The role of tubulins in secondary cell wall deposition in woody tree species

Larissa Machado Tobias

ORCID: https://orcid.org/0000-0002-5197-7589

Master of Philosophy

April 2020

School of Ecosystem and Forest Sciences

Faculty of Science

The University of Melbourne

Submitted in total fulfilment of the requirements of the degree of Master of Philosophy

Abstract

Woody trees are an essential source of timber, pulp, paper and biofuel, and advances in biotechnology provide opportunities for the improvement of traits of interest for specific end uses. Cellulose microfibrils, the basic structural component of plant cell walls, are responsible to a large degree for wood mechanical and physiological properties. The angle between the direction of the helical windings of cellulose microfibrils in plant secondary cell walls, or microfibril angle (MFA), plays critical roles in a tree's development and has become a subject of major interest in forest biotechnology, particularly in detailed studies of the secondary cell wall of xylary (wood) cells. While our knowledge of how exactly the cellulose synthase complex (CSC) acts in response to environmental and genetic cues remains sketchy, guidance of cellulose deposition has been repeatedly accredited to microtubules, a cytoskeleton component formed of protein dimers of α- and β-tubulin. Nevertheless, few studies explore the cytoskeleton roles in secondary cell wall deposition in woody tree species. Reaction wood (RW) develops in response to gravitational stimulus through a series of changes at the cellular and molecular levels. Tubulin genes have been previously reported to be upregulated during RW formation and differences in their expression might lead to differences in microtubule assembly. This differential microtubule organisation might be related to changes on cell wall morphology, including MFA. In this study, cortical microtubule array organisation was therefore assessed in samples from trees forming RW and stems growing upright (normal wood). To further investigate if perturbation of microtubule organisation would impact wood formation, microtubule-interacting drugs were applied to wood tissue depositing SCW *in vivo* and *in vitro*. Together, results indicate that tubulins play an essential role in cellulose deposition in the secondary cell wall of woody tree species to ensure appropriate microfibril orientation.

Declaration

This is to certify that:

I. This thesis comprises only my original work

II. Due acknowledgement has been made in the text to all other material used

III. This thesis is less than 50,000 words in length, exclusive tables, figures, appendices and bibliographies.

Larissa Machado Tobias

The University of Melbourne

April 2020

Preface

The publication status of all chapters presented in article format within this work are as follows.

Chapter 1 was published by *Plants* (ISSN 2223-7747; CODEN: PLANCD) on 10 January 2020 as:

Tobias, L.M.; Spokevicius, A.V.; McFarlane, H.E.; Bossinger, G. The Cytoskeleton and Its Role in Determining Cellulose Microfibril Angle in Secondary Cell Walls of Woody Tree Species. *Plants* **2020**, *9*, 90.

Chapters 2 and 3 comprehend unpublished material not submitted for publication at the time of thesis submission.

Acknowledgements

This research was funded by a Melbourne Research Scholarship provided by the University of Melbourne. The project also received support from the Madeleine Selwyn-Smith Memorial Fund Scholarship (MSSM).

The Melbourne Advanced Microscopy Facility was essential in providing all necessary training and research support in confocal microscopy and scanning electron microscopy. In particular I would like to thank Dr. Allison van De Meene, Dr. Gabriela Segal and Mr. Roger Curtain.

I am mostly thankful to my supervisors Prof. Gerd Bossinger, Dr. Antanas Spokevicius and Dr. Heather McFarlane for their dedication, guidance and encouragement at all times during the past 2 years. To Antanas, thank you for your thought-provoking questions and your enthusiasm in solving the puzzle. Heather, thank you for always making time to discuss this project and other matters; and, Gerd, thank you for your supervision, your love for science and for always reminding me to breathe. Without you three this project could never have been completed and my research skills would never have developed as far.

Thanks to Nadeeshani Karannagoda, Motjaba Khorrami Raad, Sachinthani Karunarathne and other members of the Forest Molecular Biology and Genetics group for your support and friendship. You have been true friends both in and out of the lab and I could not have expected better lab mates, you surely made the entire process of moving to a different country and developing a research project a lot easier and fun. For that I will always be thankful.

I would like to thank all my family members and friends in Brazil for their encouragement and support. A very special mention to my parents, Jefferson Tobias and Clarice Machado Tobias, who have always believed in me and supported me throughout my research journey and life. I dedicate this thesis to you.

Last but not least, to my partner Matthew Girdler, thank you so much! You were always there to encourage and love me through the ups and downs of this research project. You have never stopped believing in me and it means a lot. Thank you.

Table of contents

List of tables

List of figures

Thesis Outline

This thesis is composed of a theoretical and an experimental part. Chapter 1 is theoretical in nature and combines the thesis introduction and a literature review. It summarises the current understanding of cytoskeleton roles in guiding secondary cell wall deposition in xylary cells and puts forward a model of cellulose microfibril orientation in woody trees in response to gravitational stimulus. Chapters 2 and 3 comprise the experimental part of the present thesis and were written in article format aiming for future publication, therefore overlap across introductory sections to some degree is expected. In chapter 2, immunolabelling techniques were employed to visualise cortical microtubules and investigate whether changes in array organisation are correlated to reaction wood formation in trees subjected to gravitational stimulus. Chapter 3 investigates the use of microtubule-interacting drugs in both *in vivo* and *in vitro* system to assess their effect on wood formation and cellulose microfibril orientation in secondary cell walls of wood cells. Finally, key findings of each chapter are put in perspective and are discussed together in Chapter 4.

Chapter 1: The cytoskeleton and its roles in determining cellulose microfibril angle in secondary cell walls of woody species

Abstract

Recent advances in our understanding of the molecular control of secondary cell wall (SCW) formation have shed light on molecular mechanisms that underpin domestication traits related to wood formation. One such trait is the cellulose microfibril angle (MFA), an important wood quality determinant that varies along tree developmental phases and in response to gravitational stimulus. The cytoskeleton is mainly composed of microtubules and actin filaments, which collectively contribute to plant growth and development by participating in several cellular processes, including cellulose deposition. Studies in Arabidopsis have significantly aided our understanding of the roles of microtubules in xylem cell development during which correct SCW deposition and patterning are essential to provide structural support and allow for water transport. In contrast, studies relating to SCW formation in xylary elements performed in woody trees remain elusive. In combination, the data reviewed here suggest that the cytoskeleton plays important roles in determining the exact sites of cellulose deposition, overall SCW patterning and more specifically, the alignment and orientation of cellulose microfibrils. By relating the reviewed evidence to the process of wood formation, we present a model of microtubule participation in determining MFA in woody trees forming reaction wood (RW).

1.1 Introduction

The differentiation of a vascular cambium and secondary tissues are amongst the most important events in the evolution of higher plants and a prerequisite for the existence of woody trees. Since its first appearance in the middle Devonian (~390 Ma), secondary xylem – wood – has adapted to transport water and minerals from the roots to the shoots and to support the plant's upright position (Carlquist 2012) . Forest trees are important as a source of timber, pulp for paper production, biofuel and other wood products and significant effort has been directed at functionally characterising specific genes that control wood and secondary cell wall (SCW) formation and underpin related domestication traits.

Ultimately, wood features are defined by polysaccharide-rich cell walls that determine shape, growth and rigidity of cells and tissues. Plant cell walls largely consist of cellulose, which is synthesised by cellulose synthase (CESA) proteins, integral membrane glycotransferases arranged into a unique hexagonal "rosette" structure, the cellulose synthase complex (CSC) (Herth 1985). CSCs move through the plasma membrane synthesising many individual β-1,4-glucan chains per complex that associate to form cellulose microfibrils, which are laid down into the extracellular space forming the primary cell wall (PCW) framework. Cellulose microfibrils are cross-linked to each other within a matrix formed by pectins and hemicellulose (mainly xyloglucan in PCW) (McFarlane *et al.* 2014; Lampugnani *et al.* 2018). In some cell types, including tracheary elements and xylem fibres, a lignified secondary cell wall (SCW) is deposited within the primary wall after the completion of cell expansion. The SCW has distinct types of hemicellulose (xylan and glucomannan) and a minor amount of structural proteins and enzymes when compared to the PCW (Sakamoto *et al.* 2018; Zhong *et al.* 2019). In addition, in the SCW the differential angle of cellulose microfibrils with respect to the long axis of a cell – a feature known as microfibril angle (MFA) – is well documented. This is especially true in the S_2 layer, the large middle layer in a usually three-layered SCW, in which MFA is the main determinant of cell architecture and mechanical properties of fibres and tracheids (Almeras & Clair 2016). While cellulose microfibrils represent the basic structural component of plant cell walls, the presence of hydrophobic lignin in SCWs results in dehydration that provides strength and resistance to negative pressure from water transport (Lampugnani *et al.* 2018; Meents *et al.* 2018). The organisation and/or patterning of these microfibrils and their

interaction with the matrix to a large degree determine the mechanical and physiological properties of the cell wall.

The relationship between the microtubule cytoskeleton and cellulose deposition has been extensively investigated, however much of our knowledge still relies on model organisms like Arabidopsis, while the molecular machinery behind secondary cell wall formation in woody species is largely understudied. Therefore, we reviewed aspects of the involvement of microtubules and actin filaments in secondary cell wall formation in xylary cells of woody trees and put forward a model of cellulose microfibril angle (MFA) determination in trees forming reaction wood (RW) as a response to gravitational stimulus.

1.2 MFA as a Key Feature of SCW Formation

During primary growth, cell expansion largely depends on microfibril reorientation in the PCW. Once SCW deposition commences, the cell has reached its final size and shape and the pattern of microfibril orientation is fixed (Baskin 2005; McFarlane *et al.* 2014). Green (1962) hypothesised that the shape of plant cells is determined by the orientation of cortical microtubules and many studies reported the effects of microtubule disruption on cell growth (Itoh 1976; Hardham & Gunning 1979; Arioli *et al.* 1998; Fagard *et al.* 2000; Chan 2012). For example, Pierce *et al.* (2019) demonstrated the effect of microtubule disruption on the diameter of fibre tips in cotton (*Gossypium spp.*). Data from hypocotyl epidermal cells of Arabidopsis demonstrated that microtubules rearrange in a growth-sensitive manner as they only switch to a transverse alignment during the acceleration in growth rate (Baskin 2001). However, this transverse orientation is not required to be maintained throughout the entire fast-growing phase, and once the growth rate slows microtubules rearrange to an oblique orientation (Chan *et al.* 2011; Crowell *et al.* 2011). Preston (1934) originally suggested a mathematical equation expressing the correlation between the inclination of cellulose microfibrils and the length of *Pinus* tracheids. An inverse relationship between MFA and cell length is generally accepted and has been reported in a number of studies (Barnett & Bonham 2004; Donaldson 2008; Mansfield *et al.* 2009). However, reports are not consistent across the scientific literature as some authors argue that tracheid length is not related to MFA (Matsumura & Butterfield 2001; Myszewski *et al.* 2004) while Evans *et al.* (2000) demonstrated a clear correlation between MFA, density and fibre cell wall thickness. On balance, these data suggest that cell length is possibly mediated by microtubules, however

since MFA is an important feature of SCW formation, it is unlikely that it influences cell size after cell elongation has ceased.

Wood stiffness, often referred to as longitudinal modulus of elasticity (MOE), is a combined effect of wood density and MFA; MFA accounts for up to 85% of MOE variation, making it the major determinant of this important wood feature (Evans & Ilic 2001; Cramer *et al.* 2005; Burgert 2006; Hein & Lima 2012). Tracheids or fibres in the centre of a tree, produced during the early stages of development and frequently referred to as juvenile wood, feature higher MFA and are markedly different from mature wood in strength, stability and stiffness (Mansfield *et al.* 2009; Sattler *et al.* 2014; Wessels *et al.* 2016; Ishikura 2017). Moore *et al.* (2014) showed that 68% of the variation in MFA in *Pinus radiata* is due to radial variation, consistent with the notion that differential MOE is required during the development of a woody tree. Elasticity provided by large MFA values allows young trees to bend with the wind and avoid damage, whereas cells produced later, usually have low MFA and provide the stiffness required to support the increasing weight of the canopy (Barnett & Bonham 2004; Hein & Lima 2012). In some investigations, MFA in the 10 inner rings showed large variability between trees (Mansfield *et al.* 2009) suggesting that featuring a high MFA value during juvenile wood formation is not as critical as exhibiting the wood properties resulting from a low MFA in mature wood. In a commercial context, faster growth rates and short-rotation cropping techniques, therefore, often result in negative implications for wood quality due to a high proportion of juvenile wood (Barnett & Bonham 2004).

MFA variation is also an important feature of RW, which forms in response to gravitational stimulus, caused by wind or load, where stems or branches deviate from a vertical orientation. Under such conditions, trees respond by reorienting branches, reinforcing stress points and maintaining branch angles (Bastien *et al.* 2013; Groover 2016). In tension wood (TW), at the upper side of angiosperm branches, the tension generated results in low MFA and, hence, the longitudinal alignment of cellulose microfibrils helps to support the leaning branch, whereas in compression wood (CW), found at the lower side of gymnosperm branches, large MFA is observed in response to compressive forces and it has been suggested to act by "pushing" the leaning branch upright (Bamber 2001). Indeed, molecular dynamics simulations showed an inverse relation between MFA and MOE when compressive strength was applied (Deng *et al.* 2012). Similarly, Wang *et al.* (2017b) found a negative correlation between longitudinal tensile wood properties and MFA. The wood

formed at the opposite side in each case is referred to as opposite wood (OW) and it is subjected to tensile and compressive forces in gymnosperms and angiosperms, respectively. In addition, wood formed in stems growing upright is subjected solely to vertical gravitational forces with respect to the long axis of xylogenic cells and it is often referred to as normal wood (NW), featuring intermediate MFA values when compared to RW and OW (Spokevicius *et al.* 2007; Almeras & Clair 2016; Roignant *et al.* 2018).

1.3 Cellulose Properties and the CSC

A recent comprehensive investigation of CSC structure revealed that three CESA proteins would fit best in each triangular lobe of the rosette, totalling 18 CESAs per complex (Nixon *et al.* 2016). Earlier, coexpression data in Arabidopsis identified specific CESA isoforms responsible for cellulose synthesis in PCW and in SCW (Taylor *et al.* 2003; Persson *et al.* 2007). Both primary wall- and secondary wall-specific CESAs coexist during the transition period (Watanabe *et al.* 2018) and PCW-CESAs possibly play a role in cell wall thickening during earlier stages of SCW development (Xi *et al.* 2017) The existence of six CESA classes is broadly accepted and each class corresponds to one of the six isoforms (Carroll & Specht 2011). While three different classes of CESA coexisting in a CSC at a time might lead to the assumption that each isoform occupies a specific position within the complex, Nixon *et al.* (2016) acknowledges the possibility of homomeric lobes in the rosette structure, especially since all PCW-CESAs are able to homodimerise (Carroll *et al.* 2012). In this context, Zhang *et al.* (2018) studied the stoichiometry of CESAs in aspen (*Populus tremula*) SCW CSCs and found a 3:2:1 ratio for CESA8:CESA4:CESA7 in normal wood that changed to 8:3:1 in TW, also coinciding with an increase in crystalline cellulose microfibril diameter. These results support alternative CSC models and suggest that homomeric CESA8 complexes might be the main CESA responsible for cellulose biosynthesis during TW formation. This implies that the composition of the CSC in developing xylem fibres potentially plays specific roles with consequences for cellulose microfibril properties.

While MFA in SCWs is particularly relevant during wood formation in tree species, much of our current knowledge still relies on experimental model organisms and systems. Arabidopsis mutants with aberrant SCW patterning for example feature changes in MFA (Schneider *et al.* 2017) and differential composition of the CSC results in alterations in cellulose microfibril polymerisation (Kumar *et al.* 2018; Turner & Kumar 2018). Even

though CSC plays the same role in synthesising cellulose both in primary and secondary walls, variations in the composition and structure of CSCs appears to impart structural variations in cellulose microfibrils (Turner & Kumar 2018). Nevertheless, our current understanding remains sketchy and more studies investigating the complex molecular relations with the CSC during SCW deposition are required**.**

1.4 Cytoskeleton Roles in SCW Biosynthesis

The cytoskeleton is an intracellular framework present in all cellular organisms composed of filamentous polymers characterised by high dynamicity. In plant cells, the main components of the cytoskeleton are microtubules and actin filaments that form an intricate array in plant cells and collectively assume key roles in cell division, cytoplasmic streaming and organelle transport. Both elements, particularly microtubules, are touted to participate in cell wall biosynthesis with different degrees of involvement. A number of studies investigate the cytoskeleton dynamics in xylary cells undergoing SCW deposition employing different imaging techniques (Abe *et al.* 1995; Chaffey *et al.* 1999; Chaffey *et al.* 2000). Microfilaments align longitudinally in fusiform cambial cells and their derivatives in both angiosperms and gymnosperms, while microtubules switch from a transverse to an oblique orientation during SCW formation (Chaffey *et al.* 2002; Begum *et al.* 2019). Furthermore, the cytoskeleton arrangement differs in fibres depositing a G-layer where both microfilaments and microtubules are arranged axially (Chaffey *et al.* 2002). Finally, microfilaments and microtubules associate with pit formation in tracheids and fibres (Chaffey *et al.* 2002; Begum *et al.* 2019). Due to little overlapping observed between the two cytoskeleton elements, it seems likely that they play different but complementary roles during SCW formation. This review, therefore, investigates and summarises our current understanding of the role played by the cytoskeleton in guiding SCW deposition in xylary cells before proposing a mechanistic molecular model for MFA determination in response to gravitational stimulus

1.4.1 Microtubules Guide the CSC and Play a Role in SCW Patterning

The primary components of microtubules are globular proteins called tubulins that form heterodimers comprised of α and a β monomers which bind head to tail, forming protofilaments (Lowe *et al.* 2001). Commonly, 13 protofilaments bind together to form the hollow cylindric structure of the microtubule that undergoes stochastic changes between

growing and shrinking phases based on GTP hydrolysis, resulting in the well-recognised dynamic microtubule network (Heald & Nogales 2002). In contrast to other eukaryotes, plant cells do not have a well-defined microtubule organising centre, which contributes to their high responsiveness to environmental triggers (Wasteneys 2002). In plants, microtubules are arranged into super-structures such as the preprophase band, the spindle and the phragmoplast during cell division (Wasteneys 2002; Mirabet *et al.* 2018) and the interphasic cortical array, where microtubules organised into a parallel array associated with the plasma membrane are believed to regulate the cellulose deposition pattern (Green 1962).

The relationship between microtubules and cellulose deposition has been studied by genetic and pharmacological techniques, and, more recently, live cell imaging has been used to assess the dynamics of these structures. Inhibition of cortical microtubule organisation or cellulose microfibril deposition by pharmacological and genetic approaches suggests that cortical microtubules control the movement of CSCs. Baskin *et al.* (2004) reported variable cellulose microfibril orientation with increasing concentrations of oryzalin, a microtubule polymerisation inhibitor, at the cellular level but not at a local subcellular level (< 10 μm^2). Paredez *et al.* (2008) described two oryzalin hypersensitive Arabidopsis mutants of PROCUSTE1 and KORRIGAN, genes previously shown to be involved in cellulose biosynthesis, indicating a bidirectional flow of information between microtubules and CESAs. Paredez *et al.* (2006) characterised CSC behaviour in PCWs, demonstrating that microtubules share spatiotemporal locations with CSCs that move along tracks delineated by them. Later, Watanabe *et al.* (2015) demonstrated the existence of the same mechanism in SCW. These findings provided fundamental evidence for interactions between microtubules and the CSC, consistent with the hypothesis that the cortical microtubule organisation in plant cells, directly or indirectly, offers cues for cellulose synthesis.

Two models have been proposed to explain the alignment between cellulose microfibrils and microtubules: the direct guidance model and the constraint or bumper model (Heath 1974; Giddings & Staehelin 1991). These models differ with respect to the requirement of a physical linker between CSCs and cortical microtubules. Following the direct guidance model, many microtubule-associated proteins (MAPs) were investigated for their potential roles as linkers between CSC and microtubules. The fragile fibre 1 (FRA1) kinesin motor protein, for example, was suspected to play a role in CSC and microtubule binding based on the aberrant cellulose microfibril deposition observed in *fra1* mutants

(Zhong *et al.* 2002). Further characterisation demonstrated the function of this protein as a bona fide motor protein that binds to microtubules, however it remains questionable if FRA1 is indeed required to guide CSC movement since *in vitro* it moves in a unidirectional fashion much faster than CESA (Paredez *et al.* 2006; Zhu & Dixit 2011). Also, the activity of Cellulose Synthase Interactive Protein 1 (CSI1) and *pom-pom2* mutants, which are allelic, were identified to physically link microtubules and CSC (Bringmann *et al.* 2012; Li *et al.* 2012a). CSI1/POM2 interacts with SCW CESAs in a similar way as with PCW CESAs and its downregulation causes abnormal SCW deposition (Schneider *et al.* 2017). Cells within stems of *pom2-4* mutants showed significantly higher MFA when compared to wild-type (Schneider *et al.* 2017), demonstrating that correct interaction between CSC and microtubules is necessary to determine cellulose microfibril orientation. Kesten *et al.* (2019) showed that the companion of cellulose synthase 1 (CC1) is part of the CSC and links to microtubules, playing a critical role in 're-establishing' the microtubule array after perturbations caused by salt stress. Conversely, in accordance with the bumper model, a recent study using near-TIRF microscopy and high-throughput particle-tracking analysis concluded that microtubules affect CSC speed by mechanisms that are independent of direct physical association (Woodley *et al.* 2018). More recently, Chan and Coen (2020) demonstrated the existence of an autonomous system that maintains the alignment of cellulose microfibrils based on CSCs following trails left by previous complexes. These observations and the fact that CSCs rapidly recover after microtubule organisation disruption by oryzalin (Paredez *et al.* 2006; Schneider *et al.* 2017) suggest that both models are correct and that it is likely that physical association of CSCs and microtubules is critical at the start of cellulose synthesis, but guidance is not strictly necessary to ensure continuous movement in straight lines during the late phases of cell development.

Microtubule organisation is also believed to play a role in determining SCW pattern in xylem cells. The SCW pits present in metaxylem cells appear to be guided by localised microtubule depolymerisation. ROP11, a plant-specific small GTPase from the Rac/Rho family, localises at the plasma membrane of metaxylem differentiating cells within pit areas and recruits Microtubule Depletion Domain 1 Protein (MIDD1), which is responsible for microtubule depolymerisation from these cellulose-depleted regions (Oda & Fukuda 2012). Simultaneously, cell wall growth is promoted at pit boundaries through the ROP-BDR-WAL-actin pathway allowing for precise control of bordered pit formation (Sugiyama *et al.*

2019). In addition, Cortical Microtubule Disordering Protein 1 (CORD1) affects microtubule organisation, branching angle and microtubule attachment to the plasma membrane, resulting in abnormally enlarged SCW pits (Sasaki *et al.* 2017). Other MAPs belonging to MAP20 and MAP70 families were also suggested to play a role in cellulose synthesis and SCW patterning in xylem cells (Rajangam *et al.* 2008; Pesquet *et al.* 2010). Microtubules undoubtedly play a role in cell wall biosynthesis, and reasonably exert influence on MFA, however further research is needed to elucidate the exact nature of this control at the molecular level.

1.4.2 Actin Filaments and Microtubules Act Together to Deliver CSC to the Plasma Membrane

The actin cytoskeleton is composed of stranded filaments of globular actins and its main functions in plant cells are related to cellular growth, cytoplasmic streaming, cell division and organelle movement (Szymanski & Staiger 2018). Monomers of globular actin (G-actin) self-assemble into filaments in a similar way as tubulin dimers do. However, in contrast to the GTP dependent assembly of microtubules, this process is ATP dependent. In terms of organisation, the actin cytoskeleton can exhibit a much more diverse organisation when compared to microtubules. During interphase, the actin cytoskeleton appears to assume two different arrangements: a cortical array and subcortical axial bundles that are established and maintained by actin binding proteins (ABPs) and actin-related proteins (ARPs) (Sampathkumar *et al.* 2013; Szymanski & Staiger 2018). The most reported function of actin filaments in all cellular organisms is to control cell polarisation, as has been especially well established for tip-growing plant cells, such as pollen tubes and root hairs (Sampathkumar *et al.* 2013). Notably, relative to microtubules, actin roles in cell wall biosynthesis have been discovered at a slower pace.

Kobayashi *et al.* (1988) were amongst the first to notice an influence of the actin cytoskeleton on cellulose deposition in SCWs; cultured Zinnia mesophyll cells differentiated into tracheary elements and treatment with the actin inhibitor cytochalasin B, produced abnormal cell wall patterning. Later, Sampathkumar *et al.* (2013), using both pharmacological and genetic approaches, reported that defects in the organisation of the actin cytoskeleton resulted in cellulose deficient Arabidopsis seedlings with cell walls of variable thickness. CSC distribution in the plasma membrane likely depends on actin-based

long-distance transport, since CSC delivery rate is limited, and proximity is increased when actin cytoskeleton organisation is disrupted (Sampathkumar *et al.* 2013). Therefore, fast and even distribution of CSCs to the plasma membrane depends on actin filaments (Nebenfuhr & Dixit 2018). CSC-containing organelles are longitudinally transported by actin cables around the cell to sites marked by transverse actin filaments and CSCs are then incorporated into the plasma membrane and kept at SCW depositing sites by bundles of microtubules (Wightman & Turner 2008). These organelles have been characterised as small CESA-containing compartments (SmaCCs) and Golgi, and CSC insertion are confined to SCW thickenings associated with microtubule bands (Watanabe *et al.* 2015). To further test if actin filaments, and not microtubules, mark the CESA delivery sites, Gutierrez *et al.* (2009) treated seedlings with latrunculin B to disassemble the actin array. They found severe disruption in CESA distribution with many cells showing areas completely depleted, even though this treatment did not directly disrupt CESA delivery to the plasma membrane. Taken together, the results of these two studies suggest that distribution of Golgi, Golgi-independent and Golgi-associated SmaCCs by actin cables and filaments are required for the correct global positioning of the CSC whereas microtubules act on a smaller scale by positioning CSCs once they are in the plasma membrane (Figure 1).

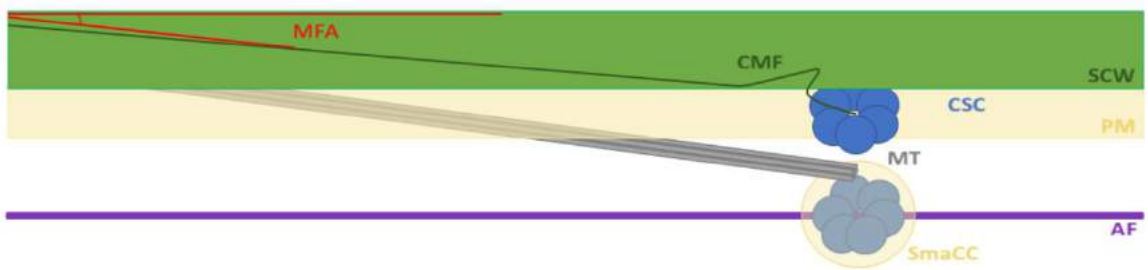

Figure 1.1: ***Model of cytoskeleton roles in MFA determination during xylem cell development.*** *SmaCCs movement is affected by actin filaments and CSCs are delivered to the plasma membrane at SCW depositing sites marked by bundles of microtubules. Microtubules also influence the angle at which cellulose microfibrils are deposited within the cell wall. AF, actin filament; CMF, cellulose microfibril; CSC, cellulose synthase complex; MFA, microfibril angle; MT, microtubule; PM, plasma membrane; SCW, secondary cell wall; SmaCC, small CESA-containing compartments.*

F-actin also plays an important role in patterned cell wall deposition in Arabidopsis metaxylem. Sugiyama *et al.* (2019) demonstrated that during pit formation in the SCW of xylem vessels ROP11 and/or other ROPs recruit wallin (WAL) to the plasma membrane via the boundary of ROP domain 1 (BDR1) and BDR3. WAL localises at pit boundaries promoting actin assembly and cell wall ingrowth (Sugiyama *et al.* 2019). This regulatory pathway has opposite effects on SCW growth in comparison to the ROP-MIDD1-microtubule pathway (Oda & Fukuda 2012). Together, they play a crucial role in efficient

water transport by allowing a tight control of pit formation during SCW deposition in xylem vessels.

The seemingly coordinated actions of microtubules and actin filaments during cell wall synthesis and other cellular processes imply a level of direct or indirect communication between these two cytoskeleton components. Live cell imaging of actin filaments and microtubules clearly showed coincidence between the arrays and interdependence for reassembly after drug treatment (Sampathkumar *et al.* 2011), indicating interactions between microtubules and actin filaments either directly or via associated proteins. Indeed, some proteins classically associated with microtubule regulation interact with actin filaments. This is the case for Arabidopsis microtubule associated protein 18 (MAP18) and microtubule destabilising protein 25 (MDP25) and several conventional microtubule motors like kinesin-like proteins (Preuss *et al.* 2004; Xu *et al.* 2009; Zhu *et al.* 2013; Qin *et al.* 2014). Conversely, conventional actin-binding proteins (ABPs) have also been found to interact with microtubules (Deeks *et al.* 2010; Rosero *et al.* 2013; Wang *et al.* 2013). Besides the intercommunication between the two arrays, the cytoskeleton also interacts with the cell wall via transmembrane proteins that possess an extracellular cell wall- and an intracellular cytoskeleton-binding domain in what is called the cytoskeleton-plasma membrane-cell wall continuum (Liu *et al.* 2015). This continuum is presumably responsible for transmitting cell wall perturbations to the cytoskeleton resulting in reorganisation. In this respect, Tolmie *et al.* (2017) reported an increase in actin network stability in plasmolysed cells or in cells treated with isoxaben, a cellulose synthesis inhibitor, and normal dynamics were recovered in re-hydrated cells, demonstrating changes in actin cytoskeleton dynamics were highly correlated with cell wall disruption. In combination, these findings suggest a role of the plant cytoskeleton in regulating cell wall biosynthesis by determining cellulose deposition sites and cellulose microfibril orientation through the cytoskeleton-plasma membrane-cell wall continuum.

1.5 Molecular Control of MFA

The orientation of cellulose microfibrils within the cell wall determines to a large degree cell architecture and mechanical properties with significant implications for plant development (Donaldson 2008; Hein & Lima 2012). The molecular machinery behind MFA determination is still unclear, however many quantitative trait loci (QTL) have been

identified for wood and fibre properties, including MFA (Thamarus *et al.* 2004; Freeman *et al.* 2009; Thumma *et al.* 2010), and some genes have been identified as candidates (Gonzalez-Martinez *et al.* 2007; Dillon *et al.* 2010; Thumma *et al.* 2010; Beaulieu *et al.* 2011; Li *et al.* 2012b). Reaction wood (RW) has proved to be a useful model system in attempts to better understand the molecular basis of wood formation (Pilate *et al.* 2004) and several studies have published transcriptomes of angiosperm and gymnosperm RW (Paux *et al.* 2005; Li *et al.* 2013; Chen *et al.* 2015). A large number of genes were reported to be highly expressed in RW, among them some encoding arabinogalactan proteins (AGPs), fasciclin-like arabinogalactan proteins (FLAs) and α and β-tubulins (Lafarguette *et al.* 2004; Andersson-Gunneras *et al.* 2006; Qiu *et al.* 2008; MacMillan *et al.* 2010; Li *et al.* 2013; Azri *et al.* 2014).

TW, developed on the upper side of angiosperms branches, is characterised by extremely low MFA values and this is often associated with upregulation of many of cytoskeleton component and secondary wall formation genes including those listed previously (Lafarguette *et al.* 2004; Paux *et al.* 2005; Andersson-Gunneras *et al.* 2006; Bhandari *et al.* 2006; Qiu *et al.* 2008; Azri *et al.* 2014; Bygdell *et al.* 2017; Pomies *et al.* 2017; Roignant *et al.* 2018; Zinkgraf *et al.* 2018). Accordingly, Li *et al.* (2011b) found the same genes overexpressed in high stiffness wood in *Pinus radiata*, which also has lower MFA, when compared to low stiffness wood. Furthermore, Li *et al.* (2011a) compared gene expression in mature wood of the same species with juvenile wood featuring a 10° larger MFA and found both α- and β-tubulin genes were upregulated in mature wood, once more associating high expression levels of these genes with low MFA. In contrast, some studies linked high expression of tubulin and arabinogalactan genes in CW of loblolly pine, maritime pine and *Chamaecyparis obtusa* Siebold & Zucc, to large MFA (Zhang *et al.* 2000; Whetten *et al.* 2001; Le Provost *et al.* 2003; Yamashita *et al.* 2008; Li *et al.* 2013). Changes in MFA, be it due to RW formation, stress response or wood maturation, involve coordinated changes in gene expression to produce context-specific outcomes. Research results published over the last decade shed light on the roles of many genes in controlling this important feature, however functional confirmation of their collective action to modulate MFA remains to be documented in most cases. Here we relate evidence of the involvement of several genes in MFA determination in different species and summarise their combined

action in a model of the molecular control of cellulose orientation in woody trees forming RW.

1.5.1 Arabinogalactans

The Arabinogalactan protein (AGP) family is characterised by highly glycosylated hydroxyproline-rich glycoproteins (HRGPs) mainly expressed in plant cell walls. In general, AGPs are expressed in various organs and tissues, but individual members with different core proteins exhibit organ-, tissue- and/or developmental-specificities (Showalter 2001). Because of their location in the cell wall and the presence of a predicted glycophosphotidyl inositol (GPI) anchor domain in many AGPs, which would localise them to the outer leaflet of the plasma membrane, it was suggested that their function is to transmit information between cell wall and cytoplasm (Driouich & Baskin 2008). AGPs are believed to play an important role in tracheid differentiation (Putoczki *et al.* 2007) and pharmacological and genetic studies provide strong evidence for a link between AGP and microtubules (Andeme-Onzighi *et al.* 2002; Sardar *et al.* 2006; Nguema-Ona *et al.* 2007). The existence of lipid rafts in the plasma membrane or a third protein or protein complex have been suggested as physical linkers between AGPs and the microtubule cortical array (Sardar *et al.* 2006; DeBolt *et al.* 2007). Alternatively, Driouich and Baskin (2008) proposed a model in which diffuse AGPs (not possessing GPI) interact with receptor kinases and another machinery of signal transduction on the plasma membrane that leads to microtubule disorganisation. Indeed, other GPI-anchored proteins have been implicated in receptor-like kinase trafficking from within the cell to the plasma membrane, possibly as cofactors or chaperones for these receptors (Xiao *et al.* 2019). Considering their position in the plant cell and how microtubules and arabinogalactan proteins seem to communicate, it can be speculated that cortical microtubules and the CSC might associate via AGPs, however, further work is needed to demonstrate the actual effects of AGPs on cellulose deposition and microfibril orientation.

The fasciclin (FAS) domain is a cell adhesion domain found in proteins of both eukaryotes and prokaryotes and, despite its considerable level of conservation, it assumes a number of different functions across kingdoms (Gaspar *et al.* 2001; Moody & Williamson 2013). In plants, FAS-containing proteins belong to the superfamily of AGPs and form FAS-like arabinogalactan proteins (FLAs) (Seifert & Roberts 2007). 21 FLAs have been

described in Arabidopsis (Johnson *et al.* 2003), 27 in rice (Ma & Zhao 2010), 19 in cotton (Huang *et al.* 2008), 18 in *Eucalyptus grandis* (MacMillan *et al.* 2015) and 15 in poplar (Lafarguette *et al.* 2004). *FLA* genes are classified in four groups according to the number of FAS domains (Johnson *et al.* 2003) and, specifically, a single-FAS group (group A) shows stem-preferential expression, particularly in developing xylem cells (MacMillan *et al.* 2010). This expression of group-A *FLA*s was reported to be 3- to 27-fold higher on the upper side of eucalypt branches compared to lower branch wood (Qiu *et al.* 2008; MacMillan *et al.* 2015) suggesting that *FLA*s might have a function in SCW biosynthesis in RW and in determining stem biomechanical properties.

Genetic studies revealed FLA effects on cellulose biosynthesis and deposition. Persson *et al.* (2005) reported high levels of co-regulation of *FLA11* and *12* with secondary cell wall-associated *CESA4, 7* and 8 in Arabidopsis and a 2° increase in MFA was found in At*fla11*/*fla12* Arabidopsis mutants (MacMillan *et al.* 2010). Dahiya *et al.* (2006) reported a putative homolog of *AtFLA11* in *Zinnia* (*ZnFLA11*) to be exclusively expressed in metaxylem with reticulate SCW thickening. Furthermore, the Arabidopsis *FLA4*, also called *SALT OVERLY SENSITIVE 5* (*SOS5*) was shown to act on a pathway that regulates the synthesis of cellulose in Arabidopsis roots similarly to FEI1/FEI2 receptor-like kinases (Xu *et al.* 2008). Similarly, Huang *et al.* (2018) identified the AtVRLK1 (*Vascular-Related Receptor-Like Kinase1*), that is specifically expressed in xylem cells undergoing SCW differentiation and seems to promote cell elongation and restrain cell wall thickening in those cells. In trees, a correlation between *FLA* expression, MFA and wood properties was established (Lafarguette *et al.* 2004; Qiu *et al.* 2008; Wang *et al.* 2015; Wang *et al.* 2017a) and MacMillan *et al.* (2015) demonstrated that overexpression of *EfrFla* genes led to a 3° reduction in MFA in eucalypt fibres and biomechanically impact tobacco stem cells. Adhesion domains (FAS) may function by binding structural components of the cell wall and the extracellular matrix or by interacting with extracellular signals, to alter microtubule orientation (MacMillan *et al.* 2015). In poplar, *FLA*s were specifically linked to the formation of the gelatinous layer of TW cells, probably as a result of tension generation. (Wang *et al.* 2017a). Together, these results suggest a role of FLA proteins in SCW formation.

1.5.2 Tubulins

High expression levels of tubulin genes, encoding the primary components of microtubules, are also associated with RW formation. Oakley *et al.* (2007) assessed transcript expression levels in cambial tissue of bent stems in poplar and found tubulin genes, *TUA1*, *TUA5*, *TUB9* and *TUB15*, to be specifically up-regulated 2- to 4-fold when compared to cambial tissue of upright stems. In plants, tubulins are encoded by multigene families and the expression of different isoforms is tissue specific and varies throughout plant development, with tissue-preferential clusters grouping separately in phylogenetic analyses (Oakley *et al.* 2007; Breviario *et al.* 2013). For instance, the Arabidopsis genome contains six *TUA*s, one of them specifically expressed in pollen tubes, and nine *TUBs* with differential expression in roots, leaves and floral tissue (Snustad *et al.* 1992; Cheng *et al.* 2001). Rice (*Oryza sativa*) also has a pollen-specific β-tubulin isoform and another seven which are differentially expressed in vegetative tissue (Yoshikawa *et al.* 2003). In cotton (*Gossypium hirsutum*), five *TUA*s are expressed in elongating fibres and only two remain highly expressed once SCW deposition is initiated (Whittaker & Triplett 1999). In Poplar, the TUA family has eight members and the TUB family has undergone significant expansion and contains 20 members as 10 pairs of highly homologous *TUB*s (Oakley *et al.* 2007). The large number of tubulin isoforms poses the question why all of them are maintained. The multi-tubulin hypothesis interprets tubulin diversity as a requirement for differential microtubule formation (Wilson & Borisy 1997). Conversely, tubulin gene redundancy might ensure expression of a fundamental protein (Breviario *et al.* 2013). Besides the large number of isoforms, it is believed that post-translational modifications (PTM) in tubulins mark microtubules with distinct stability and association with MAPs and motor proteins, thereby altering their sensitivity to microtubule-disrupting drugs (Breviario *et al.* 2013). Hence, selective expression of tubulin genes and PTM accumulation could, collectively, finely adjust microtubule assembly and/or dynamics in specific tissues to perform required functions (Parrotta *et al.* 2014).

Few studies have uncovered functional links between tubulins and MFA. Spokevicius *et al.* (2007) established that β-tubulin affects cellulose microfibril orientation in fibre SCWs of young eucalypt trees. In their study, phenotype-based evidence indicates that downregulation of an *Eucalyptus grandis* β-tubulin gene (*EgrTUB1*) causes a significant increase in MFA in transgenic fibres suggesting that this β-tubulin isoform is directly involved in determining cellulose microfibril orientation during xylogenesis, possibly via

changes to microtubule structure. Swamy *et al.* (2015) studied post-translational modifications in poplar TUA1 but did not find any effects on MFA of transgenic trees. This study demonstrated that non-cellulosic polysaccharides – deposited early during cell wall biosynthesis – are more sensitive to this type of tubulin manipulation in wood cells than cellulose content and its organisational features like MFA or crystallinity. Therefore, these efforts made it evident that modifications of microtubule organisation by targeting tubulins have potential to significantly affect the mechanical properties of plant cell walls. Nevertheless, studies attempting to understand how microtubules coordinate microfibril orientation directed towards specific tubulin roles are still elusive and virtually nothing is known about the molecular mode of action of these specific protein isoforms in MFA determination.

1.5.3 Other Cell Wall-Related Genes

Other genes known to have effects on cell wall formation and influence MFA include the *KORRIGAN* (*KOR*) gene. *KOR* was isolated from the Arabidopsis mutant *kor1-1*, which is characterised by abnormal PCW formation in the absence of light (Nicol *et al.* 1998) and further characterisation demonstrated that *KOR* plays roles in cytokinesis, cell elongation and cellulose synthesis (Maloney & Mansfield 2010) and influences pectin metabolism (His *et al.* 2001). Despite the majority of roles having been described in PCW synthesis, Szyjanowicz *et al.* (2004) report *KOR* functions also in the SCW. In poplar, two families of glycosyl hydrolase genes were shown to be similar to Arabidopsis *KOR* (Hertzberg *et al.* 2001) and these genes were found to be up-regulated in cells undergoing secondary wall formation (Takahashi *et al.* 2003; Bhandari *et al.* 2006). Maloney and Mansfield (2010) demonstrated architectural alterations in poplar wood due to downregulation of *PaxgKOR* with transgenic trees exhibiting lower cellulose content, changes in cellulose composition, an increase in cellulose crystallinity and significantly lower MFA.

Finally, xylan-acting enzymes were found to be upregulated during xylem SCW formation in aspen and to affect MFA and other aspects of plant development. Xylans, the main hemicellulose of SCW, are polymers with a β-1,4-D-xylopyranose backbone (Scheller & Ulvskov 2010) and the downregulation of an aspen gene encoding the endo-1,4-β-xylanase belonging to the glycoside hydrolase family 10 (*PtxtXYN10A*) resulted in a clear

reduction of MFA of transgenic fibres, indicating a role for this gene in orientating cellulose microfibrils in secondary walls (Derba-Maceluch *et al.* 2015).

1.5.4 A Molecular Model for MFA Alterations in Response to Gravitational Stimulus

While the roles of *KOR* and *XYN* have not been demonstrated in other species, *TUB*s and *AGP*s, *FLA*s among them, have shown a consistent expression profile in several studies across species and deserve to be more carefully examined here to understand their combined actions during cellulose biosynthesis. Stress has been shown to either up- or downregulate AGP expression (Mareri *et al.* 2019) with some AGPs being upregulated in TW while others are downregulated (Qiu *et al.* 2008). The perception of gravity, a mechanical stimulus in nature, and a plant's capability to respond to it are not fully understood. However, the roles of different cell wall components and the secondary messenger calcium (Ca^{2+}) have been proposed (Landrein & Hamant 2013; Monshausen & Haswell 2013). Due to their position and structure, AGPs are strong candidates to function as mechanical sensors, sensing both tension and compression within the cell wall (Showalter & Basu 2016). Moreover, AGP can bind and release Ca^{2+} in the cell wall under certain conditions (Lamport & Varnai 2013). Ca^{2+} plays critical roles during stress response activating important signal-mediators such as phosphatases and phospholipases (Li *et al.* 2009). The rapid activation of phospholipase D (PLD) during stress cleaves the GPI anchor and releases AGP from the plasma membrane into the cell wall (Mareri *et al.* 2019) and might function as another signalling molecule in the cascade of events that culminates in the transcription of *AGP* genes. In addition, one of the products of PLD, phosphatidic acid (PA), is known to activate the mitogen-activated kinase protein (MPK6) which can phosphorylate the microtubule associated protein MAP65-1 leading to destabilisation of the microtubule array in dividing cells (Smertenko *et al.* 2006). Conversely, PA binds to MAP65-1 promoting microtubule reorganisation (Zhang *et al.* 2012). MAP65-1 ensures array stabilisation by promoting microtubule bundling (Stoppin-Mellet *et al.* 2013) and it plays an important role in different types of stress, such as salt and cold response (Wang *et al.* 2007; Endler *et al.* 2015; Wang & Nick 2017; Kesten *et al.* 2019). Ca^{2+} also binds to the protein phosphatase PP2A and one Arabidopsis gene belonging to its regulatory subunit B" is involved in microtubule nucleation (Lillo *et al.* 2014).

Another important stress signalling mediator is auxin. The plasma membrane protein family PIN-FORMED (PIN) acts in auxin efflux and is important for intercellular auxin signaling. PIN1 has been reported to localise to membranes adjacent to cell walls subjected to the highest stress and correlates to cortical microtubule array orientation in Arabidopsis shoot apical meristems (Heisler *et al.* 2010). While changes in auxin balance seem not to contribute to RW formation (Hellgren *et al.* 2004), they activate the ROP6-RIC1 pathway through auxin binding protein 1 (ABP1) (Xu *et al.* 2010). RIC1 binds to the p60 subunit of the Arabidopsis katanin 1 (KTN1), a microtubule severing protein involved in mechanical stress response (Uyttewaal *et al.* 2012; Lin *et al.* 2013; Li *et al.* 2017). Finally, microtubule nucleation and severing of nascent microtubules followed by depolymerisation of mother-microtubules are key steps in cortical microtubule array shift from a transverse to an oblique reorientation (Lindeboom *et al.* 2013).

Due to the gene expression profile of RW, we hypothesise that the perception of gravitational stimulus by AGPs, PINs, and other proteins, causes Ca^{2+} influx that activates PP2A. In turn, PP2A acts on promoting nucleation of microtubules. Branched nucleation followed by severing of newly formed microtubules promoted by KTN1 results in reorientation of the microtubule array. Moreover, other Ca^{2+} targets include PLD, which acts on microtubule associated proteins (MAPs) responsible for promoting microtubule stabilisation through bundling of microtubules. Shifts on microtubule orientation of differentiating wood cells lead to MFA changes during RW formation (Figure 1.2). Nevertheless, further work is required to elucidate the fine-tuning mechanisms responsible for correct tissue and function specific cellulose microfibril deposition angles.

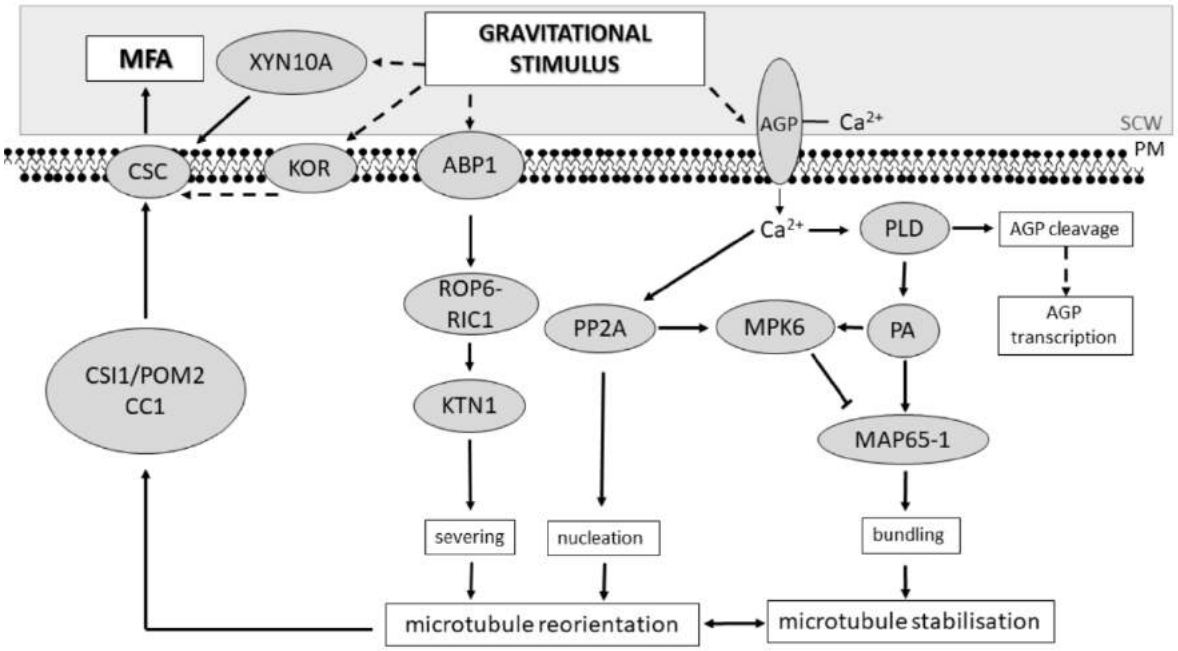

*Figure 1.2**: Model of MFA determination in response to gravitational stimulus.** Gravitational stimulus is potentially sensed by AGP proteins, which could result in release of calcium into the cell. Ca2+ activates PLD and PP2A that act on downstream targets and promote cortical microtubule array reorientation and stabilisation. Microtubules interact with the CSC through CSI1/POM2 and CC1 to determine MFA. ABP1, auxin binding protein 1; AGP, arabinogalactan protein; CC1, companion of cellulose synthase 1; CSC, cellulose synthase complex; CSI1/POM2, cellulose synthase interacting 1; KOR, korrigan; KTN1, katanin 1; MAP65-1, microtubule associated protein 65-1; MPK6, mitogen activated kinase 6; PA, phosphatidic acid; PLD, phospholipase D; PM, plasma membrane; PP2A, protein phosphatase 2A; ROP6, plants Rho-related GTPase; RIC1, ROP-interactive CRIB motif-containing protein 1; SCW, secondary cell wall; XYN10A, endo-1,4-β-xylanase glycoside hydrolase 10. Solid lines indicate experimentally determined interactions, while dashed lines indicate hypothesised connections.*

1.6 Conclusions

The cytoskeleton plays an important role in determining CSC delivery sites during SCW deposition and, specifically in xylary cells, it has been demonstrated to be critical for proper microfibril orientation. Both microtubules and actin filaments are involved in patterning secondary wall deposition of xylem cells, which ensures essential features of wood related to upright support and water transport. These features are of special interest particularly in woody trees because they impact on wood quality.

Studies in Arabidopsis have been essential in aiding our understanding of the roles of cytoskeleton in xylem cell development. The roles of microtubules and actin filaments coordinated by MAPs and ABPs within the cytoskeleton-plasma membrane-cell wall continuum have been revealed, but these only form part of a robust regulatory pathway that encompasses environmental perception and signal transduction. Demonstrations of similar

processes in woody trees are still wanting. In their absence and in utilising all available evidence, we have put forward a hypothesis for how microtubules might participate in MFA determination in response to gravitational stimulus which can be used as a molecular model for future studies that will investigate how tubulins interact with other molecular components to shape xylary cells in response to environmental changes in woody trees.

Chapter 2: Reaction wood as a model for studying tubulin roles in wood formation

Abstract

The wood formed in response to gravitational stimulus, reaction wood (RW), is often studied as a model of wood formation due to its well-defined cellular and molecular modifications. Tubulin genes, for example, are known to be differentially expressed in RW and to be involved in secondary cell wall deposition, affecting the cellulose microfibril angle (MFA) of xylem fibres. The aim of this study is to better understand how tubulin proteins participate in wood formation. Towards this end, this study describes, as a first step, microtubule organisation and orientation in fixed cells of xylem excised from reaction, opposite and normal wood from branches and induced systems in eucalypt, poplar and radiata pine. Microtubule organisation was found to vary between reaction, normal and opposite wood, indicating that tubulins play a significant role in determining the cellular phenotypes featured in RW – cell wall thickening and MFA. In addition, tension and compression wood seem to be formed by opposite mechanisms, considering microtubule angle and length. Some of the aspects of the microtubule array organisation behind tension wood formation are similar between the two angiosperms and the two states of RW analysed, nevertheless in some cases different mechanisms seem to be in place. These results imply that changes at molecular and cellular levels assist woody trees in coping with stress and that different species employ different strategies of RW formation.

2.1 Introduction

Microtubules are macromolecules that are part of the cytoskeleton of eukaryotic cells and play key roles in cell division, cytoplasmic streaming and organelle transport (Komis *et al.* 2015). The primary component of microtubules are globular proteins named tubulins, that form heterodimers comprised of α and β monomers (Lowe *et al.* 2001). They bind head to tail to form protofilaments, which go through stochastic changes between growing and shrinking phases based on GTP hydrolysis (Heald & Nogales 2002). The impact of microtubules on cellulose microfibril orientation has been acknowledged since their first observations (Green 1962; Ledbetter & Porter 1963; Baskin 2001). Studies inhibiting cortical microtubule organisation (Whittington *et al.* 2001; Baskin *et al.* 2004) or cellulose microfibril deposition (Williamson *et al.* 2001; Paredez *et al.* 2008) by pharmacological and genetic approaches suggest that cortical microtubules control the movement of the cellulose synthase complex (CSC). The CSC is a membrane-embedded enzymatic complex containing many cellulose synthases (CesAs) (Herth 1985; Haigler *et al.* 1986) that move through the plasma membrane synthesising individual cellulose chains that associate to form microfibrils in the primary and secondary cell wall (McFarlane *et al.* 2014; Lampugnani *et al.* 2018). In the secondary cell wall particularly, cellulose microfibrils act as the main load-bearing cell wall component (Polko & Kieber 2019) and their arrangement has great influence on cell wall physical properties (Hein & Lima 2012; Meents *et al.* 2018). Ultimately, Paredez *et al.* (2006) demonstrated that microtubules share spatiotemporal locations with CSCs that move along tracks delineated by them in the primary cell wall and the same mechanism has been established for the secondary cell wall (Watanabe *et al.* 2015). These findings helped to shed light on the ongoing debate about the involvement of the microtubule array in guiding the movement of CSCs, yet little is known about the means by which this occurs.

Reaction wood (RW) develops in response to gravity associated stress, which can be caused by wind or load, and it has been used as a model in studies of wood formation (Pilate *et al.* 2004). The perception of gravitational stimulus (e.g. load or changes in stem orientation) triggers a quick response that acts generating strain that acts in returning the stem to an upright position, reorienting branches, reinforcing stress points or maintaining branch angles (Scurfield 1973; Bastien *et al.* 2013; Almeras & Clair 2016; Groover 2016). This response, so called RW, is known as compression wood (CW), usually found at the

lower side of gymnosperms branches, and tension wood (TW), commonly found at the upper side of angiosperms branches. The wood formed opposite to RW is referred to as opposite wood (OW) and differs from the normal wood (NW) formed in upright stems (Wardrop & Dadswell 1948, 1950; Fisher & Stevenson 1981). RW is formed through a series of changes that occur at cellular and molecular levels.

Cellulose microfibril angle (MFA), measured as the deviation of microfibrils from the long axis of the cell in the S_2 layer of the secondary cell wall (Long *et al.* 2000; Donaldson 2008) and consequently cell architecture and mechanical properties of fibres and tracheids, to a large degree are determined by the orientation of cellulose microfibrils (Clair et al. 2011). Changes in MFA are a distinguishable characteristic of RW: CW features a large MFA (30˚– 40˚), while NW and OW are characterised by intermediate values (10˚ to 20˚) and TW has very low values (less than 5˚) (Washusen *et al.* 2005; Spokevicius *et al.* 2007; Li *et al.* 2013; Almeras & Clair 2016; Roignant *et al.* 2018). Other cellular modifications in TW include the development of a gelatinous layer (GL) in the secondary cell wall, inside the S_3, replacing the S_3 or replacing S_3 and partially or entirely replacing the S_2 in a number of angiosperms species, such as poplar (Wardrop & Dadswell 1948; Andersson-Gunneras *et al.* 2006; Abedini *et al.* 2015); differences in vessel size; cell differentiation, expressed by differences in the ratio of fibres to vessels in the xylem, and the width of cellulose crystallite (Jourez *et al.* 2001; Washusen & Evans 2001; Washusen *et al.* 2005; Mellerowicz & Sundberg 2008). On the other hand, CW cells are rounded with large intercellular spaces, lack the S_3, have helical cavities in their lumen, higher lignin content and a comparatively low cellulose content (Wardrop & Dadswell 1950; Tarmian & Azadfallah 2009; Li *et al.* 2013; Almeras & Clair 2016).

At the molecular level, the involvement of several genes in the regulation of RW formation has been identified. Gene expression studies have revealed α and β-tubulins to be highly expressed in tension and compression wood (Lafarguette *et al.* 2004; Andersson-Gunneras *et al.* 2006; Qiu *et al.* 2008; MacMillan *et al.* 2010; Li *et al.* 2013; Azri *et al.* 2014). Downregulation of an *Eucalyptus grandis* β-tubulin gene (*EgrTUB1*) causes a significant increase in MFA in transgenic fibres (Spokevicius et al. 2007) suggesting that β-tubulin is directly involved in the determination of cellulose microfibril orientation during xylogenesis, possibly via changes to microtubule structure. OW also plays an active role in responding to gravity associated stress, suggested by the number of genes reported to be

differentially expressed in OW versus NW (Chen *et al.* 2015; Groover 2016). Therefore, it is evident that modifications in tubulin content significantly affect MFA, possibly via changes to microtubule structure, thereby impacting the mechanical properties of plant cell walls and ultimately wood.

Cortical microtubule organisation and cellulose microfibril deposition are closely associated and microtubules influence cell size and shape. Therefore, it is hypothesised that differences in the expression of tubulin genes might lead to differences in microtubule assembly. This differential microtubule orientation and/or organisation might impact on cell wall morphology, including cell wall thickness, cell length and MFA. In this sense, the objective of this study is to investigate the role played by tubulin genes during the deposition of secondary cell walls in woody trees using RW as a model. To do so, cortical microtubule arrays were visualised using immunolabeling in fixed cells of xylem excised from reaction, opposite and normal wood of eucalypt, poplar and radiata pine and several aspects of microtubule organisation were assessed. Two different states of RW have been used in this study: (i) induced, where seedlings were tipped to their side at 90˚ and, therefore, actively produced RW in response to a dynamic load; and (ii) RW formed in branches constantly subjected to a static load imposed by branch development and position. These two RW models are known to produce different cell phenotypes, providing an opportunity to draw conclusions on the relationship between cortical microtubule array features and cellular morphology in each state, condition, and species.

2.2 Material and methods

2.2.1 Plant growth and reaction wood sampling

As a model of long-term response to gravity-associated stress, branch wood was sourced from mature trees growing at the University of Melbourne Creswick Campus. Three species of economic and ecological interest, two angiosperms and one gymnosperm, were chosen for this study: *Eucalyptus globulus* Labill., *Populus alba* L., and *Pinus radiata* D. Don. Branches were collected with the aid of a pole pruner.

An induced RW development treatment was used as a model for short term response to gravitational stimulus under controlled glasshouse conditions. Seedlings were grown to approximately 0.5-meter height under (exposed to 15 h photoperiod and 25˚C day

temperature) and tipped on their side at 90°. They were then allowed to grow for eight weeks under the same conditions before samples were collected for assessment.

A total of nine branches and stems of each species were used in this study: three branches (B), three induced stems (I) and three normal (upright) stems (N) also grown in the glasshouse.

2.2.2 Tubulin visualisation and microtubule organisation analysis

A small portion (~1 cm^3) of each side of the branch/stem was harvested (20-25 cm up the stems and ~10 mm branch diameter) and identified as tension (TW), compression (CW), opposite (OW) or normal wood (NW) and, using a razor blade, radially hand-cut from bark to pith into ~0.5 mm sections. Samples were then fixed according to Collings and Wasteneys (2005) in PME solution [50 mmol·L^{-1} Pipes (P7643, www.sigmaaldrich.com/australia) pH 7.2, 2 mmol·L^{-1} EGTA (E3889), 2 mmol·L^{-1} $MgSO_4$ (AJA1548, www.ajaxfinechem.com), and 0.1% Triton X-100 (X-100, www.sigmaaldrich.com/australia)] containing 2 mmol·L^{-1} phenylmethylsulfonyl fluoride (PMSF, P7626), 400 µmol·L^{-1} maleimidobenzoyl-N-hydroxysuccinimide ester (MBS, M2786), 4% formaldehyde (FA010, www.chemsupply.com.au) and 1% glutaraldehyde (G5882, www.sigmaaldrich.com/australia). After fixing, samples were washed in PME twice, extracted for 1 h in PME solution containing 1% Triton X-100 and washed in PME once more.

Cell walls were digested in 1% Cellulase from *Aspergillus niger* (C1184) and 0.1% Pectolyase from *Aspergillus japonicus* (P3026) dissolved in PME containing 1% BSA (A2153) and 0.4 mol·L^{-1} mannitol (M4125). After two washing steps in PME, samples were permeabilised in methanol (-20˚C, 10 minutes) and rehydrated in PBS [131 mmol·L^{-1} NaCl (S7653), 5.1 mmol·L^{-1} Na_2HPO_4 (S0876), 1.56 mmol·L^{-1} KH_2PO_4 (PL009, www.chemsupply.com.au) pH 7.2]. Free aldehyde groups were reduced with sodium borohydride (4528825, mg·mL^{-1}) in PBS for 30 minutes and after three rinses in PBS, sections were incubated with blocking buffer [PBS containing 1% BSA and 50 mmol·L–1 glycine (G8898)] for 30 minutes.

Monoclonal anti-α-tubulin produced in mouse (clone B-512, T6074; Thermo) was used as primary antibody diluted 1:1000 in blocking buffer and anti-mouse IgG1 (γ1), CF™488A antibody produced in goat (SAB4600237, www.sigmaaldrich.com/australia) as

secondary antibody diluted 1:100. Antibody assay was performed as described in Appendix A of this thesis. Antibody incubations were kept overnight at 4°C and material was washed in PBS containing 50 mmol·L^{-1} glycine (4 × 15 minutes) in between incubations. After removal of secondary antibodies, sections were again washed in PBS glycine (3 x 15 minutes) and mounted on glass slides in glycerol (AJA242, www.ajaxfinechem.com) and cover slips were sealed with nail polish.

Samples were viewed under a Nikon C2 confocal microscope (www.nikon.com/products/microscope-solutions/lineup/confocal/c2) with 40x numerical aperture 1.3 oil-immersion lens with 488.6 nm laser and fluorescence was collected from 500–580 nm. Stacks of optical sections were generally collected at 0.5 µm intervals. All images were processed with Fiji-ImageJ (www.fiji.sc) using standard adjustment tools and skeletonisation of microtubule arrays. Microtubule organisation for each treatment was determined as a combination of microtubule orientation (given by the microtubule angle in relation to the long axis of the cell), microtubule density (given by the number of distinguishable units within an area of 320 μm^2), microtubule bundling (given by the thickness of said units), microtubule branching (given by the number of microtubules branching events within the said area) and microtubule average length. Measurements were taken from several planes with variable z-depth.

2.2.3 Cellulose microfibril visualisation and MFA analysis

Cell walls were stained with Fluorescent Brightener 28 (Sigma) at 10 µg/mL and examined under a Nikon $C2^+$ confocal microscope (www.nikon.com/products/microscope-solutions/lineup/confocal/c2) with 40x numerical aperture 1.3 oil-immersion lens. Samples were excited with a 404.8nm laser, and fluorescence was collected from 410-440 nm. MFA was calculated as the angle formed between the cell's longer axis and the cellulose microfibril either directly or using the aperture method described in Cockrell (1974). For MFA analysis, five fibres were selected per treatment and the MFA attributed to each fibre was determined from the median value of three independent MFA measurements of that fibre.

2.2.4 Cell length analysis

Wood tissue was processed as described in Spokevicius *et al.* (2007). Fibres were excised from samples as small blocks of wood (~1 mm^3), which were macerated using a master mix

of 1:1 hydrogen peroxide and glacial acetic acid solution for two hours at 90˚C, mounted on glass slides and stained with methylene blue. Pictures were taken with a digital microscope camera (Leica DFC450 C, www.leica-microsystems.com/products/microscope-cameras/p/leica-dfc450-c). The digital software Leica Application Suite (www.leica-microsystems.com/products/microscope-software/p/leica-application-suite) was used to assess the length of ten fibres excised from each individual.

2.2.5 Scanning electron microscopy

Transverse sections were visualised using a FEI Teneo VolumeScope scanning electron microscope (www.fei.com/products/sem/teneo) at 10.8 mm working distance with a low vacuum detector (LVD) using 5 kV beam and 50 pA current at 2500x magnification. For this analysis, five fibres located at similar distance from the cambium on transverse sections, and, therefore, assumedly being at the same developmental stage of each individual were measured once for cell wall thickness, lumen and cell wall area utilising Fiji-ImageJ software (www.fiji.sc).

2.2.6 Pith eccentricity calculation

Pith eccentricity was calculated according to Roignant *et al.* (2018) as:

$$E(\%) = \frac{e}{r} X100$$

where *E* represents the pith position in the stem, *e* is the distance between the geometrical centre of the stem and the centre of the pith and *r* is the mean radius of the stem transverse section.

2.2.7 Data analysis

Two samples were taken from each branch or stem (from the upper and the lower side) and stems growing upright were sampled randomly only once. Five fibres per were collected at between 5 to 10 cell files from the cambial zone for all analysis sample (except when noted otherwise), totalling 15 data points per condition. Outliers were excluded from the analysis after a Grubbs' test with α=0.05 and means were compared using one-way ANOVA assuming equal variances with α=0.05. Confidence intervals were calculated at 95% using Minitab 19 statistics package.

2.3 Results

2.3.1 Microtubules form distinct structures during wood formation

To investigate the roles of tubulin in wood formation, microtubule organisation was assessed in radial sections of RW, OW and NW of eucalypt, poplar and radiata pine. Microtubules were observed to be in association with pit apertures in eucalypt samples of induced OW, NW and TW (Fig 2.1 A-C). Furthermore, we observed the formation of microtubule bands (Fig 2.1 D-F), microtubule bundles in close association with each other forming distinct groups (bands) within the cell, in all three species. Bands were more frequent in poplar TW than in NW and branch OW but were absent in poplar induced OW (Table 2.1). In eucalypt, microtubule bands were more frequent in OW from both induced and branch states, and for this species the lowest occurrence of microtubule bands was recorded in NW samples. Finally, in pine, microtubule bands were presented equally across treatments except for induced OW where it was found to be less numerous.

A second phenomenon of microtubule behaviour was observed in which microtubules in different planes intertwine creating a braiding pattern (Fig 2.1 F-J). In contrast, microtubule encounters at steep angles in the same plane produce crossovers, which were also observed (Fig 2.1 F-L). In poplar, microtubule braiding was predominantly observed in NW, being present in ~47% of samples, followed by induced OW (~33%) and TW (~27%), while this pattern was observed in 20% of branch OW and in only ~13% of TW samples. Similarly, braiding was more prevalent in eucalypt NW samples (~47%), while it was present in 40% of induced TW and in 7% of OW samples. In branch samples, braiding was more prevalent in OW when compared to TW (27 and 20% of the samples, respectively). In pine, microtubule braiding was observed in ~53% of induced CW samples and in 47% of branch CW, while in OW it was present in ~33% of induced samples and in 40% of branch samples and only in ~13% of NW samples.

Table 2.1: Percentage of samples exhibiting microtubule bands and microtubule branching

Species	RW state		Microtubule bands (%*)	Microtubule branching (%*)
Eucalyptus globulus		NW	13	47
	Induced	TW	20	40
		OW	33	7
	Branch	TW	27	20
		OW	33	27
Populus alba		NW	7	47
	Induced	TW	34	27
		OW	0	33
	Branch	TW	40	13
		OW	7	20
Pinus radiata		NW	33	13
	Induced	CW	33	53
		OW	13	33
	Branch	CW	33	47
		OW	33	40

*Percentage of the total samples per condition (n=15).

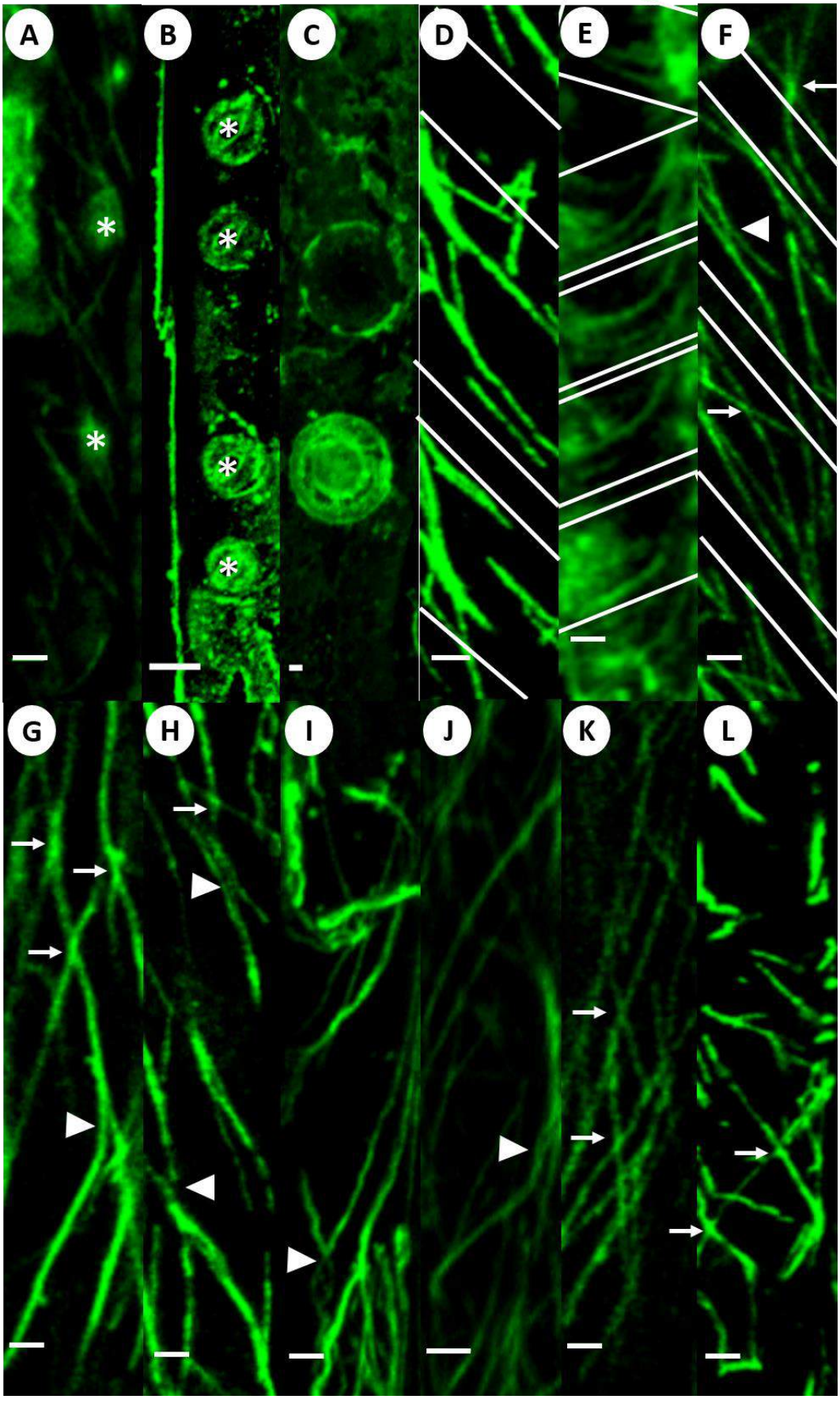
A
B
C
D
E
F
G
H
I
J
K
L

Figure 2.1: ***Microtubule structures.*** *Confocal images of microtubules in association with pit apertures in eucalypt I-OW (A), pine B-CW (B) and pine B-OW (C). Microtubule bands formation in pine I-CW (D), poplar NW (E) and pine I-OW (F). F-J, microtubule braiding in pine I-OW (F and G), pine I-CW (H), eucalypt I-TW (I) and poplar I-TW (J). Microtubule crossover in pine I-OW (K) and pine I-CW (L). Arrowheads mark braiding events; arrows mark crossover events; asterisks mark apertures; lines mark different bands. Bars measure 2µm.*

2.3.2 MTA and MFA are strongly correlated in poplar and eucalypt

To better understand how closely microtubule and microfibril orientation are aligned during wood cell development, correlation studies of microtubule angle (MTA) and MFA were performed for all three species. In poplar, the Pearson correlation coefficient (PCC) was 0.883 (P-value<0.001), indicating a strong correlation between MTA and MFA in poplar fibres from all treatments (Fig 2.2 A). The variance in MFA was larger than in MTA (81.034 and 42.805, respectively), however, the difference was not significant (P=0.467). In eucalypt, the PCC for MTA and MFA was 0.602 (P=0.018, Fig 2.2 B) and the variances of MTA and MFA were significantly different (P=0.039). Conversely, MTA variance was higher in comparison to MFA (101.172 and 67.841). Finally, MTA and MFA were found to be moderately correlated in pine samples (Fig 2.2 C; PCC=0.410; P=0.145) and their variances were not statistically different (σMTA=37.13; σMFA=54.45; P=0.214).

2.3.3 Tension wood is related to lower MTA, longer microtubules, fewer microtubule bundles and thicker cell walls

Microtubules were visualised using immunofluorescence to evaluate whether they are reorganised during TW formation. Because the two RW states investigated here are known to produce different cell phenotypes, cell morphology was also assessed to better understand the role played by microtubules in cell morphogenesis. MTA values were lower in both poplar and eucalypt TW samples when compared to OW in both branch and induced states (Fig 2.3 A and B; Table B.1 of Appendix B) and significantly different between eucalypt induced TW and OW (41.7˚ and 60.8˚, respectively, P=0.027). In addition, in poplar, MTAs were particularly low in samples of NW and significantly different from induced OW (29.6˚ and 45˚, respectively, P=0.009). Microtubule average length was larger in TW samples when compared to OW of poplar and eucalypt in samples from both branch and induced states (Fig 2.3 C and D). Moreover, TW samples of both species presented fewer microtubule bundles when compared to OW (Figure 2.3 E and F) and differences between eucalypt branch TW and OW were statistically significant (0.57 µm and 0.77 µm, respectively,

P=0.029). Finally, TW samples generally presented thicker cell walls in relation to OW samples resulting in larger cell wall areas (Figure 2.4 A-D).

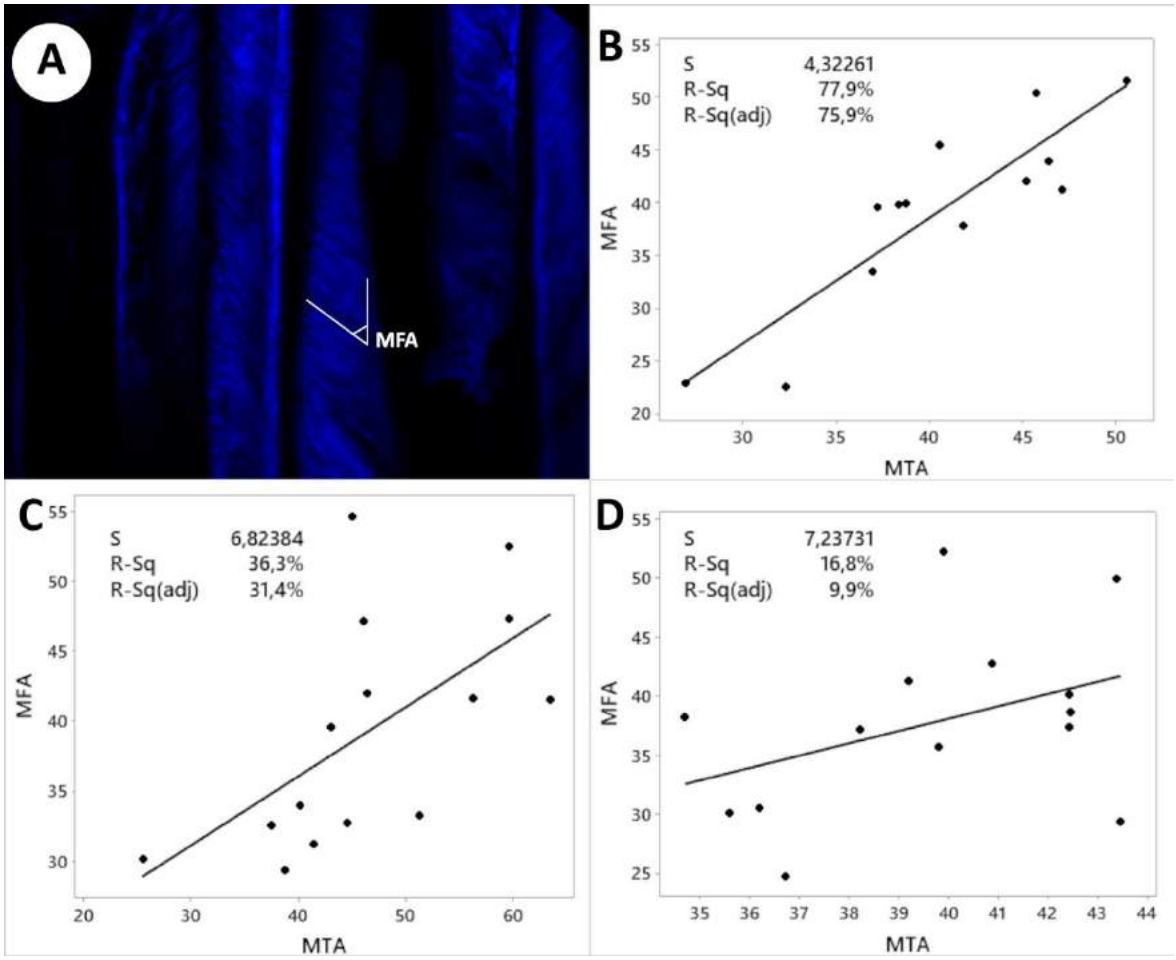

Figure 2.2: ***MTA and MFA correlation.*** *Confocal image of FB28 stained tracheids (A). Scatterplots of average MFA and MTA values (15 cells measured per treatment) with regression line and R^2 for poplar (B), eucalypt (C) and pine (D).*

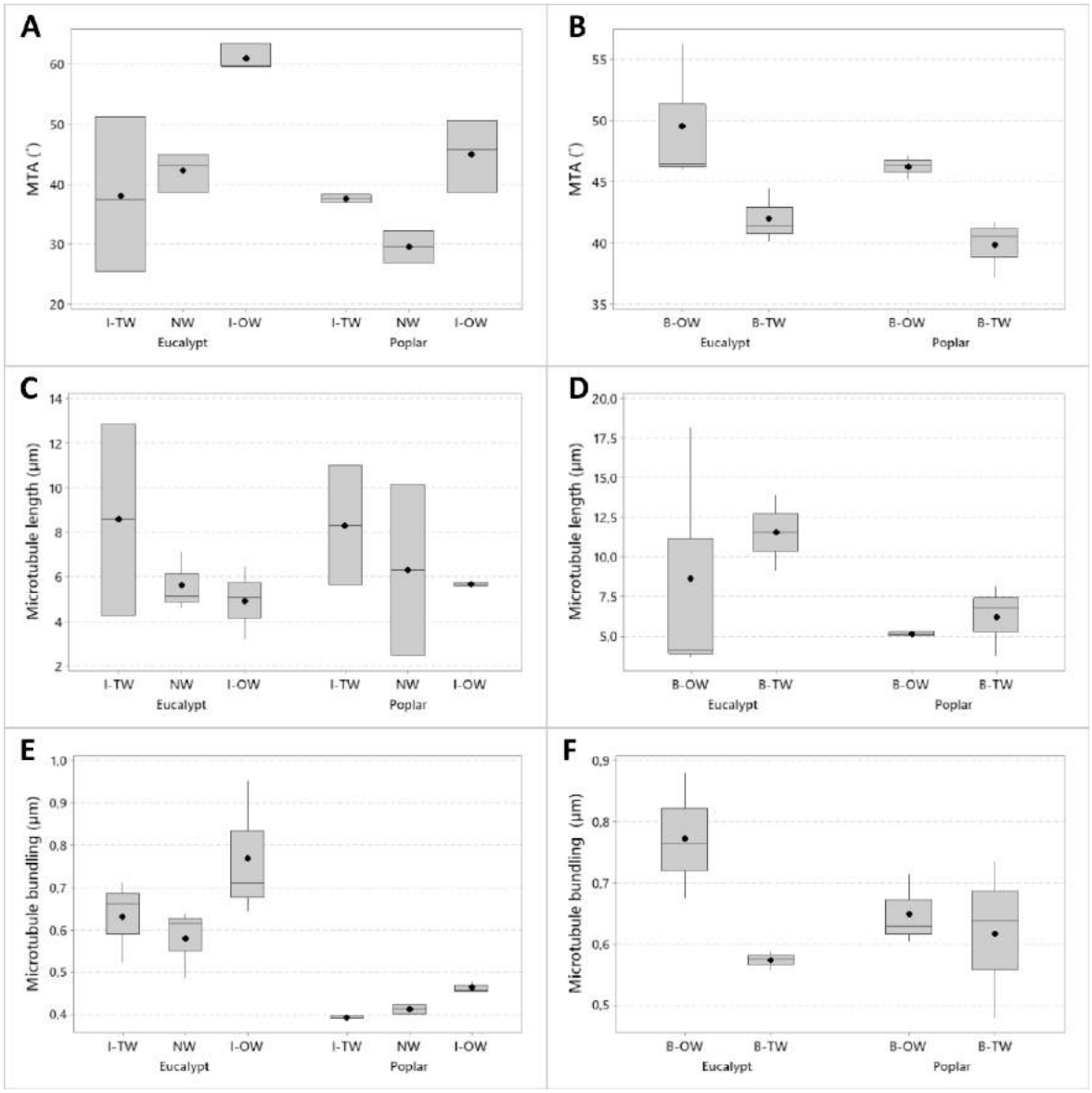

Figure 2.3: ***Tension wood traits****. Boxplot with median (crossing line) and mean (dot). Microtubule angle (MTA, A-B), microtubule length (C-D), microtubule bundling (E-F). B-OW, branch opposite wood; B-TW, branch tension wood; I-OW, induced opposite wood; I-TW, induced tension wood; NW, normal wood.*

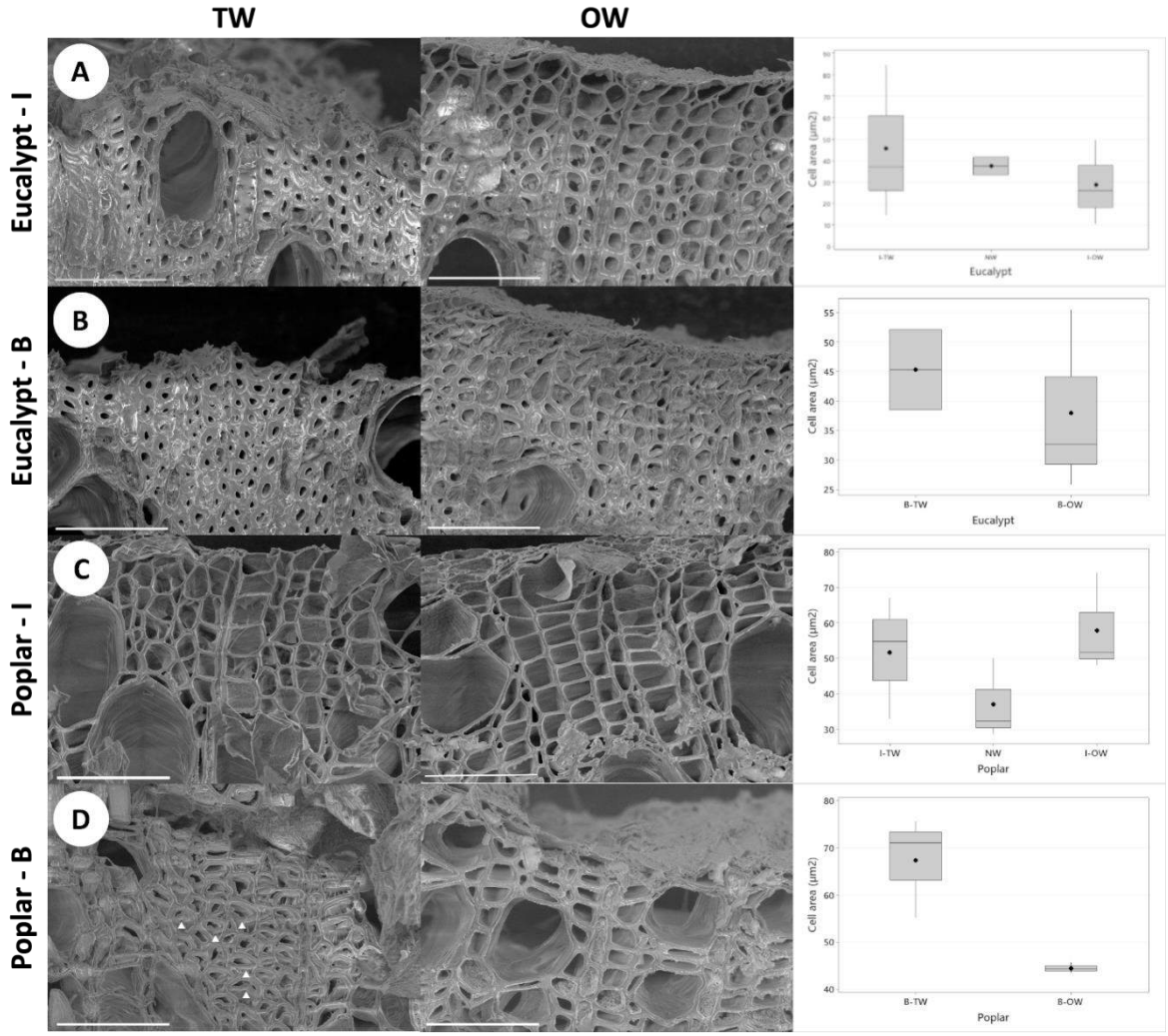

Figure 2.4: ***Cell wall thickness in tension wood****. Scanning electron micrographs of transverse sections of eucalypt (A-B) and poplar (C-D) stems (A and C) and branches (B and D) exhibiting tension and opposite wood. Notice gelatinous layer (G-layer) deposition in poplar B-TW indicated by arrowheads. Bars measure 50 µm. Boxplot with median (crossing line) and mean (dot) for cell wall area of eucalypt stems (A) and branches (B) and poplar stems (C) and branches (D).*

Microtubule branching events were found to be increased in poplar TW when compared to OW and were significantly increased in branch TW (31, P=0.035), whereas no differences were found in eucalypt samples (Fig 2.5 A and B). In addition, poplar induced TW samples featured significantly higher microtubule density (37.2, P=0.003) while this feature did not seem to be altered between TW and OW in branches of this species (Fig 2.5 C and D). Interestingly, with regards to microtubule density, eucalypts showed opposite trends in induced and branch states, with average density found to be lower in induced TW but higher in branch TW when compared to OW samples (Fig 2.5 C and D). Transverse cell area was generally larger in branch TW samples in comparison to OW in both angiosperm species (Fig 2.5 E). This trend, however, was not observed in the induced state: TW poplar

samples presented cell area values similar to OW while TW eucalypt samples generally featured smaller cell area when compared to OW (Fig 2.5 F). Moreover, eucalypt TW samples from both induced and branch states featured shorter fibres in relation to OW while in poplar, induced TW fibres were longer than OW, but no difference was observed between branch TW and OW samples (Fig 2.5 G and H). While these results demonstrate similarities in microtubule organisation during TW formation between the two angiosperm species, different microtubule traits or arrangements (e.g. microtubule branching in poplar TW) might have different functions in branch and induced states and in different species.

2.3.4 Compression wood is related to larger MTA, thicker cell walls and smaller tracheids

Microtubule organisation and cell phenotypes in compression wood (CW) formed in pine branches and bent stems were determined to investigate whether microtubules play a role in CW formation in these two experimental states. CW samples presented larger cell wall areas when compared to OW (Fig 2.6 A-E; Table B.1 of Appendix B) and especially in branch CW samples it was possible to observe typically rounded tracheids (Fig 2.6 A). Generally, tracheids were shorter and showed smaller transverse areas in CW when compared to OW (Fig 2.6 F and G). In addition, CW showed increased MTA, however no significant differences were found (Fig 2.6 H). CW showed contrasting trends for microtubule bundling, which was lower in the induced state but higher in the branch state when compared to OW (Fig 2.6 I). Density of microtubules was significantly higher in branch CW samples in comparison to OW (15.46 and 17.8, respectively; P=0.021) but no differences were found between induced CW and OW (Fig 2.6 J). More microtubule branching was observed in induced CW when compared to OW, but branch samples presented similar values for CW and OW (Fig 2.6 K). Finally, in average, microtubule length was higher in induced OW samples in comparison to CW but similar between branch OW and CW samples (Fig 2.6 L). Pine samples from induced and branch states appear to share some of the cellular mechanisms of CW formation, yet different strategies might be employed by the trees at different states.

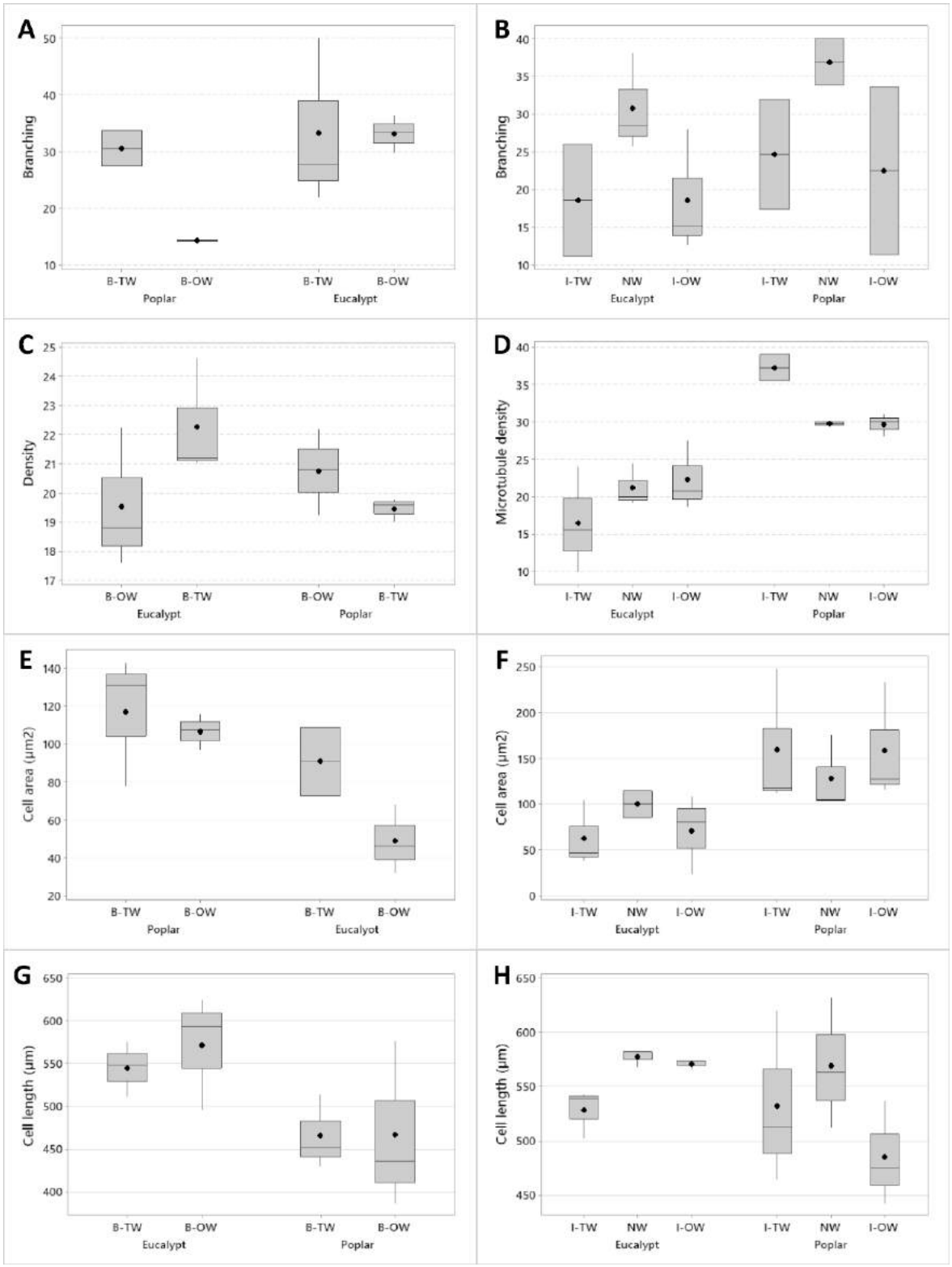

Figure 2.5: ***Branch and induced tension wood traits****. Boxplot with median (crossing line) and mean (dot) for microtubule branching (A-B), microtubule density (C-D), cell area (E-F) and cell length (G-H).*

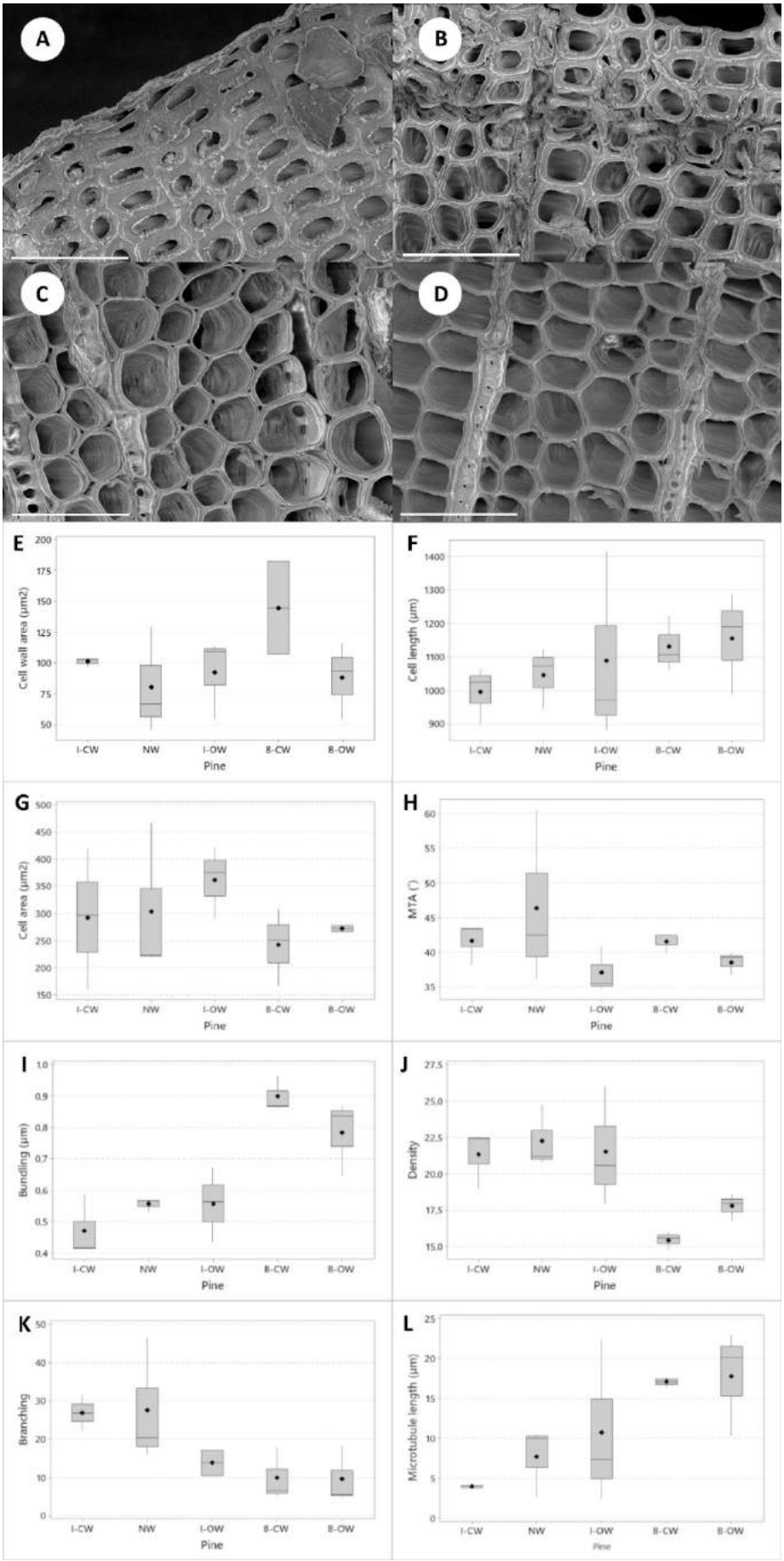
A
B
C
D
E
Cell wall area (µm2)
F
Cell length (µm)
G
Cell area (µm2)
H
MTA (°)
I
Bundling (µm)
J
Density
K
Branching
L
Microtubule length (µm)
I-CW
NW
I-OW
B-CW
B-OW
Pine

Figure 2.6: ***Compression wood traits****. Scanning electron micrographs of transverse sections of pine branches (A and B) and stems (C and D) exhibiting compression (A and C) and opposite wood (B and D). Bars measure 50 µm. Boxplot with median (crossing line) and mean (dot) for cell wall area (E), cell length (F), cell area (G), MTA (H), microtubule bundling (I), microtubule density (J), microtubule branching (K) and microtubule length (L).*

2.3.5 Reaction wood formation results in asymmetrical growth of the stem

To verify if RW formation would lead to increased wood formation, branches and stems were assessed for asymmetrical growth. Stems growing upright (NW) did not exhibit distinguishable asymmetrical growth (Fig 2.7 A, D and G) while enhanced wood formation was visually verified on the upper side of branches and bent stems of eucalypt and poplar (Fig 2.7 B-C and E-F) and on the lower side of pine branches and bent stems (Fig 2.7 H and I), being more prominent in induced RW (Fig 2.7 B, E and H).

To quantify the asymmetrical growth, pith position was calculated and further confirmed enhanced wood formation in RW samples. Pith eccentricity (*E*) increased as the pith moved further from the geometrical centre of the branch or stem. Positive values represent a geometrical centre on the upper side of the branch/stem while negative values were obtained when the geometrical centre was on the lower side of the branch/stem. *E* values were especially higher in induced samples and eucalypt induced TW samples showed the highest values while the lowest value was found in pine NW (Table 2.2).

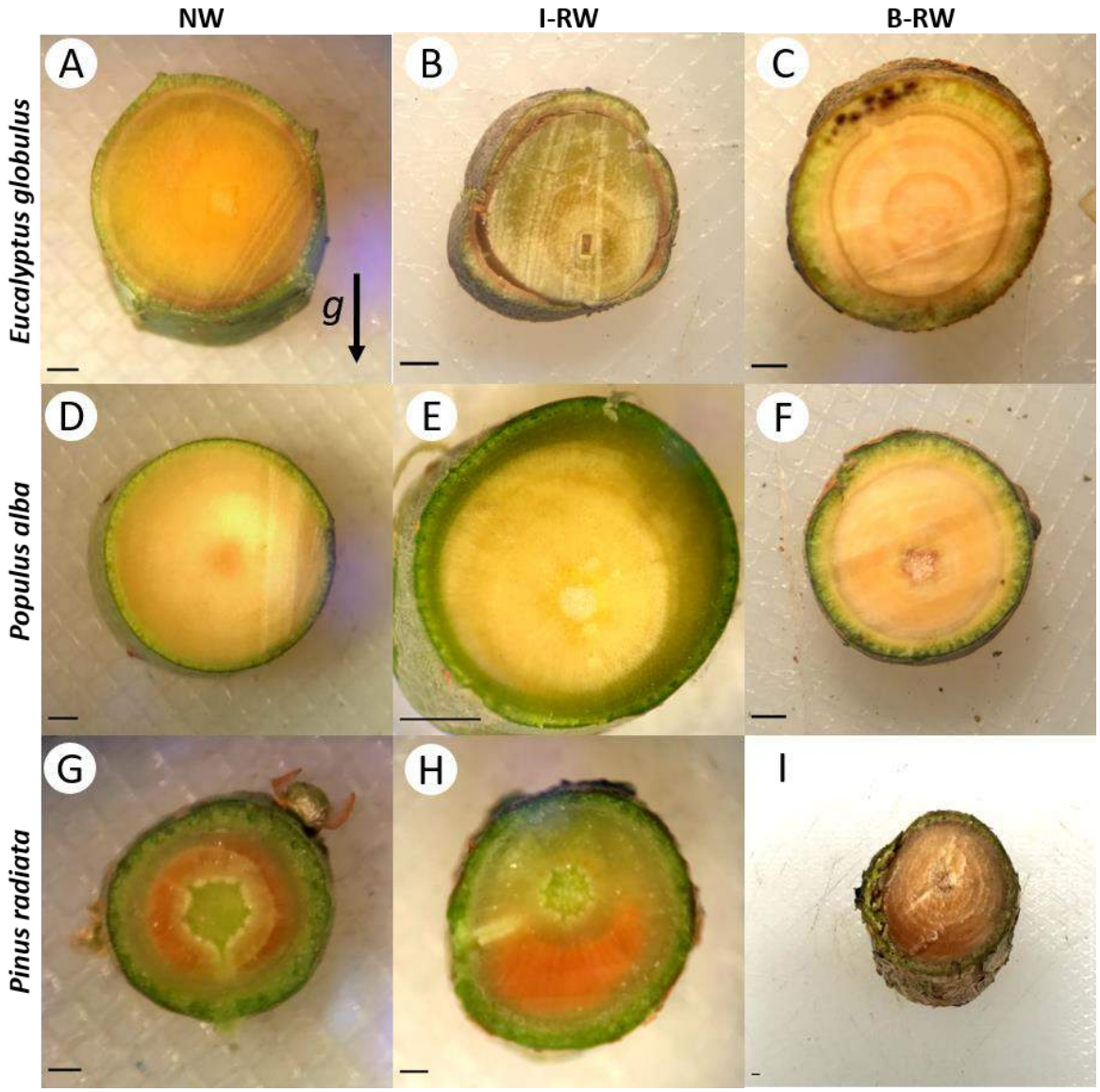

Fig. 2.7: ***Transverse sections of stems and branches showing different degrees of pith eccentricity****. Eucalyptus globulus NW (A), I-TW (B) and B-TW (C). Populus alba NW (D), I-TW (E) and B-TW (F). Pinus radiata NW (G), I-CW (H), B-CW (I). Notice the orange coloration in pine samples. NW, normal wood; I-RW, induced reaction wood; B-RW, branch reaction wood. Arrow indicates gravity (g) vector direction in relation to branch and stem sections. Bars measure 1mm.*

Table 2.2: E values for pith eccentricity (%)

	Populus alba	*Eucalyptus globulus*	*Pinus radiata*
NW	4.15	5.37	-0.27
Induced RW	29.73	43.95	-29.83
Branch RW	21.75	19.78	-21.31

2.3.6 Results summary

A summary of results for microtubule organisation and cell morphology of all three species and for each condition can be found in Table 2.3. While the statistical comparisons were made within the same state for each species (branch RW versus branch OW; induced RW and induced OW versus NW), one could still infer other biological implications from these results. For example, TW and CW seem to be formed by opposite mechanisms, considering aspects of microtubule organisation in those conditions such as MTA and microtubule length. Moreover, some of the aspects of the microtubule array organisation behind TW formation are similar between the two angiosperms and the two states of RW analysed in this study, nevertheless, different mechanisms seem to be in place for each species. These results suggest that RW is formed through a series of cellular and molecular changes in wood cells, including cortical microtubule array reorganisation, which can be different for each species and between different RW states.

Table 2.3: Results summary

Species	RW state		Microtubules					Cell morphology			
			Angle	Density	Bundling	Branching	Length	Cell wall area	Cell length	Lumen area	MFA
Populus alba	Induced	TW	+	+	-	+	+	-	+	+	-
		OW	+	-	+	-	-	+	-	+	+
	Branch	TW	-	0	0	+	+	+	0	-	-
		OW	+	0	0	-	-	-	0	-	+
Eucalyptus globulus	Induced	TW	-	-	+	-	+	+	-	-	-
		OW	+	+	-	-	-	-	-	0	+
	Branch	TW	-	+	-	0	+	+	-	+	-
		OW	+	-	+	0	-	-	+	-	+
Pinus radiata	Induced	CW	+	+	-	+	-	+	-	-	+
		OW	-	-	+	-	+	-	+	+	+
	Branch	CW	+	-	+	0	0	+	-	-	-
		OW	-	+	-	0	0	-	+	+	+

Key results of microtubule organisation and cell morphology for each species in each condition. Symbols indicate that values are higher (+), lower (-) or do not show any trend (0) in comparison to the other treatment of the same state. Red symbols indicate statistically significant results.

2.4 Discussion

2.4.1 Distinct microtubule structures might have specific functions during wood formation in woody trees

For a distinct microtubule organisation observed in this study, the term microtubule braiding was introduced. To current knowledge, this is the first report of such microtubule behaviour in any species, which suggests the possibility of a specific function for these braids during wood formation in woody trees. This braided pattern results from microtubule intertwining in different planes which is distinct from the previously reported crossover behaviour (Dixit & Cyr 2004b). Crossover encounters result from single microtubules interacting at steep-angles within the same plane, producing the “cross” pattern observed in immunofluorescent images. By contrast, braiding is a result of microtubules interlacing more than once producing a pattern recognisable on immunofluorescent images as double helices. Braiding

was most frequently observed in eucalypt and pine RW. Cellulose microfibrils have been reported to highly aggregate in the secondary cell wall, improving wall rigidity (Li *et al.* 2016). If CSCs could be guided by braiding microtubules, the existence of a mechanism allowing cellulose microfibrils to intertwine with each other could be speculated. Cellulose microfibrils deposited in the same orientation but being capable of forming higher level structures in a braided pattern would have the potential to generate stiffer or stronger cell walls.

In some instances, microtubule bundles were found to aggregate in bands that could be easily observed and were especially frequent in RW. Microtubule band formation has also been reported as a typical feature of Arabidopsis epidermal cells transdifferentiated into xylem tracheary elements induced by *VASCULAR-RELATED NAC-DOMAIN6* (*VND6*) and *VND7* transcription factors (Kubo *et al.* 2005; Yamaguchi *et al.* 2010) and their presence has been suggested to function as a template for helical secondary cell wall thickenings in both fibres and tracheids (Funada *et al.* 2001; Lloyd 2011). Formation of microtubule bands might be involved in patterned secondary cell wall thickening in RW.

2.4.2 MTA plays an important role in MFA determination during wood formation

The angle at which cellulose microfibrils are deposited in the secondary cell wall is an important characteristic of RW formation and TW has often been described to feature very low MFA while OW reportedly presents larger angles (Washusen *et al.* 2005; Almeras & Clair 2016; Roignant *et al.* 2018). In this study, such low values of MFA were not recorded, however MFA, as well as microtubule angle, was lower in TW than in OW, even though no statistical difference was found. It might well be the case that young seedlings like the ones used for this analysis usually feature larger MFAs than mature trees (Barnett & Bonham 2004). Microfibrils are described to shift their orientation to lower angles towards the end of secondary cell wall deposition (Prodhan *et al.* 1995; Abe & Funada 2005). For these analyses, fibres were collected at between 5 to 10 cell files from the cambial zone, and therefore, they might have been at different stages of development. Finally, while NW is frequently described to have intermediate MFA values, in a gradient between TW and OW (Almeras & Clair 2016), in poplar samples NW presented the lowest values for both microtubule angle and MFA.

It is generally accepted that microtubules guide the CSC (Paradez *et al.* 2006; Lei *et al.* 2014; Komis *et al.* 2015; Watanabe *et al.* 2015) and Chan and Coen (2020) recently demonstrated that microtubule guidance overrides autonomous CSC trajectories in response to environmental and developmental cues. Therefore, it seems reasonable to expect that MFA will reflect MTA. Changes in microtubule orientation in response to bending have been reported (Furusawa *et al.* 1998) and previous studies have shown correspondence between microtubule and microfibril orientation (Abe *et al.* 1995; Prodhan *et al.* 1995; Furusawa *et al.* 1998). However, due to the dynamics of microtubule arrays, which undergo constant reorganisation in response to environmental cues (Nick 2013), it is unlikely that they remain unchanged following the deposition of cellulose microfibrils in the S_2 layer of the secondary cell wall. Furthermore, microfibril orientation does not exclusively result from microtubule guidance and it might change due to interaction with adjacent microfibrils and non-cellulosic components (McFarlane *et al.* 2014). Therefore, MFA might have to be seen as the result of a combination of different interactions with the constantly shifting microtubule cortical array and with other cell wall components. Results show a strong positive correlation between MFA and MTA in poplar samples, which suggests that even though other factors might influence the orientation of newly deposited cellulose microfibrils, MTA plays an important role in MFA determination during wood formation.

2.4.3 Microtubule branching is important for array reorientation

While animal cells have a microtubule organising centre, called a centrosome, in plant cells the formation of new microtubules, nucleation, occurs dispersed at the cell cortex, throughout the cytoplasm, and near the nucleus (Dixit & Cyr 2004a; Hashimoto 2015). Visualisation of microtubule dynamics in live cells of Arabidopsis seedlings revealed that nucleation centres, formed by γ-tubulin-containing ring complexes (γTuRC), are activated along the sides of cortical microtubules, where they instantly promote microtubule nucleation (Nakamura *et al.* 2010; Nakamura 2015). In the present study, nucleation events were counted as branching occurrences in the microtubule array. Branching was more prevalent in NW of all three species and occurred more frequently in RW in comparison to OW samples. Computer simulation models showed that branched nucleation increased array polarity (i.e. microtubule growth towards the same direction) and that changes in the mean branch angle increased the probability of the array to shift at least 20° from the initial transverse orientation (Eren *et al.* 2010). Indeed, several studies have demonstrated that

branched nucleation followed by depolymerisation of mother microtubules is the main mode of array reorientation (Nakamura 2015; Oda 2015; Chen *et al.* 2016). Observations of more frequent nucleation events in poplar samples, which featured the lowest MTA and MFA are in agreement with such models, since it is important that microtubules are correctly orientated during RW formation.

2.4.4 Microtubule reorganisation during reaction wood formation might have a role in cell wall thickening

Because microtubules play an important role in diffusely growing cells (Wasteneys & Galway 2003), changes in fibre and tracheid length and transverse area were investigated. No statistical differences were found across the different treatments in all three species. However, except for poplar, shorter cells were usually associated with RW formation. Because cell elongation occurs prior to secondary cell wall deposition, and despite smaller cells being a common feature of RW, the absence of a clear relationship between cell size and microtubule organisation in cells depositing a secondary cell wall is not surprising.

Finally, tubulin genes are highly expressed in RW. In TW, for example, expression levels of some tubulin genes can increase as much as 3.4-fold in comparison to OW (Qiu *et al.* 2008). While, as a result, there may be more of these globular proteins available in RW cells, exactly how the quantity of tubulins might determine microtubule assembly in those cells is not known. In this study the quantity of tubulins assembled into microtubules was assessed by measuring the average length of microtubules, the number of branching events, the thickness of microtubule units (microtubule bundling) and microtubule density. However, those measurements account only for tubulins polymerised into microtubules while gene expression reports are likely to have included free tubulins, or the augment in tubulin expression in these reports may be a mere result of a larger number of cells on the side of the branch/stem producing RW.

Generally longer microtubules were observed in TW and also density of microtubules in poplar induced state was significantly increased, which might be a result of such an augment in tubulin content. This is in alignment with Prodhan *et al.* (1995), who reported that microtubules at a high density play an important role during secondary cell wall deposition in woody trees, especially during TW formation. RW formation was associated with thicker cell walls, which suggests greater CSC activity in those cells and,

hence, increased cellulose deposition. In this case, more or longer microtubules, as observed in poplar and eucalypt TW samples, would be required to ensure the guidance of more CSCs in the plasma membrane at a given time. Indeed, changes in microtubule orientation in the Arabidopsis mutant *fra2* have been shown to affect cell wall thickness and cellulose biosynthesis (Burk & Ye 2002). The development of a gelatinous layer (G-layer) observed in branch TW poplar samples is a common response to gravitational stimulus and it is believed to rapidly generate tension stress (Andersson-Gunneras *et al.* 2006; Abedini *et al.* 2015). Changes in microtubule organisation might well be related to the deposition of a G-layer in poplar TW cells. Although eucalypts do not develop a G-layer, samples from induced TW were almost twice as thick as OW, suggesting an alternative strategy employed by this species to cope with sudden exposure to gravitational stimulus since branch wood samples did not present such sharp differences. In pine, CW samples presented thicker cell walls and branch CW samples featured tracheids with walls twice as thick as OW samples. However, microtubule length and bundling are lower in induced CW and microtubule density appears not be affected by RW formation and only microtubule branching was found increased in CW. In combination, these results suggest that cell wall thickening is related to RW formation and that reorganisation of the cortical microtubule array might have a role in promoting cellulose deposition through increases in CSC activity.

2.4.5 Asymmetrical growth acts in readjusting the stem to an upright position and in maintaining branch angles

Here, induced RW formation resulted in more eccentric piths, and sudden exposure to gravitational stimulus generated by tilting the stems caused enhanced tissue growth on the RW side. These results are consistent with other studies which demonstrated that RW is typically associated with increased wood formation on the upper or the lower sides of leaning branches and stems, in angiosperms and gymnosperms, respectively, leading to pith eccentricity (Wardrop & Dadswell 1950; Fisher & Stevenson 1981; Jourez *et al.* 2001). Interestingly, in this study, this response was found to be less pronounced in branch wood, which presented only mild pith eccentricity. In this respect, Roignant *et al.* (2018) reported higher pith eccentricity in permanently leaning stems when compared to stems subjected to single unidirectional bending and postulated that the degree of pith eccentricity is related to an increase in the applied strain. Results suggest that when tilted to their side, the stem rapidly grows asymmetrically in response to the elevated strain attempting to readjust to an

upright position through an increase in wood formation which, in combination with cellular alterations, acts by either “pushing” or “pulling” the leaning stem back upright in compression and tension wood, respectively. In branches, on the other hand, wood cells are exposed to gravitational stimulus from the moment of their initiation, which means that these branches are not necessarily trying to regain an upright position and, thus, the asymmetrical response is less prominent. In this state, the role of RW is restricted to solely maintaining the angle of a branch in relation to the main stem.

2.5 Conclusions

Microtubule organisation and cell morphology varied across all treatments, indicating a response to gravitational stimulus. Microtubules were demonstrated to play important roles in determining cellular features in RW to some degree in respect to cell wall thickening, but mainly in determining the MFA. RW formation was demonstrated to occur by different processes in angiosperms and gymnosperms and in different angiosperm species. Differences were also found between artificially induced RW and RW spontaneously formed in branches. In summary, the results demonstrate that changes at molecular and cellular levels assist woody trees in coping with gravitational stimulus and that different species employ different strategies of RW formation. Unravelling exactly how differential cortical microtubule array organisation is triggered during RW formation, how changes in this organisation determine morphogenesis of xylary cells in response to environmental signalling, and whether different tubulin isoforms have different roles in wood formation will be the subject of further investigations.

Chapter 3: Microtubule-interacting drugs affect cellulose microfibril angle of woody tree species *in vivo* and *in vitro*

Abstract

Microtubule reorganisation appears to be a key response to gravitational stimulus, since formation of tension wood (TW) occurs through a series of cellular modifications coordinated, to some degree, by the cortical microtubule array. Microtubule-interacting drugs were employed to study microtubule dynamics during secondary cell wall (SCW) deposition in wood cells. Specifically, paclitaxel, a secondary metabolite produced by yews that reduces the dynamicity of the microtubule array, was injected *in vivo* on the upper side of horizontal poplar stems to study the effects of cortical microtubule array stabilisation on TW development in poplar trees. In response, microtubule angle (MTA) and cellulose microfibril angle (MFA) were found to be larger when compared to the upper side of untreated stems. In addition, an apical stem segment system for *in vitro* wood formation was developed for eucalypt, poplar and radiata pine to assess the effects of oryzalin, a well-known microtubule polymerisation inhibitor, on MFA. The unique conditions promoted by *in vitro* culture were advantageous to investigating changes in cellulose microfibril orientation in SCW in response to cortical microtubule array disruption. Cells cultured in medium containing oryzalin exhibited destabilised microtubule arrays, however cellulose microfibrils were capable of self-alignment independently from microtubules in lower angles when compared to those cultured in control medium. Altogether, these results provide evidence that perturbation of cortical microtubule array organisation leads to changes in microfibril orientation, implying that microtubules are involved in MFA determination of developing wood cells.

3.1 Introduction

Plasma membrane embedded cellulose synthase complexes (CSCs) move along tracks delineated by cortical microtubules in the primary cell wall (PCW) (Paredez *et al.* 2006). Studies in Arabidopsis elongating cells have provided important insights into the role of the cortical microtubule array in determining cellulose microfibril orientation, which play a critical role in anisotropic growth (Baskin 2005). When compared to the PCW, the secondary cell wall (SCW) presents differences in composition and mechanical properties, however cellulose deposition occurs in a similar manner (Watanabe *et al.* 2015). In woody trees, tubulin gene expression has been demonstrated to be enhanced on the upper side of angiosperm branches forming tension wood (TW) (Qiu *et al.* 2008) and an *Eucalyptus grandis* β-tubulin gene was implicated in directly contributing to the determination of cellulose microfibril angle (MFA) in transgenic eucalypt fibres *in vivo* (Spokevicius *et al.* 2007). Such gene expression studies often involve the use of reaction wood (RW) as a model, and the same system can also be used to study the roles played by microtubules during wood formation as demonstrated in Chapter 2. Studies employing pharmacological approaches have provided compelling evidence regarding cytoskeleton functions in Arabidopsis (Wang & Mao 2019), encouraging their utilisation to investigate microtubule roles in wood formation in trees by adding microtubule-interacting drugs to both *in vivo* and *in vitro* systems.

Paclitaxel is a taxane produced by plants within the genus *Taxus* (yews) that binds to polymerised tubulins and enhances microtubule polymerisation by lowering the critical concentration (*C*c) of the tubulin heterodimer, below which tubulin assembly does not occur spontaneously (Schiff *et al.* 1979). Treatment with paclitaxel reduces microtubule kinetics and, consequently, the dynamicity of the cortical array (Kumar 1981; Morejohn & Fosket 1984) and it has been suggested to enhance microtubule nucleation events (Bokros *et al.* 1993). Microtubule reorientation does not occur in the presence of paclitaxel (Seagull 1990) and cells subjected to paclitaxel-induced microtubule array stabilisation are more susceptible to cold and hyperosmotic conditions, suggesting that microtubule reorganisation is a key response to stress (Wang *et al.* 2007; Wang & Nick 2017). Nucleation of microtubules followed by depolymerisation of mother microtubules are the main mechanisms by which microtubules shift their orientation within an array (Lindeboom *et al.* 2013; Nakamura 2015; Chen *et al.* 2016), making them important steps of TW formation.

Oryzalin (3,5-dinitro-N^4,N^4-dipropylsulfanilamide) is a dinitroaniline herbicide, a large class of herbicides that produce morphological abnormalities in plants and disrupt cell division (Morejohn *et al.* 1987). Bartels and Hilton (1973) were the first to report that oryzalin interferes with microtubule synthesis and its mode of action was then further characterised by Strachan and Hess (1983), who reported that oryzalin binds to plant tubulin and forms a stable complex which is incapable of polymerising into microtubules. This microtubule inhibitor has been extensively used in different plant organs and species as an antagonist of paclitaxel effects (McNally & Vale 1993; Baskin *et al.* 1994; Sugimoto *et al.* 2003). Microtubule behaviour was investigated in a number of species and cell types treated with oryzalin, resulting in important findings regarding microtubule-dependent microtubule nucleation (Tian *et al.* 2004; Murata *et al.* 2005), cell ploidy (Lam *et al.* 2014), cell morphogenesis (Baskin *et al.* 1994; Hashimoto 2013; Swamy *et al.* 2015) and plant physiological response to stress (Wang *et al.* 2007; Ban *et al.* 2013; Endler *et al.* 2015). The functions of microtubule associated proteins (MAPs) (Xu *et al.* 2009; Mitra *et al.* 2019) and the roles of microtubules in guiding the CSC (Gardiner *et al.* 2003; Schneider *et al.* 2017; Yang *et al.* 2019) were also studied with the aid of this microtubule inhibitor. Furthermore, drug-dependent microtubule disruption resulted in loss of localised SCW deposition pattern in Arabidopsis developing vessels (Gardiner *et al.* 2003), suggesting that cytoskeleton roles in wood development studies could be further elucidated by employing oryzalin treatments.

To further investigate the roles of microtubule dynamics in wood formation, previously described microtubule-interacting drugs, paclitaxel and oryzalin, were applied to wood tissue depositing SCW. Because reorganisation of the cortical microtubule array seems to be a key step during TW formation, the effect its stabilisation on TW development in poplar trees was studied by applying paclitaxel to an *in vivo* TW induction system using trunk injection. This technique has been used since the 15th century to deliver pesticides, plant growth regulators and other chemicals directly to a tree's vascular system (Berger & Laurent 2019). Additionally, to monitor changes to cellulose microfibril orientation in response to cortical microtubule array disruption, a technique first described by Leitch (1999) in *Eucalyptus globulus* Labill, the apical stem segment (ASS) system of *in vitro* culture, was employed. Although other studies have used young apical stem tissues, this system represents the first validated method for culturing fully differentiated xylogenic cells and allows for investigations into cambial development and wood formation under *in vitro*

conditions. Due to its reliable production of close-to-normal wood, this method has been successfully used for genetic transformation experiments with promising results (Spokevicius *et al.* 2005). It allowed us to assess the effects of oryzalin on microtubule organisation and MFA determination in eucalypt, poplar and radiata pine woody tissue grown in culture.

3.2 Material and Methods

3.2.1 In vivo studies

1-year-old *Populus alba* L. trees were grown under glasshouse conditions and TW formation was induced (see chapter 2.2.1 for details on growth conditions and RW formation induction). Four trees growing upright were injected with DMSO and 0.1% safranin (W387520 and S2255, respectively, www.sigmaaldrich.com/australia) containing 30 μM paclitaxel (T7402, www.sigmaaldrich.com/australia) at 120 cm height (Fig 3.1 A). A syringe containing 1 mL was introduced through the bark, so the solution was in contact with the vascular cambium. The syringe was left in place during the entire experiment to allow the solution to move passively through the vascular tissue and gentle thumb pressure was applied daily to assist uptake. Three control trees were injected with the dye solution only. After 24 h, all trees were tipped on their side at 90˚ to promote TW formation and were allowed to grow seven days in this position (Fig 3.1 B). A small portion (~1 cm^3) of the upper side of the stems was collected from 1 cm and 10 cm above the injection point and microtubule organisation, MFA and cell wall thickness were assessed in each sample.

Microtubule organisation, MFA, cell wall thickening, and gelatinous layer (G-layer) deposition were assessed in each sample according to Chapter 2.2. The uplifting response (Fig 3.2) was given by the difference in degrees between the inclinations of the stem apex measured on day 1 (right after pots were tipped to the side) and on day 7 (before sample collection). Pith eccentricity was calculated as described in Chapter 2.2.6.

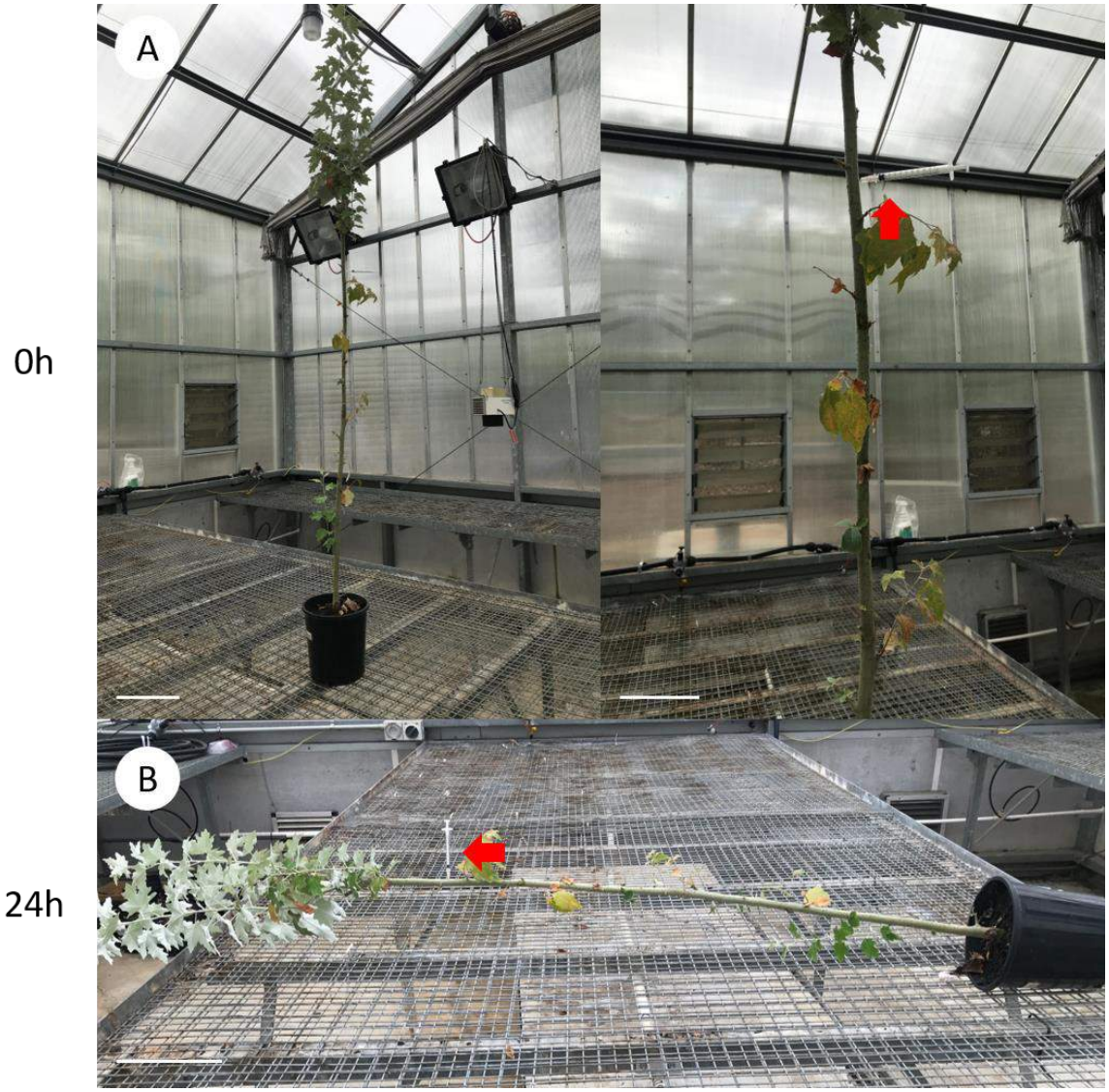

Figure 3.1: ***Stem Injection.*** *DMSO 0.1% safranin solution containing 30 µM paclitaxel was injected in stems growing upright (A). After 24 h pots were tipped on their side and trees were allowed to grow for seven days in this position (B). Red arrow marks the syringe. Bars measure 10 cm.*

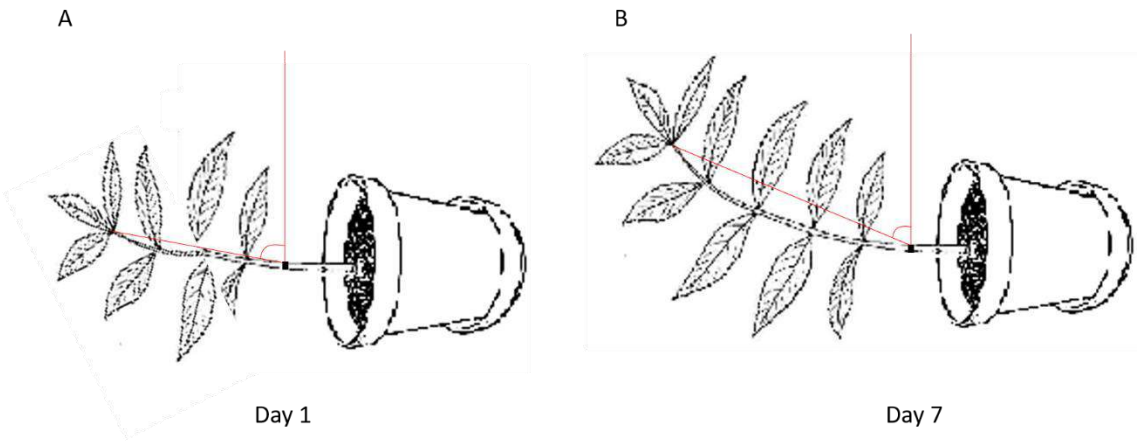

Figure 3.2: ***Uplifting response.*** *Inclination of the leaning stems on day 1 (A) and day 7 (B) measured by the angle between the apex of the stem and a point marked on the horizontal portion of the stem. Uplifting response is given by the difference (in degrees) of the angles on D1 and on D7.Free clipart used to compose this scheme were sourced from D G Mackean www.biology-resources.com and www.pinclipart.com.*

3.2.2 In vitro studies

In preparation for the establishment of callus cultures, apical stem sections of up to 30 cm were harvested from young stems of *Eucalyptus grandis x camaldulensis*, *Populus alba* and *Pinus radiata* D.Don. that had been cultivated under controlled glasshouse conditions (Chapter 2.2.1). These stem sections were sterilised using detergent (3% Decon 90, www.decon.co.uk/products/decon90) for 10 minutes followed by another 30 minutes. Stems were then immersed in 0.4% sodium hypochlorite for 10 minutes twice before being immersed in 0.9% hydrogen peroxide for 30 minutes. Finally, they were washed in double-distilled water for one hour. Double-distilled water was also used for rinsing between each treatment.

Sterilised and washed stems were transferred to a laminar flow cabinet, dipped in 70% ethanol and left to dry on sterile filter paper. Stems were then cut into segments of about 1 cm in length using a sterilised razor blade and transferred to solidified culture medium (Table 3.1) in sterile Petri dishes with their apical ends inserted into the medium and left to produce wound callus and new xylogenic tissue under continuous light (standard cool white fluorescent, 36 Watts) at room temperature (~24°) with a 2-week subculture interval. In the sixth week, half of the segments which had produced new growth were transferred to media containing 3 µM of Oryzalin (36182, www.sigmaaldrich.com/australia) and after 72 h, 20 segments per species (10 oryzalin

treated and 10 control) were harvested, cut longitudinally and fixed as described in Chapter 2.2.2.

Table 3.1. Composition of tissue culture medium

Lloyd & McCown woody plant basal salt mixture[1] (g/L)	2.3
Murashige & Skoog modified vitamins (x1000)[1] (ml/L)	1
D-glucose (g/L)[2]	25
NAA (mg/L)[1]	0.5
GA (mg/L)[2]	2.5
Phytagel (g/L)[2]	3

[1]: Austratec, Australia (www.austratec.com.au)
[2]: Sigma, Australia (www.sigmaaldrich.com/australia)

3.2.3. Data analysis

Five fibres per sample were analysed except where otherwise stated. Means were compared using one-way ANOVA assuming equal variances with α=0.05 and a Tukey pairwise comparison. Confidence intervals were calculated at 95% using Minitab 19 statistics package.

3.3 Results

3.3.1 Microtubule organisation was affected by paclitaxel injection in tension wood forming poplar trees

Microtubule organisation was studied as described in Chapter 2.2.2 to investigate if stabilisation of the cortical array by paclitaxel injection had occurred and affected microtubule organisation.

Microtubule bundling was found to be higher in fibres treated with paclitaxel (P=0.251, Figure 3.3 A) as were branching events (P=0.866, Fig 3.3 B). Density of microtubules was the highest in control samples collected at 10 cm above the injection point and the lowest in paclitaxel injected samples collected at the same height, however similar values were obtained between control and paclitaxel at 1 cm above the injection point (P=0.456, Fig 3.3 C). Finally, microtubule length was generally similar in all treatments, but microtubules were slightly longer in paclitaxel treated fibres at the height of 1 cm (P=0.834,

Fig 3.3 D). In summary, treatment with paclitaxel possibly enhanced microtubule bundling and branching occurrence in xylem fibres.

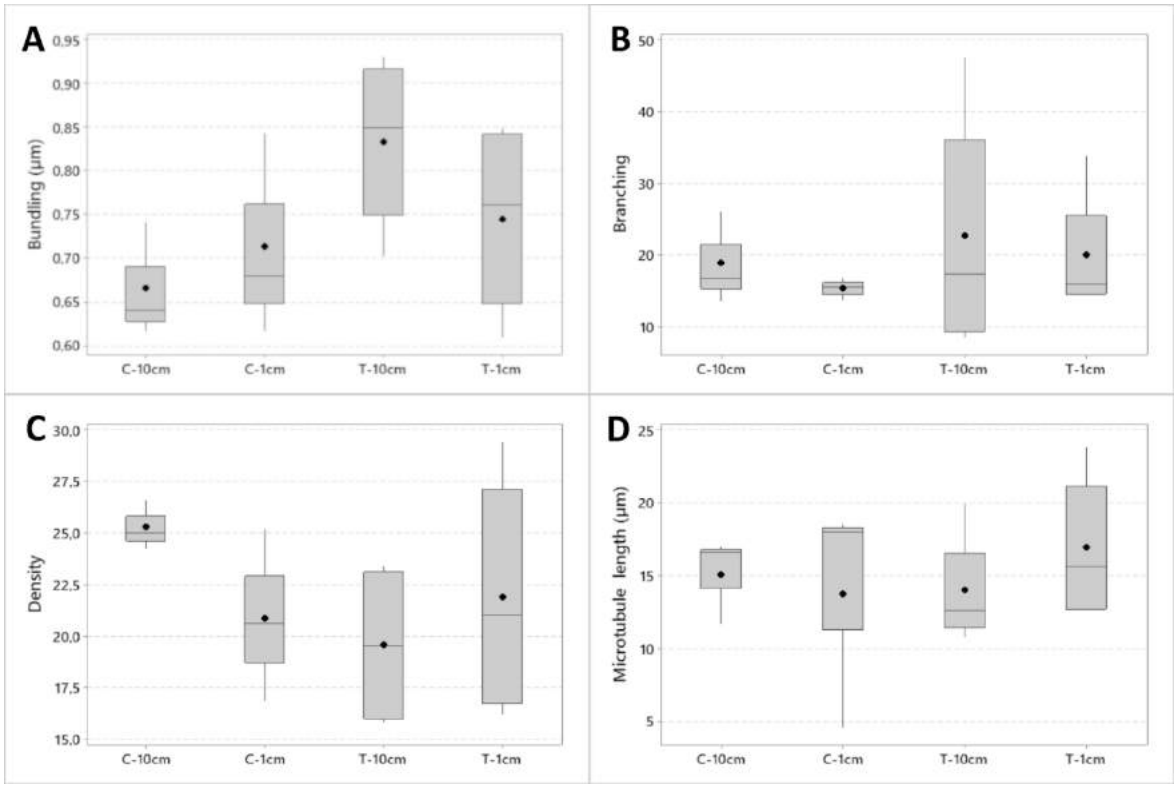

Figure 3.3: ***Microtubule organisation.*** *Boxplot with median (crossing line) and mean (dot) for microtubule bundling (A), microtubule branching (B), microtubule density (C) and microtubule length (D). C, control; T, 30 µM paclitaxel at 1 cm and 10 cm from injection point.*

3.3.2 Paclitaxel affected MFA of developing fibres

Typical changes of TW formation in poplars include decrease in MFA, increase in cell wall thickness and G-layer deposition. To investigate if stabilisation of microtubules would impact TW formation, MFA and SCW development were analysed in samples from stems injected with paclitaxel using confocal and scanning electron microscopy (see Chapter 2.2 for details).

MFA was found to be larger in fibres treated with the paclitaxel solution when compared to control fibres by ~11.9° at 1 cm above the injection point and by ~5.4°at 10 cm (P=0.102, Fig 3.4 A). In addition, MTA was generally larger in fibres treated with paclitaxel in comparison to control fibres at both heights by ~5.8° at 1 cm above the injection point and ~5.2° at 10 cm, however no significant difference was found (P=0.165, Fig 3.4 B). Furthermore, a high correlation between MFA and MTA in all samples was verified (Fig

3.4 C). Pearson coefficients are: Control – 1 cm: 0.970, Control – 10 cm: 0.970, paclitaxel – 1 cm: 0.920 and paclitaxel – 10 cm: 0.937. Paclitaxel promoted microtubule alignment at larger angles, which possibly impacted cellulose microfibril orientation, given by the large MFA of fibres treated with the drug.

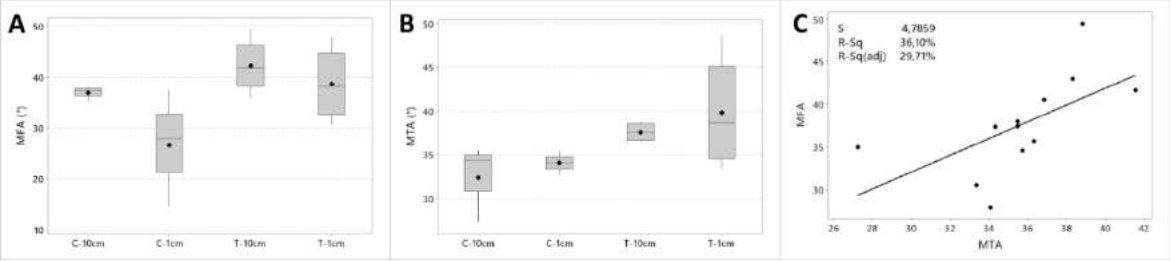

Figure 3.4: ***MFA and MTA in fibres treated with taxol.*** *Boxplot with median (crossing line) and mean (dot) for microfibril angle (A) and microtubule angle (B). C, control; T, 30 μM paclitaxel at 1 cm and 10 cm from injection point. Scatterplots of average MFA and MTA values (15 cells measured per treatment) with regression line and R^2 (C).*

Cell wall area and cell wall thickness were mostly reduced in paclitaxel injected samples in comparison to controls at both sampling points, and generally smaller at 10 cm above the injection point under both conditions (P=0.136 and P=0.336, respectively; Fig 3.4 B and C). Finally, G-layer formation was documented both in control and paclitaxel treated samples to the same extent (Fig 3.5). Treatment with paclitaxel resulted in larger MFA and reduced cell wall thickness, but no effects were found in relation to G-layer development.

3.3.3 Stems injected with paclitaxel demonstrated minor uplifting response but no asymmetrical growth

Asymmetrical growth, often determined by pith eccentricity (*E*), and uplifting of leaning stems are commonly associated with TW formation in *Populus* (Matsuzaki *et al.* 2007; Groover 2016). Therefore, the impact of paclitaxel on these stem responses was investigated. paclitaxel treatment did not affect pith eccentricity during the time of the experiment since control and paclitaxel treated stems demonstrated similar *E* (P=0.45, Fig 3.6). Control trees were able to uplift their stems by 2.6±1.3 degrees within seven days while trees injected with paclitaxel were uplifted by 0.87±0.5 degrees. However, no significant difference was found between these means (P=0.14). Stems injected with paclitaxel showed diminished uplifting response but, given similar *E* values, no evident changes on asymmetrical lateral growth.

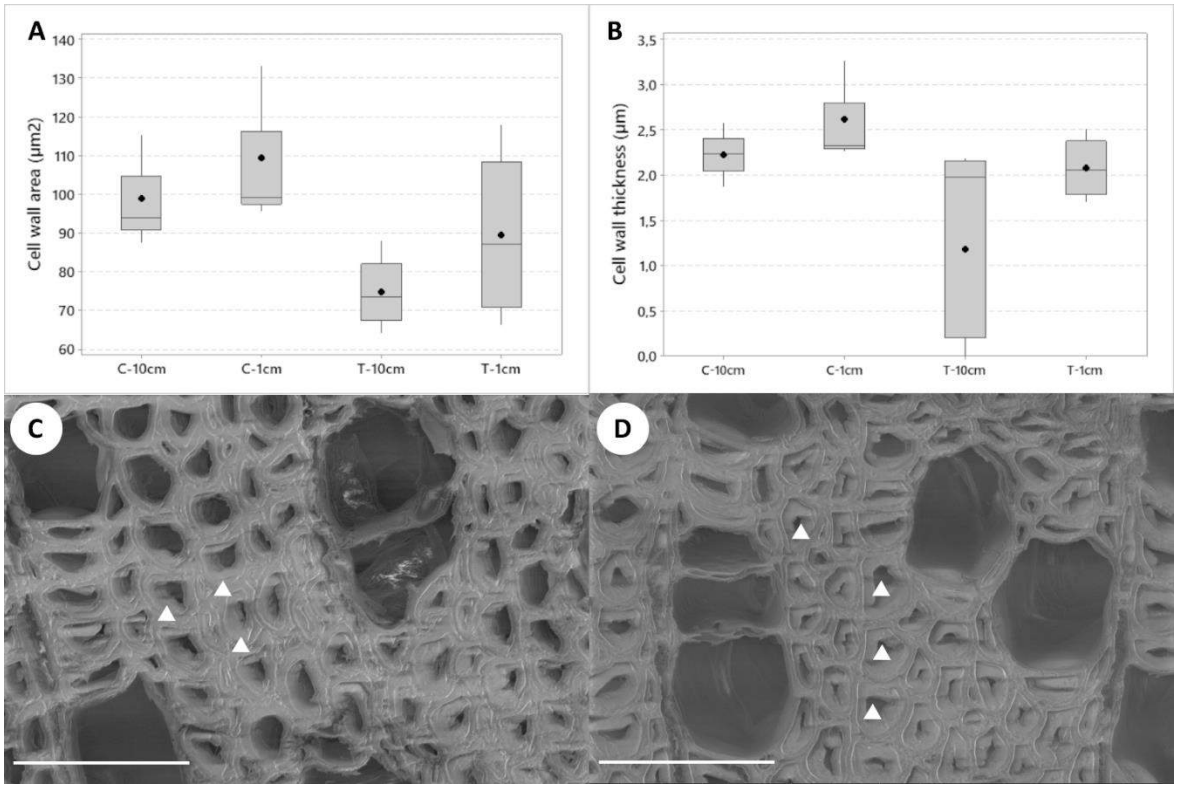

Figure 3.5: ***Cell wall traits.*** *Boxplot with median (crossing line) and mean (dot) for cell wall area (A) and cell wall thickness (B). C, control; T, 30 µM paclitaxel at 1 cm and 10 cm from injection point. Scanning electron micrographs of transverse sections of poplar stems (C-D). Notice gelatinous layer (G-layer) deposition in control (C) and paclitaxel treated (D) samples indicated by arrowheads. Bars measure 50 µm.*

3.3.4 Callus growth and wood formation were detectable in apical stem segments cultured in vitro

Apical stem segments were used as explants to promote callus growth and wood formation *in vitro*. Callus started growing on the exposed ends of segments above the media after approximately two weeks. Calluses were pale brown or green to white (Fig 3.7 A-C). The concentration of plant growth regulators used in the culture medium had been demonstrated to promote wood formation (Leitch 1999). *In vitro* wood formation occurred following the re-establishment of cambium activity. Wound parenchyma was formed between cells produced prior to culture and those produced in culture. This region was distinguishable in transverse and longitudinal hand-sections as cells with different sizes and thin cell walls in a disorganised pattern (Fig 3.7 D-K). Where cambial activity was regained, organised layers of xylogenic cells resulting from periclinal division were observed. In some cases, like in eucalypt stems (Fig 3.7 F-G), it was not possible to observe a discernible cambial zone and a severe wound was visible before the beginning of new wood development and callus growth. In other cases, observed in pine and poplar, the cambium was re-established shortly

after development of an initial wound and promoted new wood formation in bundles surrounded by disorderly callus mass (Fig 3.7 H-K). Differentiation of all xylogenic cell types were observed to the extent of 2 to 8 eight cells in a radial file with normal SCW deposition observed in longitudinal sections stained with Fluorescent Brightener 28 (Fig 3.7 L-M). Therefore, the described system promoted wood formation *in vitro* in different woody tree species after 6 weeks in culture.

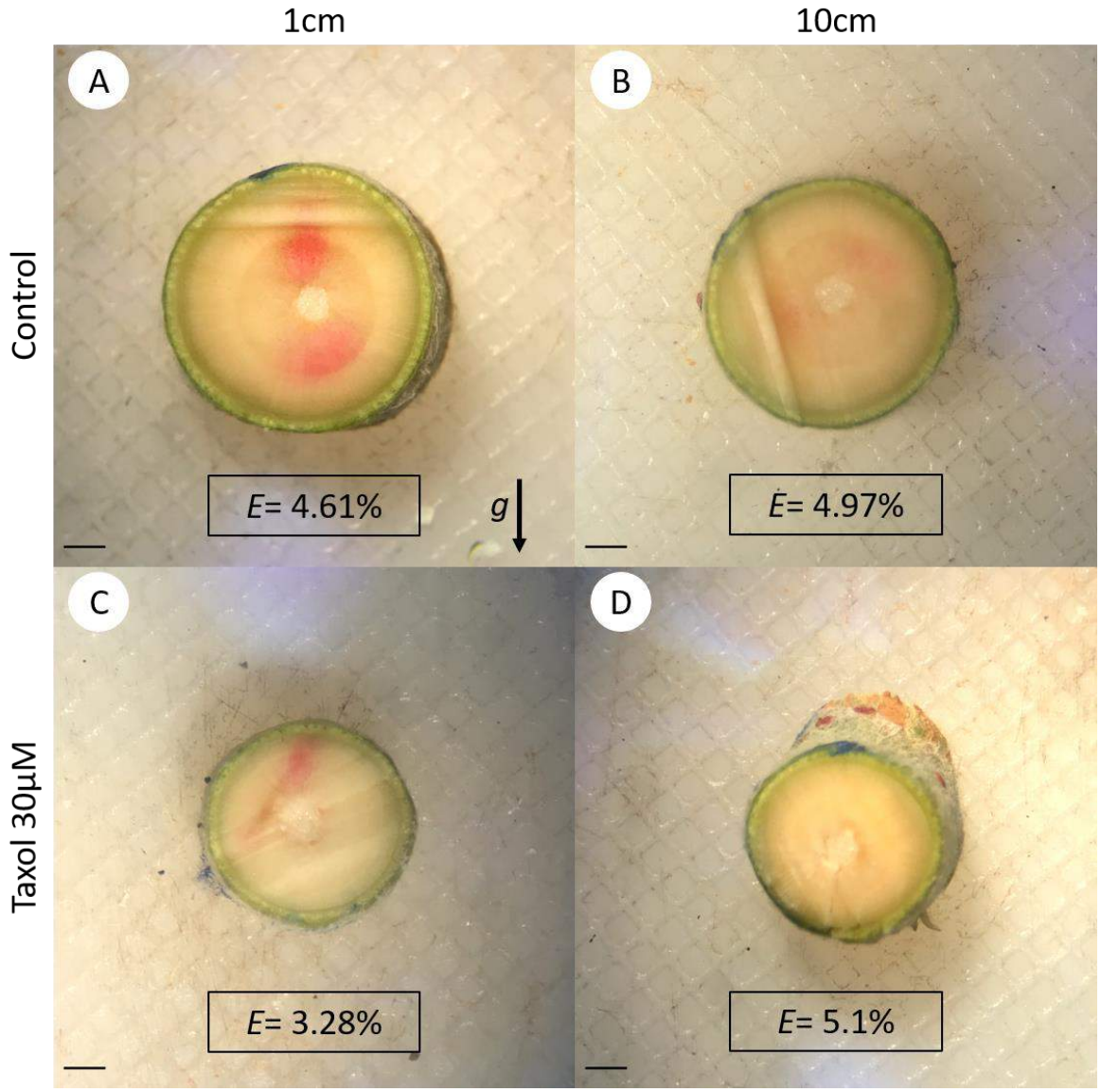

Figure 3.6: ***Pith eccentricity.*** *Transverse sections of poplar stems treated with control solution (A-B) or paclitaxel 30 µM solution (C-D) collected at 1 cm or 10 cm above the injection point with respective average E. . Arrow indicates gravity (g) vector direction in relation to stem sections. Bars measure 2 mm.*

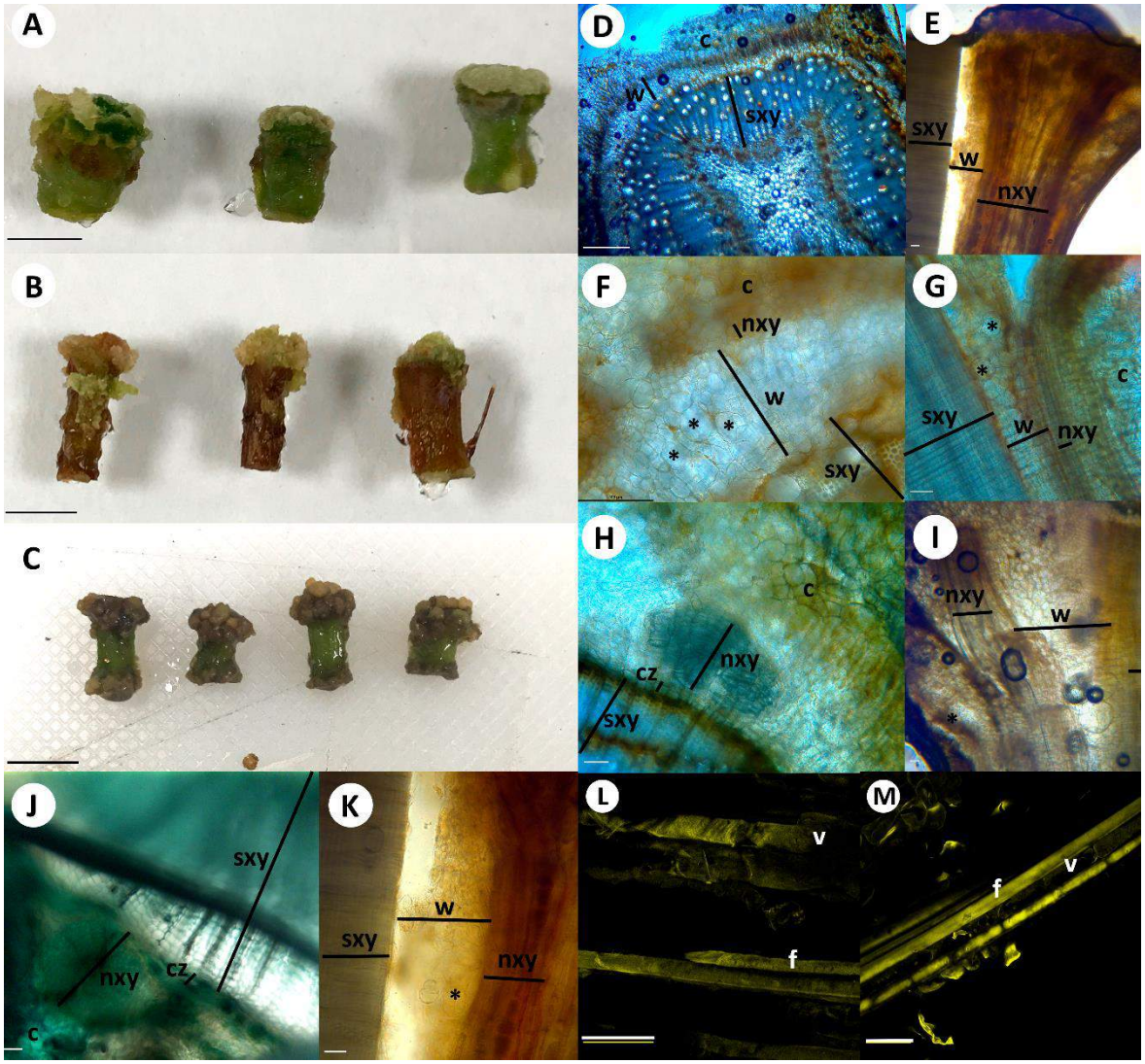

Figure 3.7: ***Callus growth and wood formation in vitro****. Apical stem segments cultured for 6 weeks exhibiting callus growth in poplar (A), pine (B) and eucalypt (C) explants. Bars measure 1 cm. Unstained transverse (D, F, H and J) and longitudinal (E, G, I and K) hand sections of stem segments of eucalypt (D, F-G), poplar (H-I) and pine (E, J-K) exhibiting callus growth and new wood formation after reestablishment of cambium activity. Arrows indicate enlarged wound parenchyma cells. Fluorescent brightener 28 stained wood cells formed in vitro from eucalypt (L) and poplar samples (M). C, callus; cz, cambium zone; f, fibre; P, pith; sxy, secondary xylem; v, vessel; nxy, newly formed xylem; w, wound. Bars measure 50 µm*

3.3.5 Microtubule disruption led to decrease in MFA of wood cells formed in vitro

To determine the extent of cortical microtubule array disruption in the presence of oryzalin, microtubules were visualised using immunofluorescence and the MFA of newly developed wood cells as described in Chapter 2.2.2. As anticipated, in samples subjected to the oryzalin treatment, the cortical microtubule array was disrupted in almost the entire extent of all observed cells (Fig 3.8). MFA of newly formed wood fibres was further assessed to determine whether it was affected by the absence of microtubules. Wood formed in the presence of oryzalin produced cells with generally lower MFA in all three species (Table

3.2). MFA was approximately 5.6˚ lower in poplar samples treated with oryzalin, 4.4˚ in eucalypt samples and 8.9˚ in pine tracheids. However, no significant difference was found when control and oryzalin treatments were compared for eucalypt, poplar and pine (P values = 0.216, 0.240 and 0.134, respectively).

Finally, correlation between MTA and MFA was assessed in samples of wood cells formed *in vitro* in the absence of oryzalin. Indeed, MTA and MFA showed a strong correlation indicated by the Pearson coefficient in pine, poplar and eucalypt (0.891, 0.957 and 0.986, respectively). Microtubules and cellulose microfibrils strongly co-aligned in control samples and disruption of microtubules caused by oryzalin led to shift in cellulose microfibril orientation to a more longitudinal alignment.

Table 3.2: MFA values

	Control	**3 µM Oryzalin**
Poplar	46.1±3.02[a]	40.53±3.5[a]
Eucalypt	43.9±2.5[a]	39.54±2.4[a]
Pine	45.85±3.1[a]	36.94±3.9[a]

Means (±s.e.) of MFA for control and oryzalin treatments. Means that do not share a letter within each row are significantly different.

3.4 Discussion

3.4.1 Cortical microtubules affect MFA and the rate of cellulose deposition but not G-layer development

Paclitaxel injection affected microtubule dynamics and TW formation in an induced system. On average, more branching events occurred in fibres treated with the drug, this was also observed by Bokros *et al.* (1993) in maize and tobacco. Moreover, fibres treated with paclitaxel presented more microtubule bundles. Microtubule bundling is often reported as the most stable form of microtubule assembly (Mollinari *et al.* 2002; Dixit & Cyr 2004a; Wicker-Planquart *et al.* 2004), and prominent bundles of microtubules were reported in paclitaxel treated cells (Baskin *et al.* 1994). Hence, paclitaxel appears to enhance microtubule polymerisation but also favours bundle formation to promote array stabilisation.

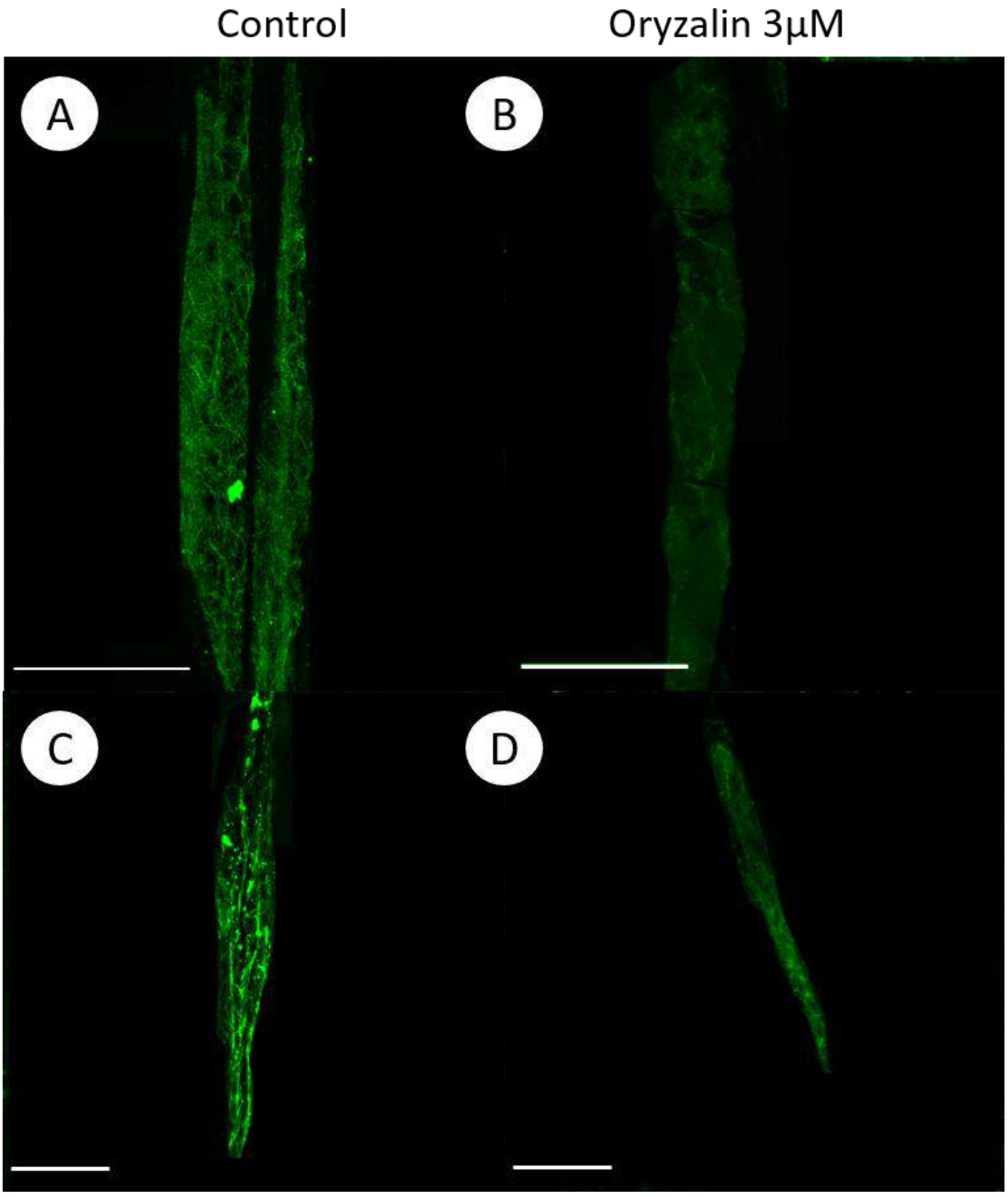

Figure 3.8: ***Effect of oryzalin on cortical microtubule array.*** *Cortical microtubule array of pine tracheids (A-B) and poplar fibres (C-D) visualised using immunolabelling cultured in control medium (A and C) and in medium containing 3 µM oryzalin for 72 h (B and D). Bars measure 50 µm.*

Furthermore, results demonstrated that stems treated with paclitaxel for 24 h before TW induction presented fibres with larger MTA and MFA after one week of an induced TW treatment, compared to control stems that were treated with a dye solution only. TW is commonly associated with low MFA values (Almeras & Clair 2016) that are likely to be determined by a shift of the cortical microtubule array from a transverse to a longitudinally

aligned position (Chapter 2). Because paclitaxel has been reported to prevent reorientation of microtubules in developing cotton fibres (Seagull 1990) it was hypothesised that injection of paclitaxel during induced TW formation might affect MTA and, consequently, MFA determination. When treated with the stabilising drug paclitaxel prior to TW induction, microtubules become "locked" in their previous orientation, therefore re-orientation during TW formation is hindered resulting in higher MTA and MFA in this condition.

Thicker cell walls are normally associated with TW formation (Almeras & Clair 2016) however when treated with paclitaxel, stems forming TW showed a decrease in cell wall thickness and cell wall area, suggesting that changes to microtubule dynamics affect cellulose deposition to some degree. It is well established that microtubules play an essential role in guiding the CSC and, therefore, they can affect cellulose deposition and cell wall development (Emons & Mulder 2000). By studying the cellulose-deficient Arabidopsis *rsw1* mutant, Sugimoto *et al.* (2001) postulated a relation between cellulose synthesis and microfibril alignment. Additionally, Gutierrez *et al.* (2009) demonstrated that CSCs are preferentially delivered to the plasma membrane at sites coinciding with cortical microtubules. Here, evidence provided by promoting microtubule array stabilisation suggests that microtubules play different roles during wood formation, not only determining the orientation of newly formed cellulose microfibrils but also controlling the rate of cellulose deposition itself.

Moreover, presence of a G-layer was equally frequent across all treatments. The G-layer is a chemical and morphologically distinct layer of the SCW characterised by extremely low MFA, a highly mesoporous matrix and the ability to generate axial strain during TW formation in poplar (Clair *et al.* 2018). While development of a G-layer can be understood as part of the process of cell wall thickening during TW formation (Clair *et al.* 2018), results suggest that microtubules do not substantially participate in G-layer formation, or at least their organisation is not crucial for its development. Even though it seems that microtubules organisation can impact cellulose deposition, G-layer formation occurs through a whole different set of transcription factors that affect cell wall composition (Gorshkov *et al.* 2017), which are more likely to be responsible for the development of distinct chemical properties in this special layer.

Lastly, it is worth mentioning that some of the studied parameters were also influenced by the height on the stem at which the sample was collected and/or the distance from the injection point. For instance, microtubule density peaked in control samples collected 10 cm above the injection point while the number of microtubules in other conditions were similar to each other. Differences in ontogenetic development between the two different sample collection points and severity of wound response to the needle, which presumably less severe further from the injection site, can account for these variations.

3.4.2 Microtubules play a role in fine-tuning the orientation of cellulose microfibrils

MFAs of fibres and tracheids cultured *in vitro* in the presence of oryzalin were determined to investigate whether the disruption of microtubule arrays would have an impact on the orientation of newly deposited cellulose microfibrils in SCW. Cells analysed in this study presented large MFAs (>40˚) in all three species, an expected result since newly developed wood cells naturally feature large MFAs (Barnett & Bonham 2004). Moreover, large MFAs might be a result of the unique conditions provided by *in vitro* culturing of young stems. Tissue cultured *in vitro* is subjected to different concentrations of phytohormones when compared to natural conditions (Leitch & Savidge 2000). While gravitational stimulus as an environmental cue has little influence on promoting cell wall stiffening, changes in stem integrity and reduction of the external pressure may also influence the final MFA of wood cells cultured *in vitro* (Brown 1964).

Results showed that when the cortical microtubule array was disrupted through the action of oryzalin, cells featured lower MFA and, therefore, cellulose microfibrils were more longitudinally aligned. Related studies in Arabidopsis root epidermal and cortical cells demonstrated that drug-dependent disruption of microtubules does not alter microfibril parallel alignment locally, but microfibrils showed considerable variation in their orientation within the same cell (Sugimoto *et al.* 2003; Baskin *et al.* 2004). These results suggest that cellulose deposition into well-ordered parallel patterns can be generated by a self-assembly mechanism with little or no microtubule influence and they seem to spontaneously align. In this respect, Emons (1994) first proposed a geometrical model to explain the self-ordering mechanism of cellulose microfibrils that has been further supported by other studies (Sugimoto *et al.* 2001; Himmelspach *et al.* 2003). This model considers the density of CSCs in the plasma membrane, the distance between newly deposited cellulose microfibrils and

the overall cell geometry as factors influencing microfibril orientation in SCW (Emons & Mulder 2000). Other models incorporated the notion of a scaffold of proteins and/or polysaccharides built on the plasma membrane, which is based on microtubule orientation but capable to sustain cellulose synthesis in an independent manner (Baskin 2001). Recently, Chan and Coen (2020) reported a dual mechanism of CSC guidance in Arabidopsis leaves: autonomous CSCs follow trails generated by previous complexes that are overridden when a CSC encounters a microtubule. Therefore, microtubule guidance seems to be dominant, but an autonomous system takes place in the absence of microtubules. Based on these findings, microtubules must play a role in fine-tuning the orientation of cellulose microfibrils deposition, ensuring their alignment at appropriate angles.

3.4.3 Tissue culture is a valuable tool to study molecular aspects of wood formation

In vitro practices have been widely applied in forestry for plant propagation, biomass and secondary metabolite production, breeding and conservation programs and biotechnology studies (Leitch & Savidge 2000; Thorpe 2007; Garcia-Gonzales *et al.* 2010). However, in wood formation studies such techniques are not broadly utilised due to concerns regarding differences between wood formed *in vitro* and wood naturally grown in trees (Leitch & Savidge 2000). The culture of apical stem segments is advantageous because it can produce a cellular response that leads to cambial activity and wood formation (Leitch & Bossinger 2004). Therefore, it can be easily employed to investigate the effects of nutrition, growth regulators and other chemicals, as well as test the impact of different genotypes or genetic transformation on a given trait. In addition, it allows a set of cellular or sub-cellular mechanisms to be studied separate from the influence of whole organism signalling pathways. So far, this specific method had only been applied to *Eucalyptus globulus* (Leitch 1999) and, here, culture of apical stem segments worked equally well in *Eucalyptus grandis x camaldulensis*; *Populus alba*, the model organism for woody trees; and in *Pinus radiata*, a conifer of economic relevance. Therefore, the use of this system can be readily employed for a wide range of woody tree species, facilitating propagation and genetic transformation.

3.5 Conclusion

Perturbation of cortical microtubule array organisation led to changes in microfibril orientation, providing evidence for microtubule involvement in determining MFA of wood cells. The stabilisation of the cortical array by paclitaxel *in vivo* affected TW formation by

preventing the shift of microtubule orientation and resulted in larger MTA and MFA and other cell wall alterations. Simultaneously, a tissue culture system that promotes wood formation *in vitro* was applied to different woody tree species and, using this system, the capacity of cellulose microfibrils to self-align with little or no microtubule influence was demonstrated. In summary, results suggest that reorganisation of microtubules and reorientation of the cortical array act to fine-tun MFA, ensuring appropriate cellulose microfibril alignment and/or realignment, which can be critical to respond to environmental stimulus.

Chapter 4: Thesis summary and future directions

This thesis aimed to shed light on the role played by microtubules in wood formation, in particular during the deposition of secondary cell wall (SCW) by investigating different aspects of cortical microtubule array organisation during several SCW formation systems. For more than 50 years, microtubules have been accredited for directly or indirectly guiding the cellulose synthase complex (CSC) through the plasma membrane (Heath 1974; Giddings & Staehelin 1991; Paredez *et al.* 2006; Watanabe *et al.* 2015) and recent surveys revealed the activity of microtubule associated proteins (MAPs) in physically linking microtubules and CSC (Li *et al.* 2012a; Schneider *et al.* 2017; Kesten *et al.* 2019). However, other studies demonstrated that microtubules are critical in reorientating cellulose deposition rather than guiding the CSC throughout the entire process of cellulose deposition (Woodley *et al.* 2018; Chan & Coen 2020). This feature is especially important when responding to environmental signals and/or stress conditions. Microtubules seem to play an important role in determining the sites of CSC delivery to the plasma membrane which consequently affects cellulose microfibril orientation and cell morphogenesis. Furthermore, tubulin genes are upregulated in response to gravitational stimulus, during reaction wood (RW) formation, which can result in changes to microtubule assembly.

In this study, RW was employed as a model to investigate whether microtubules reorganise during wood formation. In Chapter 2, microtubules were found to differently organise on different sides of branches and stems subject to gravitational stimulus and in stems growing upright. Changes in microtubule organisation were correlated to changes in cell wall morphology, for instance cells forming RW in all species presented thicker cell walls. Tension wood (TW) was often related to lower microtubule angle (MTA), longer microtubules, fewer microtubule bundles and lower microfibril angle (MFA). In contrast, compression wood (CW) was correlated with larger MTA, larger MFA and generally smaller cells. However, some microtubule traits, especially microtubule density and bundle formation, were observed to differ between different species and/or between induced and branch states within a species. This implies that these arrangements might have diverse functions. Together, the results suggest that microtubule reorganisation is a cellular response to gravitational stimulus, and it could be part of the mechanism promoting changes in cell wall properties for coping with mechanical forces.

The role of microtubules in RW formation was further investigated with the aid of paclitaxel. This chemical acts by enhancing microtubule polymerisation and reducing microtubule kinetics. Paclitaxel was injected into poplar stems subsequently induced to form TW. Results reported in Chapter 3 were consistent with the notion that microtubules shift their orientation during RW formation. Fibres treated with paclitaxel prior to TW induction showed larger MTA, which resulted in larger MFA in this condition. This might indicate a reduced ability to shift the orientation of their cortical microtubules. Finally, the capability of cellulose microfibrils to self-organise in the absence of microtubules was tested in apical stem segments cultured *in vitro* using media containing oryzalin, a chemical known to inhibit microtubule polymerisation. Wood cells treated with oryzalin presented lower MFA compared to cells cultured in control medium. These results provide evidence that deposition of parallel aligned cellulose microfibrils in wood cells can occur in the absence of microtubules, however this autonomous mechanism is secondary to microtubule guidance (Chan & Coen 2020), which is, therefore, required for fine-tuning the orientation of cellulose microfibrils. In summary, findings presented in this study suggest that reorientation of the cortical microtubule array affects cellulose deposition during SCW by ensuring appropriate orientation of cellulose microfibrils and determining MFA in wood cells. Moreover, environmental signals, such as gravitational stimulus, can act in wood formation resulting in alterations in the cell wall, possibly through interaction with microtubules.

It remains to be determined how exactly microtubule organisation participates in the determination of wood cell wall properties. An issue to be overcome is our current ability to observe microtubule dynamics in xylary cells via live cell imaging. Immunolabelling techniques employed in the present study are practical tools to observe intracellular structures, however due to the nature of the material and the position of xylem cells in a tree's body, the use of fixed cells was required. Microtubules are dynamic polymers, thus images of the array in fixed cells are, at best, snapshots of a continuous process of reorganisation. Evidence of the occurrence of such dynamic process during RW formation was presented here, indicated by differences in the number of branched nucleation events and microtubule angles, for example. In the future, it will be worthwhile to observe these structures in live wood cells, either in cell culture or *in vivo*, using a time-lapse approach. So far, this has been achieved using transdifferentiation of Arabidopsis epidermal cells into tracheary elements (Kubo *et al.* 2005; Kondo *et al.* 2015), which does not necessarily

represent the processes occurring in woody trees. Therefore, live wood cells cultured *in vitro* or live cells *in arbor* transformed with a fluorescent microtubule-marker could be employed to study microtubule dynamics during wood development in woody tree species.

In addition, it will be necessary to investigate how differential gene expression and interactions between microtubules and MAPs cause the cortical microtubule array to reorganise in response to environmental signals. Recently, Smertenko *et al.* (2020) described how MAP20 supresses microtubule depolymerisation in a similar manner to paclitaxel. Therefore, overexpression of *MAP20* might impact microtubule reorganisation during RW formation, presenting an opportunity to study cellular characteristics controlled by microtubule dynamics. A promising technique is Induced Somatic Sector Analysis (ISSA) (Spokevicius *et al.* 2016), which allows direct comparison between transformed wood sectors and neighboring non-transgenic tissue. Moreover, tissue sectors transformed using ISSA and cultured *in vitro* can potentially be used to produce full transgenic plants for the study of microtubule dynamics in live cells of plants expressing fluorescent microtubule markers in combination with differentially regulated MAPs.

Reorientation of cortical microtubules is an important step in RW formation and a common response to other environmental cues and stress conditions (Wang & Mao 2019). Future work may use molecular techniques to determine the exact mechanism by which wood cells sense extracellular stimuli and how this signal is transduced within the cell. At least two types of mechanosensing molecules were identified in plant cell walls – for extensive review see Landrein and Ingram (2019) – characterisation of these molecules and their specific function in wood cells will improve our knowledge of mechanoperception in plants.

Finally, upregulation of tubulin genes during RW formation has been consistently reported in both angiosperms (Paux *et al.* 2005; Qiu *et al.* 2008; Azri *et al.* 2014) and gymnosperms (Villalobos *et al.* 2012; Li *et al.* 2013), however exactly how this differential expression influences microtubule organisation in those cells is not known. In this study, microtubule organisation was assessed as microtubule density, length of microtubules, nucleation events and microtubule bundling, but the presented results were not sufficient to explain upregulation of tubulin genes during RW formation. The method employed here could not differentiate between tubulin isoforms, but it might be the case that preferred

isoforms are expressed in RW cells and differently affect microtubule properties and, consequently, cellular features. For example, *EgrTUB1* has been implicated in affecting MFA of transgenic fibres (Spokevicius *et al.* 2007). Therefore, protein expression studies would be necessary to investigate whether some tubulin isoforms are preferred expressed during RW development. Additionally, dynamics of microtubules formed by different ratios of preferred RW isoforms could be assessed via living cell imaging to determine whether microtubules with distinct features play specific roles during SCW deposition.

This study started to unveil the role of tubulins in wood formation by exploring cortical microtubule array organisation in trees subjected to gravitational stimulus. Results presented in this thesis provide evidence that microtubules reorganise and reorientate in response to environmental signals affecting aspects of cell morphology, MFA for instance. However, other aspects of microtubule dynamics in live cells, how the mechanical stimulus is sensed by the cell wall and the role of MAPs and tubulins isoforms are awaiting further investigation (Fig 4.1).

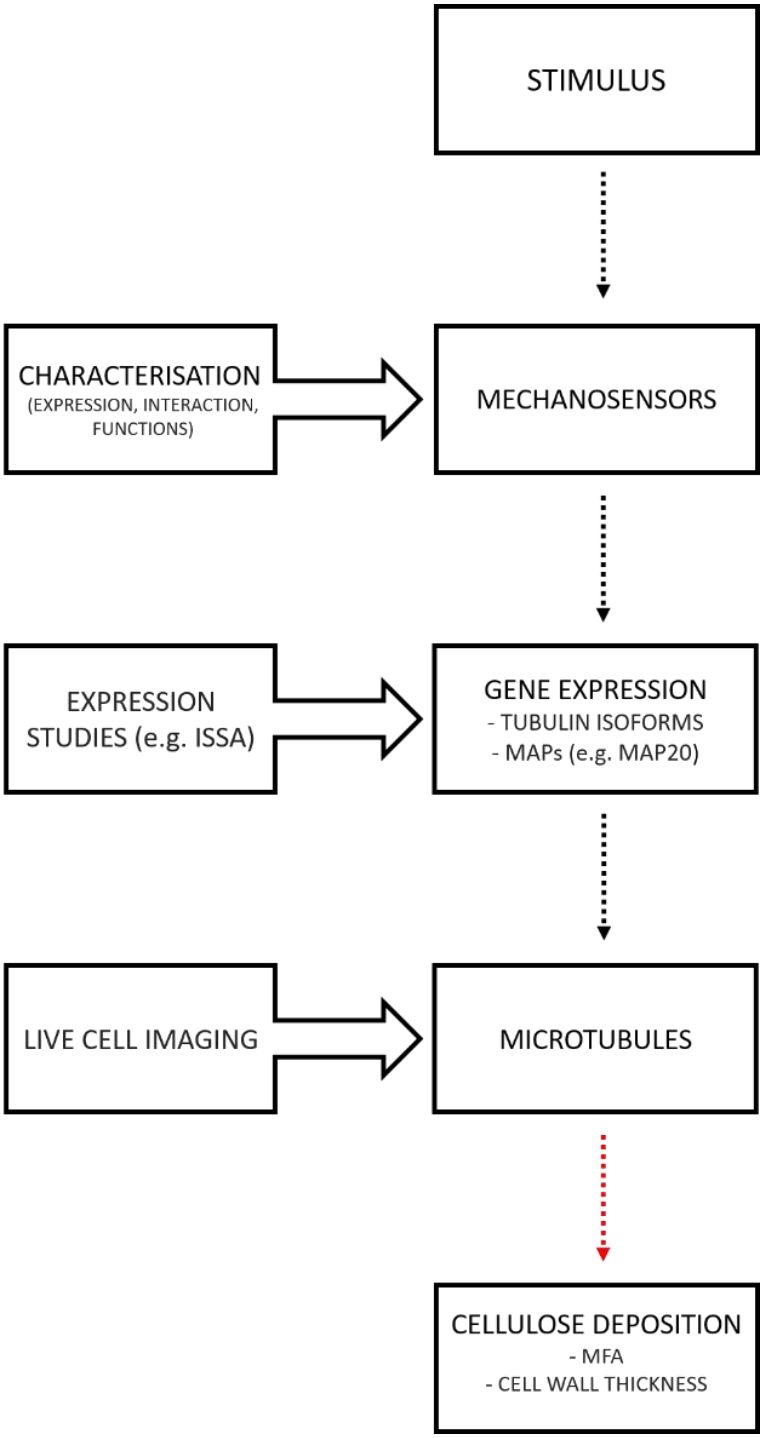

Figure 4.1: ***Thesis summary.*** *Environmental signals can impact wood formation resulting in alterations in the cell wall. To further understand this process, the present study investigated microtubule organisation in response to gravitational stimulus and reported changes in cell wall features, MFA and cell wall thickness for instance (red arrow). The participation of mechanosensing molecules, microtubule associated proteins (MAPs) and tubulin isoforms are still to be investigated (black arrows). Arrowed callouts contain suggested approaches for the investigation of these processes.*

References

Abe, H & Funada, R 2005, 'Review - The orientation of cellulose microfibrils in the cell walls of tracheids in conifers', *Iawa Journal*, vol. 26, no. 2, pp. 161-74.

Abe, H, Funada, R, Imaizumi, H, Ohtani, J & Fukazawa, K 1995, 'Dynamic changes in the arrangement of cortical microtubules in conifer tracheids during differentiation', *Planta*, vol. 197, no. 2, pp. 418-21.

Abedini, R, Clair, B, Pourtahmasi, K, Laurans, F & Arnould, O 2015, 'Cell wall thickening in developing tension wood of artificially bent poplar trees', *Iawa Journal*, vol. 36, no. 1, pp. 44-57.

Almeras, T & Clair, B 2016, 'Critical review on the mechanisms of maturation stress generation in trees', *Journal of the Royal Society Interface*, vol. 13, no. 122.

Andeme-Onzighi, C, Sivaguru, M, Judy-March, J, Baskin, TI & Driouich, A 2002, 'The reb1-1 mutation of Arabidopsis alters the morphology of trichoblasts, the expression of arabinogalactan-proteins and the organization of cortical microtubules', *Planta*, vol. 215, no. 6, pp. 949-58.

Andersson-Gunneras, S, Mellerowicz, EJ, Love, J, Segerman, B, Ohmiya, Y, Coutinho, PM, Nilsson, P, Henrissat, B, Moritz, T & Sundberg, B 2006, 'Biosynthesis of cellulose-enriched tension wood in Populus: global analysis of transcripts and metabolites identifies biochemical and developmental regulators in secondary wall biosynthesis', *Plant Journal*, vol. 45, no. 2, pp. 144-65.

Arioli, T, Peng, LC, Betzner, AS, Burn, J, Wittke, W, Herth, W, Camilleri, C, Hofte, H, Plazinski, J, Birch, R, Cork, A, Glover, J, Redmond, J & Williamson, RE 1998, 'Molecular analysis of cellulose biosynthesis in Arabidopsis', *Science*, vol. 279, no. 5351, pp. 717-20.

Azri, W, Ennajah, A, Nasr, Z, Woo, SY & Khaldi, A 2014, 'Transcriptome profiling the basal region of poplar stems during the early gravitropic response', *Biologia Plantarum*, vol. 58, no. 1, pp. 55-63.

Bamber, RK 2001, 'A general theory for the origin of growth stresses in reaction wood: How trees stay upright', *Iawa Journal*, vol. 22, no. 3, pp. 205-12.

Ban, Y, Kobayashi, Y, Hara, T, Hamada, T, Hashimoto, T, Takeda, S & Hattori, T 2013, 'Alpha-tubulin is rapidly phosphorylated in response to hyperosmotic stress in rice and Arabidopsis', *Plant and Cell Physiology*, vol. 54, no. 6, pp. 848-58.

Barnett, JR & Bonham, VA 2004, 'Cellulose microfibril angle in the cell wall of wood fibres', *Biological Reviews*, vol. 79, no. 2, pp. 461-72.

Bartels, PG & Hilton, JL 1973, 'Comparison of trifluralin, oryzalin, pronamide, propham and colchicine treatments on microtubules', *Pesticide Biochemistry and Physiology*, vol. 3, no. 4, pp. 462-72.

Baskin, TI 2001, 'On the alignment of cellulose microfibrils by cortical microtubules: a review and a model', *Protoplasma*, vol. 215, no. 1-4, pp. 150-71.

—— 2005, 'Anisotropic expansion of the plant cell wall', *Annual Review of Cell and Developmental Biology*, vol. 21, pp. 203-22.

Baskin, TI, Beemster, GTS, Judy-March, JE & Marga, F 2004, 'Disorganization of cortical microtubules stimulates tangential expansion and reduces the uniformity of cellulose microfibril alignment among cells in the root of Arabidopsis', *Plant Physiology*, vol. 135, no. 4, pp. 2279-90.

Baskin, TI, Wilson, JE, Cork, A & Williamson, RE 1994, 'Morphology and microtubule organization in Arabidopsis roots exposed to oryzalin or taxol', *Plant and Cell Physiology*, vol. 35, no. 6, pp. 935-42.

Bastien, R, Bohr, T, Moulia, B & Douady, S 2013, 'Unifying model of shoot gravitropism reveals proprioception as a central feature of posture control in plants', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 2, pp. 755-60.

Beaulieu, J, Doerksen, T, Boyle, B, Clement, S, Deslauriers, M, Beauseigle, S, Blais, S, Poulin, P-L, Lenz, P, Caron, S, Rigault, P, Bicho, P, Bousquet, J & MacKay, J 2011, 'Association Genetics of Wood Physical Traits in the Conifer White Spruce and Relationships With Gene Expression', *Genetics*, vol. 188, no. 1, pp. 197-U329.

Begum, S, Furusawa, O, Shibagaki, M, Nakaba, S, Yamagishi, Y, Yoshimoto, J, Rahman, MH, Sano, Y & Funada, R 2019, 'Localization of actin filaments and cortical microtubules in wood-forming tissues of conifers', *Iawa Journal*, vol. 40, no. 4, pp. 703-20.

Berger, C & Laurent, F 2019, 'Trunk injection of plant protection products to protect trees from pests and diseases', *Crop Protection*, vol. 124, p. 9.

Bhandari, S, Fujino, T, Thammanagowda, S, Zhang, DY, Xu, FY & Joshi, CP 2006, 'Xylem-specific and tension stress-responsive coexpression of KORRIGAN endoglucanase and three secondary wall-associated cellulose synthase genes in aspen trees', *Planta*, vol. 224, no. 4, pp. 828-37.

Bokros, CL, Hugdahl, JD, Hanesworth, VR, Murthy, JV & Morejohn, LC 1993, 'Characterization of the reversible taxol-induced polymerization of plant tubulin into microtubules', *Biochemistry*, vol. 32, no. 13, pp. 3437-47.

Breviario, D, Giani, S & Morello, L 2013, 'Multiple tubulins: evolutionary aspects and biological implications', *Plant Journal*, vol. 75, no. 2, pp. 202-18.

Bringmann, M, Li, EY, Sampathkumar, A, Kocabek, T, Hauser, MT & Persson, S 2012, 'POM-POM2/CELLULOSE SYNTHASE INTERACTING1 is essential for the functional association of cellulose synthase and microtubules in Arabidopsis', *Plant Cell*, vol. 24, no. 1, pp. 163-77.

Brown, CL 1964, *The influence of external pressure on the differentiation of cells and tissues cultured in vitro*, M. H. Zimmerman, Editor. The Formation of Wood in Forest Trees. A Symposium.

Burgert, I 2006, 'Exploring the micromechanical design of plant cell walls', *American Journal of Botany*, vol. 93, no. 10, pp. 1391-401.

Burk, DH & Ye, ZH 2002, 'Alteration of oriented deposition of cellulose microfibrils by mutation of a katanin-like microtubule-severing protein', *Plant Cell*, vol. 14, no. 9, pp. 2145-60.

Bygdell, J, Srivastava, V, Obudulu, O, Srivastava, MK, Nilsson, R, Sundberg, B, Trygg, J, Mellerowicz, EJ & Wingsle, G 2017, 'Protein expression in tension wood formation monitored at high tissue resolution in Populus', *Journal of Experimental Botany*, vol. 68, no. 13, pp. 3405-17.

Carlquist, S 2012, 'How wood evolves: a new synthesis', *Botany-Botanique*, vol. 90, no. 10, pp. 901-40.

Carroll, A, Mansoori, N, Li, SD, Lei, L, Vernhettes, S, Visser, RGF, Somerville, C, Gu, Y & Trindade, LM 2012, 'Complexes with mixed primary and secondary cellulose synthases are functional in Arabidopsis plants', *Plant Physiology*, vol. 160, no. 2, pp. 726-37.

Carroll, A & Specht, CD 2011, 'Understanding plant cellulose synthases through a comprehensive investigation of the cellulose synthase family sequences', *Frontiers in Plant Science*, vol. 2, p. 11.

Chaffey, N, Barlow, P & Barnett, J 2000, 'A cytoskeletal basis for wood formation in angiosperm trees: the involvement of microfilaments', *Planta*, vol. 210, no. 6, pp. 890-6.

Chaffey, N, Barlow, P & Sundberg, B 2002, 'Understanding the role of the cytoskeleton in wood formation in angiosperm trees: hybrid aspen (Populus tremula x P-tremuloides) as the model species', *Tree Physiology*, vol. 22, no. 4, pp. 239-49.

Chaffey, N, Barnett, J & Barlow, P 1999, 'A cytoskeletal basis for wood formation in angiosperm trees: the involvement of cortical microtubules', *Planta*, vol. 208, no. 1, pp. 19-30.

Chan, J 2012, 'Microtubule and cellulose microfibril orientation during plant cell and organ growth', *Journal of Microscopy*, vol. 247, no. 1, pp. 23-32.

Chan, J & Coen, E 2020, 'Interaction between autonomous and microtubule guidance systems controls cellulose synthase trajectories', *Current biology : CB*.

Chan, J, Eder, M, Crowell, EF, Hampson, J, Calder, G & Lloyd, C 2011, 'Microtubules and CESA tracks at the inner epidermal wall align independently of those on the outer wall of light-grown Arabidopsis hypocotyls', *Journal of Cell Science*, vol. 124, no. 7, pp. 1088-94.

Chen, H., J, Chen, BB & Zhang, DQ 2015, 'Transcript profiling of Populus tomentosa genes in normal, tension, and opposite wood by RNA-seq', *Bmc Genomics*, vol. 16, no. 164.

Chen, X, Wu, S, Liu, ZY & Friml, J 2016, 'Environmental and endogenous control of cortical microtubule orientation', *Trends in Cell Biology*, vol. 26, no. 6, pp. 409-19.

Cheng, ZG, Snustad, DP & Carter, JV 2001, 'Temporal and spatial expression patterns of TUB9, a beta-tubulin gene of Arabidopsis thaliana', *Plant Molecular Biology*, vol. 47, no. 3, pp. 389-98.

Clair, B, Dejardin, A, Pilate, G & Almeras, T 2018, 'Is the G-Layer a Tertiary Cell Wall?', *Frontiers in Plant Science*, vol. 9, p. 4.

Cockrell, RA 1974, 'Comparison of latewood pits, fibril orientation and shrinkage of normal and compression wood of giant sequoia', *Wood Science and Technology*, vol. 8, no. 3, pp. 197-206.

Collings, DA & Wasteneys, GO 2005, 'Actin microfilament and microtubule distribution patterns in the expanding root of Arabidopsis thaliana', *Canadian Journal of Botany-Revue Canadienne De Botanique*, vol. 83, no. 6, pp. 579-90.

Cramer, S, Kretschmann, D, Lakes, R & Schmidt, T 2005, 'Earlywood and latewood elastic properties in loblolly pine', *Holzforschung*, vol. 59, no. 5, pp. 531-8.

Crowell, EF, Timpano, H, Desprez, T, Franssen-Verheijen, T, Emons, A-M, Hoefte, H & Vernhettes, S 2011, 'Differential regulation of cellulose orientation at the inner and outer face of epidermal cells in the Arabidopsis hypocotyl', *Plant Cell*, vol. 23, no. 7, pp. 2592-605.

Dahiya, P, Findlay, K, Roberts, K & McCann, MC 2006, 'A fasciclin-domain containing gene, ZeFLA11, is expressed exclusively in xylem elements that have reticulate wall thickenings in the stem vascular system of Zinnia elegans cv Envy', *Planta*, vol. 223, no. 6, pp. 1281-91.

DeBolt, S, Gutierrez, R, Ehrhardt, DW, Melo, CV, Ross, L, Cutler, SR, Somerville, C & Bonetta, D 2007, 'Morlin, an inhibitor of cortical microtubule dynamics and cellulose synthase movement', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 14, pp. 5854-9.

Deeks, MJ, Fendrych, M, Smertenko, A, Bell, KS, Oparka, K, Cvrckova, F, Zarsky, V & Hussey, PJ 2010, 'The plant formin AtFH4 interacts with both actin and microtubules, and contains a newly identified microtubule-binding domain', *Journal of Cell Science*, vol. 123, no. 8, pp. 1209-15.

Deng, Q, Li, S & Chen, YP 2012, 'Mechanical properties and failure mechanism of wood cell wall layers', *Computational Materials Science*, vol. 62, pp. 221-6.

Derba-Maceluch, M, Awano, T, Takahashi, J, Lucenius, J, Ratke, C, Kontro, I, Busse-Wicher, M, Kosik, O, Tanaka, R, Winzell, A, Kallas, A, Lesniewska, J, Berthold, F, Immerzeel, P, Teeri, TT, Ezcurra, I, Dupree, P, Serimaa, R & Mellerowicz, EJ 2015, 'Suppression of xylan endotransglycosylase PtxtXyn10A affects cellulose microfibril angle in secondary wall in aspen wood', *New Phytologist*, vol. 205, no. 2, pp. 666-81.

Dillon, SK, Nolan, M, Li, W, Bell, C, Wu, HX & Southerton, SG 2010, 'Allelic variation in cell wall candidate genes affecting solid wood properties in natural populations and land races of Pinus radiata', *Genetics*, vol. 185, no. 4, pp. 1477-U545.

Dixit, R & Cyr, R 2004a, 'The cortical microtubule array: from dynamics to organization', *Plant Cell*, vol. 16, no. 10, pp. 2546-52.

—— 2004b, 'Encounters between dynamic cortical microtubules promote ordering of the cortical array through angle-dependent modifications of microtubule behavior', *Plant Cell*, vol. 16, no. 12, pp. 3274-84.

Donaldson, L 2008, 'Microfibril angle: measurement, variation and relationships - a review', *Iawa Journal*, vol. 29, no. 4, pp. 345-86.

Driouich, A & Baskin, TI 2008, 'Intercourse between cell wall and cytoplasma exemplified by arabinogalactan proteins and cortical microtubules', *American Journal of Botany*, vol. 95, no. 12, pp. 1491-7.

Emons, AMC 1994, 'Winding threads around plant cells - a geometrical model for microfibril deposition', *Plant Cell and Environment*, vol. 17, no. 1, pp. 3-14.

Emons, AMC & Mulder, BM 2000, 'How the deposition of cellulose microfibrils builds cell wall architecture', *Trends in Plant Science*, vol. 5, no. 1, pp. 35-40.

Endler, A, Kesten, C, Schneider, R, Zhang, Y, Ivakov, A, Froehlich, A, Funke, N & Persson, S 2015, 'A Mechanism for Sustained Cellulose Synthesis during Salt Stress', *Cell*, vol. 162, no. 6, pp. 1353-64.

Eren, EC, Dixit, R & Gautam, N 2010, 'A three-dimensional computer simulation model reveals the mechanisms for self-organization of plant cortical microtubules into oblique arrays', *Molecular Biology of the Cell*, vol. 21, no. 15, pp. 2674-84.

Evans, R & Ilic, J 2001, 'Rapid prediction of wood stiffness from microfibril, angle and density', *Forest Products Journal*, vol. 51, no. 3, pp. 53-7.

Evans, R, Stringer, S & Kibblewhite, RP 2000, 'Variation of microfibril angle, density and fibre orientation in twenty-nine Eucalyptus nitens trees', *Appita Journal*, vol. 53, no. 6, pp. 450-7.

Fagard, M, Desnos, T, Desprez, T, Goubet, F, Refregier, G, Mouille, G, McCann, M, Rayon, C, Vernhettes, S & Hofte, H 2000, 'PROCUSTE1 encodes a cellulose synthase required for normal cell elongation specifically in roots and dark-grown hypocotyls of arabidopsis', *Plant Cell*, vol. 12, no. 12, pp. 2409-23.

Fisher, JB & Stevenson, JW 1981, 'Occurence of reaction wood in branches of dicotyledons and its role in tree architecture', *Botanical Gazette*, vol. 142, no. 1, pp. 82-95.

Freeman, JS, Whittock, SP, Potts, BM & Vaillancourt, RE 2009, 'QTL influencing growth and wood properties in Eucalyptus globulus', *Tree Genetics & Genomes*, vol. 5, no. 4, pp. 713-22.

Funada, R, Miura, H, Shibagaki, M, Furusawa, O, Miura, T, Fukatsu, E & Kitin, P 2001, 'Involvement of localized cortical microtubules in the formation of a modified structure of wood', *Journal of Plant Research*, vol. 114, no. 1116, pp. 491-7.

Furusawa, O, Funada, R, Murakami, Y & Ohtani, J 1998, 'Arrangement of cortical microtubules in compression wood tracheids of Taxus cuspidata visualized by confocal laser microscopy', *Journal of Wood Science*, vol. 44, no. 3, pp. 230-3.

Garcia-Gonzales, R, Quiroz, K, Carrasco, B & Caligari, P 2010, 'Plant tissue culture: current status, opportunities and challenges', *Ciencia E Investigacion Agraria*, vol. 37, no. 3, pp. 5-30.

Gardiner, JC, Taylor, NG & Turner, SR 2003, 'Control of cellulose synthase complex localization in developing xylem', *Plant Cell*, vol. 15, no. 8, pp. 1740-8.

Gaspar, Y, Johnson, KL, McKenna, JA, Bacic, A & Schultz, CJ 2001, 'The complex structures of arabinogalactan-proteins and the journey towards understanding function', *Plant Molecular Biology*, vol. 47, no. 1-2, pp. 161-76.

Giddings, TH & Staehelin, LA 1991, 'Microtubule-mediated control of microfibril deposition: a re-examination of the hypothesis', in CW Lloyd (ed.), *The Cytoskeletal Basis of Plant Growth and Form*, Academic Press, London, pp. 85-99.

Gonzalez-Martinez, SC, Wheeler, NC, Ersoz, E, Nelson, CD & Neale, DB 2007, 'Association genetics in Pinus taeda L. I. Wood property traits', *Genetics*, vol. 175, no. 1, pp. 399-409.

Gorshkov, O, Mokshina, N, Gorshkov, V, Chemikosova, S, Gogolev, Y & Gorshkova, T 2017, 'Transcriptome portrait of cellulose-enriched flax fibres at advanced stage of specialization', *Plant Molecular Biology*, vol. 93, no. 4-5, pp. 431-49.

Green, PB 1962, 'Mechanism for plant cellular morphogenesis', *Science*, vol. 138, no. 3548, pp. 1404-5.

Groover, A 2016, 'Gravitropisms and reaction woods of forest trees - evolution, functions and mechanisms', *New Phytologist*, vol. 211, no. 3, pp. 790-802.

Gutierrez, R, Lindeboom, JJ, Paredez, AR, Emons, AMC & Ehrhardt, DW 2009, 'Arabidopsis cortical microtubules position cellulose synthase delivery to the plasma membrane and interact with cellulose synthase trafficking compartments', *Nature Cell Biology*, vol. 11, no. 7, pp. 797-U43.

Haigler, H., C & Brown, RM 1986, 'Transport of rosettes from the golgi-apparatus to the plasma membrane in isolated mesophyll-cells of Zinnia elegans differentiation to tracheary elements in suspension culture', *Protoplasma*, vol. 134, no. 2-3, pp. 111-20.

Hardham, AR & Gunning, BES 1979, 'Interpolation of microtubules into cortical arrays during cell elongation and differentiation in roots of Azolla pinnata', *Journal of Cell Science*, vol. 37, no. JUN, pp. 411-42.

Hashimoto, T 2013, 'Dissecting the cellular functions of plant microtubules using mutant tubulins', *Cytoskeleton*, vol. 70, no. 4, pp. 191-200.

Hashimoto, T 2015, 'Microtubules in plants', *The arabidopsis book*, vol. 13, pp. e0179-e.

Heald, R & Nogales, E 2002, 'Microtubule dynamics', *Journal of Cell Science*, vol. 115, no. 1, pp. 3-4.

Heath, IB 1974, 'A unified hypothesis for the role of membrane-bound enzyme complexes and microtubules in plant cell wall synthesis', *Journal of Theoretical Biology*, vol. 48, no. 2, pp. 445-9.

Hein, PRG & Lima, JT 2012, 'Relationships between microfibril angle, modulus of elasticity and compressive strength in Eucalyptus wood', *Maderas-Ciencia Y Tecnologia*, vol. 14, no. 3, pp. 267-74.

Heisler, MG, Hamant, O, Krupinski, P, Uyttewaal, M, Ohno, C, Jonsson, H, Traas, J & Meyerowitz, EM 2010, 'Alignment between PIN1 polarity and microtubule orientation in the shoot apical meristem reveals a tight coupling between morphogenesis and auxin transport', *Plos Biology*, vol. 8, no. 10, p. 12.

Hellgren, JM, Olofsson, K & Sundberg, B 2004, 'Patterns of auxin distribution during gravitational induction of reaction wood in poplar and pine', *Plant Physiology*, vol. 135, no. 1, pp. 212-20.

Herth, W 1985, 'Plasma membrane rosettes involved in localised wall thickening during xylem vessel formation of Lepidium sativum L.', *Planta*, vol. 164, no. 1, pp. 12-21.

Hertzberg, M, Aspeborg, H, Schrader, J, Andersson, A, Erlandsson, R, Blomqvist, K, Bhalerao, R, Uhlen, M, Teeri, TT, Lundeberg, J, Sundberg, B, Nilsson, P & Sandberg, G 2001, 'A transcriptional

roadmap to wood formation', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 25, pp. 14732-7.

Himmelspach, R, Williamson, RE & Wasteneys, GO 2003, 'Cellulose microfibril alignment recovers from DCB-induced disruption despite microtubule disorganization', *Plant Journal*, vol. 36, no. 4, pp. 565-75.

His, I, Driouich, A, Nicol, F, Jauneau, A & Hofte, H 2001, 'Altered pectin composition in primary cell walls of korrigan, a dwarf mutant of Arabidopsis deficient in a membrane-bound endo-1,4-beta-glucanase', *Planta*, vol. 212, no. 3, pp. 348-58.

Huang, C, Zhang, R, Gui, J, Zhong, Y & Li, L 2018, 'The receptor-like kinase AtVRLK1 regulates secondary cell wall thickening', *Plant Physiology*, vol. 177, no. 2, pp. 671-83.

Huang, GQ, Xu, WL, Gong, SY, Li, B, Wang, XL, Xu, D & Li, XB 2008, 'Characterization of 19 novel cotton FLA genes and their expression profiling in fiber development and in response to phytohormones and salt stress', *Physiologia Plantarum*, vol. 134, no. 2, pp. 348-59.

Ishikura, Y 2017, 'Structural and bending properties of juvenile and mature softwood', *Cellulose Chemistry and Technology*, vol. 51, no. 9-10, pp. 879-87.

Itoh, T 1976, 'Microfibrillar orientation of radially enlarged cells of coumarin- and colchicine-treated pine seedlings', *Plant and Cell Physiology*, vol. 17, no. 2, pp. 385-98.

Johnson, KL, Jones, BJ, Bacic, A & Schultz, CJ 2003, 'The fasciclin-like arabinogalactan proteins of arabidopsis. A multigene family of putative cell adhesion molecules', *Plant Physiology*, vol. 133, no. 4, pp. 1911-25.

Jourez, B, Riboux, A & Leclercq, A 2001, 'Anatomical characteristics of tension wood and opposite wood in young inclined stems of poplar (Populus euramericana cv 'Ghoy')', *Iawa Journal*, vol. 22, no. 2, pp. 133-57.

Kesten, C, Wallmann, A, Schneider, R, McFarlane, HE, Diehl, A, Khan, GA, van Rossum, BJ, Lampugnani, ER, Szymanski, WG, Cremer, N, Schmieder, P, Ford, KL, Seiter, F, Heazlewood, JL, Sanchez-Rodriguez, C, Oschkinat, H & Persson, S 2019, 'The companion of cellulose synthase 1 confers salt tolerance through a Tau-like mechanism in plants', *Nature Communications*, vol. 10, p. 14.

Kobayashi, H, Fukuda, H & Shibaoka, H 1988, 'Interrelation between the spatial disposition of actin-filaments and microtubules during the differentiation of tracheary elements in cultured Zinnia cells', *Protoplasma*, vol. 143, no. 1, pp. 29-37.

Komis, G, Luptovciak, I, Doskocilova, A & Samaj, J 2015, 'Biotechnological aspects of cytoskeletal regulation in plants', *Biotechnology Advances*, vol. 33, no. 6, pp. 1043-62.

Kondo, Y, Fujita, T, Sugiyama, M & Fukuda, H 2015, 'A novel system for xylem cell differentiation in Arabidopsis thaliana', *Molecular Plant*, vol. 8, no. 4, pp. 612-21.

Kubo, M, Udagawa, M, Nishikubo, N, Horiguchi, G, Yamaguchi, M, Ito, J, Mimura, T, Fukuda, H & Demura, T 2005, 'Transcription switches for protoxylem and metaxylem vessel formation', *Genes & Development*, vol. 19, no. 16, pp. 1855-60.

Kumar, M, Mishra, L, Carr, P, Pilling, M, Gardner, P, Mansfield, SD & Turner, S 2018, 'Exploiting CELLULOSE SYNTHASE (CESA) class specificity to probe cellulose microfibril biosynthesis', *Plant Physiology*, vol. 177, no. 1, pp. 151-67.

Kumar, N 1981, 'Taxol-induced polymerization of purified tubulin - mechanism of action', *Journal of Biological Chemistry*, vol. 256, no. 20, pp. 435-41.

Lafarguette, F, Leple, JC, Dejardin, A, Laurans, F, Costa, G, Lesage-Descauses, MC & Pilate, G 2004, 'Poplar genes encoding fasciclin-like arabinogalactan proteins are highly expressed in tension wood', *New Phytologist*, vol. 164, no. 1, pp. 107-21.

Lam, HK, Harbard, JL & Koutoulis, A 2014, 'Tetraploid induction of Acacia crassicarpa using colchicine and oryzalin', *Journal of Tropical Forest Science*, vol. 26, no. 3, pp. 347-54.

Lamport, DTA & Varnai, P 2013, 'Periplasmic arabinogalactan glycoproteins act as a calcium capacitor that regulates plant growth and development', *New Phytologist*, vol. 197, no. 1, pp. 58-64.

Lampugnani, ER, Khan, GA, Somssich, M & Persson, S 2018, 'Building a plant cell wall at a glance', *Journal of Cell Science*, vol. 131, no. 2, p. 6.

Landrein, B & Hamant, O 2013, 'How mechanical stress controls microtubule behavior and morphogenesis in plants: history, experiments and revisited theories', *Plant Journal*, vol. 75, no. 2, pp. 324-38.

Landrein, B & Ingram, G 2019, 'Connected through the force: mechanical signals in plant development', *Journal of Experimental Botany*, vol. 70, no. 14, pp. 3507-19.

Le Provost, G, Paiva, J, Pot, D, Brach, J & Plomion, C 2003, 'Seasonal variation in transcript accumulation in wood-forming tissues of maritime pine (Pinus pinaster Ait.) with emphasis on a cell wall glycine-rich protein', *Planta*, vol. 217, no. 5, pp. 820-30.

Ledbetter, MC & Porter, KR 1963, 'A microtubule in plant cell fine structure', *Journal of Cell Biology*, vol. 19, no. 1, pp. 239-50.

Lei, L, Li, SD, Bashline, L & Gu, Y 2014, 'Dissecting the molecular mechanism underlying the intimate relationship between cellulose microfibrils and cortical microtubules', *Frontiers in Plant Science*, vol. 5, p. 8.

Leitch, MA 1999, 'The development of tissue culture techniques to study wood formation in Eucalyptus globulus Labill.', PhD thesis, The University of Melbourne.

Leitch, MA & Bossinger, G 2004, 'In vitro systems for the study of wood formation', *Molecular Genetics and Breeding of Forest Trees*, pp. 193-211.

Leitch, MA & Savidge, RA 2000, *Cell, tissue and organ culture for the study of cambial activity and wood formation - a resurgence of interest in an old technique*, Cell and Molecular Biology of Wood Formation, Bios Scientific Publishers Ltd, Oxford.

Li, S., Bashline, L, Zheng, Y, Xin, X, Huang, S, Kong, ZS, Kim, SH, Cosgrove, DJ & Gu, Y 2016, 'Cellulose synthase complexes act in a concerted fashion to synthesize highly aggregated cellulose in secondary cell walls of plants', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 113, no. 40, pp. 11348-53.

Li, CJ, Lu, HM, Li, W, Yuan, M & Fu, Y 2017, 'A ROP2-RIC1 pathway fine-tunes microtubule reorganization for salt tolerance in Arabidopsis', *Plant Cell and Environment*, vol. 40, no. 7, pp. 1127-42.

Li, M, Hong, Y & Wang, X 2009, 'Phospholipase D- and phosphatidic acid-mediated signaling in plants', *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids*, vol. 1791, no. 9, pp. 927-35.

Li, S, Lei, L, Somerville, CR & Gu, Y 2012a, 'Cellulose synthase interactive protein 1 (CSI1) links microtubules and cellulose synthase complexes', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 1, pp. 185-90.

Li, X, Wu, HX & Southerton, SG 2011a, 'Transcriptome profiling of wood maturation in Pinus radiata identifies differentially expressed genes with implications in juvenile and mature wood variation', *Gene*, vol. 487, no. 1, pp. 62-71.

Li, XG, Wu, HX & Southerton, SG 2011b, 'Transcriptome profiling of Pinus radiata juvenile wood with contrasting stiffness identifies putative candidate genes involved in microfibril orientation and cell wall mechanics', *Bmc Genomics*, vol. 12, p. 16.

Li, XG, Wu, HX & Southerton, SG 2012b, 'Identification of putative candidate genes for juvenile wood density in Pinus radiata', *Tree Physiology*, vol. 32, no. 8, pp. 1046-57.

Li, XG, Yang, XH & Wu, HX 2013, 'Transcriptome profiling of radiata pine branches reveals new insights into reaction wood formation with implications in plant gravitropism', *Bmc Genomics*, vol. 14, no. 768.

Lillo, C, Kataya, ARA, Heidari, B, Creighton, MT, Nemie-Feyissa, D, Ginbot, Z & Jonassen, EM 2014, 'Protein phosphatases PP2A, PP4 and PP6: mediators and regulators in development and responses to environmental cues', *Plant Cell and Environment*, vol. 37, no. 12, pp. 2631-48.

Lin, D, Cao, L, Zhou, Z, Zhu, L, Ehrhardt, D, Yang, Z & Fu, Y 2013, 'Rho GTPase signaling activates microtubule severing to promote microtubule ordering in Arabidopsis', *Current Biology*, vol. 23, no. 4, pp. 290-7.

Lindeboom, JJ, Nakamura, M, Hibbel, A, Shundyak, K, Gutierrez, R, Ketelaar, T, Emons, AMC, Mulder, BM, Kirik, V & Ehrhardt, DW 2013, 'A mechanism for reorientation of cortical microtubule arrays driven by microtubule severing', *Science*, vol. 342, no. 6163.

Liu, ZY, Persson, S & Zhang, Y 2015, 'The connection of cytoskeletal network with plasma membrane and the cell wall', *Journal of Integrative Plant Biology*, vol. 57, no. 4, pp. 330-40.

Lloyd, C 2011, 'Dynamic microtubules and the texture of plant cell walls', *International Review of Cell and Molecular Biology, Vol 287*, vol. 287, pp. 287-329.

Long, JM, Conn, AB, Batchelor, WJ & Evans, R 2000, 'Comparison of methods to measure fibril angle in wood fibres', *Appita Journal*, vol. 53, no. 3, pp. 206-9.

Lowe, J, Li, H, Downing, KH & Nogales, E 2001, 'Refined structure of alpha beta-tubulin at 3.5 A resolution', *Journal of Molecular Biology*, vol. 313, no. 5, pp. 1045-57.

Ma, HL & Zhao, J 2010, 'Genome-wide identification, classification, and expression analysis of the arabinogalactan protein gene family in rice (Oryza sativa L.)', *Journal of Experimental Botany*, vol. 61, no. 10, pp. 2647-68.

MacMillan, CP, Mansfield, SD, Stachurski, ZH, Evans, R & Southerton, SG 2010, 'Fasciclin-like arabinogalactan proteins: specialization for stem biomechanics and cell wall architecture in Arabidopsis and Eucalyptus', *Plant Journal*, vol. 62, no. 4, pp. 689-703.

MacMillan, CP, Taylor, L, Bi, YD, Southerton, SG, Evans, R & Spokevicius, A 2015, 'The fasciclin-like arabinogalactan protein family of Eucalyptus grandis contains members that impact wood biology and biomechanics', *New Phytologist*, vol. 206, no. 4, pp. 1314-27.

Maloney, VJ & Mansfield, SD 2010, 'Characterization and varied expression of a membrane-bound endo-beta-1,4-glucanase in hybrid poplar', *Plant Biotechnology Journal*, vol. 8, no. 3, pp. 294-307.

Mansfield, SD, Parish, R, Di Lucca, CM, Goudie, J, Kang, KY & Ott, P 2009, 'Revisiting the transition between juvenile and mature wood: a comparison of fibre length, microfibril angle and relative wood density in lodgepole pine', *Holzforschung*, vol. 63, no. 4, pp. 449-56.

Mareri, L, Romi, M & Cai, G 2019, 'Arabinogalactan proteins: actors or spectators during abiotic and biotic stress in plants?', *Plant Biosystems*, vol. 153, no. 1, pp. 173-85.

Matsumura, J & Butterfield, BG 2001, 'Microfibril angles in the root wood of Pinus radiata and Pinus nigra', *Iawa Journal*, vol. 22, no. 1, pp. 57-62.

Matsuzaki, J, Masumori, M & Tange, T 2007, 'Phototropic bending of non-elongating and radially growing woody stems results from asymmetrical xylem formation', *Plant Cell and Environment*, vol. 30, no. 5, pp. 646-53.

McFarlane, HE, Doring, A & Persson, S 2014, 'The cell biology of cellulose synthesis', *Annual Review of Plant Biology, Vol 65*, vol. 65, pp. 69-+.

McNally, FJ & Vale, RD 1993, 'Identification of katanin, an ATPase that severs and disassembles stable microtubules', *Cell*, vol. 75, no. 3, pp. 419-29.

Meents, MJ, Watanabe, Y & Samuels, AL 2018, 'The cell biology of secondary cell wall biosynthesis', *Annals of Botany*, vol. 121, no. 6, pp. 1107-25.

Mellerowicz, EJ & Sundberg, B 2008, 'Wood cell walls: biosynthesis, developmental dynamics and their implications for wood properties', *Current Opinion in Plant Biology*, vol. 11, no. 3, pp. 293-300.

Mirabet, V, Krupinski, P, Hamant, O, Meyerowitz, EM, Jonsson, H & Boudaoud, A 2018, 'The self-organization of plant microtubules inside the cell volume yields their cortical localization, stable alignment, and sensitivity to external cues', *Plos Computational Biology*, vol. 14, no. 2, p. 23.

Mitra, D, Klemm, S, Kumari, P, Quegwer, J, Moller, B, Poeschl, Y, Pflug, P, Stamm, G, Abel, S & Burstenbinder, K 2019, 'Microtubule-associated protein IQ67 DOMAIN5 regulates morphogenesis of leaf pavement cells in Arabidopsis thaliana', *Journal of Experimental Botany*, vol. 70, no. 2, pp. 529-43.

Mollinari, C, Kleman, JP, Jiang, W, Schoehn, G, Hunter, T & Margolis, RL 2002, 'PRC1 is a microtubule binding and bundling protein essential to maintain the mitotic spindle midzone', *Journal of Cell Biology*, vol. 157, no. 7, pp. 1175-86.

Monshausen, GB & Haswell, ES 2013, 'A force of nature: molecular mechanisms of mechanoperception in plants', *Journal of Experimental Botany*, vol. 64, no. 15, pp. 4663-80.

Moody, RG & Williamson, MP 2013, 'Structure and function of a bacterial Fasciclin I Domain Protein elucidates function of related cell adhesion proteins such as TGFBIp and periostin', *Febs Open Bio*, vol. 3, pp. 71-7.

Moore, JR, Cown, DJ & McKinley, RB 2014, 'Modelling microfibril angle variation in New Zealand-grown radiata pine', *New Zealand Journal of Forestry Science*, vol. 44, p. 11.

Morejohn, LC, Bureau, TE, Molebajer, J, Bajer, AS & Fosket, DE 1987, 'Oryzalin, a dinitroaline herbicide, binds to plant tubulin and inhibits microtubule polymerization in vitro', *Planta*, vol. 172, no. 2, pp. 252-64.

Morejohn, LC & Fosket, DE 1984, 'Taxol-induced rose microtubule polymerization in vitro and its inhibition by colchicine', *Journal of Cell Biology*, vol. 99, no. 1, pp. 141-7.

Murata, T, Sonobe, S, Baskin, TI, Hyodo, S, Hasezawa, S, Nagata, T, Horio, T & Hasebe, M 2005, 'Microtubule-dependent microtubule nucleation based on recruitment of gamma-tubulin in higher plants', *Nature Cell Biology*, vol. 7, no. 10, pp. 961-U52.

Myszewski, JH, Bridgwater, FE, Lowe, WJ, Byram, TD & Megraw, RA 2004, 'Genetic variation in the microfibril angle of loblolly pine from two test sites', *Southern Journal of Applied Forestry*, vol. 28, no. 4, pp. 196-204.

Nakamura, M 2015, 'Microtubule nucleating and severing enzymes for modifying microtubule array organization and cell morphogenesis in response to environmental cues', *New Phytologist*, vol. 205, no. 3, pp. 1022-7.

Nakamura, M, Ehrhardt, DW & Hashimoto, T 2010, 'Microtubule and katanin-dependent dynamics of microtubule nucleation complexes in the acentrosomal Arabidopsis cortical array', *Nature Cell Biology*, vol. 12, no. 11, pp. 1064-U49.

Nebenfuhr, A & Dixit, R 2018, 'Kinesins and myosins: molecular motors that coordinate cellular functions in plants', *Annual Review of Plant Biology, Vol 69*, vol. 69, pp. 329-61.

Nguema-Ona, E, Bannigan, A, Chevalier, L, Baskin, TI & Driouich, A 2007, 'Disruption of arabinogalactan proteins disorganizes cortical microtubules in the root of Arabidopsis thaliana', *Plant Journal*, vol. 52, no. 2, pp. 240-51.

Nick, P 2013, 'Microtubules, signalling and abiotic stress', *Plant Journal*, vol. 75, no. 2, pp. 309-23.

Nicol, F, His, I, Jauneau, A, Vernhettes, S, Canut, H & Hofte, H 1998, 'A plasma membrane-bound putative endo-1,4-beta-D-glucanase is required for normal wall assembly and cell elongation in Arabidopsis', *Embo Journal*, vol. 17, no. 19, pp. 5563-76.

Nixon, BT, Mansouri, K, Singh, A, Du, J, Davis, JK, Lee, JG, Slabaugh, E, Vandavasi, VG, O'Neill, H, Roberts, EM, Roberts, AW, Yingling, YG & Haigler, CH 2016, 'Comparative structural and computational analysis supports eighteen cellulose synthases in the plant cellulose synthesis complex', *Scientific Reports*, vol. 6, p. 14.

Oakley, RV, Wang, YS, Ramakrishna, W, Harding, SA & Tsai, CJ 2007, 'Differential expansion and expression of alpha- and beta-tubulin gene families in Populus', *Plant Physiology*, vol. 145, no. 3, pp. 961-73.

Oda, Y 2015, 'Cortical microtubule rearrangements and cell wall patterning', *Frontiers in Plant Science*, vol. 6, p. 7.

Oda, Y & Fukuda, H 2012, 'Initiation of cell wall pattern by a Rho- and microtubule-driven symmetry breaking', *Science*, vol. 337, no. 6100, pp. 1333-6.

Paradez, A, Wright, A & Ehrhardt, DW 2006, 'Microtubule cortical array organization and plant cell morphogenesis', *Current Opinion in Plant Biology*, vol. 9, no. 6, pp. 571-8.

Paredez, R., A, Somerville, CR & Ehrhardt, DW 2006, 'Visualization of cellulose synthase demonstrates functional association with microtubules', *Science*, vol. 312, no. 5779, pp. 1491-5.

Paredez, AR, Persson, S, Ehrhardt, DW & Somerville, CR 2008, 'Genetic evidence that cellulose synthase activity influences microtubule cortical array organization', *Plant Physiology*, vol. 147, no. 4, pp. 1723-34.

Parrotta, L, Cresti, M & Cai, G 2014, 'Accumulation and post-translational modifications of plant tubulins', *Plant Biology*, vol. 16, no. 3, pp. 521-7.

Paux, E, Carocha, V, Marques, C, de Sousa, AM, Borralho, N, Sivadon, P & Grima-Pettenati, J 2005, 'Transcript profiling of Eucalyptus xylem genes during tension wood formation', *New Phytologist*, vol. 167, no. 1, pp. 89-100.

Persson, S, Paredez, A, Carroll, A, Palsdottir, H, Doblin, M, Poindexter, P, Khitrov, N, Auer, M & Somerville, CR 2007, 'Genetic evidence for three unique components in primary cell-wall cellulose synthase complexes in Arabidopsis', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 39, pp. 15566-71.

Persson, S, Wei, HR, Milne, J, Page, GP & Somerville, CR 2005, 'Identification of genes required for cellulose synthesis by regression analysis of public microarray data sets', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 24, pp. 8633-8.

Pesquet, E, Korolev, AV, Calder, G & Lloyd, CW 2010, 'The microtubule-associated protein AtMAP70-5 regulates secondary wall patterning in Arabidopsis wood cells', *Current Biology*, vol. 20, no. 8, pp. 744-9.

Pierce, ET, Graham, BP, Stiff, MR, Osborne, JA & Haigler, CH 2019, 'Cultures of Gossypium barbadense cotton ovules offer insights into the microtubule-mediated control of fiber cell expansion', *Planta*, vol. 249, no. 5, pp. 1551-63.

Pilate, G, Dejardin, A, Laurans, F & Leple, JC 2004, 'Tension wood as a model for functional genomics of wood formation', *New Phytologist*, vol. 164, no. 1, pp. 63-72.

Polko, JK & Kieber, JJ 2019, 'The regulation of cellulose biosynthesis in plants', *Plant Cell*, vol. 31, no. 2, pp. 282-96.

Pomies, L, Decourteix, M, Franchel, J, Moulia, B & Leblanc-Fournier, N 2017, 'Poplar stem transcriptome is massively remodelled in response to single or repeated mechanical stimuli', *Bmc Genomics*, vol. 18, p. 16.

Preston, RD 1934, 'The organization of the cell wall of the conifer tracheid', *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, vol. 224, pp. 131-45.

Preuss, ML, Kovar, DR, Lee, YRJ, Staiger, CJ, Delmer, DP & Liu, B 2004, 'A plant-specific kinesin binds to actin microfilaments and interacts with cortical microtubules in cotton fibers', *Plant Physiology*, vol. 136, no. 4, pp. 3945-55.

Prodhan, A, Funada, R, Ohtani, J, Abe, H & Fukazawa, K 1995, 'Orientation of microfibrils and microtubules in developing tension wood fibers of Japanese ash (Fraxinus mandshurica var japonica)', *Planta*, vol. 196, no. 3, pp. 577-85.

Putoczki, TL, Pettolino, F, Griffin, MDW, Moller, R, Gerrard, JA, Bacic, A & Jackson, SL 2007, 'Characterization of the structure, expression and function of Pinus radiata D. Don arabinogalactan-proteins', *Planta*, vol. 226, no. 5, pp. 1131-42.

Qin, T, Liu, XM, Li, JJ, Sun, JB, Song, LN & Mao, TL 2014, 'Arabidopsis Microtubule-Destabilizing Protein 25 Functions in Pollen Tube Growth by Severing Actin Filaments', *Plant Cell*, vol. 26, no. 1, pp. 325-39.

Qiu, D, Wilson, IW, Gan, S, Washusen, R, Moran, GF & Southerton, SG 2008, 'Gene expression in Eucalyptus branch wood with marked variation in cellulose microfibril orientation and lacking G-layers', *New Phytologist*, vol. 179, no. 1, pp. 94-103.

Rajangam, AS, Kumar, M, Aspeborg, H, Guerriero, G, Arvestad, L, Pansri, P, Brown, CJL, Hober, S, Blomqvist, K, Divne, C, Ezcurra, I, Mellerowicz, E, Sundberg, B, Bulone, V & Teeri, TT 2008, 'MAP20, a microtubule-associated protein in the secondary cell walls of hybrid aspen, is a target of the cellulose synthesis inhibitor 2,6-dichlorobenzonitrile', *Plant Physiology*, vol. 148, no. 3, pp. 1283-94.

Roignant, J, Badel, E, Leblanc-Fournier, N, Brunel-Michac, N, Ruelle, J, Moulia, B & Decourteix, M 2018, 'Feeling stretched or compressed? The multiple mechanosensitive responses of wood formation to bending', *Annals of Botany*, vol. 121, no. 6, pp. 1151-61.

Rosero, A, Zarsky, V & Cvrckova, F 2013, 'AtFH1 formin mutation affects actin filament and microtubule dynamics in Arabidopsis thaliana', *Journal of Experimental Botany*, vol. 64, no. 2, pp. 585-97.

Sakamoto, S, Somssich, M, Nakata, MT, Unda, F, Atsuzawa, K, Kaneko, Y, Wang, T, Bagman, AM, Gaudinier, A, Yoshida, K, Brady, SM, Mansfield, SD, Persson, S & Mitsuda, N 2018, 'Complete substitution of a secondary cell wall with a primary cell wall in Arabidopsis', *Nature Plants*, vol. 4, no. 10, pp. 777-83.

Sampathkumar, A, Gutierrez, R, McFarlane, HE, Bringmann, M, Lindeboom, J, Emons, AM, Samuels, L, Ketelaar, T, Ehrhardt, DW & Persson, S 2013, 'Patterning and lifetime of plasma membrane-localized cellulose synthase is dependent on actin organization in Arabidopsis interphase cells', *Plant Physiology*, vol. 162, no. 2, pp. 675-88.

Sampathkumar, A, Lindeboom, JJ, Debolt, S, Gutierrez, R, Ehrhardt, DW, Ketelaar, T & Persson, S 2011, 'Live Cell Imaging Reveals Structural Associations between the Actin and Microtubule Cytoskeleton in Arabidopsis', *Plant Cell*, vol. 23, no. 6, pp. 2302-13.

Sardar, HS, Yang, J & Showalter, AM 2006, 'Molecular interactions of arabinogalactan proteins with cortical Microtubules and F-actin in bright yellow-2 tobacco cultured cells', *Plant Physiology*, vol. 142, no. 4, pp. 1469-79.

Sasaki, T, Fukuda, H & Oda, Y 2017, 'CORTICAL MICROTUBULE DISORDERING1 is required for secondary cell wall patterning in xylem vessels', *Plant Cell*, vol. 29, no. 12, pp. 3123-39.

Sattler, DF, Comeau, PG & Achim, A 2014, 'Within-tree patterns of wood stiffness for white spruce (Picea glauca) and trembling aspen (Populus tremuloides)', *Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere*, vol. 44, no. 2, pp. 162-71.

Scheller, HV & Ulvskov, P 2010, 'Hemicelluloses', *Annual Review of Plant Biology, Vol 61*, vol. 61, pp. 263-89.

Schiff, PB, Fant, J & Horwitz, SB 1979, 'Promotion of microtubule assembly in vitro by taxol', *Nature*, vol. 277, no. 5698, pp. 665-7.

Schneider, R, Tang, L, Lampugnani, ER, Barkwill, S, Lathe, R, Zhang, Y, McFarlane, HE, Pesquet, E, Niittyla, T, Mansfield, SD, Zhou, YH & Persson, S 2017, 'Two complementary mechanisms underpin cell wall patterning during xylem vessel development', *Plant Cell*, vol. 29, no. 10, pp. 2433-49.

Scurfield, G 1973, 'Reaction wood - its structure and function', *Science*, vol. 179, no. 4074, pp. 647-55.

Seagull, RW 1990, 'The effects of microtubule and microfilament disrupting agents on cytoskeletal arrays and wall deposition in developing cotton fibers', *Protoplasma*, vol. 159, no. 1, pp. 44-59.

Seifert, GJ & Roberts, K 2007, 'The biology of arabinogalactan proteins', *Annual Review of Plant Biology*, vol. 58, pp. 137-61.

Showalter, AM 2001, 'Arabinogalactan-proteins: structure, expression and function', *Cellular and Molecular Life Sciences*, vol. 58, no. 10, pp. 1399-417.

Showalter, AM & Basu, D 2016, 'Extensin and arabinogalactan-protein biosynthesis: glycosyltransferases, research Challenges, and biosensors', *Frontiers in Plant Science*, vol. 7, p. 9.

Smertenko, AP, Chang, HY, Sonobe, S, Fenyk, SI, Weingartner, M, Bogre, L & Hussey, PJ 2006, 'Control of the AtMAP65-1 interaction with microtubules through the cell cycle', *Journal of Cell Science*, vol. 119, no. 15, pp. 3227-37.

Smertenko, T, Turner, G, Fahy, D, Brew-Appiah, RAT, Alfaro-Aco, R, Engler, JD, Sanguinet, KA & Smertenko, A 2020, 'Brachypodium distachyon MAP20 functions in metaxylem pit development and contributes to drought recovery', *New Phytologist*, p. 15.

Snustad, DP, Haas, NA, Kopczak, SD & Silflow, CD 1992, 'The small genome of Arabidopsis contains at least 9 expressed beta-tubulin genes', *Plant Cell*, vol. 4, no. 5, pp. 549-56.

Spokevicius, A, Southerton, SG, MacMillan, CP, Qiu, D, Gan, S, Tibbits, JFG, Moran, GF & Bossinger, G 2007, 'Beta-tubulin affects cellulose microfibril orientation in plant secondary fibre cell walls', *Plant Journal*, vol. 51, no. 4, pp. 717-26.

Spokevicius, A, Taylor, L, Melder, E, Van Beveren, K, Tibbits, J, Creux, N & Bossinger, G 2016, 'The use of induced somatic sector analysis (ISSA) for studying genes and promoters involved in wood formation and secondary stem development', *Jove-Journal of Visualized Experiments*, no. 116, p. .

Spokevicius, A, Van Beveren, K, Leitch, MM & Bossinger, G 2005, 'Agrobacterium-mediated in vitro transformation of wood-producing stem segments in eucalypts', *Plant Cell Reports*, vol. 23, no. 9, pp. 617-24.

Stoppin-Mellet, V, Fache, V, Portran, D, Martiel, JL & Vantard, M 2013, 'MAP65 coordinate microtubule growth during bundle formation', *Plos One*, vol. 8, no. 2, p. 17.

Strachan, SD & Hess, FD 1983, 'The biochemical mechanism of action of the dinitroaniline herbicide oryzalin', *Pesticide Biochemistry and Physiology*, vol. 20, no. 2, pp. 141-50.

Sugimoto, K, Himmelspach, R, Williamson, RE & Wasteneys, GO 2003, 'Mutation or drug-dependent microtubule disruption causes radial swelling without altering parallel cellulose microfibril deposition in Arabidopsis root cells', *Plant Cell*, vol. 15, no. 6, pp. 1414-29.

Sugimoto, K, Williamson, RE & Wasteneys, GO 2001, 'Wall architecture in the cellulose-deficient rsw1 mutant of Arabidopsis thaliana: microfibrils but not microtubules lose their transverse alignment before microfibrils become unrecognizable in the mitotic and elongation zones of roots', *Protoplasma*, vol. 215, no. 1-4, pp. 172-83.

Sugiyama, Y, Nagashima, Y, Wakazaki, M, Sato, M, Toyooka, K, Fukuda, H & Oda, Y 2019, 'A Rho-actin signaling pathway shapes cell wall boundaries in Arabidopsis xylem vessels', *Nature Communications*, vol. 10, p. 10.

Swamy, PS, Hu, H, Pattathil, S, Maloney, VJ, Xiao, H, Xue, LJ, Chung, JD, Johnson, VE, Zhu, YY, Peter, GF, Hahn, MG, Mansfield, SD, Harding, SA & Tsai, CJ 2015, 'Tubulin perturbation leads to unexpected cell wall modifications and affects stomatal behaviour in Populus', *Journal of Experimental Botany*, vol. 66, no. 20, pp. 6507-18.

Szyjanowicz, PMJ, McKinnon, I, Taylor, NG, Gardiner, J, Jarvis, MC & Turner, SR 2004, 'The irregular xylem 2 mutant is an allele of korrigan that affects the secondary cell wall of Arabidopsis thaliana', *Plant Journal*, vol. 37, no. 5, pp. 730-40.

Szymanski, D & Staiger, CJ 2018, 'The actin cytoskeleton: functional arrays for cytoplasmic organization and cell shape control', *Plant Physiology*, vol. 176, no. 1, pp. 106-18.

Takahashi, H, Kawahara, A & Inoue, Y 2003, 'Ethylene promotes the induction by auxin of the cortical microtubule randomization required for low-pH-induced root hair initiation in lettuce (Lactuca sativa L.) seedlings', *Plant and Cell Physiology*, vol. 44, no. 9, pp. 932-40.

Tarmian, A & Azadfallah, M 2009, 'Variation of cell features and chemical composition in spruce consisting of opposite, normal and compression wood', *Bioresources*, vol. 4, no. 1, pp. 194-204.

Taylor, NG, Howells, RM, Huttly, AK, Vickers, K & Turner, SR 2003, 'Interactions among three distinct CesA proteins essential for cellulose synthesis', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 3, pp. 1450-5.

Thamarus, K, Groom, K, Bradley, A, Raymond, CA, Schimleck, LR, Williams, ER & Moran, GF 2004, 'Identification of quantitative trait loci for wood and fibre properties in two full-sib pedigrees of Eucalyptus globulus', *Theoretical and Applied Genetics*, vol. 109, no. 4, pp. 856-64.

Thorpe, TA 2007, 'History of plant tissue culture', *Molecular Biotechnology*, vol. 37, no. 2, pp. 169-80.

Thumma, BR, Southerton, SG, Bell, JC, Owen, JV, Henery, ML & Moran, GF 2010, 'Quantitative trait locus (QTL) analysis of wood quality traits in Eucalyptus nitens', *Tree Genetics & Genomes*, vol. 6, no. 2, pp. 305-17.

Tian, GW, Smith, D, Gluck, S & Baskin, TI 2004, 'Higher plant cortical microtubule array analyzed in vitro in the presence of the cell wall', *Cell Motility and the Cytoskeleton*, vol. 57, no. 1, pp. 26-36.

Tolmie, F, Poulet, A, McKenna, J, Sassmann, S, Graumann, K, Deeks, M & Runions, J 2017, 'The cell wall of Arabidopsis thaliana influences actin network dynamics', *Journal of Experimental Botany*, vol. 68, no. 16, pp. 4517-27.

Turner, S & Kumar, M 2018, 'Cellulose synthase complex organization and cellulose microfibril structure', *Philosophical Transactions of the Royal Society a-Mathematical Physical and Engineering Sciences*, vol. 376, no. 2112, p. 11.

Uyttewaal, M, Burian, A, Alim, K, Landrein, BT, Borowska-Wykret, D, Dedieu, A, Peaucelle, A, Ludynia, M, Traas, J, Boudaoud, A, Kwiatkowska, D & Hamant, O 2012, 'Mechanical Stress Acts via Katanin to Amplify Differences in Growth Rate between Adjacent Cells in Arabidopsis', *Cell*, vol. 149, no. 2, pp. 439-51.

Villalobos, DP, Diaz-Moreno, SM, Said, ESS, Canas, RA, Osuna, D, Van Kerckhoven, SHE, Bautista, R, Claros, MG, Canovas, FM & Canton, FR 2012, 'Reprogramming of gene expression during compression wood formation in pine: Coordinated modulation of S-adenosylmethionine, lignin and lignan related genes', *Bmc Plant Biology*, vol. 12, p. 17.

Wang, C, Li, JJ & Yuan, M 2007, 'Salt tolerance requires cortical microtubule reorganization in arabidopsis', *Plant and Cell Physiology*, vol. 48, no. 11, pp. 1534-47.

Wang, H, Jiang, CM, Wang, CT, Yang, Y, Yang, L, Gao, XY & Zhang, HX 2015, 'Antisense expression of the fasciclin-like arabinogalactan protein FLA6 gene in Populus inhibits expression of its homologous genes and alters stem biomechanics and cell wall composition in transgenic trees', *Journal of Experimental Botany*, vol. 66, no. 5, pp. 1291-302.

Wang, H, Jin, YL, Wang, CT, Li, B, Jiang, CM, Sun, ZC, Zhang, ZP, Kong, FJ & Zhang, HX 2017a, 'Fasciclin-like arabinogalactan proteins, PtFLAs, play important roles in GA-mediated tension wood formation in Populus', *Scientific Reports*, vol. 7, p. 13.

Wang, H, Yu, ZX, Zhang, XX, Ren, D & Yu, Y 2017b, 'The combined effects of initial microfibrillar angle and moisture contents on the tensile mechanical properties and angle alteration of wood foils during tension', *Holzforschung*, vol. 71, no. 6, pp. 491-7.

Wang, JJ, Zhang, Y, Wu, J, Meng, L & Ren, HY 2013, 'At FH16, an Arabidopsis Type II Formin, Binds and Bundles both Microfilaments and Microtubules, and Preferentially Binds to Microtubules', *Journal of Integrative Plant Biology*, vol. 55, no. 11, pp. 1002-15.

Wang, LX & Nick, P 2017, 'Cold sensing in grapevine-Which signals are upstream of the microtubular "thermometer"', *Plant Cell and Environment*, vol. 40, no. 11, pp. 2844-57.

Wang, XF & Mao, TL 2019, 'Understanding the functions and mechanisms of plant cytoskeleton in response to environmental signals', *Current Opinion in Plant Biology*, vol. 52, pp. 86-96.

Wardrop, AB & Dadswell, HE 1948, 'The nature of reaction wood. 1. The structure and properties of tension wood fibres', *Australian Journal of Scientific Research Series B-Biological Sciences*, vol. 1, no. 1, pp. 3-16.

—— 1950, 'The nature of reaction wood. 2. The cell wall organization of compression wood tracheids', *Australian Journal of Scientific Research Series B-Biological Sciences*, vol. 3, no. 1, pp. 1-13.

Washusen, R & Evans, R 2001, 'The association between cellulose crystallite width and tension wood occurrence in Eucalyptus globulus', *Iawa Journal*, vol. 22, no. 3, pp. 235-43.

Washusen, R, Evans, R & Southerton, S 2005, 'A study of Eucalyptus grandis and Eucalyptus globulus branch wood microstructure', *Iawa Journal*, vol. 26, no. 2, pp. 203-10.

Wasteneys, GO 2002, 'Microtubule organization in the green kingdom: chaos or self-order?', *Journal of Cell Science*, vol. 115, no. 7, pp. 1345-54.

Wasteneys, GO & Galway, ME 2003, 'Remodelling the cytoskeleton for growth and form: An overview with some new views', *Annual Review of Plant Biology*, vol. 54, pp. 691-722.

Watanabe, Y, Meents, MJ, McDonnell, LM, Barkwill, S, Sampathkumar, A, Cartwright, HN, Demura, T, Ehrhardt, DW, Samuels, AL & Mansfield, SD 2015, 'Visualization of cellulose synthases in Arabidopsis secondary cell walls', *Science*, vol. 350, no. 6257, pp. 198-203.

Watanabe, Y, Schneider, R, Barkwill, S, Gonzales-Vigil, E, Hill, JL, Jr., Samuels, AL, Persson, S & Mansfield, SD 2018, 'Cellulose synthase complexes display distinct dynamic behaviors during xylem transdifferentiation', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 115, no. 27, pp. E6366-E74.

Wessels, CB, Crafford, PL, Du Toit, B, Grahn, T, Johansson, M, Lundqvist, SO, Sall, H & Seifert, T 2016, 'Variation in physical and mechanical properties from three drought tolerant Eucalyptus species grown on the dry west coast of Southern Africa', *European Journal of Wood and Wood Products*, vol. 74, no. 4, pp. 563-75.

Whetten, R, Sun, YH, Zhang, Y & Sederoff, R 2001, 'Functional genomics and cell wall biosynthesis in loblolly pine', *Plant Molecular Biology*, vol. 47, no. 1-2, pp. 275-91.

Whittaker, DJ & Triplett, BA 1999, 'Gene-specific changes in alpha-tubulin transcript accumulation in developing cotton fibers', *Plant Physiology*, vol. 121, no. 1, pp. 181-8.

Whittington, AT, Vugrek, O, Wei, KJ, Hasenbein, NG, Sugimoto, K, Rashbrooke, MC & Wasteneys, GO 2001, 'MOR1 is essential for organizing cortical microtubules in plants', *Nature*, vol. 411, no. 6837, pp. 610-3.

Wicker-Planquart, C, Stoppin-Mellet, V, Blanchoin, L & Vantard, M 2004, 'Interactions of tobacco microtubule-associated protein MAP65-1b with microtubules', *Plant Journal*, vol. 39, no. 1, pp. 126-34.

Wightman, R & Turner, SR 2008, 'The roles of the cytoskeleton during cellulose deposition at the secondary cell wall', *Plant Journal*, vol. 54, no. 5, pp. 794-805.

Williamson, RE, Burn, JE, Birch, R, Baskin, TI, Arioli, T, Betzner, AS & Cork, A 2001, 'Morphology of rsw1, a cellulose-deficient mutant of Arabidopsis thaliana', *Protoplasma*, vol. 215, no. 1-4, pp. 116-27.

Wilson, PG & Borisy, GG 1997, 'Evolution of the multi-tubulin hypothesis', *Bioessays*, vol. 19, no. 6, pp. 451-4.

Woodley, M, Mulvihill, A, Fujita, M & Wasteneys, GO 2018, 'Exploring microtubule-dependent cellulose-synthase-complex movement with high precision particle tracking', *Plants (Basel, Switzerland)*, vol. 7, no. 3.

Xi, W, Song, D, Sun, J, Shen, J & Li, L 2017, 'Formation of wood secondary cell wall may involve two type cellulose synthase complexes in Populus', *Plant Molecular Biology*, vol. 93, no. (4-5), pp. 419-29.

Xiao, Y, Stegmann, M, Han, ZF, DeFalco, TA, Parys, K, Xu, L, Belkhadir, Y, Zipfel, C & Chai, JJ 2019, 'Mechanisms of RALF peptide perception by a heterotypic receptor complex', *Nature*, vol. 572, no. 7768, pp. 270-+.

Xu, SL, Rahman, A, Baskin, TI & Kieber, JJ 2008, 'Two leucine-rich repeat receptor kinases mediate signaling, linking cell wall biosynthesis and ACC synthase in Arabidopsis', *Plant Cell*, vol. 20, no. 11, pp. 3065-79.

Xu, T, Qu, Z, Yang, XY, Qin, XH, Xiong, JY, Wang, YQ, Ren, DT & Liu, GQ 2009, 'A cotton kinesin GhKCH2 interacts with both microtubules and microfilaments', *Biochemical Journal*, vol. 421, pp. 171-80.

Xu, TD, Wen, MZ, Nagawa, S, Fu, Y, Chen, JG, Wu, MJ, Perrot-Rechenmann, C, Friml, J, Jones, AM & Yang, ZB 2010, 'Cell surface- and rho GTPase-based auxin signaling controls cellular interdigitation in Arabidopsis', *Cell*, vol. 143, no. 1, pp. 99-110.

Yamaguchi, M, Goue, N, Igarashi, H, Ohtani, M, Nakano, Y, Mortimer, JC, Nishikubo, N, Kubo, M, Katayama, Y, Kakegawa, K, Dupree, P & Demura, T 2010, 'VASCULAR-RELATED NAC-DOMAIN6 and VASCULAR-RELATED NAC-DOMAIN7 effectively induce transdifferentiation into xylem vessel elements under control of an induction system', *Plant Physiology*, vol. 153, no. 3, pp. 906-14.

Yamashita, S, Yoshida, M, Yamamoto, H & Okuyama, T 2008, 'Screening genes that change expression during compression wood formation in Chamaecyparis obtusa', *Tree Physiology*, vol. 28, no. 9, pp. 1331-40.

Yang, B, Voiniciuc, C, Fu, LB, Dieluweit, S, Klose, H & Usadel, B 2019, 'TRM4 is essential for cellulose deposition in Arabidopsis seed mucilage by maintaining cortical microtubule organization and interacting with CESA3', *New Phytologist*, vol. 221, no. 2, pp. 881-95.

Yoshikawa, M, Yang, GX, Kawaguchi, K & Komatsu, S 2003, 'Expression analyses of beta-tubulin isotype genes in rice', *Plant and Cell Physiology*, vol. 44, no. 11, pp. 1202-7.

Zhang, Q, Lin, F, Mao, TL, Nie, JN, Yan, M, Yuan, M & Zhang, WH 2012, 'Phosphatidic acid regulates microtubule organization by interacting with MAP65-1 in response to salt stress in Arabidopsis', *Plant Cell*, vol. 24, no. 11, pp. 4555-76.

Zhang, X, Dominguez, PG, Kumar, M, Bygdell, J, Miroshnichenko, S, Sundberg, B, Wingsle, G & Niittyla, T 2018, 'Cellulose synthase stoichiometry in Aspen differs from Arabidopsis and Norway spruce', *Plant Physiology*, vol. 177, no. 3, pp. 1096-107.

Zhang, Y, Sederoff, RR & Allona, I 2000, 'Differential expression of genes encoding cell wall proteins in vascular tissues from vertical and bent loblolly pine trees', *Tree Physiology*, vol. 20, no. 7, pp. 457-66.

Zhong, RQ, Burk, DH, Morrison, WH & Ye, ZH 2002, 'A kinesin-like protein is essential for oriented deposition of cellulose microfibrils and cell wall strength', *Plant Cell*, vol. 14, no. 12, pp. 3101-17.

Zhong, RQ, Cui, DT & Ye, ZH 2019, 'Secondary cell wall biosynthesis', *New Phytologist*, vol. 221, no. 4, pp. 1703-23.

Zhu, L., Zhang, Y, Kang, EF, Xu, QY, Wang, MY, Rui, Y, Liu, BQ, Yuan, M & Fu, Y 2013, 'MAP18 Regulates the Direction of Pollen Tube Growth in Arabidopsis by Modulating F-Actin Organization', *Plant Cell*, vol. 25, no. 3, pp. 851-67.

Zhu, CM & Dixit, R 2011, 'Single Molecule Analysis of the Arabidopsis FRA1 Kinesin Shows that It Is a Functional Motor Protein with Unusually High Processivity', *Molecular Plant*, vol. 4, no. 5, pp. 879-85.

Zinkgraf, M, Gerttula, S, Zhao, S, Filkov, V & Groover, A 2018, 'Transcriptional and temporal response of Populus stems to gravi-stimulation', *Journal of Integrative Plant Biology*, vol. 60, no. 7, pp. 578-90.

Appendix A

Visualisation of cortical microtubules was performed using immunolabelling in which anti-α-tubulin produced in mouse was used as primary antibody and anti-mouse IgG1 CF™488A antibody produced in goat was used as secondary antibody. To assess whether there was non-specific binding of the secondary antibody to the sample, longitudinal sections of poplar wood were incubated with the secondary antibody alone. In addition, incubations with no antibody and the primary antibody alone were performed to assess the level of lignin autofluorescence in the samples, slides were visualised under confocal microscope with 404.8 nm laser (Alexa Fluor 430). Samples exhibited some degree of autofluorescence (Fig A.1 b, e, h and k) but no signal was received with the 488.6 nm laser (Alexa Fluor 488) when samples were incubated with no antibody or with the primary or the secondary antibodies alone (Fig A.1 c, f and i). Samples incubated with both antibodies as described in Chapter 2.2.2 presented strong signal at 488.6 nm (Fig A.1 l). Therefore, anti-mouse IgG1 CF™488A specifically bonded to anti-α-tubulin allowing the study of cortical microtubules.

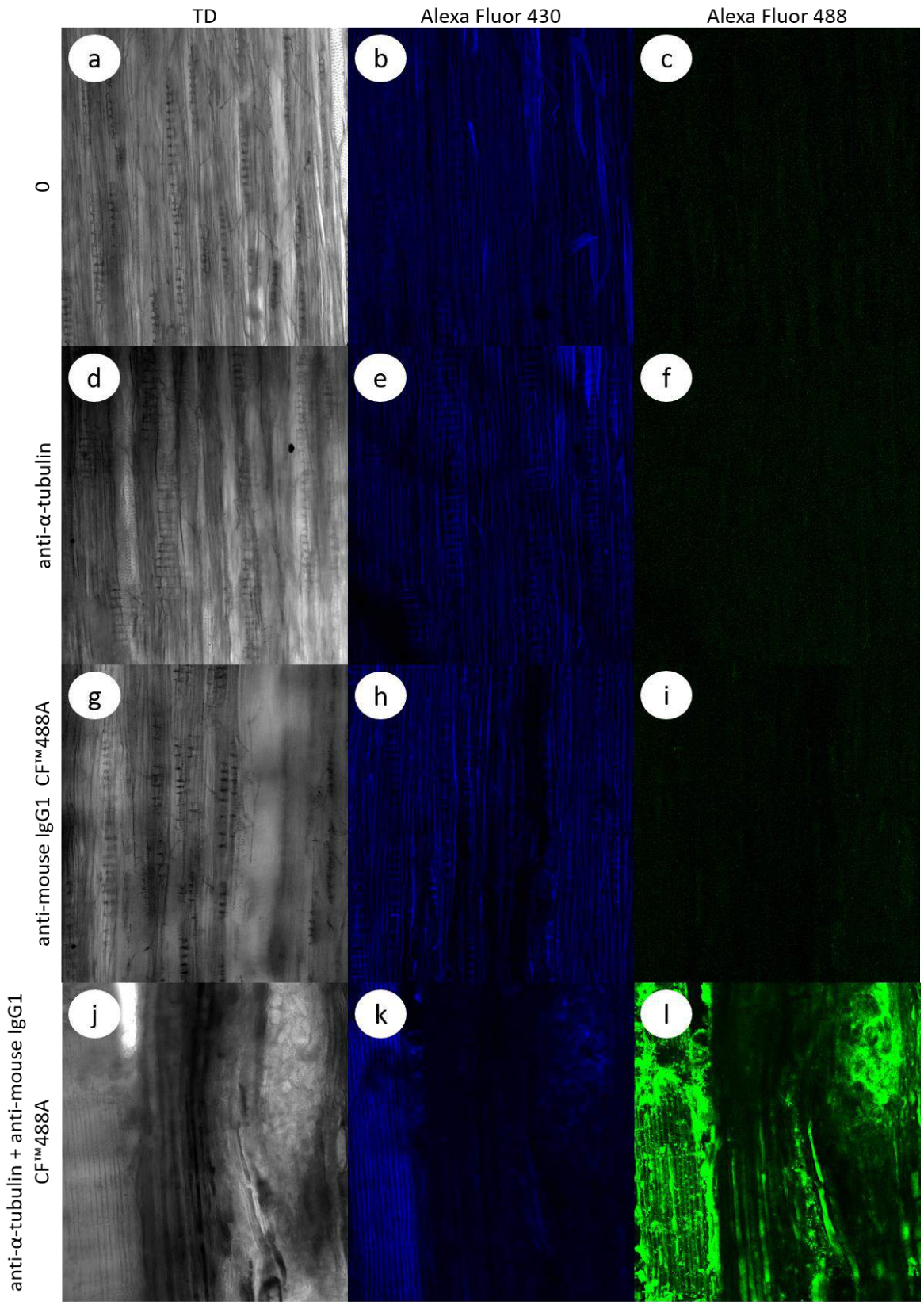

Figure A.1: ***Antibody assay.*** *Longitudinal sections of poplar wood (a-i) and pine apical stem segment (j-l) incubated with different combinations of primary and secondary antibodies. TD, transmitted light.*

Appendix B

Absolute values for tension (Table B.1) and compression wood traits (Table B.2) regarding microtubule organisation and cell morphology are compiled here.

Table B.1: Tension wood traits

Species	Trait	Cell Morphology					*Microtubules*				
	Sample	MFA (˚)	Cell wall area (µm²)	Fibre length	Lumen area (µm²)	Fibre area (µm²)	Length (µm)	Bundling (µm)	Branching events	Density	*MTA (˚)*
Populus alba	**I-TW**	38.06±2.[a]	51.7±10[a]	532.46±45.[a]	108.73±45.[a]	159.4±44.2[a]	8.33±2.7[a]	0.394[a]	25±7.3[a]	37.2±1.8[a]	*37.6±0.6[ab]*
	NW	23.3±0.7[b]	37.1±6.7[a]	569±43.5[a]	91±17.6[a]	128.2±24.1[a]	6.34±3.84[a]	0.41±0.01[a]	37±3.1[a]	29.7±0.25[b]	*29.63±2.7[b]*
	I-OW	47.23±3.7[a]	58±8.1[a]	485.2±27.7[a]	105.9±42.2[a]	159.2±37.4[a]	5.7±0.1[a]	0.46[a]	23±11.1[a]	29.67±0.88[b]	*45.03±3.4[a]*
	B-TW	40.9±2.3[a]	67.4±6.2[a]	465.6±25.2[a]	38.5±14.5[a]	117.25±20.11[a]	6.24±1.29[a]	0.617±0.07[a]	31±3.12[a]	37.2±1.8[a]	*39.85±1.3[a]*
	B-OW	42.4±0.8[a]	44.5±0.7[a]	466.9±56.9[a]	59.5±16[a]	106.81±5.6[a]	5.18±0.14[a]	0.65±0.03[a]	14±0.15[b]	20.7±0.85[a]	*46.2±0.57[a]*
Eucalyptus globulus	**I-TW**	32±0.94[a]	45.51±20.6[a]	528±13.2[a]	19.5±2.7[a]	63.52±21[a]	8.6±4.3[a]	0.63±0.05[a]	18.6±7.4[a]	17±4[a]	*41.7±1.7[a]*
	NW	41.16±7.3[a]	37.35±4.3[a]	577.3±5[a]	62.9±10.2[b]	100.3±14.5[a]	5.63±0.8[a]	0.57±0.04[a]	30.8±3.8[a]	21.2±1.6[a]	*42.2±1.8[ab]*
	I-OW	47±3.17[a]	28.5±11.3[a]	570.8±2.7[a]	46.12±11[ab]	71.4±25[a]	4.93±0.9[a]	0.7±0.09[a]	18.6±4.7[a]	22.1±2.6[a]	*60.8±1.2[b]*
	B-TW	32.63±0.8[a]	45.33±6.9[a]	544.67±18.9[a]	45.76±11.17[a]	91.1±18[a]	11.57±1.36[a]	0.57[a]	33.26±8.53[a]	22.26±1.16[a]	*42.01±1.31[a]*
	B-OW	43.6±1.8[a]	38±9[a]	571±38.5[a]	10.9±2.5[b]	48.95±10.7[a]	8.69±4.75[a]	0.77±0.05[b]	33.2±1.9[a]	19.55±1.39[a]	*49.57±3.34[a]*

Means (±s.e.) for each of the microtubule organisation and cell morphology aspects investigated in tension wood (TW) normal wood (NW) and opposite wood (OW) of the induced (I) and branch (B) states for poplar and eucalypt. Means that do not share a letter within each column for each species and state are significantly different.

Table B.2: Compression wood traits

Trait	**Cell Morphology**					***Microtubules***				
Sample	**MFA (°)**	**Cell wall area (µm²)**	**Tracheid length**	**Lumen area (µm²)**	**Tracheid area (µm²)**	**Length (µm)**	**Bundling (µm)**	**Branching events**	**Density**	***MTA (°)***
I-CW	38.8±5.9[a]	101.32±2.08[a]	996.34±49[a]	189.99±76[a]	291.32±74.6[a]	3.9±0.17[a]	0.4±0.05[a]	27±2.7[a]	21.3±1.1[a]	*41.7±1.7[a]*
NW	36.53±3[a]	80.83±25.08[a]	1047.2±53[a]	222.36±58[a]	303.2±81.83[a]	7.72±2.6[a]	0.56±0.01[a]	27.6±9.5[a]	22.27±1.27[a]	*46.4±7.3[a]*
I-OW	37±3.7[a]	92.63±19[a]	1089.8±164.5[a]	269.16±47[a]	361.8±38.4[a]	10.75±6[a]	0.57±0.06[a]	13.85±3.35[a]	21.53±2.35[a]	*37.06±1.93[a]*
B-CW	37.75±1.3[a]	144.8±37.5[a]	1131.75±49[a]	127.7±43.6[a]	241.9±41.2[a]	17.09±0.39[a]	0.89±0.03[a]	9.93±4.05[a]	15.46±0.35[a]	*41.55±0.87[a]*
B-OW	39.5±8[a]	88.2±17.8[a]	1155.5±88[a]	153.65±52[a]	272.4±6.1[a]	17.83±3.38[a]	0.78±0.06[a]	9.66±4.26[1]	17.8±0.52[b]	*38.61±0.96[a]*

Means (±s.e.) for each of the microtubule organisation and cell morphology aspects investigated in compression wood (CW), normal wood (NW) and opposite wood (OW) of the induced (I) and branch (B) states pine samples. Means that do not share a letter within each column for each state are significantly different.