Gene expression associated with N-induced shifts in resource allocation in poplar

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ABSTRACT

Surprisingly little is known about molecular mechanisms by which nitrogen (N) availability acts to modulate the growth of forest trees. To address this issue, differential display was used in conjunction with filter-based arrays to identify 52 partial cDNA clones that were significantly regulated within days in response to limiting or luxuriant levels of NH3NO3 fertilization in Populus trichocarpa Torr. & Gray × deltoides Bartr. ex Marsh. A subset of these cDNAs also demonstrated shifts in expression patterns in stem-girdled trees, a manipulative physiology technique that disrupts phloem transport. Stem girdling also induced changes in glutamine and asparagine pools which were correlated with the observed changes in expression profiles for these genes. The identity of these genes provides insight into biochemical processes that are altered by N availability in poplar. Carbon–nitrogen interactions appear to figure prominently in the N-response. The gene expression data suggest that N availability modulates the partitioning of C and N resources into metabolic fates that have the potential to alter both wood quality and quantity, including synthesis of vegetative storage proteins, cell wall components, and terpenoids.

Key-words: carbon-nitrogen balance; cell walls; defence; lignin; one-carbon metabolism; terpenoids; vegetative storage proteins.

INTRODUCTION

Nitrogen has dramatic effects on plant growth and architecture. These changes in growth are a manifestation of changes in the expression of a myriad of genes that modulate resource utilization. Together, these changes in gene expression can influence how C and N resources are allocated amongst plant parts to determine plant proportions, and how these resources are partitioned into biochemical pathways that ultimately determine the characteristics of a tissue (e.g. Scheible et al. 1997a; Nielsen et al. 1998).

Comparatively little is known about the effect of N availability on gene expression in perennial species, and how N-associated changes in gene expression mediate changes in growth. However, perennial species presumably contend with considerable variation in N availability over the course of the plant’s lifespan, and this is likely reflected in the overall size and form of the plant. Poplars are an ideal system to study the effects of N on the growth of a perennial species because they have a remarkable ability to take up and utilize N from their surroundings (Min et al. 2000). Furthermore, poplars display great plasticity in growth and architecture in response to changing N availability (e.g. Liu & Dickmann 1992; Heilman & Xie 1993; Coleman, Dickson & Isebrands 1998).

The N resources not only provide N moieties to biosynthetic pathways; inorganic and organic N compounds can also mediate changes in gene expression (Stitt 1999; Coruzzi & Zhou 2001). Recent genomic surveys of nitrate-responsive gene expression patterns in Arabidopsis (Wang et al. 2000) and tomato (Wang, Garvin & Kochian 2001) indicate that many genes respond rapidly to changes in N. The N-responsive genes identified in these and other studies encode for a diverse array of proteins involved in many cellular processes, including metabolism and signal transduction.

Phloem transport is an important means of allocating nutrients, including N compounds, among plant parts, and also provides a vital conduit for communication between tissues (Oparka & Turgeon 1999). Poplars are a good system for studying the role of phloem transport and phloem-transmissible compounds in the N-response. Stem phloem is relatively accessible in the bark of poplars and other trees, and makes up a sizeable percentage of the cells constituting young bark (Teleswki, Aloni & Sauter 1996). In addition, patterns of transport for both C and N compounds have been well studied in poplar (reviewed in Dickson 1989). Glutamine (gln) is the major organic N transport compound in poplar (Dickson 1979; Sauter & van Cleve 1992), and is preferentially transported up the stem to developing leaves via a xylem-to-phloem transfer facilitated by ray cells (Dickson, Vogelmann & Larson 1985). Gln modulates the
expression of a number of genes in herbaceous plants (Sivasankar, Rothstein & Oaks 1997; Lam, Hsieh & Coruzzi 1998; Oliveira & Coruzzi 1999; Rawat et al. 1999). Recently, the promoter of a poplar gene encoding bark storage protein (bspA) was demonstrated to be transcriptionally regulated by gln (Zhu & Coleman 2001a, 2001b).

In this study, our objective was to identify genes whose expression patterns were correlated with N-induced shifts in resource allocation. We used differential display in conjunction with filter arrays to identify 52 cDNAs whose corresponding transcript levels are significantly modulated by nitrogen availability in *Populus trichocarpa* Torr. & Gray × deltoides Bartr. ex Marsh. Stem-girdling experiments indicate that expression of a subset of these genes is also altered by phloem-transmissible compounds. Changes in levels of the phloem-transmissible amino acids gln and asparagine (asn) are correlated with the girdling-induced changes in gene expression patterns. The identities of these genes, together with their expression profiles, suggest N-modulated processes involved in the allocation and partitioning of resources that potentially impact both wood quality and wood quantity.

**MATERIALS AND METHODS**

**Plant material and cultivation**

Rooted cuttings of *Populus trichocarpa* Torr. & Gray × deltoides Bartr. ex Marsh hybrid UCC-1 (provided by Union Camp Corporation, Savannah GA, USA in 1997) were grown as described in Lawrence et al. (1997). The plants used in the differential display experiments were grown in growth chambers (Conviron Model E15; Controlled Environments Ltd, Winnipeg, MB, Canada) under a 12 h photoperiod at 25 °C. All other experiments were carried out in greenhouses maintained at 20–35 °C under natural lighting with day lengths of 12–14 h. Plants were fertilized every 3 days with Hocking’s complete nutrient solution (Hocking 1971) supplemented with 5 mM ammonium nitrate (molarity calculated with respect to NH₄NO₃) prior to experimentation.

**N-availability experiments**

Plants that were approximately 60–80 cm tall were ranked according to height. Each cohort was then divided equally and individuals of a cohort assigned randomly between treatments such that there were at least three plants per treatment in each experiment. At day 0, the plants were fertilized daily with Hocking’s solution supplemented with 0 mM, 2 mM, or 50 mM NH₄NO₃. Harvested tissues were quick frozen in liquid nitrogen and stored at −80 °C until analysed. Leaves were numbered according to leaf plastochron index (LPI; Larson & Isebrands 1971), with LPI 0 being approximately 2 cm long with a one-half expanded lamina. Shoot tip samples included the shoot apex, all leaves up to and including LPI 1, and the adjoining stem segment. Tissue samples for differential display and RNA analysis included source leaves collected at approximately one-third of the total plant height (generally, LPI 15 and LPI 16 were harvested), stems (sampled at internodes between LPI 14 and LPI 17), and total root mass.

**Stem-girdling experiments**

Plants at least 60 cm in height were assigned to treatments as described above. Daily fertilization with Hocking’s solution plus 2 mM NH₄NO₃ was started 2 weeks prior to girdling and continued through the duration of the experiment. Girdling was performed at day 0 by mechanically removing a 1 cm strip of bark just above the midpoint of the stem, which corresponded to between LPI 8 and LPI 11, depending on plant height. Source leaves were present both above and below the girdle. A single internode of stem section was harvested one internode away from the girdle. A region corresponding to the girdle was marked on the stems of control plants so that comparable tissues could be harvested. This control non-girdled tissue is referred to in the text as a ‘girdle-equivalent’. Stem sections were separated into wood and bark for subsequent analyses. These are referred to as xylem and phloem, respectively, since these are the predominant tissues in the wood and bark at this stage of development. Harvests were carried out at the midpoint of the day.

**Carbon and nitrogen analyses**

Total C and N content of tissues was determined using a NCS 2500 automatic elemental analyser (CE Instruments, ThermoQuest Italia SpA, Milan, Italy). The instrument was calibrated with a pine needle standard (National Institute for Standards and Testing, Gaithersburg, MD, USA).

**Photosynthesis measurements**

CO₂ exchange was measured with a portable infrared gas analyser (Li 6400P photosynthesis system; Li-Cor Inc., Lincoln, NE, USA). Photosynthetically active radiance was maintained at 1000 µmol m⁻², airflow rate at 500 µmol s⁻¹ and leaf temperature between 27 and 30 °C. The chamber CO₂ was adjusted to 400 µmol mol⁻¹.

**Amino acid analyses**

Amino acids extracts were prepared according to King & Gifford (1997). Samples were derivatized with phenylisothiocyanate on an ABI420 derivatizer and analysed with an ABI172 high-performance liquid chromatograph (HPLC; Applied Biosystems, Foster City, CA, USA).

**RNA extraction and differential display**

Total RNA was extracted using a CTAB method (Chang, Puryear & Cairney 1993). All steps of the differential display [differential display reverse transcriptase (DDRT)-polymerase chain reaction (PCR)] process were conducted.
essentially according to the manufacturer’s instructions (GenHunter, Nashville, TN, USA). Briefly, reverse transcrip-
tion was carried out with DNase-treated RNA using MMLV-RT from RNAimage (GenHunter) or Gibco-BRL
(Gaithersburg, MD, USA) with identical results. PCR was
performed with factorial combinations of the three
anchored primers and 31 random primers (no. 9 to 39), for
a total of 93 reactions (GenHunter); 74 KBq α-[32P]dATP
(NEN, Boston, MA, USA) was included in each 10 µL reac-
tion. The PCR products were separated in 6% denaturing
polyacrylamide gels (HR1000 gel; Genomyx, Foster City,
CA, USA) subjected to electrophoresis under 60 W con-
stant power, and visualized via autoradiography. Differential
displayed products were recovered, re-amplified by
PCR, and ligated into pGEM-T (Promega, Madison, WI,
USA) for transformation into competent E. coli DH5α
(Gibco BRL). Sequencing was carried out by the Uni-
versity of Florida DNA Sequencing Core Laboratory
(Gainesville, FL, USA). Sequences were analysed using BLAST
utilities (Altschul et al. 1997) at NCBI

Array Construction cDNA inserts were PCR-amplified
from plasmids using SP6 and T7 primers and purified with
Qiaex resin (Qiagen, Valencia, CA, USA). PCR products
were checked on agarose gels to ensure appropriate size,
quantity and integrity. Loading dye was added to the cDNA
to facilitate spotting.

The filter arrays were produced using a procedure mod-
ified from Desprez et al. (1998). cDNAs were arrayed onto
Hybond N membranes using a 96-pin colony replicator
together with an alignment template that allowed for a total
of 384 spots per membrane (V & P Scientific, San Diego,
CA, USA). Each spot contained approximately 4 ng DNA.
After arraying the cDNAs, the membranes were incubated
DNA side up on 1.5 M NaCl/0.5 M NaOH for 10 min, fol-
lowed by 0.5 M Tris-HCl (pH 7.4)/0.5 M NaOH for 5 min.
Membranes were rinsed in 2× NaCl/sodium citrate buffer
(SSC)/0.1% (w/v) sodium dodecyl sulphate (SDS) before
air-drying.

Each cDNA was arrayed as a quartet; namely a group of
four spots. Each quartet was replicated at random three
times on a membrane. A total of four membranes were used
to accommodate all cDNAs. Two cDNAs (pni79 and
pot178), shown by Northern blot analysis to be expressed
at relatively constant levels in all tissues under different
nitrogen regimes, were included on every membrane for
comparison purposes. In addition, a cDNA (pni171–4)
known to be a low abundance transcript based on Northern
analysis was included on each blot.

Probe preparation and array hybridization

Arrays were probed with first-strand cDNA synthesized
from 8 or 10 µg total RNA using MMLV-RT (Gibco-BRL),
with 40 pmol (4.44 MBq) α-[32P]dCTP, and dATP, dTTP
and dGTP in excess. Labelled cDNA probes were sepa-
rated from unincorporated nucleotides using Sephadex
G50 columns (Amersham Pharmacia Biotech, Ithaca, NY,
USA), and quantified by liquid scintillation (Beckman
Coulter, Fullerton, CA, USA). Hybridization and washing
were carried out at 65 °C using standard protocols
described in Davis et al. (1991).

Array data acquisition and analysis

Spot intensities on the arrays were visualized with a phos-
phorimager and quantified using the manufacturer’s ImageQuant software (Molecular Dynamics, Amersham Biosciences Corp., Piscataway, NJ, USA). Data were
imported into Microsoft Excel (Microsoft Corp., Redmond,
WA, USA) for further analyses.

Defective spots, including those with high local back-
ground, were removed from the analysis. To facilitate com-
parisons between blots, data were scaled by dividing each
spot value by the standard deviation of all spots on a blot
(x = x/s), a slight modification of the scaling function used
by Schenk et al. (2000). As an independent check, data were
also scaled by dividing each spot value by the average value
of the two ‘constitutive’ cDNAs included on every blot.
There was an overall 85% agreement between the two
scaling methods in the statistical analysis. However,
because scaling with standard deviations is a more widely
applicable and objective technique, the data in this paper
are presented as x = x/s. A low signal cut-off value for each
blot was determined as the mean value plus one standard
deviation (x + s) of the low-expressing cDNA described
above. A cDNA that did not exhibit a mean value greater
than this cut-off value in at least one treatment of the
statistical analysis was considered below detectable limits.

Statistics

Statistical analyses of the array data and physiological data
were essentially the same: t-tests, ANOVA, and multiple
comparisons of means (with least squared means option)
were carried out using Excel and SAS (SAS Institute, Cary,
NC, USA).

RNA blots

RNA blots were used to confirm expression patterns for
several cDNAs. The cDNAs for labelling were prepared by
PCR as described above, and radiolabelled with α-
[32P]dCTP using random primer labelling (GibcobRL).
Formaldehyde-agarose gel electrophoresis of total RNA, as
well as hybridization and washing of the RNA blots, was
carried out essentially as described in Davis et al. (1991).
The final wash [1% (w/v) SDS/1 mM EDTA/40 mM Na
phosphate buffer (pH 7.4)] was performed at 55 or 65 °C.

RESULTS

Nitrogen availability affects resource allocation in poplar within days

In order to determine the time frame in which shifts in
resource allocation occur in *Populus trichocarpa × deltoides*
in response to N fertilization, we examined total N and C content of leaves, stems and roots over a 4 week time course treatment with limiting (0 mM), adequate (2 mM), or luxuriant (50 mM) NH₄NO₃. The mean height of the trees at day 0 was 74.7 ± 9.2 cm (mean ± SD). After 28 d of treatment with 0, 2 or 50 mM NH₄NO₃, the tree heights were 146.7 ± 10.9, 172.7 ± 14.8 and 168.3 ± 10.4 cm, respectively. The trees did not exhibit symptoms of deficiencies for other nutrients, such as P, K, or Mg over the course of the experiment (data not shown).

The trees showed perceptible differences in total N content after 7 d of treatment (Fig. 1a–c). The difference in N content of stems and roots grown with 0 versus 50 mM NH₄NO₃ was statistically significant at 7 d (Fig. 1b–c). There were significant differences in leaf N content at 14 d (Fig. 1a). These differences were even greater by 28 d of treatment. When expressed on a percentage dry weight basis, differences in N content between 0- and 50 mM NH₄NO₃-treated plants were statistically significant in leaves, stems and roots by 7 d of treatment (data not shown). By 28 d, there were 2.5-, 4.9- and 3.0-fold differences in percentage N content between 0- and 50 mM NH₄NO₃-treated plants (data not shown).

Total C allocated to roots did not differ significantly between treatments at 7, 14 or 28 d (Fig. 1f). Although the roots never stopped growing during these experiments, containment of roots by the pots may have limited root growth to some extent, which is an inherent limitation of pot studies. Total C allocated to leaves steadily increased in 2 and 50 mM NH₄NO₃-treated plants, but remained relatively constant in 0 mM NH₄NO₃-treated plants (Fig. 1d). Total C content of leaves from 2 and 50 mM NH₄NO₃-treated plants was significantly different from total C of leaves from 0 mM NH₄NO₃-treated plants by 14 d, and the differences were more pronounced at 28 d. Increased allocation of C resources to leaves under adequate and luxuriant N conditions was mainly due to increased size and number of leaves (data not shown). Differences in C allocation to stems of 0 versus 50 mM NH₄NO₃-treated plants were significant only by 28 d of treatment (Fig. 1e).

Increasing N availability caused a shift in the C : N ratio of all tissues (Fig. 1g–i), even though increasing N availability caused an increase in both N and C resources to leaves and stems. Differences in C : N ratios between treatments were statistically significant by 7 d of treatment. By 28 d of treatment, there were 2.5-, 5.2- and 3.9-fold differences between the C : N ratios of 0 and 50 mM NH₄NO₃-treated plants in leaves, stems and roots, respectively.

Using DDRT-PCR and arrays to identify N-associated genes

DDRT-PCR was carried out with RNA from plants treated daily for 10 d with either 0 or 50 mM NH₄NO₃, namely during the window in which shifts in allocation were occurring. RNA from shoot tips, mature leaves and stems was used in the DDRT-PCR reactions. Ninety-three primer
combinations were used for differential display, suggesting that approximately one-third of the transcriptome was sampled (RNAimage; Liang & Pardee 1997). Altogether, 97 unique DD cDNA products were successfully amplified, cloned and sequenced. These cDNAs were designated pni (poplar nitrogen-responsive), based on their putative regulation. These pni clones, as well as additional cDNAs from our collection relevant to N utilization and metabolic processes (designated po or ppot), were used for analysis of mRNA abundance with filter arrays. Since pni34, pni67 and pni207 were 98% identical to bsp, encoding bark storage protein (BSP), and exhibited an expression pattern identical to that of bsp in Northern blot analysis (data not shown), the full-length bsp was used on the arrays instead of the partial cDNAs.

Arrays were probed with first-strand cDNA synthesized from mRNA extracted from shoot tips, leaves, stems or roots of plants treated with either 0 or 50 mM NH4NO3 daily for 14 d. Transcript abundance for each of the 116 cDNA clones in each of the different tissues from 0 versus 50 mM NH4NO3-treated plants were statistically compared using t-tests. Because of the variation associated with measuring transcription abundance from experiment to experiment, a relatively permissive α = 0.1 was chosen as the rejection level. Altogether, 52 of the 116 cDNA clones analysed by filter arrays demonstrated significantly different transcript levels in 0 versus 50 mM NH4NO3-treated plants in at least one tissue (Table 1; Fig. 2). This included 49 pni clones identified by DDRT-PCR. Ten of the 116 cDNA clones displayed transcript abundances below the threshold set for reliable detection in all tissues (see Materials and methods for details). Of the 52 cDNAs showing a significant N response, eight pni clones show no similarity to GenBank sequences. Another 17 pni clones show similarities to genes or ESTs with unknown function.

Northern blots were used to confirm the expression patterns for 24 pni clones, representing both significantly and not significantly responsive cDNAs (data not shown). In all cases, the array expression patterns were confirmed using Northern blots. In five of the 24 comparisons, differences between 0 and 50 mM NH4NO3 treatments observed with Northern blots were not found to be statistically significant in the array data, although the transcript abundance patterns were consistent. This was mainly due to variance associated with the array data.

Twenty of the 26 cDNAs that displayed a significant response to N levels in stems (Fig. 2) also showed significant differences (P < 0.01) in transcript abundance in xylem versus phloem. Of these, 12 showed higher expression in phloem, whereas the remainder showed higher expression in xylem. A subset of these cDNAs is depicted in Fig. 3.

Girdling as a tool to disrupt phloem transport

Removing a strip of bark from the circumference of a tree stem can be used to disrupt phloem transport (e.g. Day & DeJong 1990; Sauter & Neumann 1994) (Fig. 4a). Xylem-to-phloem transfer of gln has been shown to occur along the entire stem length in poplar, with a higher proportion occurring between LPI 8 and 13 (Dickson et al. 1985). Accordingly, we positioned the girdle between LPI 8 and LPI 11. Photosynthesis was used as an indicator of plant vigour in the girdling experiments. Photosynthetic rates of leaves above or below the girdle or girdle-equivalent did not show significant changes over the duration of the experiment (data not shown).

Although girdling did not significantly affect percentage N (α = 0.05) above the girdle or girdle-equivalent, it stimulated a significant increase below the girdle within 3 d (Fig. 4). By 6 d, there was a 2.4-fold and 1.8-fold greater percentage N content in girdled stem xylem (Fig. 4b) and phloem (Fig. 4c), respectively, relative to control tissues below the girdle or girdle-equivalent. This shifted the C:N ratio by approximately two-fold in both xylem and phloem tissues (Fig. 4d).

HPLC analysis of soluble amino acid pools demonstrated that stem girdling resulted in a statistically significant increase (α = 0.05) in total amino acids in both the xylem and phloem below the girdle compared to control plants (data not shown). Although there was a decrease in total amino acids in xylem and phloem above the girdle compared to control plants, this difference was not statistically significant (data not shown).

HPLC analyses also established that gln and asn are the major components of the soluble amino acid pool of both xylem and phloem for Populus trichocarpa × deltoides fertilized with 2 mM NH4NO3. Gln and asn made up 65 ± 7 and 19 ± 3% of the xylem amino acid pool, and 62 ± 4 and 23 ± 4% of the phloem amino acid pool of ungirdled plants (mean ± SD), respectively.

Gln and asn levels increased significantly in both xylem and phloem below the girdle in comparison with control plants (Fig. 5). A significant increase in gln in xylem below the girdle was detected by 1 d after girdling. Increases in gln in phloem below the girdle, and increases in asn in both xylem and phloem below the girdle were significant by 3 d after girdling. Gln and asn levels did not significantly change in xylem or phloem of the control plants.

The accumulation of gln and asn in tissues sampled below the girdle was significantly greater than the 2.8-fold (xylem) and 1.5-fold (phloem) increases observed in the pool of 17 other amino acids combined (excluding arginine, see below) by 6 d after girdling (data not shown). By 6 d after stem girdling, there was a 5.3- and 5.6-fold increase in gln and asn, respectively, in xylem below the girdle compared with its control. There was a 2.5- and 3.2-fold increase in gln and asn, respectively, in xylem below the girdle compared with its control. There was a 2.5- and 3.2-fold increase in gln and asn, respectively, in xylem below the girdle. Gln levels also decreased above the girdle in xylem (2.0-fold) and phloem (2.8-fold). Only the difference in phloem gln content was statistically significant at α = 0.05. Asn levels did not appreciably decrease above the girdle in either xylem or phloem.

Arginine also showed a statistically significant increase in both xylem and phloem below the girdle relative to tis-
Table 1. Nitrogen-responsive cDNA clones identified by differential display in Populus trichocarpa × deltoides

<table>
<thead>
<tr>
<th>Clone</th>
<th>Length (bp)</th>
<th>Accession no</th>
<th>Related sequence</th>
<th>E value</th>
</tr>
</thead>
<tbody>
<tr>
<td>bsp</td>
<td>1153</td>
<td>CAA49669</td>
<td>Populus bark storage protein</td>
<td>–</td>
</tr>
<tr>
<td>win4</td>
<td>1112</td>
<td>AAI16342</td>
<td>Populus vegetative storage protein</td>
<td>–</td>
</tr>
<tr>
<td>pni288</td>
<td>934</td>
<td>AF330050</td>
<td>Populus win4 vegetative storage protein (AAA16342)</td>
<td>$3 \times 10^{-41}$</td>
</tr>
<tr>
<td>pni69</td>
<td>695</td>
<td>BU791162</td>
<td>Lycopersicon ADP-glucose pyrophosphorylase large subunit (U85497)</td>
<td>$3 \times 10^{-39}$</td>
</tr>
<tr>
<td>pni88</td>
<td>271</td>
<td>BU791222</td>
<td>Lycopersicon osmotin-like protein (L76632)</td>
<td>$3 \times 10^{-4}$</td>
</tr>
<tr>
<td>pni95</td>
<td>201</td>
<td>BU791224</td>
<td>Arabidopsis similar to protein phosphatase 2C (ABH1-like) (AAD46006)</td>
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</tr>
<tr>
<td>pni102</td>
<td>387</td>
<td>BU791128</td>
<td>Medicago histone H3 (U09460)</td>
<td>$1 \times 10^{-4}$</td>
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<tr>
<td>pni107</td>
<td>537</td>
<td>BU791129</td>
<td>Solanum soluble starch synthase (P93568)</td>
<td>$2 \times 10^{-28}$</td>
</tr>
<tr>
<td>pni122</td>
<td>835</td>
<td>BU791131</td>
<td>Arabidopsis similar to bHLH regulatory proteins (AL023094)</td>
<td>$3 \times 10^{-46}$</td>
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<td>pni134</td>
<td>430</td>
<td>BU791225</td>
<td>Brassica pollen coat protein similar to cold-induced kin1 (BAB10133)</td>
<td>$7 \times 10^{-7}$</td>
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<tr>
<td>pni145</td>
<td>592</td>
<td>BU791172</td>
<td>Arabidopsis similar to chloroplast 31 kDa RNA-binding protein (CAP78028)</td>
<td>$8 \times 10^{-46}$</td>
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<tr>
<td>pni150</td>
<td>1250</td>
<td>BU791137</td>
<td>Arabidopsis glutathione-S-transferase (U70672)</td>
<td>$3 \times 10^{-17}$</td>
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<tr>
<td>pni159</td>
<td>705</td>
<td>BU791139</td>
<td>Citrus thiazole biosynthetic enzyme (Z28983)</td>
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<td>pni164</td>
<td>267</td>
<td>BU791176</td>
<td>Nicotiana kinesin-like calmodulin-binding protein</td>
<td>$2 \times 10^{-2}$</td>
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<td>pni183</td>
<td>731</td>
<td>BU791143</td>
<td>Arabidopsis similar to recA and LRR proteins (AAG51016)</td>
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<td>pni212</td>
<td>583</td>
<td>BU791147</td>
<td>Glycine SE50 sulfur-rich protein similar to proteinase inhibitor II (Z18359)</td>
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<td>Arabidopsis ABC transporter-like protein (BAB10074)</td>
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<td>$4 \times 10^{-39}$</td>
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<td>pni240</td>
<td>540</td>
<td>BU791152</td>
<td>Lavatera LTC011 similar to GASA, gibberellin-regulated proteins (AF00784)</td>
<td>$9 \times 10^{-24}$</td>
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<td>771</td>
<td>BU791154</td>
<td>Gossypium sesquiterpene cyclase (U88318)</td>
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<td>pni275</td>
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<td>BU791195</td>
<td>Arabidopsis similar to set hexomythyltransferase (AAC40343)</td>
<td>$5 \times 10^{-29}$</td>
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<tr>
<td>pni278</td>
<td>776</td>
<td>BU791156</td>
<td>Arabidopsis similar to vacuolar sorting receptor (AAF98196)</td>
<td>$3 \times 10^{-31}$</td>
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<td>pni282</td>
<td>492</td>
<td>BU791157</td>
<td>Lycopersicon histone H1 (AF253416)</td>
<td>$5 \times 10^{-3}$</td>
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<td>pni287</td>
<td>461</td>
<td>BU791158</td>
<td>Malus proline rich protein (T71107)</td>
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<td>pni289</td>
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<td>BU925847</td>
<td>Pyrus Rubisco ss (D00572)</td>
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<td>AY166668</td>
<td>Populus photosystem Q(B) protein (P36491)</td>
<td>$3 \times 10^{-26}$</td>
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<tr>
<td>pto164</td>
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<td>AY166669</td>
<td>Fragaria hydroxyproline rich protein (AAD01800)</td>
<td>$4 \times 10^{-34}$</td>
</tr>
<tr>
<td>pot171</td>
<td>536</td>
<td>AY161276</td>
<td>Populus Caffeyl-CoA 3-O-methyltransferase (AIJ224895)</td>
<td>$3 \times 10^{-39}$</td>
</tr>
</tbody>
</table>

The length of the 3’ cloned partial cDNA is given in base pairs. Sequence similarities based on BLASTN (ESTs) or BLASTX (others) (NCBI). The GenBank accession number of the sequence demonstrating the highest similarity is indicated in parentheses.
Figure 2. A nitrogen-response roadmap in poplar. Filters arrayed with cDNAs were probed with $^{32}$P-labelled first-strand cDNA synthesized from 10 µg total RNA extracted from tissues of plants treated with 0 or 50 mM NH$_4$NO$_3$ for 16 d. t-tests were used to statistically compare scaled transcript abundance values in 0 versus 50 mM NH$_4$NO$_3$ for each tissue for each cDNA. The cDNAs showing significantly greater transcript abundance with 0 mM NH$_4$NO$_3$ treatment than with 50 mM NH$_4$NO$_3$ treatment were considered to be low-N induced, and are shown in yellow ($P=0.01–0.1$) or red ($P<0.01$). The cDNAs showing significantly greater transcript abundance in 50 mM NH$_4$NO$_3$ treatment than with 0 mM NH$_4$NO$_3$ treatment were considered to be high-N induced, and are shown in light green ($P=0.01–0.1$) or dark green ($P<0.01$). Light grey indicates no significant difference in transcript abundance was detected between treatments. Dark grey indicates that the mean transcript abundance in both 0 and 50 mM NH$_4$NO$_3$ treatments was below the mean + 5D transcript abundance for a low-abundance reference cDNA. Individual cDNAs were replicated three times.
sues sampled from control plants below the girdle equivalent. However, arginine accounted for only 1–2% of the total soluble amino acid pool of tissues from control plants, and 2–3% of the total soluble amino acid pool of tissues sampled below the girdle (data not shown).

A subset of N-associated genes is also girdling responsive

Filter arrays were used to compare gene expression profiles in xylem and phloem of girdled versus control plants sampled both above and below the girdle or girdle-equivalent. We used 6 d girdled plants to maximize the probability of

Figure 3. Several stem N-responsive cDNAs are preferentially expressed in xylem or phloem. Trees were treated for 3 weeks with 2 mM NH4NO3. Filters were probed with 32P-labelled cDNA synthesized from 8 µg total RNA. Transcript abundance is expressed as the log$_{10}$ values of $T = 10^{(X_i/s)}/n$ (see Materials and methods). Individual cDNAs were replicated three times. Plotted values for xylem (cross-hatched bars) and phloem (solid bars) are means ± SD. Values to the right of each bar pair indicate the fold difference in expression of a cDNA between xylem and phloem.

Figure 4. Disrupting phloem transport by stem girdling causes an increase in percentage N below the girdle. (a) A schematic representation of stem girdling, providing a legend used for the bar fills in b–d. (b) Xylem percentage N ratios of girdled plants to control plants, sampled above (striped bars) or below (grey bars) the girdle or girdle-equivalent. (c) Phloem percentage N ratios of girdled plants to control plants, sampled above (striped bars) or below (grey bars) the girdle or girdle-equivalent. (d) C : N ratios at 6 d after girdling of control tissues above (diagonally-hatched bars) or below (open bars) the girdle-equivalent; girdled tissues above (cross-hatched bars) or below (solid bars) the girdle. Results are the means of three separate experiments, with at least two plants per day per experiment. ANOVA and multiple comparison of means were used to determine significant differences ($\alpha = 0.05$). Means assigned the same letter are not significantly different. In (b), (c), lowercase letters are used to denote significance within tissue.
detecting statistically significant differences. The data were
analysed by multifactor ANOVA. cDNAs that showed a
significant treatment × position or treatment × position × tissue
interaction were further analysed using multiple compari-
son of means to determine which of the eight different
samples displayed significantly different transcript abun-
dance.

The cDNAs were considered positively N-responsive if
they demonstrated increased transcript abundance below
the girdle relative to above the girdle and to control plants.
The cDNAs were considered negatively N-responsive if
they demonstrated either increased transcript abundance
above the girdle relative to below the girdle and to control
plants, or decreased transcript abundance below the girdle
relative to above the girdle and to control plants. cDNAs
whose expression profiles showed differences mainly attrib-
utable to wounding (approximately equal induction in tis-
ues above and below the girdle) or development
(increased expression either above or below the girdle/gir-
dle-equivalent in both girdled and ungirdled tissues) were
removed from further analysis. Sixteen cDNAs identified
as significantly girdling-responsive by the multifactor
ANOVA showed a pattern of expression that was congruous
with their response to 0 versus 50 mM NH₄NO₃ fertilization.
Expression patterns for 10 cDNAs are depicted in Fig. 6.

As with the N-responsive cDNAs, expression patterns for
several girdling-responsive cDNAs were confirmed by
Northern blot analysis. The cDNAs that were not girdling-
responsible by array analysis were also assessed to test for
false negatives. In total, 14 cDNAs were examined by
Northern blots; the patterns of expression for all the
cDNAs were consistent between Northern blots and arrays
(data not shown).

**DISCUSSION**

**The N-response roadmap**

Availability of inorganic N is often cited as a major factor
limiting forest tree productivity (Dickson 1989). Many
studies conducted at the whole plant level have demon-
strated that N fertilization increases above-ground biomass
of forest trees (e.g. Teskey, Gholz & Cropper 1994; Samuel-
son 1998). Despite the economic and ecological importance
of forest trees, however, few tangible links have been made
between production physiology and molecular physiology
in forest tree species. Accordingly, we adopted a gene dis-
covey strategy as our starting point to identify molecular
mechanisms by which N availability impacts resource allo-
ocation and partitioning in poplar trees. The whole plant
allocation data allowed us to determine that shifts in
resource allocation in *Populus trichocarpa × deltoides* occur
primarily between 7 and 14 d of treatment with either lim-
iting or luxuriant levels of NH₄NO₃. To capture changes in
gene expression linked to shifts in resource allocation prior-
ity, DDRT-PCR was carried out with RNA from plants
treated daily for 10 d with either 0 or 50 mM NH₄NO₃.

The spatial representation of genes whose expression
profiles are altered in response to limiting versus luxuriant
N can be thought of as a roadmap of the response of a
woody plant to N availability at the transcriptional level. It
needs to be stressed that because we sampled for changes
in gene expression at 10–14 d post-treatment, many of the

genes that exhibit differential gene expression are probably not regulated \textit{sensu stricto} by inorganic or organic N compounds. For these genes, there are likely to be several degrees of separation between the perception of N compounds by the plant and the signal transduction networks that mediate their expression. Thus, within the set of genes on the N-response roadmap, some genes may be directly responsive to N compounds, whereas other genes are likely to be only indirectly responsive to N compounds. Nevertheless, differential expression of both subsets of genes coincides with changes in allocation of resources, and directly and indirectly N-responsive genes both have the potential to contribute to the overall changes in growth and architecture that are part of the global response to N availability.

The putative identities of genes shown to respond to limiting versus luxuriant N provide important clues to biochemical processes that may be altered by N availability in forest trees. At the same time, the number of genes on the roadmap for which there is no known function – 26 out of 52 or one-half of the genes – is noteworthy. Fourteen of these genes show a significant response to N in the woody tissues of the plant. Of these 14 genes, nine show no sequence similarity to genes with assigned function or to genes from the \textit{Arabidopsis} database. Also noteworthy is that 33 of the 52 genes on the roadmap are up-regulated under limiting N conditions. Previous studies on N-responsive gene expression have tended to focus upon genes that are positively responsive to N.
**Phloem-transmissible compounds alter the expression of some N-responsive genes**

We used experimental manipulation of phloem transport as the next step in characterizing the response of cDNAs on the N-response roadmap to N availability in poplar. Dickson et al. (1985) showed that gln is transported up the stem to developing leaves via a xylem-to-phloem transfer in poplars. We predicted that disrupting phloem transport by stem girdling would result in an accumulation of amino acids in stems below the girdle. Our data indicate that levels of both gln and asn are altered by stem girdling, mainly via an accumulation of gln and asn below the girdle. Thus, stem girdling is an experimental technique that can be used to manipulate the in planta N-status of trees over relatively short time frames.

The expression of a subset of stem-expressed genes on the N-response roadmap was found to be responsive to stem girdling. The expression patterns of these genes were correlated with altered gln and asn levels in the girdled plants, and were also consistent with their expression patterns in limiting versus luxuriant NH$_4$NO$_3$ applications. These data suggest that this subset of genes may be responding to altered N status of the tissues, and as such are putative N-responsive genes. We recognize that stem girdling alters pools of other phloem-transmissible compounds, and we cannot rule out the possibility at this point that these compounds may be affecting the observed changes in expression. Further studies, particularly with shorter time courses, will be essential to determine which of the genes that are responsive both to NH$_4$NO$_3$ application and to stem girdling are bona fide N-responsive genes. The remaining stem-expressed genes identified in the limiting versus luxuriant N availability study are either not girdling-responsive, or are girdling-responsive in a pattern inconsistent with tissue N status. The transcriptional activation of this subset of genes is probably not N-mediated, but rather a consequence of growth-associated parameters that result from increased N availability.

Promoter discovery and analysis of both directly and indirectly N-responsive genes will be integral components of a functional genomics approach to dissecting the N-response in poplar. Directly N-responsive genes ensuing from this work can serve as ‘anchors’ for studies of signal transduction networks in trees that are mediated by organic or inorganic N moieties. Cloning and analysis of directly N-responsive gene promoters may lead to the discovery and/or further definition of promoter motifs important in N-mediated signal transduction. Investigating the transcriptional activation of these promoters in trees may help us to understand how N moieties regulate N utilization strategies that are important for the perennial lifestyle, such as seasonal N cycling. Analysis of the bsp promoter in poplar has already yielded important insight into this process (Zhu & Coleman 2001a, b). No less important, promoter analysis of genes that are indirectly N-responsive may lead to the identification of motifs common to these promoters, and ultimately to the transcription factors that bind these motifs. In this way, these genes will help us to elucidate the far-reaching signal transduction ‘web’ that is initiated by N perception, helping to form a global picture of how N availability mediates plant growth and development. On a more applied level, N-responsive genes that exhibit tissue specificity make especially attractive targets for promoter discovery, since one of the constraints in woody plant transgenic research at present is the paucity of tissue-specific promoters with characterized triggers.

**N availability alters expression of genes that potentially impact wood properties**

In addition to identifying genes that may be useful for dissecting N-mediated signal transduction networks, the putative identities of genes that respond to limiting versus luxuriant N provide clues to cellular processes that are altered by N availability in forest trees. Based on these putative identities, we can make predictions about physiological events and metabolic pathways that may be altered in response to N availability in forest trees. This insight allows us to formulate specific hypotheses, so that we can begin to dissect the N-response at the biochemical level. In particular, we can explore how these changes at the biochemical level are affected by transcriptional regulation of the genes identified in this study.

By taking advantage of stem girdling as a means of altering the N status of poplars, we focused our attention on putative N-responsive genes in stems; that is, wood. Increased N fertilization has been correlated with altered wood properties such as reduced specific gravity (Blankenhorn et al. 1992), a utilitarian measure that reflects cell size, cell wall thickness, cell wall composition, and proportion of early wood to late wood (Koch 1972). However, we know very little about the mechanisms that link N availability to changes in the cellular structure or composition of wood. The putative identities of some of the girdling-responsive, N-associated cDNAs reveal hints as to how N availability may alter both wood quantity and wood quality.

Interactions between C and N metabolism and cross-talk between C- and N-signalling networks are emerging as important themes in how C and N resource availability affects growth and development (Koch 1997; Coruzzi & Zhou 2001). Putative identities of some of the girdling-responsive, N-associated genes suggest that limiting versus luxuriant N availability can shift the relative partitioning of C and N resources into C-intensive versus N-intensive biosynthetic pathways that may affect wood. We highlight a few examples that illustrate this concept, along with speculation as to how modulation of these genes by N availability may alter wood quantity or wood quality.

Vegetative storage proteins (VSPs) are one means that plants have evolved to store and recycle nutrients in response to changing N availability (Staswick 1994; Cole-
VSPs play an especially prominent role in perennials, in which seasonal N cycling is an important process associated with overwintering (Coleman 1997). Up-regulation of the poplar vsp5 win4, bsp and pni288 by increased N availability and wounding has been well documented (Coleman, Pilar Bañados & Chen 1994; Lawrence et al. 1997; Lawrence et al. 2001). Zhu & Coleman (2001a) have demonstrated that the bspA promoter is responsive to exogenously applied gln. In this study, win4, bsp and pni288 all demonstrated a positive N-response, while exhibiting somewhat different spatial expression patterns. Lawrence et al. (2001) suggested that VSPs such as BSP, WIN4 and PNI288 act as molecular determinants of N sink strength within the plant, rather than passive consequences of luxuriant N levels within the cells. If this is true, then reducing individual VSP levels in the plant should alter resource allocation patterns in a manner consistent with their spatial expression pattern. This hypothesis is supported by preliminary analysis of bsp-down-regulated poplars: bsp is preferentially synthesized in stems, and transgenic plants showed reduced stem height and increased leaf area, with a correspondingly increased leaf:stem biomass ratio (G.D. Coleman, personal communication).

We identified four candidate N-responsive cDNAs that suggest limiting N conditions can increase the partitioning of both C and N resources into pathways contributing to cell wall synthesis, a C-intensive process. Lignin is a major component of cell walls, making up as much as 35% of the dry weight of wood (Baucher et al. 1998). Lignin imparts strength and hydrophobicity to the cell walls in secondary xylem, but causes discoloration and reduced brightness of pulp products (Whetten, MacKay & Sederoff 1998), pot171, which was negatively N-responsive in this study, encodes a protein similar to caffeoyl-CoA 3-O-methyltransferase (CCoAOMT) of the lignin biosynthetic pathway (Baucher et al. 1998). Experiments with transgenic plants indicate that CCoAOMT down-regulation can alter lignin content and composition (Meyermans et al. 2000; Zhong et al. 2000; Guo et al. 2001; Pinçon et al. 2001). Thus, modulation of pot171 expression represents a potential mechanism by which N availability might regulate the partitioning of resources into C-intensive pathways, and impact wood quality.

Methylation of lignin intermediates by CCoAOMT and other OMTs involves transfer of one-carbon units from S-adenosylmethionine (Vander Mijnsbrugge et al. 2000). Hanson & Roje (2001) estimate that in woody plants, lignin biosynthesis demands 10-fold more one-carbon units than all of primary metabolism combined. pni275 encodes a protein similar to serine hydroxymethyltransferase, one of the enzymes involved in S-adenosylmethionine biosynthesis. pni275 was negatively N-responsive in this study, suggesting that the availability of one-carbon units for metabolism, including lignin biosynthesis, may be reduced under luxuriant N conditions. The use of amino acids as a source of one-carbon units, a pathway that involves serine hydroxymethyltransferase, represents an intriguing point of intersection between C and N metabolism.

Some cell wall proteins may also respond to N availability. pol64 and pni287 encode proteins similar to hydroxyproline-rich and proline-rich proteins, respectively, which are components of both primary and secondary cell walls (Loosestra 2000). Like pot171 and pni275, pni287 and pol164 were up-regulated by limiting NH4NO3 fertilization. Whereas pol164 was negatively N-responsive in the girdling experiments, pni287 was also responsive to the girdling-associated wounding (data not shown) Zhu & Coleman (2001a) have demonstrated that the bspA promoter is responsive to exogenously applied gln.

Terpenoids constitute a large and complex family of C-based secondary compounds (Trapp & Croteau 2001). Lipophilic compounds such as terpenoids can influence wood quality as well as wood processing (Martínez et al. 1999). pni263 shows strong sequence similarities to terpene cyclases (synthases). pni263 was negatively N-responsive in both the NH4NO3 application and girdling studies, suggesting that N availability might potentially decrease terpenoid concentration or alter terpenoid composition in poplar. Other studies with woody plants have shown only a weak correlation between N availability and terpenoid content (e.g. Lerdau et al. 1997; Powell & Raffa 1999; Lamontagne, Margolis & Bauce 2000). However, most of these studies have surveyed foliar terpenoids rather than stem terpenoids, and have been conducted under field conditions in which several environmental variables could potentially confound the results. More detailed studies are required to determine whether N-induced changes in terpenoid biosynthetic gene expression can alter terpenoid concentration or composition.

**CONCLUSIONS**

The gene discovery approach that we have described in this paper has allowed us to identify candidate N-responsive genes that play potential roles in allocating C and N resources to different plant parts and in partitioning those resources into different biosynthetic pathways. The putative identities of these candidate N-responsive genes suggest that C : N interactions might play an important role in the N-response of poplar. Some of these candidate N-responsive genes are putatively involved in the biosynthesis of vegetative storage proteins, cell wall components, and terpenoids. Each of these processes has the potential to alter the cellular composition of wood, thereby impacting wood properties. Most studies to date that have examined the effect of N availability on gene expression have focused on genes involved in primary metabolism. Our results have revealed candidate N-responsive genes implicated in secondary metabolism. This study provides us with the tools and testable hypotheses to further examine mechanisms by which N can influence wood quality and quantity. Such knowledge will be vital in understanding forest tree nutrient relations in natural and managed environments.
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REFERENCES


Sauter J.J. & van Cleve B. (1992) Seasonal variation of amino acids
Pinçon G., Maury S., Hoffmann L., Geoffroy P., Lapierre C., Pollet
Powell J.S. & Raffa K.F. (1999) Sources of variation in concentra-
Poplar
Zhong R., Morrison W.H. III, Himmelsbach D.S., Poole F.L. II.
© 2003 Blackwell Publishing Ltd, Plant, Cell and Environment, 26, 757–770