Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2025:88
Faculty of Forest Sciences

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SLU
SWEDISH UNIVERSITY
OF AGRICULTURAL
SCIENCES

DOCTORAL THESIS
Umeå 2025

Acta Universitatis Agriculturae Sueciae
2025:88

ISSN 1652-6880
ISBN (print version) 978-91-8124-072-6
ISBN (electronic version) 978-91-8124-118-1
https://doi.org/10.54612/a.86bpiki3tb

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Print: SLU Grafisk service, Uppsala 2025

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Abstract

Nitrogen is vital for tree growth. Recent declines in Swedish forest productivity and a growing interest in forest fertilisation highlight the urgent need to understand the mechanisms governing tree nitrogen uptake and utilisation. While it is well established that nitrogen availability influences the quantity, structure, and chemistry of wood, less is known about the effects of different nitrogen forms, the underlying molecular mechanisms, and the short-term nitrogen responses in the developing xylem tissues of forest trees.

To investigate the effects of nitrogen, I developed a strategy to systematically vary both the sources and levels of nitrogen supplied to hybrid aspen (*Populus tremula* × *P. tremuloides*) trees. In addition, a near-infrared (NIR) spectroscopy method was established to characterise the wood's physical and chemical properties. Controlled nitrogen additions showed that increased nitrogen availability enhances biomass accumulation and radial expansion of xylem cells, while decreasing lignin content and wood density. Among the nitrogen sources tested, nitrate was unique in that it significantly stimulated shoot biomass accumulation and increased the proportion of *p*-hydroxyphenyl units (H-type lignin) in the wood.

Dynamic, time-resolved transcriptome analyses revealed that nitrate is transported in the xylem sap of hybrid aspen stems and taken up into differentiating xylem tissues, rapidly inducing the expression of genes related to xylem cell expansion. Functional assays indicated that nitrate promotes xylem cell expansion via the transcription factor CYTOKININ RESPONSE FACTOR 4 (CRF4). A population-wide analysis of nitrogen transport and metabolism-related gene expression suggested that nitrogen uptake into differentiating xylem tissues has to be tightly regulated, since excessive nitrogen retrieval from the xylem sap seemed to suppress radial tree growth.

Altogether, these studies elucidate the mechanisms by which nitrogen—and nitrate in particular—regulates wood formation in *Populus* trees. They also provide advanced imaging and molecular tools that can be used to optimise nitrogen use and promote forest tree growth.

Keywords: cambial growth, wood formation, nitrogen responses, nitrate signalling, wood characterisation, NIR imaging, single cell RNA sequencing

Fysiologiska och molekylära aspekter av kvävestimulerad kambieaktivitet i asp

Sammanfattning

Kväve är avgörande för träds tillväxt. De senaste minskningarna i svensk skogsproduktivitet och ett växande intresse för skogsgödsling understryker det akuta behovet av att förstå de mekanismer som styr trädens upptag och användning av kväve. Det är väl etablerat att kvävetillgänglighet påverkar vedens mängd, struktur och kemi, men mindre är känt om effekterna av olika former av kväve, de underliggande molekylära mekanismerna och de kortsiktiga kväveresponserna i xylemet.

För att undersöka kvävets effekter utvecklade jag en strategi för att systematiskt variera både kvävekällor och kvävenivåer som tillförs hybridasp (*Populus tremula* × *P. tremuloides*). Dessutom etablerades en nära-infraröd (NIR) spektroskopimetod för att karakterisera vedens fysiska och kemiska egenskaper. Kontrollerad kvävetillförsel visade att ökad kvävetillgänglighet främjar ackumuleringen av biomassa och radiell expansion av xylemceller, samtidigt som lignininnehåll och veddensitet minskar. Bland de testade kvävekällorna var nitrat unikt då det signifikant stimulerade ackumulering av biomassa och ökade andelen *p*-hydroxyphenyl enheter (H-typ lignin) i veden.

Dynamiska och tidsupplösta transkriptomanalyser visade att nitrat transporteras via xylemsaven i hybridaspens stam och tas upp i differentierande xylemvävnad, vilket snabbt inducerade uttryck av gener relaterade till xylemcellernas expansion. Funktionella analyser visade att nitrat främjar xylemcellernas expansion via transkriptionsfaktorn CYTOKININ RESPONSE FACTOR 4 (CRF4). En populationsomfattande genuttrycksanalys av genuttryck kopplat till kvävetransport och metabolism antydde att upptaget av kväve i differentierande xylemvävnad är strikt reglerat, eftersom överdrivet upptag av kväve från xylemsaven verkade hämma trädens radiella tillväxt.

Sammantaget klargör dessa studier de mekanismer genom vilka kväve – och särskilt nitrat – reglerar vedbildning i poppelträd. De tillhandahåller även avancerade avbildning- och molekylärbiologiska verktyg som kan användas för att optimera kväveanvändning och främja tillväxt hos skogsträd.

Nyckelord: kambieaktivitet, vedbildning, kväveresponser, nitratsignalering, vedkarakterisering, NIR-avbildning, RNA-sekvensering av enskilda celler

Preface

> “It is simply this: do not tire, never lose interest, never grow indifferent - lose your invaluable curiosity and you let yourself die. It's as simple as that, isn’t it?”
>
> - Tove Jansson, Fair Play

Dedication

To my family, my partner Emanuel, and my son Eli, who give me invaluable perspective on what is important in life.

I also dedicate this work to Hannele Tuominen, that have inspired me along the way with your curiosity, dedication to science and supportive character.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Renström, A., Choudhary, S., Gandla, M.L., Jönsson, L.J., Hedenström, M., Jämtgård, S. & Tuominen, H. (2024). The effect of nitrogen source and levels on hybrid aspen tree physiology and wood formation. *Physiologia Plantarum,* 176(1), e14219. https://doi.org/https://doi.org/10.1111/ppl.14219

II. Renström, A., Scheepers, G., Yassin, Z., Grahn, T., Sivan, P., Niittylä, T., Mellerowicz, Ewa J., & Tuominen, H. (2025). High-resolution imaging of the physical and chemical properties of Populus wood using SilviScan™ and near-infrared spectroscopy. *IAWA Journal*, 1-16. Online publication 14 Feb 2025. https://doi.org/https://doi.org/10.1163/22941932-bja10179

III. Zhang, Y., Choudhary, S., Renström, A., Luomaranta, M., Chantreau, M., Fleig, V., Gaboreanu, I., Grones, C., Nilsson, O., Robinson, K. M., & Tuominen, H. (2025). Unraveling Nitrogen Uptake and Metabolism: Gene Families, Expression Dynamics, and Functional Insights in Aspen (*Populus tremula*). *Tree Physiology*. In press. https://doi.org/10.1093/treephys/tpaf099

IV. Choudhary S., Renström A., Miskolczi P., Kochakarn T., Piombo E., Jämtgård S., Henriksson J., Tuominen H. (2025). Xylem cells rapidly sense and respond to nitrate to promote cell expansion. 28 October 2025, PREPRINT (Version 2) available at Research Square. https://doi.org/10.21203/rs.3.rs-7685028/v2

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The contribution of Anna Renström to the papers included in this thesis was as follows:

I. AR performed all growth experiments and collected and prepared the plant material for all analyses. AR wrote the first draft of the manuscript.

II. AR provided material for the different analyses and contributed to the pyrolysis-GC/MS analyses. AR wrote the manuscript together with GS.

III. AR named genes in the phylogenetic analyses and performed the nitrate and ammonium treatments.

IV. AR performed plant experiments, collected and prepared plant material for RNA sequencing (RNAseq) and wood histology, and contributed to the writing.

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Abbreviations

$^{15}NO_3$	isotope-labeled nitrate
4CL	4-COUMARATE-COA LIGASE
AA	amino acid
AAP	AMINO ACID PERMEASE
ABA	abscisic acid
AFM	Atomic Force Microscopy
AMT	AMMONIUM TRANSPORTER
ANT	AINTEGUMENTA
AP	ASPARTIC PROTEASE
AQP	AQUAPORIN
ARAC	ARABIDOPSIS RAC-LIKE
ARF	AUXIN RESPONSIVE FACTOR
Arg	arginine
ARR	ARABIDOPSIS RESPONSE REGULATOR
AS/ASN	ASPARAGINE SYNTHETASE
Asn	asparagine
ATP	adenosine triphosphate
Aux	auxin
BSP	BARK STORAGE PROTEIN
bZIP/BZIP	BASIC LEUCINE ZIPPER PROTEIN
C/N	carbon/nitrogen

C4H	CINNAMATE 4-HYDROXYLASE
CAD	CINNAMYL ALCOHOL DEHYDROGENASE
CAld5H	CONIFERALDEHYDE 5-HYDROXYLASE
$CaSO_4$	calcium sulfate
CBF	C-REPEAT BINDING FACTOR
CDF	CYCLING DOF FACTOR
CDKB	CYCLIN-DEPENDENT KINASE B
CEP/CEPD	C-TERMINALLY ENCODED PEPTIDE, CEP DOWNSTREAM
CesA/CESA	CELLULOSE SYNTHASE A
CK	cytokinin
Cl^-	chloride
CLE	CLAVATA3/EMBRYO SURROUNDING REGION
CLV	CLAVATA
CML	compound middle lamella
COBL	COBRA-LIKE
COMT	CAFFEIC ACID O-METHYLTRANSFERASE
CPIK	CBL-INTERACTING PROTEIN KINASE
CPK	Ca^{2+} SENSOR PROTEIN KINASE
CRF	CYTOKININ RESPONSE FACTOR
Csl, CSL	CELLULOSE SYNTHASE-LIKE
CYC	CYCLIN
DEGs	differentially expressed genes
EA-IRMS	Elemental Analyzer–Isotope Ratio Mass Spectrometry
EBF	EIN3-BINDING F BOX PROTEIN

eQTL	expression quantitative trait locus
ERF	ETHYLENE RESPONSE FACTOR
ET	ethylene
EXP/EXPA	ALPHA-EXPANSIN
EXPB	BETA-EXPANSIN
FA	ferulate
FBH	FLOWERING BHLH
FT-IR	Fourier-Transform Infrared Spectroscopy
G2	GOLDEN 2
GA	gibberelic acid
GC	Gas Chromatography
GDH	GLUTAMATE DEHYDROGENASE
GH	GLYCOSIDE HYDROLASE
GID	GIBBERELLIN INSENSITIVE DWARF
Gln	glutamine
GLP	GERMIN-LIKE PROTEIN
GNC	GATA NITRATE-INDUCIBLE CARBON-METABOLISM-INVOLVED
GO	Gene Ontology
GOGAT	GLUTAMINE-2-OXOGLUTARATE AMINO-TRANSFERASE
GRN	Gene Regulatory Network
GS	GLUTAMINE SYNTHETASE
GS	Genome Selection
GT	GLYCOSYLTRANSFERASE
GWAS	Genome-Wide Association Study

HAT	HOMEODOMAIN-LEUCINE ZIPPER PROTEIN
HB	HOMEOBOX-LEUCINE ZIPPER PROTEIN
HCT	HYDROXYCINNAMOYL-CoA:SHIKIMATE HYDROXYCINNAMOYL TRANSFERASE
HD-ZIP III	CLASS III HOMEODOMAIN-LEUCINE ZIPPER
HIK	HINKEL
IAA	INDOLE-3-ACETIC ACID (auxin)
INV	INVERTASE
IPT	ISOPENTENYL TRANSFERASE
IRX	IRREGULAR XYLEM
JA	jasmonate
JAR	JASMONATE RESISTANT
JAZ	JASMONATE ZIM DOMAIN
K^+	potassium
KNAT	KNOTTED-LIKE HOMEOBOX OF ARABIDOPSIS THALIANA
KNO_3	potassium nitrate
K_s	hydraulic conductivity
LAC	LACCASE
LAX	AUXIN RESISTANT 1/LIKE-AUX
LBD	LATERAL ORGAN BOUNDARY DOMAIN
LCM	Laser Capture Microdissection
LHT	LYSINE HISTIDINE TRANSPORTER
LM	light microscopy
LMX	LIM HOMEOBOX
lncRNA	long non-coding RNA

m/z	mass-to-charge ratio
MALDI-MS	Matrix-Assisted Laser Desorption Ionisation-Mass Spectrometry
MAN	ENDO-BETA-MANNANASE
MC	METACASPASE
MFA	microfibril angle
miRNA	microRNA
mM	millimolar
MOE	modulus of elasticity
mRNA	messenger RNA
MS	Murashige and Skoog
MYB	MYELOBLASTOSIS
MYC	MYELOCYTOMATOSIS
N	nitrogen
N_2	molecular nitrogen (dinitrogen)
N_2O	nitrous oxide
NAC	NAC DOMAIN CONTAINING PROTEIN
NADH	nicotinamide adenine dinucleotide (reduced form)
NAGs	NITROGEN ASSIMILATION GENEs
NERD	NOVEL ETHYLENE RESPONSE FACTOR DNA-BINDING
NFYA	NUCLEAR FACTOR Y, SUBUNIT A
NH_4^+	ammonium
NH_4NO_3	ammonium nitrate
NIN	NODULE INCEPTION
NIP	NOD26-LIKE INTRINSIC PROTEIN

NIR	near-infrared
NIR	NITRITE REDUCTASE
NLP	NIN-LIKE PROTEIN
nm	nanometer
NMR	Nuclear Magnetic Resonance
NO_3^-	nitrate
NPF	NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER FAMILY
NPK	nitrogen, phosphorus, potassium (fertilizer ratio)
NR	NITRATE REDUCTASE
NRE	NITRATE-RESPONSIVE CIS-ELEMENTS
NRT	NITRATE TRANSPORTER
NST	NAC SECONDARY WALL THICKENING PROMOTING FACTOR/SECONDARY WALL ASSOCIATED NAC DOMAIN
NUE	Nitrogen Use Efficiency
PAL	PHENYLALANINE AMMONIA-LYASE
PARVUS	gene family name
pBA	*p*-hydroxybenzoate
pCA	*p*-coumarate
PCA	Principal Component Analysis
PCD	Programmed Cell Death
PDLP	PLASMODESMATA-LOCATED PROTEIN
PGSIP	PLANT GLYCOGENIN-LIKE STARCH INITIATION PROTEIN
pHB	*p*-hydroxybenzoic acid
PIP	PLASMA MEMBRANE INTRINSIC PROTEIN

PLE	PLEIADE
PLS	Partial Least Squares
PNR	primary nitrate response
PS	PHOTOSYSTEM
PXY	PHLOEM INTERCALATED WITH XYLEM
Py-GC/MS	Pyrolysis-Gas Chromatography/Mass Spectroscopy
QTL	Quantitative Trait Loci
RISE	Research Institutes of Sweden
RNAseIII	RIBONUCLEASE III
RNAseq	RNA sequencing
ROPGAP	RHO GTPase-ACTIVATING PROTEIN
RuBisCO	RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE
S, G, H	syringyl, guaiacyl, and *p*-hydroxyphenyl (types of lignin monomers)
S/G	syringyl/guaiacyl ratio
S1, S2, S3	names for the three layers of the secondary cell wall
SA	salicylic acid
scRNAseq	single-cell RNA sequencing
SCW	secondary cell wall
SEM	Scanning Electron Microscopy
SHM	SERINE HYDROXYMETHYLTRANSFERASE
SND	SECONDARY WALL ASSOCIATED NAC DOMAIN
SNPs	single-nucleotide polymorphisms
snRNAseq	single-nucleus RNA sequencing

SNZ	SCHNARCHZAPFEN
spp	specie plural (used for multiple species in a genus)
SuSy	SUCROSE SYNTHASE
T89	clone name (not an acronym, but used as a label)
TCA	tricarboxylic acid
TDIF	TRACHEARY ELEMENT INHIBITORY FACTOR
TDR	TDIF RECEPTOR
TEM	Transmission Electron Microscopy
TF	transcription factor
tZ	*trans*-Zeatin
UMAP	Uniform Manifold Approximation and Projection
UPSC	Umeå Plant Science Centre
VND	VASCULAR RELATED NAC DOMAIN
VPE	VACUOLAR PROCESSING ENZYME
VSP	VEGETATIVE STORAGE PROTEIN
WOX	WUSCHEL-RELATED HOMEOBOX
WRKY	WRKY (transcription factors)
XCP	XYLEM CYSTEINE PEPTIDASE
XSP	XYLEM SERINE PEPTIDASE
XET/XTH	XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE
ZF-HD	ZINC FINGER HOMEODOMAIN
µm	micrometre

1. Introduction

For trees to contribute to the mitigation of climate change, an increase in forest growth would be required. Trees are important as they can capture and store carbon from the atmosphere and provide alternatives to fossil-based products. In ecosystems where nitrogen (N) availability is limited, such as in many Swedish forests, nitrogen fertilisation has been identified as a possible way to increase carbon sequestration and forest growth within a relatively short period (Hyvönen *et al.* 2008; Jämtgård *et al.* 2023). In 2023, less than 1% of Swedish production forests were fertilised, and only about one fifth of the added nitrogen was estimated to be taken up by the trees (Jämtgård *et al.* 2023). Perhaps due to the recent decrease in Swedish forest growth (Laudon *et al.* 2024), there is a rising interest in forest fertilisation (Drott *et al.* 2025). The risks of increasing nitrogen fertilisation are biodiversity losses and leaching (Jämtgård *et al.* 2023; Drott *et al.* 2025).

So, we know that we can increase tree growth and stem volumes by supplying nitrogen, but how can we do it sustainably? We need to know more about the underlying molecular mechanisms of nitrogen-stimulated growth. In my project, the focus lies on the cambium and the radial growth of trees, the basis of wood formation. I use hybrid aspen (*P. tremula* x *P. tremuloides*) and controlled nitrogen supply as a model system to examine the effect on wood as a tissue, looking at cell types, cell sizes, its chemical composition, and molecular responses.

I aimed to understand these processes on a fundamental level, which may, in the long run, facilitate increased forest growth while limiting the use of fertilisers.

1.1 Cambial growth

Wood is formed by the activity of the lateral meristem of the stem – the vascular cambium. The vascular cambium is composed of meristematic cells that divide periclinally to form secondary phloem tissues outwards and secondary xylem tissues inwards. Anticlinal divisions are required for increased radial growth. Different from the primary growth of the plants, which contributes to the elongation of a plant's axes (roots and shoots), secondary growth allows the thickening of the stem in the lateral direction by the activity of the cork and the vascular cambia. For the sake of this thesis, I will focus mostly on the secondary growth of the stem and mainly on what has been shown in *Populus spp*.

The cambial activity is controlled by molecular mechanisms that are partially understood. One of the central mechanisms includes the action of the phloem-derived CLAVATA3/EMBRYO SURROUNDING REGION (CLE) peptides CLE41 and CLE44, also known as TRACHEARY ELEMENT INHIBITORY FACTORs (TDIFs), which move towards the cambium and bind there to the PHLOEM INTERCALATED WITH XYLEM (PXY)/TDIF RECEPTOR (TDR) to stimulate cell division activity, control vascular patterning, and suppress xylem differentiation (Etchells *et al.* 2016; Fatz *et al.* 2023). Downstream of the TDIF-PXY/TDR peptide signalling, WUSCHEL-RELATED HOMEOBOX 4 (WOX4) acts to regulate cambial cell proliferation (Kucukoglu *et al.* 2017; Fatz *et al.* 2023). The expression of *WOX4* also seems to be under epigenetic (Dai *et al.* 2023) and post-translational (Tang *et al.* 2022) control. Other CLE peptides, shown to regulate the cambial activity in *Populus* trees, are CLE20 as a negative regulator (Zhu *et al.* 2020), and CLE47 as a positive regulator (Kucukoglu *et al.* 2020). In addition to the TDIF-PXY/TDR regulation, plant hormones such as auxin, cytokinins (CKs), and brassinosteroids coordinate the functioning of these pathways to regulate cell division activity and to create precise boundaries between the cambium and the differentiating phloem and xylem tissues (Tuominen *et al.* 1997; Nieminen *et al.* 2008; Immanen *et al.* 2016; Fischer *et al.* 2019; Fu *et al.* 2021; Fatz *et al.* 2023). CK peaks in the developing secondary phloem, and the CK levels influence the rate of cell division in the cambium (Immanen *et al.* 2016). It has also been shown that CK signalling acts in a non-cell-autonomous way to regulate cambial activity, affecting both the periclinal and anticlinal divisions (Fu *et al.* 2021). Auxin, on the other hand, peaks in the cambial region and, apart from influencing the cambial activity, affects the duration of the developmental processes such as xylem fibre expansion (Tuominen *et al.* 1997). The auxin signalling works through the degradation of AUXIN/INDOLE-3-ACETIC ACID (IAA) proteins that repress the transcriptional activity of their associated AUXIN RESPONSE FACTOR (ARF) proteins (Mockaitis & Estelle 2008). In *P. tomentosa*, an IAA9-ARF5 module was shown to be involved in the regulation of xylem development. ARF5.1 could bind to the promoters of two CLASS III HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIP III) genes, *HOMEOBOX-LEUCINE ZIPPER PROTEIN 7* and *8* (*HB7, HB8)* (Xu *et al.* 2019), which in turn control xylem formation (Zhu *et al.* 2013). The local auxin maxima and the expression of the HD-ZIP III transcription factors also define the stem cell organiser of the vascular cambium (Smetana *et al.* 2019). Gradients of auxin and TDIF have been shown to determine the size of the cambium and the localisation of the stem cells (Eswaran *et al.* 2024). Moreover, microRNAs (miRNAs) have also been shown to influence xylem formation (Hou *et al.* 2020; Wang *et al.* 2023; Guo *et al.* 2024). A module of MIR166c-ENOYL-COA HYDRATASE 2 (ECH2) was recently identified in *P. alba x P. glandulosa*. This module was shown to regulate cambial differentiation via auxin signalling pathways (Zhao *et al.* 2025).

The third constitutively present hormone, known to impact xylem development, is the bioactive gibberellins (GAs). GAs have been shown to peak in the developing xylem and affect xylem differentiation (Eriksson *et al.* 2000; Israelsson *et al.* 2005; Immanen *et al.* 2016; Fischer *et al.* 2019). The gaseous hormone ethylene (ET) is also important in cambial growth (Etchells *et al.* 2012; Fischer *et al.* 2019) even though its role seems to be most prominent in response to external stimuli (Love *et al.* 2009; Vahala *et al.* 2013; Seyfferth *et al.* 2019).

What is less well understood is how cambial growth and the involved molecular pathways are influenced by environmental factors such as nitrogen availability. Potential crosstalk between plant hormones and nitrogen has been discussed in connection to the members of the NITRATE TRANSPORTER1/PEPTIDE TRANSPORTER (NPF) gene family since they can transport not only nitrate but also GAs, IAA, abscisic acid (ABA), and jasmonates (JAs) (Krouk 2016; Corratge-Faillie & Lacombe 2017; Fischer *et al.* 2019; Kanstrup & Nour-Eldin 2022). In addition, interactions between the signalling pathways of nitrogen and cytokinins have been reported

(Takei *et al.* 2004; Dluzniewska *et al.* 2006; Sakakibara *et al.* 2006; Osugi *et al.* 2017; Gu *et al.* 2018; Poitout *et al.* 2018; Zhang *et al.* 2020), and will be covered further in the "N as a plant signal" section of the thesis.

1.1.1 Wood formation

Wood formation is a process that starts with the cambial cell divisions and is followed by the differentiation of xylem elements. In each radial file, the stem cells, also called initials, divide and give rise to a xylem mother cell that can divide and differentiate into fibres, tracheids, vessel elements, or axial/radial parenchyma cells (Bossinger & Spokevicius 2018; Smetana *et al.* 2019). There are two types of initials: fusiform and ray initials. The fusiform initials give rise to the axial arrangement of cells, and the ray initials to the radial one. The xylem cell types in aspen are fibres, vessel elements, and ray parenchyma cells. The process of xylem differentiation is divided into three phases: cell expansion, secondary cell wall (SCW) formation and lignification, and lastly, programmed cell death (PCD) and autolysis (Fromm 2013; Ye & Zhong 2015; Sundell *et al.* 2017; Meents *et al.* 2018).

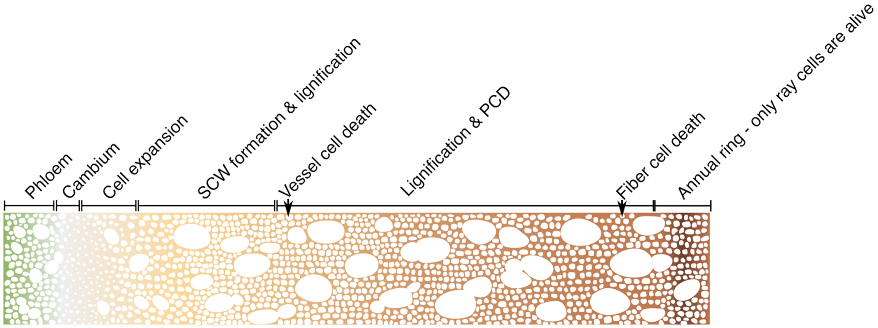

Figure 1. A schematic representation of the different wood formation phases in a transverse section of a *Populus* stem. The image is adapted from https://plantgenie.org/exImage.

Cell expansion

All xylem cell types undergo expansion in the cell expansion phase. For cells to enlarge, the primary cell wall must expand, and the orientation of the cellulose microfibrils in the primary wall determines the direction of the expansion. This, together with cell differentiation, determines the wood structure (Fromm 2013). The major factors facilitating cell wall expansion are turgor pressure and cell wall stress relaxation (Fromm 2013; Cosgrove 2016a; Chebli & Geitmann 2017). Cell wall stress relaxation implies that the walls loosen and that the turgor pressure reduces, which allows for water uptake, leading to physical enlargement of the cell and decreased interface between the cell wall polymers (Cosgrove, 1997). Cell wall relaxation can be caused by acidification of the cell walls, cell wall loosening enzymes, and expansins. Cell wall loosening enzymes such as xylanases, glucanases, endoglucanases, and XYLOGLUCAN ENDOTRANSGLUCOSYLASES/HYDROLASES (XET/XTH) can act on the cell wall polymers, changing the chemical bonding between cellulose and hemicelluloses (mostly xyloglucan), which in turn changes the structure of the cell walls (Cosgrove 1997; Mellerowicz *et al.* 2001; Cosgrove 2005; Fromm 2013). The XTH activity as a cell wall loosening agent is debated (Cosgrove 2016b), but the expression has been found to correlate with cell growth (Chebli & Geitmann 2017). Even though the underlying mechanism is unclear, the *Populus XET16-34,* was found to influence the expansion of vessel elements (Nishikubo *et al.* 2011), similarly to the *Arabidopsis XTH4* and *XTH9* (Kushwah *et al.* 2020).

Additionally, a pH-dependent mechanism known as acid growth affects wall expansion. The acidification causes the weakening of bonds between cell wall polymers and is partially regulated by plasma membrane-located H^+-ATPases (Cosgrove 1997; Cosgrove 2000; Fromm 2013). Acidification, coupled with the action of expansins, also impacts wall relaxation. Expansins are wall-associated proteins that can reversibly disrupt the non-covalent bonds between cell wall polymers in a pH-dependent way (Cosgrove 2000). Thus, they can disrupt hydrogen bonding but have no hydrolytic or enzymatic activity. It has been suggested that expansins bind to polysaccharides and locally loosen the polymer from the matrix, allowing for other wall relaxation mechanisms to act on the site (Cosgrove 2000; Cosgrove 2005). Expansins explored in poplar wood include the *ALPHA-EXPANSIN 1-8 (EXP1-8)* and the *BETA-EXPANSIN 1 (EXPB1)* (Gray-Mitsumune *et al.* 2004). *EXP1* was reported to be expressed in the cambium and the expanding zones of wood and implicated in both radial and longitudinal expansion of the cell walls (Gray-Mitsumune *et al.* 2004). It was also confirmed that the active EXP1 affected the radial growth of fibres in *P. tremula x P. tremuloides* wood (Gray-Mitsumune *et al.* 2008).

Nitrogen fertilisation affects radial expansion of both vessels and fibres. Application of ammonium nitrate to *P. trichocarpa x P. deltoides* trees induced wider xylem elements along with axially shorter fibre lengths (Pitre *et al.* 2007a; Hacke *et al.* 2010; Plavcová *et al.* 2013). Similarly, in Paper I, the lumen areas of xylem cells in hybrid aspen increased in diameter with the increasing availability of nitrogen. The relationship between nitrogen availability and cell expansion has been linked to the function of aquaporins, which facilitate the transport of water and other substrates across membranes (Hacke *et al.* 2010; Plavcová *et al.* 2013). Increased aquaporin (AQP) activity may affect not only nitrogen uptake and transport (Gao *et al.* 2018), but also xylem cell expansion by directing water flow to the expanding zone (Plavcová *et al.* 2013).

Secondary cell wall formation

While the primary cell wall has a high water content, is porous, and consists mainly of cellulose, hemicelluloses, pectin, and proteins, the secondary cell wall is hydrophobic, rigid, and chemically composed mainly of cellulose, hemicelluloses, and lignin. The secondary cell wall of a fully matured xylem cell has a layered structure. The compound middle lamella (CML) is the outermost layer, which consists of the middle lamella and the primary cell wall. This is followed by three layers of secondary walls: S1, S2, and S3. These layers have different thicknesses and distinct angles of the cellulose microfibrils, which influence the mechanical properties of the wall (Figure 2). The S2 layer is the thickest and constitutes most of the woody biomass, thus extensively influencing the properties of wood (Fromm 2013).

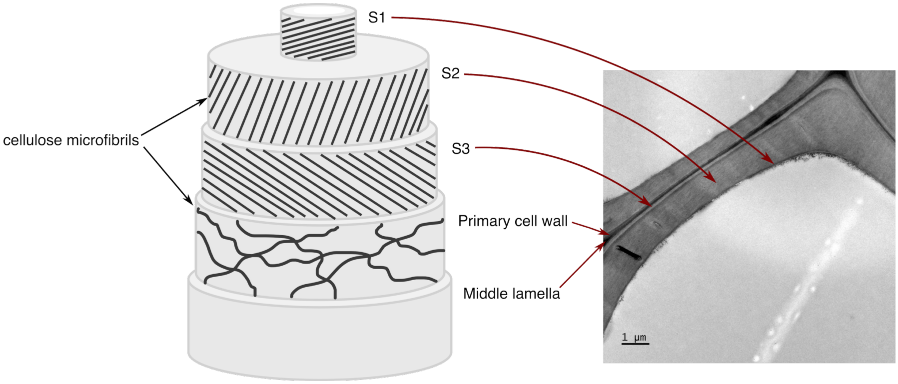

Figure 2. Structure of the secondary cell walls. A schematic drawing of a secondary cell wall together with a corresponding TEM image of a secondary cell wall in wood.

At a molecular level, the transcriptional programming of SCW formation in wood cells involves key regulatory factors such as the VASCULAR RELATED NAC DOMAIN proteins VND6 and VND7, and NAC SECONDARY WALL THICKENING PROMOTING FACTOR/SECONDARY WALL ASSOCIATED NAC DOMAIN proteins NST1, NST2, SND1/NST3, SND2 and SND3, characterised in *Arabidopsis thaliana* (from here on referred to as only *Arabidopsis*) (Zhu & Li 2021; Hussey 2022). The expression of *VND6* and *VND7* is specific to the vessels in *Arabidopsis* (Kubo *et al.* 2005), but their homologs in *P. trichocarpa* are expressed in both vessels and fibres (Ohtani *et al.* 2011). The *Arabidopsis SND1/NST3* and its homologs seem to be quite specific or have at least much higher expression in the fibres than in the vessel elements (Zhong *et al.* 2006; Mitsuda *et al.* 2007; Takata *et al.* 2019). Moreover, the SCW formation is regulated by an additional layer of transcription factors and the expression of cell wall biosynthetic genes. The *VND/NST/SND* genes regulate the expression of a set of transcription factors, such as the *Arabidopsis MYB46* and *MYB83*, as well as the expression of the genes for cellulose, hemicellulose, and lignin biosynthesis (Hussey 2022). Also in *P. trichocarpa,* the orthologs of *Arabidopsis* MYB46/83, MYB2, MYB3, MYB20, and MYB21, could activate secondary wall biosynthetic genes (Zhong *et al.* 2013).

The onset of secondary cell wall formation differs between the different cell types. Vessels are known to have an earlier onset compared to the fibres to allow rapid initiation of water transport (Fromm 2013). Important for proper water conductivity is also the deposition of lignin, which adds hydrophobicity to the cell walls (Chantreau & Tuominen 2022). Lignification is an irreversible process where lignin monomers, via radical coupling, form the lignin polymer. The monomers, also called monolignols, are *p*-coumaryl, sinapyl, and coniferyl alcohol, which, after polymerisation, form *p*-hydroxyphenyl (H), syringyl (S) and guaiacyl (G) type lignin, respectively. They differ in their degree of methoxylation and, hence, impact the type of chemical bonds formed in the lignin polymer. The occurrence of these monolignols differs in different plant species. In *P. trichocarpa* wood, the dominating monolignol is S (67.8%), then G (31.9%), and finally H (0.3%) type lignin (Wang *et al.* 2018). In general, the lignin composition in hardwoods ranges from 46-75% for sinapyl alcohol, 25-50% for coniferyl alcohol, and 0-8% for *p*-coumaryl alcohol (Ek *et al.* 2009b). The polymerisation of the monolignols is facilitated by laccases and peroxidases (Boerjan *et al.* 2003). To add to the complexity, acyl groups, such as *p*-coumarate (pCA), ferulate (FA), and *p*-hydroxybenzoate (pBA), to mention a few, can be incorporated by ester bonds into the lignin polymer as pendant groups (Ralph 2009; Zhao *et al.* 2024). In *P. trichocarpa*, *P. tremula x P. alba,* and *P. nigra*, these decorations of lignin can contribute substantially to the total lignin composition (Wang *et al.* 2018; Goacher *et al.* 2021). The chemical and structural composition of the different xylem cell types varies depending on their function. For example, the water-transporting vessels are enriched in G-type lignin while S-type lignin prevails in the fibres (Chapple *et al.* 1992; Gorzsas *et al.* 2011; Chantreau & Tuominen 2022). Lignification has also been shown to respond to environmental factors such as light and temperature, abiotic and biotic stress, and nutrient availability (Moura *et al.* 2010; Cesarino 2019; Chantreau & Tuominen 2022; Han *et al.* 2022; Peng *et al.* 2024). It has been shown in controlled nutritional studies that the lignin composition of poplar wood can be altered in response to nitrogen availability (Cooke *et al.* 2003; Pitre *et al.* 2007b; Goacher *et al.* 2021; Renström *et al.* 2024). The purpose of this chemical alteration is, however, not known.

Programmed cell death

The last phase in xylem differentiation is programmed cell death (PCD). The vessel elements differentiate and die very quickly to allow instantaneous water transport in the hollow conduits that are left behind after death and autolysis. The lifetime of fibres is longer, but they also die even though they do not transport water. Finally, the ray parenchyma cells also die, many times only after several years. In vessels, the vacuole bursts, and rapid autolytic hydrolysis of cell contents occurs. In contrast, death is a slower process in fibres, where gradual hydrolysis of the cytoplasm is followed by vacuolar rupture (Moreau *et al.* 2005; Courtois-Moreau *et al.* 2009; Bollhöner *et al.* 2012). Molecular players of PCD are poorly understood, but several hydrolytic enzymes, such as XYLEM CYSTEINE PEPTIDASEs (XCPs), nucleases, VACUOLAR PROCESSING ENZYMES (VPEs), and METACASPASE9 (MC9) homologs have been implicated (Bollhöner *et al.* 2012; Olvera-Carrillo *et al.* 2015). In *Arabidopsis*, *XCP1*, *XCP2* and *MC9* are specifically expressed in the vessel elements and therefore commonly used as markers of vessels in the single-cell RNAseq studies Tung *et al.* (2023). But in *Populus,* the homologous genes are also expressed in the fibres (Bollhöner *et al.* 2012; Sundell *et al.* 2015). The explanation for the differences in gene expression between the herbaceous *Arabidopsis* plants and the *Populus* trees is that the fibres in *Arabidopsis* do not die and therefore do not produce the hydrolytic enzymes in the fibres (Bollhöner *et al.* 2012). It has also been shown that cell wall lignification continues after cell death at least in the vessel elements (Pesquet *et al.* 2013; Chantreau & Tuominen 2022).

These details demonstrate the complexity of wood formation and the need for precise spatiotemporal regulation of the different phases of xylem differentiation.

1.1.2 Methods for studying wood: from molecular to physical characterisation

Single-cell and spatial transcriptomics in Populus

Recent advances in understanding the process of wood formation include the use of single-cell transcriptomics (Chen *et al.* 2021b; Du *et al.* 2023b; Li *et al.* 2023; Tung *et al.* 2023) and single-nucleus transcriptomics (Gomez-Soto *et al.* 2025; Schmidt *et al.* 2025). Single-cell RNA sequencing (scRNAseq) relies on the isolation of cells by protoplasting, while the single-nucleus RNA sequencing (snRNAseq) is based on nuclei isolated from intact tissues. Hence, snRNAseq is suitable for difficult tissues such as wood and does not involve bias due to protoplasting. However, the drawback is the loss of cytoplasmic RNA and thus comprehension of the full transcriptome (Ding *et al.* 2020). The scRNAseq and snRNAseq methods facilitate the characterisation of gene expression in different cell types and stages of differentiation. The results from scRNAseq reveal clusters of cells that are formed in an unsupervised manner based on similarities of the transcriptomes because of either similar identity or the developmental stage of the cells.

Identification and annotation of clusters are commonly based on known marker genes, combined with cluster-enriched genes (Seyfferth *et al.* 2021a). In the study by Chen *et al.* (2021b), the *VND1* gene was mentioned as a marker of vessels. However, this gene was expressed only in a part of the cells within a cluster of cells that was annotated as SCW-forming cells. This is a representative example of when a transcriptional program, in this case SCW formation, overrides the cell-specific transcriptome in terms of cell clustering. Some genetic markers used for different cell types and cell stages are presented in Table 1.

Table 1. A compilation of genetic markers for cell type or cell state identity from different references and different cellular transcriptomic methods.

Genetic markers	**Identity**	**Reference**	**Method**
SND1, NST1, MYB46, MYB83	xylem cell population	(Chen *et al.* 2021b)	scRNAseq
IRX9, IRX14-L, IRX15-L, COBL4, CesA4, CesA7, CesA8, LAC4, LAC17, KNAT7	xylem SCW thickening		
VND1	vessel cell		
CAD7	xylem parenchyma cells		
WOX4, PXY, MP, PIN1, ANT, CLE47	cambial cells		
HB4, HB7	xylem mother cells		

CSLD5, PLE, HIK, CYCA1;1, CYCA2;1, CYCA2;4, CYCB1;4, CYCB2;1, CYCB2;3, CYCB2;4, CYCB3;1, CDKB1;2, CDKB2;1	dividing cells		
CesA4, CesA7, CslA1, CslA2, PARVUS-1, PARVUS-2, 4CL5, C4H2, CAD1, CAld5H1, HCT1, PAL1, PAL2 PAL3, IRX15-L, DUF579-9, AP66	libriform fibre cell cluster	(Tung *et al.* 2023)	scRNAseq and laser capture microdissection (LCM)
Plastocyanin 1, PS II lightharvesting complex gene B1B2, *PS II oxygen-evolving complex 1,* *Lactate/malate dehydrogenase family protein,* *Photosystem II reaction center protein C,* *Photosynthetic electron transfer C,* *PS I light harvesting complex gene 3,* *RuBisCO family protein*	ray parenchyma cells		
EXPA6	vessel elements		
ARR4, LAC6	xylem precursor cells	(Li *et al.* 2023)	spatial transcriptomics
PIP2;3	secondary growth tissues		
LAC17a, LAC17d, PAL1a, GLP7, PIP2	vessels		
LAC17c, IRX14a, ROPGAP3, PGSIP1, PDLP6	fibres		
MYB48, PRN2	vessel-associated cells	(Schmidt *et al.* 2025)	snRNAseq
ANT, HB7	cambium cells		
WOX4, PXY	cambium cells, differentiating xylem		

HB4, HB8	xylem mother cells		
SND1-A1/NST3, NST1, MYB46, MYB83	secondary cell wall formation in fibres		
VND1	secondary cell wall formation in vessels		

Another advancement in studying the molecular regulation of wood formation includes spatial transcriptomics (Du *et al.* 2023b; Li *et al.* 2023). This is a method that captures gene expression in microscopic sections via positional barcoding of mRNA (Marx 2021). In a study by Li *et al.* (2023), scRNAseq was combined with spatial transcriptomics in order to dissect differences between primary and secondary growth of stem, and developmental trajectories of vessel and fibre cells. New potential regulators of vessels and fibres were also described. For example, several members of the NPF gene family (*NPF6.3/NRT1.1, NPF4.4a,* and *NPF5.2a*) were expressed in different stages of vessel development (Li *et al.* 2023).

Morphological characterisation of wood and cell walls

The most common method for studying wood anatomy and xylem cell morphology is light microscopy (LM). Thin (~30 µm) sections of wood can, via LM, be magnified and imaged to be further analysed by image processing programs such as ImageJ and Fiji, amongst others. Image processing programs usually aid in quantifying anatomical features such as cell sizes, cell frequencies, and cell wall area, to mention a few. The wood sections can also be stained for histochemical determination of the chemical components. Autofluorescence is also often used to visualise cell walls and can be captured via fluorescence microscopy, a type of LM (Karannagoda *et al.* 2020).

While LM offers a rapid way to determine wood anatomy, higher resolution is required to analyse the ultrastructure of cell wall. In this case, transmission electron microscopy (TEM) or scanning electron microscopy (SEM) based techniques are used. TEM has the highest resolution and can, for example, detect the thickness of the different layers (S1, S2, S3) of cell walls, and reveal cellulose microfibril orientation and lignification patterns (Donaldson & Baas 2019). The method can also be used to assess different cell wall constituents and chemical components by immunogold-labelled cell wall polymers or other metal-staining techniques (Karannagoda *et al.* 2020).

Chemical characterisation of wood and cell walls

Apart from the histological methods for chemical analysis of wood, there are numerous ways to assess wood chemical composition. Some of the most frequently used include crude wet chemistry methods and microanalytical methods such as Pyrolysis-Gas Chromatography/Mass Spectroscopy (Py-GC/MS), Matrix-Assisted Laser Desorption Ionisation-Mass Spectrometry (MALDI-MS), Fourier-Transform Infrared (FT-IR) spectroscopy, Raman spectroscopy, and nuclear magnetic resonance (NMR).

Wet chemistry, in this case, is the collective term for analytical methods that rely on the identification and quantification of chemical compounds in lignocellulosic materials, often based on the sequential extraction of the different polymers using solvents and hydrolytic reactions (Sluiter *et al.* 2010; Balakshin *et al.* 2014). These methods are often laborious and require relatively large sample amounts for good accuracy. Common wet chemistry methods involve measurement of acid-soluble and acid-insoluble (Klason) lignin, acetyl bromide lignin, monosaccharide composition, carbohydrate content, composition of extractives, and protein content (Sluiter *et al.* 2010).

The microanalytical methods are often semi-quantitative and require small sample amounts and minimal sample preparation. However, the spectroscopic techniques require multivariate methods for the identification and determination of the chemical compounds using the complex and vast amount of spectral data that is typically produced in the process (Karannagoda *et al.* 2020). The use of Raman spectroscopy, FT-IR spectroscopy, and MALDI-MS can, similar to the histological methods, also provide spatial information within a sample (Karannagoda *et al.* 2020). Methods such as Py-GC/MS and MALDI-MS can provide sensitive chemical fingerprints and are excellent for comparing treatment effects or genotypic effects on lignocellulosic materials (Persson *et al.* 2010; Gerber *et al.* 2016). Furthermore, solution-state 2D NMR methods can be used to elucidate the inter-unit linkages within the cell wall polymers (Mansfield *et al.* 2012; Terrett *et al.* 2019).

There are several advantages and limitations to the analytical methods mentioned here, and it is often advised to include complementary techniques. To conclude, the most suitable method depends on the sample type and the research question.

Physical characterisation of wood and cell walls

The physical properties of wood can also be assessed in many ways. One instrument, developed for this purpose, is the SilviScan^{TM}. This instrument can analyse several physical variables such as microfibril angle (MFA), modulus of elasticity (MOE), wood density, stiffness, and coarseness. Wood density and MFA are measured by X-ray methods, while properties like stiffness and coarseness are estimated from these measurements and an optical cell scanner (Schimleck *et al.* 2019). At a cell wall level, atomic force microscopy (AFM) has been shown to aid in the determination of physical characteristics such as MFA, adhesion, and Young's modulus (Lou *et al.* 2023).

Recent advances in NIR technology allow the analysis of the physical properties with a high throughput and resolution using NIR spectroscopy and hyperspectral imaging (Schimleck *et al.* 2019). The NIR spectra need to be calibrated with other methods, and SilviScan^{TM} has been one of the methods when it comes to the physical properties of wood. Thus, NIR models are based on correlations between a NIR spectrum and data from a reference method, which enables high-throughput predictions of sample characteristics from the NIR spectra alone (Schimleck *et al.* 2019). However, the use of NIR is not limited to physical characterisation, as NIR spectroscopy can also provide information on the spatial distribution of wood chemical components (Poke & Raymond 2006; Thumm *et al.* 2016; Fahey *et al.* 2019). In Paper II, we demonstrate how this method can be applied to both small greenhouse-cultivated trees and larger field-grown trees with high throughput. Still, there are some limitations to the technology. It requires sensitive calibration methods, and the NIR models must be trained on large amounts of data to achieve high prediction accuracy (Schimleck *et al.* 2019).

1.2 Nitrogen, the environment, and plant nutrition

Nitrogen is an essential part of the two most important molecules for living organisms: nucleic acids and proteins. It is also the fifth most abundant element on Earth. However, most of the nitrogen is present in the form of the inert dinitrogen (N_2) gas that needs to be fixed by prokaryotic organisms and converted into accessible forms to be taken up by plants. Anthropogenic interference has doubled the rate of terrestrial nitrogen fixation, creating an imbalance in the biogeochemical nitrogen circuit (Canfield *et al.* 2010). Anthropogenic interference involves industrial fixation of atmospheric nitrogen by the Haber-Bosch process, the burning of fossil fuels, and cultivation-induced nitrogen fixation. The use of nitrogen fertilisers increased between 1960 and 2000 by approximately 800% to meet the demands of food and feed for a growing population (Canfield *et al.* 2010). However, an excess in nutrient supply and a low nutrient use efficiency of crops cause nitrogen loss to the environment, disturbing ecosystems and fuelling climate change (Erisman *et al.* 2008).

In the paper by Erisman *et al.* (2008) it is mentioned that the need for bioenergy and biofuel may further exacerbate the use of nitrogen fertilisers. This highlights the importance of improving nitrogen use efficiency, either by changing fertilisation practices, targeted breeding, or plant productivity in all settings where nitrogen availability is considered limited (Vitousek & Howarth 1991). This applies especially well to many Swedish forests (Högberg *et al.* 2021), where the situation is exacerbated by an increasing pressure to increase forest fertilisation as a short-term means to increase carbon sequestration and forest growth (Hyvönen *et al.* 2008; Jämtgård *et al.* 2023). For these reasons, it is of utmost importance to increase the understanding of the fundamentals of plant nutrient biology.

1.2.1 Nitrogen as a tree macronutrient

Nitrogen is a plant-essential macronutrient, an element necessary for growth (see Figure 3). It is a component of building blocks such as nucleic and amino acids, and a prerequisite for photosynthesis as a constituent of chlorophyll and RuBisCO. However, the availability is often limited, and plants cannot assimilate nitrogen from the atmosphere but depend on nitrogen in organic or inorganic forms confined in the root-soil area and/or nitrogen-fixing symbionts. Plants have therefore developed strategies to ensure sufficient uptake and allocation according to the surrounding conditions. These strategies are often mechanisms that rely on complex networks of nutrient signalling, nitrogen-specific transporters, and gene activation.

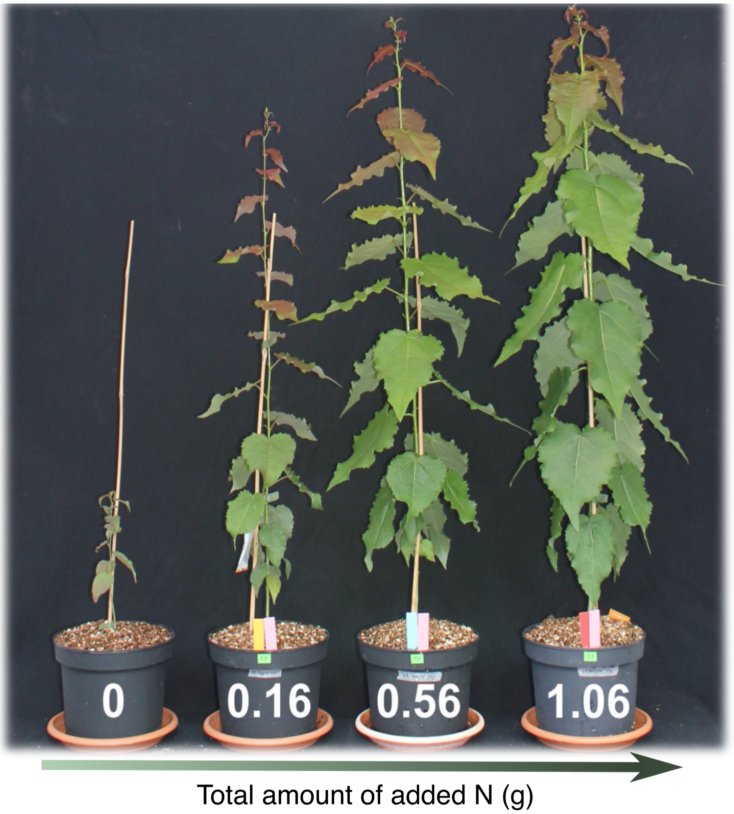

Figure 3. The growth of hybrid aspen trees in response to added nitrogen after 10 weeks of cultivation. The figure is adapted from Paper I.

Nitrogen is taken up by trees as nitrate (NO_3^-), ammonium (NH_4^+), amino acids (AA) and small peptides (Näsholm *et al.* 2009; Rennenberg *et al.* 2010). Conditional circumstances such as tree species and growth environment dictate the preferential form of inorganic nitrogen (NO_3^- and NH_4^+) (Rennenberg *et al.* 2010). Uptake, assimilation, and distribution are facilitated by low- and high-affinity transporters to ensure acquisition under fluctuating nitrogen availabilities (Castro-Rodríguez *et al.* 2017). Membrane-bound ammonium transporters (AMTs) aid in acquiring NH_4^+ ions from soils and NH_4^+ distribution within the plant. The members of the NPF gene family can have dual roles in both nitrogen transport and sensing (Castro-Rodríguez *et al.* 2017).

In addition to the uptake of inorganic forms, amino acids, and small peptides can also be taken up by plants (Näsholm *et al.* 2009). Important amino acid uptake proteins include the *Arabidopsis* LYSINE HISTIDINE TRANSPORTER 1 (LHT1), AMINO ACID PERMEASE 1 (AAP1), and AAP5 (Näsholm *et al.* 2009). The hybrid aspen LHT1.2 has been confirmed as a functional homolog of the *Arabidopsis* LHT1 (Gratz *et al.* 2021). However, amino acid transport within plants is essential for many biological processes, and the transporters constitute a large gene family to facilitate distribution throughout the plant and within cells, depending on different affinities towards neutral, acidic, or basic types of amino acids, and in response to internal and external conditions (Wu *et al.* 2015; Yang *et al.* 2020; Yao *et al.* 2020). Regulation of amino acid transport can also influence the translocation of amino acids from source to sink tissues, which in turn can impact the carbon/nitrogen balance and nitrogen use efficiency (Zhang *et al.* 2010; Perchlik & Tegeder 2018). Functional studies and genetic manipulations of amino acid transporters are fewer in *Populus* wood. However, AAP11, described in *P. tremula x P. alba,* was suggested to have a role in xylogenesis (Couturier *et al.* 2010). *AAP11* was expressed specifically in differentiating xylem and showed in yeast assays a high affinity for proline. Thus, it was proposed that AAP11 could provide proline necessary for cell wall protein synthesis (Couturier *et al.* 2010). Moreover, storage and mobilisation of nitrogen within the tree is necessary to sustain growth and cope with seasonality. The major storage forms of nitrogen are proteins, such as VEGETATIVE STORAGE PROTEINS (VSP) and

BARK STORAGE PROTEINS (BSP), and amino acids, such as arginine (Arg), glutamine (Gln), and asparagine (Asn) (Rennenberg *et al.* 2010). The complex interactions in the rhizosphere and adaptations to the surrounding environment have probably caused the variety of nitrogen uptake mechanisms, nitrogen assimilation strategies, and responses to nitrogen availability seen among tree species. The different types of nitrogen available to plants may influence biomass partitioning. As an example, organic nitrogen has been shown to stimulate root growth, thereby promoting the partitioning of biomass to belowground structures (Cambui *et al.* 2011; Jiao *et al.* 2018). In Paper I, no such trend was seen when comparing the growth of hybrid aspen trees in response to organic versus inorganic nitrogen sources. However, there was a stimulatory effect of nitrate, particularly on shoot growth. Nitrate as a signal for growth allocation has been explored previously, showing that there is an intricate connection between nitrate accumulation and changes in allocation, favouring shoot growth under certain circumstances (Scheible *et al.* 1997).

Furthermore, it seems that the location of nitrate assimilation within the tree can vary between poplar species (Wang *et al.* 2024). It has been suggested that fast-growing, pioneer species would favour assimilation in the shoots rather than in the roots (Stewart *et al.* 1992). In hybrid aspen (*P. tremula x P. alba*) trees, nitrate was shown to be partially assimilated in roots and stems, but the majority of the assimilation occurred in young leaves (Black *et al.* 2002). In *P. trichocarpa,* most of the nitrate assimilation occurred in roots (Hu & Guy 2020). In any case, nitrate uptake is light-dependent, having a diurnal rhythm (Delhon *et al.* 1995), and nitrate assimilation must be balanced with the requirements for carbon skeletons and energy (van Gelderen 2021). Leaf assimilation is therefore convenient, although light-independent assimilation can also occur in non-photosynthetic tissues (Yoneyama & Suzuki 2019; Yoneyama & Suzuki 2020).

The assimilation of nitrate requires a two-step reduction process, facilitated by NITRATE REDUCTASE (NR) and NITRITE REDUCTASE (NIR) for the conversion of NO_3^- to NO_2^- and NO_2^- to NH_4^+, respectively (Figure 4). NH_4^+ can be subsequently incorporated into amino acids via the GLUTAMINE SYNTHETASE/GLUTAMINE-2-OXOGLUTARATE AMINO-TRANSFERASE (GS/GOGAT) pathway (Masclaux-Daubresse *et al.*

2006; Xu *et al.* 2012; Krapp 2015; Li *et al.* 2024). GS catalyses the reaction where glutamate and ammonia form glutamine, and GOGAT catalyses the reaction where glutamine and 2-oxoglutarate form two glutamate molecules (Masclaux-Daubresse *et al.* 2010). This is where carbon and nitrogen metabolism meet, the assimilation of nitrogen requiring reducing power and energy from ferredoxin, nicotinamide adenine dinucleotide (NADH), and adenosine triphosphate (ATP), as well as carbon skeletons (2-oxoglutarate) produced in the tricarboxylic acid (TCA) cycle (Masclaux-Daubresse *et al.* 2010; Nunes-Nesi *et al.* 2010). Other important enzymes involved in nitrogen assimilation are asparagine synthetase (AS), which catalyses the formation of glutamate and asparagine, and GLUTAMATE DEHYDROGENASE (GDH), which primarily catalyses the deamination of glutamate. These enzymes have also been shown to have the ability to assimilate NH_4^+ under certain circumstances and can, therefore, compensate for or complement the GS activity (Masclaux-Daubresse *et al.* 2010). The assimilation takes place in the cytosol (NR, AS, and GS1), in plastids/chloroplasts (NIR, GS2 and GOGAT), and partially in the mitochondria (GDH) (Masclaux-Daubresse *et al.* 2010; Castro-Rodríguez *et al.* 2011).

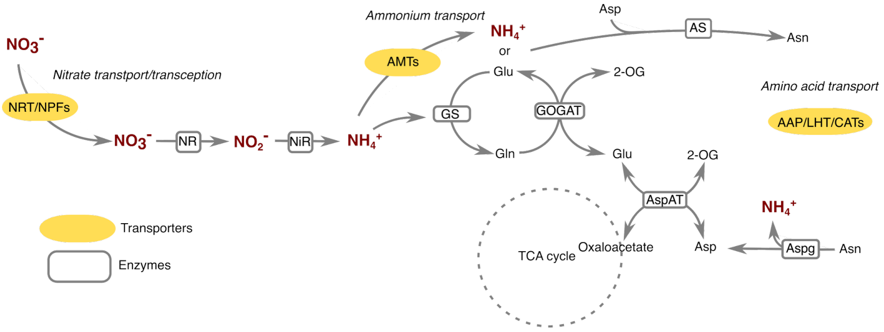

Figure 4. Nitrogen assimilation pathways, showing important transporters and enzymes involved in the process. The figure is adapted from Paper IV, where we show how several of the nitrogen assimilation genes along this pathway were significantly differentially expressed in response to nitrate versus control treatment.

1.2.2 Nitrogen functions as a plant signal

Despite the importance of nitrogen for plant growth, the underlying molecular mechanisms behind nitrogen sensing and signalling are not well understood. Most research is done in *Arabidopsis,* where ample evidence shows the role of nitrate as a signalling molecule (Medici & Krouk 2014; Vidal *et al.* 2020; Zhang *et al.* 2020; Courrèges-Clercq & Krouk 2022; Ruffel *et al.* 2025). Nitrate can, apart from regulating nitrogen uptake and assimilation, induce changes in carbon metabolism and root-to-shoot allocation, impact the proliferation of lateral roots, and affect flowering time, seed dormancy, and even senescence (Nunes-Nesi *et al.* 2010; Zhang *et al.* 2020; Fataftah *et al.* 2022). Other forms of nitrogen, mainly ammonium and certain amino acids, have also been suggested as plant signals even though less is known about their signalling pathways (Forde & Lea 2007; Gent & Forde 2017; Liu & von Wiren 2017).

NPF6.3/NRT1.1 is a central protein in nitrate signalling (Zhang *et al.* 2020). It functions as a 'transceptor' having a function in both nitrate transport and sensing. The plasma membrane-bound NPF6.3/NRT1.1 can sense a wide range of nitrate concentrations (Ho *et al.* 2009). Phosphorylation of the T101 residue via CBL-INTERACTING PROTEIN KINASE 23 (CIPK23) triggers conformational changes of the protein, leading to the different primary nitrate responses(Ho *et al.* 2009; Bouguyon *et al.* 2015; Zhang *et al.* 2020). The simple definition of primary nitrate responses (PNR) is that they are early events triggered by nitrate itself, rather than by its assimilation products (Medici & Krouk 2014). Downstream of NPF6.3/NRT1.1, subgroup III Ca^{2+} SENSOR PROTEIN KINASES (CPK) CPK10, CPK30, and CPK32 phosphorylate NLP7 and promote its nuclear retention in the presence of nitrate (Liu *et al.* 2017). NLP7 is often referred to as a master regulator in nitrate signalling, activating a transcriptional response (Alvarez *et al.* 2020), and has recently been suggested to be an intracellular nitrate sensor itself (Liu *et al.* 2022). In addition to phosphorylation of NLP7, nitrate can act by binding to the N-terminus of NLP7 and trigger a transcriptional activation (Liu *et al.* 2022). Moreover, all the investigated NLPs (NLP1-9) showed the capacity to activate marker genes in the primary nitrate response and could, in different ways, impact the nitrate-mediated transcriptional reprogramming (Liu *et al.* 2022). The fact that NLPs bind to

NITRATE-RESPONSIVE CIS-ELEMENTS (NREs) and thus translate nitrate signals to transcriptional regulation has been described previously (Konishi & Yanagisawa 2013). However, only *nlp2* and *nlp7* single mutants have displayed plant growth defects, suggesting that NLP2 and NLP7 are key factors in nitrate signalling (Konishi *et al.* 2021; Liu *et al.* 2022). It is believed that NLP2 is activated, like NLP7, by nitrate-dependent phosphorylation. However, unlike *nlp7*, the *nlp2* growth phenotype could not be rescued by supplementation of ammonium or glutamine as nitrogen sources, indicating a unique role of NLP2 in the stimulation of shoot growth despite having overlapping targets with NLP7 (Konishi *et al.* 2021).The NLPs are less explored in *Populus.* In Paper III, we perform an *in silico* expression analysis of the NLP gene family members in wood. While the *Arabidopsis NLP7* homolog was found to be expressed in the cambium and the expanding xylem, an *NLP8* homolog showed the highest expression in the wood. Interestingly, the *NLP8* homologs were recently identified as candidate genes for improved NUE in a quantitative trait loci (QTL) analysis (Du *et al.* 2023a).

Nitrate induces rapid changes in the shoot transcriptomes and metabolomes of *Arabidopsis* (Wang *et al.* 2003; Scheible *et al.* 2004; Krouk *et al.* 2010). The corresponding changes were coupled mainly to carbon, iron, sulphate, phenylpropanoid and flavonoid metabolism, cellular growth and protein synthesis. Important genes and transcription factors identified as part of the primary nitrate response (PNR) include transient-, temporal-, and secondary-induced genes (Varala *et al.* 2018; Brooks *et al.* 2019; Alvarez *et al.* 2020). Known regulators in nitrate signalling are *NLP7/8, TGACG MOTIF-BINDING FACTOR 1/4 (TGA1/4), NAC DOMAIN CONTAINING PROTEIN 4 (NAC4), LATERAL ORGAN BOUNDARY DOMAIN 37/38/39 (LBD37/38/39),* amongst others. These genes were validated by the machine learning method, and new candidates were explored in Varala *et al.* (2018). One of these candidates was CYTOKININ RESPONSE FACTOR 4 (CRF4), which could regulate nitrogen uptake *in planta*, probably via its downstream targets *SCHNARCHZAPFEN (SNZ)* and *CYCLING DOF FACTOR 1 (CDF1)* (Varala *et al.* 2018). Another study, using a method called TARGET (Transient Assay Reporting Genome-wide Effects of Transcription factors), showed that CRF4 targets a set of transcription factors (TFs), such as *GATA TRANSCRIPTION FACTOR 17 (GATA17), NAC-LIKE ACTIVATED BY AP3/PI (NAP), HY5-HOMOLOG (HYH), and MYB DOMAIN PROTEIN 34 (MYB34),* and suggested that these TFs could indirectly regulate 87% of the genes that respond to *CRF4* overexpression *in planta* (Brooks *et al.* 2019). The TARGET method was also used for validating *CDF1*, *TGA4* and *LDB37/38* as direct targets of NLP7, highlighting their importance in conveying the early nitrate responses (Alvarez *et al.* 2020).

For plants to coordinate the exogenous and endogenous nitrogen status, some kind of systemic nitrogen signalling and sensing is needed. Due to its complexity, this subject is far less explored. However, two types of long-distance signalling have been investigated, one involving CKs and the other peptides (Zhang *et al.* 2020). CKs have been proposed to play a role in long-distance signalling since they are transported from the roots to the shoots and their biosynthesis is promoted by nitrate (Rahayu *et al.* 2005; Sakakibara *et al.* 2006; Osugi *et al.* 2017; Gu *et al.* 2018). Also, plants defective in cytokinin biosynthesis or root-to-shoot transport have difficulty coping with the spatial heterogeneity of nitrate availability in the soil (Poitout *et al.* 2018). The work of Poitout *et al.* (2018) suggested that the accumulation of *trans*-Zeatin (tZ) in shoots, in response to long-distance nitrate signalling, likely mediates the transcriptional changes in the roots. The second group of putative long-term nitrogen signalling compounds, the C-TERMINALLY ENCODED PEPTIDE (CEPs), can be produced in nitrogen-limited roots and be transported to the shoots, where they, after being perceived by the CEP receptors, induce translocation of phloem-specific glutaredoxins, the CEP DOWNSTREAMs (CEPDs), back to roots to regulate nitrogen uptake (Tabata *et al.* 2014; Ohkubo *et al.* 2017). Furthermore, a short-distance nitrogen signalling mechanism was found to be facilitated by CLAVATA 1 (CLV1) peptide, which repressed lateral root growth in low nitrogen conditions (Araya *et al.* 2014).

How poplar trees sense their nitrogen status is partially explained by shoot-to-root signals in the form of reduced nitrogen compounds such as amino acids (Rennenberg *et al.* 2010). When glutamine was exogenously supplied to *P. tremula x P. alba* trees, nitrate uptake decreased and assimilation and accumulation in leaves were reduced (Dluzniewska *et al.* 2006). As in *Arabidopsis*, cytokinins also seem to be involved. In the paper by Dluzniewska *et al.* (2006), they showed that trees had reduced nitrate uptake when applied with the active CK form of tZ riboside.

1.2.3 NUE in poplar

There are different ways to express plant nitrogen use efficiency. Simply put, it can be described as plant productivity based on the amount of added or available nitrogen taken up by the plant. In practice, however, it is more complex, and the definition varies depending on the context. The concept of NUE has been mostly applied in agriculture. In a perspective paper, Congreves *et al.* (2021) compiled common NUE indices. They categorise the different NUE indices as fertiliser-, plant-, soil-, isotope-, ecology-, and system-based. The plant- and ecology-based indices centred around plant tissue allocation, plant growth rates, and productivity in response to internal and external nitrogen, and are, therefore, perhaps most useful from a plant breeding perspective.

With the current need for sustainable bioresource production, the concept of NUE is also becoming increasingly relevant in forestry. Most studies have aimed at understanding the molecular mechanisms related to nitrogen utilisation and uptake to find candidate genes and traits for selection (Luo & Zhou 2019; Hu *et al.* 2020; Chen *et al.* 2021a; Shen *et al.* 2022). Hu *et al.* (2020) found that leaf area and leaf nitrogen allocation were central traits for nitrogen utilisation efficiency. Chen *et al.* (2021a) showed in *P. deltoides* genotypes that leaf area, fine root growth, chlorophyll content and NR activity in leaves were traits that correlated with increased NUE in low nitrogen conditions. Shen *et al.* (2022) demonstrated that *P. tremula x P. alba* trees overexpressing the *GATA NITRATE-INDUCIBLE CARBON-METABOLISM-INVOLVED (GNC)* transcription factor had improved NUE in low nitrate conditions due to improved nitrate uptake, remobilisation and assimilation. Moreover, genes encoding major nitrogen assimilation enzymes, sometimes called as *NITROGEN ASSIMILATION GENEs* (*NAGs*), have recently been suggested as suitable targets to improve NUE in poplar (Li *et al.* 2024).

Genetic association studies focusing on NUE have been performed in a few poplar species (Novaes *et al.* 2009; Liu *et al.* 2019; Du *et al.* 2023a; Zhou *et al.* 2023). Liu *et al.* (2019) suggested *AMT1;2* as a marker gene for high NUE in *P. nigra*. In this study, they specifically focused on allelic variations in ammonium (AMT) and nitrate (NRT) transporters and their association with seedling height and ground diameter. In *P. cathayana,* two NAC DOMAIN CONTAINING PROTEINS (NAC123 and NAC025) were proposed on the basis of genome-wide association studies (GWAS) and genomic selection (GS) assistance to be involved in the regulation of wood formation and growth via modulation of nitrogen metabolism levels (Zhou *et al.* 2023). Through quantitative trait loci (QTL) analyses of several traits for biomass yield, candidate genes related to nitrogen transport (*NRT3.1*, *NPF5.1*, *NPF5.10*, and *NPF8.1*), transcription factors (*NLP8.1*, *NLP8.2*, and *NLP2),* amino acid metabolism (*GDH2* and *SERINE HYDROXYMETHYLTRANSFERASE 2 (SHM2)*), and carbon metabolism were identified (Du *et al.* 2023a).

To conclude, this is still a relatively unexplored research area compared to what has been done in the agricultural field. The complexity lies within the different definitions of NUE, the choice of experimental setup, and the correlation of growth to nitrogen uptake and assimilation.

1.3 The effect of nitrogen on cambial growth

1.3.1 Physiological responses

Nitrogen fertilisation stimulates the activity of the cambium, hence increasing stem volumes. Hybrid aspen, *P. tremula x P. tremuloides*, has been shown to exhibit strong nitrogen responsiveness. When treated with 2 millimolar (mM) versus 0.2 mM potassium nitrate (KNO_3), young trees showed a threefold increase in stem volumes and almost doubled the stem biomass (Euring *et al.* 2012). In addition to the control of meristem activity, nitrogen availability also influences the wood structure and chemical composition. Euring *et al.* (2012) observed a drastic decrease in wood density and a reduction of G-type lignin in wood in response to increased nitrogen availability. However, the influence of nitrogen on wood formation varies among different poplar species and seems to be, therefore, influenced by genetic adaptation to their environment (Luo *et al.* 2005; Euring *et al.* 2012; Li *et al.* 2012).

Several studies have investigated the effect of nitrogen on wood properties. In *P. xiaohei,* increased availability of ammonium nitrate (NH_4NO_3) led to a wider xylem area with an increased number of cell layers, thinner cell walls of fibres, a decrease in lignin content, increased S/G lignin ratio, and an increase in cellulose content (Cao *et al.* 2024). The short-term influence of increased NH_4NO_3 availability in *P. trichocarpa x P. deltoides* correlated with shorter and wider xylem fibres (Pitre *et al.* 2007a), decreased lignin content with a slight increase in frequencies of condensed bonds (i.e., less β-O-4 ether bonds), and a lower S/G lignin ratio (Pitre *et al.* 2007b). Noteworthy, in the study of Pitre *et al.* (2007b), higher levels of applied NH_4NO_3 resulted in lignin with a higher frequency of *p*-hydroxyphenyl units (H-type lignin) and more ester-linked *p*-hydroxybenzoic acid (pHB) groups. These results are similar to Paper I where nitrate was shown to increase the level of *p*-hydroxybenzoylated lignin in *P. tremula x P. tremuloides* trees.

Findings supporting the typical pattern of lower lignin content and S/G ratio in response to higher NH_4NO_3 treatments were also shown by Novaes *et al.* (2009) in an interspecific pseudo-backcross pedigree (*P. trichocarpa x P. deltoides*) composed of 396 genotypes. In this paper, they also explored the nitrogen-dependent genetic control and found a genetic correlation within the pedigree between plant growth and wood chemistry. Furthermore, they showed that increased nitrogen availability favoured shoot over root growth and supported the hypothesis that growth covaries with wood chemistry. The link between nitrogen availability, tree resource allocation, and its effect on wood quality was also explored previously by Cooke *et al.* (2003). By girdling *P. trichocarpa x P. deltoides* clones under high and low NH_4NO_3 treatments, they obtained gene expression data that supported the idea that the partitioning of carbon and nitrogen resources into different metabolomic pathways, such as vegetative storage proteins, cell wall components and terpenoids, can alter wood quality and quantity. Furthermore, Li *et al.* (2012) demonstrated that growth, C- and N-physiology, and wood properties in fast-growing poplar saplings responded more strongly to nitrogen fertilisation than slow-growing ones due to shifts in resource allocation. Alongside the effect on C/N partitioning, the alterations in wood chemistry and lignin composition due to nitrogen availability could also relate to the spatiotemporal control of wood formation in combination with increased or decreased cambial activity (Pitre *et al.* 2007b; Paper I). Apart from the basic understanding of the nutrient effect on wood chemistry and morphology, it is important regarding the utilisation and end use of wood as a bioresource.

There seems to be a consensus that high nitrogen availability leads to wood with lower density, and structural changes such as thinner cell walls and wider xylem cells (Luo *et al.* 2005; Pitre *et al.* 2007a; Novaes *et al.* 2009; Hacke *et al.* 2010; Plavcová *et al.* 2013; Cao *et al.* 2024; Renström *et al.* 2024). However, the physiological explanation for this phenotypic response appears less clear. Changes in xylem structure, such as the diameter and frequency of vessels, have been shown to impact the hydraulic conductivity (K_s) of the tissue (Hacke *et al.* 2010; Plavcová *et al.* 2013; Hacke *et al.* 2017). Increased K_s and water transport as a response to high nitrogen treatments serve perhaps to support a fast-growing tree with an adequate water supply. However, a higher K_s increases the vulnerability to xylem cavitation (Hacke *et al.* 2010; Hacke *et al.* 2017). The relations between nitrogen and water uptake are an interesting area for research and are important for developing sustainable fertilisation strategies. Testing whether different nitrogen sources would have an impact on water transport could be a potential area of expansion from this work. However, the topic will not be further touched upon in this thesis.

1.3.2 Molecular responses

Despite the clear and economically significant impact of nitrogen on cambial activity and wood formation, the underlying molecular mechanisms are relatively unexplored. Early transcriptomic analyses in cambial tissues focused on transcriptomic changes that resembled those occurring during tension wood formation (Pitre *et al.* 2010) and those related to xylem water transport (Plavcová *et al.* 2013) in response to high nitrogen treatment in hybrid poplar (*P. trichocarpa x P. deltoides*). Differentially expressed genes (DEGs) overlapping with those for the tension wood formation and high nitrogen conditions were associated with osmotic changes or water transport, such as the aquaporin *NOD26-LIKE INTRINSIC PROTEIN 5.1 (NIP5.1)* (Pitre *et al.* 2010). Some of the most interesting patterns found in the transcriptomic analysis of Plavcová *et al.* (2013) were the up-regulation of many *GLYCOSIDE HYDROLASE* (*GH*) genes as a response to the high nitrogen treatment. Elevated activity of GH enzymes is common for expanding tissues and was suggested to, via primary cell wall loosening, explain the wider lumens of xylem elements found in the cambial tissue of the tested plants. Also, downregulation of the NAC TF genes *NAC157, NAC105,* and *NAC150* was found. These genes are related to secondary cell wall thickening and were therefore speculated to be involved in the decreased cell wall thickness seen in the high nitrogen-treated trees. Another transcription factor involved in the early nitrogen responses, *LDB38*, was shown to be upregulated in response to high nitrogen availability. Moreover, two important genes involved in PCD, *XYLEM SERINE PEPTIDASE (XSP1)* and *XCP2*, were less expressed in high nitrogen-treated trees. As in the work of Pitre *et al.* 2010, there were several aquaporins differentially expressed in response to nitrogen treatments, suggesting their role as water channels to facilitate turgor-driven cell expansion. Nitrogen-responsive aquaporins in *Populus* xylem were also identified in a transcriptomic analysis focusing on primary growth in *P. trichocarpa* and the nitrogen-driven stem elongation (Euring *et al.* 2014). Apart from the aquaporins, the overlap between differentially expressed genes in the secondary compared to the primary growth was very low. The primary cell wall showed almost the opposite response to high nitrogen treatments, with higher lignin content and thicker cell walls. *ARABIDOPSIS RAC-LIKE 2.1 (ARAC2.1)* was identified as a regulatory gene connected with cell wall-related genes such as *FASCICLIN-LIKE* genes *(FLAs)*, *CELLULOSE SYNTHASES (CESAs)*, the xylan biosynthetic gene *IRREGULAR XYLEM 9 (IRX9)* and *LACCASE 4* (*LAC4)*. Euring *et al.* (2014) also found that the nitrogen-driven elongation seemed to be related to biotic (*JASMONATE RESISTANT 1 (JAR1), JASMONATE ZIM DOMAIN 5 (JAZ5), MYELOCYTOMATOSIS 2 (MYC2)*) and abiotic (*WRKY40, OSMOTIN 34*) stress, possibly due to the mechanical strain caused by rapid growth.

More recent transcriptional analyses include molecular and hormonal characterisation of *P. canescens* wood acclimated to low nitrogen availability (Lu *et al.* 2019), and a network analysis of the nitrogen-responsive metabolome and transcriptome in the *P. xiaohei* cambium (Zhang *et al.* 2024). The hormonal characteristics in *P. canescens* wood under low NH_4NO_3 treatment were high levels of GA_3 and salicylic acid (SA), and low levels of GA_1, GA_4 and JA. These characteristics were related to anatomical and chemical properties such as narrow xylem elements, thick cell walls and high lignin content (Lu *et al.* 2019). Moreover, Lu *et al.* (2019) also modelled a hierarchical genetic network, which defined *GIBBERELLIN INSENSITIVE DWARF 1B* (*GID1B)* as a first-layer regulator, and *ENDOGLUCANASE 10 (EG10),* EIN3-BINDING F BOX PROTEIN 1 (*EBF1)*, *ASPARAGINE SYNTHASE 1* (*ASN1*), and *BASIC LEUCINE ZIPPER PROTEIN 1* (*BZIP1)* as the second-layer regulators of the response. Furthermore, they identified two long non-coding RNAs (lncRNAs), *MSTRG.24415.1* and *MSTRG.8196.1,* that were downregulated and thought to regulate the target genes *TIP1.1* and *FLA12,* which in turn were suggested to impact cell size and cellulose deposition, respectively. Lu *et al.* (2019) proposed that low nitrogen availability triggers complex interactions among mRNA, lncRNA, and miRNA for the coordination of nitrogen acclimation processes. In the paper by Zhang *et al.* (2024), they found similarly low JA concentrations in the wood of low NH_4NO_3-treated *P. xiaohei* trees. Their results also showed that genes in the hormonal signal transduction pathway, as well as starch and sucrose metabolism, phenylpropanoid biosynthesis, and the phenylalanine pathway, responded differentially to exogenous NH_4NO_3 concentrations, correlating with the metabolomic data.

Another recent study of NH_4NO_3-treated *P. xiaohei* trees focused on the transcriptional regulation of genes controlling wood chemistry and composition via nitrogen (Cao *et al.* 2024). They found, like in Paper I, that several lignin-biosynthetic genes were responsive to nitrogen. They also suggested that nitrogen regulates wood chemical composition through directing the flow of carbon within different metabolic pathways, and hence stimulating, for instance, the carbon-intensive lignin biosynthesis over cellulose biosynthesis in low nitrogen conditions.

Temporal transcriptomic analyses, linking nitrogen-responsive genes to regulatory TFs, lead to the identification of CRF4 as an important regulator of time-dependent nitrogen-responsive genes in *Arabidopsis* (Varala *et al.* 2018; Brooks *et al.* 2019). The overexpression of *CRF4* resulted in repression of the high-affinity nitrate transporter *NRT2.1* and lowered nitrate uptake under low nitrogen conditions (Varala *et al.* 2018), which supports the function of CRF4 in suppressing nitrogen uptake and/or sensing. The closest homolog of the *Arabidopsis CRF4* in *P. tremula*, *CRF4* (a.k.a. ERF85), shows similar expression patterns to the *Arabidopsis CRF4* and shares homologous targets in a gene regulatory network (Seyfferth *et al.* 2021b). The *Populus* CRF4 was suggested to function as a transcriptional switch between cell expansion and SCW formation since the overexpression of *CRF4* upregulated genes involved in cell growth and expansion while downregulating the expression of the SCW-related genes. In line with this, overexpression of *CRF4* under the *pLMX5* promoter resulted in increased cell size of xylem fibres and reduced SCW thickness in woody tissues of hybrid aspen. Interestingly, these changes are reminiscent of those occurring in response to high nitrogen treatments of poplar (Pitre *et al.* 2007a; Hacke *et al.* 2010; Plavcová *et al.* 2013). The role of the *Arabidopsis* CRF4 in nitrogen signalling and the similarities in the expression and phenotypic response to overexpression in *Populus* lead to a question on how CRF4 mediates the transition from cell expansion to SCW formation, whether it is affected by nitrogen availability and whether it has a nitrogen-sensing role in *Populus*. We found in paper IV that *CRF4* responds to nitrogen treatments and specifically to nitrate. In paper IV, we attempted to decipher the time-sensitive as well as cellular specificity of CRF4 action, aspects that are poorly understood for any nitrogen-responsive gene in cambial tissues.

2. Aims and Objectives

The main goals of this project were to investigate:

(1) How cambial activity and xylem differentiation are affected by different forms of nitrogen fertilisation.
(2) How to study and analyse nitrate responses in wood.
(3) How the nitrate signal is transduced in the stem, and what are the corresponding genetic networks.
(4) Whether *CRF4* is nitrogen responsive or involved in a primary nitrate response network in the vascular cambium.

With these goals, I aimed to provide basic knowledge about nitrogen-stimulated cambial growth to support a broader objective of the sustainable use of nitrogen-based fertilisers for increased tree biomass production and to understand the effects on wood quality.

3. Materials and Methods

3.1 Hybrid aspen

Hybrid aspen (*P. tremula* L. *x P. tremuloides* Michx.), clone T89, was used as the model system to study the physiological and molecular responses to nitrogen stimulated cambial growth. *Populus*, as a model system for tree and wood biology, is now well established due to the advanced and available genomic and molecular resources (Jansson & Douglas 2007; Sundell *et al.* 2017). The cultivation and breeding of hybrid aspens in Sweden also has a history for both experimental and commercial interests because of their very high productivity (Tullus et al. 2012).

3.1.1 CRF4

The transgenic *CRF4* overexpressor (CRF4-OX) lines used in this thesis carry the *P. tremula CRF4* (*Potra2n7c16584)* under the control of the pLMX5 promoter (Love *et al.* 2009). The generation of the transgenic lines is described in Vahala et al. (2013), and the transformation of hybrid aspen *(P. tremula L. x P. tremuloides* Michx.), clone T89, is described in detail in Nilsson et al. (1992).

3.2 N-experiments

3.2.1 N-treatments: N-levels and N-sources

Extensive efforts were made to optimise the experimental setup to find physiologically relevant N-addition levels. Literature studies on optimal plant nutrition (Ingestad 1979) and application strategies (Coleman *et al.* 1998) laid the foundation for these experiments.

N-levels and application method

The foundation for establishing an application method started with estimations of the N uptake of hybrid aspen trees cultivated in a controlled environment. The estimation of N uptake assumed that:

- The shoot biomass contains approximately 2% nitrogen per dry weight in g (defined by actual measurements).
- The N content of the soil is negligible at day 1 of the experiment.
- The dry weight of the trees is 20% of the fresh weight.
- The growth rate and N uptake are exponential (when N is not a limiting factor).

The following calculations are based on the fresh weight of shoot biomass after 10 weeks of growth in greenhouse conditions and non-limiting N availability. The average fresh weight of shoot biomass was 244 g ± 5 g (n =40). Thus, the dry weight would be approximately

$$Shoot\ biomass_{dry\ weight} \Rightarrow 244\ g \cdot 0.2 = 48.8\ g,$$

and the resulting estimated N content per plant would be

$$N_{content} \Rightarrow 48.8\ g \cdot 0.02 = 0.976\ g.$$

Based on the above calculations, the N content in plants would be approximately 0.976 g after 10 weeks. Hence, at least 0.976 g should have been available to the plant up to that point, representing an estimate of the total N to be added to pots to sustain normal growth. How to distribute this ~1g of N over the experimental period was calculated using the following formula by Ingestad (1979) :

$$(N_t - N_s) = N_s\left(e^{R_N(t-s)} - 1\right)$$

This is also described in Paper I, but the main idea was to design a fertilisation regime that kept the plants' internal nitrogen levels relatively stable during the experimental period. The overall growth rate (height) was higher in the first experiment compared to the second due to seasonal changes in the ambient light and temperature conditions in the greenhouse. We defined the nitrogen levels based on the nitrogen content of newly expanded leaves and the overall growth of the trees. This showed that a total nitrogen addition of less than 0.5

g per plant restricted plant growth (Figure 5A), while levels above 1.5 g did not further stimulate growth (Figure 5A and B). The nitrogen content of the leaves increased steadily until a total nitrogen addition of approximately 1 g, then showed a trend of saturation at 1.5 g added nitrogen (Figure 5C). Based on the growth restriction, lack of further growth rate increase, and leaf nitrogen saturation, the following nitrogen levels used in this thesis work were defined as limited, sub-optimal, optimal, and excessive.

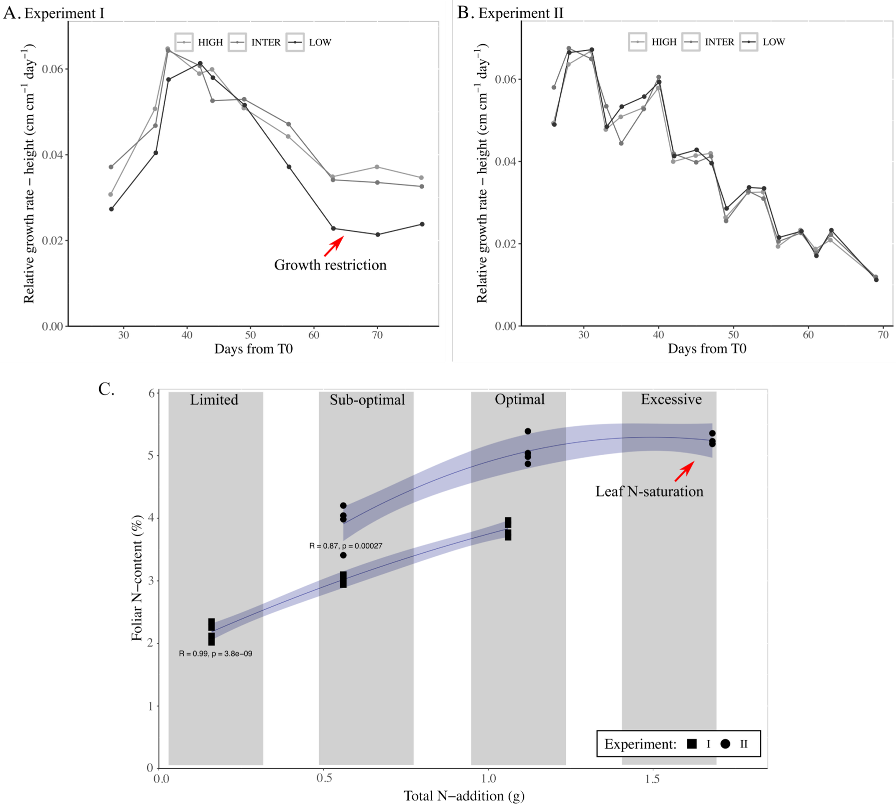

Figure 5. The relative growth rate (height) and leaf nitrogen content in response to different nitrogen applications. (A) relative growth rate in Experiment I, (B) relative growth rate in Experiment II, and (C) nitrogen content in leaves in response to nitrogen additions. HIGH, INTER and LOW correspond to 0.16, 0.56, and 1.06 g added nitrogen in Experiment I and 0.56, 1.12, and 1.68 g in Experiment II.

N sources

The most conventional nitrogen source used for fertilisation experiments is NH_4NO_3. This is also the nitrogen source commonly applied as a fertiliser in agricultural and forestry practices. Ammonium nitrate was therefore selected as one of the forms of nitrogen to use in Paper I. However, the inclusion of several forms of nitrogen was of interest since previous studies have shown that the nitrogen form can influence certain plant properties. For example, it has been suggested that organic nitrogen in the form of glutamine or arginine can enhance plant NUE (Franklin *et al.* 2017), and that phenylalanine can stimulate root growth in *P. tremula x P. alba* (Jiao et al. 2018). Moreover, ample evidence points to nitrate, an inorganic nitrogen form, as being a potent signal to modulate shoot-to-root ratio as well as lateral root proliferation (Nunes-Nesi *et al.* 2010). Consequently, the potential of the different nitrogen forms to modulate plant growth led to the inclusion of four nitrogen sources in Paper I (Table 2). Arginine was selected as the organic nitrogen form due to its high nitrogen content (four nitrogen atoms per molecule), and the use of arginine in forestry-related research (Öhlund & Näsholm 2001; Wilson *et al.* 2012; Häggström *et al.* 2021; Schneider *et al.* 2024). Two additional inorganic forms were nitrate (NO_3^-) in the form of KNO_3 and ammonium (NH_4^+) in the form of NH_4Cl. Apart from having different molecular charges, there are also different assimilation costs associated with the selected nitrogen sources, nitrate having the highest and amino acids the lowest according to Franklin *et al.* (2017).

Table 2. Selected N-sources included in the studies, together with molar N content and their respective charges.

N-source	Molar N content	Charge
Arginine	4	+
Ammonium	1	+
Ammonium nitrate	2	+/-
Nitrate	1	-

Nutritional compositions were kept as similar as possible. Importantly, the ratios of the macronutrients were never lower than the suggested optimum of NPK 7:1:5 (Jia & Ingestad 1984). The counterion to ammonium was chloride (Cl^-) and potassium (K^+) for nitrate, leading to a surplus of chloride in the ammonium treatment, and potassium in nitrate-containing treatments (Table 3). The overall composition of the nutrient solutions was based on the “Rika-S” liquid fertiliser solution (SW HORTO AB). A full description is found in Appendix A.

Table 3. NPK ratios of the different nitrogen sources in Paper I.

N-source	N:P:K ratios
Arginine	7:1:5
Ammonium	7:1:5
Ammonium nitrate	7:1:9
Nitrate	7:1:21

3.2.2 Nitrate uptake experiment

Hybrid aspen cuttings (T89) and transgenic CRF4-overexpression lines were selected for the analysis of nitrate uptake of the roots. The protocol was adapted from Delhon *et al.* (1995) and is also described in Paper IV. Plant cuttings were first cultivated in low nitrogen media (1 mM KNO_3) until rooted. Then, the rooted cuttings were washed three times in 0.1 mM calcium sulphate ($CaSO_4$) solution before being transferred from the growth media to an isotope-labelled nitrate solution (0.2 mM $^{15}NO_3$ in ½ Murashige and Skoog (MS) media) with the same pH of 5.6 as the growth media. Roots were submerged and incubated for 35 minutes in the ^{15}N-labelled solution. After the incubation time, the roots were washed again in $CaSO_4$ solution three times to remove excess isotope-labelled nitrate (Figure 6). Roots were then dried and homogenised. The nitrogen content of the tissue was measured by an elemental analyser–isotope ratio mass spectrometry (EA-IRMS) instrument for both natural and isotopic nitrogen composition.

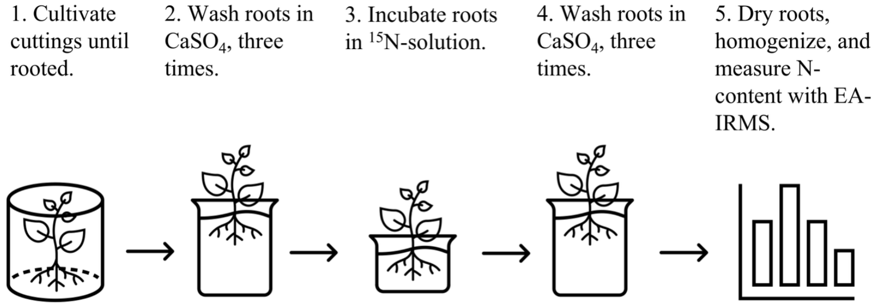

Figure 6. Schematic representation of the uptake assay protocol.

3.2.3 Nitrate injection experiment

To evaluate how nitrate influences gene expression and cell expansion in differentiating xylem tissues of hybrid aspen trees, a method for injecting nitrate directly into stems was developed. The developed method is relatively non-invasive and circumvents the root systems, allowing controlled application of nitrate into the tissue of interest. A full description is available in Paper IV. Nitrate concentrations of 1.42 ± 0.08 mM in the xylem sap of *P. trichocarpa* have previously been reported (Hu & Guy 2020). Based on this, we concluded that the injected nitrate concentration should be slightly higher since it will be diluted in the transported sap and therefore used a concentration of 5 mM. The method itself was adapted from Sano *et al.* (2005), and the method evaluation, using a dye to track the spread of the injected solution within the stems, was inspired by Umebayashi *et al.* (2007). The conclusion, after several tests, was that the spread of the solution within the stem had reached the whole circumference of the stems after 2 h at a 60 cm distance from the injection point. It was pivotal that the solution would reach the cells in the cambium and differentiating xylem since both bulk and single-cell transcriptomic analyses were conducted on RNA from these tissues. No dye was used in the experiments with the nitrate treatments in Paper IV.

3.3 Analytical methods

3.3.1 Py-GC/MS

Pyrolysis, the decomposition of materials by heat in the absence of oxygen, combined with gas chromatography and mass spectrometry (Py-GC/MS), is an analytical method that has been applied to complex biological polymers for chemical characterisation (Meier & Faix 1999; Gerber *et al.* 2012). The volatile degradation products from the pyrolysis are separated by capillary gas chromatography, and then the separated compounds are identified by mass spectroscopy. In an ideal scenario, the heat applied to the material is evenly distributed and thermally excites the molecules, resulting in instantaneous decomposition. The volatile degradation products of low molecular weight will then pass through the gas chromatography (GC) column. However, if the ideal scenario is not met, there will be low molecular weight fragments that can recombine with those of higher weight (Meier & Faix 1999). In Faix *et al.* (1990), the typical thermal degradation products found in wood are listed together with their expected GC retention time and the key features of their mass spectra.

The separation of volatile degradation compounds by GC is facilitated by the transportation of these compounds by an inert gas through a capillary column, which takes place in a time-temperature-regulated oven, allowing for maximal separation. The time it takes for a compound to pass through the column with the specific settings of transportation gas, type of column used, and time-temperature program is the retention time that is indicative of that compound (Faix *et al.* 1990; Lourenço *et al.* 2018). When coupled to a mass spectrometry unit, the separated compounds are ionised, analysed according to the mass-to-charge ratio (m/z), and detected, giving rise to a mass spectrum. The mass spectra of a compound are viewed as a histogram, showing the m/z ratio on the x-axis and the relative intensity of each peak on the y-axis. The relative intensity and the number of peaks depend on the ionisation and fragmentation of the compound. These are characteristic of the different compounds and constitute, together with the GC retention time, the basis for the compound identification (Glish & Vachet 2003).

The full analytical setup and the protocol used for Py-GC/MS analysis in this thesis work are described in detail in Gerber *et al.* (2012) and in Paper I. Important to mention, the heat used in the Py-GC/MS analyses was set to 450°C. This is crucial for the analysis of lignocellulosic material since temperatures above 450°C might impact the substitution pattern of lignin degradation products (Meier & Faix 1999). Proper analysis of lignin monomeric composition requires an intact substitution pattern. The degradation products from cellulose and hemicelluloses, in comparison to lignin, are less specific. The two groups of polysaccharides can produce the same degradation products, and the products are easily fragmented (Lourenço *et al.* 2018). In our analyses, we have grouped all compounds that originate from cell wall polysaccharides as carbohydrate (C) compounds to evaluate the proportion between lignin- and polysaccharide-derived cell wall components (Renström *et al.* 2024).

The high-throughput Py-GC/MS method (Gerber *et al.* 2012; Gerber *et al.* 2016), developed to include data processing for the spectral output, is very useful for sensitive chemical discrimination between, for example, cell wall composition of different genotypes or treatments. However, the classification of compound origin is not always straightforward, as the complexity of cell walls increases with the increasing power of analytical methods. The compound origins and the classification of cell wall components are presented in Table 4.

Table 4. Classification of cell wall components based on compound origin.

Classification	**Compound origin**
C	Carbohydrates
G	Guaiacyl units
S	Syringyl units
H	*p*-hydroxyphenyl units (*p*-cresol & phenol)
pHB	*p*-hydroxybenzoylated lignin
P	Generic Benzen derivatives (i.e., benzenediols, most probably originates from lignin)
U	known spectra, unknown identification (can be C or L, similar spectra but not possible to identify)
L	total lignin, G+S+H+P+ pHB

Specific compounds included in the cell wall analyses by Py-GC/MS are based on the work Faix *et al.* (1990), and their respective classification is presented in Table 5. Each mass spectra from each peak in the chromatograms of the analysis were manually matched with MS-Search, a software provided by the National Institute of Standards and Technology's Mass Spectrometry Data Center (NIST MSDC), which is used to search and match mass spectra with pure spectra of standard compounds in libraries (Gerber *et al.* 2012).

Table 5. Specific compounds included in the Py-GC/MS analyses and their classification.

Compound	**Classification**
Phenol, 2-methoxy-/Guaiacol	G
3-methyl-guaiacol	G
4-methyl-guaiacol	G
4-ethyl-guaiacol/3-ethyl-guaiacol	G
4-vinyl-guaiacol	G
Isoeugenol (cis)	G
Homovanillin	G
Vanillin	G
Isoeugenol (trans)	G
Homovanillin	G
Guaiacyl acetone	G
4-(oxy-allyl)-guaiacol	G
4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	G
Benzeneacetic acid, 4-hydroxy-3-methoxy-, methyl ester	G
Coniferaldehyde	G
Coniferyl alcohol (trans)	G
Guaiacyl acetone	G
4-propyl-guaiacol	G
x-oxy-propioguaiacone	G
Unknown-3	G
Phenol	H
p-Cresol	H
Styrene	P
Resorcinol?	P
2,3-Dihydroxybenzaldehyde	P
1,2-Benzenediol, 3-methoxy-/ 3-methoxy-catechol	P

Catechol/Hydroquinone	P
Benzaldehyde, 3-hydroxy-	P
4-hydroxy-benzoic acid	pHB
Syringol	S
4-methyl-syringol	S
4-ethyl-syringol	S
4-vinyl-syringol	S
Phenol, 2,6-dimethoxy-4-(2-propenyl)-	S
4-propyl-syringol	S
3,5-Dimethoxycinnamic acid	S
Benzaldehyde, 4-hydroxy-3,5-dimethoxy-	S
4-propyl-syringol	S
4-propenyl-syringol (trans)	S
Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)-	S
Propiosyringone	S
Syringyl acetone	S
3,5-Dimethoxy-4-hydroxycinnamaldehyde	S
4-ethyl-syringol	S
Propiosyringone	S
4-(oxy-allyl)-syringol	S
3,5-Dimethoxy-4-hydroxyphenylacetic acid	S
Sinapyl alcohol (trans)	S
Sinapyl alcohol (trans)	S
3,5-Dimethoxy-4-hydroxycinnamaldehyde	S
3,5-Dimethoxy-4-hydroxycinnamaldehyde	S
Benzenemethanol, 2,5-dimethoxy-, acetate	S

3.3.2 NIR imaging and SilviScan

NIR spectroscopy is a method that utilises the reflectance or transmittance of electromagnetic wavelengths between 780 and 2526 nm, measuring the interaction between light and the analysed material. This span of wavelengths is called the near-infrared region and lies beyond the region of visual light and can, therefore, not be detected by human eyes. The recorded absorption of light by NIR spectroscopy, the resulting spectra, show the characteristics of the reflectance or transmittance exchanged from the analysed material. Light absorption in the NIR region arises from molecular vibrations from functional groups such as –CH, –NH, –OH, etc. The spectral bands recorded in this region are often broad, overlapping, and weak compared to other spectroscopy methods and require analytical, mathematical, and statistical processing in order to relate sample properties with the corresponding spectral information for increased sensitivity. On the other hand, weaker absorption allows for the analysis of highly absorbing and light-scattering samples. Thus, this method has a dual capacity, detecting both the chemical and physical properties of a sample due to signals of absorption and scatter effects (Reich 2005).

To determine the qualitative and quantitative characteristics of wood using NIR spectroscopy entails multivariate analysis in combination with calibration. The calibration relates the NIR spectra with known wood characteristics, and the resulting NIR model can thereby be used to make predictions for samples or materials not used in the calibration. Variants of Principal Component Analysis (PCA) and Partial Least Squares (PLS) regressions are the common multivariate methods used to reduce the variables in the NIR spectra and statistically relate the absorption bands or scatter effects to a certain wood property (Reich 2005; Hein *et al.* 2017).

Since wood originates from perennial growth, there is spatiotemporal information within each wood sample. This information can be exploited with the combination of NIR spectroscopy and hyperspectral imaging (NIR imaging). Hyperspectral imaging refers to the acquisition of NIR spectra for each pixel in an image (Hein *et al.* 2017). This results in a three-dimensional dataset, two spatial dimensions, and one spectral dimension. Further, the obtained NIR spectra must be calibrated against a reference value for that specific area. This requires spatially collected reference material. In this work, we have utilised the SilviScan technology to measure the physical properties of wood and the analytical Pyrolysis-GC/MS for the collection of chemical information to use as reference material for the training and calibration of the NIR imaging models.

The SilviScan technology is based on a combination of instruments: an optical cell scanner, an X-ray densitometer, and an X-ray diffractometer. A polished, transversal wood segment, trimmed to 2 mm × 7 mm (tangential × longitudinal) dimensions, is then run through the system of instruments. The optical cell scanner measures wood anatomical features at 25 µm resolution, the X-ray densitometer measures the density profile at 25 µm, and finally, the X-ray diffractometer can measure the microfibril angle (MFA) of crystalline cellulose down to 0.2 mm resolution, mapped onto the same 25 µm scale as the other profiles. Other physical wood characteristics, such as MOE and wall thickness, to mention a few, can be derived from these direct measurements (Schimleck *et al.* 2019).

The technical development of NIR imaging was facilitated by RISE Research Institutes of Sweden[1] and the Biopolymer Platform at UPSC[2]. NIR prediction models were based on linear PLS (Partial Least Squares) methodology and were constructed with the help of the multivariate data analysis tools Evince and Breeze (Prediktera AB, Umeå, Sweden). Physical and chemical properties of wood were predicted at 30 µm or 0.5 mm resolution, depending on the resolution of the NIR image.

3.4 Transcriptomic analysis

3.4.1 Single-cell RNA sequencing

To deepen our understanding of molecular nitrate responses in wood, we applied scRNAseq in Paper IV. This technique enables the capture of short-lived cell states and different cell types in a heterogeneous sample via its transcriptome. The method has been developed since 2009 (Tang *et al.* 2009) and can today be applied even to woody tissues (Grones *et al.* 2024).

The single-cell RNA sequencing procedure, in general, involves a first step of protoplast isolation when the cell walls are degraded. This step requires a cell viability test of some sort to ensure the quality of the cells. In Paper IV, we used trypan blue staining to check protoplast concentration and viability. Secondly, cells need to be separated. The two most common methods are either based on microfluidic droplet cell assortment (e.g., 10 x Genomics Chromium) or micro/nanowells

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[1] https://www.ri.se/en/what-we-do/expertises/high-resolution-wood-characterisation. Accessed: 2025-10-30.

[2] https://www.upsc.se/platforms/cell-wall-analysis/4845-biopolymer-analytical-platform.html Accessed: 2025-10-30.

(e.g., Takara ICELL8 or BD Rhapsody) (Grones *et al.* 2024; Cho *et al.* 2025). There is also a new system called Evercode, provided by Parse Bioscience, that is neither based on droplets nor micro/nanowells but on combinatorial barcoding. We used in Paper IV the Chromium GEM-X Technology[3]. One of the problems with this technology is that the microfluidic channels are <100 µm wide while plant cells can be up to ~80 µm (Grones *et al.* 2024). However, testing the method for use in Paper IV, we never found cells larger than 50 µm after protoplast isolation. After the mRNA sequencing of the single cells, a substantial amount of data processing is required. The sequencing reads need to be mapped to a genome and to the cell identity provided by the cellular barcode via different bioinformatic tools. Then, cells are clustered together based on similarities in the transcriptome. The clustering is based on algorithms that, depending on set parameters, decide the level of similarity to form a cluster of cells. Clustered data are then visualised using projection algorithms to reduce the dimensionality of the data. This could be, for example, Principal Component Analysis (PCA) or Uniform Manifold Approximation and Projection (UMAP), which are based on linear versus non-linear projections, respectively (Grones *et al.* 2024). Next, these clusters should then be interpreted as cell types or cell stages. The cluster identity can be described by known marker genes for a certain cell type or state. This step can be more or less of a challenge depending on what is known about the specific plant species or tissue studied. In paper IV, the cell clusters were identified based on known marker genes and cluster-enriched genes. Gene expressions of the different clusters were also correlated to known data sets: RNAseq data from the AspWood database (Sundell *et al.* 2017), spatial profiles (Du *et al.* 2023b), single nuclei profiles (Conde *et al.* 2022), and laser capture microdissected cells (Shi *et al.* 2017; Tung *et al.* 2023). In this way, fourteen out of twenty-one clusters could be assigned to a cell type or state (Table 6). The remaining seven unannotated clusters show that there is still plenty to learn about this highly complex woody

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[3] https://www.10xgenomics.com/support/universal-three-prime-gene-expression/documentation/steps/sample-prep/single-cell-protocols-cell-preparation-guide. Accessed: 2025-10-24.

tissue. For all details about the method and for the complete list of marker genes used, see Paper IV.

Table 6. Summary of the cluster annotation in Paper IV.

Cluster	**Markers/data correlations**	**Identity (cell type/stage)**
5, 7, 18	Photosynthesis related genes	Rays
4, 12, 15, 17	*CELLULOSE SYNTHASES* (*CESA*) *IRX9, IRX14, IRX15* *GLYCOSYLTRANSFERASE* (*GT43A*) Lignin-biosynthetic genes (*LAC*)	Early xylem maturation Cell differentiation
4, 12, 15	Fiber LCM, Tung *et al.* (2023)	Fibres
17	*XCP2a, XCP2b* *RIBONUCLEASE III* (*RNAseIII*) *ENDO-BETA-MANNANASE 6* (*MAN6*)	Vessels
14, 16	Early expanding zone AspWood	Fusiform initials
6	Developmental trajectories; gene ontology (GO) terms	Vessel precursors
1, 10	Developmental trajectories; GO terms	Fiber precursors
19, 20	*AINTEGUMENTA* (*ANT*) *CYCB1;2, CDKB1;2*	Cambial cells Cell divison
0, 2, 3, 8, 9, 11, 13	Unclear correlations with published datasets	Unknown
3, 8, 11, 13	Transcription correlation with rays Early cambial zone AspWood Correlation with the LCM ray sample, Tung *et al.* (2023)	Possibly early rays
9	High expression of *PIRIN2*	Possibly ray cells next to vessel elements

3.4.2 Bulk RNA sequencing

The full description of the bulk RNA sequencing procedure is described in Papers I and IV. In Papers I, III and IV, secondary xylem tissues were scraped from the surface of wood after removal of the bark until the death of the xylem fibres, identified through a change in the texture and the colour of the wood. The most obvious difference between the different RNAseq methods is the resolution of the gene expression data. While scRNAseq offers cellular resolution, bulk RNAseq provides tissue-specific resolution and thus shows the average gene expression of all cell types and cell stages included in the sample. This naturally provokes a bias towards the dominating cell type/cell state of the tissue. This is good to keep in mind when analysing woody tissues and interpreting the transcriptomic data. Features that can cause bias are, for example, the dominance of fibres over the vessels, quicker differentiation of the vessel elements than the fibres, and the presence of rays throughout the whole wood. In Papers I, III and IV, we used bulk RNAseq to observe transcriptional responses in wood to nitrogen treatments on a whole tissue level.

4. Results and Discussion

4.1 Physiological responses to nitrogen-stimulated cambial growth (Paper I)

In contrast to other studies exploring the relationship between nitrogen and wood formation, we applied a range of nitrogen sources and concentrations to elucidate both the general nitrogen responses and the ones specific to a certain nitrogen source and concentration. We also tried to match the application of nutrients according to the growth rate to avoid risks of fluctuating nitrogen status. Not surprisingly, we observed clear stimulatory effects of nitrogen on biomass accumulation and could show that a decline in wood density in response to increasing nitrogen additions did not outweigh the increase in dry stem biomass (Paper I). Even though there were only small differences in growth between the selected nitrogen sources, nitrate seemed unique in stimulating shoot biomass accumulation more than the other nitrogen sources (Paper I). This was a somewhat surprising result since earlier field studies have shown that nitrate-based fertilisation led to less biomass accumulation compared to ammonium-based fertilisation (DesRochers *et al.* 2007). The contradictory results are most likely related to the high mobility of nitrate in soil, which did not occur in the greenhouse conditions of our study, where we prevented any leaching of the nutrients. In any case, these results highlight the importance of the chemical properties of the applied nutrients, which influence soil retention and, consequently, nitrogen availability to the plant (Inselsbacher *et al.* 2011; Lim *et al.* 2022).

Increasing nitrogen availability also increased the cumulative water use efficiency (relationship reviewed in Brueck (2008)) as well as the diameter of the xylem vessels, implicating an enhanced water transport. Similar results have been obtained in previous studies (Hacke *et al.* 2010; Plavcová *et al.* 2013), where they found higher specific conductivities (K_s), thus altered hydraulic conductivities in the xylem of high nitrogen-treated trees.

Furthermore, similar to other studies (Cooke *et al.* 2003; Pitre *et al.* 2007b; Novaes *et al.* 2009), there was a general trend of decreased lignin content in response to increased nitrogen availability (Paper I). The exception to this trend was observed in the nitrate-treated trees, where the lignin content remained constant across a wide range of nitrate additions. Like in Pitre *et al.* (2007b), we observed an increase in pHB and H-type lignin content in response to elevated nitrogen levels. However, the increase in H-type lignin was specific to the nitrate-based N-sources (Paper I). We discuss two possible reasons for this increase in Paper I: one being the accumulation of "stress-lignin" and the other a higher proportion of primary to secondary cell wall material in the woody tissues of nitrate-treated trees. Yet another reason could be the lower energetic cost for H-type lignin production (Luomaranta 2025), which could compensate for the higher costs of nitrate assimilation (Franklin *et al.* 2017). Overall, our findings emphasise the nuanced and source-specific effects of nitrogen on wood formation and highlight the importance of considering both the chemical characteristics of nitrogen sources and their interactions with plant physiological processes when evaluating fertilisation strategies.

4.2 Analytical methods to study nitrogen effects on wood formation (Papers I & II)

4.2.1 Lignin determination by Py-GC/MS (Paper I)

Lignin characterisation is a multifaceted process and often laborious. Many of the analytical methods, such as the Klason method, require relatively large sample amounts. This is not always a luxury we have in plant science. While the Klason lignin method is well established and widely used in industrial settings, it is often too crude for research purposes, as was the case in our studies.

In Paper I, we show the outstanding sensitivity of Py-GC/MS in terms of showing the chemical footprint of a certain treatment in the wood. The high resolution enabled us to distinguish between H-type lignin and pHB units, known to be difficult (Ralph *et al.* 2019; Goacher *et al.* 2021). It also showed the low but very specific increase of H-type lignin in response to nitrate treatments, information that might have been lost using cruder methods. This was also confirmed using NMR.

Another superior feature of the method is that it provides simultaneous information on both the relative lignin content and its individual components. However, the complex native structure of lignin still poses difficulties in interpreting the large amount and convoluted data produced by mass spectrometry-based methods (Letourneau & Volmer 2023). New decorations and constituents of lignin are constantly being discovered (Vanholme *et al.* 2019), which necessitates continuous efforts in developing both the existing and new methods.

4.2.2 Wood characterisation by NIR spectroscopy imaging (Paper II)

In Paper II, we presented a NIR spectroscopy method for wood characterisation. This method is based on the near-infrared reflectance/transmission of electromagnetic waves from the analysed material. In our case, we calibrated the chemical NIR models using Py-GC/MS. The low quantity (50 ± 10 µg wood powder) requirement of the input sample enabled spatially resolved calibration.

The image resolution was improved without any loss in throughput capacity, and routines were established for the handling of samples down to 4 mm in diameter. These changes enabled large-scale analysis of both field- and greenhouse-grown material.

The intended use of the method is to provide spatial information within a transverse wood section. Thus, it is appropriate for the analysis of any kind of deviation found in wood. A common deviation is the formation of reaction wood, in angiosperm trees called tension wood. Tension wood is also known to prevail in response to nitrogen treatments (Pitre 2007a; Pitre *et al.* 2010). We therefore produced a NIR model predicting the probability of tension wood (Paper II). Spatial variation was found in the abundance of tension wood in stem transverse sections of nitrogen-treated, greenhouse-grown trees that was not detectable in homogenised bulk samples. In summary, NIR spectroscopy imaging can be a useful tool for screening large amounts of materials produced in experimental investigations of wood in response to different treatments and environmental conditions.

4.3 Molecular regulation of nitrogen-stimulated cambial growth (Papers I, III & IV)

4.3.1 Global nitrogen responses in *Populus* wood (Papers I and III)

The nitrogen-induced molecular response in wood has previously focused on the effect on cell walls and the related genes, such as lignin biosynthetic genes, laccases (Cooke *et al.* 2003), and aquaporins (Hacke *et al.* 2010; Plavcová *et al.* 2013). In line with the earlier studies, we identified several aquaporins, lignin-biosynthetic genes and laccases (*LAC2, 4, 6, 11,* and *17*) as differentially expressed in response to increasing availability of nitrogen.

Moreover, the expression of the cytokinin biosynthetic *ISOPENTENYL TRANSFERASE* (*IPT5a, 5b,* and *6b*) was stimulated by increased nitrate availability (Paper I), supporting the interplay between cytokinin and nitrate signalling (Takei *et al.* 2004; Sakakibara *et al.* 2006; Gu *et al.* 2018). The increased expression of the cytokinin-biosynthetic genes may indicate a cytokinin-mediated negative feedback mechanism regulating nitrate uptake, similar to findings previously reported in *Populus* trees (Gu *et al.* 2018). We also propose an interesting candidate in mediating nitrate responses in wood, the MYB-like helix-turn-helix transcription factor *(Potra2n16c29671).* This TF is expressed in the transition zone from cell wall formation to cell death and could potentially affect lignification in response to the nitrate signal. The data also supported the importance of the nitrate-sensing/signalling gene *NIN LIKE PROTEIN 2 (NLP2)* in wood, being downregulated in response to higher nitrate levels. Surprisingly, *NLP7*, a well-established nitrate sensor in *Arabidopsis*, did not show up in the global transcriptomic analysis (Paper I).

Several nitrate assimilation genes were also found among the DEGs in Paper I. Nitrogen assimilation in wood was further explored in Paper III, where we analysed fourteen gene families related to nitrogen uptake, transport and assimilation in the *P. tremula* genome annotation. Phylogeny as well as the structure of the genes in these families were analysed. Then the expression profiles of these genes in wood were identified using the AspWood database, which contains high-resolution expression profiles across the woody tissues of *Populus* stem (Sundell *et al.* 2017). Many of the examined genes exhibited two distinct peaks in wood: one in the phloem/cambium region and the next in the zone of xylem cell death (Paper III). We propose that this biphasic expression pattern relates to the transport and sensing of nitrogen moving in the phloem and the transport/sensing of nitrogen taken up from xylem sap to the ray parenchyma cells. A closer look at the various members of the NLP gene family showed that the *NLP7* homolog (*PotraNLP7a*) had an interesting expression profile with high expression throughout the phloem, cambium and xylem expansion zone, showing low expression in the zone of secondary cell wall formation and then peaking again in the cell death zone. However, the NLP member with the highest expression was *PotraNLP8b.* As mentioned earlier in the thesis, *NLP8* homologs have recently been identified in a QTL analysis as candidate regulators of NUE in *P. deltoides* (Du *et al.* 2023a), emphasising their importance in nitrogen-regulated growth in tree species.

Another key factor in nitrate signalling is *NRT1.1/NPF6.3*, a known nitrate sensor (Krouk *et al.* 2010a; Gojon *et al.* 2011). This gene showed a monophasic expression pattern in wood, peaking in the cell expansion zone in Paper III. Additionally, a large proportion of the *NRT/NPF* genes was shown to be expressed in woody tissues, and many of those in a biphasic manner. This was the case with *PotraNPF5.4a, PotraNPF5.4b* and *PotraNPF5.6b*, whose corresponding homologs in *Arabidopsis* have not been functionally characterised, perhaps indicating on specificity of these transporters in the woody tissues.

Interestingly, also in Paper III, we found all the necessary genes in the nitrogen assimilation pathway expressed in the woody tissues. The main players, the glutamate synthase *PotraGLT1b* and the glutamine synthetases, *PotraGLN1.2a* and *PotraGLN1.2b*, were highly expressed in all developmental stages of wood. Nitrogen assimilation in trees has been described as a process occurring in either roots or leaves, and the partitioning seems to vary between species and the available nitrogen sources (Black *et al.* 2002; Li *et al.* 2012; Cánovas *et al.* 2018; Meng *et al.* 2018). The stem has merely been described as a transport route and perhaps a storage place for nitrogenous compounds in this context. Our results, however, indicate a significant contribution of the stem to the overall nitrogen assimilation of the trees, which should not be overlooked.

Furthermore, in Paper III, we were able to distinguish between a general nitrogen response and nitrate-specific upregulation of genes by comparing ammonium- and nitrate-treated trees. Apart from the obvious differences in the expression of the nitrate and nitrite reductases, we found *PotraNRT1.1/NPF6.3*, *PotraNRT2.5c*, *PotraNRT2.1b*, *PotraNLP7a,* and *PotraNLP7b* significantly upregulated in response to nitrate but not ammonium.

Besides the gene expression analysis, expression quantitative trait locus (eQTL) data from the SwAsp collection, described in Luomaranta *et al.* (2024), were examined for the nitrogen-related genes in an aspen population. From this data, we found that the single-nucleotide polymorphisms (SNPs) in *PotraNLP2e* showed association with the expression of 34 different genes (Paper III). Moreover, a whole-transcriptome co-expression analysis and stem diameter data from the SweAsp collection were used to infer the subnetwork of nitrogen-related genes and their correlation with stem diameter. A negative correlation was found between members in the AAP and NPF gene families and stem diameter (Paper III). We propose that these genes could indirectly affect cambial growth via modulation of nitrogen allocation, impeding photosynthetic nitrogen use efficiency. I conclusion, these findings demonstrate that the molecular response to nitrogen in wood is highly dynamic and complex, involving both general and nitrate-specific pathways, and underscore the significant, yet often underappreciated, role of the stem in nitrogen sensing, assimilation, and allocation in trees.

4.3.2 Cellular responses to nitrate signalling: secondary cell wall formation, cell expansion, and *CRF4 (*Paper IV)

To further understand nitrate signalling in wood, we explored the temporal and spatial dimensions of nitrate-induced gene expression in differentiating xylem tissues of hybrid aspen in Paper IV. We performed a time course experiment measuring the global gene expression response after 2, 4, 8, 12, 24, and 48 h of nitrate or control injections to the stem. Application of nitrate induced the strongest changes in transcriptomes at 2 and 4 h after the injections with 142 and 372 differentially expressed genes (DEGs), respectively (Paper IV, Figure 2B, Table S1A-D). We identified early responders, such as *NRT1.1/NPF6.3* and *NLP7a*, which were transiently expressed only in the 2 h time point, followed by nitrate assimilation genes that were differentially expressed at 2 to 4 h, and finally ammonium and amino acid transporters that responded at 4 to 8 h.

Besides the DEG analysis, a whole-transcriptome co-expression network analysis, together with a sub-network of differentially expressed TFs, inferred the extent of the temporal regulation by nitrate. The TFs were also indexed by their nitrogen specificity, as described in Varala *et al.* (2018), resulting in the identification of novel nitrate-responsive TFs, such as *LBD19 (Potra2n14c26863)*.

Next, dependent on when in the transcriptome and where in the wood the DEGs appeared, we could relate the responses to different phases of wood formation. The analysis revealed that nitrate influenced gene expression first in the expanding xylem elements and then in the maturing xylem elements in a manner that indicated a role for nitrate in stimulating xylem cell expansion and suppressing the secondary cell wall formation. Taken together, the 2 h time point seemed to capture well the steps in the primary nitrate responses found in *Arabidopsis* (Medici & Krouk 2014; Ruffel *et al.* 2025).

As a second step, scRNAseq was performed to increase the spatial resolution of gene expression. The 2 h time point was selected for this analysis, comparing the effects of nitrate and the control treatment. All identified clusters, representing different cell types or stages of xylem differentiation, responded to nitrate. The nitrate sensor/transporters *NPF6.3/NRT1.1* and *NRT2.1* were, for example, specifically expressed in clusters 1, 7 and 10 (fibre precursors and ray cells) (Paper IV).

In line with nitrate-stimulating xylem cell expansion, we saw the up-regulation of cell wall-modifying enzymes and aquaporins. But we also recognised suppressed expression of the lignin-biosynthetic genes, which could prolong the xylem cell expansion phase and thereby affect cell sizes. A refined search for involved TFs narrowed down the number of putative candidate genes and led to further investigation of the *CYTOKININ RESPONSE FACTOR 4* (*CRF4*). Overexpression of *CRF4* in *P. tremula* x *P. tremuloides* resulted in changes in xylem cell morphology similar to those of nitrate-treated trees in Paper I. *CRF4* was also co-expressed with *ERF4,* which in turn has been shown to impact cell size in *Arabidopsis* (Ding *et al.* 2022), supporting the role of CRF4 in xylem element expansion. Other novel

TFs responding to nitrate and potentially involved in cell expansion were: *ERF1* (Potra2n13c25901), *ERF4* (Potra2n17c31814), *ERF110* (Potra2n2c5831), *HB-8* (Potra2n18c32867), *FLOWERING BHLH 4* (*FBH4)* (Potra2n1c2330), *HOMEODOMAIN-LEUCINE ZIPPER PROTEIN 22 (HAT22)* (Potra2n2c5385), bZIP TF (Potra2n2c5816), *NOVEL ETHYLENE RESPONSE FACTOR DNA-BINDING (NERD)* (Potra2n4c9878), *WRKY48* (Potra2n8c17556), and *C-REPEAT BINDING FACTOR 1* (*CBF1)* (Potra2n9c18829). Together, these findings demonstrate that nitrate rapidly and specifically alters gene expression and transcriptional networks in differentiating xylem of hybrid aspen, promoting cell expansion and delaying secondary cell wall formation through coordinated regulation of transporters, transcription factors, and cell wall-modifying enzymes.

5. Conclusions and Future Perspectives

In this thesis, I have tried to summarise all that I learned during my time as a PhD student. I started with assessing the physiological aspects of cambial growth and found that nitrogen influenced both the morphology and the chemical composition of wood. At this point, I could already see the specific effects of nitrate when it was given as a sole nitrogen source. It stimulated the growth slightly more than the other nitrogen sources, had a bigger impact on cell morphology, and influenced both lignin content and its composition. By following the growth of the trees after applications with both organic and inorganic nitrogen, we could distinguish between general nitrogen responses and a specific nitrate response. Knowing that nitrate acts as a plant signal, these results are perhaps not surprising but important nonetheless, and quite unexplored in the context of wood formation. Even though nitrate stimulated growth of the hybrid aspen trees somewhat more than the organic forms of nitrogen, I found that organic nitrogen would suffice and perhaps be a more sustainable option due to environmental risks linked to the application of inorganic nitrogen.

Studying the wood-specific responses has been a very interesting and, at times, demanding task. The rigidity of wood is equally fascinating and frustrating. I found that the analytical methods often require a lot of material and pre-processing steps. As for lignin characterisation, no method is perfect. The Py-GC/MS method applied in this thesis is remarkably sensitive and requires only ~50 ± 10 µg. However, it could be developed further, deepening the understanding of compound origin and its classifications. Another interesting aspect of wood chemical composition is the extractives, which constitute about ~1-3% in *Populus* wood (Ek *et al.* 2009a). Because of the low percentages of extractives, it is often overlooked, but it could be interesting to investigate in response to nitrogen additions. These are also potential areas of development of the NIR spectroscopy method presented in this thesis. The NIR imaging was shown to be useful for high-throughput analyses of both field- and greenhouse-cultivated trees and gave additional spatial information that is otherwise lost when working with homogenised material.

Finally, time-sensitive and cellular responses to nitrate were explored in cambial tissues on a transcriptomic level in a way previously not described. The vast amount of data produced will hopefully continue to deliver insights into the complexity of the genetic regulation of cambial growth. I hope that this work, in the future, is used for wood-specific manipulation of nitrate sensing and signalling via transgenic technology in forest trees. This could further elucidate the role, and specifically, the importance of nitrate signalling in cambial growth and cell expansion and perhaps contribute to providing methods for breeding novel varieties of trees with improved NUE, for instance.

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Popular science summary

Trees, just like all plants, need nitrogen to grow. Recently, forests in Sweden haven't been growing as well as before, and there is an increasing interest in fertilising the forest with nitrogen. We therefore urgently need more research to understand how trees take up and use nitrogen. We know that nitrogen affects how much wood trees make and wood's chemical composition, but we don't know much about how this is affected by different types of nitrogen and what happens inside the tree on a molecular level.

In my research, I looked at what happens when young hybrid aspen trees get different kinds and amounts of nitrogen. I also used a special kind of technology to capture any changes in wood caused by nitrogen additions. The results showed that when these trees get more nitrogen, they grow bigger and their wood develops differently. With nitrate as a sole nitrogen source, the trees grew larger shoots and had wood with an altered composition of a substance called lignin.

By studying the tree cells more closely, I discovered that nitrate actually travels up the tree and into growing wood tissues, quickly turning on genes that help the cells expand and grow. One important gene called *CRF4* played a big part in this process.

Further analysis of nitrogen-responsive genes in wood revealed that wood is not only a transportation route for nitrogen, but nitrogen can also be taken up and processed in the wood cells. In short, this research helps explain how nitrogen—especially nitrate—shapes tree growth and wood quality. These insights could help forests grow better in the future.

Populärvetenskaplig sammanfattning

Träd, precis som alla växter, behöver kväve för att växa. På senare tid har skogarna i Sverige inte vuxit lika bra som tidigare, och intresset för att gödsla skogen med kväve har ökat. Därför behövs det mer forskning för att förstå hur träd tar upp och använder kväve. Vi vet att kväve påverkar hur mycket ved ett träd producerar och att det kan påverka vedens kemiska sammansättning, men vi vet inte mycket om hur detta påverkas av olika former av kväve och vad som händer inne i veden på molekylär nivå.

I min forskning har jag undersökt vad som händer när unga hybridaspar får olika typer och mängder av kväve. Jag använde också en speciell teknik för att fånga upp förändringar i veden orsakade av kvävetillförsel. Resultaten visade att när dessa träd får mer kväve, växer de sig större och deras ved utvecklas annorlunda. Med nitrat som enda kvävekälla fick träden större skott och ved med en förändrad sammansättning av ett ämne som kallas lignin.

Genom att studera vedcellerna närmare upptäckte jag att nitrat faktiskt transporteras upp i trädet och in i växande vedvävnader, vilket snabbt sätter i gång gener som hjälper cellerna att expandera och växa. En viktig gen, kallad *CRF4*, spelade en stor roll i denna process.

Ytterligare analys av kväveresponsiva gener i veden visade att träet inte bara är en transportväg för kväve, utan kväve kan också tas upp och bearbetas i vedcellerna. Sammanfattningsvis hjälper denna forskning till att förklara hur kväve—särskilt nitrat—påverkar trädens tillväxt och vedkvalitet. Dessa insikter kan bidra till att våra skogar växer bättre i framtiden.

Acknowledgements

I want to acknowledge the importance of the highly skilled people who have supported me along this journey. Hannele Tuominen, I am so thankful for having you as my supervisor. My assistant supervisor, Sandra Jämtgård, with her profound knowledge in plant-nutrient interactions and contagious curiosity, thank you. Junko Takahashi Schmid, an expert in plant cell wall chemistry, taught me everything I know about Py-GC/MS and stayed extra hours in the lab with me. Marta Derba-Maceluch, an expert in wood anatomy, taught me a great deal about image analysis using ImageJ. Finally, Shruti Choudhary, an excellent molecular biologist and a great friend. Àngela Carrio Seguí and Marta Pérez Alonso, experts not only in plant biology and biotechnology but also in how to make a new PhD student feel included. Alice Marcon, brilliant both in plant science and as a friend.

Also, my other assistant supervisor, Leif Jönsson, and everyone in Hannele Tuominen's research group, extra thanks to Pal Miskolczi and Maxime Chantreau, who helped me a lot in the lab, Mikko Luomaranta, and Yupeng Zhang. All co-authors in the included papers, especially Gerhard Scheepers, for a great collaboration on the NIR paper. Members of the C/N group for nice science discussion and afterworks.

All fellow PhD students at UPSC, great fikas, AWs, and discussions. Tuuli Aro, Sonja Viljamaa, Sara Häggström, Tinkara Bizjak-Johansson, Laura Tünnermann and Ellen Svennson, to mention a few.

Thanks to my family for your support and for motivating me every day to do my best and to be kind.

Appendix A

The chemical composition of the concentrated nutrient solutions used in the experiments described in this thesis is presented in Table 7.

Table 7. Constituents of stock solutions prepared for the different nitrogen treatments.

Solution	**Chemical formula**	**(g/mol)**	**Molar ratio**		**(mol/L)**
ARG	L-arginine	174,2	4	N	1,4989
				N	5,9957
NH_4NO_3	NH_4NO_3	80,04	2	N	2,3126
	KNO_3	101,1	1	N	1,3704
			1	K	
				N	5,9957
NO_3^-	KNO3	101,1	1	N	5,9957
			1	K	
NH_4^+	NH_4Cl	53,49	1	N	5,9957
			1	Cl	
MAKRO	KH_2PO_4	136,09	1	P	
			1	K	
				P	0,3871
MAKRO	K_2HPO_4	174,18	1	P	
			2	K	
				K	1,4425
MAKRO	K_2SO_4	174,27	2	K	
			1	S	
MAKRO	$MgSO_4 * 7\ H_2O$	246,48	1	Mg	0,2963
			1	S	
				S	0,2991
MAKRO	$CH_2N(CH_2\ COO)_2)_2FeNa$	367,05	1	Fe	0,0150
			2	N	
			1	Na	
MIKRO	H_3BO_3	61,83	1	B	0,0111

MIKRO	$MnSO_4 \ast H_2O$	169,02	1	Mn	0,0087
			1	S	
MIKRO	$ZnSO_4 \ast 7\ H_2O$	287,54	1	Zn	0,0018
			1	S	
MIKRO	$CuSO_4 \ast 5\ H_2O$	249,68	1	Cu	0,0004
			1	S	
MIKRO	$Na_2MoO_4 \ast 2\ H_2O$	241,96	1	Mo	0,0003
			2	Na	

Received: 5 October 2023 | Revised: 26 January 2024 | Accepted: 5 February 2024
DOI: 10.1111/ppl.14219

ORIGINAL RESEARCH

Physiologia Plantarum

The effect of nitrogen source and levels on hybrid aspen tree physiology and wood formation

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Funding information
Swedish Research Foundation Formas, Grant/Award Number: 2021-00992; Trees and Crops for the Future, Grant/Award Number: 2022.3.2.5-426; BIO4ENERGY, Grant/Award Number: B4E3-FM-2-06

Edited by B. Neuhaeuser

Abstract

Nitrogen can be taken up by trees in the form of nitrate, ammonium and amino acids, but the influence of the different forms on tree growth and development is poorly understood in angiosperm species like *Populus*. We studied the effects of both organic and inorganic forms of nitrogen on growth and wood formation of hybrid aspen trees in experimental conditions that allowed growth under four distinct steady-state nitrogen levels. Increased nitrogen availability had a positive influence on biomass accumulation and the radial dimensions of both xylem vessels and fibers, and a negative influence on wood density. An optimal level of nitrogen availability was identified where increases in biomass accumulation outweighed decreases in wood density. None of these responses depended on the source of nitrogen except for shoot biomass accumulation, which was stimulated more by treatments complemented with nitrate than by ammonium alone or the organic source arginine. The most striking difference between the nitrogen sources was the effect on lignin composition, whereby the abundance of H-type lignin increased only in the presence of nitrate. The differential effect of nitrate is possibly related to the well-known role of nitrate as a signaling compound. RNA-sequencing revealed that while the lignin-biosynthetic genes did not significantly (FDR <0.01) respond to added NO_3^-, the expression of several laccases, catalysing lignin polymerization, was dependent on N-availability. These results reveal a unique role of nitrate in wood formation and contribute to the knowledge basis for decision-making in utilizing hybrid aspen as a bioresource.

KEYWORDS
H-type lignin, lignin composition, N-nutrition, organic vs. inorganic N, *Populus tremula x* P. *tremuloides*, Pyrolysis-GC/MS, xylogenesis

1 | INTRODUCTION

Nitrogen (N) fertilization is a common practice to boost net primary production, especially in terrestrial ecosystems suffering from N limitation. In boreal forests, N fertilization can increase severalfold the volume production of trees (Bergh et al. 1999). Also, short-rotation plantations of various poplar and willow species gain significant increases in biomass production when fertilized with N (Rodrigues et al. 2021). The downside of fertilization is leaching of N to the surrounding environment and the risks of nutrient imbalance in aquatic systems

(Canfield et al. 2010). To avoid the overuse of fertilizers, we need better knowledge on the effects of N on both qualitative and quantitative properties of angiosperm trees such as *Populus* sp.

It is well established that N increases tree volume production by stimulating the activity of the vascular cambium through an unknown mechanism that most likely includes cytokinins (Aloni 2021). Functional studies with cytokinin biosynthetic and catabolic genes in both *Arabidopsis thaliana* and hybrid aspen supported the role of cytokinins in the regulation of cambial activity (Matsumoto-Kitano et al. 2008; Nieminen et al. 2008; Immanen et al. 2016), but the link to N status of the plants was not studied. Nitrogen also influences the basic density of wood. Increased N availability reduced wood density in poplar (Luo et al. 2005; Hacke et al. 2010), most probably as a consequence of increased growth rate (Pretzsch et al. 2018). In addition to wood density, addition of N (in the form of ammonium nitrate, NH_4NO_3) has been shown to increase vessel diameter (Hacke et al. 2010), and causes shorter and wider fibers (Pitre et al. 2007a), thinner secondary cell walls (Plavcová et al. 2013) and tension wood formation (Luo et al. 2005; Pitre et al. 2010) in poplar. These responses vary between *Populus* species of different growth rates (Li et al. 2012), necessitating further studies in these species.

Changes in xylem properties influence the hydraulic properties of the xylem. Treatment with a high NH_4NO_3 concentration increased the diameter of xylem vessels as well as specific conductivity (K_s) compared to treatment with a moderate level of NH_4NO_3 in hybrid poplar (Hacke et al. 2010). Increased stem hydraulic capacity coincided with increased expression of several aquaporins that have been functionally characterized as water channels, suggesting that aquaporins mediate N-stimulated vessel expansion through increased water transport (Hacke et al. 2010).

In addition to the control of meristem activity and wood morphology, N status can influence wood chemistry. Greenhouse experiments with elevated levels of NH_4NO_3 decreased both the S/G lignin ratio and lignin content compared to low levels or lack of NH_4NO_3 in one specific genotype (Pitre et al. 2007b) and across 396 clonally replicated genotypes (Novaes et al. 2009) of hybrid poplar. Increased N availability has also been shown to stimulate the abundance of *p*-hydroxybenzoylated (Goacher et al. 2021) and *p*-hydroxyphenyl (Pitre et al. 2007b) lignin. Lignin changes were shown to depend on the position in the stem (Euring et al. 2014), and it seems, therefore, that N responses in wood chemistry vary depending on the level of the available N as well as the age of the trees.

Another factor influencing tree responses to N availability is the source of N (Yan et al. 2019). Nitrogen is taken up by trees as nitrate or ammonium ions depending on tree species and growth environment (Rennenberg et al. 2010; Song et al. 2015; Zhou et al. 2021). Plants can acquire N also in organic forms as amino acids or small peptides (Näsholm et al. 2009; Inselsbacher & Näsholm 2012). However, the effects of the different N sources on the physiology and wood formation of trees have not been explored yet. In this study, we established a physiologically relevant strategy to investigate the effect of four different N sources, arginine, ammonium (NH_4^+), nitrate (NO_3^-) and ammonium nitrate (NH_4NO_3), at four different levels on plant growth, cambial activity and xylem differentiation in clonally propagated hybrid aspen (*P. tremula x P. tremuloides*) clone T89 trees. Increasing levels of N stimulated cambial activity, reduced wood density, and increased xylem cell dimensions regardless of the N-source. However, significant differences were observed in wood chemistry depending on the source of N, which can have a bearing on the type of N to be used in forest practices.

2 | MATERIALS AND METHODS

2.1 | Plant growth and fertilization strategy

Approximately 10-cm-tall *in vitro* hybrid aspen (*Populus tremula* x *P. tremuloides*) cuttings were transplanted in 3-L pots with a soil mixture of peat:perlite (10:1) that was mixed with 2 g L^{-1} lime prior to use to increase the pH. Trees were cultivated in greenhouse for 10 weeks with 18:6 h day/night cycles, with an average daily temperature of ~22°C and a relative humidity of 50–60%. In addition to the ambient light, trees were illuminated with Fionia FL300 Sunlight LED lamps (Senmatic A/S) with a maximal irradiance of 150–250 μmol m^{-2} s^{-1}. Trees were supported with stakes during their growth.

The fertilisation regime was designed to maintain a stable internal N-level in the trees by adjusting the fertilisation according to their growth. We followed the formula by (Ingestad 1979) $N_t - N_s = N_s\left(e^{R_N(t-s)} - 1\right)$ which determines the amount of N to be added each day on the basis of the estimated N content at the start (N_s) of the application and at each day (N_t). Rate of increase in plant N content (R_N) was calculated on the basis of the estimated total N content (~1 g) of hybrid aspen trees cultivated for 10 weeks in non-N-limiting conditions of pilot studies. Thus, ($N_t - N_s$) is the calculated amount of N to be added from day *s* to day *t* to allow plant N content to follow R_N. The fertilizer was based on "Rika-S" (SW HORTO AB). The pH was adjusted with either KOH or HCl to approximately 5.6. Nitrogen was supplied in four different forms. See Table S1 for complete nutrient composition and the amounts of added N.

Two separate experiments (Experiment I and II) were performed with a slightly different steady-state fertilization regime. In experiment I, N-levels were defined as limited, sub-optimal and optimal, corresponding to approximately 0.16, 0.56 and 1.06 g of N supplied in total to each tree by the end of the experiment. The pot substrate was "Solmull" (Hasselfors Garden), which contains naturally abundant nutrients and low level of N (approximately 20 mg N per litre). After three weeks of growth without fertilization, the trees were supplemented, with one or two days intervals, with nutrient solution until the end of the experiment. Fertilizer solution was added directly to the top of the soil and the potential runoff was captured by trays placed underneath each pot. The soil was kept humid by adding water to the trays to prevent potential nutrient runoff.

In Experiment II, the saplings were potted to the same pot substrate as in Experiment I, but this time supplemented to contain 40 mg N per liter (either in the form of arginine, NH_4^+, NH_4NO_3 or NO_3) to avoid initial plant stress due to low N level. This increased the total amount of N applied to each tree. Thus, in Experiment II, the N-levels were assigned as sub-optimal, optimal, and excessive, corresponding to a total N-addition of 0.56 g, 1.12 g and 1.68 g, respectively. The residual N content in the soil at the end of the experiment II was 0.0055 ± 0.0001 g in the suboptimal, 0.0194 ± 0.0098 g in the optimal, and 0.1155 ± 0.0295 g in the excessive N conditions, indicating that most of the applied N was taken up by the trees.

The results are presented in main figures for Experiment I and in supplemental figures for Experiment II. Experiment I took place in the summer and Experiment II in the late autumn, which is probably the reason for the differences in the overall growth rate of the trees in the two different experiments. RNA sequencing, saccharification and root biomass results pertain only to Experiment I, while all other analyses were performed for material collected from both experiments. Raw data for both experiments is shown in Table S1.

2.2 | Analyses of tree growth and physiological parameters

Stem height and diameter at the base of the tree were measured on a weekly basis. At the end of the experiment, after 10 weeks of growth, fresh weight of the shoot and roots was recorded. The dry weight of wood was estimated according to the formula $1/3\,x\,\pi\,x\,\mathit{stem\ height}\,x\,(\mathit{stem\ diameter}/2)^2\,x\,\mathit{wood\ density}$ (Escamez et al. 2017). Instantaneous gas-exchange measurement was made on a mid-stem leaf at the end of the experiment using a LI-6400XT (LI-COR Biosciences, Inc.). Parameter settings used for the LI-6400XT were: flow rate $250\,\mu\mathrm{mol\,s^{-1}}$, CO_2 reference $380\,\mu\mathrm{mol\,mol^{-1}}$, Tleaf 25°C and PAR $800\,\mu\mathrm{mol\,m^{-2}\,s^{-1}}$.

For carbon (C) and N analysis, three newly expanded leaves were collected at the end of the experiment. C-and N-analysis was done on dried, milled leaves with Elemental Analyzer Isotope Ratio Mass Spectrometry (EA-IRMS) at the SLU Stable Isotope Laboratory. The instrument setup consisted of an elemental analyser (Flash EA 2000, Thermo Fisher Scientific) connected to a continuous flow isotope ratio mass spectrometer (DeltaV, Thermo Fisher Scientific), which allowed detection of C- and N-content and the isotopic composition of these elements.

The $^{13}C/^{12}C$ isotopic composition in planta is usually lower than in the atmosphere since the kinetics of CO_2 incorporation discriminate against the heavier isotope. The discrimination is related to the rate of water loss (decreased duration/abundance of water retention in the leaves increasing the ^{13}C discrimination), and the $^{13}C/^{12}C$ ratio can therefore be used as a proxy for plant water-use efficiency (Farquhar et al. 1989).

2.3 | Wood density, chemistry and anatomy

Samples for measurement of wood density were taken 5–10 cm from the base of the stem and assessed using the water displacement method whereby a newly harvested and debarked wood sample was submerged under water in a flask placed on a balance. The weight of water (in grams at room temperature) displaced by the sample corresponds to the volume of the sample (in cm^3). Dry weight of the sample was obtained after drying in oven for two days at 50°C. Wood density was calculated as dry weight of wood divided by the volume of wood.

Stem pieces for wood chemistry assessment were collected 10–20 cm from the base of the stem. The stem pieces were debarked, and differentiating xylem layers were removed by scraping from the surface of the stem. The remaining mature part of the wood was freeze-dried and, after removal of the pith and the leaf traces, ball-milled into a fine powder. Extractives were removed from the wood powder according to Gandla et al. (2015). Extractive-free, ball-milled wood powder (50 μg ± 10 μg) was applied to a pyrolyzer equipped with an autosampler (PY-2020iD and AS-1020E, Frontier Lab) connected to a GC/MS (7890A/5975C; Agilent Technologies). The pyrolysis-GC/MS (Py-GC/MS) conditions were the same as in Gerber et al. (2012). The pyrolysate was separated and analysed. Py-GC/MS provides sensitive chemical fingerprints whereby the contents of carbohydrates, lignin subunits (S, G, H), total lignin, other phenolic compounds, and unidentified compounds in the secondary cell walls are derived as a signal% of the integrated GC peak area (Gerber et al. 2012). *p*-hydroxybenzoylated (pHB) lignin was identified on the basis of the fragmentation pattern of *p*-hydroxybenzoic acid (base peak m/z 121 (100), 138 (80), 93 (26), 65 (25), 39 (20), 43 (14), 55 (10), 63 (8), 122 (7), 53 (6)) (Faix et al. 1990). Even though the values obtained with the Py-GC/MS method are not absolute and influenced by the sensitivity of the instrument for the different compounds, they are proportional to weight%. In this study, all samples were run at the same time, allowing detection of even small differences between the relative levels of the cell wall components. The same starting material as for Py-GC/MS was used for quantification of cell wall monosaccharides from hemicelluloses and amorphous cellulose by acidic methanolysis followed by trimethylsilyl (TMS) derivatization according to Gandla et al. (2015).

Wood anatomy samples were taken 20–25 cm from the base of the stem. The tissues closest to the pith were excluded from the anatomy analysis. Sections were cut with Vibratome VT100 (Leica Microsystems) at a thickness of 30 μm. Radial sections were always taken above the node. Eight pictures per section were taken with Leica Dmi8 microscope with colour Leica DFC 7000 T colour camera and LasX software (Leica Microsystems). Sections were stained with 0.05% Toluidine blue O and imaged in bright field mode. Images were then processed in ImageJ2 version 2.14.0/154f by the "analyze particle" function counting numbers of vessels, fibers and their lumen area. The macros for these analyses can be found in Table S1.

2.4 | NMR spectroscopy

Lignin was extracted from pre-grinded wood powder (the same as for Py-GC/MS) according to Mottiar et al. (2023). In short, extractives were removed by washing with ethanol and acetone. Extractive-free wood powder was ball-milled and enzymatically treated with cellulase (Cellulase "Onozuka" RS, Yakult Pharmaceutical Industry Co., Ltd.) to remove carbohydrates. The residual enzyme-lignin was rinsed and freeze-dried. Enzyme-lignin powder (12–15 mg) was added to 5 mm NMR tubes followed by addition of 550 μL of a 4:1 dmso-d_6:pyridine-d_5 mixture and thorough mixing. 2D ^{1}H-^{13}C Heteronuclear Single Quantum Coherence NMR experiments (HSQC) were recorded on a Bruker 850 MHz Avance III HD spectrometer equipped with a cryo-TCI probe, using the hsqcetgpsisp2.2 pulse sequence. All experiments were performed at 298 K using a relaxation delay of 0.75 s and by recording 40 transients for each of the 196 data-points in the indirect dimension. Processing was performed using Gaussian window functions with GB 0.001 in both dimensions, and LB -0.1 Hz in F2 and $-$ 1 Hz in F1. Peaks in the aromatic region originating from syringyl (S), guaiacyl (G), *p*-hydroxyphenyl (H) and *p*-hydroxybenzoyl units (pHB) were integrated and used for calculating the relative amounts according to Mottiar et al. (2023). Processing was performed in Topspin 3.6 (Bruker Corp.).

2.5 | Saccharification

The remaining stem parts from the harvest (i.e., after samples were collected for anatomy, wood chemistry and RNA analysis) were peeled and oven-dried. After drying, the stems were homogenized with a centrifugal mill (Retsch), and the 100–500 μm particle size fraction was collected and used for saccharification with and without a pretreatment according to Gandla et al. (2021). Briefly, the dry wood fraction [50 mg; solids loading of 5% (w/w)] of each sample was subjected to analytical scale saccharification after moisture analysis (HG63 moisture analyser, Mettler-Toledo). Acid pretreatment (1% w/w sulfuric acid per gram of total reaction mixture) was performed using an Initiator single-mode microwave instrument (Biotage Sweden AB). The solid residue after acid pretreatment was separated from the hemicellulosic hydrolysate (Pretreatment liquid; PL) by centrifugation (20600 *g*, 15 min; Eppendorf) and subjected to enzymatic saccharification (solids loading of 5% (w/w)) after washing sequentially twice with deionized MilliQ water and twice with sodium citrate buffer (pH 5.2, 50 mM). The enzymatic saccharification was performed using liquid enzyme preparation of 5 mg of Cellic CTec-2 (Sigma-Aldrich) for 72 h at 45°C in an orbital shaking incubator with a shaking speed of 170 rpm (Ecotron incubator shaker). Samples were collected 2 and 72 h after incubation. For the samples collected after 2 h, the glucose production rate (GPR) was determined using an Accu-Chek® Aviva glucometer (Roche Diagnostics Scandinavia AB) after calibration with a set of glucose standard solutions. For the samples collected after 72 h, monosaccharide sugar yields (arabinose, galactose, glucose, xylose, and mannose) were determined using an Ion Chromatography System ICS-5000 high-performance anion-exchange chromatography system (HPAEC) with pulsed amperometric detection (Dionex; Tang et al., 2022).

2.6 | Statistical analyses

Statistical analysis was performed in R version 4.2.2 (2022-10-31) and RStudio version 2023.03.0 + 386. Growth response, wood anatomy and wood chemistry were all analysed using two-way ANOVA (aov function) with N-source and N-level as factors, followed by Tukey's 'Honest Significant Difference' method (TukeyHSD function). PCA analysis was performed to reduce the dimensionality of multivariate data (prcomp function). PCA results were then visualized using a PCA-biplot (fviz_pca_biplot function) where both individual trees and response variables are shown in relation to the dimensions. Correlation matrix was calculated using the 'rcorr' function and 'Hmisc' package. Pearson's correlation coefficients and p-values from the correlation matrix was used in 'corrplot' function and 'corrplot' package R-studio version 2023.06.0 + 421.

2.7 | RNA sequencing and data analysis

A stem piece (25–35 cm from the base) was peeled, and a sample collected from the exposed surface of the wood by scraping until the mature wood. Four biological replicates were included for each of the three concentrations of applied NO_3^- from Experiment I. The sample was homogenized to fine powder in liquid nitrogen using mortar and pestle. RNA extraction was done using Sigma Spectrum™ Plant Total RNA Kit followed by the on-column DNase treatment according to manufacturer's instructions. RNA was quantified with Nanodrop 1000, and the quality was assessed with an Agilent 2100 Bioanalyzer. The RIN-values ranged from 7.9 to 9.20.

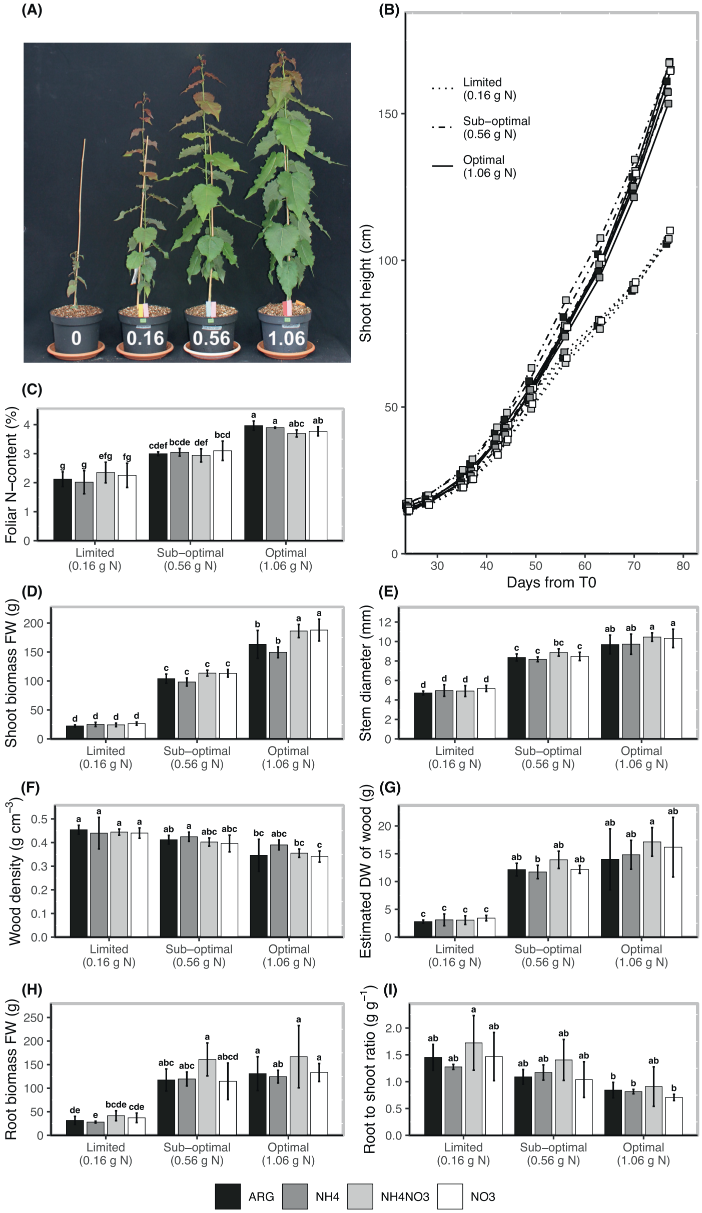
Following sequencing library generation and paired-end (2x 150 bp) sequencing using Illumina NovaSeq 6000, the raw reads were pre-processed to remove sequencing adapters using Trimmomatic (v0.39). The trimmed read pairs were quantified with Salmon (v1.9) using transcriptome index based on *P. tremula* (v2.0). After variance stabilizing transformations (VST) of the data, differential gene expression analysis was done using DESeq2 (v1.38.3) in R. Differentially expressed genes (DEGs) between the three NO_3^- concentrations were selected following the criteria of $\log_2$ fold change (lfc) >1 and a false discovery rate (FDR) <0.01 (Table S3). Heatmaps were generated in R with the heatmap.2 of gplots package (v3.1.3). The gene ontology (GO) enrichments and gene co-expression networks were obtained from the respective tools available at https://plantgenie.org/ using all genes expressed in the Aspwood database (plantgenie.org; (Sundell et al. 2017) as the background. The *P. tremula* genes were annotated according to the closest *A. thaliana* homologs from TAIR or from *P. trichocarpa*.

3 | RESULTS

3.1 | The effect of the different nitrogen sources on tree biomass accumulation

Nitrogen has been shown to influence plant growth and development in different ways depending on whether it is provided in the form of NO_3^-, NH_4^+, alone or in combination, or in organic form such as arginine (Cambui et al. 2011; Gruffman et al. 2014; Kasper et al. 2022; Lim et al. 2022). For detailed comparison of these different sources of N on wood formation of hybrid aspen trees, we

FIGURE 1 Tree growth in response to nitrogen application. (A) NO_3^--treated hybrid aspen trees grown for 2.5 months in the greenhouse. Trees were treated, from left to right, with 0 g, 0.16 g, 0.56 g, 1.06 g of N. Similar phenotypes were observed for all N-sources. **(B)** Shoot height growth. T0 indicates the start of the experiment. The points represent means. n = 6. **(C)** Foliar N-content in newly expanded leaves. n = 3. **(D-F)** Growth traits at the end of the experiment, including fresh weight of the shoot **(D)**, stem diameter **(E)**, wood density **(F)** and estimated dry weight of wood **(G)**. n = 6. Root biomass **(H)** and root to shoot ratio on a fresh-weight basis **(I)**. n = 3. Data was collected from Experiment I. Bars with error bars represent means ± SD. Statistical significance is tested by two-way ANOVA and Tukey post-hoc test. Means not sharing any letter are significantly different at the 5% level of significance.



devised a fertilization strategy that depended on the growth rate of the trees (Ingestad 1979; Kelly & Ericsson 2003) and allowed comparison of tree growth and physiology in conditions of varying N availability (Figures 1 and S1). The addition of approximately 1 g of N was expected to be optimal based on earlier measurements of hybrid aspen trees grown for 10 weeks in optimal growth conditions. The height growth of the trees (Figures 1B and S1B) corresponded to these expectations. Also, the foliar N-content increased steadily with increasing levels of N until it saturated at the optimal N level (Figures 1C and S1C).

Increasing availability of N increased total shoot biomass, stem height and stem diameter (Figures 1B-E and S1B-E) but decreased

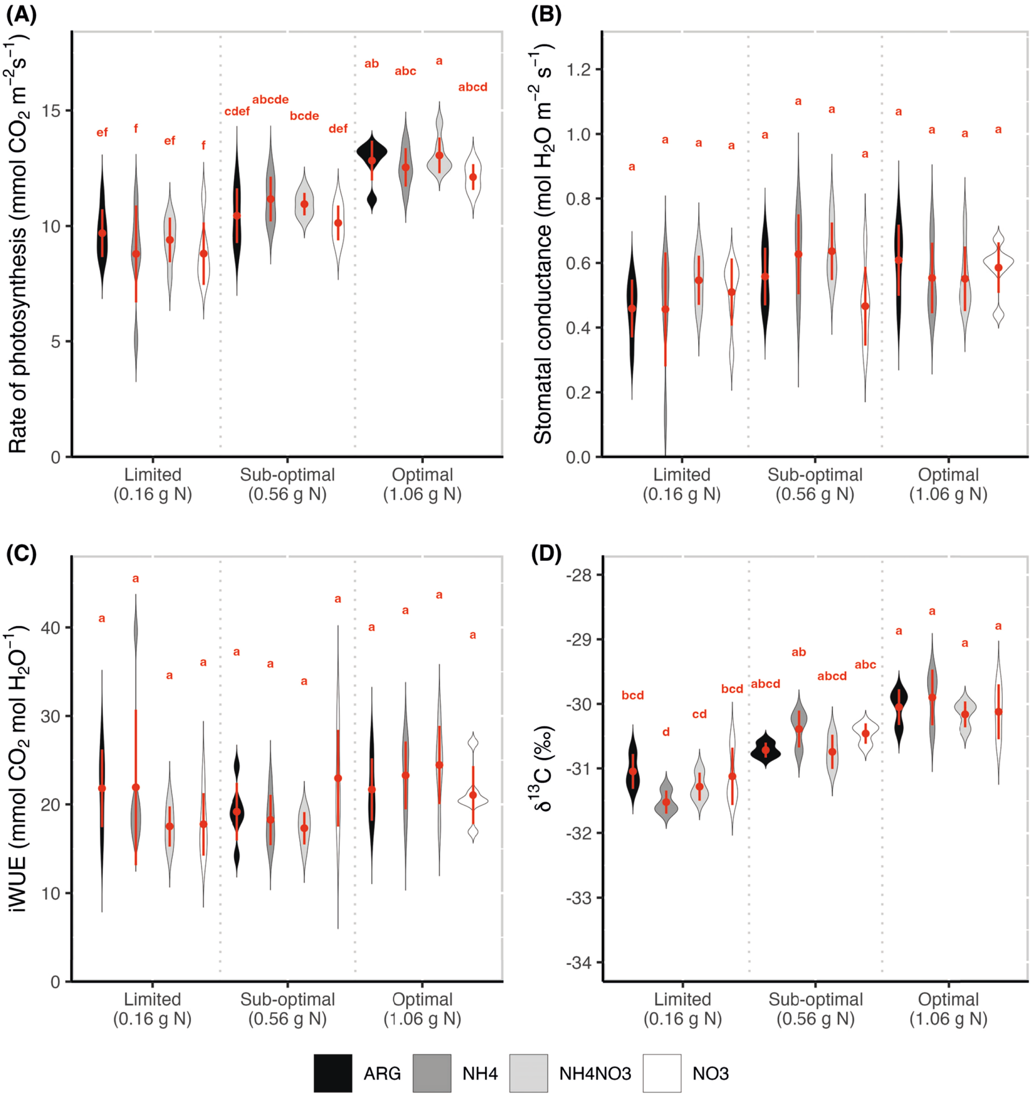


FIGURE 2 Gas exchange and ^{13}C discrimination (δ^{13}C) in response to nitrogen application. **(A-C)** Rate of photosynthesis **(A)**, stomatal conductance **(B)** and instantaneous water use efficiency (iWUE) **(C)**, analysed by gas exchange measurements with LICOR-6400XT from one mid-stem leaf. n = 6. **(D)** ^{13}C discrimination (δ^{13}C). δ^{13}C, providing an estimate of cumulative WUE, was measured by isotopic composition analysis with EA-IRMS of a newly formed leaf. n = 3. Data was collected from Experiment I. The violin plots show data distribution. Red dots and error bars indicate mean values ± SD. Statistical significance is tested by two-way ANOVA and Tukey's HSD test. Means not sharing any letter are significantly different at the 5% level of significance.

the density of wood (Figure 1F) after 10 weeks of growth. Even though the density decreased, the estimated dry weight of the stem did not decrease but tended to increase in response to increasing N-levels (Figures 1G and S1G). The weight of the roots increased slightly while the root-to-shoot biomass ratio tended to decrease in response to increasing N levels (Figure 1H, I). The positive effect on shoot biomass accumulation and radial growth was observed up to the optimal level of applied N. These trends were similar irrespective of the source of N, even though trees applied with arginine and NH_4^+ tended to be more similar to each other than trees

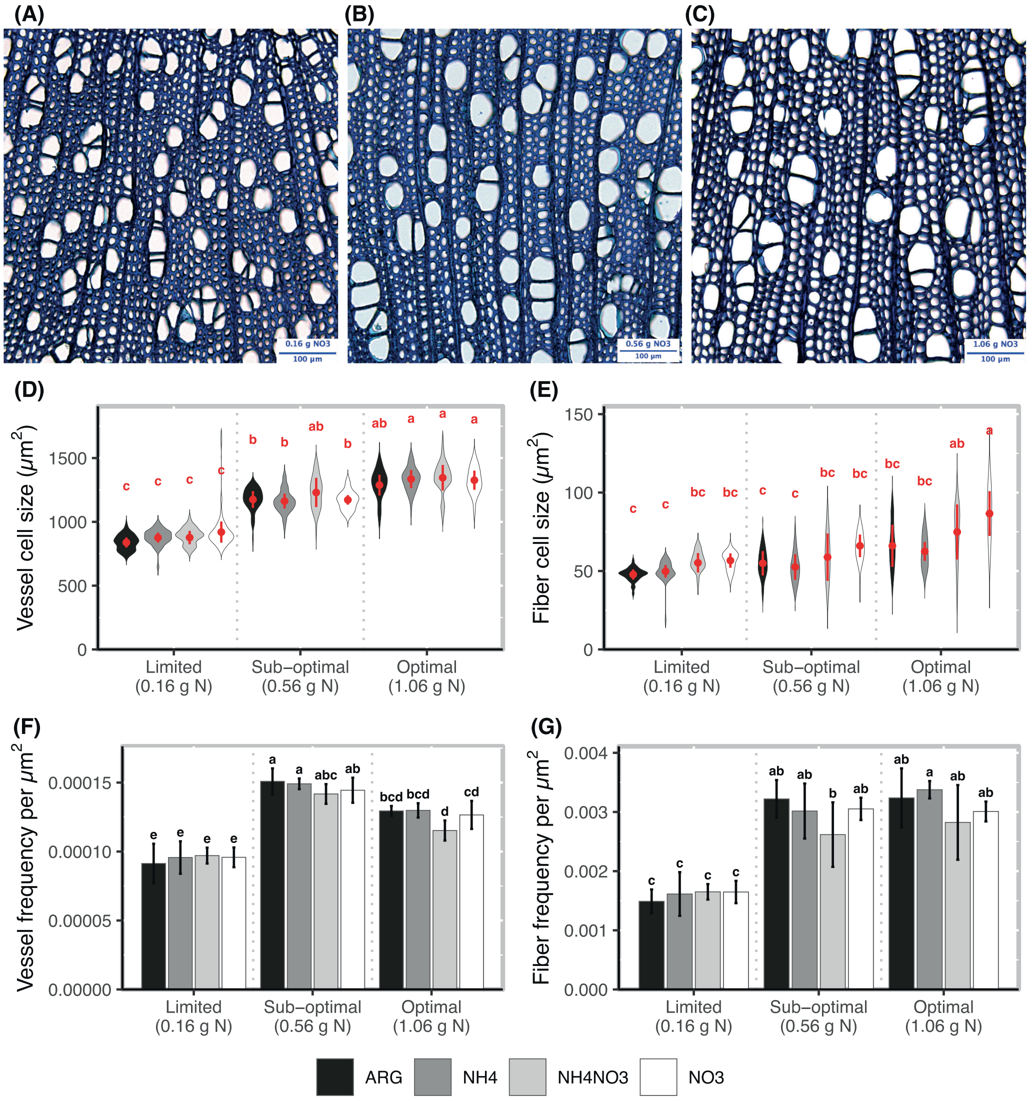


FIGURE 3 Wood anatomy in response to nitrogen application. (A-C) Cross sections of wood from NO_3^--treated trees in limited **(A)**, sub-optimal **(B)** and optimal **(C)** N-conditions. **(D-G)** Vessel cell size **(D),** fiber cell size **(E),** vessel frequency **(F),** and fiber frequency **(G).** Xylem cell sizes and frequencies were calculated per representative area in radial sections of wood. n = 6. Data is collected from Experiment I. The violin plots show data distribution. Red dots, bars and error bars display mean values ± SD. Statistical significance was tested by two-way ANOVA and Tukey's HSD test. Means not sharing any letter are significantly different at the 5% level of significance.

applied with NH_4^+ or NO_3^- in terms of growth within the same N-level (Figures 1D, E and S1D, E). The biggest difference between the different N-sources was the higher potency of NO_3^- and NH_4NO_3 than of NH_4^+ or arginine to stimulate growth and in particular the total shoot biomass accumulation of the trees in the conditions of the optimal N-level (Figure 1D).

3.2 | The effect of nitrogen on xylem cell morphology and stem hydraulic conductivity

Instantaneous gas exchange measurements showed a tendency towards increased photosynthetic rate with increased N-level but no difference between the different N-sources within each N-level

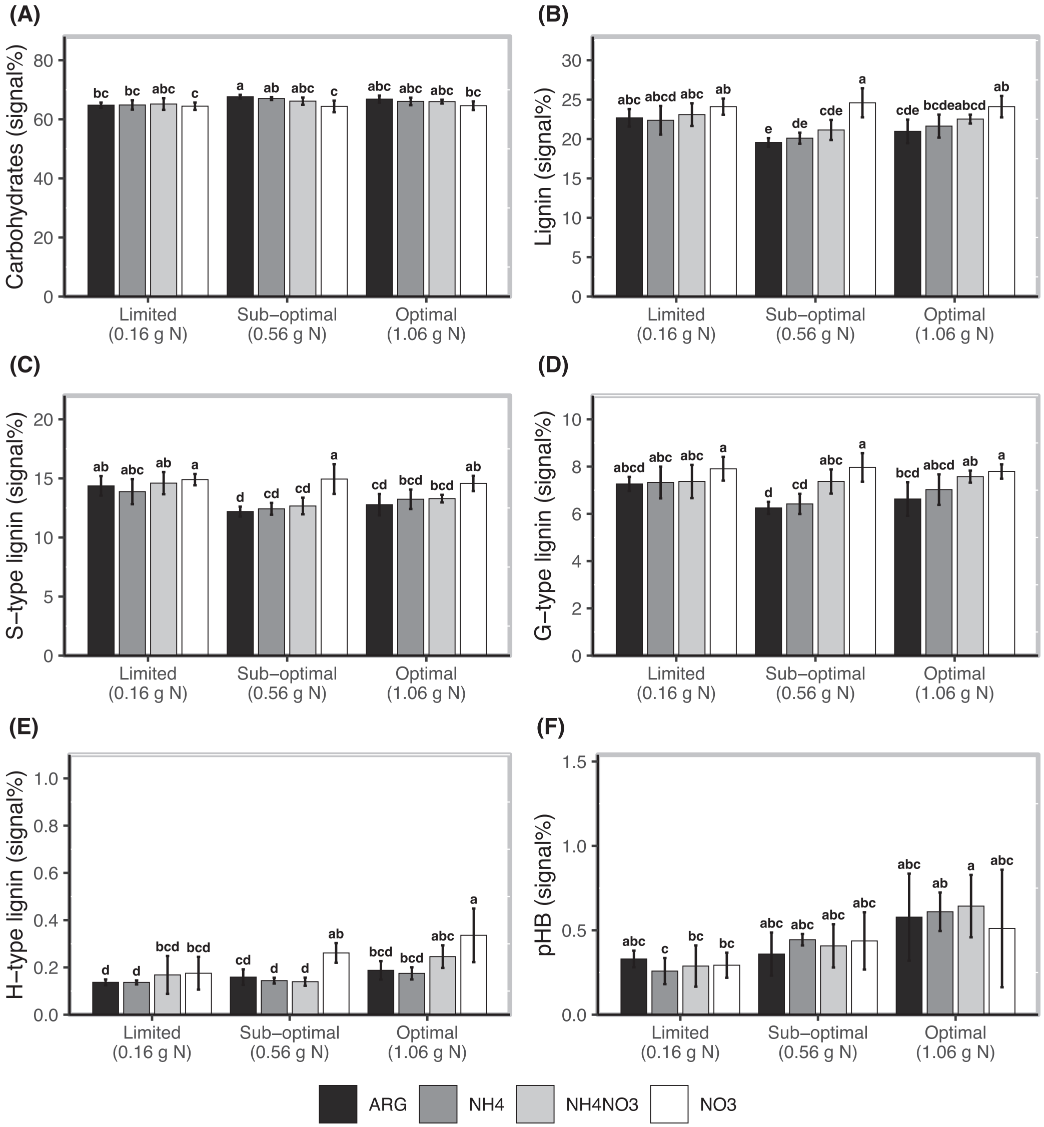


FIGURE 4 Wood chemical composition in response to nitrogen application. (**A-F**) The analysis of the chemical components of wood by pyrolysis-GC/MS analysis. Relative contents are shown for carbohydrates (**A**), total lignin (**B**) S-type lignin (**C**), G-type lignin (**D**), H-type lignin (**E**) and pHB-lignin units (**F**). n = 6. Bars with error bars indicate means ± SD. Statistical significance was tested by two-way ANOVA and Tukey's HSD test. Means not sharing any letter are significantly different at the 5% level of significance.

(Figures 2A and S2A). Instantaneous water use efficiency (iWUE) and stomatal conductance were not influenced by the source or the availability of N (Figures 2B, C and S2B, C).

Carbon discriminant analysis was used to analyse the cumulative, rather than instantaneous, effect of N. A decrease in ^{13}C discrimination with increasing N-availability indicated elevated photosynthetic efficiency even though the effect levelled off with the highest N-levels (Figures 2D and S2D). As carbon discrimination can also be used as a proxy for plant water use efficiency (Farquhar et al. 1989), the observed trends of ^{13}C discrimination were indicative of increased water use efficiency in response to increased N availability.

In line with the increase in water use efficiency, the analysis of wood anatomy (Figure 3A-C) revealed that the radial dimensions of the xylem vessel elements increased with increasing N-levels, independent of the N-source (Figure 3D). Also, xylem fibers showed a tendency towards increased cell size in response to increasing N-levels (Figure 3E). The frequencies of both vessels and fibers increased from limited to sub-optimal N-level (Figure 3F, G). Neither cell frequencies nor cell sizes changed further with increasing N-availability (Figure S3).

3.3 | The effect of nitrogen on wood chemistry

The effect of the different N-sources was studied on wood chemistry by Py-GC/MS (Table S2), which provides an estimate of the relative contents of the cell wall chemical components (Gerber et al. 2012). The relative content of carbohydrates did not change in any of the N conditions (Figures 4A and S4A). The relative content of total lignin as well as S- and G-type lignin decreased slightly from limited to suboptimal N-level for all N-sources except for NO_3^- (Figures 4B-D and S4B-D). In NO_3^--treated trees, however, lignin content did not decrease in response to increasing NO_3^--level (Figures 4B and S4B). Most strikingly, increasing levels of NO_3^- and, to a lesser extent, NH_4NO_3 increased the relative content of H-lignin (Figures 4E and S4E). *p*-hydroxybenzoylated lignin levels tended to increase with increasing N-levels for all N-sources (Figures 4F and S4F). NMR analysis revealed similar results for the levels and composition of lignin in a subset of NO_3^--treated trees (Table S1).

Saccharification efficiency was analysed in conditions of limited, sub-optimal and optimal N availability to analyse whether changes in lignin content influence feedstock processability in our conditions. There were no statistically significant differences in saccharification yields of any of the sugars (glucose, xylose, mannose, arabinose, galactose) after mild acidic pretreatment. Without the pretreatment, the release of xylose increased slightly with increasing N-level but statistically significantly only for the arginine treatment (Table S1). The potential influence of wood hemicelluloses on the saccharification efficiency was excluded on the basis of wood monosaccharide composition that did not differ between either N-sources or N-levels (Table S1).

3.4 | The effect of nitrate application on global gene expression

Since NO_3^- has been shown to have a signaling function *in planta*, this treatment was selected for transcriptional analysis. Transcriptome profiles from RNA-sequencing data were analysed to assess the gene expression changes in developing xylem under limited, sub-optimal and optimal NO_3^- availabilities. The PCA plot, based on the normalized gene counts, showed a clear separation of samples treated with limited NO_3^- from sub-optimal and optimal ones that grouped together (Figure 5A). The GO analysis presented significant enrichment of 'oxidation–reduction process' among the upregulated and the 'transcription factor complex' among the downregulated differentially expressed genes (DEGs) in optimal NO_3^- conditions (Figure 5B). The differential gene expression analysis showed a total of 832 DEGs (FDR <0.01) between three pairwise comparisons (a higher NO_3^- vs a lower NO_3^--level) (Figure 5C; Table S3). The spatial expression pattern of the DEGs that were common for the pairwise comparisons varied across the secondary vascular tissues in the AspWood database, suggesting that many processes of wood formation were affected by changes in N availability (Figure 5C). For instance, *MYB-like* helix-turn-helix transcription factor (Potra2n16c29671) was upregulated in all three pairwise comparisons and could be involved in NO_3^--mediated lignification based on its AspWood expression in the transition zone between xylem cell wall formation and cell death (Figure 5C). Three genes homologous to the *A. thaliana YELLOW STRIPE-LIKE 3* (*YSL3*) were likewise upregulated in all pairwise comparisons. *A. thaliana YSL3* is a plasma membrane-localised metal transporter (Chu et al. 2010) that was shown to be NO_3^--induced in an earlier study (Poovaiah et al. 2019). Further support for the connection between YSL3 and NO_3^- was provided by co-expression of the *YSL3* homologs with oligopeptide transporters (*OPT*) and a nitrate transporter family member (*NPF2.11*) (Table S3).

Significant changes were observed in the expression of key NO_3^- sensing, signaling, assimilation, and transporter genes, including homologs of the *A. thaliana NIN LIKE PROTEIN 2* (*NLP2*), *NITRILASE 4* (*NIT4*), *NITRITE REDUCTASE 1* (*NIR1*), *NITRATE REDUCTASE 2* (*NR2*) and nitrate transporters *NPF7.1* and *PTR3* (Figure 5D). Although *NIN LIKE PROTEIN 7* (*NLP7*), recently identified as a NO_3^- sensor (Liu et al. 2022), did not show any significant change, 20 potential targets of NLP7 were found among the DEGs, including *NIR*, *LIK1*, *MTP10*, *OSCA1.5*, *MLKL1* and *MTP10* (Table S3).

The possible role of cytokinins in NO_3^- signaling and cambial activity was supported by increased expression of the cytokinin-biosynthetic isopentenyltransferases (*IPT5a*, *5b*, and *6b*) in response to increasing NO_3^- level (Figure 5E).

The effect of NO_3^- on cell expansion has been proposed to be mediated by induction of the expression of aquaporin family genes (Hacke et al. 2010), and seven aquaporin genes displayed significant expression changes across the three NO_3^- concentrations in our RNAseq data (Figure 5F).

Among the lignin-biosynthetic machinery, significant changes were evident for the laccases *LAC2*, *4*, *6*, *11* and *17*, but not for any of the lignin-biosynthetic genes (Figure 5E). However, most of the lignin-biosynthetic genes, including *HCT1* and *C3H3* that act in the branch-point towards G and S-type lignin biosynthesis, showed trends of lower expression in the optimal NO_3^- level compared to the suboptimal and limited levels, while *CSE2* and *CCoAOMT1* and *2* as well as the *pHBMT* acyltransferases, involved in lignin *p*-hydroxybenzoylation, showed the opposite trends (Figure 6).

4 | DISCUSSION

4.1 | Nitrogen in the form of nitrate surpasses other N-sources in stimulating tree biomass accumulation

The type of N-source available to the plants influences N assimilation and plant growth. The organic forms have energetic assimilation

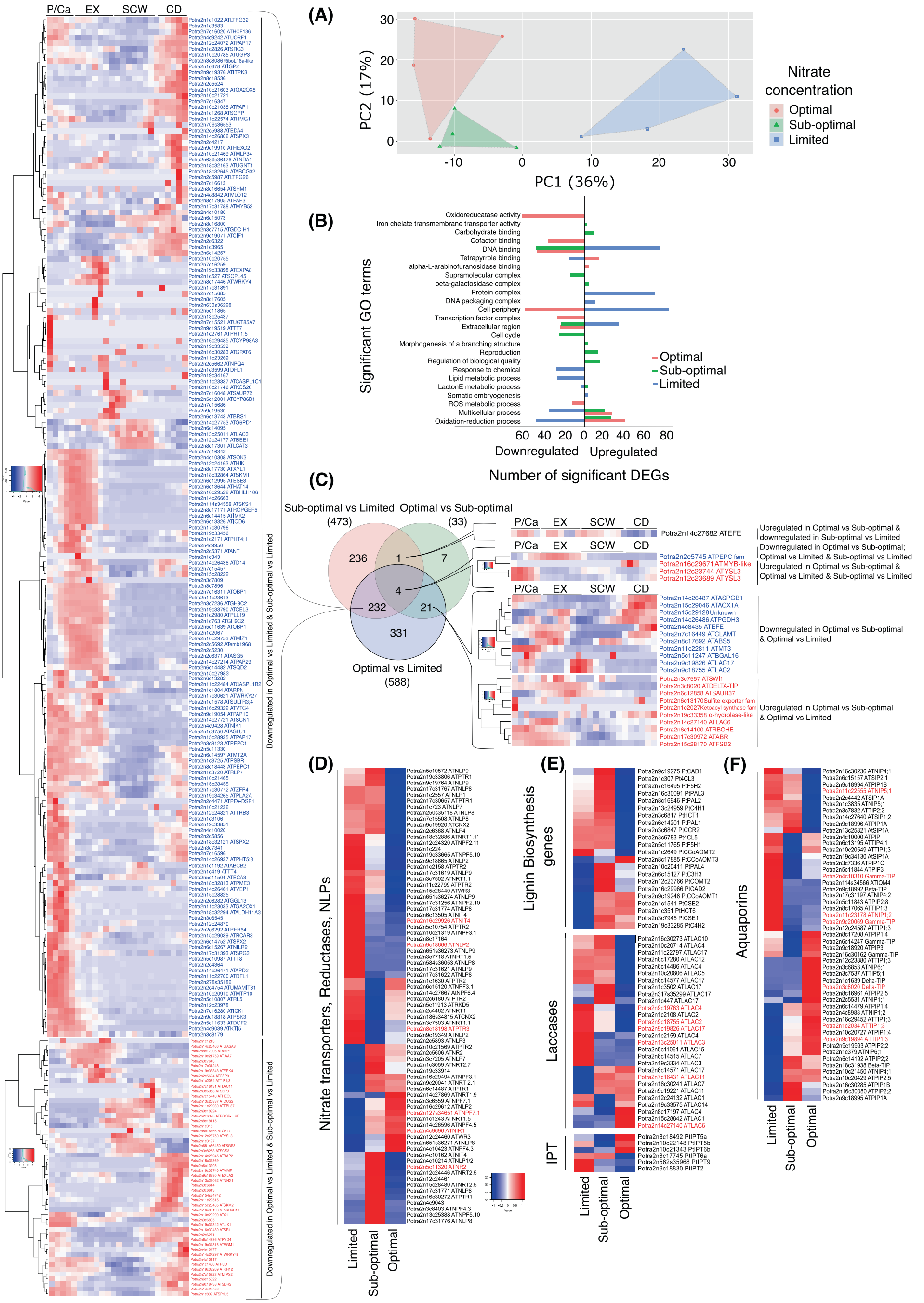


FIGURE 5 Legend on next page.

advantages over inorganic N (Franklin et al. 2017). Nitrogen is also, at least in boreal forest soils, mostly available in organic form (Inselsbacher & Näsholm 2012). On the other hand, plant species seem to differ in their preference for the N form they take up (Scott & Rothstein 2011). In the current work, the N-source had an influence on tree growth and wood properties, even though it was less pronounced than the effect of the N-level (Figure S5A-B). The organic N, applied in the form of arginine, stimulated growth and biomass accumulation of the hybrid aspen trees but less than the inorganic N forms of NH_4NO_3 and NO_3^- (Figures 1 and S1). The effect of arginine was similar to the effect of NH_4^+, another positively charged N-form (Figure 1C-D). Similar results were earlier obtained in *Populus x canescens*, where shoot growth was stimulated more by the inorganic NH_4NO_3 than the organic N-source of phenylalanine (Jiao et al. 2018).

A well-documented effect of organic N is the influence on plant biomass partitioning and stimulation of biomass accumulation primarily in the roots, which leads to an increased root-to-shoot ratio compared to inorganic N sources (Cambui et al. 2011; Franklin et al. 2017). This may be due to the direct incorporation of organic N into the roots (Cambui et al. 2011) or differences in the cost of assimilation in different plant parts (Franklin et al. 2017). Therefore, the question arises whether, in our experiments, the lower biomass stimulation of shoots by arginine, compared to NO_3^- and NH_4NO_3, was due to preferential accumulation of root biomass in response to arginine. Arginine did, however, not increase the root growth of hybrid aspen trees compared to the inorganic N-sources (Figure 1H). Organic N has been shown to primarily stimulate the rooting of young seedlings (Lim et al. 2022), and it is possible that the inability of arginine to stimulate root growth in our experiments was due to the fact that we used *in vitro*-propagated saplings that had pre-formed roots before exposure to the different N conditions in the soil. It is also possible that arginine and NH_4^+ are less accessible to the roots due to their positive charge and, hence, attraction to the negatively charged surfaces of organic soil particles, as proposed earlier (Inselsbacher et al. 2011; Lim et al. 2022). In any case, in our experimental conditions, it is the NO_3^- complemented, inorganic forms that seem to stimulate biomass accumulation more than the organic N-source in hybrid aspen trees.

Increased biomass accumulation is commonly associated with decreased wood density (Lindström 1996; Luo et al. 2005; Hacke et al. 2010). In our experimental setup, all measured biomass-related traits showed increases in response to increasing N-level, independent of the source of N, up to the application level of 1 g per tree (Figures 1 and S1). In the same range of increasing N levels, wood density decreased in the tree stem (Figure 1F), confirming the frequently observed negative relationship between biomass accumulation and wood density also in our experimental conditions. Consequently, wood density correlated negatively with N-level as well as leaf N-content (Figure S6). Interestingly, even though wood density decreased with increasing N-level, the overall biomass accumulation on a dry weight basis continued to increase (Figures 1G and S1G), supporting the potential of increased nitrogen availability in stimulating biomass accumulation without a drastic negative impact on wood density in hybrid aspen trees.

4.2 | Water transport and use efficiency are influenced by nitrogen level

Stems' hydraulic conductivity can be estimated theoretically according to the Poiseuille equation according to which the volumetric flow of water is proportional to the 4th power of the radius of the water-conducting tubes (Tyree & Ewers 1991). This means that the diameter of the vessel elements has a significant influence on water transport and, hence, nutrient uptake of tree stems. Interestingly, N is known to stimulate vessel expansion (Hacke et al. 2010; Plavcová et al. 2013) and, hence, increase its own uptake and transport potential along with the increase in mass flow (McMurtrie & Näsholm 2018). Increased N-level increased the diameter of the vessels also in our experiments (Figures 3 and S3) in a manner that did not depend on N-source. Increased vessel diameter correlated also with increased water-use efficiency according to the ^{13}C-discriminant analysis (Figures 2D and S6). Therefore, we can conclude that N had a positive influence on the water transport capacity of our trees. On another note, the positive influence of N on water transport capacity and xylem expansion entails risk for a trade-off with xylem safety in conditions of limited water availability (Pratt & Jacobsen 2017). Increased vessel diameter makes N-fertilised trees presumably more susceptible to cavitation, and it will therefore be necessary to explore, in future experiments, the effects of N on xylem cavitation as well as drought resistance of trees.

4.3 | Nitrate has a unique influence on lignin accumulation and composition

Lignification is a developmentally regulated process that is also influenced by biotic and abiotic stresses as well as limitations in nutrients,

FIGURE 5 Differential gene expression in response to three NO_3^- levels. **(A)** Principal component analysis of RNA-sequencing data from differentiating xylem tissues of hybrid aspen trees exposed to optimal, sub-optimal and limited NO_3^--levels. n = 4. **(B)** Significantly represented gene ontology (GO) terms for differentially regulated genes (DEGs). DEGs were defined on the basis of up- or downregulation in at least one of the three pairwise comparisons (optimal vs sub-optimal, optimal vs limited, sub-optimal vs limited; see Table S3 for additional information). **(C)** Venn diagram of up- and down-regulated DEGs (fitting the criteria of lfc >1 and FDR <0.01) in pairwise comparisons between the three NO_3^- levels. Heatmaps for DEGs that overlap between pairwise comparisons display their expression profile during cambial growth according to the AspWood database (Sundell et al. 2017; plantgenie.org). P/Ca, phloem/cambium; Ex, expanding xylem; SCW, secondary cell wall formation; CD, cell death. **(D-F)** Heatmaps for expression profiles for selected gene families, including Nitrate transporters (NRT/NPF), Nitrite reductase (NIR) Nitrate reductase (NR), and NIN-Like Proteins (NLPs) **(D)**, lignin biosynthetic genes, laccases (LAC), and isopentenyl transferase (IPT) **(E)** and aquaporins **(F)**. The DEGs are shown in red. The gene annotations in (C-F) are based on the closest *A. thaliana* gene from TAIR, except for the lignin biosynthetic and the *IPT* genes that are annotated according to the closest homolog in *Populus trichocarpa* (Shi et al. 2010; Immanen et al. 2016). Data was collected from Experiment I.

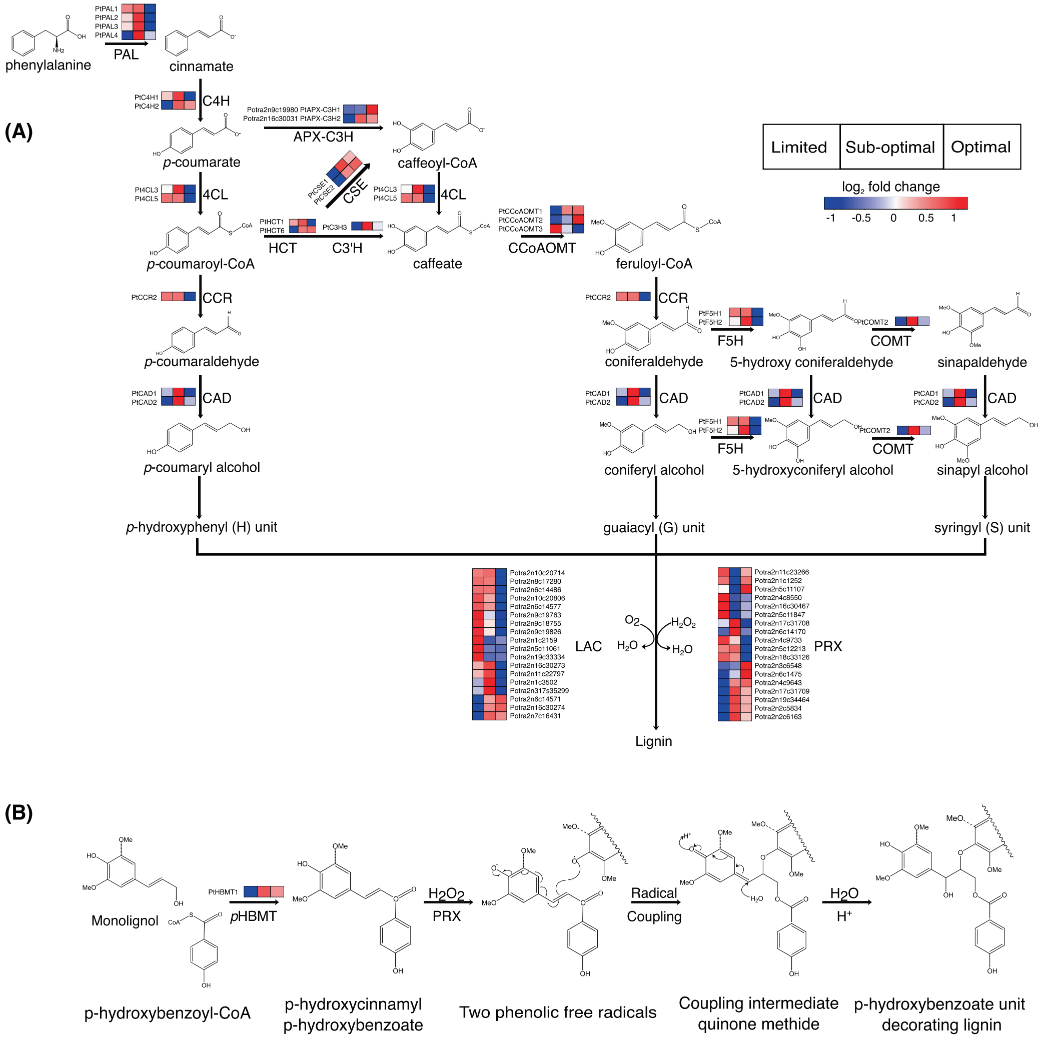


FIGURE 6 The expression of genes involved in lignin biosynthesis and polymerization in response to three NO_3^- levels. **(A)** Lignin biosynthesis and polymerization. The monomer biosynthesis represents the dominating pathway in *Populus* according to Wang et al. (2019). The gene annotations are based on the closest homologs of the *Populus trichocarpa* (Pt) lignin-biosynthetic genes from Shi et al. (2010) and the *Populus tremula* (Potra) laccases and peroxidases from Sundell et al. (2017). Only highly expressed laccases and peroxidases (VST >3) in the secondary xylem tissues, according to the Aspwood database (plantgenie.org), are shown. **(B)** Incorporation of monolignol–pHB conjugates into lignin polymer. The gene annotations were based on the closest *Populus trichocarpa* (Pt) *pHBMT* genes from de Vries et al. (2022). PAL, L-phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; C3H, 4-coumarate 3-hydroxylase; COMT, caffeic acid/5- hydroxyconiferaldehyde *O*-methyltransferase; F5H, ferulate-5-hydroxylase/coniferaldehyde 5-hydroxylase; 4CL, 4-coumarate:CoA ligase; HCT, *p*-hydroxycinnamoyl-CoA:quinate shikimate *p*-hydroxycinnamoyltransferase; C3H, 4-coumarate 3-hydroxylase; CSE, caffeoyl shikimate esterase; CCoAOMT, caffeoyl-CoA 3-*O*-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; pHBMT, p-hydroxybenzoyl-CoA monolignol transferase; LAC, laccase; PRX, peroxidase.

such as iron, phosphorus and N (Liu et al. 2018; Cesarino 2019; Chantreau & Tuominen 2022). Increased availability of N inhibits, in general, the lignin-biosynthetic pathway, whereas limiting N stimulates lignification (Fritz et al. 2006). In woody tissues of *Populus* trees, increased N supply resulted in wood properties having a juvenile character with decreased lignin and S/G content (Pitre et al. 2007b). We detected, similarly, that the lignin content decreased at optimal and sub-optimal levels of N compared to limited N but did not further decrease with excessive N-levels (Figures 4 and S4). This holds true for all N-sources tested except for NO_3^-, which did not significantly influence lignin content in any of the applied N-levels (Figures 4B and S4B). Even though increased NO_3^- availability did not influence the total lignin content, there was a clear stimulatory effect on the accumulation of the H-type lignin (Figures 4E and S4E). Also, the other NO_3^- containing N-source, NH_4NO_3, increased H-type lignin accumulation in the higher N-levels. An increase in the relative content of H-lignin was also observed by Pitre et al. (2007b) after application of high (10 mM) NH_4NO_3 compared to adequate (1 mM) NH_4NO_3 in hybrid poplar. The fact that H-type lignin is mainly deposited in the middle lamella and primary cell walls (Fukushima & Terashima 1990) suggests that a larger proportion of the xylem tissues were in the stage of early differentiation and/or having thinner secondary cell walls, which in turn could be due to changes in cambial activity or the spatio-temporal regulation of xylem differentiation. Increased H-lignin could also be related to H-type lignin functioning as a "stress lignin" (Lange et al. 1995; Cesarino 2019) and hence excessive NO_3^- being experienced as some kind of stress (Plavcová et al. 2013) even though it should be noted that stress-related symptoms were not observed in terms of growth penalty of the trees and that the effect was not only related to excessive NO_3^- but discernible already at optimal level.

Lignin biosynthesis is known to be transcriptionally regulated in response to N-availability both at the level of lignin monomer biosynthesis and lignin polymerization (Cooke et al. 2003; Poovaiah et al. 2019; Zhao et al. 2022). Our RNAseq analysis revealed that the lignin-biosynthetic genes did not significantly (FDR <0.01) respond to added NO_3^-. Suppression of lignin-polymerising laccases, in particular the homologs of the *A. thaliana LAC17* and *LAC2*, have been linked to reduced lignification in high-N-treated plants (Plavcová et al. 2013). We also detected suppression of several homologs of the *A. thaliana* secondary cell wall-related *LAC4* and *17* in the optimal and sub-optimal NO_3^- compared to the limited NO_3^--level (Figures 5E and 6; Table S3). Interestingly, homologs of *LAC6* and *11* were upregulated in the optimal NO_3^- level, and the question is whether the expression of these laccases could be related to increased deposition of H-type lignin in response to increased availability of NO_3^-. Furthermore, a *MYB-like* transcription factor was upregulated by increasing NO_3^- availability (Figure 5C). As MYBs are widely recognized as central regulators of lignin biosynthesis, it is possible that this previously uncharacterized transcription factor is related to the specific changes observed in response to increased NO_3^- availability.

5 | CONCLUSIONS

Increased availability of N affected growth and wood formation of hybrid aspen trees in many ways. We observed increased diameter of xylem elements and decreased wood density in response to increasing availability of N. We also identified N-level that was optimal for biomass accumulation of the trees in our experimental conditions. Biomass accumulation was not significantly influenced by N-source even though the NO_3^--containing N-sources were somewhat more efficient. However, lignin composition was influenced by the N-source, as increased levels of NO_3^- had a stimulatory effect on the accumulation of H-type lignin. This is an interesting observation from an applied point of view as lignin composition is known to influence both wood pulping and bioprocessing (Ragauskas et al. 2014). H-lignin, in particular, can confer a high abundance of condensed-type linkages and decrease the degree of lignin polymerization (Ziebell et al. 2010; Sangha et al. 2014). Targeted fertilization with NO_3^--containing compounds could be used in cases when wood with these lignin properties is desired. On the other hand, avoiding NO_3^--containing fertilization of forest feedstocks could be advantageous for downstream applications where relatively low H-lignin content is preferred.

AUTHOR CONTRIBUTIONS

AR, SJ and HT conceived the project. AR performed all growth experiments, collected and prepared the plant material for all analyses. MH performed the NMR analysis. MLG performed saccharification experiments under supervision of LJJ. SC analysed the RNAseq data. AR wrote the first draft of the manuscript, and all authors contributed to the writing.

ACKNOWLEDGEMENTS

We acknowledge Junko Takahashi Schmid at the Biopolymer Analytical Platform (BAP) at UPSC/SLU (supported by Bio4Energy and TC4F), Marta Derba-Maceluch for technical assistance and analyses at the UPSC Microscopy Facility, the Stable Isotope Lab at SLU Umeå, and the UPSC in vitro and growth facility. We thank Àngela Carrió Seguí, Marta Pérez Alonso and Pal Miskolczi for help in sample preparations.

FUNDING INFORMATION

The work was funded by the Swedish Research Foundation Formas (grant 2021–00992), Bio4Energy (www.bio4Energy.se, grant B4E3-FM-2-06), and Trees for the Future post doc program (diarie 2022.3.2.5–426).

DATA AVAILABILITY STATEMENT

All raw data is included in Supplemental Table S1. The RNAseq data is available at NCBI with the project ID PRJNA975342. (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA975342).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Renström, A., Choudhary, S., Gandla, M.L., Jönsson, L.J., Hedenström, M., Jämtgård, S. et al. (2024) The effect of nitrogen source and levels on hybrid aspen tree physiology and wood formation. *Physiologia Plantarum*, 176(1), e14219. Available from: https://doi.org/10.1111/ppl.14219

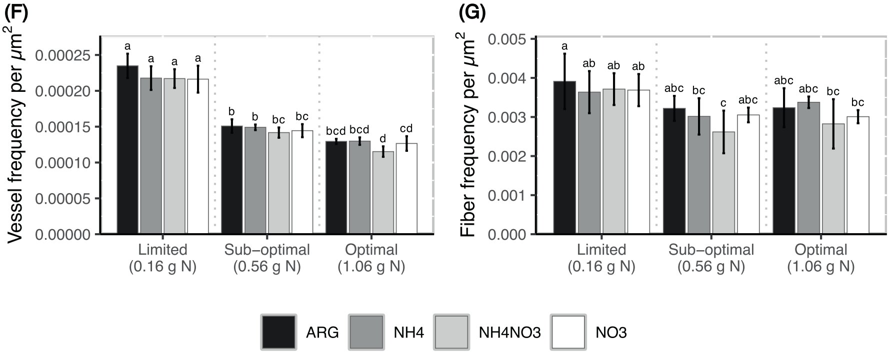
DOI: 10.1111/ppl.14655

CORRIGENDUM

Correction to “The effect of nitrogen source and levels on hybrid aspen tree physiology and wood formation”

Renström, A., Choudhary, S., Gandla, M.L., Jönsson, L.J., Hedenström, M., Jämtgård, S., et al. (2024) The effect of nitrogen source and levels on hybrid aspen tree physiology and wood formation. *Physiologia Plantarum*, 176(1), e14219. https://doi.org/10.1111/ppl.14219

In the “Results” section, Figures 3F and 3G, an incorrect area was used to calculate the vessel and fiber frequencies in the wood of trees grown at a limited N-level. In the new versions of Figures 3F and 3G, this is corrected. From this follows that the phrase “The frequencies of both vessels and fibers increased from limited to sub-optimal N-level (Figure 3F, G).” in the “Results” section is incorrect and should read: “The frequencies of vessels decreased from limited to sub-optimal N-level while the effect on fibers was less pronounced (Figure 3F, G).”



The correction does not change the conclusions of this paper. We apologize for this error and any inconvenience this might have caused the reader.

Supporting information to “The effect of nitrogen source and levels on hybrid aspen tree physiology and wood formation”
Volume 176 Issue 6 Physiologia Plantarum First Published online: December 9, 2024

Figure S1. Tree growth in response to different nitrogen additions in Experiment II.

Figure S2. Gas exchange and 13C discrimination (delta13C) in response to different nitrogen additions in Experiment II.

Figure S3. Wood anatomy in response to different nitrogen additions in Experiment II.

Figure S4. Wood chemical composition in response to different nitrogen additions in Experiment II.

Figure S5. PCA plots with all measured parameters in response to N-addition, N-source, and Experiment.

Figure S6. Correlation plot with all measured parameters and data from Experiment I and II.

Table S1. Supplementary data including N-application strategy and a compilation of all raw data used in figures with corresponding statistical output.

Table S2. Supplementary raw data from Pyrolysis-GC/MS analysis, including non-normalized values.

Table S3. Supplementary data for differentially expressed genes (DEGs) from RNA-sequencing of NO3− treated hybrid aspen for three selected doses (limited 0.16 g, sub-optimal 0.56 g and optimal 1.06 g).

Only supplementary figures is included in the thesis, tables can be found online.

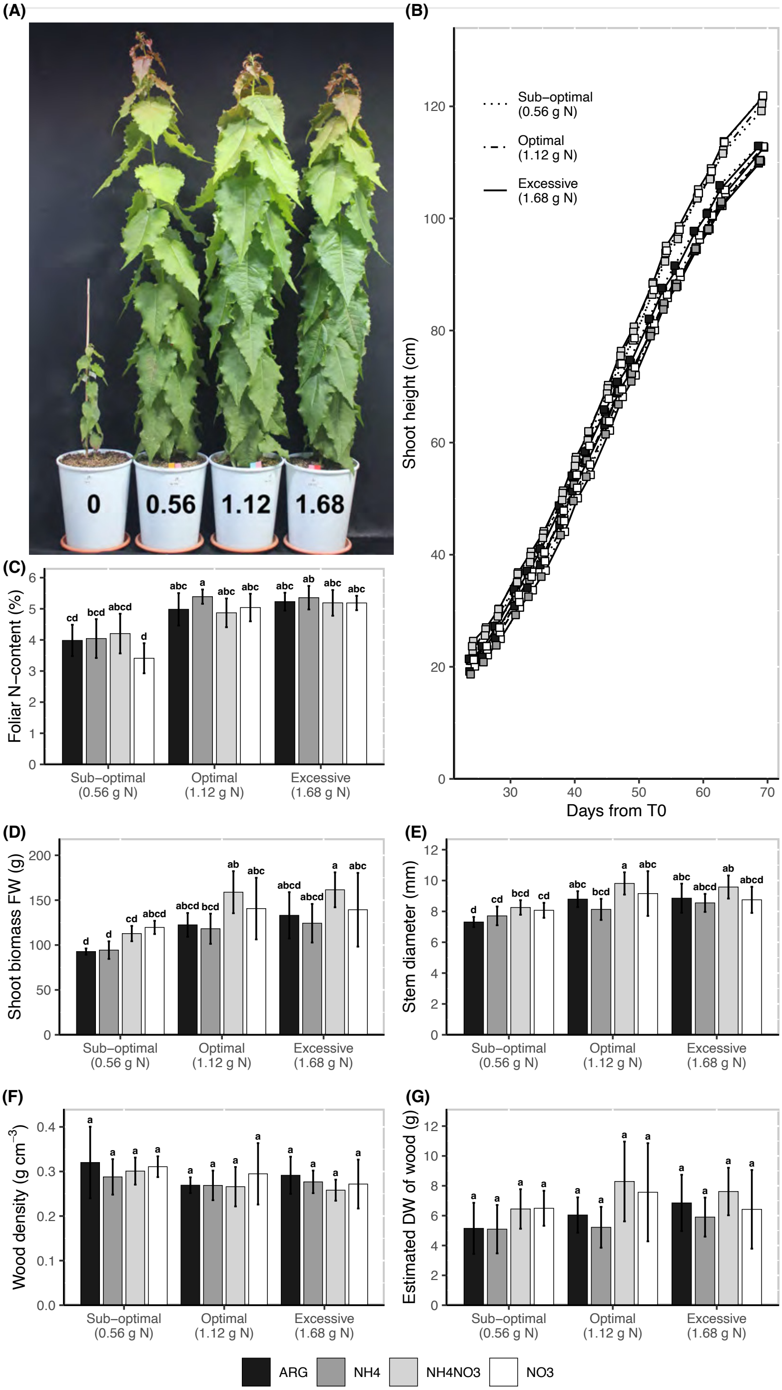


Figure S2. Tree growth in response to different nitrogen additions in Experiment II. (A) NO_3^- -treated hybrid aspen trees grown for 10 weeks in greenhouse conditions. Trees were treated, from left to right, with 0 g, 0.56 g, 1.12 g and 1.68 g N. Similar phenotypes were observed for all N-sources. **B)** Shoot height growth. T0 indicates the start of the experiment. The points represent means. n=6. **(C)** Foliar N-content in newly expanded leaves. n=3. **(D-F)** Growth traits at the end of the experiment, including fresh weight of the shoot **(D)**, stem diameter **(E),** wood density **(F)** and estimated dry weight of wood **(G)**. n= 6. Bars with error bars represent means ± SD. Statistical significance was tested by Two-way ANOVA and Tukey post-hoc test. Means not sharing any letter are significantly different at the 5% level of significance.

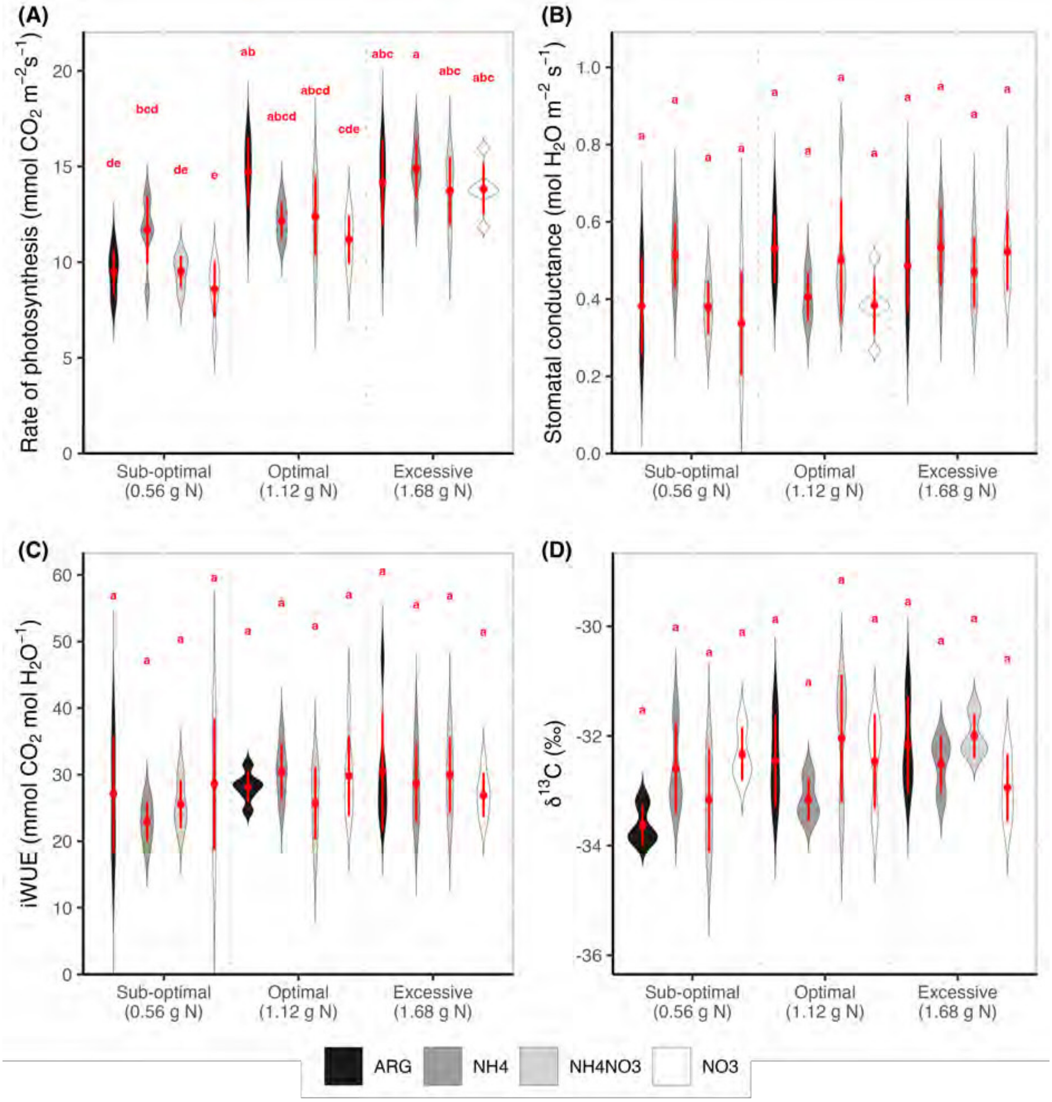


Figure S2. Gas exchange and ^{13}C discrimination ($\delta^{13}C$) in response to different nitrogen additions in Experiment II. (A)-(C) Rate of photosynthesis **(A)**, stomatal conductance **(B)** and instantaneous water use efficiency (iWUE) **(C)**, analysed by gas exchange measurements with LICOR-6400XT from one mid-stem leaf. n = 6. **(D)** ^{13}C discrimination ($\delta\ ^{13}C$). $\delta\ ^{13}C$, providing an estimate of cumulative WUE, was measured by isotopic composition analysis with EA-IRMS of a newly formed leaf. n=3. The violin plots show data distribution. Red dots and error bars indicate mean values ± SD. Statistical significance was tested by Two-way ANOVA and Tukey's HSD test. Means not sharing any letter are significantly different at the 5% level of significance.

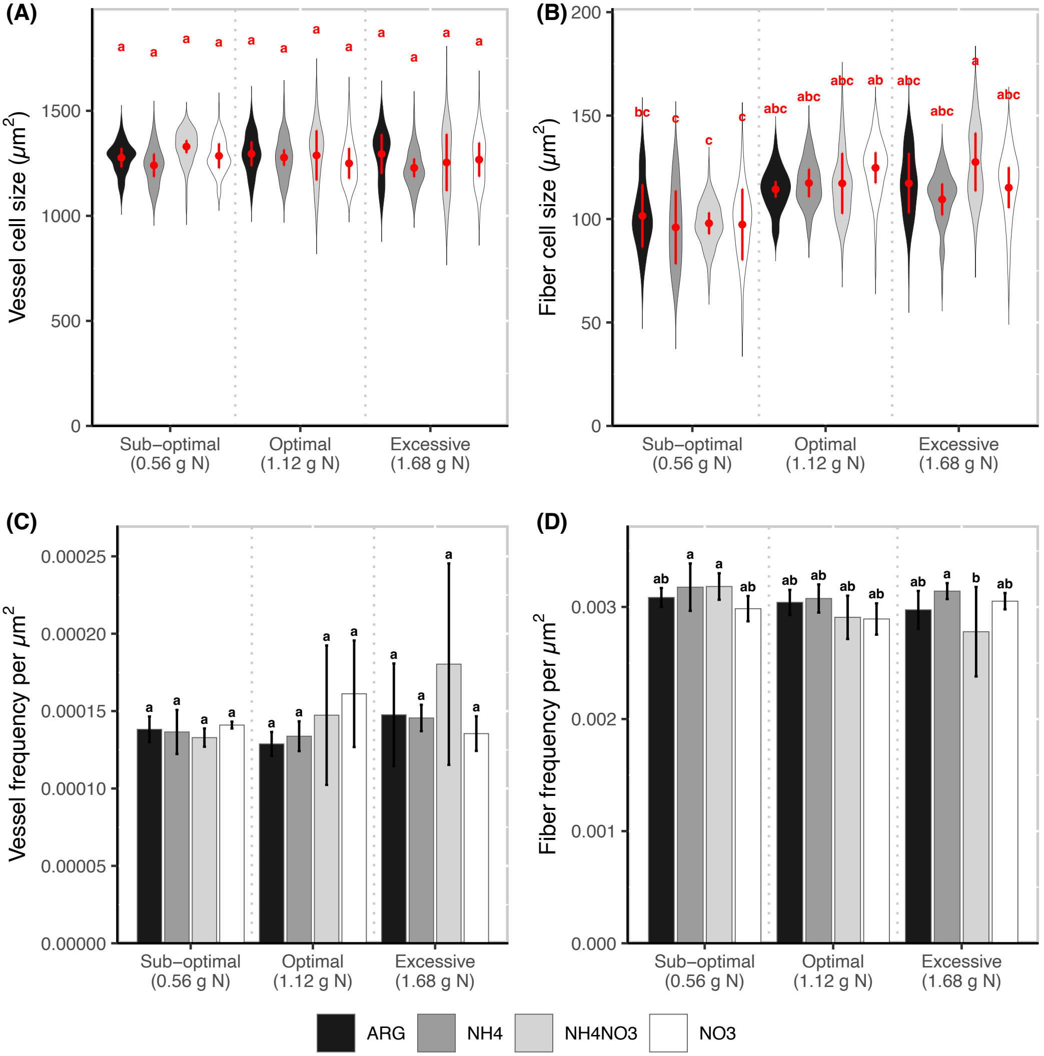


Figure S3. Wood anatomy in response to different nitrogen additions in Experiment II. (A-D) Vessel cell size **(A)**, fiber cell size (**B)**, vessel frequency **(C),** and fiber frequency **(D).** Xylem cell sizes and frequencies are calculated per representative area of wood. n = 6. The violin plots show data distribution. Red dots, bars and error bars display mean values ± SD. Statistical significance was tested by Two-way ANOVA and Tukey's HSD test. Means not sharing any letter are significantly different at the 5% level of significance.

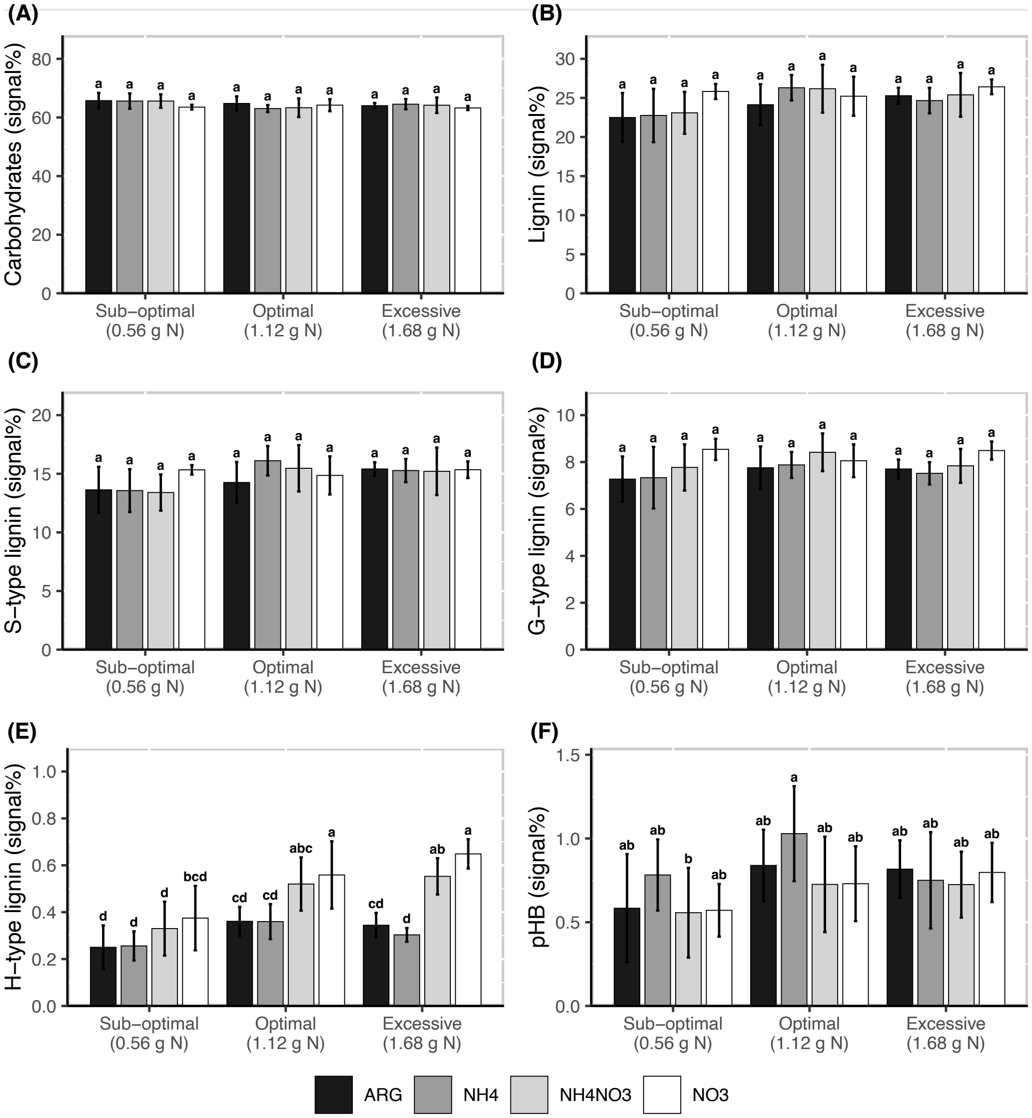


Figure S4. Wood chemical composition in response to different nitrogen additions in Experiment II. (A-F) The analysis of the chemical components of wood by pyrolysis-GC/MS analysis. Relative contents are shown for carbohydrates **(A),** total lignin **(B)** S-type lignin **(C)**, G-type lignin **(D)**, H-type lignin **(E)**, and pHB-lignin units **(F)**. n = 6. Bars with error bars indicate means ± SD. Statistical significance was tested by Two-way ANOVA and Tukey's HSD test. Means not sharing any letter are significantly different at the 5% level of significance.

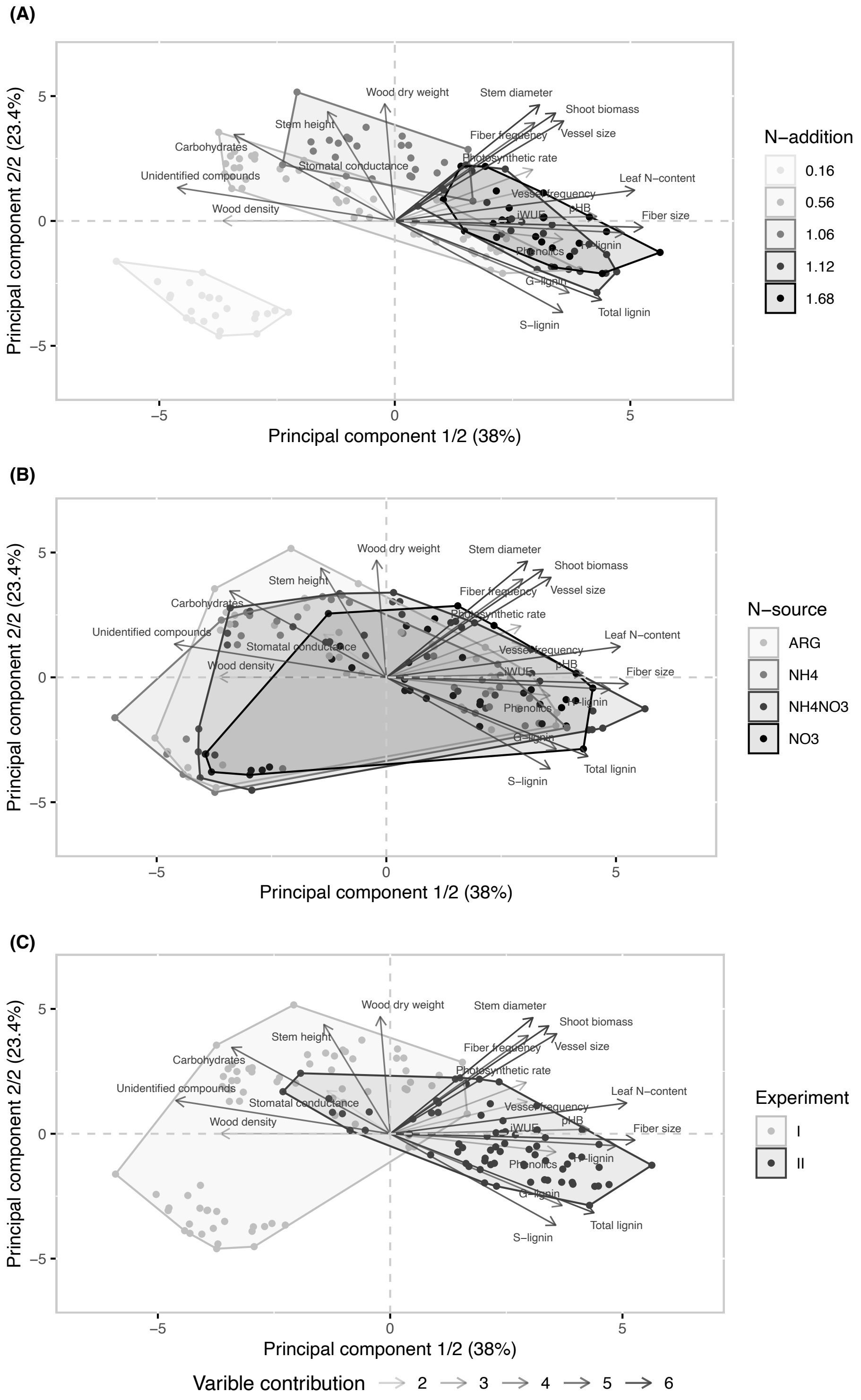


Figure S5. PCA plots with all measured parameters in response to N-addition, N-source, and Experiment. (A-C) Principal component analysis of all measured parameters in Experiment I and II. Individual trees and response variables are shown for the two first components. Data is clustered by N-addition **(A)**, N-source **(B)** and experiment **(C)**.

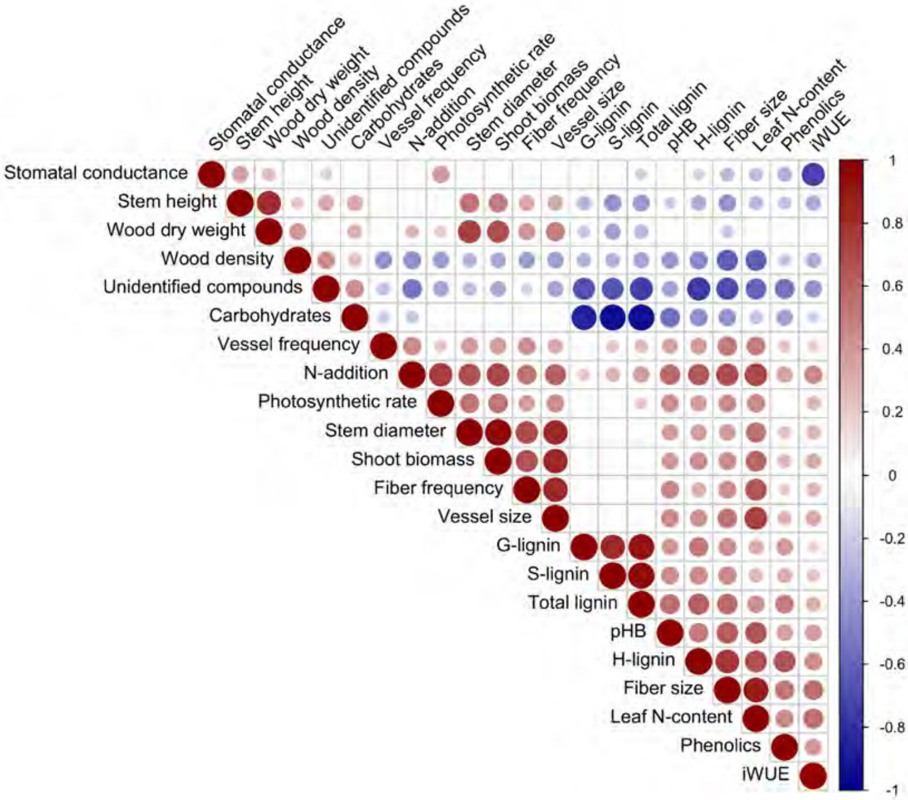


Figure S6. Correlation plot with all measured parameters and data from Experiment I and II. Size of the dot corresponds to the significance value i.e., larger dot equals to lower p-value. Correlation matrix was calculated using the ‘rcorr’ function and ‘Hmisc’ package. Correlation coefficients and p-values from the correlation matrix was used in ‘corrplot’ function and ‘corrplot’ package. R-studio version 2023.06.0+421. Since the correlations are based on combined data from the two experiments the results need to be treated with caution.

III

Tree Physiology, 2025, tpaf099
https://doi.org/10.1093/treephys/tpaf099
Advance access publication date 11 August 2025
Research paper

Unraveling nitrogen uptake and metabolism: gene families, expression dynamics and functional insights in aspen (*Populus tremula*)

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Handling Editor: Gary Coleman

The influence of nitrogen on wood formation is well established. To gain insight into the underlying molecular mechanism, we first identified genes in 14 gene families that are involved in nitrogen uptake and metabolism in European aspen (*Populus tremula* L.) genome annotation. Gene expression data from a de novo RNA sequencing (RNA-seq) analysis and data available from the AspWood database (plantgenie.org) provided putative candidate genes for the uptake of nitrate, ammonium and amino acids from the xylem sap as well as their further assimilation in the secondary xylem tissues of the stem. For a population-wide analysis of the nitrogen-related genes, we utilized RNA-seq data from the cambial region of the stems of 5-year-old aspen trees, representing 99 natural aspen accessions, and compared the expression of the nitrogen-related genes to stem diameter. Novel regulatory interactions were identified in expression quantitative loci and co-expression network analyses in these data. The expression of certain nitrate and amino acid transporters correlated negatively with stem diameter, suggesting that excessive nitrogen retrieval from the xylem sap suppresses radial growth of the stem. The expression of a glutamine synthetase correlated with the expression of these transporters, a link further supported by increased plant growth in transgenic glutamine synthetase overexpressing trees. This study provides insight into the genetic basis of nitrogen uptake and assimilation and its connection to wood formation, providing interesting targets for improving nitrogen-use efficiency and growth of aspen trees.

Keywords: genetic variation, nitrogen assimilation, nitrogen reallocation, wood development.

Introduction

Nitrogen is an essential macronutrient for plant growth and development, playing a critical role in a variety of physiological and biochemical processes, including photosynthesis, amino acid and protein synthesis, metabolic regulation, growth and biomass production. Nitrogen availability also influences wood formation, either directly or indirectly, in many ways (for a recent review, see Lu et al. 2024). In *Populus* trees, high nitrogen availability has been reported to increase vessel and fiber lumen area and to reduce secondary cell wall thickness, lignin content and wood density (Luo et al. 2005, Pitre et al. 2007, Novaes et al. 2009, Cao et al. 2024, Renström et al. 2024).

Nitrogen-use efficiency, which refers to the ability of a plant to acquire and utilize nitrogen to maximize growth and yield, is a key aspect of modern agriculture and forestry (Congreves et al. 2021, Q.Liu et al. 2022). Increased understanding of the molecular mechanisms that underpin nitrogen uptake, transport and metabolism can provide candidate genes to increase nitrogen-use efficiency of plants and to improve sustainability and minimize environmental impacts caused by excessive use of nitrogen fertilizers (Cánovas et al. 2018, Stevens 2019, Waqas et al. 2023). While significant progress has been made in studying nitrogen-related pathways in model plants like *Arabidopsis thaliana* (Arabidopsis), there is still much to learn about how these mechanisms operate in long-lived woody species. Recent advances in genomics and transcriptomics have enabled investigation of the genes and pathways involved in nitrogen metabolism in various plant species, including woody species. In black cottonwood (*P. trichocarpa*), nitrogen-related gene families, such as amino acid permeases (AAPs), nitrate transporters (NRTs), nitrite reductases (NIRs) and NIN-like proteins (NLPs) have been identified (Couturier et al. 2010, Bai et al. 2013, Léran et al. 2014, von Wittgenstein et al. 2014, Wu et al. 2015, Xu et al. 2017, Du et al. 2022, Han et al. 2022, Cao et al. 2023, Yu et al. 2023, Li et al. 2024, Guan et al. 2025). These gene families often exhibit substantial variation in gene number, structure and function across plant species, reflecting evolutionary pressures and ecological adaptations. However, the extent of this variation remains poorly characterized in other *Populus* species.

Received: February 18, 2025. **Accepted:** August 4, 2025

In this study, we investigated nitrogen-related gene families in European aspen (aspen, *Populus tremula*), which is the only native *Populus* species in Sweden and extensively used as a deciduous tree model for population genetics and molecular studies (Escamez et al. 2023, Luomaranta et al. 2024, Robinson et al. 2024). We identified the members of the following gene families: amino acid transporters (AAT), ammonium transporters (AMT), asparagine synthetases (ASN), asparaginases (ASPG), alanine aminotransferases (AlaAT), aspartate aminotransferases (AspAT), cationic amino acid transporters (CAT), glutamate dehydrogenases (GDH), glutamate synthases, glutamine synthetases (GS), NIR, nitrate reductases (NR), NLP and NRT in aspen, and constructed phylogenetic trees including genes from aspen and Arabidopsis. We also revealed tissue specific expression patterns and specific responses to nitrate vs ammonium in developing xylem tissues of hybrid aspen trees. Additionally, expression quantitative trait loci (eQTL) mapping and gene co-expression networks highlighted links between nitrogen metabolism and radial growth of the stem.

Materials and methods

Gene family identification in aspen

Arabidopsis thaliana (Arabidopsis) genes from 14 gene families encoding AAT, AMT, ASN, ASPG, AlaAT, AspAT, CAT, GDH, glutamate synthases (GOGAT), GS, NIR, NR, NIN-like transcription factors (NLP) and NRT were extracted from The Arabidopsis Information Resource (TAIR; https://www.arabidopsis.org). These sequences were utilized as queries for a BLASTP search against the *Populus tremula* v2.2 genome annotation on plantgenie.org by using default settings (Sundell et al. 2015, Robinson et al. 2024). As a second step, the nitrogen-related genes from Arabidopsis were analyzed for conserved functional motifs searching the Pfam database via the HMMER v3.4 tool (hmmscan, Potter et al. 2018). The identified motifs served as seed motifs for HMMER (hmmsearch, Potter et al. 2018) against the annotated peptide sequences in *Populus tremula* v2.2, resulting in an initial gene list. Genes from the first BLASTP output that, on the basis of the second screening, lacked the conserved functional motifs were excluded from the final list of nitrogen-related genes in aspen. The aspen genes were named after Arabidopsis genes with the highest sequence similarity according to BLASTP.

Phylogenetic tree construction and the analyses of the gene structures and conserved domains

The peptide sequences of identified genes were aligned using default settings of MAFFT v7.526 (Katoh and Standley 2013). The aligned sequences were trimmed by trimal v 1.5.rev0 with default settings (Capella-Gutiérrez et al. 2009). The aligned sequences were then employed to construct a maximum likelihood phylogenetic tree with 1000 bootstrap replicates using IQ-TREE2 v2.3.6 (Minh et al. 2020). The resulting phylogenetic trees were visualized with MEGA11 (Tamura et al. 2021). The identified motifs from hmmerscan and the structure of the genes were visualized by TBTOOL2 (Chen et al. 2023).

Gene expression in the AspWood database

The Aspen Wood (AspWood) resource of high-resolution gene expression data from the developing wood of aspen trees (Sundell et al. 2017) were downloaded from PlantGenIE (https://plantgenie.org, Sundell et al. 2015). The expression data were presented as log2(TPM + 1) values and visualized as heatmap using ComplexHeatmap v2.20.0 (Sundell et al. 2017, Gu 2022).

RNA-sequencing of ammonium treated hybrid aspen trees

The experiment is described in full in Renström et al. (2024). Briefly, hybrid aspen (*P. tremula* L. × *P. tremuloides* Michx, clone 'T89') trees were cultivated in greenhouse conditions for 10 weeks under controlled nutrient additions. Trees were fertilized with ammonium-based nutrient solution at three different addition levels: limited, sub-optimal and optimal, corresponding to total nitrogen amounts of 0.16, 0.56 and 1.06 g, respectively. At the end of the experiment, 10-cm-long stem pieces were collected from the base of the plant and immediately frozen in liquid nitrogen. The developing xylem part of the stem piece was then scraped off and homogenized using a mortar and pestle in liquid nitrogen. RNA was extracted from the homogenized material using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich Co. LLC). RNA was quantified with a Nanodrop 1000 Spectrophotometer (Thermo Scientific), and the quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Following sequencing library generation and paired-end (2 × 150 bp) sequencing using Illumina NovaSeq 6000, the raw reads were pre-processed to remove sequencing adapters using Trimmomatic (v0.39). The trimmed read pairs were quantified with Salmon (v1.9) using the transcriptome index based on aspen genome annotation (Robinson et al. 2024). For exploratory analysis, including sample clustering and principle component analysis (PCA) visualization, we applied variance stabilizing transformation (VST) to the normalized counts using DESeq2's vst() function. Formal differential expression testing was subsequently performed on raw counts using DESeq2 (v1.46.0 in R 4.4.2), which internally handles count normalization via median-of-ratios scaling (Love et al. 2014). Statistical significance between the RNA-Seq results for the ammonium-treated (analyzed here) and nitrate-treated trees (published in Renström et al. 2024) was tested using Tukey's Honestly Significant Difference (HSD) post-hoc tests on the normalized expression values. The cld() function (multcompView v0.1–10) generated the compact letter display, indicating significant differences (adjusted $P < 0.01$) between all treatment combinations (3 concentrations × 2 nitrogen treatments).

Expression quantitative trait loci analysis in the Swedish aspen collection

The eQTL data for the Swedish aspen (SwAsp) trees were retrieved from Luomaranta et al. (2024). Briefly, the eQTL analysis was based on RNA-seq analysis of developing xylem tissue collected from 5-year-old stems, representing 99 SwAsp genotypes. The eQTL analysis was based on 6,806,717 bi-allelic SNPs (Robinson et al. 2024). Mean normalized gene expression values were used for each genotype. The false discovery rate threshold of 0.05 was applied to exclude non-significant eQTLs. eQTLs were categorized as local (within 1 Mbp) or distant.

The subnetwork of nitrogen-related genes

The whole-transcriptome co-expression network from Luomaranta et al. (2024) was used to extract a sub-network of nitrogen-related genes using Cytoscape v3.10.2 (Shannon et al. 2003). The sub-network was clustered by using INFOMAP (v.1.8.0; Rosvall and Bergstrom 2008). Stem diameter of the 99 different SwAsp genotypes (Luomaranta et al. 2024) was added as a node in the sub-network. Correlation of stem diameter to each gene in the sub-network was then represented according to the calculated Spearman rank correlation ($R > 0.3$) with only edges.

Overexpression of the *Populus* glutamine synthetase *GLN1.2a* and the growth of the transgenic lines

A DNA fragment corresponding to the coding sequence of *Populus tremula PotraGLN1.2a* (Potra2n12c24087), flanked by the Gateway attL1 and attL2 cloning sequences, was synthesized and inserted into pUC57 by the GenScript company. The resulting vector was recombined in an LR reaction with the destination vector pK2GW7. The destination vector, directing the expression of *PotraGLN1.2a* under the control of the 35S promoter, was transformed into hybrid aspen (*Populus tremula* × *P. tremuloides*), as described in Nilsson et al. (1992).

The transgenic trees were grown in an automated phenotyping platform (WIWAM Conveyor, Eeklo, Belgium) for 7.5 weeks under long-day conditions (18 h/6 h day/night) with 160–230 μmol m^{-2} s^{-1} light intensity from white light (FL300 LED Sunlight v1.1) and far red light (FL100 LED custom-made, 725–735 nm) lamps (Senmatic A/S, Søndersø, Denmark), 22 °C/18 °C temperature regime, and 60% relative humidity, as described in Wang et al. (2022). The expression of the transgene was analyzed in in vitro material (whole shoots) from tissue culture by quantitative reverse transcription polymerase chain reaction (qPCR). Briefly, 1 μg RNA of at least three biological replicates was taken for cDNA synthesis using the iScript™ cDNA Synthesis Kit (Bio-Rad, USA). Primers were designed using the Primer3 web server (v.4.1.0; Untergasser et al. 2012). Forward and reverse sequences, for the *PotraGLN1.2a* (Potra2n12c24087) were GTCTGACTGGTCGCCATGAA and AGCTTTCTCTGTGTCCCTGC, respectively, with ubiquitin (Potra2n1c3635; 5′-AGATGTGCTGTTCATGTTGTCC-3′, 5′-ACAGCCACTCC AAACAGTACC-3′) as a reference. qPCR was performed in 10 μL reaction volume, comprising of 1 μL of 5× diluted cDNA, 1× PowerTrack™ SYBR Green Master Mix (Thermo Fisher Scientific), 100–200 nmol each of forward and reverse primer. All reactions were performed in 96-well plates on a C1000Touch™ Thermal Cycler (Bio-Rad) with an initial denaturation at 95 °C for 3 min, followed by 40 cycles, each of denaturation at 95 °C for 10s, and annealing at 60 °C for 10 s. The C_q values were acquired from the CFX96™ Maestro software (Bio-Rad) and average dCq shown for each sample were calculated relative to ubiquitin.

Results

Identification, phylogeny and expression analysis of nitrogen uptake and metabolism related gene families in aspen

In order to facilitate molecular analyses underlying nitrogen-mediated responses in wood formation, we first identified the aspen members of 14 gene families related to nitrogen uptake and metabolism (Figure 1, Table S1 available as Supplementary Data at *Tree Physiology* Online). Phylogenetic trees containing aspen and Arabidopsis were constructed to provide insight into the evolutionary relationship (Figure 1). In addition, the overall gene structure was analyzed for all genes (Figure 2).

We also used the publicly available AspWood dataset to extract information of gene expression in the woody tissues of aspen for the nitrogen-related genes identified in this study. AspWood is a high-resolution gene expression database in the woody tissues of aspen stems (Sundell et al. 2017), including data from the phloem/cambium region, and the different phases of xylem expansion, xylem maturation and xylem cell death (plantgenie.org; Sundell et al. 2015). Several members of the nitrogen-related gene families were according to the AspWood database expressed in a biphasic manner in aspen, with a first peak in the phloem/cambium and a second peak in the phase of xylem cell death (Figure 3). The expression in the phloem/cambium is likely related to the transport and sensing of nitrogen that is being transported in the phloem, while the expression in the phase of cell death is most likely related to the transport and sensing of nitrogen compounds that have been taken up from the xylem sap to ray parenchyma (van Bel 1984, Tegeder 2014, Cánovas et al. 2018).

NIN-like proteins

The NIN-like transcription factors (NLPs) initiate nitrate signaling and coordinate gene expression related to nitrate transport and metabolism, and plant development (Vidal et al. 2020). Arabidopsis has nine *NLP* genes, and the AtNLP7 has been shown to both directly bind nitrate and act as a transcription factor (K.H.Liu et al. 2022). We identified 12 *NLP* genes in aspen (Figures 1 and 2, Table S1A available as Supplementary Data at *Tree Physiology* Online), most of them having the biphasic expression pattern in woody tissues (Figure 3). The aspen *PotraNLP7a*, homologous to the Arabidopsis *AtNLP7,* was expressed in phloem/cambial tissues and during xylem expansion. The highest expressed gene was *PotraNLP8b,* which has the highest sequence similarity to the Arabidopsis *AtNLP8* and *AtNLP9* (Figure 1).

Nitrate transporters

Nitrate transporters are vital for the uptake and partitioning of nitrate in plants. They can be found in several different families, including the Nitrate transporter 1/Peptide transporter Family (NRT1/NPF) (Léran et al. 2014), the Nitrate Transporter 2 (NRT2) (von Wittgenstein et al. 2014) and Nitrate Transporter 3 (NRT3) (Wang et al. 2018) families. The NRT1/NPF family members are typically so-called low-affinity transporters (Corratgé-Faillie and Lacombe 2017) that are active at nitrate concentrations exceeding 250 μM, while members of the NRT2 family seem to represent high-affinity transporters that are generally active at nitrate concentrations in the range of 10–250 μM (Xu et al. 2024). The first described nitrate transporter, the Arabidopsis AtNRT1.1 (also called CHL1 or NPF6.3), is an exception in that it can operate as a dual affinity transporter (Liu et al. 1999). NRT3 is necessary for NRT2 stability and targeting to the plasma membrane (Wang et al. 2018).

The NRT1/NPF gene family was one of the largest families in aspen. We found 57 aspen genes corresponding to the 53 Arabidopsis genes (Figures 1 and 2, Table S1B available as Supplementary Data at *Tree Physiology* Online). The aspen NRT2 gene family contained six aspen genes corresponding to the seven genes in Arabidopsis while the NRT3 family had

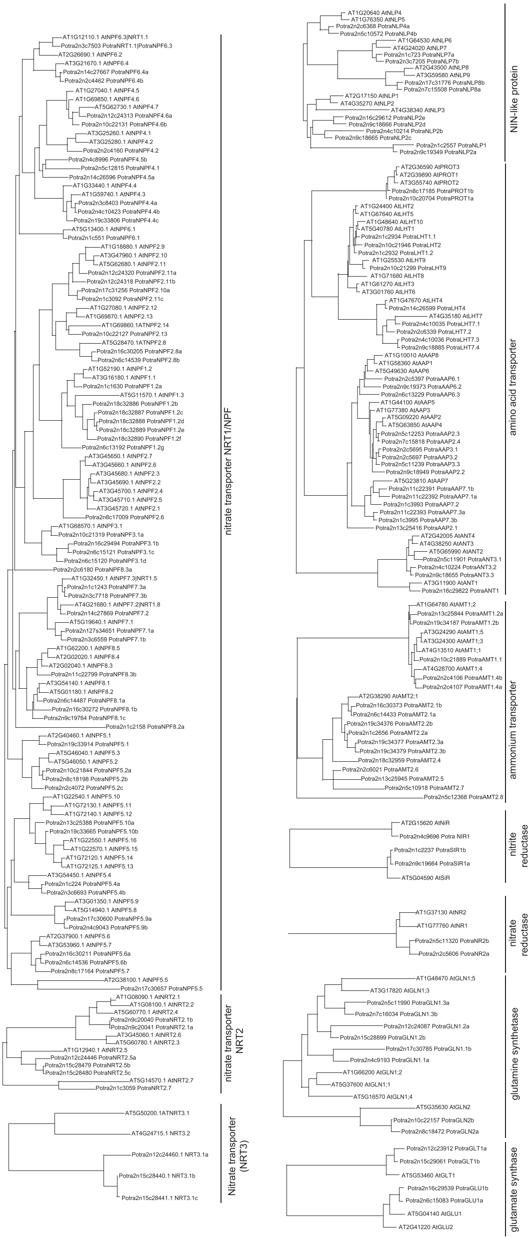

Figure 1. Identification of nitrogen uptake and metabolism-related genes in aspen (*P. tremula*). Phylogenetic trees include genes encoding members of the nitrate transporter families, NIN-like transcription factors, AAT, AMT, glutamate synthases, glutamine synthetases, NIR and NR in aspen and Arabidopsis. The peptide sequences of genes in corresponding gene families were aligned by MAFFT. The maximum likelihood tree with 1000 bootstraps was constructed by IQTREE2 and visualized by MEGA11.

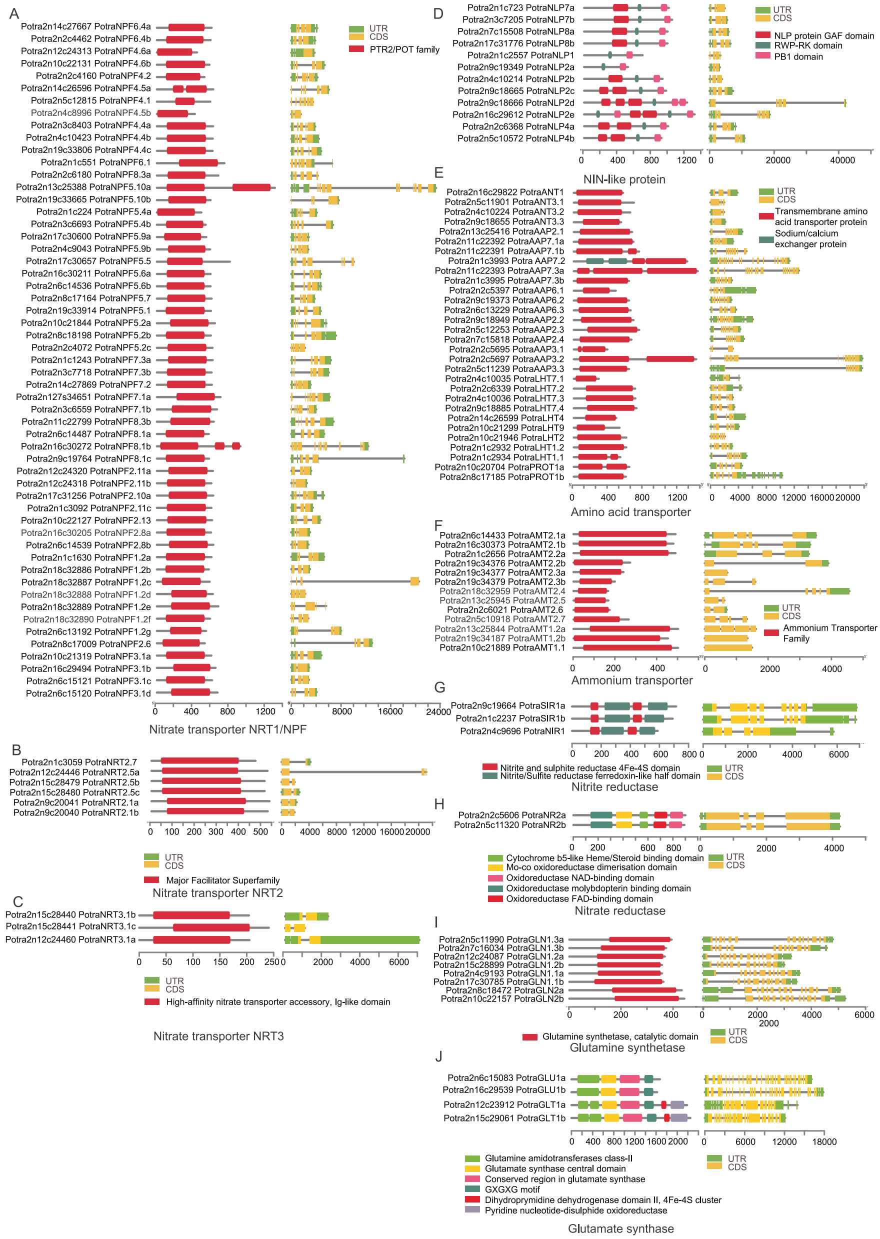

Figure 2. Gene structure and conserved protein motifs of nitrogen uptake and metabolism-related gene families in aspen. Distribution and the relative genomic positions of conserved motifs, identified by hmmscan, are shown for nitrate transporter (A, B, C), NIN-like protein (D), amino acid transporter (E), ammonium transporter (F), nitrite reductase (G), nitrate reductase (H), glutamine synthetase (I) and glutamate synthase (J) gene families. Exons are shown as yellow boxes, introns as black lines and untranslated regions as green boxes. Gene lengths are indicated at the x axis relative to the transcription start site (0 bp).

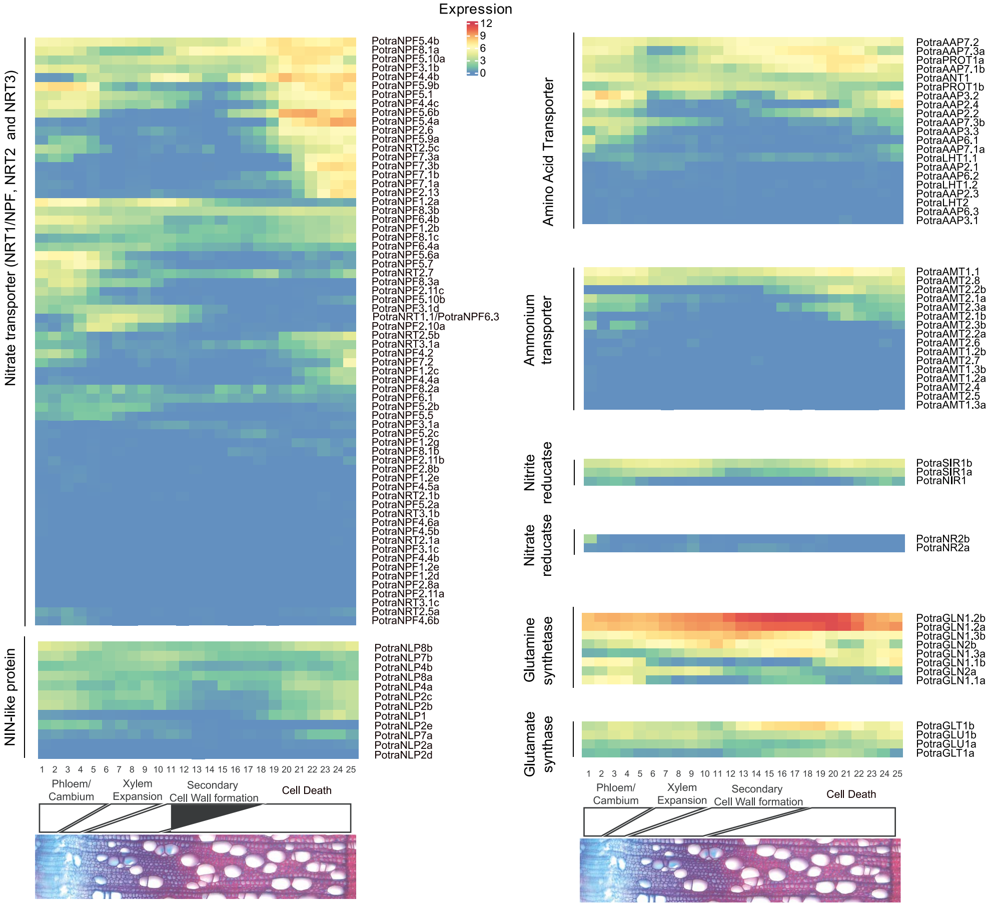

Figure 3. The expression of the nitrogen uptake and metabolism-related genes in aspen. The heatmap represents log(TPM + 1) values for the different genes in the AspWood database (plantgenie.org). Horizonal axis indicates the identity of the samples (in the AspWood tree 1) and the different zones of the cambial region (the phloem, the phase of xylem expansion, the phase of secondary cell wall formation and cell death) in aspen stem. The cambium (>85% of the cell population) is in samples 3–6 and the expanding xylem (>85% of the cells) is in samples 7–11. According to Sundell et al. (2017), the vessels die and autolyze around the sample number 18 while the fibers are dead only in the last sample where the only living cells are the rays in tree 1 of the AspWood database. The colors indicate relative expression levels according to the scale on the right.

three aspen genes corresponding to the two Arabidopsis genes (Figures 1 and 2, Table S1B available as Supplementary Data at *Tree Physiology* Online).

A large proportion of the genes in the aspen nitrate transporter gene families were expressed in the woody tissues (Figure 3). Several of them showed the biphasic expression pattern (Figure 3). Examples of such genes included *PotraNPF4.4c* and *PotraNPF7.3a*, homologs of which are well characterized in Arabidopsis, as well as *PotraNPF5.4a*, *PotraNPF5.4b* and *PotraNPF5.6b*, which have not been functionally characterized as nitrate transporters in Arabidopsis. *PotraNRT1.1/NPF6.3* had a distinct expression pattern within the family: its expression was not biphasic but peaked in the zone of xylem cell expansion (Figure 3). The two aspen homologs of the high-affinity *AtNRT2.1* both had very low expression in the woody tissues (Figure 3).

Ammonium transporters

AMTs mediate the uptake of ammonium from the soil, but also ammonium transport from root to shoot and within leaves, and ammonium acquisition into other organs (Hao et al. 2020). The *AMT* genes in Arabidopsis are divided in two phylogenetic groups; one containing five genes (*AtAMT1;1—AtAMT1;5*) and the other one containing the single *AtAMT2;1*. Both AMT1 and AMT2 show high affinity to ammonium (Yuan et al. 2007).

The AMT gene family showed clear clustering into two main clades (Figure 1, Table S1C available as Supplementary Data at *Tree Physiology* Online). The clade corresponding to Arabidopsis *AtAMT1;1–1;5* contained five aspen genes. *PotraAMT1.1* showed a strong, biphasic expression in the woody tissues (Figure 3). The second clade, containing the Arabidopsis *AtAMT2;1*, has undergone copy number expansion with 11 aspen genes (Figure 1, Table S1C available as Supplementary Data at *Tree Physiology* Online). Several of the aspen *AMT2* clade members were expressed at a low level primarily in the cell death zone except for *PotraAMT2.8* that had a constant, low expression in the woody tissues (Figure 3).

Amino acid transporters

Transport of amino acids and other nitrogenous compounds in plants is primarily mediated by two major superfamilies: the amino acid-polyamine-organocation (APC) superfamily and the amino acid/auxin permease (AAAP) superfamily (Pratelli and Pilot 2014). We focused on four functionally characterized subfamilies within these superfamilies: the AAP and the lysine/histidine transporters (LHT) from the AAAP superfamily, and the proline transporters (ProT) and the aromatic and neutral amino acid transporters (ANT) from the APC superfamily (Tegeder and Hammes 2018, Yang et al. 2020). Aspen contained 15 *AAP*, 9 *LHT*, 2 *ProT* and 4 *ANT* family members (Figures 1 and 2, Table S1D available as Supplementary Data at *Tree Physiology* Online). They were expressed primarily in the phloem/cambium (*PotraANT1, PotraAAP3.2, PotraAAP3.3, PotraAAP6.1, PotraAAP7.3b*), the expansion zone (*PotraPROT1b*), secondary cell wall formation (*PotraAAP7.2*, *PotraAAP7.3a*) and the cell death zone (*PotraProT1a*, *PotraAAP*7.1b) (Figure 3).

Nitrogen assimilation and metabolism

Nitrate is reduced to nitrite by the cytoplasmic enzyme nitrate reductase (NR). Two *NR* genes, clustering together with the Arabidopsis *AtNR1* (also known as *NIA1*) and *AtNR2* (also known as *NIA2*), were annotated in the aspen genome (Figures 1 and 2, Table S1F available as Supplementary Data at *Tree Physiology* Online). *PotraNR2a* and *PotraNR2b* had a low expression in the woody tissues (Figure 3).

Nitrite is subsequently imported into plastids, where nitrite reductase (NIR) reduces it to ammonium. One aspen homolog was found for the Arabidopsis nitrite reductase *AtNIR1* while two homologs were found for a sulfite reductase (*AtSIR*, AT5G04590) which both contained a Nitrite/Sulfite reductase ferredoxin-like domain (Figures 1 and 2, Table S1E available as Supplementary Data at *Tree Physiology* Online). *PotraNIR1* had a low, biphasic expression in the phloem/cambium and cell death zone while *PotraSIR1a* and *PotraSIR1b* had a broader and higher expression, including expression in the xylem expansion zone (Figure 3).

Ammonium is assimilated in both the cytosol and plastids to produce amino acids in a set of interconnected reactions. In the cytosol, ammonium can react with glutamate to produce glutamine by the cytosolic glutamine synthetase (GS) or asparagine by asparagine synthetase (ASN). In the plastids, a plastidic GS catalyses conversion of ammonium to glutamine, which can be then converted to glutamate by glutamate synthase (GOGAT) in the so-called GS/GOGAT cycle. Asparaginase (ASPG), alanine aminotransferase (AlaAT), aspartate aminotransferase (AspAT) and glutamate dehydrogenase (GDH) are additional enzymes involved in nitrogen assimilation.

In the aspen genome, we identified eight glutamine synthetase, four glutamate synthase, three asparagine synthetase, four glutamate dehydrogenase, three asparaginase, two alanine aminotransferase and nine aspartate aminotransferase genes (Figures 1 and 2, Table S1G–N available as Supplementary Data at *Tree Physiology* Online). Each of these gene families had at least one gene that was highly expressed in the woody tissues (Figure 3). A few members of both the glutamate synthase (*PotraGLT1b*) and the glutamine synthetase (*PotraGLN1.2a* and *PotraGLN1.2b*) families had a constant, high expression throughout wood development (Figure 3).

The expression of the nitrogen uptake and metabolism-related genes under NH_4^+ and NO_3^- treatment

Populus species can take up nitrogen in the forms of both ammonium (NH_4^+) and nitrate (NO_3^-), but the site of assimilation and hence the transported form of nitrogen in the stem varies depending on the genotype and the environmental conditions (Black et al. 2002). In this study, we aimed to shed light on nitrate assimilation and subsequent nitrogen metabolism by exploring the effect of both NH_4^+ and NO_3^- on the expression of the nitrogen-related genes in developing xylem tissues of wood. For this purpose, we utilized material and data from our earlier study (Renström et al. 2024) where hybrid aspen trees were grown for 2 months in three different levels (suboptimal, low and optimal) of either NH_4^+ or NO_3^-. The nitrogen levels were defined in Renström et al. (2024) on the basis of known amounts of total nitrogen present in trees reaching maximal growth rates. The RNA-seq data in the nitrate-fertilized trees were retrieved from Renström et al. (2024) while the RNA-seq experiments in the ammonium-fertilized trees were performed in this study (see Table S2 available as Supplementary Data at *Tree Physiology* Online).

Almost all members of the nitrate transporter gene families were expressed in at least one of the experimental conditions (Figure 4). Half of them were expressed at a higher level when plants were fertilized with nitrate compared with ammonium (see Figure S1 available as Supplementary Data at *Tree Physiology* Online) even though statistically significant differences were present only for a few of them, including *PotraNRT1.1/NPF6.3*, *PotraNRT2.5c* and *PotraNRT2.1b* (Figure 4). Also nitrate and nitrite reductases as well as the two homologs of the *AtNLP7* nitrate sensors (*PotraNLP7a* and *PotraNLP7b*) were significantly more expressed in response to nitrate than ammonium (Figure 4). The AMT family members were rather equally expressed even though tendencies toward higher expression of a few of them were observed in response to fertilization with nitrate (Figure 4). The amino acid transporter families contained specifically responding genes but also genes that responded similarly to both nitrogen sources (Figure 4).

Expression quantitative trait loci analysis of nitrogen-related gene expression in the Swedish aspen collection

Members of the NLP gene family are interesting since they function in sensing of nitrate but also as transcription factors. We were interested in the regulatory aspects of this gene family, and utilized eQTL data from a population of aspen trees (the SwAsp collection) to investigate variation in the expression and regulation of the *NLP* genes in woody tissues (Luomaranta et al. 2024). The eQTLs were classified either as local or distant using 1 Mbp as a threshold (Luomaranta et al. 2024). The different members of the NLP gene family contained 333 local eQTLs and 336 distant eQTLs, corresponding to association with 28 and 179 genes, respectively

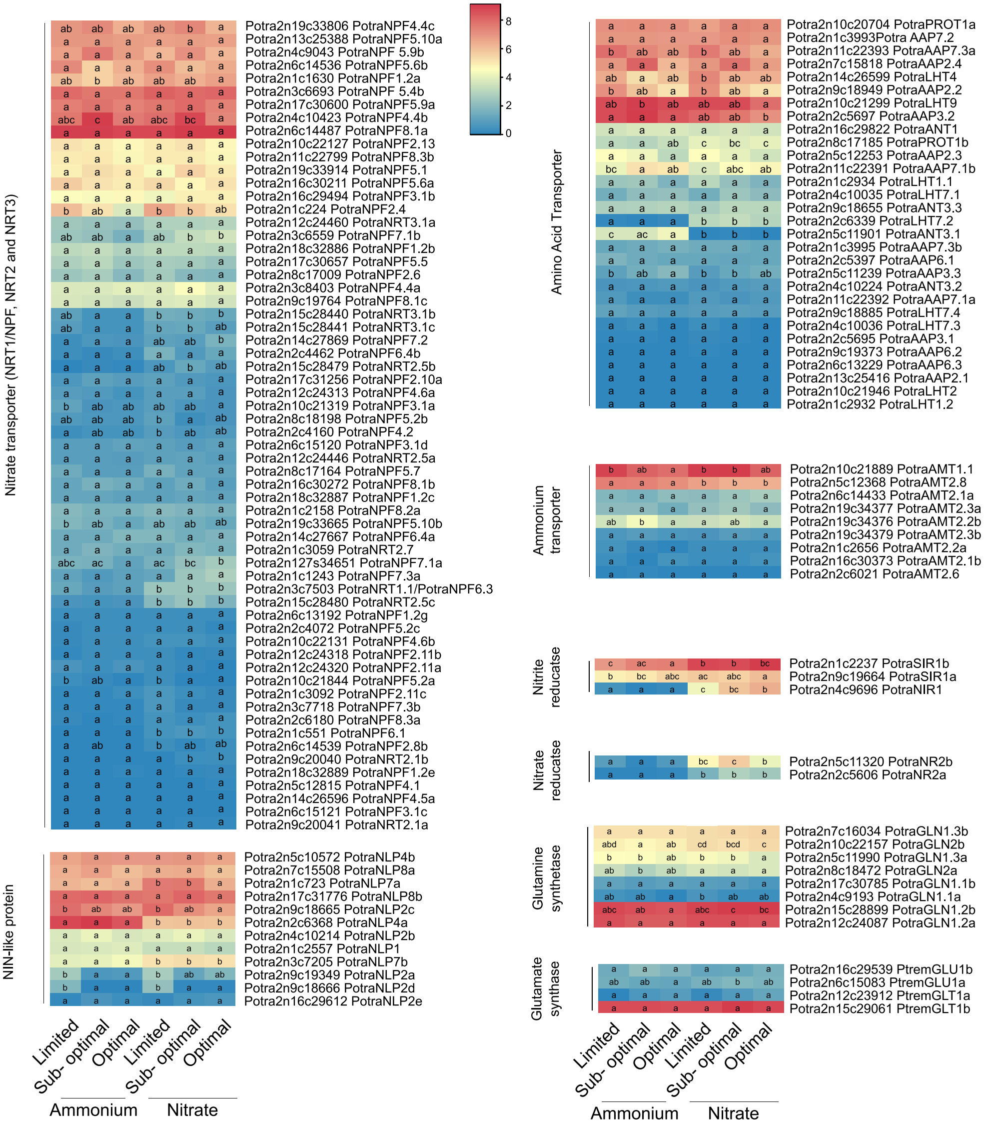

Figure 4. The expression of the members of aspen gene families related to nitrogen uptake, sensing and assimilation in response to fertilization with either nitrate or ammonium. The heatmaps represent VST normalized gene expression data from RNA-sequencing of developing xylem tissues of hybrid aspen after long-term treatment with optimal, sub-optimal and limited doses of ammonia (NH_4^+, this study) and nitrate (NO_3^-, reported earlier in Renström et al. 2024). Different letters indicate statistically significant differences using two-way ANOVA model and Tukey's HSD test with adjusted *P*-value < 0.01. The number of the trees in each condition is three or four.

(see Table S3 available as Supplementary Data at *Tree Physiology* Online). The SNPs in *PotraNLP2e* showed association with the expression of 34 different genes, including homologs of Arabidopsis *GA 20-oxidase* and *UMAMIT34* as distant eQTLs and *PotraGLU1b* as a local eQTL (see Table S3 available as Supplementary Data at *Tree Physiology* Online). Distant eQTLs for *PotraNLP7a* and *PotraNLP7b* were found in association with the expression of 12 and 13 genes, respectively, several of them having predicted functions in signaling and transcriptional regulation (see Table S3 available as Supplementary Data at *Tree Physiology* Online).

To broaden the scope of the expression analysis, we retrieved co-expression data for the nitrogen-related genes from a whole-transcriptome co-expression analysis in woody tissues of young aspen stems from Luomaranta et al. (2024), and constructed a subnetwork with these values together with the Pearson correlation values for the expression of these genes with the stem diameter of the same set of trees that were used for the RNA-Seq analysis.

A large proportion of the genes (91) were not co-expressed at all, a few genes were co-expressed with only one or two genes, and 54 genes were found in four clusters containing more than three co-expressed genes (Figure 5A). The largest cluster contained 26 genes, including the NPF family members *PotraNPF5.4a*, *PotraNPF5.4b* and *PotraNPF5.6b* that were also highly expressed in the woody tissues according to the AspWood database (Figure 3). This cluster also contained several AAP family members, such as *PotraAAP2.2*, *PotraAAP3.2*, *PotraAAP6.1* and *PotraANT1*, and the NRT1/NPF family members *PotraNPF5.6b* and *PotraNPF2.13*, which all correlated negatively with stem diameter, and glutamine synthetases *PotraGLN1.2a* and *PotraGLN1.2b* (Figure 5A).

To gain more understanding on the significance of the co-expression patterns in the largest cluster, we analyzed the effect of *PotraGLN1.2a* overexpression on tree growth in greenhouse conditions. *PotraGLN1.2a* was selected due to its high expression in the secondary xylem tissues (Figure 3). Its expression is also specific to wood (plantgenie.org). Three hybrid aspen (*Populus tremula* × *P. tremuloides*) overexpression lines of *PotraGLN1.2a* were grown in controlled conditions. After 2 months of growth, the *PotraGLN1.2a* overexpression lines were significantly taller than the wild-type trees (Figure 5B and C). Positive influence of *GS* overexpression on the growth of *Populus* trees has also been reported in several earlier studies (Gallardo et al. 1999, Fu et al. 2003, Jing et al. 2004, Castro-Rodríguez et al. 2016). Altogether, these results suggest that while tree growth is positively influenced by the expression of glutamine synthetases, it is counteracted by the expression of AAP and NRT1/NPF family members in the secondary xylem tissues of the stem.

Discussion

Identification of nitrogen uptake and metabolism related gene families in aspen

Here, we performed identification and analysis of 14 nitrogen uptake and metabolism associated gene families in aspen. Even though the function of many of these genes is known in Arabidopsis, little is known about their role in woody tissues of trees. Our analyses did not always identify the best characterized members of the gene families as those that seemed most important in aspen stem based on gene expression. For instance, several *NRT1/NPF* genes, such as *PotraNPF5.4*a, *PotraNPF5.4b* and *PotraNPF5.6b* which were highly expressed in the woody tissues of aspen stem (Figure 3), have not been established as nitrate transporters in any other species. Their role in nitrate sensing or transport is supported by their co-expression in the population of the SwAsp trees (Figure 5A), and in particular their co-expression with *PotraNPF4.4* which is a homolog of the Arabidopsis nitrate-binding protein *AtNPF4.4/NRT1.13* (Chen et al. 2021). The protein sequences of PotraNPF5.4a, PotraNPF5.4b and PotraNPF5.6b also contain the proline residue that is required for nitrate transport activity (see Figure S2 available as Supplementary Data at *Tree Physiology* Online; Ho et al. 2009, Chen et al. 2021). We therefore propose involvement of these aspen *NPF* genes in nitrate uptake and/or sensing in secondary xylem tissues of the stem. Recently, a cassava homolog of *PotraNPF5.4* was shown to be specifically expressed in the stem and decrease the efflux of nitrate in the root when overexpressed in rice, supporting the role of NPF5.4 in nitrate uptake from the xylem sap (Ji et al. 2024).

The highest expressed *NLP* gene in aspen wood was *PotraNLP8b* (Figure 3), which is most similar in sequence to Arabidopsis *AtNLP8* and *AtNLP9*. *AtNLP8* and *AtNLP9* are not very well characterized in Arabidopsis (Konishi et al. 2021). *AtNLP8* gene has been reported to be expressed in imbibed seeds and to promote seed germination (Yan et al. 2016). *AtNLP9* is expressed, similar to *PotraNLP8*, in the seeds, but also in procambium of the root (Brady et al. 2007). Two *Populus NLP8* homologs were recently mapped to QTL loci associated with tree biomass related traits in *P. deltoides* × *P. simonii* F1 population although their direct involvement was not demonstrated (Du et al. 2023). Taken together, the homologs of *AtNLP8* and *AtNLP9* genes seem to have pivotal roles in nitrate signaling of woody tissues in *Populus* trees.

Nitrogen uptake and assimilation in the developing xylem tissues of *Populus* stems

Populus trees, such as aspen, are known to be able to utilize both nitrate (NO_3^-) and ammonium (NH_4^+) even though nitrate is the predominant source of nitrogen (Rennenberg et al. 2010). We showed earlier that the growth of hybrid aspen trees was comparable when using either ammonium or nitrate as the sole nitrogen source, demonstrating that both sources can be equally utilized (Renström et al. 2024). However, it is not clear where in the tree nitrogen that is taken up in the form of nitrate is being assimilated. Preferential assimilation of nitrate in both the shoots (Black et al. 2002) and the roots (Gessler et al. 2004) have been reported. We observed that fertilization with nitrate activated the so-called primary nitrate response (Krouk et al. 2010), including induction of the expression of both nitrate and nitrite reductases, in developing xylem tissues of the stem (Figure 4). This observation demonstrates that nitrate that is being taken up by the roots is not necessarily assimilated in the roots but transported in the xylem sap of the stem and taken up in the stem where it stimulates the expression of nitrate-assimilating genes. However, nitrate treatment also tended to increase the expression of a few members of the AMT gene family (Figure 4), which could reflect regulation of these *AMT*s by nitrate or that at least a part of the applied nitrate is assimilated in the roots and transported in the form of ammonium. Furthermore, the expression of amino acid transporters in the secondary xylem tissues in response to nitrate fertilization supports the assimilation of nitrate before reaching the xylem tissues of the stem (Figure 4). It is therefore likely that nitrate can be assimilated in both the roots and the above ground parts of the trees.

The capacity of applied nitrate to induce the expression of *NR* and *NIR* in the secondary xylem tissues raises the question of how nitrate is removed from the xylem sap. The tissue-specific expression pattern and nitrate responsiveness indicated on the action of specific *NRT1/NPF* genes, such as *PotraNPF4.4b* and *PotraNPF7.2,* in the lateral transport of nitrate from the xylem sap to the surrounding parenchymatic cells (Figures 3 and 4). *PotraNPF4.4b* is homologous to the Arabidopsis *AtNRT1.13/NPF4.4* which is expressed in the parenchymatic cells next to the xylem elements, but

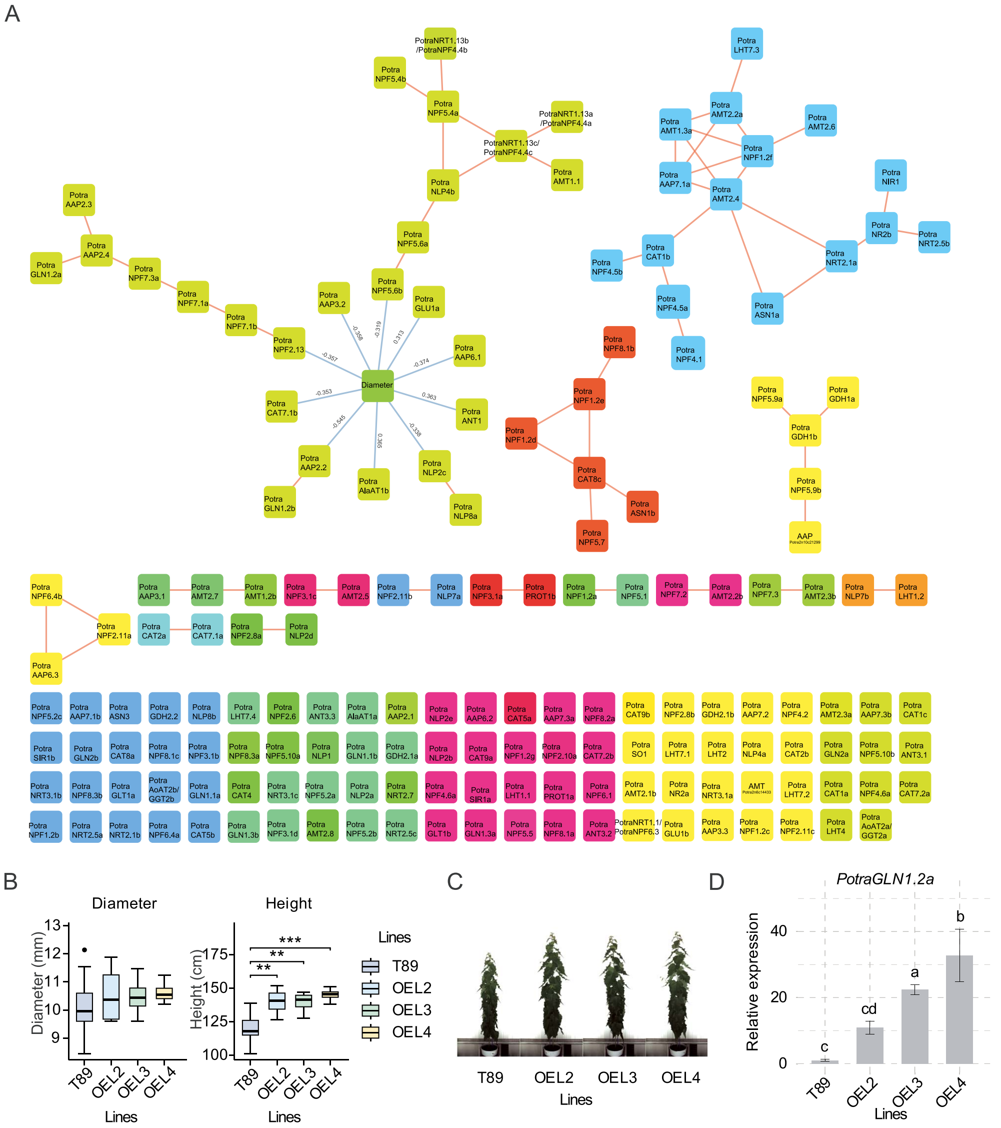

Figure 5. Co-expression of the nitrogen uptake and metabolism-related aspen genes in stem tissues of a population of SwAsp trees. (A) A subnetwork was calculated by extracting the co-expression values for the aspen nitrogen-related genes from a whole-transcriptome network, published in Luomaranta et al. (2024), that was based on gene expression in developing xylem tissues of 5-year-old aspen trees representing 99 genotypes of the SwAsp population. Correlation between genes in the Seidr whole-transcriptome network are indicated by orange lines. The Spearman correlation values ($R > 0.3$) between the diameter data of the stems and gene expression were included as edges, indicated as blue lines in the figure. (B) Phenotypic characterization of glutamine synthetase *PotraGLN1.2a* overexpression lines in hybrid aspen. Data for stem diameter and height of the trees is shown for the wild type (T89) and three *PotraGLN1.2a* overexpression lines grown for 2 months in an automated phenotyping platform. (C) Photos of representative trees in each of the wild type (T89) and transgenic *PotraGLN1.2a* overexpressor lines at the end of the growth period. (D) The relative expression of *PotraGLN1.2a* in in vitro tissues of wild type (T89) and three transgenic *PotraGLN1.2a* overexpressing lines (L2, L3, L4). Different letters indicate statistically significant differences using two-way ANOVA model and Tukey's HSD test with adjusted P-value < 0.01. The number of trees in each condition is three.

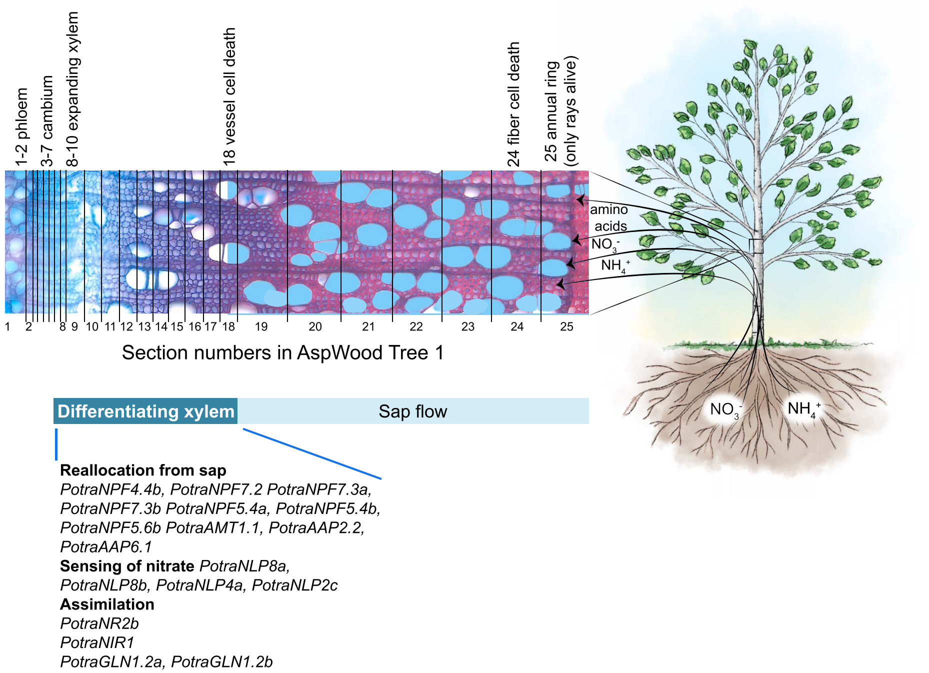

Figure 6. A proposed scheme of nitrogen uptake, reallocation and assimilation in the stem of aspen trees. The scheme is based on data presented on the expression of the aspen genes in AspWood (plantgenie.org) and gene co-expression patterns in the SwAsp population of aspen, presented in Figures 3 and 5. The microscopic image depicts the location of the tangential sections used for the gene expression analysis in the aspen stem in tree 1 in the AspWood data. The majority (>50%) of the cells in each section was estimated to reside in the phloem (sections 1 and 2), in the vascular cambium (sections 3–6), in the expanding xylem (sections 7–10) and in maturing xylem (the rest of the sections) according to Sundell et al. (2017). Vessel cell death was estimated to occur around the section number 18, and sap flow therefore takes place from sections 18–25. Fiber cell death was estimated to occur around section number 24. The only living cell type in section number 25 is therefore the rays. We provide evidence that both nitrate and ammonium is taken up by aspen roots at least in greenhouse conditions, and that each of nitrate, ammonium and amino acids are reallocated from the xylem sap to the parenchymatic ray cells and assimilated in the developing xylem tissues of the stem. Some of the proteins that seem to operate in nitrogen reallocation, nitrate sensing and nitrogen assimilation in the developing xylem, based on gene expression, are listed in the schematic representation.

which cannot transport nitrate from the sap since it lacks the proline residue crucial for nitrate transport (Chen et al. 2021). Interestingly, PotraNPF4.4b contains the proline residue (see Figure S2 available as Supplementary Data at *Tree Physiology* Online), supporting its function in nitrate uptake from the xylem sap. *PotraNPF7.2* is a promising candidate for having a role in nitrate retrieval from the sap to the xylem parenchyma on the basis of its strictly ray cell specific expression (Figure 3 and Figure S3 available as Supplementary Data at *Tree Physiology* Online, Tung et al. 2023) and homology to *AtNRT1.8/NPF7.2* which is expressed in xylem parenchyma cells and supposedly removes nitrate from the xylem vessels (Li et al. 2010). Additionally, the aspen homologs of *AtNRT1.5/NPF7.3* which participates in nitrate reallocation in Arabidopsis (Lin et al. 2008) could play a role in nitrate reallocation in tree stems.

Assimilation of nitrogen and radial growth of tree stems

Xylem-to-phloem allocation of amino acids has been demonstrated in lupin (Pate et al. 1975) and tomato (van Bel 1984). We identified several members of the amino acid transporter families that were highly expressed in the developing xylem tissues of aspen trees (Figure 3) and that could therefore have a function in the uptake of amino acids from the xylem sap into the parenchymatic ray cells of the secondary xylem. Interestingly, the results from the co-expression analysis in the SwAsp population of aspen trees showed that the expression of three AAPs, *PotraAAP2.2, PotraAAP3.2* and *PotraAAP6.1*, correlated negatively with stem diameter (Figure 5A). *PotraAAP2.2* is homologous to *AtAAP2* (AT5G09220) which mediates root-derived amino acid transport from xylem to phloem (Zhang et al. 2010) and coordinates partitioning of nitrogen and carbon within the plants (Perchlik and Tegeder 2018). The loss of AtAAP2 function in Arabidopsis resulted in increased allocation of nitrogen into the leaves as well as increased plant growth, which suggests that nitrogen allocation to leaves improves carbon fixation and, vice versa, that nitrogen allocation to other parts of the plant than leaves, such as stem tissues, suppresses carbon fixation and growth of the plants. Based on these results, it seems possible that amino acid permeases, such as PotraAAP2.2, act to divert amino acids from the xylem sap, negatively influencing carbon fixation in the leaves and concomitantly carbon allocation to the stem and the radial growth. However, the expression of *PotraAAP2.2* in the xylem parenchyma as well as in the phloem (Figure 3, Figure S3 available as Supplementary Data at *Tree Physiology* Online) is different from the strictly phloem-specific expression of the Arabidopsis *AtAAP2* (Zhang et al. 2010). *PotraAAP2.2* expression is actually more similar to *AtAAP6* which is expressed in the xylem parenchyma cells (Okumoto et al. 2002). AtAAP6 has been proposed to have a role in xylem-to-phloem transport of amino acids (Okumoto et al. 2002), supporting function of PotraAAP2.2 in amino acid uptake from the sap. Future work is needed to clarify the function of these AAPs in amino acid reallocation in the stem and their effect on tree growth.

Conclusions

We present a comprehensive analysis of nitrogen-related gene families in aspen, highlighting the genetic and functional dynamics essential for nitrogen-use efficiency. High-resolution gene expression analyses, together with co-expression analyses in a population of aspen trees, revealed a set of genes that are not typically associated with nitrogen uptake or assimilation and might therefore encode proteins that have specific functions in nitrogen reallocation and assimilation in the developing xylem tissues of the stem (Figure 6). The expression of these genes provide evidence on nitrate and ammonium uptake from the soil, uptake of nitrate, ammonium and amino acids from xylem sap into the parenchymatic ray cells, and assimilation in the developing xylem tissues of the stem (Figure 6).

We suggest on the basis of our gene expression analyses that even though nitrogen assimilation seems to be highly active in the developing xylem tissues of the stem, it needs to be suppressed by limiting the uptake of nitrate and amino acids from the xylem sap for the benefit of the photosynthetic nitrogen-use efficiency in the leaves. The negative correlation between the expression of specific members of the AAP and NRT1/NPF family members with plant diameter emphasizes their potential role in modulating nitrogen allocation, photosynthetic nitrogen-use efficiency and tree growth. Future investigations are warranted to validate these findings, paving the way for sustainable practices that optimize plant productivity in variable environmental conditions.

Acknowledgments

The authors thank Nathaniel Street for critical reading and commenting of the manuscript, Nicolas Delhomme at the UPSC Bioinformatic facility, Elin Nordin for qPCR primer design and Daria Chrobok (DC SciArt) for the illustration of the Figure 6. The authors acknowledge support from the National Genomics Infrastructure in Stockholm funded by Science for Life Laboratory, the Knut and Alice Wallenberg Foundation and the Swedish Research Council, and NAISS/Uppsala Multidisciplinary Center for Advanced Computational Science for assistance with massively parallel sequencing.

Author contributions

H.T. initiated the project. Y.P.Z. and M.C. performed the phylogenetic analyses, A.R. named genes in the phylogenetic analyses and performed the nitrate and ammonium treatments, M.L. produced the data for the eQTL analyses and the co-expression network, S.C. analyzed the RNA-seq data for the nitrate vs ammonium treatments, C.G., V.F., I.G. and O.N. created and analyzed the *PotraGLN1.2a* overexpressor lines, K.M.R. provided the data on the diameter of the SwAsp population, Y.P.Z. extracted the data from the AspWood database and the eQTL analysis and wrote the first draft of the manuscript. All authors contributed to the writing of the manuscript.

Supplementary Data

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

Conflict of interest

None declared.

Funding

This work was supported by grants from the Knut and Alice Wallenberg Foundation (KAW 2016.0352 and KAW 2020.0240), the Swedish Research Foundation Formas (grant 2021–00992), the Swedish Research Foundation VR (grant 2020–03799), Bio4Energy (www.bio4Energy.se, grant B4E3-FM-2-06) and Trees for the Future post doc program (diarie 2022.3.2.5–426).

Data availability

The raw reads from RNA-Sequencing of ammonia treated hybrid aspen xylem are available under the NCBI project ID# PRJNA1169771. The script for DESeq analysis and heatmaps can be found at https://github.com/shruti281989/nitrogenDESeq.

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Tree Physiology, 2025, 45, tpaf099
https://doi.org/10.1093/treephys/tpaf099
Research paper

Supplementary data

Figure S1. The expression of the members of aspen gene families related to nitrogen uptake, sensing and assimilation in response to fertilization with either nitrate or ammonium.

Figure S2. Presence of the proline residue in selected members of the NPF family in Arabidopsis and *Populus tremula*.

Figure S3. The expression of the members of the nitrogen uptake and metabolism related gene families in xylem rays, fibers, and vessels.

Table S1. Nitrogen uptake and metabolism related gene families in aspen (*Populus tremula)* and Arabidopsis.

Table S2. Gene expression analysis from RNA-sequencing of xylem tissue from hybrid aspen fertilized with either ammonium or nitrate.

Table S3. eQTL analysis of the NIN-like and NRT transcription factor family in a population of Swedish aspen trees.

Only supplementary figures is included in the thesis, tables can be found online.

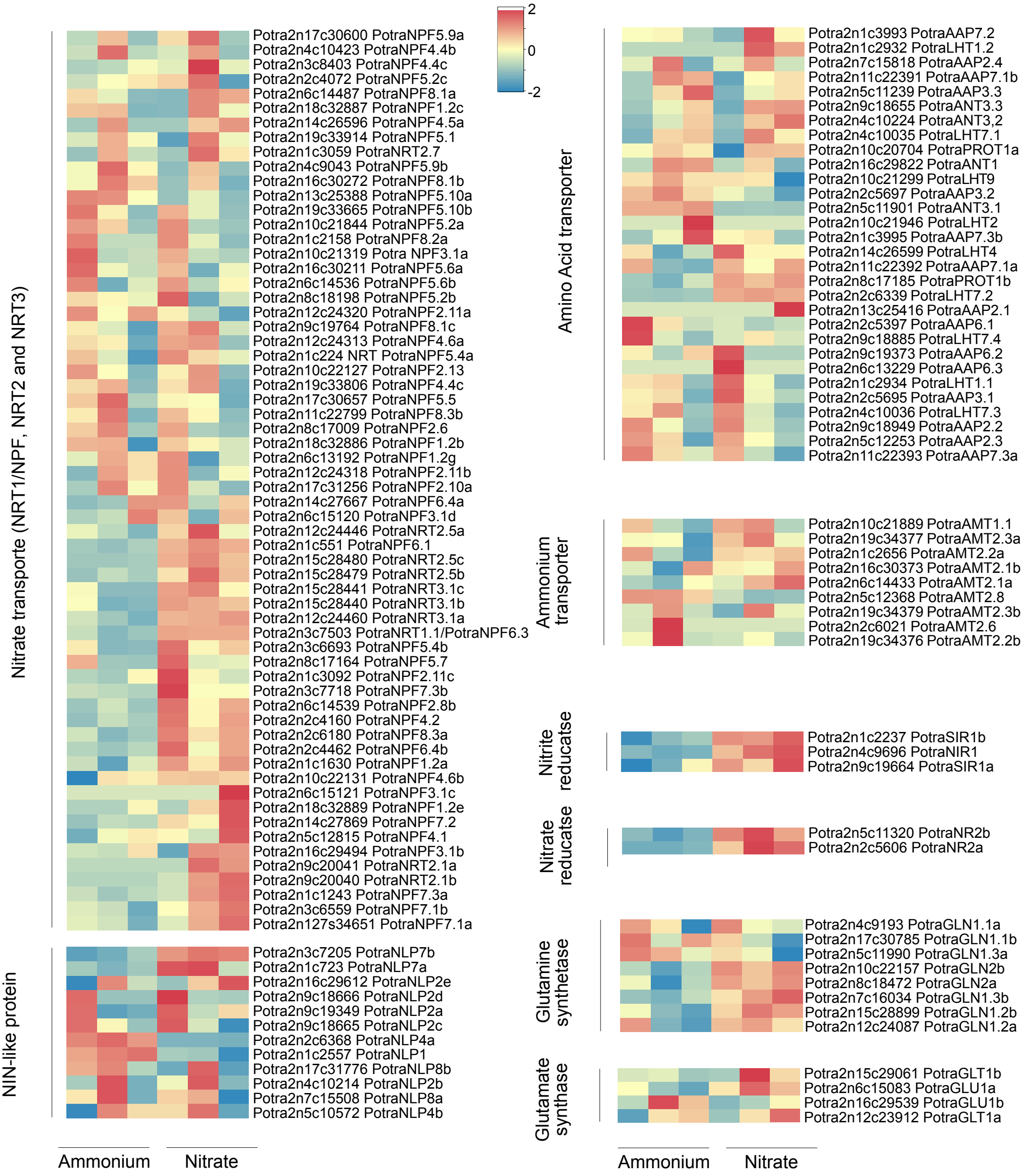

Figure S1. The expression of the members of *P. tremula* gene families related to nitrogen uptake, sensing and assimilation in response to fertilization with either nitrate or ammonium. The heatmaps represent gene expression data from RNA-sequencing of differentiating xylem tissues of hybrid aspen (*Populus tremula* × *P. tremuloides*) after long-term treatment with optimal, sub-optimal and limited doses of ammonia (NH_4^+, this study) and nitrate (NO_3^-, reported earlier in Renström et al. 2024). The data are VST normalized expression values scaled within each row (gene).

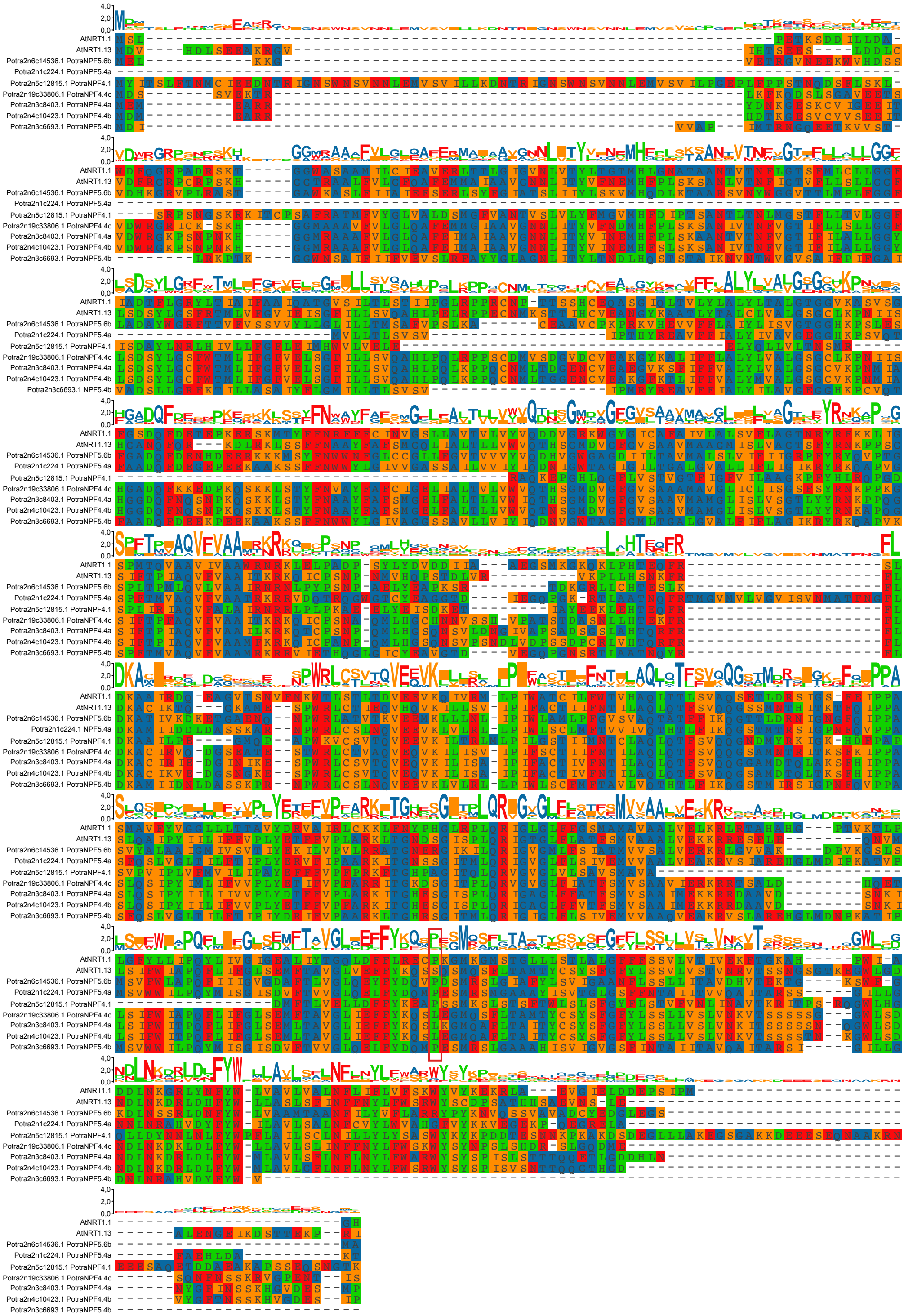

Figure S2. Presence of the proline residue in selected members of the NPF family in *Arabidopsis* and *Populus tremula*. The amino acid alignment was performed using the MAFFT. The aligned sequences were visualized by TBTOOL2. The red box indicates the proline residue in the aligned sequences.

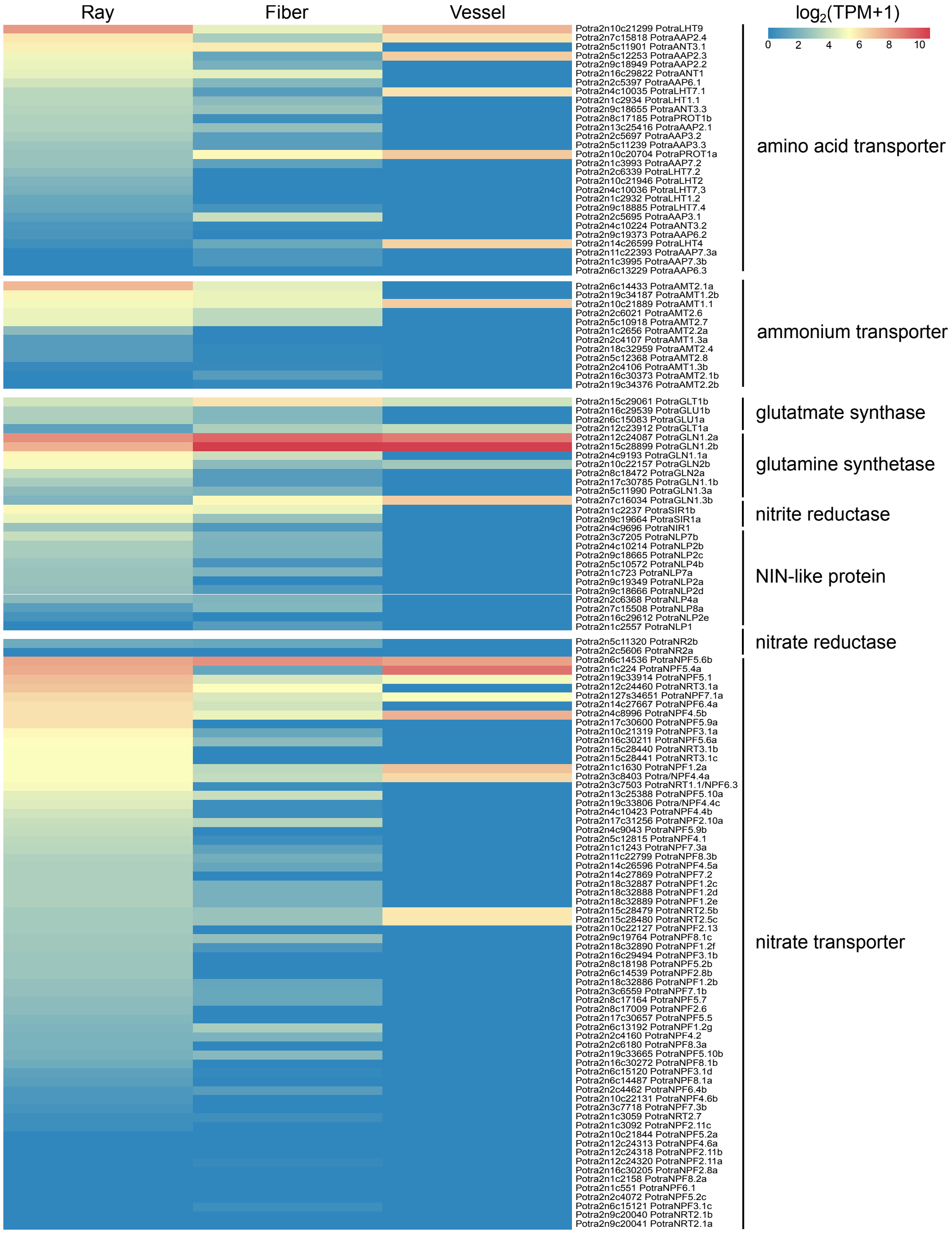

Figure S3. The expression of the members of the nitrogen metabolism related gene families in xylem rays, fibers, and vessels. The data is derived from RNA-sequencing of laser capture microdissected xylem cells from *P. trichocarpa*, performed by Tung et al. (2023). It should be noticed that the vessel sample (sample number 2 shown here) is likely to contain signal also from the other cell types, in particular the rays, as demonstrated in Tung et al. (2023). The gene expression data shown are log2(TPM+1) values

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Nitrogen is essential for tree growth and wood formation. While it is well established that nitrogen affects the quantity, structure, and chemistry of wood, less is known about the effects of different forms of nitrogen, the underlying molecular mechanisms, and the short-term responses in developing xylem. This research in hybrid aspen demonstrates that nitrate, in particular, stimulates shoot growth and the accumulation of H-type lignin. Advanced imaging and transcriptome analyses reveal nitrate's rapid impact on gene expression and xylem development.

Anna Renström received her graduate education at the Department of Forest Genetics and Plant Physiology at the Swedish University of Agricultural Sciences. She received her Master of Science degree in Bioresource Engineering at Umeå University.

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ISSN 1652-6880
ISBN (print version) 978-91-8124-072-6
ISBN (electronic version) 978-91-8124-118-1