High nitrogen fertilization and stem leaning have overlapping effects on wood formation in poplar but invoke largely distinct molecular pathways

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Summary Previous studies indicated that high nitrogen fertilization may impact secondary xylem development and alter fibre anatomy and composition. The resulting wood shares some resemblance with tension wood, which has much thicker cell walls than normal wood due to the deposition of an additional layer known as the G-layer. This report compares the short-term effects of high nitrogen fertilization and tree leaning to induce tension wood, either alone or in combination, upon wood formation in young trees of Populus trichocarpa (Torr. & Gray) × P. deltoides Bartr. ex Marsh. Fibre anatomy, chemical composition and transcript profiles were examined in newly formed secondary xylem. Each of the treatments resulted in thicker cell walls relative to the controls. High nitrogen and tree leaning had overlapping effects on chemical composition based on Fourier transform infrared analysis, specifically indicating that secondary cell wall composition was shifted in favour of cellulose and hemicelluloses relative to lignin content. In contrast, the high-nitrogen trees had shorter fibres, whilst the leaning trees had longer fibres that the controls. Microarray transcript profiling carried out after 28 days of treatment identified 180 transcripts that accumulated differentially in one or more treatments. Only 10% of differentially expressed transcripts were affected in all treatments relative to the controls. Several of the affected transcripts were related to carbohydrate metabolism, secondary cell wall formation, nitrogen metabolism and osmotic stress. RT-qPCR analyses at 1, 7 and 28 days showed that several transcripts followed very different accumulation profiles in terms of rate and level of accumulation, depending on the treatment. Our findings suggest that high nitrogen fertilization and tension wood induction elicit largely distinct and molecular pathways with partial overlap. When combined, the two types of environmental cue yielded additive effects.

Keywords: FQA, FT-IR, gene expression, G-layer, microarray RNA profiling, secondary cell wall, secondary xylem.

Introduction

Wood is produced through successive seasons of secondary xylem formation, a developmental process that is modulated by genotype as well as environmental conditions (Mellerowicz et al. 2001). Once harvested, wood serves many purposes, including lumber for construction and pulp and paper products. Also, it is potentially an important source of renewable biomass feedstock for the production of bioenergy (for a review, see Karp and Shield 2008). Many of the studies that have been conducted to date of secondary xylem cell wall properties and the developmental and biochemical processes that contribute to wood formation have been carried out using Populus spp. (Jansson and Douglas 2007), including examination of the molecular events regulating the deposition of cellulose, hemicelluloses (matrix glycans) and lignin (Tuskan et al. 2006, Mellerowicz and Sundberg 2008). Silvicultural methods are increasingly used...
to augment forest productivity, and nitrogen fertilization is one of the most widely used silvicultural practices that have been employed to achieve this goal (Macdonald and Hubert 2002). Nitrogen is often limiting for plant growth in terrestrial environments (Vitousek and Howarth 1991); therefore, the addition of nitrogen-rich fertilizers can have profound effects on tree physiology and biomass accumulation (Coleman et al. 1998, Gessler et al. 2004, Cooke et al. 2005).

Wood formation is highly responsive to environmental changes (Mellerowicz et al. 2001, Mellerowicz and Sundberg 2008). Modifications at anatomical and compositional levels have frequently been described in relation to N–P–K fertilization, water stress and mechanical stimuli (Peszlen 1994). Generally, increased growth rates have been found to increase the relative proportion of juvenile wood compared with more desirable mature wood (for a review, see Macdonald and Hubert 2002). In poplar, it has been shown that nitrogen fertilization affects wood properties by reducing its density, altering cell wall thickness and influencing fibre length in a genotype-dependent manner in coppiced trees (Luo et al. 2005). Luo et al. (2005) showed that high N availability increased fibre lengths for *Populus alba* L. and *P. nigra* L., but decreased fibre lengths for *Populus deltoides* Bartr. ex Marsh. × *P. nigra* L. Using controlled growth conditions, we previously observed that trees fertilized with high N produced slightly shorter but wider fibres with significantly thicker cell walls compared with low-N trees; this response was observed within a 28-day period of treatment (Pitre et al. 2007a). The thicker cell walls were mainly composed of cellulose and hemicelluloses and were reminiscent of the G-layer of tension wood. Lignin content in the newly formed wood decreased and its structure was similar to that of tension wood lignin, with a decreased proportion of p-coumaric acid (lignin H) units and other structural modifications (Pitre et al. 2007b).

At the molecular level, we have shown that specific genes are differentially regulated in stems by high nitrogen availability (Cooke et al. 2003). Genes whose transcript abundance was found to be altered in response to N availability included a caffeoyl-CoA O-methyltransferase (CCoAOMT), together with other sequences that may be associated with cell wall development, such as leucine-rich repeat proteins. More recently, the relationship between phenylpropanoid metabolism and nitrate availability has been elucidated using a tobacco (*Nicotiana tabacum* L.) model where NIA-deficient (nitrate reductase) mutants provided metabolic and transcriptional evidence of increased lignin formation under low nitrogen supply (Fritz et al. 2006).

One of the best documented examples of environmental stimuli effects on wood formation is that of tension wood. When forced to grow in a leaning or bent position, angiosperm trees adapt to changes in the orientation of their stems by producing reaction wood (known as tension wood), which has altered cell wall morphology and composition compared with those of unstressed wood (Wardrop 1965). Formation of reaction wood has been posited as a mechanism by which trees can recover from leaning and restore the orientation of their stems to the vertical (Scourfield 1973, Bowling and Vaughn 2008). Xylem fibres formed in poplar tension wood develop thicker cell walls and contain more cellulose and less lignin than in normal wood (Timell 1969, Pilate et al. 2004, Bowling and Vaughn 2008). An important feature of poplar tension wood fibres is the production of a characteristic gelatinous layer, which is referred to as the G-layer and was shown to be composed of ~70% crystalline cellulose (Timell 1969, Pilate et al. 2004, Bowling and Vaughn 2008). Transcript profiling in poplar tension wood has identified molecular markers of tension wood formation, including several fasciclin-like arabinogalactan proteins (FLAs) (Lafarguette et al. 2004) and several carbohydrate-active enzymes (CAZy) (Aspeborg et al. 2005, Andersson-Gunneras et al. 2006, Geisler-Lee et al. 2006). Several transcripts encoding monolignol biosynthesis enzymes were down-regulated in tension wood of eucalyptus, including phenylalanine ammonia-lyase (PAL) and CCoAOMT (Paux et al. 2005), and in poplar (Andersson-Gunneras et al. 2006). These observations on transcriptional up-regulation and down-regulation are consistent with the decreased lignin and increased cellulose content that has been observed in tension wood.

Based on previously reported similarities between leaning-induced tension wood and wood formed under high nitrogen concentrations in poplar (Pitre et al. 2007a, 2007b), we compared xylem formed under these two environmental cues in *Populus trichocarpa × deltoides* saplings. The goal of this study was to further investigate to what extent wood formation under high nitrogen conditions is analogous to wood formation under tension. We tested the hypothesis that inducing tension wood in young trees by leaning and fertilizing young trees with high concentrations of nitrogen elicits similar modifications to cell wall formation. Accordingly, we conducted a factorial experiment to assess and compare the effects of these two factors, alone and in combination, on xylem morphology, chemical composition and gene expression profiles.

**Materials and methods**

**Plant material and treatments**

Rooted cuttings of *P. trichocarpa* (Torr. & Gray) × *P. deltoides* Bartr. ex Marsh. (clone H11-11) were produced and maintained in a greenhouse as described by Pitre et al. (2007a). Prior to the experiments, young trees were grown under natural light that was supplemented with full-spectrum lights (metal halogen lamps) to maintain an 18-h photoperiod and were fertilized weekly with soluble 20–20–20 NPK fertilizer. A complete randomized design was implemented with single tree as the experimental unit. The experiment used 42 trees that were selected to be similar in size and developmental stage, i.e. 1 m in height, at the onset of the experiment. Each tree was randomly assigned to one of the following four treatments (three trees per treatment for each time point): 1, low-N, vertical trees receiving 1 mM NH$_4$NO$_3$
(Vw); 2, high-N, vertical trees receiving 10 mM NH₄NO₃ (Fw); 3, low-N, leaning-induced tension wood receiving 1 mM NH₄NO₃ (Tw); 4, high-N, leaning-induced tension wood receiving 10 mM NH₄NO₃ (FTw). Nitrogen treatments were applied using a complete nutrient solution (Hocking 1971) in which NH₄NO₃ levels were adjusted to concentrations of 1 and 10 mM (respectively described as adequate and luxuriant levels of fertilization in Cooke et al. 2003 and Pitre et al. 2007a). Nitrogen treatments were applied daily for 28 days through watering to field capacity. Tension wood formation was induced by leaning the pots at 45° from the vertical (Figure 1A). The leaning trees were supported with a block installed under the pots, and the stems were maintained with bamboo stick to regulate growth at 45° (as described in Lafarguette et al. 2004). Differentiating xylem tissues were collected after 0, 1, 7 and 28 days of treatment. The developmental stage of the sampled stem sections was determined using the leaf plastochron index (LPI; Larson and Isebrands 1971). LPI₀ was defined as the first developing leaf that had at least a half-expanded lamina and was longer than 2 cm. As described by Pitre et al. (2007a, 2007b), all analyses were made on secondary xylem from stem samples harvested at LPI25 so that similar developmental stages were being compared. Secondary growth in this portion of the stem had commenced well before treatments were initiated, so that differences in stem characteristics would be due solely to differences in secondary xylem development. After removing the bark, differentiating secondary xylem tissues were scraped (2–3 mm in depth) from both the upper side (tension wood) and the lower side (opposite wood) of the leaning stems, as well as from corresponding positions on the vertical trees. Xylem samples were flash-frozen in liquid nitrogen and stored at −80 °C before use.

**Histological analysis**

For scanning electron microscopy (SEM), samples were prefixed overnight in formalin–acetic acid–alcohol solution, transferred to 0.1 M cacodylate buffer for 20 min, then over-fixed with 1% OsO₄ in 0.1 M cacodylate buffer. After 90 min, the tissues were washed in 0.1 M cacodylate buffer overnight at 4 °C. Dehydration was achieved by moving the samples though an ethanol series (30, 50, 70, 95 and 99%), with changes every 10 min. The dehydrated samples were dried 2 × 20 min in 100% hexamethyldisilazane, followed by 15 min of air-drying. The samples were deposited onto carbon-coated copper grids and SEM observations were made at 13 kV using a JEOL JSM 360LV microscope (JEOL, Tokyo, Japan).

**Wood fibre characterization**

Stem composition was determined for extract-free wood (EFW) from 28-day samples by Fourier transform infrared spectroscopy (FT-IR). Wood samples were dried and ground in a Wiley Mill to pass a 60-mesh sieve. EFW was obtained by exhaustively extracting wood samples in a Soxhlet apparatus (2:1 [v/v] toluene:ethanol, ethanol, hot water). An area of 50 μm × 50 μm in xylem fibres was selected for spectral analysis and five different areas were acquired per tree. Spectra were collected from the surface of each wood fibre sample using a Nexus FT-IR instrument (ThermoNicolet, Madison, WI) equipped with a continuum microscope accessory. The resulting interferograms were co-added to improve the signal-to-noise ratio of the spectrum. Spectra were then baseline-corrected and normalized, as described by Mouillle et al. (2003) and Robin et al. (2003). Given the resolution of the spectra, there were 1000+ possible absorbance peaks that could be registered in each scan. We confined chemical profiling of these scans to 14 bands where certain classes of components most strongly associated with the cell wall were known to absorb infrared radiation (Mouillle et al. 2003). Characterization of wood fibre dimensions (arithmetic mean fibre lengths (ln) and fibre widths (w)) was performed on samples from three trees per treatment (after 28 days) using a fibre quality analyser (OpTest Equipment, Hawkesbury, ON, Canada) as previously described by Pitre et al. (2007a). Brief-
ly, fresh wood scrapings were macerated for 48 h at 65 °C in Franklin’s solution (30% H2O2:glacial acetic acid, 1:1 v/v) to separate the individual fibres (Franklin 1945). Arithmetic mean fibre lengths (In) and fibre widths (w) were obtained by automated cytometric flow and detection under circular polarized light (fiber quality analyser; OpTest Equipment, Hawkesbury, ON, Canada). Statistical differences between the treatments were determined based on a two-way analysis of variance (ANOVA) using GLM performed in SAS, as described in the next section.

This experiment included two fixed factors (N and wood type), each having two levels (low N vs high N; leaning vs vertical) (Figure 1). Except where otherwise noted, the data were not transformed and each tree was considered an individual experimental unit. Before analysis, the normality of the data was confirmed, and ANOVAs were carried out using procGLM in SAS (Version 9.01, SAS Institute Inc., Cary, NC). For FT-IR data, ANOVAs were performed at each wave number. Except where noted, all ANOVAs followed this format.

Gene expression analyses

To follow transcript accumulation, stem segments were sampled at the base of the poplar trees (LPI25) after 0, 1, 7 and 28 days of fertilizer application. Total RNA was extracted from ~100 mg (Fw) of ground samples using the protocol described by Chang et al. (1993), modified according to Pavy et al. (2008). RNA integrity was checked with a Bioanalyzer 2001 using an RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA), and concentrations were determined by measuring absorbance at 260 and 280 nm. The 28-day samples were used in microarray analyses, whilst all time points were used for RT-qPCR.

Transcript accumulation by microarray analyses

A poplar 3.4 K cDNA microarray was used for analyses and described in Supplementary File 1. One microgram of RNA for each sample was amplified using the SuperScript™ Indirect RNA Amplification System (Invitrogen Canada, Burlington, ON) and 5 µg of aRNA were labelled with Alexa Fluor® 555 and 647 dyes (Invitrogen) for use in dye-swap experiments. Microarray slides were pre-hybridized (2 h at 42 °C) in a solution containing 5 × SSC, 0.1% SDS, 0.02% BSA (m/v), 0.01% herring sperm DNA (m/v) and 50% formamide. The slides were then washed twice in 0.1 × SSC, once in water, rinsed in 2-propanol and finally dried by centrifugation. The labelled targets (3.5 µl) were mixed with 52.5 µl of hybridization solution containing 5 × SSC, 0.1% SDS, 0.01% herring sperm DNA (m/v) and 50% deionized formamide. The mixture was heat-denatured for 4 min at 95 °C and cooled for 5 min on ice prior to hybridization with the microarray. The microarray was then covered with a LifterSlip (Erie Scientific Company, Portsmouth, NH) and placed in a hybridization chamber (Corning, Lowell, MA) and incubated (12 h at 45 °C) in a hybridization oven (Shel Lab, Cornelius, OR). After hybridization, the slides were iteratively washed for 15 min in 2 × SSC + 0.5% SDS, 0.5 × SSC + 0.1% SDS and 0.1 × SSC solutions at 45 °C.

Slides were scanned using a ScanArray™ Express scanner (Packard BioScience, Meriden, CT) and the image files were analysed using QuantiArray® software (Packard BioScience). Scan intensities were comparable between each set of slides for a given hybridization. Data analysis was carried out using Bioconductor packages (http://www.bioconductor.org) in R (R Development Core Team 2008). Data processing, quality assessment, normalization and statistical analyses were carried out as described by Pavy et al. (2008). Median foreground intensity minus median background intensity was used for the statistical analysis. Briefly, data quality was assessed using the marray and olin packages and by assessment of within- and between-slide Pearson correlation (r) coefficients calculated from both raw and normalized data. Background-corrected intensities were normalized using a composite method with the functions maNorm2D and maNormLoess in the marray package (Dudoit et al. 2002, Yang et al. 2002). We identified differentially expressed sequences with the Limma package (Release 2.0.7). P-values were adjusted for multiple testing by using the false discovery rate (FDR) approach of Benjamini and Hochberg (1995). Data reported in the article are log2 ratios of Alexa Fluor® 555/647 which are denoted as M. Genes were considered as differentially expressed based on P-value <10^-4 and a log2-fold difference (M) greater than ± 0.5. Similar statistical cut-off criteria (P-values and M) were previously shown to be robust, based on RT-qPCR validation (Pavy et al. 2007, 2008). Visualization of the data was done by hierarchical clustering using MeV, part of the TM4 suite (Saeed et al. 2003).

Analysis of transcript accumulation by RT-qPCR

Total RNA (2 µg) treated with DNase I (Sigma-Aldrich, Oakville, ON) was retro-transcribed (RT) using SuperScript™ II RNase H- and oligo(dT)12–18 (Invitrogen) in a 20-µl reaction according to the manufacturer’s instructions. Each resulting cDNA sample was diluted 1:10 in sterile ddH2O. Gene-specific primers were designed with the Primer3 software to generate amplicons with a calculated Tm of between 63 and 65 °C, and ranging between 100 and 200 bp in length. Quantitative PCR was performed with QuantiTect™ PCR SYBR® Green Kit (Qiagen, Mississauga, ON) in a final reaction volume of 15 µl. Each PCR reaction contained 1 × master mix, 2 µl of diluted cDNA and 0.3 µM each primer. Amplifications were carried out in a LightCycler® 480 System (Roche Diagnostics, Laval, QC). Initial denaturing time was 15 min at 95 °C, followed by 40 cycles consisting of 94 °C for 10 s and 62 °C for 2 min, with a single fluorescence measurement taken at the end of each cycle. A melting curve analysis was performed at the end of cycling to verify amplification specificity.

The number of molecules amplified in each sample was determined using the linear regression of efficiency (LRE) procedure (Rutledge and Stewart 2008), with slight modifications. Fluorescence background was removed from the raw
fluorescent reads prior to LRE analysis. Quantification was carried out according to Eq. (1):

\[
F_0 = \frac{E_{\text{max}}}{-\Delta E (1 + (E_{\text{max}} + 1)^{C_{1/2}})}
\]

where \(F_0\) is the initial target quantity expressed in fluorescence units, \(E_{\text{max}}\) is the maximal efficiency that occurs at the beginning of cycling, \(C_{1/2}\) is the reaction cycle located at the inﬂection point of the fluorescence curve where fluorescence is half of \(F_{\text{max}}\) (the maximal fluorescence reached at the plateau phase where the efficiency of the PCR reaction reaches 0), efficiency is half of \(E_{\text{max}}\) and \(\Delta E\) represents the rate of loss of efficiency. For each amplification reaction, \(\Delta E\) and \(E_{\text{max}}\) were determined using the LRE method (Rutledge and Stewart 2008) and \(C_{1/2}\) was calculated by taking the first derivative of the fluorescent readings. \(F_0\) was then transformed to molecules (\(N_0\)) with equations described in Rutledge and Stewart (2008). To facilitate data comparisons across treatments and time points, the data were normalized using several reference transcripts with the GeNorm procedure as described by Boyle et al. (2009). The statistical analyses were done as described previously in this report except that the number of RNA molecules calculated from the RT-qPCR was \(\log_2\) transformed.

Results

Comparing fertilization and tension effects on wood structure and composition

The short-term effects of tension induction by leaning and nitrogen fertilization on wood formation were studied in young (i.e. 1–2 m tall) \(P.\) trichocarpa × deltoides over a period of 28 days under controlled conditions.

The two treatment factors exerted contrasting effects on tree growth and wood formation (Table 1), as determined by two-way ANOVA. First, high N fertilization had a large impact on tree growth and morphology, consistent with previous experiments (Cooke et al. 2005, Pitre et al. 2007a). The trees were taller, had greener and larger leaves and produced more sylleptic branches than control individuals, regardless of whether the former were grown in a vertical or a leaning position (Figure 1A). Second, tension wood was clearly induced in the leaning trees, consistent with previous reports (for a review, see Pilate et al. 2004). The wood fibres were 9.1% longer on average in the leaning trees relative to the vertical controls (main effect of wood type: \(P = 0.005\)), considering both fertilization levels. Third, in contrast, the wood fibres were significantly shorter with high N fertilization (decreased by 7.2% on average relative to controls; main effect of fertilization: \(P = 0.020\)). Fourth, the largest response was to the treatment combining high N and tension induction by leaning, which resulted in a 12.3% increase in fibre length in tension-induced plants (Table 1), whilst mean fibre length of the tension-induced, high-N plants was most similar to the control plants. However, the interaction between the two factors was not significant (tension wood × fertilization: \(P = 0.233\)); therefore, the effects of the two factors could be considered additive (i.e. independent of one another). Furthermore, the two treatment factors were found not to have significant main or interaction effects on wood fibre diameter (\(P > 0.277\)).

Cell walls of fibres in the secondary xylem examined by SEM showed that leaning and high-N trees developed thicker cell walls compared with the control vertical trees (Figure 2). Elevated nitrogen fertilization moderately increased cell wall thickness compared with the controls (Figure 2A vs B), whilst tension induction produced highly thickened cell walls that were characteristic of reaction wood in angiosperms (Figure 2C; Pilate et al. 2004, Pitre et al. 2007a, 2007b). Moreover, the cell walls of xylem fibres in the leaning trees that had received either low or high N fertilization appeared to be identical, i.e. the lumen was nearly completely ﬁlled with cell wall materials that had wide lumens (Figure 2C), making them morphologically similar to the G-fibres of tension wood.

The composition of secondary xylem that differentiated over the 28 days of treatment was assessed by FT-IR microspectroscopy of the outer layer of the stem in order to target the forming wood (Figure 3; Table 2). FT-IR provides a sensitive means to rapidly assess the nature of major cell wall constituents (Mouille et al. 2003). It was applied to EFW primarily composed of xylem cell walls. Wave numbers that varied signiﬁcantly for at least one growth condition (relative to the control) were identiﬁed by two-way ANOVA (Table 3) and were mainly observed in two regions of the spectrum that are characteristic of cell wall compounds. Absorbance in the region between 1500 and 1200 cm\(^{-1}\) was generally lower in control trees than in the tension wood samples and in high-N trees. These wave numbers are associated with aromatic rings and phenolic compounds, which are presumed to represent lignins (Owen and Thomas 1989). This observation was consistent with the additive effects of high-N and tension wood, which both contributed to decreased lignin contents observed in our previous studies (Pitre et al. 2007b). Tension wood dis-

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**Table 1. Effects of nitrogen fertilization and wood type on fibre dimensions, as determined by FQA. Values are means ± standard deviations of three trees per treatment.**

<table>
<thead>
<tr>
<th>Wood type(^1) (degrees from vertical)</th>
<th>Nitrogen fertilization ((\text{NH}_3\text{NO}_4)) (mM)</th>
<th>Length(^2) (mm)</th>
<th>Width (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal 1</td>
<td>1</td>
<td>0.382 ± 0.01</td>
<td>0.020 ± 0.00</td>
</tr>
<tr>
<td>Tension (45°) 1</td>
<td>1</td>
<td>0.429 ± 0.02</td>
<td>0.021 ± 0.00</td>
</tr>
<tr>
<td>Normal 10</td>
<td>10</td>
<td>0.368 ± 0.01</td>
<td>0.022 ± 0.00</td>
</tr>
<tr>
<td>Tension (45°) 10</td>
<td>10</td>
<td>0.390 ± 0.03</td>
<td>0.021 ± 0.00</td>
</tr>
</tbody>
</table>

\(^{1}\)Normal wood was obtained from trees grown in the vertical position and collected from all sides of the stem; tension wood was obtained from trees grown in a leaning position (45° from vertical) and collected from the upper side of the stem only.

\(^{2}\)Arithmetic mean fibre length.
played the most altered composition, as reflected by decreased absorbance at specific wave numbers between 1550 and 1774 cm\(^{-1}\), relating to lignin content and structure. The 1774-cm\(^{-1}\) wave number, which is attributed to ester linkages in lignin, gave a higher absorbance in both high-N and tension wood. Higher absorbances were observed for wave numbers between 1100 and 830 cm\(^{-1}\) and attributed to increased cell wall polysaccharides (cellulose and hemicelluloses) in the high-N and leaning trees. Overall, the FT-IR spectra clearly indicated groups of wave numbers that responded either to one of the factors (N fertilization or tension induction) or to both, providing further evidence of additive effects when the two factors were combined.

**Transcript accumulation profiles and functional classifications**

Microarray RNA profiling provided an overview of transcriptome-level differences in the secondary xylem tissue near the base of the trees (LP125), after 28 days of treatment (see the ‘Materials and methods’ section and Supplementary Material 1). A total of 181 distinct sequences gave differential transcript accumulation levels that were statistically significant in at least one pair-wise comparison (Supplementary Table 2). The number and distribution of the differentially accumulated transcripts provided several indications of the overall effects of each treatment factor, either alone or in combination (Figure 4). First, the abundance of transcripts corresponding to several sequences varied within each individual treatment, such that differential transcript accumulation was observed for 57 sequences following the high N treatment, 81 sequences in the tension wood and 171 sequences in high N combined with tension wood induction. Second, about half of the sequences (89 out of 181) overlapped for two treatments that had one factor in common (i.e. either high-N or tension wood). Hierarchical clustering illustrated this overall consistency within the data that were obtained with the different treatments (Figure 4). Only 18 of the 181 sequences were differentially expressed in all three treatments (Figure 5; Table 3) and none overlapped uniquely between the tension wood and high nitrogen treatment. These observations suggested there is limited overlap between the responses to high-N and tension wood induction after 28 days of treatment. Third, >30% of the sequences that showed differential transcript abundance were unique to the treatment combining high-N and tension wood, suggesting that, in combination, the two factors trigger or enhance transcriptional responses that are not otherwise detected. Interestingly, most of the sequences that were unique to the combined treatment were weakly down-regulated (Figures 4 and 5).

We grouped the 181 differential transcripts into six major clusters according to their patterns of accumulation (Figure 7). Functional classification of the differentially accumulated transcripts within these clusters could be used to infer physiological or biochemical responses (Figure 6), in that about half (48%) of the sequences could be attributed to a physiological or biochemical function, according to the KOG classification. The classes that were represented the most were associated
with amino acid transport and metabolism (12 sequences), post-translational modification of proteins (12), carbohydrate transport and metabolism (8), transcription (6) and general functions (11). Sequences in the same KOG class generally clustered together (Figure 7). For example, clusters 2, 3 and 5 were largely composed of down-regulated transcripts involved in amino acid metabolism. Clusters 3 and 6 included sequences that were involved in carbohydrate metabolism.

Transcriptional markers of nitrogen response and tension wood formation

The putative functional assignments helped to identify markers of tension wood formation and high N availability amongst the differentially accumulated transcripts, which were consistent with previous reports (detailed below). Tension wood up-regulated sequences included a fasciclin-like arabinogalactan protein (FLA10) (Lafarguette et al. 2004), proline-rich cell wall proteins and pectate lyases (Andersson-Gunneras et al. 2003). Down-regulated transcripts included PAL2 and bHLH DNA binding proteins, such as those found by Andersson-Gunneras et al. (2006). CCoAOMT transcript abundance was decreased, in agreement with a report in eucalyptus (Paux et al. 2005). Each of these sequences was similarly differentially expressed in tension wood, regardless of N level, but differential expression did not occur in control wood with high N alone. All of the tension wood samples (including those with high N) also had a group of unexpectedly up-regulated sequences, which appear to be related to photosynthesis. These include genes encoding proteins of Photosystems I and II, chloroplast ribosomal RNAs or protein and cytochrome encoding sequence. This response likely represents a

Figure 3. FT-IR absorbance spectra of newly formed wood after 28 days of treatment. Each of the spectra represents the average from three trees. Values above the curves represent wave numbers that varied significantly between treatments (Table 2). Vw, vertical wood (control); Tw, tension wood; Fw, vertical wood with high nitrogen supply; FTw, tension wood with high nitrogen supply. This figure appears in color in the online version of Tree Physiology.

<table>
<thead>
<tr>
<th>Wave number (cm⁻¹)</th>
<th>Assigned chemical function¹</th>
<th>ANOVA summary (P-value)²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wood type</td>
</tr>
<tr>
<td>830</td>
<td>Polysaccharides/aromatic rings</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>841</td>
<td>Polysaccharides</td>
<td>0.318</td>
</tr>
<tr>
<td>922</td>
<td>Polysaccharides</td>
<td>0.395</td>
</tr>
<tr>
<td>1011</td>
<td>Polysaccharides</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1130</td>
<td>Polysaccharides</td>
<td>0.890</td>
</tr>
<tr>
<td>1230</td>
<td>Polysaccharides/aromatic rings</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1365</td>
<td>Polysaccharides/xylans</td>
<td>0.003</td>
</tr>
<tr>
<td>1466</td>
<td>Aromatic rings</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1500</td>
<td>Lignin/aromatic rings</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1516</td>
<td>Lignin/aromatic rings</td>
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<tr>
<td>1589</td>
<td>Lignin/aromatic rings</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1674</td>
<td>Lignin/aromatic rings</td>
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</tr>
<tr>
<td>1720</td>
<td>Lignin-ester linkages</td>
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<tr>
<td>1774</td>
<td>Lignin-ester linkages</td>
<td>0.005</td>
</tr>
</tbody>
</table>

¹Wave number assignment as described by Owen and Thomas (1989), Faix et al. (1994) and Mouille et al. (2003).
²Statistical differences were determined by two-way ANOVA.

Table 2. Variation in FT-IR absorbance and chemical functions assigned to wave numbers for tension wood spectral scans after 28 days. A two-way ANOVA was computed based on data obtained from three trees per treatment. P-values in bold are significant based on Benjamini and Hochberg’s multiple comparison procedure.

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metabolic reaction to increased light penetration on the upper side of the stem of leaning trees.

Reporter genes related to N availability were previously identified in poplar (Cooke et al. 2003) as well as Arabidopsis and tobacco (Fritz et al. 2006, Geiger et al. 1999, Scheible et al. 1997). Likely, N-related markers were up-regulated under high N, including a photosynthesis (Qb) protein, a glutathione S-transferase, vegetative storage proteins and an EST specific for the cambium (Table 4). The strongly induced BSPa (bark storage protein, also referred to as BSP1; Cooke and Weih 2005) is known to be a positive marker of high N availability in poplar (Coleman et al. 1991, 1998, Cooke et al. 2003). Several sequences encoding for amino acid and one-carbon metabolism enzymes, including serine hydroxymethyltransferase, glycine dehydrogenase and methionine synthase isoenzymes, showed differential transcript abundance in both treatments with high N, but not in tension wood alone.

Genes differentially expressed across all treatments

A set of 18 sequences showed significant differential transcript abundance in trees that had been subjected to each of the different treatments (Figure 4; Table 3). Eight sequences were down-regulated in the three treatments, whilst six sequences were systematically up-regulated compared with the vertical control trees. The remaining five sequences gave different profiles, depending on the treatment. Several of the sequences that were differentially expressed in all treatments were related to osmotic changes or water transport. The sequences coded for putative DNA-J, AP2/EREBP transcription factors, dehydrins, ripening-related proteins, LEA proteins and aquaporins.

Time course analysis of selected genes by RT-qPCR

A separate RT-qPCR analysis monitored transcript accumulation for selected genes at different time points over the course of the 28-day experiment (0, 1, 7 and 28 days) and expanded the samples analysed to include opposite sides of the stem of leaning trees. We targeted genes that were either co-ordinately differentially expressed amongst all treatments in the microarray results or represented putative gene markers related to N fertilization, tension wood formation or secondary cell wall formation (Pitre et al. 2007a, 2007b and this report). RT-qPCR results obtained with the 28-day time point RNA samples were consistent with the microarray results, thereby providing technical validation of the microarray data (Tables 3 and 4; Supplementary Table 4).

Statistical analyses of the data showed that sampling time significantly influenced most of the transcripts (13 out of 18 sequences) (Table 4; P ≤ 0.0062) and condition-specific patterns of transcript accumulation were observed at 1, 7 and 28 days of treatment (Figure 8; Supplementary Table 3). Statistically significant interactions between time and wood type (P ≤ 0.0070) and between time and N level (P = 0.0001) were observed for most genes, but not time interacting with both of the latter factors (Table 4). The large differentials ob-
served between tension wood and opposite wood highlight the strong effect of gravitropic imbalance on the transcriptome.

Together, the RT-qPCR data clearly showed that elevated N treatment and tension wood induction influence an overlapping set of genes, but their effects vary in magnitude and with time. For example, most of the genes that were up-regulated in tension wood showed higher levels of transcript abundance (larger differential) than the genes that were up-regulated by N availability. Many of the tension wood-induced genes showed transcript abundance that was detected after 1 day of treatment and varied as a function of time (time \( \times \) wood type interaction), whilst fewer of the genes affected by N availability showed transcript abundance that varied significantly across time (Supplementary Table 4). These observations suggest that microarray profiling over a time course may reveal many more overlapping transcripts than the single point in time that we used in this study.

Four of the genes are putatively involved in the cellular response to osmotic changes (AP2/EREBP, dehydrin, ripening-related proteins and late embryogenesis abundant (LEA) proteins). All were accumulated under high N, and all but one also accumulated in tension wood (Figure 8; Table 4). However, they varied in regard to rapidity of their response (1 vs 7 days) and their accumulation levels in opposite wood. Interestingly, two other transcripts that were not related to osmotic changes followed similar patterns (ARP1 and lateral organ boundary [LOB] domain). The transcripts encoding a LOB domain protein clearly accumulated in response to elevated N and their level in leaning trees was enhanced by elevated N (synergistic effect). Transcripts corresponding to ARP1 also accumulated under elevated N, but in contrast, they had a reduced response in the leaning trees under high N.

Amongst the genes encoding cell wall-related enzymes that we examined, differential transcript accumulation was observed in leaning trees, but the responses were variable with respect to N level. The transcripts corresponding to xyloglucan endotransglycosylase (XET) and FLA12 were rapidly down-regulated in tension wood (at Day 1) and FLA10 was strongly up-regulated after 7 days, but none were influenced by N level. Transcripts encoded by PAL1 and PAL2 were down-regulated in tension wood by Day 7; however, PAL1 was up-regulated by high N, whereas PAL2 was slightly down-regulated in tension wood.

**Discussion**

Direct comparison of young poplar trees subjected to high nitrogen fertilization and leaning to induce tension wood for-
Information showed that these two types of environmental factor had overlapping effects on wood structure and composition, as previously predicted (Pitre et al. 2007a, 2007b). The altered fibre morphology, increased cellulose and decreased lignin content are consistent with altered carbon partitioning to the secondary cell wall. We hypothesized that these two types of response could invoke common or shared physiological and molecular pathways but our gene expression data point to molecular pathways that are largely distinct with minor overlapping components.

Figure 5. Venn diagram illustrating the distribution of differentially expressed sequences identified by microarray hybridizations comparing vertical control trees compared with Tw, Fw or FTw. For each group of sequences, the number of up-regulated (+) or down-regulated (−) sequences is indicated relative to the control vertical trees. Vw, vertical wood (control); Tw, tension wood; Fw, vertical wood with high nitrogen supply; FTw, tension wood with high nitrogen supply. This figure appears in color in the online version of Tree Physiology.

Figure 6. Functional classification (KOG) of differentially accumulated transcript. This figure appears in color in the online version of Tree Physiology.
Physiological and molecular wood formation pathways affected by N fertilization and tension wood formation

The shared or overlapping properties of newly formed secondary xylem led us to ask whether common physiological or molecular pathways could be linked with responses to both high N fertilization and tension wood formation. A microarray transcript profiling experiment of trees subjected to high N fertilization and/or tension wood-inducing treatments for 28 days showed that transcript accumulation in the newly formed secondary xylem was influenced by each of the factors taken individually, and for a small set of transcripts, by both the high-N and leaning treatments. Interestingly, a large...
set of transcripts only accumulated differentially (mostly down-regulation) in the trees receiving a combined treatment of high N and leaning, suggesting that the two factors had additive effects (Figure 7). Results from the RT-qPCR analyses also indicated that additive effects on transcript accumulation were highly time dependent (Figure 8).

As evidence that core N metabolism was affected by high N availability, transcripts corresponding to *BSPa* were significantly increased, whilst transcripts corresponding to a cytosolic glutamine synthetase (*GS1*) decreased. This response is typical of high nitrogen fertilization, i.e. investment of N resources in storage protein accumulation and lower GS1 levels indicative of reduced protein turnover (Serapiglia et al. 2008). Amongst the differentially expressed genes to which we were able to assign a functional classification, the most abundantly represented classes were amino acid transport and metabolism, post-translational protein modifications and carbohydrate transport and metabolism, after transcription and other general functions.

The relative increase in transcripts related to photosynthesis in the tension wood of leaning trees (including those with high N) was likely a metabolic reaction to the increased light penetration reaching the xylem tissue (after 28 days growing at a 45° angle). Differentiating secondary xylem is not considered a photosynthetic tissue as such, but Du et al. (2006) showed that enhanced exposure to light during bark regeneration resulted in the accumulation of light-inducible proteins in the xylem. An intriguing observation was that transcripts corresponding to a LOB domain protein were up-regulated in the high-N trees and transiently up-regulated in the leaning high-N trees (tension wood and opposite wood at Day 1). Assuming that the gene product plays a role in lateral organ development in poplar, it is not surprising that its expression may be conditioned by a factor like high N availability, known to stimulate sylleptic branch growth (Cooke et al. 2005). Lateral organ development is also likely to be influenced by light intensity, by gravity and by apical dominance, which are altered in the xylem of leaning trees. Considering lateral branches in particular, their development requires a local reorganization of the vasculature (from a pre-existing leaf trace) to enable increased sap flow from the main stem into the nascent branch. Perhaps, LOB domain proteins could play a role in this reorganization.

Increased aboveground biomass accumulation was previously described in young poplar trees grown under very similar conditions to those used here (Cooke et al. 2005) and was observed in this report (not shown) and prior analyses of wood development (Pitre et al. 2007a, 2007b). It could be hypothesized that the increased load caused by the biomass accumulation could augment structural strain on the stem and increase the formation of tension wood. The study reported here did not test this hypothesis as such; therefore, further experimentation would be needed to draw any conclusions. The time course of transcript accumulation in response to high N was somewhat delayed relative to tension wood. This observation is consistent with an indirect effect such as increased biomass altering wood development; however, many of the differential transcripts were clearly detected by 7 days of high N fertilization (Figure 8; Supplementary Table 4), before aboveground biomass had accumulated significantly. Overall, the tension wood and fertilized wood had relatively reduced overlap in transcript abundance, which we were able to assign a functional classi-

### Table 4. ANOVA of the transcript accumulation results. The factors analysed were time point (1, 7 or 28 days), wood type (tension vs opposite) and N levels (low vs high).

<table>
<thead>
<tr>
<th>Sequence ID</th>
<th>Functional annotation</th>
<th>Time</th>
<th>Wood</th>
<th>N level</th>
<th>Wood × time</th>
<th>Wood × N</th>
<th>N × time</th>
<th>Wood × N × time</th>
</tr>
</thead>
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<td>Ferulate 5-hydroxylase</td>
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<td>0.0001</td>
<td>0.0537</td>
<td>0.0070</td>
<td>0.9035</td>
<td>0.6706</td>
<td>0.9213</td>
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<td>Dehydrin</td>
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<td>0.2723</td>
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<td>0.0001</td>
<td>0.0024</td>
<td>0.2817</td>
<td>0.6688</td>
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<tr>
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<td>0.0001</td>
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accumulation profiles (Figure 5; Table 3), suggesting that the high-N trees in fact formed relatively little tension wood. The lack of response of known markers of tension wood response like FLA10 (Lafarguette et al. 2004) and for transcripts such as XET (poplar XTH-9; Nishikubo et al. 2007) is consistent with this interpretation.

Based on our results, another hypothesis to consider is that the overlap between N fertilization and tension wood formation by leaning may involve transcripts related to salt stress and dehydration, which are more generally classified as osmotic stress-related. Transcripts of DNA-J, which encodes a homologue to HSP70 (Mayer and Bukau 2005), accumulated in response to both N and in leaning trees. In willows, DNA-J mRNA was shown to rapidly accumulate in response to NaCl stress, but its precise role has not been elucidated (Futamura et al. 1999). The accumulation of transcripts encoding LEA proteins and dehydrins was also observed. It is unclear whether and how these changes in transcript accumulation may be related to a putative dehydration response or to the altered cell wall formation; however, digital profiling in Pinus taeda L. showed that related transcripts accumulated in secondary xylem once it had become lignified (including several LEA proteins and aquaporins) and in compression wood (dehydrin) (Pavy et al. 2005). Evidence linking wood formation and osmotic stress was recently provided in a study showing that poplar wood anatomy was directly affected by salt stress (NaCl) (Escalante-Perez et al. 2009). A 2-week-long salt stress reduced transport of nutrients to the cambium, lowered shoot K+ content and affected cambium physiology such that the development of full-size vessels was impaired and the lumen diameter of xylem fibres was slightly decreased. It was also shown that bending and salinity stress stimulated the promoter activity of the auxin inactivation GH3 gene in xylem cells in poplar (Teichmann et al. 2008).
Taken together, this evidence, including our transcript accumulation data, form a starting point to test whether osmotic stress may take part in responses such as those described here.

Accelerated growth in the secondary xylem impacts cell wall structure

High N fertilization and the leaning treatments have well-characterized impacts on tree growth and development. Elevated N fertilization increases the growth rate of above-ground organs and tissues, affects overall tree morphology, enhances carbon assimilation and alters carbon allocation (Geiger et al. 1999, Cooke et al. 2005). The major changes observed in leaning trees are related to the gravitropic response by which the trees attempt to realign the growth axis to the vertical position, resulting in the formation of tension wood on the upper side of the stem (Pilate et al. 2004). In both cases, there is localized accelerated growth in the secondary xylem, i.e. the wood-forming tissues; however, these effects on wood formation have not previously been directly compared as such.

Compared with tension wood, relatively little is known about the wood properties of trees that have been fertilized with high N, although it appears that high-N trees grown in field conditions may have increased reaction wood formation based on their altered wood structure and composition. Luo et al. (2005) observed larger vessels in poplar xylem and described what appeared to be localized tension wood in the stems of field-coppiced poplars grown under elevated nitrogen fertilization. Our previous results (Pitre et al. 2007b) and the present study indicate that wood formed following high N fertilization is similar to tension wood with respect to enhanced fibre cell wall thickness and altered wood composition. Specifically, fibres produced under high-N conditions exhibit a substantially thickened cell wall layer, and high-N-treated secondary xylem contains increased cellulose and decreased lignin content. Our findings in the present study related to fibre length and gene expression, however, provide evidence that there is only partial overlap between the two responses.

Whilst there are strong similarities between the cell wall properties of fibres produced under high N fertilization and under tree leaning, there are also differences. For example, SEM carried out in the present study revealed that thicker cell walls developed in the fibres of newly formed wood in both high-N and leaning trees, compared with the vertical, normal-N trees, and that the morphologies of these thicker cell walls were similar. The observation of thicker cell walls under high-N conditions was also previously described by Pitre et al. (2007a, 2007b). However, we found that the increase in secondary cell wall thickness in the high-N-treated trees was not as significant as the typical G-layer observed in leaning trees (Timell 1969, 1986). It is well established that the G-layer of tension wood is enriched in pure cellulose and decreased in lignin content (Timell 1969, 1986). Overall chemical composition determined by FT-IR indicated marked similarities between wood generated under high N or tree leaning. The largest cell wall compositional changes that we observed by FT-IR were in two regions of the spectrum known to represent aromatic rings of phenolic compounds or lignins and cell wall polysaccharides, respectively. The data from our study strongly suggested that newly formed tension wood and xylem of high-N trees both accumulated less lignin, but more cellulose and hemicelluloses. These observations are consistent with our previous report in which we showed that high-N poplars had modified lignin (Pitre et al. 2007b) and increased cellulose (Pitre et al. 2007a). Together, these findings support our hypothesis that both lignin and cell wall carbohydrate metabolism were affected by nitrogen fertilization, as has already been established for tension wood. Gierlinger et al. (2008) also used FT-IR to compare the properties of tension-induced and normal wood. They observed changes in absorbance affecting wave numbers that were related to cellulose and hemicelluloses that were similar to those we observed in both tension wood and high-N trees. In contrast to tension wood, however, where fibre cell wall composition is altered only on one side of the tree, our previous studies show that high N alters cell wall composition around the entire circumference of the stem (Pitre et al. 2007a).

The results of the present study, taken together with results of our previously published studies (Pitre et al. 2007a, 2007b), suggest that the cell walls of fibres from high-N-treated trees could be analogous to the G-layer of tension wood fibres. However, the lesser degree of thickness suggests that this N-induced cell wall layer is not identical to a G-layer. Reduced microfibril angle is another characteristic of tension wood which could be used to further ascertain to what extent this high-N-induced thickened cell wall layer is similar to the G-layer of tension wood.

We also found that fibre length data (fibre quality analysis [FQA]) indicated that N fertilization and tree leaning may have contrasting effects on other aspects of fibre morphology such as fibre elongation. The high-N trees had shorter fibres, whereas the tension wood fibres were longer than the control. It is postulated that XETs are involved in breaking the bridges between xylloglucan and cellulose (reviewed in Mellerowicz et al. 2001); therefore, we could hypothesize that shifts in fibre length are, at least in part, mediated through the expression of XET enzymes. The report of Nishikubo et al. (2007) examined transcript profiles and protein localization of a large set of XTH16 genes and showed that most were up-regulated during tension wood formation with the exception of XTH16–34 and 35. The authors linked XET protein accumulation and G-layer formation. We found that XHT16-9 (not surveyed by Nishikubo et al. 2007) was down-regulated most strongly in tension wood and moderately affected in high-N trees, but did not survey other XTH16 transcripts. Further investigation is needed to evaluate whether and how XETs may play a role in the shifts in fibre lengths such as those reported here.

At the metabolic level, there is evidence that accumulation of phenylpropanoid compounds may be inversely proportion-
al to N availability or to the growth rate. For example, an N-depleted tobacco mutant accumulated several phenylpropanoid compounds and more lignin accumulation compared with the wild type (Fritz et al. 2006). Our findings that high N fertilization and assimilation led to reduced lignin content also suggested an inverse relationship. Harding et al. (2009) compared a fast-growing vs a slow-growing clone of poplar and showed that the fast-growing genotype accumulated significantly less lignin and more cellulose, consistent with what we observed with high N fertilization. Interestingly, the authors found that the fast-growing genotype had lower total nitrogen, suggesting that it may have higher N-use efficiency. Further links between N metabolism, rate of growth and cell wall formation were described by He et al. (2008) in cotton (Gossypium hirsutum L.) by comparing genotypes that differed in fibre strength. The genotypes that produced stronger fibres were also those with higher cytosolic glutamine synthetase (GS1) transcript levels and GS enzyme activity. These authors hypothesized that higher GS activity could increase ammonium incorporation into the developing seed, and thus, stimulate the rate of fibre growth; however, they did not find a correlation between GS enzyme activity and the accumulation of cellulose.

Several cell wall-related transcripts had similar down- or up-regulation in the differentiating tension wood and in the xylem of high-N trees, but they gave different accumulation profiles, responding more or less strongly to the treatments and at different points in time (Table 4 and see Supplementary Table 4). Transcript accumulation related to the induction of tension wood has been well characterized and markers like FLA10 and FLA12 have been identified (Lafarguette et al. 2004). Consistent with our observations, the transcripts that differentially accumulated in tension wood corresponded to genes that included a large proportion of CAZy, cell wall-related proteins and transcription factors, as previously shown (Andersson-Gunneras et al. 2006). These authors reported that changes in gene expression were consistent with decreased accumulation of sucrose and arabinose, as well as increased xylose and xylitol. We observed the down-regulation of transcripts linked to the formation of lignin S-unit precursors, namely, FSH and COMT, as was reported in Eucalyptus gunnii Hook (Paux et al. 2005). This behaviour is consistent with the shift in S/G that we have reported previously (Pitre et al. 2007b).

Conclusion

We investigated the extent to which the different factors (high N, tension wood formation) result in similar or overlapping secondary xylem phenotypes including thicker secondary cell walls. On one hand, growth of tree stems at 45° from vertical produced what is known as tension wood with highly thickened cell walls (with a characteristic G-layer) and longer fibres. On the other hand, high nitrogen fertilization induced the formation of wood with moderately thicker cell walls and with shorter fibres. Both wood types had increased cellulose and decreased lignin content. Transcript accumulation data were presented, indicating the pathways and mechanisms that may lead to altered growth and development. The results clearly suggested that the partly overlapping xylem phenotypes result from the modulation of largely distinct molecular pathways and different response intensities (level and speed of transcript accumulation) within the xylem tissue. A minor overlap was observed between the sets of differentially accumulating xylem transcripts; it included genes related to cell wall formation and to osmotic response. The additive effect of the combined treatment on transcript accumulation (mainly lowered transcript levels) may indicate that the shared osmotic response is amplified when the two factors are combined.

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Supplementary data

Supplementary data mentioned in the text are available to subscribers in Tree Physiology online.

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References


