Original research article

Identification of nitrogen responsive genes in poplar roots grown under two contrasting nitrogen levels

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Abstract: We investigated the short-term response of poplar roots to low and high nitrogen availability in order to elucidate the mechanisms involved in nutrient acquisition. After 28 days of fertilization with low versus high ammonium nitrate, an increase in aboveground biomass was observed accompanied by a decrease in root biomass, reducing the root: shoot ratio after 28 days. These changes in biomass allocation were accompanied by changes in root architecture and altered gene expression. The gene expression response was evaluated after 7 days using a custom cDNA micrarray following transfer to low and high nitrogen supply. We found that 56 sequences were differentially expressed in poplar roots. Many of these 56 genes could be associated with putative roles in development or response to biotic and abiotic stress. A time course analysis of selected cell wall-related genes by RT-qPCR confirmed the expression patterns obtained by microarray and also showed the timing of this differential response. Our results show that patterns of transcript accumulation in roots of poplars are influenced by nitrogen supply, providing evidence of unique nitrogen-adaptative mechanisms.

Keywords: genes, microarray, N metabolism, nitrogen, poplar, roots, transcriptome

Introduction

Species of the *Salicaceae* family, such as *Populus* spp., have a high demand for water and nutrients to sustain their considerable growth rates (Tuskan 1998) . Because of these relatively rapid growth rates, many *Populus* spp. are amenable to intensive culture practices aimed at decreasing rotation times and/or increasing biomass production. Intensively managed plantations of *Populus* spp. trees are receiving increasing attention with the recent focus on bioenergy production. Nitrogen (N) fertilizers are often recommended and applied in order to increase biomass production (Coleman et al. 1998, Oren et al. 2001, Gessler et al. 2004). An elegant series of experiments demonstrated that young *Populus tremuloides* Michx. trees exhibit relatively high affinity for uptake of both nitrate and ammonium from the rhizosphere, and that uptake increases with increasing N availability (Min et al. 1999, Min et al. 2000). The high N absorption capacity of *Populus* spp. also makes them suitable for the removal of excess N – particularly nitrate – from the soil (O'Neill and Gordon 1994, Licht and Isebrands 2005). The systematic planting of poplars in hedgerows on agricultural lands to serve both as a riparian buffer functioning to remove fertilizer-derived nitrates and as a source of biomass production is a promising approach for multi-purposes agrosystems (Licht and Isebrands 2005).

In poplar, much of the inorganic N that is taken up by the roots is transported to the shoots, where the

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majority of primary N assimilation takes place, as estimated by nitrate reductase activity (Min et al. 1998, Black et al. 2002). Nitrate reductase activity in leaves can be more than an order of magnitude greater than that of roots, the latter reaching significant levels only at elevated concentrations of external N (Black et al. 2002). The relative proportion of inorganic N that is transported to aerial parts of the plant increased with increasing N availability (Min et al. 1998, Black et al. 2002). Perhaps because of this, C: N ratios in both roots and shoot significantly decreased just days after the onset of fertilization (Cooke et al. 2003).

Several studies have evaluated the impact of increasing N availability on growth and development of fast-growing poplars (Liu and Dickmann 1992, Heilman et al.., 1994, Ibrahim et al. 1997, Cooke et al. 2005a, Luo et al. 2005, Liberloo et al. 2006). N fertilization dramatically affects poplar biomass accumulation, notably through increased sylleptic branching and enhanced allocation of carbon (C) resources to shoots, particularly leaves (Cooke et al. 2003, Cooke et al. 2005a). High N also modified poplar secondary xylem development and the partition of C resources to the cell wall (Cooke et al. 2003, Cooke et al. 2005a). We previously reported high N altered lignin content and structure and that total cellulose content was increased (Pitre et al. 2007a, Pitre et al. 2007b). *Populus* roots also exhibit considerable developmental plasticity, and soil nutrient availability has been shown to have large effects on poplar root growth and architecture (Friend et al. 1999, Woolfolk and Friend 2003).

Studies in herbaceous plants such as *Arabidopsis* and tomato have shed considerable light on how N availability reconfigures the root transcriptome (Wang et al. 2001, Gutierrez et al. 2007). These studies illustrated that large-scale changes in transcript profiles occur rapidly in roots upon exposure to high N, and that some of these differentially expressed genes are uniquely expressed in roots (Gutierrez et al. 2007). Differential N availability not only affects genes associated with N uptake, transport, assimilation and metabolism, but many other biosynthetic and metabolic processes. In particular, inorganic N has dramatic effects on root system architecture (Malamy, 2005, Forde and Walch-Liu, 2009) which appear to be mediated at least in part by auxin (Krouk et al. 2010, Vidal et al., 2010).

Despite a growing body of literature describing the N response of poplar at the molecular level in aboveground tissues (Cooke et al. 2003, Gutierrez et al. 2007, Ramírez-Carvajal et al. 2008, Pitre et al. 2010) there have been surprisingly few investigations of molecular responses to N fertilization in poplar roots. In one such study, researchers (Cooke et al., 2003) used filter arrays containing 116 genes to identify 29 genes that showed differential transcript abundance in roots of *Populus trichocarpa* x *P. deltoides* subjected to low versus high levels of NH_4NO_3 for 16 days. Recently, we described the transcriptomics response of poplar xylem to high N availability (Pitre et al., 2010). This study allowed us to identify N-specific transcript that differentially accumulated in response to nitrogen but was limited to xylem. Given how little is known about the effect of differential N availability on poplar roots, particularly on the molecular response to N, the objective of the present study was to use transcript profiling to identify additional N-responsive genes in roots, and use the putative identity of these genes to infer molecular processes that are altered in roots in response to differential N availability. Building on our previous studies on whole plant (Cooke et al., 2005a) and gene expression responses to N availability in roots (Cooke et al., 2003), we have used a custom 3.4K cDNA microarray together with quantitative RT-PCR to identify short-term changes in transcript accumulation in response to limiting versus luxuriant levels of N fertilization.

Materials and Methods

Plant Material

All experiments were conducted with plants propagated from rooted cuttings of poplar (*Populus trichocarpa* x *P. deltoides* clone H11-11) produced essentially as described in Cooke et al. (2003) and Pitre et al. (2007a). Experiments 1 and 2 were conducted with plants grown in 4 litres pots in standard greenhouse conditions as previously described (Pitre et al. 2007a). Experiment 3 was conducted in controlled environment growth rooms with 16 h, 25°C day/8 h, 18°C night, ca. 70% relative humidity, and photosynthetically active radiation of ca. 250 μmol s^{-1} m^{-2}. Plants were grown in 5.5 L pots containing Sunshine Mix #4, prior to commencing the experiments, plants were fertilized weekly by alternating 0.5 g l^{-1} of 20-20-20 with 0.5 g l^{-1} each of 10-52-10 and 15.5-0-0 19% Ca. Experiments were initiated when plants reached a uniform height of 0.65-0.80 m. For all experiments, the treatments consisted of daily applications of a complete water-soluble fertilizer where nitrogen (N) was supplied as ammonium nitrate (NH_4NO_3) (Hocking, 1971), as described in Cooke et al. (2003).

Experiment 1. The effects of N supply on poplar tree biomass partitioning were monitored over the course of 28 days' application of Hocking's solution supplemented with either 0 mM or 25 mM of N by determining the dry weight (DW) of the shoots and

roots. For each sampling time, seven (7) trees were used per N level (0, 7, 28 days: n= 56). The shoots consisted of the complete above-ground biomass (stems and leaves) while the roots consisted of all below-ground biomass, with the exception of fine roots. The stem section used for the rooted cuttings was removed for measurements and the roots were rinsed under tap water and briefly dried using paper towels. After harvest, the organs were immediately dried for 48 hours in the oven at 65°C. The DW is defined as the mass measured in grams (g).

Experiment 2. Trees were treated with Hocking's solution supplemented with either 0 mM or 25 mM NH_4NO_3 for seven days. Total roots were quickly harvested under running water – likely removing fine roots – visually inspected for absence of mycorrhizae, and rapidly frozen in liquid nitrogen. Two trees per condition were harvested. Tissues were stored at -80°C until analysis.

Experiment 3. Plants were maintained as described in experiment 1, then fertilized with 0 mM or 10 mM NH_4NO_3 for 1, 3, 7, or 14 days using a complete randomized block design, and harvested as described previously. A total of six biological replicates were harvested per treatment.

Gene expression analyses

Plant material harvested as previously described was used for microarray analyses. Total RNA was extracted as described (Chang et al., 1993) , and RNA concentration and quality were determined with a BioAnalyser 2100 (Agilent Technologies, RNA 6000 Nano Assay Kit). RNA was reverse transcribed to cDNA and then labelled using aminoallyl indirect labelling (Invitrogen). Hybridization and washing steps were performed as described (Pavy et al., 2008). Hybridization was carried out with the Arborea custom poplar cDNA array that contains 3400 unigenes, as described in Pitre et al. (2010). cDNAs used to produce this array were cloned and sequenced from shoot tips, leaves, and woody stems of both 0 mM and 25 mM NH_4NO_3-treated plants of Experiment 2. This custom microarray was successfully used by Côté et al. (2010) and Pitre et al. (2010) to identify genes differentially expressed in response to gravity, N availability, and mis-expression of transcriptional regulators (NCBI, GEO Platform GPL11055). Including dye-swap, a total of 8 microarray slides were analysed for this experiment.

Unless mentioned otherwise, all analyses and data mining were performed as described in Pitre et al. (2010). Data acquisition was conducted with a ScanArray Express scanner and image analysis performed with the QuantArray software (Packard BioScience, US). Data analysis was performed using packages in Bioconductor for the R environment (R Foundation for Statistical Computing, Vienna, Austria, www.r-project.org). Quality control was achieved by using the *marray* and *olin* packages in Bioconductor (www.bioconductor.org). Intensity and spatial normalization were performed with the functions *maNormLoess* and *maNorm2D* of *marray*. Fold change estimates between the sample intensities were determined using *Limma* (Smyth and Speed, 2003). The sequences identified as differently expressed between high and low nitrogen fertilization were selected on the basis of a minimum log-ratio value of 0.4 (i.e. fold change High/Low of 1.3). As previously described (Pavy et al. 2008, Pitre et al. 2010), p-values were adjusted for multiple testing by using a false discovery rate (FDR) of 5% (Benjamini and Hochberg, 1995) with a cutoff of 0.01.

This relatively permissive fold-change filter was used because of the comparatively high degree of data compression that we observed using this amino allyl indirect labelling technique compared to other labelling techniques. Sequence analyses were performed with comparison with the *Populus trichocarpa* genome v.2.0 (www.phytozome.net) as described in Pitre et al. (2010). Functional classification was performed by comparing the sequences' gene ontologies (GO terms). We manually attributed the 256 differentially expressed sequenced to GO categories using the annotations obtained by our group using BlastX on GenBank non-redundant database. The entire dataset is available upon request and on GEO.

For the RT-qPCR analyses, RNA was extracted from roots obtained in Experiment 3. Gene-specific primers were designed using Primer Express (v3.0, Applied Biosystems, ON, Canada, Table 1). Two micrograms of total RNA was treated with DNaseI (Invitrogen) prior to cDNA synthesis using Superscript II reverse transcriptase (Invitrogen). PCR reactions were performed in 10 µl, containing SYBR Green master mix (0.2 mM dNTPs, 0.3 U Platinum Taq Polymerase (Invitrogen), 0.25X SYBR Green, and 0.1X ROX), 20 ng of cDNA and 300 nM of each primer. Six biological replicates were analyzed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) with a first step of 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curves were generated using the following program: 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 15 s at 60 °C. Transcript abundance was quantified using standard curves for both target and reference genes, which were generated from serial dilutions of PCR products from corresponding cDNAs. Of the 33 candidate reference genes that were tested, a member of the EF1α family (corresponding to POPTR_0006s13310.1, *P. tricho-*

Table 1. Primers used for the qRT-PCR analysis.

Gene	Sequence ID	Forward	Reverse
PP2A	POPTR_0012s06210.1	AAGTTAGGCGCATGTGTGGAT	GCACTACTGCCAATACCAATG
TIF5A	POPTR_0018s11660.1	CCTCGGTAAGTCTCGAATTGCT	TTTCTCTGGAGCCC AAATTTC
Fascilin-like arabinogalactan protein 1	MN5184154	ACGGATGTTAAAGGAGGGAAAGT	AAACACATCAAGGTTCCCTCCAT
Proline rich cell wall protein	MN5184232	GGGTTGTGCAGCTCTTGGAA	AAGGATATGCGCCACACAATC
Putative xyloglucanase inhibitor	MN5185332	CGATTAAAGATGGGTTTGGTGAA	CTCTCCCATGGCAGACATGA
EF1 α	POPTR_0006s13310.1	CTTGGTGTGAAGCAGATG	CCTCTCAGGTCATCATCA

carpa genome assembly v2.0) was chosen as the reference gene for these experiments, since ANOVA indicated that this gene showed no significant differences in Ct values across all samples ($p = 0.074$).

Results and Discussion

Morphology and Root Growth

Poplars exhibit considerable phenotypic plasticity, including plasticity in response to N availability (Cooke et al., 2003). N-responsive plasticity in root architecture not only has ramifications for poplar fitness in natural ecosystems (O'Neill and Gordon, 1994), but also for the utility of poplars in multi-purpose agrosystems (Licht and Isebrands, 2005). Nitrogen availability affected poplar morphology by increasing shoot DW and decreasing root development (Table 2). At the end of the experiment (28 days), the DW of the shoots produced under high N was significantly higher than the biomass produced under low N (36.4 ± 4.2 g and 22.4 ± 1.8 g, respectively). As was previously reported in our experi-experimental system for aerial organs (stems, branches and leaves; (Cooke et al. 2003, Cooke et al. 2005b) ammonium nitrate fertilization induced rapid (7 days) and persistent developmental and physiological alterations in poplar. These modifications were linked to rapidly altered resource allocation patterns (Cooke et al. 2003, Cooke et al. 2005b, Pitre et al. 2007b). This report adds to previous studies by showing that, as early as 7 days, root DW was significantly lower in the high N poplar trees and no increase was observed during the 28 days of analysis. Also, we visually followed the overall morphology of the roots produced during the N fertilization experiment and differences were observed (Fig. 1). Under low N, the root mass is denser and more ramified. Moreover, when we removed the roots from the pots we had higher order root branching and the root tips were whiter, when compared to the high N trees. Our experimental system enabled us to maintain a constant N level in the pot environment. Hence, the arrested root development may be explained by the high levels of ammonium nitrate fed to the plant. It is possible that soil exploration and directed root formation toward nutrient patches (Cahill et al. 2010) may have slowed or stopped as the root mass filled the pot. On the other hand, the increased root DW and increased branching we observed (Table 2, Fig. 1) in the low N trees are suggestive of an increased development of the roots. High nitrate (NO_3) decreased the amount of lateral roots in Arabidopsis (Bao et al. 2007). Interestingly, a study of *Populus* showed that high N availability increased the total biomass of the fine roots produced but also significantly increased their rate of mortality (Pregitzer et al. 1995). The results of Pregitzer et al. (1995) is in agreement with our observations that high N fertilization decreased the biomass of roots recovered at the end of the experiment.

The same mechanisms involved in the arrest of root formation could suggest that the lack of available N in the pot environment increased root development in order to maximize the nutrient capture (here, N). Similar conclusions were drawn in *Arabidopsis* systems, in which lateral root formation was greatly reduced by high nitrate levels in the growth medium (Zhang and Forde 2000). The altered morphology and development we observed in response to low and high nitrogen availability may help to identify molecular mechanisms involved in modulating root development in response to variations in N availability.

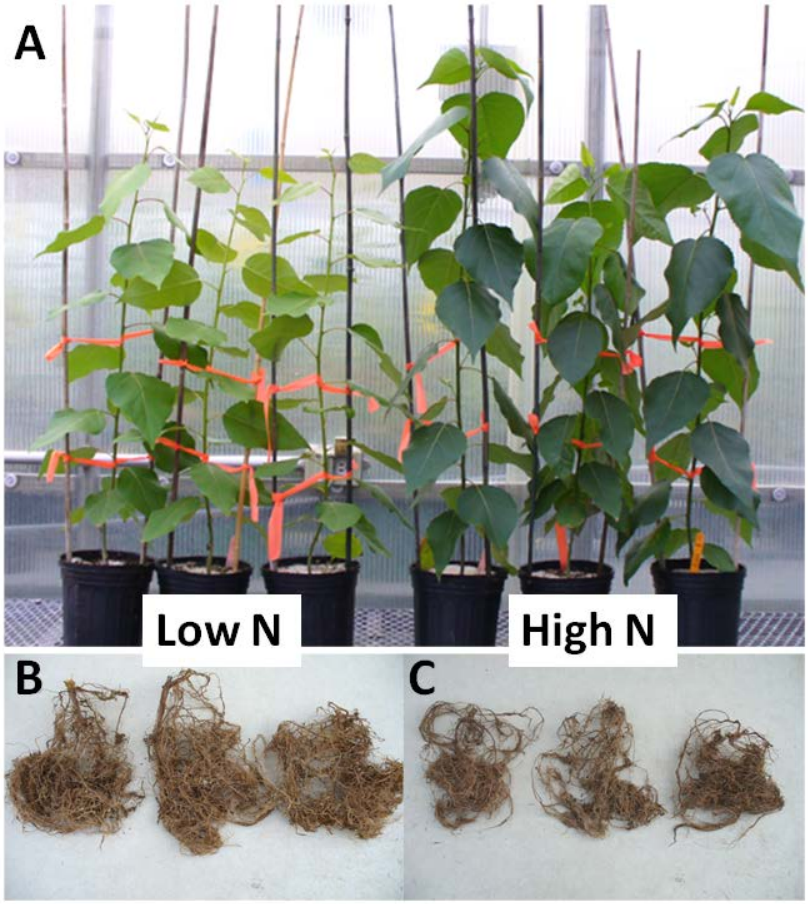

Fig. 1. Effects of N fertilization on poplar development. (A) Poplar trees were treated with Low and High levels of ammonium nitrate and sampled after 28 days. Poplar root morphology in response to Low N (B) and high N (C) availability.

Table 2. Short term effects (Mean ± S.D.) of nitrogen availability on poplar biomass partitioning. Above (Shoot) and below (Root) ground dry weight (DW in gram) biomass were determined 48 hours after drying at 65°C [a].

	Initial	Nitrogen	7 days	28 days
Shoot	15.3 ± 4.6	Low	16.7 ± 4.4	22.4 ± 1.8
		High	18.4 ± 1.1	36.4 ± 4.2 * *
Root	13.3 ± 5.1	Low	10.3 ± 3.1	10.9 ± 1.8
		High	7.3 ± 1.2 *	6.1 ± 0.9 * *
Root: Shoot	0.9	Low	0.6	0.5
		High	0.4	0.2

[a] Statistical significance between Low N and the High N treatment for each time point, is based on Student's t-test (* $p < 0.05$; ** $p < 0.01$).

Microarray survey of N-associated differential transcript accumulation

The above data indicate that N availability alters root development, impacting both morphology and biomass. As such, we hypothesized that genes associated with growth and development would show changes in transcript abundance under high and low N fertilization. Based on the findings of some researcher (Min et al. 1998, Black et al. 2002) and a plethora of other studies in herbaceous plants, we further hypothesized that N availability would also alter transcript abundance corresponding to genes encoding enzymes of stress and osmotic metabolism. To test these hypotheses, a survey of differential transcript levels in poplar roots – including roots with secondary growth, suberinized roots, and white root tips, but likely lacking the majority of fine roots – following nitrogen fertilization was conducted using a custom cDNA microarray comprising a total of 3400 genes, similar to the approach taken by Pitre et al. (2010). We applied a linear model (*LIMMA*) to the

data obtained by comparing low and high N treated samples after seven days of application as described in Pitre et al. (2010). A volcano plot was used to represent the magnitude and intensity of the expression response to high nitrogen supply. We identified 56 sequences differentially expressed in poplar roots, using a threshold p-value ≤ 0.01 on the log-ratios (M values, Fig. 2 A). Among the differentially-expressed sequences, 26 showed an increased level of transcripts in response to high N while 30 showed a decreased level of transcripts.

The putative identity of each N-responsive differentially-expressed sequence is indicated in Table 3, while functional classification of these differentially-expressed transcripts is shown in Fig. 2B. More than 25% of the 56 differentially expressed sequences identified (15/56) did not show similarities to genes with known functions and 5/56 exhibited no sequence similarities to NCBI sequences at all. Additionally, several of the annotations that were obtained for differentially expressed sequences were weak, and did not provide the ability to predict function of the encoded protein in the N response. For example, of the differentially expressed sequences, MN183383 showed the greatest magnitude response to differential N availability. Sequence analysis by BLASTX revealed the presence of an Al-induced domain and suggested homologies with the stem-specific protein TSJT1 (Seurinck et al. 1990), which does not have a defined function. These data identifying significant differences in transcript abundance corresponding to genes of unknown function indicate that potential mechanisms of the N response are yet to be uncovered or, alternatively, that the sequences are not well conserved between plant species.

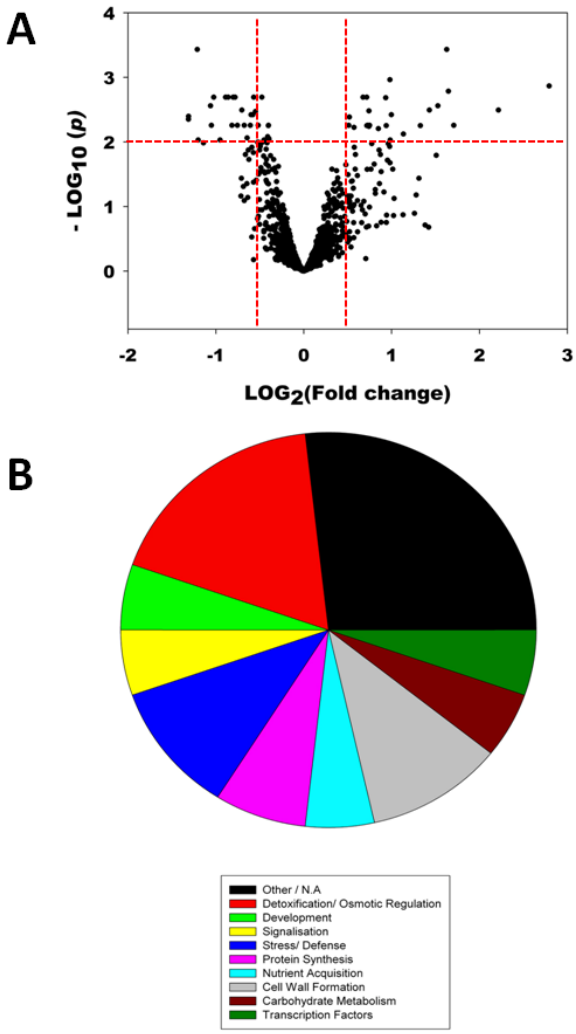

Fig. 2. Transcript profile of poplar roots in response to nitrogen fertilization. Data are based on an in-house cDNA microarray comprising 3400 different genes analyzed after 7 days. (A) Volcano plot of significance (y axis) against transcript accumulation (x axis) between low and high nitrogen supply. For each microarray spot, the differences between normalized expression values are plotted against the negative log10 of the p-values. The horizontal line is the significance threshold (p-value ≤ 0.01) and the vertical lines are the minimal High N/Low N expression ratio (> 1.3). Highly significant genes are toward the top. (B) Functional classification of the sequences transcripts differentially expressed in roots in response to nitrogen, based on the comparison of the sequences GO term revealed by BlastX on GenBank non-redundant database.

Table 3. Genes differentially expressed in poplar roots 7 days after high nitrogen fertilization. Transcript level of High N/Low N represent LOG_2 expression ratios of fold change between Low H and High N [a].

Sequence ID	Annotation	J.G.I Poplar ID (Blast Search)	Unigene ID	Sequence Up (High L/ Low N)	Sequence Down (High L/ Low N)	*Adjusted* p- value
MN5183383	Putative Aluminum-induced protein	POPTR_0011s04960.1	61936	6.93		0.001361
MN5186226	Putative DNA J protein	POPTR_0001s03470.1	58076	4.64		0.003215
MN5185371	Aquaporin / putative ethylene receptor	POPTR_0003s04930.1	58793	3.27		0.005544
MN5187806	Putative pathogenesis related-1 (PR1) protein	POPTR_0001s29530.1	58262	3.13		0.001638
MN5189761	Dormancy associated family protein; similar to Aux-in-repressed protein			3.09		0.000370
MN5185332	Putative xyloglucanase inhibitor	POPTR_0008s20730.1	58146	2.88		0.002758
MN5184667	Putative proteinase inhibitor se60-like protein, Thio-nin-like	POPTR_0019s00770.1	58877	2.69		0.003215
MN5185577	Putative signal-transduction protein with CBS domains	POPTR_0013s15730.1	57748	2.51		0.005592
MN5187617	zinc finger (CCCH-type) family protein	POPTR_0006s25080.1	62750	2.20		0.007542
MN5189525	similar to bZIP family transcription factor	POPTR_0010s15280.1		2.00		0.003787
MN5189366	Putative treha-lose-6-phosphate synthase	POPTR_0018s10680.1		1.98		0.009345
MN5185635	Photosystem I reaction center subunit II	POPTR_0008s15100.1	58953	1.97		0.001086
MN5183597	No Hit	POPTR_0005s25020.1	62372	1.92		0.005592
MN5186658	F-box family protein / lectin-relatedCyclin-like	POPTR_0014s07190.1	62854	1.91		0.002070
MN5187491	Polyamine oxidase ammonia formation	POPTR_0015s08590.1	62487	1.80		0.006227
MN5182963	Phospholipase D delta isoform 1a	POPTR_0005s26730.1	62444	1.68		0.005544
MN5185051	Peroxidase	POPTR_0005s21740.1	58971	1.67		0.005758
MN5183604	BURP domain containing protein - RD22-like protein	POPTR_0009s11750.1	58796	1.67		0.003306
MN5183075	Constans-like protein 2	POPTR_0006s28350.1	58717	1.65		0.005544
MN5185182	No Hit	POPTR_0015s03670.1	58794	1.65		0.005592
MN5188514	Pyruvate dehydrogenase E1 alpha subunit	POPTR_0008s19680.1	59730	1.64		0.002030
MN5184130	No Hit		57791	1.64		0.002030
MN5189709	Late Embryogenesis Abundant Protein	POPTR_0010s01590.1		1.60		0.002030
MN5185841	No Hit	POPTR_0014s17440.1	58028	1.49		0.005966
MN5184481	DNA J-like protein homo-log-root specific/chaperone	POPTR_0002s02240.1	57368	1.43		0.004124
MN5187076	Photosystem I reaction center subunit	POPTR_0006s27030.1	58670	1.43		0.005544
MN5183211	similar to chloroplast nucleoid DNA-binding protein-related	POPTR_0002s10480.1	62918		2.48	0.004464

Table3. (continued)

MN5187417	Putative cytochrome P450	POPTR_0003s06460.1	59043	2.48	0.004012
MN5186984	Folded petals membrane protein	POPTR_0019s09950.1	63016	2.32	0.000370
MN5185357	Phosphate induce protein Phi-1	POPTR_0004s21650.1	57634	2.30	0.009345
MN5188397	Heat shock protein 70, cognate	POPTR_0008s05480.1	58208	2.09	0.002758
MN5185534	NA	POPTR_0015s14910.1	61729	2.07	0.005544
MN5188747	Cytochrome P450 DDWF1	POPTR_0003s06510.1	57546	2.03	0.002030
MN5189377	Al-induced protein			1.94	0.009345
MN5187347	Putative defence protein	POPTR_1031s00220.1	62588	1.85	0.002030
MN5183230	Defence, similar to progesterone 5-beta-reductase	POPTR_0002s12170.1	57816	1.82	0.002030
MN5184232	PtxA proline rich cell wall protein	POPTR_0006s01020.1	58592	1.76	0.005541
MN5183785	Cytochrome P450 monooxygenase CYP704G7	POPTR_0014s06820.1	58844	1.75	0.002030
MN5184996	UDP-glucose glucosyltransferase	POPTR_0016s01620.1	58811	1.72	0.002030
MN5184691	similar to A thaliana DUF538	POPTR_0005s01470.1	58886	1.69	0.005544
MN5182927	Putative beta-tubulin 4/5	POPTR_1455s00200.1	57862	1.63	0.003215
MN5183717	Phenylcoumaran benzylic ether reductase	POPTR_0002s03580.1	59045	1.60	0.005544
MN5183270	Calcium-binding EF hand family protein	POPTR_0019s04410.1	57440	1.56	0.008683
MN5186380	similar to Arabinogalactan protein 13	POPTR_0001s04130.1	62960	1.53	0.005544
MN5184657	Benzoyl coA: benzyl alcohol transferase/ similar to hypersensitivity-related gene product HSR201	POPTR_0013s07240.1	58477	1.52	0.003787
MN5184847	Cytochrome p450	POPTR_0009s04830.1	62309	1.49	0.003787
MN5185542	CCoAOMT-1	POPTR_0009s10270.1	58182	1.48	0.002030
MN5185017	Similar to root apyrase-like APY1	POPTR_0019s04670.1	59034	1.46	0.003377
MN5184154	Fasciclin-like arabinogalactan-protein 1		58840	1.43	0.005544
MN5186724	Fasciclin-domain protein	POPTR_0001s37650.1	63122	1.39	0.002030
MN5189817	Cytochrome P450-like protein	POPTR_0014s06820.1		1.37	0.009345
MN5184995	Cytochrome P450 71A1	POPTR_0016s14440.1	58392	1.33	0.008288
MN5187748	ATP sulfurylase [Glycine max]	POPTR_0008s15880.1	57516	1.32	0.005592
MN5184216	Myb-putative	POPTR_0003s19470.1	62973	1.32	0.005544
MN5184080	Similar to fiber protein Fb34	POPTR_0001s41410.1	57795	1.32	0.008893
MN5188290	2,4-D inducible Glutathione transferase	POPTR_0878s00210.1	58246	1.32	0.009345

[a] Details of the sequences on the microarrays used are described in Pitre et al. 2010.

We did not detect significant differential expression of sequences related to primary nitrogen acquisition and assimilation. This may reflect the fact that primary assimilation occurs mainly in leaves at higher levels of N (Black et al. 2002). It may also be due to the make-up of the custom cDNA used for this array, which only represents a subset of the genome, and was constructed with cDNA obtained from sequencing of shoot tips, leaves and woody stems from Experiment 2, described in Materials and

Methods (Pitre et al. 2010). Consequently, this array does not contain sequences corresponding to inorganic N transporters or several other genes associated with N acquisition in roots. Interestingly, no vegetative storage protein genes showed statistically significant upregulation by high N at 7 days in this study. However, in a separate study, we have found using RT-qPCR that *BSP* exhibited increased transcript abundance in roots under high N fertilization at 7 days (Ryan and Cooke, unpublished).

Many of the other differentially expressed sequences assigned putative functions were associated with growth and development processes, consistent with our hypothesis. Several of the differentially expressed genes encoded putative metabolic enzymes, also supporting our hypothesis. The majority of these putative metabolism-associated genes were associated with secondary (natural products) metabolism. Although annotation of genes encoding enzymes of natural products metabolism is challenging since small changes in the coding sequence of closely related genes can drastically alter substrate specificity, some of the differentially expressed genes belong to families containing members associated with biotic and abiotic stress responses. Interestingly, a number of genes classically associated with stress responses were also found to be differentially expressed in response to N availability.

N-responsive genes in poplar roots putatively associated with growth and development.

Several sequences with similarity to transcription factors and other developmental regulators were found to be differentially expressed under high versus low N availability. For example, MN5185357, a sequence similar to the tobacco *PHI-1* (Sano et al. 1999) and *Arabidopsis EXORDIUM* (Farrar et al. 2003), was repressed by high N. In Arabidopsis, *EXORDIUM* affects meristem function and root growth, possibly through mediating cell division or cell expansion (Farrar et al. 2003, Schroder et al. 2009). Of particular interest, a CONSTANS (CO)-like gene, MN5183075, showed increased transcript abundance under high N supply. CO genes are implicated in photoperiodic regulation of developmental events via the CO/FT regulon, such as controlling time of flowering in *Arabidopsis* and seasonal growth in poplar (Izawa et al. 2002, Böhlenius et al. 2006). Other *CONSTANS-like* genes have been shown to regulate other developmental processes: for example, *CONSTANS-like 3* promoted lateral root development in Arabidopsis (Datta et al. 2006). Other differentially expressed genes can be assigned to *bZIP*, *F-box*, *FOLDED PETALS*, and *MYB* gene families that in other species contain members encoding developmental regulators. However, the sequences do not have sufficient annotation to more accurately predict a more defined function in development. These genes are putative regulators of developmental processes and are attractive candidates for functional characterization to determine if they are indeed involved in mediating molecular events that are manifested as changes in root morphology.

Morphological changes of the extent observed in this study are undoubtedly accompanied by changes in cell wall biosynthesis. Such changes could include differences attributable to differential cell maturation rates, as well as differences in final cell wall composition. A number of genes associated with cell wall biosynthesis were found to be differentially expressed in response to N availability, including sequences exhibiting similarity to arabinogalactan proteins (MN5186380, MN5184154), a proline rich cell wall protein (MN5184232), the lignin biosynthetic enzyme caffeoyl Co-A O-methyltransferase (MN5185542), and a putative xyloglucanase inhibitor (MN5185332). Xyloglucans are a main constituent of cell wall matrix glycans (Nishikubo et al. 2011). The putative xyloglucanase inhibitor was upregulated under high N conditions, raising the possibility that a reduction in xyloglucan cleavage is indicative of a slowdown in cellular elongation.

Suberin, a complex and poorly characterized biopolymer comprising both aliphatic (fatty acid derivative) and aromatic (phenylproponoid derivative) domains, is an important cell wall component of endo- and exodermis cells, as well as cork cells of the periderm (Soler et al. 2007, Ranathunge et al. 2011). The morphological differences between low N and high N roots suggest that there could be differences in suberin content between rot masses from the two different treatments, with corresponding differences in transcript abundance of suberin biosynthetic genes. While the identity of these genes is mostly unknown in poplar, recent advances made in suberin biosynthesis of other species implicates enzymes from families such as the cytochrome P450s, peroxidases, and acyltransferases, in addition to genes of the phenylpropanoid pathway (Ranathunge et al. 2011, Soler et al. 2007). Several cytochrome P450s were detected as differentially expressed in this study (MN5187417, MN5188547, MN5183785, and MN5189817), as was a peroxidase (MN5185051), and the aforementioned caffeoyl Co-A O-methyltransferase. While these data are insufficient to connect these genes to suberin biosynthesis, we hypothesise that they provide attractive candidates to test for a potential role in synthesis of this complex molecule.

N-responsive genes in poplar roots putatively associated with biotic and abiotic stress responses

Changes in cell wall composition can be induced by a variety of biotic and abiotic stresses (Moura et al. 2010, Pinot and Beisson 2011). Besides cell wall biosynthetic enzymes, other differentially expressed genes encoding enzymes of secondary (natural products) metabolism could potentially function in biotic and abiotic stress responses, such as BAHD acyltransferases (MN5184657, benzoyl coA:benzyl alcohol transferase-like; Tuominen et al. 2011), phenylcoumaran benzylic ether reductases (MN5183717; (Min et al. 2003)), and cytochrome P450s (MN5187417, MN5188547, MN5183785, and MN5189817; Pinot and Beisson 2011). Differential expression of stress response genes under contrasting N regimes has previously been reported elsewhere. (Wang et al. 2001, Wei et al. 2013), and a number of other differentially expressed genes show similarity to genes commonly implicated in stress responses, such as a thionin-like protein (MN5184667, high N induced), RD22-like dehydration responsive protein (MN5183604, high N induced), pathogenesis related-1 protein (MN5187806, high N induced), heat shock protein 70 (MN5188397, low N induced), glutathione S-transferase (MN5188290, low N induced; Foyer and Noctor 2009), and late embryogenesis abundant protein (MN5189709, high N induced). Other N-responsive genes could also be implicated in stress responses, such as a putative defense protein (MN5187347, low N induced), DUF538 domain protein (MN5184691, low N induced), apyrase (MN5185917, low N induced; Navarro-Gochicoa et al. 2003), and DNA-J chaperone-like (MN5184481, high N induced. As previously reported in Pitre et al. (2010), MN5184481 was differentially expressed in secondary xylem in response to both high N fertilization and stem leaning, suggesting that the transcript levels of this putative DNA-J protein are affected by environmental factors. This collection of stress-associated genes does not exhibit a concerted pattern of up- or down-regulation, suggesting that N availability modulates a suite of stress responsive mechanisms rather than direct a wholesale increase or decrease in stress response capacity.

Differentially expressed genes such as *DUF538* and *glutathione S-transferase* may be implicated in redox regulation commonly associated with stress responses (Foyer and Noctor 2009). Also related to redox regulation, at least three genes upregulated under high N were putatively associated with oxidative stress and hydrogen peroxide signalling, including the aforementioned peroxidase. MN5187491 belongs to the polyamine oxidase family comprising enzymes oxidizing polyamines and concomitantly generating hydrogen peroxide (Fincato et al. 2011). Recently, both polyamines and hydrogen peroxide have been implicated not only in stress response signalling, but also in differentiation and development of xylem and roots (Tisi et al. 2011). Similarly, *phospholipase D delta isoform* (MN5182963) mediates responses to hydrogen peroxide in stress responses, but also is implicated in regulation of development (Wang 2005, Munnik and Testerink 2009, Zhang et al. 2009).

Temporal transcript profiling of N-responsive cell wall-associated genes

The microarray experiment was conducted on roots harvested 7 days following commencement of Low N/High N treatments, and thus provides us with a snapshot of gene expression occurring at this particular time. To illustrate the temporal response to low or high N availability of transcript accumulation for a subset of genes associated with cell wall biosynthesis, we carried out quantitative RT-PCR to investigate transcript abundance profiles for these genes over a two week time course of differential N availability (Fig. 3). The qRT-PCR transcript abundance profiles are in agreement the microarrays results, and illustrate that N-induced changes to the root transcriptome can occur quickly, as in the case of the putative xyloglucanase inhibitor (MN5185332). The putative xyloglucanase inhibitor exhibited increased transcript levels after just 1 day of high N availability, while depletion of N in the potting mix resulted in decreased transcript levels. An opposite response was obtained for FLA 1 (Fasciclin-like arabinogalatan protein 1; MN5184154) where high N decreased the levels of transcript from day 1. As reported for poplar wood formation, this decreased transcript levels correlates with xylem maturation (Lafarguette et al. 2004). The proline-rich cell wall protein we studied (MN5184232) had a tendancy to show lower levels of transcripts in response to N availability irrespective of the level of N and of the time of the analysis. The RT-qPCR results suggested that after 14 days of high N availability the proline-rich cell wall protein had lower transcript level than the control, similar to the microarray data.

A comparison between the sequences identified as differentially expressed in poplar roots (this study) with the transcripts DE accumulated in xylem (data not shown, modified from Pitre et al. 2010) clearly illustrate that genes are specifically induced in roots in response to nitrogen availability (Fig. 3).

Fig. 3. Venn diagram presenting the genes differentially expressed in response to nitrogen in xylem, in roots and shared between the two tissues.

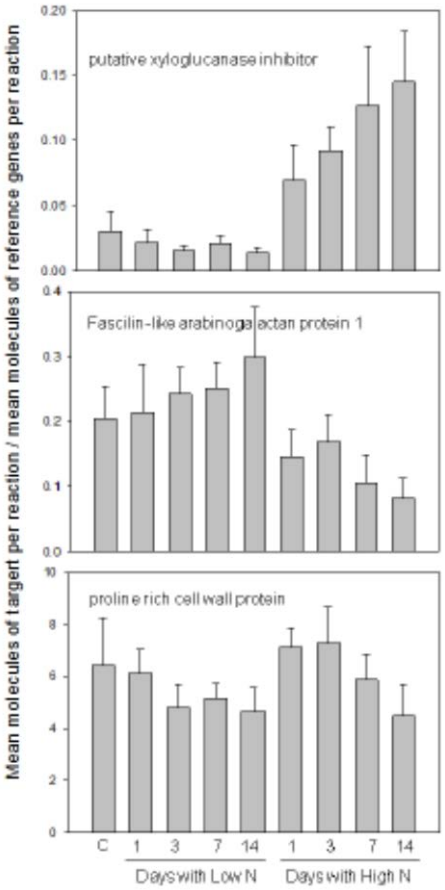

Fig. 4. Quantitative transcript accumulation (RT-qPCR) of targeted sequences over 14 days. Time-course analysis of MN5185332 (Putative xyloglucanase inhibitor); MN5184154 (Fascilin-like arabinogalactan protein 1); and MN5184232 (Proline rich cell wall protein). Results are presented as mean molecules of target sequence/mean molecules reference sequence (i.e. POPTR_0012s06210.1[PP2A] and POPTR_0018s11660.1[TIF5A]). Means ± S.D. of six biological replicates are shown. The capital C is for the control at t= 0 day.

Conclusion –new sequences

Very few studies to date have documented the effects of nitrogen availability on gene expression in tree roots. As such, new insights on poplar root-expressed N-responsive genes have been revealed through interrogation with this modest-scale custom cDNA array. Wei et al. (2013) used poplar microarray to indentify genetic networks involved in root growth in response to low nitrogen. Our results are in agreement with the biological processes they identified as significantly affected, namely 1) metabolic processes such as organic compound processes, secondary metabolic processes and oxidation-reduction processes, 2) response to stimulus such as chemical stimulus, abiotic stimulus and stress, 3) cellular localization such as establishment of localization and transport.

These results indicate that changing N availability modulates the poplar root transcriptome within days, including altered transcript levels of genes associated with growth and developmental processes and stress responses. Extrapolating from these gene expression data suggests N-responsive molecular processes that may mediate the observed changes in root architecture and other characteristics that translate into altered soil exploration patterns and dynamics by poplar roots in response to differential N availability (Cahill et al. 2010). The gene expression data also suggest that, in parallel to these developmental changes, transcription-level changes are being orchestrated in response to N availability to alter the plant's constitutive array of responses to abiotic and/or biotic stresses. These findings thus form the basis of a conceptual model that can now be explicitly tested in future investigations. Several of the N-responsive genes identified in this study have limited annotation, but possibly encode products that condition traits such as nitrate removal, nitrogen use efficiency and soil exploration, i.e. traits that are important in the application of poplar in multi-purpose agrosystems. As such, future studies will also focus on characterization of the function of the products encoded by these weakly annotated genes.

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