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Research paper

# Global poplar root and leaf transcriptomes reveal links between growth and stress responses under nitrogen starvation and excess

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**Nitrogen (N) starvation and excess have distinct effects on N uptake and metabolism in poplars, but the global transcriptomic changes underlying morphological and physiological acclimation to altered N availability are unknown. We found that N starvation stimulated the fine root length and surface area by 54 and 49%, respectively, decreased the net photosynthetic rate by 15% and reduced the concentrations of $NH_4^+$, $NO_3^-$ and total free amino acids in the roots and leaves of *Populus simonii* Carr. in comparison with normal N supply, whereas N excess had the opposite effect in most cases. Global transcriptome analysis of roots and leaves elucidated the specific molecular responses to N starvation and excess. Under N starvation and excess, gene ontology (GO) terms related to ion transport and response to auxin stimulus were enriched in roots, whereas the GO term for response to abscisic acid stimulus was overrepresented in leaves. Common GO terms for all N treatments in roots and leaves were related to development, N metabolism, response to stress and hormone stimulus. Approximately 30–40% of the differentially expressed genes formed a transcriptomic regulatory network under each condition. These results suggest that global transcriptomic reprogramming plays a key role in the morphological and physiological acclimation of poplar roots and leaves to N starvation and excess.**

***Keywords***: nutrient physiology, phytohormone, *Populus*, RNA sequencing, woody plants.

## Introduction

Nitrogen (N) is an essential macronutrient for plant growth and development. In plants, N is a major nutrient because it is required in many biomolecules, such as amino acids, proteins, chlorophylls, phytohormones and nucleic acids. High availability of N is required to ensure the high productivity of crops, including woody crops such as fast-growing *Populus* species (Mamashita et al. 2015). However, N availability is often limited in natural and agricultural ecosystems. Thus, to satisfy the growing global population's increasing demands for food, raw materials and biofuels, large amounts of N fertilizer (~120 Tg N $year^{-1}$) are applied to agricultural soils (Robertson and Vitousek 2009). However, up to 75% of the N fertilizer applied is not absorbed by plants and it is lost to the environment, thereby leading to the eutrophication of water and enrichment of $NO_x$ gases in the atmosphere (Gutierrez 2012). To sustain high productivity and decrease the rate of N application, it is important to obtain a better understanding of the molecular regulatory mechanisms underlying morphological and physiological acclimation to N availability in crops (Rennenberg et al. 2010, Xu et al. 2012).

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Although both organic and inorganic N can be utilized by plants (Näsholm et al. 2009, Gruffman et al. 2014), inorganic N resources such as nitrate ($NO_3^-$) and ammonium ($NH_4^+$) are the major N forms in soil (Rennenberg et al. 2009). These N ions are taken up by specific transporters, such as nitrate (NRTs) and ammonium (AMTs) transporters, and they can be assimilated locally, stored in vacuoles or transported to other parts of plants (Rennenberg et al. 2010, Xu et al. 2012). For instance, little $NO_3^-$ is assimilated in roots and most $NO_3^-$ assimilation occurs in leaves of poplars (Black et al. 2002). With the help of nitrate reductase (NR), cytosolic $NO_3^-$ is reduced to $NO_2^-$, which can be further converted into $NH_4^+$ in plastids (Rennenberg et al. 2010). Glutamine synthetase (GS) and glutamate synthase (GOGAT) use $NH_4^+$ to synthesize glutamine (Gln) and glutamate (Glu) in plastids (Rennenberg et al. 2010). In the mitochondria, $NH_4^+$ can also be converted into Glu with the help of glutamate dehydrogenase (GDH). Glutamine and Glu can be further converted into other organic N forms, thereby providing precursors for the biosynthesis of N-containing compounds in plants. Nitrogen uptake, assimilation and metabolism in plants are tightly regulated according to the internal N status and external N availability via phytohormone-mediated signalling pathways (Tian et al. 2008, Krouk et al. 2010, Gojon et al. 2011, Kiba et al. 2011, Krouk et al. 2011, Ruffel et al. 2011, Ma et al. 2014*a*).

Phytohormones such as abscisic acid (ABA) and auxin are considered to be signalling molecules, which combine with N signals to modulate root growth and development, thereby adjusting N acquisition according to the demand (Kiba et al. 2011, Vidal et al. 2013). Abscisic acid is involved in regulating lateral root growth and development in *Arabidopsis thaliana* and *Medicago truncatula* in response to altered N levels (Signora et al. 2001, Zhang et al. 2007, Yendrek et al. 2010). In addition, auxin has been demonstrated to play a role in modulating lateral root initiation and development in response to a low N supply (Fukaki and Tasaka 2009, Ma et al. 2014*a*). In maize, reduced root growth is correlated with low auxin concentrations in the presence of a high nitrate supply (Tian et al. 2008). However, little is known about the involvement of phytohormones in mediating the responses of woody plants to changes in the N supply (Rennenberg et al. 2010).

Significant progress has been made in understanding the transcriptomic networks that underlie morphological and physiological acclimation to low and/or high N supply levels in herbaceous plants, such as *A. thaliana* and *Zea mays* (Peng et al. 2007, Wang et al. 2007, Krapp et al. 2011, Ruffel et al. 2011, Bi et al. 2014, Canales et al. 2014). For instance, transcriptomic analysis has shown that up-regulated gene transcripts are mainly enriched in the categories of amino acid metabolism, transport and stress, whereas repressed transcripts are overrepresented in the categories of hormone metabolism and redox control in the roots of *A. thaliana* under N starvation (Krapp et al. 2011). In contrast, limited information is available about the global transcriptomic responses to changes in N supply levels in woody plants. Indeed, only two recent studies have investigated transcriptomic reprogramming in poplar roots in response to low N (50 μM $NO_3^-$) (Wei et al. 2013*a*, 2013*b*), one report has investigated global transcriptomic regulation in the developing xylem of poplar during acclimation to a high N (7.5 mM $NH_4NO_3$) supply (Plavcová et al. 2013), and one study reports that the N-responsive transcriptomic network in the elongation zone of *Populus trichocarpa* stem is related to stress, photosynthesis and cell-wall formation (Euring et al. 2014). Currently, we have a poor understanding of transcriptomic reprogramming in the roots and leaves of woody plants in response to N starvation and excess.

*Populus* species are fast-growing woody plants, which have great potential applications in $CO_2$ mitigation and as a bioenergy crop to produce biofuels (Luo and Polle 2009, Rennenberg et al. 2010, Polle et al. 2013). Poplar plantations are often established on marginal lands where the soil N is limited (Balasus et al. 2012). Thus, poplar plantations require N fertilization to achieve high biomass production (Novaes et al. 2009, Bilodeau-Gauthier et al. 2011). Several studies have reported the morphological and physiological responses of poplars, as well as the regulation of distinct genes in response to low or high N levels (Cooke et al. 2003, 2005, Hacke et al. 2010, Pitre et al. 2010, Euring et al. 2012, 2014, Li et al. 2012, Luo et al. 2013*a*, Plavcová et al. 2013, Wei et al. 2013*a*, 2013*b*). Thus, it is well known that N availability affects the photosynthetic rate and leaf area in poplar, which increases in the presence of high N levels but decreases with low N levels (Cooke et al. 2005, Li et al. 2012, Luo et al. 2013*a*). It has also been shown that leaf area is correlated with biomass production in the stem in poplar (Cooke et al. 2005). Low and high N levels lead to contrasting morphological changes in the roots, where root length and biomass are decreased with high N availability but increased with low N availability (Li et al. 2012, Luo et al. 2013*a*). The transcript levels of genes involved in the N uptake, assimilation and metabolism, as well as root morphogenesis, are differentially regulated depending on the N supply in poplars (Cooke et al. 2003, Li et al. 2012, Luo et al. 2013*a*, Wei et al. 2013*a*, 2013*b*, Euring et al. 2014). However, the global poplar root and leaf transcriptomic changes that underlie morphological and physiological acclimation to N starvation and excess are still unknown. Nitrogen starvation and excess are contrasting N supply regimes, which have distinct effects on the N uptake and metabolism in the roots and leaves of plants. In this study, we hypothesized: (i) that N starvation and excess would induce the distinct and common transcriptomic regulation pathways that underlie morphological and physiological acclimation in poplar roots and leaves; and (ii) that poplars would mobilize their stress pathways to regulate the growth of roots and leaves under N starvation and excess. To test both hypotheses, we used *Populus simonii* Carr., which often grows in

nutrient-deficient soils (Zhao et al. 2010). The poplar saplings were exposed to 0, 2 or 10 mM $NH_4NO_3$ for 4 weeks. We determined morphological (e.g., root characteristics) and physiological (e.g., photosynthesis, accumulation of $NH_4^+$, $NO_3^-$, amino acids, total N and phytohormones) characteristics, as well as the nutrient levels and related global transcriptomic changes. Based on multivariate and bioinformatics analyses, we elucidated the major transcriptomic regulation pathways that underlie morphological and physiological acclimation to N starvation and excess in poplar roots and leaves.

## Materials and methods

### *Plant cultivation and N treatments*

Cuttings (~15 cm in length, 1 cm in diameter, 1-year-old stem) of *P. simonii* were rooted and planted in plastic plots (10 l) filled with fine sand. The plants were cultivated in a greenhouse for 9 weeks (see Method S1 available as Supplementary Data at *Tree Physiology* Online for details). Next, 36 plants with similar height (~45 cm) and growth performance were selected and assigned to three groups with 12 plants in each group. Plants were irrigated with modified Long Ashton solution (see Method S1 available as Supplementary Data at *Tree Physiology* Online for details) containing one of the following $NH_4NO_3$ concentrations: 0 mM (N starvation), 2 mM (control) and 10 mM (N excess). The N treatments were maintained for 4 weeks until distinct morphological differences were observed among the treatments.

### *Measurements of gas exchange and leaf chlorophyll fluorescence*

Gas exchange and leaf chlorophyll fluorescence were analysed before harvesting (see Method S1 available as Supplementary Data at *Tree Physiology* Online for details). Six plants from each group were randomly selected for determination of gas exchange and leaf chlorophyll fluorescence. For each plant, three mature leaves [leaf plastochron index (LPI) = 5–7] formed during N treatment were selected to measure gas exchange. The net photosynthetic rates ($A$), stomatal conductance ($g_s$) and transpiration rates ($E$) were determined as described by He et al. (2011). Leaf chlorophyll fluorescence was determined according to a previously described method, with minor modifications (Luo et al. 2009*b*).

### *Analysis of root and leaf characteristics, and harvest*

The plant height was measured before the harvest. The root system of each plant was carefully washed and dabbed with tissue paper to remove water on the root surface. Lengths of main roots and fresh weights of roots were recorded. A portion of the roots (~0.5 g) of each plant was excised, scanned and analysed by the WinRHIZO root analyser system (WinRHIZO version 2012b, Regent Instruments, Montreal, Canada) as described by Ma et al. (2014*b*). The shoot (formed during the N treatments) above and below the mark was separated, and each part was further divided into wood, bark and leaves. Fresh weight of each tissue was recorded. For leaves formed during the N treatments, leaf discs (LPI = 5–7) for the determination of specific leaf area were also collected and specific leaf area was calculated according to Cao et al. (2012). Lengths and widths of six leaves (LPI = 7–12) from each plant were determined with a digital caliper. Afterwards, the six leaves were scanned by a scanner (V700, Epson, Long Beach, CA, USA) and analysed for the leaf area using ImageJ 1.42q (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). Harvested samples were wrapped with tinfoil and immediately frozen in liquid N. Frozen samples were ground into fine powder in pre-cooled jars (liquid $N_2$) in a ball mill (MW400, Retsch, Haan, Germany) and stored at –80 °C for further analysis. Fine powder (~100 mg) from each tissue of each plant was dried at 60 °C for 72 h to calculate the fresh-to-dry-mass ratio. Biomass of each tissue was calculated using fresh-to-dry-mass ratio and the fresh weight of the plant fraction. Only samples of roots and leaves formed during the N treatments were used in the molecular and biochemical analyses, unless stated otherwise. For each tissue, equal weights of fine-powdered samples obtained from two plants that received the same treatment were pooled to form a well-mixed sample, thereby yielding six mixed samples for each tissue in each treatment group.

### *Determination of photosynthetic pigments and carbohydrates*

The concentrations of chlorophyll and carotenoids in the leaves were determined spectrophotometrically, according to the method described by He et al. (2011). The concentrations of soluble sugars and starch in roots and leaves were determined by the anthrone method of Yemm and Willis (1954) with minor modifications (He et al. 2013*b*).

### *Determination of $NH_4^+$ and $NO_3^-$*

The $NH_4^+$ concentration in the roots and leaves was determined based on the Berthelot reaction (Bräutigam et al. 2007) with minor modifications, as described by Luo et al. (2013*b*). In brief, ~100 mg fine power was extracted in an extraction solution (1 ml 100 mM HCl and 500 µl chloroform), then the extraction solution was shaken at 4 °C for 15 min. Afterwards, the solution was centrifuged (10,000*g*, 4 °C, 10 min) and the aqueous phase was transferred to a new tube mixing with 50 mg activated charcoal, and centrifuged (12,000*g*, 4 °C, 5 min). Then 100 µl extraction solution was mixed with 500 µl 1% (w/v) phenol–0.005% (w/v) sodium nitroprusside solution. Subsequently, 500 µl 1% (v/v) sodium hypochlorite–0.5% (w/v) sodium hydroxide solution was added. The mixture was incubated at 37 °C for 30 min and measured at 620 nm spectrophotometrically.

The $NO_3^-$ concentration was measured according to the method reported by Patterson et al. (2010). Fine powder (~100 mg) was extracted in 1 ml deionized water at 45 °C for 1 h. After centrifugation (5000*g*, 20 °C, 15 min), 0.2 ml supernatant was mixed with 0.8 ml 5% (w/v) salicylic acid (SA) in concentrated $H_2SO_4$. After incubation at room temperature for 20 min, 19 ml of 2 M NaOH was added to increase the pH >12. The absorbance of the solution was determined spectrophotometrically at 410 nm after being cooled to room temperature.

### Determination of total N and other mineral nutrients

The total N concentrations in poplars were determined according to the Kjeldahl method with a minor modification, as described by Li et al. (2013).

The total phosphorus (P) concentrations in roots and leaves were analysed spectrophotometrically, according to the method of Parkinson and Allen (1975).

The concentrations of other minerals (K, Na, Ca, Mg, Cu, Zn, Fe and Mn) in roots and leaves were determined by flame atomic absorbance spectrometry (Hitachi Z-2000, Hitachi Ltd, Tokyo, Japan) using $HNO_3$–$HClO_4$ digested solution as described by He et al. (2011).

### Determination of free amino acids, soluble protein and phenolics

Free amino acids were analysed according to the method of Cao et al. (2014). Briefly, ~50 mg fine powder was mixed with 100 µl of 50% ethanol containing 0.1 M HCl. After centrifugation (13,800*g*, 4 °C, 20 min), the supernatant was filtered through a 0.22 µm organic membrane. Then 600 µl supernatant was used to analyse free amino acids with an LC/MS (LTQ-XL, Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Total free amino acids were calculated as the summed concentrations of individual free amino acid.

The soluble protein contents of roots and leaves were analysed based on the Bradford method (Bradford 1976). Soluble phenolics were determined according to the method of Luo et al. (2008).

### Enzyme activity assays

The NR (EC 1.7.99.4) activity levels in roots and leaves were determined according to the method of Ehlting et al. (2007) with minor modifications (see Method S1 available as Supplementary Data at *Tree Physiology* Online for details) (Luo et al. 2013*a*). The GOGAT (EC 1.4.7.1) and GDH (EC 1.4.1.2) activities in roots and leaves were assayed spectrophotometrically at 340 nm, as described by Lin and Kao (1996) (see Method S1 available as Supplementary Data at *Tree Physiology* Online for details).

### Ribonucleic acid isolation and sequencing

Total RNA was isolated from roots and leaves using a plant RNA extraction kit (R6827, Omega Bio-Tek, Norcross, GA, USA). Genomic DNA in the RNA extract was digested using DNase I (E1091, Omega Bio-Tek), according to the manufacturer's instructions. Total RNA samples were prepared from roots or leaves in six independent experiments. Equal amounts of total RNA from each preparation were pooled for subsequent library construction and RNA sequencing. One library per tissue per treatment was established. Prior to mRNA isolation and purification, the quality of the total RNA was checked using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

The sequencing libraries were constructed as described in the TruSeq RNA sample preparation guide (Illumina, San Diego, CA, USA). Briefly, poly-A-containing mRNA in the total RNA samples was isolated using RNA purification beads (15027078, Illumina). The mRNA was fragmented by incubation in Elute-Prime-Fragment Mix (15026782, Illumina) at 94 °C for 8 min to obtain 120–200 bp fragments. The fragmented mRNA was used to synthesize the first-strand cDNA and then the second-strand cDNA with a TruSeq RNA sample preparation kit (15027387, Illumina). Next, the double-stranded cDNA was end repaired, adenylated and ligated with RNA Adapter using a TruSeq RNA sample preparation kit (15025062, Illumina). Polymerase chain reaction (PCR) was performed for 15 cycles to enrich the DNA fragments with adapter molecules on both ends and to amplify the amount of DNA in the library. The purified libraries were quantified using a Qubit 2.0 Fluorometer (Invitrogen, Life Technologies Inc., Waltham, MA, USA) and validated with an Agilent 2100 Bioanalyzer (Agilent Technologies). Clusters were generated using the cBot cluster generation system (SY-301-2002, Illumina) and subsequently sequenced by an Illumina Genome Analyzer (HiSeq 2500, Illumina) obtaining reads with 50 bp length. Library construction and Illumina sequencing were performed at the Shanghai Biotechnology Corporation (Shanghai, China). High-quality reads that passed the Illumina quality filters were selected for further sequence analysis. The sequencing data sets are available at NCBI Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra/, accession number: SRP042609).

### Sequence analysis

High-quality reads were mapped to the mRNA reference sequence of *P. trichocarpa* (ftp://ftp.jgipsf.org/pub/compgen/phytozome/v9.0/Ptrichocarpa/assembly/Ptrichocarpa_210.fa.gz) using the spliced mapping algorithm in tophat (version 2.0.9) (Trapnell et al. 2009) where the settings allowed two mismatches and multihits ≤1. The gene expression levels were calculated using the fragments per kilobase of exon model per million mapped reads (FPKM) method in cufflink (version 2.1.1) (Trapnell et al. 2010). The differential expression of genes was calculated based on the normalized FPKM using the DEGseq package (Wang et al. 2010) implemented in R (www.r-project.org).

The statistical significance of the differential gene expression levels was tested using Fisher's exact test and the likelihood ratio test. The *P*-values of the statistics were adjusted as described by Benjamini and Hochberg (1995) and the false discovery rate (FDR) was used to determine the threshold *P*-value in multiple test analyses. The FPKM values obtained by applying these criteria to *P. simonii* roots (R) and leaves (L) exposed to 0 mM (R0 and L0), 2 mM (R2 and L2) or 10 mM (R10 and L10) $NH_4NO_3$ were used in further analyses. The fold changes in differentially expressed genes were calculated by comparing the FPKM values for R0 vs R2, R10 vs R2, L0 vs L2 and L10 vs L2. Significant differentially expressed genes in *P. simonii* roots and leaves were determined by FDR values <0.05 and fold changes ≥2 (or less than −2).

### Annotation, gene ontology, functional categorization and co-expression analysis

Differentially expressed genes were annotated as described by He et al. (2013*a*). Briefly, the coding sequences of differentially expressed genes were retrieved from the *P. trichocarpa* database (version 3.0). The closest *Arabidopsis* (*A. thaliana*) homologue (AGI identification) of a *P. simonii* gene was determined by translated nucleotide BLAST (BLASTX) of the coding sequence for the best *P. trichocarpa* hit against the *Arabidopsis* protein sequence data set. The annotations were taken from the latest release of The *Arabidopsis* Information Resource genome database (TAIR10).

To investigate significantly enriched GO terms for the differentially expressed genes obtained under the four comparison conditions (R0 vs R2, R10 vs R2, L0 vs L2 and L10 vs L2), AGIs derived from each comparison condition were used for singular enrichment analysis (SEA) in the agriGO database (http://bioinfo.cau.edu.cn/agriGO/index.php) and the session ID of each SEA was recorded. Subsequently, cross-comparisons of the SEA results (SEACOMPARE) were performed among the four comparison conditions (Du et al. 2010).

To identify significant differentially expressed overlapping poplar genes, which were consistently up- or down-regulated in both tissues under N starvation or excess (R0 vs R2 ∩ L0 vs L2, R10 vs R2 ∩ L10 vs L2), or in the roots or leaves in response to N variation (R0 vs R2 ∩ R10 vs R2, L0 vs L2 ∩ L10 vs L2), we used the 'VLOOKUP' function in Excel (version 2010) to obtain the poplar gene IDs in the list of differentially expressed genes. Subsequently, the identified overlapping genes were used for MapMan analysis with the Classification SuperViewer tool via the Bio-Analytic Resource (BAR, http://bar.utoronto.ca/) developed by Provart and Zhu (2003). The relative abundance in a MapMan bin was calculated based on the method of Provart and Zhu (2003).

To categorize differentially expressed genes according to their biological functions, poplar genes with unique AGIs (only the poplar gene with the lowest *P*-value was selected when multiple poplar genes corresponded to an AGI) were analysed using MapMan (Thimm et al. 2004).

Gene co-expression analysis was performed as suggested by He et al. (2013*a*). Briefly, for each comparison, all genes with functional categorizations assigned by MapMan were subjected to co-expression analysis as described by Wei et al. (2006) using an online open resource (http://cressexpress.org/index.jsp). Next, the gene interaction networks were visualized in Cytoscape version 3.0.2 as described by Shannon et al. (2003), with minor modifications as suggested by Saito et al. (2012).

### Analysis of cis-elements in conserved motifs of hub genes

To identify the conserved motifs in the promoter regions of hub genes (which are defined as highly connected genes depending on the number of edges) derived from the co-expression analysis, we performed motif analysis based on the method described by Wang et al. (2013) with minor modifications (Bai et al. 2013). In brief, the 5′ upstream sequences of 1000 bp from the translation start sites of hub genes were retrieved from the poplar database phytozome (http://www.phytozome.net/). The retrieved sequences were then submitted to the motif database MEME (http://meme.nbcr.net/meme/) to identify possible motifs, as described by Bailey et al. (2009). Subsequently, the significance of each motif identified was evaluated using a $\chi^2$ test based on comparisons with randomly selected genes in the genome of *P. trichocarpa* with the FIMO program, according to the method of Grant et al. (2011). The logos of the conserved motifs were visualized in WebLogo (http://weblogo.berkeley.edu/) according to Crooks et al. (2004). Finally, the sequences of significantly conserved motifs were submitted to the PLACE database (http://www.dna.affrc.go.jp/htdocs/PLACE/) to search for *cis*-elements, as suggested by Higo et al. (1999).

### Validation of RNA sequencing data by real-time quantitative PCR

To validate the differentially expressed genes obtained from the RNA sequencing analysis, we performed real-time quantitative PCR (RT-qPCR) according to the method of Li et al. (2012). The PCR products were sequenced and aligned with homologues from other model plants to ensure their validity. *Actin2/7* and *polyubiquitin* (*UBQ*) were selected as reference genes (Brunner et al. 2004). PCR was performed in triplicate together with a dilution series of the reference genes. The efficiencies of all PCRs ranged between 100 and 108% (see Table S1 available as Supplementary Data at *Tree Physiology* Online).

### Determination of phytohormone concentrations

Plant hormones, i.e., auxin (specifically indole-3-acetic acid; IAA), ABA, gibberellin ($GA_3$), jasmonic acid (JA) and SA, were extracted according to the method of Walker-Simmons (1987) with minor modifications (Shi et al. 2015). Briefly, fine powder

of fresh samples (~500 mg) was extracted with 4 ml of 80% methanol (containing 200 mg $l^{-1}$ butylated hydroxytoluene and 500 mg $l^{-1}$ citric acid monohydrate) on ice. After shaking at 4 °C overnight, the mixture was centrifuged (10,000*g*, 4 °C, 15 min) and the supernatant was collected. The extraction was repeated twice for 30 min each. The supernatants were pooled and dried under gas $N_2$. The dried compounds were dissolved in 800 µl of 80% methanol and filtered through a 0.22 µm organic membrane (Xinya, Shanghai, China). Two-microlitre samples were separated within a C18 column (4.6 × 150 mm, 5 µM; WondasilTM, Shimadzu, Kinh Do, Japan) and the phytohormone concentrations were analysed using a high-performance liquid chromatography (LC-20AT, Shimadzu) and an electrospray tandem mass spectrometry (API 2000TM, Allen-Bradley, Milwaukee, WI, USA). Abscisic acid (A1049), IAA (I2886), GA3 (G7645), JA (J2500) and SA (S7401) were purchased from Sigma (St Louis, MO, USA) for the preparation of standard curves to quantify hormone concentrations in the samples.

### Statistical analysis

Statistical tests were performed with Statgraphics (STN, St Louis, MO, USA), where the data were tested to confirm their normality before the statistical analyses. For experimental variables, one-way analysis of variance (ANOVA) was used with N treatment as a factor. Differences between means were considered significant when $P < 0.05$ according to the ANOVA *F*-test. The $C_t$ values obtained from qPCR were normalized and the relative fold changes in transcripts were calculated using the relative expression software tool, REST (Pfaffl et al. 2002). In the principal components analysis (PCA), the data were standardized and computed using the command prcomp() in R (http://www.r-project.org/). The heatmap representing the hierarchical clustering of differentially expressed genes involved in metabolism of N and amino acids was computed using the heatmap.2() command with the package 'gplots' in R.

## Results

### Nitrogen affects the morphological, photosynthetic and growth characteristics of poplar

After 4 weeks exposure to 0 mM (N starvation), 2 mM (N control) or 10 mM (N excess) $NH_4NO_3$ in sand culture, changes were observed in the morphology, photosynthesis and growth of *P. simonii* saplings (Table 1; Table S2 available as Supplementary Data at *Tree Physiology* Online). In particular, the length, surface area and volume of the fine roots of *P. simonii* were stimulated significantly by N starvation, whereas they were inhibited by N excess. The net photosynthetic rates, concentrations of photosynthetic pigments and the leaf biomass of *P. simonii* were lower under N starvation, but higher with excess N. Stomatal conductance, transpiration and leaf area were not affected by N starvation, whereas they increased in response to excess N.

### Nitrogen has major effects on the concentrations of nutrients and carbohydrates

The availability of N in the soil also affected the nutritional status of the roots and leaves in *P. simonii* (Table 2; Table S2 available as Supplementary Data at *Tree Physiology* Online). The concentrations of both $NH_4^+$ and $NO_3^-$ were affected greatly by N starvation or excess in the roots, but to a lesser extent in the leaves (Table 2). Nitrogen starvation led to great decreases in the concentrations of nitrate, soluble amino acids and total N in the roots, but it had no effects on the concentrations of $NH_4^+$ and protein. The availability of excess N led to significant increases in all N-containing compounds in the roots. The changes induced by N starvation or excess were less pronounced in the leaves compared with those in the roots, with the exception of $NH_4^+$. Foliar $NH_4^+$ was decreased greatly in response to N starvation, whereas it was unaffected by excess N.

Table 1. Morphological, physiological and growth responses to N starvation and excess. Net photosynthetic rates (µmol $CO_2$ $m^{-2}$ $s^{-1}$), stomatal conductance (mol $H_2O$ $m^{-2}$ $s^{-1}$), transpiration (mmol $H_2O$ $m^{-2}$ $s^{-1}$), photosynthetic pigments (mg $g^{-1}$ DW), biomass (g DW) of root and leaf, leaf area ($cm^2$ $leaf^{-1}$), fine root length (m $root^{-1}$), fine root surface area ($cm^2$ $root^{-1}$) and root to shoot ratio are shown. Data indicate means ± SE ($n = 6$). Values labelled with different letters in the same row indicate significant difference between the N treatments. *P*-values of the ANOVAs are indicated. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

| | Tissue | $NH_4NO_3$ (mM) | | | *P*-value |
|---|---|---|---|---|---|
| | | 0 | 2 | 10 | |
| Fine root length | Root | 206.89 ± 13.19c | 134.51 ± 9.91b | 80.15 ± 11.19a | ** |
| Fine root surface area | Root | 672.93 ± 58.80c | 453.15 ± 26.23b | 247.31 ± 29.12a | ** |
| Root to shoot ratio | Plant | 0.29 ± 0.01b | 0.17 ± 0.01a | 0.17 ± 0.01a | *** |
| Net photosynthetic rates | Leaf | 11.62 ± 0.49a | 13.72 ± 3.30b | 15.10 ± 0.06c | *** |
| Stomatal conductance | Leaf | 0.36 ± 0.03a | 0.38 ± 0.04a | 0.55 ± 0.02b | * |
| Transpiration | Leaf | 6.91 ± 0.01a | 7.34 ± 0.31a | 9.29 ± 0.22b | ** |
| Chlorophyll (*a* + *b*) | Leaf | 5.59 ± 0.35a | 7.69 ± 0.23b | 9.03 ± 0.37c | *** |
| Root biomass | Root | 2.23 ± 0.11b | 1.54 ± 0.22a | 1.52 ± 0.07a | * |
| Leaf biomass | Leaf | 1.67 ± 0.16a | 2.56 ± 0.15b | 3.18 ± 0.21c | *** |
| Leaf area | Leaf | 67.81 ± 1.39a | 72.55 ± 2.94a | 84.84 ± 1.62b | *** |

Table 2. N- and C-bearing compounds in roots and leaves of *P. simonii* exposed to 0, 2 or 10 mM $NH_4NO_3$. N concentration (mg $g^{-1}$ DW), concentrations of ammonium (μmol $g^{-1}$ DW), nitrate (μmol $g^{-1}$ DW), total amino acids (μmol $g^{-1}$ DW), soluble protein (mg $g^{-1}$ DW), soluble sugars (mg $g^{-1}$ DW), starch (mg $g^{-1}$ DW) and soluble phenolics (mg $g^{-1}$ DW) are shown. Colour codes are given as the percentage of the value in *P. simonii* treated with 2 mM $NH_4NO_3$. Data indicate means ± SE ($n = 6$). Values labelled with different letters in the same row for each tissue indicate significant difference between the N treatments.

| Colour code (%) | <50 | 69–50 | 89–70 | 90–109 | 110–150 | 151–200 | >200 |
|---|---|---|---|---|---|---|---|
| | Roots | | | | Leaves | | |
| Compounds | 0 mM | 2 mM | 10 mM | | 0 mM | 2 mM | 10 mM |
| N concentration | 7.3 ± 0.8a | 9.7 ± 0.4b | 13.4 ± 0.4c | | 13.6 ± 0.8a | 18.3 ± 0.5b | 24.6 ± 1.0c |
| Ammonium | 3.7 ± 0.7a | 4.1 ± 0.3a | 6.8 ± 0.6b | | 1.7 ± 0.1a | 2.4 ± 0.3b | 2.5 ± 0.1b |
| Nitrate | 20.6 ± 2.7a | 38.0 ± 4.5b | 131.7 ± 1.7c | | 62.2 ± 5.5a | 74.0 ± 6.2ab | 88.8 ± 4.2 b |
| Total amino acids | 1.1 ± 0.1a | 2.4 ± 0.2b | 8.8 ± 0.4c | | 1.4 ± 0.2a | 2.0 ± 0.1b | 2.9 ± 0.2c |
| Soluble protein | 63.0 ± 0.6a | 66.7 ± 2.4a | 83.6 ± 6.8b | | 365.8 ± 28.5a | 421.1 ± 40.7a | 362.2 ± 13.4a |
| Soluble sugars | 25.8 ± 3.6b | 15.4 ± 1.8a | 16.8 ± 1.2a | | 42.7 ± 2.2a | 44.3 ± 3.1a | 46.4 ± 4.5a |
| Starch | 7.4 ± 0.5a | 10.4 ± 0.5b | 6.1 ± 0.6a | | 10.7 ± 0.8b | 9.3 ± 0.5b | 7.1 ± 0.5a |
| Soluble phenolics | 171.6 ± 6.9 | 161.4 ± 11.4b | 97.1 ± 9.3a | | 259.4 ± 9.8c | 226.2 ± 5.8b | 177.5 ± 3.4a |

Differences in the availability of N also affected the status of other mineral nutrients and carbohydrates in the roots and leaves of *P. simonii* (Table 2; Table S3 available as Supplementary Data at *Tree Physiology* Online). For example, the P concentrations in the roots were lower under N starvation but higher when more N was available. The concentrations of potassium (K) in the roots and leaves increased with N starvation but decreased with N excess. The soluble sugar concentrations were elevated by 68% in N-starved roots compared with the control roots (Table 2). The starch levels were reduced in the roots of plants exposed to low or high N levels (Table 2), which was probably attributable to the lower production of photosynthates under N starvation, whereas more carbohydrates were consumed for growth with excess N. The concentrations of soluble phenolics were lower in the roots and leaves of *P. simonii* when treated with excess N (Table 2).

### *RNA sequencing analysis to elucidate the global transcriptomic co-expression patterns in roots and leaves with N starvation and excess*

We conducted genome-wide transcriptional analyses based on RNA sequencing using the roots and leaves of *P. simonii* to identify the transcriptomic regulation mechanisms that underlie the morphological and physiological responses to N starvation and excess. A total of 191 million raw reads were generated, and each library produced 22.6–42.2 million reads (see Table S4 available as Supplementary Data at *Tree Physiology* Online). After sequence trimming, the number of clean reads per library still ranged from ~21.9 to 41.0 million (see Table S4 available as Supplementary Data at *Tree Physiology* Online). About 17.7–36.1 million clean reads per library were mapped to the genome of *P. trichocarpa*, and the mapping ratios ranged from 71.2 to 87.9% (see Table S4 available as Supplementary Data at *Tree Physiology* Online).

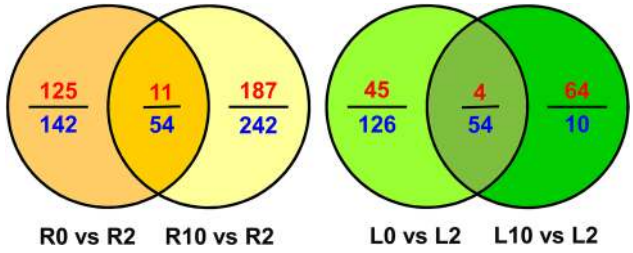


Figure 1. Overlapping significant differentially expressed genes in the roots (R) and leaves (L) of *P. simonii* when exposed to 0 mM (R0 and L0), 2 mM (R2 and L2) or 10 mM (R10 and L10) $NH_4NO_3$. The upper and lower numbers in each fraction indicate the numbers of up- and down-regulated genes, respectively. The Venn diagram was drawn using VENNY (http://bioinfogp.cnb.csic.es/tools/venny/).

Compared with the control roots (R0 vs R2), the transcript levels of 136 genes increased significantly in N-starved roots, whereas those of 196 genes decreased significantly (see Table S5 available as Supplementary Data at *Tree Physiology* Online; Figure 1). Compared with the control roots, the transcript levels of 198 genes increased in response to excess N, whereas those of 296 genes decreased (R10 vs R2, Table S5 available as Supplementary Data at *Tree Physiology* Online; Figure 1). Among these genes, 65 genes exhibited regulatory responses to both treatments, where 11 were up-regulated and 54 were down-regulated in response to N starvation and excess, respectively (see Table S5 available as Supplementary Data at *Tree Physiology* Online; Figure 1).

Compared with the control conditions, the mRNA levels of 49 genes were up-regulated in the leaves, whereas those of 180 genes were down-regulated under N deficiency (L0 vs L2, Table S5 available as Supplementary Data at *Tree Physiology* Online; Figure 1). Compared with the control, the transcript levels of 68 genes were increased in the leaves, whereas those of 64 genes were repressed in the presence of excess N (L10 vs L2, Table S5

available as Supplementary Data at *Tree Physiology* Online; Figure 1). In response to N starvation and excess, four common genes were up-regulated and 54 were down-regulated (see Table S5 available as Supplementary Data at *Tree Physiology* Online; Figure 1).

These results were validated by analysing a subset of 10 randomly selected differentially expressed genes using RT-qPCR (see Figure S1 available as Supplementary Data at *Tree Physiology* Online). Overall, the transcriptomic analyses demonstrated that more genes exhibited significant differences in expression in the roots compared with those in the leaves in response to N starvation or excess, thereby indicating a greater responsiveness to changes in N availability in the roots than that in the leaves, which is consistent with the changes in N-containing compounds induced by N deprivation or excess.

***Gene ontology term analysis to determine tissue- and/or N-level-specific gene enrichment, as well as common enriched GO terms for tissues and/or N treatments*** To obtain functional insights into the differential expression of genes in response to N starvation and excess in roots and leaves, SEA was applied to identify significant GO terms, which were then compared in different conditions (R0 vs R2, R10 vs R2, L0 vs L2 and L10 vs L2) using SEACOMPARE in AgriGO (see Tables S5 and S6 available as Supplementary Data at *Tree Physiology* Online). This analysis showed that there was a major difference in the category 'cellular components' where GO terms in the roots were enriched with respect to mitochondria, whereas those in the leaves were enriched for chloroplasts (see Table S6 available as Supplementary Data at *Tree Physiology* Online).

More GO terms related to molecular functions were significantly enriched in the roots than the leaves when *P. simonii* was exposed to differences in N availability, especially in response to N excess (see Table S6 available as Supplementary Data at *Tree Physiology* Online). In particular, a high number of GO terms was related to transport activities in roots exposed to different N levels. The only exclusive GO terms in leaves were enzyme activities related to the hydrolysis of *O*-glycosyl compounds and tetrapyrrole binding, i.e., photosynthesis (see Table S6 available as Supplementary Data at *Tree Physiology* Online).

Similarly, GO terms for biological processes were also found more frequently in roots than leaves (see Table S6 available as Supplementary Data at *Tree Physiology* Online). The transcriptome of N-starved roots was specifically enriched with respect to GO terms for amino acid and carboxylic acid metabolism, whereas that of roots exposed to excess N exhibited an overabundance of GO terms related to stress and plant hormones (see Table S6 available as Supplementary Data at *Tree Physiology* Online). An exception was the GO term ABA stimulus, which was found exclusively in leaves and not in roots (see Table S6 available as Supplementary Data at *Tree Physiology* Online). The GO term for the response to SA stimulus was specifically enriched in both leaves and roots treated with excess N. This global GO term analysis supported the physiological and biochemical data in terms of the responsiveness of photosynthesis and energy metabolism to change in N. Moreover, this analysis demonstrated that stress responses were induced by N starvation or excess.

In more specific analyses, we focused on overlapping genes with transcript abundances that were regulated in the same direction under either N starvation or excess in both the roots and leaves. Nitrogen starvation was related to numerous categories, including stress, carbohydrate metabolism and amino acid metabolism (see Table S7 available as Supplementary Data at *Tree Physiology* Online; Figure 2). Excess N was associated with the unidirectional regulation of genes in roots and leaves for the stress, minor C metabolism and cell-wall categories (see Table S7 available as Supplementary Data at *Tree Physiology* Online; Figure 2).

We also expected that genes exhibiting responses to the N status in a tissue would exhibit opposite transcript abundances in the presence of low or high N in the roots or leaves. In the roots, we identified common gene sets in the stress, hormone and lipid metabolism, and transport categories, as well as amino acid and N metabolism, whereas only a set of stress-related genes fulfilled this criterion in the leaves (see Table S7 available as Supplementary Data at *Tree Physiology* Online; Figure 2).

***Co-expressed genes with common cis-regulatory elements in the conserved motifs of hub genes play roles in acclimation to N starvation and excess*** In addition to GO term analysis, we identified co-expression networks of differentially expressed genes in the roots and leaves of *P. simonii* when exposed to N starvation or excess (see Table S8, Figure S2 available as Supplementary Data at *Tree Physiology* Online). In N-starved roots, 121 (~36%) of the 332 differentially expressed genes formed an N-starvation-responsive co-expression network with 867 edges (see Table S8, Figure S2a available as Supplementary Data at *Tree Physiology* Online). In the roots of plants supplied with excessive N, 199 (~40%) of the 494 differentially regulated genes formed a high-N-responsive co-expression network with 1392 edges (see Table S8, Figure S2b available as Supplementary Data at *Tree Physiology* Online). In the leaves, 70 (~31%) of the 229 genes formed an N-starvation-responsive co-expression network with 285 edges (see Table S8, Figure S2c available as Supplementary Data at *Tree Physiology* Online), whereas 35 (~27%) of the 132 genes established a high-N-responsive co-regulation network with 160 edges (see Table S8, Figure S2d available as Supplementary Data at *Tree Physiology* Online). The network analysis showed that ~30% of the significant differentially expressed genes were highly co-regulated in either the roots or leaves of *P. simonii* when exposed to N starvation or excess.

To identify the key players in the co-expression networks, we also analysed the highly interconnected hub genes, where each

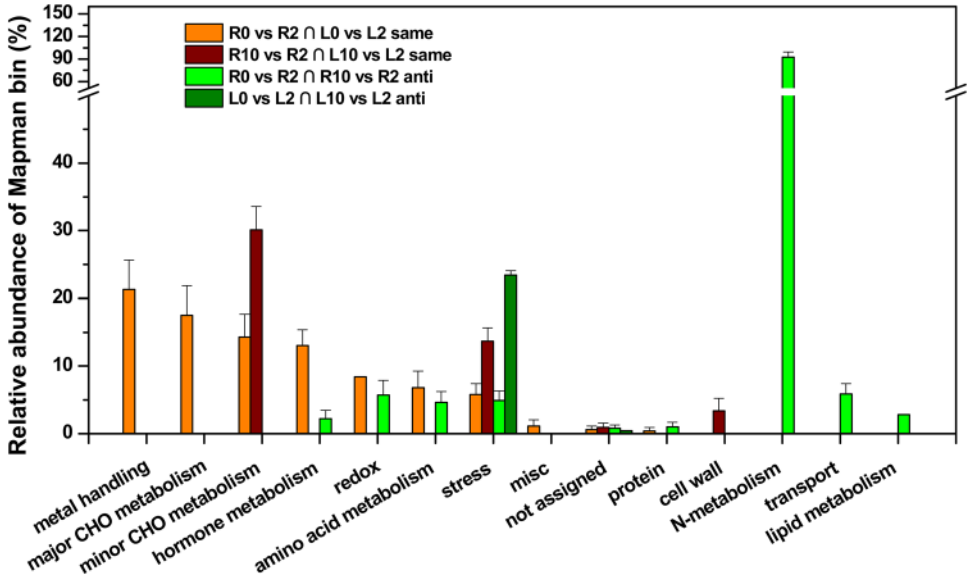


Figure 2. Categorization of genes that exhibited consistent or opposite transcript regulation in roots and leaves under N starvation and excess, which were sorted after MapMan categorization. R0 vs R2 ∩ L0 vs L2 same: overlapping genes from roots and leaves under N starvation that exhibited consistent transcript regulation in roots and leaves; R10 vs R2 ∩ L10 vs L2 same: overlapping genes from roots and leaves under N excess that exhibited consistent transcript regulation in roots and leaves; R0 vs R2 ∩ R10 vs R2 anti: overlapping genes from roots under N starvation and excess that exhibited opposing transcript regulation effects with N starvation and excess; L0 vs L2 ∩ L10 vs L2 anti: overlapping genes of leaves under N starvation and excess that exhibited opposing transcript regulation effects with N starvation and excess.

gene had >30 edges in roots or 10 edges in leaves (see Table S8 available as Supplementary Data at *Tree Physiology* Online; Figure 3). According to this criterion, in roots exposed to N starvation, 29 of the co-expressed genes were identified as hub genes, which were linked directly by 382 edges to form a highly interconnected sub-network (Figure 3a). In roots supplied with excessive N, a similar number was found under N starvation, i.e., 32 hub genes with 385 edges (Figure 3b). In leaves, 29 and 19 hub genes were detected under N starvation and excess conditions, respectively (Figure 3c and d).

Highly connected hub genes can play pivotal roles in essential biological processes. The regulation of nutrient uptake in roots is critical for plants during acclimation to changes in N availability. Indeed, we identified genes with putative roles in transport in the hubs: in N-starved roots, *TIP2;3* involved in $NH_3/NH_4^+$ transport, *NRT 1;5* associated with $NO_3^-$ transport and *PHO1* involved in the transfer of phosphate from root epidermal and cortical cells to the xylem (Hamburger et al. 2002); and in roots exposed to N excess, *ABCB4* encoding an auxin efflux transmembrane transporter (Kubeš et al. 2012) and *PDR9* regulating auxin distribution and homeostasis (Ruzicka et al. 2010) (see Table S8 available as Supplementary Data at *Tree Physiology* Online; Figure 3a and b). In leaves, photosynthesis provides the carbon skeletons required for the biosynthesis of N metabolites in poplars exposed to altered N levels. Accordingly, in the light reaction to photosynthesis category, we found 8 of the 29 hub genes in N-starved leaves, such as *PSBA* and *PSBC*, and 6 of the 19 hub genes in leaves with N excess, including *PSAA*, *ATPH* and *RBCL* (see Table S8 available as Supplementary Data at *Tree Physiology* Online; Figure 3c and d).

It is likely that transcriptionally co-regulated genes will share conserved motifs as their regulatory elements (Wang et al. 2013). Therefore, we analysed the promoter regions of hub genes (see Table S9 available as Supplementary Data at *Tree Physiology* Online; Figure 4). For hub genes in N-deprived roots, we predicted five candidate motifs but only two motifs could be confirmed based on sequence comparisons with known *cis*-regulatory motifs in the PLACE database (see Tables S9 and S10 available as Supplementary Data at *Tree Physiology* Online; Figure 4a1 and a2). One conserved motif was the 'TGAC' motif, a core binding site of rice WRKY71, which is a transcriptional repressor of the gibberellin signalling pathway (Xie et al. 2005). However, the role of the second motif in N metabolism is still unknown. In roots supplied with excess N, five candidate motifs were predicted and four were present in the PLACE database (see Tables S9 and S10 available as Supplementary Data at *Tree Physiology* Online; Figure 4b1–b4). The 'AAAG' and 'TTATTT' motifs are particularly relevant to N metabolism in plants. The 'AAAG' motif is a core site required for the binding of Dof proteins in maize (Yanagisawa and Schmidt 1999, Yanagisawa 2000). In plants, Dof proteins are known to be involved in N metabolism (Rueda-López et al. 2008). The 'TTATTT' motif belongs to the TATA box, which is found in the 5′ upstream region of a pea (*Pisum sativum*) gene that encodes GS (Tjaden et al. 1995). In the leaves of *P. simonii* subjected to N deprivation, three motifs were predicted, but only the 'GATA' motif from the CaMV 35S promoter (Reyes et al. 2004) matched with sequences in the PLACE database (see Tables S9 and S10 available as Supplementary Data at *Tree Physiology* Online; Figure 4c). In the leaves of *P. simonii* treated with excess N, two motifs were

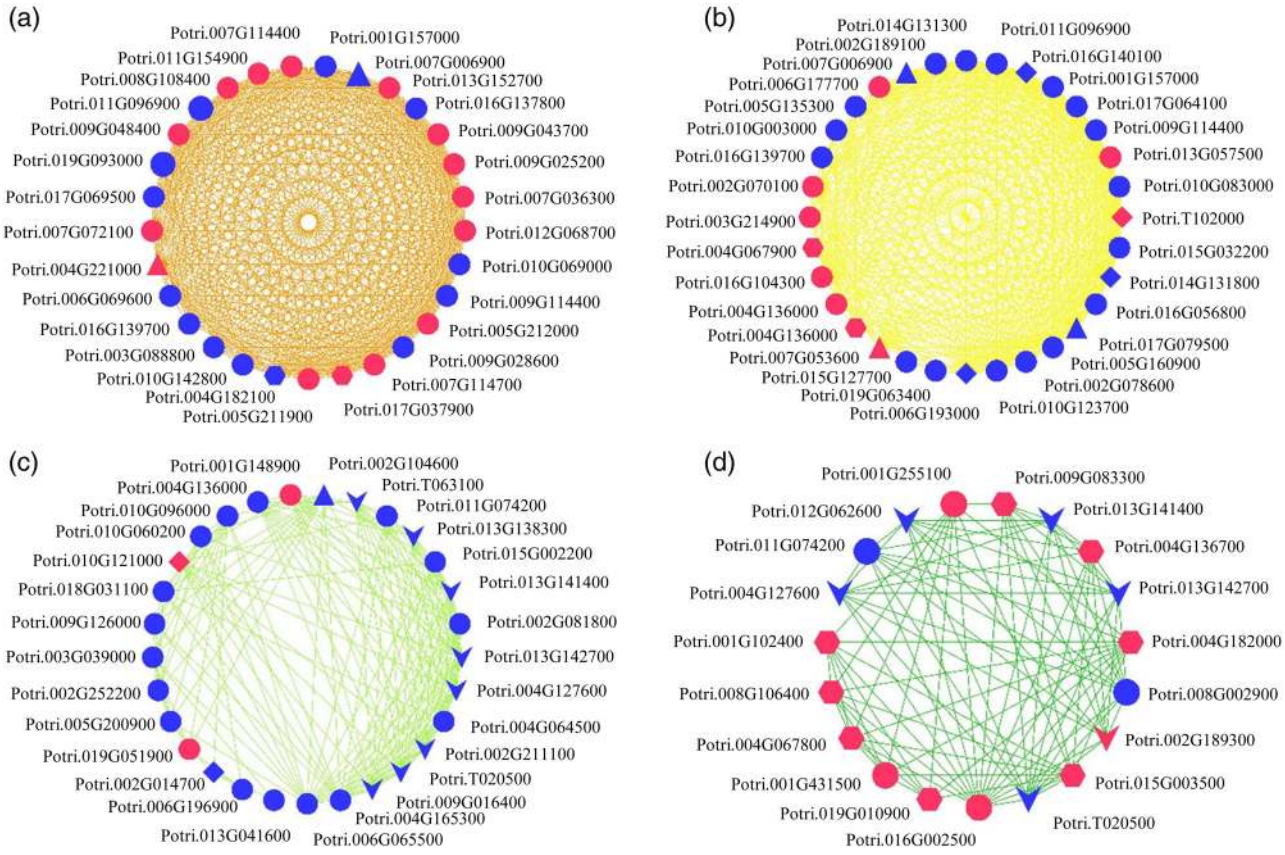


Figure 3. Networks of hub genes derived from co-expression networks (see Figure S2 available as Supplementary Data at *Tree Physiology* Online) of significant differentially regulated genes in each comparison condition: R0 vs R2 (a), R10 vs R2 (b), L0 vs L2 (c) and L10 vs L2 (d). Networks were generated by setting a cut-off value of 0.15 for the Kolmogorov–Smirnov quality control statistics and an $r^2$ value of 0.36, which yielded reliable results for the co-expressed genes in CressExpress, as suggested by Wei et al. (2006). An edge indicates the co-expression of two genes. Triangle nodes: genes from the RNA regulation category; diamond nodes: genes from the signalling category; hexagon nodes: genes from the stress category; 'V' shaped nodes: genes from photosynthesis category; circle nodes: genes from other categories. Red and blue nodes represent up- and down-regulated genes, respectively.

predicted and confirmed in the PLACE database (see Tables S9 and S10 available as Supplementary Data at *Tree Physiology* Online; Figure 4d1 and d2). The 'AAAG' motif was detected, which is a core sequence for the binding of Dof proteins, as mentioned above.

### *Nitrogen starvation and excess affect N metabolism in the roots and leaves of P. simonii*

Nitrogen availability affected the internal N pools of *P. simonii*, such as the concentrations of $NH_4^+$, $NO_3^-$ and total free amino acids (Table 2). Consistently, our analyses of changes in the transcript levels highlighted genes involved in primary N metabolism, particularly genes encoding NR, GOGAT and GDH, and enzymes implicated in biosynthesis of amino acids in the roots and leaves of *P. simonii* when exposed to N starvation or excess (see Table S8 available as Supplementary Data at *Tree Physiology* Online; Figure 5).

The activities of key enzymes involved in N metabolism were affected by N availability in *P. simonii* (Figure 5). The activity of NR was inhibited by N starvation but induced by excess N in the roots and leaves. In agreement with these changes, N deprivation decreased and N excess increased the transcript levels of the gene encoding nitrate reductase 2 (NIA2) in roots. In contrast, N deficiency increased the expression level of *NIA2* in leaves. The activity of GOGAT was induced under N starvation, but inhibited by high N in roots. In contrast, the gene encoding NADH-dependent glutamate synthase (GLT1) was down-regulated in roots under N starvation. The foliar activity of GOGAT remained unaltered under N starvation, but it was stimulated by ~50% with excess N. The activity of GDH was elevated with decreased mRNA levels of the genes encoding GDH1 and GDH2 under N starvation, whereas it was repressed with down-regulated *GDH1* transcript levels in the presence of excess N in the roots. The foliar activity of GDH exhibited the opposite pattern with respect to N availability compared with that in the roots.

In addition, the concentrations of individual amino acids and the transcript levels of genes involved in amino acid metabolism were also affected by N starvation and excess in the roots and leaves of *P. simonii* (Figure 5). Among the free amino acids

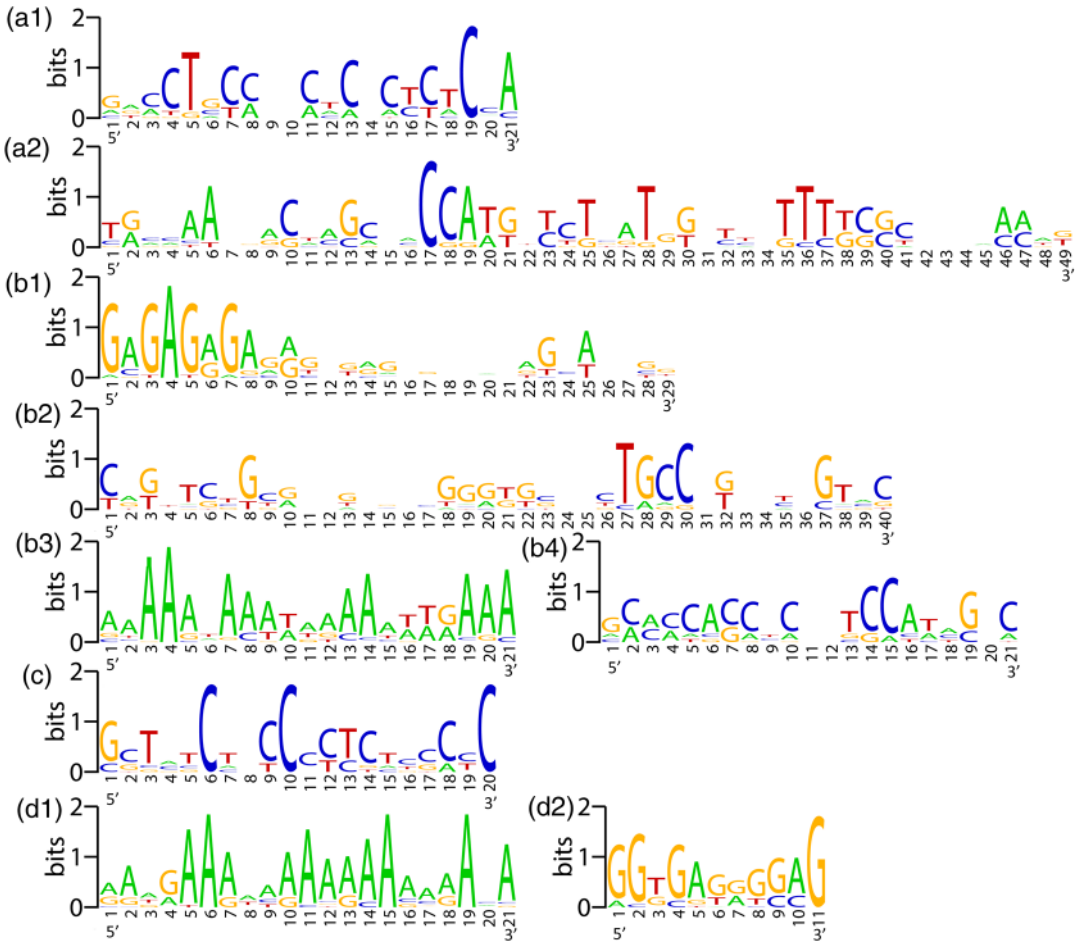


Figure 4. Conserved motifs in the 5′ upstream sequences of hub genes in each comparison condition: R0 vs R2 (a1–a2), R10 vs R2 (b1–b4), L0 vs L2 (c) and L10 vs L2 (d1–d2). Detailed data about the conserved motifs and their matched *cis*-regulatory elements are presented in Tables S9 and S10 available as Supplementary Data at *Tree Physiology* Online, respectively.

detected, we found that Lys, Glu, His and Arg had the highest concentrations in roots in the control N supply conditions (data not shown). In most cases, the concentrations of individual amino acids in the roots and leaves of *P. simonii* were decreased under N starvation, whereas they increased with excess N. In particular, Arg contains four N atoms and it is an efficient means of storing assimilated N as an amino acid, which can be degraded to release N under conditions of N deprivation. In *P. simonii*, the Arg levels were increased by ~25- and 6.5-fold in roots and leaves, respectively, with excess N compared with those in the control N conditions. In contrast, the Arg concentrations were decreased by ~70% in roots and leaves under N starvation, thereby suggesting that this amino acid is degraded to release N for poplar growth and defence in conditions of N deprivation. Furthermore, in the roots, N deprivation led to decreased transcript levels of genes encoding aspartate aminotransferase 2 (ASP2), ASP5, alanine aminotransferase 2 (ALAAT2), alanine : glyoxylate aminotransferase 3 (AGT3) and glutamine-dependent asparagine synthase 1 (ASN1). In roots, excess N induced the mRNA levels of *ASN1*, but decreased the transcript levels of genes encoding ASP5 and L-asparaginase. In leaves, N deficiency increased the transcript levels of genes encoding NIA1, NIA2, NIR1 and L-asparaginase, but repressed the mRNA level of *ASN1*.

### *Nitrogen starvation and excess affect the levels of phytohormones related to growth regulation and stress responses*

Plant hormones play key roles in regulating growth and development, as well as sensing changes in environmental conditions, e.g., nutrient availability. Therefore, we analysed changes in the concentrations of IAA, ABA, $GA_3$, JA and SA in the roots and leaves of *P. simonii* when treated with N starvation or excess (Table 3). The IAA concentration remained unchanged in the roots and leaves of *P. simonii* when exposed to N deficiency, but it decreased by ~32 and 19% in the roots and leaves of plants exposed to N excess compared with the control conditions, respectively (Table 3). Decreased transcript levels of four genes involved in auxin-mediated signal transduction (*CYP711A1*, *PIN5*, *auxin-responsive family protein* and *JAR1*) were detected in

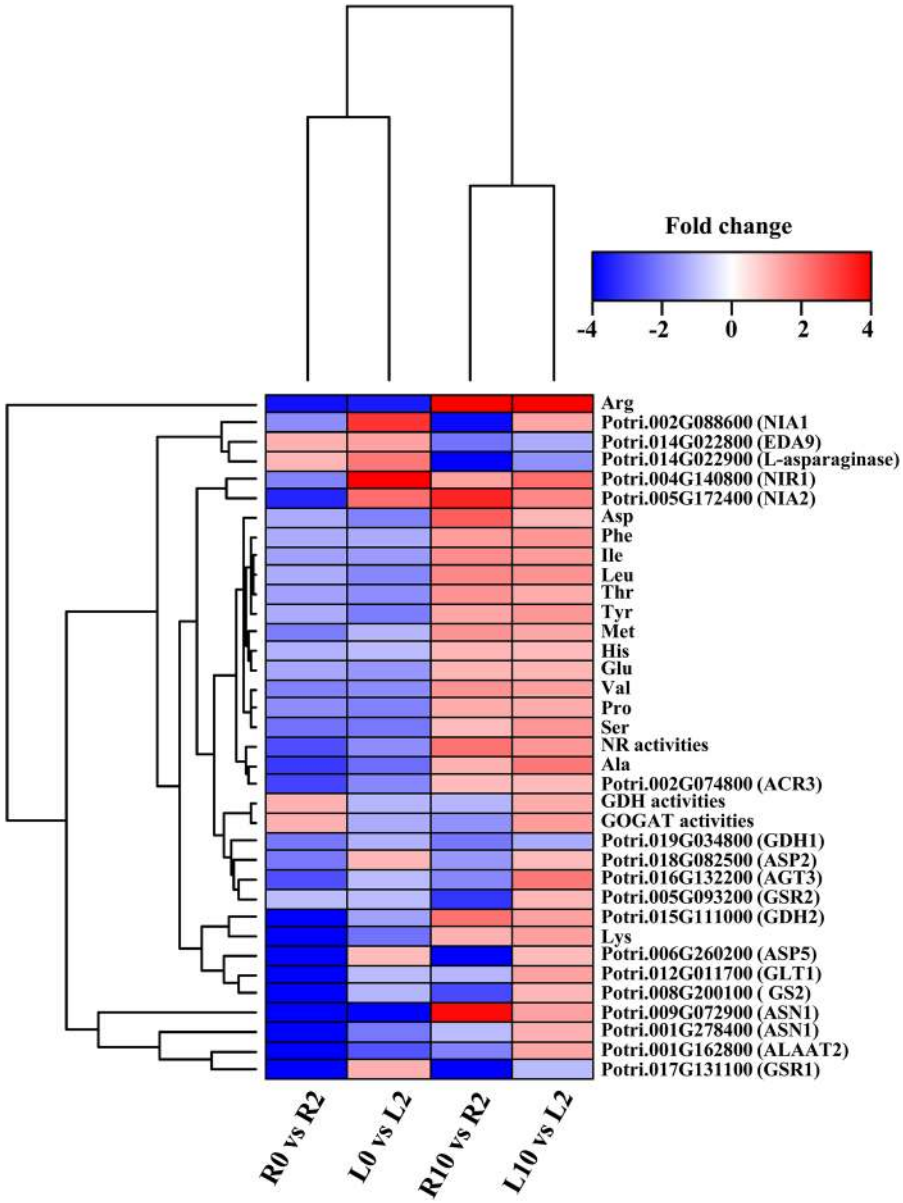


Figure 5. Heatmap representing the hierarchical clustering of differentially expressed genes involved in N metabolism, as well as relative changes in the enzymatic activities and amino acid concentrations in the roots and leaves of *P. simonii* when exposed to N starvation and excess. Detailed information for each gene is provided in Tables S5 and S8 available as Supplementary Data at *Tree Physiology* Online.

Table 3. Concentrations (ng $g^{-1}$ DW) of IAA, ABA, $GA_3$, JA and SA in roots and leaves of *P. simonii* exposed to 0, 2 or 10 mM $NH_4NO_3$. Data indicate means ± SE ($n = 6$). Values labelled with different letters in the same column for each tissue indicate significant difference between the N treatments. *P*-values of the ANOVAs are indicated. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

| Tissues | $NH_4NO_3$ (mM) | IAA | ABA | $GA_3$ | JA | SA |
|---|---|---|---|---|---|---|
| Roots | 0 | 2017.2 ± 77.7b | 17.6 ± 2.7a | 218.5 ± 11.6a | 157.7 ± 7.2a | 3707.4 ± 49.5c |
| | 2 | 2226.4 ± 138.3b | 35.0 ± 6.5b | 357.1 ± 15.7b | 288.8 ± 23.9b | 1945.4 ± 163.4b |
| | 10 | 1514.7 ± 32.5a | 57.4 ± 4.5c | 400.3 ± 15.5b | 379.9 ± 17.7c | 942.6 ± 25.6a |
| | *P*-value | ** | ** | *** | *** | *** |
| Leaves | 0 | 2271.8 ± 190.1b | 82.3 ± 9.2a | 154.1 ± 12.1a | 82.1 ± 5.5a | 477.7 ± 99.6a |
| | 2 | 1932.5 ± 128.8ab | 170.7 ± 12.1b | 211.4 ± 20.0b | 120.5 ± 8.0b | 835.2 ± 15.9b |
| | 10 | 1566.2 ± 28.3a | 396.0 ± 49.3c | 243.0 ± 22.4b | 195.7 ± 13.7c | 888.7 ± 88.1b |
| | *P*-value | * | *** | * | *** | * |

roots treated with excess N (see Table S8 available as Supplementary Data at *Tree Physiology* Online).

The ABA concentrations were decreased by ~50% under N starvation and stimulated by 64–132% with excess N in roots and leaves compared with those under the control N conditions (Table 3). In agreement with these results, the transcript level was down-regulated for *aldehyde oxidase 2* (*AAO2*), which is involved in ABA biosynthesis, whereas the gene encoding ABA 8′-hydroxylase (*CYP707A1*), which is involved in ABA degradation, was up-regulated in N-starved roots (see Table S8 available as Supplementary Data at *Tree Physiology* Online). However, two genes encoding carotenoid cleavage dioxygenase 7 (CCD7) and GRAM domain-containing protein, which are involved in ABA metabolism, were down-regulated in roots supplied with excess N (see Table S8 available as Supplementary Data at *Tree Physiology* Online).

The $GA_3$ concentrations were decreased by 39% in roots and by 27% in leaves under N deficiency, whereas they remained unaltered in the roots and leaves of plants exposed to excess N (Table 3). Interestingly, in N-deprived roots of poplars, there were increases in the transcript levels of an *Arabidopsis* homologous gene encoding a GASA domain-containing protein, which regulates increases in plant growth via GA-induced and DELLA-dependent signal transduction, and that can increase abiotic stress resistance by reduced ROS accumulation (Sun et al. 2013) (see Table S7 available as Supplementary Data at *Tree Physiology* Online). Moreover, in the N-starved leaves of *P. simonii*, we detected decreased transcript levels of three *Arabidopsis* homologous genes involved in gibberellin biosynthesis and signal transduction (see Table S8 available as Supplementary Data at *Tree Physiology* Online).

The main function of JA is the regulation of plant responses to abiotic and biotic stresses, as well as controlling plant growth and development, including growth inhibition, senescence and leaf abscission (Delker et al. 2006). In the roots and leaves of *P. simonii*, the JA concentration exhibited a similar response to ABA to changes in the external N levels, i.e., the JA concentration was reduced in N-deprived roots or leaves, but elevated in roots or leaves when supplied with excess N (Table 3). In contrast to the decrease in the concentration of JA, the mRNA level of an *Arabidopsis* homologous gene (*LOX3*) involved in JA biosynthesis was elevated in the roots of *P. simonii* with N deficiency (see Table S8 available as Supplementary Data at *Tree Physiology* Online).

Salicylic acid is a phenolic phytohormone with roles in plant growth and development, photosynthesis, transpiration, ion uptake and transport. Salicylic acid is also involved in endogenous signalling, where it mediates plant defence against pathogens. In the roots of *P. simonii*, the SA concentrations were elevated by 91% under N deprivation but reduced by 52% with excess N (Table 3). In leaves, the SA concentration decreased by 43% under N starvation (Table 3). Interestingly, in roots treated with N excess, we detected decreased mRNA levels of an *Arabidopsis* homologous gene encoding a SABATH methyltransferase, which methylates both SA and benzoic acid, and which is involved in direct defence mechanisms (see Table S8 available as Supplementary Data at *Tree Physiology* Online). These results suggest that the SA level in roots that received excess N was linked to the transcript level of *SABATH methyltransferase*. However, the GO term for response to SA stimulus was enriched in the roots and leaves with excess N (see Table S6 available as Supplementary Data at *Tree Physiology* Online), thereby suggesting that more genes may be differentially expressed under excess N in response to decreased SA levels.

### *Principal components analysis of physiological responses indicates that roots are more sensitive to changes in N availability than leaves*

To elucidate the key parameters involved in the response patterns of roots and leaves of *P. simonii* to changes in the N supply level, PCA was conducted using N-related physiological data (see Table S11 available as Supplementary Data at *Tree Physiology* Online; Figure 6), which showed that PC1 and PC2 accounted for 52 and 32% of the variation, respectively. PC1 accounted for the effects of the N supply levels and PC2 clearly separated the variation in tissue effects. The concentrations of Val and total N were key contributors to PC1, whereas the concentrations of soluble protein, His and IAA were essential components of PC2. In the PCA plot, the greater distance between components associated with the N supply levels support the stronger responsiveness of physiological parameters to changes in N levels. Therefore, the greater distance between components related to the control N level and N starvation or excess in roots compared with those in leaves suggests that the roots of *P. simonii* are more sensitive than the leaves to changes in the N levels.

## Discussion

### *Nitrogen starvation and excess induce distinct and common transcriptomic regulation processes that underlie morphological and physiological acclimation in poplar roots and leaves*

Several studies have demonstrated that changes in N supply levels can induce transcriptomic reprogramming in herbaceous species (Peng et al. 2007, Krapp et al. 2011, Canales et al. 2014), but little information is available about the transcriptomic regulation that underlies morphological and physiological acclimation to N starvation and excess in woody plants (Wei et al. 2013*b*, Euring et al. 2014). In poplars, including *P. simonii*, N deprivation induces root biomass production and inhibits the growth of aerial organs such as leaves, whereas a high supply of N represses root growth and stimulates the shoot biomass (Cooke et al. 2005, Li et al. 2012, Luo et al. 2013*a*, Wei et al. 2013*b*, Euring et al. 2014). Moreover, previous studies in

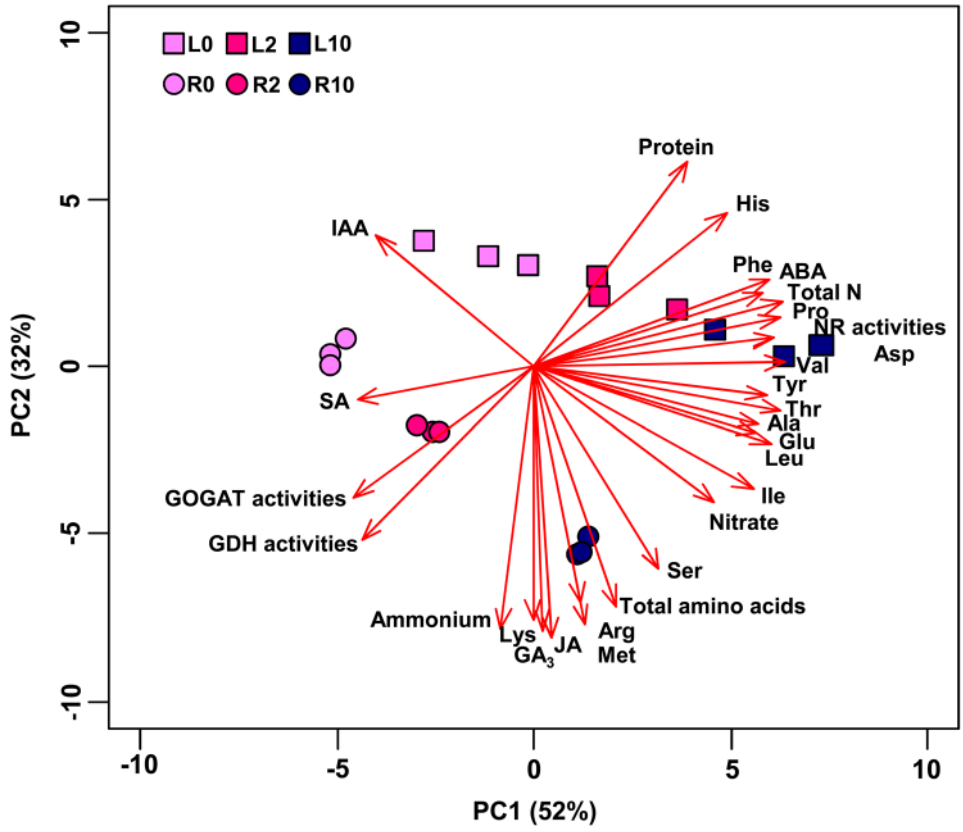


Figure 6. Principal components analysis plot of the first two principal components based on N-related parameters in the roots and leaves of *P. simonii* when exposed to 0, 2 or 10 mM $NH_4NO_3$. The PCA loadings are given in Table S11 available as Supplementary Data at *Tree Physiology* Online.

other poplar species have demonstrated decreased N intake and assimilation under a limiting N supply (Luo et al. 2013*a*) and accelerated N metabolism processes in high N conditions (Cooke et al. 2005, Li et al. 2012). The decreased levels of $NO_3^-$ and total free amino acids, as well as the reduced NR activity levels observed in *P. simonii*, have also been found in the roots and shoots of *A. thaliana* when exposed to N starvation for up to 10 days (Krapp et al. 2011). Clearly, physiological and morphological adaptation to environmental conditions must be driven by changes in gene regulation. Indeed, our global transcriptome analysis identified major changes in the N uptake and translocation systems, for instance, aquaporins involved in the transport of $NH_3/NH_4^+$ in plant roots (Coskun et al. 2013). In *Arabidopsis*, TIP2;1, which is induced under high N, is located on the tonoplast membrane where it functions as a transporter for $NH_3/NH_4^+$ into vacuoles (Loqué et al. 2005). Our finding that the poplar orthologue *TIP2;1* increased with high N and decreased with low N suggests that this gene is responsive to the intrinsic N concentration. This was also the case for *NIA2*, *GDH2* and *ASN1*, which are genes involved in the metabolism of N and amino acids. Nitrogen-responsive *NIA2* regulation has been observed previously in the roots of other *Populus* species (Li et al. 2012, Luo et al. 2013*a*). *GDH2* is an isogene that encodes GDH, which is involved in the biosynthesis of glutamate, a central precursor used in the biosynthesis of other amino acids in plants (Turano et al. 1997). Glutamine-dependent asparagine synthase is an essential enzyme for asparagine biosynthesis (Lam et al. 2003) and asparagine is important for N storage because 1 mol of asparagine contains 2 mol of N. Consistent with our results, the transcript levels of *ASN* were shown to be repressed by N deficiency and induced with high N supply in the roots of *Pinus pinaster* (Canales et al. 2010). Our results demonstrate that N utilization and storage are adjusted by transport and the rates of biosynthesis in response to N availability.

Nitrogen metabolism and hormonal regulation are tightly linked. We found two poplar *EXO* orthologues among the genes that were responsive to N. The *Arabidopsis EXO* (*EXORDIUM*) gene is involved in the control of brassinosteroid responses and the mediation of cell expansion in roots and leaves (Schröder et al. 2009). The elevated transcript levels of two poplar genes that are homologous to *AtEXO* in roots and the decreased mRNA levels of these genes in leaves under N starvation suggest that the contrasting transcriptional regulation of *EXO* in the roots and leaves of *P. simonii* may contribute to the stimulation of root growth and the inhibition of leaf expansion in N-deficient conditions. A similar behaviour was determined for the *ELIP1* orthologue in poplar. The *ELIP1* gene encodes an early light-inducible

protein 1 in *Arabidopsis* and the transcript level of *ELIP1* increases in response to high light (Casazza et al. 2005). We observed N-induced transcriptional changes in roots, so our results indicate that this gene is also regulated by other factors in addition to light, which are currently unclear.

In addition, N deficiency and excess resulted in common transcriptomic reprogramming effects in the roots and leaves of *P. simonii*, i.e., the transcript abundance of some genes decreased (or increased) when the N level changed compared with the controls, regardless of whether the N availability increased or decreased. For example, the transcript levels of five genes involved in N metabolism were down-regulated in the roots of *P. simonii* when exposed to N starvation or excess. These genes included two genes homologues of *AtGSR1* (glutamine synthetase 1;1), two homologues of *AtGS2* (glutamine synthase) and one homologue of *AtGDH1* (GDH). Moreover, under N starvation or excess, the roots and leaves also exhibited common transcriptomic regulation in *P. simonii*. The repressed *ASN1* mRNA levels in the roots and leaves of *P. simonii* when exposed to N starvation agreed with the limited N status in poplars, thereby suggesting that N storage is inhibited in N-deprived poplars. Extensins are abundant in the cell walls of higher plants and the cross-linking of extensins enhances the strength of the cell wall. In *Arabidopsis*, *AtEXT4* is a member of the extensin gene family, which is involved in the biosynthesis of cell-wall hydroxyproline-rich glycoproteins and in the control of stem thickness (Roberts and Shirsat 2006). Up-regulation of the transcript levels of homologous *AtEXT4* in the roots and leaves of *P. simonii* under N excess probably led to changes in cell extension, thereby contributing to morphological changes in the roots and leaves of poplars when excess N was supplied.

In addition to the specific responses discussed above, we found that ~30–40% of the differentially expressed genes formed a transcriptomic network in each condition that we compared, where many of the genes appeared to be hub genes because they were highly co-expressed with other genes in $NH_4NO_3$-treated *P. simonii*. Recently, co-regulation networks of transcriptomes have been identified in nitrate-treated roots of *Arabidopsis* (Canales et al. 2014). A genetic regulatory network has also been documented in the roots of *Populus tremula* × *P. alba* in response to low N (50 μM $NO_3^-$) supply (Wei et al. 2013*b*). These studies have focused on the transcriptomic regulation networks that underlie changes in the root architecture and/or the physiological status in response to nitrate treatments in plants. However, for the first time, we uncovered the co-expression networks that mediate global transcriptomic reprogramming to facilitate morphological and physiological acclimation to N starvation and excess in the roots and leaves of *P. simonii*. Furthermore, our results demonstrate that the hub genes identified in N-treated poplar roots were overrepresented in fundamental processes related to transport and responses to extracellular stimuli, whereas the genes identified in the leaves were enriched in biological processes such as photosynthesis and the generation of metabolic intermediates. Our analysis of *cis*-regulatory elements indicates that the hub genes in each transcriptomic network are co-regulated by *cis*-regulatory elements in the conserved motifs of their promoter regions, and that these elements act in an orchestrated manner during transcriptomic regulation in the roots and leaves of *P. simonii* in response to N depletion or excess. These results suggest that the hub genes in the co-expression network play central roles in cross-talk among distinct biological processes.

### *Poplars employ stress pathways to regulate the growth of roots and leaves in response to N starvation and excess*

As a macronutrient, N plays essential roles in plant growth and development. Thus, an appropriate N level is essential for normal plant growth, whereas N starvation or excess can expose plants to N stress. Under N starvation, *P. simonii* employs stress response pathways to inhibit the growth of its aerial parts and to stimulate root growth. With N deprivation, the decreased photosynthates in *P. simonii* must be channelled to roots for growth and to secondary compounds for stress defence. Indeed, our data demonstrated the stimulation of soluble sugars in N-starved roots, which can be used for root growth, and of soluble phenolics in N-starved leaves, which are secondary compounds required for defence. In general, there is a trade-off between growth and defence in plants (Walters and Heil 2007). Thus, plant growth is limited in N-deficient conditions and more carbohydrates are used to synthesize secondary metabolites, thereby leading to enhanced stress resistance (Massad et al. 2012). Our results indicate that N starvation can induce stress resistance in *P. simonii*. Moreover, the transcriptomic data obtained using the roots and leaves of *P. simonii* exposed to N starvation or excess also indicated the enhancement of stress pathways to modulate the growth of roots and leaves. For example, among 83 GO terms related to biological processes, 20 GO terms were associated with stress in the N-starved roots of *P. simonii*. In particular, a number of genes involved in modulation of root and leaf growth exhibited differential expression in the roots and leaves under N deficiency or excess. The *CLE* (*CLAVATA3*/*ESR-related*) genes play crucial roles in the morphological plasticity of *Arabidopsis* root systems in response to N deficiency (Araya et al. 2014). Thus, the decreased transcript levels of *Arabidopsis* homologous *CLE* genes with N starvation or excess may contribute to alterations in the root characteristics of *P. simonii* under changing N levels. Repressed mRNA levels of a large number of genes involved in photosynthesis have been detected previously in the leaves of *A. thaliana* when exposed to N starvation or limiting N levels (Peng et al. 2007, Krapp et al. 2011). Similarly, under N deprivation, the decreased mRNA levels of *PSAA*, *PSBA*, *PSBC*, *PSBN* and *YCF9*, which are involved in the light reaction in photosynthesis, as well as the increased transcript level of *LHCB1.4* with excess N, probably contribute to alterations in the

photosynthetic rates and leaf growth with these N supply levels. These transcriptomic data suggest that N starvation or excess can induce changes in the transcript levels of genes involved in stress response, thereby enhancing stress resistance and modulating the growth of *P. simonii*.

Consistent with these stress responses in the roots and leaves of *P. simonii*, we found that changes in the concentrations of phytohormones, particularly stress hormones such as ABA, JA and SA, in the roots and/or leaves of *P. simonii* treated with N starvation or excess indicated that these poplars experienced stress. Phytohormones play essential roles in regulating plant growth and stress responses in various environmental conditions (Kiba et al. 2011). Many studies have demonstrated that the concentrations of IAA, ABA, GA, JA and SA can be modified in plants in response to abiotic and biotic stresses, including N stress (Tian et al. 2008, Luo et al. 2009*a*, 2009*b*, Kiba et al. 2011, Shi et al. 2015). In herbaceous species, experimental evidence has shown that N and phytohormone signals are integrated to facilitate the morphological and physiological acclimation of plants in response to N deficiency or excess (Tian et al. 2008, Gojon et al. 2011, Kiba et al. 2011, Krouk et al. 2011, Ruffel et al. 2011). Indole-3-acetic acid is a key player in signalling pathways, but it is also an N-containing compound that can be affected by N availability. The decreased IAA levels found in the roots and leaves of *P. simonii* treated with excess N indicate that IAA metabolism is linked to N physiology. Indeed, previous studies suggest that the reduced root growth in the presence of a high supply of N is due to decreased IAA levels (Tian et al.



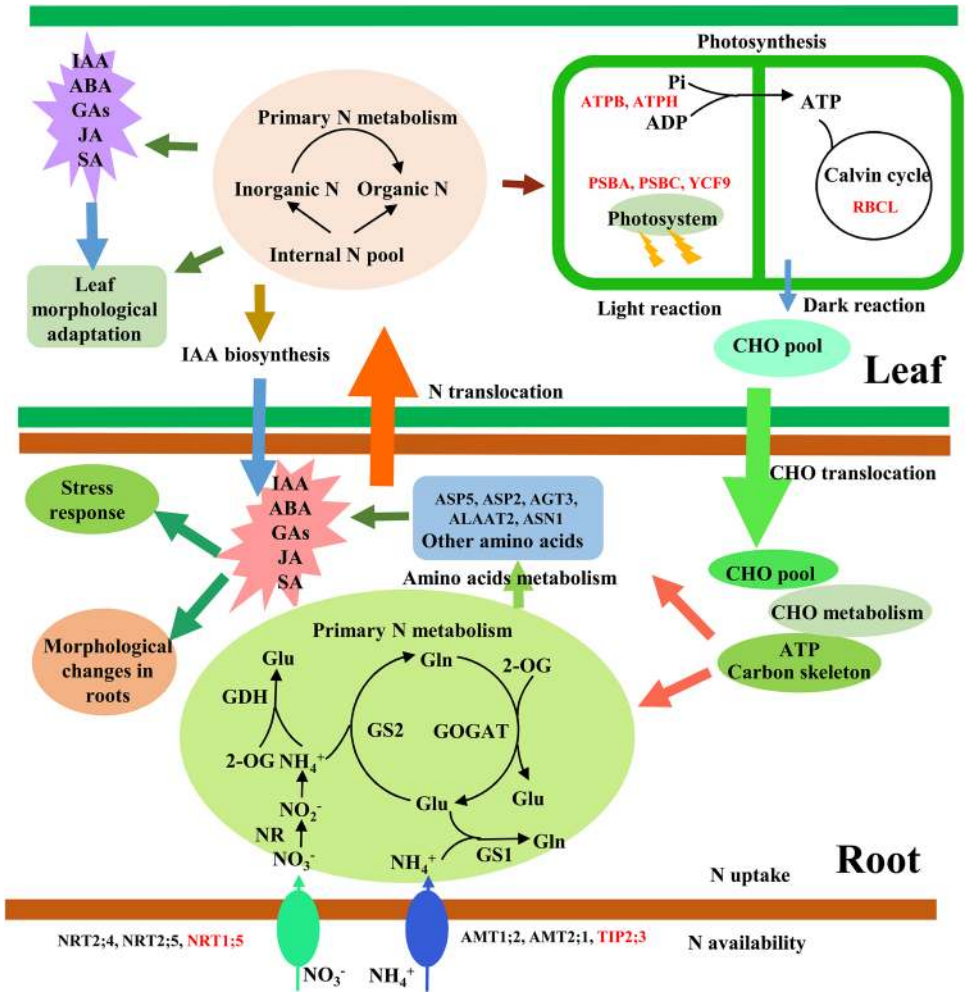


Figure 7. Schematic model representing the growth and stress responses in the roots and leaves of *P. simonii* when exposed to N starvation and excess. $NH_4^+$ and $NO_3^-$ are absorbed via AMTs and NRTs, which are differentially regulated in response to changes in N availability. The $NH_4^+$ and $NO_3^-$ absorbed by the roots are converted into glutamate (Glu) and glutamine (Gln) via primary N metabolism. Subsequently, Glu and Gln in plants are converted into other amino acids. The amino acids and some inorganic N from roots are translocated into the leaves where they are assimilated into organic N to form the internal foliar N pool. Under N starvation, more CHO compounds are translocated into the roots to support root growth and development, whereas excessive N has the opposite effect. N availability affects the concentrations of hormones (e.g., IAA, ABA, $GA_3$, JA and SA), thereby triggering the corresponding signalling cascades and modulating the morphological characteristics of roots and leaves. Changes in the N status in poplars may act as an internal stress stimulus to activate phytohormone-mediated signalling pathways and trigger stress responses. Differentially expressed hub genes (highlighted in red) are involved in N-mediated morphological and physiological responses.

2008), whereas induced lateral root growth with low N levels is associated with the function of the IAA biosynthetic gene *tryptophan aminotransferase related 2* (Ma et al. 2014*a*). Recently, it was shown that the nitrate transporter NRT1;1 can also transport IAA in *Arabidopsis* roots, where it plays a role in N sensing in plants (Krouk et al. 2010). This suggests that IAA and N signals are integrated in plants to modulate root growth in response to changes in N availability.

Abscisic acid inhibits lateral root growth in the presence of a high nitrate supply (Signora et al. 2001) and there is a link between the levels of ABA and the N status of plants (Brewitz et al. 1995, Kiba et al. 2011). In the current study, overexpression of the mRNA of the *Arabidopsis* homologue of ABA-degraded *CYP707A1* gene (Okamoto et al. 2009) was consistent with the decreased ABA level found in the N-starved roots of *P. simonii*. Similarly, down-regulation of the *Arabidopsis* homologous gene *carotenoid cleavage dioxygenase 7* (Booker et al. 2004) may have contributed to the high ABA level in poplars roots when supplied with excess N. These results highlight the role of ABA-mediated signalling pathway in fine tuning N acquisition by the roots of *P. simonii* in response to N deficiency and excess. Although little is known about the involvement of GA, JA and SA in N signalling and metabolism in plants (Peng et al. 2007, Rennenberg et al. 2010), changes in levels of $GA_3$, JA and SA, and the altered transcript levels of genes associated with $GA_3$- JA- and SA-mediated signalling pathways in N-treated *P. simonii* suggest that these phytohormones also play roles in sensing changes in the availability of N and in modulating the growth of poplar roots and leaves.

As summarized in Figure 7, N deprivation reduced the concentrations of $NH_4^+$, $NO_3^-$, total free amino acids and total N, as well as modifying the levels of other mineral nutrients in the roots and leaves of *P. simonii*. In contrast, N excess had the opposite effects in most cases. Our global transcriptomic analysis showed that N starvation and excess N induce distinct and common transcriptomic regulation processes that underlie morphological and physiological acclimation in poplar roots and leaves. GO terms related to ion transport, response to auxin stimulus and aromatic compound biosynthetic process were specifically enriched in roots, whereas the GO term for response to ABA stimulus was specifically overrepresented in leaves. The common GO terms enriched with all N treatments were related to development, N metabolism, response to stress and hormone stimulus. Among all 992 genes that exhibited differential expression, ~30–40% formed a transcriptomic network in each condition. Hub genes in each transcriptomic network were co-regulated via *cis*-regulatory elements in the conserved motifs of their promoter regions. Consistent with the differentially expressed genes involved in phytohormone metabolism, N deficiency led to decreased concentrations of ABA, $GA_3$, JA and SA in roots, and of ABA and JA in leaves, whereas N excess led to reduced levels of IAA and SA in roots, and of IAA in leaves, as well as elevated concentrations of ABA and JA in the roots and leaves of *P. simonii*. These results suggest that the co-expression network that mediates global transcriptomic reprogramming plays a key role in the morphological and physiological acclimation of the roots and leaves of *P. simonii* in response to N starvation and excess. *Populus simonii* can activate stress pathways via elevated secondary compounds and by integrating N and phytohormonal signalling pathways to modulate root and leaf growth in response to changes in N availability.

## Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

## Conflict of interest

None declared.

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