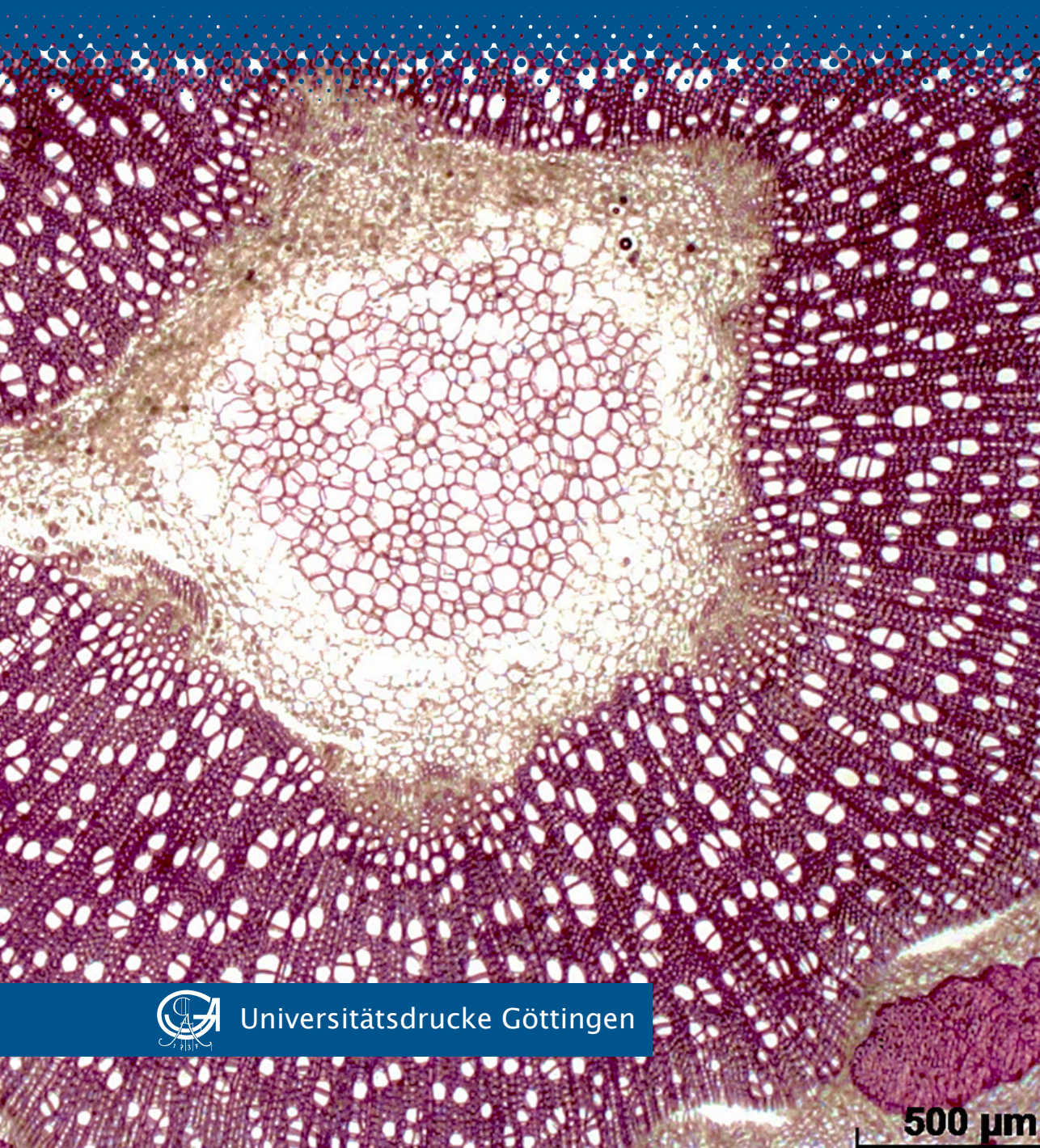
Aileen Kogel

Effects of Ammonium or Nitrate Nutrition on Wood Formation of *Populus* sp. and *Arabidopsis thaliana*



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List of abbreviations

°C .....Degrees Celsius
% .....Percent
% (v/v) .....Percent (volume/volume)
% (w/v) .....Percent (weight/volume)
A. thaliana .....*Arabidopsis thaliana*
a.m.....Ante meridiem
Am.....Ammonium
bp .....Base pair(s)
BP reaction .....Recombination reaction between PCR fragment and donor vector
BLAST .....Basic Local Alignment Search Tool
CCD .....*CAROTENOID CLEAVAGE DIOXYGENASE*
CCoAOmt1.....*CAFFEOYL COENZYME A DEPENDEND O-METHYL-TRANSFERASE 1*
CDS .....Coding sequence
cm .....Centimeter
Col-0.....Columbia 0
Cq.....Quantification cycle value of sample for gene
Dev. xylem .....Developing xylem
DNA .....Deoxyribonucleic acid

DNase ..... Deoxyribonuclease
dx ..... Developing xylem
EDTA ..... Ethylenediaminetetraacetic acid
EF1α ..... *ELONGATION FACTOR1-ALPHA*
F0 ..... Untransformed mother plant
F1 ..... The progeny of the F0 (parent plants), heterozygous genotype
F2 ..... Progenies from self-pollinated F1 transgenic plants, 1:2:1 genotype
F3 ..... Progenies from self-pollinated F2 transgenic plants
F4 ..... Self-pollinated F3 transgenic plants, homozygous genotype
Fig. ..... Figure
g ..... Gravitational force
GFP ..... Green Fluorescent Protein
g ..... Gram
GATA12 ..... *GATA TRANSCRIPTION FACTOR 12*
h ..... Hour(s)
HAm ..... High ammonium
HN ..... High nitrogen
HNA ..... High ammonium-nitrate
HNi ..... High nitrate
HT ..... Heterozygous
HM ..... Homozygous
IRX1 ..... *IRREGULAR XYLEM 1/ CELLULOSE SYNTHASE A CATALYTIC SUBUNIT 8*
IP ..... Insert primer
Kan ..... Kanamycine sulphate monohydrate
kb ..... Kilobases (1.000 bases)
L ..... Liter
LA ..... Long-Ashton
LAm ..... Low ammonium
LB ..... Luria Broth
LN ..... Low nitrogen
LNA ..... Low ammonium-nitrate
LNi ..... Low nitrate
LP ..... Primer flanking left border of T-DNA
LR reaction ..... Recombination reaction between attL and attR sites
m ..... Meter
M ..... Molar, mol/L
MAm ..... Moderate ammonium
Mat. xylem ..... Mature xylem
mg ..... Milligram
min ..... Minute(s)

mL .....Milliliter
mm .....Millimeter
mM .....Millimolar
MN.....Moderate nitrogen
MNi.....Moderate nitrate
mRNA .....Messenger RNA
MS medium .....Murashige & Skoog medium
mx.....Mature xylem
µ .....Micro
µg .....Microgram
µL .....Microliter
µm .....Micrometer
µM .....Micromolar
N_2 .....Nitrogen
Ni .....Nitrate
ng .....Nanogram
nm .....Nanometer
OE.....Overexpression
ODx .....Optical density at x nm wavelength
p. .....Page
P. trich. .....*Populus trichocarpa*
P. trichocarpa .....*Populus trichocarpa*
P. x *can.* .....*Populus* x *canescens*
P. x *canescens* .....*Populus* x *canescens*
p.m. .....Post meridiem
PAR .....Photosynthetically active radiation
PCR .....Polymerase chain reaction
PPR_2.....*PPR REPEAT FAMILY 2*
qRT-PCR .....Quantitative reverse transcription polymerase chain reaction (real-time)
r.h. .....Relative humidity
rcf .....Relative centrifugal force (1 rcf is equivalent to 1 g = 9.81 m/s)
Rif .....Rifampicin
RNA .....Ribonucleic acid
RNase .....Ribonuclease
RNase_P_Rpp14 ....*Rpp14/Pop5 RNASE FAMILY GENE*
RP.....Primer flanking right border of T-DNA
rpm .....Revolutions per minute
RT .....Reverse Transcriptase
Tab. .....Tab.
TAE .....Tris-acetate-EDTA
Taq .....*Thermus aquaticus*
TE .....Tris-EDTA buffer

TF .....Transcription factor
Tris .....Tris(hydroxymethyl)aminomethane
TUBULINß2..... *TUBULIN BETA-2 CHAIN*
UBC9.....*SUMO-CONJUGATING ENZYME UBC9*
UBQ10 .....*POLYUBIQUITIN 10*
V .....Volt
VND7.....Vascular related NAC-domain protein 7
VNS07..... *VASCULAR RELATED NAC-DOMAIN TRANSCRIPTION FACTOR 7*
VNS08 ..... *VASCULAR RELATED NAC-DOMAIN TRANSCRIPTION FACTOR 8*
WT .....Wild type
WND6A..... *WOOD-ASSOCIATED NAC DOMAIN TRANSCRIPTION FACTOR 6A*
WND6B ..... *WOOD-ASSOCIATED NAC DOMAIN TRANSCRIPTION FACTOR 6B*
YEB .....Yeast Extract Broth

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Summary

Nitrogen is an essential nutrient taken up by plants from the soil mainly as nitrate and ammonium. Poplars are fast-growing woody species that use both nitrogen forms for their nutrition. However, little is known how different forms of nitrogen nutrition influence biomass production and wood formation in poplar.

The goal of this study was to characterize growth, biomass production, anatomical effects and the regulation of genes involved in wood formation of poplars in response to nitrate or ammonium feeding. A further goal was to characterize a selected candidate gene *PtGATA12* functionally using poplar and Arabidopsis as model plants.

Poplars and Arabidopsis were exposed to low, intermediate and high nitrogen levels, administered in the forms of either ammonium or nitrate or ammonium nitrate. Secondary cell wall formation and cell lumina expansion of vessels and fibers in poplar stems and Arabidopsis hypocotyls were analysed histologically by phloroglucinol staining. High nitrate (8 mM) supply resulted in fast secondary growth and wood formation in poplar. But anatomically, the cell walls of vessels in the secondary xylem were much thinner and the vessel cell lumina were larger than under low nitrogen supply. These developmental effects were not found in response to ammonium. High ammonium (8 mM) fertilized plants displayed a higher wood density as response to high vessel cell wall thickening with enhanced lignin production and smaller vessel cell lumina compared with high nitrate fed poplars.

The expression values of transcription factors known to regulate wood formation were determined under different nitrogen treatments. High nitrate fertilized poplars exhibited less transcript abundances of *PtGATA12* and *PtWND6B*, a master regulator of wood formation, than under high ammonium supply. The transcript abundances of two genes involved in secondary cell formation, *PtCCoAOMT1* (CCoAOMT1 catalyzes a step in lignification) and *PtIRX1* (part of a cellulose synthase), were not increased under 8 mM ammonium although vessel cell walls were thicker than those of high nitrate fed poplars. Since the genes are members of larger gene families, we speculate that other cell wall biosynthetic genes might have been upregulated to achieve the formation of thicker walls.

PtGATA12 is a putative upstream regulator of *PtWND6B*. Therefore, PtGATA12 was chosen as the candidate for an overexpression study in poplar and Arabidopsis. Vessel cell walls of poplars fertilized with high nitrogen (8 mM) and fiber cell walls of Arabidopsis fed with low nitrogen (1 mM) were thicker in the *PtGATA12* overexpressing lines (*35S:PtGATA12*) than in the wildtype. No secondary xylem tissue was formed in *35S:PtGATA12* Arabidopsis plants fertilized with high nitrogen (8 mM). AtGATA12 knock-down plants formed smaller secondary cell lumina as well as thinner vessel cell walls when fertilized with 8 mM ammonium, while secondary xylem formation of AtGATA12 knock-down plants fed with 1 mM nitrate was unaffected. Therefore, we concluded that the impact of GATA12 on cell walls and wood density is dependent on the nitrogen form and level. It is possible that GATA12 plays a role as upstream regulator of vessel cell wall thickening under ammonium excess.

Zusammenfassung

Stickstoff ist ein essentieller Pflanzennährstoff, der hauptsächlich in Form von Nitrat oder Ammonium über die Wurzeln aufgenommen wird. Pappeln, die als schnellwachsende Baumarten gelten, können sich von beiden Stickstoffformen ernähren. Dennoch ist wenig darüber bekannt, wie die Ernährung von unterschiedlichen Stickstoffformen die Biomasseproduktion und Holzbildung in der Pappel beeinflusst.

Das Ziel dieser Studie war, Wachstumsverhalten, Biomasseproduktion, anatomische Effekte und Genregulation in der Holzbildung von Pappeln aufgrund von Nitrat- oder Ammoniumgabe zu charakterisieren. Ein weiteres Ziel stellte die funktionelle Charakterisierung eines gewählten Kandidatengens *PtGATA12* in Pappel- und Arabidopsis-Modellpflanzen dar.

Die Pappeln und Arabidopsis-Pflanzen wurden niedrigen, mittleren und hohen Stickstoffkonzentrationen ausgesetzt, die in Form von Ammonium, Nitrat oder Ammoniumnitrat verabreicht wurden. Die sekundäre Zellwandzunahme und Zellvolumenvergrößerung der Gefäß- und Faserzellen wurde anhand von Querschnitten der Pappelstämme und der Hypokotyle der Arabidopsis-Pflanzen histologisch mit Phloroglucine angefärbt und unter dem Mikroskop ausgemessen. Pappeln, die mit einer hohen Nitratkonzentration (8 mM) gedüngt wurden, zeigten ein schnelles Dickenwachstum und eine ausgeprägte Holzbildung. Anatomisch betrachtet, jedoch, waren die Gefäßzellwände im sekundären Xylem dieser Pflanzen viel dünner

und die Gefäßlumina voluminöser ausgeprägt als in den Pflanzen, die mit niedrigen Stickstoffkonzentrationen ernährt wurden. Diese Effekte in der Holzentwicklung zeigten sich allerdings nicht in den Pappeln, die mit Ammonium gedüngt wurden. Bei der Zugabe hoher Ammoniumkonzentrationen (8 mM), entwickelten die Pflanzen ein viel dichteres Holz, bedingt durch dickere Gefäßzellwände mit einem höheren Lignin-Gehalt, als unter einer hohen Nitratkonzentrationszugabe und kleineren Gefäßzelllumina.

Die Genexpression von Transkriptionsfaktoren, die bekannt dafür sind an der Holzbildung beteiligt zu sein, wurde in den unterschiedlich behandelten Pappeln bestimmt. Pappeln, die mit hohen Nitratkonzentrationen gedüngt wurden wiesen geringere Expressionswerte von *PtGATA12* und *PtWND6B*, eines der Hauptregulatoren im Holzbildungsprozess auf, als unter hoher Ammonium-Zugabe. Die Expressionswerte von zwei Genen, die an der sekundären Zellwandbildung beteiligt sind, *PtCCoAOMT1* (CCoAOMT1 katalysiert einen Schritt in der Lignifizierung) und *PtIRX1* (Teil der Zellulose-Synthese) waren in Pappeln, die mit 8 mM Ammonium ernährt wurden nicht erhöht, obwohl diese dickerer Zellwände aufwiesen als die, Pflanzen, die mit 8 mM Nitrat gedüngt wurden. Da diese Gene Teil einer größeren Genfamilie sind, nehmen wir an, dass womöglich andere biosynthetische Zellwand-Gene, die nicht getestet wurden, in diesen Pflanzen hochreguliert wurden, um dickere Zellwände zu erzielen.

PtGATA12, ist ein mutmaßlicher vorgeschalteter Regulator von *PtWND6B*. Deshalb wurde PtGATA12 als Kandidat für Überexpressionsstudien in der Pappel und in Arabidopsis ausgewählt. Die Gefäßzellwände der Pappeln die mit hohen Stickstoffkonzentrationen gedüngt wurden (8 mM), sowie die Faserzellwände der Arabidopsis-Pflanzen, die mit niedrigen Stickstoffkonzentrationen gedüngt wurden (1 mM), waren in den transgenen *PtGATA12*-Überexpressionslinien (*35S:PtGATA12*) stärker ausgeprägt als im Wildtyp. Die transgenen Arabidopsis-Pflanzen, denen hohe Stickstoffkonzentrationen zugegeben wurden (8 mM), zeigten generell keine Ausprägung des sekundären Xylems. Arabidopsis-Mutanten, die eine mangelnde Expression ihres eigenen *GATA12*-Gens (*AtGATA12*) aufwiesen, bildeten kleinere sekundäre Zelllumina und dünnere Gefäßzellwände, sobald sie mit 8 mM Ammonium gedüngt wurden. Währenddessen wurde die sekundäre Xylembildung in diesen *AtGATA12*-Mutanten unter 1 mM Nitrat-Zugabe nicht beeinflusst. Deshalb schlussfolgern wir, dass der Einfluss von GATA12 auf die Ausprägung der Zellwanddicke und Holzdichte abhängig von der Stickstoffform und -konzentration ist, die zugegeben wird. Es ist möglich, dass GATA12 als vorangeschalteter Transkriptionsfaktor einen Einfluss auf die Gefäßzellwandentwicklung hat, wenn Ammonium im Überschuss geboten wird.

1 Introduction

1.1 Nitrogen as a plant nutrient

1.1.1 Nitrogen effects on plant physiology

Nitrogen is an essential nutrient, which is involved in several plant physiological processes. It is necessary to form amino acids as the basic constituents of proteins and as precursor of purines and pyrimidine in the DNA. Furthermore, nitrogen can influence the activity of hormones like auxin, salicylic acid, abscisic acid, jasmonic acid, gibberellic acid, indole-3-acetic acid (IAA) and cytokinin in stress signaling pathways of plants (Aloni 2007; Luo et al. 2015; Nafisi et al. 2015). For example, Luo et al. (2015) observed reduced levels of IAA and salicylic acid in *P. simonii* roots and a decrease of IAA in *P. simonii* leaves under nitrogen excess. These findings imply that N has not only a direct effect on growth as an important building unit for metabolic compounds but also indirectly by its effect on growth hormones.

Nitrogen is taken up by plants from the soil mainly in the form of nitrate and ammonium (Rennenberg et al. 2010). Benefits depend on the ability of a plant species to use a certain nitrogen form; for example, ammonium is preferred by a number of woody plants, like conifers and broadleaved trees grown on acid soil (Zhang and Bai 2003; Wang et al. 2012). The presence of nitrogen fixing bacteria

or mycorrhizal infection can improve the nitrogen absorption of plants (Rennenberg et al. 2010).

1.1.2 Plant growth is influenced by different nitrogen levels

In agricultural areas, there is often a nitrogen surplus in soil (Bouraoui et al. 2009). Soil with nitrogen fixing bacteria contains usually 45 kg N/ha (Chamber of Agriculture Lower Saxony, 2018), a concentration which sustains plant growth. Because nitrogen availability is not uniform across different regions, plants need to adapt to varying nitrogen conditions. Due to intensive agriculture (potatoes, crop, rape, maize) and high animal density, many regions contain too much nitrogen, leading to a problem of excessive nitrogen content in soil. On the one hand, high nitrogen availability is leading to a faster plant growth and more biomass, including increased secondary growth (Martin and Carter 1967; Euring et al. 2014; Camargo et al. 2014). Furthermore, high nitrogen supply causes low wood density (Euring et al. 2014). On the other hand, nitrogen limitation leads to increased root proliferation and nitrogen uptake capacity (Rothstein et al. 2000). Nitrogen uptake is regulated, among others, by nitrate transporters (NRTs), for instance NRT1.1 and NRT2.1; these systems have been well studied in Arabidopsis and were found to be responsible for nitrate sensing as well as for the cross-talk with hormonal signalling (Ho et al. 2009; Krouk et al. 2010a; Krouk et al. 2010b). Nitrate sensing is important for lateral root growth (Wei et al. 2013; Remans et al. 2006a; Remans et al. 2006b), by initiating an early activation of cell growth associated genes in response to low nitrogen conditions (Wei et al. 2013). While ammonium is directly assimilated in roots after uptake (**Fig. 1.1**; Márquez et al. 2005), nitrate can be stored in the vacuole without

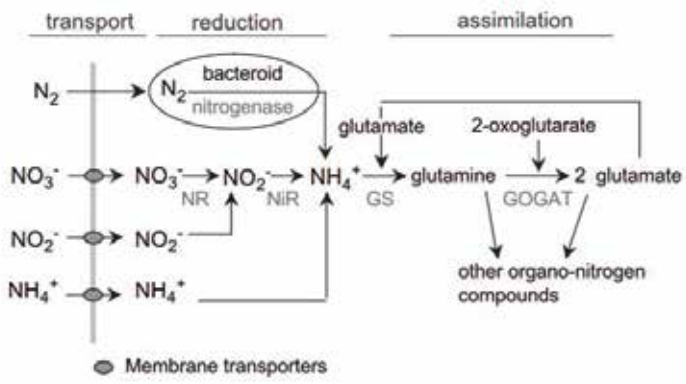


Fig. 1.1: *Nitrogen assimilation in plants. Ammonium can be directly used for glutamine synthesis. Nitrate first needs to be reduced by nitrate reductase (NR) and nitrite reductase (NiR) to ammonium. GS: glutamine synthase, GOGAT: glutamine oxoglutarate aminotransferase (taken from: Márquez et al. 2005).*

immediate assimilation. Nitrate is reduced to nitrite and then to ammonium by two plant enzymes, nitrate reductase (NR) and nitrite reductase (NiR) (**Fig. 1.1**). These differences in ammonium and nitrate assimilation pathways result in different responses in plant physiology and growth (Hachiya et al. 2012). In particular, the uptake and assimilation of nitrate (Temple et al. 1998) is more energy costly than direct uptake and utilization of ammonium in plants (Bassirirad et al. 1997). In poplar, nitrate is mainly assimilated in leaves (Black et al. 2002, Luo et al. 2015).

1.1.3 Wood density is influenced by different nitrogen levels

When plants sense high nitrogen availability, their metabolism is reprogramed (Rennenberg et al. 2010). In poplar, gene expression of NR, GOGAT (glutamine oxoglutarate aminotransferase) and GDH (glutamate dehydrogenase) is changed (Li et al. 2012; Luo et al. 2013). Furthermore, the expression of genes encoding enzymes implicated in amino acid biosynthesis is changed (Luo et al., 2015). Phenotypically these alterations affect elongation growth, developing xylem and cell wall formation (Plavcová et al. 2013; Euring et al. 2014). It has been shown that wood characteristics are influenced by nitrogen nutrition (Euring et al., 2014). Under nitrogen limitation, secondary cell wall related transcription factors such as members of MYB, basic helix-loop-helix (bHLH), WRKY and WD40 gene families, are up-regulated, leading to increased cell wall formation (van den Broeck et al., 2008; Camargo et al. 2014). In contrast to low nitrogen availability, the secondary cell walls of xylem are thinner and the vessel lumina are more expanded under high nitrogen levels in poplar (Plavcová et al. 2013; Camargo et al. 2014; Euring et al. 2014). Transcriptomic analyses of ammonium-fed Arabidopsis plants show down-regulation of the expression of cell wall related genes that regulate cell expansion (Patterson et al. 2010; Podgórska et al. 2015). It is therefore, likely that the impact of ammonium and nitrate on wood properties differ profoundly.

Under high ammonium-nitrate supply lignin biosynthesis is inhibited leading to a shift of the cellulose:lignin ratio (10 mM NH4NO3: Pitre et al. 2007a; Pitre et al. 2007b, 2 mM KNO3: Euring et al 2012), but underlying molecular mechanisms leading to these alterations have not been clarified. Lignin is an essential chemical cell wall component providing mechanical strength and stiffness of wood (Zhang et al. 2013). A relative reduction in lignin under high nitrogen may cause loss in stability, which can be compensated by an increasing amount of gelatinous layers of cellulose in fiber cells; consequently tension wood is distributed all over the stem (Camargo et al. 2014).

1.2 Poplar, a model tree to study the effects of NO3- and NH4+ on wood characteristics

1.1.1 Secondary growth and wood formation

Mitchell (1961) defined wood quality as physical and chemical properties of wood. The cell density is an indicator for a number of physical properties, like mechanical strength and compression. The ratio of cell wall components such as lignin and cellulose represents chemical properties. Wood formation is regulated by genetic network, involving the key transcription factors WNDs (wood-associated NAC domain transcription factors) and NACs (NAC domain transcription factors) (Zhong et al. 2011) (**Fig. 1.2**).

The process of secondary growth in dicotyledonous plants leads to wood production and starts when plant elongation is completed (Eckert et al. 2019). Here, phloem and xylem mother cells are formed by periclinical division from vascular cambium. Phloem cells differentiate in direction towards the outer part of the shoot. Xylem mother cells divide in direction to the pith, the inner part of the shoot. During wood formation, xylem cells pass through stages of cell wall thickening and eventually programmed cell death, to produce a functioning water transport system (Plomion et al. 2001; Courtois-Moreau et al. 2009; Ye and Zhong 2015). Consequently, the diameter of the stem increases (Jansson and Ingvarsson 2010).

Nitrogen has an effect on this secondary cell wall formation process in poplars (Euring et al. 2014). Under high nitrogen, the xylem cells form thinner secondary cell walls and synthesize less lignin (Plavková et al. 2013). The genetic regulation of this phenomenon is still unclear. Therefore, it was a goal of this study, to compare the characteristics of wood formed under the influence of high or low ammonium or nitrate and to study the transcriptional abundance of genes involved in secondary cell wall formation

1.2.2 Molecular network of wood formation in Arabidopsis and poplar

Wood formation is regulated by a cascade of transcription factors, denoted as first-layer master switches (Zhong et al. 2011, Nakano et al. 2015, Endo et al. 2015, **Fig. 1.2**). AtVND7 is a NAC domain transcription factor that can directly activate biosynthetic genes of cellulose, lignin and xylan and control wood formation by acting on MYB transcription factors and KNAT7 (Kubo et al. 2005, Zhong et al. 2006, Mitsuda et al. 2007, Zhong et al. 2008, Ohashi-Ito et al. 2010, Wang et al. 2014). The poplar enzyme PtrWND6B is a VND7 homologue and induces cell wall thickening, when overexpressed in Arabidopsis (Endo et al. 2015) and poplar (Zhong et al. 2010; Zhong et al. 2011). Gene expression analyses showed higher transcript abundances of cell wall biosynthetic genes (*CesA4*, *CesA7*, *CesA8*/ *IRX1*, *FRA8*, *IRX8*, *IRX9*, *4CL1* and *CCoAOMT1* Zhong et al. 2011) and of secondary

cell wall master switch regulators in both Arabidopsis and poplar overexpressing *PtrWND6B* (poplar: *PtrMYB3*, *PtrMYB21*, *PtrNAC157*, *PtrMYB128*, *PtrKNAT7*, *PtrLBD15*, *PtrNAC118*, Zhong et al. 2011).

But, the expression of the key gene *PtrWND6B* under various nitrogen conditions was still unknown. In this study, we determined the expression of this gene in nitrate and ammonium fertilized poplars. Additionally, the expression of the cellulose gene *PtrIRX1* (*PtrCesA8)* and the lignin gene *PtrCCoAOMT1*, highly expressed in *PtrWND6B* overexpression study (Zhong et al. 2011) were measured, to get an overview about lignin and cellulose biosynthesis regulation under different nitrogen supply. *PtrCCoAOMT1* synthesizes feruloylated polysaccharides, which are important for lignin formation. *PtrIRX1* catalyzes beta-1,4-glucan microfibril crystallization, a major step of cellulose formation.

In addition to the master switches regulating wood formation, upstream transcription factors are involved in the fine-tuning of secondary growth (Endo et al. 2015). For example, distinct GATA transcription factors play a role in regulating cell wall formation of secondary xylem tissue in plants (Endo et al. 2015). GATA transcription factors are zinc fingers specifically binding 5'-GATA-3' or 5'-GAT-3' motifs in gene promoters (Endo et al. 2015). GATA5 and GATA12 function as upstream regulators of the master switch for secondary cell wall formation AtVND7 in Arabidopsis (**Fig. 1.2B**, Endo et al. 2015). The Arabidopsis overexpressing *GATA12* exhibited an ectopic xylem vessel formation and secondary cell wall deposition (Endo et al. 2015). This finding suggests that GATA12 homologs may also be important for the modulation of cell wall thickness in poplar.

GATA transcription factors are also interesting because they might be links between cell wall properties and nitrogen physiology in plants. GATA transcription factors are involved in the nitrate assimilation pathway by functioning in NIR-gene regulation (Rastogi et al. 1997; Zhang et al. 2015). Additionally, the nitrate transporter genes NRT1 and NRT2 contain a GATA binding domain, suggesting a control of NRT expression by GATA transcription factors (Zhang et al. 2015).

Comprehensive analyses of GATA genes in plants are lacking. However, several studies imply a role of *GATA12* in nitrogen physiology and secondary cell wall formation in plants. First, GATA12 activates *VND7* expression in Arabidopsis (**Fig. 1.2B**, Endo et al. 2015). Second, the GATA12 homolog GmGATA60 in soybean is highly expressed in stem (Zhang, 2015), pointing to a putative function in secondary growth. Third, *GmGATA60* expression is upregulated in leaves and roots in response to 5 mM nitrogen supply within three days, before it comes back to moderate expression levels. Under limited nitrogen conditions (0.5 mM), the expression of *GmGATA60* is downregulated in roots, but initially upregulated in leaves and then downregulated as well.

Based on this background information, *GATA12* expression was measured in *P. trichocarpa* plants fertilized with different nitrogen forms and levels in this study. Additionally, the function of GATA12 in wood formation was clarified by genetic approaches.

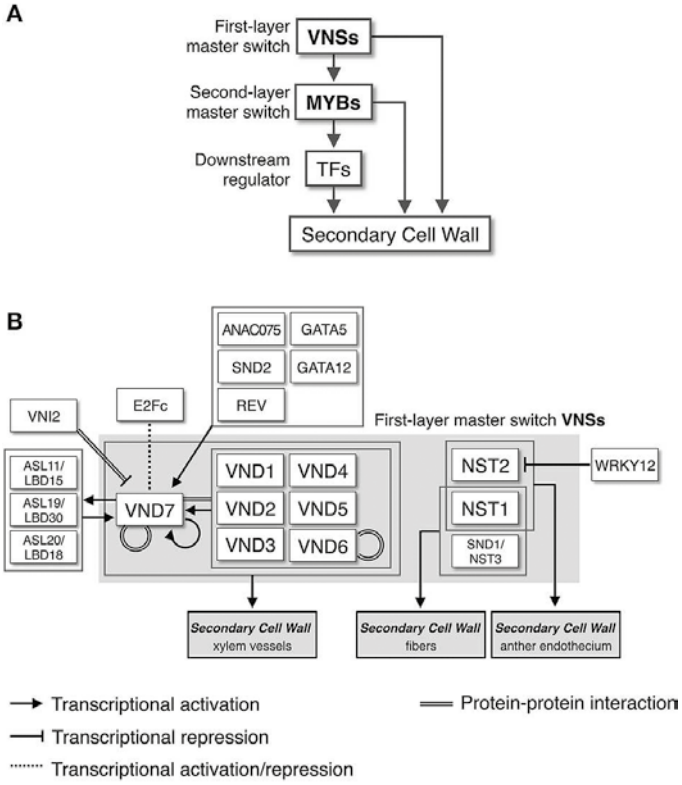


Fig. 1.2: *NAC-MYB-based transcriptional regulation of secondary cell wall biosynthesis in land plants (taken from: Nakano et al. 2015). A: First-layer master switches are regulating second-layer master switches, directly influencing downstream regulators. (VNSs: vascular related NAC-domain transcription factors, MYBs: MYB family transcription factors, TFs: transcription factors) B: Upstream regulators influence expression of first-layer master switches. (LBD15/LBD30/LBD18: lateral organ boundaries domain 15/30/18; VNI2: NAC domain transcription factor VND-INTERACTING2; E2Fc: member of a transcription factor family conserved in eukaryotes; ANAC075: Arabidopsis NAC DOMAIN containing protein 075; REV: Class III homeobox-leucine zipper protein; GATA5/GATA12: GATA-domain family member 5/12; VND1-7: vascular related NAC-domain 1-7; SND2: secondary wall-associated NAC domain 2; NST1-3: NAC secondary wall thickening promoting factor 1-3; WRKY12: WRKY-type transcription factor).*

1.3 Objectives

The main goal of this study was to characterize wood formation in poplar in response to nitrate and ammonium and to different nitrogen concentrations. Poplars (Family: Salicaceae) were chosen because they are excellent model plants for studying wood characteristics and wood formation. They are fast-growing tree species, with completely sequenced genome for *Populus trichocarpa* (Tuskan et al. 2006). Therefore,

this poplar species was chosen for experiments. Additionally, *Populus* x *canescens* (*P. alba* × *tremula*, Sm. clone INRA 717-1B4) was used in parallel, based on its easily transformation suitability (Stettler et al. 1996; Klopfenstein 1997). The biogenesis of wood is intensively being studied in poplar species (Plomion et al. 2001; Novaes et al. 2010; Ye and Zhong 2015; Rodriguez-Zaccaro and Groover 2019; Eckert et al. 2019). In addition, *Arabidopsis thaliana* was used in this study, because it is a small, well established model plant for understanding plant growth and development (Koornneef and Meinke 2010). The different plant species were propagated in different ways. *Populus trichocarpa* and *Populus* x *canescens* cuttings were cultivated in soil to imitate naturally conditions, in one experiment. Additionally, *in-vitro* poplars were grown as hydroponic culture under controlled conditions, in another experiment, to exclude phenotypical differences, which occurred just in the first experiment. Differences in poplar growth found in both experiments were considered as result of different nitrogen conditions.

This thesis had the following specific aims:

- To investigate growth and biomass production of poplar under low and high supply with ammonium or nitrate
- To characterize wood properties of ammonium- or nitrate-fed poplars
- To determine the expression of genes involved in wood formation
- To clarify the function of GATA in wood formation by genetic approaches.

2 Materials and Methods

2.1 Chemicals

Chemicals, kits and their manufacturers are listed in Tab. 2.1.

Tab. 2.1: *Chemicals and kits used for this work.*

Chemical name/Kit	Chemical formula	Supplier
1-naphthylamine	$C_{10}H_9N$	Merck KGaA, Darmstadt, Germany
Acetanilide	$C_6H_5NH(COCH_3)$	Elemental Microanalysis, Okehampton, Devon, United Kingdom
Acetic acid (96 % v/v)	CH_3COOH	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Acetone	C_3H_6O	Merck KGaA, Darmstadt, Germany
Acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone)	$C_{10}H_{12}O_4$	Sigma-Aldrich Corporation, St. Louis, U.S.A.

Chemical name/Kit	Chemical formula	Supplier
Acetyl bromide	C_2H_3BrO	Sigma-Aldrich Corporation, St. Louis, U.S.A.
Agar, BD Bacto TM, No. 214010		Becton, Dickinson and Company, Sparks, U.S.A.
Ammonium chloride	NH_4Cl	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
ß-mercaptoethanol	C_2H_6OS	Sigma-Aldrich Corporation, St. Louis, U.S.A.
Beef extract, BD Difco TM, No. 212610		Becton, Dickinson and Company, Sparks, U.S.A.
Boric acid	H_3BO_3	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Calcium chloride	$CaCl_2$	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Calcium chloride dehydrate	$CaCl_2 \cdot 2H_2O$	Merck KGaA, Darmstadt, Germany
Calcium nitrate tetrahydrate	$Ca(NO_3)_2 \cdot 4H_2O$	Merck KGaA, Darmstadt, Germany
Cefotaxime sodium	$C_{16}H_{16}N_5O_7S_2Na$	Duchefa Biochemie B.V, Haarlem, The Netherlands
Chlorine dioxide	ClO_2	DanKlorix, Colgate-Palmolive GABA GmbH, Hamburg, Germany
Chloroform	$CHCl_3$	Merck KGaA, Darmstadt, Germany
Cobalt(II) sulfate heptahydrate	$CoSO_4 \cdot 7H_2O$	Merck KGaA, Darmstadt, Germany
Coniferyl alcohol	$C_{10}H_{12}O_3$	Sigma-Aldrich Corporation, St. Louis, U.S.A.
Copper(II) sulfate	$CuSO_4$	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Dipotassium phosphate	K_2HPO_4	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethanol (96 % v/v)	C_2H_6O	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethanol (99.6 % v/v)	C_2H_6O	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethidium bromide	$C_{21}H_{20}BrN_3$	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethylenediaminetetraacetate	$C_{10}H_{16}N_2O_8$	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

Chemical name/Kit	Chemical formula	Supplier
Ferric ethylenediaminetetraacetic acid	$C_{10}H_{13}FeN_2O_8$	Sigma-Aldrich Corporation, St. Louis, U.S.A.
Gateway™ BP Clonase™ II Enzyme mix, No. 11789020		Invitrogen™, Carlsbad, United States
Gateway™ LR Clonase™ Enzyme mix; No. 11791100		Invitrogen™, Carlsbad, United States
Gentamycin sulphate	$C_{60}H_{125}N_{15}O_{25}S$	Duchefa Biochemie B.V, Haarlem, The Netherlands
Glufosinate (BASTA)	$C_5H_{12}NO_4P$	Bayer Cropscience Deutschland GmbH, Langenfeld, Germany
Glycerol (v/v)	$C_3H_8O_3$	Sigma-Aldrich Corporation, St. Louis, U.S.A.
Hydroxylamine	NH_2OH	Sigma-Aldrich Corporation, St. Louis, U.S.A
innuPREP Plant DNA Kit, No. 845-KS-1060250		Analytik Jena AG, Jena, Germany
innuPREP Plasmid Mini Kit 2.0, No. 845-KS-5041050		Analytik Jena AG, Jena, Germany
Isopropanol	C_3H_8O	Merck KGaA, Darmstadt, Germany
Kanamycine sulphate monohydrate	$C_{18}H_{36}N_4O_{11}\cdot H_2O$	Duchefa Biochemie B.V, Haarlem, The Netherlands
Magnesium sulfate	$MgSO_4$	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Magnesium chloride for PCR (25 mM)	$MgCl_2$	Thermo Scientific, Thermo Fisher Scientific, Braunschweig, Germany
Manganese(II) sulfate	$MnSO_4 \cdot 2H_2O$	Merck KGaA, Darmstadt, Germany
Methanol	CH_4O	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Monopotassium phosphate	KH_2PO_4	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
MS medium including vitamins, No. M0222.0005		Duchefa Biochemie B.V, Haarlem, The Netherlands
Muriatic acid (25 %)	$HCl \cdot 6H_2O$	Merck KGaA, Darmstadt, Germany
Natrium chloride	NaCl	Duchefa Biochemie B.V, Haarlem, The Netherlands
Nessler reagent	1:1, $K_2[HgI_4]$: NaOH	Merck KGaA, Darmstadt, Germany

Chemical name/Kit	Chemical formula	Supplier
NucleoSpin® Plasmid, No. 740499.50		Macherey - Nagel GmbH & Co. KG, Düren, Germany
Peptone, BD Bacto TM, No. 211677		Becton, Dickinson and Company, Sparks, U.S.A.
Phloroglucinol	$C_6H_6O_3$	Sigma-Aldrich Corporation, St. Louis, U.S.A.
Phusion HF Buffer 5X		Thermo Scientific, Thermo Fisher Scientific, Braunschweig, Germany
Phusion High-Fidelity DNA Polymerase (2 U/µL)		Thermo Scientific, Thermo Fisher Scientific, Braunschweig, Germany
PCR Buffer $(NH_4)_2SO_4$ 10X		Thermo Scientific, Thermo Fisher Scientific, Braunschweig, Germany
Potassium nitrate	KNO_3	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
QIAquick gel extraction kit, No. 28706		Qiagen, Venlo, Netherlands
RevertAid First Strand cDNA Synthesis Kit, No. K1621		Thermo Scientific, Thermo Fisher Scientific, Braunschweig, Germany
Rifampicin	$C_{43}H_{58}N_4O_{12}$	Duchefa Biochemie B.V, Haarlem, The Netherlands
RNeasy Plant Mini Kit, No. 74904		Qiagen, Venlo, Netherlands
Silwet® L-77, Vac-In-Stuff, No. VIS-01		Lehle Seeds, Texas, U.S.A.
Sodium chloride	NaCl	Merck KGaA, Darmstadt, Germany
Sodium chlorite	$NaClO_2$	Sigma-Aldrich Corporation, St. Louis, U.S.A.
Sodium dodecylsulphate	$C_{12}H_{25}NaO_4S$	Sigma-Aldrich Corporation, St. Louis, U.S.A.
Sodium hydroxide	NaOH	Merck KGaA, Darmstadt, Germany
Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	Merck KGaA, Darmstadt, Germany
Spectinomycin HCl pentahydrate	$C_{14}H_{24}N_2O_7 \cdot 2HCl \cdot 5H_2O$	Duchefa Biochemie B.V, Haarlem, The Netherlands
Sucrose	$C_{12}H_{22}O_{11}$	Duchefa Biochemie B.V, Haarlem, The Netherlands

Chemical name/Kit	Chemical formula	Supplier
SYBR Green I Master kit, No. 04707516001		Roche Diagnostics, Mannheim, Germany
Taq Polymerase (5 U/µL)		Thermo Scientific, Thermo Fisher Scientific, Braunschweig, Germany
Thidiazuron	$C_9H_8N_4OS$	Duchefa Biochemie B.V, Haarlem, The Netherlands
Ticarcillin dissodium/clavulanate potassium, No.T0190.0002		Duchefa Biochemie B.V, Haarlem, The Netherlands
Triptone, BD Bacto TM, No.211705		Becton, Dickinson and Company, Sparks, U.S.A.
Tris hydrochloride	Tris-HCl	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Tris(hydroxymethyl)-aminomethane hydrochloride	$C_4H_{11}NO_3$	Sigma-Aldrich Corporation, St. Louis, U.S.A.
Triton X-100 (t-octylphenoxypolyethoxy-ethanol)	$C_{14}H_{22}O(C_2H_4O)n$ (n = 9-10)	Sigma-Aldrich Corporation, St. Louis, U.S.A.
Turbo DNA free™ kit, No. AM1907		Invitrogen^{TM}, Carlsbad, United States
Yeast extract, BD Bacto TM, No. 212750		Becton, Dickinson and Company, Sparks, U.S.A.
Zinc powder	Zn	Merck KGaA, Darmstadt, Germany
Zinc sulfate heptahydrate	$ZnSO_4 \cdot 7H_2O$	Merck KGaA, Darmstadt, Germany

2.2 Plant materials

2.2.1 P. trichocarpa and Populus x canescens cuttings

P. trichocarpa Torr. and A. Gray ex Hook (Malpighiales: Salicaceae) cuttings (Muhle Larsen, Nordwestliche Versuchsanstalt Hannoversche Münden) and *Populus x canescens* (*P. alba* × *tremula*, Sm. clone INRA 717-1B4) cuttings were planted in 1 L pots with 20 % (w/v) low nutrient soil (Null type, Fruhstorfer 0-Erde, Industrieerdenwerk Erich Archut, Lauterbach/Fulda, Germany) and 80 % (w/v) washed sand (20 % (w/v) fine sand Ø 0.4 to 0.8 mm and 60 % (w/v) coarse sand Ø 0.71 to 1.25 mm; Interseroh Dienstleistungs GmbH, Cologne, Germany) (Euring et al. 2014; Müller et al. 2013) and placed in greenhouse. The plants were grown for 2 months

in a greenhouse at ambient temperatures ranging from 18 °C to 24 °C at 60 to 70 % r.h. and additional light with PAR of 150 µmol photons m^{-2} s^{-1} (fluorescent lamps L58W/25 and 58W/840, Osram, Munich, Germany and TLD 58W/840, Philips, Amsterdam, Netherlands) for 16 h per day (6:00 a.m. to 10:00 p.m.).

2.2.2 Populus x canescens micropropagation

In vitro Populus x *canescens* (*P. alba* × *tremula*, Sm. clone INRA 717-1B4) plantlets were used as wildtype reference and as host for production of transgenic overexpression lines (*35S:PtGATA12*). They were multiplied by micropropagation in WECK glasses (580 mL) containing ½ MS agar (0.22 % (w/v) MS medium including vitamins (Murashige and Skoog, 1962), 2 % (w/v) sucrose and 0.7 % (w/v) plant agar, pH: 5.8)). A Paramoll N260/200 fleece ring (Mank GmbH, Dernbach, Germany) was placed between the glass and the lid for gas exchange. The WECK glass was closed with Micropore surgical (3 M GmbH, Neuss, Germany) and grown in an air-conditioned room (22 °C, 20 to 40 % r.h.; 70 µmol photons m^{-2} s^{-1}, fluorescent lamps L18W/840, Osram, Munich, Germany, 16/8 h light). For the experiments, approximately 6 cm tall *in vitro* grown rooted plants were transferred in 3 L pots with 20 % (w/v) low nutrient soil (Null type, Fruhstorfer 0-Erde, Industrieerdenwerk Erich Archut, Lauterbach/Fulda, Germany) and 80 % (w/v) washed sand (20 % (w/v) fine sand Ø 0.4 to 0.8 mm and 60 % (w/v) coarse sand Ø 0.71 to 1.25 mm; Interseroh Dienstleistungs GmbH, Cologne, Germany) (Euring et al. 2014; Müller et al. 2013) and transferred to a greenhouse. Plants were covered with a transparent lid, lifted up regularly for acclimatization during one week, before the experimental treatments started.

2.2.3 Arabidopsis thaliana seeds

Arabidopsis thaliana Col-0 wildtype plants were used as reference and as host for production of transgenic overexpression lines (*35S:PtGATA12*). Additionally, *A. thaliana* AtGATA12 (At5g25830) T-DNA loss-of-function mutants SALK_112752, SALK_143606 and SALK_012501 were ordered from The Nottingham Arabidopsis Stock Center (NASC, University of Nottingham, Loughborough, United Kingdom). All *Arabidopsis thaliana* seeds were stored in a dark and dry place until they were needed for experiment. When seeds were required for germination, they were first sterilized by the following procedure. 500 µL deionized H_2O, 500 µL chlorine dioxide and 2 µL of 2 % (v/v) Triton X-100 were mixed with approximately 100 seeds. After an incubation period of 12 to 15 min, five washing steps followed in 500 µL deionized H_2O each. Seeds were stratified for three days at 4 °C and then sown individually for germination in 250 mL pots with T-type soil (Fruhstorfer Erde Typ T, Industrieerdenwerk Erich Archut, Lauterbach/Fulda, Germany) placed in growth

chambers (Percival Scientific, Perry, Iowa, U.S.A.) at 20 to 22 °C. They were grown for one week under short-day conditions (8 h light, 120 to 130 µmol photons m^{-2} s^{-1}, fluorescent lamps L18W/840, Osram, Munich, Germany) and covered with a plastic dome to keep the relative humidity at 60 to 80 %. Then, the seedlings were grown under long-day conditions (16 h light, 140 to 150 µmol photons m^{-2} s^{-1}, fluorescent lamps L18W/840, Osram, Munich, Germany, r.h. 60 %) without the plastic dome.

2.3 Experimental treatments and harvests

2.3.1 Nitrogen treatments of P. trichocarpa and P. x canescens in soil

P. trichocarpa and *Populus* x *canescens* plantlets of cuttings were potted in 3 L pots with 20 % (w/v) low nutrient soil (Null type, Fruhstorfer 0-Erde, Industrieerdenwerk Erich Archut, Lauterbach/Fulda, Germany) and 80 % (w/v) washed sand (20 % (w/v) fine sand Ø 0.4 to 0.8 mm and 60 % (w/v) coarse sand Ø 0.71 to 1.25 mm; Interseroh Dienstleistungs GmbH, Cologne, Germany) (Euring et al. 2014; Müller et al. 2013). The plants were grown in a greenhouse at ambient temperatures ranging from 18 °C to 26 °C at 60 to 70 % r.h. and additional light with PAR of 150 µmol photons m^{-2} s^{-1} (fluorescent lamps L58W/25 and 58W/840, Osram, Munich, Germany and TLD 58W/840, Philips, Amsterdam, Netherlands) for 16 h per day (6:00 a.m. to 10:00 p.m.) for approximately one month without nutrient supply. After this period, selected plants of similar height were grown for five to seven weeks with fertilizer (modified Long Ashton (Hewitt, 1966) (LA) nutrient solution). The LA nutrient solution contained either 0.2 mM (LN) or 8 mM nitrogen (HN), in form of nitrate, ammonium or ammonium-nitrate (**Tab. 2.2**). For this purpose, a LA nutrient stock solution was prepared in a 100 L container as follows: 500 mL of 37 g/L $MgSO_4$, 500 mL of 40.82 g/L KH_2PO_4, 500 mL of 3.6 g/L K_2HPO_4, 500 mL of 1.84 g/L Fe-EDTA and 500 mL micronutrients (309 mg/L H_3BO_3, 169 mg/L $MnSO_4 \cdot 2H_2O$, 846 mg/L $Na_2MoO_4 \cdot 2H_2O$, 5.6 mg/L $CoSO_4 \cdot 7H_2O$, 28.8 mg/L $ZnSO_4 \cdot 7H_2O$ and 16 mg/L $CuSO_4$). To achieve the NH_4^+ and NO_3^- target concentrations, further nutrients were added as indicated in Tab. 2.2 and then the volume was adjusted to 100 L by adding ddH_2O. The amount of nutrient solution supplied per plant was increased depending on the mean size of the plants. The amount of daily nutrient solution input per pot was 100 mL, when the plants were smaller than 30 cm and increased to 200 mL, 300 mL and 400 mL, respectively, when the plants reached 30 cm, 50 cm, and 75 cm in height. The respective treatment period was six weeks for *P. trichocarpa*, seven weeks for *P.* x *canescens* under HN treatment and five weeks for *P.* x *canescens* under LN treatment. At harvest, stem pieces of 5 cm (S1) were collected from basal parts of six to eight plants per treatment for anatomical examination (**Fig. 2.1**). For ammonium and nitrate determination, the second and third fully expanded leaf and an upper stem part of 5 cm (S3), as well as a basal stem part of 5 cm (S2) were

harvested (**Fig. 2.1**). Ten centimeter stem parts (S4) were debarked and developing xylem (dx) tissue was scratched with Apollo-ever-sharp-blades (Apollo-Herkenrath, Solingen, Germany) directly into a sterile 2 mL reaction tube (**Fig. 2.1**). All samples were immediately shock-frozen in liquid nitrogen and stored at -80 °C.

Tab. 2.2: *Modified Long Ashton (Hewitt, 1966) stocks for A: 0.2 mM (LN) or B: 8 mM nitrogen (HN) supplied in form of nitrate (Ni), ammonium (Am) or ammonium-nitrate (NA). Further nutrients are listed in the text. Treatment solution of 100 L contains 0.2 mM nitrogen in the LN and 8 mM nitrogen in the HN solutions, respectively.*

	0.2 mM nitrogen (LN)		
A	**LNA**	**LNi**	**LAm**
	g/L	g/L	g/L
KNO_3	2.02	4.04	
KCl	58.15	56.66	59.64
NH_4Cl	1.07		2.14
$CaCl_2 \cdot 2H_2O$	58.81	58.81	58.81
$Ca(NO_3)_2 \cdot 4H_2O$			
	8 mM nitrogen (HN)		
B	**HNA**	**HNi**	**HAm**
	g/L	g/L	g/L
KNO_3	80.89	80.89	
KCl			59.64
NH_4Cl	42.80		85.60
$CaCl_2 \cdot 2H_2O$	58.81		58.81
$Ca(NO_3)_2 \cdot 4H_2O$		94.70	

2.3.2 Nitrogen treatments of P. trichocarpa and P. x canescens in hydroponics

Plantlets of *P. trichocarpa* (Weser 6) and *P.* x *canescens* were grown in ½ MS agar including vitamins with 2 % (w/v) sucrose and 0.7 % (w/v) plant agar, pH 5.8 in an air-conditioned room at standard *in vitro* conditions (22 °C, 20 to 40 % r.h.; 70 µmol photons m^{-2} s^{-1}, fluorescent lamps L18W/840, Osram, Munich, Germany, 16/8 h light). Plantlets of similar height, were transferred from sterile culture in liquid LA culture medium to a greenhouse and grown at a temperature of 24 °C, 60 to 70 % r.h., and additional light with PAR of 140 µmol photons m^{-2} s^{-1} (fluorescent lamps 3071/400 HI-I, IP55, 230 V, 50 Hz, Adolf Schuch GmbH, Worms, Germany) for 16 h per day (6:00 a.m. to 10:00 p.m.). The plants were grown in hydroponic culture using 1.8 L pots with modified LA nutrient solution containing 2 mM

nitrogen as of nitrate, ammonium or ammonium-nitrate (adapted from **Tab. 2.2**). The hydroponic solutions in 1.8 L pots were changed twice a week until the plants were 10 cm tall and thereafter daily. After ten weeks all plants were harvested and samples were prepared as described in chapter 2.3.1.

2.3.3 Nitrogen treatment of genetically modified P. x canescens plants in soil

Line 8 and line 10 of positively transformed *35S:PtGATA12 P.* x *canescens* plantlets as well as wildtype *P.* x *canescens* were potted in 3 L pots containing 20 % (w/v) low nutrient soil (Null type, Fruhstorfer 0-Erde, Industrieerdenwerk Erich Archut, Lauterbach/Fulda, Germany) and 80 % (w/v) washed sand (20 % (w/v) fine sand Ø 0.4 to 0.8 mm and 60 % (w/v) coarse sand Ø 0.71 to 1.25 mm; Interseroh Dienstleistungs GmbH, Cologne, Germany) (Euring et al. 2014; Müller et al. 2013). The transformation of the plants is described below (chapter 2.12.2). The plants were grown in a greenhouse at ambient temperatures ranging from 19 °C to 24 °C at 60 to 70 % r.h. and additional light with PAR of 140 µmol photons m^{-2} s^{-1} (fluorescent lamps 3071/400 HI-I, IP55, 230 V, 50 Hz, Adolf Schuch GmbH, Worms, Germany) for 16 h per day (6:00 a.m. to 10:00 p.m.). The plants were grown for approximately one month without nutrient supply. After this period, selected plants of similar height were grown for eight weeks with fertilizer (8 mM LA nutrient solution, **Tab. 2.2**). The LA nutrient solution contained 8 mM nitrogen, in form of nitrate, ammonium or ammonium-nitrate. The treatment solution was prepared in a 100 mL container as described in 2.3.1. The amount of nutrient solution supplied per plant was increased depending on the mean size of the plants. The amount of daily nutrient solution input per pot was 25 mL, when the plants were smaller than 5 cm and increased to 50 mL, 100 mL, 200 mL, and 300 mL, respectively, when the plants reached 5 cm, 12.5 cm, 30 cm and 45 cm in height. After the treatment period all plants were harvested, as described in chapter 2.3.1.

2.3.4 Nitrogen treatment of transgenic A. thaliana lines: OE-PtGATA12 and SALK mutants

Transformed *A. thaliana* F4-seeds from three lines, which are homozygous for the *PtGATA12* overexpressing insert, termed as line g4-2B3, line g4-2G1 and line g4-8C1; (*35S:PtGATA12*, glufosinate resistant; **Tab. 2.3**), as well as wildtype seeds (Col-0) and homozygous AtGATA12 SALK seeds of line 58 and 60 (origin: SALK_012501, **Tab. 2.3**), were harvested, sterilized and sown as previously described (chapter 2.2.3). Ordered AtGATA12 SALK seeds of origin 143606C were not homozygous for the insert and SALK_112752 seeds did not survive. Therefore the experiment was performed with plants of origin SALK_012501 only. The generation of the transgenic plants is described below (chapter 2.12.3 and 2.12.4). After stratification,

seeds were placed individually for germination in 250 mL pots with 20 % (w/v) low nutrient soil (Null type, Fruhstorfer 0-Erde, Industrieerdenwerk Erich Archut, Lauterbach/Fulda, Germany) and 80 % (w/v) washed sand (20 % (w/v) fine sand Ø 0.4 to 0.8 mm and 60 % (w/v) coarse sand Ø 0.71 to 1.25 mm; Interseroh Dienstleistungs GmbH, Cologne, Germany) (Euring et al. 2014; Müller et al. 2013), covered with a plastic dome. The seeds germinated in a greenhouse at ambient temperatures ranging from 18 °C to 23 °C at 60 to 70 % r.h. and additional light with PAR of 150 µmol photons m^{-2} s^{-1} (fluorescent lamps L58W/25 and 58W/840, Osram, Munich, Germany and TLD 58W/840, Philips, Amsterdam, Netherlands) for 8 h per day (9:00 a.m. to 5:00 p.m.). After one week, the plastic dome was removed and the conditions were changed to long-day (16 h light, 6:00 a.m. to 22:00 p.m., 150 µmol photons m^{-2} s^{-1}, r.h. 60 - 70 %) and nutrient supply started. Twenty plants per line and treatment with two cotyledons were selected for nutrient experiments. The plants were fertilized for eight weeks with modified LA nutrient solution containing either 1 mM (LN) or 8 mM nitrogen (HN) as nitrate or ammonium (**Tab. 2.4**). The treatment solution was prepared in a 100 mL container as described in 2.3.1. The amount of nutrient solution input per pot started with 12.5 mL once at the beginning and continued with 25 mL every second day. After the treatment period, hypocotyls were collected and shock-frozen in liquid nitrogen from basal parts of four plants per treatment and line for anatomical examination and stored at -80 °C.

Tab. 2.3: *Homozygous 35S:PtGATA12 lines g4-2B3, g4-2G1 and g4-8C1, as well as homozygous AtGATA12 SALK mutant lines 58 and 60 are listed with their background information and respective plant resistance marker.*

Line	Background	Gene name	Potri-ID	Promotor	Resistance
g4-2B3	*35S:PtGATA 12*	*PtGATA 12*	Potri.018G044900	35S, over-expression	glufosinate
g4-2G1	*35S:PtGATA 12*	*PtGATA 12*	Potri.018G044900	35S, over-expression	glufosinate
g4-8C1	*35S:PtGATA 12*	*PtGATA 12*	Potri.018G044900	35S, over-expression	glufosinate
SALK 58	SALK_012501, pROK2-AtGATA12	*AtGATA 12*	At5G25830	pROK2 T-DNA insertion	kanamycine sulphate monohydrate
SALK 60	SALK_012501, pROK2-AtGATA12	*AtGATA 12*	At5G25830	pROK2 T-DNA insertion	kanamycine sulphate monohydrate

Tab. 2.4: *Long Ashton stocks for 1 mM (LN) or 8 mM nitrogen (HN) supply in form of nitrate (Ni) or ammonium (Am). Further nutrients are listed in 2.3.1. Treatment solution of 100 L contains 1 mM nitrogen in the LN and 8 mM nitrogen in the HN solutions, respectively.*

	1 mM nitrogen (LN)		8 mM nitrogen (HN)	
	LNi	LAm	HNi	HAm
	g/L	g/L	g/L	g/L
KNO_3	20.22		80.89	
KCl	44.73	59.64		59.64
NH_4Cl		10.70		85.60
$CaCl_2 \cdot 2H_2O$	58.81	58.81		58.81
$Ca(NO_3)_2 \cdot 4H_2O$			94.70	

2.4 Growth measurements

2.4.1 Growth measurements of poplar

During each poplar experiment (chapter 2.3.1. to 2.3.3), plant heights and stem diameters were measured weekly with a folding ruler and a caliper (Tchibo GmbH, Hamburg, Germany). Every plant tip was marked at treatment start and the height was noticed as starting point. The diameter was measured during experimental growth on three defined positions per plant: +1 cm, +5 cm and +10 cm above the marked position. Furthermore, leaf numbers and relative chlorophyll content were examined once a week. The relative chlorophyll content was measured on six positions of the second fully expanded poplar leaf with a chlorophyll-meter, Dualex Scientific TM (FORCE-A, Orsay Cedex, France). Instrument calibrations of the optical sensor were performed without a leaf between the closed clips, before each measurement. The recorded values were not validated by photometric measurements and therefore are considered as relative values. Relative chlorophyll content was displayed as an index calculated as leaf chlorophyll content (µg/cm²). Before harvest, first fully expanded poplar leaves of three biological replicates per treatment were measured with a chlorophyll fluorometer, Imaging-PAM (Walz GmbH, Effeltrich, Germany) using increasing light pulses of increasing light intensities from 0 PAR to 701 PAR

At harvest, fresh biomass of each poplar plant was determined with a precision balance (Sartorius AG, Göttingen, Germany), separately for roots, leaves and stem. The plant tissues were dried for one week in a drying oven at 60 °C (Memmert GmbH + Co. KG, Schwabach, Germany) and weighed again with a precision balance (Sartorius AG, Göttingen, Germany) for dry biomass determination:

Dry biomass total (g) = fresh biomass total (g) / remaining fresh biomass (g) x remaining dry biomass (g)
Four 5 cm parts of the dry basal stem tissue were debarked and stored for measurements of wood composition and wood density.

In order to determine the total leaf area, three to four leaves from upper, middle and bottom positions were collected and photographed on a flat sheet of paper together with a ruler using a digital camera (Olympus, C-770). Their fresh weight was determined for calculating fresh weight based leaf area. The digital images were analysed with Image J (https://imagej.nih.gov/ij/; Wayne Rasband, Maryland, USA). Total plant leaf area (m²) was determined as follows:

Area per leaf (cm²) = area of photographed leaves / number of photographed leaves
Leaf area per plant (cm²) = total fresh leaf weight (g) x area per leaf of photographed leaves (cm²) / fresh weight of photographed leaves (g)
Leaf loss per plant (%) = loosed leaves / fully expanded leaves x 100

2.4.2 Growth measurements of Arabidopsis

Five Arabidopsis plants were pooled as one biological replicate per treatment. This resulted in four replicates per treatment. Fresh biomass was determined with a precision balance (Sartorius AG, Göttingen, Germany), separately for roots and leaves. These plant tissues were dried for one week in a drying oven at 60 °C (Memmert GmbH + Co.) and weighed again with a precision balance (Sartorius AG, Göttingen, Germany) for dry biomass measurements. All data were divided by ten to determine biomass per plant.

2.5 Chemical analysis of harvested material

2.5.1 Measurements of ammonium and nitrate concentrations in plant tissues

To determine ammonium and nitrate concentrations, stem (200 mg), leaves (200 mg) and dx (20 mg) were used (n = 3 per tissue). The following extraction solution volumes were used for stem and leaf tissues. The volumes used for dx are defined in brackets.

The frozen samples were homogenized in a mortar with liquid N_2 and a pestle. The powder was extracted in 5 mL (500 µL) methanol and chloroform mix (3.5:1.5) (modified after Stitt et al. 1983). The extract was mixed and cooled on ice for 30 min. Afterwards, 4 mL (400 µL) nuclease-free water was added and the solution was mixed for 2 min. For phase separation, the extracts were centrifuged for 5 min at 2800 x *g* at RT (Eppendorf AG, Hamburg, Germany). The hydrophilic upper phase was transferred to a new 15 mL tube (Falcon tube; Sigma-Aldrich Corporation, St. Louis, U.S.A.) (1.5 mL reaction tube (Eppendorf AG, Hamburg, Germany)) and adjusted to 20 mL (2 mL) with nuclease-free water. The remaining amount was mixed again for 2 min with 3 mL (300 µL) nuclease-free water. After centrifugation (5 min, 2800 x *g*, RT), the hydrophilic upper phase was combined with the first one and stored at -80 °C.

Nitrate content was measured colorimetrically using the 1-naphthylamine reaction with nitrite ions (Pfeiffer and Pecher 1997; Schmidt 2004). A calibration curve was created with 326 mg/L KNO_3 solution (200 mg/L NO_3^-) for 0, 10, 15, 20 and 25 mg/L NO_3^- (**Fig. 2.2**). In detail, 40 mg Zn and 80 µL 1 M NaOH were added to 2 mL extract solution, mixed intensely and incubated for 1 min at RT. Afterwards, 800 µL of the solution was transferred to a new 1.5 mL reaction tube. 200 µL 96 % (v/v) acetic acid and 40 µL 1-naphthylamine reagent were added. After a 5 min incubation period at RT, nitrite ions were detected spectrophotometrically at 535 nm (DU®640, Beckman Coulter, Brea, U.S.A.). Calculations of nitrate content (nmol/g) in three technical replicates were done by using calibration curves as follows:

y = 0.044*x*
x = y/0.044 x dilution factor
x = concentration in extract (mg/L);
y = mean extinction of technical replicates;
Dilution factor = 0.02 for leaves and stem samples or 0.002 for dx samples
Concentration in tissue (mol/g) = *x* mg L^{-1} / fresh weight (mg) / molar mass of NO_3^- (g mol^{-1})

Ammonium content was measured colorimetrically using the Nessler reagent reaction with ammonium ions (Neßler, 1856). A calibration curve was created with 594 mg/L NH_4Cl solution (200 mg/L NH_4^+) for 0, 10, 15, 20 and 25 mg/L NH_4^+ (**Fig. 2.3**). In detail, each extract solution was mixed 1:20 with Nessler reagent and incubated for 3 min at RT. Ammonium ions were detected spectrophotometrically at 436 nm (DU®640, Beckman Coulter, Brea, U.S.A.). Calculations of ammonium content (µmol/g) in three technical replicates were done by using calibration curves as follows:

y = 0.03*x*
x = y/0.03 x dilution factor
x = concentration in extract (mg/L);
y = mean extinction of technical replicates;
Dilution factor = 0.02 for leaves and stem samples or 0.002 for dx samples
Concentration in tissue (mol/g) = *x* mg L^{-1} / fresh weight (mg) / molar mass of NH_4^+ g mol^{-1}

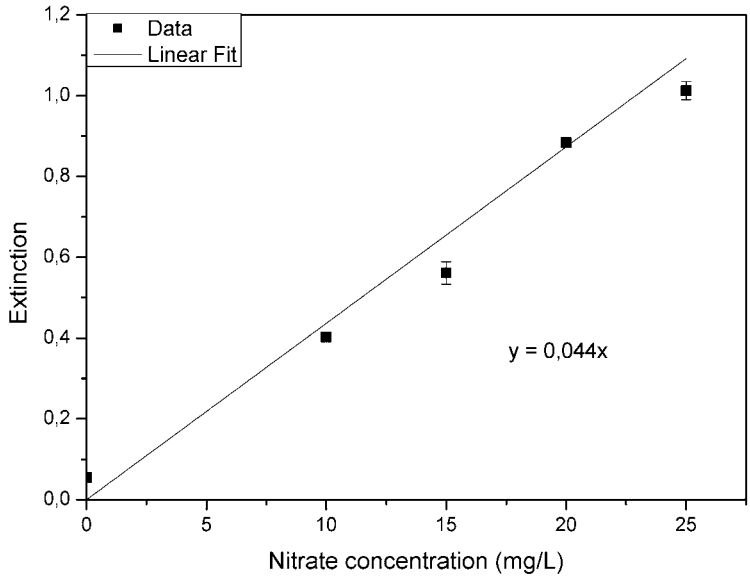


Fig. 2.2: *Calibration curve for KNO_3 for nitrate ions determination (n = 2 ± SE).*

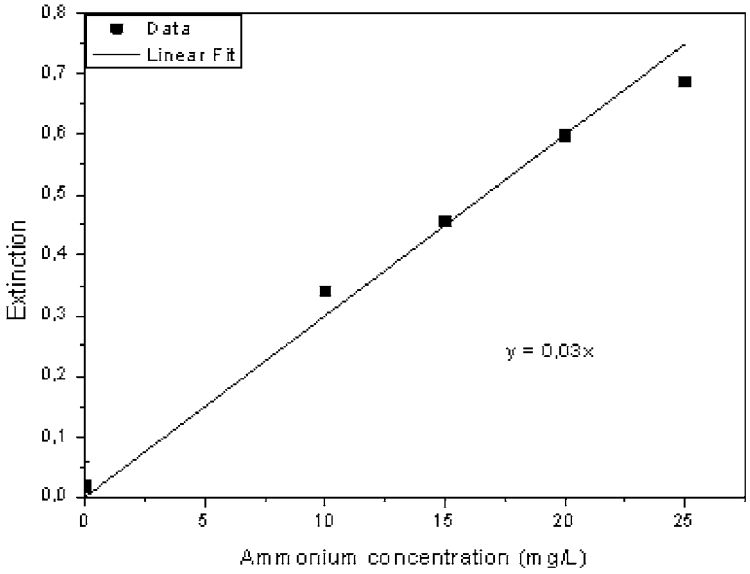


Fig. 2.3: *Calibration curve of NH_4Cl for ammonium ions determination (n = 3).*

2.5.2 Measurements of nitrogen and carbon content in dry wood

Dry wood of five biological replicates was milled to a fine powder in a ball mill with metal beads (Ø 5mm) for 5 min at RT with 30 U/s (Mixer Mill MM 200, Retsch GmbH, Haan, Germany) and weighed on a high precision balance (Sartorius, Göttingen, Germany). Five milligram milled wood per sample were filled into a tin capsule (4x6 mm, IVA Analysentechnik, Meerbusch, Germany) and placed in a microtiter plate. Three technical replicates were prepared for each sample. Two milligram acetanilide were used as the standard. Carbon and nitrogen contents were determined in an elemental analyzer (Vario MICRO Cube, Elementar Analysensysteme GmbH, Hanau, Germany).

2.5.3 Determination of lignin and holocellulose content in dry wood

Holocellulose was determined as the total amount of polysaccharides in wood, consisting of cellulose and hemicellulose (after a method of Wise et al. 1946). The holocellulose content was measured in three technical and five biological replicates. Sixty milligram milled dry wood was mixed with 600 µL ddH_2O in a 2 mL reaction tube (Eppendorf AG, Hamburg, Germany), before 7.5 µL 96% (v/v) acetic acid and 22.5 mg $NaClO_2$ were added. Subsequently, the reaction tubes were placed in a water bath (60 °C, 45 min; GFL Gesellschaft für Labortechnik mbH, Burgwedel,

Germany) for bleaching. Afterwards 600 µL ddH_2O, 7.5 µL 96 % (v/v) acetic acid and 22.5 mg $NaClO_2$ were added and the reaction tubes were placed in the heated water bath again (60 °C, 45 min). Then, the samples were cooled in ice-cold water and filtered through a pre-weighed empty paper filter. The flow-through was discarded and the residual in the filter was washed with 3 mL ice-cold water, then with 750 µL 100 % (v/v) acetone. The filter with the residual material was dried overnight in an oven at 100 °C (Memmert GmbH + Co.). Thereafter, the filter with the holocellulose was determined as follows:

Amount of holocellulose (mg) = filter with residual material (mg) - empty filter (mg)
Holocellulose (mg^{-1} wood) = amount of holocellulose (mg)/wood starting material (mg)
Holocellulose (%) = holocellulose (mg^{-1} wood) x 100

The lignin content in dry wood samples was determined by the acetyl bromide method (Blaschke 1999, modified by Brinkmann et al. 2002) and measured in three technical and five biological replicates. 30 mmol coniferyl alcohol was used to create a calibration curve for 0, 100, 250, 500, 1000, 1250 and 1500 nmol in 50 µL (**Fig. 2.4**). The concentrations were adjusted with 99.6 % (v/v) ethanol. The highest concentration of 1500 nmol / 50 µL contained 30 nmol coniferyl alcohol, whereby 1 mol coniferyl alcohol is 180 g/L (1 nmol coniferyl alcohol = 0.18 µg). In detail, 3 mg milled wood sample was mixed with 250 µL acetyl bromide solution (1:3, acetyl bromide:acetic acid 96 % (v/v)) in a 2 mL screw-cap reaction tube (Eppendorf AG, Hamburg, Germany) and heated in a water bath at 70 °C for 30 min (GFL Gesellschaft für Labortechnik mbH, Burgwedel, Germany). During incubation, the samples were mixed after 10 min and 20 min. Then, the samples were cooled on ice, 250 µL 2 M NaOH was added and the samples were mixed. The samples were centrifuged for 5 min at 4 °C (11000 G, Eppendorf AG, Hamburg, Germany) and the supernatant was transferred to a new 2 mL reaction tube. Then, 3 µL 50 % (v/v) hydroxylamine and 1.25 mL 96 % (v/v) acetic acid were added and mixed. The samples were diluted 4-fold with 96 % (v/v) acetic acid and measured spectrophotometrically at 280 nm (DU®640, Beckman Coulter, Brea, U.S.A.). Calculations of lignin content (%) were done by using the coniferyl alcohol calibration curve (**Fig. 2.4**) as follows:

$y = 0.000528x$
$x = 0.000528/y$ x dilution factor
x = concentration of coniferyl alcohol (nmol);
y = mean extinction of standard component;
Dilution factor = 4
Amount of lignin (µg) = coniferyl alcohol (nmol) x 0.18 µg $nmol^{-1}$ coniferyl alcohol
Lignin (μg^{-1} wood) = amount of lignin (µg)/wood starting material (µg)
Lignin (%) = lignin (μg^{-1} wood) x 100
Undefined extractives were not measured but calculated as follows:
Undefined extractives (%) = 100 - Holocellulose (%) - Lignin (%)

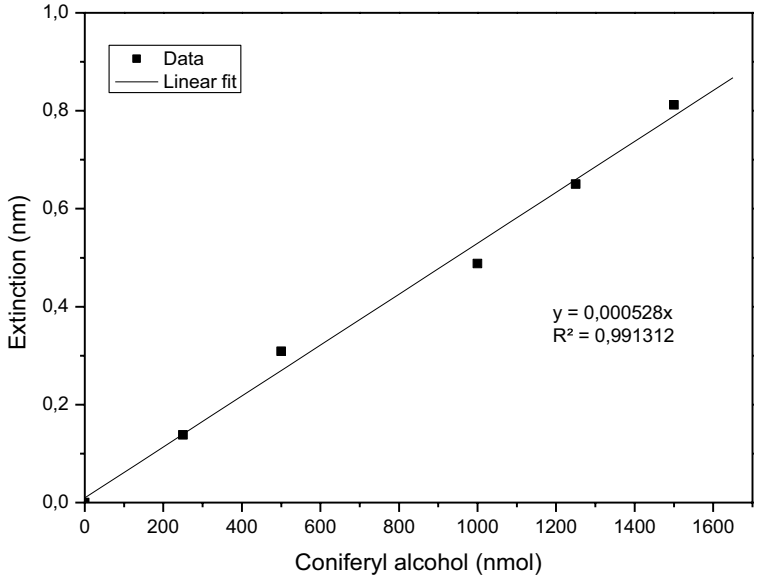


Fig. 2.4: *Calibration curve of coniferyl alcohol for lignin determination. Formula of linear fit from data points is shown. Symbols indicate means of n = 3. R^2 = coefficient of linear regression.*

2.6 Wood density determination

Dry basal debarked wood pieces of 5 cm length were weighed and completely submersed in 10 mL distilled water in a graded measuring cylinder with a thin wire. The difference of water displacement was noted. Based on the Archimedes principle wood density was calculated as follows:

Wood density (mg cm^{-3}) = dry wood biomass (mg)/volume of water displacement (cm^3)

2.7 Wood anatomical analysis

Cross sections of 30 µm thickness were cut with a freezing microtome (Frigocut-2800 E, Reichert-Jung, Leica, Bensheim, Germany) from frozen basal poplar stem or frozen hypocotyl samples of Arabidopsis. The cross sections were stained for 3 min with phloroglucinol-muriatic acid (0.525 gr phloroglucinol, 35 mL 96 % (v/v) ethanol, 17.5 mL 25 % (v/v) muriatic acid), washed with distilled water and observed under a light microscope (Axio Vision 4.9, Zeiss AG, Oberkochen, Germany), directly after the cutting. Pictures were taken with an integrated digital camera (Axiocam, Zeiss AG, Oberkochen, Germany). Image J (https://imagej.nih.gov/ij/; Wayne Rasband, National Institutes of Health, USA) was used to analyze wood anatomy.

2.7.1 Anatomical analysis of poplar stem cross sections

Cell layer width of poplar bark, phloem, cambial zone, developing xylem, secondary xylem and pith were measured on three different positions of a cross section (**Fig. 2.5**). The percentages of cell layer widths based on the radius of a cross section and were determined as follows:

Tissue (%) = cell layer width (µm) x 100 (%) / radius of cross section (µm)
Radius of cross section (µm) = diameter of cross section (µm) / 2

Moreover, numbers of cell types were counted at 20-fold magnification. Vessel cells were counted in an area of 300 x 300 µm and fiber cells were counted in an area of 100 x 100 µm. Cell lumen area and cell wall thickness of vessels and fibers were analyzed at 40-fold magnification on poplar developing and mature xylem tissue (**Fig. 2.5**). Fiber cell wall thickness was determined by measuring double cell wall thickness between two adjacent cells and the value was divided by a factor of two. Three to five biological and three technical replicates were analyzed for each sample.

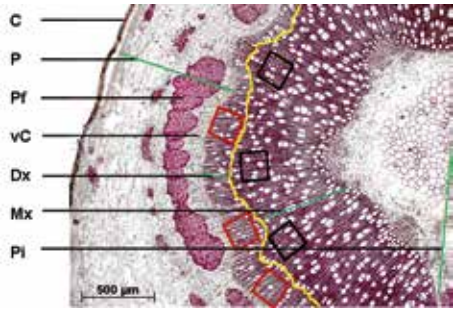


Fig. 2.5: *Typical stem cross section of P. trichocarpa. Three areas (300 x 300 µm) were selected for wood anatomy analysis in developing (red marked squares) and mature xylem tissue (black marked squares). Within these squares, areas of 100 x 100 µm were selected for fiber anatomical measurements. The border between developing and mature xylem tissue is marked in yellow and was distinguished by differences in phloroglucinol staining intensity of secondary xylem cell walls. The green lines indicate cell layer width measurements. C = cortex, P = phloem, Pf = phloem fibers, vC = vascular cambium, Dx = developing xylem, Mx = mature xylem and Pi = pith. Scale bar = 500 µm.*

2.7.2 Anatomical analysis of Arabidopsis hypocotyl cross sections

In Arabidopsis the hypocotyl cross section radius, cell layer width of cortex, secondary growing zone and primary growing zone were determined (**Fig. 2.6**). The percentages of cell layer widths based on the radius of a cross section and were determined as described in chapter 2.7.1. Moreover, five vessel and fiber cells were measured at 40-fold magnification on primary and secondary xylem tissue (**Fig. 2.6**).

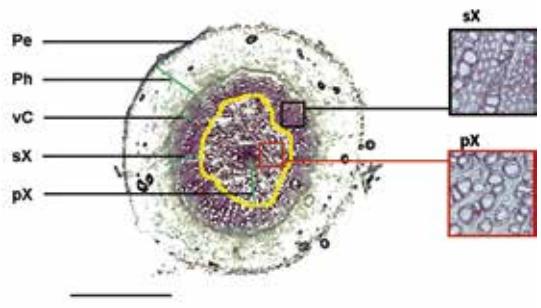


Fig. 2.6: *Typical hypocotyl cross section of A. thaliana. Five vessel and fiber cells were selected in primary (pX) and secondary (sX) growth zone on three different positions, each. 40-fold magnification of primary (red marked square) and secondary xylem tissue (black marked square) is displayed on the right side of the figure. The border between primary and secondary xylem tissue is marked in yellow and was distinguished by differences in phloroglucinol staining intensity of secondary xylem cell walls. The green lines indicate cell layer width measurements. Pe = periderm, Ph = phloem, vC = vascular cambium, sX = secondary xylem and pX = primary xylem. Scale bar = 500 µm.*

2.8 RNA isolation, cDNA synthesis and gene expression analysis

2.8.1 Total poplar RNA isolation and cDNA synthesis

Frozen poplar developing xylem tissue or frozen Arabidopsis leaves were homogenized in a mortar with a pestle in liquid nitrogen. Then, 20 to 50 mg homogenized dx sample was used to extract RNA, according to Chang et al., 1993, with minor modifications. Instead of spermidine, 2% (v/v) ß-mercaptoethanol was used in the extraction buffer. The isolated RNA was dissolved in 20 µl nuclease-free H_2O. DNA contamination was removed with Ambion® Turbo DNA free™ kit, according to the instructions in the manual. Thereafter, the purified RNA was controlled on a 1.2% (w/v) agarose gel including ethidium bromide by gel electrophoresis (15 to 20 min, 120 V, Bio-Rad Laboratories, Inc., Hercules, U.S.A). If there was no remaining DNA band visible, the concentration and quality of purified RNA were

measured spectrophotometrically (BioPhotometer, Eppendorf AG, Hamburg, Germany). Samples with more than 100 ng/µL RNA concentration and an absorbance ratio of A_{260}:A_{280} of 2.1±0.2 and A_{260}:A_{230} of 2.3±0.2 were used for further analyses. One microgram purified RNA was reversely transcribed into cDNA with the RevertAid First Strand cDNA Synthesis Kit using 1 µL oligo(dT)-primers (20 pmol) in 20 µL reaction mix. The synthesized cDNA with the concentration of 0.1 µg/µL was diluted 1:10 for gene expression analysis by quantitative real-time PCR (qRT-PCR; chapter 2.8.4) or used to generate the attB-PCR-product of *PtGATA12* gene (chapter 2.11).

2.8.2 Total Arabidopsis RNA isolation and cDNA synthesis

100 mg frozen leaf powder of Arabidopsis were used to extract RNA with the RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands), according to the instructions in the manual. Two microgram purified RNA was reversely transcribed into cDNA and used for gene expression analysis by qRT-PCR (chapter 2.8.4) or for monitoring on a gel (next chapter).

2.8.3 Gene expression analysis by PCR

Two microliters of approximately 0.05 µg/µL cDNA of *AtGATA12* loss of function mutants was used in a 20 µL reaction mix for PCR amplification with 2 µL PCR Buffer $(NH_4)_2SO_4$, 1.6 µL $MgCl_2$ (25 mM), 0.4 µL dNTPs (10 mM), 12.9 µL nuclease-free water, 0.1 µL Taq Polymerase (5 U/µL) and 2 µL specific *AtGATA12* primer, which were designed with Primer3 (http://primer3.ut.ee/; Rozen and Skaletsky 2000) and tested by using Oligo Analyzer (Gene Link, Hawthorne, NY, USA). *AtACTIN1* was used as positive control (**Tab. 2.5**). The PCR program started with pre-incubation at 95 °C for 60 s and continued with 45 cycles of amplification (95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s). Thereafter, PCR was terminated at 72 °C for 10 min. The amplified *AtGATA12* cDNA was monitored on a 1.2% (w/v) agarose gel including ethidium bromide by gel electrophoresis (20 to 25 min, 120 V, Bio-Rad Laboratories, Inc., Hercules, U.S.A).

Tab. 2.5: *AtGATA12 primer pair used to test for AtGATA12 expression in putative loss of function mutants. AtACTIN1 primer pair was selected as positive control.*

Gene name	Number	Primer forward (5` to 3`)	Primer reverse (5` to 3`)	Product (bp)
AtGATA12	At5G25830	TCTCCGGTG ACCTTTGTA TACCTTC	CCACAATGT TCGAAAGCC ACTC	69
AtACTIN1	At2G37620	TGCGACAAT GGAACTGG AATG	GGATAGCAT GTGGAA GTGCATACC	499

2.8.4 Gene expression analysis by quantitative real-time PCR (qRT-PCR)

To determine expression values of candidate genes involved in secondary vessel cell wall formation, cDNA of *P. trichocarpa* plants from experiment 2.3.1 were used. Expression analysis for five genes related to wood formation: *PtCCoAOMT1*, *PtIRX1*, PtVNS07/*PtWND6A*, *PtVNS08/PtWND6B* and *PtGATA12* (**Tab. 2.6**) was performed by qRT-PCR in a LightCycler 480® (Roche Diagnostics, Mannheim, Germany). Gene specific primers were designed with Primer3 (http://primer3.ut.ee/; Rozen and Skaletsky 2000) and tested by using Oligo Analyzer (Gene Link, Hawthorne, NY, USA) as well as by creating a three-fold dilution series for checking, melting temperature (Tm), primer dimers, and primer loops. For each gene, three technical and five biological replicates were analyzed.

The following protocol was also used for analyzing target gene expression (*PtGATA12*) in *35S:PtGATA12* poplar and Arabidopsis lines.

The reaction mix for qRT-PCR was prepared with 10 µL SYBR Green I Master kit, 2 µL of forward and reverse primers, each (10 µM; Microsynth Austria GmbH, Vienna, Austria; Tab. 2.11), 1 µL nuclease-free water and 5 µL cDNA-solution (0.01 µg/µL). The qRT-PCR program was adapted from Carsjens et al. (2014). Pre-incubation started at 95 °C for 5 min, followed up by 55 cycles of amplification (95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s) and finished with a melting process (95 °C for 5 s, 65 °C for 1 min, then to 97 °C at a rate of 0.11 °C s^{-1}) to assess primer specificity. To calculate primer efficiencies, the raw data output from LightCycler 480® instrument was converted into an Excel file using LC480 conversion software (version 2014.1; http://www.hartfaalcentrum.nl/index.php?main=files&fileName=LC480Conversion.zip&description=LC480 %20Conversion&sub=LC480Conversion) and loaded into LinRegPCR (version 2017.0; Ruijter et al. 2009) for baseline correction. The mean primer efficiencies were calculated across all samples and ranged between 1.9 and 2.2 (**Tab. 2.6**). The detected quantification cycle values (Cq) were used to determine the transcript level of each sample and candidate gene with normalizing to the housekeeping genes

PtTUBULINβ2 and *PtEF1β* for *P. trichocarpa*, *PtRNase_P_Rpp14* and *PtPPR_2* for *35S:PtGATA12 P.* x *canescens* and *AtUBC9* and *AtUBQ10* for *35S:PtGATA12* Arabidopsis cDNA.

Relative Expression = $\sqrt{E(Ref1)^{Cq} \times E(Ref2)^{Cq} / E(GOI)^{Cq}}$ (Hellemans et al. 2007)
E = primer efficiency;
Cq = cycle threshold;
Ref = reference gene;
GOI = gene of interest

Tab. 2.6: *Genes used for qRT-PCR with the Arabidopsis homologs and the primer binding efficiency numbers (E) are shown. The respective primer sequences and expected product lengths of PCR are listed in the last columns. Pt = P. trichocarpa, Pc = P. x canescens.*

Gene name	ID	*A. thaliana* homolog	Primer efficiency (E)	Primer forward (5` to 3`)	Primer reverse (5` to 3`)	Product size (bp)
Pt TUBULINβ2	Potri.001G272800	At1G20010	1.96	AGGTCACTAACTGTCCCAGAAC TC	ATGGCAGAGGCAGTCAGATAGC	104
PtEF1α	Potri.006G130900	At1G07940	2.11	GGCAAGGAGAAGGTACACAT	CAATCACACGCTTGTCAATA	115
Pt GATA 12	Potri.018G044900	At5G25830	2.02 for Pt 2.25 for Pc	ACGCCTTCTTGTGCTCTCTC	CCTTACACCACACGCATTGC	241
Pt VNS07/ PtWND6A	Potri.013G113100	At1G71930	1.97	CACCAGGCTTTAGGTTTCATCC	TTGCACTTGGCTTGGATGTC	148
Pt VNS08/ PtWND6B	Potri.019G083600	At1G71930	1.91	GCTTGGAGTCATGCTTATTATCTC	TAAGTCAGGAAAGCAGTCAAGGA	446

Gene name	ID	*A. thaliana* homolog	Primer efficiency (E)	Primer forward (5` to 3`)	Primer reverse (5` to 3`)	Product size (bp)
PtIRX1	Potri.011G069600	At5G44030	2.10	ACCCACTGAAAGAGCCTCCATTG	CAGCACCATCATCAGACACATAGC	108
Pt CCoAOMT1	Potri.009G099800	At4G34050	2.04	GGCAGGAAGGCACCAGGAAG	GCATTCAGGCTCTCTTGGATACAC	103
Pt RNase_P_Rpp14	Potri.015G001600	At1G04635	2.08	ATCGTTCCAAGTCAAGTATGT	TCAAGGCAGCAACTTTACAG	92
PtPPR_2	Potri.012G141400	At1G55630	2.24	GCAATGTGAGGAGTTTAGGG	TATTAAATGTCTGTGCTGTAG	86
AtUBC9	At4G27960		2.12	TCACAATTTCCAAGGTGCTGC	TCATCTGGGTTTGGATCCGT	61
AtUBQ10	At4G05320		2.24	GGCCTTGTATAATCCCTGATGAATAAG	AAAGAGATAACAGGAACGGAAACAGT	61

2.9 Phylogenetic analyses of GATA transcription factors in Arabidopsis and poplar

The GATA12 transcription factor was pointed out in chapter 1 as a candidate involved in nitrogen-mediated wood formation. To identify a homolog poplar candidate gene, expressed in secondary xylem tissue, GATA family members in Arabidopsis and poplar were compared by CLUSTAL Omega(1.2.1) (EMBL-EBI 2018, Sievers et al. 2011) multiple protein sequence alignment, combined with a percent

identity matrix created in Clustal2.1 (EMBL-EBI 2018, Larkin et al. 2007). The resulting neighbour-joining tree without distance corrections was clustered in four subfamilies (Reyes et al. 2004). Relative expression levels of poplar GATA genes in wood tissue were inspected in PopGenIE.org (Sjödin et al. 2009) and relative expression levels of Arabidopsis GATA genes in stem tissue viewed in Arabidopsis eFP Browser 2.0 (bar.utoronto.ca, Winter et al. 2007). The selected *PtGATA12* homolog gene was characterized functionally in *P.* x *canescens* via Gateway overexpression system.

2.10 Producing competent Escherichia coli and Agrobacterium tumefaciens cells

Competent *E. coli* cells were produced according to the $CaCl_2$ method (Dagert and Ehrlich, 1979) with minor modifications. After growing a starter culture overnight in 2 mL liquid LB medium (1 % Bacto™ tripton, 0,5 % Bacto™ yeast extract, 1 % NaCl and 1,5 % (w/v) Bacto™ agar) at 37 °C (oven, Memmert GmbH + Co. KG, Schwabach, Germany) under constant shaking (~250 rpm, GFL Gesellschaft für Labortechnik mbH, Burgwedel, Germany), the resulting bacteria culture was filled into 200 mL of fresh liquid LB medium and grown in a shaking incubator (~120 rpm, Sanyo Gallenkamp PLC IOC400.XX2.C, Loughborough, United Kingdom) at 37 °C until the culture reached an OD_{600} of 0.31 to a maximum of 0.49 (Biophotometer 6131, Eppendorf AG). The cells were chilled on ice for 20 min by occasionally swirling before harvesting by centrifugation for 10 min at 1800 g and 4 °C in a cooling centrifuge (J2-HS, Beckman Coulter, Brea, U.S.A.). After discarding the supernatant, the bacterial cells were resuspended in 20 mL ice-cold 50 mM $CaCl_2$ solution. The cells were incubated on ice for 20 min again and harvested as described above. Afterwards, the pellet was resuspended in 8 mL ice-cold solution of 50 mM $CaCl_2$ including 15 % (v/v) glycerol 100 µL aliquots were prepared, snap-frozen in liquid nitrogen and stored at -80 °C or kept on ice for immediate transformation.

Competent *A. tumefaciens* cells of GV3101-pMP90 strain were produced, according to the $CaCl_2$ method, similar as described for *E. coli* cells, but with minor changes. Instead of LB medium, YEB medium (0,5 % Difco™ beef extract, 0,1 % Bacto™ yeast extract, 0,5 % Bacto™ peptone, plus 0,5 % sucrose and 1,5 % (w/v) Bacto™ agar), containing rifampicin (50 µg/mL) and gentamycin sulphate (25 µg/mL), were used. *A. tumefaciens* cells were incubated in a shaking incubator at 28 °C (~90 rpm, Sanyo Gallenkamp PLC IOC400.XX2.C, Loughborough, United Kingdom) and 200 µL aliquots of competent cells were prepared.

2.11 Cloning of PtGATA12 gene via Gateway system

2.11.1 Creating an entry vector

AttB recognition sites were added to 1.5 µL synthesized and diluted *PtGATA12* cDNA in a 50 µL reaction mix by PCR amplification (Thermocycler, SensoQuest GmbH, Göttingen, Germany) with 10 µL Phusion HF Buffer, 1 µL dNTPs (10 mM), 34 µL nuclease-free water, 0.5 µL Phusion Polymerase (2 U/µL) and 1.5µL of 10 mM of each Gateway primers (**Tab. 2.7**), which were designed using the SnapGene program (http://www.snapgene.com/resources/gateway_cloning, GSL Biotech LLC, Chicago, U.S.A.). The program for the PCR reaction started with pre-incubation at 98 °C for 30 s and continued with 35 cycles of amplification (98 °C for 10 s, 57 °C for 30 s and 72 °C for 90 s). Thereafter, a final elongation step at 72 °C for 10 min was performed. The product was tested by gel electrophoresis (15 to 10 min, 120 V, Bio-Rad Laboratories, Inc., Hercules, U.S.A) on a 1.2 % (w/v) agarose gel including ethidium bromide (3 µl of a 1 % solution per 100 ml gel). The *attB*-PCR-product was cut out of the gel, purified by QIAquick gel extraction kit and eluted in 30 µL nuclease-free H_2O. The quality and the concentration were measured spectrophotometrically (BioPhotometer, Eppendorf AG) at A_{260} and A_{280}. The designed *attB*-PCR-fragment of *PtGATA12* was inserted into the donor vector pDONR™ 201 (Thermo Scientific, Thermo Fisher Scientific, Braunschweig, Germany) by BP reaction. The Gateway reaction mix was prepared on ice with approximately 20 ng of *attB*-PCR-insert, 75 ng pDONR™ 201, 1 µL BP Clonase II of Gateway™ BP Clonase™ II Enzyme mix and TE buffer (10 mM Tris hydrochloride, 1 mM ethylenediaminetetraacetate, pH 8.0 in 5 µL volume). The cloning reaction took place overnight at room temperature (25 °C). The product was either used directly for transformation or stored in the fridge (4 °C) and used within one week after preparation. 100 µL of chemically competent TOP10 *E. coli* cells were transformed with the created entry vector, according to the instructions in the manual (Gateway™ BP Clonase™ II Enzyme mix, Invitrogen™, Carlsbad, United States, p.3) with minor modifications. Cells were subjected to heat-shock at 42 °C for 90 seconds, but instead of S.O.C medium, 800 µL sterile liquid LB medium was used. Colonies were grown on LB plates containing kanamycine sulphate monohydrate (50 µg/mL) at 37 °C. On the next day, kanamycine resistant colonies were picked with a sterile pipette tip under sterile conditions (sterile bench SAFE 2020, Thermo Scientific, Thermo Fisher Scientific, Braunschweig, Germany) and transferred in 5 mL liquid LB medium. Each culture was grown at 37 °C overnight on a shaker (~250 rpm).

To detect transformed colonies, a PCR was performed with 0.2 µL of each liquid breeding colony in a 10 µL reaction volume with 1 µL PCR Buffer $(NH_4)_2SO_4$, 0.8 µL $MgCl_2$ (25 mM), 0.2 µL dNTPs (10 mM), 5.9 µL nuclease-free water, 0.1 µL Taq Polymerase (5 U/µL) and either 1 µL of 10 mM forward and reverse gene

specific primers or 1 µL forward and reverse vector specific pDONR201 primers (Microsynth Austria GmbH, Vienna, Austria; **Tab. 2.8**). Specific primers were designed with (http://primer3.ut.ee/; Rozen and Skaletsky, 2000) and tested by using Oligo Analyzer (Gene Link, Hawthorne, NY, U.S.A.). The same PCR program used for creating an attB-PCR-product was applied. The transformed cell products were monitored on a 1.2 % (w/v) agarose gel including ethidium bromide, as described above. The plasmid was extracted from transformed TOP10 *E. coli* cells using the NucleoSpin® Plasmid kit. A PCR was performed with the same master mix in a 10 µL reaction volume with 0.2 µL plasmid instead of colony cells and the same PCR program as mentioned above was applied. The amplified product was monitored by gel electrophoresis (15 to 20 min, 120 V), cut out of the gel and purified by QIAquick gel extraction kit. The isolated plasmid was eluted with 30 µL nuclease-free H_2O and its quality and the concentration were measured spectrophotometrically (BioPhotometer, Eppendorf AG) at A_{260} and A_{280}. 2.5 µL of approximately 100 ng/µL purified plasmid was sequenced by GATC Biotech AG (Konstanz, Germany) using 2.5 µL pDONR201-reverse primer (5 mM; Microsynth Austria GmbH, Vienna, Austria; **Tab. 2.8**).

Tab. 2.7: *Gene specific Gateway primers with attB recognition sites for creating an attB-PCR-product designed by SnapGene.*

Gene name	Potri. Number	*A. thaliana* homolog	Gateway primer *attB*-forward (5` to 3`)	Gateway primer attB-reverse (5` to 3`)	Product size (bp)
PtGATA 12	Potri.018 G044900	At5G258 30	**GGGGAC AAGTTT GTACAA AAAAGC AGGCTT A**ATGGA AGCACC AGAATT CTATGGG	**GGGGAC CACTTT GTACAA GAAAGC TGGGTT C**TAGAT CATCTG CCTAAA ATCGGG TCC	1143

Tab. 2.8: *Gene specific primers, as well as vector specific pDONR201 and p7WGF2 primers to perform a PCR. Product sizes of empty vectors are indicated in the last column.*

Gene/ vector name	Potri. Number	*A. thaliana* homolog	Primer forward (5` to 3`)	Primer reverse (5` to 3`)	Product size (bp)
PtGATA 12	Potri.018 G044900	At5G258 30	CCCAC GACTT CATCCT CC GATAC	CGCAAC CTTCAAC ACCACC ATC	124
pDONR 201	–	–	TAACG CTAGC ATGGA TCTC	GTGCAA TGTAAC ATCAGA GAT	2494
p7WGF2	–	–	CACAA TCCCA CTATCC TT CGCA	CATGAG CGAAAC CCTATA AGAACC	2671

2.11.2 Creating an expression vector

The designed entry vector including PCR-fragment of *PtGATA12* was subcloned into the destination vector pK7WGF2.0, obtained from VIB, Gent, Belgium (Karimi et al. 2002) by LR reaction. The Gateway reaction mix of 5 µL was prepared on ice with approximately 75 ng of pDONR201-entry vector clone, 75 ng pK7WGF2.0, 1 µL LR Clonase II of Gateway™ LR Clonase™ Enzyme mix and filled up with TE buffer (pH 8.0) to the final volume. The cloning reaction took place overnight at room temperature (25 °C). The resulting vector was designated as *35S:PtGATA12* and was either used directly for transformation or stored in fridge (4 °C) and used within one week after preparation. 100 µL of chemically competent DH5α *E. coli* cells were transformed with the *35S:PtGATA12* vector, according to the instructions in the manual (GatewayTM BP ClonaseTM II Enzyme mix, InvitrogenTM, Carlsbad, United States, p.3) with minor modifications (chapter 2.11.1). Colonies were grown on LB plates containing spectinomycin HCl pentahydrate (50 µg/mL) at 37 °C. On the next day, spectinomycin HCl pentahydrate resistant colonies were picked with a sterile pipette tip under sterile conditions and transferred in 5 mL liquid LB. Each picked colony was grown at 37 °C overnight on a shaker (~250 rpm). To detect transformed colonies, a PCR was performed with 0.2 µL of each liquid breeding colony in a 10 µL reaction volume with 1 µL PCR Buffer $(NH_4)_2SO_4$, 0.8 µL $MgCl_2$ (25 mM), 0.2 µL dNTPs (10 mM), 5.9 µL nuclease-free water, 0.1 µL Taq Polymerase (5 U/µL) and either 2 µL of 10 mM forward and reverse gene specific primers or 2 µL of 10 mM forward and reverse vector specific

p7WGF2 primers (**Tab. 2.8**). The same PCR program used for creating an attB-PCR-product was applied (chapter 2.11.1). The amplified products were monitored on a 1.2 % (w/v) agarose gel including ethidium bromide. The plasmid was extracted from transformed DH5α *E. coli* cells with the innuPREP Plasmid Mini Kit. The same master mix in a 10 µL reaction volume and the same PCR program as mentioned above was applied (chapter 2.11.1). The amplified product was monitored by gel electrophoresis (15 to 20 min, 120 V) on a 1.2 % (w/v) agarose gel including ethidium bromide. The plasmid was cut out of the gel, purified by QIAquick gel extraction kit and eluted in 30 µL nuclease-free H_2O. The quality and the concentration were measured spectrophotometrically (BioPhotometer, Eppendorf AG) at A_{260} and A_{280}. 2.5 µL of approximately 100 ng/µL purified plasmid was sequenced by GATC Biotech AG (Konstanz, Germany) using 2.5 µL p7WGF2-reverse primer (5 mM; Tab. **2.8**).

2.12 Generation of PtGATA12 overexpressing plants

2.12.1 Preparation of transgenic A. tumefaciens cells containing the 35S:PtGATA12 vector

Two hundred microliters of chemically competent *A. tumefaciens* cells (strain GV3101-pMP90) were transformed with approximately 3 µg of designed *35S:PtGATA12* vector by gently mixing, chilling on ice for 30 min, freezing in liquid nitrogen for two minutes and thawing in a water bath for 5 min at 37 °C. Afterwards, the cells were incubated in 800 µL liquid YEB medium, containing spectinomycin HCl pentahydrate (50 µg/mL), rifampicin (50 µg/mL) and gentamycin sulphate (25 µg/mL), at 28 °C in a shaking incubator for at least four hours. Colonies were grown on pre-warmed sterile YEB plates (28 °C), containing spectinomycin HCl pentahydrate (50 µg/mL), rifampicin (50 µg/mL) and gentamycin sulphate (25 µg/mL). After two to three days of growing at 28 °C, resistant colonies were picked with a sterile pipette tip under sterile conditions and cultivated individually in 2 mL liquid YEB at 28 °C overnight in a shaking incubator (~90 rpm). Colony PCR was performed with 1 µL of those colonies in a 20 µL reaction volume with 2 µL PCR Buffer $(NH_4)_2SO_4$, 1.6 µL $MgCl_2$ (25 mM), 0.4 µL dNTPs (10 mM), 12.9 µL nuclease-free water, 0.1 µL Taq Polymerase (5 U/µL) and either 2 µL of 10 mM forward and reverse gene specific primers or 2 µL of 10 mM forward and reverse vector specific p7WGF2 primers (**Tab. 2.8**). The same PCR program was used as in chapter 2.11.1. The amplified products were monitored on a 1.2 % (w/v) agarose gel including ethidium bromide to confirm a positive transformation.

2.12.2 Transformation of P. x canescens by A. tumefaciens

The *A. tumefaciens* culture from 2.12.1 was transferred from 2 mL liquid YEB to 4 mL YEB media with spectinomycin HCl pentahydrate (50 µg/mL), rifampicin (50 µg/mL) and gentamycin sulphate (25 µg/mL). The overnight grown bacterial cells (28 °C, ~90 rpm) were transferred into pre-warmed 100 mL liquid YEB medium (28 °C) without antibiotics and incubated at 28 °C by continuously shaking (~90 rpm) until the culture reached an OD_{600} of 0.25 to a maximum of 0.8. Then, acetosyringone was added to a final concentration of 20 µM. The Agrobacterium were further incubated for 30 min (28 °C, ~90 rpm).

The stem segments (2 to 3 cm) of four- to five-weeks-old *P.* x *canescens in vitro* plants were co-cultivated with the *A. tumefaciens* culture in liquid YEB medium. After 30 min inoculation period at 28 °C (~120 rpm), *P.* x *canescens* stem pieces were separated from excess bacteria culture by sieving and dabbed softly with filter paper. The *P.* x *canescens* explants were distributed on medium containing ½ MS agar (0.22 % (w/v) MS medium including vitamins, 2 % (w/v) sucrose and 0.7 % (w/v) plant agar, pH: 5.8) and were incubated for three to four days at 25 °C in darkness.

When the bacterial cells were overgrowing the explants, the incubation was stopped by the following washing procedure. All *P.* x *canescens* explants were collected and washed once in 150 mL sterile demineralized water for 2 min and three times in 150 mL sterile ticarcillin dissodium / clavulanate potassium (400 µg/µL) solution for 2 min each and then in 150 mL sterile demineralized water for 2 min. Between each step, explants were dabbed softly with sterile filter paper. Finally, the explants were distributed on medium containing ½ MS agar with thidiazuron (0.22 % (w/v) MS medium including vitamins, 2 % (w/v) sucrose, 0.0022 % thidiazuron, 0.7 % (w/v) plant agar, pH: 5.8), cefotaxime sodium sodium (150 mg/L, Sigma-Aldrich) and ticarcillin dissodium / clavulanate potassium (200 mg/L). One plate, containing ½ MS agar without these antibiotics was used as regeneration control. The plates were incubated for three to five weeks at 23 °C in a dark air-conditioned room with very low light (~10 µmol photons m-2 s-1, fluorescent lamps L18W/840, Osram, Munich, Germany, 16/8 h light; 20 to 40 % r.h.). As soon as 3-5 cm tall green plantlets regenerated, they were transferred to medium containing ½ MS agar with cefotaxime sodium, ticarcillin dissodium / clavulanate potassium and kanamycine sulphate monohydrate (0.22 % (w/v) MS medium including vitamins, 2 % (w/v) sucrose, 0.015 % cefotaxime sodium, 0.02 % ticarcillin dissodium / clavulanate potassium, 0.5 % kanamycine sulphate monohydrate, 0.7 % (w/v) plant agar, pH: 5.8) in small WECK glasses (370 mL, Ø108 mm) under sterile conditions and grown at 22 to 23 °C and weak light (~20 µmol photons m^{-2} s^{-1}, fluorescent lamps L18W/840, Osram, Munich, Germany, 16/8 h light; 20 to 40 % r.h.). After four to eight weeks putatively genetically modified plantlets exhibited root growth and green shoot growth. These plantlets were labeled and placed in one large WECK glass (580 mL, Ø108 mm) per line, containing ½ MS agar with kanamycine sulphate monohydrate

(0.22 % (w/v) MS medium including vitamins, 2 % (w/v) sucrose, 0.5 % kanamycine sulphate monohydrate, 0.7 % (w/v) plant agar, pH: 5.8). Cuttings were adapted to standard *in vitro* conditions (22 °C, 20 to 40 % r.h.; 70 µmol photons m^{-2} s^{-1}, fluorescent lamps L18W/840, Osram, Munich, Germany, 16/8 h light) and propagated every four weeks in new WECK glasses, containing ½ MS agar with kanamycine sulphate monohydrate (0.22 % (w/v) MS medium including vitamins, 2 % (w/v) sucrose, 0.5 % kanamycine sulphate monohydrate, 0.7 % (w/v) plant agar, pH: 5.8). One leaf per line was sampled and used to test for a positive transformation event by quantitative RT-PCR (chapter 2.12) with *PtGATA12* forward and reverse primer (**Tab. 2.8**). Another leaf was used for DNA extraction and sequencing (chapter 2.13.1) by GATC Biotech AG (Konstanz, Germany) using 2.5 µL p7WGF2-reverse primer (**Tab. 2.8**).

2.12.3 Transformation of A. thaliana by floral dip with A. tumefaciens

A. tumefaciens containing the recombinant pB7WGF2.0-*35S:PtGATA12* plasmid (chapter 2.12.1), were grown overnight at 28 °C (~90 rpm) in 4 mL liquid YEB media with spectinomycin HCl pentahydrate (50 µg/mL), rifampicin (50 µg/mL) and gentamycin sulphate (25 µg/mL). Then, the cells were transferred into pre-warmed 150 mL liquid YEB medium without antibiotics and incubated at 28 °C by continuously shaking (~90 rpm) until the culture reached an OD_{600} of 0.5 to a maximum of 0.8. The culture was centrifuged at 4 °C and 1800 g for 25 min (J2-HS, Beckman Coulter, Brea, U.S.A.). The supernatant was discarded and the bacterial cells were resuspended in 300 mL solution of sucrose (5 % (v/v)) and Silwet® L-77 (0.03 % (v/v)).

Transformation of *A. thaliana* was performed according to the floral-dip method (Bechtold and Pelletier 1998; Clough and Bent 1998). Two to 10 cm long second inflorescence stems with half opened flowers (stage 4) of short-day grown *A. thaliana* plants (Col-0) (250 mL pots; T-type soil, Fruhstorfer Erde; 140 µmol photons m^{-2} s^{-1}, fluorescent lamps 3071/400 HI-I, IP55, 230 V, 50 Hz, Adolf Schuch GmbH; 9:00 a.m. to 5:00 p.m., r.h. 40 %) were dipped into bacterial solution for 30 s. Thereafter, plants were placed horizontally and covered with a plastic foil. After 24 h incubation, plants were put upright again and placed in a climate chamber with a defined temperature of 24 °C and long-day conditions for seed production (140 µmol photons m^{-2} s^{-1}, fluorescent lamps 3071/400 HI-I, IP55, 230 V, 50 Hz, Adolf Schuch GmbH; 6:00 a.m. to 10:00 p.m., r.h. 60 %). The inflorescence stems of eight-week-old plants were bundled and covered with a paper bag for seed production. The seeds of the untransformed mother plant (F0) were termed as F1-seeds and were collected after four weeks.

2.12.4 Selection of homozygous A. thaliana OE-PtGATA12 and AtGATA12 SALK mutant lines

The selection procedure was conducted according to Harrison et al. 2006.

F1-seeds (glufosinate resistant) were sterilized, stratified and sown as previously described (chapter 2.2.3).

Germinated seeds of *35S:PtGATA12* F1-lines were sprayed with glufosinate (200 µg/mL) on day 9, day 12 and last on day 15. Surviving plants were grown separately in greenhouse at a temperature of 24 °C and under long-day conditions for silique production (140 µmol photons m^{-2} s^{-1}, fluorescent lamps 3071/400 HI-I, IP55, 230 V, 50 Hz, Adolf Schuch GmbH; 6:00 a.m. to 10:00 p.m., r.h. 60 %). One leaf per plant was sampled and used to test for a positive transformation by PCR with *PtGATA12* forward and reverse primer (**Tab. 2.8**) and gel electrophoresis. The inflorescence stems of eight-week-old transformed heterozygous F1-plants were bundled and covered with a paper bag for seed production. The seeds of the regenerated transgenic plants (F1) were termed as F2-seeds and were collected after four weeks.

35S:PtGATA12 F2-seeds were sterilized, stratified and sown as previously described. The *35S:PtGATA12* F2-seeds are either homozygous or heterozygous for glufosinate resistance or homozygous for glufosinate sensitivity (=wildtype genotype) (1:2:1). Therefore, the phenotypes of the F2 plants are either glufosinate resistant or glufosinate sensitive (3:1). Germinated seeds were sprayed with glufosinate (200 µg/mL) on day 10, day 11 and last on day 14. Surviving plants were selected and grown separately in greenhouse at a temperature of 24 °C and under long-day conditions for silique production. The inflorescence stems of eight-week-old F2-plants, which could be homozygous or heterozygous for glufosinate resistance, were bundled and covered with a paper bag for seed production. The seeds of the self-pollinated F2 transgenic plants were termed as F3-seeds and were collected after four weeks.

To identify seeds, which are homozygous for the transgene, one hundred seeds were germinated on T-type soil (Fruhstorfer Erde Typ T, Industrieerdenwerk Erich Archut, Lauterbach/Fulda, Germany) and sprayed with glufosinate (200 µg/mL) on day 7, day 8 and last on day 14. If some plants per line died, then most probably the F2 parent of this F3 population was heterozygous. These heterozygous lines were discarded. If all plants for one line survived, then most probably the F2 parent of this F3 population was homozygous. These progeny of F2-plants exhibiting 100 % glufosinate resistances were kept. One leaf per line was sampled and used to test the level of overexpression by quantitative RT-PCR (chapter 2.8) with *PtGATA12* forward and reverse primer (**Tab. 2.8**). Another leaf was used for DNA extraction and sequencing (chapter 2.13.2) by GATC Biotech AG (Konstanz, Germany) using 2.5 µL p7WGF2-reverse primer (**Tab. 2.8**). The inflorescence stems of eight-week-old transformed homozygous F3-plants were bundled and covered with a paper bag for seed production. The seeds of the regenerated transgenic plants (F3) were termed as F4-seeds and were collected after four weeks and used for the experiments.

Moreover, *A. thaliana* seeds of AtGATA12 (At5g25830) T-DNA loss-of-function mutants SALK_112752, SALK_143606 and SALK_012501 were ordered from The Nottingham Arabidopsis Stock Center (NASC, University of Nottingham, Loughborough, United Kingdom) and handled as *35S:PtGATA12 A. thaliana* F1-seeds. But, *A. thaliana* SALK mutant plants were selected with kanamycine sulphate monohydrate (50 µg/mL). Sixteen to 20 kanamycine resistant plants were grown separately under long-day conditions for silique production. After five weeks, one leaf per plant sampled and used to test the genotype for homozygosity by gel electrophoresis (chapter 2.13.3). Another leaf was used to extract *AtGATA12* RNA. The RNA was reverse transcribed to cDNA (as described in chapter 2.8.1) and amplified by PCR (as described in chapter 2.11.1). The amplified cDNA of loss-of-function mutants was monitored by gel electrophoresis (chapter 2.8.2-2.8.3).

2.13 DNA extraction and genotyping

2.13.1 Poplar DNA extraction

For DNA extraction, one leaf per plant was ground to a fine powder in a 2 mL reaction tube (Eppendorf AG, Hamburg, Germany) with glass beads (Ø 0.5 mm and 1 mm, Carl Roth GmbH & Co. KG) in a ball mill (30 Hz, 1.5 min, Mixer Mill MM 200, Retsch GmbH, Haan, Germany), which was cooled with liquid nitrogen to keep the plant material frozen. Poplar DNA was extracted with the innuPREP Plant DNA Kit (Analytik Jena AG, Jena, Germany) and used for gene sequencing by GATC Biotech AG (Konstanz, Germany).

2.13.2 Arabidopsis DNA extraction

After milling, Arabidopsis leaf DNA was extracted according to the following protocol (Edwards et al. 1991). The frozen leaf powder was lysed in 600 µL extraction buffer (**Tab. 2.9**) by tilting the tube several times. After centrifugation (10 min, 19000 x *g*, RT), the supernatant was transferred to a new 1.5 mL reaction tube. Then, 350 µl isopropanol was added and the tube was tilted properly mix the solution, before it was incubated for 5 to 10 min at -20 °C. Then, the sample was centrifuged (10 min, 19000 x *g*, RT) and the supernatant was discarded. Subsequently, 500 µL of 70 % (v/v) ethanol was added, mixed and centrifuged (10 min, 19000 x *g*, RT). The supernatant was discarded and the DNA-pellet was dried overnight at RT. Thereafter, 50 µL deionized and autoclaved H_2O was added to solve the pellet at 65 °C for 10 min under vigorous shaking (1.000 rpm, Thermomixer, Eppendorf AG). The resuspended DNA was used directly for PCR (see next chapter) or gene sequencing by GATC Biotech AG (Konstanz, Germany).

Tab. 2.9: *Composition of 150 mL autoclaved DNA extraction buffer used for Arabidopsis leaf DNA extraction.*

Chemical	Stock solution	pH	Amount for 100 mL in g	Amount for 150 mL extraction buffer in mL	Final concentration in 150 mL
Tris (hydroxymethyl)-amino methane hydro chloride	1 M	1.5	12.114	30.0	200 mM
Sodium chloride	5 M		29.220	7.5	250 mM
EDTA	0.5 M	8.0	14.612	7.5	25 mM
Sodium dodecyl sulphate	10 %		10.000	7.5	0.5 %
Water				97.5	

2.13.3 Genotyping of Arabidopsis SALK mutants

The Arabidopsis SALK mutants were tested for homozygosity by PCR with two different primer combinations. The Insert Primer (IP) is binding to the T-DNA region and used with another primer (RP), flanking right border of T-DNA in the genomic sequence. As second primer pair, the RP primer was combined with LP, the flanking left border of T-DNA in the gene (**Tab. 2.10, Fig. 2.7**). One microliter of DNA solution was used in a 20 µL reaction mix for PCR amplification with 2 µL PCR Buffer $(NH_4)_2SO_4$, 1.6 µL $MgCl_2$ (25 mM), 0.4 µL dNTPs (10 mM), 12.9 µL nuclease-free water, 0.1 µL Taq Polymerase (5 U/µL) and either 2 µL IP and RP or 2 µL RP and LP, which were designed with Primer3 (http://primer3.ut.ee/; Rozen and Skaletsky 2000) and tested by using Oligo Analyzer (Gene Link, Hawthorne, NY, USA). The program for the PCR reaction started with pre-incubation at 95 °C for 60 s. Then, 35 cycles of amplification (94 °C for 15 s, 54 °C for 30 s and 72 °C for 30 s) followed and the reaction was terminated at 72 °C for 5 min. If the T-DNA insertion was homozygous (on both alleles), one single band of specific size (**Tab. 2.10**) was monitored on a 2 % (w/v) agarose gel including ethidium bromide for IP and RP primer combination. Heterozygous T-DNA insertions (on one allele) were resulting in an additional band of specific size for RP and LP region (**Tab. 2.10**). In case, the T-DNA was crossed out (two wildtype alleles), one single band of specific size for RP and LP region was monitored (**Fig. 2.7**).

Tab. 2.10: *SALK mutants of A. thaliana with inserted T-DNA in the AtGATA12 gene sequence. Expected product sizes of RP and LP or RP and IP are listed in the last two columns.*

Gene name	ID	SALK line	Back-ground	Primer flanking left border of T-DNA (LP) (5` to 3`)	Primer flanking right border of T-DNA (RP) (5` to 3`)	Insert Primer (IP) (5` to 3`)	LP + RP Product size (bp)	IP + RP Product size (bp)
AtGATA12	At5G25830	SALK_112752	Col-0	CATATTCAAGTTCGAGTGTCGC	CTTTTATTGCGCTTGAAGGG	ATTTTGCCGATTTCG-GAAC	439-739	29-629
		SALK_012501		AAAGGTGGTGTCGGTACTGC	AGGTCATCAACGGCAAAATC		469-769	59-659
		SALK_143606		AAAGGTGGTGTCGGTACTGC	GTCACCGGAGAAGCTAGTGC		590-890	180-780

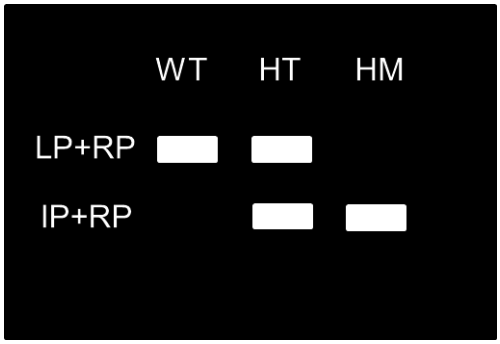


Fig. 2.7: *Schematic picture of the resulting gel after PCR with the three primers LP, RP and IP to test T-DNA insertion lines. WT = wildtype, HT = heterozygous and HM = homozygous.*

2.14 Statistical analyses

An unpaired two sample T-test and one-way ANOVA with Tukey's HSD were performed in Origin Pro 8.5 (Origin Lab Corporation, Northampton, U.S.A.) with raw data to test for differences between treatments and transgenic lines. All mean values that resulted in a p-value < 5 % were considered as significantly different. Data are displayed as mean ± standard error (SE) with n = 3 to 20, as indicated. Additionally, a two-way ANOVA was used to test for significant differences between the nitrogen forms, among different nitrogen levels or between nitrogen forms and genotypic background. Correlations of anatomical examinations and gene expression mean values were calculated using Pearson´s test in Origin Pro 8.5.

3 Results

3.1 Different nitrogen forms affect growth of poplars

P. trichocarpa plants, fertilized with high concentrations of nitrate were significant higher (**Fig. 3.1A**) and thicker (**Fig. 3.1B**) than plants, fed with 0.2 mM nitrogen or with high concentrations of ammonium. The growth with ammonium-nitrate had a similar effect as nitrate alone (**Fig. 3.1**). A significant height increment of 8 mM nitrogen fed *P. trichocarpa* plants started after the second week of treatment, while 0.2 mM nitrogen fertilized plants grew more slowly (**Fig. 3.1, Tab. 3.1**). After the third week of treatment, plants fertilized with 8 mM nitrate or 8 mM ammonium-nitrate were higher than 8 mM ammonium-fed ones. A significantly higher rate of secondary growth followed one week later. Additionally, 8 mM nitrate and 4 mM ammonium-nitrate-fed *P. trichocarpa* plants developed bigger leaves (**Fig. 3.2A**) and exhibited less leaf loss (**Fig. 3.2B**) than plants fertilized with 0.2 mM nitrogen or 8 mM ammonium. Moreover, *P. trichocarpa* plants, fertilized with 8 mM nitrate or 4 mM ammonium-nitrate displayed relatively high chlorophyll contents, whereas 0.2 mM nitrogen fed plants had a consistent slightly decrease (**Fig. 3.3**). Poplars, fed with high ammonium concentrations presented a strong decrease in chlorophyll content after one month treatment (**Fig. 3.3, Tab. 3.2**).

To test whether there were differences in photosynthetic electron transport, light response curves of the quantum yield of PSII were determined. The quantum yield

of PSII of 8 mM nitrate-fed *P. trichocarpa* plants was lower and showed a stronger decline in response to increasing light than that of the treatments (**Fig. 3.4, Tab. 3.3**).

The root biomass of 8 mM ammonium-fed *P. trichocarpa* plants was similar to 0.2 mM nitrogen fertilized ones, but more stem and leaf biomass was developed (**Fig. 3.5**). Plants fertilized with 8 mM nitrate or 4 mM ammonium-nitrate exhibited similar high root biomass and differed only in fresh stem production and fresh and dry leaf biomass, to each other. *P. trichocarpa* plants fertilized with high nitrate concentrations produced more biomass in total and more leaves. The nitrogen level had an influence on relative stem water content of ammonium-fed poplars.. Plants, fed with 8 mM ammonium contained more water in stem tissue than poplars fertilized with 0.2 mM nitrogen (**Tab. 3.4**), apparent as a loss of significant difference in dry stem biomass (**Fig. 3.5B**). In general, the relative water content in whole plant was similar between the various treatments (**Tab. 3.4**: 77-80 %, **Tab. 3.5**). There were significant differences in distinct plant tissues. *P. trichocarpa* plants, fed with high nitrate concentrations contained more water in leaves and roots, accompanied by the lowest standard derivations than plants fertilized with high ammonium concentrations (**Tab. 3.4**).

P. x *canescens* plants, grown under similar conditions as the *P. trichocarpa* plants, exhibited similar growth effects under high nitrate fertilization. Plants fertilized with 8 mM nitrate were significantly higher (**Fig. 3.6A**) and thicker (**Fig. 3.6B**) than plants, fed with 8 mM ammonium. The growth with ammonium-nitrate had the same effect as nitrate alone (**Fig. 3.6**). But, plants fertilized with 0.2 mM nitrogen grew up quite slowly and did not show diameter increment; even those plantlets were already taller and bigger at the beginning of the experiment than plantlets chosen for high nitrogen treatment. Most probably, 0.2 mM nitrogen fertilization was too low for already 35 to 40 cm high plants. There was no growth difference between nitrate, ammonium nitrate or ammonium fertilization of already 35 to 40 cm high plants under limited nitrogen conditions. The significant height and diameter increment of *P.* x *canescens* plants fed with high nitrogen concentrations occurred three weeks later than in *P. trichocarpa* plants grown under the same conditions (**Fig. 3.1**). Additionally, 8 mM nitrate and 4 mM ammonium-nitrate-fed *P.* x *canescens* plants had larger leaves (**Fig. 3.7A**), less leaf loss (**Fig. 3.7B**) and relatively high chlorophyll contents (**Fig. 3.8**), than plants fed with 0.2 mM nitrogen or 8 mM ammonium, similar to 8 mM nitrate-fed *P. trichocarpa* plants (**Fig. 3.2, 3.3**). *P.* x *canescens* plants fed with 8 mM ammonium presented a strong decrease in chlorophyll content upon one month treatment (**Fig. 3.8**) as already found in *P. trichocarpa* plants grown under these conditions (**Fig. 3.3**). In contrast to *P. trichocarpa* plants, there was no significant difference of the quantum yield of fluorescence in 8 mM nitrate-fed *P.* x *canescens* plants compared to high ammonium treatment (**Fig. 3.9**). Additionally, *P.* x *canescens* plants, fertilized with 8 mM ammonium, exhibited less biomass of each plant organ, than plants, fed with 8 mM nitrate or 4 mM ammonium-nitrate (**Fig. 3.10**). In general, the relative water content in whole plant was very similar between the various treatments (**Tab. 3.6**: 78-82 %). In contrast to *P. trichocarpa*

plants, *P.* x *canescens* plants, fertilized with 8 mM nitrate or 4 mM ammonium-nitrate had more water in fine roots, than 8 mM ammonium-fed plants (**Fig. 3.10B**).

Interestingly, *P. trichocarpa* growth was different to that of *P.* x *canescens* in response to different nitrogen forms in hydroponic solution (**Fig. 3.11E**), too. *P. trichocarpa* plants, fed with 2 mM nitrate in hydroponic, were significantly taller than the 2 mM ammonium-fed ones, after eight weeks of fertilization treatment (**Fig. 3.11A**). *P. trichocarpa* plants fertilized with 1 mM ammonium-nitrate in hydroponic differed to 2 mM ammonium-fed plants, after one additional week. *P.* x *canescens* plants, fed with 2 mM nitrate in hydroponic were significantly taller than the 2 mM ammonium-fed plants (**Fig. 3.11B**), like *P. trichocarpa* in hydroponic (**Fig. 3.11A**). But, a significant thicker diameter of *P.* x *canescens* plants, fed with 2 mM nitrate in hydroponic occurred already after five weeks of fertilization treatment (**Fig. 3.11D**), while there was no significant difference in secondary growth of *P. trichocarpa* plants after ten weeks under the same conditions (**Fig. 3.11C**). Only 2 mM nitrate-fed *P. trichocarpa* and *P.* x *canescens* plants in hydroponic developed larger leaves (**Fig. 3.12A-B**) and did not show leaf loss at all (**Fig. 3.12C-D**). Additionally, 2 mM ammonium-fed *P.* x *canescens* plants in hydroponic produced similar amounts of total biomass as *P. trichocarpa* plants, whereas *P.* x *canescens* plants fertilized with 2 mM nitrate or 1 mM ammonium-nitrate, exhibited most biomass in total, compared to *P. trichocarpa* and 2 mM ammonium-fed *P.* x *canescens* plants (**Fig. 3.13**).

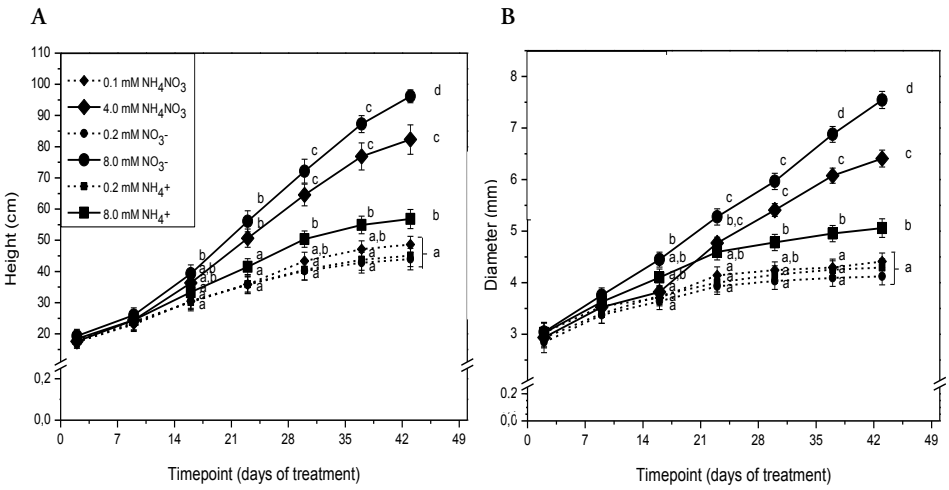
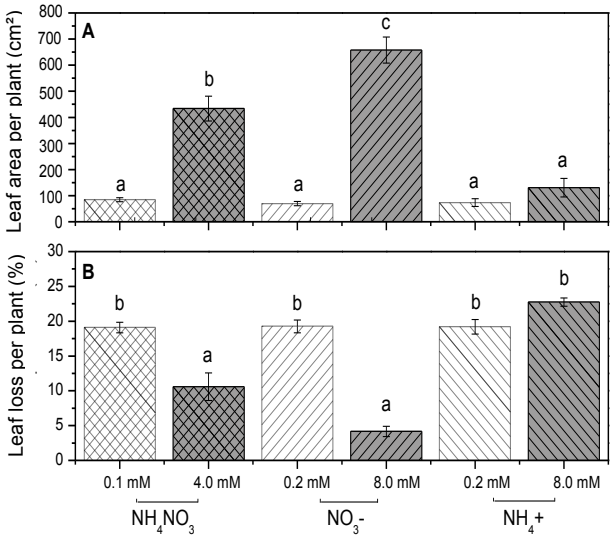


Fig. 3.1: *Mean height (A) and diameter (B) growth of P. trichocarpa in response to fertilization with 0.2 mM or 8 mM nitrogen in different forms (n = 12 per treatment, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (One-way ANOVA and T-test, p < 0.05).*

Tab. 3.1: *Different P. trichocarpa plant height and diameter as response to fertilization with different nitrogen forms under different nitrogen levels started on day 23 of treatment for height and on day 30 of treatment for diameter. No significant differences are darkened (Two-way ANOVA, p < 0.05).*

	Height			Diameter		
Time point	**N-level**	**N-form**	**Interaction**	**N-level**	**N-form**	**Interaction**
(d)	(p-value)	(p-value)	(p-value)	(p-value)	(p-value)	(p-value)
2	0.64	0.90	0.93	0.72	0.98	0.98
9	0.40	0.90	0.82	0.18	0.94	0.60
16	< 0.01	0.45	0.02	0.01	0.29	0.02
23	< 0.01	0.01	< 0.01	< 0.01	0.35	< 0.01
30	< 0.01	0.01	< 0.01	< 0.01	0.02	< 0.01
37	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
43	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01



Factor	p-value
N-level	< 0.01
N-form	< 0.01
Interaction	< 0.01

Fig. 3.2: *Mean leaf area per plant (A) and relative leaf loss (B) of P. trichocarpa were determined after six weeks fertilization with 0.2 mM or 8 mM nitrogen in different forms (n = 8 per treatment, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. B: 100 % = all leaves were lost; 50 % = number of new leaves formed are the same number of lost leaves; 0 % = no leaf was lost. Different letters indicate significant differences (One-way ANOVA, Two-way ANOVA and T-test, p < 0.05).*

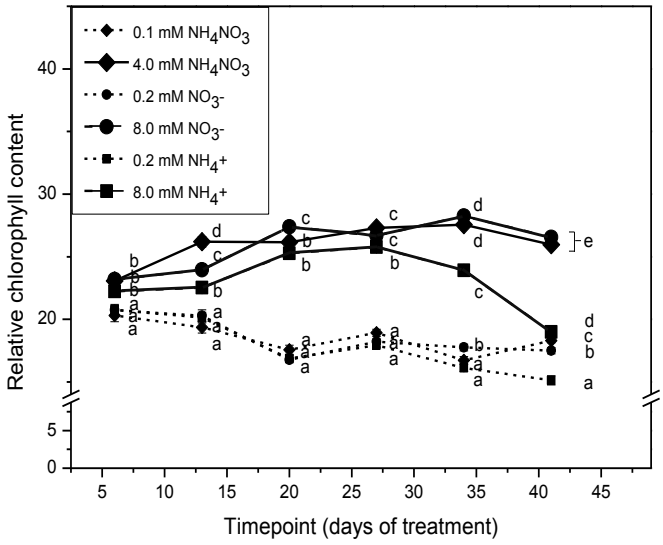


Fig. 3.3: *Relative chlorophyll content in P. trichocarpa leaves grown with 0.2 mM or 8 mM nitrogen in different forms (n = 12 per treatment, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (One-way ANOVA and T-test, p < 0.05).*

Tab. 3.2: *Different chlorophyll content of P. trichocarpa leaves as response to fertilization with different nitrogen forms under different nitrogen levels starting on day 13 of treatment. No significant differences are darkened (Two-way ANOVA, p < 0.05).*

Timepoint	N-level	N-form	Interaction
(d)	(p-value)	(p-value)	(p-value)
6	< 0.01	0.50	< 0.01
13	0.00	< 0.01	0.00
20	0.00	< 0.01	0.00
27	0.00	< 0.01	0.00
34	0.00	0.00	0.00
41	0.00	0.00	0.00

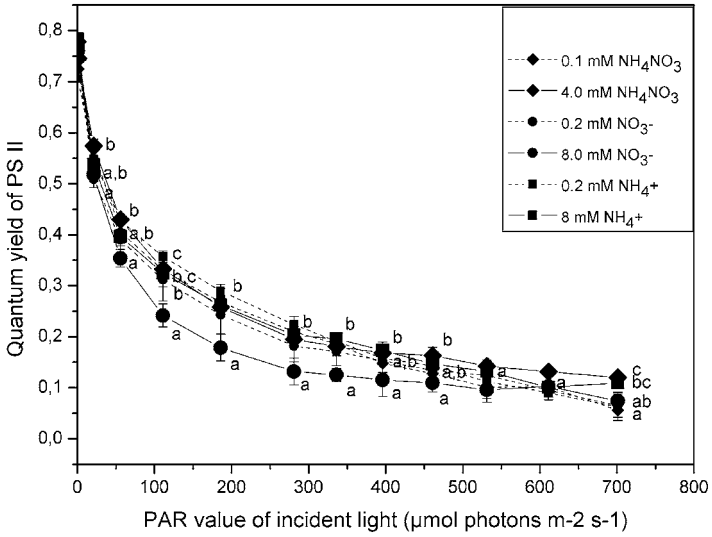
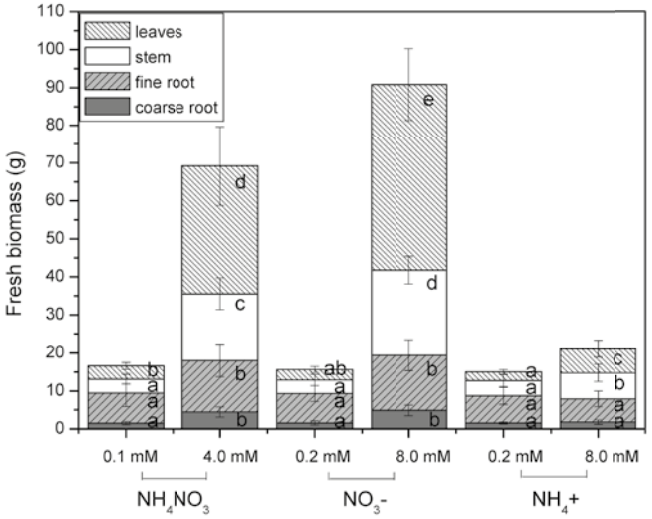


Fig. 3.4: *Quantum yield of Photosystem II (PS II) in youngest fully expanded P. trichocarpa leaves after six weeks fertilization with 0.2 mM or 8 mM nitrogen in different forms (n = 3 per treatment, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (One-way ANOVA, p < 0.05).*

Tab. 3.3: *Different quantum yield of PS II in P. trichocarpa leaves as a result of different nitrogen forms at defined PAR pulse values. No significant differences are darkened (Two-way ANOVA, p < 0.05).*

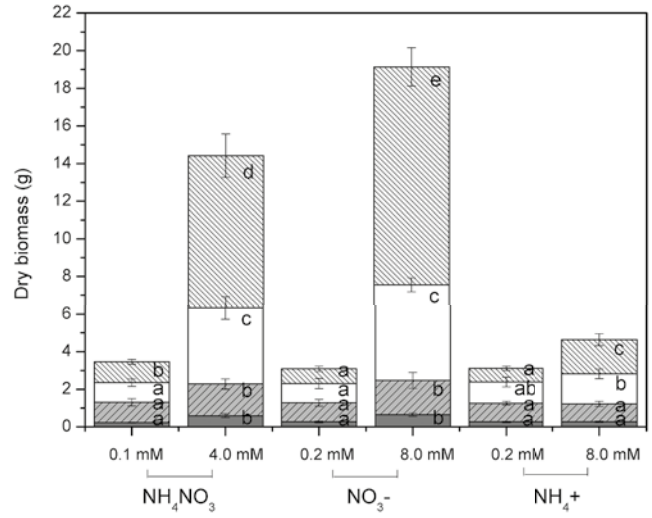
PAR pulse value	N-level	N-form	Interaction
	(p-value)	(p-value)	(p-value)
0	< 0.01	< 0.01	< 0.01
1	< 0.01	0.31	< 0.01
21	0.42	< 0.01	< 0.01
56	0.62	0.06	0.10
111	0.11	< 0.01	0.01
186	0.05	< 0.01	< 0.01
281	0.04	< 0.01	< 0.01
336	0.20	< 0.01	< 0.01
396	0.85	0.16	0.28
461	0.41	0.26	0.33
531	0.24	0.72	0.55
611	0.11	0.02	0.02
701	< 0.01	0.35	< 0.01

A



Factor	p-value
N-level	< 0.01
N-form	< 0.01
Interaction	< 0.01

B



Factor	p-value
N-level	< 0.01
N-form	< 0.01
Interaction	< 0.01

Fig. 3.5: *Fresh (A) and dry (B) biomass of P. trichocarpa leaves, stem, fine and coarse roots after six weeks fertilization with 0.2 mM or 8 mM nitrogen in different forms (n = 8 per treatment, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (One-way ANOVA, Two-way ANOVA and T-test, p < 0.05).*

Tab. 3.4: *Relative water content of P. trichocarpa plants after six weeks fertilization with 0.2 mM or 8 mM nitrogen in different forms (n = 8, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (One-way ANOVA and T-test, p < 0.05). 100 % = complete plant organ consists of water.*

Tissue	0.1 mM NH_4NO_3	4 mM NH_4NO_3	0.2 mM NO_3	8 mM NO_3	0.2 mM NH_4	8 mM NH_4
Leaves	60.65 % ± 3.10 (a)	73.90 % ± 0.92 (bc)	66.01 % ± 6.65 (ab)	74.96 % ± 0.84 (c)	64.70 % ± 5.73 (ab)	66.28 % ± 4.56 (ab)
Stem	47.20 % ± 4.45 (a)	65.74 % ± 5.32 (b)	34.48 % ± 10.19 (a)	67.69 % ± 2.07 (b)	44.78 % ± 8.78 (a)	62.39 % ± 4.11 (b)
Fine root	82,99 % ± 1.92 (bc)	83,62 % ± 2.53 (bc)	82,18 % ± 1.96 (bc)	85,25 % ± 1.08 (c)	81,06 % ± 1.54 (ab)	76,11 % ± 3.50 (a)
Coarse root	83,85 % ± 1.39 (a)	86,47 % ± 0.70 (b)	82,87 % ± 1.04 (a)	86,61 % ± 0.61 (b)	82,85 % ± 1.56 (a)	84,53 % ± 0.98 (a)
Whole plant	78,77 % ± 1.20 (a)	79,10 % ± 1.16 (a)	80,21 % ± 1.32 (a)	78,90 % ± 0.52 (a)	79,22 % ± 1.79 (a)	77,82 % ± 1.40 (a)

Tab. 3.5: *No difference in relative water content of soil-grown P. trichocarpa plants fertilized with different nitrogen forms and levels (Two-way ANOVA, p > 0.05).*

Factor	p-value
N-level	0,17
N-form	0,44
Interaction	< 0.01

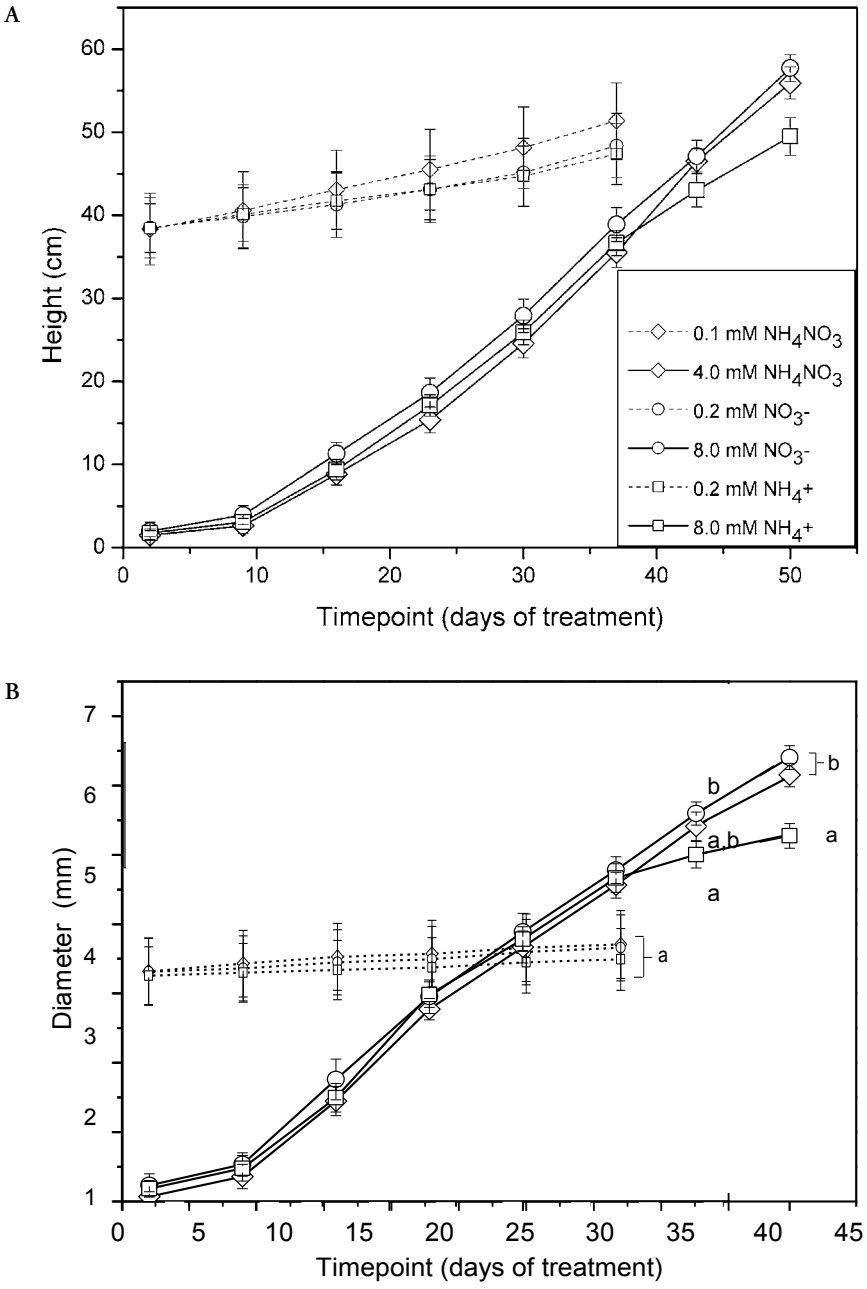


Fig. 3.6: *Mean height (A) and diameter (B) growth of P. x canescens in response to fertilization with 0.2 mM (relatively flat curves) or 8 mM nitrogen (increasing curves) in different forms (n = 6 per treatment, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (One-way ANOVA and T-test, p < 0.05).*

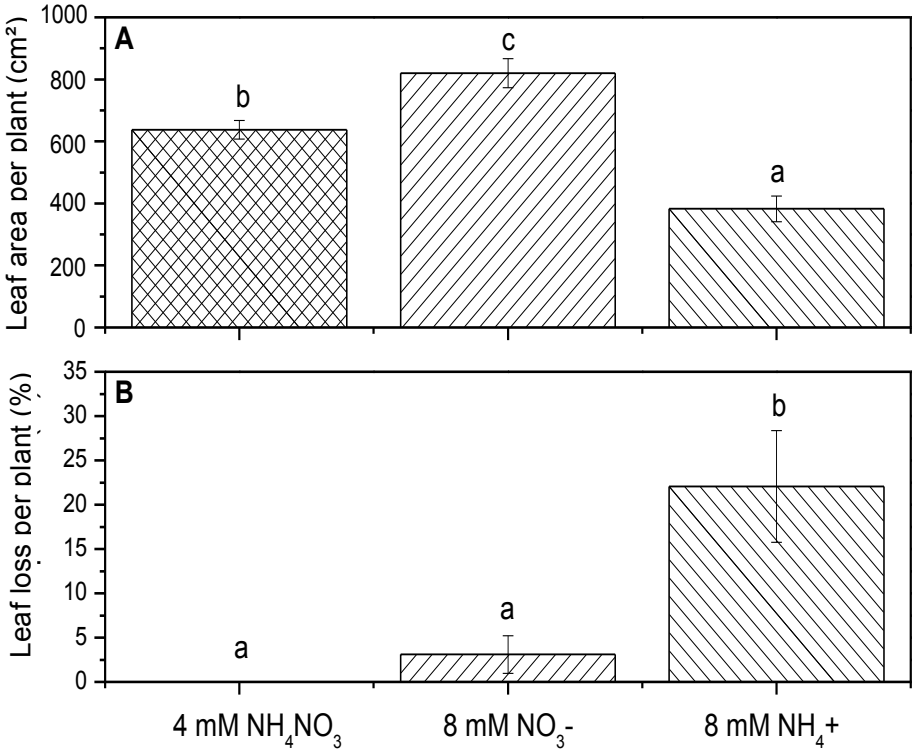


Fig. 3.7: *Mean leaf area per plant (A) and relative leaf loss (B) of P. x canescens were determined after seven weeks fertilization with 8 mM nitrogen in different forms (n = 6 per treatment, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. B: 100 % = all leaves were lost; 50 % = number of new leaves formed are the same number of lost leaves; 0 % = no leaf was lost. Different letters indicate significant differences (One-way ANOVA, Two-way ANOVA and T-test, p < 0.05).*

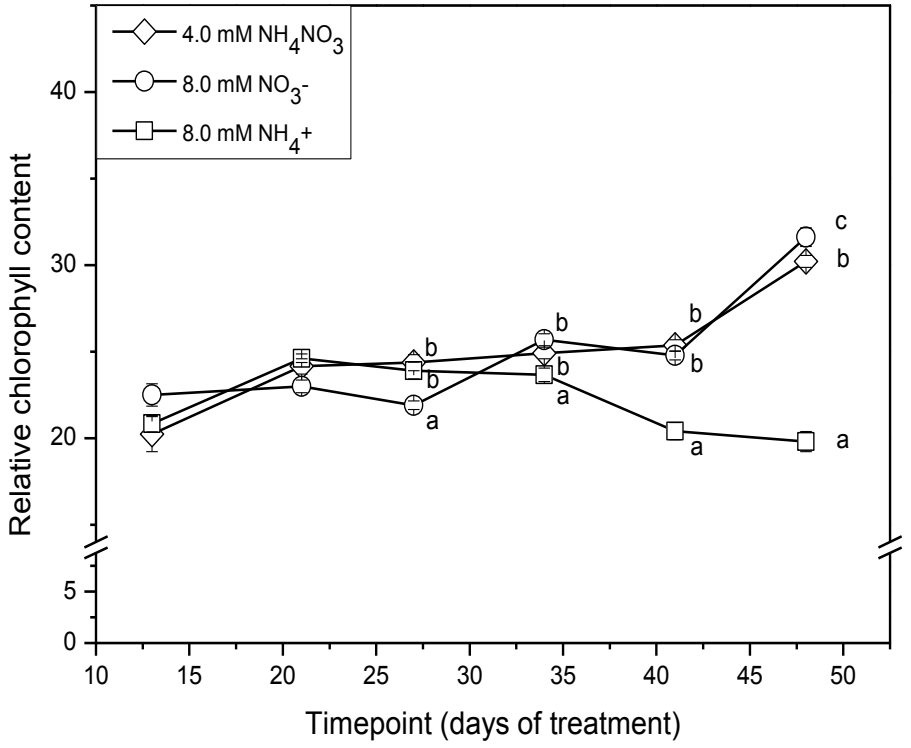


Fig. 3.8: *Relative chlorophyll content in P. x canescens leaves grown with 8 mM nitrogen in different forms (n = 6 per treatment, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (One-way ANOVA and T-test, $p < 0.05$).*

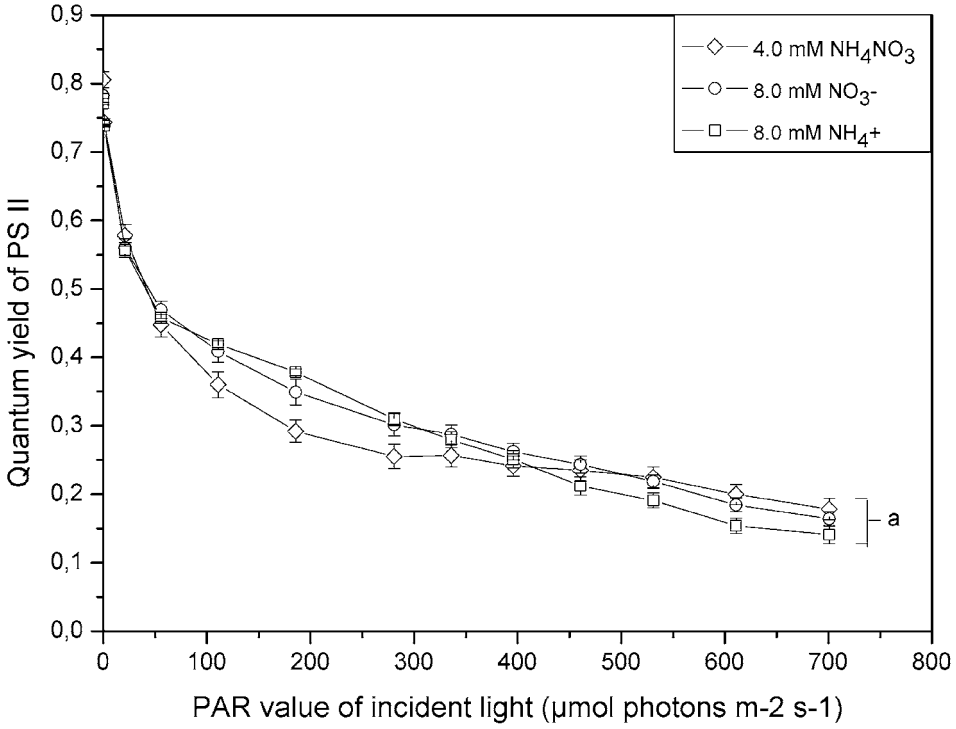


Fig. 3.9: *Quantum yield of Photosystem II (PS II) in youngest fully expanded P. x canescens leaves after seven weeks fertilization with 8 mM nitrogen in different forms (n = 3 per treatment, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (One-way ANOVA, $p < 0.05$).*

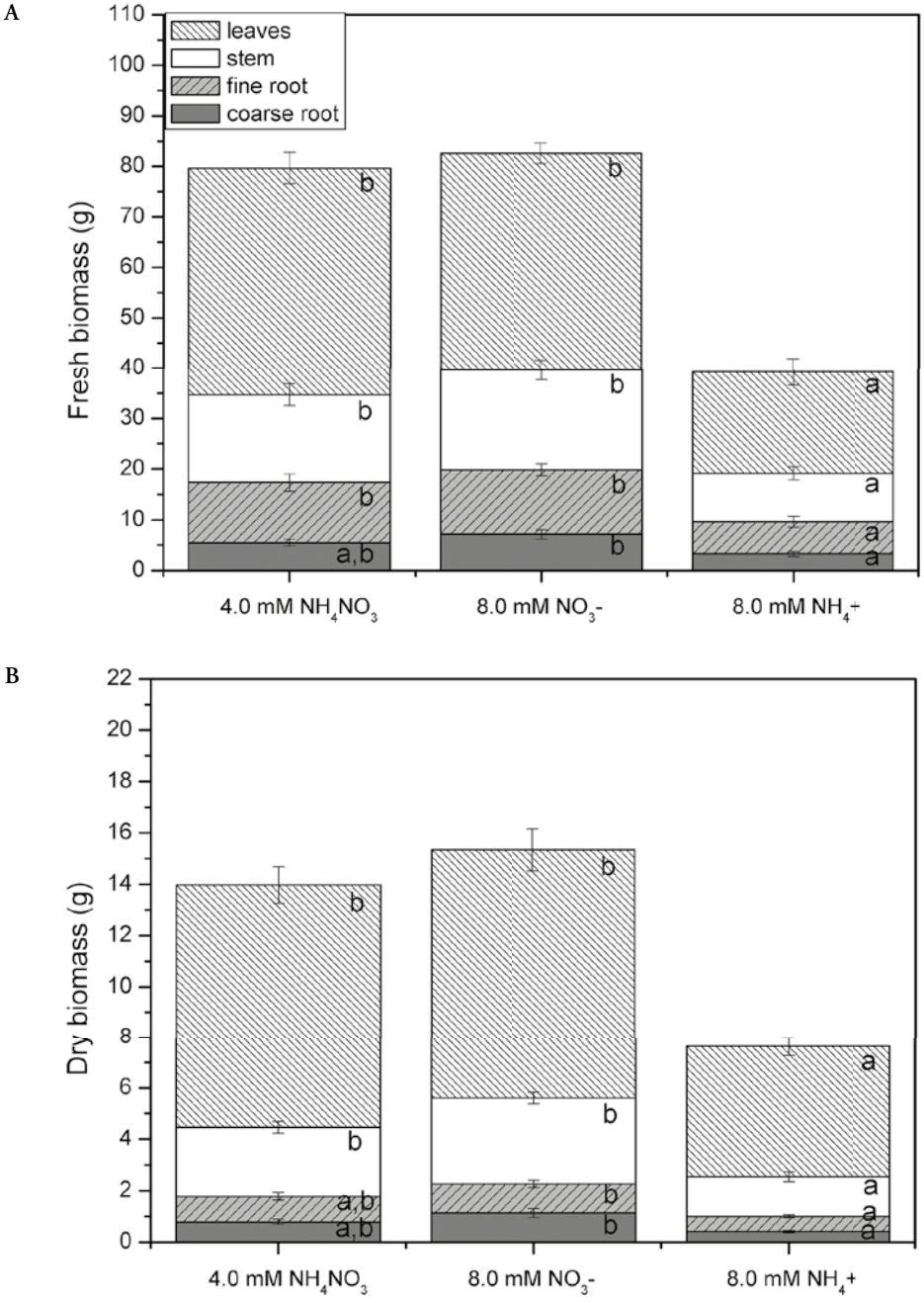
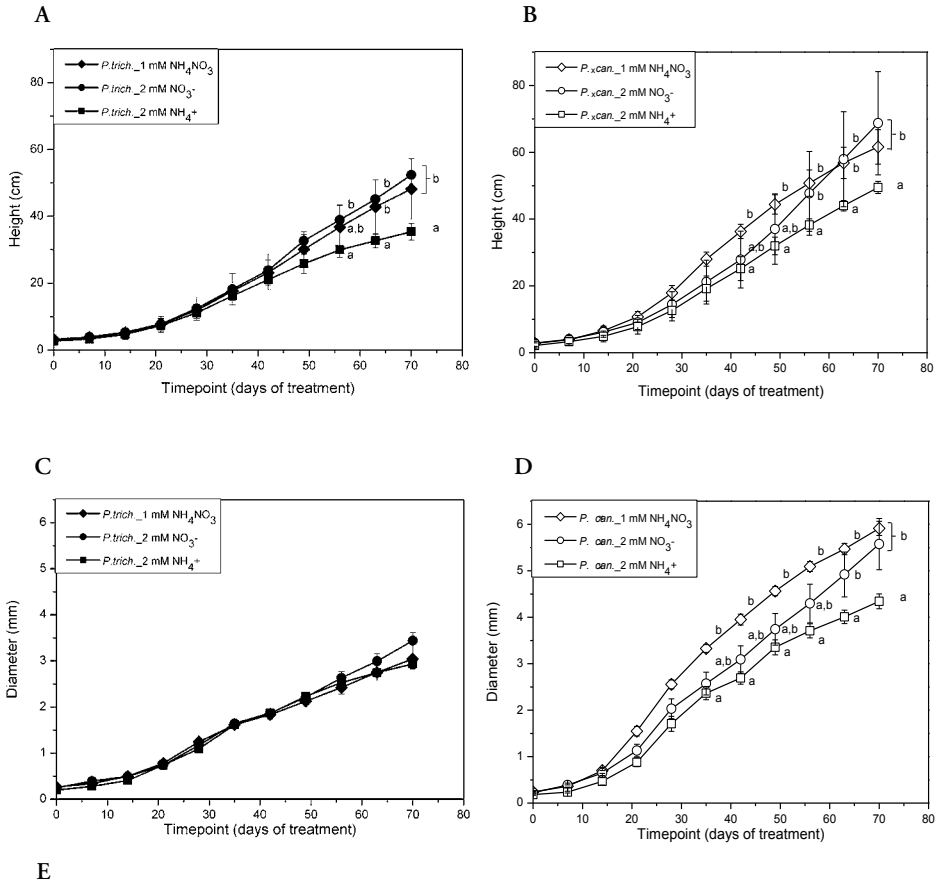


Fig. 3.10: *Fresh (A) and dry (B) biomass of P. x canescens leaves, stem, fine and coarse roots after seven weeks fertilization with 8 mM nitrogen in different forms (n = 6 per treatment, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (One-way ANOVA and T-test, p < 0.05).*

Tab. 3.6: *Relative water content of P. x canescens after seven weeks fertilization with 8 mM nitrogen in different forms (n = 6 mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (One-way ANOVA and T-test, p < 0.05).*

Tissue	**4 mM NH_4NO_3**			**8 mM NO_3**			**8 mM NH_4**		
Leaves	76.32 %	± 0.38	(a)	73.09 %	± 0.62	(a)	63.75 %	± 5.94	(a)
Stem	65.42 %	± 0.75	(a)	63.37 %	± 0.87	(a)	64.19 %	± 1.08	(a)
Fine root	88,97 %	± 1.13	(b)	88,76 %	± 0.90	(b)	84,39 %	± 1.47	(a)
Coarse root	85,49 %	± 0.72	(a)	84,58 %	± 1.00	(a)	86,89 %	± 0.97	(a)
Whole plant	81,59 %	± 0.23	(a)	79,61 %	± 0.47	(a)	78,85 %	± 1.93	(a)



E

	Height			Diameter		
Timepoint	**Species**	**N-form**	**Interaction**	**Species**	**N-form**	**Interaction**
(d)	(p-value)	(p-value)	(p-value)	(p-value)	(p-value)	(p-value)
70	< 0.01	< 0.01	0.87	< 0.01	< 0.01	0.03

Fig. 3.11: *Mean height (A-B) and diameter (C-D) growth of P. trichocarpa (A, C) and P. x canescens (B, D) in response to fertilization with 2 mM nitrogen in different forms (n = 10 per treatment, mean ± SE). Plants were grown in hydroponic culture. Hydroponic nutrient solutions were changed twice a week until the plants were 10 cm tall and thereafter daily. Different letters indicate significant differences (One-way ANOVA, p < 0.05). E: Two-way ANOVA on last day of treatment, p < 0.05. No significant differences are darkened: P. trichocarpa growth is different to P. x canescens growth as response to different nitrogen forms.*

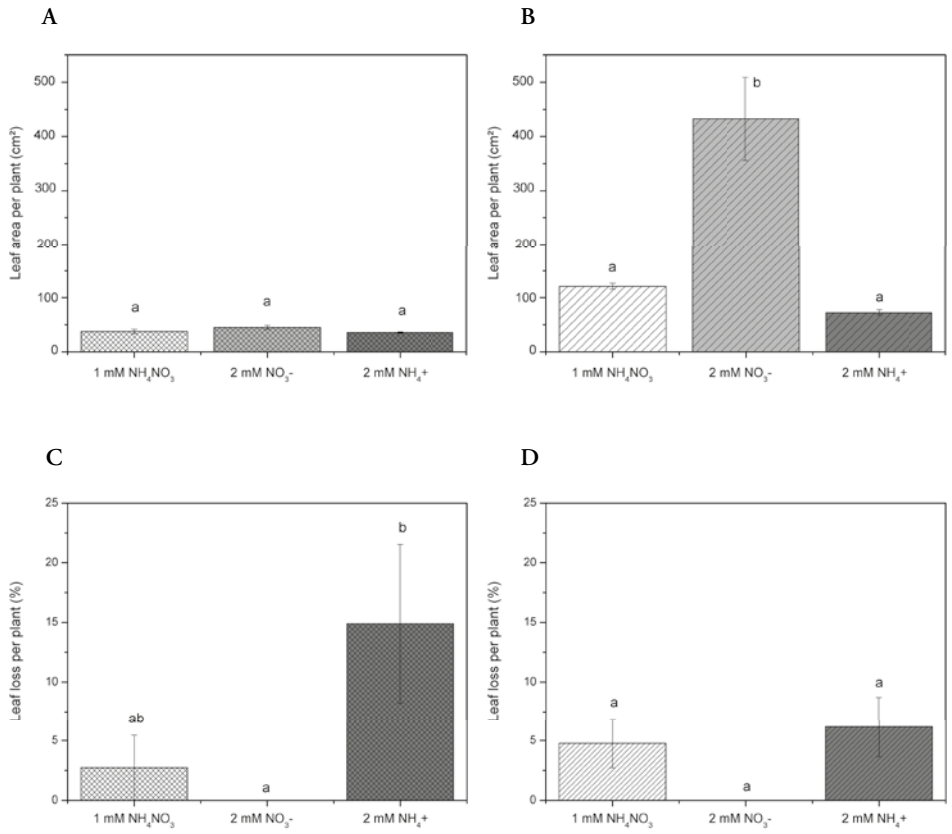


Fig. 3.12: *Mean leaf area per plant (A-B) and relative leaf loss (C-D) of P. trichocarpa (A, C) and P. x canescens (B, D) were determined after ten weeks fertilization with 2 mM nitrogen in different forms (n = 10 per treatment, mean ± SE). Plants were grown in hydroponic culture. Hydroponic nutrient solutions were changed twice a week until the plants were 10 cm tall and thereafter daily. C-D: 100 % = all leaves were lost; 50 % = number of new leaves formed are the same number of lost leaves; 0 % = no leaf was lost. Different letters indicate significant differences (One-way ANOVA, p < 0.05).*

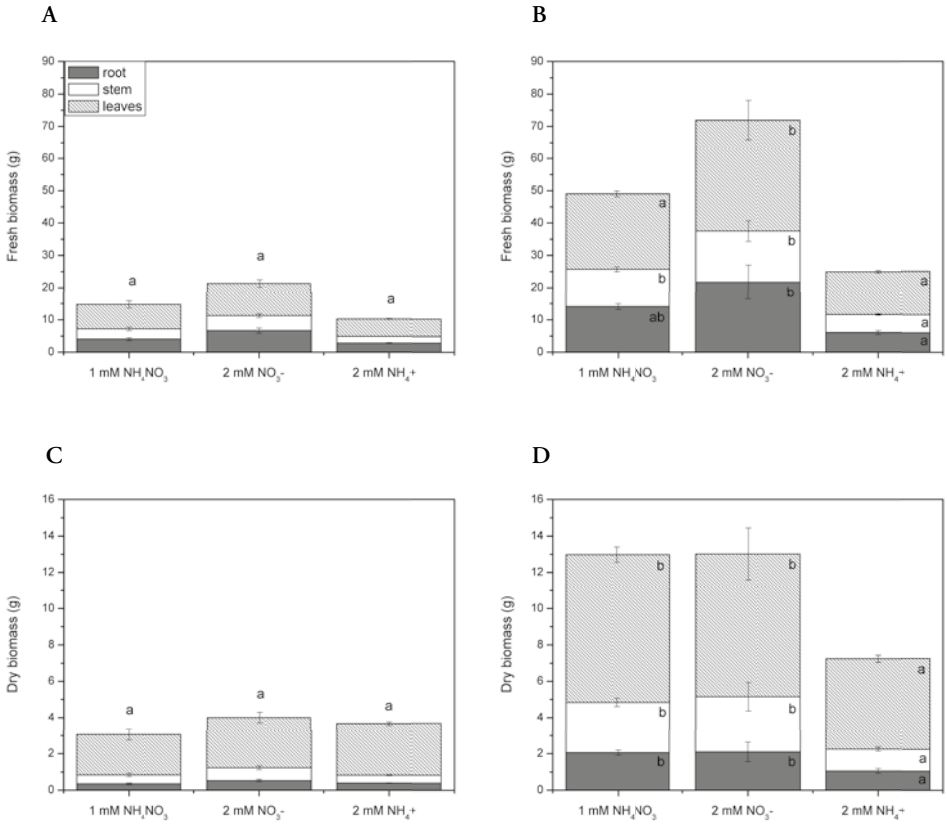


Fig. 3.13: *Fresh (A-B) and dry (C-D) biomass of of P. trichocarpa (A, C) and P. x canescens (B, D) leaves, stem and roots were determined after ten weeks fertilization with 2 mM nitrogen in different forms (n = 10 per treatment, mean ± SE). Plants were grown in hydroponic culture. Hydroponic nutrient solutions were changed twice a week until the plants were 10 cm tall and thereafter daily. Different letters indicate significant differences (One-way ANOVA, p < 0.05).*

3.2 Ammonium and nitrate concentrations in poplar plant tissues

Soil-grown *P. trichocarpa* plants fertilized with 8 mM nitrogen exhibited neither significant different concentrations of ammonium in young leaves, stem and developing xylem tissue (**Fig. 3.14A, C, E**), nor significant different concentrations of nitrate in stem (**Fig. 3.14D**). 8 mM nitrate-fed *P. trichocarpa* plants contained significantly more nitrate in young leaves and developing xylem tissue, than 8 mM ammonium-fertilized plants (**Fig. 3.14B, F**).

Additionally, 8 mM nitrate-fed *P.* x *canescens* plants showed a significantly higher nitrate content in young leaves, developing xylem tissue and stem than 8 mM ammonium-fed *P.* x *canescens* plants (**Fig. 3.15B, D, F**). The nitrate concentration in young leaves of *P.* x *canescens* plants fertilized with 8 mM ammonium-nitrate was as high as in 8 mM nitrate-fed ones (**Fig. 3.15B**). In stem and developing xylem tissue of these plants, the nitrate content was much lower than in these tissues of 8 mM nitrate-fed *P.* x *canescens* plants (**3.15D, F**). Besides, the ammonium concentration in developing xylem tissue of 8 mM nitrate-fertilized *P.* x *canescens* plants was significantly higher than of other nutrient feeding treatments (**Fig. 3.15E**). The ammonium content in stem tissue (**Fig. 3.15C**) and young leaves of these plants (**Fig. 3.15A**) was very low, instead. 8 mM ammonium-fed *P.* x *canescens* plants showed the highest concentration of ammonium in stem tissue (**Fig. 3.15C**), whereas the ammonium content of young leaves was highest in 8 mM ammonium-nitrate plants (**Fig. 3.15A**).

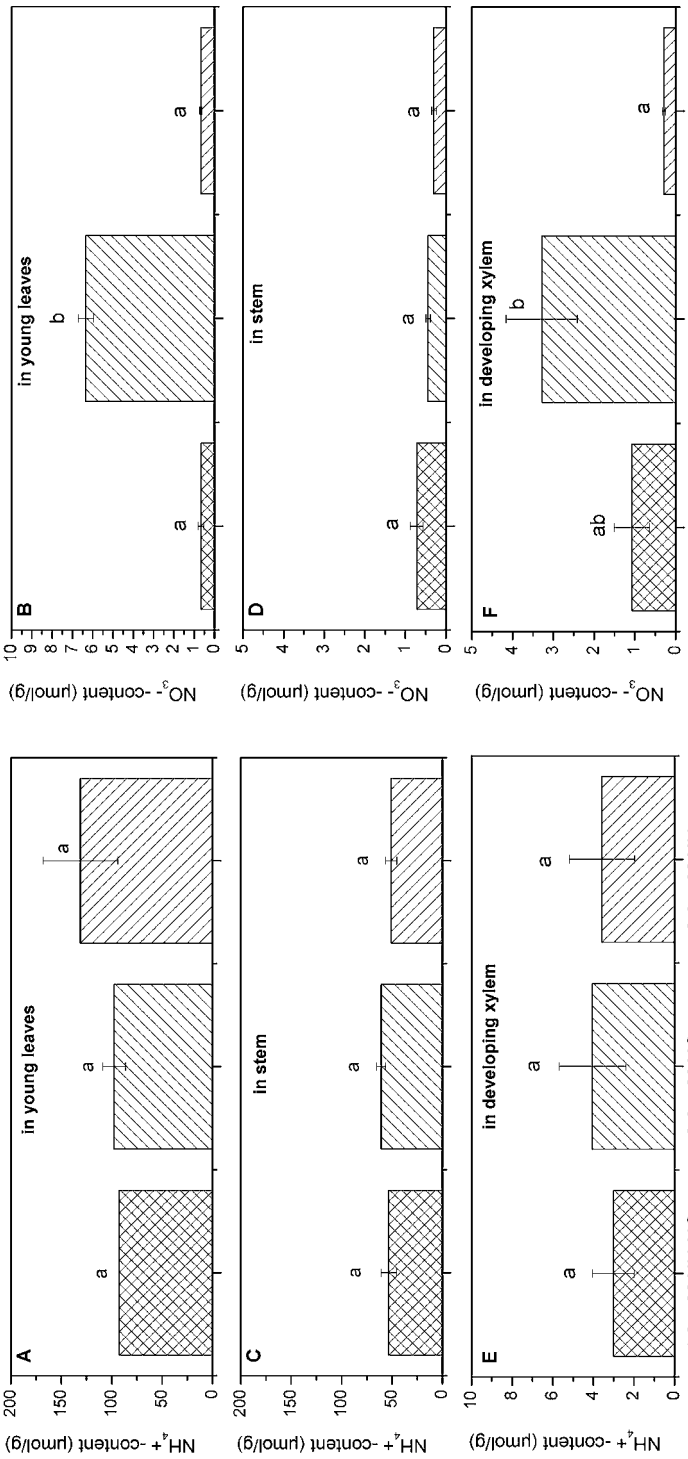


Fig. 3.14: *Mean ammonium ion (A, C, E) and mean nitrate content (B, D, F) were determined in young leaves (A-B), stem (C-D) and developing xylem of basal stem tissue (E-F) of P. trichocarpa plants after six weeks fertilization with 8 mM nitrogen in different forms (n = 3, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (One-way ANOVA and T-test, p < 0.05).*

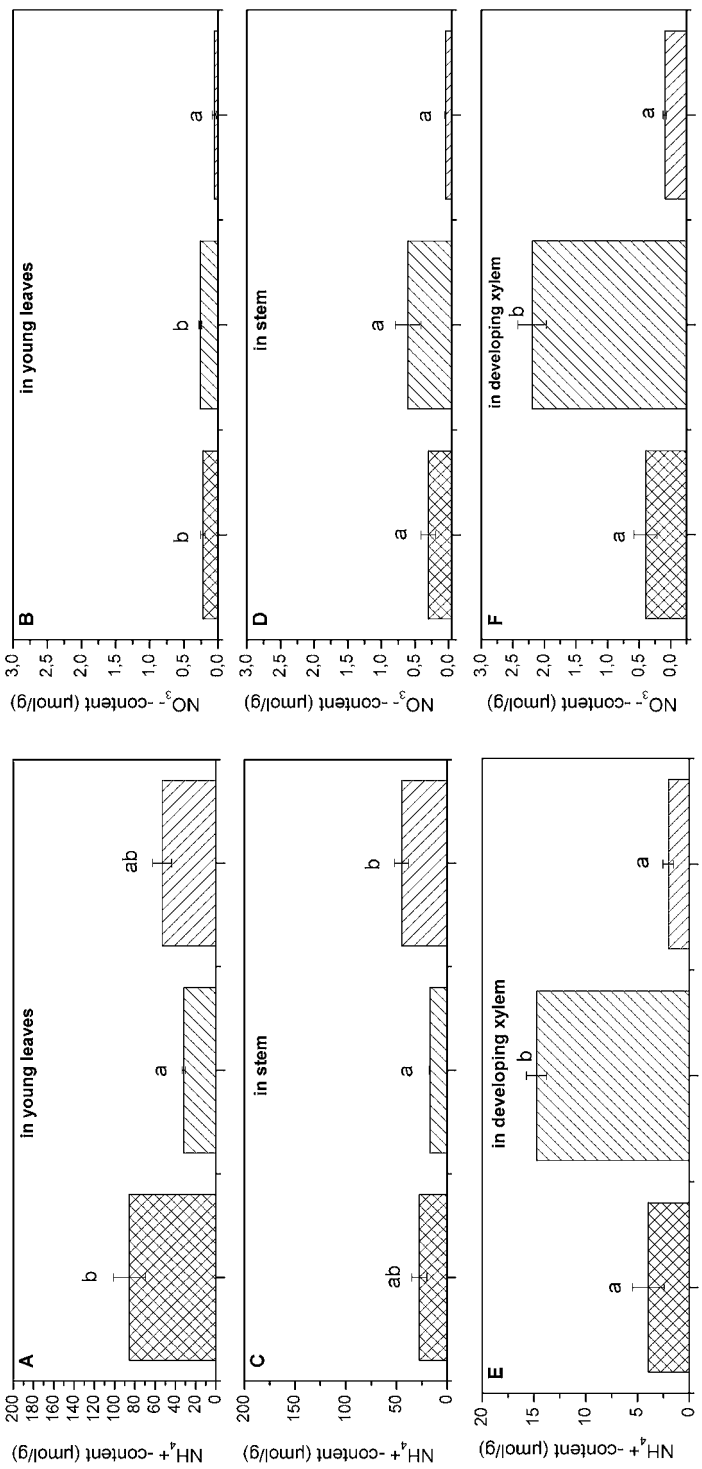


Fig. 3.15: *Mean ammonium ion (A, C, E) and mean nitrate content (B, D, F) were determined in young leaves (A-B), stem (C-D) and developing xylem of basal stem tissue (E-F) of P. x canescens plants after seven weeks fertilization with 8 mM nitrogen in different forms (n = 3, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (One-way ANOVA and T-test, p < 0.05).*

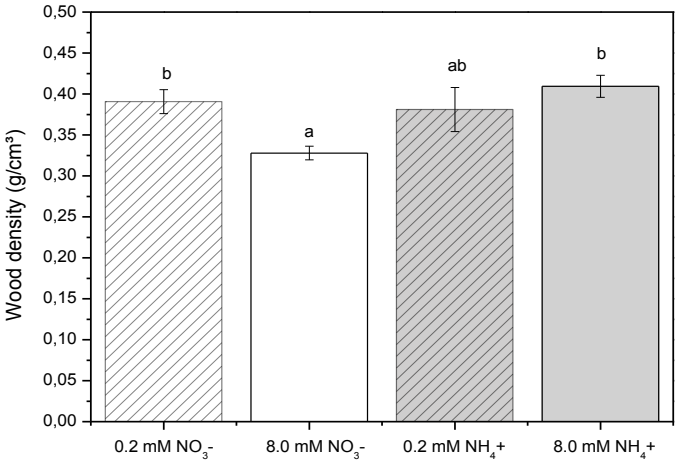
3.3 Different nitrogen forms affect wood properties and wood formation of poplars

The wood density of poplars was affected by high and low nitrogen (**Fig. 3.16-3-17**). The lowest wood density exhibited *P. trichocarpa* and *P.* x *canescens* plants fertilized with 8 mM nitrate (**Fig. 3.16-17**). The highest wood density was shown by 8 mM ammonium-fed *P. trichocarpa* (**Fig. 3.16**).

The C/N ratio in dry wood material of 8 mM ammonium-fed *P. trichocarpa* plants was significantly higher than of other nutrient feeding treatments (**Fig. 3.18A**). Beside, significantly lower nitrogen content was measured in dry wood material of these plants than in plants from other nitrogen treatments (**Fig. 3.18B**). *P. trichocarpa* plants fertilized with 8 mM nitrate contained more nitrogen in dry wood material, but still significantly less than in low nitrogen fed plants. However, these changes did not affect the C/N ratio between 8 mM and 0.2 mM nitrogen fertilized plants (**Fig. 3.18A**).

When holocellulose content in dry wood material was measured, a significant difference could be detected just in low nitrogen fed *P. trichocarpa* plants (**Fig. 3.19**). 0.2 mM nitrate-fed *P. trichocarpa* plants contained significantly less holocellulose than 0.2 mM ammonium-fertilized ones. Holocellulose content in dry wood of 8 mM nitrate or 8 mM ammonium-fed plants was similar, but lignin content was significantly different, instead. The dry wood material of 8 mM ammonium-fertilized *P. trichocarpa* plants contained the most lignin, whereas wood of 8 mM ammonium-fed *P. trichocarpa* plants contained the least amounts of lignin (**Fig. 3.19**).

Besides, 8 mM ammonium-fed poplars developed a wider cambium than 0.2 mM ammonium-fed ones, but similar width of developing xylem tissue (~6%; **Tab. 3.7**). 8 mM nitrate-fertilized *P. trichocarpa* plants showed more mature and developing xylem tissue than in response to other nitrogen treatments. Additionally, the stem cross section of those plants consisted in more than a half of xylem tissue in these plants. Thus, there was relatively less phloem, bark and pith tissue developed. Moreover, plants fertilized with 8 mM nitrate had significant bigger fibers and especially vessels in xylem tissue (**Fig. 3.20c-d**), accompanied by a fewer vessels per area unit than in response to other nitrogen treatments (**Tab. 3.8**). While vessel lumen area increases during maturation of secondary xylem, fiber lumen area decreased, because of secondary fiber cell wall thickening, particularly under high nitrate levels (**Fig. 3.21**). Plants fed with low nitrogen levels, showed the thickest fiber cell walls (1.63 ±0.05 µm to 1.75 ±0.07 µm, **Tab. 3.8**). There was no vessel cell wall increment from developing to mature xylem neither under low, nor under high nitrate levels. Thus, high nitrate-fed *P. trichocarpa* plants exhibited the thinnest vessel cell walls in mature xylem (0.9 ±0.03 µm, **Tab. 3.8**; **Fig. 3.21c**).



Factor	p-value
N-level	0.326
N-form	0.045
interaction	0.01

Fig. 3.16*: Mean wood density of P. trichocarpa plants after six weeks fertilization with 0.2 mM or 8 mM nitrate or ammonium (n = 8, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (One-way ANOVA, Two-way ANOVA and T-test, p < 0.05).*

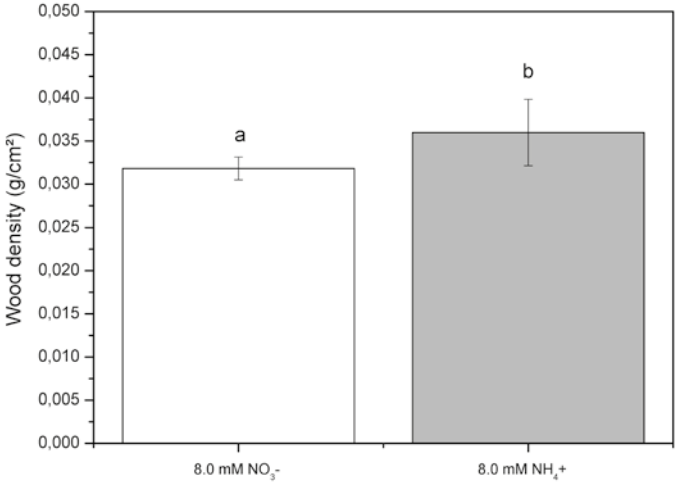
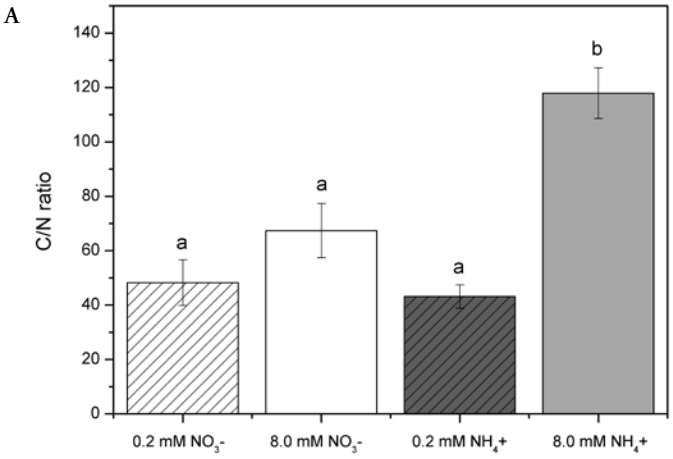
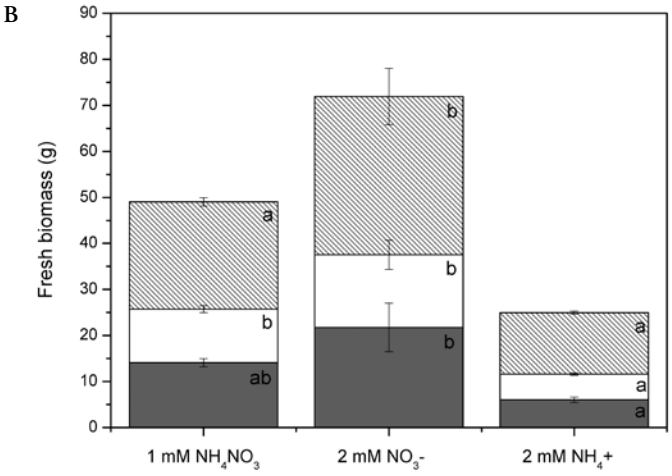


Fig. 3.17*: Mean wood density of P. x canescens plants after seven weeks fertilization with 8 mM nitrate or ammonium (n = 6, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (One-way ANOVA and T-test, p < 0.05).*

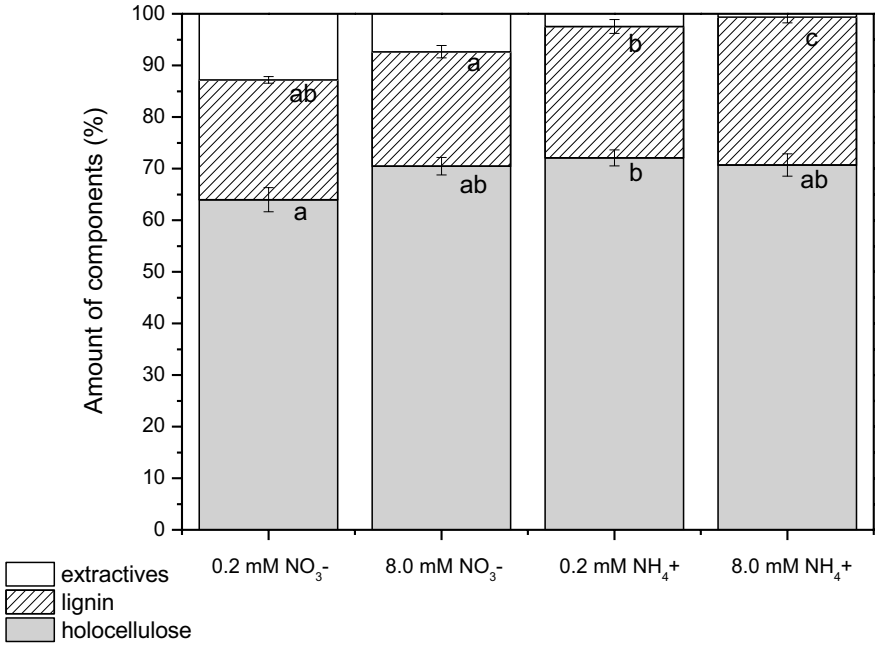


Factor	p-value
N-level	< 0.01
N-form	< 0.01
Interaction	< 0.01



Factor	p-value
N-level	< 0.01
N-form	< 0.01
Interaction	0.3

Fig. 3.18*: A: C/N ratio and B: relative nitrogen content (%) in dry wood of P. trichocarpa plants after six weeks fertilization with 0.2 mM or 8 mM nitrate or ammonium (n = 5, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (One-way ANOVA and Two-way ANOVA p < 0.05).*



Factor	p-value Holocellulose	p-value Lignin
N-level	0.210	0.34
N-form	0.049	< 0.01
Interaction	0.06	< 0.01

Fig. 3.19*: Secondary xylem cell wall components in relative mean amounts of P. trichocarpa dry wood after six weeks fertilization with 0.2 mM or 8 mM nitrate or ammonium (n = 5, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. 100 % = complete wood composition. Measured lignin (patterned bars) and holocellulose (grey bars) content is shown. Undefined extractives were not analyzed (white bars). Different letters indicate significant differences (One-way ANOVA and Two-way ANOVA p < 0.05).*

Tab. 3.7: *Tissue distributions of P. trichocarpa stem cross sections after six weeks plant fertilization with 0.2 mM or 8 mM nitrate or ammonium (n = 5, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Percentages are calculated from cell layer width and radius of cross sections. Distinct letters per row indicate significant differences (One-way ANOVA and T-test, p < 0.05).*

Tissue in %	0.2 mM NO_3^- (LNi)			8 mM NO_3^- (HNi)			0.2 mM NH_4^+ (LAm)			8 mM NH_4^+ (HAm)		
Bark	5.09	± 0.27	(b)	3.10	± 0.17	(a)	3.54	± 0.20	(a)	3.74	± 0.24	(a)
Phloem	21.60	± 1.22	(ab)	18.61	± 0.79	(a)	23.01	± 0.59	(b)	20.25	± 1.06	(ab)
Cambium	1.49	± 0.20	(ab)	1.68	± 0.11	(b)	1.10	± 0.08	(a)	1.92	± 0.11	(b)
Developing xylem	6.35	± 0.69	(a)	12.73	± 0.53	(b)	6.55	± 0.43	(a)	5.76	± 0.46	(a)
Mature xylem	30.46	± 2.74	(a)	51.89	± 1.43	(c)	27.71	± 1.09	(a)	39.21	± 1.35	(b)
Pith	33.00	± 6.88	(c)	17.71	± 2.26	(a)	29.85	± 6.99	(bc)	26.91	± 7.37	(b)

Tab. 3.8: *Detailed xylem cell anatomy of P. trichocarpa stem cross sections after six weeks plant fertilization with 0.2 mM or 8 mM nitrate or ammonium (n = 5, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Percentages are calculated from cell layer width and radius of cross sections. Distinct letters per row indicate significant differences (One-way ANOVA and T-test, p < 0.05).*

Tissue	0.2 mM NO_3^- (LNi)			8 mM NO_3^- (HNi)			0.2 mM NH_4^+ (LAm)			8 mM NH_4^+ (HAm)		
Developing xylem fibers												
Number (cells/mm²)	4140	± 193.4	(ab)	3673	± 256.6	(a)	4173	± 167.5	(ab)	4720	± 249.8	(b)
Lumen/fiber (µm²)	65.60	± 3.56	(a)	103.79	± 4.64	(b)	68.44	± 3.89	(a)	68.14	± 4.39	(a)
Cell wall thickness (µm)	1.16	± 0.06	(ab)	0.91	± 0.07	(a)	1.33	± 0.08	(b)	0.97	± 0.06	(a)
Developing xylem vessels												
Number (cells/mm²)	209	± 19.55	(b)	137	± 8.45	(a)	211	± 13.00	(b)	185	± 14.45	(ab)
Lumen/vessel (µm²)	538.36	± 33.47	(a)	1275.05	± 55.56	(b)	613.35	± 32.93	(a)	668.33	± 42.60	(a)
Cell wall thickness (µm)	1.26	± 0.07	(b)	0.96	± 0.05	(a)	0.89	± 0.06	(a)	1.00	± 0.05	(a)
Mature xylem fibers												
Number (cells/mm²)	4513	± 344.3	(ab)	4153	± 248.4	(a)	4560	± 232.8	(ab)	5353	± 252.2	(b)
Lumen/fiber (µm²)	67.47	± 4.33	(b)	80.82	± 3.61	(c)	49.96	± 2.91	(a)	60.56	± 2.34	(a)
Cell wall thickness (µm)	1.63	± 0.05	(b)	1.56	± 0.05	(ab)	1.75	± 0.07	(b)	1.37	± 0.11	(a)
Mature xylem vessels												
Number (cells/mm²)	221	± 11.21	(b)	143	± 8.39	(a)	204	± 9.75	(b)	204	± 12.73	(b)
Lumen/vessel (µm²)	888.99	± 40.93	(a)	1229.47	± 71.35	(b)	879.71	± 64.01	(a)	896.83	± 52.89	(a)
Cell wall thickness (µm)	1.28	± 0.05	(b)	0.90	± 0.03	(a)	1.07	± 0.06	(a,b)	1.21	± 0.09	(b)

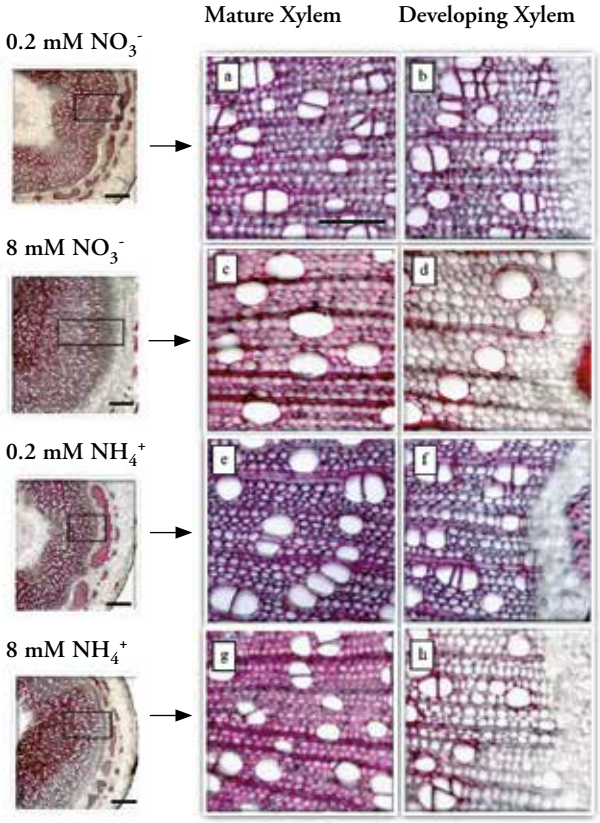


Fig. 3.20: *Phloroglucinol staining of P. trichocarpa stem cross sections. Vessel lumen and cell wall thickness were measured in developing (b, d, f, h) and mature xylem tissue (a, c, e, g) after six weeks fertilization with 0.2 mM (a-b, e-f) or 8 mM (c-d, g-h) nitrate (a-d) or ammonium (e-h). Scale bar: 100 µm/small overview pictures: 500µm.*

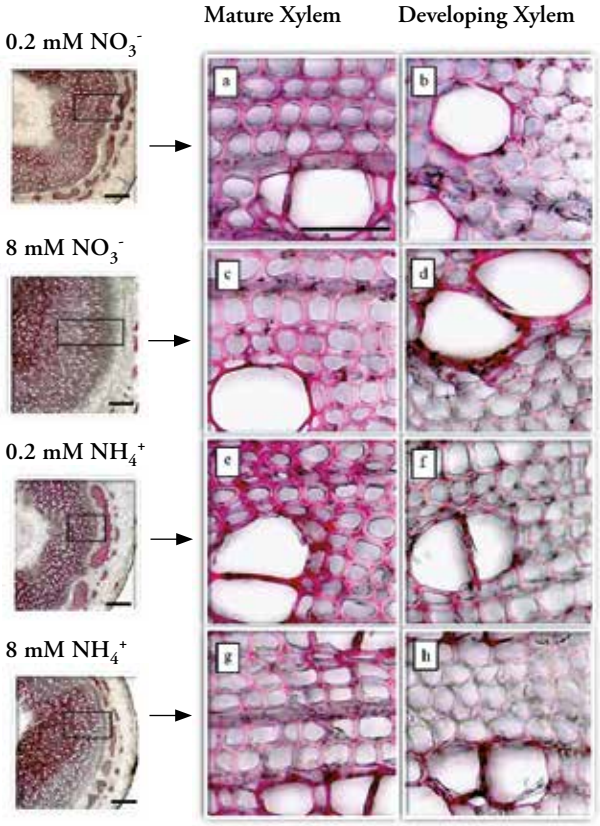


Fig. 3.21: *Phloroglucinol staining of P. trichocarpa stem cross sections. Fiber lumen and cell wall thickness were measured in developing (b, d, f, h) and mature xylem tissue (a, c, e, g) after six weeks fertilization with 0.2 mM (a-b, e-f) or 8 mM (c-d, g-h) nitrate (a-d) or ammonium (e-h). Scale bar: 50 µm/small overview pictures: 50µm.*

3.4 Candidate genes involved in secondary vessel cell wall formation are differentially expressed under different nitrogen conditions

PtGATA12, PtWND6B, PtWND6A, PtCCoAOMT1 and *PtIRX1* are genes which are involved in the control of secondary vessel cell wall formation and in the production of cell wall materials. The transcript levels of these genes were determined in developing xylem tissue of *P. trichocarpa* plants fertilized with low or high nitrogen levels. The relative transcript abundances of these genes differed among the treatments. The expression levels of the putative upstream regulator gene *PtGATA12* and the first layer master switch *PtWND6B* were positively correlated (Pearson correlation: 0.72,

$p < 0.05$). They were significantly stronger expressed in developing xylem tissue of 0.2 mM and 8 mM ammonium-fed plants than in response to other nitrogen treatments (**Fig. 3.22-3.23**). Besides, there was nearly no transcript level of any of both genes measured in 0.2 mM nitrate-fed poplar. Additionally, *PtWND6B* correlated positively with the biosynthetic gene for cellulose *PtIRX1* (Pearson correlation: 0.52, $p = 0.02$) and lignin *PtCCoAOMT1* (Pearson correlation: 0.56, $p = 0.01$). *PtIRX1* and *PtCCoAOMT1* were highly positively correlated, too (Pearson correlation: 0.75, $p < 0.01$). The highest relative expression values of both biosynthetic genes were found in developing xylem tissue of *P. trichocarpa* plants fertilized with 0.2 mM ammonium (**Fig. 3.24-3.25**). The transcriptional abundance of these genes differed slightly in high nitrogen fertilized plants, while 8 mM nitrate-fed plants showed a trend towards lower expression of the cellulose biosynthetic gene *PtIRX1* (**Fig. 3.25**). Additionally, this gene was even less expressed in developing xylem tissue of 0.2 mM nitrate-fed plants, but not significant. The relative expression of the lignin biosynthetic gene *PtCCoAOMT1* in developing xylem tissue of these plants was low (**Fig. 3.24**).

The relative expression of the *PtWND6B* homolog gene, *PtWND6A* was different. This gene was highly positively correlated with vessel (Pearson correlation: 0.80, $p < 0.01$) and fiber lumina (Pearson correlation: 0.67, $p < 0.01$) in developing xylem tissue, but negatively with vessel cell wall thickness in mature xylem (Pearson correlation: 0.44, $p = 0.05$). The vessel cell wall thickness in mature xylem correlated positively with wood density (Pearson correlation: 0.48, $p = 0.03$). In detail, the highest *PtWND6A* expression was found in developing xylem tissue of plants fertilized with 8 mM nitrate, even three- to four-fold more than it was detected in 0.2 mM and 8 mM ammonium-fed plants (**Fig. 3.26**). The lowest transcriptional abundance of *PtWND6A* was found in 0.2 mM nitrate-fed ones.

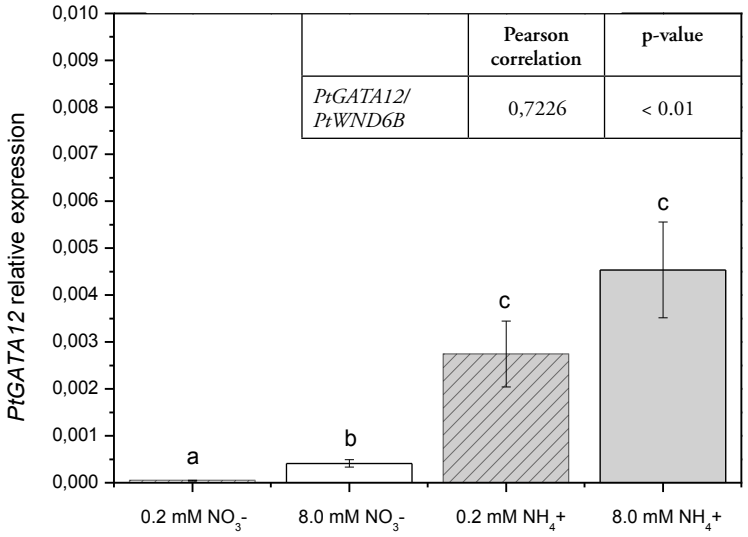


Fig. 3.22: *Relative PtGATA12 expression in P. trichocarpa developing xylem after six weeks fertilization with 0.2 mM or 8 mM nitrate or ammonium (n = 5, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (T-test, p < 0.05).*

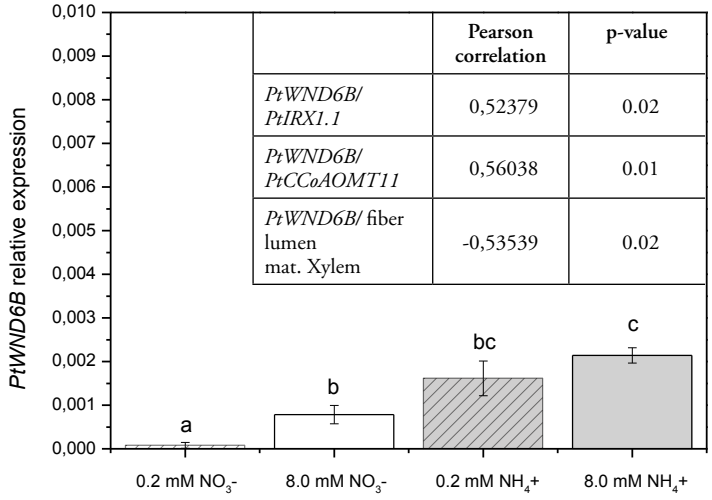
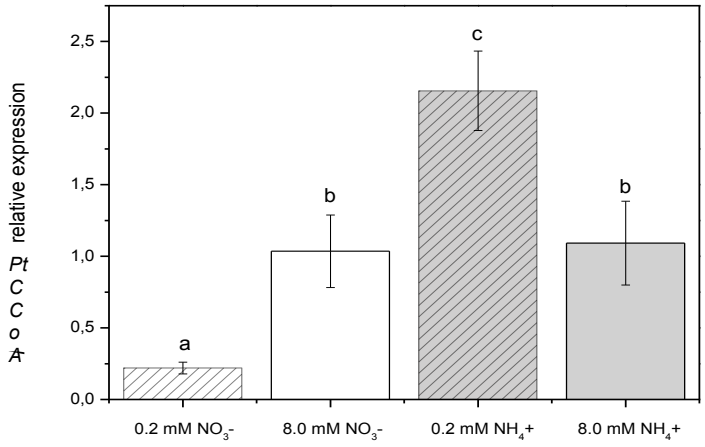
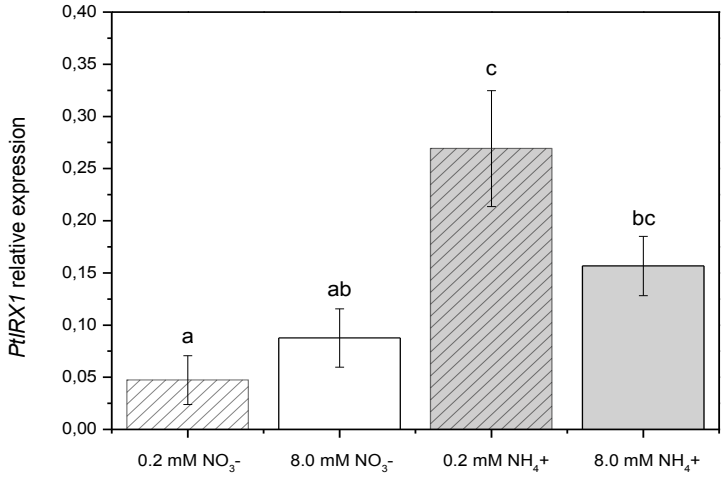


Fig. 3.23: *Relative PtWND6B expression in P. trichocarpa developing xylem after six weeks fertilization with 0.2 mM or 8 mM nitrate or ammonium (n = 5, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (T-test, p < 0.05).*



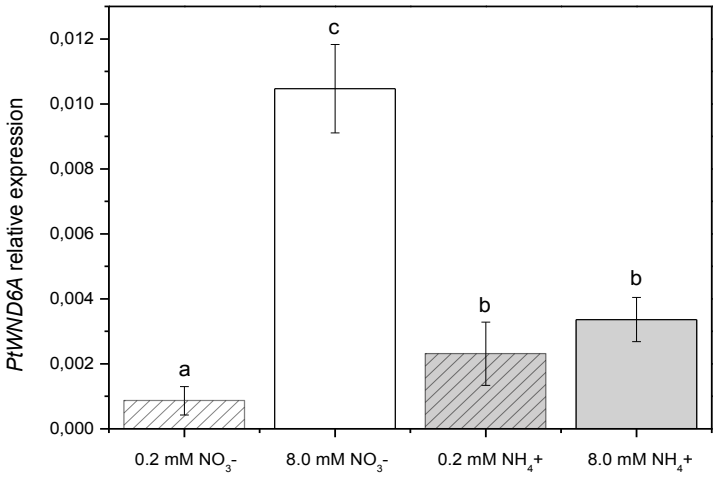
	Pearson correlation	p-value
PtCCoAOMT1/ *PtIRX1.1*	0,75144	< 0.01
PtCCoAOMT1/ vessel cell wall dev. Xylem	-0,59338	0.01
PtCCoAOMT1/ fiber lumen mat. Xylem	-0,5237	0.02

Fig. 3.24: *Relative PtCCoAOMT1 expression in P. trichocarpa developing xylem after six weeks fertilization with 0.2 mM or 8 mM nitrate or ammonium (n = 5, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (T-test, p < 0.05).*



	Pearson correlation	p-value
PtIRX1.1/ vessel cell wall dev. Xylem	-0,45607	0.04

Fig. 3.25: *Relative PtIRX1.1 expression in P. trichocarpa developing xylem after six weeks fertilization with 0.2 mM or 8 mM nitrate or ammonium (n = 5, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (T-test, p < 0.05).*



	Pearson correlation	p-value
PtWND6A/ vessel lumen dev. xylem	0,80264	< 0.01
PtWAND6A/ vessel lumen mat. xylem	0,53956	0.01
PtWND6A/ fiber lumen dev. xylem	0,66478	< 0.01
PtWND6A/ fiber lumen mat. xylem	0,56326	0.01

Fig. 3.26: *Relative PtWNDA expression in P. trichocarpa developing xylem after six weeks fertilization with 0.2 mM or 8 mM nitrate or ammonium (n = 5, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (T-test, p < 0.05).*

3.5 Identification of AtGATA12 homologs in Populus trichocarpa

The GATA transcription factor family in Arabidopsis and poplar clustered in four subfamilies (**Fig. 3.27**). AtGATA12 is present in subfamily one, strongly expressed in inflorescence stem (**Fig. 3.27** red colour in legend) and homolog to AtGATA9. The AtGATA12 amino sequence alignment results in three *P. trichocarpa* homologues (**Fig. 3.28**), which are nearly identical with GmGATA60 and EucalyptusGATA12 (~60 %): POPTR_0006s251410g, POPTR_0018s08090g.1 and POPTR_0018s08090g.2. The two transcripts POPTR_0018s08090.2 and

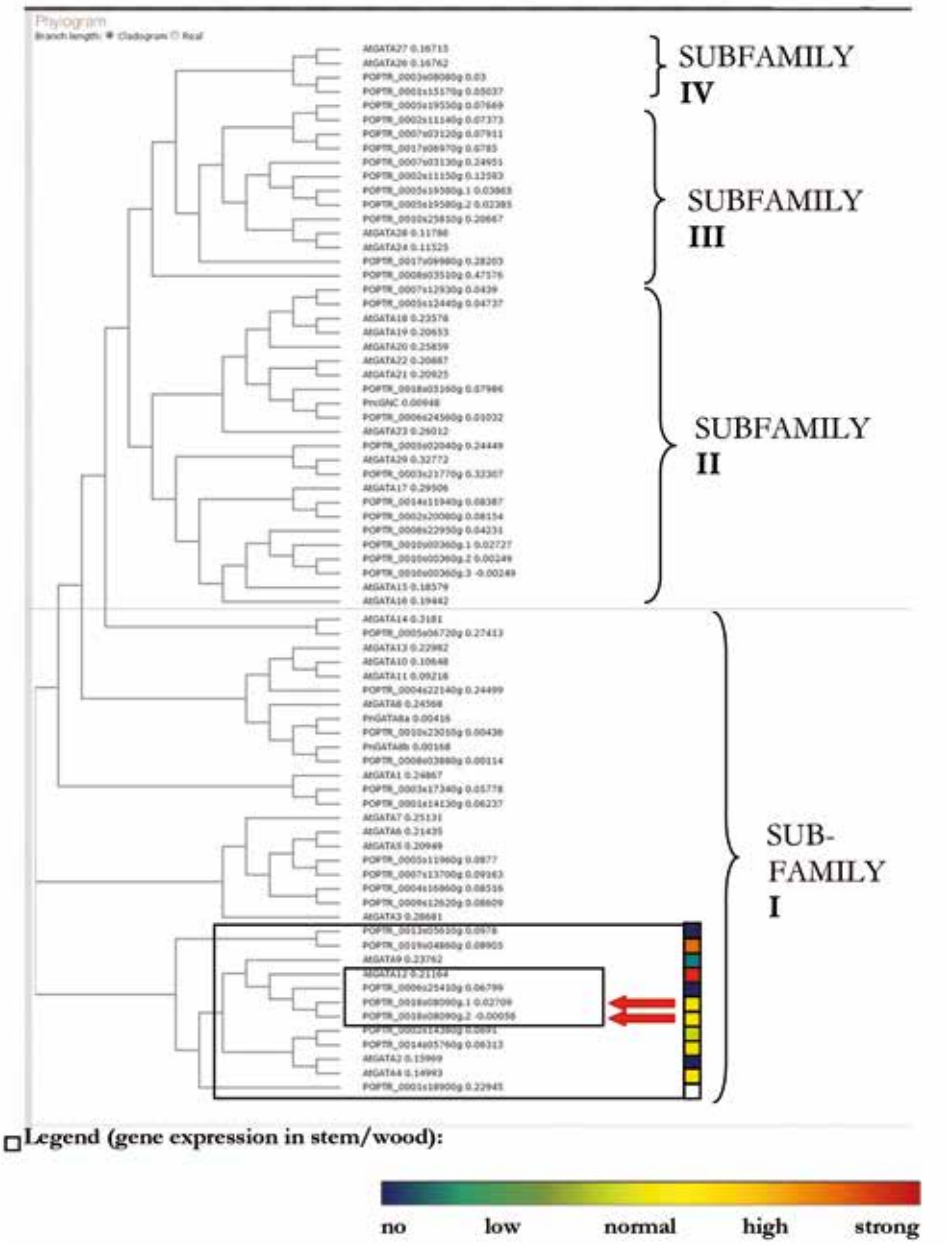
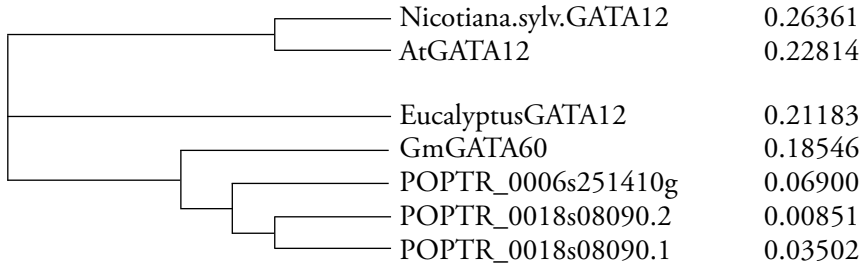


Fig. 3.27: *Phylogenetic Tree (Cladogram) of GATA transcription factors in Arabidopsis and P. trichocarpa, clustered in four subfamilies. Gene expression in wood or stem tissue is shown in a colour code for selected candidates. Arrows are indicating two poplar homologs of AtGATA12, expressed in wood. Neighbour-joining tree without distance corrections (Clustal Omega). Legend of gene expression in Arabidopsis stem (eFP Browser 2.0; bar.utoronto.ca, Winter et al. 2007) or poplar wood (PopGenIE.org, Sjödin et al. 2009).*

POPTR_0018s08090.1 represent two splicing variants of one gene (http://plants.ensembl.org; CLUSTAL Omega (1.2.1) multiple nucleotide sequence alignment). The two variants were named *PtGATA12.1* and *PtGATA12.2.*, which are expressed in wood tissue (see arrows in **Fig. 3.27**, yellow colour code). But, it was not possible to separate both genes in expression analysis (qRT-PCR). Consequently, they were named as one single gene *PtGATA12*. As this gene is differentially expressed in developing xylem under different nitrogen conditions (**Fig. 3.22**), it was selected as candidate gene to examine the wood characteristics resulting of *PtGATA12* (transcript POPTR_0018s08090.1 and POPTR_0018s08090.2) overexpression. POPTR_0006s251410g is not expressed in wood tissue (**Fig. 3.27** blue colour code) and will be not considered any further for this study, therefore.

A



B

	Nic.	AtGATA12	Euca.	GmGATA12	B9H8Y3	B9NEK3	B9IKH6
Nicotiana.sylv.GATA12	100.00	50.83	51.36	54.46	54.97	56.38	52.72
AtGATA12	50.83	100.00	55.73	57.05	56.86	59.84	56.90
EucalyptusGATA12	51.36	55.73	100.00	58.26	60.98	66.27	59.17
GmGATA60	54.46	57.05	58.26	100.00	64.05	67.82	61.66
POPTR_0006s251410g	54.97	56.86	60.98	64.05	100.00	88.03	86.13
POPTR_0018s08090.2	56.38	59.84	**66.27**	**67.82**	**88.03**	100.00	**97.35**
POPTR_0018s08090.1	52.72	56.90	**59.17**	**61.66**	**86.13**	97.35	100.00

Fig. 3.28: *AtGATA12 amino sequence alignment as A: cladogram and B: identity matrix (Clustal2.1).*

3.6 Genotyping of transgenic poplar and Arabidopsis lines

The expression of *GATA12* was genetically modified in *P.* x *canescens* plants by transformation with the *35S:PtGATA12* gene construct, to identify its function in secondary vessel cell wall formation. The generation of the plants has been reported

in 2.12. Eleven poplar lines were generated and tested for *PtGATA12* overexpression. Two of them, named L8 and L10, displayed the successful integration of this construct, because they show a construct product size of 2671 bp (**Fig. 3.29**). In leaves of these lines a thousand-fold higher transcriptional abundance of *PtGATA12* was found than in the leaves of wildtype (**Fig. 3.30**). These two lines were selected for a nitrogen experiment, which is explained in chapter 3.7

Additionally, the *35S:PtGATA12* gene construct was integrated in Arabidopsis. Three homozygous lines of F4-generation show a successful integration of the construct, because they show a construct product size of 2671 bp (**Fig. 3.31**). These lines exhibited about 100 to 300-fold higher expression levels of *PtGATA12* than the wildtype and were selected (**Fig. 3.32**). These lines were named L2B3, L2G1 and L8C1 and used to identify their phenotype under different nitrogen conditions (chapter 3.8).

Moreover, additional *AtGATA12* SALK mutant lines L58 and L60 of strain SALK_012501 were selected for that experiment. They were homozygous for the T-DNA insertion, displayed as one band for IP and RP primer (**Fig. 3.33B**), but not resulting in a product for LP and RP primer (**Fig. 3.33A**). *AtGATA12* expression was less in these lines (**Fig. 3.34**). Homozygous L23, L25, L26, L37, L38, L55, L56, L62 and L73 with potentially suppressed *PtGATA12* expression were kept as reserve.

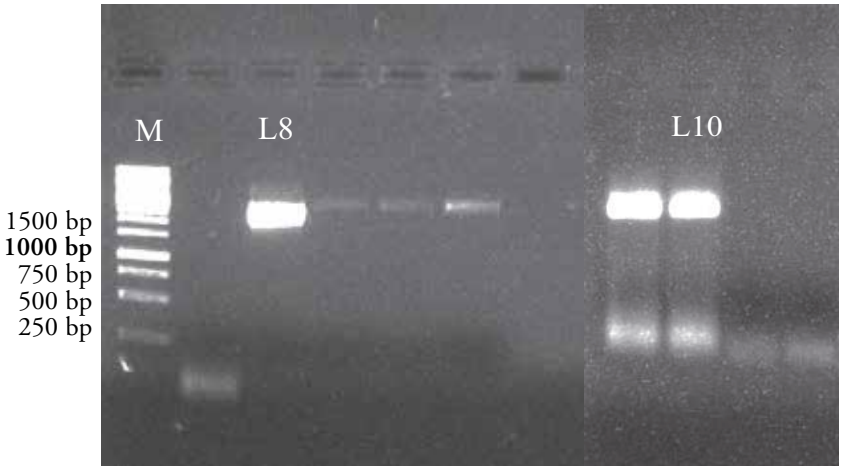


Fig. 3.29: *Amplified leaf DNA of genetically modified P. x canescens plantlets. 35S:PtGATA12 line 8 (L8) and line 10 (L10) were selected. Primer: p7WG2, product size: ~2671 bp, M = 1 kb DNA ladder, Fermentas, Thermo Fisher Scientific, Braunschweig, Germany.*

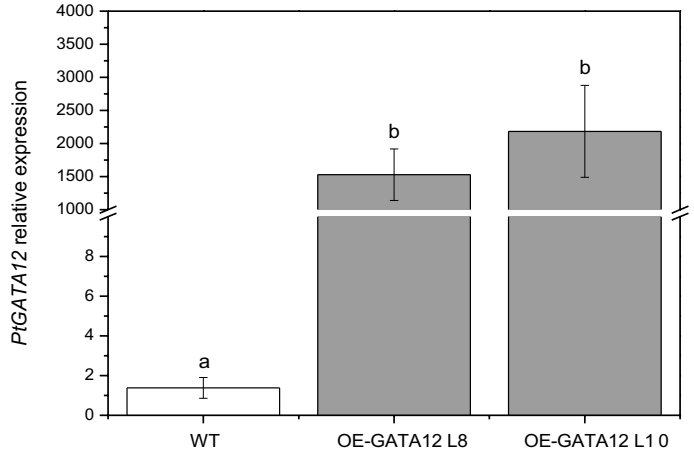


Fig. 3.30: *Relative expression of PtGATA12 in leaf tissue of wildtype (WT) and genetically modified (35S:PtGATA12 line 8 and 10) Populus x canescens plants (n = 3, mean ± SE). Different letters indicate significant differences (T-test, p < 0.05).*

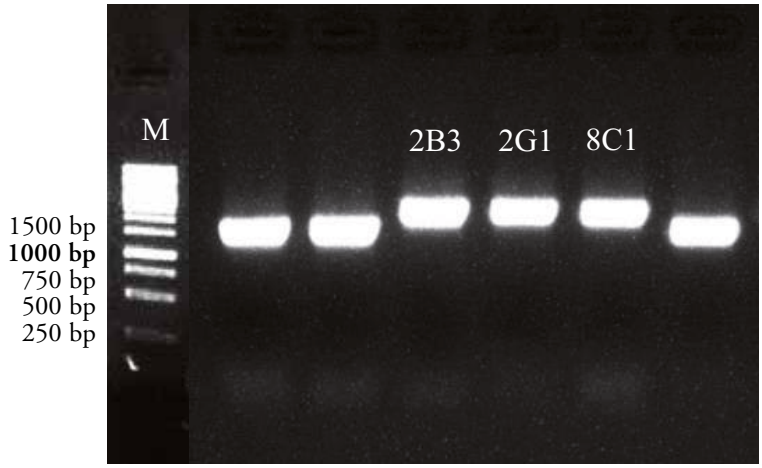


Fig. 3.31: *Amplified leaf DNA of genetically modified Arabidopsis thaliana F4-plants. 35S:PtGATA12 line 2B3, line 2G1 and line 8C1 were selected. Primer: p7WG2, product size: ~2671 bp, M = 1 kb DNA ladder, Fermentas, Thermo Fisher Scientific, Braunschweig, Germany.*

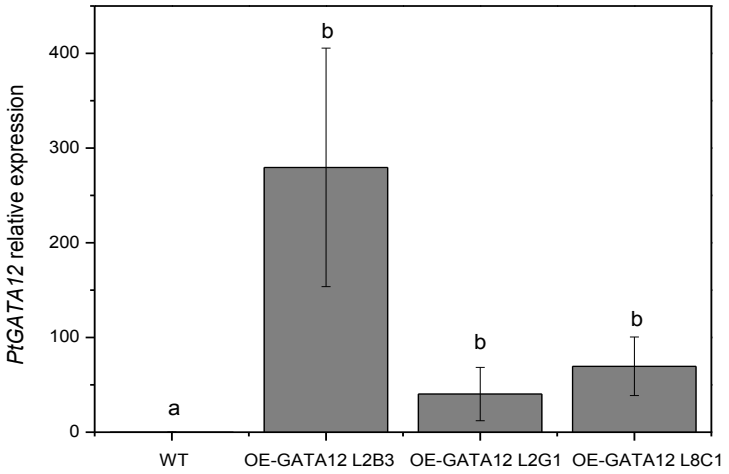


Fig. 3.32: *Relative expression of PtGATA12 in leaf tissue of wildtype (WT) and genetically modified (35S:PtGATA12 line 2B3, line 2G1 and line 8C1) Arabidopsis thaliana plants (n = 3, mean ± SE). Different letters indicate significant differences (One-way ANOVA, $p < 0.05$).*

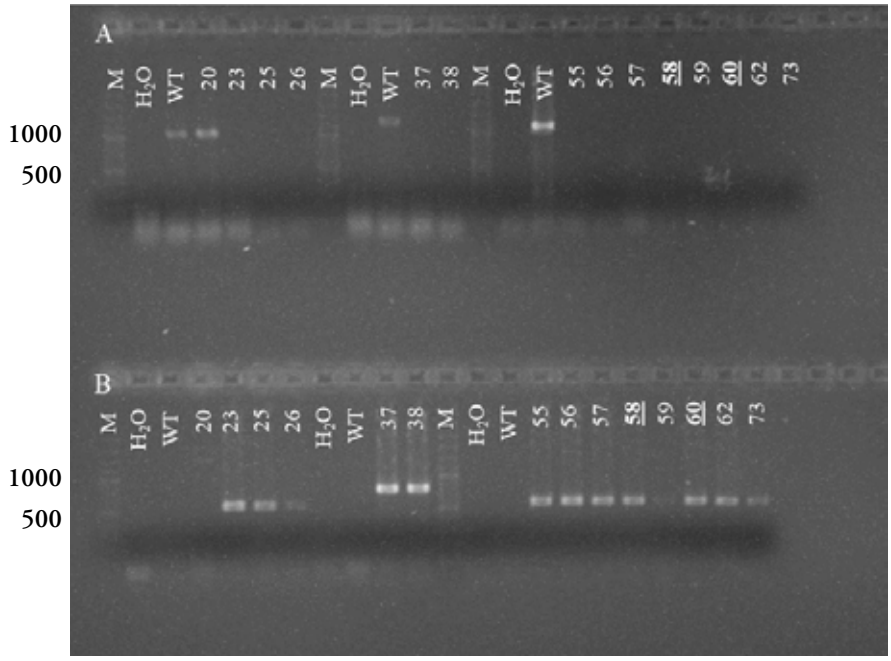


Fig. 3.33*: Amplified leaf DNA of A. thaliana SALK mutants for identifying homozygous T-DNA insertion lines. A: LP and RP primer, B: IP and RP primer. L20-26 = SALK_112752, L37-38 = SALK_143606, L55-73 = SALK_012501; L58 and L60 were selected for experiment. M: 100 bp Plus DNA ladder Fermentas, Thermo Fisher Scientific, Braunschweig.*

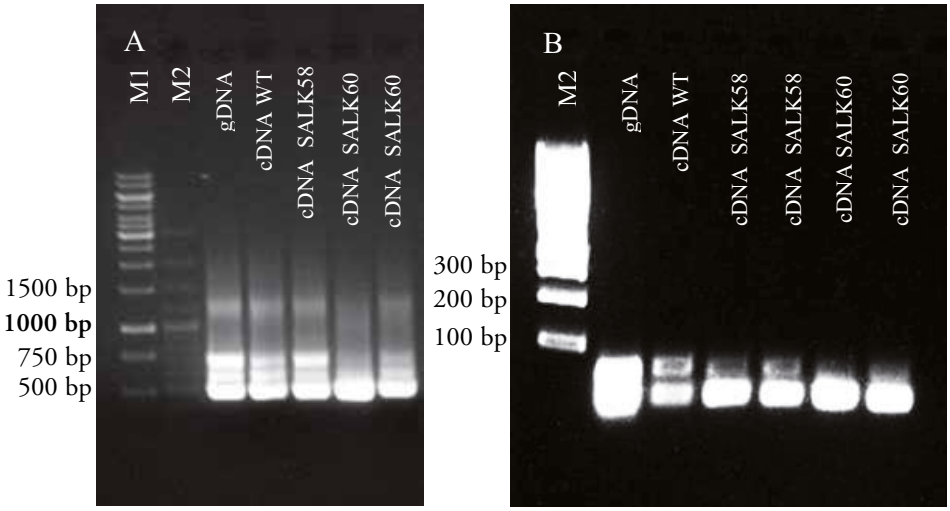


Fig. 3.34*: Amplified leaf cDNA of A. thaliana SALK mutants L58 and L60 for testing loss-of-function of AtGATA12 gene. A: AtACTIN1 as positive control (product size: 499 bp), B: AtGATA12. (product size: 69 bp) M1: 1 kb DNA ladder, M2: 100 bp Plus DNA ladder Fermentas, Thermo Fisher Scientific, Braunschweig.*

3.7 Growth and wood anatomy of OE-GATA12 poplar lines are different under high nitrate levels

35S:PtGATA12 poplar lines fertilized with 8 mM nitrate or ammonium grew similar to 8 mM nitrate-fed *P.* x *canescens* wildtype plants in height (**Fig. 3.35A**) and diameter (**Fig. 3.35B**). 8 mM ammonium-fertilized *P.* x *canescens* wildtype plants were significantly smaller than transgenic and wildtype plants fertilized with 8 mM nitrate, as well as 8 mM ammonium-fed *35S:PtGATA12* L8 plants after seven weeks treatment (**Fig. 3.35A, Tab. 3.9**), but without a change in diameter (**Fig. 3.35B**). The leaves of 8 mM ammonium-fed *35S:PtGATA12* poplar lines were much larger than 8 mM ammonium-fertilized wildtype ones, especially these leaves of L8 (**Fig. 3.36**). Leaves of 8 mM nitrate-fed *35S:PtGATA12* poplars were as small as leaves of 8 mM ammonium-fed wildtype plants. In contrast, leaf biomass of 8 mM nitrate-fed *35S:PtGATA12* poplar lines was higher than that of 8 mM ammonium-fertilized plants, especially leaf biomass of 8 mM nitrate-fed *35S:PtGATA12* L8 plants (**Fig. 3.37**). This controversial observationy of larger leaves but less biomass was the result of very flat and thin leaf phenotype from 8 mM ammonium-fertilized transgenic poplars. Root and leaf biomass of 8 mM nitrate-fed plants was higher than that of poplars fertilized with 8 mM ammonium. Interestingly, 8 mM nitrate-fed *35S:PtGATA12* L10 plants showed most dry stem biomass (**Fig. 3.37B**)

and significantly higher wood density than wildtype plants fertilized with 8 mM nitrate (**Fig. 3.38**). In general, wildtype plants fertilized with 8 mM nitrate had the lowest wood density of all, while that of the *35S:PtGATA12* lines fed with nitrate was higher. There was no significant difference in wood density of 8 mM nitrate-fed transgenic poplars and also not in 8 mM ammonium-fed transgenic and wildtype plants.

Moreover, *35S:PtGATA12* L10 plants showed a relatively higher fraction of cambial cell tissue (2.0 % ±0.1, **Tab. 3.10**) and mature xylem tissue (55.5 % ±2.9) than 8 mM fed wildtype and 8 mM ammonium-fertilized poplars. The relative fraction of cambium, developing xylem and mature xylem tissue was similar in 8 mM ammonium-fed transgenic and wildtype plants, while the amounts of developing xylem tissue of these plants were significantly less than in *P.* x *canescens* wildtype plants. Both *35S:PtGATA12* lines fertilized with 8 mM nitrate exhibited smaller vessel lumen in mature xylem (**Tab. 3.11**, **Fig. 3.39e, i**) and significantly thicker vessel cell walls (**Fig. 3.39c, e**) than 8 mM nitrate-fed wildtype plants (**Fig. 3.39a-b**, **Fig. 3.40a**). Additionally, the vessel cell walls were already thicker in developing xylem tissue of those plants (**Fig. 3.39f, j**). 8 mM ammonium-fed transgenic *P.* x *canescens* plants (**Fig. 3.39g-h**, **k-l**, **Fig. 3.40d, f**) showed similar differences in vessel lumen and vessel cell wall to 8 mM ammonium-fertilized wildtype plants (**Fig. 3.39c-d**, **Fig. 3.40b**). Besides, the mature vessel and fiber cell walls of 8 mM ammonium-fed *35S:PtGATA12* line 8 plants (**Fig. 3.40d**) were significantly thicker than those of the wildtype plants grown under these conditions (**Fig. 3.40b**).

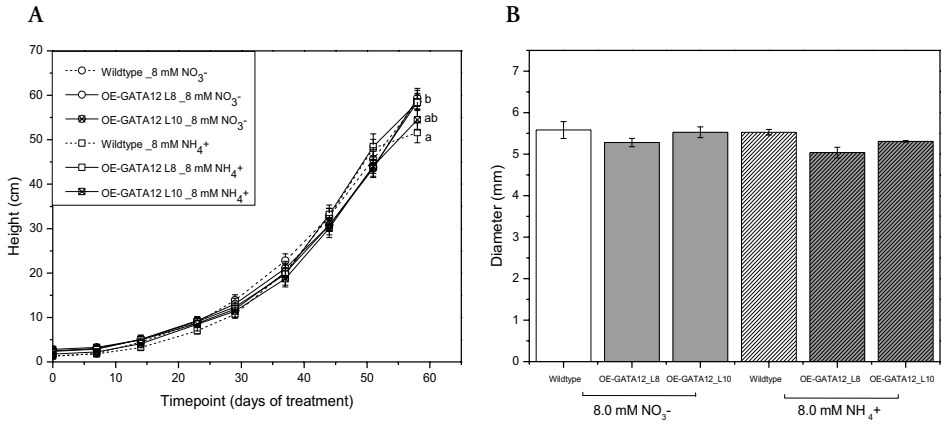


Fig. 3.35: *Mean height (A) and diameter (B) growth of P. x canescens wildtype and 35S:PtGATA12 lines in response to fertilization with 8 mM ammonium or nitrate (n = 6 per treatment, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. B: Diameter after seven weeks fertilization treatment. Different letters indicate significant differences (One-way ANOVA and T-test, p < 0.05).*

Tab. 3.9: *Different P. x canescens wildtype and transgenic plant heights in response to fertilization with different nitrogen forms and nitrogen levels No significant differences for genetic background (Two-way ANOVA, p < 0.05).*

Factor	p-value
Genotype	0.23
N-form	0.03
Interaction	0.10

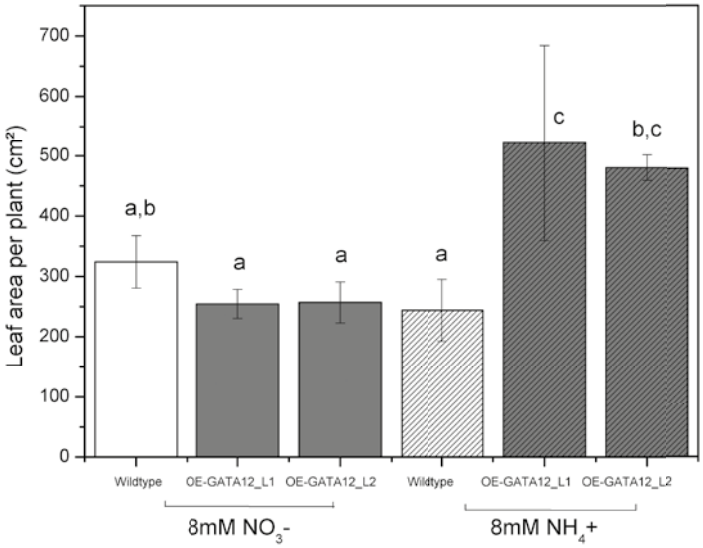


Fig. 3.36: *Mean leaf area per plant of P. x canescens wildtype and two 35S:PtGATA12 lines were determined after seven weeks fertilization with 8 mM ammonium or nitrate (n = 6 per treatment, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (One-way ANOVA and T-test, p < 0.05).*

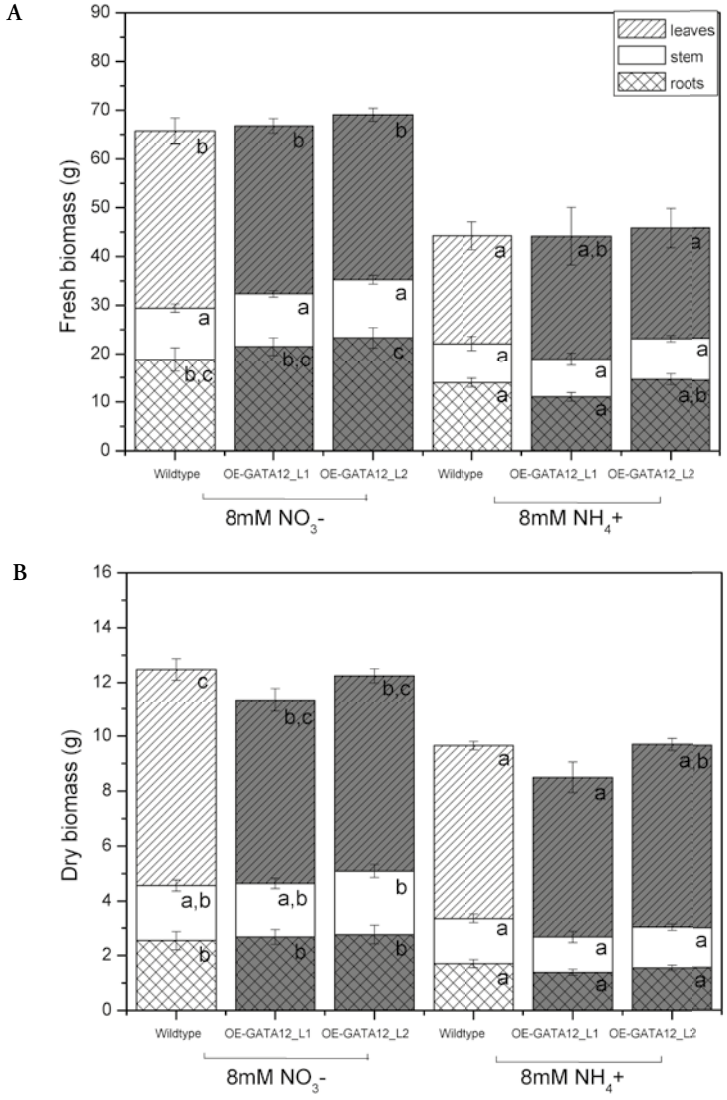


Fig. 3.37: *Fresh (A) and dry (B) biomass of leaves, stem, and roots from P. x canescens wildtype and 35S:PtGATA12 line 8 and 10 plants after seven weeks fertilization with 8 mM nitrate or ammonium (n = 6 per treatment, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (One-way ANOVA and T-test, p < 0.05).*

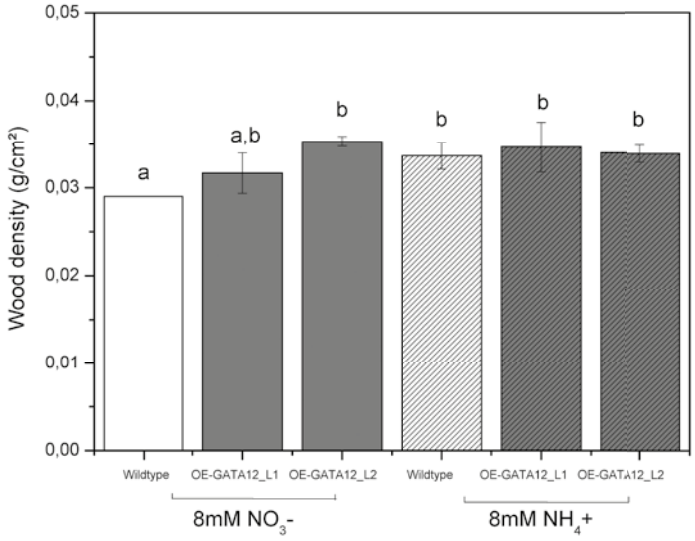


Fig. 3.38*: Wood density of P. x canescens wildtype and 35S:PtGATA12 line 8 and 10 plants after seven weeks fertilization with 8 mM nitrate or ammonium (n = 6 per treatment, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (One-way ANOVA and T-test, p < 0.05).*

Tab. 3.10: *Tissue distribution of P. x canescens wildtype and transgenic stem cross sections after seven weeks plant fertilization with 8 mM nitrate or ammonium (n = 5, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Percentages are calculated from cell layer fraction and radius of cross sections. Distinct letters per row indicate significant differences (One-way ANOVA and T-test, p < 0.05).*

Tissue in %	8 mM NO_3^- (HNi) wildtype	8 mM NO_3^- (HNi) OE-GATA12 L8	8 mM NO_3^- (HNi) OE-GATA12 L10	8 mM NH_4^+ (HAm) wildtype	8 mM NH_4^+ (HAm) OE-GATA12 L8	8 mM NH_4^+ (HAm) OE-GATA12 L10
Bark	3.45 ± 0.13 (d)	3.08 ± 0.20 (cd)	2.55 ± 0.15 (ab)	2.42 ± 0.18 (ab)	2.73 ± 0.10 (bc)	2.07 ± 0.11 (a)
Phloem	15.57 ± 0.59 (b)	16.29 ± 0.77 (bc)	19.82 ± 1.65 (bc)	12.53 ± 0.68 (a)	13.05 ± 0.77 (a)	21.10 ± 2.07 (c)
Cambium	1.50 ± 0.09 (a)	1.61 ± 0.11 (ab)	2.03 ± 0.08 (b)	1.35 ± 0.09 (a)	1.41 ± 0.12 (a)	1.58 ± 0.08 (a)
Developing xylem	6.42 ± 0.56 (b)	6.22 ± 0.53 (ab)	7.04 ± 0.40 (b)	4.54 ± 0.42 (a)	4.56 ± 0.46 (a)	5.26 ± 0.43 (ab)
Mature xylem	55.13 ± 1.30 (a)	57.17 ± 1.24 (a)	65.54 ± 2.91 (b)	51.29 ± 0.93 (a)	53.38 ± 2.15 (a)	53.60 ± 1.36 (a)
Pith	26.38 ± 0.93 (b)	20.84 ± 1.17 (a)	21.20 ± 1.31 (a)	24.28 ± 0.91 (ab)	22.01 ± 1.02 (a)	24.98 ± 0.52 (ab)

Tab. 3.11: *Detailed xylem cell anatomy of P. x canescens wildtype and transgenic stem cross sections after seven weeks plant fertilization with 8 mM nitrate or ammonium (n = 5, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. (One-way ANOVA and T-test, p < 0.05).*

Tissue	8 mM NO_3^- (HNi) wildtype	8 mM NO_3^- (HNi) OE-GATA12 L8	8 mM NO_3^- (HNi) OE-GATA12 L10	8 mM NH_4^+ (HAm) wildtype	8 mM NH_4^+ (HAm) OE-GATA12 L8	8 mM NH_4^+ (HAm) OE-GATA12 L10
Developing xylem fibers						
Number (cells/mm²)	2133 ± 131.2 (a)	2244 ± 124.8 (a)	2933 ± 286.3 (b)	2000 ± 135.4 (a)	2800 ± 229.7 (ab)	3478 ± 225.3 (b)
Lumen/fiber (µm²)	79.94 ± 5.51 (c)	41.47 ± 3.66 (a)	50.79 ± 3.31 (a)	59.69 ± 2.56 (ab)	76.45 ± 7.12 (bc)	80.92 ± 4.64 (c)
Cell wall thickness (µm)	1.69 ± 0.14 (a)	1.77 ± 0.07 (a)	1.96 ± 0.29 (ab)	1.80 ± 0.10 (ab)	1.97 ± 0.15 (ab)	2.47 ± 0.15 (b)
Developing xylem vessels						
Number (cells/mm²)	184 ± 7.20 (a)	169 ± 9.76 (a)	180 ± 15.80 (a)	182 ± 9.07 (a)	164 ± 13.83 (a)	190 ± 13.54 (a)
Lumen/vessel (µm²)	1268.49 ± 34.00 (c)	1099.46 ± 90.46 (b)	757.41 ± 69.39 (a)	976.37 ± 27.49 (ab)	1007.15 ± 63.84 (ab)	927.63 ± 57.77 (ab)
Cell wall thickness (µm)	0.82 ± 0.03 (a)	1.35 ± 0.09 (b)	1.37 ± 0.05 (b)	1.39 ± 0.10 (b)	1.48 ± 0.09 (b)	1.72 ± 0.19 (b)
Mature xylem fibers						
Number (cells/mm²)	4456 ± 151.9 (b)	4611 ± 196.1 (b)	4289 ± 187.4 (ab)	3411 ± 193.3 (a)	3644 ± 365.6 (a)	4189 ± 209.8 (ab)
Lumen/fiber (µm²)	74.59 ± 2.59 (b)	54.09 ± 4.39 (a)	77.14 ± 5.41 (b)	71.64 ± 4.12 (ab)	58.18 ± 3.90 (ab)	70.64 ± 4.33 (ab)
Cell wall thickness (µm)	2.91 ± 0.08 (a)	3.05 ± 0.16 (a)	2.70 ± 0.10 (a)	2.74 ± 0.09 (a)	3.82 ± 0.22 (b)	3.15 ± 0.09 (a)
Mature xylem vessels						
Number (cells/mm²)	167 ± 4.90 (a)	195 ± 12.98 (ab)	211 ± 18.89 (ab)	183 ± 12.02 (ab)	194 ± 12.85 (ab)	232 ± 11.92 (b)
Lumen/vessel (µm²)	1356.73 ± 64.32 (b)	915.42 ± 62.15 (a)	868.50 ± 82.36 (a)	985.43 ± 77.15 (a)	946.12 ± 57.12 (a)	1070.93 ± 28.66 (a)
Cell wall thickness (µm)	1.77 ± 0.08 (a)	2.57 ± 0.09 (cd)	2.16 ± 0.06 (b)	2.32 ± 0.07 (bc)	2.78 ± 0.12 (d)	2.41 ± 0.06 (bc)

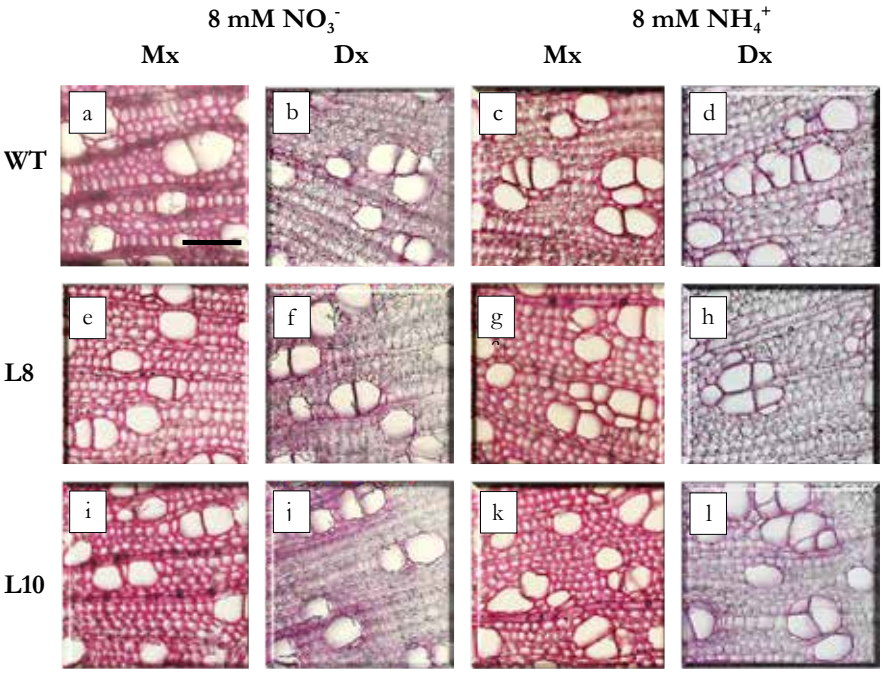


Fig. 3.39: *Phloroglucinol staining of P. x canescens wildtype (a-d) and transgenic 35S:PtGATA12 lines: L8 (e-h) and L10 (i-l) stem cross sections. Vessel lumen and cell wall thickness were measured in developing (b, d, f, h, j, l) and mature xylem tissue (a, c, e, g, I, k) after seven weeks fertilization with nitrate (a-b, e-f, i-j) or ammonium (c-d, g-h, k-l). Scale bar: 80 µm.*

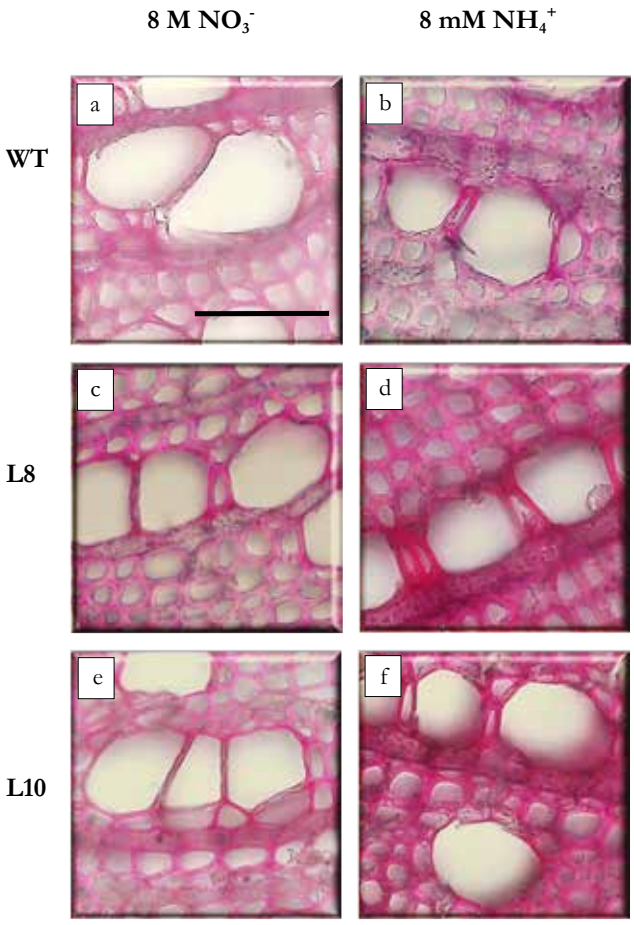


Fig. 3.40: *Phloroglucinol staining of P. x canescens wildtype (a-b) and transgenic 35S:PtGATA12 lines: L8 (c-d) and L10 (e-f) stem cross sections. Fiber lumen and cell wall thickness were measured in mature xylem tissue after seven weeks fertilization with nitrate (a, c, e) or ammonium (b, d, f). Scale bar: 50 μm.*

3.8 Different nitrogen forms affect growth and hypocotyl anatomy of transgenic A. thaliana lines (OE-GATA12 & SALK)

Arabidopsis plants fertilized with 8 mM nitrogen regardless the nitrogen form, developed more biomass than 1 mM nitrogen fed plants (**Fig. 3.41**), with the exception of L2G1. This line did not produce more biomass under high nitrogen conditions and was therefore excluded from the following analyses.

35S:PtGATA12 L2B3 fertilized with 1 mM nitrogen produced more secondary xylem and cortex tissue than the other 1 mM nitrogen fed transgenic and wildtype plants (**Fig. 3.42A-B**). There was even more primary xylem tissue developed in these plants fertilized with 1 mM ammonium (**Fig. 3.42B**). Besides, there was no secondary xylem tissue developed in both SALK *AtGATA12* lines under same conditions. There was as well no secondary xylem tissue produced in these lines fertilized with 8 mM nitrate, equally to *35S:PtGATA12* lines (**Fig. 3.42C**). Just 8 mM nitrate-fed Arabidopsis wildtype plants showed secondary xylem tissue after eight weeks. Also, both *35S:PtGATA12* lines did not produce secondary xylem tissue, when they were fertilized with 8 mM ammonium (**Fig. 3.42D**).

The primary vessel lumina of both 1 mM nitrate-fed SALK *AtGATA12* lines were larger and the primary vessel cell walls were bigger than wildtype plants (**Tab. 3.12**). *35S:PtGATA12* L2B3 plants showed bigger primary vessel cell walls under same conditions (1.49 µm ±0.09). Both 1 mM ammonium-fed *35S:PtGATA12* lines developed significantly thicker secondary fiber cell walls than wildtype plants and bigger primary vessel lumen. Furthermore, both SALK *AtGATA12* lines fertilized with 8 mM ammonium showed less secondary fiber and vessel lumina and thinner vessel cell walls than wildtype plants, additionally.

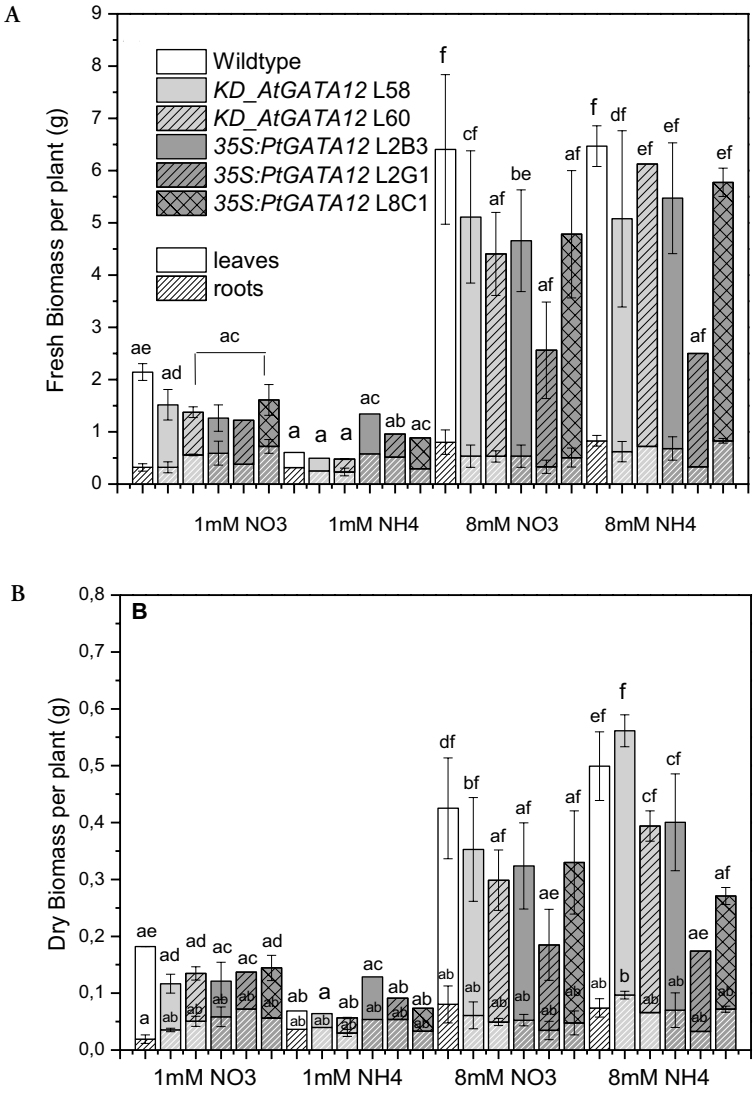


Fig. 3.41: *Fresh (A) and dry (B) biomass of leaves and roots from genetically modified knockdown GATA12 (SALK AtGATA12, 2 lines) and overexpressing GATA12 (35S:PtGATA12, 2 lines) Arabidopsis thaliana plants in comparison to wildtype after eight weeks fertilization with 1 mM or 8 mM nitrogen in form of ammonium or nitrate (n = 20 per line and treatment, mean ± SE).. Plants in soil were irrigated with nutrient solution once a day. Different letters indicate significant differences (One-way ANOVA, p < 0.05).*

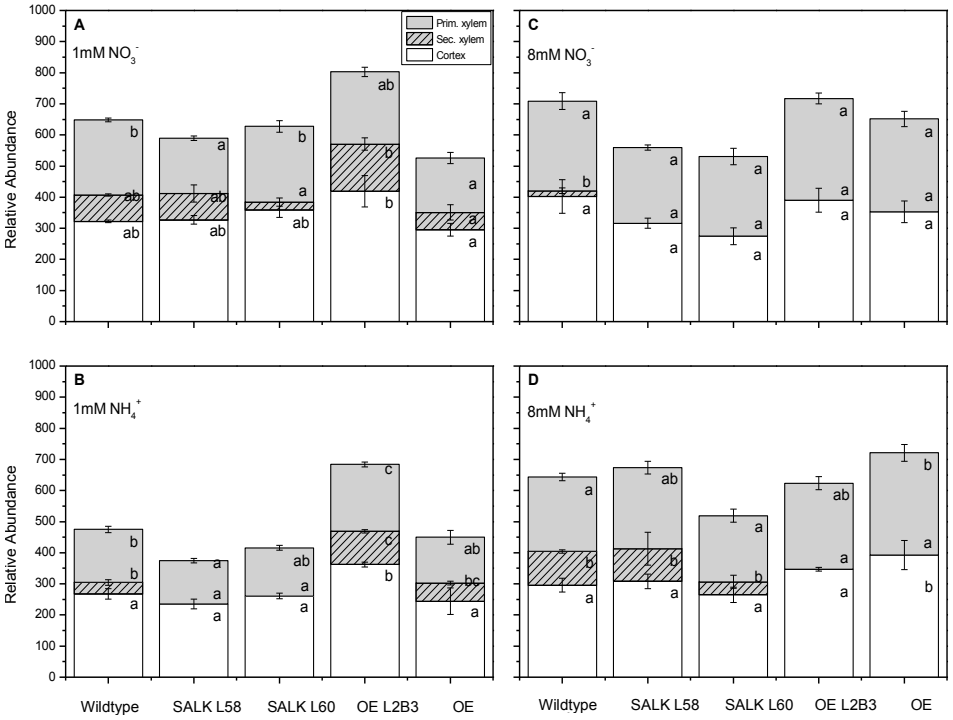


Fig. 3.42: *Tissue distribution of A. thaliana wildtype and transgenic (SALK AtGATA12 and 35S:PtGATA12) hypocotyl cross sections after eight weeks plant fertilization with 1 mM or 8 mM nitrogen in form of ammonium or nitrate. A: 1 mM nitrate, B: 8 mM nitrate, C: 1 mM ammonium, D: 8 mM ammonium (n = 3, mean ± SE). Plants in soil were irrigated with nutrient solution once a day. Relative abundances are calculated from cell layer width and radius of cross sections. Different letters per indicate significant differences (T-test, $p < 0.05$).*

Tab. 3.12: *Detailed xylem cell anatomy of A. thaliana wildtype and transgenic (SALK AtGATA12 and 35S:PtGATA12) hypocotyl cross sections after eight weeks plant fertilization with 1 mM or 8 mM nitrogen in form of ammonium or nitrate (n = 3, mean ± SE). Different letters per column indicate significant differences (One-way ANOVA, p < 0.05).*

Treatment & Genotype	Secondary xylem tissue				Primary xylem tissue	
	Vessel lumen/ cell (µm²)	Vessel cell wall/ cell (µm)	Fiber lumen/ cell (µm²)	Fiber cell wall/ cell (µm)	Vessel lumen/ cell (µm²)	Vessel cell wall/ cell (µm)
1 mM NO_3^- wildtype	109.80 ± 3.07 (b)	1.16 ±0.06 (b)	34.41 ±0.95 (b)	0.92 ±0.07 (a)	99.77 ±12.71 (a)	0.88 ±0.07 (a)
1 mM NO_3^- *KD-AtGATA12* L58	80.77 ±21.24 (ab)	0.97 ±0.24 (b)	23.50 ±6.14 (ab)	1.16 ±0.30 (ab)	158.30 ± 9.00 (b)	1.13 ±0.08 (b)
1 mM NO_3^- *KD-AtGATA12* L60	39.28 ±19.75 (a)	0.13 ±0.13 (a)	9.19 ±4.62 (a)	0.55 ±0.28 (a)	162.38 ±14.21 (b)	1.36 ±0.05 (c)
1 mM NO_3^- *35S:PtGATA12* L2B3	88.67 ± 5.05 (ab)	1.40 ±0.08 (b)	35.44 ±2.17 (b)	1.46 ±0.10 (b)	89.36 ±6.12 (a)	1.49 ±0.09 (c)
1 mM NO_3^- *35S:PtGATA12* L8C1	67.55 ±17.59 (ab)	0.77 ±0.20 (ab)	25.65 ±6.63 (ab)	0.94 ±0.26 (ab)	88.05 ±4.06 (a)	1.05 ±0.07 (ab)
1 mM NH_4^+ wildtype	64.78 ±18.48 (b)	0.79 ±0.21 (b)	22.17 ±6.12 (b)	0.71 ±0.18 (b)	73.39 ±9.44 (a)	1.06 ±0.07 (a)
1 mM NH_4^+ *KD-AtGATA12* L58	0.00 ± 0.00 (a)	0.00 ±0.00 (a)	0.00 ±0.00 (a)	0.00 ±0.00 (a)	117.95 ±5.40 (b)	1.18 ±0.03 (ab)
1 mM NH_4^+ *KD-AtGATA12* L60	0.00 ± 0.00 (a)	0.00 ±0.00 (a)	0.00 ±0.00 (a)	0.00 ±0.00 (a)	114.78 ±10.44 (b)	1.07 ±0.04 (a)
1 mM NH_4^+ *35S:PtGATA12* L2B3	81.31± 5.04 (b)	1.21 ±0.10 (b)	31.75 ±1.83 (bc)	1.14 ±0.10 (c)	107.74 ±9.15 (b)	1.17 ±0.08 (ab)
1 mM NH_4^+ *35S:PtGATA12* L8C1	111.11 ±10.41 (c)	1.21 ±0.05 (b)	37.00 ±3.46 (c)	1.31 ±0.10 (c)	114.30 ±13.14 (b)	1.35 ±0.08 (b)
8 mM NO_3^- wildtype	73.45 ±36.83 (b)	0.39 ±0.20 (b)	15.87 ±7.95 (b)	0.30 ±0.16 (b)	141.02 ± 9.69 (bc)	1.24 ±0.08 (a)
8 mM NO_3^- *KD-AtGATA12 L58*	0.00 ± 0.00 (a)	0.00 ±0.00 (a)	0.00 ±0.00 (a)	0.00 ±0.00 (a)	166.77 ±11.37 (c)	1.45 ±0.11 (a)
8 mM NO_3^- *KD-AtGATA12 L60*	0.00 ± 0.00 (a)	0.00 ±0.00 (a)	0.00 ±0.00 (a)	0.00 ±0.00 (a)	213.20 ±15.86 (d)	1.31 ±0.07 (a)
8 mM NO_3^- *35S:PtGATA12* L2B3	0.00 ± 0.00 (a)	0.00 ±0.00 (a)	0.00 ±0.00 (a)	0.00 ±0.00 (a)	104.72 ±7.29 (a)	1.40 ±0.14 (a)
8 mM NO_3^- *35S:PtGATA12* L8C1	0.00 ± 0.00 (a)	0.00 ±0.00 (a)	0.00 ±0.00 (a)	0.00 ±0.00 (a)	118.35 ±7.86 (ab)	1.30 ±0.05 (a)
8 mM NH_4^+ wildtype	136.89 ± 9.38 (c)	1.39 ±0.08 (c)	47.87 ±2.73 (c)	0.96 ±0.05 (b)	151.83 ±7.86 (b)	1.30 ±0.12 (a)
8 mM NH_4^+ *KD-AtGATA12 L58*	38.63 ±19.59 (b)	0.54 ±0.27 (b)	7.12 ±3.72 (b)	0.46 ±0.24 (b)	167.00 ±13.10 (bc)	1.46 ±0.06 (a)
8 mM NH_4^+ *KD-AtGATA12 L60*	37.76 ±19.63 (b)	0.38 ±0.19 (b)	6.53 ±3.37 (b)	0.47 ±0.24 (b)	204.20 ±14.91 (c)	1.26 ±0.06 (a)
8 mM NH_4^+ *35S:PtGATA12* L2B3	0.00 ± 0.00 (a)	0.00 ±0.00 (a)	0.00 ±0.00 (a)	0.00 ±0.00 (a)	97.59 ± 8.56 (a)	1.32 ±0.07 (a)
8 mM NH_4^+ *35S:PtGATA12* L8C1	0.00 ± 0.00 (a)	0.00 ±0.00 (a)	0.00 ±0.00 (a)	0.00 ±0.00 (a)	108.06 ± 5.87 (a)	1.47 ±0.04 (a)

4 Discussion

4.1 Influence of nitrogen availability on poplar growth

In agreement with previous studies (*P. trichocarpa*, Euring et al., 2014; *P. deltoides*, Martin et al., 1967), poplar growth was stimulated by high nitrogen supply. The fertilization with 8 mM nitrate led to faster poplar height and diameter growth than with 8 mM ammonium. This different growth caused of different nitrogen forms was seen in poplars fertilized just with 2 mM nitrogen in hydroponic culture, as well, but later, especially in *P. trichocarpa*. A better growth under nitrate than under ammonium conditions were also found in ammonium-sensitive cucumber, but not in ammonium-tolerant rice plants (Zhou et al. 2011). Apparently, poplars behaved more similar as ammonium-sensitive than ammonium-tolerant plant species under increased nitrogen conditions.

Poplar biomass is positively affected by high nitrogen conditions (Euring et al. 2014; Coyle et al. 2013). A novel observation of this study was that high nitrate stimulated biomass production more than high ammonium, with stronger effects in *P.* x *canescens* than in *P. trichocarpa*. When the plants were cultured with 8 mM or 2 mM ammonium for more than one month biomass production declined, which might be the result of ammonium toxicity, faster occurring in *P.* x *canescens* plants. In Arabidopsis, nitrate supply can alleviate negative ammonium symptoms, but not ammonium accumulation in tissues (Hachiya et al. 2012). The shoot-to-root ratio

was shifted in favor of shoot biomass in poplars fertilized with high ammonium-nitrate and high nitrate. Obviously, the high availability of nitrogen allowed more aboveground biomass production, but not when high ammonium was applied to poplars as the single nitrogen source. Ammonium-fed poplars showed a restricted shoot growth and an unaffected shoot-to-root ratio. The shoot-to-root ratio was also stable under low nitrogen conditions, most likely as the result of limited nitrogen supply.

There is a close link between chlorophyll and nitrogen content (Ghasemi et al. 2017; Bojović and Marković 2009). In the present study, especially nitrate feeding had a positive effect on the chlorophyll content in leaves. High nitrate also led to bigger leaves and higher nitrate contents in the leaves.

Besides the biochemical acclimation, leaves of *P. trichocarpa* plants fertilized with 8 mM nitrate were able to adapt to strong light faster than leaves of plants cultured with 8 mM ammonium (**Fig. 3.4**). Nitrate assimilation is coupled to photosynthetic electron transport (Zhou et al. 2011), while photosystem II and electron transport chain are inhibited by increasing free ammonium concentrations (Markou et al. 2016). Since ammonium is the reduced form, electrons produced by photosystem electron transport chains, which cannot be consumed by assimilatory processes under strong light, result in oxidative stress and damage to leaves (Demidchik 2015). In contrast, the free electrons can be used for nitrate reduction, which leads to less oxidative stress in nitrate- than in ammonium-fed poplars.

Furthermore, we found higher nitrate contents in developing xylem tissue of 8 mM nitrate-fed *P.* x *canescens* and *P. trichocarpa* plants than in these tissues of 8 mM ammonium- or ammonium nitrate-fed plants (**Fig. 3.14F, 3.15F**). Only, *P.* x *canescens* plants show additionally higher nitrate content in stem wood (**Fig. 3.15D**) and higher ammonium contents in developing xylem (**Fig. 3.15E**) under high nitrate feeding. It is speculative, if *P.* x *canescens* plants stored nitrate in the stem and reduced it to ammonium in developing xylem tissue, whereas *P. trichocarpa* plants reduced nitrate only in roots. The ammonium concentration in stem of 8 mM ammonium-fed *P.* x *canescens* plants was higher (**Fig. 3.15C**) and in developing xylem lower (**Fig. 3.15E**), than in nitrate-fed *P.* x *canescens*. This observation may suggest that ammonium was quickly metabolized for example into amino acids such as glutamine in developing xylem of 8 mM ammonium-fed poplars. As a result of significantly lower total nitrogen content in stem of 8 mM ammonium-fed *P. trichocarpa* plants, the C/N ratio of these plants was very high (**Fig. 3.18**).

In summary, these results support that ammonium and nitrate assimilations have a distinct influence on plant growth and biomass, when supplied at high concentrations. The level used here (8 mM) reflects actually a moderate nitrate fertilization level in agricultures (Chamber of Agriculture Lower Saxony, March 2018), but would be considered as excessive compared to free nitrate or ammonium concentrations in forest soil solution (nitrate: 0.04-0.5 mM (Falk and Stetter 2010; Chamber of agriculture Lower Saxony 2018; Voß and Blanca 2019); ammonium: 0.06-0.1 mM (NLWKN Cloppenburg 2016; Voß and Blanca 2019)). Particularly, nitrate fertiliza-

tion led to better poplar growth and harvesting results than ammonium. However, the difference in growth was not detected when the two nitrogen compounds were applied at concentrations (0.2 mM) reflecting concentration levels in forest soil (Falk and Stetter 2010).

Although, fertilization with nitrate is promising to achieve more profitable harvesting results than that with ammonium, it should be noticed that fertilization is recommended only in later cycles of the poplar plantation's lifetime (Schweier et al. 2017). Thereby, it is attempted to minimize the negative impact of nitrogen fertilization on the environment, such as increased nitrate leaching and N_2O emissions (Schweier et al. 2017). Fabio and Smart (2018) suggested that the best harvest time would be during dormancy and after litter-fall, when most nitrogen is stored in plants (Millard and Grelet, 2010; Babst and Coleman, 2018). It is clear that field experiments are necessary to study the trade-offs between enhanced biomass production and environmental pollution caused by nitrate fertilization.

4.2 Impact of fertilization with different nitrogen compounds on wood anatomy in poplar

Fertilization with 8 mM nitrate led to higher wood production in *P. trichocarpa* plants than application of lower nitrogen concentrations because high nitrogen availability was inducing the cambial activity (**Tab. 3.7**). Since nitrate is increasing cytokinin levels (sunflower: Salama and Wareing 1979; barley: Samuelson et al. 1992; Arabidopsis: Takei et al. 2001) and. cytokinins are the physiological mediators of cambial growth (Matsumoto-Kitano et al. 2008), it is likely that nitrate influenced cambial cell production in poplar via cytokinins.

Secondary fiber lumina were already increased in poplars grown under 2 mM nitrate and the secondary vessel lumina were increased at 8 mM nitrate (**Tab. 3.8**). Bigger vessel lumina allow the plant to take up more water and nutrients, supporting rapid plant growth and wood formation. Thin secondary vessel cell walls in these plants could be the consequence of fast xylem tissue formation and slower cell wall thickening under high 8 mM nitrate. Contrarily, smaller secondary xylem cell lumina and less fiber cell wall thickening were observed in 8 mM ammonium-fed poplars. These results indicate a possible time-delay of either vessel or fiber cell wall thickening under high nitrogen conditions, depending on the nitrogen form.

As wood density is changing as the result of cell wall thickening and cell lumen growth (Thomas et al. 2004) thin vessel cell walls and big xylem cell lumina cause lower wood density in 8 mM nitrate-fed poplars than in poplars exposed to other nitrogen treatments. Here, the measured wood densities of ammonium-treated poplars were ranging within the span of reported values from 0.35 to 0.6 g/cm³ (Heimpold 2018). Whereas, the 8 mM nitrate-fertilized plants showed values under 0.35 g/cm³. The critical development of fast-growing poplar wood with less wood density causes

wind or storm susceptibility and pathogen sensitivity. Despite this fact, low density wood shows the proficiency of thermic isolation and resiliency. High density wood is stiffy and weatherproofed

Wood density and lignification may be correlated in poplar similar as suggested by Maiti et al. (2016). Here we found the lignin:cellulose ratio was shifted towards an enhanced lignin content, accompanied by higher wood density for 8 mM ammonium and the 0.2 mM nitrate-fed poplars in this study (**Fig. 3.19**). Stem wood in hardwoods contains 40-50 % cellulose (Fengel and Grosser, 1975), 15-40 % hemicelluloses and 18-25 % lignin (Silvy et al. 2018). In this study, 8 mM ammonium-fertilized poplars showed 5 % higher lignin content in dry wood than plants of other treatments, reaching values comparable to other softwoods (25-30 %, Silvy et al. 2018). Wood with higher lignin content is more pressure-resistant, whereas more cellulose content has a positively effect on breaking stress. But, the cellulose amount is critical in this study, because the material was not combusted afterwards to clarify, if it contained solid compounds like Calcium.

Overall, the present results highlighted that ammonium and nitrate nutrition have strongly diverging effects on wood anatomy, density and chemical composition in fast-growing poplars.

4.3 Ammonium-regulated GATA12 pathway in poplar wood

A central study question was to characterize GATA12 in response nitrate and ammonium in Arabidopsis and poplar. This study showed that a knock-down of *AtGATA12* had no influence on biomass production of Arabidopsis. However, biomass in Arabidopsis is dominated by leaves and therefore lacking secondary xylem formation in *AtGATA12* SALK mutants did not influence total biomass. Apparently, the whole pathway of secondary xylem formation was downregulated by *AtGATA12* knock-down, suggesting that *AtGATA12* expression is necessary for secondary xylem formation, when nitrogen levels were changed. Secondary xylem formation of *AtGATA12* knock-down plants fed with 1 mM nitrate was unaffected. The mutants formed smaller secondary cell lumina as well as thinner vessel cell walls when fertilized with 8 mM ammonium. It is therefore possible that GATA12 plays a role as upstream regulator of vessel cell wall thickening (Nakano et al. 2015; Endo et al. 2015) under ammonium excess. There was no secondary xylem tissue developed in SALK AtGATA12 lines and *35S:PtGATA12* lines fertilized with 8 mM nitrate, in contrast to wildtype plants under same conditions. Consequently, secondary xylem formation is dependent on a fine-tuned expression value of *PtGATA12* under nitrate excess at all.

The expression of genes possibly involved in putative *PtGATA12* pathway (**Fig. 1.2**, Nakano et al. 2015) were analyzed in differently fertilized *P. trichocarpa* plants. Poplars fertilized with ammonium exhibited higher transcript abundances of *PtGATA12* and *PtWND6B* than poplars fed with nitrate (**Fig. 3.22-3.23**). This

shows that the expression of these genes is dependent on the nitrogen compound. *PtGATA12* and *PtWND6B* were positively correlated, supporting that *PtGATA12* may function as an upstream regulator of *PtWND6B* (**Fig. 1.2**, Nakano et al. 2015; Endo et al. 2015), but a feedback loop is also possible. Moreover, the fiber or vessel cell wall thickening were dependent on the ammonium level (**Tab. 3.8**). Thicker fiber cell walls were formed in poplars fed with 0.2 mM ammonium most likely the result of increased transcriptional abundances of *PtCCoAOMT1* (**Fig. 3.24**) and *PtIRX1* (**Fig. 3.25**), which were induced by PtGATA12 and PtWND6B (**Fig. 1.2**, Nakano et al. 2015; Endo et al. 2015; Zhong et al. 2011). However, the transcript abundances of the selected lignin biosynthetic gene *PtCCoAOMT1* and the cellulose biosynthetic gene *PtIRX1* were not increased under 8 mM ammonium (**Fig. 3.24-3.25**) although vessel cell walls were thicker. Since *PtGATA12* and *PtWND6B* transcript level were enhanced, we assume that other biosynthetic genes were regulated.

Interestingly, thick vessel cell walls had a positive effect on wood density under 0.2 mM nitrate and 8 mM ammonium conditions (**Fig. 4.1A,D**). Fiber cell wall formation was induced and vessel lumen decreased under both 0.2 mM nitrogen forms (**Tab. 3.8**). The *PtGATA12* pathway was downregulated in poplars fertilized with 0.2 mM nitrate (**Fig. 4.1A**). We speculate that nitrate recruits different pathways to regulate cell wall formation.

Besides *PtWND6A* was upregulated under 8 mM nitrate supply (**Fig. 3.26**). Wood of these plants showed increased vessel and fiber cell lumen sizes (**Tab. 3.8**). While fiber cell walls developed normally, the 8 mM nitrate-fed plants were characterized by thin cell walls and low wood density (see chapter 4.2) (**Fig. 4.1B**). This may suggest that WND6A has a greater effect on vessels than on fibers.

Consequently, ammonium and nitrate had a distinctive influence on wood forming gene expression at low and high nitrogen concentrations. The results are in agreement with a role of GATA12 as an upstream regulator of *PtWND6B* in poplar. Interestingly, the GATA12 expression was induced by ammonium (**Fig. 3.22**). The pathway of ammonium inducing *GATA12* expression could be studied more in detail in the future.

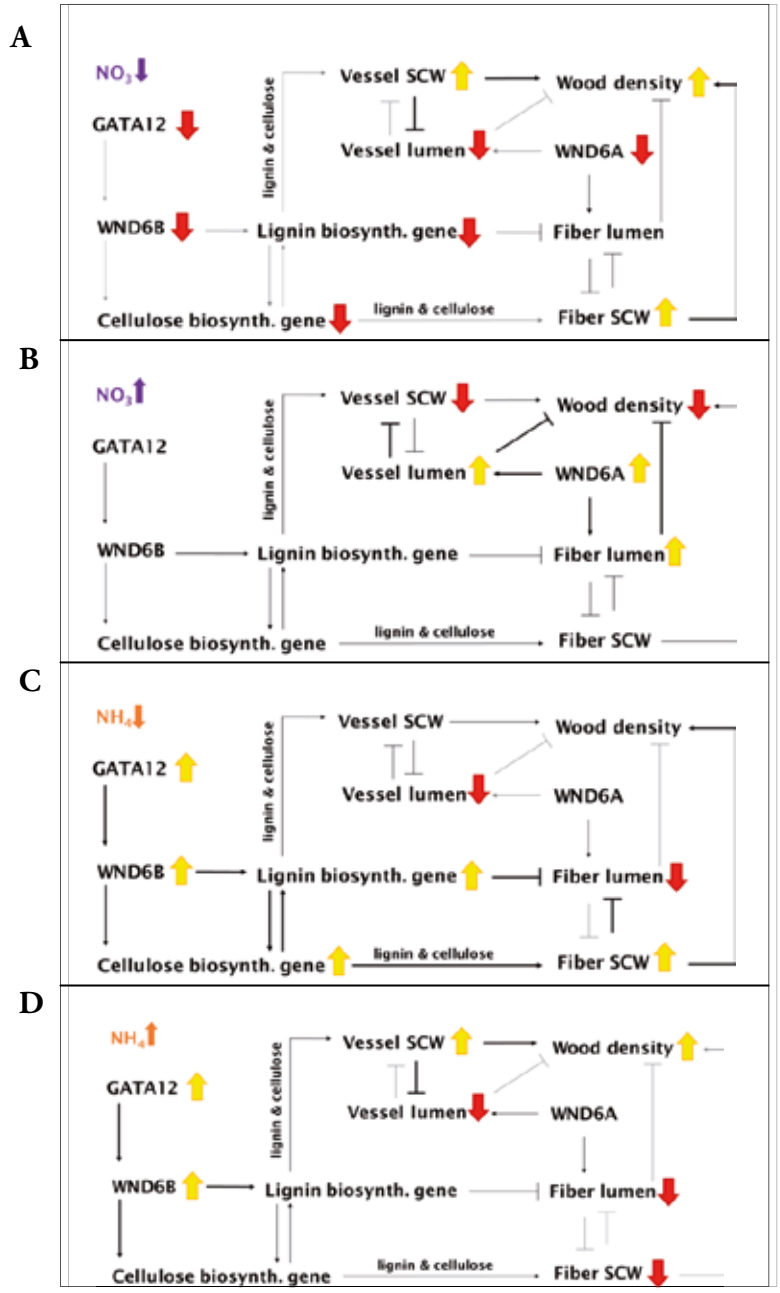


Fig. 4.1.: *Putative genetic model of poplar wood formation under A: 0.2 mM nitrate, B: 8 mM nitrate, C: 0.2 mM ammonium and D: 8 mM ammonium fertilization. SCW: secondary cell wall. Arrows up: significant high concentration/expression value; arrows down: significant low concentration/expression value (T-test, $p < 0.05$).*

4.4 Impact of PtGATA12 overexpression on poplar and Arabidopsis xylem

PtGATA12 overexpression prevented the inhibiting effect of high nitrate fertilization on poplar wood density by decreased vessel lumen size and thicker vessel cell walls in both transgenic lines (**Tab. 3.11**). Especially the *35S:PtGATA12* L10 showed significantly increased wood density (**Fig. 3.38**), more cambial cell layers and more mature xylem tissue than the wildtype (**Tab. 3.10**). In general, both *35S:PtGATA12* poplar lines exhibited thicker vessel cell walls than the wildtype, independent of the applied nitrogen form. This result strongly underpins that GATA12 is a positive regulator for vessel cell wall formation (Nakano et al. 2015; Endo et al. 2015).

Furthermore, *PtGATA12* overexpression prevented the inhibiting effect of 8 mM ammonium on poplar elongation growth (**Fig. 3.35A**) and leaf area size (**Fig. 3.36**). Endo et al. (2015) found ectopic differentiation of xylem vessel elements in leaves of *AtGATA12* overexpressing Arabidopsis lines. It is possible that such a phenotype was also occurring in the *35S:PtGATA12* poplar lines L8 and L10. Ectopic xylem vessel cells in different parts of plant organs could be a reason for an increased leaf area size and an increased elongation growth of *PtGATA12* overexpressing poplar lines. Unfortunately, this was not tested in this study.

In contrast to 1 mM nitrogen fertilized plants, no secondary xylem tissue was formed in *35S:PtGATA12* Arabidopsis plants fertilized with 8 mM nitrogen (**Fig. 3.42**). It is speculative, if *PtGATA12* had an inhibitory effect or no function in Arabidopsis under high nitrogen conditions. However, more secondary xylem tissue was formed in *35S:PtGATA12* L2B3 fed with low nitrogen concentrations, especially with 1 mM ammonium. Our results on the 1 mM fed *35S:PtGATA12* and *AtGATA12* SALK mutant Arabidopsis plants revealed the importance of GATA12 as a key regulator for secondary xylem formation under low ammonium levels. In general, the cell walls of secondary fibers were increased in *35S:PtGATA12* Arabidopsis lines, compared to wildtype fed with 1 mM nitrogen (**Tab. 3.12**). This may reflect a different role of GATA12 in fiber cell wall formation of non woody plants grown under nitrogen starvation.

Based on our study, we conclude that GATA12 has a positive function on cell wall thickness. Vessel cell walls of poplars fertilized with 8 mM nitrogen and fiber cell walls of Arabidopsis fed with 1 mM nitrogen were thicker in the *PtGATA12* overexpressing lines than in the wildtype. Therefore, our results support only partially the hypothesis that *35S:PtGATA12* plants exhibit thicker secondary cell walls than wildtype under high nitrate supply because the beneficial effects were confined to vessel cell walls. The impact of GATA12 on cell walls and wood density is obviously dependent on the nitrogen form and level.

In conclusion, this study provides novel insights into wood formation and its regulation in poplar. Ammonium has positive effects on wood density. To combine a fast plant growth and high wood density formation, either moderate levels of ammonium should be applied or soils with high nitrate concentrations could be afforested

with *35S:PtGATA12* poplars. However, it needs to be tested exactly, which genes are upregulated as the result of *PtGATA12* overexpression and if there were any disadvantages for the plant, like sensitivity to drought stress or herbivores. It should also be tested, if there is ectopic xylem vessel formation in different parts of plant organs, for example in the leaves and if this had an influence on vulnerability to pathogens. Therefore, field testing is necessary; before these genetically modified plants can be used for biomass production. It should be kept in mind that this study did the first step of analyzing *PtrGATA12* in poplar. This opens up a wide field of potential cases of removing difficulties in the methodological part.

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Appendix

GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGAAGCACCAGAATTCTATGGGGCATCCGGGTTTTTCAGC
TCACAATTCACAAGTAATGAAAAGCACCACTCTTTGGACTCTAATAATAAGTCCATTGGTGGTGGTGACCATTT
CATTGTTGAAGACCTTTTGGACTTCTCTAATGAAGATGATGATGCCATGATAACTGACCCAAACAATAATAATAC
TATCGTCACTGCCACCACCAACTCCACGGACTCTTCCACCGTAACTATTAAAGAAACACATCAATATGCTAATTA
TGGTTTCTGTTGTTATGTGCAGTATGATGATTTAGCGGAACTAGAATGGCTGTCGAATTTTGTGGAGGAATCG
TTCTCCAGCGAGGACTTGCAAAGGCTTCAGCTAATATCCGGCATGAAAGCTCGGCCAGACGAATCATCCAAGA
GTCGACACTTCCGAACTCATGGAGACACTGATGACAATAACAATGGCGATGTCTCCAATATTAGTAACATTAACA
ACACAATGTTCAATCCTGAAACGGCTGTCCCCGCTAAGGCTCGGAGCAAACGGTCTCGGGCAGCGCCGGGCAA
TTGGGCCTCACGCCTTCTTGTGCTCTCTCCCACGACTTCATCCTCCGATACTGAAATTATTGCTGGGCCGACC
CCACATCCGAATTCTGGGAAAAAGACAATTAAGGTTGAGGCGCGGCAGAAGAAAAGAGATGGTGGTGTTGAAG
GTTGCGATGGTCGCAAGTGCCTGCATTGTGCCACGGATAAGACGCCGCAGTGGCGGACTGGACCCATGGGGC
CAAAGACGCTGTGCAATGCGTGTGGTGTAAGGTACAAGTCGGGCCGGCTTGTGCCTGAGTACAGGCCTGCAG
CTAGCCCAACATTTATGCTCACTAAACACTCAAATTCGCACCGTAAGGTGCTTGAGCTCAGGCGACAAAAGGAG
ATGGTAAGAGCTCAGCAGCAACACCAGCACCAGCAATTTCTTCATCACCATCAGAATATGGTGTTTGGTGTATC
AAACGGTGGTGATGATTACTTGATTCACCAACATGTGGGACCCGATTTTAGGCAGATGATCTAGAACCCAGCTT
TCTTGTACAAAGTGGTCCCC

Fig. A1*: 35S:PtGATA12 gene construct. AttB-Primers are underlined. Yellow marked: Forward attB-recognition side. Green: Reverse attB-recognition side. Red: ATG = PtGATA12 start codon, TAA = PtGATA12 stop codon.*

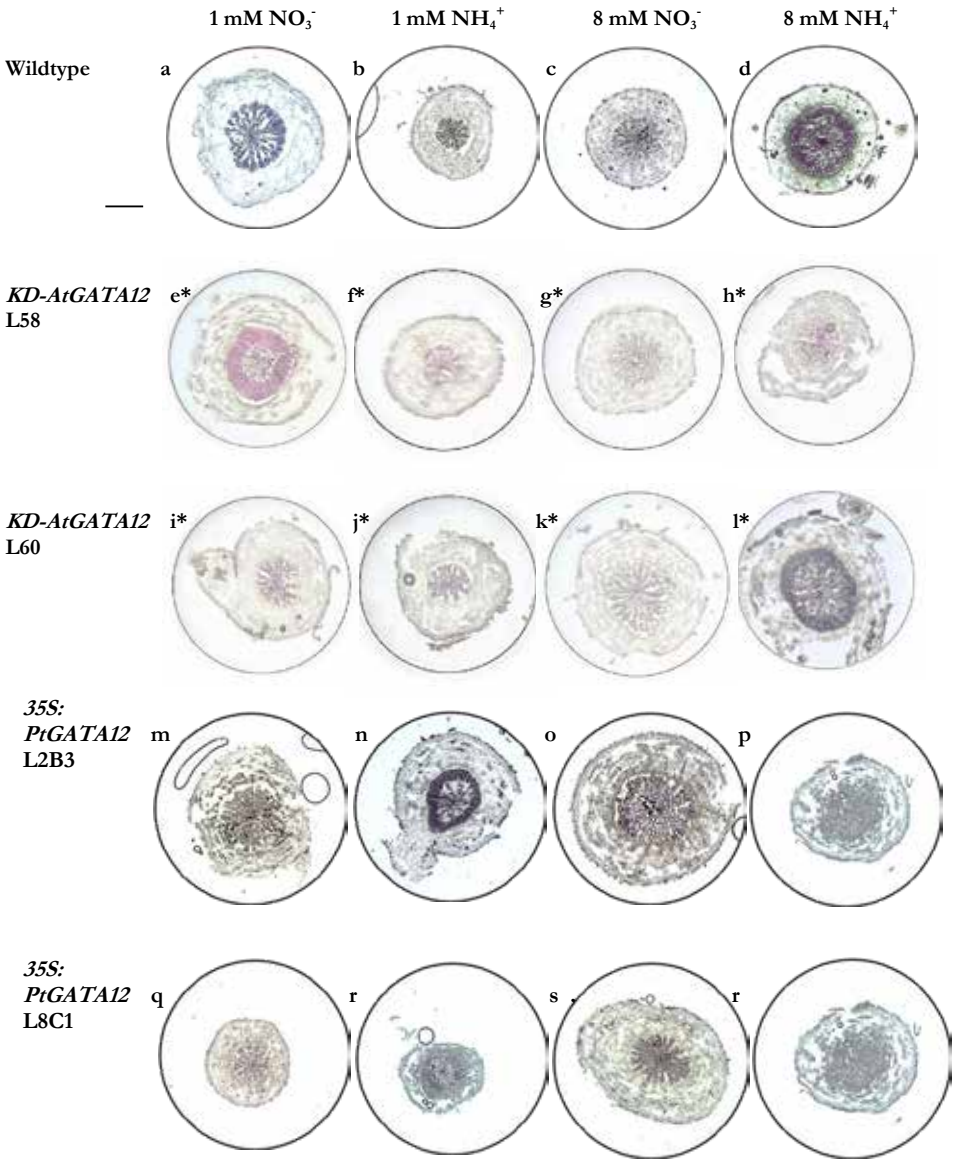

Fig. A2*: Overview pictures as examples for wildtype and transgenic (35S:PtGATA12 and KD-AtGATA12) Arabidopsis-Hypocotyl cross sections in response to different nitrogen levels and forms. Scale bar: 500 µm. Pictures with stars are produced by Ashkan Amirkhosravi und Felix Häffner*

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Nitrogen is an essential nutrient taken up by plants from the soil mainly as nitrate and ammonium. Poplars are fast-growing woody species that use both nitrogen forms for their nutrition. However, little is known how different forms of nitrogen nutrition influence biomass production and wood formation in poplar.
The goal of this study was to characterize growth, biomass production, anatomical effects and the regulation of genes involved in wood formation of poplars in response to nitrate or ammonium feeding. A further goal was to characterize a selected candidate gene PtGATA12 functionally using poplar and Arabidopsis as model plants.

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