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Membrane transport and long-distance translocation of urea in *Arabidopsis thaliana*

Dissertation

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1 Summary/Zusammenfassung

1.1 Summary

Urea is a soil nitrogen (N) form available to plant roots and a secondary N metabolite liberated in plant cells by protein degradation, especially during senescence. Despite the fact that urea also represents the most widespread form in N fertilizers used in agricultural plant production, membrane transporters that might contribute to urea uptake in plant roots or urea retranslocation in senescent leaves have so far been characterized only in heterologous systems.

The first part of the thesis investigated a role of the H^+/urea cotransporter AtDUR3 in N nutrition of *Arabidopsis thaliana* plants. T-DNA insertion lines with a defective expression in *AtDUR3* showed impaired growth on urea as a sole nitrogen source. In transgenic lines expressing an *AtDUR3-promoter-GFP* construct, promoter activity was upregulated under N deficiency and localized to the rhizodermis, including root hairs, as well as to the cortex in more basal root zones. The AtDUR3 protein accumulated in plasma membrane-enriched protein fractions, and *AtDUR3* gene expression in N-deficient roots was repressed by ammonium and nitrate but induced after supply of urea. Higher urea accumulation in roots of wild-type plants relative to the T-DNA insertion lines confirmed that urea was the transported substrate of AtDUR3. Influx of ^{15}N-labeled urea allowed the calculation of an affinity constant of 4 µM. These results indicated that AtDUR3 is the major transporter for high-affinity urea uptake in Arabidopsis roots and suggested that the high substrate affinity of AtDUR3 reflects an adaptation to the low urea levels usually found in unfertilized soils.

A physiological function of urea and its transporters in leaves was investigated in the second part of the thesis. Currently it is unclear whether transport and metabolism of urea might limit the overall retranslocation of N during senescence. *AtDUR3* transcript levels were only slightly de-repressed under N starvation, but strongly increased in senescent leaves. Urea concentrations in leaf samples of different plant and leaf age showed a strong increase after plants turned into generative growth. In parallel, mRNA as much as the protein abundance of

AtDUR3 increased with leaf age. The analysis of leaf petiole exudates revealed that urea was indeed a translocated N form and urea-N represented approx. 13% of the total amino acid-N irrespective of the N status of the plant. Urea concentrations determined in apoplastic wash fluids supported a role of AtDUR3 in urea retrieval from the leaf apoplast, and transgenic *AtDUR3*-promoter-GUS lines indicated a localization of *AtDUR3* promoter activity in the vasculature of old leaves. Thus, AtDUR3 might keep internal urea in the cytosol by urea retrieval from the apoplast, allowing urea to be transported to the vascular bundle, where it is either passively loaded to the phloem or converted into amino acids for long-distance N translocation.

A strong daytime-dependent phenotype with shorter leaf petioles of an Arabidopsis line overexpressing *AtDUR3* led to an *in silico* analysis of the *AtDUR3* promoter sequence revealing that salicylic acid (SA) appears to induce *AtDUR3* gene expression in senescent leaves. SA is well known for its involvement in the initiation of senescence. A strongly enhanced uptake capacity for ^{15}N-labeled urea in N-sufficient Arabidopsis roots after SA pretreatment indicated that SA might be able to mimic N-deficiency conditions, paving the way to the possibility that SA builds a regulatory link between developmental and N deficiency-induced senescence.

1.2 Zusammenfassung

Harnstoff ist eine im Boden vorkommende, für Pflanzenwurzeln verfügbare Stickstoffform und ein sekundärer Stickstoffmetabolit, der durch Proteindegradation in Pflanzenzellen freigesetzt wird. Trotz der Tatsache, dass es sich bei Harnstoff um die am weitesten verbreitete Stickstoffdüngerform der agrarwirtschaftlichen Pflanzenproduktion handelt, sind Membrantransporter, die zur Harnstoffaufnahme in Pflanzenwurzeln oder zur Harnstoffretranslokation in seneszenten Blättern beitragen, bisher nur in heterologen Systemen charakterisiert.

Der erste Teil der Arbeit untersucht die Rolle des H^+/Harnstoff - Cotransporters AtDUR3 in der Stickstoffernährung in *Arabidopsis thaliana.* T-DNA Insertionslinien mit einer fehlenden Expression des *AtDUR3* Gens zeigten vermindertes Wachstum, wenn Harnstoff als einzige Stickstoffquelle angeboten wurde. Die

Promoteraktivität in transgenen Linien, die ein *AtDUR3-promoter-GFP* Konstrukt exprimieren, war unter Stickstoffmangel hochreguliert und in der Rhizodermis inklusive der Wurzelhaare lokalisiert, sowie auch im Cortex in den basaleren Wurzelzonen. Das AtDUR3 Protein wurde vorwiegend in der mit Plasmamembranen angereicherten Proteinfraktionen detektiert. In unter N-Mangel kultivierten Wurzeln war die *AtDUR3* Genexpression durch Ammonium und Nitrat reprimiert, wurde aber durch Zugabe von Harnstoff induziert. Des Weiteren bestätigte eine höhere Harnstoffakkumulation in Wurzeln von Wildtyppflanzen im Vergleich zu den T-DNA Insertionslinien, dass Harnstoff das transportierte Substrat von AtDUR3 war. Eine Affinitätskonstante von 4 µM konnte mithilfe von Influxexperimenten mit ^{15}N-markiertem Harnstoff berechnet werden. Diese Ergebnisse zeigen, dass AtDUR3 der Haupttransporter für die hochaffine Harnstoffaufnahme in Arabidopsiswurzeln ist und weisen darauf hin, dass die hohe Substrataffinität von AtDUR3 eine Adaption an die normalerweise in ungedüngten Böden vorherrschenden niedrigen Harnstoffgehalte ist.

Im zweiten Teil der Arbeit wurde die physiologische Funktion von Harnstoff und seinen Transportern in Blättern untersucht. Derzeit ist nicht klar, ob der Transport und Metabolismus von Harnstoff die gesamte Stickstoffretranslokation während der Seneszenz limitiert. Unter Stickstoffmangel waren die *AtDUR3* Transkriptmengen nur leicht de-reprimiert, stattdessen aber in seneszenten Blättern stark erhöht. Nach dem Wechsel der Pflanzen in die generative Wachstumsphase waren die Harnstoffkonzentrationen in Blattproben unterschiedlichen Pflanzen- und Blattalters stark erhöht. Parallel dazu erhöhte sich mit zunehmendem Pflanzenalter die AtDUR3 mRNA- und Proteinmenge gleichermaßen. Die Analyse von Blattstielexudaten hat gezeigt, dass Harnstoff tatsächlich eine translozierte Stickstoffform ist und Harnstoff-N circa 13% des totalen Aminosäurestickstoffs darstellt, unabhängig vom Stickstoffstatus der Pflanze. Die Harnstoffkonzentrationen im apoplastischen Wasser weisen auf eine Rolle von AtDUR3 in der Harnstoffrückgewinnung aus dem Blattapoplasten hin, und transgene *AtDUR3*-promoter-GUS Linien zeigten eine Lokalisation der *AtDUR3* Promoteraktivität nahe der und im Leitbündel von alten Blättern. Demnach hält AtDUR3 vermutlich den internen Harnstoff durch Harnstoffrückgewinnung aus dem Apoplasten im Zytosol, damit er in die Nähe des Leitgewebes transportiert werden kann. Dort wird er entweder passiv ins Phloem

geladen oder für den Langstreckentransport von Stickstoff in Aminosäuren umgewandelt.

Eine *AtDUR3*-Überexpressionslinie in Arabidopsis, die einen stark tageszeiten-abhängigen Phänotyp mit kürzeren Blattstielen zeigt, führte zu einer *in silico* Analyse der *AtDUR3* Promotersequenz. Diese zeigte, dass Salizylsäure (SA) für die *AtDUR3* Geneinduktion in seneszenten Blättern benötigt zu werden scheint. SA ist bekannt für seine Beteiligung an der Initialisierung der Seneszenz. Eine stark erhöhte ^{15}N-Harnstoffaufnahme nach SA-Vorbehandlung in mit ausreichend Stickstoff versorgten Arabidopsiswurzeln impliziert, dass SA in der Lage ist, Stickstoffmangelbedingungen zu imitieren. Damit könnte SA die Verbindung zwischen der Regulation der entwicklungsbedingten und der stickstoffmangelinduzierten Seneszenz sein.

2 Introduction

2.1 Importance of nitrogen for plant growth

In natural ecosystems and in most agricultural systems nitrogen (N) is often considered to be one of the most important factors limiting plant growth since it represents up to 80% of the total mineral elements found in plants (Marschner, 1995). The strong increases in crop yields that have been obtained for most crops since the middle of last century are strongly based on elevated N fertilizer application together with improved plant protection measures and breeding efforts (Millder and Crane, 2005). However, N is one of the most expensive nutrients to supply and the major running cost in plant production (Singh, 2006). On the other hand it has been reported that the recovery of fertilizer N by the grain is relatively low (Raun and Johnson, 1999). In agricultural systems, where plants rely on fertilizers to meet their N demand, the low N recovery by plants may cause problems by the negative environmental impact mainly linked to nitrate leakage, affecting biodiversity, air and water quality, and the global climate (Bacon, 1995; Lawlor *et al*., 2001). A major challenge of modern agriculture is therefore to reduce the excessive input of N fertilisers by enhancing fertilizer use efficiency and, at the same time, to improve grain quality without negatively affecting yield. One possible way to achieve this goal is by improving the acquisition of fertilizer N by crops and/or by increasing their N utilization efficiency including assimilation, translocation and remobilisation when grown with reduced N supply. This requires a deeper understanding of the genetic basis of N assimilation and N use at different stages of plant development (Lourde *et al*., 2003).

The soil represents an extremely heterogeneous medium affecting the availability of minerals and thus plant nutrition (Hodge, 2004). Nutrients dissolved in the soil solution need to be transported to the root surface by diffusion and mass flow (Marschner, 1995). A mixture of forms of inorganic N (nitrate and ammonium) as well as to a lower extent of organic forms, such as amino acids, peptides or urea, is available in most natural soils, but especially in agricultural soils the concentration of different N forms can vary three to four orders of magnitude

(Wolt, 1994; Miller *et al*., 2007). Plants therefore constantly sense their nutrient environment and respond to it by modifying their uptake and metabolism using an intricate system of sensors, receptors, transporters, signal transduction components and gene expression regulators that collectively lead to changes in growth rates and development (Coruzzi and Zhou, 2001). The amount of N necessary for a plant to complete its life cycle varies among species. Additionally, the N demand of a plant varies according to its developmental stage being high during vegetative growth and decreasing during the reproductive phase, when N reserves are remobilized.

2.2 Primary nitrogen uptake and assimilation

On most arable soils, nitrate (NO_3^-) is the major form of N uptake. Two gene families, the *NRT1* and *NRT2* families representing low and high-affinity nitrate uptake systems, respectively, mediate nitrate uptake by roots (e.g. Muller *et al*., 1995; Devienne *et al*., 1994; Trueman *et al*., 1996, Crawford and Forde, 2002, Miller *et al*., 2007). Although depending on the plant species and growth conditions, a large proportion of the nitrate ions taken up from the soil are immediately translocated to the leaf and transiently stored in the vacuole. Nitrate reductase in the cytosol as well as nitrite reductase in the chloroplast need reductants such as NADH and ferredoxin (Fd) from photosynthesis to reduce nitrate to ammonium, which is then incorporated into amino acids via the glutamin-synthetase/glutamin-oxoglutarat-aminotransferase (GS/Fd-GOGAT) cycle (Lea *et al.,* 1992; Campell, 1999).

The average ammonium concentration in well-aerated agricultural soils is often much lower than that of nitrate, rarely exceeding 50 µM (Marschner, 1995; Miller *et al.,* 2007). However, the difference in soil concentration does not necessarily reflect the uptake ratio of the two N forms. Since ammonium is already a reduced N form, it is preferentially taken up when both N forms are present in particular as long as N concentrations are below 1 mM (Gazzarini *et al*., 1999, Xu *et al.*, 1992).

Similar to nitrate, the uptake of ammonium from the soil is mediated by low (LATS) and high-affinity transport systems (HATS). Ammonium transporters of the AMT1 family thereby play a major role in HATS, while members of the AMT2 as well as

members of the aquaporin families have been shown to mediate low-affinity transport (Gazzarini *et al*., 1999; Loqué *et al*., 2005; Yuan *et al.*, 2007).

Following its uptake ammonium is in general immediately assimilated in the roots by GS1 and NADH-GOGAT and the generated glutamine (Gln) is translocated to the shoot via the xylem (Coruzzi, 2003). Other amino acids such as glutamate (Glu), aspartate (Asp) and asparagine (Asn) may also be produced and translocated (Lam *et al*., 1995). Thus, ammonium is assimilated in the root, while nitrate is preferentially assimilated in the shoot.

Glutamine synthetase (GS) transfers the ammonium to Glu, generating Gln using one molecule of ATP (Lea and Miflin, 1974, Miflin and Lea 1980, Lea *et al*., 1992). The affinity of GS for ammonium is extremely high ($K_m \approx$ 20-40 µM). In plants, there are two GS isoforms: GS1 is encoded by five nuclear genes in Arabidopsis and targeted to the cytosol, while GS2 is encoded by a single nuclear gene and targeted to both the plastid and the mitochondria (Taira *et al*., 2004). While the latter is mainly expressed in mesophyll cells being part of the primary N assimilation and photorespiratory ammonia fixation, GS1 is present in phloem companion cells and roots where it is thought to synthesize Gln for long-distance transport (Edwards and Coruzzi, 1990; Carvalho *et al*., 1992; Coruzzi, 2003). Since GS1 is also induced by wounding and during senescence, differential roles for GS1 (synthesis of amino acids for long-distance transport) and GS2 (synthesis of amino acids for protein biosynthesis) have been suggested (Masclaux *et al*., 2000).

Much less attention has been given to uptake mechanisms of other nitrogenous compounds, especially organically bound N in the form of proteins, amino acids or urea. Even though nitrate and ammonium are the predominant N forms available to plants in most soils, urea and not nitrate is the most commonly used N fertilizer. Due to its high N content and cheaper production costs, urea fertilizers make up more than 50% of the total N fertilizers used worldwide (http://www.fertilizer.org/ifa/statistics/statsind). Urea fertilizers combine the advantages of rapid availability to plants and of a retarded microbial transformation into nitrate, which is the N form most prone to leaching. The microbial degradation process of urea can even be further slowed down by the co-application of urease

inhibitors that have been demonstrated to further reduce N losses from urea-fertilized plots (Xu *et al*., 2000).

In soils, urea is rapidly degraded to ammonium and CO_2 by urease, a nickel-dependent enzyme, which amongst others is synthesized and secreted by microorganisms (Watson *et al*., 1994). Therefore, the concentration of urea in lakes or natural soils is usually low and ranges between 0.1 - 3 μM (Cho *et al*., 1996; Mitamura *et al*., 2000a; Mitamura *et al*., 2000b), but up to 70 μM in fertilized crop-planted soils (Gaudin *et al*., 1987). With regard to this very low concentration it has been assumed that plants take up urea-derived N mainly in the form of ammonium (Polacco and Holland, 1993; Marschner, 1995).

Due to its low molecular weight and neutral character urea was believed for a long time to enter plant cells via diffusion through plant membranes (Galluci *et al*., 1971). Only a very limited number of physiological studies indicated that urea uptake may be plant-regulated, for example, because ammonium and nitrate exerted adverse effects on urea uptake (Bradley *et al*., 1989). However, all *in planta*-studies lacked verification whether urea itself or its degradation product ammonium had been transported across plant membranes. The first reliable experimental evidence for protein-mediated urea uptake by plant cells has been obtained by Wilson *et al.* (1988), who reported that short-term influx of ^{14}C-labeled urea in algal cells was dependent on the ATPase inhibitor DCCP or the protonophore CCCP and therefore appeared to be coupled to the proton gradient across the plasma membrane. Since concentration-dependent uptake followed bi- or even multiphase kinetics, the authors suggested the combined action of a high- and a low-affinity urea transport system *in planta*. Kinetically and energetically, these transport systems are clearly set apart from a passive, diffusion-like transport mechanism (Wilson *et al*., 1988). Later, CCCP-sensitive urea uptake was also confirmed in Arabidopsis suspension cells (Liu *et al*., 2003a).

2.3 The role of senescence in nitrogen efficiency

Besides an efficient N uptake and utilization during vegetative growth, N remobilization and retranslocation from senescing organs into the grain in the reproductive growth phase are further limiting factors for an efficient recovery of fertilizer N. Although N uptake after anthesis or during the onset of leaf

senescence contributes to seed filling to a varying extent (Niu *et al*., 2007) it has been estimated that > 70% of the seed N in annual crop species is derived from the retranslocation of N from senescing vegetative tissues into reproductive organs (Peoples and Dalling, 1988; Patrick and Offler 2001). In wheat or maize grains the contribution of leaf N remobilisation to N grain content was shown to be more than 50% (Kichey *et al*., 2007) and in field-grown rice even 60% (Mae, 2004), suggesting that the extent of N remobilization varies with plant species and growth system (Lohaus and Moellers, 2000). Improving N remobilisation during senescence would contribute to plant N economy and limit the requirement for exogenous N uptake after onset of flowering, therefore being a key process that directly affects protein quality and grain yield.

Senescence is a stage of plant development which is commonly defined as the sequence of biochemical and physiological events comprising the final stage of development until plant death (Smart, 1994). Senescence-associated nutrient mobilization is probably common to all plants (Hill, 1980; Mauk and Noodén, 1992; Hocking, 1994; Nieminen and Helmisaari, 1996) and the nutrient salvage from older or damaged leaves has the obvious adaptive value of recycling nutrients that may be limiting in the environment or that are energetically costly to acquire (Leopold, 1961). In contrast to aging, senescence represents only the final stage of vegetative plant development and is characterized by the transition from nutrient assimilation to nutrient remobilization in order to support growth of generative organs. Senescence is therefore often associated with the onset of bolting.

Senescence is induced by endogenous signals including age, developmental cues and plant growth regulators (Raghothama *et al*., 1991; Gan and Amasino, 1995; Grbic and Bleecker, 1995; Noodén and Penney, 2001; Riefler *et al*., 2006). A first visible symptom correlated to N mobilization and therewith protein degradation in leaf senescence in general is the yellowing of the leaves. Genetic and environmental factors that interfere with yellowing tend to modify protein degradation; however, protein degradation can be induced prematurely by a number of exogenous environmental stresses, including light or temperature stress, dehydration, nutrient stress or pathogen attack (Beers and McDowell, 2001; Pic *et al*., 2002; Xiong *et al*., 2005). Premature senescence induced by abiotic stress is estimated to be a primary cause for crop losses worldwide with the potential to reduce average crop yields by more than 50%.

These endogenous and exogenous signals inducing senescence appear to be coordinated through a common signaling network (Buchanan-Wollaston *et al*., 2003; Lim *et al*., 2003; Thomas *et al*., 2003). Unfortunately, the network of interaction between all inducing factors shows no clear hierarchy but rather hubs of more or less important factors, which complicates to define specific effects of single hubs. Recent transcriptome analysis indicated that signalling pathways involving ethylene, jasmonic acid and salicylic acid seem to play a crucial role in the network as they regulate the expression of genes required for developmental senescence (Buchanan-Wollaston *et al*., 2005). In addition, transcription factors from at least 20 different gene families are expressed during developmental leaf senescence (Guo *et al*., 2004; Buchanan-Wollaston *et al*., 2005). Among these are members of the MYB, AP2, NAC and WRKY transcription factor families. Several members of the WRKY transcription factor family are thought to be involved in senescence (Eulgem *et al*., 2000; Robatzek and Somssich, 2001, 2002). WRKY53, for example, regulates other WRKY transcription factors as well as stress genes and senescence-associated genes (SAGs) (Hinderhofer and Zentgraf, 2001; Miao *et al*., 2004). But also the overexpression of NAC transcription factors has been shown to alter plant senescence (Balazadeh, 2011).

2.3.1 Nitrogen remobilisation during senescence

A first step of the leaf senescence program is the breakdown of leaf cell components resulting in the mobilization of nutrients. In most plant tissues the largest fraction of organic N is contained in proteins. In the photosynthetic active tissue of C3 plants more than 50% of N is found in chloroplast proteins (Ellis, 1979), of which ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) alone makes up to 50% of the total plastidial N (Mae *et al*., 1983; Feller and Fischer, 1994). Therefore, the initial target of senescence is the chloroplast as it represents an enormous source of N; its recovery is of vital importance for the plant to gain full benefit from the photosynthetic loss of its leaves (Smart, 1994; Noodén *et al*., 1997). The advantage of chloroplast breakdown initiation during the early stages of senescence is seen in maintenance of cellular metabolism ensuring a successful remobilization of the N reserves localized in this organelle (Matile *et al*., 1996; Masclaux *et al*., 2000; Thomas and Donnison, 2000; Dangl *et al*., 2000). However, chloroplast breakdown comprises the photosynthetic

productivity of crops (Krupinska and Humbeck, 2004) causing the dilemma for agronomists that efficient N remobilisation is associated with an early loss of CO_2 fixation, which is one of the reasons for the negative correlation of seed protein content and grain yield (Beninati and Busch, 1992).

The dismantling of the chloroplast needs to be tightly regulated as to prevent cell damage due to the highly photodynamic nature of some of the breakdown products (Hörtensteiner, 2004). While chloroplasts show the first symptoms of senescence–associated impairment in leaves, other organelles such as mitochondria and nuclei remain intact to provide energy and to allow the expression of specific genes required for senescence while total RNA levels drop (Lim *et al*., 2003; Diaz *et al*., 2005). As the central vacuole remains intact, cellular compartmentation can be maintained during leaf senescence (Matile, 1997; Noodén, 2004).

In higher plant cells, most hydrolytic activities reside in the central vacuole, which typically contains 50-100% of the acid nuclease and 80-100% of the acid protease activity of a cell (Matile 1997). Searches for senescence–associated genes allowed to identify a number of cysteine proteases (Buchanan-Wollaston *et al*., 2003), in particular *SAG12* (Guo *et al*., 2004), which has been extensively used as a molecular marker for late senescence ever since. Also chloroplasts contain active proteases, but whether they are responsible for RuBisCO degradation is still unclear. However, there is evidence for the generation of small senescence-associated vacuoles (SAVs) with high-proteolytic activities in senescing leaf cells; these SAVs contain chloroplast proteins and merge with the central vacuole at a later stage.

2.3.2 Senescence-associated urea metabolism

Protein cleavage by peptidases leads to an accumulation of oligopeptides and free amino acids in senescing leaves (Brouquisse *et al*., 2001; Fischer, 2007). However, no general increase of all amino acids was observed in senescent Arabidopsis leaves. While levels of Arg, Ser, Tyr, Leu, Ile and GABA increased several fold, Glu, Asp, Asn and Gln slightly decreased or remained unchanged (Diaz *et al*., 2005). The arginine increase in senescing leaves (Polacco and Holland, 1993, Diaz *et al*., 2005) went along with a steady increase of arginase

activities with leaf age (Paschalidis and Roubelakis-Angelakis, 2005). After being transported to the mitochondria, arginine is re-assimilated within the ornithine cycle (urea cycle) by degradation into ornithine and urea, a process mediated by arginase (Polacco and Holland, 1993; Marschner, 1995). Urea concentrations in senescing leaves have so far been poorly characterized, but a subsequent accumulation of urea following arginine degradation is to be expected. Although transcriptome and metabolome analyses have described enzymes, transporters and nitrogenous compounds active during N retranslocation, and even though amino acid-N is widely accepted as the most important N form for phloem transport, experimental evidence for the significance and efficiency of different N forms for retranslocation processes is poor. In particular amino acids with a narrow C/N ratio are synthesized for an efficient long-distance transport of N, making urea a promising candidate for a not yet investigated role in N retranslocation.

Next to a possible direct export of urea, it can be hydrolysed in the cytosol by urease to ammonium. Since the chloroplast is becoming increasingly unable to assimilate N as chloroplast localized N-assimilation enzymes such as NiR, GS and GOGAT decrease, ammonium assimilation is shifted into the cytosol. In a large variety of plants, the induction of cytosolic glutamine synthetase (*GS1*) and glutamate dehydrogenase (*GDH)* genes has been detected during leaf senescence (Bernhard and Matile 1994; Masclaux *et al*., 2000; Cabello *et al*., 2006; Martin *et al*., 2006). GDH is ubiquitous in all organisms, its exact role in plants, however, is still not understood. It has been reported to be specifically present in phloem companion cells and the cytosol of senescing leaves (Tercé-Laforgue *et al*., 2004). Thus, a role of GDH in Glu catabolism during senescence and N-limitation appears likely (Dubois *et al*., 2003).

Taken together, besides by acquisition from the soil, urea can also accumulate in plant cells as a consequence of secondary N metabolism. Besides being released from the ornithine cylce, urea may also be liberated during the catabolism of purins or ureides, in particular allantoin and allantoate. Leguminous plant species employ ureides for long-distance translocation of N, and thus shuttle potentially larger amounts of N through urea in their sink tissues (Stebbins and Polacco, 1995). However, plant species like Arabidopsis appear to have the enzymatic capacity to degrade ureides without generating urea as an intermediate, leaving arginine catabolism as the only confirmed source for the generation of urea (Witte, 2011).

Since urea accumulation is particularly high in source leaves of older plants and in germinating seeds (Zonia *et al*., 1995), it has been proposed that urea accumulation mainly reflects N recycling as a consequence of protein and in particular arginine catabolism, which is emphasized by an increase in arginase protein levels relative to total seed protein (Bailey and Boulter, 1971, Zonia *et al*., 1995). These stages of enhanced N re-mobilization are further characterized by increased activities of cytosolic glutamine synthetase (GS1), probably as a prerequisite for the generation and re-fixation of urea-derived N (Masclaux *et al*., 2000; Witte *et al*., 2002). Although tissue aging and seed germination are accompanied by massive increases in urea concentrations, the routes of intracellular urea synthesis and the size of intracellular urea pools in different plant compartments have not yet been characterized in quantitative terms. Thus, it is currently unclear whether internal urea accumulation has a meaning for the short-term storage and long-distance transport of N, whether and to what extent urea is transported across intracellular membranes and to what extent transport systems are required to do so.

2.4 Molecular mechanisms of urea transport in plants (partially published J. Membrane Biol. 2006, 212 (2), 83-91)

2.4.1 Yeast as a model to study urea transport systems

Short-term uptake experiments using ^{14}C-labeled urea identified two major pathways for urea uptake into yeast cells: one is an active, energy-dependent transport system with a rather low Km of 14 μM, while the other is a passive transport system that operates at concentrations above 0.5 mM (Cooper and Sumrada, 1975). Screening of EMS-mutagenized yeast cells on media with different N sources allowed identification of a yeast strain that did not or poorly grow on urea or ureidoglycolate (Sumrada *et al*., 1976). Transformation of this mutant by a genomic library from yeast and screening of transformants with complemented urea uptake led to the identification of *ScDUR3* (ElBerry *et al*., 1993). Supply of ^{14}C-labeled urea to a liquid culture of the *ScDUR3*-complemented yeast mutant and trapping of liberated $^{14}CO_2$ indicated that the reintroduced gene, which encodes a hydrophobic protein with 15 putative trans-membrane domains (Turk and Wright, 1997; Saier, 2000), mediated or at least assisted in urea uptake.

Under adequate N supply, *ScDUR3* expression was repressed in a manner similar to that of other genes in the allantoin pathway, suggesting a regulatory link between *ScDUR3* and other genes involved in urea-releasing N catabolism (ElBerry *et al*., 1993).

2.4.2 Plant aquaporins can act as urea transporters

To isolate genes encoding urea transporters in plants, the *ScDUR3* gene was disrupted in an ura$^-$ yeast background and transformed with a cDNA library from Arabidopsis seedlings. Subsequent screening of the resulting transformants on < 5 mM urea as a sole N source allowed the isolation of four genes, *AtTIP1;1*, *AtTIP1;2*, *AtTIP2;1* and *AtTIP4;1*, that all encoded members of the tonoplast intrinsic protein (TIP) subfamily of aquaporins (Liu *et al*., 2003b). Growth complementation of the *ura*$^-$ *dur3*$^-$ mutant by the isolated TIPs from Arabidopsis was insensitive to pH but inhibited in the presence of phloretin [3-(4 hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)- 1-propanone], which is commonly used as a transport inhibitor for various classes of urea transporters including aquaporins (You *et al*., 1993; Ishibashi *et al*., 1994; Tsukaguchi *et al*., 1998). In a parallel approach, *AtTIP2;1* was heterologously expressed in *Xenopus laevis* oocytes where it enhanced the accumulation of radio-labeled urea in oocytes independent of external pH in a range from pH 5 to 8, confirming the pH-independent growth complementation in yeast (Liu *et al*., 2003b). Urea accumulation in *AtTIP2;1*-expressing oocytes increased linearly with external urea supply even when raising urea concentrations from 100 µM to 30 mM. Such linear concentration-dependent kinetics is typical for channel-mediated substrate transport and commonly observed for low-affinity transporters, although discrete substrate affinities cannot be calculated. A function of TIPs in urea transport had been previously observed, when the TIP homologue from tobacco NtTIPa, that is sequence-wise closely related to AtTIP2;1, was expressed in Xenopus laevis oocytes, where it was permeable to radiolabeled glycerol and urea besides water (Gerbeau *et al*., 1999). Moreover, oocyte expression also allowed the demonstration that plasma membrane intrinsic proteins (PIPs), too, may be permeable to urea. Functional expression of *NtAQP1* and, to a lesser extent, of Arabidopsis *PIP2;1*, facilitated significantly the accumulation of radiolabeled urea relative to water-injected oocytes (Eckert *et al*., 1999). In another yeast

complementation approach, which used an expression library from zucchini for complementation of the *dur3* mutant, CpNIP1, an NOD26-like intrinsic protein was isolated (Klebl *et al*., 2003). This emphasized that not only members of the TIP subfamily but also PIP- and NIP-like aquaporins may permeate urea, thus suggesting a comparable situation to that in mammals, where 4 out of 11 AQPs transport urea besides water (King *et al*., 2004).

2.4.3 Urea transport across tonoplast and plasma membranes

The high abundance of TIPs that were found to mediate urea transport suggested that their host membrane, the tonoplast, should exhibit high urea permeabilities. Indeed, recording volume changes in membrane vesicles from wheat roots in response to external urea revealed an up to 3-fold higher and mercury-sensitive permeability of vesicles enriched with endosomal membranes compared to the lower, mercury-insensitive urea permeability of plasma membrane-enriched vesicles (Tyerman *et al*., 1999). By stopped-flow spectrofluorimetry tonoplast vesicle preparations from tobacco were reported to be permeable to urea at a 75-fold higher rate than plasma membrane vesicles (Gerbeau *et al*., 1999). Whether these differences are caused by a higher activation state and/or density of urea-transporting TIPs in tonoplast membranes or by a higher ratio of urea-transporting versus urea-impermeable aquaporins is currently unresolved. A prerequisite for tackling this question is a reliable membrane localization of the diverse urea-transporting aquaporins. A membrane assignment based on the sequence-dependent classification into TIPs and PIPs, at least, appears not reliable, because some aquaporins can be found in both types of membranes, as put in evidence by Western blot analysis with membrane fractions from the ice plant *Mesembryanthemum crystallium* (Barkla *et al*., 1999), by GFP-tagging of TIP proteins and expression in Arabidopsis protoplasts (Liu *et al*., 2003b), or by systematic sequencing of membrane proteins (Marmagne *et al*., 2004). Nevertheless, for certain TIPs, such as AtTIP2;1, a major localization in the tonoplast has been reported by several independent approaches (Daniels *et al*., 1996; Saito *et al*., 2002; Carter *et al*., 2004) and thus appears very reliable. Taken together, these observations suggest that urea transport across the tonoplast is of greater physiological significance for a plant cell than that across the plasma

membrane, supposing that urea transport is not just a non-specific side activity of TIPs.

2.4.4 Possible physiological roles of TIPs in urea transport in plants

Although some TIPs show distinct organ- and cell type-specific expression patterns (Ludevid *et al*., 1992; Daniels *et al*., 1996; Ma *et al*., 2004), systematic gene expression analyses in different Arabidopsis organs indicated that all plant organs express a certain individual subset of TIPs (AtGen-Express; Schmid *et al*., 2005), per se not allowing to derive information on their possible function in urea transport.

Vacuolar compartmentation mediated by TIPs could be beneficial to transiently store or to detoxify an excess amount of urea that otherwise would accumulate in the cytoplasm. Under natural conditions this might be a rare event, but it is expected to become relevant in leaf-fertilized crop plants. An addition of the urease inhibitor phenylphosphorodiamidate (PPD) to foliar-applied urea increased leaf tip necrosis and increased the urea content but decreased ammonia levels and urease activity in soybean leaves (Krogmeier *et al*., 1989). Moreover, enhanced urea levels in necrotic areas indicated that leaf tip necrosis in response to leaf fertilization resulted from accumulation of excess urea rather than from the formation of excess ammonia. It will certainly be interesting to test whether an increased expression level of urea-transporting TIPs in urea-fertilized leaves of transgenic lines will diminish or delay leaf damage via an enhanced vacuolar loading capacity for urea. To further elucidate a physiological role of urea-transporting TIPs in different plant tissues, a separate determination of urea concentrations in different intracellular compartments seems required, since it is still unclear whether urea can accumulate in the vacuole or in other organelles. Theoretically, TIPs will mediate urea transport independently of the proton gradient across the membrane, just following the direction of the substrate concentration gradient.

Due to a rather constitutive expression and activity of urease in almost any cell type (Holland *et al*., 1987; Polacco and Holland, 1993), cytoplasmic urea concentrations should be low. With respect to micromolar K_m values of plant ureases (Kerr *et al*., 1983), urea-transporting TIPs will rather move low amounts of

urea *in planta*, while their urea-transporting function would increase whenever leaves are sprayed with urea fertilizers or urease activity is lost, i.e., under nickel deficiency (Gerendás and Sattelmacher, 1997).

2.4.5 The Arabidopsis urea transporter AtDUR3 belongs to the sodium solute symporter protein family

A genome-wide *in silico* search indicated that the Arabidopsis gene At5g45380 showed 41% sequence identity to the putative yeast urea transporter gene *ScDUR3*. Homologous EST clones were also found in maize, rice, soybean, barley, wheat, and oilseed rape. It is interesting to note, however, that in all plant species investigated so far only one *DUR3* homolog could be identified on the basis of database search in EST collections and genomic DNA. An Arabidopsis EST clone matching the genomic sequence of *AtDUR3* was then used for functional complementation of the urea uptake-defective yeast mutant *ura*$^-$ *dur3*$^-$. Heterologous expression of *AtDUR3* conferred yeast growth on 2 mM urea but only at a medium pH of 6 or lower, indicating that protons might stimulate AtDUR3-dependent urea transport (Liu *et al*., 2003a). AtDUR3 was predicted to encode an integral membrane protein with 14 transmembrane-spanning domains with its N and C termini protruding into the apoplasmic space (Schwacke *et al*., 2003). A phylogenetic analysis of AtDUR3 and the 22 most homologous and best characterized amino acid sequences derived from a BLAST search revealed a relatively high similarity among DUR3 proteins from plants and yeast and that all these sequences belong to the superfamily of sodium solute symporters (SSS) (Liu *et al*., 2003a). The SSS family includes currently more than one hundred members of pro- and eukaryotic origin (Jung, 2002) and some of them have been described to transport sugars, amino acids, nucleosides, myoinositols, vitamins, ions, phenyl acetate, water and urea (Reizer *et al*., 1994; Turk and Wright, 1997; Saier, 2000). Among all SSS proteins, DUR3 members showed closest relation to bacterial sodium pantethonate or sodium proline symporters (Figure 1). Since all these substrates appear to be structurally quite different, it will be exciting to uncover how substrate selectivity is determined in this transporter class. Leung *et al*. (2000) reported that SSS transporters such as the low-affinity sodium-glucose transporters from pig (pSGLT3) or rabbit (rbSGLT1), the sodium-iodide transporter from rat (rNIS), or the human sodium-chloride-GABA transporter can act as urea

channels in the absence of substrate but as urea cotransporters in the presence of substrate. This raises the possibility that in SSS transporters urea is just an alternative substrate for water that usually maintains the bulk flow of the substrates.

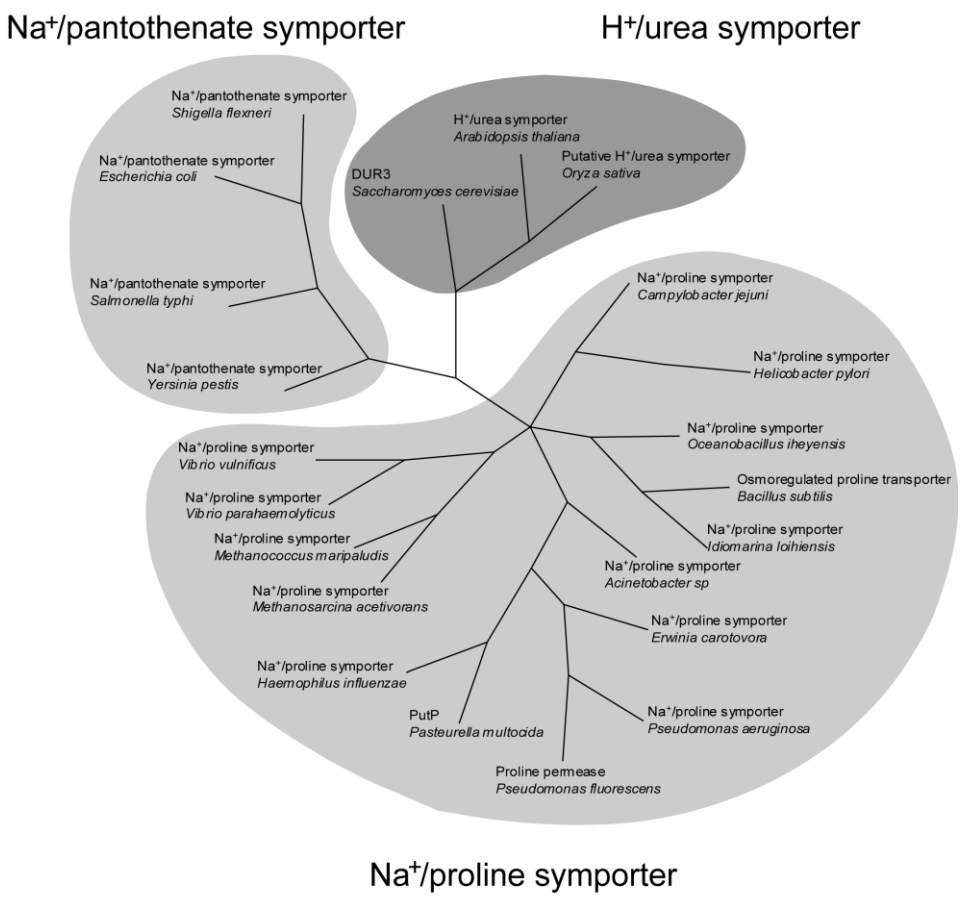

Figure 1: Phylogenetic tree of characterized SSS family proteins with highest sequence similarity to AtDUR3.

2.4.6 Substrate specificity and transport mechanism of AtDUR3

To study its transport mechanism in more detail, *AtDUR3* was expressed in *X. laevis* oocytes and substrate transport was investigated by two-electrode voltage clamp. Although urea itself is neutral, the presence of urea induced a weak inward current of positive charge, indicating the cotransport of cations (Liu *et al.*, 2003a). To increase sensitivity of urea transport measurements in oocytes the accumulation rate of ^{14}C-labeled urea was determined. It was found that low pH

strongly stimulated urea import. This observation was in good agreement with an improved yeast complementation at acidic pH and suggested that AtDUR3 co-transported mainly protons. Even though urea accumulation was not altered in the presence of 3 mM sodium in the bathing solution (Liu *et al*., 2003a), it might be too early to exclude that also alternative cation gradients might drive uphill transport of urea. For example, in the SSS member OpuE, a sodium/proline cotransporter, substrate transport strongly depended on the external sodium concentration in the medium (von Blohn *et al*., 1997).

Since SSS family members have been described to mediate the transport of a large variety of solutes, substrate specificity of AtDUR3 was tested, but no current was observed in response to glucose, galactose, myo-inositol or proline. Only in the presence of thio-urea an even weaker current was observed than for urea, suggesting a rather high urea specificity of AtDUR3. In most of the cases, SSS proteins were suggested to preferentially transport one substrate or a structurally closely related substrate group (Turk and Wright, 1997), whereas SSS proteins with a broad range of different substrates seem to be the exception (Leung *et al*., 2000).

Similar to urea-induced currents, radiolabeled urea uptake into *AtDUR3*-expressing oocytes was concentration-dependent and saturated around 50 µM urea (Liu *et al*., 2003a). Both transport assays allowed to calculate a K_m value of approx. 3 µM, indicating that AtDUR3 mediates high-affinity transport of urea. Thus, AtDUR3 exhibited a relatively high-affinity compared to most other urea transporters that saturate at millimolar concentrations (Leung *et al*., 2000). As pointed out by Liu *et al*. (2003a), uptake studies with AtDUR3 in yeast cells were difficult to perform due to the rapid degradation of urea by endogenous yeast urease. Acid trapping and determination of $^{14}CO_2$ released after the supply of radiolabeled urea, as described in ElBerry *et al*. (1993), does not allow determination of short-term substrate influx like it is required for the biochemical characterization of membrane transporters.

Studies in cyanobacteria showed that substrate degradation can be successfully avoided when high-affinity urea influx was investigated in the absence of any urease activity (Valladares *et al*., 2002). Only after disruption of the UreG gene, which encodes an accessory protein required for the synthesis of active urease,

high-affinity urea influx could be reliably determined in *Synechocystis*. The corresponding ABC-type urea transporter Urt was then shown to transport urea against a concentration gradient at a substrate affinity of 1 µM (Valladares *et al*., 2002). Thus, at least in plants, fungi and cyanobacteria measurements of high-affinity urea import can be easily perturbed if urease-dependent urea degradation remains uncontrolled.

2.4.7 Regulation of *AtDUR3* gene expression in Arabidopsis

To collect evidence for the physiological function of AtDUR3-mediated urea transport *in planta*, changes of AtDUR3 mRNA levels were examined under different conditions (Liu *et al*., 2003a). When Arabidopsis plants were cultured hydroponically and then subjected to N deficiency for 3 days *AtDUR3* mRNA levels strongly increased in roots but not in shoots. This transcriptional response was reminiscent of the N deficiency-induced derepression of high-affinity ammonium (AMT) and nitrate (NRT) transporters in Arabidopsis roots (Gazzarrini *et al*., 1999; Lejay *et al*., 1999), both of which are considered to contribute to N uptake under N-deficient growth conditions.

Gene expression levels of *AtDUR3* were further examined in mature leaves and germinating seeds of Arabidopsis (Liu *et al*., 2003a), since it is known that in both of these developmental stages high amounts of urea are liberated via the degradation of storage proteins. Indeed, *AtDUR3* mRNA was abundant in germinating seeds peaking five days after germination, suggesting a direct and/or indirect role of AtDUR3 in the transport of endogenously synthesized urea. Since arginase appeared to be localized in mitochondria, whereas urease is localized in the cytoplasm (Faye *et al*., 1986; Goldraij and Polacco, 2000), urea is supposed to pass the mitochondrial membrane on its way into the cytoplasm by a yet unknown transport mechanism (Figure 2). As AtDUR3-mediated transport of urea depends on a proton gradient, it is difficult to imagine that AtDUR3 participates in this transport step.

Presuming a localization in the plasma membrane does not yet point to an obvious role of AtDUR3 in mature leaves. Based on the observation that AtDUR3 gene expression in seedlings and leaves coincides with developmental stages, in which urea is liberated from internal N sources, it is tempting to speculate that AtDUR3

participates in transport processes that are linked to the recycling of urea-N in plants.

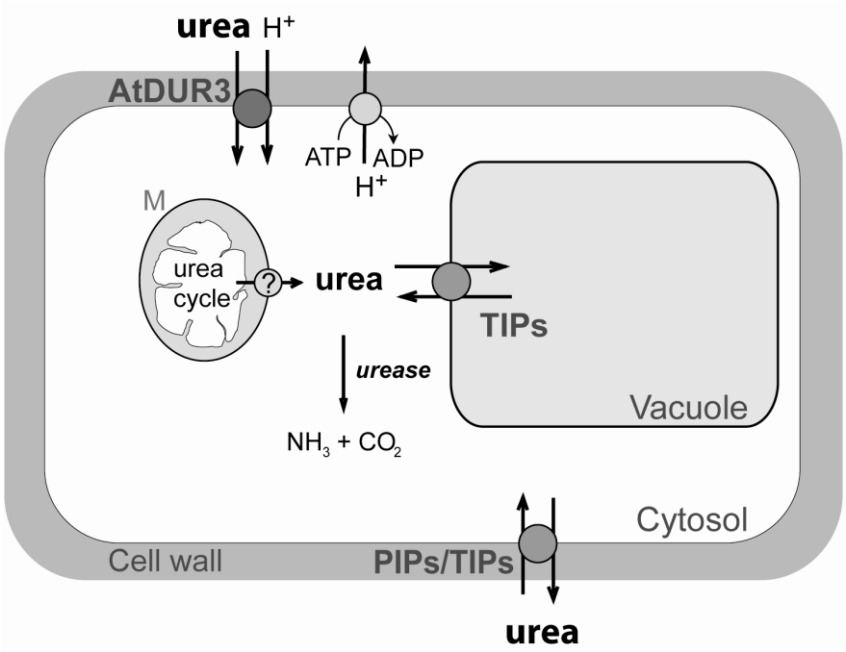

Figure 2: Model for protein-mediated urea transport pathways in plant cells.

AtDUR3 mediates secondary active, high-affinity urea transport across the plasma membrane, while aquaporins of the PIP or TIP subfamilies mediate low-affinity urea transport. In particular TIPs might further transport urea across the tonoplast for transient storage in the vacuole or remobilization. M, mitochondrion.

2.4.8 Physiological roles of high- and low-affinity urea transporters in plants

As a proton/urea symporter AtDUR3 is able to transport urea into root cells even when external concentrations are low. With regard to the almost ubiquitous occurrence of ureases in soil substrates and their millimolar affinity constants (Dalal, 1985) AtDUR3 might find its role in the lower micromolar concentration range of urea that might be left over in the soil solution after microbial degradation. The particularly low K_m of AtDUR3 might therefore reflect an evolutionary adaptation directed to use this diluted but valuable N source.

Plasma membrane-localized aquaporins of the PIP or, depending on their membrane localization, even of other MIP families might increasingly contribute to urea import with increasing concentrations of external urea. Their linear concentration-dependent transport kinetics makes it likely that this transporter

class serves in low-affinity uptake of urea. Under agricultural conditions, this transport system might become relevant in roots after urea application to soil or in leaves after foliar spray with liquid urea fertilizers. With respect to the tonoplast localization of certain or even most TIPs, a low-affinity transport pathway would also allow plant cells to load urea into the vacuole for transient storage of this N source (Figure 2). To what extent this transport path depends on cytoplasmic urease requires further studies determining urea in different subcellular compartments of plant lines with altered urease activities.

In conclusion, plants possess different types of urea transporters for passive and secondary active transport of urea across different cellular membranes. Together with a large number of transport systems for other N forms these membrane transporters appear to enable plants optimizing their N intake and compartmentation in dependence of the N forms being available in the medium and being synthesized endogenously.

2.5 Aim of this study

Characterizing the molecular basis of soil-to-plant and whole-plant fluxes of N has been the challenge for the past decade and requires a deep understanding of the molecular basis of N uptake, assimilation and N use at different stages of plant development. To improve plant N use efficiency it requires a better knowledge on the regulation of plant N metabolism during senescence which is regulating this terminal phase of development. The enormous and rapid progress in plant functional genomics has already uncovered some of the molecular components involved in these complex pathways and research efforts need to be made to develop genotypes that use N more efficiently.

Despite the importance of urea as a fertilizer and an intermediate in leaf N metabolism, kinetic and molecular aspects of urea transport in leaf cells have only been investigated to a limited extent. A deeper understanding of urea transport processes in plants and their regulation by N will not only allow a better understanding of the importance of urea for plant N nutrition but also an improvement of its utilization as N fertilizer for soil and foliar application in agricultural crop production. Since urea is also an important intermediate in secondary N metabolism of higher plants, urea transporters such as AtDUR3 and

AtTIPs might be promising candidates to carry out a physiological function in urea acquisition, in long-distance urea translocation or in intracellular urea compartmentation. Some information is already available, but there are important gaps related to the molecular basis of urea transport across the plasma or mitochondrial membrane. The latter ties with the general lack of data on urea concentrations in different cell compartments.

The aim of the first part of the thesis was therefore to investigate and quantify the contribution made by AtDUR3 to urea uptake in Arabidopsis roots. For this purpose, plant growth on urea as the sole N source, urea concentrations in the root tissue and root uptake capacities for ^{15}N-labeled urea were determined in wild-type *Arabidopsis* plants and in two independent T-DNA insertion lines defective in *AtDUR3* gene expression. Furthermore, promoter–reporter gene fusions were expressed in transgenic plants to localize AtDUR3 promoter activity in roots, gene expression studies under different N regimes were performed and immunological approaches were undertaken to determine membrane localization.

In the second part of the thesis a focus was laid on urea as a prominent N form generated during senescence, its relative contribution to N retranslocation from senescing leaves into sinks and on the relative contribution of the urea transporter AtDUR3 in these processes. This required first a quantitative characterization of urea pools in senescent leaves of different plant or leaf age and their correlation to the expression of urea transport proteins. To further describe the physiological function of urea transporters *in planta*, metabolites were measured in the apoplastic washing fluid and in leaf petiole exudates in a series of Arabidopsis mutant and wild-type plants with de-regulated expression of the AtDUR3 urea transporter or of urease, the enzyme catalysing the hydrolysis of urea. An *in silico* approach indicated the involvement of salicylic acid (SA) in the transcriptional regulation of *AtDUR3*. Therefore, uptake studies using ^{15}N-labelled urea were undertaken to verify a regulatory role of SA in urea transport.

3 Materials and Methods

3.1 Isolation of T-DNA insertion lines and growth test on agar plates

The T-DNA insertion lines for *AtDUR3* SALK_042649 (*atdur3-1*) and SALK_036318 (*atdur3-3*) were obtained from the Salk Institute collections, selfed and selected for homozygosity of the T-DNA insertion by PCR using primers for the left border of the insert (5´-GCGTGGACCGCTTGCTG-3´) and for the *AtDUR3* gene (5´-GGAAGAAACGTTAAGACAGGA-3´). The PCR products from the T-DNA insertion lines were cloned and sequenced to confirm the positions of the insertions. Homozygous lines were analyzed by RNA gel blots. For the growth test (Figure 3c), modified half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) without N was supplemented with 1 µM $NiSO_4$ and 50 µM KNO_3. Plates containing no further N served as a negative control. Either 500 and 5000 µM NH_4NO_3 or 50, 500, 1000 and 5000 µM urea were added as N sources. Columbia-0 and *atdur3* insertion lines were cultured for 3 weeks in a growth chamber (Percival, http://www.percival-scientific.com) under a 10 h/22°C light and 14 h/19°C dark regime.

3.2 Promoter:GFP and GUS analysis

For the *AtDUR3*-promoter::GFP fusion, a 1046 bp fragment of the genomic region of *AtDUR3* upstream of the translation initiation site was amplified by PCR using Pfu Turbo DNA polymerase (Stratagene) and the DNA sequence was verified. The gene-specific primers DUR3pro5 (5´-AAAAGCTTAAGGTAAAGAAAGGATACTTGTA-3´) and DUR3pro3 (5´-AAACCATGGTTCCTCTTCTTCTTTACGTTTT-3´) were used to generate a *Hind*III restriction site at the 5´-end, and a *Nco*I restriction site at the 3´-end, respectively. The *Hind*III–*Nco*I fragment of the *AtDUR3* promoter sequence was used to replace the CaMV 35S promoter in CaMV35S–SGFP–TYG–nos (Chiu *et al.*, 1996) and directly fused with sGFP. Then the *AtDUR3* promoter–sGFP–nos gene cassette was subcloned into pGreen0029 (Hellens *et al.*, 2000) as a *Hind*III–

EcoRI fragment. The *Hind*III–*Nco*I fragment of the *AtDUR3* promoter sequence was also cloned into pBI101 (Clonetech) for promoter-GUS fusion. *Agrobacterium tumefaciens* (strain GV3101:pMP90)-mediated Arabidopsis transformation was conducted as described previously (Loqué *et al.*, 2006). N-deficient medium for microscopic observation of promoter:GFP plants (Figure 5) was prepared by replacing nitrate in MGRL medium (Fujiwara *et al.*, 1992) with an equivalent of chloride salts (Inaba *et al.*, 1994). Fluorescence of GFP in transgenic plants was observed under an inverted fluorescence microscope equipped with an apotome (Zeiss Axiovert 200 M, http://www.zeiss.com). Transgenic plants expressing the *AtDUR3pro::GUS* construct were either cultured on soil under long-day conditions and old leaves showing first symptoms of senescence were used for analysis. Additionally, plants were cultured on ½ MS medium for 4 weeks before transfer to N-deficient medium for 4 days.

3.3 Whole-mount immunohistochemistry

The procedure for whole-mount preparations was modified from Lauber *et al.* (1997). Fourteen-day-old Col-0 and *atdur3-1* roots were fixed in 4% paraformaldehyde dissolved in microtubule-stabilizing buffer (MTSB; 50 mM 1,4-piperazinediethanesulfonic acid (PIPES), 5 mM EGTA, 5 mM $MgSO_4$, pH 6.9-7.0) at room temperature for 30 min and washed 3 times in phosphate-buffered saline buffer (PBS; 0.02 M sodium phosphate buffer with 0.15 M sodium chloride, pH 7.4) and two times in ultra-pure water. Roots were placed onto silane-coated microscopic slides (Histo Bond, Marienfield) with a drop of water. Coverslips were removed after dipping the slides into liquid N. After drying for 1 h, specimens were rehydrated in PBS for 10 min. Cell walls were partially digested with 2% driselase (Sigma) for 30 min. The plasma membrane was permeabilized with 0.4% Nonident P40 in 10% DMSO–PBS for 1 h.

Non-specific interactions of antiserum were blocked with 1% BSA in PBS overnight, and antisera were diluted in 3% BSA in PBS and incubated overnight. Primary antiserum raised against AtDUR3 was used at the dilution of 1:500. Cy3-conjugated anti-rabbit-IgG was employed as a secondary antibody (Dianova; http://www.dianova.de) at a dilution of 1:200. Apotome scanning of specimens was

performed with an inverted fluorescence microscope (Zeiss), equipped with appropriate filters for Cy3, using the AxioVision software (version 4.5; Zeiss).

3.4 Hydroponic plant culture

Arabidopsis thaliana seeds were germinated in the dark for 4 days and cultured on rockwool moistened with tap water. After 1 week, tap water was replaced by half-strength nutrient solution containing 1 mM KH_2PO_4, 1 mM $MgSO_4$, 250 µM K_2SO_4, 250 µM $CaCl_2$, 100 µM Na–Fe–EDTA, 50 µM KCl, 30 µM H_3BO_3, 5 µM $MnSO_4$, 1 µM $ZnSO_4$, 1 µM $CuSO_4$, and 1 µM $NaMoO_4$, pH adjusted to 5.8 by KOH. N was supplied as 2 mM NH_4NO_3. To suppress exogenous degradation of urea by urease liberated from decaying root cells, urea was supplied with 75 µg l^{-1} of the urease inhibitor phenylphosphorodiamidate (PPD; Martens and Bremner, 1984; Pedrazzini *et al.*, 1987) whenever indicated in the legend. The nutrient solution was renewed once a week during the first 3 weeks, twice in the fourth week and every 3 days for the following weeks. Plants were grown hydroponically under non-sterile conditions in a growth cabinet. If not otherwise indicated the following conditions were applied: 10 h/14 h (short day) light/dark; light intensity 200-280 µmol m^{-2} sec^{-1}; temperature 22°C/18°C and 60-70% humidity.

Senescence was induced either by a switch from short to long day (16 h /8 h light/dark) conditions and/or by applying N starvation for 4 days to induce N remobilisation. Additionally, senescence was induced by shading of a single leaf of the plant (leaf number (no) 7) by wrapping aluminium foil around it for a periode of up to 3 days.

Next to wild-type and the two *atdur3* T-DNA insertion lines a *ureG* mutant lacking a functional urease was employed which was kindly provided by C.P. Witte (Witte *et al*., 2005). Seeds of the crossing of *ureGA* with *atdur3-1* leading to the double mutant line *dur3ureGA* as well as a 35S-overexpression line of AtDUR3 (*35SDUR3*) in the wild-type background Col-0 were kindly provided by Soichi Kojima (RIKEN, Japan).

3.5 RNA gel blot analysis

Total RNA was isolated by phenol-guanidine extraction followed by lithium chloride precipitation according to Logemann *et al.* (1987) or by extraction with TRIzol (Invitrogen) following the manufacturer's protocol. Total RNA (10–20 μg per lane) was separated by electrophoresis on 3-(N-morpholino) propanesulfonic acid (MOPS)-formaldehyde agarose gels, blotted onto Hybond-N^+ nylon membranes (Amersham) and cross-linked to the membrane by incubation at 80°C for 2 h. The coding sequences of *AtDUR3, AtAMT1;1, AtTIP2;1 and SAG12* were used as probes for hybridization to total RNA. Hybridization to a randomly primed ^{32}P-radiolabeled probe was performed at 42°C in 50% (v/v) formamide, 1% (w/v) sarcosyl, 5x SSC and 100 µg ml^{-1} yeast t-RNA. Membranes were washed at 42°C once in 2x SSC, 0.1% (w/v) SDS for 40 min and once in 0.2x SSC, 0.1% (w/v) SDS for 40 min. A 25S- RNA probe was used as a RNA loading control.

3.6 ^{15}N influx and retranslocation analysis

Urea influx measurements in plant roots were conducted after rinsing the roots in 1 mM $CaSO_4$ solution for 1 min, followed by incubation for 10 min in nutrient solution containing different concentrations of ^{15}N-labeled urea (95-98 at.% ^{15}N) as the sole N source. After a final rinse in 1 mM $CaSO_4$ solution, roots and shoots were separated and stored at -70°C before freeze-drying. Each sample was ground and 1.0–2.5 mg sample powder was used for ^{15}N determination by isotope ratio mass spectrometry (Finnigan; http://www.thermo.com). Values obtained for concentration-dependent urea influxes up to 200 µM urea were directly fitted to the Michaelis–Menten equation. For salicylic acid (SA) treatments, plant roots were incubated in nutrient solution containing 500 µM SA 3 h prior to the uptake experiment (Figure 28). Uptake experiments were repeated two or three times, and representative results are shown.

For retranslocation studies 10 µl of a 2% ^{15}N-labeled urea (96 at.% ^{15}N) solution blended with 0.05% detergent (Silwet gold) were applied for 3 days on leaf no. 6 in 6 week-old hydroponically grown plants under long day conditions. For this purpose a 1.5 ml tube was cut, its border lubricated with Vaseline (Balea) and stamped onto the middle of the leaf to create a defined area of uptake.

3.7 Preparation of microsomal and plasma membrane fractions

Fresh root or shoot tissue was ground in a buffer containing 250 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)–HCl (pH 8.5), 330 mM sucrose, 25 mM EDTA, 5 mM ß-mercaptoethanol (ß-ME), 2 mM dithiothreitol (DTT) and 1 mM phenylmethyl-sulfonyl fluoride (PMSF). Homogenates were centrifuged at 10.000 *g* for 15 min. Supernatants were filtered through nylon mesh (58 µm) and centrifuged at 100.000 *g* for 30 min to pellet microsomal membrane fractions. The pellet was resuspended in conservation buffer (5 mM Bis-TRIS propane-MES, pH 6.5, 250 mM sorbitol, 20% w/v glycerol, 1 mM DTT, 2 mM PMSF) and gently homogenized in a potter, as previously described in Loqué *et al.* (2006). Plasma membrane fractions were prepared by aqueous two-phase partitioning based on Larsson *et al.* (1987). A microsomal pellet was resuspended in microsomal buffer (5 mM KH_2PO_4, pH 7.8, 330 mM sucrose) and added to dextran-polyethylene glycol buffer (6.4% dextran T-500, 6.4% PEG 3350, 5 mM KH_2PO_4, 3 mM KCl and 330 mM sucrose). The two phases were mixed and centrifuged at 1500 *g* for 5 min. Upper and lower phases were collected and re-partitioned twice with fresh washing buffer (5 mM KH_2PO_4, pH 7.8, 330 mM sucrose, 1 mM PMSF). The upper and lower phases were diluted with washing buffer and centrifuged at 100 000 g for 60 min to pellet the membranes, respectively. The pellet was re-suspended in conservation buffer and gently homogenized in a potter. Protein concentrations were determined using a Bradford protein assay kit (Bio-Rad, http://www.bio-rad.com) with BSA as a standard.

3.8 Protein gel blot analysis

Polyclonal antibodies were raised against an oligopeptide representing the C-terminal 14 amino acids of AtDUR3 (n-LLELEKTKKNDEEG-c). The antiserum was affinity-purified using a nitrocellulose membrane for peptide binding as described in Ludewig *et al.* (2003). Sodium dodecyl sulfate-PAGE and protein gel blot analysis were performed as described previously (Loqué *et al.*, 2006) always using 5µg protein per lane. Antiserum raised against AtDUR3 was diluted in blocking solution at 1:5000 or 1:10.000 (Figure 4b, c). The secondary antibody was diluted 1:100.000. The dilution of the antibodies against AHA2 and DET3 has

been described in Yuan *et al.* (2007) and was performed accordingly. Rainbow marker (Amersham) or Magic marker (Invitrogen) were used as molecular weight markers.

3.9 Determination of urea concentrations

Root urea concentrations were determined based on a colorimetric reaction described by Kyllingsbæk (1975). Approximately 50 mg of freeze-dried plant tissues was milled and suspended in 1 ml of cold 10 mM formic acid. After centrifugation at 16000 *g* and 4°C for 15 min, 30 µl of the supernatant were incubated with 1 ml of a color development reagent (4.6 mM diacetylmonoxime, 1.28 mM thiosemicarbazide, 6.6% H_2SO_4, 14.6 µM ferric chloride hexahydrate and 0.006% orthophosphoric acid) at 99°C for 15 min, and then cooled down at 4°C for 5 min. Absorbance at 540 nm was measured with a photometer. The ureides allantoin, ornithine, arginine, and uric acid did not interfere with urea determinations, but other ureides were not tested.

3.10 Leaf petiole exudates

Exudates from leaf petioles were collected in 500 µl 10 mM EDTA solution pH 8.5 as described by Corbesier *et al.* (2001). In brief, petioles were detached as low as possible and were cut a second time inside EDTA solution without crushing the stem. After rinsing for 1 min 2-3 leaves were pooled and transferred into the collection tubes with petioles immersed in the EDTA solution. Exudates were collected over a time period of 6-8 h in a closed system to prevent transpiration under controlled light and temperature. Exudates were analysed for urea, sugar and amino acid concentration.

3.11 Apoplastic wash fluid

Apoplasmic wash fluids (AWF) were collected using the infiltration–centrifugation method described by Lohaus *et al*. (2001). Briefly, leaves of different ages (1-6, 7-12, >12) from approximately 100 six week old wild type or *atdur3-1* plants grown under long day conditions and starved for N for 4 days were pooled

corresponding to ~3-4 g fresh weight each. Leaves were washed in ice-cold milli-Q water (Milipore, Schwalbach, Germany) and infiltrated with ice-cold milli-Q water by five applications of a pressure of 0.8 bar for 2 min and wiped dry with tissues. AWF was collected by centrifugation for 20 min at 100 *g*. The volume of the collected liquid was measured, and samples were stored at – 20°C before urea analysis.

3.12 Analytical methods

Amino acids were measured as described by Zurbriggen *et al*. (2009). To detect primary and secondary amino acids the fluorescing reagent AQC (6-aminoquinolyl-N-hydroxysuccinimidylcarbamat) was used. The separation was carried out with a reversed phase HPLC system (Waters) consisting of a gradient pump, a degasing module, an autosampler and a fluorescence detector. Chromatograms were recorded using the software program Millennium 32 or Empower. The gradient was accomplished with a buffer containing 140 mM sodium acetate, pH 5.8 (Suprapur, Merck) and 7 mM triethanolamine (Sigma). Acetonitril (Roti C Solv HPLC, Roth) and purest HPLC water (Baker) were used as eluents.

3.13 In silico data analysis

The eFP Browser of the Bio-Array Resource (BAR) by Winter *et al*. (2007) was employed to extract data from transcriptome analysis. The microarray dataset dealing with SA treatment was obtained from the *AtGenExpress* project of the Schmid, Lohmann and Weigel labs (2005) as presented on the TAIR website (http://www.arabidopsis.org). The subset provided by Buchanan-Wollaston *et al*. (2005) used the Affymetrix ATH1 Arabidopsis Genome Array.

For an analysis of the promoter of AtDUR3 the Arabidopsis Gene Regulatory Information Server (*AGRIS*) was used as an information resource for Arabidopsis promoter sequences, transcription factors and their target genes (Davuluri *et al*., 2003; Yilmaz *et al*., 2011). The coexpression analysis was performed in Atted-II which allowed the mining of coexpressed gene networks (Obayashi *et al*., 2009).

Visualization of coexpressed genes was carried out using Genevestigator (Hruz *et al.,* 2008).

4 Results

4.1 AtDUR3 represents the major transporter for high-affinity urea transport across the plasma membrane of nitrogen-deficient Arabidopsis roots (published in The Plant Journal (2007), 52, 30-40)

4.1.1 Disruption of AtDUR3 causes growth inhibition on urea when it is supplied as a sole nitrogen source

To investigate the contribution made by AtDUR3 to urea uptake in Arabidopsis roots, two independent T-DNA insertion lines in the Columbia-0 (Col-0) background were obtained from the insertion mutant collections of the Salk Institute Genomic Analysis Laboratory (Alonso *et al*., 2003). The T-DNAs were found to be inserted either 520 or 586 bp downstream of the start codon (Figure 3a), and the lines were named *atdur3-1* and *atdur3-3*, respectively. Roots of hydroponically grown Col-0, *atdur3-1* and *atdur3-3* plants were then harvested for the extraction of total RNA and subsequent RNA gel blot analysis. Transcript levels of *AtDUR3* were low in N-sufficient but strongly increased in N-deficient roots of wild-type plants, whereas no *AtDUR3* mRNA was detected in roots of homozygous progenies from either T-DNA insertion line (Figure 3b).

The two *atdur3* insertion lines did not show any visible growth difference on soil or nutrient solution supplemented with nitrate or ammonium as the N source. When grown on sterile agar plates supplemented with urea as a sole N source, however, growth differences between Col-0 and *atdur3* plants became apparent. While wild-type plants developed green leaves when supplied with 1 mM, and even as little as 0.5 mM urea, both insertion lines became chlorotic and accumulated more anthocyanins than the wild type, this being another visible sign of N deficiency (Figure 3c). Shoot biomass production was little affected, if at all. With a supply of ammonium nitrate, shoot growth of all lines was better than with urea and no phenotypical differences were observed among the different lines (Figure 3c).

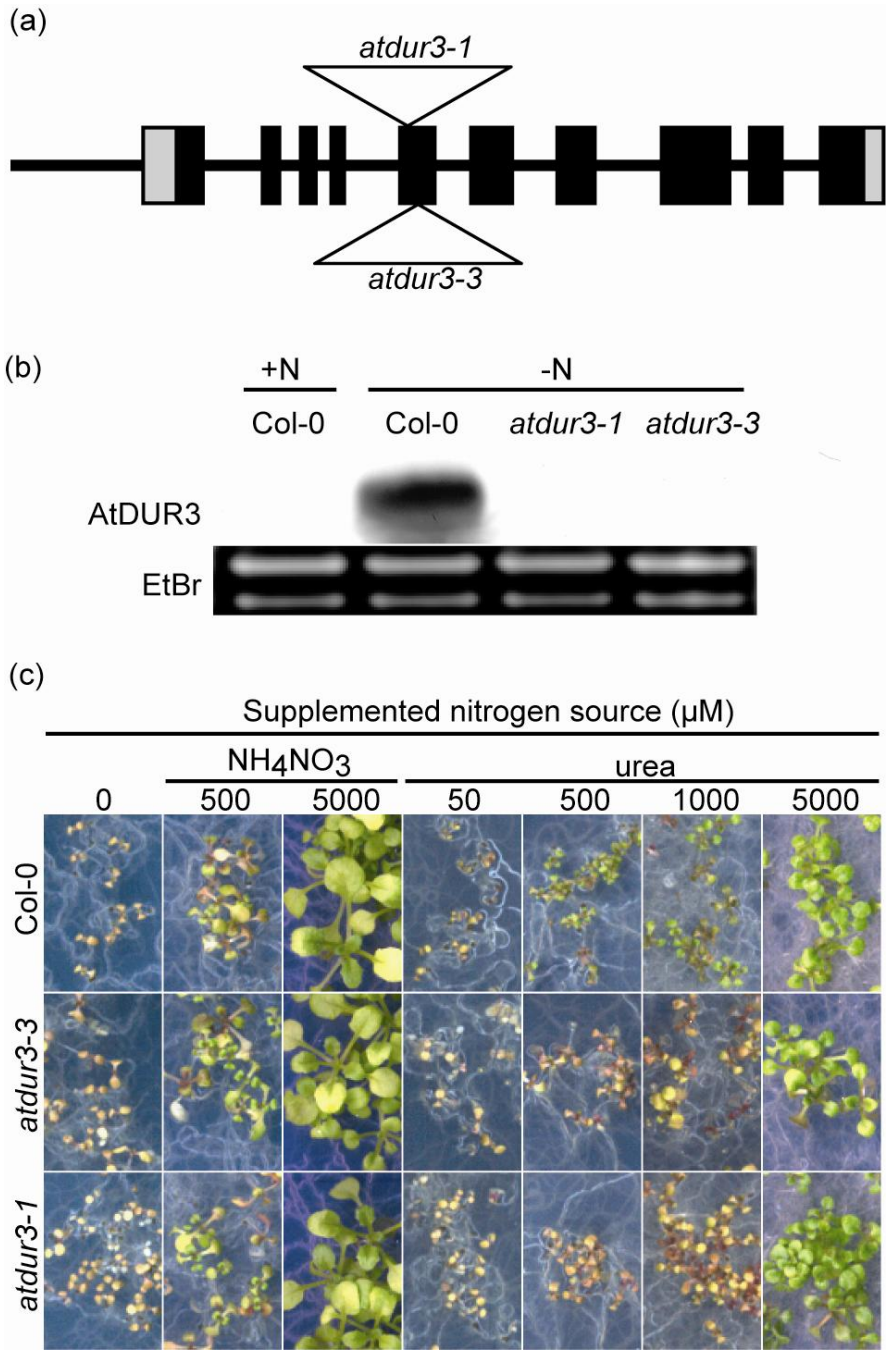

Figure 3: Disruption of AtDUR3 leads to impaired growth under urea supply.

(a) Schematic representation of the positions of the T-DNA insertions in *atdur3-1* and *atdur3-3*. The insertions in the *AtDUR3* gene are located 520 and 586 bp, respectively, downstream of the putative translation start. (b) Ribonucleic acid gel blot analysis of RNA from N-starved roots from wild-type Col-0, *atdur3-1* and *atdur3-3* plants using the coding sequence of *AtDUR3* as a probe. (c) Growth of the insertion lines *atdur3-1* and *atdur3-3* and their corresponding wild-type (Col-0) on sterile half-strength MS medium supplied with 1 µM $NiSO_4$ and 50 µM KNO_3 and different concentrations of ammonium nitrate or urea as a sole N source.

4.1.2 Plasma membrane localization of AtDUR3

The intracellular localization of AtDUR3 was investigated employing protein gel blot analysis of membrane protein fractions from Arabidopsis roots. An antibody raised against 14 amino acids of the C-terminus of AtDUR3 detected a single band at approximately 55 kDa in the microsomal membrane fraction of Col-0 roots (Figure 4a). This was somewhat lower than the calculated molecular weight of 75 kDa for AtDUR3, but corresponded to the expected size with respect to the hydrophobicity of the protein. No signal was detected in microsomal membrane protein fractions from roots of the *atdur3-1* and *atdur3-3* insertion lines, confirming the specificity of the antibody. In wild-type roots accumulation of the AtDUR3 protein strongly increased during N deficiency (Figure 4b). Microsomal fractions from roots that were starved of N for a period of 3 days were then separated by two-phase partitioning into fractions enriched with plasma membranes or endosomal membranes (Larsson *et al*., 1987). In a subsequent protein gel blot analysis (Figure 4c), use of an antibody raised against the Arabidopsis plasma membrane H^+-ATPase (AHA2) confirmed enrichment of plasma membrane proteins in the upper phase (U), while enrichment of endosomal membrane proteins in the lower fraction (L) was verified by detection of DET3, a subunit of the endosomal V-type H^+-ATPase (Schumacher *et al*., 1999). A highly preferential enrichment of AtDUR3 in the upper phase of N-deficient protein fractions indicated plasma membrane localization of AtDUR3.

In a whole-mount immunohistochemical approach, fluorescence imaging assisted by an apotome was directed to root hairs, which are usually less vacuolated and densely filled with cytoplasm (Figure 4d-A). After whole-mount specimens of root hairs from N-starved roots of Col-0 plants were incubated with AtDUR3-specific antiserum and a Cy3-conjugated, secondary fluorescent antibody, red fluorescence was observed along the border of individual root hair cells (Figure 4d-C). Due to the absence of a large vacuole in the hair tip (Figure 4d-A), localization of AtDUR3 could be assigned to the plasma membrane. No AtDUR3 signal was detected in root hairs of the *atdur3-1* insertion line (Figure 4d-D). Taken together, both independent experimental approaches indicated that AtDUR3 accumulated in the plasma membrane of root cells of N-deficient Arabidopsis plants.

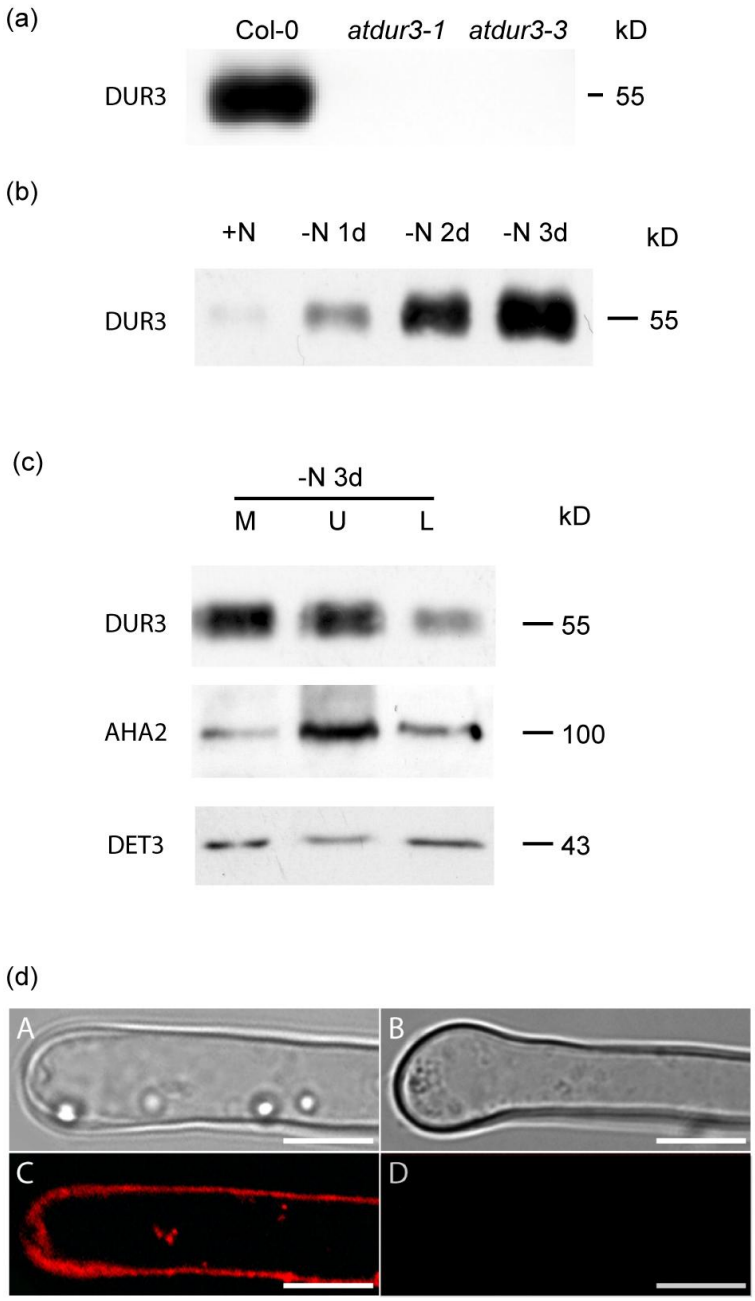

Figure 4: AtDUR3 localizes to the plasma membrane.

(a) Protein gel blot analysis of microsomal membrane fractions from roots of wild-type (Col-0) or *atdur3-1* and *atdur3-3* plants cultured under N deficiency. For AtDUR3 protein detection a polyclonal antibody raised against the C-terminus of AtDUR3 was employed. An AtDUR3-specific signal was detected at approximately 55 kDa. (b) Protein gel blot analysis of root membrane fractions from roots of wild-type (Col-0) plants cultured under a continuous supply of 2 mM ammonium nitrate (+N) or under N deficiency for 1, 2 or 3 days (-N). (c) Microsomal fractions (M) from N-deficient roots (-N 3d) of wild-type (Col-0) plants were separated by aqueous two-phase partitioning into a plasma membrane-enriched upper phase (U) and an endosomal membrane-enriched lower phase (L). Protein gel blot analysis was conducted with antibodies against AtDUR3, an Arabidopsis plasma membrane H^+-ATPase (AHA2) and a subunit of an endosomal H^+-ATPase (DET3). (d) Intracellular localization of the AtDUR3 protein in N-starved root hairs from Col-0 (a and c) or from *atdur3-1* (b and d) plants. Whole-mount root samples were stained with an anti-AtDUR3 antiserum and a Cy3-conjugated red-fluorescing secondary antibody. Whole-mount images were taken by an inverted fluorescence microscope equipped with an apotome. The bar represents 10 µm.

4.1.3 Localization of AtDUR3 promoter activity in outer root cells

Cell type-specific expression of *AtDUR3* was investigated in Arabidopsis plants transformed with an *AtDUR3* promoter:GFP construct containing 1046 bp of the 5'-upstream region of AtDUR3. Green fluorescent protein-dependent fluorescence in the roots of several independent transformants cultured under N-sufficient conditions was detected at a low level in the meristem of root apices and in the root cap as well as in basal root hair zones, but was absent from the younger root hair zone (Figure 5a,c,e). Green fluorescent protein-derived fluorescence strongly increased after plants were transferred to N deficiency (Figure 5b,d) when AtDUR3 transcripts and protein abundance also increased (Figure 3b, 2b). The N deficiency response of *AtDUR3* promoter activity was confined to epidermal and cortical cells in the basal root hair zone but limited only to epidermal cells in the younger root hair zone (Figure 5b,d). In the root hair zone, promoter activity was also observed near the xylem in vascular tissues, although at a comparatively low intensity (Figure 5b).

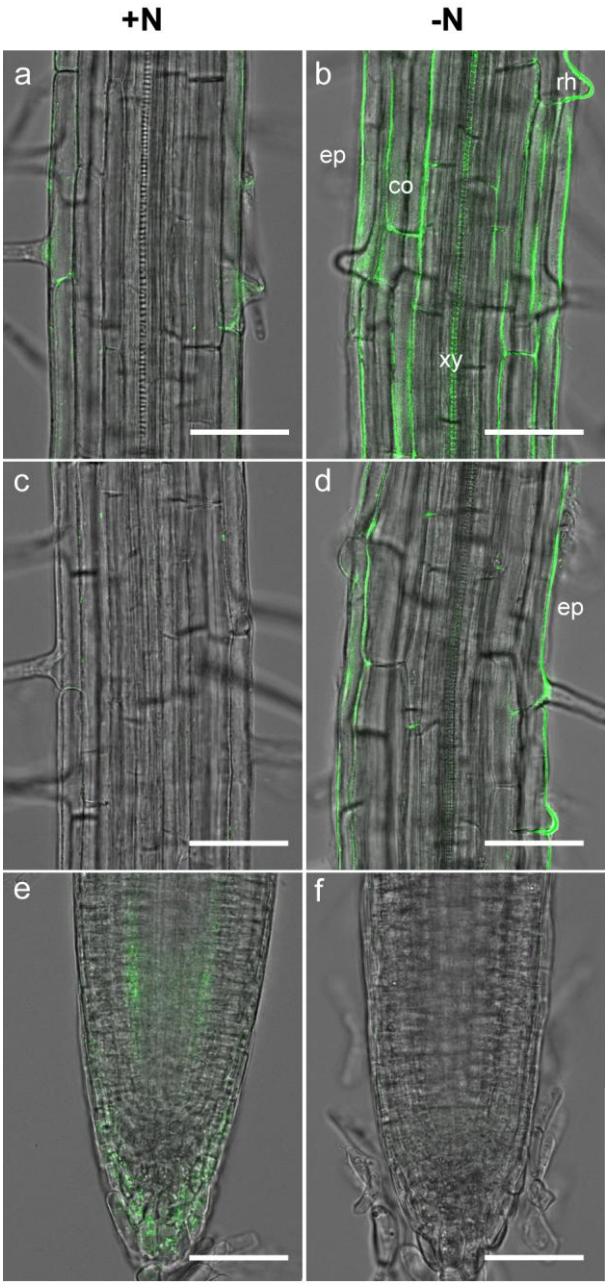

Figure 5: Localization of the promoter activity of AtDUR3.

Transgenic plants expressing an *AtDUR3-promoter::GFP* construct were grown on MGRL agar plates under N-sufficient (+N) or N-deficient conditions (-N). Images from upper (a and b) and lower (c and d) root hair zones and root tips (e and f) were taken by an apotome-equipped fluorescence microscope. Bars represent 50 µm. rh, ep, co and xy indicate root hair, epidermis, cortex and xylem, respectively.

4.1.4 Regulation of *AtDUR3* gene expression by nitrogen availability

Changes in *AtDUR3* gene expression in response to different N treatments were monitored in roots following RNA extraction from hydroponically grown Col-0 plants. While levels of *AtDUR3* mRNA were high in N-deficient roots, they were rapidly repressed after resupply of ammonium or nitrate (Figure 6). Both forms of

N, ammonium and nitrate, seemed to act with high efficiency even though residual mRNA levels were slightly higher after the ammonium treatment. Thus, upregulation of *AtDUR3* gene expression under N deficiency most likely reflected a de-repression from ammonium, nitrate or other N sources, similar to that reported for the ammonium transporter genes *AtAMT1;1* and *AtAMT1;3* (Gazzarrini *et al*.,1999; Rawat *et al*., 1999).

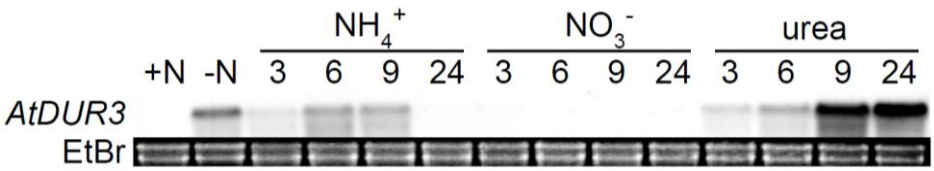

Figure 6: Regulation of *AtDUR3* gene expression in Arabidopsis roots in dependence of the N supply.

Ribonucleic acid gel blot analysis of total root RNA from hydroponically grown 6-week-old Arabidopsis plants using the coding sequence of *AtDUR3* as a probe. Plants were cultured under continuous supply of 2 mM ammonium nitrate (+N) or under N deficiency for 4 days (-N). N-deficient plants were resupplied with 2 mM NH_4^+ or NO_3^- or with 1 mM urea in the presence of the urease inhibitor phenylphosphorodiamidate (PPD) for up to 24 h. Ethidium bromide-stained RNA (EtBr) served as a loading control.

Resupply of urea to N-deficient plants led to a dramatic upregulation of levels of *AtDUR3* mRNA in roots (Figure 6), which exceeded even the transcript levels present in N-deficient plants. Thus, *AtDUR3* gene expression was also substrate inducible, similar to the transcriptional regulation of the high-affinity nitrate transporter *AtNRT2;1* by nitrate (Lejay *et al*., 1999).

4.1.5 Role of AtDUR3 in the accumulation of urea in roots

Urea is highly sensitive to enzymatic degradation by urease, which is among the most persistent enzymes in nature and almost ubiquitously expressed by most organisms (Polacco and Holland, 1993). Influx of urea into the roots of N-deficient wild-type plants was therefore determined in the presence of the urease inhibitor phenylphosphorodiamidate (PPD). To exclude any interference of this inhibitor on anything other than urea hydrolysis in the outside medium, urea concentrations and *AtDUR3* gene expression in roots were determined in wild-type and the two T-DNA insertion lines in the presence or absence of PPD under adequate or

deficient N supply (Figure a,c). Additionally, urea influx of 200 µM ^{15}N-labeled urea into roots and translocation to shoots of wild-type plants under N-deficient conditions was compared under different PPD pretreatment (Figure 7b). However, neither urea influx and translocation to shoots nor *AtDUR3* gene expression or urea concentrations in the roots of N-sufficient or N-deficient plants significantly differed in the presence or absence of PPD under our growth conditions (Figure 7). Moreover, an influx analysis of ^{15}N-labeled urea was performed with Arabidopsis plants grown in a sterile medium in Magenta boxes. A comparison with influx analyses conducted under non-axenic growth conditions provided no indication of urea degradation prior to short-term urea uptake from nutrient solutions (Gassert, 2006).

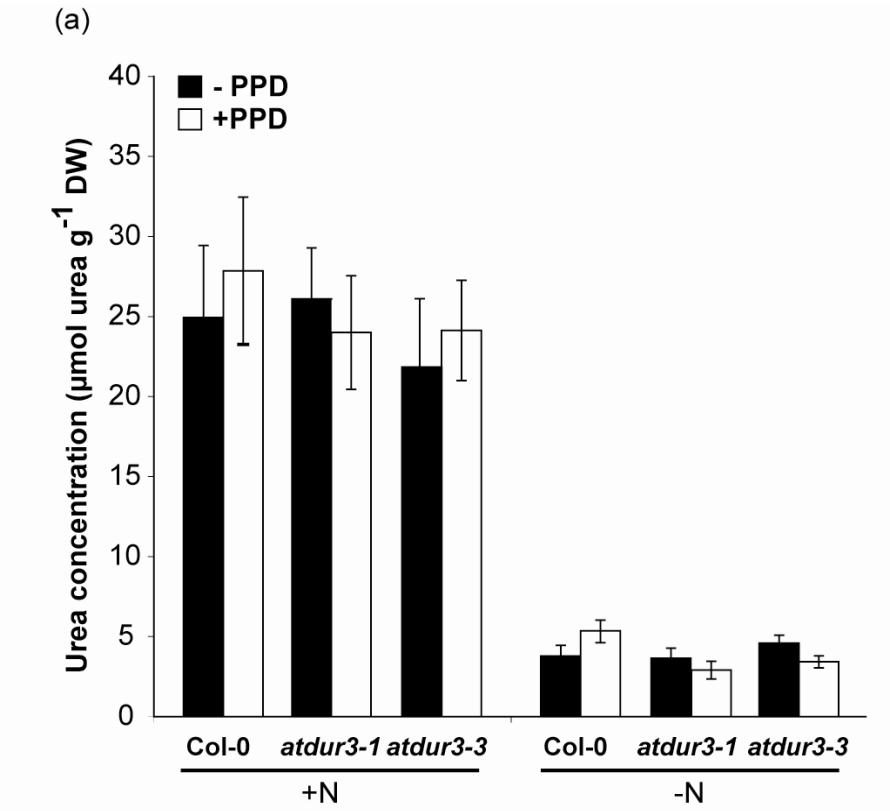

Figure 7: No effect of the urease inhibitor PPD on urea uptake and translocation or *AtDUR3* gene expression. Continuation next page.

(a) Urea concentrations in roots of Col-0, *atdur3-1* or *atdur3-3* plants grown hydroponically for 38 d under continuous supply of 2 mM ammonium nitrate or, alternatively, for a period of 4 d under N deficiency. Plants were grown in the presence or absence of phenylphosphorodiamidate (PPD) for 3 h. Bars indicate means ± SD, n =10. (b) Influx of ^{15}N-labeled urea into roots and translocation to shoots of wild-type Col-0 plants grown hydroponically for 38 d under continuous supply of 2 mM ammonium nitrate before culture under N deficiency for 4 d. ^{15}N-labelled urea was supplied at 200 µM in the presence or absence of PPD. Bars indicate means ± SD, n =10. (c) RNA gel blot analysis of the wild-type root samples from (b) using the coding sequence of *AtDUR3* as a probe. Ethidium bromide-stained RNA (EtBr) served as a loading control.

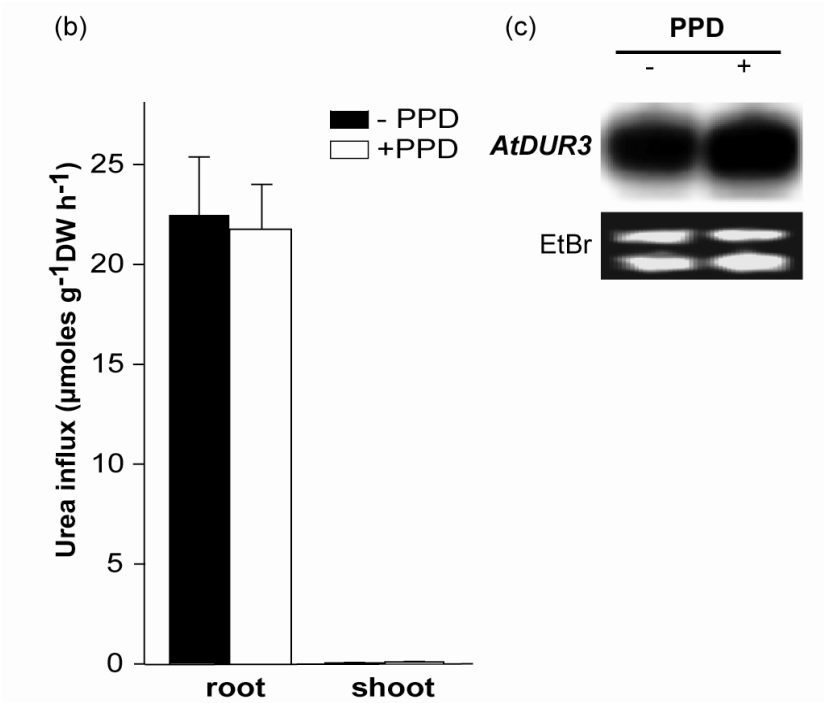

Figure 7 continued.

In order to independently verify whether urea is taken up as an intact molecule or else in the form of its degradation product (ammonium), the concentration of urea in the root tissue of plants subjected to different N treatments was determined. To avoid any possible degradation of urea, nutrient solutions were supplemented with PPD. When Col-0 plants and the two insertion lines *atdur3-1* and *atdur3-3* were cultured with a continuous supply of 2 mM ammonium nitrate, root urea concentrations were around 25 µmol g^{-1} dry weight (DW) and did not differ significantly between lines (Figure 8). Under conditions of N deficiency, urea levels in all lines decreased by approximately 80%. After resupply of 50 or 100 µM urea for 3 h to N-deficient plants, urea accumulated only in wild-type roots and reached three- to fivefold higher levels than in the two insertion lines (Figure 8). Thus, functional expression of AtDUR3 enhanced the accumulation of intact urea molecules, suggesting the acquisition of externally fed urea by AtDUR3 *in planta*. However, after supply of 1 mM urea, accumulation of urea in both *atdur3* insertion lines increased to a level corresponding to 60% of the wild type, suggesting that other low-affinity transport systems then contributed to urea uptake.

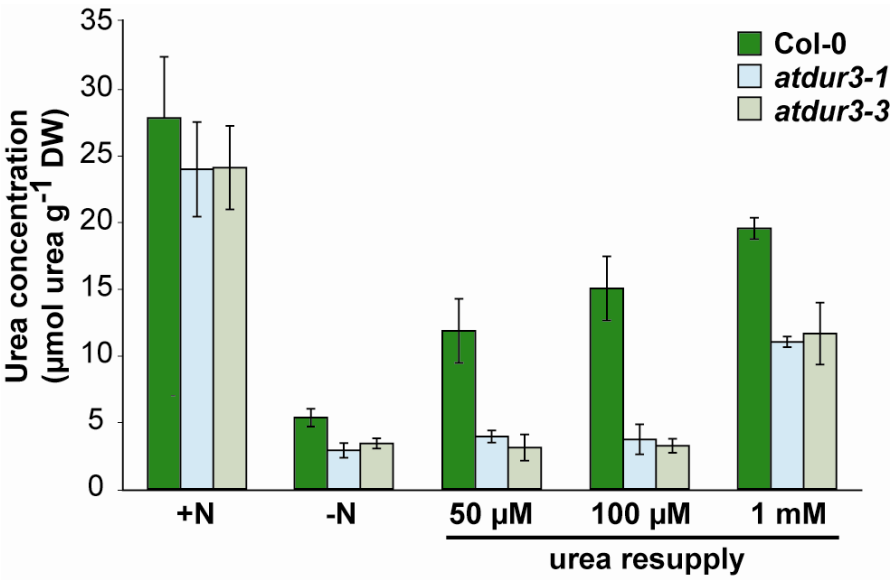

Figure 8: Urea accumulation in roots of wild-type plants and *atdur3* insertion lines in response to urea resupply.

Plants were cultured hydroponically for 6 weeks under continuous supply of 2 mM ammonium nitrate (+N). After a N starvation treatment for 4 days (-N), plants were resupplied with different concentrations of urea for 3 h in the presence of the urease inhibitor phenylphosphorodiamidate (PPD). Urea concentrations were determined in freeze-dried root samples.

4.1.6 High-affinity urea uptake by AtDUR3 in Arabidopsis roots

For a concentration-dependent influx analysis of ^{15}N-labeled urea in roots, the two insertion lines *atdur3-1* and *atdur3-3* were grown together with their wild-type for 38 days on nutrient solution containing 2 mM ammonium nitrate before they were subjected to N deficiency for another 4 days. In a concentration range of 3-200 µM, urea influx in Col-0 roots approached saturation at approximately 17 µM (Figure 9a). In contrast, urea influx into roots of *atdur3-1* and *atdur3-3* plants was between 70 and 90% lower than in the wild-type and identical in both insertion lines, showing no saturable kinetics within this concentration range. A low-affinity uptake analysis conducted with the wild-type Col-0 showed that urea influx continued to increase with an almost linear concentration dependency between 200 and 1200 µM (Figure 9b). Urea influx in *atdur3-1* increased with increasing external supply at a similar rate as in the wild-type. At 1.2 mM urea, uptake rates in *atdur3-1* were only 20–30% lower than in the wild-type. Thus, kinetic analysis revealed a dominant role for AtDUR3 only in high-affinity urea uptake in Arabidopsis roots and allowed calculation of an apparent affinity constant (K_m) for urea transport by AtDUR3 of 4.0 µM.

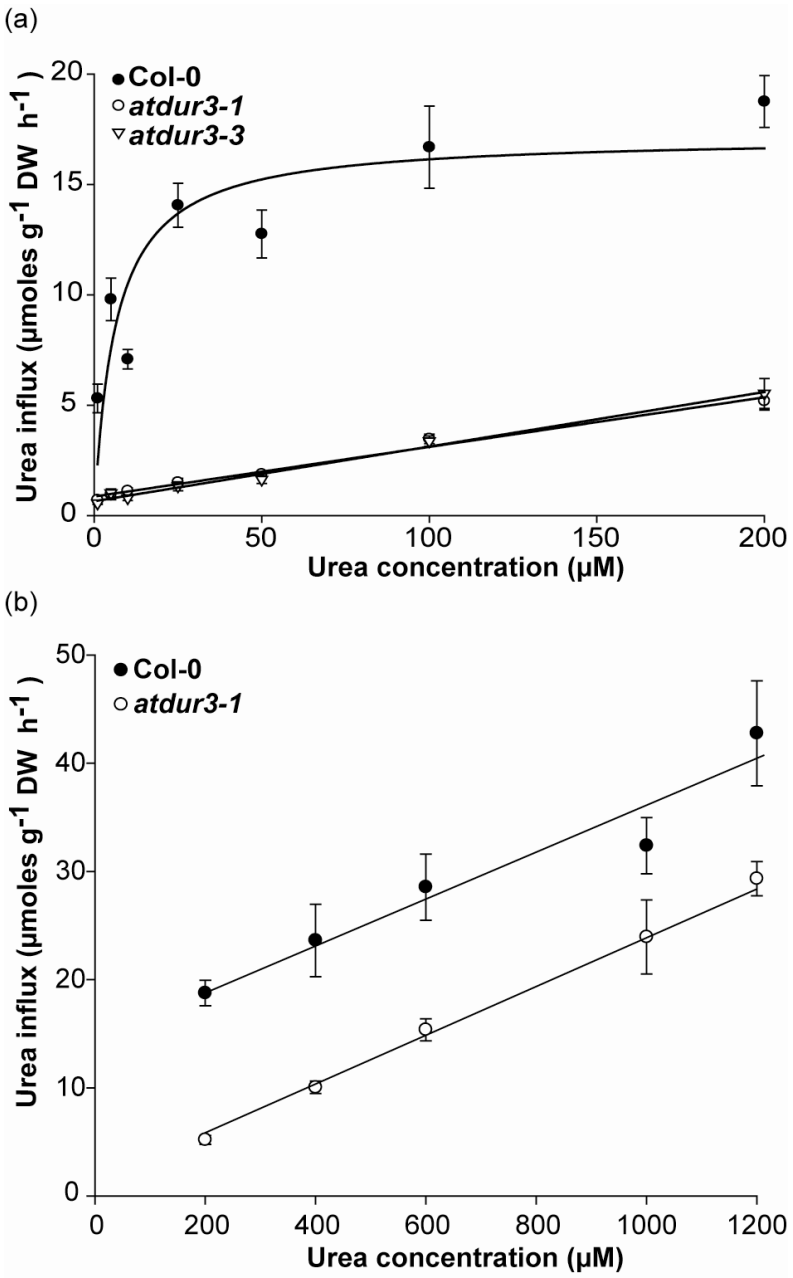

Figure 9: Disruption of *AtDUR3* decreases the capacity for high-affinity uptake of ^{15}N-labeled urea.

Concentration-dependent influx of ^{15}N-labeled urea into roots of Col-0, *atdur3-1* and *atdur3-3* plants. Plants were cultured in nutrient solution containing 2 mM ammonium nitrate for 38 days before transfer to N deficiency for 4 days. (a) Urea influx into wild-type and *atdur3-1* or *atdur3-3* plants at an external supply of 3–200 µM urea. (b) Urea influx into roots of wild-type and *atdur3-1* plants at an external supply of 200-1200 µM urea. Dots indicate means ± SD, n = 10.

4.2 A role of AtDUR3 in urea accumulation and urea translocation out of senescent leaves

The studies on the high-affinity urea transporter AtDUR3 in the first part of this thesis focused on its physiological function in primary uptake of urea from the soil. The second part will elucidate its role in intercellular transport and N retranslocation during senescence in shoots.

4.2.1 In vegetative growth stages *AtDUR3* expression and urea concentration in shoots are only weakly affected by the nitrogen status

To obtain an insight into the physiological relation between urea and *AtDUR3* gene expression in different organs of Arabidopsis, RNA gel blot analysis and urea concentration measurements were performed in roots and shoots of hydroponically-grown Arabidopsis plants, that were precultured under adequate or deficient N supply (Figure 10).

Under N-sufficient growth conditions *AtDUR3* mRNA could not be detected in roots or shoots (Figure 10a). In agreement with previous gene expression analysis (Figure 6) *AtDUR3* was strongly de-repressed in roots of N-starved plants. In view of these high expression levels in roots, *AtDUR3* gene expression in shoots showed only a slight increase under N-deficient conditions (Figure 10a).

Urea concentrations under N-sufficient conditions were three times higher in roots than in shoots (Figure 10b). In roots, N deficiency led to a strong decrease in urea concentrations by about 85%, while urea concentrations in shoots did not change at all. This indicated a rapid depletion of the root urea pool as a N reserve under N-limiting conditions, while the urea pool in the shoot remained unaffected and thereby may operate in a different physiological context. Interestingly, the level of urea in roots under N deprivation was similar to the concentration found in shoots suggesting that this reflects a background level of urea generated by protein turnover that is not serving as a N source under N-limiting conditions.

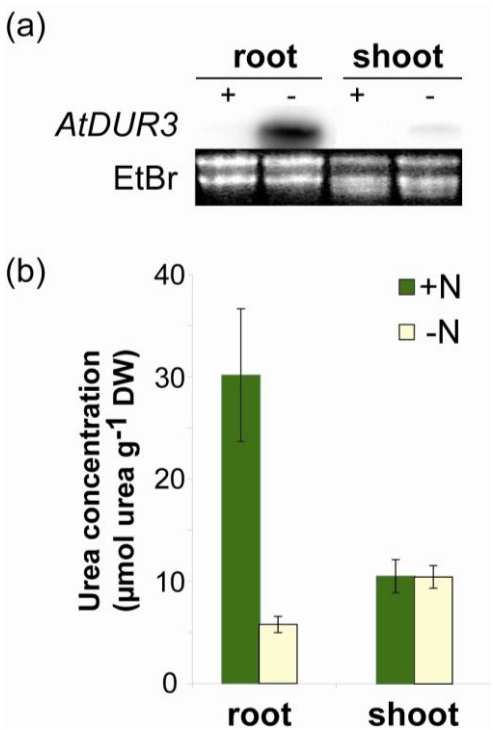

Figure 10: *AtDUR3* gene expression and urea concentrations in roots and shoots of Arabidopsis wild-type plants in dependence of the N nutritional status.

(a) RNA gel blot analysis of total root and shoot RNA from 6 week-old wild-type Arabidopsis plants cultured hydroponically on 2 mM NH_4NO_3 continuously (+N) or for 4 days on N-deficient medium (-N). (b) Urea accumulation in roots and shoots of wild-type Arabidopsis plants grown hydroponically for 6 weeks under permanent NH_4NO_3 supply (+N) or N-deficient medium for 4 days (-N). Bars indicate means ± SD, n=6. DW, dry weight. Ethidium bromide (EtBr)-stained gel was used as RNA loading control.

Daytime-dependent changes in either urea concentrations or *AtDUR3* and *AtTIP2;3* gene expression in wild-type plants cultured under adequate N supply were monitored over a 24h time period (Figure 11). While *AtTIP2;3* showed a strong diurnal regulation which was in accordance to findings of Loqué *et al*. (2005), mRNA abundance of *AtDUR3* was below the detection limit (Figure 11). This is clearly different to the expression pattern observed for root transporters such as ammonium transporters, which showed a distinct diurnal regulation with stronger expression levels during the day due to their co-regulation by photoassimilates or related metabolites from shoots (Gazzarrini *et al*., 1999, Lejay *et al*., 2003). Furthermore, neither in root nor in shoot tissues there was any significant daytime-dependent change in the urea concentration (Figure 11b) indicating that urea pools are only affected under N deficiency in roots, but seem to play only a minor role in N distribution and balance under adequate N nutrition.

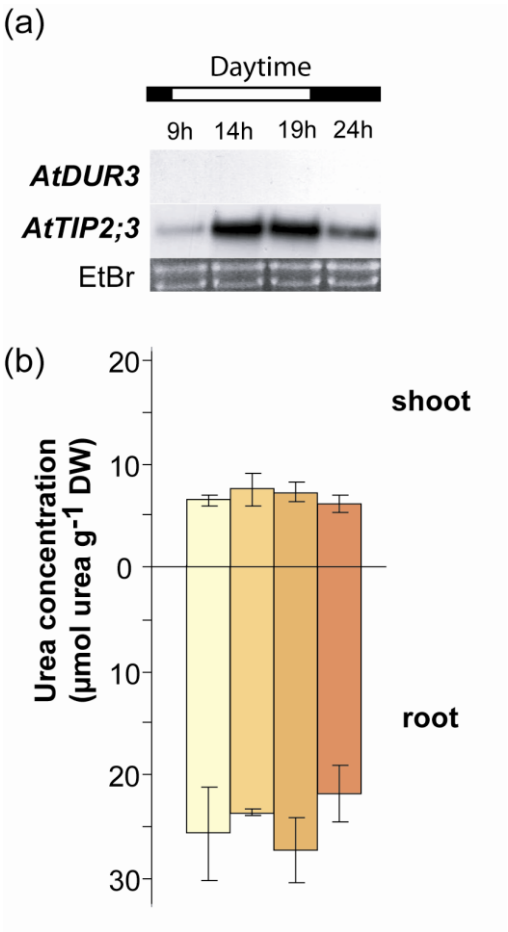

Figure 11: *AtDUR3* gene expression in roots and urea concentration in dependence of daytime.

(a) Total root RNA was used for RNA gel blot analysis applying *AtDUR3* and *AtTIP2;3* as probes. (b) Urea concentrations as determined in root and shoot samples harvested at different daytimes. 6 week-old Col-0 plants grown on 2 mM NH_4NO_3 under short-day conditions were harvested at 9 (onset of light), 14, 19 (beginning of dark phase) and 24 o´clock. EtBr-stained gel served as loading control for the RNA gel blot analysis. Bars indicate means ±SD, n=3.

4.2.2 The influence of leaf and plant age on urea accumulation and the regulation of urea transporters

In silico analysis confirmed that the urea transporter *AtDUR3* was only weakly expressed in leaves of plants during the vegetative growth phase. However, several independent transcriptome studies revealed that *AtDUR3* was highly de-repressed in senescent and cauline leaves (Figure 12).

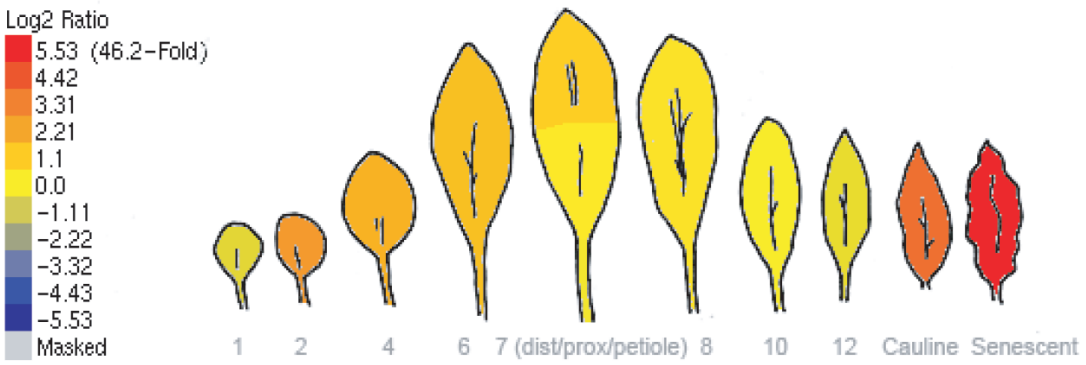

Figure 12: Microarray analysis indicates an elevated expression of AtDUR3 in senescent leaves.

Data were obtained from the Arabidopsis eFPBrowser (at bar.utoronto.ca, Winter *et al.*, 2007). All data were produced using the Affymetrix ATH1 Arabidopsis Genome Array.

To further investigate the role of AtDUR3 in senescent leaves, leaf material of mature Arabidopsis plants was assayed that corresponded either to different leaf age or to different developmental stages. During vegetative plant growth of up to 6.5 weeks, urea concentrations were similar between old and young leaves (Figure 13a).

With the beginning of bolting after 7.5 weeks a weak increase of urea concentration in older leaves (7-14) was observed. After 8.5 weeks the urea level in all leaves steeply increased reaching 4-5 times higher levels in week 10.5 than in younger plants. The concentration of urea thereby increased gradually from young leaves to old leaves in week 8.5, while in the middle of the 9th or 10th week the highest urea concentration was shifted to younger leaves as the oldest leaves started to decay.

The expression levels of *AtDUR3* in the same samples mostly followed the urea concentrations in the leaf fractions (Figure 13b). Thus, *AtDUR3* transcript levels were regulated by plant age as well as by leaf age. The overall level of *AtDUR3* expression rose especially after the plants turned into the generative growth phase between weeks 6.5 and 7.5 and the increase in urea accumulation. While in week 5.5 highest *AtDUR3* expression was in the oldest leaf, it was almost undetectable in 6.5 week-old leaves and then increased especially in the middle-agedd leaves up to the young leaves at 9.5 weeks. RNA levels of the ammonium transporter *AMT1;1* were similarly regulated as those of *AtDUR3* increasing with plant age.

AMT1;3, however, was differently expressed and rather derepressed in younger plants.

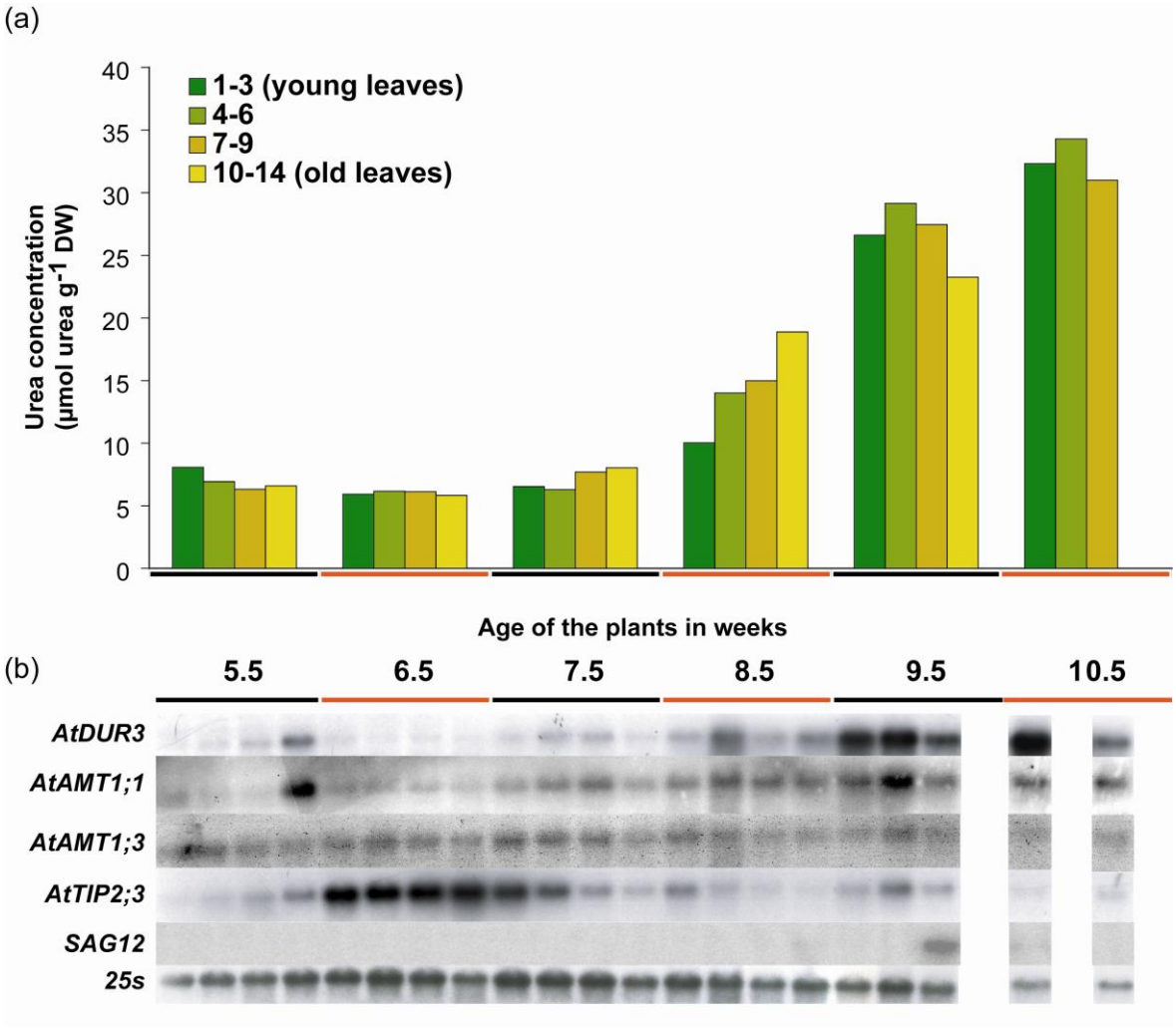

Figure 13: Urea concentrations and corresponding gene expression analysis of *AtDUR3, AtAMT1;1, AtAMT1;3*, *AtTIP2;3* and *SAG12* in leaves in dependence of plant and leaf age.

(a) Urea concentrations and (b) RNA gel blot analysis of *AtDUR3*, *AtAMT1;1, AtAMT1;3*, *AtTIP2;3* and *SAG12* in Arabidopsis leaves of different plant age. To generate four pools of different leaf age, leaves no. 1-3 (young leaves), 4-6, 7-9 and 10-14 (older leaves) were harvested separately and pooled for analysis. Plants were grown on soil under low light conditions and harvested between 5.5-10.5 weeks always 4 h after the onset of light. The leaf material was kindly provided by Ulrike Zentgraf, ZMBP Tübingen, Germany. A probe against 25S was used as loading control for RNA gel blot analysis. RNA quality of old leaves after 9.5 and 10.5 weeks was too poor to yield reproducible results. Bars indicate means of 3 technical replica.

In contrast, the tonoplast-localized aquaporin *AtTIP2;3*, which can also act as a low-affinity urea transporter, showed a different transcriptional regulation to that of *AtDUR3*. *AtTIP2;3* was most upregulated in all leaves in week 6.5. Before, at 5.5 weeks, *AtTIP2;3* was preferentially expressed in the old leaves. Thereafter, *AtTIP2;3* expression levels decreased in particular in the older leaves and approached the detection limit. The higher the age of the plant was, the weaker

was the expression of *AtTIP2;3*, and it was therefore almost an opposite regulation to *AtDUR3*.

As a marker for senescence the expression of *SAG12* (senescence-associated gene 12, encoding a cysteine protease) was investigated as well. Due to several rounds of hybridisation and stripping the membrane quality was already negatively affected, but expression was detected in old leaves of 9.5 week-old plants and weakly also in young leaves of 10.5 week-old plants. This was in agreement with studies of Zentgraf *et al*. (2004) who showed in comparable samples an induction of transcript levels for *SAG12* in old leaves of 8 week-old plants but not in young leaves.

4.2.3 Different triggers of senescence induce *AtDUR3* gene expression and urea accumulation

As urea concentrations and *AtDUR3* gene expression were elevated in senescent leaves (Figure 13) a possible contribution of the transporter to urea accumulation was addressed by comparing wild-type plants with the T-DNA insertion line *atdur3-1* (Figure 14). Senescence was induced by a switch from short to long days after 4 weeks of growth and urea concentrations were measured in three different leaf fractions pooling leaf number 1-6 (old leaves), 7-12 or all leaves above (>12, young leaves). After 5 weeks urea concentrations were still low in all leaf fractions being at a similar level than shown before (Figure 10 and Figure 13), and there was no significant difference between the wild type and *atdur3-1*. However, there was a tendency to slightly higher urea concentrations in the oldest leaves (1-6) in both lines.

One week later urea concentrations rose in all leaf fractions to a twofold higher level in young leaves (>12) and up to a fourfold higher level in old leaves (1-6). Most interestingly, while in the young (>12) and middle (7-12) aged leaves urea concentrations in the two lines were similar, urea concentrations in old wild-type leaves were approximately 40% higher than those in *atdur3-1* leaves. Nevertheless, urea concentrations in the *atdur3-1* insertion line also increased with the age of the leaves, but did not exceed the level found in middle-aged leaves (7-12).

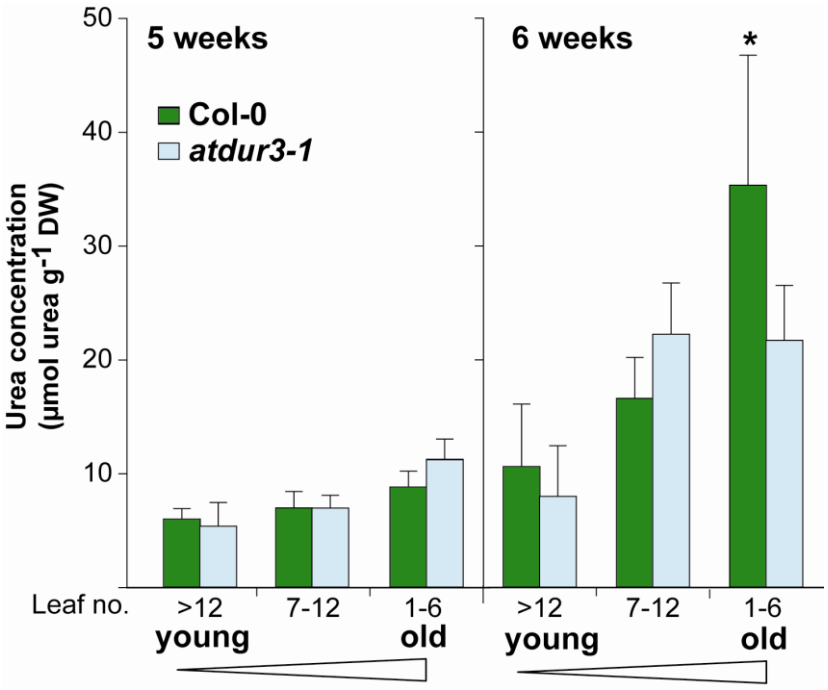

Figure 14: In the generative phase AtDUR3 contributes to elevated urea concentrations in old leaves.

Urea concentrations determined in 5 or 6 weeks-old wild type (Col-0) and *atdur3-1* plants. Plants were grown on nutrient solution and transferred after 4 weeks of culture in short days to long-day conditions. Bars represent means ±SD, n=10. The asterisk denotes significant differences among means at $p<0.05$ according to Tukey´s test.

Since there are many external signals to trigger senescence, *AtDUR3* expression and urea accumulation were also investigated in plants under short day conditions when senescence was induced either by N deprivation (Figure 15) or by shading of single leaves (Figure 16).

Northern analysis comparing *AtDUR3* gene expression in young and old leaf tissues (Figure 15a) of the N-starved plants were as well in agreement with microarray data from developmental series (Figure 12). In wild-type shoots the transcript level of *AtDUR3* was more induced in old than in young leaves. As expected, *AtDUR3* transcripts were absent in the *atdur3* insertion line. Thus, elevated urea concentrations in old leaves of Arabidopsis plants coincided with an enhanced expression level of *AtDUR3*.

In N-starved plants the concentrations of urea (Figure 15b) in young leaves showed no difference between the wild-type and the *atdur3-1* insertion line but were 2-3 fold lower than in old leaves. These concentrations were similar to those in shoots during the vegetative growth stage (Figure 14). In old leaves of plants subjected to N-limiting conditions urea concentrations were 20% lower in *atdur3-1*

compared to the wild type, but still rose above the level in young leaves. Thus, the differences in urea concentration of old leaves between wild type and *atdur3-1* showed a similar pattern in developmental as in N starvation-induced senescence.

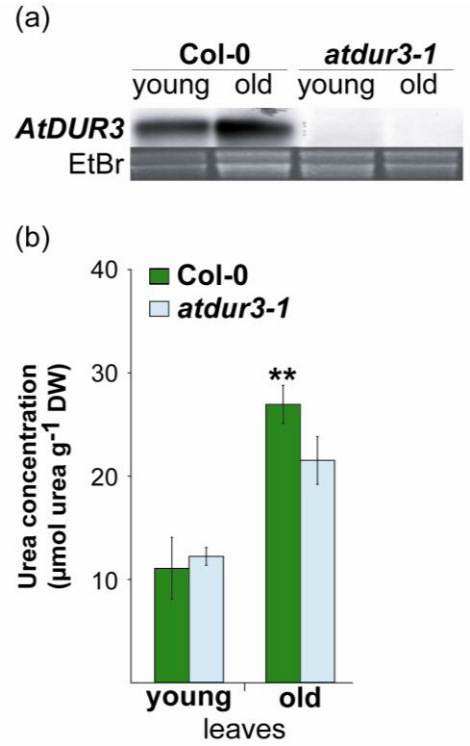

Figure 15: *AtDUR3* gene expression and leaf urea concentrations in wild-type and in *atdur3-1* under prolonged N deprivation.

(a) RNA gel blot analysis of *AtDUR3* expression and (b) urea concentrations in shoots of wild-type (Col-0) and *atdur3-1* plants precultured on 2 mM NH_4NO_3 for 6 weeks in short-day conditions and transferred on N-deprived medium for 9 days. Bars indicate means ±SD, n=6. The asterisk denote significant differences among means at $p<0.001$ according to Tukey´s test.

Leaf shading for up to three days also lead to an increase in urea concentrations (Figure 16b). Within the first day of shading urea concentrations in wild-type leaf no. 7 (middle-aged leaf) did not differ significantly, even though it tended to increase within the first 12h. After three days a significant rise of around 50% up to 40 µmol g^{-1} DW were observed which was accompanied by first visible symptoms of leaf yellowing (data not shown). RNA gel blot analysis showed a weak expression of *AtDUR3* without shading and an upregulation reaching its highest expression after 1 day of shading (Figure 16a).

Urea concentrations in the transgenic line *ureGA* also increased due to shading. Starting from a concentration reached by the wild type after 3 days of shading, urea concentrations in *ureGA* were about one third higher at all times. In the first 24 h urea concentrations stayed the same and significantly increased after 3 days.

AtDUR3 gene expression in *ureGA* leaves started as well at a higher expression intensity than the wild-type but stayed constant up to 1 day. Only after 3 days of shading RNA levels increased reflecting the development of urea concentrations (Figure 16) Interestingly, *SAG12* expression was induced only after 3 days of shading in both lines, but was more prominent in Col-0.

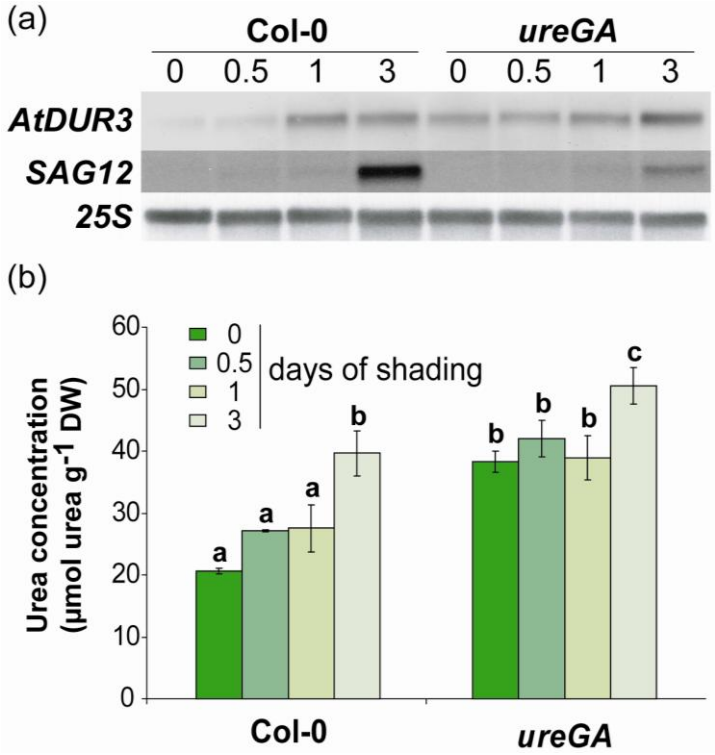

Figure 16: *AtDUR3* and *SAG12* gene expression and urea concentration in leaf no. 7 in dark induced senescence.

(a) RNA gel blot analysis of *AtDUR3* and *SAG12* expression and (b) urea concentrations in leaf no. 7 of wild-type (Col-0) and *ureGA* plants after shading. Plants were cultured on 2 mM NH_4NO_3 for 6 weeks when leaf no. 7 was shaded with aluminium foil for 0.5- 3 days. Bars indicate means ±SD, n=3. Significant differences among means at $p<0.05$ according to Tukey´s test are denoted by different letters. A probe against *25S* served as loading control for RNA gel blot analysis.

Taken together urea concentrations and *AtDUR3* gene expression levels rose after onset of senescence irrespective of the means by which senescence was induced. Thereby, both measures were elevated in the oldest leaves and progressed with time to the younger leaves as senescence evolved.

4.2.4 A role of AtDUR3 in urea retrieval

High urea accumulation in senescent leaves might lead to a loss of urea from the cytoplasm into the apoplast by e.g. aquaporins of the NIP, PIP and/or TIP families located at the plasma membrane. Since AtDUR3 was up-regulated simultaneously

with increasing urea concentrations and since it is functional at the plasma membrane, a role in urea retrieval of AtDUR3 was tested. Therefore, urea concentrations in apoplastic wash fluids from N-starved leaves of different age in wild-type and *atdur3-1* plants were determined as well as the urea levels in the corresponding leaves (Figure 17).

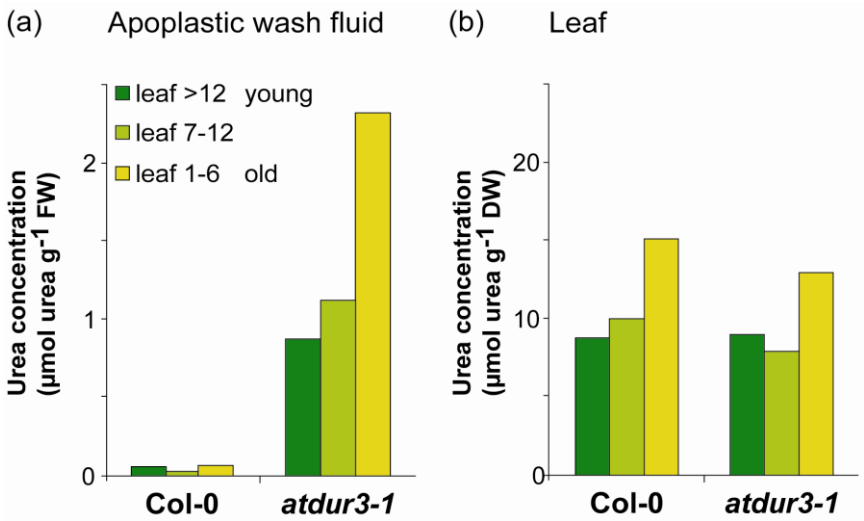

Figure 17: Urea determination in the apoplastic wash fluid and its corresponding leaves.

Urea concentrations in (a) the apoplastic wash fluid of pooled leaf fractions and (b) in the pooled leaf fractions itself. Wild-type and *atdur3-1* plants were cultured hydroponically under short-day conditions on 2mM NH_4NO_3 for 5 weeks before transfer to long-day conditions. The different leaf fractions were collected from 6 week-old plants that were deprived of N for 4 days. Bars show means of 3 technical replica.

Preliminary results showed that in wild-type leaves the urea concentrations in the apoplastic wash fluid were low and did not differ in the different leaf fractions (Figure 17a). In contrast, urea concentrations in the apoplastic solution of the *atdur3-1* insertion line were much higher than in the wild type and additionally increased with leaf age, reaching more than 30-fold higher levels in senescent leaves corresponding to approximately 1 mM urea.

For comparison, urea concentrations in leaf extracts were analysed as well (Figure 17b). As expected, urea accumulated to highest levels in the oldest leaves. There was no obvious difference between the two lines. This suggests a major role of AtDUR3 in recapturing urea from the apoplast during leaf senescence.

4.2.5 Urea as a translocated nitrogen form in senescent leaves and the contribution of AtDUR3 to urea translocation

The intercellular localisation of a protein often provides an important indication of its physiological function. Transgenic lines expressing the *AtDUR3pro::GUS* construct were analysed for their GUS activity in senescent leaves. For this purpose plants were cultured on soil under long-day conditions and old leaves showing first symptoms of leaf yellowing were used for GUS staining (Figure 18). Preliminary results indicated that the *AtDUR3* promoter is active in the leaf vasculature, suggesting that the transporter might be either involved in the intercellular transport of urea from neighbouring cells towards the vascular bundle or even contributes to phloem loading. When senescence was induced by N deficiency, a similar localization was observed showing stronger expression towards the base of the leaf and getting less pronounced towards the tip, where senescence was already too far advanced.

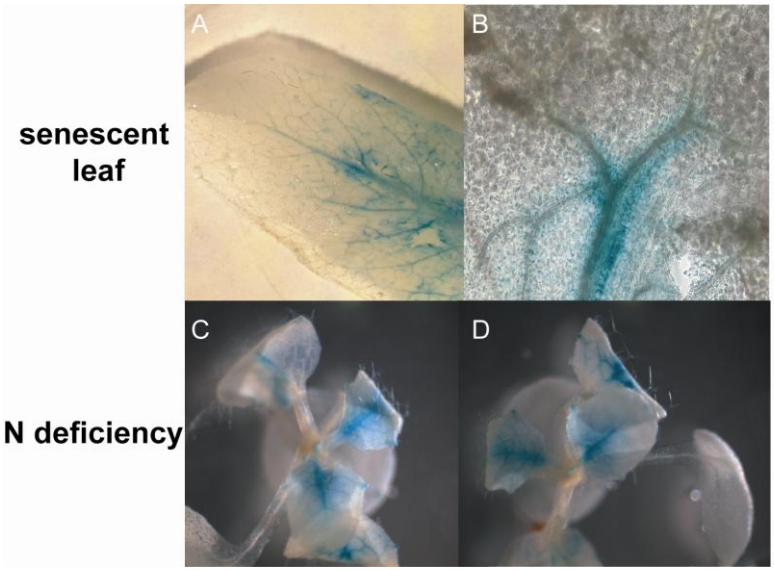

Figure 18: Gus staining in transgenic lines expressing *AtDUR3pro::GUS*.

Transgenic plants expressing the *AtDUR3pro::GUS* construct were cultured for 8 weeks on soil under long day conditions (A, B) and leaves showing first symptoms of senescence were analysed for GUS activity. Transgenic plants grown under axenic conditions and short-day conditions for 4 weeks under adequate N supply were subjected to N deficiency for 4 days and GUS staining was performed (C, D).

To test the hypothesis that urea is a suitable form for N retranslocation in Arabidopsis, a preliminary experiment was performed in leaves of senescing wild-type plants precultured under adequate or N-deficient conditions. Leaf petiole exudates, as an approximation for the composition of the phloem sap, were

collected from leaves of different age and urea concentrations therein were compared to those in whole leaves (Figure 19). In whole leaves that were grouped to obtain sufficient amount of biomass for analysis, urea concentrations increased with the age of the leaf irrespective of the N status of the plant (Figure 19a). Urea concentrations in the leaf petiole exudates followed that tendency observed in the whole leaves and showed highest urea concentrations in the exudates of the oldest leaf (Figure 19b). Interestingly, even if urea concentrations in the oldest leaf fraction under N-deficiency were slightly lower in the whole leaves, urea concentrations in the corresponding leaf petiole exudates tended to increase compared to an adequate N status of the plants indicating an increase of urea translocation under N limiting conditions only from the oldest leaves. Cauline leaves showed low urea concentrations in the whole leaves as well as in leaf petiole exudates and were therefore more similar to a young leaf then to an old leaf.

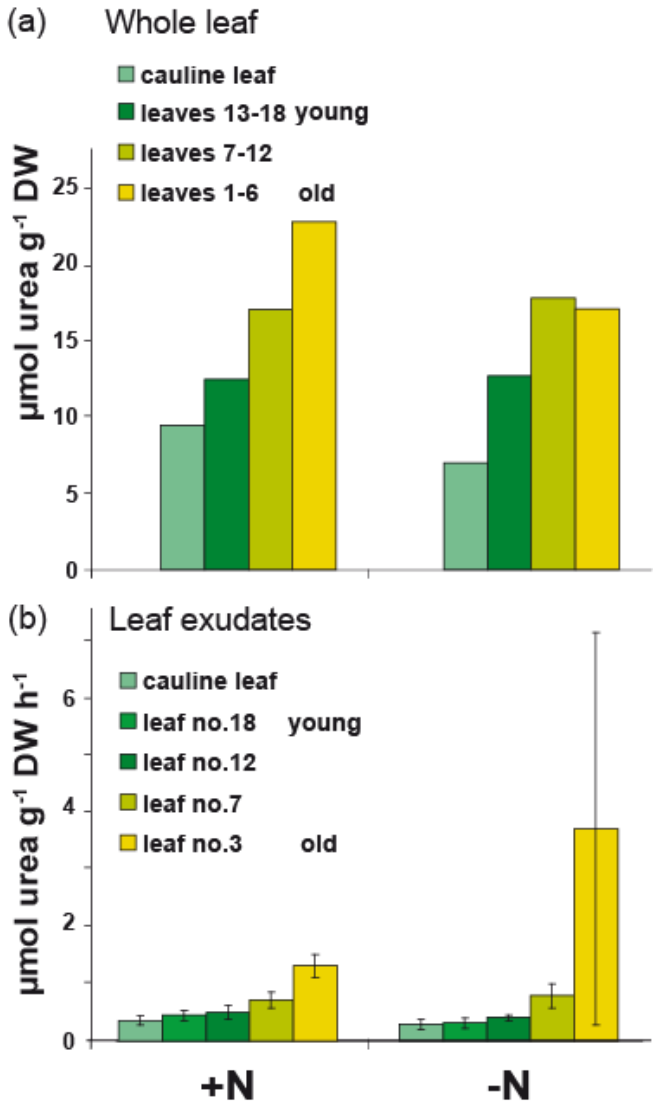

Figure 19: Dependence of urea concentration in leaves and leaf petiole exudates on leaf age and N status

(a) Urea concentrations in whole leaf fractions of wild type plants and (b) urea concentrations in leaf petiole exudates in leaves of defined age collected for 6h in 10 mM EDTA solution. Cauline, young (leaf no. 18), middle-aged (leaf no.12 and 7) and old leaves (leaf no. 3) of three plants were pooled for analysis. Plants were cultured hydroponically under adequate N supply (2 mM NH_4NO_3) for 4 weeks before transfer to long-day conditions for another 2 weeks to induce senescence. N deficiency was induced 4 days prior to harvest (-N). Bars indicate means ± SD, n=4.

In order to enhance urea accumulation in leaves, the *atdur3-1* line was crossed with *ureGA*. In addition, AtDUR3 over-expression lines were examined. To test these lines for their urea uptake capacity, influx analysis at 100 µM ^{15}N-labelled urea was performed in roots precultured under N-sufficient or N-deficient conditions (Figure 20). The overexpressor line showed the highest urea influx in N-sufficient roots indicating that the protein is not only strongly expressed in this line (Figure 21a) but is also functional. Under N-limiting conditions also the wild

type and *ureGA* showed elevated urea influx, while in the overexpressor the uptake capacity was even doubled, suggesting that in addition to the ectopically expressed AtDUR3 protein, also the native AtDUR3 protein was upregulated, leading to a strong increase in urea uptake capacity.

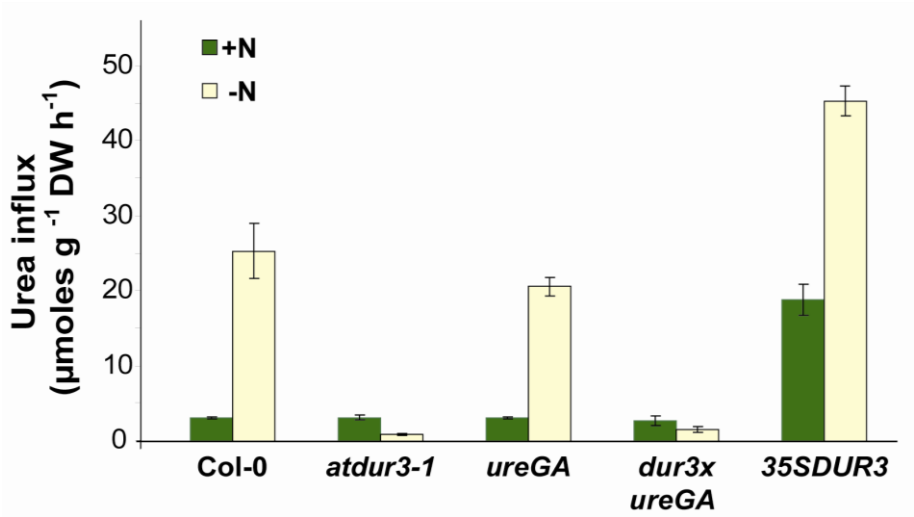

Figure 20: Urea uptake analysis in dependence of the nitrogen status in different mutants defective in urea transport or metabolism.

Influx of ^{15}N-labeled urea into Arabidopsis roots of wild-type Col-0, *atdur3-1*, *ureGA*, *dur3ureGA* and *35SDUR3* in dependence of the N status. Plants were grown hydroponically for 38 d under continuous supply of 2 mM NH_4NO_3 (+N) or subjected to N deprivation for 4 days (-N) prior to the experiment. 100µM ^{15}N-labeled urea was supplied for 10 min. Bars indicate means ± SD, n=8.

Using these mutant lines impaired either in urea transport and/or metabolism, plants were cultured hydroponically under adequate or deficient N supply after induction of senescence by transfer to long-day conditions and urea concentrations were compared in leaf petiole exudates and the corresponding leaf extracts (Figure 21 and Figure 22).

In wild-type plants precultured under N-sufficient conditions Western blot analysis of microsomal membrane fractions in leaf samples of different leaf age revealed a very low amount of the AtDUR3 protein in the youngest leaves that increased with the age of the leaf (Figure 21a). As expected, in *atdur3-1* and *dur3ureGA* plants no AtDUR3 protein was detectable. In the urease-defective *ureGA* mutant AtDUR3 abundance in each leaf fraction was higher than in the wild type and showed a similar increase towards the oldest leaf. *35SDUR3* showed very high protein levels of AtDUR3 which were not affected by leaf age and were much higher than in any other line.

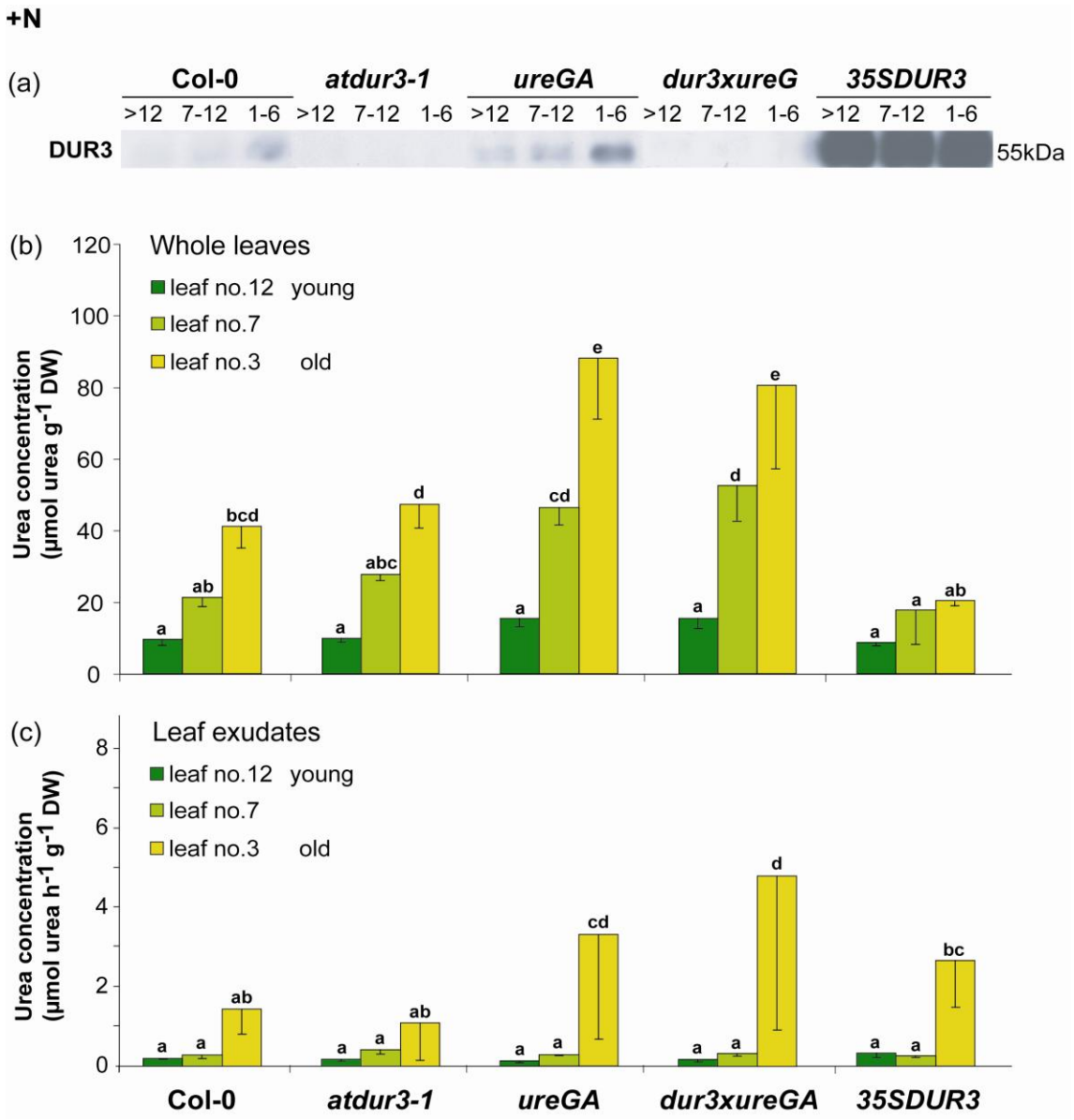

Figure 21: Contribution of AtDUR3 and *UreG* to urea accumulation and urea export in leaves of N sufficient plants.

(a) Protein gel blot analysis of AtDUR3 in leaves of different age from wild type (Col-0), *atdur3-1*, *ureGA*, *dur3ureGA* and *35SDUR3*. Microsomal membrane fractions (MMF) were prepared from N-sufficient plants and 5µg MMF per lane were assayed by anti-DUR3. (b) Urea concentrations in whole leaves and (c) urea concentrations in leaf petiole exudates collected for 6h in 10 mM EDTA solution. Young (leaf no. 12), middle-aged (leaf no. 7) and old leaves (leaf no. 3) of three plants were pooled for analysis. Plants were cultured hydroponically under adequate N supply (2 mM NH_4NO_3) for 4 weeks before transfer to long day to induce senescence. Bars indicate means ± SD, n=5. Different letters denote significant differences among means at $p<0.05$ according to Tukey´s test.

Urea concentrations in wild-type leaf samples of different leaf age showed significant differences with low urea concentrations in younger or sink leaves and up to 4 times higher concentrations in leaves of more advanced leaf age (Figure 21b). Unexpectedly, urea concentrations in the oldest leaves of *atdur3-1* did not

differ from the wild type, which might be due to a reduced light intensity causing a delayed development compared to earlier experiments (Figure 14). In *ureGA*, urea concentrations in all leaf samples nearly doubled compared to the wild type, though not significantly in the youngest leaf. In all lines the effect of leaf age on urea concentration was prominent. The leaves in the *dur3ureGA* line showed the same increase in urea concentrations as its background line *ureGA*. Interestingly, the *AtDUR3* overexpressor line *35SDUR3* showed only a tendency of a leaf age-dependent increase in urea accumulation.

As an approximation for phloem sap composition leaf petiole exudates were collected and analyzed for urea concentrations (Figure 21c). In general, urea concentrations in leaf petiole exudates followed a similar pattern as in their corresponding leaves, increasing with leaf age. Unlike urea concentrations in leaves, there was no significant change in urea levels in the exudates of young and middle-aged leaves in any line. Only the old leaves showed a varying degree of urea in their exudates. Again, there was no significant difference between the urea concentration in the sap of the oldest leaf between Col-0 and *atdur3-1. UreGA* showed a significantly higher urea concentration in the exudates of leaf no. 3, which was also found in *dur3ureGA*. Urea export from older leaves in *35SDUR3* was slightly higher than in wild-type plants.

In addition to the plant culture under N-sufficient (+N) conditions another set of plants was starved for N for 4 days before harvest to provoke the N remobilization processes (Figure 22). Under these conditions, AtDUR3 protein levels in Col-0 slightly increased relative to N-sufficient conditions and were highest in the oldest leaf. In the *ureGA* mutant, the protein was more abundant and still showed an increase towards the oldest leaves. Again, AtDUR3 abundance in *35SDUR3* was not dependent on the age of the leaf and was high in all three leaf fractions.

The effect of nitrogen depletion on urea accumulation was strongest in the oldest and middle-aged leaves emphasising the importance of N remobilization processes on urea accumulation. In the wild-type, young and middle-aged leaves were not affected, but in the oldest leaf urea concentrations rose by about 25% relative to N-sufficient plants. Nitrogen starvation had little impact on the oldest leaves of *atdur3-1* and therefore tended to be slightly lower compared to wild type.

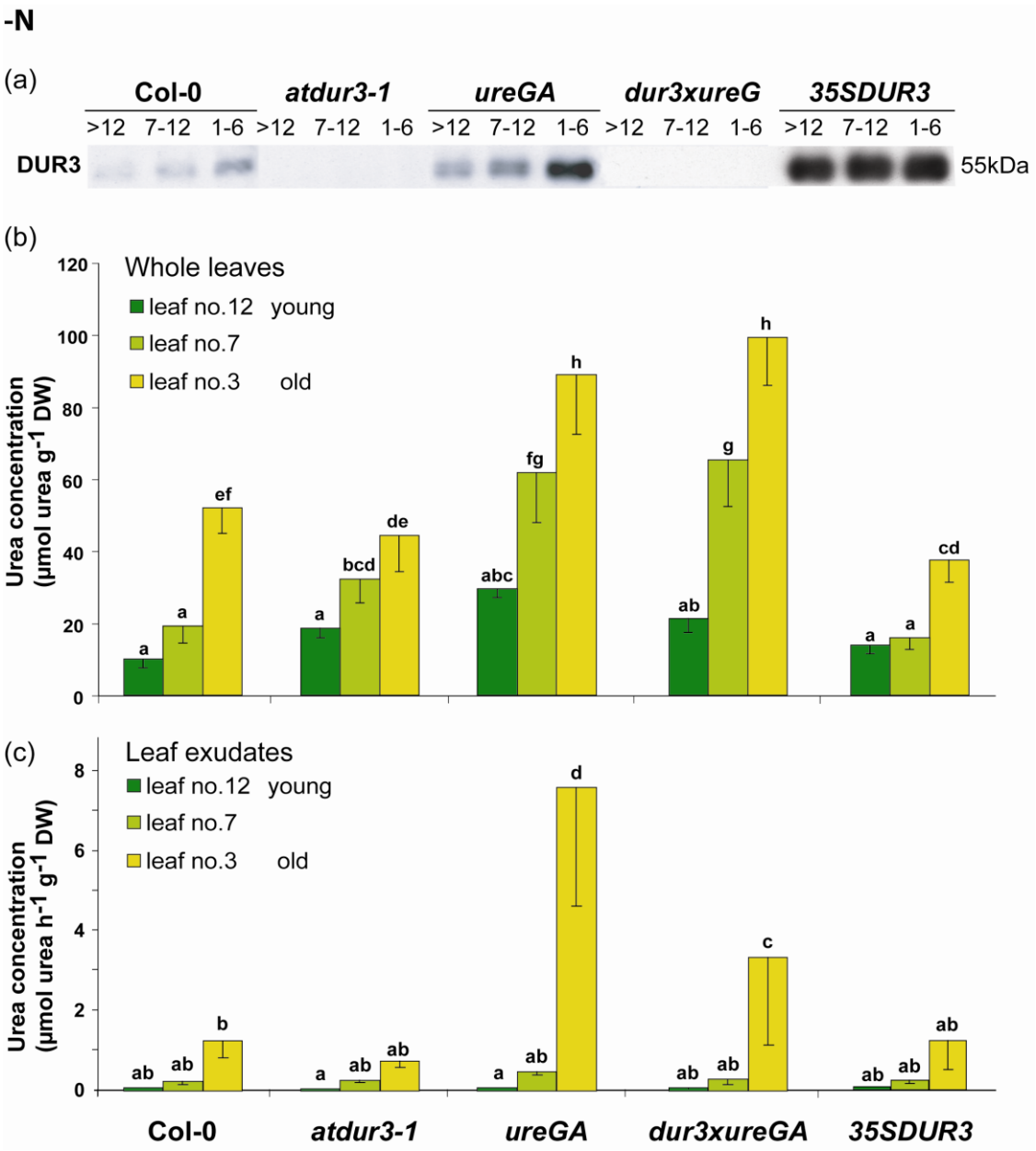

Figure 22: AtDUR3 contributes to urea re-translocation under nitrogen limiting conditions.

(a) Protein gel blot analysis of AtDUR3 in leaves of different age from wild type (Col-0), *atdur3-1*, *ureGA*, *dur3ureGA* and *35SDUR3*. Microsomal membrane fractions (MMF) were prepared from N-depleted plants and 5µg MMF per lane were assayed by anti-DUR3. (b) Urea concentrations in whole leaves and (c) urea concentrations in leaf petiole exudates collected for 6h in 10 mM EDTA solution. Young (leaf no. 12), middle-aged (leaf no. 7) and old leaves (leaf no. 3) of three plants were pooled for analysis. Plants were cultured hydroponically under adequate N supply (2 mM NH_4NO_3) for 4 weeks before transfer to long day to induce senescence. N starvation was applied for 4 days prior to harvest. Bars indicate means ± SD, n=5. Different letters denote significant differences among means at $p<0.05$ according to Tukey´s test.

However, compared to wild type *atdur3-1* had a significantly higher level of urea in the middle-aged leaves. *UreGA* and *dur3ureGA* showed highest levels of urea in all leaf samples compared to N-sufficient conditions. While in the oldest leaf there was only a small increase under N starvation, urea concentrations the younger

leaves increased by 30%. In the overexpressor line *35SDUR3* N depletion led to a significant increase in urea concentration in the oldest leaf which was still less than in the wild type.

Urea concentrations in leaf petiole exudates in young and middle-aged leaves were not affected by the N status of the plant in any line. Also in the oldest leaves of the wild type and *atdur3-1* N deficiency had no considerable impact. In *ureGA*, however, urea concentrations in the leaf petiole exudate from the oldest leaves doubled under N deficiency and created an even larger difference to the wild type. Unexpectedly, in *dur3ureGA* there was no increase under N deficiency which resulted in a significant lower urea concentration compared to *ureGA,* indicating a contribution of AtDUR3 to net urea export out of older leaves. Urea concentrations in leaf exudates of the oldest leaf of *35SDUR3* plants were similar to those in the wild type.

Taken together, AtDUR3 protein abundance and urea concentrations in leaves as well as in leaf petiole exudates increased with leaf age, but a contribution of the transporter to urea export and thus phloem loading could only be detected in the absence of urease and when plants were grown under N-limiting conditions.

In order to quantify the amount of N that was exported out of the leaves in the form of urea, amino acid concentrations were determined in the leaf petiole exudates (Table 1). High standard deviations partially caused by the analysis of very small sample volumes made a clear interpretation difficult. However, the most abundant amino acid was glutamine followed by asparagine, serine, glutamate and aspartic acid. This is in agreement with previous reports on amino acid abundance in phloem exudates of *Arabidopsis* plants (Hirner *et al*., 2006). In general there were more amino acids translocated under +N conditions than under N deficiency, but this did not change the general order of abundance of amino acids. Additionally, amino acids were exported in larger amounts from the oldest leaves when subjected to N deprivation. In old leaves of wild-type plants urea represented approximately 10% of total amino acids independent of the N status of the plant (Table 2). Therefore, urea-N represents approximately 13% of the total amino acid-N indicating that urea is a quantitative important N-form for N utilized for phloem loading.

Table 1: Amino acid concentrations in leaf petiole exudates under N-sufficient and N-deficient conditions.

Amino acid concentrations in leaf petiole exudates of Col-0, *atdur3-1*, *ureGA*, *dko* and *35Sdur3* plants collected for 6h in 10 mM EDTA solution. Young (leaf no. 12), middle-aged (leaf no. 7) and old leaves (leaf no. 3) of three plants were pooled for analysis. Plants were cultured hydroponically under adequate N supply (2 mM NH_4NO_3) for 4 weeks before transfer to long day to induce senescence. N starvation was applied for 4 days prior to harvest. Values are means +/-SD. Different letters denote significant differences among each amino acid (aa) at p<0.05 according to Tukey´s test, n=5.

µmol g^{-1} DW h^{-1}

+N	AA Leaf	Total	Gln	Ser	Asn	Glu	Thr	Pro	Val	Asp	Leu
Col-0	young (>12)	17,8 +/- 18,3 ac	7,38 +/- 7,61 a	1,28 +/- 1,23 ab	1,56 +/- 1,67 a	0,99 +/- 0,95 a	0,94 +/- 1,04 a	0,59 +/- 0,54 a	0,53 +/- 0,54 a	0,8 +/- 0,94 a	0,43 +/- 0,4 a
	middle (7-12)	15,9 +/- 3,95 ac	6,13 +/- 0,98 a	1,33 +/- 0,45 ab	1,48 +/- 0,29 a	0,88 +/- 0,2 a	0,85 +/- 0,14 a	1,15 +/- 0,71 a	0,59 +/- 0,17 a	0,53 +/- 0,12 a	0,43 +/- 0,15 a
	old (1-6)	12,6 +/- 7,28 ac	4,05 +/- 2,56 a	1,41 +/- 0,84 ab	0,71 +/- 0,41 a	0,87 +/- 0,39 a	0,66 +/- 0,38 a	0,49 +/- 0,24 a	0,76 +/- 0,42 ab	0,36 +/- 0,18 a	0,84 +/- 0,49 ab
atdur3-1	young	6,2 +/- 4,01 a	2,59 +/- 1,56 a	0,3 +/- 0,19 b	0,47 +/- 0,3 a	0,4 +/- 0,24 a	0,25 +/- 0,17 a	0,22 +/- 0,17 a	0,17 +/- 0,13 a	0,37 +/- 0,18 a	0,19 +/- 0,15 a
	middle	23,8 +/- 15,3 c	9,5 +/- 6,64 a	1,6 +/- 0,72 ab	1,85 +/- 1,21 a	1,46 +/- 0,76 a	1,13 +/- 0,65 a	2,02 +/- 2,54 a	0,91 +/- 0,38 ab	0,66 +/- 0,11 a	0,72 +/- 0,28 a
	old	12,8 +/- 7,26 ac	4,09 +/- 2,66 a	1,42 +/- 0,89 ab	0,6 +/- 0,33 a	0,77 +/- 0,5 a	0,69 +/- 0,26 a	0,64 +/- 0,19 a	0,75 +/- 0,44 ab	0,39 +/- 0,12 a	0,88 +/- 0,5 ab
ureGA	young	5,46 +/- 3,68 a	2,14 +/- 1,27 a	0,37 +/- 0,23 b	0,42 +/- 0,24 a	0,39 +/- 0,32 a	0,24 +/- 0,15 a	0,34 +/- 0,25 a	0,16 +/- 0,12 a	0,28 +/- 0,21 a	0,15 +/- 0,13 a
	middle	15,7 +/- 9,31 ac	5,93 +/- 3,23 a	1,17 +/- 0,62 ab	1,23 +/- 0,64 a	1,21 +/- 0,83 a	0,78 +/- 0,43 a	0,95 +/- 0,85 a	0,53 +/- 0,28 a	0,71 +/- 0,4 a	0,46 +/- 0,24 a
	old	24 +/- 10,3 c	7,83 +/- 2,93 a	2,26 +/- 1,11 a	1,27 +/- 0,37 a	1,66 +/- 0,57 a	1,3 +/- 0,69 a	0,66 +/- 0,11 a	1,55 +/- 0,77 b	0,71 +/- 0,24 a	1,75 +/- 0,91 b
dur3xureGA	young	8,85 +/- 3,69 ab	3,33 +/- 1,31 a	0,68 +/- 0,18 ab	0,67 +/- 0,25 a	0,61 +/- 0,22 a	0,39 +/- 0,15 a	0,5 +/- 0,3 a	0,26 +/- 0,11 a	0,41 +/- 0,18 a	0,26 +/- 0,14 a
	middle	22,8 +/- 14,5 bc	9,67 +/- 6,53 a	1,49 +/- 0,48 ab	1,86 +/- 1,07 a	1,34 +/- 0,47 a	1,15 +/- 0,51 a	1,29 +/- 0,55 a	0,69 +/- 0,39 ab	0,91 +/- 0,89 a	0,55 +/- 0,52 a
	old	16,2 +/- 14,7 ac	5,05 +/- 5,2 a	1,41 +/- 1,13 ab	0,77 +/- 0,84 a	1,19 +/- 1,14 a	0,92 +/- 0,68 a	0,85 +/- 0,87 a	0,88 +/- 0,79 ab	0,77 +/- 0,6 a	1,01 +/- 0,75 ab
35SDUR3	young	14,5 +/- 4,62 ac	6,08 +/- 1,4 a	0,96 +/- 0,4 ab	1,06 +/- 0,25 a	0,72 +/- 0,22 a	0,63 +/- 0,19 a	0,52 +/- 0,19 a	0,36 +/- 0,15 a	0,78 +/- 0,34 a	0,32 +/- 0,16 a
	middle	12,4 +/- 4,39 ac	4,57 +/- 1,57 a	0,94 +/- 0,11 ab	0,89 +/- 0,32 a	0,91 +/- 0,17 a	0,6 +/- 0,18 a	1,26 +/- 1,05 a	0,36 +/- 0,05 a	0,56 +/- 0,21 a	0,25 +/- 0,06 a
	old	14,1 +/- 8,68 ac	3,8 +/- 2,94 a	1,58 +/- 1,27 ab	0,59 +/- 0,48 a	0,74 +/- 0,59 a	1,11 +/- 0,48 a	1,01 +/- 0,57 a	0,69 +/- 0,21 ab	0,6 +/- 0,32 a	0,72 +/- 0,17 a

µmol g^{-1} DW h^{-1}

+N	AA Leaf	GABA	Ala	Ile	Lys	Phe	Arg	His	Gly	Met	Tyr
Col-0	young (>12)	0,89 +/- 0,99 a	0,66 +/- 0,68 a	0,36 +/- 0,35 a	0,38 +/- 0,4 ab	0,25 +/- 0,25 a	0,4 +/- 0,43 ab	0,16 +/- 0,15 ab	0,06 +/- 0,05 a	0,04 +/- 0,03 ab	0,05 +/- 0,03 a
	middle (7-12)	0,52 +/- 0,12 a	0,44 +/- 0,07 a	0,39 +/- 0,12 a	0,32 +/- 0,12 ab	0,28 +/- 0,08 a	0,28 +/- 0,13 ab	0,17 +/- 0,04 ab	0,05 +/- 0,03 a	0,03 +/- 0,01 ab	0,06 +/- 0,02 a
	old (1-6)	0,27 +/- 0,15 a	0,32 +/- 0,17 a	0,56 +/- 0,33 ab	0,43 +/- 0,22 ab	0,36 +/- 0,21 a	0,12 +/- 0,07 b	0,15 +/- 0,1 ab	0,13 +/- 0,06 a	0,1 +/- 0,06 ab	0 +/- 0 a
atdur3-1	young	0,4 +/- 0,28 a	0,24 +/- 0,16 a	0,13 +/- 0,1 a	0,13 +/- 0,11 b	0,09 +/- 0,07 a	0,16 +/- 0,12 b	0,04 +/- 0,03 b	0,02 +/- 0,01 a	0,01 +/- 0,01 b	0,03 +/- 0,03 a
	middle	1 +/- 0,62 a	0,68 +/- 0,41 a	0,61 +/- 0,24 ab	0,43 +/- 0,15 ab	0,47 +/- 0,17 ab	0,26 +/- 0,14 ab	0,25 +/- 0,11 ab	0,1 +/- 0,08 a	0,07 +/- 0,03 ab	0,08 +/- 0,02 a
	old	0,26 +/- 0,11 a	0,32 +/- 0,18 a	0,57 +/- 0,31 ab	0,36 +/- 0,29 ab	0,47 +/- 0,17 ab	0,13 +/- 0,03 b	0,16 +/- 0,05 ab	0,17 +/- 0,17 a	0,14 +/- 0,06 ac	0,01 +/- 0,01 a
ureGA	young	0,25 +/- 0,19 a	0,18 +/- 0,14 a	0,12 +/- 0,1 a	0,11 +/- 0,12 b	0,09 +/- 0,06 a	0,11 +/- 0,09 b	0,05 +/- 0,03 b	0,02 +/- 0,01 a	0,01 +/- 0,01 b	0,02 +/- 0,02 a
	middle	0,66 +/- 0,49 a	0,5 +/- 0,34 a	0,37 +/- 0,19 a	0,34 +/- 0,26 ab	0,29 +/- 0,13 a	0,28 +/- 0,2 ab	0,18 +/- 0,09 ab	0,05 +/- 0,04 a	0,04 +/- 0,02 ab	0,06 +/- 0,04 a
	old	0,54 +/- 0,22 a	0,73 +/- 0,4 a	1,15 +/- 0,58 b	0,76 +/- 0,42 a	0,84 +/- 0,42 b	0,24 +/- 0,14 ab	0,3 +/- 0,15 a	0,18 +/- 0,1 a	0,25 +/- 0,15 c	0,05 +/- 0,06 a
dur3xureGA	young	0,41 +/- 0,2 a	0,31 +/- 0,15 a	0,21 +/- 0,1 a	0,22 +/- 0,1 ab	0,15 +/- 0,07 a	0,24 +/- 0,13 ab	0,07 +/- 0,04 bc	0,04 +/- 0,02 a	0,02 +/- 0,01 b	0,04 +/- 0,04 a
	middle	1,11 +/- 0,87 a	0,69 +/- 0,46 a	0,48 +/- 0,33 a	0,38 +/- 0,3 ab	0,35 +/- 0,23 a	0,43 +/- 0,59 ab	0,18 +/- 0,09 ab	0,13 +/- 0,07 a	0,04 +/- 0,02 ab	0,08 +/- 0,1 a
	old	0,42 +/- 0,34 a	0,53 +/- 0,46 a	0,71 +/- 0,55 ab	0,49 +/- 0,41 ab	0,52 +/- 0,37 ab	0,14 +/- 0,11 b	0,26 +/- 0,18 ac	0,13 +/- 0,12 a	0,12 +/- 0,07 ab	0,02 +/- 0,04 a
35SDUR3	young	0,76 +/- 0,42 a	0,49 +/- 0,19 a	0,25 +/- 0,12 a	0,3 +/- 0,13 ab	0,16 +/- 0,07 a	0,78 +/- 0,21 a	0,09 +/- 0,03 ab	0,16 +/- 0,12 a	0,03 +/- 0,01 ab	0,03 +/- 0,04 a
	middle	0,39 +/- 0,16 a	0,33 +/- 0,1 a	0,24 +/- 0,04 a	0,26 +/- 0,04 ab	0,17 +/- 0,04 a	0,37 +/- 0,19 ab	0,1 +/- 0,01 ab	0,14 +/- 0,07 a	0,03 +/- 0,01 ab	0,03 +/- 0,01 a
	old	0,64 +/- 0,41 a	0,54 +/- 0,32 a	0,54 +/- 0,15 ab	0,43 +/- 0,24 ab	0,36 +/- 0,12 ab	0,21 +/- 0,11 ab	0,17 +/- 0,07 ab	0,2 +/- 0,16 a	0,1 +/- 0,01 ab	0,03 +/- 0,05 a

µmol g^{-1} DW h^{-1}

-N	AA Leaf	Total	Gln	Ser	Glu	Asn	Val	Leu	Pro	Thr	Ile
Col-0	young (>12)	5,01 +/- 1,28 ab	1,63 +/- 0,25 b	0,32 +/- 0,06 b	0,53 +/- 0,16 ab	0,28 +/- 0,06 bc	0,25 +/- 0,08 ab	0,21 +/- 0,09 b	0,1 +/- 0,05 a	0,22 +/- 0,05 bc	0,21 +/- 0,08 bc
	middle (7-12)	13,4 +/- 4,28 ae	4,99 +/- 1,18 ab	1,09 +/- 0,49 ab	1,11 +/- 0,1 ab	0,95 +/- 0,2 ab	0,61 +/- 0,3 ab	0,49 +/- 0,29 ab	0,51 +/- 0,26 a	0,65 +/- 0,21 abc	0,44 +/- 0,24 ab
	old (1-6)	13,1 +/- 7,27 ae	3,82 +/- 2,56 ab	1,19 +/- 0,6 ab	0,93 +/- 0,5 ab	0,53 +/- 0,24 bc	0,9 +/- 0,5 bc	1,04 +/- 0,56 bc	1,05 +/- 0,54 a	0,73 +/- 0,34 abc	0,68 +/- 0,35 ab
atdur3-1	young	3,84 +/- 2,07 a	1,02 +/- 0,53 b	0,17 +/- 0,15 b	0,42 +/- 0,23 ab	0,18 +/- 0,1 b	0,24 +/- 0,14 b	0,21 +/- 0,12 b	0,09 +/- 0,03 a	0,17 +/- 0,09 b	0,21 +/- 0,12 bc
	middle	15,7 +/- 3,08 ce	5,82 +/- 0,73 ab	1,22 +/- 0,25 ab	1,04 +/- 0,18 ab	1,05 +/- 0,12 ac	0,87 +/- 0,18 bc	0,72 +/- 0,19 bc	0,67 +/- 0,47 a	0,77 +/- 0,13 abc	0,61 +/- 0,13 ab
	old	14,7 +/- 8,14 be	4,2 +/- 2,6 ab	1,37 +/- 0,77 ab	1,13 +/- 0,7 ab	0,62 +/- 0,37 bcd	1,03 +/- 0,56 bc	1,1 +/- 0,6 bc	0,88 +/- 0,35 a	0,85 +/- 0,39 bd	0,76 +/- 0,4 ab
ureGA	young	5,7 +/- 2,16 ac	2,07 +/- 0,76 ab	0,45 +/- 0,13 bc	0,39 +/- 0,16 b	0,38 +/- 0,13 bc	0,31 +/- 0,1 ab	0,25 +/- 0,08 b	0,21 +/- 0,21 a	0,29 +/- 0,09 bc	0,22 +/- 0,07 bc
	middle	17,4 +/- 11,1 de	4,53 +/- 3 ab	1,04 +/- 0,66 ab	1,88 +/- 1,21 a	0,84 +/- 0,58 ab	1,05 +/- 0,69 bc	0,9 +/- 0,58 bc	0,34 +/- 0,25 a	0,8 +/- 0,51 abc	0,92 +/- 0,59 ac
	old	19,1 +/- 13,1 ef	5,53 +/- 3,81 ab	1,77 +/- 1,19 a	1,49 +/- 0,96 ab	0,9 +/- 0,58 ab	1,19 +/- 0,81 ac	1,37 +/- 0,91 ac	1,08 +/- 1,37 a	1,11 +/- 0,69 ad	0,95 +/- 0,62 ad
dur3xureGA	young	4,51 +/- 1,07 ab	1,37 +/- 0,27 b	0,3 +/- 0,11 b	0,58 +/- 0,11 ab	0,24 +/- 0,05 bc	0,22 +/- 0,03 b	0,16 +/- 0,04 b	0,12 +/- 0,07 a	0,19 +/- 0,05 bc	0,18 +/- 0,04 b
	middle	18,8 +/- 9,65 ef	6,95 +/- 3,19 ac	1,65 +/- 0,64 ac	1,61 +/- 1,17 ab	1,46 +/- 0,7 a	0,83 +/- 0,39 bc	0,68 +/- 0,36 bc	0,61 +/- 0,47 a	0,96 +/- 0,48 cd	0,59 +/- 0,29 ab
	old	8,19 +/- 5,43 ad	1,9 +/- 1,48 ab	0,8 +/- 0,51 ab	0,57 +/- 0,52 ab	0,34 +/- 0,2 bc	0,51 +/- 0,34 ab	0,65 +/- 0,36 bc	0,39 +/- 0,17 a	0,54 +/- 0,32 abc	0,42 +/- 0,25 bcd
35SDUR3	young	7,62 +/- 3,31 ad	1,7 +/- 0,77 ab	0,54 +/- 0,23 ab	1,04 +/- 0,42 ab	0,31 +/- 0,11 bc	0,49 +/- 0,25 ab	0,32 +/- 0,19 b	0,16 +/- 0,05 a	0,37 +/- 0,15 abc	0,38 +/- 0,2 bcd
	middle	13,8 +/- 4,15 ae	5,06 +/- 2,09 ab	0,97 +/- 0,11 ab	1,38 +/- 0,24 ab	0,95 +/- 0,28 ab	0,54 +/- 0,16 bc	0,34 +/- 0,09 ab	0,84 +/- 0,47 a	0,73 +/- 0,16 bd	0,36 +/- 0,08 ab
	old	28,3 +/- 12,1 f	11,5 +/- 5,17 c	1,94 +/- 0,96 a	1,61 +/- 1,16 ab	1,47 +/- 0,69 ad	1,68 +/- 0,64 c	1,58 +/- 0,65 c	0,91 +/- 0,19 a	1,65 +/- 0,51 d	1,2 +/- 0,46 a

µmol g^{-1} DW h^{-1}

-N	AA Leaf	Lys	Asp	Ala	GABA	Phe	His	Arg	Met	Gly	Tyr
Col-0	young (>12)	0,22 +/- 0,09 bc	0,31 +/- 0,08 ab	0,18 +/- 0,05 b	0,25 +/- 0,08 a	0,09 +/- 0,03 b	0,07 +/- 0,02 bc	0,08 +/- 0,03 a	0,01 +/- 0 b	0,01 +/- 0 a	0,03 +/- 0,02 a
	middle (7-12)	0,44 +/- 0,21 ab	0,46 +/- 0,15 ab	0,37 +/- 0,09 ab	0,48 +/- 0,16 a	0,3 +/- 0,17 ab	0,18 +/- 0,09 ab	0,18 +/- 0,07 a	0,03 +/- 0,02 ab	0,03 +/- 0,01 a	0,07 +/- 0,05 a
	old (1-6)	0,53 +/- 0,31 ab	0,37 +/- 0,14 ab	0,36 +/- 0,16 ab	0,18 +/- 0,06 a	0,39 +/- 0,19 ab	0,11 +/- 0,05 bde	0,08 +/- 0,04 a	0,15 +/- 0,08 ace	0,08 +/- 0,04 a	0 +/- 0 a
atdur3-1	young	0,17 +/- 0,1 b	0,28 +/- 0,09 ab	0,14 +/- 0,08 b	0,24 +/- 0,13 a	0,1 +/- 0,05 b	0,06 +/- 0,03 b	0,08 +/- 0,05 a	0,01 +/- 0,01 b	0,01 +/- 0,01 a	0,04 +/- 0,02 a
	middle	0,46 +/- 0,1 ab	0,51 +/- 0,1 ab	0,4 +/- 0,06 ab	0,51 +/- 0,18 ab	0,49 +/- 0,11 ab	0,25 +/- 0,04 ad	0,16 +/- 0,04 a	0,07 +/- 0,03 abd	0,03 +/- 0,01 a	0,09 +/- 0,02 ab
	old	0,54 +/- 0,31 ab	0,48 +/- 0,24 ab	0,39 +/- 0,2 ab	0,27 +/- 0,08 a	0,52 +/- 0,31 ab	0,2 +/- 0,06 ab	0,1 +/- 0,04 a	0,16 +/- 0,09 ac	0,06 +/- 0,05 a	0,01 +/- 0,03 a
ureGA	young	0,16 +/- 0,05 b	0,21 +/- 0,07 b	0,15 +/- 0,06 b	0,2 +/- 0,1 a	0,19 +/- 0,06 b	0,09 +/- 0,03 bd	0,06 +/- 0,02 a	0,02 +/- 0,01 be	0,01 +/- 0,01 a	0,03 +/- 0,01 a
	middle	0,79 +/- 0,53 ac	1,24 +/- 0,54 c	0,64 +/- 0,41 a	1,04 +/- 0,67 b	0,48 +/- 0,31 ab	0,24 +/- 0,14 ad	0,37 +/- 0,25 b	0,05 +/- 0,03 abd	0,04 +/- 0,03 a	0,19 +/- 0,13 b
	old	0,63 +/- 0,44 ab	0,67 +/- 0,38 bc	0,54 +/- 0,28 ab	0,41 +/- 0,18 a	0,77 +/- 0,51 a	0,27 +/- 0,11 ae	0,12 +/- 0,06 a	0,2 +/- 0,15 c	0,08 +/- 0,08 a	0,02 +/- 0,03 a
dur3xureGA	young	0,18 +/- 0,04 b	0,28 +/- 0,07 ab	0,17 +/- 0,03 b	0,26 +/- 0,04 a	0,07 +/- 0,04 b	0,05 +/- 0,02 b	0,08 +/- 0,02 a	0,01 +/- 0 b	0,02 +/- 0,02 a	0,03 +/- 0,01 a
	middle	0,51 +/- 0,26 ab	0,64 +/- 0,46 ab	0,54 +/- 0,31 ab	0,64 +/- 0,35 ab	0,45 +/- 0,23 ab	0,23 +/- 0,12 acd	0,21 +/- 0,1 ab	0,07 +/- 0,04 abd	0,13 +/- 0,06 ab	0,07 +/- 0,05 a
	old	0,39 +/- 0,21 ab	0,27 +/- 0,15 b	0,4 +/- 0,24 ab	0,29 +/- 0,21 a	0,29 +/- 0,17 ab	0,1 +/- 0,06 bde	0,11 +/- 0,07 a	0,09 +/- 0,06 abc	0,12 +/- 0,07 a	0,01 +/- 0,02 a
35SDUR3	young	0,41 +/- 0,21 ab	0,64 +/- 0,19 bc	0,31 +/- 0,12 ab	0,32 +/- 0,12 a	0,2 +/- 0,1 b	0,1 +/- 0,04 bde	0,16 +/- 0,06 a	0,03 +/- 0,01 ab	0,06 +/- 0,06 a	0,07 +/- 0,04 a
	middle	0,34 +/- 0,02 ab	0,58 +/- 0,08 bc	0,4 +/- 0,07 ab	0,47 +/- 0,04 ab	0,23 +/- 0,05 ab	0,15 +/- 0,04 ab	0,2 +/- 0,04 ab	0,05 +/- 0,01 abd	0,13 +/- 0,09 ab	0,05 +/- 0,02 a
	old	0,9 +/- 0,31 a	0,89 +/- 0,36 ac	0,59 +/- 0,17 ab	0,59 +/- 0,13 ab	0,76 +/- 0,26 a	0,35 +/- 0,12 a	0,24 +/- 0,05 ab	0,18 +/- 0,06 cd	0,27 +/- 0,2 b	0 +/- 0 a

Table 2: Comparison of urea and total amino acids in leaf petiole exudates in dependence of the N supply.

	+N [µmol $g^{-1}DWh^{-1}$]	-N [µmol $g^{-1}DWh^{-1}$]
Old leaves		
Total aa	12.6	13.3
Urea	1.3 (10.3%)	1.3 (9.7%)
Middle leaves		
Total aa	15.9	13.4
Urea	0.3 (1.9%)	0.2 (1.5%)
Young leaves		
Total aa	17.8	5.1
Urea	0.2 (1.1%)	0.1 (1.9%)

4.2.6 Retranslocation of ^{15}N-labelled urea from senescing leaves

A quantification of the contribution of AtDUR3 to urea re-translocation was investigated by short-term re-translocation assays with ^{15}N-labelled urea in wild-type and *atdur3-1* plants precultured under different N regimes. In wild-type plants, the natural abundance was determined in N-sufficient leaves of different age (Figure 23). Surprisingly, there was a clear gradient in the $^{15}N/^{14}N$ ratio from old to young leaves. While an adequate N supply yielded a higher abundance of ^{15}N in the old leaves, N deprivation led to an opposite pattern with heavier N in the younger leaves. Taking into account remobilization processes of N from old to young leaves when plants are grown under N deficiency, this suggests that remobilised N is heavier than newly assimilated one.

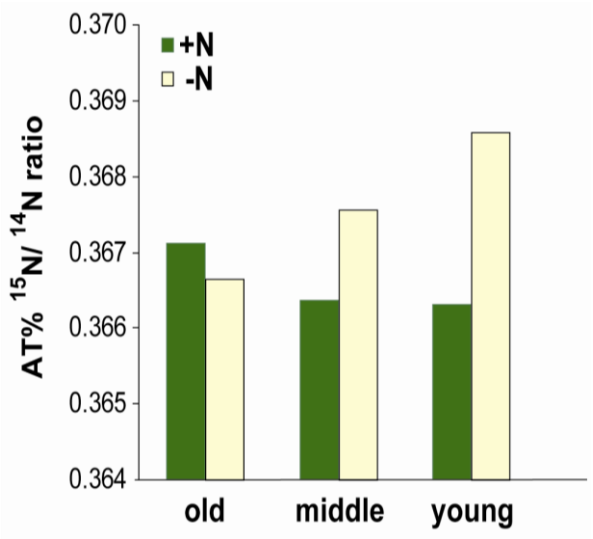

Figure 23: The natural ^{15}N abundance in leaves of N-sufficient and N-deficient Arabidopsis plants.

Natural abundance of ^{15}N in different leaf fractions of wild-type plants. Plants were cultured for 6 weeks under long-day conditions at either continuous supply of 2 mM NH_4NO_3 (+N) or 4 days N starvation prior to harvest. Old (leaf no. 1-5), middle (leaf no. 7-12) and young (leaf no. >12) leaves were separately harvested and analysed by IR-MS. Bars represent mean of two biological replica.

Short-term labelling of leaf no. 6 of 6 week-old wild-type or *atdur3-1* plants cultured under long days either under N-sufficient (+N) or N-deficient (-N) conditions for 4 days conditions was performed by applying 10 µl of a 2% solution of ^{15}N-labelled urea on a defined area for a period of 3 days. Changes in $^{15}N/^{14}N$ ratio were monitored in old (1-5), middle (7-12) and young (>12) leaves as well as in the roots (Figure 24) after subtraction of the natural abundance found in each fraction prior to labelling.

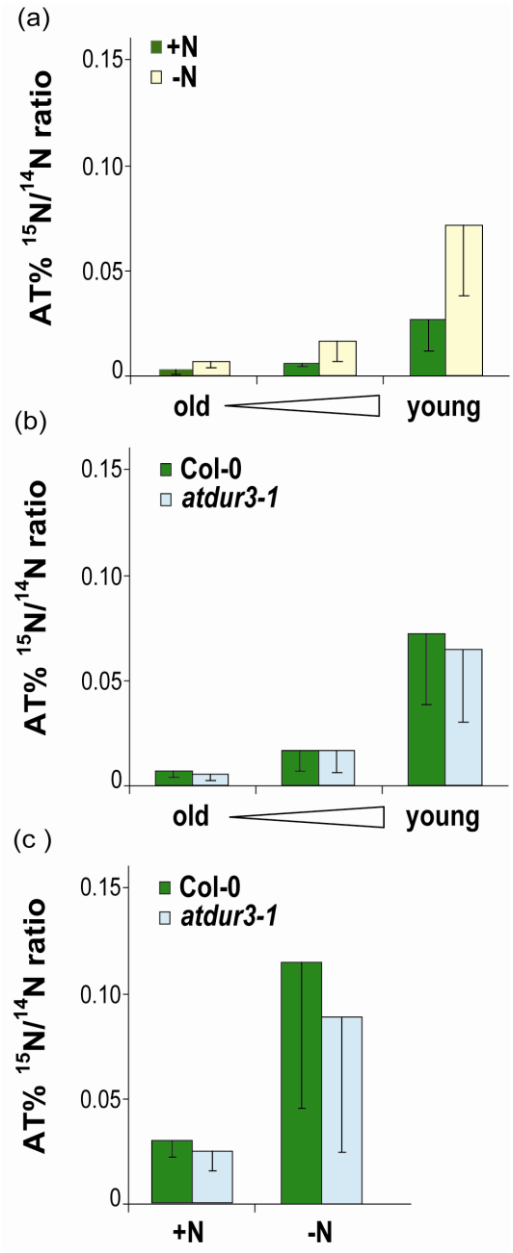

Figure 24: Changes in $^{15}N/^{14}N$ ratios after short-term labelling of a single leaf.

$^{15}N/^{14}N$ ratio in (a) wild-type leaves from plants precultured under adequate or deficient N supply, (b) in wild-type and *atdur3-1* leaves from N-deficient plants and (c) in roots of wild-type and *atdur3-1* precultured under adequate or deficient N supply. 6 week-old wild-type and *atdur3-1* plants were cultured under long-day conditions under either continuous supply of 2 mM NH_4NO_3 (+N) or 4 days of N starvation prior to harvest. Leaf no.6 was labelled with 10 µl of 2% ^{15}N-labelled urea solution for 3 days. Old (leaf no. 1-5), middle (leaf no. 7-12) and young (leaf no. >12) leaves were separately harvested and analysed by IR-MS. Bars indicate means ± SD, n=7.

In N-sufficient wild-type plants the $^{15}N/^{14}N$ ratio increased from the old and middle-aged leaves to young leaves (Figure 24a). N starvation reinforced this pattern and the ratio increased now also in the middle-aged leaves, indicating a preferential translocation of the heavier N to younger leaves. Comparing the changes in $^{15}N/^{14}N$ ratio between the wild-type and *atdur3-1* plants from

N-deficient preculture confirmed the preferential allocation of heavy N into younger parts of the plant (Figure 24b). However, there was no difference between the two lines indicating that a loss of AtDUR3 had no or such a weak effect that it could not be detected. Interestingly, the $^{15}N/^{14}N$ ratio also increased in roots when plants were precultured under N-limiting conditions (Figure 24c), indicating that even during early senescence roots create a sink for N from source leaves.

4.2.7 Day-length specific phenotype of plants over-expressing *AtDUR3* and regulatory elements in its promoter region

35SDUR3 plants showed no differences in phenotype compared to Col-0 as long as plants were grown under short-day conditions (Figure 25). However, when plants were cultured in long days, *35SDUR3* shoots clearly altered their morphology. The petiole length was reduced, therefore the rosette appeared more compact. The leaf shape changed as well, became curly and a bit broader, indicating that the actual shade avoidance phenotype of the wild type with elongated petioles got lost in the overexpressor line. Fresh weight as well as flowering time were not altered in the transgenic line (Kriegel, 2011). It is important to note here that the *atdur3-1* insertion line did not show any phenotypical differences to the wild type under any of the growth conditions tested so far. Additionally, only one *AtDUR3* overexpressing line (B3) could be tested due to a very low germination rate of the other overexpressing lines. To rule out an influence by the insertion per se, other lines have to be analysed in near future.

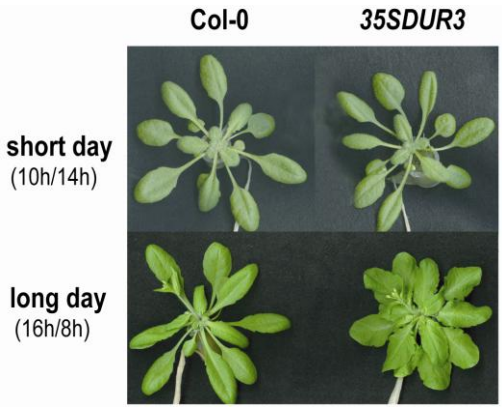

Figure 25: A day length-dependent phenotype of a transgenic line ectopically expressing *35S::AtDUR3*.

Shoot phenotype of wild-type (Col-0) and *35SDUR3* as influenced by short-day conditions (10h/ 14h light/dark) or long days (16h/ 8h light/dark). Plants were cultured hydroponically for 6 weeks on adequate N supply (2 mM NH_4NO_3) under continuous day length.

To better understand this extraordinary performance of the overexpressor the properties of the promoter region of *AtDUR3* were analysed (Figure 26). 1000 bp of the genomic DNA sequence upstream of the transcription star of *AtDUR3* contained several binding sites and predicted motifs for known transcription factors (TF) (Figure 26). Promoter elements indicated regulation of *AtDUR3* expression by different signals such as light, ABA or UV-B. Related to the subject of senescence two binding sites for transcription factors of the WRKY family (W-box) as well as a bZIP binding site appeared to be interesting. Subsequent coexpression analysis via Atted-II (Obayashi *et al*., 2009) identified, among others, *WRKY60* (At2g25000) as being closely related with *AtDUR3* expression with a correlation coefficient of 0.65 as well as *bZIP50* (At1g77920) with a correlation coefficient of 0.61.

Comparison of the expression levels of *AtDUR3*, *WRKY60* and *bZIP50* at different developmental stages indicated that coexpression of *AtDUR3* and *bZIP50* matched even closer at different developmental stages that of *AtDUR3* and *WRKY60* (Figure 26).

(a)

Binding site (BS) Name	BS genome start	BS genome end	BS Sequence	BS Family/TF	Function
ATB2/AtbZIP53/AtbZIP44/ GBF5 BS in ProDH	18408197	18408202	actcat	**bZIP**	
W-box promoter motif	18408252	18408257	ttgact	**WRKY**	
W-box promoter motif	18408276	18408281	ttgact	**WRKY**	
MYB4 binding site motif	18407707	18407713	aacaacc	**MYB**	UV-B response
RAV1-A binding site motif	18407905	18407909	caaca	**ABI3VP1**	ABA-responsive
RAV1-A binding site motif	18407920	18407924	caaca	**ABI3VP1**	
RAV1-A binding site motif	18407967	18407971	caaca	**ABI3VP1**	
GATA promoter motif [LRE]	18407769	18407774	agataa	...	light responsive elements
GATA promoter motif [LRE]	18407944	18407949	agatag	...	
GATA promoter motif [LRE]	18408076	18408081	agatag	...	
GATA promoter motif [LRE]	18408035	18408040	agataa	...	
GATA promoter motif [LRE]	18407926	18407931	agataa	...	

(b)

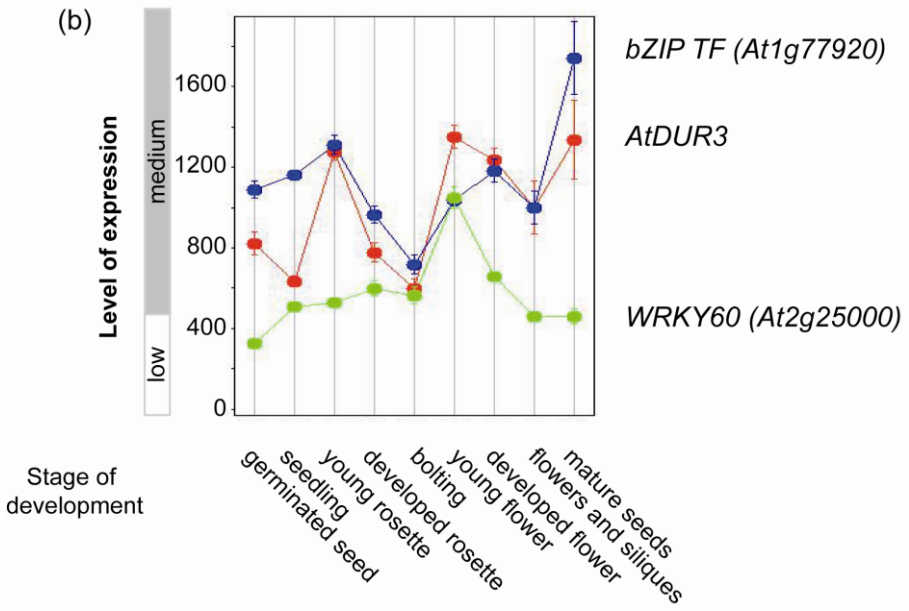

Figure 26: *In silico* analysis of the transcriptional regulation of AtDUR3.

(a) Promoter elements in *AtDUR3* predicting the regulation by several *TFs.* The Arabidopsis Gene Regulatory Information Server (*AGRIS*, Davuluri *et al*., 2003) was used as information resource of Arabidopsis promoter sequences, transcription factors and their target genes, using 1000bp of the genomic DNA sequence upstream of the transcription start of *AtDUR3*. (b) Coexpression analysis of *AtDUR3* with *WRKY60* and *bZIP50.* Genevestigator (Hruz *et al*., 2008) was used to visualize the levels of coexpressed genes found via Atted-II (Obayashi *et al*., 2009).

4.2.8 Salicylic acid is required for induction *of AtDUR3* gene expression during senescence and mimics N-deficiency effects on AtDUR3 transport activity

In silico data mining of relative expression levels confirmed that *AtDUR3* was induced after the onset of flowering in Col-0 leaves (Figure 27). However, in the transgenic line *NahG*, in which salicylic acid (SA) accumulation is prevented by expression of the SA-degrading enzyme salicylate hydroxylase, no induction of *AtDUR3* occurred in leaves after the onset of flowering. This dependency on SA seemed to be exclusive, since mutants with e.g. a defect in either the ethylene signalling pathway (*ein2*) or jasmonic acid pathway (*coi1*) showed no altered *AtDUR3* expression levels in senescent leaves. This pointed to a prominent role of SA in the induction of AtDUR3 gene expression in senescent leaves.

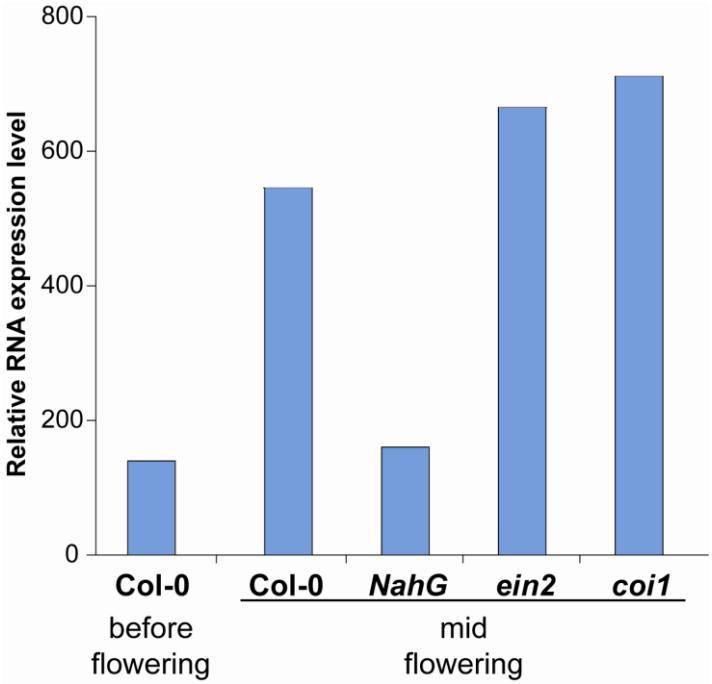

Figure 27: *AtDUR3* expression during senescence in leaves of mutants defective in phytohormone signaling.

Relative expression levels of *AtDUR3* in Col-0 before and at flowering as well as in the transgenic line *NahG* (unable to accumulate SA due to salicylate hydroxylase expression), *ein2* (ethylene insensitive mutant) and *coi1* (defective in jasmonic acid signalling). The microarray dataset was obtained from AtGenExpress (Schmid *et al*., 2005) using a subset of Buchanan-Wollaston *et al*., (2005).

An uptake experiment with ^{15}N-labelled urea in wild-type roots was then performed in the absence or presence of salicylic acid to validate the putative effect of SA on *AtDUR3* expression and AtDUR3 activity. For this purpose, roots of 6 weeks-old

plants cultured under continuous supply of 2 mM NH_4NO_3 (+N) or subjected to N deprivation for 4 days (-N) were treated with 500 µM SA for 3h before the uptake experiment. To separately assess the contribution of high-affinity (HATS) and low-affinity transport systems (LATS) 200 and 2000 µM ^{15}N-labelled urea, respectively, were used (Figure 28).

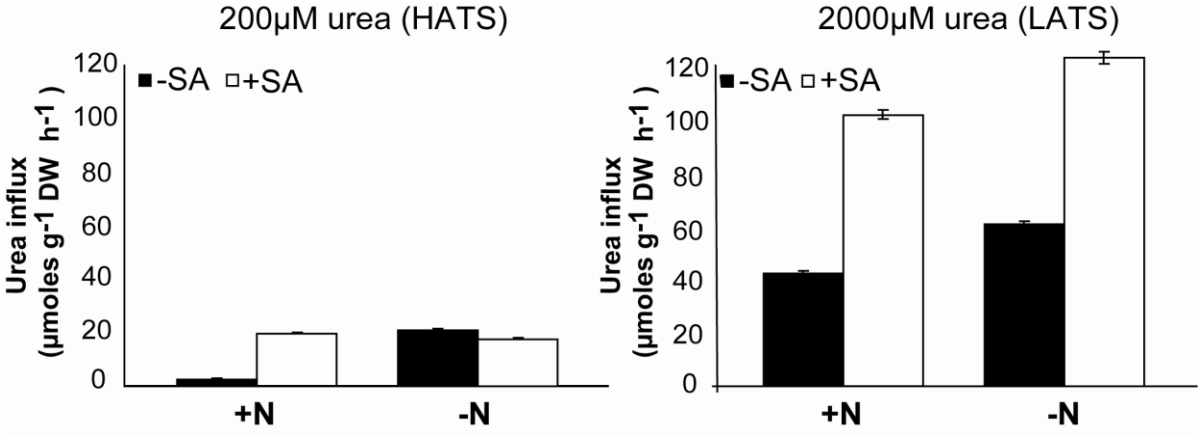

Figure 28: Root incubation with salicylic acid increases urea uptake capacity.

Urea influx into roots of hydroponically-grown Arabidopsis wild-type plants. Plants were grown for 6 weeks under continuous supply of 2 mM NH_4NO_3 (+N) or subjected to N deprivation for 4 days (-N) prior to the experiment. 500µM salicylic acid was applied 3 h before the uptake experiment with either 200 µM (for high-affinity transport, HATS) or 2000 µM (for low-affinity transport, LATS) ^{15}N-labelled urea for 10 min. Bars indicate means ± SD, n=8.

As expected, urea uptake in the high-affinity range (200 µM) was low when plants were precultured under N-sufficient conditions. N deficiency led to a strong increase in urea influx as observed previously (Figure 9). Under adequate N supply the application of SA led to an increase in urea uptake capacity that was comparable to that under N-deficient conditions, indicating that SA may mimic N deficiency in roots. Under N-deficient conditions, however, the preincubation with SA had no further stimulatory effect on urea uptake.

However, not only the HATS was affected by SA, but also the LATS showed a doubling of urea influx after SA application. In addition, the low-affinity transport capacity was slightly increased in the absence of SA, which probably reflected the contribution of the HATS. This suggested that SA stimulates besides HATS for urea also the LATS.

5 Discussion

5.1 AtDUR3 represents the major transporter for high-affinity urea transport across the plasma membrane of nitrogen-deficient Arabidopsis roots (published in The Plant Journal (2007), 52, 30-40)

Heterologous expression in yeast and oocytes allowed identification of AtDUR3 as a high-affinity urea transporter in Arabidopsis (Liu *et al.,* 2003a). Belonging to the SSS superfamily, AtDUR3-mediated urea transport depends on co-transport with protons. Of the broad range of neutral and charged solutes that have been reported as substrates for SSS-type transporters in different organisms (Reizer *et al*., 1994), heterologous expression studies with AtDUR3 permitted the testing of only a few (Liu *et al*., 2003a). Furthermore, these studies could not exclude the possibility that urea permeation might reflect a physiologically irrelevant transport activity of AtDUR3. In other words, the physiological nature of this unique transport system as to its significance in plant N nutrition has been poorly understood so far. The first part of the present study set out to investigate the physiological role of AtDUR3 in urea transport *in planta* and shows that AtDUR3 does indeed represent the major transporter for high-affinity urea uptake in Arabidopsis roots.

5.1.1 AtDUR3 acts as a nitrogen-regulated urea transporter at the root plasma membrane

A general feature of membrane transporters that fulfil a function in nutrient uptake by roots is their transcriptional upregulation under limiting supply of the corresponding nutrient (Ahn *et al*., 2004; Gazzarrini *et al*., 1999; Rausch and Bucher, 2002; Takahashi *et al*., 2000). In the case of the high-affinity nitrate and ammonium transporter genes in Arabidopsis roots, *NRT2;1* and *AMT1;1*, transcriptional upregulation under N deficiency reflected a de-repression most likely due to decreasing root concentrations of glutamine or other reduced N forms (Nazoa *et al*., 2003; Rawat *et al*., 1999; Vidmar *et al*., 2000). Corresponding with such a N-dependent regulation, transcript levels of *AtDUR3* increased in N-

deficient roots and were strongly repressed after resupply of ammonium or nitrate (Figure 6). Since nitrate-dependent repression of *AtDUR3* mRNA levels was even stronger than that of ammonium, it is possible that nitrate per se is a signal for transcriptional repression in addition to a repression by its downstream metabolites (Wang *et al*., 2000, 2001). Moreover, levels of *AtDUR3* transcript were strongly induced after resupply of urea to N-deficient roots (Figure 6). Substrate induction was even stronger than the preceding de-repressive effect by N deficiency in root cells. This suggests that AtDUR3 is not exclusively a component of the N deficiency stress response in Arabidopsis but also represents a substrate-inducible transport system, similar to NRT2;1 for nitrate (Lejay *et al*., 1999; Zhuo *et al*., 1999).

Two independent experimental approaches indicated that the AtDUR3 protein resides predominantly in the plasma membrane of root cells. First, protein gel blot analysis employing a specific antibody raised against the C-terminus of AtDUR3 documented a several-fold higher abundance of the protein in a plasma membrane-enriched protein fraction from N-deficient roots relative to a protein fraction depleted of plasma membrane proteins (Figure 4c). The fact that a small portion of AtDUR3 protein was also detected in endosomal membrane fractions was most likely due to an incomplete separation of the two fractions. In addition, a minor fraction of AtDUR3 may have resided in endosomal compartments, reflecting, for example, proteins that were trafficking to or from the plasma membrane (Takano *et al*., 2005). Secondly, whole-mount immunohistochemistry allowed tracing of AtDUR3-dependent fluorescence along the border of root hair cells (Figure 4d). Since these cells were densely filled with cytoplasm, the observed fluorescence could be assigned to the plasma membrane. Also in this approach, only a minor portion of the labeled protein appeared to be localized inside the cells. Taken together, the highly predominant localization of AtDUR3 in the root plasma membrane strongly supports a role for the protein in substrate exchange between root cells and the external medium.

A role for AtDUR3 in urea uptake from the external medium was indicated by a phenotypical analysis of two T-DNA insertion lines, in which no *AtDUR3* mRNA or protein could be detected (Figure 3b and Figure 4a). The absence of any visual symptoms or growth defects when ammonium nitrate was supplied as the sole N source suggested that gene disruptions in *AtDUR3* did not interfere with the

acquisition of ammonium or nitrate in general. When grown on agar medium supplied with 500-1000 µM urea as a sole N source, both insertion lines *atdur3-1* and *atdur3-3* exhibited chlorotic leaves and appeared to accumulate increased levels of anthocyanins (Figure 3c). These two symptoms are highly indicative of N deficiency (Marschner, 1995; Steyn *et al*., 2002) and suggest that AtDUR3 also contributed to urea uptake in the millimolar concentration range. In fact, concentration-dependent uptake studies confirmed that urea influx in the *atdur3* mutant was still significantly lower than in the wild-type in a concentration range from 0.5 to 1.2 mM urea, although the contribution of AtDUR3 to urea influx did not change in absolute terms (Figure 9b). As there are no close homologs to AtDUR3 within the SSS superfamily of Arabidopsis (Figure 1) it is likely that AtDUR3 performs a unique physiological function in urea-based N nutrition.

Urea concentrations in the roots of hydroponically grown wild-type plants decreased approximately fivefold under conditions of N deficiency (Figure 8). Although our approach to determine urea cannot rule out the interference of other ureides, this observation supports the notion that internal accumulation of urea depends on provision of N to the roots and that root urea pools are also broken down under N deficiency during vegetative growth (Walker *et al*., 1985). As N-dependent changes in internal urea pools were similar in wild-type and insertion lines, the loss of AtDUR3 function did not substantially impair the internal utilization of urea. However, slightly lower urea concentrations in the two insertion lines after a period of N starvation pointed to the possibility that AtDUR3 contributes to the retrieval of urea lost by efflux, e.g., via urea-transporting aquaporins in the plasma membrane (Eckert *et al*., 1999; Gerbeau *et al*., 1999). The strong increase in urea concentrations in roots after resupply of urea to N-deficient wild-type plants relative to the insertion lines (Figure 8) clearly indicated that AtDUR3 mediated a significant accumulation of urea when supplied in the micromolar concentration range; at millimolar concentrations of external urea, additional transport processes must have contributed to urea uptake.

Transient storage of urea inside the cell will most likely depend on urea transport into the vacuole, because urease is a cytoplasmic enzyme (Witte *et al*., 2002). Several fold higher urea transport capacities across the tonoplast membrane than across the plasma membrane have been reported for tobacco and wheat, and the mercury sensitivity of this transport process pointed to an involvement of

aquaporins (Tyerman *et al*., 1999), most likely represented by homologs of the TIP subfamily of aquaporins (Liu *et al*., 2003b). With regard to the size of the vacuole relative to the cytoplasm, such a high vacuolar loading capacity could then create an intracellular sink that withdraws a large amount of urea from urease-mediated degradation and might finally increase the driving force for AtDUR3-dependent and -independent urea transport across the plasma membrane.

5.1.2 Physiological role of AtDUR3-mediated urea transport in Arabidopsis roots

Transgenic plants expressing an AtDUR3-promoter::GFP fusion construct showed high promoter activity in rhizodermal cells including root hairs as well as in cortical cells of mature root hair zones (Figure 5). Promoter activity became more restricted to outer root cells in younger root zones but was absent in the root apex. This N deficiency-enhanced fluorescence was supported by increasing mRNA levels of *AtDUR3* in N-deficient roots (Figure 6; Liu *et al*., 2003a). Such a cell-type-specific expression pattern was reminiscent of that found for high-affinity ammonium and nitrate transporters which also contribute to import of N from the external medium (Guo *et al*., 2002; Loqué *et al*., 2006; Nazoa *et al*., 2003). Interestingly, *AtDUR3* promoter activity was also observed in the stele of N-deficient roots (Figure 5b,d), most likely reflecting developing xylem vessels or underlying xylem parenchyma cells. Indeed, earlier studies reported that urea was translocated in the xylem sap when urea was supplied as a dominant N form or when nickel deficiency prevented urease from degrading urea prior to translocation (Gerendás *et al*., 1998; Hine and Sprent, 1988). Thus, xylem-associated *AtDUR3* expression might reflect an involvement of the transporter in xylem loading or in retrieval of urea to xylem parenchyma cells.

A quantitative determination of the uptake capacity of AtDUR3 was obtained by influx studies using ^{15}N-labeled urea. Concentration-dependent influx of urea in *atdur3-1* and *atdur3-3* showed a linear concentration dependency (Figure 9a), which is typical for channel-mediated urea transport, as has been demonstrated for AtTIP2;1 when expressed in oocytes (Liu *et al*., 2003b). In contrast, urea influx in wild-type plants was steeply elevated relative to the increase in external urea supply at concentrations below 50 µM, reaching up to 10-times the activity of

atdur3. Between 50 and 200 µM urea wild-type plants showed no further significant increase in urea influx (Figure 9a). Taking into account the fact that urea influx in wild-type and *atdur3-1* plants showed the same concentration-dependent increase at higher urea supply (Figure 9b), this study demonstrated that AtDUR3 is the major high-affinity urea transporter in Arabidopsis roots. Subtracting the urea influx in *atdur3-1* from that in wild-type plants allowed calculation of a substrate affinity of AtDUR3 of 4 µM. This value accords well with the K_m value of 3 µM as determined by ^{14}C-urea transport assays and electrophysiological studies in *AtDUR3*-expressing *Xenopus oocytes* (Liu *et al*., 2003a). Considering the average urea concentrations of below 70 µM in natural or agricultural soils (Cho *et al*., 1996; Gaudin *et al*., 1987; Mitamura *et al*., 2000a, b), this particularly high substrate affinity is very likely to allow saturation of AtDUR3-mediated substrate transport into root cells in most soils. The low K_m of AtDUR3 may be seen as an adaptation to the ubiquitous occurrence of microbial ureases. These enzymes usually have an affinity for their substrate in the millimolar range (Dalal, 1985) and are therefore unable to completely deplete soil urea. We propose a role for AtDUR3 as a unique transport system in Arabidopsis, allowing the direct use of urea, a limited but highly valuable N source in soils.

5.2 AtDUR3 retrieves apopolastic urea for nitrogen remobilization in senescent Arabidopsis leaves in dependence of the salicylic acid regulatory pathway

Reducing fertilizer input and at the same time increasing seed yield and quality by enhanced N fertilizer use efficiency is one of the major goals of agriculture today, especially when considering that these traits are negatively correlated (Krupinska and Humbeck, 2004). Next to primary uptake of N by roots the mobilization and retranslocation of N is one of the most limiting steps in N use efficiency. Cellular processes including the chemical transformation of N forms or the intracellular trafficking of N reserves are involved in delivering N from their sites of origin (e.g. the chloroplast undergoing disassembly) to the sites where long-distance transport of recycled N starts off (Gregersen *et al.,* 2008; Mascleaux and Chardon, 2011). To elucidate bottlenecks in the efficient recycling of N, an even more detailed understanding at the whole-plant as well as at the cellular level is required.

Transcriptome analyses in senescent leaves have already provided significant insights into the nature and role of changes in gene expression during senescence and a lot of differentially expressed genes have been identified (Smart, 1994; Buchanan-Wollaston, 1997; Nam, 1997; Quirino *et al.,* 2000, Gao, 2004). Some of these genes have proven to be apparent bottlenecks in efficient N metabolism and underlined the potential of increasing N use efficiency in plants by modulating their expression. For example, overexpression of a cytosolic *GS1* isoform, which has been shown to be strongly upregulated in senescent leaves led to an increased kernel number under high and low N fertilization (Martin *et al*., 2006). However, modulating just one gene might not be sufficient in the long run, especially since the regulatory network governing senescence with its multiple interactions of the molecular components is so complex that a regulatory hierarchy is hard to figure out and might even not exist. Therefore, a combination of physiological, biochemical, genetic and molecular approaches is required to fully elucidate the regulation of both, the senescence initiation and execution.

Experimental evidence for the significance and efficiency of different N forms for retranslocation processes is poor. Based on the first part of this study that has demonstrated the contribution of urea transporters for N uptake in roots, the second part of the thesis aimed at elucidating their role in N retranslocation as well as the role of urea as an intermediate in N remobilization during leaf senescence.

5.2.1 Urea as a senescence-induced metabolite

Organ-specific urea determinations in roots and shoots of wild-type plants indicated that in the vegetative growth stage urea accumulation in shoots is low and not affected by the N nutritional status of the plant (Figure 10). Moreover, this urea concentration is similar to concentrations found in roots under N deficiency, when root urea pools are depleted. Together with the observation that urea concentrations exhibit no daytime-dependent changes (Figure 11), shoot urea pools only seemed to have a minor or no function as N storage or intermediate and rather described a background urea level derived by general protein turnover processes. As this urea cannot be accessed under N-limiting conditions and since urease activity in the cytosol is not a rate-limiting factor (Witte, 2011), this background might reflect urea that is compartmentalized in the mitochondria,

where urea is generated in the ornithine cycle as a consequence of arginine catabolism (Polacco and Holland, 1993).

A first quantitative characterization of the available urea in senescent leaves showed that urea concentrations started to increase with the switch of the plant into the generative growth phase being highest in the oldest leaves (Figure 13 and Figure 21). This was in agreement with an increase in arginine concentrations and arginase activity reported previously (Polacco and Holland, 1993; Goldraij and Polacco, 1999; Diaz *et al*., 2005; Paschalidis and Roubelakis-Angelakis, 2005). As in roots, leaf vacuoles might serve as transient storage compartments, whenever urea production exceeds the capacity of urease and urea (Witte, 2011; Britto and Kronzucker, 2002). Additionally, urea might also leak out of the cell via aquaporins located at the plasma membrane, indicating that the apoplast might also serve as a short-term reservoir for urea as anticipated previously by Sattelmacher (2001). However, urease is a very stable enzyme and might even be present in the apoplast (Polacco and Holland, 1993), which is in agreement with low urea concentrations found in apoplastic wash fluids (Figure 17).

An accumulation of urea in senescent leaves could be observed independent of the way senescence was induced (Figure 14, Figure 15 and Figure 16). In the mutant line *ureGA,* that is unable to hydrolyse urea, the increase in leaf urea concentration after induction of senescence by shading was higher than in the wild type (Figure 16 and Figure 21). Comparing urea concentrations in different leaf fractions indicated that the elevation in overall urea concentration in *ureGA* was mostly due to an increase in old and middle-aged leaves, but not in young leaves (Figure 21). Interestingly, N deficiency, which had no impact on urea concentrations in the different wild-type leaf fractions, led to elevated urea concentrations in the youngest leaves in *ureGA,* while in the old and middle-aged leaves urea concentrations were just slightly increased in this mutant (Figure 21 and Figure 22). This indicates that in young leaves of *ureGA* urea is either increasingly generated under N deficiency, a feature that in wild-type plants is masked by a functional urease. Or, urea is increasingly translocated into young leaves from the source leaves, in which the strong urea accumulation might lead to an increased leakage into the apoplast. Indeed urea concentrations in leaf petiole exudates tended to be enhanced in the oldest leaves of the wild type (Figure 21c), indicating that urea was not only increasingly accumulating in

senescent leaves, but may also have serveed as a translocated N form in Arabidopsis. In the oldest leaves of *ureGA*, urea concentrations in leaf petiole exudates were significantly higher, indicating that the high urea concentrations of the leaves were reflected in the exudates. However, this holds true only for old leaves, since in middle and young leaves with urea concentrations comparable to old leaves of wild type this could not be observed, suggesting that controlled loading of the phloem might have taken place.

Taken together, urea represents an intermediate in N remobilization processes liberated during senescence. Based on its leaf- and plant age-dependent increase during senescence, urea might be suitable as a metabolic marker for early stages of leaf senescence, since its appearance is common for senescence. Additionally, urea can be transported in the phloem sap, indicating that urea potentially contributes to N retranslocation during senescence.

5.2.2 Role of AtDUR3 in retrieval of apoplastic urea

In shoots, the expression of the high-affinity urea transporter *AtDUR3* correlated with urea concentrations. N deficiency just slightly induced *AtDUR3* expression, which was not subject to a daytime-dependent regulation (Figure 10 and Figure 11). This emphasizes a less important physiological function of AtDUR3 in leaves during the vegetative growth phase (Schmid *et al*., 2005, Figure 12). However, gene expression increased with the onset of senescence and since AtDUR3 is localized to the plasma membrane, an import function of apoplastic urea into the cytosol became likely (Figure 29).

Several lines of evidence indicated that AtDUR3 takes over a retrieval function of apoplastic urea in senescent leaves: i) As urea accumulated over time in old leaves and urea concentrations were closely related to *AtDUR3* gene expression levels (Figure 15), urea accumulation appeared to be directly stimulated by AtDUR3 expression and activity. ii) Under high cellular urea accumulation, urea-transporting aquaporins localized at the plasma membrane are capable of urea permeation as a consequence of passive transport along a concentration gradient. Correlating urea concentrations in the apoplastic wash fluid to *AtDUR3* gene expression in wild-type and *atdur3-1* leaves indicated that a functional expression of *AtDUR3* decreased apoplastic pools of urea (Figure 17). The concentration

found in the apoplast of the *atdur3-1* mutant line accounted for approximately 1 mM urea (Figure 17). This value matches the apoplastic concentrations reported for ammonium, which is also supposed to leak from the cytosol to the apoplast via NH_3-transporting aquaporins (Schjoerring *et al.,* 2002).

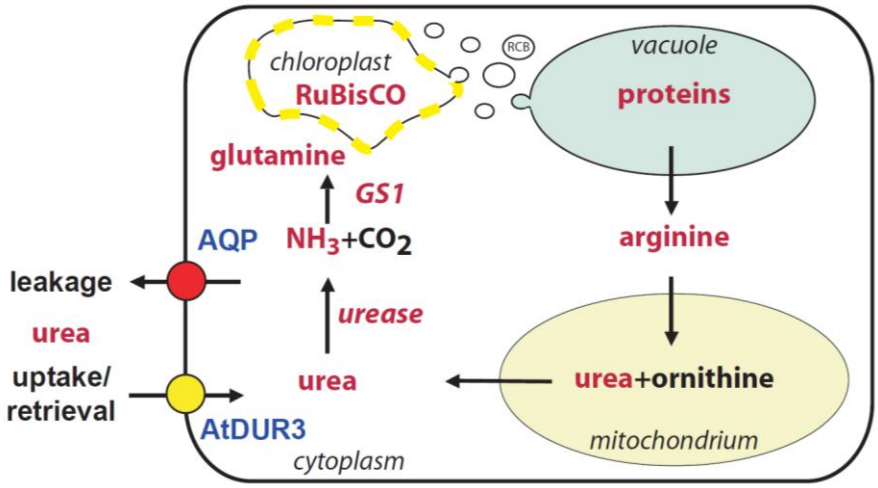

Figure 29: Urea metabolism in senescent leaves.

The senescence-induced degradation of proteins especially from the chloroplast generates large amounts of urea. As soon as urea is liberated via arginine catabolism during senescence, urea is accumulating in the cytosol. Some urea may be leaking to the apoplast via aquaporins (AQPs) located in the plasma membrane. Urea, which is lost into the apoplastic space, can then be retrieved by AtDUR3. RCB, RuBisCO-containing body.

With regard to the fact that AtDUR3 cotransports urea with protons (Liu *et al*., 2003a) and that the leaf apoplastic pH is approximately 1.5-2 pH units lower, i.e. more acidic than the cytosol (Sattelmacher, 2001; Britto and Kronzucker, 2002), an urea import function of AtDUR3 from the apoplast into the cytosol of leaf cells is proposed. iii) The collection of leaf petiole exudates from N-deficient leaves allowed recovering largest amounts of urea when urease activity was lost, but AtDUR3 was functional, as in *ureGA* (Figure 22c). Thus, AtDUR3 was able to increase the phloem loading capacity, at least via the apoplastic phloem loading pathway. Actually, this hypothesis could be supported by the preliminary localization of the *AtDUR3* promoter activity confirming *AtDUR3* promoter activity in the vasculature of senescent leaves (Figure 18). Additionally, overexpression of AtDUR3 did not lead to an increase in urea concentrations in the phloem sap, indicating that it might not be involved directly in phloem loading (Figure 21).

Thus, AtDUR3 may find its precise role in a back-up function to keep as much urea as possible in the cytoplasm under growth conditions, in which large amounts of urea are generated via protein catabolism.

Such a regulation of apoplastic metabolites has also been shown for ammonium and its transporters (Nielsen and Schjoerring, 1998; Sattelmacher, 2001; Sohlenkamp *et al*., 2002). NH_4^+ is constantly generated in large quantities in plant leaves by processes such as photorespiration, nitrate reduction, protein turnover, and lignin biosynthesis (Joy, 1988; Leegood *et al*., 1995). Aquaporins might be responsible for a leakage into the apoplastic space since they were shown to transport ammonia as well (Loqué *et al*., 2005; Dynowski *et al*., 2008), and ammonium transporters of the high-and low-affinity systems have been shown to play an orchestrated role in keeping pH, N status and ammonium retrieval in balance (Schjoerring *et al*., 2002; Sohlenkamp *et al*., 2002).

5.2.3 A Role of AtDUR3 in nitrogen retranslocation

Long-distance transport of nutrients from senescing leaf tissue to the seeds or other parts of the plant is thought to take place via the phloem (Hill, 1980). Hence, also the phloem accessibility of a nutrient influences the efficiency with which it is mobilized from senescing leaves (Bukovac and Wittwer, 1957). Preliminary localisation studies of *AtDUR3promoter::GUS* plants (Figure 18) pointed to a localisation close to or even in the vascular bundle. It has been shown that *GS1* and *GDH* are strongly induced in senescent leaves, and GDH especially in phloem companion cells (Pérez-Garcia *et al*., 1998; Olea *et al*., 2004; AbuQamar *et al*., 2006). AtDUR3 might therefore contribute to an increase of urea concentration in those cells that mediate urea transport to the site of phloem loading which are the companion cells in the vascular bundle. The primary function of urea may not be to serve as a N form for phloem loading, but rather to be hydrolysed, so that its N can be re-assimilated and translocated in another form such as glutamine or asparagine, which have been shown to be the most dominant amino acids found in the petiole exudates (Table 1). This would explain why urea was only visible in the *ureGA* background, since elevated urea concentrations may have led to a stronger phloem loading, and, as a consequence, the contribution of AtDUR3 could only be observed in the double knock out *dur3ureGA*. Since urea

concentrations were always measured in whole leaf samples, a localized accumulation of urea in the vascular tissue was not observed. In fact, urea concentrations in whole leaf samples of *dur3ureGA* plants appeared similar to those found in the *ureGA* mutant (Figure 21 and Figure 22). Furthermore, this would also explain the slightly higher total amino acid exudation of old leaves of *35SDUR3* plants under –N conditions (Table1), since urease is not the limiting step of urea metabolism (Gerendás and Sattelmacher, 1997). In future, it would be highly interesting to also exploit downstream genes of *AtDUR3* such as urease, *GS* and *GDH* encoding genes to further strengthen this theory.

Glutamine appears as a preferential N form for phloem loading (Caputo and Barneix, 1997). This view, however, mainly builds on the correlation of amino acid concentrations in leaves with that in the phloem sap, while it is still unclear to what extent enzymatic or transport steps determine the synthesis of low molecular-weight N compounds required for phloem loading. As a N form with a narrow C/N ratio urea would actually be an ideal transport form. In order to clarify if a retrieval function of AtDUR3 and subsequent urea accumulation is also related to the availability for phloem loading and retranslocation, Arabidopsis mutant lines with altered expression of genes involved in the transport or assimilation of urea were investigated (Figure 21 andFigure 22). When N deficiency was applied for 4 days to trigger the remobilization of N, urea levels in the petiole sap of *ureGA* strongly increased which was not observed in *dur3ureGA* (Figure 22), pointing to a quantitative contribution of AtDUR3 to phloem loading that was only visible in the *ureGA* background, but otherwise masked in the wild-type background.

Interestingly, the *AtDUR3* over-expressing line showed no significant increase in urea retranslocation also under N deficiency. Uptake experiments with ^{15}N-labelled urea, however, proofed an enhanced AtDUR3 transport activity in this line, irrespective of its N nutritional status (Figure 20 and Figure 21). Thus, the lack of retranslocation in *35SDUR3* was not due to a limited transport capacity but rather implies that the transport activity in the wild type is not limiting as long as ammonium is generated by an active urease and long-distance transport forms (in particular glu and asn) are generated.

In order to put the amount of urea translocated into perspective, amino acid levels were determined in the petiole exudates as well. Previous metabolite profiling

studies performed on Arabidopsis leaves over time showed that the concentration of individual amino acids fluctuated independently from each other with leaf ageing. As shown there, glutamate and aspartate, the most abundant amino acids in young leaves of Arabidopsis, decreased with senescence. In contrast, less abundant amino acids like tyrosine, leucine, isoleucine and the non-proteinogenic amino acid, γ-aminobutyric acid (GABA), accumulated with ageing (Diaz *et al*., 2008). In general, the results found in this study confirmed the general order of abundance reported before with glutamine being most abundant over glutamate, serine, asparagine and aspartate (Table 1). A rough estimation showed that urea-N represented approximately 13% of total amino acid-N indicating that urea is a quantitatively important N form utilized for phloem loading.

The retranslocation studies could not identify an explicit role of AtDUR3 in urea retranslocation (Figure 24). Surprisingly, roots seemed to be able to still create a sink for N, especially under N starvation. In literature, it is often referred to that roots do not create a sink anymore as soon as plants enter the generative phase supporting solemnly the development of the seed (Mattsson *et al*., 1993). However, an interesting discovery was made regarding the natural abundance of ^{15}N in the different leaf fractions that changed with the leaf age as well as in dependence of the N nutritional status of the plant (Figure 23). Under N-sufficient conditions the $^{15}N/^{14}N$ ratio was higher in the old leaves most likely due to the ^{15}N discrimination by nitrate reductase, which is about 15‰ (Tcherkez and Farquhar, 2006). Under N starvation, however, ^{15}N accumulated in younger leaves indicating that ^{15}N transiently accumulated in old leaves, which was then retranslocated to young leaves. This leads to the conclusion that remobilized N is heavier than freshly assimilated N which might be a future tool to distinguish these two N pools. More experiments determining the progression of the natural abundance of ^{15}N during different developmental stages in sink and source leaves have to be performed to verify this hypothesis.

5.2.4 AtDUR3 gene expression in leaves is regulated by salicylic acid

35SDUR3 plants developed a strong conditional phenotype as soon as plants were cultured under long-day conditions (Figure 25). To learn more about the regulation of *AtDUR3* expression, the promoter region of *AtDUR3* was analysed

(Figure 26), yielding several predicted binding sites for transcription factors of the WRKY, MYB, RA, GATA and bZIP families. As an independent *in silico* approach coexpression analysis was performed and pointed to two genes, whose expression pattern closely matched that of *AtDUR3*, namely *WRKY60* and *bZIP50*.

WRKY60 belongs to the WRKY superfamily of TFs, which have been shown to be involved in the regulation of various physiological and developmental programs including pathogen defense and senescence (Eulgem *et al*., 2000, Rushton *et al*., 2010). Besides a documented interaction of WRKY60 with WRKY40, WRKY18 or itself (Xu *et al.,* 2006), it has been reported to be inducible by SA (Li *et al*., 2004). The TF *bZIP50*, however, belongs to a large family of TFs that regulate processes including pathogen defence, light and stress signalling, seed maturation and flower development (Jakoby *et al*., 2002). *BZIP50* has mostly been reported to be involved in defence responses to pathogens. Moreover, in a transcriptome analysis performed to identify genes affected by long-term N deprivation or short-term nitrate nutrition in Arabidopsis*, bZIP50* turned out to be de-repressed under N-limiting conditions and was repressed after 3h of nitrate resupply (Scheible *et al*., 2004). Additionally, the subcellular localisation of the corresponding protein was predicted to reside not only in the nucleus, but also in mitochondria, even though at a lower likelihood (Heazlewood *et al.*, 2007). A few studies have reported that certain metabolic pathways, activated during stress or pathogen infections, are also induced during senescence (Quirino *et al.,* 1999). Strong evidence is lacking but it has been shown that pathogen-related genes can also be upregulated under sterile conditions in the absence of pathogens when plants undergo senescence, pointing to a common regulatory pathway.

A search for conditions causing and for mutants with altered *AtDUR3* gene expression clearly indicated that *AtDUR3* derepression in senescent leaves depended on SA. *AtDUR3* expression was not induced in *NahG*, a transgenic line incapable of SA accumulation (Figure 27). SA is one of the superior regulatory hubs involved in the induction of senescence (Morris *et al*., 2000), but it has been mostly studied in dependence of pathogen attack, where the SA pathway is responsible for systemic acquired resistance (Durrant and Dong, 2004). Arabidopsis mutants defective in SA signaling (*npr1*, *pad4*, *eds5*, and *sid2 (eds16)*) or transgenic plants (*NahG*) that cannot accumulate SA show enhanced susceptibility to pathogens (Cui *et al*., 2002) and a delay in the onset of

developmental leaf senescence in Arabidopsis (Buchanan-Wollaston *et al*., 2005). However, all these findings are only based on reverse genetic approaches and therefore have to be treated with care.

To get an idea of the effect of SA on AtDUR3 activity *in planta,* ^{15}N-labelled urea influx was analyzed in dependence of the N nutritional status of the plants and in the presence of SA (Figure 28). SA clearly showed a strong stimulation of urea transport rates at N-sufficient conditions, where the high-affinity uptake systems usually are not activated. In this experiment, therefore, no additional induction could be obtained by a SA treatment under N-deficient conditions. Surprisingly, this inducing effect was not limited to high-affinity urea uptake, but also applied to low-affinity urea uptake. The low-affinity uptake system is usually differently regulated by the N status, and rather downregulated as soon as strong N deficiency is induced. However, the low-affinity system could be strongly induced irrespective of the N nutritional status, indicating that SA might have a wide range of action, which would be in agreement with its role as a dominant bottleneck in the regulatory network (Buchanon-Wollaston *et al*., 2005).

Further studies have to confirm these findings, but an attractive hypothesis is that salicylic acid might be the signaling link between developmental senescence and N deficiency-induced senescence, merging for a combined initiation of target genes (Figure 30). SA by itself has been shown to be responsible for the expression of approximately 20% of the senescence-induced genes (Buchanan-Wollaston *et al*., 2005). In the present working model, *AtDUR3* represents one of these genes being indirectly regulated by SA, putatively via WRKY60 or bZIP50 (Figure 30).

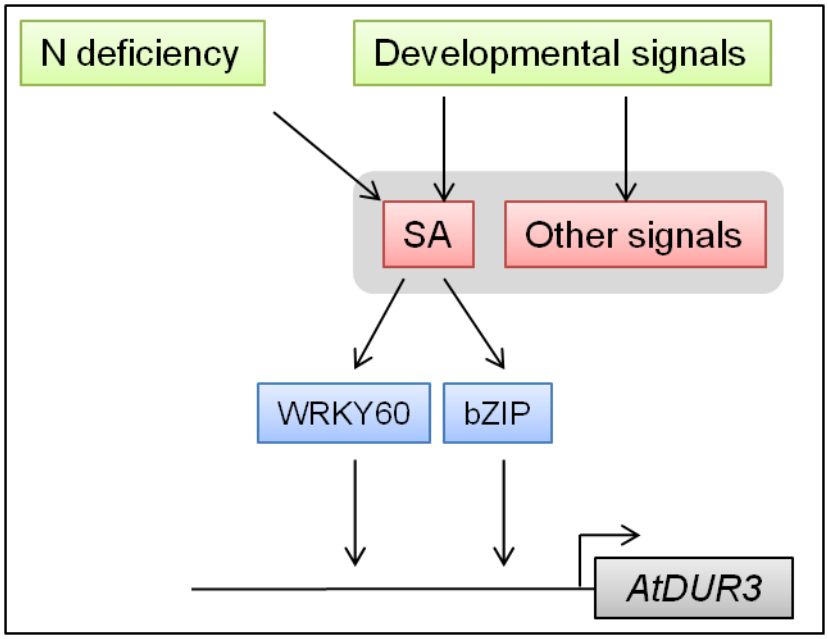

Figure 30: Working model of the regulation of AtDUR3 by salicylic acid (SA).

Senescence is induced by several regulatory mechanisms. One is the salicylic acid (SA)-dependent pathway. N-deficiency might induce senescence by SA, thereby engaging in the regulatory network of SA. SA in turn induces transcription factors, e.g. WRKY60 and bZIP50, that are upstream of the transcriptional regulation of *AtDUR3*.

In further studies, mutants will be employed to alter internal SA concentrations or to directly impair *WRKY60* or *bZIP50* expression. As the N deficiency signal is starting in the root (Walch-Liu *et al*, 2005) and if SA is this N deficiency signal, SA application to the root and a time-dependent analysis of N deficiency responses in the shoot will help to better understand how these two regulatory pathways are entangled.

Taken together, urea is a prominent N intermediate generated during senescence that serves as a mobile transient storage form for N before its conversion into amino acids for long-distance transport. Most likely, AtDUR3 fulfills a urea retrieval function in the vasculature, keeping urea levels high in the cells and contributing only in an indirect way to phloem loading. Primary root uptake, the mobilization and the retranslocation of N are major limiting factors for N use efficiency. Since urea and AtDUR3 are involved in all of these processes they are promising targets to improve N use efficiency. As yield parameters were not included in this work and the overexpression of the transporter did not result in higher urea retranslocation, but indicated a higher amino acid efflux from older leaves, further studies on the impact of altered urea levels and *AtDUR3* expression especially in crop plants should be conducted. Additionally, the impact of SA on the

N-regulatory network opens another door to investigate up- and downstream situated genes and metabolites, whose regulation might have a more powerful impact on N use efficiency.

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8 Curriculum vitae

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Kojima, S., **Bohner, A**. (contributed equally), Gassert, B., Yuan, L., and von Wiren, N. (2007). AtDUR3 represents the major transporter for high-affinity urea transport across the plasma membrane of nitrogen-deficient Arabidopsis roots. Plant J 52, 30-40.

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Oral presentations

XVI. International Plant Nutrition Colloquium 2009, Sacramento, USA, "Physiological functions of the urea transporter AtDUR3 in Arabidopsis thaliana"

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