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Gene expression patterns underlying changes in xylem structure and function in response to increased nitrogen availability in hybrid poplar

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ABSTRACT

Nitrogen availability has a strong influence on plant growth and development. In this study, we examined the effect of nitrogen availability on xylogenesis in hybrid poplar (*Populus trichocarpa* × *deltoides* H11-11). Saplings of hybrid poplar were fertilized for 33 d with either high or adequate levels of ammonium nitrate. We observed enhanced radial growth, wider vessels and fibres and thinner fibre walls in the secondary xylem of high N relative to adequate N plants. These anatomical differences translated into altered hydraulic properties with xylem being more transport efficient but also more vulnerable to drought-induced cavitation in high N plants. The changes in xylem structure and function were associated with differences in gene expression as revealed by the transcriptome analysis of the developing xylem region. We found 388 genes differentially expressed (fold change ±1.5, *P*-value ≤ 0.05), including a number of genes putatively involved in nitrogen and carbohydrate metabolism and various aspects of xylem cell differentiation. Several genes encoding known transcriptional regulators of secondary cell wall deposition were down-regulated in high N plants, corresponding with thinner secondary cell walls in these plants. The results of this study provide us with gene candidates potentially affecting xylem hydraulic and structural traits.

Key-words: phenotypic plasticity; transcriptome analysis; wood; xylogenesis.

INTRODUCTION

Wood (i.e. secondary xylem produced by cambial activity) of poplars represents an important raw material of great economic value (Balatinecz, Kretschmann & Leclercq 2001). Poplar biomass also provides a promising bioenergy feedstock that could help to reduce our dependency on fossil fuels (Sannigrahi, Ragauskas & Tuskan 2010). From a biological perspective, wood serves three main functions that are fundamental for plant growth. These functions are (1) long-distance transport of water and nutrients from roots to transpiring leaves, (2) providing mechanical support to the plant body and (3) storage of carbohydrates, water and various other specialized compounds. In a typical hardwood such as poplar, these three functions are divided between three different cell types – vessel elements, fibres and living parenchyma.

Vessel elements and fibres represent 85–90% (vol/vol) of mature wood in poplar (Mellerowicz *et al*. 2001). Hydraulic and mechanical properties of wood are closely associated with the physical structure of these cells. For instance, xylem hydraulic conductivity is proportional to the vessel diameter to the fourth power as predicted by the Hagen–Poiseuille equation (Tyree & Zimmermann 2002), and mechanical parameters such as modulus of rupture have been linked with wood density and fibre lumen diameters (Woodrum, Ewers & Telewski 2003; Pratt *et al*. 2007; Onoda, Richards & Westoby 2010). Moreover, hydraulic and mechanical functions appear to be closely integrated as strong mechanical support may be required to prevent implosion of xylem conduits under high xylem tension (Hacke *et al*. 2001; Jacobsen *et al*. 2005; Pittermann *et al*. 2006; Coleman *et al*. 2008). Furthermore, wood density and fibre length together with the chemical composition of wood are critical factors that determine its material properties and hence its suitability for a specific end use in the wood-processing industry.

Wood structure is established during xylogenesis as a result of cambial activity. Wood is formed through a series of precisely regulated developmental steps that include cell division, cell expansion, secondary cell wall deposition and programmed cell death (Samuels, Kaneda & Rensing 2006). Many genes influencing xylem differentiation in poplar have been recently identified, including several key regulatory and structural genes (e.g. Aspeborg *et al*. 2005; Groover *et al*. 2010; Zhong *et al*. 2011). The process of xylogenesis and the resulting xylem phenotype are strongly affected by environmental conditions such as water (Arend & Fromm 2007), nutrient (Lautner *et al*. 2007; Hacke *et al*. 2010) and

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light availability (Plavcová, Hacke & Sperry 2011). The developmental programme giving rise to a specific physiological and anatomical xylem phenotype is underpinned by changes in gene expression as demonstrated by recent studies describing transcriptional changes in developing xylem of poplars subjected to drought (Berta *et al*. 2010) and high salinity (Janz *et al*. 2012). However, more research is needed to better understand the molecular mechanisms underlying xylem phenotypic plasticity as it is likely that expression of different genes is altered by different environmental triggers.

In this study, we used nitrogen (N) fertilization to perturb the xylem phenotype of hybrid poplar (*Populus trichocarpa* × *deltoides*, clone H11-11) saplings and to investigate corresponding changes in gene expression. Nitrogen fertilization has a profound effect on poplar growth and development including xylogenesis (Harvey & van den Driessche 1999; Pitre, Cooke & Mackay 2007a; Pitre *et al*. 2007b; Hacke *et al*. 2010). The influence of nitrogen supply on the expression of selected genes has been evaluated in poplar leaves, roots, phloem and bulk xylem (Cooke *et al*. 2003; Cooke, Martin & Davis 2005; Ehlting *et al*. 2007; Hacke *et al*. 2010). To our knowledge, there is only one genome-wide study focused on the expression of nitrogen availability-related genes in the cambial region of poplar (Pitre *et al*. 2010), and this study was specifically designed to compare the effects of nitrogen fertilization and stem leaning on wood formation. In contrast, our study was designed to explore changes in gene expression that may underlie traits related to xylem water transport. Our goal was to identify candidate genes that may be linked with increased radial growth, wide vessel diameters and decreased wood density that we expected to be differentially expressed in poplars growing under high N availability.

MATERIALS AND METHODS

Plant material

Seedlings of hybrid poplar (*P. trichocarpa* × *deltoides* H11-11) were produced from rooted cuttings and maintained in a growth chamber under the following conditions: 16/8 h day/night cycle, 24/18 °C day/night temperature, c. 75% daytime relative humidity, photosynthetically active radiation of c. 400 μmol m^{-2} s^{-1}. During the initial phase of sapling establishment, plants were kept in 6 L pots filled with a standard gardening soil and fertilized once a week with 500 mL of N-P-K: 20-20-20 fertilizer (1 g L^{-1} dilution; Plant Products Co. Ltd., Brampton, Ontario, Canada). After 6 weeks, when the saplings were c. 70 cm in height, plants were randomly assigned to either adequate or high nitrogen (N) treatments. The fertilizer was applied every other day as either 0.75 mM or 7.5 mM NH_4NO_3 in 0.5 × Hocking's complete nutrient solution (Hocking 1971) for adequate and high N plants, respectively. To avoid drought stress, plants were irrigated with tap water on the days when the fertilizer was not applied. The fertilization treatment was applied for 33 d after which plants were harvested. The total amount of N added to each plant over the course of experimental treatments was 0.12 and 1.2 g for adequate N and high N plants, respectively. Final height and stem basal diameter (D_{stem}) of each sapling were assessed with a measuring tape and a caliper. Leaf area (A_L) was measured with an area meter (LI-3100, Li-Cor, Lincoln, NE, USA). Stem segments c. 25 cm in length were excised 5 cm above the root collar, placed in a dark plastic bag with a wet paper towel and stored at 4 °C until hydraulic measurements were conducted. The same stem segments were later used for anatomical measurements. For RNA extraction, developing xylem tissue was collected from 50 cm long stem segments distally adjacent to the segments used for hydraulic and anatomical measurements. In both treatments, the secondary xylem was well developed and no obvious differences in stem maturation were apparent in this region of the stem. First, the bark was peeled from the stem and discarded. To obtain the tissue for the microarray analysis, the developing secondary xylem tissue was scraped from the exposed surface of the woody core using a fresh razor blade until resistance could be felt and the colour of the scraping changed, indicating that fully mature xylem cells were reached. The scraped tissue was immediately frozen in liquid nitrogen and stored at –80 °C until used for RNA extraction. Careful preliminary observations using a light microscope revealed that the bark separates from the stem in the cambial region. Thus, the tissue scraped from the outermost layer of the exposed xylem contained newly formed expanding xylem cells and cells that underwent secondary wall thickening. Although this sampling method did not allow sampling-specific developmental stages, it has been widely used to analyse transcript and protein levels in developing secondary xylem (Gray-Mitsumune *et al*. 2004; Berta *et al*. 2010; Song *et al*. 2011).

Xylem anatomy

Xylem anatomy was analysed in stem segments sampled at a height of 10–20 cm above the root collar using light microscopy as described previously (Plavcová *et al*. 2011). Five or six individual stems were measured for each treatment. The cross-sectional surface of stems was photographed with a digital camera attached to a stereomicroscope (MS5; Leica Microsystems, Wetzlar, Germany) at 10–16× magnification. Xylem cross-sectional area (A_X), excluding pith and bark, was measured with image analysis software (ImagePro Plus version 6.1, Media Cybernetics, Silver Spring, MD, USA). Vessel lumen diameters were measured in two radial sectors on stem cross sections prepared with a sliding microtome and stained with toluidine blue. Between 300 and 400 vessels observed at 200× magnification were measured from each stem. Since fibres are much narrower than vessels, resin-embedded samples and 400× magnification were used to produce reliable measurements of fibre lumen diameters. 200–300 fibres from at least three different randomly selected areas were measured for each stem. In addition, measurements of fibre double wall

thickness were conducted on at least 80 fibre pairs. Only fibres that did not have a conspicuous gelatinous layer were selected for measurements of lumen diameters and double wall thickness. Vessel element and fibre length measurements were conducted on macerated xylem tissue. At least 100 individual cells were photographed under 100× magnification and measured for each stem. Vessel length was assessed using the silicone injection method (Sperry, Hacke & Wheeler 2005). Finally, wood density was measured by the water displacement method. Debarked stem segments c. 2 cm in length were longitudinally split with a razor blade and the pith was removed. Samples were submersed in a beaker of water on a balance to measure the wood fresh volume. Samples were then oven-dried at 70 °C for 48 h and weighted. Wood density was expressed as dry weight per fresh volume. For statistical analysis, an independent two-sample *t*-test was used to compare the differences in means between the treatments. Prior to the analysis, normality and homogeneity of variances were graphically checked.

Hydraulic measurements

Stem hydraulic conductivity and vulnerability to cavitation were measured following the standard methodology described in detail in Hacke *et al*. (2010). Stem segments 14.2 cm in length were flushed with standard measuring solution (20 mM KCl +1 mM $CaCl_2$ filtered at 0.2 μm) for 20 min at 50 kPa. Subsequently, the gravity-driven flow through the segments was recorded using an electronic balance (CP225, Sartorius, Göttingen, Germany) interfaced with a computer. The value of maximal conductivity was normalized by xylem cross-sectional area (A_X) to calculate the xylem-specific hydraulic conductivity (K_S). After measuring maximal conductivity, stem segments were fixed into a custom-built centrifuge rotor and spun to progressively more negative pressures. Hydraulic conductivity was measured after each pressure increment. The percentage loss of hydraulic conductivity was plotted against the corresponding xylem pressure to generate vulnerability curves. Data points were fitted with a Weibull function and the xylem pressure corresponding to 50% loss of conductivity (P_{50}) was calculated for each segment.

Microarray analysis

Total RNA was extracted from six individual poplar saplings per treatment using the hexadecyltrimethylammonium bromide (CTAB) extraction protocol of Chang, Puryear & Cairney (1993). Total RNA quality for each individual sample was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) prior to microarray analysis. Samples with an RNA integrity number value of greater than or equal to 8.0 were deemed to be acceptable for microarray analysis. Samples were prepared following NimbleGen's Arrays User Guide (Gene Expression Analysis version 3.2; Roche NimbleGen Inc., Madison, WI, USA). 10 μg of each total RNA was converted to double-stranded (ds) cDNA with the Invitrogen SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA), 1 μg of each ds cDNA was fluorescently labelled using the NimbleGen One-Color DNA Labeling Kit, and 4 μg of each Cy3-labelled sample was hybridized on Roche NimbleGen poplar gene expression microarrays (Design Name Populus 135K EXP HX12 090828). These arrays target 55 794 gene models predicted in the *P. trichocarpa* genome with each gene model represented by three unique 60-mer probes. Arrays were scanned at 5 μm resolution with a Molecular Devices GenePix 4200AL scanner (Molecular Devices LLC, Sunnyvale, CA, USA). NimbleScan version 2.5 was used for quantitation and robust multichip average (RMA) normalization of data which included quantile normalization and background subtraction. Agilent's GeneSpring 7.3.1 was used to analyse the normalized data. To find significantly differentially regulated genes, fold changes between the compared groups and *P*-values gained from *t*-test between the same groups were calculated. The *P*-values were further corrected for multiple testing (MTC) using the method of Benjamini & Hochberg (1995). The *t*-tests were performed on normalized data that had been log transformed and the variances were not assumed to be equal between sample groups. The genes with MTC *P*-value ≤ 0.05 and fold change ± 1.5 were considered to be significantly differentially regulated. The robustness of the selected cut-off criteria was validated with quantitative real-time PCR (qRT-PCR) analysis. The gene model names and annotation is based on the Phytozome v2.2 version of the *Populus* genome (ftp://ftp.jgi-psf.org/pub/JGI_data/phytozome/v7.0/Ptrichocarpa/annotation/).

Microarray qRT-PCR validation

One microgram of total RNA was treated with DNase I (Invitrogen) and used for first strand cDNA synthesis using oligo(dT)23VN (IDT, Coralville, IA, USA) and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocols. Four potential reference genes were identified by screening the microarray data for cDNAs whose signals remained apparently unchanged (fold difference ratios between 0.97 and 1.05). Eight candidate genes were also selected in the microarray and included in the validation assay. Gene-specific qRT-PCR primers were designed mainly in the 3′ untranslated (UTR) region using Primer Express v3 (Applied Biosystems, Foster City, CA, USA; Supporting Information Table S1). For each gene, PCR efficiency (E) was determined from a four-point cDNA serial dilution, according to $E = 10^{[-1/\text{slope}]}$. The stability of the gene expression profile of the four potential reference genes was evaluated in three biological replicates for each of the two fertilization treatments. The two most stable reference genes were selected for the qRT-PCR assay [*protein phosphatase 2A* (*PP2A*), *POPTR_0010s13760*; and *yellow-leaf-specific gene 8* (*YLS8*), *POPTR_0007s07660*]. RT-PCR was performed on a 7900 HT Fast Real-Time PCR system (Applied Biosystems). Assays were carried out in 384-well plates. Three biological replicates, each with three technical replicates, were assayed for high N and adequate

N nitrogen treatments. A negative control (no cDNA template) was included for every gene. PCR was carried out in a final volume of 10 μL including a final concentration of 20 ng of cDNA, 0.4 μM of each primer (IDT), 1× master mix containing 0.2 mM dNTPs, 0.3 U Platinum Taq polymerase (Invitrogen), 0.25× SYBR Green and 0.1× ROX. PCR conditions were as previously described (Almeida-Rodriguez *et al*. 2010). Samples were subjected to auto Ct (cycle threshold) for analysis, and dissociation curves were verified for each of the genes. Changes in gene expression of nine target genes (including *YLS8*) were calculated according to Pfaffl (2001), relative to the reference gene *PP2A*.

RESULTS AND DISCUSSION

Nitrogen availability affects xylem structure and function in hybrid poplar

We exposed clonally propagated hybrid poplar ramets (*P. trichocarpa* × *deltoides*, clone H11-11) to either adequate or high levels of ammonium nitrate. As expected, nitrogen fertilization enhanced growth of poplar saplings in both height and girth (Table 1). High N availability influenced not only the amount but also the structure of the secondary xylem produced. Substantial differences in xylem cell dimensions and wall reinforcement were identified when adequate versus high N plants were compared (Table 2, Fig. 1). More specifically, vessel and fibre lumens were significantly wider (Fig. 2) and the average vessel element length was higher in high N plants (Table 2). In contrast, the mean vessel length was not significantly different (Table 2). However, like vessel element length, a slight tendency towards longer vessels was identified in high N plants (Supporting Information Fig. S1b), suggesting that there may be a subtle effect. Similarly, the average fibre length did not significantly differ between the treatments (Table 2); however, a decrease in the proportion of fibres longer than 700 μm was apparent in high N plants (Supporting Information Fig. S1c). Lower fibre length in high N-treated plants has been previously reported as one of the hallmarks of nitrogen fertilization in this hybrid poplar genotype (Pitre *et al*. 2007a, 2010). These studies have also reported increased occurrence of fibres with thick secondary cell walls resembling a gelatinous layer typical for the reaction wood in high N-treated plants of this genotype. While we noticed the gelatinous fibres in our plant material as well, we found such layers in plants treated with both high N and adequate N. Furthermore, the gelatinous fibres were found only in certain regions of cross sections usually forming a distinct band around the stem. In regions where fibres lacked the gelatinous layer, the regular secondary cell wall was thinner in high N in comparison with adequate N plants (Fig. 1, Table 2). In agreement with these results, lower secondary cell wall thickness has been reported in three poplar species subjected to nitrogen fertilization (Luo *et al*. 2005). Thus, while nitrogen fertilization may stimulate gelatinous layer production (Pitre *et al*. 2007a, 2010), it also negatively affects the deposition of the regular secondary cell wall. Increased vessel and fibre lumen diameters and lower wall reinforcement in fibres translated into lower wood density in high N plants (Table 2).

The changes in xylem structure were paralleled by differences in hydraulic properties, which have important

Table 1. Growth characteristics of poplar saplings receiving 0.75 mM (adequate N) versus 7.5 mM (high N) levels of ammonium nitrate

	Adequate N	High N
Height (cm)	160 ± 2.3	171.3 ± 2.2**
D_{stem} (mm)	8.9 ± 0.2	9.5 ± 0.1*
A_X (mm^2)	40.3 ± 1.3	51.3 ± 1.8**
A_L (m^2)	0.63 ± 0.03	0.99 ± 0.03**

Parameters shown are final height of saplings (height), stem diameter at 10 cm above the root collar (D_{stem}), xylem cross-sectional area at ~10 cm above the root collar (A_X) and plant total leaf area (A_L). Mean ± SE ($n = 8$). Results of independent two-sample *t*-tests, testing for differences between adequate and high N plants, are indicated (*$P \leq 0.05$, **$P \leq 0.01$).

Table 2. Xylem characteristics of poplar saplings receiving 0.75 mM (adequate N) versus 7.5 mM (high N) levels of ammonium nitrate

	Adequate N	High N
Vessel lumen D (μm)	38.9 ± 0.2	42.6 ± 0.7**
Vessel element length (μm)	234 ± 2	255 ± 4**
Vessel length (cm)	5.2 ± 0.2	5.8 ± 0.2 ns
Fibre lumen D (μm)	9.5 ± 0.4	11.2 ± 0.5*
Fibre length (μm)	579 ± 9	561 ± 7 ns
Fibre double wall thickness (μm)	3.3 ± 0.1	2.7 ± 0.2*
Wood density (g cm^{-3})	0.37 ± 0.01	0.31 ± 0.01**

Mean ± SE ($n = 5$–6). Results of independent two-sample *t*-tests, testing for differences between adequate and high N plants, are indicated (*$P \leq 0.05$; **$P \leq 0.01$; ns, non-significant).

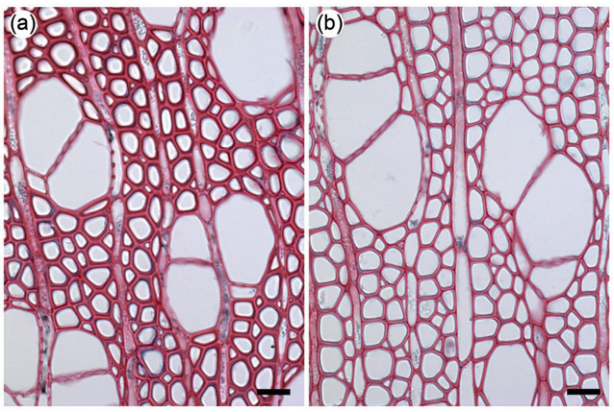

Figure 1. Cross sections of stem xylem from (a) adequate N and (b) high N plants. High N plants had wider fibres and vessels and thinner fibre walls than adequate N plants. Scale bar = 20 μm.

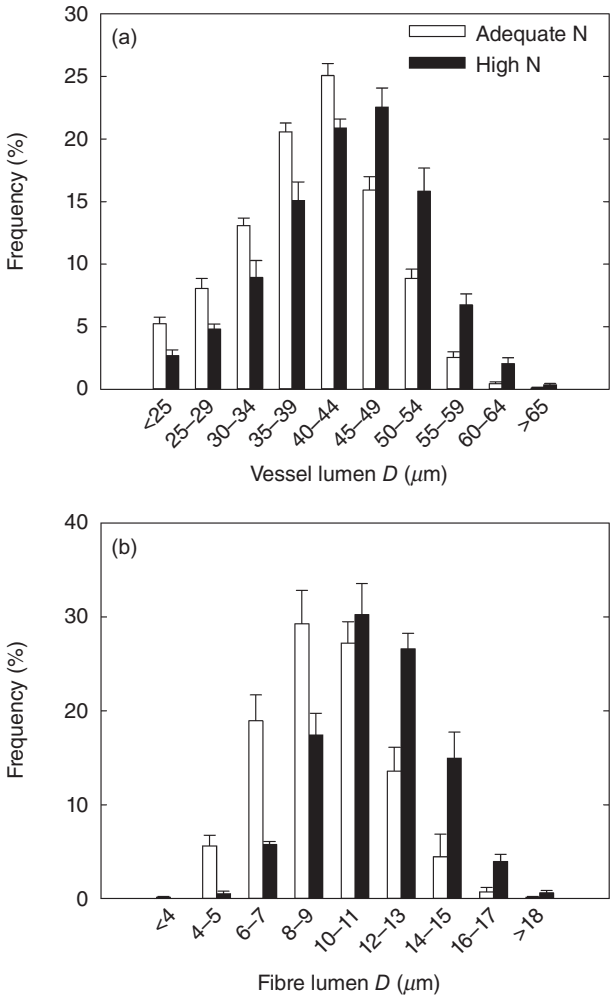

Figure 2. Frequency histograms of (a) vessel and (b) fibre lumen diameters for adequate N (open bars) and high N (black bars) plants. Means and SE ($n = 5$–6).

implications for plant water use. High N plants with larger vessel diameters transported water more efficiently, as measured by average xylem area-specific conductivity (Fig. 3a), than adequate N plants with narrower vessel diameters. More efficient water transport is correlated with the necessity to sustain the larger areas of transpiring leaves in high N-treated plants (Table 1). However, high N plants were more vulnerable to cavitation than adequate N plants, as shown by the less-negative P_{50} values in high N plants (Fig. 3b). Thus, although more efficient, water transport in high N plants was more prone to dysfunction under drought conditions. This finding is in agreement with previous work (Harvey & van den Driessche 1999; Hacke *et al*. 2010).

Nitrogen availability evokes transcriptional changes in developing xylem

To investigate the molecular basis for the developmental changes leading to the xylem phenotypes described above, we carried out gene expression profiling of developing xylem isolated from high N- and adequate N-treated plants. RNA was isolated from developing secondary xylem, exposed by peeling bark from stem segments adjacent to segments used for hydraulic and anatomical measurements, and hybridized to NimbleGen poplar full genome microarrays. Six biological replicates per treatment were analysed. Expression of 49 476 gene models was detected; of those 388 non-redundant genes showed statistically significant ($P \leq 0.05$) 1.5-fold or greater changes in transcript abundance between the treatments. Out of these genes, 243 were up- and 145 down-regulated (Supporting Information Table S2). We used quantitative qRT-PCR to independently assay expression of nine selected genes, which confirmed the reliability of the microarray analysis (Supporting Information Fig. S2).

Microarray results suggest that extensive remodelling of the poplar transcriptome accompanies changes in xylem development and secondary cell wall deposition associated with increased nitrogen availability. The potential functional roles played by differentially regulated genes in xylem development were further analysed based on the

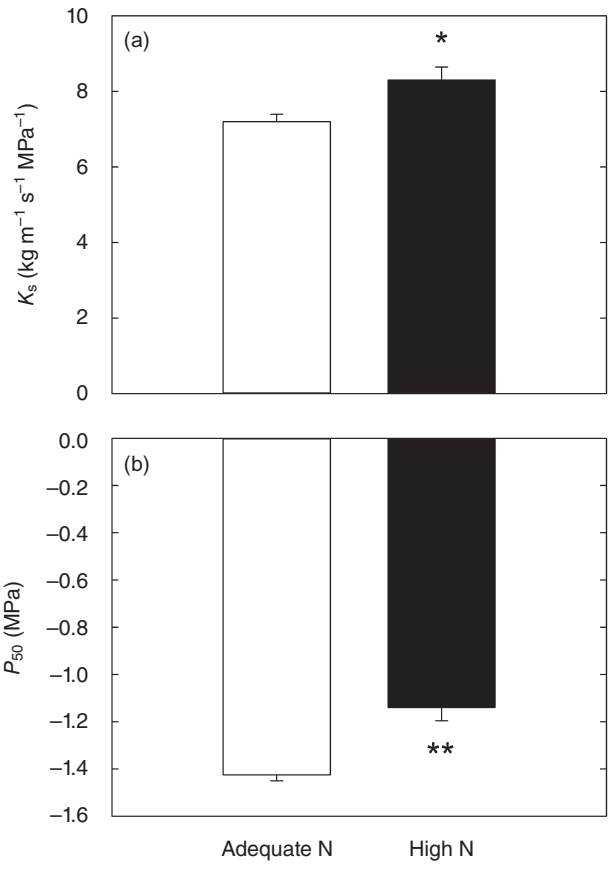

Figure 3. Average (a) xylem area-specific conductivity (K_S) and (b) xylem pressure causing 50% loss of conductivity (P_{50}) of stem segments from adequate N (open bars) and high N (black bars) plants. Error bars are SE ($n = 8$). * indicates the means are significantly different at $P \leq 0.05$; ** indicates significant differences at $P \leq 0.01$ (independent two-sample *t*-test).

annotations of the differentially regulated poplar genes or their presumed *Arabidopsis* orthologs. Genes of particular interest that might be associated with various aspects of stem growth and development including xylem and secondary wall development are presented in Table 3 and discussed below.

Nitrogen metabolism

Several poplar genes with a putative function as amino acid transporters were differentially regulated in this study. Amino acids and amides represent the principal long-distance transport form of organic nitrogen. They are abundant in phloem and xylem sap of woody plants (Sauter & Van Cleve 1992; Weber *et al*. 1998) from which they can be translocated radially into the cambial zone where they are required for protein and lignin biosynthesis. Among the differentially regulated genes encoding amino acid transporters were an uncharacterized amino acid transporter, a homolog of *Arabidopsis* amino acid exporter *GLUTAMINE DUMPER 3* (*GDU3*) and a homolog of *BIDIRECTIONAL AMINO ACID TRANSPORTER 1* (*BAT1*). The importance of *GDU3* and *BAT1* genes in vascular tissue physiology is reinforced by the fact that they are highly expressed in the *Arabidopsis* vasculature (Dundar 2009; Pratelli *et al*. 2010). Two other amino acid transporter genes, namely genes homologous to *Arabidopsis LYSINE HISTIDINE TRANSPORTER 1* (*LHT1*) and *AMINO ACID PERMEASE 3* (*AAP3*), were down-regulated in high N plants. The specific role of the differentially regulated transporters cannot be elucidated from this study; nonetheles, these genes represent interesting candidates for future research on xylogenesis and nitrogen cycling in poplar. Increased expression during secondary xylem formation in poplar has been already demonstrated for the *GDU3* and *AAP3* homologs (Dharmawardhana, Brunner & Strauss 2010).

Furthermore, we found several genes involved in organic acid metabolism up-regulated in high N plants. Organic acids such as malate, citrate and α-oxoglutarate are required as carbon skeletons for amino acid synthesis. The genes up-regulated in this study included two genes encoding glucose-6-phosphate dehydrogenase of the pentose phosphate pathway, and several other enzymes (phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxykinase, alanin aminotransferase and NADP-malic enzyme) related to organic acid metabolism. In agreement with our findings, an increased nitrate supply has been previously shown to induce genes involved in the synthesis of organic acids in *Arabidopsis* (Scheible *et al*. 1997; Stitt 1999). The observed patterns in gene expression suggest that due to an increased need for nitrogen assimilation and amino acid synthesis, relatively more carbon is channelled towards the synthesis of organic acids in the high N plants relative to controls. Organic acid metabolism hence represents an important intersection between nitrogen and carbon metabolism.

Carbohydrate metabolism

The microarray analysis revealed extensive changes in the transcription of genes involved in carbohydrate metabolism. Sucrose synthase (*Pt-SUS2.2*) and plant-neutral invertase genes were up-regulated in high N plants. These enzymes break down sucrose into fructose and glucose monomers (Koch 2004) and are important in carbon partitioning into cellulose biosynthesis during secondary wall develoment (Hauch & Magel 1998; Coleman, Yan & Mansfield 2009). Furthermore, a gene encoding hexokinase (*Pt-HXK1.1*), an enzyme required for activation of non-phosphorylated sugar monomers, was up-regulated. Thus, these three enzymes could act together to provide free phosphorylated sugar monomers for the biosynthesis of cellulose and/or hemicellulose. Enhanced expression of enzymes involved in sucrose metabolism is expected in fast-growing high N plants (Table 1) considering that the developing xylem acts as a major carbon sink and carbohydrates for the production of new cells are delivered from photosynthetic source organs via the phloem mainly in the form of sucrose.

Three trehalose-phosphatase genes showed increased expression in high N plants. Up-regulation of genes involved in trehalose metabolism in response to increased nitrate availability was previously reported in *Arabidopsis* (Wang *et al*. 2003; Scheible *et al*. 2004), and it has been suggested that trehalose could act as a regulator of the pentose phosphate pathway (Wang *et al*. 2003). Simple sugars such as trehalose or glucose represent effective signalling molecules, and a pivotal role of sugar-mediated signalling during many developmental processes has been recognized (Rolland, Baena-Gonzalez & Sheen 2006). To our knowledge, the potential role of sugar sensing and signalling during xylogenesis has not yet been investigated. Nevertheless, it might provide a promising avenue for future research considering the complex dynamics of sugars (Schrader & Sauter 2002), hormones (Tuominen *et al*. 1997) and transcription factors (Du & Groover 2010) and their interplay that occurs in the cambial zone of trees.

The poplar genes potentially involved in metabolism of cell wall polysaccharides have been previously studied (Aspeborg *et al*. 2005); however, the specific functions of many of them still remain elusive. Only few genes encoding polysaccharide synthases and glycosyl transferases, enzymes responsible for cell wall polysaccharide synthesis, were differentially expressed in our dataset. Among them, two closely related cellulose synthase-like D genes (*Pt-ATCSLD5.1*, *Pt-ATCSLD5.2*) both showed more than twofold up-regulation in high N plants. These genes are likely involved in the biosynthesis of xylan (Aspeborg *et al*. 2005; Bernal *et al*. 2007), which represents a hemicellulosic polysaccharide that is abundantly present in poplar wood. Furthermore, two putative UDP-glucosyl transferase genes and a homolog to an *Arabidopsis* gene encoding plant glycogenin-like starch initiation protein 1 (*PGSIP1*) were down-regulated in high N plants. The protein encoded by poplar *PGSIP1* may have a priming function for cell wall

Table 3. Genes differentially expressed in the developing xylem of hybrid poplar growing under high N versus adequate N availability. The poplar gene names indicated in the brackets were obtained from (1) Phytozome (ftp://ftp.jgi-psf.org/pub/JGI_data/phytozome/v7.0/Ptrichocarpa/annotation/), (2) Zhong *et al.* (2011), (3) Gupta & Sankararamakrishnan (2009). Fold change values represent a ratio between the normalized averaged values of high N relative to adequate N plants. Multiple testing corrections adjusted *P*-values of *t*-tests comparing adequate and high N plants are indicated

Populus trichocarpa Phytozome v2.0 gene name	*Arabidopsis thaliana* homologous gene	TAIR description	Fold change	*P*-value
Amino acid transport				
POPTR_0008s03620	*At2g39130*	Transmembrane amino acid transporter family protein	4.75	0.014
POPTR_0006s18790	*At5g57685 (GDU3)*	Glutamine dumper 3	2.35	0.021
POPTR_0008s12400	*At2g01170 (BAT1)*	Bidirectional amino acid transporter 1	1.69	0.031
POPTR_0001s36330 (Pt-LHT1.2)[1]	*At5g40780 (LHT1)*	Lysine histidine transporter 1	–1.87	0.037
POPTR_0002s07960 (PtrAAP5)[1]	*At1g77380 (AAP3)*	Amino acid permease 3	–3.10	0.041
Organic acid metabolism				
POPTR_0008s11330	*At1g68750 (PPC4)*	Phosphoenolpyruvate carboxylase 4	2.52	0.015
POPTR_0001s13510 (Pt-G6PD.2)[1]	*At5g13110 (G6PD2)*	Glucose-6-phosphate dehydrogenase 2	2.51	0.032
POPTR_0001s16300 (Pt-ALAAT1.1)[1]	*At1g72330 (ALAAT2)*	Alanine aminotransferase 2	1.82	0.037
POPTR_0013s00660	*At1g09420 (G6PD4)*	Glucose-6-phosphate dehydrogenase 4	1.69	0.028
POPTR_0007s14250	*At4g37870 (PCK1)*	Phosphoenolpyruvate carboxykinase 1	1.68	0.047
POPTR_0006s25280	*At5g25880 (NADP-ME3)*	NADP-malic enzyme 3	1.57	0.032
Carbohydrate metabolism				
POPTR_0006s06460	*At5g20250 (DIN10)*	Raffinose synthase family protein	5.21	0.018
POPTR_0011s00480	*At1g11580 (PMEPCRA)*	Methylesterase PCR A	3.84	0.017
POPTR_0007s05670	*At5g65140*	Haloacid dehalogenase-like hydrolase (HAD) superfamily protein/trehalose-phosphatase family protein	3.42	0.029
POPTR_0014s12000 (Pt-ATCSLD5.1)[1]	*At1g02730 (CSLD5)*	Cellulose synthase-like D5	2.88	0.015
POPTR_0002s21700	*At2g05790*	O-Glycosyl hydrolases family 17 protein	2.74	0.030
POPTR_0002s20130 (Pt-ATCSLD5.2)[1]	*At1g02730 (CSLD5)*	Cellulose synthase-like D5	2.46	0.045
POPTR_0009s01210 (Pt-XTR8.1)[1]	*At2g36870 (XTH32)*	Xyloglucan endotransglucosylase/hydrolase 32	2.46	0.023
POPTR_0008s20870	*At5g18670 (BMY3)*	Beta-amylase 3	2.42	0.029
POPTR_0008s13200 (Pt-CEL1.3)[1]	*At1g70710 (GH9B1)*	Glycosyl hydrolase 9B1	2.07	0.042
POPTR_0008s09380 (Pt-HIUHASE.1)[1]	*At1g26560 (BGLU40)*	Beta glucosidase 40	2.06	0.023
POPTR_0003s07040 (Pt-PE3.4)[1]	*At3g14310 (PME3)*	Pectin methylesterase 3	2.05	0.033
POPTR_0004s03830	*At5g08370 (AGAL2)*	Alpha-galactosidase 2	2.01	0.019
POPTR_0006s08030	*At5g58090*	O-Glycosyl hydrolases family 17 protein	1.99	0.023
POPTR_0010s13560 (Pt-XYL1.1)[1]	*At1g68560 (XYL1)*	Alpha-xylosidase 1	1.98	0.011
POPTR_0011s09660	*At5g55180*	O-Glycosyl hydrolases family 17 protein	1.94	0.048
POPTR_0019s09740	*At1g71380 (CEL3)*	Cellulase 3	1.86	0.032
POPTR_0002s20340 (Pt-SUS2.2)[1]	*At4g02280 (SUS3)*	Sucrose synthase 3	1.79	0.049
POPTR_0010s26170	*At5g08380 (AGAL1)*	Alpha-galactosidase 1	1.75	0.046
POPTR_0002s09450	*At1g78060*	Glycosyl hydrolase family protein	1.73	0.022
POPTR_0008s10090	*At3g06500*	Plant neutral invertase family protein	1.69	0.043
POPTR_0018s09560 (Pt-HXK1.1)[1]	*At4g29130 (HXK1)*	Hexokinase 1	1.69	0.014
POPTR_0018s14730	*At5g58090*	O-Glycosyl hydrolases family 17 protein	1.65	0.041
POPTR_0013s05620	*At1g64760*	O-Glycosyl hydrolases family 17 protein	1.61	0.021
POPTR_0010s11510	*At1g68020 (ATTPS6)*	UDP-Glycosyltransferase/trehalose-phosphatase family protein	1.58	0.015
POPTR_0008s13590	*At1g68020 (ATTPS6)*	UDP-Glycosyltransferase/trehalose-phosphatase family protein	1.56	0.042
POPTR_0003s13940	*At1g64390 (GH9C2)*	Glycosyl hydrolase 9C2	1.50	0.033

Table 3. *Continued*

Populus trichocarpa Phytozome v2.0 gene name	*Arabidopsis thaliana* homologous gene	TAIR description	Fold change	*P*-value
POPTR_0005s06280	*At3g18660 (PGSIP1)*	Plant glycogenin-like starch initiation protein 1	–1.55	0.023
POPTR_0004s06840	*At3g16520 (UGT88A1)*	UDP-glucosyl transferase 88A1	–1.62	0.023
POPTR_0006s11040	*At5g01930 (MAN6)*	Glycosyl hydrolase superfamily protein	–1.76	0.029
POPTR_0004s06910	*At3g16520 (UGT88A1)*	UDP-glucosyl transferase 88A1	–1.79	0.009
POPTR_0016s02640	*At3g10740 (ASD1)*	Alpha-L-arabinofuranosidase 1	–1.79	0.009
POPTR_0016s14550	*At5g01930 (MAN6)*	Glycosyl hydrolase superfamily protein	–1.85	0.023
POPTR_0002s11090	*At1g09890*	Rhamnogalacturonate lyase family protein	–1.95	0.041
POPTR_0016s02620	*At3g10740 (ASD1)*	Alpha-L-arabinofuranosidase 1	–1.95	0.040
POPTR_0001s08750	*At1g11260 (STP1)*	Sugar transporter 1	–2.02	0.025
POPTR_0002s02550	*At1g19940 (GH9B5)*	Glycosyl hydrolase 9B5	–2.02	0.025
POPTR_0016s02690	*At3g10740 (ASD1)*	Alpha-L-arabinofuranosidase 1	–2.04	0.011
POPTR_0005s27680	*At4g35300 (TMT2)*	Tonoplast monosaccharide transporter2	–2.07	0.047
Fasciclin-like arabinogalactan				
POPTR_0014s16610	*At4g12730 (FLA2)*	FASCICLIN-like arabinogalactan 2	2.07	0.010
POPTR_0073s00210	*At5g06390 (FLA17)*	FASCICLIN-like arabinogalactan protein 17 precursor	–1.67	0.047
Laccase				
POPTR_0004s16370	*At5g03260 (LAC11)*	Laccase 11	2.65	0.026
POPTR_0011s12090	*At5g60020 (LAC17)*	Laccase 17	–1.67	0.045
POPTR_0011s12100	*At5g60020 (LAC17)*	Laccase 17	–1.91	0.040
POPTR_0009s03940	*At2g29130 (LAC2)*	Laccase 2	–4.98	0.045
Transcription factor				
POPTR_0009s01110	*At5g22920*	CHY-type/CTCHY-type/RING-type Zinc finger protein	3.37	0.035
POPTR_0009s09270	*At3g49940 (LBD38)*	LOB domain-containing protein 38	3.21	0.019
POPTR_0003s12240	*At1g63100*	GRAS family transcription factor	2.52	0.028
POPTR_0001s08850	*At1g63100*	GRAS family transcription factor	2.5	0.009
POPTR_0017s06590	*At4g26400*	RING/U-box superfamily protein	2.38	0.011
POPTR_0018s10510	*At5g57660 (COL5)*	CONSTANS-like 5	2.35	0.036
POPTR_0014s11940	*At5g49300 (GATA16)*	GATA transcription factor 16	1.82	0.029
POPTR_0005s05470 (Pt-BZO2.3)[1]	*At5g28770 (BZO2H3)*	bZIP transcription factor family protein	1.69	0.040
POPTR_0005s20890 (Pt-SCL1.1)[1]	*At1g21450 (SCL1)*	SCARECROW-like 1	–1.61	0.021
POPTR_0017s12740 (PtrZF1)[2]	*At1g26610*	C2H2-like zinc finger protein	–1.63	0.041
POPTR_0018s06790 (PtrNAC150)[2]	*At4g29230 (NAC075)*	NAC domain containing protein 75	–1.65	0.028
POPTR_0006s27570	*At5g25220 (KNAT3)*	KNOTTED1-like homeobox gene 3	–1.68	0.032
POPTR_0011s05740 (PtrNAC105)[2]	*At4g28500 (NAC073)*	NAC domain containing protein 73	–1.72	0.023
POPTR_0003s11120	*At2g30590 (WRKY21)*	WRKY DNA-binding protein 21	–1.73	0.022
POPTR_0006s04770 (Pt-HSFB3.1)[1]	*At2g41690 (HSFB3)*	Heat shock transcription factor B3	–1.73	0.011
POPTR_0010s22320	*At2g28550 (RAP2.7)*	Related to AP2.7	–1.83	0.017
POPTR_0013s08040	*At2g40470 (LBD15)*	LOB domain-containing protein 15	–1.83	0.041
POPTR_0009s16590	*At1g08320 (TGA9)*	bZIP transcription factor family protein	–1.89	0.041
POPTR_0004s04900 (PtrNAC157)[2]	*At4g28500 (NAC073)*	NAC domain containing protein 73	–1.91	0.033
POPTR_0005s14860	*At2g01275*	RING/FYVE/PHD zinc finger superfamily protein	–2.01	0.018
POPTR_0009s03240 (Pt-MYB111.1)[1]	*At3g46130 (MYB48)*	myb domain protein 48	–2.28	0.038
POPTR_0006s21640	*At5g06510 (NF-YA10)*	Nuclear factor Y, subunit A10	–2.91	0.010
POPTR_0016s06860	*At5g06510 (NF-YA10)*	Nuclear factor Y, subunit A10	–4.12	0.022
Cell cycle				
POPTR_0001s27890	*At2g26760 (CYCB1;4)*	Cyclin B1;4	3.26	0.041
POPTR_0009s16730	*At1g76310 (CYCB2;4)*	Cyclin B2;4	2.87	0.047
Phosphatases				
POPTR_0019s04670 (Pt-APY1.2)[1]	*At5g18280 (APY2)*	Apyrase 2	5.97	0.023

Table 3. *Continued*

Populus trichocarpa Phytozome v2.0 gene name	*Arabidopsis thaliana* homologous gene	TAIR description	Fold change	*P*-value
POPTR_0001s19180	*At4g25150*	HAD superfamily, subfamily IIIB acid phosphatase	5.22	0.020
POPTR_0012s09940 (Pt-PPD3.2)[1]	*At5g50400 (PAP27)*	Purple acid phosphatase 27	3.82	0.023
POPTR_0004s16720 (Pt-PAP.2)[1]	*At2g16430 (PAP10)*	Purple acid phosphatase 10	1.64	0.018
Aquaporins				
POPTR_0010s21700 (PtTIP1;3)[3]	*At4g01470 (TIP1;3)*	Tonoplast intrinsic protein 1;3	2.26	0.047
POPTR_0008s05050 (PtTIP1;4)[3]	*At4g01470 (TIP1;3)*	Tonoplast intrinsic protein 1;3	2.25	0.041
POPTR_0001s18730 (PtTIP2;1)[3]	*At3g16240 (DELTA-TIP)*	Delta tonoplast integral protein	1.87	0.029
POPTR_0019s04640 (PtSIP1;2)[3]	*At3g04090 (SIP1A)*	Small and basic intrinsic protein 1A	–1.70	0.033
POPTR_0001s45920 (PtNIP3;3)[3]	*At4g10380 (NIP5;1)*	NOD26-like intrinsic protein 5;1	–1.80	0.040
POPTR_0006s09920 (PtPIP1;4)[3]	*At4g00430 (PIP1;4)*	Plasma membrane intrinsic protein 1;4	–3.27	0.040
Programmed cell death				
POPTR_0008s03480	*At3g10410 (SCPL49)*	Serine carboxypeptidase-like 49	–1.53	0.032
POPTR_0002s00720	*At1g20850 (XCP2)*	Xylem cysteine peptidase 2	–1.69	0.043
POPTR_0014s07050	*At4g00230 (XSP1)*	Xylem serine peptidase 1	–1.85	0.030
POPTR_0001s13140	*At1g28110 (SCPL45)*	Serine carboxypeptidase-like 45	–1.99	0.040
POPTR_0002s15330	*At4g00230 (XSP1)*	Xylem serine peptidase 1	–2.56	0.023

polysaccharides (Aspeborg *et al*. 2005). The fact that only few polysaccharide biosynthetic genes, and especially no genes related to cellulose synthesis, were down-regulated in high N plants is surprising, considering the thin cell wall phenotype of high N plants. However, the faster radial growth in high N plants (Table 1) could lead to thinner cell walls even under constant biosynthetic activity. Alternatively, other processes such as the perturbation of overall carbon partitioning, polysaccharide remodelling and alternations in lignin metabolism can drive the lower cell wall thickness in high N versus adequate N plants.

Up-regulation of a plethora of glycoside hydrolase (GH) genes in high N plants was one of the most apparent patterns revealed in this study. Five 1,3-β-D-glucan endohydrolases (GH17 family), two α-galactosidase, three endo-1,4-β-D-glucanase (GH9 family, cellulase), α-xylosidase, β-glucosidase and xyloglucan endotransglucosylase/hydrolase all showed increased transcript abundance in high N plants. High activity of GH enzymes is typical for growing and expanding tissue (Cosgrove 2005). The wide lumen diameters of vessels and fibres in high N plants (Fig. 2) indicate that more intensive cell expansion took place in the cambial region of high N-treated plants. Thus, a role of at least some of these genes in primary cell wall loosening conferring increased cell wall extensibility can be implied. Three α-L-arabinofuranosidases, two glycosyl hydrolases with a putative function of endo-β-mannanases and one endo-1,4-β-D-glucanase (GH9) showed lower expression levels in high N plants. α-L-arabinofuranosidases are most likely involved in modification of the carbohydrate moieties of arabinogalactan proteins (AGPs; Geisler-Lee *et al*. 2006; Kotake *et al*. 2006). AGPs in turn are involved in a number of developmental processes and their importance during secondary cell wall deposition has been reported in poplar (Dharmawardhana *et al*. 2010) and pine (Zhang *et al*. 2003). In our dataset, two genes encoding fasciclin-like AGPs (FLAs) were differentially expressed, one up- and the other down-regulated. Several *FLAs* have been previously shown to be involved in tension wood formation (Lafarguette *et al*. 2004; Andersson-Gunneras *et al*. 2006). Gelatinous layer formation is affected by nitrogen fertilization (Pitre *et al*. 2007a); hence, it is possible that the two genes differentially expressed in this study are involved in this process.

Substantial changes in pectin composition occur during xylogenesis (Guglielmino *et al*. 1997a; Hafren, Daniel & Westermark 2000). We found two pectin methyl esterase (*PME*) genes up-regulated and one rhamnogalacturonate lyase gene down-regulated in high N plants. PMEs catalyse de-esterification of pectin molecules. In the cambial zone of poplar, PME activity has been localized predominantly in cell corners (Guglielmino *et al*. 1997b) and a role of PMEs in xylem cell expansion and fibre elongation has been demonstrated (Siedlecka *et al*. 2008). Alternatively, some of the genes encoding GH and PME enzymes may play a role in the secondary cell wall deposition or production of gelatinous fibres as extensive remodelling involving carbohydrate hydrolysis occurs during the secondary cell wall biosynthesis as well (Aspeborg *et al*. 2005).

Lignin-related genes

Surprisingly, only few genes with a predicted function in secondary cell wall lignification were differentially regulated in this study. Among the few were three laccase (*LAC*) genes that were down-regulated in high N plants. These included two homologs of Arabidopsis *LAC17* and a closely related homolog of *LAC2*. *LAC17* is known to be directly involved in lignin monomer polymerization, and *lac17* mutants have reduced lignin deposition (Berthet *et al*. 2011). Thus, reduced laccase polymerization activity may be one of the mechanisms underlying reduced lignin deposition in high N-treated plants previously reported (Pitre *et al*. 2007b).

Transcription factors

A number of transcription factors were differentially expressed in high N versus adequate N plants indicating extensive changes in regulation of gene transcription in response to fertilization. A gene homologous to *LATERAL ORGAN BOUNDARY DOMAIN 38* (*AtLBD38*) showed 3.2-fold up-regulation in high N plants. Expression of *AtLBD38* is strongly induced by nitrate addition in *Arabidopsis* and an important regulatory role for this and two other closely related genes (*AtLBD37*, *AtLBD39*) in response to nitrate availability has been reported (Rubin *et al*. 2009). These three LBD transcription factors act as repressors of many N-responsive genes such as nuclear factor Y subunit A-10 (*NF-YA10*; Rubin *et al*. 2009). In agreement, we found two poplar genes homologous to *AtNF-YA10* strongly down-regulated in high N plants. At least some members of the LBD family of transcription factors have been recently identified as regulators of secondary growth in poplar (Yordanov, Regan & Busov 2010). A homolog of *AtLBD15* that was down-regulated in high N plants in this study could fulfil such function as its paralog has been recently characterized to be part of the transcriptional network involved in secondary xylem patterning (Zhong *et al*. 2011).

Furthermore, three genes encoding NAC domain transcription factors, PtrNAC157, PtrNAC105 and PtrNAC150, and a zinc finger transcription factor PtrZF1were down-regulated in high N plants. These genes have been implicated as intermediate regulators in a transcriptional network governing secondary cell wall biosynthesis in poplar (Zhong *et al*. 2011). Thus, their lower transcript abundance could be correlated with the significantly thinner secondary cell walls observed in fibres of high N plants. Work in *Arabidopsis* and poplar showed that expression of many regulatory and structural genes related to xylem differentiation, including several genes differentially expressed in this study, is under the control of NAC domain transcription factor master regulators such as SND1, NST1, VND6 and VND7 (Kubo *et al*. 2005; Yamaguchi *et al*. 2011) and their homologs in poplar (Ohtani *et al*. 2011; Zhong *et al*. 2011). These regulators are characterized by the ability to ectopically activate secondary cell wall biosynthesis and deposition in vessels, fibres and other secondarily thickened cells (Yamaguchi *et al*. 2010; Ohtani *et al*. 2011). Five genes encoding these master regulators, namely *PtVNS01/PtrWND5A*, *PtVNS03/PtrWND4A*, *PtVNS04/PtrWND4B*, *PtVNS06/PtrWND3B*, *PtVNS10/PtrWND2B* (gene names according to Ohtani *et al*. 2011; Zhong *et al*. 2011) showed more than 1.5-fold down-regulation in high N plants but did not meet our criteria for significance ($P \leq 0.05$). Nevertheless, it is reasonable to expect that even such potentially subtle changes in their expression are biologically significant, considering the central position of these transcription factors in the regulatory cascade controlling secondary cell wall biosynthesis. In addition, down-regulated in high N plants was the poplar homolog of *Arabidopsis WRKY21*, a gene of undefined function that is regulated by *Arabidopsis* VND7 in transgenic poplar (Ohtani *et al*. 2011). A homolog of the *Arabidopsis KNOTTED-like* gene *KNAT3* was also down-regulated in high N plants. While the function of *KNAT3* is unknown, this poplar gene is up-regulated in response to osmotic stress treatments (Bae *et al*. 2010), suggesting that it could play a role in adjustment of xylem to water stress.

Cell division, expansion and death

The processes of cell division, cell expansion and programmed cell death and their dynamics are important during xylem development. The increased radial stem growth of high N plants suggests that increased cell proliferation may have occurred in the cambial region, leading to more cambial derivatives. Transcription of several genes encoding important cell cycle regulators was elevated in high N plants. Two cyclin (*CYC*) genes were strongly up-regulated. At least some poplar *CYC* genes have been previously shown to have specific expression maxima on the xylem side of the cambium; and hence, have been linked with xylem cell proliferation (Schrader *et al*. 2004).

Another gene strongly up-regulated in high N plants was an apyrase (*Pt-APY1.2*). Apyrases displaying nucleoside triphosphate diphosphohydrolytic activity are involved in cell growth regulation and are highly expressed in rapidly growing tissues and/or tissues that accumulate auxin at high levels (Clark & Roux 2011). Interestingly, we also found three genes encoding acid phosphatases strongly up-regulated in high N plants. While acid phosphates are involved in a variety of physiological functions, they are also involved in xylem development. Their specific functions during xylem differentiation are not clear, but putative roles in secretion and resorption of sugars, secondary cell wall deposition (Charvat & Esau 1975), and/or degradation of cellular content (Gahan 1978) have been proposed.

Water uptake is essential to drive cell expansion. Three genes encoding aquaporins of the tonoplast intrinsic protein (TIP) class were up-regulated in high N plants, *PtTIP1;3*, *PtTIP1;4* and *PtTIP2;1*. In contrast, three other aquaporin genes were down-regulated, a plasma membrane intrinsic protein *PtPIP1;4*, a small basic intrinsic protein *PtSIP1;2* and a nodulin-like intrinsic protein *PtNIP3;3*

(gene names according to Gupta & Sankararamakrishnan 2009). Expression of *PtTIP2;1* and *PtSIP1;2* in response to high N availability was previously studied in the bulk xylem of poplar stems (Hacke *et al*. 2010) and the change in expression was in the same direction as in the present study. Expression of several aquaporin genes has been shown to peak in the radial expansion zone of poplar cambium (Schrader *et al*. 2004) and it has been speculated that aquaporins may play a role in xylogenesis by facilitating the flow of water into the zone of expanding cells (Groover *et al*. 2010; Hacke *et al*. 2010; Almeida-Rodriguez & Hacke 2012). Following this hypothesis, the up-regulation of the three TIPs observed in this study may be linked with the wider vessels in high N plants. Many TIPs and PIPs have been functionally characterized as water channels (Chaumont *et al*. 1998; Almeida-Rodriguez 2009; Sade *et al*. 2009; Secchi *et al*. 2009). In a recent study, an elevated hydraulic conductance of root tips was associated with a decreased expression of *PtPIP1;4* suggesting that the *PtPIP1;4* may not be a water channel (Almeida-Rodriguez, Hacke & Laur 2011). Similarly, NIPs usually display low water permeability but are permeable to small solutes such as urea and glycerol (Gomes *et al*. 2009) and the transport specificity of SIPs is largely unknown. Thus, it is reasonable to expect that the down-regulated aquaporins may fulfil roles other than water transport within the developing xylem region.

Programmed cell death (PCD) is a crucial process during xylogenesis, as xylem vessels and fibres are dead and hollow at maturity. Two genes homologous to *Arabidopsis XYLEM SERINE PEPTIDASE 1* (*XSP1*) and a homolog of *XYLEM CYSTEIN PEPTIDASE* 2 (*XCP2*), known to be involved in PCD during xylem differentiation in *Arabidopsis* (Funk *et al*. 2002) showed significantly lower expression in high N plants. In addition, two other PCD-related genes, homologs of *Arabidopsis SERINE CARBOXYPEPTIDASE-LIKE 45* and *49* (*SCPL45* and *49*), were down-regulated in high N plants indicating that cell maturation and death was suppressed. The down-regulation of genes involved in PCD in high N plants supports previous findings that nitrogen fertilization results in wood with more juvenile characteristics (Pitre *et al*. 2007b) and supports a recent notion that programmed cell death and secondary cell wall formation are correlated and governed by common regulatory mechanisms involving PtVNS/PtrWND transcription factors (Bollhöner, Prestele & Tuominen 2012).

CONCLUSIONS

In this study, we showed that fertilization with ammonium nitrate evokes changes in growth, anatomy and hydraulic properties of secondary xylem in the stems of hybrid poplar (*P. trichocarpa* × *deltoides*, clone H11-11). These anatomical and physiological differences were underpinned by changes in transcription of hundreds of genes in the developing xylem region. Our results revealed that several transcriptional patterns previously observed in *Arabidopsis* roots and shoots in response to high nitrate availability are also elicited in the developing secondary xylem of poplar. An example of such common responses is increased expression of genes encoding the transcription factor *LBD38* and enzymes involved in organic acid and trehalose metabolism. Such comparisons between a short-lived annual herb and a relatively long-lived perennial tree as well as between the different types of tissues suggest that metabolic and regulatory pathways controlled by the corresponding proteins may represent evolutionary conserved aspects of plant responses to high N availability. However, future studies are needed to confirm this finding in a broader range of species and experimental conditions and to better understand the specific roles of these genes and processes in plant responses to high N.

Furthermore, the data presented in this study shed light on molecular mechanisms that underlie the phenotypic plasticity of xylem hydraulic and structural traits in this hybrid poplar genotype. We identified gene candidates that may affect xylem cell dimensions and cell wall thickness, although detailed functional characterization of these genes in poplar is required to corroborate the proposed function. Based on our results, wider lumens of vessels and fibres in high N plants might be linked with increased expression levels of genes encoding cell wall loosening enzymes such as various glycoside hydrolases and genes encoding aquaporins that may facilitate increased water uptake into expanding xylem cells. Our results also suggest that the changes in xylem development and secondary wall deposition in response to N availability may, at least in part, be mediated by the differential expression of several transcription factors that are part of a core transcriptional cascade governed by the recently characterized master regulators of xylem cell differentiation, the NAC domain transcription factors (PtVNS/PtrWND). Future research will reveal if the same genes that were identified in this study underlie changes in xylem phenotype under different environmental conditions, or if distinctly different suits of genes are regulated by various environmental cues, yet resulting in a similar xylem phenotype.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Frequency histograms of (a) vessel element length, (b) vessel length, and (c) fiber length for adequate N (open bars) and high N (black bars) plants. Means and SE ($n = 6$).

Figure S2. qRT-PCR validation of microarray results for nine selected genes: Xylem serine peptidase 1 (*XSP1*, POPTR_0014s12000) (1), LOB domain-containing protein 15 (*LBD15*, POPTR_0013s15220) (2), Bifunctional nuclease I (*BFN1*, POPTR_0011s04430) (3), NAC domain containing protein 75 (*NAC150*, POPTR_0018s06790) (4), No apical meristem (NAM) protein (*WND2B*, POPTR_0002s17950) (5), Myb-like binging domain (*MYB26*, POPTR_0005s06410) (6), Yellow-leaf-specific gene 8 (*YSL8*, POPTR_0007s07660) (7), Glycosyl hydrolase 9C2 (*GH9C2*, POPTR_0003s13940) (8), and Cellulose synthase-like D5 (*CSLD5*, POPTR_0014s12000) (9).

Table S1. List of qRT-PCR gene-specific primers used in this study. Gene names were given according to annotation. Gene models correspond to the Phytozome database (http://www.phytozome.net/search.php).

Table S2. List of all genes differentially regulated (Fold change ± 1.5, P value ≤ 0.05) in hybrid poplar saplings fertilized with 7.5 mM (high N) versus 0.75 mM (adequate N) ammonium nitrate in $0.5 \times$ Hocking's complete nutrient solution.