directed DNA methylation of either the coding sequence or the promoter region creating a physical barrier to transcription (Bernstein *et al.* 2001b). Methylation also triggers PTGS with siRNAs targeting methylated regions of DNA:RNA sequence, recruiting the RICE:Dicer complex which starts the process of degradation (Jones *et al.* 2001; Matzke *et al.* 2004). As methylation patterns have been shown to be heritable, gene silencing can continue to occur in multiple cellular generations (Jones *et al.* 2001).

The mechanism of PTGS involves the targeted degradation of double stranded (ds)RNA by the RNA inducing silencing complex (RISC) which binds to dsRNA and recruits the enzyme

Dicer, which then cuts the dsRNA into small 21-23 bp fragments, known as short interfering RNA (siRNA) (Zamore *et al.* 2000; Jones *et al.* 2001; Vance 2001; Miki *et al.* 2005). The RISC complex retains the 21-32 bp sense strand of the cut fragment and in doing so the RISC:RNA complex continues to bind to complementary sequences. The process of degradation continues until little or no complementary RNA remains (Matzke and Matzke 1995; Vance 2001; Matzke *et al.* 2004).

A range of down-regulation vectors are available, including antisense, hairpin (RNAi) and microRNA vectors. These vectors result in the formation of double stranded RNA hairpin structures which through RNA induced silencing complexes targets homologous mRNA transcripts for gene silencing *via* degradation (Sui and Shi 2005). Both antisense and RNAi vectors use full length cDNA sequence or partial DNA sequence which can vary in size from 100 bp to 1 Kb (Meyer *et al.* 2004; Miki and Shimamoto 2004; Sui and Shi 2005). The advantage of microRNA vectors over the other two vectors is that they target a small specific region of the candidate gene. This specificity allows down-regulation of individual genes from within closely related gene families.

RNA molecules were originally believed to be involved exclusively in protein biosynthesis e.g. messenger RNA, ribosomal RNA, transfer RNA. However, in 1993 the first microRNA was discovered and it targeted the *Lin-14* gene in *C. elegans* (Lee *et al.* 1993). microRNAs and other regulatory RNA molecules have since been discovered in both plants and animals. These regulatory RNAs are involved in gene silencing and are referred to as silencing RNAs (sRNAs), and include short interfering RNAs (siRNAs), and microRNAs (miRNAs) (Ossowski *et al.* 2008). As regulatory molecules miRNAs act on transcription factors, promoters, mRNA stability and translation efficiency *via* a negative feedback mechanism (gene silencing), affecting gene expression in plants and animals (Lu *et al.* 2005; Schwab *et al.* 2006).

Both siRNAs and miRNAs differ in their biosynthesis however, they are similar with regards to their size and function. siRNAs are derived from long complementary double stranded RNA (dsRNA) sequences, and can form from both endogenous genes and transgenes (Schwab *et al.* 2006). These siRNAs combine with RNA-induced silencing complexes (RISCs), which in plants have been shown to contain Argonaute (AGO) proteins as catalytic subunits, which direct site dependent cleavage of the target transcripts (Ossowski *et al.* 2008). These

double stranded regions are targeted by Dicer RNase (predominantly DCL1 in plants) which cleaves them into short ~21 bp sequences (Schwab *et al.* 2006).

The process of siRNA gene regulation has been harnessed through the development of RNA-interference technology (RNAi technology), where double stranded DNA hairpin constructs are created with homology to the target candidate gene. When transcribed these sequences form a hairpin loop structure which is targeted and cleaved by the DICR RNase (DCL1) complex (Schwab *et al.* 2006). The DICR complex actively down regulates gene expression through the repeated production of siRNAs.

On the other hand, miRNA formation and processing is more complex and also more specific than siRNAs (Bartel 2004). In plants, primary miRNA transcripts are recognised and cleaved by a Dicer RNase (DCL1) complex in the nucleus to produce a 60 to 300 bp hairpin structure known as the miRNA precursor (Lu *et al.* 2005) (Figure 5.1). The miRNA precursor consists of a seed region which contains the target sequence, a 21 bp specific sequence, and a backbone which forms a secondary hairpin and loop structure (Bartel 2004). The precursor backbone influences the recognition and processing of the miRNA by DCL1 and therefore the effectiveness of the miRNA to suppress the target transcript (Kertesz *et al.* 2007). The more closed or complex the secondary structure of the backbone, the greater the reduction of seed site accessibility, leading to reduced effectiveness of the miRNA in down-regulating gene expression (Kertesz *et al.* 2007).

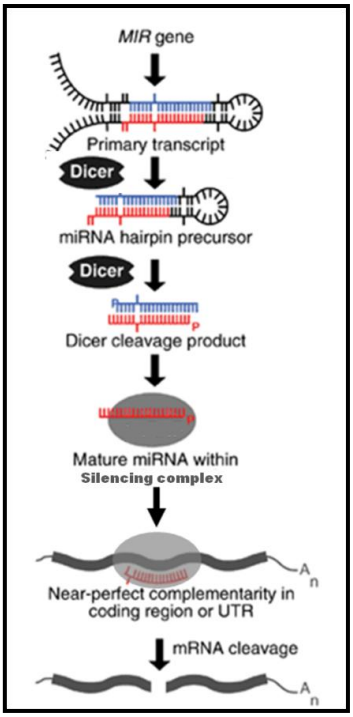

Figure 5.1 A diagrammatic representation of the stages of microRNA production. Firstly, microRNA primary transcript forms a stem-loop structure (hairpin), which is cleaved by the enzyme Dicer. This is followed by the 21 bp sequence being incorporated in the silencing complex. The miRNA has near-perfect complementation to a mRNA. Lastly the mRNA sequence is cleaved. Picture adapted from Steinberg, *et al* (2003).

The specific 21 bp nucleotide sequence of the seed site, once incorporated into the Dicer complex, then directs site-dependent cleavage of the target transcripts (Lu *et al.* 2005). miRNAs are strand-specific unlike siRNAs, in that the strand with the lower thermodynamic stability at the 5' end is preferentially active in the Dicer complex (Schwab *et al.* 2006). The highly specific activity of plant miRNA is governed by the fewer than five mismatches found between the miRNA and their target transcript (Schwab *et al.* 2006). For example, for pairing of the 5' portion nucleotide positions two to twelve must be exactly the same as the target sequence, or have only one mismatch and have very specific 3' end pairing. Furthermore, the first nucleotide at the 5' end is predominantly a uridine base and the tenth an adenine in most endogenous plant miRNAs (Schwab *et al.* 2006; Ossowski *et al.* 2008). Nucleotides two, twelve and twenty are believed to be important for sequence recognition (Bowman pers com. 2007). This high sequence recognition is preserved with clusters of more than two mismatches in the 3' end being uncommon (Schwab *et al.* 2006; Ossowski *et al.* 2008). Due to this high sequence specificity, microRNAs and synthetic microRNA lend

themselves to being used as genetic tools for the specific down-regulation of candidate genes.

The use of microRNAs as genetic tools can be either *via* the use of endogenous microRNAs or through the use of artificial microRNAs (amiRNA). The advantage of amiRNAs is that they can be designed for any gene under investigation. At present the use of amiRNA as a tool for the functional characterization of genes is still in its infancy.

amiRNAs have a wide range of applications, as they act on transcription factors, promoters, and affect mRNA stability and translation efficiency (Schwab *et al.* 2006). Their applications range from assessing gene function (Alvarez *et al.* 2006; Schwab *et al.* 2006) and regulation of developmental changes in plants (McConnell *et al.* 2001; Rhoades *et al.* 2002; Hunter and Poethig 2003), to engineering disease resistance (Niu *et al.* 2006; Qu *et al.* 2007; Ossowski *et al.* 2008). Importantly, for non-model plant species amiRNAs can overcome some of the limitations resulting from incomplete genomic information.

Other methods for the down-regulation of genes include antisense constructs and RNAi where a full or partial gene is inserted in a hairpin configuration (Smith *et al.* 2000; Meyer *et al.* 2004). The disadvantage of using either of these methods compared to amiRNA is that it can be difficult or impossible to target individual genes within a highly conserved gene family, and down-regulation of many genes at once could be detrimental to cell viability. Furthermore, specific information on the function of individual genes could be unattainable.

In recent studies both endogenous and artificial microRNAs (amiRNAs) have been used successfully to down-regulate gene expression in arabidopsis, tomato and tobacco plants (Alvarez *et al.* 2006; Niu *et al.* 2006; Schwab *et al.* 2006; Qu *et al.* 2007; Koundal and Praveen 2010). With regard to woody plant species endogenous miRNAs have also been extracted from *Populus trichocarpa* (Lu *et al.* 2005) however, to date none have been functionally characterised. Synthetic microRNAs however, have recently been successfully used to target a KNOX homebox gene in *Populus alba* (Du *et al.* 2009).

5.5 Summary

Agrobacterium mediated transformation through the use of Induced Somatic Sector Analysis was selected as the method of investigating candidate gene function in this project. Also the selection of the two different vector based delivery systems, used to manipulation gene expression being over-expression and down-regulation *via* synthetic miRNAs was outlined.

Chapter 6 Functional characterisation of alpha and beta-tubulin genes in eucalypts

6.1 Introduction

Early breeding efforts in tree, including eucalypts, focused on the improvement of tree growth, survival, stem straightness and branch quality, and more recently emphasis was also directed at including fitness (ability to withstand environmental challenges) and wood quality traits (Greaves *et al.* 1997; Raymond 2002). Wood quality traits such as density, cellulose content, fibre length and modulus of elasticity are predicted to increase with tree age, while microfibril angle and lignin content decrease with age (Raymond 2002). This project specifically focuses on the economically important wood quality trait cellulose microfibril angle (MFA), which has profound effects on wood stiffness (Barnett and Bonham 2004) and the suitability for timber products for solid wood products or pulp and paper production (Plomion *et al.* 2001a). Wood fibres with small MFA in the S2 layer of their secondary cell walls have increased tensile strength, while large MFA increases the elasticity of individual wood fibres (Barnett and Bonham 2004).

From a biological perspective trees produce wood fibres with altered MFA and other cell wall characteristics in order to withstand environmental stresses also allowing them to grow to immense heights. For example, smaller MFAs are observed in tension wood which forms on the upper side of branches and leaning stems and, like tension wood in mature wood, this smaller MFA results in wood fibres that are much stiffer and better able to withstand the forces of wind and gravity (Barnett and Bonham 2004; Burgert and Fratzl 2010). In contrast, larger MFA are observed in wood fibres in the stems of young trees (juvenile wood) which make them more flexible thereby reducing the impact of strong wind.

Eucalypts were selected as the model tree species for investigation of wood formation in this project for a number of reasons. The tubulin candidate gene family available for investigation was from *Eucalyptus grandis* (Qiu *et al.* 2008), and eucalypts have been effectively used for similar transgenic studies in the past (Spokevicius *et al.* 2007a). Furthermore, globally, eucalypt fibre is considered superior, which is reflected by the decision of the US Department of Energy to sequence the eucalypt genome in an effort to

harness it for fibre production, bio-energy, carbon sequestration and other industrial applications (www.EUCAGEN.org). This makes eucalypts an ideal species for the investigation of wood formation and wood fibre quality traits, with any finding relevant to a wide range of tree species.

The strong link between tubulin gene expression, cortical microtubules and cellulose microfibrils was outlined in Chapters 2-4. Qiu *et al.* (2008) identified two tubulin genes that showed differential expression correlating with changes in MFA. These were *Eucalyptus grandis ALPHA TUBULIN 1* (*EgrTUA1*) (Genbank ID EF534224) and *BETA TUBULIN 1* (*EgrTUB1*) (Genbank ID EF534219). The *EgrTUB1* gene showed a 1.47 fold increase in expression on the upper side (low MFA) versus lower side (high MFA) of *E. nitens* branches, whereas, *EgrTUA1* showed a 1.53 fold increase in expression on the upper side (low MFA) versus lower side (high MFA) of branches (Qiu *et al.* 2008).

The recent successful demostration of *EgrTUB1* involvement in altering MFA further supports the selection of *E. grandis* tubulins as excellent candidates for investigating the molecular control of cellulose microfibril angle (MFA) determination (Spokevicius 2006; Spokevicius *et al.* 2007a). In this chapter the following research questions are addressed:

- Does alpha tubulin gene activity, like beta tubulin gene activity influence MFA?
- How does down-regulation of tubulin genes alter MFA? And what does this tell us with regard to the over-expression vector phenotypes observed?
- Does altering alpha and beta tubulin activity together, have a different or greater effect on MFA?

6.2 Materials and Methods

6.2.1 Plant material

Eucalyptus camaldulensis X grandis and *globulus X grandis* clonal seedlings from Narromine Transplants (www.transplants.com.au) were used in the experiments undertaken in this chapter. The seedlings were potted up and allowed to grow for approximately four months prior to inoculation. All experimental trees were grown and maintained in a glasshouse with

supplemented 16 hr light period and temperature control (24^{o}C +/- 3^{o}C). Plants were watered three times daily *via* a dripper system.

6.2.2 Candidate gene selection

The cDNA sequence for all known *Eucalyptus grandis* alpha and beta-tubulin genes were kindly provided by Dr Simon Southerton (CSIRO Plant Industry) as part of an ongoing collaboration between the University of Melbourne and CSIRO laboratories (Appendix A).

6.2.3 Vector selection and development

In addressing these research questions both new and established molecular techniques were used. The achievement of these objectives required the selection of an appropriate vector-based delivery system, including over-expression and down-regulation *via* synthetic microRNA vectors, and the *Agrobacterium* based transformation methodology Induced Somatic Sector Analysis (ISSA) (described in Chapter 5). The vectors created during this project included entry vectors which are used at multiple cloning steps throughout vector development and destination vectors which are the final expression vector used for inoculation. Their different features and development are outlined below.

The destination vector developed for this project was adapted from the pCAMBIA1305.1GusPlus (www.cambia.org). The pCAMBIA1305.1GusPlus vector was modified through the ligation of a gateway cassette (www.invitrogen.com) into the multi-cloning site to create the destination vector called 1305.1GWGUS+, Figure 6.1. This task was undertaken jointly with other members of the Forest Molecular Biology and Genetics research group, including Dr. Antanas Spokevicius, Dr. Josquin Tibbits and Ms Emma Crewdson.

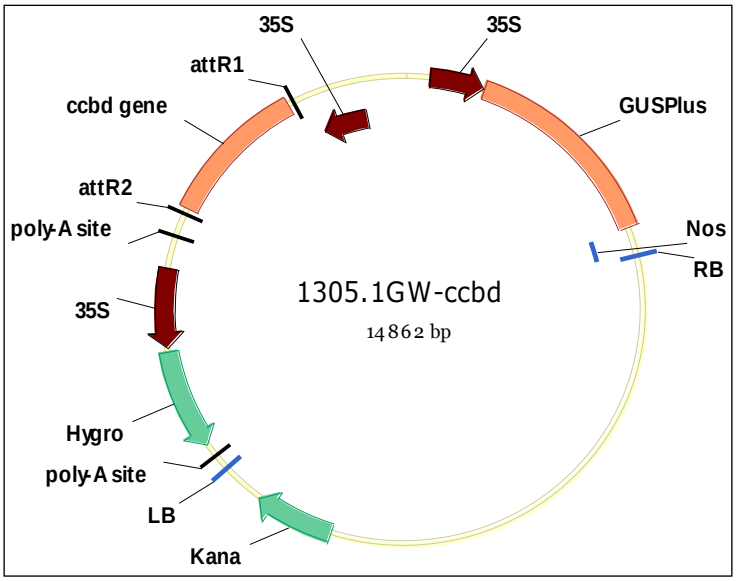

Figure 6.1 Destination vector 1305.1GW-GusPlus. Main vector features include left (LB) and right (RB) boarders, gateway recognition sites (attR1 and attR2), GUSPlus reporter gene, ccdB gene, and 35SCaMV promoters. Picture created using the Vector NTI 10 program.

Important features of this destination vector include the gateway recognition sites (attR1 and attR2) for cloning of entry vectors into this destination vector, the ccdB-gene for negative selection of candidate gene insertion, and the β-glucuronidase (GUSPlus) reporter gene (Figure 6.1).

Transgene destination vectors used in this study were created by recombination of one or two common entry vectors; the pGEM-T-Easy Vector System (www.promega.com) and the pCR8-GW-TOPO TA cloning entry vector (www.invitrogen.com). The following primers were used for the PCR of *EgrTUB1* for the over-expression vector (1305.1GWTUB1+) 5'GTCTAGAGCTCAAGATGAGAGAAAT 3' and 5' CATACTGCAGTTTCCCCTGTTCAATC 3', for the *EgrTUA1* PCR reaction (1305.1GWTUA1+) 5' GGTACCATGAGGGAGTGCATC 3' and 5' TCTAGATCAATACTCTTCTCCCTCA 3'. The double over-expression destination vector (1305.1GWTUA1/TUB1+) was created by ligation of the 1305.1GWTUB1+ destination vector with the pGEMTEasyTUA1 entry vector, which were digested with restriction enzyme ApaI.

The down regulation entry vectors were created using the Puc-57 vector (www.cambia.org). miRNAs were selected for the down-regulation of these tubulin genes because of their high sequence homology (Appendix A). GenScript (www.genscript.com) synthesised the synthetic-microRNAs 21 bp DNA sequences (miRNAs) into two different endogenous

Arabidopsis backbones called MIR164A and MIR164B (Reinhart *et al.* 2002). For the single miRNA vectors created (1305.1GWmiTUA2- and 1305.1GWmiTUB1-) the MIR164A backbone was used and for the double vector MIR164A and MIR164B were both used (Appendix B). In all miRNA vectors the following specific gateway recombination sites were included at the start and end of the miRNA backbone sequence.

attL1 5'CAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATGAGCAA TGCTTTTTTATAATGCCAAGTTTGTACAAAAAAGCAGGCT3'

attL2 5'ACCCAGCTTTCTTGTACAAAGTGGGCATTATAAGAAAGCATTGCTTATCAATTTGTTGCA ACGAACAGGTCACTATCAGTCAAAATAAAATCATTATTTG3'

Or

attL5 5'CAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATGAGCA ATGCTTTTTTATAATGCCAACTTTGTATACAAAAGTTG3'

attR5 5'ACAACTTTGTATACAAAAGTTGAACGAGAAACGTAAAATGATATAAATATCAATATAT TAAATTAGATTTTGCATAAAAAACAGACTACATAATACTGTAAAACACAACATATCCA GTCACTATG3'

For the development of the synthetic miRNAs used in this study, the guidelines recommended below were used. These are guidelines only as exceptions and new recommendations are still appearing in the literature as the development of amiRNAs technology is in its infancy (Franco-Zorrilla *et al.* 2007; Ossowski *et al.* 2008) (www.wmd2.weigelworld.org)(John Bowman *pers com*, 2007).

- Starting at the 5' end the 2nd to the 10thnucleotides must exactly match the target sequence, or have only one mismatch and very specific 3' end pairing.
- Starting at the 5' end the 1st nucleotide is predominantly a uridine and the 10th an adenine.
- Nucleotide positions 2, 12 and 20 are believed to be important for sequence recognition.
- Seldom clusters of more than two mismatches in the 3' end are observed.

- The 5’ strand (two sided seed region) with the lower GC content preferentially forms the mature miRNA.

The sequence selected for the seed 21 bp miRNAs contained all the above features and was specific to the target candidate gene only. The mature miRNA sequences are presented in Table 6-1. The nucleotide similarity between the mature *Eucalyptus grandis* tubulins miRNAs are presented in Appendix B.

Table 6-1 Mature miRNA sequences for *EgrTUB1*, *EgrTUA1* and *EgrTUA2*. Letters in bold are nucleotide positions 1 and 10; underlined letters are positions 2, 12 and 20.

Candidate gene	Mature amiRNA sequence 5’ to 3’
EgrTUB1	**U**C C AGU CGU **A**G U AGG CCA G C C
EgrTUA1	**U**A U GAG AAG **A**G G GAG UAC U G G
EgrTUA2	**U**G G UAG UCC **A**G G UAC GGU A C C

The cloning steps taken for each transgene destination vector, including the entry vectors used and the gateway or ligation reactions involved are shown in Table 6-2.

Table 6-2 Vector construction step by step protocol. PCR or Gene synthesis was used for each vector. The different entry vectors are listed, and the different types of reactions used BP and LR, or LR or Ligation. rxt =reaction. Over-expression vectors are denoted with a (+), Down-regulation vectors are denoted with a (-).

Candidate gene	PCR or synthesis	Entry vector	BP rxt	LR rxt	Ligation	Destination vector
EgrTUA1	**PCR**	**pCR8TOPO**	**Yes**	**Yes**	**N/A**	**1305.1GWTUA1+**
EgrTUA1	**Synthesis**	**Puc57**	**N/A**	**Failed**	**N/A**	**Not completed**
EgrTUA2	**Synthesis**	**Puc57**	**N/A**	**Yes**	**N/A**	**1305.1GWmiTUA2-**
EgrTUB1	**PCR**	**pCR8TOPO**	**Yes**	**Yes**	**N/A**	**1305.1GWTUB1+**
EgrTUB1	**Synthesis**	**Puc57**	**N/A**	**Yes**	**N/A**	**1305.1GWTUB1-**
EgrTUA1 ***EgrTUB1***	**PCR** **N/A**	**pGEMTEasy** **1305.1GWTUB1+**	**N/A**	**N/A**	**Yes**	**1305.1GWTUA1/TUB1+**
EgrTUA1 ***EgrTUB1***	**Synthesis** **Synthesis**	**Puc57** **Puc57**	**N/A**	**Yes**	**N/A**	**1305.1GWmiTUA1/miTUB1-**

6.2.4 Experimental inoculation rounds

Three inoculation rounds containing different vectors were undertaken over the course of two growing seasons. Each round commenced within weeks of each other or at approximately the same time of the year but a year apart. All inoculation rounds were

maintained under the same glasshouse conditions. These multiple inoculation rounds (experiments) were undertaken in order to answer the different experimental questions for both this chapter and for Chapter 7. In cases were multiple rounds used the same vectors, results from these multiple independent observations are presented together. Inoculation round one (R1) included two control GUS reporter gene vectors, the 1305.1GUS+ (CaMV35S:GUS+) used by Spokevicius *et al*.(2007a) and the new destination vector created for this project 1305.1GWGUS+ (CaMV35SGW:GUS+), as well as 1305.1GWTUB1+ (CaMV35S:*EgrTUB1*), 1305.1GWmiTUB1- (CaMV35S:miTUB1), 1305GWTUA1+ (CaMV35S:*EgrTUA1*) and 1305.1GWmiTUA1/TUB1- (CaMV35S:mirTUA1/TUB1). Inoculation round two (R2) included 1305.1GWGUS+, 1305.1GWmiTUB1-, 1305.1GWmiTUA2- (CaMV35S:miTUA2) and other miRNA vectors presented in Chapter 7. Finally, inoculation round three (R3) contained 1305.1GWGUS+, 1305.1GWmiTUB1-, 1305.1GWTUA1/TUB1+ (CaMv35S:*EgrTUA1/TUB1*), 1305.1GWmiTUA1/TUB1-. The different inoculation rounds were undertaken to address the different research questions proposed in this thesis. Some vectors were re-used in multiple inoculation rounds.

6.2.5 *Agrobacterium tumefaciens* preparation

All destination vectors listed in Table 6-2, were electroporated into *A. tumefaciens* (AGL1), and grown on agar plates containing kanamycin [50 μg/ml] for binary vector selection and rifampicin [25 μg/ml] for *Agrobacterium* selection. A final PCR amplification of the transgene expression cassette, using 0.5 μl of *Agrobacterium* culture, was undertaken for each vector to confirm insertion within the individual *Agrobacterium* colonies selected to be used for inoculation.

For inoculation the *Agrobacterium* culture containing the destination vector was grown for 48 hrs at 28^{o}C in 5 ml of Luria Bertani (LB) medium containing 25 μg/ml of rifampicin and 50 μg/ml kanamycin. On the morning of inoculation the culture was diluted 1:40 with fresh LB medium (50 ml Falcon tube), and grown to an optical density (OD) of O.4. The culture was then centrifuged at 4^{o}C at 2500 rmp for 15 mins, and the LB poured off. The pellet was then immediately resuspended in 0.5 to 1 ml of minimal salts (MS) medium and used directly on the plants. This method was adapted from Spokevicius *et al.* (2007a). In this study once the *Agrobacterium* was resuspended in MS medium it was maintained on ice during inoculation

in order to prolong the viability of the *Agrobacterium*, as the process of inoculation often took a number of hours.

6.2.6 Induced Somatic Sector Analysis

Induced somatic sector analysis (ISSA) was developed by the University of Melbourne Forest Molecular Biology and Genetics research group and has been successfully used to transform eucalypt, poplar and pine trees (Spokevicius 2006; Spokevicius *et al.* 2006; Van Beveren *et al.* 2006; Spokevicius *et al.* 2007a). This transformation methodology involves the inoculation of cambium cells in plant stem by *Agrobacterium*, followed by a three to four month growing period, and finally GUS staining and sector screening.

For inoculation cambial windows are created by making three incisions on the plant stem using a scalpel (1 cm^2 area of the stem). For each window the bark is pealed upwards exposing the cambium. *A. tumefaciens* containing one of the destination vectors outlined in Table 6-2 or the control vector 1305.1GW-GUS+ were pipetted onto the stem (5 µl). The bark flap was then replaced and Parafilm™ tightly wrapped around the cambial window to seal the transformation site aiding the reattachment of the bark (Figure 6.2).

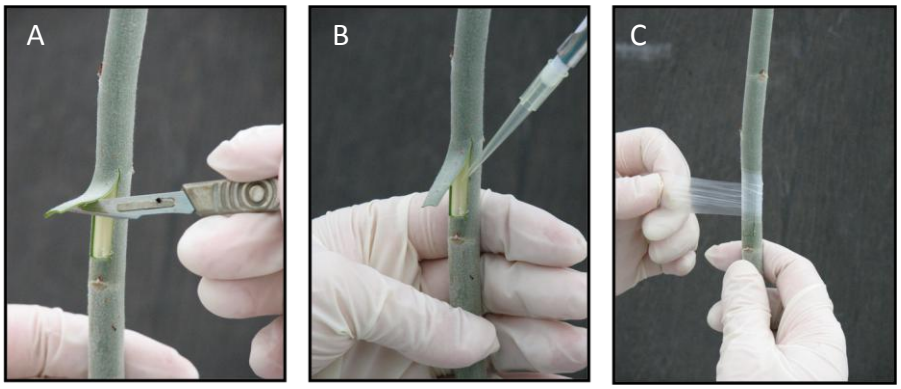

Figure 6.2 The three stages of ISSA transformation procedure. The cambium is exposed by cutting the stem (A), then inoculated with *Agrobacterium* (B) and reattached and sealed with Parafilm (C).

Although each inoculation round used a different number of trees (13-23) the same clonal trees were used for all (Table 6-3). Each tree was inoculated with a minimum of 2 cm^2 of the control 1305.1GWGusPlus vector (2x cambial windows) and subsequent destination vectors (between 2-4x cambial windows). The trees were always inoculated on both sides of the

stem and the order of the inoculation (vectors up the stem) was alternated evenly so as to eliminate any positional effect during data analysis.

The height and diameter at 10 cm of each tree were recorded at the commencement and end dates of round. These measurements were also recorded during the experiment, allowing an assessment of how actively the trees were growing in order to determine an ideal harvest date.

After one to two weeks a single tree from each experiment was assessed for transient expression for all vectors. The remaining trees were harvested three to four months later. Excised cambial windows (1 cm^2 stem segments) were cut to size (~2 cm long stem segment) with secateurs, the bark was peeled off. Stem segments were then placed into a 12 ml Falcon tube. While harvesting was in progress samples were kept moist with phosphate buffer (pH7.0). Each sample then was washed twice in 100 mM sodium phosphate buffer and incubated at 55^{o}C for 10 min in X-gluc staining solution (1 mM X-gluc, 100 mM phosphate buffer, 1% triton X-100, 10 mM EDTA, 0.5 mM potassium ferricyanide III and 0.5 mM potassium ferrecyanide II) in the dark. Samples were then further incubated overnight at 37^{o}C on a rotary shaker at 150 rpm. The next day the X-gluc solution was poured off and the samples stored in 70% ethanol. The pH of some samples was checked before the X-gluc solution was poured off as high pH was expected to potentially lead to endogenous staining (Spokevicius 2006). Endogenous staining was not observed for any of the experiments presented in this thesis.

The number of sectors found for each cambial window was recorded, providing an indication of the transformation efficiency of each individual destination vector relative to the control vector (1305.1GWGusPlus). For each vector the xylem stem segments were assessed for transformation events (sectors), using an Olympus SZ-PT light microscope.

6.2.7 Statistical analysis

A preliminary sample collection was undertaken to determine the ideal sample size for assessing MFA using this methodology (power analysis). It was also decided that a paired T-test for analysing MFA was the most suited statistical analysis for this study. The variance around different sample size was calculated and with the results from the power analysis an

ideal sampling strategy was determined (Appendix C). In addition, a one way ANOVA was used in the analysis of similarity between selected vectors.

6.2.8 MFA assessment

A number of methods are available for the determination of MFA in wood fibres (see Chapter 4 for a description). An indirect method for assessing MFA through the visualisation of pit apertures was selected as the most suitable for this project, as reviewed by Batchelor *et al.*(2000) and used by Pleasents *et al.* (1998) and more recently by Spokevicius *et al.* (2007a).

This method involves measuring the angle of the long ellipse of the pit aperture relative to the longitudinal axis of fibre cells. According to Spokevicius *et al.* (2007a) optimisation sampling strategies, pair-wise sampling of transgenic fibres (GUS stained; treatment), and control fibres from near the cambium was optimal and this strategy was followed through this project (Figure 6.3). Preference was given to sampling the largest transformed sectors under a stereo microscope. For each transgene treatment paired xylem tissue samples were excised from treatment (GUS-stained) and control regions (untransformed). In all cases the distance between the treatment site and the start of the control sampling site was approximately 1 mm. The excised fibre blocks were placed in 1.5 ml reaction tubes and macerated with 1:1 hydrogen peroxide and glacial acetic acid in a 90^{o} heat block for 1 hour or until soft. The fibres were then washed with distilled water before being mounted onto glass slides with Gelvastab mounting medium also known as Polyvinyl alcohol mounting medium with DABCO (www.sigma-aldrich.com). A small drop of Gelvastab was placed on the fibres and a cover slip was placed on top. The cover slip was then moved in a gentle circular motion to separate the fibres. Following this the edge of the cover slip was sealed with nail polish to increase the longevity of the slides and a gentle weight was applied to the slides overnight while the Gelvastab set. In some cases the maceration process resulted in a loss or reduction of visible Gus staining in the transformed wood fibres.

Slides were then assessed under an Olympus BX41 microscope (Olympus Australia, Sydney) at 40x magnification and photos of individual fibres were taken with an Olympus Color View III camera. Polarised light was used to enhance the appearance of the pit apertures and cell

wall striation in the wood fibres. The digital imaging program analySIS FIVE was used to assess the angle of the pit apertures and calculate the corresponding MFA values.

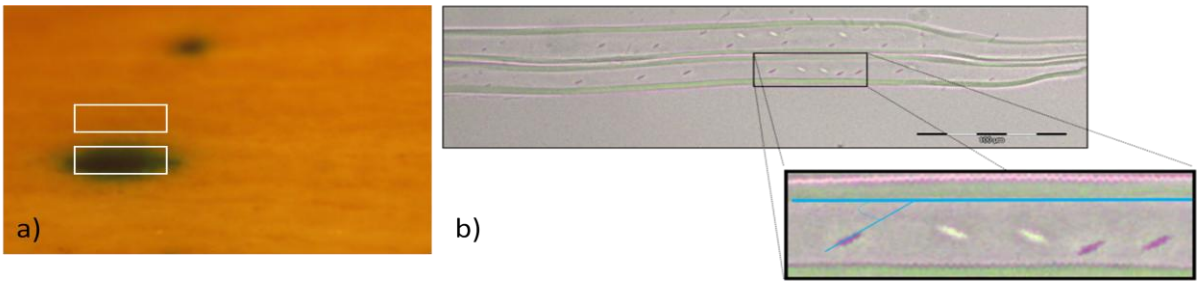

Figure 6.3 Methodology for MFA assessment. a) The white outlined boxes (0.5mm length) show an example of paired sampling sites used in this project. b) Magnifications of individual macerated eucalypt wood fibres showing pit apertures. Pits can appear black or white in colour depending on the plane of focus and under polarised light. Blue lines indicate longitudinal axis of the fibre and the angle of the long ellipse of the pit which was used as a measure of MFA.

For each slide, five fibres were selected at random for assessment of MFA, and the median angle of three pits per fibre was recorded. For treatment slides priority was given to fibres that contained GUS staining or occurred in close proximity to GUS stained ray cells. The average value of the angle of three pit apertures was recorded for each fibre. Statistical significance testing was undertaken using a paired t-test with $\alpha = 0.05$ indicating a significant difference. For each vector treatment and from each trial an optimum of twenty two sectors were sampled.

6.3 Results

6.3.1 Plant growth

All experiments commenced during the spring/summer growing season and all plants were observed to be actively growing (Table 6-3). The plants increased in height daily by 3.6 to 10.8 mm and radius by 0.02 to 0.03.

Table 6-3 Plant growth and inoculation round information. The number of plants harvested for MFA assessment is shown. Daily growth rates are presented in italics. Inoculation round R1, R2 or R3.

Inoculation round	No of Plants	Season	Growth period (days)	Total area of cambial windows (cm^2)	Average growth height (mm)	Average growth diameter at 10cm (mm)
R1	23	Jan-May	100	184	360 (*3.60*)	2.8 (*0.028*)
R2	21	Dec-Mar	92	336	1000 (*10.86*)	3 (*0.032*)
R3	13	Jan-Apr	78	130	680 (*8.71*)	2.7 (*0.034*)

6.3.2 GUS sector analysis

An example of a cambial window from a tree harvested for assessment of transient expression is shown together with a window showing GUS sectors is shown in Figure 6.4. Frequent transient transformation was observed (Figure 6.4 a), while for stable transformation and the average number of sector per window was 35 (average for all vectors) (Table 6-4).

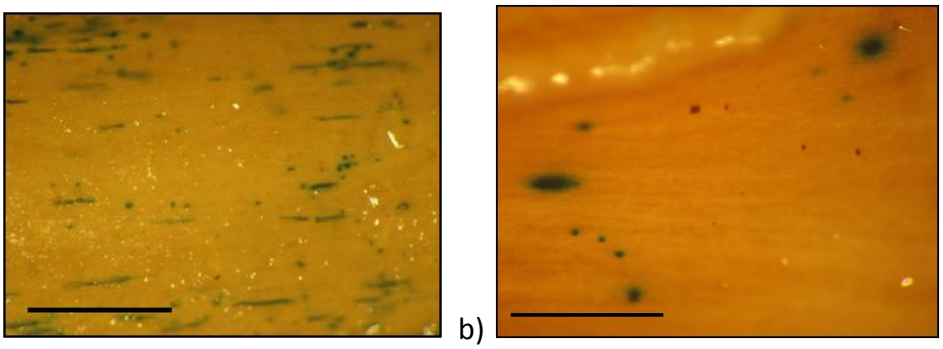

Figure 6.4 Transient and stable expression of GUS in eucalypts. Examples shown from experiment one, a) GUS staining one week after inoculation showing transient expression and b) Stable expression and GUS staining 3-4 months after inoculation at final harvest time, Scale bar 1 mm.

Xylem sectors were observed in all plants. The number of sectors was recorded for each cambial window (1 cm^2) and for each vector treatment. The average number of transformed sectors per cm^2 (ATS^{-2}) varied between experiments, treatments and plants. In round one (R1) the CaMV35SGW:GUS+ control vector compared to the control vector CaMV35S:GUS+, showed a comparative or a slightly greater transformation efficiency ($ATScm^{-2}$) (Table 6-4). All transgene vectors in round one showed equal or higher expression than the control

vector (CaMV35SGW:GUS+). The vectors used in the other two rounds also showed high levels of transformation efficiency (Table 6-4).

Table 6-4 Transformation rates. For each vector from round 1, round 2 and round 3. Average transformed sectors (ATScm^{-2}).

Inoculation round	Vector	ATScm^{-2}	Inoculation round	Vector	ATScm^{-2}
R1	CaMV35S:GUS+	24.48	R2	CaMV35S:miTUB1	35.35
R1	CaMV35SGW:GUS+	34.44	R2	CaMV35S:miTUA2	49.32
R1	CaMV35S:*EgrTUB1*	52.67	R3	CaMV35SGW:GUS+	12.50
R1	CaMV35S:miTUB1	35.91	R3	CaMV35S:miTUB1-	35.83
R1	CaMV35S:*EgrTUA1*	46.15	R3	CaMV35S:*EgrTUA1/TUB1*	19.46
R1	CaMV35S:miTUA1/TUB1	40.31	R3	CaMV35S:miTUA1/TUB1	29.27
R2	CaMV35SGW:GUS+	16.58			

6.3.3 Cellulose microfibril angle (MFA) assessment

Wood fibres were analysed for changes in MFA using a paired T-test and 95% confidence intervals. This statistical analysis allowed easy detection of a significant change in MFA between the control and transgenic fibres (mean difference). In Figure 6.5, 95% confidence intervals which did not cross the zero axes (bold line) indicate a statistically significant change in MFA.

In all experiments the control destination vector (empty vector) containing only the GUS+ reporter gene (CaMV35SGW:GUS+) was used to determine the effect if any of GUS enzyme on MFA (Figure 6.5, a). No significant difference from zero was observed for CaMV35SGW:GUS+ transformed sectors at the 95% confidence interval for R1 with a mean difference in MFA of 0.104^{o} and P-value of 0.26. Likewise in R2 a mean difference of -0.877^{o} (P-value 0.316) and in R3 a mean difference of 0.476^{o} (P-value 0.636) was found. Thus the activity of the GUS enzyme did not significantly alter MFA in any of the three rounds.

The CaMV35S:*EgrTUB1* vector designed to investigate the role of *E. grandis BETA TUBULIN 1* was observed to significantly increase MFA. In R1 twenty-two sectors were assessed for this vector and a mean difference of 3.350^{o} in MFA was observed (P-value 0.001) (Figure 6.5, b). The CaMV35S:miTUB1 vector which also targets the *EgrTUB1* gene, similarly and significantly increased MFA in all rounds. Increased mean differences were 2.327^{o} (P-value

0.034) in R1, 2.164^{o} (p-value 0.014) in R2 and 1.269^{o} (P-value 0.049) in R3 (Figure 6.5, b). In these silencing experiments twenty-two sectors from R1 and R2 and twenty-one sectors for R3 were assessed.

Similar to *EgrTUB1*, the CaMV35S:*EgrTUA1* vector designed to alter *E. grandis ALPHA TUBULIN 1* also significantly increased MFA. In R1 the mean difference in MFA observed for this vector was 4.136^{o} (P-value zero), from twenty-two sectors (Figure 6.5, c). As construction of the miRNA vector designed to target *EgrTUA1* was never successfully completed, the miRNA targeting a closely homologous *E. grandis ALPHA TUBULIN 2* gene (*EgrTUA2*) was assessed instead (Appendix A and B). In R2 the 1305.1miTUA2- vector resulted in a significant positive increase in MFA, with a mean difference of 2.537^{o} (P-value 0.019) from twenty sectors (Figure 6.5, c).

Two vectors CaMV35S:*EgrTUA1/TUB1* and CaMV35S:miTUA1/TUB1, were used to investigate the combined effect of *EgrTUA1* and *EgrTUB1* perturbation on MFA. Use of the CaMV35S:miTUA1/TUB1- vector produced a significant increase in MFA of 1^{o} or 4^{o}, in R3 and R1, respectively (Figure 6.5, d). In contrast, a significant decrease in MFA (2^{o}) was observed when transforming with CaMV35S:*EgrTUA1/TUB1* (R3) (Figure 6.5, d).

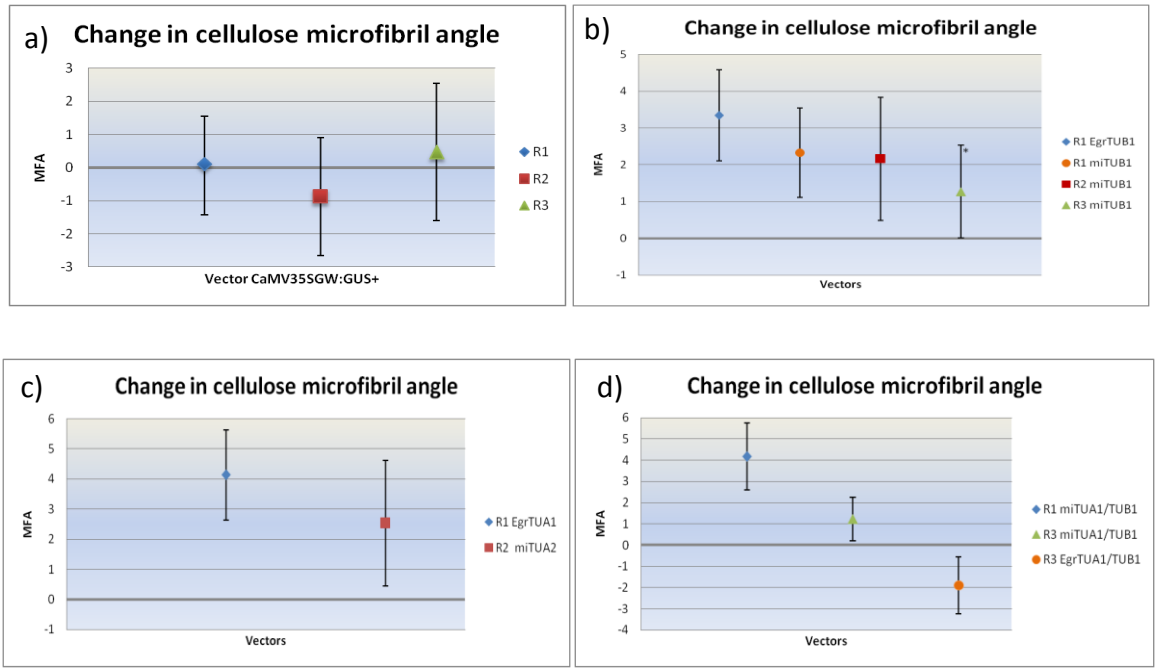

Figure 6.5 Change in microfibril angle a) Results from CaMV35SGW:GUS+ pair-wise comparisons from R1, R2 and R3, all showed no significant difference from zero. b) Results from CaMV35S:*EgrTUB1* and CaMV35S:miTUB1 pair-wise comparisons from R1, R2 and R3, both vectors resulted in a significant positive change in MFA. *Confidence intervals for the CaMV35S:miTUB1- from R3 are 0.008 to 2.531. c) Results from CaMV35S:*EgrTUA1* and CaMV35S:miTUA2 pair-wise comparisons from R1 and R2, both vectors resulted in a significant positive change in MFA. d) Results from CaMV35S:*EgrTUA1/TUB1* and CaMV35S:miTUA1/TUB1 pair-wise comparisons from R1, R2 and R3, both vectors resulted in a significant change in MFA, positive and negative, respectively. All Error bars are 95% confidence intervals.

Further analysis was undertaken to determine if the increased MFA phenotype observed for both over-expression and down-regulation vector was similar or different. A one-way ANOVA using MiniTab15 was conducted and comparisons between vectors were made (Table 6-5). The increase in MFA observed for the CaMV35S:*EgrTUB1* and the CaMV35S:miTUB1 vectors across all experiments was the same/ not significantly different (P-value 0.179). Similarly the increase in MFA observed for the CaMV35S:*EgrTUA1* and CaMV35S:miTUA2- vectors was also the same/ not significantly different.

Table 6-5 One-way Anova for vector treatments of MFA mean differences.

Factors	DF	P-value
R1 *EgrTUB1*, R1 miTUB1, R2 miTUB1, R3 miTUB1	3	0.179
R1 *EgrTUA1*, R2 miTUA1	1	0.194

6.4 Discussion

6.4.1 GUS staining and transformation efficiency

The transient GUS staining observed after one to two weeks is indicative of successful plant cell inoculation and T-DNA processing. The later stable GUS staining after three to four months indicates stable insertion of the T-DNA into the plant genome and transcription of the T-DNA within the plant cell. The number of stable GUS sectors per cambial window ($ATScm^{-2}$) was used to determine the transformation efficiency of each vector. In the first inoculation round undertaken (R1) the CaMV35S:GUS+ vector was included as a control for the newly developed destination vector CaMV35SGW:GUS+ which was used as the control for the transgene destination vectors in this and future experiments. The transformation efficiency of the CaMV35SGW:GUS+ was comparable. Hence, a high transformation rate of 15 plus sectors per cambial window was expected and observed for all the transgene vectors made from this new destination vector. Previously an average transformation rate of two to five sectors per cambial window (for cambium sectors) with the CaMV35S:GUS+ vector had been observed (Van Beveren *et al.* 2006).

Within each inoculation round each vector had differing transformation efficiencies ranging from 12 to 52 $ATScm^{-2}$. This is likely to be due to a number of reasons, which include a slight differences in the final optical density (OD) of each *Agrobacterium* culture (Appendix D), order of inoculation, as the viability of the *Agrobacterium* is expected to decrease over time (Appendix D), time of year, which influences the availability of actively growing cells available for transformation (Spokevicius *pers com*.), as well as the exact insertion position of the T-DNA, which has been shown to influence gene expression and cell viability (Hobbs *et al.* 1990). The ideal OD of 0.4 was always aimed for to ensure transformation with a high quantity of live *Agrobacterium* cells and, in this project during inoculation the *Agrobacterium* culture was kept on ice and mixed between inoculations in an effort to maintain equal quantities of live bacterium at the beginning and end of inoculation rounds, as larger experiments took a number of hours to complete.

6.4.2 GUS activity does not affect MFA

GUS was used as a reporter gene to identify transgenic sectors where the T-DNA portion of the destination vector had been integrated into the plant genome. The GUS reporter gene has been used widely in plant species and has been shown to be a highly effective reporter system (Jefferson 1989). GUS as a reporter has also been shown to have little to no effect on secondary cell wall MFA in eucalypts (Spokevicius 2006; Spokevicius *et al.* 2007a). This was again confirmed in this project, as all 'GUS only' samples showed no significant change in MFA. From these results it can be concluded that GUS does not alter fibre MFA.

6.4.3 Alpha and Beta tubulin activity and influence on MFA

Here transformation with *E. grandis* alpha or beta-tubulin over-expression vectors was shown to produce wood fibres with altered MFA, strengthening the link demonstrated in the literature (Chapters 3 and 4), that cortical microtubules and hence tubulin gene expression, are involved in the molecular control of cellulose deposition within the cell walls of plants (See for example Oda and Hasezawa 2006; Paradez *et al.* 2006a; Oakley *et al.* 2007).

The hypothesis that over-expression of *EgrTUB1* gene in *Eucalyptus* xylogenic tissue could result in a lower MFA was tested by Spokevicius *et al.* (2007a). In their study, they used the ISSA methodology to transform eucalypt stem tissue with the overexpression construct 1303TUB1+. They obtained five sectors from two trees which were assessed and found to have an average increase in MFA of 5^{o}. In *Eucalyptus grandis BETA TUBULIN 1* has been shown to be up-regulated on the upper side of a branches with a lower MFA (12 to 25^{0}), and was observed to be down-regulated on the lower side where average fibre MFA was higher ($38\text{-}52^{0}$) (Qiu *et al.* 2008). Hence, in the Spokevicius *et al.*(2007a) study the direction of MFA change was opposite than expected from the Qiu *et al.*(2008) study. The authors hypothesised that the most likely explanation for the unexpected increase in MFA was homology dependent gene silencing (HDGS) which may have resulted in lower beta-tubulin expression thus causing an increased in MFA. In the this present project, results from R1 with EgrTUB1 (CaMV35S:*EgrTUB1*) confirmed the phenotype observed by Spokevicius *et al.* (2007a). In this project 22 sectors from 11 trees showed an average increase in MFA of 3^{o}. It

is therefore likely that gene silencing is also occurring in this project as the EgrTUB1 over-expression vector design was very similar to that used by Spokevicius *et al.* (2007a).

In the Qiu *et al.* (2008) microarray study *Eucalyptus grandis ALPHA TUBULIN 1* was observed to be down-regulated on the lower sides of branches which contain higher MFAs (38-52^0). From this report, in this project it was hypothesised that, as with beta-tubulin, altering the expression of alpha-tubulin may also alter MFA. Here transformation with *EgrTUA1* (CaMV35S:*EgrTUA1*) in R1 resulted in fibres with an average increase in MFA of 4^o. Because the increase in MFA was similar to that observed with the over-expression vector containing *EgrTUB1* in this study, it was hypothesised further that this might again be due to gene silencing, resulting in a decrease in alpha-tubulin in these fibres. Homology dependent gene silencing is likely to be responsible for the observed phenotype, as the plant species selected for transformation were *Eucalyptus camaldulensis X grandis* and *globulus X grandis* and transformation with an additional copy of an *E. grandis* tubulin gene sequence is a possible trigger for HDGS due to high (complete) sequence homology (Napoli *et al.* 1990; Van Der Krol *et al.* 1990; Tsai *et al.* 1998; Moller *et al.* 2003).

To follow on from these results, in this project *Eucalyptus* trees were transformed with dual 35SCaMV promoters driving the expression of both *EgrTUA1* and *EgrTUB1* genes (CaMV35S:*EgrTUA1/TUB1*) in combination, to test the hypothesis that up-regulation of both alpha and beta tubulin genes simultaneously would elevate the gene silencing response (increase in MFA). A mean significant decrease of 2^o in MFA was observed in the resulting in wood fibres. This decrease in MFA is consistent with observations previously mentioned, by Qiu *et al.*(2008) who found up-regulation of *EgrTUB1* and *EgrTUA1* correlated with lower MFA in branch wood. These results suggest that a balanced increase in abundance of alpha- and beta-tubulin protein may elevated the gene silencing trigger which was previously suggested to be responsible for the observed phenotype.

Tubulin transgenes have been expressed in a wide range of plants. In maize for example loss of cell viability due to tubulin over-expression, and similar to the hypothesis gene silencing observed in this project and by Spokevicius *et al.*(2007a), was overcome by simultaneously over-expressing both alpha and beta-tubulin genes (Anthony and Hussey 1998; Anthony *et al.* 1999). In these studies vectors with dual 35SCaMV or MFS14 promoters driving both

alpha- and beta-tubulin gene expression resulted in stable transformants capable of callus proliferation (Anthony and Hussey 1998). In these maize cells the introduced (transfected) alpha- and beta-tubulin proteins were shown to be incorporated into cortical, spindle and phragmoplast microtubule arrays, confirming that these transfected tubulin proteins were assembled into functional microtubules (Anthony and Hussey 1998). Similar observations were also reported for tobacco (Anthony *et al.* 1999), where seed transformation with combined alpha- and beta-tubulin genes germinated, giving rise to stably transformed root, leaf, stem, bud and flower tissue. Transfected tubulin proteins were again found to have been incorporated into functional, interphase, pre-prophase, spindle, phragmoplast and cortical microtubule arrays (Anthony *et al.* 1999). In both studies the rate of increased expression of transfected tubulin genes and resulting protein production led to an equivalent decrease in endogenous tubulin expression (Anthony and Hussey 1998; Anthony *et al.* 1999).

Individual over-expression of either alpha or beta-tubulin genes has also been undertaken in *Saccharomyces cerevisiae* (yeast) (Katz *et al.* 1990; Weinstein and Solomon 1990) and *Arabidopsis* (Burk *et al.* 2006). In yeast, the over-expression of beta-tubulin resulted in reduced cell viability, while the over-expression of alpha- or alpha- and beta-tubulin in combination had less of an effect on cell viability (Katz *et al.* 1990; Weinstein and Solomon 1990). These authors suggested that a large cytoplasmic pool of free beta-tubulin might be cytotoxic, explaining the loss of cell viability. They also suggested that alpha-tubulin regulates the ratio of alpha/beta tubulin and that when alpha-tubulin was in excess that it was broken down rapidly (Katz *et al.* 1990; Weinstein and Solomon 1990). In an *Arabidopsis* study the tubulin protein GFP-*ArathTUA6* was shown to be readily incorporated into cortical microtubules and not to adversely affect their organisation, however over-expression of GFP-*ArathTUA6* caused aberrant microtubule organisation, reduced cell wall synthesis and altered cell morphogenesis (Burk *et al.* 2006). In contrast to previous studies over-expression of alpha-tubulin in arabidopsis plants was not found to be cytotoxic, and, unlike in this project, alpha tubulin over-expression did not affect cellulose microfibril organisation (Burk *et al.* 2006).

6.4.4 Use of miRNAs to target tubulin genes for down-regulation

To further investigate the hypothesis that HDGS was likely to have caused a decrease in alpha and beta-tubulin expression, constructs were designed aimed at down-regulating the expression of *EgrTUB1* and *EgrTUA1* both singularly and in combination. Synthetic microRNA was created to target *EgrTUB1* and *EgrTUA1*, however, difficulties during the cloning step with miRNATUA1- necessitated the construction of another synthetic microRNA targeting the closely related *EgrTUA2*, which was successfully cloned into the destination vector.

miRNAs were selected to down-regulate the *E. grandis* tubulin genes under investigation in this study because of their high sequence similarly (Appendix A). miRNAs are processed by the host organism and specifically target the gene(s) of interest. Although synthetic miRNAs have not been widely utilised in non-model plant species, their potential is considerable. The microRNA backbones used in this study (MIR164A and MIR164B backbones) have been successfully expressed in tomato and tobacco plants (Alvarez *et al.* 2006). Furthermore, synthetic miRNAs have been successfully used to target a KNOX homeobox gene *ARBARKNOX2* (*ARK2*) *in Populus alba* (Du *et al.* 2009).

In this project, for the microRNA vector CaMV35S:miTUB1- vector at least 20 sectors were recovered from each of the three independent inoculation rounds (R1, 2 and 3) across 21 trees. In all experiments a significant increase in average MFA of between 1^{o} and 2.5^{o} was observed. This result confirms that the observed phenotype for the CaMV35S:*EgrTUB1* vector in this study and in that of Spokevicius *et al.* (2007a) is most likely to have resulted from a decrease and not an increase in *EgrTUB1* expression, which is consistent with the microarray study by Qiu *et al.*(2008). Likewise the CaMV35S:miTUA2- vector resulted in wood fibres with a significant average increase of MFA of 2.5^{o} providing further evidence that down-regulation of TUA2 leads to an increase in MFA in a similar way as the over-expression vector indicating HDGS.

A double miRNA vector (CaMV35S:miTUA1/miTUB1) was used to contrast the effect of the double over-expression vector (CaMV35S:*EgrTUA1/TUB1*), which, as previously described resulted in wood fibres with an average decrease in MFA of 2^{o}. The dual targeted down-

regulation of two alpha and beta tubulin gene alone, however, resulted in an increase in MFA of 1^{o} and 4^{o}, respectively. These results support the correlation between increased and decreased *EgrTUA1* and *EgrTUB1* gene activity and increased and decreased MFA observed in the microarray study by Qiu *et al.*(2008) and other studies that have linked increased alpha and beta tubulin gene activity with tension wood formation, which is known to contain wood fibres with smaller MFA values (Oakley *et al.* 2007).

6.4.5 Gene expression

GUS staining is indicative of T-DNA transfer and this does not fully confirm over-expression of the target transgene itself but only the GUS reporter gene. Multiple attempts were made to confirm transgene expression *via* RNA extraction from both transient and stable sectors, however, due to methodological difficulties associated with destructive GUS staining and extracting RNA from small amounts of live tissue meant that actual changes in candidate gene expression could not be assessed.

The problem associate with GUS as a reporter gene has been overcome in other species whereby UV activated fluorescent reporter genes such as green fluorescent protein (GFP) and yellow fluorescent protein (YFP) are commonly used. At present using a different reporter gene such as GFP is not practical in tree species as green auto-fluorescence from lignin in the plant cell walls makes easy and high-through put visual detection of potential transgenic sectors difficult. Towards this end, current experiments in our research group have been testing the use of AS-RED as reporter gene in tree species, which hopefully will give a better contrast in fluorescence and allow for easier RNA extraction and down-stream phenotyping in the future.

6.4.6 Wood fibre strength properties

Wood fibres with smaller microfibril angle are known to have greater tensile strength while wood fibres with larger MFA are more elastic (Page and El-Hosseiny 1983; Batchelor *et al.* 2000; Burgert *et al.* 2002; Barnett and Bonham 2004). The magnitude of the changes in MFA observed in this study raises the question of what is a biologically significant change in MFA? For example wood fibres with MFA of $5\text{-}10^{0}$ compared to 40^{0} have shown a reduction in tensile strength of approximately one third and stiffness of approximately one fifth in a

study by Batchelor et al. (2000), while in another report an MFA increase of only 2^{o} was predicted to reduce tensile strength by 1-3% (Burgert *et al.* 2002).

It is therefore hypothesised that altering the angle of cellulose microfibrils within wood fibres by a few degrees may recognisably alter their strength properties. Testing of this hypothesis was not originally within the scope of this PhD project, however, a preliminary study was undertaken as part of this thesis in collaboration with researchers at the Max Planck Institute in Golm, Germany (Appendix F). To address this question this preliminary study involved the adaptation of a fibre tensile testing method developed for individual gymnosperm fibres (3-5 mm long) to eucalypt wood fibres (approximately 1 mm long). Because of the smaller length of angiosperm wood fibres it was decided to use bundles (segments) of transgenic fibres for tensile strength assessment (approximately 3 mm long). Difficulties with sample preparation and uneven surface area meant that results from this preliminary study are so far inconclusive.

6.5 Summary and future directions

In this project a range of vectors have been created and proven to be effective in the determination of candidate gene function, increasing our understanding of the molecular control of cellulose microfibril angle determination in wood fibres. The use of synthetic miRNA vectors for example suggests that observed increases in MFA, for both single and double over-expression vectors, was most likely a result of gene silencing. It was also demonstrated that MFA could be influenced by perturbing both alpha (*EgrTUA1* and *EgrTUA2*) and beta tubulin (*EgrTUB1*) genes, coding for the major constituents of cortical microtubules. Furthermore, transgenic wood fibres were created with both increased and decreased MFA showing that tubulin transgenes can drive cellulose microfibril orientation in either direction (larger or smaller angle), identifying tubulin genes as candidates for the development of molecular markers (Thavamanikumar 2009), aiding breeding of tree species with desirable wood quality traits such as cellulose microfibril angle, a priority of the timber and fibre based industries (Walker and Butterfield 1996; Greaves *et al.* 1997; Raymond 2002).

The observation that both *EgrTUA1* and *EgrTUA2* influence MFA, as well as *EgrTUB1* raises the question if also other tubulin genes can influence MFA in eucalypts. The *Eucalyptus grandis* genome at present is known to contain at least four alpha and six beta tubulin genes (Southerton *pers com*.) and the specificity of *E. grandis* beta tubulin gene family members towards influencing MFA is investigated in the following chapter.

Chapter 7 Specificity of eucalypt tubulin genes in controlling cellulose microfibril orientation

7.1 Introduction

The *Eucalyptus grandis* tubulin gene family is known to contain at least four alpha and six beta tubulin genes (Qiu *et al.* 2008; Southerton *pers com*.). In work described in Chapter 6 in this thesis the *Eucalyptus grandis* alpha (*EgrTUA1* and *EgrTUA2*) and beta tubulin genes (*EgrTUB1*) were shown to influence cellulose microfibril angle (MFA) in eucalypt wood fibres. Here the role of an additional five beta tubulin genes (*EgrTUB2-6*) in controlling cellulose MF orientation is examined.

The spatial and temporal expression of members of tubulin gene families in plants (summarised in Chapter 3) suggests that individual members of tubulin gene families are expressed in a tissue-specific manner and that some genes are specific to particular cellular processes (Ueda *et al.* 1999). Examples from two species are outlined below. In *Gossypium hirsutum* (cotton), which contains 19 beta-tubulin genes, the *Goshi*TUB4 protein for example was found to accumulate in leaves and roots, while *Goshi*TUB6 and *Goshi*TUB7 were specific to cotton fibres undergoing secondary cell wall formation (Dixon *et al.* 1994; Dixon *et al.* 2000). In *Populus trichocarpa* (poplar), which contains 20 beta-tubulin genes, *Poptr*TUB9 , *Poptr*TUB15 and *Poptr*TUB16 were expressed in xylem tissue and up-regulated during tension wood formation (Oakley *et al.* 2007). Furthermore, within the *E. grandis* tubulin gene family, differential expression of five of the ten members was observed on the upper side of branches (small MFA) compared to the lower side (large MFA). As previously described, *EgrTUA1* and *EgrTUB1* expression was 1.53 and 1.47 fold increased on the upper side compared to the lower side, respectively. In addition, *EgrTUA3*, *EgrTUB4* and *EgrTUB6* also showed increased expression on the upper side of branches; 1.25, 1.45 and 1.49 fold, respectively (Southerton *pers com*.). A preliminary phylogenetic analysis of the *E. grandis* beta-tubulin protein family was undertaken which showed that each of the proteins was represented within each of the different clades (as per Oakley *et al.* 2007), from which it was hypothesised that these *E. grandis* TUBs might have different functional roles.

It was further argued that these *E. grandis* TUBs have specificity with respect to the cellular processes controlling the orientation of cellulose microfibrils. The experiment described in this chapter investigated the role of individual beta-tubulin genes using both transgenic and phylogenetic analyses. The candidate genes selected for this study were kindly supplied by Dr. Simon Southerton (CSIRO Plant Industry), and these genes had previously been found to be differentially expressed on the upper and lower sides of eucalypt branches (Qiu et al 2008).

7.2 Methods

7.2.1 Phylogenetic analysis selection

Phylogeny is the study of evolutionary relatedness, and phylogenetic comparisons between related protein sequences can shed light on their function. The type of phylogenetic analysis can influence the interpretation of results. Therefore, it is important to use the right distance matrixes. Distance matrixes such as the neighbour-joining and minimum-evolution algorithms are frequently used for reconstructing phylogenies (Gascuel 2005). The neighbour-joining algorithm (NJ) (Saitou and Nei 1987) works on the principle of identifying pairs of proteins with the lowest distance (Q value) connecting them, then creating a node in the tree branch and then adding the next protein. This algorithm is described as a bottom up clustering methodology. NJ matrixes are considered reasonably accurate, especially if coupled with a re-sampling strategy such as bootstrapping (Saitou and Nei 1987). In contrast, the minimum-evolution (ME) algorithm (Kidd and Sgaramella-Zonta 1971; Rzhetsky and Nei 1992), works by assigning branch length between proteins for all possible topologies. The tree with the smallest sum of branch lengths is selected as the true tree (Nei and Kumar 2000). This method can be considered more reliable than NJ-trees because the NJ-trees are constructed in a stepwise manner.

Both the NJ and ME matrixes can be statistically enhanced by including methods for testing the reliability of branch patterns, such as a bootstrap test or the interior branch test (Nei and Kumar 2000). If the percentage of support for a given branch is 95-99% it is considered to be significant. However, trees with lower values are still considered reliable as long as a sufficient sequence length and number of subunits have been used (Nei and Kumar 2000). Another important feature of a phylogenetic tree is the rooting of a tree with an ancestral

outgroup (Nei and Kumar 2000). It is important to select an outgroup that is not too distantly related as to infer no genetic information, nor to select one that is too closely related and hence is not a true outgroup. In the absence of a good outgroup, un-rooted trees can be used effectively to assess phylogeny (Nei and Kumar 2000). It is therefore important to select an appropriate phylogenetic matrix for the proteins being analysed.

Phylogenetic trees for plant alpha- and beta-tubulin proteins created in this chapter were generated using the MEGA4 and SplitsTree programs. Multiple trees were created using different analyses, and variability between phylogenetic trees was compared. The phylogenetic trees presented in the results section are minimum evolution trees employing the interior branch test algorithm and were produced using the MEGA4 program.

7.2.2 Vector construction and experimental design

All constructs created were synthetic miRNA vectors synthesised by GenScript (www.genscript.com) and cloned into the Puc57 entry vector. Each vector underwent an LR reaction resulting in the creation of five new destination vectors (Table 7-1). The original 1305.1miTUB1 (CaMV35S:miTUB1) vector was included as a control and the vector 1305.1GWGUS+ (CaMV35SGW:GUS+) was also included as a positive control for the GUS reporter gene. The procedure for microRNA vector development outlined in Chapter 6 was again used in the experiment described in this chapter. Details of miRNA site selection and sequence differences between all miRNAs are presented in Appendix B.

Table 7-1 Synthetic miRNA vectors created for six *Eucalyptus grandis* beta tubulin genes. GW stands for Gateway. The sequences of the different mature miRNAs are presented, letters in bold are nucleotide positions 1 and 10, underlined letters are positions 2, 12 and 20, which are all important nucleotides involved in microRNA recognition (see Chapter 6). The *Arabidopsis* MIR164A backbone was used for all miRNAs.

Candidate gene	Entry vector	LR rxt	Destination vector	Mature miRNA sequence 5' to 3'
EgrTUB1	Puc57	Yes	1305.1GWmiTUB1	**U**CC AGU CGU **A**GU AGG CCA GCC
EgrTUB2	Puc57	Yes	1305.1GWmiTUB2	**U**AU GUG AGC **A**GC CGU AAG AGC
EgrTUB3	Puc57	Yes	1305.1GWmiTUB3	**U**GG UUG ACU **A**CG UGA CUC UCC
EgrTUB4	Puc57	Yes	1305.1GWmiTUB4	**U**AU GUA AGU **A**GU CGU AAG AGU
EgrTUB5	Puc57	Yes	1305.1GWmiTUB5	**U**AC GUG AGU **A**GU CGU AAU AGG
EgrTUB6	Puc57	Yes	1305.1GWmiTUB6	**U**GC UCA ACU **A**CC UGU CUU UCC

Transformations and analyses for the 6 constructs presented in this chapter were carried out simultaneously in inoculation round two (R2), using the same methodology described in detail in Chapter 6. Round two commenced with inoculation of plants in December and transformed sectors were harvested in March. The sectors underwent GUS assay and xylem sectors containing wood fibres were used for MFA assessment. Twenty two paired sectors of transgenic and untransformed wood fibres underwent comparative analysis using paired T-tests to determine the effect of the transgene on MFA for each of the vectors.

7.3 Results

7.3.1 Phylogenetic analysis of plant alpha and beta-tubulin gene families

Phylogenetic analysis of alpha tubulin (TUA) and beta-tubulin (TUB) proteins from algae and plants was undertaken. Phylogenetic analyses using neighbour-joining and minimum-evolution methods with both boot-strapping and interior branch test values gave similar topologies. Here only the minimum-evolution trees are presented. Interior branch test values of 80% and above are shown, however, for most nodes the values were low. This is likely to be due to the high level of sequence similarity between plant tubulins.

Phylogenetic analysis of TUA proteins from algae and plants is shown in Figure 7.1. TUAs from *Eucalyptus grandis* (eucalypt), *Populus trichocarpa* (poplar), *Arabidopsis thaliana*,

Gossypium hirsutum (cotton), *Zea mays* (maize) and *Oryza sativa* (rice) separate into two Classes in accordance with a previously published phylogeny (Oakley *et al.* 2007) (Chapter 3, Figure 3.1). The alga TUA protein forms an ancestral branch and root of the tree. Within the designated poplar Class I and poplar Class II subdivisions a number of separate branches can be observed. The *Eucalyptus grandis* TUA proteins all fall into the poplar Class II clade. *Egr*TUA1 and *Egr*TUA2 cluster with the poplar *Poptr*TUA3 and *Poptr*TUA7 proteins and their respective genes expressed in xylem and shoots, and xylem and leaves, respectively (Oakley *et al.* 2007) (Chapter 3). The other two *Eucalyptus grandis* TUA proteins (*Egr*TUA3 and *Egr*TUA4) form a separate branch within a different clade. Part of the cDNA sequence for *EgrTUA4* is missing (approximately 131 bases; 43 amino acids at the beginning), however the full length gene is likely to share the highest sequence similarity to *EgrTUA3*. For all other *EgrTUA* genes full length cDNA and amino acid sequences were analysed.

Phylogenetic analysis of TUB proteins from algae, and plants is presented in Figure 7.2, TUB proteins from *Eucalyptus grandis* (eucalypt), *Populus trichocarpa* (poplar), *Arabidopsis*, *Gossypium hirsutum* (cotton), *Zea mays* (maize) and *Oryza sativa* (rice) separate in accordance with a previously published phylogeny (Oakley *et al.* 2007) (Chapter 3, Figure 3.1). The alga and moss TUB proteins form an ancestral branch and root of the tree. The TUB phylogeny is divided into five clades previously classified as poplar TUB Class I (*Egr*TUB5 and *Egr*TUB6), Class I-like (*Egr*TUB1), Class II (*Egr*TUB2), Class III (*Egr*TUB3) and Class IV (*Egr*TUB4). An in-complete EST sequence was the only sequence available for EgrTUB6 (approximately 789 bases missing; 263 amino acids), therefore the *Egr*TUB6 position within the phylogeny may not be exactly correct, however, for all the other *EgrTUB* genes full length cDNA and amino acid sequences were used.

As multiple tubulin genes are found in all plants and different spatial and temporal patterns of expression have been observed for tubulin genes within a species, it is possible that related tubulins have conserved functions. To examine this further, tissue-specific gene expression profiles and protein abundance information, where known, was overlayed using coloured symbols on the phylogenetic analyses of plant TUAs and TUBs (Figure 7.1 and Figure 7.2).

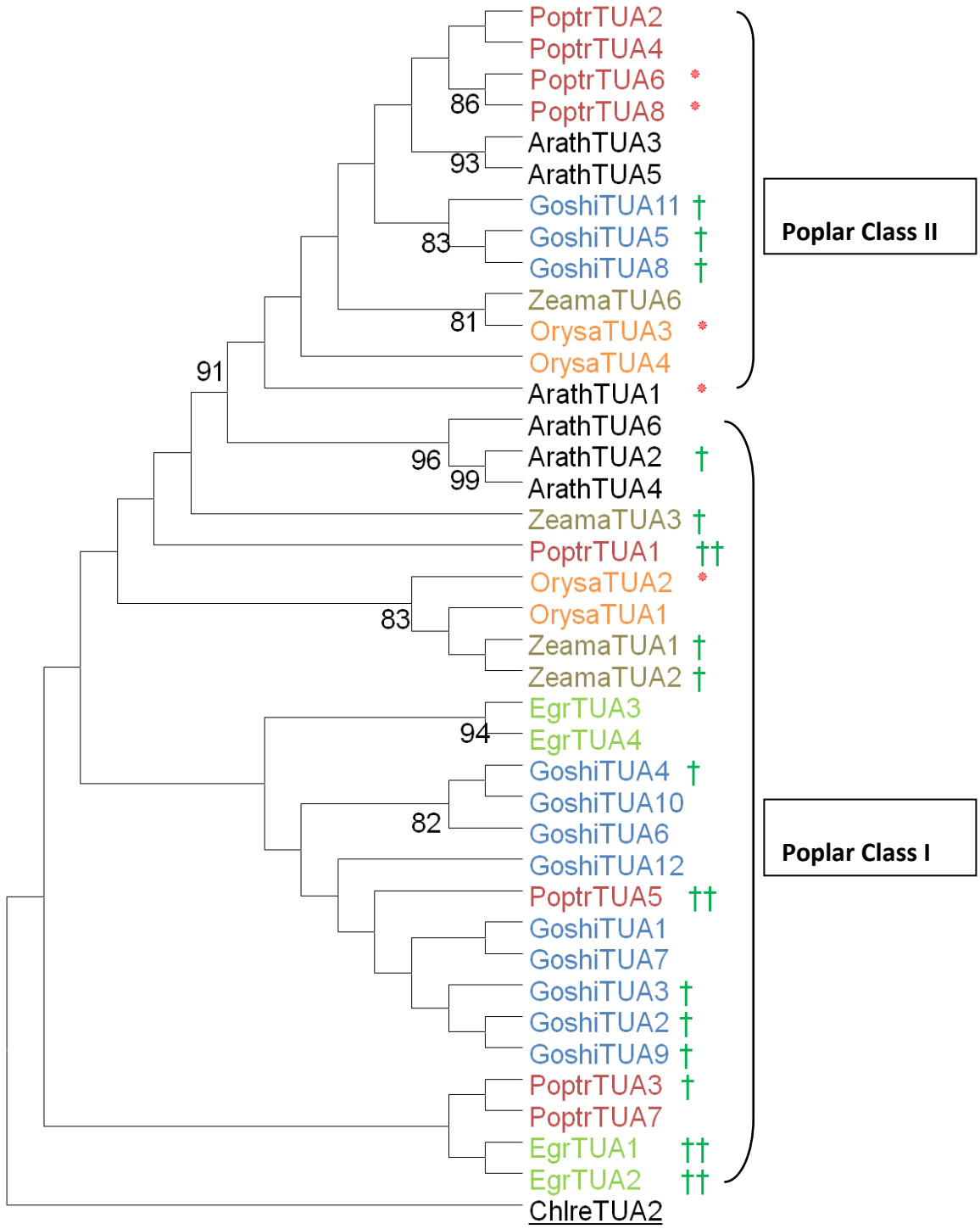

Figure 7.1 Phylogenetic analysis of plant alpha-tubulin proteins. Minimum evolution tree with interior branch test. Branch values with >80% confidence are shown. The ancestral alga (ChlreTUA2) used as an outgroup to root the tree is underlined. Protein names coloured the same are derived from the same species. Labelled groupings as per the poplar Class phylogeny are shown (Oakley *et al.* 2007). Proteins are also marked according to their tissue-specific expression if known; pollen ( ), xylem (†) or active in xylem during tension wood formation or secondary cell wall formation processes (††). For Genbank accession numbers see Appendix E.

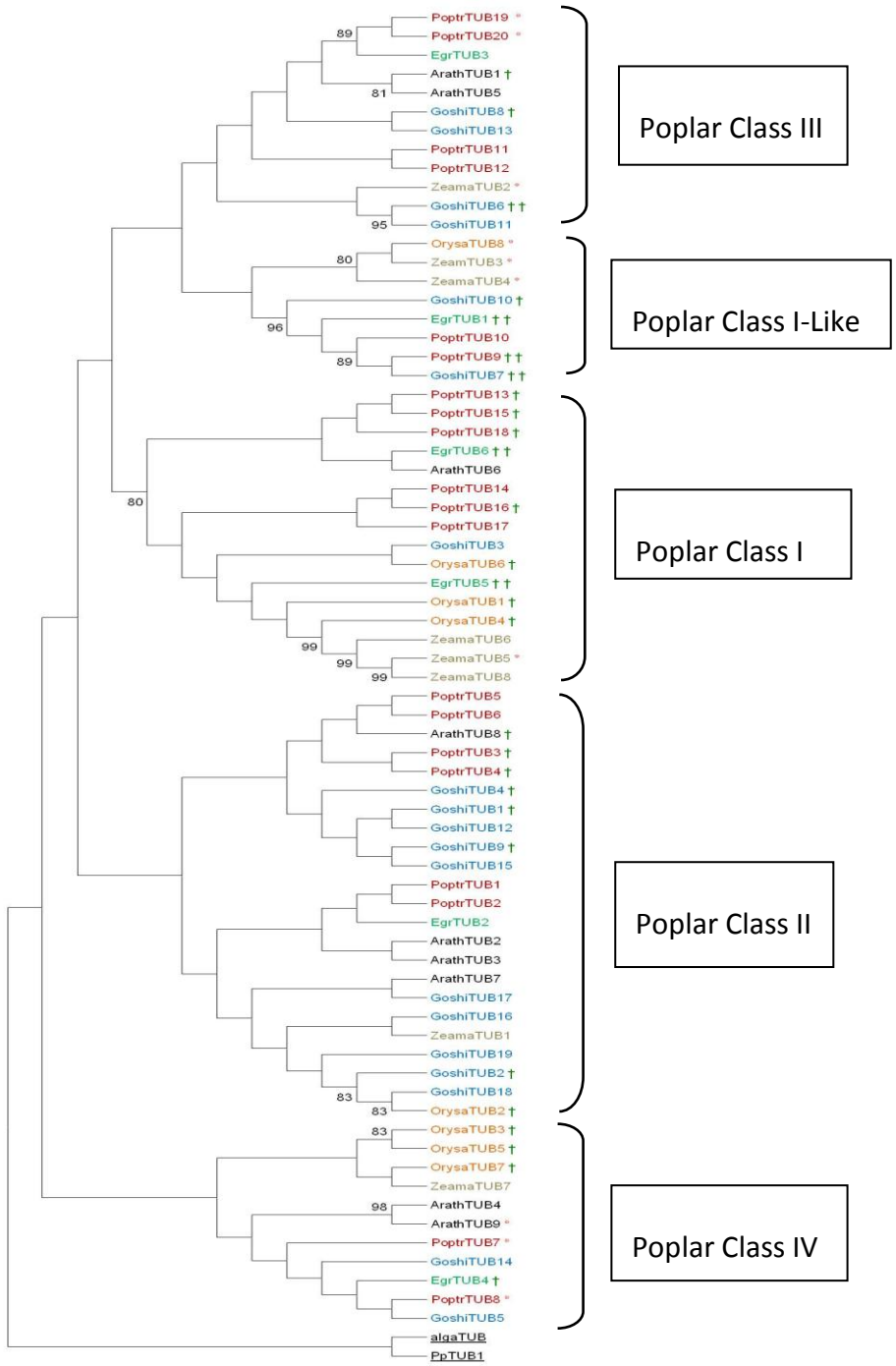

Figure 7.2 Phylogenetic analysis of plant beta-tubulin proteins. Minimum evolution tree with interior branch test, branch values at <80% confidence shown. The ancestral alga (AlgaTUB) and moss (PpTUB1) outgroup proteins used to root the tree are underlined. Protein names coloured the same are derived from the same species. Labelled groupings as per the poplar phylogeny are shown. Proteins are also marked according to their tissue-specific expression if known; pollen ( ), xylem (†) or active in xylem during tension wood formation or secondary cell wall formation processes (††). For Genbank accession numbers see Appendix E.

7.3.2 Plant growth and GUS assay

Plant growth data for R1, R2 and R3 was presented in Chapter 6 for all plants (Table 6.4). Here the results for five additional miRNA vectors from trial Exp 2 are presented. For each vector the number of GUS sectors was recorded for each cambial window as the average number of transformed sectors per cm^2 ($ATScm^{-2}$). $ATScm^{-2}$ varied between trials and treatments. A consistently high rate of transformation was observed for all miRNA vectors compared to the control CaMV35SGW:GUS+ vector (empty vector) (Table 7-2).

Table 7-2 Transformation rates and GUS sector patterns for miRNA vectors. Some of the data presented in this table was also presented in Table 6.5 and is presented again here to allow direct comparisons. $ATScm^{-2}$ = average transformed sectors cm^2.

Inoculation round	Vector	$ATScm^{-2}$	Inoculation round	Vector	$ATScm^{-2}$
R1	CaMV35SGW:GUS+	34.44	**R2**	CaMV35S:miTUB4	42.02
R1	CaMV35S:miTUB1	35.91	**R2**	CaMV35S:miTUB5	51.45
R2	CaMV35SGW:GUS+	16.58	**R2**	CaMV35S:miTUB6	48.41
R2	CaMV35S:miTUB1	35.35	**R3**	CaMV35SGW:GUS+	12.50
R2	CaMV35S:miTUB2	30.45	**R3**	CaMV35S:miTUB1	35.83
R2	CaMV35S:miTUB3	37.71			

7.3.3 Specificity of EgrTUB2-6 to MFA determination

In summary, in Chapter 6, the CaMV35SGW:GUS+ vector was used as a control vector, as it only contains the GUS reporter gene, and the CaMV35S:miTUB1 vector was assessed. For the CaMV35SGW:GUS+ vector, 22 sectors in R1 and 20 sectors in R2 and R3 have been analysed *via* a paired T-test. The MFA values presented are the mean difference between the transformed and untransformed wood fibres, and in all rounds GUS+ was shown to have no significant effect on MFA (See Figure 7.3). For the CaMV35S:miTUB1 vector 22 sectors in R1 and R2, and 21 sectors in R3 have been measured (Chapter 6), and in all rounds the presence of the CaMV35S:miTUB1 vector resulted in a significant increase in MFA (Figure 7.3).

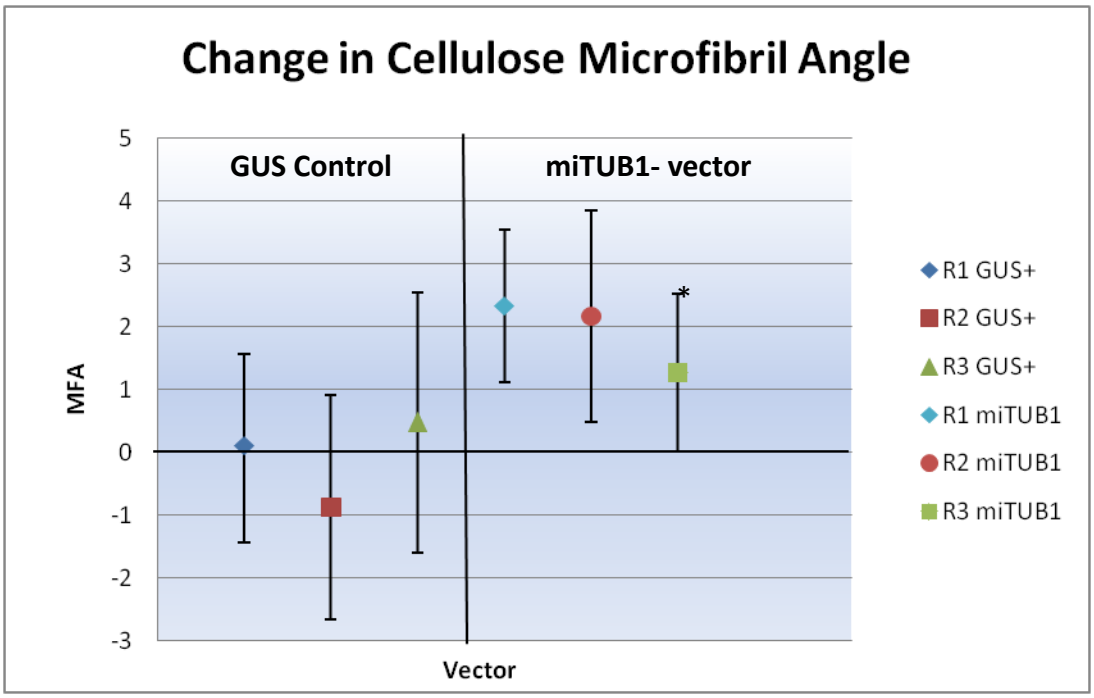

Figure 7.3 Effect of expression of GUS or over-expression of TUB1 on cellulose microfibril angle (MFA). Pair-wise comparisons from R1, R2 and R3. MFA values are mean difference between transformed and untransformed wood fibres. Error bars are 95% confidence intervals. All GUS+ vectors show no-significant difference from zero and therefore did not alter MFA. The miTUB1- vectors showed a significant positive change in MFA. * Confidence intervals for R3 miTUB1- are 0.008 to 2.531.

miRNA vectors were used to investigate the influence of five *E. grandis* beta-tubulin genes on MFA. For vectors CaMV35S:miTUB2, CaMV35S:miTUB4 and CaMV35S:miTUB5, 22 sectors were analysed and 21 and 20 sectors were analysed for CaMV35S:miTUB3 and miTUB6, respectively, *via* paired T-tests. The MFAs presented are the mean difference between the transformed and untransformed wood fibres. Both CaMV35S:miTUB5 and CaMV35S:miTUB6 showed a significant increase in MFA of 1.9^{o} (P-value 0.024) and 1.6^{o} (P-value 0.014), respectively (Figure 7.4). The other vector treatments had no significant effect on MFA. For CaMV35S:miTUB2 a change of -0.3^{o} (P-value 0.707) was calculated, for CaMV35S:miTUB3 a change of 0.8^{o} (P-value 0.297) and for CaMV35S:miTUB4 a change of 0.2^{o} (P-value 0.74) (Figure 7.4).

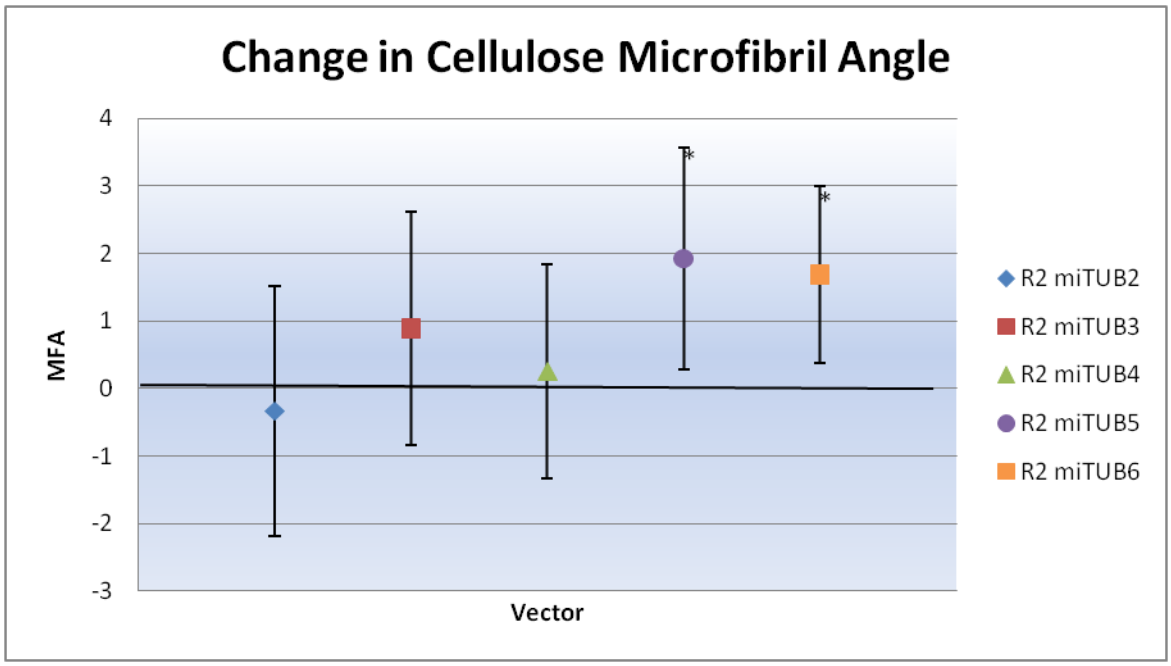

Figure 7.4 Effect of over-expression of TUB2 to TUB6 on cellulose microfibril angle (MFA). From T-test MFA values are mean difference between transformed and untransformed wood fibres. Error bars are 95% confidence intervals. * The miTUB5- and miTUB6- vectors caused a significant increase in MFA.

With the identification of specificity of individual members of the *E. grandis* beta tubulin family, further analysis of the alignment of their protein sequences was undertaken. The clustalW alignment of their sequences (Bioedit) did not reveal any amino acid changes that were conserved in only the three proteins which have been shown to alter MFA (*Egr*TUB1, *Egr*TUB5 and *Egr*TUB6) (Appendix G).

7.4 Discussion

7.4.1 Phylogenetic analysis of TUAs

The phylogenetic analysis of plant alpha-tubulin proteins revealed that *Eucalyptus grandis* alpha-tubulin genes are paired, with *Egr*TUA1 and 2 and *Egr*TUA3 and 4 being most similar to each other, respectively, and that they are more similar to other *P. trichocarpa* and *G. hirsutum* alpha-tubulin genes. Generally, the monocotyledonous TUA proteins (*Zea mays* and *Orysa sativa*) cluster separately from the dicotyledonous TUAs, while most of the dicotyledonous TUA proteins tend to cluster by species (Figure 7.1). All *Eucalyptus grandis* TUA proteins fall into the poplar TUA class I. *Egr*TUA1 and *Egr*TUA2 cluster with poplar *Poptr*TUA3 and *Poptr*TUA7. Both of these poplar genes are expressed in xylem as well as shoots (*PoptrTUA3*) and leaves (*PoptrTUA7*) (Oakley et al. 2007) (Chapter 3). The other two

Eucalyptus grandis TUA proteins (*Egr*TUA3 and *Egr*TUA4) form a separate branch within the same Class.

A range of spatial and temporal expression patterns have been observed for tubulin genes within plant species as shown for plant TUAs in Figure 7.1. A number of TUA proteins that are associated with pollen formation (red) are represented in both classes. Likewise, TUA proteins active in xylem tissue are also widely distributed within the phylogenetic tree. Two *P. trichocarpa* TUA genes, *PoptrTUA1* and *PoptrTUA5*, which are both expressed in xylem have been shown to be highly up-regulated in tension wood (Oakley *et al.* 2007). *EgrTUA1* and *EgrTUA2* are also up-regulated in tension wood in eucalypts (Qiu *et al.* 2008) and both genes have been shown to affect MFA (Chapter 6). However, phylogenetic analysis does not suggest that these poplar and eucalypt TUAs are more closely related at the protein sequence level (Figure 7.1).

7.4.2 Phylogenetic analysis of TUBs

Phylogenetic analysis of plant beta-tubulin proteins shows that the *Eucalyptus grandis* TUBs are generally more similar to tubulin proteins from other species than to each other, with at least one *Egr*TUB represented within each clade (poplar Classes). The exception was poplar Class I which contains both *Egr*TUB5 and *Egr*TUB6.

With the release of the *Arabidopsis* genome, Snustad *et al.* (1992) classified the nine *ArathTUB* members into three sub-classes with *ArathTUB1* and *ArathTUB5* belonging to Class I, *ArathTUB4* and *ArathTUB9* to Class II and *ArathTUB2*, *AarthTUB3*, *ArathTUB6*, *AarthTUB7* and *ArathTUB8* to class III. They suggested that these genes might have evolved from at least three putative ancestral genes (Snustad *et al.* 1992). Oakley *et al.* (2007) published another plant beta-tubulin phylogeny based on the beta-tubulin genes found in the *Populus* genome. The *Arabidopsis* TUB Class I aligned with *Populus* TUB Class III, and *Arabidopsis* TUB class II falls within *Populus* Class IV, while *Arabidopsis* TUB class III divided between *Populus* Classes I and II. No *Arabidopsis* TUB genes are represented in the *Populus* Class I-like TUB clade (Figure 7.2). Unlike Snustad *et al.* (1992), Oakley *et al.* (2007) argued that the distribution of *Arabidopsis* and other dicotyledonous and monocotyledonous tubulin genes represented within the *Populus* Classes (phylogenetic clades) suggests that angiosperm TUB genes evolved from a single ancestral TUB gene. This view is further

supported by the phylogenetic analysis conducted in this thesis (Figure 7.2) which shows the both monocot and dicot tubulin genes are represented within all phylogenetic clades.

Tissue-specific gene expression profiles and protein abundance information, where known, was overlayed using coloured symbols in the phylogenetic analyses of plant TUBs (Figure 7.2). At the branch level there are clear groupings of TUB proteins, coding sequences for which are known to be expressed in pollen tissue (red), both within species such as *Poptr*TUB19 and *Poptra*TUB20 and between species, *Zeama*TUB3, *Zeama*TUB4 and *Orysa*TUB8. Genes coding for the *P. trichocarpa* proteins *Poptr*TUB9, *Poptr*TUB15 and *Poptr*TUB16 up-regulated in xylem during tension wood formation (Oakley *et al.* 2007), while the *G. hirsutum GoshiTUB6* and *GoshiTUB7* genes are active in fibres during secondary cell wall formation (Dixon *et al.* 1994; Dixon *et al.* 2000). Furthermore, the *E. grandis EgrTUB1* gene which is highly up-regulated in xylem tissue during tension wood formation (Qiu *et al.* 2008), has been shown to influence MFA (Spokevicius *et al.* 2007a), as well as *EgrTUB5* and *EgrTUB6* which have also been shown to be involved in MFA determination. Generally, there appeared to be little correlation between tissue-specific expression patterns and the phylogeny of related proteins (Figure 7.2).

While phylogenetic analysis can provide a useful framework for identifying related proteins based on sequence homology, care must be taken when extrapolating sequence homology to functional homology. Sequence similarity, especially across species that diverged millions of years ago, can be the result of program limitations whereby “best available matches” between proteins arise and pairing of proteins that are not in fact orthologous (evolved from a common ancestral gene and retaining the same function) but merely similar (Paterson et al. 2000). This is because gene loss after genome duplication events complicates the identification of orthologs, when amino acid-based phylogenetic analysis is conducted (Paterson et al. 2000). If two species lost different paralogs (proteins related by duplication within a genome and which have gained new functions) of an ancestral protein that was duplicated before species divergence, the remaining proteins will have the highest sequence identity, yet could have differences in expression and/or function (Paterson et al. 2000). Hence, Paterson et al. (2000) argued that orthologs within multigene families could be very difficult to identify. Therefore, in these instances, if chromosomal organisation

information is available, it should also be taken into account when assessing protein and gene homology (Paterson et al. 2000; Bauer et al. 2004).

Chromosomal arrangement of TUA and TUB genes may be contributing to the complexity observed in this phylogenetic tree. The chromosomal arrangement of *Arabidopsis* alpha and beta tubulins was readily available, and this information was overlayed with their phylogeny (Appendix H), however, this analysis did not explain the complexity of *Arabidopsis* TUA or TUB protein phylogenies observed. The complexity of poplar TUB gene arrangements within each class is due to recent and ancient genome duplication events, genome expansion events as well as causative mutations (SNPs; single nucleotide polymorphisms) (Tuskan *et al.* 2006). A chromosomal map showing the specific arrangements of *P. trichocarpa* and *E. grandis* TUA and TUB genes was not readily available and creating one fell outside the scope of this thesis. As more expression and genomic information becomes available for more of the proteins included in this phylogenetic analysis the association between these proteins and their profiles may become clearer.

7.4.3 Plant growth and GUS assays

For all inoculation rounds including R2 glasshouse conditions were found to be favourable to plant growth and transformation. In R2 the miRNA vectors (miTUB2-, miTUB3-, miTUB4-, miTUB5- and miTUB6-) all showed high transformation rates with 30 plus sectors per cambial window. The higher rate of transformation for these vectors compared to other experiments is most likely due to the commencement of this experiment at the optimum time for inoculation e.g. late spring / early summer, and therefore the higher transformation rate maybe due to the greater cambial activity in spring, which is characterised by more competent cells and tissue suitable for transformation.

GUS was used as a reporter gene to identify tissue sectors where the T-DNA from the destination vectors had been integrated into the plant genome. In all experiments including Exp 2 MFA values showed no significant change due to GUS expression, therefore, GUS expression is not considered to alter wood fibre MFA in this thesis. This is consistent with earlier studies in eucalypts where the GUS gene had been shown to have little to no effect on MFA (Spokevicius 2006; Spokevicius *et al.* 2007a).

7.4.4 Specificity of *E. grandis* beta-tubulin genes to MFA

Eucalyptus grandis BETA TUBULIN 1 has been shown to have a 1.47 fold increase in expression on the upper (MFA 12-25^{o}) compared to the lower side (38-52^{o}) of eucalypt branches (Qiu *et al.* 2008). In the same microarray study *Eucalyptus grandis BETA TUBULIN 4* and *BETA TUBULIN 6* also showed a 1.45 and 1.49 fold increase in expression on the upper compared to the lower side, respectively (Simon Southerton *pers com*.). It was therefore hypothesised that other *EgrTUBs* apart from *EgrTUB1* may also be able to influence MFA. Therefore, synthetic -microRNAs targeting *EgrTUB2*, *EgrTUB3*, *EgrTUB4*, *EgrTUB5* and *EgrTUB6* were developed. This decision was also based on a preliminary phylogenetic analysis of the *E. grandis* beta tubulin protein family which showed that each of the proteins was represented in each of the different clades and poplar Classes, from which it was hypothesised that they might have different functional roles. The miTUB1- vector had previously been shown to cause an increase in MFA of 1^{o} to 2.5^{o} in R1, and subsequently in R2 and R3. From the T-test results of the five new miRNAs, the miTUB5 and miTUB6 had caused a significant increase in MFA. For both vectors between 20 and 22 sectors from six to seven trees were assessed. These results implicate changes in *EgrTUB1*, *EgrTUB5* and *EgrTUB6* expression with changes in secondary cell wall cellulose microfibril angle, that in the future could be confirmed *via* RNA extraction as outlined in Chapter 6. Interestingly, presumed changes in expression of *EgrTUB2*, *EgrTUB3* and *EgrTUB4* did not cause a change in MFA.

Different spatial and temporal expression of members of tubulin gene families in plants has been observed in Arabidopsis, *Gossypium hirsutum* , *Zea mays* , *Oryza sativa* and *Populus trichocarpa* (summarised in Chapter 3), which suggests that individual members of tubulin gene families are expressed in a tissue-specific manner and that some genes are specific to particular cellular processes (Ueda *et al.* 1999). For example in *Arabidopsis*, *ArathTUA1* and *ArathTUB9* genes have been identified as pollen specific isoforms (Carpenter *et al.* 1992; Cheng *et al.* 2001). Another example, is the *PwTUA1* pollen specific isoform which has been characterised in *Picea wilsonii* and in *Arabidopsis* pollen (Yu *et al.* 2009). The resultant pollen tubes had increased growth rates. The *PwTUA1* gene was proposed to confer increased stability in microtubules at the tip of pollen tubes, which resulted in increased pollen tube tip growth. Likewise, in *Zea mays*, immunofluorescence microscopy has shown alpha and

beta tubulin isoforms to be preferentially incorporated into microtubules during specific molecular processes such a sexual reproduction and within specific cell types (Eun and Wick 1998).

The gene members of the *E. grandis* alpha and beta-tubulin gene family have likewise been shown to be differentially expressed on the upper side of branches compared to the lower side, and where associated with changes in MFA characteristics (Qiu *et al.* 2008; Southerton *pers com*.). This project has shown that different *E. grandis* tubulin isotypes have different specificity towards MFA determination. Therefore it can be hypothesised that specific *E. grandis* tubulin isoforms form functional cortical microtubule arrays within the cytoplasm of wood fibres, giving these arrays specific functionality towards governing cellulose microfibril deposition and orientation within their secondary cell walls.

The size of tubulin genes families appears to differ between plants that undergo primary growth and those that undergo secondary growth (See Chapter 3). Large tubulin gene families have been observed for plants that undergo secondary growth such as poplar and cotton with 28 and 31 tubulin genes, respectively (Dixon *et al.* 2000; Tuskan *et al.* 2006; Oakley *et al.* 2007; He *et al.* 2008). The complete sequences of the *E. grandis* genome are expected to be published in 2011 (http://web.up.ac.za/eucagen/viewnews). The *E. grandis* tubulin gene family is expected to contain 14 or more members (Alexander Myburg *pers com*.; Simon Southerton *pers com*.). These larger sized tubulin families could be explained by the additional functional roles of such tubulin genes during secondary cell wall formation.

7.5 Summary and Future directions

Here members of the *E. grandis* beta tubulin gene family have been shown to have different specificity toward MFA determination, and this finding suggests that different E. *grandis* tubulin isotypes may have different functions. In future studies in a model species such as *Arabidopsis* or *N. tabaccum*, florescent tagging of *EgrTUB* and *EgrTUA* genes could be used to elucidate their role in specific microtubule array formation and function during different molecular processes as has been shown for Zea mays.

Preliminary analysis of phylogenetic and tissue-specific expression patterns of plant alpha and beta tubulin proteins suggests little correlation between tubulin protein evolution and

function, however, as more genomic information and spatial and temporal expression information accumulates it may become possible to detect examples of conservation of sequence homology and function.

Chapter 8 Summary and future directions

8.1 Thesis summary

This thesis aimed to improve our understanding of wood formation by investigating the molecular control of the ecologically and economically important wood trait microfibril angle (MFA). Wood fibre properties are important to both the timber industry and pulp and paper manufactures. Wood fibres with smaller MFA have increased tensile strength while fibres with larger MFA have increased flexibility and both these traits are important characteristics (Plomion *et al.* 2001a; Burgert *et al.* 2002). Over the past 40 years many publications have suggested a potential role for cortical microtubules and tubulin genes in governing cellulose microfibril deposition within plant cell walls and recent research in tree species has further revealed a link between tubulin gene expression, wood formation and MFA (Burk *et al.* 2006; Oakley *et al.* 2007; Spokevicius *et al.* 2007a; Qiu *et al.* 2008).

Eucalyptus grandis tubulin genes were chosen for investigation in this thesis. The influence of *E. grandis BETA TUBULIN 1* (*EgrTUB1*) on MFA, which had been studied in earlier experiments by Spokevicius *et al.* (2007a), was re-assessed using a similar vector CaMV35S:*EgrTUB1* and experimental design to the original study confirming that *EgrTUB1* activity influences wood fibre MFA. Using over expression constructs as well as newly developed synthetic-microRNAs for the down-regulation of beta-tubulin gene, genetic transformation experiments described in this thesis resulted in the creation of transgenic tissue sectors all of which were characterised by increased MFA, indicative of down-regulation of beta tubulin. These results provide strong evidence that not only microRNA constructs but also the use of over-expression vectors containing *EgrTUB1* resulted in gene silencing as previously hypothesised by Spokevicius *et al.*(2007a). The microRNA experiments presented in this thesis to our knowledge represent one of the first examples of the successful application of synthetic-microRNA technology in a perennial forest tree species.

Similar results were obtained by using the *E. grandis ALPHA TUBULIN* genes, *EgrTUA1* and *EgrTUA2*, which also led to subtle increases in MFA in transgenic wood fibres. Again, not only transformation with the synthetic-microRNA vector (CaMV35S:miTUA2) but also the

use of the over-expression vector (CaMV35S:*EgrTUA1*) resulted in increased MFA and again the latter is likely to be the result of homology dependent gene silencing. This finding confirms that both tubulin heterodimers that are required to form cortical microtubules are equally able to influence wood fibre properties and that the balance between them plays a vital role in finetuning the determination of MFA.

This statement is further supported by experiments altering the expression of alpha and beta tubulin genes in combination. MFA was found to be increased when both genes were down regulated by synthetic-microRNAs simultaneously targeting *EgrTUA1* and *EgrTUB1*, at the same time MFA was found to be decreased following simultaneous over-expression. First efforts to quantify the physical attributes of these resulting transgenic fibres and fibre bundles (tensile strength and flexibility) are described in Appendix F.

In an attempt to identify potential links between specific beta tubulin gene function and the phylogenetic relationship between their products, all known *E. grandis* beta tubulin gene family members were down regulated using synthetic-microRNAs. Analysis of resulting transgenic tissue sectors revealed that targeting *EgrTUB1*, *EgrTUB5* or *EgrTUB6* produced wood fibres with increased MFA, while down regulation of any other family members did not have any effect. This is consistent with other studies that found differential expression of tubulin genes and protein accumulation in a wide range of plant species (Carpenter *et al.* 1992; Dixon *et al.* 1994; Cheng *et al.* 2001; Oakley *et al.* 2007; He *et al.* 2008). In accordance with these studies, also in *E. grandis* a number of tubulin isoforms were identified which are likely to be differentially regulated as they show specificity towards determining microfibril angle during secondary cell wall formation in wood fibres.

8.2 Future work

Further confirmation of molecular changes underlying altered MFA phenotypes e.g. through analysing gene expression in transgenic tissue sectors, was hindered by difficulties in extracting RNA from the minute amounts of transgenic tissue produced, and possible the GUS assay (required to identify the transgenic regions) which maybe degrading RNA before extraction. Further method development in this regard should therefore be a high priority for any future ISSA based studies.

The subtlety of the observed changes of MFA in wood fibres developed as part of this thesis raises the question of what constitutes a biologically significant change in MFA. An increase in MFA from 5-10^0 to 40^0 has been shown to result in reduced tensile strength (approximately one third) and reduced stiffness (approximately one fifth) (Batchelor *et al.* 2000), while an increase of only 2^o is predicted to reduce tensile strength by 1-3% (Burgert *et al.* 2002). To further address this question a preliminary study was undertaken in collaboration with researchers at the Biomaterials Department of the Max Planck Institute of Colloids and Interfaces in Germany, assessing the tensile strength of fibres from transgenic wood sectors produced as part of this thesis. This assessment only commenced during the final year of this PhD project and, while 90% completed, the final analysis of results falls outside the scope of this thesis and is expected to be published independently.

The demonstrated link between tubulin genes and MFA in wood fibres identifies specific tubulin genes as strong candidates for genetic association studies in an effort to develop molecular markers for tree improvement programs. This approach has been successful in loblolly pine where allelic variation in a loblolly pine TUA gene was found to be significantly associated with earlywood cellulose microfibril angle (Gonzalez-martinez *et al.* 2007). In this study the α-tubulin M10 SNP explained ~2-4% of the total phenotypic variation in earlywood MFA in an association discovery population.

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Appendix A

Here the Genbank ID codes for the *E. grandis* genes used in this thesis are provided (Table A1). For the alpha tubulin genes which have not yet been published the sequence are provided below. Following this Table A2 presents *E. grandis* alpha and beta tubulin pair wise nucleotide and amino acid sequence similarities.

Table A1, Genbank ID codes for gene supplied by Simon Southerton.

Gene	Genbank ID	Gene	Genbank ID
EgrTUB1	**EF534219**	***EgrTUB4***	**EF534222**
EgrTUB2	**EF534220**	***EgrTUB5***	**EF534223**
EgrTUB3	**EF534221**	***EgrTUA1***	**EF534224**

E. grandis BETA TUBULIN 5 (EgrTUB5)

GTGAGAAAGGAGGCTGAGAACTGCGACTGTCTTCAAGGTTTCCAAGTATGCCATTCACTTGGTGGA
GGAACTGGCTCTGGAATGGGCACTTTGCTCATCTCAAAAATCAGGGAGGAATATCCTGATAGGATG
TTGATGACATTCTCTGTGTTCCCTTCTCCTAAGGTTTCTGATACAGTGGTCGAGCCTTACAATGCCACC
CTTTCTGTCCATCAACTCGTGGAGAATGCAGATGAATGCATGGTTCTGGACAATGAAGCTTTGTATG
ATATTTGCTTCCGGACTCTCAAGCTGACAACTCCCAGCTTTGGCGATCTGAACCACTTGATCTCTGCA
ACCATGAGTGGAGTTACTTGTTGCCTCCGTTTCCCGGGTCAACTGAACTCTGACCTGAGGAAACTTG
CTGTTAACCTGATTCCCTTCCCTCGCCTGCACTTCTTTATGGTTGGTTTTTCCCCTCTGACCAGTCGCG
GTTCCCAGCAGTATCGTGCATTGACAGTGCCGGAACTCACACAACAGATGTGGGATTCCAAGAACAT
GATGTGTGCTGCTGACCCAAGGCACGGCCGCTACCTTACTGCATCAGCGATGTTCAGGGGCAAGAT
GAGTACCAAGGAGGTTGATGAGCAAATGATAAATGTACAGAACAAGAACTCTTCCTACTTTGTGGA
ATGGATTCCTAACAATGTGAAGTCAAGCGTGTGTGACATTCCTCCCAAGGGGCTTTCCATGGCTTCC
ACATTCATCGGGAATTCAACTTCCATCCAGGAAATGTTTAGGAGGGTGAGCGAGCAGTTCACTGCCA
TGTTCA

Translation

VRKEAENCDCLQGFQVCHSLGGGTGSGMGTLLISKIREEYPDRMLMTFSVFPSPKVSDTVVEPYNATLSV
HQLVENADECMVLDNEALYDICFRTLKLTTPSFGDLNHLISATMSGVTCCLRFPGQLNSDLRKLAVNLIPF
PRLHFFMVGFSPLTSRGSQQYRALTVPELTQQMWDSKNMMCAADPRHGRYLTASAMFRGKMSTKEV
DEQMINVQNKNSSYFVEWIPNNVKSSVCDIPPKXAFHGFHIHRXNSTSIQEMFRRVSEQFTAMFX

E. grandis ALPHA TUBULIN 2 (*EgrTUA2*)

CCCTCTCTCTCCGGATCCTTCCGTCTCCGTCCTCGGCCCTCCTCTCCGGACCGTCGAAGAAG**ATG**AGA
GAGTGCATCTCGATTCACATTGGCCAGGCCGGCATTCAGGTCGGCAACTCCTGCTGGGAGCTCTACT
GTGTCGAGCACGGCATTCAGCCTGATGGCCAGATGCCAAGTGACAAGACGGTTGGCGGAGGCGAT
GATGCTTTCAACACCTTCTTCAGCGAGACCGGCGCTGGCAAGCACGTCCCTCGCGCTGTGTTTGTGG
ATCTGGAGCCTACTGTGATTGATGAAGTGAGGACCGGCACTTACCGCCAGCTCTTTCACCCTGAGCA
GCTCATCAGCGGCAAAGAAGATGCCGCCAACAACTTCGCCCGTGGTCATTACACAATTGGAAAGGA
GATCGTGGATCTCTGCTTGGACAGGATCCGCAAGCTTGCAGACAACTGCACTGGTCTCCAGGGCTTC
CTTGTCTTTAACTCTGTCGGAGGCGGCACTGGTTCTGGTCTCGGATCACTCCTCTTGGAGCGACTCTC
CGTTGACTATGGAAAAAAATCAAAGCTCAGTTTCACCGTTTATCCCTCTCCCCAGGTTTCCACCTCTGT
CGTGGAACCTTACAACAGCGTCCTTTCTACCCACTCCCTCCTTGAGCACACTGATGTTGCCGTGCTTCT
CGATAATGAAGCCATCTATGACATCTGCCGAAGATCCCTCGATATTGAGCGTCCCACATACACTAATC
TGAACCGGTTGATCTCTCAGGTGATCTCATCCTTGACTGCCTCTCTGAGGTTTGATGGAGCCTTGAAT
GTGGATGTCACTGAATTCCAGACCAACCTGGTCCCTTACCCCAGGATCCACTTTATGCTTTCTTCATAT
GCTCCAGTCATCTCAGCCGAGAAAGCACATCACGAGCAGCTCTCGGTGGCTGAGATCACCAACAGT
GCATTCGAGCCATCATCTATGATGGCCAAGTGTGATCCTCGCCATGGCAAGTACATGGCTTGCTGTC
TCATGTACCGAGGAGATGTCGTTCCAAAGGATGTGAATGCAGCTGTGGGCACCATCAAGACCAAAC
GGACTATCCAATTTGTTGACTGGTGCCCAACAGGATTCAAGTGTGGTATTAACTACCAACCTCCTACT
GTCGTTCCAGGAGGTGACCTTGCCAAGGTGCAGAGGGCTGTCTGCATGATCTCCAACTCAACCAGT
GTTGCCGAGGTGTTCTCTCGCATCGACCACAAGTTTGACCTCATGTATGCCAAGCGTGCCTTTGTGCA
TTGGTACGTCGGTGAGGGTATGGAAGAGGGAGAGTTCTCCGAGGCTCGGGAGGATCTTGCTGCCCT
GGAGAAGGACTATGAAGAAGTCGGTGCTGAGTCTGCTGAGGGCGAGGATGGTGATGAAGGAGAG
GACTACTGATGCGGTTATGAATTTCGCTGGCTTGTTTGCTTCATATGTATGGGTTTTATGTTTGTCCTA
TTAAAACTGATTGTGTGCGCACGGGGATTTCCATGGCATGGACCTGATGGTTTTTTGTCATGAATTG
CTATCAAATCATTATCTAAGTATTCCGCAGCTTCTCCCTAAAAAAAAAAAAAAAA

Translation

MRECISIHIGQAGIQVGNSCWELYCVEHGIQPDGQMPSDKTVGGGDDAFNTFFSETGAGKHVPRAVFV
DLEPTVIDEVRTGTYRQLFHPEQLISGKEDAANNFARGHYTIGKEIVDLCLDRIRKLADNCTGLQGFLVFNS
VGGGTGSGLGSLLLERLSVDYGKKSKLSFTVYPSPQVSTSVVEPYNSVLSTHSLLEHTDVAVLLDNEAIYDIC
RRSLDIERPTYTNLNRLISQVISSLTASLRFDGALNVDVTEFQTNLVPYPRIHFMLSSYAPVISAEKAHHEQL
SVAEITNSAFEPSSMMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVGTIKTKRTIQFVDWCPTGFKC
GINYQPPTVVPGGDLAKVQRAVCMISNSTSVAEVFSRIDHKFDLMYAKRAFVHWYVGEGMEEGEFSEA
REDLAALEKDYEEVGAESAEGEDGDEGEDY

E. grandis ALPHA TUBULIN 3 (*EgrTUA3*)

GTTCTCCAGATCTAGAGATAGAGAGAGAGACATTCTCCAAGAAGGCGTCTTCATAAACGCCTTCGAC
CTCCCCTCCGGCCCACTCAATCTCCGATCTCCCCTCCGGCGAAGCCTCCTCCGGTCCCTTTTCCGGCGA
CGAAG**ATG**AGGGAGTGCATCAGCATCCACATCGGCCAGGCCGGTATCCAGGTCGGGAACGCCTGCT
GGGAGCTGTACTGTCTCGAGCACGGTATTCAGCCTGATGGCCAGATGCCGAGTGACAAGACTGTTG
GCGGAGGTGATGATGCCTTCAACACCTTCTTCAGCGAAACTGGAGCTGGGAAGCACGTCCCTCGCG
CTGTCTTTGTAGATCTAGAGCCCACCGTCATTGACGAGGTCAGGACCGGAACCTACCGCCAGCTCTT
CCACCCTGAGCAGCTCATCAGTGGCAAGGAAGATGCTGCCAACAACTTTGCTCGTGGCCACTACACA
ATTGGGAAGGAAATTGTCGATCTCTGCTTGGACCGCATCAGAAAGCTTGCTGACAACTGCACTGGTC
TTCAAGGGTTCCTTGTGTTCAATGCTGTTGGTGGAGGCACTGGTTCAGGGCTTGGTTCCCTTCTGTTG
GAGCGCCTGTCTGTGGATTATGGCAAGAAATCCAAGTTGGGTTTCACTGTCTACCCATCCCCTCAGG
TGTCCACGTCTGTTGTCGAGCCCTACAACAGTGTCCTCTCGACCCACTCCCTTCTGGAGCATACCGAT
GTTGCTGTGCTTCTGGACAATGAGGCCATTTATGATAT*CTGCAG*GCGTTCTCTTGACATTGAGCGTCC
CACGTACACCAACCTGAACCGTCTCGTTTCACAGGTCATTTCCTCCTTGACTGCCTCTCTAAGGTTTGA
TGGTGCCCTGAATGTGGATGTGACTGAATTCCAGACCAACTTGGTCCCATACCCCAGAATCCACTTC
ATGCTTTCATCCTATGCCCCTGTCATCTCAGCTGAGAAGGCCTACCATGAGCAGCTCTCAGTGGCTGA
GATCACCAACAGTGCTTTCGAGCCATCCTCTATGATGGCCAAGTGCGACCCTCGCCATGGGAAATAC
ATGGCATGCTGCCTCATGTACCGTGGTGATGTGGTGCCCAAGGACGTGAACGCAGCTGTGGCAACC
ATCAAGACCAAGCGCACCATCCAGTTCGTCGACTGGTGCCCCACCGGATTCAAGTGCGGTATCAACT
ACCAGCCGCCCACAGTTGTCCCTGGAGGCGACCTCGCCAAGGTCCAGAGGGCTGTGTGCATGATCT
CCAACTCAACCAGCGTTGCTGAGGTCTTCTCGCGCATTGACCACAAGTTCGATCTGATGTATGCCAA
GCGTGCCTTTGTGCACTGGTATGTGGGTGAGGGTATGGAAGAGGGAGAGTTTTCTGAGGCTCGGG
AGGATCTCGCTGCCCTCGAGAAGGATTATGAGGAGGTCGGTGCAGAGTTGGCTGAGGGCGAGGAT
GATGAGGGTGACGAGTATTAGAGGGTGTCTGCGCCATGATCTCTATTGGCTGTTGATGTGTTGGGT
TGTATGTGTGTCTTCGTATTCATGCTCTGTGGCTCAAAGCACTGCGTGTCTGTATCCTATATGAGTGA
CTGTGATCTAGTAAAAAAAAAAAAA

Translation

MRECISIHIGQAGIQVGNACWELYCLEHGIQPDGQMPSDKTVGGGDDAFNTFFSETGAGKHVPRAVFV
DLEPTVIDEVRTGTYRQLFHPEQLISGKEDAANNFARGHYTIGKEIVDLCLDRIRKLADNCTGLQGFLVFN
AVGGGTGSGLGSLLLERLSVDYGKKSKLGFTVYPSPQVSTSVVEPYNSVLSTHSLLEHTDVAVLLDNEAIYD
ICRRSLDIERPTYTNLNRLVSQVISSLTASLRFDGALNVDVTEFQTNLVPYPRIHFMLSSYAPVISAEKAYHE
QLSVAEITNSAFEPSSMMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTIQFVDWCPTGFK
CGINYQPPTVVPGGDLAKVQRAVCMISNSTSVAEVFSRIDHKFDLMYAKRAFVHWYVGEGMEEGEFSE
AREDLAALEKDYEEVGAELAEGEDDEGDEY

E. grandis ALPHA TUBULIN 4 (*EgrTUA4*)

CGGAGGTGATGATGCCTTCAACACCTTCTTCAGCGAAACTGGCGCTGGGAAGCACGTCCCTCGTGCA
GTCTTTGTAGATCTCGAGCCCACCGTCATCGATGAGGTCAGGACCGGAACCTACCGCCAGCTCTTCC
ACCCTGAGCAACTCATCAGTGGCAAGGAAGATGCTGCCAACAACTTTGCTCGTGGCCACTACACAAT
TGGGAAGGAAATTGTCGATCTCTGCTTGGACCGCATCAGAAAGCTTGCTGACAACTGCACTGGTCTT
CAAGGGTTCCTTGTGTTCAATGCTGTCGGTGGAGGCACTGGTTCGGGGCTTGGTTCCCTTTTGTTGG
AGCGCCTGTCTGTGGATTATGGCAAGAAATCCAAGTTGGGTTTCACTGTCTACCCATCCCCTCAGGT
GTCCACATCTGTTGTTGAGCCCTACAACAGTGTCCTCTCGACCCACTCCCTTCTGGAGCACACCGATG
TTGCTGTGCTTCTGGACAATGAGGCCATTTATGATATCTGCAGGCGTTCTCTTGACATTGAGCGTCCC
ACGTACACCAACCTGAACCGTCTCGTTTCGCAGGTCATTTCCTCCTTGACTGCCTCTCTAAGGTTTGAT
GGTGCCCTGAATGTGGATGTGACTGAATTCCAGACCAACTTGGTCCCATACCCCAGAATCCACTTCA
TGCTTTCATCCTATGCCCCCGTCATCTCAGCTGAGAAGGCCTACCATGAGCAGCTCTCAGTGGCTGA
GATCACCAACAGCGCTTTCGAGCCATCCTCTATGATGGCCAAGTGCGACCCTCGCCATGGGAAATAC
ATGGCATGCTGCCTCATGTACCGTGGTGATGTGGTGCCCAAGGACGTGAACGCAGCTGTGGCAACC
ATCAAGACCAAGCGCACCATCCAGTTCGTCGACTGGTGCCCCACCGGATTCAAGTGCGGTATCAACT
ACCAGCCGCCCACAGTTGTCCCTGGAGGCGACCTCGCCAAGGTCCAGAGGGCTGTGTGCATGATCT
CCAACTCAACTAGTGTTGCTGAGGTCTTCTCGCGCATTGACCACAAGTTCGATCTGATGTATGCCAAG
CGTGCCTTTGTGCACTGGTACGTGGGTGAGGGTATGGAGGAGGGAGAGTTCTCTGAGGCTCGGGA
GGATCTCGCTGCCCTCGAGAAGGATTATGAGGAAGTCGGTGCAGAGTTGGCCGAGGGCGAGGATG
ATGAGGGTGACGAGTATTAGAGGGTGTCTGCGCCATGATCTCTATTGGCTGTTGATGTTGGGTTCGT
ATGTGTTTTCTTTCTTTTTTCATTTCTCTGTGGCTTCAAAGACACCTGCGTGTTCTGTTATTCTATATGA
AGTGAACTGGTGATTCTAGTATGCTGCTTTATCTTGCGTTTACATTCTCGTCCTTAATTGTTATATCTG
GGGTTTTCCGAAGAATGTTGAGCTGTTTTGTGTGGCCGCTGGATATGTATGTTTTTTCCATCAAGTCG
TGTAGTTACTCAGCTGAATAACAAAAATAAGACATTTCAAATTCAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Tranlation

GGDDAFNTFFSETGAGKHVPRAVFVDLEPTVIDEVRTGTYRQLFHPEQLISGKEDAANNFARGHYTIGKE
IVDLCLDRIRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGKKSKLGFTVYPSPQVSTSVVEPY
NSVLSTHSLLEHTDVAVLLDNEAIYDICRRSLDIERPTYTNLNRLVSQVISSLTASLRFDGALNVDVTEFQTN
LVPYPRIHFMLSSYAPVISAEKAYHEQLSVAEITNSAFEPSSMMAKCDPRHGKYMACCLMYRGDVVPKD
VNAAVATIKTKRTIQFVDWCPTGFKCGINYQPPTVVPGGDLAKVQRAVCMISNSTSVAEVFSRIDHKFDL
MYAKRAFVHWYVGEGMEEGEFSEAREDLAALEKDYEEVGAELAEGEDDEGDEY

Table A2, *Eucalyptus grandis* alpha and beta-tubulin nucleotide and amino acid sequence identity. Pair-wise homologies were determined using BioEdit and the BLOSUM62 algorithm. # missing approximately the first 131 nucleotide bases. *missing approximately 789 nucleotide bases. White background nucleotide identity and grey background amino acid identity comparisons.

Eucalyptus grandis tubulin sequence identity pair wise percentages (%)										
	***Egr*TUA1**	***Egr*TUA2**	***Egr*TUA3**	***Egr*TUA4#**	***Egr*TUB1**	***Egr*TUB2**	***Egr*TUB3**	***Egr*TUB4**	***Egr*TUB5**	***Egr*TUB6***
EgrTUA1		82	81	66	32	33	30	32	33	19
EgrTUA2	75		79	68	33	34	31	33	34	20
EgrTUA3	76	73		70	32	32	30	32	32	19
EgrTUA4#	60	61	70		27	28	29	27	27	20
EgrTUB1	43	46	43	38		74	71	70	76	47
EgrTUB2	43	45	43	40	69		74	76	77	46
EgrTUB3	40	43	40	42	65	69		70	72	47
EgrTUB4	44	44	44	37	63	69	65		75	44
EgrTUB5	44	45	43	36	67	69	63	68		47
EgrTUB6*	23	24	24	25	39	38	39	38	41	

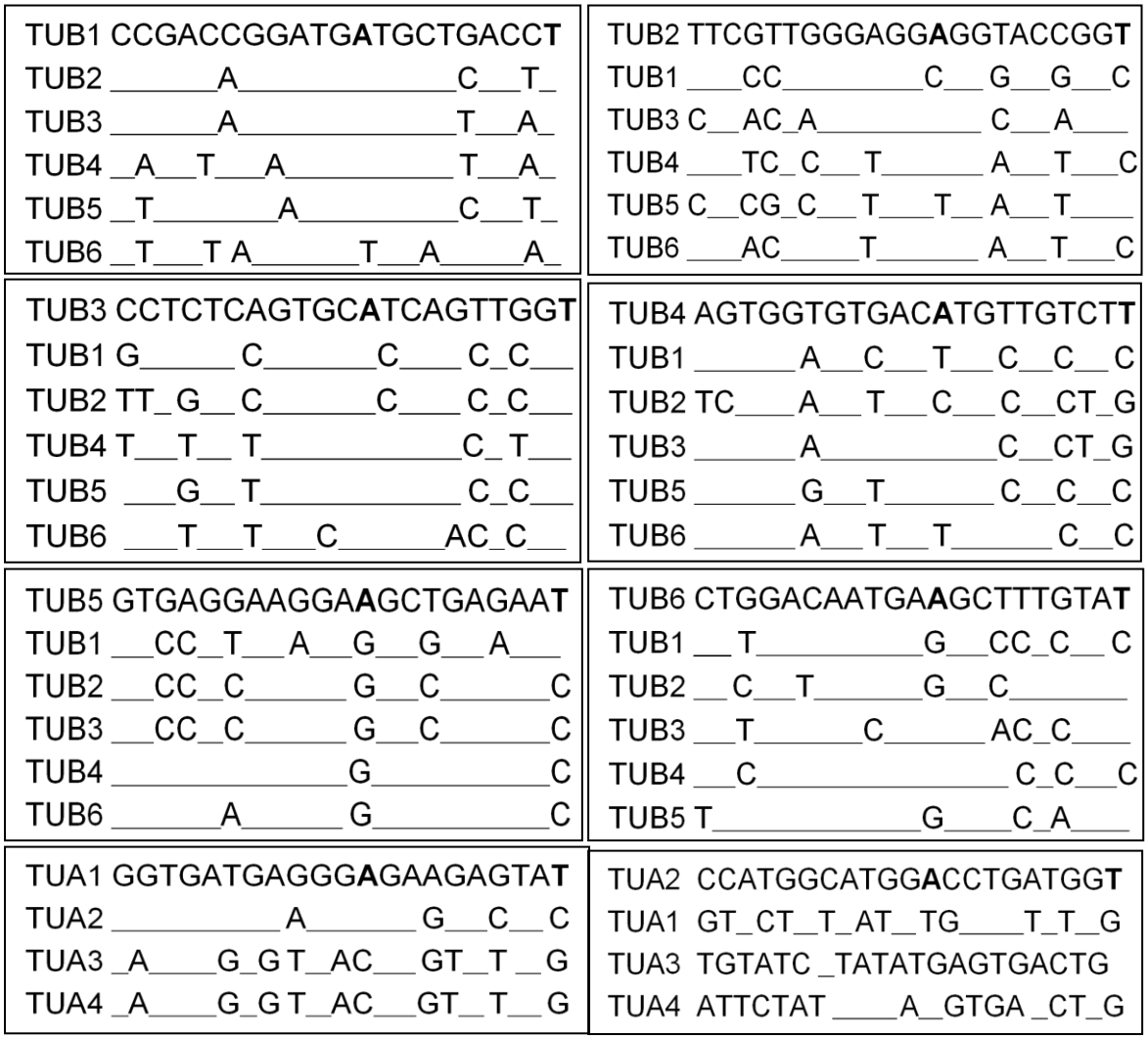
Appendix B

The *Arabidopsis* microRNA backbones used in this project are presented in Table B1. The nucleotide sequence similarities between the different microRNA 21 base pair seed sites, for each of the *E. grandis* gene sequences are presented in Figure B1.

Table B1, *Arabidospis* backbone sequences for MIR164A and MIR164B. The N..N denotes the site of the endogenous mirRNA 21bp region.

Arabidopsis backbones	Arabidopsis backbones 5' to 3'
MIR164A	**GCGGGTGAGAATCTCCATGTN..NAACCAACAAACACGAAATCCGTCTCATTTGCTTA TTN..NACATGAGCTCTTCACCCATTGATGTTATATATCCCTTAAACCTTATTGATAATG TGTTTTTCGTTGTTCTTGACTAGCTCTTCGTTGTTTGTGTGTATATGTGTGTTAAATCAT TTGTGTTCGAC**
MIR164B	**AGAATGATGAAGGTGTGTGATGAGCAAGAN..NTTACTAGCTCATATATACACTCTCA CCACAAATGCGTGTATATATGCGGAATTTTGTGATATAGATGTGTGTGTGTGTTGAGT GTGATGATATGGATGAGTTAGTTCN..NTCATGACCACTCCACCTTGGTGACGATGACG ACGAGGGTTCAAGTGTTACGCACGTGGGAATATACTTATATCGATAAACACACACGTGC**

Figure B1, microRNA seed site selection. cDNA sequences are 5' to 3'. Only the nucleotides that are different between the different E. grandis tubulin genes selected for synthetic microRNA development (21 base pair region).



Appendix C

Cellulose microfibril angle (MFA) sample size determination

Preliminary assessment of nine paired samples from five trees was used for this power analysis to determine the fibre sample size required for the assessment of MFA in this study. In this preliminary study the assessment of fibres were sampled from xylem tissue excised from stem segments. The variance around MFA measurements was observed to decrease with increasing sample number (Figure C1). Through the power analysis it was determined that increasing the number of sectors and not the number of fibres was the best way to increase the statistical power of this study. The standard deviations per number of fibres sampled was calculated to be 3.7 (5 fibres), 3.6 (ten fibres) and 3.3 (fifteen fibres). The power analysis was solved for differing sector sample sizes for these three selected fibre sample sizes, and for power (type error I), alpha values (type error II) and effect sizes (Table C1). It was concluded that sampling 22 paired sectors per vector with five fibres measured per sector (110 fibres measured in total per vector) would give a 95% confidence level of detecting a 3 degree change in MFA.

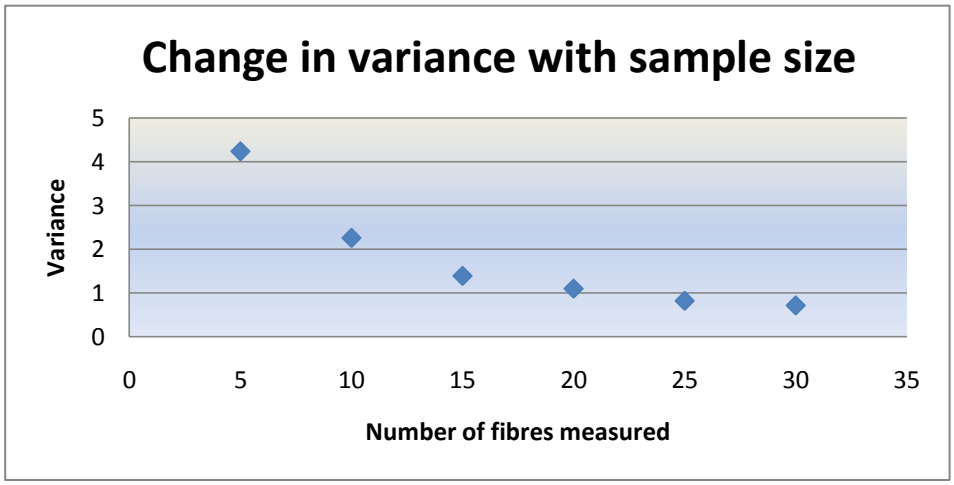


Figure C1, Change in variance depending on the number of fibres sampled per sector. Here nine sector pairs were used in this analysis of variance of fibre sample size, across five different plants.

It has been previously shown in a similar study, that the altered activity of *E. grandis BETA TUBULIN 1* using a similar vector, resulted in a five degree change in wood fibre MFA (Spokevicius *et al.* 2007a), therefore being able to detect a change in MFA of three degrees (Effect size) was determined to be sufficient. Furthermore, from this power analysis (Table C1), at a power of 95% or 98% and respective alpha levels of 0.05 and 0.01 the same 22 sectors and five fibres measured per sector are predicted to be able to pick up a change of 3 degrees. Therefore in Chapters 6 and 7 approximately 22 sectors and five fibres per sector were assessed for each vector treatment.

Table C1, Power analysis for MFA sample size determination.

Sample size (fibres)	No paired sectors	SD	Total No fibres	Power	Alpha	Effect size (change in MFA)
5	22	3.7	110	0.95	0.05	3
10	21	3.6	210	0.95	0.05	3
15	18	3.3	270	0.95	0.05	3
5	180	3.7	900	0.95	0.05	1
10	170	3.6	1700	0.95	0.05	1
15	143	3.3	2145	0.95	0.05	1
5	18	3.7	90	0.95	0.1	3
10	17	3.6	170	0.95	0.1	3
15	15	3.3	225	0.95	0.1	3
5	11	3.7	55	0.8	0.1	3
10	10	3.6	100	0.8	0.1	3
15	9	3.3	135	0.8	0.1	3
5	5	3.7	25	0.8	0.2	3
10	5	3.6	50	0.8	0.2	3
15	4	3.3	60	0.8	0.2	3
5	22	3.7	110	0.98	0.1	3
10	21	3.6	210	0.98	0.1	3
15	18	3.3	270	0.98	0.1	3

Appendix D

The optical densities for each *Agrobacterium* culture for each inoculation round are presented in Table D1, including vector used in Chapter 6 and 7. In round three the ideal OD was not reached as the cultures grew more slowly. For R1 and R2, the higher the OD of the bacterial culture at inoculation, the greater number of sectors observed (Average transformed sectors; ATScm^{-2}) however this relationship does not hold for R3. In R3 variable transformation rate may have been influenced by other factors as well.

Table D1, Optical density of each vector and transformation efficiency.

Inoculation round	Vector	OD_{600}	ATScm-^{2}
R1	1305.1GUS+	0.351	24.48
R1	1305.1GWGUS+	0.397	34.44
R1	1305GWTUB1	0.413	52.67
R1	1305.1GWmiTUB1	0.389	35.91
R1	1305GWTUA1	0.377	46.15
R1	1305.1GWmiTUA1/TUB1	0.386	40.31
R2	1305.1GWGUS+	0.414	16.58
R2	1305.1GWmiTUB1	0.406	35.35
R2	1305.1GWmiTUB2	0.431	30.45
R2	1305.1GWmiTUB3	0.420	37.71
R2	1305.1GWmiTUB4	0.419	42.02
R2	1305.1GWmiTUB5	0.439	51.45
R2	1305.1GWmiTUB6	0.478	48.41
R2	1305.1GWmiTUA2	0.442	49.32
R3	1305.1GWGUS+	0.304	12.50
R3	1305.1GWmiTUB1	0.235	35.83
R3	1305.1GWTUA1/TUB1	0.239	19.46
R3	1305.1GWmiTUA1/TUB1	0.216	29.27

The order of inoculation from the base of the plant stem upwards was recorded in order to see if stem position influences transformation efficiency. A trend is evident in R2 only however. In this case optical density increased with stem position, therefore this trend can be most likely attributed to the effect of increased live bacteria in these cultures. Furthermore, the proposed effect of stem position has not been observed by other researchers from this lab group (Spokevicius *pers com*. ; Crewdson *pers com*.).

Table D2, Order of inoculation from the base of the plant stem upwards.

Order of inoculation	
R1	**1305.1GUS+, 1305.1GWGUS+, 1305GWTUA1, 1305GWTUB1, 1305.1GWmiTUB1, 1305.1GWmiTUA1/TUB1**
R2	**1305.1GWGUS+, 1305.1GWmiTUB1, 1305.1GWmiTUB2, 1305.1GWmiTUB3, 1305.1GWmiTUB4, 1305.1GWmiTUB5, 1305.1GWmiTUB6, 1305.1GWmiTUA2**
R3	**1305.1GWGUS+, 1305.1GWTUA1/TUB1, 1305.1GWmiTUA1/TUB1, 1305.1GWmiTUB1**

Appendix E

Genbank accession numbers for proteins used in Phylogenetic trees.

Species	Protein ID	Genbank No
Arabidopsis thaliana	**ArathTUA1**	**At1g64740**
	ArathTUA2	**At1g50010**
	ArathTUA3	**At5g19770**
	ArathTUA4	**At1g04820**
	ArathTUA5	**At5g19770**
	ArathTUA6	**At4g14960**
	ArathTUB1	**At1g75780**
	ArathTUB2	**At5g62690**
	ArathTUB3	**At5g62700**
	ArathTUB4	**At5g44340**
	ArathTUB5	**At1g20010**
	ArathTUB6	**At5g12250**
	ArathTUB7	**At2g29550**
	ArathTUB8	**At5g23860**
	ArathTUB9	**At4g20890**
Chlamydomonas reinhardtii	**ChlreTUA2**	**AAN87017**
	ChlreTUB	**P04690**
Gossypium hirsutum	**GoshiTUA1**	**AAQ92661**
	GoshiTUA2	**AAQ92662**
	GoshiTUA3	**AAD50627**
	GoshiTUA4	**AAQ92663**
	GoshiTUA5	**AAD50629**
	GoshiTUA6	**EF151302**
	GoshiTUA7	**EF151303**
	GoshiTUA8	**EF151304**
	GoshiTUA9	**EF151305**
	GoshiTUA10	**EF151306**
	GoshiTUA11	**EF151307**
	GoshiTUA12	**EF151308**
	GoshiTUB1	**AAL92118**
	GoshiTUB2	**EF464668**
	GoshiTUB3	**FJ594488**

Species	Protein ID	Genbank No
Gossypium hirsutum	GoshiTUB4	AF521243
	GoshiTUB5	AAQ92665
	GoshiTUB6	AAQ92666
	GoshiTUB7	AAQ92667
	GoshiTUB8	AF521247
	GoshiTUB9	AY345610
	GoshiTUB10	AAN32988
	GoshiTUB11	EU375996
	GoshiTUB12	DQ023526
	GoshiTUB13	EU375997
	GoshiTUB14	EU375998
	GoshiTUB15	EU375999
	GoshiTUB16	EU376000
	GoshiTUB17	EU376001
	GoshiTUB18	EU376002
	GoshiTUB19	EU376003
Oryza sativa	OrysaTUA1	Os03g0726100
	OrysaTUA2	Os11g0247300
	OrysaTUA3	Os07g0574800
	OrysaTUA4	Os03g0219300
	OrysaTUB1	Os01g0282800
	OrysaTUB2	Os03g0105600
	OrysaTUB3	Os06g0671900
	OrysaTUB4	Os01g0805900
	OrysaTUB5	Os02g0167300
	OrysaTUB6	Os05g0413200
	OrysaTUB7	Os03g0780600
	OrysaTUB8	Os03g0661300
Zea Mays	ZeamaTUA1	X15704
	ZeamaTUA2	CAA33733
	ZeamaTUA3	CAA44861
	ZeamaTUA6	CAA44863
	ZeamaTUB1	CAA37060

Species	Protein ID	Genbank No
	ZeamaTUB2	**CAA37061**
	ZeamaTUB3	**CAA52718**
	ZeamaTUB4	**CAA52719**
	ZeamaTUB5	**CAA52720**
	ZeamaTUB6	**Q41783**
	ZeamaTUB7	**Q41784**
	ZeamaTUB8	**Q41785**
Populus trichocarpa	**PoptrTUA1**	**AY229881**
	PoptrTUA2	**AY229882**
	PoptrTUA3	**EF583813**
	PoptrTUA4	**EF584828**
	PoptrTUA5	**EF583814**
	PoptrTUA6	**EF583816**
	PoptrTUA7	**EF584829**
	PoptrTUA8	**EF583815**
	PoptrTUB1	**EF584830**
	PoptrTUB2	**EF584831**
	PoptrTUB3	**EF584832**
	PoptrTUB4	**EF584833**
	PoptrTUB5	**EF584834**
	PoptrTUB6	**EF584835**
	PoptrTUB7	**EF584836**
	PoptrTUB8	**EF584837**
	PoptrTUB9	**EF584838**
	PoptrTUB10	**EF584839**
	PoptrTUB11	**EF584840**
	PoptrTUB12	**EF584841**
	PoptrTUB13	**EF584842**
	PoptrTUB14	**EF584843**
	PoptrTUB15	**EF584844**
	PoptrTUB16	**EF584845**
	PoptrTUB17	**EF584846**

Species	Protein ID	Genbank No
	PoptrTUB18	**EF584847**
	PoptrTUB19	**EF584848**
	PoptrTUB20	**EF584849**
Physcomitrella patens	**PpTUB1**	**AY382287**

Appendix F A preliminary study on *E. grandis* wood fibre tensile strength

Introduction

Microfibril angle (MFA) has a major influence on both wood fibre tensile strength and modulus of elasticity (Page and El-Hosseiny 1983; Batchelor *et al.* 2000; Burgert *et al.* 2002). In this study a method for measuring the tensile strength and elasticity of *Eucalyptus* wood fibres was developed in collaboration with the Biomaterials Department of the Max Planck Institute for Colloids and Interfaces in Golm, Germany. This involved sample preparation both at the University of Melbourne and the MPI, which was undertaken during a one month intensive research visit to Germany. Following this initial study additional sample assessment was required and was undertaken principally by Dr. Michaela Eder and Dr. Markus Rueggeberg at the MPI.

Transgenic wood fibres created as part of this PhD project (Chapter 6), that contained subtle changes in MFA of -2^{o} to $+4^{o}$, were assessed for their influence on wood fibre strength. An increase of 2^{o} MFA has been predicted to reduce the tensile strength of a fibre by 1-3% (Burgert *et al.* 2002). Here an introduction to the influence of MFA on wood fibre strength properties and the development of tensile testing of handsheets and micro-tensile testing of individual wood fibres is presented.

Wood quality traits

Wood fibre strength, flexibility and MFA are important wood quality traits in both the pulp and paper and sawn timber products industries (Greaves et al. 1997; Raymond 2002). For example, the early selection of trees for propagation with desirable wood quality traits, such as density, MFA and modulus of elasticity, has been successfully undertaken by breeders of *Pinus radiata* in two separate populations in Australia and New Zealand (Dungey *et al.* 2006). The heritability and genetic variation of these three wood quality traits has been shown to be age dependent, and it can be positively selected for in core wood (juvenile wood) for timber quality improvement. Similarly, in eucalypts, density, MFA, modulus of elasticity, pulp yield, fibre length, cellulose and lignin content have also been shown to be age dependent (Raymond 2002). In general, the continued reduction in rotation age means

that core wood is becoming a larger proportion of logs at harvest age. It is now suggested that core wood and not just outer wood (mature wood) should be targeted for improvement (Walker and Butterfield 1996; Dungey *et al.* 2006).

Pulp and paper manufacturers are interested in MFA as it has a strong influence on fibre mechanical properties. For example, selective chipping of trees to produce chip stocks with different average MFA values can be sold to mills based on their expected end uses. Low MFA fibres are stronger and stiffer and can be used to make containerboard grade pulp where strong paper sheets are required (French *et al.* 2000). In addition, apart from MFA, other wood fibre properties also influence paper sheet properties including fibre length, fibre wall thickness and fibre diameter (French *et al.* 2000). These other fibre quality traits are also important in paper production as paper products are made up of a network of wood fibres, and the mechanical properties of paper are therefore a function of both the network characteristics and the mechanical properties of the individual fibres (French *et al.* 2000).

Influence of cellulose microfibril angle on plant tissues

Plant cell wall structure and function (reviewed in Chapter 4) requires a complex assembly of a variety of macromolecules, such as cellulose, hemicellulose, pectin and lignin. Plants are able to adapt these cell wall properties and cell geometry in response to environmental conditions (Burgert and Fratzl 2010). For example, plants use the orientation of cellulose microfibrils within their cell walls to generate different physical properties which are required for specific mechanical functions. In plant secondary cell walls the pre-stressing (orientation of cellulose microfibrils) of wood fibre cell walls during tension wood formation and addition of G-layers enables the plant to withstand gravity and reorientate stems and branches (Burgert and Fratzl 2010). Furthermore, this orientation of cellulose microfibrils can influence the movement of other plant organs upon swelling or shrinking of the cell wall, for example controlling movement of wheat awns during seed dispersal and pine cone opening (Dawson *et al.* 1997; Elbaum *et al.* 2007).

The influence of different cellulose microfibril orientations in fibres extracted from woody tissues with differing mechanical properties has been demonstrated by Burgert *et al.* (2002).

These authors used *Picea abies* single wood fibres from different woody tissue types which are known to be characterised by different MFA values. These wood tissues are involved in different developmental processes during plant growth. The tissue types used were; compression wood and opposite wood from the upper- and lower-side of gymnosperm stems and branches, juvenile wood from young trees and early wood, which is produced at the start of the growing season and compression wood fibres with higher MFA compared to the stem. Juvenile wood is characterised by higher MFA and more flexible fibres (Barnett and Bonham 2004; Burgert and Fratzl 2010). Early wood contains larger cells and thinner cell walls than late wood fibres, both are characterised by smaller MFA, with respect to juvenile wood, and stiffer fibres (Barnett and Bonham 2004). In their study, Burgert *et al.*(2002) observed that fibres with low MFA (0^o- 10^o from earlywood and latewood samples) displayed the highest tensile strength characteristics (Figure F1). Opposite wood and juvenile wood fibres (20^o-30^o MFA range) displayed midrange strength characteristics and compression wood fibres (50^o-60^o MFA range) displayed the greatest elasticity (Figure F1).

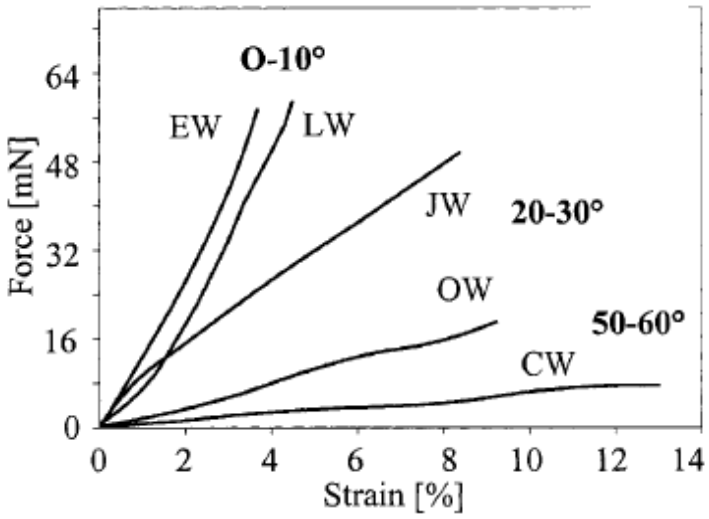


Figure F1, Load strain diagram of single *Picea abies* fibres (Burgert *et al.* 2002). Early wood (EW), Late wood (LW), Juvenile wood (JW), Opposite wood (OW) and Compression wood (CW), MFA range displayed.

The development of micro-tensile testing methods

Research into the stress-strain properties of single wood fibres and handsheets over the past 40 years has continued because of the importance of fibre properties in paper, board and other composite structures (Page *et al.* 1971). The methodologies for handling and

mounting of wood fibres for determining stress-strain curves has changed as more sophisticated microscopic techniques and analysis has become available. An outline of the development of handsheet tensile testing followed by single fibre methods is reviewed here.

The influence of MFA of hardwood fibres on handsheet properties has been investigated in *Eucalyptus nitens* and *E. globulus*. Six fibre samples were collected from three trees from the bottom, middle and upper region of the log and from the inner and outer regions (French *et al.* 2000). The average MFA was determined using confocal microscopy and ranged from 0 to 13^{o}. The average reduction in MFA between the inner and outer segments was approximately 5^{o} (French *et al.* 2000). In this study the small differences in fibre MFA (+/-5^{o}) was shown to have a negligible impact on handsheet tensile strength, and likewise, fibre length was shown to have only a slight impact on tensile strength (French *et al.* 2000).

In another study fibre samples from six *E. globulus* clones were assessed for fibre length, MFA and wood density and their direct effects on handsheet properties (Wimmer *et al.* 2002). Wimmer *et al.* (2002) found that longer fibres were linked to higher pulp yield, increased tear index and bending stiffness and pulp freeness (water drainage time during pulping). Smaller MFA was linked to increased tensile strength and bending stiffness while larger MFA was linked to increased light scattering coefficients (indicator of free surface area and fibre bonding) and opacity (brightness or reflectivity). Lower density increased handsheet strength and reduced optical properties, paper porosity (air flow) and tear index. Wimmer *et al.*(2002) also suggested that as inter-relationships exist between these three fibre traits (a negative relationship between MFA and fibre length, density and fibre length, and a positive relationship between MFA and density) that selective breeding for one of these traits without considering its effect on other fibre traits could have a negative impact.

The influence of MFA in individual softwood fibres on tensile properties and modulus of elasticity has been investigated in a range of species. In *Picea sitchensis* (Cowdrey and Preston 1966), *Pinus radiata* (Cave 1968), *Picea mariana* and *Picea glauca* (Page *et al.* 1977), as fibre MFA increased their modulus of elasticity decreased. The method these authors used involved gluing a single fibre to two glass tabs which were held together by a stiff piece of cardboard. The tabs were then mounted into the jaws of a tensile tester and the

cardboard was burnt away with a hot wire, leaving the fibre as the only link between the jaws.

In a subsequent study with *P. mariana* fibres, Page and El-Hosseiny (1983) showed that changes in MFA of individual fibres not only alter fibre tensile strength and modulus of elasticity but that these changes alter the entire shape of the stress (force) vs strain (sample displacement) curve (Figure F2), suggesting that fibres with different MFA deform differently. Fibres with low MFA produce steep almost linear curves before sample failure, whereas fibres with high MFA reach a yield point after which plastic deformation occurs. This stretching event can be as much as 20% before failure (Page and El-Hosseiny 1983).

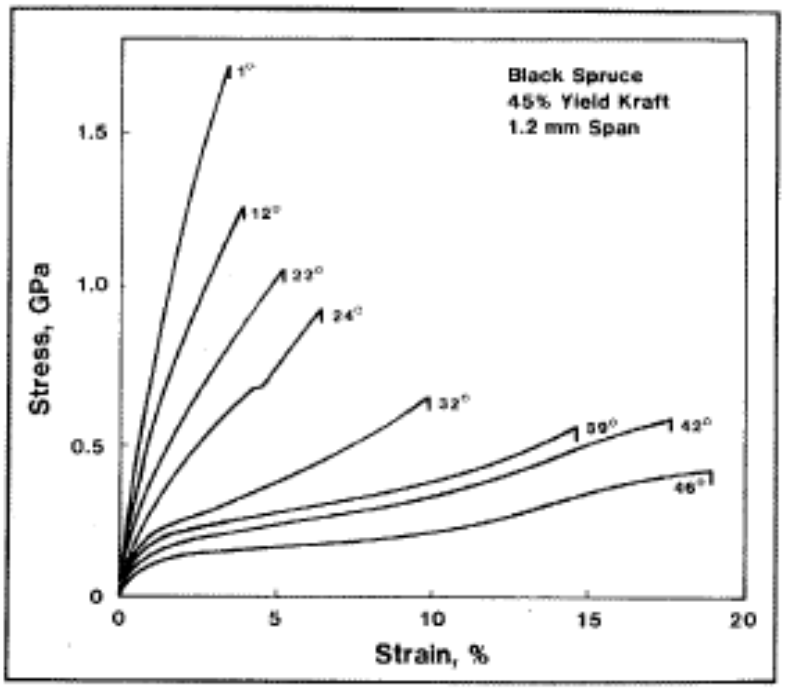


Figure F2, Single fibre *Picea mariana* stress strain curves (Page and El-Hosseiny 1983). Low MFA fibres show almost linear curves. High MFA fibres show a yield point followed by elastic deformation. MFA values of each fibre curve are labelled.

In another study changes in the molecular structure of the internal cell wall matrix of single *Picea abies* fibres during tensile testing has given insight onto the micromechanical role of these molecules (Gierlinger *et al.* 2006). In this study by Gierlinger *et al.* (2006) single fibres were isolated by pulling them apart with tweezers. Fibres then were air dried and glued into foliar frames which stabilised the fibres during the mounting process. Once the frames were mounted into the tensile testing machine a hot wire was used to burn the frame bridges

away. During testing, synchronised force and elongation data was recorded along with spectra acquired from confocal Raman microscopy. After tensile testing one broken part of the fibre was dipped into liquid polyethylene glycol which solidifies at room temperature. The fractured surface was then cut and the cross sectional area of the fibre measured using electron microscopy.

The main features in the observed Raman spectra arose from cellulose and lignin, whereas hemicelluloses were found to have a minor contribution (Gierlinger *et al.* 2006). The strongest band in the spectra was from lignin at 1602 cm-1 (see also Agarwal, 1999) and was attributed to the stretching of lignin. Another strong band was detected at 1097 cm-1 which is contributed by the C-C and C-O bond stretching of cellulose (Agarwal 1999). Along with increased stress and strain of the fibres during testing the 1097 cm-1 band (Grey rectangles) decreased to lower wave numbers, whereas the 1602 cm-1 band (Black circles) stayed at the same position (Figure F3). The absence of a shift in the lignin band has also been observed in cotton, flax and hemp fibres (Eichhorn *et al.* 2000; Eichhorn *et al.* 2001; Eichhorn and Young 2003). In all of these studies it was concluded that lignin acts as a low-modulus, non-load-bearing polymer within fibre cell walls. The shift in the cellulose band with increasing strain is attributed to the stretching of the load bearing cellulose at the C-O-C linkage of the glucose rings and the O(3)H ..O(5) intramolecular hydrogen bond, which has also been observed in flax and hemp fibres (Eichhorn *et al.* 2000).

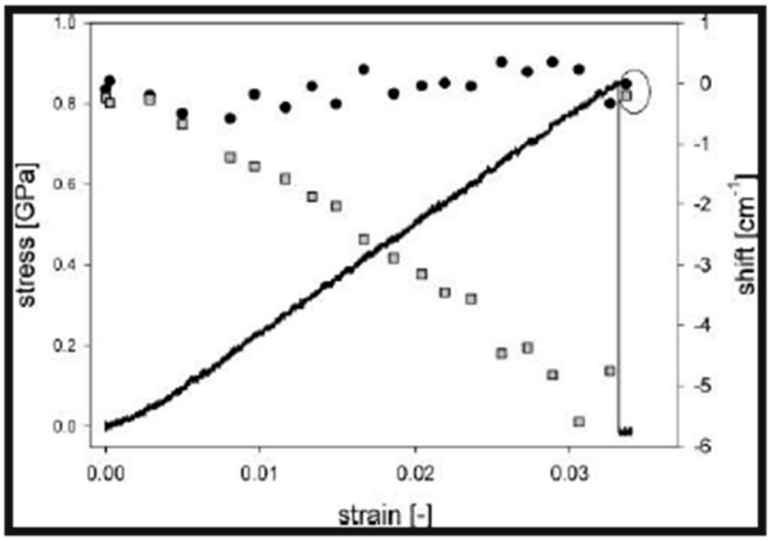


Figure F3, Single spruce fibre stress stain curve with Raman spectra (Gierlinger *et al.* 2006). The solid line is the stress strain curve of the fibre. Grey rectangles are cellulose spectra and black circles are lignin spectra.

The use of real time imagery (video extensometry) has been applied to wood fibre stress-strain testing (Burgert *et al.* 2003). Here Burgert *et al.* (2003) mechanically isolated single fibres from *Picea abies* mature wood using tweezers and the fibres were glued onto foliar frames. The length of the fibre available for testing was approximately the distance of the gap in the foliar frames (0.9mm). In addition, the cross sectional area of the fibres was also taken into account. Two black lines drawn on the frame close to the fibre ends allowed for easy visualisation of elongation while using video extensometry during testing. In Figure F4 an example of a stress-strain graph from the Burgert *et al.* (2003) study demonstrates how the simultaneous recorded load (force) and elongation (strain) of the fibre allows for a synchronised combination of the two curves as a function of time. Burgert *et al.*(2003) proposed that, although the addition of video extensometry based on the displacement of the frame line marks was an important development in the investigation of wood fibre mechanical behaviour, measuring the displacement of the fibre directly from the fibre would be better.

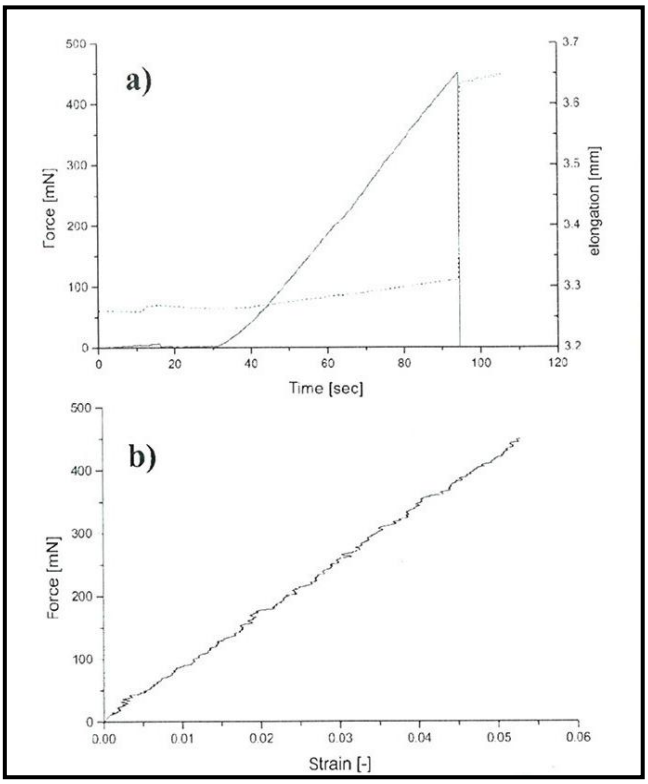


Figure F4, a) Load time and elongation of an individual fibre. Force is shown as a solid line and elongation as a dotted line. b) Synchronised combination of both curves as a function of time (Burgert *et al.* 2003).

In general, a microtensile tester consists of a sample holder, a load cell and a motor which stretches the sample at a given deformation rate. The measurements that come from this type of test are indicating force and displacement, and a diagram of a typical force displacement curve which could be expected for this study is shown below (Figure F5). Typically, a stress-strain curve (esp. metals) can be divided into two regions; the elastic region corresponding to reversible deformation and the plastic region corresponding to irreversible deformation. In the engineering field, materials and structures are very often designed to undergo relatively small deformations. This involves only the first portion of the stress-strain curve which is often linear in shape. This initial part of the stress-strain curve is termed the "elastic region" as the material returns to its initial shape after unloading. In 1676 Robert Hooke (1635 – 1703) made this observation from a series of tensile tests giving rise to the linear relationship between stress and strain:

In the equation ($\sigma = E * \varepsilon$) σ equals stress (force), E equals modulus of elasticity and ε equals strain (displacement).

Beyond the proportional limit of the stress-strain curve the material starts to deform plastically or irreversibly. If a material shows plastic deformation it does not return to its initial shape, even when the load is completely removed. Within the plastic region the slope changes and the material will eventually reach a maximum stress. This maximum stress is used to define the strength of the material where the highest stress is the greatest force tolerable by the wood fibre for example.

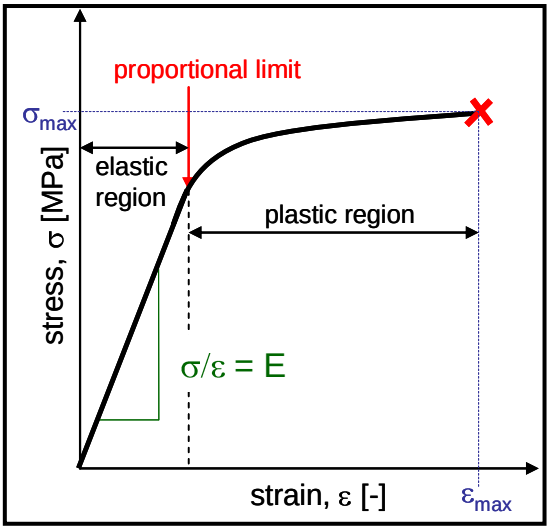


Figure F5, Stress-strain diagram, showing linear elastic and plastic behaviour and the proportional limit of wood fibre behaviour during tensile testing. Stress (Force), Strain (Displacement). The tangent of the line gives the modulus of elasticity of the fibre.

Materials and Methods

Sample selection

Transgenic wood fibres with differing average MFA values were selected for tensile testing. Sectors from the following vector transformations were selected, control 1305.1GWGUS+ fibres with unaltered MFA values, 1305.1GWTUB1 average fibre MFA of plus 3^{o}, 1305.1GWmirTUB1- average fibre MFA of plus 2^{o}, 1305.1GWTUA1+ average fibre MFA of plus 4^{o} and 1305.1GWTUA1/TUB1+ average fibre MFA of minus 2^{o}. Sectors were selected according to size, position and surrounding tissue. Preference was given to the largest sectors in order to make downstream analysis easier. Sectors were sampled a minimum of 3 mm from the edge of the stem segments, in order to provide flat untransformed surrounding tissue so that they could be cut into 3 mm long fibre bundles.

Sample preparation

A sledge microtone was used to section the samples at a thickness of 40 µm. Samples were kept moist while being sectioned and were stored in distilled water so that the fibres did not become dehydrated. For each vector treatment ten to twenty samples were sectioned in the first round of experiments, and an additional eight samples for vector 1305.1GWTUA1/TUB1+ were analysed in round two. A micro laser was used to cut out small

bundles of fibre approximately 200 µm wide by 40 µm thick by 3000 µm long for samples analysed in round one. Sample widths of less than 100 µm were used in round two. Rectangle cutting diagrams for the laser were prepared leaving room for the radius of the laser beam. The rectangle was also aligned with the plane of the fibres. Fine tweezers were then used to collect the samples. Two fibre bundles were cut out for each sample containing both transformed and untransformed fibre bundles. Control fibre bundles were sampled from within a few millimetres of the transformed bundle in round one, while only transformed fibre bundles were used in round two (Figure G6). The fibre bundles were placed in a 1% glycerol solution overnight at 4^{o}C to keep them in their hydrated condition.

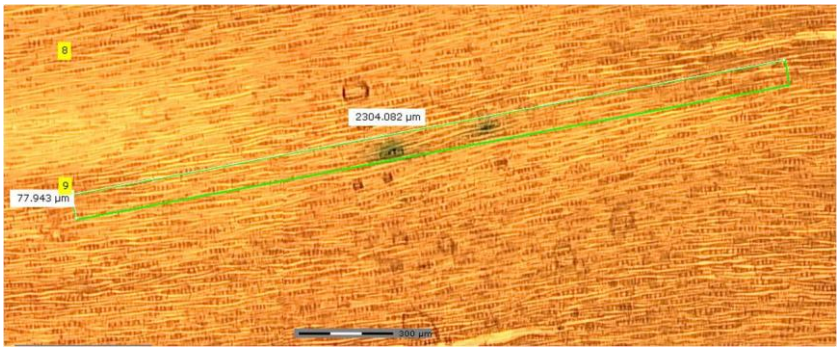
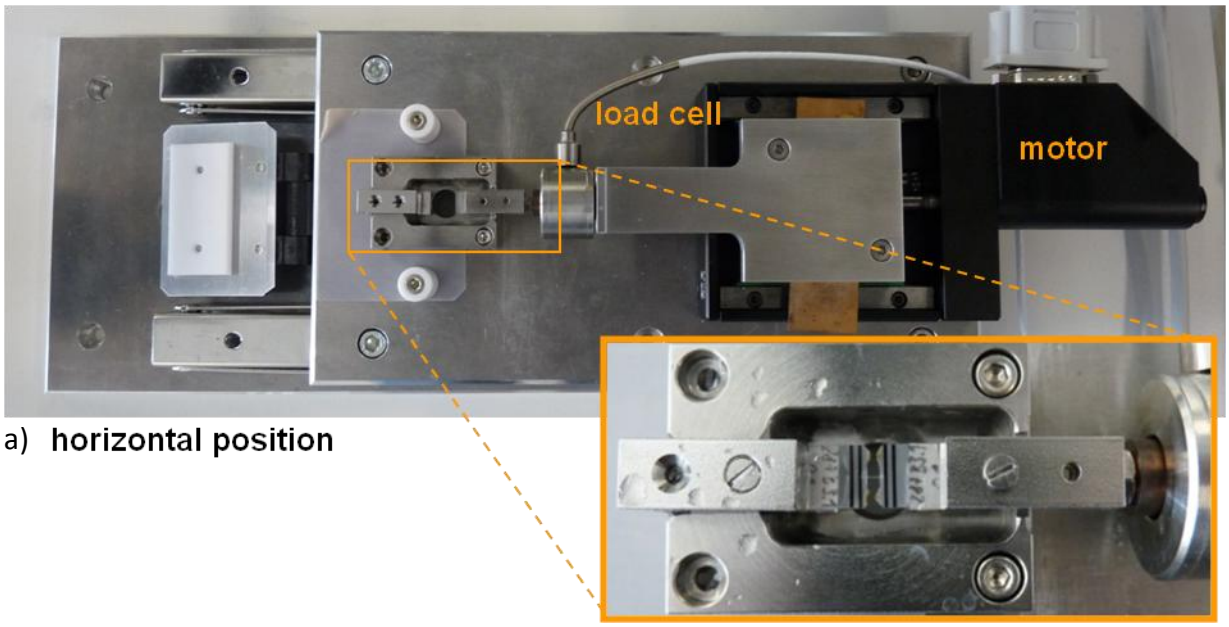


Figure F6, Outline of fibre bundle showing where the microlaser is about to cut (Green rectangle).

After sectioning fibre bundles were glued onto a polyester frame using cyanacrylate glue (Loctite 454). After curing of the glue the foliar frame carrying the sample was glued onto the clamps of a tensile tester which was operated in its horizontal position (Figure F7, a). The frames contained support bridges which were cut by a hot wire. The tensile tester was tilted by 90 °, and a water filled cuvette (optical glass) was used to prevent the sample from drying and to ensure stable optical conditions (Figure F7, b). During testing the samples were strained at a constant speed (1 µm/s in round one and 2 µm/s in round two), and real time images of the sample were captured with a microscope camera (Video extensometry). Simultaneously, the force was recorded by a highly sensitive load cell with a maximum capacity of 500 mN (Honeywell Sensotec, Model 31E). Subsequent strain analysis was performed using the LabView^{TM} program. Video extensometry of the fibre sample was developed in order to overcome the inherent variability associated with strain measurements. Real time imagery linked to the movement of a marked line on the frame, and not directly on the sample, was performed as described by Burgert *et al.* (2003).



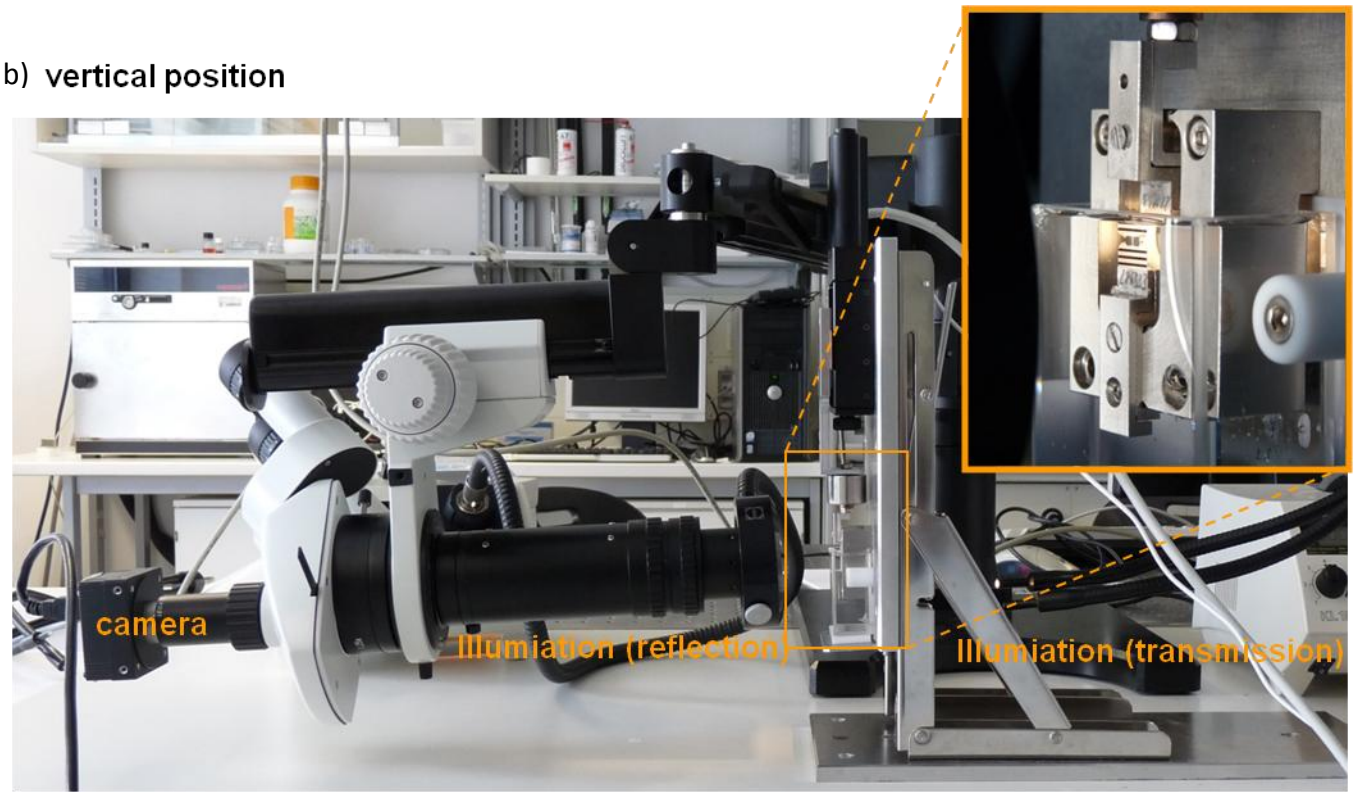


Figure F7, Microtensile apparatus set up. a) Hrizontal view showing a sample that has been glued onto the clamps, with a close up showing fibre bundles spanning the gap. b) In tensile testing mode (vertical view), showing camera set up and close up of the sample in the water filled cuvette.

Strain analysis

The strain analysis was performed on the series of captured images, in a method similar to the procedure described in Benecke *et al.* (2009) on bone samples. Multiple local strains along the sample were calculated for the whole sample length (red line) and internal regions of transformed (blue line) and untransformed tissue (1-2 control regions, green line) (Figure F8). For each of these regions stress (MPa) versus strain (-) graphs were calculated.

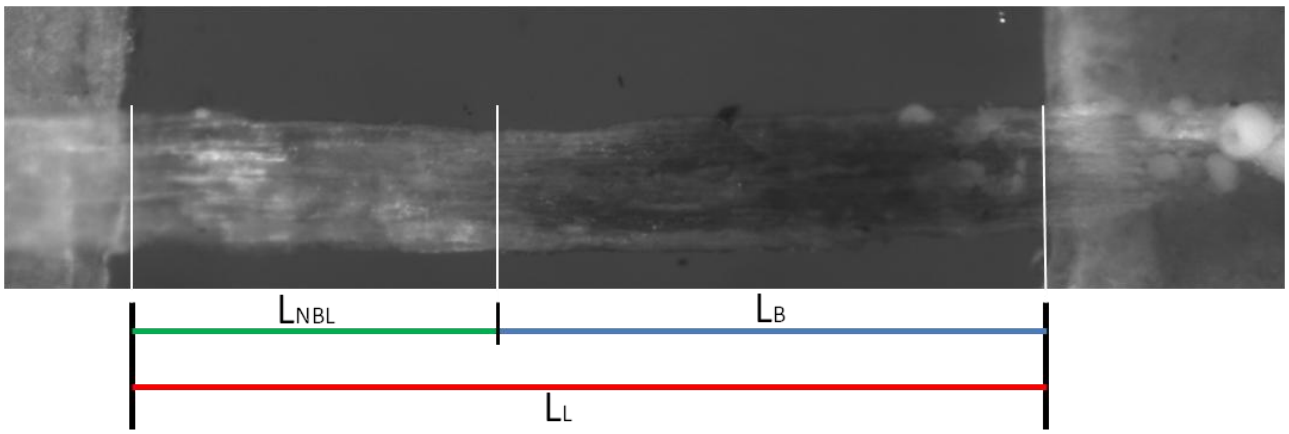


Figure F8, Example of regions selected for local tensile strain analysis. L_L = whole sample length, L_{NBL} = non-blue region left-hand side and/or L_{NBR} = non-blue region right-hand side (not shown), L_B = length of blue region.

Results

Sample section for analysis: Round one

The tensile tests carried out in round one were performed jointly with Dr. Michaela Eder using a microtensile tester at the Max Planck Institute of Colloids and Interfaces, Golm, Germany. For each transgene treatment a minimum of ten paired fibre bundles were prepared. At the gluing stage fibre bundles with irregular form, either damaged before or after gluing, or non-parallel placement within the frame were discarded. For paired bundles where only the control fibre bundle was discarded, testing of the transformed bundles was still performed, as this fibre bundle could still be analysed for internal changes in local strain. After initial total force and whole sample tensile analysis on a number of samples was completed it was decided that due to the high variability in force between the control and transformed fibre bundles it would be better to analyse local tensile strain within the

transformed fibre bundles themselves, using one or two non-transformed regions (non-blue regions) of tissue within the bundle as a control.

After tensile testing, analysis of the shape of the stress strain-curve and the video of the sample during testing were used to reject samples that showed deformations which would have negatively impacted their tensile strength measurement. This may have been due to uneven sample diameter, premature failure (glue gave way before sample), non-parallel alignment or other damage. These rigorous selection criteria meant that more than half of the fibre bundles prepared were not included in the final analysis (Table F1). Local tensile stress and strain graphs were produced for all samples from round one (data not presented). At this point a number of issues arising from sample preparation and testing were noticed including variability in the data due to the variable width of sample (from 100 µm to 1200 µm) and the surface area along the fibre bundles. In addition, the force values were not in the range expected for wood fibres.

Table F1, Number of fibre bundles tensile tested and analysed per transgene treatment for round one.

Vector Treatment	No pairs of fibre bundles made	No fibre bundles used for local tensile strain analysis
1305.1GWGUS+	**16**	**7**
1305.1GWTUB1+	**15**	**6**
1305.1GWmiTUB1-	**11**	**5**
1305.1GWTUA1+	**12**	**6**
1305.1GWTUA1/TUB1+	**12**	**7**

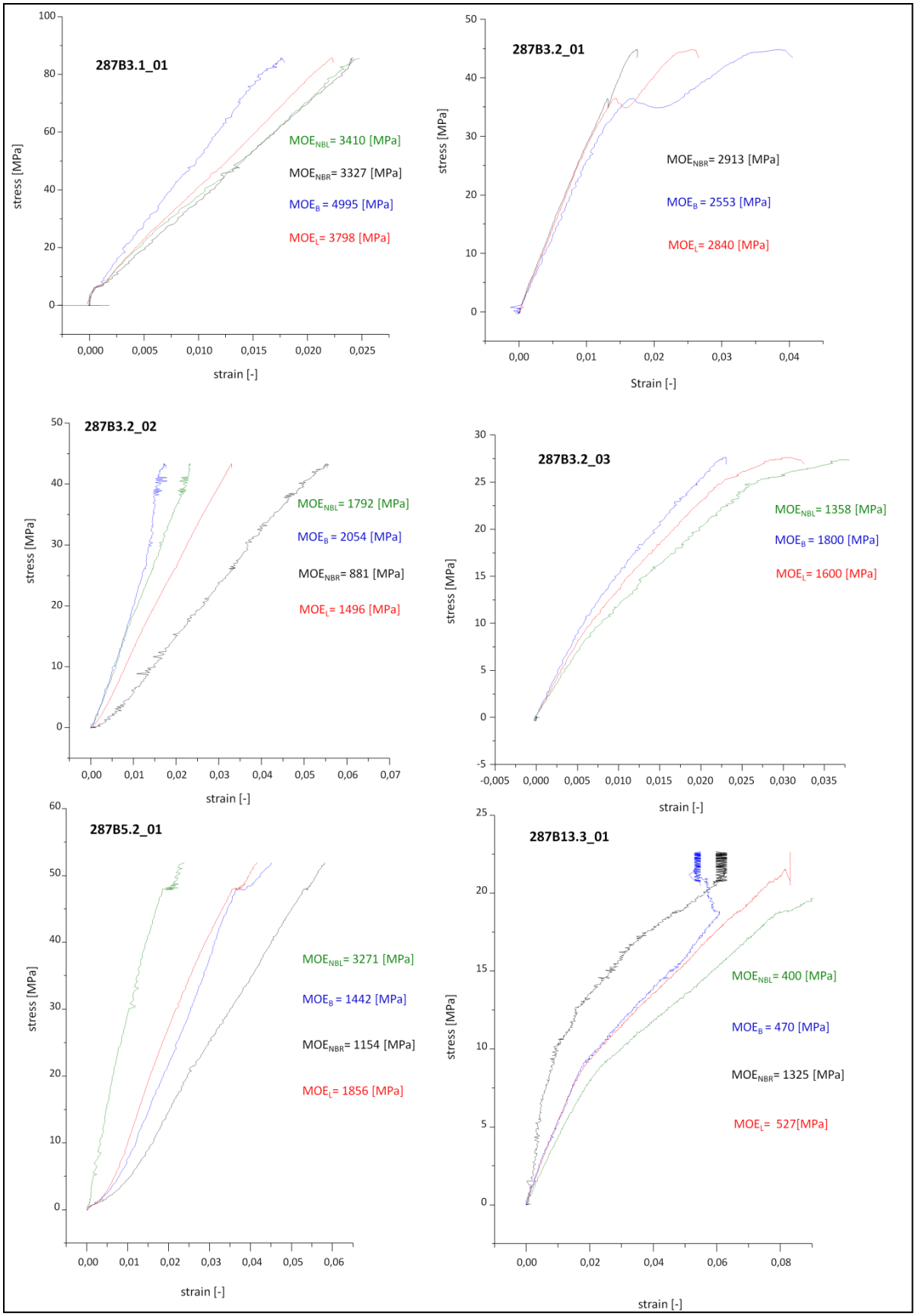
Tensile Tests for Alpha and Beta-tubulin1+: Round two

A second round of samples (eight) was assessed for the impact of the 1305.1GWTUA1/TUB1+ vector treatment on fibre tensile properties. The micro laser sectioning of these samples was completed by Dr. Markus Rueggeberg and the microtensile testing was performed by Dr. Michaela Eder at the MPI. All sample widths (micro laser cutting width) were below 100 µm, and the stiffness values were in the expected range for wood fibres (1500 - 3000 MPa) Michaela Eder *pers com.* (2010). The stiffness values (modulus of elasticity) in Table F2 were calculated on the assumption that the sample thickness was constant, however, this is unlikely to be true. At present, three of the eight samples show a trend towards the transgenic fibres being stronger, however two appear to

be weaker, and the other three show no difference, as the transgenic fibres are stronger than one of the control sample regions and weaker than the other. Accurate cross sectional surface area determination is therefore needed before any real inferences can be made regarding this data. The modulus of elasticity was calculated as described above. The stress-strain graphs for these samples from round two are presented in Figure F9, and show the same trends in sample strength and the sample stiffness calculations.

Table F2, Analysis of TUA1/TUB1+ wood fibre bundle stiffness (modulus of elasticity). The left-hand side control region was not analysed for sample 287B3.2 01 as there were no untransformed wood fibres to the left of the transgenic region.

Sample ID	Sample width (um)	Stiffness whole length (MPa)	Stiffness transformed region (Mpa)	Stiffness untransformed region(s) (Mpa)
287B3.1_02	75	1653	1847	1282, 1872
287B3.3_02	81	1560	1085	1019, 1879
287B3.2_02	79	1902	1812	1511, 813
287B3.1_01	72	3817	4923	2586, 3764
287B5.2_01	78	1869	2349	2436, 2242
287B3.2_03	80	1398	1969	1401, 1339
287B3.2_01	79	2862	2482	3190
287B5.1_03	80	3874	2091	5397, 5480


287B3.1_01
MOE$_{NBL}$= 3410 [MPa]
MOE$_{NBR}$= 3327 [MPa]
MOE$_{B}$ = 4995 [MPa]
MOE$_{L}$= 3798 [MPa]
stress [MPa]
strain [-]
287B3.2_01
MOE$_{NBR}$= 2913 [MPa]
MOE$_{B}$ = 2553 [MPa]
MOE$_{L}$= 2840 [MPa]
stress [MPa]
Strain [-]
287B3.2_02
MOE$_{NBL}$= 1792 [MPa]
MOE$_{B}$ = 2054 [MPa]
MOE$_{NBR}$= 881 [MPa]
MOE$_{L}$= 1496 [MPa]
stress [MPa]
strain [-]
287B3.2_03
MOE$_{NBL}$= 1358 [MPa]
MOE$_{B}$ = 1800 [MPa]
MOE$_{L}$= 1600 [MPa]
stress [MPa]
strain [-]
287B5.2_01
MOE$_{NBL}$= 3271 [MPa]
MOE$_{B}$ = 1442 [MPa]
MOE$_{NBR}$= 1154 [MPa]
MOE$_{L}$= 1856 [MPa]
stress [MPa]
strain [-]
287B13.3_01
MOE$_{NBL}$= 400 [MPa]
MOE$_{B}$ = 470 [MPa]
MOE$_{NBR}$= 1325 [MPa]
MOE$_{L}$= 527[MPa]
stress [MPa]
strain [-]

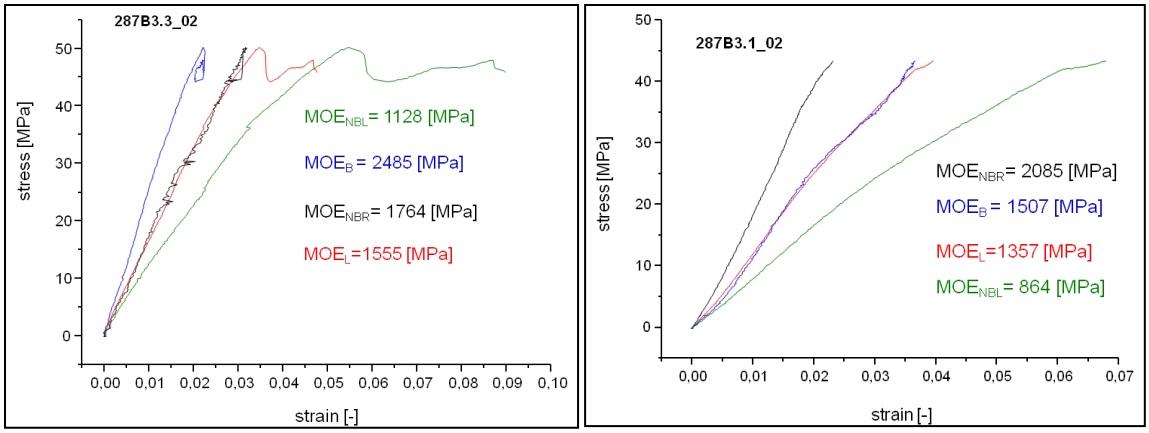


Figure F9, Stress-strain analysis of 1305.1GWTUA1/TUB1+ fibre bundles. Modulus of elasticity (MOE), MOE_L whole sample length (red line), MOE_{NBL} non-blue region left-hand side (green line), MOE_{NBR} non-blue region right-hand side (black line), MOE_B Blue region (blue line).

Discussion & Future directions

Method development

In this study tensile testing of individual wood fibres was not practical as eucalypt wood fibres are smaller (~1 mm length) than the *Picea abies* fibres (3-5 mm) used in other tensile fibre strength studies (Burgert *et al.* 2002; Burgert *et al.* 2003). Instead, it was decided to measure the strength of small bundles of eucalypt wood fibres. A potential advantage of using fibre bundles is that while individual fibre properties have been related to paper and board properties the behaviour of free fibres have been suggested in some cases to be different from the behaviour of tightly bounded fibres (Page *et al.* 1971; French *et al.* 2000).

Here optimisation of the Burgert *et al.*(2003) method for eucalypt fibres was undertaken and substantial progress made. Genetically modified wood fibres were assessed and the potential to detect changes in strength compared to untransformed fibres was demonstrated. Furthermore, the use of local tensile measurement within the fibre bundles was applied using a method adapted from Benecke *et al.* (2009).

A disadvantage of using wood fibre bundles compared to individual wood fibres is the highly variable nature of the cross sectional surface area of the fibre bundles. Determining the cross sectional area is an important step required for calculating the modulus of elasticity. An initial attempt was made to use a high resolution µCT machine to determine accurate

cross sectional area along the whole length of the samples, however, difficulties were encountered with the low resolution of the instrument and with the sample moving slightly throughout the measurement procedure. Development of a device to hold the samples will be needed before a second attempt could be undertaken. An alternative method would be to cut the samples into several slices and use a SEM-microscope to determine cross sectional areas. Due to the high variability of the results from round one this has not yet been undertaken. Furthermore, this method is highly time consuming and less accurate than the µCT machine. The more promising round two results were completed in the final few weeks of thesis preparation and cross sectional area measurements will still be undertaken at the MPI in the coming months.

For round two, sample widths were kept below 100 µm because from past experience (Michaela Eder *pers com.* 2010), when the cross sectional area of a sample becomes too large with respect to the test span, the values recorded are believed to represent sample shear more than tensile strength. This is believed to be one of the reasons that the tensile strength values from round one were aberrant.

Fibre bundle crack propagation

Wood is much stronger in its longitudinal direction than in the transverse plane. Hence, when a wood sample is placed under tensile strain it first undergoes longitudinal elastic deformation before inelastic failure (Klaus *et al.* 2003). Theoretically, when a wood sample fails under tensile force the crack should propagate transversely, tearing the fibres perpendicularly, however, it is easier for a crack to propagate along fibres than tearing them transversely (Klaus *et al.* 2003). A perfectly transverse crack is therefore theoretical, however, Klaus *et al.* (2003) showed that maintaining the moisture content of the sample during tensile testing improves the likelihood of a transverse crack, as the sample does not undergo any distortion dimensionally due to dehydration once mounted into the chamber. Changes in humidity have also been shown to affect the tensile and bending strength of kraft linerboard, copy paper and single *Pinus radiata* wood fibres (Denis and Parker 1994). This is why a water filled cuvette was used as part of this project methodology.

Future perspectives

From the promising results obtained from round two, it can be said that an effective methodology for assessing changes in wood fibre properties of angiosperm species has been developed through collaboration between the Max Plank Institute and the University of Melbourne as part of this PhD project. In order to complete this study the final cross sectional surface area measurements will be undertaken in the following month. A more extensive study with increased sample size and a range of transgene treatments (including GUS reporter gene as a control) should be undertaken in the near future.

Appendix H

Bio-Edit alignment of *E. grandis* beta tubulin proteins, showing amino acid comparisons; no SNPs were observed to be specific to all three proteins shown in the project to be involved in MFA orientation (EgrTUB1, EgrTUB5 and EgrTUB6).

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
EgrTUB1         ---------- ---------- ---------- ----XPFLKD SHL-A*FILL
EgrTUB6         ---------- ---------- ---------- ---------- ----------
EgrTUB5         ---------- ------XSSS RNPDRASLPV ARSPSPRSRS GIX--XRR*T
EgrTUB2         ---------- ---------- ------XLSL SRFESHFSNP LRX--XIVRA
EgrTUB3         ---------- ---------- ---------- ---------- ----------
EgrTUB4         --XIIFPSIF PALSRENSSQ KNRHPPPPPP PPPPQPPPES ESARETQRLA


                ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
EgrTUB1         KMREILHVQA GQCGNQIGGK FWEVVCEEHG IDAAGNYTGD --SHVQLERV
EgrTUB6         ---------- ---------- ---------- ---------- ----------
EgrTUB5         KMREILHVQG GQCGNQIGSK FWEVVCDEHG IDPTGRYTGT --SDLQLERV
EgrTUB2         KMREILHIQG GQCGNQIGAK FWEVVCAEHG IDATGRYHGD --SELQLERV
EgrTUB3         ----ILHIQG GQCGNQIGSK FWEVICDEHG IDPTGKFTGD ASSDLQLERI
EgrTUB4         KMREILHIQG GQCGNQIGAK FWEVICDEHG IDATGKYNGD --SELQLERI
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
EgrTUB1         NVYYNEASGG RYVPRAVLMD LEPGTMDSLR TGPYGRIFRP DNFVFGQNGA
EgrTUB6         ---------- ---------- ---------- ---------- ----------
EgrTUB5         NVYYNEASCG RFVPRAVLMD LEPGTMDSVR TGPYGQIFRP DNFVFGQSGA
EgrTUB2         NVYYNEASCG RFVPRAVLMD LEPGTMDSVR SGPYGQIFRP DNFVFGQSGA
EgrTUB3         NVYYNEASGG RYVPRAVLMD LEPGTMDSIR SGPFGQIFRP DNFVFGQSGA
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EgrTUB4      NVYYNEASGG RYVPRAVLMD LEPGTMDSVR SGPFGQIFRP DNFVFGQSGA

             ....|....| ....|....| ....|....| ....|....| ....|....|

                    160        170        180        190        200

EgrTUB1      GNNWAKGHYT EGAELIDSVL DVVRKEAENC DCLQGFQICH SLGGGTGSGM

EgrTUB6      ---------- ---------- --VRKEAENC DCLQGFQVCH SLGGGTGSGM

EgrTUB5      GNNWAKGHYT EGAELIDAVL DVVRKEAENC DCLQGFQICH SLGGGTGSGM

EgrTUB2      GNNWAKGHYT EGAELIDSVL DVVRKEAENC DCLQGFQVCH SLGGGTGSGM

EgrTUB3      GNNWAKGHYT EGAELIDAVL DVVRKEAENC DCLQGFQVCH SLGGGTGSGM

EgrTUB4      GNNWAKGHYT EGAELIDAVL DVVRKEAENC DCLQGFQVCH SLGGGTGSGM


             ....|....| ....|....| ....|....| ....|....| ....|....|

                    210        220        230        240        250

EgrTUB1      GTLLISKIRE EFPDRMMLTF SVFPSPKVSD TVVEPYNATL SVHQLVENAD

EgrTUB6      GTLLISKIRE EYPDRMLMTF SVFPSPKVSD TVVEPYNATL SVHQLVENAD

EgrTUB5      GTLLISKIRE EYPDRMMLTF SVFPSPKVSD TVVEPYNATL SVHQLVENAD

EgrTUB2      GTLLISKIRE EYPDRMMLTF SVFPSPKVSD TVVEPYNATL SVHQLVENAD

EgrTUB3      GTLLISKIRE EYPDRMMLTF SVFPSPKVSD TVVEPYNATL SVHQLVENAD

EgrTUB4      GTLLISKIRE EYPDRMMLTF SVFPSPKVSD TVVEPYNATL SVHQLVENAD


             ....|....| ....|....| ....|....| ....|....| ....|....|

                    260        270        280        290        300

EgrTUB1      ECMVLDNEAL YDICFRTLKL INPSFGDLNH LISTTMSGVT CCLRFPGQLN

EgrTUB6      ECMVLDNEAL YDICFRTLKL TTPSFGDLNH LISATMSGVT CCLRFPGQLN

EgrTUB5      ECMVLDNEAL YDICFRTLKL TTPSFGDLNH LISATMSGVT CCLRFPGQLN

EgrTUB2      ECMVLDNEAL YDICFRTLKL TTPSFGDLNH LISATMSGVT CCLRFPGQLN

EgrTUB3      ECMVLDNEAL YDICFRTLKL STPSFGDLNH LISATMSGVT CCLRFPGQLN

EgrTUB4      ECMVLDNEAL YDICFRTLKL ATPTFGDLNH LISATMSGVT CCLRFPGQLN
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350
EgrTUB1         SDLRKLAVNL IPFPRLHFFM VGFAPLTSRG SQQYRSLTIP ELTQQMWDAK
EgrTUB6         SDLRKLAVNL IPFPRLHFFM VGFSPLTSRG SQQYRALTVP ELTQQMWDSK
EgrTUB5         SDLRKLAVNL IPFPRLHFFM VGFAPLTSRG SQQYRALTVP ELTQQMWDAK
EgrTUB2         SDLRKLAVNL IPFPRLHFFM VGFAPLTSRG SQQYRALTVP ELTQQMWDSK
EgrTUB3         SDLRKLAVNL IPFPRLHFFM VGFAPLTSRG SQQYISLTVP ELTQQMWDSK
EgrTUB4         SDLRKLAVNL IPFPRLHFFM VGFAPLTSRG SQQYRALTVP ELTQQMWDAK
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        360        370        380        390        400
EgrTUB1         NMMCAADPRH GRYLTASAMF RGKMSTKEVD EQMLNVQNKN SSYFVEWIPN
EgrTUB6         NMMCAADPRH GRYLTASAMF RGKMSTKEVD EQMINVQNKN SSYFVEWIPN
EgrTUB5         NMMCAADPRH GRYLTASAMF RGKMSTKEVD EQMINVQNKN SSYFVEWIPN
EgrTUB2         NMMCAADPRH GRYLTASAMF RGKMSTKEVD EQMLNVQNKN SSYFVEWIPN
EgrTUB3         NMMCAADPRH GRYLTASAMF RGKMSTKEVD EQMINVQNKN SSYFVEWIPN
EgrTUB4         NMMCAADPRH GRYLTASAMF RGKMSTKEVD EQMINVQNKN SSYFVEWIPN


                ....|....| ....|....| ....|....| ....|....| ....|....|
                        410        420        430        440        450
EgrTUB1         NVKSSVCDIP PTX-AAHV-V DLHGX----- NSTSIQEMFR RVSEQFTVMF
EgrTUB6         NVKSSVCDIP PKX-AFHG-F HIHRX----- NSTSIQEMFR RVSEQFTAMF
EgrTUB5         NVKSSVCDIP PRX-TLHG-I DFHWX----- NSTSIQEMFR RVSEQFTAMF
EgrTUB2         NVKSTVCDIP PTX-P*DG-V DIHRX----- NSTSIQEMFR RVSEQFTAMF
EgrTUB3         NVKSSVCDIP PKX-PEDG-V HVCGX----- NSTSIQEMFR RVSEQFTAMF
EgrTUB4         NVKSSVCDIP PKX-FEDG-I DLHRX----- NSTSIQEMFR RVSEQFTAMF
```

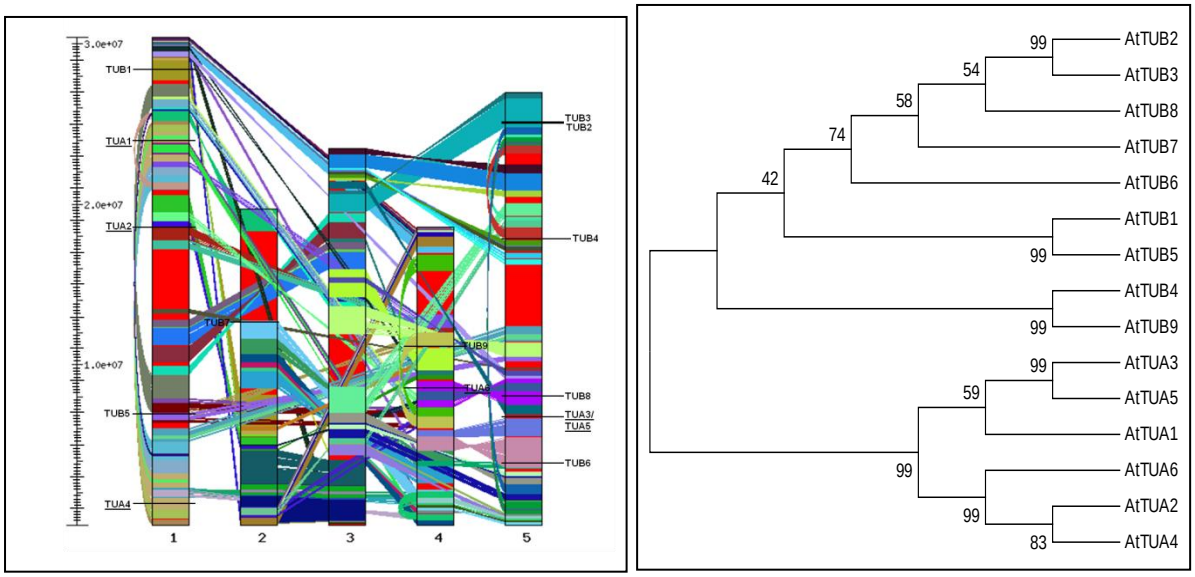
```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        460        470        480        490        500
EgrTUB1         RRKAFLHWYT GEGMDEMEFT EAESNMNDLV SEYQQYQDAV ADEVEEYDED
EgrTUB6         X--------- ---------- ---------- ---------- ----------
EgrTUB5         RRKAFLHWYT GEGMDEMEFT EAESNMNDLV SEYQQYQDAT ADEELEYEDE
EgrTUB2         RRKAFLHWYT GEGMDEMEFT EAESNMNDLV SEYQQYQDAT ADDE-EYEEE
EgrTUB3         RRKAFLHWYT GEGMDEMEFT EAESNMNDLV AEYQQYQDAT ADDEAEYEEG
EgrTUB4         RRKAFLHWYT GEGMDEMEFT EAESNMNDLV AEYQQYQDAT ADDEEEYEED
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        510        520        530        540        550
EgrTUB1         EVAEN*LNRG N*SMVTPFFW LFM*VLWGFF VCPSYGLHDC SYV*YAFSKX
EgrTUB6         ---------- ---------- ---------- ---------- ----------
EgrTUB5         EDAEQDM*MC YREG*YTFX- LQQXLWWLMS KXLXSGEGV* RXXPFFXFXX
EgrTUB2         EGDYQEEEM* **GTSDWLEK LLSXL*QPFF LXLNFQLLVC RXX*S*LMXX
EgrTUB3         EEAYED*CFR SSSIISTCIE FRTWMYFLYL LLLLMSVFV* ILLPCCFPRL
EgrTUB4         EEEDLVEVHA *GVVFSVLX- YLSXLL*TEF MXMXVKLSFR LXXSHLXIXX
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        560        570        580        590        600
EgrTUB1         RISPMRVARY GAGGPKKKKK KKKX------ ---------- ----------
EgrTUB6         ---------- ---------- ---------- ---------- ----------
EgrTUB5         *SQYLDMEKL LLXXTKK-XK KKX------- ---------- ----------
EgrTUB2         GRKLSTIF*L FL*PAIMLDK SLGIPIIFYK LILHSVRLKK KKKK------
EgrTUB3         WSSWLIVLRF *LSLAFQTRK AANCSCIFTC RRVKPCTR*F SYSQCDV*VM
EgrTUB4         VHYCFAIR*L DLXXAVV-XI IMVVPIK*MK VLLEAKKKKK X---------
```

```
                ....|....| ....|....| ....|
                        610        620
EgrTUB1         ---------- ---------- -----
EgrTUB6         ---------- ---------- -----
EgrTUB5         ---------- ---------- -----
EgrTUB2         ---------- ---------- -----
EgrTUB3         KKKKKKX--- ---------- -----
EgrTUB4         ---------- ---------- -----
```

Appendix I

A comparison of *Arabidopsis* chromosomal structure with tubulin gene position and tubulin protein phylogeny (Figure I1). The phylogenetic structure observed does not entirely complement the chromosomal structure.

Figure I1, Chromosomal structure and phylogeny. The phylogenetic tree contains both arabidopsis alpha and beta tubulin genes and is a minimum-evolution tree with interior branch test.



TAIR http://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp