



Cortical microtubule dynamics during reaction wood formation ensures context-appropriate cellulose microfibril angle in woody trees

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Abstract

Key message Cortical microtubule arrays are the primary mechanism for guiding the re-orientation of cellulose microfibrils and determining MFA in secondary cell wall of wood fibre and tracheid cells in reaction wood.

Abstract Microtubules are directly and indirectly involved in guiding cellulose synthase complexes (CSCs) through the plasma membrane. The angle of cellulose deposition is a critical response to environmental signals and/or stress conditions, and particularly crucial during reaction wood formation, a process in which woody plants deposit additional cell wall material to counteract gravitational forces. Tubulin genes are upregulated in response to gravitational stimulus during reaction wood formation, which can result in changes to microtubule assembly. In this study, microtubules were visualised in three woody tree species (two angiosperms: *Eucalyptus globulus* Labill., *Populus alba* L., and one gymnosperm: *Pinus radiata* D. Don.) using immunofluorescence to quantitatively evaluate microtubule organisation during reaction wood formation. Our results suggest that reorientation of the cortical microtubule array affects secondary cell wall deposition, even across different types of reaction wood, by ensuring context-appropriate orientation of cellulose microfibrils and determining MFA in wood cells. Pharmacological studies conducted on in vitro cultured stem segments or in vivo during reaction wood formation corroborated these important roles for microtubules during wood development. This study starts to unveil the role of tubulins during wood formation by exploring cortical microtubule array organisation in trees subjected to gravitational stimulus and it sheds light on cellular and molecular mechanisms behind cellulose deposition in tree species.

Keywords Cellulose deposition · Microfibril angle · Microtubules · Reaction wood · Secondary cell wall · Wood formation

Introduction

In woody trees, reaction wood develops in response to gravity-associated stress, which can be caused by wind or load, and it has been frequently 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 response that returns the stem to an upright position by reorientating branches, reinforcing stress points or maintaining branch angles (Almeras and Clair 2016). The resulting tissue, called reaction wood, is categorised as compression wood, usually found at the lower side of gymnosperm branches or tension wood, commonly found at the upper side of angiosperm branches. The wood formed opposite to reaction wood is referred to as opposite wood and differs from normal wood formed in upright stems (Fisher and Stevenson 1981). Gravitropic and mechanical signals initiate response in cambial stem cells that regulate changes in wood properties during xylogenesis (Fisher et al. 2019).

Cell wall architecture and mechanical properties of fibres and tracheids are largely determined by the orientation of cellulose microfibrils. For instance, changes in the cellulose microfibril angle (MFA), measured as the deviation of microfibrils from the long axis of the cell in the S₂ layer of the secondary cell wall (Donaldson 2008), is a

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distinguishing characteristic of reaction wood: compression wood features a large MFA (30° – 40°), while normal wood and opposite wood are characterised by intermediate values (10° – 20°) and tension wood has very low values (less than 5°) (Almeras and Clair 2016). Other cellular modifications in tension wood include the development of a gelatinous layer (G-layer) in the secondary cell wall in a number of angiosperm species, such as poplar (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 crystalline cellulose microfibrils (Jourez et al. 2001; Washusen and Evans 2001; Washusen et al. 2005). On the other hand, compression wood cells are rounded with large intercellular spaces, lack the S_3 layer, have helical cavities in their lumen, higher lignin content and a comparatively low cellulose content (Tarmian and Azadfallah 2009; Wardrop and Dadswell 1950).

At the molecular level, several genes are involved in the regulation of reaction wood formation. Gene expression studies have revealed that α - and β -tubulins are highly expressed in tension and compression wood (Li et al. 2013; Qiu et al. 2008). Downregulation of a *Eucalyptus grandis* β -tubulin-encoding gene (*EgrTUB1*) results in 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. Opposite wood also likely plays an active role in responding to gravity associated stress, suggested by the number of genes reported to be differentially expressed in opposite versus normal wood (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.

Plasma membrane-localised cellulose synthase complexes (CSCs) often move along tracks delineated by cortical microtubules in the primary cell wall, and CSC-microtubule interactions govern the organised deposition of cellulose synthesis (Paredes et al. 2006). When compared to the primary cell wall, the secondary cell wall presents differences in composition and mechanical properties. In the secondary cell wall particularly, cellulose microfibrils act as the main load-bearing cell wall component and their arrangement has great influence on cell wall physical properties (Hein and Lima 2012); however, cellulose deposition occurs in a similar manner to the primary cell wall (Watanabe et al. 2015). The impact of microtubules on cellulose microfibril orientation has been acknowledged since their first observations (Green 1962). Studies inhibiting cortical microtubule organisation (Baskin et al. 2004; Whittington et al. 2001) or cellulose microfibril deposition (Paredes et al. 2008; Williamson et al. 2001) in *Arabidopsis* by pharmacological and

genetic approaches suggested that cortical microtubules guide the movement of CSCs. Recent studies revealed the activity of microtubule associated proteins (MAPs), such as cellulose interactive protein 1 (CS11) and companion of cellulose synthase 1 (CC1), in physically linking microtubules and CSCs (Kesten et al. 2019; Schneider et al. 2017). It seems that microtubule-CSC interactions are particularly important during CSC reorientation (Chan and Coen 2020). These results imply that microtubules may play an important role in the reorganisation of cell wall deposition during reaction wood formation (Tobias et al. 2020). However, the molecular mechanisms that govern reaction wood formation remain poorly defined, chiefly because these systems are less amenable to techniques that are commonly used to study *Arabidopsis* primary cell wall deposition. Woody trees are less genetically tractable and fluorescent protein marker lines do not exist to monitor microtubule organisation or cell wall synthesis in these species. Furthermore, since wood forms deep within tissues, it is less accessible to microtubule inhibitors or microscopy.

Here, we overcome several of these technical hurdles using complementary approaches and report on the role played by microtubules during the deposition of secondary cell walls in three species of woody trees (two angiosperms: *Eucalyptus globulus* Labill. and *Populus alba* L., and one gymnosperm: *Pinus radiata* D. Don.) using reaction wood as a model. Divergent woody species were investigated to understand whether microtubule dynamics mechanisms were universal across tree species or whether specific differences existed between tension (angiosperm) and compression (gymnosperms) wood producing species or between genus' producing tension wood (*Populus* and *Eucalyptus*). We examined both induced reaction wood, where seedlings actively produced reaction wood in response to a change in orientation and load; and branches, where reaction wood formed while branch stems are subjected to a constant orientation and static load imposed by branch angle, development and position. Together, our results provide a comprehensive overview of microtubule organisation during reaction wood formation across three woody tree species that show both conserved and species-specific responses. Our success in employing both in vitro and in vivo techniques to test the effects of drug treatments on growing xylogenetic cells provides useful tools for future cell and molecular biology studies in wood formation within appropriate tissue context.

Results

Microtubule orientation and microfibril angle

To study secondary cell wall formation, we examined *in planta* reaction wood in leaning stems and branches.

In planta, MFA values observed in fibres and tracheids (Fig. 1A, B) ranged from $\sim 15^\circ$ to $\sim 65^\circ$ and were significantly lower in tension wood compared to opposite wood (eucalypt $P < 0.001$; poplar $P = 0.003$). By contrast, no significant differences were observed in compression wood when compared to opposite wood (pine $P = 0.695$). Average MFA values in vertical stems (normal wood) of eucalypt were intermediate between tension and opposite wood, while in poplar and pine normal wood MFA were the lowest values from these species. When MFA was compared with MTA across all conditions, a moderate-to-strong correlation between the two was observed within all three species (Fig. 1C–E, Pearson correlation coefficients (PCC) are:

eucalypt = 0.868, poplar = 0.827 and pine = 0.662) indicating that microtubules (MTA) and microfibrils (MFA) co-align in the cell walls of developing wood cells.

In addition to changes in MFA, increases in cell wall thickness and G-layer formation are also observed during reaction wood formation; therefore, we measured these properties using scanning electron microscopy. Induced tension wood cell walls in eucalypts were significantly thicker compared to opposite wood samples, whereas no significant changes were observed between tension and opposite wood in branch samples (Fig. 2A, B). No significant differences were observed in cell wall thickness in poplar within induced or branched tension wood samples,

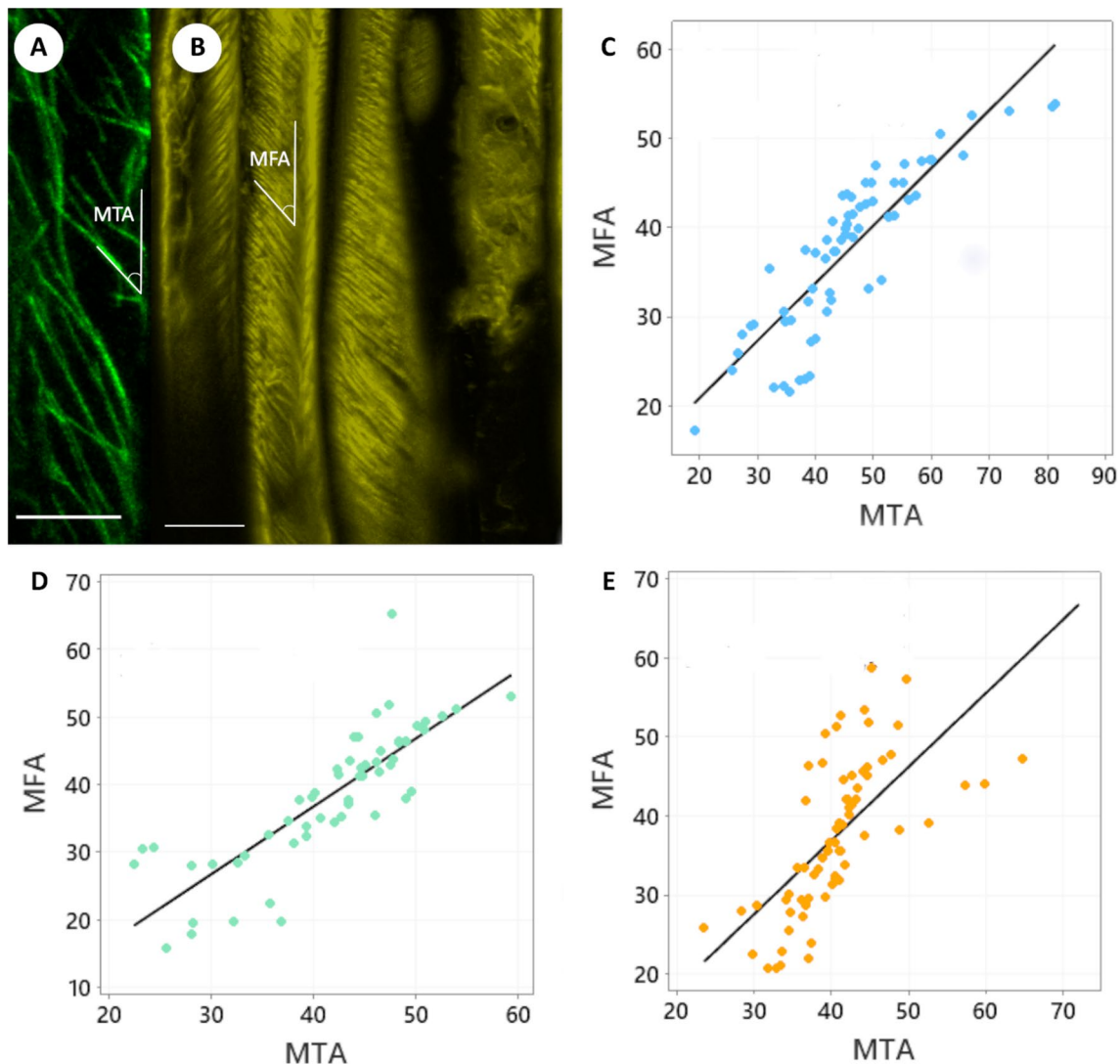


Fig. 1 Microtubule angle (MTA) and microfibril angle (MFA) correlation for normal and reaction wood. Confocal image of immunolabelled poplar fibre microtubules (A) and fluorescent brightener 28 stained microfibrils in pine tracheids (B). Scatterplots of average MFA and MTA values per sample (15 cells measured per treatment in

reaction and normal wood) with regression line for eucalypt (C), poplar (D) and pine (E) and respective Pearson Correlation Coefficients of 0.868, 0.827 and 0.662. MFA microfibril angle, MTA microtubule angle. Scale bars represent 20 µm

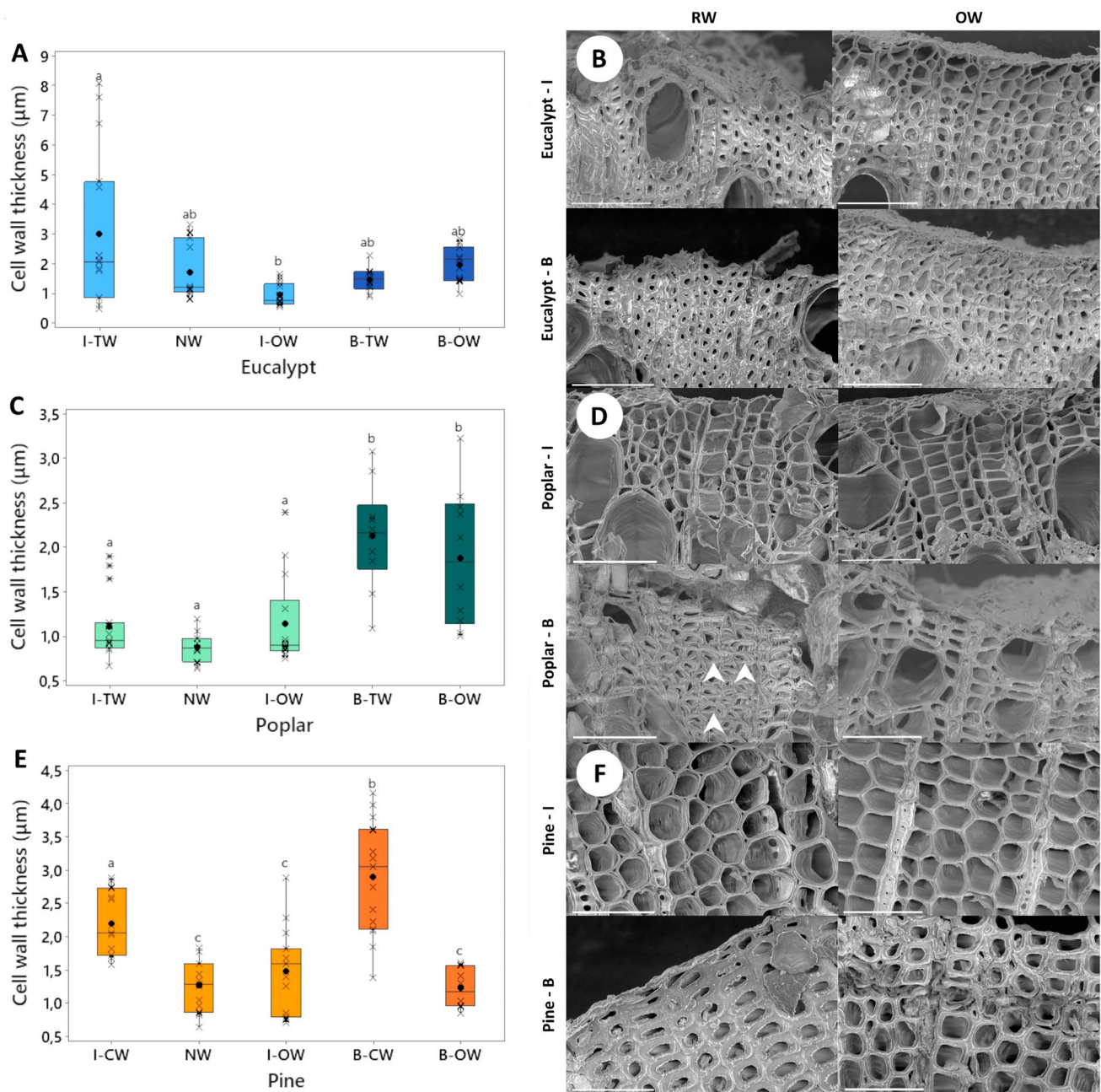


Fig. 2 Cell wall thickness in reaction and opposite wood in all species. Quantification of cell wall thickness of stems and branches from eucalypt (A), poplar (C) and pine (E). Boxplots indicate interquartile range, median (crossing line), mean (dot) and individual data points (crosses). Samples that do not share a letter are significantly different (ANOVA with Tukey post-hoc test). Representative scanning electron

micrographs of transverse sections of eucalypt (B), poplar (D) and pine (F) induced stems and branches exhibiting reaction and opposite wood. Gelatinous layer (G-layer) deposition in poplar (B-TW) is indicated by white arrow heads on selected cells. Scale bars represent 50 μm. B branch, I induced, RW reaction wood, NW normal wood, OW opposite wood

however, G-layer formation was observed in tension wood and not in opposite wood (Fig. 2C, D). G-layers were not observed in any of the samples assessed in other species. In pines, significantly thicker cell walls were observed in both induced and branch compression wood, compared to opposite and normal wood (Fig. 2E, F). Overall, in most

cases cell wall thicknesses were significantly increased in reaction wood, particularly in comparison to opposite wood.

Gravitational and mechanical stimuli and microtubule properties

Because cortical microtubule arrays can impact CSCs trajectories, and therefore MFA and other secondary cell wall properties (Watanabe et al 2015), we examined cortical microtubules in induced and branch reaction wood across all three species using immunofluorescence microscopy and quantified several properties of the microtubule cytoskeleton, including density, branching, bundling and length (Fig. 3A–C). Developing wood cells in eucalypt induced and branch tension wood presented significantly thinner microtubule bundles when compared to opposite wood (Fig. 3D, $P = 0.042$ and $P < 0.001$, respectively). However, no significant differences were observed in microtubule bundling amongst poplar samples (Fig. 3E). The number of microtubule branching events, microtubule density and microtubule length showed no significant differences between wood samples in either angiosperm species (Fig. 3D–G, Fig S1). Interestingly, the microtubule bundling results (Fig. 3D, E) are consistent with the changes observed in cell wall thickness between samples collected from both angiosperm species (Fig. 2), with significant increase in eucalypt cell wall thickness correlated with a significant decrease in microtubule bundling in in tension wood compared to opposite wood (Figs. 2A, 3D), but no significant differences in microtubule bundling or cell wall thickness in poplar (Figs. 2C, 3E).

Pine compression wood and opposite wood also followed this correlation, with a significant decrease in microtubule bundling in induced compression wood, compared to opposite wood (Fig. 4A), consistent with the significant increase in cell wall thickness observed in induced compression wood, compared to opposite wood (Fig. 2E). Intriguingly, microtubules in pine induced compression wood were also significantly more branched and shorter, compared to opposite wood (Fig. 4B, C), but did not show any significant differences in density (Fig. 4D).

No significant differences in microtubule organisation were observed between branch reaction and opposite wood across any of the species (Figs. 3D–G, 4, Fig S1). Together, these results suggest an inverse correlation between microtubule bundling and reaction wood cell wall thickness that is consistent across all three species. A summary of findings can be found in Supplementary Table 1.

Microtubule dynamics and cellulose microfibril alignment

To directly test whether an organised microtubule array is required during wood cell wall formation, an apical stem segment culture system was employed to produce wood under controlled in vitro conditions in the presence and absence of the microtubule destabilising agent, oryzalin,

across all three species. MTA and MFA co-alignment was strongly correlated in cultured developing wood cells in the absence of oryzalin (PCC of eucalypt = 0.934, poplar = 0.995 and pine = 0.959). Wood formed under in vitro conditions is typically free of significant stresses that may be present in vertically orientated stems *in planta*, so it is possible that the transfer of apical stem segments from *in planta* to in vitro conditions could result in changes in MFA values. Compared to normal wood, MTA and MFA were generally higher in cultured tissues across all species, and significantly higher in poplar and pine samples (eucalypt $P = 0.08$, poplar $P < 0.001$ and pine $P = 0.01$) indicating that a move from *in planta* to in vitro leads to increases in MFA.

In in vitro cultured segments treated with oryzalin, the cortical microtubule array was disrupted in almost the entire extent of all observed cells (Fig S2); however, wood cells continued to develop. In the presence of oryzalin, wood cells had a trend toward lower MFA compared to control in all three species; however, no statistically significant differences were observed between oryzalin treatment and control (Fig. 5; eucalypt $P = 0.195$, poplar = 0.289 and pine = 0.134). Given the shift observed to higher MFA when *in planta* stems are move to in vitro conditions across all species, the lower-than-expected MFA observed in the presence of oryzalin could be a result of microtubule destabilisation hindering MFA realignment.

To assess the role of microtubule dynamics reorganisation during reaction wood formation *in planta*, poplar stems were injected with the microtubule stabilising agent paclitaxel, reaction wood was induced by leaning stems so that they grew horizontally, and microtubule and cell wall properties were assessed. Microtubule bundles were typically thicker in developing tension wood fibres treated with paclitaxel when compared to control developing wood fibres (Fig. 6A). Additionally, microtubule density was significantly lower in tension wood fibres examined at 10 cm above the paclitaxel injection point when compared to control tension wood cells at that same point (Fig. 6B, $P = 0.002$). However, an opposite effect was observed at the 1 cm injection point where microtubule density was slightly but not significantly higher in tension wood fibres treated with paclitaxel. Microtubule length and branching did not exhibit significant differences between developing fibres treated with paclitaxel and those that were not (Fig S1).

Fibre MFA and MTA were then assessed in samples harvested from 1 and 10 cm above the injection site after 7 days of reaction wood formation. MTA values were significantly higher in fibres treated with paclitaxel at both sampling points when compared to control fibres (Fig. 7A). Similarly, MFA in wood fibre cells was significantly higher at 1 cm above the injection point; however, this was not the case at the 10 cm above the injection point where values between treatment and controls were similar (Fig. 7B), suggesting

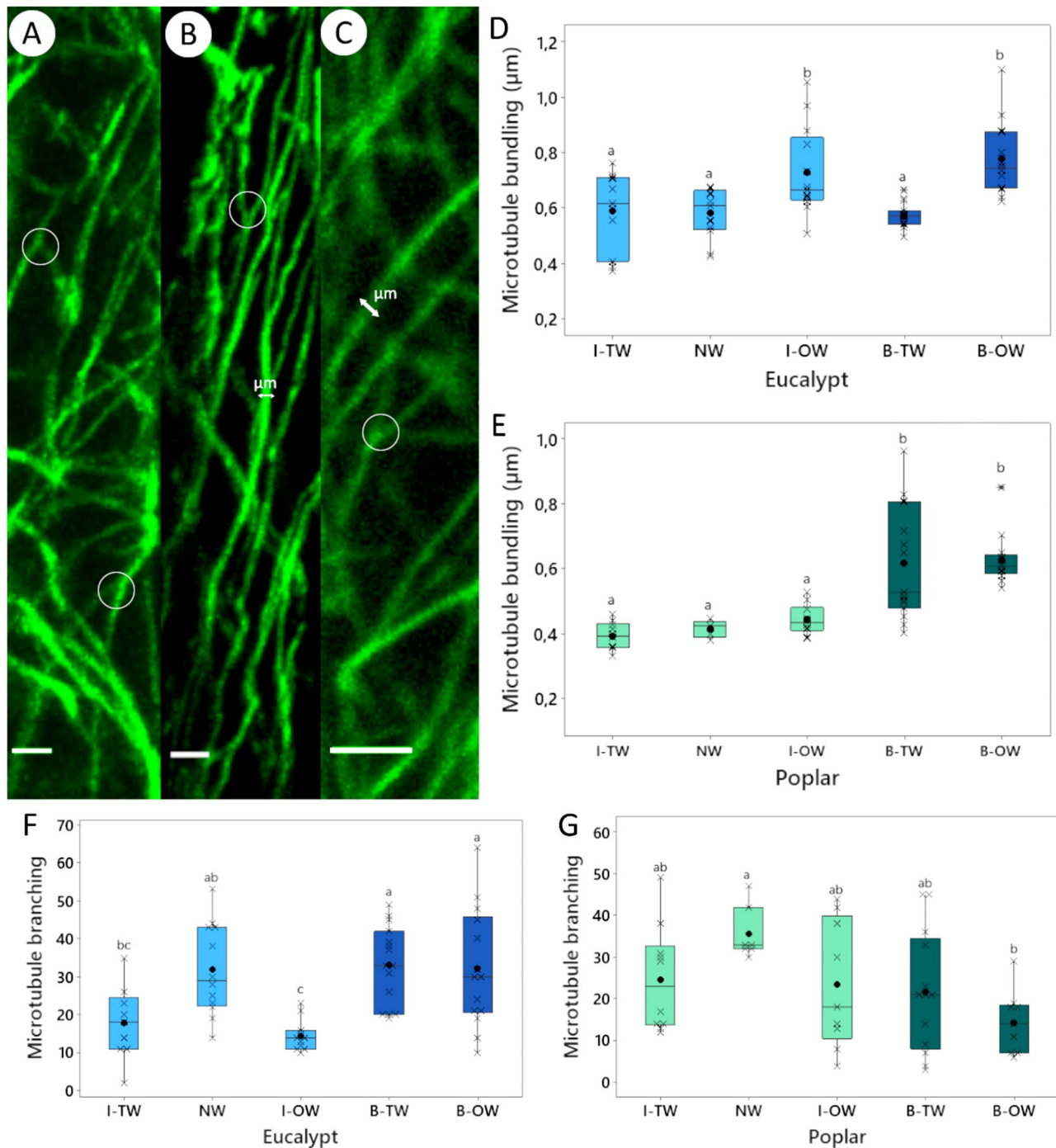


Fig. 3 Microtubule bundling and branching in branch and induced in tension wood of eucalypt and poplar. Representative confocal images of immunolabelled microtubules in developing poplar fibres indicating where measurements were undertaken (A–C) with circles marking what was deemed a branching event, and double arrows indicating where an example microtubule bundle thickness measurement was taken. Quantification of microtubule properties, includ-

ing bundling (D, E) and branching (F, G) in developing fibres from eucalypt (D, F) and poplar (E, G). Boxplots indicate interquartile range, median (crossing line), mean (dot) and individual data points (crosses). Samples that do not share a letter are significantly different (ANOVA with Tukey post-hoc test). *B* branch, *I* induced, *NW* normal wood, *OW* opposite wood, *TW* tension wood. Scale bars represent 5 μm

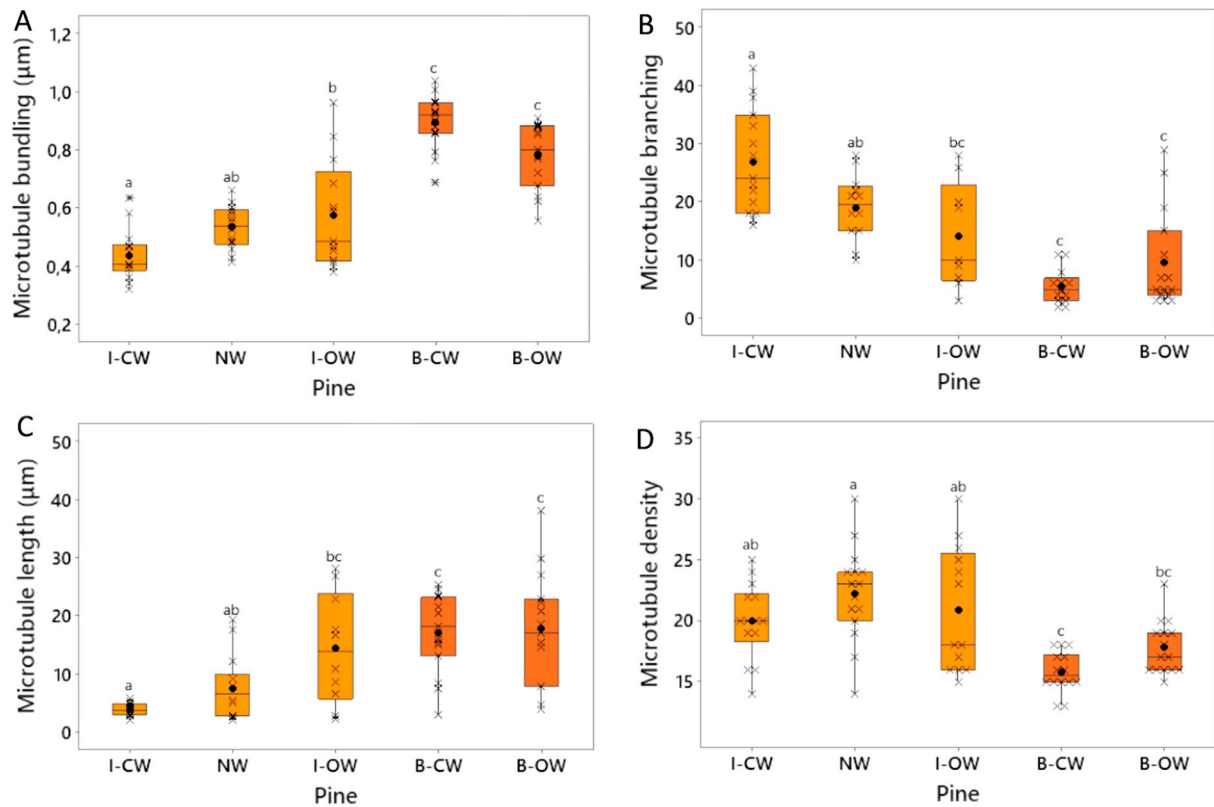


Fig. 4 Microtubule organisation in compression wood tracheids of pine. Quantification of microtubule properties from developing pine tracheids, including bundling (A), branching (B), length (C) and density (D). Boxplots indicate interquartile range, median (crossing line),

mean (dot) and individual data points (crosses). Samples that do not share a letter are significantly different (ANOVA with Tukey post-hoc test). *B* branch, *I* induced, *CW* compression wood, *NW* normal wood, *OW* opposite wood

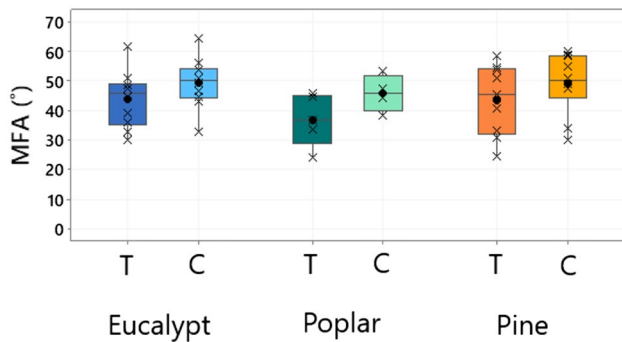


Fig. 5 Microfibril angle of developing fibres and tracheids in oryzalin-treated stem explants. Quantification of MFA in stem explants of eucalypt, poplar and pine. Boxplots indicate interquartile range, median (crossing line), mean (dot) and individual data points (crosses). No statistically significant differences were detected between oryzalin treatment and control for any species (ANOVA with Tukey post-hoc test). *C* control, *MFA* microfibril angle, *T* oryzalin treatment

decreased efficacy of the inhibitors further from the injection site. A high correlation between the alignment of MFA and MTA was observed in developing wood cells in all samples (Fig. 7C, $PCC = 0.741$), further demonstrating strong microtubule and microfibril co-alignment. Overall, microtubule stabilisation in developing wood cells resulted in significantly higher MTA when compared to control wood cells under induced tension wood conditions, whereas this difference was only observed in developing wood cells closer to the injection point in relation to MFA.

Paclitaxel effects on cell wall thickness

To investigate whether changes to MTA, MFA and microtubule organisation were coupled to increases in cell wall area and G-layer formation, developing wood tissue in poplar stems was injected with the microtubule stabilising drug paclitaxel while undergoing an induced reaction wood formation and cell wall properties were measured using scanning electron microscopy. Fibre cell wall thickness was significantly reduced in paclitaxel-injected stems in comparison to control stems at both sampling points (Fig. 8A, *P*

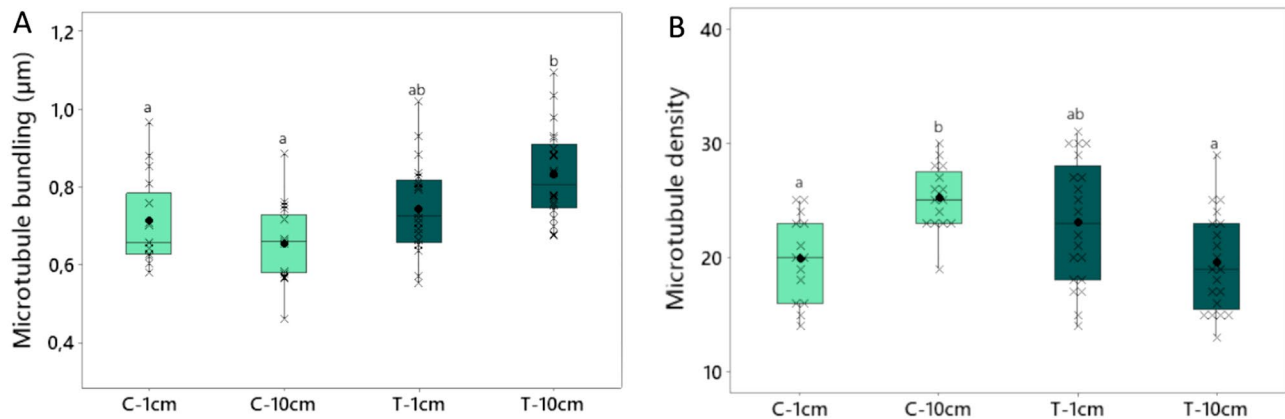


Fig. 6 Microtubule organisation in poplar fibres treated with paclitaxel. Quantification of microtubule properties, including bundling (A) and branching (B) from developing poplar fibres at two distances (1 cm and 10 cm) from the paclitaxel-injection site. Boxplots indi-

cate interquartile range, median (crossing line), mean (dot) and individual data points (crosses). Samples that do not share a letter are significantly different (ANOVA with Tukey post-hoc test). *C* control, *T* paclitaxel treatment

=0.007), which is consistent with earlier results that documented an inverse correlation between microtubule bundling and cell wall thickness. Interestingly, G-layer development was frequently observed in both control and paclitaxel-treated stems to the same extent (Fig. 8B, C) and a slight decrease in the distance of uplifting of a leaning stem was observed in treated stems ($2.6 \text{ cm} \pm 1.3$ degrees uplift control, $0.9 \text{ cm} \pm 0.5$ degrees uplift treatment, $P=0.14$). These results suggest that cortical microtubule arrays influence both MFA and cell wall thickness in poplar in a coordinated way and may impact stem reorientation; however, dynamic cortical microtubules do not appear to be required for efficient G-layer formation.

Discussion

Microtubules play an important, but not vital role, in MFA determination and reorientation during wood formation.

MFA, the angle at which cellulose microfibrils are deposited in the secondary cell wall, is an important characteristic of reaction wood formation. In angiosperms, tension wood features very low MFA while opposite wood reportedly presents larger angles (Almeras and Clair 2016). At the upper side of angiosperm branches, the alignment of cellulose microfibrils at low angles gives support to non-vertical stems by opposing tensional forces, whereas in gymnosperm compression wood, large MFA acts by opposing compression forces (Bamber 2001). While many studies focus on understanding tension wood, active re-modelling of opposite wood was also implied by transcriptomic studies, which found substantial transcriptional changes occurring in opposite

wood compared to normal wood (Chen et al. 2015; Groover 2016). In our experiments, both MFA and MTA were lower in tension wood when compared to opposite wood in both eudicots, which is consistent with expectations. By contrast, normal wood usually displays intermediate MFA values between tension wood and opposite wood (Almeras and Clair 2016). We observed this relationship for normal wood in eucalypt and pine; however, in our study poplar normal wood presented the lowest values for both MTA and MFA. This discrepancy might relate to the species and the effects of glasshouse conditions. Environmental conditions were favourable for fast growth, free of forces such as wind, which can lead to poplar stems growing longer and thinner relative to other species as they competed for light, altering load dynamics within the stem. Consequently, this fast competitive stem growth may have induced tension near the base of vertical stems leading to the observed lower-than-expected MTA and MFA values.

Tubulin genes are highly expressed in reaction wood. In tension wood, for example, expression levels of some tubulin genes can increase as much as 3.4-fold in comparison to opposite wood (Qiu et al. 2008), while in Arabidopsis, genes related to microtubule formation were downregulated under microgravity conditions which corresponded with suppression of reorientation processes (Kato et al. 2022). It seems that transcriptional, rather than translational regulation was more influential in determining the composition of tubulin isotypes in woody tissue, suggesting isotypes may play a role in this aspect of microtubule function (Hu et al. 2015). While these results indicate that there are more tubulin proteins present in reaction wood cells, exactly how the quantity of tubulin might determine microtubule assembly and reorientation is unknown. Here, high correlations between MTA and MFA were observed across all studies,

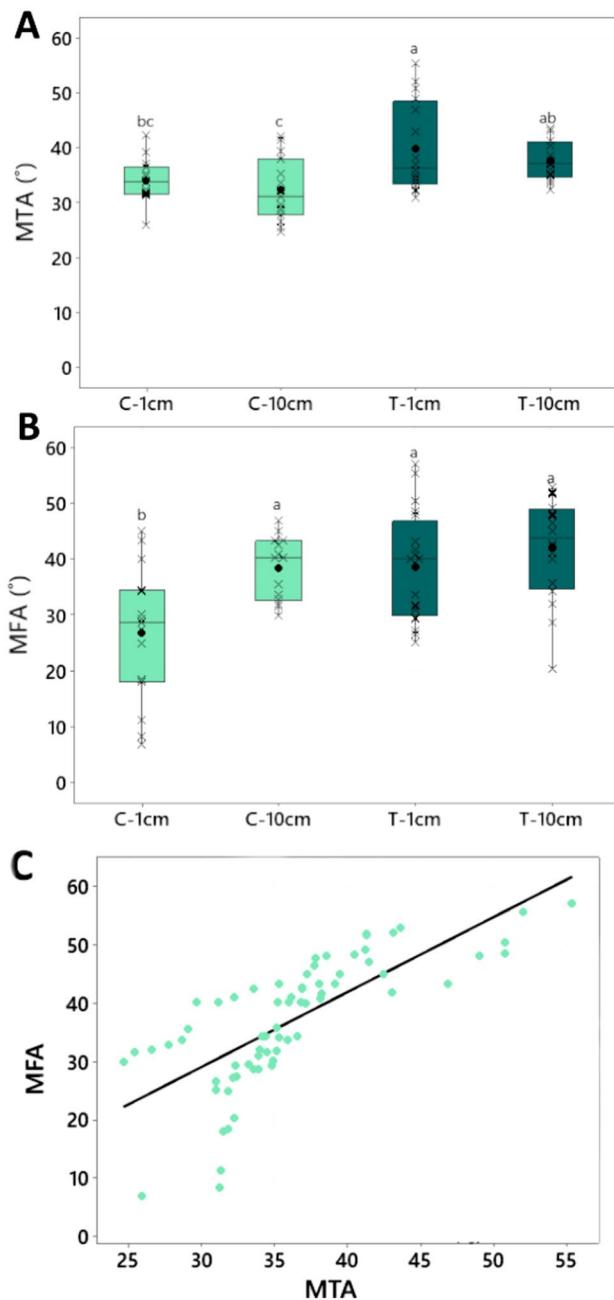


Fig. 7 MTA and MFA of poplar fibres cells treated with paclitaxel. Quantification of MFA (A), MTA (B), and the correlation between these measurements (C) from developing poplar fibres at two distances (1 cm and 10 cm) from the paclitaxel-injection site. Boxplots indicate interquartile range, median (crossing line), mean (dot) and individual data points (crosses). Samples that do not share a letter are significantly different (ANOVA with Tukey post-hoc test). Scatterplots of average MFA and MTA of poplar fibres treated with paclitaxel and with dye only (control) values with regression line and a Pearson's Correlation Coefficient of 0.741. *C* control, *MFA* microfibril angle, *MTA* microtubule angle, *T* paclitaxel treatment

which also correlated with cell wall thickness. Loss of an organised microtubule array via oryzalin treatment perturbed the reorientation of cellulose microfibril deposition in *in vitro* cultured wood cells, while stabilisation of microtubule arrays in planta presumably halted re-alignment of cellulose synthesis in reaction wood, resulting in cell wall changes. Taken together these findings suggest an important for cortical microtubule arrays in the re-alignment of cellulose microfibril deposition.

Using microtubule-disrupting and -stabilising drugs, we documented that microtubule dynamics are important, but not essential, for reorientation of microfibril deposition during wood formation. Here, we showed lower MFA in fibres and tracheids cultured *in vitro* in the presence of oryzalin, although these results were not statistically significantly different from control *in vitro* stem segments. A move to from *in planta* to *in vitro* conditions subjects stems to different concentrations of phytohormones, gravitropic stimuli, environmental cues and inter- and intracellular forces, leading to *in vitro* derived wood and xylem fibres with distinct changes in cell wall properties (Leitch et al. 1995; Decou et al. 2020), which may have masked some of the changes in microtubule organisation and therefore, MFA. We, therefore, focused on injection of the microtubule-stabilising drug paclitaxel into live, whole trees undergoing reaction wood formation. Previous studies have shown that paclitaxel stabilisation of microtubules prevents their reorientation in the primary cell wall of developing cotton fibres (Seagull 1990). Here, higher MTA and MFA were observed in poplar wood fibre cells treated with paclitaxel when compared to untreated cells, consistent with the idea that microtubules stabilisation may have prevented reorientation of cellulose deposition in tension wood.

Microtubules guide the CSC in secondary cell walls (Watanabe et al. 2015) and microtubule reorganisation and CSC realignment are particularly important in response to environmental and developmental cues (Kesten et al. 2019; Schneider et al. 2017). Microtubule arrays undergo constant reorganisation in response to environmental cues. Changes in microtubule orientation and microfibril deposition also occur during tracheary element development in woody stems (Abe and Funada 2005) and bending (Bisgrove 2008). In tension wood, low MFA is likely orchestrated by a shift of the cortical microtubule array from a transverse to a longitudinal alignment with the reverse taking place in opposite wood. We document strong coalignment between MFA and MTA in wood fibres and tracheids across a number of states and species, in line with these expectations. Studies of cells with only primary cell walls suggest that microtubule reorientation can be controlled by mechanical stresses (Landrein and Hamant 2013) and it is therefore likely that mechanical forces in the stem and gravitropic stimuli contribute to changes in MTA and consequently, MFA. Overall, these data

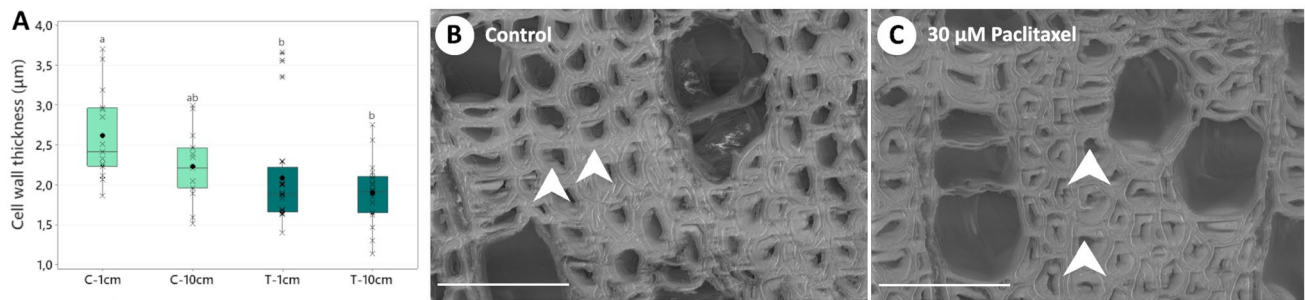


Fig. 8 Cell wall thickness and G-layer formation in poplar fibres treated with paclitaxel. Quantification of cell wall thickness from developing poplar fibres at two distances (1 cm and 10 cm) from the paclitaxel-injection site. Boxplots indicate interquartile range, median (crossing line), mean (dot) and individual data points (crosses). Samples that do not share a letter are significantly different (ANOVA with

Tukey post-hoc test). Representative scanning electron micrographs of transverse sections of poplar induced tension wood from control (**B**) and paclitaxel-treated (**C**) samples. Gelatinous layer (G-layer) deposition is indicated by white arrow heads on selected cells. Scale bars represent 50 μm. *C* control, *T* paclitaxel treatment

supports the premise that microtubule arrays play an important primary role in MFA determination during secondary cell wall formation in woody stems in response to environmental and mechanical cues.

While microtubules play a primary role in microfibril orientation, microfibril orientation does not exclusively result from microtubule guidance, and can be influenced by interactions with adjacent microfibrils and non-cellulosic components (McFarlane et al. 2014), particularly when MT arrays are disrupted. In primary cell walls of *Arabidopsis* root epidermal and cortical cells, drug-dependent microtubule disruption does not alter parallel microfibril deposition alignment locally, but microfibrils showed considerable variation in their orientation within the same cell (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 based on the density of CSCs in the plasma membrane, the distance between newly deposited cellulose microfibrils and the overall cell geometry, which has been further supported by other studies (Himmelsbach et al. 2003; Sugimoto et al. 2001). Other models incorporated the notion of a scaffold of proteins and/or polysaccharides built on the plasma membrane, which is based on microtubule orientation (Baskin 2001). For example, Schneider et al (2017) found that microtubule-driven patterns become 'imprinted' in cell walls during the xylem vessel development to the point where they are sufficient to sustain the continued development of wall thickening, suggesting once the cell wall patterning is established, there does not appear to be a direct need for ordered microtubules. 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, playing a role in reorientation of cellulose microfibril deposition, but an autonomous system can take over in the absence of microtubules to ensure cell wall formation continues, which is consistent with our observations.

Microtubule alignment changes are coupled with changes in cell wall thickness but not G-layer formation

Thicker secondary cell walls with higher cellulose content are normally associated with reaction wood formation (Almeras and Clair 2016) and transcriptional studies have shown upregulation of cellulose synthase genes in these tissues (Li et al. 2013; Chen et al. 2015), demonstrating increased CSC activity or abundance in cells during reaction wood development. Throughout the diversity of samples and treatments we observed, we consistently documented an inverse relationship between microtubule bundling and reaction wood cell wall thickness. Eucalypt and pine samples both showed significantly thicker cell walls and decreased microtubule bundling in induced reaction wood compared to opposite wood, while neither of these properties were significantly different between poplar reaction and opposite wood. These findings are consistent with a recently proposed molecular model of MFA determination in trees forming reaction wood, which underlined the action of microtubule associated proteins (MAPs) in reorganizing the cortical microtubule array by promoting bundling of microtubules (Tobias et al. 2020). Specifically, MAP65 associates with microtubules to facilitate bundling (Meng et al. 2010) by acting as a spacer and structural element that promotes the bundling of microtubules that encounter each other at shallow angles (Tulin et al. 2012), while katanin, a microtubule severing enzyme, is involved in promoting microtubule array alignment by

removing discordant microtubules (Deinum et al. 2017; Yagi et al. 2021). Interestingly, MAP65 inhibits katanin binding in vitro leading to increasing bundling and crosslinking in the cortical microtubule arrays in primary cell walls (Burkart and Dixit 2019). It is therefore possible that bundling may assist in microtubule reorientation through MAP65/katanin interactions where decreased bundling favours katanin action, supporting reorientation and facilitating parallel alignment as well as potentially influencing the extent of organized cellulose deposition leading to changes in cell wall thickness. Consequently, less microtubule bundling would therefore be expected in cells undergoing cellulose deposition re-orientation and reaction wood formation, as observed here.

The G-layer is a chemically and morphologically distinct layer of the secondary cell wall characterised by extremely low MFA, a highly mesoporous matrix, and the ability to generate axial strain during tension wood formation in poplar (Clair et al. 2018). The development of a G-layer in poplar is a common response to gravitational stimuli and it is believed to rapidly generate tension stress (Abedini et al. 2015). Here, thicker cells wall in tension wood of poplar where not consistently observed, however G-layers were. While *Eucalyptus globulus* has been reported to produce typical G-fibres (Washusen et al. 2005), in our study this feature was not observed and is consistent with other reports of low or no G-layer formation across eucalypt species (Qiu et al. 2008). While development of a G-layer is part of the process of secondary cell wall thickening during tension wood formation (Clair et al. 2018), our results suggest that microtubules do not substantially participate in G-layer formation, or at least microtubule (re)organisation is not crucial for G-layer development, since G-layer development was unaffected by paclitaxel-mediated microtubule stabilization. Indeed, G-layer formation is regulated by a different set of transcription factors that affect secondary cell wall composition (Gorshkov et al. 2017). These G-layer transcription factors are more likely to be responsible for the development of distinct chemical properties in this layer and appear to be transcriptionally regulated via ethylene (Seyfferth et al. 2019).

Comparing wood formation across diverse species reveals conserved and distinct roles for microtubules

Dynamic microtubule reorganisation is an important component of reorganizing microfibril deposition during reaction wood formation. Computer simulations 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, some studies have demonstrated that branched nucleation followed by depolymerisation of mother microtubules is the main mode of array reorientation in plants (Nakamura 2015). This process appears to be facilitated by the action of both Augmin, a complex involved in recruiting gamma tubulin to initiate nucleation (Liu et al. 2014) and prevents severing (Wang et al. 2018), and katanin, a microtubule severing enzyme (Deinum et al. 2017; Yagi et al. 2021). These findings suggest a model in which branching, followed by severing, could shift microtubule orientation and then maintain parallel alignment of the whole array. Here, we inferred new microtubule formation (nucleation) by the number of branching events observed in the microtubule arrays. In angiosperms, the amount of microtubule branching was lower in tension and opposite wood fibres compared to normal wood, while in the gymnosperm, branching in tracheids was significantly higher in compression wood when compared to opposite wood, demonstrating inconsistent responses between different species. One possible explanation for this low degree of branching could be that at the time at which the samples were fixed, reorientation in induced stems had been completed and therefore microtubule branching was no longer required to actively realign microtubule arrays and that the most discordant MTs have already been severed. The higher degree of branching observed in vertical stems could be due to the increased need for more frequent reorientation in response to subtle shifts in dynamic load as a result of the spindly growth form previously noted. This would also explain why no significant differences were observed in branch wood across all species, as load in branch samples would be relatively static.

Other microtubule properties showed either unique, contrasting or no changes between induced and branch reaction wood when compared to normal and opposite wood across species. This lack of consistent responses across states and between divergent woody plant species raises questions regarding if and/or how these structures are involved in reorientation processes and whether different mechanisms exist amongst woody plant species. Such differences between gymnosperm and angiosperm subcellular dynamics are not unprecedented; for example, microtubule organisation is strikingly different between gymnosperm and angiosperm pollen tubes, resulting in different dynamics of intracellular transport and cell wall secretion (Chebli et al. 2013).

Immunolabelling techniques employed in the present study are practical tools to observe microtubules; however, due to the nature of the material and the position of xylem cells in a tree's stem, the use of fixed cells was unavoidable. Microtubules are dynamic polymers, thus images of these arrays in fixed cells are, at best, snapshots of a continuous process of reorganisation at the time of sampling. So far, observation of microtubules in live xylary cells has been achieved, for example, using transdifferentiation of

Arabidopsis epidermal cells into tracheary elements (Kubo et al. 2005). While this system has provided valuable insight into secondary cell wall formation, transdifferentiating epidermal cells do not necessarily fully represent xylogenetic processes in woody stems across diverse species, especially responses to mechanical stresses or gravitropic change such as reaction wood formation. However, our success in employing an in vitro technique to test the effects of drug treatments on growing xylogenetic cells provides useful tools for future cell and molecular biology studies in wood formation within appropriate tissue context.

Together, our results provide new insights into microtubule organisation during reaction wood formation across three woody tree species where both conserved and species-specific responses were observed. Cortical microtubules were shown to play a primary role in guiding the alignment of microfibrils in secondary cell walls in all species, however, other mechanisms appear to be able to assume this role in the absence of microtubules. Similarly, changes in microtubule/microfibril alignment were coupled with adjustments in cell wall thickness and in the amount of microtubule bundling. These findings suggest that changes in microtubule alignment and bundling could play an important role in determining both the angle of microfibrils and the extent of cellulose deposition to create secondary cell walls capable of withstanding the mechanical stresses imparted on the fibre or tracheid at the time of its formation. Microtubule branching is thought to be the primary driver of re-alignment (Tobias et al. 2020); however, this could not be confirmed in this study due to the timing of tissue sampling post reorientation. Future studies would therefore benefit by sampling tissue immediately after re-orientation to establish the role of tubulin branching in their re-alignment. Unravelling exactly how differential cortical microtubule array organisation is triggered during reaction wood 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.

Materials and methods

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 (37.42490° S, 143.90261° E). 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 during the growing season.

An induced reaction wood development treatment was used as a model for short-term response to gravitational stimulus under controlled glasshouse conditions using the same three tree species. Seedlings were grown to approximately 0.5-m height exposed to 15 h photoperiod and 25 °C day temperature, then tipped on their side at 90°. They were then allowed to grow for 8 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, three induced stems and three normal (upright) stems also grown in the glasshouse. For microtubule organization, MFA and fibre/tracheid morphological assessments, a small portion (~ 1 cm³) of each side of the branch/stem was harvested (20–25 cm up the stems and ~ 10 mm branch diameter) and identified as tension, compression, opposite or normal wood. Using a razor blade, samples were radially hand-cut from bark to pith into ~ 0.5 mm sections. 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.

Trunk injection

1-year-old *Populus alba* L. trees were grown under glasshouse conditions with four trees growing upright 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. 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. 24 h after injection, all trees were tipped on their side at 90° to promote tension wood formation and were allowed to grow seven days in this position. A small portion (~ 1 cm³) of the upper side of the stems was collected from 1 and 10 cm above the injection point to determine if there were any efficacy differences as a result of longitudinal transport and microtubule organization, MFA and fibre/tracheid morphology were assessed in each sample. Two samples were taken from each stem from the upper and the lower side and five fibres per sample were analysed.

Apical stem segment in vitro culture

In preparation for the establishment of callus cultures, apical stem sections of up to 30 cm in length were harvested from young stems of *Eucalyptus grandis* × *camaldulensis*,

Populus alba and *Pinus radiata* D.Don. that had been cultivated under controlled glasshouse conditions. These stem sections were sterilised using detergent (3% Decon 90, www.decon.co.uk/products/decon90) for 10 min followed by another 30 min. Stems were then immersed in 0.4% sodium hypochlorite for 10 min twice before being immersed in 0.9% hydrogen peroxide for 30 min. 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 1) in sterile Petri dishes with their apical ends inserted into the medium and left to produce wound callus and new xylogenetic tissue under continuous light (standard cool white fluorescent, 36 W) at room temperature ($\sim 24^\circ$) 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 prior to microtubule visualization, MFA and fibre/tracheid morphology assessments were conducted on five fibres/tracheids per sample.

Tubulin visualisation and microtubule organisation analysis

A small portion ($\sim 1 \text{ cm}^3$) of each side of the branch/stem was harvested (20–25 cm up the stems and $\sim 10 \text{ mm}$ branch diameter) and identified as tension, compression, opposite or normal wood and, using a razor blade, radially hand-cut from bark to pith into $\sim 0.5 \text{ mm}$ sections. Samples were then fixed according to Collings and Wasteneys (2005) in PME solution [$50 \text{ mmol}\cdot\text{L}^{-1}$ Pipes (P7643, www.sigma

aldrich.com/australia) pH 7.2, $2 \text{ mmol}\cdot\text{L}^{-1}$ EGTA (E3889), $2 \text{ mmol}\cdot\text{L}^{-1}$ MgSO_4 (AJA1548, www.ajaxfinechem.com) and 0.1% Triton X-100 (X-100, www.sigmaaldrich.com/australia)] containing $2 \text{ mmol}\cdot\text{L}^{-1}$ phenylmethylsulfonyl fluoride (PMSF, P7626), $400 \mu\text{mol}\cdot\text{L}^{-1}$ 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 $0.4 \text{ mol}\cdot\text{L}^{-1}$ mannitol (M4125). After two washing steps in PME, samples were dehydrated in methanol (-20°C , 10 min) and rehydrated in PBS [$131 \text{ mmol}\cdot\text{L}^{-1}$ NaCl (S7653), $5.1 \text{ mmol}\cdot\text{L}^{-1}$ Na_2HPO_4 (S0876), $1.56 \text{ mmol}\cdot\text{L}^{-1}$ KH_2PO_4 (PL009, www.chemsupply.com.au) pH 7.2]. Free aldehyde groups were reduced with sodium borohydride ($4528825, \text{mg}\cdot\text{mL}^{-1}$) in PBS for 30 min and after three rinses in PBS, sections were incubated with blocking buffer [PBS containing 1% BSA and $50 \text{ mmol}\cdot\text{L}^{-1}$ glycine (G8898)] for 30 min.

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 ($\gamma 1$), CFTM488A antibody produced in goat (SAB4600237, www.sigmaaldrich.com/australia) as secondary antibody diluted 1:100. Antibody incubations were kept overnight at 4°C and material was washed in PBS containing $50 \text{ mmol}\cdot\text{L}^{-1}$ glycine ($4 \times 15 \text{ min}$) in between incubations. After removal of secondary antibodies, sections were again washed in PBS glycine ($3 \times 15 \text{ min}$) 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 $40\times$ numerical aperture 1.3 oil-immersion lens with 489 nm laser and fluorescence was collected from 500 to 580 nm. Stacks of optical sections were generally collected at $0.5 \mu\text{m}$ intervals. All images were processed with Fiji-ImageJ (www.fiji.sc) using standard adjustment tools and skeletonization of microtubule arrays. Cortical microtubule structures were assessed across all treatments which included: microtubule angle (MTA), determination of the mean angle of individual cortical microtubules in relation to the long axis of the cell with in an area of $320 \mu\text{m}^2$; microtubule density, the number of cortical microtubules in distinguishable within an area of $320 \mu\text{m}^2$; microtubule bundling, the mean thickness of cortical microtubules within an area of $320 \mu\text{m}^2$; microtubule branching, the number of microtubule branching events present within

Table 1 Composition of tissue culture medium

Lloyd and McCown woody plant basal salt mixture ^a (g/L)	2.3
Murashige and Skoog modified vitamins ($\times 1000$) ^a (mL/L)	1
D-glucose (g/L) ^b	25
NAA (mg/L) ^a	0.5
GA (mg/L) ^b	2.5
Phytigel (g/L) ^b	3

^aAustratec, Australia (www.austratec.com.au)

^bSigma, Australia (www.sigmaaldrich.com/australia)

an area of 320 μm^2 ; and microtubule length, the average length of cortical microtubules. Measurements were taken from several planes with variable z-depth.

MFA analysis

Cell walls were stained with Fluorescent Brightener 28 (Sigma), which is also known as calcofluor white, at 10 $\mu\text{g}/\text{mL}$ and examined under a Nikon C2 confocal microscope (www.nikon.com/products/microscope-solutions/lineup/confocal/c2) with 40 $\times$ numerical aperture 1.3 oil-immersion lens. Samples were excited with a 404.8 nm laser, and fluorescence emission was collected from 410 to 440 nm. MFA was calculated as the angle formed between the cell's longer axis and the cellulose microfibril either measured directly, in cases where it was possible to detect microfibril bundles or using the aperture method described in Cockrell (1974) and demonstrated in Fig. 1A, B. For MFA analysis, five fibres were selected per treatment in branch, induced and pharmaceutical trails and the MFA attributed to each fibre was determined from the median value of three independent MFA measurements of that fibre.

Fibre and tracheid morphology assessment

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 using 5 kV beam and 50 pA current at 2500 $\times$ magnification. For this analysis, five fibres were measured per treatment in branch, induced and pharmaceutical trails at between 5 and 10 cell files from the cambial zone for cell wall thickness, lumen and cell wall area utilising Fiji-ImageJ software (www.fiji.sc). Measured fibres were located at similar distance from the cambium on transverse sections, and, therefore, assumed to be at similar developmental stages.

Data analysis

For all data analysis undertaken, outliers were excluded from the analysis after a Grubbs' test where $\alpha = 0.05$ and means were compared using one-way ANOVA assuming equal variances with $\alpha = 0.05$.

Author contribution statement All authors contributed to the study conception and design. Material preparation, data collection, analysis and interpretation were performed by Larissa Machado Tobias with support from Heather E. McFarlane, Antanas V. Spokevicius and Gerd Bossinger. The first draft of the manuscript was written by Larissa Machado Tobias and all authors commented on previous

versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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