

Wood formation from the base to the crown in *Pinus radiata*: gradients of tracheid wall thickness, wood density, radial growth rate and gene expression

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Abstract

Wood formation was investigated at five heights along the bole for two unrelated trees of *Pinus radiata*. Both trees showed clear gradients in wood properties from the base to the crown. Cambial cells at the base of the tree were dividing 3.3-fold slower than those at the crown, while the average thickness of cell walls in wood was highest at the base. Cell wall thickness showed an overall correlation coefficient of >0.7 with wood density in both genotypes. Microscopic examination of developing tracheids showed that 33% of cells had formed secondary cell walls at the base of the tree, reducing to 3% at the crown. In total, 455 genes differentially expressed in developing xylem tissue from either the base or the crown were identified using modified differential display. RT-PCR analysis of 156 genes confirmed differential expression for 77%. Of the genes tested, 73% showed gradients in transcript abundance either up or down the bole of the tree, although the steepness of the gradients differed between genes. Genes involved in cell division and expansion tended to be more highly expressed in the crown of the tree, and two putative cell-cycle repressor genes were expressed 2-fold higher at the base. Conversely, transcripts of genes involved in secondary wall thickening were more abundant at the base of the tree. These results suggest that differences in the rate of cambial cell division, differences in the rate and duration of tracheid wall thickening, and differences in gene expression underpin the gradients of wood properties found in pines.

Introduction

In conifers, wood properties vary spatially within a single tree, both from the pith to the bark as well as from the base to the apex of the tree. Burdon *et al.* (2004) recently proposed a nomenclature to describe both the radial variation in wood properties (corewood vs. outerwood) and the vertical variation pertaining to the maturation state of the

plant (juvenile vs. mature), which is most evident in the lower region of the trunk. In general, corewood (defined as the first 10 growth rings from the pith) tends to have distinct properties from wood in subsequent rings, termed outerwood (Zobel and Sprague, 1998). In particular, xylem in corewood tends to have thinner cell walls, a lower specific gravity, larger cellulose microfibril angles and larger spiral-grain angles than outerwood

(Zobel and Sprague, 1998; Burdon *et al.*, 2004). Many of the anatomical characteristics of corewood adversely affect solid wood product performance through effects on strength, stiffness, and warping upon drying (Zobel and Sprague, 1998; Burdon *et al.*, 2004). Pulp and paper manufacturing characteristics are also distinct; corewood tends to yield less pulp per unit volume (using the kraft process) and the paper produced has a lower tearing strength and is more difficult to bleach (Zobel and Sprague, 1998; Burdon *et al.*, 2004). Because of a strong trend towards decreasing the harvest age in plantation forests due to economic factors, faster growth rates, and changing silvicultural practices (Zobel and Sprague, 1998), there is a growing interest in understanding and manipulating corewood formation.

In gymnosperms, changes in gene expression that underlie the phenotypic differences between crown and base wood have recently been investigated, along with those that affect commercially important wood properties such as wood density and microfibril angle. To date, the expression patterns of over 50 *Pinus* genes and proteins have been studied and confirmed to be differentially expressed using Northern analysis between xylem tissue from developing vertical wood (normal wood), compression wood (the wood formed on the underside of inclined stems), opposite wood (wood from the upper side of inclined stems) and side wood (wood from the sides of inclined stems) (Allona *et al.*, 1998; Plomion *et al.*, 2000; Zhang *et al.*, 2000; Le Provost *et al.*, 2003). The differentially expressed genes identified in these studies included cell-wall-associated proteins and enzymes, and regulatory proteins. In total, over 230 genes have been shown to be differentially expressed between wood laid down in spring (earlywood), and wood laid down later in the growing season (latewood) in *P. pinaster* and *P. taeda* using RT-PCR and cDNA microarray analysis (Le Provost *et al.*, 2003; Egertsdotter *et al.*, 2004; Yang and Loopstra, 2005). A comprehensive study of gene expression patterns in crown and base wood in *P. taeda* was performed by Lorenz and Dean (2002), who isolated 150 855 SAGE tags (up to 42 641 unique) from lignifying xylem in the crown and base of a 10-year-old loblolly pine tree. Correlations between putative function and differential expression of individual SAGE tags has become possible recently, when 12 918 of these

unique SAGE tags were deposited in the GEO profiles database in Genbank, and annotated based on 100% sequence similarity to ESTs in the *P. taeda* unigene set.

Here we present a detailed comparison of wood properties and gene expression patterns in developing xylem tissue sampled from the base to the crown of two individual trees of *P. radiata*. This sampling therefore provided simultaneous comparison of both the radial and vertical components of wood formation. We used a modified differential display approach that can distinguish between closely related members of gene-families and also individual alleles of the same gene (unpublished observation). Individual trees were used in preference to pools of individuals, in order to obtain information about genetic differences between individual trees and about the expression of individual genes and alleles that might be useful for subsequent genetic mapping and QTL detection experiments for wood property traits. Our data revealed strong gradients for both wood properties and gene expression patterns from the base to the crown. In part these gradients reflected fundamental differences in the rate of cambial cell division and the rate and duration of cell wall thickening along the bole of the tree.

Methods

Isolation of RNA

Two 24-year-old *Pinus radiata* trees (Tree 188 and Tree 93) in Kaingaroa Forest in Rotorua, New Zealand, were harvested in late summer (March), and two clonal replicates of Tree 93 (Tree 93i and 93ii) were harvested from the same site in the following spring (November). Cambium and developing xylem tissue were collected from two heights along the bole of Tree 188 (5.5 and 30 m) and at five heights from the three clonal replicates of Tree 93 (1.4, 5.5, 14, 22 and 30 m). Tissue was collected from one side of each tree, from an area of 50 cm², using a paint scraper or a potato peeler that had been washed with RNase AWAYTM (Molecular BioProducts) and then 96% ethanol. All soft tissue down to the hardened lignified layer beneath was collected. The tissue was immediately frozen by immersion in iso-pentane (that had been pre-chilled on dry ice) and was then placed on dry

Table 1. Differential display adapter and primer sequences.

Name	Sequence
Biotinylated T-primer ^a	5'biotin GCTGCAGACTTGTAGTTTTTTTTTTTTTVV
Taq-adapterF	CCGGATGAGTCCTGAC
Taq-adapterR	5'phosphate CCGTCAGGACTCATCCGG
Non-selective Taq-primer	CCGGATGAGTCCTGACCGA
Non-selective T-primer	GCTGCAGACTTGTAGTTTTTTTTTTTTTT
Taq-primerNNN ^a	CCGGATGAGTCCTGACCGANN
T-primerVVN ^a	GCTGCAGACTTGTAGTTTTTTTTTTTTTVV

^a V = A/C/G and N = A/C/G/T.

ice in transit to storage at -80°C . Total RNA was extracted from 2 g of frozen tissue using LiCl precipitation (Chang *et al.*, 1993). mRNA was isolated from total RNA using Dynabeads[®] oligo (dT)₂₅ (Dynal Biotech) following manufacturer's recommendations.

Cross-sectional tracheid dimensions

Wood discs were taken from three heights (1.4, 5.5 and 30 m) in Tree 188 and five heights (1.4, 5.5, 14, 22, and 30 m) in all three replicates of Tree 93. Xylem cell dimensions (including radial and tangential diameter, and cell perimeter) and wood density were measured at 50- μm intervals (across two radii from pith to bark in opposite directions) using SilviScan-2 (Evans *et al.*, 1995) in Tree 188 and one replicate of Tree 93. These measurements included the fully lignified xylem in the current incomplete growth ring. Coarseness, wall thickness and external specific surface area were calculated from the measurements (Evans *et al.*, 1995). Microfibril angle was also measured at 5 mm intervals using SilviScan-2 (Evans, 1999).

Microscopy

Cambial scrapings were dehydrated in an acetone series and embedded in Spurr resin. Samples were sectioned with a glass knife at a thickness of 2 μm , stained with toluidine blue and examined with a Zeiss Axioscope equipped with a digital camera, using either bright field or polarized light illumination.

Modified differential display method

We used a modified differential display approach to detect differential gene expression. The approach

incorporates features from both the ordered differential display (Matz *et al.*, 1997) and AFLP techniques (Bachem *et al.*, 1996). However, unlike AFLP, it specifically analyses only the 3' ends of mRNA transcripts. In addition, it utilizes non-denaturing polyacrylamide gels (Bolshoy *et al.*, 1991), which can detect both fragment length and sequence composition differences between transcripts.

First strand cDNA was synthesised from 500 ng mRNA using a biotinylated T-primer (Table 1) and Superscript II reverse transcriptase (Invitrogen) following manufacturer's instructions. The T-primer was composed of four separate components as follows (5'-3'): a 5' biotin label; a 15-bp arbitrary PCR primer sequence; a 13-bp polyT sequence (that can anneal to poly-A-rich regions including the poly-A tails of mRNA), and a 2-bp 'VV' sequence (V = A, C or G) (that promotes T-primer annealing to the 5' end of poly-A tails in mRNA). Double stranded cDNA was synthesised using *E. coli* DNA ligase, DNA polymerase I, RNase H and T4 DNA polymerase (Invitrogen) according to manufacturer's instructions.

Three hundred ng of double stranded cDNA was digested with 5 U of *TaqI*, in 40 μl of 1x OPA+ buffer (Pharmacia) for 1 h at 65°C according to manufacturer's instructions. Next, 10 μl of a solution containing 50 pmol of Taq-adapter, 1U T4 DNA ligase, 5 mM ATP in 1x OPA+ buffer (Pharmacia) was added and incubated at 15°C overnight. The Taq-adapter was prepared by adding equimolar amounts of Taq-adapterF and Taq-adapterR (Table 1), then incubating the mixture at 37°C for 10 min.

The cDNA fragments containing a biotinylated T-primer (i.e. fragments containing a *TaqI* site and a polyA tail) were isolated from non-biotinylated fragments (i.e. *TaqI*-*TaqI* and 5'cDNA-*TaqI*

fragments) using Dynabeads® M-280 streptavidin (DynaL Biotech) following manufacturer's instructions. The streptavidin beads containing the biotinylated cDNA fragments were resuspended in 100 μ l of TE_{0.1} buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and the cDNA quantified by fluorescence using picogreen dye (Molecular Probes) and a fluorometer (Polar Star Galaxy, BMG) following manufacturer's recommendations.

cDNA fragments were amplified using a non-selective TaqI and T-primer (Table 1) (PCR1). PCR amplifications were carried out in a total volume of 25 μ l, using 350 pg of biotinylated cDNA, 0.4 μ M of each primer, 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 1.5 mM MgCl₂, 0.2 mM each of dATP, dCTP, dGTP, dTTP in 1x AmpliTaq Gold buffer (Applied Biosystems). Samples were overlaid with paraffin oil and amplified in 96-well plates on a Techne PHC-3 thermal cycler. PCR amplifications were preceded by a 9-min incubation at 92 °C. Amplifications were performed for 35 cycles with the following cycling profile: 30 s at 94 °C, a 30-sec annealing step, 60 s at 72 °C. The annealing temperature in the first cycle was 65 °C; this was reduced by 1 °C for the next five cycles and was continued at 60 °C for 30 cycles. PCR amplifications were followed by a 5-min incubation at 72 °C. After the first amplification, PCR products were diluted 50-fold with TE_{0.1} and a 5- μ l aliquot was used as a template for subsequent PCRs.

To increase the yield of PCR product, a second amplification reaction (PCR2) was performed as described above using a 5- μ l aliquot of 50-fold diluted PCR1 product, and a non-selective Taq- and T-primer. PCR amplifications were preceded by a 9-min incubation at 92 °C. Amplifications were performed for 20 cycles with the following cycle profile: 30 s at 94 °C, 30 s at 60 °C, and 60 s at 72 °C. PCR amplifications were followed by a 5-min incubation at 72 °C. PCR products were diluted 50-fold with TE_{0.1} and a 5- μ l aliquot was used as a template for the selective amplification reaction.

Subsets of cDNA fragments were amplified using a Taq-primer and a T-primer that each contained three selective nucleotides at their 3' end (Table 1). PCR amplifications were carried out in a total volume of 20 μ l, using 5 μ l of template (either from PCR1 or PCR2), 0.4 μ M of each primer, 2.5 U AmpliTaq Gold DNA polymerase,

1.5 mM MgCl₂, 0.2 mM each of dATP, dCTP, dGTP, dTTP in 1x AmpliTaq Gold buffer. PCR amplifications were preceded by a 9-min incubation at 92 °C. Amplifications were performed for 37 cycles with the following cycle profile: 30 s at 94 °C, 30 s at 66 °C, and 60 s at 72 °C. PCR amplifications were followed by a 5-min incubation at 72 °C. Ten- μ l aliquots of PCR product were electrophoresed through 6% non-denaturing acrylamide gels (20 cm long) at 350 V using 1 x TBE as electrophoresis buffer, and then stained with ethidium bromide (0.25 ng/ μ l) (Sambrook *et al.*, 1989). cDNA fragments that were differentially amplified between tissues, and were thought to represent differentially expressed genes, were excised from the gel using a scalpel blade, added to 50 μ l of 1x PCR buffer (20 mM Tris-HCl, 100 mM KCl, pH 8.3) and incubated at 94 °C for 90 min (Frost and Guggenheim, 1999).

A 10- μ l aliquot of the eluted PCR product was re-amplified in a total volume of 50 μ l as described above using AmpliTaq Gold DNA polymerase and a non-selective Taq- and T-primer. PCR amplifications were preceded by a 9-min incubation at 92 °C. Amplifications were performed for 40 cycles with the following cycle profile: 30 s at 94 °C, 30 s at 62 °C, and 60 s at 72 °C. PCR amplifications were followed by a 5-min incubation at 72 °C. The PCR product was purified using a PEG precipitation protocol (Rosenthal *et al.*, 1993). Briefly, a 40- μ l aliquot of PCR product was added to 40 μ l of PEG precipitation solution (0.6 M sodium acetate, 26.2% polyethylene glycol 8000, 6.6 mM MgCl₂, pH 5.5) and incubated at room temperature for 60 min. The mixture was then centrifuged at 13 000 rpm (Eppendorf 5417R) for 15 min and the supernatant was removed. The pellet was washed once with 100 μ l of 70% ethanol, dried in a vacuum centrifuge (Hetovac VR-1) for 10 min, and resuspended in 40 μ l of distilled water.

Purified PCR products were sequenced in both directions using a cycle sequencing Big Dye™ Terminator V2.0 kit (Applied Biosystems) and a non-selective Taq- or T-primer. Sequencing reactions were purified using sodium acetate-ethanol precipitation (Applied Biosystems) and were electrophoresed through capillaries on a 3100 DNA sequencer (Applied Biosystems). The cDNA sequences were edited using Sequencher V.4.1.4 (<http://www.Genecodes.com>, Ann Arbor, USA)

and were analysed for sequence similarity to other differential display fragments and existing sequences in the public databases 'nr' and 'est' using the NCBI BlastN and BlastX programmes (Altschul *et al.*, 1990).

Validation of differential amplification

In order to confirm that differential amplification between tissues (detected using differential display) reflected differences in transcript levels, PCR primers were designed (using PRIMER3 software (Rozen and Skaletsky, 2000)) to 156 differentially amplified fragments, and to a constitutively expressed *P. radiata* gene, ubiquitin-activating enzyme (UAE) (unpublished data). Prior to analysis, the cDNA from each tissue was quantified using fluorescence and diluted to a 1 – ng/ μ l stock. In Tree 93, the cDNA was generated from total RNA prepared independently to the RNA used for differential display (i.e. from an independent 2-g aliquot of tissue). In Tree 188, due to a lack of available tissue, cDNA was generated from the same total RNA that was used for differential display. Aliquots of xylem cDNA from five heights (1.4, 5.5, 14, 22 and 30 m) were diluted from 70 pg/ μ l, to 14, 2.8, and 0.56 pg/ μ l (or in the case of Tree 188 from 10 pg/ μ l to 2, 0.4, and 0.08 pg/ μ l) and used as templates for PCR. cDNA dilutions were performed independently and in duplicate from aliquots of cDNA stocks derived from the same RNA extraction. Real-time PCR was performed on a LightCycler (Roche) in a total volume of 10 μ l, using 2.5 μ l of template, 0.5 μ M of each primer, 3 mM MgCl₂ and 1 μ l of FastStart DNA Master SYBR Green I reagent (Roche). PCR amplifications were preceded by a 10-min incubation at 95 °C. Amplifications were performed for 45 cycles with the following cycle profile: 5 s at 95 °C, 4 s at 60 °C, and 1 s at 72 °C per 25 bp of expected product. Fluorescent signal was acquired at the 72 °C extension step. PCR amplifications were followed by 1 s at 95 °C, 30 s at 65 °C, and then slowly heated to 95 °C using a ramp rate of 0.2 °C per second. Fluorescent signal was acquired continuously during the temperature gradient melting phase to generate a melting curve. Amplification of the expected product size was verified using melting curve and gel-based analysis. For quantitative analysis, the crossing point for each sample was calculated. The crossing points repre-

sent cycle numbers where the fluorescence level is the same in all samples. The crossing points were calculated during the logarithmic amplification phase using the second derivative maximum method as implemented by the Lightcycler software (Rasmussen, 2001). The crossing points for the 30 m cDNA dilutions were plotted against log concentration to generate a standard curve. The slope of this curve was used to calculate the PCR efficiency (Rasmussen, 2001) and the relative template concentration of all samples. For each gene tested, a two-tailed student's *t*-test (degrees of freedom = $n - 2$, n = number of replicates per tissue (independent cDNA dilutions)) was used to calculate the statistical significance of rejecting the null hypothesis that there was no difference in gene expression between tissues. UAE primers were also used to compare UAE expression levels in independent RNA extractions, as well as independent cDNA preparations from each height. This data was used to calculate the statistical significance of testing the first null hypothesis that there was no difference in UAE gene expression between independent RNA and cDNA preparations collected from the same tissue, and the second null hypothesis that there was no difference in UAE gene expression between heights, genotypes and tissue origins.

Results

Cross sectional tracheid dimensions

Cellular wood properties were measured at 50- μ m increments across two radii in the outermost growth ring of two trees, namely 188 and 93, using SilviScan-2 (Evans *et al.*, 1995; Evans, 1999). Only fully lignified xylem is measured using this approach. The cellular properties of a single radial for each tree are shown graphically in Figure 1A and B. As expected from the literature, wood properties showed a gradient from the base to the crown (Zobel and Sprague, 1998; Burdon *et al.*, 2004). Results summarised in Table 2 show that tracheids in the outer growth ring at the lowest sample of each tree (heights of 1.4 m in Tree 93 and 5.5 m in Tree 188, corresponding to growth rings 22 and 20 respectively) had significantly thicker cell walls, a higher density, increased coarseness, decreased specific-surface area, and

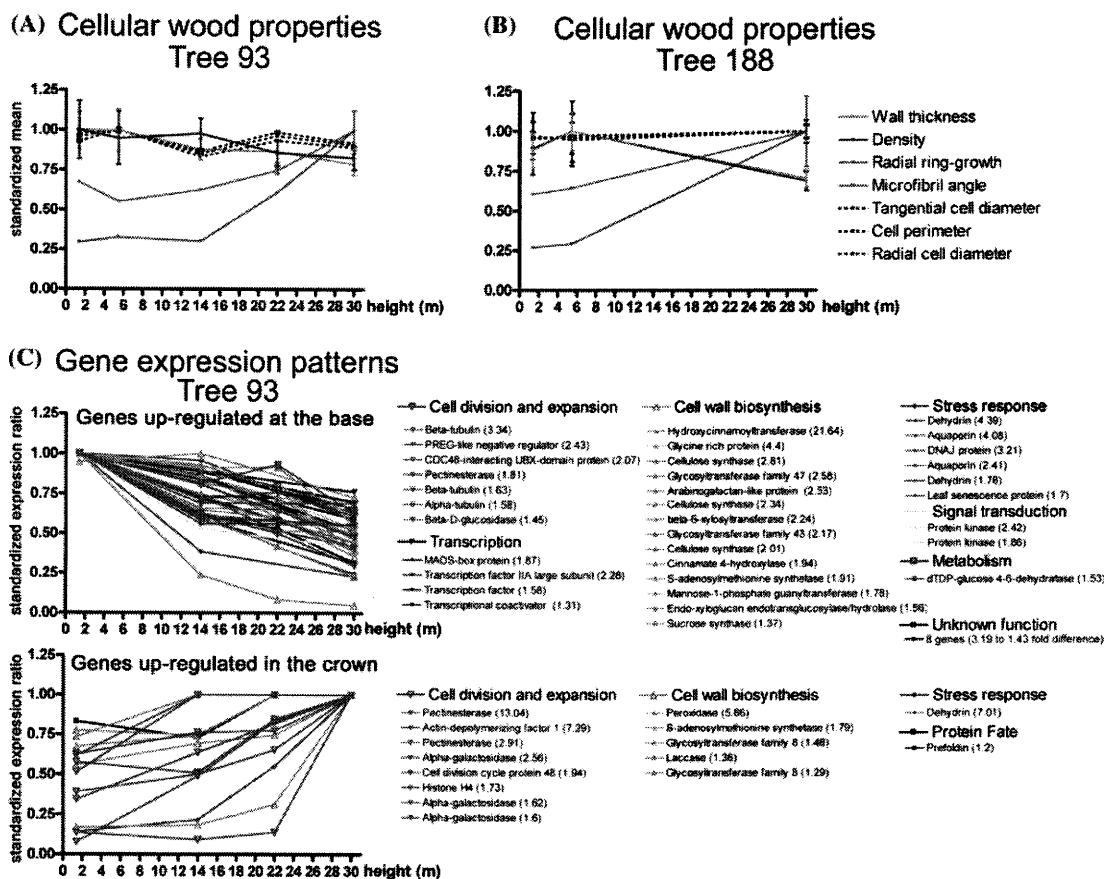


Figure 1. Average cellular wood properties in the outer growth ring in (A) Tree 93 at 1.4, 5.5, 14, 22 and 30 m, and (B) Tree 188 at 1.4, 5.5 and 30 m. For ease of comparison, the wood property values were standardized. Each mean wood property value is given as a fraction from 0 to 1 with 1 representing the greatest value that was observed at all heights assayed (all other heights were divided by this value). Gene expression patterns classified by functional category for (C) 57 selected genes at three or four heights (1.4, 14, 22 and 30 m) in Tree 93. Expression fold differences are also given as a fraction from 0 to 1 with 1 being the greatest fold difference that was observed between tissues. Genes in each category are listed in order from the largest to smallest fold-difference between 1.4 and 30 m (the fold difference is given in brackets).

smaller microfibril angles than tracheids at the crown (a height of 30 m, corresponding to growth ring 7). Cellular measurements were also taken at 50- μ m increments across two complete radii (from bark to bark) in each tree at each height; similar wood property trends were seen between all three heights, in both earlywood and latewood, in the outer six rings across two independent radii (data not shown). These wood property differences correspond to those previously published for corewood and outerwood (Zobel and Sprague, 1998; Burdon *et al.*, 2004); therefore the xylem collected from both 1.4 and 5.5 m in height at the

base was deemed 'outerwood', and the xylem tissue collected from 30 m in the crown was deemed 'corewood'. Xylem collected from 14 m was termed intermediate outerwood, and xylem collected from 22 m termed transition outerwood. The wood in the outer growth ring at all heights was also deemed to be in the earlywood phase of growth, based on both the trends and absolute values of average earlywood and latewood measurements in the preceding rings (from pith to bark across the two independent radii) for each tree.

In the outer growth ring, the rate of cell division was 3.3 times higher at 30 m than at

Table 2. Average cellular wood properties across a radial in the outer growth ring at 1.4, 5.5, and 30 m (growth rings 22, 20 and 7 respectively) in tree 188 and tree 93.

Parameter	Tree 188				Tree 93			
	5.5 m mean \pm sd (n ^a = 47)	30 m mean \pm sd (n ^a = 160)	Ratio of means 5.5/30 m	Student's <i>t</i> -test <i>p</i> -value	1.4 m mean \pm sd (n ^a = 47)	30 m mean \pm sd (n ^a = 159)	Ratio of means 1.4/30 m	Student's <i>t</i> -test <i>p</i> -value
Radial cell diameter (μm)	38.0 \pm 6.5	40.2 \pm 2.9	0.95		42.9 \pm 6.0	40.3 \pm 2.3	1.06	
Tangential cell diameter (μm)	31.8 \pm 0.8	32.6 \pm 1.2	0.97		31.6 \pm 1.8	30.4 \pm 1.1	1.04	
Density (kg m^{-3})	508.2 \pm 96.4	350.6 \pm 30.0	1.45	<0.0001	436.1 \pm 79.5	361.1 \pm 32.5	1.21	<0.0001
Wall thickness (μm)	3.1 \pm 0.4	2.2 \pm 0.2	1.42	<0.0001	2.8 \pm 0.3	2.2 \pm 0.2	1.27	<0.0001
Coarseness ($\mu\text{g m}^{-1}$)	595.5 \pm 45.4	457.9 \pm 38.8	1.30	<0.0001	576.7 \pm 29.6	441.2 \pm 34.7	1.31	<0.0001
Specific surface area ($\text{m}^2 \text{kg}^{-1}$)	234.8 \pm 21.5	319.7 \pm 22.4	0.73	<0.0001	258.8 \pm 24.1	322.2 \pm 23.6	0.80	<0.0001
Microfibril angle ($^\circ$)	11.9 (<i>n</i> = 1)	18.5 \pm 4.1 (<i>n</i> = 2)	0.64	NA	13.0 (<i>n</i> = 1)	19.8 \pm 1.6 (<i>n</i> = 2)	0.66	NA
Cell perimeter (μm)	139.6 \pm 14.1	145.7 \pm 6.6	0.96		149.1 \pm 14.1	141.4 \pm 5.4	1.05	
Rings from the pith	20	7	NA	NA	22	7	NA	NA
Ring width (mm)	2.4	8.0	0.3	NA	2.4	8.0	0.3	NA
Increase in circumference (cm) from the previous growth ring	1.6	5.2	0.31	NA	1.9	6.5	0.3	NA
Ring sectional area (cm^2) (calculated)	26.5	30.6	0.87	NA	29.8	31.0	0.96	NA

^a (*n* = number of 50- μm increments measured across a single radial from pith to bark).

1.4 m or 5.5 m in Tree 93 and 188 respectively. This conclusion is based on comparisons of the radius and circumference of ring growth, which were both 3.3-fold higher at 30 m than at the base (Table 2), and the observation that there was no significant difference in the tangential and radial diameters of the tracheids at all three heights. Total ring sectional area was similar at all heights. The average wood density (based on 2 radii) was calculated for every ring (from pith to bark at all heights assayed), and then compared to radial ring growth. Regression analysis showed that in Tree 188, wood density was strongly negatively correlated with radial ring growth, irrespective of ring number or height ($R^2 = 0.71$) (Table 3). However, in Tree 93, this correlation was weaker ($R^2 = 0.39$). Similar trends were observed in clonal replicates of each tree grown at the same geographical location (Tree 188i and 93i respectively) and three unrelated genotypes growing at the same site (Table 3).

Using regression analysis, correlations were estimated between wood density, radial and tangential diameter, and wall thickness within both the outer growth ring and the entire outerwood and corewood zones at 5.5 and 30 m in Tree 188 (Table 4). Notably, wall thickness was strongly correlated with wood density at all heights and rings assayed ($R^2 > 0.7$). In the outerwood zone at 5.5 m, radial diameter and wood density were strongly negatively correlated (average $R^2 = 0.78$), but in the corewood zone at 30 m this correlation was weaker (average $R^2 = 0.34$) (Table 4). In Tree 93, correlations between cellular wood properties in the outer growth ring, and across the entire corewood and outerwood zones

(data not shown), were similar to those obtained in Tree 188 (Table 4).

Over 30% of developing tracheids at the base were producing secondary cell walls compared to 3% in the crown

In Tree 93, xylem scrapings at 1.4, 5.5, 14, 22 and 30 m were used to isolate mRNA for gene expression (see below). The scrapings at 1.4, 14, 22, and 30 m were collected using a paint scraper, which removed all soft tissue down to the harden lignified layer beneath. Samples were collected in late summer. Microscopy was performed on these samples so that comparisons could be made between the observed gene expression patterns, and the proportion of cells in each phase of growth (i.e. cell division and expansion vs. secondary wall formation), at each height, at the time of sampling. Ten 1–2 mm strips of this scraped tissue (chosen at random from hundreds of scrapings at each height) were analysed using both bright field and polarised light microscopy at 1.4 and 30 m, and a single scraping was analysed at 5.5, 14, and 22 m (Figure 2).

The bright field images showed that each scraping contained, on average, 16 cells per radial file, and that there were approximately 200–300 cells per image. Secondary walls appear white in colour under polarised light due to birefringence (Figure 2D, H, J), and this enabled us to estimate the percentage of cells producing secondary cell walls at 1.4 and 30 m. We found that 33% of developing tracheids at the base (1.4 m) were producing secondary cell walls, with an average of

Table 3. Correlations between radial ring growth and average wood density per ring for seven trees.

Tree ^a	Radial ring growth vs. average wood density R^2	<i>p</i> -value	Number of rings (radii assayed)	Heights assayed (m)
188	0.71	3.60E-24	43 (86 radii)	1.4, 5.5, 30
188i	0.66	4.80E-09	34	1.4, 5.5, 14
93	0.39	3.70E-14	58 (116 radii)	1.4, 5.5, 14, 22, 30
93i	0.26	0.16	9	1.4
3	0.34	8.00E-02	10	14
6	0.68	7.50E-07	20	1.4
59	0.79	1.80E-07	20	1.4

^a Tree 188i is a clonal replicate of tree 188. Tree 93i is a clonal replicate of tree 93. All seven trees were grown in the same geographical location.

Table 4. Correlations between cellular wood properties at the base (5.5 m) and crown (30 m) of tree 188.

Height and wood type	Ring number from the bark ^a	Number of 50 µm increments	Wood density		Wood density		Wood density		Wall thickness		Wall thickness	
			vs. radial diameter R^2 b (sign of R)	vs. tangential diameter R^2 b (sign of R)	vs. radial diameter R^2 b (sign of R)	vs. tangential diameter R^2 b (sign of R)	vs. radial diameter R^2 b (sign of R)	vs. tangential diameter R^2 b (sign of R)	vs. radial diameter R^2 b (sign of R)	vs. tangential diameter R^2 b (sign of R)	vs. radial diameter R^2 b (sign of R)	vs. tangential diameter R^2 b (sign of R)
5.5 m	0	47	0.81 (-) ^c	0.43 (-)	0.90 (+)	0.57 (-)	0.30 (-)	0.22 (+)	0.23 (-)	0.21 (+)	0.30 (-)	0.22 (+)
30 m	0	160	0.37 (-) ^c	0.01	0.73 (+)	0.07	0.62 (-)	0.23 (-)	0.03 (-)	0.21 (+)	0.30 (-)	0.22 (+)
5.5 m outerwood	0-9	2158	0.78 (-)	0.34 (-)	0.95 (+)	0.62 (-)	0.23 (-)	0.21 (+)	0.30 (-)	0.22 (+)	0.30 (-)	0.22 (+)
30 m corewood	0-6	2523	0.34 (-)	0.08 (-)	0.78 (+)	0.03 (-)	0.21 (+)	0.30 (-)	0.22 (+)	0.21 (+)	0.30 (-)	0.22 (+)

^a Ring 0 is the outer-incomplete growth ring and was deemed to be in the earlywood phase of growth (see text).

^b R^2 values in bold have p -values <0.0001 .

^c In the outer growth ring at 5.5 m, the strong correlation between radial diameter and wood density in the earlywood zone appeared to be due the presence of a false ring (radially flattened cells), which coincided with a period of drought earlier in the growing season (data not shown). Radial diameter did not decrease during the drought period at 30 m.

4.4 cells producing secondary walls per radial file (Figure 2J). At 30 m, only 3% of the tracheids examined were producing secondary cell walls, with less than one cell per radial file producing secondary walls; no secondary cell walls were seen in the photographed image (Figure 2B). At 22 m, the corresponding number of tracheids producing secondary cell walls per radial file was also less than one; out of 20 files examined from a single scraping, only six files showed one or two tracheids producing secondary cell walls (Figure 2D). The scrapings at 5.5 m were collected with a potato peeler and these contained on average only seven cells per radial file. At 5.5 m, on average, four cells per radial file were producing secondary cell walls (Figure 2H), similar to the number seen per radial file at 1.4 m. Xylem scrapings at 5.5 m were not analysed by RT-PCR in Tree 93. In Tree 188, all of the xylem tissue collected was used for gene expression analysis so this tissue could not be analysed by microscopy.

Analysis of gene expression during xylogenesis

We used a modified differential display technique (see Methods for details) to detect differences in gene expression between developing wood from the base and the crown in late summer. Gene expression was compared at two heights in the same two unrelated trees (188 and 93). Tissue from the crown (30 m) in both trees was compared with tissue from the base (5.5 m in Tree 188, and at 1.4 m in Tree 93). In total, 486 primer pairs were used to screen approximately 3900 amplified cDNA fragments. Differences in amplified fragment intensity between tissues were assumed to reflect differences in the relative abundance of each transcript in the cDNA population. In total 455 unique transcripts were isolated that showed putative expression level differences between xylem tissue from the base and crown (Supplementary Table 1). Of the 455 differentially expressed fragments, 423 were assayed in both trees and a further 32 were assayed only in Tree 188. Of the 423 fragments, 396 (94%) were up-regulated at the base or the crown in both trees (Supplementary Table 1), and 27 transcripts were up-regulated in either Tree 93 or 188 at the base or the crown, but were not differentially expressed in the other tree.

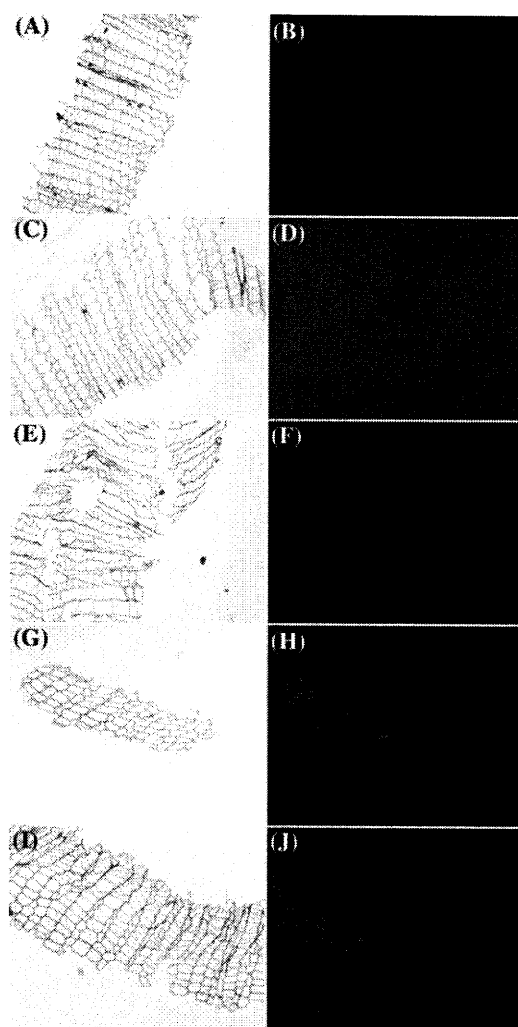


Figure 2. Cross-sectional images of cambial scrapings isolated from 1.4, 5.5, 14, 22 and 30 m in Tree 93. The left hand microscopy images are bright field images stained with toluidine blue, right hand images are the same field of view under polarised light showing the presence or absence of secondary wall formation in each sample. Secondary walls (the S1 layer) appear white in colour due to birefringence and were observed in images, (D) (22 m), (H) (5.5 m) and (J) (1.4 m). The S2 layer typically appears darker than the S1 layer due to birefringence, but was not observed in any of the photographed images. (A), 30 m – bright field. (B), 30 m – polarised light. (C), 22 m – bright field. (D), 22 m – polarised light. (E), 14 m – bright field. (F), 14 m – polarised light. (G), 5.5 m – bright field. (H), 5.5 m – polarised light. (I), 1.4 m – bright field. (J), 1.4 m – polarised light. Each image has a field of view of $700 \times 550 \mu\text{m}$.

Validation of differential expression using real-time RT-PCR

Expression levels of the putative constitutively expressed *P. radiata* gene, ubiquitin-activating enzyme (UAE), was assayed by real-time RT-PCR using a LightCycler (Roche). The UAE gene had previously been isolated from needle, root, shoot, and male cone tissue from an unrelated genotype using cDNA-AFLP and appeared to be expressed at the same level in all tissues assayed (unpublished data). UAE expression was assayed at four heights in Tree 93 (1.4, 14, 22, and 30 m) and two heights in Tree 188 (5.5 and 30 m). For comparison, UAE gene expression was also assayed in two clonal replicates of Tree 93 (Tree 93i and 93ii) collected in spring from the same site, as well as in embryogenic cultured cells from five cell lines from an unrelated genotype (XGLn7). All of the samples tested at a $1\text{ng}/\mu\text{l}$ dilution had similar crossing points for UAE (20.44 ± 0.23) irrespective of height (1.4, 5.5, 14, 22, 30), genotype (188, 93, 93i, 93ii, and XGLn7) or tissue origin (xylem or embryogenic tissue). Thus, we found that if the cDNA was quantified and diluted to a predetermined cDNA concentration prior to analysis, little or no post-PCR normalization was required using UAE as a reference, regardless of tissue-type assayed. For further comparisons it was assumed that equal crossing points represented equal expression (in terms of relative template abundance) between all tissues.

Gene specific primers were designed in the 3'UTR to 156 of the putative differentially expressed transcripts. For each gene, expression levels for at least two heights were re-assayed by real-time RT-PCR in one or both trees. Based on the consistent results obtained for UAE, a single RNA preparation was used to assess expression levels at each height. In total, 113 genes were assayed in Tree 93 and 67 genes were assayed in Tree 188 (with 24 assayed in both trees). RT-PCR analysis confirmed that 77% (120/156) of the genes were differentially expressed in either tree (p -value < 0.05) (Supplementary Table 1 and Figure 1C). Of 81 genes that showed differential expression between the base and crown of Tree 93, RT-PCR of samples at three or four heights along the bole (Supplementary Table 1) showed gradients in gene

expression for 60 (73%) (Figure 1C). Of 24 genes assayed in both trees, 19 showed the same expression patterns (Supplementary Table 1), with 13 genes up-regulated at the base in both trees, four genes up-regulated in the crown in both trees, and two that were not differentially expressed in both trees. Three transcripts (T69T2317030, T69T6018006, and T31T3925006) were differentially expressed in Tree 188 but were not differentially expressed in Tree 93. However, these findings were expected based on the differential display data.

For genes verified with RT-PCR, the expression differences ranged from 1.2-fold to 21.6-fold between tissues (p -value < 0.05). Almost all of the differentially expressed fragments isolated using differential display had shown staining intensity differences between tissues (e.g. faint vs. medium) rather than presence/absence differences. Thus, in many cases the expression difference between tissues was expected to be small; indeed, the average expression difference detected between the base and the crown using RT-PCR was 2.8-fold.

The putative functions of genes up-regulated at the base or crown in summer

Of the 455 unique transcripts that showed putative differential expression between the base and the crown in summer using differential display, 368 (81%) shared $>90\%$ sequence identity to expressed sequence tags (ESTs) from other *Pinus* or *Picea* species in Genbank. Of these ESTs, 231 could be assigned a putative function based on amino acid similarity to known or predicted proteins (Supplementary Table 1). Each of the 231 fragments was assigned to a functional category (Figure 3; Supplementary Table 1). Of 114 such genes more highly expressed in the crown, 26% were involved in metabolism, 13% were involved in cell division and expansion, 9% were involved in cell wall biosynthesis, and 3% were involved in signalling (Figure 3). Among the 117 genes (with a putative function assigned) that were more highly expressed at the base, the percentage of genes assigned to each category differed; more of the differentially expressed genes were involved in cell wall biosynthesis (21%), and signalling (13%), with fewer related to metabolism (16%), or to cell division and expansion (5%) (Figure 3).

All genes assigned to the categories of cell division and expansion, or cell wall biosynthesis were re-assayed by RT-PCR in Tree 93 and/or Tree 188 (Supplementary Table 1). RT-PCR results confirmed that 1.8 times as many genes involved in cell division and expansion were up-regulated in the crown compared to the base, and 2.6 times as many genes involved in cell wall biosynthesis were up-regulated at the base compared to the crown (Supplementary Table 1). Thus, using RT-PCR analysis, we found that the ratio of genes up-regulated at the base or crown in each functional category was similar to those obtained with differential display.

Expression differences between gene family members

In several instances, our modified differential display technique identified different members of the same gene family as being differentially expressed (Supplementary Table 1). Real-time RT-PCR confirmed differential expression for a number of these gene family members. For example, three alpha-galactosidase genes were isolated that were up-regulated 1.6-, 1.6-, and 2.6-fold respectively in the crown in Tree 93. Four pectinesterase genes were isolated, of which two were up-regulated at the crown (3- and 13-fold), one at the base (1.8-fold), and one was not differentially expressed in Tree 93. Similarly, three dehydrin genes were isolated, of which two were up-regulated at the base (1.6- and 4.9-fold in Tree 93, and 3.1- and 11.1-fold in Tree 188, respectively). The third dehydrin gene was up-regulated at the crown (7.0-fold) in Tree 93, but was not expressed at detectable levels in Tree 188 at either 5.5 or 30 m.

*Comparison of gene expression profiles with SAGE tag frequencies in *P. taeda**

Comparisons were made between the 455 differentially expressed fragments identified in this study, and 12 918 annotated SAGE tags in GenBank isolated from the base and crown of a 10-year old *P. taeda* tree (Lorenz and Dean, 2002). Exact sequence matches were found for 274 of the 455 differentially expressed fragments. A number of the *P. radiata* fragments (93) matched multiple SAGE

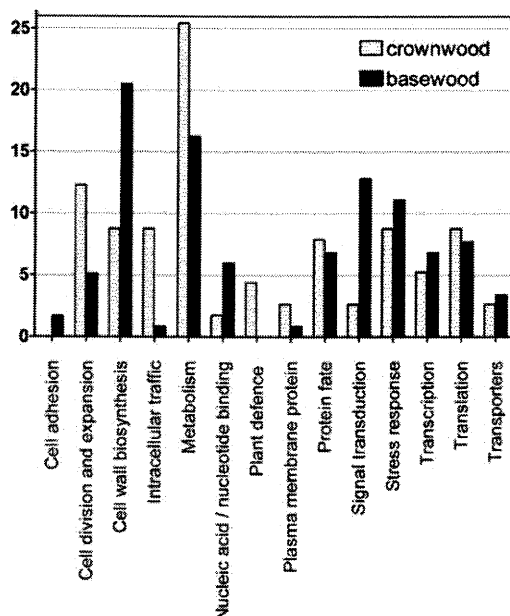


Figure 3. Comparison of differentially expressed fragments (isolated by differential display) between the base and the crown of the tree, classified by cellular functional categories. In each tissue, the percentage of genes in each category is relative to the total number of fragments that were assigned a putative function in that tissue (based on a BLASTX e-value $<10^{-5}$, or $>70\%$ positive identity, to amino acid sequences in the databases 'nr' and 'swissprot' in Genbank). In many cases, differential display fragments were assigned to a category based on the putative function of their longer *P. taeda* EST homologues. The criterion for EST homology was $>90\%$ identity in the 3'UTR.

tags (i.e. 207 tags in total), and often these SAGE tags gave conflicting expression profiles (i.e. some were more frequent in the crown library, while others were more frequent in the base library). We noted a difference between SAGE tags located in the coding region and those located within the 3' UTR of genes. For example, we obtained two different β -tubulin genes from *P. radiata*, one up-regulated in basewood and the other in crownwood in Tree 188 (Supplementary Table 1). The sequence of the 3' UTR of each β -tubulin gene matched two different SAGE tags (Supplementary Table 1). These two SAGE tags showed the same expression pattern as its respective *P. radiata* fragment (in Tree 188), and was presumed to be specific for that gene family member. However, the coding region of both the *P. radiata* β -tubulin genes also matched a single SAGE tag (i.e. the same SAGE tag (aatgatttgg) matched both genes). This SAGE tag was more frequent in the crown library than the base library, and thus only matched the expression pattern of one of the *P. radiata* transcripts. It is presumed that the SAGE results for this tag

represent the sum of at least two gene family members. We note that 19% of the SAGE tags analysed had exact matches to two or more unrelated *P. taeda* ESTs in GenBank and so were discarded from further analysis.

The expression patterns of *P. radiata* transcripts were therefore only compared to those SAGE tags in the 3'UTR. Of 91 transcripts compared, with a putative function assigned, between *P. radiata* and SAGE (*P. taeda*), 69% (63/91) showed similar patterns of differential expression (Supplementary Table 1).

Discussion

Wood from the crown has a lower density due to a decrease in cell wall thickness

Consistent with previously published wood properties in pines, detailed investigations of wood properties in two individual trees showed gradients in cell wall thickness, wood density, and microfi-

bril angle along the bole from the base to the crown (Figure 1A and B). In gymnosperms, wood density is affected by two cellular components: the radial diameter of the tracheids, and the thickness of the cell wall in each tracheid (Whitmore and Zahner, 1966; Shelbourne *et al.*, 1997; Kibblewhite *et al.*, 2003). Based on ring averages, no significant difference in the average radial or tangential diameter of the tracheids was seen between all heights assayed. Conversely, regression analysis showed that variation in wood density and wall thickness were tightly and consistently linked within and between growth rings at all heights assayed ($R^2 > 0.7$). In addition, the ranges of variation shown between wood density and cell wall thickness were similar. In Tree 188, cell wall thickness was on average 1.42 times greater at 5.5 m than at 30 m (Table 2), similar to the ratio of wood density between the two (1.45 times). A similar trend was seen for Tree 93 at 1.4 and 30 m (Table 2). This study implies that the primary manner in which density is moderated in this species, both within or among trees, is by altering cell wall thickness. These results are consistent with correlations observed in genetic studies where cell wall thickness is strongly associated with wood density (Shelbourne *et al.*, 1997).

Wood density is negatively correlated with radial growth rate in some genotypes

Wood formation is initiated in the vascular cambium. Cambial derivatives develop into tracheid cells through the process of cell division, cell expansion, secondary wall formation, lignification and programmed cell death (Harris, 1991). Growth ring measurements led to the conclusion that the rate of cell division was 3.3 times higher in cambium in the crown than at the base of the tree. This faster growth at the crown was associated with reduced cell wall thickness (measured by SilviScan-2 analysis), which was evident throughout the growing season. In the developing xylem tissues, which were harvested in late summer, we observed that the proportion of cells undergoing secondary thickening in the crown was significantly lower than at the base (measured by microscopy). However association does not neces-

sarily imply causation. Whole-tree trends showed that in Tree 188 (and a clonal replicate), radial ring growth was strongly associated with cell wall thickness ($R^2 = 0.71$), but in Tree 93 (and a clonal replicate) this correlation was weak ($R^2 = 0.39$). Three other unrelated genotypes had R^2 values similar to either Tree 188 or Tree 93, suggesting that the strength of the relationship between wood density and radial ring growth may be genotype specific. Interestingly, Burdon and Low (1992) previously showed that in two New Zealand *P. radiata* populations, both phenotypic and genetic correlations observed between density and radial diameter growth were weak ($R = -0.4$), although there was significant variation between individuals, and confidence intervals were wide.

Whitmore and Zahner (1966) proposed that cell wall thickness in *P. resinosa* was related to the rate of cell division and therefore the duration of the secondary wall thickening phase of growth. Measurements were taken at two heights across an entire growing season, in twelve drought-stressed, irrigated or bud-clipped trees, and showed that an increase in the rate of cell division led to a decrease in the duration of the wall thickening phase, and a decrease in wall thickness. This proposal was supported by Uggle *et al.* (2001) who found a lack of obvious seasonal changes in auxin supply and carbohydrate availability in *P. sylvestris* and concluded that it was the duration, and not the rate of wall material deposition, that caused the thicker cell walls of latewood tracheids. Our results in *P. radiata* also show that growth rate is tightly associated with wall thickness but only in some genotypes. It is likely that variation in cell wall thickness, within and between genotypes, is moderated by changes in the timing of onset, the rate and the duration of the wall-thickening phase. The relative contribution of each variable may be genotype specific.

Gene expression differences from the base to the crown reflect differences in radial growth rate and the proportion of developing tracheids in the expansion and wall thickening phases

In Tree 93, 73% of differentially expressed transcripts (confirmed by RT-PCR) showed gradients in gene expression from the base to the crown

(Figure 1C), parallel to the gradients observed for both growth rate and cellular wood properties (including wood density, wall thickness and microfibril angle) (Figure 1A and B). The slope of the expression gradient differed for different genes. Therefore, these gradients cannot simply reflect differences in the relative proportion of cells in the expansion and secondary wall formation phase at each height. There must also be expression level differences between cells at similar stages of differentiation at different heights.

Microscopy and SilviScan-2 analysis showed that cell division was faster at the crown of the tree, while a higher proportion of tracheids at the base were producing secondary cell walls, which were 1.2–1.4 times thicker than cell walls at the crown. Functional classification of differentially expressed transcripts by homology revealed close parallels with the conclusions from analysis of wood properties. First, three putative cell cycle genes were isolated whose transcript levels differed between the base and the crown. It is noteworthy that two of these genes, up-regulated 2-fold at the base, were putative cell cycle repressor genes (Supplementary Table 1). One encoded a putative PREG-like negative regulator (containing a cyclin-N domain) that may be involved in cell cycle regulation. The other gene encoded a putative *CDC48* repressor protein, with 74% amino acid similarity to the *PUX1* gene, which has been shown to inhibit growth rate in *Arabidopsis thaliana* (Rancour *et al.*, 2004). The third gene, encoding a putative *CDC48*-like protein, was down-regulated 2-fold at the base of the tree.

Second, the microscopy results had shown that fewer developing tracheid cells at the crown showed secondary thickening, consistent with the hypothesis that the phase of cell wall formation may be shorter in the crown and that more cells would be undergoing expansion. Six genes that were up-regulated in the crown were putatively involved in cell expansion and/or cell wall modification. These included three α -galactosidase genes, an endo-xyloglucan endotransglycosylase, and two pectinesterases. α -Galactosidase enzymes catalyze the hydrolysis of α -1, 6-linked galactosyl residues from galacto-oligosaccharides and polymeric galacto-(gluco)mannans in the cell wall and are known to be involved in the mobilization of sugars and cell growth (Feurtado *et al.*, 2001). Likewise, pectinesterases catalyze the

hydrolysis of pectin into pectate and methanol, and are thought to modulate radial cell expansion as well as cell-to-cell cohesion (Micheli *et al.*, 2000).

Three endo-xyloglucan endotransglycosylase family members were isolated. RT-PCR showed that one family member was not differentially expressed, while another was up-regulated in the crown, and the third was up-regulated at the base. Endo-xyloglucan endotransglycosylase enzymes can catalyze both xyloglucan endohydrolysis (XEH) and endotransglycosylation (XET) and are involved in both cleaving and religating xyloglucan polymers (Darley *et al.*, 2001). Hence they can participate in both cell wall loosening, and hemicellulose biosynthesis. Interestingly, Allona *et al.* (1998) also isolated two endo-xyloglucan endotransglycosylase family members in *P. taeda* and found that one was more abundant in xylem from vertical stems (normal wood), and the other more abundant in xylem from the sides of inclined stems (side wood). It is possible that these gene family members have different functions, and perhaps XEH and XET activities, in different wood tissues.

Eighteen genes that were up-regulated at the base (1.2- to 21.6-fold) were putatively involved in cell wall biosynthesis, compared to only seven in the crown. Furthermore, five-times as many genes putatively involved in signalling were up-regulated at the base compared to the crown. These included eight putative protein kinases, three WD-40 repeat proteins, and two calmodulin related proteins, that may be involved in signalling processes related to cell wall deposition.

Three cellulose synthase genes and a sucrose synthase gene were up-regulated at the base of the tree. Cellulose is the major constituent of the S2 secondary wall layer, the thickest wall layer in gymnosperms. The three cellulose synthase genes showed gradients of gene expression with similar slopes along the bole (-0.019 ± 0.001). Their 3' UTR regions shared >90% sequence similarity to three *Pinus taeda* cellulose synthases, PtCesA1, -2 and -3 (Supplementary Table 1), which in turn show the highest amino acid identity among the *Arabidopsis thaliana* cellulose synthase genes to AtCesA8, -4 and -7 (Nairn and Haselkorn, 2005); these three genes are involved in secondary wall formation in *Arabidopsis* (Taylor *et al.*, 2003). Uggle *et al.* (2001) found that, in *P. sylvestris*, sucrose synthase enzyme activity was lowest in the

primary walled tissues and peaked in the zone of maturing tracheids where the secondary wall is formed. Sucrose synthase can exist as a cytoplasmic or a membrane-associated enzyme and in the latter form it has been suggested to associate with cellulose synthase and directly supply UDP-glucose to growing glucan chains (Amor *et al.*, 1995). Interestingly, the sucrose synthase gene and two of the cellulose synthase genes, up-regulated in basewood (Supplementary Table 1), were also significantly up-regulated in latewood tissue from *P. taeda* trees sourced from the South Arkansas region in the USA (Yang and Loopstra 2005). Both latewood and basewood have significantly thicker cell walls than earlywood from the crown in both species.

Three putative glycosyltransferase genes were also isolated that were up-regulated at the base. One gene shared 90% amino acid identity to a *Populus* glycosyltransferase family 47 gene (PttGT47A) that was highly expressed during secondary wall formation in poplar (Aspeborg *et al.*, 2005). The other two glycosyltransferases belonged to family 43; members of this family are highly expressed during fibre development in cotton, *Arabidopsis*, and poplar (reviewed by Aspeborg *et al.*, 2005).

Five lignin-related genes were up-regulated at the base of either Tree 188 or 93, including two cinnamate 4-hydroxylase (C4H) genes, a hydroxycinnamoyltransferase (HCT), a laccase and an S-adenosylmethionine synthetase gene. Two laccase genes, a peroxidase, and an S-adenosylmethionine synthetase gene were up-regulated in the crown, however these genes perform a variety of functions in the cell, and their up-regulation in the crown may be unrelated to lignin biosynthesis. Two other genes involved in lignin biosynthesis, a cinnamyl-alcohol dehydrogenase, and a caffeoyl-CoA O-methyltransferase gene, were not differentially expressed between the base and the crown. However, Anterola *et al.* (2002) showed that these two enzymes were not rate-limiting in lignin biosynthesis in *P. taeda* cell suspension cultures. The putative HCT was up-regulated 21.6-fold at 1.4 m, and expression levels decreased along the bole from the base to the crown. This gene has recently been shown to be involved in lignin biosynthesis (Hoffmann *et al.*, 2004) and may be a rate limiting step in the synthesis of syringyl and guaiacyl units in angiosperms and gymnosperms respectively (Hoffmann *et al.*, 2004). C4H cata-

lyzes the 4-hydroxylation of cinnamic acid, the first oxidative step in the general phenylpropanoid pathway. C4H enzymes, also up-regulated in basewood (and latewood in *P. taeda* from the South Arkansas region (Yang and Loopstra 2005)), were found to be a rate-limiting step in the synthesis of lignin in *P. taeda* cell suspension cultures (Anterola *et al.*, 2002).

The differential expression patterns observed here between xylem from the base and the crown of the tree were not confined to *P. radiata* species. A comparison with *P. taeda* SAGE tags showed that 69% of transcripts assayed with a putative function assigned, showed the same patterns of differential expression between the base and the crown of both species. A moderate level of variation between the two studies was expected given that the two species were grown in opposite hemispheres, the tissues were collected in different seasons (spring vs. summer), at different ages (10 vs. 24 years), and considering the evolutionary distance between the two species (21–22 million years) (Millar, 1993; Krupkin *et al.*, 1996). However, it appears that gene expression differences were conserved to a large extent across sites, seasons, and pine species boundaries.

Conclusion

This work has revealed a number of strong gradients from the base to the crown of the tree during wood formation in *Pinus radiata*. The crown cambial layer underwent cell division 3.3 times more frequently than the base cambial layer, and the rate of cell division decreased along the bole. In addition, fewer cells were undergoing secondary wall thickening in the crown, and the cell walls were significantly thinner. The reduced cell wall thickness of tracheids at the crown of the tree correlated closely with lower wood density, and is likely to be moderated by decreases in both the rate and duration of wall thickening in the developing tracheid cells. Genes putatively involved in cell division and expansion were preferentially up-regulated at the crown, while genes putatively involved in secondary wall formation were up-regulated at the base of the tree. RT-PCR analysis of differentially expressed genes also revealed gradients in gene expression levels in developing xylem tissue along the bole of *Pinus*

radiata. Such gradients in gene expression are likely to underpin differences in cellular wood properties along the bole of the tree.

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