New Phytologist

Gene expression in *Eucalyptus* branch wood with marked variation in cellulose microfibril orientation and lacking G-layers

Deyou Qiu[1], Iain W. Wilson[2], Siming Gan[1], Russell Washusen[3], Gavin F. Moran[1] and Simon G. Southerton[1]

[1]CSIRO Forest Biosciences, PO Box E4008, Kingston ACT 2604, Australia; [2]CSIRO Plant Industry, PO Box 1600, Canberra ACT 2001, Australia; [3]CSIRO Forest Biosciences, Private Bag 10, Clayton South VIC 3168, Australia

Author for correspondence:
Simon G. Southerton
Tel: +61 2 6281 8209
Fax: +61 2 6281 8312
Email: simon.southerton@csiro.au

Received: *30 November 2007*
Accepted: *7 February 2008*

Summary

- In response to gravitational stresses, angiosperm trees form tension wood in the upper sides of branches and leaning stems in which cellulose content is higher, microfibrils are typically aligned closely with the fibre axis and the fibres often have a thick inner gelatinous cell wall layer (G-layer).
- Gene expression was studied in *Eucalyptus nitens* branches oriented at 45° using microarrays containing 4900 xylem cDNAs, and wood fibre characteristics revealed by X-ray diffraction, chemical and histochemical methods.
- Xylem fibres in tension wood (upper branch) had a low microfibril angle, contained few fibres with G-layers and had higher cellulose and decreased Klason lignin compared with lower branch wood. Expression of two closely related fasciclin-like arabinogalactan proteins and a β-tubulin was inversely correlated with microfibril angle in upper and lower xylem from branches.
- Structural and chemical modifications throughout the secondary cell walls of fibres sufficient to resist tension forces in branches can occur in the absence of G-layer enriched fibres and some important genes involved in responses to gravitational stress in eucalypt xylem are identified.

Key words: β-tubulin, cellulose, cellulose microfibril angle, *Eucalyptus*, fasciclin-like AGP, G-layer, lignin, tension wood.

New Phytologist (2008) **179**: 94–103

doi: 10.1111/j.1469-8137.2008.02439.x

Introduction

Many angiosperm trees form wood with distinct properties in response to gravitational stresses incurred in leaning stems or branches or in response to wind or load (Scurfield & Wardrop, 1962; Prodhan *et al.*, 1995; Mellerowicz *et al.*, 2001). Known as tension wood (TW) or reaction wood, it functions in the recovery of the vertical position by leaning stems and branches or maintenance of an established stem orientation. Anatomically, TW tends to have fewer vessels that are also of smaller diameter, and a greater proportion of thicker-walled fibres with a higher cellulose and reduced lignin content (Wilson & White, 1986). Tension wood fibres frequently have an additional inner layer of the secondary wall, called the gelatinous layer or G-layer, which in many cases completely fills the cell cavity. The G-layer is characteristically unlignified and is composed principally of highly crystalline cellulose microfibrils aligned closely with the cell axis. The presence of G-layers is regarded by many to be diagnostic of TW (Wardrop, 1964); however, there are several reports of TW where G-layers were either rare or absent. Fisher & Stevenson (1981) did not detect G-layers in xylem fibres from the upper sides of branches in 56 out of 122 species, while Clair *et al.* (2006) failed to detect G-layers in 13 out of 21 rainforest species. Most gymnosperms also form reaction wood, known as compression wood, in the lower sides of stems. Compression wood is characterized by

short, rounded, thick-walled tracheids with a higher lignin content and higher cellulose microfibril angle (MFA; the overall angle that cellulose MFs make with the longitudinal axis of the fibre cell) in the S2 layer (Timell, 1986).

Genomics research is beginning to reveal the molecular mechanisms underlying TW formation in trees. Global transcript analysis in poplar has revealed increased sucrose synthase activity and thus carbon flow to cellulose biosynthesis; however, expression changes in different secondary wall-specific cellulose synthase genes was variable (Andersson-Gunneras *et al.*, 2006). Increased expression of genes encoding fasciclin-like arabinogalactan proteins (AGPs) has been observed in poplar TW (Lafarguette *et al.*, 2004; Andersson-Gunneras *et al.*, 2006). Genes encoding proteins involved in pectin degradation were also more active in poplar TW while decreased activity was observed in genes involved in the pentose phosphate pathway as well as biosynthesis of lignin and matrix carbohydrates. Xyloglucan endo-transglycosylase (XET) activity was recently detected in developing G-layers in poplar where they are believed to repair xyloglucan cross-linkages (Nishikubo *et al.*, 2007). Paux *et al*. (2005) examined TW in bending experiments in *Eucalyptus grandis* and identified a cellulose synthase gene that is more strongly expressed in TW. Several genes in the lignin biosynthesis pathway were transiently repressed in TW but their activity returned to normal a week after bending. Genes directly involved in cell wall biogenesis which are more highly expressed in the compression wood of gymnosperms include three MYB genes (Bedon *et al.*, 2007), genes encoding several arabinogalactan proteins, α- and β-tubulin and genes involved in lignin biosynthesis (Plomion *et al.*, 2000; Whetten *et al.*, 2001; Koutaniemi *et al.*, 2007).

Recently Washusen *et al*. (2005) observed that cellulose MFA was dramatically altered in wood forming in *Eucalyptus globulus* and *E. grandis* branches oriented at 80° from vertical. Compared with vertical stems, MFA was markedly lower in the upper side of branches and higher in the lower side of branches, suggesting an active role of MFs in both tension wood and opposite (lower branch) wood. Abundant G-layers were detected in upper branches of both species.

Here we report investigations of TW formation in *Eucalyptus nitens* branches oriented at 45° from vertical. We examined morphological, chemical and physical properties of TW and opposite wood and gene expression in these tissues using *Eucalyptus* microarrays containing approx. 4900 xylem cDNAs. This provides important clues to the possible role specific classes of genes and pathways may play in xylem development and responses to gravitational stresses in *Eucalyptus.*

Materials and Methods

Plant materials

Five 9-yr-old unrelated genotypes of *E. nitens* (Deane et Maiden) Maiden and two of *E. grandis* W. Hill ex. Maiden growing in Canberra, Australia, were used as a source of biological material. Two *E. nitens* trees, tree 347 and tree Tall6, were selected for detailed study after preliminary analysis for the presence of G-layers. RNA was isolated from xylem from the vertical main stem and from the upper and lower quarter of adjacent branches oriented at approx. 45° from vertical. A bark window approx. 400 × 60 mm was removed from the stem using a hammer and chisel and young xylem tissue scraped from the exposed wood. Branch samples were harvested approx. 200 mm from the vertical main stem, and branches extended *c.* 2 m beyond the sampling point. A single 9-yr-old *E. grandis* tree growing in the same orchard was used as a source of xylem tissue for RNA isolation and cDNA library construction. Tissues were placed on ice immediately after harvesting and then frozen in liquid nitrogen within 20 min and stored at −80°C.

Wood sampling, X-ray diffraction and chemical measurements

Two 1-cm wood cores were drilled through vertical stems and four cores through branches at the sites of xylem sampling of each *E. nitens* tree. Wood cores were treated as described in Washusen *et al.* (2005) to produce two matching diametral strips. One strip was used for X-ray diffraction analysis (SilviScan-2, CSIRO, Clayton, Australia) and the other was used for anatomical analysis by light microscopy.

Wood samples for lignin and cellulose analysis were collected by cutting 200–250 mm segments from vertical stems and branches; bark to pith wedges were sampled from the upper and lower quarters of branches using a band saw. Wood samples for WinCel analysis of cellular morphology and additional histochemical staining tests were sampled from six positions along a 400 mm length of two separate branches and a 200 mm section of vertical stem of each *E. nitens* tree.

Wood density and MFA measurements were determined using X-ray diffraction patterns obtained using SilviScan-II and the methods of Downes *et al.* (1997). Before chemical measurements, wood was ground in a Wiley mill to pass through a screen with 0.75 mm holes. Cellulose was measured in the Diglyme–HCl method (Macfarlane *et al.*, 1999). Klason lignin was estimated after digesting 1.0 g woodmeal with 72% v : v sulphuric acid, followed by boiling in 3% v : v sulphuric acid. Acid soluble lignin in the hydrolysate was measured by spectrophotometry at 206 nm (APPITA, 1978).

Microtechniques

The matching diametral wood strips were saturated in water and 12 μm transverse sections cut on an American Optical sliding microtome model 860 (AO Scientific, Warner-Lambert Tech. Inc., Buffalo, NY, USA) along the entire transverse face of each strip on the face closest to the sample used for SilviScan analysis. The sections were stained with 1%

aqueous Alcian blue and examined microscopically to identify the location of TW. Alcian blue stains insoluble carbohydrates blue (Gahan, 1984) and is used to stain cellulose in gelatinous fibres. Additional transverse sections were cut from fresh stem segments using a sliding microtome model 860 (AO Scientific) and stained with a 1:2 mixture of 1% safranin solution in 50% ethanol and 1% aqueous Alcian blue (Gurr, 1960). Safranin stains strongly lignified tissue carmine (crimson-red), moderately lignified tissue red and lightly lignified tissue light pink (Aufseß, 1973). After staining, sections were washed in deionized water before microscopic examination on a Leica DMR Light Microscope fitted with a Leica DC500 Camera (Leica Microsystems, Wetzlar, Germany). Photomicrographs were taken of sections in the outer 5 mm of stem and branch segments and images analysed for cell dimensional properties using WinCel V 5.4A software (Regent Instruments Inc., Blain, Canada) following the manufacturer's protocols.

Array construction, labelling and hybridization

A cDNA library of genes expressed in differentiating xylem tissue from a vertical stem of *E. grandis* was constructed using the Stratagene lambda ZAP®II pre-digested vector kit (Stratagene, La Jolla, CA, USA) with poly (A)+mRNA isolated by methods described previously (Southerton *et al.*, 1998). The primary library was amplified before pBluescript phagemids were mass excised using the ExAssit/SOLR system (Stratagene). Randomly picked phagemid colonies were cultured in a 96-well plate and the inserts amplified by polymerase chain reaction (PCR) directly using forward, 5′-GTAAAACGACGGCCAGTG-3′ and reverse, 5′-GGAAA-CAGCTATGACCATG-3′ primers using standard PCR conditions. The PCR products were purified using the TIGR's filtration protocol (Hegde *et al.*, 2000). Approximately 4900 cDNAs were printed onto Superaldehyde glass slides (TeleChem, Sunnydale, CA, USA) using a Bio-Rad (Hercules, CA, USA) VersArray Chipwriter Pro with SMP 3 stealth microspotting quill pins (TeleChem). Each cDNA was spotted twice by different pins on a different area of the same slide. Glass slides were processed according to the TeleChem protocols. Cy3- and Cy5-labeled (Amersham Pharmacia Biotech, Uppsala, Sweden) cDNA probes were generated using the two-step labelling method described by Schenk *et al.* (2000). Application of the probe to microarray slides, hybridization and subsequent washes of the slides were performed according to Schenk *et al.* (2000). Two unrelated *E. nitens* trees in which G-layers were not detected were sampled for microarray analysis. For each tree, slides were hybridized with probes synthesized from vertical xylem and one or other of upper or lower branch xylem. A technical replication was made from the same RNA and the Cy3 and Cy5 labels were swapped to minimize any possible impact of inequalities in DNA incorporation and photo bleaching of the fluorescent dyes. Microarrays were scanned with a GenePix TM4000A laser scanner (Axon Instruments, Inc., Foster City, CA, USA). Spot intensities from scanned slides were quantified using GENEPIX PRO 3.0 software. Grids were predefined and manually adjusted to ensure optimal spot recognition. Spots with dust or locally high background were discarded, as well as spots that were not detected by the software in at least half the slides.

Microarray analysis

All microarray data analysis were performed in R (http://www.r-project.org) using the Bioconductor package Limma (Smyth, 2005). Microarrays were normalization within each array using the printtiploess function (Smyth & Speed, 2003). All data was then normalized between arrays using scale normalization. After normalization, each experiment was analysed using a simple comparison methodology. Genes selected from each experiment as being significantly differently expressed were based on a stringent cut-off of possessing a P-value ≤ 0.05 and a ratio value of ± 1.75. All microarray data is available in a format that meets the minimal information about microarray experiments (MIAME) standards (Brazma *et al.*, 2001) at both the public NCBI Gene Expression Omnibus (GEO accession# = GSE8816, http://www.ncbi.nlm.nih.gov/geo) and at http://www.pi.csiro.au/gena.

Sequence and expression analysis

The cDNA clones were sequenced using the CEQ 2000XL DNA Analysis System (Beckman Coulter, Fullerton, CA, USA). After manual correction and removal of vector sequences, the remaining sequences were reduced to a 47 unigene set by BLAST comparisons between all expressed sequences tag (EST) sequences. The unigenes were compared with the *Arabidopsis* genomic sequence using the TBLASTX search program in November 2007. Genes for which a putative function was identified were assigned to functional groups using the MIPS search facility (http://mips.gsf.de/proj/thal/db/search/search_frame.html).

Full-length cDNA clones of *EgrFLA1*, *2* and *3* were identified on the microarrays and fully sequenced. Deduced amino acid sequences were used to search DNA sequence databases using the TBLASTN search algorithm (Altschul *et al.*, 1990). Sequences with close similarity were aligned using the CLUSTAL W program (Thompson *et al.*, 1994) and the alignments were used as input into MEGA 3.1 (Kumar *et al.*, 2004) to generate an unrooted phylogenetic tree using the neighbour-joining method. All analyses were performed using the default parameters specified by the authors.

Northern analysis was carried out using standard methods (Sambrook *et al.*, 1989). Full-length cDNA clones of each of the three *FLA* genes were used as probes. A 281 bp probe for *EgrTUB1* containing 105 bp of the *C*-terminal and 176 bp of the 3′ untranslated region was amplified using the

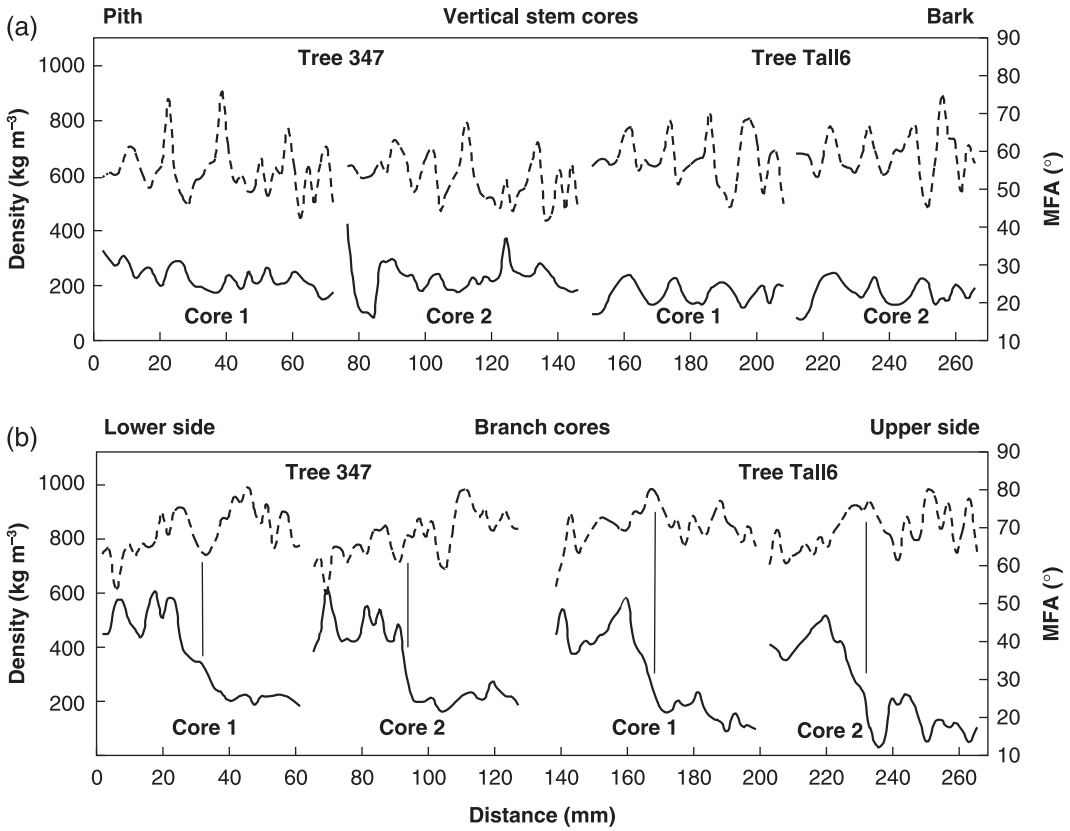

Fig. 1 Cellulose microfibril angle (MFA, solid line) and density (dashed line) in wood from two *Eucalyptus nitens* trees. Data is 2 mm means obtained using SilviScan-2 from (a) pith to bark in vertical stems and (b) through the entire branch from the upper to the lower side. The position of the pith in branches is indicated by a vertical line. Two traces from each tree are shown.

left primer 5′GCGGAGAGCAACATGAACG3′ and right primer 5′ACCACCAGCCCCATATC3′. All probes gave single bands when hybridized to Southern blots of eucalypt genomic DNA indicating hybridization at a single locus (data not shown).

Results

X-ray diffraction

Wood density and MFA in branches and vertical stems of two *E. nitens* trees are shown in Fig. 1. Both traits fluctuated throughout the length of wood cores taken through vertical stems. Density did not appear to change markedly from pith to bark; however, there was a slight decrease in MFA from pith to bark reaching approx. 25° in the outer wood in both trees (Fig. 1a). Similar fluctuations in density and MFA were observed across cores taken from *E. nitens* branches (Fig. 1b). In both trees, wood forming on the upper side of branches appeared to have a slightly higher density than wood forming on lower sides of branches. Striking variation in MFA was observed in branches compared with vertical stems. Upper branch wood had lower (12–25°) and lower branch wood had higher (38–52°) MFA compared with vertical wood (25°). This contrast was apparent immediately from when the branch was formed, as revealed by the divergence in MFA near the pith. There was no clear difference in cellulose crystallite width between upper and lower branch wood (data not shown).

Microscopy

Preliminary analysis of wood sections sampled from 400 mm branch segments revealed abundant (*c*. 50% of fibres) blue-stained G-layers in the two *E. grandis* genotypes and few (*c*. 10% of fibres) in one of the five *E. nitens* genotypes (data not shown). G-layers were absent in the remaining four *E. nitens* genotypes, which included the further analysed trees 347 and Tall6. In branch wood samples from both *E. nitens* trees no evidence of carbohydrate-rich G-layers in fibres was observed at four positions along a second branch at positions where xylem was sampled for expression analysis. To investigate further the presence of G-layers and lignin and to measure cell wall and vessel areas, sections taken from 10 sites along a further two branches oriented at 45° from the vertical in each *E. nitens* tree were examined.

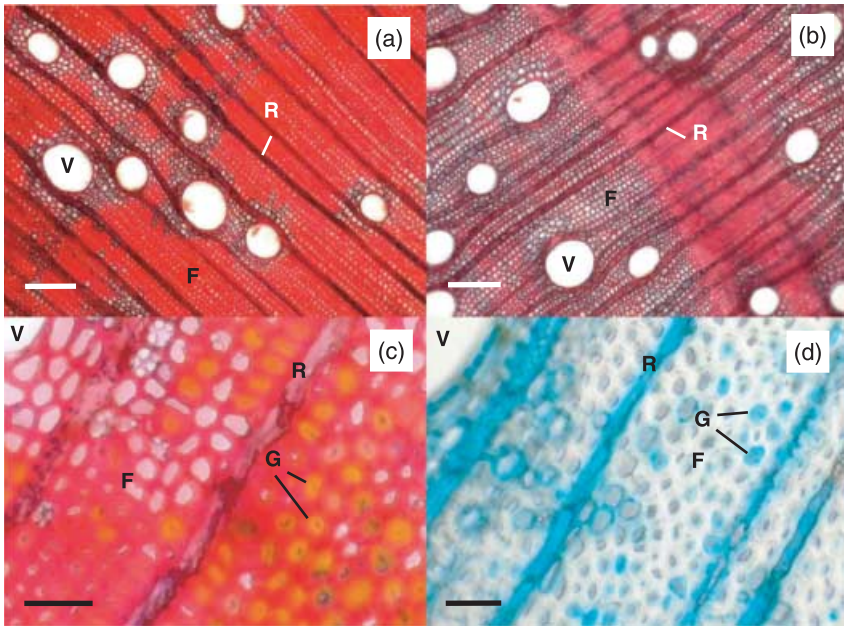

Fig. 2 Microphotographs of cross-sections taken through wood forming in the upper (a,c,d) and lower (b) sides of *Eucalyptus nitens* branches from tree 347 (c,d) and tree Tall6 (a,b) stained with safranin and Alcian blue (a–c) or Alcian blue (d). Safranin stains strongly lignified fibres crimson and moderately lignified fibres red, while Alcian blue stains insoluble carbohydrates blue. Bar: (a,b) 100 μm; (c,d) 20 μm. F, fibres; G, G-layer; R, ray; V, vessel.

Table 1 Cell wall and vessel area in *Eucalyptus nitens* wood formed in the upper and lower sides of branches

	Branch	
Tree	Upper side	Lower side
Tall6		
Cell walls	93.6 ± 1.4†	90.2 ± 1.4†
Vessels	4.4 ± 0.8†	7.1 ± 0.7†
347		
Cell walls	91.3 ± 2.7†	86.7 ± 2.0†
Vessels	5.9 ± 1.4†	9.0 ± 2.1†

Values are expressed as percentage means ± SD. Trait measurements sharing the same symbol (†) are significantly different at $P = 0.01$.

In both trees, fibre cell walls from the upper side of branches tended to stain bright red in large regions of the tissue (Fig. 2a), whereas fibre cells from lower branches typically stained a crimson-red colour (Fig. 2b), suggesting higher lignification in lower branch wood. In all branch wood samples from both *E. nitens* trees, Alcian blue stained ray cell walls and portions of fibre cell walls adjacent to xylem vessels blue indicting lower lignification in these cells. In both trees a very small number of fibre cells from the upper side of the branch that had very thick cell walls were stained orange with safranin (Fig. 2c). In tree 347, the inner cell walls of a small subset of these cells stained blue, indicating the presence of G-layers containing insoluble carbohydrates (Fig. 2c,d).

Cellular traits were examined in cross-sections taken from wood forming in the upper and lower sides of branches of both *E. nitens* trees. The area occupied by vessels was significantly higher (50–60%) in wood from lower sides of branches, while the area occupied by cell walls was significantly higher in wood forming on upper sides of branches (Table 1).

Chemical composition

In both *E. nitens* trees, wood forming in vertical stems and either side of branches had distinct chemical properties (Table 2). Cellulose content was 17–24% higher and Klason lignin content was 8–12% lower in wood forming on the upper sides of branches compared with wood forming on the lower sides of branches. Cellulose content of lower branch wood was also significantly lower than levels observed in vertical wood. In both trees, wood forming in vertical stems had cellulose and Klason lignin contents that were intermediate between upper and lower branch wood. Total lignin was not significantly different between any of the three wood tissues examined. This was because changes in acid-soluble lignin levels were typically opposite to the observed variation in Klason lignin.

Gene expression analysis

The cDNA clones that had a P-value ≤ 0.05 and a ratio value of ± 1.75 in both *E. nitens* trees in comparisons between vertical and branch xylem were considered to be differentially expressed. Using this criterion, expressed sequence tags were obtained for 113 clones. Fig. 3 shows a breakdown into functional groups of the 47 unigenes obtained from the ESTs. About 36% of the genes differentially expressed in branch xylem compared with vertical xylem were involved in biogenesis of the cell wall. A large number coded for proteins that shared

Table 2 Cellulose and lignin content of *Eucalyptus nitens* wood formed in vertical stems and the upper and lower sides of branches

		Branch	
Tree	Vertical stem	Upper side	Lower side
347			
Cellulose§	40.2 ± 0.16*	45.7 ± 2.37†	36.8 ± 0.24*†
Klason lignin	23.1 ± 0.37*	22.3 ± 0.43†	25.2 ± 0.62*†
Acid-soluble lignin	7.6 ± 0.52	8.6 ± 0.91	7.0 ± 0.55
Total lignin	30.7 ± 0.16	31.0 ± 1.18	32.2 ± 0.29
Tall6			
Cellulose	37.7 ± 0.03*‡	41.1 ± 0.83†‡	35.1 ± 0.04*†
Klason lignin	21.7 ± 0.19	20.5 ± 0.20†	22.5 ± 0.48†
Acid-soluble lignin	6.4 ± 0.28*	7.6 ± 0.34*	6.9 ± 0.26
Total lignin	28.0 ± 0.47	28.1 ± 0.37	29.4 ± 0.55

Values are means ± SD. Trait measurements sharing the same symbol are significantly different at the following levels of probability; *, $P = 0.05$; † or ‡, $P = 0.01$. §Cellulose and lignin content are expressed as a percentage of total weight.

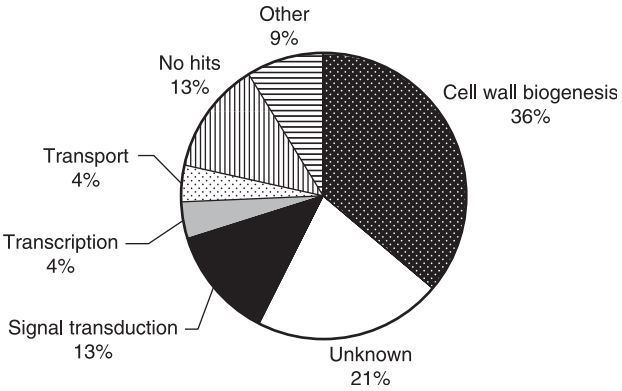

Fig. 3 Genes found to be differentially expressed in eucalypt branches distributed into functional groups.

significant homology with genes identified in other plant species; however, their function remains unknown (21%) and *c.* 13% of the interesting transcripts had no homologues in the *Arabidopsis* genome. When compared with vertical xylem, most genes differentially expressed in upper branch xylem were upregulated (71%) and most genes differentially expressed in lower branch xylem were downregulated (78%). Two genes described below (*EgrTUB1* and *EgrFLA2*) were upregulated in upper branch xylem and downregulated in lower branch xylem.

Differentially expressed genes coding for proteins likely to be involved in cell wall biogenesis in xylem are listed in Table 3. A complete list of the differentially expressed genes is shown in the Supplementary Material (Table S1). The most differentially expressed gene identified in our microarrays was a gene with homology to genes coding for fasciclin-like arabinogalactan proteins, which we named *EgrFLA2.* This gene was upregulated in upper xylem and downregulated in lower xylem and its expression was 27-fold higher in upper versus lower xylem. Other genes with similar patterns of expression included a closely related FLA gene, which we named *EgrFLA1* (2.7-fold), and a β-tubulin, which we named *EgrTUB1* (3.4-fold). These three genes were also among the most redundant clones identified on our microarrays (*EgrFLA1*, 32 copies; *EgrFLA2*, 10 copies; *EgrTUB1*, 8 copies). An extensin was identified that was downregulated in both upper and lower xylem. Also of note was the downregulation in lower branch xylem of several cell-wall loosening enzymes including a xyloglucan endotransglycosylase protein and five genes involved in the degradation of pectin, including a polygalacturonase, a pectinesterase and three pectate lyases.

The expression of *EgrTUB1* and *EgrFLA1* and *EgrFLA2* were examined by northern blot analysis using RNA from an additional branch from tree 347. An increase in expression of *EgrTUB1* (Fig. 4a) and *EgrFLA1* and 2 (Fig. 4b) was detected in upper branch xylem and a decrease in lower xylem compared with vertical xylem, which was consistent with the microarray data (Table 3).

Phylogenetic analysis of FLAs

Fig. 4(c) shows the position of EgrFLA1 and 2 in a phylogenetic tree of fasciclin-like AGPs from *Arabidopsis* and poplar that contain a single fasciclin domain. The eucalypt FLAs cluster with AtFLA12 (EgrFLA1 and EgrFLA2) and AtFLA 11 (EgrFLA3). EgrFLA1 and EgrFLA2 do not appear to fall within the large poplar FLA12 clade which is most closely related to AtFLA 12 and contains genes generally upregulated in TW.

Discussion

We investigated changes in structural and chemical properties of wood fibres in eucalypt branches and used eucalypt xylem microarrays to investigate the underlying molecular mechanisms of xylem responses to gravitational stresses. In contrast to several reports in eucalypts (Scurfield & Wardrop, 1962; Paux *et al.*, 2005; Washusen *et al.*, 2005) and other angiosperms (Wilson & White, 1986; Prodhan *et al.*, 1995; Pilate *et al.*, 2004), we observed very few tension wood fibres in the upper side of branches with G-layers enriched in insoluble carbohydrates. Some thick-walled fibres were observed in which the inner layer of the cell wall stained orange, perhaps indicating altered or reduced lignin, but these fibres were extremely rare. The paucity of fibres containing G-layers strongly suggests that they are not essential for resisting the tension forces experienced in upper branch wood in most *E. nitens* branches, which are typically oriented at *c.* 45° or less. The lack of G-layers in leaning stems and branches of many other Angiosperm species (Fisher & Stevenson, 1981; Clair *et al.*, 2006) raises questions about their importance in resisting tension forces in many natural situations. It should be pointed out that there

New Phytologist

Table 3 Genes differentially regulated in eucalypt branches likely to be involved in cell wall biosynthesis

Gene name	Genbank ID	*Arabidopsis* homolog ID	Branch xylem expression ratio	*P*-value
			Upper/vertical	
Fasciclin-like arabinogalactan protein (EgrFLA2)	EF534217	At5g60490	3.56	0.00
Cellulose synthase (EgCesA2)	EW688454	At5g17420	2.63	0.00
Shikimate hydroxycinnamoyltransferase	EW688399	At5g48930	1.91	0.01
Fasciclin-like arabinogalactan protein (EgrFLA1)	EF534216	At5g60490	1.91	0.01
β-tubulin (EgrTUB1)	EF534219	At5g62700	1.80	0.00
			Upper/vertical	
Leucine-rich repeat family protein	EW688250	At3g20820	0.56	0.00
Extensin	EW688456	At5g14920	0.54	0.00
			Lower/vertical	
Pectinesterase	EY195995	At5g66920	2.34	0.03
Laccase	EY195997	At5g21105	2.22	0.02
			Lower/vertical	
Pectinesterase	EW688528	At1g21850	0.57	0.04
Myo-inositol oxygenase	EY195985	At1g14520	0.56	0.02
Polygalacturonase	EW688503	At3g61490	0.56	0.01
Peptidoglycan-binding LysM domain-containing protein	EW688545	At1g21880	0.56	0.03
Pectate lyase	EW688303	At1g67750	0.54	0.01
β-tubulin (EgrTUB1)	EW688268	At5g62700	0.54	0.02
Myosin heavy chain like, putative	EW688490	At2g40480	0.52	0.00
Pectate lyase	EY195993	At1g04680	0.50	0.00
Xyloglucan endotransglycosylase protein	EW688386	At5g13870	0.49	0.01
Leucine-rich repeat transmembrane protein	EW688358	At2g36570	0.49	0.00
Leucine-rich repeat transmembrane protein	EW688497	At2g36570	0.48	0.00
Pectate lyase	EW688219	At1g04680	0.44	0.00
Extensin	EW688456	At5g14920	0.41	0.04
Fasciclin-like arabinogalactan protein (EgrFLA2)	EW688462	At5g60490	0.13	0.00

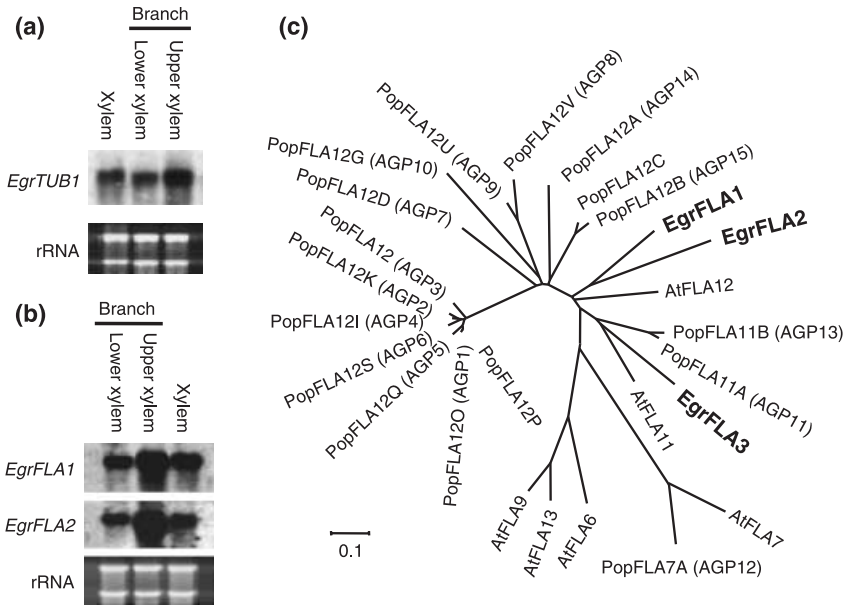

Fig. 4 Northern analysis of *EgrTUB1* and *EgrFLA1, 2* and phylogenetic analysis of group A fasciclin-like arabinogalactan proteins (AGPs). (a) Expression of *EgrTUB1* in vertical and branch xylem. (b) Expression of *EgrFLA1* and *EgrFLA2* in vertical and branch xylem. (c) Phylogenetic tree of group A FLAs from *Arabidopsis*, *Populus* spp. and *Eucalyptus grandis*. CLUSTAL W was used to align protein sequences and the unrooted dendrogram was obtained using MEGA 3.1. *Arabidopsis* Genome Initiative (AGI) codes, GenBank accession numbers and PU IDs for the genes used were as follows: *Arabidopsis thaliana*, AtFLA6 (At2g20520), AtFLA7 (At5g65390), AtFLA9 (At1g03870), AtFLA11 (At5g03170), AtFLA12 (At5g60490), AtFLA13 (At5g44130); *Populus* gene names are consistent with the names assigned in Andersson-Gunneras *et al*. (2006); *Eucalyptus grandis*, EgrFLA1 (EF534216), EgrFLA2 (EF534217), EgrFLA3 (EF534218).

are differences in the experimental approaches used in recent molecular studies, which may affect the stresses experienced in the TW formed. In some studies previously vertical stems were tied to adjacent trees at 45° (Paux *et al*., 2005) while in others the pots were inclined at 45° (Lafarguette *et al*., 2004; Andersson-Gunneras *et al*., 2006). This may result in significantly higher stresses than those in our branches, which may result in increased G-layer synthesis.

The lack of G-layer fibres leads us to conclude that the major changes to wood properties observed across branches, including changes in microfibril orientation, cellulose content and Klason lignin levels, are occurring during most or all stages of secondary cell wall deposition in the majority of fibre cells. The most striking phenotype observed in *E. nitens* branches was the markedly divergent orientation of cellulose microfibrils in branch wood forming above (*c*. 19°) or below (*c*. 45°) the pith. A similar divergence in MFA has been observed through branches in *E. globulus* (13°/40°) and *E. grandis* (10°/50°) oriented at *c*. 80° to the vertical of the main stem (Washusen *et al*., 2005). The much closer alignment of cellulose microfibrils with the fibre axis in upper branch wood would be expected to dramatically increase the stiffness of upper branch wood. A lowering of cellulose MFA is a major factor contributing to increased wood stiffness in eucalypts (Evans & Ilic, 2001) and some softwoods (Cave & Walker, 1994). It is also interesting that compared with vertical wood, MFA increased dramatically in lower branch wood. This suggests that lower branch wood also plays an active role in maintaining the mechanical stability of the branch.

Conspicuous among the differentially expressed genes was a β-tubulin (*EgrTUB1*) which was upregulated in low MFA wood (upper branch) and downregulated in high MFA wood (lower branch). The α- and β-tubulin proteins form heterodimers, which are the major structural component of the protofilaments within microtubules (MTs). There is growing circumstantial evidence that plant MTs influence cellulose microfibril orientation (Lloyd *et al*., 2000) and hence MFA in trees (Chaffey, 2000). Cortical MTs are typically parallel to cellulose microfibrils in many plant cells and recent evidence has been presented that cortical microtubules guide CesA complexes in primary cell walls (Paredez *et al*., 2006). There are six expressed α-tubulins and nine expressed β-tubulins in the *Arabidopsis* genome and expression of tubulin isoforms is both tissue and developmental stage specific (Cheng *et al*., 2001) and their role appears to be related to structural variations observed between α and β isoforms (Richards *et al*., 2000). It is possible that the differential expression of the *EgrTUB*1 gene may influence the arrangement of cortical MTs in developing secondary walls in wood fibre cells and that this in turn influences the direction of movement of the cellulose synthesizing machinery at the plasma membrane. This view is supported by the observation that MFA was significantly altered in eucalypt tissues as a consequence of stable transformation with *EgrTUB1* (Spokevicius *et al*., 2007).

Other differentially expressed genes in branches included two coding for closely related fasciclin-like arabinogalactan proteins, *EgrFLA1* and *EgrFLA2*. These eucalypt FLAs are most homologous to *AtFLA12*, a FLA belonging to subgroup A (Johnson *et al*., 2003) that possesses a single fasciclin domain flanked by two proline-rich domains. The FLAs belonging to this group have also been found to be strongly expressed in tissue undergoing secondary cell wall synthesis in angiosperms (Sterky *et al*., 1998; Lafarguette *et al*., 2004; Pilate *et al*., 2004; Andersson-Gunneras *et al*., 2006) and gymnosperms (Loopstra & Sederoff, 1995; Loopstra *et al*., 2000). In poplar there are 25 group A FLAs, owing to the occurrence of a large number (22) most closely related to *AtFLA12* (Andersson-Gunneras *et al*., 2006). Most members of the latter group are induced in TW in poplar (Lafarguette *et al*., 2004) just as *EgrFLA1* and *EgrFLA2*, the eucalypt FLAs most closely resembling *AtFLA12*, are induced in TW in eucalypts. A third FLA identified on our arrays, *EgrFLA3*, has two putative poplar orthologues (*PopFLA11A* and *B*) and *PopFLA11B* was observed to be more strongly expressed in opposite wood, the equivalent of lower branch wood in this work (Lafarguette *et al*., 2004). These observations suggest a level of functional conservation among FLAs between poplar and eucalypts. Like *EgrTUB1*, the expression of *EgrFLA1* and *EgrFLA2*, was also strongly correlated with changes in cellulose microfibril orientation, most dramatically in the case of *EgrFLA2* where a 27-fold expression differential was observed between upper and lower branch xylem. This suggests that these AGPs may also contribute to the orientation of cellulose microfibrils within the secondary wall. Arabinogalactan proteins have been observed to be associated with pectic (1→4)-β-galacturonan (Nothnagel, 1997) and they could interact with hemicellulosic (1→4)-β glucans and cellulose (Seifert & Roberts, 2007). It was recently demonstrated that *AtFLA11* and *AtFLA12* are strongly expressed in *Arabidopsis* inflorescence stems (Ito *et al*., 2005) and that both genes are coregulated with secondary wall-specific *CesA* genes (Persson *et al*., 2005). Increased expression of a secondary cell wall *CesA* gene in the upper sides of branches of eucalypt branches was observed; however, this same gene was not downregulated on the lower side of branches.

The most substantial contrast in cell wall chemistry between upper and lower branch wood was the increased cellulose content of upper wood and increased Klason lignin content of lower wood. As with the changes in MFA, the changes in cellulose and Klason lignin are likely to be caused by changes in most fibres and not the occurrence of a few G-layer-containing fibres greatly enriched in cellulose and devoid of lignin. Interestingly, lignin has recently been detected in G-layers of polar tension wood (Gierlinger & Schwanninger, 2006). Few genes involved in cellulose and lignin biosynthesis were identified in our experiments. Three secondary cell wall *CesAs* have been identified in *E. grandis* (Ranik & Myburg, 2006) and at least two (*EgCesA2* and *EgCesA3*) were represented on our microarrays; however, only *EgCesA2* was upregulated in xylem from the upper sides of branches. Paux *et al*. (2005) detected increased expression of *EgCesA1*, the third secondary cell wall *CesA*, in eucalypt TW. It is possible that increased expression of specific cellulose synthase isoforms influences the behaviour of cellulose synthase complexes in eucalypt xylem cells and that this affects the orientation of

cellulose deposition. In *Arabidopsis* it has been demonstrated that all three secondary cell wall CesA proteins are required for correct arrangement of cellulose synthase complexes in the plasma membrane of developing xylem cells (Gardiner *et al.*, 2003). These authors also observed that microtubules were necessary for correct targeting of secondary CesA proteins to regions of cell wall thickening.

The expression of genes encoding cell wall loosening/hydrolytic enzymes was mostly downregulated in branches. In lower branch xylem a xyloglucan endotransglycosylase (XET) and five genes involved in the breakdown of pectin (including a polygalacturonase, a pectinesterase and three pectate lyases) were less strongly expressed. The only gene implicated in wall loosening, which was upregulated, was a pectinesterase in the lower side of branches. These observations suggest that less remodelling of primary and/or secondary walls is occurring during early stages of xylem development in the lower side of branches. Genes involved in pectin breakdown were observed to be less strongly modulated in TW than genes involved in secondary cell-wall formation in *E. grandis* (Paux *et al.*, 2005). In poplar, a strong increase in transcript abundance of genes involved in pectin degradation was observed in TW compared with vertical wood (Andersson-Gunneras *et al.*, 2006). Increased pectate lyase transcript abundance in tissues where secondary wall deposition is occurring suggests that in poplar they are involved in wall remodelling at the early stages of secondary wall biosynthesis (Geisler-Lee *et al.*, 2006).

Eucalypt xylem responds to tension by increased synthesis of cellulose microfibrils more closely aligned with the direction of the tension force and reduced synthesis of Klason lignin. We have identified genes likely to play an important role in responses to tension and gravitational stresses in eucalypt xylem. Among these, we have observed a strong negative correlation between expression of two closely related fasciclin-like arabinogalactan proteins and a β-tubulin and lowered microfibril angle. The products of these genes appear to play a key role in increasing the stiffness of TW in eucalypts where a strong correlation between microfibril angle and this trait has been observed.

Acknowledgements

We thank Washington Gapare and Emlyn Williams for help with statistical analysis and Andrew Spriggs and Gavin Kennedy, CSIRO Plant Industry, for bioinformatics support. We thank Linda Ballard from the Cooperative Research Centre for Sustainable Production Forestry in Hobart who carried out the lignin and cellulose analyses and Tim Longley and Rosemary White, CSIRO Plant Industry, for assistance with wood sample preparation and microscopy respectively. D.Q. and S.G. were supported by postdoctoral fellowships jointly funded by CSIRO and the Australian Government Department of Agriculture, Fisheries and Forestry (DAFF).

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic Local Alignment Search Tool. *Journal of Molecular Biology* **215**: 403–410.

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* **45**: 144–165.

APPITA. 1978. Australian standard: klason lignin in wood and pulp. *Appita Journal* **31**: 11–78.

Aufseß HV. 1973. Mikroskopische darstellung des verholzungsgrades durch färbemethoden. *Holz als Roh-und Werkstoff* **31**: 24–33.

Bedon F, Grima-Pettenati J, Mackay J. 2007. Conifer R2R3-MYB transcription factors: sequence analyses and gene expression in wood-forming tissues of white spruce (*Picea glauca*). *Bmc Plant Biology* 7: 17.

Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P. 2001. Minimum information about a microarray experiment (MIAME) – towards standards for microarray data. *Nature Genetics* **29**: 365–371.

Cave ID, Walker JCF. 1994. Stiffness of wood in fast-grown plantation softwoods: the influence of microfibril angle. *Forest Products Journal* **44**: 43–48.

Chaffey N. 2000. Microfibril orientation in wood cells: new angles on an old topic. *Trends in Plant Science* **5**: 360–362.

Cheng ZG, Snustad DP, Carter JV. 2001. Temporal and spatial expression patterns of TUB9, a beta-tubulin gene of *Arabidopsis thaliana*. *Plant Molecular Biology* **47**: 389–398.

Clair B, Ruelle J, Beauchene J, Prevost MF, Fournier M. 2006. Tension wood and opposite wood in 21 tropical rain forest species 1. Occurrence and efficiency of the G-layer. *Iawa Journal* **27**: 329–338.

Downes GM, Hudson IL, Raymond CA, Dean GH, Michell AJ, Schimleck LS, Evans R, Muneri A. 1997. *Sampling plantation eucalypts for wood and fibre properties*. Melbourne, Australia: CSIRO Publishing.

Evans R, Ilic J. 2001. Rapid prediction of wood stiffness from microfibril angle and density. *Forest Products Journal* **51**: 53–57.

Fisher JB, Stevenson JW. 1981. Occurrence of reaction wood in branches of dicotyledons and its role in tree architecture. *Botanical Gazette* **142**: 82–95.

Gahan PB. 1984. *Plant histochemistry and cytochemistry*. New York, NY, USA: Academic Press.

Gardiner JC, Taylor NG, Turner SR. 2003. Control of cellulose synthase complex localization in developing xylem. *Plant Cell* **15**: 1740–1748.

Geisler-Lee J, Geisler M, Coutinho PM, Segerman B, Nishikubo N, Takahashi J, Aspeborg H, Djerbi S, Master E, Andersson-Gunneras S *et al.* 2006. Poplar carbohydrate-active enzymes. Gene identification and expression analyses. *Plant Physiology* **140**: 946–962.

Gierlinger N, Schwanninger M. 2006. Chemical imaging of poplar wood cell walls by confocal Raman microscopy. *Plant Physiology* **140**: 1246–1254.

Gurr E. 1960. *Encyclopaedia of microscopic stains*. London, UK: Leonard Hill Books.

Hegde P, Qi R, Abernathy K, Gay C, Dharap S, Gaspard R, Hughes JE, Snesrud E, Lee N, Quackenbush J. 2000. A concise guide to cDNA microarray analysis. *Biotechniques* **29**: 548–562.

Ito S, Suzuki Y, Miyamoto K, Ueda J, Yamaguchi I. 2005. AtFLA11, a fasciclin-like arabinogalactan-protein, specifically localized in screlenchyma cells. *Bioscience Biotechnology and Biochemistry* **69**: 1963–1969.

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* **133**: 1911–1925.

Koutaniemi S, Warinowski T, Karkonen A, Alatalo E, Fossdal CG, Saranpaa P, Laakso T, Fagerstedt KV, Simola LK, Paulin L *et al.* 2007.

Expression profiling of the lignin biosynthetic pathway in Norway spruce using EST sequencing and real-time RT-PCR. *Plant Molecular Biology* **65**: 311–328.

Kumar S, Tamura K, Nei M. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* **5**: 150–163.

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* **164**: 107–121.

Lloyd CW, Himmelspach R, Nick P, Wymer C. 2000. Cortical microtubules form a dynamic mechanism that helps regulate the direction of plant growth. *Gravitational and Space Biology Bulletin* **13**: 59–65.

Loopstra CA, Sederoff RR. 1995. Xylem-specific gene expression in loblolly pine. *Plant Molecular Biology* **27**: 277–291.

Loopstra CA, Puryear JD, No EG. 2000. Purification and cloning of an arabinogalactan-protein from xylem of loblolly pine. *Planta* **210**: 686–689.

Macfarlane C, Warren CM, White DA, Adams MA. 1999. A rapid and simple method for processing wood to crude cellulose for analysis of stable carbon isotopes in tree-rings. *Tree Physiology* **19**: 831–835.

Mellerowicz EJ, Baucher M, Sundberg B, Boerjan W. 2001. Unravelling cell wall formation in the woody dicot stem. *Plant Molecular Biology* **47**: 239–274.

Nishikubo N, Awano T, Banasiak A, Bourquin V, Ibatullin F, Funada R, Brumer H, Teeri TT, Hayashi T, Sundberg B *et al.* 2007. Xyloglucan endo-transglycosylase (XET) functions in gelatinous layers of tension wood fibers in poplar – a glimpse into the mechanism of the balancing act of trees. *Plant & Cell Physiology* **48**: 843–855.

Nothnagel EA. 1997. Proteoglycans and related components in plant cells. *International Review of Cytology* **174**: 195–119.

Paredez AR, Somerville CR, Ehrhardt DW. 2006. Visualization of cellulose synthase demonstrates functional association with microtubules. *Science* **312**: 1491–1495.

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* **167**: 89–100.

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, USA* **102**: 8633–8638.

Pilate G, Dejardin A, Laurans F, Leple JC. 2004. Tension wood as a model for functional genomics of wood formation. *New Phytologist* **164**: 63–72.

Plomion C, Pionneau C, Brach J, Costa P, Bailleres H. 2000. Compression wood-responsive proteins in developing xylem of maritime pine (*Pinus pinaster* Ait.). *Plant Physiology* **123**: 959–969.

Prodhan A, Ohtani J, Funada R, Abe H, Fukazawa K. 1995. Ultrastructural investigation of tension wood fiber in *Fraxinus mandshurica* Rupr Var *Japonica* Maxim. *Annals of Botany* **75**: 311–317.

Ranik M, Myburg AA. 2006. Six new cellulose synthase genes from *Eucalyptus* are associated with primary and secondary cell wall biosynthesis. *Tree Physiology* **26**: 545–556.

Richards KL, Anders KR, Nogales E, Schwartz K, Downing KH, Botstein D. 2000. Structure–function relationships in yeast tubulins. *Molecular Biology of the Cell* **11**: 1887–1903.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory.

Schenk PM, Kazan K, Wilson I, Anderson JP, Richmond T, Somerville SC, Manners JM. 2000. Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. *Proceedings of the National Academy of Sciences, USA* **97**: 11655–11660.

Scurfield G, Wardrop AB. 1962. The nature of reaction wood; the reaction anatomy of seedlings of woody perennials. *Australian Journal of Botany* **10**: 93–105.

Seifert GJ, Roberts K. 2007. The biology of arabinogalactan proteins. *Annual Review of Plant Biology* **58**: 137–161.

Smyth GK. 2005. Limma: linear models for microarray data. In: Gentleman R, Carey V, Dudoit S, Irizarry R, Huber W, eds. *Bioinformatics and computational biology solutions using R and Bioconductor*. New York, NY, USA: Springer, 397–420.

Smyth GK, Speed T. 2003. Normalization of cDNA microarray data. *Methods* **31**: 265–273.

Southerton SG, Marshall H, Mouradov A, Teasdale RD. 1998. Eucalypt MADS-box genes expressed in developing flowers. *Plant Physiology* **118**: 365–372.

Spokevicius AV, Southerton SG, MacMillan CP, Qiu D, Gan S, Tibbits JFG, Moran GF, Bossinger B. 2007. Beta-tubulin affects cellulose microfibril orientation in plant secondary fibre cell walls. *Plant Journal* **51**: 717–726.

Sterky F, Regan S, Karlsson J, Hertzberg M, Rohde A, Holmberg A, Amini B, Bhalerao R, Larsson M, Villarroel R *et al.* 1998. Gene discovery in the wood-forming tissues of poplar: analysis of 5692 expressed sequence tags. *Proceedings of the National Academy of Sciences, USA* **95**: 13330–13335.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W – improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673–4680.

Timell TE. 1986. *Compression wood in gymnosperms*. Berlin, Germany: Springer-Verlag.

Wardrop AB. 1964. The formation of wood in forest trees. In: Zimmermann MH, ed. New York, NY, USA: Academic Press, 405–456.

Washusen R, Evans R, Southerton S. 2005. A study of *Eucalyptus grandis* and *Eucalyptus globulus* branch wood microstructure. *Iawa Journal* **26**: 203–210.

Whetten R, Sun YH, Zhang Y, Sederoff R. 2001. Functional genomics and cell wall biosynthesis in loblolly pine. *Plant Molecular Biology* **47**: 275–291.

Wilson K, White DJB. 1986. Reaction wood: its structure, properties and functions. In: Wilson K, White DJB, eds. *The anatomy of wood: its diversity and variability*. London, UK: Stobart and Son Ltd, 222–250.

Supplementary Material

The following supplementary material is available for this article online:

Table S1 Genes differentially expressed in eucalypt branches

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1469-8137.2008.02439.x

(This link will take you to the article abstract).
Please note: Blackwell Publishing are not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the journal at *New Phytologist* Central Office.