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Transcriptional and proteomic responses towards early nitrogen depletion in *Arabidopsis thaliana*

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“If you torture the data long enough, it will confess”

R.H. Coase

ABSTRACT

Plant roots acquire nitrogen predominantly as ammonium and nitrate, which besides serving as nutrients, also have signaling roles. Re-addition of nitrate to starved plants rapidly and directly transcriptionally re-programs the metabolism and induces root architectural changes, but the earliest responses to nitrogen deprivation are unknown. In this thesis, the early transcriptional response of developed roots to nitrate or ammonium deprivation were analyzed in two *Arabidopsis* ecotypes contrasting in their nitrogen use efficiency: the inefficient genotype Col-0 and the efficient Tsu-0. The rapid transcriptional repression of known nitrate-induced genes proceeded the tissue NO_3^- concentration drop, with the transcription factor genes *LBD37/38* and *HRS1/HHO1* among those with earliest significant change. Some transcripts were stabilized by nitrate, but similar rapid transcriptional repression occurred in *loss-of-function* mutants of the nitrate response factor *NLP7*. In contrast, an early transcriptional response to ammonium deprivation was almost completely absent. In Col-0, the analysis was extended with the proteome and phospho-proteome resulting in a rapid and transient perturbation of the proteome induced by ammonium deprivation and a differential phosphorylation pattern in proteins involved in adjusting the pH and cation homeostasis, plasma membrane H^+, NH_4^+, K^+ and water fluxes. Fewer differential phosphorylation patterns in transporters, kinases and other proteins occurred with nitrate deprivation. The deprivation responses are not just opposite to the resupply responses, identify NO_3^--deprivation induced mRNA decay and signaling candidates potentially reporting the external nitrate status to the cell. Transcriptome comparison revealed only few N-nutrition related genes between both ecotypes contributing the increased NUE of Tsu-0, which probably relies on higher biomass accumulation. Besides, Tsu-0 confirmed the transcriptional depletion response of Col-0.

ZUSAMMENFASSUNG

Pflanzen nehmen Stickstoff vorwiegend in Form von Ammonium und Nitrat auf. Neben Nährstoffen fungieren diese auch als Signalstoffe. Wenn nach Mangel Pflanzen wieder mit Nitrat versorgt werden, wird der Stoffwechsel direkt und innerhalb kürzester Zeit umprogrammiert und die Wurzelarchitektur verändert. Die frühesten Reaktionen einer Pflanze auf Stickstoffmangel sind jedoch unbekannt. In dieser Arbeit wurden die frühesten transkriptionellen Antworten von voll entwickelten Wurzeln gegenüber Ammonium und Nitratmangel in zwei *Arabidopsis*-Ökotypen untersucht, die sich in ihrer Stickstoffnutzungseffizienz unterscheiden: dem ineffizienten Genotyp Col-0 und dem effizienten Tsu-0. Die schnelle transkriptionelle Repression bekannter nitratinduzierter Gene, darunter die Transkriptionsfaktoren *LBD37/38* und *HRS1/HHO1* mit den frühesten signifikanten Veränderungen, gingen mit dem Absinken des Nitratgehalts im Gewebe einher. Manche Transkripte wurden durch Nitrat stabilisiert, aber eine ähnlich schnelle Repression trat in auch Funktionsverlustmutanten des bekannten Transkriptionsfaktors *NLP7* der Nitrat-Antwort auf. Gegenüber Ammoniumentzug fehlte eine frühe Mangelantwort hingegen nahezu vollständig. In Col-0 wurde die Analyse um das Proteom und Phosphoproteom erweitert. Eine schnelle, jedoch transiente Veränderung des Proteoms wurde durch Ammoniummangel ausgelöst. Zudem wurden differentielle Phosphorylierungen in Proteinen festgestellt, die an der Regulation des pH- und des Kationengleichgewichts, sowie dem Austausch von H^+, NH_4^+, K^+ und Wasser durch die Plasmamembran beteiligt sind. Mit Nitratmangel traten weniger differentielle Phosphorylierungen auf, hauptsächlich in Transportern, Kinasen und anderen Proteinen. Die Nitratmangelantwort entspricht nicht einfach der gegenteiligen Nitratantwort. Möglicherweise übertragen mangelinduzierter mRNA-Abbau und potenzielle Signalproteine den externen Stickstoffstatus in die Zelle weiter. Der Vergleich der Transkriptome zwischen Col-0 und Tsu-0 ergab nur wenige differentiell exprimierte stickstoffrelevante Gene, welche zur höheren Stickstoffnutzungseffizienz von Tsu-0 beitragen könnten.

Diese ist vermutlich auf höhere Biomassebildung zurückzuführen. Anhand der von Tsu-0 gewonnenen Transkriptomdaten wurden zudem die Stickstoffmangelantworten von Col-0 bestätigt.

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ABBREVIATIONS

ActD	Transcriptional inhibitor Actinomycin-D
AGI	Arabidopsis Genome Initiative Identifier
AHA1/2	Arabidopsis ATPases (Proton pumps)
AMT	Ammonium Transporter
AS	Asparagine synthetase
Asn	Asparagine
ATP	Adenosine triphosphate
bp	base pairs
Col-0/8	*Arabidopsis* Ecotype Columbia-0/8
DEG	Differentially expressed Genes
DM	Dry Matter
FDR	Benjamini-Hochberg False Discovery Rate
FNR	Ferredoxin-NADP(+)-Oxidoreductase
GARP	Myb-Transcription factor family (GOLDEN2, ARR, PSR2)
Gln	Glutamine
GOGAT	Glutamine oxoglutarate aminotransferase
GS	Glutamine Synthethase

HPLC	High Performance Liquid Chromatography
LBD	Lateral Organ Boundary Domain Transcription Factor
LC-MS/MS	Liquid chromatography coupled with two mass spectrometries
LFC	Log_2 Fold Change
limma	Linear Models for Microarray Data
LRR-RK	Leucine-Rich Repeat - Receptor Kinase
*m*RNA	*messenger* Ribonucleic Acid
miRNAs	Noncoding regulatory Micro Ribonucleic Acids
-N	Nitrogen depletion condition(s)
NADP(H^+)	Nicotinamide adenine dinucleotide phosphate
NAE	Nitrogen assimilation efficiency
NIA	Nitrate Reductase
NIR	Nitrite Reductase
NLP	NIN-like Protein Transcription Factor
NRT	Nitrate Transporter
NUE	Nitrogen Use Efficiency
OPPP	Oxidative Pentose-Phosphate Pathway
PCA	Principal Components Analysis
PC	Principal Component
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
QTL	Quantitative Trait Locus
RIL	Recombinant Inbred Line
SAUR	Small Auxin Responsive RNA
SD	Standard Deviation
SPAD	single-photon avalanche diode
TAIR10	The *Arabidopsis* Information Resource - Genome Release 10
TFA	Trifluoroacetic acid
Thr	Threonine
Tsu-0	*Arabidopsis* Ecotype Tsushima-0

1 INTRODUCTION

1.1 Nitrogen in Plants

For the fulfillment of a plants life-cycle, nitrogen (N) is one of the most limiting nutrients. N is an essential structural component for genetic and metabolic compounds including amino acids, nucleic acids and many secondary metabolites. With a worlds average of 86 kg applied per hectare, nitrogen is the nutrient applied as fertilizer in the highest amounts in agricultural systems (FAO, 2015). Use of mineral nitrogen fertilizers greatly increased humankind's food production, but it is also cause for detrimental environmental effects like eutrophication of terrestrial and aquatic systems and global acidification (Gruber and Galloway, 2008). Moreover, the production of nitrogen fertilizers via the Haber-Bosch process requires high temperatures and high pressures. More than 1 % of world's total energy consumption flows into N-fertilizer production (Kitano et al., 2012).

Besides organic N bound in urea (H_2N-CO-NH_2), peptides and amino acids, the inorganic compounds nitrate (NO_3^-) and ammonium (NH_4^+) are the major nitrogen sources available for plants. Some families, like most leguminous plants have the ability to fix atmospheric N_2 by symbioses with rhizobacteria (*e.g.* rhizobia). In plants, different uptake systems between low- and high-affinity concentration ranges and physiological adaptations evolved towards the two inorganic nitrogen forms (von Wirén et al., 2000; Miller and Cramer, 2005). The availability of nitrate and ammonium in soils depends on a wide and dynamic range of environmental variables including soil pH, temperature and other factors (Sarasketa et al., 2014). Ammonium is highly abundant anaerobic wet soils at low temperatures (Xu et al., 2012). Nitrate is the major N form in aerobic soils (Marschner, 2011). Both nitrogen forms have different impacts on metabolism and physiology of the plant. Plants develop toxicity symptoms to higher concentrations of ammonium (Britto et al., 2001) while nitrate supplied in the same concentrations does not.

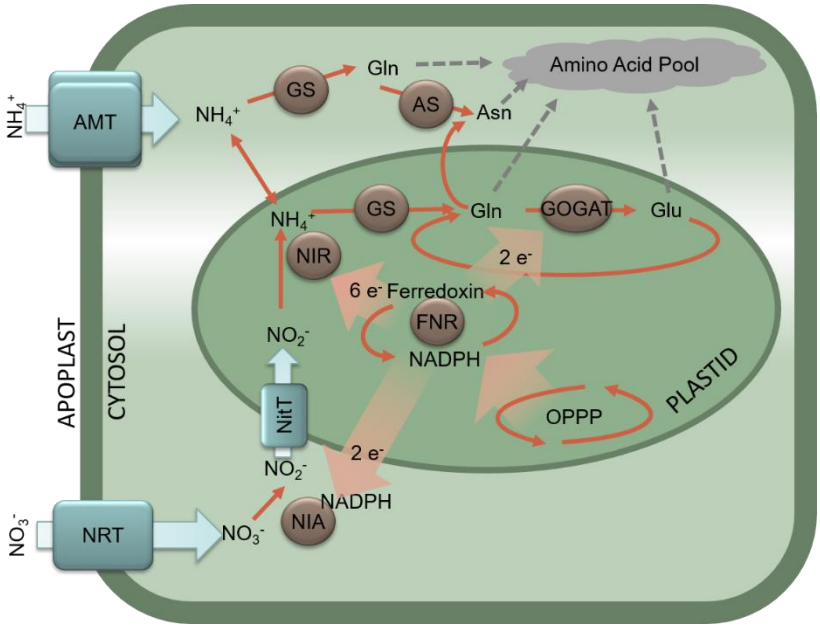

Figure 1-1: Schematic, simplified overview of N-uptake and assimilation in plant cells
Nitrate (NO_3^-) uptake into the cytosol is facilitated by different nitrate transporters (NRTs). Nitrate reductases (NIA) reduce nitrate to nitrite (NO_2^-) with electrons (e^-) derived from NADPH. Nitrite is transferred into plastids by a nitrite transporter (NitT), where it is subsequently reduced to ammonium (NH_4^+) by ferredoxin dependent nitrite reductase (NIR). Ammonium, derived from nitrate reduction or by uptake over ammonium transporters (AMT), is assimilated to glutamine (Gln) by plastidial and cytosolic glutamine synthases (GS). Both, NADPH and ferredoxin dependent GOGAT enzymes, synthesize glutamate (Glu) from glutamine in a cyclic manner with 2-oxoglutarate (not shown). Cytosolic Asparagine Synthase (AS) synthesizes asparagine (Asn) with glutamine as substrate and contributes to the amino acid pool in which nitrogen is organically bound. Reduction equivalents (NADPH as electron donors) are recycled over the oxidative pentose phosphate pathway (OPPP). Electrons are exchanged between ferredoxin and NADPH by oxidoreductases (FNR).

The uptake of nitrate from the soil solution occurs against an electrochemical gradient by nitrate transporter proteins (NRTs) in the plasma membrane of root cells. Subsequently, nitrate is compartmented, translocated and remobilized over the whole plant with numerous proteins involved (Figure 1-1) (Dechorgnat et al., 2011). To make the nitrogen accessible for biochemical reactions, nitrate has to be reduced into ammonium by two energetically expensive enzymatic steps. Nitrate is reduced to nitrite (NO_2^-) over the cytosolic nitrate reductases (NIA1/2). The cell-toxic nitrite is subsequently transferred to chloroplasts where it is rapidly reduced by the nitrite reductase (NiR) into ammonia (Coruzzi, 2003). The reducing equivalents (*e.g.* NADH and ferredoxin) for nitrate and nitrite reduction are recycled over the oxidative

pentose-phosphate pathway (OPPP) (Stitt, 1999). Gene expression of enzymes involved in this pathway were shown to be strongly regulated in response to nitrate (Wang et al., 2003).

The passage of ammonium through the plasma membranes into the plant is facilitated by ammonium transporters (AMTs) (von Wirén et al., 2000; Ludewig et al., 2007). Ammonium derived from both, nitrate or directly from ammonium acquisition is then assimilated into amino acids via the glutamine synthase (GS) / glutamine-2-oxoglutarate aminotransferase (GOGAT) cycle to carbonic compounds derived from photosynthesis (Figure 1-1) (Xu et al., 2012). The amino acids glutamate, glutamine, aspartate and asparagine are the N-containing end products of nitrogen assimilation and available for subsequent biochemical reactions in the plants (Coruzzi, 2003).

1.2 Nitrogen Signaling

Sensing the availability and distinction between different nitrogen forms in order to adjust the gene expression and consecutively metabolism, root system architecture, physiology and transport to the N-forms available in the environment is an essential ability of plants.

Many studies have already focused on NO_3^--responsive factors that are involved in signaling the presence of NO_3^-. Other than ammonium, NO_3^- is always absorbed from the environment as it cannot be produced by plants and hence resembles a potent signaling molecule. In the model plant *Arabidopsis thaliana* the transcriptomic changes upon severe NO_3^- deficiency and re-supply have been studied in much detail (Wang and Guegler, 2000; Scheible et al., 2004; Wang et al., 2004; Gutiérrez et al., 2007). More than 1000 genes were induced or repressed after 20 minutes in N-starved Arabidopsis roots by the addition of 250 µM NO_3^- (Wang et al., 2003). Processes such as the biosynthesis of amino and nucleic acids, transcription and RNA processing, ribosome and hormone biosynthesis, N assimilation, reductant supply (OPPP), and trehalose metabolism respond within 20 min to 3 h of NO_3^- induction (Castaings et al., 2011). A core set of genes responsive to nitrate responses robustly in various nitrate resupply experiments with changes in expression (Canales et al., 2014).

By use of a NIA1/NIA2 null mutant it was demonstrated that the NO_3^- molecule itself leads to transcriptomic changes of ~600 genes (Wang et al., 2004) and decomposed the NO_3^- dependent response from downstream metabolites like NO_2^-, NH_4^+ and amino acids. Numerous transcriptome and systems biology approaches in the recent years extensively increased the knowledge in the nitrate response (Vidal et al., 2015). The findings include the transcription factors ANR1, LBD37/38/39, NLP6 and NLP7, the protein kinases CIPK8 and CIPK23, microRNAs and the NO_3^- transporter and receptor NRT1;1/NPF6.3 and several other players (Castaings et al., 2011; Gutiérrez, 2012). The so-called primary nitrate response (PNR) is well studied but still holds several open questions (Medici and Krouk, 2014). Furthermore, signaling peptides as shown for CEPs (C-terminally encoded peptides) (Tabata et al., 2014) and CLEs (CLAVATA3/ESR-related) (Araya et al., 2014) are associated with the nitrate response. These peptides are secreted in roots and transmit the plants N status to the shoot, where they are received by leucine-rich receptor kinases (LRR-RKs) (Araya et al., 2014; Tabata et al., 2014).

Nitrate is apparently directly sensed externally by the nitrate transporter and sensor NRT1.1/NPF6.3 (Ho et al., 2009). NRT1.1/NPF6.3 regulates several aspects of the response to nitrate, including root morphological and metabolic adjustments (Bouguyon et al., 2015). Phosphorylation of the Thr-101 switches the transceptor from low- to high-affinity uptake (Figure 1-2) and is necessary for the sensing function (Liu and Tsay, 2003; Ho et al., 2009) . The transcriptional response to nitrate re-addition is strongly reduced in mutants lacking this NRT1.1 “transceptor” (Muños et al., 2004), but mutants lacking this plasma membrane nitrate receptor still respond to nitrate with activation of nitrate reductase expression (Konishi and Yanagisawa, 2010).

A prominent role is imputed to the NIN(*N*odule *In*ception)-LIKE Protein transcription factors NLP6/7. In response to nitrate supply NLP7 is accumulated in the nucleus within minutes (Marchive et al., 2013). Moreover, NLPs, as shown for NLP6, bind to NREs (nitrate regulatory elements) of various key genes in the nitrate response and regulate their transcription (Konishi and Yanagisawa, 2013; Konishi and Yanagisawa, 2014). Nitrate modulates the N-terminal re-

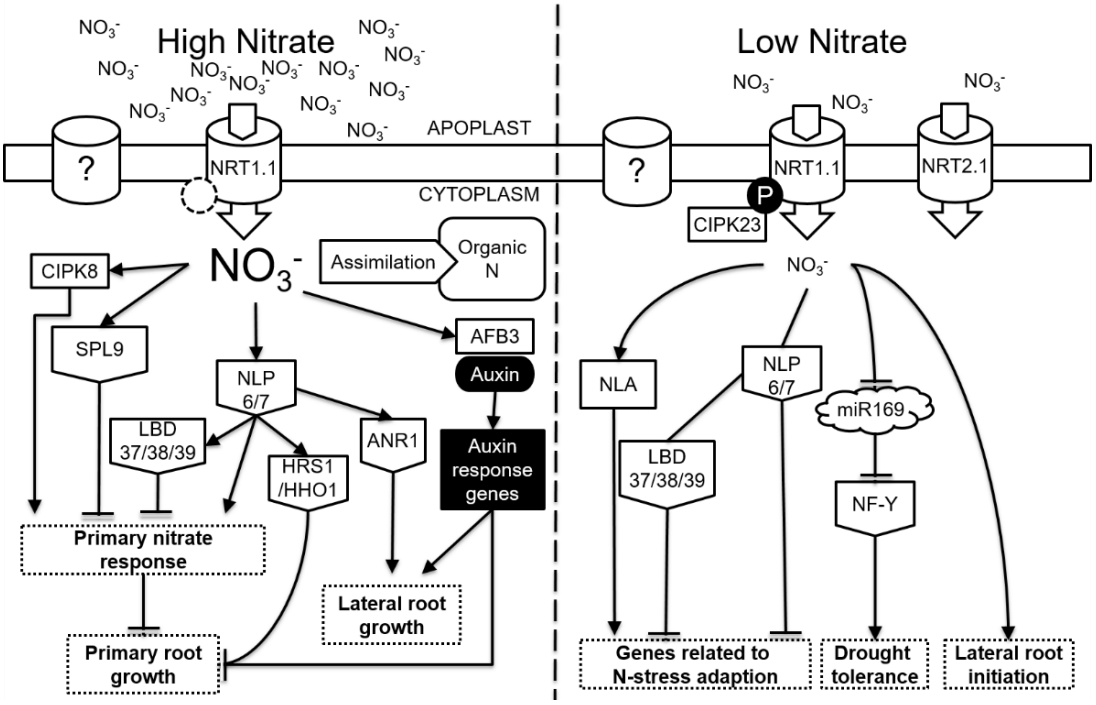

Figure 1-2: Simplified overview over regulatory components of the responses to nitrate

NRT1.1/NPF6.3 is the best-characterized nitrate-sensor. It is likely that also other receptors for nitrate (membrane-borne and intracellular) exist. Response networks correspond to metabolic, physiological and growth and developmental pathways activated for adaptive responses to changes in N-availability. Only responses that can be connected to upper regulatory components are shown. Under high-nitrate NRT1.1 acts as low-affinity transporter. Under low nitrate, NRT1.1 is phosphorylated at T101 and functions as high-affinity nitrate transporter. NRT2.1 may play a role in nitrate signaling. (Gutiérrez, 2012). Recently, NLP6/7 were shown to act as "master regulators" of the nitrogen response and to act upstream of LBD37-39, HRS1/HHO1. (Konishi and Yanagisawa, 2014). Rectangles represent regulatory proteins; pentagons are transcription factors.

(adopted and modified after Gutiérrez, 2012; with modifications after Konishi and Yanagisawa, 2014; Medici et al., 2015; Vidal et al., 2015)

gion of NLP6 (Konishi and Yanagisawa, 2013), but whether upstream signals or direct binding of nitrate act as signal is ambiguous.

Although the position and function within the signaling cascade is still uncertain for many components, much less is known about the molecular responses towards ammonium as the downstream metabolite of NO_3^-. However, the transcriptomic response to NH_4^+ re-supply is markedly different from that of NO_3^-, and consists mainly of genes associated with biotic stresses (Patterson, et al., 2010). Recently, in rice the transcription factor *Indeterminate domain* (IDD), was shown to be involved in induction of major ammonium-related genes like

*Os*AMT1.2 and *Os*GDH2 (Xuan et al., 2013). Little is also known about the primary targets of ammonium. High concentrations of ammonium were proposed to be sensed directly at the plasma membrane by ammonium transporter AMT1.1. The transporter is phosphorylated at Thr-460 and its ammonium transport ability consequently inactivated (Lanquar et al., 2009). Also so far unknown sensor are considered for ammonium sensing. The ammonium transporter AMT1.3 has been associated with the root branching response to local ammonium availability (Lima et al., 2010). On proteome level, phosphorylation patterns in various proteins of ammonium resupplied plants differ distinctly from nitrate resupplied plants (Engelsberger and Schulze, 2012).

1.3 Responses to low N

Physiological and metabolic adaptations to low nutrient concentrations are important for a sessile plant to accomplish their life-cycle even under harder conditions. For example, by growing into nitrogen-depleted soil patches or due to rainfall induced leaching of nitrate and other short-termed fluctuations in the N-supply require a plant to plastically adjust their root morphology and metabolism to the changing conditions (Hodge, 2004). Long-term nitrogen deficiency affects the whole plant: thus, the physiology and biochemistry of the roots and the shoot is adjusted to limiting N-conditions. This includes increasing expression of high-affinity N uptake systems (*e.g.* AMTs and NRTs), reduction of growth and photosynthesis, the remobilization of N from old, mature tissues to actively growing ones and the accumulation of abundant photodamage-protecting anthocyanin pigments (Peng et al., 2007). Nitrogen deprivation for few to several days leads to severe starvation symptoms in plant roots and shoots, with remobilization of N and allocation of carbon to the growing roots, which further precedes the depletion of the internal N stores in *Arabidopsis* (Krapp et al., 2011).

Precedent to adaptations towards low N conditions are transcriptome adjustments. With microarray-experiments transcriptome responses of *Arabidopsis* and rice-plants to low N conditions were elucidated (Hirai et al., 2004; Scheible et al., 2004; Bi et al., 2007; Peng et al.,

2008; Krapp et al., 2011). Within two days after withdrawal of N hundreds of genes are regulated in roots and shoots (Krapp et al., 2011). N-depletion leads to a coordinate repression of the majority of genes assigned to photosynthesis, chlorophyll synthesis, plastid protein synthesis, induction of many genes for secondary metabolism, and reprogramming of mitochondrial electron transport (Scheible et al., 2004). This involves also changes in the expression of regulatory *miRNAs* upon N-starvation (Liang et al., 2012). The adaptation to limiting N is concentration dependent as mild N-stress triggers transcriptional down-regulation only of a small gene set (Bi et al., 2007).

Remarkably, in most studies a prevalent down-regulation of gene expression with advancing N-deficiency was common. Moreover, in most cases the N-depletion was equated with nitrate-depletion. Transcriptomic impacts of long-termed ammonium depletion to prior ammonium supply are rather unspecified. Even less is known about the earliest responses to depletion of each nitrogen compound from the nutrient solution. Nutrient deprivation from roots can elicit rapid responses: for example, the deprivation of phosphorus elicits rapid and robust up- and down-regulation of multiple genes already within a few minutes, although not all genes are related to P-deficiency (Lin et al., 2011). Such rapid responses may be also expected for the withdrawal of nitrogen.

1.4 Nitrogen Use Efficiency

Due to the detrimental environmental effects of inadequate nitrogen fertilization, numerous efforts are made to decrease the amount of N losses to the environment. One approach is to increase the nitrogen use efficiency (NUE) of crop plants. Breeding crops with a better NUE results in decreased nitrogen input per biomass or yield gain to make crop farming systems more sustainable.

Different definitions of NUE are common: Grain yield per available soil N or fresh or dry-matter, respectively, produced per N-content (Good et al., 2004). In both cases, NUE is the product of nitrogen uptake efficiency (NupE) and nitrogen utilization efficiency (NUtE), which

is the optimal combination between nitrogen assimilation efficiency (NAE) and nitrogen remobilization efficiency (Chardon et al., 2010). While an increasing number of genes involved in improved nitrogen use efficiency have been proposed in crops and *Arabidopsis* (Hirel et al., 2007) causal relationships with NUE are often lacking. But it is obvious that genes involved in root architecture, nitrogen uptake, assimilation, N-storage and retranslocation as well as genes involved in regulation of these processes, such as transcription factors, have a critical impact on NUE (Xu et al., 2012).

1.5 Ecotype Differences in NUE in *Arabidopsis*

Arabidopsis thaliana offers a high quantity of genetic variation as its natural range extends over the whole European continent, parts of Northern Africa, eastern Asia including the Himalaya and Japan and North America (Koornneef et al., 2004). A high genetic diversity is derived from publicly available *Arabidopsis* accessions collected from different geographic sites. This variation is greatly suitable for analyzing variation in adaptive traits (Koornneef et al., 2004).

Recombinant inbred lines (RILs), which are usually plants derived from crossings of two genetically distinct accessions, were used to identify growth-related quantitative trait loci (QTL) in response to varying nitrogen sources (Rauh et al., 2002). By growing such RILs under contrasting N environments (high and low soil nitrate), the natural variation can be employed in order to identify NUE-related QTLs (Loudet et al., 2003). Furthermore, several studies characterized accessions from of core collections of selected *Arabidopsis* accessions with highest genetic diversity (McKhann et al., 2004) grown under ample and limiting nitrogen supply (North et al., 2009; Chardon et al., 2010; Ikram et al., 2012). Their findings helped to classify the genotypes into nitrogen use-efficient and -inefficient genotypes (Chardon et al., 2010). Surprisingly, the accession Columbia (Col-0), which was the basis for the *Arabidopsis* reference genome (The Arabidopsis Genome Initiative, 2000) and which is used as wild-type in numerous studies, has a low responsiveness to different N-environments and a low NUE (Chardon et al., 2010). In contrast, the accession Tsushima (Tsu-0) has, mainly at high N environments,

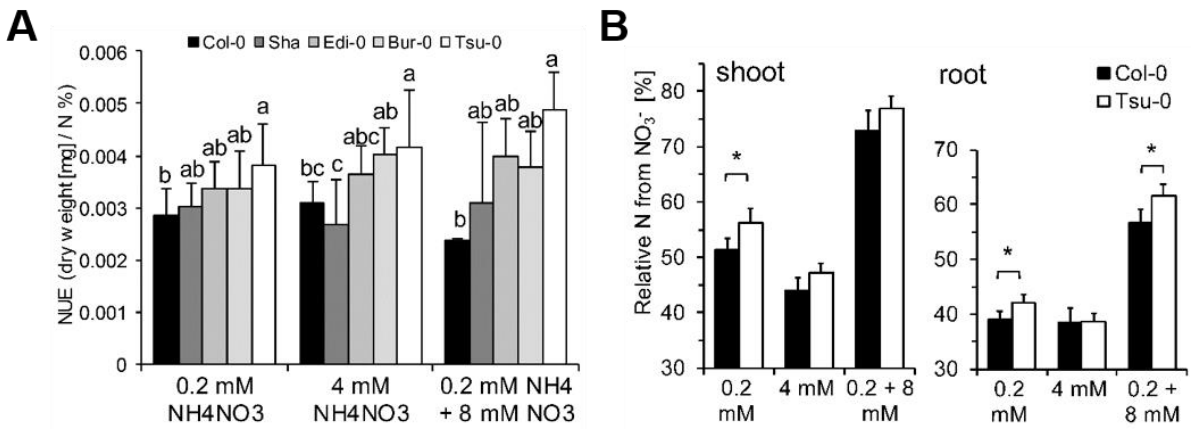

Figure 1-3: Tsu-0 has a higher NUE than Col-0 and prefers nitrate over ammonium

A NUE of different *Arabidopsis* ecotypes at different N-environments (B.Sc. thesis, J. Trini, 2014) **B** Relative N from Nitrate in Col-0 and Tsu-0 in these N-environments (M.Sc. thesis, T. Range, 2013)

a high NUE, which is prevailingly based on a disproportionate biomass gain in response to N-supply (Chardon et al., 2010). Tsu-0 is therefore a favorable genotype for further nitrogen nutrition studies (Chardon et al., 2012).

The higher NUE of Tsu-0 was also underpinned by findings of the Ludewig workgroup. By growing these genotypes in hydroponic culture at different N-forms and concentrations Tsu-0 was confirmed as a genotype accumulating high biomass at different N-conditions which is one reason for the increased NUE. Additionally, Tsu-0 and Col-0 are distinct in their preference for nitrate as N-source (Figure 1-3, personal communication).

1.6 Aims and methodology in this work

The main objective of this thesis was the investigation of the rapid transcriptional, proteomic and phospho-proteomic responses of mature *Arabidopsis* roots being deprived for nitrogen with focus on the research question:

> *"What are the earliest transcriptional and proteomic changes, that occur under ammonium and nitrate deprivation in Arabidopsis thaliana?"*

To answer this question, a N-depletion experiment with a hydroponic culture of *Arabidopsis thaliana* plants was conducted. Two time-points were chosen to trace the progression of the

putative nitrogen depletion responses. The identification of very early (after 15 minutes) molecular signals in N-deficiency helped to separate the direct effects from indirect, downstream effects of these signals. Such effects were expected already after intermediate duration (after 3 hours) of N-depletion. Furthermore, by distinguishing between the effects of nitrate and ammonium depletion by a previous acclimation of the plants to one of the two N-forms it was expectable that their lack leads to distinct transcriptomic and proteomic adjustments.

In this study microarray technology was used to acquire the transcriptome data. In the last two decades, gene expression arrays have established as the gold-standard to obtain reliable and comparable large-scale gene expression data of almost all annotated (and undescribed) genes in *Arabidopsis* and various other organisms in research. There are only few limitations like the missing representation of (mature) *microRNAs*, incomplete data about splice variants or a limited dynamic range in which relative gene-expression changes can be documented. Relative protein abundance changes of membrane proteins of the *Arabidopsis* roots and shifts in protein modifications, such as phosphorylations, were quantified with a stable label-free mass spectrometric approach (Steen et al., 2005). These kinds of approach were already utilized to detect protein phosphorylation changes upon nitrogen resupply (Engelsberger and Schulze, 2012) or protein abundance changes of maize root hairs in response to several macro- and micronutrient deficiencies (Li et al., 2015).

The results of this study contribute to the identification of new, and the verification of known key genes of a nitrogen-form specific depletion response on transcriptome and proteome level as well as it increases the resolution of the time-course dependent dynamics of these responses. The transcriptome response was investigated in the two *Arabidopsis* genotypes Tsu-0 and Col-0. As these ecotypes are at contrasting ends of the NUE (Chardon et al., 2010), a higher responsiveness in the depletion response was hypothesized for the N-efficient ecotype Tsu-0. A comparative analysis of the transcriptional responses with the inefficient genotype Col-0 may consequently elucidate genes involved in the better NUE of Tsu-0.

Furthermore, the findings of this work will increase our knowledge about plant responses towards nutrient stresses and will possibly supply breeders with information about new genes and markers to improve the NUE of crops.

2 MATERIALS AND METHODS

2.1 Equipment & Consumables

2.1.1 Equipment

- **Auer Packaging GmbH**, Amerang, Germany; Gray Boxes 40 x 30 x 13.5/23.5 cm with ~11/22 L volume for hydroponic culture, Custom Lids for 32 Falcon tubes
- **Biometra,** Göttingen, Germany; T3 Thermocycler
- **BioRad**, Hercules, USA; CFX384 Realtime-PCR Detection System/C1000 Thermal Cycler set
- **Eppendorf**, Hamburg, Germany; Microcentrifuges: 5415D /-R, 5417C, Thermomixer compact
- **Retsch**, Haan, Germany; Shaker Mill MM2
- **Seal Analytical GmbH**, Norderstedt, Germany; AA3 HR, Autoanalyzer
- **Thermo Fisher Scientific**, Waltham, USA; NanoDrop 2000c Spectrophotometer; NanoFlow Easy-nLC1000 HPLC system; Q Exactive Plus Orbitrap hybrid mass spectrometer

2.1.2 Consumables and Chemicals

- **AppliChem**, Darmstadt, Germany; Minerals for nutrient solutions, Chemicals for protein preparation
- **BioRad;** 384 Well PCR plates (white opaque), Microseal 'B' adhesive seals
- **Duchefa Biochemie**, Haarlem, The Netherlands; Phytoagar
- **Greiner Bio-One,** Frickenhausen, Germany; CELLSTAR® 50 ml tubes which were modified for hydroponic culture
- **Roth**, Karlsruhe, Germany; Lids detached from black Rotilabo® reaction tubes (1.5 ml), Minerals for nutrient solution
- **Sigma Aldrich,** Munich, Germany; Actinomycin D from *Streptomyces spp.* ≥ 95%
- **Th. Geyer**, Renningen, Germany; Minerals for nutrient solutions

- **Thermo Fisher Scientific,** PepMan C18 tips, C8 extraction disks, Invitrogen Standard Oligonucleotides Primers for RT-PCR
- **Wako Chemicals**, Neuss, Germany; Lys-C endoproteinase

2.1.3 Kits

- **Analytik Jena**, Jena, Germany; innuPREP Plant RNA Kit
- **KAPA Biosystems**, Wilmington, USA; KAPA SYBR FAST qPCR 2x Supermix
- **QIAGEN GmbH**, Hilden, Germany; RNase-Free DNAse Set; QuantiTect Reverse Transcription Kit

2.1.4 Services

- **Oaklabs GmbH**, Henningsdorf, Germany; Microarray Analysis with Array XS Arabidopsis

2.2 Plant material

- *Arabidopsis thaliana* (L.) Heynh., Ecotype Col-0 (Columbia)
- *Arabidopsis thaliana* (L.) Heynh., Ecotype Tsu-0 (Tsushima)

Seeds were derived from the European Arabidopsis Stock Center (NASC) and propagated in the institute's greenhouse.

- *Arabidopsis thaliana* (L.) Heynh., Ecotype Col-8 (Columbia)
- nlp7-1
- nlp7-3

The aforementioned three genotypes were obtained from Anne Krapp, INRA France and were genotypes originally used in the in the published studies about NLP7 (Castaings et al., 2009; Marchive et al., 2013).

2.3 Growth Conditions

Plants were grown in a climate chamber with controlled short day conditions with 10 h light at 22 °C and 14 h darkness at 18 °C temperature. Light intensity was approximately 200 µmol m^{-2} sec^{-1}. Relative Humidity was held constantly on 55 %.

2.4 Nutrient solution for hydroponic culture

Arabidopsis plants were grown hydroponically in a modified ¼ strength Hoagland's nutrient solution (Hoagland, D.R., Arnon, 1950) nutrient solution. The solution was mixed from 100× concentrated stock solutions of macro nutrients and 1000× concentrated stock solution of all micronutrients. pH of the nutrient solution was adjusted to pH 6 with KOH.

Table 2-1: Final concentrations of nutrients in different solutions

	NN	**+NO_3^-**	**+NH_4^+**	**-N**
KH_2PO_4	1 mM	1 mM	1 mM	1 mM
$MgSO_4$	0.5 mM	0.5 mM	0.5 mM	0.5 mM
Na_2EDTA-Fe	0.1 mM	0.1 mM	0.1 mM	0.1 mM
$CaCl_2$	1 mM	1 mM	1 mM	1 mM
NH_4NO_3	1.5 mM	1.5 mM	1.5 mM	1.5 mM
KNO_3	-	3 mM	-	-
$(NH_4)_2SO_4$	-	-	1.5 mM	-
K_2SO_4	-	-	-	1.5 mM
Micronutrients	*	*	*	*

* 1x concentration: 9 µM $MnSO_4$; 0.765 µM $ZnSO_4$; 0.32 µM $CuSO_4$; 0.016 µM Na_2MoO_4; 46 µM H_3BO_3

2.5 Plant growth in hydroponic culture

Arabidopsis seeds were imbibed on filter paper with 1-2 mL of Hoagland's solution and stored in a refrigerator for 3 days at 4 °C for stratification of the seeds. For growing one plant in hydroponic culture approximately 400 µl of Hoagland's (NN) was solidified by 0.8 % (w/v) Phytoagar (Duchefa, Haarlem, The Netherlands) in each lid of black colored opaque 1.5 mL microcentrifuge tubes, similarly as described in by Conn et al., 2013. These lids served as seed/plant carriers. For each experiment 144 – 216 seed carriers were prepared. Three seeds were transferred to solidified nutrient medium in small cavities on top of every carrier. The carriers were placed in a microcentrifuge tube rack (with 72 or 144 holes) in which each hole was filled with Hoagland's (NN) solution in every hole and placed in a box which was covered with plastic wrap to keep humidity high for germination and the first days after. The box was placed into a growth chamber where seeds germinated.

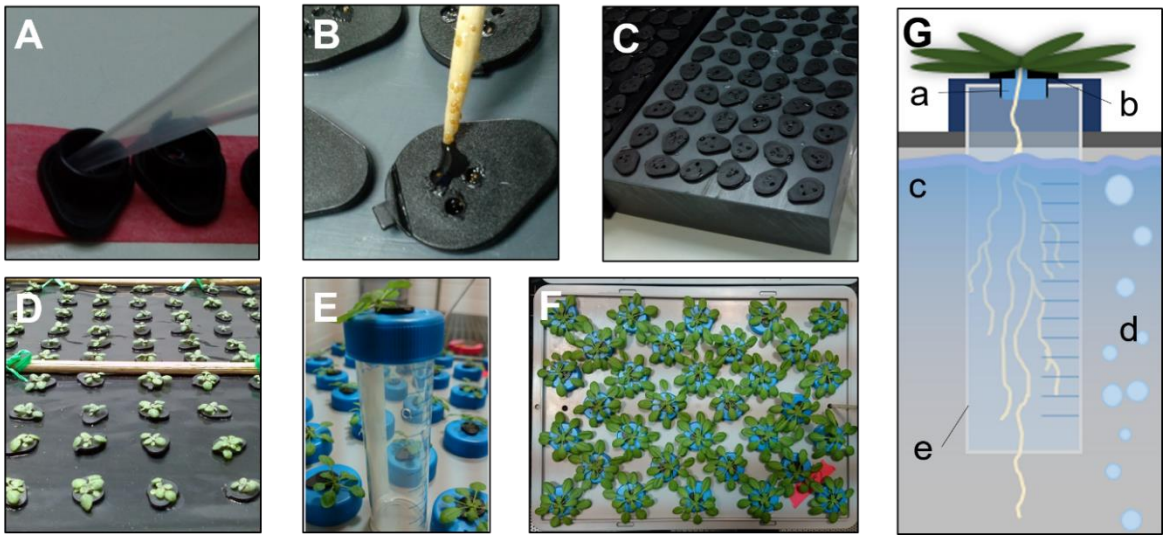

Figure 2-1: Different steps of the hydroponic culture of Arabidopsis

A Filling the agarose into seed holders sticking on a laboratory tape; **B** Transfer of imbibed *Arabidopsis* seeds into the holes of the seed carrier with a toothpick; **C** Seed holders in a microcentrifuge tube rack; **D** 3 week old plants in a polystyrene float (covered by a black foil to suppress algae growth); **E** Side-view of a modified 50 mL reaction tube with an approximately 4-week old plant; F 32 5-week old plants growing in a 22 L box with aerated nutrient solution; **G** Schematic side-view of one *Arabidopsis* plant in hydroponic solution (c). **a** – agarose plug, **b** – seed holder, **c** – nutrient solution, **d** – aeration, **e** – modified 50 mL reaction tube

After germination, plants were grown for approximately 10-14 days in the box until the rootlets penetrated the plug of solidified Hoaglands (NN). The plant holders were placed on a self-made polystyrene float (for up to 216 seed carriers) swimming in a box with approximately 11 L of Hoaglands (NN). Degenerated, damaged or weak plants were removed with a forceps and plants were reduced to one plant per holder. When plant roots had a length of approximately 4-5 cm, usually 3 weeks after germination, the plants were transferred to boxes with 11 L volume. The covers of the boxes had 32 holes. In each hole a 50 mL reaction tube was placed. The conical bottom of these tubes was previously removed. Plant carriers were placed in screw-caps of the 50 mL tubes. These caps were prepared with a hole in which the plant holders fitted well. Hence, every plant root was growing in a separate tube as a root protection in the same nutrient solution. The protection was important to keep avoidable influences of damages by hooking or during regular replacement of nutrient solution on transcriptome low. With the transfer to the box also an aeration of the nutrient solution was installed for each box.

This consisted of a membrane air pump and piping into the nutrient solution of each box with air stones at its ends. During the last two weeks the nutrient solution was replaced every 4-5 days. 5 Days before harvest (Day 30 after germination) one half of all plants was transferred on Hoaglands with 3 mM KNO_3 and the other half of plants on Hoagland with 1.5 mM $(NH_4)_2SO_4$ to adapt the plants to the respective nitrogen-form.

2.6 N-depletion experiment

For induction of N-depletion, roots were rinsed in two boxes of N-free Hoaglands (-N) and immediately placed in a third box for the desired time span of 15 minutes or 3 hours. Roots were harvested at time-point 0 in the respective N-solution ($+NH_4^+$ or $+NO_3^-$) and 15 minutes and 3 hours after transferring the roots to N-free medium. Additional controls were harvested to detect possible transcriptional effects caused by the unavoidable mechanical stimulus, minor variation in pH or salt concentrations. The controls were conducted by transferring the root transfer to another box containing fresh Hoaglands ($+NH_4^+$ or $+NO_3^-$). Controls were harvested after 15 min. and 3 h in fresh $+NH_4^+$- or $+NO_3^-$ Hoaglands.

To avoid confounding regulation by the circadian rhythm and diurnal gene regulation, the root harvest was always carried out at 4 h, 4 h+15 min. and 7 h after onset of daytime. For each replication of the experiment, roots of six individual plants per experimental treatment were pooled and subsequently frozen in liquid nitrogen and stored at -80 °C until RNA-extraction was conducted.

2.7 Transcriptional inhibition with Actinomycin-D

Roots of five-week old nitrate-adapted plants were incubated in 250 mL nutrient solution (3mM KNO_3) supplemented with 100 µg/mL ActD (Sigma-Aldrich, dissolved in DMSO) for one hour. Subsequently, plants were placed in N-depleted (3 mM KCl) or N-supplied (3 mM KNO_3) nutrient solutions. Incubated root parts of 3-4 plants were collected directly after ActD treatment

(0 h) and after 1, 2, and 3 hours in the N-treatments, rinsed in 1 mM $CaSO_4$ and were frozen instantly in liquid N_2.

2.8 RNA-Extraction

Frozen root material was ground in a pre-cooled mortar in liquid nitrogen and separated into approximately 100 mg aliquots. Total RNA was extracted out of these aliquots with innuPREP Plant RNA Kit (Analytik Jena, Jena, Germany) according to the manual with an additional in-column DNAse digestion-step (RNase-Free DNAse Set, QIAGEN). Concentration and potential ethanol or salt contaminations in every RNA extraction was measured with a spectrophotometer (NanoDrop 2000c, Thermo Scientific). Quality and integrity of RNA samples was tested by running 1 µL of every RNA sample in a bleach gel according to Aranda et al., 2012 with minor modifications (commercial bleach was replaced by 1% (v/v) sodium hypochlorite). Total RNA aliquots were then stored at - 80 °C for further use.

2.9 Analysis of Transcriptome and Gene Expression

2.9.1 Microarray analysis

Microarray analysis is an established method to identify differential gene expression in *Arabidopsis* RNA-samples. Briefly, a dye-labelled *c*DNA of a tissue sample is hybridized to glass slides on which DNA-fragments are printed on which are representative for a subset of known genes of an organism – so called DNA probes. After hybridization, the array is scanned and dye fluorescence intensity of each probe on the array is measured. The sequence and position of every probe on the microarray are known. The fluorescence intensity of every probe correlates with the amount of dye labelled *c*DNA, which hybridized to this probe. After subtraction of background intensities and normalization procedures, this fluorescence intensity can be related to the corresponding *m*RNA amount of every gene in the analyzed tissue and hence, its gene expression.

The microarray analysis in this work was conducted with total RNA samples of three independent replicates for each treatment. RNA was diluted to a concentration of 150 ng/µl and 15-20 µl were sent to Oaklabs GmbH (Henningsdorf, Germany) on dry ice. Analysis was carried out by Oaklabs on experimentally validated custom microarrays (ArrayXS Arabidopsis) based on Agilent 8×60K microarray-chips (Agilent Technologies, Inc., Santa Clara, USA). A single-channel approach was chosen, which means, every RNA sample was labelled with only one dye (Cy5). RNA-Quality tests, cDNA-synthesis, dye-binding, hybridization and array scanning was conducted by Oaklabs. Agilent raw-intensity data were returned and analyzed with R and Bioconductor (Gentleman et al., 2004).

2.9.2 Quantitative RT-PCR

Of each sample 1 µg RNA was transcribed into cDNA with QuantiTect Reverse Transcription Kit (QIAGEN GmbH, Hilden, Germany) according to the manual. Heating steps of reverse transcription reactions were conducted in a thermocycler (Biometra T3). 1:10 dilutions of the cDNA were used for the PCR reactions. The reactions usually contained 7.5 µL 2× KAPA SYBR FAST qPCR Supermix (KAPA Biosystems, Wilmington, USA), 5 µL cDNA (5 ng/µL) and 200 nM of each gene-specific primer for a final volume of 15 µL. For each primer-pair at least one no-template-control (NTC) was run, in which 5 µl cDNA was replaced by ddH_2O. The two-step thermal cycling protocol included 40 cycles at 95°C for 3 s and 60°C for 20 s. Melt curves were recorded after the final cycle heating from 65°C to 95°C with an increment of 0.5 °C for 5 s per step, to confirm a single amplicon. PCR-reactions were analyzed with the CFX384 Real-time-PCR Detection System/C1000 Thermal Cycler set (BioRad, Hercules, USA). Data analysis was conducted with CFX Manager Software v3.1 according to MIQE standards. The relative gene expression values (using the $\Delta\Delta$Ct method) were normalized towards the expression of the reference genes *SAND* (*At2g28390*) and *PDF2* (*At1g13320*) (Czechowski et al., 2005). Averaged values were shown for three biological replicates of each condition with each sample containing three technical replicates per target gene.

2.9.3 Primers used for qRT-PCR

For genes of some transcription factors and reference genes primer sequences were adopted from the respective publication. Generally, all primer sequences were designed with the curated *m*RNA/*c*DNA-sequence of the respective gene in the database of the National Center for Biotechnology Information (NCBI). With NCBI's PrimerBLAST specific primers were designed with a melting temperature (T_m) closely around 60 °C, a product-size of between 70 and 150 bp and a specificity check against the whole *Arabidopsis* transcriptome (Refseq mRNA).

Table 2-2: Primers used for qRT-PCR

Name	AGI	Forward Primer Sequence 5'-3'	Reverse Primer Sequence 5'-3'	
SAUR6	At2g21210	AGTGCTGCAATCATCAAAGCAA	TCCTACGTAAACCGCAAGGT	[d]
MSC	At5g26200	AATGAGTTTAGGTGCTTTGATGGAA	GGAGAAAAGAGCAGCACCGA	[d]
LBD37	At5g67420	ATGGATTGAAACCGCCGATG	CAAAGCAGGACGTTGAGAATCC	[a]
LBD38	At3g49940	GCCCTGCTTTGTTTCAGTCTT	ACATTCCAATTCCCCGTCCAC	[a]
LBD39	At4g37540	CTCCAACGTCCTGCTTTGTTTC	AGTTCCTGGTCCACAACATACC	[a]
NIA1	At1g77760	GGCTACGCTTATTCTGGAGGAGGT	TGGTGGTCAAGCTCACAAACACTC	[d]
NIR1	At2g15620	AACCGTTTCTCCCCTGAACC	CTTGTCCGCAGAACTGGCTA	[d]
HRS1	At1g13300	AACGAGAGAGATGTGGGCAA	CGTCGTCATCTCCGGTAACTC	[b]
HHO1	At3g25790	GATGATGAAGAACACCAGTC	TATTAGAAACAACTGCGTCG	[b]
NLP7	At4g24020	GCCCTTGAGGCGGTAAATCT	GTCTGAGCGAGAGGCAAGTT	[d]
AMT1;1	At4g13510	TATGGGCGGTGGAGGAAAAC	CTCGGACGATATCCGCAACA	[d]
AMT1;2	At1g64780	CGACTCCTACACCGACCTTG	TTTGGTGCCCGAACTCTTGT	[d]
AMT2	At2g38290	AAGGGACAAGCAAAGATCCCA	ATCCCGCCACAAGTATCGTC	[d]
GLN1;1	At5g37600	TGTGAAGTGGCCTGTTGGTT	TGTCTGCTCCAATACCGCAA	[d]
GLN1.3	At3g17820	AGCGTCGTCTCACTGGAAAG	CACGTCCCACTCTCACTGAC	[d]
NRT2.4	At5g60770	CTGGTGGAAACTTCGGCTCT	CCATCCATGTCAGCCCTTGT	[d]
Reference genes:				
PDF2	At1g13320	TAACGTGGCCAAAATGATGC	GTTCTCCACAACCGCTTGGT	[c]
SAND	At2g28390	AACTCTATGCAGCATTTGATCCACT	TATTGCATATCTTTATCGCCATC	[c]
UBC10	At4g05320	GGCCTTGTATAATCCCTGATGAA-TAAG	AAAGAGATAACAGGAAC-GGAAACATAGT	[c]

Primer Source: [a] Rubin et al., 2009, [b] Medici et al., 2015, [c] Czechowski et al., 2005, [d] own design

2.10 Quantitation of root nitrate- and ammonium concentration

Roots of hydroponically grown *Arabidopsis* plants were rinsed in 1 mM $CaSO_4$ solution to wash remaining nutrient solution away. Roots were dried on paper towels and snap-frozen in liquid

nitrogen in 2 mL reaction tubes after removal of shoot tissue. The roots were freeze dried for 5 days. After determination of dry weight roots were ground in a shaker mill with steel beads (Retsch, Haan, Germany). 100 mg root powder per sample was incubated in 1.5 mL water over night at 4 °C. 1 ml of each solution was 1:4 diluted, filtered and colorimetrically analyzed for nitrate and ammonium concentration with an autoanalyzer (AA3 HR, Seal Analytical GmbH, Norderstedt, Germany).

2.11 Analysis of the Proteome

2.11.1 Protein Preparation for LC-MS/MS

The microsomal protein fraction was extracted from approximately 1 g of frozen roots according to the method described (Pertl et al., 2001) with minor modifications. Extracted protein was redissolved in 6 M urea and 2 M thiourea, pH 8 and predigested with endoproteinase Lys-C (0.5µg/µL Wako Chemicals, Neuss, Germany) at room temperature for 3 h. After 4-fold dilution in 10 mM Tris-HCl, pH 8, protein samples were digested with 1 µL trypsin per 50 µg protein over night at room temperature. To stop digestion, trifluoroacetic acid (TFA) was added until pH was below 3. Samples were desalted with C18 tips (PepMan, ThermoScientific) and dissolved in 100 µL 5% acetonitrile and 0.1% TFA before enrichment for phosphopeptides.

2.11.2 Phosphopeptide Enrichment

Phosphopeptides were enriched over TiO_2 as described elsewhere (Larsen et al., 2005) with minor modifications. TiO_2 beads were equilibrated with 100 µL of 5% acetonitrile and 0.1% TFA. In acetonitrile and TFA dissolved, desalted samples were incubated for about 1 min with 2 mg equilibrated TiO_2 beads. The solution with the beads was then placed into a self-made microcolumn in a 200-µL pipette tip with a C8 disc as a plug. In these microcolumns phosphopeptides were eluted from TiO_2 beads using 5% ammonium hydroxide and 5% piperidine. By adding 50 µl 20% phosphoric acid to reach a pH value less than 3, elutes were immediately acidified. Again, samples were desalted with C18 tips prior to subsequent LC-MS/MS analysis.

2.11.3 LC-MS/MS Analysis

Digested peptide-samples were analyzed by LC-MS/MS by the use of a nanoflow Easy-nLC1000 HPLC system (Thermo Scientific) connected to a quadrupole Orbitrap hybrid mass spectrometer (Q Exactive Plus, Thermo Scientific) to analyze peptide masses. Peptides were sprayed directly into the mass spectrometer after elution from a 75 µm × 50 cm C18 analytical column (PepMan, Thermo Scientific) on a linear gradient from 4 to 64% acetonitrile over 120 min. Proteins were identified by MS/MS using information-dependent acquisition of fragmentation spectra of multiple charged peptides. Up to 12 data-dependent fragment spectra were acquired in the linear ion trap for each full-scan spectrum at a resolution of 70 000 and with an isolation width of 1.2 *m/z*. The normalized collision energy was set to 25%. Singly charged ions were excluded, and ions were dynamically excluded for 30 s within a 5 ppm mass window.

2.12 Bioinformatic Analyses

2.12.1 Analysis of microarray data

The derived microarray raw data files contained besides annotation data the median fluorescence intensity and median background signal intensity values. These values were obtained for every of the approximately 60,000 probes of each of the 8 sections on a microarray chip. Every section on the array represented one RNA sample and was stored in a separate file.

The microarray raw-data were imported to the R (R Core Team, 2016) with the *read.maimages* function of the LIMMA package (Smyth, 2004). According to the LIMMA User's guide (Smyth et al., 2015), the median signal intensities were background corrected with the *normexp* method and an additional offset of 16. This offset parameter added a constant value to each intensity value to suppress the high variances in $\log_2$-ratios (see below) derived from intensities with values close to zero. All microarrays were quantile-normalized by what the value distribution of every microarray was forced to be identical (Bolstad et al., 2003). Probes which had intensities not at least 10% higher than the negative-control probes of each chip

were filtered out. After filtering, approximately 23,400 transcripts remained detectable. Furthermore, intensities of probes with the same corresponding gene were averaged. All microarray data were fitted to a linear model with a combination of ecotype + pre-treatment + sampling time point (*e.g.* Col-0_Nitrate_15min for all Nitrate adapted Col-0 roots after 15 min in -N) as factor for the linear model with. Thus, replications of the experiments were averaged. The LIMMA package uses an empirical Bayes moderated statistics to calculate the significance of the ratios of differentially expressed genes. For an example R-code, see Appendix.

For identification of early differential expression in contrasts of 15 min vs. 0 min datasets a comparatively low threshold for the log_2-fold-change (log_2-FC) of > 1 or < -1 was chosen. To detect differentially expressed genes after 3 h, the threshold was increased to > 1.5 or < -1.5. P-values were corrected with the Benjamini-Hochberg False Discovery Rate (*FDR*) method (Benjamini and Hochberg, 1995). Genes with a *FDR* < 0.05 were generally defined as significantly differentially expressed. The earliest responsive genes (15 min) were additionally filtered against (even non-significant) transcripts in controls or the opposite N-form with a log_2-FC > 1 and < -1, to obtain the N-form-specific responsive genes.

2.12.2 Mass Spectrometric Data Analysis and Statistics

The mass-spectra derived from the MS/MS Analysis were loaded into MaxQuant, v.1.4.1.2 (Cox and Mann, 2008) for peptide identification. Mass spectra were annotated against Arabidopsis proteome (TAIR10) with Andromeda (Cox et al., 2011). Methionine oxidation and protein N-terminal acetylation, serine, threonine and tyrosine phosphorylation were set as variable modifications while carbamidomethylation of cysteine was set as fixed modification. Mass tolerance for database search was set to 20 ppm on full scans and 0.5 Da for peptide-fragment ions. Multiplicity was set to 1. For label-free quantitation, retention time matching between runs was chosen into a time-window of 2 min. Peptide and protein *FDR* were set to 0.01 and site *FDR* to 0.05.

With help of the site-scanning algorithm of Andromeda the location of phosphorylation sites was determined. Contaminants (e.g. keratins) were excluded from further analysis. Peptide ion intensity values derived from MaxQuant (evidence.txt) were utilized for further analysis. The software cRacker (Zauber and Schulze, 2012) was used to conduct a label-free data analysis of (phospho-)peptide ion intensities. All phosphopeptides and non-phosphopeptides were used for quantitation. Fraction of total normalization was used to normalize intensity data separately for each sample and replication (peptide ion intensity/total sum of ion intensities). Averages of ion intensities were calculated for each peptide from three replications of every sampling condition and time-point in the experiment. All proteotypic peptide-intensities were summarized to calculate protein-intensity for each sample. Similar to transcriptome analysis the use of the cRacker software allowed us to calculate $\log_2$ fold-changes of the protein levels. Changes in phosphorylation were expressed as $\log_2$ fold-changes of phosphopeptides ion intensity of respective sampling time-point versus time-point 0 min. in the respective treatment (for example $\log_2$(15 min/0 min)). The same approach was conducted for protein-intensities to express change of protein abundances between different sampling time-points. Automated pairwise t-tests were applied to test for statistical significance of each change in protein/phosphopeptide abundance. Where applicable, p-values were Benjamini-Hochberg corrected.

2.12.3 Principal Components Analysis

PCA was conducted with the normalized fluorescence intensity-data of all microarray experiments of the N-depletion experiment of both, Col-0 and Tsu-0 ecotypes, except the control datasets in R with the *prcomp* function and *ggord* plugin for *ggplot2* graphics library (Wickham, 2009; Beck, 2015).

2.12.4 Over representation analysis of genes/proteins

Of BAR for Plant Biology the Classification SuperViewer Tool was used to identify transcripts for over-representation of specific biological pathways (Provart and Zhu, 2003). The latest MapMan bin-classification (Ath_AGI_LOCUS_TAIR10_Aug2012) was chosen for functional

classification of the genes. A few of the detected transcripts identified as significant were not assigned to any of the known functional MapMan classes. In addition, PageMan application of MapMan version 3.5.1 (Usadel et al., 2009) was used to test for over-represented transcripts or proteins by using lists with the log_2-fold ratios. Over-representation was analyzed by use of Fisher's exact test and Benjamini-Hochberg corrected p-values.

2.12.5 Software used

- Andromeda (Cox et al., 2011)
- BioConductor Version 3.1; http://bioconductor.org; (Gentleman et al., 2004)
- BioRad CFX Manager™ Software Version 3.1
- cRacker Version 1.498 (Zauber and Schulze, 2012)
- MapMan Version 3.5.1 (Usadel et al., 2009)
- MaxQuant, Version 1.4.1.2 (Cox and Mann, 2008)
- Microsoft Excel 2010
- Notepad++ Version 6.8.8
- R Version 3.2.1 (2015-06-18) - "World-Famous Astronaut" - (R Core Team, 2016)
- Online Tools:
    - BAR Classification SuperViewer Tool (Provart and Zhu, 2003): http://bar.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi
    - ClustalW2 Protein Sequence Alignment: http://www.ebi.ac.uk/Tools/msa/clustalw2/
    - jVENN (Bardou et al., 2014): http://bioinfo.genotoul.fr/jvenn/index.html
    - EnsemblPlants for *Arabidopsis* database queries: http://plants.ensembl.org/Arabidopsis_thaliana/Info/Index
    - The *Arabidopsis* Information Resource (TAIR) for gene information: https://www.arabidopsis.org/index.jsp
    - ARAMEMMNON - Plant membrane protein database: http://aramemnon.uni-koeln.de/index.ep

3 RESULTS

3.1 The N-depletion Experiment

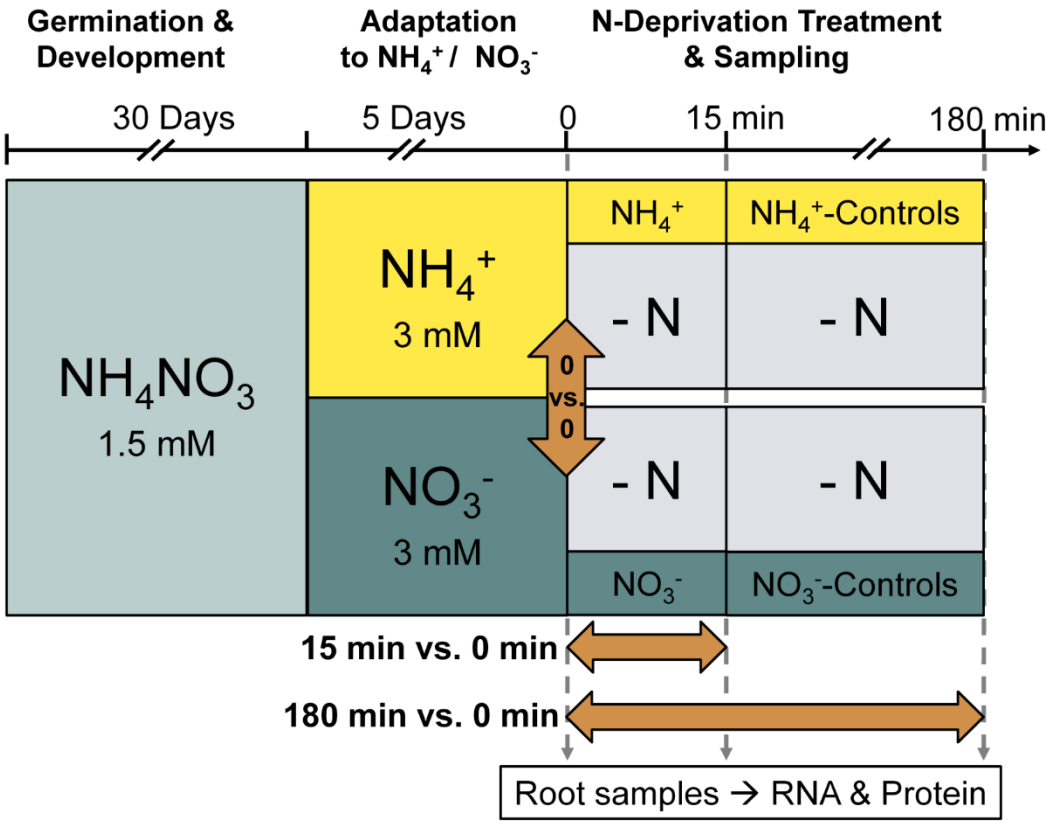

Figure 3-1: Experimental design

Experimental design and schematic time points of harvest. Orange arrows show time-points of informative transcript-/protein-abundance changes which were analyzed more in detail. The experiment was conducted three times for Col-0 and Tsu-0 at short day conditions (10 h light/14 h dark with 22°/18°C and 55% rel. humidity). Plants were supplied with 3 mM nitrogen which was given in equimolar amounts in form of either 1.5 mM NH_4NO_3, 3 mM KNO_3 or 1.5 mM $(NH_4)_2SO_4$. In N-depleted nutrient solution (-N) Nitrogen was replaced by 1.5 mM K_2SO_4. Root samples for the proteome analysis were collected in a separate experiment with Col-0 under the same experimental conditions but without collection of control-samples.

3.1.1 The growth conditions and induction of nitrogen deprivation

Arabidopsis thaliana plants of the Columbia-0 (*Col-0*) and Tsushima-0 (*Tsu-0*) ecotypes were grown in hydroponic culture for 30 days with 1.5 mM NH_4NO_3 in Hoagland's nutrient solution (Figure 3-1).

Figure 3-2: Habitus of *Arabidopsis* plants grown with different photoperiods

Left: 25 days old plant with constant light (24 h). **Right**: short day conditions (10 h light) after 30 days of growth

For the transcriptome experiment this hydroponic culture, as described above (Chapter 2.5), was introduced as novel method. Single plants were easy to handle as all steps were conducted under non-sterile conditions and transcriptome disturbances by root damages and mechanical stimuli were as far as possible suppressed by growing each plant in a separate 50 mL reaction tube but with contact to the same nutrient solution. In an initial experiment, constant light and temperature conditions were chosen in order to suppress circadian oscillations in gene expression which would have interfered with the expected changes in transcriptome caused by N-depletion. But these plants grew too fast with abnormal leaf shapes, darker leaves and very early shoot/flowering initiation after already 2.5 weeks of growth (Figure 3-2). Consequently, for the microarray analysis, plants were grown in short day conditions which led to decreased, but homogeneous growth and sufficient plant size and root size at harvest after 5 weeks of growth. By using control plants and harvest of each experiment to the same time after onset of light, interferences of circadian gene expression were mostly alleviated.

As nitrogen concentration, 3 mM of was chosen to supply the plants with an adequate, intermediate amount of nitrogen, without fully filling and saturating the N-storage pools. The plants were then exposed to nutrient solutions for five days in which 3 mM nitrogen was either given as KNO_3 or $(NH_4)_2SO_4$, respectively (Figure 3-1). The transfer to specific NO_3^- or NH_4^+ nutrition had negligible influence on their size and phenotype. All other nutrients were given in sufficient amounts for optimal plant growth and were frequently refreshed by exchange of nutrient solution.

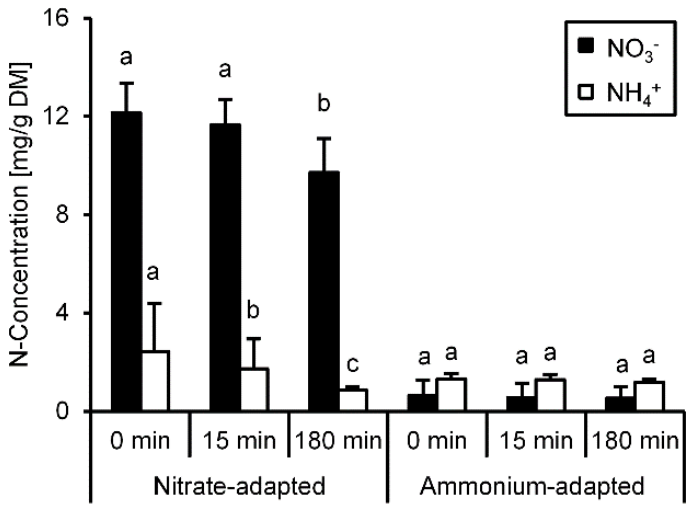

Figure 3-3: Nitrate and ammonium concentration in N-depleted roots

Nitrate and ammonium concentration of plants at different time-points of the N-deprivation experiment. Error bars indicate SD of mean (n=10). Tukey's HSD test was used to test for significant differences of values of the same N-adaptation and in the same N-form.

3.1.2 Nitrogen concentration in roots of Col-0 plants

The nitrate-adapted roots contained around 12 mg of nitrate per g dry mass and 2 mg × (g DM)$^{-1}$ of ammonium. The nitrate concentration was drastically reduced in the ammonium-adapted plants to about 0.6 mg×(g DM)$^{-1}$, while the ammonium concentrations were similar to those of the nitrate-adapted plants (Figure 3-3). The NO_3^- concentrations in tissues of nitrate-adapted roots had not dropped after 15 min. of nitrate deprivation, but had significantly decreased by 3 h after transfer of the plants to a medium without nitrate (Figure 3-3). In contrast, the tissue NH_4^+ concentration in the ammonium-adapted roots remained at almost the same low level, even after three hours of NH_4^+-deprivation. This suggested that 15 min. and 3 h of nitrate deprivation reflect meaningful time points for the transcriptional response, corresponding to situations when tissue nitrate had not yet dropped or was already significantly reduced, respectively. For consistency, the same harvest time points were chosen for the ammonium deprivation time course.

3.2 Transcriptome Analysis of *Arabidopsis* Ecotypes to N-deprivation

The whole root transcriptome was analyzed in order to identify gene transcripts, which responded to the stimulus of a rapid deprivation of external nitrogen in the nutrient solution surrounding the roots. This was established with a microarray-analysis by using arrays which

cover 30,541 of all known gene loci in *Arabidopsis* and three independent replications of the N-depletion experiment for two *Arabidopsis* ecotypes (Col-0 and Tsu-0) with contrasting nitrogen use efficiencies (Chardon et al., 2010).

3.2.1 Principal components analysis of all microarray data

A principal component analysis (PCA) helped to gain a first overview over the major influencing factors on gene expression. All normalized microarray intensity-data derived from Tsu-0 and Col-0 adapted to nitrate or ammonium, respectively, were taken into account (Figure 3-4). Surprisingly, the highest fraction of variance with 40.7% described by PC1 did not describe the expected impact of the different nitrogen forms on gene expression, but rather reflected the differential gene expression unrelated to N metabolism between the ecotypes. However, the adaptation to different nitrogen forms was reflected by the PC2, which described 16.3 % of the variance. Besides, a clear shift in the variance of nitrate depleted plants after 3 hours was recognizable. Principal component 3 and those of higher magnitudes further divided the data in time-points of N-depletion experiment and the three replications of the experiment. The effects of treatment and genotypes were hierarchically higher-ranked than the independent replications, which indicates a high reproducibility of the experiments and only few disruptive factors in the experimental set-up.

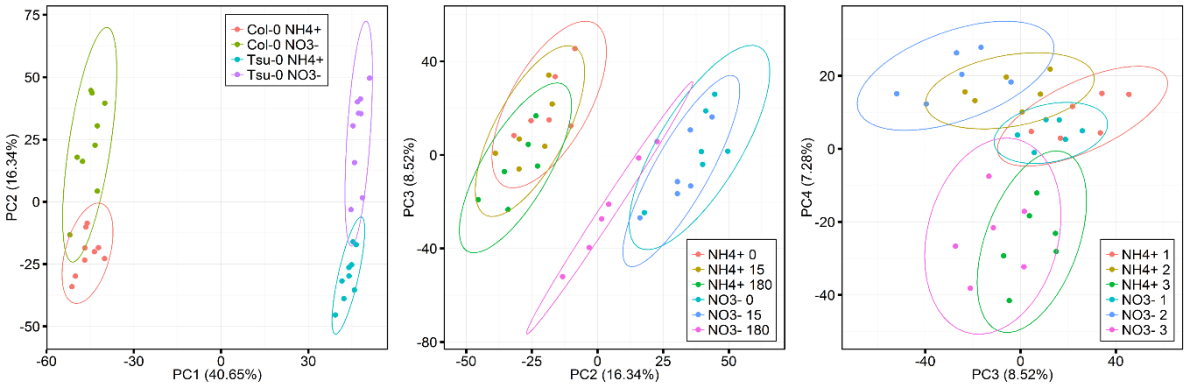

Figure 3-4: Principal Components Analysis of gene expression data
PCA of normalized microarray fluorescence intensity data of all N-depletion experiments conducted with the ecotypes Col-0 and Tsu-0 after both, nitrate and ammonium pre-treatment. **left:** PC1 vs. PC2, data points are grouped for ecotype and pre-treatment, **center:** PC2 vs. PC3, data grouped after pre-treatment and time-point, **right:** PC3 vs. PC4 grouped for replication and pre-treatment.

Using PageMan, overrepresented MapMan bins, which represent functional gene categories between the two ecotypes were identified (Figure 3-5). As expected from the PCA, clear differences in gene expression between the ecotypes were identified. But gene categories related to nitrate assimilation, nitrogen-uptake, -translocation and -metabolism were not among the over-represented categories. In many cases the gene categories were similar between the two N-forms, but differed between the ecotypes. For example, stress related genes were overrepresented in Col-0 and secondary metabolism related genes in Tsu-0. Protein synthesis genes were underrepresented in Tsu-0 and transcriptional regulators less expressed in Col-0.

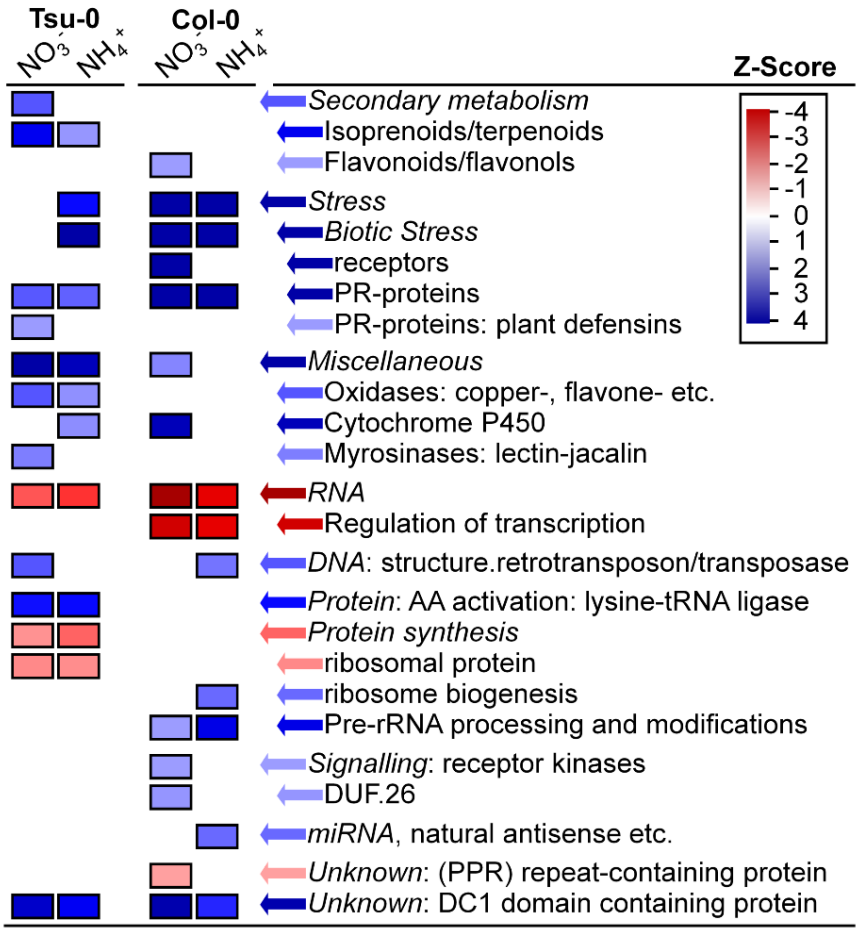

Figure 3-5: MapMan overrepresentation analysis of Col-0 and Tsu-0

Overrepresented MapMan bins of 35 days old *Col-0* or *Tsu-0* plants grown on nitrate or ammonium as sole nitrogen source for five days. Comparisons were taken between both ecotypes grown with the same N-form in the nutrient solution (e.g. Col-0 NH_4^+ vs. Tsu-0 NH_4^+). Conducted with Fisher's exact test and Benjamini-Hochberg corrected p-values, which were Z transformed (*e.g.* p = 0.05 ≙ Z = 1.96).

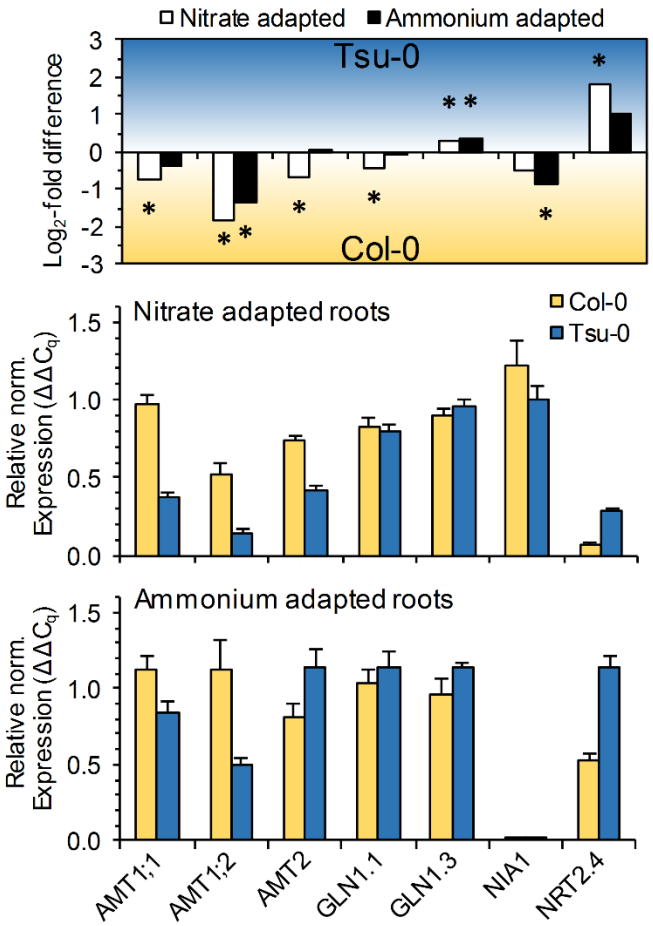

Figure 3-6: Differentially expressed N-related genes in Col-0 and Tsu-0

N-related genes which were significantly differentially expressed between Tsu-0 and Col 0 (Asterisk: *FDR* < 0.05) and their validation by qRT-PCR. Error Bars: Standard error of mean (n=3).

Genes coding for major nitrogen-nutrition responsive transcription factors like *ANR1*, *HRS1*, *HHO1*, *LBD37-39*, *NLP6-7* and signaling proteins (e.g. *CIPK8*, *CIPK23*) were not visibly differentially expressed between both genotypes (Figure 3-7). A number of genes were higher expressed at nitrate adapted conditions, particularly *HRS1*, confirming the typical nitrate dependency of their expression. *LBD38* was the only transcription factor which showed a higher expression in ammonium adapted conditions. The differences in expression of N-uptake and assimilation related genes were rather negligible between the two accessions. But the differential expression of these few genes could at least contribute to the preferential nitrate or ammonium uptake and different NUE between Tsu-0 and Col-0, but does not explain it entirely.

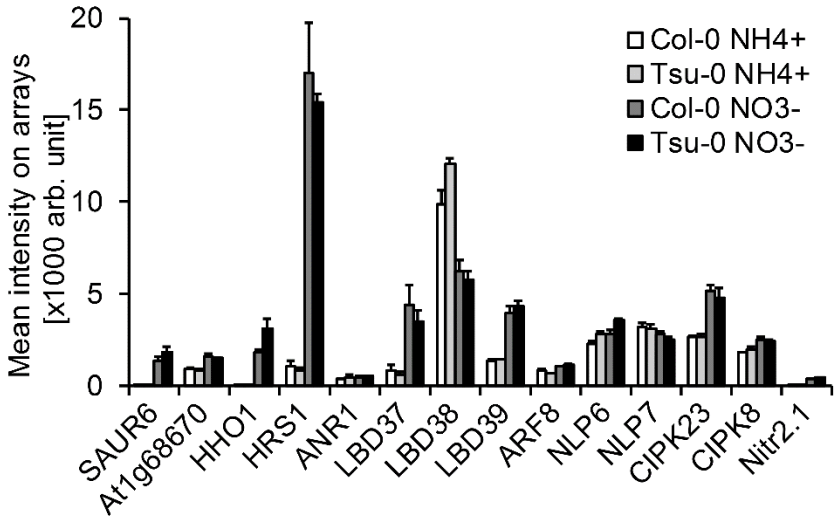

Figure 3-7: Nitrogen nutrition related gene expression in Col-0 and Tsu-0

Mean expression values derived from normalized microarray intensities at time-point 0 of each replication, genotype and N-treatment. Error bars indicate standard deviation (n=3). At1g68670: HHO2.

3.2.2 Transcriptome adaptations to ammonium and nitrate in Col-0

Due to the little differences in nitrogen related genes observed between Tsu-0 and Col-0, further analyses were focused on the ecotype Col-0. Although its lower NUE (Chardon et al., 2010), Col-0 is the reference genotype in most studies. Thus, results which were derived from this genotype have a better comparability with other studies. Results from the transcriptome analysis derived from Tsu-0 were taken to confirm the findings in Col-0.

In the roots, only 85 genes were differentially expressed after 5 days in nitrate- or ammonium-adapted plants ($log_2FC > 2$ or < -2, FDR < 0.05). Among them, 53 were higher expressed under nitrate nutrition (Suppl. Table A-1). As expected, a significant over-representation of differentially expressed genes involved in the nitrate metabolism, including both nitrate reductases and nitrite reductase, the oxidative pentose phosphate pathway (glucose-6-phosphate dehydrogenase 3), amino acid metabolism, as well as transcripts involved in development and transport was found (Figure 3-8). Over-represented categories of differentially regulated transcripts in the ammonium-adapted plants involved amino acid degradation (Figure 3-8), in accordance with earlier results (Scheible et al., 2004; Patterson et al., 2010).

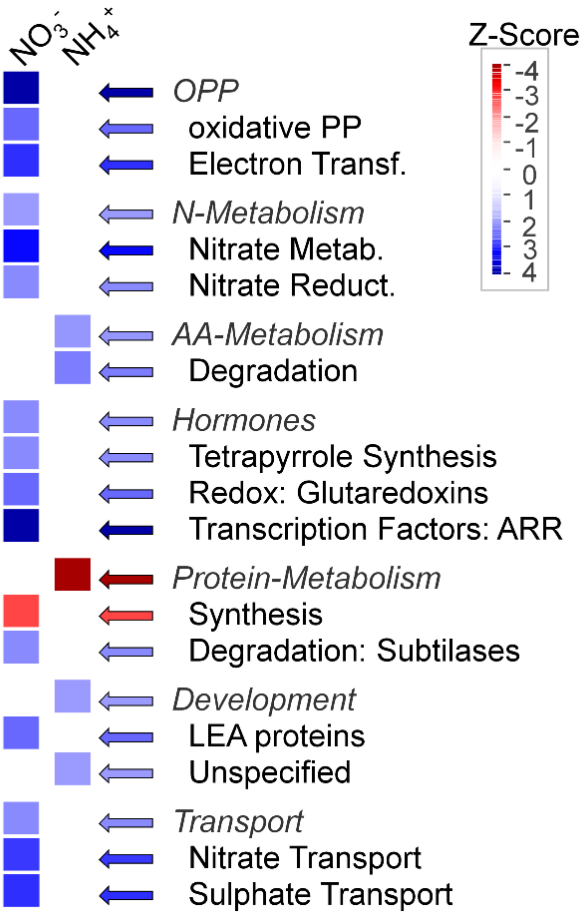

Figure 3-8: Adaptation towards different N-forms

Over-represented gene categories of plant roots adapted for 5 days to ammonium or nitrate, respectively. Conducted with Fisher's exact test and Benjamini-Hochberg corrected p-values which were transformed to Z-scores (*e.g.* p = 0.05 ≙ Z = 1.96)

3.2.3 Transcriptional adjustments to nitrate and ammonium deprivation

76 genes in nitrate-adapted roots were at least 2-fold up- or down-regulated ($log_2FC > 1$ or < -1, FDR < 0.05) 15 min. after transfer of the roots to nitrogen-free nutrient solution. However, when these 76 responsive genes were filtered for transcripts also responsive in ammonium-adapted roots and for the mechanical stress induced by the transfer of the roots to a new pot (i.e. they were also regulated in the 15 min. controls), only 22 unique genes remained (Figure 3-9). These were enriched with calcium- and ethylene-signaling genes and most were only transiently regulated, as no significant expression difference for these genes was detected after three hours of N-deprivation. Only six of these 22 genes were down-regulated and continued to decrease by 3 hours of nitrate deprivation. These six genes are likely the earliest genes specifically responsive to nitrate deprivation (Table 3-1).

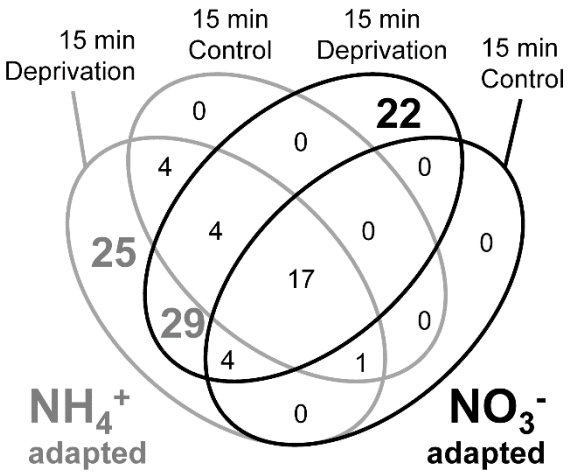

Figure 3-9: Venn diagram of early responsive genes (15 min) in –N and in controls

Venn diagram of significantly differentially expressed genes (FDR<0.05, log2FC > 1 or < -1 to detect very early changes) of ammonium and nitrate adapted plants after 15 min in nitrogen deprivation or the respective control treatment. Boldfaced, colored numbers are of special interest.

A transcript coding for the small auxin-responsive protein SAUR6 *(At2g21210)* showed the highest, 11-fold down-regulation (log_2-FC = -3.5). Transcripts coding for two members of the *LATERAL ORGAN BOUNDARY DOMAIN*-containing protein-family, *LBD37 (At5g67420)* and *LBD38 (At3g49940),* were also down-regulated. These transcription factors are well-known nitrate-responsive negative regulators of the anthocyanin synthesis and the nitrate assimilation pathway (Rubin et al., 2009). Further strongly down-regulated transcripts encoded a mitochondrial substrate carrier *(At5g26200)* and an unknown protein *(At5g19970).* Most of these transcripts were predominating with nitrate as exclusive N-source, confirming their strong dependency on nitrate. Their rapid down-regulation of expression was confirmed by *qRT-PCR* (Figure 3-10). The *qRT-PCR* confirmed also the immediate down-regulation of the GARP-transcription factors *HRS1 (At1g13300)* and its homologue *HHO1 (At3g25790)* which were slightly above the set significance threshold. Both were recently identified as integrators of the N and P-status and are usually rapidly induced with nitrate supply (Medici et al., 2015). A large number of transcripts was differentially expressed after 3 h in nitrate-deprived solution (Figure 3-11, Table 3-1). After filtering for unspecific (and diurnal) transcripts by the use of the control-treatment (Figure 3-11), a list of 60 specific down-regulated genes and only four up-regulated transcripts

remained. Among the up-regulated transcripts, the most responsive one encoded an unknown protein *(At4g39795)*. This gene was predominantly expressed in ammonium-adapted plants and was up-regulated under nitrate-deprivation, but was suppressed by nitrate. Similar observations were made for the high-affinity nitrate transporter 2.5 transcript (*NRT2.5, At1g12940*), which was the second strongest up-regulated transcript after 3 h of nitrate-deprivation. *NRT2.5* is constitutively expressed in roots, particularly under limiting nitrate (Orsel, 2002; Okamoto, 2003). This is in agreement with the finding that this gene was 32-fold higher expressed in ammonium-adapted plants, compared to nitrate-adapted plants (Appendix: Table A-1). The 60 specifically down-regulated transcripts were over-represented in the OPPP-genes *(At5g13110, At1g24280, At1g64190, At4g05390, At5g41670, At3g60750),* genes involved in nitrate metabolism and transport (*At2g15620, At1g77760),* nitrite transport (*At5G62720*), transcripts involved in other solutes transport (e.g. boron transporter *BOR1*, sulfate transporters *SULTR1.3* and *3.5*), as well as developmental genes. Interestingly, the transcript levels of three members of the recently identified *CLE*-family (*CLAVATA3/ESR*-related) signaling pep tides, *CLE-1, CLE-7* and *CLE-4 (AT1G73165, At2g31082, At2g31081),* were significantly decreased (-1.95, -2.2 and -3.9 log_2-fold, respectively) at 3 hours of nitrate deprivation. This is surprising, as particularly these members of the *CLE*-family were previously found to be in-

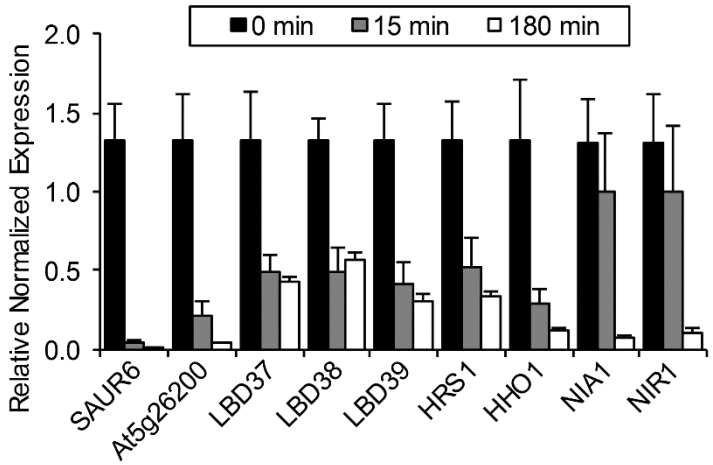

Figure 3-10: qRT-PCR validation of key genes

Gene Expression of selected earliest nitrate-depletion responsive transcripts validated with *qRT-PCR*. Error Bars indicate Standard Error of Mean (n=3). At5g26200: Mitochondrial Substrate Carrier

duced in nitrogen-depleted conditions (Araya et al., 2014). In contrast, *CLE-3* (*At1g0622*) was very low expressed in the presence of nitrate, but highly abundant under ammonium nutrition.

A homolog of the rapidly responding *LBD37*, *LBD39,* also had reduced expression levels at 3 h of nitrate deprivation. Furthermore*, HRS1* and *HHO1* were 5-fold and 13-fold down-regulated, respectively. Two of the major nitrate assimilation genes, the nitrate reductase *NIA1 (At1g77760)* and nitrite reductase *NiR (At2g15620),* were also among the strongest down-regulated transcripts, 13- and 9-fold, respectively. In contrast, none of the *NLP7*-related *NIN-like transcription factor* genes were changed in expression levels over the measured time-period*. RT-PCR* analyses of regulated genes confirmed the strong early down-regulation of *HRS1/HHO1*, although these genes were not yet significantly down-regulated in the microarrays at 15 min. (Figure 3-10).

Figure 3-11: Visualization of gene expression results
Gene expression ratios versus time point 0 of nitrate and ammonium adapted roots after 15 min and 3 hours in N-depleted medium (-N) and the respective control-treatment (C) in which plants were placed into fresh medium containing ammonium or nitrate Only genes which were significantly (*FDR*<0.05) differentially expressed in the –N-treatment and genes with a $\log_2$ fold-change < -0.5 or > 0.5 were compared with the ratio in the control-treatment and were sorted from lowest to the highest value. Of special interest were those transcripts which were stably expressed in the control-treatment (White spaces at C of each column). Most of these N-depletion responsive genes are listed in Table 3-1.

Table 3-1: N-Form specific early differentially expressed genes to -N

N-Form specific early responsive transcripts (LFC > 1 and < -1 for 15 min or > 1.5 and < -1.5 for 180 min) filtered for control conditions. Significant (*FDR*<0.05) changes versus time point 0 are boldfaced. Asterisks mark transcripts which respond also at mid-/long-term N-depletion conditions (Krapp et al., 2011). Transcripts which belong to the "Top 50" most consistent nitrate responsive genes (Canales et al., 2014) are shown in italic letters. Positive values in column "NO_3^- vs. NH_4^+ 0 min" indicate the gene is up-regulated under nitrate-adapted conditions while negative values show an up-regulation under ammonium-adapted conditions.

AGI Locus ID	Transcript Description	Log$_2$-fold change (LFC)				
		NO_3^- vs. NH_4^+ 0 min	NO_3^- -adapted		NH_4^+-adapted	
			15 min	180 min	15 min	180 min
Nitrate adapted plants: earliest responsive transcripts (15 min)						
At2g21210.1*	SAUR-like auxin-responsive protein, SAUR6	5.04	**-3.50**	**-4.69**	0.08	-0.24
At5g26200.1*	Mitochondrial substrate carrier family protein	6.53	**-2.30**	**-4.91**	0.01	1.25
*At5g67420.1**	*LOB domain-containing protein 37, LBD37*	2.56	**-2.01**	**-2.45**	0.39	-0.48
At3g49940.1	*LOB domain-containing protein 38, LBD38*	-0.67	**-1.31**	**-1.45**	0.03	**-1.68**
*At5g19970.1**	*Unknown protein*	0.74	**-1.42**	**-2.10**	-0.27	**-1.24**
At1g13245.1	ROTUNDIFOLIA like 17	-0.02	**-1.10**	**-0.79**	0.38	-0.41
Nitrate adapted plants: responsive transcripts after 180 min in N-depleted solution						
At5g26200.1*	Mitochondrial substrate carrier family protein	6.53	**-2.30**	**-4.91**	0.01	1.25
*At5g01740.1**	*Nuclear transport factor 2, NTF2*	6.45	**-1.27**	**-4.83**	0.25	0.21
At2g21210.1*	SAUR-like auxin-responsive protein, SAUR6	5.04	**-3.50**	**-4.69**	0.08	-0.24
At2g31081.1*	CLAVATA3/ESR-RELATED 4	4.48	-0.62	**-3.90**	0.08	-0.15
*At3g25790.1**	*MYB-like transcription factor, HHO1*	5.51	-2.01	**-3.73**	-0.09	-0.24
*At1g77760.1**	*Nitrate reductase 1, NIA1*	6.49	-0.20	**-3.71**	-0.02	**1.17**
*At2g15620.1**	*Nitrite reductase 1, NIR1*	3.56	-0.34	**-3.24**	0.17	0.15
*At1g24280.1**	*Glucose-6-phosphate dehydrogenase 3*	2.74	-0.39	**-3.17**	0.01	-0.28
At2g22122.1*	Unknown protein	5.11	-0.20	**-2.87**	0.28	0.20
At1g02820.1*	Late embryogenesis abundant 3, LEA3	3.13	-0.33	**-2.75**	0.44	0.03
*At1g80380.2**	*P-loop containing nucleoside triphosphate hydro-lase*	3.18	-0.47	**-2.59**	0.76	0.59
At3g19030.1	Unknown protein	0.97	-0.84	**-2.57**	0.91	-1.36
At3g46880.1	Unknown protein	2.72	-0.29	**-2.54**	0.14	-0.37
*At4g02380.1**	*Senescence-associated gene 21*	2.95	-0.30	**-2.53**	0.60	0.08
At5g63160.1*	BTB and TAZ domain protein 1	4.06	-1.19	**-2.53**	1.14	0.51
At5g52790.1*	Unknown protein	3.71	-0.35	**-2.48**	0.05	-0.12
At3g02850.1	*STELAR K+ outward rectifier*	3.22	-0.35	**-2.45**	0.03	0.08
*At5g67420.1**	*LOB domain-containing protein 37, LBD37*	2.56	**-2.01**	**-2.45**	0.39	-0.48
At5g07680.1*	NAC domain containing protein 80	2.56	-0.52	**-2.41**	-0.14	-0.33
At1g22150.1	Sulfate transporter 1;3	1.34	-0.29	**-2.41**	-0.01	-1.14
At4g29905.1*	Unknown protein	4.07	-0.72	**-2.33**	0.35	-0.89
At1g68238.1	Unknown protein	4.02	-0.55	**-2.31**	0.19	-0.14
At1g13300.1	*MYB-like transcription factor, HRS1*	4.06	-1.17	**-2.29**	-0.04	0.08
At4g13620.1	Integrase-type DNA-binding superfamily protein	1.70	-0.98	**-2.26**	0.01	-1.33
*At2g26980.4**	*CBL-interacting protein kinase 3*	2.62	-0.47	**-2.25**	-0.05	-0.13
At5g62430.1	Cycling DOF factor 1	1.47	-0.25	**-2.20**	0.05	**-1.15**
*At5g10210.1**	*Unknown Protein*	5.69	-1.32	**-2.20**	1.35	1.37
*At5g62720.1**	*Nitrite transporter Nitr2.1*	3.53	-0.32	**-2.19**	0.11	0.33
At2g31082.1	CLAVATA3/ESR-RELATED 7	2.55	-0.31	**-2.19**	0.21	-0.07
At3g30415.1	Putative urophorphyrin III methylase	2.05	-0.46	**-2.12**	-0.01	-0.26
At3g57157.1	other RNA	1.06	-0.79	**-2.11**	-0.17	-0.97

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*At5g19970.1**	*Unknown protein*	0.74	-1.42	**-2.10**	-0.27	**-1.24**
At5g65980.1	Auxin efflux carrier family protein (AtPILS7)	1.23	-0.50	**-2.09**	0.16	-0.68
At1g78050.1	*phosphoglycerate/bisphosphoglycerate mutase (PGM)*	1.69	-0.54	**-2.04**	0.13	-0.06
At5g19600.1*	Sulfate transporter 3;5	3.40	-0.14	**-2.01**	-0.44	-1.04
*At3g16560.1**	*Protein phosphatase 2C family protein*	1.77	-0.59	**-2.01**	0.10	-0.49
*At5g41670.1**	*6-phosphogluconate dehydrogenase family protein*	1.64	-0.41	**-2.00**	0.10	-0.10
*At5g13110.1**	*Glucose-6-phosphate dehydrogenase 2 (G6PD2)*	1.79	-0.34	**-1.97**	-0.04	-0.13
At5g40850.1	*Urophorphyrin methylase 1*	1.83	-0.42	**-1.95**	0.30	0.06
At1g73165.1	CLAVATA3/ESR-RELATED 1	2.59	-0.32	**-1.95**	-0.13	-0.04
*At4g37540.1**	*LOB domain-containing protein 39, LBD39*	1.50	-1.31	**-1.94**	0.20	-1.21
At3g60750.1	*Transketolase*	1.37	-0.28	**-1.93**	-0.18	-0.37
At3g63110.1*	Isopentenyltransferase 3	2.71	-0.41	**-1.89**	0.08	-0.25
At3g30405.1	Transposable element gene	2.00	-0.30	**-1.80**	0.12	0.08
At2g33550.1	Homeodomain-like superfamily protein	1.92	-0.43	**-1.77**	-0.02	-0.13
At1g63940.2	Monodehydroascorbate reductase 6	1.65	-0.15	**-1.76**	-0.11	-0.24
At1g16170.1	*Unknown protein*	1.95	-0.38	**-1.75**	0.04	-0.09
At4g25835.1	*P-loop containing nucleoside triphosphate hydrolases superfamily protein*	1.46	-0.66	**-1.74**	-0.08	-0.57
At1g70780.1	Unknown protein	1.75	-0.24	**-1.73**	-0.17	-0.15
*At1g49500.1**	*G2-like transcription factor family protein*	3.84	-0.90	**-1.65**	0.28	0.13
At4g05390.1	*Root FNR 1*	1.35	-0.19	**-1.60**	-0.07	-0.18
At1g64190.1	6-phosphogluconate dehydrogenase	1.54	-0.29	**-1.59**	-0.10	-0.20
At2g47160.2*	Boron Transporter (BOR1)	1.99	-0.34	**-1.59**	-0.17	0.89
At4g03500.1	Ankyrin repeat family protein	2.23	-0.42	**-1.58**	0.18	0.23
At3g50660.1	Cytochrome P450 superfamily protein	1.43	-0.55	**-1.58**	-0.11	-0.11
At4g29990.1	LRR-transmembrane protein kinase	1.84	-0.05	**-1.56**	-0.01	-0.22
At2g25090.1	CBL-interacting protein kinase 16	1.25	-0.19	**-1.55**	0.49	-0.32
At5g19040.1*	Isopentenyltransferase 5	2.05	-0.56	**-1.54**	-0.12	-0.31
At3g15650.2	alpha/beta-Hydrolases superfamily protein	1.87	-0.07	**-1.54**	0.05	0.39
At4g25190.2	Unknown protein	0.82	-0.61	**-1.51**	0.53	-0.18
At3g22370.1*	Alternative oxidase 1A	-1.20	-0.04	**1.53**	0.17	0.50
At2g03570.1	Unknown protein	-0.02	0.49	**1.73**	-0.55	1.01
At1g12940.1*	Nitrate transporter 2.5	-5.03	-0.15	**1.73**	0.36	1.02
At4g39795.1*	Unknown Protein	-3.46	-0.07	**1.75**	0.03	-0.16
Ammonium adapted plants: earliest responsive transcripts (15 min)						
At3g15870.1	Fatty acid desaturase family protein	0.45	0.14	-0.18	**1.42**	0.18
At5g66730.1	C2H2-like zinc finger protein	-0.06	0.60	0.31	**1.29**	0.21
At1g21281.1	Unknown protein	0.08	0.22	-0.01	**1.15**	-0.02
At3g52010.1	serine carboxypeptidase-like 37	-0.48	0.98	0.31	**1.48**	-0.53
Ammonium adapted plants: responsive transcripts after 180 min in N-depleted solution						
At3g49940.1	*LOB domain-containing protein 38, LBD38*	-0.67	**-1.31**	**-1.45**	0.03	**-1.68**
At1g77160.1	Unknown Protein	-0.26	0.15	0.91	0.14	**1.58**
At5g66110.1	Heavy metal transport/detoxification superfamily	-0.18	0.11	0.15	-0.28	**1.79**

3.2.4 Nitrate depletion response in comparison with other studies

The list of transcripts with a significant response to 3 h nitrate deprivation was then compared to the “top 50 list” of nitrate responsive transcripts recently defined in a meta-analysis of various nitrate-resupply transcriptome experiments (Canales et al., 2014) . As expected, there was a high overlap of nitrate supply responsive and nitrate deprivation responsive genes. A total of

27 nitrate-deprivation responsive transcripts were also found in the “top 50 list” of nitrate-responsive genes. These were found to be regulated in the opposite direction compared to what was found in nitrate-resupply experiments (*italic letters* in Table 3-1, Figure 3-12). However, about half of the genes responsive to nitrate deprivation were not found among the top 50 genes induced by nitrate. This indicates that nitrate resupply and nitrate deprivation are not exactly opposite transcriptional responses, but involve further genes.

Furthermore, 30 genes that were transcriptionally repressed at 3 h of nitrate deprivation overlapped with transcripts that were repressed under intermediate and long-term acclimation to nitrogen starvation in roots (Krapp et al., 2011). Of only four genes that were up-regulated after 3 h of nitrate deprivation, three genes (*At4g39795, At1g12940* and *At3g22370)* were still up-regulated in intermediate and long-term response to N-starvation (asterisks in Table 3-1, Appendix: Figure A-0-1).

Under conditions of ammonium deprivation, 84 transcripts were at least 2-fold up- or down-regulated after 15 minutes ($log_2FC > 1, < -1$, FDR < 0.05). However, after clearance for unspecific genes, only 25 transcripts remained (Figure 3-11, Figure 3-9), which were mostly also found transiently regulated in the controls (see Materials & Methods). Only 4 transiently up-regulated genes remained specifically responsive to short-term ammonium deprivation (Table 3-1). Neglecting diurnally regulated transcripts that were also regulated in control datasets, only two up-regulated genes and one down-regulated gene remained significantly changed after three hours of ammonium deprivation. Thus, on a global scale, gene expression in ammonium-adapted roots was not responsive to the depletion of external ammonium.

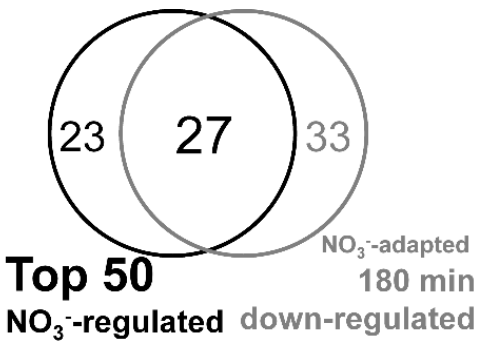


Figure 3-12: Comparison with “Top-50” nitrate responsive genes

Comparison of Top 50 most consistent nitrate responsive genes (Canales et al., 2014) with the down-regulated transcripts of our experiment with nitrate adapted plants after 3 hours in N-deprivation ($log_2FC < -1.5$, Control-$log_2FC < -1$).

3.2.5 N-depletion response in Tsu-0 verified the Col-0 results

As mentioned above (Chapter 3.2.2), the N-depletion experiment and the transcriptome analysis was conducted identically with the ecotype Tsu-0. Early nitrate depletion led also in Tsu-0 already after 15 min. to a rapid decrease of transcript levels of *SAUR6* (-3.47 Log_2-fold) and the *mitochondrial substrate carrier* (-2.2 Log_2-fold). Correspondingly, transcript levels of both, *LBD37* and *LBD39*, were significantly decreasing within 15 min. (-2 and -1.6 Log_2-fold). Up-regulated genes (after filtering for control-genes) were also in Tsu-0 transient and in consequence apparently absent. Thus, the earliest nitrate-depletion responsive genes were confirmed by the findings in Tsu-0. After 3 hours in nitrate depletion the response was highly similar to Col-0 (Figure 3-13, Appendix: Table A-2).

The majority of genes (80 of 103 with a Log_2-FC > 1.5 or < -1.5, FDR< 0.05, Controls > -1 and < 1) were down-regulated. More genes were regulated in comparison with Col-0. Among the

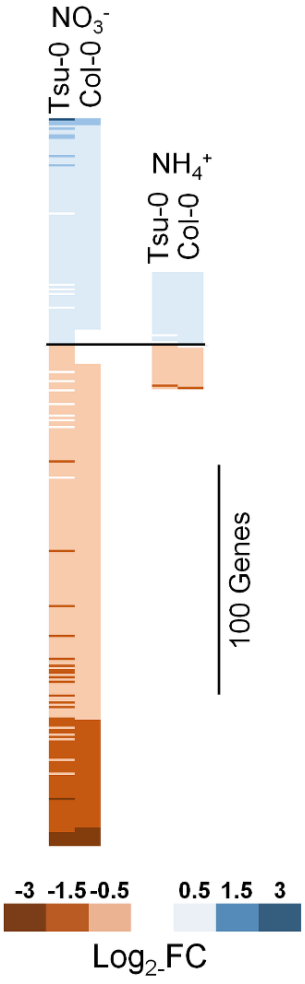


Figure 3-13: Differentially expressed genes in Tsu-0 and Col-0 after 3 h in -N

Each column shows all significant (*FDR*<0.05) differentially expressed transcripts after 3 h in -N in the contrasting ecotypes Tsu-0 and Col-0 which were previously adapted to nitrate (NO_3^-) or ammonium (NH_4^+), respectively. Genes of the Col-0 genotype were sorted by their Log_2-FC value in descending order. Additionally, genes were filtered for control treatments: genes with a Log_2-FC > 1 and < -1 in any of the respective control treatments were neglected. Note that more genes were stronger up- and also down-regulated in nitrate adapted Tsu-0 roots.

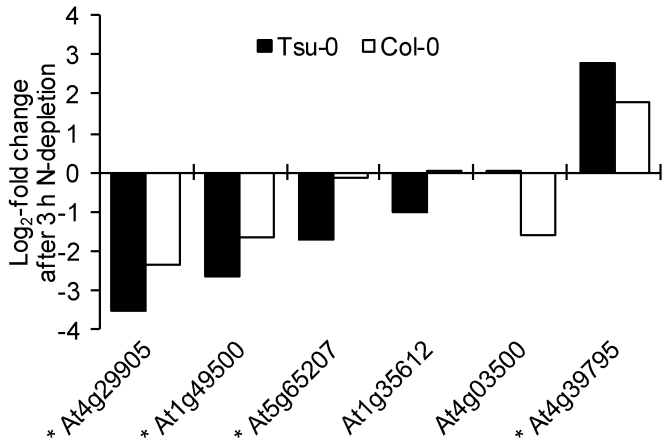


Figure 3-14: Ecotype differences after 3 h nitrate depletion

Significant (*FDR*<0.05) in at least one (Tsu-0 or Col-0) LFC (3h $-NO_3^-$ vs. NO_3^- 0h) and in controls LFC < 1 and > -1.

Unknown protein coding *mRNAs* are marked with an asterisk (*).

At1g35612: transposable element

At4g03500: Ankyrin repeat family protein

few up-regulated transcripts the cation/H^+ exchanger *CHX17* (*At4g23700*) transcript was surprisingly the strongest up-regulated gene (3.5 log_2-fold). This gene was regulated in Col-0 as well, but slightly above the used significance threshold (FDR<0.05). In addition, also its homologue *CHX18* (*At5g41610*) was up-regulated with N-deprivation in nitrate adapted Tsu-0. Also several non-annotated proteins, a potassium transporter and the constitutively regulated nitrate transporter *NRT2.5* were higher expressed after three hours. A few significant genes coding for an ankyrin protein, unknown proteins or transposable elements differed in the rate in which they were up- or down-regulated (Figure 3-14).

In ammonium adapted Tsu-0 a very similar observation to Col-0 was made: only two transcripts were down-regulated after 3 hours. The transcripts of Beta-Amylase 3 (*At5g18670*) and the *AtCIR1* (*At5g37260*, a myb-transcription factor involved in circadian regulation) were significantly decreased within the course of 3 hours (-1.54 and -1.78 Log_2-fold, respectively). *LBD38*, which was the sole down-regulated transcript in Col-0, was to a lesser extent (-1.43 Log_2-fold) also significantly down-regulated in Tsu-0. Hence, the observation of an almost absent response towards ammonium deprivation was confirmed with the observations of the Tsu-0 transcriptome.

3.2.6 Early nitrate-depletion overrides NLP7 promoted gene expression

Due to the rapid down-regulation of numerous genes involved in transcriptional regulation (*LBDs, HRS1, HHO1, etc.*), nitrate uptake and assimilation during early nitrate depletion it is

expectable that a major transcriptional regulator, *e.g.* a transcription factor, could be involved in the depletion response of nitrate adapted roots. In the recent years the transcription factor NLP7 was identified as a key regulator of the physiological adaptation towards nitrate in *Arabidopsis* and its regulatory function was positioned upstream the LBDs and HRS1/HHO1 transcription factors (Castaings et al., 2009; Konishi and Yanagisawa, 2013; Marchive et al., 2013; Vidal et al., 2015). NLP7 protein is rapidly transferred to the nucleus to directly activate target genes upon nitrate resupply. Thus, it was hypothesized that a rapid nuclear release or degradation of NLP7 in response to the withdrawal of nitrate leads to the down-regulation of these genes. Its loss might not only lead to reduced steady state activation of nitrate-induced genes, but also different time course of the response to nitrate withdrawal. Hence, in plants lacking NLP7, the N-depletion response should differ. This was tested using the two T-DNA insertion lines *nlp7-1*, *nlp7-3* (Castaings et al., 2009), and the wild-type Col-8 (thanks to Anne Krapp, INRA France) which were grown identically to previous experiments in hydroponic culture with a 5-day adaptation to nitrate and a 15 min and 3 h –N treatment.

Due to the repression of the NLP7 transcription factor in the mutants, differences in the overall gene expression of nitrate- and NLP7-dependent genes and a delayed down-regulation of the early N-depletion responsive genes were expected. With *qRT-PCR* the gene expression of several early down-regulated genes during early nitrate depletion and the absence of *NLP7*-transcript were tested in the *nlp7*-mutants (Figure 3-15). The overall gene-expression of the *LBD37-39* transcription factors, *HRS1* and the nitrate reductase gene *NIA1* were clearly reduced in the mutants already before onset of N-depletion. Also the earliest responsive genes, *SAUR6* and the *mitochondrial substrate carrier (MSC)*, which were not shown to be NLP7-dependent yet, were considerably down-regulated in both mutant-lines. The difference between the expression in Col-8 and the mutants can be considered as the NLP7 regulated component. This component was missing in nitrite reductase *NIR* and the *HRS1* homologue *HHO1*, which both exhibited almost identical expression to the wild-type Col-8.

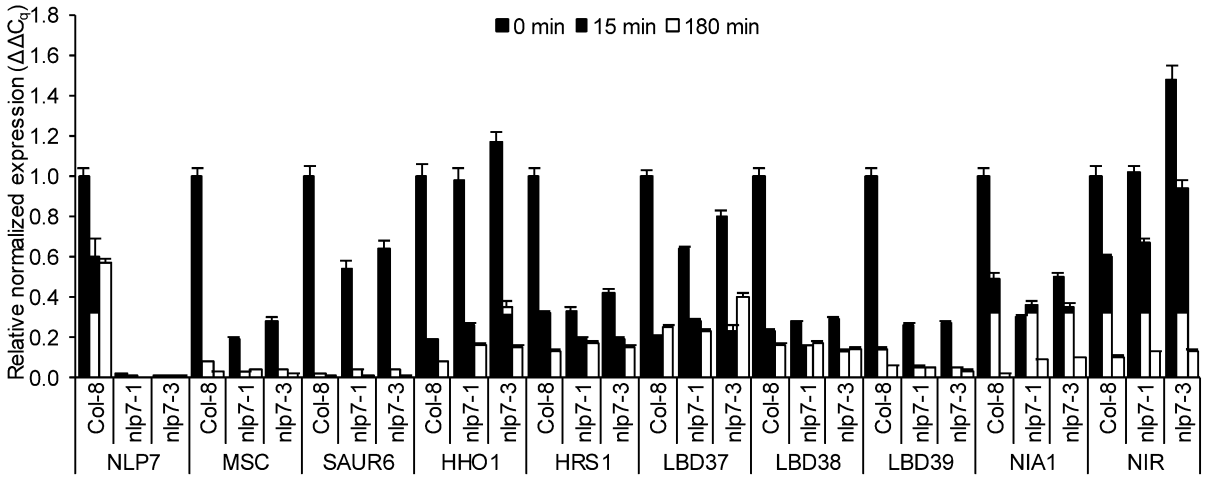


Figure 3-15: qRT-PCR of nitrate depletion responsive genes in *nlp7* mutants

The expression values are relative to control genotype Col-8 at time-point 0. Error bars indicate standard error of mean (n=3). RNA was extracted from 3 pooled roots of the N-depletion experiment. Plants were harvested at 0, 15 min, and 180 min after onset of N-depletion. Due to the genetic background of both nlp7-mutants, Col-8 plants were taken as reference genotype. MSC: mitochondrial substrate carrier.

The time-resolved N-depletion response of the respective genes in *nlp7*-mutants was largely identical to the wild-type. Already after 15 minutes in –N the expression of all tested transcripts was highly reduced and remained in most instances also within the 3 hours on a basal level. Although transcript abundances were in most genes in the *nlp7* mutants already decreased, they aligned with withdrawal of nitrate to the same levels as detected in the wild-type.

Considering the high similarity between the *nlp7*-mutants and the wild-type, the N-depletion response can be regarded (for these genes) as NLP7-independent. Interestingly, also *NLP7* expression was declining in response to N-depletion in the wild-type Col-8. This was not observed in the microarray data. Additionally, *NLP7* expression in Col-0 and Tsu-0 was in accordance to previous findings (Konishi and Yanagisawa, 2013) stable in –N treatments (data not shown) or in plants which were adapted to ammonium (Figure 3-7).

3.2.7 Nitrate-depletion accelerates transcript degradation

Due to the rapid drop of mRNA abundance in many genes upon nitrate withdrawal, we hypothesized that this results from an active process such as *mi*RNA dependent transcript degradation. The rapid decay of some genes, *e.g. HRS1* and *NIR,* contrasted their known *m*RNA stability in cell suspension culture under constant nitrate supply (Narsai et al., 2007). Hence, their transcript stability was tested after incubation of the root with actinomycin-D (ActD), a transcriptional inhibitor. Most studies with *Arabidopsis thaliana* in which actinomycin-D was utilized, used young (1-3 weeks) plantlets grown on solid medium or in liquid culture (Johnson et al., 2000; Ali et al., 2003; Narsai et al., 2007; Schult et al., 2007). It is unclear, how effective an ActD treatment is in adult plants (5 weeks old), especially in roots grown in hydroponic culture and when applied dissolved in the hydroponic solution. The roots were incubated for 1 hour in nitrate-containing (3 mM) Hoagland's with a ActD concentration of 100 µg/ml. The concentration was thereby in the upper range of commonly used concentrations plants were

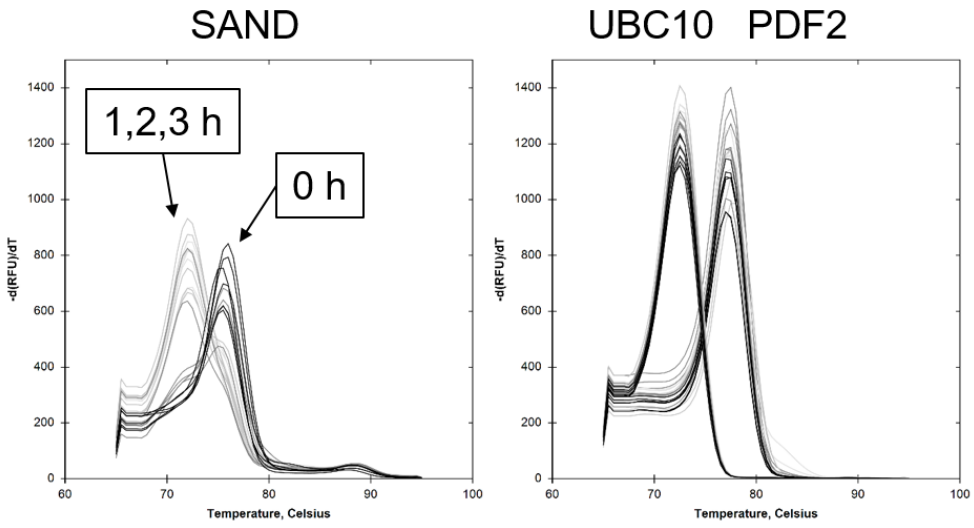


Figure 3-16: Melt peaks of qRT-PCR reference genes after ActD treatment

ActD inhibits transcription efficiently in *Arabidopsis* roots. Note the shift of melt peaks from 0 h (black lines) to 1-3 h after ActD treatment (grey lines) in the reference gene SAND with a very short mRNA half-life ($T_{½}$ = 2.39, Narsai et al., 2007). This change in the PCR product indicates a degradation of the SAND transcript in the samples and an inhibited *de novo* transcription due to the ActD treatment. In comparison, reference genes UBC10 (left peaks) and PDF2 (right peaks) have a high mRNA half-life ($T_{½}$ ~ 10 h). Even after 3 hours their stability results a stable PCR product.

treated with (50-100 µg/ml). After treatment, incubated plant parts were stained red due to the intense red color of the chemical. The staining remained also after several rinse steps in $CaSO_4$ (1mM), indicating that the roots absorbed the ActD from the nutrient solution.

By *qRT-PCR*, changes in transcript abundance due to nitrate depletion in the ActD treated plants were analyzed. Surprisingly, transcripts with very short half-lives, such as the initially used reference gene *SAND*, decayed rapidly, validating that the chemical was inhibiting transcription (Figure 3-16). Consequently, *SAND* was replaced by the reference gene *UBQ10*, which had similar as *PDF2*, a high *mRNA* half-life of approximately 10 hours (Narsai et al., 2007). Several genes were chosen being representative for different half-lives and were tested in the presence of ActD. The transcription factor gene *HRS1* (> 3 h) with an intermediate and nitrite reductase *NiR* with a rather stable (> 6 h) transcript half-life (Narsai et al., 2007). Upon nitrate deprivation, both transcripts declined rapidly (Figure 3-17). In contrast, both transcripts were remarkably stable in the presence of nitrate, similar as in previous studies with constant nitrate supply in cell cultures (Narsai et al., 2007).

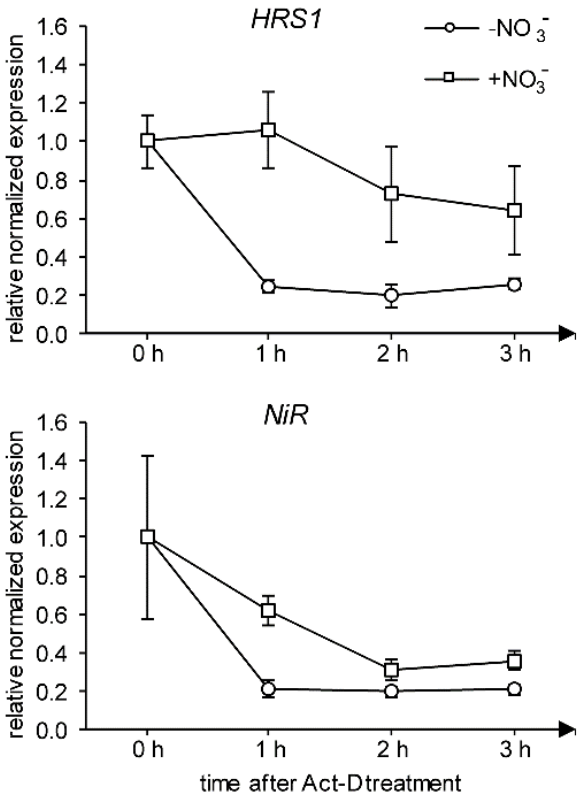


Figure 3-17: NO_3^--dependent *mRNA* decay
mRNA decay in actinomycin-treated roots for *HRS1* (top) and *NIR1* (bottom) in the presence (squares) and absence (circles) of nitrate. Error bars show SD, derived from two experimental replications.

3.2.8 Concluding remarks to the transcriptome analysis

Conclusively, the observed nitrate-depletion response was shown independently in three different genotypes (*Col-0, Col-8, Tsu-0*) and the two *nlp7* mutant lines, which all were previously adapted to nitrate. Given that NLP7 plays only a minor role in the early depletion response, the rapid *m*RNA decline depends on another, unknown mechanism. A putative signal of the external drop of the nitrate concentration is translated into the observed transcriptional response. Nitrate depletion has either a negative effect on *mRNA* stability/turnover or several transcripts are actively degraded upon nitrate depletion, *e.g.* by *miRNA* dependent regulation. In contrast, external drop of the ammonium concentration led in the measured time-period and in two contrasting genotypes to a rather unspecific, almost absent transcriptome response.

3.3 Proteome Analysis of the Nitrogen Depletion Response

Since transcriptional differences do not necessarily reflect the same differences in functional proteins, and protein modifications are involved in the perception and coordination of the nitrogen deficiency status, the proteomic changes in the roots of the Col-0 genotype were also analyzed at the same time points. In addition, these protein samples were enriched for phospho-peptides to identify posttranslational modifications in nitrogen deprivation induced signaling cascades. The proteome analysis was conducted in collaboration with Prof. Dr. Waltraud Schulze and Zhi Li of the Plant Systems Biology Group of the Institute of Plant Physiology and Biotechnology (260).

3.3.1 Adaptations to Nitrate and Ammonium on Proteome Level

About 20.000 peptide sequences were identified, which represent 5341 unique protein sequences (Figure 3-18). 4831 proteins were identified in ammonium as well as nitrate-adapted plants. Only very few proteins were specifically identified at a certain time point of nitrogen deprivation (Figure 3-18), indicating good coverage during proteome analysis.

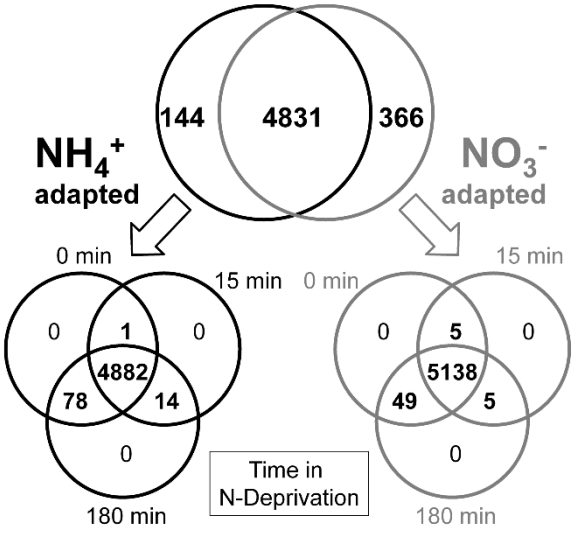


Figure 3-18: Distribution of identified proteins in the samples

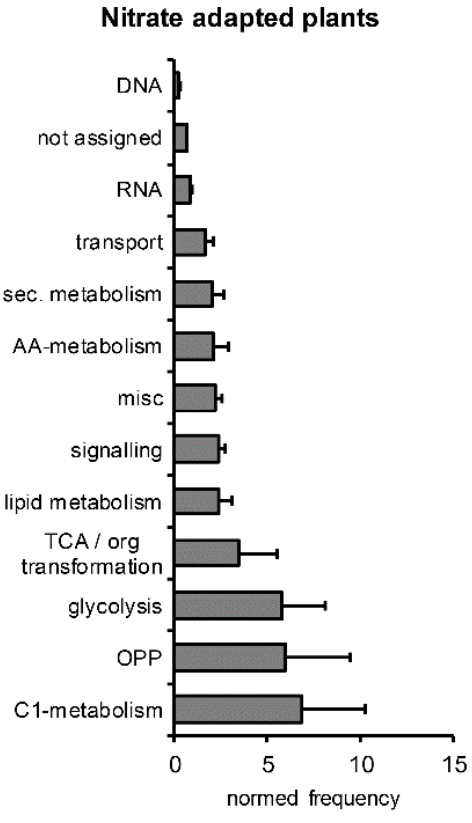
Number of common and specific protein sequences were further separated into time-points of N-deprivation treatment.

144 proteins were specifically identified in ammonium-adapted plants (Figure 3-18), with an over-representation of proteins involved in amino acid metabolism, transport, or nucleotide metabolism (Figure 3-19). Among transport proteins, Nitrate-transporter 2.5 (AT1G12940), Cation exchanger CHX18 (AT5G41610), Aquaporin PIP1.4 (AT4G00430) and the K^+-transporters HAK5 (AT4G13420) and a K^+ Efflux antiporter (AT5G51710) were exclusively identified in ammonium-adapted plants.

366 proteins were only identified in nitrate-adapted roots (Figure 3-18), with an over-representation of C1-metabolism, OPP-pathway, glycolysis, tricarboxylic acid pathway, lipid-metabolism, transport, signaling and *RNA*-related processes (Figure 3-19). Nitrate reductase NIA1 (AT1G77760) was exclusively detected in the nitrate-adapted plants. The root tip located auxin efflux-carrier proteins, PIN3 (AT1G70940) and PIN4 (AT2G01420), were also specifically detected in nitrate-adapted plants, potentially reflecting the stimulatory effect of nitrate on root growth. At 15 minutes of nitrate-deprivation, only 3 proteins were found with a larger than 2-fold decrease in protein abundance. These include jasmonate responsive AT3G16470, calmodulin-like AtCAM3 (AT3G56800) and an unknown protein (AT2G22795). In contrast, two of those aforementioned proteins (AtCAM3 and AT3G16470) were found to be rapidly significantly up-regulated by ammonium deprivation.

In general, 15 minutes of ammonium deprivation led to a larger, but transient protein abundance changes than found under nitrate deprivation (Figure 3-20). Ammonium deprivation resulted in larger than 2-fold abundance changes of 64 proteins, 23 being up- and 41 down-regulated. These were over-represented in calcium signaling, hormone metabolism, tetrapyrrole synthesis, mitochondrial electron transport, N-metabolism and nucleotide-metabolism.

Surprisingly, not one protein with a significant, more than two-fold abundance change at 3 h of N-deprivation was detected, irrespective of the nitrogen-form the plants were adapted to. The differential protein abundance changes observed at 15 minutes of N-deprivation must therefore reflect transient changes, likely overlapped by transient responses to the mechanical stimulus of root transfer to the new medium. Despite the immediate and profound transcriptomic repression of nitrate-responsive genes after nitrate-deprivation (see above), little changes in abundance of the proteins (e.g. NIA) encoded by strongly repressed genes were identified, indicating substantial protein stability.



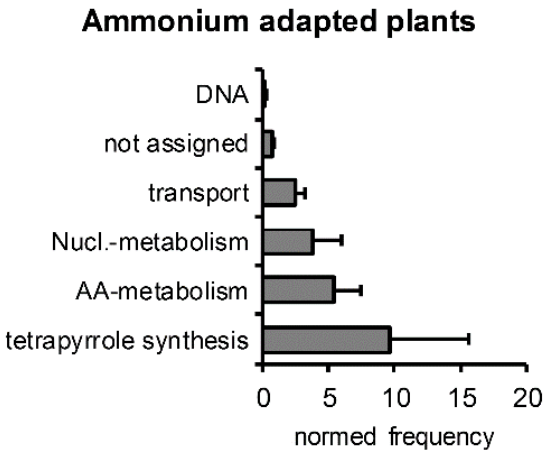


Figure 3-19: Overrepresented functional MapMan bins

Significantly ($p<0.05$) over represented MapMan bins of proteins which were identified either in nitrate or ammonium adapted roots (*cf.* Figure 3-18) (derived from BAR Classification Super Viewer Tool)

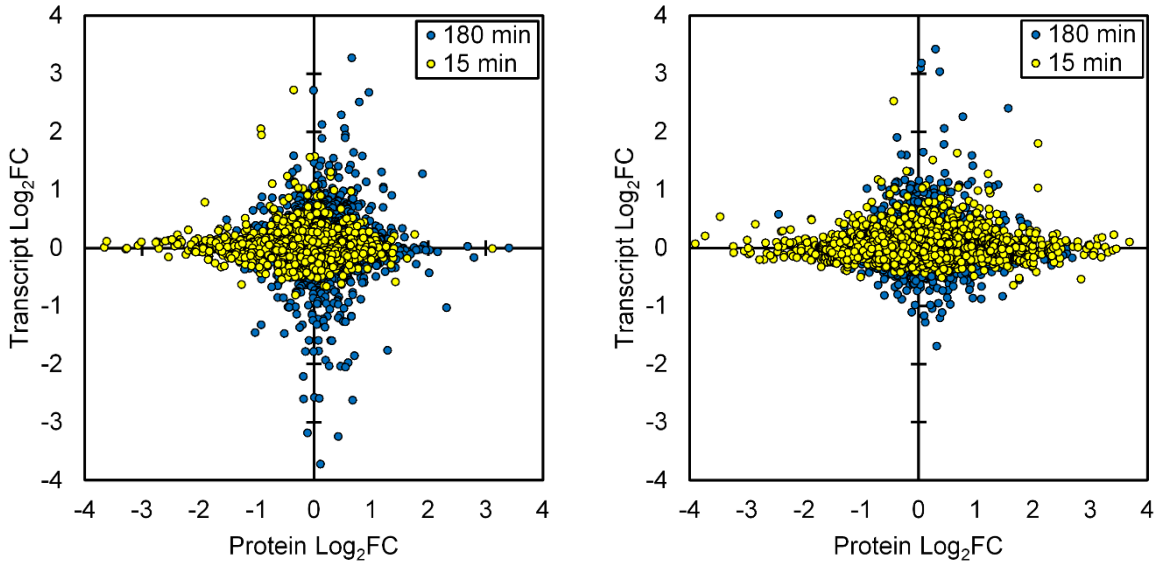


Figure 3-20: Comparison of transcriptome and proteome data after 15 min and 3 h in -N
Comparison of the changes in transcript and protein abundances for nitrate- (left) and ammonium-adapted plants (right) upon N-deprivation. Note the large transient proteomic differences with ammonium-deprivation, which are not mirrored by differential gene expression.

An overall lack of congruency in these rapid responses on the transcriptomic and proteomic level was observed (Figure 3-20). The transcriptional repression of nitrate assimilation genes was not found to result in similar alterations of nitrate assimilatory protein abundance in the observed time period (Figure 3-20). Transient proteome abundance changes observed after ammonium deprivation were also not mirrored on the transcriptomic level (Figure 3-20), suggesting that the primary targets for signaling of nitrate- and ammonium-deprivation differ considerably. These findings suggest posttranslational modifications as major signal transducers within the investigated time course of three hours.

3.3.2 N-form specific phospho-proteomes

Of the 632 phospho-peptides identified, only 364 were commonly identified under all conditions, while several phosphorylation-sites were only identified in ammonium- or nitrate-preadapted roots or at specific time points (Figure 3-21). However, for many phospho-peptides that were robustly detected under several conditions, a strong bias in abundance was observed regarding the predominant occurrence under ammonium- or nitrate-adapted conditions.

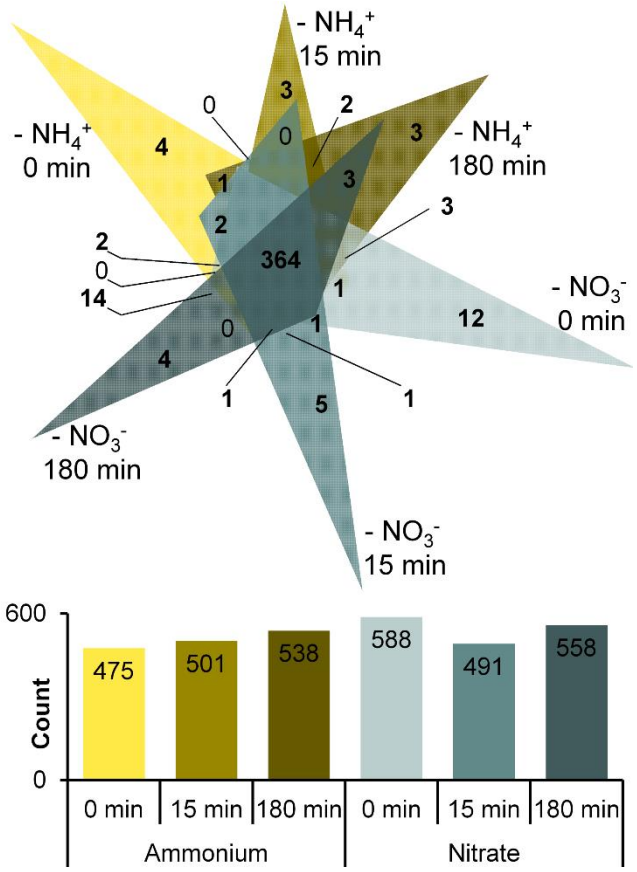


Figure 3-21: Venn diagram and counts of phospho-peptides

of all six sampling time points derived from phospho-peptides normalized intensity-data. Venn diagram generated with jvenn (Bardou et al., 2014)

Nine phosphorylation sites were identified to be significantly (p<0.01) enriched after adapting roots to ammonium nutrition (Table 3-2). For example, the two isoforms of the plasma membrane proton pumps AHA1 (AT2G18960) and AHA2 (AT4G30190) were phosphorylated at the C-terminal threonines (Thr-948 and Thr-947, respectively) and at Thr-881 (Table 3-2). Furthermore, the phosphoenolpyruvate carboxylase AtPPC1 (AT1G53310) was found to be phosphorylated at the conserved and regulatory Ser-11 in the N-terminus. The root specific glutamate decarboxylase AtGAD1 (AT5G17330) was phosphorylated at Ser-8 and the phosphorylation of the ABC-transporter AtABCA7 (AT3G47780) at Ser-576 was found to be highly significant in the ammonium-adapted plants (Table 3-2).

In contrast, 25 phosphorylated proteins were found to be significantly more abundant in nitrate-adapted roots (Table 3-2). For example, nitrate-pretreatment strongly favored phosphorylation of the nitrate transporter NRT2.1 (AT1G08090) at Ser-11 and Ser-28. Furthermore,

phosphorylation of two membrane proteins of the major facilitator family (putative xylose transporter AT5G17010 and putative monosaccharide transporter AT1G19450) was promoted by nitrate pre-treatment at Ser-26 and Ser-2, respectively. Differential protein-phosphorylation between ammonium and nitrate-adapted plants were also found in the kinase domains of two putative LRR-protein kinases, at Ser-919 of AT5G49770 and at Ser-533 of AT1G51850. Both kinases are highly expressed in roots, but their *mRNA* levels neither differed under adaptations to different N sources nor varied after N-deprivation. Finally, the nitrate reductase NIA2 (AT1G37130) was phosphorylated at the highly conserved and regulatory Ser-534. Several phosphorylation sites in uncharacterized or unknown proteins were also found specifically or predominantly under the nitrate-adapted conditions (Table 3-2).

Two unknown, highly similar proteins (AT1G80180, AT1G15400, 72.59% identity according to ClustalW2) were represented by two highly similar phosphopeptides in nitrate-adapted conditions (Table 3-2, Figure 3-22:). The motif P-P-S-P-R is found in both proteins and is targeted by the MAP-kinases MPK3 and MPK6 (Sörensson et al., 2012). The target motif was highly phosphorylated under nitrate adapted-conditions.

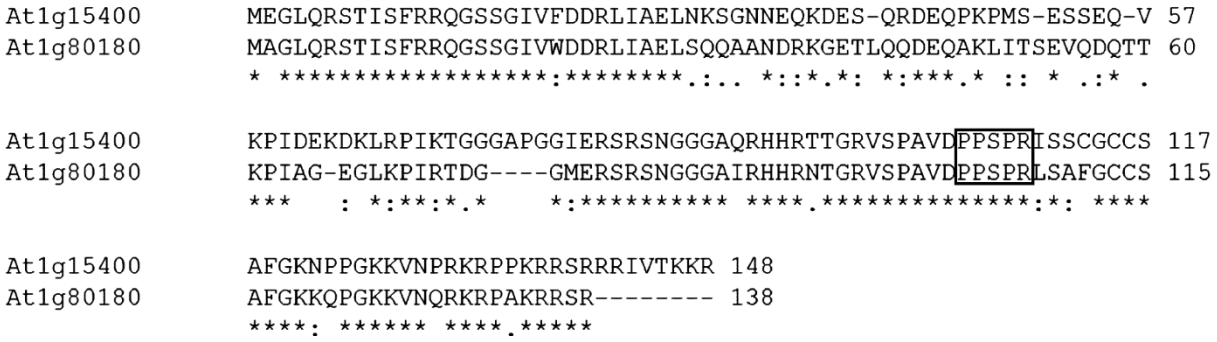


Figure 3-22: Sequence alignment of two phosphorylated proteins

ClustalW2-sequence alignment of two proteins (AT1G80180, AT1G15400). Box: MPK6-Motif P-P/S-S-P-R.

Table 3-2: Phosphopeptides predominating under nitrate or ammonium nutrition

Phospho-peptides which were predominantly identified in ammonium- or nitrate-adapted roots (p<0.01). Log_2-FC values indicate the intensity differences between nitrate and ammonium-adapted roots at time point 0. Proteins indicated with an asterisk are represented by more than one peptide.

AGI Locus ID	Protein Description	Phosphopeptide Sequence	Log_2-FC	p-Value
Ammonium adapted plants				
At3g47780	ABC2 homolog 6	SPSLRRPS*(ph)*LQR	-2.82	8.00E-03
At4g30190*	**H(+)-ATPase 2, AHA2**	LKGLDIETPSHYT*(ph)*V	-2.06	2.64E-04
At1g32400	Tobamovirus multiplication 2A	APTLDQRPSRS*(ph)*DPWSAR	-2.00	9.00E-03
At2g47485	Unknown Protein	LSSNSILLS*(ph)*PIR	-1.45	2.03E-03
At2g18960	**H(+)-ATPase 1, AHA1**	LKGLDIDTAGHHYT*(ph)*V	-1.27	3.30E-03
At4g30190*	**H(+)-ATPase 2 , AHA2**	EAQWALAQRT*(ph)*LHGLQPK	-1.16	5.37E-04
At1g53310	Phosphoenolpyruvate carboxylase 1, PPC1	MAS*(ph)*IDVHLR	-1.14	7.88E-03
At3g26560	ATP-dependent RNA helicase	MSS*(ph)*PERWEAK	-0.63	1.83E-03
At5g17330	**Glutamate decarboxylase, GAD1**	VLSHAVS*(ph)*ESDVSVHSTFASR	-0.49	5.92E-03
Nitrate-adapted plants				
At2g43680	IQ-domain 14	LDAPRPT*(ph)*TPKPPS*(ph)*PR	0.77	8.37E-03
At3g55320	P-glycoprotein 20	SHS*(ph)*QTFSRPLSSPDDTK	0.89	2.07E-03
At5g17010	Major facilitator superfamily protein	SSGEIS*(ph)*PEREPLIK	1.04	8.41E-03
At1g37130	**nitrate reductase 2, NIA2**	SVS*(ph)*TPFMNTTAK	1.35	3.70E-03
At4g37070	Acyl transferase/acyl hydrolase/ lysophospholipase	SDT*(ph)*MIKDSSNESQEIK	1.50	2.20E-05
At1g52320	Unknown Protein	VSS*(ph)*PPRVPNPAIQK	1.51	2.45E-03
At4g26130*	Unknown Protein	VKS*(ph)*INMPYFK	1.53	1.50E-03
At1g05150	Calcium-binding tetratricopeptide family protein	TAAWAVS*(ph)*PNHGIVFDETWK	1.83	9.36E-03
At5g04930	aminophospholipid ATPase 1 , ALA1	EVTFGDLGS*(ph)*KR	1.86	3.04E-03
At3g49590	Autophagy-related protein 13	IITDYVGS*(ph)*PATDPMR	1.89	6.47E-03
At2g45820	**Remorin, AtREM1.3**	ALAVVEKPIEEHT*(ph)*PK	2.01	2.30E-04
At1g80180*	Unknown Protein	NTGRVS*(ph)*PAVDPPS*(ph)*PR	2.04	6.50E-03
At4g18030	S-adenosyl-L-methionine-dependent methyltransferase	IEGIAES*(ph)*LCWEK	2.08	3.83E-04
At1g51850	LRR-protein kinase	VEGPPPSYMQASDGRS*(ph)*PR	2.13	1.54E-04
At4g32285	ENTH/ANTH/VHS superfamily	S*(ph)*RSFGDVNEIGAR	2.25	5.30E-04

Continued on next page

At1g19450	Major facilitator super-family protein	(ac)S*(ph)*FRDDNTEEGRNDLR	2.30	2.70E-03
At1g76040	Calcium-dependent protein kinase 29	LGS*(ph)*KLTESEIK	2.41	9.51E-03
At4g26130*	Unknown Protein	FGRPPS*(ph)*LIDR	2.46	8.87E-03
At1g15400*	Unknown Protein	TTGRVS*(ph)*PAVDPPS*(ph)*PR	2.47	9.31E-03
At1g15400*	Unknown Protein	RQGSS*(ph)*GIVFDDR	2.77	1.85E-03
At1g80180*	Unknown Protein	RQGSS*(ph)*GIVWDDR	2.91	5.85E-03
At1g08090*	**Nitrate transporter 2.1**	EQSFAFSVQS*(ph)*PIVHTDK	3.09	9.22E-09
At2g34310	Unknown Protein	SDEKEEILS*(ph)*PR	3.11	1.07E-03
At5g49770	LRR-protein kinase	LVGLNPNADS*(ph)*ATYEEASGDPYGR	3.22	7.36E-03
At1g08090*	**Nitrate transporter 2.1**	(ac)GDSTGEPGSS*(ph)*MHGVTGR	4.54	4.82E-10

3.3.3 Time-resolved phosphorylation responses of nitrogen deprivation

A large number of phospho-peptides was detected in all conditions (Figure 3-21), but some were not detected in all samples, but at only a single or few time-points of a specific pre-treatment with ammonium or nitrate. For these peptides, without a reference at time point 0 no ratio differences or true abundance ratios could be defined and calculated. Due to overall low abundance (Figure 3-21) some of such peptides may have escaped detection in some conditions. For this reason, these phospho-peptides were listed separately (Table 3-3).

In the nitrate pre-treated roots, 42 specific phospho-peptides were identified specifically and responded with *de novo* phosphorylation or *de novo* dephosphorylation events upon nitrate deprivation (Figure 3-23, Table 3-3). These included peptides from several receptor-like kinases (AT3G14840, AT1G18390, AT1G66880, AT4G35600, AT5G58950) that may transduce a nitrate or nitrate deficiency signal from the external apoplast to the cell interior. Nitrate

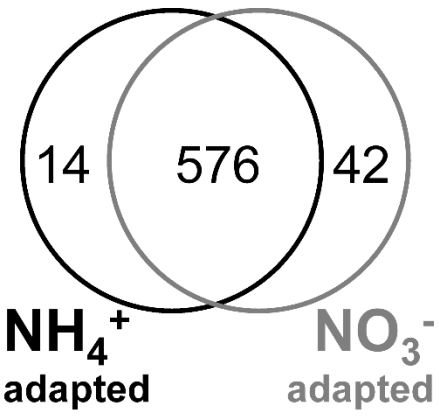


Figure 3-23: Venn diagram of N-form specific phospho-peptides

Number of common and N-form specific phospho-peptides. All phospho-peptides were taken in account which were detected in at least one of the three analyzed time-points of each N-form adaptation.

deprivation resulted in a slight, but not significant increase in phosphorylation of nitrate reductase (NIA1, AT1G7760 and NIA2, AT1G37130).

Table 3-3*: De novo* de-/phosphorylation events in response to -N

Intensity values of phospho-peptides that were only detected at certain time points and specifically only in ammonium- or in nitrate-adapted roots (*cf.* Figure 3-23) and resemble thereby (partially) transient *de novo* phosphorylation or *de novo* dephosphorylation events specifically for the N-form. *nd* = not detected. Bold peptide names are of special interest.

AGI Locus ID	Protein Description	Phospho-peptide Sequence	Intensity		
			0 min	15 min	180 min
Ammonium-adapted plants					
At1g22530	PATELLIN 2	EILQS*(ph)*ESFKEEGYLASELQEAEK	*nd*	1.00	*nd*
At1g48920	nucleolin like 1	GFDASLS*(ph)*EDDIKNTLR	*nd*	*nd*	1.00
At3g01780	ARM repeat protein	IEEES*(ph)*ENEEEEEGEEEDDDEEVKEKK	*nd*	1.00	*nd*
At3g11820	syntaxin of plants 121	AS*(ph)*SFIRGGTDQLQTAR	*nd*	*nd*	1.00
At3g20550	SMAD/FHA domain-containing protein	HDEGS*(ph)*NARGGSEEPNVEEDSVAR	1.00	*nd*	*nd*
At3g28180	Cellulose-synthase-like C4	RNSES*(ph)*GLELLSK	*nd*	1.00	*nd*
At3g58640	MAP-Kinase	TASS*(ph)*SPEHLSFR	*nd*	0.63	1.02
At4g21160	Calcium-dependent ARF-type GTPase activating protein	TPAFLSSSLS*(ph)*KK	1.00	*nd*	*nd*
At4g23700	**Cation/H+ exchanger 17, CHX17**	NVTTEESLVEDSES*(ph)*P	1.24	*nd*	0.97
At4g31880	Unknown Protein	TSGDETANVS*(ph)*SPSMAEELPEQSVPKK	*nd*	1.10	0.90
At4g35780	ACT-like tyrosine kinase	VQTESGVMT*(ph)*AETGTYR	1.00	*nd*	*nd*
At5g40930	translocase of outer membrane 20-4	SLTLASKAPELHTGGTAGPS*(ph)*SNSAK	1.00	*nd*	*nd*
At5g45380	**urea transmembrane transporters, DUR3**	VVEAYAS*(ph)*GDEDVDVPAEELREEK	0.88	2.67	1.24
At5g60660	**plasma membrane intrinsic protein 2.4, PIP2.4**	(ac)AKDLDVNES*(ph)*GPPAAR	*nd*	*nd*	1.00
Nitrate adapted plants					
At1g01960	SEC7-like guanine nucleotide exchange family protein	SDS*(ph)*QSELSSGNSDALAIEQR	1.00	*nd*	1.00
At1g08090	**nitrate transporter 2.1**	VRSAAT*(ph)*PPENTPNNV	1.10	0.69	1.55
At1g16860	Ubiquitin-specific protease C19	S*(ph)*GSFAGTAQSGPGAPMATGR	1.05	0.77	*nd*

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At1g18390	**Protein kinase, LRK10L1**	SGPLVAQS*(ph)*PDSVIVK	5.36	*nd*	0.84
At1g18880	**Nitrate transporter 1.9**	TSAEFDKVS*(ph)*V	1.07	*nd*	0.81
At1g20840	Tonoplast monosaccharide transporter1	PVPEQNS*(ph)*SLGLR	0.99	*nd*	1.21
At1g53310	**Phosphoenolpyruvate carboxylase 1 , PPC1**	M(ox)AS*(ph)*IDVHLR	*nd*	1.00	*nd*
At1g66880	Protein kinase superfamily	NPTSTTISSSSNHSLLPSIS*(ph)*NLANR	1.00	*nd*	*nd*
At1g68070	Zinc finger, RING finger family	(ac)SSPES*(ph)*PSGSDSSTPLLR	0.95	*nd*	1.05
At1g69070	Unknown Protein	MQETEELS*(ph)*DGDEEIGGEESTKR	*nd*	1.00	*nd*
At1g77760	**Nitrate reductase 1, NIA1**	SVS*(ph)*SPFMNTASK	1.87	1.29	1.52
At2g16850	**plasma membrane intrinsic protein 2.8, PIP2.8**	ALAS*(ph)*FRS*(ph)*NPTN	5.12	0.53	2.65
At2g19385	zinc ion binding	KLETLDETS*(ph)*EGEEAK	1.05	*nd*	0.95
At2g30530	Unknown Protein	AFLDEDDPNQLPQS*(ph)*PK	1.50	*nd*	0.50
At2g32240	Unknown Protein	DIDLSFSS*(ph)*PTK	6.23	*nd*	1.00
At2g34660	multidrug resistance-associated protein 2	EIAES*(ph)*LEEHNISR	*nd*	*nd*	1.00
At2g34680	Outer arm dynein light chain 1 protein	PVIS*(ph)*SNLIK	*nd*	*nd*	1.00
At3g01290*	SPFH/Band 7/PHB domain-containing membrane-associated	SSAVFIPHGPGAVS*(ph)*DVAAQIR	0.86	*nd*	*nd*
At3g01290*	SPFH/Band 7/PHB domain-containing membrane-associated	YLS*(ph)*GLGIAR	2.51	1.05	0.77
At3g07020	UDP-Glycosyltransferase superfamily protein	VWT*(ph)*MPLEGSSSSDKAESSSTNQPR	1.00	*nd*	*nd*
At3g13100	multidrug resistance-associated protein 7	VSNDEEKQEEDLPS*(ph)*PK	1.00	*nd*	*nd*
At3g14840*	LRR-transmembrane protein kinase	LDEEENTHIS*(ph)*TR	*nd*	*nd*	1.00
At3g14840*	LRR-transmembrane protein kinase	LDEEENTHIST*(ph)*R	1.00	*nd*	*nd*
At3g19820	DWARF1	S*(ph)*DLQTPLVRPK	1.01	*nd*	0.97
At3g55600	Membrane fusion protein Use1	IEDEPRS*(ph)*PTSPQLR	1.10	*nd*	*nd*
At4g06534	Unknown Protein	AASPIRSDS*(ph)*FQAR	*nd*	*nd*	1.00
At4g19860	alpha/beta-Hydrolases superfamily protein	INVIS*(ph)*HSM(ox)GGLLVK	*nd*	1.00	*nd*

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At4g20780	Calmodulin like 42	LRS*(ph)*PSLNALR	*nd*	1.00	*nd*
At4g22980	Unknown Protein	FTSQES*(ph)*LPR	1.76	*nd*	0.82
At4g31160	DDB1-CUL4 associated factor 1	VHEGAPDTEVLLAS*(ph)*PR	*nd*	1.00	*nd*
At4g33400	Vacuolar import/ degradation Vid27	S*(ph)*PSSSLDDVEAK	0.78	*nd*	1.85
At4g35600	Protein kinase superfamily protein	NFKPDS*(ph)*MLGQGGFGK	1.00	*nd*	*nd*
At4g37070	Acyl transferase/ lysophospholipase	LRS*(ph)*DTMIKDSSNESQEIK	1.00	*nd*	*nd*
At5g13260	Unknown Protein	LSDIELKS*(ph)*PGGPK	*nd*	0.73	1.27
At5g13890	Unknown Protein	T*(ph)*GSYEALPTNNADSNHIQMK	1.00	*nd*	*nd*
At5g14050	Transducin/WD40 repeat-like superfamily protein	KQYEDVEDEEEIGS*(ph)*DDDLTR	1.00	*nd*	*nd*
At5g19050	alpha/beta-Hydrolases superfamily protein	S*(ph)*SSMAGGGSGSGDYGGPIKGK	1.00	*nd*	*nd*
At5g20490	Myosin family protein	QQALAIS*(ph)*PTSR	1.22	1.31	0.68
At5g44240	aminophospholipid ATPase 2 , ALA2	SPVYEPLLSDS*(ph)*PNATRR	0.88	*nd*	1.12
At5g49890	chloride channel C	TTFGS*(ph)*QILR	1.29	*nd*	0.56
At5g57110	autoinhibited Ca2+ -ATPase, isoform 8	SEHADS*(ph)*DSDTFYIPSK	1.00	*nd*	*nd*
At5g58950	Protein kinase	SVS*(ph)*PSPQMAVPDVFK	1.02	*nd*	0.98

Only seven phospho-peptides, over-representing membrane and transporter proteins, were rapidly (within 15 min.) significantly dephosphorylated after withdrawal of nitrate. Among these were Ser-49 of the plasma-membrane localized aminophospholipid translocase ALA1 (AT5G04930) and Ser-35 of phosphatidylserine decarboxylase 3 (AT4G25970) as the most rapidly dephosphorylated (-2.52 $\log_2$-FC) peptide. One phosphorylation site at Ser-8 of At-SYP122, a vesicle transport syntaxin-type t-SNARE protein (AT3G52400, Table 3-4) was identified exclusively with nitrate deprivation after 15 min and 3 hours. Phosphorylation of the syntaxin AtSYP121 (AT3G11820) however, was detected exclusively in 3 hours N-depleted ammonium adapted plants (Table 3-3).

At 3 h of nitrate deprivation, 10 peptides were dephosphorylated and 3 increased in phosphorylation status. The unknown protein AT1G16170, which was dephosphorylated by 18 % after 3 hours, was the only protein, of which the transcript abundance was also reduced at 3 hours of nitrate deprivation (-1.76 $\log_2$-FC). A member of the major facilitator group

(AT1G64650) was dephosphorylated at Ser-444 with a log_2-FC of nearly 3. Interestingly, a splice variant of AT1G64650 has a shortened amino-acid sequence lacking Ser-444. A putative receptor protein kinase, AtLRK10L1 (AT1G18390), recently described to be involved in ABA signaling and drought resistance (Lim et al., 2015) was dephosphorylated at Ser-637 with a log_2-FC of -2.67. Among the three up-regulated phosphorylation-sites, none appeared specific for nitrate-adapted plants.

Overall, 14 phospho-peptides were specifically identified in the ammonium-adapted roots, but not at all time points (Figure 3-23, Table 3-3). Among these, several belonged to transporter proteins. For example, the phosphorylation of Ser-568 in urea transporter DUR3 (AT5G45380) was 3-fold increased only in 15 min. ammonium-depleted roots and decreased after 3 hours. This was accompanied by a high protein and transcript abundance of *DUR3* only observed under ammonium-adapted conditions. In addition, an ammonium-specific phosphorylation site (Ser-366) in a putative MAP-kinase (AT3G58640) appeared in plants only at 15 minutes and 3 hours of ammonium-deprivation.

Several phospho-peptides, however, were robustly found under both, ammonium and nitrate adapted conditions, but showed differential responses to the withdrawal of either nitrogen form. For these, transient or persistent phosphorylation changes could be monitored by abundance ratios derived from the normalized ion intensities. Significant ratio changes ($p \leq 0.01$) with respect to the onset of N-starvation (time-point 0) are shown in Table 4. In ammonium-adapted plants, strong changes of 37 phospho-peptides were identified. Out of these, phosphorylation levels decreased for 30 phosphorylation sites during the ammonium deprivation. Notably, these proteins appeared to be particularly plasma membrane proteins involved in the transport of H^+, K^+, NH_4^+, NO_3^- and water, in addition to global enzymes involved in primary metabolism (Table 3-4). Several phospho-peptides with known phosphorylation sites of the ammonium transporter AMT1.1 (AT4G13510) were among the significantly regulated peptides. Two phospho-peptides containing the C-terminal Ser-488 were significantly down-

regulated, indicating a regulatory trend for this site which was also detected after 3 h. Furthermore, the known conserved regulatory phosphorylation site of AMT1.1 Thr-460 (Lanquar et al., 2009) was identified, which was two-fold up-regulated (inhibiting transport) at 15 min of ammonium-deprivation. In the C-terminus of ammonium transporter AMT1.3 (AT3G24300), another responsive phosphorylation site at Ser-487 was increased 2.8-fold during the first 15 min of ammonium deprivation. In ammonium-adapted plants, the phosphorylation of residual nitrate transporter NRT2.1 (AT1G08090) at the N-terminal Ser-10 was further reduced after depriving plants for ammonium. The strongest decrease in phosphorylation (more than 200-fold) was found in the C-terminus of the plasma membrane aquaporins AtPIP2.2 (AT2G37170, Ser-278) and AtPIP2.7 (AT4G35100, Ser-273) at the respective sites responsible for channel opening.

Table 3-4: Time-resolved phosphorylation change ratios in response to -N

Significantly regulated phospho-peptides by ammonium or nitrate deprivation and log_2-fold changes. Boldfaced LFC-values are significant (p<0.01). Asterisks indicate that several proteotypic phospho-peptides were found significantly changed. Boldfaced descriptions highlight peptides of special interest.

AGI Locus ID	Protein Description	Phospho-peptide Sequence	Log2 fold-change (LFC)			
			NH_4^+ adapted		NO_3^- adapted	
			15 min	180 min	15 min	180 min
Ammonium adapted: significantly different at 15 min and to some extent also after 180 min						
At2g37170	**plasma membrane intrinsic protein 2, PIP2.2**	SLGS*(ph)*FRSAANV	**-7.88**	-0.13	1.36	0.63
At1g02520	P-glycoprotein 11	TSELSSGS*(ph)*SFRNSNLKK	**-4.30**	-0.85	1.33	1.25
At4g26630	DEK domain-containing chromatin associated prot.	AVVAAKS*(ph)*SPPEKITQK	**-2.87**	-0.21	0.01	0.62
At4g35100	**Plasma membrane intrinsic protein 3, PIP3**	ALGS*(ph)*FRSNATN	**-2.79**	-0.66	0.69	0.30
At1g59870	ABC-2 and Plant PDR ABC-type transporter	SLS*(ph)*TADGNRRGE-VAMGR	**-2.46**	-0.29	0.64	0.46
At1g59359	Ribosomal protein S5	AVS*(ph)*ATKVITEGEDQA	**-2.38**	0.12	0.00	0.62
At4g01480	pyrophosphorylase 5	ILS*(ph)*SLSKR	**-2.37**	-1.13	0.35	-0.43
At3g17420	glyoxysomal protein kinase	SNATT*(ph)*LPVTQSPR	**-2.29**	0.27	-1.31	-0.66
At3g47780	ABC2 homolog 6	SPSLRRPS*(ph)*LQR	**-2.08**	-0.51	2.32	2.71
At3g10260	Reticulon family protein	IPS*(ph)*GKFGLK	**-2.05**	-0.10	0.02	0.70

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At4g38470	ACT-like protein tyrosine kinase family protein	VKAQTGVMT*(ph)*AETGTYR	**-2.03**	-0.57	-0.31	-0.97
At1g59820	aminophospholipid ATPase 3, ALA3	VRS*(ph)*GSFSVDSSATHQR	**-1.90**	-0.03	-0.85	-0.17
At5g47690	Unknown Protein	SLS*(ph)*LEHEKVESR	**-1.81**	-0.37	0.69	0.58
At5g51060	NADPH/respiratory burst oxidase protein D	LAS*(ph)*VSHELKR	**-1.79**	-0.79	0.17	0.39
At4g13510*	**Ammonium transporter 1.1**	RVE-PRS*(ph)*PSPSGANTTPTPV	**-1.77**	-0.35	0.56	-0.14
At1g08090	**Nitrate transporter 2.1**	(ac)GDSTGEPGSS*(ph)*MHG VTGR	**-1.71**	-0.32	-1.25	-1.15
At2g18730	diacylglycerol kinase 3	(ac)MDS*(ph)*PVSKTDASKEK	**-1.70**	-0.40	-0.08	-0.25
At4g30190*	**H(+)-ATPase 2, AHA2**	LKGLDIETPSHYT*(ph)*V	**-1.59**	**-1.07**	0.38	-0.27
At4g26130	Unknown Protein	LQRLDS*(ph)*FLR	**-1.59**	-0.60	-0.66	-0.45
At5g63490	Unknown Protein	SLS*(ph)*VTTASLHGK	**-1.56**	-0.50	0.14	0.23
At5g47910	respiratory burst oxidase homologue D	RGNS*(ph)*SNDHELGILR	**-1.42**		0.15	-0.38
At3g62700	multidrug resistance-associated protein 10	SIS*(ph)*IESPRQPK	**-1.40**	0.07	-0.28	-0.51
At4g13510*	**Ammonium transporter 1.1**	VE-PRS*(ph)*PSPSGANTTPTPV	**-1.33**	-0.85	0.45	-0.38
At4g37100	Pyridoxal phosphate (PLP)-dependent transferase	LLGVEDEHPS*(ph)*KGR	**-1.32**	0.28	-0.24	-0.25
At1g53310	**Phosphoenolpyruvate carboxylase 1, PPC1**	MAS*(ph)*IDVHLR	**-1.23**	**-0.93**	-0.40	-0.08
At2g18960	**H(+)-ATPase 1, AHA1**	LKGLDIDTAGHHYT*(ph)*V	**-1.18**	**-0.82**	0.17	-0.14
At5g07920	diacylglycerol kinase1	TGS*(ph)*FGQKEYHALR	**-0.92**	0.78	-0.13	0.20
At4g30190*	**H(+)-ATPase 2, AHA2**	EA-QWALAQRT*(ph)*LHGLQPK	**-0.77**	**-0.55**	0.09	-0.25
At1g52200	PLAC8 family protein	GRVTTPSEEDSN-NGLPVQQPGT*(ph)*PNQR	**-0.76**	-0.32	-0.28	-0.55
At3g02880	LRR-protein kinase	LIEEVSHSSGS*(ph)*PNPVSD	**-0.72**	-0.03	-2.43	-1.06
At5g17330	**Glutamate decarboxylase, GAD1**	VLSHAVS*(ph)*ESDVSVHSTF AS	**0.43**	**-0.50**	-0.06	-0.03
At3g24300	**Ammonium transporter 1.3**	VDPGS*(ph)*PFPR	**0.58**	0.40	-1.79	-1.14
Atmg00510	NADH dehydrogenase subunit 7	IDELEEMS*(ph)*TGNR	**0.90**	**0.59**	0.34	0.32
At4g13510*	**Ammonium transporter 1.1**	ISSEDEMAGMDMT*(ph)*R	**1.01**	0.13	-1.54	-0.81
At4g35785	RNA-binding (RRM/RBD/RNP motifs) family protein	T*(ph)*PTPGHYLGLK	**1.31**	**1.04**	-1.04	-0.07
At2g45820	**Remorin AtREM1.3**	ALAVVEKPIEEHT*(ph)*PK	**1.74**	**1.91**	-0.93	-0.08
At5g59010	Protein kinase with tetra-tricopeptide repeat	ETDIP-SHVLMGIPHGAAS*(ph)*PK	**2.27**	2.60	-	-0.03

Ammonium adapted: 180 min specific phosphopeptides

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At3g18450	PLAC8 family protein	GRPVGQTNQAQPSVQHT*(ph)*ASPSNK	-1.30	**-0.63**	-0.84	0.56
At3g47200	Unknown Protein	(ac)AD-KTDIISSSSDKAS*(ph)*PPPPSAFR	-0.40	**-0.62**	0.43	-0.22
At4g31700	ribosomal protein S6	SRLS*(ph)*SAAAKPSVTA	-	**0.45**	0.25	-0.71
At1g51850	LRR-protein kinase	VEGPPP-SYMQASDGRS*(ph)*PR	1.62	**1.49**	-0.56	-0.74
Nitrate adapted: significantly different at 15 min and to some extent also after 180 min						
At4g25970	phosphatidylserine de-carboxylase 3	LSRPGS*(ph)*GSVSGLASQR	-1.40	-1.82	**-2.52**	-
At5g04930*	aminophospholipid ATPase 1, ALA1	EVTFGDLGS*(ph)*KR	0.98	1.10	**-1.64**	-0.19
At1g20840	tonoplast monosaccha-ride transporter1	YYLKEDGAES*(ph)*R	1.34	1.90	**-1.27**	-0.42
At3g25610	ATPase E1-E2 type	SSFQEDHS*(ph)*NIGGPGFSR	0.13	0.21	**-0.99**	-0.16
At1g26630	Eukaryotic translation initiation factor 5A-1	(ac)S*(ph)*DDEHHFEASESGASK	-0.36	0.08	**-0.93**	-0.57
At3g55320	P-glycoprotein 20	SHS*(ph)*QTFSRPLSSPDDTK	0.48	0.56	**-0.61**	**0.41**
At1g19870	IQ-domain 32	RTS*(ph)*FGYDQEAR	-	-0.21	**-0.44**	**-0.54**
At3g52400	syntaxin of plants 122	(ac)MNDLLSGS*(ph)*FKTSVADGS*(ph)*SPPHSHNIEMSK	-	-	**1.42**	2.60
Nitrate adapted: significantly different after 180 min						
At1g64650	Major facilitator super-family	S*(ph)*QEWSAEKEMTSEADPLNP	-	-	-	**-2.96**
At1g18390	**Protein kinase, LRK10L1**	SGPLVAQS*(ph)*PDSVIVK	-	-	-	**-2.67**
At4g32285	ENTH/ANTH/VHS su-perfamily protein	S*(ph)*RSFGDVNEIGAR	-0.06	0.78	-2.42	**-1.44**
At5g61150	leo1-like family protein	SNRYS*(ph)*DE-DEEEEEVAGGR	-	-	-	**-1.31**
At4g37070	Acyl transferase/acylhy-dro-lase/lysophospho-lipase	SDT*(ph)*MIKDSSNESQEIK	0.66	0.95	-0.73	**-1.16**
At3g58170	BET1P/SFT1P protein 14A	RLS*(ph)*GDINEEVDTHNR	-0.03	0.27	0.13	**-0.77**
At5g04930*	aminophospholipid ATPase 1, ALA1	DNKEVTFGDLGS*(ph)*KR	1.02	1.11	-	**-0.73**
At1g16170	Unknown Protein	VLDGLVS*(ph)*SPSRR	-	1.22	-0.07	**-0.28**
At4g37100*	Pyridoxal phosphate (PLP)-dependent trans-ferase	LLGVEDEHPS*(ph)*KGR	**-1.32**	0.28	-0.24	**-0.25**
At1g09770	cell division cycle 5	IGLT*(ph)*PSRDGSSFSMTPK	-0.18	0.32	0.11	**0.77**
At1g16610	arginine/serine-rich 45	VSS*(ph)*PPKPVSAAPK	-1.31	-0.53	-0.93	**1.26**

It is notable that most of the aforementioned changes in phosphorylation were transient. After 3 hours of ammonium deprivation, most sites returned to their previous phosphorylation state. Only 11 phosphorylation sites showed a significant change also after 3, but also in these

cases a transient tendency was observable. Only for two peptides, a different regulation was apparent: Phosphorylation of Ser-8 of the glutamate decarboxylase AtGAD1 (AT5G17330) was decreased after 3 h, while after 15 min., it was one of the few up-regulated sites. A remorin, AtREM1.3 (AT2G45820), potentially involved in plant-microbe interactions and signaling, membrane integrity and scaffolding (Benschop et al., 2007; Jarsch and Ott, 2011; Marín et al., 2012), was rapidly phosphorylated in Thr-68, resembling the strongest up-regulation of all phosphopeptides. This phosphorylation site in the remorin was also one of the predominating phosphorylation sites in found in nitrate-adapted plants (Table 3-2).

Another strong and continuous phosphorylation was identified for a putative LRR-type receptor protein kinase (AT1G51850) at Ser-533, a site that was also slightly dephosphorylated by nitrate deprivation after 3 h. The C-terminal threonines (Thr-948/947) of proton pumps AHA1 (AT2G18960) and AHA2 (AT4G30190) were significantly de-phosphorylated over time with ammonium deprivation. In addition, phosphorylation on another site in AHA2 (Thr-881) decreased over time in ammonium depletion. Finally, the conserved phosphorylation site at Ser-11 of the phosphoenolpyruvate carboxylase AtPPC1 (AT1G53310) was down-regulated already after 15 min and remained low abundant also after 3 h.

4 DISCUSSION

This extensive analysis of the earliest transcriptional and (phospho-)proteomic responses of roots to deprivation of nitrate or ammonium identified large differences in the early deprivation responses. Ammonium- or nitrate-adapted *Arabidopsis* roots expressed a distinct transcriptome and (phospho)proteome, largely in agreement with previous analyses (Patterson et al., 2010; Engelsberger and Schulze, 2012) and confirming nitrate as the major regulator to adjust the metabolism to nitrogen availability.

Additionally, the nitrogen use efficient *Arabidopsis* genotype Tsu-0 and the contrasting, inefficient reference genotype Col-0 were compared in their global root transcriptome and on potential differences in their nitrogen deprivation response. Their transcriptome differences revealed a rather unambiguous effect of nitrogen nutrition related genes on the contrasting N use efficiencies.

4.1 Responses to nitrate deprivation

Overall, the gradual transcriptional repression of genes upon nitrate deprivation overlapped well with transcripts that were repressed under mid- and long-term acclimation to nitrate starvation (Suppl. Figure A-0-1) (Krapp et al., 2011). However, the response to nitrate deprivation is more than the opposite of what is observed after nitrate resupply to roots, although the majority of early nitrate-activated (Wang et al., 2003) were repressed by nitrate deprivation. Major players of the nitrate-signaling cascade were identified in this work (Figure 3-10) among the earliest responding genes. This includes several transcription factors, such as *LBD37/38/39*, *HRS1* and *HHO1,* a small auxin-responsive gene (*SAUR6*) and a gene encoding a mitochondrial substrate carrier. All of these genes had reduced transcript abundance after deprivation of nitrate (Table 3-1,Figure 3-11).

LBD TRANSCRIPTION FACTORS

Interestingly, *LBD37/38/39* had been identified as direct regulators (repressors) of the nitrate uptake and assimilation pathway, and suppressors of the anthocyanin biosynthesis (Rubin et al., 2009). Given that *LBD37/38/39* are repressors of nitrate assimilation, the reduction of their gene products levels should promote *NRT* and *NIA* transcription. However, this was not observed here, as nitrate uptake and assimilation genes were also repressed. The nitrate deprivation observed here thus bypassed the *NRT/NIA* gene activation by repressed *LBD37/38/39*, suggesting that the regulatory gene module may only be functional as long as residual nitrate is available. The repression of *LBDs* preceded the repression of nitrate uptake and assimilation genes, suggesting that mild nitrate deficiency or early deprivation response via *LDBs* may be a plant strategy to rescue some nitrate acquisition and assimilation, but severe nitrate deprivation bypasses this strategy. In addition, there are indications for a post-transcriptional regulation of some *LBDs.* Even in LBD over expressors under full nitrogen nutrition transcript abundance is drastically reduced (Rubin et al., 2009; Medici and Krouk, 2014).

The expression pattern of *LBD38* was highly remarkable: its transcript was slightly higher expressed in ammonium adapted plants (Tsu-0 and Col-0) (Figure 3-7, Table 3-1) and decreased with withdrawal of both N-forms, even in plants in which nitrate was absent for already 5 days. Such common, nitrate-independent regulation was to a lesser extent observed for two other genes, coding an unknown protein (*At5g19970*) and the *cycling DOF1 Factor (At5g62430)*, while the latter one is associated with circadian regulation (Seaton et al., 2015). Responding to the lack of nitrogen, irrespective of the N-form, suggests the integration of the external N-status over the *LBD38* transcription factor into downstream gene expression responses.

HRS1 AND HHO1 TRANSCRIPTION FACTORS

HRS1, together with *HHO1*, were identified as critical GARP-transcription factors for integrating the nitrate and phosphate starvation response and the adaptation of root architecture to different nutrient availabilities (Medici et al., 2015). *HRS1/HHO1* act downstream of the nitrate transceptor NRT1.1/NPF6.3, as their transcriptional activation by nitrate is strongly reduced in

a mutant lacking this receptor (Muños et al., 2004). *HRS1/HHO1* in the presence of nitrate also represses primary root growth in phosphorus deficiency (Medici et al., 2015). As *HRS1/HHO1* were found among the earliest genes repressed by nitrate deprivation, root growth may be stimulated via the repression of these transcription factors. In this N-depletion dataset of nitrate adapted roots, only little changes in direct target genes of these transcription factors were detected, possibly due to their localization restricted to the root meristem.

NLP TRANSCRIPTION FACTORS

Recently, NIN-like transcription factors were shown to regulate nitrate-responsive transcription. The proteins directly bind *cis*-regulatory elements of nitrate metabolism genes in a nitrate-dependent way (Konishi and Yanagisawa, 2013). In this work, the *NLP7* transcript was only weakly regulated in the early transcriptional response and was not in the proteome. NLP7 protein exerts its response not by alterations in abundance, but through different, nuclear compartmentation upon nitrate availability (Marchive et al., 2013). A mild decrease of internal nitrate concentration may already release NLP7 from nitrate-responsive DNA elements (Konishi and Yanagisawa, 2014) but many NLP7 downstream targets may not be regulated after 15 min, as internal nitrate had not dropped yet clearly. Consequently, *NLP*-dependent genes such as *NIA1, NIR1* and others, were in the transcriptome data only regulated after 3 h of nitrate deprivation, after nitrate in the tissue had considerably dropped.

In contrast, the observations made in *nlp7*-mutants indicate, that N-depletion response in nitrate adapted plants is obviously independent of NLP7. The gene expression of NLP7 target genes, even starting from a lower (NLP7-dependent) mRNA level, decreased during N-depletion within minutes to the same level as in wild-type plants. Potentially NLP6 or other NLPs are involved in the depletion response, as for example both, NLP6 and NLP7, have numerous common target genes (Konishi and Yanagisawa, 2014).

Due to the decreased expression of nitrate uptake and assimilation genes, nitrate concentration is slightly higher in *nlp7*-mutants compared with wild-type plants (Castaings et al., 2009). This may favor the existence of a fast responding membrane-borne nitrate sensor, such

as NRT1.1/NPF6.3, as a drop of internal nitrate concentration and the subsequent release of NLP7 from its targets may not be sufficiently fast enough for the observed, rapidly declining, gene expression. Thus, the withdrawal of N is probably sensed externally and rapidly translated over an unknown regulatory mechanism into the early nitrate-depletion response. According to these findings, the early regulation of *LBDs*, the *Mitochondrial Substrate Carrier* and *SAUR6* (the latter one had not previously been associated with the nitrate response) may thus rely on another regulatory mechanism in response to external nitrate. Expression of *SAUR6* and the *Mitochondrial Substrate Carrier* was also in *nlp7*-mutants decreased, pushing both genes in focus for further studies.

SAUR6

The *Arabidopsis* genome harbors 82 SAUR coding genes (Markakis et al., 2013) of which many respond within minutes to auxin treatment (Hagen and Guilfoyle, 2002). Only few of these very short proteins were functionally described. For instance, the highly identical proteins SAUR19-24 are extremely unstable and were shown to be involved in cellular expansion (Spartz et al., 2012). Relative to other SAUR proteins, sequence homology of SAUR6 to SAUR19-24 is comparatively high. This offers the possibility, that SAUR6 may be involved in root cell elongation which is then inhibited by nitrate depletion. Furthermore, some SAUR proteins, as shown for maize, bind *in vitro* to calmodulin proteins (Yang, 2000; Knauss et al., 2003), which provides a possible link of SAURs to the Ca^{2+}/calmodulin second messenger system. Interestingly, the earliest responding genes to nitrate deprivation (15 min.) were enriched in calcium- and ethylene-signaling genes, but these were only transiently regulated. Also the calmodulin CML42 (At4g20780) was transiently phosphorylated in nitrate deprivation after 15 min. Recently, calcium signaling was suggested to be involved in nitrate signaling (Riveras et al., 2015), which is supported by these data. However, many ethylene- and calcium-signaling related genes were also rapidly responding in control-treatments and are thereby responding to the unavoidable mechanical stimulus in the experiments (Suppl. Figure A-0-2). Notably, *SAUR6* was not, which was specifically responsive to nitrate depletion and

strongly up-regulated upon nitrate supply. Accordingly, this protein should be further studied with respect to the N-response.

MITOCHONDRIAL SUBSTRATE CARRIER

The *Arabidopsis* genome contains 58 genes encoding mitochondrial substrate carriers which transport diverse substrates like nucleotides/dinucleotides, di-/tri-carboxylates and keto acids, and amino acids in the inner organelle membranes of mitochondria and even with non-mitochondrial localization (Palmieri et al., 2011). The expression patterns and the substrate of the mitochondrial substrate carrier coding gene *At5g26200,* which was one of the earliest and durably down-regulated genes in the nitrate depletion response not reported yet. The transcript is also in mid- and long-term nitrogen depleted conditions down-regulated (Krapp et al., 2011) and the reduced expression in the *nlp7*-mutants enhances its relevance in nitrate nutrition while its function still remains unclear.

CLE-PEPTIDES

CLAVATA3/ESR-related signaling peptides were recently identified as regulators of the root architecture and are N-starvation induced (Araya et al., 2014). These peptides may be also involved in rapid N-form-specific root growth responses, as *CLE-1, -4,* and *-7* transcripts were present with nitrate, but rapidly decreased with nitrate deprivation. *CLE-3,* in contrast, was low in nitrate, but highly expressed with ammonium nutrition. The short-termed down regulation of *CLE* transcripts may be explained with a local root response to a signal of the externally sensed nitrate decline by the putative transceptor NRT1.1/NPF6.3 or the identified receptor kinases whereas the transcript levels increase with an internal, systemic “low N” signal – when plants grow for longer periods in N starved conditions.

NITRATE DEPENDENT RNA STABILITY

Most regulated transcripts were repressed by nitrate deprivation, opening the possibility that active degradation contributes to the lower transcript levels. The steady state expression of a gene is determined by the equilibrium between gene transcription and transcript degradation. Rapid *m*RNA decay rates from cell culture experiments using a transcriptional inhibitor (Narsai

et al., 2007) were largely in agreement with the hypothesis that nitrate deprivation rapidly inhibits transcription of rapidly degraded *mRNAs*, such as the *LBD* transcription factors, without major influence on the *mRNA* decay rates. However, some rapidly decreased transcripts under nitrate deprivation, such as the transcription factor *HRS1* or the nitrite reductase gene *NiR1 (AT2G15620),* were remarkably stable under full nitrate nutrition ($t_{1/2}$ > 6 h), in agreement with previous experiments (Narsai et al., 2007). Their *mRNA* decay rates in the presence of a transcriptional repressor identified a much faster transcript decay after nitrate deprivation (Figure 3-17), which may be explained by active *mRNA* degradation under nitrate deprivation or a possible *m*RNA stabilization in the presence of nitrate. Though, *microRNAs* that specifically target these *mRNAs* for degradation have not yet been identified. A number of studies identified *Arabidopsis microRNAs* that are regulated by nitrogen (Gifford et al., 2008; Pant et al., 2009; Zhao et al., 2011; Liang et al., 2012), but most *microRNAs* are down-regulated by nitrogen starvation. Only few, such as *miR160, miR826, miR839,* and *miR846,* with primary targets in root architecture genes, but not targeting *Nitr2.1* and *NIR1*, were up-regulated when N is missing (Liang et al., 2012). Due to their small size of *miRNAs*, the microarray technology and RNA-extraction method used in this work were not suitable to detect differences of mature *miRNA* abundances.

PROTEIN PHOSPHORYLATION

Remarkably, although the considerable nitrate depletion transcriptome response, the proteomic response to nitrate depletion on proteome level was less definite, particularly in comparison with the strong perturbations observed in ammonium depleted roots. A number of interesting, early responding phosphorylation sites in metabolic and assimilatory genes were also identified. Protein phosphorylation was shown to be involved in the nitrate response using inhibitors (Sueyoshi et al., 1999) and phosphorylation and de-phosphorylation events in nitrate assimilation genes were also detected with nitrate resupply to starved seedlings (Engelsberger and Schulze, 2012). Most notable were the phosphorylation sites in NRT2.1, the major high affinity nitrate transporter in roots that requires nitrate for expression, which had a moderately transiently decreased phosphorylation, followed by an increase. Other phosphorylation sites

were unexpectedly also responding to ammonium depletion in NRT2.1. Furthermore, the nitrate reductase NIA2 was phosphorylated in the highly conserved Ser-534. This phosphorylation site has a well-known inhibitory function, as it allows the binding of an inhibitory 14-3-3 protein (Bachmann et al., 1996; Su et al., 1996), indicating that part of nitrate reductase was inhibited already under sufficient nitrate supply, which did not massively change after nitrate deprivation.

Several receptor-like kinases were identified with abundance changes or phosphorylation changes in response to the lack of external nitrate (Table 3-4). These, in addition to the transceptor NRT1.1/NFP6.3 (Figure 4-1), are candidates for further nitrate signaling across the plasma membrane, whose phosphorylation changes were not detected in this data. Under nitrate nutrition, several unknown phospho-peptides significantly differing in abundance from ammonium-adapted plants were identified. A closer view on these proteins (AT1G80180, AT1G15400) identified similar phospho-peptide target sequences (Figure 3-22), which were reported as substrates of MAP-kinases (Sörensson et al., 2012), uncovering a possible link to a nitrate-dependent MAP-Kinase signaling cascade.

4.2 Responses to ammonium deprivation

The responses in ammonium adapted plants, irrespective of the ecotype used, were clearly differing from those observed in nitrate adapted roots.

TRANSCRIPTOME RESPONSES

The deprivation of ammonium induced only a minor, partially transient transcriptional response, which was identified specifically after clearing for unspecific genes, which were probably regulated due to the mechanical stress elicited by the transfer of the roots to new nutrient solutions (Table 3-1, Figure 3-11). Although a signaling role of local ammonium in lateral root growth in barley is evident from classical experiments (Drew, 1975) and AMT1.3 was identified as being involved in “sensing” ammonium and positioning of higher order lateral root primordia

in *Arabidopsis* (Lima et al., 2010), a rapid transcriptional response after "sensing" the lack of external ammonium is apparently lacking, even after 3 h of deprivation (Table 3-1).

It should be noted that the withdrawal of external ammonium did not reduce the tissue ammonium concentrations (Figure 3-3), so it is assumable that the cytoplasmic ammonium concentrations remained constant at least over the time period analyzed in this work. Probably deaminase reactions and passive ammonium release from the vacuole via aquaporins (Loqué et al., 2005) buffer cytoplasmic (and tissue) ammonium concentrations, despite ongoing ammonium assimilation. Thus, the observations made in ammonium adapted plants argue against a rapid sensing of external ammonium, e.g. by AMTs, as no rapid transcriptional response was observed compared to the withdrawal of nitrate. If gene expression is regulated by internal ammonium status, this was possibly hampered by the stability of the internal ammonium concentration during the three hours of the experiment. On the other hand, if AMTs were involved in sensing the loss of external ammonium, the C-terminal phosphorylated serine residues (Table 3-4), which have no known function yet, could be candidates for being involved in the (slow) transmission of such a putative ammonium deprivation signal.

(PHOSPHO-)PROTEOME RESPONSES

In contrast to the transcriptional response, ammonium deprivation elicited a large transient effect on the (phospho)-proteome, which was strongly associated with transmembrane pH-adjustments, maintenance of the cation-anion homeostasis and osmotic adjustments. The response to ammonium deprivation targeted H^+, NH_4^+ and K^+ transporters, in addition to water channels and a nitrate transporter, but also respiratory NADPH oxidases, which are involved in K^+ homeostasis signaling (Table 3-4).

With ammonium deprivation, the two isoforms of the plasma membrane proton pumps AHA1 (At2g18960) and AHA2 (At4g30190) were inhibited by dephosphorylation at the C-terminal threonines (Thr-948 and Thr-947, respectively, Table 3-2). These phosphorylation sites are involved in activating the proton pumps, and serve as binding site for 14-3-3 proteins (Duby et al., 2009). In AHA2, a second ammonium responsive phosphorylation site (Thr-881) was

also down-regulated by ammonium deprivation. This site is known to be phosphorylated by the kinase PSY1R, which also results in an activation of the proton pump (Fuglsang et al., 2014). Plasma membrane proton pumps are thus massively activated during sole ammonium supply, but they are immediately post-translationally shut off when ammonium is omitted from the external medium (Table 3-2). This is well in agreement with the acidifying role of ammonium nutrition on the rhizosphere. Active proton pumps act in concert with plasma membrane K^+/H^+ exchangers, such as CHX17 and CHX18, which were predominantly or exclusively identified under ammonium nutrition. These endomembrane and plasma membrane localized K^+/H^+ exchangers link intracellular pH with potassium homeostasis (Chanroj et al., 2013). The N-terminal half of CHX17 protein harbors the ion transport protein, while the C-terminus is involved in the subcellular localization of the protein (Chanroj et al., 2013). Potential transient differential phosphorylation of CHX17 at Ser-819 in the C-terminus after ammonium deprivation may be related to trafficking and subcellular localization of CHX17, rather than its transport activity. Besides their potential relevance for ammonium nutrition, *CHX17* and *CHX18* transcripts were both up-regulated with nitrate deprivation (significantly in Tsu-0, Appendix: Table A-2) although ammonium was not re-supplied. Together with the nitrate transporter *NRT2.5*, which was also up-regulated, these transcripts were seemingly suppressed by nitrate availability rather than being induced by ammonium.

Interestingly, the inhibitory phosphorylation site in the C-terminus of the ammonium transporter AMT1.1 was found to be phosphorylated after adaptation to ammonium nutrition, but the phosphorylation at this site was even increased after short-term ammonium deprivation (Table 3-4). As phosphorylation of this site inhibits ammonium transport (Lanquar et al., 2009), this may indicate that roots are able to diminish ammonium efflux and NH_4^+ release (via AMT1.1) to the apoplast, after withdrawal of external ammonium.

The differential phosphorylation in glutamate decarboxylase 1 (GAD1) after removal of ammonium may also be related to immediate responses to combat cation and pH stress. GADs

catalyze the conversion of L-glutamate to gamma-aminobutyric acid, a potential stress-signaling compound that accumulates with several stresses (Kinnersley and Turano, 2010). Enzymes in the primary metabolism were also targeted by phosphorylation. The conserved Ser-11 of the phosphoenolpyruvate carboxylase AtPPC1 (At1g53310) in the N-terminus was dephosphorylated under ammonium deprivation. It is known that this site acts as regulatory, activating site of the important enzyme involved in glycolysis (Gregory et al., 2009).

Although the plants were never supplied with urea, the urea transporter DUR3 was strongly up-regulated in ammonium adapted plants (Table A-1). Nitrate and ammonium supply usually suppress DUR3 expression (Kojima et al., 2007). Moreover, a phosphorylation site of DUR3 which was already identified in *Arabidopsis* cell suspension cultures (Hem et al., 2007), responded rapidly to ammonium withdrawal, supposing a function of this transporter in ammonium signaling.

It is worth mentioning that the major perturbation of the proteome and phospho-proteome by ammonium deprivation was only transient (Figure 3-20, Table 3-4) and had little consequence for ammonium-related gene expression, although a minor transient perturbation of gene expression was detected (Figure 3-11, Table 3-1). Although plasma membrane receptors might recognize the loss of ammonium, it is also possible that the coupled pH and cation imbalances are causal for the observed transient phospho-proteomic responses. Overall, these datasets provide little evidence for direct external ammonium recognition, but candidate receptors for the transient adjustment of the plasma membrane pH gradient, cation balance and osmotic homeostasis may be represented by the newly identified, differentially phosphorylated leucin-rich repeat protein kinase members.

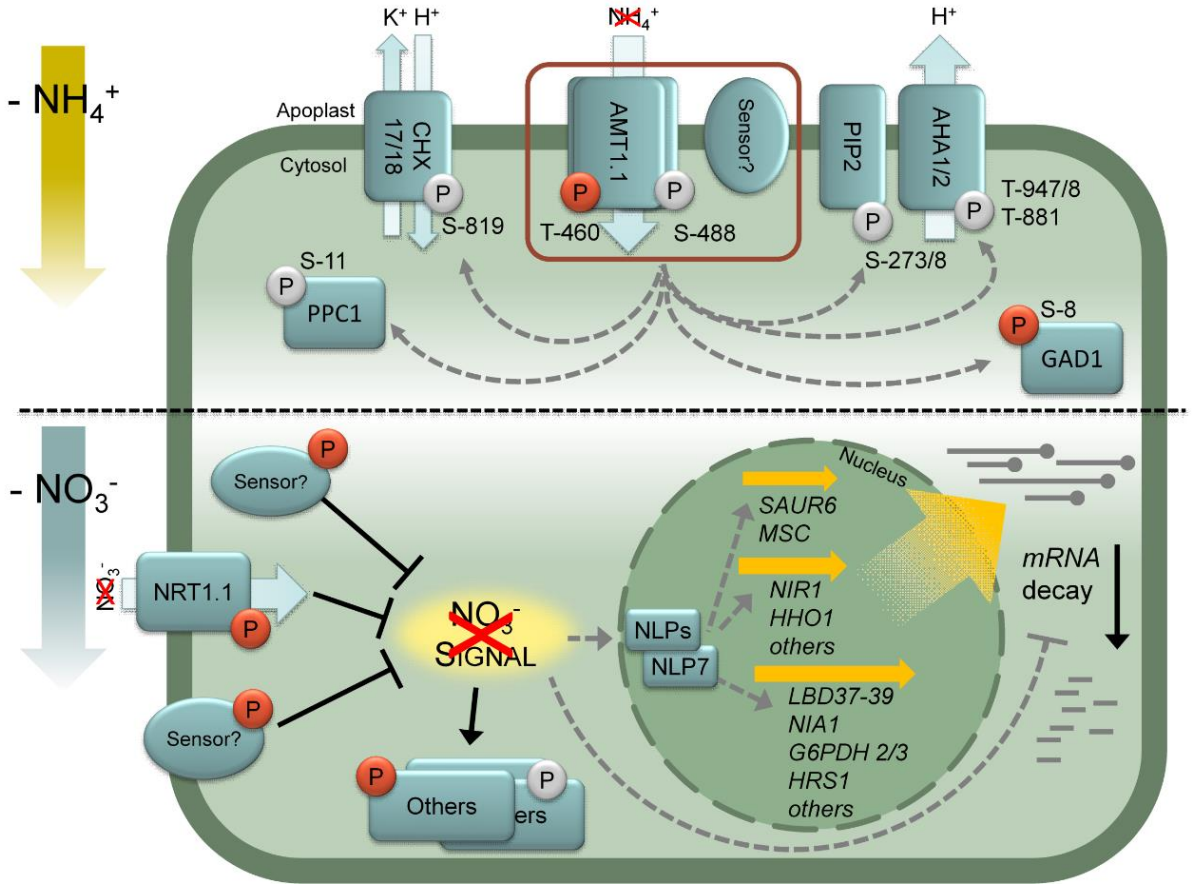

Figure 4-1: Schematic summary of the cellular responses to NH_4^+- or NO_3^--deprivation.
External nitrate deprivation and the early drop of internal nitrate concentration is sensed by membrane-bound (e.g. NRT1.1/NPF6.3) and putative internal/membrane-borne sensors and results in a decline of the nitrate signal. Consequently, well-known nitrate responsive genes, including LBD37/38/39, HRS1/HHO1, a mitochondrial substrate carrier (MSC), SAUR6 are rapidly and sustainably repressed. During 3 hours more genes, like Nitrate/Nitrite reductase 1 and Glucose-6-Phosphate dehydrogenases 2/3 (G6PDH) are suppressed. The missing nitrate signal overrides the nitrate dependent, NLP6/7 promoted expression of these genes. Some transcripts are possibly stabilized by nitrate; of these, mRNA decay is increased by nitrate depletion. Moreover, the depletion response is transduced to other proteins over phosphorylation-/dephosphorylation events (red/grey P) for integration and alleged metabolic adjustments. Ammonium deprivation does not affect gene expression levels, but has a major transient effect on the plasma membrane transporter phospho-proteome. Although there is few evidence for direct external sensing (e.g. by AMT1.1 or receptors), ammonium deprivation is rapidly transduced (dashed arrows) into phosphorylation-/dephosphorylation events of proton pumps (AHA1/2), aquaporins (PIP2.2/2.7) and K+/H+-exchangers (CHX17/18). Phosphorylation of glutamate decarboxylase GAD1 and dephosphorylation of phosphoenolpyruvate carboxylase PPC1 form putative regulatory links of ammonium depletion sensing to stress signaling and major carbohydrate metabolism, respectively.

4.3 Transcriptome differences in Tsu-0 and Col-0

The root transcriptomes of the two most contrasting accessions Col-0 and Tsu-0 were compared to identify genes determining differences of NUE. Differentially expressed genes involved in nitrogen uptake, assimilation, metabolism, signaling and regulation would provide causes for a better nitrogen uptake and use efficiency of Tsu-0. For example, ecotype specific differential expression of several nitrate transporters and assimilation genes was observed in the ecotypes Col-0, Ga-0, Sha and Ws-0 (North et al., 2009). In the PCA the high transcriptome variance explained by the accessions demonstrated, that many transcripts were identified specifically overrepresented in Tsu-0 or Col-0, respectively. But the differences were mostly identified in other functional gene categories than the ones mentioned above. Thus, the transcriptome-analysis provided a complex view considering nitrogen nutrition and NUE. Clearly, differences in NUE between Col-0 and Tsu-0 are to a lesser extent determined by the N-content (N-%) and the genes influencing this factor. Tsu-0 has been characterized as an ecotype with an exceptional biomass production with highly efficient carbon fixation as possible reason for its high NUE (Chardon et al., 2010). Differences would be expected rather in biomass influencing gene categories. But biomass is a complex, quantitative trait which is influenced by thousands of genes, which makes it challenging to decipher single contributing genes and their function.

In this work only plant root transcriptomes were compared in order to identify genes in the root which serves as interface between soil and plant, where nutrients are exchanged and translocated. In fact, several functional gene classes were identified to be overrepresented in to the opposite ecotype. Secondary metabolism related genes involved in isoprenoid/terpenoid synthesis were over-represented in Tsu-0 while in Col-0 stress-related genes were over-represented. But any influence of these functional gene classes expressed in plant roots on NUE in form of influencing N-% or biomass related processes stays speculative. While biomass is mainly determined by genes involved in the primary metabolism with photosynthesis, tricarboxylic acid cycle and hormone related processes (Lisec et al., 2008) in aboveground tissues

of the plants it is likely that clearer alterations between both ecotypes would be detectable in an analysis of their shoot transcriptomes.

Nevertheless, few N-nutrition related genes had different steady-state expression in the roots of Col-0 and Tsu-0. The high-affinity nitrate transporter *NRT2.4* (Kiba et al., 2012) was higher expressed in nitrate adapted roots of Tsu-0 and may have an influence on its higher nitrate preference in high-affinity ranged nitrate concentrations. In contrast, in Col-0 the half of all annotated *Arabidopsis* high-affinity ammonium transporters (*AMT1;1, AMT1;2* and *AMT2*) were higher expressed in both, nitrate and ammonium fed plants. A former M.Sc. thesis showed Col-0 having a slightly higher preference for ammonium, when given in the same amounts as nitrate (see Figure 1-3), which may be explained by the higher expression of these AMTs. However, differences in two glutamine synthase genes (*GLN1.1* and *GLN1.3*) were significant but negligible. But as these findings in the transcriptomes resemble only snapshots of an adaptation after 5 days to a single nitrogen-form it is ambiguous to which extent these differences in gene expression explain the nitrate preference of Tsu-0.

In the nitrate depletion response within 3 hours very few differences of generally unknown genes were detected between both genotypes. The chosen time-course was apparently too short to identify here clear alterations. Probably the time-points were too early for identification of alterations, otherwise it is also possible that differences were simply not existing.

5 CONCLUSION AND OUTLOOK

The identification of the earliest responses of plants with nitrogen deficiency has a high value for agriculture. When transferred to crop plants, some of these findings could help improve agricultural systems by supplementing a needs-based N-fertilization with an additional indicator: the plant itself. To some extent this is done by getting information about the N status over the chlorophyll content derived from SPAD readings (single-photon avalanche diode). These measurements vary widely depending on environmental factors and the crop plant measured (Xiong et al., 2015). By recognizing the N-status of the plant even earlier would help to improve the plant long time before clearly visible severe nitrogen deficiency symptoms, *e.g.* pale green leaves and yellowing of older leaves appear. The earlier symptoms of a beginning nutrient deficiency are recognized in a plant, the faster a farmer can react and the lesser the negative effects of the deficiency affect the crop yield. Numerous genes respond within hours towards the withdrawal of nitrogen, particularly towards nitrate depletion. Unexpectedly, most of them are down-regulated. But some, like genes coding for two unknown proteins or the nitrate transporter NRT2.5 are rapidly up-regulated with N-depletion. By using the bioreporter principle in a biotechnological approach, promoter regions of these genes could be utilized to express an appropriate reporter gene depending on the N-status of the plant. Ideally, these promoter sequences are ammonium or nitrate specific and the reporter genes are easily to detect. But these concepts are far away from development.

Data gathered with –omics approaches are highly complex and the information hidden within leaves great scope for interpretation. But they offer also a great number of open questions and thus starting points for further experiments, projects or whole studies. As a consequence, this work is understood as cutting-edge for further studies of the nitrogen or nitrate-depletion response, respectively.

The approaches of this work led to unexpected results and not all observations made can be explained in this work. Initially, a characterization of loss-of-function mutant lines of

early responding genes were planned as follow-up experiments. Due to their unexpected down-regulation, results of experiments are challenging to interpret with plants in which such down-regulated genes are defective. Obviously, most genes are relevant for processes where the nitrogen, in most situations, nitrate is present – and not directly related for a N-form specific depletion response. Nevertheless, it would be of great interest to ascertain the roles of *SAUR6*, the *mitochondrial substrate carrier* and several undescribed proteins play with respect to the nitrate response and why these genes are so rapidly degraded with nitrate withdrawal. Here, loss-of-function mutants are suitable for their characterization.

The question, why these genes are down-regulated with such rate is not sufficiently answered. Two possibilities may explain the observed processes: active degradation over *miRNAs* or decline of a putative nitrate dependent *m*RNA stabilization. The extraction method used in this work was not suitable for the preparation of small RNAs and the microarrays were not suitable for detection of (mature) *miRNAs* (precursor *miRNA*-probes are spotted on these arrays). To gather clearer information about the possible functions of these in the depletion response, sequencing of the RNA degradome of nitrate adapted roots followed by starvation would help to identify target sequences of *miRNAs*. Also further bioinformatics approaches, *e.g.* binding-motif searches may help to reveal possible regulatory *miRNAs* involved in the early nitrate depletion response.

In many transcriptome studies hundreds of transcripts appear which code for unassigned proteins with only few or without known sequence motifs, pseudogenes or transposable elements. Although hundreds of these transcripts are differentially expressed to certain stimuli, such as in this work against nitrogen depletion, from most their function is completely unknown. Thus, the resulting proteins may also have a function in structural adaptations or the pathways related to the stimulus. In future work, these transcripts should be analyzed more in detail, as they still keep many open questions which are deemed to become answered.

To date, the functions and the regulation of LBD37-39 transcription factors are not completely clarified. In particular, the deviant expression pattern of *LBD38* after ammonium depletion, which differs from the other related LBDs and the cause for the rapid down-regulation in the course of nitrate drop are remarkable observations which should be studied in the future. LBD knock-out mutant lines are available. Hence, crossbreeding these lines to double and triple LBD-knock-outs could diminish redundancy effects and will help to elucidate the LBD positions in the nitrate response.

The (phospho-)proteomic analyses added an additional dimension of complexity to the observations. Surprisingly, the severe transcriptomic impact of nitrate depletion was not mirrored on proteomic level. Several interesting phosphorylation changes in receptor kinases and nitrate transporters and other membrane proteins may be involved in the transmission of depletion signals or concentration. Phosphorylation changes were not observed in NRT1.1/NPF6.3, the major nitrate receptor. But differences in the nitrate depletion response would be expectable in a *nrt1.1* loss-of-function-mutant or with mutations in certain signaling amino acid residues, such as Thr-101 (Ho et al., 2009). Plants which lack this sensor have distinct expression patterns upon nitrate re-supply (Hu et al., 2009), which is also expectable in the nitrate depletion response. The NRT1.1/NPF6.3 dependency of the early depletion responsive genes should be ascertained with further transcriptome studies.

The abundant phosphorylation changes in transporters, channels, aquaporins and proton pumps of ammonium adapted plants stay in contrast to the almost absent transcriptome response, but also towards rather weak changes in nitrate adapted plants. A functional characterization of some of these phosphorylation sites, as reported for AMTs, H^+-ATPases and their interactors (Duby et al., 2009; Lanquar et al., 2009; Fuglsang et al., 2014) would increase our knowledge in the perception and signal transduction of changes in the nitrogen supply.

Although the profound transcriptome differences between Tsu-0 and Col-0 rather few genes in the root transcriptome were considered for the higher NUE observed for Tsu-0. Due to missing transcriptome data of the shoot, where most biomass related processes occur, such

data should be surveyed for both genotypes under different nitrogen regimes. In addition, metabolome analyses would add more detailed information to specific pathways which differentiate between both ecotypes. Such information would probably confirm the use Tsu-0 as a reference genotype for future nitrogen related studies with *Arabidopsis thaliana*.

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APPENDIX

Supplemental Tables

Table A-1: Genes up-regulated in nitrate or ammonium adapted plants

Significantly differentially expressed genes between ammonium and nitrate adapted Col-0 plants at time-point 0 (log2FC > 2 or < -2, FDR<0.05). Positive log_2-FC values indicate genes higher expressed in nitrate adapted plants, negative log_2-FC values were stronger expressed in ammonium adapted plants.

AGI Locus ID	Gene Description	NO_3^- vs. NH_4^+ 0 min	FDR
Upregulated under nitrate nutrition			
At5g26200.1	Mitochondrial substrate carrier family protein	6.53	1.000E-06
At1g77760.1	nitrate reductase 1	6.49	0.000E+00
At5g01740.1	Nuclear transport factor 2 (NTF2) family protein	6.45	1.000E-06
At5g10210.1	Unknown Protein	5.69	8.000E-06
At3g25790.1	myb-like transcription factor family protein HHO1	5.51	1.600E-05
At2g22122.1	Unknown Protein	5.11	1.000E-06
At2g21210.1	SAUR-like auxin-responsive protein family SAUR6	5.04	0.000E+00
At2g31081.1	CLAVATA3/ESR-RELATED 4	4.48	2.600E-05
At4g39675.1	Unknown Protein	4.34	2.561E-03
At4g29905.1	Unknown Protein	4.07	1.100E-05
At1g13300.1	myb-like transcription factor family protein HRS1	4.06	2.600E-05
At5g63160.1	BTB and TAZ domain protein 1	4.06	4.790E-04
At1g68238.1	Unknown Protein	4.02	2.300E-05
At3g48360.1	BTB and TAZ domain protein 2	4.01	8.800E-05
At1g49500.1	Unknown Protein	3.84	2.500E-05
At5g52790.1	Unknown Protein	3.71	1.000E-06
At2g15620.1	nitrite reductase 1	3.56	6.000E-06
At5g62720.1	Nitrite transporter Nitr2.1	3.53	0.000E+00
At5g19600.1	sulfate transporter 3;5	3.40	1.400E-05
At3g02850.1	STELAR K+ outward rectifier	3.22	6.800E-05
At1g80380.2	P-loop containing nucleoside triphosphate hydrolases superfamily protein	3.18	6.500E-05
At1g02820.1	Late embryogenesis abundant 3 (LEA3) family protein	3.13	1.120E-04
At1g73600.2	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	3.09	4.310E-04
At1g73602.1	conserved peptide upstream open reading frame 32	3.06	4.790E-04
At5g53980.1	homeobox protein 52	3.02	2.940E-04
At3g17180.1	serine carboxypeptidase-like 33	3.00	1.000E-05
At4g02380.1	senescence-associated gene 21	2.95	1.670E-04
At4g33960.1	Unknown Protein	2.76	2.150E-04
At1g24280.1	glucose-6-phosphate dehydrogenase 3	2.74	2.384E-03
At3g46880.1	Unknown Protein	2.72	2.550E-04
At2g16060.1	hemoglobin 1	2.72	4.565E-03
At3g63110.1	isopentenyltransferase 3	2.71	3.570E-04
At2g26980.4	CBL-interacting protein kinase 3	2.62	1.400E-05
At2g44220.1	Unknown Protein	2.59	2.055E-03
At1g73165.1	CLAVATA3/ESR-RELATED 1	2.59	3.000E-06
At5g67420.1	LOB domain-containing protein 37	2.56	1.627E-03
At5g07680.1	NAC domain containing protein 80	2.56	1.620E-04
At2g31082.1	CLAVATA3/ESR-RELATED 7	2.55	1.060E-04
At4g18510.1	CLAVATA3/ESR-related 2	2.55	3.900E-03
At1g60050.1	Nodulin MtN21 /EamA-like transporter family protein	2.44	8.800E-05
At5g15830.1	basic leucine-zipper 3	2.36	5.860E-03

Continued on next page

At4g30110.1	heavy metal ATPase 2	2.34	2.600E-04
At5g14120.1	Major facilitator superfamily protein	2.27	1.982E-02
At4g03500.1	Ankyrin repeat family protein	2.23	3.300E-05
At5g08360.1	Unknown Protein	2.22	4.760E-04
At4g31330.1	Unknown Protein	2.17	2.550E-04
At1g37130.1	nitrate reductase 2	2.15	1.700E-05
At5g24120.1	sigma factor E	2.15	1.188E-03
At3g26960.1	Pollen Ole e 1 allergen and extensin family protein	2.12	2.267E-02
At5g23980.1	ferric reduction oxidase 4	2.12	9.743E-03
At4g26050.1	plant intracellular ras group-related LRR 8	2.10	3.464E-03
At5g19040.1	isopentenyltransferase 5	2.05	8.870E-04
At3g30415.1	pseudogene, putative urophorphyrin III methylase	2.05	4.500E-04
Upregulated under ammonium nutrition			
At3g45130.1	lanosterol synthase 1	-2.02	2.561E-03
At5g50760.1	SAUR-like auxin-responsive protein family	-2.04	5.620E-04
At4g02280.1	sucrose synthase 3	-2.04	3.246E-02
At4g25760.1	glutamine dumper 2	-2.09	6.800E-05
At1g21890.1	nodulin MtN21 /EamA-like transporter family protein	-2.10	1.804E-02
At2g17500.1	Auxin efflux carrier family protein	-2.13	7.200E-04
At1g03106.1	Unknown Protein	-2.17	3.565E-03
At1g35186.1	transposable element gene	-2.23	1.000E-06
At1g80320.1	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily	-2.25	1.504E-02
At1g64370.1	Unknown Protein	-2.32	4.790E-04
At4g33040.1	Thioredoxin superfamily protein	-2.47	9.200E-05
At5g60770.1	nitrate transporter 2.4	-2.51	3.565E-03
At5g64550.1	loricrin-related	-2.53	1.900E-05
At3g47340.1	glutamine-dependent asparagine synthase 1	-2.59	4.744E-02
At1g06225.1	CLAVATA3/ESR-RELATED 3	-2.65	1.538E-03
At2g33710.1	Integrase-type DNA-binding superfamily protein	-2.82	1.250E-04
At1g68880.1	basic leucine-zipper 8	-2.83	2.150E-04
At2g43500.1	Plant regulator RWP-RK family protein	-2.87	5.564E-03
At2g26695.1	Ran BP2/NZF zinc finger-like superfamily protein	-2.96	3.442E-03
At2g33710.2	Integrase-type DNA-binding superfamily protein	-3.07	4.100E-05
At4g13420.1	high affinity K+ transporter 5	-3.37	1.710E-04
At4g39795.1	Protein of unknown function (DUF581)	-3.46	1.900E-05
At5g04120.1	Phosphoglycerate mutase family protein	-3.93	2.550E-04
At4g01390.1	TRAF-like family protein	-4.00	1.800E-05
At4g32950.1	Protein phosphatase 2C family protein	-4.14	0.000E+00
At4g28040.1	Nodulin MtN21 /EamA-like transporter family protein	-4.16	6.600E-05
At5g41610.1	Cation/H+ exchanger 18	-4.50	0.000E+00
At2g39510.1	nodulin MtN21 /EamA-like transporter family protein	-4.54	2.550E-04
At5g45380.1	Urea transmembrane transporter, DUR3	-4.79	0.000E+00
At1g12940.1	nitrate transporter2.5	-5.03	0.000E+00
At4g23700.1	cation/H+ exchanger 17	-5.22	8.660E-04
At1g73220.1	organic cation/carnitine transporter1	-5.45	9.420E-04

Table A-2: Early differentially expressed genes to –N in *Tsu-0*

N-Form specific early responsive transcripts (LFC > 1.5 and < -1.5 for 180 min) filtered for control conditions. Significant (*FDR*<0.05) changes versus time point 0 are boldfaced. Separate filtering for 15 min was omitted. Positive values in column "NO_3^- vs. NH_4^+ 0 min" indicate the gene is up-regulated under nitrate-adapted conditions while negative values show an up-regulation under ammonium-adapted conditions. LBD38 is shown for demonstrative purpose although its LFC is > -1.5.

AGI Locus ID	Transcript Description	Log$_2$-fold change (LFC)				
		NO_3^- vs. NH_4^+ 0 min	NO_3^- - adapted		NH_4^+-adapted	
			15 min	180 min	15 min	180 min
Nitrate adapted plants						
AT4G23700.1	Cation/H+ exchanger 17, CHX17	**-6.33**	0.69	**3.51**	-0.14	-1.16
AT5G26920.1	Cam-binding protein 60-like G	-1.71	1.27	**2.89**	0.83	1.51
AT4G39795.1	unknown protein	**-3.69**	0.46	**2.79**	-0.10	-0.17
AT1G64590.1	NAD(P)-binding Rossmann-fold superfamily protein	**-1.83**	0.65	**2.60**	-0.35	0.06
AT5G37840.1	unknown protein	-0.42	0.35	**2.43**	-0.01	0.81
AT1G67810.1	sulfur E2	**-2.43**	-0.01	**2.32**	-0.12	0.44
AT2G02990.1	ribonuclease 1	-1.32	0.25	**2.22**	-0.21	1.79
AT4G33040.1	Thioredoxin	**-2.93**	0.37	**1.99**	0.04	0.24
AT5G26690.1	Heavy metal transport/detoxification	**-1.47**	0.39	**1.96**	-0.10	1.00
AT1G17170.1	glutathione S-transferase TAU 24	-1.46	0.86	**1.83**	-0.12	1.04
AT1G12940.1	nitrate transporter2.5	**-4.84**	0.23	**1.83**	0.02	0.64
AT1G79320.1	metacaspase 6	**-1.46**	-0.11	**1.79**	-0.23	0.65
AT1G17180.1	glutathione S-transferase TAU 25	-0.96	0.35	**1.76**	0.05	0.71
AT3G28210.1	zinc finger (AN1-like) family protein	-0.47	1.05	**1.73**	0.41	1.36
AT4G13420.1	high affinity K+ transporter 5	**-4.73**	0.09	**1.67**	0.19	-0.12
AT1G22070.1	TGA1A-related gene 3	**-1.17**	0.20	**1.66**	0.04	**0.78**
AT5G52670.1	Copper transport protein family	0.44	1.46	**1.65**	1.32	1.77
AT5G59490.1	Haloacid dehalogenase-like hydrolase	-0.74	0.97	**1.62**	0.49	0.95
AT4G17670.1	unknown protein	**-1.18**	-0.08	**1.62**	-0.42	0.86
AT3G08040.1	MATE efflux family protein	-0.63	0.51	**1.58**	-0.42	0.57
AT5G41610.1	Cation/H+ exchanger 18, CHX18	**-5.16**	-0.01	**1.56**	-0.02	-0.28
AT5G49850.1	Mannose-binding lectin superfamily protein	-0.55	0.25	**1.56**	0.22	**1.72**
AT5G25930.1	LRR-Protein kinase family	-0.92	0.63	**1.52**	0.43	0.87
AT3G14060.1	unknown protein	-0.27	-0.09	**-1.50**	0.11	**-1.37**
AT1G66130.1	NAD(P)-binding Rossmann-fold	**0.89**	-0.29	**-1.51**	-0.26	-0.74
AT1G22500.1	RING/U-box superfamily protein	**1.65**	-0.46	**-1.51**	-0.13	-0.10
AT5G47560.1	tonoplast dicarboxylate transporter	0.12	-0.36	**-1.51**	-0.10	-0.72
AT2G31082.1	CLAVATA3/ESR-RELATED 7	**3.15**	-0.23	**-1.51**	0.04	0.06
AT1G49860.1	glutathione S-transferase (class phi) 14	**1.82**	-0.03	**-1.52**	0.03	0.24
AT4G33960.1	unknown protein	**3.26**	-0.39	**-1.53**	-0.24	0.30
AT4G02920.2	unknown protein	**1.10**	-0.41	**-1.53**	-0.08	-0.17
AT3G49760.1	basic leucine-zipper 5	0.69	-0.61	**-1.55**	-0.14	-0.77
AT5G01840.1	ovate family protein 1	0.75	-0.26	**-1.55**	-0.13	-0.24
AT3G57040.1	response regulator 9	**2.05**	-0.75	**-1.55**	-0.18	-0.12
AT5G19040.1	isopentenyltransferase 5	**2.00**	-0.65	**-1.55**	-0.29	-0.32
AT1G78050.1	Phosphoglycerate mutase	**1.38**	-0.41	**-1.55**	0.20	0.25
AT5G58360.1	ovate family protein 3	**1.03**	-0.76	**-1.56**	-0.10	-0.56
AT5G27360.1	Major facilitator superfamily protein	**1.63**	-0.38	**-1.58**	-0.25	-0.21
AT2G41310.1	response regulator 3	**1.56**	-0.69	**-1.58**	-0.12	-0.41
AT5G52790.1	unknown protein	**2.79**	-0.18	**-1.60**	-0.22	0.07
AT1G19050.1	response regulator 7	**1.44**	-0.46	**-1.60**	0.26	-0.11
AT1G70780.1	unknown protein	**1.72**	-0.37	**-1.63**	-0.08	-0.30
AT1G03850.1	Glutaredoxin family protein	**1.19**	-0.09	**-1.64**	-0.05	-0.69
AT5G27350.1	Major facilitator superfamily protein	**2.00**	-0.40	**-1.66**	-0.20	-0.28
AT5G65207.1	unknown protein	**1.90**	-0.45	**-1.69**	0.42	0.60
AT3G13404.1	unknown protein	**2.34**	-0.33	**-1.69**	-0.17	-0.32
AT1G23160.1	Auxin-responsive GH3 family protein	**1.76**	-0.26	**-1.70**	-0.19	-0.58
AT2G43445.1	F-box containing protein	**2.08**	-0.63	**-1.71**	0.06	-0.06
AT5G26010.1	Protein phosphatase 2C family protein	0.88	-0.45	**-1.71**	0.14	-0.22
AT5G15830.1	basic leucine-zipper 3	**1.88**	-0.81	**-1.73**	-0.23	-0.65

Continued on next page

AT3G30405.1	transposable element gene	**1.90**	-0.55	**-1.78**	0.43	0.31
AT3G46880.1	unknown protein	**2.19**	-0.15	**-1.78**	-0.18	-0.03
AT1G11080.2	serine carboxypeptidase-like 31	0.32	-0.36	**-1.79**	0.06	-0.26
AT5G62920.1	response regulator 6	**1.16**	-1.08	**-1.80**	-0.38	-0.33
AT2G17820.1	histidine kinase 1	**1.50**	-0.38	**-1.81**	-0.05	-0.06
AT2G25090.1	CBL-interacting protein kinase 16	0.26	-0.45	**-1.81**	0.35	-0.85
AT3G63110.1	isopentenyltransferase 3	**2.90**	-0.68	**-1.82**	0.13	-0.01
AT3G48100.1	response regulator 5	**1.59**	-0.52	**-1.84**	0.04	-0.42
AT3G30415.1	unknown protein	**2.18**	-0.37	**-1.85**	0.28	0.34
AT1G16170.1	unknown protein	**2.43**	-0.36	**-1.86**	0.05	-0.08
AT5G19970.1	unknown protein	0.72	**-1.24**	**-1.86**	-0.62	**-1.29**
AT1G03850.2	Glutaredoxin family protein	**0.88**	-0.42	**-1.87**	-0.01	-0.61
AT2G47160.2	HCO3- transporter family	**2.50**	-0.50	**-1.87**	-0.06	**0.75**
AT5G24120.1	sigma factor E	**2.42**	0.09	**-1.88**	-0.09	-0.22
AT5G13110.1	glucose-6-phosphate dehydrogenase 2	**2.13**	-0.31	**-1.89**	0.20	0.43
AT4G25835.1	unknown Protein	**1.67**	**-0.81**	**-1.90**	0.12	-0.53
AT1G63940.2	monodehydroascorbate reductase 6	**2.06**	-0.24	**-1.93**	0.10	0.19
AT5G10580.2	unknown protein	**2.48**	-0.11	**-1.93**	-0.17	0.10
AT3G48360.1	BTB and TAZ domain protein 2	**4.53**	-0.94	**-2.00**	0.10	-0.18
AT5G67420.1	LOB domain-containing protein 37, LBD37	**2.53**	**-2.00**	**-2.03**	0.89	-0.39
AT2G33550.1	Homeodomain-like superfamily protein	**1.83**	-0.79	**-2.09**	-0.02	-0.11
AT2G26980.4	CBL-interacting protein kinase 3	**2.75**	-0.47	**-2.10**	-0.06	0.11
AT3G19030.1	unknown protein	0.58	-0.49	**-2.11**	0.82	**-1.33**
AT1G78000.1	sulfate transporter 1;2	**1.15**	-0.14	**-2.12**	-0.06	-0.35
AT4G02380.1	senescence-associated gene 21	**2.70**	-0.29	**-2.13**	0.64	0.17
AT1G22150.1	sulfate transporter 1;3	**1.12**	-0.27	**-2.13**	-0.14	-0.37
AT5G62720.1	Integral membrane HPP family protein, Nitr2.1	**3.97**	-0.35	**-2.15**	-0.19	0.44
AT5G37260.1	Homeodomain-like superfamily protein	**1.16**	-0.72	**-2.17**	-0.17	**-1.79**
AT5G10210.1	unknown protein	**6.39**	-1.04	**-2.17**	1.00	0.78
AT4G37540.1	LOB domain-containing protein 39, LBD39	**1.57**	**-1.59**	**-2.20**	0.09	-0.79
AT1G68238.1	unknown protein	**5.40**	-0.51	**-2.25**	0.01	0.58
AT3G16560.1	Protein phosphatase 2C family protein	0.88	-0.66	**-2.26**	-0.11	-0.53
AT3G02850.1	STELAR K+ outward rectifier	**2.86**	-0.53	**-2.28**	-0.19	-0.09
AT5G62430.1	cycling DOF factor 1	**2.05**	-0.35	**-2.33**	-0.22	-0.63
AT5G19600.1	sulfate transporter 3;5	**4.34**	-0.44	**-2.33**	-0.29	-1.01
AT1G02820.1	Late embryogenesis abundant 3 (LEA3)	**3.06**	-0.33	**-2.36**	0.62	0.20
AT5G65030.1	unknown protein	**1.49**	**-1.59**	**-2.38**	0.54	0.16
AT1G13300.1	MYB-like transcription factor, HRS1	**4.19**	-1.22	**-2.41**	0.12	-0.15
AT5G63160.1	BTB and TAZ domain protein 1	**3.60**	-0.89	**-2.45**	1.09	-0.34
AT1G73165.1	CLAVATA3/ESR-RELATED 1	**3.67**	-0.35	**-2.48**	-0.02	0.18
AT1G80380.2	Nucleoside triphosphate hydrolases	**2.33**	-0.59	**-2.57**	0.69	-0.55
AT1G49500.1	unknown protein	**5.09**	-0.78	**-2.65**	0.48	-0.57
AT1G24280.1	glucose-6-phosphate dehydrogenase 3	**3.03**	-0.30	**-2.82**	0.46	0.40
AT2G15620.1	nitrite reductase 1, NIR1	**3.86**	-0.16	**-2.85**	0.62	0.84
AT5G07680.1	NAC domain containing protein 80	**2.22**	-0.76	**-2.91**	-0.14	-0.88
AT2G22122.1	unknown protein	**4.84**	-0.32	**-2.98**	-0.10	0.11
AT2G31081.1	CLAVATA3/ESR-RELATED 4	**5.04**	-0.33	**-3.45**	0.28	0.32
AT4G29905.1	unknown protein	**3.99**	-0.74	**-3.52**	0.07	-1.35
AT3G25790.1	MYB-like transcription factor, HHO1	**6.19**	**-1.69**	**-3.54**	0.18	-0.01
AT1G77760.1	nitrate reductase 1, NIA1	**6.83**	-0.35	**-3.76**	0.46	0.93
AT2G21210.1	SAUR-like auxin-responsive protein SAUR6	**6.02**	**-3.50**	**-4.29**	0.27	0.07
AT5G01740.1	Nuclear transport factor 2 (NTF2)	**6.44**	-1.37	**-4.50**	-0.14	0.20
AT5G26200.1	Mitochondrial substrate carrier	**6.33**	**-2.20**	**-4.51**	0.32	1.37
Ammonium adapted plants						
AT3G49940.1	*LOB domain-containing protein 38, LBD38*	***-1.06***	*-1.22*	***-1.42***	*0.05*	***-1.42***
AT5G18670.1	beta-amylase 3	-0.24	0.29	-0.96	0.43	**-1.54**
AT1G13080.1	cytochrome P450 family protein	0.14	-0.24	-1.11	-0.28	**-1.55**
AT5G37260.1	Homeodomain-like superfamily protein, AtCIR1	**1.16**	-0.72	**-2.17**	-0.17	**-1.79**

Supplemental Figures

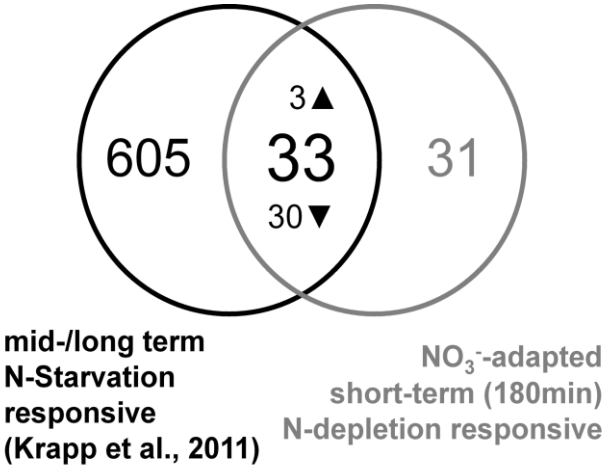

Figure A-0-1: Identified nitrate depletion responsive genes in mid-/long term -N

Comparison of genes responsive in mid-/long term N-depletion (Krapp et al., 2011) with the 64 (up- and down-regulated) responsive genes after 3 h in the dataset of Col-0 nitrate-adapted roots. Common up and down-regulated transcripts genes are separately visualized.

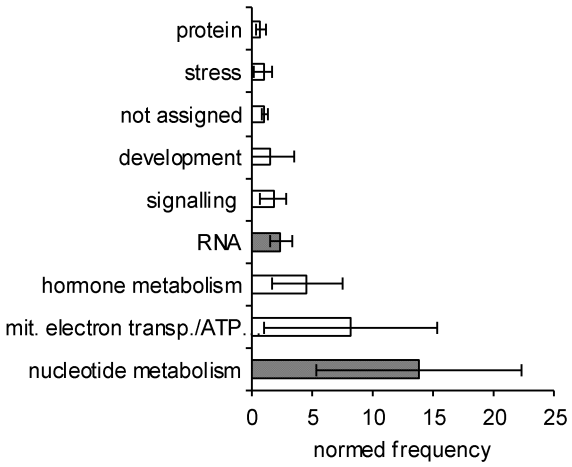

Figure A-0-2: Overrepresented functional MapMan bins in 15 min controls

All transcripts, which significantly responded after 15 min in any of the controls of ammonium and nitrate adapted Tsu-0 and Col-0 were taken into account (27 transcripts). Significantly ($p<0.05$) over-represented MapMan bins are colored grey. These involved several ethylene responsive transcription factors, calcium signaling proteins and numerous genes coding unassigned proteins. (derived from BAR Classification Super Viewer Tool)

Abbreviated R-Code for Microarray Analysis with LIMMA

```
source("http://bioconductor.org/biocLite.R")
biocLite("limma")
library("limma")

targets        <-readTargets("raw_data/targets.txt", row.names=NULL)
Rohdaten       <-read.maimages( targets_selected, path="raw_data/", source="agilent",
               green.only=TRUE, annotation=c("ProbeName", "ControlType", "SystematicName","De-
               scription", "Row", "Col", "Status"))

Rohdaten.bc    <-      backgroundCorrect(Rohdaten, method="normexp", offset=16)
Rohdaten.bc.n  <-      normalizeBetweenArrays(Rohdaten.bc, method="quantile")

Neg95          <- apply(Rohdaten.bc.n$E[Rohdaten.bc.n$genes$ControlType==-      1,],2,func-
               tion(x) quantile(x,p=0.95))
cutoff         <- matrix(1.1*neg95, nrow(Rohdaten.bc.n), ncol(Rohdaten.bc.n), byrow=TRUE)
isexpr         <- rowSums(Rohdaten.bc.n$E > cutoff) >= nrow(targets)%/%2)

y0             <- Rohdaten.bc.n[Rohdaten.bc.n$genes$ControlType==0 & isexpr,]
yave           <- avereps(y0,ID=y0$genes[,"SystematicName"])

Treatment      <- paste(y0$targets$Ecotype,y0$targets$Treatment,y0$targets$Timepoint,sep=".")
Treatment      <- factor(Treatment,levels=unique(Treatment))

Design         <- model.matrix(~0+Treatment)
colnames(design)<-unique(Treatment)

contrast.matrix<-makeContrasts(
       "Nitrate vs. Ammonium"        ="C0.NO.0-C0.NH.0",
        Col0_NO15                    ="C0.NO.15-C0.NO.0",
        Col0_NO180                   ="C0.NO.180-C0.NO.0",
        Col0_NO15vsCtrl              ="C0.NO.15-C0.NON.15",
        #[…],
        levels=design)

contrasts      <-      colnames(contrast.matrix)
fit2           <-      lmFit(yave,design)
fit2           <-      contrasts.fit(fit2,contrast.matrix)
fit2           <-      eBayes(fit2)

write.fit(fit2, file="Gene_Expression_Data_Col-0_vs_Tsu-0.txt, adjust="BH", method="separate"
```

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